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INHIBITORY EFFECT OF ESSENTIAL OILS FROM SOME *LAURACEAE* SPECIES ON THE GROWTH OF *PENICILIUM COMMUNE*

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ABSTRACT

The aim of this study was to determine the inhibitory effect of four essential oils (EOs) on the growth of seven strains of *Penicillium commune* isolated from moldy milk products by contact vapour. Next objective was to determine the minimum inhibitory doses (*in vitro* and probit analyses) of EOs, which at concentration 625 $\mu l.l^{-1}$ of air completely inhibited the growth of all strains. The antifungal activity was evaluated by the micro-atmosphere method. Cinnamon, cinnamon bark and litsea cubeba EOs completely inhibited the growth of strains during cultivation at 25 °C and 5 °C. Laurel EO had no effects on the growth of *P. commune* strains. EOs that completely inhibit the growth of all strains were used to determine their minimum inhibitory doses (MIDs). The best results were obtained for cinnamon EO (MIDs 15.625 $\mu l.L^{-1}$ of air) after all days at 25 °C of incubation. Litsea cubeba EO had the highest MIDs value (from 250 to 62,5 $\mu l.l^{-1}$ of air). It was found that EOs have different effects on individual strains of *P. commune*. The tested strains (KMi 370 and 402) showed differences among themselves even though they came from the same products (sour cream). According to probit analyses, the most effective tested EOs were cinnamon EO and the least effective was litsea cubeba EO. The antitoxinogenic effect of tested EOS was evaluated by TLC method. Also in this study, cinnamon EO was the most effective against CPA production by *P. commune*. This oil affected the production of CPA by strain KMi177 at 31.25 $\mu l.l^{-1}$ of air completely, and by strain KMi 403, respectively.

Keywords: *P. commune*, essential oils, antifungal activity, vapor phase, cyclopiazonic acid

INTRODUCTION

Mould growth on cheese represents both a quality and a food safety problem and several moulds may destroy cheese. The genus *Penicillium* is the major contaminant followed by the genus *Aspergillus* (**Kure and Skaar, 2019**). *Penicillium* spp. is the predominant contaminant of dairy products (**Hansen *et al.*, 2003**). It is because it's well-adapted to grow in the cheese matrix. The growth of molds in cheese is associated with undesirable taints and odors, liquefaction of the curd and in some cases, production of mycotoxins. *P. commune* (42%) is one of the most widespread microscopic filamentous fungus from genus *Penicillium*, which is able to involved the degradation of cheese (**Kure *et al.*, 2001**). Moreover, *P. commune* is able to produce cyklopiazonic acid. Some of the mycotoxins have been shown to be stable under normal processing conditions of cheese (**Martín and Liras, 2017; Casquete *et al.*, 2019**). Natural preservatives have proven popularity such that interest continues in substituting chemical additives with natural. Therefore, production of safe food without or with low amounts of synthetic preservatives is one of the most important challenges in the food industry (**Khorshidian *et al.*, 2018**). Essential oils (EOs) are mixtures of aromatic oily liquids extracted from different parts of plants, usually by steam distillation. Several of them are characterized by antibacterial and antifungal activities. This activity is depending on their constituents, mainly due to their content in terpenes and phenylpropanoids (**Seow *et al.*, 2014; Nikkhah *et al.*, 2017**). Several essential oils can be applied as natural antimicrobial agents in order to inhibit microbial deterioration of cheeses and extending the shelf-life. Major compounds including thymol, carvacrol, eugenol, carvone and cinnamaldehyde are mainly responsible for exerting antimicrobial activity through various mechanisms such as increasing the cell permeability, change of membrane fatty acids and effect on membrane proteins. Few data are available about the application of EOs for their antifungal activity in food such as cheese. Despite of the suitable efficacy of essential oils in restriction of growth and survival of microorganisms in cheese, some limitations have been recognized in their application (**Khorshidian *et al.*, 2018**). Essential oils constituents could be interacting with food components such as fat, carbohydrate and proteins. So their antimicrobial effect can be reduced, and this is the reason for use height concentration of EOs in food application. But EOs have intense aroma and their utilization at high concentrations in order to compensate their interaction with food components could result in sensory defects (**Hyldgaard *et al.*, 2012**). In order to rectify this shortcoming, various approaches have been proposed. For these reasons, the aim of the presented study is to assess the antimycotic activity of some EOs against *P. commune* strains spoiling selected dairy products from Slovakia and evaluating their activity at lowest concentration by using Minimum inhibitory doses.

