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ARTICLE

Isolation and Identification of Polymethoxyflavones from the Hybrid *Citrus*, Hallabong

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Seven polymethoxyflavones (PMFs) were isolated from the dried peels of hallabong, the hybrid *Citrus*, by a repeated column chromatography. The structures of PMFs were identified as 5,6,7,3′,4′-pentamethoxyflavone (**1**), 6,7,8,3′,4′-pentamethoxyflavone (**2**), 3-hydroxy-5,6,7,4′-tetramethoxyflavone (**3**), 5,6,7,8,3′,4′-hexamethoxyflavone (**4**), 3,6,7,4′-tetramethoxyflavone (**5**), 3,5,6,7,8,3′,4′-heptamethoxyflavone (**6**), and 5,6,7,8,4′-pentamethoxyflavone (**7**) using ^{1}H and ^{13}C NMR in combination with mass spectrometry. Among these compounds, **5** was isolated for the first time from nature. The content of PMFs **1**−**7** in hallabong was determined by HPLC-UV. The major PMFs of hallabong are **5** in the dried peels (15.4 mg/g) and **7** in the dried leaves (12.2 mg/g).

KEYWORDS: Hallabong ([*Citrus unshiu* × *C. sinensis*] × *C. reticulata*); dekopon; polymethoxyflavone; quantitative analysis; HPLC

INTRODUCTION

The fruits of *Citrus* species are important crops because of their industrial value, especially in foods and cosmetics (*1*). Dekopon, a cross between Kiyomi (*Citrus unshiu* Marcov. × *Citrus sinensis* Osbeck) and Ponkan (*Citrus reticulata* Blanco), was first bred at the Kunchinotsu Branch of the Fruit Tree Research Station in Nagasaki Prefecture, Japan, in 1972. Dekopon was brought from Japan to Republic of Korea and renamed hallabong in 1998 (*2*). The commercial value of hallabong is due to its sweet taste and pleasant aroma (*3*).

The total production of hallabong in Republic of Korea was 23544 tons in 2009 (Korea Rural Economic Institute, Republic of Korea), and hallabong is now widely cultivated in Republic of Korea (*4*). Hallabong fruits are used for juice manufacture, with peels produced as byproduct. In some regions of the world, the peels of *Citrus* species are used in traditional medicine to treat stomach upsets, cough, skin inflammation, muscle pain, and ringworm infections, as well to lower blood pressure (*5*). Phytochemical investigations of *Citrus* species have shown that the peels contain flavonoids (*6*, *7*), limonoids (*8*, *9*), and coumarins (*10*). Polymethoxyflavones (PMFs) are major constituents in *Citrus* peels (*6*, *11*) and have attracted considerable attention because of their anti-inflammatory (*12*), anticarcinogenic (*13*), and antiatherogenic properties (*14*). Other flavonoids from the genus *Citrus* have significant biological activities, including antioxidant (*15*, *16*) and antitumor properties (*17*). Common PMFs have been isolated from various parts of *Citrus* plants, including the parents (*C. sinensis* and *C. reticulate*) of hallabong (*18*, *19*). However, no previous study has reported isolation of phytochemical constituents from hallabong.

Our aim was to isolate PMFs from hallabong peels by a repeated column chromatography and to determine the quantities of these compounds present in the different parts of the plant by HPLC-UV.

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MATERIALS AND METHODS

Plant Materials. The peels, leaves, and stems of hallabong ([*C. unshiu* Marcov. × *C. sinensis* Osbeck] × *C. reticulata* Blanco) were collected in Jeju, Republic of Korea. All samples were sliced and dried in an oven at 45 °C until use. Voucher specimens (No. LEE 2009-01, 2009-02, and 2009-03) were deposited at the Herbarium of Department of Applied Plant Science, Chung-Ang University, Republic of Korea.

