



## Comparative Study on the Content and Cytotoxicity of Pseudolaric Acid B in the Five Plant Parts of *Pseudolarix kaempferi*

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**Abstract** – Pseudolaric acids of *Pseudolarix kaempferi* (Pinaceae) have been known as diterpenoids with potent anti-fungal-, anti-microbial, and cytotoxic activities. In the present study, the five MeOH extracts were prepared from the five plant part (root bark, stem bark, leaf, the inner part of root, and cone) to find the relation between the concentration of pseudolaric acids and cytotoxicity. Pseudolaric acids B and C were isolated from the root bark of *P. kaempferi* to use them as standard compounds. The five extracts were tested on cytotoxicity against six cancer cell lines, A549 (lung), HCT116 (colon), MDA-MB-231 (breast), SNU638 (stomach), and SK-hep-1 (liver) by SRB assay, but against K562 (leukemia) by SRB- or MTT assay. HPLC quantification were performed on a Shisheido Capcell PAK C18 column (5  $\mu$ m, 4.6 mm  $\times$  250 mm) using 254 nm wavelength. The cytotoxicity (IC<sub>50</sub>, 0.36  $\mu$ g/ml on K562 cell lines) of the root bark extract was potent and the content (101.1 mg/g extract) of pseudolaric acid B was very high in the root bark. These results suggest that the MeOH extract obtained from the root bark could be developed as the anti-cancer agent with a high quantity of pseudolaric acid B.

**Keywords** – *Pseudolarix kaempferi*, Pinaceae, Pseudolaric acid B, Cytotoxicity, Quantification, HPLC

### Introduction

The barks of root and trunk of *Pseudolarix kaempferi* have been used to treat fungal skin disease in China.<sup>1</sup> This plant is now cultivated because its therapeutic effect became famous in Korea. This plant is a woody plant belonging to the family Pinaceae. Many diterpenoids belonging to a series of pseudolaric acids have been known from *P. kaempferi*<sup>1,2</sup> since Zhou et al.<sup>3</sup> determined the structure of pseudolaric acids A, B, and C. Studies on antifungal activity against fungi including *Candida albicans* are more common than on other biological activities.<sup>1,4,5</sup> It was also reported that pseudolaric acids have cytotoxic, anti-microbial,<sup>3,6</sup> and anti-tumor activities.<sup>7</sup> Moreover, the promising activities of pseudolaric acid B have been reviewed.<sup>8</sup>

Anti-cancer activities can be expected since pseudolaric acids are highly cytotoxic on cancer cell lines.<sup>9</sup> However, application of natural anti-cancer compounds are limited

because the phytochemical isolation from natural sources are required. Therefore, the extract comprising potent cytotoxic agents can be also expected as the anti-cancer agent. In this study, pseudolaric acids B and C were isolated from *P. kaempferi* to use it as standard compounds in cytotoxicity assay and in HPLC analysis. After the five extracts were prepared from the root bark, stem bark, leaf, the inner part of root, and cone of this plant, the cytotoxic activities were determined. The content of pseudolaric acids B and C were also comparatively studied using HPLC. Pseudolaric acids have not been analyzed in every part of *P. kaempferi*, though the HPLC quantification method was known from this plant by Liu et al.<sup>10</sup>

### Experimental

**Instrument and reagents** – The HPLC system used for quantification consisted of HPLC 210 pump, Prostar 325 UV-vis detector, and Shisheido Capcell Pak C18 column (5  $\mu$ m, 4.6 mm  $\times$  250 mm) equipped with a MetqTherm temperature controller. Solvents used for the

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HPLC eluent were HPLC grade purchased from J.T.Baker (Philisburg, NJ, USA).

**Plant material** – The plant materials of the five plant parts (root bark, stem bark, leaf the inner part of root, and cone) were purchased in an herb store in Wonju. This plant material was identified by Dr. Nam-Tae Woo (Department of Oriental medicine, Sangji University). A voucher specimen (natchem-#122) was deposited in the Laboratory of Natural Products Chemistry, Department of Pharmaceutical Engineering, Sangji University).

