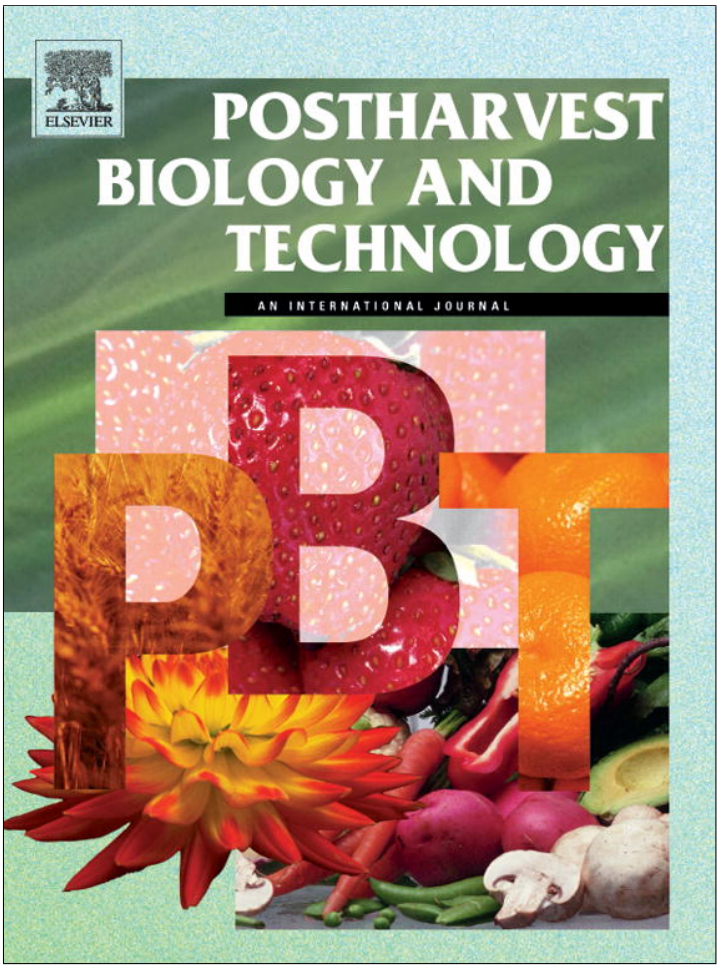

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Control of postharvest green mold of citrus fruit with yeasts, medicinal plants, and their combination

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ABSTRACT

The use of bio-fungicides and a plant extracts to control postharvest disease was investigated as an alternative to chemical control. The combination of a promising plant extract and yeast were selected through *in vitro* and *in vivo* techniques. A combination of *Candida utilis* TISTR 5001 and *Eugenia caryophyllata* crude extract was the best combination to attain a reduction in disease incidence and disease severity of *Penicillium digitatum* on citrus fruit. Colonization was the lowest on fruit treated with the combination of *E. caryophylata* crude extract and *C. utilis* TISTR 5001, and survival of *C. utilis* TISTR 5001 was the highest. The combination of *E. caryophylata* crude extract and *C. utilis* TISTR 5001 significantly reduced the natural development of green mold of citrus fruit, and had no effect to fruit quality. The active compound of *E. caryophylata* was found to be eugenol, based on HPLC and NMR (^{1}H and ^{13}C). Hence, the results indicate that a combination of plant extracts and yeasts posses antifungal activity that can be exploited as an ideal treatment for future plant disease management.

1. Introduction

Green mold rot disease caused by *Penicillium digitatum* is the most important postharvest disease of citrus fruit (Kinay et al., 2007). This pathogen has a relatively short disease cycle: 3–5 days at 25 °C. Each conidial head is able to produce up to 2 billion conidia and can be spread by wind (Holmes and Eckert, 1999). Harvested fruit are very susceptible to the pathogen (Sommer et al., 2002), and thus the greatest loss of citrus fruit by *P. digitatum* can occur after harvest, during transport, storage and marketing (Eckert and Eaks, 1989). *P. digitatum* is responsible for 90% of citrus fruit loss (Macarisin et al., 2007), and minimizing fruit injury, precise temperature management, and postharvest fungicide treatments, are the primary methods to reduce these losses (Eckert and Eaks, 1989).

In the many citrus packing-houses, fungicides are used to treat the fruit. Continuous use of a fungicide such as imazalil, *o*-phenylphenol, or thiabendazole for more than three decades has resulted in pathogen resistance (Holmes and Eckert, 1999). Kinay et al. (2007) reported that isolates of *P. digitatum* collected from California packing-houses and orchards were resistant to two or more fungicides. Efforts to find an effective control technique which is safe for workers, the environment and consumers, are needed (Janisiewicz and Korsten, 2002). Biological control (Pimenta et al., 2010), plant extracts (Mekbib et al., 2007), low toxicity chemicals (Smilanick et al., 1999), and physical treatments (Porat et al., 2000) are possible methods to control *P. digitatum*.The use of antagonistic bacteria and fungi are often used in biological control methods. Wilson and Wisniewski (1989) summarized that antagonist microbes could be potential antagonists; the antagonist should have the ability to colonize and survive in the commodity, and be compatible with other postharvest practices, processes, and other control methods including chemicals. In addition, organisms must be suitable for large-scale production using low cost substrates. Among modes of actions, competition for nutrients and space between pathogen and antagonist is considered a desirable mode of action in postharvest control (Ippolito et al., 2000).

