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Antifungal Efficacy and Convenience of *Krameria lappacea* for the Development of Botanical Fungicides and New Alternatives of Antifungal Treatment

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Abstract: The support of trends in agriculture with limited or restricted use of pesticides is linked to the difficulty of protection against pathogenic and toxigenic fungi. Therefore, it is a great challenge to find alternatives to these dangerous fungi. These alternatives include using safe antifungal plant substances of medicinal or aromatic plants as components of botanical pesticides. Within 69 plant species, only 13 were selected as potentially of interest. However, the species *Krameria lappacea*, whose extraction yield (economic factor) achieved 17.6% and minimum inhibitory concentrations (MIC_{50}) 0.11–1.24 mg mL^{-1}, was found to be enormously advantageous. Extraordinary efficacy on a set of dangerous filamentous fungi, comparable to expensive essential oils or active phenolic compounds, was demonstrated. In the most effective extract fraction, two main substances from the group of neolignans, analogues of kramerixin, were detected by using GC-MS and LC-MS analysis, and their molecular structure was determined. The advantage of *K. lappacea* was discussed on the basis of the mode of action and chemical properties of the detected neolignans. *K. lappacea* could be a suitable source for environmentally friendly preparations, thanks to its high yield in simple extraction, excellent antifungal activity, broad antifungal spectrum, harmlessness, and assumed lower volatility of active compounds.

Keywords: krameria; neolignans; aromatic plants; filamentous fungi; toxigenic fungi; botanical fungicides

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1. Introduction

Modern agriculture, strict inspections of final foods, and environmental protection are linked to the current innovative trends, whereas consumption of synthetic fungicides is restricted and inspected. Very marked changes are currently occurring regarding the support of organic farming with zero tolerance of synthetic pesticides. Even though these trends are becoming more popular on the market and among ordinary consumers worldwide, farming in such a situation is not simple given the higher infection pressure and development of fungal pathogens. Toxigenic and pathogenic fungi currently pose a very serious health risk, mainly due to their ability to produce highly toxic secondary metabolites. Apart from their potential toxigenic and health risks to the consumer, fungi are one of the major factors capable of significantly decreasing the yield and quality of food and agricultural products. In terms of food safety, species of the *Fusarium*, *Penicillium*, and *Aspergillus* genera represent the most significant groups of toxigenic and pathogenic fungi, mainly on account of their worldwide distribution and ability to produce a great majority of the known mycotoxins [1–3]. In addition, the *Aspergillus* and *Fusarium* genera in particular are known to include species which are even able to cause very dangerous systemic human and animal mycoses [4–6]. Pathogenic and toxigenic fungi are mostly controlled by applying synthetic fungicides, but this can be very complicated in many cases due to high toxicity to mammals or other side effects, along with residual persistence. This fact is obvious in the case of antifungal treatment of stored products such as food [7–10]. Treatment of the mentioned

human mycoses can be considered the most problematic and questionable issue. In these cases, synthetic fungicides are often the only means of suppressing the pathogenic fungal species. Antifungal treatment is commonly connected with direct toxicity and various side effects of the synthetic fungicides used [11,12]. In addition, the number of resistant fungal pathogenic and toxigenic fungal species is increasing [13–16]. Therefore, the need for new antifungal substances and alternative treatments is becoming more and more obvious in many areas. One of the most promising and ecologically safe possibilities could be based on taking advantage of a plant's natural antifungal properties. Thanks to long evolution, plants possess an effective defence system, making them the richest natural source of bioactive compounds that could provide natural alternatives to synthetic chemical fungicides. The promising biological activities of many plant extracts or essential oils has recently become a focal point of research dealing with seeking new, environmentally safe botanical fungicides based on plant active substances. Many previous studies have demonstrated promising fungicidal effects [17–20]. Their mild toxicity confirms the correctness of the hypotheses stating the necessity to study the effects of plant extracts on significant toxigenic fungal pathogens. The primary goal of this study was to find the most suitable candidate, among various significant medicinal or aromatic and commercially used plant species, having potential during production of botanical pesticides intended for environmentally friendly inhibition of dangerous and problematic filamentous fungi. This study focuses on significant toxigenic plant and human fungal pathogens, with a primary focus on *Fusarium oxysporum*, *F. verticillioides*, *Penicillium expansum*, *P. brevicompactum*, *Aspergillus flavus,* and *A. fumigatus*. As the supporting selective factor during commercial production of botanical pesticides, the final yield was compared for all 69 tested species of plant candidates. By using targeted experiments, a group of 13 usable species with high antifungal efficacy was selected. From the aspect of yield and, especially, antifungal efficacy, we established the dominance of the significant and commercially valuable species *Krameria lappacea* (Dombey) from Burdet and B.B. Simpson. *K. lappacea* is a slow-growing hemiparasitic shrub reaching a height of up to one meter. Its procumbent branches, growing outward along the ground are covered with little hairs. Branches bear yellowish-white oblong-ovate leaves approximately one centimeter in length [21]. In order to gain more understanding of the origin of the antifungal activity of the extract of *K. lappacea,* chemical analyses of the active fractions and identification of the key antifungal-active compounds were performed by using chromatographic and mass spectrometry methods.

2. Materials and Methods

2.1. Plant Material and Extraction

Fresh plant material from each of the selected species (Table 1) was collected in flowering season. The plant material was shade-dried at 40 °C. Samples were subsequently homogenized by means of cutting mill (CM-1000; Laarmann, Roermond, The Netherlands) into particles with a size of 2–5 mm. The dry powder was extracted with 100% pure methanol (500 mL of MeOH for 100 g of plant powder) for 24 h. The crude extracts were separately filtered and evaporated under reduced pressure in a rotary evaporator (R-200; Büchi, Flawil, Switzerland). The yield was determined by percentage ratio to the dry weight of the original plant material. The crude extracts were stored at 7 °C until further assay.

Table 1. Plants used in this study, their part used, origin, and yield of extracts.

