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Composition and Antimicrobial Activities of Secondary Metabolites in Tomato and Strawberry Plants

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ABSTRACT

This thesis is consisted of Part I and II in tomato and strawberry researches, respectively. Chapter I and III deal with the secondary metabolites profiling and Chapter II and IV deal with antimicrobial activity of extracts from various parts of tomato and strawberry plants, respectively.

In Chapter I, contents of carotenoids, phenolics, volatile organic compounds, and alkaloids in leaves, intermodal stems, fruits, and roots of 'Bacchus' tomato at different developmental stages were measured. Lycopene content in red fruits was 196.2 $\mu g \cdot g^{-1}$ FW. β-Carotene and lutein contents in the 24th leaves were 23.2 and 25.6 $\mu g \cdot g^{-1}$ FW, respectively, and were greater than those in the other parts. Content of chlorogenic acid in the 18th leaves was 40.1 $\mu g \cdot g^{-1}$ FW, while that in the other parts was lower than 31.0 $\mu g \cdot g^{-1}$ FW. Contents of caffeic and vanillic acids in the 24th leaves were 9.2 and 1.6 $\mu g \cdot g^{-1}$ FW, respectively, and were greater than those in the other parts. Moreover, younger leaves contained more diverse

volatile organic compounds including mono- and sesquiterpenes. Contents of dehydro- and α-tomatine were greatest in leaves, followed by internodal stems, roots, and fruits. Younger leaves and internodal stems contained more dehydro- and α-tomatine than older leaves and internodal stems. Contents of dehydro- and α-tomatine in the 24th leaves were 889.1 and 1,417.9 $\mu g \cdot g^{-1}$ FW, respectively, and were greatest among all parts tested. These results indicated that, except lycopene, tomato leaves contained greater secondary metabolites than red fruits.

In Chapter II, antimicrobial activity was confirmed in methanol, acetone, dichloromethane, and hexane extracts from various parts of 'Bacchus' tomato plants including non-edible parts. Minimum inhibitory concentration of actetonic extract from tomato leaves on *Fusarium oxyspourum* f. sp. *lycopersici, Colletotrichum coccodes*, *Phytophthora capsici*, *Rhizoctonia solani*, and *Glomerella cingulata* was lowest. The actetonic extracts also reduced the mycelial growth of *F. oxysporum* f. sp. *lycopersici* and *R. solani*. Mycelial growth of *R. solani*, especially, was significantly inhibited by the actetonic extracts. Bioautography on thin layer chromatography showed that the acetonic extract included two antimicrobial compounds against *R. solani*. The dominant antimicrobial compounds in the chromatogram were linolenic and caffeic acids. Linolenic acid had greater inhibitory effect on mycelial growth of *R. solani* than caffeic acid.

In Chapter III, a comparative chemical analysis was performed on the compounds found in roots, leaves, petioles, runners, and green and red fruits during vegetative propagation and reproductive growth of 'Seolhyang' strawberry. Contents of ellagic and gallic acids in leaves of runner plants during vegetative

propagation were 7.4 and 5.1 mg·g^{-1} FW, respectively, and were higher than those in the other parts. The main volatile organic compound was identified as 3-hexen-1-ol, and it was detected mostly in leaf parts. Content of ellagic acid in leaves during reproductive growth was 13.0 mg·g^{-1} FW, while that in the other parts was below 6 mg·g^{-1} FW. Content of gallic acid in green fruits was 2.8 mg·g^{-1} FW and was higher than that in the other parts. Red fruits contained the most diverse volatile organic compounds, including sesquiterpenes, among the tested plant parts but contained the lowest contents of ellagic and gallic acids.

In Chapter IV, antimicrobial activity was confirmed in methanol, acetone, dichloromethane, and hexane extracts from various parts of 'Seolhyang' strawberry plants including non-edible parts. Minimum inhibitory concentration value of methanolic extract from strawberry leaves was lowest on all tested microorganisms (*Fusarium oxyspourum* f. sp. *lycopersici, Colletotrichum coccodes*, *Phytophthora capsici*, *Rhizoctonia solani*, *Glomerella cingulate*, and *Phytophthora cactorum*). The methanolic extracts also inhibited the mycelial growth of *R. solani*, *G. cingulate*, and *C. coccodes*. Mycelial growth of *C. coccodes*, especially, was significantly inhibited by the methanolic extracts. Moreover, the methanolic extracts inhibited mycelial growth of the other *Colletotrichum* spp. such as *C. caudatum, C. higginsianum, C. liliacearum, C. lindemuthianum, C. musae, C. orbiculare,* and *C. truncatum*. Bioautography on thin layer chromatography showed that the methanolic extract included two antimicrobial compounds against *C. coccodes*. The dominant antimicrobial compounds in the chromatogram were tyrosol and β-sitosterol. Tyrosol showed greater inhibitory effect on mycelial growth of *C. coccodes* than β-sitosterol.

In conclusion, non-edible parts of tomato and strawberry, especially leaves, contained greater secondary metabolites contents than edible parts and contained antimicrobial compounds against phytopathogenic microorganisms. The results could be a useful database for utilizing of the non-edible parts which were dumped after last harvest and could provide information for developing natural antimicrobial agents against phytopathogens.

Keywords: alkaloids, antimicrobial compounds, carotenoids, *Fragaria* × *ananassa*, phenolics, *Solanum lycopersicum*, volatile organic compounds

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LIST OF ABBREVIATIONS

A	acetic acid
B	benzaldehyde
BA	benzoic acid
1BA	1,2-benzenedicarboxylic acid
Ba	butanal
2Ba	2-butenal
Ben	benzene
Bi	bicyclo[4.3.0]nona-3,7-diene
1Bo	1-butanol
2Bo	2-butanol
Bu	butanoic acid
Ca	caryophyllene
δCa	δ-carene
1Ch	1-chlorohepta-fluorobut-2-ene
Chd	1,4-cyclohexanedimethanol
Cy	cyclohexene
Cyo	cyclohexanol
D	decane
DA	n-decanoic acid
De	decanal
Do	2,6-dimethyl-1,3,5,7-octatetraene
Ea	ethyl acetate

EC	2-ethyl crotonaldehyde
Eo	*Z* and *E* epoxy-ocimene
Etl	ethanol
Etr	ether
Fa	*E*,*E*-α-farnesene
Fo	formic acid
Fu	furan
2Fu	2(5H)-furanone
Fua	2-furancarboxaldehyde
Hd	heptadecane
1He	1-hexene
2He	2-hexenal
3He	3-hexenal
Hep	heptane
Hex	hexane
2Ho	2-hexen-1-ol
3Ho	3-hexen-1-ol
Hpn	4-heptanone
Ht	*Z*-3-hexenyl tiglate
Hu	α-humulene
Hxa	hexanoic acid
Hxn	hexanal
L	di-limonene
MC	methylene chloride

Me	methane
Mho	6-methyl-5-hepten-2-one
Mi	4,7-methano-1H-inden-1-ol
My	6-methyl-5-hepten-2-one
Na	nonanal
Ne	nonane
Noa	nonanoic acid
O	octane
Oa	octanal
Oc	octadecane
Ot	1,3,6-octatriene
P	pentane
P3l	1-penten-3-ol
P3n	1-penten-3-one
Pd	pentadecane
Phe	phenol
Pn	4-pentenal
αPh	α-phellandrene
βPh	β-phellandrene
Pi	α-pinene
Pr	propanoic acid
S	sabinene
Sa	sulfurous acid.
T	α-terpinene

Td	tetradecane
U	undecanene.

GENERAL INTRODUCTION

Fungi represent an increasing problem both in crop production and human health as reduction factors of crop yield and quality, spoilage organisms in food products, in-house surface contaminants, and important lethal human pathogens (Brul and Klis, 1999). Occurred fungi in the field cause quality deterioration, economic loss, and lowing of labor efficiency. A number of prevention methods including application of artificial synthetic fungicides have been developed to solve these problems. The amount of the fungicides used has been increased for last few decades, because the fungicide application is an easy and fast prevention method. Since the mid-1990s, new artificial synthetic fungicides with good activity against phytopathogenic fungi have been commercialized (Cuppen et al., 2000; Staub et al., 1998).

However, strategies to control the fungi with synthetic fungicides may induce side effects, notable environmental contamination, and the development of multi-resistant fungal strains. For example, synthetic fungicides contaminate air, water, and soil environment, while fungicides suspended in the air as particles are moved by wind to other areas. The fungicides are one of the reasons for water pollution, and some fungicides are continuous pollutants and cause soil pollution. Some of these fungicides including procymidones and dicarboximides are persistent enough to be discovered after several weeks of application in vegetables (Aplada-Sarlis et al., 1994) and soil (Paris-Palacio et al., 1998).

In addition, use of synthetic fungicides reduces biodiversity, decreases nitrogen fixation (Schnelle and Hensley, 1990), causes pollinator decline (Brittain

et al., 2010), destroys animal's habitat (Fraser, 2012), and threatens endangered species (Lawler et al., 2002) because of their residual toxicity. Most of the fungicides are not biodegradable and thus possess the potential to disrupt the ecological balance. Development of the fungicide resistance in microorganisms is also a major problem in today's agriculture which subsequently induces to an application of higher dose of the fungicide to counteract the resistance (Matson et al., 1997), aggravating the air pollution problem. Because of growing concerns about environmental and health safety, the use of environmentally damaging, toxic, and carcinogenic fungicides is recently being discouraged. Therefore, alternative agents should be developed for controling phytopathogenic fungal diseases in plants.

Biological control could be considered as the alternative of synthetic fungicides. Hegazi and El-Kot (2010) reviewed that successful biological control of foliar diseases has been performed by numerous researchers under greenhouse and field trials using fungal and bacterial antagonists. Plant extracts have also been used to control many phytopathogens and diseases. Historically, diverse plant extracts have been examined to find out their antimicrobial properties. Earlier, many fungicides have been derived from plants and the plants have been applied widely as sources of natural fungicides. However, synthetic fungicides largely replaced plant derived natural fungicides as the key commercial fungicides. Recently, the development of safer antimicrobial agents than synthetic fungicides has been interested. Plant-based natural substances are generally considered as non-phytotoxic and potentially effective against microorganisms. Plant extracts containing natural substances have been reported to induce host resistance through

increased activity of a number of enzymes including peroxidase and polyphenol oxidase which have a defense role against invading phytopathogens (Caruso et al., 2001; Nawar and Kuti, 2003). More infromation on plant extracts for antimicrobial effect and environmental safety is required to meet the demand of consumers.

Higher plants produce diverse compounds to defend themselves against phytopathogenic fungi, bacteria, and viruses. Antimicrobial substances such as phenolics, terpenoids, and alkaloids are secondary metabolites. Extracts of many higher plants have been tested for their antibacterial, antifungal, and insecticidal activities under laboratory trials (Beckman, 2000; Bisogno et al., 2007; Kim et al., 2003).

Phenolics are synthesized by plants in response to abiotic and biotic stresses. The compounds are present in different quantities in all plant parts, depending on the environment influence and plant developmental stage (Milenković-Andjelković et al., 2015). Phenolics are mainly represented by simple phenolics, flavonoids, isoflavonoids, anthocyanins, and stilbenes. These compounds are recognized as potential antioxidants and antimicrobial agents with possible applications as natural pesticides and food ingredients. Fruit is recognized as part of plants which are rich in diverse phenolics and have been used in folk medicine for centuries (Milenković-Andjelković et al., 2015).

Numerous terpenoids have been examined as toxins, growth inhibitors, and deterrents to microorganisms and animals that defense against enemies. For example, various monoterpenes are toxic to insects (Lee et al., 2003), fungi (Hammer et al., 2003), and bacteria (Friedman et al., 2002), and serve as feeding

deterrents to mollusks (Frank et al., 2002), insects (Szczepanik et al., 2005), and mammals (Vourc'h et al., 2002). Among the monoterpenes, thymol, carvacrol, and eugenol showed antifungal activity (Isman, 2000). Moreover, (-)-gossypol, sesquiterpene, showed anticancer (Oliver et al., 2005; Wolter et al., 2006), antiherbivore (Stipanovic et al., 2006), and antifungal activity (Puckhaber et al., 2002).

Alkaloids are a variety group of low-molecular-weight, nitrogen-containing compounds found in about 20% of plant species (Facchini, 2001). Many of the ~12,000 alkaloids for which structures have been described function in the defense of plants against herbivores and phytopathogens (Caporale, 1995). For instance, flindersine, anhydroevoxine, and haplamine which possessed antifungal activity against *Colletotrichum fragariae*, *C. gloeosporioides*, and *C. acutatum* were extracted from *Haplophyllum sieversii* Lincz et Wed. (Rutaceae) (Cantrell et al., 2005).

The specific type of secondary metabolites can be differed by spatio distribution. For instance, glandular trichomes on the leaf surface synthesize volatile mono- and sesquiterpenes. These volatile organic compounds are generally released from aerial parts of plants and play an important role in the interaction between plants and environment (e.g., defense against phytopathogens and herbivores) (Aharoni et al., 2003). α-Tomatine, tomato steroidal glycoalkaloid with antifungal activity, was mainly included in leaves and flowers (Friedman and Levin, 1998). Flavonoids, one class of phenolics, can be presented in most plant tissues while individual flavonoid sub-classes show a much more specialized distribution. For example, flavan-4-ol, 3-deoxyflavonoids, apiferol, and luteoferol

are presented in the floral tissues of monocots and isoflavones, antimicrobial and antiherbivore comounds, are accumulated in the embryo and seed coat of soybean (Lepiniec et al., 2006).

In the area of secondary metabolite characterization of fruit, the studies on two fruit vegetables, strawberry and tomato, dominate the field. These two family show two highly unique fruit structures both physiologically and biochemically, and represent distinct fruit development. While tomato fruit is a true fruit having a peel tissue surrounding the fleshy pericarp and seeds, strawberry fruit is called "false fruit", with the achenes developing on the surface of the swollen receptacle. Composition of major secondary metabolism of the two fruit vegetables is quite different as their structural distinction. While main secondary metabolites of tomato fruit were lycopene, β-carotene, lutein, ferulic acid, caffeic acid, chlorogenic acid, *E*-2-hexenal, *Z*-3-hexen-1-ol, benzaldehyde, 6-methyl-5-heptene-2-one, 2-phenylethanol, geranyl acetone, and dehydro- and α-tomatines (Buttery et al., 1987; Friedman and Levin, 1998; Moco et al., 2007; Slimestad and Verheul, 2009; Tieman et al., 2006), strawberry fruit mainly contained ellagic acid, 50.9% of the total phenolics in red fruit, caffeic acid, *trans*-cinnamic acid, *p*-coumaric acid, p-benzoic acid, gallic acid, kaempferol, morin, anthocyanin, methyl butanoate, ethyl butanoate, methyl hexanoate, ethyl hexanoate, and 2-heptanone (Hakala et al., 2002; Häkkinen et al., 1999).

However, little information is available on contents of secondary metabolites in non-edible parts of tomato and strawberry plants, because the compounds in the plants have been mainly studied focusing on edible parts. The experiments in the first step of this thesis were conducted in attempts to obtain the basic data of

secondary metabolites in various parts of tomato (Chapter I) and strawberry plants (Chapter III). The second step was focused on antimicrobial activity of extracts from various parts of tomato (Chapter II) and strawberry (Chapter IV) prior to the identification of antimicrobial compounds to develop a natural antimicrobial agent.

LITERATURE REVIEW

Secondary metabolites in various parts of tomato plants

Tomato is one of most cultivated fruit vegetable in the world and also serves as a model for research on fleshy fruit development (Giovannoni, 2007; Giovannoni et al., 1995), including for metabolic studies (Carrari and Fernie, 2006). The most dramatic change in tomato fruit development occurs during the transition to the ripening procedure with the accumulation of innumerous secondary metabolites. The transition to ripening, moreover, is accompanied by an enormous change to metabolism as a result of the de-greening procedure, in which the photosynthetically active chloroplasts are differentiated to chromoplasts containg β-carotene and lycopene. The phenomenon can be a reason which red fruit have more chromoplasts than green fruit.

As exemplified for tomato fruit peel and flesh, these tissues vary in terms of transcript and metabolic profiles (Mintz-Oron et al., 2008; Moco et al., 2007). Mintz-Oron et al. (2008) reported a comparative transcriptome and metabolome analyses of peel and flesh tissues during five stages of tomato fruit development (i.e., green, breaker, turning, pink, and red stage). More than 45 secondary metabolites were identified to increase at least 2- fold in the peel than in the flesh, and among them 30 secondry metabolites were derived from the phenylpropanoid pathway. Slimestad and Verheul (2009) reviewed that skin and pulp of tomato fruit possess numerous hydroxycinnamic acids along with their conjugates. These simple phenolics are predominantly present as a family of esters formed between hydroxycinnamic acids and quinic acid. These esters are often collectively known

as chlorogenic acids, but chlorogenic acid is also the specific name of the most common ester found in nature. Walker (1962) reported that chlorogenic, caffeic, *p*-coumaric, and ferulic acids were found in tomato fruits, and contents of chlorogenic and caffeic acids decreased during the fruit development process. Wardale (1973) reported that chlorogenic acid is the main simple phenolics contained in fruits of various tomato cultivars, accounting for 75% of the total phenolics in mature green fruit and 35% in red fruit.

Except phenylpropanoids, numerous glycoalkaloids having antimicrobial activity were identified in tomato peel. α-Tomatine, glycoalkaloids of tomato, was accumulated to high concentrations in early fruit development and decreased in the mature stages, and ends with lycoperosides that display an opposite profile of accumulation during fruit development. Moco et al. (2007) investigated different compound classes such as carotenoids, xantophylls, chlorophylls, tocopherols, flavonoids, simple phenolics, glycoalcaloids, saponins, and a few primary metabolites in tomato fruit.

Antimicrobial activity of secondary metabolites in various parts of tomato plants

Chlorogenic acids and related compounds are the main simple phenolics in tomato plants (Slimestad and Verheul, 2009). They are structurally ester between caffeic acid and l-quinic acid and occurs in high amounts in solanaceous plants including tomato and potato plants (Ahmad et al., 2013). Chlorogenic acid is an important fungus defense compound of tomato plants (Wojciechowska et al., 2014). Important antifungal secondary metabolites from tomato plants have often been reported (Atanasova-Penichon et al., 2012; Ruelas et al., 2006). Ruelas et al.

(2006) investigated the simple phenolics composition of tomato fruits and found that chlorogenic, caffeic, *p*-coumaric, and ferulic acids were the most important simple phenolics in diverse cultivars of tomatoes. Chlorogenic acid has significant concentration-dependent antimicrobial activities. Bostock et al. (1999) described that the resistance of peach against *Monilia fructicola* fungus decreased with the reduction of chlorogenic content. Terry et al. (2004) also reported that strawberry fruits were more susceptible towards infection by *Botrytis cinerea* with decreasing concentrations of phenolics. Chlorogenic acid, therefore, has definitely antifungal activity and protects tomato plants from fungal attack.

In *Solanum* species such as tomato, the main saponin is α-tomatine, the steroidal glycoalkaloid which has potent antifungal activity (Arneson and Durbin, 1968; Roddick, 1974). Sandrock and VanEtten (2001) proved that α-tomatine showed antifungal effect against *Alternaria alternate* f. sp. *lycopersici*, *Aspergillus nidnulans*, *Botrytis cinerea*, *Colletotrichum coccodes*, *Cryphonectria parasitica*, *Fusarium moniliforme*, *Fusarium oxysporum* f. sp. *lycopersici*, *Macrophomina phaesolina*, *Magnaporthe grisea*, *Nectria haematococca*, *Neurospora crassa*, *Phytophthora infestans*, *Pythium aphanidermatum*, *Stemphylium botryosum*, *Stemphylium solani*, *Verticillium dahliae*, *Verticillium albo-atrum*, and *Verticillium dahliae*. The fungicidal mode of α-tomatine is that it forms a complex with fungal membrane sterols with free 3β-hydroxyl groups, resulting in pore formation and loss of membrane integrity followed by leakage of cell components and cell death (Friedman, 2002; Morrissey and Osbourn, 1999).

Secondary metabolites in various parts of strawberry plants

Strawberry belongs to the subfamily Rosoideae of the Rosaceae family that includes other known fruit species such as apple, pear, plum, peach, blueberry, and raspberry. Fruits of the Rosaceae family are known to contain exceptionally diverse secondary metabolite composition upon ripening. The stages of fruit development were well reported with respect to its metabolite composition (Hannum, 2004; Koponen et al., 2007; Puupponen-Pimiä et al., 2005).

The largest metabolite group consists of compounds from central phenylpropanoid and flavonoid pathway such as simple phenolics derivatives, flavonols, condensed tannins, and anthocyanins. In addition to the usually occurring phenolics, remarkable quantity of ellagic acid and ellagitannins are present in plants of the Rosaceae family (Kähkönen et al., 2001), including in strawberry (Hukkanen et al., 2007; Mullen et al., 2003). Ellagitannins are polyphenols that are not from the phenylpropanoid pathway, but rather synthesized through the shikimate pathway via the 5-dehydroshikimate precursor (Werner et al., 2004). The composition of ellagitannins in strawberry fruits varies between the receptacle tissue and the achenes (Aaby et al., 2005). In strawberry fruits, the phenylpropanoid pathway is changed during the ripening process. Proanthocyanidins and flavonoids are actively induced and accumulated to high levels in receptacle of immature fruits, thereby giving green and white fruits an astringent flavor (Aharoni et al., 2002; Almeida et al., 2007; Cheng and Breen, 1991), contributing to plant defense (Halbwirth et al., 2006; Hukkanen et al., 2007; Terry et al., 2004).

Antimicrobial activity of secondary metabolites in various parts of strawberry

plants

Several studies have indicated that proanthocyanidins in strawberry fruits showed antifungal activity against *Botrytis cinerea*. Jersch et al. (1989) found that aqueous extracts of immature fruits of 'Chandler' cultivar had antifungal activity against conidial germination and mycelial growth of *B. cinerea*. They also reported that the proanthocyanidins concentration was higher in the less susceptible strawberry cultivars. Di Venere et al. (1998) reported that the proanthocyanidin content in immature fruits of diverse cultivars and the activity of *B. cinerea* showed an inverse relationship. Puhl and Treutter (2008) observed that the accumulation of catechin-derived procyanidins was a key factor to inhibit the growth of *B. cinerea* in immature fruits. Gray mold symptoms by *B. cinerea*, in fact, occur only in red fruits. The phytopathogen changed the concentration of flavanols in developing fruits by inhibiting flavanone 3-hydroxylase, a prominent dioxygenase of the flavonoid pathway, which is involved in the biosynthesis of catechin precursors. Therefore, a higher increment of the antifungal compounds was found after flowering, during the stage of green fruits, but showing no effect thereafter. The increasing catechin and procyanidins concentrations at the green fruits restricted fungal development. The latent infections on immature fruits, becoming quiescent until fruits is mature, has also been studied for other strawberry pathogens including *Colletotrichum* spp. (Guidarellim et al. 2011; Prusky, 1996).

Strawberry leaves also contain preformed antimicrobial compounds. Vincent et al. (1999) reported the unidentified antifungal compounds providing the strawberry resistance to *Colletotrichum fragariae*. They found that the amount of

the preformed compounds varied between mild resistant and susceptible cultivars to *Colletotrichum*. For instance, antifungal activity of 'Sweet Charlie' (resistant cultivar) presents approximately 15 times higher than 'Chandler' (susceptible cultivar). The resistance of diverse strawberry cultivars against *C. fragariae* may be mediated by these preformed antifungal compounds (Vincent et al., 1999). Terry et al. (2004) also reported that the antifungal compounds found in green stage fruits might be similar to the preformed antifungal compounds. Yamamoto et al. (2000) observed that protective agent, catechin, preformed in strawberry leaves inhibited growth of *Alternaria alternata*, and Hanhineva et al. (2009) showed that decreased contents of flavonols in strawberry leaves increased susceptibility to *Botrytis cinerea*. Therefore, the role of flavonols in strawberry leaves is important for plant defense (Halbwirth et al., 2006; Hukkanen et al., 2007; Terry et al., 2004). Filippone et al. (1999) isolated a new type of antimicrobial compound, fragarin, constitutively contained in strawberry leaves. The compound showed high antimicrobial activity against bacterial and fungal phytopathogens isolated from strawberry (*Colletotrichum gloeosporioides*, *C. fragariae*, and *C. acutatum*) and other plants (*Clavibacter michiganensis* subsp. *sepedonicus*, strain C5, and *Pseudomonas corrugata*, isolated from tomato; *Pseudomonas syringae* isolated from onion; and *Erwinia* spp. isolated from rose leaves). The authors reported that fragarin showed antifungal activity by dissipating membrane potential of *C. michiganensis*, and suggested its action precedes or was contemporaneous with cell death by changing the membrane permeability and interrupting function of *C. michiganensis* (Filippone et al., 2001). Quantitative differences of several phenolics were also found in strawberry roots,

and this appeared to be critical in giving moderate resistance to rot diseases of strawberry root caused by *Pythium irregulare*, *Rhizoctonia solani*, and *Alternaria alternata* (Nemec, 1973, 1976).

