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Biological evaluation of 32 different essential oils against *Acidovorax citrulli*, with a focus on *Cinnamomum verum* essential oil

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Bacterial fruit blotch (BFB) of watermelon caused by *Acidovorax citrulli* (ACC) is one of the most severe diseases of watermelon worldwide. Antibacterial activity of 32 essential oils (EOs) was evaluated against ACC using disk-diffusion assays. The oil from cinnamon exhibited the greatest antibacterial activity. Using gas chromatography-mass spectrometry (GC-MS), the major components of cinnamon oil were analyzed. Among the various components of cinnamon oil, benzaldehyde and cinnamaldehyde exhibited the effective antibacterial activities against ACC. The minimum inhibitory concentrations (MICs) and the minimum bactericidal concentrations (MBCs) of benzaldehyde and cinnamaldehyde were measured using broth dilution assays. The MICs against ACC of benzaldehyde and cinnamaldehyde were 0.1 and 0.01% (v/v), respectively. The MBCs of benzaldehyde and cinnamaldehyde against ACC were 0.2 and 0.02% (v/v), respectively. Also, 0.2% (v/v) levels of cinnamon oil, benzaldehyde and cinnamaldehyde completely killed ACC cells artificially contaminating watermelon seeds. This study suggests that cinnamon oil and its bioactive components, benzaldehyde and cinnamaldehyde, have potential for application as natural agents for the prevention and treatment of BFB.

Key words: *Acidovorax citrulli*, bacterial fruit blotch, cinnamon oil, essential oil.

INTRODUCTION

One of the most severe diseases of watermelon is bacterial fruit blotch (BFB), which is caused by *Acidovorax citrulli* (ACC). This disease is one of the major factors limiting yields worldwide (Burdman and Walcott, 2012). The disease was devastating and accounted for 100% loss of marketable fruit (Latin and Hopkins, 1995). Both watermelon seedlings and fruit are highly susceptible to BFB. A lag period occurs between infection and symptom development, and plants may thus remain asymptomatic for several days or more after infection (Burdman and Walcott, 2012).

A. citrulli can be introduced into watermelon fields in

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various ways, including contaminated seeds (Hopkins and Thompson, 2002) and infected transplants (Dutta et al., 2012), or via natural spread from alternate hosts such as wild cucurbits (Isakeit et al., 1998). *A. citrulli* may over-winter on infected wild cucurbits, volunteer plants or diseased plant debris. In the greenhouse, physical structures, equipment, and greenhouse supplies may be contaminated with the pathogen. The dense plant populations and high relative humidity, which is characteristic of greenhouse production facilities favor the spread of ACC. Overhead irrigation facilitates the spread of the pathogen among plants and can rapidly cause a large section of a greenhouse to become infected with ACC. Machinery, field workers, and wind-driven rain can spread ACC in the field. Hot and wet conditions in the field or greenhouse are critical environmental factors facilitating the spread of ACC and disease development. Grafting significantly increases the risk of ACC transmission (Burdman and Walcott, 2012).

The best form of control is to prevent the introduction of ACC into the field (Latin, 1996). Intensive efforts have been made by the seed and transplant industries to produce seeds and transplants that are free of ACC; such efforts have reduced the incidence of BFB. Despite these efforts, however, BFB outbreaks continue to occur every year, and BFB remains a significant problem worldwide (Hopkins, 1991; Burdman and Walcott, 2012). Currently, plant-derived essential oils (EOs) are highlighted as new generation antibacterial agents instead of antibiotics, which cause the appearance of antibiotics-resistance (Fabio et al., 2007; Samie et al., 2012; Seow et al., 2014; Hamedo, 2015). EOs are naturally occurring terpenic or aromatic mixtures, whose insecticidal and microbicidal actions against some plant pathogens have been reviewed (Isman, 2000).

The aim of the present study was to screen plant essential oils showing antibacterial activity against ACC and evaluate antibacterial activity of essential oils selected as active against ACC. *In vitro* antibacterial tests showed that cinnamon oil was the most effective against ACC. The major active constituents of cinnamon oil were determined via GC-MS, and the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of the two most effective constituents were calculated. In addition, the *in vivo* antibacterial activities of these materials against ACC were investigated.

