

Antifatigue Effects of Ethanol Extracts and Polysaccharides Isolated from *Abelmoschus esculentus*

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ABSTRACT

Background: The aim of this study is to determine the antifatigue active fraction from *Abelmoschus esculentus*. The *in vivo* antifatigue effects of ethanol extracts and polysaccharides from *A. esculentus* fruit have been determined. The polysaccharides of *A. esculentus* were determined as the best effective fractions of antifatigue effects. **Materials and Methods:** About 360 Kunming male mice were randomly divided into nine subgroups: normal control subgroup, model subgroup, positive subgroup and the ethanol extracts of *A. esculentus* with high dose (3.2 g/kg) subgroup, medium dose (1.6 g/kg) subgroup and low dose (0.8 g/kg) subgroup, the polysaccharides of high dose (3.2 g/kg) subgroup, medium dose (1.6 g/kg) subgroup, and the low dose (0.8 g/kg) subgroup. The antifatigue effects of ethanol extracts and polysaccharides from *A. esculentus* were measured by comparing body weight, food intake, swimming time, liver glycogen, serum urea, blood lactic acid as well as visceral parameter in mice.

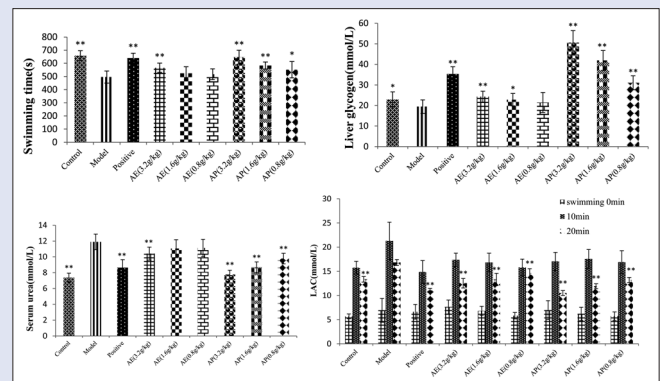
Results: Compared with the model subgroup, other subgroups significantly prolonged swimming time, and high dose polysaccharides administration was the most effective ($P < 0.01$). High dose polysaccharides significantly increased liver glycogen, serum lactic acid, and serum urea ($P < 0.01$) in mice. In contrast with model group, the high dose polysaccharides administration could also significantly elevated the parameters of testicles and epididymis ($P < 0.01$). The study established that the ethanol extracts and polysaccharides of *A. esculentus* both have antifatigue effects. **Conclusions:** The results demonstrated that both the ethanol extracts and polysaccharides of *A. esculentus* have antifatigue effects. The high dosage polysaccharides have significant antifatigue properties. The results will provide the basis for further development and utilization of this plant.

Key words: *Abelmoschus esculentus*, antifatigue effects, ethanol extracts, polysaccharides

SUMMARY

- The high dosage polysaccharides have restoration ability on kidney yang deficiency mice.
- The high dosage polysaccharides have significant effects of relieving body fatigue of mice.

- The polysaccharide of *Abelmoschus esculentus* showed better antifatigue effects than the ethanol extracts.



Abbreviations used: *A. esculentus*: *Abelmoschus esculentus*, BUN: Blood urine nitrogen, LD: Lactic Acid dehydrogenase, AE: *Abelmoschus esculentus* ethanol extracts, AP: *Abelmoschus esculentus* polysaccharides, LAC: Lactic acid content.

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INTRODUCTION

Abelmoschus esculentus (L.) Moench is an annual herbal plant, belonging to the okra genus, Malvaceae family, with different names of sword weed and kidney tonifying herb, etc., *A. esculentus* is suitable to be planted in tropic and sub-tropic regions, and therefore, it is widely seen in both South and North parts of China. *A. esculentus* contains rich functional components and nutrients. Therefore, it is designated by many countries as the first choice of the health food for the old people and the athletes. It is nicknamed as the "plant Viagra" and "green ginseng," and it is called a new health vegetable with a relatively high nutritional value.^[1-4]

Through literature retrieval, *A. esculentus* is rich in functional constituents such as flavonoids,^[5] polysaccharides, pectin of fruit, trace elements, and amino acid. It also has the medical effects of antifatigue, antiageing, immune strengthening, liver protecting, blood sugar

reducing, eyesight improving, internal fever removing, digestion helping, stomach invigorating, intestine lubricating, kidney strengthening, blood lipid reducing, etc. We can enhance its added value and its utilization ratio if we make deep processing and development for this edible

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and medicinal plant. From the literature retrieval, *A. esculentus* is greatly appreciated in strengthening human endurance and improving motorsports function. There have been some researching reports that the water extracts of *A. esculentus* can obviously improve the body endurance, anoxia-resistance, cold-resistance, and heat-resistance of the experiment mice,^[6] which shows that the water extracts of *A. esculentus* have certain antifatigue activity. However, there have been no reports on the experiment research of establishing kidney-yang deficiency model of the mice and adopting multi-index method to study the antifatigue activity of the different fractions of the *A. esculentus*.