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CHAPTER I

Secondary Metabolites Profiling in Various Parts of Tomato Plants

ABSTRACT

Contents of carotenoids, phenolics, volatile organic compounds, and alkaloids were measured in leaves, internodal stems, fruits, and roots of 'Bacchus' tomato plants at different developmental stages. Lycopene, β-carotene, and lutein were detected in all parts tested except roots and immature green fruits. Lycopene content in red fruits was 196.2 $\mu g \cdot g^{-1}$ FW. β-Carotene and lutein contents in the 24th leaves were 23.2 and 25.6 $\mu g \cdot g^{-1}$ FW, respectively, and were greater than those in the other parts. Caffeic, chlorogenic, and vanillic acids were detected in all tested parts except roots. Chlorogenic acid content in the 18th leaves was 40.1 $\mu g \cdot g^{-1}$ FW, while that in the other parts was lower than 31.0 $\mu g \cdot g^{-1}$ FW. Contents of caffeic and vanillic acids in the 24th leaves were 9.2 and 1.6 $\mu g \cdot g^{-1}$ FW, respectively, and were greater than those in the other parts. Moreover, younger leaves contained more diverse volatile organic compounds including monoterpenes and sesquiterpenes. Contents of dehydro-tomatine and α-tomatine were greatest in leaves, followed by internodal stems, roots and fruits. Younger leaves and internodal stems contained more dehydro-tomatine and α-tomatine than older ones. Contents of dehydro-tomatine and α-tomatine in the 24th leaves

were 889.1 and 1,417.9 $\mu g \cdot g^{-1}$ FW, respectively, and were greatest among all parts tested. These results indicated that, except lycopene, tomato leaves included greater secondary metabolites contents than red fruits. The results suggest that non-edible parts of tomato plants can be used as raw material for antioxidants, anti-inflammatory agents, fungistats, and pesticides.

INTRODUCTION

Secondary metabolites are diverse compounds found in terrestrial and marine plant species. Plants produce secondary metabolites under biotic or abiotic stresses because the compounds have antimicrobial, anti-herbivory, and allelopathic effects (Dixon, 2001). Secondary metabolites also have color, scent, and flavor attractants (Frydman et al., 2004; Rohloff and Bones, 2005; Verdonk et al., 2003), UV-protectants, antioxidants, signaling factors, and nutraceutical and pharmacological efficacies (Aerts et al., 1999; Bagchi et al., 2000; Deavours and Dixon, 2005; D'Haeze and Holsters, 2002; Gidley, 2004; Manach et al., 2004; Merz-Demlow et al., 2000; Oldroyd, 2001; Relić et al., 1994; Setchell and Cassidy, 1999).

Tomato (*Solanum lycopersicum* L.) plants contain many secondary metabolites. Profiling of secondary metabolites in tomato plants, particularly in fruits, has been performed by analyses of carotenoids (Moco et al., 2007), phenolics (Slimestad and Verheul, 2009), volatile organic compounds (Buttery et al., 1987), and alkaloids (Friedman and Levin, 1998).

The carotenoids are important color compounds in food, flowers, and fruits

(Verpoorte and Memelink, 2002). Lycopene is the most abundant secondary metabolites in fully red ripe tomato fruits. This carotenoid is a natural pigment synthesized by plants to protect cells against oxidative damage (Rao and Agarwal, 1999) and to attract pollinators (Moco et al., 2007). Moreover, lycopene has been reported to induce communication between cells (Zhang et al., 1991) and to control immune systems and other metabolic pathways (Fuhrman et al., 1997; Astorg et al., 1997). Tomato plants also include β-carotene and lutein. β-Carotene is known for its provitamin A activity and lutein for its anticancer activity against lung cancer (Di Mascio et al., 1991).

Phenolics are known to possess antimicrobial and antiviral properties (Dixon, 2001; French and Neil Towers, 1992). Many phenolics in tomato fruits have also been found (Slimestad and Verheul, 2009). Walker (1962) reported that caffeic, chlorogenic, *p*-coumaric, and ferulic acids were found in tomato fruits, and contents of caffeic and chlorogenic acids decreased during fruit development process. Wardale (1973) reported that chlorogenic acid was the major phenolics contained in fruits of various tomato cultivars, accounting for 75% of the total phenolics in mature green fruits and 35% in red fruits.

Volatile organic compounds in tomato plants affect the behaviors of pests and pollinators (Buttery et al., 1987). Andersson et al. (1980) identified volatile mono- and sesquiterpenes in tomato leaves. Urbasch (1981) additionally identified hexanal, (*E*)-2-hexenal, (*Z*)- and (*E*)-β-ocimenet erpinolene, linalool, neral, geranial, methyl salicylate, nerol, geraniol, and 2-tridecanone. Buttery et al. (1987) improved upon the detection and isolation of mono- and sesquiterpenes, and aliphatic and aromatic compounds in tomato leaves.

Tomatine from tomato was firstly isolated in 1948 (Fontaine et al., 1948) and Friedman and Levin (1998) identified dehydro- and α-tomatine in various parts of tomato plants. These tomato glycoalkaloids are of interest because of their implication in host-plant resistance. In particular, α-tomatine is expected to protect tomato leaves against attack by microorganisms. (Morrissey and Osbourn, 1999). Sandrock and VanEtten (1998) reported that growth of eight saprophytic fungi and the tomato pathogens *Stemphylium solani 11128* and *Verticillium dahlia* were greatly inhibited by α-tomatine.

Non-edible parts of vegetables have been indicated to contain higher contents of secondary metabolites than their edible parts. For instance, the strawberry and carrot leaves included higher contents of phenolics than their edible parts (Kähkönen et al., 1999; Kim et al., 2013). However, few studies have reported the metabolites in non-edible parts of tomato plants such as leaves, internodal stems, immature green fruits, and roots. The objective of this study was to identify the carotenoids, phenolics, volatile organic compounds, and alkaloids present in leaves, internodal stems, fruits, and roots of tomato plants.

MATERIALS AND METHODS

Plant materials

'Bacchus' tomato plants (Monsanto Korea, Jochiwon, Korea) were transplanted in a greenhouse located in Gwangju (E 126.7^{o}, N 35.2^{o}) on August 19, 2011. The plants were fertigated with Yamasaki nutrition solution (N, 7.0 me·L^{-1}; P, 2.0 me·L^{-1}; K, 4.0 me·L^{-1}; Ca, 3.0 me·L^{-1}; Mg, 2.0 me·L^{-1}; Fe, 15.4 mg·L^{-1}, B, 1.14 mg·L^{-1}; Mn, 0.81·mg L^{-1}; Zn, 0.09 mg·L^{-1}; Cu, 0.04 mg·L^{-1}; Mo, 0.01 mg·L^{-1})

using an automatic drip fertigation system (pH, 5.5-6.0; EC, 2.0-2.5 $dS \cdot m^{-1}$) and were pinched off at the two leaves above the the 6th flower truss. Roots, the 18th (above the 4th flower truss), the 21th (above the 5th flower truss), and the 24th (above the 6th flower truss) leaves and internodal stems, and immature green, pink, and red fruits were harvested on May 15, 2012.

Fruits were classified into three ripening stages by percentage of red color: 0%, immature green; 30-60%, pink; > 90%, red. Samples at each stage were separately stored at $-20^{o}C$ until phytochemical analysis.

Analysis of carotenoids

The hexane extraction method was performed as described by Sadler et al. (1990). Samples (4 g) were weighed into 125 mL flasks covered with aluminum foil to block light. A hexane:acetone:ethanol (50:25:25, v/v) solvent (100 mL) was added to the flask and then agitated for 10 min. Water (15 mL) was added and agitated for additional 5 min. The solution was separated into distinct polar (65 mL) and non-polar hexane (50 mL) layers. The carotenoids in the upper hexane layer were measured using a spectrophotometer (UV-2550, Shimadzu, Kyoto, Japan). Lycopene, β-carotene, and lutein contents in each sample were then estimated using absorbance (A) 503, 455, 446 nm, respectively. Contents of β-carotene and lycopene were calculated according to the following equations (Barros et al., 2007).

Lycopene (mg/100 mL) = $-0.0458\ A_{663} + 0.372\ A_{503} - 0.0806\ A_{455}$

β-Carotene (mg/100 mL) = $0.216\ A_{663} - 0.304\ A_{503} + 0.452\ A_{455}$

Analysis of phenolics

Phenolics were extracted and hydrolyzed according to the process described by Nuutila et al. (2002). Various parts (5 g FW) of tomato plants were homogenized with 5 mL of 1.2 M HCl in 50% (v/v) aqueous methanol. Ascorbic acid (8 mg) was added to the mixture as an antioxidant. After incubating at 35^{o}C for 16 h, the extract was cooled, filled up to 10 mL, and sonicated for 3 min. The extract was filtered through a syringe filter (0.45 μm pore size) before injection to an HPLC system (Ultimate 3000, Dionex, Sunnyvale, CA, USA). A Zorbax SB C-18 column (150 × 4.6 mm, i.d., 5 μm, Agilent, New York, NY, USA) was used. The mobile phase was programmed as 20-60% gradient in 25 min of methanol in water containing 300 mL·L^{-1} trifluoroacetic acid at a flow rate of 0.8 mL·min^{-1}. The eluted components were monitored at 280 and 340 nm using a UV/Vis detector. Analytical standards were *trans*-cinnamic acid, *p*-coumaric acid, caffeic acid, chlorogenic acid, gallic acid, ellagic acid, *p*-hydrobenzoic acid, 2,5-hydrobenzoic acid, 3,4-hydrobenzoic acid, vanillic acid, kaempferol, quercetin, and morin.

Analysis of volatile organic compounds

Volatile organic compounds were determined using the procedure described by Isleten and Karagül-Yüceer (2008). The sample gas was gathered by Tenax-TA (Perkin Elmer Life and Analytical Sciences, Waltham, MA, USA), a dynamic thermal extractor chamber system, which consisted of a 26 mL glass tube and an air control system, containing an air supply unit and pumps. Purified nitrogen gas (39 mL·min^{-1}) was used for ventilation. Volatile organic compounds in various

parts of tomato plants were analyzed by thermal desorption gas chromatograph mass spectrometry (TDS-GC MSD and TDS2, Gerstel GmbH & Co. KG, Mülheim, Germany; 6890N and 5975, Agilent Technologies, Santa Clara, CA, USA).

Analysis of tomatines

Tomatines were isolated and identified by the method of Friedman and Levin (1998). Samples (1 g) in 20 mL of 1% acetic acid were extracted by stirring for 2 h. The suspension was centrifuged at 13,300 *g* for 10 min. The supernatant was filtered through a Whatman GF/C filter and the pellet was resuspended, centrifuged, and filtered. The two extracts were pooled and then purified using SPE tubes (Supelco, Bellefonte, PA, USA). The C18 SPE tubes were conditioned with methanol (5 mL) followed by water (5 mL). The extract (about 30 mL) was allowed to gravity drip. When the sample was fully absorbed, the tube was washed with water (10 mL), subsequently with 5 mL of 30:70 (acetonitrile:1% NH_4OH), and then water (5 mL). The dehydrotomatine and α-tomatine were eluted with 10 mL of 70:30 (acetonitrile:1 mM HCl). The sample was dried on a rotary evaporator and the residue was suspended in 1 mL of 50% methanol/0.1% acetic acid. The two samples were combined and filtered through a HV membrane (0.45 μm pore size) before HPLC injection.

Commercial tomatine (Tokyo Chemical Industry Co., Tokyo, Japan) was separated into dehydrotomatine and α-tomatine by preparative HPLC using UV detection. Conditions were as follows: 3 mL of eluent/min was passed through a 150 × 4.6 mm, i.d. 5 μm, Zorbax SB C-18 column; the eluent consisted of 25%

acetonitrile and 100 mM ammonium phosphate brought to pH 3 with phosphoric acid. Tomatine (2 mg) in 1 mL of 50% methanol and 0.1% acetic acid was injected to the column; the two peaks were collected from the UV detector, which was monitored at 200 nm. The structures of the dehydrotomatine and α-tomatine were confirmed by mass spectrometry. The HPLC eluent for tomatine analysis was prepared by combining buffer (100 mL) with polished water (550 mL), acetonitrile (200 mL), and methanol (150 mL). The concentrated buffer was prepared by combining disodium phosphate (29.0 g) and citric acid (93.7 g) in water (1 L). This buffer was filtered through a nylon membrane (0.45 μm pore size), passed through a 3 × 1 cm bed of Chelex 100, and then passed through a C18 SPE. The chromatography column was a 150 × 4.6 mm, i.d. 5 μm, Zorbax SB C-18. Flow rate was set to 1.0 $mL \cdot min^{-1}$. Tomatine was monitored at 200 nm.

Statistical analysis

Statistical analyses were performed using SAS statistical software version 9.2 (SAS Inst., Cary, NC, USA). Duncan's multiple range test was used to assess differences in contents of carotenoids, phenolics, and alkaloids at $P \leq 0.05$.

RESULTS AND DISCUSSION

Contents of carotenoids

Lycopene was only detected in red fruits, with a content of 196.2 $\mu g \cdot g^{-1}$ FW (Table I-1). Kozukue and Friedman (2003) and Lenucci et al. (2006) reported that

Table I-1. Contents of carotenoids in different parts of 'Bacchus' tomato plants.

Part		Lycopene	β-Carotene	Lutein
		($\mu g \cdot g^{-1}$ FW)		
Leaf	18th	-[z]	14.6 c[y]	17.9 b
	21th	-	20.1 b	23.9 a
	24th	-	23.2 a	25.6 a
Internodal stem	18th	-	1.2 d	1.5 d
	21th	-	1.5 d	1.9 d
	24th	-	1.5 d	1.8 d
Fruit	Immature green	-	-	-
	Pink	-	-	1.1 d
	Red	196.2	3.4 d	5.0 c
Root		-	-	-

[z]-, not detected.

[y]Mean separation within columns by Duncan's multiple range test at $P = 0.05$.

lycopene content ranged from 58 mg·kg^{-1} FW in 'Momotaro' tomato cultivar to 253 mg·kg^{-1} FW in 'Kalvert' cultivar. Accumulation of lycopene generally begins at the 'breaker' stage after tomato fruits has reached the 'mature green stage'. Lycopene is mainly included in red-ripe tomato fruits (approximately 80 to 90% of total pigment) (Lenucci et al., 2006). Ronen et al. (1999) reported that mRNA of CrtL-b which encodes lycopene β-cyclase and CrtL-e which encodes lycopene β-cyclase decrease at the 'breaker' stage.

Contents of β-carotene were 23.2, 20.1, 14.6, 3.4, 1.5, 1.5, and 1.2 μg·g^{-1} FW in the 24th leaves, the 21th leaves, the 18th leaves, red fruits, the 21th internodal stems, the 24th internodal stems, and the 18th internodal stems, respectively (Table I-1). β-Carotene content in the 24th leaves was 6.9 times greater than that in red fruits. Contents of lutein were 25.6, 23.9, 17.9, 5.0, 1.9, 1.8, 1.5, and 1.1 μg·g^{-1} FW in the 24th leaves, the 21th leaves, the 18th leaves, red fruits, the 21th internodal stems, the 24th internodal stems, the 18th internodal stems, and pink fruits, respectively (Table I -1). Lutein content in the 24th leaves was 5.1 times greater than that in red fruits. Fraser et al. (1994) also showed that tomato leaves contained β-carotene and lutein but not lycopene. Lycopene plays an attractant role in seed dispersal that can affect the further propagation (Moco et al., 2007) and β-carotene and lutein, major carotenoids in green leafy vegetables, are potent antioxidants (Jiménez-Escrig et al., 2000). The results show that tomato plants contain more antioxidants and attractants in leaves and reed fruits, respectively.

Contents of phenolics

Caffeic, chlorogenic, and vanillic acids were detected in all parts tested except

root (Table I-2), while *trans*-cinnamic acid, *p*-coumaric acid, gallic acid, ellagic acid, *p*-hydrobenzoic acid, 2,5- hydrobenzoic acid, 3,4- hydrobenzoic acid, kaempferol, quercetin, and morin were not found. Chen et al. (2006) and Slimestad and Verheul (2009) reported that chlorogenic acid and its derivatives are the main simple phenolics in tomato leaves and fruits. Slimestad and Verheul (2009) reviewed that chlorogenic acid content ranged from 1.7 $mg \cdot kg^{-1}$ FW in 'Izabella' cultivar to 32.8 $mg \cdot kg^{-1}$ FW in 'Liso' and 'Senior' cultivars. Results from the present study also showed that content of chlorogenic acid was higher than those of caffeic and vanillic acids. Contents of chlorogenic acid were 40.1, 30.3, 25.9, 15.6, 10.0, 9.7, 8.7, 7.7 $\mu g \cdot g^{-1}$ FW in the 18th leaves, the 21th leaves, the 24th leaves, the 24th internodal stems, the 18th internodal stems, immature green fruits, the 21th internodal stems, and red fruits, respectively (Table I-2). Chlorogenic acid content in the 18th leaves was 5.2 times greater than that in red fruits.

The contents of the chlorogenic acid in leaves and internodal stems may be different due to their senescence. Chlorogenic acid that presents in tomato leaves related to insect resistance decreases growth and development of several tomato herbivores (Elliger et al., 1981; Stamp and Yang, 1996). Moreover, the compound is produced in response to greenbug (Todd et al., 1971) and black rot infection in resistant sweet potato roots (Akazawa and Wada, 1961; Uritani and Akazawa, 1955). Chlorogenic acid also has numerous beneficial properties related to potent antioxidant activities such as hepatoprotective, hypoglycemic, and antiviral activities (Farah and Donangelo, 2006). Chlorogenic and caffeic acids are

Table I-2. Contents of phenolics in different parts of 'Bacchus' tomato plants.

Part		Chlorogenic acid	Caffeic acid	Vanillic acid
		($\mu g \cdot g^{-1}$ FW)		
Leaf	18th	40.1 a[y]	6.1 b	-
	21th	30.3 ab	5.1 b	0.5 c
	24th	26.0 ab	9.2 a	1.6 a
Internodal stem	18th	10.0 c	0.5 c	0.9 b
	21th	8.7 c	0.4 c	0.9 b
	24th	15.6 bc	0.3 c	0.8 b
Fruit	Immature green	9.7 c	9.0 a	0.1 d
	Pink	-[z]	3.8 b	-
	Red	7.7 c	6.5 ab	0.2 d
Root		-	-	-

[z]-, not detected.

[y]Mean separation within columns by Duncan's multiple range test at $P = 0.05$.

oxidized to more toxic quinones by polyphenol oxidase from the host or parasite, which then are polymerized to insoluble non-toxic melanins. Orthoquinones are very unstable, but the supplement of amino acids to the reactive center after orthophenol oxidation might prevent polymerization and could explain how the quinones provide disease resistance (Clack et al., 1959; Johnson and Schaal, 1957). Contents of caffeic and vanillic acids in the 24th leaves were 9.2 and 1.6 $\mu g \cdot g^{-1}$ FW, respectively, and were greater than that of the older leaves and other parts (Table I-2). Brown et al. (2003) reported that young leaves of *Arabidopsis* had more diverse secondary metabolites compared to old leaves, probably due to the re-allocation of secondary metabolites to decrease feeding by herbivores. Young leaves of strawberry plants also included higher content of phenolics than old leaves (Kim et al., 2013). Antimicrobial activity of caffeic acid has been tested against various fungi and bacteria (Harrison et al., 2003; Kim et al., 2012; Rauha et al., 2000; Widmer and Laurent, 2006). The results show that tomato leaves include more antimicrobial and antihervibore phenolics than other parts.

Contents of volatile organic compounds

'Bacchus' tomato plants included 40 volatile organic compounds (Tables I-3, I-4). Contents of total volatile organic compounds were 444, 206, 152, 111, 108, 82, 37, 30, 26, and 18 $\mu g \cdot g^{-1}$ FW in red fruits, pink fruits, the 24th leaves, the 18th leaves, the 21th leaves, immature green fruits, the 18th internodal stems, the 21th internodal stems, the 24th internodal stems, and roots, respectively. The 2-butenal produced by the aldol condensation of acetaldehyde was the predominant volatile organic compound in red fruits; however, it was not detected in roots, leaves,

Table I-3. Contents of volatile organic compounds (hydrocarbon fraction compounds) in different parts of 'Bacchus' tomato plants.

Part		Ben[z]	Ca	Cy	D	Hd	Hex	Hu	L	αPh	βPh	Pi	S	T	U
		($\mu g \cdot g^{-1}$ FW)													
Leaf	18th	3	11	-[y]	-	-	14	-	5	-	36	-	-	15	-
	21th	2	10	2	-	-	-	-	5	-	-	-	36	16	8
	24th	6	15	-	9	6	6	-	8	3	3	4	50	22	6
Internodal	18th	-	-	-	-	-	-	3	2	-	-	-	18	8	-
stem	21th	2	-	-	-	-	-	-	2	-	-	-	18	8	-
	24th	-	-	-	-	-	-	-	2	-	-	-	16	6	-
Fruit	Immature green	2	-	-	-	-	-	-	-	-	-	-	-	-	-
	Pink	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Red	-	-	2	-	-	-	-	-	-	-	-	-	-	-
Root		-	-	-	-	-	-	-	-	-	-	-	-	-	-

[z]Ben, benzene; Ca, caryophyllene; Cy, cyclohexene; D, decane; Hd, heptadecane; Hex, hexane; Hu, α-humulene; L, di-limonene; α-Ph, α-phellandrene; β-Ph, β-phellandrene; Pi, α-pinene; S, sabinene; T, α-terpinene; U, undecanene.

[y]-, not detected.

Table I-4. Contents of volatile organic compounds (oxygenated fraction compounds) in different parts of 'Bacchus' tomato plants.

Part		B[z]	1BA	Ba	BA	1Bo	2Ba	DA	Ea	EC	Fo	2Fu	Fu	Fua	Hep
		($\mu g \cdot g^{-1}$ FW)													
Leaf	18th	-[y]	-	-	-	-	-	-	-	-	-	-	-	-	-
	21th	-	-	-	-	-	-	7	-	-	-	-	-	-	2
	24th	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Internodal	18th	-	-	2	-	-	-	-	-	-	-	-	-	-	-
stem	21th	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	24th	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fruit	Immature green	-	-	3	-	-	47	-	-	-	-	-	-	-	-
	Pink	-	-	-	-	3	-	-	25	136	-	8	-	-	-
	Red	2	-	-	5	39	288	-	-	-	5	7	2	-	-
Root		-	9	-	-	-	-	-	-	-	-	-	-	4	-

(continued)

Part		2He	3He	3Ho	Mho	Na	Ne	Noa	O	P	P3l	P3n	Sa
		($\mu g \cdot g^{-1}$ FW)											
Leaf	18th	-	-	2	-	1	9	-	7	-	-	-	8
	21th	-	-	-	-	-	12	-	8	-	-	-	-
	24th	2	6	6	-	-	-	-	-	-	-	-	-
Internodal	18th	-	-	-	-	2	-	2	-	-	-	-	-
stem	21th	-	-	-	-	-	-	-	-	-	-	-	-
	24th	-	-	-	-	2	-	-	-	-	-	-	-
Fruit	Immature green	8	-	19	-	3	-	-	-	-	-	-	-
	Pink	15	-	16	-	3	-	-	-	-	-	-	-
	Red	28	-	46	4	5	-	-	-	3	4	4	-
Root		-	-	-	-	5	-	-	-	-	-	-	-

[z]B, benzaldehyde; 1BA, 1,2-benzenedicarboxylic acid; BA, benzoic acid; Ba, butanal; 2Ba, 2-butenal; 1Bo, 1-butanol; DA, n-decanoic acid; Ea, ethyl acetate; EC, 2-ethyl crotonaldehyde; Fo, formic acid; 2Fu, 2(5H)-furanone; Fu, furan; Fua, 2-furancarboxaldehyde; Hep, heptane; 2He, 2-hexenal; 3He, 3-hexenal; 3Ho, 3-hexen-1-ol; Mho, 6-methyl-5-hepten-2-one; Na, nonanal; Ne, nonane; Noa, nonanoic acid; O, octane; P, pentane; P3l, 1-penten-3-ol; P3n, 1-penten-3-one; Sa, sulfurous acid.

