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Antifungal and antibacterial activities of essential oil from Som Keaw (*Citrus nobilis*) in Thailand

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Abstract The essential oil from *Citrus nobilis* leave was displayed antibacterial activity higher than that from the fruit's peel, with inhibition zone against *Erwinia chrysanthemi* and *Xanthomonas axonopodis* at 10,000 ppm as 10.67 mm and 11.33 mm, respectively. The pure essential oil from the peel 20 µL, however, displayed the highest antibacterial activity against *E. chrysanthemi* and *X. axonopodis* with the inhibition zone 20.00 mm and 14.00 mm, respectively. The antifungal activity was tested by vapour phase technique against five plant pathogenic fungi *Colletotrichum gloeosporioides*, *Curvularia lunata*, *Fusarium sacchari*, *Pythium* sp. and *Rhizoctonia solani* at 5, 10 and 20 µL/disc. Results show the essential oil from the peel extract completely inhibited mycelial growth of *Pythium* sp. and strongly inhibited *R. solani*. Additionally, the essential oil from leaf strongly inhibited mycelial growth of *Pythium* sp. and *R. solani*. The results suggested the essential oil from leaves and peel of Som Keaw (*C. nobilis*) serve as a high effective natural deterrent against all of the tested fungi.

Keywords: Essential oil, Orange, Plant pathogens, Som Keaw (*Citrus nobilis*)

Introduction

Agriculture crop production is susceptible to plant pathogens, especially fungi and bacteria, which can severely diminish both quality and yield (Oreke *et al.*, 1994). *Colletotrichum gloeosporilides* (antracnose), *Curvularia lunata*, *Fusarium sacchari* (rice grain discolorationdis), *Pythium* sp. (damping off) and *Rhizoctonia solani* (sheath blight) are caused diseases in many economic plants. In addition, plant pathogenic bacteria including *Erwinia* sp. and *Xanthomonas* sp. have been reported to cause diseases such as bacteria blight, canker and vegetable soft rot (Agrios, 2005). Chemical pesticides have traditionally been used for suppressing these pathogens. However, these pesticides will be accumulated and affected on ecological food chains, human and animals;

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therefore, alternative substances are searched to prevent the existing excessive use of chemical pesticides (Bernabo *et al.*, 2016; Hong-Thao *et al*., 2016).

Essential oils (EO) are complex mixtures of volatile secondary metabolites, which are biosynthesized by plants and known as "volatile oils" for their ability to vaporise. They are produced from various plant parts including buds, flowers, seeds, fruits, leaves and stems (Burt, 2004). The constituent chemical compounds, acting in synergism or additive, influence antimicrobial activity of the EO (Songsamoe *et al.*, 2017).

Rutaceae consist of about 160 genera, of which citrus is the most important. *Citrus* genus is one of the commercially grown species, this crops are nowadays cultivated worldwide, mainly in tropical, subtropical and temperated regions (Xiao *et al.*, 2017). Citrus includes several fruits of high economic value such as grapefruits, lemons, limes, mandarins, and oranges (Kamal *et al*., 2013). Its EO is obtained as a by-products of citrus processing, and is used in the manufacture of a variety of products including: perfumery, aromatherapies, cosmetic, and aroma flavouring (Dharmawan *et al*., 2009). Citrus essential oil is also used in the manufacture of natural medicines, it has anti-inflammatory, antioxidant, anticancer, antiviral, antiproliferative and antimicrobial activities (Hong-Thao *et al.*, 2016; Mandalari *et al.*, 2017). Due to the abundance of active substances such as terpenes, sesquiterpenes, taxinols, aldehydes, ketones and esters are found in citrus essential oil (Wu *et al.*, 2017).

Som Keaw (*Citrus* nobilis) belongs to the Rutaceae in mandarin group. In Thailand, Samutsongkhram Province is generally considered the best growing areas for this citrus fruit because the land and weather are highly suitable area for Som Keaw cultivation. The Province has adopted Som Keaw as its official crop and it produceds a crop of high-quality, high-yield fruit, Som Keaw fruit resembles oblate shape with 8-9 cm diameter, 7-8 cm hight and 300-310 g/fruit (Sawbangkhae, 2014). Normally, these citrus trees were cultivated together with other plants, such as pomelo or lychee trees (Anonymous, 2015). This kind of citrus is a popularly used to worship and homage in Chinese New Year, and in various festivals, because it has smooth, golden-yellow peel, appealing shape and size larger than a tangerine but smaller than pomelo fruit (Vetchakit, 2015). The fruit is especially valued for its easy-peeling, large petals, small seeds, and sweet and tangy taste. However, after squeezing for preparing orange juice, the peel becomes organic agricultural waste residues. The peel of this orange has large oil glands, therefore it is interesting to use their peel as a source of essential oil.

