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Original Research

The anti-fatigue potential of water-soluble polysaccharides of *Semen cassiae* on BALB/c mice

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Abstract: Fatigue syndrome is a major health problem that affects the voluntary activities of an individual. Particularly, exercise-induced fatigue has become a serious concern in people's health. Since polysaccharides from various medicinal plants have been reported for anti-fatigue effect, the current study deals with the anti-fatigue potential of water-soluble polysaccharides of the Chinese medicinal plant *Semen cassiae* (*Cassia obtusifolia* L.) in BALB/c mice. Water-soluble polysaccharides from *Semen cassiae* were extracted using aqueous solvent (water). An orthogonal test design was employed for the optimization of polysaccharide extraction. The conditions optimized through this design unveiled the raw materials to solvent ratio as 1:30. The optimal temperature and time duration were found to be 80°C and 3.5 h, respectively. The yield of soluble polysaccharides at these specified conditions was 5.42%. Strikingly, the water-soluble polysaccharide from *S. cassiae* exhibited strong anti-fatigue activity at 100 mg/kg in BALB/c mice. *S. cassiae* polysaccharide extended the weight-loaded swimming duration in BALB/c mice. In addition, it ameliorated the level of antioxidant enzymes (SOD, GPX) while decreased the blood urea nitrogen, creatine phosphokinase, triglyceride, lactic acid, lactate dehydrogenase, and malondialdehyde levels in blood serum. Moreover, the assessment of the immunomodulatory effect of *S. cassia* polysaccharides unveiled the enhancement of B-cell and T-cell lymphocytes, denoting the positive effect on physical immunity.

Key words: *S. cassiae*; Water-soluble polysaccharides; Anti-fatigue; Immunomodulatory.

Introduction

The use of herbal/medicinal products over the last three decades has increased enormously due to the side effects of modern medicines. Medicinal plants have diverse chemical compounds with high medicinal values and thus it has been used to treat several diseases. In the current scenario, public interest in herbal medicine has gained attraction and people started ingesting herbal medicines in the form of supplementary diets (also known as nutraceuticals or bioceuticals) and it has been witnessed through the robust increase in the production of natural medicines. The global herbal medicine market is anticipated to reach US$ 550 billion by 2030 from US$ 83 billion (2019) at a compound annual growth rate of 18.9% (1). Rashrash et al., (2) report that the use of herbal medicine has become more prevalent among adults in the United States (US). Residents of the US are mostly devouring herbal supplements for diseases such as cancer, stroke, and arthritis. In addition, it was also stated that people with chronic diseases are largely consuming herbal medicines. People all over the world are favouring natural medicines to get rid of unwanted side effects, to improve health and well-being, to get a safer and effective therapy. In fact, more than 110,000 literatures have been published from 1960 to 2019 describing the benefits of medicinal plants (3).

Fatigue is explained as a lack of energy with tiredness. The individual with fatigue syndrome experiences extreme tiredness, weakness and malaise. It may be physical or mental or a combination of both. The main causes of fatigue are anaemia, thyroid, diabetes, chronic obstructive pulmonary disease, heart disease, and disorders in sleep. Chronic fatigue syndrome (CFS) otherwise known as myalgic encephalomyelitis (ME) is a complicated illness. In this case, the condition is not improved through rest; it severely affects the usual activities of an individual. If the condition persists for longer periods it may lead to serious disability. It occurs commonly in people greater than 40 years old while all age groups are vulnerable to this illness. Women are mostly affected than men. According to information of medicine (IOM), 836,000 to 2.5 million US residents are anticipated to have CFS. The causative agents and diagnosis of CFS is not clearly defined. Also, there is no accepted treatment regimen for CFS (4). The other cause that gives rise to fatigue effect in the modern population is exercise. Excessive and intensive exercise practice can lead to muscle fatigue in humans (5). Several studies are striving to find a promising antifatigue agent for exercise-induced fatigue syndrome (6-8).

