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Effect of Methyl Jasmonate on Secondary Metabolites of Sweet Basil (*Ocimum basilicum* L.)

HYUN-JIN KIM,[†] FENG CHEN,*[,†] XI WANG,[§] AND NIHAL C. RAJAPAKSE[#]

Department Food Science and Human Nutrition, Department of Genetics and Biochemistry, and Department of Horticulture, Clemson University, Clemson, South Carolina 29634

The effect of methyl jasmonate (MeJA) in terms of its induction of inherent bioactive chemicals in sweet basil (*Ocimum basilicum* L.) was evaluated after MeJA was sprayed on healthy basil plants. The total phenolic content of the sweet basil significantly increased after 0.1 and 0.5 mM MeJA treatments compared with the control not subjected to MeJA. Two phenolic compounds, rosmarinic acid (RA) and caffeic acid (CA), were identified as strong antioxidant constituents of the sweet basil. Their amounts also significantly increased after the MeJA treatment. In addition, eugenol and linalool increased 56 and 43%, respectively, by the 0.5 mM MeJA treatment. Due to the accumulation of RA, CA, and eugenol, which possess strong 2,2-diphenyl-1-picrylhydrazyl (DPPH•) free radical scavenging activities, the antioxidant activity of the sweet basil extract was 2.3-fold greater than that of the control after the 0.5 mM MeJA treatment. In the DPPH• assay, the EC_{50} values of RA, CA, and eugenol were determined as 23, 46, and 59 μM, respectively, which indicated they were 6-, 3-, and 2.4-fold more efficient than BHT (140 μM). Besides, an unidentified HPLC peak in the methanolic extract of the sweet basil was 4.3-fold higher than that of the control after the 0.5 mM MeJA treatment.

KEYWORDS: Basil; MeJA; elicitor; antioxidant; phenolics

INTRODUCTION

Under various biotic and abiotic stresses, plants can respond to produce not only direct defensive compounds such as proteinase inhibitors (PIs), polyphenol oxidase (PPO), and α-amylase inhibitors to protect themselves from stresses but also secondary metabolites such as phenolic compounds, terpenoids, and alkanoids (*1*−*4*). Even though some secondary metabolites have been reported to be involved in defense systems such as lignification and serving as phytoalexins, most of them, which are not related to the directive defense systems, have been confirmed to possess many bioactive functional properties (*5*−*7*). As knowledge of the biological functionalities of secondary metabolites has been accumulated, many methods for inducing secondary metabolites have been investigated. It has been reported that various elicitors such as chitosan, β-glucan, and yeast extracts and plant hormonal chemicals such as jasmonic acid (JA) and methyl jasmonate (MeJA) can induce secondary metabolites in various plants. Those elicitors can act like biotic and abiotic stresses such as wounding, pathogen attack, UV-light exposure, and temperature upon plants (*1*, *8*−*14*).

After the octadecanoid pathway, which begins with phospholipase A to release linolenic acid from chloroplast membrane, was clearly demonstrated to be involved in a major wounding signal transduction in plants, endogenous JA and MeJA that were derived principally from linolenic acid had been considered as controllers of secondary metabolites as well as defense systems (*15*−*17*). Under stresses such as wounding and pathogen attack, volatile MeJA could be released into the air from wounded plants, and simultaneously the wounding signal was transferred to other healthy plants. In turn, the healthy plants that receive the signal often respond through their defensive systems with increasing secondary metabolites. Although MeJA has been shown to be a powerful inducer of secondary metabolites in various plants (*18*−*22*), on the basis of our knowledge, the effect of MeJA on secondary metabolites in sweet basil plants has not been investigated. Therefore, the main objective of this study was to estimate the effect of MeJA on the induction of phenolic compounds and terpenoids in sweet basil. In addition, antioxidant activities of the basil extract and its major constituents were measured using the DPPH• free radical scavenging assay.