Species	Family	Plant Part Assayed	Yield (%)	Origin
Acanthopanax senticosus (Rupr. & Maxim.) Harms	Araliaceae	Roots	5.4	Cicenice, Czech Republic
Acer campestre L.	Aceraceae	Leaves	9.8	Prague, Czech Republic
Acer capillipes Maxim.	Aceraceae	Leaves	12.5	Prague, Czech Republic
Acer platanoides L.	Aceraceae	Leaves	9.3	Prague, Czech Republic
Achillea ageratum L.	Asteraceae	Stem	11.8	Prague, Czech Republic

Table 1. *Cont.*

Species	Family	Plant Part Assayed	Yield (%)	Origin
Achillea collina Heimerl	Asteraceae	Stem	7.0	Prague, Czech Republic
Achillea nobilis L.	Asteraceae	Stem	10.9	Prague, Czech Republic
Aegopodium podagraria L.	Apiaceae	Stem	5.6	Dobre, Czech Republic
Ajuga chamaepitys (L.) Schreber	Lamiaceae	Stem	14.8	Prague, Czech Republic
Alpinia purpurata K. Schum.	Zingiberaceae	Roots	7.0	Cicenice, Czech Republic
Anethum graveolens L.	Apiaceae	Stem	12.4	Prague, Czech Republic
Angelica archangelica L.	Apiaceae	Fruits	6.1	Cicenice, Czech Republic
Angostura trifoliata (Willd.) T.S.Elias	Rutaceae	Bark	11.7	Cicenice, Czech Republic
Asarum europaeum L.	Arisrolochiaceae	Stem	7.5	Vranov, Czech Republic
Astragalus glycyphyllos L.	Fabaceae	Stem	9.1	Prague, Czech Republic
Bistorta officinalis Delarbre	Polygonaceae	Roots	7.2	Cicenice, Czech Republic
Borago officinalis L.	Boraginaceae	Stem	4.5	Prague, Czech Republic
Buddleja davidii Franch.	Buddlejaceae	Stem	21.0	Prague, Czech Republic
Cinchona officinalis L.	Rubiaceae	Bark	10.4	Cicenice, Czech Republic
Citrus × *sinensis* (L.) Osbeck	Rutaceae	Pericarp	8.3	Cicenice, Czech Republic
Citrus aurantium L.	Rutaceae	Pericarp	5.6	Cicenice, Czech Republic
Citrus bergamia (Risso) Wright & Arn	Rutaceae	Pericarp	9.1	Cicenice, Czech Republic
Daucus carota L.	Apiaceae	Stem	8.1	Prague, Czech Republic
Dracocephalum moldavica L.	Lamiaceae	Stem	12.7	Prague, Czech Republic
Foeniculum vulgare Mill.	Apiaceae	Seeds	5.9	Prague, Czech Republic
Galega officinalis L.	Fabaceae	Stem	14.8	Prague, Czech Republic
Galium sylvaticum L.	Rubiaceae	Stem	5.2	Vranov, Czech Republic
Geum urbanum L.	Rosaceae	Roots	13.6	Cicenice, Czech Republic
Gonolobus condurango Decne.	Apocynaceae	Bark	12.6	Cicenice, Czech Republic
Guaiacum officinale L.	Zygophyllaceae	Xylem	5.3	Cicenice, Czech Republic
Harpagophytum procumbens (Burch.) DC ex Meissn.	Pedaliaceae	Roots	5.2	Cicenice, Czech Republic
Hyssopus seravschanicus (Dub.) Pazij	Lamiaceae	Stem	11.0	Prague, Czech Republic
Inula magnifica Lipsky	Asteraceae	Stem	11.8	Prague, Czech Republic
Krameria lappacea (Dombey) Burdet & B.B.Simpson	Krameriaceae	Roots	17.6	Cicenice, Czech Republic
Lamium argentatum(Smejkal) Henker ex G. H. Loos	Lamiaceae	Stem	15.1	Vranov, Czech Republic
Lathyrus tuberosus L.	Fabaceae	Stem	13.5	Znojmo, Czech Republic
Lavandula angustifolia Mill.	Lamiaceae	Stem	9.4	Prague, Czech Republic
Lavandula canariensis Mill.	Lamiaceae	Stem	6.2	Prague, Czech Republic
Leuzea carthamoides DC.	Asteraceae	Roots	2.3	Cicenice, Czech Republic
Lotus corniculatus L.	Fabaceae	Stem	7.3	Dobre, Czech Republic
Lythrum virgatum L.	Lythraceae	Stem	9.6	Prague, Czech Republic
Melilotus albus Medik.	Fabaceae	Stem	10.7	Dobre, Czech Republic
Mentha arvensis L.	Lamiaceae	Stem	6.2	Prague, Czech Republic
Mentha longifolia (L.) L.	Lamiaceae	Stem	9.2	Prague, Czech Republic
Mentha suaveolens Ehrh.	Lamiaceae	Stem	16.9	Prague, Czech Republic
Nepeta pannonica L.	Lamiaceae	Stem	8.5	Prague, Czech Republic
Ononis arvensis L.	Fabaceae	Stem	12.6	Prague, Czech Republic
Orlaya grandiflora (L.) Hoffm.	Apiaceae	Stem	11.4	Prague, Czech Republic
Picramnia excelsa (Swartz) Planch.	Simaroubaceae	Xylem	10.7	Cicenice, Czech Republic
Plantago lanceolata L.	Plantaginaceae	Stem	18.6	Prague, Czech Republic
Potentilla anserina L.	Rosaceae	Stem	7.3	Prague, Czech Republic
Potentilla fruticosa L.	Rosaceae	Stem	21.6	Prague, Czech Republic
Potentilla hirta L.	Rosaceae	Stem	2.1	Prague, Czech Republic
Potentilla reptans L.	Rosaceae	Stem	12.2	Prague, Czech Republic
Quercus robur L.	Fagaceae	Bark	4.6	Cicenice, Czech Republic
Rhamnus frangula L.	Rhamnaceae	Bark	9.7	Cicenice, Czech Republic
Rheum officinale L.	Polygonaceae	Roots	8.0	Cicenice, Czech Republic
Salix alba L.	Salicaceae	Bark	3.5	Cicenice, Czech Republic
Salvia officinalis L.	Lamiaceae	Stem	14.0	Prague, Czech Republic
Stachys palustris L.	Lamiaceae	Stem	8.0	Prague, Czech Republic
Stachys recta L.	Lamiaceae	Stem	8.3	Prague, Czech Republic
Symphytum officinale L.	Boraginaceae	Roots	7.5	Cicenice, Czech Republic
Tabebuia impetiginosa (Mart. ex DC.) Standl.	Bignoniaceae	bark	11.2	Cicenice, Czech Republic
Tanacetumparthenium (L.) Schlutz-Bip.	Asteraceae	Stem	12.3	Prague, Czech Republic
Teucrium botrys L.	Lamiaceae	Stem	4.6	Prague, Czech Republic
Teucrium capitatum L.	Lamiaceae	Stem	9.7	Prague, Czech Republic
Uncaria tomentosa (Willd. ex Schult.) DC	Rubiaceae	Bark	8.3	Cicenice, Czech Republic
Urtica dioica L.	Urticaceae	Roots	3.6	Cicenice, Czech Republic
Valeriana officinalis L.	Valerianaceae	Roots	11.7	Prague, Czech Republic

2.2. Fungal Strains

All target pathogenic and toxigenic fungal strains were obtained from the collection of pathogenic fungi maintained in the Crop Research Institute (Prague, Czech Republic).

