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Anti-fatigue activity of polysaccharides from the fruits of four Tibetan plateau indigenous medicinal plants

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ABSTRACT

Ethnopharmacological relevance: The fruits of *Hippophae rhamnoides* L., *Lycium barbarum* L., *Lycium ruthenicum* Murr. and *Nitraria tangutorum* Bobr. are traditional medicinal food of Tibetans and used to alleviate fatigue caused by oxygen deficiency for thousands of years. The present study focused on exploiting natural polysaccharides with remarkable anti-fatigue activity from the four Qinghai-Tibet plateau characteristic berries.

Materials and methods: The fruits of *Hippophae rhamnoides*, *Lycium barbarum*, *Lycium ruthenicum* and *Nitraria tangutorum* were collected from Haixi national municipality of Mongol and Tibetan (N 36.32°, E98.11°; altitude: 3100 m), Qinghai, China. Their polysaccharides (HRWP, LBWP, LRWP and NTWP) were isolated by hot-water extraction, and purified by DEAE-Cellulose ion-exchange chromatography. The total carbohydrate, uronic acid, protein and starch contents of polysaccharides were determined by a spectrophotometric method. The molecular weight distributions of polysaccharides were determined by gel filtration chromatography. Their monosaccharide composition analysis was performed by the method of 1-phenyl-3-methyl-5-pyrazolone (PMP) pre-column derivatization and RP-HPLC analysis. HRWP, LBWP, LRWP and NTWP (50, 100 and 200 mg/kg) were orally administrated to mice once daily for 15 days, respectively. Anti-fatigue activity was assessed using the forced swim test (FST), and serum biochemical parameters were determined by an autoanalyzer and commercially available kits; the body and organs were also weighted.

Result: LBWP, LRWP and NTWP were mainly composed of glucans and some RG-I pectins, and HRWP was mainly composed of HG-type pectin and some glucans. All the four polysaccharides decreased immobility in the FST, and the effects of LBWP and NTWP were demonstrated in lower doses compared with HRWP and LRWP. There was no significant difference in liver and heart indices between non-treated and polysaccharide-treated mice, but the spleen indices were increased in LBWP and NTWP (200 mg/kg) group. Moreover, the FST-induced reduction in glucose (Glc), superoxide dismutase (SOD) and glutathione peroxidase (GPx) and increase in creatine phosphokinase (CK), lactic dehydrogenase (LDH), blood urea nitrogen (BUN), triglyceride (TG) and malondialdehyde (MDA) levels, all indicators of fatigue, were inhibited by HRWP, LBWP, LRWP and NTWP to a certain extent while the effects of LBWP and NTWP were much better than that of HRWP and LRWP at the same dosage.

Conclusion: Water-soluble polysaccharides HRWP, LBWP, LRWP and NTWP, from the fruits of four Tibetan plateau indigenous berry plants, significantly exhibited anti-fatigue activities for the first time, through triglyceride (TG) (or fat) mobilization during exercise and protecting corpuscular membrane by prevention of lipid oxidation via modifying several enzyme activities. Moreover, it is demonstrated that LBWP and NTWP are more potent than HRWP and LRWP, which were proposed to be applied in functional foods for anti-fatigue and antioxidant potential.

1. Introduction

Fatigue is best defined as the difficulty in initiating or sustaining voluntary activities, and can be classified into mental and

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physical fatigue (Tanaka et al., 2008). Fatigue, an important factor influencing physical performance, often occurred in aging, cancer, depression, HIV infection, multiple sclerosis and Parkinson's disease (Belluardo et al., 2001; Tharakan et al., 2006; Huang et al., 2011). To date, pharmacological drugs or therapies effective for treating fatigue cannot yet satisfy the need of the people. So, people pay more attention to seek natural anti-fatigue compounds without side effect to improve athletic ability, postpone fatigue and accelerate the elimination of fatigue in human beings (Kim et al., 2002; Uthayathas et al., 2007). Recently, polysaccharides from medicinal plants are considered to be a new sort of natural anti-fatigue substances (Wang et al., 2010; Chen and Zhang, 2011; Sheng et al., 2011; Hu et al., 2012; Tan et al., 2012).

Tibetan plateau, due to its high altitude and big diurnal amplitude, is referred to as the Earth's third pole. Under the environment of plateau hypoxia, it is easy to get tired. So, in Tibetan medical system, lots of native plants and their fruits were used to alleviate fatigue caused by oxygen deficiency (Ma et al., 2011). In these folk native anti-fatigue medicines, the fruits of *Hippophae rhamnoides* L., *Lycium barbarum* L., *Lycium ruthenicum* Murr. and *Nitraria tangutorum* Bobr. are traditional medicinal food of Tibetans for thousands of years, which are recorded in the classic Tibetan pharmacological book "Crystal Pearl of Materia Medica" (Chinese pinyin: jingzhubencao; Dierma, 2012).

Recent studies have revealed that *Hippophae rhamnoides* fruit is considered to be a good source of large number of bioactive substances like vitamins, carotenoids, phytosterols, organic acids, polyunsaturated fatty acids and some essential amino acids (Kanayama et al., 2012); *Lycium barbarum* fruit and polysaccharide from it possess a range of biological activities, including anti-aging, neuroprotection, increased metabolism, glucose control in diabetics, glaucoma, anti-oxidant properties, immunomodulation, anti-tumor activity and cytoprotection (Amagase and Farnsworth, 2011; Jin et al., 2013); *Lycium ruthenicum* fruit contains abundant anthocyanins and a highly branched arabinogalactan protein (Zheng et al., 2011; Peng et al., 2012); *Nitraria tangutorum* fruit contains several kinds of alkaloids and flavones including allantoin, MTCCA, and quercetin (Wang et al., 2007; Suo and Wang, 2010). However, previous studies mainly focused on the structural characterization and pharmacological activity evaluation of small molecular compounds from *Nitraria tangutorum*, *Hippophae rhamnoides* and *Lycium ruthenicum*. Even the studies on *Lycium barbarum* polysaccharide, one of the hotspots in the polysaccharide research field, lack anti-fatigue activity assay. To our knowledge, there is no report on the anti-fatigue activity of polysaccharides from the fruits of four Tibetan plateau indigenous berry plants to date.