MATERIAL AND METHODS

Eseential oils analysis

The essential oils (EOs) used in this study were obtained from cinnamon (*Cinnamomum zeylanicum* L.), cinnamon bark (*Cinnamomum zeylanicum* L.), laurel (*Laurus nobilis* L.) and litsea cubeba (*Litsea deccanensis* L.) by hydrodistillation. The essential oils were supplied by Calendula company a.s. (Nová Ľubovňa, Slovakia) and Hanus (Nitra, Slovakia). All EOs were stored in air-tight sealed glass bottles at 4°C. Essential oils constituent were identified and the relatively composition of the oil was determined by gas chromatography followed by mass spectrometry (GC-MS) s described by **Božik *et al.* (2017**). The identification of constituents was based on a comparison of their mass spectra and relative retention indices (RI) against the National Institute of Standards and Technology Library (NIST, USA), as well as authentic analytical standards and data from the literature. Relative proportions were calculated by dividing individual peak area by total area of all peaks. The response factor was not taken into account. Only compounds over 1% were included. Peaks under 1% were not counted. The used standards are listed in Table 2.

Fungal isolation and identification

Seven isolates (Table 1) from different moldy milk products were used. Isolates of the genus *Penicillium* were identified to the species level according to morphological characteristics based on microscopy. To determine particular species, diagnostic literature was used as follows: **Blakeslee** (**1915**), **Frisvad and Thrane** (**1995**), **Pitt and Hocking** (**2009**), **Samson *et al*.,** (**2010**). Isolates were inoculated in three points on the identification media CYA (Czapek Yeast Extract Agar). MEA (Malt extract agar), YES (Yeast extract agar) and CREA (Creatine sucrose agar). Inoculated media were incubated at 25 ± 1 °C, 7-17 days in the dark. After identification the strains belong to *Penicillium commune* were saved to the Collection of Fungi of Department of Microbiology; Faculty of Biotechnology and Food Sciences SUA in Nitra, Slovakia. For further analysis the 5 days old culture of *Penicillium commune* strains were used.

Table 1 Origin of the strains *Penicillium commune* and identification media and temperature used

Strains	Origin	Cultivation temperature and identification media used
P. commune KMi 177	cheese flavored with pepper	CYA25: incubation period 7 – 14 days in the dark at 25 ± 1°C MEA: incubation period 7 – 14 days in the dark at 25 ± 1°C CREA: incubation period 7 – 14 days in the dark at 25 ± 1°C YES: incubation period 7 – 14 days in the dark at 25 ± 1°C
P. commune KMi 270	smoked cheese (block)	
P. commune KMi 276	smoked cheese (slices)	
P. commune KMi 277	smoked cheese (slices)	
P. commune KMi 370	sour cream	
P. commune KMi 402	sour cream	
P. commune KMi 403	parenica (pasta filata)	

Antifungal activity of essential oils and minimum inhibitory doses (MIDs)

The antifungal activity of tested EOs was evaluated by using the vapour phase of oils. The test was performed in sterile Petri dishes (PD) (Ø 90 mm) for the first analysis and three sectors Petri dishes for the MIDs analysis. The PDs contained 15 mL of CYA. The used method was modified from **Guynot *et al*.** (**2003**) and is described in **Císarová *et al*.** (**2016**). Firstly EOs were tested in higher concentration (625 µl/L^{-1} of air). After preparation the PDs were tightly saled with parafilm and incubated for 14 days at 25 ± 1°C and 35 days at 5 ± 1°C. The diameters (in mm) of the growing colonies were measured at the 3rd, 7th, 11th, 14th, 21th, 28th and 35th day and strains incubated at 25 ± 1°C were measured at 3rd, 7th, 11th, 14th day with a digital caliper. After incubation, the minimum inhibitory doses (MIDs) of EOs with the most significant activity were recorded. EOs dissolved in DMSO were prepared at different concentrations (500, 250, 125, 62.5, 31.25 and 15.625). The used method and MIDs evaluation are described in **Tančinová *et al.*** (**2019**).For each fungal strain the inoculum was prepared. The spore suspension contained 10^6 spore/.ml^{-1}. 5 µl of this suspension was inoculated on PDs with CYA medium. Cultivation was carried out at the 25 ± 1°C and measured after 7th and 14th day.