Semen cassiae, a dry seed of *Cassia obtusifolia* L. known as 'Juemingzi' in Chinese, is a plant used in traditional folk Chinese medicine for centuries and has been consumed broadly in the regions of China, Korea, Japan, and Southeast Asia. As of now, more than 70

diverse compounds have been identified from *S. cassiae*. Among them, anthraquinones are the predominant phytocompounds. Other notable phytochemicals are naphthopyrones, flavonoids, sterols, aspidinol, campesterol, malvalic acid, mandelic acid, sterculic acid, 5,7-dihydroxychromone, and volatile oil. The list of phytocompounds obtained from *S. cassiae* extracts as well as the pharmacological functions associated with *S. cassiae* phytocompounds were reviewed by Dong et al., (9). Innumerable pharmacological activities have been described from *S. cassia* which includes antidiabetic, antihyperlipidemic, hepatoprotective, neuroprotective, dizziness, headache, antioxidant, anti-inflammation, antibacterial, photophobia, and hypotensive activities (9-15). A recent report evidences the potential role of *S. cassiae* in lung disease (16). Water-soluble polysaccharides from *S. cassiae* have been shown to possess antioxidant properties (17). In addition, it has been shown to act against retinitis pigmentosa, an eye disorder that causes loss of vision (18). Polysaccharides extracted from alcohol have shown to be effective against hyperlipidemia and diabetes-induced oxidative stress in rats (19, 20). Researchers are fascinated by polysaccharides of *S. cassiae* due to their multifarious pharmacological activities. Anti-fatigue properties have been reported from polysaccharides of different medicinal plants of China (21-23). Therefore, the current research aims to assess the anti-fatigue ability of water-soluble polysaccharides extracted from *S. cassiae*.

Materials and Methods

Reagents

All the reagents used in this work were purchased from Sigma-Aldrich, Shanghai. Assay kits (BUN, TG, CK, LA, LDH, MDA, SOD, GPX, and hepatic and muscle glycogen) were procured from Jiancheng Bioengineering Inc. Cell proliferation assay kit (OneSolution Cell Proliferation Assay) were procured from Sigma-Aldrich, Shanghai.

Ethics

All the experiments in BALB/c mice were performed according to the standard principles. The experiments were performed after obtaining ethical approval from the committee of the Institute of Physical Education, Hebei Normal University, Shijiangzhuang Hebei Province.

Animals

BALB/c male mice, around 40-60 days old, were used for the experiments. Animal maintenance was carried out according to the standard guidelines. Standard food and water were provided to the animals and acclimatized to laboratory conditions. Animals were cared for under standard conditions with exposure to 12 h light/dark cycles.

Polysaccharide extraction from *S. cassiae*

To extract the water-soluble polysaccharides from *S. cassiae,* about 2 g of powdered *S. cassiae* was weighed, dissolved in water, and filtered. The resultant filtrate was mixed with a five-fold volume of ethanol to precipitate the polysaccharides. The solution was then maintained for 24 h at 4°C. The resultant polysaccharides after being spun at 10000 rpm for 15 min were thoroughly resuspended in milli-Q water (24). The content of polysaccharide in the aqueous extract was estimated through the phenol-sulphuric acid method. The absorbance of the sample was performed at 481 nm. Percentage of polysaccharide content and yield were calculated according to the following Eq. 1 and 2.

$$\%\ \text{of polysaccharide content (w/w)} = \frac{\text{concentration x sample volume x dilution}}{\text{extract sample weight}} \text{x100\%} \quad [1]$$

$$\%\ \text{of polysaccharide yield (w/w)} = \frac{\text{polysaccharide content x extract total weight}}{\textit{S.cassiae}\ \text{sample weight}} \text{x100\%} \quad [2]$$

Purification of *S. cassiae* water-soluble polysaccharide

Gel column purification technique was employed to purify the polysaccharides. diethylaminoethyl cellulose (DEAE-C) resins were soaked into Milli-Q water. Resins were then soaked in sodium hydroxide solution (0.5 mol/L, 1000 mL) for 30 min and cleansed with sterile and purified water. The process was repeated three times until neutral. The polysaccharides were loaded into the DEAE-C column and left undisturbed. Elution was achieved through the sequential addition of milli-Q and 0.1 M sodium chloride. Around 9 fractions of eluates (0.5 mL in each tube) were collected and labelled as SCPE-1 (*S. cassiae* polysaccharide eluate-1), SCPE-2, SCPE-3, SCPE-4, SCPE-5, SCPE-6, SCPE-7, SCPE-8, and SCPE-9, respectively. The polysaccharide content in the sample was estimated through the phenol-sulfuric acid method.

Experimental design

After acclimatization to laboratory conditions, mice were trained for swimming experiments for one week (10 min). The mice were segregated into three groups. The group-I BALB/c mice was the control (normal mice, fed with normal food and water). The animals in group-II were the fatigue control group, where it is fed with water (negative control). While group-III mice were supplied with polysaccharide extracts (100 mg/kg/day).