**Extraction and fractionation** – To prepare pseudolaric acids B and C, the root bark (553 g) was extracted with MeOH, under reflux, for 5 h, three times. The extracted solution was filtered and evaporated under reduced pressure on a rotatory evaporator to produce 18.6 g of MeOH extract. To fractionate the MeOH extract, it was suspended in H<sub>2</sub>O and fractionated three times with n-

hexane. The residual aqueous layer was further fractionated three times with EtOAc. The EtOAc-soluble portion was concentrated to dryness to yield an EtOAc extract (5.78 g).

**Isolation** – The EtOAc fraction was subjected to silica gel column chromatography using a High-flash column (48 × 170 mm, 40 μm, 165 g Yamazen, Japan) with the eluent CHCl<sub>3</sub>-MeOH-H<sub>2</sub>O (8:1:1, lower phase). The mobile phase was eluted with the flow rate of 1.5 mL/min, and collected by each 80 ml. After TLC checking, the fractions containing compounds **1** and **2** were combined, respectively, to give the two fractions PK-1 and PK-2. These two fractions (PK-1 and PK-2) were further purified by column chromatography with the eluent CHCl<sub>3</sub>-MeOH-H<sub>2</sub>O (8:1:1, lower phase), respectively, to yield compounds **1** and **2**.

**Compound 1 (pseudolaric acid B)** – Mp. 165 - 167 °C,  $[\alpha]_D^{27}$  -37.5 (MeOH, 0.0241); UV 258 nm (log ε =

**Table 1.** <sup>1</sup>H- and <sup>13</sup>C- NMR data of compounds **1** (pseudolaric acid B) and **2** (pseudolaric acid C) measured in CDCl<sub>3</sub>

Position	Compound <b>1</b>		Compound <b>2</b>	
	<sup>13</sup> C	<sup>1</sup> H	<sup>13</sup> C	<sup>1</sup> H
1	33.3	1.86 (2H, m)	33.3	1.86 (2H, m)
2	24.3	1.81 (1H, m) 2.92 (1H, 6.0, 13.8)	24.5	1.76 (1H, m) 2.16 (1H, m)
3	49.3	3.34 (1H, d, 6.0)	54.1	2.23 (1H, d, 6.0)
4	90.2	-	80.6	-
5	30.7	1.76 (1H, m) 3.10 (1H, dd, 6.0, 13.8)	35.2	2.01 (1H, m) 2.14 (1H, m)
6	20.2	2.17 (1H, m) 2.92 (1H, 6.0, 15.0)	19.8	2.59 (1H, m) 2.93 (1H, 6.0, 15.6)
7	134.5	-	134.1	-
8	141.6	7.23 (1H, dd, J=4.2, 9.0 Hz)	142.7	7.26 (1H, dd-like)
9	27.8	2.63 (1H, dd, J=4.2, 15.0 Hz) 2.78 (1H, dd, 9.0, 15.0)	27.3	2.60 (1H, dd-like) 2.71 (1H, dd, 8.4, 14.4)
10	55.3	-	55.1	-
11	83.7	-	80.6	-
12	28.5	1.62 (3H, s)	28.6	1.60 (3H, s)
13	144.5	5.94 (1H, d, 14.4)	145.0	5.96 (1H, d, 14.4)
14	121.8	6.58 (1H, dd, 11.4, 14.4)	121.5	6.59 (1H, dd, 11.4, 14.4)
15	138.7	7.29 (1H, d, 11.4)	138.8	7.28 (1H, d, 11.4)
16	127.8	-	127.4	-
17	12.6	1.99 (3H, s)	12.6	2.00 (3H, s)
18	169.4	-	168.2	-
19	173.2	-	173.9	-
20	168.0	-	168.2	-
19-OCH <sub>3</sub>	52.3	3.74 (3H, s)	52.0	3.75 (3H, s)
4-OCHOCH <sub>3</sub>	172.8	-		
4-OCOCH <sub>3</sub>	21.7	2.15 (3H, s)		

4.40); IR  $\nu_{\max}$   $\text{cm}^{-1}$  KBr: 2952, 2874 (C-H), 1741 (lactonic C=O), 1710 ( $\alpha,\beta$ -unsaturated ester) 1645 ( $\alpha,\beta$ -unsaturated carboxylic acid), 1610 (olefinic C-H), 1441 ( $\text{CH}_2$ ), 1361 ( $\text{CH}_3$ ), 1208 (C-O);  $^1\text{H-NMR}$  (600 MHz,  $\text{CDCl}_3$ ) and  $^{13}\text{C-NMR}$  (150 MHz,  $\text{CDCl}_3$ )  $\delta$ : Table 1.