Anti-toxicogenic effect of essential oils

The inhibitory of essential oils on the production of mycotoxins of tested strains was studied after 7 days of cultivation at 25 °C on individual species treated by the gas diffusion method with EOs. The concentrations of EOs used depended on the activity of the concrete EO at the results from minimum inhibitory doses (MIDs). The range of 625 to 15.625 µl.l^{-1} of air was used. We used only those samples of treated strains by EOs whose visible colony growth was not completely inhibited. We performed all analyses in three replicates by TLC method followed the methodology described in **Císarová *et al*.** (**2015**).

Statistical analysis

The antifungal effect of EOs at concentration 625 µl.l^{-1} of air was performed in triplicate and the MIDs were performed in six repetitions. The results were expressed as the mean of the data obtained in each replicate. Statistical analyses were performed with descriptive statistics.
The ability of strains to grow in the presence of EO was coded to binomial scale (1 - growth observed, 0 - without growth). All data were processed by probit analysis in Statgraphics Centurion XVI (Statgraphics) software. Doses that inhibit the growth in 50 % respectively 90 % of cases (MID_{50} and MID_{90}) were reversely predicted from regression equation.

RESULTS AND DISCUSSION

Chemical analysis of essential oils

In this study, the antifungal properties of four essential oils from family *Lauraceae* were evaluated. Essential oils are a mixture of many complex compounds, which may vary depending on individual chemical compounds or their concentrations. Their antimicrobial activity also depends on their chemical composition (**Omonijo *et al*., 2018**). The most effective components of essential oils are predominantly terpenes, terpenoids and phenolic substances (**Wiese *et al*., 2018; Olmedo *et al*., 2018; Pichersky a Raguso, 2018**). Based on the above we evaluated the chemical composition of tested EOs by the GC-MS analysis. The identified compounds of EOs are listed in Table 2.

Table 2 Essential oils - chemical composition (in %) determined by gas chromatography coupled with mass spectrometry

	Component	Cinnamon	Cinnamon bark	Laurel	Litsea cubeba
1	**α-pinene**[a]			5.4	1.5
2	β-phellandrene			9.8	1.6
3	**β-Pinene**[a]			4.0	1.1
4	**p-Cymene**[a]			2.2	
5	**(R)-(+)-Limonene**[a]				14.6
6	**1,8-cineole**[a]			52.5	1.6
7	**linalol**[a]		1.9		1.1
8	**mentol**[a]			2.6	
9	α-terpineol			1.5	
10	asarone				1.0
11	**Cinnamaldehyde**[a]	76.0	63.33		
12	β-citrall				31.4
13	**α-citrall**[a]				38.0
14	α-terpineole acetate			12.2	
15	**Eugenol**[a]		20.0		
16	**β-caryophyllene**[a]		4.2	2.6	1
17	cumarine	2.4			
18	**Citronelyl propionate**[a]	4.2	1.6		
19	o-methoxycinnamaldehyde	11.6	2.2		
total		**94.8**	**94.9**	**96.9**	**96.0**

Legend: [a] – Identification confirmed by co-injection of authentic standard

The major components according to the concrete essential oil were: cinnamon - Cinnamaldehyde (76.00 %); cinnamon bark - Cinnamaldehyde (63.3 %) and Eugenol (20.00 %); laurel - Eucalyptol (52.5 %) and litsea cubeba - α-citrall (38.00 %) and limonene (14.6 %). Our results are similar with other authors (**Lins *et al*., 2019; Wang *et al*., 2018; Fidan *et al*., 2019, Huang *et al*., 2019**).

Antifungal activity of essential oils

The antifungal activity of four EOs against seven strains of *P. commune* was determined, using micro-atmosphere method, firstly at the concentration of 625 µl.l^{-1} of air. Three essential oils: cinnamon, cinnamon bark and litsea cubeba completely inhibited the growth of all strains during cultivation at 25 °C and 5 °C. Laurel essential oil had different effects on the growth of *P. commune* strains.