Our research focuses on the antifatigue effects of ethanol extracts and polysaccharides of the *A. esculentus* fruit in kidney yang deficiency mice. Through comparing a series of parameters changed *in vivo* of mice, such as weight, food intake, swimming time and liver glycogen, the antifatigue effects of the ethanol extracts and polysaccharides of *A. esculentus* fruit in mice was determined. Furthermore, the polysaccharides of *A. esculentus* fruits were determined as the best fractions of effective antifatigue. These results will provide the basis for *A. esculentus* further development and utilization.

MATERIALS AND METHODS

Reagents and instruments

Infinite M 200 Microplate Reader (Swiss Tecan), ultraviolet (UV)-2102 PCS UV and Visible Spectrophotometer (Shanghai Unico Instrument Corp., Ltd.); KQ-250B Supersonic Cleaner (Kunshan Supersonic Instrument Corp., Ltd.); R201B Rotary Evaporator (Shanghai Shensheng Biotechnology Corp., Ltd.); LG10-2.4A High Speed Centrifuge (Beijing Medical Centrifuge Factory); HH-2 Digital Thermostat Water Bath (Shanghai Changsi Industry and trade Corp., Ltd.); Vacuum thin film concentration device (self-installation).

All the kits including glycogen, blood urea nitrogen (BUN), lactic acid dehydrogenase (LD) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Dexamethasone sodium phosphate injection (Hubei, China), JA31001 Analytical Balance (Shanghai, China), Swimming Tank (specifications: 40 cm × 40 cm × 50 cm).

Extraction and preparation of the samples

Take the fresh fruit 15 kg of *A. esculentus*, and use 2 times amount 70% ethanol to cold-soak 4 times, each time 8 h,^[7] combine the extracted liquid, and use the vacuum thin film concentration device to make the pressure-reducing concentration. The resulting material was vacuum dried at low temperature to powder; we will get the ethanol extracts of *A. esculentus* of yield 275 g. Total flavonols content was determined by NaNO₂-Al(NO₃)₃ colorimetric method. The absorbance was measured at 510 nm with UV-2102 PCS spectrophotometer. Total flavonols content of the ethanol extracts of *A. esculentus* was calculated as 312.51 mg/g using the calibration curve of rutin standard solution as contrast.

Take the fresh fruit 15 kg of *A. esculentus*, and use 3 times of the amount of water as the solvent to make cooking extracting for 3 times, each time 1 h. Combine the extracted liquids and use the vacuum thin film concentration device to make the pressure-reducing concentration. Add ethanol into the concentration and mix it until its ethanol content reaches 80% when there is a large amount of deposition, and then transfer it into the refrigerator for the night. After the sediment comes out completely, remove the upper clear liquid, and we will get the sediment and dry it into powder by vacuum low-temperature drying method, and this is just the polysaccharides of *A. esculentus* of 306 g. Total polysaccharides content was determined by C₆H₅OH-H₂SO₄ colorimetric method. The absorbance was measured at 490 nm with UV-2102 PCS spectrophotometer. Total polysaccharides content of *A. esculentus* was calculated as 536.32 mg/g using the calibration curve of glucose standard solution as contrast.

Animals and treatment

About 360 male Kunming mice weight 18–22 g were provided by the experimental animal center, Zhejiang Academy of Medical Science, China. Animals were allowed to adapt to their surroundings for 1 week before starting the experiments. Mice were at room temperature (23°C ± 1°C) and moderate humidity (50 ± 5) %, with a 12/12-h light – dark cycle. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals (National Institute of health) and approved by the Committee on the Ethics of Animal Experiments at Zhejiang Agriculture and Forestry University. The mice were sacrificed by decapitation under anesthesia (i.p., chloral hydrate), and the blood was collected and centrifuged.

