Anti-fatigue Effects of Ginseng Antler Yam Tang Modulation of Oxidative Stress Signaling in a Mice Model

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Research

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Abstract

Background: *Ginseng Antler Yam Tang* (GAYT), believe to invigorate “Qi” (vital energy), nourish “Blood” (body circulation) and engender “liquid” (body fluid), is a traditional Chinese medicine formula derived from the traditional prescription and Chinese traditional medicine partner theory.

Methods: In this study, we aimed to evaluate the anti-fatigue effects of GAYT and its mechanisms are related to oxidative stress signaling using GAYT composition, in vitro and in vivo antioxidant, and biochemical index detection. Chemical components analysis of GAYT was performed by high performance liquid chromatography (HPLC) and ultraviolet spectrophotometry (UV).

Results: The results show that the GAYT is rich in protein, total flavonoids, total polysaccharide and saponin. The mice model was treatment by GAYT (0.9, 1.8 and 3.6g/kg) for 4 weeks. GAYT treatment enhanced antioxidant activities. GAYT significantly enhances the exercise performance in weight-loaded swimming, rotating rod, and forced running test. Biochemical index levels showed that these effects were closely correlated with inhibiting the depletion of glycogen, blood lactic acid (LD) and adenosine triphosphate (ATP) stores, regulating oxidative stress-related parameters (superoxide dismutase (SOD), glutathione peroxidase (GSH-PX) and malonaldehyde (MDA)) in serum and liver of mice. Moreover, the results show that the effects of GAYT may be related with its regulation on the activations of AMP-activated protein kinase and protein kinase B in liver of mice.

Conclusions: GAYT can induce recovery from fatigue in mice via the activation of the AMPK and AKT/mTOR pathways. Provide a theoretical basis for the study of GAYT's anti-fatigue effect

Introduction

Fatigue is one of the most common physiological reactions. The main physiological function of fatigue is the impact on energy metabolism during muscle exercise [1]. The body is in fatigue state for a long time can make the body produce chronic fatigue syndrome (CFS), tender lymphadenopathy, body aches, headaches, unrefreshing sleep, inattention, and lower work efficiency[2]. Therefore, Fatigue can have a negative impact on work efficiency and life[3-4]. The etiology of fatigue has not yet been fully clarified. Current studies have shown that the pathogenesis of fatigue is mainly related to energy metabolism, immunity, secretion system, inflammation, and antioxidant defense dysfunction[5-8], Oxidative stress, a well-characterized factor, has received widespread attention as a bridge between fatigue and CFS. Intense exercise results in the depletion of glycogen and adenosine triphosphate (ATP) in the body, while a large number of oxidative reactions occur in the body, leading to the production of excessive reactive oxygen species (ROS)[9]. Moreover, under the physiological condition of oxidative stress, excess ROS can directly react with protein and DNA, as well as lipid to damage proteins and biological films[10], leading to cell death and aging[11-12]. Therefore, the antioxidant system protects against exercise-induced oxidative damage, and reduced physical fatigue and hypoxias [13-14]by controlling the body's oxidative stress response. 5'-ampk-activated protein kinase (AMPK) regulates the role of the body in the incoming and input response of the carotid

artery during hypoxia, thereby regulating the hypoxia and oxygenation response [15]. AMPK provides oxygen and ATP to the whole body through the regulation of respiration. Therefore, AMPK is a key regulator of cellular and whole body energy balance [16]. In molecular level, AMPK acts to suppress anabolic ATP-consumption pathways, and stimulates catabolic ATP-generating pathways [17].

Fatigue is becoming a serious public health problem, and current medicines or treatments are far from meeting the needs of patients. Additionally, the majority of the broad-spectrum drugs exhibit adverse effects [18]. The Traditional Chinese Medicine formula is developed under the guidance of traditional Chinese medicine theory, which contains Chinese herbal medicine. *Ginseng Antler Yam Tang* (GAYT), according to the classic prescription “pilose antler wine” and “dushen soup” have the effect of treating weak Yang and weak vitality. GAYT consists of Ginseng, Common Yam Rhizome and Velvet antler, and has been used for replenishing vital energy, restoring vital energy, generating fluid and nourishing blood function. *Ginseng*, Panax *ginseng* C. A. Meyer of the Araliaceae family, has over 2000 years history of extensive uses as TCM and functional supplements. Ginsenosides are common pharmacological components in ginseng herbs. Substantial experimental evidences have suggested that ginseng extracts and active ingredients can alleviate physical fatigue and disease fatigue such as cancer-related fatigue [19-20]. Many of the pharmacological actions of ginseng extract are produced by ginsenosides which belong to a common type of glycosides, and have been demonstrated by intensively studied to possess a pivotal role in the pharmacological activities of ginseng [21]. As a traditional herbal medicine, Chinese yam(dioscoreaceae, dry rhizome), “*Compendium of Materia Medica*” records “Yam, Strengthen the spleen and invigorate deficiency, Nourish the kidney, Cure all wound”. Chinese yam polysaccharide is a fundamental active component, can significantly improve the exercise ability of mice, and relieve physical fatigue and antioxidant effect [22]. Velvet antler (V A, Cornu Cervi Pantotrichum) has been a precious traditional Chinese medicine for 2,000 years. “*Compendium of Materia Medica*” states that antler is “nourishing kidney and aphrodisiac, prospering blood, nourishing the bone marrow”. Modern pharmacological studies have revealed that Velvet antler displays a wide range of activities including immune-enhancement, anti-aging, anti-fatigue, anti-oxidation [23-26]. Experiments show that antler water extraction, peptide and proteins can significantly relieve physical fatigue and fatigue in mice [27].

This study explored the anti-fatigue effect of GAYT extract. The antioxidant activity and the anti-fatigue of GAYT were investigated in vitro and in vivo. And we established a fatigue model to evaluate the anti-fatigue and anti-oxidation effects of GAYT and investigate the AMPK antioxidant pathway for exploring the molecular mechanism. This study may provide a new insight on the Anti-fatigue effects and molecular mechanism of GAYT.

Methods

Reagents and chemicals

Ginseng, Common Yam Rhizome and Velvet antler were bought from Department of Pharmacy, the Affiliated Hospital of Changchun University of Chinese Medicine, Jilin, China. Identification and

authentication of the herb materials were carried out at Changchun University of Chinese Medicine. GAYT (self-made in laboratory); Rhodiola Capsule (RHO); Ginsenoside Rg1 (110818-201507), Ginsenoside Re (110754-201520) and Ginsenoside Rb1 (110704-201625) were purchased from China food and drug testing and research institute. Adenosine triphosphate kit (ATP); Blood lactic acid kit (LD); Superoxide dismutase (SOD); glutathione peroxidase (GSH-Px); malonaldehyde (MDA) were purchased from Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China.

Animals

Kun Ming mice (6-week-old; 18-22g; n=50/group; male) were purchased from the Liaoning Changsheng Biotechnology Co., Ltd, Shenyang, Liaoning, China (Certificate No. SCXK (JI) 2016 - 0008) and acclimatized for 7 days. Mice were kept in controlled ambient temperature (24 ± 2°C) and humidity (60 ± 10%) under 12 hour's light-dark cycles; water was available ad libitum. The animal protocol was approved by the Animal Care Ethics Committee of Changchun University of Chinese Medicine (Changchun, Jilin, China, Approval No. 20171011).

preparation of GAYT

Velvet antler, Ginseng and Yam Rhizome plus 8 times the amount of water to cook 3 times, each time 1.5h, decoction filtered, the filtrate combined dried and crushed to get GAYT.

Determination of total polysaccharide, total saponins, total protein and total flavonoids

$NaN0_2$-Al $(N0_3)_3$-NaOH colorimetric method [28], vanillin colorimetric method [29], anthrone-sulfuric acid colorimetric method [30], Coomassie brilliant blue method [31] the content of total flavonoids, total saponins, total polysaccharides, and total protein, with rutin, ginsenoside Re, anhydrous glucose, and bovine serum albumin as controls, determine the absorbance value at the detection wavelength with a spectrophotometer, and draw a standard curve. Calculate the content of each component, Table 1 below for details.

Identification of phytochemical constituents using HPLC

High performance liquid chromatography (HPLC) analysis for GAYT sample solution (0.05g/ml) was performed on an Agilent 1260 Infinity HPLC system equipped with a UV detector. Chromatographic separation was conducted on an Sepax Bio-C18 (4.6×250 mm, 5μm). The solvent system composed of solvent A (acetonitrile) and solvent B (water) in the following variable wavelength gradient elution: 0-10min, 2%A; 10-20min, 2%-4%A; 20-35min, 4%A; 35-45min, 4%-19%A; 45-60min, 19%A; 60-90min, 19%-29%A; 90min-105min, 29%A; 105-125min, 29%-40%A; 125-135min, 40%A. Operating conditions were as follows: variable wavelength, 260nm (0-30min) and 203nm (30-135min); flow rate, 0.8ml/min; column temperature, 30°C; injection volume, 20μl. Ten batches of GAYT were prepared and measured separately to identify phytochemical constituents.