Many studies using plant extracts such as essential oils and pure compounds against plant pathogenic fungi have been conducted. A number of antifungal compounds have been found in plants, derived from secondary metabolites such as tannins, terpenoids, alkaloids, and flavonoids (Arif et al., 2009).

The combination of different methods could provide more effective control and consistent results compared with single treatments (El-Ghaouth et al., 2004). Treatments with mixed aqueous leaf extracts of *Solonum virginionum* and *Trichoderma viridae* were found to effectively control anthracnose disease of tomato by 70% (Mogle, 2011). A treatment comprising *Bacillus amyloliquefaciens*

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HF-01 combined with 50 μg mL^{-1} tea saponin was as effective as a fungicide treatment and subsequently resulted in excess of 90% control of green and blue molds and sour rot (Hao et al., 2011). The application of carnauba wax formula containing either yeast *Saccharomyces cerevisiae* or the commercial backing yeast of *S. cerevisiae* mixture (CBY) combined with peppermint oil (1%) had a superior effect in reducing gray mold, soft rot and black rot incidence in tomatoes (Abd-Alla et al., 2009).

The objective of the present research was to select plant extracts and yeasts both *in vitro* and *in vivo* to control *P. digitatum* in citrus fruit.

2. Materials and methods

2.1. Pathogen inoculum

A highly virulent strain of *P. digitatum* was obtained from decaying "Sai Num Pung" tangerine fruit. The pathogen was grown on potato dextrose agar (PDA) at 25 °C for seven days and a conidial suspension was prepared. Conidia concentration was determined by a haemacytometer and adjusted to 1×10^5 conidia mL^{-1} with sterile distilled water.

2.2. Antagonists

Seven antagonist yeasts were used in this research, including *Candida tropicalis* TISTR5010/ATCC13803 (http://www.biotec.or.th/tncc/dbstore/detail.asp?DB=TISTR) used for production of xylitol (Choi et al., 2000), *Pichia membranefaciens* TISTR 5093 isolated from coconut toddy and used for ester production (http://www.tistr.or.th/tistr_culture/list_en.php?type=y&key=P), *Candida utilis* TSITR 5001, used for production of single cell protein from cassava (Trien et al., 2000), *Aureobasidium pullulans* TISTR 3384 (http://www.straininfo.net/strains/505092) used for fructo-oligosaccharide (FOS) production for prebiotic purposes (Fungsin et al., 2012), *Candida guillermondii* BCC 5389 (http://www.biotec.or.th/tncc/dbstore/detail.asp?pg=113&wr=&DB=BCC), *Candida sake* TISTR 5143 from decayed fruit (http://www.biotec.or.th/tncc/dbstore/StrainDetails.asp?Genus=Candida&Species=sake&id=376&DB=TISTR), and *Debaryomyces hansenii* TISTR 5155 (http://www.tistr.or.th/tistr_culture/list_en.php?type=y&key=D) used for economical kefiran production (Cheirsilp and Radchabut, 2011). All the above information was accessed on 18 December 2012.

The yeasts were acquired from the National Science and Technology Development Agency (NSTDA) and the Thailand Institute of Scientist and Technological Research (TISTR). The yeast cells were cultured on yeast malt extract agar (YMA) and incubated at 28 °C for 48 h. The cell suspension was then prepared and added to 10 mL of sterile distilled water and counted with a haemocytometer. The cell suspension was adjusted to 1×10^8 cells/mL (Nunes et al., 2001; Yu et al., 2007).

2.3. Fruit preparation

"Sai Num Pung" tangerine fruit (*Citrus reticulata* Blanco) were obtained from a commercial orchard in Fang, Chiang Mai province, Thailand, and fruit free of defects were chosen. The fruit surfaces were disinfected by immersion in 1% sodium hypochlorite for 3 min, rinsed with sterile water, and drying in a sterile chamber. The semi-commercial test did not immersion in 1% sodium hypochlorite.

2.4. Plant extract materials

The following plant extracts: *Cymbopogon citratus* stem, *Zingiber officinale* rhizome, *Momordica charantia* fruit, *Curcuma longa* rhizome, dried *Eugenia caryophyllata* flower bud, *Cinnamomum cassia* bark, and *Tinospora crispa* bark were used in this research. The fresh plants were dried under shade and blended to powder. Powdered material with a mass of 300 g was soaked in 400 mL 96% ethanol for three days with frequent agitation. The mixture was filtered through Whatman no. 1 filter paper and the crude extract was collected. The crude extract was distilled at 40 °C with a rotavapor at 200 mbar. An extract was collected and mixed in a ratio of 1:3 of CH_2Cl_2 and left for 30 min prior to filtration. The filtrate was dried in a rotary evaporator and subsequently added to 20% ethanol and kept at −20 °C until used.

2.5. Plant crude extract screening

The screening of the plant crude extracts for their efficacy against the fungal pathogen *P. digitatum* was conducted with the poisonous food technique. For this purpose, potato dextrose agar was amended with plant crude extract at a concentration of 0 (no plant extract added), 5000, 10,000, 15,000, and 20,000 mg/L plus 2% citrus juice. A small block (2 cm × 2 cm) was aseptically cut and inoculated with the *P. digitatum* on the sides of an agar block and then incubated on a moist plate for 48 h. *P. digitatum* growth was investigated by adding lactophenol cotton blue (Harris, 1986; Woo et al., 2010). Five replicates were used for each plant extract. Inhibition (%) of fungal radial growth was calculated using the formula: ((radius of control − radius of treatment)/radius of control) × 100% (Skidmore and Dickinson, 1976).

