Mycobiology

Research Article

Chemical Constituents of the Culture Broth of *Phellinus linteus* and Their Antioxidant Activity

Myeong-Seok Lee[1], Byung Soon Hwang[1], In-Kyoung Lee[1], Geon-Sik Seo[2] and Bong-Sik Yun[1,*]

[1]*Division of Biotechnology and Advanced Institute of Environment and Bioscience, Chonbuk National University, Iksan 570-752, Korea*
[2]*Korea National College of Agriculture and Fisheries, Hwaseong 445-760, Korea*

Abstract The medicinal fungus *Phellinus linteus*, in the family Hymenochaetaceae, has been used as a traditional medicine for the treatment of various diseases. In this study, the chemical constituents of the culture broth of *P. linteus* were investigated. *P. linteus* was cultured in potato dextrose broth medium, and the culture broth was extracted with ethyl acetate. The ethyl acetate-soluble portion was concentrated and subjected to ODS column chromatography, followed by Sephadex LH-20 column chromatography. Six compounds (**1**~**6**) were purified by preparative reversed-phase high-performance liquid chromatography. Spectroscopic methods identified their structures as caffeic acid (**1**), inotilone (**2**), 4-(3,4-dihydroxyphenyl)-3-buten-2-one (**3**), phellilane H (**4**), (2*E*,4*E*)-(+)-4'-hydroxy-γ-ionylideneacetic acid (**5**), and (2*E*,4*E*)-γ-ionylideneacetic acid (**6**). Compounds **1**, **2**, and **3** exhibited potent dose-dependent antioxidant activity.

Keywords Antioxidant, Caffeic acid, Inotilone, Medicinal fungus, *Phellinus linteus*

The inherent chemical diversity of natural products has provided opportunities for new drug discoveries [1]. Mushrooms are very important sources of the bioactive compounds that produce various classes of secondary metabolites with interesting biological activities and thus have the potential to be used as valuable chemical resources for drug discovery [2]. *Phellinus* species have been used for the treatment of inflammation, diabetes, gastrointestinal cancer, cardiovascular disease, stomach ailments, and tuberculosis [3-5]. *P. linteus*, belonging to Hymenochaetaceae, is indigenous to tropic America, Africa, and East Asia and has been widely used in East Asia, especially Korea, China, and Japan as a health booster and an ancient herbal medicine [6, 7]. *P. linteus*, known as Sangwhang in Korea, produces abundant bioactive compounds with various biological activities, such as anti-cancer, anti-oxidative,

Mycobiology 2015 March, **43**(1): 43-48
http://dx.doi.org/10.5941/MYCO.2015.43.1.43
pISSN 1229-8093 • eISSN 2092-9323

*Corresponding author
E-mail: bsyun@jbnu.ac.kr

Received October 30, 2014
Revised December 5, 2014
Accepted February 4, 2015

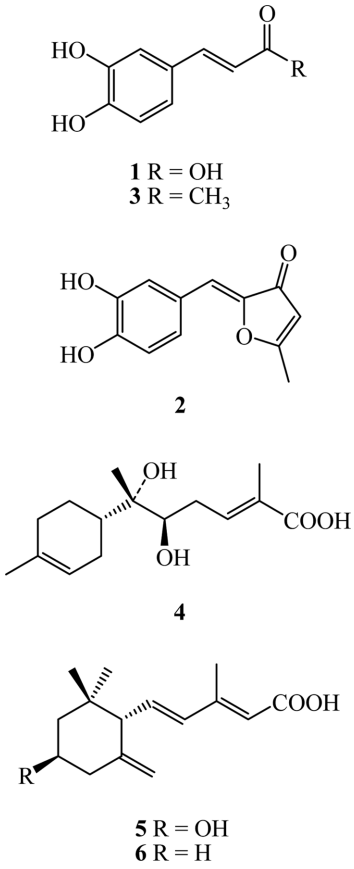

Fig. 1. Structures of compounds **1**~**6**. **1**, caffeic acid; **2**, inotilone; **3**, 4-(3,4-dihydroxyphenyl)-3-buten-2-one; **4**, phellilane H; **5**, (2*E*,4*E*)-(+)-4′-hydroxy-γ-ionylideneacetic acid; **6**, (2*E*,4*E*)-γ-ionylideneacetic acid.

