

Antioxidant effect of *Phyllanthus amarus* after moderate-intensity exercise in sedentary males: a randomized crossover (double-blind) study

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Abstract. [Purpose] We aimed to evaluate the effects of *Phyllanthus amarus* (PA) on oxidative stress and damage, inflammation, and soreness in muscle after a single session of moderate-intensity exercise. [Subjects and Methods] Twelve men randomly participated in 2, three-day phases with a one-week washout period. On the first day, participants consumed two capsules of PA or placebo control (CTL) before 20 min of cycling. They then consumed four capsules on the same day after exercise and six capsules/day for the next two days. Blood samples were obtained before, immediately after exercise and 24 h and 48 h after exercise. The pain tolerance was measured at both legs. [Results] Plasma vitamin C levels in the PA group were higher than those in the CTL group after exercise. At 48 h after exercise, vitamin C levels were higher in the PA group, but those in the CTL group were lower than the pre-exercise levels. However, plasma levels of creatine kinase were increased in both groups after exercise compared with the pre-exercise levels. The neutrophil count was higher immediately after exercise than the pre-exercise levels in the CTL group. [Conclusion] Acute supplementation with PA improves antioxidant status after a single session of moderate-intensity exercise.

Key words: Exercise, Vitamin C, Muscle damage

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INTRODUCTION

The World Health Organization (WHO) introduced the Global Recommendations on Physical Activity for Health in 2010¹⁾, which encourages people of all ages to carry out moderate levels of physical activity. However, after their first session of moderate-intensity exercise, individuals often experience muscle soreness because they do not have sufficient time to adapt to various factors, such as increased mitochondrial activity leading to malondialdehyde (MDA) production from lipid peroxidation of the plasma membrane^{2, 3)}. This phenomenon can cause muscle damage, leakage of creatine kinase (CK)^{2, 4)} into the circulation, and inflammation^{5, 6)}. Then, C-reactive protein (CRP) is generated to stimulate the influx of inflammatory cells to repair muscular damage⁷⁾.

Neutrophils, lymphocytes, and monocytes are inflammatory cells with important roles in the damage/repair of muscle tissue due to exercise-induced injury. Neutrophils and monocytes repair muscle *via* oxidative or proteolytic modification to remove tissue debris in injured muscle⁸⁾. This process (“acute inflammatory response”) causes delayed onset of muscle soreness after exercise⁹⁾. The inflammation seems to be important for healing, but rapid recovery from previous exercise allows continuation of exercise training. Therefore, the search for an antioxidant supplement that can scavenge excess exercise-induced free radicals, resulting in reduced muscle soreness after a single session of moderate-intensity exercise, is important so that novice trainers can continue to exercise to improve their health¹⁰⁾.

Phyllanthus amarus (PA) is a plant that grows in tropical/subtropical areas, including Thailand. It comprises phytochemicals such as alkaloids, flavonoids, hydrolyzable tannins, major lignans, and polyphenols^{5, 11)}. These compounds can increase antioxidant status, which may reduce muscle damage. Several studies have revealed that PA has anti-inflammatory¹²⁾ and analgesic properties¹³⁾. Recently, we reported on the antioxidant and antinociceptive effects of PA on recovery after a single session of high-intensity exercise

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in sedentary male subjects⁵). However, a single session of moderate-intensity exercise was also shown to produce lipid peroxidation, inflammation, and damage in muscle^{2, 4}). We suspected that antioxidant supplementation is needed to reduce the risk of these adverse processes. Accordingly, we hypothesized that PA may confer beneficial effects on recovery after a single session of moderate-intensity exercise (which is recommended for health promotion in sedentary individuals). Hence, we investigated the acute effect of PA on oxidative stress and damage, inflammation, and soreness in muscles after a single session of moderate-intensity exercise.

