

Effects of Tropical Citrus Essential Oils on Growth, Aflatoxin Production, and Ultrastructure Alterations of *Aspergillus flavus* and *Aspergillus parasiticus*

Kadsarin Rammanee · Tipparat Hongpattarakere

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Abstract Ethyl acetate extracts and hydrodistilled essential oils from five cultivars of tropical citrus epicarps were evaluated for their inhibitory activities against *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus parasiticus*, and *Penicillium* sp. using disk diffusion and broth microdilution assays. Essential oils prepared from kaffir lime (*Citrus hystrix* DC) and acid lime (*Citrus aurantifolia* Swingle) epicarps exhibited stronger antifungal activity to all fungi than their ethyl acetate extracts with minimum inhibitory concentration and minimum fungicidal concentration values of 0.56 and 1.13 mg/ml (dry matter), respectively, against aflatoxin-producing *A. flavus* and *A. parasiticus*. The dominant components of the essential oil from kaffir lime were limonene, citronellol, linalool, *o*-cymene, and camphene, whereas limonene and *p*-cymene were major components of acid lime essential oil. Pure limonene, citronellal, and citronellol were five to six times less fungicidal than the natural essential oils, indicating the synergistic activity of many active compounds present in the oils. Kaffir and acid lime essential oils significantly reduced aflatoxin production of *A. flavus* and *A. parasiticus*, particularly lime essential oil, which completely inhibited growth and aflatoxin production of *A. flavus* at the concentration of 2.25 mg/ml. Target cell damage caused by acid lime essential oil was investigated under transmission electron microscopy. Destructive alterations of plasma and

nucleus membrane, loss of cytoplasm, vacuole fusion, and detachment of fibrillar layer were clearly exhibited in essential-oil-treated cells.

Keywords Citrus essential oils · *Aspergillus flavus* · *Aspergillus parasiticus* · Aflatoxin · Antifungal · TEM

Introduction

Mycotoxins are well known for their health-hazardous effect in humans and animals. Among all mycotoxins, aflatoxin is the most toxic form for mammals and presents hepatotoxic, teratogenic, and mutagenic properties, causing damage such as toxic hepatitis, hemorrhage, edema, immunosuppression, and hepatic carcinoma (Reddy et al. 2004). Aflatoxin-producing fungi are widely distributed in nature and frequently contaminate human food resources. Aflatoxin is a secondary metabolite produced by several species of *Aspergillus*, particularly *Aspergillus flavus* and *Aspergillus parasiticus* which are the most common spoilage fungi in food and feedstuffs (Egal et al. 2005).

Use of synthetic chemicals as fungicidal preservatives to extend shelf life of food products and to ensure food safety has been widely practiced, leading to health and environmental problems as well as development of synthetic fungicide-resistant microorganisms. As a result of the increase of consumer's demand for safe and natural preservatives, natural fungicides have been intensively investigated. Among natural antimicrobials, plant essential oils have exhibited potential inhibition against both bacteria and fungi (Baratta et al. 1998; Caccioni et al. 1998; Lanciotti et al. 2004; Mahmud et al. 2009; Melendez and Capriles 2006; Samy 2005; Soliman

K. Rammanee · T. Hongpattarakere (✉)
Department of Industrial Biotechnology,
Faculty of Agro-Industry, Prince of Songkla University,
Hat Yai,
Songkhla 90112, Thailand
e-mail: tipparat.h@psu.ac.th

and Badeaa 2002; Wannissorn et al. 2005). Recently, the potential antimicrobial uses of citrus essential oils were extensively reviewed (Fisher and Phillips 2008). Pronounced antifungal activity of essential oils from many citrus cultivars grown in temperate climate has been extensively recognized. Matan and Matan (2007) reported that lime essential oil displayed antifungal activities against *Aspergillus niger* and *Penicillium* sp. Viuda-Martos et al. (2008) found that the essential oils of lemon (*Citrus lemon* L.), mandarin (*Citrus reticulata* L.), grapefruit (*Citrus paradisi* L.), and orange (*Citrus sinensis* L.) showed antifungal activity against *A. flavus*, *A. niger*, *Penicillium chrysogenum*, and *Penicillium verrucosum*. Antifungal activities of hydrodistilled essential oils from orange (*C. sinensis* cv. “Washington navel,” “Sanguinello,” “Tarocco,” “Moro,” “Valencia late,” and “Ovale”), bitter (sour) orange (*Citrus aurantium*), mandarin (*Citrus deliciosa* cv. “Avana”), grapefruit (*C. paradisi* cv. “Marsh seedless” and “Red Blush”), citrange (*C. sinensis* × *Poncirus trifoliata* cv. “Carrizo” and “Troyer”), and lemon (*Citrus limon* cv. “Femminello”) were shown against *Penicillium digitatum* and *Penicillium italicum* (Caccioni et al. 1998). Essential oils from *C. sinensis* (L.), *C. aurantium* L. (Rutaceae), *Citrus bergamia* Risso and Poit (Rutaceae), *C. limon* (Linn.) Burm.f. (Rutaceae), and *Citrus bigaradia* Hook.f. (Rutaceae) inhibited growth of *A. niger* (Pawar and Thaker 2006; Sharma and Tripathi 2008).

