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Recovery of Orange Peel Essential Oil from 'Sai-Namphaung' Tangerine Fruit Drop Biomass and Its Potential Use as Citrus Fruit Postharvest Diseases Control

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Abstract: In this study, we assessed the quality of essential oil recovered from fruit drop biomass and assessed its usefulness in preventing postharvest diseases in the tangerine 'Sai-Namphaung'. Greening was the primary cause of the fruit drop, based on the enduring symptoms and occurrence of the disease in the area. Limonene, together with the presence of β-pinene and linalool, was discovered to be prevalent in essential oils of tangerine fruit peel, particularly that of 'Sai-Namphaung'. Through isolation of citrus postharvest fungi, we were able to identify four genera which were later DNA sequenced using Internal Transcribed Spacer: ITS and subjected to Basic Local Alignment Search Tool (BLAST), with a high possibility (>98% similarity) of being *Penicillium digitatum*, *Colletotrichum gloeosporioides*, *Fusarium sarcochrum* and *Geotrichum candidum*. Essential oil from 'Sai-Namphaung' and 'Fremont' peel biomass positively inhibited green mold rot and citrus anthracnose caused by *P. digitatum*, *C. gloeosporiodes*, but were less effective than the commercial citrus oil and *Zanthoxylum myriacanthum* oil. This is the first evidence of 'Sai-Namphaung' postharvest diseases caused by these two fungi and their controls using citrus essential oil.

Keywords: antifungal; citrus greening; green mold rot; limonene; *Penicillium digitatum*

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1. Introduction

Tangerine is an economic fruit crop that is widely cultivated around the world with global production reaching 29 million tons per year [1]. In Thailand, the cultivation in the northern region of 'Sai-Namphaung-Namphaung' tangerine alone accounts for more than 104,581 rais (167.33 km^2). During pre-harvesting, it was estimated that the total loss of fruit was ~20% of the total production yield, and plant diseases such as stem-end rot and citrus decline are the main causes of losses in the orchard [2,3]. Dropped fruit was the major biomass that was left to decompose naturally due to high management costs. However, this practice has led to the accumulation of disease pathogens in the orchard that are difficult to eliminate and, in fact, cause an even higher maintenance levy. Fruit biomass

can be used as raw material for extracting various valuable components. Citrus peel can be used for essential oil, fiber and pectin recovery and these components are highly demanded in the food and medicine industries [4–6]. Orange peel essential oil is the most sought-after natural food additive in the world [7]. The global citrus oil market was valued at USD 6.31 billion in 2018 and is expected to grow up to 6.8% between 2019 and 2025 [8]. Volatile components such as aliphatic hydrocarbons, alcohols, aldehydes, acids, esters and some aromatic compounds represent more than 80% of the essential oil, with D-limonene being the principal component [9,10]. In addition, the citrus essential oil possesses the ability to inhibit postharvest fungal diseases, especially in tropical fruit production [11,12]. Citrus polysaccharides such as pectin are used as food additives, fat replacers or pharmaceutical ingredients [13,14]. Considering the volume and feasibility of biomass recovery in the citrus industry, continued efforts are made to explore further applications, particularly since bio-circular green production is a major concern [15–18].

Harvested 'Sai-Namphaung' fruits are especially susceptible to fungal and microbial invasion which contribute up to 35% of the total postharvest loss. The major diseases are primarily green mold caused by *Penicillium digitatum*, and blue mold generated by *P. italicum* [19]. Fruit rots in citrus are caused by a variety of fungi, including *Penicillium, Alternaria, Aspergillus, Colletotrichum, Botryodiplodia* and *Phomopsis* [20]. To control these infections, the use of natural products, such as essential oils, has been shown to be effective in reducing the physiological activities of fruits during storage while also reducing overall qualitative and quantitative losses [21]. With this in mind and in line with the zero-waste production concept, the ultimate aims of this work are to analyse the quality of the essential oil recovery from fruit drop biomass and to evaluate its efficacy in controlling postharvest diseases of 'Sai-Namphaung' tangerines. The overall outcome of this study can hopefully be an immersing step forward to the Sustainable Development Goals (SDGs) by reducing the volume of agricultural waste and providing alternative uses for the Thai fruit industry.

2. Materials and Methods

2.1. Study Site and Survey of Preharvest Losses

A survey of fruit loss during the pre-harvesting period was conducted in a 0.128 sq. km 'Sai-Namphaung' monocrop orchard (19.9301 N, 99.1325 E), located in Mon Pin sub-district, Fang district, Chiang Mai province, Thailand. The survey began after 7 months of fruit set from September to November 2021. Six plots of 12 m × 12 m each were laid out randomly in two separated alleviations within the site. Three 5-year-old citrus trees were selected from each plot, and losses (i.e., dropped fruits) were gathered from nylon sheets laid underneath the tree canopy (Figure 1). Loose fruits were collected twice a month until the beginning of the harvest season and transferred to the laboratory at the Faculty of Agriculture, Chiang Mai University, immediately. In the lab, fruits were weighed and were visually determined for the cause of fruit loss including physical damage or citrus greening disease. Fruits were peeled and juiced, and each type of biomass was separated, weighed and used for the calculation of the total biomass [22,23].

