International Food Research Journal 25(2): 534-539 (April 2018)
Journal homepage: *http://www.ifrj.upm.edu.my*

Potential of *Melodorum fruticosum* Lour. essential oil against aflatoxigenic *Aspergillus* strains

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Article history

Received: 18 November 2016
Received in revised form: 15February 2017
Accepted: 18 February 2017

Keywords

Melodorum fruticosum Lour.
Essential oil
Aflatoxin
Aspergillus flavus
Aspergillus parasiticus

Abstract

The essential oil from *Melodorum fruticosum* Lour was investigated for an inhibitory effect on the mycelial growth, sporulation and production of aflatoxin B1 by *Aspergillus flavus* IMI 242684 and *Aspergillus parasiticus* IMI 283883. Essential oil of *Melodorum fruticosum* Lour. obtained by hydrodistillation extraction, significantly ($P < 0.05$) inhibited both sporulation and aflatoxin production by these two *Aspergillus* strains and also reduced mycelial growth. Essential oil of *Melodorum fruticosum* Lour. at 1000 ppm showed the inhibition activities on mycelial growth (> 50 %) more than sporulation and aflatoxin B1 production by poison food technique. The IC_{50} of mycelial growth, sporulation and aflatoxin production were 812.64-826.61 ppm, 1,358.59-1,412.43 ppm and 1,043.84-1,302.08 ppm, respectively.

Introduction

Aflatoxins produced by *Aspergillus* species are among the most toxic and carcinogenic compounds in nature. Four aflatoxins, i.e., aflatoxins B1, B2, G1, and G2 are the major toxins among 16 structurally related toxins, of which aflatoxin B1 is particularly important as it is the most hazardous and potent hepatocarcinogenic compound (Bennett and Klich, 2003). Some synthetic chemicals used to control mycotoxin production can cause harmful effects on consumers (Paranagama *et al*., 2003). Plant essential oils, a potential source of antimicrobial agents, are of interest as an alternative way to prevent food or feed from fungal contamination. Many approaches have been investigated on the use of essential oils to inhibit aflatoxin production (Bullerman *et al*., 1974; Thanaboripat, 2003; Thanaboripat *et al.,* 2004, 2007; Kumar *et al*.,2010; Nogueira *et al.*, 2010; Sakuda, 2010). To our knowledge, no documented records on antifungal activity of *Melodorum fruticosum* Lour essential oil against the aflatoxigenic strains, are available. Thus, the objective of this study is to determine an inhibitory effect of essential oil of *Melodorum fruticosum* Lour. at different concentrations against the aflatoxigenic strains such as *Aspergillus flavus* IMI 242684 and *Aspergillus parasiticus* IMI 283883 by Poison food technique as well as MIC and MFC values by the broth microdilution method.

Materials and Methods

Plant material

The flower of *Melodorum fruticosum* Lour. was collected from Ayutthaya Province, Thailand. The flower specimens defined as RU001, was deposited at the Faculty of Science, Ramkhamhaeng University, Bangkok, Thailand.

Extraction of essential oil

The flowers of *Melodorum fruticosum* Lour. were washed twice with distilled water, subsequently, air dried before homogenizing to a fine powder and then stored in airtight bottles. Air-dried plant materials (100 g) were placed in a 1 L round-bottom distillation flask and 300 mL double distilled water was added. Essential oil was then obtained by hydrodistillation for 3 hr, using a Clevenger-type apparatus according to the method recommended by British Pharmacopoeia (1988). The oily layer collected on top of the aqueous distillate was separated and dried over anhydrous sodium sulfate, then stored in a tightly closed dark vial at 4°C until further studies.

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Preparation of spore suspension

Two aflatoxigenic strains, i.e. *Aspergillus flavus* IMI 242684 and *Aspergillus parasiticus* IMI 283883 obtained from the International Mycological Institute (Egham, Surrey, UK) were used throughout this study. The strains were cultured on potato dextrose agar (PDA, Merck, Darmstadt, Germany) slope for 7-10 days at 28±1°C. Spores were harvested aseptically by adding 10 ml of sterile 0.05% (v/v) Tween 80 solution to culture and gently scraping the mycelia 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 106 ml^{-1}.

