

Improving Antioxidant Activity of *Ophioglossum thermale* Kom. by Fermentation with *Talaromyces purpurogenus* M18-11

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Ophioglossum thermale Kom. was fermented with several fungi. The total phenolic and flavonoid contents (TPCs and TFCs) and antioxidant activities of fermented and non-fermented *O. thermale* (FOT and NFOT) were investigated. The results showed that *Talaromyces purpurogenus* M18-11 fermented *O. thermale* possessed significantly improved TPC and TFC and exhibited significantly stronger 1,1-diphenyl-2-picrylhydrazyl (DPPH) (half maximal inhibitory concentration (IC₅₀) = 75.7 ± 2.1 µg mL⁻¹) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (IC₅₀ = 20.52 ± 1.68 µg mL⁻¹) free-radical scavenging activities, ferric reducing antioxidant power (0.585 ± 0.045 mmol g⁻¹), and reducing power (half maximal effective concentration (EC₅₀) = 30.52 ± 1.91 µg mL⁻¹) than original material. The determination of the contents of representative flavonoids and their glucosides revealed that the improvements are attributed to the hydrolysis of homoisoflavonoid and flavonoid glucosides, glycometabolism, as well as fungal metabolites. This paper is the first to report the fermentation of *O. thermale* with pure strains and *T. purpurogenus* is an effective strain to process *O. thermale* for improving the antioxidant activity.

Keywords: *Ophioglossum thermale*, *Talaromyces purpurogenus*, fermentation, antioxidant activity, flavonoid

Introduction

The genus *Ophioglossum* (Ophioglossaceae) is a small terrestrial plant that is distributed worldwide.¹ Phytochemical researches revealed that the herb of *Ophioglossum* contains massive fatty acids and their esters,^{2,3} amino acid,⁴ flavonoids,⁵⁻¹¹ and polysaccharoses.^{12,13} *Ophioglossum thermale* Kom., “Yizhijian” in traditional Chinese medicine (TCM), is a plant of the genus *Ophioglossum*, which is frequently used as a herbal medicine for clearing heat and detoxicating. Its main chemical constituents are flavonoids^{2,14,15} which possess significant antioxidant activity.⁷

Herbal fermentation processing began approximately 4000 years ago in China, which is frequently used to produce secondary metabolites from domestic plants in bulk by utilizing the metabolic mechanisms of microorganisms. Solid-state natural fermentation (SSF)

was very popular in the processing of herbal medicines in ancient China, such as *Semen Sojae Praeparatum*, *Mass Galla chinensis et camelliae Fermentata*, *Massa Medicata Fermentata*, and *Rhizoma Pinelliae Fermentata*, etc. With the development of microbial technology, pure strain fermentation has become increasingly acceptable and reliable for processing herbal medicines in modern times. In recent years, some fermented traditional medicines (FTMs) have been reported. FTMs exhibit stronger biological activities or higher bioavailability in the human body compared with raw materials. For example, Hsu *et al.*¹⁶ reported that *Bacillus subtilis* fermented *Radix astragali* could stimulate the biosynthesis of type I procollagen in a dose-dependent manner in both aged and young human dermal fibroblast cells. *Aspergillus oryzae*-fermented *Curcuma longa* L. could effectively prevent CCl₄-induced hepatic damage in rats.¹⁷ Wang *et al.*¹⁸ improved bioactivities of polyphenol extracts from *Psidium guajava* L. leaves by fermentation of *Monascus anka* and *Saccharomyces cerevisiae*.

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In the present study, the herb of *O. thermale* was fermented with several fungi. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical-scavenging activities, reducing power, and ferric reducing antioxidant power (FRAP) of non-fermented and fermented *O. thermale* (FOT) were determined for evaluating their antioxidant activities. *Talaromyces purpurogenus* M18-11 was screened to process *O. thermale* for improving the antioxidant activity. Further total phenolic and flavonoid contents (TPCs and TFCs) determination and high-performance liquid chromatography (HPLC) analysis were used for explaining the changes of antioxidant.

