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A new polysaccharide isolated from *Dendrobium offcinale*, stimulates aquaporin-3 expression in human keratinocytes

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Abstract

Polysaccharides are the major components of the water extract from *Dendrobium officinale*, which have been thoroughly studied and found to have favorable moisturizing properties on mammalian skin. However, the structures of polysaccharides on skin moisturizing properties remains unknown. The aim of this study was to extract and characterize *D. officinale* polysaccharides, which have anti-oxidant activity and the role of promoting effects of aquaporin-3 (AQP3). Two crude polysaccharide fractions were extracted using water and biological fermentation, respectively. The crude polysaccharides were further purified by graded alcohol precipitation, and finally purified polysaccharides DOP-S and DOP-F were obtained by Sephacryl S-300 column chromatography. The average molecular weights of DOP-S and DOP-F were 3.604×10^5 and 1.027×10^5 Da, respectively. Monosaccharide units and structures of DOP-S and DOP-F were further elucidated by gas chromatography-mass spectrometry (GC-MS) and Fourier Transform infrared spectroscopy (FTIR). At the same time, DOP-S and DOP-F induced aquaporin-3 (AQP3) expression was evaluated by immuno-fluorescence analysis. The antioxidant activities of the polysaccharides were evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging capacities, and ferric reducing antioxidant power (FRAP). The results showed that biological fermentation effectively achieved the hydrolysis of the polysaccharides and stimulated antioxidant activity and AQP3 expression.

Keywords: dendrobium officinale; polysaccharides; aquaporin-3.

Practical Application: Antioxidant activity of Dendrobium officinale polysaccharide and promotion of aquaporin-3.

1 Introduction

Dendrobium candidum is a perennial herb of Orchidaceae in China (Li et al., 2008). It is listed as a top grade in the traditional medical book "Sheng Nong's Herbal Classic". It has played a very good medicinal value in clinical applications for hundreds of years (Shen et al., 2017).

D. candidum contains a variety of active substances, and polysaccharides are the main components. At present, the main extraction methods are water extraction, ultrasonic assisted extraction, microwave assisted extraction, high pressure extraction, flash extraction, and enzyme extraction (Tang et al., 2017). *D. candidum* has numerous pharmacological and cosmetic effects. For example, they significantly attenuated the dry mouth symptom in patients with Sjögren syndrom (Xiao et al., 2011).

It has been reported that *Dendrobium* polysaccharide activates the M3 muscarinic acetylcholine receptor (M3R) through calcium influx, thereby inducing aquaporin-5 (AQP5) translocation to the apical plasma membrane (APM), which explains the transition of water to saliva (Lin et al., 2011, 2015; Zha et al., 2007). *D. candidum* polysaccharides also play an important role in myocardial protection by reducing ROS production, enhancing the antioxidant enzyme system, maintaining mitochondrial function and regulating apoptosis-related proteins (Zhang et al., 2017). Recent research has also revealed that *D. candidum* polysaccharides can fight tumors (Wei et al., 2018), reduce diabetes (Wang et al., 2018b), regulate immunity (Wang et al., 2018a; Liu et al., 2018), protect liver (Liang et al., 2017) and treat colitis (Liang & Chen et al., 2018). In addition, *D. candidum* is widely used in cosmetics due to its moisturizing and antioxidant activity (Liang et al., 2018a, b).

Aquaporins (AQPs) are a highly selective family of water channel specific proteins with 13 family members (AQP0–12). They mediate transmembrane water transport of different cell types. It is generally believed that the channel is only permeable to water and has a certain selectivity for glycerin and urea. Aquaporin 3 (AQP3) is the most important channel protein in mammalian skin, and its regulation in the body has been extensively studied. AQP3 regulates the differentiation of keratinocytes by affecting Notch signal transduction and Notch1's reciprocal negative feedback loop (Guo et al., 2013). Deletion of AQP3 in mice results in impaired hydration of the stratum corneum, which prove that AQP3 expression may affect epidermal moisture content and water loss (Ma et al., 2002). The expression of AQP3 can affect skin homeostasis (Kitchen et al., 2015), which can affect wound healing in burned skin (Sebastian et al., 2015). In the present study, we elucidate the purification method, chemical structures and antioxidant activity of the purified polysaccharides from *D. candidum*. In the same time, we used keratinocytes to study the effects of the new extracted DOP-S and DOP-F on AQP3 expression to explore the moisturizing function of these two polysaccharides.