In the present paper, the water-soluble polysaccharides from *Hippophae rhamnoides*, *Lycium barbarum*, *Lycium ruthenicum* and *Nitraria tangutorum* were isolated, purified and performed composition analyses, anti-fatigue and antioxidant activity assays, for exploiting natural polysaccharides with remarkable anti-fatigue activity from the Qinghai-Tibet plateau characteristic berries.

2. Materials and methods

2.1. Plant materials and chemicals

The fruits of *Hippophae rhamnoides* L., *Lycium barbarum* L., *Lycium ruthenicum* Murr. and *Nitraria tangutorum* Bobr. were collected from Dongshangen of Dulan Country (N 36.32°, E98.11°; altitude: 3100 m), Haixi national municipality of Mongol and Tibetan, Qinghai, China. Materials were identified by Prof. Xuefeng Lu, Northwest Plateau Institute of Biology, Chinese Academy of Sciences in Xining, China. The herbarium samples were numbered as HR20110805 for *Hippophae rhamnoides*, LB20110802 for *Lycium barbarum*, LR20110719 for *Lycium ruthenicum* and NT20110802 for *Nitraria tangutorum*, and deposited at Qinghai Key Laboratory of Tibetan Medicine Pharmacology and Safety Evaluation.

DEAE-Cellulose and Sepharose CL-6B were purchased from Amersham Pharmacia Biotech. Standard dextrans and the monosaccharides (D-Galactose, D-Arabinose, D-Fucose, D-Rhamnose, D-Mannose, D-Xylose, D-Glucose, Glucuronic acid, and Galacturonic acid) were purchased from Sigma Co. (St. Louis, USA). All other reagents were of analytical grade made in China.

2.2. Experimental animals

Male BALB/c male mice (8 weeks old) were procured from the Pharmacology Experimental Center of Jilin University (Changchun, China). The mice were housed on a 12/12-h light–dark cycle at room temperature and allowed free access to standard rodent food and water during the experiments. Animal handling procedures were conducted under National Institutes of Health animal care and use guidelines. The Institution Animal Ethics Committee reviewed the entire animal protocol prior to conducting the experiments.

2.3. Polysaccharide preparation

Plant material was air-dried, and then exhaustively extracted with 95% ethanol under reflux for 12 h to remove hydrophobic compounds. This step was repeated three times. After filtration through a gauze (100 mesh), the residue was dried at room temperature, and then extracted with hot water (90 °C, 1:20 w/v) three times (6 h for each). The aqueous filtrates were combined and concentrated, subsequently 95% ethanol was added to the aqueous filtrates up to 80% to precipitate the polysaccharides which were collected by centrifugation and dried in vacuum. The precipitate was dissolved in water (5% w/v) and the insoluble substances are removed by centrifugation. The supernatant was loaded to a DEAE-Cellulose column, and then eluted with 0.5 M NaCl. The eluent was collected, concentrated to a small volume, dialyzed, and finally lyophilized to obtain the polysaccharide.

The water-soluble polysaccharide from the four characteristic Qinghai-Tibet plateau berries were respectively HRWP from *Hippophae rhamnoides*, LBWP from Qaidam *Lycium barbarum*, LRWP from *Lycium ruthenicum* and NTWP from *Nitraria tangutorum*. Contaminant endotoxin was analyzed by a gel-clot Limulus amebocyte lysate assay. The endotoxin level in each polysaccharide solution was less than 0.5 EU (endotoxin units)/mL.

2.4. Analytical methods

The total carbohydrate content was determined by the phenol-H_2SO_4 method using glucose as standard (Dubois et al., 1956). All gel filtration chromatography was monitored by assaying carbohydrate content. Uronic acid content was determined by the m-hydroxydiphenyl colorimetric method, using galacturonic acid as standard (Blumenkrantz and Asboe Hansen, 1973). Protein content was determined by the method of Sedmak and Grossberg (1977), with Coomassie brilliant blue reagent and bovine serum albumin as the standard. Starch content was determined according to the method of Gur et al. (1969), using soluble starch as the standard. Molecular weight distributions were determined by Sepharose CL-6B (85 cm × 1.5 i.d.) gel filtration chromatography. Contaminant endotoxin was analyzed in a limulus amebocyte lysate (LAL) assay using an E-TOXATE kit (Sigma, St. Louis, USA) according to the manufacturer's instructions. Dialysis was carried out using tubing with Mw cut-off 3500 Da (for globular protein).

Monosaccharide analysis was performed as described by Honda et al. (1989). Briefly, sample (2 mg) was hydrolyzed with 2 M CF_3COOH (1.0 ml) at 120 °C for 3 h. The hydrolyzed products (monosaccharides) were derivatized with 0.5 M 1-phenyl-3-methyl-5- pyrazolone (PMP) and 0.3 M NaOH. After neutralization with 0.3 M HCl, the PMP-derivatives were analyzed in an Agilent 1100 HPLC system equipped with an Agilent Eclipse XDB-C_{18} column (250 mm × 4.6 mm, 5 μm) with a guard column and monitored by UV absorbance at 245 nm.

2.5. Experimental design for anti-fatigue assay

Mice were divided into the following experimental groups (6 mice/group). The intact group was treated with saline and not subjected to a swimming test (Mizoguchi et al., 2011); the p.s. group was treated with physiologic saline and subjected to a swimming test. The test groups were treated with different doses of polysaccharides (HRWP, LBWP, LRWP and NTWP) (50, 100 and 200 mg/kg) and subjected to a swimming test. Saline/polysaccharides were administrated orally (7:00 am) to mice for 15 days and the forced swim test was conducted on the last day, 1 h after compound administration.