Swimming experiments

Shortly after oral administration (1 h) of *S. cassiae* polysaccharide extract through gavage, the animals were allotted to swim in a swimming pool (tank dimension of 50x50x40 cm length x depth x width; water dimension of 30 cm depth and the temperature of the water is 25°C). Mice were recorded for changes in body weight every seven days. The tails of the mice (group-II and III) were tied to a wire, 5% body weight. The mice were allowed to swim until it experiences struggle and become exhausted. When the animals were not able to rise ahead for 5 s, the mice were removed from the tank and allowed to recover. The mice were allowed to perform swimming experiments every day for 4 weeks.

Biochemical analysis

At the end of the fourth week, the mice were allowed to swim and recovered as described above. The blood sample was collected from mice of each group after

anesthetizing with chloral hydrate. Serum from the blood sample was separated after spun at 2800 rpm at 4°C for 10 min and analyzed for blood urea nitrogen (BUN), creatine phosphokinase (CK), triglyceride (TG), lactic acid (LA), and lactate dehydrogenase (LDH). Antioxidant enzymes such as superoxide dismutase (SOD), and glutathione peroxidase (GPX) were analyzed. The lipid peroxidation marker malondialdehyde (MDA) was also evaluated. Glycogen levels in mice liver and muscles were analyzed. Organs (heart, kidney, liver, and spleen) were collected, weighed and reckoned for organ index as Eq. 3.

$$Organ\ index = \frac{organ\ weight\ (mg)}{body\ weight\ (g)} \quad [3]$$

Lymphocyte proliferation assay

Spleens were collected from all the experimental groups after cervical dislocation and immersed in 70% ethanol for disinfection (2 min). The splenocyte suspension was prepared by cutting the spleen into pieces and passed through a cell strainer. The cells were then mixed with red cell lysis buffer for 3 min and spun at 1200 rpm for 3 min. The top supernatant free from red blood cells was collected and further incubated in a 6-well plate to remove the adherent cells. After incubation, the free suspension was collected and diluted to a cell suspension of $5x10^6$ cells/mL using the growth medium RPMI-1640. The suspension was then assessed for cell viability. The assay was carried out in a 96-well plate in which an aliquot of 100 µL cells suspension was seeded. To this, LPS and concanavalin A (ConA) was added, final concentrations were 20 and 2.5 µg/mL, respectively. In each well 20 µL of a cell, proliferation reagent was added and incubated according to the manufacturers' instructions. Absorbance was recorded at 490 nm.

Statistics

Experiments were carried out in triplicate. Values were indicated as mean±SD. Statistical significance was analyzed through t-test and one-way ANOVA (Fisher's LSD multi-comparison method). $P<.05$ were considered significant.

Results and discussion

Orthogonal test and polysaccharide estimation

Three-level orthogonal design, $L_9(3^3)$, was employed to optimize the conditions for polysaccharide extraction from *S. cassiae*. Variables such as raw material to the solvent ratio (A), temperature (B) and duration of time (C) are used for orthogonal test design. The list of variables and levels used in the experiment is charted in Table 1. Table 2 represents the percentage yield of polysaccharides from *S. cassiae*.

K1, K2, and K3 represent the average value of each variable (A, B, and C) at levels 1, 2, and 3, respectively. k1 represents K1/test time at their respective level. R-value represents the range difference of extraction (between maximal k1, k2, k3 and minimal k1, k2, k3). From Table 2, it is clear that A has the maximal R-value of 1.52 and B has the minimal R-value of 0.07. Through

Table 1. Variables used in the orthogonal test design.

Level	Variables		
	Raw material to solvent ratio A	Temperature (°C) B	Duration (h) C
1	1:20	70	2.5
2	1:30	80	3.0
3	1:40	90	3.5

Table 2. Percentage yield of polysaccharides from *S. cassiae.*

Analysis No.	Variables			Polysaccharide yield (%)
	Raw material to solvent ratio A	Temperature (°C) B	Duration (h) C	
1	1	1	1	3.02
2	2	1	2	3.97
3	3	1	3	4.72
4	3	2	1	4.56
5	2	2	3	5.42
6	1	2	2	1.98
7	1	3	3	4.12
8	2	3	1	4.27
9	3	3	2	3.53
K1	9.12	11.75	11.13	
K2	13.53	11.87	9.98	
K3	12.87	11.80	12.27	
k1	3.04	3.92	3.71	
k2	4.51	3.96	3.32	
k3	4.29	3.93	4.09	
R	1.47	0.04	0.77	

orthogonal test design experiments the optimized extraction conditions such as raw material to solvent ratio, temperature and time duration were found to be 1:30, 80°C, and 3.5 h, respectively. Under the $A_2B_2C_3$ condition, the polysaccharide extraction was found to be the maximal with a percentage yield of 5.42. Polysaccharides were estimated using glucose as the standard.

