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ANTIFUNGAL ACTIVITY OF ESSENTIAL OILS FROM TWO VARIETIES OF SWEET BASIL (*OCIMUM BASILICUM* L.)

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Summary

The aim of this work was an evaluation of the antifungal activity of essential oils from two cultivars of sweet basil (*Ocimum basilicum* L.): 'Wala' and 'Fine Verde'. The essential oils from dried, ground herbs were extracted with the hydro-distillation method using Deryng apparatus. The composition of the oils was analyzed by gas chromatography. The antifungal activity of the oils was tested against the following strains: *Alternaria* sp., *Aspergillus flavus*, *Botrytis cinerea*, *Cladosporium herbarum*, *Eurotium amstelodami* and *Eurotium chevalieri*. The disc diffusion agar method was applied. Each oil was used in two doses, 10 and 5 µl/disc. A disc (6 mm dia.), soaked with oil, was placed on agar plates, which had been previously inoculated with spores of the different strains. The diameters of fungal growth inhibition zones were measured after 72h of incubation at 25°C and expressed in millimeters. The obtained results showed that the diameter of the mycelium growth inhibition zone significantly depended on the source of oil, the dose of oil, and the species of fungi. The essential oil from the 'Wala' variety was more effective against the tested fungi than the oil from 'Fine Verde' and the inhibition zones were greater when the dose of the oils was higher. *E. chevalieri* was the most sensitive to both of the oils and *A. flavus* was the most resistant. The differences in the antifungal activity of the tested oils were probably the result of their different chemical compositions. In comparison with the 'Fine Verde' oil, the oil from 'Wala' had more than twice the amount of linalool, more 1,8-cineole, and contained geraniol and methyl chavicol. In the conditions of the experiment both oils showed greater antifungal activity than cycloheximide (actidion) and methyl thiophanate (Topsin M 500 SC) used as controls.

key words: sweet basil, essential oil, antifungal activity

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INTRODUCTION

Synthetic pesticides are usually applied to control pests and diseases of agricultural food commodities. However, indiscriminate use has resulted in several problems such as pest resistance to pesticides, toxic residues in food (causing health hazards), pollution of water and soil, and disruption of the eco-system (Shahi *et al.* 2003). Natural plant products are an alternative to the use of chemical substances because they are safer and eco-friendly (Pawar & Thaker 2006). Essential oils are one of the most promising groups of vegetal compounds for the development of natural antifungal and antibacterial agents (Feng & Zheng 2007).

Sweet basil (*Ocimum basilicum*) essential oil contains biologically active constituents, belonging mainly to monotherpens and phenylpropanoids. Out of the phenoyl components, eugenol, methyl eugenol, chavicol, estragol, methyl-cinnamate are often constituents of basil oil. They are combined with various amounts of monotherpene derivatives such as mainly linalool, but also geraniol, 1,8-cineole and limonene (Labra *et al.* 2004). However, the composition of sweet basil oil (absence or presence, and the amount of particular components) shows significant differences depending on the chemotype and origin of basil varieties (Telci *et al.* 2006). The genotype, plant development stage and environmental conditions also influence the chemical composition of basil oil (Kalemba & Kunicka 2003, Seidler-Łożykowska & Król 2008, Seidler-Łożykowska *et al.* 2008).

Studies conducted earlier proved the high activity of sweet basil oil against pathogenic bacteria: *Staphylococcus aureus*, *Escherichia coli*, *Yersinia enerocolitica*, *Shigella* sp., *Pseudomonas* sp., *Enterococcus* sp. (Ozcan & Erkmen 2001, Opalchenova & Obreshkova 2003, Bagamboula *et al.* 2004, Moreira *et al.* 2005). It was also stated that basil oil effectively inhibited growth of fungi such as: *Fusarium verticillioides* (Fandohan *et al.* 2004), *Aspergillus ochraceus* (Basilico & Basilico 1999), *Apergillus niger* (Pawar & Thaker 2006), *Eurotium* sp. (Guynot *et al.* 2003). However, results of some studies indicate low antifungal activity of basil oil (Ozcan & Erkmen 2001).

The majority of previous studies described antifungal properties of sweet basil oil without considering the variety of basil from which the oil was extracted. The aim of this work was an evaluation of antifungal activity of essential oils from two varieties of sweet basil (*Ocimum basilicum* L.). The results of this study indicate which variety is a source of the oil that has a stronger effect and thus is more likely to be used as a natural fungicide.