Determination of the minimum inhibition concentration and minimum fungicidal concentration

Minimum inhibitory concentrations (MICs) were determined using the broth microdilution method modified from CLSI (2006). Serial dilutions of the essential oil (0.02-2.5 mL/mL) were prepared in sterile 96-well microtitre plates (Nest Biotechnology) using PDB media. The *Melodorum fruticosum* Lour essential oil was solubilized in 10% dimethyl sulfoxide (DMSO) and diluted to obtain a final concentration of 10 mg ml^{-1}. PDB (100 µL) was added to all 96 wells of a sterile plastic microtitre plate using a multichannel pipette. The solubilized essential oil (100 µL) was added into each of the first well and then serially diluted as two-fold dilution. The spore suspension, prepared in PDB (100 µL), was added to each well. The final concentrations were 2.5, 1.25, 0.64, 0.32, 0.16, 0.08, 0.04 and 0.02 mg mL^{-1}. A solvent control of DMSO: water (1:9) was used as negative control, and ketoconazole was included as positive control. Spore suspensions of the respective fungus without the addition of essential oil in the wells were served as a comparison. The covered microtitre plates were then incubated at 30°C and examined visually every 24 hr up to 72 hr. Microtiter plate reader was used to determine the growth of fungi by measuring the optical density at 620 nm (OD 620). All tests were performed in triplicate.

The minimum concentration at which no visible growth was observed and defined as the MIC and expressed in mg/mL. After MIC determination, the microtitre plates were shaken automatically for ten seconds. One hundred µL from each well that showed no growth was then placed on PDA. These plates were incubated at 30°C for 72 hr. The lowest concentration of *Melodorum fruticosum* Lour essential oil capable of inhibiting fungal growth was considered to be the MFC.

Poisoned food technique

The antifungal activity of *Melodorum fruticosum* Lour essential oil against *A. flavus* IMI 242684 and *Aspergillus parasiticus* IMI 283883 was based on mycelial growth inhibition using the poisoned food technique described by Grover and Moore (1962), with slight modification. The *Melodorum fruticosum* Lour essential oil concentrations including 0 100 200 300 600 and 1000 ppm were prepared by adding appropriate volumes of essential oil to the sterilized molten PDA medium and then poured into sterilized plates. A 6 mm sterile diameter Whatman No 1 filter paper disc was placed at the center of each PDA plate and inoculated with 10 µl of spore suspension (10^6 spore ml^{-1}). Agar plates were incubated for 120 hr at 30 ±1 °C in the darkness and the average of two perpendicular diameters of each colony was daily calculated. Proper control (PDA without essential oil) was also maintained. The relative growth inhibition of the treatment compared to the control (RGI%) was calculated as a percentage, using the equation : RGI (%) = [(dc-dt)/dc] x 100, where dc is the diameter of fungal colony on the control Petri dish and dt is the diameter of fungal colony on the essential oil-treated Petri dish. A linear regression analysis of percent growth inhibition versus *Melodorum fruticosum* Lour essential oil concentration, estimated as 50% inhibition (IC_{50}), was determined from the regression equation. After growth was evaluated, all samples were used for the analyses of fungal sporulation and aflatoxin B1 (AFB1) qualification.

Effect of Melodorum fruticosum *Lour. essential oil on sporulation of* A. flavus *IMI 242684 and* A. parasiticus *IMI 283883*

Spore production of *Aspergillus* strains was determined using the modified method of Tzortzakis and Economakis (2007). Spores from colonies of *A. flavus* IMI 242684 and *A. parasiticus* IMI 283883 previously incubated for 120 hr and exposed to *Melodorum fruticosum* Lour essential oil by Poisoned food technique, 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 collected and 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] x100. Where Nc is the number of spores in control sample and Ns is the number of spores in

treated sample.