DPPH Radical-Scavenging Assay

DPPH radical scavenging activity of GAYT was determined using the previously reported method [32] with some modifications. The absorbance was read against blank at 517nm. Vitamin C

(Vit.C) was used as a positive control. Inhibition rate (% I) on the DPPH radical was calculated using the formula as below:

Percentage DPPH inhibition (% I) = (A0-AS)/A0×100

Hydroxyl Radical-Scavenging Assay

OH-scavenging activity of GAYT was measured as described by L.You[33]. The absorbance was read at 536 nm. Vit.C was considered as a positive control.

% OH-scavenged = (A0-AS)/A0×100

Superoxide Anion Radical-Scavenging Assay

Superoxide anion radical-scavenging activity of GAYT was assessed by the method reported by Li et al [34].The absorbance of the mixture was read at 560nm (As), mixture without GAYT samples was used as blank control (A0). Vit.C was considered as a positive control.

Superoxide anion−scavenging rate%= (A0-AS)/A0×100

A0 is the absorbance of the control containing all the reagents except the test compound while, AS is the absorbance of the test compound.

Experimental procedures

Before experiment, mice swam twice a day (10 min each time) within one week to accustom themselves to swimming; mice that failed to learn swimming were eliminated. Then mice were divided into five groups randomly and orally administrated with Normal Saline (0.4ml/20g) (CTRIL mice), 0.06g/kg RHO (0.2 ml/20g), and GAYT at doses of 0.9g/kg, 1.8g/kg, 3.6g/kg (5, 10 and 20 times the recommended dose for humans) once per day for 4 weeks. (The detailed Model experimental protocol and drug administration are shown in Figure1). During 4-weeks GAYT treatment, the mice were not subjected to any physical efforts and the body weight, diet, and social behavior were monitored. In 28 day, the following animal behavior tests and biochemical indicators were conducted.

Weight-Loaded Forced Swimming Test

The weight loaded force swimming test was performed as described previously [35-36] but with some modifications. Briefly, 30min after the last dose of extract on day 28 of treatment, mice taken from each group were subjected to the force swimming exercise. Mice was supplied with a constant load (corresponding to 10% of the body weight) tagged to the tail and placed individually in a swimming pool

(height: 35cm, diameter: 25cm) at 25℃±1℃, mice were assessed to be exhausted when they failed to rise to the surface of water to breathe within a period of 8s. Their exhaustive swimming time was recorded.

Rota-Rod Test

Thirty minutes after GAYT administration, the rota-rod test was conducted as previous research with minor modification [37]. Before the formal test, mice were trained twice on rota-rod at 15 rpm for 60s to adapt to the instrument. And, then, mice were placed inside a rota-rod spinning and allowed to run at speed of 15 rpm until they were exhausted and dropped from the rod. The total running time was recorded.

Forced Running Test

Thirty minutes after GAYT administration, mice were trained on the runway at 20 rpm for 1 min to adapt to the tread mill. Following three training exercises, the mice were emplaced on the treadmill at the 20mph speed. The number of shocks received from an electrode, touched when the mice cannot run at the set speed, in a 5min period was used to evaluate running performance.

Sample Preparations and Analysis of Biochemical Parameters

At 30min following the final treatment, 10 mice in each group were forced to swim for 60min and recess for 10 min, following which 0.2ml blood samples were collected from mice orbit. Serum was isolated by centrifugation at 4000rpm for 15min at room temperature. One part of the liver and muscle was homogenized to 10% solution with normal saline at 4℃. The levels of blood urea glycogen; Adenosine triphosphate kit (ATP); Blood lactic acid kit (LD); Superoxide dismutase (SOD); glutathione peroxidase (GSH-Px); malonaldehyde (MDA) in serum, liver, and muscle were detected by ELISA method according to the manufacturer's instructions.

Organ Weight Analysis

After the mice were sacrificed, the main visceral organs, namely the liver, muscles, kidney, heart, lung, EFP, and BAT, were accurately excised and weighed. Relative organ weight was calculated according to the following formula:

Relative organ weight (%) = organ weight/body weight*100

Western Blot Analysis

One part of liver tissues obtained from CFS mice was extracted with lysis buffer (RIPA with protease and phosphatase inhibitor) for 30min on ice and then centrifuged at 12000rpm for 10min at 4℃ to remove the precipitate. The concentration of total protein was determined by a bicinchoninic acid (BCA) protein assay kit (Merck Millipore, USA). An equal amount of denatured protein samples (40g) was loaded per well for 10% SDS polyacrylamide gel electrophoresis and transferred to PVDF membranes. The membranes were blocked using 5% bovine serum albumin (BSA) at room temperature for 2h. The blots were incubated with the appropriate concentration of specific antibody overnight at 4℃. Primary antibodies AKT1+2+3 (bs-

6951R), p-AKT (bs-0876R), AMPKα1 (bs-1115R), P-AMPKα2(bs-4002R), mTOR (bs-1992r), p-mTOR (bs-3495r) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (GB-12002) were diluted at 1:2000. The bonds were washed with TBS buffer plus 0.1% Tween-20 for five times and then incubated with horseradish hydroxide-conjugated goat anti-rabbit secondary antibody (sc-3836) (Santa Cruz Biotechnology, Santa Cruz, USA) for 4h at 4°C. The bands were established and fixed by an ECL Advance kit. The quantification of protein expression was determined using the Image J 1.46 software (Rasband, Bethesda, MD, USA).

Statistical Analysis

The data were analyzed using SPSS 16.0 software (IBM Corporation, USA). The results were presented as means±standard deviation (SD), and the statistical significance of each difference was determined using a one way analysis of variance (ANOVA) followed by Dunn's test. In the analysis results, $P \leq 0.05$was considered to indicate significant differences.

Results

General Chemical Analysis

The content of each component was calculated by standard curve method. The results of total flavonoids, total saponins,total polysaccharides and total proteins are shown in the table 2.

Component analysis

GAYT typical chromatograms from 10 batches with good quality control were shown in Fig.2 by HPLC analysis. Nine peaks of GAYT were identified on HPLC fingerprints. Nine peaks, including Pilose antler (No.1、2、3、4、5); Adenosine (No.2); Tyrosine (No.3); ginsenoside Rg1 (No.6), ginsenoside Re (No.7), ginsenoside Rb1 (No.8), Common Yam-Allantoin (No.9); in GAYT were identified by comparing with corresponding reference standards (Fig.2). The relative content of the individual constituent for five peaks in GAP was presented in Table 3.

The in vitro scavenging capacities of GAYT against hydroxyl, DPPH, and superoxide anion radicals were shown in Figure 3. When the GAYT was at 0.41mg/ml, the DPPH clearance rate reached 93.8%, and the clearance rate no longer increased. The clearance rate of Vit.C at 0.1mg/ml for DPPH reached 83.8%, which suggest at 0.21 mg/mL. Produces approximate scavenging effect as Vit.C at 0.1mg/ml (Fig.3A). In the range of 0~20mg/ml, hydroxyl radical scavenging activity of GAYT increased with the increase of sample concentration, the highest scavenging rate against hydroxyl radical of GAYT was 91.9%, and the IC50 value was 1.19mg/ml, which was much lower than that of positive control (VC) with the IC50 value of 0.043mg/ml (Fig.3C, E). In the range of 1~6mg/ml, GAYT exhibited scavenging activity against superoxide anion radical in a good linear relationship to sample concentration (R=0.9871), and the highest scavenging rate was 93.8%, its activity was still much lower than VC; the corresponding IC50 values were 2.7mg/ml and 0.029mg/ml.

Effects of GAYT on body weight and relative organ indexes

Behavior was monitored daily during GAYT administration, and the behavior was normal among the groups. As presented in Figure 4, the treatment main effect did not demonstrate a significant difference in terms of body weight, but GAYT were significantly higher in body weight than the other groups. The body weights of mice increased gradually during the study period, when compared with CTRIL group, no significant differences in body weight changes were observed. Moreover, no significant difference was noted in the diet and energy intake among GAYT-treated groups.