[y]-, not detected.

internodal stems, and pink fruits. Leaves included six monoterpenes such as α-terpinene, α-phellandrene, β-phellandrene, di-limonene, α-pinene, and sabinene. Buttery et al. (1987) reported that the leaves of the red cherry tomato contain eight monoterpenes (2-carene, myrcene, terpinolene, α-pinene, limonene, α-phellandrene, β-phellandrene, and α-terpinene) and three sesquiterpenes (δ-elemene, caryophyllene, and humulene). Contents of β-phellandrene in the 18th leaves and sabinene in the 24th leaves were higher than those in other parts. Tomato plants lay in volatile organic compounds in their glandular trichomes and the compounds are emitted by damage of the trichomes (van Schie et al., 2007). Herbivores increase the emission of the compounds from tomato trichomes when they have damage the trichomes (Gibson, 1971). In addition, sesquiterpene (α-humulene) was only detected in the 18th internodal stems. Degenhardt et al. (2003) reported that mono- and sesquiterpene are volatile compounds that attract herbivore enemies when plants are damaged by herbivores. The results show that the red fruits of tomato contained the highest contents of total volatile organic compounds, and the leaves of tomato contained the most diverse volatile organic compounds to attract herbivore enemies.

Contents of alkaloids

Contents of dehydrotomatine were 889.1, 852.3, 817.1, 358.3, 269.7, 242.7, 59.1, 11.3, 7.9, and 7.5 $\mu g \cdot g^{-1}$ FW in the 24th leaves, the 21th leaves, the 18th leaves, the 24th internodal stems, the 21th internodal stems, the 18th internodal stems, roots, immature green fruits, pink fruits, and red fruits, respectively (Table I-5). Contents of α-tomatine were 1,417.9, 1,321.3, 1,275.0, 307.4, 214.4, 167.5,

Table I-5. Contents of alkaloids in different parts of 'Bacchus' tomato plants.

Part		Dehydro-tomatine	α-Tomatine
		($\mu g \cdot g^{-1}$ FW)	
Leaf	18th	817.1 c[z]	1,275.0 c
	21th	852.3 b	1,321.3 b
	24th	889.1 a	1,417.9 a
Internodal stem	18th	242.7 e	167.5 d
	21th	269.7 e	214.4 d
	24th	358.3 d	307.4 d
Fruit	Immature green	11.3 g	43.0 e
	Pink	7.9 g	15.5 e
	Red	7.5 g	13.1 e
Root		59.1 f	56.6 e

[z]Mean separation within columns by Duncan's multiple range test at $P = 0.05$.

56.6, 43.0, 15.5, and 13.1 $\mu g \cdot g^{-1}$ FW in the 24th leaves, the 21th leaves, the 18th leaves, the 24th internodal stems, the 21th internodal stems, the 18th internodal stems, roots, immature green fruits, pink fruits, and red fruits, respectively (Table I-5). Contents of dehydro- and α-tomatine were greatest in leaves, followed by internodal stems, roots, and fruits. Younger leaves and internodal stems contained higher contents of dehydro- and α-tomatine than older leaves and internodal stems. Friedman and Levin (1998) reported that leaves of tomato contained greatest dehydro- and α-tomatine and the compounds in immature green fruits were partly degraded during fruit development. Dehydro- and α-tomatine in plants serve as natural defenses against fungi, bacteria, insects, and mammals. Especially, α-tomatine that disrupts cell membrane by lysing of liposome has been implicated in plant defense (Morrissey and Osbourn, 1999). Boulogne et al. (2012) reviewed insecticidal and fungicidal activities of tomatine against *Macrosiphum euphorbiae*, *Leptinotarsa decemlineata*, *Melanopus bivittatus*, *Heliothis zea*, *Hyposoter exiguae*, *Spodoptera exigua*, *Tribolium castaneum*, *Sitophilus oryzae*, *Fusarium solani*, *Nomuraea rileyi*, and *Fusarium oxysporum*. The results showed that young leaves of tomato include more pesticidal alkaloids than other parts.

Pichersky and Gang (2000) reported that the ability to produce secondary metabolites has been selected throughout the process of evolution in different plant lineages when the compounds addressed specific needs. For instance, floral scent volatiles and pigments have evolved to attract pollinators and thus increase fertilization rates. The ability to synthesize toxic metabolites has evolved to ward off microorganisms and herbivores (from bacteria and fungi to insects and mammals) or to suppress the growth of other plants. The metabolites found in

fruits prevent spoilage, and their color, aroma, and flavor signal the presence of potential rewards such as sugars, vitamins, and amino acids to animals who eat the fruits and thereby help seed dispersal. Other chemicals serve cellular functions that are unique to the particular plant in which they occur, such as resistance to salt or drought. Non-edible parts (e.g., leaves, internodal stems, and immature green fruits) of tomato plants mainly included compounds toxic to microorganisms and insects (chlorogenic acid, caffeic acid, and tomatine) and edible parts contained pollinator attractants (lycopene and volatile compounds). Thus, after harvesting of fruits, non-edible parts of tomato plants could be used as a raw material for antioxidants, anti-inflammatory agents, fungistats, and pesticides.

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CHAPTER II

Antimicrobial Activity of Extracts from Various Parts of Tomato Plants

ABSTRACT

The objective of this chapter was to confirm antimicrobial activity of acetone, hexane, dichloromethane, or methanol extracts from leaves, stems, immature green fruits, or red fruits of 'Bacchus' tomato plants. The antimicrobial activity of 'Bacchus' tomato extracts was tested against six phytopathogens including *Colletotrichum coccodes*, *Fusarium oxysporum*, *Glomerella cingulate*, *Phytophthora cactorum*, *P. capsici*, and *Rhizoctonia solani*. Minimum inhibitory concentration (MIC) of acetone extracts was lower than those of the other solvents. Tomato leaves had higher antimicrobial activity than the other parts against tested phytopathogens. Acetonic extract from tomato leaves, hence, was selected as the source of antimicrobial substance. Acetonic extract from tomato leaves inhibited mycelial growth of *Fusarium oxysporum*, *Glomerella cingulata*, and *Rhizoctonia solani.* Mycelial growth of *R. solani* showed more susceptibility to the acetonic extract than to the other phytopathogens. With 0.31 $mg \cdot mL^{-1}$ of acetonic extract from tomato leaves, mycelial growth of *R. solani* on day 1st, 2nd, and 3rd decreased by 50.0, 52.1, and 64.0% as compared with acetone solvent treatment, respectively. When the amount of acetonic extract from tomato leaves used was 8 times, mycelia of *R. solani* did not grow until day 1st and mycelial growth on day

2nd and 3rd decreased by 83.0 and 88.0% as compared with acetone solvent treatment, respectively. Two antimicrobial compounds against *R. solani* were found to be linolenic and caffeic acids by bioautography and GC-MS. The compounds were treated to the six phytopathogens to confirm antimicrobial activity of linolenic and caffeic acid. Linolenic acid inhibited mycelial growth against *R. solani*, while caffeic acid showed a little antimicrobial activity. Tomatine was also included in acetonic extract from tomato leaves, but its content was not enough to inhibit mycelial growth of *R. solani*. Based on the antimicrobial activity, antimicrobial compound of acetonic extract from tomato leaves was linolenic acid. The results indicated that tomato leaves might include antimicrobial compounds against strawberry and tomato pathogenic microorganisms, and the extracts could be considered as potential sources of natural antimicrobial agents.

INTRODUCTION

Over the years, many studies have been conducted to analyze plant-microorganism interactions. Plants have evolved many strategies to protect themselves against their pathogens. Among the strategies by which plants can control the phytopathogen, the induction of secondary metabolites acting as defense compounds is the most common trait. Among secondary metabolites, phytoanticipins that are preformed chemical barriers occur constitutively in plants before provocation by biotic and abiotic stresses (Lambert et al., 2011). Phenolics, terpenoids, and alkaloids make up the chemical barrier and perform locally at the

very early stages of phytopathogen attack. Plants synthesize a broad range of the secondary metabolites, many of which have a proven antimicrobial effect against pathogens (Dixon, 2001).

Since tomato fruit is subject to metabolites analyses for several decades, a huge number of compounds, representing a diversity of biosynthetic pathways and covering the entire array from volatile to highly polar and non-polar, has been identified. These biochemicals contain sugars, amino acids, organic acid, hormones, phenolics, alkaloids, fatty acids, and terpenes like carotenoids and volatile compounds. Tomato fruits may include several thousands of secondary metabolites, many of which are still unidentified. For example, using high-resolution accurate mass MS coupled to C_{18}-reversed phase LC, more than 100 secondary metabolites could be annotated in aqueous-methanol extracts of peel from ripe fruits (Moco et al., 2006), while using fruits at different ripening stages about 500 compounds were detected in the cultivar Ever (Moco et al., 2007) and more 800 compounds in the cultivar Micro-Tom (Iijima et al., 2008). In addition, secondary metabolites strongly vary between the various fruit parts like peel, pericarp, placenta, seeds, and jelly parenchyma.

Tomato plants also include their own phytoanticipins. Tomato fruits include various phenolics (naringenin, kaempferol, quercetin, myricetine, *p*-coumaric acid, vanillic acid, caffeic acid, chlorogenic acid, ferulic acid, and sinapic acid), carotenoids (lycopene, α- and β-carotene, and lutein) and alkaloids (dehydro- and α-tomatine) (Fraser et al., 1994; Friedman and Levin, 1998; Slimestad and Verheul, 2009). Chlorogenic acid is the main phenolic in tomato fruits besides flavonoids (Slimestad and Verheul, 2009). The compounds are reported to inhibit

the colonization of *Alternaria alternate* and *Sclerotinia fructigena* (Fawcett and Spencer, 1968; Wojciechowska et al., 2014). Dehydro- and α-tomatine in tomato serve as natural defenses against fungi, oomycetes, bacteria, insects, and mammals. α-Tomatine that disrupts cell membrane by lysing of liposome has been related in plant defense (Morrissey and Osbourn, 1999).

In Chapter I, 'Bacchus' tomato cultivar was found to include lycopene, β-carotene, lutein, chlorogenic acid, caffeic acid, vanillic acid, β-phellandrene, sabinene, α-terpinene, dehydro-tomatine, and α-tomatine. In addition, contents of the secondary compounds in non-edible parts such as leaves, stems, and immature green fruits were higher than those in red fruits (Kim et al., 2014). Some studies also indicated that non-edible parts of vegetables are available because they contain higher contents of secondary metabolites than edible parts. For instance, strawberry and carrot leaves include higher contents of phenolics than their edible parts (Kähkönenet et al., 1999; Maas et al., 1991). However, a few studies have reported on the activities of the metabolites from non-edible parts using various extraction methods. The objective of this study was to identify antimicrobial compounds from three organs of tomato plants above ground, including leaf, stem, and fruit and confirm antimicrobial activity of the compounds.

MATERIALS AND METHODS

Plant materials

'Bacchus' tomato plants (Monsanto Korea, Jochiwon, Korea) were transplanted in a greenhouse located in Gwangju (N 35.26°, E 126.74°) on August

19, 2011. Their leaves, stems, immature green stage fruits and red stage fruits were harvested on May 15, 2012.

Fruits were classified into two ripening stages by percentage of red color: 0%, immature green; > 90%, red. Samples at each stage were separately stored at –20^{o}C until antimicrobial and phytochemical analysis.

Extraction procedure

The extraction was performed according to the method of Mahlo et al. (2010). Finely ground materials of tomato plant sample (4 g) were extracted with 40 mL of hexane, dichloromethane, acetone, or methanol in polyester plastic tubes, while shaking strongly for 5 min on a shaker. The solvent polarity parameters of hexane, dichloromethane, acetone, and methanol were 0.1, 3.1, 5.1, and 5.1, respectively, and the solvent strength parameters for these solvents on alumina were 0.01, 0.42, 0.56, and 5.1, respectively. After centrifuging at 13,300 *g* for 5 min, the supernatants were decanted into weighed glass vials. The process was repeated three times on the marc and the extracts were mixed. The solvents were removed under a stream of cold air at room temperature.

Microorganisms

Pathogenic fungi and oomycetes of tomato plants *Colletotrichum coccodes* (KACC No. 40802), *Fusarium oxysporum* f. sp. *lycopersici* (KACC No. 40043), and *Phytophthora capsici* (KACC No. 40177) and those of strawberry plants *Glomerella cingulata* (KACC No. 40300), *Phytophthora cactorum* (KACC No. 40183), and *Rhizoctonia solani* (KACC No. 40115) were determined. They were

obtained from the Genebank Information Center, Rural Development Administration, Jeonju, Korea. All microbial strains were maintained on potato dextrose (PD) agar in a chamber maintained at 25°C.

Antimicrobial assays

Microdilution assay

A serial microdilution assay (Masoko et al., 2007) was performed to measure the minimum inhibitory concentration (MIC) values for tomato plant extracts using *p*-iodonitrotetrazolium violet reduction as an indicator of microorganism growth. This method developed only for antibacterial activities by Eloff (1998) but was later modified for antifungal activity test by Masoko et al. (2005). Residues of the four extracts were dissolved to a concentration of 10 $mg \cdot mL^{-1}$. The plant extracts of 100 μL were repeatly diluted in an 1:1 ratio by volume with distilled water in 96-well microtiter plates. Microbial cultures of 100 μL transferred into PD broth was added to each well. *p*-Iodonitrotetrazolium violet of 40 μL with 0.2 $mg \cdot mL^{-1}$ was dissolved in distilled water and was added to each of the microtiter plate wells an indicator of microbial growth. The covered microtiter plates were incubated for 2 to 3 days at 35°C and 100% relative humidity (RH). The MIC was registered as the lowest concentration of the extracts that inhibited microbial growth after 24 to 48 h.

Agar dilution method

The method used in this study was previously described by Kim et al. (2012). PD agar medium of 100 mL was made by solvents or the tomato plant extracts

adding distilled water. The mixtures were placed into petri dishes. Mycelia of the fungi and oomycetes were placed in the center of each petri dish and incubated in a growth chamber at 26^{o}C with 50% RH. The mycelial diameter was measured with a ruler for 7 days, and the two measurements were averaged.

Bioautography

Bioautography using thin layer chromatography (TLC) was performed by the method of Mahlo et al. (2010). The TLC plates were loaded with 100 μg of each of the extracts in a line 10 mm wide. The prepared plates were developed using eluent consisted of toluene:ethyl acetate:methanol:formic acid (6:4:2:1, v/v/v/v). The developed chromatograms were dried at room temperature to remove the remaining solvent. Plant pathogenic microorganisms were grown on PD agar for 7 to 10 days. The cultures were transferred into PD broth from agar with sterile swabs. The developed plates were sprayed with a concentrated suspension containing 1.0×10^{6} cells·mL^{-1} of actively growing microorganisms. After overnight incubation, the plates were sprayed with 2 mg·mL^{-1} solution of *p*-iodonitrotetrazolium violet and incubated further overnight in a chamber at 35^{o}C with 100% RH in darkness. White areas indicated where tetrazolium salts were not reduced to the colored formazan because of the presence of compounds that inhibited the growth of the test microorganisms. The plates were sealed in plastic to prevent the spreading of the microorganisms and to retain the humidity and then scanned to record of the results.

GC-MS identification

The extracts of 10 μL were spotted onto four TLC plates and developed with eluents. The three plates were sprayed with anisaldehyde-sulfuric acid, Dragendorff's reagent, natural product A spraying solution, and phosphomolybdic acid to visualize the compounds. The plates were inspected at 254 and 366 nm, and then used for GC-MS identification. The active compounds were scraped off and placed in vials with solvent. The vials were shaked for 10 min and centrifuged for separation of the compounds and silica-gel. The supernatant was collected and placed in other clean vials. After evaporation of the solvents under nitrogen gas, 100 μL of pyridine and 100 μL of N,O-bis (trimethylsilyl) trifluoro-acetamide:trimethylchlorosilane (99:1, v:v) were mixed and heated for 30 min to derivatize.

The solution of 1 μL was injected into the GC-MS under splitless mode. The GC-MS spectra were recorded on a Thermo TRACE1310 equipped with Thermo ISQ LT (Thermo Fisher Scientific, Waltham, MA, USA), operating under EI mode at 70 eV. An HP-5 MS column (30 m × 0.25 mm × 0.5 μm film thickness) was used to separate the unknown antimicrobial compounds. The temperature program was at 50°C for 2 min, followed temperature gradient of 50-325°C by 10°C·m^{-1} and at 325°C for 10 min. The injector temperature was 300°C. The flow rate of helium as carrier gas was 1.5 mL·min^{-1}. Plant extracts were also subjected to GC-MS analysis using the above-described chromatographic conditions for characterization of their composition. One μL of an aliquot from of the extracts was injected using a split ratio of 1:10 (v:v). The mass spectra were deconvoluted by AMDIS® (NIST, Gaithersburg, MD, USA) software, and the compounds identified by comparing their mass spectral fragmentation and retention time with

those of reference compounds isolated in my laboratory, supplied from other laboratories or with standard reference spectra from the database of National Instrumentation Center for Environmental Management, Seoul National University (Seoul, Korea).

HPLC conditions

Quantitative analysis of the antimicrobial compounds was performed by an HPLC system. A Zorbax ODS C-18 column (150 × 4.6 mm i.d. 5 μm; Youngjinbiochrom, Seongnam, Korea) was used for the analysis. The extract was filtered through 0.45 μm pore size of a syringe filter before injection to an HPLC apparatus (Ultimate 3000, Dionex, Sunnyvale, CA, USA). Mobile phase was consisted of 0.3% trifluoroacetic acid (phase A) and acetonitrile (phase B). Separation was carried out for 40 min under the following conditions: from 0 to 25 min, 90% (A) and 10% (B); from 25 to 30 min, 40% (A) and 60% (B); from 30 to 35 min, 100% (B); from 35 to 40 min, 90% (A) and 10% (B). The eluted components were monitored using a UV/Vis detector at 280 and 340 nm. β-Sitosterol, tyrosol, caffeic acid were detected at 210, 280, and 320 nm, respectively.

RESULTS AND DISCUSSION

Selection of the most antimicrobial extract by minimum inhibitory concentration

MIC was chosen for testing the antimicrobial activities of tomato extracts and

then determing the most active antimicrobial extract. MIC is important to confirm resistance of microorganisms to antimicrobial compounds and also to monitor the antimicrobial activity of unidentified substances (Andrews, 2001). An electron is transferred during the active growth of fungi from NADH to *p*-iodonitrotetrazolium violet resulting in a formazan dye, which is purple in color. Therefore, the clear zone on the microplate wells indicates areas of inhibition (zone where microorganisms do not actively grow). MIC values were measured by checking growth after 48 h. The MIC values of acetone extract from tomato leaves were very low (Table II-1). Previous researches also showed that plant leaves have antimicrobial activity against phytopathogens. For example, leaf extract of sweet William catchfly (*Silene armeria* L.) inhibited growth of *Botrytis cinerea*, *Colletotrichum capsici*, *Fusarioum solani*, *F. oxysporum*, *Phytophthora capsici*, and *Rhizoctonia solani* (Bajpai et al., 2008).

Among the phytopathogens, *Rhizoctonia solani* which is strawberry pathogenic fungus was the most susceptible microorganism to the acetonic extract with MIC less than 0.31 $mg \cdot mL^{-1}$ (Table II-1). Tomato leaves might contain antimicrobial compounds against *R. solani*. The candidates of antimicrobial compounds might be phenolics because acetone is a solvent for phenolics (Cowan, 1999). Osorio et al. (2010) showed that phenolic extracts of creosote bush leaves (*Larrea tridentata* Cov.) showed antifungal activities against *R. solani*. Chapter I showed that tomato leaves contained high concentration of chlorogenic acid, vanillic acid, caffeic acid, and tomatines.

In Chapter I, tomato leaves included greater content of chlorogenic acid, caffeic acid, vanillic acid, mono- and sesquiterpenes, and α- and dehydro

Table II-1. Minimum inhibitory concentrations (MIC) of extracts from tomato plants with various solvents against six pathogenic microorganisms.

	Average MIC ($mg \cdot mL^{-1}$)															
	Leaf				Internodal stem				Immature green fruit				Red fruit			
Microorganism	H[z]	D	A	M	H	D	A	M	H	D	A	M	H	D	A	M
C. coccodes	>5.00	2.50	2.50	2.50	-[z]	2.50	-	2.50	>5.00	2.50	2.50	2.50	>5.00	2.50	2.50	2.50
F. oxyspourum	2.50	1.25	1.25	1.25	-	1.25	-	2.50	2.50	1.25	2.50	5.00	1.25	1.25	2.50	2.50
G. cingulata	5.00	1.25	1.25	1.25	-	5.00	-	5.00	5.00	5.00	1.25	2.50	5.00	1.25	5.00	5.00
R. solani	1.25	1.25	<0.31	1.25	-	2.50	-	2.50	2.50	1.25	5.00	5.00	2.50	1.25	5.00	5.00
P. cactorum	>5.00	1.25	1.25	1.25	-	2.50	-	5.00	>5.00	1.25	2.50	2.50	2.50	1.25	1.25	<0.31
P. capsici	>5.00	>5.00	1.25	2.50	-	>5.00	-	>5.00	>5.00	5.00	5.00	2.50	>5.00	>5.00	2.50	2.50

[z]H, hexane; D, dichloromethane; A, acetone; M, methanol

[y]-, not obtained.

tomatines than the other parts. The secondary metabolites are known for antimicrobial compounds. Among the solvents used for leaf extraction, the acetonic extract showed lower MIC values than the other solvent extracts (Table II-1). Jayaraman et al. (2008) reported that the greater antimicrobial activity of the acetone extract might be due to the higher solubility of the extract in organic solvents. Acetone, moreover, can be a solvent used for polar and non-polar compounds because solvent strength of acetone is 5.1 that is median value of 0 of hexane and 10.2 of water. Acetonic extract from tomato leaves may contain more diverse biochemicals including antimicrobial compounds than the other solvent extracts of the other parts. Therefore, the acetonic extract from tomato leaves could be recommended as an antimicrobial agent.