2.6. Plant crude extract screening on citrus fruit

Ten μL of conidial suspension (1×10^5 conidia/mL) was added, using a sterile pipette, to the citrus wound to a depth of 3 mm and allowed to dry under aseptic conditions. Then 10 μL of plant crude extract or 10 μL of sterile water (control treatment) was applied to the wound. The inoculated fruit were incubated in a 100% RH chamber at 25 °C for 7 days. Each treatment had three replications and the experiment was arranged in a randomized block design. Disease incidence and disease severity were observed after 7 days of incubation. To calculate disease incidence, the number of infested citrus fruit was observed. The disease incidence was calculated using the formula:

Disease incidence

= (number of infected fruit/total number of fruit assessed) × 100.

The disease severity was determined according to the portion of the infected area of the fruit. The disease severity was calculated using the formula (Masood et al., 2010):

Disease severity = (infected tissue area/total tissue area) × 100.

2.7. Antagonist screening

Interaction between yeasts and *P. digitatum* hyphae was assessed for direct parasitism in 90 mm diameter Petri dishes which contained PDA medium with dual cultures segregated by 3 cm. The radial growth of the mycelium toward the yeast strip was measured after storage at room temperature for 7 days. Ten Petri dishes per treatment were used. The percentage of inhibition of radial growth (PIRG) was recorded. The screening tests were performed to select two promising yeasts. The formula of PIRG was defined as: ((radius of control – radius of treatment)/radius of control) × 100% (Skidmore and Dickinson, 1976).

2.8. Antagonist and plant crude extracts to control P. digitatum on citrus fruit

Two promising plant crude extracts and two yeasts were tested on citrus fruit both individually and in combination. Ten μL of conidial suspension (1×10^5 conidia/mL) of *P. digitatum* were added using a sterile pipette to the citrus wound to a depth of 3 mm, made with a sterile needle, and allowed to dry under aseptic conditions. Ten μL of cell suspension of yeast at 1×10^8 cells/mL were also added on the wound. After drying, 10 μL of the plant crude extract were added to the wound site, and then incubated under a 100% RH, at 25 °C. Disease incidence and disease severity were observed after 7 days incubation. Each treatment had three replications and the experiment was arranged in a randomized block design.

2.9. Effects of antagonist and plant extracts on P. digitatum and yeast colonization on citrus fruit

The colonization ability of *P. digitatum* and yeast on citrus wounds was observed in the treated fruit (added with 10 μL of conidial suspension of *P. digitatum* at 1×10^5 conidia/mL, then 10 μL of cell suspension of yeast at 1×10^8 cells/mL, and finally, 10 μL of plant crude extract). In addition, the colonization ability was observed in the control (add with 10 μL of sterile water). Both the treated and the control fruit were incubated at 100% RH and 25 °C. The colonization ability was observed 2, 4, 6, and 8 h after treatment. The method used was based on the method described by Pimenta et al. (2010); 1 cm^2 of citrus peel around pricked area was cut into small pieces (0.5 mm × 5 mm) and then subsequently cultured in both PDA and YMA media. The number of *P. digitatum* and yeast colonized pieces were observed during 7 days incubation. Each treatment had 5 replications and the experiment was arranged in a randomized block design. Colonization of *P. digitatum* and yeast was determined with a formula defined as: number of colonized of citrus peel/total pieces of citrus peel × 100%.

2.10. Efficacy of yeast and plant extracts in reducing natural decay development on citrus fruit

The effectiveness of yeast and plant extracts to control naturally infected citrus fruit was determined in semi-commercial experiment. The best combination of plant extract and yeast was tested and compared to Imazalil (150 mg/L), water and 20% ethanol. All the treatments 200 fruit were dipped in the suspension of yeast and plant extract, imazalil, water or 20%ethanol for 2 min, then dried and packed into plastic boxes and cover with plastic bags. After storage at 25 °C, 95% RH for 2 weeks, disease incidence and disease severity were recorded. Each treatment had four replications and the experiment was arranged in a randomized block design.

2.11. Effects of yeast and plant extracts on postharvest quality of citrus fruit

The effect of yeast and plant extract on postharvest quality of citrus fruit was examined. Weight loss (%), fruit firmness (kg), total juice percentage (%), total soluble solids (%), titratable acidity (%), and ascorbic acid content (mg/100 g) of fruit were determined from 20 symptomless fruit, randomly selected from each replication after storage at 25 °C for three weeks. For weight loss, fruit were weighed before treatment (A) and after storage (B), and the weight loss calculated as $(A/B)/A$. Fruit firmness was measured at four points of the equatorial region using a firmness tester (N.O.W., FHR-5) with a 5 mm probe. Total juice percentage was calculated using the formula: (juice weight/fruit weight) × 100. The total soluble solids in juice were determined with a digital refractometer (N1-E, Atago Co., Tokyo, Japan) (Lacey et al., 2009). The titratable acidity was measured by titration with 0.1 M NaOH pH 8.3 (Hernández et al., 2006).