MATERIALS AND METHODS

Bacterial strains and culture conditions

A. citrulli strain ACC02, which is highly pathogenic to watermelon plants, was used. The strain was cultured in Luria-Bertani (LB) medium (10 g tryptone, 5 g yeast extract and 10 g NaCl in 1 L distilled water) with or without 1.5% (w/v) agar at 28°C. The strain was stored at -80°C for long-term storage.

Essential oils

Thirty-two EOs were purchased from HerbMall Co. Ltd., (Seoul, Korea). Supplementary Table S1 provides a list of the EOs used in the present study and the plant parts extracted.

Disk-diffusion assay

A bacterial suspension was prepared from an overnight-grown culture, and adjusted to an optical density at 600 nm (OD_{600}) of 0.5 (~1.0×10^8 CFU mL^{-1}) (or 0.5 McFarland turbidity units). A sterile swab immersed in the bacterial suspension was used to spread the entire surface of a LB agar plate. A total of 10 µL of each EO was applied to a sterile paper disc aseptically placed on the center of the inoculated plates. After 36 h of incubation at 28°C, the diameter of the zone of growth inhibition was measured in centimeters. Kanamycin served as a positive control. All experiments were carried out in triplicate. Average values of inhibition diameters were calculated to classify the EOs as follows: the strains were termed not sensitive (0) for a diameter smaller than 0.8 cm, moderately sensitive (+) for a 0.8–2.5 cm diameter, sensitive (++) for a 2.5–5 cm, and very sensitive (+++) for a diameter greater than 5 cm.

Gas chromatography-mass spectrometry (GC-MS) analysis

The active chemical constituents of the cinnamon oil were determined using gas chromatography-mass spectrometry (GC-MS), a GC puls-2010 coupled with GC-MS-QP2010 (Shimadzu, Japan), which was fitted with a HP-Innowax column (30 mm × 0.25 mm i.d. × 0.25 µm, J & W Scientific Co., USA). The temperature program started at 40°C for 1 min and increased to 250°C at 6°C min^{-1}, and then held for 4 min. Split injection (1:5 ratios) was performed with a 1-µL sample volume. The mass detector was fitted with an electron ionization source operated at 70 eV with a source temperature of 230°C. Helium was the carrier gas at a flow rate of 1 mL min^{-1}. Identification of EO compositions was based on the mass spectral information in a mass spectra library (McLafferty, 2000), and sample peaks were confirmed by comparison with the retention indices (RI) and mass spectra of authentic standards.

β-Phellandrene was prepared as described previously (Kang et al., 2013). Benzaldehyde, hydrocinnamic aldehyde and cinnamaldehyde were synthesized from the corresponding alcohol by PCC oxidation (Corey and Suggs, 1975). Hydrocinnamyl acetate and cinnamyl acetate were obtained by acetylation of the corresponding alcohol. Hydrocinnamyl alcohol was synthesized by hydrogenation of cinnamyl alcohol with Pd on the carbon.

Determination of MIC and MBC

MIC of the test compounds was determined using the broth dilution method in LB broth as described by Sfeir et al. (2013). Briefly, each compound was first diluted to 40% (v/v) in dimethyl sulfoxide (DMSO). Serial dilutions were carried out in sterile distilled water at concentrations of 0.01-0.5% (v/v). One milliliter of bacterial suspension (10^6 CFU mL^{-1}) and 0.1 mL of each compound showing antibacterial activity were added to 2.9 mL of LB broth. Controls without test compounds were prepared. After 24 h of incubation at 28°C under agitation in culture tubes, the MIC was determined as the lowest concentration that visibly inhibited bacterial growth.

To determine the MBC, 10 µL of bacterial inoculums were removed from tubes that had not presented visible turbidity and spread onto LB agar. These plates were incubated at 28°C for 48 h. The MBC was considered as the lower concentration that showed no bacterial growth on LB agar plates. Each MIC and MBC value was obtained from three independent experiments.

