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Morinda citrifolia leaf enhanced performance by improving angiogenesis, mitochondrial biogenesis, antioxidant, anti-inflammatory & stress responses

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ABSTRACT

Morinda citrifolia fruit, (noni), enhanced performances in athletes and post-menopausal women in clinical studies. This report shows the edible *noni* leaves water extract enhances performance in a weight-loaded swimming animal model better than the fruit or standardized green tea extract. The 4 weeks study showed the extract (containing scopoletin and epicatechin) progressively prolonged the time to exhaustion by threefold longer than the control, fruit or tea extract. The extract improved (i) the mammalian antioxidant responses (MDA, GSH and SOD2 levels), (ii) tissue nutrient (glucose) and metabolite (lactate) management, (iii) stress hormone (cortisol) regulation; (iv) neurotransmitter (dopamine, noradrenaline, serotonin) expressions, transporter or receptor levels, (v) anti-inflammatory (IL4 & IL10) responses; (v) skeletal muscle angiogenesis (VEGFA) and (v) energy and mitochondrial biogenesis (via PGC, UCP3, NRF2, AMPK, MAPK1, and CAMK4). The ergogenic extract helped delay fatigue by enhancing energy production, regulation and efficiency, which suggests benefits for physical activities and disease recovery.

1. Introduction

Morinda citrifolia fruit, (called noni in USA, Mengkudu in Malaysia) has been traditionally consumed by Polynesians to maintain health and vigor besides combating fatigue or diseases (Thaman, 1990). Two clinical studies on athletes and post-menopausal women demonstrated the noni juice effect on improving endurance (Langford, Doughty, Wang, Clayton, & Babich, 2004; Palu, Seifulla, & West, 2008). Another in vivo study on aged mice, given increasing doses of Tahitian Noni Juice orally (10, 20 and 40 mL/kg body weight) showed significantly longer average time in both the swim test and the rotarod test when compared with young and aged control (Ma et al., 2007). However, the fruit have been associated with liver toxicity (Millonig, Stadlmann, & Vogel, 2005), while the leaves are consumed as vegetables after blanching since ancient times. *M. citrifolia* leaves reportedly have antioxidant, liver-protective and wound healing properties without any acute, sub-acute and sub-chronic oral toxicity (West, Tani, Palu, Tolson, & Jensen, 2007). The *M. citrifolia* leaves extract oral no observed-adverse-effect level (NOAEL) is 1000 mg/kg (Lagarto, Bueno, & Merino, 2013).

Ergogenic functional foods help improve physical performance or suppress fatigue, by enhancing energy production, regulation or efficiency. It is not only useful in sports but also for recovery from illnesses. Compounds with ergogenic potential include vitamins, protein, amino acid, sodium bicarbonate (Shelton & Kumar, 2010), caffeine, creatine monohydrate and herbs (Chen, Muhamad, & Ooi, 2012). Caffeine for example has anti-fatigue and alertness effects and the ergogenic anabolic steroids such as amphetamines have been abused and banned for athletes.

Fatigue may be influenced or caused by (i) excessive levels of reactive oxygen species (ROS) accumulating in the contracting muscles, that inhibit force production (Reid, 2001), (ii) muscle

Abbreviations: AMPK, AMP-activated protein kinase; CAMK4, calcium/calmodulin-dependant protein kinase IV; DRD2, dopamine receptor D2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSH, glutathione; H6PD, hexose-6-phosphate dehydrogenase; HPA, hypothalamic–pituitary–adrenal axis; HSP90AB1, heat shock protein 90 kDa alpha (cytosolic) class B member 1; IL10, interleukin-10; IL4, interleukin-4; MAPK1, mitogen-activated protein kinase 1; MDA, malondialdehyde; NADPH, nicotinamide adenine dinucleotide phosphate-oxidase. NR3C1; NRF2/NFE2L2, nuclear factor erythroid derived 2 like 2; PGC, peroxisome proliferative activated receptor gamma; PRKAA1, protein kinase AMP-activated alpha 1 catalytic subunit; ROS, reactive oxygen species; SLC6A2, solute carrier family 6 (neutransmitter transporter noradrenaline) member 2; SLC6A4, solute carrier family 6 (neutransmitter transporter serotonin) member 4; SOD2, superoxide dismutase 2; UCP3, uncoupling protein 3; VEGFA, vascular endothelial growth factor A.

