J Am Oil Chem Soc (2010) 87:1341–1349
DOI 10.1007/s11746-010-1623-9

ORIGINAL PAPER

Antibacterial Activity of Essential Oil and Extracts of *Cleistocalyx operculatus* Buds Against the Bacteria of *Xanthomonas* spp.

Vivek K. Bajpai · Nguyen Thi Dung · Hwa-Jin Suh · Sun Chul Kang

Received: 5 November 2009 / Revised: 3 June 2010 / Accepted: 8 June 2010 / Published online: 26 June 2010

Abstract This study was undertaken to assess the antibacterial efficacy of the essential oil and extracts of *Cleistocalyx operculatus* buds against plant pathogenic bacteria of *Xanthomonas* spp. The diameter of inhibition zones of oil (1,000 μg/disc) and extracts (1,500 μg/disc) against the tested bacteria were found in the range of 7–23 mm. The MIC and MBC values of the oil and extracts against the tested *Xanthomonas* spp. ranged from 31.25–125 to 62.5–250 μg/ml and 125–500 and 250–1,000 μg/ml, respectively. The cell viability study demonstrated a potential detrimental effect of the oil (1,000 μg/ml) and hexane extract (250 μg/ml) on the tested *Xanthomonas* spp. Also the oil displayed significant antibacterial effects in vivo against *Xoo* KX019 and *Xsp* SK12 conducted on greenhouse-grown oriental melon plants (*Cucumis melo* L. var. *makuwa*). The results of this study suggest that *C. operculatus*-derived essential oil and extracts could be used as natural bactericides in the food and agriculture industries.

Keywords *Xanthomonas* spp. · *Cleistocalyx operculatus* buds · Antibacterial efficacy · Essential oil · Oriental melon (*Cucumis melo* L. var. *makuwa*)

V. K. Bajpai and N. T. Dung equally contributed to this research.

V. K. Bajpai · N. T. Dung · H.-J. Suh · S. C. Kang (✉)
Department of Biotechnology, Daegu University,
Kyoungsan, Kyoungbook 712-714, Republic of Korea
e-mail: sckang@daegu.ac.kr

N. T. Dung
Department of Plant Biochemistry, Institute of Biotechnology,
Vietnamese Academy of Science and Technology,
Hanoi, Vietnam

Introduction

Plant diseases constitute an emerging threat to global food security. Many of the currently available antimicrobial agents for agriculture are highly toxic, non-biodegradable, and cause extended environmental pollution [1]. Plants are constantly exposed and threatened by a variety of pathogenic microorganisms present in their environments. Diseases caused by pathogens including bacteria and fungi significantly contribute to the overall loss in crop yields worldwide [2]. In an effort to combat diseases, plants have devised various mechanisms and compounds to fend off microbial invaders. Despite the existence of plant defense mechanisms, a major difficulty encountered is the lack of effective control agents against some severe plant bacterial diseases. On the other hand, application of chemical derivatives has effectively controlled the plants from bacterial disease but this threatens to contaminate the environment, hindering the management of diseases in crops and agricultural products [3].

Bacterial diseases caused by *Xanthomonas* has devastated various host-plants, resulting in considerable losses in productivity and quality of harvests [4]. Pathovars of *Xanthomonas* are reported to have developed resistance to several antibiotics such as kanamycin, ampicillin, penicillin, and streptomycin [5]. In addition, control of the disease is difficult, often requiring expensive and complex integrated pest management, including the use of contamination-free seeds, sanitization practices, and the use of chemicals. Moreover, antibiotics and synthetic pesticides are forbidden in many countries as they exert a negative impact such as high and acute toxicity, long degradation periods and accumulation in the food chain. Consequently, there is an obvious need to search for alternative natural antimicrobial agents or biopesticides to control bacterial

plant diseases for agricultural applications, which are nontoxic and nonpolluting [6]. Compounds are specially sought that have activity against plant pathogenic bacteria that have acquired resistance to commercial compounds.

Researches focused on plant-derived natural bactericides and their possible applications in agriculture to control plant bacterial diseases has intensified as this approach has enormous potential to inspire and influence modern agro-chemical research. Naturally occurring and biologically active plant products such as essential oils and organic extracts could be a source of alternative classes of natural biopesticides to serve as templates for new and more effective compounds in controlling plant pathogenic microorganisms.

Melon crops are much diversified and comprise six subvarieties, among which the oriental melons (*Cucumis melo* L. var. *makuwa*) are commercially important. They are widely grown in Korea, China and Eastern Asian countries and favored by consumers, largely due to their high qualities, special flavors and consumer demands.

Cleistocalyx operculatus (Roxb.) Merr and Perry, is a well known traditional medicine, widely distributed and propagated in China, Vietnam and some other tropical countries. Various in-vitro and in-vivo biological activities such as anticancer, antitumor, antihyperglycemic and cardiotonic actions of the extract and components of *C. operculatus* buds have been reported [7–10]. Previously, we have reported the chemical composition and antibacterial properties [11] of the essential oil and various organic extracts of *C. operculatus* buds against food-borne pathogens and multiantibiotic-resistant bacteria as well as the antiinflammatory property of its oil [12]. The GC/MS analysis of the leaf essential oil of *C. operculatus* has also been reported [13]. In the present study, a detailed in-vitro and in-vivo investigation was conducted to test the efficacy of essential oil and various organic extracts of *C. operculatus* buds against plant pathogenic bacteria of *Xanthomonas* spp. on greenhouse-grown oriental melon plants.