To date, antifungal activities of tropical citrus oils against aflatoxin-producing fungi regarding their chemical composition as well as mode of action have not been extensively investigated. Among a few studies on essential oils from tropical citrus, *Citrus hystrix* DC (kaffir lime) and *Citrus aurantifolia* exhibited antibacterial activity against *Bacillus cereus*, *Staphylococcus aureus*, and *Salmonella typhi* (Chaisawadi et al. 2003). Similarly, the antimicrobial activities against *S. aureus*, *B. cereus*, *Listeria monocytogenes*, *Saccharomyces cerevisiae*, and *Aspergillus fumigatus* of ethyl acetate extracts from *C. hystrix* DC were also reported (Chathaphon et al. 2008). Hydrodistilled essential oil from *Citrus reticulata* Blanco inhibited growth to fungal sporulation of plant pathogenic fungi, such as *Alternaria alternate*, *Rhizoctonia solani*, *Helminthosporium oryzae*, *Curvularia lunata*, and *Fusarium oxysporum* (Chutia et al. 2009). The oil from tropical sour lime (*C. aurantifolia* Swingle) showed 47.8% growth inhibition and suppressed aflatoxin production of *A. parasiticus* (Razzaghi-Abyaneh et al. 2009).

In this study, antifungal activities against *A. flavus* and *A. parasiticus* and their aflatoxin production of essential oils and ethyl acetate extracts from five tropical citrus cultivars grown in Thailand were evaluated and compared according to the National Committee for Clinical Laboratory Standards standard method in parallel with chemical composition analysis. Morphological alterations of the fungal cells caused

by the tropical citrus essential oil were also revealed through transmission electron microscopy (TEM).

Materials and Methods

Plant Materials

Five tropical citrus cultivars of kaffir lime (*C. hystrix* DC), acid lime (*C. aurantifolia* Swingle), shogun mandarin (*C. reticulata* cv. Shogun), pomelo (*Citrus maxima* Merr.), and acidless orange (*C. paradisi*) were collected at the mature stage from three fruit orchards located around the Songkhla and Nakornsri-thumarat area during November 2007 to January 2008.

Extraction Procedures

Chopped citrus epicarps (500 g) were subjected to 4 h of hydrodistillation performed using a simple quick-fit apparatus, in which a 2,000-ml steam generator flask, a distillation flask coupled with a condenser, and a receiving vessel were assembled. The steam generator flask filled with 500 ml of water was heated with a heating mantle. The water vapor passed to the distillation flask containing citrus peel and then condensed. The distillate was collected in the receiving flask and dried over anhydrous sodium sulfate. The essential oils were stored under N₂ in tightly sealed vials at 4°C.

Ethyl acetate extracts were prepared by grinding 500 g of citrus epicarps to fine powder, then soaking in 2 l of ethyl acetate and mixing at the shaking speed of 130 rpm for 8 h. The epicarp residue was removed by filtration through Whatman filter paper no.4. The solvent extracts were dried over anhydrous sodium sulfate. Ethyl acetate was removed using a rotary vacuum evaporator. The concentrated extract was transferred for complete drying with nitrogen evaporator to yield crude ethyl acetate extract, which was stored under nitrogen gas in sealed vials at 4 °C. The percentage of essential oil and extract yield (w/w) was calculated based on dry matter of citrus epicarp.

Fungal Strains and Culture Condition

Aflatoxin-producing fungi including *A. flavus* Thailand Institute of Scientific and Technological Research (TISTR) 3041 and *A. parasiticus* TISTR 3018 obtained from the culture collection of the TISTR, Phatumthani, Thailand, were grown on Difco™ potato dextrose agar (PDA) at 35 °C for 7 days. The spore inoculum was prepared by suspending in sterile 0.1% Tween 80. The spore count was performed using hemacytometer before being diluted to achieve the concentration of 10⁶ spores per milliliter with potato dextrose

broth (PDB). The diluted fungal spore inoculum was spread over the PDA surface using sterile cotton swab to obtain uniform fungal growth for antifungal determination using agar disk diffusion assay.

Determination of Antifungal Activity by Agar Disk Diffusion Assay

All hydrodistilled essential oils and ethyl acetate extracts of citrus epicarps from five citrus cultivars were tested for antifungal activity against *A. flavus* and *A. parasiticus* by disk diffusion assay. The essential oils and the citrus extracts were dissolved in dimethyl sulfoxide (DMSO) to achieve a concentration of 50 mg/ml and then were sterilized by filtration through a 0.45- μ m Syringe filter sterile-EO (Sartorius-Minisart, Germany) before addition (10 μ l) to a sterilized paper disk (6-mm diameter) and left to dry for 10 min in a biosafety cabinet. The paper disk contained the essential oil and DMSO (control) on the agar plate inoculated with fungal spore. The plates were then incubated at 35 °C for 48 h. Diameters of inhibition zones were measured with a vernier caliper. All treatments were performed in three replicates.

