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IN VITRO CONTROL OF PLANT PATHOGENIC *XANTHOMONAS* SPP. USING *PONCIRUS TRIFOLIATA* RAFIN

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ABSTRACT

The secondary metabolites such as essential oil and pure compounds (limonin and imperatorin) from *Poncirus trifoliata* Rafin were tested for *in vitro* control of phytopathogenic bacteria of *Xanthomonas* spp. *In vitro* studies showed that the oil had inhibitory effect on *Xanthomonas campestris* pv. *compestris* KC94-17-XCC, *Xanthomonas campestris* pv. *vesicatoria* YK93-4-XCV, *Xanthomonas oryzae* pv. *oryzae* KX019-XCO and *Xanthomonas* sp. SK12 with their inhibition zones and minimum inhibitory concentration (MIC) values ranging from 13.1~22.1 mm and 62.5~125 µg/ml, respectively. Limonin and imperatorin also had *in vitro* antibacterial potential (MIC: 15.62~62.5 µg/ml) against all the tested *Xanthomonas* spp. Furthermore, the SEM studies demonstrated that limonin and imperatorin caused morphological changes of *Xanthomonas* sp. SK12 at the minimum inhibitory concentration (15.62 µg/ml). These results of this study support the possible use of essential oil and natural compounds from *P. Trifoliata* in agriculture and agro-industries to control plant pathogenic microorganisms.

Keywords: *Xanthomonas* spp., *Poncirus trifoliata* Rafin, oriental tomato plants, antibacterial efficacy, secondary metabolites, essential oil

INTRODUCTION

Bacterial diseases represent some recalcitrance and difficulties to manage the pest problems affecting commercial vegetable production. Spraying with antibiotics and pesticides, usually suggested controlling bacterial diseases, have never been satisfactory. Antibiotics and synthetic pesticides are forbidden in many countries because of their exerting a negative impact such as high and acute toxicity, long degradation periods and accumulation in the food chain. Therefore, losses from bacterial diseases can be substantial all over the world. Plant diseases caused by bacteria are factors in several plant family members (Aktar et al., 2009). In addition; bacterial diseases caused by pathovars of *Xanthomonas* also result in a decreased yield of harvest. Pathovars of *Xanthomonas* are reported to have developed resistance to several antibiotics such as kanamycin, ampicillin, penicillin, and streptomycin (Rodriguez et al., 1997). This seriously hinders the management of diseases of crops and agricultural products (Satish et al., 1999). Besides, control of the disease is difficult, often requiring expensive and complex integrated pest management (IPM), including the use of contamination-free seeds, sanitization practices, and the use of chemicals (Araujo et al., 2003). Naturally occurring biologically active plant products such as plant-based essential oils and organic extracts, pure compounds could be a source of new eco-friendly pesticides to serve as templates for new and more effective compounds in controlling plant pathogenic microorganisms. Furthermore, bio-pesticides have been suggested as effective substitutes for chemical pesticides (Gan-Mor and Matthews, 2003).

Poncirus trifoliata Rafin (Rutaceae), also known as trifoliate orange, is a close relative to the *Citrus* trees. It is a decidious or semi-decidious shrub, a native of China and Korea, and is also known as the Korean bitter orange. Traditionally, trifoliata oranges (*P. trifoliata*) have been widely used in folk medicine as a remedy for gastritis, dysentery, inflammation, digestive ulcers, etc. A scientific investigation into the health-maintaining properties of trifoliate orange fruit has revealed its anti-inflammatory, antibacterial and anti-anaphylactic activities (Kim et al., 1999). In Korea, fruit extracts of *P. trifoliate* are used in some over-the-counter drugs for the treatment of a variety of gastrointestinal (GI) disorders (Lee et al., 2005). Another researcher reported that poncirus fruit is a potent antileukaemic agent by promoting apoptosis of cancer cells (Yi et al., 2005). Several compounds such as poncirin, coumarins, auraptine, hesperidin and naringin have been identified from poncirus fruits (Avula et al., 2005). Previously, we reported the chemical compositions of seed essential oil of *Poncirus trifoliata* Rafin by GC-MS and evaluated antibacterial potential of the oil and organic seed extracts of *P. trifoliata* Rafin against foodborne pathogens (Rahman et al., 2009). Further, we have previously reported on the isolation, characterization and anti-listerial potential of naturally occurring limonin and imperatorin from *P. trifoliata* seed (Rahman et al., 2012). However, there is no report available in the literature on antibacterial properties especially on *Xanthomonas* spp. including morphological changes using volatile essential oil, isolated natural compounds of *P. trifoliata* seeds.