The mice were randomly divided into four groups (*n* = 90), and each group was used for the loaded swimming test, hepatic glycogen test, serum urea test, and the lactic acid of blood test, respectively. Each large group was randomly divided into nine subgroups: Normal control subgroup, model subgroup, positive subgroup and the ethanol extracts of *A. esculentus* fruit with high dose (3.2 g/kg) subgroup, medium dose (1.6 g/kg) subgroup and low dose (0.8 g/kg) subgroup, the polysaccharides of high dose (3.2 g/kg) subgroup, medium dose (1.6 g/kg) subgroup, the low dose (0.8 g/kg) subgroup, with 10 mice each. All mice were given dexamethasone sodium phosphate injection 0.5 mg by intramuscular injection for 7 days, except for control subgroup. Samples were administered by the oral gavage once a day according to the dose design for 2 weeks.

The experiment of the mouse loaded-swimming

First, train the mice for 2 times before the official swimming begins, and all the mice are forced to swim in the swimming-tank after 30 min of the last medicine-administration,^[8] with the water depth of 40 cm, temperature of 25°C, the mouse tail part loaded with lead sheet of 5% of the mouse body weight. Record the time from the beginning of the swimming to the time when their heads sank completely into water and regard this time as the final exhaustion swimming time (s).^[9] If the swimming time in the medicine-giving group is obviously longer than those in the model group, and the difference is evident (*P* > 0.05), it then can be judged that this medicine has the function of lengthening the mouse exhaustion swimming time.

The determination of hepatic glycogen

Blood glucose is the main energy source of the muscular activity. The long, tense activity and its ensuing physical exhaustion always synchronize with the exhaustion of the muscular glycogen, and the storage volume of glycogen can directly affect the sports ability of the organs.^[10] Therefore, the glycogen content can influence the how soon the fatigue will arise. The measuring principle of hepatic glycogen: Under the action of the concentrated sulfuric acid, the glycogen will dehydrate to produce furfural derivatives, and the latter will again be combined with anthrone to become blue compound, and make a colorimetric analysis for the standard glucose solution, which is treated with the same method. The glycogen is very stable in the concentrated sulfuric acid; so before the color development, we should first put the tissues into the concentrated alkali for heating in order to destroy the other ingredients and retain the glycogen.

After 30 min of the last medicine-administration, put the mice into the swimming tank (water temperature is 25°C) to make unloaded swimming for 90 min, each time with the constant temperature of 25°C before going into water. After swimming, strip off the cervical spine and take out the liver,^[11] weigh out liver of 100 mg, and rinse them with normal saline, and then suck dry them. Finally, determine the content of hepatic glycogen with glycogen test box and UV-visible spectrophotometer, and by using the classic anthrone method.^[12,13] If the hepatic glycogen level in the medicine-administration group is obviously higher than that in the model group, and the difference

is significant ($P > 0.05$), it then can be decided that this medicine has the function of improving the mouse reserve of hepatic glycogen.

The determination of serum urea

Urea is the main product of human protein metabolism, which constitutes the bulk of nonprotein nitrogen in human blood. The urea nitrogen in blood comes from liver, and it will be discharged with urine from the body through kidney. As a result, the nonfunction of kidney and nephritis can make the content of urea nitrogen in the blood rise up. Use a large amount of glucocorticoid to make an intraperitoneal injection for a week, and then make them into a kidney-yang deficiency model, and the mouse serum urea value will naturally rise. In the process of the medicinal curing, observe whether the serum urea will decrease or not.^[14]

After 30 min of the last medicine-administration, put the mice into the swimming tank (water temperature is 25°C) to make unloaded swimming for 90 min. After 60 min rest, get the blood by taking out their eyeballs and put them at 4°C for 3 h in standing, and centrifuge them for 10 min at 2500 r/min; take out the serum and use urea nitrogen test box (BUN) and microplate reader to determine the content of serum urea. If serum urea content in the medicine-administration group is obviously lower than that in the model group, and the difference is significant ($P > 0.05$), it then can be decided that this medicine has the function of reducing the fatigued mouse serum urea production.

