



Characterization of phenolic compounds from normal ginger (*Zingiber officinale* Rosc.) and black ginger (*Kaempferia parviflora* Wall.) using UPLC–DAD–QToF–MS

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Abstract

Zingiberaceae is the large diverse family comprised of rhizomatous plants with a higher concentration of phenolic compounds. The normal ginger (*Zingiber officinale* Rosc.) and black ginger (*Kaempferia parviflora* Wall.) belongs to this family and have similar morphological characteristics of rhizome, but their phenolic composition revealed differently. On this study, the phenolic composition of normal ginger (from Ethiopia and Korea) and black gingers (from Korea and Thailand) were profiled using ultra performance liquid chromatography coupled with diode array detector, quadrupole time-of-flight mass spectrometry (UPLC–DAD–QToF–MS). The result revealed that gingerol-related phenolic acid was detected only in normal gingers while, the methoxyflavones were identified exclusively in black gingers. 6-gingerol, 8-gingerol, 10-gingerol, 1-dehydro-6-gingerdione, and diacetoxy-8-gingerdiol were the major constituents among 18 phenolic acids detected from normal gingers, and 3,5,7,3',4'-pentamethoxyflavone and 5,7,4'-trimethoxyflavone were confirmed as predominant constituents among 13 methoxyflavones from black gingers. The total phenolic content was 434.7 and 698.1 mg/100 g dry weight (DW) in Korean and Ethiopian sample, respectively. The total methoxyflavone concentration was 4388.0 mg/100 g DW and 3940.2 mg/100 g DW in Korean and Thai black ginger sample, respectively. The higher concentration of methoxyflavones is from advanced biosynthetic pathway related to accumulated color of black ginger. In addition, new possible structural fragmentation was proposed for gingerol homologous series as additional pathway under the positive ionization mode, and further rationalized that ferulic acid moiety is the biosynthetic precursor. The result suggested that despite the morphological similarity, normal and black ginger showed distinct biosynthetic difference to synthesis their major secondary metabolites.

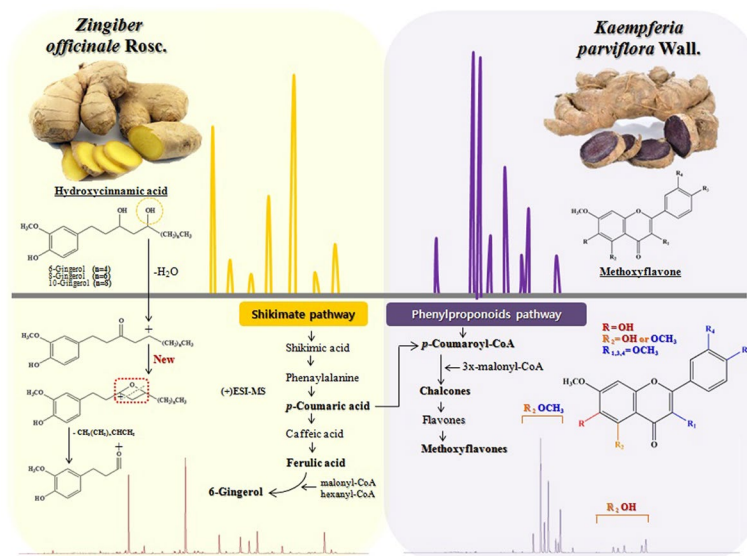
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Graphical Abstract



Keywords *Kaempferia parviflora* Wall. · Phenolic compounds · UPLC–DAD–QToF–MS · Zingiberaceae · *Zingiber officinale* Rosc.

Introduction

The Zingiberaceae family comprises about 53 genera and a total of over 1200 species [1]. It is widely distributed in the tropical region of Asia, Africa, and America. These plants are taxonomically characterized as perennial, aromatic, tuberous and non-tuberous rhizomes [2]. The rhizome is the main part of the plant which is economically important and a rich source of effective phytoconstituents for biological activities [2, 3].

Ginger (*Zingiber officinale* Rosc.) is one of the most popular species belonging to this family. China, Thailand, Vietnam, India, and Indonesia are considered to be center of origin. It has been mainly utilized as a food seasoning ingredient and medicinal resource particularly for treating diseases related to inflammation and oxidative stress [4–8]. For its wide range of health functionality, the presence of higher concentration of phenolic derivatives plays an important role [5, 8–10].

Over the last three decades, extensive studies have been conducted to understand the detailed chemical composition and biological activities of normal ginger. Phenolic acids, diarylheptanoids, terpenoids, and flavonoids were reported to exist in ginger rhizomes [10–19]. In Supplementary Table 1, comprehensive library containing a total of 72 gingerols and diarylheptanoids derivatives from ginger rhizomes is presented as summary. Gingerol- and shogaol-related derivatives are the principal medicinally active

components contributing to the characteristic pungent flavor of ginger together with essential oil major component, zingerone [19, 20]. The maturation state, environment, cultivar, and processing steps are major factors to influence the biosynthesis and concentration of the chemical composition in the rhizomes [21].

Black ginger (*Kaempferia parviflora* Wall.), Kra-chai-Dum (in Thai), is another species with deep purple-colored rhizomes under the Zingiberaceae family, and shares a morphological resemblance with normal ginger. Thailand is recognized as a center of diversity, and distribution to different region of Asia [22]. In previous studies, 25 methoxyflavones and their glycosides were identified and quantified as predominant flavonoids from rhizome extracts (Supplementary Table 2) [23–26]. The presence of these compounds contributed to a wider range of medicinal effects such as anti-inflammatory [27], antioxidant [28, 29], antiproliferative activity [30], hepatoprotective effect [31], and anticancer [32].

In Ethiopia, the history of ginger cultivation and utilization dates back from thirteenth century, and it is one of the spices contributing significantly to the country economy [33]. However, regardless of the significant cultivation, the status of phenolic constituents and its contribution to product quality standardization remains unstudied. Also, no studies have been reported on phenolic composition comparisons between normal and black ginger. Hence, in this study, the phenolic composition of gingers cultivated in

Ethiopia, Korea, and Thailand were profiled in reference to constructed LC–MS libraries. Furthermore, the different phytochemical biosynthesis between normal and black ginger were considered.

The aims of the present study were to characterize the phenolic compounds of the normal and black ginger using ultra performance liquid chromatography coupled with diode array detector, quadrupole time-of-flight mass spectrometry (UPLC–DAD–QToF–MS), and to conduct quantitative comparison between the test samples.

Materials and methods

Chemicals

Acetonitrile, methanol, and water (HPLC-grade) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid was purchased from Junsei Chemical (Tokyo, Japan). 6-gingerol, 8-gingerol, 10-gingerol, 6-shogaol, 8-shogaol, and 10-shogaol were used as pure standards for identification. Galangin and 2,4,5-trimethoxycinnamic acid were used as internal standard. All standard chemicals were purchased from Sigma Aldrich Co. (St. Louis, MO, USA).

