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Control of postharvest anthracnose of mango using pomelo albedo extracts

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ABSTRACT

The *in vitro* and *in vivo* antifungal activities of pomelo albedo extract cvs. 'Khao Tang Gwa' and 'Tub Tim Siam' on the mango anthracnose pathogen *Colletotrichum gloeosporioides* were studied. Under *in vitro* condition, ethanol extracts of pomelo albedo suppressed mycelial growth as indicated by mycelial dry weight and inhibited spore germination of *C. gloeosporioides*. Spore germination was significantly reduced ($p < 0.05$) in the treatments of 'Khao Tang Gwa' (28.77%) and 'Tub Tim Siam' (35.65%) ethanol extracts in comparison to the control (77.47%). Results of *in vivo* study showed that the percentage of disease incidence was not significantly different among the treatments. However, average lesion diameters in 'Mahajanaka' mango treated with 'Tub Tim Siam' (2.42±0.86 cm) and 'Khao Tang Gwa' (2.54±0.64 cm) extracts were slightly smaller than those in the control (2.76±0.63 cm), showing the effect in delaying anthracnose development in artificially inoculated mango. Total phenolic compound of pomelo albodo extracts was 28.73±0.06 and 22.46±0.26 mg GAE/100g dry weight for 'Khao Tang Gwa' and 'Tub Tim Siam', respectively.

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INTRODUCTION

Anthracnose disease caused by *Colletotrichum gloeosporioides* (Penz.) Sacc. is one of the major postharvest diseases in mango. The disease causes spoilage and rotting in mango, resulting in considerable losses of postharvest quality and quantity. Chemical fungicides have been intensively used in control of anthracnose in mango; however, the use of chemicals has led to the development of chemical resistance by pathogens, and has harmed human health and environment due to its toxic residues. Increasing public concerns over chemical residues on fruits and environmental pollution, there have been several attempts to look for natural alternative treatments.

Plants produce secondary metabolites such as flavonoids, saponins, alkaloids, tannins and phenols as a defend mechanism against attack and damage by microorganisms, insects, and herbivores (Spelman *et al.*, 2006). Thus, the screening of antimicrobial activity of plant extracts has been conducted for decades. Several plant extracts have shown their efficacy against postharvest fungal pathogens. The spore germination of *C. gloeosporioides*, for example, was inhibited by extracts of custard apple (*Annona reticulate* L.) (Bautista-Banos *et al.*, 2000), papaya (Bautista-Banos *et al.*, 2003), citrus (*Citrus aurantium* and *Citrus aurantifolia*) (Hernández-Albíter *et al.*, 2007) and chaplu (*Piper sarmentosum*) (Bussaman *et al.*, 2012). However, the search for antimicrobial property of other natural sources including residues of agricultural produces has been promoted due to its benefit to both the economy and the environment. The agricultural residues or industrial-by-products, like peel and seed, are proven to have high antioxidant level with the presence of high phonolics content (Ling and Palanisamy, 2012). The extracts derived from agricultural residues have been reported in control of plant and foodborne pathogens, e.g., lemon peels against *Penicillium digitatum* (Ben-Yehoshua *et al*., 1992) and pomelo peels against *Cladosporium cladosporioides* (Srisajjalertwaja, 1996), the yeasts *Zygosaccharomyces rouxii* (Suklampoo *et al.*, 2012), *Bacillus cereus* and *Staphylococcus aureus* (Chaisawadi *et al.*, 2007; Suklampoo *et al.*, 2012). Antimicrobial activity was also found in pomelo albedo against bacteria (Mokbel and Suganuma, 2006) and yeasts (Suklampoo *et al.*, 2012). Preliminary study on effect pomelo albedo extracts was conducted *in vitro* and showed a promising result in control of the fungal pathogen *C. gloeosporioides*.

The objectives of this study were, therefore, (i) to investigate the *in vitro* and *in vivo* antifungal activities of pomelo albedo extract cvs. 'Khao Tang Gwa' (white flesh) and 'Tub Tim Siam' (red flesh) on the mango anthracnose pathogen and (ii) to determine total phenolic content of the albedo extracts.

