J Food Sci Technol (August 2017) 54(9):2929–2935
DOI 10.1007/s13197-017-2731-4

ORIGINAL ARTICLE

Control of aflatoxigenic strains *by Cinnamomum porrectum* essential oil

Oratai Sukcharoen[1] · Pramote Sirirote[2] · Dusanee Thanaboripat[2]

Revised: 18 May 2017 / Accepted: 30 May 2017 / Published online: 9 June 2017

Abstract Essential oil from the bark of *Cinnamomum porrectum* (Roxb.) Kosterm was obtained by hydrodistillation using a Clevenger-type system for 3 h and identified by gas chromatography–mass spectrometry. The compounds were safrole (93.9%), elemicine (4.3%) and methyl eugenol (1.7%). The effect of essential oil on mycelial growth, sporulation, and aflatoxin B1 production of *Aspergillus parasiticus* IMI 283883 and *Aspergillus flavus* IMI 242684 was evaluated using contact and vapor treatments. Aflatoxin B1 was determined using the Enzyme-linked immunosorbent assay. The results showed that *C. porrectum* (Roxb.) Kosterm essential oil at concentrations more than 200 ppm exhibited inhibition effect on mycelial growth, sporulation, and aflatoxin B1 production of both *Aspergillus* strains as compared with control. The fumigation activities via vapor treatment showed higher inhibition than contact treatment. This study suggests that *C. porrectum* (Roxb.) Kosterm essential oil represents a good alternative in eco-friendly control of aflatoxigenic strain on food and agricultural commodities.

Keywords Aflatoxigenic strain · Aflatoxin · *Cinnamomum porrectum* (Roxb.) Kosterm · Essential oil

✉ Oratai Sukcharoen
osukcharoen@hotmail.com

[1] Department of Biotechnology, Faculty of Science, Ramkhamhaeng University, Bangkok 10240, Thailand

[2] Department of Biology, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang, Bangkok 10520, Thailand

Introduction

Aflatoxins are toxic and carcinogenic secondary metabolites that are produced by several species of *Aspergillus* such as *Aspergillus flavus* and *Aspergillus parasiticus* (Ellis et al. 1991). There are four major aflatoxins: B1, B2, G1, and G2; aflatoxin B1 is the most toxic and potent hepatocarcinogenic compound (Bennett and Klich 2003). Aflatoxin management by controlling aflatoxin contamination in agricultural farming foods and feeds have been applied to reduce impacts on human and animal health and also economic losses. Over the last few decades, various synthetic fungicides are commonly used for protection of food commodities from fungal deterioration as well as mycotoxin contaminations. However, undesirable side effects in the food chain, i.e., environmental persistence, residual toxicity, fungal and pest resistance, and mammalian toxicity are increased (Isman 2006).

Considering this, plant-based essential oils are gaining interest as a source of natural antioxidants, insecticidal, and antimicrobial properties (Miyake and Hiramitsu 2011; Adiani et al. 2015; Kasrati et al. 2015; Kumar et al. 2016) which are safe to the environment as well as to human (Prakash et al. 2012). Thus, many research work have been applied essential oils to prevent and control the growth of aflatoxin-producing fungi as an alternative to synthetic fungicides. The essential oils have high hydrophobicity and high viscosity, therefore theirs can cause an irregular distribution through the liquid and solid medium. In contrast, the essential oils in vapor are more the advantage for microbial inhibition and application for food and feed protection (Tian et al. 2012). *Cinnamomum porrectum* (Roxb.) Kosterm, locally known as Thep-ta-ro, belonging to the same family with cinnamon (Lauraceae). It is mainly grown in Southern Thailand including Krabi, Phang Nga,

Pattalung and Trang provinces. Its essential oil has been used as flavoring agent in food and beverages industry, the component of perfumes, soap and in Thai traditional medicine as an anti-inflammatory activity and antimicrobial activity (Palanuvej et al. 2006; Phongpaichit et al. 2006; Buru et al. 2014). To our knowledge, no documented reports on the antifungal activity of *C. porrectum* (Roxb.) Kosterm essential oil against the aflatoxigenic strains is available. In this study, the chemical composition of the *C. porrectum* (Roxb.) Kosterm essential oil was investigated by gas chromatography–mass spectrometry (GC–MS) and evaluated for its effect on mycelial growth, sporulation and aflatoxin B1 production against the aflatoxigenic strains *A. flavus* IMI 242684 and *A. parasiticus* IMI 283883 by comparison between the contact and the vapor treatments.

