EFFECT OF THE VOLATILE SECONDARY METABOLITES OF *MONARDA DIDYMA* L., *LAMIUM ALBUM* L. AND *MYRRHIS ODORATA* L. PLANTS AGAINST MICROMYCETES OF INDOOR ENVIRONMENTS OF ANIMALS

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Abstract. The aim of this study was to carry out quantitative and qualitative analyses of essential oils, determine the in *vitro* antifungal activities of essential oils against fungi present in animals indoor environments. Quantitative and qualitative analyses of essential oils of *Monarda didyma* L., *Lamium album* L., and *Myrrhis odorata* L. obtained by the supercritical carbon dioxide extraction method were carried out using gas chromatography with mass spectrometric detection. The minimal inhibitory concentrations were determined in *vitro* by means of two methods, namely the standard method of broth dilution for antifungal susceptibility testing and the micromycetes inhibition by volatile compounds assay against some fungi dominant in the indoor environments. Using broth dilution method, essential oil of *Lamium album* L., showed minimal inhibition concentration (MIC)>25 (%v/v), $P>0.05$. Essential oils from *Monarda didyma* L. and *Myrrhis odorata* L. showed MIC values 0.5-15 (%v/v), $P<0.05$. The results revealed that the antifungal activity of essential oils depended on the assay used. The inhibiting effects of essential oils in vapour phase were generally higher than those in liquid phase MIC=0.05-23.0 (%v/v). According to both methods, *Monarda didyma* L., *Myrrhis odorata* L., and *Lamium album* L. essential oils were found to be of wide spectrum of activity against all fungi tested.

Keywords: plant essential oils, antifungal activity, indoor environment, poultry, broiler, *Monarda didyma* L., *Myrrhis odorata* L., *Lamium album* L.

Introduction. The plant essential oils as natural substances represent a potential source of new antifungal and antibacterial agents. During the past years, a number of studies have been carried out concerning the application of essential oils as antimicrobial agents. Particularly essential oils obtained from aromatic herbs and spice plants are known to be inhibitive or lethal to microorganisms, depending on the concentration used. Essential oils are volatile natural complex compounds and are biosynthesized by aromatic plants as secondary metabolites. The complexity of essential oils is attributed to the variety of terpene hydrocarbons, alcohols, aldehydes, ketones, acids and esters (Tzortazakis et al., 2007).

Due to their bactericidal and fungicidal properties, pharmaceutical and food uses of essential oils are increasingly widespread as alternatives to synthetic chemical products to protect the ecological equilibrium.

The European consumers show growing concerns regarding the welfare of domestic animals. Many health problems are likely related to high levels of dust and airborne microorganisms in poultry houses (Bakutis et al., 2011). Today's indoor environments are often accompanied by high concentrations of airborne fungi. Fungi can cause damage to the structures, decoration of buildings and are also responsible for their indoor air quality (Radon et al., 2004; Verma et al., 2011). Fungi are present everywhere in indoor environments. The dominant fungi found in indoor environments are species from the genera *Aspergillus*, *Penicillium* and *Cladosporium* (Tsai et al., 2007; Chao et al., 2002; Takahashi, 1997). *Alternaria, Aspergillus, Penicillium, Trichoderma species* (Kumar et al., 2010) have been found in the air and surface samples from indoor environments. Many fungi are toxigenic and produce toxins as their secondary metabolites (Nielsen et al., 2001). These fungi grow rapidly on a variety of natural substrates and may cause various human and animal health problems. Fungal spores are a well-known cause of allergic reactions and are identified as one of the major indoor allergens. Exposure to high concentrations of fungal spores has been shown to exacerbate symptoms of asthma and allergic rhinitis in sensitive individuals (Li and Yang, 2004).

Mycotoxins can cause serious diseases in farm animals. Aflatoxin B1 is a highly toxic and carcinogenic metabolite produced by *Aspergillus* species on agricultural commodities (Allameh et al., 2001; Bakutis et al., 2005). Production of essential oils by plants is believed to be predominantly a defence mechanism against pathogens and pests and it has been shown that essential oils possess fungicidal properties (Cakir et al., 2004; Voda et al., 2003). Essential oils and their components are gaining an increasing interest because of their relatively safe status, wide acceptance by consumers, and their exploitation for potential multi-purpose functional use. Therefore, essential oils are one of the most promising groups of natural compounds for the development of safer antifungal agents. The increasing resistance to antifungal compounds and the reduced

number of available drugs led us to search for the new alternatives among medicinal aromatic plants and their essential oils, used for their antifungal properties. The antifungal activity can be attributed to the presence of some components such as carvacrol, cymene, thymol, pinene and other (Mishra and Dubey, 1994; Tabassum and Vidyasagar, 2013).