Regulation effect of the extracts on mycelial growth

Acetonic extracts from tomato leaves was chosen as the most antimicrobial extract by MIC value. Thereafter, antimicrobial activity of the acetonic extract was confirmed by agar dilution method against six microorganisms with the concentration of 0.31 $mg \cdot mL^{-1}$ where the concentration showing the lowest MIC. Acetone was treated as a negative control. Acetone decreased mycelial growth of the tested six phytopathogens. The results indicated that acetone can be an antimicrobial solvent for the tested phytopathogens. Acetonic extract from tomato leaves inhibited mycelial growth of *Fusarium oxysporum*, *Glomerella cingulata*, and *Rhizoctonia solani* compared to acetone solvent treatment (Fig. II-1). *F. oxysporum* and *R. solani* are ubiquitous soil inhabitants that can colonize plant roots and stems. Therefore, acenoic extract from tomato leaves can be used as a

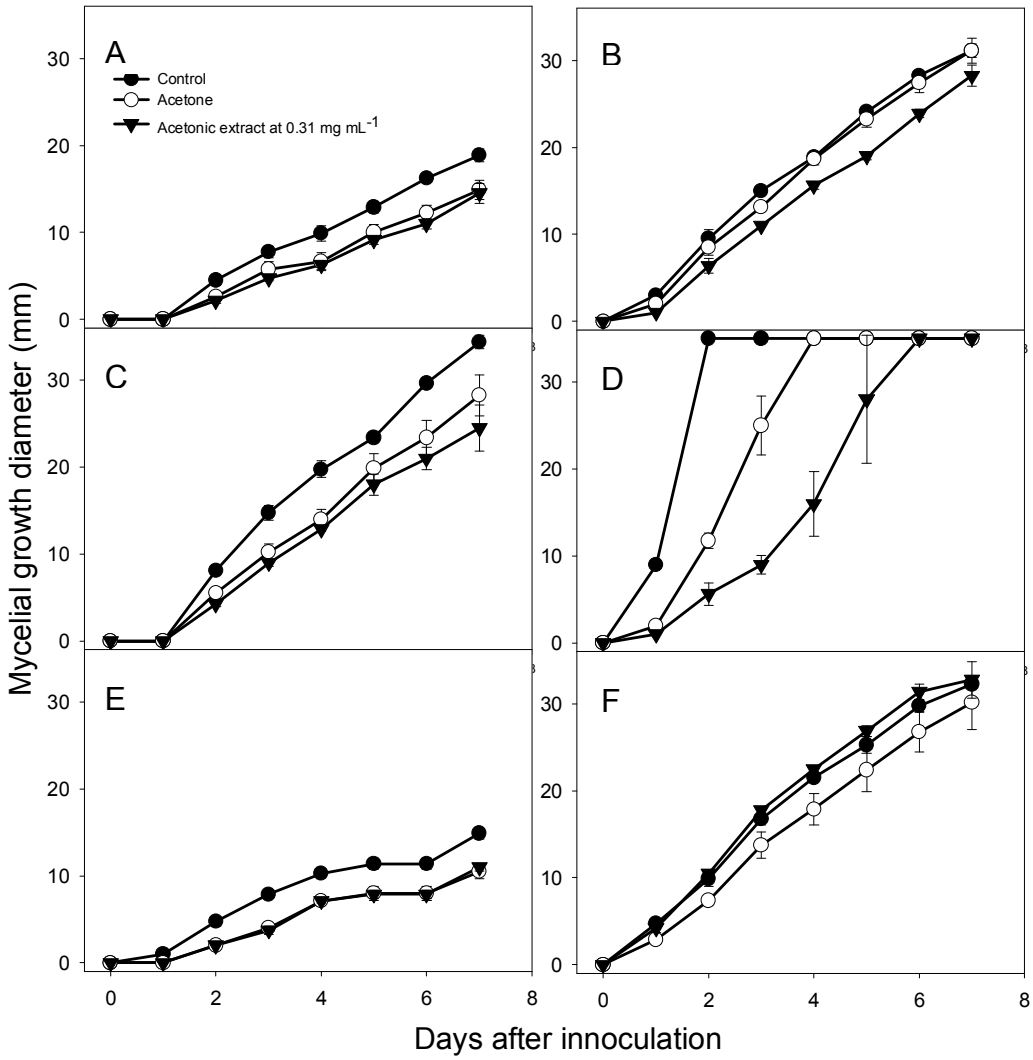

Fig. II-1. Inhibitory effect of acetonic extract from tomato leaves on mycelial growth of six microorganisms. A, *Colletotrichum coccodes*; B, *Fusarium oxyspourum*; C, *Glomerella cingulata*; D, *Rhizoctonia solani*, E, *Phytophthora cactorum*; F, *P. capsici*. Vertical bars represent standard errors of the means.

soil disinfectant. The acetonic extract showed the greatest antimicrobial activity against *R. solani* among the treatment of the solvent extracts. This is in agreement with the MIC data that MIC value of *R. solani* to the acetonic extract was lowest. With acetonic extract from tomato leaves of 0.31 $mg \cdot mL^{-1}$, mycelial growth of *R. solani* on day 1st, 2nd, and 3rd decreased by 50.0, 52.1, and 64.0% as compared with acetone solvent treatment, respectively (Fig. II-1). The result indicated that inhibitory effect of acetonic extract from tomato leaves on mycelial growth of *R. solani* increased along the time course. When *R. solani* was treated with eight times concentration of acetonic extract from tomato leaves (2.5 $mg \cdot mL^{-1}$), mycelia of *R. solani* did not grow until day 1st and mycelial growth on day 2nd and 3rd decreased by 83.0 and 88.0% as compared with acetone solvent treatment, respectively (Fig. II-2). Acetonic extract from tomato leaves did not inhibit completely mycelial growth of *R. solani*, but higher concentration of the acetone extract treatment every other day with the acetone extract may stop mycelial growth of *R. solani*. Thus, the antimicrobial compounds against *R. solani* were confirmed to exist in acetonic extract from tomato leaves.

Detection of antimicrobial compounds

Tomato leaves contained caffeic acid of 2.0 $\mu g \cdot g^{-1}$ DW and tomatine of 3,175.8 $\mu g \cdot g^{-1}$ DW that was dissolved by acetone only and chlorogenic acid of 3.3 $\mu g \cdot g^{-1}$ DW that was dissolved by methanol. However, hexanic and dichloromethanic extracts did not extract the secondary metabolites presented in tomato leaves (Table II-2). Among the secondary metabolites in the acetonic extract, tomatine may be an important antimicrobial compound because it exhibits

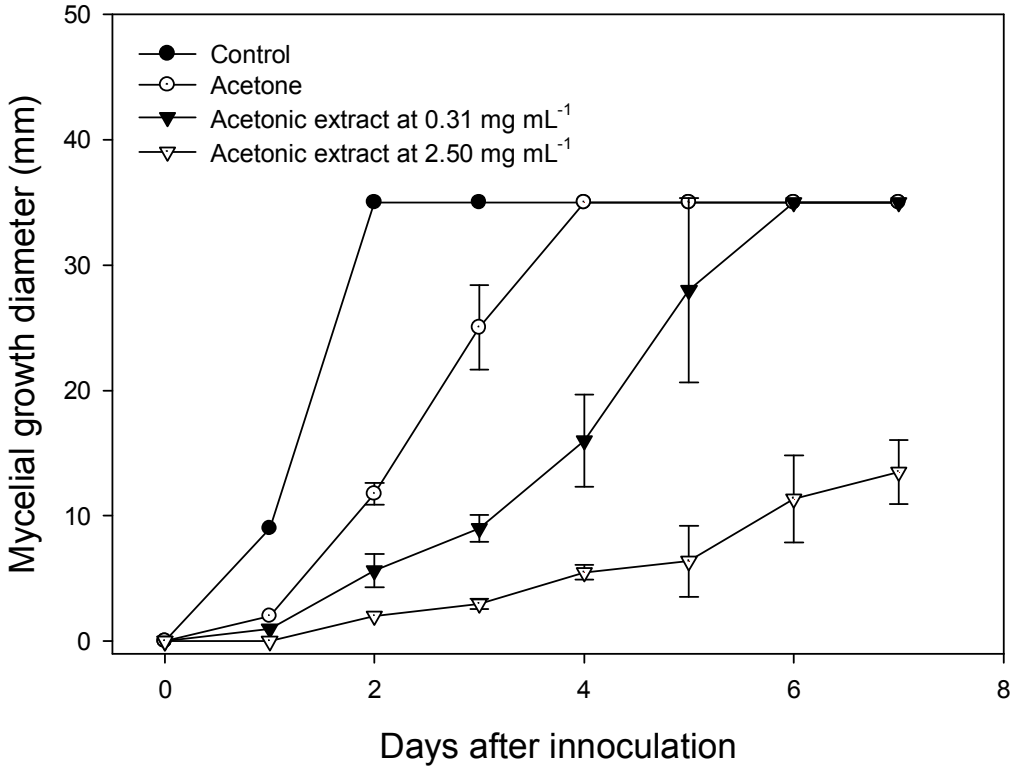

Fig. II-2. Inhibitory effect of acetonic extract from tomato leaves on mycelial growth of *Rhizoctonia solani.* Vertical bars represent standard errors of the means.

Table II-2. Contents of chlorogenic acid, caffeic acid, vanillic acid, and tomatine in hexanic, dichloromethanic, acetonic, and methanolic extracts from tomato leaves.

	Chlorogenic acid	Caffeic acid	Vanillic acid	Tomatine
Solvent	($\mu g \cdot g^{-1}$ DW)			
Hexane	-[z]	-	-	-
Dichloromethane	-	-	-	-
Acetone	-	2.0		3,195.8
Methanol	3.3	-	-	-

[z]-, not detected.

the highest content of secondary metabolite detected in the acetonic extract and is often considered as a toxic compound (Rietjens et al., 2005).

Bioautography was used to identify antimicrobial compounds from acetonic extracts of tomato plant. Inhibition zones (white area) were found in all tested microorganisms by organs of tomato plants (Fig. II-3). Acetonic extracts of tomato leaves possessed 1-3 antimicrobial compounds, but extracts of immature green fruits and red fruits contained only one compound for microbials used in this study. Lane of *R. solani*, the most susceptible microorganism to actetonic extract from tomato leaves, showed two inhibition zones. The *R. solani* inhibition spots were analyzed by GC-MS. Linolenic acid of 16.1 $mg \cdot g^{-1}$ and caffeic acid of 2.0 $\mu g \cdot g^{-1}$ were found to be the dominant compounds in the chromatogram from both inhibition zones (Fig. II-4). However, tomatine was not detected from this experimental method. Linolenic acid, acetone soluble lipid, is the major components of the total lipids in tomato leaves (Conconi et al., 1996), but is not a secondary metabolite. However, its antimicrobial activity has often been demonstrated. For instance, Walters et al. (2004) reported that linolenic acid decreased mycelial growth and fungal biomass of *R. solani*, *Pythium ultimum*, *Pyrenophora avenae*, and *Crinipellis perniciosa*. Hamberg (1999) reported that linolenic is a substrate for the production of a range of trihydroxy oxylipins, which are known to possess antifungal activity. San Francisco and Cooper-Driver (1984) confirmed that caffeic acid showed antifungal activity against fungi (*R. solani*, *Pythium debaryanum*, *P. middletonii*, and *P. ultimum*) and bacteria (*Corynebacterium poinsettiae*, *C. fasciens*, *Erwinia amylovora*, and *E. carotovora*).

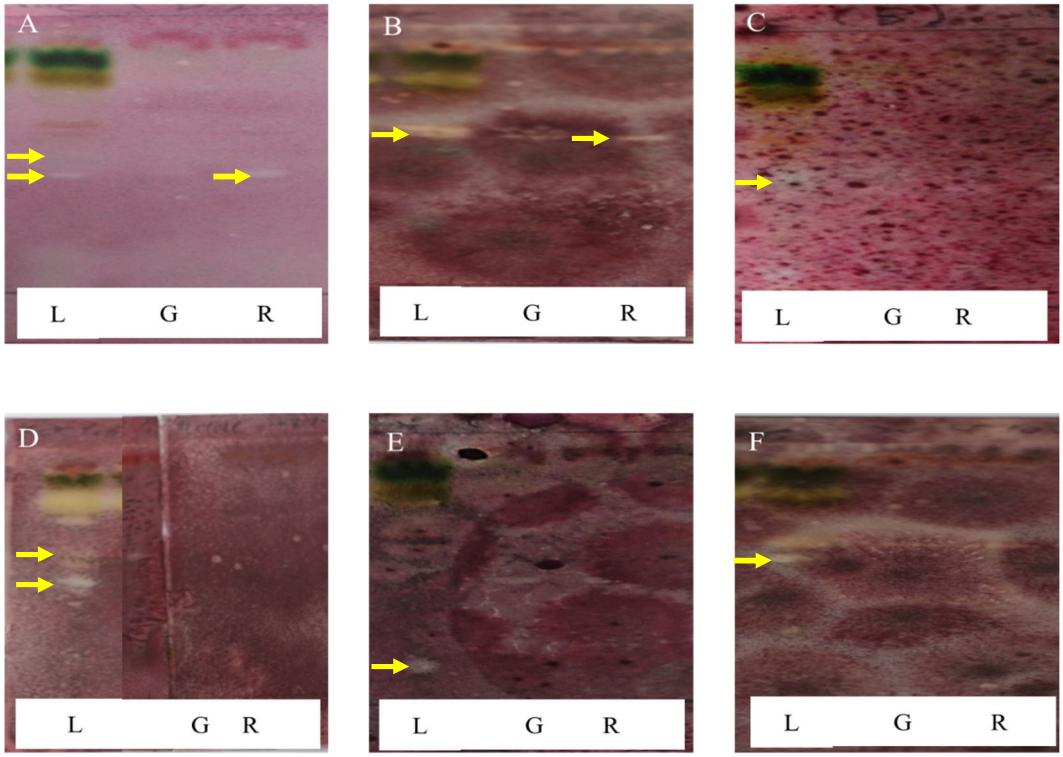

Fig. II-3. Bioautogram of acetonic extracts from leaves (L), immature green fruits (G), and red fruits (R) of tomato plants. White areas indicate inhibition of microbial growth. A, *Colletotrichum coccodes*; B, *Fusarium oxyspourum*; C, *Glomerella cingulata*; D, *Rhizoctonia solani*; E, *Phytophthora cactorum*; F, *P. capsici*.

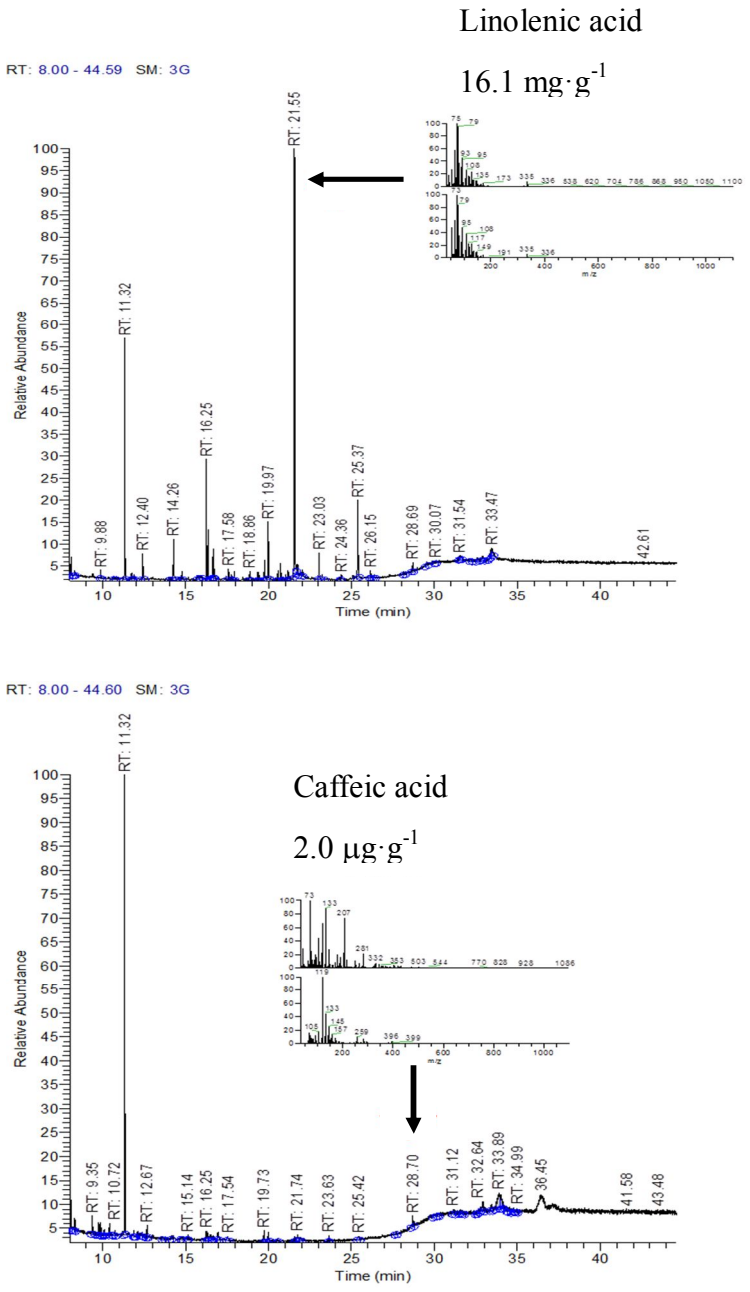

Fig. II-4. GC-MS chromatogram of preparative TLC-isolated compounds in acetonic extract from tomato leaves.

To confirm the antimicrobial compounds, acetonic extract from tomato leaves was developed on a TLC plate and then the plate was sprayed with phosphomolybdic acid or anisaldehyde-sulfuric acid. Linolenic and caffeic acids were visualized on a TLC plate and located to the same line with the antimicrobial compounds (Fig. II-5), but burnamonine, hexadecanoic acid, hexanedioic acid, hexanoic acid, inositol, levoglucosan, octadecanoic acid, pentonic acid, pregnane, and propanoic acid which were detected by GC-MS were not visualized by phosphomolybdic acid or anisaldehyde-sulfuric acid, and not located to the same line with the antimicrobial compounds (data not shown). The results indicated that linolenic and caffeic acids from the tomato extract are the antimicrobial compound against *R. solani*. Therefore, the acetonic extract from tomato leaves was confirmed to contain marker compounds for commercialization.

Antimicrobial activity of caffeic acid, chlorogenic acid, linolenic acid, and tomatine detected in extract from tomato leaves

The antimicrobial compounds of tomato leaves against *R. solani* were compared and confirmed to be linolenic and caffeic acids. To determine the mycelial inhibition by the compounds, linolenic and caffeic acid of 0.01, 0.1, and 1 $mg \cdot mL^{-1}$ were treated. Chlorogenic acid, vanillic acid, and tomatine which were detected in acetonic and methanolic extract from tomato leaves were also treated on the microorganisms. With linolenic acid of 1 $mg \cdot mL^{-1}$, mycelial growth of *R. solani* on day 3rd was reduced by 54.5% as compared with acetone solvent treatment. Caffeic acid showed little antimicrobial activity against *R. solani* (Fig. II-6). Walters et al. (2004) reported that linolenic acid of 100 and

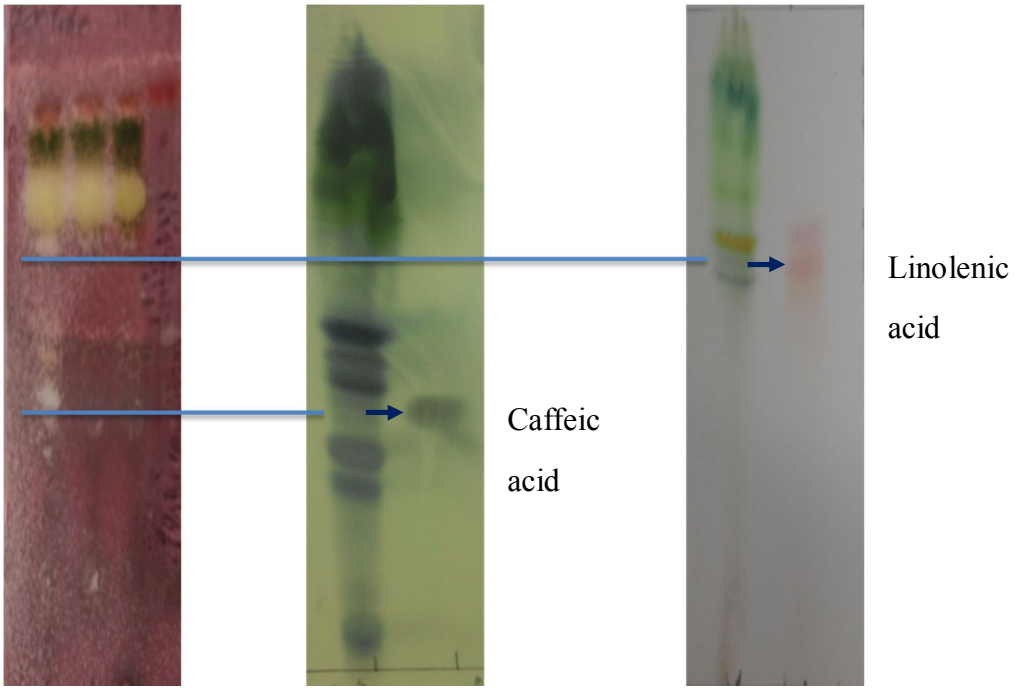

Fig. II-5. Linolenic and caffeic acids visualized on TLC using phosphomolybdic acid and anisaldehyde-sulfuric acid.

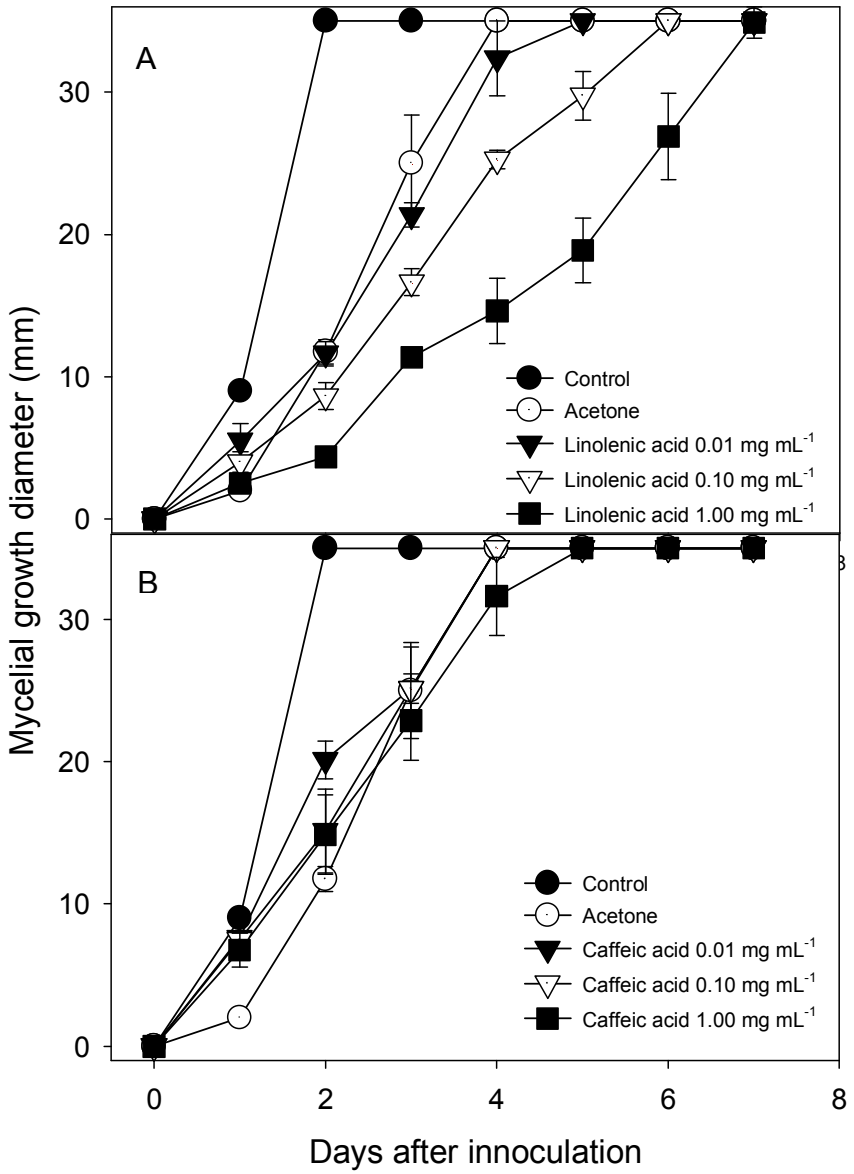

Fig. II-6. Inhibitory effect of linolenic (A) and caffeic acids (B) on mycelial growth of *Rhizoctonia solani*. Vertical bars represent standard errors of the means.

1,000 μM decreased mycelial growth of *R. solani* by 51.2 and 69.8%, respectively. Francisco and Cooper-Driver (1984) confirmed that caffeic acid showed antifungal activity against *R. solani*.