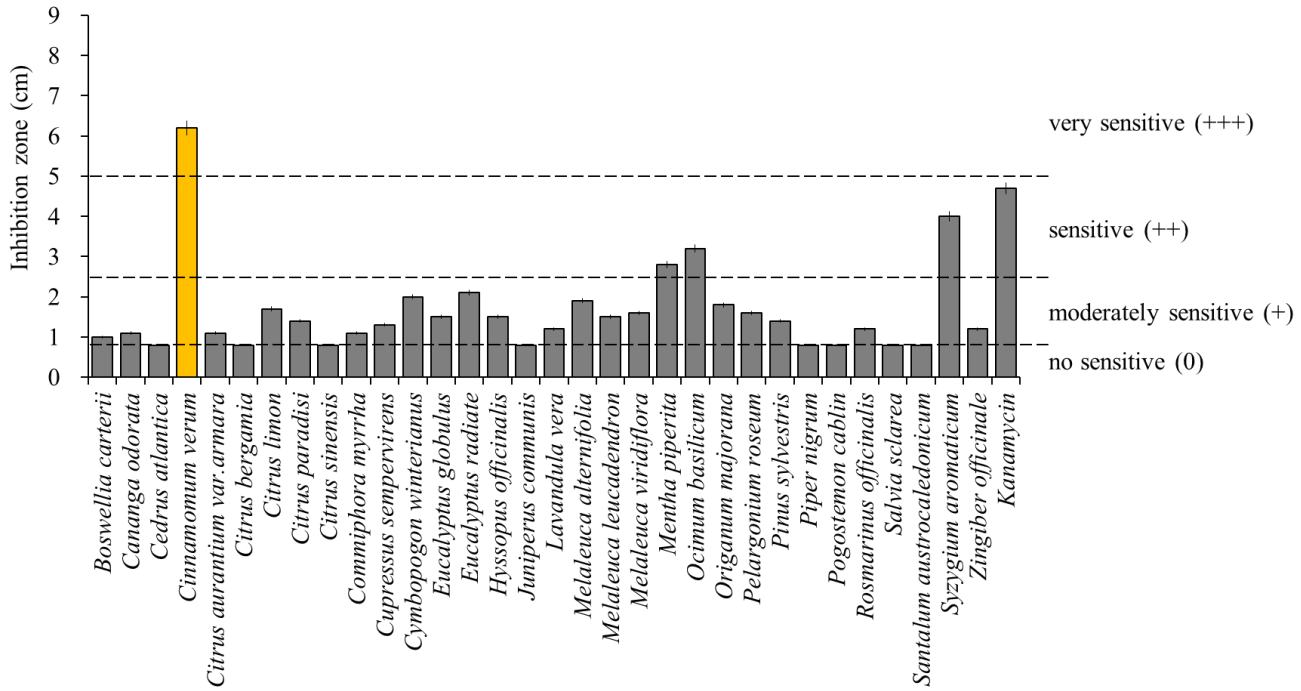

Figure 1. Inhibition zone diameters of the various essential oils against *A. citrulli* (means ± SD). Kanamycin (50 µg mL^{-1}) was used as the positive control. The experiments were carried out in triplicate. Average inhibition diameters were calculated to classify the EOs as follows: the strain was termed not sensitive (0) for a diameter smaller than 0.8 cm, moderately sensitive (+) for a 0.8–2.5 cm diameter, sensitive (++) for a 2.5–5 cm, and very sensitive (+++) for a diameter greater than 5 cm.

Antibacterial activity in watermelon seeds

Two chemical components (benzaldehyde and cinnamaldehyde) of cinnamon oil exhibited potent *in vitro* inhibition of ACC02 growth and were evaluated in terms of inhibition of ACC growing on artificially inoculated watermelon seeds. As an inoculum, a bacterial suspension was prepared from an overnight-grown culture, and adjusted to an optical density at 600 nm (OD_{600}) of 0.5 (1.0×10^8 CFU mL^{-1}) (or 0.5 McFarland turbidity units). Watermelon seeds (vr. Speed; Nongwoo Bio, Co. Ltd., Suwon, Korea) were soaked in the bacterial suspension for 30 min and dried.