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2 Materials and Methods

2.1 Materials and reagents

Plants (D. officinale) were sampled in Yunnan Province (China) and identified by Prof. Lijia Xu, Institute of Medicinal Plant Development. Sephacryl S-300 was purchased from the American Sigma Company (Beijing, China). MTT (Sigma, Aldrich), Anti-Aquaporin 3 antibody, Goat Anti-Rabbit IgG, and Goat Anti-Mouse IgG, were all purchased from Abcam (USA). Ethylbenzothiazoline-6-sulphonic acid (ABTS; Sigma-Aldrich Co., Ltd., USA), 1,1-diphenyl-2-picrylhydrazyl (DPPH; Tokyo Chemical Industry, Tokyo, Japan), potassium peroxydisulfate (Xilong Chemical Co., Ltd., Guangdong, China), iron (II) sulfate heptahydrate (Xilong Chemical Co., Ltd., Guangdong, China), and L-ascorbic acid (J&K Scientific Co., Ltd., Beijing, China) were all of analytical grade.

2.2 Extraction and purification of DOP-S and DOP-F

Dried stems of D. officinale were extracted with distilled water at 1:100 w/v ratio. After soaking for 1 h, the decoction was boiled for 2 h. The filtered supernatant of the extract was concentrated and freeze-dried to a powder (crude DOP-S). The filtered supernatant of water extraction was fermented by Saccharomyces cerevisiae. An 8% volume of S. cerevisiae was inoculated into the water extract. This preparation was then incubated at 28 °C and pH 4.5 for 54 h, under constant agitation at 170 r/min. After fermentation, the filtered supernatant was concentrated and freeze-dried to a powder (crude DOP-F).

The DOPs (crude DOP-S and crude DOP-F) were converted to aqueous solutions in deionized water at a 1:4 w/v ratio. Subsequently, appropriate volumes of ethanol (concentrations of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, and 90%) were added to the water extraction and allowed to stand for 24 h at 4 °C to achieve final ethanol precipitants.

The crude DOP-S from 20% ethanol precipitation and crude DOP-F from 40% ethanol precipitation were then deproteinized by sevage solvent, and put into column Sephacryl S-300 (50×1.6 cm), with a continuously water elution at 0.5 mL/min. The water elution was collected each 5.0 mL and made the chromogenic reaction at 490 nm with the solvent phenol sulfuric acid. The purities of DOP-S and DOP-F were then tested by GPC-MALS system, respectively.

2.3 Measurement of molecular weight of DOP-S and DOP-F

The monosaccharide composition of DOP-S and DOP-F was analysed by GC–MS. First, DOP-S and DOP-F were hydrolyzed with 2.0 M trifluoroacetic acid, and then the hydrolysate was evaporated to dry and recovered. A small amount of acetic acid was added to the methyl solution (0.1%, v/v) to remove excess KBH4. Finally, the reduced product of dry acid hydrolysis is acetylated in acetic anhydride and pyridine (1:1, v/v) and converted into acetate derivative dissolved in trichloromethane. The IR spectra of DOP-S and DOP-F were obtained at a resolution of 4 cm^{-1} on S pectrum 400 infrared spectrophotometer (American, PE Company, Waltham, MA, USA).

2.4 Cell culture

Keratinocytes were purchased from Guangdong Boxi Biological Technology Co., Ltd. The catalog numbers for all cell lines is EP140805. Each cell type was grown in a culture flask and maintained at 37 °C, 95% RH and 5% CO2, in keratinocyte prime media. Cells from each type were counted with a hemocytometer.

2.5 Cytotoxicity analysis

MTT assay was used to analyse the cytotoxicity of DOP-S and DOP-F. Cells were seeded in 24-well, flat-bottom tissue culture plates at a density of approximately 104 cells/well and allowed to attach for 24 h at 37 °C. The cells were then incubated with DOP-S and DOP-F at a concentration of 0.025, 0.05, 0.1, 0.2, 0.5, 1.0, or 2.0 mg/g for 24 h. MTT reagent was then added to each well, and the plate was incubated in the dark at 37 °C. After 4 hours, the supernatant was discarded and 150 μL of dimethyl sulfoxide was added to each well. Absorbance measurements were made at 490 nm. Cytotoxicity was expressed as the percentage of treated cells surviving relative to untreated cultures. All experiments were performed in triplicate.