Plant material

Four ginger rhizome samples (each two samples of *Zingiber officinale* Rosc. and *Kaempferia parviflora* Wall.) were obtained in 2017. The voucher specimens were deposited in the Department of Agro-food Resources, National Institute of Agricultural Sciences, Rural Development Administration (Korea). Korean ginger (RDAZO117) was purchased from the local market (Wanju province, Korea). Ethiopian ginger (variety name; Bozia mau32/b 79, RDAZO217) was obtained from the local market (Ziway city, Ethiopia). Black ginger (RDAKP117) was obtained from Jeju Island (Korea). Thai black ginger (RDAKP217) was purchased from NEXTOBE co. LTD (Thailand). All ginger samples were lyophilized, pulverized and stored below $-60\text{ }^{\circ}\text{C}$ prior to analysis.

Extraction of phenolic acids and methoxyflavones

Sample extraction was conducted according to the method described by Kim et al. [34] with minimum modification. The powdered rhizomes (1 g) were mixed with 10 mL of extraction solvent (methanol: water: formic acid=80:15:5, v/v/v) containing 250 ppm of 2,4,5-trimethoxycinnamic acid as internal standard. The mixture was vortexed, stirred with an orbital shaker for 5 min at 200 rpm and centrifuged for 15 min at 3000 rpm, $10\text{ }^{\circ}\text{C}$. The supernatant was filtered using syringe filter (0.45 μm , PVDF, Whatman, Kent, England). 0.5 mL of the filtrate was diluted with water to 5 mL

of final volume. The crude phenolic extract was then isolated by solid phase extraction method using Sep-Pak C₁₈ cartridge (Waters Co., Milford, MA, USA). The cartridge was activated by washing with 2 mL of methanol, followed by 2 mL of water for conditioning. Then the diluted phenolic extract was loaded in to the cartridge, and impurities were removed by washing with 2 mL of water. Finally, the crude phenolic acid was eluted from the cartridge using 3 mL of methanol. The purified extract was concentrated using N₂ gas, and then re-dissolved with 0.2 mL of the extract solvents without internal standard prior to instrumental analysis. For identification of methoxyflavones from black ginger, 1 g of powdered sample was taken and extracted with solvent system (methanol: water: formic acid=50:45:5, v/v/v) containing 100 ppm of galangin as an internal standard. The extraction and purification steps were followed as described above. All experimental analyses were performed in triplicates.

UPLC–DAD–QToF–MS analysis

Ultra performance liquid chromatography with photodiode array detector (Waters Co., Milford, MA, USA) coupled with quadrupole time-of-flight mass spectrometry (Waters Micromass, Manchester, UK) was used for analysis. UV spectra were taken in the region of 210–400 nm (representative wavelengths of 280 nm, 350 nm for phenolic acid and methoxyflavone, respectively). Chromatographic condition was conducted: column, Cortexs[®] UPLC[®] T3 1.6 μm C18, 2.1 mm \times 150 mm (Waters Co.); pre-column, Security Guard ULTRA Cartridges, UHPLC C18 for 2.1 ID column (Phenomenex, Torrance, CA, USA); oven temperature $30\text{ }^{\circ}\text{C}$; for phenolic acid analysis, mobile phase was used 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B); flow rate 0.30 mL/min (the elution gradient used as follow: 0–15 min, 5% B; 15 min, 35% B; 20 min, 50% B; 35–40 min, 75% B; 45–50 min, 5% B). For methoxyflavone analysis, 0.5% formic acid in water (A) and 0.5% formic acid in acetonitrile (B); flow rate 0.30 mL/min (the elution gradient used as follow: 0–15 min, 5% B; 15 min, 38% B; 30 min, 50% B; 35–40 min, 80% B; 45–50 min, 5% B). The mass spectrometric settings used were: ion source temperature $140\text{ }^{\circ}\text{C}$, desolvation temperature $500\text{ }^{\circ}\text{C}$, desolvation gas 1020 L h^{-1} , capillary voltage 3.5 kV, and sampling cone voltage 40 V. Mass analysis was run in positive ion mode using an electrospray ionization (ESI) source, and their range measured 50–1000 m/z in full scan mode.

Identification and quantification of phenolic compounds

The identification of phenolic compounds was conducted for the pure standard available, by comparing their UV and

mass spectral data. The rest of the peaks were determined by comparing retention time, UV spectra, and mass fragmentation presented in the library constructed from previous literatures (Supplementary Tables 1 and 2). The quantification was performed using internal standard without considering relative response factor.

Statistical analysis

The data analysis was performed by SPSS statistical data analysis software (PASW statistic 18). All data expressed as a mean \pm standard error of the mean (SEM). Data were considered statistically significant at a value of $p < 0.05$.

Results and discussion

Several analytical approaches were developed to identify phytochemicals and authenticating ginger samples from possible contaminating plant material having similar appearance with ginger (adulterants) [12]. High-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (HPLC–ESI–MS/MS) has shown better sensitivity for identification of compounds from ginger rhizomes [11, 13, 19, 21]. Recently, with the current analytical technology advancement, UPLC–DAD–QToF–MS becomes a powerful tool in providing excellent sensitivity and resolution for determination of natural products. Moreover, it provides best separation capability in higher throughput and time saving to run complex sample matrix compared to HPLC–ESI–MS/MS [35]. With this merit, in the current study, we identified phenolic compounds from normal and black ginger samples using UPLC–DAD–QToF–MS.

The phenolic acids and methoxyflavones characterized in the normal and black gingers are presented in Tables 1 and 2, respectively, including UV spectra, mass fragmentation (m/z), and contents. Especially, the positive ionization mode was used to obtain clear mass fragmentation as well as sodium (Na^+ , m/z 23) and potassium (K^+ , m/z 39) adducts ion which helps to confirm the parent ion of the peak unambiguously [11, 36]. Gingerol-related phenolic acid derivatives (wavelength at 280 nm) were identified only from normal ginger, while black ginger showed different spectral peaks on UPLC–DAD chromatograms (Figs. 1, 3).