SUBJECTS AND METHODS

Subjects provided written consent to participate in the study after receiving a written and verbal explanation of its requirements. The study protocol was approved by the Ethics Committee of Khon Kaen University (Khon Kaen, Thailand). The study conformed to the standards set by the Declaration of Helsinki in 2010 (HE531029). This study had a randomized crossover (double-blind) design. Twelve healthy, sedentary, male participants aged 22±2.90 (mean±SD) years were the study cohort. They were blinded to the composition of the PA or placebo control (CTL) they were given and to which treatment they would receive. Supplements were prepared by a researcher who was not involved in the remainder of the study. None of the participants were smokers; had neuromuscular, cardiovascular, orthopedic, renal, or liver diseases; had taken antioxidant supplements or medications; or undertook exercise regularly. Subjects underwent a physical examination and electrocardiography before enrollment in the study. To ascertain health status before participation in this study, blood samples were collected after a 12-h overnight fast to measure levels of glucose, creatinine, lipid profiles, and alanine aminotransferase.

WinPepi was used to calculate the sample size of the present study based on the method described in the study of Fenercioglu et al¹⁴). Previously, we reported that antioxidant supplementation prevented increases in MDA levels after exercise. We decided that we required 80% power at $p=0.05$. Therefore, the proposed sample size was 12 participants per treatment. The supplements used were PA and CTL. Aerial parts of PA were collected from the campus of Khon Kaen Province (Thailand) during August and September. The plant was identified by taxonomic means by Professor B.S. (Faculty of Pharmacy, Khon Kaen University). For preparation of PA, aerial parts were washed thoroughly under distilled water, oven-dried at 50 °C, and powdered in a mechanical grinder. Then, the plant was tested for microbial contamination. One-hundred milligrams of dried PA powder was placed into each capsule containing excipients. Each placebo control capsule contained Avicel® PH101 (microcrystalline cellulose; FMC BioPolymer, Philadelphia, PA, USA), Aerosil® (desiccant; Evonik Industries, Beijing, PR China), and artificial colors. The Center for Research and Development of Herbal Health Products of Khon Kaen University manufactured and controlled the contents of PA and CTL capsules. They were produced in the same manufacture lot. PA dose was based on recommendations for the product in

Thailand (G 357/42; Khaolaor Laboratories Co. Ltd., Samut Prakan, Thailand). Each PA capsule contained total polyphenol compounds (33 mg/g) and vitamin C (1.60 mg/100 g) as measured by the Central Laboratory (Thailand) Co., Ltd. (Bangkok, Thailand).

Participants undertook an incremental exercise test on a electromagnetic cycle ergometer (Corival, Lode, The Netherlands) fitted with a gas analysis system (ML206; ADInstruments, Bella Vista, NSW, Australia). Peak oxygen uptake ($VO_{2, peak}$) was determined if any of the following was achieved: VO_2 reached a plateau with increased workload; a respiratory gas exchange ratio >1.15; heart rate (HR) reached a maximum HR (calculated using the equation: $220 - \text{age}$); and maximal symptoms of dyspnea and fatigue using the ratings of perceived dyspnea and perceived exertion scales. Also, $VO_{2, peak}$ was determined if the participant could not maintain a cycling speed of 60 rpm. The relationship between workload and VO_2 from the test was used to determine the workload for the subsequent exercise session. Room temperature and humidity were 25±0.5 °C and 48±1.8%, respectively.

One week after the $VO_{2, peak}$ test, participants returned to the laboratory to participate randomly in three-day phases at one-week apart to avoid the carryover effect. On the first day of both phases, they consumed two capsules of PA or CTL 20 min before undertaking exercise with 3-min of warming up on the cycle ergometer. Then, they carried out moderate-intensity exercise (65% $VO_{2, peak}$) for 20 min. Subsequently they consumed four capsules of the same supplement (two capsules each after lunch and dinner) on the same day of the exercise. This supplementation regimen was continued for the next two days after exercise (two capsules/time; t.d.s.). At each visit, a 12-mL blood sample was collected from the antecubital vein of each participant immediately before and after exercise, and 12-mL blood samples were also collected at 24 h and 48 h after exercise. One week before and during the experiment, subjects were asked to drink no more than one glass of fruit juice and two cups of coffee/tea per day. Fruit juices especially rich in antioxidants (e.g., orange juice) were to be avoided.