Purification of water-soluble polysaccharides

Eight fractions of eluate 0.5 mL each were collected after loading the crude polysaccharide into the DEAE-C column. The fraction containing tubes were labelled as SCPE1, SCPE2, SCPE3, SCPE4, SCPE5, SCPE6, SCPE7, SCPE8, and SCPE9. Here, SCPE1, SCPE2, SCPE3, SCPE4, SCPE5, and SCPE6 represent the fractions eluted from water (Figure 1) while SCPE7, SCPE8, and SCPE9 represent the fractions eluted from 0.1M sodium chloride (Figure 2). The highest polysaccharide content was found in SCPE1, followed by SCPE2 (Figure 1).

Swimming endurance

In order to evaluate the anti-fatigue potential of water-soluble polysaccharides from *S. cassiae*, the animals were loaded with weight after oral administration of polysaccharides and allowed to swim daily for 4 weeks. Weight-loading has been shown to hasten continuous leg movement (25). Here, the degree of anti-fatigue activity on polysaccharide-treated mice corresponds to the endurance in the swimming test. Remarkably, group-III mice treated with polysaccharides showed longer swimming time (836.5±61.07 s, p<.01) in comparison to group-II mice (507±54.23 s, p<0.05) which received only water, on day 20 (Figure 3). These results denote the strong anti-fatigue activity of *S. cassiae* polysaccharides. The experiments extended for another 10 more days unveiled the significant anti-fatigue effect of polysaccharides. The control group showed decreased swimming time (426±41.03 s, p<0.05), indicating the effect of fatigue on mice. While, polysaccharide treated group showed a striking increase in swimming duration (912±13.58 s, p<0.01), indicating the positive effect of polysaccharides on swimming-induced fatigue syndrome (Figure 3). Many bioactive such as polysaccharide, flavonoid, and the marine nutritious product have been shown to enhance the swimming time in weight-loaded mice (24, 26-30). Chang et al., (31) studied the effect of pulse current on fatigue syndrome triggered by exercises. They reported the endurance ability in rat models as well as significant recovery from fatigue effect. The results suggest that *S. cassiae* polysaccharide may serve as a better anti-fatigue agent in the treatment of exercise-induced fatigue.

Effect of *S. cassiae* polysaccharide on body weight

The body weight of the animal on all groups was weighed every seven days. In all the cases, the body weight was increased in comparison to the body weight of the animal on the first day of the experiment. However, mice administered orally with polysaccharides showed a gradual and slight increase in body weight when compared to the animals from the control groups (Figure 4A). The food intake rate of mice was monitored throughout the experiment. The results revealed a slight increase in the food intake rate of negative control and polysaccharide-treated mice when compared to the control group (Figure 4B).

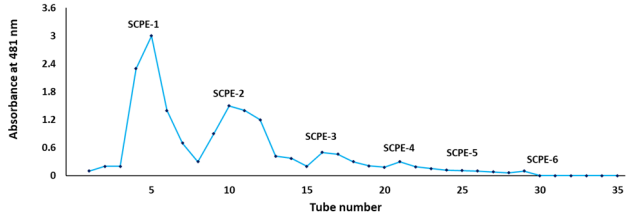

Figure 1. Elution profile of *S. cassiae* polysaccharide fractions on DEAE-C. Elution was achieved through water.

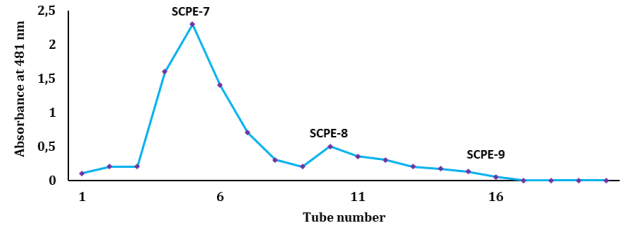

Figure 2. Elution profile of *S. cassiae* polysaccharide fractions on DEAE-C. Elution was achieved through 0.1 M sodium chloride.