The effects of GAYT on relative weight of vital organs including liver, Muscle, Kidney, Heart, Lung, EFP and BAT were demonstrated in Table 4. No significant differences in relative organ weight were noted between CTRIL and GAYT-treated groups. But GAYT were significantly higher in body weight than the other groups.

GAYT Enhancing Exercise Capacities of fatigue Mice

The anti-fatigue activities of GAYT were detected via weight-loaded forced swimming, rota-rod and forced running test. Compared to no-treated mice, both GAYT and RHO have significantly enhanced the exercise endurance indicated by longer movement duration in all three behavior test ($p<0.05$, Figure5). GAYT treatment significantly enhanced swimming duration, with a maximum recording of 10.9min, compared with the duration of 4.5min in CTRL group ($p<0.01$,Figure5A). The duration for which the mice remained on rotating rod were recorded to evaluate the anti-fatigue activities of GAYT. Compared with the mice in the CTRL group, 3.6g/kg GAYT treatment enhanced the duration remaining on the rod by almost 96.01% ($p<0.01$, Figure 5B).In the forced running test, number of shocks were significantly reduced following the treatment of 1.8 and 3.6g/kg GAYT for two weeks, compared with the control ($p<0.05$, Figure 5C).

Effects of GAYT on the Levels of LD, ATP, and Glycogen in Serum and Liver

The accumulation of LD interferes with nerve impulses and muscle contraction, thus resulting in fatigue [38]. Before swimming, no significant differences on LD levels in serum and live were noted in mice among all groups (Figure 6). After swimming exercise, Compared with CTRL group, 25.06% reduction on LD level in serum were observed in 3.6g/kg GAYT treated mice, respectively ($P<0.5$, Figure 6.A). There were significant differences in LD content between the medium and high dose groups in the liver tissues, and LD content in the liver decreased by 19.81% and 22.89% respectively after 0.6g/kg and 0.8g/kg GAYT treatment compared with the CTRL group (*$p<0.05$, **$p<0.01$, Figure 6.A).

ATP is the most direct and rapid energy source to exercise. The higher level of ATP protects the muscle against membrane damage [39]. Interestingly, compared with swimming mice, ATP levels in serum and liver were enhanced after 60min swimming (Figure 6.B). In mice without swimming, GAYT at dose of 3.6g/kg resulted in over 30% increment of ATP levels in serum and liver ($p<0.05$, Figure 6.B). After swimming GAYT significantly enhanced the ATP levels in serum and live. GAYT at 3.6g/kg resulted in 21.2% enhancement in serum ($p<0.05$, Figure 6.B) and 34.9% enhancement in live ($p<0.05$, Figure 6.B).

Glycogen is the primary factor in fatigue and exhaustion during exercise [40-41]. Compared to unexercised mice, low hepatic glycogen levels were observed in mice after swimming (P<0.05, Figure 6.C). However, no significant difference on muscle glycogen were noted in exercised mice (P>0.05, Figure 6.C). GAYT strongly enhanced the levels of hepatic glycogen and muscle glycogen in mice. In mice with swimming, GAYT at 3.6g/kg enhanced 52.09% of hepatic glycogen and 10.01% of muscle glycogen.

Effects of GAYT on the Levels of Oxidative Stress Factors in Serum and Live of Mice.

Antioxidant enzymes including GSH-PX and SOD play important roles in preventing oxidative injury in animals [42]. MDA, a peroxide degradation product, indirectly reflects the degree of cellular attack and damage by free radicals. To investigate the effect of GAYT on oxidative system, the content of MDA, activities of SOD and GSH-Px in serum and liver were determined. 4-weeks GAYT treatment enhanced GSH-Px and SOD activity in mice. Treatment with 3.6g/kg GAYT increased serum SOD levels by 42.31% following swimming, compare with CTRL mice (p<0.01, Figure 7, A). Additionally, treatment with 3.6g/kg GAYT enhanced the levels of SOD in liver by 8.78% and 21.10% prior to and following swimming, respectively (*p<0.05, **p<0.01, Figure 7, A). The same enhanced trend was noted in the serum and liver tissues, in determent the levels of GSH-PX prior to and following swimming. In the serum, 3.6g/kg GAYT treatment resulted in increased of 58.22% and 34.3% prior to and following swimming. Compared with CTRL group, 35.22% and 30.76% increased on GSH-PX level in liver were observed in 3.6g/kg GAYT treated mice at prior to and following swimming, respectively (P<0.5,Figure7.B). Compared to no swimming mice, the overproduction of MDA in liver and serum were observed in mice with 60 min swimming (P<0.05, Figure 7, C). Compared with CTRL group, the downward trend was noted in serum and liver tissues.4-weeks treatment with 3.6g/kg GAYT downward the levels of MDA in serum by 30.43% and 20.65% prior to and following swimming, respectively (*p<0.05, Figure 7, C). In liver, treatment with 3.6g/kg GAYT downward serum MDA levels by 13.48% following swimming.

The Regulation Effect of GAYT on Protein Expression in Liver

To evaluate the potential mechanism of GAYT on regulating energy metabolism and physical fatigue, the activation of AMPK, Akt, and mTOR in liver of mice after 60min swimming were detected via western blot. In the RHO treated group, no significant effects on the expression levels of p-AKT, p-AMPK or p-mTOR were observed (Figure 8). Treatment for 4 weeks with GAYT significant effects on the expression levels of p-mTOR. 3.6g/kg GAYT enhanced the expression levels of p-AMPK, p-AKT, p-mTOR in the liver by 191.89%, 190.86% and 561.17%, compared with the CTRL mice (p<0.05; Figure 9).

Discussion

Herbs turn out to be a valuable reservoir for novel drugs selection to alleviate the symptoms of fatigue [43]. GAYT of ginseng, yam and pilose antler nourish the Qi and promote the growth of body fluid. It nourishes the deficiency of liver and kidney, spleen and lung. It can nourish the Qi and blood of the body, the deficiency of viscera, and has the effect of delaying physical fatigue. The current study systematically

investigated the potential effects of GAYT on anti-fatigue performance to prevent exercise-induced damage.

In this paper, the extract of *Ginseng Antler Yam Tang* (GAYT) was prepared and analyzed. The results indicated that 15.01±0.15% total polysaccharide, 1.12±0.06% total protein and 1.35±1.71% total saponins which may contain active hydrogen. The free radical scavenging activity of GAYT may be contributed by some of its hydroxide radical. Therefore, the in vitro evaluation was firstly conducted to obtain the antioxidant potential of GAYT. The results showed that GAYT exhibits certain scavenging capacities against hydroxyl, DPPH, and superoxide anion radicals.

The physiological effect of fatigue can be attributable to energy metabolism, metabolite accumulation, and muscle glycogen deletion, which are also associated with hypoxia [44]. In our group, GAYT extract exhibiting exercise enhancement. The enhanced exercise endurance of GAYT treated mice in weigh-loaded swimming, forced running, and rotating rod test revealed the anti-fatigue activities of GAYT. LD, known as glycolysis product of carbohydrate under anaerobic conditions, is one of the major factors responsible for physical exercise-induced fatigue [45].In hematologic system; LDH oxidizes LD, changes the pH value, and further reduces LD caused damage [46]. Therefore, LD can be served as an indicator for fatigue determination. GAYT regulated LD level activity in serum and liver, similarly, GAYT that accelerated the clearance of LD may be involved in its alleviating fatigue phenomenon in experimental mice. ATP is known as the most direct and rapid energy source to exercise. Exercise elevates muscle [H] and depresses muscle function by inhibiting myofibrillar ATP, which leads to the decrease of ATP synthesis [47]. GAYT at chosen doses enhanced ATP concentration in both liver and serum. Oxidative stress regulates the activity of the glycogen synthase kinase-3 and results in abnormalities of glucose and lipid metabolism, which is believed to be deeply involved in glycogen synthesis [48]. Glycogen is commonly subdivided into two types, hepatic glycogen that supports blood glucose concentration and muscle glycogen that provides muscle contraction for energy [49]. Exercise, hepatic glycogen metabolizes into glucose to support blood glucose consume; and muscle glycogen metabolizes into lactic acid, which arrives in the liver and converts to hepatic glycogen or glucose used to replenish liver glycogen. 60min swimming exercise can cause glycogen consumption, and GAYT strongly enhanced the levels of glycogen in muscle and liver of mice with or without swimming.