anti-angiogenic, anti-inflammatory, and anti-viral effects [2, 8-14]. This mushroom produces a bundle of yellow antioxidant pigments composed of polyphenols [2]. In a previous study, nine compounds were isolated from the ethyl acetate-soluble fraction of its fruiting body and identified as protocatechuic acid, protocatechualdehyde, caffeic acid, ellagic acid, hispidin, davallialactone, hypholomine B, interfungin A, and inoscavin A. Interfungin A has been reported as a potent inhibitor of protein glycation [15]. In this study, we investigated the chemical constituents of the culture broth of *P. linteus* and isolated six compounds, caffeic acid (**1**), inotilone (**2**), 4-(3,4-dihydroxyphenyl)-3-buten-2-on (**3**), phellilane H (**4**), (2E,4E)-(+)-4′-Hydroxy-γ-ionylideneacetic acid (**5**), and (2E,4E)-γ-ionylideneacetic acid (**6**) (Fig. 1).

MATERIALS AND METHODS

General methods. Electrospray ionization (ESI) mass spectra were taken using Agilent Technologies 6410 Triple Quad LC/MS spectrometer (Agilent Technologies, Santa Clara, CA, USA) in positive and negative modes. Nuclear magnetic resonance spectra were obtained on a JEOL JNM-ECA600 NMR spectrometer (JEOL, Tokyo, Japan) with ^{1}H NMR at 600 MHz and ^{13}C NMR at 150 MHz in $CDCl_3$ or CD_3OD. Chemical shifts were given in ppm (δ) using tetramethylsilane as an internal standard.

Microorganism and fermentation. The *P. linteus* was obtained from the Korea National College of Agriculture and Fisheries (Korea). The strain was cultured for 30 days in 1-L flasks with 400 mL of potato dextrose broth (26 L) at 27°C.

Extraction and isolation. The mycelium of *P. linteus* was extracted with acetone, and partitioned with ethyl acetate. The ethyl acetate-soluble portion was concentrated under reduced pressure, and then was subjected to reversed-phase (ODS) column chromatography and Sephadex LH-20 (Pharmacia, Uppsala, Sweden) column chromatography for the isolation and purification of compounds. Finally, compounds were purified by preparative reversed-phase high-performance liquid chromatography (HPLC).

ABTS radical scavenging activity. Evaluation of free radical scavenging activity was carried out using 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation decolorization assay [16]. ABTS radical scavenging activity was evaluated using the method with minor modification [2, 17]. ABTS was dissolved in H_2O to a concentration of 7 mM. The ABTS cation radical was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) after allowing the mixture to stand in the dark at room temperature for 12 hr. After the addition of 190 μL of the ABTS radical cation solution (A734 nm, 0.700) to the dimethyl sulfoxide (DMSO) solution of the compound (10 μL) and mixing for 7 min, the absorbance was measured by a microplate reader using VERSAmax (Molecular Devices Co., Sunnyvale, CA, USA). Assays were carried out in triplicate, and antioxidants Trolox and BHA were used as positive controls.

DPPH radical scavenging activity. α,α-Diphenyl-β-picrylhydrazyl (DPPH) radical scavenging activity was evaluated using the method with minor modification [2, 17, 18]. The sample was dissolved in 10 μL of DMSO and added to 90 μL of 320 μM DPPH ethanol solution. After vortexing, the mixture was incubated for 10 min at room temperature, and the absorbance was measured at 517 nm using a microplate reader (Molecular Devices Co.). The differences in absorbance between the test sample and control (DMSO) were measured. Assays were carried out in triplicate, and antioxidants Trolox and BHA were used as positive controls.

Reducing power. Reducing power was evaluated using the potassium ferricyanide reduction method with minor modification [19]. Sample (20 μL) was mixed with 50 μL of 200 mM potassium phosphate buffer (pH 6.6) and 50 μL of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. After addition of 50 μL of 10% trichloroacetic acid (w/v), the mixture was centrifuged at 650 rpm for 10 min. The upper layer (100 μL) was mixed with 100 μL distilled water and 20 μL of 0.1% ferric chloride, and absorbance was measured at 700 nm. Assays were carried out in triplicate, and antioxidants Trolox and BHA were used as positive controls.