Determination of the Minimum Inhibitory Concentration and Minimum Fungicidal Concentration

The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values were determined by using broth microdilution assays performed in 96-well microtiter plate according to the National Committee for Clinical Laboratory Standards (2002) and Sugar and Liu (1995) with some modification. Twenty microliters of fungal spore suspension (culture and diluted as mentioned above to yield 10^6 spores per milliliter) was added to 180 μ l PDB to achieve a final concentration of 10^5 spores per milliliter in each well. Citrus essential oil/extract was added at the twofold dilution manner to achieve the final concentration of essential oils ranging from 0.07 to 2.25 mg/ml (dry matter). The plate was then incubated at 35 °C, and fungal growth was determined visually at 48 h of incubation. The lowest concentration of citrus essential oil/extract required to completely inhibit fungal growth (optically clear or absence of growth compared with a control well without fungal inoculum) after incubation for 48 h (endpoint) was reported as the MIC value defined by the National Committee for Clinical Laboratory Standards (2002). Fungal viability in the well which showed no growth was determined by transferring and spreading 100 μ l of the culture broth on PDA and incubating it at 35 °C for 48 h. The lowest concentration of ethyl acetate extract or hydrodistilled essential oil required to completely destroy the test fungi (no growth on the agar plate) after incubation at 35 °C for 48 h was defined as the MFC. Each

ethyl acetate extract or essential oil was evaluated for its antimicrobial activity in triplicate on two separate runs.

Chemical Composition of Citrus Essential Oils

The analysis of the essential oil composition was performed by gas chromatography–mass spectrophotometry (GC–MS) on a Hewlett Packard 5890 Gas chromatograph with an INNOWax column (30 m \times 0.25 μ m ID, 0.25) and a Hewlett Packard 5972 Mass Detector. For MS detection, an electron ionization system with ionization energy of 70 eV and MS transfer line at temperature of 260 °C was used. Column temperature was initially maintained at 80 °C for 1 min and gradually increased at a rate of 5 °C/min until reaching 250 °C, at which the temperature was held for 2 min. Helium was used as carrier gas at a flow rate of 1 ml/min. The sample of 1 μ l was injected in the acquisition mode. The components were identified based on the comparison of their relative retention times and fragmentation pattern of mass spectra compared with those reported in the literature as well as the Wiley, 275.L data library of the GC–MS system (Agnihotri et al. 2004).

Effects of Citrus Essential Oils/Extracts on Growth and Aflatoxin Production of *A. flavus* and *A. parasiticus* in Liquid Medium

Semi-synthetic yeast extract–sucrose (YES) containing 2 g yeast extract and 15 g sucrose in 100 ml of distill water was used for aflatoxin production (Nogueira et al. 2010; Vergopoulou et al. 2001). Spore suspension (in sterile 0.1% Tween 80) was prepared from *A. flavus* and *A. parasiticus* cultivated in YES agar at 30 °C for 7 days. The spore suspensions (500 μ l) of the fungi containing 10^6 spores per milliliter were transferred to 20 ml of YES broth (with addition of 0, 0.28, 0.56, and 1.13 mg/ml of essential oils). Each flask was incubated at 30 °C for 72 h under shaken speed of 200 rpm. Fungal growth was determined by measurement of dried fungal mycelium according to Razzaghi-Abyaneh et al. (2009). The culture broth was filtered through filter paper (Whatman no. 1). The mycelia were washed with sterile water and then allowed to dry at 80 °C until a constant weight was obtained. Aflatoxin content in culture supernatant was determined by adding 100 ml of 70% methanol to 20-ml supernatant. The supernatant–methanol mixture was then shaken at 300 rpm for 30 min and then filtered through filter paper. The obtained aliquot was further analyzed for aflatoxin.

Aflatoxin Assay

Aflatoxin B₁ (AFB₁) content was determined using the Department of Agriculture enzyme-linked immunosorbent

assay (ELISA) test kit (Department of Agriculture, Thailand). Direct competitive ELISA was employed for this measurement, and the analysis procedure was performed according to the manufacturer's instruction (Chinaphuti et al. 2002). A sufficient number of the microwell strips coated with purified polyclonal antibody against AFB₁ supplied by the manufacturer were inserted into the holder. Fifty microliters of Sigma AFB₁ standard solutions or diluted sample (1:20) was dispensed into each antibody-coated well, followed by addition of 50 μ l AFB₁ horseradish peroxidase conjugate. The microwell strips were then incubated at room temperature for 30 min. The content was later discarded, and the microwell holder was tapped upside down vigorously against an absorbent paper. All wells were subsequently washed three to five times with 0.01 M phosphate buffer saline+0.5% Tween 20 in a similar manner. The substrate (100 μ l of tetramethylbenzidine, KPL, Inc.) was then added into each well. After incubation at room temperature for 10 min, stopping solution of 0.3 M phosphoric acid (100 μ l) was finally added. The absorbance was measured at 450 nm in BioTek[®] microplate reader (model Power Wave Xs, BioTek Instruments, Inc., USA). The AFB₁ level (ppb) was estimated from OD₄₅₀, corresponding to the AFB₁ standard curve.

Transmission Electron Microscopy

A. flavus and *A. parasiticus* (10⁸ spores per milliliter inoculum) were grown in PDB with and without kaffir lime essential oil (MIC) at 35 °C for 48 h before being subjected to TEM analysis according to Rasooli et al. (2006). Fungal mycelia were pre-fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 3 h at room temperature. After washing three times in 0.1 M phosphate buffer (5 min each time), the specimens were post-fixed in 1% osmium tetroxide (OsO₄) for 1–2 h at room temperature and washed three times in water (5 min each). Samples were dehydrated in a graded series of ethanol (30%, 50%, 70%, 80%, and 100%, two times for 5 min each). Fixed mycelia were processed in graded propylene oxide twice (15 min each), propylene oxide/Epon 812 (1:1) for 1–2 h, and finally were embedded in 100% Epon 812. The polymerization to form a specimen block was achieved at 70–80 °C overnight. The specimen blocks were sectioned using an ultramicrotome (Drukker International, Netherland) with 80–90-nm thickness (golden yellow section). The ultrathin section was placed on 200-mesh copper grids and stained with 5% uranyl acetate (dissolved in 70% methanol) for 20 min and then with lead citrate (dissolved in 70% methanol) for 15 min. The grids were dried and examined under JOEL Electron Microscope, model JEM-2010 (Japan).