Exercise produce of a large amount reactive oxygen species (ROS), in turn, leads to oxidative stress by causing imbalance between oxidant and antioxidant defense system (SOD, MDA, GSH-PX) which carry the body in a risk of injury via affecting the homeostatic environment [50]. GAYT treatment increased the levels of SOD and GSH-PX in the serum and liver, prior to and following exercise. SOD and GSH-PX, the major components of enzymatic antioxidant defense systems combined together to scavenge free radicals. SOD catalyzes the conversion of superoxide into hydrogen peroxide and oxygen; meanwhile, GSH-Px scavenges the hydroxyl radical to maintain reduction-oxidation homeostasis [51]. SOD and GSH-Px combined together to scavenge free radicals, especially ROS, and inhibit lipid peroxidation by decreasing the production of MDA, thus to protect the cellular structures from destruction and further help to reducing fatigue [52]. All these work together to prevent lipid peroxidation and further protect cells from oxidative injury via

suppressing hyperlevel of MDA and ROS and enhancing SOD and GSH-Px activities, which are involved in GAYT mediated anti-fatigue effect on enhancing exercise endurance.

In liver, 4-weeks GAYT administration enhanced the phosphorylation of mTOR, AKT and AMPK after 60min swimming. AMPK is known to be important in energy homeostasis, as a cell energy regulator [53]. AMPK maintains ATP balance via inhibiting the synthesis of glycogen, cholesterol, and fat and promoting fatty acid oxidation and glucose transporter [54]. In starvation, hypoxia and oxidative stress, activated AMPK promotes cell survival [55]. In the liver, AMPK activates catabolic pathways to regulate ATP generation and consumption [56]. In the GAYT-treated mice in the study, the enhanced ATP concentration in the serum and liver following 60-min swimming may have combined with AMPK phosphorylation. Furthermore, as a major switch of energy metabolism, AMPK activation counteracts oxidative stress by inhibiting NAD(P)H oxidase-derived ROS accumulation [57].research indicates that AMPK activation counteracts oxidative stress via suppressing ROS accumulation and increasing SOD and GSH-Px activities in liver [58].AMPK contributed to the antioxidant activity via regulating the levels of SOD and GSH [59].

Moreover, we fail to explain the relationship between AMPK and AKT/mTOR. In research, GAYT administration enhanced the expressions of P-AKT and P-mTOR. mTOR is considered as a downstream target protein of AKT, being sensitive to regulate growth factors and energy metabolism [60]. During body exercise, AKT/mTOR signaling is activated to promote protein synthesis and translation [59]. Results suggest AKT/mTOR signaling is involved in the anti-fatigue activities of GAYT investigations performed in cancer cells, inhibited the phosphorylation of AMPK by upregulating the activation of AKT and mTOR [61]. However, GAYT treatment enhanced the phosphorylation of AMPK, mTOR and AKT. Further investigations are required to elucidate the underlying mechanism.

Conclusion

GAYT, a traditional Chinese herbal formula, improves the exercise ability of mice correlated with inhibiting the depletion of glycogen stores and ATP, regulating oxidation related enzymes. GAYT induced recovery from fatigue in mice, at least partially via the activation of the AMPK and AKT/mTOR pathways. These data provide experimental evidence supporting the clinical use of GAYT as an effective agent against fatigue.

Abbreviations

GAYT: Ginseng Antler Yam Tang;

HPLC: high performance liquid chromatography ;

UV: ultraviolet spectrophotometry;

LD: lactic acid ;

ATP: adenosine triphosphate ;

SOD: superoxide dismutase;

GSH-PX: glutathione peroxidase;

MDA: malonaldehyde;

CTRIL: control group ;

RHO: positive control;

AMPK: 5'-ampk-activated protein kinase

Declarations

Availability of data and materials

Not applicable.

Ethics approval and consent to participate

The animal protocol was approved by the Animal Care Ethics Committee of Changchun University of Chinese Medicine (Changchun, Jilin, China, Approval No. 20181011).

Consent for publication

The manuscript is approved by all authors for publication.

Availability of data and materials

The data sets supporting the conclusions of this article are included within the article and its additional files.

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Competing interests

We declare that there are no conflicts of interest associated with this manuscript and no significant financial support that would influence our findings.

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epilepsy and depressive-like behavior,involves interplay between AMPK,AKT/mTOR pathways and neuroinflammatory cytokine release," Brain Behav Immun 42:157-168,2014.

Tables

Table 1 Determination of active ingredients in GAYT

Component	Standard	Standard concentration（mg/ml）	Sample size（g）	Fixed volume（ml）	Wavelength（nm）
Total Flavonoids	Rutinum	39.88	1.0	50	510
Total Polysaccharide	Anhydrous Glucose	34.20	1.0	20	582
Total Saponins	Ginsenoside Re	4.09	0.1	25	560
Total Protein	Bovine albumin	1.00	0.2	10	595

Table2 The results of total saponins, total polysaccharides and total proteins

Component	standard curve	R^2	linearity and range（mg）	content
Total Flavonoids	y=1.18x+0. 24	0.9998	0.14~0.84	2.15±0.07%
total saponins	Y=0.03x+0.268	0.9991	0.02~0.12	1.35±0.12%
Total polysaccharide	Y=0.046x+0. 393	0.9995	0.1~0.6	15.01±0.15%
total protein	y=5.12x+1.15	0.9998	0.01~0.06	1.12±0.06%

Table3. Identification and determination of the compounds in the GAYT by HPLC

Peak No	Retention time (min)		Compounds	Contents (mg/g)
	Reference standard	GAYT		
2	12.497±0.013	12.575±0.007	Adenosine	0.28±0.021
3	16.243±0011	16.410±0.013	Tyrosine	0.39±0.012
6	82.251±0.010	82.281±0.012	ginsenoside R_{g1}	0.15±0.008
7	82.512±0.012	82.524±0.008	ginsenoside R_e	0.13±0.006
8	120.246±0.008	120.524±0.06	ginsenoside R_{b1}	0.01±0.005
9	125.118±0.015	125.089±0.009	Allantoin	0.08±0.010

Table 4. Effects of GAYT on relative organ weight in mice.

Characteristic	CTRIL	RHO	GAYT		
			0.9g/kg	1.8g/kg	3.6g/kg
Liver (%)	5.50±0.04	5.51±0.07	5.49±0.05	5.51±0.10	5.53±0.06
Muscle (%)	0.98±0.02	0.98±0.02	0.99±0.03	1.00±0.04	1.01±0.01
Kidney (%)	1.48±0.05	1.48±0.03	1.49±0.02	1.48±0.06	1.51±0.08
Heart (%)	0.55±0.03	0.57±0.08	0.57±0.04	0.58±0.07	0.59±0.05
Lung (%)	0.53±0.01	0.55±0.04	0.55±0.02	0.56±0.05	0.56±0.06
EFP□%)	0.65±0.08	0.67±0.05	0.65±0.06	0.68±0.03	0.68±0.02
BAT (%)	0.28±0.01	0.29±0.03	0.27±0.01	0.29±0.02	0.30±0.01