Mice were anesthetized with ether and blood samples were collected from each treatment group. Serum samples were obtained by centrifugation (3000*g*, 10 min, 4 °C) and stored at −80 °C for further analysis. The spleens, hearts, and livers were weighed and their weights relative to the final body weights (organ index) were calculated.

2.6. Forced swim test

FST (forced swim test, FST) used was the same as described by Porsolt et al. (1978). Briefly, ICR mice were placed individually in glass cylinders (height: 25 cm, diameter: 10 cm) containing 10 cm of water at 23–25 °C for a 6 min session. At the end of the session, mice were removed from the water, dried with a paper towel, and placed back in their home cage. Water in the container was changed after each session. The duration of immobility was manually recorded by a trained experimenter, blind to the experimental conditions, during the last 4 min of the testing period and immobility was defined when mice ceased struggling and were floating motionless in the water, making only those movements necessary to keep their head above water.

2.7. Serum analysis

Mice were anesthetized with intraperitoneal injection of ketamine (80 mg/kg) and xylazine (4 mg/kg). After anesthetization, bloods were withdrawn from heart of forced swimming-treated mice into syringes. Then, serum was prepared by centrifugation at 3000 rpm at 4 °C for 10 min. Contents of glucose (Glc), lactic dehydrogenase (LDH), creatine phosphokinase (CK) and triglyceride (TG) were determined by an autoanalyzer (Hitachi 7060, Hitachi, Japan). Levels of blood urea nitrogen (BUN), superoxide dismutase (SOD), malondialdehyde (MDA) and glutathione peroxidase (GPx) were determined using commercially available kits from the Jiancheng Bioengineering Institute (Nanjing, China).

2.8. Statistical analysis

Statistical significance was determined by one-way ANOVA followed by Dunnett's post hoc comparisons. All values were expressed as mean ± S.D. A *P*-value of <0.05 was considered to be significant.

3. Results and discussion

3.1. Analyses of medicine-food berry polysaccharides

Four polysaccharide fractions were isolated from the fruits of *Hippophae rhamnoides*, *Lycium barbarum*, *Lycium ruthenicum* and *Nitraria tangutorum* by hot water extraction and 80% ethanol precipitation, which were respectively named after HRWP, LBWP, LRWP and NTWP. HRWP is a yellowish-brown powder, and accounted for 5.5% of *Hippophae rhamnoides* fruit dry fruit weight; LBWP is a light brown powder, and accounted for 4.0% of *Lycium barbarum* fruit dry weight; LRWP is a dark brown powder, and accounted for 10.3% of *Lycium ruthenicum* fruit dry fruit weight; NTWP is a light brown powder, and accounted for 4.8% of *Nitraria tangutorum* fruit dry fruit weight.

Carbohydrate contents of the four polysaccharide fractions were determined to be above 95% by the phenol-H_2SO_4 method. Due to the process of removing protein by DEAE-Cellulose ion-exchange chromatography, the protein contents of polysaccharide fractions were all below 1.2%. Their UV spectra contained no peaks at 260 nm, which indicated that they did not contain nucleic acid. Limulus amebocyte lysate (LAL) assays revealed less than 0.05 EU/mg, which indicates negative results. The total carbohydrate, uronic acid, protein, starch contents and yield of each polysaccharide are listed in Table 1, and their monosaccharide compositions and molecular weight distributions are listed in Fig. 1 and Table 2.

The results exhibited that the fractions LBWP, LRWP and NTWP were mainly composed of glucans, and the absence of starch in them, so the glucose was not α-(1,4) or α-(1,6) linked. Besides glucans, they also contained some rhamnose and galacturonic acid. The ratios of Rha/GalUA determined for LBWP, LRWP and NTWP were 0.33, 0.21 and 0.38, respectively, which are in the rhamnogalacturonan I (RG-I) range from 0.05 to 1.0 defined by Schols and Voragen (1996). This suggested that they might contain RG-I pectin. RG-I has been reported to be composed of α-(1,4)-linked D-galacturonic acid and α-(1,2)-linked L-rhamnose, which are alternatively combined with each other in the backbone; and some of the rhamnose residues contained side chains, such as arabinan, galactan and arabinogalactan at 4-O-rhamnose (McNeil et al., 1980). Therefore, we deduced that galactose and arabinose of the above polysaccharide fractions might be composed of arabinans, galactans and/or arabinogalactans, which are associated with their RG-I domains in non-covalent form or as side chains of RG-I. However, their structures needed to be further characterized.

The ratio of Rha/GalUA of HRWP was determined to be 0.02, which is below the lower limit of the RG-I range, suggesting that

Table 1
The yield, total carbohydrate, uronic acid, protein and starch contents of polysaccharide fractions from four medicine-food berries.

Fraction	Yield[a] (%)	Total carbohydrate content[b] (%)	Uronic acid content[c] (%)	Protein content[d] (%)	Starch content[e] (%)
HRWP	5.5	95.2	61.8	1.3	0.01
LBWP	14.0	97.5	1.9	0.01	0.01
LRWP	10.3	96.8	3.3	0.03	0.01
NTWP	4.8	98.0	2.6	1.9	0.02

[a] Yield was calculated based on dried materials mass.
[b] The total carbohydrate content was determined by the phenol-H_2SO_4 method using glucose as standard.
[c] Uronic acid content was determined by m-hydroxydiphenyl colorimetric method, using galacturonic acid as standard.
[d] Protein content was determined according to the method of Sedmak and Grossberg (1977), with Coomassie brilliant blue reagent and bovine serum albumin as the standard.
[e] Starch content was determined according to the method of Gur et al. (1969), using soluble starch as the standard.

