

Research Article

ISSN 2320-4818
JSIR 2014; 3(2): 168-172
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Received: 16-01-2014
Accepted: 04-03-2014

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Isolation and antioxidant activity (in vitro and in vivo) of the flavonoid from *Tartarian-buckwheat*

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Abstract

The total flavonoid was isolated from tartarian-buckwheat through reflux extraction. In order to explore the antioxidant activities, in the present study, our study aimed to examine the antioxidant activity of the total flavonoid using different assays including: reducing power, 2,2-diphenyl 1-picrylhydrazyl (DPPH) radical scavenging assay, ABTS radical scavenging assay and hydroxyl radical scavenging activity. The results exhibited that the total flavonoid has significant reductive ability and radicals scavenging effects. In vivo antioxidant activities assays, the flavonoid was found to increase the levels of antioxidant enzymes (SOD) in blood serum. Therefore, the flavonoid should be explored as novel potential antioxidants.

Keywords: Free radicals, Tartarian-buckwheat, Flavonoid, SOD.

Introduction

Previous studies have indicated that free radicals are able to attack lipid membranes, proteins and DNA, and lead to some detrimental effects, such as lipid peroxidation of cell membranes, alteration of lipid-protein interactions, enzyme inactivation, DNA breakage¹, even result in cell death². So, the oxidative stress induced cell damage triggers both the physiological process of aging and many pathological progressions that eventually lead to serious health problems.³ Antioxidants can reduce the cellular oxidative stress by inhibiting the formation of superoxide anions, and by detoxification of reactive oxygen reactive nitrogen species through up regulation of cellular defense mechanisms, such as superoxide dismutase, catalase, or glutathione peroxidase.⁴ Therefore, research on antioxidants, especially exploration of potent natural compounds with low cytotoxicity has become an important branch of biomedicine. Since the relieving hypertension effect of rutin has been found, flavonoids attracted more and more researchers' attention. The researches about the medicinal value and biological activities of flavonoids have been widely done, but there is few about the flavonoids in tartarian-buckwheat. The most of researches are about their chemical compositions. Over 4000 structurally unique flavonoids have been identified in plant sources.⁵ It has been reported that they have various biological activities and are used in health-care food and drugs, especially the activities like antibacterial⁶, Antimycobacterial⁷, antiviral⁸ and antioxidant⁹.

Therefore, the purpose of the present investigation was to elucidate the isolation the flavonoid from tartarian-buckwheat, as well as to evaluate its antioxidant activities in vitro and in vivo.

Materials and Methods

MDA Assay Kit A003 was purchased from the Institute of Biological Engineering of Nanjing Jianchen (Nanjing, China). 1,1-diphenyl-2-picryl-hydrazyl (DPPH), potassium ferricyanide [$K_3Fe(CN)_6$], trichloroacetic acid (TCA), polyoxyethylenesorbitan monolaurate (Tween-20), and Flavonoid were purchased from Sigma (Sigma- Aldrich GmbH, Sternheim, Germany). BTH and Vitamin C were purchased from Sigma Chemical Co. Thiobarbituric acid (TBA), sodium dodecyl sulphate, nitroblue tetrazolium (NBT), nicotinamide adenine dinucleotide (NADH), and phenazine methosulphate (PMS) were purchased from Applichem. ABTS radical was purchased from Merck. And all other chemicals were analytical grade and were made in China.

Extraction of flavonoids

Extraction the antioxidants by using fractionated extraction, the method was described by Shi *et al.*¹⁰, with some modifications. The powder (100 g) was extracted with 95% and 70% ethanol (300 mL) at boiling point for 1.5 h respectively, filtered, repeat the process thrice. Then the two filtrates were collected and combined, followed by concentrated using rotary evaporation at 50 °C under vacuum to give a solid extract. Solvents from each fraction were removed with a rotary evaporator at 50 °C under vacuum, and the residues were freeze-dried to yield the flavonoids.

Determination of total flavonoids content

The total flavonoid content was determined by the $AlCl_3$ method described by Zuo *et al.*¹ with some modifications, Briefly, 0.25 ml of each fraction and rutin was mixed with 1.25 ml of distilled water in a test tube, followed by addition of 75 μ l of a 5% (w/v) $NaNO_2$ solution. After 6min, 150 μ l of 10% (w/v) $AlCl_3$ solution was added, and the mixture was allowed to stand for a further 5 min before 0.5 ml of 1 mol/L NaOH was added. The mixture was made up to 2.5 ml with distilled water and mixed well. The absorbance was measured immediately at 510 nm. The results of samples were expressed as mg of rutin equivalents of total extractable compounds. Each sample was measured in triplicate. The total flavonoid content was 2.6%.

