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Research Article

Prismatomeris glabra Increases Forced Swimming Time in Mice

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ABSTRACT

Roots of *Prismatomeris glabra* (*P. glabra*), family Rubiaceae, have been traditionally used by rural indigenous people for wellness and stamina purposes. However there is no scientific evidence to support the folkloric use of this plant. Ergogenic effects were studied in weight-bearing mice (load: 10% of body weight) by a modified forced swim test (FST) following treatment with PG. Control groups were given vehicle or L-arginine. Effect was studied on exercised and non exercised groups. Nine exercise bouts were performed with each bout comprising of 3 consecutive days of FST till exhaustion and a rest day between bouts. Body weight, water and food intake were recorded. Mice were killed immediately after the final FST and blood was collected for glucose, lactate and serum assays. Serum corticosteroid was measured using enzyme immunoassay (EIA) kits. Results showed that mice treated with *P. glabra* exhibited significantly greater exercise performance than control ($p=0.000$) or L-arginine ($p=0.001$) groups. Effect of *P. glabra* on post-exercise blood glucose was greater than control exercised group ($p=0.011$) but similar to control non-exercised and L-arginine groups. Effect of *P. glabra* on blood lactate was similar to all other exercised groups. Serum cortisterone of *P. glabra*-treated group was significantly higher ($p<0.001$) than that of control non-exercised group, but similar to control exercised and L-arginine groups. In conclusion, findings of this study provide evidence to confirm the traditional use of *P. glabra* roots for improving stamina and physical performance.

Keywords *Prismatomeris glabra*, forced swim test (FST), ergogenic, mice

INTRODUCTION

Prismatomeris glabra (*P. glabra*) is a tropical plant which normally grows on hillsides and ridges of tropical forests at altitudes up to 700 m. The plant is occasionally used by indigenous people in wellness remedies. A decoction of its roots is traditionally used for various purposes such as enhancing freshness, resistance to tropical diseases and physical stamina. However there has been no scientific work to prove these claims as yet.

Consumption of plants for ergogenic effects have been reported by many[1-5]. In these studies, ability of plants to enhance exercise performance was associated with various biological mechanisms[1] including hormonal functions[3], antioxidative functions[4] and lipid utilization[5]. The ability of a substance to sustain the test animal for relatively long periods in a given regime of exercise, i.e. forced swim test (FST) can indicate anti-fatigue effects of the test substance[6].

MATERIALS AND METHODS

Preparation of P. glabra aqueous extract

P. glabra plants (voucher code of PT/UiTM/AS2) were collected from tropical jungle in the Peninsular of Malaysia. Fresh roots of the plants were chipped into small pieces within 24-48 h of collection and dried at 45ºC in the oven for three days. Dry root chips were grounded to crude powder before every 100 g of them were boiled in 1 L of distilled water for 10 minutes. The suspension from the boiling process was filtered using filter paper. The filtrate was collected and dried using laboratory spray dryer (Büchi Mini Spray Dryer B-290). PG aqueous extract powder was kept in -20ºC freezer until use.

Animals

Thirty-two male young adult *Mus musculus* mice (25-35 g, 9 weeks old) from Institute of Charles River (ICR) breed were used. Animals were maintained under standard conditions of 12h/12h of dark/light cycle with food and water supplied *ad libitum*, in ventilated 25ºC room. Mice were placed in semi-transparent plastic cages with 8 mice per 82 sq inch cage. Animals were acclimatized for a week before assigned to any treatment. The use of mice in this experiment was approved by the university's ethical committee.

FST apparatus

The pool: A transparent plastic tank container sized 28x46x29 cm was modified as a swimming pool for FST. This was an open pool whereby water was supplied directly from tap water supply. A controllable outlet was constructed at the bottom of the side wall of the pool to allow water to flow out from the pool. During FST, the pool was filled with water and maintained at 26 cm in depth by circulating water from the tap towards the outlet. The circulation managed to maintain pool temperature at 29±1°C. B)

The weight load: Iron weight (10% of mouse body weight) was used to provide resistance to mice during FST. The

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iron weight was attached to mouse tail using masking tape. The weight caused mice to swim vertically towards the surface by continuously kicking downwards using all limbs until the mice became exhausted and were rescued. Without weight load, mice would float on the surface due to buoyancy effect of air trapped in the fur.