Results and Discussion

The extraction yields of hydrodistilled essential oils and ethyl acetate extracts from fresh peels of *Citrus* spp. widely varied, depending on citrus cultivars. For each cultivar, the production yield of the essential oil was much lower than its ethyl acetate extract (Fig. 1). The production yields of ethyl acetate extract from kaffir lime, lime, pomelo, acidless orange, and shogun epicarps were 2.36%, 1.53%, 1.91%, 1.08%, and 2.15% (w/w dry basis), whereas only 0.77%, 0.45%, 0.2%, 0.15% and 0.49% (w/w dry basis) of essential oil were obtained from hydrodistillation, respectively. Kaffir lime epicarp yielded the highest amount of ethyl acetate extract and essential oil compared with other citrus cultivars. The lowest yields of both extract and essential oil were obtained from acidless orange epicarp. The result was correlated to Chathaphon et al. (2008) who reported that the production yield of hydrodistilled essential oil was much lower than that obtained from ethyl acetate extraction with the highest yield obtained from kaffir lime.

Antifungal activity of essential oil and ethyl acetate extracts from fresh citrus epicarps was determined using the disk diffusion assay. Among all citrus cultivars, only essential oil and ethyl acetate extract from kaffir lime inhibited the growth of all fungi tested including aflatoxigenic *A. flavus* and *A. parasiticus*, *A. fumigatus*, *A. niger*, and *Penicillium* sp. Such broad inhibitory spectrum was also observed in the treatment of essential oil from acid lime (Table 1). No antifungal activity was detected from pomelo, acidless orange, and shogun essential oils, whereas their ethyl acetate extracts was inhibitory to *A. fumigatus* which was most sensitive to all citrus oils and extracts (data not shown). Among aflatoxin-producing fungi, *A. parasiticus* was more sensitive to kaffir lime essential oil than *A. flavus*, which was more susceptible to acid lime essential oil in disk diffusion

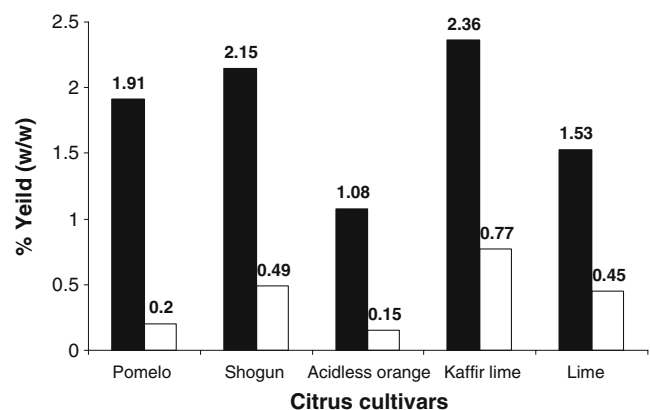


Fig. 1 Extraction yields (w/w of dry matter) of ethyl acetate extracts (black square) and essential oils (white square) from epicarps of various citrus cultivars

Table 1 Antifungal activity of essential oils and ethyl acetate extracts of citrus epicarps (0.5 mg)

| Fungi | Inhibition zone (mm) | | | |
|------------------------|----------------------|------------|------------------------|-----------|
| | Essential oils | | Ethyl acetate extracts | |
| | Kaffir lime | Lime | Kaffir lime | Lime |
| <i>A. flavus</i> | 17.33±0.28 | 24.13±0.32 | 13.33±0.57 | NI |
| <i>A. parasiticus</i> | 29.63±0.23 | 18.13±0.32 | 15.13±0.32 | NI |
| <i>A. fumigatus</i> | 29.85±0.25 | 28.63±1.09 | 14.78±0.73 | 9.65±0.56 |
| <i>A. niger</i> | 27.38±0.24 | 22.82±0.42 | 12.98±0.02 | NI |
| <i>Penicillium</i> sp. | 24.91±0.38 | 22.85±6.15 | 12.25±0.25 | NI |

The activity was determined by disk diffusion assay, and diameters of zones of inhibition were measured and expressed as millimeters

assay. Both kaffir and acid lime essential oils exhibited the same MIC and MFC values of 0.56 and 1.13 mg/ml, respectively, against aflatoxin-producing *A. flavus* and *A. parasiticus* (Table 2) in broth dilution assay, indicating their potent fungicidal effects.