2.2. Essential Oil and Chemical Analysis

The peel was shade dried at room temperature (~30 °C) until a constant weight was reached (<10% RH). The dried peel was ground to powder using a food processor (Mawin Quality Produce, Bangkok, Thailand) at high speed prior to essential oil extraction in a Clevenger's apparatus [24,25]. The essential oil was collected after 2 h of distillation and thereafter dried using anhydrous sodium sulphate. The oil yield was also recorded. Similarly, essential oil of citrus fruit cv. 'Fremont' was extracted using peel biomass collected from commercial-stage fruit from the same farmer. The essential oil was stored at 4 °C in air-tight sealed glass vials until used. In addition, two additional essential oil viz., makhwaen (*Zanthoxylum myriacanthum*) and commercial orange essential oil were obtained from Plant Bioactive Compound laboratory, Chiang Mai University, Thailand and Royal Project shop, Chiang Mai, Thailand, respectively. The volatile components were

analyzed by Gas Chromatography-Mass Spectrometry (GC-MS) of Agilent Technologies 7890A GC System and Agilent Technologies 5975C inert XL El/Cl MSD outfitted with 30 m × 0.25 mm × 0.25 µm, HP-5MS UI column. Essential oil samples (2 µL at the dilution of 1%, v/v, in ethyl acetate) were injected in a split mode (1:200). The oven temperature was set at 60 °C for 0 min, then increased by 5 °C/min until it reached 220 °C, where it was kept at this temperature for 0 min. Helium was used as the carrier gas, with a constant flow rate of 1 mL/min. The MS interface was maintained at 230 °C, and mass spectra were collected in electron impact ionization mode at 70 eV. The volatile compositions were identified by comparing mass spectra from NIST 0.5a.L libraries with >70% similarity. The volatile retention indexes (RI) were confirmed with the standard injection of N-alkane (C8–C20) [11]. The standard substance of limonene (Sigma-Aldrich, St. Louis, MO, USA) was also used to extrapolate the amount of limonene present in the essential oil.

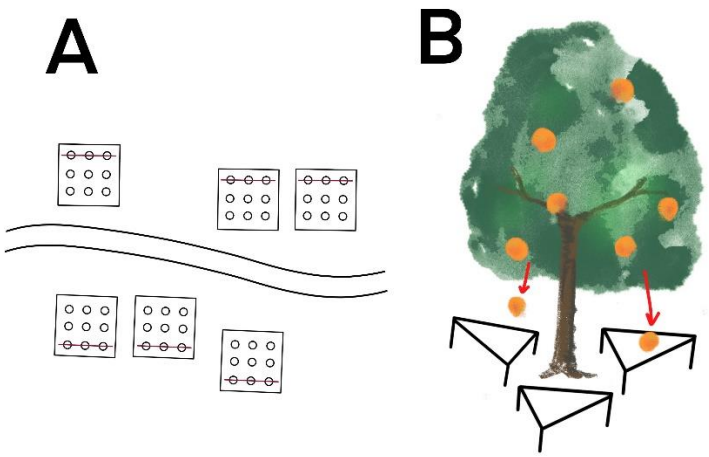

Figure 1. Survey citrus fruits cv. 'Sai-Namphaung' loss during preharvest (**A**) Six plots of three 5-year-old citrus trees were selected from each plot (**B**) and losses (i.e., dropping fruits) were gathered from nylon sheets laid underneath the tree canopy.

2.3. Isolation of Fungal Causing Post-Harvest Disease

Defective fruits (20 kg) were collected from a commercial postharvest warehouse located within 5 km of the orchard. They were visually sorted, and only infected fruits were collected and brought back to the Plant pathology clinic center, Department of Entomology and Plant Pathology, Faculty of Agriculture, CMU, for disease evaluation and isolation of fungi that were causing diseases. The fungal isolations was performed by the single spore isolation method [26], and the fungal strains were consequently confirmed by DNA sequencing. In addition, the fungal specimens with the desired structure were mounted on lactic acid and microphotographs that were taken with a Canon EOS 6D digital camera and an Axiovision Zeiss Scope-A1 microscope (Zeiss, Jena, Germany) (Canon, Tokyo, Japan). The Tarosoft (R) Image Frame Work application was used to perform the morphological measurements (Tarosoft, Bangkok, Thailand).

The genomic DNA from the fungal mycelia was extracted according to the manufacturer's instructions using the DNA Extraction Mini Kit (FAVORGEN, Ping Tung, Taiwan). Polymerase chain reaction (PCR) was used to amplify the isolated DNA. Internal transcribed spacer [18] gene sections were amplified using the primer pairs ITS5/ITS4. [27]. The PCR was carried out according to the procedure given by Haituk et al. [28]. The sequences were received from 1st BASE Company, a commercial sequence source (Kembangan, Malaysia). The sequence data were deposited in the GenBank database.