2.12. Determination of active ingredients of plant extracts

A thin layer chromatography (TLC) method was used for fractionation of plant crude extracts. The solvent combinations of toluene, dichloromethane, and acetone were tested to obtain the best separation of active ingredients. Purity of the isolated compounds was confirmed by high performance liquid chromatography (HPLC). The HPLC measurement was carried out using an Agilent 1100 series with UV-DAD detection at 230 nm with a reference wavelength at 600 nm. The separation was accomplished by using a Hypersil BDS C-18 column with the dimensions of 250 mm × 4.6 mm and 5 μm particle size. The mobile phase was an aqueous buffer containing 15 mM ortho-phosphoric acid and 1.5 mM tetrabutylammonium hydroxide (A) and methanol (B) with a linear gradient 0–15 min: 20–90% B followed by 100% B for 5 min. This was kept for a further 8 min. The flow rate was set to 0.8 mL/min and the injection volume was 10 μL. Identification of the chemical formula of the compound was obtained using nuclear magnetic resonance spectroscopy (NMRs), using a Bruker DRX-400 AVANCE spectrometer (Bruker, Rheinstetten, Germany) equipped with 400.13 MHz (^{1}H) or 100.61 MHz (^{13}C), and Topspin 1.2 software was used.

2.13. Statistical analysis

All data were analyzed by statistical analysis of variance (ANOVA) and regression analysis using Statistical Analysis System (SAS) software. Statistical significance was assessed at $p < 0.05$ and Tukey'S HSD multiple range test was used to separate means.

3. Results and discussion

3.1. Effects of plant crude extracts on pathogen growth (in vitro)

The plant crude extracts at concentrations of 10,000–20,000 mg/L reduced *P. digitatum* hyphal growth. The results showed *E. caryophyllata* and *C. longa* crude extracts were effective to reduce *P. digitatum* hyphal growth by 100% and up to 70%, respectively, more than the other plant crude extracts (Table 1). As such, *E. caryophyllata* and *C. longa* crude extracts were established as the most promising crude extracts to control *P. digitatum in vitro*. The antifungal activity of clove may be due to its active compound, eugenol, which is the main constituent of clove oil. The mechanism of eugenol as an effective antagonist is in increasing the permeability of the cells and causing irregular branching of hyphae in the apical parts and a loss of linearity, with collapsing and squashing due to the lack of cytoplasm (Xing et al., 2012). Laksanaphisut and Sangchot (2010) reported that a crude extract of turmeric powder in 20% of ethanol at 30,000 ppm reduced disease incidence of green mold disease on treated fruit by 25%.

3.2. Effects of yeast on P. digitatum growth (in vitro)

C. tropicalis TISTR 5010 and other *Candida* spp. were found to inhibit mycelial growth of *P. digitatum* by 78.0% and *P. membranefaciens* TISTR 5093 by 69.3% (Fig. 2). Further tests revealed that both *C. utilis* TITR 5001 and *C. tropicalis* TISTR 5010 were promising yeasts for the potential application to control *P. digitatum* individually or in combination (Fig. 3).

Table 1
In vitro screening of seven plant crude extracts dissolved in 20% ethanol at different concentrations mixed with PDA plus 2% citrus juice on their inhibition of hyphal growth of *P. digitatum* at 24 h at 25 °C.

Treatment	Percentage of inhibition of *P. digitatum* hyphal growth (%)			
	5000 mg/L	10,000 mg/L	15,000 mg/L	20,000 mg/L
Cymbopogon citratus	31.35c	40.08e	49.21fg	53.97h
Zingiber officinale	53.97h	55.95hi	55.30hi	69.05m
Momordica charantia	23.89b	33.73d	58.89j	64.29kl
Curcoma longa	46.67f	64.68k	73.41m	78.97o
Eugenia caryophyllata	100.00p	100.00p	100.00p	100.00p
Cinnamomum	0.00a	0.00a	50.86g	75.00n
Tinospora crispa	42.86e	55.95hi	56.75ij	65.87l
20% ethanol	0.00a	0.00a	0.00a	0.00a

Values with the same letters were not significant different from each other based on the Tukey'S HSD multiple range test ($p = 0.05$). Each value was mean of five replicates.

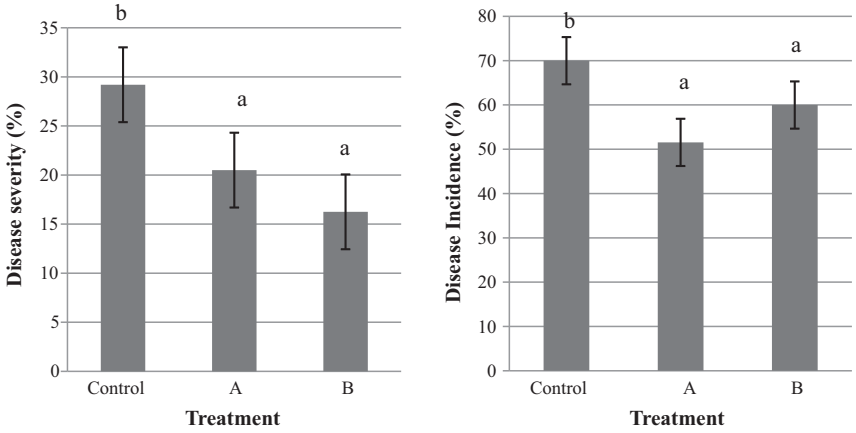

Fig. 1. Effect of plant crude extracts on disease severity (%) and disease incidence (%) of green mold on citrus fruits which were treated with *P. digitatum* (1×10^5 conidia/mL, 10 μL), incubated at 25 °C and 95% RH for seven days. (Control) ethanol 20% (A) crude extract of *Eugenia caryophyllata* (15,000 mg/L, 10 μL); (B) *Curcuma longa* (30,000 mg/L, 10 μL). Each value is mean of three replicates. Bars represent the standard deviations of the mean. Statistical significance determined at $p < 0.05$ according to Tukey's HSD test.