MATERIALS AND METHODS

Chemicals. MeJA, 2,2-diphenyl-1-picrylhydrazyl (DPPH•), gallic acid, caffeic acid (CA), and Folin−Ciocalteu's reagent were purchased from Sigma Chemical Co. (St. Louis, MO). Eugenol, methyleugenol, dodecane, 1,8-cineole, and L-linalool were purchased from Aldrich Chemical Co. (Milwaukee, WI). Rosmarinic acid (RA) was obtained from Cayman Chemical Co. (Ann Arbor, MI), and BHT (2,6-di-*tert*-butyl-4-methylphenol) was obtained from Acros (Fair Lawn, NJ). All HPLC analytical grade solvents were from Fisher Scientific (Suwanee, GA).

* Author to whom correspondence should be addressed [e-mail fchen@clemson.edu; telephone (864) 656-5702; fax (864) 656-0331].
[†] Department of Food Science and Human Nutrition.
[§] Department of Genetics and Biochemistry.
[#] Department of Horticulture.

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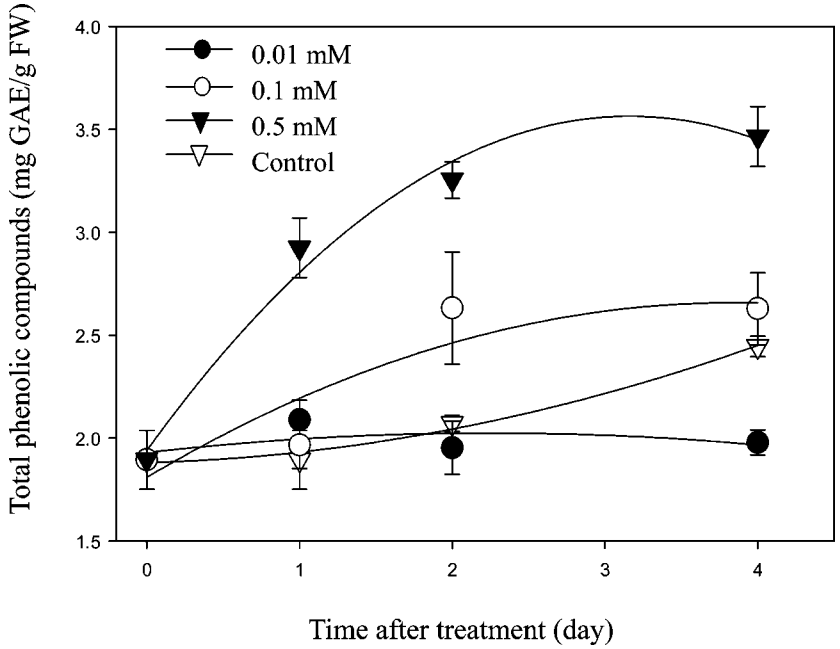

Figure 1. Time course of total amount of phenolic compounds of the sweet basil treated with different MeJA concentrations. The total amount of phenolic compounds extracted by 80% methanol from sweet basil was spectrophotometerically determined at 735 nm and expressed as gallic acid equivalent. Error bars in the figure are standard deviations of triplicate experiments.

Plant Culture and MeJA Treatment. Sweet basil (*Ocimum basilicum* L.) seeds purchased from a local grocery (Clemson, SC) were sown into 48-cell trays containing a commercial potting mixture (Fafard 3-B Mix, Fafard Inc., Anderson, SC). The sweet basil was grown under natural light conditions in the greenhouse located at Clemson University at Clemson, SC. It was watered every other day with addition of fertilizer once a week. Greenhouse cooling/heating set points were 27/25 °C. At the second leaf stage, the sweet basil plants were transplanted into 0.4 L plastic pots. Before harvest, the basil plants having the fourth to fifth leaves were sprayed with different MeJA concentrations (0.01, 0.1, and 0.5 mM) that were mixed with water using a sonicator and then zipped with a vinyl pack for 1 h. After removal of the vinyl pack, the plants treated with MeJA were isolated from the control plants treated with only water. The treated plants were left in the open air for 2 h to completely remove the remaining MeJA.

