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ORIGINAL ARTICLE

Content Determination and Anti-fatigue Effect of the Purified Anthocyanin from Purple *Daucus carota*

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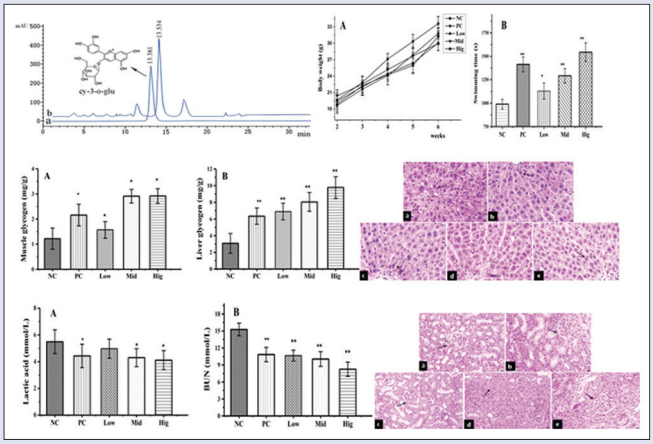
ABSTRACT

Background: The content and anti-fatigue effect of the purified anthocyanin from purple *Daucus carota* (PAPD) were studied. **Materials and Methods:** The content of total anthocyanins in PAPD was determined by the pH trial-difference method, and the content of cyanidin-3-o-glucoside in PAPD was determined by high-performance liquid chromatography (HPLC) method. Hundred ICR mice were randomly divided into five groups with 20 mice in each group: Negative control (NC) group, positive control (PC, red bull drink of 1.0 g/kg BW) group, PAPD dose group of low, middle, high (Low, 0.2 g/kg; Mid, 0.4 g/kg; Hig, 0.8 g/kg). Mice were intragastric injected continuously for 4 weeks; once a day, the NC group received an equal volume of saline, the PC group was given 1.0 g/kg BW red bull drink. After the last administration, the time of weight-bearing exhaustion swimming of 10 mice in each group was determined, the contents of lactic acid and urea nitrogen in the serum and glycogen in the liver and muscle tissues after 30 min of no-weight-bearing swimming of ten mice in each group were determined and the weight of mice during the experiment was also determined and the pathological analysis of the liver and kidney tissues in mice was conducted. **Results:** The content of total anthocyanin in PAPD was determined by the pH-differential method to be 132.46 mg/g DW, which was converted into fresh weight as 1.146 mg/g. The content of cyanidin-3-O-glucoside in PAPD was determined by HPLC, which was 31.45 mg/g DW, accounting for 21.37% of the total anthocyanin. Compared with the NC group, the exhausted swimming time of the PC group and PAPD group was significantly prolonged ($P < 0.05$), the contents of muscle glycogen and liver glycogen in the tissues of mice were significantly increased ($P < 0.05$), the contents of lactic acid and urea nitrogen in the serum of mice were significantly reduced ($P < 0.05$), and the lesions of the liver and kidney tissues were enhanced to some extent. **Conclusion:** The content of anthocyanin is rich in PAPD and has an observable anti-fatigue effect; its potential mechanism could improve the metabolism of sugar and lipid in muscle and liver tissues, reduce the oxidative damage of cells, improve the adaptability and endurance of the body to strenuous exercise, and enhance the immunity and antioxidant ability of cells, to play an anti-fatigue role.

Key words: Anthocyanin, anti-fatigue effect, cyanidin-3-o-glucoside, high performance liquid chromatography, purple *Daucus carota*

SUMMARY

- The content of anthocyanin in the purified anthocyanin from purple *Daucus carota* (PAPD) is rich
- PAPD has obvious anti-fatigue effect.



Abbreviations used: PAPD: The purified anthocyanin from purple *Daucus carota*; PDR: Purple *Daucus carota* L.; NC: Negative control; PC: Positive control; HPLC: High-performance liquid chromatography; Low: PAPD (0.2 g/kg); Mid: PAPD (0.4 g/kg); Hig: PAPD (0.8 mg/kg).