F. oxysporum (MZL/21215) and *F. verticillioides* (MZL/100415) strains were isolated originally from an infected corn-cob, whereas *P. brevicompactum* (MZL/270215), *P. expansum* (MZL/280912), *A. flavus* (LS/25702), and *A. fumigatus* (LS/2206) were isolated from contaminated stored corn. Strains were preserved on slant agar (potato carrot agar) at 4 °C. Subcultivations on Petri dishes and other manipulations with these strains were carried out in the Bio Security Level 2 (BSL 2) laboratory, given the BSL of the *Fusarium* and *Aspergillus* species used in our experiment.

2.3. Experimental Design Used for Determination of Inhibitory Effect

The inhibitory effect of methanol extracts on the growth of fungi was tested by the agar dilution method. Dried plant extracts were dissolved in an equal volume of methanol. The dissolved extracts were properly diluted in potato dextrose agar (PDA) at concentration 2 mg mL^{-1}. The final concentration of the solvent (methanol) in PDA was 0.75% (v/v). The prepared Petri dishes (9.0 cm diameter) were aseptically inoculated with assay disc (0.4 cm) cuts from the periphery of a seven-day-old culture of the target fungi. The control sets were subsequently prepared by using an equal volume of methanol without extracts. Incubation was carried out in the dark at 21 °C for seven days. The percent inhibition of the radial growth of the target fungi was calculated according to the following formula: Percent inhibition = (DC − DT) / DC × 100, where DC is the colony diameter of the control sets and DT is the colony diameter of the treated sets. Extracts whose inhibitory effect on mycelial growth was higher than 50% at the basic concentration 2 mg mL^{-1} were chosen for further testing for evaluation of median inhibitory concentration (MIC_{50}). The value of MIC_{50} was determined by the method of graded concentration of the plant extracts (0.10, 0.25, 0.50, 1.00, 1.50, 2.00 mg mL^{-1}) in the PDA. Cultivation was carried out in the same way as before (in the dark at 21 °C, for 7 days). The MIC_{50} was regarded as the concentration of plant extract that results in a 50% inhibition of visible growth when compared with control sets [17,22]. The fungicide propiconazole (high purity grade-Pestanal® from Sigma Aldrich, St. Louis, MO, USA) was used as a reference compound.

2.4. Statistical Analysis

Probit analysis was applied to assess the MIC_{50} values for each effective compound associated with 95% confidence limits (CI^{95}) [23]. The EPA Probit Analysis Program (Version 1.5) was used for statistical evaluation. The MIC values were statistically calculated and associated with Chi square values significant at the $p < 0.05$ level. MIC_{50} were assessed for each extract showing a basic fungal growth inhibitory effect higher than 50% at the basic concentration of 2 mg/mL. The Welch's T-test was used for the most effective extracts.

2.5. Purification and Preparation of K. lappacea Extract Fractions

For the purposes of the purification and separation of a large amount of extract, silica gel column chromatography was utilised. A silica gel (Merck *Silica gel* 60, 70–230 mesh ASTM) column (50 cm × 4 cm diameter) was prepared. A sample of crude *K. lappacea* extract was then loaded with a Pasteur pipette. The column with sample was then washed with dichloromethane: methanol mobile phase. Different fractions for bioassays were obtained with a step gradient (from 100:0 v/v up to 80:20 v/v) [24,25]. The obtained fractions were then evaporated to dryness and preserved at 4 °C until the bioassays. The inhibitory effect of the acquired fractions was tested by using the same method used to test the original extracts mentioned above. Fractions demonstrating the highest antifungal activity were subsequently examined by using GC/MS and LC/MS analytical methods.

2.6. Derivatization

The aliquots of obtained extracts were evaporated to dryness in 100 µL ethylacetate-acetone (95:5, *v*/*v*). Derivatization of extracts was performed by using 0.5 mL of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA): trimethylsilane (TMS) (99:1, *v*/*v*) at 70 °C. Samples were then cooled to room temperature, the derivatization agent was removed by using a nitrogen stream, and samples were analyzed by using GC/MS.

2.7. GC/MS Analysis

GC/MS analyses were performed by using a Scion SQ gas chromatogram with MS detection equipped with a CP-8400 autosampler and MS workstation 8.0 software (Bruker, Bremen, Germany). Separations of non-derivatized and derivatized extracts were separately performed by using an Rxi-5ms column (30 m × 0.25 µm, 0.25 mm ID; Restek, Bellefonte, PA, USA). Helium 5.0 (99.999% purity; Linde, Prague, Czech Republic) was used as the carrier gas with the constant flow 1 mL/min. The injector was operated in split/splitless mode, with the splitless time 1 min. The injector temperature was 240 °C and the EI source, transferline and manifold temperatures were 250, 280, and 50 °C, respectively. The GC oven temperature program started from 60 °C (for 1 min), then heated up to 120 °C at 25 °C/min, and finally to 240 °C at 2.5 °C/min, and was held isothermally for 28 min. The injection volume was 1 µL.

The mass spectra were recorded at 3 scans/min under electron impact 70 eV. For qualitative analysis, the full scan mode (50–750 amu) was used. Mass spectrometry workstation software (8.0, Bruker, Germany) equipped with the NIST 08 library was used for verification of structure identity.

2.8. UHPLC-ToFMS Analysis

UHPLC-ToF analyses were performed by using a Waters Acquity UPLC System (Waters; Prague, Czech Republic) consisting of Acquity UPLC Sample Manager, Acquity UPLC Solvent Manager, Acquity UPLC Column Heater and Waters LCT Premier XE orthogonal accelerated ToFMS (Water MS; Manchester, UK). MassLynx V4.0 software was used for data processing.

For the ionization of analytes, an ESI interface was employed (operating in the positive ion mode) by using the following parameters: cone voltage, 40 V; capillary voltage, +2800 V; ion source block temperature, 120 °C; nitrogen desolvation gas temperature, 350 °C; desolvation gas flow, 800 L/h; cone gas flow, 50 L/h. Full scan spectra were acquired in the range of 100–1000 m/z, with a scan time of 0.15 s and an interscan delay of 0.01 s. Mass accuracy was maintained by lock spray by using leucine-enkephalin (5 ng/µL; 5 µL/min).

