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ANTIMICROBIAL ACTIVITY OF EXTRACTS FROM *IN VIVO* AND *IN VITRO* PROPAGATED *LAMIUM ALBUM* L. PLANTS

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Abstract

The antimicrobial activity of 18 different extracts from *in vivo* and *in vitro* grown *L. album* L. plants was evaluated against clinical bacteria and yeasts using the well diffusion method. All the used extracts demonstrated antibacterial activity, whereas only the water extracts from leaves (*in vivo*) possessed antifungal activity against *Candida albicans* NBIMCC 72 *and Candida glabrata* NBIMCC 8673 (14 and 20 mm diameter of inhibition zones and MIC 10 mg/ml, respectively). The methanol and ethanol extracts obtained from the *in vitro* propagated plants had a broader spectrum of antibacterial activity than those from *in vivo* plants, while the opposite tendency was observed for the chloroform extracts. All tested flower extracts possessed antimicrobial activity. The chloroform extract from *in vivo* flowers demonstrated the highest activity against *E. faecalis* NBIMCC 3915, *S. aureus* NBIMCC 3703, *P. hauseri* NBIMCC 1339 and *P. aeruginosa* NBIMCC 3700 (22 mm, 13 mm, 11 mm, 23 mm zone diameter of inhibition and MIC 0.313 mg/ml, respectively). The water extracts from leaves (both *in vivo* and *in vitro*) possessed higher antibacterial activity than extract from flowers. The obtained results showed that both *in vivo* and *in vitro* propagated *L. album* L. could be used as a source of antibacterial substances.

Keywords: *Lamium album*, *in vitro* propagation, plant extracts, antimicrobial activity

Introduction

Medicinal plants contain a variety of chemical substances and have been reported to possess many useful properties, including anti-inflammatory activity, oestrogenic activity, enzyme inhibition, antimicrobial activity (Havsteen, 1983; Harborne and Baxter, 1999; Madox et al., 2010), antiallergic activity, antioxidant activity (Middleton and Chithan, 1993; Velioglu et al., 1998; Re et al., 1999), vascular activity, and cytotoxic antitumour activity (Harborne and Williams, 2000). These plants can serve as a possible source for new antimicrobials to which pathogen strains are not resistant (Ali-Shtayeh et al., 1998; Augustin and Hoch, 2004; Aslim and Yucel, 2008).

Genus *Lamium* includes almost 40 species distributed throughout Europe, Asia and Africa, some of which are well-known: *Lamium album* L. (white dead-nettle), *L. purpureum* L. (purple dead-nettle), and *L. maculatum* L. (spotted dead-nettle) (Yalçin and Kaya, 2006). Some *Lamium* plants have been used in traditional medicine for treatment of disorders, such as trauma, fracture, paralysis, hypertension, menorrhagia, and uterine hemorrhage (Bisset, 1994; Weiss, 1988). In particular, *L. album* is considered as the most popular species that contains a variety of compounds: phenols, iridoids, triterpens, saponines, fatty acids, phytoecdysteroids, essential oils, tannins, amines (Chudnicka and Matysik, 2005; Alipieva et al., 2007; Paduch et al., 2006). Valyova et al. (2011) showed that *L. album* could be a source of natural antioxidants with potential use in food supplements.

Since there was no available data about the cultivation of *L. album* L. *in vitro*, the aim of this study was to evaluate the antibacterial activity of wild *L. album* plants cultivated in both, *in vivo* and *in vitro* conditions.

Material and Methods

Plant material

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

Plant extracts

Soxhlet extraction

Samples of 3 g from *in vivo* (leaves and flower) and *in vitro* powdered air-dried *L. album* plants were used. The material was subjected to Soxhlet extraction by chloroform and methanol as solvents. Afterwards, the solvents were removed by a rotary evaporator, and the extracts were concentrated, dried, and kept in dark at 4^{o}C.

Thermostat extraction

Samples of 2 g from *in vivo* (leaves and flower) and *in vitro* air-dried *L. album* plants were extracted according to the method described by Schinella et al. (2002) for 24 h at 40^{o}C by the following solvents – chloroform, ethanol, methanol, and water. After filtration the raw materials were extracted twice in the same conditions. The solvents were removed under a vacuum evaporator, and the extracts were concentrated, dried, and kept in dark at 4^{o}C.