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INTRODUCTION

Purple *Daucus carota* L. root (PDR) is the most popular vegetable, usually orange in color, though purple, red, black, white, and yellow cultivars exist. It is a great source of nutrients such as anthocyanins, Vitamin C, carotene, and a variety of minerals. PDR is a rare food ingredient with the performance of vegetables and medicinal food; it is mild in nature and sweet and spicy in taste. Because it can relieve eye fatigue, prevent hypertension, regulate metabolism, and enhance resistance and has strong antioxidant effect, it is popular among consumers.[1] Anthocyanins are colored water-soluble natural pigments.[2] Anthocyanins are typical polyphenolic flavonoids. A variety of anthocyanins are formed due to different types, quantities and positions of substituents connected on aromatic rings.[3]

Purple food containing anthocyanins not only can cause their own bright colors, but also they have many biological activities, due to their specific structure and chemical composition, such as strong antioxidant, anti-inflammatory and bacteriostatic, liver and vision protection, an

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anti-aging and anticancer effects.[4-6] With the development of agriculture and biotechnology, the research on the breeding, development, and utilization of purple and black plants has attracted attention, and a diet rich in fruits, vegetables, tea, whole grains, and other plants in anthocyanins has appeared on the market,[7] and these foods of fruits, vegetables, grains, and other plants are directly related to our lives. Fatigue is a general physiological and pathological phenomenon of the body caused by long period of heavy, intense physical, or mental work under definite environmental condition. It mainly includes mental and sports fatigue. Fatigue is accompanied by decreased immunity and endocrine imbalance of the body, which can cause a variety of diseases, such as diabetes, kidney disease, hepatitis, and tumor.[8] At present, although many drugs can relieve fatigue, they also impede the overall function of the body. A large number of studies have also shown that the active components of flavonoids in natural plants have anti-aging and antioxidation effects and can improve the body function without toxic side effects.[9-11] Therefore, the search for natural plants and their active ingredients can not only eliminate fatigue, but also regulate the immune function of the body, which is a research hotspot in the field of exercise physiology and sports medicine. In this article, the purified anthocyanin from purple *Daucus carota* (PAPD) was extracted from the PDR and its anthocyanin content was analyzed. At the same time, anti-fatigue experiments were conducted, and the anti-fatigue effect and mechanism of PAPD were discussed preliminary.

MATERIALS AND METHODS

Instrument and materials

Instruments most commonly used in this study were High-Performance Liquid Chromatography (HPLC) instrument (Waters 2695 HPLC, Milford, MA, USA); ultraviolet (UV)-2102 PCS UV-Visible Spectrophotometer (Shanghai Unik Instrument Co., Ltd.); Infinite M200 Microplate Reader (Tecan, Switzerland); Optical Microscope (BX20, Olympus, Tokyo, Japan); LG10-2.4A High Speed Centrifuge (Beijing Medical Centrifuge Factory, China); KQ-250B Ultrasonic Cleaner (Kunshan Ultrasonic Instrument Co., Ltd., China); Concentration Device of Flash Distillation (Independent Research and Development);[12] R201B Rotary Evaporator (Shanghai Shensheng Biotechnology Co., Ltd., China); Diaion HP2 MGLMacroporous Adsorbent Resin (Mitsubishi Corporation, Japan).

PDR root, also known as black ginseng, was harvested from agricultural production base in Liaocheng, Shandong Province, China, in May 2018. Clean grade ICR male mice (18–22 g) were provided by the Experimental Animal Center of Zhejiang Academy of Medical Sciences, and the number of production licenses of the experimental animal was SCXK 2015-0033. Red bull beverage was obtained from Red Bull Vitamin Beverage Co., Ltd., China; liver glycogen, muscle glycogen, urea nitrogen, lactic acid kit was obtained from Nanjing Jiancheng Technology Co., Ltd., China.

Preparation of the purified anthocyanin from purple *Daucus carota*

According to the properties of anthocyanins which are unstable and easy degradation when exposed to light and heat, in this study, the methods of crushing extraction and ultrasonic extraction were used to complete the extraction of PDR. Using the optimized extraction method, the fresh PDR was accurately weighed and was extracted by the crushing method for 1.5 min with 50% ethanol of pH = 3.0 ± 0.1 according to the 1:6 (W: V) ratio of the plant material to extracting solution, filter by reducing pressure. The filter residue was added to 50% ethanol of pH = 3.0 ± 0.1 according to 1:3 (W: V) ratio of plant material to extracting solution for ultrasonic extraction for 30 min and then filtered. The obtained filtrate was combined and concentrated at 40°C by the concentration device of flash distillation until the concentrate was alcohol-free. The concentrate was loaded into Diaion HP 2MGL macroporous resin column for enrichment and purification of anthocyanin. First, after the sample was added, column chromatographic elution was performed with deionized water until the elution solution is clarified, then 60% ethanol of pH = 3.0 ± 0.1 was used for elution, and elution solution of the purplish red ribbon was collected and vacuum-concentrated to dry powder at 40°C by rotary evaporator. PAPD was obtained, weighed, and placed in a low temperature and dry place for reserve.