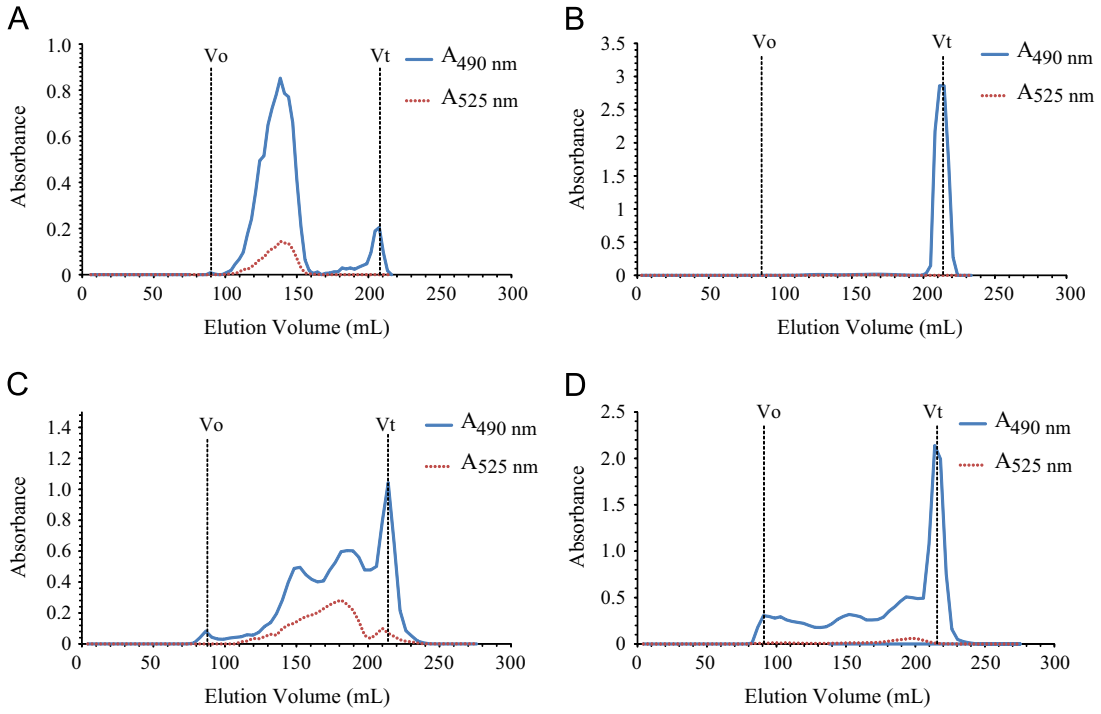

Fig. 1. Sepharose CL-6B elution profiles of polysaccharide fractions from four medicine-food berries: A, HRWP; B, LBWP; C, LRWP and D, NTWP.

Table 2
The molecular weight distributions, monosaccharide components and probable types of polysaccharide components of polysaccharide fractions from four medicine-food berries.

Fraction	Mw (kD)	Monosaccharide component[a] (%)								Type of polysaccharide component[b]
		Man	Rha	GlcUA	GalUA	Glc	Gal	Xyl	Ara	
HRWP	194.2, ≤10.0	4.2	1.3	0.7	59.4	20.1	9.7	1.1	3.5	Homogalacturonan and Glucan
LBWP	≤10.0	2.1	0.6	n.d.	1.9	86.8	3.0	n.d.	5.6	Glucan and Rhamnogalacturonan I
LRWP	≥1000.0, 78.7, 9.4	1.6	1.2	n.d.	5.7	82.3	2.9	0.7	6.2	Glucan and Rhamnogalacturonan I
NTWP	≥1000.0, 79.7, ≤10.0	3.9	1.8	0.2	3.3	70.6	7.6	n.d.	13.1	Glucan and Rhamnogalacturonan I

[a] Sugar-1-phenyl-3-methyl-5-pyrazolone (PMP) derivatives obtained after methanolysis combined with CF3COOH hydrolysis followed by PMP-precolumn derivation and analyzed by HPLC.
[b] Type of polysaccharide component was deduced according to monosaccharide compositions and ratios of Rha/GalUA.

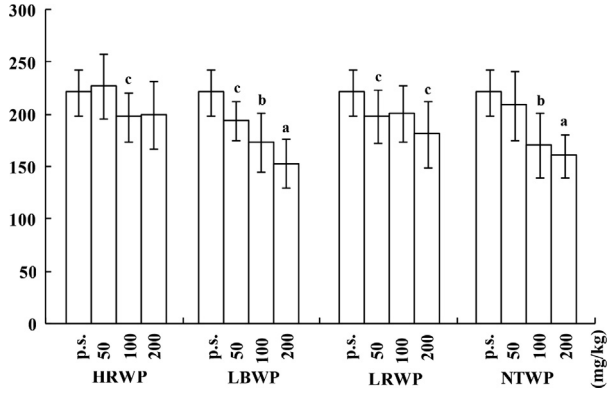

Fig. 2. Effects of polysaccharide fractions on immobility in the FST. Immobility time recorded during 4 min in the FST in intact group or polysaccharide. Values are expressed as mean ± S.D.; $n=6$ in each group, a, b and c represent significant difference from p.s. group. $^{a}P<0.001$, $^{b}P<0.01$, $^{c}P<0.05$.

galacturonic acid of HRWP might mainly be α-(1,4) linked, which exists in homogalacturonan (HG)-type pectin form (Schols and Voragen, 1996). Besides galacturonic acid, HRWP also contained some glucose. The starch content assay indicated that glucose of HRWP was not α-(1,4) or α-(1,6) linked, thus the glucan is of non-starch nature. Based on its elution profile, it is deduced that HG-type pectin might be present in the high molecular weight compounds, and glucan present in the low. This deduction needs to be validated in our further research.

3.2. *Effects of polysaccharides in the forced swim test.*

A forced swimming test, which has been widely used as anti-fatigue test was selected to evaluate the anti-fatigue of these four polysaccharides (Dubovik and Bogomazov, 1987; Ozturk et al., 2002). The immobility time indicates the degree of fatigue (Tanaka et al., 2003). As expected, in comparison with the saline-treated mice, all four polysaccharides decreased immobility times in the FST (Fig. 2). However, the effects of LBWP, LRWP and NTWP were demonstrated at 50 mg/kg, and HRWP reduced immobility at 100 mg/kg ($P<0.05$). While LBWP and NTWP were demonstrated at 100 and 200 mg/kg which exhibited an extreme significance ($P<0.01$), indicating that LBWP and NTWP showed better anti-fatigue abilities than those of HRWP and LRWP.