Content of tomatine in tomato leaves was 3,195.8 $\mu g \cdot g^{-1}$, and content of tomatine in acetonic extract from tomato leaves of 0.31 $mg \cdot mL^{-1}$ was estimated to be approximately 0.03 $mg \cdot mL^{-1}$. Tomatine detected in acetonic extract from tomato leaves inhibited mycelial growth of *R. solani* from 0.01 $mg \cdot mL^{-1}$. With tomatine of 0.01 and 0.1 $mg \cdot mL^{-1}$, mycelial growth of *R. solani* on day 3rd was reduced by 24.3% and 63.5% as compared with acetone solvent treatment, respectively. Mycelial growth of *R. solani* was completely inhibited by tomatine of 1 $mg \cdot mL^{-1}$ for 1 week (Fig. II-7). With vanillic acid of 1 $mg \cdot mL^{-1}$, mycelial growth of *R. solani* on day 3rd was reduced by 41.3% as compared with acetone solvent treatment (Fig. II-7). Korukluoglu et al. (2007) reported that vanillic acid has antifungal activity against *Alternaria* spp., *Fusarium* spp., and *Penicillium* spp. However, vanillic acid was not detected in acetonic extract from tomato leaves. Chlorogenic acid detected in methanolic extract from tomato leaves was major phenolic of 'Bacchus' tomato, but its antimicrobial activity against *R. solani* was not strong (Fig. II-7). Antimicrobial activity of antimicrobial compounds included in acetonic extract from tomato leaves was compared to the acetone extract. Contents of caffeic acid, tomatine, and linolenic acid (CTL) in acetone extract from tomato leaves 0.31 $mg \cdot mL^{-1}$ were approximately 1.0 $\mu g \cdot mL^{-1}$, 3.0 $mg \cdot mL^{-1}$, and 6.0 $mg \cdot mL^{-1}$ (yield: 3%), respectively. Inhibitory effect of CTL on mycelial growth of *R. solani* was lower than that of the acetone extract (Fig. II-8). The result indicated that acetone extract from tomato leaves may contain other

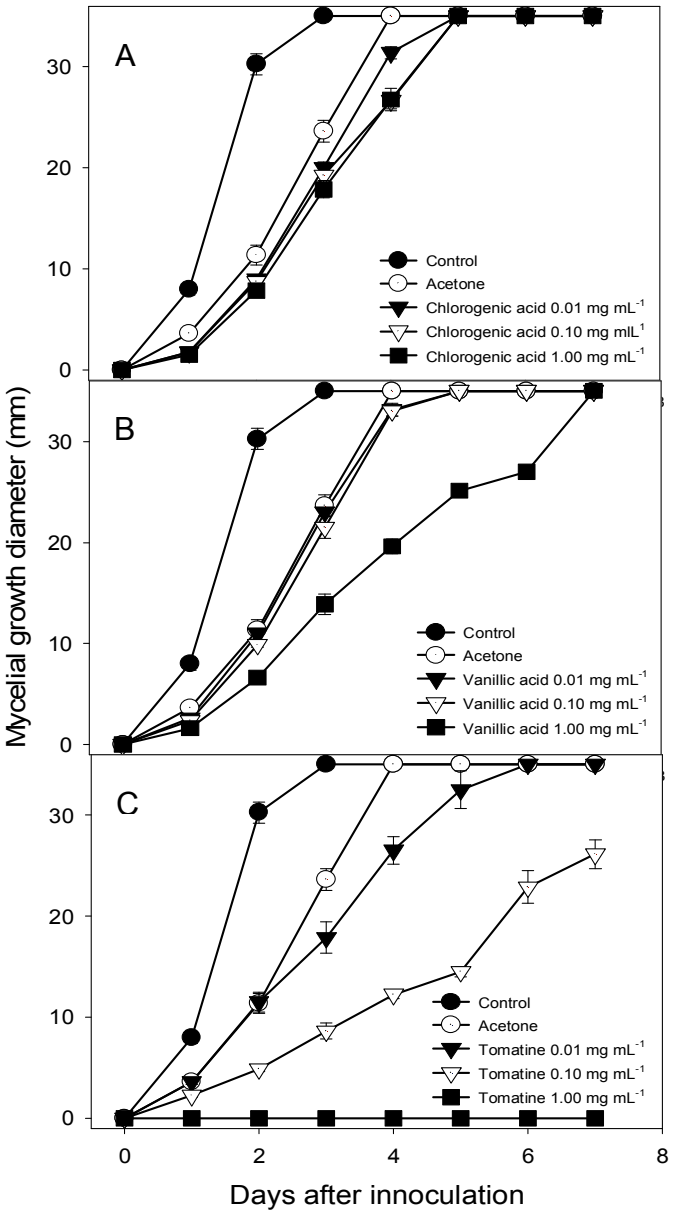

Fig. II-7. Inhibitory effect of chlorogenic acid (A), vanillic acid (B), and tomatine (C) on mycelial growth of *Rhizoctonia solani*. Vertical bars represent standard errors of the means.

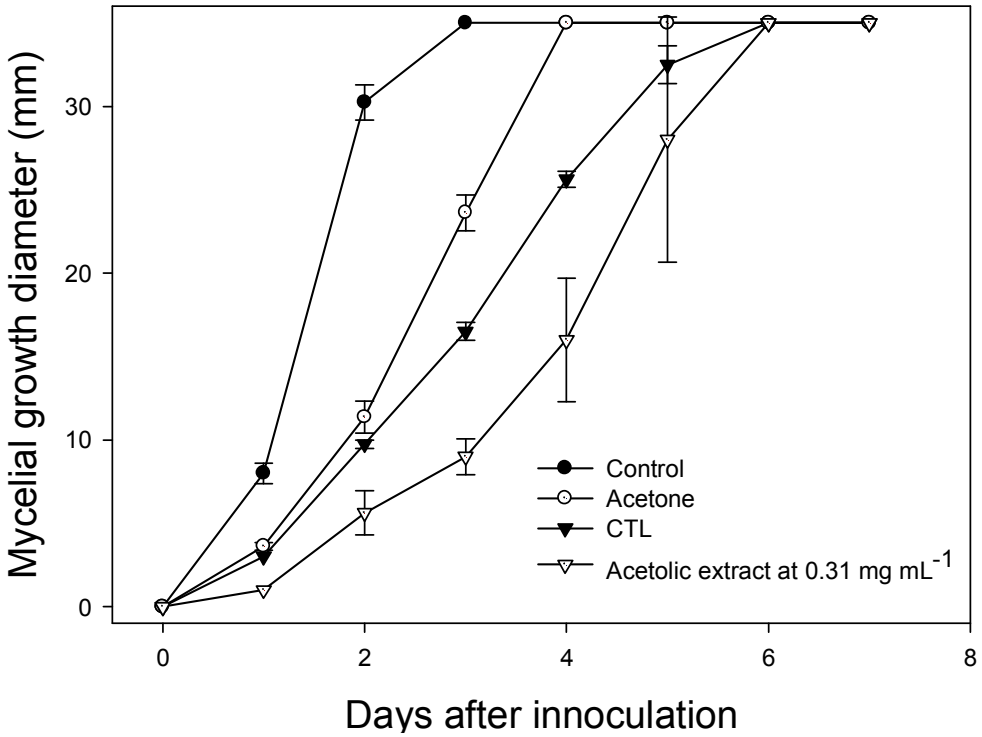

Fig. II-8. Inhibitory effect of antimicrobial compounds on mycelial growth of *Rhizoctonia solani*. Vertical bars represent standard errors of the means. CTL, caffeic acid 1.0 μg·mL^{-1} + tomatine 3.0 mg·mL^{-1} + linolenic acid 6.0 mg·mL^{-1}.

antimicrobial compounds. Consequently, combined effect of several compounds in the acetone extract can be expected although the individual compounds vary greatly with respect to biological effect.

In MIC tests, acetonic extract from tomato leaves had the highest antimicrobial activity against tested phytopathogenic microorganisms. The result indicated that acetone was the best solvent, since it extracted active antimicrobial compounds from tomato plants and tomato leaves have promising antimicrobial activity. Among the tested phytopathogens, mycelial growth of *Rhizoctonia solani* was greatly inhibited by acetonic extract from tomato leaves. Based on bioautography results, linolenic and caffeic acids were antimicrobial compounds against *R. solani*. Linolenic acid, especially, was more active compound than other secondary metabolites included in tomato leaves. This study showed the potential of tomato leaves as sources of extracts or pure compounds with activity against *R. solani*. Profiling and MIC results showed that the other parts of tomato plant also had antimicrobial activity due to the presence of antimicrobial compounds although their activities were comparatively lower. Results suggested that the tomato leaves, trashed after training braches and at the end of cultivation, with other aerial parts could be potential sources for natural antimicrobial agents.

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CHAPTER III

Secondary Metabolites Profiling in Various Parts of Strawberry Plants

ABSTRACT

The objective of this study was to identify content of phenolic and volatile organic compounds in edible and non-edible parts of 'Seolhyang' strawberry plants. I performed a comparative chemical analysis of the compounds found in roots, leaves, petioles, runners, and green and red fruits during vegetative propagation and reproductive growth. Contents of ellagic and gallic acid in leaves of runner plants during vegetative propagation were 7.4 and 5.1 $mg \cdot g^{-1}$ FW, respectively, and were higher than that in the other parts. The main volatile organic compound was identified as 3-hexen-1-ol, and it was mostly detected in leaf parts. Content of ellagic acid in leaves during reproductive growth was 13.0 $mg \cdot g^{-1}$ FW, while that in the other parts was below 6 $mg \cdot g^{-1}$ FW. Content of gallic acid in green fruits was 2.8 $mg \cdot g^{-1}$ FW and was higher than that in the other parts. Red fruits contained the lowest contents of ellagic and gallic acids but contained the most diverse volatile organic compounds, including sesquiterpenes, among the tested plant parts. The results indicate that non-edible parts (e.g., leaves and green fruits) of strawberry plants can be used as a raw material for antioxidant and anti-

inflammatory agents, and edible parts (i.e., ripe fruits) can be available for making an essential oil.

INTRODUCTION

Plants contain a wide variety of phenolics, and approximately 10,000 individual compounds have been identified in various plants (Fiehn, 2001). The phenolics range from simple molecules such phenolic acids to highly polymerized compounds such as tannins. Those compounds, reported to have various biological effects, are important for both plant and animal health (Dixon et al., 1999, 2002; Dixon and Sumner, 2003). Strawberry (*Fragaria* × *ananassa* Duch.) fruit is a valuable vegetable to intake various nutrients such as vitamins, minerals, and phenolics (Hannum, 2004; Meyers et al., 2003; Prior and Cao, 2000). Machiex et al. (1990) reported that strawberry fruits contained innumerable phenolics including hydroxycinnamic acid derivatives, hydroxybenzoic acid derivatives, flavonols, flavanols, anthocyanins, and tannins. Especially, ellagic acid, which is a phenolic lactone compound (dimeric derivative of gallic acid), possessed 51% of total phenolics in red strawberry fruits (Häkkien et al., 2000).

The volatile organic compounds emitted from plant tissues are not only fundamental flavor and fragrance constituents, as an indicator of ripeness and an attractant of pollinators, but also antimicrobial agents against pathogenic fungi and bacteria (Arroyo et al., 2007). These volatile compounds can be extracted from a variety of parts of plants, such as buds, fruits, seeds, leaves, peel, wood,

and roots. They are generally classified into 12 groups including organic acids, aldehydes, ketones, alcohols, esters, lactones, sulfur compounds, acetals, furans, phenols, terpenes, and epoxides (Aharoni et al., 2004), whereas the metabolic pathways of many volatile compounds were not fully studied. More than 300 volatile compounds including methyl butanoate, ethyl butanoate, methyl hexanoate, hexyl acetate and ethyl hexanoate have been identified in red strawberry fruits (Zabetakis and Holden, 1997).

The majority of these studies has been focused on the edible parts only and, therefore, little information is available on contents of phenolic and volatile compounds in the non-edible parts (e.g., leaf, petiole, runner, root, and green fruit) of strawberry plants. Various non-edible parts of strawberry plants, which has a harvest index below 30 (Fernandez et al., 2001), are dumped after last harvest. Those non-edible parts of vegetables include higher contents of phenolic or volatile compounds than edible parts do. For example, the leaf of carrot was reported to contain 12 times higher contents of total phenolic acid than the root did (Kähkönen et al., 1999), and the leaf of red spring onion contained kaempferol, which was not found in the edible bulb (Nuutila et al., 2002). With these research background, the objective of the present study was to identify contents of secondary metabolites which can be used as raw materials for antioxidant and anti-flammatory agents and essential oils from edible and non-edible parts of strawberry plants.

MATERIALS AND METHODS

Plant materials

'Seolhyang' strawberry plants were grown in a greenhouse located in Suwon, Korea (E 127.0^{o}, N 37.3^{o}). The strawberry plants were grown in 800 × 300 × 200 mm (L × W × H, outside) plastic containers filled with a mixture (1:1, v/v) of peatmoss (BM-4, Berger Peat moss, Quebec, Canada) and perlite (Part No. 3, Kyung Dong Ceratech, Seoul, Korea). The plants were fertigated with 450 mL d^{-1}/plant with Yamasaki nutrition solution (N; 5.5 me·L^{-1}, P; 1.5 me·L^{-1}, K; 3.0 me·L^{-1}, Ca; 2.0 me·L^{-1}, Mg; 1.0 me·L^{-1}, S; 1.0 me·L^{-1}) using an automatic drip fertigation system (pH; 5.8-6.2, EC; 1.4-1.6 dS m^{-1}). Roots, unfolded leaves, and petioles of mother plants, runners, and folded leaves of runner plants were harvested on Sep. 3, 2011 (when only the first runner plants were produced and before the second runner plants); unfolded leaves, petioles, and green and red fruits were harvested on Nov. 16, 2012.

The strawberry fruits were classified into two ripening stages by percentage of red color: 0% red color (Green); 100% red color (Red). Then, surface color of the classified fruits was measured using a chromameter (CR-300, Minolta Camera Co., Osaka Japan) and chromaticity a* (red-green) in the CIE scale was measured on a representative point at the middle of the fruit height from three fruits. Samples at each stage were separately stored at –20^{o}C until phytochemical analysis.

Analysis of phenolics

Phenolics were extracted and hydrolyzed according to the procedure of Nuutila et al. (2002). Different parts (200 mg FW) of strawberry plants were homogenized with 5 mL of 1.2 M HCl in 50% (v/v) aqueous methanol. As an antioxidant, ascorbic acid of 8 mg was added to the hydrolysis mixture and this process was performed in triplicate. After refluxing at 80^{o}C for 2 h, the extract was cooled, filled up to 10 mL, and sonicated for 3 min. The extract was filtered through a 0.45 μm syringe filter before injection to an HPLC system (Ultimate 3000, Dionex, Sunnyvale, CA, USA). A Zorbax SB C-18 column (150 × 4.6 mm, i.d., 5 μm, Agilent, New York, NY, USA) used. The mobile phase was programmed as 0-60% gradient in 25 min of methanol in water containing 300 mL·L^{-1} trifluoroacetic acid, eluted at a flow rate of 0.8 mL·min^{-1}. The eluted components were monitored at 280 and 340 nm using a UV/Vis detector. Analytical standards were *trans*-cinnamic acid, *p*-coumaric acid, caffeic acid, gallic acid, ellagic acid, 2,5-hydrobenzoic acid, 3,4-hydrobenzoic acid, kaempferol, and quercetin, which were purchased from Sigma-Aldrich Co. (St. Luis, MO, USA) and *p*-hydrobenzoic acid was purchased from Samchun Pure Chemical Co. (Pyeongtaek, Korea).

Analysis of volatile organic compounds

Volatile organic compounds were measured using the method of Isleten and Karagül-Yüceer (2008). The sample gas was collected by Tenax-TA (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA), the dynamic thermal extractor chamber system, which is composed of a 26 mL glass tube and the air control

system, including an air supply unit and pumps. Purified nitrogen gas (39 mL min^{-1}) was used for ventilation. Volatile organic compounds in various parts of strawberry plants were analyzed by thermal desorption gas chromatograph mass spectrometer (TDS-GC MSD and TDS2, Gerstel GmbH, KG, Mülheim, Germany; 6890N and 5975, Agilent Technologies, Santa Clara, CA, USA).

Statistical analysis

Statistical analyses were performed using the SAS statistical software, release 9.2 (SAS Inst., Cary, NC, USA). Dunkan's multiple range test was used to assess differences in contents of ellagic and gallic acids at $P \leq 0.05$.

RESULTS AND DISCUSSION

Contents of phenolics

Parts of 'Seolhyang' strawberry plants during vegetative propagation showed significant differences in content of ellagic and gallic acid. Other phenolics, however, were not detected. Häkkinen et al. (1998) reported that extraction and hydrolysis method influence the diversity of the phenolics extracted from strawberry fruits. Contents of ellagic acid were 2.3, 4.3, 2.7, 3.7, and 7.4 $mg \cdot g^{-1}$ FW in roots, leaves and petioles of mother plants, runners, and leaves of runner plants, respectively (Table III-1). Content of ellagic acid in leaves and petioles of mother plants was 2-3 times lower than that in leaves and petioles of strawberry plants during reproductive growth (Tables III-1 and III-2). Contents of gallic acid

Table III-1. Contents of phenolics in different parts of 'Seolhyang' strawberry plants during vegetative propagation.

	Ellagic acid	Gallic acid
Part	($mg \cdot g^{-1}$ FW)	
Root of mother plant	2.3 d[z]	0.9 b
Leaf of mother plant	4.3 b	2.1 b
Petiole of mother plant	2.7 cd	1.3 b
Runner	3.7 bc	1.6 b
Leaf of runner plant	7.4 a	5.1 a

[z]Mean separation within columns by Duncan's multiple range test at $P = 0.05$.

Table III-2. Contents of phenolics in different parts of 'Seolhyang' strawberry plants during reproductive growth.

	Ellagic acid	Gallic acid
Part	($mg \cdot g^{-1}$ FW)	
Leaf	13.0 a[z]	1.9 b
Petiole	5.4 b	0.9 c
Green fruit	5.1 bc	2.8 a
Red fruit	2.0 d	0.4 d

[z]Mean separation within columns by Duncan's multiple range test at $P = 0.05$.

were 0.9, 2.1, 1.3, 1.6, and 5.1 $mg \cdot g^{-1}$ FW in roots, leaves and petioles of mother plants, runners, and leaves of runner plants, respectively (Table III-1). The results indicated that contents of ellagic and gallic acid in leaves of runner plants were higher than those in other parts. The difference of contents of the phenolics between leaves of mother and runner plants may be the cause of leaf age (unfolded or folded). Wang and Lin (2000) reported that young leaves (from the upper part of shoots or stems) of strawberry plants contained higher content of total phenolics than old leaves (from the lower part of shoots or stems). Nantitanon et al. (2010) found that young leaves (from the apex of the branch) of guava (*Psidium guajava* L.) contained higher contents of ellagic and gallic acids than old leaves (from the branch position close to the stem) did. Young leaves of Arabidopsis also have very diverse secondary metabolites compared to old leaves, presumably due to the re-allocation of metabolites to reduce feeding by herbivores (Brown et al., 2003).

Parts of 'Seolhyang' strawberry plants during reproductive growth showed significant differences in contents of ellagic and gallic acid. Hunter's a* value of green and red fruits was −13.2 and 36.6, respectively. Contents of ellagic acid were 13.0, 5.4, 5.1, and 2.0 $mg \cdot g^{-1}$ FW in leaves, petioles, and green and red fruits fruits, respectively (Table III-2). Content of ellagic acid in leaves and fruits of 'Seolhyang' strawberry plants was higher than in those of other cultivars. The red fruits, moreover, included higher ellagic acid than those of same cultivar (Kim et al., 2011). The results showed that different hydrolysis conditions significantly change content of ellagic acid obtained (da Silva Pinto et al., 2008). Content of

ellagic acid was higher in vegetative organs than in reproductive organs. Maas et al. (1991) found that content of ellagic acid in leaves of 35 strawberry cultivars was higher than that in green and red fruits fruits. Blackberry and raspberry also showed similar trend (Wang and Lin, 2000). Among the vegetative organs, leaves contained higher content of ellagic acid than petioles. There were significant differences among reproductive organs. Content of ellagic acid in green fruits was 2.5 times higher than that in red fruits. Previous studies have indicated that, in most cases, content of ellagic acid in green fruit were higher than that in red fruit (Fait et al., 2008; Maas et al., 1991; Williner et al., 2004). Content of gallic acid was 1.9, 0.9, 2.8, and 0.4 $mg \cdot g^{-1}$ FW in leaves, petioles, and green and red fruits, respectively (Table III-2). Content of gallic acid, in each part, was lower than content of ellagic acid. Content of gallic acid in green fruits was 7.9 times higher than that in red fruits. This was in agreement with previous research for content of gallic acid in strawberries (Fait et al., 2008). The results indicated that red fruits, the edible part of strawberry plants, presented lower values for contents of ellagic and gallic acid than the other parts. Fait et al. (2008) also observed that contents of ellagic and gallic acid decreased through the fruit development process.

Ellagic and gallic acid is derived from ellagitannin. Some researchers reported that tannin is a feeding repellent comound because ingestion of tannins can have strong negative effects on herbivorous insects. For example, Feeny (1968) showed that the inhibitory effect of oak tannin on the growth of the winter moth (*Operophtera brumata*) was correlated with tannin levels. Ayres et al. (1997) reported that tannin of *Betula resinifera* Britt. had a significant dosage effect on

the growth of *Chrysomela falsa* Brown and tannin of *Populus tremuloides* Michx. had a significant dosage effect on the growth of *Pyrrhalta luteola*. These examples can explan why leaves and green fruits include higher ellagic and gallic acid than red fruits. Leaves and green fruits protect themselves from herbivores while red fruits, except achenes, allow to be fed to their pulp for seed spread. Maas et al. (1991) reported that more ellagic acid was found in achenes than in fruit pulp.

Contents of volatile organic compounds

'Seolhyang' strawberry plants during vegetative propagation included 32 volatile organic compounds. Contents of total volatile organic compounds were 90, 782, 45, 38, and 916 $\mu g \cdot g^{-1}$ FW in roots, leaves and petioles of mother plants, runners, and leaves of runner plants, respectively (Table III-3 and III-4). The 3-hexen-1-ol, a naturally occurring alcohol, was the predominant volatile organic compound in the leaves of mother and runner plants; however, it was not detected in roots and petioles of mother plants. Leaves of mother and runner plants included two sesquiterpenes such as *E*,*E*-α-farnesene and *trans*-caryophyllene which are not found in leaves during reproductive growth. *E*,*E*-α-Farnesene that is biosynthesized via the mevalonate pathway (Pechous and Whitaker, 2004) is a floral volatile organic compound and is known as the predominant terpene produced during storage of apple fruits (Huelin and Murray, 1966). However, in this study, content of *E*,*E*-α-farnesene in leaves of mother plants was 2.6 times higher than that in red fruits. In addition, monoterpene (δ-3-carene; 20 $mg \cdot m^{-3}$)

Table III-3. Contents of volatile organic compounds (hydrocarbon fraction compounds) in different parts of 'Seolhyang' strawberry plants during vegetative propagation.

	Ben[z]	Bi	δCa	Ca	1Ch	Cyo	Do	Eo	Etl	Etr	Fa	Hd	Ht	Me
Part	($\mu g \cdot g^{-1}$ FW)													
Root of mother plant	-[y]	-	-	-	-	24	18	-	6	20	-	-	-	4
Leaf of mother plant	-	-	20	15	-	-	-	3	-	-	153	3	6	-
Petiole of mother plant	3	-	-	-	4	-	-	-	-	-	-	-	-	6
Runner	3	6	-	-	-	-	-	-	-	-	-	-	-	-
Leaf of runner plant	-	-	-	4	-	-	-	-	-	-	59	-	-	-

(continued)

Part	MC	Oc	Ot	Pd	Pn	Td
	($\mu g \cdot g^{-1}$ FW)					
Root of mother plant	-	-	-	-	-	-
Leaf of mother plant	4	5	30	-	57	-
Petiole of mother plant	3	-	-	6	-	9
Runner	6	-	-	-	-	-
Leaf of runner plant	3	-	16	-	22	-

[z]Ben, benzene; Bi, bicyclo[4.3.0]nona-3,7-diene; δCa, δ-carene; Ca, *trans*-caryophyllene; 1Ch, 1-chlorohepta-fluorobut-2-ene; Cyo, cyclohexanol; Do, 2,6-dimethyl-1,3,5,7-octatetraene; Eo, *Z* and *E* epoxy-ocimene; Etl, ethanol; Etr, ether; Fa, *E*,*E*-α-farnesene; Hd, heptadecane; Ht, *Z*-3-hexenyl tiglate; Me, methane; MC, methylene chloride; Oc, octadecane; Ot, 1,3,6-octatriene; Pd, pentadecane; Pn, 4-pentenal; Td, tetradecane.

