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Variation in essential oil composition and antimicrobial activity among different genotypes of *Perilla frutescens* var. crispa

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Abstract *Perilla frutescens* var. crispa (Pfc), a herb belonging to the mint family (Lamiaceae), has been used for medicinal and aromatic purposes. In the present study, we analyzed the variation in the chemical composition of essential oils (EOs) obtained from five different genotypes of Pfc collected from different regions. Based on principal component analysis (PCA) and hierarchical cluster analysis (HCA), we identified three groups: PA type containing perillaldehyde, PP type containing dillapiole, and 2-acetylfuran type. To assess the correlation between EO components and antimicrobial activities, we compared classification results generated by PCA and HCA based on antimicrobial activity values. The findings suggested that the major compounds obtained from EOs of Pfc are responsible for their antimicrobial activities. Chemotypes of Pfc plants are essentially qualitative traits that are important for breeders. The present findings provide potential information for breeding Pfc as an antimicrobial agent.

Keywords Antimicrobial activity · Chemotype · Essential oil · *Perilla frutescens* var. crispa · Principal component analysis

Introduction

Essential oils (EOs) obtained from plant materials, including flowers, leaves, stems, and roots, are complex natural mixtures of volatile compounds [1]. In plants, EOs play an important role as the defense system against pathogens and herbivores and are responsible for characteristic smells and flavors, which can attract some insects to disperse pollen and seeds. The major components of EOs are terpenes/terpenoids, aromatic compounds, and aliphatic compounds [2]. Usually, these major compounds determine their biological properties, including antimicrobial, antioxidant, anti-inflammatory, antiviral, antimuta-genic, and anticarcinogenic properties [2]. Therefore, EOs have become a target substance in medical and clinical microbiology; pharmaceutical botany; and fragrance, food flavoring, and preservation industries and are being used in various applications or as alternatives [3]. However, the yield and composition of EOs are affected by several environmental factors and by genetic diversity [4]. Cis-sesquisabinene hydrate, curzerenone, β-bisabolol, and farnesol have been identified as potential chemical markers to distinguish the CIM-Pitamber variety from different genotypes of Curcuma longa [5]. Thus, the chemical identification of EOs provides basic information for the development of chemical markers, which can be used to characterize plant species.

Perilla frutescens, an annual self-fertilizing crop belonging to the Lamiaceae family, has been used for flavor and fragrance and as an oil, vegetable, and medicine. Two major varieties, P. frutescens var. frutescens (Pff) and P. frutescens var. crispa (Pfc), are extensively cultivated in Asian countries, such as China, Korea, and Japan. Pff is used as an oil (seeds) and vegetable (leaves), while Pfc is often used as a spice and medicinal herb [6]. Pfc has pharmaceutical properties, such as antiallergic, anti-inflammatory, antioncogenic, anti-tumor, antiaging, and antidepressant properties [7,8]. Although this indicates the potential of Pfc as a functional resource, Pfc genotypes have been reported to vary in terms of their morphology, cultivation, and genetic diversity [6]. Therefore, several approaches, such as random amplified polymorphic DNA, amplified fragment length polymorphism, and sequence-tagged microsatellite, have been introduced and developed to distinguish subspecies of Pfc. Perilla varieties are

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classified into seven chemotypes based on the main component of EO, while Pfc plants are commonly divided into two forms, red and green, using chemotypes differing in anthocyanin accumulation [9,10]. This indicates that the metabolic compositions and chemotypes of different genetic resources can be used to improve the assessment of genetic diversity.

Considering the pharmaceutical properties of EOs, it is important to investigate the chemotype and composition of EOs of Pfc. In the present study, we analyzed and compared the compositions of EOs obtained from five different genotypes of Pfc. To determine the possible chemotype, we processed the data using multivariate statistical methods, namely principal component analysis (PCA) and hierarchical cluster analysis (HCA). In addition, we investigated the effect of chemotypes on antimicrobial activity.