MATERIALS AND METHODS

Plant materials

Mango cv. 'Mahajanaka' was selected to use in this study due to its high susceptibility to postharvest disease. The mangoes at the stage of 119 days after full bloom were collected from a commercial orchard in Chiang Mai province and transported to laboratory at Mae Fah Luang University, Chiang Rai province. Individual fruit weighing 200 to 300 g without visible defects were selected and randomly allocated to treatments prior to experimentation.

C. gloeosporioides isolation and maintenance

An isolate of *C. gloeosporioides* was obtained from an infected mango and was cultured on Potato Dextrose Agar (PDA). The pure culture was then identified based on their morphological and cultural characters. Pathogenicity was verified by inoculating the fungus into mango fruit before use in this study. Stock cultures were maintained by transferring mycelial plugs from the edge of actively growing cultures onto fresh PDA every 2 weeks.

Preparation of spore suspension

A two-week-old sporulating culture of *C. gloeosporioides* was flooded with Tween-80 solution (sterile distilled water containing 0.01% (v/v) Tween-80) and gently agitated to dislodge the spores. Spore suspension was then filtered through sterile cheesecloth, washed three times by centrifugation at 10,000 rpm for 10 min and resuspended in fresh Tween-80 solution. The concentration of spores was counted by using haemacytometer (Hirschmann, Germany) and adjusted to the concentration used in the experiment.

Extraction of pomelo albedo

The extracts were prepared according to the method described by Sukorini *et al.* (2013) with some modifications. Pomelo albedo (*Citrus grandis* L. Osbeck) cvs. 'Khao Tang Gwa' and 'Tub Tim Siam' from pomelo at 80% maturity were cut into small pieces and were then dried in an oven at 40°C for 7 days or until constant weight obtained. The ovendried albedo was blended into powder by a blender before extraction by soaking in 95% ethanol (EtOH) at the ratio of 1:5 for 3 days with frequent agitation. The mixture was then filtrated through Whatman No.1 filter paper and the crude extract was collected and dried under a vacuum on a rotary evaporator (EYELA-CCA1110, Japan) at 40°C. An extract was then collected and mixed with dichloromethane at the ratio of 1:3, and left for 30 minutes before filtration. The filtrate was dried with a rotary evaporator, subsequently added with 20% EtOH and kept in air-tight bottles and in the refrigerator at 4°C until used. The extracts at 200 mg/mL of 95% EtOH was prepared and used throughout the experiments.

In vitro antifungal assays of pomelo albedo extracts

Mycelial growth. The mycelial growth was observed by measuring mycelial dry weight according to the method given by Vicedo *et al.* (2006) with slight modification. One mL spore suspension at 10^6 spores/mL was added to 100 mL of Potato Dextrose Broth (PDB) in the flask before adding 1 mL of either albedo extract (making a final concentration of 2 mg/mL), 95% EtOH or sterile water (as a control). The mixture was then incubated at 25°C with gentle shaking for 96 h. After incubation, the content of the each flask were poured into a pre-weighed Whatman No.1 filter paper. The filter paper with the mycelia was dried in an oven at 60°C until a constant weight was obtained. The dry weight of the mycelia was determined by subtracting the weight of the filter paper from the total weight of the filter paper with mycelia. There were 3 replications for each treatment. The percentage reduction over the control (water) was calculated.

Spore germination test. The spore germination test was carried out by the cavity slide technique modified from the method given by Maqbool *et al.* (2011). Ten µL of spore suspension (10^6 spores/mL) was transferred to the cavity slide and a drop of 10 µL of albedo

extract was added, making a final concentration of extract at 100 mg/mL in each slide. Three replications of 5 slides were used for each albedo crude extracts. Sterile water and 95% EtOH were used as controls. After incubation at 25±2°C for 6 h, slides were fixed in lactophenol cotton blue and germination of 100 spores per slide was examined microscopically. A spore was considered germinated when the length of germ tube equaled or exceeded the length of spore. The percentage inhibition of spore germination over the control (water) was calculated.