The aliquots of obtained extracts were evaporated to dryness and reconstituted in acetonitrile-acetone (95:5, *v*/*v*). Analytes were separated on an Acquity UPLC C18 column (50 mm × 2.1 mm × 1.7 µm) with the mobile phase consisting of (A) formic acid-water (0.1:99.9, *v*/*v*), and (B) formic acid-acetonitrile (0.1:99.9, *v*/*v*). A linear gradient elution program was employed as follows (min/%B): 0/5; 15/80; 18/99 followed by 1.5 min step with 100% B and 2.0 min equilibration step. The mobile phase flow rate was 0.4 mL/min, the column temperature was 40 °C and the injection volume was 5 µL.

For verification of the compounds' identity, the parameters set for the Elementary Composition editor were: mass measurement, 5 mDa; i-FIT (norm) error, 5; CHNO algorithm.

3. Results

The observed percentage yield of extracts from individual plant species of aromatic and other medicinal plants is listed in Table 1. The lowest percentage yield of the extract was in the case of the species *P. hirta* with a value of 2.1%. The highest yield of 21.6% was measured in the species *P. fruticosa. K. lappacea* showed extract yield of 17.6%.

The inhibitory effects of all 69 different plant methanolic extracts on the mycelial growth of target pathogenic and toxigenic fungi are listed in Table 2.

Table 2. Inhibition effect of plant extracts on pathogenic and toxinogenic fungi at concentration 2 mg mL^{-1}.