The determination of lactic acid of blood

Lactic acid is the metabolite of the sugar anaerobic oxidation (glycolysis). Lactic acid comes from skeleton, muscles, brain, and red blood cells and will be discharged by kidney secretion after liver metabolism. The determination of lactic acid of blood can show the conditions of organic oxygen supply and metabolism, and whether the perfusion is enough or not.^[15]

After 30 min of the last medicine-administration, collect 40 μ L of blood from angular veins, and have unloaded swimming for 10 min at 30°C in the water tank, and later, in the same way as above, collect 40 μ L of blood from angular veins at 0 min and 20 min, respectively, after swimming activity and then centrifuge them for 10 min at 2500 r/min; take out the blood serum and use lactic acid test box (LD) and microplate reader to determine the content of lactic acid of blood. If lactic acid content in the medicine administration group is obviously lower than that in the model group after resting for 20 min, and the difference is significant ($P > 0.05$), it then can be decided that this medicine has the function of removing the mouse lactic acid of blood.

The determination of the mice visceral index

Put all the experimented mice to death, dissect them, take out their testicles and epididymis. Weigh the separated testicles and epididymis and calculate the visceral indexes of testicle/body weight and epididymis/body weight through observing and comparing the visceral indexes to study whether the medicine has influence on the size of testicles and epididymis.

Statistical treatment

The data are processed by the statistical software of SPSS 11.0 (Statistical Package for the Social Science, SPSS company, USA)^[16] and are based on the significance of difference and great notable significance of difference of $P < 0.05$ and $P < 0.01$.

RESULTS AND DISCUSSION

The influence on the mouse body mass

It can be seen from the weight change of the mice in the manufacturing model period that the weight of all mice in the model group decrease

during modeling, but during the medicine administration period, the weight of all the mice in all the groups has the tendency of increasing, only with a slight difference. Through “t” checking, there is no clear difference between the weight of mice ($P > 0.05$) in the normal contrast group, positive group, different medicine-dosage group, and model group, which shows that the ethanol extracts and polysaccharides of *A. esculentus* have no obvious influence on the mice weight.

The influence on the mouse food-intake

It can be seen from the food-intake change of the mice in the experiment period that generally the food-intake of all mice in all the groups has the tendency of increasing, only with a slight difference. Through “t” checking, there is no clear difference ($P > 0.05$) in the normal contrast group, positive group, different medicine-dosage group, and model group, which shows that the ethanol extracts and polysaccharides of *A. esculentus* have no obvious influence on the mice food-intake.

The influence on the mouse's loaded swimming time

Compared with normal contrast group, the mouse loaded swimming time in the kidney yang deficiency group decreased obviously by 24.7% ($P < 0.01$). Compared with the model group, with the increasing of dosage, the mouse loaded swimming time will all gradually increase. Among them, the loaded swimming time of the polysaccharides is longer than the ethanol extracts of *A. esculentus*, especially the loaded swimming time of the mice in the high-dosage group of the polysaccharides, and it is very close to that in the normal contrast group, reaching (646.8 \pm 53.57) s, which means it has very remarkable significance ($P < 0.01$) [Figure 1]. The loaded swimming time extended by 30.5%, and this is longer than that in the model group. It shows that the polysaccharides of *A. esculentus* have very good effects of relieving fatigue and obvious anti fatigue function.

The influence on the mouse reserve of hepatic glycogen

Hepatic glycogen is the storehouse of blood sugar. It can quickly decompose and release into blood when the organic blood sugar decreases for the sake of maintaining the stability of the blood sugar level. Sugar is the main energy source of muscular activity. After 2 h

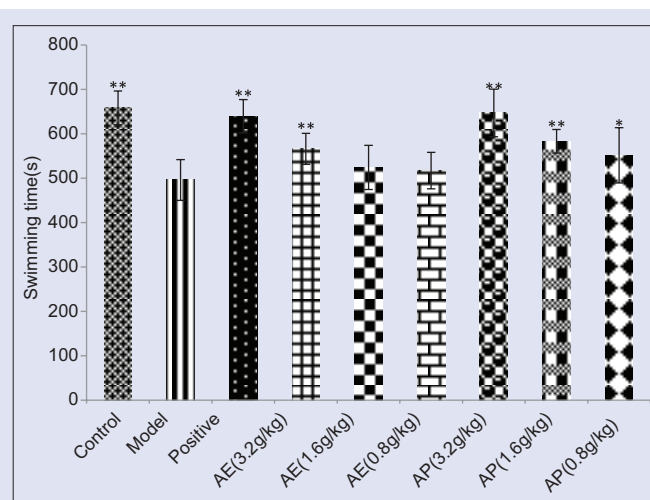


Figure 1: Effects of the ethanol extracts and polysaccharides of *Abelmoschus esculentus* on weight-loaded swimming endurance time of the mouse. Data denoted were means \pm standard deviation ($n = 10$). Compared with the model group, * $P < 0.05$, ** $P < 0.01$