2.6 Immuno-fluorescence analysis of AQP3 expression

After incubation with DOP-S and DOP-F for 24 h, the cells were taken for immune fluorescent analyses according to a reported method with a few modifcations (Wellner et al., 2005). Briefly, the monolayer was washed three times with PBS and then fixed with 4% paraformaldehyde (PFA) solution at 4 °C for 30 min. The cells were washed again with PBS followed by incubation with 0.2% bovine serum albumin in PBS, and then 0.5% goat serum, for 30 min. For immuno-fluorescent staining, AQP3 antibody (1:200) was incubated at 4 °C for 24 h followed by staining with goat anti-rabbit IgG-FITC (1: 200) for 2 h at 37 °C. Hoechst 33258 (1:500) was used as a nuclear staining dye. Image-pro Plus (IPP) software was used to analyse the green fluorescence intensity of the cells. Fluorescence intensity per unit area was then calculated.

2.7 Anti-oxidant activities

Determination of DPPH radical scavenging activity

DPPH radical scavenging rates of DOP-S and DOP-F were measured according to Brand et al. (Brand-Williams et al., 1995). Samples (1.0, 2.0, 4.0, 6.0, 8.0, and 10.0 mg/mL) were added to 1.0 mL of 0.2 mM ethanolic DPPH and shaken. After 30 min, the decrease in absorbance was measured at 517 nm. Methanol was used as a blank, and ascorbic acid was used as a positive control. Assays were performed in triplicate.

Scavenging of ABTS radicals

The radical scavenging activities of DOP-S and DOP-F against ABTS radical cations were measured using the method (Benzie & Strain, 1996) with some modifications. ABTS was dissolved in water to 7 mmol/L. ABTS radical cations were produced by reacting an ABTS stock solution with 2.45 mmol/L potassium persulfate and allowing the mixture to stand in the dark at 25 °C

for 12-16 h before use. The ABTS radical cation solution was diluted in ethanol to an absorbance of 0.70 ± 0.02 at 734 nm and equilibrated at 30 °C. Samples (0.1 mL; final concentrations of 0.1, 0.5, 1.0, 2.0, 4.0, and 6.0 mg/mL) were mixed with 3.9 mL of diluted ABTS radical cation solution, and the absorbance at 734 nm was measured after reaction for 6 min.

Total antioxidant capacity assay

The total antioxidant capacity of DOP-S and DOP-F was measured by the ferric reducing antioxidant power (FRAP) method (Benzie & Strain, 1996; Nilsson et al., 2005). We added 180 μL FRAP working liquid into each well of a 96-well plate, then added 5.0 μL of FeSO4 standard solution at concentrations of 0.15, 0.3, 0.6, 0.9, 1.2, and 1.5 mM to the standard curve detection wells. Sample wells received 5.0 μL of the extracted samples in triplicate, and a 0.15 mM sample containing 1.5 mM Trolox was used as a positive control. The plate was gently shaken and incubated at 37 °C for 5-7 minutes, and then the decrease in absorbance was measured at 593 nm. Distilled water was used as a blank. The total antioxidant capacity of each sample was expressed as the FeSO4 concentration and was calculated according to the standard curve $y = 0.411x - 0.0755$ ($R^2 = 0.9946$, x: concentration of FeSO4 (mM); y: absorption value).

2.8 Data analysis

Results are expressed as means ± SD. Data were analysed using an unpaired Student's t-test to determine differences between groups by SPSS 16.0 (*$p < 0.05$; **$p < 0.01$).

3 Results and discussion

3.1 Extraction and purification of DOP-S and DOP-F

Polysaccharides obtained by water extraction and fermentation were further separated by different concentrations of alcohol and purified in a Sephadex S-300 column. The yield of crude polysaccharides from water extraction reached 560 mg/g in the part of 20% alcohol precipitation. The yield of crude polysaccharides from fermentation reached 600 mg/g in the part of 40% alcohol precipitation. These crude polysaccharides were further deproteinized with Sevage reagent and then applied to a Sephadex S-300 column (50 × 1.6 cm i.d.) packed 46 cm high. Ultrapure water eluting solvent and a 0.5 mL/min flow rate were used to purify the crude polysaccharides. Symmetrical spikes were obtained on the Sephadex S-300 chromatogram. The corresponding main fractions were then collected, lyophilized and designated as DOP-S and DOP-F. Average molecular weights (Mw) of DOP-S and DOP-F detected by HPGPC were 3.604 × 105 and 1.027 × 105 Da, respectively. GC/MS analysis revealed the presence of Man, Glc, Gal, Ara, Xyl, Fuc, and Rha at a molar ratio of 13.6: 3.2: 3.1: 1.5: 1.3: 1.0: 0.8, in DOP-S, and of Man, Gal, Glc, Ara, Xyl, Fuc, and Rha at a molar ratio of 13.7: 4.0: 3.8: 1.4: 1.4: 1.0: 0.8, in DOP-F (Figure 1). DOP-S and DOP-F were determined to be heteropolysaccharides. The ratio of Man to Glc content decreased from 4.40 to 3.58 after fermentation.