Oil suspensions were prepared according to a previously described procedure with some modifications (Roh et al., 2011); 10 µL of cinnamon oil, benzaldehyde or cinnamaldehyde was dissolved in 1 mL ethanol followed by mixing with 9 mL distilled water to yield 0.1% (v/v) oil solutions, and Triton X-100 (0.009%, v/v) was added to each diluted solution. A mixture of ethanol (1 mL), Triton X-100 (0.009%, v/v), and distilled water (9 mL) served as a negative control. The seeds artificially contaminated with ACC were soaked in suspensions of cinnamon oil, benzaldehyde or cinnamaldehyde for 30 min, and dried. Three seeds per treatment were used in each experiment and bacterial colonies were calculated using a serial dilution plat method. The experiment was performed in triplicate.

RESULTS

Screening for antibacterial activity

Antibacterial activities of plant EOs against the ACC strain (ACC02) are presented in Figure 1. Results obtained from disk-diffusion assays showed that cinnamon oil was the most active against ACC02, with inhibition zones greater than 5.0 cm (+++). ACC02 was sensitive (++) to *Mentha piperita*, *Ocimum basilicum* and *Syzygium aromaticum* oils. Most EOs tested showed moderate inhibitory activities (+) against the tested strain. Eight EOs (those of *Cedrus atlantica*, *Citrus bergamia*, *Citrus sinensis*, *Juniperus communis*, *Piper nigrum, Pogostemon cablin, Salvia sclarea,* and *Santalum austrocaledonicum*) exhibited no significant activities (0) against the test strain. The inhibition zone of cinnamon oil was greater than that of the positive control, kanamycin.

Essential oil composition

The results of the chemical analysis are presented in Supplementary Table S2. The compounds are listed according to their elution order, which was in agreement with their RI on HP-Innowax columns (van Den Dool and Kratz, 1963). Of the 27 components of cinnamon oil, 24 were identified: α-pinene, camphene, β-pinene, 3-carene, α-phellandrene, α-terpinene, limonene, β-phellandrene, *p*-cymene, α-terpinolene, benzaldehyde, linalool, β-caryophyllene, humulene, α-terpineol, hydrocinnamic aldehyde, hydrocinnamyl acetate, caryophyllene oxide,