of intense physical exercise, the muscular glycogen will become nearly exhausted. Thus, when doing long-time exercise, the body will decrease its hepatic glycogen reserve^[17] in order to maintain the blood sugar level. Compared with the normal control contrast group, the content of hepatic glycogen in the model group decreased notably ($P < 0.05$). Compared with the model group, the content of hepatic glycogen in each medicine group are all obviously higher. The content of hepatic glycogen increases with the increase of the medicine dosage. The ethanol extracts and the polysaccharides of *A. esculentus* increase significantly, and the polysaccharides have notable and remarkable significance. The polysaccharides exhibit relatively high content of hepatic glycogen, and the content of hepatic glycogen in the high-dosage group of polysaccharides has reached (50.581 ± 5.879) mg/g, increased by 1.6 times compared with the model group. The polysaccharides of *A. esculentus* show very strong function of storing hepatic glycogen, so they have very powerful antifatigue activity ($P < 0.01$) [Figure 2].

The influence on the mouse content of serum urea

The content of BUN increases with the increasing of different work and the workload, the poorer ability to adapt to the workload, the clearer of the increase of the BUN. The amount of the serum urea nitrogen after physical exercise shows the amount of energy supplied by protein, and it indirectly reflects the fatigue of the body. A large amount of glucocorticoid is used when modeling, and this is made into kidney yang deficiency model. The exhaustion of kidney function can increase the content of BUN [Figure 3]. In Figure 3, we can see that the content of urea nitrogen in the model group is the highest, reaching (11.89 ± 0.99) mmol/L, compared with the normal control group, it increases by 61.3%. However, after medicine administration, the urea nitrogen can be discharged from the body with the urine through kidney, and thus results in obvious reduction of content of the urea nitrogen in blood, and all of them decrease is more obvious with the increasing of the medicine dosage.

Among them, the reduction of the urea nitrogen of the polysaccharides in the high-dosage group decreased to (7.76 ± 0.55) mmol/L, the lowest urea nitrogen content. Compared with the model group, it decreases by 34.7%. Therefore, the polysaccharides of *A. esculentus* have very good effects on improving the kidney function ($P < 0.01$).

The influence on the mouse content of lactic acid

LAC is the most widely used biochemical indicator for assessing the body fatigue when doing the intense physical activities. Before the mice swim, and after they made the 5% - loaded swimming for 10 min, measure the concentration of lactic acid in blood after 0 min and after resting for 20 min, respectively. Compared with the normal contrast group, the LAC of the yang deficiency mouse increased obviously after swimming, and after a short rest, the concentration of lactic acid in blood declined very slowly. Compared with the model group, the concentration of lactic acid in blood of the mouse in the medicine group rose slower than that in the model group, and after a rest, the declining ranges of the lactic acid in blood are all higher than those in the model group, and they all have very special remarkable significance ($P < 0.01$). Among them, the polysaccharides high-dosage group of *A. esculentus* can greatly reduce the LAC value of the yang deficiency mice after physical activities, and its effects of relieving fatigue is the most obvious [Figure 4].

The influence on the mouse visceral index

Anatomize the visceral of the mouse: Take out the testicles and epididymis, weigh the separated testicles and epididymis, calculate the visceral index of testicle/body weight and epididymis/body weight.

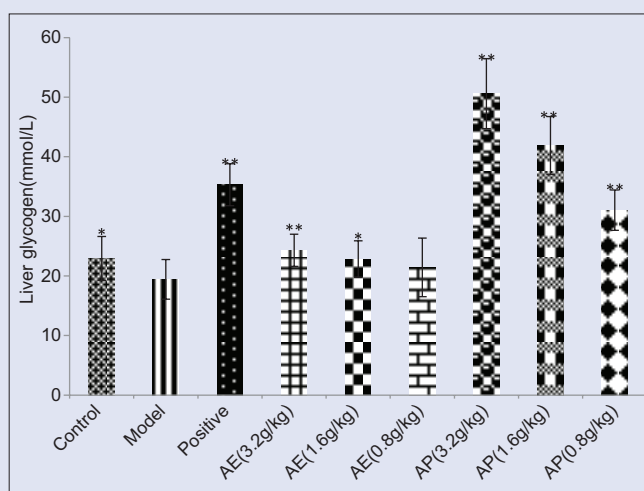


Figure 2: Effects of the ethanol extracts and polysaccharides of *Abelmoschus esculentus* on hepatic glycogen content of the mouse after exhaustive swimming. Data denoted were means \pm standard deviation ($n = 10$). Compared with the model group, * $P < 0.05$, ** $P < 0.01$

