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Purification and Properties of a Novel Phenolic Antioxidant from *Radix astragali* Fermented by *Aspergillus oryzae* M29

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Supporting Information

ABSTRACT: The Chinese herb *Radix astragalus* (RA) has been widely used as a dietary supplement in Asia, and there are numerous reports on its bioactivities. However, there are no reports to date regarding the use of *Aspergillus* spp. in the culture medium of the RA plant for the production of phenolic antioxidants. In this study, utilizing the fungus *Aspergillus* to ferment the native RA has successfully resulted in a significant increase in the phenolic contents of RA, and the fermented RA also revealed much better antioxidant activity toward 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radicals, hydroxyl radical, superoxide radical and peroxyl radical than those of unfermented RA. Among these phenolics, a potent novel antioxidant was isolated and identified as 3,4-di(4′-hydroxyphenyl) isobutyric acid with a molecular weight of 272, by ESI-MS (electrospray ionization mass), ^{1}H NMR (nuclear magnetic resonance), ^{13}C NMR, DEPT (distortionless enhancement by polarization transfer)-NMR, HMQC (heteronuclear multiple quantum coherence), and HMBC (heteronuclear multiple bond correlation) spectra. These data demonstrated that the solid-state bioprocessing strategy could be an innovative approach to enhance the antioxidant activity of RA.

KEYWORDS: *Aspergillus*, *Astragalus*, antioxidant, phenolic

INTRODUCTION

In addition to regular medicinal care, many patients also use some forms of complementary/alternative medicine to combat the development of many degenerative diseases, and traditional botanical medicine has received tremendous attention in the past few years.[1] It is a widespread belief that herbal products are safe because they are "from nature"; thus, some botanical medicines, such as *Angelicae sinensis*, *Dioscorea rhizoma*, and *Radix astragali* (RA), have for a long time been categorized for use as food. Among those herbs categorized as food, *Radix astragali* is a Chinese medicine widely used in Asia, and clinical investigation shows that it is safe as a complementary/alternative medicine.[2]

The crude extract of RA is usually taken directly as health-promoting supplements in commercial forms. RA extracts exhibited many bioactivities such as antitumoral activity,[3,4] antioxidant activity,[5−7] and antiviral activity,[8] as well as both the regulation of immunity[9] and lipid metabolism activities.[10] The active constituents of RA are flavonoids, saponins, polysaccharides, and amino acids; and they vary according to seasonal variation and age of the plant.[11] The major flavonoids present in RA are ononin, calycosin, formononetin, 9,10-dimethoxypterocarpan 3-*O*-β-D-glucoside, 2′-hydroxy-3′,4′-dimethoxyisoflavane 7-*O*-β-D-glucoside, 7,3′-dihydroxy-4′-methoxyisoflavone, 7,3-dimercapto-4,1-methoxyisoflavone, 3-dimercapto-7,4,1-methoxyisoflavone, and kumatakenin.[12−14]

When the homeostasis between the prooxidant formation and antioxidant capacity is disrupted in living systems, oxidative damage will accumulate and result in pathophysiological events. To maintain the prooxidant−antioxidant balance, the removal of free radicals and reactive oxygen species (ROS) is probably one of the most effective defenses of a living body against various diseases.[15] The phenolics have long been demonstrated to exhibit a scavenging effect for free radicals, and they are also the major bioactive components in RA responsible for pharmacological activity and therapeutic efficacy.[16,17] Many studies showed that the concentration and diversity of phenolics could be increased by the fermentation of soybean,[18] rice,[19] black bean,[20] fava bean,[21] and cranberry pomace;[22] but no reports were available on RA.

A few publications have also explored the use of the RA plant as a fermentative substrate with various microbes for functional food and preventive medicine, such as *Cordyceps militaris*,[23] *Bacillus subtilis*,[24] *Ganoderma lucidum*,[25] or chicken cecal contents.[26] However, there are no reports to date regarding the use of *Aspergillus* spp. in the culture medium of the RA plant for the production of bioactive compounds. Aspergilli with metabolic versatility are widely used in industry and biotechnology for the production of enzymes and food fermentation.[27] In some cases, the antioxidant effect could be significantly enhanced through fermentation with *Aspergillus* spp.;[18−20] therefore, it

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might be a useful tool for producing bioactive materials with health-promoting properties from RA.