Extraction of Phenolic Compounds and Terpenoids from Sweet Basil. Fresh sweet basil plants harvested at 0, 1, 2, and 4 days after the treatments were ground in liquid nitrogen. Two grams of the sweet basil powder was mixed with 20 mL of 80% methanol to extract phenolic compounds or with 20 mL of methyl *tert*-butyl ether (MTBE) to extract terpenoids from the sweet basil. The mixtures were shaken at room temperature for 12 h and then centrifuged at 2000*g* for 20 min. After centrifugation, the methanol and the MTBE supernatants were used for the determination of phenolic compounds and terpenoids, respectively.

Determination of Total Content of Phenolic Compounds. The total amount of phenolic compounds in the sweet basil plants was determined using Folin−Ciocalteu's reagent according to the method of Singleton and Rossi (*23*). Fifty microliters of the methanolic extract was mixed with 450 μL of distilled water and 250 μL of 2 N Folin−Ciocalteu reagent. The mixture added to 1.25 mL of 20% Na_2CO_3 was incubated at 25 °C for 20 min and then centrifuged at 2000*g* for 10 min. The absorbance of the supernatant was measured at 735 nm, and the standard curve was prepared using the gallic acid.

Separation and Identification of Phenolic Compounds. To separate and identify antioxidant phenolic compounds in the sweet basil extract, reverse phase C_{18} high-performance liquid chromatography (HPLC) was used. The Pinnacle II C_{18} column (150 × 4.6 mm, 5 μm; Restek, PA) was connected to the LC-10AT HPLC system (Shimadzu, Kyoto, Japan) and equilibrated with 0.05% aqueous trifluoroacetic acid (TFA). Fifty microliters of the methanolic extract was injected and eluted with 0.05% aqueous TFA (solvent A) and acetonitrile (solvent B) at a flow rate of 1 mL/min. Collected fractions of the eluant were all in 1 mL, and the absorbance of the eluant was scanned from 200 to 500 nm by a SPD-M10V photodiode array detector (PDA). Authentic standards were used to identify phenolic compounds of sweet basil.

Determination and Identification of Terpenoids. To determine the quantitative changes of terpenoids affected by MeJA, a GC-FID system (Shimadzu, Kyoto, Japan) equipped with a DB-5 capillary column (30 m × 0.25 mm, thickness = 0.25 μm; J&W Scientific, Folsom, CA) was used. The oven temperature was programmed from 60 to 300 °C at 5 °C/min and held at 300 °C for 10 min. The injector and detector temperatures were 220 and 300 °C, respectively. The injection volume was 2 μL, and the split ratio was 1:20.

To identify terpenoids in sweet basil, a GC (GC-17A)−mass spectrometer (QP 5050 MS) system (Shimadzu, Kyoto, Japan) with a DB-5 capillary column (60 m × 0.25 mm, thickness = 0.25 μm) was used. The oven temperature was programmed from 60 to 240 °C at 3 °C/min and held at 240 °C for 10 min. Injector and ion source temperatures were 200 and 250 °C, respectively. Detector voltage was 70 eV, and the MS spectra were obtained in the mass range of *m*/*z* 43−350. Helium was used as a carrier gas, and the flow rate of the carrier gas was 1.1 mL/min. The injection volume was 3 μL, and the split ratio was 1:10. Identification of compounds was based on comparison of mass spectra, retention index (RI), and authentic standards. The mass spectrum of each compound was compared with that of Wiley and NIST mass spectral databases.