Figures

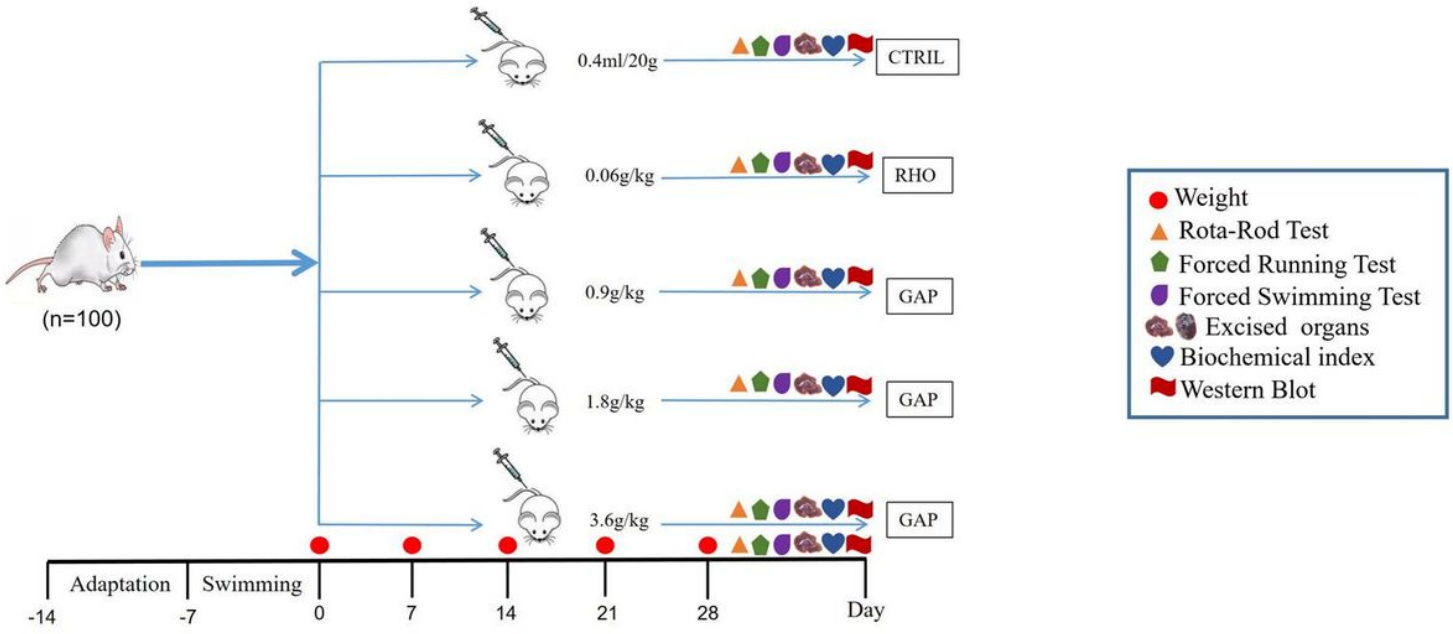

Figure 1

The flow chart for experimental design of anti-fatigue evaluation of GAYT. CTRIL: control group (Normal Saline); RHO: positive control (Rhodiola Capsule, 0.06g/kg BW); GAYT (0.9, 1.8, 3.6g/kg BW)

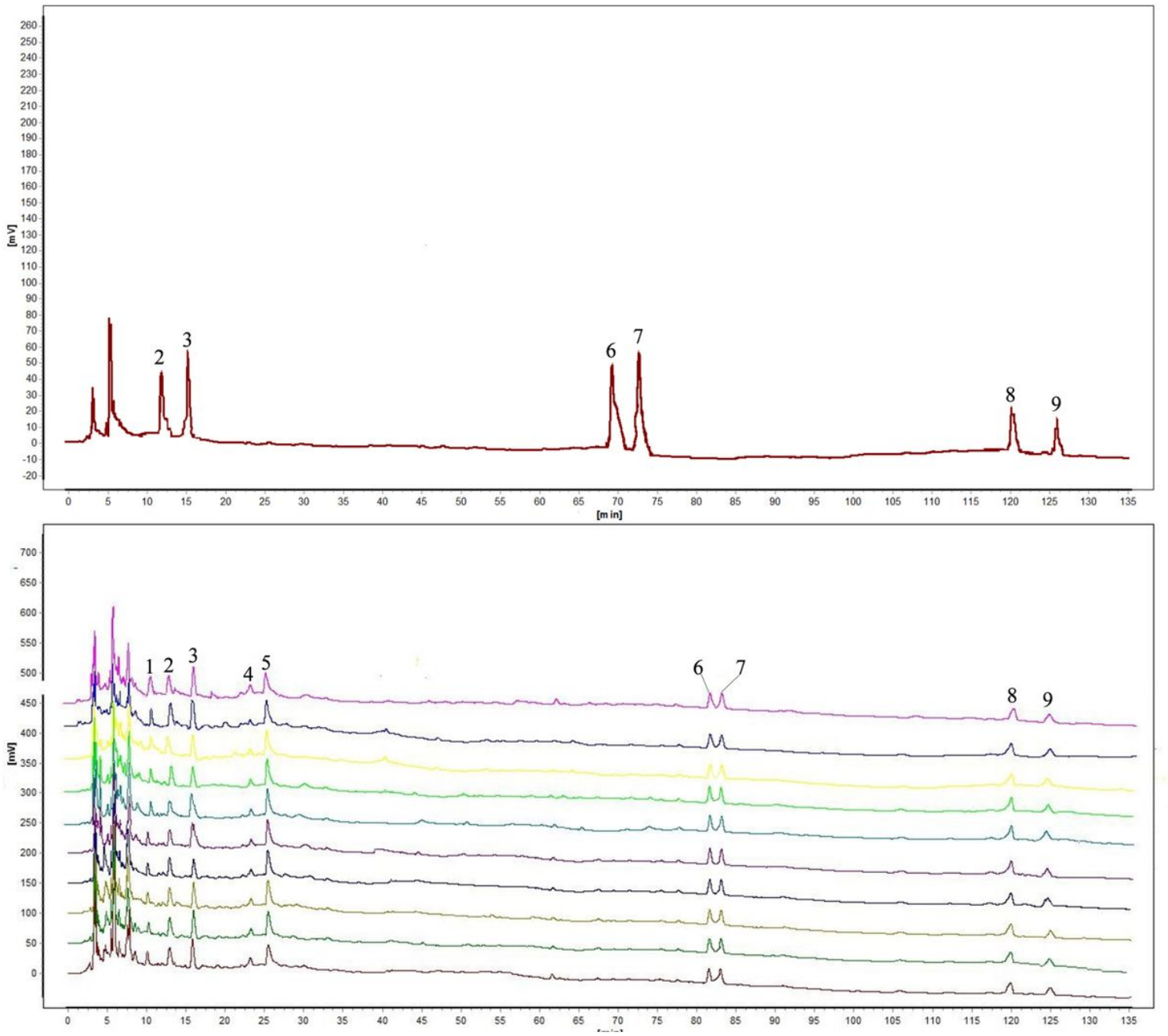

Figure 2

The Fingerprint of GAYT used high performance liquid chromatography (HPLC). In Vitro Antioxidant Activity of GAYT

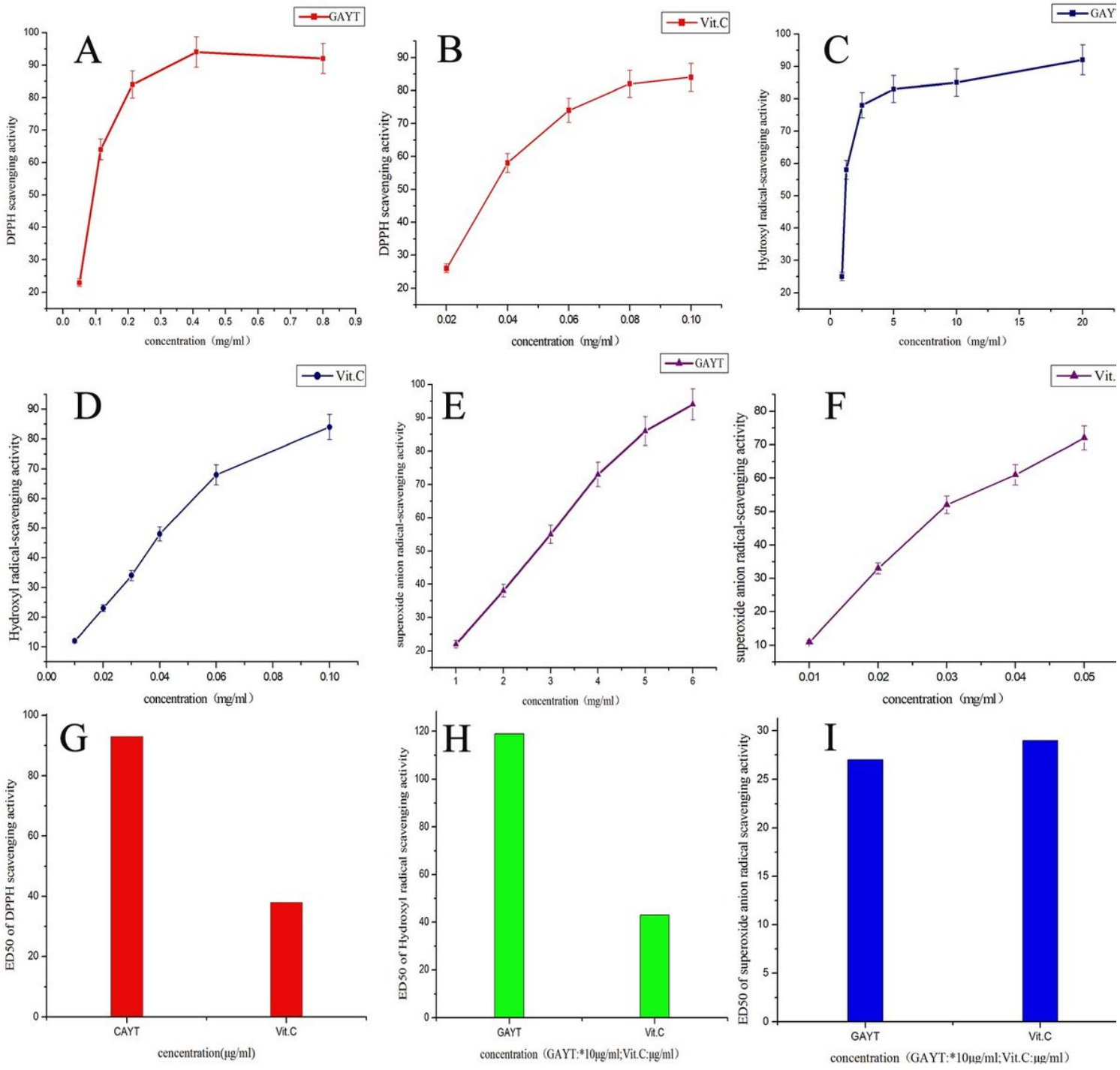

Figure 3

The in vitro antioxidant activities of GAYT using VC as a positive control. (A-B) DPPH radical-scavenging activity; (C-D) Hydroxyl radical-scavenging activity; (E-F) Superoxide anion radical-scavenging activity. (G-I) The values of 50% effective dose (ED50) for DPPH, Hydroxyl and superoxide anion radical-scavenging activities in the GAYT and Vit.C groups were calculated. Data was expressed as the mean±SD (n =3).