2.4. Pathogenicity Test

Citrus fruits cv. 'Sai-Namphaung' at the commercial harvesting stages from the same orchard were sent to the laboratory within 3 h. At the laboratory, fruits with no evident faults were washed with running tap water. Then, they were washed with 70% ethanol

for 30 s and rinsed with sterilised water. They were thereafter washed with sodium hypochlorite (0.5%) for 30 s, rinsed with three times sterilised water, and air-dried at room temperature (25–28 °C). [29]. Fruits were inoculated by scratching on the skin (3× scratches on each fruit) with a steel blade (4 mm wide) and 15 µL a conidial suspension of *P. digitatum* (10^6 spores/mL) was suspended onto each incision independently. The inoculated fruits were in a sterile moist chamber using aseptic technique. The sterilised distilled water (15 µL) was used to inoculate the control fruit. After 2–4 days at 25 °C, observations on the percentage of fruits infected by fungal isolates were made. This study used a Completely Randomised Design, in which each isolate was repeated twice [30].

2.5. In Vitro Antifungal Assay of the Essential Oil

Pure cultures of the previously confirmed isolations which were maintained on Potato Dextrose Agar (PDA) at 4 °C were used. The isolations were reactivated on the PDA that was incubated for 7 days at 28 °C, and the conidia were harvested in sterile distilled water filled with 0.1% tween 80.

A broth dilution test in the 96-well plates was used to determine the active inhibitory concentrations [31]. A 100 µL of Potato Dextrose Broth (PDB) with different concentrations of the essential oil (256, 128, 64, 32, 16 µL/mL) was mixed with 100 µL of spore suspension (10^5 CFU/mL). The 96-well plates were then covered and incubated for 72 h at 28 °C. Each test was repeated thrice. The positive control consisted of the PDB mixed with the conidial suspension, while the negative control was the PDB alone. The minimum inhibitory concentration [32] was determined as the lowest concentration that prevented visible fungus growth. The mixture from the well with no visible fungus development was then transferred onto a new PDA plate and cultivated for another 72 h. The lowest concentration that showed no fungal growth on the PDA plate was termed the minimal fungicidal concentration (MFC) [33].

2.6. In Vivo Antifungal Assay of the Essential Oil

Citrus fruits cv. 'Sai-Namphaung' were prepared as the same method in 2.4. Fruits were inoculated by scratching on the skin with a steel blade and dropped with 15 µL of essential oil solutions at the MFC for each type of essential oils onto each incision independently. After the droplets dried, a conidial suspension of the responding fungus was suspended onto each incision independently. Sterile distilled water (10 µL) was used to inoculate the control fruits. After incubation at 25 °C for 2–4 days, observations on the percentage of fruits infected by the responding fungus were made. This study used a Completely Randomised Design, in which each treatment was repeated three times and each biological replication consisted of one fruit [30]. The evidence of disease development (viz., lesion or shrunken skin) was measured. Two fruits were used for this study. Each inclusion was treated as an independent replication for statistical analyses.

2.7. Statistical Analysis

For each test, at least three duplicates of each experiment were carried out. In the in vivo test, a paired sample *t*-test was used to test the difference between two biological samples using the SPSS 23.0 program (SPSS Inc., Chicago, IL, USA). A one-way analysis of variance and Duncan's Multiple Range Test were used to compare the mean of differences of each assay using Statistical Analysis System (SAS) software (SAS Institute Inc., Cary, NC, USA). Statistical significance was defined as a *p*-value of less than 0.05.

3. Results and Discussion

3.1. Pre-Harvest Losses

Data on fruit loss was collected over 60 days (Figure 2). It was apparent that the major loss during pre-harvesting was fruit drops, and the volume increased over time with the average volume ranging from 0.60–1.50 kg/plot (each containing 3 representatives). As presented in Figure 2, the fruits mainly had uneven skin colour, sometimes greenish and

brown lesions and evident cracks present. Fruit sag was not fully developed and seeds were aborted. The fruit *Citrus* spp. are prone to various types of fungal, viral and bacterial pathogens right from nursery and pre-harvesting through post-harvesting stages to the bearing stage, resulting in incalculable losses to the plantation and its produce. The plant requires up to 5–9 months for maturity on trees. During fruit setting and until harvest, the fruits may be exposed to pathogens like *Colletotrichum gloeosporioides*, *C. acutatum*, *Botryodiplodia theobromae*, *Alternaria citri* and *Phomopsis citri* resulting in considerable damage to its production and quality of the fruit [34]. Post bloom fruit drop (PFD) caused by *C. acutatum* fungal disease that is linked to wet weather during the bloom of citrus flowers produces orange-brown lesions on flower petals, abscission of fruits and eventually causes fruitlets to fall off trees prematurely; however, the calyx remains attached to the branch [3,35]. Later, *C. gloeosporioides* was also identified as an initiator of these symptoms [36]. Furthermore, the shortage of carbon sources, nutrient imbalances and plant hormones, as well as insects, have all been mentioned as the possible causes of fruit drop in citrus species [37]. Citrus greening is one of the world's most serious diseases in the citrus industry. The disease is predominately caused by the bacteria *Candidatus Liberibacter* species that can be transmitted either by grafting or insect carrier [38]. The phloem tissues of the leaf and root area are dramatically obstructed due to this bacteria's translocation. As a result of the blockage of the nutrients and sugar flows, citrus plants lose leaves, have uneven fruit size, which can impair the flavor and texture of the fruit, and premature fruit drop [39]. We believe that based on the persisting symptoms and occurrence of the disease in the areas, greening may be the primary causes of fruit drop.