Experimental

Chemicals

ABTS, DPPH, 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were obtained from J&K Scientific Ltd. (Beijing, China). Rutin and gallic acid were purchased from Aladdin-Reagent (Shanghai, China). Luteolin (**1**), quercetin (**2**), 3-methoxyquercetin (**3**), ophioglonol (**4**), ophioglonol 4'-O- α -D-glucopyranoside (**5**), and pedunculumoside B (**6**) used in the present study were isolated from *O. thermale*.¹⁴ The water (resistivity ≥ 18.25 M Ω cm) used was purified with a purity water system (Chengdu, China). All other chemicals used were of analytical grade.

Plant material

The herbs of *O. thermale* were collected from Wenshan, Yunnan, China, in February 2014, and were identified by Assistant Professor Shu-Da Yang from School of Pharmacy, Kunming Medical University, Kunming, China. A voucher specimen (2014-0t-01) has been deposited in School of Chemical Science and Technology, Yunnan University, Kunming, China.

Microorganisms and fermentation

All strains including *T. purpurogenus* M18-11, *Aspergillus niger* YIM3029, *Geomyces luteus* P18-5, and *Penicillium swiecickii* F2-7 were obtained from Yunnan Institute of Microbiology, Yunnan Province, China.

Potato dextrose agar (PDA; 1 L water, 200 g potato, 20 g dextrose, and 15 g agar) slant culture mediums were inoculated with fungi above and incubated in a constant temperature incubator at 28 °C for 5 days. Five grams of

O. thermale were added to a 100 mL Erlenmeyer flask to function as the fermentation culture medium. After being infiltrated with 15 mL water and sterilized at 121 °C for 30 min, the mature slants culture mediums were added and incubated at 28 °C for 30 days.

Extraction

O. thermale (5.0 g) and each of the four individual FOTs were immersed in 50 mL acetone for 24 h and ultrasonicated three times for 30 min each time. The extracting solution was decanted, filtered under vacuum and concentrated in a rotary evaporator to afford five extracts: ROT (raw *O. thermale*, 1.063 g), BFOT (blank FOT, 1.099 g), FOT1 (*A. niger* FOT, 0.405 g), FOT2 (*T. purpurogenus* FOT, 0.549 g), FOT3 (*G. luteus* FOT, 0.667 g), and FOT4 (*P. swiecickii* FOT, 0.475 g).

Antioxidant activities

DPPH radical-scavenging activity

The DPPH free radical-scavenging activity was estimated by the method described previously.¹⁹ DPPH radical-scavenging activity of the sample was calculated as follows:

$$\text{Inhibition (\%)} = (1 - A_{\text{sample}}^{\text{DPPH}} / A_{\text{control}}^{\text{DPPH}}) \times 100 \quad (1)$$

where $A_{\text{sample}}^{\text{DPPH}}$ is the absorbance of sample solution mixed with DPPH and $A_{\text{control}}^{\text{DPPH}}$ is the absorbance of the blank solution (ethanol). Rutin was used as a positive control. All tests were performed in triplicate. The half maximal inhibitory concentration (IC₅₀) value was defined as the effective concentration at which the DPPH radical was scavenged by 50%. Anti-radical power (ARP) was defined as 1/IC₅₀. ARP_i and ARP₀ were represented as the ARP of each sample and ROT, respectively.

ABTS assay

ABTS antioxidant capacity was measured using a previous ABTS method.^{19,20} ABTS radical-scavenging activity was calculated as follows:

$$\text{Inhibition (\%)} = (1 - A_{\text{sample}}^{\text{ABTS}} / A_{\text{control}}^{\text{ABTS}}) \times 100 \quad (2)$$

where $A_{\text{sample}}^{\text{ABTS}}$ is the absorbance of sample solution mixed with ABTS and $A_{\text{control}}^{\text{ABTS}}$ is the absorbance of the blank solution. The IC₅₀ value was defined as the effective concentration at which the ABTS radical was scavenged by 50%. ARP was defined as 1/IC₅₀. ARP_i and ARP₀ were represented as the ARP of each sample and ROT, respectively.

FRAP

The FRAP assay measures the reduction of ferric iron to the ferrous form in the presence of antioxidants. The assay protocol followed a previously reported procedure.²¹ The antioxidant capacity of the sample was expressed as μmol ferrous sulfate *per g* extract ($\mu\text{mol g}^{-1}$), which was calculated from the standard graph obtained with the ferrous sulfate solution.