Test-microorganisms

Bacterial and yeast strains were used as references for the antimicrobial susceptibility assay: *Bacillus subtilis* NBIMCC 1709, *Enterobacter*

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aerogenes NBIMCC 3699, *Enterococcus faecalis* NBIMCC 3360, *Escherichia coli* NBIMCC 3397, *Klebsiella pneumoniae* NBIMCC 3670, *Micrococcus luteus* NBIMCC 159, *Proteus hauseri* NBIMCC1339, *Pseudomonas aeruginosa* NBIMCC 3700, *Salmonella enterica* NBIMCC 3669, *Staphylococcus aureus* NBIMCC 3359 and 3703, *Staphylococcus epidermidis* NBIMCC1093, *Candida albicans* NBIMCC 72 and *Candida glabrata* NBIMCC 8673. The strains were obtained from the National Bank for Industrial Microorganisms and Cell Cultures (NBIMCC, 49 "S^{t} Kliment Ohridski" Blvd, 1756 Sofia, Bulgaria, www.nbimcc.org/bg/about.htm).

Antimicrobial activity

The antimicrobial activity was determined by the well diffusion method according to National Committee for Clinical Laboratory Standards (NCCLS – 1997). Petri plates with 20 ml of Muller Hinton Agar medium (MNB, Difco Laboratories, Detroit, MI, USA) for bacteria and Potato Dextrose Agar (Oxoid) for yeasts were inoculated with $1.5.10^{7}$ cfu/ml (0.5 McFarland standards) of 24 h bacterial or yeast suspensions. Wells were cut into agar and filled with 50 µl (10 mg/ml) of plant extracts. Standard disks (Oxoid) impregnated with tetracycline (30 µg/disk) and nystatine (100 IU/disk) were used as positive controls. Sterilized paper disks impregnated with pure solvents (10 µg/disk) served as negative controls. The plates were placed for 2 h at 4^{o}C to allow the diffusion of extracts and cultivated for 24 h at 37^{o}C. The antimicrobial activity was assessed by measuring the diameter of the inhibition zone around the well. All experiments were performed in triplicate.

Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) was determined by the micro-dilution method using paper disks. Each extract was diluted two-folds and used for preparation of serial dilutions with a final concentration from 10 mg/ml to 0.078 mg/ml. Sterile paper disks were impregnated with 50 µl of the diluted extracts, dried and placed onto agar medium which was previously inoculated with test-microorganisms. After diffusion of the extract for 2 h at 4^{o}C the plates were cultivated for 24 h at 37^{o}C. The antimicrobial activity was assessed by measuring the diameter of the inhibition zone around the disks. All experiments were performed in triplicate. The lowest concentration of the extract that formed inhibition zone was regarded as MIC.

Results and Discussion

Eighteen different *L. album* extracts were screened for antimicrobial activity (Table1). The extracts were obtained from leaves and flowers of mature *L. album* plants which were harvested either in their natural habitats, or from *in vitro* propagated plants. Four solvents (chloroform, methanol, ethanol, and water) and two methods of extraction (Soxhlet, thermostat) were used.

Table 1. Antimicrobial activity of the crude extracts of *L. album* L. to the test-microorganisms