3.2 Structural characterization of DOP-S and DOP-F

The FTIR spectra of DOP-S and DOP-F were showed in Figure 2, respectively. The broad absorption peak around 3395 and 3375 cm^{-1} were characteristic of the O-H group.

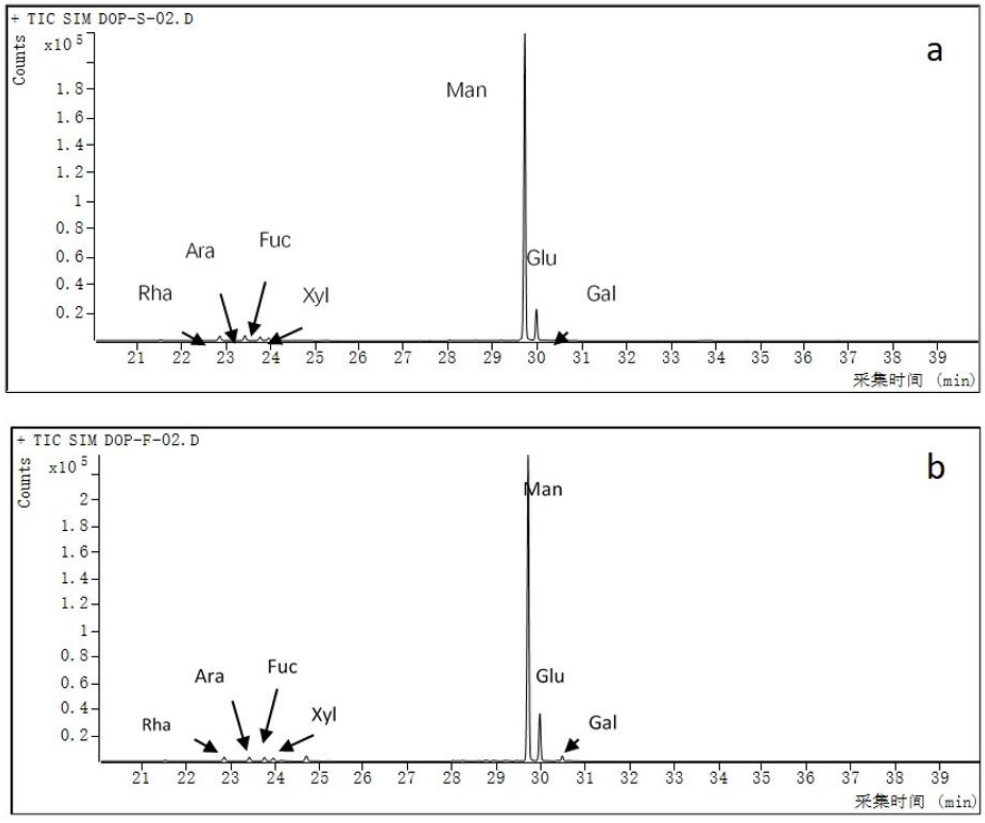

Figure 1. Acetylated derivatives of DOP-S (a) and DOP-F (b) by GC-MS.

