In vitro activity of the cinnamon essential oil against the plant pathogen *Septoria melissae* desm.

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Abstract: *Septoria* leafspot caused by the mitosporic fungus *Septoria melissae* Desm. is the most significant disease of lemon balm (*Melissa officinalis* L.). The fungus frequently appears in the plantations and causes serious yield losses or decreases the quality of the drug. At present plant protection of lemon balm is inadequate due to the lack of authorised plant protection products. The essential oil of the cinnamon bark (CEO) has a strong antimicrobial activity proved by several *in vitro* experiments. Therefore the goal of our work was to test antifungal effect of the CEO against the pathogen *Septoria melissae* Desm.

In vitro tests were carried out with three concentrations (0.3%, 0.1%, 0.03%) of the CEO against a Hungarian and a Polish isolate of the fungus. Inhibition of the germination of the conidia as well as the mycelial radial growth was investigated. Supplementary tests were carried out with colonies of the fungi transferred to growth media without CEO after an incubation period of 14 and 35 days on media containing CEO.

Our results showed that all the applied concentrations of CEO have very strong (98.07-100%) inhibitory effect on the mycelial growth of both isolates. Germination of conidia was also blocked on each medium containing CEO. However, the effect of the CEO at 0.03% concentration was reversible. Mycelium began to grow again on media without CEO after transfer.

Based on the results, further investigation of CEO as a potential plant protection product in lemon balm crops is recommended.

Keywords: *Septoria* leafspot, lemon balm, growth inhibition, environmental friendly, medicinal plants

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Introduction

Septoria leafspot caused by the mitosporic fungus *Septoria melissae* Desm. is the most important disease of lemon balm (*Melissa officinalis* L.) (Nagy et Horváth, 2010; Jadczak et Pizoń, 2017; Wielgusz et Seidler-Łożykowska, 2017). This pathogen frequently appears in lemon balm crops and causes serious yield losses expressed by the severe leaf fall as a consequence of the lack of proper pest management. Furthermore, even a moderate infection may highly influence the quality of the drug by decreasing the essential oil (EO) content and modifying the rate of the main compounds (Aulerio et al., 1995; Kowalska et al., 2014). At present, the plant protection of lemon balm is inadequate, due to the strict regulation of the maximum residue levels allowed in herbal products (Kowalska et al., 2014; Bernáth et Zámbori-Németh, 2015). In Hungary, only a few plant protection products are authorised in this crop which cannot provide reliable control of the pathogens (Ocskó et al. 2017).

In the light of the facts mentioned above, the development of new and environmental friendly methods for the protection of lemon balm is essential. EOs are potential substances for this purpose. The antimicrobial activity of these compounds has been confirmed by several *in vitro* and *in vivo* investigations in the past decades. EO of the cinnamom bark (CEO) is one of the most highly investigated EOs in the field of food science. The results of an *in vitro* experiment of Ju et al. (2018) demonstrated the efficacy of the CEO against *Penicillium* and *Aspergillus* species. The results of López et al. (2007) indicate, that *trans*-cinnamaldehyde, the main compound of this oil might be responsible for its antimicrobial activity. Another *in vitro* experiment of Feyaerts et al. (2018) carried out with 175 EOs confirmed the previous

observation. The authors' results showed, that the EOs which contain high amount of aldehydes, had the highest inhibitory effect in vapour phase.

Field trials were carried out by Hochbaum et Nagy (2013) with the combination of the EOs of *Thymus vulgaris* and *Cinnamomum zeylanicum.* Spraying a mixture of the EOs significantly decreased the disease incidence of *Monilinia* blossom blight (*Monilinia laxa*) of apricot and leafcurl (*Taphrina deformans*) of peach in the applied 0.05% and 0.1% concentrations, respectively. The wide spectrum of antimicrobial activity of CEO was also demonstrated by a small plot trial of Kovács et al. (2013). The application of this EO gave the best result against the *Fusarium* head blight (*Fusarium graminearum*, *F. culmorum*) of winter wheat on artificially inoculated plants.

In medicinal plant production only few data are available (e.g. Kovács et Nagy (2014)) about the use of EOs as plant protection product. Therefore, the goal of our recent study was to test the efficacy of the EO of *C. zeylanicum* bark *(Aetheroleum cinnamomi zeylanici corticis)* against the most important pathogen of lemon balm: *S. melissae* Desm.