Materials and Methods

Plant Material

The buds of *C. operculatus* used in this experiment were purchased from its range of habitat, a local herb supplier in Hanoi, Vietnam, and were identified by comparing its morphological features with the specimen deposited at the Plant Laboratory, Institute of Biological Ecology and Biological Resources, Vietnamese Academy of Science and Technology, Hanoi, Vietnam.

The seeds of the oriental melon (*Cucumis melo* L. var. *makuwa*) were purchased from a local seed supplier in Daegu city, Kyoungbook, Republic of Korea.

Isolation of Essential Oil

The essential oil of *C. operculatus* buds used in this study was obtained as described previously [15]. In brief, the air-dried *C. operculatus* buds were pulverized and a powder sample (250 g) was subjected to hydrodistillation for 4 h using a Clevenger type apparatus to obtain the essential oil. The oil was dried over anhydrous Na_2SO_4 and preserved in a sealed vial at 4 °C in the dark until further analysis (yield 0.68%, w/w). The chemical composition of the oil was similar to that reported previously [11].

Preparation of Organic Extracts

The air-dried buds of *C. operculatus* were pulverized and the dried powder (50 g) was successively extracted twice with hexane, chloroform, ethyl acetate and methanol at room temperature and the similar extracts were combined. The solvents from the extracts were evaporated by a vacuum rotary evaporator (EYELA N1000, Japan) and dried to yield hexane (3.2%, w/w), chloroform (4.3%, w/w), ethyl acetate (4.8%, w/w) and methanol (5.6%, w/w) extracts. Solvents (analytical grade) for extraction were purchased from Sigma-Aldrich Co., USA.

Microorganisms

The test organisms used in this study were *Xanthomonas campestris* pv. *campestris* KC94-17 (*Xcc* KC94-17), *Xanthomonas campestris* pv. *vesicatoria* YK93-4 (*Xcv* YK93-4), *Xanthomonas oryzae* pv. *oryzae* KX019 (*Xoo* KX019) and *Xanthomonas* sp. SK12 (*Xsp* SK12), which were collected from the Korean Agricultural Culture Collection (KACC), Suwon, Republic of Korea. Active cultures for experimental use were prepared by transferring a loopful of cells from stock cultures to flasks and inoculating them in a Luria–Bertani (LB) broth medium at 30 °C for 24 h. Cultures of each bacterial strain were maintained on an LB agar medium at 4 °C.

Determination of Antibacterial Activity by Disc Diffusion Method

The agar diffusion method was used for antibacterial assay [14]. Previously prepared LB agar plates were used for the antibacterial assay. A 100-µl sample of a standardized bacterial suspension containing 10^7 CFU/ml was poured and uniformly spread, and the inoculum was allowed to dry for 5 min. To prepare the stock solution of the samples, 1 ml oil was dissolved in 10 ml 5% DMSO (dimethyl sulfoxide), whereas the extracts (hexane, chloroform, ethyl acetate and methanol) were dissolved in their respective solvents. Whatman No. 1 sterile filter paper

discs (6 mm diameter) were impregnated with defined concentrations of essential oil (1,000 μg/disc) and organic extracts (1,500 μg/disc), prepared in 5% DMSO or the solvents (hexane, chloroform, ethyl acetate and methanol), respectively. The discs were allowed to dry for 15 min and placed on the inoculated agar. The standard reference drug, ampicillin (20 μg/disc) was used as a positive control, was obtained from Sigma Chemicals (St. Louis, MO). Negative controls were prepared using the same solvents employed to dissolve the samples. The plates were incubated at 30 °C for 24 h. Antibacterial activity was evaluated by measuring the diameter of zones of inhibition using a vernier caliper against the tested bacteria. Each assay in this experiment was replicated three times.

Determination of Minimum Inhibitory and Minimum Bactericidal Concentrations

Micro-dilution susceptibility assay was performed using the NCCLS method for the determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) [15]. Bacteria were cultured overnight at 30 °C. The test samples of oil and the extracts were dissolved in 5% DMSO. Dilutions were prepared in a 96-well microtiter plates to get final concentrations ranging from 0 to 4,000 μg/ml. Finally, 20 μl of inoculum (10^{6}–10^{-7} CFU/ml) was inoculated onto the microplates and the tests were performed in a volume of 200 μl. Plates were incubated at 30 °C for 24 h. The standard reference drug, ampicillin, was used as a positive control for the tested plant pathogenic bacteria. The lowest concentrations of tested samples, which did not show any visual growth after macroscopic evaluation, were determined as MICs, which were expressed in μg/ml. Using the results of the MIC assay, the concentrations showing complete absence of visual growth of bacteria were identified and 50 μl of each culture broth was transferred onto the agar plates and incubated for the specified time and temperature as mentioned above. The complete absence of growth on the agar surface in the lowest concentration of sample was defined as the MBC. Each assay in this experiment was replicated three times.