Plant Extract	% Inhibition of Target Fungi (Mean ± SE)					
	Fusarium oxysporum	***Fusarium verticillioides***	***Penicillium brevicompactum***	***Penicillium expansum***	***Aspergillus flavus***	***Aspergillus fumigatus***
Acanthopanax senticosus	31.22 ± 0.05	33.33 ± 0.08	12.20 ± 0.08	22.64 ± 0.09	13.54 ± 0.05	44.44 ± 0.05
Acer campestre	13.82 ± 0.09	17.43 ± 0.00	−8.16 ± 0.05	5.66 ± 0.05	4.71 ± 0.14	5.26 ± 0.00
Acer capillipes	24.39 ± 0.00	18.35 ± 0.05	−4.08 ± 0.00	9.43 ± 0.00	8.24 ± 0.00	28.07 ± 0.05
Acer platanoides	12.20 ± 0.00	14.68 ± 0.00	−4.08 ± 0.00	9.43 ± 0.00	0.00 ± 0.05	5.26 ± 0.00
Achillea ageratum	35.34 ± 0.00	27.59 ± 0.00	20.00 ± 0.00	32.08 ± 0.00	32.22 ± 0.05	51.72 ± 0.05
Achillea collina	19.83 ± 0.00	20.69 ± 0.00	11.11 ± 0.05	35.85 ± 0.05	23.33 ± 0.00	27.59 ± 0.00
Achillea nobilis	22.41 ± 0.00	17.24 ± 0.00	6.67 ± 0.00	26.42 ± 0.00	26.67 ± 0.00	41.38 ± 0.05
Aegopodium podagraria	0.00 ± 0.05	−5.75 ± 0.05	−2.22 ± 0.05	13.21 ± 0.05	15.56 ± 0.05	31.03 ± 0.05
Ajuga chamaepitys	−1.72 ± 0.05	−12.64 ± 0.09	15.56 ± 0.05	18.87 ± 0.05	17.78 ± 0.05	18.97 ± 0.05
Alpinia purpurata	76.19 ± 0.00	65.93 ± 0.17	21.95 ± 0.09	52.83 ± 0.05	48.96 ± 0.05	72.73 ± 0.00
Anethum graveolens	22.41 ± 0.00	27.59 ± 0.00	20.00 ± 0.00	26.42 ± 0.00	16.67 ± 0.08	15.52 ± 0.05
Angelica archangelica	82.01 ± 0.05	73.33 ± 0.00	48.78 ± 0.00	64.71 ± 0.09	50.00 ± 0.00	88.89 ± 0.05
Angostura trifoliata	58.20 ± 0.05	46.67 ± 0.00	48.78 ± 0.00	37.74 ± 0.00	16.67 ± 0.19	63.54 ± 0.05
Asarum europaeum	63.43 ± 0.05	60.75 ± 0.00	83.33 ± 0.05	55.10 ± 0.05	42.86 ± 0.00	57.81 ± 0.00
Astragalus glycyphyllos	18.70 ± 0.24	22.94 ± 0.00	0.00 ± 0.05	7.55 ± 0.05	18.82 ± 0.00	10.53 ± 0.00
Bistorta officinalis	23.81 ± 0.00	11.85 ± 0.05	−9.76 ± 0.00	11.32 ± 0.05	−33.33 ± 0.05	2.02 ± 0.24
Borago officinalis	3.25 ± 0.09	14.68 ± 0.00	18.37 ± 0.12	5.66 ± 0.05	−3.53 ± 0.05	8.77 ± 0.05
Buddleja davidii	12.20 ± 0.00	21.10 ± 0.05	−6.12 ± 0.05	9.43 ± 0.00	8.24 ± 0.00	12.28 ± 0.09
Cinchona officinalis	23.28 ± 0.05	11.11 ± 0.14	24.39 ± 0.05	15.09 ± 0.00	2.08 ± 0.33	−26.26 ± 0.05
Citrus × *sinensis*	17.99 ± 0.09	12.59 ± 0.12	−17.07 ± 0.00	3.77 ± 0.00	8.33 ± 0.05	21.21 ± 0.00
Citrus aurantium	12.17 ± 0.05	14.07 ± 0.05	−14.63 ± 0.05	15.09 ± 0.00	2.08 ± 0.05	6.06 ± 0.00
Citrus bergamia	14.81 ± 0.09	16.30 ± 0.09	−14.63 ± 0.05	20.75 ± 0.00	−1.04 ± 0.05	26.26 ± 0.05
Daucus carota	21.55 ± 0.05	13.79 ± 0.14	8.89 ± 0.05	24.53 ± 0.05	33.33 ± 0.00	34.48 ± 0.09
Dracocephalum moldavica	21.95 ± 0.00	16.51 ± 0.05	2.04 ± 0.00	9.43 ± 0.00	10.59 ± 0.05	3.51 ± 0.05
Foeniculum vulgare	65.85 ± 0.00	60.55 ± 0.05	97.96 ± 0.05	69.81 ± 0.05	78.95 ± 0.00	94.74 ± 0.00
Galega officinalis	24.39 ± 0.00	22.94 ± 0.00	−4.08 ± 0.00	5.66 ± 0.05	8.24 ± 0.00	−7.02 ± 0.05
Galium sylvaticum	10.45 ± 0.00	22.43 ± 0.05	14.58 ± 0.09	20.41 ± 0.00	23.81 ± 0.09	23.44 ± 0.05
Geum urbanum	20.63 ± 0.14	9.63 ± 0.09	17.07 ± 0.05	18.87 ± 0.09	−10.42 ± 0.05	8.08 ± 0.05
Gonolobus condurango	26.98 ± 0.00	22.22 ± 0.14	2.44 ± 0.05	28.30 ± 0.05	−10.42 ± 0.05	24.24 ± 0.00
Guaiacum officinale	64.02 ± 0.05	47.41 ± 0.05	19.51 ± 0.00	20.75 ± 0.00	3.13 ± 0.00	51.52 ± 0.00
Harpagophytum procumbens	21.69 ± 0.05	13.33 ± 0.08	0.00 ± 0.09	24.53 ± 0.05	1.04 ± 0.05	23.23 ± 0.09
Hyssopus seravschanicus	21.55 ± 0.05	18.39 ± 0.09	13.33 ± 0.00	26.42 ± 0.00	26.67 ± 0.00	24.14 ± 0.05
Inula magnifica	35.77 ± 0.05	32.11 ± 0.05	16.33 ± 0.05	16.98 ± 0.05	24.71 ± 0.05	49.93 ± 0.00
Krameria lappacea	87.30 ± 0.00	90.37 ± 0.05	63.41 ± 0.00	52.83 ± 0.05	69.38 ± 0.00	91.92 ± 0.00
Lamium argentatum	12.07 ± 0.08	2.30 ± 0.09	6.67 ± 0.00	20.75 ± 0.00	13.33 ± 0.00	17.24 ± 0.00
Lathyrus tuberosus	−2.99 ± 0.00	6.54 ± 0.09	12.50 ± 0.08	20.41 ± 0.08	10.71 ± 0.00	20.31 ± 0.00
Lavandula angustifolia	19.51 ± 0.14	31.19 ± 0.00	8.16 ± 0.00	−9.43 ± 0.09	18.82 ± 0.00	7.02 ± 0.05
Lavandula canariensis	14.66 ± 0.00	−6.90 ± 0.00	−13.33 ± 0.00	32.08 ± 0.00	23.33 ± 0.00	17.24 ± 0.00
Leuzea carthamoides	55.03 ± 0.09	37.78 ± 0.14	78.05 ± 0.00	69.81 ± 0.05	18.75 ± 0.00	37.37 ± 0.05
Lotus corniculatus	8.62 ± 0.05	−2.30 ± 0.12	−13.33 ± 0.00	9.43 ± 0.00	12.22 ± 0.05	25.86 ± 0.05
Lythrum virgatum	6.50 ± 0.05	12.84 ± 0.05	−4.08 ± 0.00	0.00 ± 0.05	8.24 ± 0.00	−5.26 ± 0.14
Melilotus albus	6.90 ± 0.00	−2.30 ± 0.09	−4.44 ± 0.05	18.87 ± 0.05	23.33 ± 0.00	37.93 ± 0.00
Mentha arvensis	51.72 ± 0.09	51.72 ± 0.00	57.78 ± 0.05	66.04 ± 0.00	32.22 ± 0.05	55.17 ± 0.09
Mentha longifolia	32.76 ± 0.00	13.79 ± 0.00	46.67 ± 0.00	58.49 ± 0.05	30.00 ± 0.00	72.41 ± 0.05
Mentha suaveolens	25.86 ± 0.09	18.39 ± 0.09	46.67 ± 0.00	54.72 ± 0.00	32.22 ± 0.05	56.90 ± 0.05
Nepeta pannonica	31.90 ± 0.09	10.34 ± 0.00	26.67 ± 0.00	45.28 ± 0.05	33.33 ± 0.00	32.76 ± 0.00
Ononis arvensis	15.52 ± 0.05	12.64 ± 0.05	6.67 ± 0.00	41.51 ± 0.05	27.78 ± 0.05	51.72 ± 0.12
Orlaya grandiflora	29.27 ± 0.08	28.44 ± 0.00	8.16 ± 0.00	18.87 ± 0.05	17.65 ± 0.05	31.58 ± 0.00
Picramnia excelsa	20.11 ± 0.25	5.93 ± 0.09	−2.44 ± 0.00	5.66 ± 0.09	−12.50 ± 0.14	18.18 ± 0.08
Plantago lanceolata	−6.90 ± 0.05	−14.94 ± 0.05	0.00 ± 0.00	20.75 ± 0.00	21.11 ± 0.47	17.24 ± 0.00
Potentilla anserina	−8.94 ± 0.17	5.50 ± 0.24	−2.04 ± 0.05	16.98 ± 0.09	18.82 ± 0.00	7.02 ± 0.05
Potentilla fruticosa	−3.25 ± 0.05	11.01 ± 0.09	0.00 ± 0.05	−1.89 ± 0.00	21.18 ± 0.05	12.28 ± 0.09
Potentilla hirta	−3.25 ± 0.05	12.84 ± 0.05	−4.08 ± 0.00	9.43 ± 0.00	18.82 ± 0.00	8.77 ± 0.19
Potentilla reptans	13.82 ± 0.05	7.34 ± 0.09	6.12 ± 0.05	5.66 ± 0.09	16.47 ± 0.19	12.28 ± 0.09
Quercus robur	17.99 ± 0.09	2.22 ± 0.08	12.20 ± 0.00	5.66 ± 0.05	0.00 ± 0.00	15.15 ± 0.00
Rhamnus frangula	36.51 ± 0.00	4.44 ± 0.00	19.51 ± 0.00	30.19 ± 0.05	−13.54 ± 0.05	9.09 ± 0.00
Rheum officinale	46.56 ± 0.05	30.37 ± 0.05	2.44 ± 0.12	37.74 ± 0.00	12.50 ± 0.00	27.27 ± 0.14
Salix alba	6.35 ± 0.08	−0.74 ± 0.09	−12.20 ± 0.05	7.55 ± 0.05	−18.75 ± 0.14	2.02 ± 0.09
Salvia officinalis	60.34 ± 0.05	47.13 ± 0.05	46.67 ± 0.00	33.96 ± 0.05	35.56 ± 0.05	82.76 ± 0.05
Stachys palustris	13.82 ± 0.05	12.84 ± 0.09	−2.04 ± 0.05	9.43 ± 0.00	1.18 ± 0.14	7.02 ± 0.09