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contraction-associated pro-inflammatory cytokines increase (Radak, Naito, Taylor, & Goto, 2012), (iii) energy source depletion and excess metabolite accumulation (You, Zhao, Liu, & Regenstein, 2011), or (iv) de-regulation of neuro-immune-endocrine dysfunction and (v) alteration of Hypothalamic–pitui tary–adrenal (HPA) axis activity (Gupta, Aslakson, Gurbaxani, & Vernon, 2007; Rajeevan et al., 2007; Watanabe, Evengård, Natelson, Jason, & Kuratsune, 2008). This research studies the ergogenic and anti-fatigue properties of *M. citrifolia* leaves water extract and the molecular mechanisms of actions involved, through weight-loaded mice swimming test.

2. Material and methodology

2.1. Plant material, aqueous extraction and High-pressure liquid chromatography (HPLC) analysis

Morinda citrifolia leaves (MCL) were obtained from the Institute of Bioscience, Universiti Putra Malaysia. A voucher specimen SK2322/14 was deposited at the Laboratory of Natural Products, Institute of Bioscience, Universiti Putra Malaysia. Leaves of *M. citrifolia* were oven-dried at 60 °C for 24 h, ground and extracted by boiling in distilled water (1:10, w/v) for 3 h. The resulting suspensions were filtered and evaporated to dryness at 60 °C. Standardized green tea water extract containing 95% polyphenol, 5% caffeine and 40% epigallocatechingallate, (Seamax Resources Sdn Bhd, Kajang, Malaysia) were used as the positive control for comparison.

The extracts were analyzed by HPLC (Waters 2996, Milford, MA). Epicatechin, scopoletin, HPLC grade methanol (MeOH), acetonitrile (MeCN) and analytical grade trifluoroacetic acid (TFA) were obtained from Merck (Darmstadt, Germany). Chromatographic separation was equipped with an Atlantis C18 column (4.6 mm × 250 mm; 5 μm, Waters Corporation, Milford, MA, USA). The pump was connected to a mobile phase system composed of three solvents: A; MeCN, B; MeOH, and C; 0.1 TFA% in H_2O (v/v). The mobile phase was programmed consecutively in linear gradients as follows: 0 min, 10% A, 10% B, and 80% C; 15 min, 20% A, 20% B, and 60% C; 26 min, 40% A, 40% B, and 20% C; 28–39 min, 50% A, 50% B, and 0% C; and 40–45 min, 10% A, 10% B, and 80% C. The elution was run at a flow rate of 1.0 mL/min. The UV spectra were monitored in the range of 210 and 450 nm. The injection volume was 50 μL for each of the sample solutions. The marker compounds scopoletin and epicatechin were present at 6 mg and 9 mg respectively per g of the leaf extract.

2.2. Animals and treatments

Female ICR (Imprinting Control Region) mice, aged 6–7 weeks, were provided by the Faculty of Veterinary Medicine, Universiti Putra Malaysia, and acclimatized for a week to the laboratory conditions before the experiment. Animal were housed in an environmentally controlled animal laboratory and maintained on a 12-h light/dark cycle at 25 ± 2 °C. They were given standard pellet food (Gold Coins from A Sapphire Enterprise, Malaysia) and water ad libitum. The experimental protocol was approved by the Institutional Animal Care and Use Committee, Universiti Putra Malaysia (Ref: UPM/IACUC/AUP-R022/2013).