DPPH• Free Radical Scavenging Activity. For the DPPH• free radical scavenging assay of the sweet basil methanolic extract, the DPPH• method of Yamaguchi et al. (*24*) was used with slight modifications. The reaction mixture containing 0.1 mL of the sweet basil methanolic extract, 0.4 mL of 0.1 M Tris-HCl (pH 7.4), and 0.5 mL of 0.3 mM DPPH• was shaken vigorously and incubated at room temperature for 10 min in the darkness. The free radical scavenging activity was spectrophotometrically measured at 517 nm, and the scavenging activity of DPPH• free radical was calculated by using the following formula:

$$\text{scavenging activity (\%)} = \left(1 - \frac{\text{absorbance of sample at 517 nm}}{\text{absorbance of control at 517 nm}}\right) \times 100 \quad (1)$$

The EC_{50} value used to evaluate the antioxidant capacity of the crude extracts and standards was the effective concentration of the crude extracts and standards at which DPPH• free radicals were scavenged by 50%.

Experimental Design and Data Analysis. To investigate the effect of MeJA on sweet basil, three MeJA concentrations and one control were adopted under a randomized complete block design. Fifteen sweet basil plants were used in each treatment for three replicates. Five sweet basil plants were randomly selected and placed at three randomly selected places in the greenhouse for each replicate. The sweet basil plants were harvested at 0, 1, 2, and 4 days after the treatments. All experiments were performed in triplicate. Data were subjected to analysis of variance and were analyzed with nonlinear regressions (SAS 9.1, SAS Institute Inc., Cary, NC). The least significant difference was used to find the difference among all sample means at $p < 0.05$.

RESULTS AND DISCUSSION

Effect of MeJA on Phenolic Compounds in Sweet Basil. Although the total phenolic content of the sweet basil significantly increased with increasing MeJA concentrations, there was no significant difference in the total phenolic content between the control and the sample with 0.01 mM MeJA treatment (**Figure 1**). Total phenolic contents of the sweet basil treated with 0.1 and 0.5 mM MeJA reached maximal values of 2.6 and 3.5 mg of gallic acid equivalent (GAE)/g of fresh weight (FW), respectively. At the second day after the treatment, these values were 27 and 57%, respectively, higher than that of the control harvested at the same day (2.1 mg of GAE/g of FW). No further increase in the total phenolic content was observed in the sweet basil at the fourth day. Although the total phenolic content of the sweet basil increased after the MeJA treatment, it was observed that, compared to the previously reported plants such as red raspberry and black raspberry, the sweet basil was less affected by higher MeJA concentration. Phenolic compounds in both raspberries significantly increased after 0.01 and 0.1 mM MeJA treatment.

To investigate the effect of MeJA on each phenolic compound in the sweet basil, the methanolic extract was analyzed by C_{18}-HPLC (**Figure 2**). Among the various phenolic compounds, RA, which has been reported to have some bioactive properties such as antioxidant, antimicrobial, and anti-inflammatory activities (*25*−*28*), was found to be a major phenolic compound in sweet basil. At the second day after treatment, its amount reached maximal values at 1.5 and 1.7 mg/g of FW in the sweet basils treated with 0.1 and 0.5 mM MeJA, respectively. Their values were 35 and 47%, respectively, higher than those of the control (**Figure 2A**). These contents were comparable with the contents of sage (1.2 mg/g of FW) and hard sweet marjoram (1.5 mg/g of FW) that also exhibited strong antioxidant activities and were used as a natural source of food flavoring and season (*29*). Unlike the effect of MeJA on the total phenolic compounds of sweet basil, RA increased 19% by low MeJA concentration (0.01 mM). CA, known as another strong antioxidant, was also identified in sweet basil, and its amount increased after the 0.5 mM MeJA treatment (**Figure 2B**). Its maximal amount (0.2 mg/g of FW) was observed in the sweet basil harvested at the second day, and the amount was 3.8-fold higher than that of the control. In addition, it was investigated that the correlations between the total phenolic compounds and both phenolic contents were very high (i.e., RA, $y = 0.406x + 0.359$, $R^2 = 0.990$; CA, $y = 0.103x - 0.145$, $R^2 = 1.000$) until the second day after the 0.5 mM MeJA treatment. An unidentified peak also increased after the 0.5 mM MeJA treatment. Its HPLC chromatogram area reached a maximal value at the fourth day, and the value was 4.3-fold greater than that of the control (**Figure 2C**), whereas at the same day RA and CA contents decreased as compared with those at the second day.