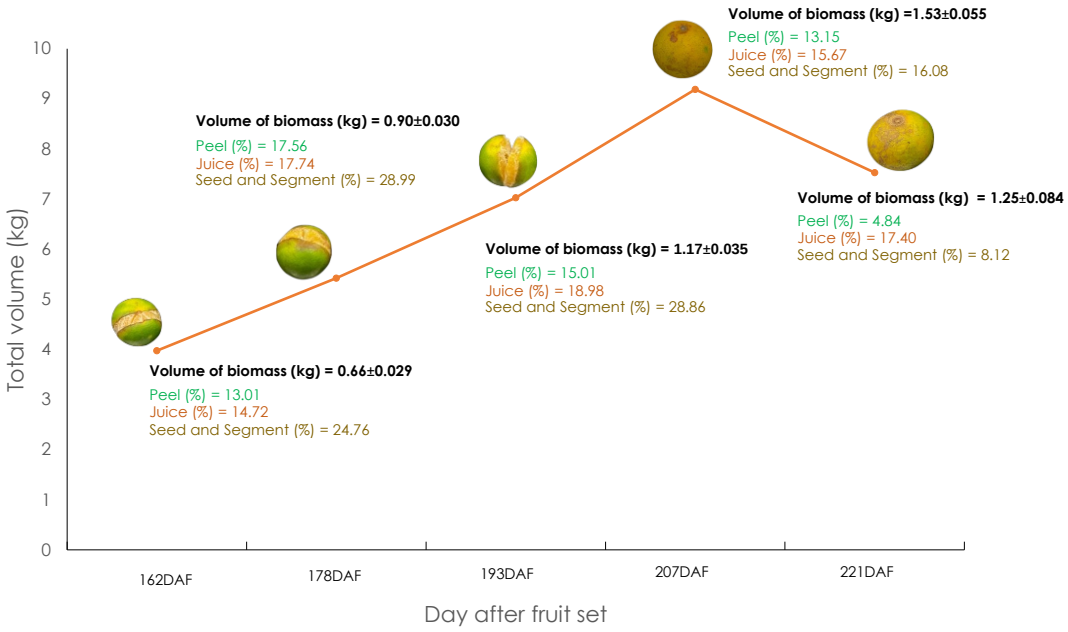

Figure 2. The average volume of biomass and percentages of each biomass type (peel, juice, and seed and segment). Data were collected from October to November 2021 from 6 plots (each containing 3 plants).

Through the fruit development process, the amount of each type of biomass viz., peel, juice, seed and segment, varied. The percentage of biomass of peels (~15%) were much higher toward the early stage of fruit development i.e., 162 days after fruit set (DAF), and 178 DAF, the amount of peel biomass dropped significantly toward the mature stage of the fruit (~5%). This pattern seems to be in accordance with the volume of seed and segment, while the amount of citrus juice remained the same (~17%) for 2 months prior to harvest. Generally, the citrus fruit peel consists of two tissues, the outer flavedo and the spongy cell, albedo. The flavedo accumulates pigments and essential oils, representing the citrus aroma, while the albedo is a rich source of pectin [40]. During the early stages of fruit development,

the albedo takes up the majority of the fruit volume, but as the juice cells in the pulp grow, it gradually thins out [41].

3.2. Volatile Components

Peel biomass from each of the collecting days was used for essential oil extraction using hydrodistillation. As illustrated in Table 1, the number of essential oils of the tangerine biomass varied from 5.0–10.0%. The 'Fremont' mandarin peel gave a significantly lower amount of essential oil when compared with 'Sai-Namphaung' peel. These amounts, nonetheless, are much higher than what have been reported by other studies, in which the amounts of citrus oil are reported to be in the range between 2.0–5.0% depending on the methods of extraction [42,43]. The volatile components of each type of essential oil were elucidated from mass spectrums derived from the GC. The essential oil of 'Sai-Namphaung' tangerine comprised of the dominant limonene followed by β-pinene and β-myrcene (Figure 3). The amount of limonene (1.2 µL/100 µL) and β-pinene (0.02 µL/100 µL) of the essential oil were higher from peel biomass collected from the early stage of fruit development, though the volatile profile were not variable through the process of fruit development. Peel biomass from the 'Fremont' was composed of limonene and β-myrcene in an almost identical amount as of the 'Sai-Namphaung' peel. Additionally, linaloo (~0.02 µL/100 µL) was also detected. The essential oil profiles of citrus oil and essential oil of makhwaen were much complex. The commercial citrus oil contained especially 1-propanol, 2-(2-hydroxypropoxy) and dipropylene glycol, while γ-terpinene, ocimene and linalool were present in makhwaen oil. Moreover, the commercial type lacked sabinene, β-pinene, β-myrcene, 1R-α-pinene and α-terpinene which again could be due to the methods of extraction applied.