The differences in these data may result from the polysaccharide compositions and structures. LBWP, LRWP and NTWP all mainly contain large amounts of glucose which are composed of glucans.

Previous studies have shown that different types of glucan with α-(1,3),(1,4), α-(1,4),(1,6) or β-(1-3)(1-6) linkages have noticeable bioactivities including immunological and antioxidant effects (Brown and Gordon, 2001; Nair et al., 2006; Liu et al., 2007; Ni et al., 2010; Gao et al., 2011; Bi et al., 2013), which are involved in anti-fatigue effect (Shin et al., 2005; Ding et al., 2011). While, HRWP contains large amount of galacturonic acid, and Rha/GalUA ratio indicated that its galacturonic acid might be composed of HG-type pectin via α-(1,4) linkage. So, it is probable that the anti-fatigue ability of HG-type pectin is weaker than that of the glucan.

3.3. Effects of polysaccharides on organ weight

Body weight was recorded before experiment (initial) and after 2 weeks (final), and weight gain was computed. As shown in Table 3, all four medicinal polysaccharides had no significant effect on the body weight, and weight gain compared to the intact group.

The association between decreased liver, heart and spleen weight was further evaluated. Orally given with HRWP, LBWP, NTWP and LRWP at 50, 100 and 200 mg/kg for 2 consecutive weeks slightly, but did not significantly, ameliorate the weight ratio of heart in mice challenged.

Energy for exercise is derived initially from the breakdown of glycogen, and as is commonly known, glucose levels are decreased immediately after exercise (Dorchy, 2006). The liver weight of saline-treated fatigue mice showed slightly, but not significantly, decrease than that of the intact group. However, the four polysaccharides increase the liver/weight ratio to an extent.

The weight ratio of spleen/body weight in fatigue mice was significantly lower than those of home cage control animals (Shin

Table 3
Effects of polysaccharide fractions on body weight and organ indices in mice.

Group	Dose (mg/kg)	Body weight (g)		Organ index		
		Day 1	Day 15	Liver	Heart	Spleen
Intact	–	22.1 ± 1.1	28.1 ± 3.9	4.4 ± 0.3	0.5 ± 0.1	0.4 ± 0.03
p.s.	–	21.5 ± 2.2	27.4 ± 3.4	4.1 ± 0.2	0.4 ± 0.06	0.4 ± 0.09
HRWP	50	18.2 ± 2.8	26.8 ± 4.2	4.1 ± 0.3	0.4 ± 0.07	0.5 ± 0.08[b]
	100	20.1 ± 3.2	30.0 ± 2.9	5.3 ± 0.3[a]	0.5 ± 0.04[b]	0.5 ± 0.09 [b]
	200	19.8 ± 3.5	28.8 ± 4.2	5.8 ± 0.2[a]	0.5 ± 0.08[b]	0.5 ± 0.06 [b]
LBWP	50	20.7 ± 2.9	27.2 ± 3.3	5.3 ± 0.4[a]	0.5 ± 0.09[b]	0.6 ± 0.05[b]
	100	21. 3 ± 2.4	29.4 ± 4.7	5.1 ± 0.2[b]	0.4 ± 0.02	0.6 ± 0.02[b]
	200	21.5 ± 3.9	29.3 ± 3.7	5.1 ± 0.6[b]	0.5 ± 0.09[b]	0.6 ± 0.01[a]
LRWP	50	19.9 ± 4.0	30.2 ± 3.5	5.0 ± 0.5	0.4 ± 0.04	0.4 ± 0.02
	100	20.2 ± 2.3	32.0 ± 5.1	5.1 ± 0.1[b]	0.4 ± 0.05	0.5 ± 0.01[b]
	200	21.1 ± 4.3	33.9 ± 4.3[b]	5.4 ± 0.4 [a]	0.5 ± 0.09[b]	0.4 ± 0.03
NTWP	50	19.4 ± 3.3	27.3 ± 3.1	5.4 ± 0.5[a]	0.4 ± 0.03	0.5 ± 0.04[b]
	100	19.3 ± 3.2	29.0 ± 3.3	4.9 ± 0.3[b]	0.4 ± 0.06	0.5 ± 0.03[b]
	200	21.3 ± 3.1	28.1 ± 4.2	5.8 ± 0.1[a]	0.5 ± 0.05[b]	0.6 ± 0.04[a]

The data were presented as means ± S.D. ($n=6$), a and b represent significant difference from p.s. group.
Organ indices = weight of organ/body weight × 100. Intact group = mice unexposed to the FST and treated with saline, p.s. = mice exposed to the FST and treated with saline.

[a] $P<0.01$.
[b] $P<0.05$.

Table 4
Effects of polysaccharide fractions on serum biochemical parameters after the forced swim test.