Materials and Methods

Plant materials and extraction of EOs

Based on the leaf color, we selected five genotypes of Pfc, and Pfc seeds were obtained from the Korean Agricultural Culture Collection (KACC) (Table S1 and Fig. S1). Several factors, including geographical location and environmental aspects, affect the EO composition. Therefore, to grow Pfc plants under the same conditions, seeds of all the genotypes were germinated and cultivated in the research farms managed by Chungbuk National University, Republic of Korea. For the extraction of EOs, freshly harvested leaves from each genotype were hydrodistilled in a Clevenger apparatus for 3 h. EOs were collected, dehydrated under anhydrous sodium sulfate, and stored in vials in the dark at 4 °C until further analysis.

Gas chromatography-mass spectrometry (GC-MS) analysis

EOs were subjected to GC-MS analysis using an Agilent 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with an Agilent 5973i inert mass selective detector operating in a 70 eV mode. The compounds were separated on a DB-5 fused-silica capillary column (30 m×250 μm, 0.25 μm film thickness). The initial oven temperature was held at 50 °C for 2 min. Following this, the oven was heated at 250 °C at a rate of 10 °C/min; the temperature was then held at 250 °C for 10 min. The carrier gas was helium at a flow rate of 1 mL/min, and the components of EOs were identified by their retention time and by computer matching with the W10N11 full library and Wiley/7n mass spectral database (Hewlett Packard, Palo Alto, CA, USA).

Determination of antimicrobial activity

The antibacterial activity of EOs of Pfc was tested against eight bacterial species: gram-positive *Kocuria rhizophila* (KACC 14744), *Micrococcus luteus* (KACC 14819), *Listeria monocytogenes* (KACC 19115), and *Staphylococcus aureus* (KACC 1916) and gram-negative *Enterobacter cloacae* (KACC 11958), *Salmonella enteritidis* (KACC 12021), *Salmonella enterica* subsp. enterica (KACC 10769), and *Pseudomonas aeruginosa* (KACC 2004). To determine the minimum inhibitory concentration (MIC), the antibacterial assay was performed by the twofold serial dilution method [11] using 96 U-bottom microtiter plates. The lowest concentration showing growth inhibition in comparison with the control was defined as the minimum inhibitory concentration (MIC).

Statistical analysis

PCA and HCA were performed to determine whether the identified components could be used to reflect the chemotype of Pfc. PCA and HCA were performed using the statistical analysis software Paleontological Statistics version 4.01. Heatmap hierarchical clustering was performed using R software (version 3.6.3).

Results and Discussion

Chemical composition of EOs

EOs were obtained from five genotypes of Pfc using the hydrodistillation method; their chemical compositions were analyzed by GC-MS analysis (Table S2 and Fig. S2). Detailed analysis of EOs led to the identification of 26 compounds (selected variables >1.0%) from the Pfc genotypes. Of these, perillaldehyde was specific to EOs of Pfc 4 and Pfc 5, while dillapiole was a major component in EOs of Pfc 1 and Pfc 2 (Table S2). Dillapiole has been identified to be a chemotype of dill seed; it has been found to inhibit aflatoxin G1 production by *Aspergillus parasiticus* [12]. In addition, dillapiole exhibits insecticidal, antimicrobial, and anti-inflammatory activities [13-15]. Perillaldehyde is a monocyclic terpenoid found in EO of *P. frutescens* leaves [16]; it shows anti-inflammatory, antidepressant, and antimicrobial properties [17-19]. Although the composition of EO differs according to the genotype of Pfc, the presence of perillaldehyde or dillapiole indicates the possible therapeutic applications of Pfc.

