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Quantification of flavonoids isolated from *Mentha spicata* in selected clones of Turkish mint landraces

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Abstract: Mint has been used extensively in traditional medicines, spices, and herbal teas throughout the world. Isolation of flavonoids from *Mentha spicata* and quantification of these compounds in selected clones of Turkish mints landraces were achieved. *M. spicata* was dried in shade and then boiled in water. After filtration, the aqueous extract was partitioned with ethyl acetate. The solvent was removed under reduced pressure to yield the extract, subjected to silica gel column chromatography to isolate 5-demethyl sinensetin (**1**), hesperidin (**2**), didymin (**3**), and linarin (**4**), the structures of which were elucidated by 1D NMR, 2D NMR, and LC-TOF/MS. The isolated compounds were employed as standard flavonoids to determine the quantitative variations in cultivated Turkish mint landraces belonging to *Mentha* × *piperita* L., *M. spicata* L., and *M.* × *villosonervata* Opiz by HPLC. The quantities of hesperidin (**2**), didymin (**3**), and linarin (**4**) in *M. spicata* were found as 11.83, 3.85, and 42.21 mg/g dried plant weight (DW), respectively, while that of 5-demethyl sinensetin (**1**) in *M. villosonervata* was 2.9 mg/g DW. Consisting of pharmaceutically valuable compounds, *M. spicata* and cultivated Turkish mint landraces could be a source of 5-demethyl sinensetin (**1**), hesperidin (**2**), didymin (**3**), and linarin (**4**).

Key words: *Mentha* species, flavonoid, quantification, isolation

1. Introduction

Plants have been a focus of science recently due to a large variety of applications in food and pharmaceutical industries, as well as including fascinating bioactive compounds.[1–7]

Mentha, belonging to the family Lamiaceae, known as mint, is a famous genus that includes 18 species and 11 hybrids.[8] The most prevailing species, *M. spicata*, *M. canadensis*, and *M. piperita*, have economic importance due to their medicinal and aromatic values.[9,10] Mint oil is the second most important product after that of the *Citrus* species in the world's essential oil trade.[11] *M. arvensis*, *M. piperita*, and *M. canadensis*, including high menthol and menthone contents, are generally cultivated for essential oil production. However, spearmint species *M. spicata*, *M. longifolia*, and *M. villosonervata* are cultivated for spice and herbal tea. The main functional components of herbal tea are phenolic compounds rather than essential oil.[12]

Flavonoids are polyphenolic compounds that constitute a large group of secondary metabolites in plants.

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They have remarkable health-promoting effects such as antioxidant,[13] antiallergic,[14] antiinflammatory, antiulcer, antibiotic, and anticarcinogenic activities.[15] Polymethoxyflavones (PMFs) have gained great interest recently due to their large variety of biological activities such as antioxidant, anticarcinogenic, and antiinflammatory effects. PMFs are found in almost all members of the genus *Citrus* and are particularly more abundant in the peel than in other edible parts of the fruit.[16]

Nobiletin and 5-demethyl nobiletin were reported to have cell growth inhibition effects in human neuroblastoma SH-SY5Y cells.[17] Hesperidin, sinensetin, and nobiletin were found in *Citrus* fruit processing waste. Most food waste has been used in animal feed. A study presented the potential use of citrus waste as a source of biologically active compounds. The extraction method developed was inexpensive and the flavonoids could be obtained on a large scale.[18] *Citrus* fruit extract including hesperidin, sinensetin, and other flavonoids displayed promising in vitro antioxidant, antidiabetic, and antibacterial activities. It was suggested that consumption of the entire fruit, rather than its single compounds, may be more beneficial for health due to the complex mixture of compounds' interactions.[19] Sinensetin and nobiletin were found to exhibit inhibitory effects on the proliferation of endothelial cells in vitro and in vivo.[20]

Quality is a relative concept varying according to requirements. While essential oil composition is responsible for quality in cultivated mint, phenolic components have vital importance for herbal tea, spice, and medicinal uses because of their biological activities (antioxidant, antimicrobial, etc.). By means of characterization of essential oil composition in cultivated Turkish mint landraces, superior plants were selected according to yield and essential oil quality. However, there are no reports on phenolic compounds of the selected clones. Herein, flavonoids were isolated from *Mentha spicata* and these flavonoids were used as standard compounds to determine the quantity in cultivated Turkish mint landraces belonging to *Mentha* × *piperita* L, *M. spicata* L., and *M.* × *villosonervata* Opiz. The study may contribute to new knowledge related to the variation of the four isolated flavonoids in cultivated mint species.

2. Results and discussion

5-Demethyl sinensetin (**1**), hesperidin (**2**), didymin (**3**), and linarin (**4**) were isolated from the ethyl acetate extract of *Mentha spicata* by chromatographic techniques and their structures were elucidated by spectroscopic methods (Figure 1).