Reducing power

The reducing power of flavonoid was quantified by the method described earlier by Raza *et al.*¹² and Yen *et*

*al.*¹³ with some modifications. Vitamin C and BHT were used as reference material. Briefly, flavonoid, Vc and BHT were used at differing concentrations (15.6- 2000 μ g/mL). 1 mL of sample was mixed with phosphate buffer (2.5 ml, 0.2 mol/l, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50 °C for 20 min. Then the reaction was terminated by 2.5 ml TCA solution (0.1%) and the mixture was centrifuged at 3000 rpm for 10 min. The supernatant (2.5 ml) was mixed with distilled water (2.5 ml) and ferric chloride (0.5ml, 6 mmol/l), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

ABTS radicals scavenging assay

The antioxidant activity of flavonoid was determined by ABTS radical cation as described by Fan *et al.*¹⁴ and Re *et al.*¹⁵, with some modifications. ABTS was dissolved in PBS (0.01 M, pH 7.4) to a 7 mM concentration. ABTS radical cation was produced by reacting 7 mM ABTS solution with 2.45 mM potassium persulphate and allowing the mixture to stand in the dark at room temperature for 16 h before use. The ABTS radical cation solution was diluted to an absorbance of 0.70 (± 0.02) at 734 nm and equilibrated at 30 °C for 30 min. Each sample (0.2 mL) with various concentrations (15.6- 2000 μ g/mL) was mixed with 2.0 ml of diluted ABTS radical cation solution. After reaction at room temperature for 6 min, the absorbance at 734 nm was measured immediately. The ABTS scavenging effect was calculated as follows:

$$\text{ABTS scavenging effect (\%)} = [A_0 - (A_s - A_b)] / A_0 \times 100$$

where A_0 : A_{734} of ABTS without sample, A_s : A_{734} of sample and ABTS, and A_b : A_{734} of sample without ABTS.

DPPH radicals scavenging assay

DPPH radical cation scavenging activity of flavonoid was carried out as described by Braca¹⁶ with minor modifications. Vitamin C and BHT were used as reference material. 3 mL of sample with various concentrations (15.6- 2000 μ g/mL) was added to 1 mL of 0.1 mM solution of DPPH. The solution was kept at room temperature for 30 min, and the absorbance at 517 nm was measured. The DPPH scavenging effect was calculated as follows:

$$\text{DPPH scavenging effect (\%)} = [A_0 - (A_s - A_b)] / A_0 \times 100$$

where A_0 is the absorbance of DPPH without sample, A_s is the absorbance of sample and DPPH, and A_b is the absorbance of sample without DPPH.

Hydroxyl radical scavenging assay

The hydroxyl radical scavenging activity of flavonoid was carried out according to the method of Wang *et al.*¹⁷, with some modifications. Various concentrations (15.6- 2000 $\mu\text{g/mL}$) samples were incubated with 2.0 mM EDTA-Fe (0.5 mL), 3% H_2O_2 (1.0 mL) and 360 $\mu\text{g/mL}$ crocus in 4.5 mL sodium phosphate buffer (150.0 mM, pH 7.4) for 30 min at 37°C, and hydroxyl radical was detected by monitoring absorbance at 520 nm. The hydroxyl radical scavenging effect was calculated as follows:

$$\text{Hydroxyl radical scavenging effect (\%)} = [(A_0 - A_s) / A_0] \times 100$$

where A_s is the absorbance of sample and A_0 is the absorbance of control. In the control, sample was substituted with distilled water, and sodium phosphate buffer replaced H_2O_2 .

SOD activity assay

Kunming mice weighing in the range of 18–22 g, were kept in separated cages at a temperature of 21 ± 1 °C and a 50–60% of relative humidity. They underwent 12-h light-and-dark cycles with free access to food and water. All of the mice were evenly and randomly divided into five groups of ten mice each. Group I was given D-galactose and normal laboratory diet; Group II was given D-galactose, Vc (100 mg/kg per day) and normal laboratory diet; Group III was given D-galactose, the sample at a dose of 200 mg/kg per day and normal laboratory diet; Group IV was given D-galactose, the test sample at a dose of 100 mg/kg per day and normal laboratory diet; Group V was given D-galactose, the sample at a dose of 50 mg/kg per day and normal laboratory diet. The dose of D-galactose of each group was 100 mg/kg/day body weight.¹⁸ Twenty-four hours after the last drug administration, blood samples were obtained from the eye pit of the mice and processed for serum. The SOD activities were determined with an SOD Assay Kit.