Considering the deleterious effects of synthetic antibiotics and pesticides on life supporting systems, there is an urgent need to search for alternative approaches for the management of plant pathogenic microorganisms. Therefore, to find out the environmentally friendly alternatives to chemical pesticides we investigated the role of secondary metabolites (essential oil and compounds limonin and imperatorin) of *P. trifoliata* as an antibacterial potential on *Xanthomonas* spp. *in vitro.*

MATERIALS AND METHODS

Micro-organisms

In this study, the used organisms were *Xanthomonas campestris* pv. *compestris* KC94-17–XCC, *Xanthomonas campestris* pv. *vesicatoria* YK93-4–XCV, *Xanthomonas oryzae* pv. *oryzae* KX019–XCO and *Xanthomonas* sp. SK12. These organisms were collected from 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 inoculated in Luria-Bertani (LB) broth medium at 28 °C for 24 h. Cultures of each bacterial strain were maintained on LB agar medium at 4 °C.

In vitro antibacterial activity assay

The antibacterial test was carried out by agar disc diffusion method using 100 µl of standardized inoculum suspension containing 10^7 CFU/ml of bacteria (Murray et al., 1995). The seed essential oil was diluted 1:5

(v/v) with methanol and appropriate amounts (5, 10 or 15 µl) were spotted onto the filter paper discs (6 mm diameter) and placed on the inoculated agar. Negative controls were prepared using the same solvent (MeOH) employed to dissolve the oil. Standard reference antibiotic, streptomycin (20 µg/disc, from Sigma-Aldrich Co., USA), was used as positive control for the tested bacteria. The plates were then sealed with parafilm and incubated at 28 °C for 24 h. Antibacterial activity was evaluated by measuring the diameter of the zones of inhibition against the tested bacteria. Each assay in this experiment was replicated three times.

Determination of minimum inhibitory concentration (MIC)

Minimum inhibitory concentrations (MICs) of seed essential oil and compounds limonin and imperatorin were tested by a two-fold serial dilution method (Chandrasekaran and Venkatesalu, 2004). The test samples of oil and pue compounds (limonin and imperatorin) were dissolved in methanol and dimethyl sulfoxide (DMSO), respectively and incorporated into LB broth medium to obtain a concentration of 1,000 µg/ml and serially diluted to achieve 500, 250, 125, 62.5, 31.25, 15.62, 7.81 and 3.9 µg/ml, respectively. The final concentration of solvent in the culture medium was maintained at 0.5 % (v/v). A 10 µl standardized suspension of each tested organism (10^7 CFU/ml approximately) was transferred to each tube. The control tubes contained only bacterial suspension, were incubated at 28 °C for 24 h. The lowest concentration of the test samples, which did not show any growth of tested organism after macroscopic evaluation, was determined as MICs, which were expressed in µg/ml.

Scanning electron microscopic (SEM) analysis on Xanthomonas spp.

To determine the antibacterial efficacy of the compounds limonin and imperatorin on the morphological changes, SEM studies were performed on *Xanthomonas* sp. SK12 treated with MIC concentrations of these compounds. Controls were prepared without samples. Further, to observe the morphological changes, the method of SEM was modified from Kockro method (Kockro et al., 2000). The bacterial samples were washed gently with 50 mM phosphate buffer solution (pH 7.2), fixed with 2.5 g/100 ml glutaraldehyde and 1 g/100 ml osmic acid solution. The specimen was dehydrated using sequential exposure per ethanol concentrations ranging from 30-100 %. The ethanol was replaced by tertiary butyl alcohol. After dehydration, the specimen was dried with CO_2. Finally, the specimen was sputter-coated with gold in an ion coater for 2 min, followed by microscopic examinations (S-4300; Hitachi).

Statistical analysis

The data obtained for antibacterial activity of seed essential oil and isolated compounds 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 effect of seed essential oil of P. trifoliata against phytopathogenic Xanthomonas spp.

Seed essential oil also showed potent *in vitro* inhibitory effect against plant pathogenic bacteria of *Xanthomonas* spp. At the concentrations of 5, 10 and 15 µl/disc of 1:5 (v/v) dilution of seed essential oil with methanol, the oil exhibited a potent inhibitory effect against all four strains of *Xanthomonas* spp. such as *X. campestris* pv. *compestris* KC94-17-XCC, *X. campestris* pv. *vesicatoria* YK93-4-XCV, *X. oryzae* pv. *oryzae* KX019-XCO and *Xanthomonas* sp. SK12 as a diameter of inhibition zones of 13.1~20.0, 14.0~22.1, 16.2~21.0 and 15.2~21.2 mm, respectively along with their MIC values ranging from 62.5 to 125 µg/ml (Table 1).