It was reported that CA and RA were produced through the phenylpropanoid pathway initiated by phenylalanine ammonia-lyase (PAL) (*26*, *30*). Many phenolic compounds produced through this pathway can be induced by stresses, elicitors, JA, and MeJA. Those phenolics were found to be induced and accumulated in some other plants such as iceberg lettuce, soybean, white lupin seedlings, birch leaves, and tomato leaves (*11*, *12*, *14*, *31*, *32*). In our previous research, the amount of RA was observed to increase with increasing PAL activity by carbohydrate elicitor, chitosan (*33*). Therefore, it was suggested that PAL might be related with induction of phenolic compounds of the sweet basil treated with MeJA.

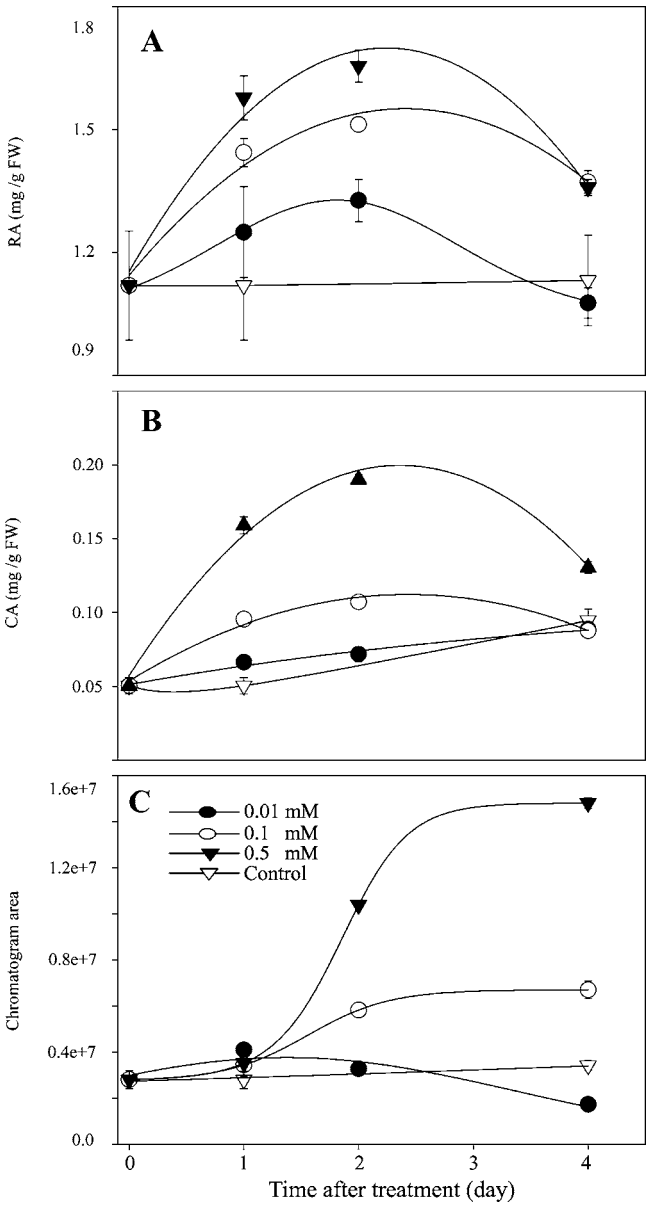

Figure 2. Time course of the effect of MeJA on phenolic compound content of sweet basil. Two identified phenolic compounds (RA and CA) and an unknown compound were separated by C_{18}-HPLC. RA and CA were determined at 316 nm, and the unknown compound was determined at 280 nm. Concentrations of RA and CA were quantitatively measured using standard RA and CA.