Test-microorganisms	Extract, No																	
	Diameter of inhibition zone (mm)																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Gram-positive bacteria																		
B. subtilis 1709	0	0	0	0	0	11	14	0	0	0	12	12	12	14	0	12	0	0
E. faecalis 3360	0	9	0	0	14	12	8	8	8	0	0	0	12	0	0	0	0	0
E. faecalis 3915	0	11	14	0	10	22	0	0	0	8	0	0	0	0	0	0	0	0
M. luteus 159	19	15	14	0	25	10	nd	16	nd	13	nd	nd	16	10	nd	nd	nd	nd
S. aureus 3359	0	10	0	0	10	0	0	10	0	0	12	12	0	0	12	0	0	0
S. aureus 3703	14	11	14	16	0	13	11	11	14	14	0	0	0	0	10	10	14	0
S. epidermidis 1093	10	0	0	0	10	0	0	15	0	11	10	0	0	0	10	10	10	0
Gram-negative Bacteria																		
E. aerogenes 3699	0	0	11	11	0	0	12	10	0	12	0	0	0	0	0	0	0	0
E. coli 3397	0	0	0	0	0	12	0	0	13	13	0	0	0	0	0	0	0	0
K. pneumoniae 3670	0	0	0	0	0	14	0	12	12	0	0	0	0	0	0	0	0	0
P. hauseri 1339	0	0	0	0	0	11	0	0	0	0	0	0	0	0	0	0	0	0
P. aeruginoisa 3700	13	0	18	20	0	23	11	12	16	15	22	20	17	15	14	23	12	25
S. enterica 3669	0	0	14	0	0	14	0	0	13	0	0	0	0	0	0	0	0	0
Yeasts																		
C. albicans 72	0	0	0	0	0	0	0	0	0	0	0	0	14	0	0	0	0	0
C. glabrata 8673	0	0	0	0	0	0	0	0	0	0	0	0	20	0	0	0	0	15
Total number of affected test-microorganisms	4	5	6	3	5	10	5	8	6	7	4	3	6	3	4	4	3	2

Table shows average zone of inhibition (in mm), nd = not determined, 1= Methanol, *in vivo* (leaves) (Soxhlet), 2= Methanol, *in vitro* (leaves) (Soxhlet), 3= Chloroform *in vivo* (leaves) (Soxhlet), 4= Chloroform *in vitro* (leaves) (Soxhlet), 5= Methanol, *in vivo* (flowers) (Soxhlet), 6= Chloroform *in vivo* (flowers) (Soxhlet), 7= Methanol, *in vivo* (leaves) (Thermostat), 8= Methanol, *in vitro* (leaves) (Thermostat), 9= Ethanol, *in vivo* (leaves) (Thermostat), 10= Ethanol, *in vitro* (leaves) (Thermostat), 11= Chloroform, *in vivo* (leaves) (Thermostat), 12= Chloroform, *in vitro* (leaves) (Thermostat), 13= H_2O, *in vivo* (leaves) (Thermostat), 14= H_2O, *in vitro* (leaves) (Thermostat), 15= Ethanol, *in vivo* (flowers)

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(Thermostat), 16= Chloroform, *in vivo* (flowers) (Thermostat), 17= Methanol, *in vivo* (flowers) (Thermostat), 18= H_2O, *in vivo* (flowers) (Thermostat).

The antimicrobial activity of screened plant extracts is shown in Table 1. All the extracts obtained by the different solvents exhibited antibacterial activity, and only the water extracts (extracts 10 and 13) possessed antifungal activity. The methanol and ethanol extracts obtained from the *in vitro* propagated plants had a broader spectrum of antibacterial activity than those obtained from the *in vivo* plants. The opposite tendency was observed for the chloroform extracts (Table 1). The chloroform extracts obtained by the Soxhlet method and the methanol extracts collected by the thermostat method showed higher activity compared to the others. All flower extracts possessed antimicrobial activity but the chloroform extract obtained by the Soxhlet method had the broadest spectrum by inhibiting ten from the eleven tested Gram-positive and Gram-negative bacteria. The activity varied among the different strains with highest sensitivity observed for *E. faecalis* 3915 and *P. aeruginosa* 3700 (Table 1). All bacterial and yeast strains were sensitive to the respective antibiotic controls. Pure solvents did not possess inhibitory effect.