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Table 1: *In vitro* antibacterial effect of seed essential oil of *Poncirus trifoliata* Rafin against phytopathogenic *Xanthomonas* spp.

Bacteria	Seed essential oil				Streptomycin	
	DD [a]	DD [b]	DD [c]	MIC	DD [d]	MIC
XCC	13.1 ± 1.1	17.3 ± 0.5	20.0 ± 0.4	125	17.1 ± 0.7	7.81
XCV	14.0 ± 0.7	18.2 ± 0.5	22.1 ± 1.1	62.5	18.0 ± 1.1	3.91
XCO	16.2 ± 1.2	19.0 ± 0.7	21.0 ± 0.5	125	18.0 ± 1.5	7.81
X. sp.	15.2 ± 0.5	17.0 ± 0.5	21.2 ± 0.6	62.5	18.2 ± 1.2	7.81

DD: Diameter of inhibition zone (mm) around the discs (6 mm). [a]Seed essential oil 5 µl/disc; [b]10 µl/disc; [c]15 µl/disc of 1:5 (v/v) dilution of oil with MeOH; [d]Streptomycin (20 µg/disc); MIC: Minimum inhibitory concentration (µg/ml); XCC: *Xanthomonas campestris* pv. *compestris* KC94-17-XCC; XCV: *Xanthomonas campestris* pv. *vesicatoria* YK93-4 - XCV; XCO: *Xanthomonas oryzae* pv. *oryzae* KX019 - XCO; X.sp.: *Xanthomonas* sp. SK12.

In vitro antibacterial activities of limonin and imperatorin against Xanthomonas spp.

Limonin and imperatorin exhibited potent antibacterial activity against phytopathogenic bacteria of *Xanthomonas* spp. (MIC value: 15.62~62.5 µg/ml) as shown in Table 2. Bacterium *Xanthomonas* spp. SK12 was found to be the most sensitive organisms toward limonin and imperatorin (MIC: 15.62 µg/ml for each), while *X. campestris* pv. *vesicatoria* YK93-4-XCV was the most sensitive to imperatorin (MIC: 15.62 µg/ml) (Table 2).

Table 2: *In vitro* antibacterial effect of limonin and imperatorin on phytopathogenic *Xanthomonas* spp.

Xanthomonas spp.	MIC (µg/ml)	
	Limonin	Imperatorin
XCC	31.25	31.25
XCV	62.5	15.62
XCO	31.25	31.25
X. sp.	15.62	15.62

MIC: Minimum inhibitory concentration (µg/mL). XCC: *Xanthomonas campestris* pv. *compestris;* KC94-17 - XCC; XCV: *Xanthomonas campestris* pv. *vesicatoria* YK93-4 - XCV; XCO: *Xanthomonas oryzae* pv. *oryzae* KX019 - XCO; X.sp.: *Xanthomonas* sp. SK12.

Scanning electron microscopic (SEM) studies on Xanthomonas sp. SK12 using compounds limonin and imperatorin

On the basis of lower MIC values (15.62 µg/ml) of both limonin and imperatorin on *X.* spp. SK12, the effect of these compounds (MIC) on the morphology of this bacterium was observed by scanning electron microscopy (SEM) and the results showed the detrimental effect of these compounds on the cell morphology of *X.* sp. SK12 (Figure 1). Control cells in the absence of limonin and imperatorin showed a regular, smooth surface as shown in Figure 1 (A1-B1). In contrast, cells inoculated with limonin and imperatorin at MIC value (15.62 µg/ml) revealed severe detrimental effect on the morphology of cell membranes, showing disruption, pore formation and analysis of the membranes integrity, as shown in Figure 1 (A2-A3 & B2-B3).