Characterization of phenolic acids from normal gingers

Figure 1 showed that peaks 3, 7, and 13 were eluted at retention time of 19.4, 23.3, and 27.7 min with high intensity at 280 nm. These peaks, respectively, were differentiated by 28 Da ($\text{CH}_2\text{--CH}_2$ moiety) for their corresponding $[\text{M}+\text{Na}]^+$, $[\text{M}+\text{H}+\text{H}_2\text{O}]^+$, and $[\text{M}+\text{H}+2\text{H}_2\text{O}]^+$. Actually,

fragment ions at m/z 259.1943, 287.2295, and 315.2657 were observed after losing of $2\text{H}_2\text{O}$ from the parent ions as well as constant patterns of m/z 179.0899, 177.1153, 145.0795, 137.0734, 117.0813, and 115.0669 from each peak. In the previous study, Jiang et al. [11] proposed the fragmentation scheme of gingerol-related derivatives. In peak 3, the predominant ion (m/z 177.1153) was formed due to the loss of a neutral alkyl moiety $[\text{CH}_3(\text{CH}_2)_4\text{CHO}$, 100 Da] from rearranged structure of $[\text{M}+\text{H}+\text{H}_2\text{O}]^+$, and further fragmented by loss of CHCHCH_2 (40 Da) to form m/z 137.0734 (Scheme 1b). However, as shown in Fig. 2a, the m/z 179.0899, 145.0795, and 117.0813 ions observed could not be rationalized from this fragmentation scheme. This suggested that peak 3 have another additional fragmentation pathway under positive ionization mode. From gingerol-related structures ($n=4,6,8$), product ions at m/z 179.0899 were formed with the remained ketone group after cleavage of bond at C3–C4 $[-\text{CH}_3(\text{CH}_2)_n\text{CHCH}_3]$ (Scheme 1a) instead of cleavage of bond at C4–C5 with the neutral alkyl chains $[-\text{CH}_3(\text{CH}_2)_n\text{CHO}]$ (Scheme 1b), and eventually produces the predominant ion at m/z 177.1153 by the loss of 2H from C2–C3 position. As the biosynthesis precursor for gingerols derivatives, it was reported that ferulic acid produced intense peak at m/z 177 after the removal of H_2O from the protonated ion ($[\text{ferulic acid}+\text{H}]^+$, m/z 195), these ions showed subsequent losses of CH_3OH and $\text{CH}_3\text{OH}+\text{CO}$ to yielded the fragment ions at m/z 145 and 117, respectively [36]. Based on these proposed fragmentation, the additional accurate ions at m/z 145.0795 and 117.0813 actually resulted from subsequent loss of methoxyl group and $-\text{CO}$ from 177.1153 ions, respectively (Scheme 1a). Therefore, the higher abundance of fragment ion at m/z 177.1153 was observed from the two fragmentation pathways. In the previous literature review, this type of fragmentation behavior for gingerol derivatives was not discussed hence we propose possible fragmentation scheme for the first time in our report. Finally, by comparing the MS data with pure standards, peak 3, 7 and 13 were identified as 6-gingerol, 8-gingerol, and 10-gingerol according to the fragmentation presented above, respectively.

Peak 4 eluted at 21.38 min and showed similar fragmentation pattern to 6-gingerol with methylene (CH_2 , 14 Da) increment on the fragment ion, which indicated that it is a methylated derivative of 6-gingerol. The position of methylation was confirmed by fragment ions at m/z 191.1283 ($[\text{M}+\text{H}+\text{H}_2\text{O}+\text{CH}_3(\text{CH}_2)_4\text{CHO}]^+$ or $[\text{M}+\text{H}+\text{H}_2\text{O}+\text{CH}_3(\text{CH}_2)_4\text{CHCH}_3]^+$) and 151.0897 $[\text{M}+\text{H}+\text{H}_2\text{O}+\text{CH}_3(\text{CH}_2)_4\text{CHO}+\text{CHCHCH}_2]^+$ due to methyl substitution in the phenyl moiety. Therefore, peak 4 tentatively identified as methyl-6-gingerol by comparing the observation with the literature [11, 14, 37].

In peak 8 (23.41 min) with a small amount, the m/z 319.3065 and 259.1956 ions were found through

Table 1 Peak assignment and quantitative data of phenolic acids presented in normal ginger samples

Peak no.	Compound name	RT (min)	UV λ_{\max} (nm)	Molecular formula	Theoretical (m/z) [M+H] ⁺ [M+Na] ⁺	Experimental (m/z) [M+H] ⁺ [M+Na] ⁺	Fragment ions (m/z)	Korea	Ethiopia
1	5-Acetoxy-1,7-bis(4-hydroxy-3-methoxyphenyl)heptan-3-one	18.56	220,280	C ₂₃ H ₂₈ O ₇	417.1919 439.1738	417.2328 ND	357.21, 259.22, 219.19, 179.09, 153.07	2.13 ± 0.01	2.22 ± 0.02
2	6-Gingerdiol	18.73	220,280	C ₁₇ H ₂₈ O ₄	297.2071 319.1891	ND 319.2247	261.21, 177.11, 163.09, 137.07	14.74 ± 0.17	11.31 ± 0.22
3 ^a	6-Gingerol	19.40	231,280	C ₁₇ H ₂₆ O ₄	295.1915 317.1734	ND 317.1999	277.21, 259.19, 179.09, 177.12, 145.08, 135.07, 117.08, 115.07	200.07 ± 1.00	376.40 ± 0.40
4	Methyl-6-gingerol	21.38	229,279	C ₁₈ H ₂₈ O ₄	309.2071 331.1891	309.1454 331.2185	291.23, 273.21, 191.13, 151.09	3.65 ± 0.02	4.86 ± 0.01
5 ^b	3-Acetoxy-6-gingerdiol/5-acetoxy-6-gingerdiol	21.63	229,272	C ₁₉ H ₃₀ O ₅	339.2177 361.1994	ND 361.2366	321.24, 261.21, 163.09, 137.07	2.15 ± 0.02	1.25 ± 0.05
6	Diacetoxy-4-gingerdiol	22.20	269,281	C ₁₉ H ₂₈ O ₆	353.1970 375.1789	ND 375.2180	293.21, 233.18, 163.09, 137.07	0.68 ± 0.00	0.84 ± 0.01
7 ^a	8-Gingerol	23.31	221,281	C ₁₉ H ₃₀ O ₄	323.2228 345.2047	323.2299 345.2388	305.24, 287.23, 179.09, 177.12, 145.08, 117.02, 115.07	52.99 ± 0.12	70.35 ± 0.34
8	Acetoxy-6-gingerol	23.41	220,281	C ₁₉ H ₂₈ O ₅	337.2020 359.1840	ND 359.2207	319.31, 259.20, 137.08	–	–
9 ^b	Methyl-3-acetoxy-6-gingerdiol/methyl-5-acetoxy-6-gingerdiol	23.74	225,279	C ₂₀ H ₃₂ O ₅	353.2333 375.2153	ND 375.2538	335.26, 275.22, 191.13, 151.09	1.28 ± 0.01	ND
10 ^a	6-Shogaol	24.12	226,281	C ₁₇ H ₂₄ O ₃	277.1809 299.1629	277.2034 ND	137.08	2.66 ± 0.02	9.10 ± 0.04
11	Diacetoxy-6-gingerdiol	26.08	227,281	C ₂₁ H ₃₂ O ₆	381.2283 403.2102	ND 403.2566	321.24, 261.21, 177.11, 163.09, 137.07	8.63 ± 0.04 ^{ns}	8.36 ± 0.15 ^{ns}
12	1-Dehydro-6-gingerdione	26.78	220,254,371	C ₁₇ H ₂₂ O ₄	291.1602 313.1421	291.2058 ND	177.07, 149.08	18.12 ± 0.10	55.72 ± 0.19
13 ^a	10-Gingerol	27.72	220,281	C ₂₁ H ₃₄ O ₄	351.2541 373.2360	ND 373.2749	333.28, 315.26, 179.09, 177.12, 145.08, 137.08, 117.08, 115.07	83.48 ± 0.05	96.08 ± 0.33
14	Methyl diacetoxy-6-gingerdiol	28.68	229,279	C ₂₂ H ₃₄ O ₆	395.2430 417.2259	ND 417.2716	412.31, 335.26, 275.23, 177.12, 151.09	1.52 ± 0.01	0.32 ± 0.01
15	Diacetoxy-8-gingerdiol	30.61	220,278	C ₂₃ H ₃₆ O ₆	409.2596 431.2415	ND 431.2866	349.28, 289.25, 177.11, 137.07	21.23 ± 0.11	34.76 ± 0.24
16	1-Dehydro-8-gingerdione	31.57	220,253,370	C ₁₉ H ₂₆ O ₄	319.1914 341.1734	319.2307 ND	177.11	3.96 ± 0.07	8.61 ± 0.20
17	Acetoxy-10-gingerol	32.43	227,281	C ₂₃ H ₃₆ O ₅	393.2646 415.2466	ND 415.2923	333.28, 315.26, 177.11, 137.08	6.73 ± 0.04	ND
18	1-Dehydro-10-gingerdione	36.31	220,253,370	C ₂₁ H ₃₀ O ₄	347.2228 369.2047	347.2707 ND	177.11, 149.09	10.65 ± 0.10	17.93 ± 0.18
Total								434.67 ± 0.22	698.11 ± 0.13