MATERIALS AND METHODS

The above-ground part of two varieties of sweet basil (*Ocimum basilicum* L.) - 'Wala' and 'Fine Verde', was the experimental material. The plants were cultivated in 2008 at the Vegetable Experimental Station of West Pomeranian University of Technology in Szczecin, Poland. They were collected at the beginning of flowering and then sorted and dried at

room temperature. Essential oils were extracted from the dried, ground material with the hydro-distillation method in Deryng apparatus (PN-ISO 6571). The hydro-distillation was carried out while maintaining the ratio of 1:10 (w/v) between the dried plant matter and the distillated water. After steam distillation (3h), the oils were isolated and dried over anhydrous sodium sulphate. Before the test, they were stored at 4°C.

The composition of the oils was analyzed by gas chromatography using Varian 4000 GC/MS/MS apparatus. The conditions of the analysis were as follows: temperature - 50°C (held for 1 min.), gradually increased (4°C/min.) to 250°C (held for 10 min.), column - VF-5ms, carrier gas - helium, flow rate 0.5 ml/min., injector - temperature 250°C, split ratio 1:100, injection - 1µl of sample, detector - Vatran 4000 MS/MS, Kovat's retention indices were calculated on the basis of n-alkenes series (C_6-C_{40}).

The antifungal activity of the oils was tested against six strains: *Alternaria* sp., *Aspergillus flavus, Botrytis cinerea, Cladosporium herbarum, Eurotium amstelodami* and *Eurotium chevalieri.* The fungi were isolated from plant material and identified according to the rules given by Samson *et al.* (1996). Before the test, stock cultures of the genus *Eurotium* strains were maintained on GC 18 Agar (Guynot *et al*. 2003) and the other strains on Malt Extract Agar [MEA] (Matan *et al.* 2006) at 4°C. The sensitivity of the strains to the essential oils was determined with the disc diffusion agar method (Pawar & Thaker 2006). 20 ml of warm medium (MEA or GC 18 Agar) was poured into 90 mm Petri dishes. After solidification, 100µl of fungal spores suspension were spread over the agar plates. The suspensions of spores had been prepared using colonies of the tested fungi which had been incubated at 25°C for 7 days. Each colony was flooded with 5 ml of sterile peptone water with 0.05% of Tween 80. The suspension was adjusted to the concentration of 10^6 CFU·ml^{-1} by diluting with sterile peptone water. A sterile paper disc (6 mm in diameter) was soaked with oil and placed on the surface of inoculated agar plates. Each oil was used in two doses - 10 and 5 µl/disc. The inoculated plates with oil-soaked discs were incubated at 25°C for 72 hours. After that time, the diameters of growth inhibition zones (including the 6 mm disc diameter) were measured and expressed in millimeters. Sterile distillated water, cycloheximide (actidion) and methyl thiophanate (Topsin M 500 SC) 30µg/disc, were used as controls.

The experiment was performed in triplicate and mean values were calculated. The obtained results were statistically analysed with a 3-way analysis of variance (factor I - basil variety = kind of oil, factor II - dose of oil, factor III - species of fungi). Significant differences between means were assessed using Tuckey's test at a significance level of P=0.05.

RESULTS AND DISCUSSION

It has already been stated that the essential oils from both varieties of basil inhibited the growth of the fungi they were tested against. The differences between the diameters of inhibition zones were significant and de-

pended on the basil variety (source of oil), the dose of oil and the strain of fungi. Interaction between these factors also had a significant influence on the size of the fungal growth inhibition zones (Table 1).

Table 1. Mean diameter (mm) of fungal growth inhibition zones as affected by the kind of oil, the dose of oil and the strains of fungi

Source of oil	Dose of oil	*Alternaria sp.*	*A. flavus*	*B. cinerea*	*C. herbarum*	*E. amstelodami*	*E. chevalieri*	Mean
'Wala' variety	10µl	45.0	7.5	56.0	90.0	90.0	90.0	63.1
	5µl	12.5	6.0	33.0	30.0	32.0	90.0	33.9
'Fine Verde' variety	10µl	34.5	8.5	44.0	43.0	32.0	90.0	42.0
	5µl	12.5	6.0	10.0	25.0	21.0	35.0	18.6
Mean for variety (factor I)	Wala	28.8	6.8	44.5	60.0	61.0	90.0	48.5
	Fine Verde	23.5	7.3	27.0	34.0	26.5	62.5	30.1
Mean for oil dose (factor II)	10 µl	39.8	8.0	50.0	66.5	61.0	90.0	52.5
	5 µl	12.5	6.0	21.5	27.5	26.5	62.5	26.1
Mean for strain (factor III)		26.1	7.0	35.8	47.0	43.8	76.2	39.3
$LSD_{0.05}$ for:								
I – basil variety		0.274	$LSD_{0.05}$ for interaction:		IxII 0.341			
II – dose of oil		0.241			IxIII 0.819			
III – strain		0.579			IIxIII 0.820			