5-Demethyl sinensetin (**1**) is a polymethoxylated flavone isolated from *Citrus reticulata*.[21] The structure of 5-demethyl sinensetin (**1**) resembled that of 5-demethyl nobiletin. However, 5-demethyl nobiletin had a methoxy group at the C-8 position. Hence, the spectral data of both compounds were compatible.[22] The molecular formula of 5-demethyl sinensetin (**1**) was established as $C_{19}H_{19}O_7$ by LC-TOF/MS (m/z 359.1159 $[M+H]^-$) (calcd. 359.1131). Nineteen lines, of which four were methoxyl, five methines, nine quaternary carbon atoms, and one carbonyl carbon, in the ^{13}C NMR spectrum approved the proposed structure. In the ^1H NMR spectrum, four methoxy groups appeared at δ 4.02, 4.01, 4.00, and 3.96 as singlets. The characteristic signal of OH bonded to C5 resonated at δ 12.78 as a broad singlet. Due to the shielding effect, H8 resonated upfield at δ 6.63 as a singlet. The signal that appeared at δ 6.58 as a singlet belonged to H3. B ring protons signals were observed at the expected location. H2', H5', and H6' gave signals at δ 7.37 (1H, d, J = 2,0 Hz, H-2'), 7.0 (1H, d, J = 8.5 Hz, H-5'), and 7.55 (1H, dd, J = 8.5 and 2.0 Hz, H-6'), respectively. The structure of the second compound isolated, hesperidin (**2**), displayed the molecular formula of $C_{28}H_{35}O_{15}$ (m/z :611.2035 $[M^+ + H]^+$) (calcd. 611.1976). In the ^1H NMR spectrum, due to the diastereotopic protons bonded at C-3,

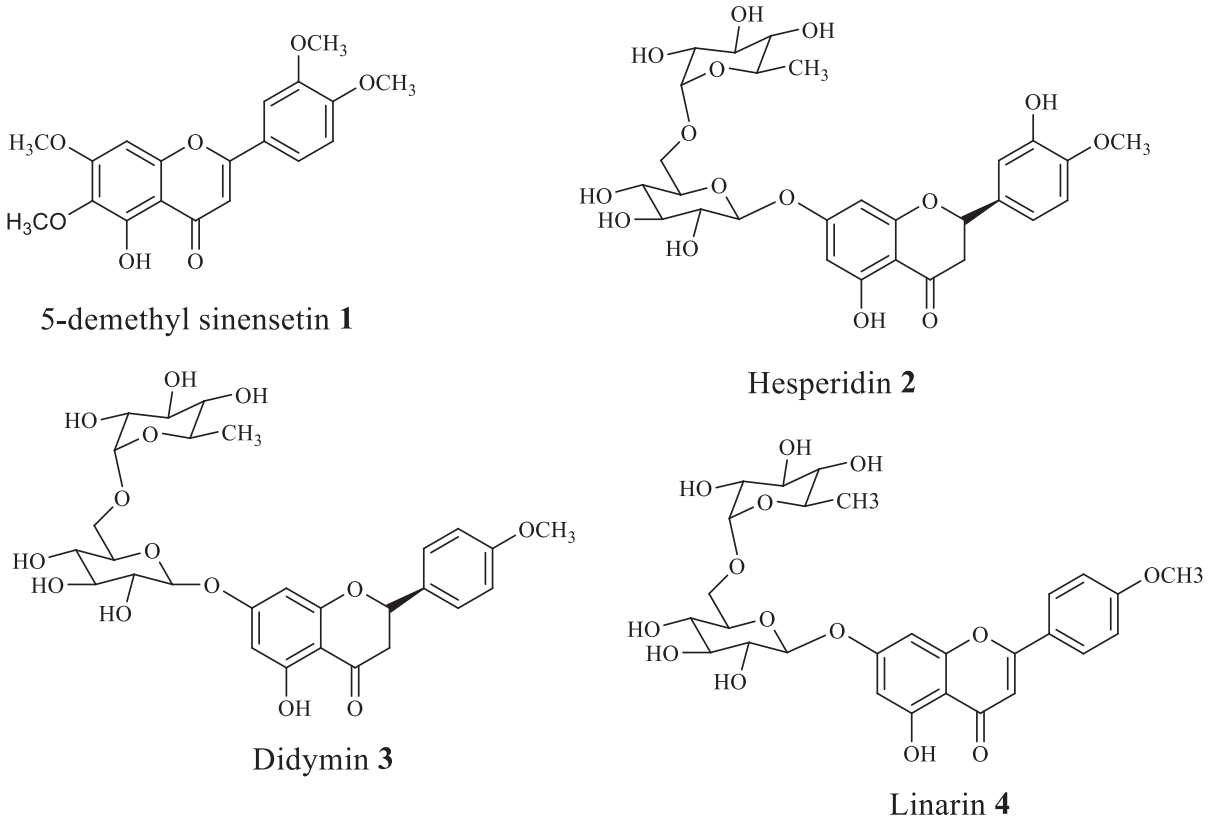

Figure 1. Isolated compounds from *Mentha spicata*.