Determination of Cell Viability Counts

Each of the tubes containing the bacterial suspension (approximately 10^{6} CFU/ml) of the test bacterial strains of *Xanthomonas* spp. was inoculated with a concentration of 1,000 and 250 μg/ml of the essential oil and hexane extract, respectively from *C. operculatus* buds in 10 ml LB medium, and kept at 30 °C. Samples for viable cell counts were taken out at 0, 5, 15, 30, 60, 120 min and 24 h time intervals. The viable plate counts were monitored as followed: 0.1 ml sample of each treatment was diluted and spread on the surface of LB agar place. The colonies were counted after 24 h of incubation at 30 °C. The controls were inoculated without test samples (essential oil and hexane extract) for each bacterial strain with the same experimental condition as mentioned above. Population levels of strains were expressed as: $\log_{10}$ CFU/ml. Each assay in this experiment was replicated three times.

Cultivation of Oriental Melon Plants (*Cucumis melo* L. var. *makuwa*) in Greenhouse and Preparation of in Vivo Test Samples

For in-vivo experimentation, the seeds of test plants (*Cucumis melo* L. var. *makuwa*) were grown in separate pots during April, 2008 in a greenhouse (24–25 °C, 60–70% of humidity and higher light intensity required for melon plants was also maintained). The tested plants possessing an average of 7–10 leaves were used as the host plants for the in vivo experiments.

The essential oil of *C. operculatus* buds was dissolved in solution of 0.5% dimethyl sulfoxide (DMSO) and 0.1% surfactant Tween 80 in distilled water to get the concentrations of the tested solutions of 0, 500, 1,000 and 2,000 μg/ml of the essential oil of *C. operculatus* buds.

In-Vivo Antibacterial Activity Assay

Among the tested strains of *Xanthomonas* spp. in vitro such as *X. campestris* pv. *campestris* KC94-17 (*Xcc* KC94-17), *X. campestris* pv. *vesicatoria* YK93-4 (*Xcv* YK93-4), *X. oryzae* pv. *oryzae* KX019 (*Xoo* KX019) and *X.* sp. SK12 (*Xsp* SK12), which cause black rot, bacterial spot, bacterial leaf blight, chlorosis and water-soaked lesions in varied host plants including oriental melon, *X. campestris* pv. *vesicatoria* YK93-4 (*Xcv* YK93-4) and *X.* sp. SK12 (*Xsp* SK12) were selected as the test organisms for the evaluation of in-vivo antibacterial activity of the essential oil of *C. operculatus* buds using the method as described previously [16].

The oriental melon plants (*Cucumis melo* L. var. *makuwa*) possessing an average of 7–10 leaves were divided to six groups. Each group consisted of three plants (24 leaves). Two control groups were treated and untreated with vehicle solution only (0.5% DMSO and 0.1% Tween 80 in distilled water) without pathogen. The other four groups were treated with different concentrations of the essential oil of *C. operculatus* buds (0, 500, 1,000 and 2,000 μg/ml, respectively) in vehicle solution and bacterial spore suspension. The bacterial spore suspension prepared in LB medium (around 10^{8} CFU/ml) of each bacterial pathogen was sprayed onto each pot at the same time.

Development of disease lesions were scored on the 12th day after pathogen inoculation. The disease lesion areas and the whole leaf area were measured in square centimeters using a vernier caliper. All tests were conducted in three replicates, and scoring was done based on the average area of diseased lesion/the area of whole leaves (L/W). A disease scale of 0–9 was used, with 0 indicating no infection of leaves; 1, $L/W < 2$; 3, $L/W = 2.5$; 5, $L/W = 5$; 7, $L/W = 7.5$ and 9 indicating complete death of leaves. A disease index (DI) was calculated using formula: DI = 100 × sum of individual scores/total leaves observed × maximum score.

The extent of disease reduction attributed to each treatment was calculated using the following formula: disease suppression efficiency = $[(DI_{control} - DI_{treatment})/DI_{control}] \times 100\%$.

Statistical Analysis

The data obtained for antibacterial activity of essential oil and various extracts were statistically analyzed and mean values were calculated. A Student's *t* test was computed for the statistical significance of the results at $p < 0.05$.

Results

In-Vitro Antibacterial Activity

As shown in Table 1, the essential oil obtained from the buds of *C. operculatus* revealed potential antibacterial effects at the concentrations utilized against plant pathogenic bacteria of *Xanthomonas* spp. such as *Xanthomonas campestris* pv. *campestris* KC94-17 (*Xcc* KC94-17), *X. campestris* pv. *vesicatoria* YK93-4 (*Xcv* YK93-4), *X. oryzae* pv. *oryzae* KX019 (*Xoo* KX019) and *X.* sp. SK12 (*Xsp* SK12). The diameter of the inhibition zones of the essential oil against the tested strains of *Xanthomonas* spp. were in the range of 13–23 mm. *Xsp* SK12 and *Xoo* KX019 were found most susceptible pathogenic bacteria to the essential oil of *C. operculatus* buds. On the other hand, hexane, chloroform, ethyl acetate and methanol extracts of *C. operculatus* buds also exhibited potent antibacterial effects against the tested strains of *Xanthomonas* spp. as a diameter of zones of inhibition, ranging from 12–22, 7–16 to 11–16, 10–13 mm, respectively (Table 1). The hexane extracted material displayed a remarkable antibacterial effect as a diameter of zones of inhibition against the tested plant pathogenic bacteria of *Xanthomonas* spp. relative to the other extracts. On the other hand, standard ampicillin showed lower antibacterial effect as compared to the oil with diameter of zones of inhibition found in the range of 10 to 13 mm. In some cases, the activity of ampicillin was also comparable with organic extracts, especially when compared with the hexane extract.