Protocol of FST

To avoid circadian variations in physical activity, swimming exercise was performed between 11:00 and 17:00, a period during which minimal variation of endurance capacity was confirmed in rodents[7]. In order to prevent mice from idle floating, a light water current was created by discharging water from water tab into the pool. Water level was maintained by displacing water input with water output through an outlet located on the bottom side of the pool. The mice were assessed to be fatigued when they failed to rise to the surface of the water to breathe within 5 seconds[8].

Work procedures

Following 2-week swimming training, mice were divided into 3 groups through stratified randomized sampling based on initial FST results. Control group (ECG, n=8) received 0.01 mL/g body weight p.o. per day of 0.9% normal saline, L-arginine-treated group (LAG, n=8) received *ad libitum* 2.5% L-arginine in drinking water in addition to 0.01 mL/g body weight p.o. per day of 0.9% normal saline whereas *P.glabra*-treated group (PGG, n=8) received 500 mg/kg body weight/day p.o. *P.glabra*. A baseline group (non-exercised (NCG), n=8) received 0.01 mL/g p.o. per day of 0.9% normal saline. All exercised groups were assigned for 9 bouts of FST with each bout comprised of 3 consecutive days of FST and 1 day rest between bouts. Body weight, food and fluid intake were recorded. On the final day, mice were killed immediately after the final FST by cervical dislocation under minimal anesthesia with diethyl ether. Blood was withdrawn immediately via cardiac puncture. Heart, lung, liver and kidneys were harvested and weighed.

Blood lactate analysis

Blood lactate was measured immediately upon completion of the 8th FST bout using Accutrend® lactate analyzer (Roche Diagnostics, USA) according to the manufacturer's instructions. Blood was withdrawn from the orbital sinus using heparin capillary tube before it was immediately discharged onto the lactate strip. Result was displayed within a few minutes.

Blood glucose analysis: Blood glucose was measured immediately after completing the final FST using Reflotron® analyzer (Roche Diagnostics, USA). Some of the blood that was withdrawn via cardiac puncture was immediately dripped onto the glucose strip before it was measured by the analyzer.

Serum assays

After incubating for 5 h at room temperature, serum was separated by centrifugation of the blood at 10000 x *g* for 10 min using Allegra® X-15R centrifuge (Beckman-Coulter, USA). Serum corticosterone was determined using enzyme immunoassay (EIA) technique (DRG ELISA kit, DRG International Inc., USA). All measurements were handled according to the manufacturers' protocols.

Statistical analysis

Data were expressed as mean±SD. One-way ANOVA was used to compare dependant variables between groups. Bonferroni post-hoc and Dunnett T3 post-hoc tests were used to determine any existence of differences in equal and unequal variances data sets, respectively, with the assistance of Levene's test. Independent-samples t-test was used for selected comparisons between samples. Alpha value was set *a priori* at $p<0.05$.

RESULTS

Effect of P. glabra on forced swim test

Mice given 500 mg/kg body weight/day p.o. *P. glabra* daily for 35 days showed better swimming (FST) performance than that of ECG ($p = 0.000$) and LAG ($p = 0.001$) groups. Exercised mice that were given *P. glabra* also showed earlier improvement of swimming time than that of the other groups. Mice given *P. glabra* recorded significantly longer swimming time than baseline (FST without dose treatment) as early as bout 4 compared to control (at bout 6) and mice given 2.5% L-arginine (at bout 5).

Effect of P. glabra on post-exercise blood glucose

One sample from each exercised and non-exercised control groups was excluded due to damage. PGG or LAG showed significantly higher post-exercise blood glucose level (8.51±1.04 mmol/L, $p = 0.011$ and 8.18±2.52 mmol/L, $p = 0.023$, respectively) as compared to ECG (4.90±2.48 mmol/L) but similar to NCG (8.42±1.60 mmol/L). Blood glucose of ECG mice was significantly lower than that of NCG ($p = 0.017$).

Effect of P. glabra on post-exercise blood lactate

P. glabra did not influence blood lactate when all exercised groups had similar levels of lactate following FST but showed significantly higher as compared to non-exercised mice.

Effect of P. glabra on corticosterone

P. glabra also did not influence corticosterone level when all exercised groups had similar levels of corticosterone. All exercised groups had greater corticosterone levels than that of NCG. Serum corticosterone was significantly increased with FST duration in all exercised groups.