**Compound 2 (pseudolaric acid C)** – Mp 220 - 222 °C ( $\text{CHCl}_3$ ); UV 258 nm ( $\log \epsilon = 4.25$ ); IR  $\nu_{\max}$   $\text{cm}^{-1}$  KBr: 3444 (O-H), 2954 (C-H), 1709 ( $\alpha,\beta$ -unsaturated ester), 1643 ( $\alpha,\beta$ -unsaturated carboxylic acid), 1610 (olefinic C-H), 1437 ( $\text{CH}_2$ ), 1381 ( $\text{CH}_3$ ), 1275, 1207, 1180 (C-O);  $^1\text{H-NMR}$  (600 MHz,  $\text{CDCl}_3$ ) and  $^{13}\text{C-NMR}$  (150 MHz,  $\text{CDCl}_3$ )  $\delta$ : Table 1.

**Cell Culture** – Human lung cancer (A549), colorectal cancer (HCT116), breast cancer (MDA-MB-231), stomach cancer (SNU-638), liver cancer (SK-HEP-1), and myeloid leukemia (K562) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in medium (DMEM for SK-HEP-1 cells and RPMI 1640 for A549, HCT116, SNU-638, MDA-MB-231, and K562 cells) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and antibiotics-antimycotics (PSF; 100 units/mL penicillin G sodium, 100 ng/mL streptomycin, and 250 ng/mL amphotericin B). The cells were maintained at 37 °C under a humidified atmosphere containing 5%  $\text{CO}_2$ .

**Cell Proliferation Assay** – The inhibitory effects of samples on cell proliferation was determined via MTT (K562) assay or sulforhodamine B (SRB) assay (A549, HCT116, SNU-638, MDA-MB-231, and SK-HEP-1).<sup>11</sup> Cells were seeded in 96-well plates with various concentrations of samples and incubated at 37 °C in a humidified atmosphere with 5%  $\text{CO}_2$  for 72 h. For MTT assay, cells were incubated in MTT (0.5 mg/mL) containing culture medium for 4h at 37 °C. Supernatant medium was carefully removed and DMSO (200  $\mu\text{L}$ ) was added to each well. Absorbance was measured at 570 nm. For SRB assay, cells were fixed with 10% trichloroacetic acid. The cellular proteins were stained with 0.4% SRB in a 1% acetic acid solution and were dissolved in 10 mM Tris buffer (pH 10.0). Absorbance was measured at 515 nm. The percentage of cell proliferation was determined according to the following formula: cell proliferation (%) =  $100 \times [(A_{\text{treated}} - A_{\text{zero day}}) / (A_{\text{control}} - A_{\text{zero day}})]$ , where A is the average absorbance. The  $\text{IC}_{50}$  values were calculated through non-linear regression analysis using TableCurve 2D v5.01 (Systat Software Inc., San Jose, CA, USA).

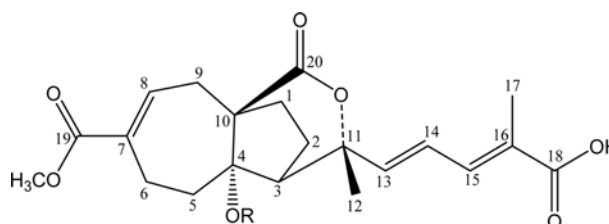
**Preparation of standard- and sample solutions** – The standard stock solutions (1,000  $\mu\text{g/mL}$ ) were prepared by dissolving compounds 1 and 2 in MeOH, and then

preserved in a refrigerator. Working standard solutions were prepared by serially diluting with MeOH. Sample solutions were prepared by dissolving every MeOH extract in MeOH and filtered through a disposable syringe filter unit (0.50  $\mu\text{m}$ , Sismic-25JP Advantec, Japan) prior to the injection to HPLC system.