Effect of MeJA on Terpenoid Content of Sweet Basil. The total amount of terpenoids significantly increased after the 0.5 mM MeJA treatment, and the amount at the fourth day was 58% greater than that of the control, whereas there was no significant difference in the total content of terpenoids among samples with 0, 0.01, and 0.1 mM MeJA treatments (**Figure 3**). This result was similar to the previously reported results

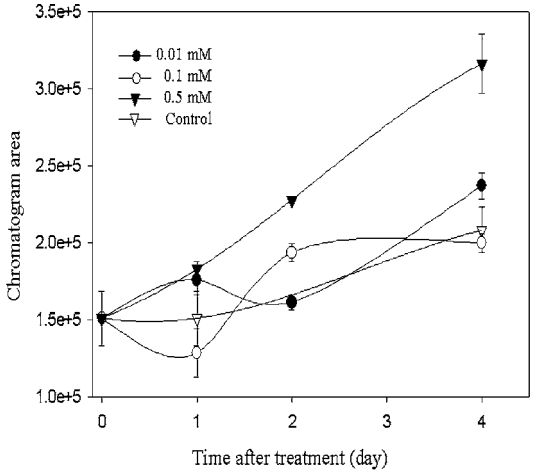

Figure 3. Time course of the total amount of terpenoids of sweet basil treated with different MeJA concentrations. The total amount of terpenoids in sweet basil was expressed by the total chromatographic area of all compounds separated by GC-FID.

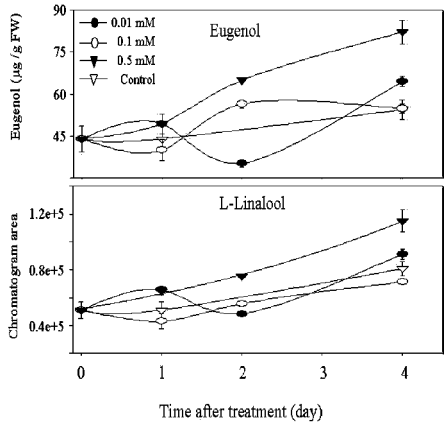

Figure 4. Time course of the effect of MeJA on eugenol and L-linalool in sweet basil. Eugenol and L-linalool were identified by GC-MS with a DB-5 capillary column. Their quantitative changes were monitored by GC-FID.

that terpenoids could be positively affected by stresses, elicitors, JA, and MeJA in various plants such as Norway spruce, lima bean, native tobacco plant, tomato, and Grand fir (*20*, *33*−*36*). Eugenol and L-linalool were identified in our research as two major flavoring terpenoids in sweet basil. They were reported to possess some functional properties such as antioxidant and antimicrobial activities (*37*−*41*). As shown in **Figure 4**, the amounts of eugenol and L-linalool could be induced by MeJA in different concentrations. Compared with the control, the amounts of eugenol and L-linalool in the sweet basil plants treated with 0.5 mM MeJA increased at the fourth day by 56 and 43%, respectively. These results were similar to the previous report that eugenol and L-linalool were induced in sweet basil by UV-B treatment (*42*).

Effect of MeJA on Antioxidant Activity of Sweet Basil. There was no significant difference in the DPPH• free radical scavenging activity between the control and the sample with 0.01 mM MeJA treatment. However, the DPPH• free radical scavenging activity of the sweet basil significantly increased by 0.1 and 0.5 mM MeJA treatments. At the fourth day their activities were 1.6 and 2.3 times, respectively, greater than that of the control (**Figure 5**).