All samples analyzed in positive ionization mode, ND; Not detected, (-) below quantification level, [M+Na]⁺ and [M+H]⁺ actual mass provided, (^a) further confirmed in comparison with authentic standards, (^b) exact position of the acetoxy substitute not confirmed, each value calculated as mean ± standard error of mean (SEM) of three replicates, content expressed in mg/100 g DW; dry weight, (^{ns}) compound showed no significant difference between the two sample means ($p > 0.05$)

Table 2 Peak assignment and quantitative data of methoxyflavones presented in black ginger sample

Peak no.	Compound name	RT (min)	UV λ_{\max} (nm)	Molecular formula	Theoretical (m/z) [M+H] ⁺	Experimental (m/z) [M+H] ⁺	Fragment ions (m/z)	Korea	Thailand
1 ^a	6-Hydroxy-7,4'-Dimethoxyflavone	14.23	229,264,330	C ₁₇ H ₁₄ O ₅	299.0925	299.1125	337.05, 321.07	24.46 ± 0.69	21.60 ± 0.59
2	5,7,3',4'-Tetramethoxyflavone (tetramethyl-luteolin)	16.86	242,265,335	C ₁₉ H ₁₈ O ₆	343.1187	343.1519	381.07, 365.10	209.56 ± 5.42	174.73 ± 5.15
3	3,5,7,3',4'-Pentamethoxyflavone (pentamethaqueretin)	17.82	249,339	C ₂₀ H ₂₀ O ₇	373.1293	373.1803	411.09, 395.11	1333.70 ± 18.05 ^{ns}	1262.70 ± 10.40 ^{ns}
4	5,7-Dimethoxyflavone	18.32	263,307	C ₁₇ H ₁₄ O ₄	283.0976	283.1452	321.056, 305.08, 268.09	385.26 ± 10.63	312.00 ± 8.78
5	5,7,4'-Trimethoxyflavone (trimethylapigenin)	18.82	230,264,326	C ₁₈ H ₁₆ O ₅	313.1081	313.1637	351.07, 335.09, 298.10	975.31 ± 19.86 ^{ns}	908.06 ± 5.23 ^{ns}
6	3,5,7-Trimethoxyflavone	20.12	262,326	C ₁₈ H ₁₆ O ₅	313.1081	313.1533	351.07, 335.09	140.08 ± 4.00	121.20 ± 3.47
7	3,5,7,4'-Tetramethoxyflavone (tetramethyl-kaempferol)	20.38	233,264,336	C ₁₉ H ₁₈ O ₆	343.1187	343.1695	381.08, 365.11, 328.10	574.50 ± 15.17 ^{ns}	559.99 ± 4.01 ^{ns}
8 ^b	5-Hydroxy-7,3',4'-trimethoxyflavone	25.11	231,267,343	C ₁₈ H ₁₆ O ₆	329.1031	329.1066	351.07, 314.08	8.78 ± 0.20	4.82 ± 0.09
9 ^b	5-Hydroxy-3,7,3',4'-tetramethoxyflavone (ayanin)	27.29	253,270,353	C ₁₉ H ₁₈ O ₇	359.1136	359.1324	381.10, 344.09, 329.07	110.39 ± 2.77	74.45 ± 1.84
10 ^{b,c}	5-Hydroxy-7-methoxyflavone (tectochrysin)	27.90	225,267,310	C ₁₆ H ₁₂ O ₄	269.0819	269.1070	254.06	95.95 ± 2.46	71.84 ± 1.80
11 ^{b,c}	5-Hydroxy-7,4'-dimethoxyflavone	28.70	231,267,331	C ₁₇ H ₁₄ O ₄	299.0925	299.1150	284.07	151.52 ± 3.39	114.73 ± 2.73
12 ^{b,c}	5-Hydroxy-3,7-dimethoxyflavone	30.99	227,265,310,350	C ₁₇ H ₁₄ O ₅	299.0925	299.1190	284.07	147.71 ± 6.82	116.14 ± 3.13
13 ^b	5-Hydroxy-3,7,4'-trimethoxyflavone	31.51	232,267,347	C ₁₈ H ₁₆ O ₆	329.1031	329.1277	351.09, 314.08	230.82 ± 13.86	197.99 ± 4.25
Total								4388.04 ± 103.32	3940.24 ± 51.46

All samples analyzed in positive ionization mode, (^a) tentatively identified, (^b) [M+K]⁺ not provided, (^c) [M+Na]⁺ not provided, fragment ions (m/z) of [M+H-CH₃]⁺ and [M+H-2CH₃]⁺ are presented. Each value calculated as mean ± standard error of mean (SEM) of three replicates, content expressed in mg/100 g DW; dry weight, (^{ns}) compound showed no significant difference between the two sample means ($p > 0.05$)

successive losses of H₂O and AcOH (60 Da) from parent ion. The base peak at m/z 137.0750 was produced by cleavage of alkyl chain including acetoxy group. Based on observed fragmentation, peak 8 was identified as acetoxy-6-gingerol [11, 13, 14]. Also, peak 17 showed characteristic fragmentation pattern similar to peak 8 with 56 Da (CH₂-CH₂-CH₂-CH₂) increment on its fragment ions at m/z 333.2766 [M+H-AcOH]⁺, hence this peak tentatively identified as acetoxy-10-gingerol.