Reducing power

Reducing power was tested using a reported method.^{22,23} 100 μL of sample at proper concentration was made up to 0.75 mL with phosphate buffer (300 mM, pH 6.6), and 1.5 mL of 1% (m/v) $\text{K}_3\text{Fe}(\text{CN})_6$ was added. The mixture was shaken vigorously and left to stand for 20 min at 50 °C. After the addition of 1.5 mL of 10% (m/v) trichloroacetic acid, the mixture was centrifuged at 3000 rpm for 10 min. A 1.5 mL of supernatant was mixed with distilled water (1.5 mL), and 0.3 mL of 0.1% (m/m) FeCl_3 was added before the absorbance was determined at 700 nm. Half maximal effective concentration (EC_{50}) was defined by the concentration at absorbance (A) = 0.50; antioxidant reducing power (ARP) was defined as $1/\text{EC}_{50}$. ARP_1 and ARP_0 were represented as the ARP of each sample and ROT, respectively.

Determination of total phenolic content

The total phenolic content was measured using the Folin-Ciocalteu method.²⁴ Folin-Ciocalteu's reagent was prepared according to the method of GB/T 23527-2009 (CN).²⁵ Fifty microliters of extract solution was added to 2.25 mL of Folin-Ciocalteu's reagent, which was pre-diluted ten-fold with distilled water. Five minutes later, 1.5 mL of Na_2CO_3 (7.5%, m/v) solution was added, and the mixture was allowed to stand for 30 min at ambient temperature. Absorbance was measured at 765 nm. The TPC was expressed as mg gallic acid equivalent *per g* extract (mg GAE g^{-1}) and calculated according to the calibration curve obtained from the standard solution of gallic acid at various concentrations.

Determination of total flavonoid content

The total flavonoid content was determined by a colorimetric assay described previously.^{23,26} The total flavonoid content was expressed as mg rutin equivalent *per g* (mg RE g^{-1}).

HPLC analysis

The determination and quantification of main

flavonoids compounds were measured using an Agilent 1260 Infinity Series HPLC system, equipped with an Agilent G1315D DAD detector, an Agilent G1311C quaternary gradient pump, and Agilent Zorbax SB-C18 (250 \times 4.6 mm i.d., 5 μm), and coupled to an Agilent OpenLab data-processing station. A gradient elution system consisting of solvent A (water containing 6 mM acetic acid) and B (acetonitrile) was used for the analysis, and the gradient program was as follows: 0-25 min, 15-65%B; 25-30 min, 65-100%B; 30-35 min, 100%B. The peaks were confirmed by the UV absorptions at 320 and 355 nm and the retention times while the flow rate was 1.0 mL min^{-1} . The column temperature was set at 35 °C, and the injection volume was 20 μL . Flavonoids were identified by using standard addition method, and quantified according to standard curves.

Results and Discussion

After fermentation of *O. thermale* with several fungi, a preliminary experiment by thin layer chromatography (TLC) was measured. Four fermented samples were selected for further experiments.

Antioxidant activities

Oxidation is ubiquitous and has pernicious effects on both food quality and human health. Previous research indicated that oxidative damage can cause browning and off-flavors, change the nutrient value of food, disrupt cellular function and attenuate the formation of compounds used to fight aging and cardio-vascular disease.²⁷ Zhang *et al.*⁷ reported that the extract of *O. thermale* possesses antioxidant activity. In the present study, therefore, the antioxidant activity of ROT, BFOT, and FOT1-4 were tested.

DPPH is a stable free radical that has been used widely to determine the free radical-scavenging activity of natural antioxidants. The DPPH radical scavenging activities shown in Figure 1 revealed that FOT1-3 exhibited more significant activities than ROT, BFOT, and FOT4, in particular, FOT2 possessed 2-fold more antioxidant activity with an IC_{50} value of $75.7 \pm 2.1 \mu\text{g mL}^{-1}$ than ROT.

ABTS is another free radical that is frequently used to study free radical-scavenging activity. Notably, the ABTS radical-scavenging activity assay is an important indicator of total antioxidant activity (TAA). As shown in Figure 2, FOT1-3 showed stronger ABTS radical-scavenging activities with the IC_{50} values of 18.61 ± 1.32 , 20.52 ± 1.68 , and $19.20 \pm 0.92 \mu\text{g mL}^{-1}$, than FOT and BFOT.