Materials and methods

Plant material and extraction

The barks of *C. porrectum* (Roxb.) Kosterm were air-dried and then powdered using a conventional blender. Dried material (100 g) was placed in a 1 L round-bottom distillation flask and 300 mL double distilled water was added. The essential oil was obtained by hydrodistillation for 3 h, using a Clevenger-type apparatus according to the method of Clevenger (1928). The oily layer was separated and dried over anhydrous sodium sulfate, then stored in a tightly closed dark vial at 4 °C until further studies.

GC–MS analysis of *Cinnamomum porrectum* (Roxb.) Kosterm essential oil

The chemical composition of essential oil was analyzed using gas chromatography–mass spectrometry (GC–MS). The GC–MS analysis was performed on Agilent 6890 gas chromatograph in electron impact (EI, 70 eV) mode coupled to an HP 5973 mass selective detector and fitted with a fused silica capillary column (HP-5MS) supplied by HP, USA (30.0 m × 0.25 mm i.d., 0.25 µm film thickness). Helium was used as the carrier gas at a flow rate of 1.0 mL/min. The GC column oven temperature was increased from 100 to 188 °C at a rate of 3 °C/min, and then 20 °C/min to 280 °C with a final hold time of 3 min. The injector and detector temperatures were maintained at 280 °C. Screening of the chromatograms was performed in scan mode, from *m/z* 50 to 500, at a rate of 3.25 scan/s, with the ionization source temperature set at 200 °C. Diluted samples (20%, in dichloromethane) of 0.2 µL was injected in the split mode ratio 1:50. Peak identification was done by comparison with the mass spectra available on the database of National Institute of Standard and Technology and Wiley libraries. The relative percentage of the essential oil constituents was expressed as a percentage by peak area normalization.

Preparation of spore suspension

Two aflatoxigenic strains, i.e. *A. flavus* IMI 242684 and *A. parasiticus* IMI 283883 obtained from the International Mycological Institute (Egham, Surrey, UK) were used throughout this study. The fungal strains were cultured on potato dextrose agar slope for 7–10 days at 28 ± 1 °C. Spores were harvested by adding 10 mL of sterile 0.05% (v/v) Tween 80 solution to culture and gently scraping the mycelial with a sterile inoculating loop to free spores. Spore concentration was determined by a hemocytometer and the spore suspension was diluted with 0.05% Tween 80 solution to give a final concentration of 10^6 spore mL^{-1}.

Effect of *Cinnamomum porrectum* (Roxb.) Kosterm essential oil on mycelial growth by the two *Aspergillus* strains

The antifungal activity of *C. porrectum* (Roxb.) Kosterm essential oil against *A. flavus* IMI 242684 and *A. parasiticus* IMI 283883 was based on mycelial growth inhibition using the contact (Soliman and Badeaa 2002) and vapor treatments (Soylu et al. 2010) with some modifications. For contact treatment, the determination of essential oil was carried out using the poisoned food technique. An appropriate volume of essential oil was added into 0.05% Tween-80 and molten PDA medium (20 mL) before pouring into sterilized Petri dishes (90 mm diameter). The final concentrations of essential oil at 100, 200, 300, 600, and 1000 ppm were obtained. Paper disc (Whatman No 1, 6 mm diameter) was placed onto the center of each PDA plate and inoculated with 10 µL of spore suspension (10^6 spore mL^{-1}) and immediately sealed with parafilm to prevent leakage of essential oil vapor. PDA plates with the same concentration were sealed in polyethylene bags and incubated for 5 days at 30 ± 1 °C in the darkness. For vapor treatment, paper disc (Whatman No. 1, 6 mm diameter) was placed onto the center of each PDA plate (20 mL of solidified PDA) and inoculated with 10 µL of spore suspension (10^6 spore mL^{-1}). PDA plate was kept in the inverted position and another sterile paper disc was placed inside at the center of the upper lid of each PDA plate, with 80 mL air space. The amount of essential oil was applied to the paper disc with the final concentrations of 100, 200, 300, 600, and 1000 ppm, and the sterile blank disc served as a control. The surface of essential oil disc was at a distance of approx. 6 mm from the growth surface of the *A. flavus* IMI 242684 and *A. parasiticus* IMI 283883.

All plates were then sealed and incubated as described above.