Content determination of the purified anthocyanin from purple *Daucus carota*

Total anthocyanins in PAPD were determined by the pH-differential method. Take PAPD of 5.45 mg, dissolve it with 10% methanol, and then transfer it to buffer solution of pH = 1 and pH = 4.5, respectively. Place it in the dark for 1 h, and then determine its absorbance value.[13] HPLC method was used to determine the content of cyanidin-3-O-glucoside in PAPD. The standard sample of cyanidin-3-O-glucoside was dissolved in a 10% methanol solution and configured into the standard solution with a particular concentration gradient. The standard curve was made by taking the concentration as the abscissa axis and the peak area value as the vertical axis, y = 16344.3x + 2134.9, R^2 = 0.9976. HPLC chromatographic condition: [14] Sample analysis was performed with SunFire-C_{18} Column (4.6 mm × 250 mm, 5 μm). The mobile phase is 2% hydrochloric acid methanol (a) and water/methanol/acetonitrile/acetic acid at a ratio of 160:90:90:40 (b) with gradient elution. 0–3 min, A: 93%, 3–6 min, A: 91%, 6–30 min, A: 70% at a flow rate of 1.0 mL/min, detection wavelength was set at 520 nm. The measurements were performed in triplicate. All samples were filtered through a 0.45-μm Millipore membrane filter before they were injected into HPLC.

Animal experiments

All experimental animals were kept in standard animal houses with ambient temperature of 23 ± 1°C and relative humidity of 50% ±5% for 1 week. Mice were randomly divided into five groups with 20 mice in each group: Negative control (NC) group, positive control (PC, red bull drink of 1.0 g/kg BW) group, PAPD dose group of low, middle, high (Low, 0.2 g/kg; Mid, 0.4 g/kg; Hig, 0.8 g/kg). Mice were intragastric injected continuously for 4 weeks, once a day; the NC group received an equal volume of saline, while the PC group was given 1.0 g/kg BW red bull drink. The mice were weighed every 5 days during the experiments. All procedures for animal experiment were in accordance with the guidelines of Chinese animal care, which conform to the international acceptance of the use of animals in experiment.

Weight-loading swimming test

Taking 5 mice in each group, only after the last Ig administration for 30 min, put the mice in swimming box (30 cm × 40 cm × 50 cm) at a water temperature of 25°C. The tail of the mice was loaded with lead sheath equivalent to 5% of the body weight of the mice; the time from the mice began to swim to the they submerged under water for 5 s and did not come out from the water was recorded, that is the exhausted swimming time of the mice.

Content determination of serum index and glycogen

Five mice were taken from each group. After the last dose, they were allowed to sit for 30 minutes and then swimming in a swimming tank with water temperature of 25°C without heavy load. The blood was collected from the eyeballs of mice. The contents of urea nitrogen and lactic acid in the serum of mice were determined according to the kit

instructions after the separation of serum. At the same time, the mice were also killed through cervical dislocation and the liver and hind leg muscles of the mice were quickly removed and washed with normal saline. After that, the contents of glycogen in the liver and muscle of the mice were measured.

Histopathological analysis

Liver and bilateral kidney tissues of the mice performed by cervical dislocation were removed, respectively, and fixed in 4% neutral formaldehyde. After 24 h of immersion, they were embedded in paraffin. Slices of 5 μm were cut and stained with hematoxylin and eosin staining (H and E).

Statistical analysis

All data are expressed as mean ± standard deviation and were analyzed using the SPSS statistical software (SPSS 19.0 Inc., Chicago, IL, USA). One-way analysis of variance with Duncan's test was used for intergroup comparison. A $P < 0.05$ was considered as statistically significant and a $P < 0.01$ was considered as extremely statistically significant.