Each blood sample was divided into three parts: 6 mL into ethylenediaminetetraacetic acid (EDTA)-lined tubes, 2 mL into lithium heparin-lined tubes, 4 mL into clotting tubes. Tubes were placed immediately on ice. $HClO_4$ (1 mol/L) was then added to one EDTA tube (2 mL) to precipitate protein for the measurement of vitamin C levels. All tubes were centrifuged at $3,000 \times g$ for 15 min at 4 °C. The upper layer was transferred to a microcentrifuge tube and stored at -80 °C until assay. Levels of CK and high-sensitivity C reactive protein (hs-CRP) and a complete blood count were ascertained in the clinical laboratory of Srinagarind Hospital (Faculty of Medicine, Khon Kaen University). Levels of MDA, vitamin C, and nitric oxide radicals (NO^{\cdot}) were measured by our research team.

The MDA level in plasma is a marker of lipid peroxidation. MDA levels were measured using the thiobarbituric acid (TBA) test according to the method of Draper¹⁵). The method of Zhang et al.¹⁶) was used to measure plasma levels of vitamin C. For measurement of plasma levels of NO^{\cdot} , the inNO Nitric Oxide Measuring System and Sensor

(Innovative Instruments Inc., Tampa, FL, USA) was used. CK activity in serum was assessed as an indicator of muscle damage using standard automated laboratory methods with a cobas® c 502 Analyzer (Roche, Basel, Switzerland). hsCRP level was determined using a particle-enhanced immunoturbidimetric assay. In this assessment, levels of human hsCRP agglutinated with latex particles coated with monoclonal anti-CRP antibodies were determined by turbidimetric means. A Mechanical Algometer (FPK/FPN; Wagner Instruments, Riverside, CT, USA) was used to measure the pressure-pain tolerance of the quadriceps muscle at the mid-thigh point. During measurement, participants were asked to indicate verbally when the pressure became intolerable. Participants were instructed to record their normal diet during the study period. They completed food records for the three days before the experiment (two weekdays and one weekend day).

Table 1. Anthropometric and physiologic characteristics of participants

	Mean ± SD
Age (yrs)	22.0±2.90
Height (m)	1.70±0.06
Body weight (kg)	63.6±10.7
BMI (kg/m ²)	22.8±3.3
BF (%)	19.5±9.0
FM (kg)	12.5±7.3
FFM (kg)	48.4±6.6
Waist circumference (cm)	76.7±7.5
Hip circumference (cm)	94.8±6.1
Waist:hip ratio	0.81±0.04
VO _{2, peak} (mL/kg body weight/min)	36.9±5.2
Work load _{max} (w)	136.1±32.4
% VO _{2, peak} during exercise in CTL treatment	70.8±10.9
% VO _{2, peak} during exercise in PA treatment	68.4±18.9

Values are the mean ± SD; n=12 for each group.

BMI: body mass index; % BF: percentage of body fat; FM: fat mass; FFM: fat free mass; VO_{2, peak}: peak oxygen consumption; CTL: placebo control; PA: *P. amarus*

Records were analyzed for dietary composition using Inmucal (Mahidol University, Bangkok, Thailand). Participants were instructed to keep records of physical activities for three days before the experimental (two weekdays and one weekend day). Records were analyzed for energy expenditure¹⁷.

Descriptive data are presented as the mean ± SD unless stated otherwise. Two-way analysis of variance with repeated measures was used to compare the PA and CTL groups at each time point during recovery. A paired t test with Bonferroni correction was used to ascertain significant differences at each time point. Statistical analyses were carried out using PASW statistics v18 (SPSS, Chicago, IL, USA). P<0.05 was considered significant.

RESULTS

Twelve healthy, sedentary males aged 22.0±2.90 years participated in this study. Anthropometric and physiological characteristics are summarized in Table 1. With regard to VO_{2, peak}, all participants carried out exercise at moderate intensity (Table 1). Physiological parameters, dietary composition, and energy expenditure were not significantly different between the PA and CTL groups (Table 2). Baseline blood parameters were within normal ranges (Table 3).