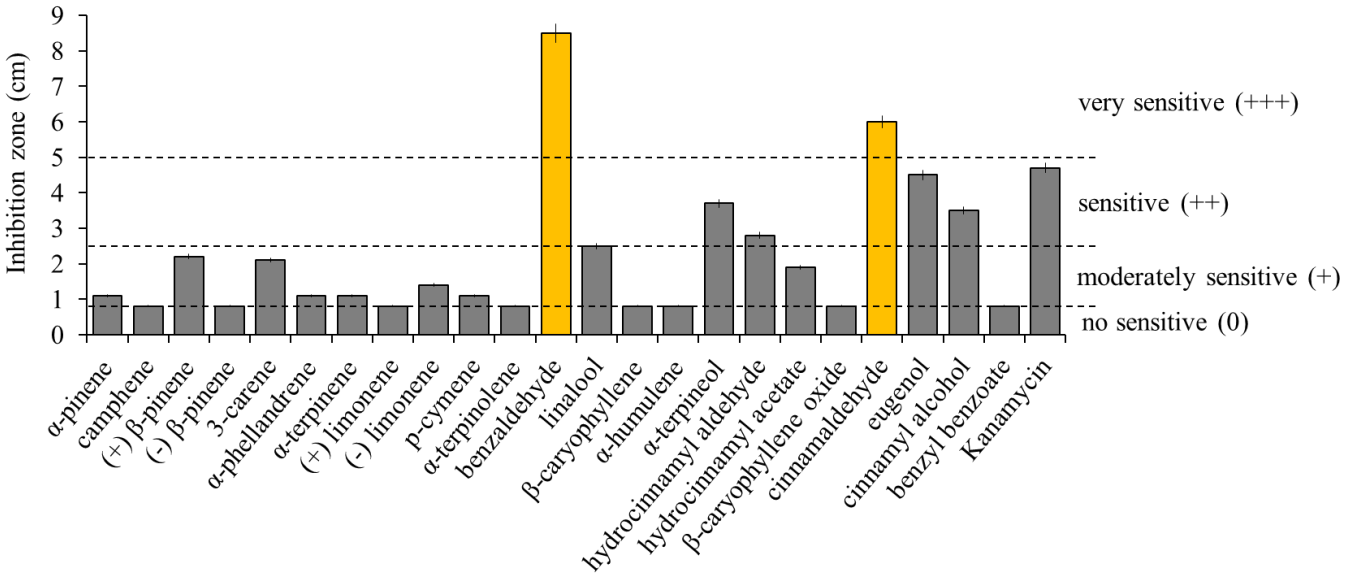

Figure 2. Inhibition zone diameters obtained for cinnamon oil components against *A. citrulli* (means ± SD). Kanamycin (50 µg mL^{-1}) was used as a positive control. The experiments were carried out in triplicate. Average inhibition diameters were calculated to classify the EOs as follows: the strain was termed not sensitive (0) for a diameter smaller than 0.8 cm, moderately sensitive (+) for a 0.8–2.5 cm diameter, sensitive (++) for a 2.5–5 cm, and very sensitive (+++) for a diameter greater than 5 cm.

cinnamaldehyde, cinnamyl acetate, eugenol, cinnamyl alcohol, methoxycinnamaldehyde and benzyl benzoate (Supplementary Table S2). Three peaks showed no match with the MS library. Cinnamaldehyde (44.35%) was the main compound in cinnamon oil, followed by β-phellandrene (9.55%) and cinnamyl acetate (8.5%) (Supplementary Table S2).

Antibacterial activities of cinnamon oil components

The antibacterial activities of the chemical constituents of cinnamon oil against ACC02 are presented in Figure 2. The results of the disk-diffusion assay showed that benzaldehyde and cinnamaldehyde were the most active against the tested bacterial strain, with inhibition zone diameters greater than 5.0 cm (+++). ACC02 was sensitive (++) to α-terpineol, hydrocinnamic aldehyde, eugenol and cinnamyl alcohol. The test strain was moderately sensitive (+) to α-pinene, (+) β-pinene, 3-carene, α-phellandrene, α-terpinene, (-) limonene, *p*-cymene, linalool and hydrocinnamyl acetate. No significant activity (0) was exhibited by eight compounds: camphene, (-) β-pinene, (+) limonene, α-terpinolene, β-caryophyllene, α-humulene, β-caryophyllene oxide and benzyl benzoate (Figure 2). The inhibition zones of benzaldehyde and cinnamaldehyde were greater than that of the positive control, kanamycin.

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) values

Disk-diffusion assays for benzaldehyde and cinnamaldehyde were used to determine the most effective compound, and the MIC values were determined by means of broth dilution assays. The MICs of benzaldehyde and cinnamaldehyde were 0.1 and 0.01% (v/v) against ACC02, respectively (Figure 3). The MBCs of benzaldehyde and cinnamaldehyde against ACC02 were 0.2 and 0.02% (v/v), respectively (Figure 3).

Antibacterial activities on watermelon seeds

Cinnamon oil, benzaldehyde and cinnamaldehyde, exhibiting strong *in vitro* antibacterial activities against ACC02, were evaluated in terms of bacterial control on watermelon seeds. Each compound at 0.2% (v/v) completely killed ACC cells. At 0.05% (v/v), each compound inhibited bacterial growth by more than 70%. At 0.1% (v/v), cinnamaldehyde inhibited bacterial growth by more than 96% as compared to the control, whereas cinnamon oil and benzaldehyde inhibited bacterial growth by 75% (Figure 4).