According to a previous report, oil and extract from kaffir lime inhibited a broad spectrum of microorganisms including bacteria, yeast, and molds. The ethyl acetate extract of kaffir lime showed potent inhibition against *S. aureus*, *B. cereus*, *L. monocytogenes*, *S. cerevisiae* var. *sake*, and *A. fumigatus* TISTR 3180, whereas its essential oil was less effective (Chathaphon et al. 2008). However, aflatoxin-producing fungi appeared to be more sensitive to the hydrodistilled oil than the ethyl acetate extract in this study. Figure 2 indicated that fungal growth inhibition depended on the concentration of acid and kaffir lime oils. The oils only delayed fungal growth at low concentration, and the complete destruction was achieved at higher concentration. At the MIC level (0.56 mg/ml), a significant inhibitory effect was apparent within 12 h, but the fungal growth increased slowly after 15 h of incubation (Fig. 2). This particular growth may be due to sub-populations of fungi that are more resistant to the oils. The addition of acid and kaffir lime oils caused complete death to *A. parasiticus* and *A. flavus* after 20- and 24-h exposure, respectively, when the concentration of 1.13 mg/ml was applied. The result was in good agreement with the experiment conducted by Razzaghi-Abyaneh et al. (2009), in which 47.8% growth inhibition of *A. parasiticus* NRRL 2999 was

reported in the presence of 1 mg/ml of acid lime (*C. aurantifolia*) essential oil. A similar effective concentration (about 1 mg/ml) of many citrus essential oils on mold inhibition was also observed in broth assay by Caccioni et al. (1998) and Viuda-Martos et al. (2008).

Many studies have reported the inhibitory activity against bacteria, yeasts, and molds of essential oils from subtropical citrus using different antifungal assays performed under various conditions (Baratta et al. 1998; Belletti et al. 2004; Chaisawadi et al. 2003; Chathaphon et al. 2008; Sharma and Tripathi 2008). Sharma and Tripathi (2008) demonstrated that *C. sinensis* (L.) essential oil inhibited *A. niger* mycelial growth (92–100% radial growth inhibition) at the concentration of 2.5–3.0 µg/ml in solid media. On the contrary, no antifungal activity against *A. parasiticus* of the essential oil from the same citrus cultivar was observed in another experiment in broth assay (Razzaghi-Abyaneh et al. 2009). As it is well-recognized that agar diffusion assay is more sensitive in detecting inhibitory activity because the extract is concentrated on the disk by drying and concentration gradient of the extract was generated around the disk, therefore, the fungal spore was exposed to a higher concentration of the extract nearby the disk than in the broth system if the same amount of the extract was applied. According to Baratta et al. (1998), 84% growth inhibition (percentage of biomass relative to control) against *A. niger* of 10 µl/ml lemon essential oil was observed in broth assay. Inhibitory activity against *P. digitatum* and *P. italicum* of essential oils from six orange cultivars, mandarin, grapefruit,

Table 2 MIC and MFC values (mg/ml) of essential oils and ethyl acetate extracts from kaffir and acid limes against food-spoilage fungi

| Fungi | MIC/MFC (mg/ml) | | | |
|------------------------|-----------------|-----------|------------------------|-------------|
| | Essential oils | | Ethyl acetate extracts | |
| | Kaffir lime | Lime | Kaffir lime | Lime |
| <i>A. flavus</i> | 0.56/1.13 | 0.56/1.13 | >2.25/>2.25 | >2.25/>2.25 |
| <i>A. parasiticus</i> | 0.56/1.13 | 0.56/1.13 | >2.25/>2.25 | >2.25/>2.25 |
| <i>A. fumigatus</i> | 0.56/0.56 | 1.13/2.25 | >2.25/>2.25 | >2.25/>2.25 |
| <i>A. niger</i> | 0.56/1.13 | 0.56/1.13 | >2.25/>2.25 | 2.25/2.25 |
| <i>Penicillium</i> sp. | 1.13/1.13 | 1.13/2.25 | >2.25/>2.25 | 2.25/>2.25 |

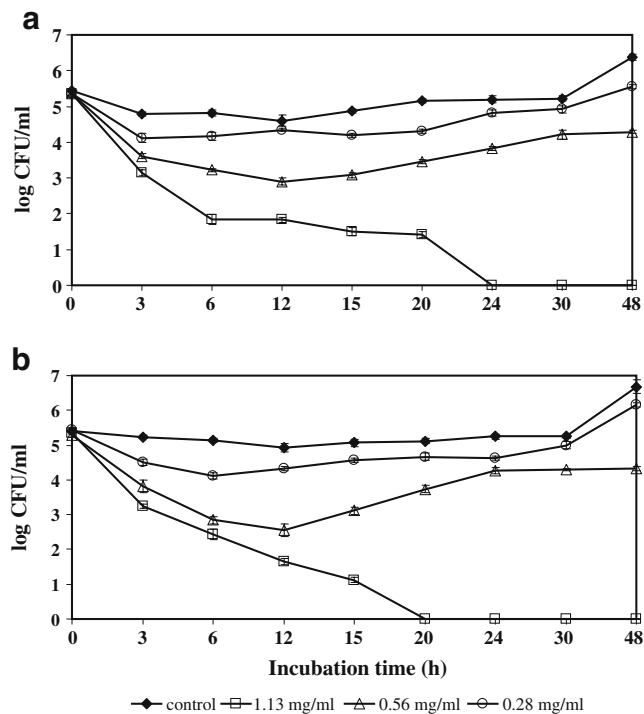


Fig. 2 Inhibitory effects of acid and kaffir lime essential oils on *A. flavus* (a) and *A. parasiticus* (b), respectively, in potato dextrose broth at 35 °C for 48 h under shaking speed of 200 rpm

bitter (sour) orange, and citrange was also reported, despite their high concentration application of >1,000 µg/ml (Caccioni et al. 1998). Similarly, antifungal activity of lemon, mandarin, grapefruit, and orange essential oils showed complete inhibition against *P. chrysogenum*, *P. verrucosum*, *A. niger*, and *A. flavus* at a concentration of 0.94% (Viuda-Martos et al. 2008). Such conflicting results may be contributed from the variations in antifungal assay, test fungal strain, assay condition, and extraction procedure. Moreover, changes of genotype, plant part, origin, climate, season, soil composition, and maturation stage strongly influenced the quantity and chemical compositions of the essential oils from citrus; hence, there is incomparability of antimicrobial activity (Caccioni et al. 1998; Lota et al. 2000; Vekiari et al. 2002).