The efficacy of the essential oil was evaluated by measuring the average of two perpendicular diameters of each colony daily. All treatments were performed in triplicate. The relative growth inhibition of the treatment compared to the control (I, %) was calculated as a percentage, using the following equation: Inhibition (I%) = [(Dc − Dt)/Dc] × 100, where Dc is the diameter of fungal colony in the control Petri dish and Dt is the diameter of fungal colony in the essential oil-treated Petri dish. The fungistatic–fungicidal nature of *C. porrectum* (Roxb.) Kosterm essential oil was tested by observing revival of growth of the inhibited mycelial disc following its transfer to non-treated PDA. A fungicidal effect was where there was no growth, whereas a fungistatic effect was where temporary inhibition of microbial growth occurred (Thompson 1989). After growth was evaluated, all samples were analyzed for both sporulation and aflatoxin B1 qualification.

Effect of *Cinnamomum porrectum* (Roxb.) Kosterm essential oil on sporulation by the two *Aspergillus* strains

Spore production of *Aspergillus* strains was determined using the modified method (Tzortzakis and Economakis 2007). Spores from colonies of *A. flavus* IMI 242684 and *A. parasiticus* IMI 283883 previously incubated for 5 days which exposed to essential oil by both contact and vapor treatments, were collected by adding 5 ml sterile water containing 0.1% (v/v) Tween 80 to each Petri dish and gently scraping the mycelial surface three times with a sterile L-shaped spreader to free spores. The spore suspension was then centrifuged and estimated using a hemocytometer slide under a light microscope. The percent inhibition of spore production was computed by the following equation: Inhibition of sporulation (%) = [(Nc − Ns)/Nc] × 100. Where Nc is the number of spores in control sample and Ns is the number of spores in treated sample.

Effect of *Cinnamomum porrectum* (Roxb.) Kosterm essential oil on aflatoxin B1 production by the two *Aspergillus* strains

After sporulation was determined, agar cultures were extracted with 10 mL of 70% methanol and shaken for 5 min before filtered by Whatman no 4. The extracts were analysed for the aflatoxin B1 (AFB1) content using DOA-Aflatoxin ELISA Test Kit from Department of Agriculture (DOA), Ministry of Agriculture and Cooperatives, Thailand, according to the method of Chinaphuti et al. (2002) by adding 50 µL of AFB1 standards into the antibody coated wells in microtitre plates and 50 µL of diluted sample into the other wells followed by adding 50 µL of AFB1-horseradish peroxidase conjugate to each well, and microtitre plates were slightly shaken before incubated at room temperature for 30 min. The contents of the well were then discarded into the appropriate waste container and washed the plate 3–5 times with 0.5% Tween 20 in 0.01 M phosphate buffer saline. One hundred µL of tetramethyl benzidine substrate was added into the well, incubated for 10 min at room temperature before adding 100 µL of stopping solution (0.3 M phosphoric acid). The solution was read at 450 nm using the automated Micro-ELISA reader. The concentration of AFB1 of samples was calculated from the slope between % maximum binding and standard AFB1 concentrations. This concentration was the sensitive upper limit of the standard curve. Percent of AFB1 inhibition was evaluated as follows: Inhibition of AFB1 production (%) = (AFB1 concentration in a control sample − AFB1 concentration in a treatment sample) × 100/AFB1 concentration in a control sample.

Statistical analysis

The results of mycelial growth, sporulation, and aflatoxin B1 production were expressed as the means ± standard deviations from three replications of each treatment. Data were analyzed for normality, and then subjected to analysis of variance (ANOVA). Significant differences between mean values were determined using a multiple comparison tests (Tukey's post hoc test) where $p < 0.05$ was considered as significant.

Results

The yield of the *C. porrectum* (Roxb.) Kosterm essential oil was 3.25–3.55% based on dry weight. The chemical composition of essential oil was qualitative and quantitative analysis by GC–MS. The identified chemical compounds, retention time, formula, molecular weight and percentage composition are presented in Table 1. Three different components of the *C. porrectum* (Roxb.) Kosterm oil, accounting for 99.9% of the total oil composition. Safrole (93.9%) was identified as the major component followed by elemicin (4.3%) and methyl eugenol (1.7%).