Antimicrobial activities of EOs of Pfc

As EOs play an important role in plant defense against insects and pathogens, direct antimicrobial activities of EOs against a range of microorganisms have been determined; the results have suggested that EOs are effective natural antimicrobial agents [20]. It is known that the antimicrobial activities of EOs are related to their hydrophobicity, which allows them to penetrate the bacterial cell membrane, thereby resulting in the distribution of the cell structure and functionality [21]. In addition, the solvent extracts and EOs of Pfc have been found to exhibit broad antimicrobial activities assumed from their use as food and cosmetic additives [22]. However, the antimicrobial activities of plant extracts or EOs vary depending on the genotype, environment, and genotype× environment interaction. To assess the variation in antimicrobial

Table 1 Antimicrobial activity of essential oils obtained from five genotype of *Perilla frutescens* var. crispa

	MIC (%)[1)]							
Extract	S.s[2)]	S.a	M.l	E.c	S.e	K.r	L.m	P.a
Pfc 1	-	-	10.00±0.0^{b}	10.00±0.0^{c}	10.00±0.0^{c}	0.83±0.2^{c}	-	10.00±0.0^{a}
Pfc 2	-	-	10.00±0.0^{b}	10.00±0.0^{c}	10.00±0.0^{c}	1.25±0.6^{c}	-	-
Pfc 3	-	-	-	10.00±0.0^{c}	10.00±0.0^{c}	2.50±0.0^{d}	-	10.00±0.0^{a}
Pfc 4	3.33±0.8^{b}	2.50±0.0^{b}	10.00±0.0^{b}	0.83±0.2^{b}	2.08±0.4^{b}	0.42±0.1^{b}	3.33±0.8^{b}	10.00±0.0^{a}
Pfc 5	1.67±0.4^{a}	1.25±0.0^{a}	6.67±1.67^{a}	0.42±0.1^{a}	1.04±0.2^{a}	0.15±0.07^{a}	1.04±0.2^{a}	10.00±0.0^{a}

[1)]MIC values against bacteria were determined by the twofold serial dilution method
[2)]S.s.: *Salmonellaentrica* sub sp.enterica 10769; S.a.: *Staphylococcus aureus* 1916; M.l.: *Micrococcus luteus* 14819; E.c.: *Enterobacter cloacae* 11958; S.e.: *Salmonella enteritidis* 12021; K.r.: *Kocuria rhizophila* 14744; L.m.: *Listeria monocytogenes* 19115; P.a.: *Pseudomonas aeruginosa* 2004

activities among the Pfc genotypes, MICs of their EOs were determined using the twofold serial dilution method; the results are expressed as the MICs (Table 1). Overall, the EO of Pfc 5 showed the highest antimicrobial activity, followed by that of Pfc 4. The EO of Pfc 5 was most active against *K. rhizophila* (MIC =0.15%) among all the bacteria tested. The antimicrobial activity against *K. rhizophila* significantly increased in a dose- and time-dependent manner when the cells were treated with the EO of Pfc 5 (Fig. S3). It is assumed that gram-negative bacteria are less susceptible to hydrophobic compounds than gram-positive bacteria because of the presence of lipopolysaccharides in their outer membrane [23]. However, EOs of Pfc 4 and Pfc 5 exhibited good antimicrobial activity against gram-negative bacteria (*E. cloacae*, *S. enteritidis*, and *S. enterica* subsp. enterica), suggesting that some constituents of these EOs can access the periplasm of gram-negative bacteria, probably via the porin proteins localized on their outer membrane [23]. Interestingly, enhanced antimicrobial activity of lysozyme or perillaldehyde against gram-positive and gram-negative bacteria has been observed when lysozyme is conjugated with perillaldehyde [24]. Although the antibacterial mechanism of perillaldehyde is not fully understood, this finding indicates that perillaldehyde should facilitate the diffusion of antimicrobial compounds across the outer membrane of gram-negative bacteria. It will be interesting to determine whether perillaldehyde interacts with porin proteins and whether this interaction is required for the diffusion of antimicrobial compounds.