Table 2. *Cont.*

Plant Extract	% Inhibition of Target Fungi (Mean ± SE)					
	Fusarium oxysporum	***Fusarium verticillioides***	***Penicillium brevicompactum***	***Penicillium expansum***	***Aspergillus flavus***	***Aspergillus fumigatus***
Stachys recta	10.34 ± 0.09	1.15 ± 0.05	−6.67 ± 0.00	18.87 ± 0.09	16.67 ± 0.00	8.62 ± 0.09
Symphytum officinale	0.00 ± 0.00	−4.44 ± 0.00	2.44 ± 0.05	24.53 ± 0.05	−14.58 ± 0.09	−24.24 ± 0.36
Tabebuia impetiginosa	50.79 ± 0.00	34.81 ± 0.09	26.83 ± 0.00	37.74 ± 0.00	−3.12 ± 0.00	36.36 ± 0.00
Tanacethum parthenium	30.17 ± 0.00	33.33 ± 0.05	20.00 ± 0.00	35.85 ± 0.05	30.00 ± 0.00	36.21 ± 0.05
Teucrium botrys	28.46 ± 0.05	28.44 ± 0.00	−8.16 ± 0.52	13.21 ± 0.05	7.06 ± 0.05	19.30 ± 0.09
Teucrium capitatum	25.20 ± 0.05	30.28 ± 0.05	4.08 ± 0.05	11.32 ± 0.05	11.76 ± 0.00	15.79 ± 0.00
Uncaria tomentosa	11.64 ± 0.05	5.19 ± 0.05	−4.88 ± 0.05	13.21 ± 0.05	−26.04 ± 0.09	6.06 ± 0.00
Urtica dioica	22.75 ± 0.09	−2.22 ± 0.49	29.27 ± 0.09	35.85 ± 0.05	−28.13 ± 0.00	13.13 ± 0.05
Valeriana officinalis	48.78 ± 0.00	28.44 ± 0.00	34.69 ± 0.05	37.74 ± 0.00	34.12 ± 0.09	43.86 ± 0.09

The results showed that all 69 plant extracts influenced fungal growth. In the end, 13 of them were evaluated as sufficiently effective. Methanolic extracts obtained from *Achillea ageratum*, *Alpinia purpurata*, *Angelica archangelica*, *Angostura trifoliata*, *Asarum europaeum*, *Foeniculum vulgare*, *Guaiacum officinale*, *Krameria lappacea*, *Leuzea carthamoides*, *Mentha arvensis*, *Mentha longifolia*, *Mentha suaveolens,* and *Tabebuia impetiginosa* exerted a growth inhibition ratio higher than 50% against the mycelial growth of target fungal species, at least in the case of one target fungal species, at the basic experimental concentration of 2 mg mL^{-1}. These 13 extracts were chosen for subsequent experiments to assignment of their MIC_{50} values. The MIC_{50} values are presented in Table 3.

Table 3. Medium inhibitory concentration of the most effective plant extracts against target fungal species (mg mL^{-1}).

Plant Species	Target Fungal Species					
	Fusarium oxysporum	***Fusarium verticillioides***	***Penicillium brevicompactum***	***Penicillium expansum***	***Aspergillus flavus***	***Aspergillus fumigatus***
	MIC_{50} (CI^{95}) [a] Chi [b]	**MIC_{50} (CI^{95}) [a] Chi [b]**	**MIC_{50} (CI^{95}) [a] Chi [b]**	**MIC_{50} (CI^{95}) [a] Chi [b]**	**MIC_{50} (CI^{95}) [a] Chi [b]**	**MIC_{50} (CI^{95}) [a] Chi [b]**
Achillea ageratum	>2	>2	>2	>2	>2	0.96 (0.75–1.50) 2.226
Alpinia purpurata	0.30 (0.24–0.38) 3.177	0.84 (0.61–1.07) 5.019	>2	0.74 (0.44–2.27) 1.615	>2	0.17 (0.13–0.20) 0.962
Angelica archangelica	0.20 (0.15–0.29) 1.579	0.17 (0.12–0.21) 0.401	>2	0.28 (0.19–0.44) 2.219	>2	0.39 (0.26–0.50) 1.487
Angostura trifoliata	1.76 (1.36–2.98) 2.251	>2	>2	>2	>2	0.29 (0.21–0.45) 1.173
Asarum europaeum	1.68 (1.01–5.05) 2.078	1.87 (1.53–2.31) 1.790	1.58 (1.03–1.73) 0.612	1.78 (1.57–2.22) 1.337	>2	1.85 (1.73–2.05) 0.868
Foeniculum vulgare	1.44 (1.27–1.67) 0.165	1.22 (1.01–1.50) 1.517	0.71 (0.63–0.79) 0.594	1.14 (0.98–1.34) 4.846	0.89 (0.49–1.10) 1.625	0.27 (0.23–0.32) 2.716
Guaiacum officinale	0.84 (0.62–1.57) 2.743	>2	>2	>2	>2	0.44 (0.34–0.72) 0.842
Krameria lappacea	0.14 (0.07–0.19) 2.190	0.12 (0.07–0.16) 0.506	0.38 (0.30–0.49) 1.317	1.24 (0.74–1.61) 3.019	0.25 (0.20–0.33) 2.175	0.11 (0.08–0.14) 0.190
Leuzea carthamoides	1.98 (1.63–2.31) 0.656	>2 2.916	1.46 (1.22–1.86) 0.491	1.32 (1.13–1.54) 1.291	>2	>2
Mentha arvensis	1.99 (1.66–2.62) 3.532	1.89 (1.60–2.82) 1.725	1.85 (1.69–2.14) 2.785	1.84 (1.66–2.14) 0.113	>2	1.86 (1.53–2.50) 1.053
Mentha longifolia	>2	>2	>2	0.82 (0.60–1.23) 0.468	>2	0.92 (0.76–1.22) 3.472
Mentha suaveolens	>2	>2	>2	0.66 (0.44–1.74) 2.941	>2	1.27 (1.03–1.69) 0.030
Tabebuia impetiginosa	1.60 (1.13–1.85) 3.150	>2	>2	>2	>2	>2
Propiconazole *	0.69 (0.47–0.93) * 0.534	0.52 (0.35–0.69) * 0.361	0.75 (0.52–1.01) * 3.339	0.53 (0.42–0.63) * 1.001	3.16 (2.19–5.23) * 1.531	0.49 (0.40–0.59) * 1.827

* Fungicide reference standard (μg/mL). [a] Median inhibitory concentration (MIC_{50}) with 95% confidence intervals. [b] Chi-square value, significant at $p < 0.05$ level.

K. lappacea and *F.vulgare* were the only species with the efficacy against all target fungal species. *K. lappachea* was then evaluated as a significantly higher effective by using the Welch's T-test comparison. The difference in fungal inhibition between *K. lappachea* (mean = 0.37; SD = 0.437) and *F. vulgare* (mean = 0.95; SD = 0.418) was significant ($t(10) = 2.3166$; $p = 0.4307$). On the basis of a statistical comparison of MIC_{50} values, the species *K. lappacea* was rated the most effective, not only in regard to the broadness of the spectrum of inhibited species of fungi, but chiefly because it had the highest inhibitory efficacy, which is represented by the lowest MIC_{50} values. In the case of the very dangerous species *A. fumigatus*, a value of 0.11 mg mL^{-1} was statistically proven.