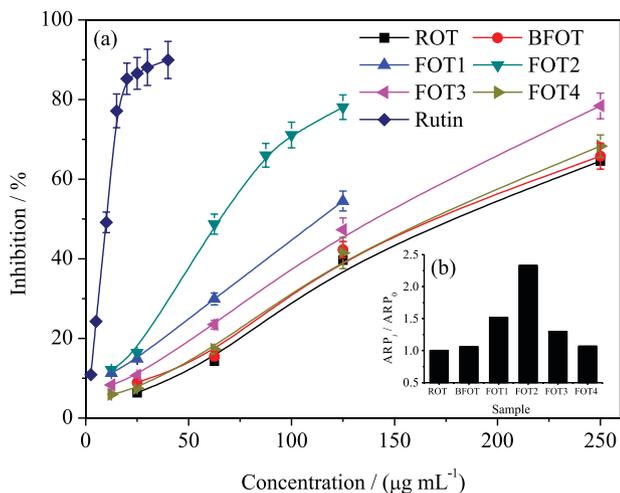


Figure 1. DPPH radical scavenging activities of the extracts of ROT, BFOT, and FOT1-4. (a) Inhibition of DPPH radical scavenging activities at different concentrations; (b) relative ARP (ARP_i/ARP_0) of BFOT and FOT1-4.

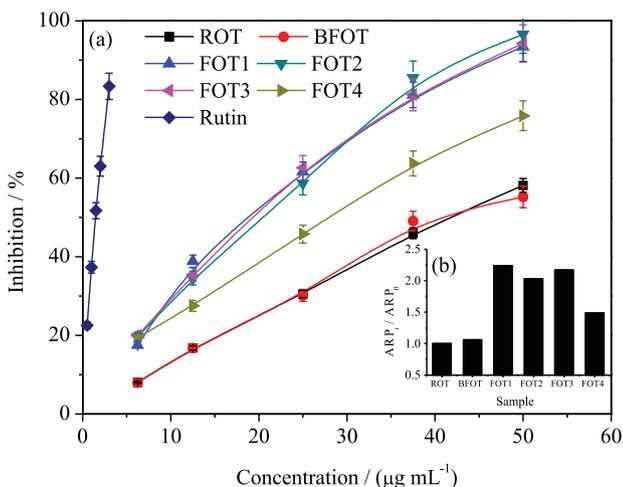


Figure 2. ABTS radical scavenging activities of the extracts of ROT, BFOT, and FOT1-4. (a) Inhibition of ABTS radical scavenging activities at different concentrations; (b) relative ARP (ARP_i/ARP_0) of BFOT and FOT1-4.

Reducing power of the natural plant extracts might be strongly correlated with their antioxidant activity. It is necessary to discuss the reducing power of a natural plant extract to elucidate the relationship between its antioxidant effect and reducing power. The results shown in Figure 3 suggest FOT2 exhibits significantly improved reducing power with an EC_{50} value of $30.52 \pm 1.91 \mu\text{g mL}^{-1}$. The FRAP is often used as an indicator of phenolic antioxidant activity as important as reducing power. The antioxidant potential of sample was estimated by their abilities, which is to reduce Fe^{III} -TPTZ to Fe^{II} -TPTZ. Similar to reducing power, FOT2 possessed improved antioxidant activity ($0.585 \pm 0.045 \text{ mmol g}^{-1}$, Figure 4).

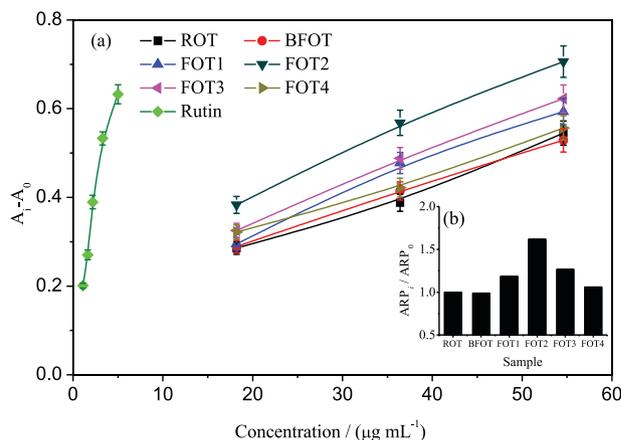


Figure 3. Reducing powers of the extracts of ROT, BFOT, and FOT1-4. (a) Reducing powers at different concentrations; (b) relative ARP (ARP_i/ARP_0) of BFOT and FOT1-4.