A preliminary study from our laboratory demonstrated that *Aspergillus* solid-state fermented RA had higher antioxidant activities compared to those of the unfermented substrate, indicating that it is suitable as a new substrate source for the selection of novel antioxidants. Herein, we screened potent *Aspergillus* spp. from soils and investigated the antioxidant activity of the extracts of solid-state fermented RA toward hydroxyl radicals, superoxide radicals, and peroxyl radicals in comparison with those of unfermented RA. In addition, we further isolated and identified the potent phenolic antioxidant from fermented RA and determined its antioxidative activity in different oxidation systems.

MATERIALS AND METHODS

Materials. *Radix astragali* (RA), the dried root of *Astragalus membranaceus* (Fisch.) *Bge. Var. mongolicus* was from an estern chinese medicine factory (Shenzhen, China) and deposited in the department of Food Science and Biotechnology, National Chung Hsing University, Taiwan. ABTS (2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, indoxyl-β-D-glucuronide (IBG), $FeSO_4 \cdot 7H_2O$, lucigenin, arginine, and methylglyoxal were from Sigma Chemical Co. (St. Louis, MO, USA). Ethylenediaminetetraacetic acid (EDTA) was purchased from Merck Co. (Darmstadt, Germany). Fluorescein, 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox), and 2.2′-azobis (2-amidinopropane) dihydrochloride (AAPH) were purchased from Aldrich (Milwaukee, WI, USA). Potato dextrose agar (PDA) was purchased from Difco Co. (Detroit, Michigan, USA). Hesperidin was purchased from Masterasia Marketing CO. (Taipei,Taiwan).

Isolated Microorganism and Fermentation. Strains of *Aspergillus* spp. were isolated from soils using a traditional plating method on yeast malt agar (YMA), adjusted to pH 3.5−4.0 with citric acid. *Aspergillus* strains were collected on the basis of morphological characteristics, and faster-growing strains were isolated and activated to potato dextrose agar (PDA) to obtain spore suspensions in sterile distilled water. RA was pulverized and sterilized at 121 °C for 15 min. Solid fermentation was performed by spraying spore suspension into sterilized RA (ca. 5×10^5 spores/g substrate), and the ioculated RA was cultured at 30 °C for 3 days.

Preparation of Methanol Extracts. The fermented samples were dried, pulverized, and extracted exhaustively with 70% methanol at a ratio of 1:10 (w/v) at 30 °C, 120 rpm for 24 h. The extract was filtrated through Whatman No. 1 filter paper, concentrated under reduced pressure, and successively lyophilized. The percentage yield was defined as weight methanol extract/weight initial fermented RA.

Trolox Equivalent Antioxidant Capacity (TEAC) Assay. The total antioxidant activity was measured using the TEAC assay as described by Arts et al.[28] Sample solution (10 μL) was mixed with 200 μL of the ABTS radicals, and absorbance was monitored at 734 nm for 2 min. The following formula was used for calculating the percent scavenging capacity.

$$(\text{OD of the blank at 734nm} - \text{OD of the sample at 734nm})/$$
$$\text{OD of the blank at734nm} \times 100$$

Hydroxyl Radical Scavenging Ability Assay. The hydroxyl radical generating system was based on the Fenton reaction ($Fe^{2+} + H_2O_2$); therefore, the reaction mixture included 1.0 mL of 3 μM IBG, 0.1 mL of 1.0 mM $FeSO_4$, 1.6 mL of 3% H_2O_2, and 0.05 mL of 10 mM EDTA. The ultraweak photon was measured using BJL-ultraweak chemiluninescence (Jye Horn Co. Taiwan).[29] A 10 μL of sample was added to the reaction mixture, and dropping degrees of the CL counts represented the hydroxyl radical scavenging abilities.

Superoxide Radical Scavenging Ability Assay. The superoxide radicals were generated by lucigenin and methylglyoxal/arginine reagents in the BJL-ultraweak chemiluninescence.[30] The reaction mixture included 1.0 mL of 2.0 mM lucigenin, 0.05 mL of 1.0 M arginine, 0.05 mL of 1.4 μM methylglyoxal, and 1.0 mL of PBS buffer (pH 7.4). A 10 μL of sample was added to the reaction mixture and dropping degrees of the CL counts represented the superoxide radicals scavenging abilities.