The aim of this study was to carry out quantitative and qualitative analyses of essential oils of *Monarda didyma* L., *Lamium album* L., and *Myrrhis odorata* L. obtained by the supercritical carbon dioxide extraction method, using gas chromatography with mass spectrometric detection and to determine the in *vitro* antifungal activities of essential oils against some environment important fungi, by means of two different methods, such as the modified standard method of broth dilution for antifungal susceptibility testing (BD) and the micromycetes inhibition by volatile compounds assay (VS).

Materials and methods

Isolation and detection of moulds. Exposure to total microorganisms was determined by means of personal sampling in the breathing zone. Samples were taken during the daily work inside the poultry houses. For the experiment three broiler houses were investigated. These moulds were isolated from the air of poultry houses, applying sedimentation method according to Standard Methods for Examination of atmospheric microorganisms described by Hadacek et al., 2000.

The microorganisms were settled from the air directly on the surface of Petri dish, sedimentation time was 10 min. Petri dish nutrient medium was Sabouraud 2% (w/v) glucose agar (SGA; Dico, USA), supplemented with 50 mg/l^{-1} of streptomycin and 20 000 IU of penicillin, and incubated at 25±2 ºC for the 5-7 days. Moulds were identified according to macroscopic and microscopic morphological characteristics after subculturing on Czapek agar (Czapek dox agar, Liofilchem, Italy)(Hoog et al., 2000). Before the experiments, the fungi were transferred to fresh media and incubated at 25±2 ºC for 5–7 days in duplicate.

Preparation of conidial suspensions

Inocula were prepared by growing *Aspergillus flavus, Aspergillus niger, Aspergillus ochraceus, Penicillium chrysogenum, Penicillium commune, Penicillium citrinum, Cladosporium cladosporioides, Alternaria alternata, Rhizopus microsporus, and Trichoderma viride* on Czapek agar (Czapek dox agar, Liofilchem, Italy) slopes as described by the reference method M38-A recommended by the National Committee for Clinical Laboratory Standards 2002 (CLSI). The slopes were flooded with 0.85% saline and conidia gently probed; the resulting suspensions were removed and vortexed thoroughly. After the settling of the larger particles, suspensions were adjusted by nephelometry and diluted in saline to obtain inocula of 2x10^4 CFU ml^{-1}, as confirmed by colony counts in triplicate on agar.

Antifungal activity testing by dilution method

With some modifications, broth dilution testing was based on the National Committee for Clinical Laboratory Standards (2002) method. The minimal inhibitory concentration (MIC) of essential oils was defined as the lowest concentration of compound that completely inhibited visible growth after 7 days of incubation.

The medium RPMI–1640 (Sigma-Aldrich, Germany), with L-glutamine and phenol red, without bicarbonate, was supplemented with glucose to a final concentration of 2 % (RPMI 2% G). Containing 3- (N-morpholino) propane sulfonic acid (MOPS) (Sigma-Aldrich, Germany) at a final concentration of 0.165 mol l^{-1} for pH 7.0 is satisfactory for RPMI 1640 media.

Doubling dilutions of the essential oils concentrations ranging from 0.5 to 50 (% v/v) were prepared in wells in RPMI-1640 with MOPS respectively. After the addition of 0.1 ml of inoculum, the trays were incubated at 25±2 ºC for 7 days. The final essential oils concentrations were ranging from 0.25 to 25.0 (% v/v). Growth controls consisting of RPMI 1640 medium and RPMI–1640 with inoculum were included for each isolate tested.

Supercritical CO_2 extraction

The plants cultivated at the experimental field of the Kaunas Botanical Garden of Vytautas Magnus University, Lithuania, were collected during the vegetation period, botanically identified by Prof. Dr. (HP) O. Ragažinskienė and immediately processed. The following plants species were tested: *Monarda didyma* L., *Lamium album* L., and *Myrrhis odorata* L. Essential oils were obtained by the supercritical carbon dioxide extraction method (Kaškonienė et al., 2011). The plant material for each experiment (0.50 ± 0.01 g) of ground raw material was used for the extraction. Supercritical CO_2 extraction was performed using Hewlett-Packard 7680T (USA) automated supercritical fluid extractor and high purity carbon dioxide 99.5% (UAB AGA, Lithuania). Extractions from the plants were performed at 9.1 and 15.1 MPa pressure (CO_2 density 0.30 and 0.70 g/ml, respectively). Other extraction parameters: static and dynamic extraction times 2 and 15 min; CO_2 flow rate 1 ml/min; extraction chamber temperature 50 ºC; volume of stainless steel extraction vessel 10 ml. The analytes were collected on octadecylsilica sorbent trap (1ml) at 5 ºC; elution of analytes was performed with 0.7 ml 75% methanol at a flow rate 0.7 ml/min at 45 ºC.