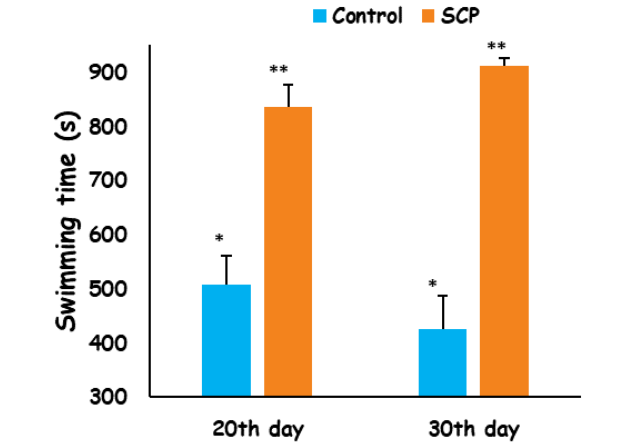

Figure 3. Effect of *S. cassiae* polysaccharide (SCP) on swimming endurance test in BALB/c mice. Control mice fed with distilled water. SCP represents the mice group fed with 100mg/kg of *S. cassiae* polysaccharide. The bar chart represents the experiments performed on the 20th and 30th day and the time taken is given in seconds. Data is analysed through the student's t-test. Values are mean±SD. Asterisk * represents p<0.05; ** represents p<0.01; *** represents p<0.001.

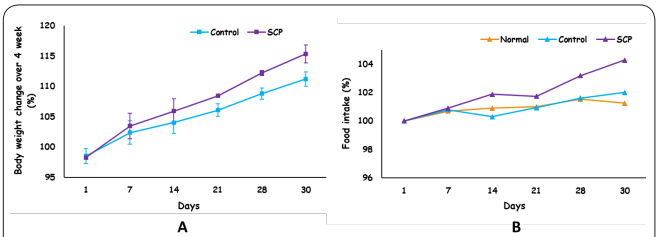

Figure 4. Effect of *S. cassiae* polysaccharide (SCP) on (**A**) Body weight - control group supplied with water; treated group fed with 100mg/kg SCP. Data is analysed through a t-test. Values are mean±SD (**B**) Food intake – consumption levels were monitored every seven day for a period of 4 weeks in normal, control and SCP-treated BALB/c mice. Data is analysed through one-way ANOVA. Values are mean±SD. Asterisk * represents p<.05; ** represents p<.01; *** represents p<.001.