Oxygen Radical Absorbance Capacity (ORAC) Assay. The ability of the sample to scavenge peroxyl radicals was measured using the ORAC assay as described by Alberto et al.[31] Fluorescein (150 μL, 96 mM) and the 20 μL sample solution were preincubated for 5 min at 37 °C, and the ORAC analyses was initiated by the addition of 30 μL of 320 mM AAPH. Fluorescein decay curves for PBS buffer and the samples were performed, and all the reaction mixtures were prepared in duplicate. The net AUC corresponding to a sample was calculated by subtracting the AUC corresponding to the PBS buffer. Different concentrations of Trolox were also carried out in each assay, and regression equations between net AUC and Trolox concentration were built. All values were expressed as Trolox equivalents.

Isolation, Purification, and Identification. The fermented RA sample was analyzed by reverse phase HPLC using an Inertsil ODS-3 C18 semiprep column (10 × 250 mm) as described by Hsu and Chiang.[24] The column was eluted with a linear gradient of acetonitrile (5−70% at 0−30 min and 70−85% at 30−45 min) containing 0.1% TFA at 1 mL/min and the pattern detected at 280 nm. Bioactive fractions with antioxidant activity in the elute were pooled and lyophilized for further purification. The strongest antioxidant fraction by the TEAC assay was selected and rechromatographied on an Inertsil ODS-3 C18 semiprep column (10 × 250 mm) eluted with a linear gradient of acetonitrile (40−60% in 30 min) containing 0.1% TFA at 1 mL/min. The purified component was collected for structure and molecular weight determination using the methods as shown below.

^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker Avance-600 and Varian VNMRS-700 NMR spectrometers, respectively, at ambient temperature with TMS (tetramethylsilane) as an internal standard. The DEPT spectrum was performed by 150 MHz C-NMR to determine the carbon type. The 2D ^{1}H,HMQC and ^{1}H,^{13}C-HMBC NMR spectra were performed using a Varian VNMRS-700 NMR spectrometer. The HMQC spectrum provided correlation between protons and their attached heteronuclei through the heteronuclear scalar coupling. The ESI-MS spectrum was recorded on a Micromass Q-Tof LC/MS/MS mass spectrometer.

RESULTS AND DISCUSSION

Strain Screening and Identification. ABTS was a stable radical source, and we applied it to samples in order to evaluate the total antioxidant activity in samples. We screened the potential *Aspergillus* spp. from the soils with the aim of identifying faster-growing characteristics and high antioxidant activity. Four out of the 20 *Aspergillus*-fermented RA samples were found to possess stronger antioxidant activity against ABTS radicals, ranging from approximately 4- to 8-fold greater than that of unfermented RA at the same concentration. The M29 strain with the highest antioxidant capacity among those tested was identified by the sequence analysis method of the rDNA ITS1-5.8S-ITS2 region and the partial β-tubulin gene, and morphological methods such as mycelia growth and spore diameter.[32] All identification data were conducted and certificated by Food

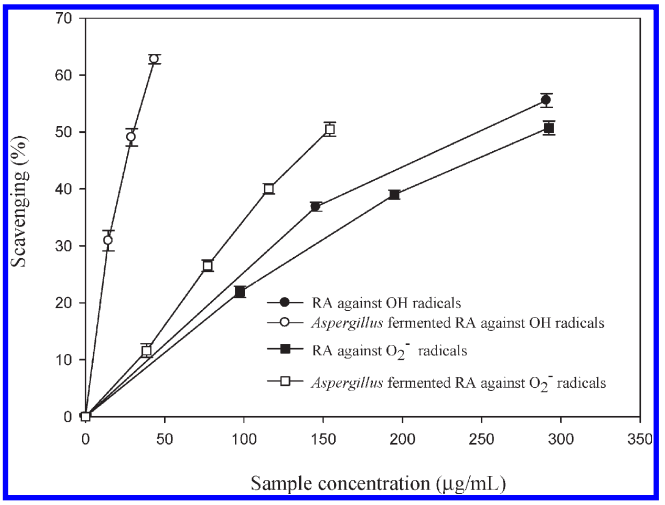

Figure 1. Radical scavenging activity of *A. oryzae* M29-fermented RA toward hydroxyl radicals and superoxyl radicals.