Effect of *Cinnamomum porrectum* (Roxb.) Kosterm essential oil on mycelial growth by the two *Aspergillus* strains

The mycelial growth of *A. flavus* IMI 242684 and *A. parasiticus* IMI 283883 resulting from the exposure to the

Table 1 Chemical composition of essential oil of *Cinnamomum porrectum* (Roxb.) Kosterm

No.	Compound	RT	Area %	Formula	Molecular weight
1	Safrole	10.26	93.92	$C_{10}H_{10}O_2$	162.1852
2	Methyl eugenol	13.89	1.79	$C_{11}H_{14}O_2$	178.2277
3	Elemicin	19.36	4.30	$C_{12}H_{16}O_3$	220.2536

different amount of essential oil during 5 days of incubation at 30 °C by the contact and vapor treatments was statistical analyzed and the results were shown in Table 2. Both treatments showed that the essential oil at 1000 ppm completely inhibited both fungal strains. When compared between contact and vapor treatments, it indicated that vapor treatment (fumigation) was significantly effective ($p < 0.05$) on mycelial growth inhibition when using the same amount of essential oils of 200 to 600 ppm. Essential oil at 1000 ppm was found to be fungistatic activity against both *Aspergillus* strains in both treatments.

Effect of *Cinnamomum porrectum* (Roxb.) Kosterm essential oil on sporulation by the two *Aspergillus* strains

The inhibitory activity of the essential oil on sporulation of *A. flavus* IMI 242684 and *A. parasiticus* IMI 283883 was shown in Table 3. At 600 and 1000 ppm essential oil completely inhibited sporulation of both *Aspergillus* strains. However, the sporulation inhibition by vapor treatment was significantly more effective than the contact treatment when the same amount of essential oil at 200 and 300 ppm were applied.

Effect of *Cinnamomum porrectum* (Roxb.) Kosterm essential oil on aflatoxin production by the two *Aspergillus* strains

The inhibitory activity of the essential oil on aflatoxin B1 production of *A. flavus* IMI 242684 and *A. parasiticus* IMI 283883 was presented in Table 4. Aflatoxin B1 production of both fungal strains was significantly inhibited ($p < 0.05$) by the essential oil at all concentrations. Treatment of essential oil at 600 and 1000 ppm by both contact and vapor methods completely inhibited aflatoxin production by *A. flavus* IMI 242684 and *A. parasiticus* IMI 283883.

Discussion

The effects of different essential oils on mycelial growth, sporulation as well as aflatoxin production have been investigated by many researchers (Sakuda 2010; Prakash et al. 2012; Thanaboripat et al. 2016). The essential oil of cassia and bay leaves caused 98% reduction in aflatoxin B1 but stimulated fungal growth. However, essential oil of anise, caraway, and cinnamon exhibited fungistatic and fungicidal activities against *A. flavus* (Patkar et al. 1993). Essential oil of lemongrass, citronella, and white wood could inhibit the growth of *A. parasiticus* IMI 102566 and aflatoxin production (Thanaboripat et al. 2007). *Nigella sativa* essential oil significantly inhibited aflatoxin B1 production of *A. flavus* by 47.9–58.3% and of *A. parasiticus* by 32–48% and no significant effect on the growth of both *Aspergillus* strains (El-Nagerabi et al. 2012). Some studies have shown that there is a direct correlation between fungal growth and aflatoxin B1 production (Rasooli and Abyaneh 2004). At 200 ppm of *Zataria multiflora* Boiss reduced the fungal growth and sporulation of *A. flavus* ATCC 15546 by 79.4 and 92.5% on PDA while essential oil at 150 ppm in YES broth could reduce the

Table 2 Effect of different amount of *Cinnamomum porrectum* (Roxb.) Kosterm essential oil on the mycelial growth of *A. flavus* IMI 242684 and *A. parasiticus* IMI 283883 inhibition using the contact and vapor treatment

Mode of inhibition (%)	Concentration (ppm)				
	100	200	300	600	1000
A. flavus					
Contact	13.1 ± 0.5^{eC}	22.3 ± 0.5^{dC}	51.8 ± 1.9^{cC}	77.6 ± 0.5^{bB}	100.0 ± 0.0^{aA}
Vapor	16.8 ± 1.0^{eB}	33.7 ± 1.1^{dB}	62.5 ± 5.0^{cA}	83.1 ± 0.0^{bA}	100.0 ± 0.0^{aA}
A. parasiticus					
Contact	13.3 ± 0.5^{eC}	20.9 ± 1.0^{dC}	52.3 ± 3.8^{cBC}	75.5 ± 1.6^{bB}	100.0 ± 0.0^{aA}
Vapor	24.1 ± 1.9^{eA}	43.8 ± 1.1^{dA}	60.5 ± 1.1^{cAB}	84.9 ± 0.5^{bA}	100.0 ± 0.0^{aA}