Group	Dose (mg/kg)	Glc (mmol/L)	BUN (mmol/L)	TG (mmol/L)	CK (U/L)	LDH (U/L)	MDA (nmol/mL)	SOD (U/mL)	GPx (U/L)
Intact	–	10.2 ± 1.1[b]	9.1 ± 2.8[c]	2.3 ± 0.2	1001 ± 220[a]	620 ± 33.0[b]	16.4 ± 2.2[a]	131.43 ± 20.4	676 ± 21[a]
p.s.	–	8.8 ± 2.2	10.1 ± 2.4	2.5 ± 0.1	1314 ± 114	847 ± 16.2	22.5 ± 1.7	96.8 ± 33.9[a]	495 ± 33
HRWP	50	10.0 ± 1.0 [b]	10.0 ± 0.9	2.5 ± 0.4	1229 ± 134[b]	812 ± 20.0	21.6 ± 2.2	96.1 ± 23.7[a]	537 ± 32[c]
	100	9.3 ± 1.2[c]	10.0 ± 1.3	2.7 ± 0.4	1102 ± 111[b]	753 ± 39.2[c]	21.2 ± 2.0	121.2 ± 11.4[c]	588 ± 24[b]
	200	10.1 ± 2.7[a]	9.3 ± 1.2[c]	2.4 ± 0.5	1004 ± 121[a]	654 ± 22.9[b]	20.3 ± 3.1	128.1 ± 26.6	658 ± 29[a]
LBWP	50	9.7 ± 2.3[b]	10.0 ± 1.5	2.2 ± 0.2	1219 ± 153[b]	698 ± 29.8[c]	19.1 ± 1.4[c]	130.0 ± 29.3	609 ± 33[a]
	100	10.2 ± 2.4[a]	9.1 ± 1.1[c]	2.0 ± 0.3	1072 ± 111[b]	612 ± 24.6[a]	19.0 ± 2.9[c]	138.9 ± 19.7	666 ± 28[a]
	200	11.5 ± 0.9[a]	9.1 ± 2.3[c]	2.1 ± 0.1[c]	999 ± 99[a]	537 ± 16.6[a]	15.5 ± 1.8[a]	149.2 ± 30.2[c]	673 ± 23[a]
LRWP	50	9.0 ± 2.3	10.0 ± 2.2	2.6 ± 0.2	1227 ± 121[b]	798 ± 19.9	21.0 ± 3.3	105.6 ± 21.8[b]	535 ± 39[c]
	100	9.2 ± 1.9[c]	9.6 ± 1.4	2.2 ± 0.2	989 ± 78[a]	676 ± 54.6[b]	21.2 ± 1.6	104.2 ± 18.9[b]	584 ± 26[b]
	200	10.1 ± 4.0[b]	9.0 ± 1.5[c]	2.1 ± 0.3	1087 ± 96[b]	612 ± 47.5[b]	18.2 ± 3.0[b]	129.0 ± 17.2	635 ± 17[a]
NTWP	50	9.1 ± 2.0[c]	10.0 ± 1.8	2.3 ± 0.2	1210 ± 129[b]	779 ± 33.6[c]	19.2 ± 2.7[c]	105.2 ± 45.2[b]	609 ± 12[a]
	100	10.0 ± 0.1[b]	9.0 ± 1.6[c]	2.1 ± 0.3	1018 ± 233[b]	639 ± 28.7[b]	17.3 ± 1.2[b]	122.3 ± 38.6	611 ± 28[a]
	200	11.1 ± 1.4[a]	8.5 ± 2.3[b]	2.1 ± 0.4	996 ± 198[a]	599 ± 22.1[a]	16.0 ± 1.1[a]	142.1 ± 18.2[c]	647 ± 37[a]

Values are expressed as mean ± S.D.; $n=6$ in each group, a, b and c represent significant difference from p.s. group.
Glc = glucose, TG = triglyceride, LDH = lactic dehydrogenase, CK = creatine phosphokinase, MDA = malondialdehyde, SOD = superoxide dismutase, GPx = glutathione peroxidase, BUN = blood urea nitrogen, intact group = mice unexposed to the FST and treated with saline, p.s. = mice exposed to the FST and treated with saline.

[a] $P<0.00$.
[b] $P<0.01$.
[c] $P<0.05$.

et al., 2005; Kuo et al., 2009). Since spleen is an immune function-related organ, these results suggested an association between physical lassitude and immunity suppression. Interestingly, supplementation with the polysaccharides however, could lead to recovery of the reduced cell proliferation in chronic fatigue-challenged group. LBWP and NTWP at 200 mg/kg exhibited extremely larger increase than that of intact group.

3.4. Effects of polysaccharides on serum biochemical parameters

In order to clarify its mechanism, blood biochemical parameters were measured in the forced swimming-treated mice. The effects of the polysaccharides in the FST are accompanied by an attenuation of the FST-induced effects on the physiological markers relevant for fatigue. As shown in Table 4, exposure to the forced swim test led to an increase in BUN, TG, CK, LDH and MDA levels in serum in comparison with the intact group. These effects were partially blocked by HRWP, LBWP, LRWP and NTWP, and all polysaccharides at a dose of 200 mg/kg restored these levels to baseline. Importantly, these effects were blocked by LBWP and NTWP, but in lower doses (50 mg/kg) compared with the other two polysaccharides (100 mg/kg). By contrast, exposure to the forced swim test led to a decrease in Glc, SOD and GPx levels. Notably, all these effects were blocked by HRWP, LBWP, LRWP and NTWP, but LBWP in lower dose (100 mg/kg) compared with the other three compounds.

The mechanism of these polysaccharides against fatigue probably included two aspects. One possible explanation is that the treated polysaccharides could involve triglyceride (TG) (or fat) mobilization during exercise, as indicated by the decrease in TG and BUN levels and the simultaneous increase in glucose (Glc) level. As is commonly known, glucose levels are decreased immediately after exercise, and later, non-esterified fatty acids released for circulating glucose (Dorchy, 2006). Such an effect might become advantageous during prolonged exercise, since better utilization of TG allows the sparing of glycogen and Glc and therefore delays fatigue (Jung et al., 2004).

Another possible explanation for the anti-fatigue effect of these polysaccharides is that it occurred probably through protection of corpuscular membrane by prevention of lipid oxidation via modifying several enzyme activities. Fatigue results in the release of reactive oxygen species (ROS) which cause lipid peroxidation of membrane structure. In fatigue conditions, MDA level is increased and is accompanied with a decrease in levels of the antioxidant enzymes SOD and GPx (Lenaz, 1998). These conditions are also marked by the release of LDH and CK into the serum, serving as an indirect index of the damage of membrane structure (Passarella et al., 2008).

Notably, the pharmacological profiles of the four polysaccharides were demonstrated in different doses, these effects were blocked by LBWP and NTWP, but in lower doses compared with the other two polysaccharides.