Table 3. Baseline blood parameters of participants

	Mean ± SD
Blood glucose (mM)	4.6±0.3
Creatinine (μmol/L)	0.01±0.001
ALT (U/L)	23.1±24.9
TG (mM)	1.1±0.3
TC (mM)	2.6±1.4
LDL (mM)	2.8±0.9
HDL (mM)	1.6±0.4

Values are the mean ± SD; n=12.

ALT: alanine transferase; TG: triglycerides; TC: total cholesterol; LDL: low-density lipoprotein; HDL: high-density lipoprotein

Table 2. Daily dietary composition and energy expenditure of participants

	CTL	PA
Protein (g)	122.9±30.6	125.4±35.6
Fat (g)	89.3±30.2	78.0±17.4
Carbohydrate (g)	254.3±71.7	239.2±62.9
Fiber (g)	5.6±2.0	7.1±2.1
Thiamine (mg)	1.3±0.4	1.2±0.5
Riboflavin (mg)	1.9±1.0	1.4±0.5
Niacin (mg)	20.7±6.6	18.6±6.5
Vitamin C (mg)	23.2±18.4	27.1±12.2
Vitamin A (RE)	746.1±1388.8	710.4±1382.4
Energy intake (kcal/day)	2289.2±475.8	2112.0±423.2
Energy expenditure (kcal/day)	2292.1±388.1	2127.9±654.6

Values are the mean ± SD; n=12 for each group.

CTL: placebo control; PA: *P. amarus*; vitamin A 1 RE = 12 μg beta carotene

Vitamin C levels in the PA group were significantly higher than those in the CTL group immediately after moderate-intensity exercise ($p<0.05$) and 24 h ($p<0.01$) and 48 h after moderate-intensity exercise ($p<0.05$, Table 4). When vitamin C levels at 48 h after moderate-intensity exercise were compared with those before exercise, they were significantly higher in the PA group but lower in the CTL group ($p<0.05$). However, MDA levels and NO \cdot levels were not significantly different between the PA and CTL groups at any time point (Table 4).

Plasma levels of CK were increased significantly immediately after exercise compared with those before exercise ($p<0.05$, Table 4) and returned to pre-exercise levels in both supplementation groups. Moreover, hsCRP levels were not significantly different between the PA and CTL groups at any time point (Table 4). Neutrophil counts were significantly higher immediately after exercise than before exercise in the CTL group ($p<0.05$). However, the lymphocyte count and monocyte count were not significantly different between the supplement treatments at any time point (Table 5). The pain

tolerance of both legs was not significantly different between the PA and CTL groups at any time point (Table 6).

DISCUSSION

This is the first study to show that acute supplementation of PA increased plasma levels of vitamin C during the recovery period after a single session of moderate-intensity exercise in healthy, sedentary participants. However, PA supplementation did not alter levels of lipid peroxidation, damage, inflammation, or soreness in muscle.

A single session of moderate-intensity exercise was shown to contribute to muscle damage as reflected by increased levels of CK. This exercise-induced muscle damage is in agreement with two previous studies^{2, 4}). Both studies reported increased levels of CK after running on a treadmill for 20–30 min at 60% VO $_{2, peak}$. Moflehi et al. (2012) demonstrated muscle damage with increased lipid peroxidation, but Seifi-Skishahr et al. (2008) showed muscle damage without increased lipid peroxidation. We found that the neutrophil

Table 4. Levels of MDA, vitamin C, NO \cdot , CK, and hs-CRP before exercise (pre-exercise), as well as immediately after moderate-intensity exercise (post-exercise) and 24 h (post-24 h) and 48 h (post-48 h) after moderate-intensity exercise