RESULTS AND DISCUSSION

Purification of chemical constituents 1~6. Compounds **1~6** were isolated by monitoring with thin-layer chromatography and HPLC (Fig. 2). The culture broth of *P. linteus* was centrifuged, and the mycelium was extracted with acetone. The acetone extract was concentrated *in vacuo* to eliminate acetone and then partitioned with ethyl acetate. The ethyl acetate-soluble portion was concentrated under reduced pressure, and the concentrate was subjected to ODS column chromatography eluted with 50%, 60%, and 80% aqueous methanol. The fraction eluted with 50% aqueous methanol was concentrated and subjected to a column of Sephadex LH-20 eluted with 70% aqueous methanol to provide compounds **1** (60.0 mg), **2** (6.8 mg), and **3** (6.3 mg). The fraction eluted with 60% aqueous methanol was subjected to Sephadex LH-20 column chromatography eluted with 70% aqueous methanol, followed by preparative HPLC equipped with reversed-phase column and eluted with 45% aqueous methanol/0.04% trifluoroacetic acid to afford compounds **4** (7.2 mg) and **5** (4.3 mg). The fraction eluted with 80% aqueous methanol was chromatographed on a column of Sephadex LH-20 eluted with methanol to isolate compound **6** (4.0 mg).

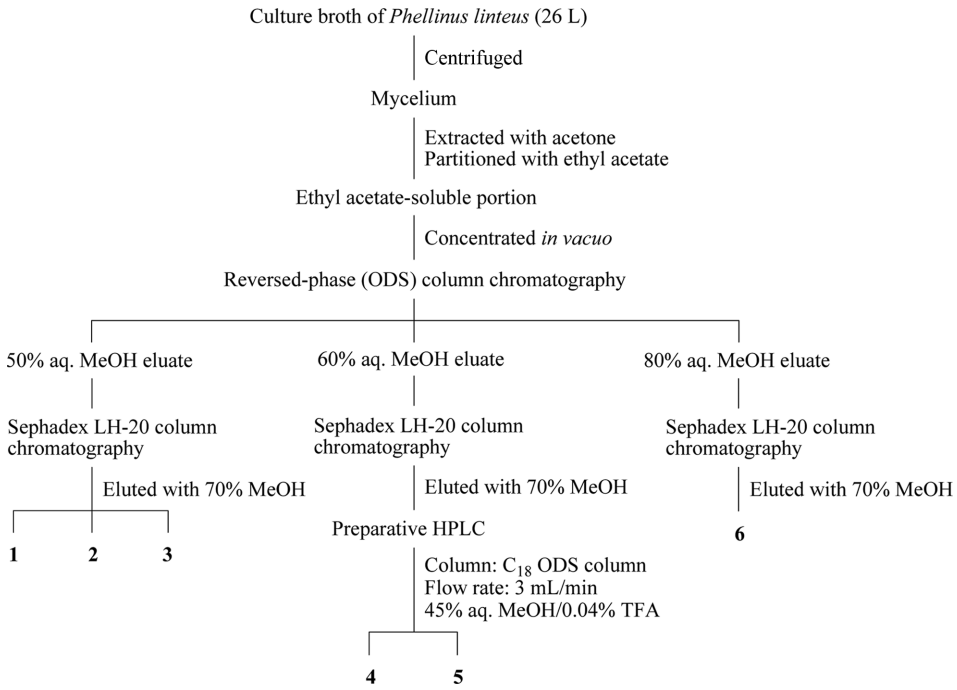

Fig. 2. Isolation procedures of compounds **1~6**. TFA, trifluoroacetic acid.

Structure determination of compounds 1~6. The chemical structure of compound **1** was determined by ^{1}H NMR spectrum and compared to the HPLC retention time of the authentic compound. The molecular weight of compound **1** was established by the ESI-mass measurement in negative mode, which provided a quasi-molecular ion peak at *m/z* 179.0 $[M-H]^-$ suggesting a molecular weight of 180. The ^{1}H NMR spectrum of compound **1** in CD_3OD exhibited signals due to five methines at δ 7.42 (d, 1H, J = 15.8 Hz), 7.02 (d, 1H, J = 2.1 Hz), 6.95 (dd, 1H, J = 2.1, 8.1 Hz), 6.75 (d, 1H, J = 8.1 Hz), and 6.17 (d, 1H, J = 15.8 Hz). This spectroscopic data was well matched with that of caffeic acid.