Effect of Melodorum fruticosum *Lour. essential oil on aflatoxin B1 production of* A. flavus *IMI 242684 and* A. parasiticus *IMI 283883*

The antiaflatoxigenic efficacy of essential oil of *Melodorum fruticosum* Lour., was studied on poisoned food technique. After sporulation was determined, agar cultures were extracted with 10 mL of 70% methanol and shaken for 5 minutes before filtered by Whatman no 4. The extracts were then analysed for the aflatoxin B1 (AFB1) content using DOA-Aflatoxin ELISA Test Kit (Chimaphuti *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 shake 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 tetramethylbenzidine substrate was added into the well, incubated for 10 min at room temperature before adding 100 µl of stopping solution (0.3M Phosphoric acid). The solution was read at 450 nm using the automated MicroELISA 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 experimental values were expressed as the means ± standard deviation from three replicate samples times of each treatment. Data analysis was assessed using a one way analysis of variance (ANOVA) followed by a multiple comparison test (Tukey's post hoc test), where $p < 0.05$ was considered as significance.

Results and Discussion

The essential oil of the *Melodorum fruticosum* Lour.(Annonaceae family) was investigated for the inhibition effect on mycelial growth, sporulation and aflatoxin B1 production of *A. flavus* IMI 242684 and *A. parasiticus* IMI 283883. The extraction yield of *Melodorum fruticosum* Lour essential oil was 0.48+0.06%.

The antifungal activity of M*elodorum fruticosum* Lour. essential oil against these two fungi was quantified in term of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) values by broth microdilution. It was found that both fungi gave MIC and MFC values of 0.64 and 2.5 mg/mL, respectively.

Effect of Melodorum fruticosum *Lour. essential oil on mycelial growth of* Aspergillus flavus *IMI 242684 and* A. parasiticus *IMI 283883*

Mycelial growth of *A. flavus* IMI 242684 and *A. parasiticus* IMI 283883 were treated with *Melodorum fruticosum* Lour. essential oil during the 120 hr incubation at 30°C by poisoned food technique, and results were showed in Tables 1-2. It indicates that fungal growth was significantly reduced with increasing concentration of *Melodorum fruticosum* Lour essential oil (i.e. 100, 200, 300, 600 and 1000 ppm) while the mycelial growth increased with incubation time (24, 48, 72, 96 and 120 hr). The radial growth rate was calculated as the slope of the radius versus time plots, and analyzed by linear regression. At 1000 ppm, the radial growth rates of *A.flavus* IMI 24684 and *A.parasiticus* IMI 283883 were reduced from 0.227 mm hr^{-1} (R^2 = 0.9463) to 0.065 mm hr^{-1} (R^2 = 0.8954), and 0.2308 mm hr^{-1} (R2 = 0.9342) to 0.065 mm hr^{-1} (R^2 = 0.8379), respectively. The growth inhibition of *A. flavus* IMI242684 and *A.parasiticus* IMI 283883 by *Melodorum fruticosum* Lour. essential oil, was between 0.55 - 63.78% and 0.4 – 63.8%, respectively, as presented in Tables 3-4.

The IC_{50} values of mycelial growth of *A. flavus* IMI 242684 and *A.parasiticus* IMI 283883 by the different amounts of *Melodorum fruticosum* Lour essential oil were calculated by the least square regression of linear relation between growth inhibition (%) against the logarithm of the essential oil concentration to achieve 50% reduction in the mycelial growth with a correlation coefficient was 0.9103 and 0.9181, respectively, as shown in Table 5. The IC_{50} values of the *Melodorum fruticosum* Lour essential oil against the mycelial growth of *A. flavus* IMI 242684 and *A.parasiticus* IMI 283883 were 826.61 ppm and 812.64 ppm, respectively.

Effect of Melodorum fruticosum *Lour. essential oil on sporulation of* Aspergillus flavus *IMI 242684 and* A. parasiticus *IMI 283883*

The inhibitory activity of the *Melodorum fruticosum* Lour. essential oil on sporulation of *A. flavus* IMI 242684 and *A. parasiticus* IMI 283883

Table 1. Effect of different amount of *Melodorum fruticosum* Lour essential oil on the growth of *A.flavus* IMI 242684 using poisoned food technique.