Values are mean (n = 3) ± standard deviation

The means followed by same small letter in the row are not significantly different according ANOVA and Tukey's multiple comparison tests ($p < 0.05$)

The means followed by same uppercase letter in the column are not significantly different according ANOVA and Tukey's multiple comparison tests ($p < 0.05$)

Table 3 Effect of different amount of *Cinnamomum porrectum* (Roxb.) Kosterm essential oil on the sporulation of *A. flavus* IMI 242684 and *A. parasiticus* IMI 283883 inhibition using contact and vapor treatment method

Mode of inhibition (%)	Concentration (ppm)				
	100	200	300	600	1000
A. flavus					
Contact	16.7 ± 3.6^{dC}	33.3 ± 3.6^{cB}	54.2 ± 3.8^{bC}	100.0 ± 0.0^{aA}	100.0 ± 0.0^{aA}
Vapor	28.6 ± 3.4^{cB}	60.0 ± 11.1^{bA}	87.7 ± 2.7^{aA}	100.0 ± 0.0^{aA}	100.0 ± 0.0^{aA}
A. parasiticus					
Contact	8.8 ± 1.7^{dC}	34.9 ± 9.1^{cB}	66.3 ± 6.5^{bB}	100.0 ± 0.0^{aA}	100.0 ± 0.0^{aA}
Vapor	44.4 ± 4.5^{dA}	57.9 ± 6.1^{cA}	80.2 ± 8.6^{bAB}	100.0 ± 0.0^{aA}	100.0 ± 0.0^{aA}

Values are mean (n = 3) ± standard deviation

The means followed by same small letter in the row are not significantly different according ANOVA and Tukey's multiple comparison tests ($p < 0.05$)

The means followed by same uppercase letter in the column are not significantly different according ANOVA and Tukey's multiple comparison tests ($p < 0.05$)

Table 4 Effect of different amount of *Cinnamomum porrectum* (Roxb.) Kosterm essential oil on the aflatoxin production of *A. flavus* IMI 242684 and *A. parasiticus* IMI 283883 inhibition using contact and vapor treatment method

Mode of inhibition (%)	Concentration (ppm)				
	100	200	300	600	1000
A. flavus					
Contact	$19.6 + 1.0^{dC}$	$41.5 + 3.9^{cC}$	$81.9 + 8.0^{bB}$	100.0 ± 0.0^{aA}	100.0 ± 0.0^{aA}
Vapor	$34.7 + 6.9^{cB}$	$68.7 + 5.0^{bA}$	100.0 ± 0.0^{aA}	100.0 ± 0.0^{aA}	100.0 ± 0.0^{aA}
A. parasiticus					
Contact	$20.0 + 4.2^{dC}$	$53.8 + 3.8^{cB}$	$75.5 + 7.2^{bB}$	100.0 ± 0.0^{aA}	100.0 ± 0.0^{aA}
Vapor	$48.5 + 2.5^{cA}$	$66.2 + 3.4^{bA}$	100.0 ± 0.0^{aA}	100.0 ± 0.0^{aA}	100.0 ± 0.0^{aA}

Values are mean (n = 3) ± standard deviation

The means followed by same small letter in the row are not significantly different according ANOVA and Tukey's multiple comparison tests ($p < 0.05$)

The means followed by same uppercase letter in the column are not significantly different according ANOVA and Tukey's multiple comparison tests ($p < 0.05$)

mycelium growth and aflatoxin production by 90 and 99.4%, respectively (Gandomi et al. 2009).

Essential oils are very complex natural mixture compounds. They can be characterized by 2 or 3 major components at fairly high concentrations (20–70%). There are a few reports on the chemical components of *C. porrectum* (Roxb.) Kosterm essential oil. In our study, the yield of *C. porrectum* (Roxb.) Kosterm essential oil was 3.25–3.55%, which was similar to 4.0% (Buru et al. 2014). Safrole (93.9%) was the main composition followed by the low level of elemicin (4.3%) and methyl eugenol (1.7%). Very few data are available regarding the antimicrobial activity of the essential oil of *C. porrectum* (Roxb.) Kosterm essential oil, in particular, there are no data of the activity on mycelial growth, sporulation, and aflatoxin B1 production. Essential oil of *C. porrectum* (Roxb.) Kosterm had high antimicrobial activity against *Candida allbicans* with a MIC (Minimum inhibition concentration) of 0.063% (Palanuvej et al. 2006) and have significant antibacterial activity against both of Gram positive and Gram negative bacteria including methicillin-resistant *Staphylococcus aureus* (Buru et al. 2014).