The aims of research were to test the effectiveness of antifungal and antibacterial activities of essential oil from Som Keaw (*C. nobilis*) against plant pathogens.

Materials and methods

Samples and preparation of essential oil extraction

The fruits and leaves of Som Keaw (*C. nobilis*) (Figure 1.) were collected from Bang Kon Tee, Samutsongkram Province, Thailand in November, 2018 (winter season). The fresh fruit was peeled, cut into small pieces, and subjected to hydrodistillation for 3 hr using a Clevenger-type apparatus. The essential oils were separated from water and dried over anhydrous Na_2SO_4 and stored at 4 ℃ to produce the stock solutions further analyses.

Figure 1. Som Keaw (*C. nobilis*) fruit (left), leaves (right)

Antifungal activity of essential oil

The mycelial growth of fungi: *Colletotrichum gloeosporioides* DOAC 2047, and *Rhizoctonia solani* DOAC 1406 were supplied by Division of Plant Disease and Microbiology Department of Agriculture, Ministry of Agriculture and Cooperative, Bangkok, Thailand. *Curvularia lunata* and *Fusarium sacchari* were isolated from grain discoloration disease of rice by Madi (2017) and *Pythium* sp. was isolated from root rot of lettuce by Adhikari *et al*. (2018). All tested fungi were maintained on Potato dextrose agar (PDA) and kept in refrigerator. They were then sub-cultured on fresh PDA for further studies. The essential oil used to test antifungal activity was extracted using a modified vapour phase technique (Boukhatem *et al*., 2014). Five millilitres (mL) of PDA was poured into plastic plate (45 mm diameter) after PDA solidified, the fungal mycelial plug (3 mm) from active growth colony was transferred and placed on the center of PDA. The essential oil (5 µL, 10 µL and 20 µL) was separately added into paper disc (6 mm diameter) which affixed on the centre of the lid and immediately sealed to avoid losses of volatile compounds. The paper disc

without essential oil served as nil control. The testing plates were incubated for 2-5 days and mycelial growth were measured and assessed as percentage mycelial growth inhibition using the formula proposed by Parveen *et al*. (2014). All treatments were replicated three times. Percentage mycelial growth inhibition = (dc-dt)/dc ×100. Where, dc=colony diameter of the mycelial growth in control, dt= colony diameter of the mycelial growth treated with essential oil.

Antibacterial activity of essential oil

The tested bacterial: *Erwinia chrysanthemi* DOA-BC 1181 and *Xanthomonas axonopodis* DOA-BC 1008 were supplied by Division of Plant Disease and Microbiology, Department of Agriculture, Ministry of Agriculture and Cooperative, Bangkok, Thailand. The efficiency of essential oil from Som Keaw against bacterial plant pathogens was tested by paper disc diffusion method (Balouiri *et al.,* 2016). Twenty millilitres of nutrient agar (NA) were poured into sterilized Petri dish (9 cm diameter) and the bacterial inoculum (McFarland No. 0.5 by 0.85% sterile NaCl) was swabbed on NA. The antibacterial activity separated into 2 experiments, the first experiment was tested by dilution of essential oil in various concentration (650, 1250, 2500, 5,000 and 10,000 ppm) and 20 µL of each concentration was added into paper disc (6 mm diameter). The second experiment was tested by non-dilution of essential oil and each volume (5 µL, 10 µL and 20 µL) was separately added into paper disc. The paper discs were placed on NA plate, incubated at 37°C for 24 hr and the diameter of inhibition zones (mm) was measured.

Statistical analysis

All data were analysed by R program (R-language and environment for statistical computing and graphics, version 3.5.1). Comparison of means was performed using Duncan's multiple range test (DMRT) and significance was accepted at the $P<0.05$ level. The experiment was designed as a Completely Randomized Design with triplications and expressed as mean±standard deviation (SD).