Apparatus and Chemicals. EI-MS spectra were obtained using a JEOL JMS-600W mass spectrometer (Tokyo, Japan). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE 300 NMR spectrometer (Rheinstetten, Germany). Chemical shifts are shown as δ values (ppm) with tetramethylsilane (TMS) as an internal standard, and coupling constants (J) are expressed in hertz. IR spectra were obtained on a JASCO FT/IR-5300 instrument. HPLC data were recorded on a Gilson HPLC equipped with a UV−vis detector (Villiers le Bel, France). TLC was performed with precoated silica gel 60 F_{254} plates (Article 5715, Merck Co., Darmstädt, Germany). The compounds on TLC plates were visualized by spraying the plate with 10% sulfuric acid in methanol followed by heating at 100 °C to detect spot color. Silica gel (200−400 mesh ASTM; Merck Co.) was used for repeated column chromatography. First-grade solvents such as water and acetonitrile (J. T. Baker, Phillipsburg, NJ) were used as elution solutions on HPLC. All other reagents such as *n*-hexane, chloroform, ethyl acetate, *n*-butanol, and methanol were of analytical grade.

Isolation of Seven PMFs. The sliced and dried peels of hallabong were extracted with methanol (8 L methanol × 7 times/3096.7 g of dried peels, 60−65 °C) under reflux. Filtrates of the extraction solution were concentrated in vacuo to produce methanol extract (1650.2 g). The methanol extract (727.4 g) thus obtained was suspended in distilled water and then partitioned in turn using *n*-hexane, chloroform, ethyl acetate, and *n*-butanol. A portion of the chloroform fraction (5.6 g) was chromatographed on a silica gel column (no. 7734, 6 × 80 cm) using a stepwise gradient of *n*-hexane/ethyl acetate and ethyl acetate/methanol solvent systems to yield 62 subfractions. Subfractions 51−52 (*n*-hexane/ethyl acetate = 25:75) led to the isolation of compounds **1** and **3**. Subfraction

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Table 1. ^{1}H NMR Spectral Data for PMFs **1**−**7** in DMSO-d_6

no.	1	2	3	4	5	6	7
H-3	6.79 (s)	6.78 (s)		6.73 (s)			6.75 (s)
H-5		7.20 (s)			7.23 (s)		
H-8	6.68 (s)		6.67 (s)		6.73 (s)		
H-2′	7.53 (d, 2.1)	7.54 (d, 2.0)	7.98 (d, 9.0)	7.42 (d, 1.8)	8.03 (d, 8.4)	7.66 (d, 2.1)	7.99 (d, 9.0)
H-3′			7.13 (d, 9.0)		7.11 (d, 8.4)		7.14 (d, 9.0)
H-5′	7.15 (d, 8.4)	7.11 (d, 8.5)	7.13 (d, 9.0)	7.00 (d, 8.7)	7.11 (d, 8.4)	7.19 (d, 8.7)	7.14 (d, 9.0)
H-6′	7.63 (dd, 8.4, 2.1)	7.65 (dd, 8.5, 2.0)	7.98 (d, 9.0)	7.59 (dd, 8.7, 1.8)	8.03 (d, 8.4)	7.71 (dd, 8.7, 2.1)	7.99 (d, 9.0)
3-OMe					3.77 (s)	3.80 (s)	
5-OMe	3.87 (s)		3.87 (s)	4.03 (s)		3.95 (s)	3.96 (s)
6-OMe	3.84 (s)	3.81 (s)	3.84 (s)	3.97 (s)	3.80 (s)	3.84 (s)	3.84 (s)
7-OMe	3.98 (s)	3.96 (s)	3.98 (s)	4.11 (s)	3.95 (s)	4.02 (s)	4.02 (s)
8-OMe		3.77 (s)		3.96 (s)		3.79 (s)	3.78 (s)
3′-OMe	3.88 (s)	3.89 (s)		4.00 (s)		3.86 (s)	
4′-OMe	3.85 (s)	3.85 (s)	3.85 (s)	3.98 (s)	3.86 (s)	3.85 (s)	3.85 (s)