the chemical shift of each proton was different. The signal observed at δ 2.77 (1H, dd, J =17.1 Hz, 3.1 Hz) belonged to H-3α. The other proton (H-3β) bonded to C-3 resonated at 3.27 (1H, dd, J = 17.1 Hz, 12.3 Hz). The methoxy group signal appeared at δ 3.78 as a singlet. The H-2 proton gave the signal at δ 5.52 (1H, dd, J = 12.3 Hz, 3.1 Hz). The signals of H-6 and H-8 were observed at δ 6.12 and 6.15 as a doublet (J = 2.2 Hz), respectively. The sugar proton signals appeared at δ 3.10–3.90. The ^{13}C NMR spectrum also confirmed the proposed structure. Didymin (**3**) was the third isolated compound. Its molecular formula was determined as $C_{28}H_{35}O_{15}$ by LC-TOF/MS (m/z 593.1966 [M -H]$^-$) (calcd. 593.1870). Its chemical structure resembled that of hesperidin (**2**). In hesperidin (**2**), there was a hydroxy at C-3'. Hence, NMR spectral values of didymin (**3**) looked like those of hesperidin (**2**). The molecular formula of linarin (**4**) was established as $C_{28}H_{35}O_{15}$ by LC-TOF/MS (m/z 591.1708 [M-H]$^-$) (calcd. 591.1714). In the ^{1}H NMR spectrum, the methoxy group resonated at δ 3.86 as a singlet. H-6 and H-8 protons gave the signals at δ 6.45 and δ 6.79 as a singlet, respectively. Due to the deshielding of the H-3 nucleus, its signal appeared more downfield (δ 6.92). The chemical shifts of B ring protons were observed at the expected location. ^{13}C NMR signals were in accord with the structure.

Sinensetin, a polymethoxy flavone found in *Citrus* peels, revealed various activities, such as antifungal, antimutagenic, anticancer, and antiinflammatory.[23] Hesperidin (**2**) is one of the common flavanone glycosides identified from mint species.[24,25] and it is found abundantly in *Citrus* fruits.[26] It has cholesterol-reduction and antiinflammatory properties.[27] Didymin (**3**) is another dietary flavanone glycoside having antioxidant and antiproliferative activity found in *Citrus* fruits.[28] Additionally, a phenolic glycoside, linarin (**4**) (acacetin-7-

$O-\beta$-D-rutinoside), was isolated from *M. arvensis.*[29] and *Lycopus lucides.*[30] The compound has a selective dose-dependent inhibitory effect on acetylcholinesterase. In addition, it reveals an inhibitory effect against *Chlamydophila pneumoniae*, an obligate intracellular bacterium.[12]

5-Demethyl sinensetin (**1**), hesperidin (**2**), didymin (**3**), and linarin (**4**) were quantified in leaf extracts of 13 selected clones from *Mentha* landraces belonging to three species: *M. piperita*, *M. spicata*, and *M. villosonervata* (Table 1). There are significant variations in the flavonoid quantities. The maximum value in 13 selected *Mentha* clones was obtained for linarin (**4**) at 42.21 mg/g. Three clones (Clone-13, -11, and -5) of *M. spicata* and one clone (Clone-9) of *M. villosonervata* had high values of linarin (**4**). On the other hand, two clones (Clone-3 and -8) of *M. piperita* had trace amounts of the compound. There are inter- and intraspecies variation for the compounds. Linarin (**4**) variation of *M. spicata* was between 2.97 and 42.1 mg/g, but hybrid species had trace amounts, except Clone-9 of *M. villosonervata* with 17.93 mg/g. Hesperidin (**2**), an isolated flavanone glycoside, was between 0.21 (in *M. piperita*) and 11.83 mg/g (in *M. spicata*) in the Turkish mint landraces. The maximum value of 11.83 mg/g was obtained from Clone-2 (*M. spicata*) and this was significant statistically compared with the data of other clones. Clone-15 (*M. spicata*) had the second highest hesperidin (**2**) amount at 9.72 mg/g, followed by Clone-3 with 7.1 mg/g (*M. piperita*) and Clone-12 with 6.16 mg/g (*M. spicata*). Contrary to linarin (**4**), the hesperidin (**2**) amount was between 1.72 and 4.28 mg/g in *M. villosonervata.*

Didymin (**3**) was the third flavone glycoside isolated from *Mentha spicata.* It was between 0.25 mg/g (*M. piperita*) and 3.85 mg/g (*M. spicata*). The maximum didymin (**3**) amounts were obtained in Clone-11 (*M. spicata*) with 3.85 mg/g and Clone-9 (*M. villosonervata*) with 3.09 mg/g.

Contrary to didymin (**3**), linarin (**4**), and hesperidin (**2**), the maximum amount of 5-demethyl sinensetin (**1**) was found in Clone-9 of *M. villosonervata* with 2.9 mg/g. Clone-13 (*M. spicata*) gave the second highest

Table 1. Quantitative analysis of isolated flavonoid in cultivated Turkish mint landraces.