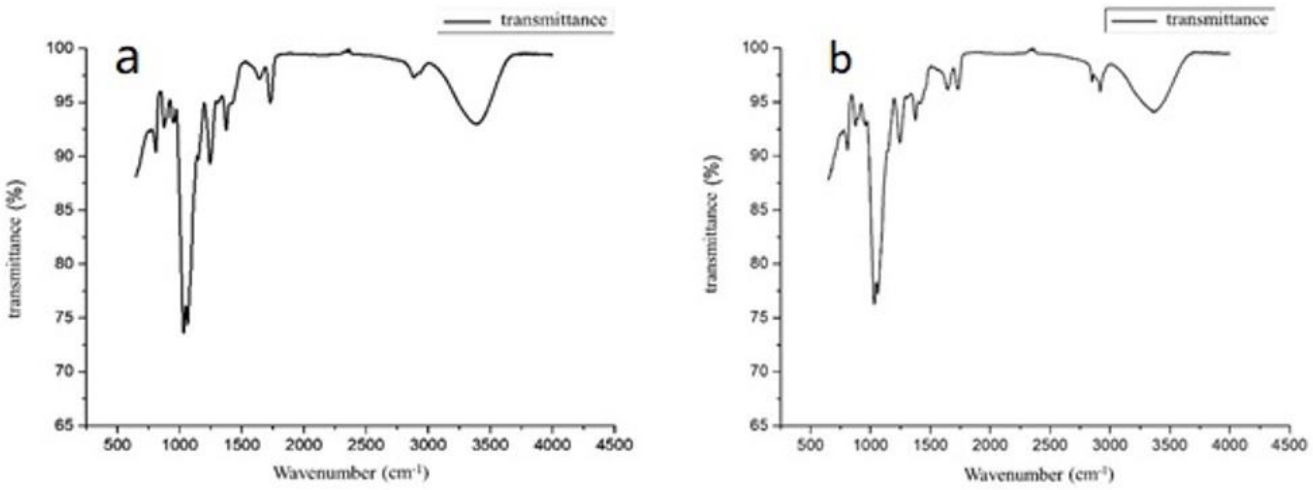

Figure 2. Infrared spectrum (IR spectrum) of DOP-S (a) and DOP-F(b).

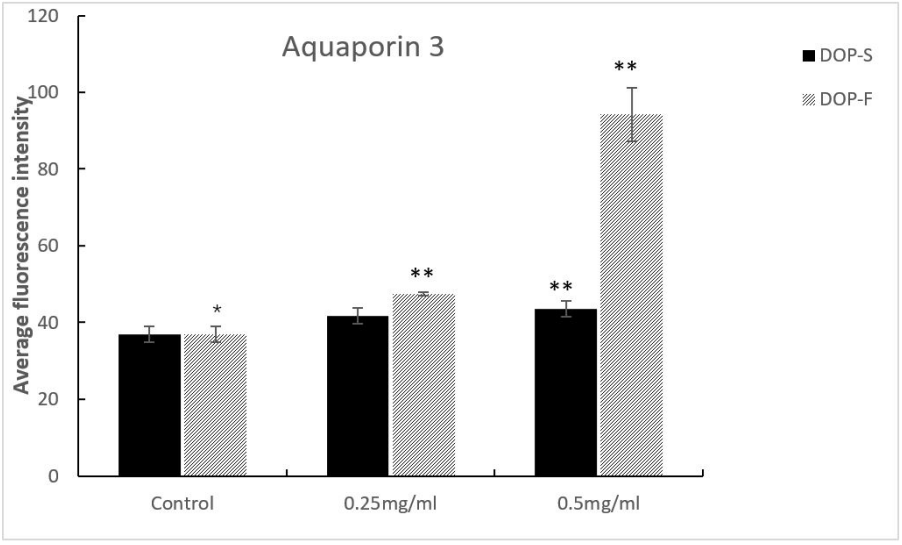

Figure 3. AQP3 promoting effects of polysaccharides DOP-S and DOP-F; $^{**}P < 0.01$, $^{*}P < 0.05$ vs blank control.

The strips in the region between 2886 and 2939 cm^{-1} (DOP-S), 2918 and 2850 (DOP-F) correspond to weak C-H bond stretching vibrations, respectively. The high-intensity absorption peaks appearing at 1733 and 1732 cm^{-1} were contributed by the valence vibration of C=O of O-acetyl groups. Furthermore, the peak at 1244 and 1242 cm^{-1} were due to the variable angular vibration of the C-O bond in the O-acetyl groups. The Significant absorption peaks at about 1378 and 1373 cm^{-1} would be assigned to bending vibrations of symmetric C-H bonds in the methyl group, respectively. The peaks in the range of 1000-1200 cm^{-1} can be attributed to the C-O-H and C-O-C bonds, thus it can be inferred that there are pyranose rings in the compounds. The important peaks at 1033 and 1063 cm^{-1} were assigned to the vibration of the C-O bond at the C-4 position of the glucose residue. The characteristic peaks at 891 and 894 cm^{-1} were caused by β-anomeric carbon, which indicated these two moieties were mainly composed of β-type glycosidic bonds (Liang & Chen et al., 2018). Peaks at 876, 810 cm^{-1} and 809, 873 cm^{-1} were caused by Man in pyranose. The structural features of DOP-S and DOP-F were further elucidated by comparing the data published in similar studies (Huang et al., 2016). DOP-S and DOP-F are composed of the C-1 atoms of (1, 4)-linked β-D-Glcp and (1,4)-linked β-D-Manp. In brief, DOP-S differed from DOP-F in the molar ratios of glucosidic bonds.