DISCUSSION

Chemical control remains the major management in the control of plant diseases. One of the most important problems against the effective use of chemical control agents is the development and spread of resistant pathogens. The application of higher concentrations of various chemicals may increase the risk of high level toxic residues in the plant products. Despite the development of control agents, bacterial and fungal

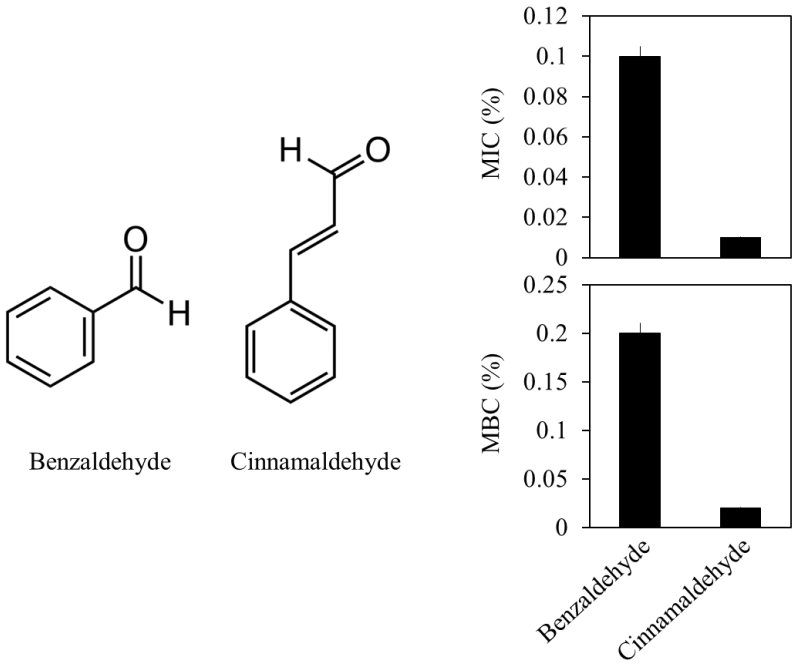

Figure 3. Chemical structure, minimum inhibitory concentrations and minimum bactericidal concentrations of benzaldehyde and cinnamaldehyde against *A. citrulli* (means ± SD).

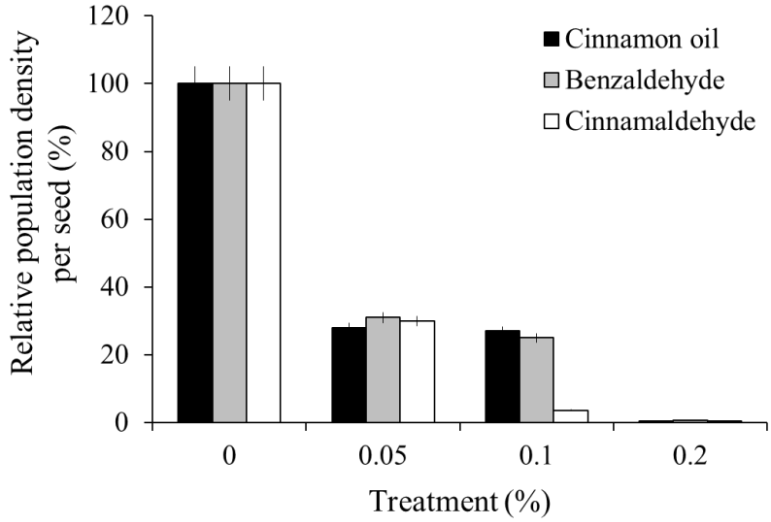

Figure 4. *In vivo* antibacterial activities of cinnamon oil, benzaldehyde and cinnamaldehyde against *A. citrulli* (means ± SD). The experiments were carried out in triplicate.

diseases are still a major problem in crop production. Therefore, there is an urgent need to develop new control agents, with higher activity, greater sensitivity, and lower toxicity. Plant-derived EOs are ideal for use in control formulations of plant diseases because they are antiseptic and environmentally friendly. In efforts to develop alternatives to synthetic chemicals for control of plant pathogens, interest in EOs has increased. However, scientific investigations to evaluate the antimicrobial activity of EOs are needed.