Results

Antifungal activity of essential oil

The antifungal effectiveness of essential oil extract from Som Keaw against plant pathogens; *C. gloeosporioides, C. lunata, F. sacchari, Pythium* sp.

and *R. solani* were evaluated by vapour phase technique at different volume (5 µL, 10 µL and 20 µL). The results showed that the essential oil obtained from peel fruit inhibited fungal mycelial growth greater effectiveness than that obtained from leave. The essential oil from peel completely inhibited significant activity against *Pythium* sp. at all treatment level as per the results shown in Table 1. In particular, the essential oil extract of Som Keaw peels displayed strong inhibition of the mycelial growth of *R. solani* (85.18-90.37% inhibition), and compleately inhibited mycelial growth of *R. solani* at 20 µL and 10 µL of leaves EO extract, respectively. In the same way, the antifungal activity of peel essential oil at 20 µL against *C. gloeosporioides, C. lunata* and *F. sacchari* showed high inhibition with results of 63.71%, 82.96% and 86.67%, respectively. The antifungal activity from peel essential oil were low concentration with an inhibition percentage of 19.26-71.85%. The peel essential oil inhibition against *C. gloeosporioides* and *F. sacchari* at 10 µL. However, result showed no significant effect at 20 µL. For the *C. nobilis* leaves essential oil extract against tested fungi, results showed completely inhibition of mycelial growth of *Pythium* sp. and *R. solani* at 20 µL. The volume of essential oil decreased the inhibition percentage of Som Keaw leave against tested fungi was decreased. The antifungal activity against *C. gloeosporioides, C. lunata*, *F. sacchari* and *R. solani* at 10 µL were not differed from 20 µL.

Table 1. The inhibition percentage of Som Keaw (*C. nobilis*) essential oil on mycelial growth of plant pathogenic fungi

Essential oil extracts	Volume (µL)	% Inhibition of mycelial growth				
		C. gloeosporioides	*C. lunata*	*F. sacchari*	*Pythium* sp.	*R. solani*
EO leaf	5	32.59 ±4.63$^{b1/}$	22.22 ±11.11^{c}	32.59 ±3.39^{c}	88.87 ±3.85^{b}	76.30 ±8.41^{c}
	10	41.48 ±12.24^{b}	67.41 ±5.59^{ab}	61.48 ±2.56^{b}	90.37 ±1.28^{b}	100.00 ±0.00^{a}
	20	44.44 ±3.85^{ab}	80.74 ±1.28^{a}	74.07 ±5.59^{ab}	100.00 ±0.00^{a}	100.00 ±0.00^{a}
EO peel	5	34.84 ±2.56^{b}	19.26 ±2.56^{c}	32.59 ±12.24^{c}	100.00 ±0.00^{a}	85.18 ±1.29^{bc}
	10	45.92 ±11.41^{ab}	56.29 ±11.40^{b}	71.85 ±7.81^{ab}	100.00 ±0.00^{a}	90.37 ±1.28^{b}
	20	63.71 ±3.39^{a}	82.96 ±1.28^{a}	86.67 ±2.23^{a}	100.00 ±0.00^{a}	100.00 ±0.00^{a}

[1]/ Indicated significant effects at $P<0.05$ (DMRT) within a column

Antibacterial activity of essential oils

The effect of essential oil from the leaf and peel of Som Keaw (*C. nobilis*) on plant pathogenic bacteria was tested using various concentrations. The results indicated the inhibition zone of essential oil from the leaf on *E. chrysanthemi* was higher than the peel. The concentration of 10,000 ppm, the

inhibition zone of as essential oil from the peel on *E. chrysanthemi* was 9.00±0.00 mm, while the essential oil from the leaf of *C. nobilis* was 10.67±0.58 mm. The antibacterial activity of essential oil from this citrus against *X. axonopodis* was evaluated. The essential oil from the leaf on growth of *X. axonopodis* exhibited greater inhibition zone than the essential oil from peel. The leaf essential oil exhibited inhibition zone against *X. axonopodis* as 11.33±2.31 mm. It was found that other concentrations (650-5,000 ppm), in both essential oil from peel and leaf, had no affected on the growth of *E. chrysanthemi* and *X. axonopodis* (data not show). Conversely, the results of antibacterial activity of essential oil in difference volume is shown in Table 2. The essential oil from the peel, when applied against plant pathogenic bacterial *E. chrysanthemi* and *X. axonopodis*, exhibited greater inhibition than the essential oil from leaf at all treatment. The peel essential oil at 20 µL was displayed the highest effectiveness against *E. chrysanthemi* and *X. axonopodis* with the inhibition zone of 20.00 ±1.00 mm and 14.00 ± 1.00 mm, respectively.

Table 2. The antibacterial activity of essential oil against *Erwinia chrysanthemi* and *Xanthomonas axonopodis* at different volumes

Essential oil extracts	Volume (µL)	Inhibition zone (mm)	
		E. chrysanthemi	***X. axonopodis***
EO leaf	5	0.00±0.00[d]	0.00±0.00[d]
	10	8.67±0.58[c]	8.33 ±0.58[c]
	20	15.67 ±1.15[b]	10.33± 0.58[b]
EO peel	5	0.00 ±0.00[d]	0.00 ±0.00[d]
	10	8.67 ±0.58[c]	8.67±0.58[c]
	20	20.00 ±1.00[a]	14.00 ±1.00[a]

[1]/ Indicate significant effects at $P<0.05$ (DMRT) within a column.