3.3 Immunofluorescence staining for AQP3 expression

Cell viability was significantly reduced at DOP concentrations exceeding 1.0 mg/mL; therefore, Immuno-fluorescence staining was used to detect the expression of AQP3 in keratinocytes under different concentrations of DOP-S and DOP-F. As shown in Figure 3, the fluorescence intensity per unit area was counted. These results indicated that DOP-S and DOP-F can stimulate the expression of AQP3, especially DOP-F. In addition, DOP-F enhances AQP3 expression levels in a dose dependent manner. 0.5 mg/mL DOP-F fluorescence intensity significantly increased

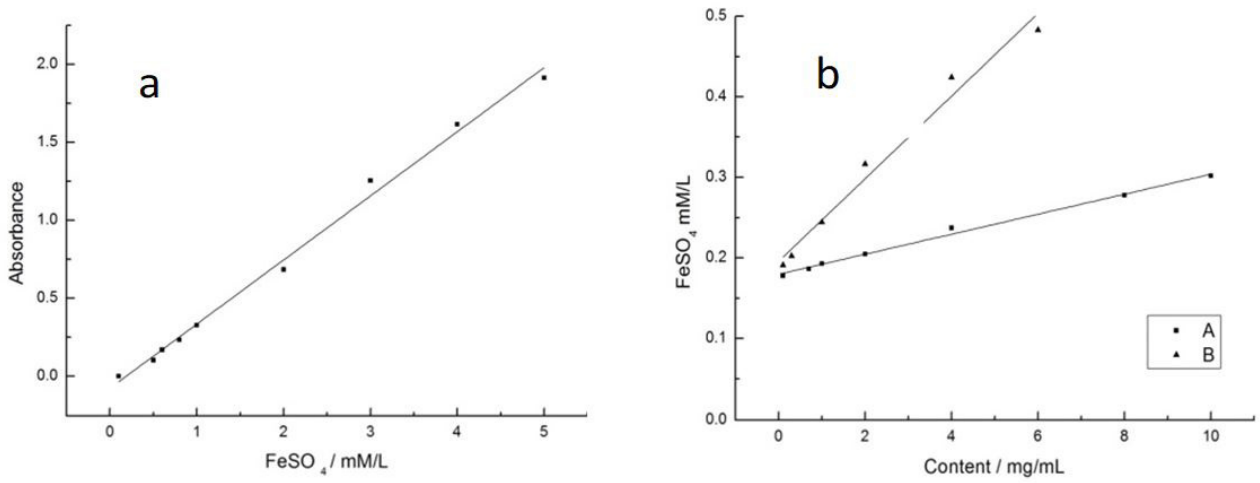

Figure 4. Standard curve of total antioxidant power (a); Total antioxidant activity of samples A and B (b). Sample A: DOP-S; Sample B: DOP-F.

to 90 units. Therefore, it is concluded that the polysaccharide extracted by bio-fermentation effectively promotes the expression of AQP3.

3.4 Antioxidant activities of DOP-S and DOP-F

The polysaccharides produced by fermentation showed better DPPH radical scavenging activity, ABTS radical scavenging activity, than from the water extraction. DOP-S and DOP-F exhibited DPPH scavenging activity with concentrations showing 50% inhibition (IC50 values) of 4.9 and 1.0 mg/mL, respectively. DOP-S and DOP-F showed ABTS radical scavenging activity, with IC50 values of 1.4 and 0.3 mg/mL, respectively. The total antioxidant capacities of DOP-S and DOP-F (0 to 10 mg/mL) were examined by FRAP. Fermentation produced better total antioxidant capacity than water extraction (Figure 4).

4 Conclusions

THighly purified novel polysaccharides DOP-S and DOP-F were obtained from D. officinale. Here, we demonstrated for the first time that the new polysaccharides could enhance the AQP3 expression in human keratinocytes. Biological fermentation effectively achieved the hydrolysis and composition difference of the polysaccharides, and promoted AQP3 expression and the anti-oxidant activities of D. officinale polysaccharides. These results provide strong evidence in support of the use of D. officinale polysaccharides as medicinal and cosmetic raw materials in future.

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