No.	Species	Code	Compounds (mg/g dried plant)			
			1	**2**	**3**	**4**
1	*Mentha piperita*	Clone-3	0.07 ± 0.02e	7.10 ± 0.39c	0.29 ± 0.06b	0.04 ± 0.01g
2		Clone-8	0.33 ± 0.01d	0.21 ± 0.01h	0.25 ± 0.00b	0.27 ± 0.02g
3	*Mentha spicata*	Clone-2	0.46 ± 0.01cd	11.83 ± 0.83a	0.85 ± 0.05b	4.46 ± 0.35e
4		Clone-5	1.30 ± 0.13bc	3.37 ± 0.08de	0.83 ± 0.02b	16.31 ± 0.47d
5		Clone-10	0.98 ± 0.04cd	2.55 ± 0.03ef	0.55 ± 0.05b	2.79 ± 0.03f
6		Clone-11	0.64 ± 0.05cd	1.68 ± 0.09f	3.85 ± 0.08a	23.65 ± 0.90b
7		Clone-12	0.65 ± 0.01cd	6.16 ± 0.01c	0.98 ± 0.02b	3.77 ± 0.01ef
8		Clone-13	2.05 ± 0.02b	3.83 ± 0.16d	0.41 ± 0.08b	42.21 ± 0.12a
9		Clone-15	0.46 ± 0.04cd	9.72 ± 0.84b	0.48 ± 0.08b	2.97 ± 0.07ef
10	*M. villosonervata*	Clone-4	1.01 ± 0.01bc	3.21 ± 0.49de	0.34 ± 0.06b	0.43 ± 0.02g
11		Clone-7	1.18 ± 0.03bc	1.84 ± 0.04gf	0.28 ± 0.02b	0.26 ± 0.04g
12		Clone-9	2.90 ± 0.09a	1.72 ± 0.09f	3.09 ± 0.09a	17.93 ± 0.66c
13		Clone-14	1.23 ± 0.03bc	4.28 ± 0.02d	0.26 ± 0.02b	0.36 ± 0.01g

Different letters in the same column indicate significant differences at P >0.01. The results are expressed as mean ± SD of each treatment. Compounds: 5-Demethyl sinensetin (**1**), hesperidin (**2**), didymin (**3**), linarin (**4**).

5-demethyl sinensetin (**1**) value with 2.05 mg/g and belonged to a different statistical group than Clone-5 of *M. spicata* and Clone 14 of *M. villosonervata*, as those two clones were in the same group statistically. The minimum values were obtained from Clone-3 and Clone-8 of *M. piperita* at 0.07 and 0.33 mg/g, respectively. HPLC chromatograms of *Mentha spicata* ethyl acetate extract and didymin (**3**) are given in Figures 2 and 3, respectively.

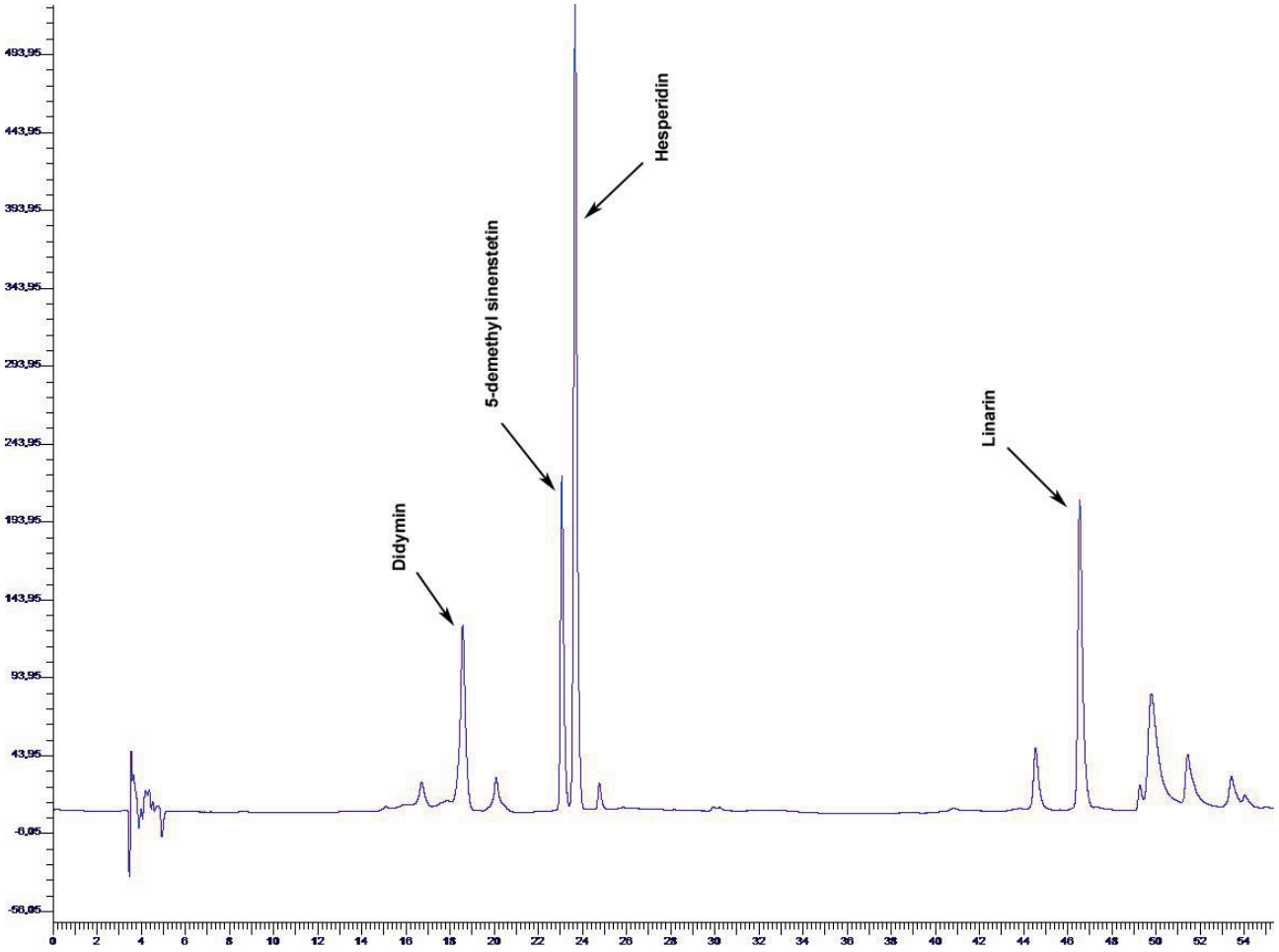

Figure 2. HPLC chromatogram of *Mentha spicata* ethyl acetate extract.