Table 2. ^{13}C NMR Spectral Data for PMFs **1**−**7** in DMSO-d_6

no.	1	2	3	4	5	6	7
C-2	159.6	160.3	159.6	160.2	160.3	150.9	160.4
C-3	106.7	106.4	156.4	106.3	106.1	140.0	106.1
C-4	176.1	175.7	175.9	175.8	175.6	172.3	175.8
C-5	151.7	97.3	151.1	143.5	97.3	143.1	147.6
C-6	131.8	139.7	138.1	137.6	139.8	137.4	143.6
C-7	156.5	157.4	161.9	150.9	161.8	151.9	162.1
C-8	94.2	151.5	98.7	147.5	157.4	138.9	137.8
C-9	155.8	153.9	155.7	147.1	153.9	147.9	147.6
C-10	111.1	111.7	114.7	114.3	114.5	114.4	114.7
C-1′	123.4	123.2	123.2	123.1	123.0	122.5	123.1
C-2′	108.9	109.2	127.6	108.9	127.8	110.0	127.8
C-3′	149.1	149.0	114.5	149.0	114.5	148.5	114.3
C-4′	151.2	151.7	151.1	151.8	151.6	154.1	151.0
C-5′	112.3	112.0	114.5	111.9	114.5	111.7	114.3
C-6′	119.3	119.4	127.6	119.3	127.8	121.5	127.8
3-OMe					61.0	61.5	
5-OMe	61.1		61.0	61.9		63.7	62.0
6-OMe	55.8	55.7	55.5	55.6	55.5	59.3	55.6
7-OMe	56.5	61.8	60.4	61.8	61.8	61.6	61.9
8-OMe		55.9		55.7		61.4	61.5
3′-OMe	56.4	61.0		61.5		56.3	
4′-OMe	55.9	56.4	56.3	61.4	56.4	55.4	61.6

36 obtained by a gradient of *n*-hexane/ethyl acetate (50:50) yielded compound **2**. Subfractions 28−34 eluting with *n*-hexane/ethyl acetate (65:35) yielded compounds **4**−**7** after recrystallization with methanol.

5,6,7,3′,4′-Pentamethoxyflavone (***1***). Compound **1** was obtained in the form of yellow needles: $C_{20}H_{20}O_7$; EI-MS (rel int, %), *m*/*z* 372 (100) $[M]^+$, 357 (98.6), 343 (14.0), 329 (11.4), 312 (1.4), 297 (1.2); UV λ_{max}, 330, 270 (sh), 247 (sh), 205 nm; ^{1}H NMR (300 MHz, DMSO-d_6), see **Table 1**; ^{13}C NMR (75 MHz, DMSO-d_6), see **Table 2**.

6,7,8,3′,4′-Pentamethoxyflavone (***2***). Compound **2** was obtained in the form of deep yellow crystals: $C_{20}H_{20}O_7$; EI-MS (rel int, %), *m*/*z* 372 (29.3) $[M]^+$, 357 (100), 326 (5.4), 313 (5.0), 296 (2.4); UV λ_{max}, 327, 264 (sh), 239 (sh), 215 nm; ^{1}H NMR (300 MHz, DMSO-d_6), see **Table 1**; ^{13}C NMR (75 MHz, DMSO-d_6), see **Table 2**.

3-Hydroxy-5,6,7,4′-tetramethoxyflavone (***3***). Compound **3** was obtained in the form of pale yellow needles: $C_{19}H_{18}O_7$; EI-MS (rel int, %), *m*/*z* 358 (0.4) $[M]^+$, 342 (100), 345 (0.6), 327 (97.5), 210 (0.9), 195 (6.3); UV λ_{max}, 313, 270 (sh), 204 nm; ^{1}H NMR (300 MHz, DMSO-d_6), see **Table 1**; ^{13}C NMR (75 MHz, DMSO-d_6), see **Table 2**.

5,6,7,8,3′,4′-Hexamethoxyflavone (***4***). Compound **4** was obtained in the form of yellow needles: $C_{21}H_{22}O_8$; EI-MS (rel int, %), *m*/*z* 402 (26.6) $[M]^+$, 387 (62.5), 373 (3.9), 355 (1.2), 342 (29.7), 327 (100.0); UV λ_{max}, 326, 268 (sh), 249 (sh), 214 nm; ^{1}H NMR (300 MHz, DMSO-d_6), see **Table 1**; ^{13}C NMR (75 MHz, DMSO-d_6), see **Table 2**.

3,6,7,4′-Tetramethoxyflavone (***5***). Compound **5** was obtained in the form of yellow crystals: $C_{19}H_{18}O_6$; HR-MS, *m*/*z* 342.1106 (calcd 342.1103); EI-MS (rel int, %), *m*/*z* 342 (25.1) $[M]^+$, 327 (100), 219 (3.5), 195 (2.8), 167 (7.7); UV λ_{max}, 320, 265 (sh), 203 nm; IR (KBr, cm^{-1}), 2937 (CH), 1637 (C═O); ^{1}H NMR (300 MHz, DMSO-d_6), see **Table 1**; ^{13}C NMR (75 MHz, DMSO-d_6), see **Table 2**.