The present work evaluated the antibacterial activities of 32 EOs specifically against ACC, responsible for BFB

outbreaks. As a results, the EO of *C. verum* exhibited the greatest antibacterial activity against ACC. In the literature, the EO of *C. verum* was active *in vitro* against the following bacteria: *Escherichia coli*, *Staphylococcus aureus* (Barnes et al., 2007), *Streptococus mutans* (Fani and Kohanteb, 2011), *S. pyogenes* (Sfeir et al., 2013), *Salmonella typhimurium*, *Bacillus subtilis*, *B. thermoacidurans*, *Pseudomonas aeruginosa* (WHO, 1999) and *Helicobacter pylori* (Dugoua et al., 2007). However, no previous publications have reported the antibacterial activity of cinnamon oil against BFB-causing bacteria.

The chemical composition of cinnamon oil showing the greatest antibacterial activity against ACC was analyzed. GC-MS analyses indicated that cinnamaldehyde (44.35%) was the principal component of cinnamon oil extracted from the bark of *C. verum* plant; other major components were β-phellandrene (9.55%) and cinnamyl acetate (8.5%). The chemical composition of the cinnamon oil used in this study was very similar to that used in previous reports (Wang et al., 2005; Jeong et al., 2014). EOs containing mainly aromatic phenols or aldehydes have been reported to exhibit considerable antimicrobial activity, whereas EOs containing terpene ethers, ketones, or oxides had weaker activities (Inouye et al., 2001; Fabio et al., 2007).

In the present study, benzaldehyde and cinnamaldehyde exhibited effective antibacterial activities against ACC. These results are in agreement with previous reports showing that benzaldehyde and cinnamaldehyde have antimicrobial properties against several species of common foodborne bacteria (Bowles and Juneja, 1998; Helander et al., 1998). Cinnamon EO damages the cellular membrane of *Pseudomonas aeruginosa*, which leads to the collapse of membrane potential and loss of membrane-selective permeability. In *Staphylococcus aureus*, cells treated with the oil showed a considerable decrease in the metabolic activity and replication capacity, leading to a viable but noncultivable state (Bouhdid et al., 2010). Cinnamaldehyde exposure causes morphological changes in foodborne pathogenic bacteria, including *S. aureus*, *S. anatum* and *B. cereus* (Shan et al., 2007). However, the mode of action of active compounds of EOs has not been verified in the present study.

The antibacterial activity of cinnamon oil was investigated by determining the MIC and MBC values. For benzaldehyde and cinnamaldehyde, the MICs against planktonic ACC02 were 0.1 and 0.01% (v/v) (Figure 3). These high activities facilitated determination of MBC values. The MIC values were indeed low, and cinnamaldehyde was more effective against planktonic cells. The MBCs of benzaldehyde and cinnamaldehyde were determined using concentrations twice those of the MIC values to verify the accuracy of the MIC testing and to determine appropriate concentrations for use. Lobo et al. (2013) found that the MIC of cinnamon oil against *Streptococcus mutans* was 0.8 mg mL^{-1}. To the best of the authors' knowledge, no previous study has calculated the MIC or MBC of cinnamaldehyde against ACC.

EOs extracted from cinnamon bark had highly effective antibacterial activities against ACC. Cinnamon oil at 0.2% (v/v) completely killed ACC cells artificially contaminating watermelon seeds. Therefore, cinnamon oil can be used to control ACC on watermelon seeds. However, in the development of Eos as alternatives to synthetic bactericides, future studies must evaluate the phytotoxicities of EOs applied to plant seeds.

This study showed the *in vitro* and *in vivo* antibacterial activities of cinnamon oil and its active components benzaldehyde and cinnamaldehyde against ACC. In addition, our study gives a support for the application of cinnamon oil to eliminate ACC under specific conditions. However, for the development of cinnamon oil as an alternative of synthetic bactericides, further investigation should be carried out to obtain information regarding the practical effectiveness to protect plants without phytotoxicity.