RESULTS AND DISCUSSION

Determination result of anthocyanin content in the purified anthocyanin from purple *Daucus carota*

The content of total anthocyanin in PAPD was determined by the pH-differential method to be 132.46 mg/g DW, which was converted into fresh weight as 1.146 mg/g. The content of cyanidin-3-O-glucoside in PAPD was determined by HPLC [Figure 1], which was 31.45 mg/g DW, accounting for 21.37% of the total anthocyanin. The results presented that the content of anthocyanin in PAPD was plentiful.

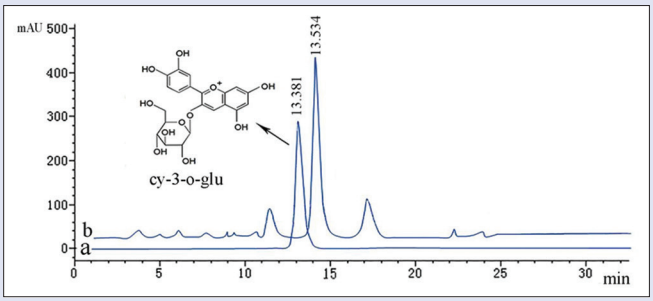

Figure 1: High-performance liquid chromatogram of reference substance (a) and sample (b). a: Reference substance of cyaniding-3-O-glucose; b: Sample of PAPD

Effects of the purified anthocyanin from purple *Daucus carota* on body mass and weight-bearing swimming time in mice

Fatigue is the most direct and objective decay in athletic endurance, resulting from physical labor or exercise. On the one hand, energy depletion causes the body to produce inhibitive protection; on the other hand, it makes the accumulation of metabolites in the body, leading to the alteration of the internal environment of the body and the deterioration of endocrine regulation function.[15,16] Exhaustion swimming time reflects the body's exercise endurance, and whether the swimming time is long or short is the most intuitive indicator of the fatigue degree of mice.

During the experiment, the body mass of mice was weighed weekly to detect the effect of PAPD on the body mass of mice. Compared with the NC, the body mass of mice in the PC and the PAPD group gradually increased over time, with a significant difference ($P > 0.05$) [Figure 2a]. Compared with the NC, both the PC and the PAPD group had significantly longer weight-bearing swimming time, with expressively difference ($P < 0.05$), especially in the high-dose group. The exhaustion swimming time of the weight-bearing mice could be significantly extended [Figure 2b]. It shows that PAPD can improve the body's exercise endurance, prolong the swimming time of weight-bearing mice, and improve the fatigue caused by swimming of weight-bearing mice by adaptable environment inside the body.

Effects of the purified anthocyanin from purple *Daucus carota* on liver glycogen and muscle glycogen content in mice

Fatigue could induce lipid peroxidation, leading to abnormal glucose and lipid metabolism in the microenvironment. The cause of exercise fatigue is due to the consumption of physical energy and cannot be supplemented, leading to energy exhaustion and fatigue. Exercise endurance is positively correlated with the storage of muscle glycogen, while liver glycogen is the key to maintain blood glucose levels during exercise.[17] Prolonged exercise lowers muscle glycogen levels, and to maintain blood sugar levels, liver glycogen decreases accordingly. Therefore, increasing the storage of glycogen can delay the occurrence of exercise fatigue.[18-20] Compared with the NC group, both the PC and PAPD groups can significantly increase the storage of liver glycogen in mice ($P < 0.01$) and increase the content of muscle glycogen ($P < 0.05$). It indicated that PAPD could significantly increase the content of liver glycogen and muscle glycogen of mice and delay the generation of exercise fatigue in mice [Figure 3].

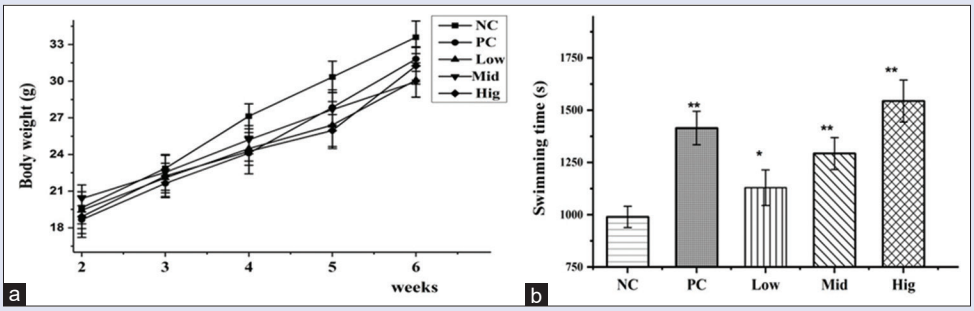

Figure 2: Effect of PAPD on body weight (a) and exhaustion swimming time (b) in mice. NC, negative control group; PC, positive control group; Low, PAPD (0.2 g/kg); Mid, PAPD (0.4 g/kg); Hig, PAPD (0.8 mg/kg). The data were expressed as mean ± standard deviation ($n = 10$), *$P < 0.05$, **$P < 0.01$ versus NC group