Time (hr)	Concentration (ppm)					
	0	100	200	300	600	1000
24	9.5 ± 0.5^{a}	9.5 ± 0.0^{a}	9.3 ± 0.3^{a}	8.5 ± 0.5^{a}	6.7± 0.6^{b}	6.7±0.3^{b}
48	14.7 ± 0.6^{a}	14.3 ± 0.3^{a}	13.8 ± 0.3^{ab}	12.3 ± 1.2^{b}	10.5 ± 0.5^{c}	7.8±0.3^{d}
72	24.7 ± 0.6^{a}	23.8 ± 0.3^{a}	22.8 ± 0.3^{ab}	21.2 ± 1.3^{bc}	19.2 ± 1.3^{c}	10.2±0.8^{d}
96	34.3 ± 0.6^{a}	34.0 ± 0.0^{a}	31.7 ± 0.6^{a}	28.2 ± 0.3^{b}	25.3 ± 0.6^{b}	12.8±0.8^{c}
120	54.3 ± 0.6^{a}	54.0 ± 0.0^{a}	50.8 ± 1.3^{b}	42.2 ± 1.8^{c}	36.3 ± 1.2^{d}	19.7±1.2^{e}

Means with different superscripts in the same row are significantly different ($p<0.05$).

Table 2. Effect of different amount of *Melodorum fruticosum* Lour essential oil on the growth of *A.parasiticus* IMI 283883 using poisoned food technique.

Time (hr)	Concentration (ppm)					
	0	100	200	300	600	1000
24	9.5 ± 0.5^{a}	9.3 ± 0.3^{a}	9.2 ± 0.3^{a}	8.7 ± 0.6^{a}	8.0± 0.0^{b}	6.5±0.0^{c}
48	14.3 ± 0.6^{a}	14.2 ± 0.3^{a}	13.2 ± 1.0^{ab}	12.0 ± 1.0^{bc}	11.2 ± 0.3^{c}	7.2±0.3^{d}
72	24.3 ± 0.6^{a}	23.8 ± 0.3^{a}	22.5 ± 0.5^{ab}	20.8 ± 1.4^{bc}	19.5 ± 0.9^{c}	9.5±0.5^{d}
96	33.7 ± 0.6^{a}	33.5 ± 0.0^{a}	31.7 ± 1.0^{a}	27.8 ± 0.8^{b}	25.7 ± 0.3^{b}	11.7±0.6^{c}
120	55.2 ± 1.0^{a}	55.0 ± 0.5^{a}	51.2 ± 1.4^{b}	42.3 ± 1.5^{c}	36.7 ± 0.6^{d}	20.0±2.0^{e}

Means with different superscripts in the same row are significantly different according ANOVA and Tukey's multiple comparison tests ($p < 0.05$).

was shown in Tables 3-4. The results indicate that sporulation inhibition was significantly ($p < 0.05$) increased with increasing amount of *Melodorum fruticosum* Lour essential oil. The sporulation of *A. flavus* IMI 242684 and *A. parasiticus* IMI 283883 after 120 hr of incubation was inhibited within the range of 0.83- 40.8% and 5.81-43.0%, respectively, when compared with control. The IC_{50} values of the *Melodorum fruticosum* Lour. essential oil against the sporulation of *A. flavus* IMI 242684 and *A.parasiticus* IMI 283883 were 1,412.43 ppm and 1,358.59 ppm with a correlation coefficient of 0.899 and 0.866, respectively, as shown in Table 5.

Effect of Melodorum fruticosum *Lour. essential oil on aflatoxin production of* Aspergillus flavus *IMI242684 and* A. parasiticus *IMI 283883*

The inhibitory activity of the *Melodorum fruticosum* Lour essential oil on aflatoxin B1 production of *A. flavus* IMI 242684 and *A. parasiticus* IMI 283883 was shown in Tables 3-4. The statistical analysis indicates that percent of aflatoxin B1 inhibition was significantly ($p <0.05$) increased with increasing amount of *Melodorum fruticosum* Lour essential oil, similar to the case of sporulation inhibition. The aflatoxin B1 production of *A. flavus* IMI 242684 and *A.parasiticus* IMI 283883 after 120 hr of incubation were inhibited within the range of 2.1-47.2% inhibition, when compared with control. The IC_{50} values of the *Melodorum fruticosum* Lour. essential oils against the aflatoxin B1 production of *A. flavus* IMI 242684 and *A.parasiticus* IMI 283883 were 1,302.08 ppm and 1,043.84 ppm with a correlation coefficient of 0.8997 and 0.8792, respectively, as shown in Table 5.