3,5,6,7,8,3′,4′-Heptamethoxyflavone (***6***). Compound **6** was obtained in the form of yellow needles: $C_{22}H_{24}O_9$; EI-MS (rel int, %), *m*/*z* 432 (65.4) $[M]^+$, 417 (100), 403 (5.1), 373 (9.1), 343 (3.7), 315 (1.3); UV λ_{max}, 341, 266 (sh), 240 (sh), 201 nm; ^{1}H NMR (300 MHz, DMSO-d_6), see **Table 1**; ^{13}C NMR (75 MHz, DMSO-d_6), see **Table 2**.

5,6,7,8,4′-Pentamethoxyflavone (***7***). Compound **7** was obtained in the form of pale yellow needles: $C_{20}H_{20}O_7$; EI-MS (rel int, %), *m*/*z* 372 (40.6) $[M]^+$, 357 (100), 343 (3.8), 312 (4.7), 297 (2.9); UV λ_{max}, 328, 268 (sh), 241 (sh), 205 nm; ^{1}H NMR (300 MHz, DMSO-d_6), see **Table 1**; ^{13}C NMR (75 MHz, DMSO-d_6), see **Table 2**.

Sample Preparation. To quantify the amounts of the seven PMFs obtained from different parts of hallabong, 20.0 g of each dried plant part was minced. The peels, leaves, and stems of hallabong were extracted with methanol (300 mL × 3) under reflux and removed in vacuo to yield crude extracts. The resultant solutions were filtered through a Whatman 0.45 μm PVDF syringe filter (catalog no. 6779) prior to HPLC.

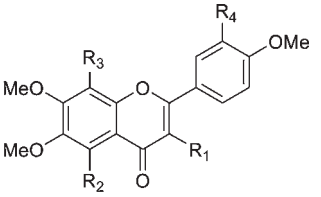
HPLC Conditions. The quantities of the seven PMFs present in the various plant parts were estimated by HPLC (*20*, *21*). A reverse phase column (Clipeus C_{18}, 5 μm, 250 × 4.6 mm, Higgins Analytical) was used, and a gradient solvent system (water/acetonitrile, v/v) was employed as the mobile phase. The gradient solvent system was 85:15 initially and was increased in linear gradients to 60:40 for 10 min, then to 45:55 for 20 min, and finally to 85:15 for 10 min. The gradient elution profile was optimized to obtain the best flavonoid resolution in the shortest analysis time. The flow rate was kept constant at 1.0 mL/min, and the peaks were identified using UV absorbance at 330 nm. The injection volume was 20 μL of the prepared methanol solutions. All solvents for HPLC analysis were degassed before use. HPLC analyses were performed in triplicate.

Calibration. Standard stock solutions (0.5 mg/400 μL) of each of the seven PMFs isolated from the peels of hallabong were prepared in a solvent mixture (chloroform/methanol/water = 1:15:4) and repeatedly blended with the same solvent. The PMF levels were ascertained by comparing the integrated peak areas of the individual compounds with those of a standard curve prepared from the corresponding standards. The peak area (Y), concentration (X, mg/mL), and mean values (n = 12) of the calibration functions of the seven PMFs were calculated.

RESULTS AND DISCUSSION

Identification of PMFs. Methanol extraction of the hallabong peels led to the isolation of compounds **1**−**7**. ^{1}H NMR signals between δ 3.5−4.3 and 6.5−8.0 indicated that all compounds had structures typical of PMFs. Their characteristics are described in detail below. The ^{1}H NMR spectra for compounds **1**, **2**, **4**, and **6** revealed that they have an ABX system (H-2′, -5′, and -6′), as demonstrated by coupling constant signals at δ 7.5−7.7 (dd, H-6′), 7.4−7.7 (d, H-2′), and 7.0−7.2 (d, H-5′) in the B-ring structure. The two methoxy groups of compounds **1**, **2**, **4**, and **6** are located in the 3′- and 4′-positions on the basis of HMBC