Statistical analysis

All data are expressed as means \pm SD ($n=3$). Data were analyzed by an analysis of variance ($P < 0.05$) and the results were processed by SPSS software.

Results and Discussion

Effect of reducing power

To measure reductive power of flavonoid, we investigated the Fe^{3+} - Fe^{2+} transformation in the presence of different concentrations sample, BHT and Vc were used as reference material. The reductive capabilities of flavonoid and reference material were exhibited as Figure 1. The references exhibited strong reducing power, especially for Vc. The reducing power of Flavonoid was also strong, at the high concentrations (from 500 to 2000 $\mu\text{g/ml}$), which was stronger than that of BHT ($P < 0.05$). From the result, reducing powers of all samples also were in a concentration-dependent manner.

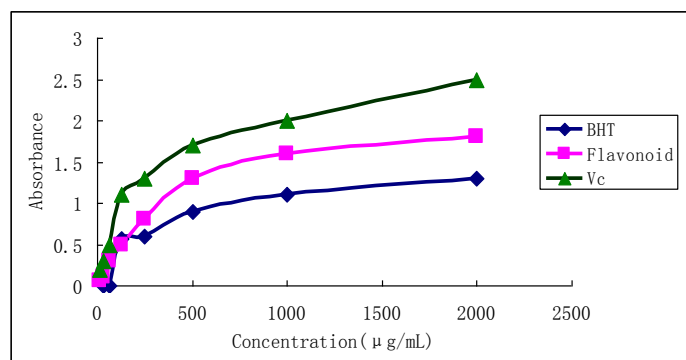


Figure 1: Reductive abilities of Flavonoid and the controls. Results are presented as means \pm standard deviations.

Effect of scavenging ABTS radicals

The results of scavenging power on ABTS free radical for the present experiment were shown in Figure 2. The scavenging power of Flavonoid was strong at the range of 1000-2000 $\mu\text{g/ml}$, which was higher than that of Vc. The results indicated that Flavonoid had excellent ABTS radical scavenging activity and should be explored as novel potential antioxidants.

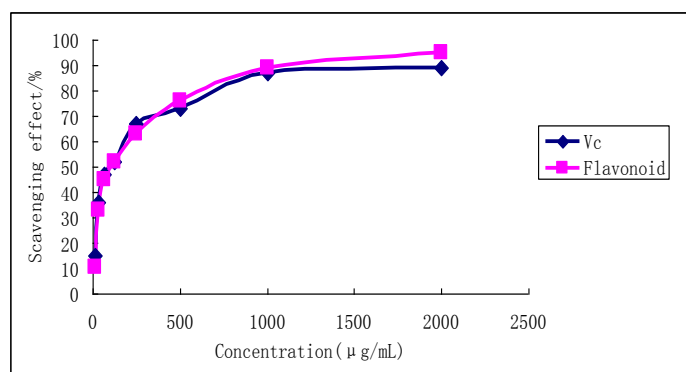


Figure 2: The scavenging effects of polysaccharides on ABTS radicals. Results are presented as means \pm standard deviations.

Effect of scavenging DPPH radicals

In this experiment, the scavenging ability of Flavonoid on DPPH free radical were examined in the concentration range of 15-2000 $\mu\text{g/mL}$ using the DPPH colorimetric assay. And the results were given in Figure 3. The results indicated that Flavonoid exhibited very significantly radical scavenging. The effect of Flavonoid was strong at the high dose 500-2000 $\mu\text{g/mL}$, which close to that of Vc, all the samples obvious scavenging activity in a concentration dependent manner. The results suggest that Flavonoid display scavenging effect on DPPH radicals generation that could help prevent or ameliorate oxidative damage.