3.3. Effects of plant crude extracts and yeasts on green mold rot control

E. caryophylata at 15,000 mg/L and *C. longa* at 30,000 mg/L reduced disease severity and disease incidence (Fig. 1). All of the combinations of plant crude extracts and yeasts reduced disease incidence in excess of 70%. The combination of *E. caryophylata* at 15,000 mg/L, 10 μL, and *C. utilis* TITR 5001 at 1×10^8, 10 μL, was established to be the best combination to attain a reduction in disease incidence by 90.3%, while the combinations of *C. longa* at 30,000 mg/L, 10 μL, and *C. utilis* TITR 5001 at 1×10^8, 10 μL, *E. caryophylata* at 15,000 mg/L, 10 μL, and *C. tropicalis* TISTR 5010 at 1×10^8, 10 μL, *C. longa* at 30,000 mg/L, 10 μL, and *C. tropicalis* TISTR 5010 at 1×10^8, 10 μL, resulted in a reduction in the disease incidence by 80.7%, 77.4%, and 71%, respectively (Fig. 4a). Likewise, for

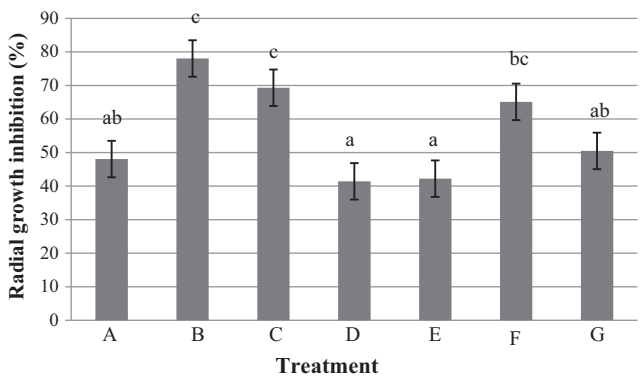

Fig. 2. Effect of yeast on hyphal radial growth inhibition of *P. digitatum* with dual cultures segregated by three centimeters, incubated at 25 °C and 95% RH for seven days. (A) *Candida sake* TISTR 5143; (B) *Candida tropicalis* TISTR 5010; (C) *P. membranefaciens* TISTR 5093; (D) *Debaryomyces hansenii* TISTR 5155; (E) *Aureobasidium pullulans* TISTR 3389; (F) *Candida guillermondii* BCC 5389; (G) *Candida utilis* TISTR 5001. Each value is mean of five replicates. Bars represent the standard deviations of the mean. Statistical significance determined at $p < 0.05$ according to Tukey's HSD test.

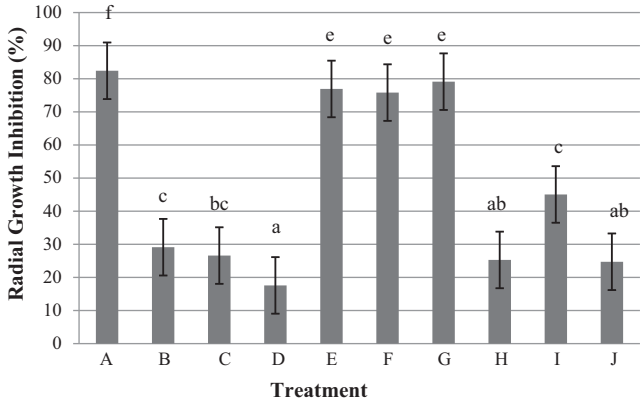

Fig. 3. Effect of yeast(s) on hyphal radial growth inhibition of *P. digitatum* with dual cultures segregated by three centimeters, incubated at 25 °C and 95% RH for seven days. (A) *Candida tropicalis* TISTR 5010 (Ct); (B) *Candida utilis* TISTR 5001 (Cu); (C) *Candida sake* TISTR 5143 (Cs); (D) *Candida guillermondii* BCC 5389 (Cg); (E) Ct and Cu; (F) Ct and Cs; (G) Ct and Cg; (H) Cu and Cs; (I) Cu and Cg; (J) Cs and Cg. Each value is mean of five replicates. Bars represent the standard deviations of the mean. Statistical significance determined at $p < 0.05$ according to Tukey's HSD test.