Comparing the amounts of compounds among the studied species, there are dramatic variations, both intraspecific and interspecific. While hesperidin (**2**) was between 0.21 and 7.10 mg/g in two clones of *M. piperita*, it was 1.68–11.83 and 1.72–4.28 mg/g in *M. spicata* and *M. villosonervata*, respectively. Despite qualitative records, there are no quantitative records of hesperidin amounts of the species, except *M. piperita* with 0.34%–1.31%.[24] Additionally, this research also contained the first record in which *M. villosonervata* contained hesperidin (**2**). Linarin (**4**) (acacetin-7-O-β-D-rutinoside) was isolated from the flower extract of *Mentha arvensis*.[29,31] and *Lycopus lucidus*,.[30] while didymin (**3**) was isolated from *Clinopodium chinense*.[32]

In contrast to other isolated compounds, the highest 5-demethyl sinensetin (**1**) amount was determined in *M. villosonervata* at 2.9 mg/g and it varied between 1.01 and 2.9 mg/g in the species, while it varied between

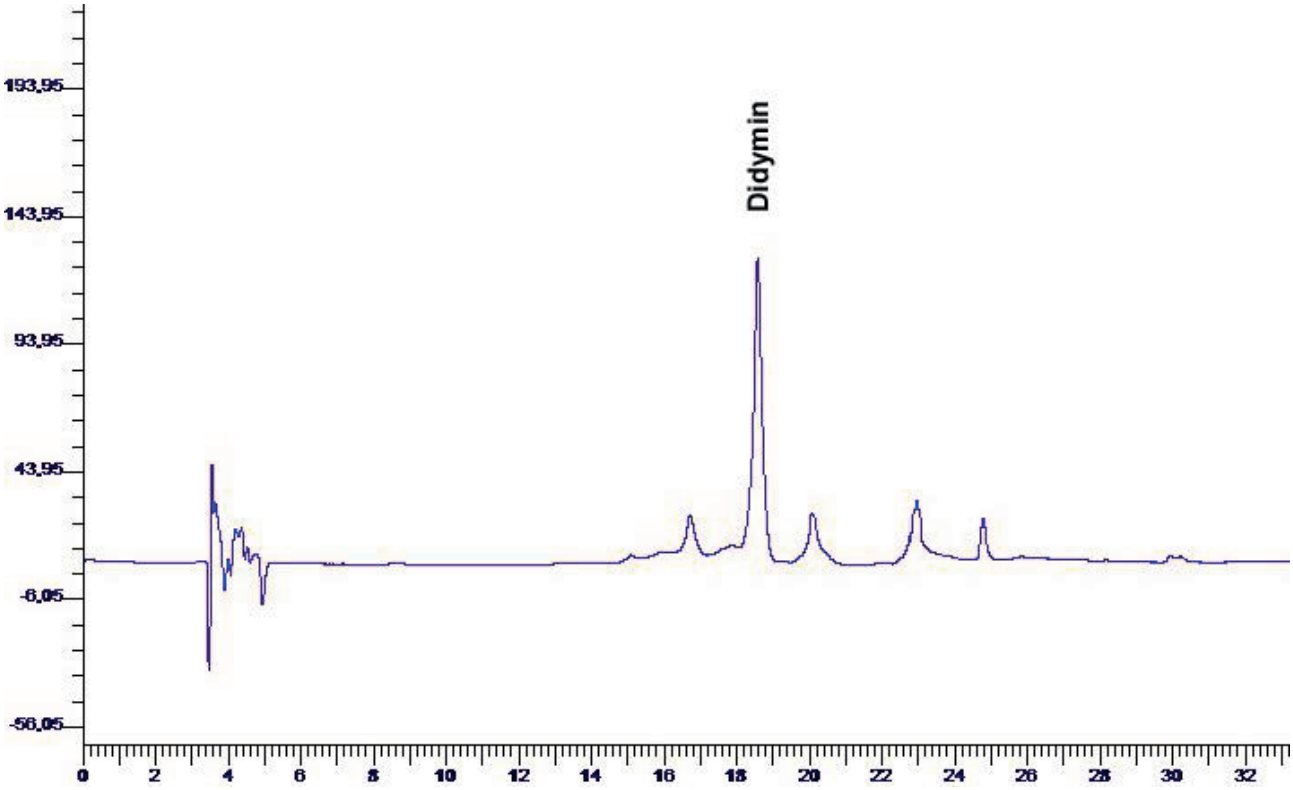

Figure 3. HPLC Chromatogram of didymin (**3**).

0.07 and 0.33 mg/g in *M. piperita* and 0.46 and 2.05 mg/g in *M. spicata*. The most 5-demethyl sinensetin (**1**) was found in Clone-9 of *M. villosonervata* and the most hesperidin (**2**) was found in Clone-14 of *M. villosonervata*. However, the most didymin (**3**) and linarin (**4**) existed in Clone-11 (*M. spicata*) and Clone-13 (*M. spicata*). If someone prefers to ingest these compounds from herbal tea, such tea should be prepared from the corresponding clones. On the other hand, due to the synergic effect of compounds in herbal tea, it is difficult to conclude which clone would be the most beneficial for herbal medicine.