analysis. The aromatic proton signals were different between these compounds depending on the numbers and locations of functional groups. The aromatic proton singlet signals at δ 6.79 and 6.68 of **1** were from protons at the 3- and 8-positions in the structure, respectively. Five methoxy group signals at δ 3.98, 3.88, 3.87, 3.85, and 3.84 were in the 5-, 6-, 7-, 3′-, and 4′-positions of the skeleton, as shown by HMBC analysis. The EI-MS spectrum showed a molecular ion peak at m/z 372. For **2**, aromatic proton singlet signals at δ 7.22 and 6.81 were at the 5- and 3-positions in the structure, respectively. Five methoxy group signals from the 6-, 7-, 8-, 3′-, and 4′-positions of the skeleton were observed at δ 3.96, 3.89, 3.85, 3.80, and 3.77, respectively. A molecular ion peak was observed at m/z 372. Compound **4** exhibited an aromatic proton singlet signal at δ 6.73 at the 3-position. Six signals at δ 4.11, 4.03, 4.00, 3.98, 3.97, and 3.95 from methoxy groups at the 5-, 6-, 7-, 8-, 3′-, and 4′-positions were observed, respectively. The EI-MS spectrum showed a molecular ion peak at m/z 402. Compound **6** had no aromatic proton singlet signal. Seven methoxy group signals at 4.02, 3.95, 3.86, 3.85, 3.84, 3.81, and 3.79 were at the 3-, 5-, 6-, 7-, 8-, 3′-, and 4′-positions of the skeleton, respectively, as revealed by HMBC analysis. The molecular ion peak was at m/z 432. Accordingly, the ABX splitting-type flavonoids of hallabong are 5,6,7,3′,4′-pentamethoxyflavone (**1**), 6,7,8,3′,4′-pentamethoxyflavone (**2**), 5,6,7,8,3′,4′-hexamethoxyflavone (**4**), and 3,5,6,7,8,3′,4′-heptamethoxyflavone (**6**) on the basis of comparisons of the spectral data with data from the literature (*22*−*24*).



1 R_1 =H, R_2 = OMe, R_3 = H, R_4 = OMe

2 R_1 = H, R_2 = H, R_3 = OMe, R_4 = OMe

3 R_1 = OH, R_2 = OMe, R_3 = H, R_4 = H

4 R_1 = H, R_2 = OMe, R_3 = OMe, R_4 = OMe

5 R_1 = OMe, R_2 = H, R_3 = H, R_4 = H

6 R_1 = OMe, R_2 = OMe, R_3 = OMe, R_4 = OMe

7 R_1 = H, R_2 = OMe, R_3 = OMe, R_4 = H

Figure 1. Chemical structures of PMFs isolated from hallabong peels.

Table 3. Linearity of Standard Curves of PMFs **1**−**7**

PMF	t_R	linear range (μg/mL)	calibration equation[a]	correlation factor, r^{2b}
1	26.9	0.048−3.125	$Y = 416623.2075X + 74.4273$	0.9995
2	29.7	0.024−1.562	$Y = 998450.0152X - 41.3359$	0.9993
3	30.0	0.048−3.125	$Y = 352925.4705X + 82.3960$	0.9991
4	32.0	0.012−3.125	$Y = 6066733.0582X - 48.4282$	0.9999
5	32.7	0.012−0.781	$Y = 661209.0053X + 12.3448$	0.9995
6	33.6	0.012−3.125	$Y = 3953208.1690X - 32.3843$	0.9997
7	35.7	0.012−3.125	$Y = 513832.4675X + 95.6221$	0.9994

[a] Y = peak area, X = concentration of standards (mg/mL). [b] r^2 = correlation coefficient for five data points in the calibration curves (n = 12).