[y]-, not detected.

Table III-4. Contents of volatile organic compounds (oxygenated fraction compounds) in different parts of 'Seolhyang' strawberry plants during vegetative propagation.

Part	A[z]	Chd	De	Fu	1He	2He	3Ho	Mi	My	Na	Phe	Pr
	($\mu g \cdot g^{-1}$ FW)											
Root of mother plants	-[y]	7	-	-	-	-	-	4	-	-	-	7
Leaf of mother plant	4	-	3	65	27	19	346	-	4	6	12	-
Petiole of mother plant	-	-	-	-	-	-	-	-	-	3	11	-
Runner	-	-	-	-	-	-	8	-	-	5	3	-
Leaf of runner plant	5	-	-	-	14	13	777	-	-	3	-	-

[z]A, acetic acid; Chd, 1,4-cyclohexanedimethanol; De, decanal; Fu, furan; 1He, 1-hexene; 2He, 2-hexenal; 3Ho, 3-hexen-1-ol; Mi, 4,7-methano-1H-inden-1-ol; My, 6-methyl-5-hepten-2-one; Na, nonanal; Phe, phenol; Pr, propanoic acid.

[y]-, not detected.

was only detected in leaves of mother plants. The results showed that leaves of runner plants contained the highest contents of total volatile organic compounds, and leaves of mother plants contained diverse volatile organic compounds.

'Seolhyang' strawberry plants during reproductive growth included 36 volatile organic compounds. Contents of total volatile organic compounds were 84, 86, 154, and 6,949 $\mu g \cdot g^{-1}$ FW in leaves, petioles, and green and red fruits, respectively (Table III-5 and 6). These results were directly opposite to content of ellagic acid in parts of strawberry plants during reproductive growth. Carboxylic acids such as acetic acid (45.40%) and butanoic acid (34.81%) were the predominant volatile organic compounds in the red fruits, while they were detected only in red fruits. On the other hand, red fruits of 'Houkouwase' and 'Suhong' cultivars mainly included butylbenzene (18.96%) and *E*-nerolidol (12.38%), respectively (Park et al., 2000). Furthermore, red fruits of 'Seolhyang' strawberry plants included monoterpene (δ-3-carene; 40 $mg \cdot m^{-3}$) and sesquiterpene (*E*,*E*-α-farnesene; 60 $mg \cdot m^{-3}$), which were not found in the other parts. Fruit tissues of strawberry showed strong upregulated expression of some terpene synthase gene homologs in the receptacle tissue during ripening; an RNA gel blot research confirmed the increase in the terpene synthase transcript levels during fruit ripening, whereas no expression could be detected in leaf tissue (Aharoni et al., 2004). Volatile organic compounds in *Nicotiana attenuata* Torr. leaves such as *cis*-3-hexen-1-ol, *cis*-3-hexenyl acetate, and *cis*-3-hexenyl butyrate derived from the octadecanoid path way are known to be emitted rapidly after

Table III-5. Contents of volatile organic compounds (hydrocarbon fraction compounds) in different parts of 'Seolhyang' strawberry plants during reproductive growth.

	Ben[z]	Bu	2Bu	δCa	Cs	Fa	Hb	Ib	Mcr	Mt	O	Pen	Pa	Td	To
Part	($\mu g \cdot g^{-1}$ FW)														
Leaf	10	6	-	-	-	-	-	-	-	-	14	-	-	-	-
Petiole	-[y]	-	-	-	-	-	-	-	-	-	-	-	-	-	6
Green fruit	-	-	-	-	-	-	-	-	-	-	6	-	-	7	-
Red fruit	-	-	30	40	8	60	9	10	6	20	-	25	10	-	-

[z]Ben, benzene; Bu, butane; 2Bu, 2-butanone; δCa, δ-carene; Cs, cyclotrisiloxane; D, disulfide; Fa, *E*,*E*-α-farnesene; Hb, *E*-2-Hexenyl butanoate; Ib, isopropyl butanoate; Mcr, methyl crotonate; Mt, methyl thiolacetate; O, octane; Pen, 2-pentanone; Pa, n-propyl acetate; Td, tetradecane; To, toluene.

[y]-, not detected.

Table III-6. Contents of volatile organic compounds (oxygenated fraction compounds) in different parts of 'Seolhyang' strawberry plants during reproductive growth.

	A[z]	1Bo	2Bo	Bu	Da	DA	Hpn	Hxa	Hxn	2He	2Ho	3Ho	My	Na	Noa
Part	($\mu g \cdot g^{-1}$ FW)														
Leaf	-[y]	-	10	-	13	-	-	-	-	-	-	-	-	23	-
Petiole	-	-	-	-	10	-	-	-	-	-	-	-	-	22	-
Green fruit	-	-	11	-	19	-	-	-	-	-	-	26	9	38	-
Red fruit	3,155	10	-	2,419	15	7	14	421	53	238	113	-	-	28	11

(continued)

Part	Oa	Phe	Pr	Sa
	($\mu g \cdot g^{-1}$ FW)			
Leaf	-	9	-	-
Petiole	-	-	-	-
Green fruit	6	26	-	6
Red fruit	-	11	109	-

[z] A, acetic acid; 1Bo, 1-butanol; 2Bo, 2-butanol; Bu, butanoic acid; De, decanal; DA, decanoic acid; Hpn, 4-heptanone; Hxa, hexanoic acid; Hxn, hexanal; 2He, 2-hexenal; 2Ho, 2-hexen-1-ol; 3Ho, 3-hexen-1-ol; My, 6-methyl-5-hepten-2-one; Na, nonanal; Noa, nonanoic acid; Oa, octanal; Phe, phenol; Pr, propanoic acid; Sa, sulfurous acid.

[y] -, not detected.

damage by hervibores (Kessler and Baldwin, 2001). As an indirect defense, the release of volatile organic compounds is known to attract parasitoids and predators to actively feeding larvae. Leaves of mother and runner plants also included more 3-hexen-1-ol than other volatile organic compounds. The result indicates that leaves of 'Seolhyang' strawberry plant may mainly release 3-hexen-1-ol after hervibore attack. Hamilton-Kemp et al. (2003) have reported the increase of 2-hexenal production in fruit tissues from the activation of lipoxygenase and hydroperoxide lyase pathway in response to phytopathogen and hervibore attack. Arroyo et al. (2007) reported that 2-hexenal were the most effective to inhibit the mycelial growth of *Colletotrichum acutatum* and decreased the development of symptoms in red fruits of 'Camarosa' strawberry plant inoculated with *C. acutatum*. Red fruits of 'Seolhyang' strawberry plant also contained high level of 2-hexenal. The result indicated that 2-hexenal could provide an alternative to chemical fungicides to control infections on strawberry fruits, as a fungistat.

Ellagic and gallic acids in strawberry fruits have been known to have antimutagenic, antioxidant, and anti-inflammatory activities in mammalian systems. In addition, volatile organic compounds in strawberry fruits can be considered as antiherbivore and antimicrobial agent (Arroyo et al., 2007; Kessler and Baldwin, 2001). However, product yield of strawberry fruits was insufficient to meet a demand. I hence confirmed that non-edible parts of strawberry plants included higher phenolic and volatile organic compounds than the other parts did, regardless of season. Thus, after production of fruits or runner plants was finished,

non-edible parts (e.g., leaves and green fruits) of strawberry plants could be used as a raw material for antioxidant or anti-inflammatory agents, and edible parts (i.e., red fruits) could be available for making an essential oil.

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CHAPTER IV

Antimicrobial Activity of Extracts from Various Parts of Strawberry Plants

ABSTRACT

The objective of this chapter was to confirm antimicrobial activity of acetone, hexane, dichloromethane, and methanol extracts from leaves, petioles, and green and red fruits of 'Seolhyang' strawberry plants. The antimicrobial activity of strawberry extracts was tested against six phytopathogens including *Colletotrichum coccodes, Fusarium oxysporum*, *Glomerella cingulate*, *Phytophthora cactorum*, *P. capsici*, and *Rhizoctonia solani*. Minimum inhibitory concentration (MIC) of methanol extracts was lower than those of the other solvents. Strawberry leaves had higher antimicrobial activity than the other parts against tested phytopathogens. Methanolic extract from strawberry leaves, hence, was selected as the source of antimicrobial substance. Methanolic extract from strawberry leaves inhibited mycelial growth of *Colletotrichum coccodes*, *Glomerella cingulate*, and *Rhizoctonia solani.* Mycelial growth of *Colletotrichum coccodes* showed more susceptibility to the methanolic extract than to the other phytopathogens. With 0.31 $mg \cdot mL^{-1}$ of methanolic extract from strawberry leaves, mycelial growth of *C. coccodes* on day 7th was reduced by 39.5% as compared with methanol solvent treatment. When the amount of methanolic extract from

strawberry leaves was used double and 8 times, mycelia of C. coccodes did not grow until day 2nd and 7th, respectively. The result indicated that methanolic extract from strawberry leaves showed strong antimicrobial activity against *Colletotrichum*. The methanolic extract was treated to find out the inhibition effect on growth of *C. acutatum, C. caudatum, C. dematium, C. higginsianum, C. musae, C. liliacearum, C. lindemuthianum, C. orbiculare,* and *C. truncatum*. Methanolic extract from strawberry leaves showed antifungal activities against seven *Colletotrichum* spp., except *C. acutatum* and *C. dematium*. The two antimicrobial compounds against *C. coccodes* were found to be tyrosol and β-sitosterol by bioautography and GC-MS. The compounds were treated to the six phytopathogens to confirm antimicrobial activity of tyrosol and β-sitosterol. Tyrosol inhibited mycelial growth against *C. cocodes*, while β-sitosterol showed a little antimicrobial activity. Ellagic acid was also included in methanolic extract from strawberry leaves, but its content was not enough to inhibit mycelial growth of *C. coccodes*. Based on the antimicrobial activity, antimicrobial compound of methanolic extract from strawberry leaves was tyrosol. The results indicate that strawberry leaves might include antimicrobial compounds against strawberry and tomato pathogenic microorganisms, and the extracts can be considered as potential sources of natural antimicrobial agents.

INTRODUCTION

Plants have evolved enormous strategies to protect themselves against their

pathogens. Among the strategies by which plants can control the phytopathogen, the induction of secondary metabolites acting as defense compounds is the most common trait. Among secondary metabolites, phytoanticipins that are preformed chemical barriers occur constitutively in plants before provocation by biotic and abiotic stresses (Lambert et al., 2011). Phenolics, terpenoids, and alkaloids make up the chemical barrier and perform locally at the very early stages of phytopathogen attack. Plants synthesize a broad range of the secondary metabolites such as phenolics, terpenoids, and alkaloids, many of which have a proven antimicrobial effect against pathogens (Dixon, 2001).

Strawberry plants also include their own phytoanticipins. Strawberry fruits contain ellagic acid, quercetin, kaempferol, gallic acid, proanthocyanins, catechin, and (*E*)-hex-2-enal. Gallic acid showed antifungal activities against *Botrytis cinerea* and *Fusarium solani* (Amil-Ruiz et al., 2011; Nguyen et al., 2013). Moreover, some unidentified compounds which were extracted from strawberry leaves and green fruits showed antifungal activities against *Botrytis cinerea*, *Cladosporium cladosporioides*, and *Colletotrichum fragariae* (Amil-Ruiz et al., 2011).

In Chapter III, 'Seolhyang' strawberry cultivar included ellagic and gallic acid, *E*,*E*-α-farnesene, and *trans*-caryophyllene. In addition, contents of the secondary compounds in non-edible parts such as leaves, petioles, and green fruits are higher than those in red fruits (Kim et al., 2013). Some studies also indicated that non-edible parts of vegetables are available because they contain higher contents of secondary metabolites than edible parts. For instance, the leaves of strawberry and

carrot include higher contents of phenolics than edible parts (Kähkönen et al., 1999; Maas et al., 1991) The previous researches, however, have studied antimicrobial activities of secondary metabolites in red fruits of strawberry plants but a few studies have reported the activities of the metabolites in non-edible parts using various extraction methods. The objective of this study was to identify antimicrobial compounds in three organs from strawberry plants above ground, including leaf, petiole, and fruit and confirm antimicrobial activity of the compounds.

MATERIALS AND METHODS

Plant materials

'Seolhyang' strawberry plants were grown in a greenhouse located in Suwon, Korea (N 37.3°, E 127.0°). Their leaves, petioles, and green and red fruits were harvested on Nov. 16, 2012.

Fruits were classified into two ripening stages by percentage of red color: 0%, green; > 90%, red. Samples at each stage were stored separately at –20^{o}C until antimicrobial and phytochemical analysis.

Extraction procedure

The extraction was performed according to the method of Mahlo et al. (2010). Finely ground materials of strawberry plant sample (4 g) were extracted with 40 mL of hexane, dichloromethane, acetone, or methanol in polyester plastic tubes,

while shaking strongly for 5 min on a shaker. The solvent polarity parameters of hexane, dichloromethane, acetone, and methanol were 0.1, 3.1, 5.1, and 5.1, respectively, and the solvent strength parameters for these solvents on alumina were 0.01, 0.42, 0.56, and 5.1, respectively. After centrifuging at 13,300 *g* for 5 min, the supernatants were decanted into weighed glass vials. The process was repeated three times on the marc and the extracts were mixed. The solvents were removed under a stream of cold air at room temperature.

Microorganisms

Pathogenic fungi and oomycetes of tomato plants *Colletotrichum coccodes* (KACC No. 40802), *Fusarium oxysporum* f. sp. *lycopersici* (KACC No. 40043), and *Phytophthora capsici* (KACC No. 40177) and those of strawberry plants *Glomerella cingulata* (KACC No. 40300), *Phytophthora cactorum* (KACC No. 40183), and *Rhizoctonia solani* (KACC No. 40115) were determined. Methanolic extract from strawberry leaves was only treated to nine species of *Colletotrichum* (*C. acutatum, C. caudatum, C. dematium, C. higginsianum, C. musae, C. liliacearum, C. lindemuthianum, C. orbiculare,* and *C. truncatum*) after the test,. They were obtained from the Genebank Information Center, Rural Development Administration, Jeonju, Korea. All microbial strains were maintained on potato dextrose (PD) agar in a chamber maintained at 25^{o}C.

Antimicrobial assays

Microdilution assay

A serial microdilution assay (Masoko et al., 2007) was performed to measure the minimum inhibitory concentration (MIC) values for strawberry plant extracts using *p*-iodonitrotetrazolium violet reduction as an indicator of microorganism growth. This method developed only for antibacterial activities by Eloff (1998) but was later modified for antifungal activity test by Masoko et al. (2005). Residues of the four extracts were dissolved to a concentration of 10 $mg \cdot mL^{-1}$. The plant extracts of 100 μL were repeatly diluted in an 1:1 ratio by volume with distilled water in 96-well microtiter plates. Microbial cultures of 100 μL transferred into PD broth was added to each well. *p*-Iodonitrotetrazolium violet of 40 μL with 0.2 $mg \cdot mL^{-1}$ was dissolved in distilled water and was added to each of the microtiter plate wells an indicator of microbial growth. The covered microtiter plates were incubated for 2 to 3 days at 35°C and 100% relative humidity (RH). The MIC was registered as the lowest concentration of the extracts that inhibited microbial growth after 24 to 48 h.

Agar dilution method

The method used in this study was previously described by Kim et al. (2012). PD agar medium of 100 mL was made by solvents or the strawberry plant extracts adding distilled water. The mixtures were placed into petri dishes. Mycelia of the fungi and oomycetes were placed in the center of each petri dish and incubated in a growth chamber at 26°C with 50% RH. The mycelial diameter was measured with a ruler for 7 days, and the two measurements were averaged.

Bioautography

Bioautography using thin layer chromatography (TLC) was performed by the method of Mahlo et al. (2010). The TLC plates were loaded with 100 μg of each of the extracts in a line 10 mm wide. The prepared plates were developed using eluent consisted of toluene:ethyl acetate:methanol:formic acid (6:4:2:1, v/v/v/v). The developed chromatograms were dried at room temperature to remove the remaining solvent. Plant pathogenic microorganisms were grown on PD agar for 7 to 10 days. The cultures were transferred into PD broth from agar with sterile swabs. The developed plates were sprayed with a concentrated suspension containing 1.0×10^6 cells·mL^{-1} of actively growing microorganisms. After overnight incubation, the plates were sprayed with 2 mg·mL^{-1} solution of *p*-iodonitrotetrazolium violet and incubated further overnight in a chamber at 35^{o}C with 100% RH in darkness. White areas indicated where tetrazolium salts were not reduced to the colored formazan because of the presence of compounds that inhibited the growth of the test microorganisms. The plates were sealed in plastic to prevent the spreading of the microorganisms and to retain the humidity and then scanned to record of the results.

GC-MS identification

The extracts of 10 μL were spotted onto four TLC plates and developed with eluents. The three plates were sprayed with anisaldehyde-sulfuric acid, Dragendorff's reagent, natural product A spraying solution, and phosphomolybdic acid to visualize the compounds. The plates were inspected at 254 and 366 nm,

and then used for GC-MS identification. The active compounds were scraped off and placed in vials with solvent. The vials were shaked for 10 min and centrifuged for separation of the compounds and silica-gel. The supernatant was collected and placed in other clean vials. After evaporation of the solvents under nitrogen gas, 100 μL of pyridine and 100 μL of N,O-bis (trimethylsilyl) trifluoroacetamide:trimethylchlorosilane (99:1, v:v) were mixed and heated for 30 min to derivatize.

The solution of 1 μL was injected into the GC-MS under splitless mode. The GC-MS spectra were recorded on a Thermo TRACE1310 equipped with Thermo ISQ LT (Thermo Fisher Scientific, Waltham, MA, USA), operating under EI mode at 70 eV. An HP-5 MS column (30 m × 0.25 mm × 0.5 μm film thickness) was used to separate the unknown antimicrobial compounds. The temperature program was at 50°C for 2 min, followed temperature gradient of 50-325°C by 10°C·m^{-1} and at 325°C for 10 min. The injector temperature was 300°C. The flow rate of helium as carrier gas was 1.5 mL·min^{-1}. Plant extracts were also subjected to GC-MS analysis using the above-described chromatographic conditions for characterization of their composition. One μL of an aliquot from of the extracts was injected using a split ratio of 1:10 (v:v). The mass spectra were deconvoluted by AMDIS® (NIST, Gaithersburg, MD, USA) software, and the compounds identified by comparing their mass spectral fragmentation and retention time with those of reference compounds isolated in my laboratory, supplied from other laboratories or with standard reference spectra from National Instrumentation Center for Environmental Management (Seoul, Korea) database.

HPLC conditions

Quantitative analysis of the antimicrobial compounds was performed by an HPLC system. A Zorbax ODS C-18 column (150 × 4.6 mm i.d. 5 μm; Youngjinbiochrom, Seongnam, Korea) was used for the analysis. The extract was filtered through 0.45 μm pore size of a syringe filter before injection to an HPLC apparatus (Ultimate 3000, Dionex, Sunnyvale, CA, USA). Mobile phase was consisted of 0.3% trifluoroacetic acid (phase A) and acetonitrile (phase B). Separation was carried out for 40 min under the following conditions: from 0 to 25 min, 90% (A) and 10% (B); from 25 to 30 min, 40% (A) and 60% (B); from 30 to 35 min, 100% (B); from 35 to 40 min, 90% (A) and 10% (B). The eluted components were monitored using a UV/Vis detector at 280 and 340 nm. β-Sitosterol, tyrosol, and caffeic acid were detected at 210, 280, and 320 nm, respectively.

RESULTS AND DISCUSSION

Selection of the most antimicrobial extract by minimum inhibitory concentration

MIC was chosen for testing the antimicrobial activities of strawberry extracts and then determing the most active antimicrobial extract. MIC is important to confirm resistance of microorganisms to antimicrobial compounds and also to monitor the antimicrobial activity of unidentified substances (Andrews, 2001). An

electron is transferred during the active growth of fungi from NADH to *p*-iodonitrotetrazolium violet resulting in a formazan dye, which is purple in color. Therefore, the clear zone on the microplate wells indicates areas of inhibition (zone where microorganisms do not actively grow). MIC values were measured by checking growth after 48 h. The MIC values of extracts of strawberry leaves were very low. Methanolic extract from strawberry leaves showed lower MIC values than the other solvent extracts of leaves (Table IV-1). Among the phytopathogens, *Colletotrichum coccodes*, tomato pathogenic fungus, was the most susceptible microorganism to the methanolic extract with MIC less than 0.31 $mg \cdot mL^{-1}$ (Table IV-1). Saponin-rich extract of Mohave yucca (*Yucca schidigera* L.) and essential oils of *Salvia gilliessi* Benth., *Satureja parvifolia* (Phil.) Epl., *Lippia polystachya* Gris., and *Lippia junelliana* (Mold.) Tronc. also inhibited growth of *C. coccodes* (Chapagain et al., 2007; Zygadlo and Grow, 1995). Essential oil of lemongrass (*Cympopogon citratus* L.), phenolic extracts of pecan nut shell (*Carya Illinoensis* L.), pomegranate husk (*Punica granatum* L.), and creosote bush leaves (*Larrea tridentata* Cov.) showed antifungal activities against both *R. solani* and *C. coccodes* (Osorio et al., 2010). Terpenoid contained in essential oil and phenolics extracted from leaves and reproductive organs of plants might include antimicrobial compounds against *C. coccodes*. The candidates of antimicrobial compounds might be phenolics or terpenoids because methanol is a solvent for phenolics and terpenoids (Cowan, 1999). Chapter III showed that strawberry leaves contained high concentration of ellagic and gallic acids and mono- and sesquiterpenes.

Table IV-1. Minimum inhibitory concentrations (MIC) of extracts from strawberry plants with various solvents against six phytopathogenic microorganisms.

	Average MIC ($mg \cdot mL^{-1}$)															
	Leaf				Petiole				Green fruit				Red fruit			
Microorganism	H[z]	D	A	M	H	D	A	M	H	D	A	M	H	D	A	M
C. coccodes	>5.00	2.50	2.50	<0.31	>5.00	2.50	2.50	0.63	>5.00	2.50	2.50	0.63	>5.00	2.50	2.50	0.31
F. oxyspourum	1.25	1.25	2.50	1.25	1.25	1.25	2.50	2.50	1.25	1.25	5.00	2.50	1.25	1.25	>5.00	2.50
G. cingulata	>5.00	1.25	1.25	1.25	>5.00	2.50	2.50	5.00	5.00	5.00	>5.00	2.50	5.00	5.00	5.00	5.00
R. solani	2.50	2.50	<0.31	<0.31	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	>5.00	5.00	>5.00	>5.00
P. cactorum	>5.00	<0.31	1.25	<0.31	>5.00	1.25	5.00	1.25	>5.00	2.50	1.25	1.25	5.00	2.50	5.00	1.25
P. capsici	>5.00	>5.00	5.00	1.25	>5.00	5.00	5.00	2.50	>5.00	5.00	5.00	1.25	>5.00	>5.00	5.00	2.50

[z]H, hexane; D, dichloromethane; A, acetone; M, methanol

Strawberry leaves included higher content of ellagic acid and gallic acid than the other parts as shown in the results in chaper III. The secondary metabolites are known for an antimicrobial compound. Moreover, solvent strength of methanol is 5.1, the same as acetone, which has median value of 0 of hexane and 10.2 of water. The result means that methanol can be a solvent used for polar and non-polar compounds. Methanol extract from strawberry may contain more diverse biochemicals including antimicrobial compounds than the other solvent extracts because methanol can extract both polar and non-polar compounds. Therefore, the methanolic extract from strawberry leaves could be recommended as an antimicrobial agent.