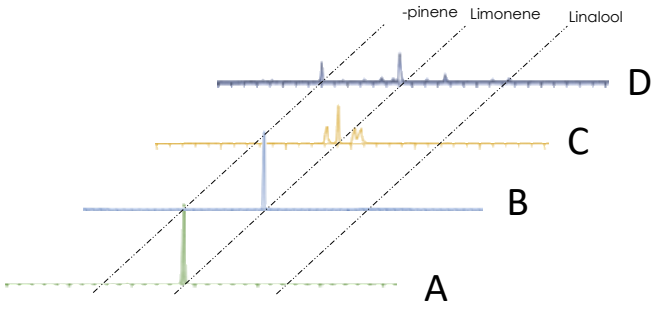

Figure 3. Volatile component chromatograms of citrus essential oils from A = peel of 'Sai-Namphaung' tangerine; B = peel of 'Fremont' mandarin; C = commercial citrus oil; D = fruit of makhwaen.

GC/MS was used to examine the chemical composition of *Citrus reticulata* Blanco essential oil. The majority of the oil was made up of monoterpene hydrocarbons [11]. The amount of essential oil extracted from orange peels depends on the type of citrus, extraction method and preliminary drying process. The essential oil content of orange peels is between 0.2–1.0% [44]. In other works, limonene was found to be dominant in the essential oil of tangerine fruit peels including that of 'Sai-Namphaung', along with the presence of β-pinene, α-pinene, 3-carene and β-phellandrene [45–47]. In the fruit peels of the 'Fremont' mandarin, the chief component was limonene, followed by linalool and β-myrcene [48]. Among all other *Zanthoxylum* species, makhwaen are known to have complex aromatic profiles comprising principally limonene, sabinene, L−phellandrene, β-ocimene, terpinen-4-ol and γ-terpene [49,50]. In addition, the composition of orange peel essential oil depends on the species, with similar main components: limonene, β-pinene and myrcene [51]. These essential oils had been proven to be potential antimicrobial agents, possibly due to the principal volatile components or their combinations [11,46,48,52].

Table 1. Volatile components of essential oils from the *cirtus* spp.

No	Compound Name	Formular	Retention Index	'Sai-Namphaung' Tangerine					'Fremont'	*Zanthoxylum myriacanthum*	Commercial
				162 DAF	178 DAF	193 DAF	207 DAF	221 DAF			
		% Yield		5.27 ± 1.87 ^{d}	10.7± 0.10 ^{a}	5.30 ± 0.30 ^{d}	9.73 ± 0.41 ^{b}	7.70 ± 0.21 ^{c}	3.30 ± 0.93 ^{e}		
1	Sabinene	$C_{10}H_{16}$	936	-	-	-	-	-	-	0.051	-
2	β-pinene	$C_{10}H_{16}$	975	0.021	0.011	0.011	0.011	0.011	-	0.004	-
3	β-myrcene	$C_{10}H_{16}$	980	-	0.012	0.012	0.012	0.012	0.012	-	-
4	1R-α-pinene	$C_{10}H_{16}$	993	-	-	-	-	-	-	0.010	-
5	α-terpinene	$C_{10}H_{16}$	1009	-	-	-	-	-	-	0.012	-
6	2-propanol, 1,1′-oxybis	$C_6H_{14}O_3$	-	-	-	-	-	-	-	-	0.198
7	o-cymene	$C_{10}H_{14}$	1027	-	-	-	-	-	-	0.008	-
8	limonene	$C_{10}H_{16}$	1032.5	1.210	0.853	0.962	0.900	0.821	1.219	0.103	0.248
9	1-propanol, 2-(2-hydroxypropoxy)	$C_6H_{14}O_3$	-	-	-	-	-	-	-	-	0.145
10	dipropylene glycol	$C_6H_{14}O_3$	-	-	-	-	-	-	-	-	0.171
11	γ-terpinene	$C_{10}H_{16}$	1046.2	-	-	-	-	-	-	0.022	-
12	Ocimene	$C_{10}H_{16}$	1089	-	-	-	-	-	-	0.005	-
13	Linalool	$C_{10}H_{18}O$	1104	-	-	-	-	-	0.024	0.009	-

The amount was reported as μL/100 μL, which was calculated corresponding to limonene running independently. In the essential oil yield the value is the mean ± SE (n = 3) with different lowercase letters being significantly different ($p < 0.05$).