Separation of the extract of *K. lappacea* into individual fractions by using the silica gel column chromatography method enabled selection and separation of the antifungally inactive or less active fractions from fractions with extreme antifungal activity (Table 4).

Of a total of seven fractions differing in the polarity of the contained substances, two were identified whose inhibitory effect far exceeded the 50% level in a concentration of just 0.5 mg mL^{-1}. Specific 3% and 5% fractions achieved a significantly higher inhibitory effect, ranging between 55.7% and 95.5% at this concentration. The abovementioned inhibitory effect naturally differed depending on the varying sensitivity of the target pathogens. Other fractions were not effective across the entire spectrum of target fungi, or did not demonstrate any antifungal activity on any pathogen. In the 3% fraction, the most abundant compound was identified as the methylated form of deoxykramexin (MW 264; trimethylsililated derivate MW 336) (Figure 1). GC/MS analysis of the significantly effective 5% fraction revealed a high abundance of the methylated form of dihydrogen deoxykramerixin (MW 266; trimethylsililated derivate MW 338) (Figure 2). The molecular properties were also obtained by using LC/MS analysis.

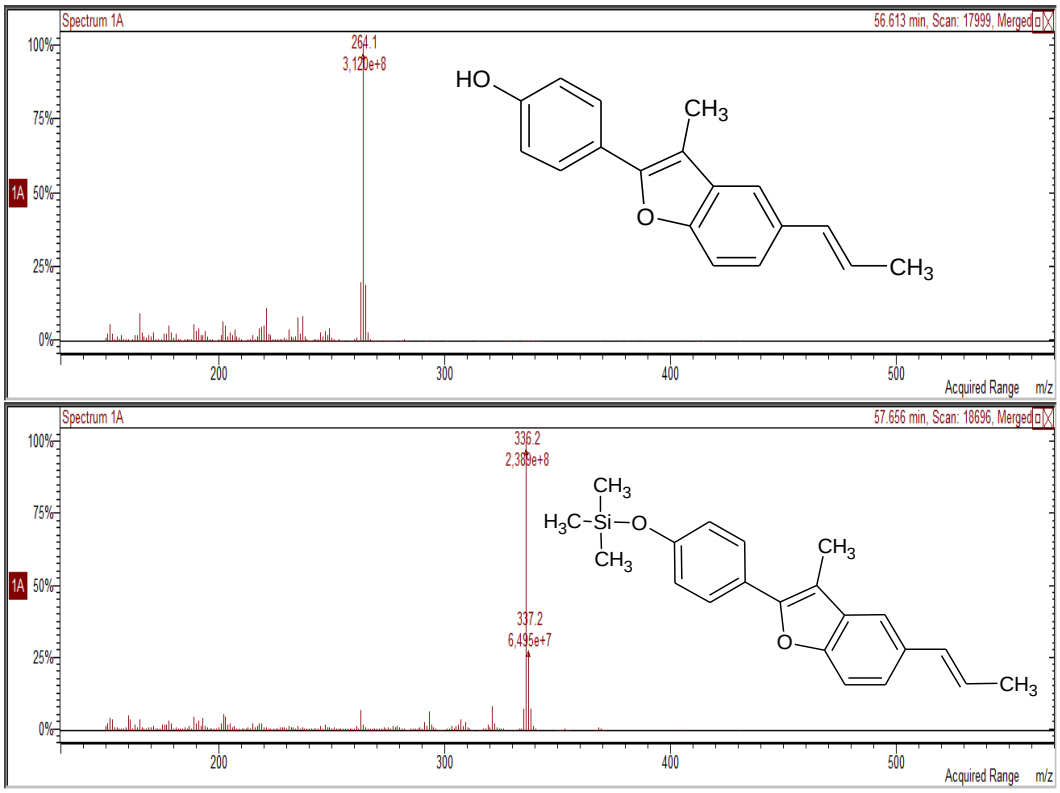

Figure 1. MS analysis of majority peak in 3% fraction 56.61 min, m/z 264 and 57.65 min, m/z 336 (trimethylsililated) corresponding to methylated form of deoxy-kramerixin.

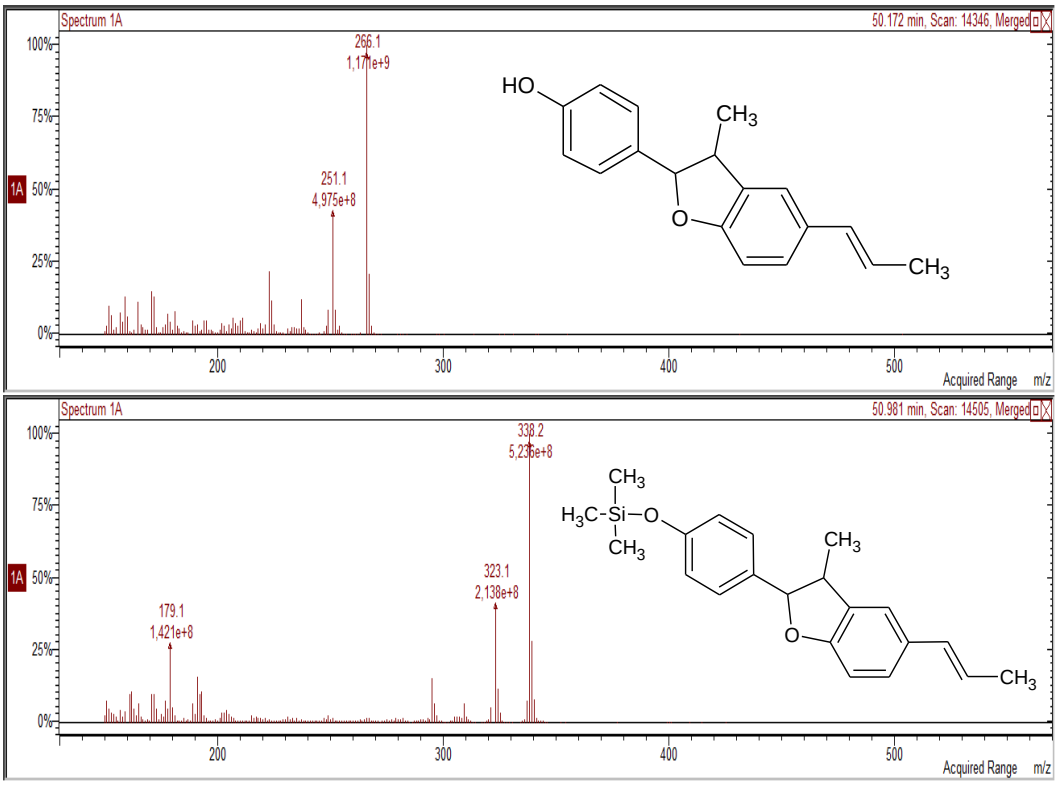

Figure 2. MS analysis of majority peak in 5% fraction 50.172 min, *m*/*z* 266 and 50.98 min, *m*/*z* 338 (trimethylsililated) corresponding to methylated form of dihydrogen-deoxy-kramerixin.