The structure of compound **2** was determined by ESI-mass measurement and ^{1}H NMR and ^{13}C NMR spectra. The molecular weight of **2** was established to be 218 by the ESI-mass measurements. The ^{1}H NMR spectrum of **2** in CD_3OD exhibited signals due to three aromatic methines at δ 7.34 (d, 1H, J = 2.0 Hz), 7.16 (dd, 1H, J = 8.4, 2.0 Hz), and 6.80 (d, 1H, J = 8.4 Hz) and two olefinic methines at δ 6.49 (s, 1H) and 5.80 (s, 1H), and one methyl at δ 2.55 (s, 3H). In the ^{13}C NMR spectrum, twelve carbons at δ 187.0, 180.9, 148.4, 145.7, 144.6, 125.0, 123.1, 118.2, 116.2, 112.3, 105.7, and 15.9 were evident. These spectral data were in good agreement with inotilone reported in the literature [20, 21]. Consequently, compound **2** was identified as inotilone. We previously isolated inotilone from the culture broth of this strain as a neuraminidase inhibitor [21].

The molecular weight of compound **3** was established by the ESI-mass measurement in negative mode, which provided a quasi-molecular ion peak at *m/z* 177.0 $[M-H]^-$, suggesting a molecular weight of 178. The ^{1}H NMR spectrum of compound **3** in CD_3OD exhibited signals of five methines at δ 7.51 (d, J = 16.4 Hz), 7.07 (d, J = 2.4 Hz), 6.98 (dd, J = 2.4, 8.4 Hz), 6.78 (d, J = 8.4 Hz), and 6.54 (d, J = 16.4 Hz) and one singlet methyl at δ 2.32. These spectroscopic data matched well with 4-(3,4-dihydroxyphenyl)-3-buten-2-one, which was previously isolated from the culture broth of this strain and identified as a neuraminidase inhibitor [21].

The molecular weight of compound **4** was established by the ESI-mass measurement, which provided a quasi-molecular ion peak at *m/z* 291.0 $[M+Na]^+$, suggesting a molecular weight of 268. The ^{1}H NMR spectrum of compound **4** in CD_3OD exhibited signals due to two olefinic methines at δ 6.97 and 5.38, one oxygenated methine at δ 3.61, one methine at δ 1.72, four methylenes at δ 2.56/2.30, 2.10/1.95, 2.00/1.95, and 1.80/1.35, three methyls at δ 1.83, 1.62, and 1.14. In the ^{13}C NMR spectrum, 15 carbon peaks including a carbonyl carbon at δ 171.6, two sp^2 methine carbons at δ 142.6 and 122.1, two sp^2 quaternary carbons at δ 134.4 and 129.8, two oxygenated carbons at δ 76.6 and 75.5, one methine carbon at δ 41.9, four methylene carbons at δ 32.0, 31.9, 26.8, and 24.9, and three methyl carbons at δ 23.5, 19.0, and 12.7. The ^{1}H-^{1}H COSY spectrum established two partial structures, =CH-CH_2-CH-CH_2-CH_2- and -CH(-O)-CH_2-CH=. These spectral data suggested that compound **4** was a sesquiterpene phelilane H, which was isolated from the fermentation broth of *P. linteus*. The chemical structure was confirmed by the HMBC spectrum, which exhibited long-range correlations which matched well to phelilane H. Compound **4** was identified as phelilane H.