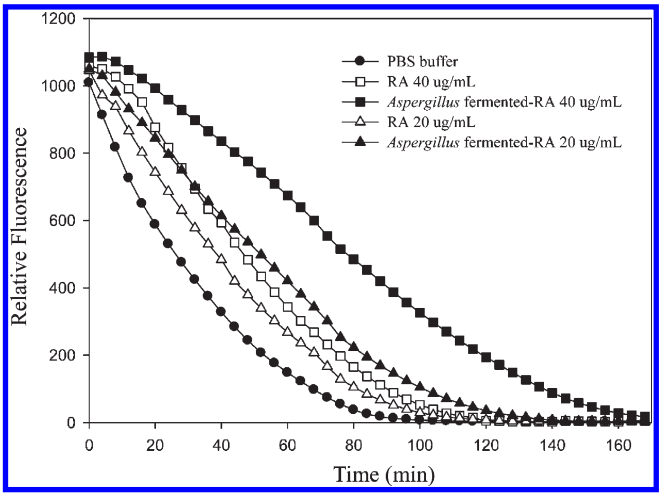

Figure 2. Radical scavenging activity of *A. oryzae* M29-fermented RA toward peroxyl radicals (ORAC assay).

Industrial Research Institute in Taiwan, and named as *A. oryzae* M29. The percentage yield of methanol extract from *A. oryzae* M29 was about 8.1%. The data demonstrated that fermentation using the fungus *Aspergillus* was necessary in order to release biological components for antioxidant activity from the low-activity RA material.

Antioxidant Characterization. The methanol extract was obtained from *A. oryzae* M29-fermented RA, and its protective effects against different free radical species including the hydroxyl, superoxide, and peroxyl radicals were investigated in vitro. The antioxidant capacities of the fungal-grown RA samples against hydroxyl and superoxide radicals are shown in Figure 1. The results showed that the antioxidant activity of the fungal-grown RA against hydroxyl radicals drastically increased with increasing sample concentration. The fungal-grown RA could effectively scavenge hydroxyl radicals (IC_{50} 28.80 μg/mL), and the scavenging activity was about 8.7-fold stronger than that of unfermented RA (IC_{50} 250.34 μg/mL). Regarding the scavenging effect of superoxide radicals, the antioxidant activity of fungal-grown RA also exhibited dose-dependent scavenging activities against superoxide radicals with varying capacities and was about two times superior to the activity observed for unfermented RA.

The antioxidant capacity of the fungal-grown RA to quench peroxyl radicals was also assayed by the ORAC test. The protective effect was measured by assessing the fluorescence decay curve (AUC) of the sample compared to PBS buffer, and the ORAC value was typically reported as a Trolox equivalent. The time-dependent decay of fluorescein induced by AAPH for RA samples is depicted in Figure 2, and the results showed that all fungal-grown RA samples had a concentration-dependent increase in the inhibition of fluorescein decay. The antioxidant activity of the fungal-grown RA against peroxyl radicals at a concentration of 20 μg/mL (4.19 μg/mL Trolox equivalent) was about 2.7-fold higher than that of unfermented RA (1.55 μg/mL Trolox equivalent).

To sum up the above antioxidant studies, the fungal-grown RA has antioxidant activity superior to that of unfermented RA against hydroxyl radicals, superoxide radicals, and peroxyl radicals.

Constituent Analysis. To evaluate constituent variation in RA under *A. oryzae* M29 fermentation, we analyzed the corresponding pattern and compared it with that of unfermented RA. The RA fermented by selected *Aspergillus* apparently increased phenolic constituents (Figure 3a), which were not present or were in significantly smaller amounts in unfermented RA (Figure 3b). It indicates that novel phenolics could be produced from the RA plant during *Aspergillus* fermentation. The finding is contrary to natto bacteria fermented RA, in which the phenolic content of RA material was reduced during fermentation as reported by Hsu and Chiang.[24]

In this study, the phenolic phytochemicals in the RA substrate were mobilized during *A. oryzae* M29 fermentation and accompanied by an increase in antioxidant functionality. These phenoxy radicals in fermented RA were much more stable and had longer lifetimes; therefore, the frequency of radical-mediated chain reaction could be minimized. This phenomenon was also observed in the antioxidant peptides containing proline, phenylalanine, or tyrosine.[33] As a result, fermented RA could be a food source of nature antioxidants.