Minimum Inhibitory and Bactericidal Concentrations

As shown in Table 2, the minimum concentrations of essential oil were found more susceptible to the tested plant pathogenic bacteria of *Xanthomonas* spp. as compared to the organic extracts. The essential oil displayed remarkable antibacterial activity against all the tested strains of *Xanthomonas* spp. such as *Xcc* KC94-17, *Xcv* YK93-4-, *Xoo* KX019 and *Xsp* SK12 with MIC and MBC values of 31.25–125 and 62.5–250 μg/ml, respectively. The oil had a detrimental effect on *Xcc* KC94-17 and *Xoo* KX019 with MIC value of 31.25 μg/ml for each bacterial strain. Also, the organic extracts (hexane, chloroform, ethyl acetate and methanol) of *C. operculatus* buds exhibited strong

Table 1 Antibacterial activity of essential oil and various organic extracts of *Cleistocalyx operculatus* buds against plant pathogenic bacteria of *Xanthomonas* spp.

Bacterial pathogen	Essential oil[a]	Organic extracts[b]				Standard[c]
		Hexane	Chloroform	Ethyl acetate	Methanol	
Xanthomonas campestris pv. *campestris* KC94-17 (*Xcc* KC94-17)	13.0 ± 0.0*	21.0 ± 0.0*	10.0 ± 0.0*	15.0 ± 0.0*	12.0 ± 0.0	12.0 ± 0.0
Xanthomonas campestris pv. *vesicatoria* YK93-4 (*Xcv* YK93-4)	13.0 ± 0.0*	22.0 ± 0.0*	16.0 ± 0.5*	16.0 ± 0.0*	13.0 ± 0.0*	10.0 ± 0.0
Xanthomonas oryzae pv. *oryzae* KX019 (*Xoo* KX019)	23.0 ± 0.0*	12.0 ± 0.0*	8.0 ± 0.0*	11.0 ± 0.0*	10.0 ± 0.0*	13.0 ± 0.0
Xanthomonas sp. SK12 (*Xsp* SK12)	15.0 ± 0.0*	12.0 ± 0.0	7. 0 ± 0.0*	14.0 ± 0.0*	13.0 ± 0.0*	12.0 ± 0.0

Data are expressed as the diameter of inhibition zones (mm); Values are given as an average of triplicate experiments

* $P < 0.05$ significant

[a] Essential oil used at 1,000 μg/disc

[b] Organic extracts used at 1,500 μg/disc

[c] Standard: Ampicillin (20 μg/ml)

Table 2 Minimum inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC) of essential oil and various extracts of *Cleistocalyx operculatus* buds against the growth of plant pathogenic bacteria of *Xanthomonas* spp.

Bacterial pathogen	Essential oil		Organic extracts								Standard[a]
			Hexane		Chloroform		Ethyl acetate		Methanol		
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC
Xanthomonas campestris pv. *campestris* KC94-17 (*Xcc* KC94-17)	31.25	62.5	250	500	500	500	500	500	250	500	500
Xanthomonas campestris pv. *vesicatoria* YK93-4 (*Xcv* YK93-4)	62.5	62.5	250	250	500	1,000	250	500	250	500	500
Xanthomonas oryzae pv. *oryzae* KX019 (*Xoo* KX019)	31.25	62.5	125	250	500	1,000	500	500	250	500	250
Xanthomonas sp. SK12 *(Xsp* SK12)	125	250	500	500	500	1,000	500	500	500	1,000	250

[a] Standard: Ampicillin (all values are in μg/ml)

antibacterial activity against the employed bacterial strains of *Xanthomonas* spp. MIC and MBC values of the extracts were found in the range of 125–500 and 250–1,000 μg/ml, respectively. However, the hexane extract exhibited stronger antibacterial effect as compared to chloroform, ethyl acetate and methanol extracts (Table 2). On the other hand, the neat essential oil displayed better antibacterial effect against the tested bacterial pathogens as MIC values as compared to standard ampicillin (MIC: 250–500 μg/ml). However, in some cases, the extracts had a higher antibacterial effect as compared to the standard antibiotic which might be due to the presence of highly bioactive compounds extracted with different solvents.

Cell Viability Counts

In the above-presented results, the neat essential oil and hexane extract of *C. operculatus* buds exhibited remarkable antibacterial activity against the tested bacteria of *Xanthomonas* spp. To visualize the antibacterial effect of the essential oil and hexane extract against the tested bacteria of *Xanthomonas* spp., a cell viability assay was performed. The effect on the cell viabilities demonstrated that exposure to essential oil at concentration of 1,000 μg/ml had a strong antibacterial effect inhibiting the cell viable counts of the tested bacteria of *Xanthomonas* spp. The exposure time of essential oil for complete inhibition of cell viabilities of the tested bacteria was found between 60 and 120 min (Fig. 1a).