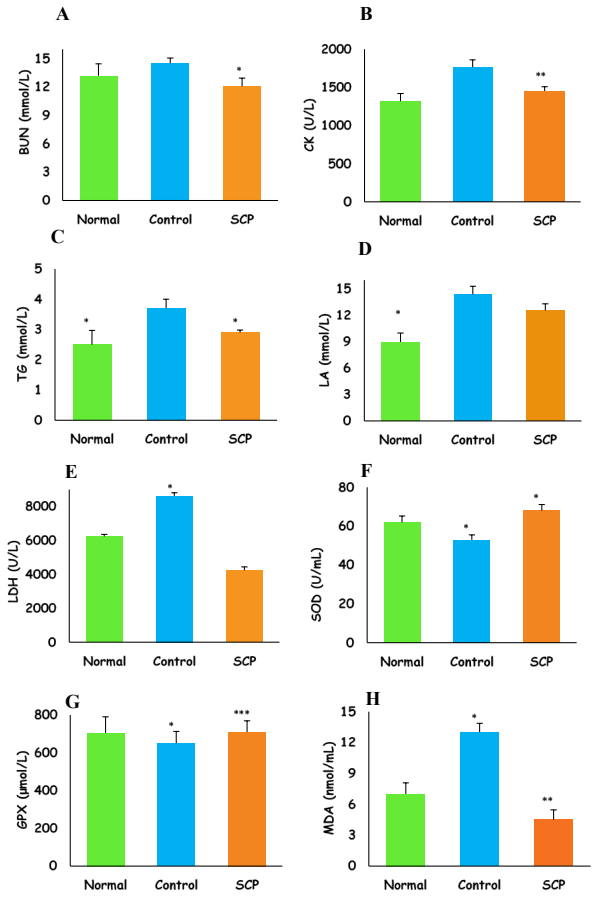

Figure 5. Effect of *S. cassiae* polysaccharide (SCP) on biochemical parameters (**A**) Blood urea nitrogen, BUN (**B**) Creatinine phosphokinase, CP (**C**) Triglycerides, TG (**D**) Lactic acid, LA and (**E**) Lactate dehydrogenase, LDH. Effect of *S. cassiae* SCP on Antioxidants (**F**) Superoxide-dismutase, SOD and (**G**) glutathione peroxidase, GPX. Effect of *S. cassiae* SCP on lipid peroxidation marker (**H**) Malondialdehyde, MDA. Data is analysed through one-way ANOVA. Values are mean±SD. Asterisk * represents p<.05; ** represents p<.01; *** represents p<.001.

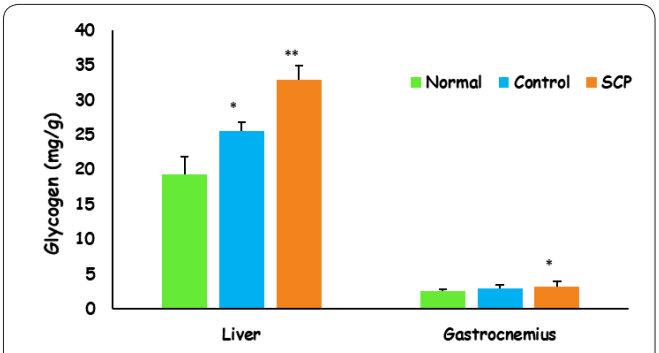

Figure 6. Effect of *S. cassiae* polysaccharide (SCP) on levels of liver and muscle glycogens. Normal, control and SCP treated mice were analysed for glycogen content. Data is analysed through one-way ANOVA. Values are mean±SD. Asterisk * represents p<.05; ** represents p<.01; *** represents p<.001.

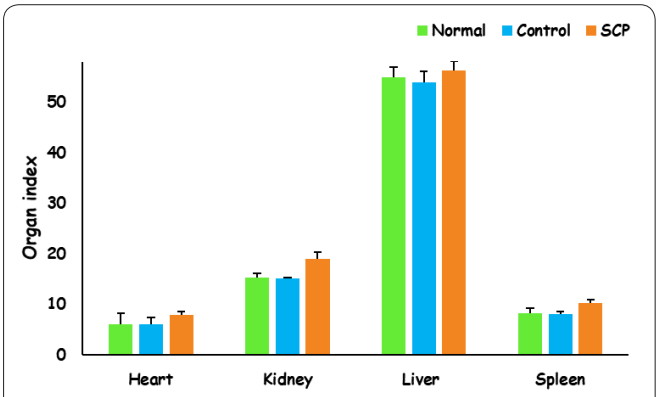

Figure 7. Effect of *S. cassiae* polysaccharide (SCP) on organ-index in BALB/c mice. Organ indexes were calculated for heart, kidney, liver and spleen, from normal, control and SCP-treated BALB/c mice. Data is analysed through one-way ANOVA. Values are mean±SD. Asterisk * represents p<.05; ** represents p<.01; *** represents p<.001.

Biochemical analysis

To confirm the anti-fatigue effect exerted by *S. cassiae* polysaccharide the blood serum levels were evaluated for BUN, CK, TG, LA, LDH, SOD, GPX, and MDA content after the weight-loaded forced swimming experiments. These biochemical parameters are commonly employed for investigating the exercise-induced fatigue effect. In Figure 6A-6F, when compared to group-I normal animals, control animals from group-II showed a significant increase in BUN, CK, TG, LA, LDH, and MDA levels due to weight-loaded force swimming. However, mice orally administered with *S. cassiae* polysaccharide showed a significant decrease in CK, TG, LA, LDH, and MDA levels. Conversely, the antioxidant enzymes such as SOD, GPX were found to decrease in control groups than in normal mice (Figure 6G & 6H). Strikingly, mice supplemented with *S. cassiae* polysaccharide have shown a considerable increase in the levels of antioxidant enzymes (Figure 6G & 6H). Studies on Chinese herbal supplements such as 'Polygonati and Notoginseng Rhizome' and 'B307' were found to exert an anti-fatigue effect by alleviating the blood serum components such as BUN, LA, and MDA. Concomitantly, it reduced oxidative stress by enhancing the antioxidant enzymes in the system (32, 33). Several studies support the role of antioxidant potential in the anti-fatigue effect (34-36).