According to previous studies, the main compound in *Melodorum fruticosum* Lour. flower was linalool (Sacchetti *et al*., 2005; Pripdeevech and Chukeatirote, 2010). Linalool has also been proved to have antifungal activities (Alginiannis *et al*., 2000; Simic *et al*., 2004; Herman *et al.*, 2006; Terzi *et al*., 2007; Singh *et al*., 2010). Although, the antimicrobial activity of essential oil is attributed mainly to its major compounds but some minor components might be involved in biological activity and synergistic interactions with other active compounds. Considering a large number of compounds in the essential oil, it may have more than one site of action and also with the different mode of action against mycelial growth, sporulation, and aflatoxin production. The potential effects of essential oils on the fungal growth, sporulation and

Table 3. Effect of different amount of *Melodorum fruticosum* Lour essential oil on the mycelial growth of *A.flavus* IMI 242684, sporulation and aflatoxin production using poisoned food technique.

Mode of Inhibition (%)	Concentration (ppm)				
	100	200	300	600	1000
Mycelial growth	0.55^{e}	6.4^{d}	22.3^{c}	33.1^{b}	63.78^{a}
Sporulation	0.83^{cb}	7.5^{bc}	10.42^{bc}	12.5^{b}	40.8^{a}
Aflatoxin production	8.1^{bc}	12.1^{bc}	17.1^{ab}	22.9^{ab}	35.5^{a}

Means with different superscripts in the same row are significantly different according ANOVA and Tukey's multiple comparison tests ($p < 0.05$).

Table 4. Effect of different amount of *Melodorum fruticosum* Lour essential oil on the mycelial growth of *A. parasiticus* IMI 283883, sporulation and aflatoxin production using poisoned food technique.

Mode of Inhibition (%)	Concentration (ppm)				
	100	200	300	600	1000
Mycelial growth	0.4^{e}	7.31^{d}	23.3^{c}	33.6^{b}	63.8^{a}
Sporulation	5.81^{cb}	7.4^{b}	8.9^{b}	12.4^{b}	43.0^{a}
Aflatoxin production	2.8^{c}	3.9^{c}	20.8^{b}	28.9^{b}	47.2^{a}

Means with different superscripts in the same row are significantly different according ANOVA and Tukey's multiple comparison tests ($p < 0.05$).

aflatoxins production have been investigated by many researchers (Rasooli and Abyaneh., 2004; Prakash *et al*., 2012; Tian *et al*., 2012). Several studies have been conducted to understand the mechanism of action of essential oils. The antifungal mechanism of essential oil was related to their low molecular weight and highly lipophilic characters which can penetrate inside the cell and interfere with cellular metabolism. Essential oils can also disturb the cellular membrane, react with active sites of enzymes or act as a hydrogen ion carrier and deplete adenosine triphosphate pool (Farag *et al*., 1989; Marino *et al*.,2001 ; Chao *et al*., 2005; Rassoli and Owlia 2005). Likewise, the aflatoxin B1 production inhibition efficacy of essential oil may be because of inhibition of carbohydrate catabolism in *Aspergillus flavus* by acting on some key enzymes, reducing its ability to produce aflatoxins (Tian *et al*., 2012). In addition, the inhibition of fungal growth may also inhibit aflatoxin production(Mahmoud, 1999).

Table 5. IC_{50} of *Melodorum fruticosum* Lour essential oil against mycelia growth, sporulation and aflatoxin production by *A.flavus* IMI 242684 and *A.parasiticus* IMI 283883

Mode of action	*A.flavus* IMI 242684 (ppm)	*A.parasiticus* IMI 283883 (ppm)
Mycelial growth	826.61	812.64
Sporulation	1,412.43	1,358.59
Aflatoxin production	1,302.08	1,043.84

Conclusion

This study reports for the first time the inhibition activity of the *Melodorum fruticosum* Lour. essential oil on the growth of the aflatoxigenic strain (*Aspergillus flavus* IMI 242684 and *A.parasiticus* IMI 283883.) and their aflatoxin production. The inhibitory effect was increased with the higher amount of *Melodorum fruticosum* Lour essential oil. *Melodorum fruticosum* Lour essential oil could be used as an alternative agent to reduce the risk of aflatoxin contamination in both animal feed and human food chains.

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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