Inhibition zones for controls:
- Topsin M 500 SC: *B. cinerea* (15 mm), *C. herbarum* (21.7 mm), *E. amstelodami* and *E. chevalieri* (20 mm)
- actidion: *Alternaria sp.* (14 mm), *B. cinerea* (21.7 mm), *E. amstelodami* (14.5 mm), *E. chevalieri* (12 mm)

The essential oil from the 'Wala' variety was generally more effective than the 'Fine Verde' oil. For the doses of 10 and 5 µl/disc of 'Wala' oil the mean diameters of growth inhibition zones for all the tested fungi were 63.1 and 33.9 mm, respectively, and for the 'Fine Verde' oil those zones measured 42 and 18.3 mm. However, both oils inhibited to the same degree the growth of *E. chevalieri* (at 10 µl/disc) and *Alternaria* sp. (at 5 µl/disc). The activity of the oil from the 'Wala' variety against the tested fungi in a decreasing order of effectiveness was as follows: *E. chevalieri, E. amstelodami, C. herbarum, B. cinerea, Alternaria* sp., *A. flavus.* In the case of the 'Fine Verde' oil, that order was different: *E. chevalieri, C. herbarum. B. cinerea, E. amstelodami, Alternaria* sp., *A. flavus.* These results show that out of the tested strains, *E. chevalieri* was the most sensitive to both of the oils (mean inhibition zone diameters for 'Wala' and 'Fine Verde' oils were 90 and 62.5 mm, respectively), while *A. flavus* was the most re-

sistant to them (the average diameters of inhibition zones were 6.8 and 7.3 mm, respectively). In effect, neither of the tested essential oils inhibited the growth of *A. flavus*. In the conditions of the experiment, both oils had a higher antifungal activity than cycloheximide and methyl thiophanate used as controls.

The zones of mycelium growth inhibition were generally greater when the dose of the oils was higher. The mean size of the growth inhibition zone for the tested fungi at a dose of 10 µl/disc was approximately twice as big as that at 5 µl/disc (the mean diameters were respectively 52.5 and 26.1 mm). However, a comparison of the individual results shows that the differences between the diameters of the inhibition zones as affected by the dose of oil were not always proportional. For example, the 'Fine Verde' oil at a dose of 10 µl/disc was four times stronger in inhibiting the growth of *B. cinerea* mycelium than it was at a dose of 5 µl/disc. On the other hand, the oil from the 'Wala' variety, regardless of the dose, completely inhibited the growth of *E. chevalieri* (the inhibition zone diameter was 90 mm).

The different levels of antifungal activity of the tested oils could have been caused by their chemical constituents (Table 2). Linalool dominated in the oils extracted from both varieties of sweet basil, but its content in the 'Wala' oil was more than twice as high (69%) as in the 'Fine Verde oil (32.29%). The oil extracted from 'Wala' basil had also more 1,8-cineole (5.53%) and was distinguished by the presence of geraniol (5.06%) and methyl chavicol (0.57%), which were not found in the 'Fine Verde' oil. On the other hand, the 'Fine Verde' oil was characterised by a higher content of mainly α-trans-bergamotene (13.13%) and also germacrene D, terpinen-4-ol, α-bulnesene and bicyclogermacrene. In this oil, in comparison with the 'Wala' oil, the presence of eugenol (6.07%) and methyl eugenol (4.11%) was confirmed. The γ-cadine-ne content was similar in both oils.