3. Experimental

3.1. General experimental procedure

^{1}H and ^{13}C NMR spectra were recorded on 400 MHz and 100 MHz Bruker instruments, respectively. Chemical shifts are in ppm from Me_4Si, generated from DMSO-$d6$. Column chromatography was performed on silica gel (Kieselgel 60, 0.063–0.200 mm, Merck). Silica gel 660 (230–400 mesh, Merck) was used for flash chromatography and Kieselgel 60 F_{254} (0.5 mm thickness, Merck) was used for thin layer chromatography (TLC). TLC spots were observed with an UV light and sprayed with ceric sulfate reagent [cerium(IV) sulfate in aqueous methanol], then heated. LC-TOF/MS analysis was performed on an Agilent 6210 TOF-LC/MS with Zorbax SB-C18 column (4.6 × 150 mm, 2.7 µm, Agilent Technologies) with injection volume of 10 µL. The mobile phase consisted of the eluent A – water with 0.1% formic acid and 10 mM ammonium formate and B – acetonitrile. The flow rate was 0.7 mL/min at 35 °C. The gradient program was fixed as follows: 0–1 min, 10% B; 1–8 min, 95% B; 8–11 min, 95% B; 11–11.1 min, 10% B; 11.1–13 min, 10% B. The column temperature was 35 °C. The total time of

evaluation was 20 min. TOF analyses were carried out in positive ion mode, gas temperature 325 °C, drying gas flow 7 mL/min, fragmentor voltage 175 V.

3.2. Plant material

Thirteen mint clones selected from Turkish landraces, belonging to three species, (*Mentha piperita*, *M. spicata*, and *M. villosonervata*) and cultivated in different regions of Turkey, were introduced to experimental areas of the Agriculture Faculty at Tokat Gaziosmanpaşa University. Rooted cuttings of each clone were planted in experimental plots with three replications. Plants were harvested at the flowering stage in mid-July 2014. After being dried in oven at 35 °C, the leaves were separated from the aerial parts of the plants before beginning the extraction procedure.

3.3. Extraction and isolation

The air-dried and powdered aerial parts of *M. spicata* (1000 g) were boiled in water for 2 h. After cooling to room temperature, aqueous extract (AE) was filtered to remove plant residue. The AE was partitioned with ethyl acetate (2.5 L × 2) to yield ethyl acetate extract (EE). After removing the solvent at 50 °C by rotary evaporator, the extract (25 g) was subjected to silica gel column chromatography, eluting with hexane, hexane-ethyl acetate, ethyl acetate, ethyl acetate-methanol, and methanol, respectively, with increasing polarity giving 300 fractions and each fraction was collected as 250 mL. Fractions 161–166 gave 5-demethyl sinensetin (**1**), fractions 230–240 gave hesperidin (**2**), fractions 258–276 gave didymin (**3**), and fractions 280–290 gave linarin (**4**). The flavonoid compounds were identified by 1D NMR, 2D NMR, and LC-TOF/MS. The isolated flavonoids were used as standard for quantitative analysis by HPLC.

5-demethyl sinensetin (**1**): LC-TOF/MS m/z: 359.1159 $[M+H]^+$, calcd. ($C_{19}H_{19}O_7$, 359.1131). UV/Vis (MeOH), λ_{max}: 340, 280. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.96 (3H, s, OCH_3), 4.00 (3H, s, OCH_3), 4.01 (3H, s, OCH_3), 4.02 (3H, s, OCH_3), 6.58 (1H, brs, H-3), 6.63 (1H, brs, H-8), 7.0 (1H, d, J =8.5 Hz, H-5'), 7.37 (1H, d, J = 2,0 Hz, H-2'), 7.55 (1H, dd, J = 8.5 and 2.0 Hz, H-6'), 12.78 (1H, s, 5-OH). ^{13}C NMR (CDCl3): δ 56.1, 56.2, 56.4, 60.9 (4 × OCH_3), 90.6 (C-8), 104.5 (C-3), 106.2 (C-10), 108.9 (C-2'), 111.2 (C-5'), 120.1 (C-6') 123.8 (C-1'), 132.7 (C-6), 149.4 (C-5), 152.3 (C-3'), 153.1 (C-4'), 153.1 (C-9), 158.8 (C-7), 164.0 (C-2), 182.6 (C-4).