Regulation effect of the extracts on mycelial growth

Methanolic extract from strawberry leaves was chosen as the most antimicrobial extract by MIC value. Thereafter, antimicrobial activity of the methanolic extract was confirmed by agar dilution method against six microorganisms with the concentration of 0.31 $mg \cdot mL^{-1}$ where the concentration showing the lowest MIC. Methanol was treated as a negative control. Methanol decreased mycelial growth of the tested six phytopathogens. The results indicated that methanol can be an antimicrobial solvent for the tested phytopathogens. The methanolic extract from strawberry leaves inhibited mycelial growth of *Colletotrichum coccodes*, *Glomerella cingulate*, and *Rhizoctonia solani* compared to acetone solvent treatment (Fig. IV-1). *Colletotrichum* (sexual stage: *Glomerella*) is a genus of fungi that are symbionts to plants as endophytes or phytopathogens.

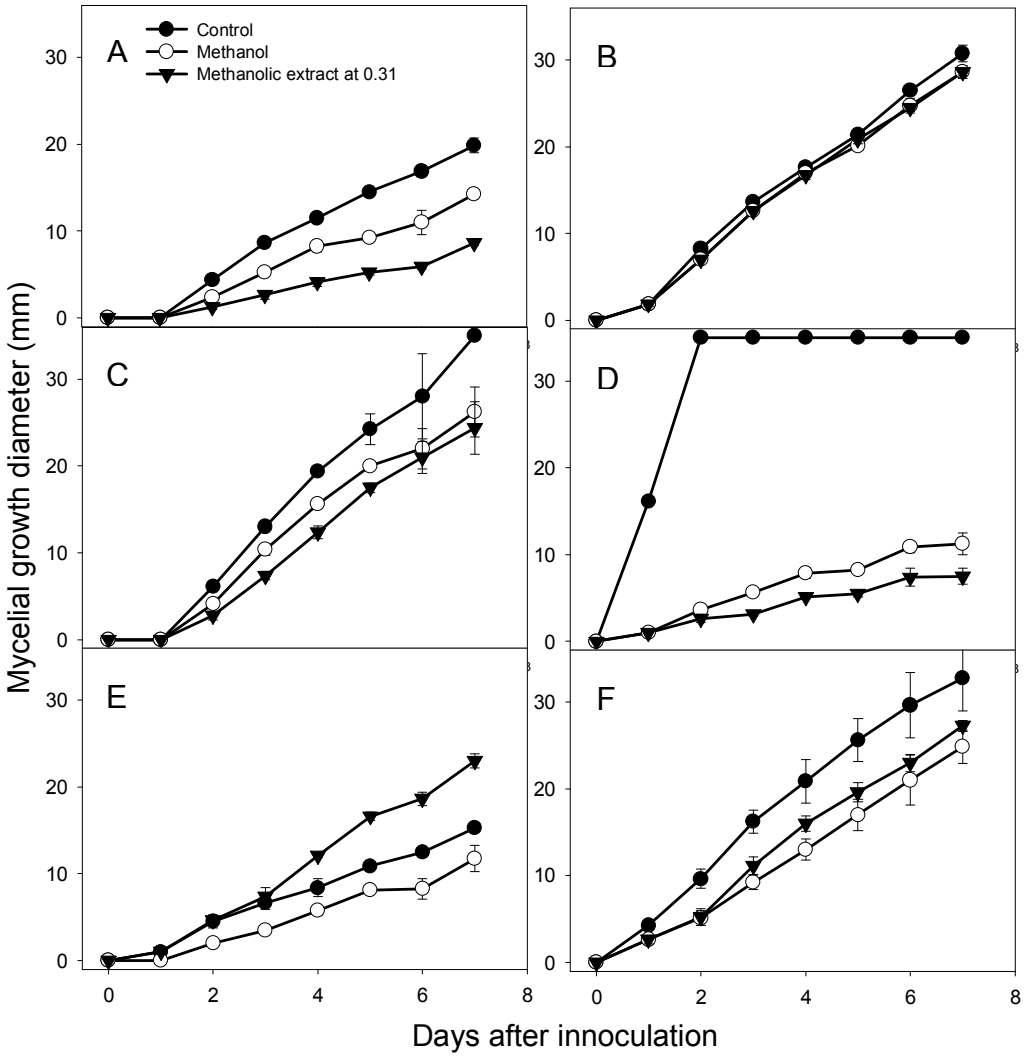


Fig. IV-1. Inhibitory effect of methanolic extract from strawberry leaves on mycelial growth of six microorganisms. A, *Colletotrichum coccodes*; B, *Fusarium oxyspourum*; C, *Glomerella cingulata*; D, *Rhizoctonia solani*, E, *Phytophthora cactorum*; F, *P. capsici*. Vertical bars represent standard errors of the means.

Colletotrichum spp., causing damping off, anthracnose, pruning wound die-back, leaf lesions, and stem dieback, can colonize aerial parts of plant. Therefore, methaolic extract from strawberry leaves can be used as a natural fungicide for *Colletotrichum* control of tomato and strawberry plants. The methaolic extractshowed the greatest antimicrobial activity against *C. coccodes* among the treatment of the solvent extracts. This is in agreement with MIC data that MIC value of *C. coccodes* to the methaolic extract was lowest. Methanolic extract from strawberry leaves showed definitely reduction of mycelial growth of *C. coccodes*. With methanolic extract from strawberry leaves of 0.31 $mg \cdot mL^{-1}$, mycelial growth of *C. coccodes* on day 7th was reduced by 39.5% as compared with methanol solvent treatment (Fig IV-1).

When *C. coccodes* was treated with double and eight times concentration of methanolic extract from strawberry leaves (0.63 and 2.5 $mg \cdot mL^{-1}$), mycelia of *C. coccodes* did not grow until day 2nd and 7th, respectively (Fig. IV-2). The results were important because complete inhibition concentration (CIC) of the methanolic extract against mycelia of *C. coccodes* was confirmed. The CIC can be used as guide line to develop natural fungicide using strawberry leaves. Zygadlo and Grow (1995) also reported that essential oils of *Salvia gilliessi* Benth., *Satureja parvifolia* (Phil.) Epl., *Lippia polystachya* Gris., and *Lippia junelliana* (Mold.) Tronc. inhibited completely mycelial growth of *C. coccodes*. *Colletotrichum* spp. is one of the most ordinary and important genera of phytopathogenic fungi. Virtually every plants grown throughout the world is susceptible to one or more species of *Colletotrichum* (Dean et al., 2012). Thus, the

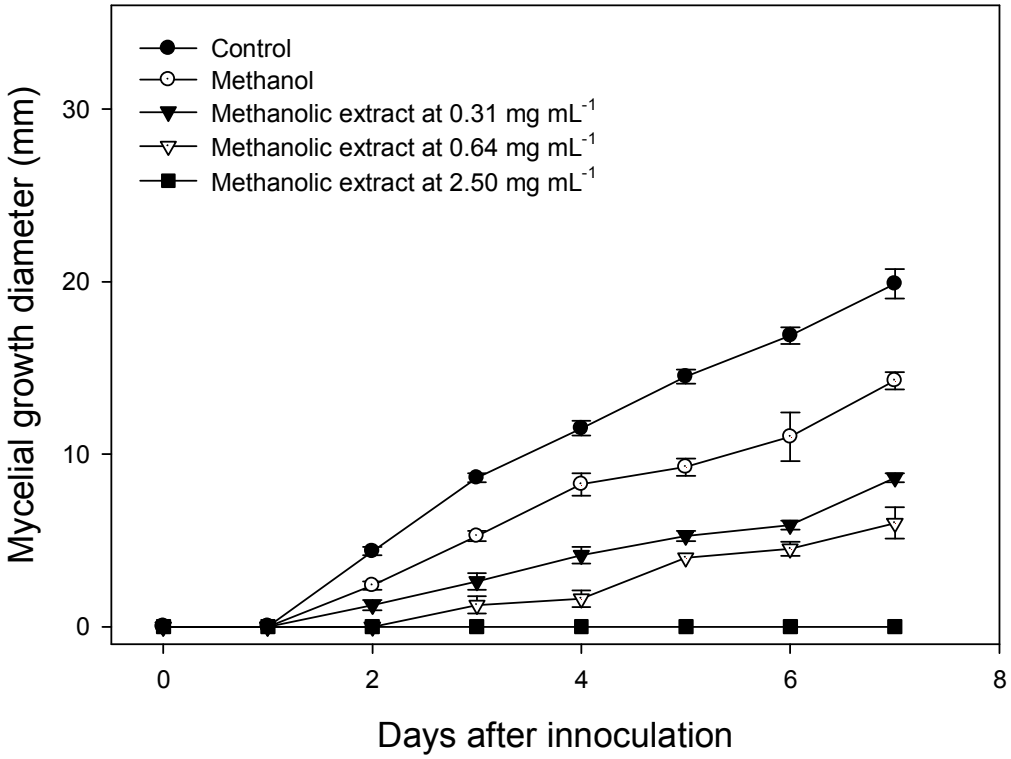


Fig. IV-2. Inhibitory effect of methanolic extract from strawberry leaves on mycelial growth of *Colletotrichum coccodes*. Vertical bars represent standard errors of the means.

antimicrobial compounds against *C. coccodes* were confirmed to exist in methanolic extract from strawberry leaves.

Methanolic extract from strawberry leaves did have antimicrobial activity against two *Colletotrichum* spp. in my research. Hence, there is a need to confirm antimicrobial test of the methanolic extract against other *Colletotrichum* spp.. I treated the methanolic extract to *C. acutatum, C. caudatum, C. dematium, C. higginsianum, C. musae, C. liliacearum, C. lindemuthianum, C. orbiculare,* and *C. truncatum*. Methanolic extract from strawberry leaves showed antimicrobial activities against seven *Colletotrichum* spp., except *C. acutatum* and *C. dematium* (Fig. IV-3). Especially, *C. caudatum* and *C. higginsianum* were more susceptible to the extract than the others. *C. acutatum* and *C. dematium* whose mycelial growth were not inhibit by methanolic extract from strawberry leaves are phytopathogens of strawberry (Freeman and Shalev, 2002; Yoshida and Shirata, 1999) indicating that the *Colletotrichum* spp. can overcome the biochemical defence by secondary metabolites in strawberry leaves.

Detection of antimicrobial compounds

Strawberry leaves were confirmed to include ellagic and gallic acid (results in Chapter III). Strawberry leaves contained ellagic acid of 14.5 $\mu g \cdot g^{-1}$ DW that was dissolved by methanol only. Gallic acid did not detected when methanol was used to dissolve. Hexanic, dichloromethanic, and acetonic extracts did not extract the secondary metabolites presented in strawberry leaves (Table IV-2). Ellagic acid may have an important antimicrobial activity because it is often considered as an

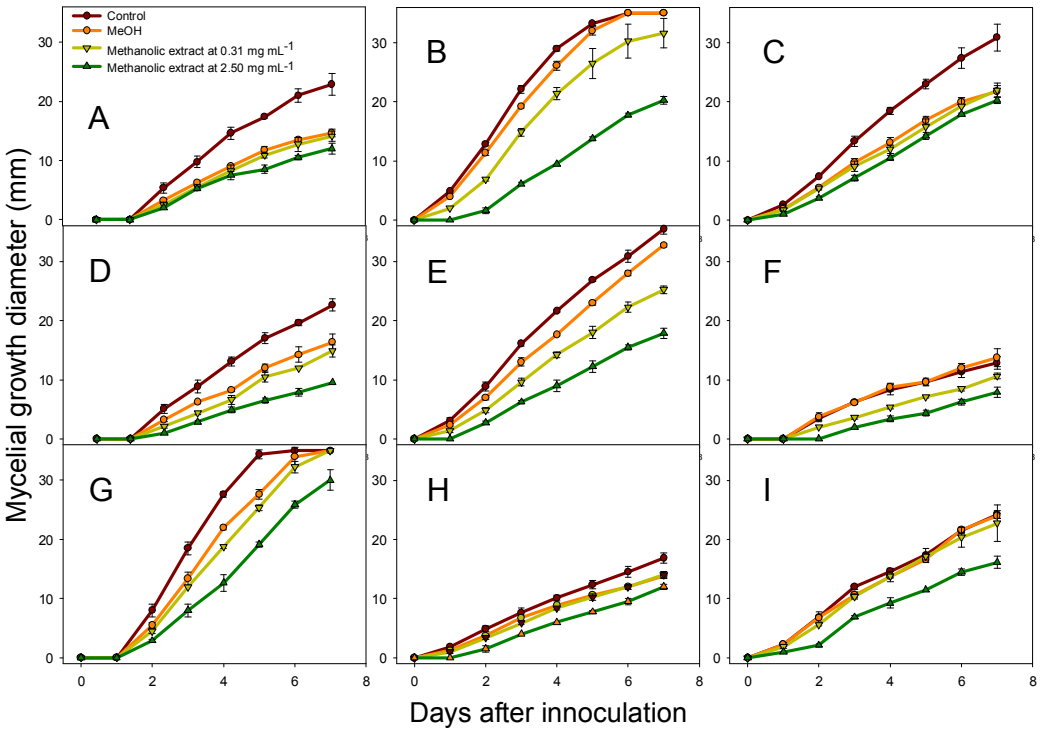


Fig. IV-3. Inhibitory effect of methanolic extract from strawberry leaves on mycelial growth of *Colletotrichum* spp.. A, *C. acutatum*; B, *C. caudatum*; C, *C. dematium*; D, *C. higginsianum*; E, *C. liliacearum*; F, *C. lindemuthianum*; G, *C. musae*; H, *C. orbiculare*; I, *C. truncatum*. Vertical bars represent standard errors of the means.

Table IV-2. Contents of ellagic and gallic acids in hexanic, dichloromethanic, acetonic, and methanolic extracts from strawberry leaves.

	Ellagic acid	Gallic acid
Solvent	($\mu g \cdot g^{-1}$ DW)	
Hexane	-[z]	-
Dichloromethane	-	-
Acetone	-	-
Methanol	14.5	-

[z]-, not detected.

antimicrobial compound (Thiem and Goślińska, 2004).

Bioautography was used to identify antimicrobial compounds of methanolic extract from strawberry plant. Inhibition zones (white area) were found in all tested microorganisms by organs of strawberry plants (Fig. IV-4). Methanolic extract from strawberry leaves and petioles included 1-2 active compounds and extract of green fruits contained only one compound, but extracts of red fruits did not possessed antimicrobial compounds for microbials used in this study. Lane of *C. coccodes*, the most susceptible microorganism to methanolic extract from strawberry leaves, showed two inhibition zones. Especially, one zone was much greater than the other zone. The *C. coccodes* inhibition spots were also analyzed by GC-MS. Tyrosol of 150.6 $\mu g \cdot g^{-1}$ and β-sitosterol of 320.6 $\mu g \cdot g^{-1}$ were found to be the dominant compounds in the chromatogram from both inhibition zones (Fig. IV-5). The compound of the greatest zone was tyrosol. However, ellagic was not detected from this experimental method. Wintoch et al. (1991) firstly found that *Fragaria* × *ananassa*, cv. Korona fruit includes tyrosol, and Raudoniūte et al. (2011) reported that 70% ethanol extract of garden strawberry (*Fragaria* × *ananassa*) leaves included tyrosol of 6.18 $mg \cdot g^{-1}$. Tyrosol showed antifungal activity against *Alternaria chevolieri, A. elegans, A. flavus, A. nidulans, A. niger, A. oryzae, A. parasiticus, A. tamari, A. versicolor, A. wentii, Neurospora crassa, Fusarium oxysporum, F. semitectum, Mucor racemosus, Penicillium chrysogenum, P. echinulatum, P. griseofulvum, P. italicum, P. roqueforti, P. verrucosum, Rhizopus oligosporus* (Korukluoglu et al., 2007). The compound, however, did not present in tomato plant (Vallverdú-Queralt et al., 2014). β-Sitosterol of

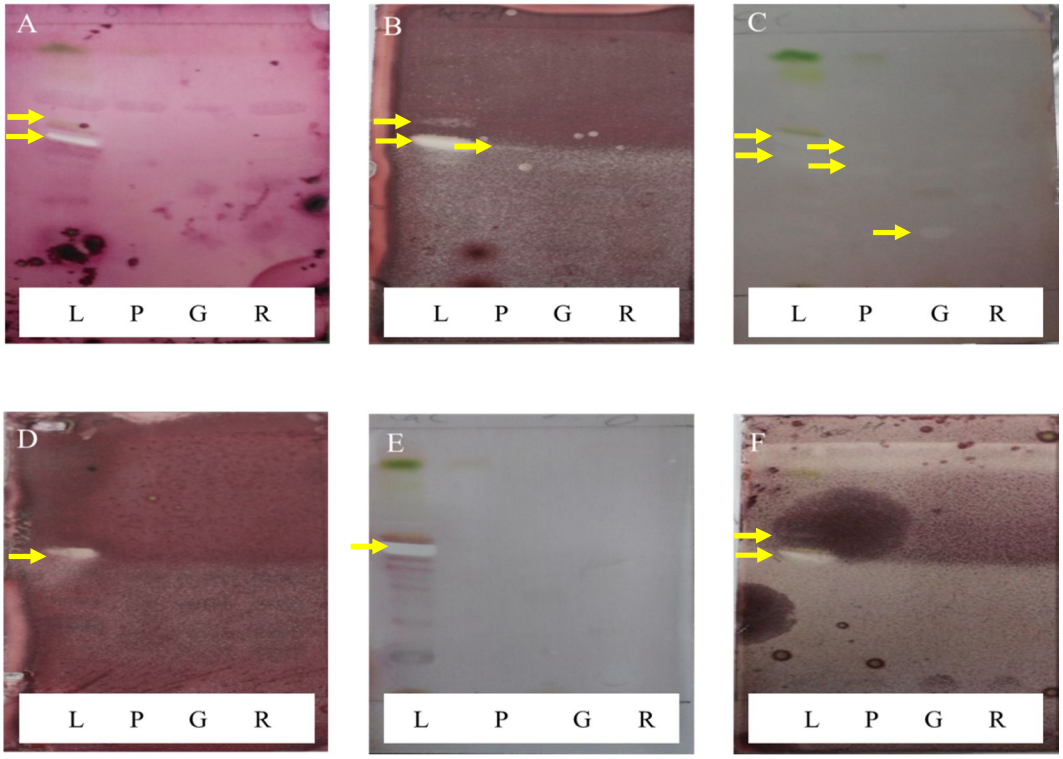
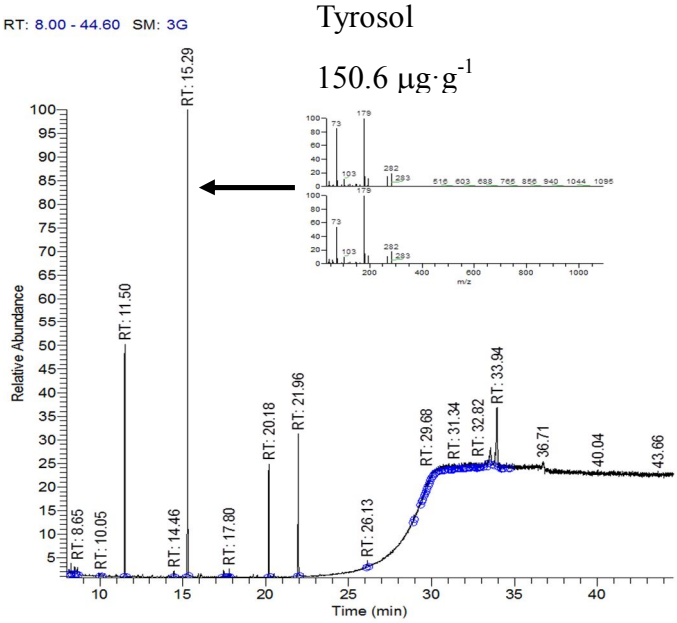


Fig. IV-4. Bioautogram of methanolic extracts from leaves (L), petioles (P), green fruits (G), and red fruits (R) of strawberry plants. White areas indicate inhibition of microbial growth. A, *Colletotrichum coccodes*; B, *Fusarium oxyspourum*; C, *Glomerella cingulata*; D, *Rhizoctonia solani*; E, *Phytophthora cactorum*; F, *P. capsici*.

Tyrosol

150.6 μg·g^{-1}



β-Sitosterol

320.6 μg·g^{-1}

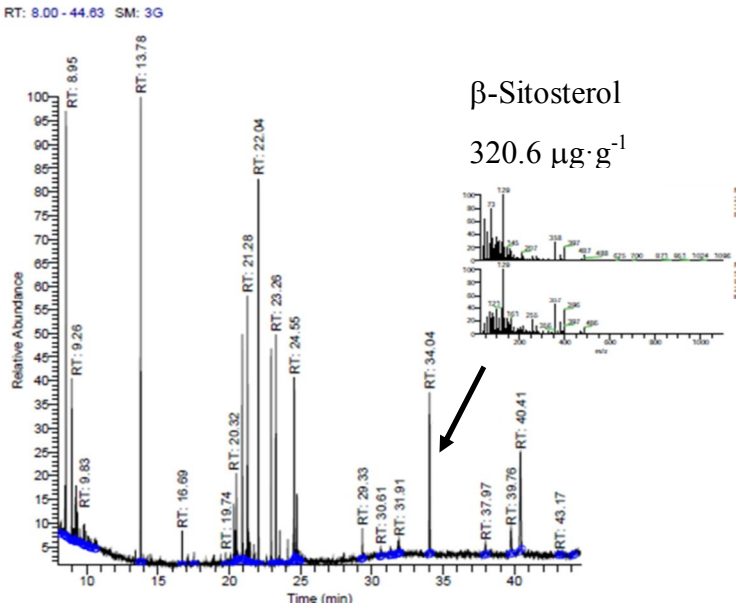


Fig. IV-5. GC-MS chromatogram of preparative TLC-isolated compounds in methanolic extract from strawberry leaves.

strawberry leaves have been examined by O'Neill et al (1981). They reported that β-sitosterol represented 95% of the total free sterols in strawberry leaves. β-Sitosterol also inhibited mycelial growth of *Aspergillus niger*, *Cladosporium cladosporioides*, and *Phytophthora* sp (Lall et al., 2006). To confirm the antimicrobial compounds, methanolic extract from strawberry leaves was developed on a TLC plate and then the plate was sprayed with anisaldehyde-sulfuric acid. Tyrosol and β-sitosterol were visualized on TLC plate and located to the same line with the antimicrobial compounds (Fig. IV-6), but benzeneacetic acid, benzoic acid, ethanedioic acid, hexadecanoic acid, hexanedioic acid, inositol, D-mannitol, octadecanoic acid, octadecatrienoic acid, phenethylamine, piperidine, pregnane, quinic acid, and tetradecanoic acid which were detected by GC-MS were not visualized by phosphomolybdic acid or anisaldehyde-sulfuric acid and not located to the same line with the antimicrobial compounds (data not shown). The results indicated that tyrosol and β-sitosterol from the strawberry extract are the antimicrobial compound against *C. coccodes*. Therefore, the methanolic extract from strawberry leaves was confirmed to contain marker compounds for commercialization.