***In vivo* antifungal assay of pomelo albedo extracts**

Mango fruits were surface sterilised by dipping in 1% sodium hypochlorite for 3 min, rinsed 3 times with sterile Reverse Osmosis (RO) water and dried at room temperature in a sterile chamber. Fruit cheeks were wound-inoculated by puncturing once to a depth of 2 mm with a 1.0-mm diameter needle. Ten µL of spore suspension (10^5 spores/mL) was dropped on a 5-mm disc of PDA previously placed on the wound. Inoculated fruits were incubated in a moist plastic container at 25±2°C, 90±5% RH for 24 h to induce fungal growth. After incubation, 10 µL of extract was placed on each inoculated wound. Sterile water and 95% EtOH were applied as controls. Lesion diameter was measured after 6 days of incubation in a moist plastic container at 25±2°C, 90±5% RH. The lesion diameters were measured horizontally and vertically, and the mean for lesion diameter was calculated and recorded in cm. There were 3 replications of 8 fruits for each treatment.

Determination of total phenolic content

Total phenolic content in albedo extracts was determined according to Folin-Ciocalteu procedure as described by Singleton and Rossi (1965) with slight modification. A standard calibration curve was prepared using different concentrations of gallic acid in EtOH (0-100 µg/L). The extract (0.1 g) was dissolved in 10 mL of 99% EtOH. One mL of diluted extract was mixed with 5 mL of Folin-Ciocalteu reagent (diluted 1:10), then 4 mL of 7.5% sodium carbonate solution was added and the tube was shaken thoroughly using a vortex. After 1 h, absorbance was measured at 765 nm with a spectrophotometer (G10S UV-Vis, Thermo Scientific, USA). For each sample, three replicate assays were performed. All determinations were carried out in triplicate. The total phenolic contents were calculated and expressed as mg gallic acid equivalent (GAE) per 100 g dry weight (DW).

Statistical analysis

For all experiments, treatments were arranged in a completely randomized design (CRD). Data were subjected to analysis of variance (ANOVA) using SPSS Statistics for Windows Version 16.0 and the differences between the means of the treatments were compared by Duncan's multiple range test at $p < 0.05$.

RESULTS AND DISCUSSION

***In vitro* antifungal assays of pomelo albedo extracts**

Mycelial growth. Inhibitory effect of pomelo albedo extracts on mycelial growth of *C. gloeosporioides* was performed in PDB medium and mycelial dry weight was determined. The results in Table 1 show that dry weight of mycelium obtained from PDB incorporated with crude ethanol extract of pomelo albedo was significantly lower ($p < 0.05$) than that of the control, suggesting EtOH extracts inhibited mycelial growth of *C. gloeosporioides* under *in vitro* condition. In contrast to our previous study (Naradisorn and Ruenkum, 2009) in which dichloromethane extracts of 'Tub Tim Siam' pomelo albedo did not inhibit radial growth of *C. gloeosporioides* on agar medium. It could be suggested that antimicrobial activity of albedo crude extract associated with solvent used in the extraction.

Table 1. Effect of pomelo albedo extracts on mycelial dry weight of *Colletotrichum gloeosporioides*, the causal agent of anthracnose of mango.

Treatment	Mycelial dry weight (mg)	Reduction over control (%)
Control	270.70 ± 14.20 a	-
95% EtOH	114.47± 3.75 c	57.71
cv. 'Khao Tang Gwa' (2 mg/mL)	169.27± 15.79 b	37.47
cv. 'Tub Tim Siam' (2 mg/mL)	163.10 ± 6.48 b	39.75

Data shown are mean ± standard deviation. Means followed by the different letters indicate significant differences among treatments according to Duncan's multiple range test at $p < 0.05$.

In agreement with the findings, Suklampoo *et al.* (2012) found that hexane extract of 'Khao Nam Phueng' albedo inhibited growth of the yeasts *Zygosaccharomyces rouxii*, whereas EtOH extract did not show any antimicrobial activity. Likewise, in the studies on bacteria, pomelo albedo methanol extract (Mokbel and Suganuma, 2006) and ethyl acetate extract (Suklampoo *et al.*, 2012) inhibited growth of *Staphylococcus aureus*, *Bacillus cereus* and *Bacillus subtilis*, but there was no inhibitory activity observed in ethanol extract treatments.