		Time			
		Pre-exercise	Post-exercise	Post-24 h	Post-48 h
MDA (nM)	CTL	1.74±0.45	3.09±0.82	3.48±0.79	2.87±0.95
	PA	1.72±0.40	2.59±0.64	2.23±0.74	2.08±0.63
Vitamin C (µg/mL)	CTL	7.97±1.21	7.18±1.05	7.45±1.20	7.35±1.06*
	PA	7.82±1.37	10.98±1.82#	11.91±1.96#	10.92±1.93*.#
NO \cdot (nM)	CTL	141.0±58.4	134.8±62.0	131.5±27.3	113.8±48.4
	PA	145.4±52.1	165.3±70.0	134.6±16.8	144.3±64.2
CK (U/L)	CTL	146.2±24.3	158.6±25.8*	167.2±28	142.3±21.4
	PA	149.3±24.4	157.9±24.3*	151.7±23.7	161.34±32.8
hs-CRP (mg/L)	CTL	0.60±0.19	0.65±0.2	0.74±0.25	0.66±0.28
	PA	0.53±0.17	0.52±0.17	0.71±0.28	0.81±0.25

Values are the mean ± SE; n=12 for each group.

CTL: placebo control; PA: *P. amarus*; MDA: malondialdehyde; NO \cdot : nitric oxide radical

* Significantly different from pre-exercise ($p<0.05$),

Significantly different from the CTL group at the same time point ($p<0.05$)

Table 5. Counts of neutrophils, lymphocytes, and monocytes before exercise (pre-exercise), as well as immediately after moderate-intensity exercise (post-exercise) and 24 h (post-24 h) and 48 h (post-48 h) after moderate-intensity exercise

		Time			
		Pre-exercise	Post-exercise	Post-24 h	Post-48 h
Neutrophils (10 3 /µL)	CTL	3.65±0.31	4.11±0.36*	3.56±0.30	3.41±0.46
	PA	3.23±0.47	3.46±0.43	3.40±0.22	3.58±0.41
Lymphocytes (10 3 /µL)	CTL	2.06±0.18	2.58±0.34	2.05±0.18	2.16±0.17
	PA	2.08±0.21	2.33±0.22	1.95±0.18	2.11±0.21
Monocyte (10 3 /µL)	CTL	0.46±0.04	0.57±0.07	0.47±0.06	0.42±0.06
	PA	0.52±0.07	0.59±0.08	0.45±0.07	0.52±0.08

Values are the mean ± SE; n=12 for each group.

CTL: placebo control; PA: *P. amarus*

*Significantly different from pre-exercise ($p<0.05$)

count increased immediately after exercise in the control group. This finding may reflect minor inflammation after exercise without PA supplementation. Exercise also decreased vitamin C levels a few days later in the control group. This observation is supported by a study that found decreased vitamin C levels after a single session of exercise without prior supplementation in untrained, healthy subjects¹⁸). That finding may show that the damage resulted from mechanical stress¹⁹). The mechanical forces occur at the sarcomere level and induce structural damage to contractile proteins and plasma membranes¹⁹). However, the muscle damage observed in the present study appeared to be small because CK leakage was minimal and the increases in neutrophil count were low, and it only occurred immediately after exercise without changes in inflammation. The unaltered inflammation may explain why there was no change in pressure pain tolerance when the CK was increased after exercise.

Our research team has examined, using a similar protocol, these affects after high-intensity exercise⁵). Our results in that study confirmed the beneficial effect of PA supplementation in subjects after exercise that produced high oxidative stress. We found that PA supplementation increased plasma levels of vitamin C immediately after high-intensity exercise, which might result in decreased lipid peroxidation and muscle soreness two days later⁵). Vitamin C levels increased over pre-exercise values throughout the recovery period in the present study but increased only immediately after exercise in our previous study⁵). This disparity in results may suggest that moderate-intensity exercise requires (as antioxidant activity) less vitamin C than high-intensity exercise. Increases in levels of other antioxidants (e.g., glutathione peroxidase, glutathione reductase, catalase) may be responsible for free radical scavenging^{20, 21}). Further research investigating the effect of PA supplementation on these antioxidants during recovery from moderate-intensity exercise is needed to confirm this hypothesis.