**HPLC analytical method** – Two solvents, solvent A ( $\text{H}_2\text{O}$  with 0.05% acetic acid, v/v) and solvent B (MeOH) were used in this method. The linear gradient elution of the solvents was programmed as follows: 0 – 20 min (40  $\rightarrow$  80% B), 20 – 21 min (80  $\rightarrow$  100% B), 21 – 25 min (100% B), 25 – 27 min (100  $\rightarrow$  40% B), and 27 – 30 min (40% B). The flow rate and column temperature was set constantly at 1.0 mL/min and 40 °C, respectively. The detection wavelength was fixed at 254 nm and monitored during 30 min for each sample. Regression equations were determined by plotting the peak areas (y axis) measured at six concentrations (x axis,  $\mu\text{g/mL}$ ). Linearity was assessed by determining the  $R^2$  value of the equation. Sensitivity was evaluated by calculation of limit-of-detection (LOD) and limit-of-quantification (LOQ). LOD and LOQ were determined by signal-to-noise (S/N), where 3 and 10 for S/N ratio were used for LOD and LOQ.

## Result and Discussion

Pseudolaric acids B and C, as the structure are shown in Fig. 1, were isolated from the root bark not only to test their cytotoxicity but also to use as the standard compound in the HPLC experiment. As shown in Table 1, the two compounds, pseudolaric acids B and C,<sup>3</sup> were identified by comparisons of  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR spectroscopic data with literature. Availability of any extracts obtained from *P. kaempferi* was examined by testing the cytotoxicity of the five plant part extracts (MeOH extracts). HPLC quantification was performed to correlate the content of the cytotoxic diterpenoids with their cytotoxicity. This biological activities were assayed by the SRB method against the five cancer cell lines (A549 (lung), HCT116 (colon), MDA-MB-231 (breast), SNU638 (stomach), and



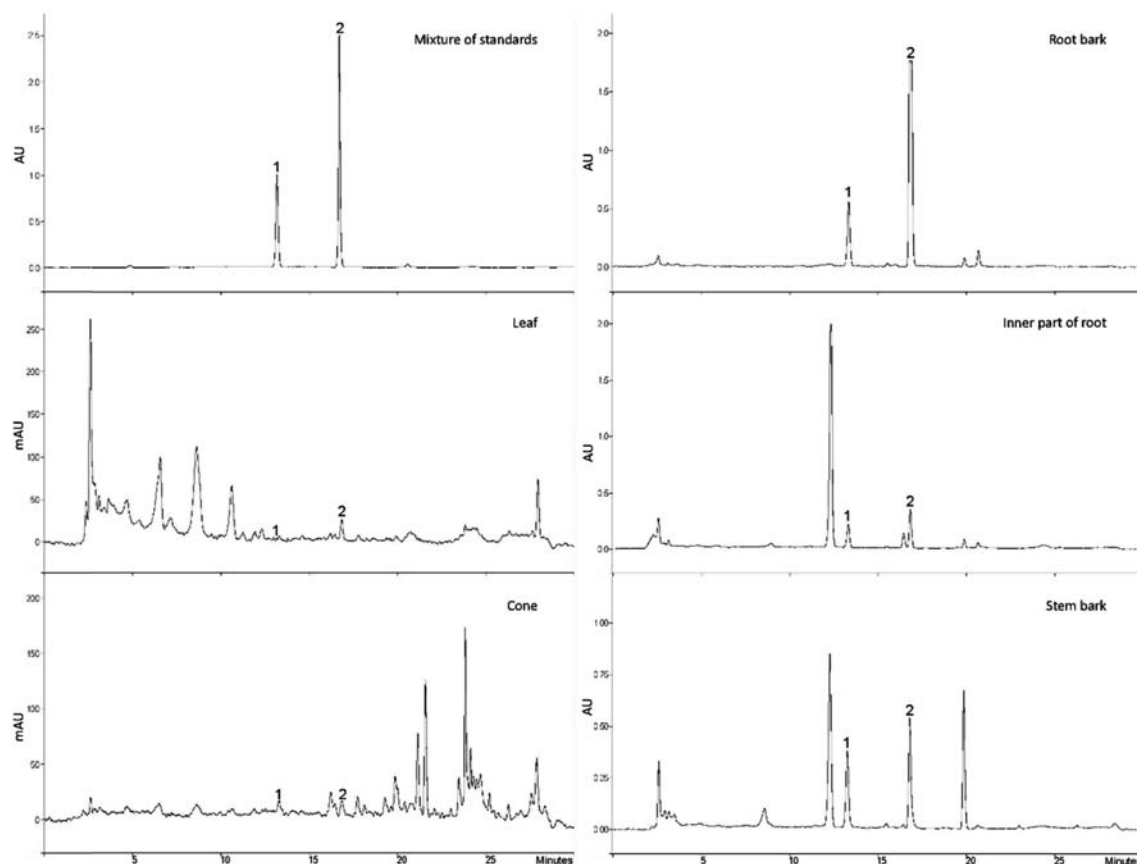
**Fig. 1.** Structure of pseudolaric acids B (R = acetyl) and C (R = H) isolated from *P. kaempferi*.