Hesperidin (**2**): LC-TOF/MS m/z: 611.2035 $[M^+ + H]^+$, calcd. ($C_{28}H_{35}O_{15}$, 611.1976). UV/Vis (MeOH), λ_{max}: 333, 269. ^{1}H NMR (DMSO-$d6$) δ 2.77 (1H, dd, J =17.1 Hz, 3.1 Hz, H-3α) 3.27 (1H, dd, J = 17.1 Hz, 12.3 Hz, H-3β), 3.78 (3H, s, OCH_3-4'), 5.52 (1H, dd, J = 12.3 Hz, 3.1 Hz, H-2), 6.12 (1H, d, J = 2.2 Hz, H-6), 6.15 (1H, d, J =2.2 Hz, H-8), 6.92 (1H, H-2'), 6.94 (2H, H-5', H-6', overlapped), 12.02 (1H, s, OH-5), [sugar moiety] 1.08 (3H, d, J = 6.2 Hz, H-6'''), 3.10–3.90 (10 sugar protons), 4.52 (1H, brs, H-1'''), 4.99 (1H, d, J = 7.4 Hz, H-1''), 5.52, 5.42, 5.20, 5.01, 4.70, 4.62 (6H, sugar hydroxyls). ^{13}C NMR (DMSO-$d6$): [aglycone moiety] δ 42.5 (C-3), 56.2 (OCH_3), 78.8 (C2), 96.0 (C-8), 96.8 (C-6), 103.8 (C-10), 112.5 (C-5'), 114.6 (C-2'), 118.4 (C-6'), 131.4 (C-1'), 146.9 (C-3'), 148.4 (C-4'), 162.9 (C-9), 165.6 (C-7), 197.5 (C-3); [sugar moiety]: glucose; 66.5 (C-6''), 70.1 (C-4''), 73.5 (C-3''), 76.0 (C-5''), 76.7 (C-2''), 99.9 (C-1''); rhamnose; 18.3 (C-6'''), 68.8 (C-5'''), 70.7 (C-3'''), 72.5 (C-2'''), 72.5 (C-4'''), 101.1 (C-1''').

Didymin (**3**): HRMS m/z: 593.1966 $[M-H]^-$, calcd. ($C_{28}H_{35}O_{15}$, 593.1870). UV/Vis (MeOH), λ_{max}: 283. ^{1}H NMR (DMSO-*d6*) [aglycone moiety] δ 2.78 (1H, dd, J = 17.1, 2.8 Hz, H-3a), 3.34 (1H, dd, J = 17.1,

12.8 Hz, H-3b), 3.77 (3H, s, OCH_3), 5.57 (1H, d, J = 12.6, 2.8 Hz, H-2), 6.14 (2H, d, brs, H-6, H-8), 6.99 (2H, d, J = 8.8 Hz, H-3', H-5'), 7.48 (2H, d, J = 8.8 Hz, H-2'-H-6'), 12.03 (1H, s, OH-5), [sugar moiety] 1.08 (3H, d, J = 6.1 Hz, rhamnose H-6'''), 4.52 (1H, brs, H-1'''), 4.97 (1H, d, J = 7.4 Hz, H-1''), 3.10–3.70 (10 sugar protons), 4.47, 4.63, 4.72, 5.00, 5.20, 5.42 (6H, sugar hydroxyls). ^{13}C NMR (DMSO-*d6*) [aglycone moiety] δ 42.3 (C-3), 55.6 (OCH_3), 78.8 (C-2), 95.9 (C-6), 96.9 (C-8), 103.8 (C-10), 114.4 (C-3', C-5'), 128.9 (C-2', C-6'), 130.8 (C-1'), 160.0 (C-4'), 163.0 (C-9), 163.0 (C-5), 165.6 (C-7), 197.5 (C-4), [sugar moiety]: glucose: 66.5 (C-6''), 71.2 (C-4''), 76.0 (C-5''), 76.7 (C-3''), 78.8 (C-2''), 99.9 (C-1''), rhamnose: 18.3 (C-6''''), 68.8 (C-5'''), 70.1 (C-2'''), 70.7 (C-3'''), 72.5 (C-4'''), 101.1 (C-1''').

Linarin (**4**): HRMS m/z: 591.1708 [M-H]?. calcd. ($C_{28}H_{35}O_{15}$, 591.1714). UV/Vis (MeOH), λ_{max}: 335, 268. ^{1}H NMR (DMSO-*d6*) δ 3.86 (3H, s, OCH_3-4'), 6.45 (1H, d, brs, H-6), 6.79 (1H, d, brs, H-8), 6.92 (1H, s, H-3), 7.14 (2H, d, J =8.8 Hz, H-3'and H-5'), 8.04 (2H, d, J = 8.8 Hz, H-2'and H-6'), 12.89 (1H, s, OH-5); [sugar moiety]: 1.07 (3H, d, J = 6.4 Hz, H-6'''), 3.10–3.90 (10 sugar protons), 4.54 (1H, brs, H-1'''), 5.04 (1H, d, J = 6.9 Hz, H-1''), 4.50, 4.69, 4.80, 5.27, 5.31, 5.55 (6H, sugar hydroxyls). ^{13}C NMR (DMSO-*d6*): [aglycone moiety] δ 56.0 (OCH_3), 95.3 (C-8), 100.4 (C-6), 104.2 (C-3), 105.9 (C-10), 115.2 (C-3', C-5'), 123.1 (C-1'), 129.0 (C-2', C-6'), 157.4 (C-9), 161.6 (C-5), 162.9 (C-4'), 163.4 (C-7), 164.5 (C-2), 182.5 (C-4); [sugar moiety] glucose: 66.5 (C-6''), 71.2 (C-4''), 73.5 (C-3''), 76.1 (C-5''), 76.7 (C-2''), 100.1 (C-1''); rhamnose: 18.2 (C-6'''), 68.8 (C-5'''), 70. 8 (C-3'''), 72.1 (C-4'''), 72.5 (C-2'''), 100.9 (C-1''').