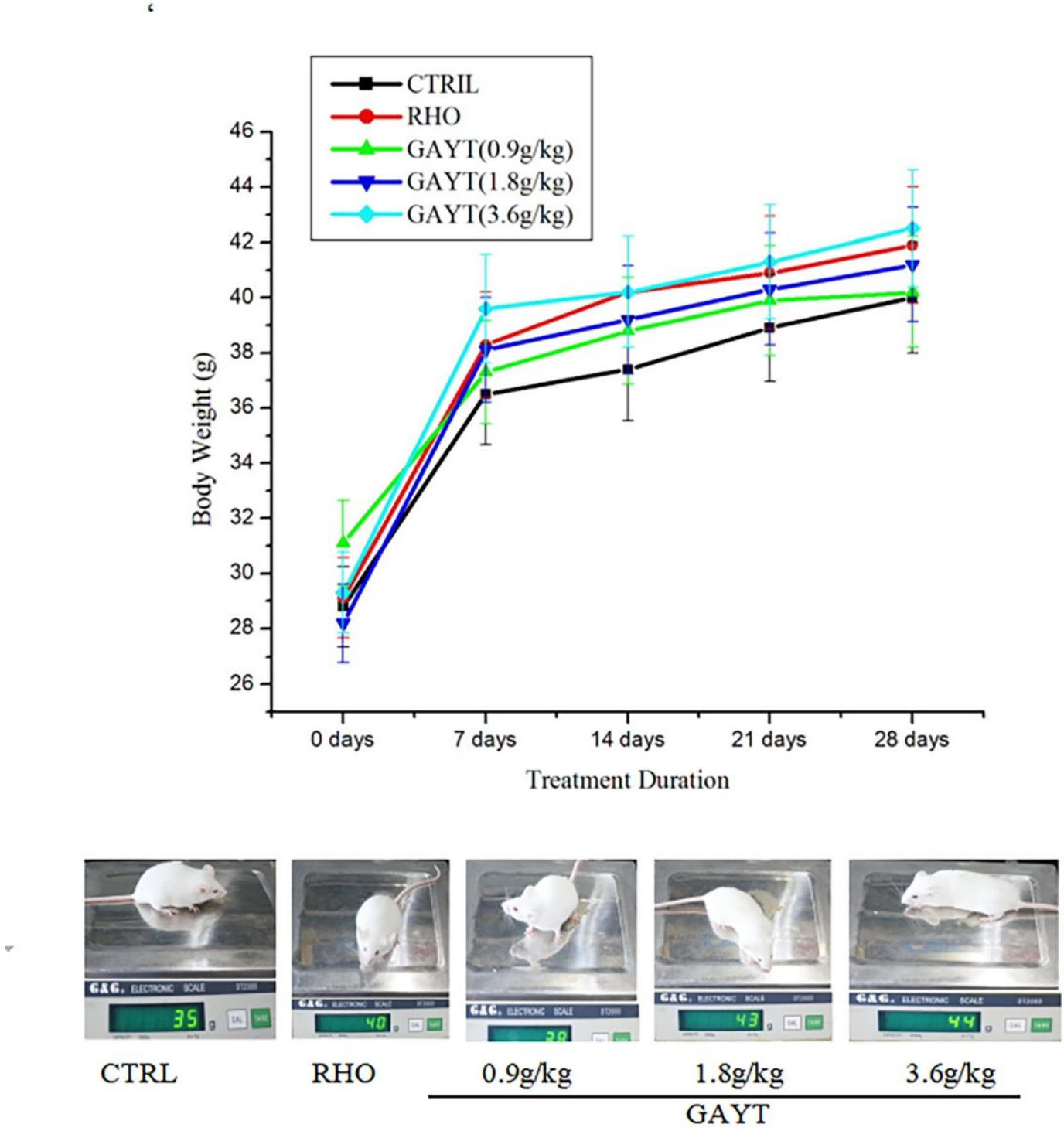

Figure 4

The effect of GAYT on body weight. The body photo of the rats from all groups was showed. CTRIL: control group (Normal Saline); RHO: positive control (Rhodiola Capsule, 0.06g/kg BW); GAYT (0.9, 1.8, 3.6g/kg BW).