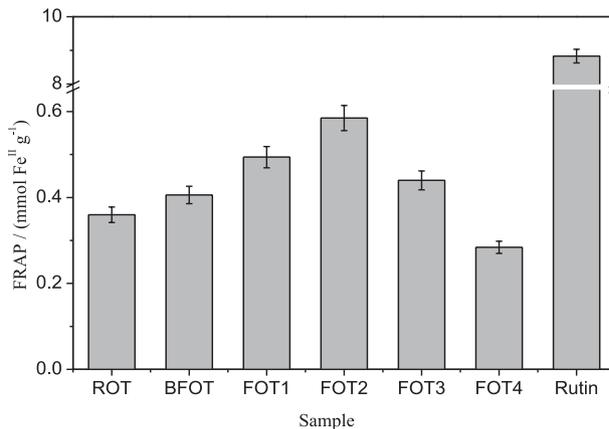


Figure 4. FRAP of the extracts of ROT, BFOT, and FOT1-4.

In addition, the DPPH antioxidant activity for extract of fungal strains *A. niger*, *T. purpurogenus*, *G. luteus*, and *P. swiecickii*, individually cultured in PDA medium was 390.8 ± 25.1 , 235.8 ± 21.1 , 234.4 ± 10.9 , $235.2 \pm 31.2 \mu\text{g mL}^{-1}$, respectively (for pure PDA medium, it was $386.5 \pm 29.1 \mu\text{g mL}^{-1}$). Therefore, the results above show that FOT2 exhibits significantly improved antioxidant activity, suggesting that fermentation of *O. thermale* with *T. purpurogenus* might be effective for improving antioxidant activity.

Total phenolic content (TPC)

Phenolic content always shows potent antioxidant activity, in particular, radical-scavenging power. In the present study, the improvement of antioxidant activity might be ascribed to changes of phenolic constituents. Hence, the TPCs of ROT, BFOT, and FOT1-4 were evaluated and the results were shown in Figure 5. The values of TPCs were expressed as

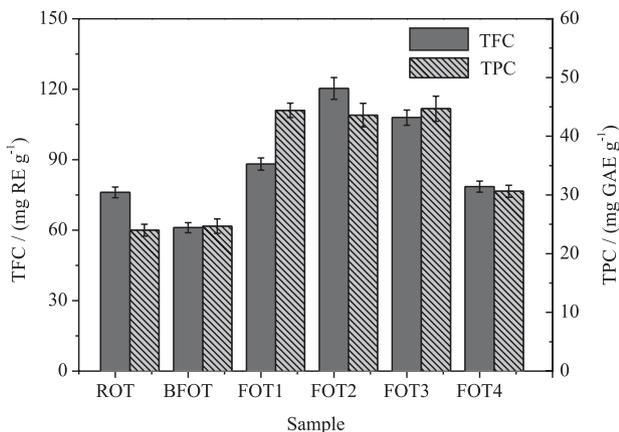


Figure 5. TPCs and TFCs of the extracts of ROT, BFOT, and FOT1-4.

mg GAE g⁻¹ and decreased in the following order: FOT1-3 (44.41 ± 1.22 , 43.63 ± 2.00 , 44.69 ± 2.13 mg g⁻¹) >> FOT4 (30.63 ± 1.03 mg g⁻¹) > ROT (24.01 ± 1.00 mg g⁻¹) ≈ BFOT (24.69 ± 1.23 mg g⁻¹), suggesting *A. niger*, *T. purpurogenus*, and *G. luteus* fermentation might improve the TPC of *O. thermale*.

Total flavonoid content (TFC)

As the main constituents of *O. thermale* are flavonoids including quercetin, 3-methoxyquercetin, ophioglonol and their esters, the TFCs of the fermented and non-fermented materials are indicators for evaluating the effects of fermentation. The TFCs of ROT, BFOT, and FOT1-4 are shown in Figure 5. FOT2 and FOT3 possessed obvious improved TFCs with the values of 120.35 ± 0.84 and 107.94 ± 4.22 mg RE g⁻¹, respectively, whereas FOT1, FOT4, and BFOT exhibited TFC with same grade as ROT.