3.3. Pathogenic Identification and Phylogenetic Species of Fungi

Four fungal isolates were isolated from 'Sai-Namphaung' tangerine fruits at the commercial packing house. They were assigned as BACI01-04 (Table 2). After 14 days, BACI01 was light green and the hyphae were segmented cells (septate) with spores of ellipsoid shape. After inoculation, we found a light green spore mass on lesions which occurred on the skin of the fruit (Figure 4A). The colony of BACI02 was white and grew in 7 days. The morphology under a microscope showed that hyphae were septate and the spores were of a long, ellipsoid shape. When this isolate was inoculated on fruit skin for 3 days, the fruit showed a shrunken and darkened lesion (Figure 4B). BACI03 had a light green colony after 7 day-subculturing; however, there was no evidence of pathogenicity. The hyphae were segmented cells and the spore is a long curved cylindrical shape with both acute ends (Figure 4C). Finally, the BACI04 had a light green colony which extended after 3 days. However, after inoculation, the fruit did not show any symptoms. Under compound microscope, the hyphae were septate, and the spores were of a small, cylindrical shape (Figure 4D). These isolates were sent to the Plant Pathology Clinique, Department of Plant Pathology, Chiang Mai University, where they were morphologically confirmed by experts as belonging to *Penicillium* sp., *Colletotrichum* sp., *Fusarium* sp. and *Geotrichum* sp., respectively. They were later confirmed by BLAST from their ITS gene sequences as being *P. digitatum*, *C. gloeosporiodes*, *F. sarcochrum* and *G. candidum* (Supplementary Figure S1). Confirming the information from BLAST, a high possibility with >98% similarity was provided. *Penicillium* sp., *Colletotrichum* sp., *Fusarium* sp. and *Geotrichum* sp. are species complexes. However, for identification at the species level, multigene phylogeny together with proper taxonomy are required.

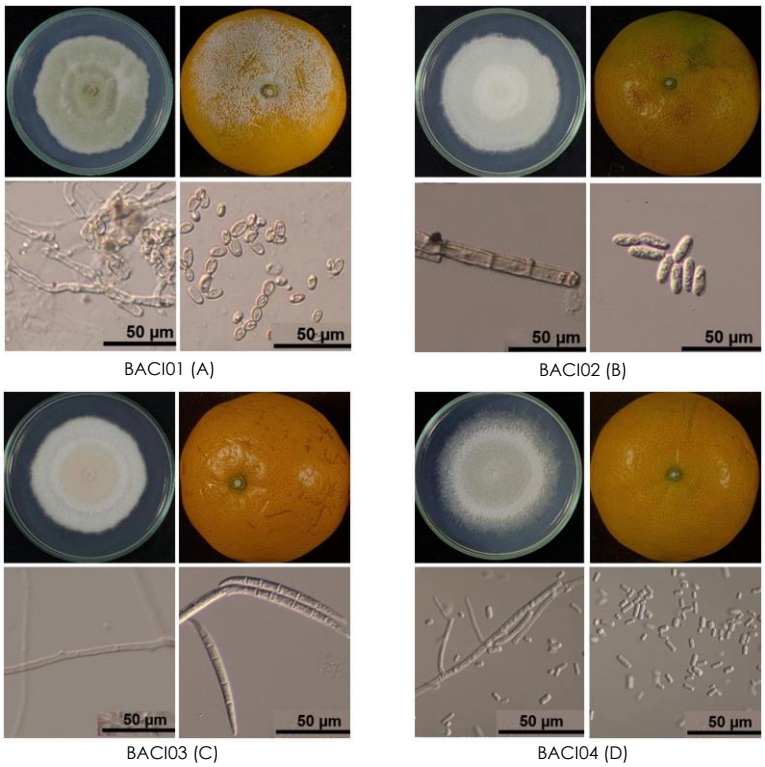

Figure 4. Morphological characteristic and pathogenicity of 'Sai-Namphaung' tangerine postharvest fungal isolates. (**A**) *Penicillium* sp. (**B**) *Colletotrichum* sp. (**C**) *Fusarium* sp. (**D**) *Geotrichum* sp.

Table 2. Morphology of fungal isolates collected from 'Sai-Namphaung' tangerine fruits during postharvest.