Table 4. Inhibition effect of *K. lapachea* extract fractions on pathogenic and toxinogenic fungi at concentration 0.5 mg mL^{-1}.

Column Fraction MeOH (%)	% Inhibition of Target Fungi (Mean ± SE)					
	Fusarium oxysporum	*Fusarium verticillioides*	*Penicillium brevicompactum*	*Penicillium expansum*	*Aspergillus flavus*	*Aspergillus fumigatus*
0.1%	NI	NI	NI	NI	NI	NI
0.5%	NI	NI	NI	NI	NI	NI
1%	13.2 ± 0.05	18.7 ± 0.05	12.6 ± 0.01	NI	NI	23.9 ± 0.05
3%	86.9 ± 0.10	84.2 ± 0.00	74.5 ± 0.00	69.3 ± 0.05	70.0 ± 0.05	95.5 ± 0.00
5%	85.0 ± 0.05	95.2 ± 0.00	79.5 ± 0.00	63.1 ± 0.00	55.7 ± 0.10	93.0 ± 0.00
10%	9.0 ± 0.00	7.9 ± 0.00	NI	NI	NI	16.8 ± 0.05
20%	NI	NI	NI	NI	NI	NI

NI—No Inhibition.

4. Discussion

Target fungal species were inhibited in the case of *K. lappacea* in very similar extremely low concentrations. Nevertheless, such significant efficacy is achieved, for instance, only by the most effective and mostly expensive essential oils or several antifungal phenolic compounds [18,22]. The percentage yield during the extraction process was also the highest in the case of *K. lappacea* compared to the aforementioned effective species (Tables 1 and 3). In relation to plants from the *Krameria* family, the most frequently mentioned is the antifungally effective kramerixin, which has significant antifungal effects and whose efficacy is comparable to that of the problematic synthetic fungicide amphotericin, which is frequently used for medical purposes [26–29]. The anti-microbially very potent kramerixin and its molecular analogues are members of the group of neolignans [30,31]. On the basis of our findings, it is clear that the antifungal efficacy of the examined extract from the root of *Krameria lappacea* is most likely not caused by kramerixin, but rather by its methylated forms. In the case of the most antifungally effective fractions, these substances were de-

tected as the substances with the greatest abundance. These majority substances have not often been described from the aspect of their antifungal efficacy. The efficacy of kramerixin and its analogues was chiefly investigated mostly against yeast fungi and not against more resistant filamentous fungi. Similar modifications of kramerixin, including the use of synthetic methods, are being intensively investigated during the search for new active substances for medical purposes as well [26,32]. However, antifungal efficacy against the complex of filamentous pathogenic and toxigenic fungi, as in our study, has seldom been mentioned in previous literature. Standard antifungals primarily act against the ergosterol component of cellular membranes, ergosterol synthesis or the mechanism of inhibition of RNA [33]. The mode of action of neolignans, such as the present analogues of kramerixin, is different, which broadens their potential in the field of antifungal treatment. This concerns blockage of 1,3-β glucan-synthase and chitin synthase and is thereby targeted at important components specific for fungi [26,34]. It therefore fulfils one factor for utilisation and does not target components occurring in higher-animal cells. In our study, the high efficacy against hygienically, agriculturally, and medically important species of the resistant filamentous species *Aspergillus, Fusarium,* and *Penicillium* was demonstrated. Obviously, individual inhibition levels based on MIC values of plant extracts were influenced also by sensitivity of the target fungi. The same effect was observed for example in other studies of different antifungals against a similar spectrum of fungal species. It was found that most of the efficient substances exhibited the highest efficacy against *A. fumigatus*. On the contrary, most substances in the mentioned studies exhibited the least efficacy against *A. flavus.* In certain cases, efficacies against *A. flavus* even several-fold lower than in the other target fungi [17,18,35] were found. The described difference in the sensitivity of the individual species of filamentous fungi used in the study was also similarly observed in the case of synthetic fungicide propiconazole used in this study as a reference compound. These species were selected on the basis of how extremely dangerous and problematic they are in practice, specifically in agriculture, in the protection of agricultural products and during the production of safe foods and other products. It must be mentioned again that some target species in this study are also dangerous human pathogens [36,37]. The extract from the root of *K. lappacea* offers the advantage of high antifungal efficacy and harmlessness to human health, because it is also significantly used in traditional medicine, chiefly in South America [38,39]. Various parts of *K. lappacea* are described to be used during stomach ailments, diarrhoea and inflammation of the oropharynx. Examples of its use for strengthening and protecting the teeth and against oral ulcers, bleeding and inflammation of the oral cavity, are also known [40–43]. This plant is currently included in the European pharmacopoeia. The photo-protective and antioxidant effects of this plant have also been described [44]. The stability and non-volatility of the antifungally active substances in the extract of *K. lappacea* represent other practical advantages compared to the frequently discussed essential oils with their active phenolic substances, such as thymol, carvacrol or eugenol [18,22]. Due to their high volatility, their efficacy quickly falls after application, which is a limiting factor [35,45]. The increasing support for eco-friendly trends in agriculture and the production of harmless agricultural products and foods is linked to limitation or strict restriction of synthetic pesticides. It is therefore necessary to find new alternative methods of protection against harmful pathogenic and toxigenic filamentous fungi. Substances and extracts from plants are one of the promising and markedly popular sources. In our study, we assessed and compared the yield and antifungal efficacy of many potential plant species. On the basis of the data we acquired about yield and the level of antifungal efficacy, it is possible to consider the species *K. lappacea* an especially suitable candidate for the purposes of natural antifungal protection. This is supported by the very high extraction yield compared to biomass, the broad antifungal spectrum, and high level of efficacy against dangerous filamentous fungi. Furthermore, the nature of the key detected antifungal substances of the extract reinforces the idea of safe and advantageous use in inhibiting the occurrence of dangerous filamentous fungi.

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