These results suggest that *T. purpurogenus* might be an effective strain for processing *O. thermale* for improving the TPC, TFC, and antioxidant activity. The improvement

of antioxidant activity might be caused by the changes of flavonoids, as well as fungal metabolites produced by fungi during fermentation.

HPLC analysis

To clarify the flavonoids, it was performed positive antioxidant activities in the fermented and non-fermented *O. thermale*. HPLC was employed to identify the changes of main flavonoids isolated from *O. thermale*. Six main flavonoid compounds isolated from *O. thermale*, luteolin (**1**), quercetin (**2**), 3-methoxyquercetin (**3**), ophioglonol (**4**), ophioglonol 4'-O- α -D-glucopyranoside (**5**), and peduncululoside B (**6**) (Figure 6) were used to identify the peaks in chromatograms of ROT, BFOT, and *T. purpurogenus* FOT. As shown in Figure 7, flavonoid glucoside **5** could be not detected in *T. purpurogenus* FOT, suggesting that flavonoid glucosides are hydrolyzed by *T. purpurogenus*-fermentation. The peaks at 30-35 min observed in ROT disappeared after fermentation, suggesting that some low-polarity constituents are metabolized leading to the enrichment of the flavonoids. The contents of three main flavonoids **3-5** were determined by HPLC. The results in Table 1 show that the contents of flavonoid aglycones **3** and **4** were enriched. This might be caused by the hydrolysis of flavonoid glucosides and glycometabolism.

Antioxidant activities of flavonoid compounds 1-6

The DPPH radical-scavenging activities of flavonoid aglycones **1-4** and glucosides **5** and **6** were determined to evaluate the difference of antioxidant activities between flavonoid aglycones and glucosides. The results shown in Table 2 reveal that flavonoid aglycones **1-4** exhibited more significant DPPH radical-scavenging power ($IC_{50} < 10 \mu\text{g mL}^{-1}$) than flavonoid glucosides

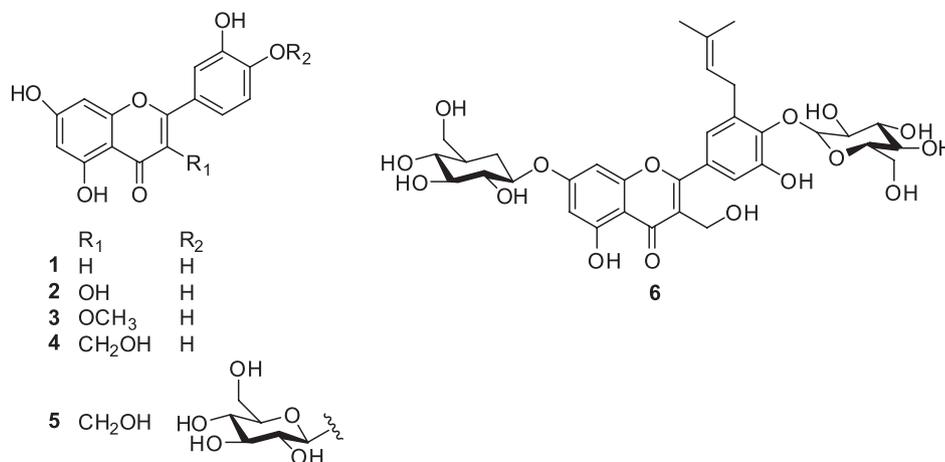


Figure 6. Structures of main flavonoid compounds.