The effects of the essential oils on aflatoxin production and dry biomass in liquid culture of YES medium were shown in Fig. 3. Obviously, a remarkable reduction in growth and aflatoxin production was dependent on the concentration of essential oils. Significant reduction of mycelial growth and aflatoxin production were associated with the increase of the essential oil concentrations from both kaffir and acid limes. It is evident that essential oils from kaffir and acid lime at the concentration of 2.25 mg/ml were effective on complete retardation of mycelial growth and aflatoxin production of *A. flavus*, while kaffir lime essential oil caused 98% mycelial reduction in *A. parasiticus*. Likewise, significant aflatoxin reduction (89%) was also observed in *A. parasiticus* NRRL

2999 treated with 1 mg/ml essential oil from *C. aurantifolia* (Razzaghi-Abyaneh et al. 2009).

Analysis of chemical components in kaffir lime and lime essential oils by GC–MS resulted in identification of 19 and three compounds from a total of 47 and 19 compounds, respectively. Kaffir lime essential oil contained multiple antimicrobial compounds with major constituents of citronellol (10.67%), limonene (7.32%), linalool (5.83%), and *o*-cymene (5.51%), correlating to its broad-spectrum inhibition. Dominant components from lime essential oil were limonene (69.11%) and *p*-cymene (12.77%). The major component (84.2%) limonene was also reported in *C. sinensis* essential oil. The antimicrobial activity of these compounds was previously reported. Pure limonene at a concentration of 25 µl/l was reported to effectively inhibit *Fusarium verticillioides* (Dambolena et al. 2008). Strong mycelial growth inhibition of *F. oxysporum* by *p*-cymene was revealed by Sekine et al. (2007). Ultee et al. (2002) elaborated the partitioning of cymene to liposomal membrane resulting in the expansion and destabilization of *B. cereus* cytoplasmic membrane without any inhibitory effect on cell growth. Vapor treatments with citronellal and linalool inhibited 98% and 97% hyphal growth and 85% and 70% spore germination of *Alternaria alternata*, respectively. Moreover, citronellal was thoroughly proven to be a major antibacterial compound in kaffir lime extract against *B. cereus* by attacking cytoplasmic membrane (data not shown). Other monoterpenes including citronellol, limonene, β-myrcene, β-pinene, α-pinene, and α-terpineol also inhibited only hyphae growth (Yamasaki et al. 2007). Also, citronellal and citronellol were reported to effectively inhibit human pathogenic fungus, *Microsporium gypseum*, which is known to cause hair and scalp infections (Saikia et al. 2001).

In this study, limonene and cymene appeared to be the major components present in the essential oils from both kaffir and acid limes, whereas a lower level of limonene and no cymene were detected in both ethyl acetate extracts (Fig. 4.),

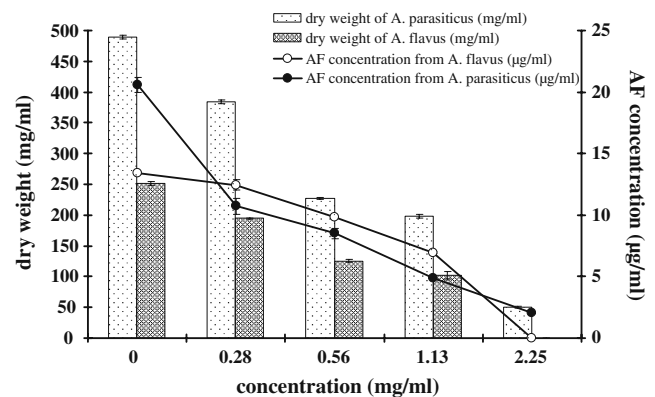


Fig. 3 Effects of kaffir lime and acid lime essential oil on growths and aflatoxin production of *A. parasiticus* and *A. flavus*, respectively, in YES broth at 30 °C for 72 h under shaking speed of 200 rpm