Peak 2 (6-gingerdiol, m/z 319) was differentiated from 6-gingerol (m/z 317) with a difference of 2 Da at [M+Na]⁺, and intense peak at m/z 261.2138 was formed

by the loss of 2H₂O from two hydroxyl groups on C3 and C5 positions of parent ion [14, 19] (Fig. 2b). As minor compound, peak 5 (21.63 min) tentatively identified as 3-acetoxy-6-gingerdiol or 5-acetoxy-6-gingerdiol by fragment ions at m/z 321.2396 and 261.2122 corresponding to the losses of H₂O and AcOH (60 Da), respectively, from gingerdiol structure. Furthermore, as methylated derivatives of acetoxy-6-gingerdiol, methyl-3- or methyl-5-acetoxy-6-gingerdiol (peak 9, 23.74 min) were presumed by fragment ions at m/z 335.2615 and 275.2209 with methylene (CH₂, 14 Da) increment from peak 5. Additionally, product ions at m/z 191.1309 and 151.0915 evidenced that

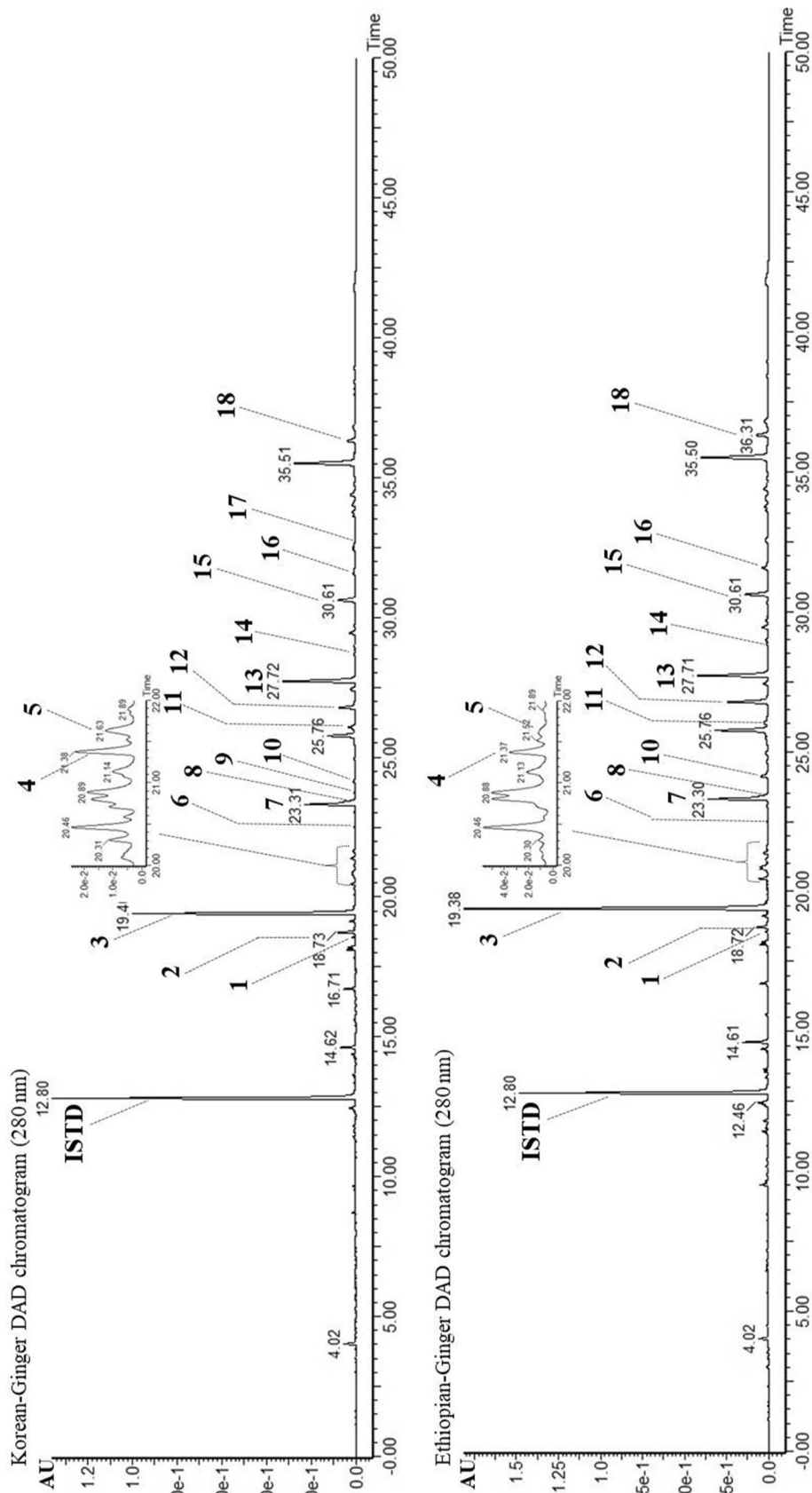
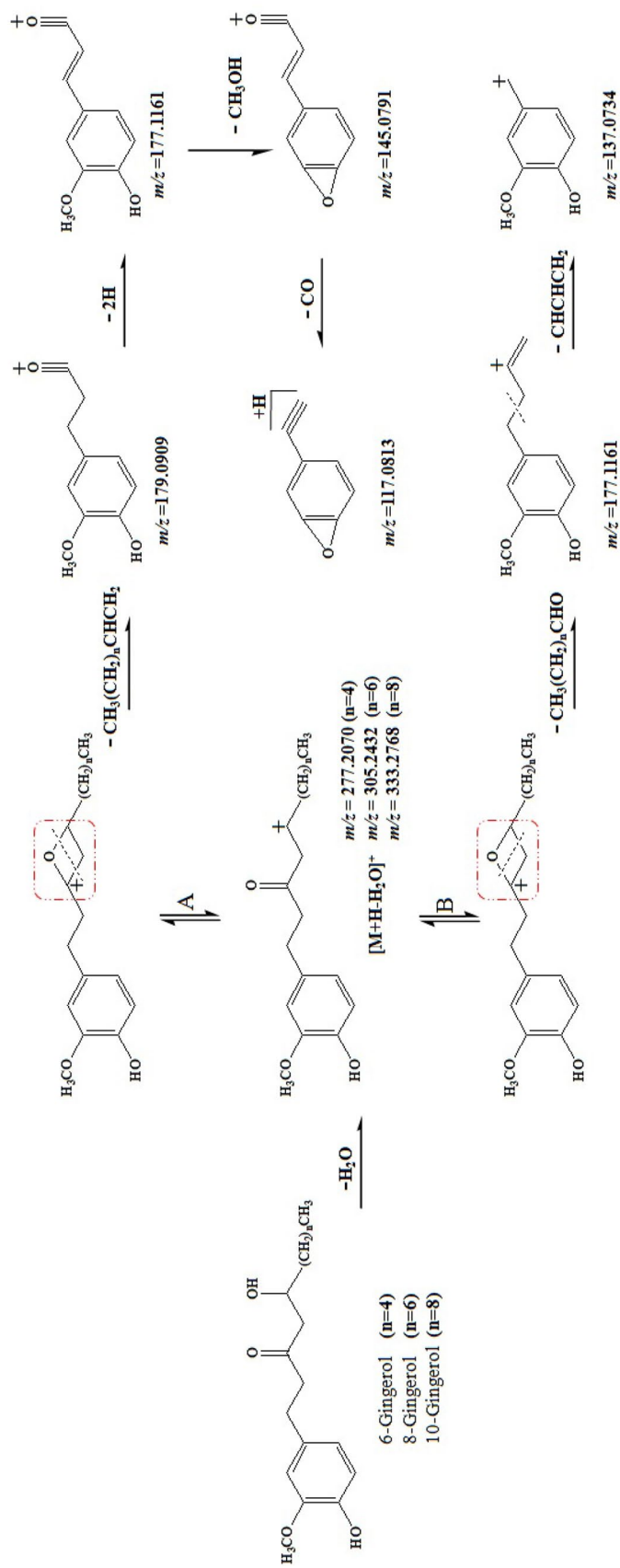