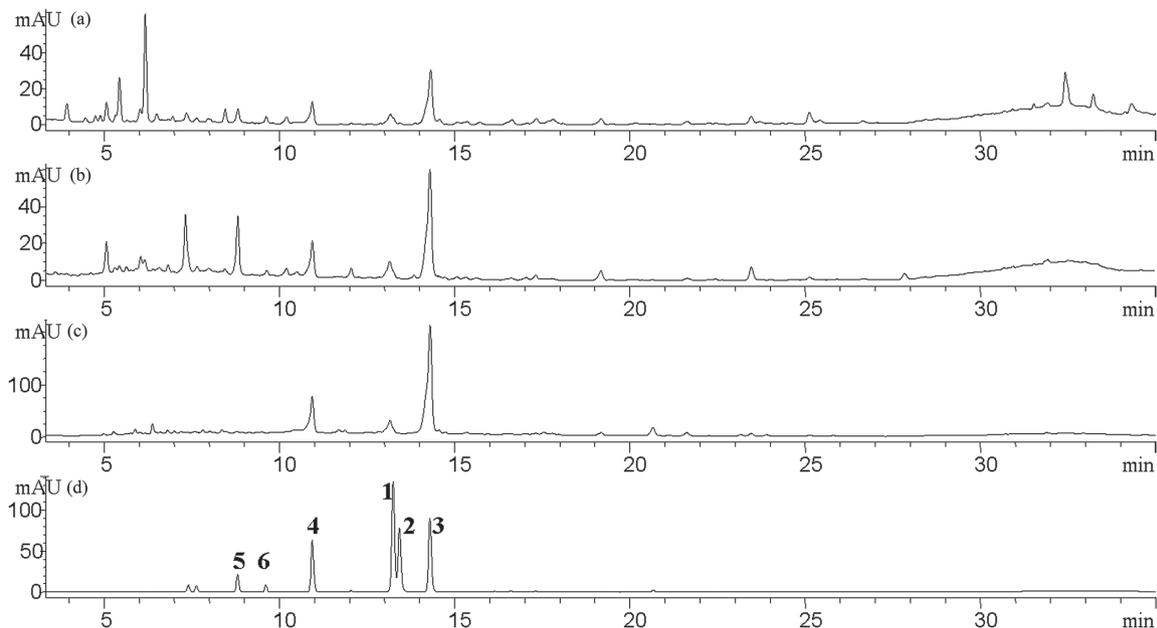


Figure 7. Chromatograms of (a) ROT; (b) BFOT; (c) *T. purpurogenus* FOT; (d) compounds **1-6**.

Table 1. The contents of compounds **3-5** in non-fermented and fermented *O. thermale*

| Sample | 3 / (mg per 100 g) | 4 / (mg per 100 g) | 5 / (mg per 100 g) |
|--------|---------------------------|---------------------------|---------------------------|
| ROT | 3.92 ± 0.94 | 16.88 ± 0.81 | 15.18 ± 1.18 |
| BFOT | 6.76 ± 0.51 | 17.08 ± 0.43 | 15.30 ± 2.55 |
| FOT2 | 17.64 ± 0.42 | 44.62 ± 0.14 | nd ^a |

^aNot detected. ROT: raw *O. thermale*; BFOT: blank fermented *O. thermale*; FOT2: *T. purpurogenus* FOT.

Table 2. DPPH radical-scavenging activities (IC₅₀) of compounds **1-6**

| Sample | IC ₅₀ / (μg mL ⁻¹) | IC ₅₀ / μM |
|--------------------|---|-----------------------|
| 1 | 7.53 ± 0.10 | 26.3 ± 0.4 |
| 2 | 2.86 ± 0.09 | 9.5 ± 0.3 |
| 3 | 9.11 ± 0.73 | 28.8 ± 2.3 |
| 4 | 8.31 ± 0.49 | 26.3 ± 1.6 |
| 5 | 56.9 ± 2.64 | 115.2 ± 5.3 |
| 6 | 61.32 ± 5.87 | 86.6 ± 8.3 |
| Rutin ^a | 8.20 ± 0.05 | 13.4 ± 0.1 |

^aPositive control. DPPH: 1,1-diphenyl-2-picrylhydrazyl; IC₅₀: half maximal inhibitory concentration.

(IC₅₀ > 50 μg mL⁻¹). Therefore, hydrolysis of flavonoid glucosides in *O. thermale* could enrich the flavonoid aglycones, improving the antioxidant activity of *O. thermale*.

Conclusions

The present study is the first to report that pure strain fermentation processing is effective in improving the TPC,

TFC, and antioxidant activity of *O. thermale*. Fermentation with *T. purpurogenus* could be an innovative approach to process *O. thermale*. TFC and antioxidant activity enhancements with *T. purpurogenus* might be attributed to hydrolysis of flavonoid glycosides, decomposition of glycoside and other low-polarity constituents, and fungal metabolites.

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