Materials and methods

Characterisation of the applied essential oil

The investigated CEO was purchased from a commercial company (Aromax Zrt., Hungary). The chemical composition was determined by gas chromatography mass spectrometric method, using an Agilent Technologies 6890N instrument equipped with HP-5MS capillary column (30 m × 0.25 mm i.d. × 0.25 μm) and an Agilent Technologies MS 5975 inert mass selective detector. The temperature program was the following: initial temperature 60°C, then by a rate of 3°C/min up to 240°C; the final temperature was kept for 5 min. Carrier gas was helium (1 mL min−1), injector and detector temperatures were 250°C. Split ratio: 30:1. Injected quantity: 0.2 μl (solvent: n-hexane). The percentage composition of the EO was computed from the GC peak areas. Ionization energy was 70 eV. The MS were recorded in full scan mode that revealed the total ion current (TIC) chromatograms (mass range m/z 50–550 uma). The components were identified by linear retention indices that were calculated using the generalized equation of Van Den Dool and Dec. Kratz (1963), and by mass spectra by using NIST MS Search 2.0 library and Adams mass spectra library (Adams, 2007).

According to the GC-MS analysis, the applied EO contains *trans*-cinnamaldehyde in 78.8% (*Table 1.*). Other components of the oil in higher amounts were the followings: *trans*-cinnamyl-acetate (3.6%), *trans*-anetol (2.8%), linalool (2.8%) and eugenol (1.3%).

Origin and maintenance of fungal isolates

Two isolates of *S. melissae* Desm. were used in our experiment. Target pathogens were

Table 1. Chemical composition of the applied cinnamon essential oil according to the GC-MS analysis

Compound	RT	LRI	%
α-Pinene	5.56	938.000	0.6
Benzaldehyde (artificial almond)	6.28	967.000	0.2
ß-Pinene	6.64	980.856	0.1
p-Cimol (para)	8.09	1025.856	1.4
Limonene	8.19	1028.529	1.5
1,8-Cineole	8.38	1033.503	2.3
Linalool	10.76	1097.246	2.8
α-Terpineol	14.55	1189.123	1.2
cis-Cinnamaldehyde	15.73	1217.000	0.1
trans-Cinnamaldehyde	17.72	1264.000	78.8
trans-Anetol	18.51	1283.175	2.8
Eugenol	21.44	1360.822	1.3
α-Copaene	22.03	1376.986	0.04
ß-Caryophyllene	23.68	1419.951	2.5
trans-Cinnamyl acetate	24.80	1448.000	3.6
α-Humulene	25.07	1454.187	0.5
orto-Methoxy Cinnamaldehyde	28.24	1536.000	0.1
Benzyl benzoate	36.83	1773.296	0.1
Total			99.9

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isolated from infected leaves of lemon balm on malt extract agar (MEA). The leaves were collected at Budapest-Soroksár (47°24'08.7"N 19°09'03.9"E) in Hungary (HBS) and at Warsaw-Wilanów (52°09'36.9"N 21°06'08.2"E) in Poland (PWW). The fungal isolates have been maintained on malt extract agar (MEA) at 24°C without light.

Experimental methods to test the antimicrobial properties

Inhibitory effect of CEO on both the *germination of conidia* and the *mycelial radial growth* were tested by the following method.

For the testing of the effect of CEO on the *germination of conidia*, suspension of conidia was prepared by suspending conidial exudates developed on the surface of monosporic cultures of the isolate PWW in distilled water. Concentration of the suspension was determined by haemocytometer. The amount of conidia in the suspension was approximately 1.6×10^6 conidia/ml. Preparation of media carried out according to the followings. CEO was evenly diluted in MEA at the 0.3%; 0.1% and 0.03% concentrations in Petri dishes. Silwet Star wetting agent in 0.02% was also applied in each treatment to improve homogeneity. Conidia growing on medium containing no CEO served as control. Besides, MEA mixed with the wetting agent were also tested. 30µl of conidial suspension was spread evenly on the surface of the media in two replications. The number of germinated conidia was counted 24 hours after inoculation on ten randomly chosen 10^6 µm^2 area of the media. Conidium was considered germinated if measured length of the germ tube was longer than the length of the conidium. Ratio of the germinated and non germinated conidia was expressed in percentage.

For the testing of the effect of CEO on the *mycelial radial growth* preparation of media and the treatments were carried out in the same way as described above. 31 days old monosporic cultures of the slow growing fungus *S. melissae* Desm. were used to obtain adequate amount of inoculum. Mycelial fragments from the margin of the cultures of both origin, HBS and PWW, were placed onto previously prepared media aseptically. The experiment was set up in 10 replicates. The radial growth of mycelium was determined by measuring the diameter of the colonies 0 and 14 days after inoculation. The ratio of inhibition was calculated by the following formula: $PI=([C_A-C_S]-[T_A-T_S])/(C_A-C_S)\times100$, where C_S is the area of the colonies on intact medium at the time of inoculation, C_A is the area of the colonies on intact medium on day 14 after inoculation, T_S is the area of colonies on the treated medium at the day of inoculation and T_A is the size of the culture on the treated medium on day 14 after inoculation. Growth inhibition was expressed in percentage comparing the growth rate of CEO treated colonies with the control ones.