Spore germination The effect of albedo extracts on spore germination of *C. gloeosporioides* was investigated 6 h after treatment. Albedo ethanol extracts significantly reduced ($p < 0.05$) spore germination of *C. gloeosporioides* compared to untreated control and 95% EtOH, with no significant difference among the pomelo cultivars (Table 2). The lowest level of spore germination (28.77%), with a 62.86% inhibition, was observed in spores treated with 'Khao Tang Gwa' crude extract. Whereas, the percentage of spore germination was 35.65, 44.37 and 77.47 for 'Tub Tim Siam' crude extract, 95% EtOH and the control, respectively. Similarly, our previous study (Naradisorn and Chaipanwiriyaporn, 2012) found that crude methanol extracts of cvs. 'Khao Yai' and 'Tub Tim Siam' exhibited in inhibition of spore germination of *C. gloeosporioides*. These findings show that spore germination was clearly affected by pomelo albedo crude extract treatment suggesting that crude extracts of pomelo albedo affected early development stage of *C. gloeosporioides*.

***In vivo* antifungal assay of pomelo albedo extracts**

There was no significant difference in rot lesion diameter on inoculated mango fruits among the treatments. However, rot lesions on fruits treated with 'Tub Tim Siam' (2.42±0.86 cm) and 'Khao Tang Gwa' (2.54±0.64 cm) extracts tended to be smaller than those treated with 95% EtOH (2.75±0.64 cm) (Figure 1).

Table 2. Effect of pomelo albedo extracts on spore germination of *Colletotrichum gloeosporioides*, the causal agent of anthracnose of mango.

Treatment	Spore germination (%)	Inhibition (%)
Control	77.47 ± 7.76 a	-
95% EtOH	44.31 ± 14.88 b	42.80
cv. 'Khao Tang Gwa' (100 mg/mL)	28.77 ± 5.94 c	62.86
cv. 'Tub Tim Siam' (100 mg/mL)	35.65 ± 7.06 c	53.98

Data shown are mean ± standard deviation. Means followed by the different letters indicate significant differences among treatments according to Duncan's multiple range test at $p < 0.05$.

It could be suggested that the EtOH extract of pomelo albedo in this study had a fungistatic rather than fungicidal effect as the extract inhibited mycelial growth and spore germination of *C. gloeosporioides*. Due to its inconsistent performance to such levels of control to be achieved by alternatives to fungicides as stand-alone treatments, plant extract has been suggested to use in integration with other treatments, e.g., heat treatment, irradiation or other natural compound, to achieve more effective (Palou *et al.*, 2008). Bazie *et al.* (2014) reported that applying aqueous plant extracts at 50°C enhanced the antifungal activity of the extracts against *Colletotrichum musae* without affecting the physico-chemical properties of banana. Likewise, Bautista-Banos *et al.* (2003) suggested that papaya fruits treated with plant extract in combination with chitosan had a lower anthracnose severity compared to those treated with plant extract alone. Additional work is needed to evaluate the efficacy of pomelo albedo extract when used as part of integrated disease management.

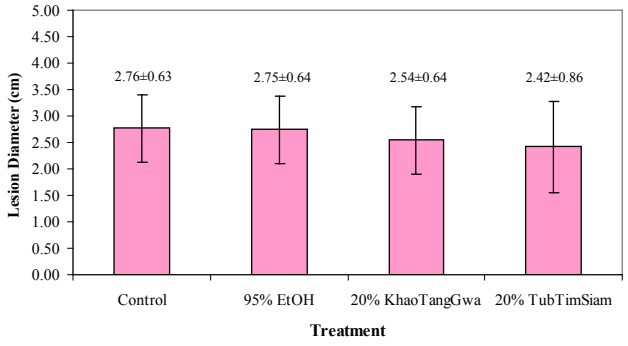

Figure 1. Effect of pomelo albedo extracts on anthracnose rot lesion of artificially inoculated 'Mahajanaka' mango fruit after storage at 25±2°C, 90±5% RH for 6 days. Data shown are mean ± standard deviation from $n = 24$.