Gas chromatographic – mass spectrometric analysis

Quantitative and qualitative analyses of essential oils were carried out using gas chromatograph GC-2010 with mass spectrometric (MS) detector GCMS-QP2010 (Shimadzu, Tokyo, Japan) (Kaškonienė et al., 2011). MS was operated in the electron impact ionization mode 70 eV. The spectra were registered within the mass range *m/z* 30-400. The compounds were separated using an RTX – 5MS column (30m × 0.25 mm i. d. ×0.25 µm film thickness) (Restec, Bellefonte, PA, USA). The aarrier gas helium was adjusted to 1.2 ml/min flow rate. For injection, a split mode was used at a split ratio of 1:10. The injector temperature was 240 ºC. The oven temperature was programmed as follows: initial temperature was maintained at 60 ºC (3 min), raised at

1ºC /min to 70 ºC, raised at 2 ºC/min to 120 ºC, raised at 20 ºC/min to 250 ºC and maintained for 5 min.

For quantitative analysis, three replicates of each sample were run by GC-MS and the results were expressed as a mean.

The components were identified by comparison of their mass spectra with commercial mass spectral libraries (NIST05) and by comparing the data with database reported results (Adams, 2007).

Vapour contact assay

The effect of essential oils on moulds was studied using an invert Petri dishes method RPMI 1640 broth supplemented with L- glutamine, phenol red and MOPS, adjusted to pH 7.0, was mixed with molten 3.0 (w/v) agar in an equal ratio immediately before the assay. The RPMI agar was poured into a 40 mm Petri dish and inoculated with 0.1 ml of spore suspension of each fungal strain tested. 100 µl of each essential oil of various concentrations, ranging from 0.05 to 25.0 v/v, was added on the filter-paper. Filter-paper was placed in the cover of each Petri dish, so that it did not directly touch surface of the agar medium. The plates were sealed with vinyl tape immediately after inoculation, and incubated at 25±5 ºC for 7 days. The control, consisting of RPMI medium, was included. The MIC (%) was determined by comparison with the control and was defined as the lowest concentration of essential oils inhibiting the visible growth.

Statistical analysis. The data were analyzed using the SPSS for Windows (version 12.0) and Microsoft Office Excel® 2010 calculating the mean values and standard deviations. The P- value of 0.05 was set as a limit for statistically significant difference in the studies.

Results and discussion

The literary data about essential oils of *Lamium album* L., *Myrrhis odorata* L. and *Monarda didyma* L. are scarce and the antifungal activity had not been evaluated before. The major components of essential oils of *Lamium album* L., *Myrrhis odorata* L., and *Monarda didyma* L., are reported in Table 1, 2, 3.

Essential oil from *Monarda didyma* L., having phenolic compounds as the major components, was found with the better spectrum of activity against all tested fungi *Aspergillus flavus, Aspergillus niger, Aspergillus ochraceus, Penicillium chrysogenum, Penicillium commune, Penicillium citrinum, Cladosporium cladosporioides, Alternaria alternata, Rhizopus microsporus, and Trichoderma viride.* Using broth dilution methods, the MICs values ranged from 1.0–11.0 (% v/v). Among the tested fungi, essential oil from *Monarda didyma* L., the most susceptible was *Alternaria alternate* and the most resistant were *Penicillium* species. Fungi *Aspergillus flavus, Aspergillus niger, Penicillium chrysogenum,* and *Penicillium commune* showed the lowest susceptibility MIC >25 (%v/v) P>0.05 and were not inhibited by *Lamium album L.* essential oil. Essential oil from *Myrrhis odorata L.,* using broth dilution method, showed MICs values ranging from 4 to 15 (%v/v) P<0.05.

In Table 4, the MICs of the essential oils tested by the two, broth dilution and vapour contact, methods are compared.