Isolate Number	Colony	Conidial Morphology	Morphological Characteristics	Pathogenic Score
BACI01	Light green fluffy colony initiated after 14 days	Ovoid, unicellular (~5 × 10 µm)	*Penicillium* sp. Branching pattern: Regular Phialide: Flask-shaped or cylindrical Conidia shape: Globose smooth-walled conidia, sometimes ellipsoidal, sometimes typical cylindrical and green [53,54]	++++
BACI02	White fluffy colony initiated after 7 days	Oblong, unicellular (~3 × 20 µm)	*Colletotrichum* sp. Branching pattern: Irregular Phialide: Simple, short and erect Conidia shape: Hyaline one celled, cylindrical, with both ends rounded. Sometimes ovoid to oblong, slightly curved or dumbbell shaped depending upon the host from which the pathogen is isolated and its area of origin [55,56]	++
BACI03	Light orange fluffy colony initiated after 7 days	Macroconidia: falcate, curved, multicellular, 6 septate (~3 × 70 µm)	*Fusarium* sp. Branching pattern: Sporodochium Phialide: A monophialide is a condio-phore with only one opening or pore through which endoconidia are extruded, while a polyphialide has two or more such openings orpores. Conidia shape: Three types ofspores-called macroconidia, microconidia, and chlamydospores which are intermediate in size and shape [57,58]	-
BACI04	White fluffy colony initiated after 3 days	Cylindrical unicellular (~1 × 2 µm)	*Geotrichum* sp. Branching pattern: Either simple (non-branched or mono-verticillate), one-stage branched (biverticillate symmetrical), two-stage branched (biverticillate asymmetrical) or three- to more-staged branched, phialide is flask-shaped, consisting of a cylindrical basal part and a distinct neck, or lanceolate (with a narrow basal part tapering to a somewhat pointed apex). Conidia shape: Oval or cylindrical sometime globose, ellipsoidal, cylindrical or fusiform, hyaline or greenish, smooth or rough-walled [59,60]	-

3.4. In Vitro Antifungal Assays of Essential Oils and In Vivo Study

In this study, we applied the microplate technique for antifungal assays. Four essential oil types (viz., 'Sai-Namphaung' 162 DAF, 'Fremont', makhwaen and citrus oil) at five concentrations (16–256 µL/mL) were tested against four fungal isolates (Table 3). By using the tangerine oil, *F. sarcochroum* and *P. digitatum* were inhibited at the highest concentration, while *G. candidum* was not. The MIC of 'Sai-Namphaung' was 64 µL/mL for *C. gloeosporioides*. The essential oil of the 'Fremont' citrus was clearly effective in the inhibition of *C. gloeosporioides* at a MIC of as little as 16 µL/mL. In fact, all other essential oil types illustrated the same pattern. The MIC of 'Fremont' for *G. candidum*, *F. sarcochroum* and *P. digitatum* was 128 µL/mL. The MIC for *G. candidum* was 64 µL/mL, while the MIC for *F. sarcochroum* and *P. digitatum* was 16 µL/mL using commercial citrus oil. The essential oil

of makhwaen showed excellent inhibitory capabilities against all fungal isolates at a MIC of 16 µL/mL. The solution at each MIC point was then suspended on the PDA plates and incubated for 72 h to test the MFC. The result revealed that none of the fungal isolates were killed with 'Sai-Namphaung' oil, while 'Fremont' oil killed *P. digitatum* and *C. gloeosporioides* at an MFC of 128 µL/mL. The commercial citrus oil and makhwaen oil were effective for *C. gloeosporioides* at 16 µL/mL, followed by *F. sarcochroum* at 64 and 32 µL/mL, respectively. *G. candidum* was completely killed by both essential oil types at 256 µL/mL and 128 µL/mL for the commercial citrus oil and makhwaen oil, respectively. Citrus essential oils which were made from indifferent varieties and genera are known to inhibit fungal pathogens such as those belonging to *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp. [61]. Moreover, in another study, it has been found that citrus peel essential oil is effective against five genera of fungi viz. *A. alternata*, *Rhizoctonia solani*, *Curvularia lunata*, *F. oxysporum* and *Helminthosporium oryzae* [46]. The composition of the volatile compounds is specific to the inhibition of plant pathogenic fungi. In the genus *Penicillium* sp., limonene, γ-terpinene and α-pinene are effective against *P. digitatum*, and β-pinene is able to inhibit *P. italicum* [11,61]. Our results seem to be in line with this work suggesting that most types of citrus oils contain limonene and β-pinene which are able to inhibit *P. digitatum*. The essential oil of the fruit of the genus *Zanthoxylum* can inhibit *A. funigatus* which is the pathogen that causes damage to commodity during storage [62]. Green mold, blue mold, and sour rot are the most serious post-harvest fungal diseases of citrus fruits caused by *Penicillium* spp. and *Geotrichum* spp. [63]. The disease causing fungi can be controlled by the use of essential oils such as those from *Thymus* sp., peels of *C. reticulata* and also chemical compounds like cinnamaldehyde, eugenol and carvacrol [11,64–66]. *Colletotrichum* spp. are reported as pathogens associated with citrus anthracnose [67]. The conidial germination of *C. gloeosporioides* was controlled by the vapor treatments of essential oil containing carvacrol, cinnamon oil, trans-cinnamaldehyde, citral, p-cymene and linalool [68]. Post-harvest Fusarium rot caused by *Fusarium* spp. was reported on *C. reticulata* Blanco and mandarin [69–71]. The alcoholic extract of chili and ginger tested positive in controlling this disease at the concentration of 300 ppm [72]. Based on the results, we only selected the essential oil types at MFC for the in vivo study of citrus diseases caused by *P. digitatum* and *C. gloeosporioides*. The essential oil of 'Sai-Namphaung' for *P. digitatum* was omitted as the MFC in the tested range was unidentified.

Table 3. Minimum inhibitory concentrations (MIC) and Minimum fungicidal concentrations (MFC) determinations.