Total phenolic content

The calibration curve showed linearity for gallic acid in the range of 20-100 µg/mL, with a correlation coefficient (R^2) of 0.9964 (Figure 2). The extract of 'Khao Tang Gwa' albedo contained higher content of phenolics (28.73±0.06 mg GAE/100g dry weight) than 'Tub Tim Siam' (22.46±0.26 mg GAE/100g dry weight) (Figure 3). It is known that antimicrobial activity of plant extract is due to phenolic compounds produced by plant. In citrus fruit, antimicrobial activity was found to be present in several parts of fruit including flavedo, albedo, juice sacs and membranes (Mokbel and Hashinaga, 2005).

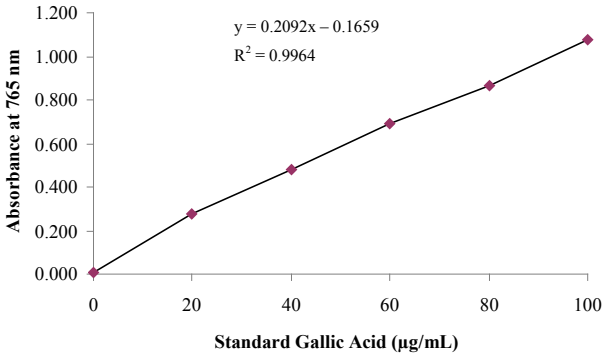

Figure 2. Standard calibration curve of gallic acid at concentrations of 20, 40, 60, 80 and 100 µg/mL. Spectrophotometric detection was at 765 nm.

Numerous studies have been carried out to compare the phenolics content in peels (flavedo and albedo) and in pulps of citrus fruit; total phenolics in peels of lemon, oranges, grapefruits and pomelo were significantly higher than in the pulps (Gorinstein *et al.*, 2001; Toh *et al.*, 2013). It was suggested that fruit's peels are an important source of phenolics (Bocco *et al.*, 1998). This present study revealed the presence of total phenolic content in pomelo albedo tissue; and this resulted in suppressing mycelial growth and inhibiting spore germination of *C. gloeosporioides*. The mechanisms of phenolic compounds in toxicity against fungi are thought to be a damage to lipids and/or proteins of microbial membrane resulting in leakage of cellular material and deactivation of microbial enzymes and other proteins (Mohammedi and Atik, 2013).

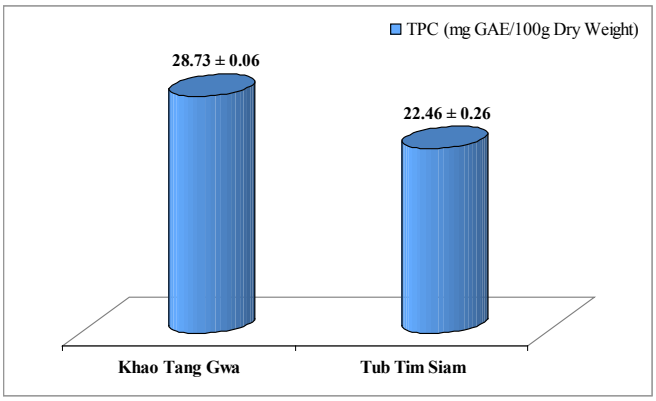

Figure 3. Total phenolic content of crude extract of pomelo albedo cvs. 'Khao Tang Gwa' and 'Tub Tim Siam' determined by Folin-Ciocalteu assay and calculated as GAE in mg/100 g dry weight. Results are the average of triplicates ± SD.

CONCLUSION

The present study revealed that albedo crude extract had antimicrobial activity against *C. gloeosporioides* based on mycelial growth and spore germination. The finding in this study indicated the possible exploitation of pomelo albedo crude extract for controlling anthracnose disease in mango. However, application of the albedo extract itself may not provide a commercially acceptable level of control of anthracnose diseases comparable to that obtained with fungicides. Pomelo albedo extract is probably considered to use as part of integrated management of anthracnose disease.

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