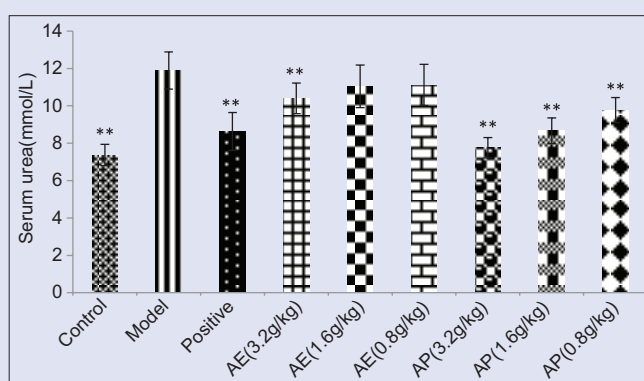


Figure 3: Effects of the ethanol extracts and polysaccharides of *Abelmoschus esculentus* on serum urea content of the mouse after exhaustive swimming. Data denoted were means \pm standard deviation ($n = 10$). Compared with the model group, * $P < 0.05$, ** $P < 0.01$

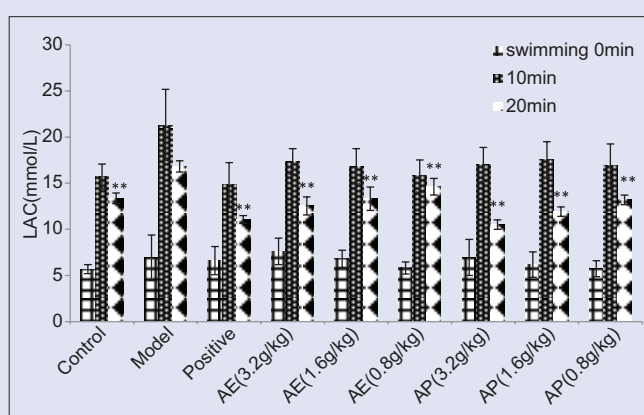


Figure 4: Effects of the ethanol extracts and polysaccharides of *Abelmoschus esculentus* on serum lactic acid content of the mouse after exhaustive swimming. Data denoted were means \pm standard deviation ($n = 10$). Compared with the model group, * $P < 0.05$, ** $P < 0.01$

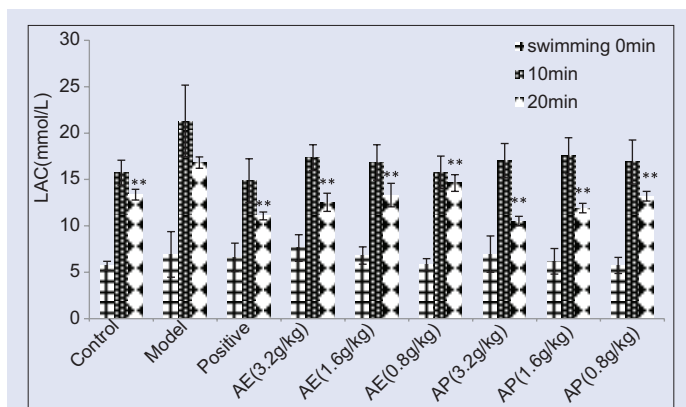


Figure 5: Effects of the ethanol extracts and polysaccharides of *Abelmoschus esculentus* on testicle index of the mouse after exhaustive swimming. Data denoted were means \pm standard deviation ($n = 10$). Compared with the model group, $P^* < 0.05$, $P^{**} < 0.01$

The experiment results show that the sizes of the mouse testicles and epididymis all have a certain degree of recovery.

The influence on the mouse testicle index

Anatomize the testicles of the mouse: Separate the testicles and weigh them, and calculate the visceral index of testicle/body weight. The experiment results can be seen in Figure 5. From Figure 5, we can know that compared with the contrast group, the testicle/body weight in the model group withered to a certain extent after successful modeling, which shows it has clear remarkable significance ($P < 0.01$). Compared with the model group, after cure of medicine administration, the sizes of the testicles all have some restoration. The restoration level of the crude extracts in the high-dosage group restored the most, the testicle index reaching (7.82 ± 0.072) mg/g, which shows it is almost near the positive group. The testicle index of the mouse increased as high as 34.8%.