Inhibitory effect of this EO on the growth of the all strains of *P. commune* during the cultivation period at 25 ± 1°C is showed in Figure 1A and during the cultivation period at 5 ± 1°C is showed in Figure 1B.

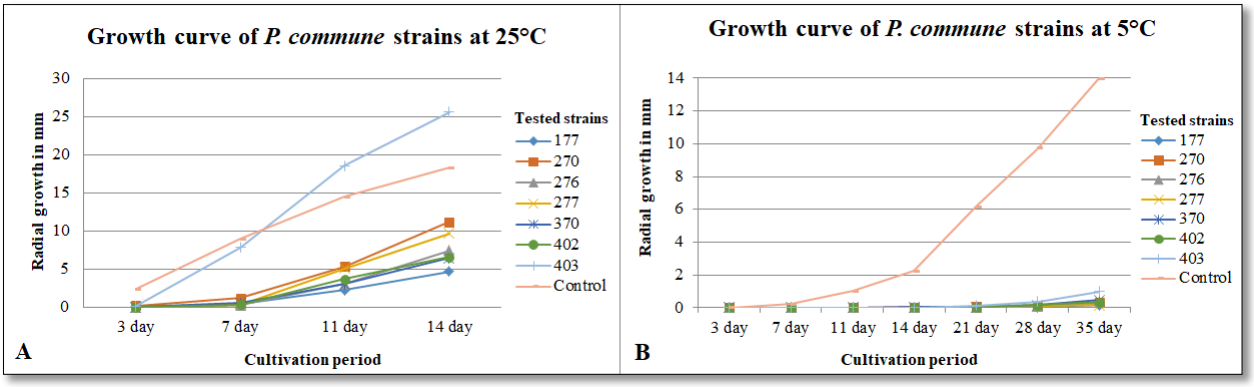

Figure 1 Growth of tested strains *P. commune* during the cultivation period at 25 ± 1°C (A) and at 5 ± 1°C (B) under treatment by laurel EO

The strain KMi 403 was the most resistant to antifungal effect of laurel EO at both, 25 °C after 14 days and at 5 °C after 35 days of cultivation. In addition its growth was after 14 days of cultivation at 25 °C stimulating in compared with control set. The most sensitive strain was KMi 177 regardless of time of cultivation temperature. The laurel EO was able to inhibit its growth significantly. But is interesting, that this strain is obtained from a cheese flavored by pepper, due this fact it can be weakened. The similar result obtained **Massa *et al.* (2018)**. They studied antifungal activity of some essential oils (included laurel EO) on growth of *C. glabrata*. The results showed that laurel EO had no inhibitory effect on the growth of this yeast. But authors **Sevindik *et al*. (2019)** tested essential oil from *Laurel nobilis* flowers and *Laurel nobilis* leaves by microdilution method against some strain of bacteria and yeast. Their results showed that those essential oils acted better against yeasts than against bacteria at relatively low concentrations (range between 0.097 - 3.125 µl/ml^{-1}).

It could be means that laurel can be able to inhibit growth of fungi but only in a contact with mycelium of tested strain.

Evaluation of minimum inhibitory doses (MIDs)

Three essential oils, concretely: cinnamon, cinnamom bark and litsea cubeba inhibited growth of tested strains completely at all temperatures (25 °C and 5 °C) during all days of cultivation. Therefore, lower concentrations (500-15.625 µl.L^{-1} of air) of these essential oils were used to determine the minimum inhibitory doses (MIDs) on the tested fungi. MIDs determined by the micro-atmosphere method are summarized in Table 3.

Table 3 Minimum inhibitory doses (µl.L^{-1} of air) of essential oils in vapor phase effective against the tested *P. commune* strains on CYA at 25 °C after 7 and 14 days, respectively.