Isolation, Purification, and Identification. The potent phenolics from RA fermented with *A. oryzae* M29 was also further isolated and identified. The methanol extract from fungal-growth RA was fractionated into four fractions (F1−F4) according to eluting time in the chromatographic profile (Figure 3a). We successively pooled, lyophilized these fractions, and detected their antioxidant capacity by the TEAC assay at the same concentration (Figure 4). Fraction 4 exhibited a higher antioxidant activity compared to that of fractions 1 and 2; however, it gave a relatively low recovery of 0.36% from the methanol extract of fungal-growth RA. Fraction 3 exhibited the highest antioxidant capacity among the four fractions and fairly displayed a good recovery; therefore, it is a potential product that warrants further purification with minimum risk on time and money resources. It was further subjected to an Inertsil *octadecylsilane* (ODS)-3 semiprep reverse phase column (10 × 250 mm) eluted with a linear gradient of acetonitrile (40−60% in 30 min) containing 0.1% TFA at 1 mL/min. A potent new phenolic antioxidant was isolated, and the recovery was approximately 2.12% from the methanol extract of fungal-growth AR using the above-described two-step purification.

The antioxidant effect of the purified compound was far superior to those of unfermented RA (Figure 5). In addition,

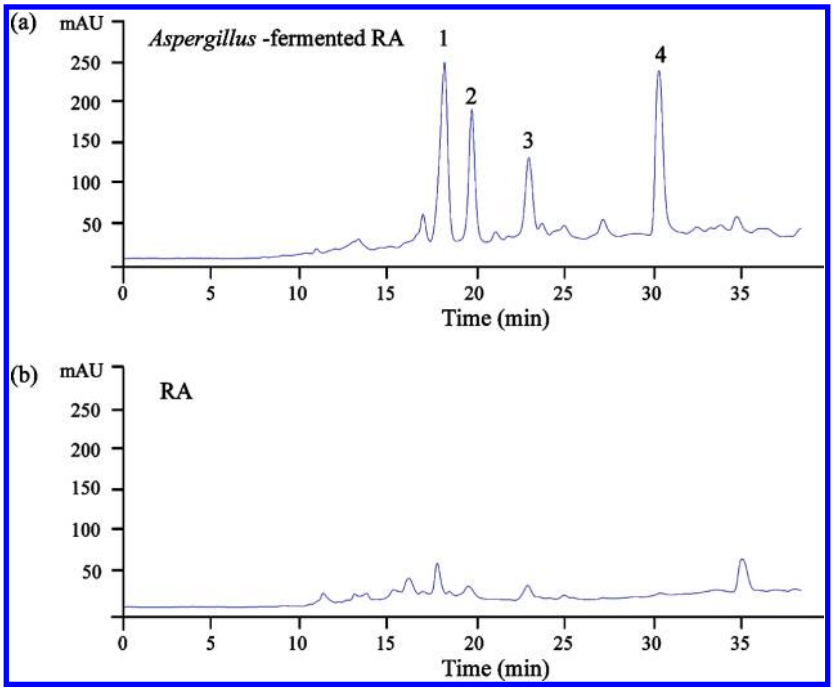

Figure 3. HPLC patterns of RA fermented with *A. oryzae* M29 (a) and RA without fermentation (b), on an ODS C18 reverse-phase column (10 × 250 mm) eluted with a linear gradient of acetonitrile (5−70% at 0−30 min and 70−85% at 30−45 min) containing 0.1% TFA at 1 mL/min and the pattern detected at 280 nm.

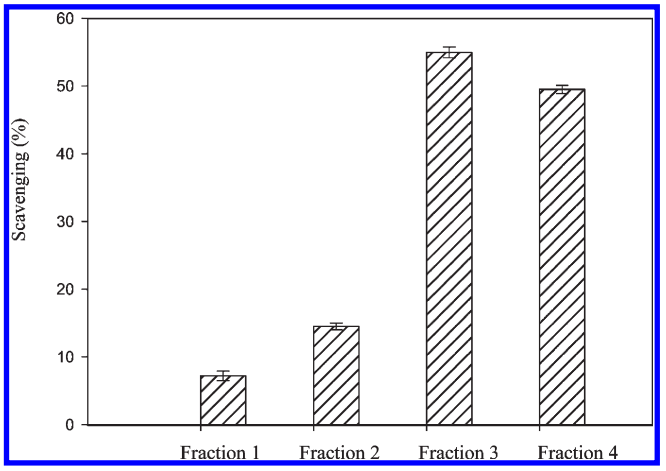

Figure 4. Resultant antioxidant activity of fractions (designated as 1 to 4) toward ABTS radicals from *A. oryzae* M29-fermented RA on an ODS C18 reverse-phase column.