The influence on the mouse epididymis index

Anatomize epididymis of the mouse, separate the epididymis and weigh them, and calculate the visceral index of testicle/body weight. The experiment results can be seen in Figure 6. From Figure 6, we can know that compared with the contrast group, the testicle/body weight in the model group has withered to (1.06 ± 0.09) mg/g after successful modeling, which shows it has clear remarkable significance ($P < 0.01$). Compared with the model group, after cure of medicine administration, the sizes of the epididymis all have some restoration. The epididymis index of polysaccharides in each dosage group all have special remarkable significance ($P < 0.01$), and the degree of the restoration of the polysaccharides in the medium-dosage group is the biggest, having restored to (1.37 ± 0.10) mg/g, so the medium-dosage group of the polysaccharides has the relatively stronger antifatigue activity.

CONCLUSIONS

Fatigue is a kind of physiological process involving many physiology-biochemical factors. It is a normal physiological phenomenon which is bound to appear when human body and brain power develop to a certain stage. This not only shows that the original organ's working ability declines temporarily but also shows that it is a sign of the body's developing into an injury- or-disease stage. The so-called "antifatigue" refers to delaying the appearance of fatigue and accelerating the removal of fatigue. The method of evaluation of fatigue is mainly about the testing of kinetic endurance and physiochemical change because fatigue is usually about the lowering of the muscular power caused by a series

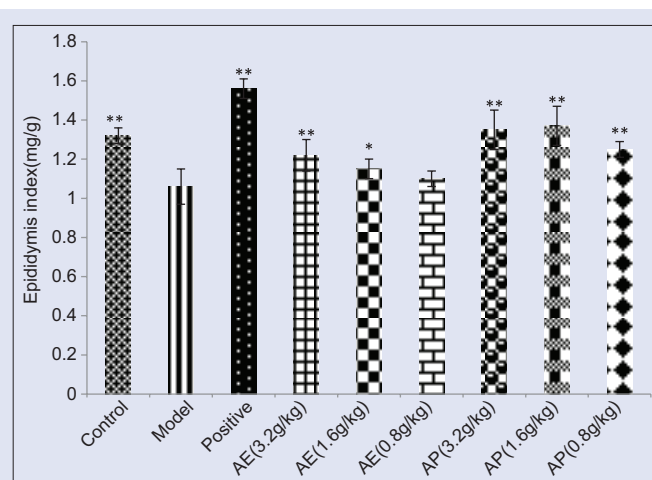


Figure 6: Effects of the ethanol extracts and polysaccharides of *Abelmoschus esculentus* on epididymis index of the mouse after exhaustive swimming. Data denoted were means \pm standard deviation ($n = 10$). Compared with the model group, $P^* < 0.05$, $P^{**} < 0.01$

of physiochemical change. The biochemical indexes are listed as follows: Energy matters are blood sugar, hepatic glycogen, muscle glycogen, and phosphoric acid creatine. Metabolic regulatory substances are enzyme and hormone. Metabolic substances are the lactic acid in the blood and muscles, the concentration of hydrogen ion, pyruvic acid, and BUN. The muscular endurance is the most direct and objective factor in showing how exhausted the organs are. Therefore, when measuring the significance of each physiology-biochemical index in anti-fatigue's functional testing, we can make a comprehensive evaluation between the test results of each physiology-biochemical index and the test results of the muscular endurance, and see their matching degree.

Through comparing the results of swimming time, liver glycogen level, blood serum urea of the mice in each group, the concentration change of the blood lactic acid, and the visceral indexes of testicles and epididymis, the two fractions of the ethanol extracts and polysaccharides of *A. esculentus* has clear effects on restoration of the kidney yang deficiency mice and relieving their body fatigue. The polysaccharides of *A. esculentus* showed better effects than the ethanol extracts, and especially the high dosage group of the polysaccharides showed the most powerful restoration ability of kidney yang deficiency and the ability of relieving physical fatigue. This study confirms that *A. esculentus* has good therapeutic effects on the patients of kidney yang deficiency, and also has the ability to restore people's physical power. These results will provide the basis for further development and utilization of this plant.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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