Effect of *S. cassiae* polysaccharide on glycogen content

To check the effect of *S. cassiae* polysaccharide on hepatic and gastrocnemius muscle glycogen, the levels of glycogens were measured using glycogen assay kits. When compared to normal mice, weight-loaded forced swimming augmented the hepatic glycogen content in control mice. In contrast, gastrocnemius glycogen was found to decrease in control mice. However, upon supplementation with *S. cassiae polysaccharide* the levels of both hepatic glycogen and gastrocnemius glycogen were found to ameliorate further (Figure 7). Similarly, an analysis of the antifatigue effect of polysaccharides extracted from *Portulaca oleracea* in liver and muscle glycogen revealed the augmented level of glycogens (37).

Effect of *S. cassiae* polysaccharide on organ index

Organs (heart, kidney, liver, and spleen) were analyzed to evaluate the effect of *S. cassiae* polysaccharide on the organ index. Mice group orally administered with *S. cassiae* polysaccharide showed modest amelioration in organ index (heart, kidney, liver, and spleen in

comparison to control groups (Figure 5).

Effect of *S. cassiae* polysaccharide on immune cell proliferation

Since polysaccharides have been reported for immunomodulatory activity (Han et al., 2019), a lymphocyte proliferation assay was carried out to assess the potential of *S. cassiae* polysaccharide on immunity. As shown in Figure 8, immune responses were elicited by LPS and conA. Bacterial LPS and conA induces B-cell and T-cell activation, respectively (38, 39). Here in Figure 8, mice treated with polysaccharides exhibited an improved lymphocyte proliferation rate in comparison to control groups. These results evidence the immunomodulatory effect of *S. cassiae* polysaccharides. Similarly, a water-soluble polysaccharide from *Hordeum vulgare* (barley) has been shown to possess immunomodulatory activity (40).

In summary, the study optimized the polysaccharide extract condition by employing an orthogonal test design. In total, three variables were considered. The design revealed the optimal raw material to solvent ratio as 1:30 while the optimal temperature and time duration were found at 80°C, and 3.5 h, respectively. Under these optimal conditions, the polysaccharide yield was 5.42%. Further, the crude polysaccharide obtained from *S. cassiae* was purified through the DEAE-C column. Although *S. cassiae* has been reported for several medicinal properties, the anti-fatigue effect is underdetermined. Therefore, the work was attempted to study the potential of water-soluble polysaccharides on exercise-induced antifatigue in BALB/c mice. Intriguingly, *S. cassiae* polysaccharide showed a marked reduction in BUN, TG, CK, LA, LDH and MDA content. Concurrently, it enhanced the SOD, GPX, and glycogen levels. Besides, the immunomodulatory effect of *S. cassia* polysaccharides was recorded. Though the work lacks an in-depth mechanism, it convincingly emphasizes the promising potential of *S. cassiae* polysaccharides on fatigue syndrome.

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Author contribution

Chenzhe Kang: Writing – original draft, Writing – review & editing, Methodology, Formal Analysis; Aiping Chi: Writing – original draft, Data Analysis; Zilin Zhang: Data analysis, investigation; Yanan Liu: Writing – review & editing, Methodology analysis and investigation.

Conflict of interest

Authors declare no conflict of interest.

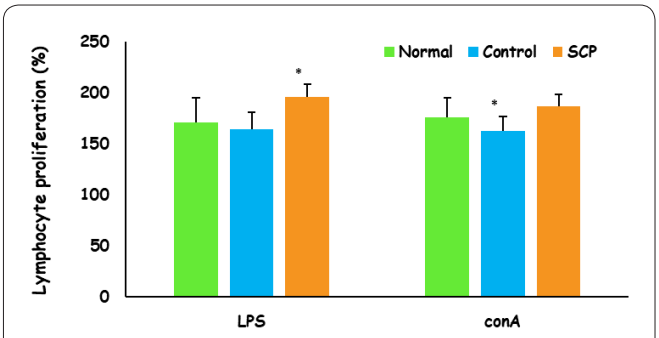

Figure 8. Evaluation of *S. cassiae* polysaccharide (SCP) on lymphocyte proliferation. The lymphocytes from spleens of normal, control and SCP treated mice were examined for LPS and conA-induced lymphocyte proliferation. Data is analysed through one-way ANOVA. Values are mean±SD. Asterisk * represents p<.05; ** represents p<.01; *** represents p<.001.

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