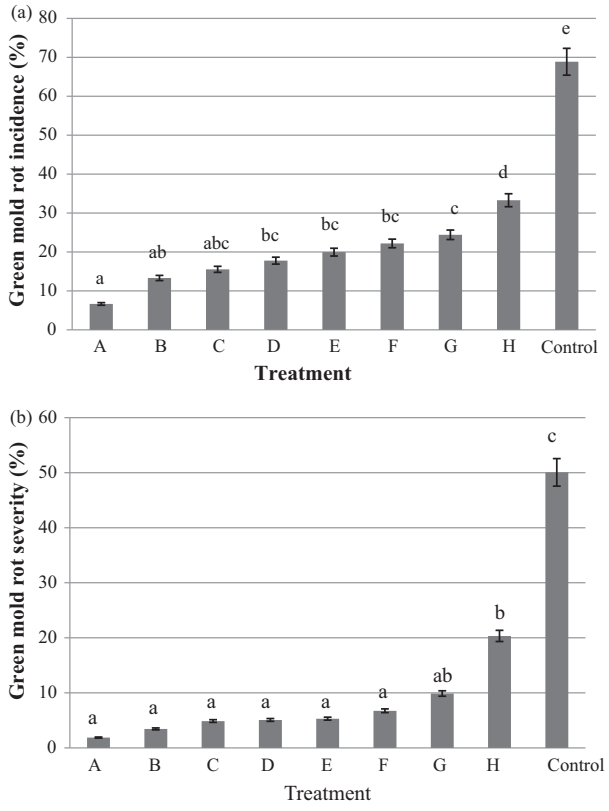

Fig. 4. Effect of plant extract (*Eugenia caryophylata* crude extract at 15,000 mg/L, and *Curcuma longa* crude extract at 30,000 mg/L) and yeast (*Candida utilis* TISTR 5001 at 1×10^8 and *Candida tropicalis* TISTR 5010 at 1×10^8) on control of green mold rot (*Penicillium digitatum*) incidence (a) and disease severity (b) in wounded citrus fruit, incubated at 25 °C and 95% RH for seven days. (A) *E. caryophylata* and *C. utilis* TISTR 5001; (B) *C. longa* and *C. utilis* TISTR 5001; (C) *E. caryophylata* and *C. tropicalis* TISTR 5010; (D) *C. utilis* TISTR 5001; (E) *C. longa* and *C. tropicalis* TISTR 5010; (F) *E. caryophylata*; (G) *C. longa*. Each value is mean of three replicates. Bars represent the standard deviations of the mean. Statistical significance determined at $p < 0.05$ according to Tukey's HSD test.

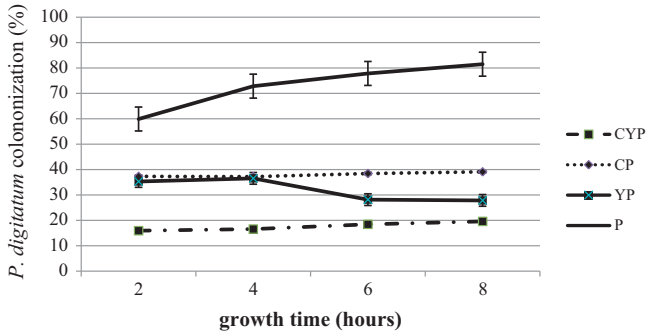

Fig. 5. *P. digitatum* colonization on citrus peel, treated with *Eugenia caryophylla* at 15,000 mg/L and *Candida utilis* TISTR 5001 at 1×10^8 at two, four, six, and eight hours after inoculation at 25 °C. Each value is mean of five replicates. C = *E. caryophylla*; Y = *C. utilis* TISTR 5001; P = *P. digitatum*.

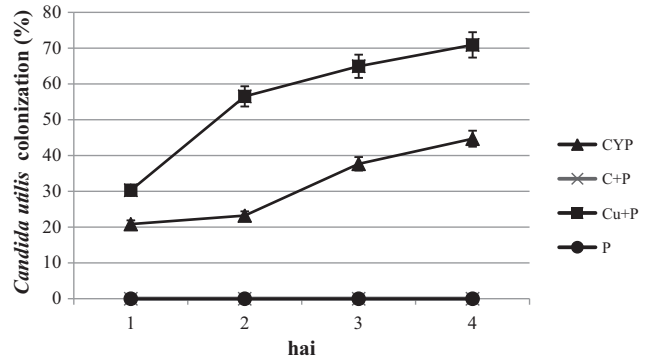

Fig. 6. *Candida utilis* TISTR 5001 colonization on citrus peel, treated with *Eugenia caryophylla* at 15,000 mg/L and *Candida utilis* TISTR 5001 at 1×10^8 at two, four, six, and eight hours after inoculation at 25 °C. Each value is mean of five replicates. C = *Eugenia caryophylla*; Y = *Candida utilis* TISTR 5001; P = *P. digitatum*.

disease severity, the combination of *E. caryophylata* at 15,000 mg/L, 10 μL, and *C. utilis* TITR 5001 at 1×10^8, 10 μL, was the best combination to attain a reduction in disease severity by 96.26%, while the combinations of *C. longa* at 30,000 mg/L, 10 μL, and *C. utilis* TITR 5001 at 1×10^8, 10 μL, *E. caryophylata* at 15,000 mg/L, 10 μL, and *C. tropicalis* TISTR 5010 at 1×10^8, 10 μL, *C. longa* at 30,000 mg/L, 10 μL, and *C. tropicalis* TISTR 5010 at 1×10^8, 10 μL resulted in reductions in disease severity by 93.2%, 90.3%, and 89.4%, respectively (Fig. 4b).