Essential oils	MIDs (µl.L^{-1} of air) after 7 days of cultivation						
	Tested strain number						
	177	**270**	**276**	**277**	**370**	**402**	**403**
Cinnamon	62.5	-	31.25	62.5	62.5	-	-
Cinnamon bark	62.5	31.25	31.25	62.5	62.5	31.25	31.5
Litsea cubeba	125	125	62.5	125	125	250	250
	MIDs (µl.L^{-1} of air) after 14 days of cultivation						
	Tested strain number						
	177	**270**	**276**	**277**	**370**	**402**	**403**
Cinnamon	125	125	62.5	62.5	62.5	-	-
Cinnamon bark	125	125	125	62.5	62.5	31.25	31.5
Litsea cubeba	125	125	125	250	125	125	250

The lowest MIDs against the tested *Penicillium* strains was found in cinnamon (MIDs 15.625 µl.L^{-1} of air) for three tested strains KMi 270, KMi 402 and KMi 403 after 7 days of cultivation and for strains KMi 402 and 403 after 14 days of cultivation at 25 °C. Also **Císarová *et al*. (2016)** reported a relatively good effect of cinnamon essential oil. They found that cinnamon EO inhibited the growth of tested *Aspergillus* strains at MIDs in the range of 31.5 µl.L^{-1} of air to 125 µl.L^{-1} of air. **Gómez *et al*. (2018)** tested *Origanum vulgare* and *Cinnamomum zeylanicum* essential oils and their major active constituents, carvacrol and cinnamaldehyde, respectively, for inhibiting species of the genus *Aspergillus* and aflatoxin production in maize extract medium under different environmental conditions. They reported that the effectiveness of EOs and their main constituents to inhibit fungal growth and aflatoxin production in contact assays was lower than in vapour phase assays.

In our study, the lowest effective essential oil was litsea cubeba with the higher MIDs for four tested strains - for 2 strains (KMi 402 and 403) after 7 days of cultivation and for 2 strains (KMi 277 and 403) after 14 days of cultivation with MIDs value 250 µl.L^{-1} of air. But many authors described Litsea cubeba EOs like the very potent antifungal and antibacterial agents (**Li *et al*., 2016**; **Hu *et al*., 2019**) Using probit analysis, predicted $MIDs_{90}$ and $MIDs_{50}$ were calculated. The MID values were calculated only for those essential oils that did not inhibit growth of the tested strains of *P. commune* completely at reduced concentrations (500-15.625 µl.L^{-1} of air). The results are shown in Table 4.

The most effective tested essential oils were cinnamon and cinnamon bark EOs, less effective litsea cubeba EO. The highest MID_{90} (162.29 µl.L^{-1} of air) was determinate for litsea cubeba against strains KMi 277 after 14 days of cultivation. Also the highest MID_{50} (150.53 µl.L^{-1} of air) was determinate for litsea cubeba, but against strains KMi 403 after 7 days of cultivation. All results obtained were statistically significant ($p < 0.005$), except MIDs values for litsea cubeba EO against strain KMi 370 (p *0.348)* after 7 and 14 days of cultivation.

Inhibitory effect of EOs on CPA production

All strains of *P. commune* were tested *in vitro* for the production of cyclopiazonic acid (CPA) by TLC method. Production of CPA was confirmed for four strains (KMi 177, 277, 370 and 403). Anti-toxinogenic effect was determined in 4 EOs, which did not inhibit the growth of the tested isolates completely at the different concentration. The used concentration was selected according the results from MIDs evaluation. The results are summarized in Table 5.

The anti-toxinogenic potential of essential oils has been already demonstrated in previous studies (**Císarová *et al*., 2015; Císarová *et al*., 2016; Foltinova *et al*., 2017**).Treatments with cinnamon EO showed some potential of fungal toxic inhibition. This oil affected the production of CPA by strain KMi177 at 31.25 µl.l^{-1} of air completely, and by strain KMi 403, respectively. Production of CPA by strains KMi 370 was completely inhibited by this oil at all tested concertation (31.25 – 15.625 µl.l^{-1}). Cinnamon bark EO was able to inhibited production of CPA completely only in strain KMi 403 at the concentration 31.25 µl.l^{-1} of air. Litsea cubeba had very low effect on the production of CPA by tested strains. Laurel was the least effective EO because its inhibitory effect on the growth of tested strains was not detected even at the highest concentration (625 µl.l^{-1} of air), as so as antitoxinogenic activity.

The strains used in our study were isolated directly from the moldy dairy products from Slovakia. For the use of EOs in food matrix, it is necessary to use very low concentrations, in order to not negatively affect the organoleptic properties of food, especially dairy products. Therefore, it is necessary to test essential oils directly on

food. Although essential oils are not always able to inhibit fungal growth at low concentrations completely, they could be used in foods to inhibit their toxic metabolites.