The wide spectrum of antibacterial activity of *L. album* plant extracts might be related to the presence of some biologically active substances. Indeed, the medicinal properties of this plant had attracted significant attention and intensive phytochemical investigations had been done. Among the secondary metabolites of *L. album* L., terpenoids (Brieskorn and Ahlborn, 1973; Eigtved et al., 1974; Damtoft, 1992; Sarker et al., 1997), phenylethanoids (Budzianowski and Skrzypczak, 1995), and phytoecdysteroids (Savchenko et al., 2001) had been reported. In a Danish population of *L. album* L. four iridoids and secoiridoids were isolated (Eigtved et al., 1974; Damtoft, 1992) whereas in a Bulgarian population several flower volatile compounds, iridoid glucosides (Alipieva, 2003; Alipieva et al., 2006), as well as phenols and flavonoids (Valyova et al., 2011) were detected. Phenols and flavonoids were found in both *in vivo* and *in vitro* propagated *L. album* plants (Valyova et al., 2011). Up to date, only the essential oil of *L. garganicum* subsp. *laevigatum* was reported to be active against some Gram-positive and Gram-negative bacteria (Roussis et al., 1996). Based on the published data, it could be assumed that in our study the antimicrobial activity of the screened *L. album* extracts is due to the presence of such biologically active compounds.

Table 2: MIC of different extracts of *L. album* L. on the test-microorganisms

Test-microorganism	Extract, No	MIC (mg/ml)
B. subtilis 1709	6	1.25
	13	2.5
	14	2.5
E. faecalis 3360	5	2.5
E. faecalis 3915	6	0.313
M. luteus 159	5	1.25
	13	2.5
S. aureus 3359	11	1.25
S. aureus 3703	4	1.25
	6	0.313
	17	1.25
S. epidermidis 1093	8	1.25
	10	2.5
E. aerogenes 3699	7	1.25
	10	0.313
E. coli 3397	6	0.625
	9	5.0
K. pneumoniae 3670	6	0.625
	9	5.00
P. hauseri 1339	6	0.313
P. aeruginoisa 3700	4	0.625
	6	0.313
	11	0.313
	16	0.313
	18	1.25
S. enterica 3669	6	0.625
	9	2.5
C. albicans 72	13	10
C. glabrata 8673	13	10

4= Chloroform *in vitro* (leaves) (Soxhlet), 5= Methanol, *in vivo* (flowers) (Soxhlet), 6= Chloroform *in vivo* (flowers) (Soxhlet), 8= Methanol, *in vitro* (leaves) (Thermostat), 9= Ethanol, *in vivo* (leaves) (Thermostat), 10= Ethanol, *in vitro* (leaves) (Thermostat), 11= Chloroform, *in vivo* (leaves) (Thermostat), 13= H_2O, *in vivo* (leaves) (Thermostat), 14= H_2O, *in vitro* (leaves) (Thermostat), 16= Chloroform, *in vivo* (flowers) (Thermostat), 17= Methanol, *in vivo* (flowers) (Thermostat), 18= H_2O, *in vivo* (flowers) (Thermostat).

The MIC was determined for the extracts with higher activity (Table 2). The MIC values varied among the extracts and the microbial strains, and ranged from 10 to 0.313 mg/ml. The lowest MIC (0.313 mg/ml) was observed as follows: in chloroform extract from flowers (by the Soxhlet method) towards *E. faecalis* NBIMCC 3915, *S. aureus* NBIMCC 3703, *P. hauseri* NBIMCC 1339, *P. aeruginosa* NBIMCC 3700; in ethanol extract from leaves (*in vitro*) towards *E. aerogenes* NBIMCC 3699; and in chloroform extracts from leaves and flowers (by the thermostat method) towards *P. aeruginosa* NBIMCC 3700.

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Conclusion

The present study revealed that all the tested *L. album* extracts possessed antimicrobial activity that varied depending on the test-microorganisms, solvents, the method of extraction, and the parts of the plants used in both *in vivo* and *in vitro* variants. The Gram-positive bacteria were more sensitive than the Gram-negative bacteria. The methanol and the ethanol leaf extracts obtained by the *in vitro* propagated *L. album* L. possessed a broader spectrum of antibacterial activity than the *in vivo* extracts. The lowest MIC was demonstrated by the chloroform extracts (from *in vivo* flowers and leaves) towards *E. faecalis*, *S. aureus*, *P. hauseri*, and *P. aeruginosa*. The obtained results showed that *L. album* L. could be of interest for biotechnology since it can be used as a natural source of antimicrobial substances as an alternative to chemical therapeutics.

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