Effect of FST on body weight, major organs, food and fluid intake: Body weight of all exercised groups was unchanged throughout the FST regime when their initial body weights (prior to experiment) and final body weights (body weight prior execution) were similar. Only NCG showed significantly increase in body weight during concurrent period. Organ to body weight ratios in lungs, heart, liver and kidney were comparable between all groups.

ECG had significantly higher food intake (0.154±0.018 g/g body weight/day) than that of NCG (0.127±0.017 g/g body weight/day; $p = 0.013$) and LAG (0.130±0.022 g/g body weight/day; $p = 0.034$) groups but similar to PGG (0.150±0.017 g/g body weight/day). At every exercise bout, food intake of ECG and PGG was always higher than the other groups. ECG also had significantly higher fluid

intake (0.151±0.023 ml/g body weight/day) than that of NCG (0.124±0.012 ml/g body weight/day; p = 0.011) and PGG (0.117±0.014 ml/g body weight/day; p = 0.001) groups but similar to LAG (0.133±0.020 ml/g body weight/day). At every exercise bout, fluid intake of ECG was always higher than the other groups.

DISCUSSION

P. glabra (500 mg/kg bodyweight) demonstrated anti-fatigue effect in mice that underwent forced swim test (FST). Ability of PGG mice to swim longer while carrying a load of 10% of body weight showed that the plant has ergogenic effect. This was proved by overall score of FST showing that PGG had greater score than that of CG and LAG groups. All groups showed similar baseline of FST at the beginning of experiment. Administration of *P. glabra* caused mice recorded earlier significant improvement of FST from the baseline compared to ECG and LAG. PGG demonstrated significantly improved FST from the baseline as early as 2 weeks time (at bout 4), which is better than that recorded by LAG (at bout 5) and CG (at bout 6). This means *P. glabra* showed faster ergogenic effect than 2.5% L-arginine. L-arginine was given *ad libitum* in drinking water, and from fluid intake analysis this means each mouse might receive 113 mg L-arginine per day. It is uncertain whether each mouse in this group consumed such amount.

Greater swimming duration in mice received *P. glabra* might be explained by higher blood glucose level. Glucose uptake increases substantially by an increase in not only intensity but also in duration of exercise[9]. Promotion of glucose uptake by skeletal muscles in response to exercise causes blood glucose concentration to increase as a result of increase in glucose delivery rate[10]. Increased movement of glucose transport and intracellular substrate flux through glycolysis during muscle contraction increase glucose delivery rate[11,12]. This may explain why mice receive *P. glabra* showed greater swimming performance when in the same time showing higher glucose level than exercised control group. Mice received 2.5% L-arginine also showed higher blood glucose level following exercise as compared to blood glucose of ECG. This may explain why the final trials of FST of LAG were significantly better (data not shown) than ECG.

Even though PGG produced greater swimming time that LAG, there was no proof as yet to elucidate mechanism of ergogenic effect of *P. glabra*. As for L-arginine, nitric oxide (NO) (and L-citrulline) is synthesized from the terminal guanidino nitrogen atom(s) of L-arginine by vascular endothelium under catalysis of NO synthase in the L-arginine/NO pathway which is present in many cells[13,14]. At rest, L-arginine increases release of plasma insulin, insulin-like growth factor-1, growth hormones, glucagon, catecholamines and prolactin[13,15-17]. Intravenously administered L-arginine stimulates insulin secretion leading to decreased output of glucose from liver[18], an effect that is also dependent on counter-action of plasma glucagon which functions to stimulate liver glucose output[19]. Small doses of oral arginine (1 mmol/kg) did not alter plasma insulin of humans while higher oral doses of 3 g/h for 10 h increased plasma insulin and inhibited liver glucose output[18]. However, aerobically trained individuals at rest showed diminished arginine-stimulated insulin secretion while arginine-stimulated increases in plasma glucagon and growth hormone were unaffected[13]. Beneficial effects of L-arginine supplementation during exercise was postulated to be due to increased glucose uptake as consequence of increased translocation of glucose transporter 4 (GLUT-4) to plasma membrane rather than to increase in skeletal muscle blood flow[13,20-22]. L-arginine supplementation to normal mice led to enhancement of exercise-induced endothelium-derived nitric oxide synthesis with consequent increase in aerobic capacity[23]. These mice showed an increase in VO_2 max and in running distance. A similar effect was seen in hypercholesterolemic mice which had reduced aerobic capacity as upon supplementation with L-arginine, exercise-induced endothelium-derived nitric oxide synthesis was restored and aerobic capacity was normalized[23]. NO promoted exercise hyperemia and has a role in cardiac and skeletal myocyte function. Moderate increases in plasma L-arginine caused vasodilation and increased oxygen utilisation by myocytes[24,25]. In excess, the converse happens as NO uncoupled mitochondrial respiration in cardiac and skeletal myocytes.