On the other hand, among the tested plant pathogenic bacteria of *Xanthomonas* spp., *Xoo* KX019 seemed to be most sensitive bacterial pathogen to hexane extract at 250 μg/ml as compared to other tested bacteria in cell viability assay. The exposure time of the hexane extract for complete inhibition of cell viability of *Xoo* KX019 was found to be 60 min (Fig. 1b). However, the hexane extract has a lesser antibacterial properties on the inhibition of cell

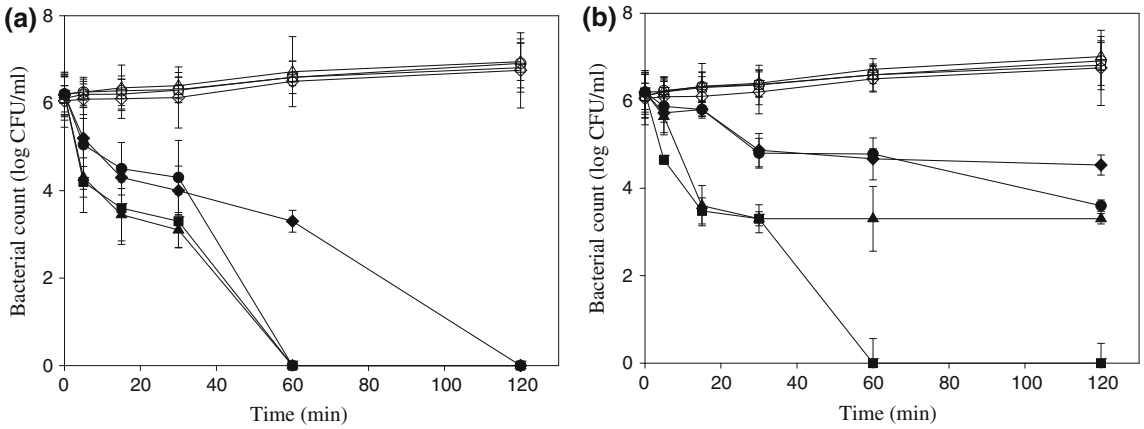

Fig. 1 Effect of essential oil (**a**) and hexane extract (**b**) of *Cleistocalyx operculatus* buds on the viability of *Xanthomonas campestris* pv. *campestris* KC94-17 (*Xcc* KC94-17, *closed circles*, *open circles*), *X. oryzae* pv. *oryzae* KX019 (*Xoo* KX019, *closed squares*, *open squares*), *X. campestris* pv. *vesicatoria* YK93-4 (*Xcv* YK93-4, *closed triangles*, *open triangles*) and *X. sp.* SK12 (*Xsp* SK12, *closed rhombus*, *open rhombus*). *Closed symbols* (*closed circles*, *closed squares*, *closed triangles* and *closed rhombus*) represent bacterial pathogens treated with essential oil (1,000 μg/ml) and hexane extract (250 μg/ml), whereas *open symbols* (*open circles*, *open squares*, *open triangles* and *open rhombus*) represent bacterial pathogens without treatment

viabilities of *Xcc* KC94-17, *Xcv* YK93-4 and *Xsp* SK12 bacterial pathogens.

In-Vivo Antibacterial Activity of the Essential Oil

For the in-vivo assay conducted on greenhouse grown oriental melon plants, two plant pathogenic bacteria *Xanthomonas* spp. *Xcv* YK93-4 and *Xsp* SK12, based on their low susceptibility to the essential oil of *C. operculatus* buds in vitro, were selected for the in vivo antibacterial study to further enrich the outcomes of in vitro study. As shown in Table 3, the essential oil of *C. operculatus* buds at the concentration of 2,000 and 1,000 μg/ml revealed potential antibacterial effect as 100% disease suppression efficiency against both the tested strains of *Xcv* YK93-4, the causal agent of bacterial spot and *Xsp* SK12 causing water-soaked lesions in oriental melon plants (Fig. 2). However, 500 μg/ml concentration of the essential oil of *C. operculatus* buds exhibited moderate activity of antibacterial efficacy against *Xcv* YK93-4 and *Xsp* SK12 with their respective disease suppression efficiency of 72.8 ± 2.9 and 83.63 ± 5.7%. As a control, vehicle solution (0.5% DMSO + 0.1% Tween 80 in distilled water) had no pathogenicity.

After 12 days, it was observed that higher essential oil concentrations (1,000 and 2,000 μg/ml) had strong antibacterial effects against the bacterial pathogenicity of *Xcv* YK93-4 and *Xsp* SK12 and no bacterial disease symptoms (bacterial blight, bacterial spot, water-soaked lesions and twig dieback) on the leaves of tested oriental melon plants caused by plant pathogenic bacteria of *Xanthomonas* spp. were observed. However, at 500 μg/ml, six leaves treated with pathogen groups were found infected by *Xcv* YK93-4 and *Xsp* SK12 (Fig. 2; Table 3).

Discussion

The increasing social and economic implications caused by plant pathogenic bacteria means there is constant striving to produce safer foods (crops, vegetables and fruits) and to develop new antibacterial agents. In general, plant-derived essential oils are considered to be non-phytotoxic compounds and potentially effective against plant pathogenic bacteria [17]. In recent years, the use of essential oil antimicrobial agents and their extracts is one of the first choices after plant bacterial disease outbreaks. Besides, interest has been generated in the development of safer antibacterial agents to control plant pathogenic bacteria in agriculture which also include essential oils and extracts.