Discussion

This study explored the antifungal and antibacterial activities of essential oil from Som Keaw (*C. nobilis*) in Thailand against plant pathogens and tested pure essential oil applied at a range of volumes. The lowest volume (5 µL) from both of peel and leave essential oil completely inhibited the mycelial growth of *Pythium* sp. and *R. solani*. The highest volume, 20 µL of the peel essential oil showed strong antifungal activity against *C. gloeosporioides*, *C. lunata* and *F. sacchari*. However, in the contrast with this research, the essential oil from *C. reticulata* had no discernible effect against *Didymella bryoniae* (Ascomycota), a pathogen which causes watermelon gummy stem blight disease (Pakchareon, 2005). This is consistent with results reported by Panthachod and co-worker (2009). This team studied the essential oil extract from sweet orange (*C.*

sinensis) for antimicrobial activity against *B. subtilis*, *B. cereus*, *B. turingensis*, *Escherichiacoli*, *Mycobaterium* sp., *Proteus* sp., *Salmonella* sp., *Staphylococcus* sp., *Aspergillus flavus*, *A. niger*, *Penicillium* sp., *Rhizopus* sp., *Pichia* sp. and *Saccharomyces cerevisae*. Their study concluded the essential oil extract from sweet oranges (*C. sinesis*) had no affected on microbial growth.

Results showed Som Keaw essential oil had more effective against *Pythium* sp. and *R. solani* than *C. gloeosporioides*, *C. lunata* and *F. sacchari*. The cell wall of *Pythium* sp.as a lower fungus is composed of cellulose, instead chitin, and contain coenocytic hyphae (Blaschek *et al.*, 1992). The lower fungi are more sensitive to essential oil than the higher fungi.

For the antibacterial activity, the essential oil from Som Keaw at low concentration (<10,000 ppm) showed no effect on the growth of tested plant pathogens bacteria (*E. chrysanthemi* and *X. axonopodis*). Result indicated that the essential oil from *C. auratium* at 8-36 $mg.mL^{-1}$ did not inhibit *B. cereus*, *Staphylococcus aureus* and *E. coli* (Worapanit *et al.*, 2014). Meanwhile, Laoongaium (2007) reported the essential oil from pomelo (*C. maxima*) peel inhibited *S. aureus*, *E. coli* and *P. aeruginosa* with MIC 1.33-21.3 $\mu g.mL^{-1}$. The consistent with this research, our study showed that pure essential oil of Som Keaw at 10 µL/disc inhibited the growth of plant pathogenic bacteria (*E. chrysanthemi* and *X. axonopodis*). However, the previous report from Bozkurt *et al*. (2017) who studied the antimicrobial activity of EO from some citrus species, the results varies according to the species of citrus and the tested bacteria. The peel EO from *C. sinensis* showed the hightest antimicrobial against *E. coli and Salmonella typhimurium*, whereas *C. meyeri*, *C. paradise*, and *C. aurantium* showed the highest against *B. cereus*, *Listeria monocytogenes*, and *S. aureus*, respectively (Bozkurt *et al*., 2017). This is consistent with the report of Cloete (2003) who described that different bacteria reacting differ to bactericides either inherent differences such as unique cell envelope compositions and non-susceptible proteins or the development of resistance by adaptation or genetic exchange. The efficacy of essential oil extract on biological activity varied on several factors including species, part of plant, time to harvest, and chemical composition of plant cells. Previous studies reported that monoterpenes were the major volatile compounds of citrus peel, with limonene usually being the dominant compound (Gursoy *et al.*, 2010; Liu *et al*., 2012; Xiao *et al.*, 2017). In a previous report, limonene demonstrated bactericidal activity against *S. aureus*, *S. epidermidis*, *Streptococcus parauberis*, *E. coli* and *B. subtilis* (Jafari *et al*., 2011; Vimal *et al*., 2013; Pathirana *et al*., 2018).

In conclusion, the antifungal and antibacterial activities of the peel essential oil extract from Som Keaw strongly inhibited the growth of tested

plant pathogens fungi and bacteria higher than the leave. It is recorded as the first report on the essential oil from Som Keaw in Thailand against plant pathogenic fungi and bacteria. The finding suggested this citrus could be further developed to be a natural fungicide for sustainable agriculture and other biological activities such as antioxidant, antityrosinase and antimicrobial activities.

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