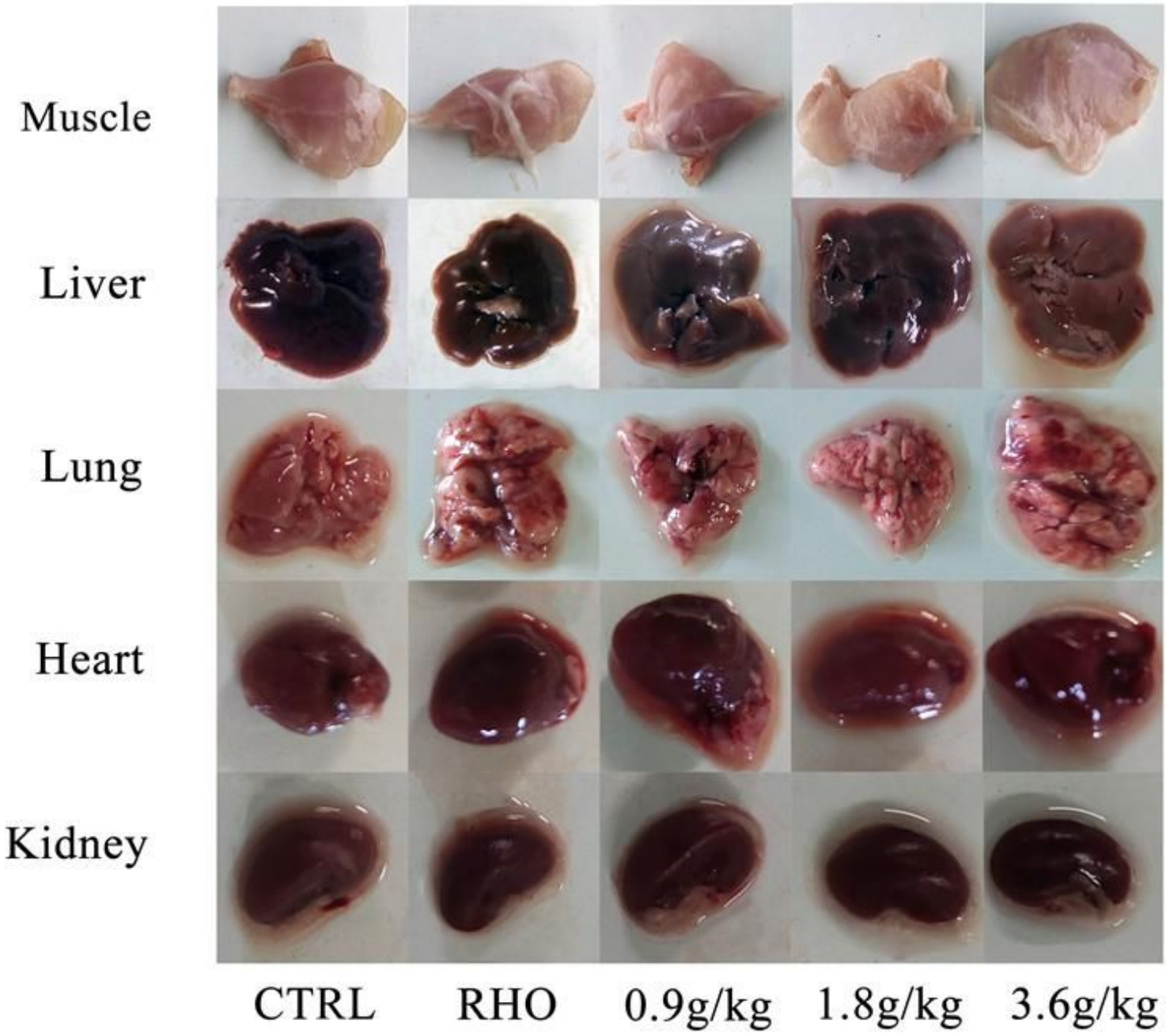

Figure 5

Effects of GAYT on relative organ in mice

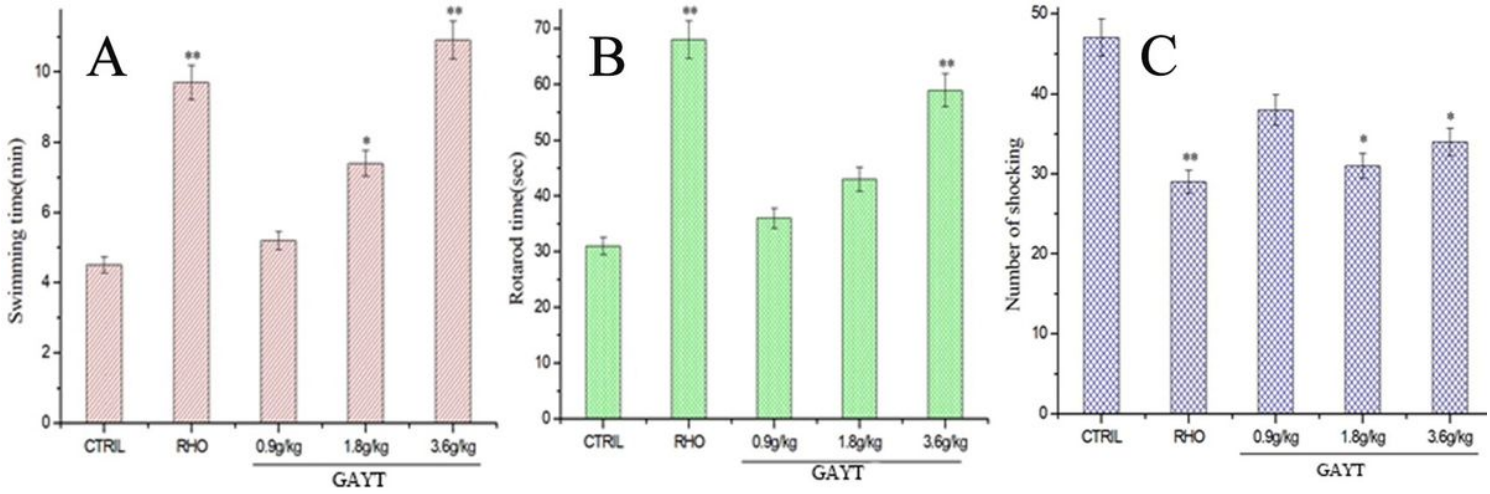

Figure 6

GAYT enhancing exercise capacities effects. The anti-fatigue effects of GAYT extract (0.9, 1.8 and 3.6g/kg) and Rhodiola capsule (0.6g/kg) treatment were analyzed by performing (A) Weight-Loaded Forced Swimming Test, (B) Rota-Rod Test, (C) Forced Running Test. Data are expressed as the mean ± standard deviation (n=20) and analyzed using one-way analysis of variance followed by Dunn's test. *p<0.05, **p<0.01. CTRIL: control group (Normal Saline); RHO: positive control (Rhodiola Capsule, 0.06g/kg BW); GAYT (0.9, 1.8, 3.6g/kg•BW).

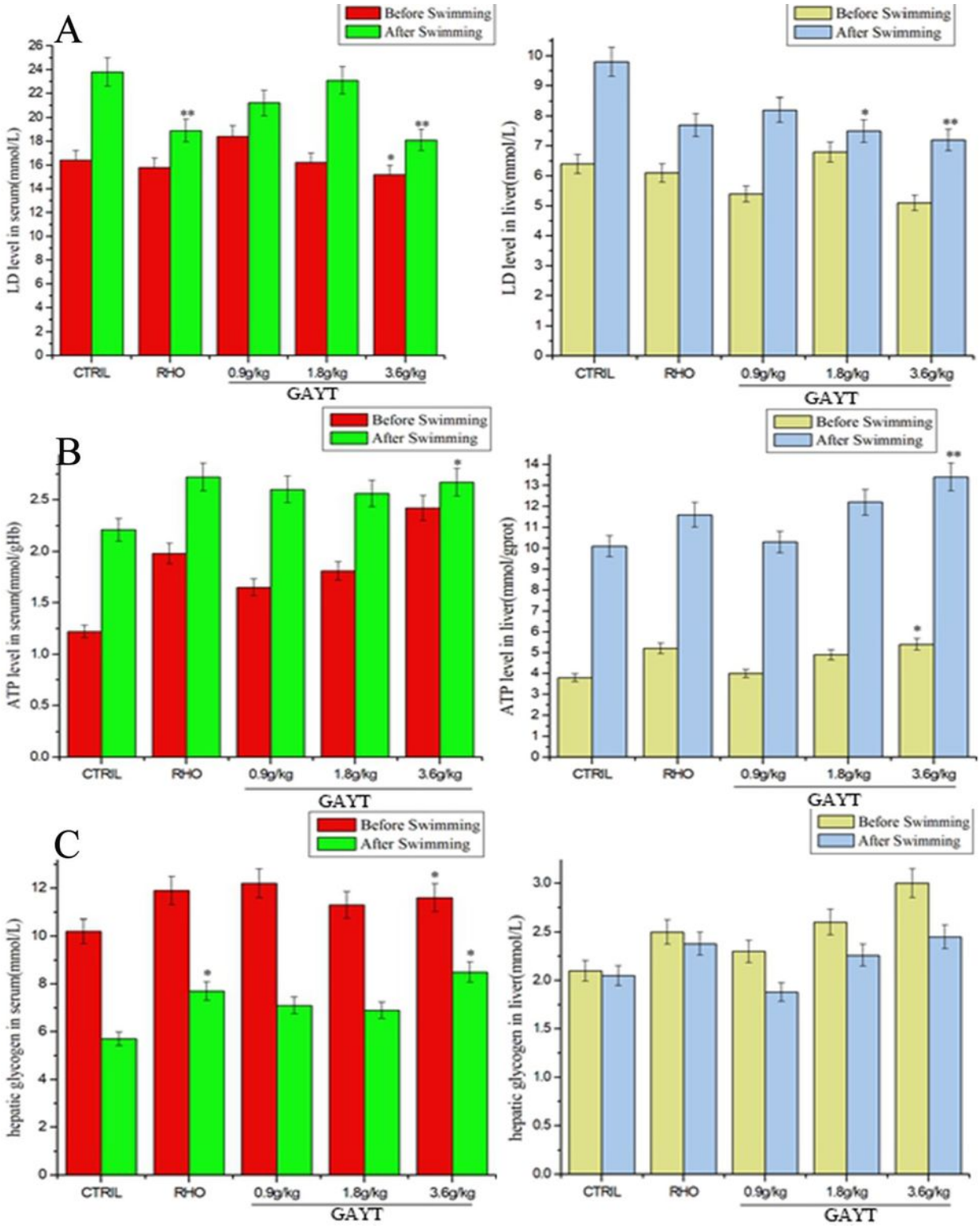

Figure 7

Treatment with GAYT extract and RHO treatment for 4 weeks, the effects on LD, ATP metabolism were analyzed in serum and live (A,B) in mice; muscle glycogen and hepatic glycogen were analyzed in serum (C) before and after 60 min swimming. Data are expressed as the mean±standard deviation (n=10) and analyzed using one-way analysis of variance followed by Dunn's test. *p<0.05, **p<0.01. CTRIL: control

group (Normal Saline); RHO: positive control (Rhodiola Capsule, 0.06g/kg BW); GAYT (0.9, 1.8, 3.6g/kg BW).