Fig. 1 UPLC-DAD chromatograms of 18 gingerol-related phenolic acids from Korean and Ethiopian normal gingers at 280 nm: Peak (1) 5-acetoxy-1,7-bis(4-hydroxy-3-methoxyphenyl)heptan-3-one, (2) 6-gingerdiol, (3) 6-gingerol, (4) methyl-6-gingerol, (5) 3-acetoxy-6-gingerdiol or 5-acetoxy-6-gingerdiol, (6) diacetoxy-4-gingerdiol, (7) 8-gingerol, (8) acetoxy-6-gingerol, (9) methyl-3-acetoxy-6-gingerdiol or methyl-5-acetoxy-6-gingerdiol, (10) 6-shogaol, (11) diacetoxy-6-gingerdiol, (12) 1-dehydro-6-gingerdione, (13) 10-gingerol, (14) methyl diacetoxy-6-gingerdiol, (15) diacetoxy-8-gingerdiol, (16) 1-dehydro-8-gingerol, (17) acetoxy-10-gingerol, (18) 1-dehydro-10-gingerdione, (ISTD) 2,4,5-trimethoxycinnamic acid



Scheme 1 The fragmentation patterns of gingerol derivatives under positive ionization mode of QToF-MS; **a** new proposed fragmentation pattern, **b** fragmentation pattern according to Jiang et al. [11]

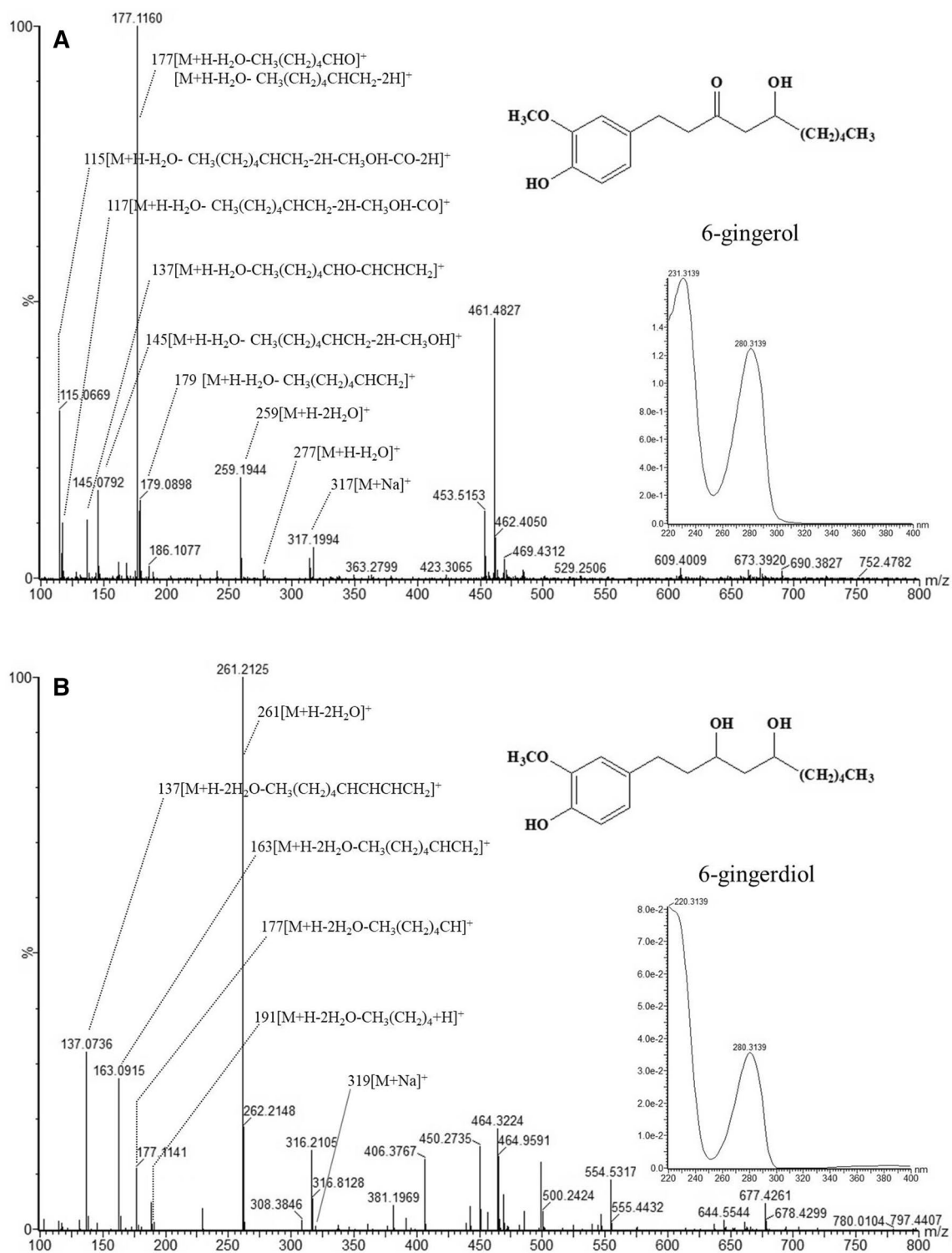


Fig. 2 (+) ESI-MS and UV spectra of 6-gingerol (a) and 6-gingerdiol (b) from normal gingers

this compound further methylated on the phenyl position like methyl-6-gingerol (peak 4) [11, 14]. The exact position of the acetoxy substitute needs to be further confirmed

from acetoxy-6-gingerdiol-related phenolics (peak 5 and 9) by NMR technique.