4. Conclusions

Four water-soluble polysaccharides were isolated from the fruits of four Tibetan plateau indigenous berry plants, including HRWP from *Hippophae rhamnoides*, LBWP from *Lycium barbarum*, LRWP from *Lycium ruthenicum* and NTWP from *Nitraria tangutorum*. Anti-fatigue activities of the four polysaccharides were shown by our results for the first time, by involving triglyceride (TG) (or fat) mobilization during exercise and protecting corpuscular membrane by preventing lipid oxidation via modifying several enzyme activities. Moreover, it is demonstrated that LBWP and NTWP are more potent for anti-fatigue than HRWP and LRWP, which were proposed to be applied in functional foods for anti-fatigue and antioxidant potentially. However, the relationships between immunology and anti-fatigue, structure and anti-fatigue activity of these polysaccharides need to be further discussed.

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References

Amagase, H., Farnsworth, N.R., 2011. A review of botanical characteristics, phytochemistry, clinical relevance in efficacy and safety of *Lycium barbarum* fruit (Goji). Food Research International 44, 1702–1717.
Belluardo, N., Westerblad, H., Mudo, G., Casabona, A., Bruton, J., Caniglia, G., Pastoris, O., Grassi, F., Ibanez, C.F., 2001. Neuromuscular junction disassembly and muscle fatigue in mice lacking neurotrophin-4. Molecular and Cellular Neurosciences 18, 56–67.
Bi, H., Gao, T., Liu, D., Tai, G., Wei, M., Zhou, Y., 2013. Structures of (1→6)-β-D-glucans from *Bulgaria inquinans* (Fries) and their immunological activities. Carbohydrate Polymers 93, 547–552.
Blumenkrantz, N., Asboe Hansen, G., 1973. New method for quantitative determination of uronic acids. Analytical Biochemistry 54, 484–489.
Brown, G.D., Gordon, S., 2001. Immune recognition: a new receptor for β-glucans. Nature 413, 36–37.
Chen, X., Zhang, G., 2011. Scavenging and anti-fatigue activity of Wu-Wei-Zi aqueous extracts. African Journal of Microbiology Research 5, 5933–5940.
Dierma, D., 2012. Crystal Pearl of Materia Medica. Shanghai Scientific and Technical Publishers, Shanghai.
Ding, J.F., Li, Y.Y., Xu, J.J., Su, X.R., Gao, X.A., Yue, F.P., 2011. Study on effect of jellyfish collagen hydrolysate on anti-fatigue and anti-oxidation. Food Hydrocolloids 25, 1350–1353.
Dorchy, H., 2006. Rational use of insulin analogues in the treatment of type I diabetic children and adolescents: personal experience. Archives De Pediatrie 13, 1275–1282.
Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Analytical Chemistry 28, 350–356.
Dubovik, B.V., Bogomazov, S.D., 1987. Multifactorial method for assessing the physical work capacity of mice. Farmakol Toksikol 50, 116–121.
Gao, T., Ma, S., Song, J., Bi, H., Tao, Y., 2011. Antioxidant and immunological activities of water-soluble polysaccharides from *Aconitum kusnezoffii* Reichb. International Journal of Biological Macromolecules 49, 580–586.
Gur, A., Cohen, A., Bravdo, B.A., 1969. Colorimetric method for starch determination. Journal of Agricultural and Food Chemistry 17, 347–351.
Honda, S., Akao, E., Suzuki, S., Okuda, M., Kakehi, K., Nakamura, J., 1989. High-performance liquid chromatography of reducing carbohydrates as strongly ultraviolet-absorbing and electrochemically sensitive 1-phenyl-3-methyl-5-pyrazolone derivatives. Analytical Biochemistry 180, 351–357.
Hu, J.H., Yan, F., Zhang, Z.Y., Lin, J.Y., 2012. Evaluation of antioxidant and anti-fatigue activities of *Ganoderma lucidum* polysaccharides. Journal of Animal and Veterinary Advances 11, 4040–4044.
Huang, L.Z., Huang, B.K., Ye, Q., 2011. Bioactivity-guided fractionation for anti-fatigue property of *Acanthopanax senticosus*. Journal of Ethnopharmacology 133, 213–219.
Jin, M.L., Huang, Q.S., Zhao, K., Shang, P., 2013. Biological activities and potential health benefit effects of polysaccharides isolated from *Lycium barbarum* L. International Journal of Biological Macromolecules 54, 16–23.
Jung, K., Kim, I.H., Han, D., 2004. Effect of medicinal plant extracts on forced swimming capacity in mice. Journal of Ethnopharmacology 93, 75–81.
Kanayama, Y., Kato, K., Stobdan, T., Galitsyn, G.G., Kochetov, A.V., Kanahama, K., 2012. Research progress on the medicinal and nutritional properties of sea buckthorn (*Hippophae rhamnoides*) – a review. Journal of Horticultural Science & Biotechnology 87, 203–210.
Kim, K.M., Yu, K.W., Kang, D.H., Suh, H.J., 2002. Anti-stress and anti-fatigue effect of fermented rice bran. Source. Phytotherapy Research 16, 700–702.
Kuo, Y.H., Tsai, W.J., Loke, S.H., Wu, T.S., Chiou, W.F., 2009. *Astragalus membranaceus* flavonoids (AMF) ameliorate chronic fatigue syndrome induced by food intake restriction plus forced swimming. Journal of Ethnopharmacology 122, 28–34.
Lenaz, G., 1998. Role of mitochondria in oxidative stress and ageing. Biochimica et Biophysica Acta 1366, 53–67.
Liu, C., Lin, Q., Gao, Y., Ye, L., Xing, Y., Xi, T., 2007. Characterization and antitumor activity of a polysaccharide from *Strongylocentrotus nudus* eggs. Carbohydrate Polymers 67, 313–318.
Ma, L., Chablat, D., Bennis, F., Zhang, W., Hu, B., Guillaume, F., 2011. Virtual Reality 15, 55–68.