Table 4 The minimum inhibitory doses (MID_{50} and MID_{90}) of tested essential oils expressed as $\mu l.L^{-1}$ of air of air estimated by probit analyses for tested strains of *P. commune*

Strains	MIDs	*Cinnamon*		*Cinnamon bark*		*Litsea cubeba*	
		7 days	**14 days**	**7 days**	**14 days**	**7 days**	**14 days**
KMi177	**MID_{50}**	27.53	21.88	27.53	21.88	66.50	66.50
	MID_{90}	32.73	52.56	32.73	52.56	78.94	78.94
	p Value	*0.000*		*0.000*		*0.000*	
KMi 270	**MID_{50}**	nd	nd	13.3	13.3	56.12	66.50
	MID_{90}	nd	nd	20.98	20.98	64.97	78.94
	p Value	*nd*		*0.005*		*0.000*	
KMi 276	**MID_{50}**	13.4	18.21	13.4	140.63	nd	66.50
	MID_{90}	20.98	45.68	20.98	162.29	nd	78.94
	p Value	*0.000*		*0.000*		*0.000*	
KMi 277	**MID_{50}**	15.87	18.21	15.87	18.21	nd	140.63
	MID_{90}	36.49	45.68	36.49	45.68	nd	162.29
	p Value	*0.000*		*0.000*		*0.000*	
KMi 370	**MID_{50}**	nd	nd	nd	nd	58.03	58.03
	MID_{90}	34.25	34.25	34.25	34.25	64.23	64.23
	p Value	*0.041*		*0.041*		*0.348*	
KMi 402	**MID_{50}**	nd	nd	13.4	18.21	119.80	119.80
	MID_{90}	nd	nd	20.98	45.68	135.99	135.99
	p Value	*nd*		*0.000*		*0.000*	
KMi 403	**MID_{50}**	nd	nd	13.4	18.21	131.21	94.47
	MID_{90}	nd	nd	20.98	45.68	150.53	104.73
	p Value	*nd*		*0.000*		*0.000*	

Legend: nd-MID not determined

Table 5 Inhibitory effect of tested EOs at different concentrations on CPA production by tested strains of *P. commune*

Essential oils	**Tested concentration ($\mu l.l^{-1}$ of air)**	Tested strain number			
		177	**277**	**370**	**403**
				CPA	
Cinnamon	**15.625**	3*/1^{n}	3/3	3/0	-
	31.25	3/0	3/3	3/0	3/0
Cinnamon bark	**15.625**	3/3	3/3	3/2	3/1
	31.25	3/3	3/3	3/1	3/0
Litsea cubeba	**15.625**	3/3	3/3	3/3	3/3
	31.25	3/3	3/3	3/3	3/3
	62.5	3/1	3/3	3/2	3/2
	125	-	-	-	3/1
Laurel	**625**	3/3	3/3	3/3	3/3

Legend: * - repetition tested, ^{n} - number of positive production

CONCLUSION

In this study, the inhibitory effect of selected essential oils cinnamon (*Cinnamomum zeylanicum* L.), cinnamon bark (*Cinnamomum zeylanicum* L.), laurel (*Laurus nobilis* L.) and litsea cubeba (*Litsea deccanensis* L.) on the growth of *P. commune* strains (7 strains) was evaluated. Our results showed that cinnamon, cinnamon bark and litsea cubeba EO had a 100% inhibition effect on the growth of all tested strains at the higher concentration (625 $\mu l.L^{-1}$ of air) throughout the cultivation days. Laurel EO had no effect on the growth of tested strains. The MIDs assay confirmed the most significant efficacy of the two oils, namely cinnamon and cinnamon bark. Litsea cubeba had no significant effect. Is interestingly, the tested strains (KMi 370 and 402) showed differences among themselves even though they came from the same products (sour cream). Strain KMi 402 was much more sensitive to essential oils than strain KMi 370. We also achieved the same results in testing the potential ability of essential oils to inhibit mycotoxin production, where cinnamon was the most effective and laurel had no effect on CPA production. Our findings confirm that although essential oils are not able to inhibit fungal growth at the lowest concentrations, they are capable of inhibiting mycotoxins production. Essential oils can be used in agro industries instead of synthetic pesticides or preservatives, to control plant diseases causing severe destruction to crops and they can be proposed as potential antimicrobial agents for food commodity preservation in the near future.

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