Essential Oil Types		Essential Oil Concentrations				
	Pathogen Isolates	256 µL/mL	128 µL/mL	64 µL/mL	32 µL/mL	16 µL/mL
'Sai-Namphaung'	G	+	+	+	+	+
	F	-	+	+	+	+
	P	-	+	+	+	+
	C	-	-	-	+	+
'Fremont'	G	-	-	+	+	+
	F	-	-	+	+	+
	P	-	-	+	+	+
	C	-	-	-	-	-
Commercial citrus oil	G	-	-	+	+	+
	F	-	-	-	-	+
	P	-	-	-	-	+
	C	-	-	-	-	-
Zanthoxylum myriacanthum oil	G	-	-	-	-	-
	F	-	-	-	-	-
	P	-	-	-	-	-
	C	-	-	-	-	-

The minimum concentration(s) at which the fungus was completely killed (MFC) at 72 h is highlighted in pink. Abbreviations; *Geotrichum candidum* (G), *Fusarium sarcochroum* (F), *Penicillium digitatum* (P), *Colletotrichum gloeosporioides* (C).

In the in vitro study, we found that green mold disease caused by *P. digitatum* was only controlled by commercial citrus oil and makhwaen oil. Essential oils of all types were able to control citrus anthracnose disease caused by *C. gloeosporioides*, and makhwaen oil is the most effective (Table 4). The result of the in vivo test is well correspondent to the in vitro analysis. All in all, this is the first report of the topical use of citrus oils from biomass during pre-harvest production in controlling post-harvest diseases of 'Sai-Namphaung' tangerines, which supports the sustainable use of by-products from agricultural production.

Table 4. The lesion size (mm^2) of pathogens (*Penicillium digitatum* and *Colletotrichum gloeosporioides*) inoculums on 'Sai-Namphaung' tangerine fruits (n = 6) over 4 days.

Citrus Causing Pathogens	**Treatments**			
		2 days	3 days	4 days
Penicillium digitatum	Control	471.16 ± 211.40 $_{ab}$	909.23 ± 70.13 $_{a}$	1142.99 ± 12.60 $_{a}$
				
	'Fremont'	729.64 ± 153.04 $_{a}$	973.17 ± 60.39 $_{a}$	1184.73 ± 0.41 $_{a}$
				
	Commercial citrus oil	341.40 ± 103.59 $_{c}$	617.82 ± 145.13 $_{b}$	930.47 ± 98.25 $_{b}$
				
	Zanthoxylum myriacanthum oil	296.62 ± 187.68 $_{c}$	514.93 ± 214.17 $_{c}$	652.59 ± 278.87 $_{c}$
				
		3 days	5 days	7 days
Colletotrichum gloeosporioides	control	137.58 ± 27.16 $_{a}$	122.19 ± 22.80 $_{a}$	128.84 ± 44.05 $_{a}$
				

Table 4. *Cont.*

Citrus Causing Pathogens	Treatments		
'Sai-Namphaung'	109.21 ± 9.11 $_{b}$	113.66 ± 9.07 $_{b}$	97.06 ± 5.25 $_{b}$
			
'Fremont'	72.41 ± 27.26 $_{c}$	79.73 ± 23.13 $_{b}$	43.39 ± 28.37 $_{d}$
			
Commercial citrus oil	99.28 ± 20.59 $_{b}$	80.54 ± 16.75 $_{b}$	80.27 ± 16.47 $_{b}$
			
Zanthoxylum myriacanthum oil	74.71 ± 4.46 $_{b}$	55.93 ± 2.91 $_{c}$	54.47 ± 4.40 $_{d}$
			

The values are mean ± SE (n = 6); where each inoculum was treated as independent replications, considering that the two fruits were subjected to a pair sample *t*-test and were not biological different (Supplementary Table S1). For each pathogen, mean values with different lowercase letters are significantly different ($p < 0.05$).

4. Conclusions

In an effort to find an alternative and environmentally friendly method to control post-harvest diseases of tangerine cv. 'Sai-Namphaung,' the efficacy of essential oil extracted from fruit drop biomass was evaluated. Based on the symptoms and prevalence of the disease in the region, greening was the major cause of fruit drop. Limonene, together with β-pinene and linalool, were detected as the principal active ingredients in the essential oils of tangerine fruit peel. Four genera were identified viz., *Penicillium digitatum, Colletotrichum gloeosporioides*, *Fusarium sarcochrum*, and *Geotrichum candidum* from infected fruits. 'Sai-Namphaung' and 'Fremont' peel essential oils were less effective in preventing green mold rot and citrus anthracnose caused by *P. digitatum* and *C. gloeosporioides* than commercial citrus oils and *Zanthoxylum myriacanthum* oil.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture12050701/s1, Figure S1: The Basic Local Alignment Search Tool (BLAST) of isolate BACI01, BACI02, BACI03 and BACI04; Table S1: Student *t*-test of 2 biological samples for in vivo study of essential oil on 'Sai-Namphaung' tangerine.

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