Their baseline initial swimming time endurance capacities were measured after acclimatization. The mice were divided into 5 groups (n = 10, 5 mice/cage): (i) MCL200 group received 200 mg *M. citrifolia* leaves extract/kg bw, (ii) MCL400 group received 400 mg extract/kg bw, (iii) GT positive control group received 200 mg green tea extract/kg bw, (iv) negative control with exercise (CE) group received the vehicle only and (v) negative control (C) without exercise group also received the vehicle only. The treatments were given by oral gavage daily for 28 days.

2.3. Weight-loaded swimming test

The swimming endurance capacity was assessed weekly for 4 weeks as the swimming time (s) from start to fatigue. A 5% weight was tied at each mice tail, to force it to keep swimming or submerge at fatigue point (and immediately rescued). The swimming pool water was maintained at 25 ± 2 °C. In the final week, the mice were sacrificed under ether anesthesia immediately after exercise. Blood samples were taken by cardiac puncture, then centrifuged at 2000*g* for 15 min to separate the serum and stored at −80 °C until further analysis. The skeletal muscle and liver tissues were isolated and stored at −80 °C for mRNA gene expression and histopathological observations.

2.4. Sample analysis

Blood glucose and blood lactate was measured using an autoanalyzer (Brand, country). Blood plasma, skeletal muscle and liver glutathione (GSH), blood cortisol and serum malondialdehyde (MDA) levels were analyzed using commercial ELISA kits (Cayman Chemical Company), according to the manufacturer's instructions.

2.5. mRNA gene expression

Quantitative RT-PCR analysis was performed using the comparative threshold cycle method to calculate fold change in gene expression of anti-inflammatory cytokines [IL-4 (Interleukin-4) and IL-10 (Interleukin-10)], angiogenesis marker [VEGFA (vascular endothelial growth factor A)], antioxidant enzyme [SOD2 (Superoxide dismutase 2)], mitochondrial biogenesis or fatty acid metabolism [UCP3 (Superoxide dismutase 2), PGC (Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha), NFE2L2 (Nuclear factor, erythroid derived 2, like 2)], anabolic kinase signalling [MAPK1 (Mitogen-activated protein kinase 1), PRKAA1 (Protein kinase, AMP-activated, alpha 1 catalytic subunit)], Ca^{2+} anabolic signalling [CAMK4 (Calcium/calmodulin-dependent protein kinase IV)], hypothalamic–pituitary–adrenal (HPA) axis function [NR3C1 (Nuclear receptor subfamily 3, group C, member 1), H6PD (Hexose-6-phosphate dehydrogenase)], and neurotransmitter [DRD2 (Dopamine receptor D2), SLC6A4 (serotonin transporter: Solute carrier family 6, member 4), SLC6A2(noradrenalin transporter: Solute carrier family, member 2)], normalized to beta-actin, GAPDH and HSP90AB1 (Heat shock protein 90 kDa Gene ID: 3326) as the reference housekeeping gene.

RNA was isolated using Trizol (Invitrogen, Carlsbad, CA). Custom RT^2 Profiler PCR Array (CAPM11988), RT^2 SYBR Green qPCRMastermix, RT^2 First Strand Kit and RNase-Free DNase Set were from SuperArray Bioscience Corporation (Frederick, MD). Quantitative RT-PCR array for differentially expressed genes was performed utilizing RT^2 Profiler PCR Array Data Analysis version 3.5 (SABiosciences; Fredrick, MD, USA), which normalized to beta-actin (NM_009793), HSP90AB1 (NM_008302) and GAPDH (NM_008084) as housekeeping genes. RT-PCR data is represented as the average relative mRNA gene expression of each experimental group (n = 3). The p values only for RT-PCR are calculated based on a *t*-test of the replicate 2^(−ΔCt) values for each gene in the control group and treatment groups.

2.6. Statistical analysis

All data are expressed as mean ± S.D. The significance of differences between groups was determined using one or 2-way analysis of variance (ANOVA). Differences between groups were further