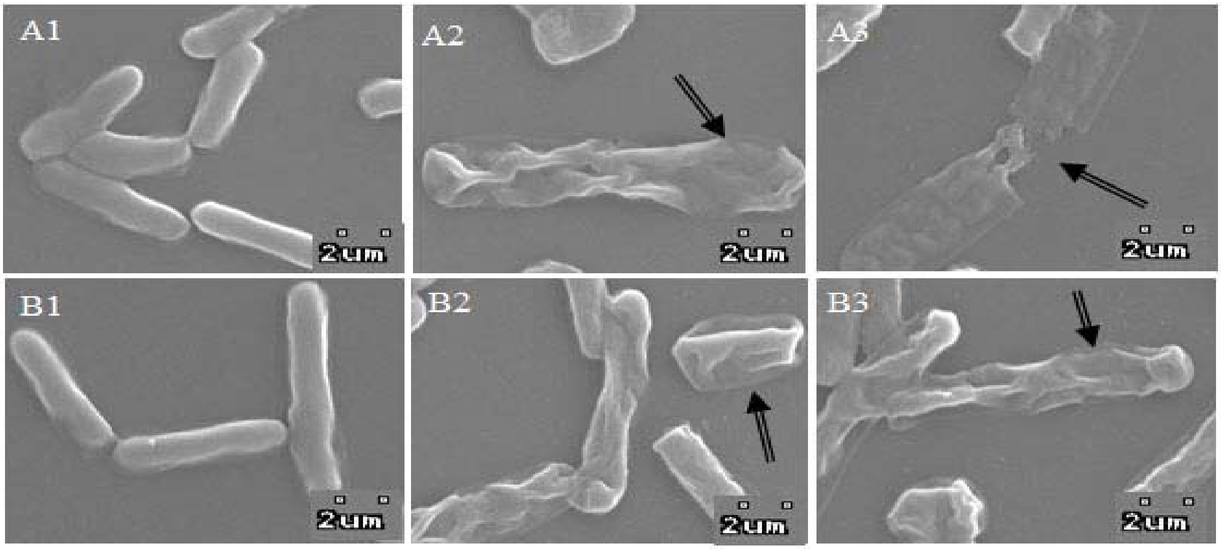

Figure 1: Effect of limonin and imperatorin on morphological changes on *Xanthomonas* sp. SK12. Morphological damages of *Xanthomonas* sp. SK12 treated with limonin and imperatorin (MIC 15.62 µg/ml) by scanning electron microscopy (SEM). A1: Control (limonin 0 µg/ml); A2 and A3: Treated with limonin (15.62 µg/ml) showing distorted cell, pore fomation and lysis of cell. B1: Control (imperatorin 0 µg/ml); B2 and B3: Treated with imperatorin (15.62 µg/ml) showing distorted cell, pore fomation and lysis of cell.

DISCUSSION

The increasing social and economic implications caused by plant pathogenic bacteria means there is a constant striving to produce safer foods (crops, vegetables and fruits) and to develop new antibacterial agents. In general, plant-derived essential oils are considered as non-phytotoxic compounds and potentially effective against pathogenic bacteria (Vasinauskiene et al., 2006; Basim and Basim, 2003). In recent years, the use of antimicrobial compounds such as essential oils extracts and natural compounds are one of the first choices after outbreaks of bacterial plant diseases. 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 (Deena and Thoppil, 2000).

Essential oils, which are odorous and volatile products of plant secondary metabolism, have wide applications to control pathogenic bacteria (Ozturk and Ercisli, 2007). Various publications have documented the antimicrobial activities of essential oils and plant extracts (Saxena et al., 2012; Shafia et al., 2002; Bougatsos et al., 2004; Jirovetz et al., 2006). Mono- and sesqueterpenes, which are phenolic in nature, have potential antimicrobial activities. It seems reasonable to assume that their antimicrobial mode of action might be related to the phenolic compounds present (Cakir et al., 2004). Most of the studies on the mechanism of phenolic compounds have focused on their effects on cellular membranes. Actually, phenolic compounds 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 (electron transport, nutrient uptake, protein, nucleic acid synthesis and enzyme activity). Thus, active phenolic compounds present in the oil of *P. trifoliata* might have several invasive targets which could lead to the inhibition of plant pathogenic bacteria.

In our study, it has become clear that seed essential oil strongly inhibited *in vitro* the growth of *Xanthomonas* spp. Bacterial leaf blight caused by *X. oryzae* pv. *oryzae* has been reported the most serious disease of rice in South East Asia, particularly since the widespread cultivation of dwarf high-yielding cultivars (Zhou et al., 2013).

Compounds limonin and imperatorin also showed a great potential of antibacterial activity against plant pathogenic bacteria of *Xanthomonas* spp. In this study, *Xanthomonas* sp. SK12 was found to be the most sensitive organism to both limonin and imperatorin. SEM study also revealed the detrimental effect of limonin and imperatorin on the cell morphology of the tested organism such as *Xanthomonas* sp. SK12. The results of this study would be worthy as an important bio-control approach to inhibit such severe plant pathogenic bacteria.

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