To investigate the relationship between the total phenolic compounds and the antioxidant activity, their correlation was investigated and antioxidants were separated and identified from

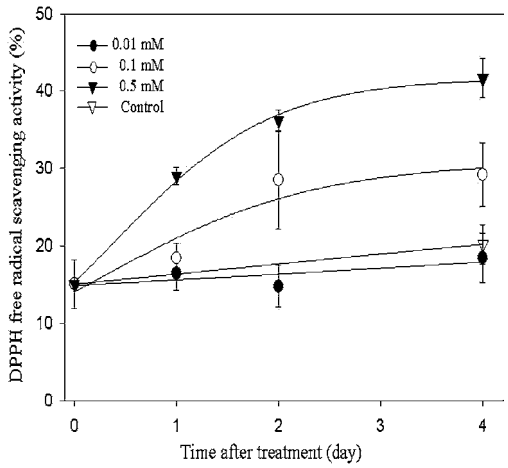

Figure 5. Time course of antioxidant activities of sweet basil treated with different MeJA concentrations. The DPPH• free radical scavenging activity of sweet basil methanol extract was spectrophotometrically measured at 517 nm.

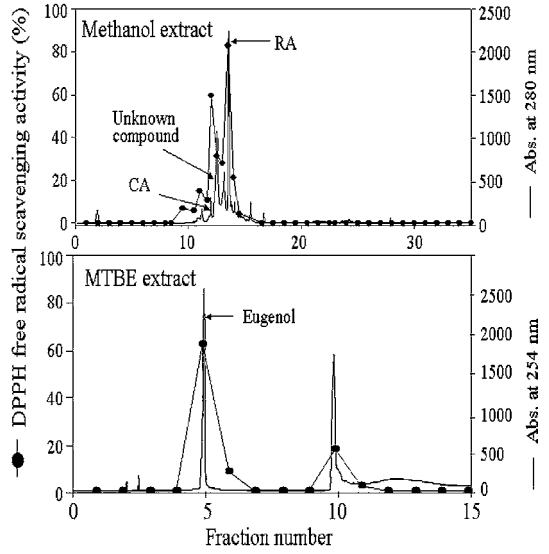

Figure 6. HPLC profiles of phenolic and terpenic compounds of sweet basil and their antioxidant activities. Antioxidant activities of all collected fractions were determined using the DPPH• assay.

the sweet basil extract. It was found that the correlation between the total phenolic compounds and the antioxidant activity was very high ($y = 16.354x + 16.701$, $R^2 = 0.980$). This result suggested that the increase of the phenolic compounds resulted in the increase of the antioxidant activity in sweet basil by MeJA treatment. Two major antioxidants, RA and CA, were found in sweet basil methanolic extract (**Figure 6**). As shown in **Figure 2**, their amounts increased after the MeJA treatment. Therefore, it was implied that the induction of antioxidant activity in the sweet basil was possibly caused by the accumulation of RA and CA after the MeJA treatment. Eugenol in the MTBE extract (**Figure 4**) was also verified as a strong antioxidant and could be induced by the MeJA (**Figure 6**). The free radical scavenging activities of three antioxidants (RA, CA, and eugenol) identified from sweet basil are shown in **Table 1**. Their EC_{50} values were 23, 46, and 59 μM, respectively and these values were 6-, 3-, and 2.4-fold greater than that of BHT (140 μM). These results strongly suggest that sweet basil can be used as an antioxidant source because sweet basil plants contain high amounts of strong antioxidants, especially RA. More importantly, sweet basil as

Table 1. DPPH• Free Radical Scavenging Activity of BHT and Three Potential Antioxidants Separated and Identified from Sweet Basil

identified potential antioxidants	free radical scavenging activity EC_{50}[a] (μM)
caffeic acid	46.1 ± 1.4b
eugenol	58.7 ± 2.9c
rosmarinic acid	23.2 ± 0.1a
BHT[b]	139.7 ± 3.4d

[a] EC_{50} value is defined as the effective concentration of the compound required to scavenge 50% of DPPH• free radical. Means with different letters were significantly different according to the least significant difference test ($p < 0.05$). [b] BHT was used as a standard antioxidant.

a common culinary spicy herb in our diet seems to deserve playing a greater health-benefiting role due to its nutraceutical values.

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