Table 2. Chemical composition of 'Wala' and 'Fine Verde' essential oils

Component	Retention time (min.)	Content (%)	
		'Wala' oil	'Fine Verde' oil
linalool	14.18	69	33.29
1,8-cineole	11.72	5.53	4.42
α-trans-bergamotene	25.52	0.77	13.13
geraniol	19.56	5.06	-
eugenol	23.12	-*	6.07
epi-α-cadinol	31.94	4.52	5.43
γ-cadinene	28.09	2.90	2.91
germacrene D	27.12	1.93	4.55
methyl eugenol	24,66	-	4.11
terpinen-4-ol	17.17	0.61	2.86
α-bulnesene	27.73	0.68	2.32
bicyclogermacrene	27.57	0.67	1.92
methyl chavicol	17.96	0.57	-

* - absent

The results obtained in this experiment correspond with previous studies which showed significant antifungal properties of basil essential oil (Basilico & Basilico 1999, Fandohan *et al*. 2004, Pawar & Thaker 2006, Atanda *et al*. 2007, Zhang *et al.* 2009). However, there is some information in the literature on the low activity of basil oil against certain fungi. Ozcan & Erkmen (2001) stated that basil oil in the concentration of 1, 10 and 15% (in a medium) did not inhibit the growth of *Aspergillus niger* and *Rhizopus oryzae.* Guynot *et al.* (2003) evaluated antifungal activity of basil oil through vapour contact and found that the oil was ineffective against *Aspargillus flavus* and *Aspergillus niger*. The results of the work presented here also confirm the resistance of *A. flavus* to basil oil. A strain of *A. flavus* demonstrated a high resistance to cinnamon and clove oils as well (Matan *et al*. 2006).

The results of the present work proved the sensitivity of the strains from the genus *Eurotium* to basil oil, especially to that extracted from the 'Wala' variety. In our experiment, *E. chevalieri* was more sensitive than *E. amstelodami*. Moreover, Guynot *et al*. (2003) stated that out of four strains they tested: *E. amstelodami, E. herbarum, E. repens* and *E. rubrum,* the least sensitive to basil oil were *E. amstelodami* and *E. rubrum,* whle the most sensitive was *E. repens.* The authors emphasized that the fungi belonging to the genus *Eurotium* could grow in products which were characterized by a relatively low level of moisture. Therefore, their growth could decrease the quality of stored seeds.

Previous studies have proved that the activity of essential oils depends on both the properties of the oils and the sensitivity of microorganisms, although the mechanisms of these two factors are not entirely known as yet. Within a species, particular strains of fungi may have different sensitivity to oils (Kalemba & Kunicka 2003). For practical use of natural plant components as botanical pesticides it is important to choose the "oil – microorganism" pair and to choose the concentration (dose) of oil or other herbal derivatives – extracts or hydrosols (Ozcan & Boyraz 2000, Boyraz & Ozcan 2006). Usually, a higher antimycological activity of an oil is recorded while its bigger dose is applied, which is confirmed also by the results of the present experiment. However, our results show that the application of a double dose of oil did not always cause twice as high inhibition of the growth of a given strain. A similar lack of relationship was observed by Singh *et al.* (2006) while examining the antifungal effectiveness of the volatile oil extracted from fennel (*Foeniculum vulgare* L.), applied in concentrations of 2, 4 and 6 μl/disc. Results of other studies also indicate that a lower dose of oil can be more effective. For example, basil oil used against *Aspergillus ochraceus* at a dose of 500 ppm, after 14 days of incubation had higher effectiveness than at 1000 ppm (Basilico & Basilico 1999). Also, basil hydrosol at a 5% concentration inhibited the growth of *Botrytis cinerea* and *Alternaria citri* more than at the doses of 10 and 15% (Boyraz & Ozcan 2005).

A composition of an essential oil and its antimicrobial activity depend on

numerous factors, including the plant species and variety (Kalemba & Kunicka 2003, Labra *et al*. 2004, Runyoro *et al.* 2010). The results of this study indicate that the tested oils, extracted from the green matter of 'Wala' and 'Fine Verde' basil cultivars, varied in chemical composition and, consequently, in their antifungal activity. Linalool was the predominant component of both oils, but its amounts varied a lot. Linalool was also among the main components of oils extracted from various varieties of sweet basil studied by other authors (Labra *et al.* 2004, Seidler-Łożykowska *et al*. 2008, Seidler-Łożykowska & Król 2008, Zhang *et al.* 2009, Carović-Stanko *et al*. 2010). By contrast, Runyoro *et al.* (2010) stated that linalool did not occur in two sweet basil oils in which 1,8-cineole and E-myroxide dominated. The composition of the 'Wala' oil presented in this work was similar to the oil from the same variety tested by Seidler-Łożykowska *et al.* (2008). On the other hand, a comparison of the chemical composition of the 'Fine Verde' oil analyzed in this study with the results presented by Labra *et al*. (2004) indicates a similarity only in the amount of linalool and the absence of geraniol and methyl chavicol.