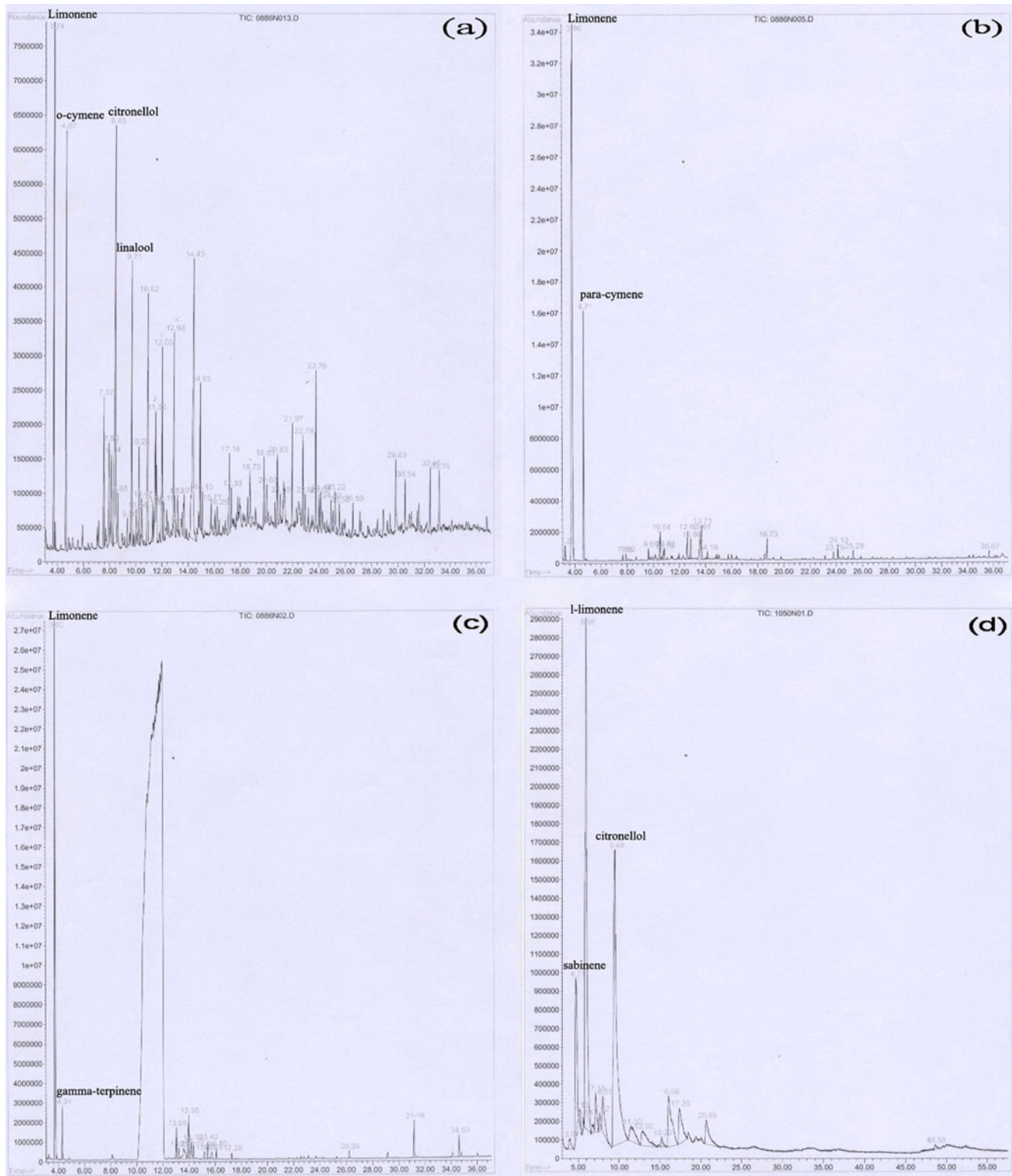


Fig. 4 GC chromatograms of essential oils (a, b) and ethyl acetate extracts (c, d) from kaffir (a, c) and acid limes (b, d)

which exhibited lower antifungal activity. According to Fig. 4, limonene could play a major contribution to the antifungal activity of acid lime essential oil, whereas combinations of limonene with other components such as

citronellol and linalool may be responsible for the antifungal activity of kaffir lime essential oil because they were present in the significant level. In addition, many other minor compounds such as *o*-cymene, sabinene, β -myrcene,

Table 3 MIC and MFC values (mg/ml) of limonene, citronellal, and citronellol against *A. flavus* and *A. parasiticus*

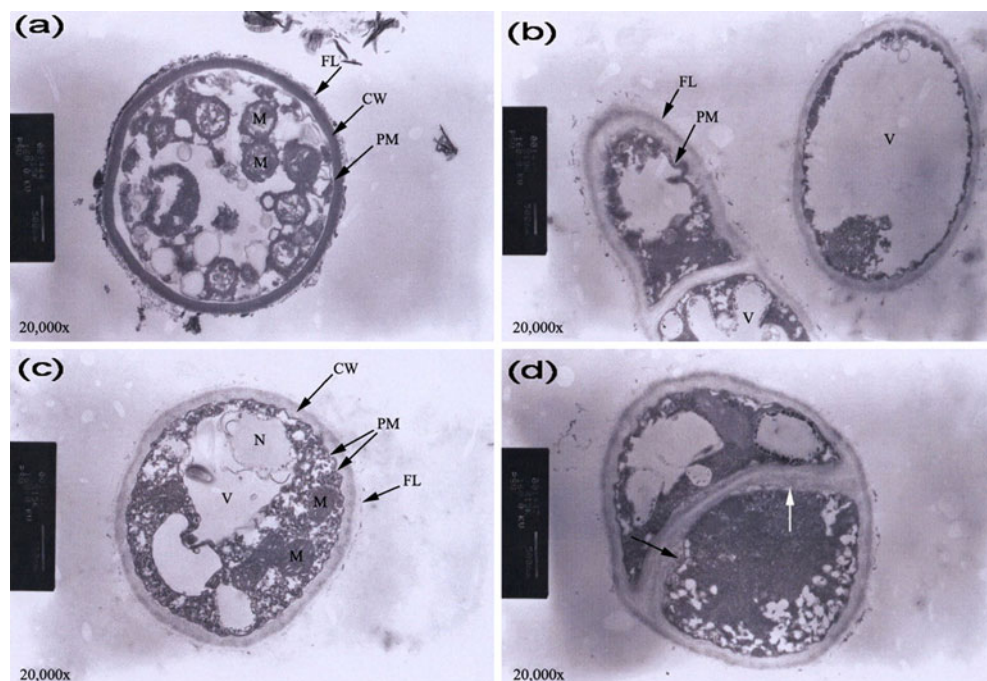
| Compounds | Fungal strain | Antifungal activity | |
|--|-----------------------|---------------------|-------------|
| | | MIC (mg/ml) | MFC (mg/ml) |
| Limonene | <i>A. flavus</i> | 2.70 | 3.15 |
| | <i>A. parasiticus</i> | 3.15 | 3.60 |
| Citronellal | <i>A. flavus</i> | 4.05 | 4.05 |
| | <i>A. parasiticus</i> | 4.05 | 4.05 |
| Citronellol | <i>A. flavus</i> | 3.60 | 4.05 |
| | <i>A. parasiticus</i> | 3.60 | 4.05 |
| Limonene + citronellal (1:1) | <i>A. flavus</i> | 4.05 | 4.05 |
| | <i>A. parasiticus</i> | 4.05 | 4.05 |
| Limonene + citronellal + citronellol (1:1:1) | <i>A. flavus</i> | 4.05 | 4.50 |
| | <i>A. parasiticus</i> | 4.05 | 4.05 |