Citrus peels were cultured both on PDA and YMA media to observe *P. digitatum* and yeasts colonization in the wound site after 2, 4, 6, and 8 h of application. *P. digitatum* colonization was the lowest on citrus treated with the combination of *E. caryophylata* crude extract at 15,000 mg/L, 10 μL, and *C. utilis* TITR 5001 at 1×10^8, 10 μL (Fig. 5). Survival of *C. utilis* TITR 5001 was the highest on fruit treated with *E. caryophylata* crude extract at 15,000 mg/L, 10 μL, and *C. utilis* TITR 5001 at 1×10^8, 10 μL (Fig. 6). In the infection site, colonization of the antagonist was higher than pathogen colonization, showing a strong relationship ($R^2 = 0.8287$) (Fig. 7).

A combination of *E. caryophylata* crude extract and *C. utilis* TITR 5001 reduced natural development of green mold rot incidence by 90% and severity by 86%, and 150 mg/L Imazalil, and 20% ethanol, by 10% and 5% of disease incidence and 25.4%, 8.76% of disease severity, respectively. The weight loss and juice content of citrus fruit before and after storage at 25 °C, 95% RH for 3 weeks were not different (Fig. 8). On the other hand, fruit firmness, total soluble solids, and ascorbic acid content were different between before and after storage (Table 2). Bardin et al. (2003), reported that a biocontrol agent and synthetic chemical or plant material combined applications, provided better plant protection than individual treatments. The combination of *Paeonia suffruticosa* (medicinal plant) and *T. harzianum* was more effective than either treatment with a single agent to control *Rhizoctonia* damping-off (Lee et al., 2008). The results agree with Lee et al. (2011) who investigated 55 species of medicinal plants for their antifungal activity against *Rhizoctonia solani* AG 2-1 to improve the biocontrol efficacy of *T. harzianum in vitro*. Six species were found to be effective and among these, *E. caryophyllata* flower bud, and *Cinnamomum loureirii* stem bark were

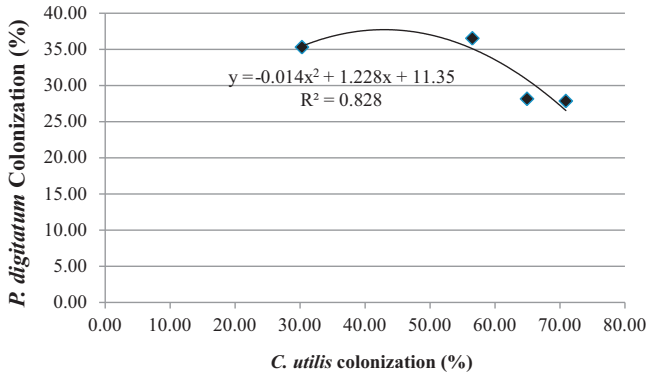

Fig. 7. Relationship between *P. digitatum* and *C. utilis* TISTR 5001 colonization on citrus peel. Each value is mean of five replicates.

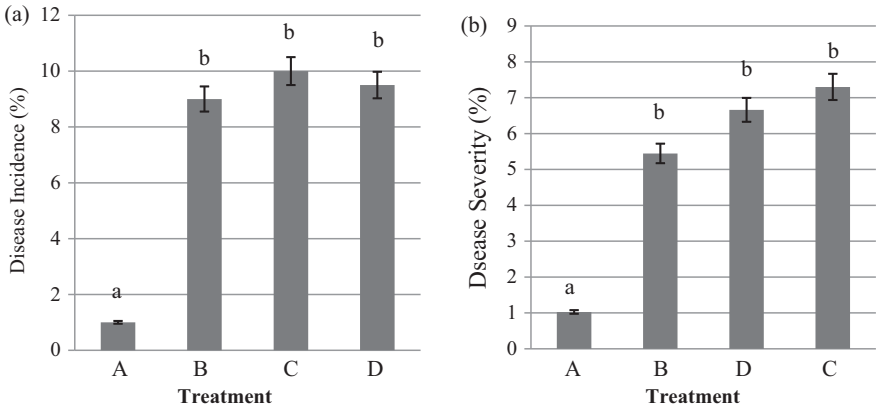

Fig. 8. Effect of *Eugenia caryophylata* crude extract at 15,000 mg/L, and yeast (*Candida utilis* TISTR 5001 at 1×10^8) for reducing natural rot development of the disease incidence (a) and disease severity (b). (A) *Eugenia caryophylata* and *Candida utilis* TISTR 5001; (B) Imazalil 150 mg/L; (C) water; (D) ethanol 20%. Each value is mean of four replicates. Bars represent the standard deviations of the mean. Statistical significance determined at $p < 0.05$ according to Tukey's HSD test.