In the present study, the in vitro antibacterial activities of essential oil and the various organic extracts of hexane, chloroform, ethyl acetate and methanol derived from *C. operculatus* buds were quantitatively assessed by the presence or absence of inhibition zones. Essential oils, which are odorous and contain volatile products of plant secondary metabolism, have wide applications to control pathogenic bacteria. In addition to this, *C. operculatus* buds mediated oil contained γ-terpinene (5.76%), *cis*-linalool oxide (5.21%), camphene (4.12%), *trans*-carveol (3.91%), α-pinene (3.45%), β-pinene (3.07%), terpinen-4-ol (2.58%), myrcene (2.40%), globulol (5.61%), acorenol (5.12%), β-himachalol (3.84%), cyclobazzanene (3.12%), 2,3-dehydro-1,4-cineol (3.01%), *trans*-dihydrocarvone (1.58%), presilphiperfol-1-ene (2.48%) and γ-amorphene (2.12%), as earlier reported the major or minor components of the various essential oils, which have enormous potential to inhibit pathogenic bacteria [17, 18]. The results of this study, obtained in vitro, showed that essential oil and

Table 3 In-vivo antibacterial activity of the essential oil of *Cleistocalyx operculatus* buds against the bacterial pathogens of *Xanthomonas* spp. conducted on greenhouse grown oriental melon plants (*Cucumis melo* L. var. *makuwa*)

Group of experiment	Treatment	Essential oil concentration (μg/ml)	*Xanthomonas campestris* pv. *vesicatoria* YK93-4		*Xanthomonas* sp. SK12	
			Number of the infected leaves/ Total leaves	Disease suppression efficiency (%)	Number of the infected leaves/ Total leaves	Disease suppression efficiency (%)
Normal control groups treated without pathogen	–	0	0/24	100.0 ± 0.0	0/24	100.0 ± 0.0
	VH[a]	0	0/24	100.0 ± 0.0	0/24	100.0 ± 0.0
Treated groups with pathogen	VH	0	24/24	0.0 ± 0.0	24/24	0.0 ± 0.0
	VH + EO[b]	500	6/24	72.8 ± 2.9	6/24	83.6 ± 5.7
	VH + EO	1,000	0/24	100.0 ± 0.0	0/24	100.0 ± 0.0
	VH + EO	2,000	0/24	100.0 ± 0.0	0/24	100.0 ± 0.0