PCA based on the constituents and antimicrobial activities of EOs of Pfc

PCA has been commonly used to extract the maximum information and to assess the relationship between large data matrices. Twenty-six EO compounds obtained from EOs of Pfc were subjected to PCA. Three principal components were found to have higher influence on the EO composition. As shown in Fig. 1A, Pfc 4 and Pfc 5 (negative dimension) were clearly separated from Pfc 1 and Pfc 2 (positive dimension) along PC 1 (74.2%), while Pfc 3 was separated from the other plants along PC 2 (25.6%). In addition, PC 1 positively correlated with dillapiole (0.73) and negatively correlated with perillaldehyde (–0.68) (Table 2). Dillapiole (0.53), perillaldehyde (0.58), and 2-acetylfuran (–0.61) correlated with PC 2, indicating that they are discriminant compounds. Perilla plants cultivated in Thailand can be classified into seven chemotypes: PA type containing perillaldehyde; EK type containing elsholtzia ketone, naginata ketone, and/or shisofuran; PK type containing perilla ketone and/or isoegomaketone; PL type containing perillene; C type containing citral; PT type containing piperitenone; and PP type containing phenylpropanoids, such as dillapiole [25]. Thus, Pfc 1 and Pfc 2 belong to the PP type, while Pfc 4 and Pfc 5 belong to the PA type (Fig. 1B). Similar to Pfc 3, 2-acetylfuran was identified as the predominant compound in EOs obtained from *P. frutescens* cultivated in China [26]. Anthocyanin is an important factor for distinguishing the red and green forms of Perilla plants [10]. However, Pfc 4 (red form) and Pfc 5 (green form) are classified into the same PA type based on the EO types (Fig. 1). This indicates that although oil type and anthocyanin are genetically controlled, they are genetically independent, as suggested by [27].

To assess the correlation between the antimicrobial activities and EOs, all the MICs were subjected to PCA and HCA. The PCA horizontal axis explained 28.9% of the total variance, while the vertical axis explained a further 60.1% (Fig. 1C). Similar to PCA involving 26 major compounds of EOs, HCA revealed three groups based on their antibacterial activities (Fig. 1D). The first principal axis separated groups A and C, while the second principal axis separated group B from group C. Group A consisted of Pfc 4 and Pfc 5, which were characterized by high antimicrobial activity against all the tested strains. Group B contained only Pfc 3, which had no or low activity against all the tested strains (Table 1). Perillaldehyde and dillapiole have been reported to act as antimicrobial agents [28,29]. Although 2-acetylfuran has been used to synthesize hydrazide-hydrazone derivatives possessing antimicrobial activity [30], it is unknown whether 2-acetylfuran acts as an antimicrobial agent. Taken together, these findings indicate that the major compounds obtained from EOs of Pfc should be responsible for the antimicrobial activities.