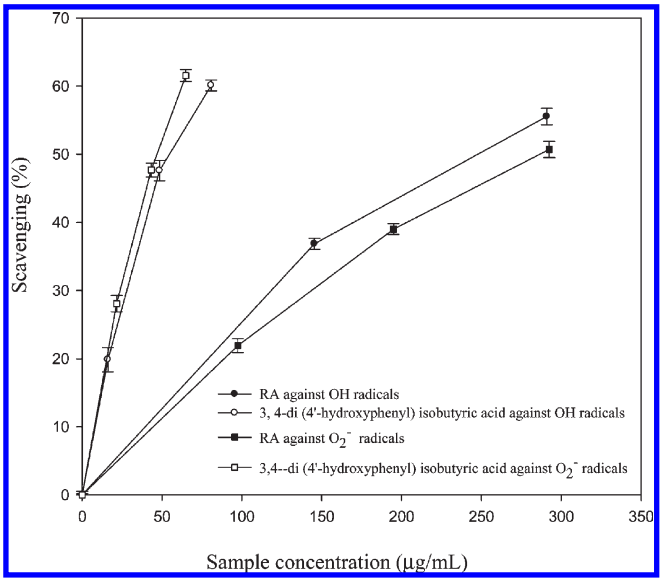

Figure 5. Radical scavenging activity of 3,4-di-(4′-hydroxyphenyl) isobutyric acid toward hydroxyl radicals and superoxyl radicals.

the IC_{50} values of the new phenolic antioxidant against hydroxyl and superoxide radicals were 52.38 and 45.81 μg/mL, respectively, and its antioxidant activity was much stronger than natural phenolic antioxidant hesperidin, which is the most important flavonoid of the *Citrus* sp., with IC_{50} values at 1.06 mg/mL for hydroxyl radicals and 2.32 mg/mL for superoxide radicals.

In this study, the novel antioxidant was identified as 3,4-di(4-hydroxyphenyl) isobutyric acid, on the basis of the following issues of ^{1}H-NMR, ^{13}C-NMR, DEPT-NMR, HMQC, and HMBC spectra. The ^{1}H-NMR spectrum (D_2O) showed two sets of benzylic proton at δ 2.95 (dd, 2H, J_{gem} = 15.0H$_Z$, J_{trans} = 8.1H$_Z$, ArC$\underline{H}_2$) and δ 3.10 (dd, 2H, J_{gem} = 15.0H$_Z$, J_{cis} = 4.8H$_Z$, ArC$\underline{H}_2$), respectively, and one methyne proton of isobutyric acid at δ 3.84 (m, 1H, H of $(CH_2)_2C\underline{H}COOH$). Moreover, the signals at δ 6.80 (d, 4H, J = 7.2H$_Z$, Ar$\underline{H}$) and δ 7.09 (d, 4H, J = 7.2H$_Z$, Ar$\underline{H}$) suggested a scaffold of two sets of four aromatic protons in a symmetrical AA′BB′ quartet system. The 175 MHz ^{13}C-NMR spectrum and different DEPT-NMR spectra revealed seven resonances at δ 35.37 ($\underline{C}H_2$), δ 55.96 ($\underline{C}H$), δ 115.77 ($\underline{C}H$), δ 126.70 ($\underline{C}q$), δ 130.67 ($\underline{C}H$), δ 154.80 ($\underline{C}q$), and δ 173.88 ($\underline{C}q$) in the structure of the new compound. The HMQC and HMBC spectra showed the following features: (1) the benzylic methylene protons at δ 2.95 and δ 3.10