The *survival rate of the treated cultures* was investigated with HBS isolates. After the maintenance of 14 and 35 days of the cultures on medium containing 0.3%, 0.1% and 0.03% CEO, mycelial fragments were placed onto media containing no CEO. The mycelial radial growth of these cultures was measured on the 3rd, 8th and 14th days after transfer. The mycelial growth was calculated by the following formula: $MReg=S_A-S_S$, where S_S is the area of the colonies on intact medium at the time of transfer and S_A is the area of the colonies on intact medium at the time of the assessment. The results of the calculations were expressed in mm^2.

Statistical analysis

Data were analysed by the IBM SPSS Statistics 22 software. Univariate ANOVA (ANOVA) was used to evaluate the significant differences among the inhibitory effect of the investigated CEO in different dilutions. Multivariate ANOVA (MANOVA) was used to detect the significant differences between the growth rates of the transferred cultures during survival rate test. The normality of the residuals was tested according to the Saphiro-Wilk and Kolgomorov-Smirnov tests. If the normality could not be justified by the mentioned analyses, it was verified by the skewness and the kurtosis. Homogeneity of variances was tested by the Levene's method. If the homogeneity assumption was not violated, Tukey *post hoc* test was used to group the genotypes. Otherwise the separation was made by the Games-Howell test.

Table 2. The rate of inhibition of mycelial radial growth of the two *Septoria melissae* Desm. isolates by cinnamon essential oil (CEO) (Legends: The abc letters refer to the significantly different groups according to the ANOVA test)

Treatment	Isolate	
	HBS	PWW
CEO 0.3%	97.68% a	100.00% a
CEO 0.1%	98.07% a	100.00% a
CEO 0.03%	99.65% a	100.00% a
Silwet Star	15.38% b	1.01% b

Table 3. The effect of cinnamon essential oil (CEO) treatments on the germination of conidia of the PWW isolate

Treatment	Conidia	
	germinated	not germinated
CEO 0.3%	0%	100%
CEO 0.1%	0%	100%
CEO 0.03%	0%	100%
Silwet Star	100%	0%
Control	100%	0%

Results and discussion

Inhibitory effect on the germination of conidia and on the mycelial radial growth

Conidia of the PWW isolate did not germinate on any of the treated medium containing CEO (*Table 3.*; *Figure 1.*). The Silwet Star wetting agent did not influence the germination of the conidia.

The CEO achieved a powerful inhibitory effect on the *mycelial growth* of both isolates of *S. melissae* Desm. (*Table 2.*) as well. All applied concentrations showed approximately 100% growth inhibition. Differences among the concentrations were not significant according to statistical analysis. The applied wetting agent showed a very low growth inhibition (1.01-15.38%) of the mycelia of both isolates.

Information about the inhibitory effect of the CEO against *Septoria* species was not found in the international literature. The effect of other EOs (thyme, fennel, rosemary, etc.) on the related fungus *Septoria tritici* (syn.: *Zymoseptoria tritici*) was investigated by Matusinsky et al. (2015). The EO of thyme inhibited mycelial growth by 100%. The strong antifungal activity of CEO was demonstrated by several authors, although against organisms other than *Septoria* spp. In the *in vitro* experiment of Kovács et al. (2013) CEO showed 100% inhibition of the mycelial growth of *Fusarium graminearum*. Another trial carried out with the pure compound *trans*-cinnamaldehyde (the main component of CEO) revealed a strong inhibitory effect on the mycelial growth of both *Aspergillus flavus* (100% inhibition) and *Penicillium islandicum* (91% inhibiton) (López et al., 2007).

Survival rate of the treated cultures

Although a good inhibitory activity of CEO was detected in each applied concentrations, survival rate trials showed that the fungal mycelium was not completely destroyed in all treatments. The transferred mycelial fragments from colonies grown for 14 days on MEA containing 0.03% CEO began to grow on MEA without CEO. The measured growth intensity of these colonies was