Safrole, which was phenylpropene, has been proved to show antifungal activities (Kubo et al. 1993). Elemicin and methyl eugenol, compounds found in relatively low concentrations, are also known to have efficient antimicrobial properties as reported by many researchers (Kubo et al. 1993; Doughari 2006). Methyl eugenol inhibited *A. flavus* and aflatoxin production on peanut pods and kernels (Sudhakar et al. 2009). Although the antimicrobial activity of essential oils from many plant species has been extensively studied, their antimicrobial mechanism has not been fully understood due to their mixture of several compounds that act on specific targets and may have synergistic interaction with other active compounds (Adegoke et al. 2000). The antifungal mechanism of essential oil and their

compounds have been proposed to their lipophilic character, which allows them to penetrate cell membranes and interfere with cellular metabolism and also react with cell processes or enzymes. (Devi et al. 2010). However, fungal cell death is reported to be mediated either by the formation of lesions of the plasma membrane or the alteration of membrane permeability (Khan et al. 2010). Methyl eugenol and eugenol induced the damage to the lipid by inhibiting ergosterol biosynthesis and induced oxidative stress causing fungal cell death (Khan et al. 2011).

When contact and vapor treatments of *C. porrectum* (Roxb.) Kosterm essential oil was compared, the results indicate that the vapor treatment showed higher inhibition of mycelial growth, sporulation, and aflatoxin B1 production than the contact treatment and can also be achieved with a smaller amount of essential oil. These could be attributed to the variation in the relative composition of the essential oil and vapors as the latter was not analyzed in that study, which can be related to many other factors, such as essential oil volatility, water solubility and general chemical complexity for the higher antifungal activity of the volatiles. Several researchers also reported that a greater antifungal activity of essential oil achieved in vapor phase than in aqueous solution or agar contact (Bluma et al. 2009; Inouye et al. 2003; Laghchimi et al. 2014). Due to the hydrophobic nature of compounds that constitute the volatile of essential oil, it could be expected that they may act mainly by accumulation on mycelium rather than the agar and inhibited three stages on germination of a conidium, elongation of vegetative mycelium and sporulation of reproductive mycelium (Inouye et al. 2003).

The inhibitory effect of essential oil against sporulation has been reported by many investigators (Gandomi et al. 2009; Laghchimi et al. 2014). The effect of three Moroccan essential oils on fungal asexual reproduction stages including spore production and found that the partial inhibition of spore production could be attributed to mycelial destruction or inhibition of fungal growth (Tataoui-Elaraki et al. 1993). The impact of *Cymbopogon citratus* L. essential oil on sporulation may reflect the effects of volatile compounds emitted by the oil on the surface of developing mycelial, and/or perception/transduction of signals involved in the switch from vegetative to reproductive development (Mahanta et al. 2007). Inhibition of the mycelial growth and spores of the aflatoxigenic fungi is one of the methods for controlling aflatoxin contamination which may proliferate under favorable conditions in the storage atmosphere and the surfaces (Tzortzakis and Economakis 2007). The mechanism of the inhibition of aflatoxin B1 production was not clear. The antiaflatoxin actions of plant essential oil may be related to inhibition of aflatoxin biosynthesis involving lipid peroxidation and oxygenation (Bluma et al. 2009). Therefore, it is apparent that *C. porrectum* (Roxb.) Kosterm essential oil contains some inhibitor substances that interfere with some steps in the metabolic pathways which control the biosynthesis of aflatoxin B1 in both *Aspergillus* strains.

Conclusion

This study reported the inhibition activity of *C. porrectum* (Roxb.) Kosterm. essential oil on the aflatoxigenic strains such as *A. flavus* IMI 242684 and *A. parasiticus* IMI 283883. The results indicated that using *C. porrectum* (Roxb.) Kosterm essential oil as a fumigant can be an effective biocontrol agent against aflatoxigenic strains contaminated in human food, animal feed, and other agricultural products. The further application in the field has to be studied.

Acknowledgements The authors would like to thank Miss Sujitra Sukonthamut for her advise on statistical analysis and Faculty of Science, King Mongkut's Institute of Technology Ladkrabang for financial support.

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