Muscle glycogen synthesis occurs in 2 phases following glycogen-depleting exercise[26]. The initial, rapid phase of of muscle glycogen synthesis lasts 30-60 minutes, it does not require insulin. In this phase, exercise-induced translocation of glucose transporter carrier protein-4 (GLUT-4) to cell surface occurs, resulting in enhanced entry of glucose through muscle membranes. The second phase lasts for several hours with much slower rate of glycogen synthesis. The rate-limiting enzyme in glycogen synthesis is glycogen synthase. Activity of glycogen synthase is increased by both muscle contraction and insulin and is regulated by muscle glycogen concentration[26]. Following exercise, low muscle glycogen concentrations are linked with increased rate of glucose transport and greater conversion of glucose into glycogen[27,28]. Since blood glucose levels were determined immediately following FST, glycogen synthesis is in the first phase which is non insulin dependent. Elevation of blood glucose level in L-arginine treated mice post-exercise may be explained by facilitation of glycogen synthesis by the amino acid [29] through enhancement of insulin release. In addition, L-arginine may have a stimulatory effect on post-exercise net muscle protein anabolism[30]. Ability of rodents to completely replenish muscle glycogen during recovery periods of repeated, high-intensity exercise bouts[31] is another explanation for elevation in blood glucose after exercise. Mice that were treated with *P. glabra* also had higher blood glucose than control group. The mechanism that explains effect of *P. glabra* on post-exercise glucose level needs to be worked out as this finding is first time reported. Logical mechanisms for elevation in plasma blood glucose following exercise in PGG may involve mechanisms of enhanced glucose transporter (GLUT4) activity,

involvement of protein kinase, glucocorticoid receptors etc.

All exercised mice, whether in treatment or control groups, showed significantly increased blood lactate levels as compared to resting lactate shown by normal, non exercised mice. Lactate is a by-product of energy metabolism during exercise[32]. High-intensity exercise training increases lactate transport in skeletal muscle and improves ability of muscle to release lactate during contractions[33]. Data from the present study showed increased exercise performance of PGG even when lactate production was similar to control provides some indication of anti-fatigue property of *P. glabra* that could be similar to effect of other herb demonstrated by a previous study[5].

FST significantly increased serum corticosterone in all exercised groups as compared to that of normal, non-exercised mice. Increase in serum corticosterone was found to be associated with increase in exercise duration[34], and following FST, as consequent to physical stress[35]. Whether *P. glabra* had any effect on the hormone is uncertain as corticosterone level at baseline (at rest) was not determined. A plausible explanation is that elevation of serum corticosterone of exercised groups occurred as a result of physical stress and as adaptation to the increased locomotor activity associated with the exercise regime[35,36] which was 5 weeks of study plus an initial 2 weeks of screening trials. Serum corticosterone levels were previously reported to respond to acute, not chronic exercise and were directly and linearly related to amount of exercise[37].

There appeared to be significant, positive correlation between serum corticosterone level and FST durations. There was better correlation between serum corticosterone level and final FST duration ($r=0.735$; $p=0.000$) than with overall FST duration. Even though secretion of corticosterone is correlated with exercise, adaptations in circadian plasma corticosterone can occur after a few weeks of voluntary exercise[38]. In other words, corticosterone response to exercise can be gradually decreased by voluntary exercise repeated for some duration of time to eventually reach sedentary levels. This may not have happened in this study as a forced exercise regime was assigned to mice instead of voluntary exercise. Further support to this is provided by findings of other studies that showed robust corticosterone response to acute exercise still occurring after 6 weeks of endurance swimming training[39].

In a study by Kimura and Sumiyoshi (2004)[6], corticosterone elevation induced by swimming stress was inhibited by a plant and the effect was associated with anti-fatigue effect of the herb. However, the intensity of swimming was not revealed nor was whether it was forced or voluntarily swimming. In that study, swimming was not conducted on a daily basis during the 9 days of experiment. In the present study, loaded mice (carrying a load of 10% of body weight) were subjected to 9 exercise bouts with each bout comprising of 3 consecutive days of FST with a day of rest in between bouts. Mice of the present study experienced more stress as compared to mice of the previous study.