The formation of fragment ions at m/z 321.2404 $[M+H-AcOH]^+$ and 261.2115 $[M+H-2AcOH]^+$ with the

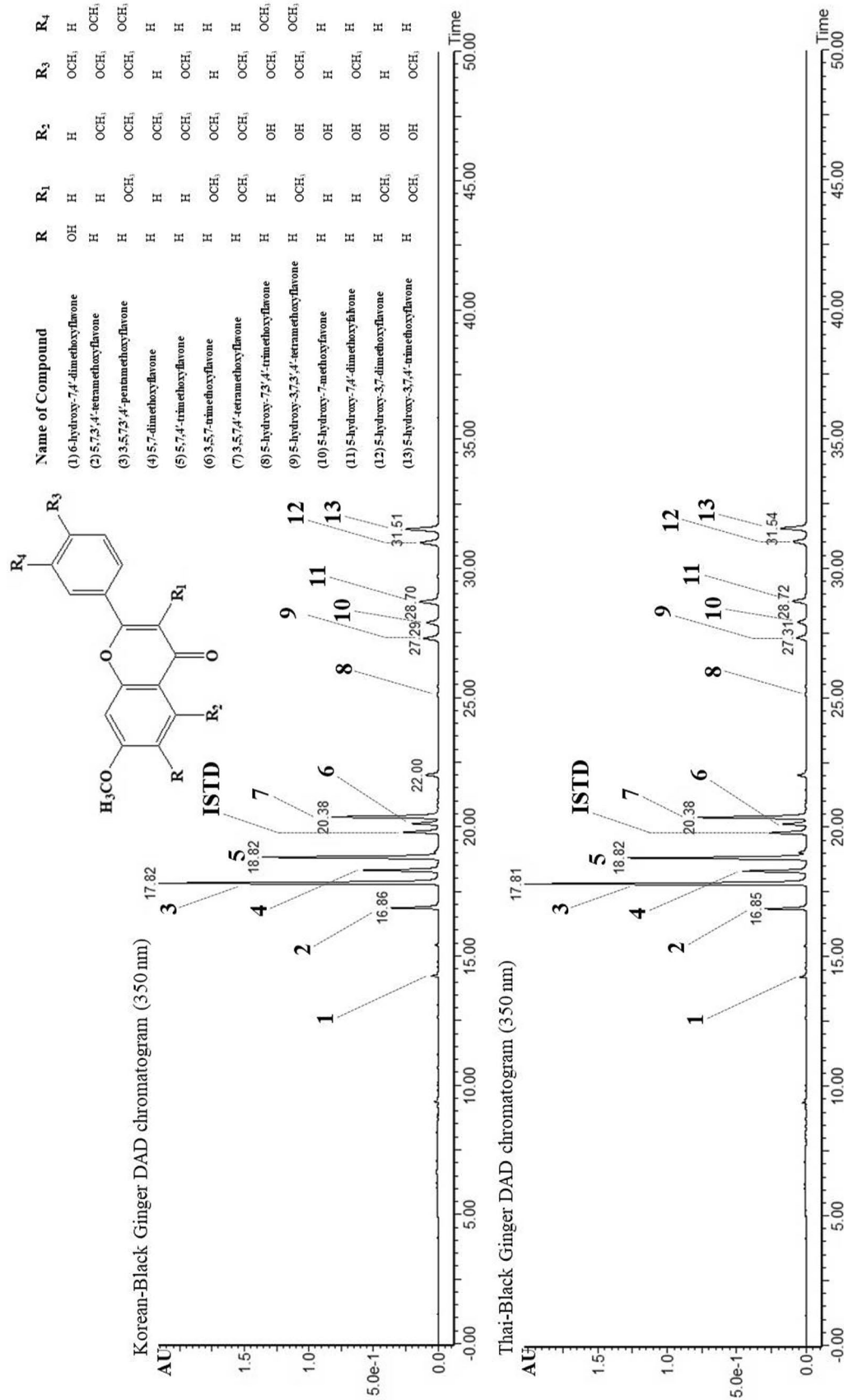


Fig. 3 UPLC-DAD chromatograms of 13 methoxyflavones from Korean and Thai black gingers at 350 nm, their basic structure and substituting R group are presented in the right side on the table

literature comparison suggested that peak 11 is di-acetylated derivative of 6-gingerdiol with low absorbance (UV spectra at 280 nm) compared to 6-gingerol (Fig. 2; Table 1). This could also be confirmed as gingerdiol structure through the absence of ketone group on the aliphatic chain. Therefore, peak 11 identified unambiguously as diacetoxy-6-gingerdiol [14]. Peaks 6 and 15 showed similar fragmentation pattern in reference to diacetoxy-6-gingerdiol. They were tentatively identified as diacetoxy-4-gingerdiol and diacetoxy-8-gingerdiol by decrease (375.2175, 293.2072, and 233.1781) and increase (431.2866, 349.2791, and 289.2470) of $\text{CH}_2\text{-CH}_2$ (28 Da) for adduct and fragment ions, respectively, corresponding to $[\text{M}+\text{Na}]^+$, $[\text{M}+\text{H}-\text{AcOH}]^+$, and $[\text{M}+\text{H}-2\text{AcOH}]^+$ from diacetoxy-6-gingerdiol. Peak 14 (28.68 min) showed similar fragmentation pattern to diacetoxy-6-gingerdiol with methylene (CH_2 , 14 Da) increment on the fragment ions, therefore, it was identified as methyl diacetoxy-6-gingerdiol [11].

Gingerdione-related compounds showed similar UV spectra at 220, 253, and 370 nm with fragment ions of $[\text{M}+\text{H}]^+$ and $[\text{M}+\text{H}-\text{CH}_3(\text{CH}_2)_n\text{OCHCH}_2, n=4,6,8]^+$ by $\text{CH}_2\text{-CH}_2$ (28 Da) increments. Therefore, peaks 12, 16, and 18 were tentatively identified as 1-dehydro-6-gingerdione, 1-dehydro-8-gingerdione, and 1-dehydro-10-gingerdione, respectively [11, 14]. Also, peak 10 was identified as 6-shogaol with intense ion at m/z 137.0762 based on pure standard. Jolad et al. [14] reported that the characteristic fragment ion at m/z 205 was produced due to C5–C6 cleavage of the aliphatic chain on 6-shogaol, but it has not been observed in this study. In peak 1, the characteristic fragmentation was confirmed from ions at m/z 259.1819, 219.1855, and 179.0881 corresponding to diarylheptanoids under positive ionization mode [12]. Especially, the fragment ion at m/z 357.2058 $[\text{M}+\text{H}-\text{AcOH}]^+$ suggested that this peak might be acetylated derivative of diarylheptanoid. Hence, peak 1 tentatively identified as 5-acetoxy-1,7-bis(4-hydroxy-3-methoxyphenyl)heptan-3-one [17].