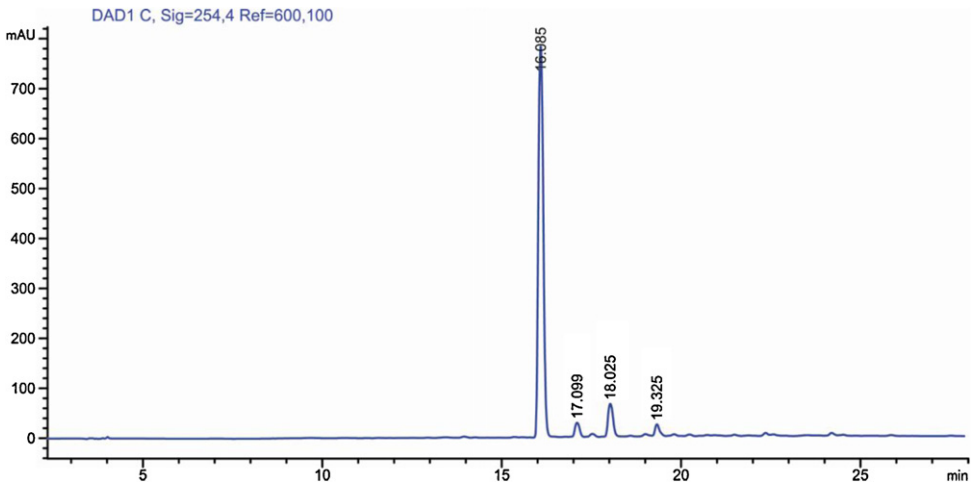

Fig. 9. HPLC chromatogram of *Eugenia caryophylla* extract, recorded at 254 nm.

Table 2
Effect of *E. caryophylata* and *C. utilis* TISTR 5001 on postharvest qualities of citrus fruits.

Treatment	Weight loss (%)	Fruit firmness (kg)	Juice content (%)	Total soluble solid (%)	Titratable acidity (%)	Ascorbic acid content (mg/100 g)
E. caryophylata and *C. utilis*	1.96a	1.59b	36.53a	9.15b	0.33a	16.61b
Imazalil 150 mg/L	1.91a	1.60b	36.73a	9.80b	0.19a	15.01b
Water	2.17a	1.51b	36.20a	9.70b	0.18a	15.09b
Ethanol 20%	3.84a	1.57b	37.74a	9.65b	0.19a	16.15b
Before storage		1.34a	40.61a	5.90a	1.73b	12.15a

Values with the same letters in column were not significant different from each other based on the Tukey'S HSD multiple range test ($p = 0.05$). Each value was mean of four replicates.

the most effective against *R. solani* AG 2-1 mycelial growth, with an inhibitory efficacy of 73.7% and 71.1%, respectively (Table 3).

3.4. Determination of active ingredients of plant extracts

The chromatograms showed the presence four peaks with retention times between 15 and 20 min. The peak of the isolated compound at 16.085 min was detected by HPLC and identified by comparison of retention time and UV spectra of the peaks in the sample solution with the standard eugenol. The standard and isolated compound showed a single peak that confirmed the compound was eugenol (Figs. 9 and 10). The ^{1}H NMR and the ^{13}C NMR provided further evidence for the confirmation of the structure of

Table 3
^{13}C and ^{1}H chemical shift of NMR spectra of *Eugenia caryophyllata* extract.

Atom number	^{13}C	^{1}H
1	143.85	–
2	146.39	–
3	111.06	6.73a
4	131.86	–
5	121.12	6.72a
6	114.21	6.89
1′	39.83	3.37
2′	137.78	6.00
3′	115.46	5.11
OMe	55.79	3.91

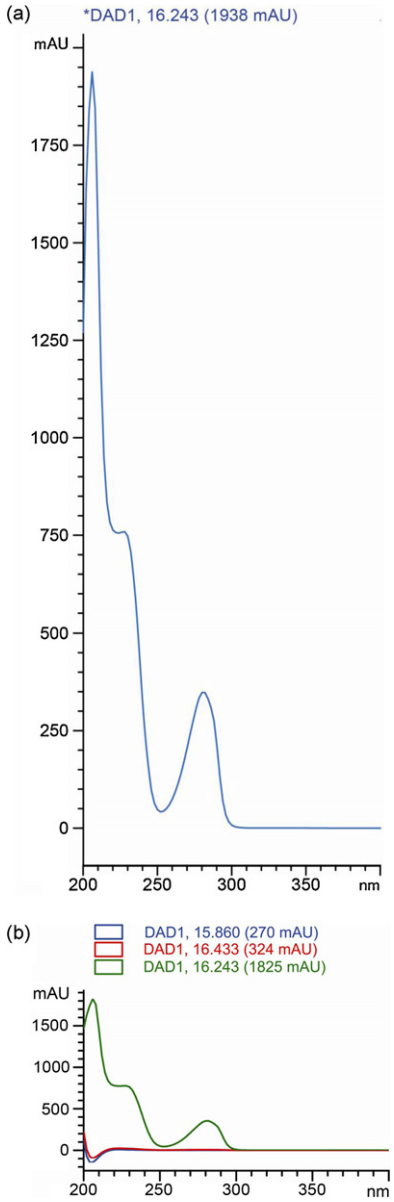

Fig. 10. UV spectra of the peaks shown in the HPLC chromatogram of *Eugenia caryophylla* extract (a); reference (b).

the isolated compound. Based on the mass spectral data, it was concluded that the isolated compound was eugenol.

4. Conclusions

The combination of *E. caryophylata* crude extract and *C. utilis* TITR 5001 had significant effects as an alternative treatment in controlling green mold. *E. caryophylata* crude extract and *C. utilis* TITR 5001 could be an ideal treatment for future plant disease management. Furthermore, further study should be focused on this combination with other control strategies, such as waxing, atmosphere conditions, carrier and adhesive materials, to develop an effective approach for postharvest disease control of citrus fruit.

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