Compounds **3**, **5**, and **7** had structural signals similar to those of the B-ring of flavones. An A_2B_2 system (H-2′, -6′ and H-3′, -5′) was revealed by coupling constant signals at δ 7.9−8.0 (d, H-2′, -6′), and 7.1−7.2 (d, H-3′, -5′) and one methoxy group at the 4′-position. The aromatic proton signals varied depending on the numbers and locations of functional groups. The aromatic proton singlet signal at δ 6.75 of **3** was at the 8-position in the structure. Four methoxy group signals at the 5-, 6-, 7-, and 4′-positions of the skeleton were observed at δ 3.98, 3.87, 3.85, and 3.84, respectively. The EI-MS spectrum showed a molecular ion peak at m/z 358. For **5**, the IR spectrum indicated a CH signal at 2937 cm^{-1} and an α,β-unsaturated C=O signal at 1637 cm^{-1}. The aromatic proton singlet signals at δ 7.23 and 6.73 were in the 5- and 8-positions in the structure, respectively. Four methoxy group signals at δ 3.95, 3.85, 3.80, and 3.77 were in the 3-, 6-, 7-, and 4′-positions of the skeleton according to HMBC analysis. The EI-MS spectrum showed a molecular ion peak at m/z 342. Compound **7** had an aromatic proton singlet signal at δ 6.75 from the 3-position. Five signals at δ 4.02, 3.96, 3.85, 3.84, and 3.78 were from methoxy groups at the 5-, 6-, 7-, 3′-, and 4′-positions. A molecular ion peak was observed at m/z 372. Accordingly, the A_2B_2-type flavonoids of hallabong are 3-hydroxy-5,6,7,4′-tetramethoxyflavone (**3**), 3,6,7,4′-tetramethoxyflavone (**5**), and 5,6,7,8,4′-pentamethoxyflavone (**7**) on the basis of comparison of the spectral data with those published (*24*, *25*).

In conclusion, seven PMFs, namely, 5,6,7,3′,4′-pentamethoxyflavone (**1**, sinensetin), 6,7,8,3′,4′-pentamethoxyflavone (**2**), 3-hydroxy-5,6,7,4′-tetramethoxyflavone (**3**), 5,6,7,8,3′,4′-hexamethoxyflavone (**4**, nobiletin), 3,6,7,4′-tetramethoxyflavone (**5**), 3,5,6,7,8,3′,4′-heptamethoxyflavone (**6**, heptamethoxyflavone), and 5,6,7,8,4′-pentamethoxyflavone (**7**, tangeretin), were isolated from the dried peels of hallabong (**Figure 1**). Among these PMFs, **5** was isolated for the first time from nature.

Quantitative Analysis of the Seven PMFs in Different Parts of Hallabong. To determine the part-specific preponderance of PMFs in hallabong, HPLC analysis was conducted using the dried peels, leaves, and stems of the plant.

The quantities of the seven PMFs in three different plant parts of hallabong were determined by HPLC. The calibration equations and retention times are shown in **Table 3**. The retention time of the expected peak of each PMF in the peels of hallabong was the same as that of the standard compounds. The major PMFs of halla bong are **5** in the dried peels (15.4 mg/g) and **7** in the dried leaves (12.2 mg/g). According to a previous study by Curtis et al. (*26*), **6** in the hybrid variety 'King Orange' was separated on a small scale (0.742 mg/g) by RP-HPLC. We detected the presence of **6** in the dried peels (0.53 mg/g), but not in the dried leaves or stems. The PMF content was higher in peels and lower in stems (**Table 4**).

The presence of PMFs in hallabong peel has important consequences; the peels of hallabong, which are an agricultural crop byproduct, can be processed to obtain natural drugs and health supplements. We developed a simple, rapid, and accurate HPLC method to simultaneously determine the presence of multiple PMFs. The proposed HPLC method can be used as a quality control method for *Citrus* species and traditional Korean medicinal preparations made from *Citrus* plants. The presence of

Table 4. Quantities of PMFs **1**−**7** Present in the Peels, Leaves, and Stems of Hallabong[a]

plant part	1	2	3	4	5	6	7
peels	0.188 ± 0.065	3.266 ± 0.071	1.357 ± 0.093	0.612 ± 0.010	15.382 ± 0.306	0.534 ± 0.015	5.160 ± 0.124
leaves	0.138 ± 0.030	7.715 ± 0.225	1.862 ± 0.215	0.608 ± 0.014	7.699 ± 0.178	ND	12.229 ± 0.073
stems	0.428 ± 0.140	2.871 ± 0.098	0.477 ± 0.058	0.260 ± 0.002	2.142 ± 0.086	ND	3.870 ± 0.167

[a] Data are the mean ± SD (n = 3) in mg/g of dried samples. ND, not detected.

several different PMFs in hallabong suggests that this product may have global distribution potential.

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