In the present study, PA supplementation increased antioxidant levels as reflected by increased vitamin C levels in plasma after moderate-intensity exercise. This finding may show PA supplementation to be an additional source of vitamin C that cannot be synthesized by the human body. The PA-induced increase in plasma vitamin C level is supported by previous studies in the human body⁵) and rat²²). Although the vitamin C content in the PA in the present study may have been very small (1.60 mg/100 g), a previous study reported that supplementation with less than 200 mg of vitamin C

increased the absorption rate to 98%²³). This may have contributed to the increased levels of plasma vitamin C in the PA group in the present study. Taking this together with the lack of inflammation and soreness in muscle, it is appropriate to recommend PA supplementation before 20 min of moderate-intensity exercise for health promotion. Exercise for 20 min is practical for healthy, sedentary Thai participants who, under normal circumstances, do not like to exercise for longer. Importantly, elderly individuals²⁴), patients (e.g., those with diabetes mellitus²⁵), cancer²⁶), or pre-hypertension²⁷), and cigarette smokers³) who have high oxidative stress even at rest may need antioxidant supplementation during exercise at this intensity. The effect of PA supplementation on oxidative stress and damage, inflammation, and soreness in muscle after a longer-duration of exercise at moderate intensity in elderly individuals, cigarette smokers, and patients with metabolic problems should be studied further.

In this study, the PA in each capsule also contained 33 mg/g of total polyphenol compounds (19.8 mg/day). The effects of polyphenol compounds on biomarkers of oxidative stress and muscle damage induced by exercise have been studied²⁸). Intra and Kuo²⁹) suggested that, even though the physiologic level (0.1–1 μ M) of dietary catechins (which contain polyphenol compounds) is much lower than that of vitamins, dietary catechins contribute to total antioxidant capacity even in the presence of vitamins. We did not measure levels of polyphenol compounds in blood in the present study. Together with other antioxidants in the PA¹¹), polyphenol compounds may have played a part as antioxidants during exercise recovery in the present study. Their actions may have preserved vitamin C levels after exercise throughout the recovery period.

In accordance with a review article examining the impact of antioxidant supplementation on high-intensity exercise³⁰), we prepared and stored the PA in the present study carefully to prevent oxidation and degradation of PA. Moreover, we assessed the diet composition and physical activity associated with both treatments, and the results confirmed that there was no difference between them. A one-week washout period was sufficient to eliminate the effect of the previous treatment because supplementation took only three days. This was confirmed by data at the start of each treatment, which were not significantly different.

A limitation of our study was that we measured only plasma levels of MDA and vitamin C to determine oxidative status, but we stored and analyzed them carefully within 3

Table 6. Levels of muscle soreness in the right leg and left leg before exercise (pre-exercise), as well as immediately after moderate-intensity exercise (post-exercise) and 24 h (post 24 h) and 48 h (post 48 h) after moderate-intensity exercise

		Time			
		Pre-exercise	Post-exercise	Post-24 h	Post-48 h
Right leg	CTL	4.7 \pm 0.4	4.8 \pm 0.4	5.0 \pm 0.6	5.3 \pm 0.6
	PA	4.4 \pm 0.2	4.5 \pm 0.5	4.9 \pm 0.6	4.8 \pm 0.5
Left leg	CTL	4.7 \pm 0.4	4.6 \pm 0.4	5.0 \pm 0.5	5.4 \pm 0.5
	PA	4.6 \pm 0.4	4.4 \pm 0.4	4.9 \pm 0.5	5.1 \pm 0.5

Values are the mean \pm SE; n=12 for each group.
CTL: placebo control; PA: *P. amarus*

months. Moreover, one might question whether vitamin C, which is located in aqueous compartments, can be used to scavenge lipophilic radicals. In fact, there is a well-described dependency between these two antioxidants: vitamin C recycles vitamin E *via* the tocopheroxyl radical³¹). Therefore, vitamin C appears to scavenge lipophilic radicals indirectly. Importantly, unaltered levels of hsCRP suggest that PA supplementation did not produce harmful effects in the male sedentary participants.

We showed that moderate-intensity exercise caused minimal muscle damage. Acute PA supplementation increased plasma levels of vitamin C throughout the two days after moderate-intensity exercise. However, PA supplementation did not affect lipid peroxidation or inflammation, damage, and soreness in muscle during the recovery period.

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