The structure of compound **5** was determined by ESI-

mass measurement and one-dimensional ^{1}H and ^{13}C NMR spectra, two-dimensional ^{1}H-^{1}H COSY, HMQC, and HMBC spectra. The molecular weight of compound **5** was established by the ESI-mass measurement in negative mode, which provided a quasi-molecular ion peak at *m/z* 249.0 $[M-H]^-$, suggesting a molecular weight of 250. The ^{1}H NMR spectrum of **5** in CD_3OD exhibited signals due to two overlapped olefinic methines at δ 6.08, one olefinic methine at δ 5.63, one terminal methylene at δ 4.76 and 4.47, one oxygenated methine at δ 3.59, one methine at δ 2.45, two methylenes at δ 2.52/1.86 and 1.65/1.22, and three methyls at δ 2.18, 0.81, and 0.73. In the ^{13}C NMR spectrum, 15 carbon peaks were evident and each carbon peak was assigned as follows; a carbonyl carbon at δ 170.6, two sp^2 quaternary carbons at δ 153.4 and 148.6, three sp^2 methine carbons at δ 137.9, 136.3, and 119.7, a terminal methylene carbon at δ 111.2, an oxygenated methine carbon at δ 68.2, a methine carbon at δ 57.7, a quaternary carbon at δ 36.7, two methylene carbons at δ 50.9 and 46.5, three methyl carbons at δ 31.2, 21.7, and 14.1. The ^{1}H-^{1}H COSY spectrum established two partial structures, -CH_2-CH(-O)-CH_2- and -CH-CH=CH-. These spectral data suggested that compound **5** was a sesquiterpene (±)-(2*E*,4*E*)-3′-hydroxy-γ-ionylideneacetic acid, which has been reported as a fungal methabolite [10, 22]. The chemical structure was confirmed by the HMBC spectrum, which exhibited long-range correlations consistent with (±)-(2*E*,4*E*)-3′-hydroxy-γ-ionylideneacetic acid.

The molecular weight of compound **6** was established by the ESI-mass measurement in negative mode, which provided a quasi-molecular ion peak at *m/z* 232.9 $[M-H]^-$, suggesting a molecular weight of 234. The ^{1}H NMR spectrum of compound **6** in CD_3OD exhibited signals due to three olefinic methines at δ 6.17 (dd, J = 15.1, 9.6 Hz), 6.10 (d, J = 15.1 Hz), and 5.74, one terminal methylene at δ 4.73 and 4.54, one methine at δ 2.56, three methylenes at δ 2.28/2.06, 1.59, and 1.53/1.37, and three methyls at δ 2.21, 0.90, and 0.83. The ^{1}H NMR spectrum was very similar to that of compound **5**, except that an oxygenated methine peak at δ 3.59 in compound **5** was replaced with a methylene peak at δ 1.59 in compound **6**, suggesting that the structure of **6** was (±)-(2*E*,4*E*)-γ-ionylideneacetic acid [10]. In the ^{13}C NMR spectrum, 15 carbon peaks including a carbonyl carbon at δ 173.5, two sp^2 quaternary carbons at δ 151.2 and 149.1, three sp^2 methine carbons at δ 137.1, 135.2, and 123.3, a terminal methylene carbon at δ 109.2, a methine carbon at δ 59.3, a quaternary carbon at δ 36.4, three methylene carbons at δ 40.1, 35.5, and 24.5, and three methyl carbons at δ 29.9, 24.0, and 14.2. The ^{13}C NMR spectral data also supported that the structure of **6** was (±)-(2*E*,4*E*)-γ-ionylideneacetic acid. The structure was confirmed by the ^{1}H-^{1}H COSY spectrum, which revealed two partial structures, -CH_2-CH_2-CH_2- and -CH-CH=CH-. Therefore, the structure of compound **6** was determined to be (±)-(2*E*,4*E*)-γ-ionylideneacetic acid.

Antioxidant activity. A majority of antioxidants are able to scavenge free radicals. Therefore, we assessed the free radical scavenging efficacy of compounds **1**~**6** by using ABTS radical cation and DPPH radical scavenging assay methods. In addition, we assessed the reducing power activity of these compounds. The ABTS and DPPH radical scavenging activity was expressed in terms of Trolox equivalent antioxidant capacity (TEAC; IC_{50} of compound/IC_{50} of Trolox). Compounds **1**~**3** exhibited potent scavenging activity against the ABTS and DPPH radicals in a concentration-dependent manner, with TEAC value of 0.52, 1.10, and 1.69, respectively (Table 1). However, compounds **4**~**6** did not show scavenging activity against these radical species.