Table 1.**Qualitative and quantitative composition of *Lamium album L.* essential oils** *(relative standard deviation did not exceed 10%)*

[a]LRI	[b]Identification	[c]Compound	[d]Peak Area	[e]Area (%)	Match (%)
927	MS, LRI	prenol	2.0	1.52	93
1169	MS,LRI	farnesene-beta- E	2.8	2.16	94
1172	MS,LRI	tridecanol n	3.1	2.32	95
1213	MS,LRI	dodecanoic acid n	2.9	2.24	92
1217	MS,LRI	hexadecane -n	3.6	2.73	87
1221	MS,LRI	squalene	6.56	9.82	85
1223	MS,LRI	tetradecanol -n	27.7	20.77	92
1228	MS,LRI	undecane -n	7.0	5.25	95
1231	MS,LRI	benzoate- isopentyl	20.3	15.24	93
1238	MS,LRI	dodecanoate -butyl	3.4	2.54	94
1259	MS,LRI	phytone	9.7	7.25	97
1295	MS,LRI	neophytadiene	4.4	3.35	95
Total peak area (a.u.)[e]			93.46		

[a]LRI, linear retention index on RTX -5MS column, experimentally determined using homologous series of C_6-C_{24} alkanes. [b]Identification method: MS, by comparison of the mass spectrum with those of the computer mass library Wiley 7 Nist; LRI, by comparison of LRI with those reported in literature. [c]Compounds are listed in order of their elution from a RTX -5MS column. [d]Values expressed in GC peak area/10^6. [e]a.u., arbitrary units.

By the vapour contact method the MICs were lower than those in liquid medium for essential oils tested (Table 4). The effect of essential oils in vapour state was probably attributable to the direct deposition of essential oils on lipophilic fungal mycelia together with an indirect effect via adsorption through the agar medium (Inouye et al. 2000). The essential oils are complex mixtures of many different aromatic components with various degrees of lipophilicity and relative hydrophilicity given by the presence of constituents with polar functional groups (Griffin et al., 1999).

Table 2. **Qualitative and quantitative composition of *Myrrhis odorata* L. essential oils** *(relative standard deviation did not exceed 10%)*

[a]LRI	[b]Identification	[c]Compound	[d]Peak Area	[e]Area (%)	Match (%)
980	MS, LRI	myrcene	1.8	0.38	96
1085	MS, LRI	estragole	3.6	0.76	94
1106	MS, LRI	thymoquinone	0.9	0.2	94
1118	MS, LRI	anethole – (Z)	76.0	15.89	97
1120	MS, LRI	thymol	6.0	1.26	83
1134	MS, LRI	bicyclogermacrene	1.7	0.37	97
1146	MS, LRI	ά- copaene	0.5	0.12	95
1160	MS, LRI	caryophyllene -E	30.4	6.36	94
1162	MS, LRI	cubebene β	1.0	0.22	89
1170	MS, LRI	humulene ά	4.2	0.9	94
1178	MS, LRI	germacrene – (D)	30.9	6.46	95
1182	MS, LRI	bicyclogermacrene	3.8	0.8	98
1185	MS, LRI	bisabolene β	24.2	5.07	93
1189	MS, LRI	cadinene δ	1.2	0.26	87
1200	MS, LRI	nerolidol – (E)	129.7	27.13	97
1204	MS, LRI	spathulenol	7.1	1.5	95
1206	MS, LRI	caryophyllene oxide	19.2	4.02	89
1209	MS, LRI	carotol	1.0	0.23	95
1212	MS, LRI	humulene epoxide	1.9	0.41	94
1239	MS, LRI	oplopanone	1.5	0.33	95
1258	MS, LRI	neophytadiene	25.2	5.28	94
1259	MS, LRI	phytone	1.9	0.41	96
1294	MS, LRI	linoleate methyl	7.6	1.6	97
1295	MS, LRI	phytol	18.2	3.82	98
Total peak area (a.u.)[e]			399.5		
[a,b,c,d,e –] as in Table 1					

Table 3. **Qualitative and quantitative composition of *Monarda didyma L.* essential oils** *(relative standard deviation did not exceed 10%)*