**Table 2.** Cytotoxic activity ( $IC_{50}$ ) of the MeOH extracts obtained from the five parts of *P. kaempferi* on six cancer cell growth in vitro

Extract	A549 <sup>1)</sup>	HCT116	MDA-MB-231	SNU638	SK-hep-1	K562
Root bark	1.58 <sup>2)</sup>	1.25	34.6	30.7	2.12	0.36
Stem bark	17.98	10.68	>50	>50	>50	2.74
Leaf	>50	>50	>50	>50	>50	>50
Inner part of root	9.49	7.26	>50	3.16	>50	2.76
Cone	16.59	17.58	31.86	27.2	>50	13.22
Pseudolaric acid B	0.63	0.14	13.17	0.13	0.93	0.11
Pseudolaric acid C	>25	>25	>25	>25	>25	>25

<sup>1)</sup> Cancer cell line: A549 (lung), HCT116 (colon), MDA-MB-231 (breast), SNU638 (stomach), SK-hep-1 (liver), and K562 (leukemia).

<sup>2)</sup> Unit:  $\mu\text{g/ml}$ .

**Fig. 2.** HPLC chromatograms of the MeOH extracts obtained from the five plant parts of *P. kaempferi*.

SK-hep-1 (liver), but by the MTT assay against K562 (leukemia). As shown in Table 2, pseudolaric acid B exhibited more potent cytotoxicity than pseudolaric acid C, where the  $IC_{50}$ s of pseudolaric acid C were not shown at the concentration lower than 50  $\mu\text{g/ml}$ . The cytotoxicity of pseudolaric acid B with 4-*O*-acetyl more potent than pseudolaric acid C with 4-OH. The  $IC_{50}$ s were shown over the range of 0.11  $\mu\text{g/ml}$ –13.17  $\mu\text{g/ml}$  against six cancer cell lines. The activities of pseudolaric acid B was highest on K562 cell lines ( $IC_{50}$ , 0.36  $\mu\text{g/ml}$ ), and lowest

on the breast cancer cell line (MDA-MB-231,  $IC_{50}$ , 34.6  $\mu\text{g/ml}$ ). The activity of the root bark extract was most potent among the five extracts, which were comparable with that of pseudolaric acid B. The three plant part extracts (the extracts of the inner part of the root-, stem bark, and cone) exhibited lower activities than the root bark extract. Furthermore, the leaf extract has no  $IC_{50}$  values at the concentration lower than 50  $\mu\text{g/ml}$ .

Using HPLC, the two diterpenoids of pseudolaric acids B and C were analyzed on a reverse-phase (ODS) column,

**Table 3.** Linearity of standard curves and detection/quantification limits for the standard compounds

Compound	$t_R$ (min)	Calibration equation (linear model) <sup>a</sup>	Linear range ( $\mu\text{g/ml}$ )	$R^2$ <sup>b</sup>	LOD <sup>c</sup> ( $\mu\text{g/ml}$ )	LOQ <sup>d</sup> ( $\mu\text{g/ml}$ )
Pseudolaric acid C	13.33	$y = 900.16x + 108.96$	7.81-250.0	0.999	0.10	0.34
Pseudolaric acid B	16.78	$y = 1045.42x + 109.95$	7.81-250.0	0.999	0.09	0.29

<sup>a</sup>y, peak area at 254nm; x, concentration of the standard ( $\mu\text{g/ml}$ ); <sup>b</sup> $R^2$ , correlation coefficient for 6 data points in the calibration curves (n = 4); <sup>c</sup>LOD, limit of detection (S/N = 3); <sup>d</sup>LOQ, limit of quantification (S/N = 10).