In the reducing power assay, the yellow color of the test solution changes to various shades of green and blue, depending on the reducing power of each compound. The presence of reducers (i.e., antioxidants) causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, measuring the formation of Perl's Prussian blue at 700 nm can monitor the Fe^{2+} concentration [19]. Results were expressed as relative activity against Trolox (absorbance value of sample/absorbance value of 10 mM Trolox). Compounds **1**~**3** exhibited higher activity than antioxidants BHA and Trolox, but compounds **4**~**5** showed marginal

Table 1. ABTS and DPPH radical scavenging activity of compounds **1**~**6**

Compounds	TEAC[a,b]	
	ABTS[c]	DPPH[d]
Caffeic acid (**1**)	0.52 ± 0.10	0.76 ± 0.10
Inotilone (**2**)	1.10 ± 0.10	1.55 ± 0.11
4-(3,4-Dihydroxyphenyl)-3-buten-2-one (**3**)	1.69 ± 0.11	2.97 ± 0.10
Phellilane H (**4**)	> 3.00	> 3.00
(2*E*,4*E*)-4′-Hydroxy-γ-ionylideneacetic acid (**5**)	>3.00	> 3.00
(2*E*,4*E*)-γ-Ionylideneacetic acid (**6**)	> 3.00	> 3.00
BHA	0.71 ± 0.10	2.39 ± 0.10

[a]Expressed as IC_{50} of compound/IC_{50} of trolox.
[b]Results presented as the mean ± SD (n = 3).
[c]2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid).
[d]α,α-Diphenyl-β-picrylhydrazyl.

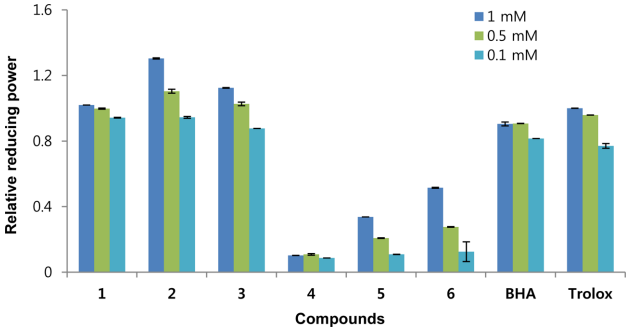

Fig. 3. Reducing power of compounds **1**~**6**. Reducing power was evaluated using the potassium ferricyanide reduction method. Results were presented as the mean ± SD (n = 3). **1**, caffeic acid; **2**, inotilone; **3**, 4-(3,4-dihydroxyphenyl)-3-buten-2-one; **4**, phellilane H; **5**, (2*E*,4*E*)-(+)-4′-hydroxy-γ-ionylideneacetic acid; **6**, (2*E*,4*E*)-γ-ionylideneacetic acid; antioxidants BHA and Trolox were used as positive controls.

activity (Fig. 3).

Mushrooms are ubiquitous in nature, and some of them are nutritionally functional foods and important sources of physiologically beneficial medicines. It is known that *Phellinus linteus* produces diverse bioactive substances, especially stylrylpyrone-class antioxidants [4, 9]. The culture broth of *P. linteus* is used as an antioxidant ingredient to make functional beverages and cosmetics in Korea. Although the antioxidant activity of *P. linteus* is well known, its antioxidant substance has yet to be determined. In a previous study, we reported four antioxidants, hispidin and its dimers, 3,14′-bihispidinyl, hypholomine B, and 1,1-distyrylpyrylethan from the culture broth of *P. linteus* [4]. In this study, we focused on chemical constituents in the culture broth of *P. linteus* and found six major constituents, caffeic acid (**1**), inotilone (**2**), 4-(3,4-dihydroxyphenyl)-3-buten-2-one (**3**), phellilane H (**4**), (2*E*,4*E*)-(+)-4′-hydroxy-γ-ionylideneacetic acid (**5**), and (2*E*,4*E*)-γ-ionylideneacetic acid (**6**). Compounds **1**~**3** exhibited potent antioxidant activity in a dose-dependent manner. Their antioxidant effects may originate from the catechol moiety [4, 23]. Other compounds **4**~**6** did not exhibit antioxidant effect. However, high accumulation of compounds **4**~**6** in the culture broth of *P. linteus* was firstly reported in this paper.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Korea Forest Service as well as support from the Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ009796), Rural Development Administration, Republic of Korea.

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