[a]LRI	[b]Identification	[c]Compound	[d]Peak Area	[e]Area (%)	Match (%)
933	MS, LRI	thujene -α	1.9	1.38	96
937	MS, LRI	pinene -α	0.8	0.56	94
971	MS, LRI	vinyl amyl carbinol	9.4	6.54	94
995	MS, LRI	carene delta-3	0.2	0.19	97
1000	MS, LRI	terpinene -α	4.4	3.07	83
1020	MS, LRI	terpinene-gamma	1.3	0.95	97
1023	MS, LRI	sabinene hydrate trans	2.4	1.71	95
1070	MS, LRI	borneol	0.3	0.25	89
1076	MS, LRI	terpinen-4-ol	0.8	0.57	94
1082	MS, LRI	terpineol -α	0.3	0.24	85
1085	MS, LRI	estragole	1.8	1.31	94
1104	MS, LRI	methyl thymol	11.3	7.86	93
1107	MS, LRI	thymoquinone	52.1	36.02	94
1120	MS, LRI	thymol	24.1	16.7	97
1146	MS, LRI	copaene appha-α	0.3	0.21	89
1160	MS, LRI	caryophyllene	1.1	0.81	94
1176	MS, LRI	murolene gamma	0.3	0.24	95
1178	MS, LRI	germacrene	1.7	1.19	94
1189	MS, LRI	cadinene δ	0.4	0.34	93
1198	MS, LRI	thymohydroquinone	2.1	1.47	95
1259	MS, LRI	phytone	0.2	0.19	96
1293	MS, LRI	Hexadecanol - n	0.4	0.3	95
Total peak area (a.u.)[e]			117.6		
[a,b,c,d,e –] as in Table 1					

Table 4. **Essential oils – minimal inhibitory concentration** (MIC) **values for fungi by the broth dilution** (BD) **and vapour contact** (VC) **methods**

Fungi strain	MIC VALUE (% v/v)			
	Method	*Myrrhis odorata L.*	*Lamium album L.**	*Monarda didyma L.*
Aspergillus flavus	BD	7.0	ND	6.0
	VC	5.0	ND	2.0
Aspergillus niger	BD	8.0	ND	6.0
	VC	7.0	ND	2.0
Aspergillus ochraceus	BD	8.0	20.0	4.0
	VC	7.0	18.0	0.5
Penicillium chrysogenum	BD	13.0	ND	11
	VC	8.0	ND	8.0
Penicillium commune	BD	15.0	ND	11.0
	VC	10.0	ND	8.0
Penicillium citrinum	BD	15.0	25.0	11.0
	VC	10.0	23.0	8.0
Cladosporiu cladosporioides	BD	15.0	25.0	9.0
	VC	8.0	10.0	5.0
Alternaria alternata	BD	4.0	15.0	1.0
	VC	2.0	10.0	0.05
Rhizopus microsporus	BD	8.0	15.0	5.0
	VC	5.0	13.0	1.5
Trichoderma viride	BD	14.0	25.0	10.0
	VC	10.0	20.0	5.0
The values are the average of three determinations; ND - not determined; The MIC was not significant (*P > 0.05)				

The essential oils distribute more or less into the aqueous phase depending on its relative hydrophilicity, when added to a medium. Essential oils with constituents of low polarity should dissolve little in aqueous medium, and consequently should show a weak activity. This is the case of *Monarda didyma* L., *Lamium album* L., and *Myrrhis odorata* L. essential oils whose constituents (n-tetradecanol, isopentyl benzoate, anethole, E-caryophyllene, D-germacrene, β bisabolene, E-nerolidol, thymoquinone, thymol) are only slightly soluble in the water, but they are highly soluble in organic solvents, their vapour accumulate in great amounts into agar layers. These essential oils showed very high activity when assayed by VC and this can be related to their high volatility (Inouye et al., 2000).

Lamium album L. essential oils containing ester compounds as the major components showed weaker vapour activity than the essential oil *Monarda didyma* L., in which phenol are the major component. According to these observations, it can be stated that strong antifungal activity of *Monarda didyma* L. essential oil could be attributed to thymol itself or could be a result of synergism of other main components in the oil. The obtained results suggest that the classification of essential oils into groups according their bioactivity based on the functional groups of the major components may be justified. Furthermore, the classification seems to be reasonable, since the inhibitory activity of essential oils has been attributed to their most abundant components (Guynot et al., 2003; Giamperi et al., 2002; Rota et al., 2004).

Conclusions

A preliminary screening of the antifungal activity of *Myrrhis odorata* L. and *Monarda didyma* L. essential oils and their components revealed that essential oils of these plants are useful as control agents. The volatile fractions of these plants possess antifungal activity against various indoor environment fungi. The essential oil from *Lamium album* L., containing the lowest amount of components, showed the weakest antifungal activity. For the practical application of these oils or their separate components as novel fungicides, further studies on the safety for humans and animals as well as development of formulations to improve the efficacy and stability and to reduce the cost are necessary.

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