**Table 4.** Content of pseudolaric acid C and pseudolaric acid B in the extracts of various parts of *P. kaempferi*

Plant parts	Extract (mg/g)		Dried plant material (mg/g)	
	Pseudolaric acid C	Pseudolaric acid B	Pseudolaric acid C	Pseudolaric acid B
Leaf	0.14 (0.01)	0.61 (0.03)	0.018 (0.002)	0.076 (0.003)
Cone	0.21 (0.02)	0.38 (0.02)	0.022 (0.002)	0.041 (0.002)
Stem bark	8.59 (0.26)	9.45 (0.21)	0.195 (0.006)	0.215 (0.005)
Root bark	27.54 (0.27)	101.05 (0.75)	0.925 (0.009)	3.39 (0.025)
Inner part of root	5.02 (0.07)	5.88 (0.16)	0.233 (0.003)	0.273 (0.007)

The data was present as average of three determinations. SD value was added in the parentheses.

which were detected by a wavelength of 254 nm. As shown in Fig. 2, pseudolaric acids B and C were shown at the retention time of 13.33 min and 16.78 min, respectively. As shown in Table 3, the two regression equations showing good linearity assessed by  $R^2$  values ( $> 0.999$ ) were established. The LOD shown at 0.09 and 0.10  $\mu\text{g/ml}$  and LOQ at 0.29 and 0.34  $\mu\text{g/ml}$  indicates that the two compounds are very sensitive at this wavelength, probably by the conjugated dienoic structure. In the root bark, the content of pseudolaric acids B and C were 101.1  $\mu\text{g/ml}$  and 27.54  $\mu\text{g/ml}$ . The content expressed in the unit of mg/g dry plant material were also shown in Table 4. However, the content of the pseudolaric acid B was very low in other extracts, and especially lowest in the leaf, demonstrating that this compound is mainly distributed in the root bark.

It looks likely that the anti-fungal substances mainly distributed in the root bark may resist fungal microorganisms in the soil. Considering the results of the HPLC chromatogram and the cytotoxicity, pseudolaric acid B may be the final product to resist fungal microorganisms. In conclusion, the root bark extract could be developed as the potential biomaterial for an anti-cancer agent since its cytotoxic potency was very high against various cancer cell lines.

## Acknowledgments

This research was supported by the Sangji University Research Fund, 2016.

## References

- (1) Yang, S. P.; Dong, L.; Wang, Y.; Wu, Y.; Yue, J. M. *Med. Chem.* **2003**, *11*, 4577-4584.
- (2) Yang, S. P.; Wu, Y.; Yue, J. M. *J. Nat. Prod.* **2002**, *65*, 1041-1044.
- (3) Zhou, B. N.; Ying, B. P.; Song, G. Q.; Chen, Z. X.; Han, J.; Yan, Y. F. *Planta Med.* **1983**, *47*, 35-38.
- (4) Li, E.; Clark, A. M.; Hufford, C. D. *J. Nat. Prod.* **1995**, *58*, 57-67.
- (5) Zhang, J.; Yan, L. T.; Yuan, E. L.; Ding, H. X.; Ye, H. C.; Zhang, Z. K.; Yan, C.; Liu, Y. Q.; Feng, G. *J. Agric. Food Chem.* **2014**, *62*, 4905-4910.
- (6) Yang, S. P.; Yue, J. M. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 3119-3122.
- (7) Li, M.; Hong, L. *Mol. Med. Rep.* **2015**, *12*, 2021-2026.
- (8) Liu, M. L.; Sun, D.; Li, T.; Chen, H. *Front. Pharmacol.* **2017**, *8*, 394.
- (9) Pan, D. J.; Li, Z. L.; Hu, C. Q.; Chen, K.; Chang, J. J.; Lee, K. H. *Planta Med.* **1990**, *56*, 383-385.
- (10) Liu, P.; Guo, H.; Sheng, Y.; Wang, W.; Xu, M.; Feng, S.; Cheng, F.; Guo, D. *J. Pharm. Biomed. Anal.* **2007**, *44*, 730-736.
- (11) Kim, W. K.; Pyee, Y.; Chung, H. J.; Park, H. J.; Hong, J. Y.; Son, K. H.; Lee, S. K. *J. Nat. Prod.* **2016**, *79*, 1097-1104.

Received August 10, 2017

Revised September 19, 2017

Accepted September 20, 2017