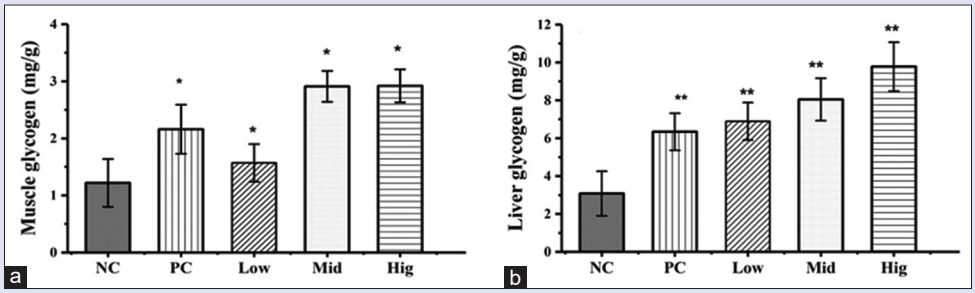

Figure 3: Effect of PAPD on content of muscle glycogen (a) and liver glycogen (b) in mice. NC, negative control group; PC, positive control group; Low, PAPD (0.2 g/kg); Mid, PAPD (0.4 g/kg); Hig, PAPD (0.8 mg/kg). The data were expressed as mean ± standard deviation ($n = 10$), *$P < 0.05$, **$P < 0.01$ versus NC group

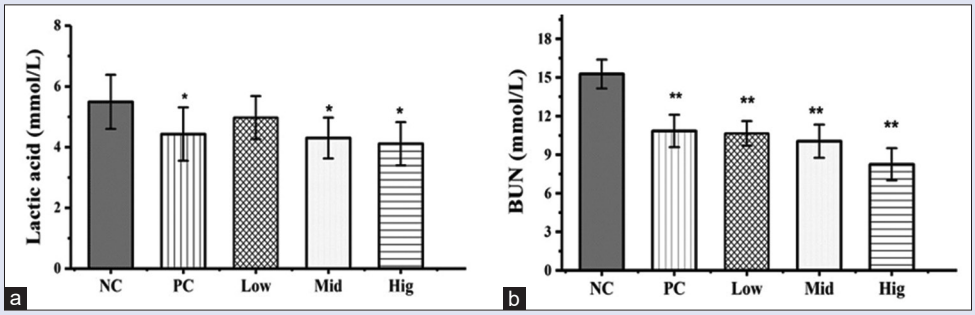

Figure 4: Effect of PAPD on the content of lactic acid (a) and urea nitrogen (b) in mice. NC, normal control group; PC, positive control group; Low, PAPD (0.2 g/kg); Mid, PAPD (0.4 g/kg); Hig, PAPD (0.8 mg/kg). The data were expressed as mean ± standard deviation ($n = 10$), *$P < 0.05$, **$P < 0.01$ versus NC group

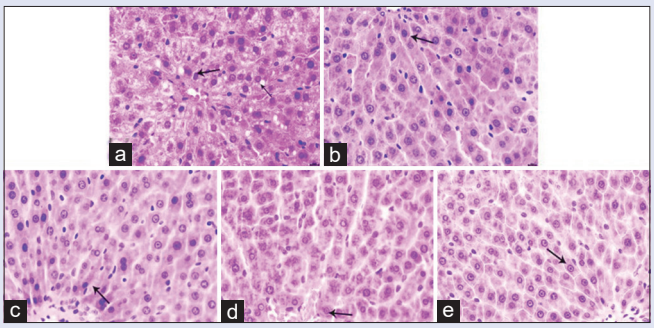

Figure 5: The effects of PAPD on the histopathological changes in the liver of mice. histological observation (a-f, H and E, ×400). (a) Normal control group; (b) positive control group; (c) PAPD (0.2 g/kg); (d) PAPD (0.4 g/kg); (e) PAPD (0.8 mg/kg); hepatocyte, →; vacuole, →