Conclusions

This study showed that the EO of *C. verum* and its major components, benzaldehyde and cinnamaldehyde, possessed considerable *in vitro* antibacterial activities against bacterial fruit blotch of watermelon caused by *A. citrulli*. The MICs against ACC of benzaldehyde and cinnamaldehyde were 0.1 and 0.01% (v/v), respectively. The MBCs of benzaldehyde and cinnamaldehyde against ACC were 0.2 and 0.02% (v/v), respectively. Also, 0.2% (v/v) levels of cinnamon oil, benzaldehyde and cinnamaldehyde completely killed ACC cells artificially contaminating watermelon seeds. This study may be useful for application as natural agents for the prevention and treatment of BFB. Experiments that evaluate the effectiveness of EOs include: the ability to penetrate the seed coat; and the assessment to decontaminate cucurbit seeds without phytotoxicity.

Conflict of interests

The authors did not declare any conflict of interest.

Abbreviations

BFB, Bacterial fruit blotch; **ACC,** *Acidovorax citrulli*; **EO,** essential oil; **GC-MS,** gas chromatography-mass spectrometry; **MIC,** minimum inhibitory concentration; **MBC,** minimum bactericidal concentration.

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Supplementary Table S1. List of essential oils used in this study.

Plant	Plant species	Plant part
Bergamot	*Citrus bergamia*	Zest
Bitter orange	*Citrus aurantium* var.*amara*	Bud
Black pepper	*Piper nigrum*	Fruit
Blue gum	*Eucalyptus globulus*	Leaf
Cajeput tree	*Melaleuca leucadendron*	Leaf
Cedarwood	*Cedrus atlantica*	Wood
Cinnamon	*Cinnamomum verum*	Bark
Citronella	*Cymbopogon winterianus*	Grass and flower
Clary sage	*Salvia sclarea*	Leaf
Clove bud	*Syzygium aromaticum*	Bud
Cypress	*Cupressus sempervirens*	Branch
Eucalyptus	*Eucalyptus radiata*	Leaf
Frankincense	*Boswellia carteri*	Sap
Geranium	*Pelargonium roseum*	Aerial part
Ginger	*Zingiber officinale*	Rhizome
Grapefruit	*Citrus paradisi*	Zest
Hyssop	*Hyssopus officinalis*	Leaf
Juniper	*Juniperus communis*	Fruit
Lemon peel	*Citrus limon*	Zest
Myrrh	*Commiphora myrrha*	Flower and wood
Niaouli	*Melaleuca viridiflora*	Leaf
Patchouli	*Pogostemon cablin*	Leaf
Peppermint	*Mentha piperita*	Leaf
Rosemary	*Rosmarinus officinalis*	Leaf and flower
Sandalwood	*Santalum austrocaledonicum*	Wood
Scotch pine	*Pinus sylvestris*	Needle
Sweet basil	*Ocimum basilicum*	Flower and leaf
Sweet marjoram	*Origanum marjorana*	Flower and leaf
Sweet orange	*Citrus sinensis*	Zest
Tea-tree	*Melaleuca alternifolia*	Leaf
True lavender	*Lavandula vera*	Leading flower
Ylang-ylang	*Cananga odorata*	Flower

Supplementary Table S2. Chemical components of *C. verum* oil.

Component	Percentage (%)
Cinnamaldehyde	44.35
β-Phellandrene	9.55
Cinnamyl acetate	8.50
p-Cymene	6.31
α-Phellandrene	3.79
β-Caryophyllene	3.10
Limonene	3.06
Linalool	2.96
α-Terpinene	2.82
Eugenol	2.78
α-Terpineol	2.08
Unknown	1.27

Supplementary Table S2. Contd.

Benzyl benzoate	1.24
α-Pinene	1.23
Humulene	1.14
Camphene	1.00
Cinnamyl alcohol	0.89
Caryophyllene Oxide	0.73
Benzaldehyde	0.54
Hydrocinnamic aldehyde	0.40
Methoxycinnamaldehyde[2]	0.39
α-Terpinolene	0.37
β-Pinene	0.36
Unknown	0.32
Unknown	0.30
3-Carene	0.25
Hydrocinnamyl acetate	0.25

Quantification of each component was estimated by area normalization.