– Normal control without treatment

[a] Vehicle solution of 0.5% DMSO + 0.1% Tween 80 in distilled water

[b] Essential oil in vehicle solution

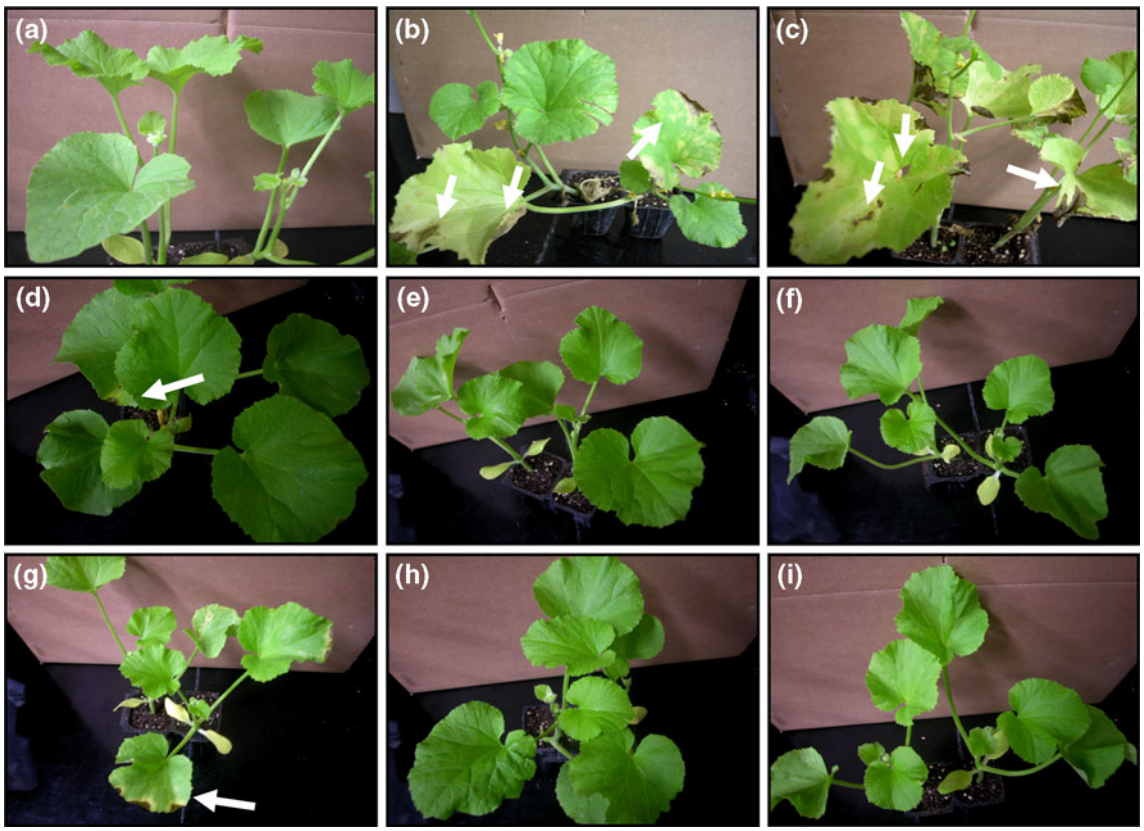

Fig. 2 In-vivo antibacterial effect of the essential oil of *Cleistocalyx operculatus* buds against plant pathogenic bacterial strains of *Xanthomonas* sp. SK12 (*Xsp* SK12) and *Xanthomonas campestris* pv. *vesicatoria* YK93-4 (*Xcv* YK93-4) on greenhouse-grown oriental melon plants. **a** No treatment; **b**, **c** treated with pathogen strains *Xsp* SK12 and *Xcv* YK93-4 in vehicle (0.5% DMSO + 0.1% Tween 80 in water); **d–f** treated with pathogen (*Xsp* SK12) and different concentrations of the essential oil (500, 1,000 and 2,000 μg/ml, respectively) in the control; **g–i** treated with pathogen (*Xcv* YK93-4) and different concentrations of the essential oil (500, 1,000 and 2,000 μg/ml, respectively) in the control. *Arrows* indicate the disease symptoms

various organic extracts of *C. operculatus* buds exerted potential antibacterial effect against *Xcc* KC94-17, *Xcv* YK93-4, *Xoo* KX019 and *Xsp* SK12, and these findings are strongly supported by our work [17].

Various publications have documented the antimicrobial activity of essential oils and plant extracts [17, 18]. In recent years, several researchers reported mono- and sesquiterpenoids as the essential components of the plant-based essential oils, which were also characterized in terms of high content in the oil of *C. operculatus* buds [11]. Mono- and sesquiterpenes are the major components of the essential oils. It seems reasonable to assume that the antimicrobial mode of the action might be related to the terpenoid components present. Terpenoids not only attack cell walls and cell membranes, thereby affecting their permeability and release of intracellular constituents (e.g. ribose, Na glutamate) but also interfere with membrane functions. Essential oils can coagulate the cytoplasm and damage lipids and proteins, leading to the leakage of macromolecules and to lysis [19].

Essential oils at the concentrations of 2,000 and 1,000 μg/ml exhibited 100% in vivo inhibitory effect against *Xcv* YK93-4 and *Xsp* SK12. Earlier in-vivo studies on the analysis of antibacterial effect of various essential oils showed that they had varying degrees of antibacterial effect against different pathogenic bacteria [20]. Our study revealed similar results of the antibacterial effect of *C. operculatus* bud oil in vivo against the tested plant pathogenic bacteria of *Xanthomonas* spp. under greenhouse conditions.

Certain essential oils and extracts act in many ways on various types of disease complex, and may be applied to the crops in the same way as other agricultural chemicals. *C. operculatus* buds mediated oil and extracts can also be used as a leading factor in a wide range of activities against many plant pathogenic bacteria, where these pathogens have developed resistance against the specific bactericides [21]. Among these pathogens, bacterial leaf blight caused by *X. oryzae* pv. *oryzae* has been reported as beeing a most serious disease of rice in South East Asia, particularly since the widespread cultivation of dwarf high-yielding cultivars [22]. In our study, it has become clear that both essential oil and extracts possessed the ability to strongly inhibit the

growth of *Xoo* KX019 along with other plant pathogenic bacteria tested.

Moreover, information on the antibacterial effects of *C. operculatus* against plant pathogenic bacteria is scant, and these results show that the oil and various extracts of *C. operculatus* buds had a substantial antibacterial effect against *Xcc* KC94-17 (black rot in radish), *Xcv* YK93-4 (bacterial spot in tomato and pepper), *Xoo* KX019 (bacterial leaf blight in rice) and *Xsp* SK12 (lesions and twig dieback in varied hosts). These pathogens are responsible for serious economic losses in various parts of the world and, although control measures are available, they are of limited effectiveness [23]. As a result, work on alternative approaches to control such pathogens is important. Thus, this research would be worthy as an important biocontrol approach to inhibit plant pathogenic bacteria in vitro and in vivo.

The results of this study provide sufficient evidences that the essential oil and various organic extracts (in *n*-hexane, chloroform, ethyl acetate and methanol) of *C. operculatus* buds represent the potential sources for their applications in the agro-industry to control plant pathogenic bacteria causing severe bacterial diseases in important agricultural plants as also shown to be evident by our previous work [17]. The use of essential oils in consumer goods is expected to increase in the future due to the rise in "green consumerism", which stimulates the use and development of products derived from plants, as both consumers and regulatory agencies are more comfortable with the use of natural antimicrobials [24]. Thus, from the above results it can be concluded that essential oil and various organic extracts derived from the buds of *C. operculatus* could be used as promising and naturally occurring bactericides to control plant bacterial diseases caused by *Xanthomonas* spp. and also to screen and develop novel types of selective and natural bactericides in the management of bacterial pathogens causing drastic losses to crops and vegetables in agriculture industry. Further study is warranted on the isolation, purification and characterization of the bioactive compounds present in various extracts of *C. operculatus* to evaluate its biological potential against plant pathogenic bacteria.

Acknowledgments This work was carried out with support of the "Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ007512)" Rural Development Administration, Republic of Korea.