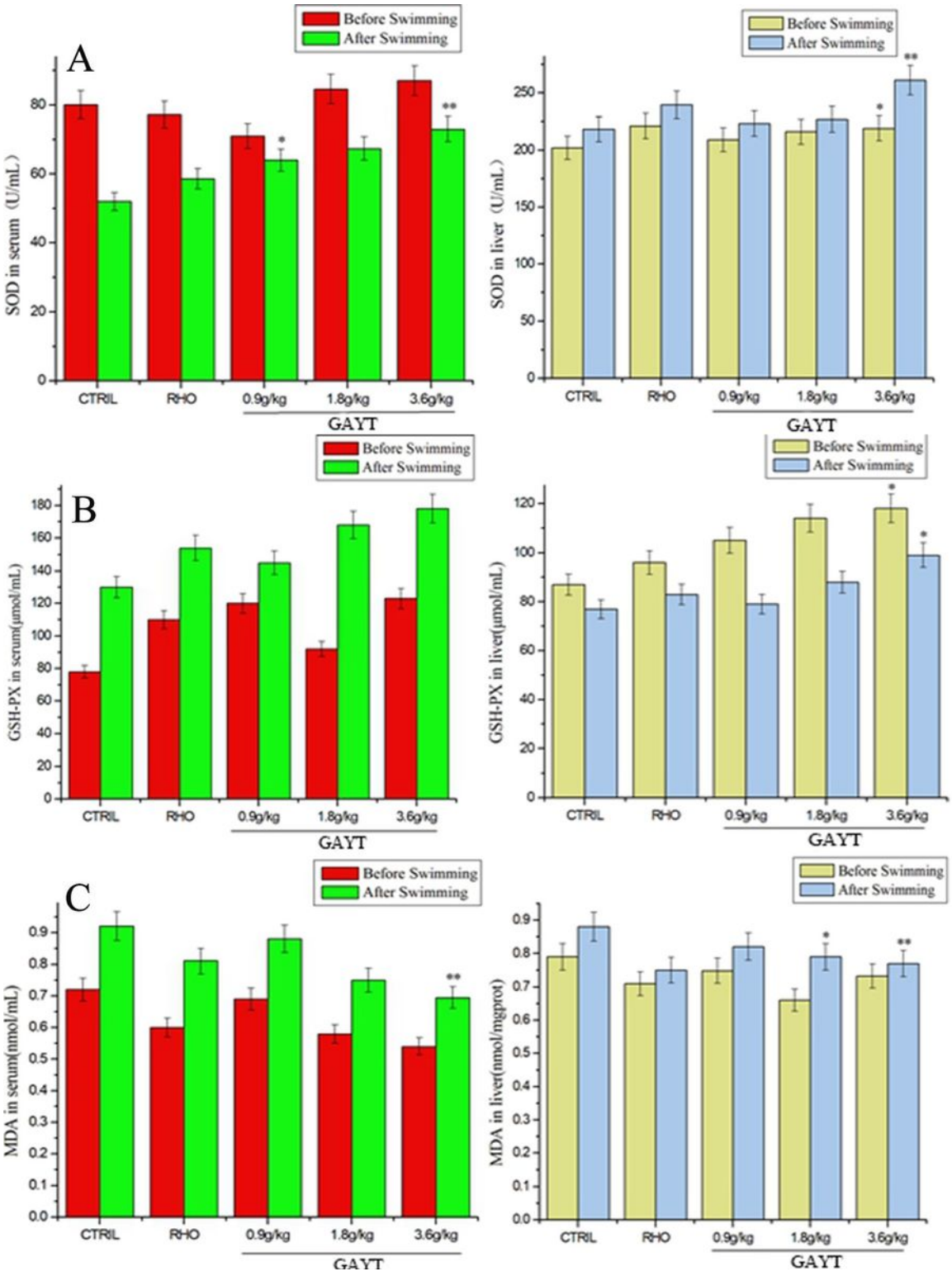

Figure 8

Effects of GAYT on the Levels of Oxidative Stress Factors in Serum and Live of Mice. Treatment with GAYT extract (0.9 g/kg, 1.8g/kg, and 3.6g/kg) and RHO (0.6g/kg) treatment for 4 weeks, the effects on SOD GSH-PX and MDA metabolism were analyzed in serum and live (A, B, C) in mice before and after 60 min

swimming. Data are expressed as the mean±standard deviation (n=10) and analyzed using one-way analysis of variance followed by Dunn's test. *p<0.05, **p<0.01 CTRIL: control group (Normal Saline); RHO: positive control (Rhodiola Capsule, 0.06g/kg•BW); GAYT (0.9, 1.8, 3.6g/kg•BW).

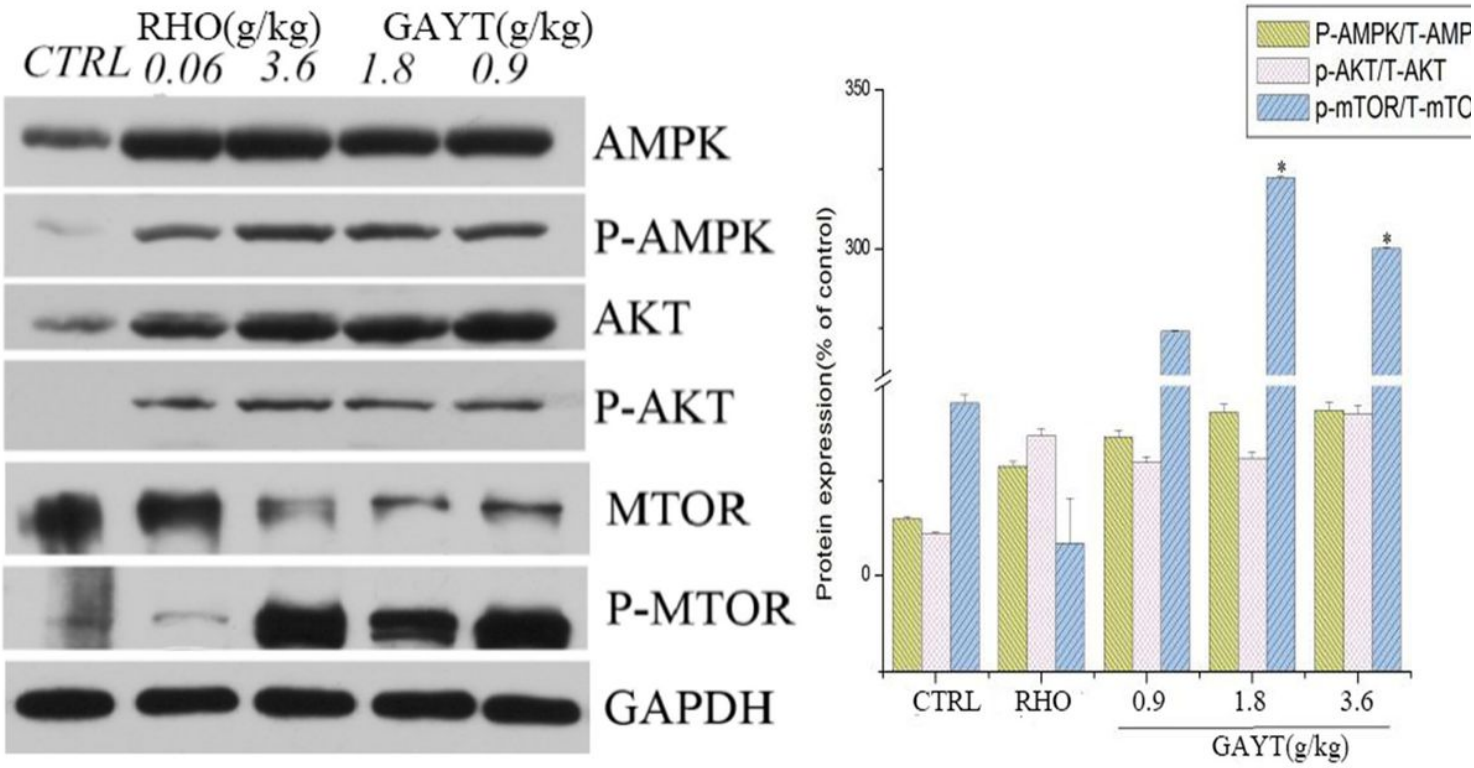

Figure 9

Mice were treated with GAYT and RHO for 14 days, following 60-min swimming, and the activation of AKT, AMPK and mTOR in the liver were analyzed using Western Blot of rapaot analysis. Quantification of expression levels of p-AKT, p-AMPK and p-mTOR were normalized by corresponding levels of t-AKT, t-AMPK and t-mTOR. Data are expressed as the mean±standard deviation (n=10) and analyzed using one-way analysis of variance followed by Dunn's test.*p<0.05, **p<0.01vs.CTRIL: control group; RHO: positive control (Rhodiola Capsule, 0.06g/kg•BW); GAYT (0.9, 1.8, 3.6g/kg•BW). AKT, protein kinase B; AMPK, 5'-monophosphate-activated protein kinase; mTOR, mammalian target of rapamycin; GAPDH glyceraldehyde-3-phosphate dehydrogenase.