β -pinene, and α -pinene should not be ignored. However, such hypothesis was not true as proven in Table 3. Although pure limonene was the most active component followed by citronellal and citronellol, its antifungal activity against *A. flavus* and *A. parasiticus* was five times lower than that of the essential oils (Table 3). However, a combination of these pure compounds did not improve antifungal activity. Therefore, the inhibitory activity was contributed from the synergistic effect of both major and minor constituents present in the essential oils.

Morphological changes of 7-day-old *A. flavus* caused by exposure to acid lime essential oil at the MIC concentration were demonstrated in comparison with that of the non-treated fungus in Fig. 5. In the non-treated sample, cell wall and plasma membrane were smooth and uniform and thoroughly surrounded by intact fibrilla layer, and all

organelles including mitochondria and vacuole have normal appearances (Fig. 5a), while in the cells treated with acid lime essential oil, plasma membranes became rough and irregular with continuous folding into cytoplasm, and detachment of fibrillar layer was also observed. Massive vacuolation of cytoplasm with vacuole fusion and formation of membrane-bounded vesicles was evident (Fig. 5b). Apart from plasma membrane disturbance, acid lime essential oil also attacked nucleus membrane but not mitochondrial membrane (Fig. 5c). Septate wall thickened with the detachment of plasma membrane (Fig. 5d). Lime essential oil was proven to stunt hypha development of *A. flavus* by attacking many targets, particularly membrane-bound structures, while the cell wall remained intact. These TEM observations were partially similar to morphological alteration in *A. parasiticus* treated with Akacid[®]plus (commercial

Fig. 5 Transmission electron micrographs of 7-day-old *A. flavus* treated with 0.56 mg/ml of acid lime essential oil. Control without essential oil treatment (a) with normal fibrillar layer (FL), cell wall (CW), plasma membrane (PM), vacuole (V), and mitochondria (M), the essential-oil-treated mycelia with vacuole fusion, detachment of fibrillar layer, crooked plasma and nucleus (N) membranes and loss of cytoplasmic content (b, c); thickening of septum (black arrow) and detachment of septum plasma membrane (white arrow)



guanidine-based polymeric disinfectants) demonstrated by Razzaghi-Abyaneh et al. (2006). Akacid^{®plus} was demonstrated to target and disrupt not only plasma membrane but also membrane of major organelle such as nuclei and mitochondria without any damage on the cell wall. Ultrastructure of *A. niger* exposed to *C. sinensis* essential oil revealed the damage of fungal mycelium, conidiophore, and conidial head through scanning electron microscopy. Cell wall was also damaged and punctured, leading to the loss of cytoplasmic content (Sharma and Tripathi 2008). Cell destruction caused by acid lime essential oil in this study was however different from that caused by *C. sinensis* essential oil, even though limonene was a major active component present in both essential oils. These observations supported the hypothesis that the minor compounds also played a very important role in antifungal activity of citrus essential oils, and limonene was not solely responsible for fungal destruction.

Conclusion

The result indicated that essential oils from tropical citrus cultivars particularly acid and kaffir limes exhibited significant antifungal activity against aflatoxin-producing strains of *A. flavus* and *A. parasiticus* as well as other common spoilage fungi. Particularly, the essential oils from kaffir lime and acid lime were most inhibitory to mold growth as well as aflatoxin production. Both essential oils exhibited fungistatic effect at their MICs, but fungicidal effect was observed at higher concentration. Natural citrus essential oils were proven to be more fungicidal than the pure active compounds. Limonene, a major active component of citrus essential oil, functioned synergistically with some minor compounds for the effective fungal inhibition. The irreversible damages and morphological alterations in membrane-bound structures of the fungal cells were clearly observed and evident. Further research on how each antifungal component damages the fungi could lead to highly effective novel system to control food spoilage or food-borne pathogens using natural compounds.

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