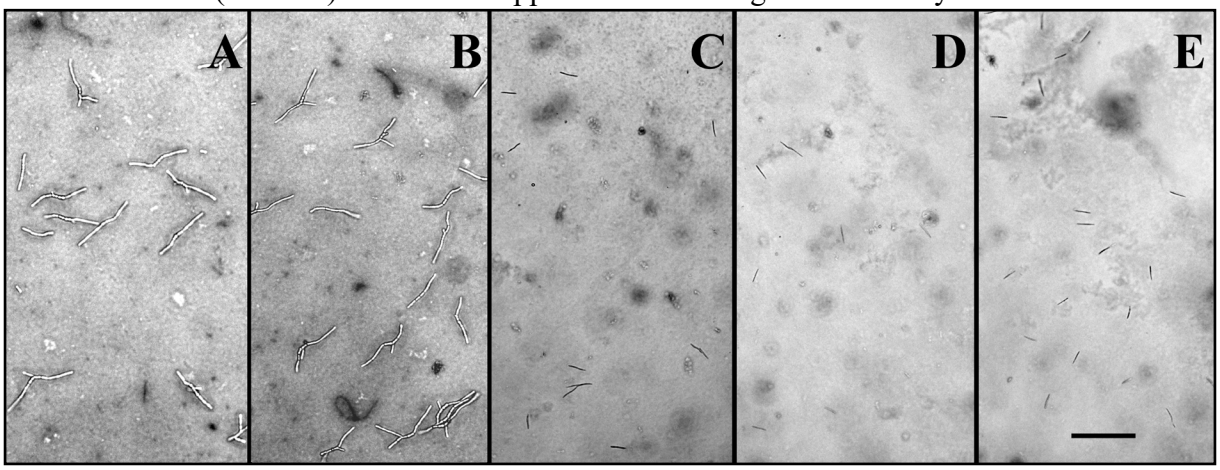

Figure 1. Inhibition of the germination of conidia by cinnamon essential oil (CEO) (Legends: Surface of the media in the following treatments: A – control; B – Silwet Star 0.02%; C – CEO 0.03%; D – CEO 0.1%; E – CEO 0.3%. Scale 100 μm)

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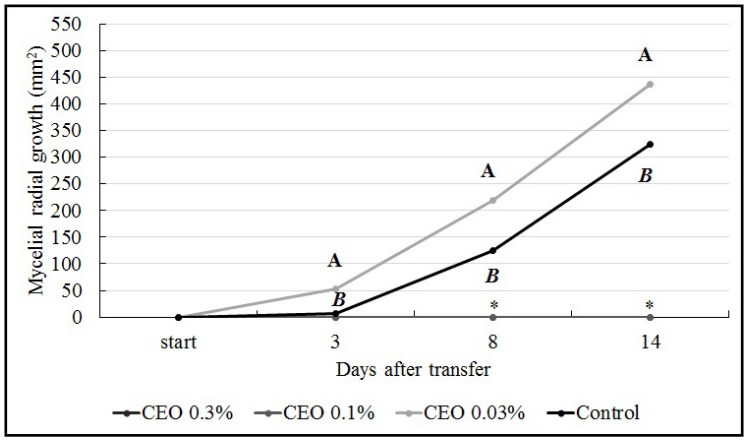

Figure 2. Mycelial radial growth of HBS colonies on MEA transferred after 14 days incubation on CEO containing MEA (Legends: The ABC letters means the significantly different groups in one time according to the MANOVA test. Normal letters belongs to the CEO 0.03% and italic letters to the control. The stars sign the CEO 0.3% and CEO 0.1% treatments where mycelial growth was not observed).

significantly higher than that of the control ones (*Figure 2.*). After a longer incubation period (up to 35 days) on MEA containing 0.03% CEO only a few colonies, which were transferred to MEA without CEO started to grow with very different intensity. This part of the experiment was not evaluated statistically. Colonies transferred from MEA containing higher CEO concentrations (0.1% and 0.3%) were not able to grow on MEA without CEO.

Conclusion

Our experiment showed that the investigated CEO had a powerful *in vitro* inhibitory effect on conidial germination and the mycelial growth of both *S. melissae* Desm. isolates. In accordance with the results of the *in vitro* experiment of Kovács et al. (2013) carried out with the wheat pathogen *Fusarium graminearum*, CEO had approximately 100% growth inhibition even in the lowest (0.03%) concentration. However, in our experiment this CEO concentration caused only a reversible inhibition and was not lethal to the mycelium of *S.melissae* Desm. Treated isolates managed to grow on MEA with no CEO after transfer.

According to Gutiérrez et al. (2010) EOs break the integrity of the cytoplasmic membrane, which leads to the collapse of fungal cell. CEO might have the same effect on the conidial cell or on the membrane of the germ tube of *S. melissae* Desm.

Based on our results CEO applied in 0.1% and 0.3% concentrations can be a potentially efficient plant protection product used for the control of *S. melissae* Desm. However, the practical application needs further investigations including *in vivo* trials.

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