Characterization of methoxyflavones from black gingers

Methoxyflavones belong to a certain flavonoid class containing 1–7 methoxyl substitutes on the 2-phenyl chromone structure, and are predominantly distributed in the peel part of *Citrus* fruits [38]. In DAD chromatogram of Fig. 3, black ginger extracts showed different spectral patterns with maximum wavelength of 350 nm compared to normal ginger. Previous studies reported that methoxyflavones of aglycone type are the major chemical constituents in black ginger [25, 39]. Also, several methoxyflavone glycosides including kaempferiaoside A, C, and D were additionally identified from this plant rhizome [24, 30, 31] (Supplementary Table 2). In the current study, a total of 13 methoxyflavone

aglycones were clearly separated and identified from black ginger extracts collected from Jeju Island, Korea and Thailand (Fig. 3), and their names, UV spectra and adduct ions ($[\text{M}+\text{K}]^+$, $[\text{M}+\text{Na}]^+$, and $[\text{M}+\text{H}]^+$) are presented in Table 2. The elution order of each peak was differentiated by number and position of adducted methoxyl group in the basic structure. In detail, peaks 2–3, 4–7, 8–9, and 10–13 were eluted slowly as the number of methoxyl group increased within their peak intervals (Fig. 3; Table 2). According to Xing et al. [38], methoxyflavones were generally fragmented by losing $n\text{CH}_3$, H_2O , CO , $\text{H}_2\text{O}+\text{CH}_3$, $\text{CO}+\text{CH}_3$, and $\text{CO}+\text{H}_2\text{O}+\text{CH}_3$ from the parent ions under positive ionization mode, however, peaks 4, 5, and 7–13 formed only minor fragment ion after losing one CH_3 from their parent ions in our study. Especially, peak 9 (5-hydroxy-3,7,3',4'-tetramethoxyflavone, ayanin) further formed fragment ion at m/z 329.0663 corresponding to $[\text{M}+\text{H}-2\text{CH}_3]^+$. Finally, these methoxyflavones were not detected in normal ginger samples.

Quantification of phenolic acid and methoxyflavone derivatives from gingers

The contents of gingerol-related phenolics and methoxyflavones were calculated using 2,4,5-trimethoxycinnamic acid and galangin as internal standard, respectively. The total phenolic acid content presented on Table 1, Ethiopian ginger has a moderate higher concentration (698.11 mg/100 g, dry weight (DW)) than that of Korean ginger (434.67 mg/100 g, DW). Except diacetoxy-6-gingerdiol, the concentration of all identified compounds showed significant difference between two samples ($p < 0.05$). Especially, 6-gingerol identified as the major constituent with 46.03 and 53.92 proportions (%) of the total content in Korean and Ethiopian ginger samples, respectively, followed by 10- and 8-gingerol. Previous studies by Jolad et al. [11], Jiang et al. [14], and He et al. [40] also supported that 6-gingerol was the major active compound in the rhizomes; however, according to Ahui et al. [37], 8-gingerol was confirmed as the major constituent from ginger of Nigeria origin. In addition, Zhan et al. [41] reported that 25.25 mg of 10-gingerol was obtained through gradient separation and purification from 100 mg of crude extract. Hence, the representative major compound among samples is affected by several factors such as plant origin, environment, and state of rhizome maturity [13]. Moreover, this variation also further supported by a metabolomics approach on characterization of ginger samples collected from five different Asian countries (China, India, Malaysia, Thailand, and Vietnam) [21].

The contents of 1-dehydro-6-gingerdione and diacetoxy-8-gingerdiol were significantly higher next to gingerol homologous series. Acetoxy-6-gingerol was detected in both sample, however, its amount was below

the quantification level. Methyl-3 or methyl-5-acetoxy-6-gingerdiol and acetoxy-10-gingerol were not detected in Ethiopian ginger, and as minor compounds, the contents of 6-gingerdiol, 3-acetoxy or 5-acetoxy-6-gingerdiol and methyl diacetoxy-6-gingerdiol were slightly higher in Korean ginger. Also, the content of 1-dehydro-6-gingerdione (55.72 mg/100 g, DW) was significantly higher in Ethiopian sample (Table 1). This might be due to environmental factors to affect the biosynthesis and the concentration level of compounds from different origins. The proportion of 6-shogaol accounts for only 1.14% of the total phenolic acid compared to 6-gingerol in Ethiopian sample. Shogaol is the dehydrated product of gingerol and this chemical change occurs in the process of thermal drying of the rhizomes and long-term storage [42]. In our treatment, the fresh samples were dried under lower temperature, hence, 6-shogaol was found to be small amount and the other shogaol derivatives were not detected.

3,5,7,3',4'-pentamethoxyflavone (PMF), 5,7,4'-trimethoxyflavone (5,7,4'-TMF), and 3,5,7,4'-tetramethoxyflavone (3,5,7,4'-TTMF) were presented as predominant aglycone among 13 methoxyflavones isolated from Korean and Thai black gingers (Table 2). Except PMF, 5,7,4'-TMF, and 3,5,7,4'-TTMF, all compounds were significantly different in concentration between both sample ($p < 0.05$). In total methoxyflavone contents, black ginger rhizomes of Korea (4388.04 mg/100 g, DW) and Thailand (3940.24 mg/100 g, DW) were much higher than the peels of *Citrus reticulata* (mandarin orange, 372.72–1547.49 mg/100 g, DW) and *Citrus sinensis* (orange, 110.05–259.07 mg/100 g, DW) reported [38]. Among 12 methoxyflavones determined from Thai black ginger, PMF (1347 mg/100 g, DW) was presented as the major compound, which is similar with our results (Korea, 1333.7; Thailand, 1262.7 mg/100 g, DW) [24]. In the other study, PMF and 5,7,4'-TMF were also confirmed as the predominant methoxyflavones in black ginger [43].

The rhizomes of normal and black ginger are rich source of phenolic compounds, and very important biologically and economically due to the presences of these compounds. Our result confirmed that even though both plants are from the same family and have similar morphological characteristics, there is a distinct biosynthetic pathway to synthesize their major chemical constituents. Hence, gingerol-related phenolics were only detected in normal ginger, while methoxyflavones were detected in black ginger. Especially, it was considered that black ginger is a major source of methoxyflavones with wide range of biological activities compared to other plants such as *Citrus* species.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

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