3.4. Preparation of samples and standard solutions

Samples of clones were powdered to a homogeneous size with a mill (Sinbo, SCM-2910, China). Powder (100 mg) was accurately weighed and extracted with 10 mL of methanol and dichloromethane (50:50; v:v) in a test tube for 6 h. After filtration over a syringe-type filter (Chromtech, 13 mm, 0.22 µm), the filtrate was injected into the HPLC system for analysis. Accurately weighed solid portions of each standard were dissolved in methanol to prepare stock solutions. Working solutions were obtained by diluting the stock solutions with methanol. The final mixed standard solution containing 100 µg/mL of each standard was used for the calibration curve with different concentrations (5, 25, 50, and 100 µg/mL). For HPLC, a PerkinElmer Series 200 liquid chromatography system (PerkinElmer, USA) equipped with a quaternary solvent delivery system and UV detector was used at 280 nm. The analytes were separated on a Phenomenex Kromasil 100 A C18 column (250 × 4.60 mm, 5 µm). The column temperature was maintained at 26 °C using the column oven. The mobile phase consisted of acetonitrile (A) and water containing 2.5% formic acid (B). The following gradient conditions were used: initial 0–3 min, held at A-B (15:85, v/v); 3–13 min, linear change from A-B (15:85, v/v) to A-B (25:75, v/v); 13–53 min, linear change from A-B (25:75, v/v) to A-B (100:0, v/v); and 53–60 min, isocratic elution A-B (100:0, v/v). The mobile phase flow rate was set at 0.7 mL/min and the injection volume was 20 µL.

3.5. Method validation

The analytical method was validated to determine the linearity, limits of detection (LODs), limits of quantification (LOQs), and precision. The relationship between peak area and concentration was found to be linear from 5 to 100 µg/mL for each compound. Linearity was evaluated using linear regression analysis of four points for each compound. Linear plots consist of three replicates per point. The correlation coefficients (R^2 values) were found to be $\geq$0.99. Linear regression equations of the compounds are presented in Table 2. The LODs and

LOQs of compounds for the HPLC method are given in Table 2. LODs and LOQs were determined by using the signal-to-noise method. A signal-to-noise ratio of three was accepted for estimation of LODs and signal-to-noise ratio of 10 was used for estimation of LOQs.[33] The repeatability of intraday values (relative standard deviation, RSD%) was determined for compounds using the corresponding peak areas of three replicate analyses at approximately 5 mg/kg concentration level. The trueness was examined as recovery of each compound from mixed stock standard solutions in cultivated Turkish mint landraces belonging to *Mentha × piperita*, *M. spicata*, and *M. × villosonervata*. The recovery was evaluated by means of three replicate measurements in a day. The average recovery data of the reported compounds were determined from the following formula and the recovery is given in Table 2.

Table 2. Method validation parameters of isolated compounds.

Comp.	Linear regression equation	R^2	LOD (µg/mL)	LOQ (µg/mL)	% RSD_r	% RSD_R	Recovery (%)
1	y = 57449x – 1305.9	0.999	1.12	3.40	3.42	3.74	96.40
2	y = 27107x – 85921	0.999	1.01	3.05	2.89	2.76	98.71
3	y = 35656x + 1342.2	0.999	0.96	2.91	3.21	3.54	95.77
4	y = 45315x + 28465	0.999	0.84	2.54	2.98	3.41	97.25

LOD: Limit of detection, LOQ: limit of quantification, RSD_r: relative standard deviation of repeatability, RSD_R: relative standard deviation of reproducibility. The standard solution was used for calibration curves with various concentrations (5, 25, 50, and 100 µg/mL). Compounds: 5-Demethyl sinensetin (**1**), hesperidin (**2**), didymin (**3**), linarin (**4**).

$$\text{Recovery } (100\%) = \left(\frac{\text{Measured concentration}}{\text{Spiked concentration}}\right) \times 100$$

The concentrations of compounds in Turkish mint landraces were obtained from one of the corresponding calibration curves. Finally, the calculated concentrations were converted to mg/g of the crude extract sample.

Expected precision (repeatability) as a function of analyte concentration of a method is the closeness of agreement between independent test results obtained under stipulated conditions. Precision is usually expressed in terms of imprecision and computed as the RSD of the test results. The imprecision of a method increases as the concentration of the analyte decreases. Predicted relative standard deviation of reproducibility ($PRSD_R$) was calculated by the Horwitz formula.

$PRSD_R = 2C^{-0.15}$

C: Mass fraction

For 5 ppm, $PRSD_R = 2 \times (5 \times 10^{-6})^{-0.15} = 12.48 = 12$ $PRSD_r = 6$

For 10 ppm, $PRSD_R = 2 \times (10 \times 10^{-6})^{-0.15} = 11.25 = 11$ $PRSD_r = 5$

After the evaluation of recovery results, the values were found to be between 95.77% and 98.71%. For recovery work, the samples were spiked with standards in which the final concentrations were 5 ppm. The recovery was evaluated using three replicate measurements per day. The average recovery data of the reported compounds are presented in Table 2.

3.6. Statistical analysis

The numerical data of flavonoids are presented as mean ± standard deviation calculated from replications. The values were subject to analysis of variance (ANOVA) using randomized block design. The mean data that were significant in variance analysis were grouped with Duncan multiple tests (P <0.01) using SPSS software.

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