In summary, we performed PCA and HCA to investigate the

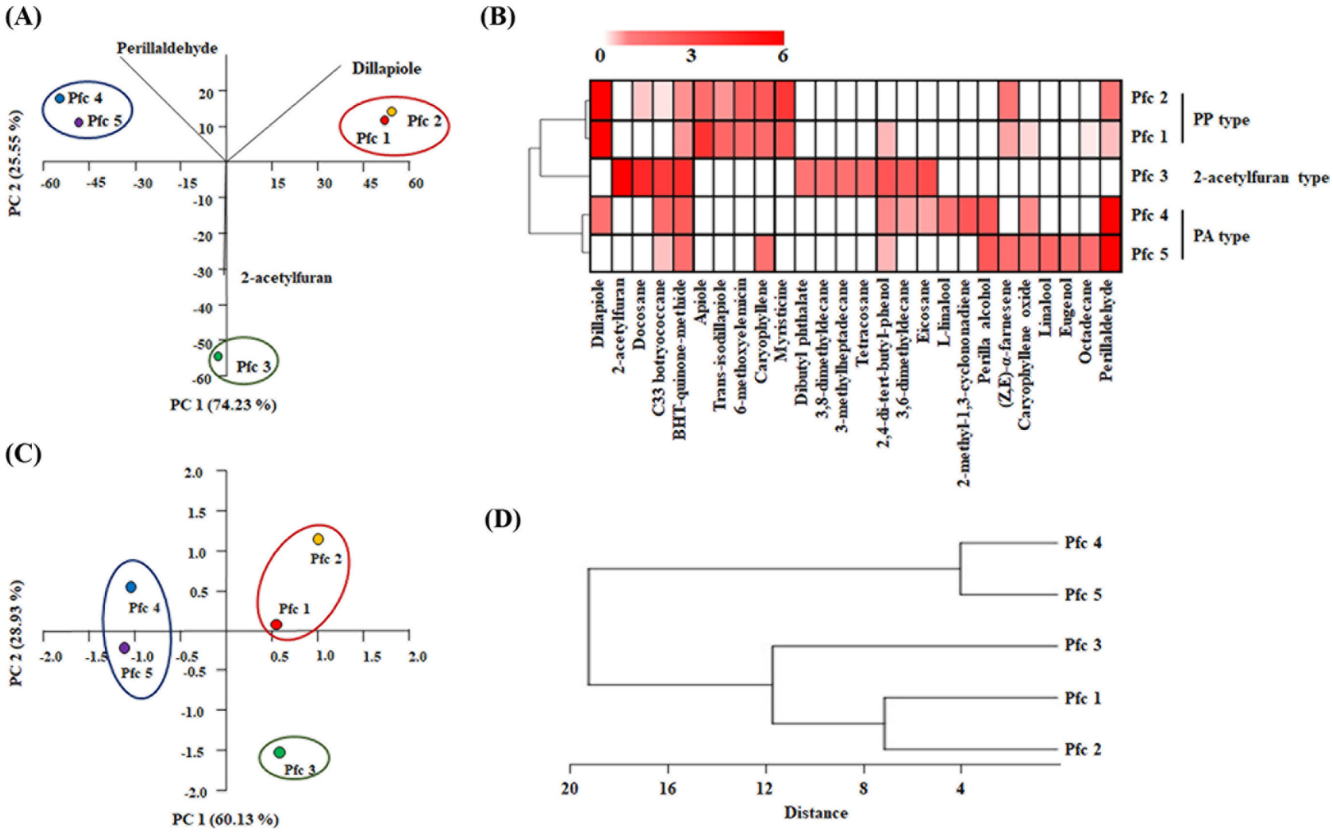

Fig. 1 Principal component analysis (PCA) and hierarchical cluster analysis (HCA) performed on essential oil compounds and antimicrobial activity of five genotype of *Perilla frutescens* var. crispa. PCA for essential oil compounds (A) and antimicrobial activity (C). HCA for essential oil compounds (B) and antimicrobial activity (D)

Table 2 Principal component coefficients of selected essential oil compounds from five genotype of *Perilla frutescens* var. crispa

Compounds	PC1	PC2
2-acetylfuran	-0.01	-0.61
Perillaldehyde	-0.68	0.56
Perilla alcohol	-0.02	0.02
BHT-quinone-methide	-0.02	-0.04
Myristicine	0.03	0.03
Dillapiole	0.73	0.53
Apiole	0.02	0.02

chemotype of EOs obtained from five different genotypes of Pfc. The results suggested that the five genotypes of Pfc grown in Korea can be classified into three chemotypes: PA type containing perillaldehyde, PP type containing dillapiole, and 2-acetylfuran type. In addition, PCA and HCA based on antimicrobial activity suggested that the variation in the antimicrobial activity of EOs of Pfc could attribute to the variation in the chemotypes. Taken together, our results support the importance of chemotype and provide useful information for breeding Perilla plants.

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