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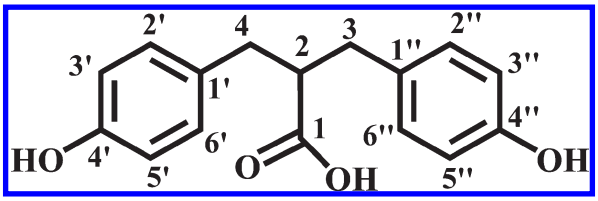
Table 1. ^{1}H- and ^{13}C-NMR Data for 3,4-Di-(4-hydroxyphenyl) isobutyric Acid in D_2O



positions	δH (J Hz)	δC (J mHz)
1		δ 173.88
2	δ 3.84, m, 1H	δ 55.96
3 and 4	δ 2.95, dd, 2H (15.0 Hz J_{gem}, 8.1 Hz J_{trans})	δ 35.37
	δ 3.10, dd, 2H (15.0 Hz J_{gem}, 4.8 Hz J_{cis})	δ 35.37
1′ and 1″		δ 126.70
4′ and 4″		δ 154.80
2′, 2″, 6′, and 6″	δ 7.09, d, 4H (7.2 Hz)	δ 130.67
3′, 3″, 5′, and 5″	δ 6.80, d, 4H (7.2 Hz)	δ 115.77

were attached to the carbons at δ 35.37 ($\underline{C}H_2$, C-3 or C-4) and coupled by ^{2}J to carbons at 55.96 ppm ($\underline{C}H$, C-2) and 126.70 ppm ($\underline{C}H$, ArC at C-1′ or C-1″), as well as by ^{3}J to carbons at 130.67 ppm ($\underline{C}H$, ArC at C-2′, C-2″, C-6′, or C-6″), 173.88 ppm ($\underline{C}q$, C-1 of carboxylic carbon), and 35.37 ($\underline{C}H_2$, C-3 or C-4); (2) the methyne proton at δ 3.84 was attached to the carbon at 55.96 ppm ($\underline{C}H$, C-2) and coupled by ^{2}J to carbons at δ 35.37 ($\underline{C}H_2$, C-3 or C-4) and 173.88 ppm ($\underline{C}q$, C-1), and by ^{3}J to the carbon at 126.70 ppm ($\underline{C}H$, ArC at C-1′ or C-1″); (3) the aromatic proton at δ 6.80 (δ_c 115.77, ArC at C-3′, C-3″, C-5′, or C-5″) or δ 7.09 (δ_c 130.67, ArC at C-2′, C-2″, C-6′, or C-6″) showed obvious connectivity with other aromatic carbons at either 115.77, 126.70, 130.67, or 154.80 ppm, respectively, indicating a symmetrical aromatic AA′BB′ system in the structure of this compound; (4) the aromatic proton at δ 7.09 also coupled with δ 35.37 by a ^{3}J connectivity, further indicating its position at C-2′, C-2″, C-6′, or C-6″ of an aromatic ring. Moreover, the hydroxyl proton of the aromatic ring was determined from the observation of spectroscopic coupling of the aromatic protons at either δ 6.80 or δ 7.09 to the proton at 4.67 ppm in a 2-D COSY-NMR spectrum. Finally, the acidic proton (10−12 ppm; broad) of carboxylic acid is normally invisible due to the rapid exchange with D_2O in the determination of the NMR spectrum. In parallel, the MS spctrum (ESI^+ MS) showed an ion at m/z 294.2, which is formulated as $C_{16}H_{15}NaO_4$ $(M+Na)^+$. These correlations established key structural features of this new isolated compound as 3,4-di(4-hydroxyphenyl) isobutyric acid (Table 1). The MS, ^{1}H-NMR, ^{13}C-NMR, HMQC, and HMBC spectra of 3,4-di(4-hydroxyphenyl) isobutyric acid are also shown in Supporting Information.

There are numerous reports on the bioactivities of RA. However, there are no reports to date regarding using the solid-state bioprocess of RA by *A. oryzae* to enhance phenolic and antioxidant profiles. The fungal-grown RA was a more effective antioxidant in quenching ABTS radicals, hydroxyl radicals, superoxide radicals, and peroxyl radicals than unfermented RA, and a potent novel antioxidant was isolated and identified as 3,4-di(4′-hydroxyphenyl) isobutyric acid. These data demonstrated that the fermented RA has the potential to be a good dietary supplement for the prevention of oxidative stress-related diseases, and the solid-state bioprocessing strategy could be an innovative approach to enhance the antioxidant activity of RA.

■ ASSOCIATED CONTENT

Supporting Information. ^{1}H-NMR, ^{13}C-NMR, ^{1}H,^{13}C-HSQC, ^{1}H,^{13}C-HMBC, and MS of 3,4-di(4′-hydroxyphenyl) isobutyric acid. This material is available free of charge via the Internet at http://pubs.acs.org.

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