The tested 'Wala' oil, in comparison with the 'Fine Verde' oil was marked by a higher level of linalool in particular, but also geraniol, 1,8-cineole and methyl chavicol. Carović-Stanko *et al.* (2010) proved a significant antimicrobial activity of linalool and geraniol. These components dominated in oils extracted from the green matter of *Ocimum basilicum* (the 'Genovese' variety). However, in the opinion of Bagamboula *et al.* (2004), antifungal and antibacterial properties of oils do not depend on one or two of their main components, but rather are the result of many components of oils acting synergistically. Thus, there would be a negligible chance for the development of resistant species of fungi caused by the application of essential oils in the protection of fruits or vegetables (Feng & Zheng 2007).

CONCLUSIONS

The essential oils from both varieties of sweet basil inhibited the growth of the tested fungi. The diameter of the growth inhibition zone significantly depended on the kind of oil, the dose of oil and the species of fungi. The essential oil from the 'Wala' variety was more effective than the 'Fine Verde' oil and the inhibition zones were generally greater when the dose of oil was higher. *E. chevalieri* was the most sensitive to both of the oils and *A. flavus* was the most resistant to them. In effect neither oil inhibited the growth of this strain.

Chemical composition of the tested oils varied. In the 'Wala' oil, there was more than twice the amount of linalool (69%) than in the 'Fine Verde' oil and the presence of geraniol and methyl chavicol was confirmed. The 'Fine Verde' oil was marked by a higher level of mainly α-trans-bergamotene and the presence of eugenol and methyl eugenol.

The results of this study show that the essential oils from both the tested varieties of sweet basil can be used as natural antifungal agents, although the 'Wala' oil is significant-

ly more effective than the 'Fine Verde' oil.

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AKTYWNOŚĆ ANTYGRZYBOWA OLEJKÓW ETERYCZNYCH DWÓCH ODMIAN BAZYLII POSPOLITEJ (*Ocimum basilicum* L.)

Streszczenie

Celem przeprowadzonych badań była ocena aktywności antygrzybowej olejków eterycznych uzyskanych z ziela dwóch odmian bazylii pospolitej (*Ocimum basilicum* L.): ‘Wala’ i ‘Fine Verde’. Olejki wyodrębniano z wysuszonego, rozdrobnionego ziela metodą hydrodestylacji prowadzonej w aparacie Derynga. Skład olejków analizowano za pomocą chromatografii gazowej. Do oceny aktywności przeciwgrzybowej olejków wykorzystano sześć szczepów: *Alternaria* sp., *Aspergillus flavus, Botryis cinerea, Cladosporium herbarum, Eurotium amstelodami* oraz *Eurotium chevalieri.* Ich wrażliwość na działanie olejków zbadano metodą agarową, dyfuzyjno-krążkową. Olejki stosowano w dwóch dawkach - 10 i 5 µl/krążek. Krążek (6 mm) nasączony olejkiem umieszczano na agarowym podłożu zaszczepionym zarodnikami danego szczepu. Po 72 h inkubacji w 25^{o}C mierzono (w mm) strefy zahamowania wzrostu grzybów. Analiza otrzymanych rezultatów wykazała, że średnica strefy inhibicji wzrostu mycelium była istotnie uza-

leżniona od rodzaju olejku (tj. odmiany, z której pochodził), dawki olejku oraz gatunku grzyba. Olejek eteryczny odmiany ‘Wala’ wykazywał, w porównaniu z olejkiem odmiany ‘Fine Verde’, wyższą efektywność hamowania wzrostu badanych grzybów. Przy wyższych dawkach olejków strefa inhibicji wzrostu mycelium była z reguły większa. Najwyższą wrażliwością na działanie obu olejków odznaczał się szczep *E. chevalieri*, natomiast *A. flavus* był najbardziej odporny. Na różnice w antygrzybowej aktywności badanych olejków wpływał zapewne ich zróżnicowany skład chemiczny. Olejek z odmiany ‘Wala”, w porównaniu z ‘Fine Verde’, zawierał ponad dwa razy więcej linalolu oraz więcej geraniolu, 1,8-cineolu i metylochawikolu. W warunkach doświadczenia oba olejki silniej hamowały wzrost badanych szczepów niż cycloheximid (actidion) i tiofanat metylowy (Topsin M 500 SC), zastosowane jako substancje kontrolne.

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