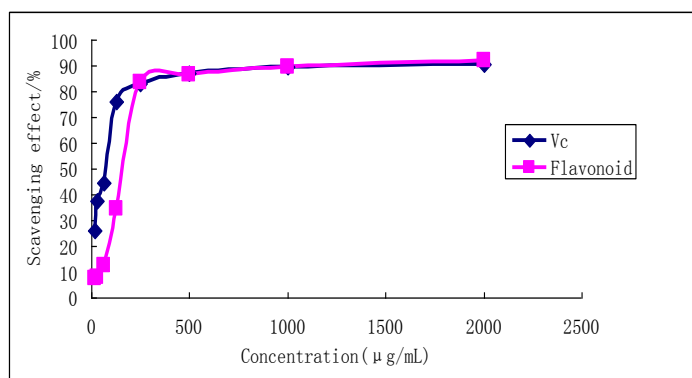


Figure 3: The scavenging effects of Flavonoid on DPPH radicals. Results are presented as means \pm standard deviations.

Effect of scavenging hydroxyl radicals

The scavenging ability of Flavonoid compared to those of vitamin C was shown in Figure 4. The scavenging activities of Flavonoid increased very significantly with increasing concentrations (500-2000 $\mu\text{g/ml}$). Especially in the high doses(2000 $\mu\text{g/ml}$), Flavonoid exhibited very high radical scavenging, which was close to that of Vitamin C ($p < 0.05$). So, it was obvious that Flavonoid has significant effects on hydroxyl radicals scavenging. The samples also exhibited obvious scavenging activities on hydroxyl radical in a concentration-dependent manner.

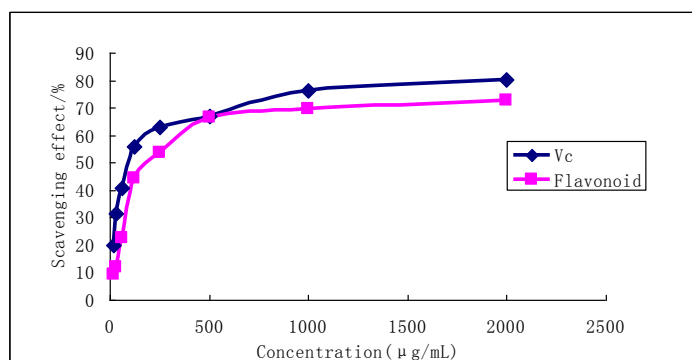


Figure 4: The scavenging effects of Flavonoid and Vc on hydroxyl radicals

SOD activity

The results are shown in Figure 5. SOD activities of different doses of Flavonoid exhibited dose-dependent behavior. At low doses(50 mg/kg and 100 mg/kg), the SOD activity value were low, but at the high dose of 100 mg/kg, the flavonoid exhibited high SOD activity, and the SOD activity value was 300.7 U/mL, which was far higher than that of the positive control. The results were therefore an indication of enhancement SOD activity of the flavonoid for high concentrations.

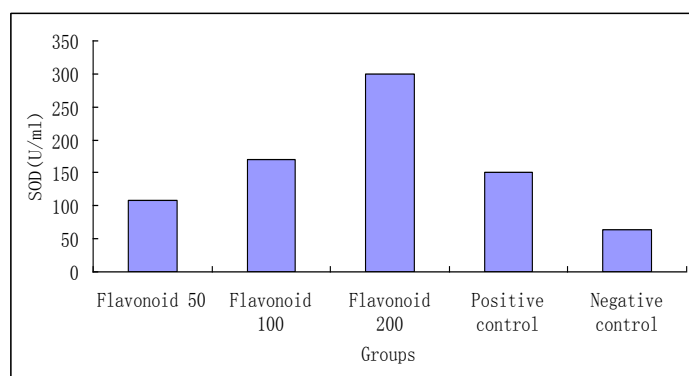


Figure 5: SOD activity analysis in mice. Results are presented as means \pm standard deviations

Conclusion

In the present study, the flavonoid was isolated from Tartarian-buckwheats by solvents extraction. Antioxidant activities in vitro indicated the flavonoid has significant radicals scavenging abilities on ABTS radicals, DPPH radicals and Hydroxyl radicals, and also exhibited strong reducing capacity. The scavenging effects were powerful, which closed to the positive control. In vivo antioxidant activities assays, the flavonoid was found to increase the levels of antioxidant enzymes (SOD) in blood serum. It was confirmed that flavonoid can protect tissues against oxidative damages. Enhanced SOD activity in mice blood serum also can be related to the in vivo antioxidant activity of flavonoid. Therefore, the flavonoid should be explored as novel potential antioxidants.

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