Effects of the purified anthocyanin from purple *Daucus carota* on serum lactic acid and urea nitrogen content in mice

Strenuous exercise of the body can lead to insufficient blood flow to some extent. The enhancement of the oxidation effect in the body can produce a large number of free radicals, leading to increased lipid peroxides in the muscle and liver and causing cell or tissue damage. As a result, the large amounts of lactic acid produced increase the concentration of hydrogen ions entering the muscle, lowering the pH value and causing a series of biochemical reactions that eventually lead to fatigue.[21,22]

Serum urea nitrogen is the metabolite of protein and amino acid. The change of its content can explain the decomposition and metabolism of nitrogen-containing substances in the body, and it is a sensitive index to assess fatigue. The worse the adaptability of the body to physical exercise load, the higher the content of blood urea nitrogen.[23] The results of Figure 4a showed that, compared with the NC group, both the PC and the high-dose groups of PAPD could significantly reduce the content of serum lactic acid ($P < 0.05$). Hence, PAPD could remove free radicals in the body, boost cellular immunity, slow down or reduce the production of lactic acid during exercise, and thereby reduce fatigue.

The results of Figure 4b showed that, compared with the NC group, the PC group and PAPD group could significantly reduce the content of serum urea nitrogen ($P < 0.01$), even more significantly than the PC group. The results showed that PAPD could suggestively reduce the content of serum urea nitrogen in mice. It diminishes the excessive oxidation and decomposition of proteins during strenuous exercise, slowing down the decomposition speed of nitrogen-containing substances in the mice body, and reduces the oxidative damage of cells, thus improving the body's adaptability to strenuous exercise, increasing endurance, and improving fatigue.

Effects of the purified anthocyanin from purple *Daucus carota* on histopathological changes in liver and kidneys tissue

Strenuous exercise can lead to a relative insufficient muscle blood flow, which quickens the fermentation of sugar and strengthens the oxidation effect in the body. It also produces a large number of free radicals, which leads to the increase of lipid peroxide in the muscle and liver and thus causes the damage of cells or tissues.[24] Fatty lesions appeared in the liver tissues of mice in the NC group and vacuoles, and a small amount of necrosis appeared in the liver cells of mice in the NC group [Figure 5]. Compared with the NC group, liver tissues of mice in the PC and PAPD group were all normal.

An essential factor of fatigue is the increase of free radicals. Fatigue can increase the production of free radicals and enhance the lipid peroxidation reaction, while the lipid peroxides and free radicals will

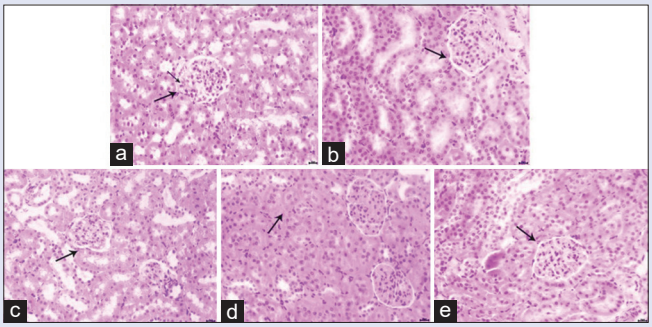

Figure 6: The effects of PAPD on the histopathological changes in the kidney of mice. Histological observation (a-f, H and E, ×200). (a) Normal control group; (b) positive control group; (c) PAPD (0.2 g/kg); (d) PAPD (0.4 g/kg); (e) PAPD (0.8 mg/kg); glomerulus, →; nephrocyte, →

promote hypertrophy of renal cells.[25] The glomerular cavity of the mice in the NC group was dilated and the boundary was blurred [Figure 6]. Compared with the NC group, the kidney tissues of the mice in the PC and the PAPD group were normal.

CONCLUSION

The content of anthocyanin constituent is rich in PAPD and has an observable anti-fatigue effect. Its potential mechanism may help to improve the metabolism of sugars and lipids in muscle and liver tissues, reduce oxidative damage to cells, improve the body's adaptability and endurance, adapt to intense load exercise, and improve the immunity and antioxidant capacity of cells, thus improving the anti-fatigue effect.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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