References

1. Vyvyan JR (2002) Allelochemicals as leads for new herbicides and agrochemicals. Tetrahedron 58:1631–1636
2. Savary S, Teng PS, Willocquet L, Nutter FW (2006) Quantification and modeling of crop losses: a review of purposes. Annu Rev Phytopathol 44:89–112
3. Burhan M, Talib S, Ishfaq M, Ahmad S (2009) Effect of various chemicals on citrus canker (*Xanthomonas campestris* pv. *citri*) in nursery plants. J Agric Res 47:465–468
4. Cavalcanti FR, Resende MLV, Carvalho CPS, Silveira JAG, Oliveira JTA (2006) Induced defence responses and protective effects on tomato against *Xanthomonas vesicatoria* by an aqueous extract from *Solanum lycocarpum* infected with *Crinipellis perniciosa*. Biol Control 39:408–417
5. Rodríguez H, Aguilar L, LaO M (1997) Variations in xanthan production by antibiotic-resistant mutants of *Xanthomonas campestris*. Appl Microbiol Biotechnol 48(5):626–629
6. Costa TR, Fernandes OFL, Santos SC, Oliveira CMA, Lião LM, Ferri PH, Paula JR, Ferreira HD, Sales BHN, Silva MRR (2000) Antifungal activity of volatile constituents of *Eugenia dysenterica* leaf oil. J Ethnopharmacol 72:111–117
7. Loi DT (1986) Vietnamese medicinal plants and herbs. Hanoi Publication House Technology, Vietnam, pp 437–438
8. Ye CL, Liu JW, Wei DZ, Lu YH, Qian F (2005) In vivo antitumor activity by 2′, 4′- dihydroxy-6′-methoxy-3′, 5′-dimethylchalcone in a solid human carcinoma xeno-graft model. Cancer Chemother Pharmacol 56:70–74
9. Mai TT, Chuyen NV (2007) Anti-hyperglycemic activity of an aqueous extract from flower buds of *Cleistocalyx operculatus* (Roxb.) Merr. and Perry. Biosci Biotechnol Biochem 71:69–76
10. Woo AYH, Waye MMY, Kwan HS, Chan MCY, Chau CF, Cheng CHK (2002) Inhibition of ATPases by *Cleistocalyx operculatus*. A possible mechanism for the cardiotonic actions of the herb. Vasc Pharmacol 38:163–168
11. Dung NT, Kim JM, Kang SC (2008) Chemical composition, antimicrobial and antioxidant activities of the essential oil and the ethanol extract of *Cleistocalyx operculatus* (Roxb.) Merr and Perry buds. Food Chem Toxicol 46:3632–3639
12. Dung NT, Bajpai VK, Yoon JI, Kang SC (2009) Anti-inflammatory effects of essential oil isolated from the buds of *Cleistocalyx operculatus* (Roxb.) Merr and Perry. Food Chem Toxicol 47:449–453
13. Dung NX, Luu HV, Khoi TT, Leclerq PA (1994) GC and GC/MS analysis of the leaf oil of *Cleistocalyx operculatus* Roxb. Merr. et Perry (Syn. *Eugenia operculata* Roxb.; *Syzygicum nervosum* DC). J Essent Oil Res 6:661–662
14. Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolke RH (1995) ASM: manual of clinical microbiology 6th edn. Washington
15. NCCLS (1993) National Committee for Clinical Laboratory Standards (NCCLS), 3rd edn. Villanova, M7-A3
16. Yoo JK, Ryu KH, Kwon JH, Ahn YJ (1998) Antifungal activities of oriental medicinal plant extracts against phytopathogenic fungi. Korean J Agric Chem Biotechnol 41:600–604
17. Bajpai VK, Cho MJ, Kang SC (2009) Control of plant pathogenic bacteria of *Xanthomonas* spp. by the essential oil and extracts of *Metasequoia glyptostroboides* Miki ex Hu in vitro and in vivo. J Phytopathol. doi:10.1111/j.1439-0434.2009.01646.x
18. Bajpai VK, Kang SC (2010) Antifungal activity of leaf essential oil and extracts of *Metasequoia glyptostroboides* Miki ex Hu. J Am Oil Chem Soc 87:327–336
19. Oussalah M, Caillet S, Lacroix M (2006) Mechanism of action of Spanish oregano, Chinese cinnamon, and savory essential oils against cell membranes and walls of *Escherichia coli* O157:H7 and *Listeria monocytogenes*. J Food Prot 69:1046–1055
20. Hevesi M, Al-Arabi K, Göndör M, Papp J, Honty K, Kasa K, Toth M (2006) Development of eco-friendly strategies for the control of fire blight in Hungry. International Society for Horticultural Science, ISHS Acta Horticulturae 704: X International Workshop on Fireblight, Bologna, Italy

21. Cooksey DA (1990) Genetics of bactericide resistance in plant pathogenic bacteria. Annu Rev Phytopathol 28:201–219
22. Ray PR, Sengupta TK (1970) A study on the extent of loss in yield in rice due to bacterial blight. Indian Phytopathol 23: 713–714
23. Dougherty DE, Powell CC, Larsen PO (1974) Epidemiology and control of bacterial leaf spot and stem rot of *Pelargonium hortorum*. Phytopathology 64:1081–1083
24. Tuley de Silva K (1996) A manual on the essential oil industry. United Nations Industrial Development Organization, Vienna