Release of corticosterone is controlled by the hypothalamic-pituitary-adrenal axis and negative feedback by serum corticosterone levels under the influence of psychological and physical stressors[40] including low intensity exercise[41]. The swimming regime that exercised mice went through was sufficiently stressful to cause elevation in serum corticosterone levels as marker of stress[42].

Corticosterone stimulates gluconeogenesis[43]. During exercise, when carbohydrate (glycogen and glucose) levels drop and becomes limited, rate of gluconeogenesis from ketone bodies, glycerol and amino acids is increased to sustain the supply of carbohydrates[44]. Hypothalamic-pituitary-adrenal axis that controls release of corticosterone is indirectly responsible for regulation of gluconeogenesis[45]. Mice treated with *P. glabra* in this study, did not show any significant difference in corticosterone levels compared to control exercised mice. Thus *P. glabra* may have had no effect on corticosterone release during exercise.

Swimming to exhaustion can cause an increase in antioxidant enzyme levels viz, an increase in superoxide dismutase[46]. *P. glabra* has moderate antioxidant capacity[47], thus could have improved performance of treated in FST via this property. To confirm this, further studies are required to determine antioxidant status of *P. glabra*-treated mice during FST.

P. glabra was claimed to contain anthraquinones[48]. Anthraquinones are a group of secondary metabolites produced by plants including those of Rubiaceae family. They are used as traditional medicine for various purposes[49] including enhancement of immune ability[50] and treatment of periodic fever and malaria[51]. Anthraquinones possess antioxidant activity[51].

Anthraquinone can stimulate contraction of muscle fibres[52]. This means that anthroquinones in PG might have produced a similar effect to promote muscular endurance thus prolong swimming duration in FST. Ability of anthraquinones to trigger skeletal muscle contraction is due to stimulating mechanisms on Ca^{2+} release from sarcoplasmic reticulum[52]. Increased in exercise endurance is not only contributed by skeletal muscle fibres but also by contraction of cardiac muscles using the same mechanism of Ca^{2+} release[53,54].

All groups showed no change in body weight throughout the experiment period. Initial body weight (measured before intervention) and final body weight (measured after intervention) was similar in all exercised groups. In a previous report, voluntarily exercised mice consumed more energy than non-exercised mice resulting in decrease in body fat and energy stores i.e. inducing negative energy balance, causing the body weight to drop[55,56]. Exercised mice in the present study did not show a decrease in body weight. All groups showed normal eating behavior did not exhibit any abnormal behavior or show any signs or symptoms. There were no changes in major organs of liver, lung, heart and kidneys during gross necropsy as well.

Exercised, control mice (ECG) showed increased food consumption during the experiment as compared to non-exercised and exercised L-arginine-treated mice. *P. glabra*

did not influence food intake as PGG taking 500 mg *P. glabra* per kilogram body weight everyday did not exhibit different food intake from other groups. Inconstant effect of exercise on food consumption in mice is supported by previous studies. Droste et al. (2003)[57] reported voluntarily exercise did not influence food consumption in mice. In contrast, Yanagidaira et al. (2003)[58] showed mice exercised voluntarily exhibited increased food consumption. FST is anaerobic endurance exercise[46] that when perform it rigorously may decrease food intake in mice[59] probably as a consequence of increase in stress level[40]. PGG performed greater FST than ECG with similar stress levels as indicated by identical corticosterone levels. Higher level of FST without decreasing food intake suggests no suppression effect of *P. glabra* on appetite thus maintaining food intake.

Exercised mice exhibited increased water consumption during experiment period as compared to non-exercised mice. This is consistent with previous studies when exercise increases water consumption in mice[57,58]. Even though exercise could attribute to psychological and physical stress as previously reported[40] that could reduce fluid consumption[60], water intake of mice was not decreased due to FST shown in this study. Water intake of PGG was significantly lower than ECG but similar to NCG. In other words, *P. glabra*-treated mice consumed less water for greater FST than exercised controls. Stress levels of all exercised groups should have been similar as shown by serum corticosterone levels, thus variation in fluid consumption did not influence by stress factor. The effect of *P. glabra* on water regulatory and homeostasis should be further studied.

In summary, these findings provide evidence to support the folkloric claim for plant *P. glabra* for increasing stamina and improve physical performance. However, further studies need to be carried out.

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