McNeil, M., Darvill, A.G., Albersheim, P., 1980. Structure of plant cell walls. X. Rhamnogalacturonan I, a structurally complex pectic polysaccharide in the walls of suspension-cultured sycamore cells. Plant Physiology 66, 1128–1134.
Mizoguchi, T., Arakawa, Y., Kobayashi, M., Fujishima, M., 2011. Influence of Chlorella powder intake during swimming stress in mice. Biochemical and Biophysical Research Communications 404, 121–126.
Nair, P.K.R., Melnick, S.J., Ramachandran, R., Escalon, E., Ramachandran, C., 2006. Mechanism of macrophage activation by (1, 4)-α-D-glucan isolated from *Tinospora cordifolia*. International Immunopharmacology 6. (1815-1524).
Ni, W., Zhang, X., Bi, H., Iteku, J., Ji, L., Sun, C., Fang, J., Tai, G., Zhou, Y., Zhao, J., 2010. Preparation of a glucan from the roots of *Rubus crataegifolius* Bge. and its immunological activity. Carbohydrate Research 344, 2512–2518.
Ozturk, N., K., Husnu Can Baser, Aydin, S., Ozturk, Y., Calis, I., 2002. Effects of *Gentiana lutea* ssp symphyandra on the central nervous system in mice. Phytotherapy Research 7, 627–631.
Passarella, S., de Bari, L., Valenti, D., Pizzuto, R., Paventi, G., Atlante, A., 2008. Mitochondria and L-lactate metabolism. FEBS Letters 582, 3569–3576.
Peng, Q., Lv, X.P., Xu, Q.S., Li, Y., Huang, L.J., Du, Y.G., 2012. Isolation and structural characterization of the polysaccharide LRGP1 from *Lycium ruthenicum*. Carbohydrate Polymers 90, 95–101.
Porsolt, R.D., Anton, G., Blavet, N., Jalfre, M., 1978. Behavioral despair in rats: a new model sensitive to antidepressant treatments. European Journal of Pharmacology 47, 379–391.
Schols, H.A., Voragen, A.G.J., 1996. Complex pectins: structure elucidation using enzymes. In: Visser, J., Voragen, A.G.J. (Eds.), Pectins and Pectinases. Elsevier Science BV, Amsterdam, pp. 3–19.
Sedmak, J.J., Grossberg, S.E., 1977. A rapid, sensitive, and versatile assay for protein using coomassie brilliant blue G250. Analytical Biochemistry 79, 544–552.
Sheng, R., Xu, X., Tang, Q., Bian, D., Li, Y., Qian, C., He, X., Gao, X., Pan, R., Wang, C., Luo, Y., Xia, Y., Dai, Y., 2011. Polysaccharide of radix pseudostellariae improves chronic fatigue syndrome induced by poly I:C in mice. Evidence-Based Complementary and Alternative Medicine: eCAM 2011, 840516.
Shin, H.Y., Park, S.J., Seo, S.W., Hong, S.H., Um, J.Y., Lee, S.H., Lee, S.H., Shin, J.Y., Shin, T.Y., Park, Y.S., Yang, D.C., Kim, H.M., 2005. Gamibojungikki-tang decreases immobility time on the forced swimming test and increases interferon-gamma production from MOLT-4 cells. Journal of Ethnopharmacology 102, 113–119.
Suo, Y.R., Wang, L.Y., 2010. Extraction of *Nitraria tangutorum* seed lipid using different extraction methods and analysis of its fatty acids by HPLC fluorescence detection and on-line MS identification. European Journal of Lipid Science and Technology 112, 390–399.
Tan, W., Yu, K.Q., Liu, Y.Y., Ouyang, M.Z., Yan, M.H., Luo, R., Zhao, X.S., 2012. Anti-fatigue activity of polysaccharides extract from *Radix Rehmanniae* Preparata. International Journal of Biological Macromolecules 50, 59–62.
Tanaka, M., Mizuno, K., Fukuda, S., Shigihara, Y., Watanabe, Y., 2008. Relationships between dietary habits and the prevalence of fatigue in medical students. Nutrition 24, 985–989.
Tanaka, M., Nakamura, F., Mizokawa, S., Matsumura, A., Nozaki, S., Watanabe, Y., 2003. Establishment and assessment of a rat model of fatigue. Neuroscience Letters 352, 159–162.
Tharakan, B., Dhanasekaran, M., Brown-Borg, H.M., Manyam, B.V., 2006. *Trichopus zeylanicus* combats fatigue without amphetamine-mimetic activity. Phytotherapy Research 20, 165–168.
Uthayathas, S., Karuppagounder, S.S., Tamer, S.B., Parameshwaran, K., Degim, T., Suppiramaniam, V., Dhanasekaran, M., 2007. Evaluation of neuroprotective and anti-fatigue effects of sildenafil. Life Sciences 81, 988–992.
Wang, H.L., Wang, X.Y., You, J.M., Suo, Y.R., Zhao, X.N., Li, Y.L., Ding, C.X., 2007. Comparative analysis of allantoin quercetin, and 1-methyl-1,2,3,4-tetrahydro-beta-carboline-3-carboxylic acid in *Nitraria tangutorum* Bobr. Seed by HPLC-APCI-MS and CE. Journal of Liquid Chromatography & Related Technologies 30, 363–376.
Wang, J., Li, S., Fan, Y., Chen, Y., Liu, D., Cheng, H., Gao, X., Zhou, Y., 2010. Anti-fatigue activity of the water-soluble polysaccharides isolated from *Panax ginseng* C. A. Meyer. Journal of Ethnopharmacology 130, 421–423.
Zheng, J., Ding, C.X., Wang, L.S., Li, G.L., Shi, J.Y., Li, H., Wang, H.L., Suo, Y.R., 2011. Anthocyanins composition and antioxidant activity of wild *Lycium ruthenicum* Murr. from Qinghai-Tibet Plateau. Food Chemistry 126, 859–865.