Antimicrobial activity of ellagic acid, gallic acid, β-sitosterol, and tyrosol detected in extract from strawberry leaves

The antimicrobial compounds of strawberry leaves against *C. coccodes* were compared and confirmed to tyrosol and β-sitosterol. To determine the mycelial inhibition of *C. coccodes* by the compounds, tyrosol and β-sitosterol of 0.01, 0.1,

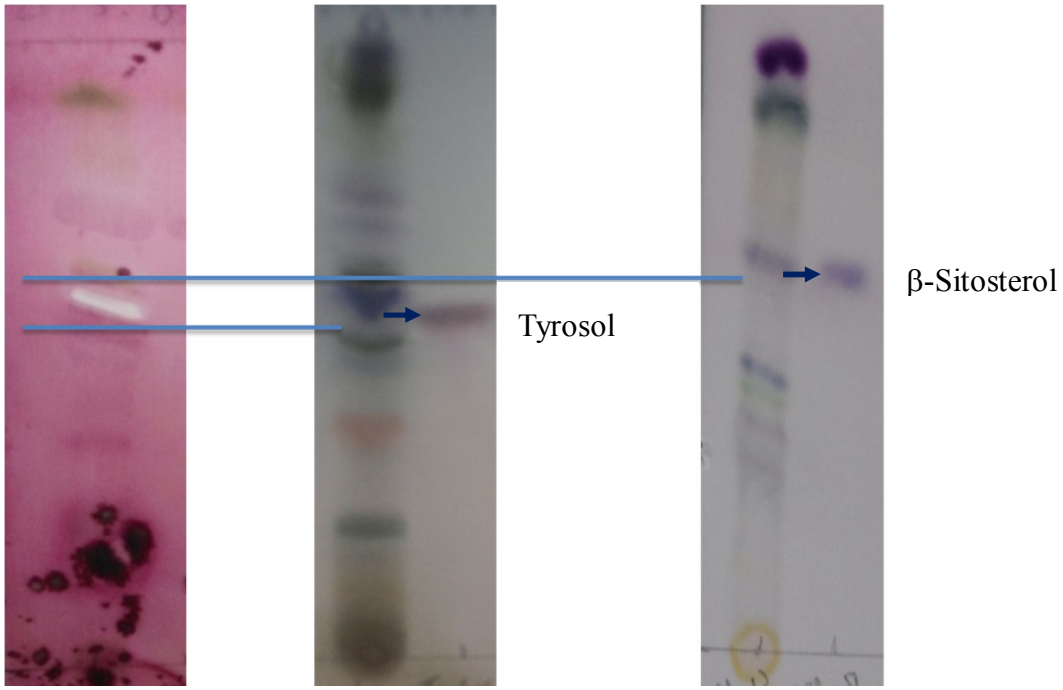


Fig. IV-6. Tyrosol and β-sitosterol visualized on TLC using anisaldehyde-sulfuric acid.

and 1.0 mg·mL^{-1} were treated. In addition, ellagic and gallic acid which were detected in methanolic extract from strawberry leaves were also treated on the microorganisms. With tyrosol of 1 mg·mL^{-1}, mycelial growth of *C. coccodes* on day 7th was reduced by 63.2% as compared with methanol solvent treatment while β-sitosterol showed little antimicrobial activity against *C. coccodes* (Fig. IV-7). Tyrosol showed antimicrobial activity against rot diseases including *Phytophthora megasperma* Drechsler (tomato root rot) and *Gibberella pulicaris* (potato dry rot) (Báidez et al., 2006; Slininger et al., 2004), while antimicrobial activity of tyrosol against *Colletotrichum* spp. was rarely known.

Ellagic acid detected in methanolic extract from strawberry leaves inhibited mycelial growth of *C. coccodes* from 0.01 mg·mL^{-1}. With ellagic acid of 0.01, 0.1, and 1 mg·mL^{-1}, mycelial growth of *C. coccodes* on day 7th was reduced by 25.5, 28.8, and 37.5% as compared with methanol solvent treatment, respectively (Fig. IV-8). Osorio et al. (2010) reported that ellagic acid of 1.0 mg·mL^{-1} inhibited fungal growth of *Colletotrichum coccodes* and *Rhizoctonia solani*. My result also showed that methanolic extract from strawberry leaves including ellgagic acid inhibited mycelial growth of *Colletotrichum coccodes* (Fig. IV-8). However, the content of ellagic acid in strawberry leaves was 14.5 μg·g^{-1}, which was too low to inhibit mycelial growth of *C. coccodes*. With gallic acid of 0.01, 0.1, and 1 mg·mL^{-1}, mycelial growth of *C. coccodes* on day 7th was reduced by 25.0, 26.0, and 28.8% as compared with methanol solvent treatment (Fig. IV-8). Gallic acid showed antimicrobial activity against *C. coccodes*, but was not included in methanol extract from strawberry leaves.

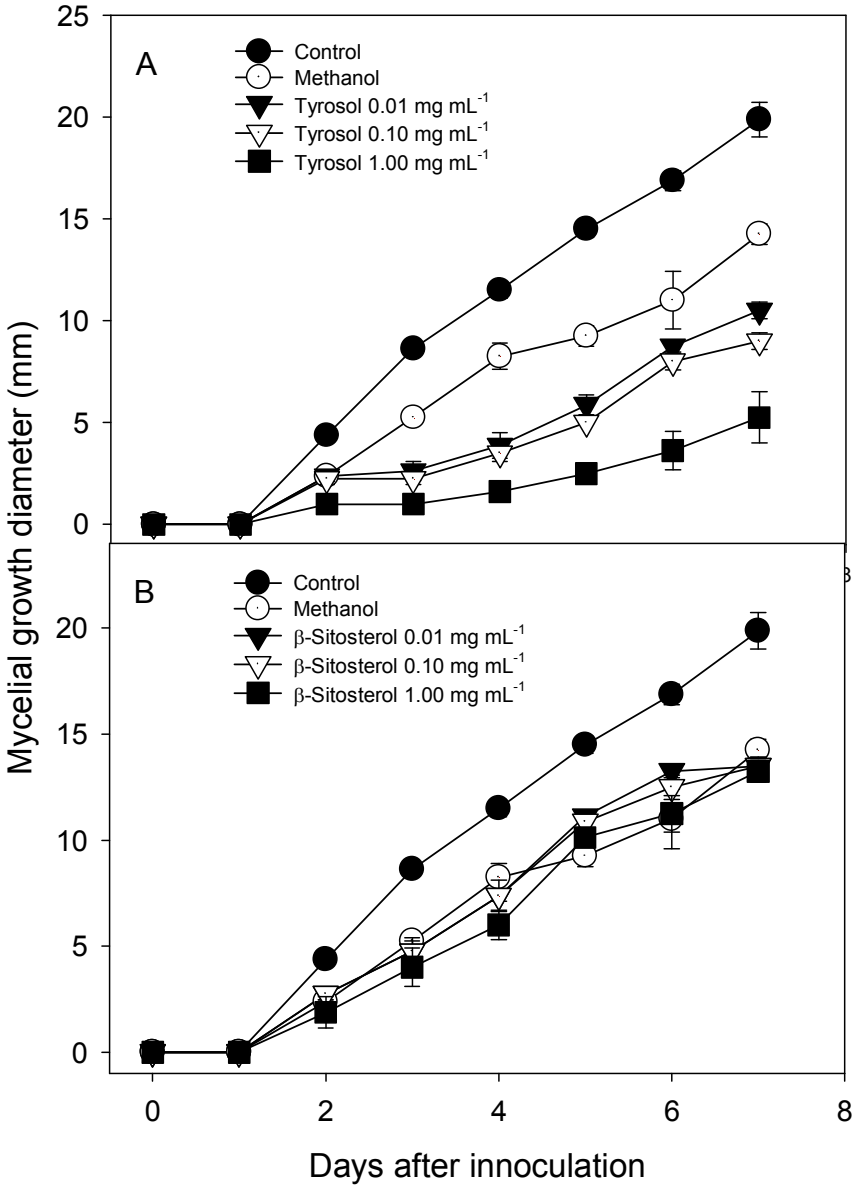


Fig. IV-7. Inhibitory effect of tyrosol (A) and β-sitosterol (B) on mycelial growth of *Colletotrichum coccodes*. Vertical bars represent standard errors of the means.

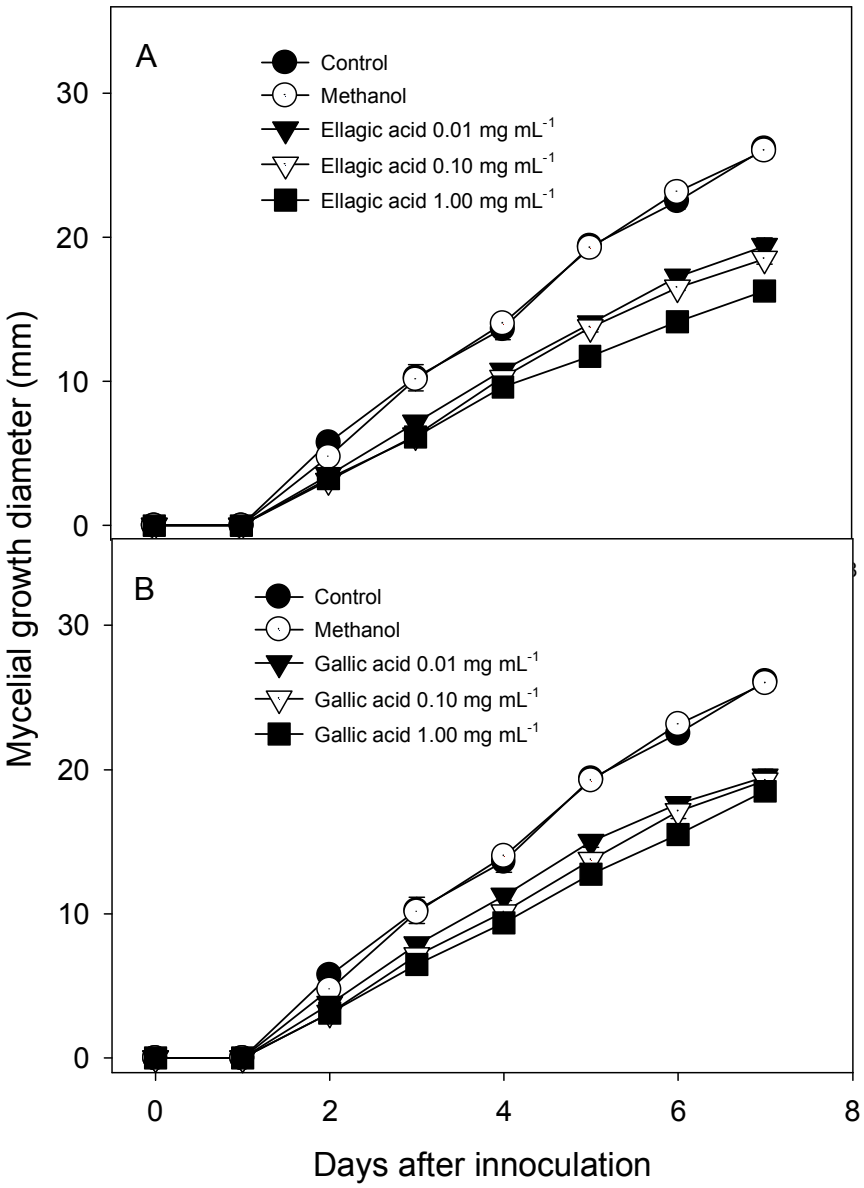


Fig. IV-8. Inhibitory effect of ellagic (A) and gallic acids (B) on mycelial growth of *Colletotrichum coccodes*. Vertical bars represent standard errors of the means.

Antimicrobial activity of antimicrobial compounds included in methanoic extract from strawberry leaves was compared to the methanolic extract. Contents of ellagic acid, tyrosol, and β-sitosterol (ETS) in methanolic extract from strawberry leaves 0.31 $mg \cdot mL^{-1}$ were approximately 50.0 $\mu g \cdot mL^{-1}$, 1.0 $mg \cdot mL^{-1}$, and 2.0 $mg \cdot mL^{-1}$ (yield: 7.5%), respectively. Inhibitory effect of ETS on mycelial growth of *C. coccodes* was lower than that of the methanolic extract (Fig. IV-9). The result indicated that methanolic extract from strawberry leaves may contain other antimicrobial compounds. Consequently, combined effect of several compounds in the methanolic extract can be expected although the individual compounds vary greatly with respect to biological effect.

In MIC tests, methanolic extract from strawberry leaves had the highest antimicrobial activity against tested phytopathogenic microorganisms. The result indicated that methanol was the best solvent, since it extracted active antimicrobial compounds from strawberry plants and strawberry leaves have promising antimicrobial activity. Some literature reports suggested that pre-formed antimicrobial compounds were found in strawberry leaves. For instance, Filippone et al. (1999) observed that extract of 'Chandler' strawberry leaves showed high antimicrobial activity against *Colletotrichum* spp. in my experiment. Methanolic extract of 'Seolhyang' strawberry leaves also inhibited mycelial growth of *Colletotrichum* spp.. Based on bioautography results, tyrosol and β-sitosterol were antimicrobial compounds against *C. coccodes*. Tyrosol, especially, was more active compound than other secondary metabolites included in strawberry leaves. This study demonstrates the potential of strawberry leaves as

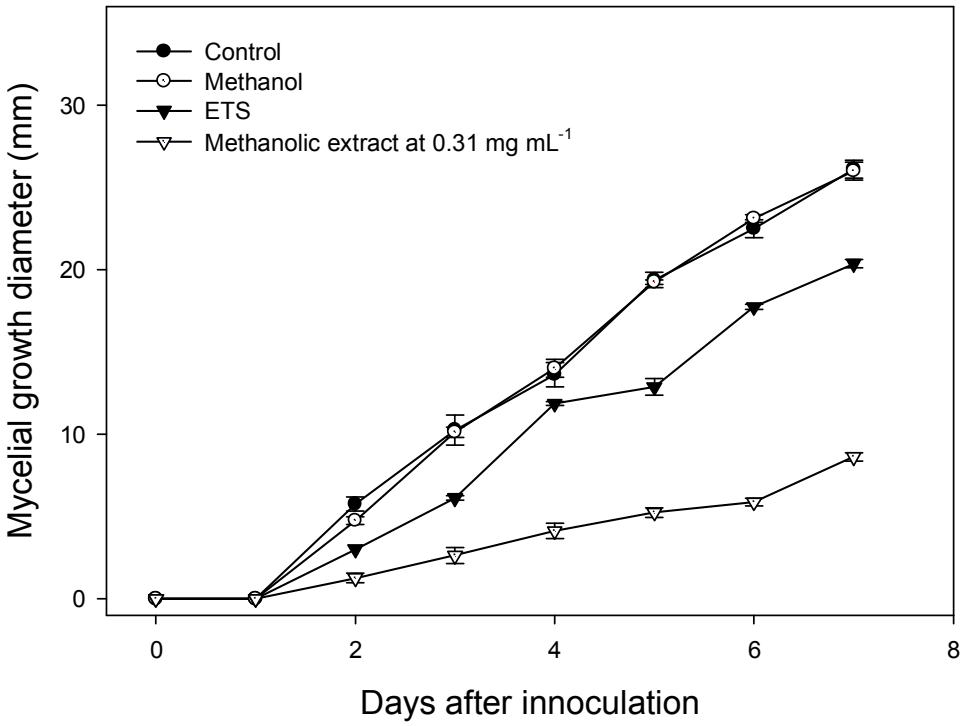


Fig. IV-9. Inhibitory effect of antifungal compounds on mycelial growth of *Colletotrichum coccodes*. Vertical bars represent standard errors of the means. ETS, ellagic acid 0.5 mg·mL^{-1} + tyrosol 10.0 mg·mL^{-1} + β-sitosterol 20.0 mg·mL^{-1}.

sources of extracts or pure compounds with activity against *C. coccodes*. Moreover, profiling and MIC results showed that the other parts of strawberry plant also had antimicrobial activity due to antimicrobial compounds although the activity was lower than leaves. Because strawberry leaves were 52.1% of aerial parts, whole plant could be considered as sources for natural antimicrobial agents.

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CONCLUSION

Higher plants synthesize diverse secondary metabolites to defend themselves against their pathogens and herbivores and to attract pollinators. Tomato and strawberry also produce the substances but composition of the compounds is distinct. For example, tomato fruit contains high level of lycopene and chlorogenic acid while strawberry fruit includes an amount of anthocyanin and ellagic acid. The compounds are known for antioxidant, antimicrobial, anti-inflammatory, and antiproliferative properties The fruit, however, is difficult to use as row material for the applications because they are expensive. Therefore, the study about non-edible parts of tomato and strawberry plants is needed.

Results of Chapter I showed that secondary metabolite in non-edible parts of 'Bacchus' tomato plants were higher than edible part except lycopene, 2-butenal, 1-butanol, and 3-hexen-1-ol. Tomato leaves, especially, included the greatest content of β-carotene, lutein, chlorogenic acid, caffeic acid, vanillic acid, caryophyllene, β-phellandrene, sabinene, and dehydro- and α-tomatines. α-Tomatine content that is known for antimicrobial compounds were higher than the other secondary metabolits content.

Results of Chapter II indicated that acetonic extract of 'Bacchus' tomato leaves showed lower minimum inhibitory concentration than other extracts. Among the tested microorganisms, mycelial growth of *Rhizoctonia solani*, strawberry pathogen, were remarkably inhibited by acetonic extract from tomato leaves. Bioautography on thin layer chromatography showed that acetonic extract

from tomato leaves contained two antimicrobial compounds against *R. solani*. GC-MS data indicated that linolenic and caffeic acid were the dominant antimicrobial compounds in the chromatogram. Inhibitory effect of linolenic acid on mycelial growth of *R. solani* was greater than caffeic acid.

Results of Chapter III showed that secondary metabolite in non-edible parts of 'Seolhyang' strawberry plants were higher than edible part except 2-hexenal, 2-hexen-1-ol, acetic acid, propanoic acid, butanoic acid and, hexanoic acid. Strawberry leaves, especially, included the greatest content of ellagic acid, gallic acid, 3-hexen-1-ol, and *E*,*E*-α -farnesene. 'Seolhyang' strawberry leaves included more ellagic acid content, known for main compound of fruit, than the other parts.

Results of Chapter IV indicated that methanolic extract of 'Seolhyang' strawberry leaves showed lower minimum inhibitory concentration than other extracts. Among the tested microorganisms, mycelial growth of *Colletotrichum coccodes*, tomato pathogen, were greatly inhibited by methanolic extract from strawberry leaves. In addition, the methanolic extracts inhibited mycelial growth of the other *Colletotrichum* spp. such as *C. caudatum, C. higginsianum, C. liliacearum, C. lindemuthianum, C. musae, C. orbiculare, and C. truncatum*. Bioautography on thin layer chromatography showed that methanolic extract from strawberry leaves included two antimicrobial compounds against *C. coccodes*. GC-MS data indicated that tyrosol acid and β-sitosterol were the dominant antimicrobial compounds in the chromatogram. Inhibitory effect of tyrosol acid on mycelial growth of *C. coccodes* was greater than β-sitosterol.

Composition of secondary metabolites of tomato and strawberry fruits have

been reported by many researchers while information about their non-edible parts is rarely studied. Some papers showed that non-edible parts of vegetables include higher contents of secondary metabolites than edible parts. However, non-edible parts of tomato and strawberry plants are usually dumped after last harvest. To remove the non-edible parts, much labor and time are spent. Also the dumped non-edible parts can cause soil and water pollution. I confirmed that content of secondary metabolites in the non-edible parts of tomato and strawberry plants was higher than that of edible part. Moreover, antimicrobial activity of extracts of tomato and strawberry leaves was confirmed and then the antimicrobial compounds were identified. The results can be used as database for utilization of the non-edible parts and can provide information for development of natural antimicrobial agents against *Rhizoctonia solani* and *Colletotrichum coccodes*. In addition, utilization of non-edible part can increase an extra income for the farmers and decrease labor force and environmental pollution.

ABSTRACT IN KOREAN

본 논문은 토마토와 딸기 연구로 구성되어 있다. 제I-1장과 II-1장은 토마토와 딸기의 부위별 이차대사산물의 조성을 분석하는 것에 대한 연구이다. 제I-2장과 II-2장은 토마토와 딸기의 부위별 추출물의 항균활성을 탐색하는 것에 대한 연구이다. 제I-1장에서는 '박커스' 토마토 품종의 발달 단계별 잎, 절간, 과실 및 뿌리에 함유된 카로티노이드, 페놀 화합물, 방향성 유기 화합물 및 알칼로이드의 함량을 조사하였다. 성숙과에 함유된 라이코펜의 함량은 196.2μg·g^{-1} FW였고 다른 부위에서는 발견되지 않았다. 24번째 잎에 함유된 베타카로틴과 루테인의 함량은 각각 23.2과 25.6μg·g^{-1} FW였고 다른 부위에서의 함량보다 많았다. 18번째 잎에 함유된 클로로제닉산의 함량은 40.1μg·g^{-1} FW였고 다른 부위에서는 31μg·g^{-1} FW 이하로 검출되었다. 24번째 잎에 함유된 카페익산과 바닐릭산의 함량은 각각 9.2 및 1.6μg·g^{-1} FW였고 이것은 다른 부위에서의 함량보다 많았다. 또한 잎은 모노테르펜 및 세스퀴테르펜을 포함한 다양한 방향성 유기 화합물을 함유하였는데 어린 잎일수록 더 많은 종류 및 양의 방향성 유기 화합물이 검출되었다. 디하이드로 토마틴과 알파토마틴은 잎에서 가장 많이 검출되었고 절간, 뿌리, 과실순으로 검출되었다. 잎과 절간은 어릴수록 토마틴의 함량이 더 많았다. 24번째 잎에 함유된 디하이드로 토마틴과 알파토마틴은 각각 889.1과 1,417.9μg·g^{-1} FW였고 이것은 다른 부위에서의 함량보다 많았다. 라이코펜을 제외하고 토마토 잎은 성숙과보다 더 많은 이차대사산물을 함유하고 있었다. 제I-2장에서는 '박커스' 토마토 품종의 부위 및

용매별 추출물의 항균 활성을 확인하였다. 토마토 잎 아세톤 추출물은 *Fusarium oxyspourum* f. sp. *lycopersici, Colletotrichum coccodes*, *Phytophthora capsici*, *Rhizoctonia solani*, *Glomerella cingulate* 균에 대한 최소 생육 제한 농도가 가장 낮았고 특히 *R. solani*의 균사 생육을 가장 많이 억제시켰다. 항균 활성 물질을 탐색하기 위해 실시한 바이오그래피는 토마토 잎 아세톤 추출물이 두 가지 항균 활성 물질을 함유하고 있다는 것을 보여 주었고 이들을 GC MS로 확인한 결과 리놀레닉산 및 카페익산이 검출되었다. 또한 리놀레닉산은 카페익산보다 *R. solani*의 균사 생육을 더 많이 저해하였다. 제II-1장에서는 '설향' 딸기 품종의 영양 생장기와 생식 생장기에 수확한 뿌리, 잎, 엽병, 런너 및 과실에 함유된 페놀 화합물 및 방향성 유기 화합물의 함량을 조사하였다. 영양 생장기에 수확한 런너의 잎에 함유된 엘라직산과 갈릭산의 함량은 각각 7.4와 5.1mg·g^{-1} FW였고 이것은 다른 부위에서의 함량보다 많았다. 영양 생장기에 수확한 딸기 식물체의 주요 방향성 유기 화합물은 3-hexen-1-ol였고 잎에서 주로 검출되었다. 생식 생장기에 수확된 잎에 함유한 엘라직산의 함량은 13.0mg·g^{-1} FW였고 다른 부위에서는 6mg·g^{-1} FW 이하로 검출되었다. 생식 생장기에 수확한 미성숙과에 함유된 갈릭산은 2.8mg·g^{-1}였고 이것은 다른 부위에서의 함량보다 많았다. 반면에 성숙과에는 세스퀴테르펜을 포함한 다양한 방향성 유기 화합물이 검출되었다. 제II-2장에서는 '설향' 딸기 품종의 부위 및 용매별 추출물의 항균 활성을 확인하였다. 딸기 잎 메탄올 추출물의 최소 생육 제한 농도는 실험에 사용된 모든 균에서 가장 낮았고 특히 *C. coccodes*의 균사 생육을 가장 많이 억제하였다. 또한 딸기 잎 메탄올

추출물은 *C. coccodes*뿐만 아니라 *C. caudatum, C. higginsianum, C. liliacearum, C. lindemuthianum, C. musae, C. orbiculare* 및 *C. truncatum*의 균사 생육을 억제하는 것을 확인하였다. 바이오그래피에서 딸기 잎 메탄올 추출물이 두 가지 항균 활성 물질을 함유하고 있다는 것을 보여 주었고 이들을 GC MS로 확인한 결과 타이로솔 및 베타사이토스테롤이 검출되었다. 또한 타이로솔은 베타사이토스테롤보다 *C. coccodes*의 균사 생육을 더 많이 저해하였다. 본 연구의 결과들을 종합해보면, 토마토 및 딸기의 비식용 부위는 식용 부위인 성숙과보다 더 많은 이차대사산물을 함유하고 있을 뿐만 아니라 식물 병원균에 대한 항균 활성도 높은 것을 확인하였다. 이 결과들은 일반적으로 수확 후에 버려지고 있는 토마토 및 딸기의 비식용 부위의 활용에 대한 기초 자료로 활용될 수 있으며, 딸기 모잘록병(*Rhizoctonia solani*) 및 가지과 탄저병(*Colletotrichum coccodes*)의 생물학적 방제제 개발을 위한 정보를 제공할 수 있다.