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ANTI-FATIGUE EFFECTS OF POLYPHENOLS EXTRACTED FROM *ARECA CATECHU* L. HUSK AND DETERMINATION OF THE MAIN COMPONENTS BY HIGH PERFORMANCE CAPILLARY ELECTROPHORESIS

MINMIN TANG, HUA CHEN, HUI WANG[1], SONGLIN ZHAO* AND JING QI[2]

Coconut Research Institute, Chinese Academy of Tropical Agricultural Sciences, 571339, Wenchang, China

Key words: Areca catechu, Husk, Polyphenols, Antioxidant, Anti-fatigue

Abstract

This study explored the antioxidant potential and anti-fatigue effects of polyphenols extracted from *Areca Catechu* L. husk (AHP). Radicals scavenging activities and reducing power were determined to evaluate the antioxidant potentials *in vitro* and a weight loaded forced swimming test (WFST) was used to estimate the anti-fatigue effect. The main components of AHP were analyzed by HPCE at last. The AHP possessed considerable antioxidant effects on scavenging DPPH, ABTS radicals and reducing power. All the tested bio parameters of the mice which received the high dose of AHP (20 mg/kg) were improved. The AHP was mainly composed of (–)-epicatechin, (+)-catechin, keampferol, naringenin, ferulic acid and chromogenic acid. These results suggest that the AHP can be a promising source as a natural antioxidant and anti-fatigue material for the use in functional foods even medicines.

Introduction

Physical fatigue, mainly caused by excessive exercise, is an important factor affecting bodily action, and often results in physical disorders (Tanaka *et al.* 2008). According to various reported theories, the most accepted one is excessive production of reactive oxygen species (ROS), as they are highly reactive particles, which can result in cytotoxicity (Chi *et al.* 2015). Compared to synthetic drugs, natural products not simply can do well on improving human's physical performance against fatigue, but also have a few side effects (Kim *et al.* 2002). Thus, people are eager to seek natural effective compounds to ease fatigue, such as phenolic extract (Wu *et al.* 2013).

Phenolic compounds play crucially in areca nut bioactivities including anti-mutagenic (Nagabhushan *et al.* 1998) and anti-aging activities (Lee *et al.* 2001). However, there are a few reports about anti-fatigue activities of phenolics from areca nut. In order to achieve a firm conclusion, we extracted AHP and determined its antioxidant activities *in vitro* and anti-fatigue effects *in vivo*. Total phenolic content of the AHP was measured and the phenolic components of the purified extraction were identified.

Materials and Methods

All phenolic standards were purchased from National Institutes for Food and Drug Control (Beijing, China). All the chemicals of analytical grade were purchased from Sigma-Aldrich Co. LLC.. Nanjing Jiancheng Bioengineering Institute (Nanjing, China) provided all the paid test kits used in this study. Experimental water was ultra-purified.

*Author for correspondence: <36491365@qq.com>. [1]Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences, 571101, Haikou, China. [2]College of Food Science, Jiangnan University, 214122, Lihu Avenue, Wuxi, China.

The *Areca catechu* fruits (cv. Reyan No. 1) were purchased from a local market and freeze-dried after pitted. Crude AHP was extracted following our previous operation (Chen *et al.* 2014). The purified AHP were obtained by removing organic acids, sugars and other non-phenolic compositions using solid-phase extraction (SPE) cartridges (Chirinos *et al.* 2007).

The scavenging activity of AHP on DPPH• was performed following the method described by Wu *et al.* 2013. Two milliliters of 0.15 mmol/l DPPH salt dissolved in 95% ethanol was added into 2.0 ml of AHP or BHT (0 - 75 μg/ml) and the mixture was shaken vigorously to be homogenous. DPPH radical solution added with the equal amount of 95% ethanol as blank control. After incubated in a dark place for 30 min, the absorbance decrease at 517 nm of the reacting solution was recorded. BHT was used for comparison. The scavenging rate was calculated followed equation below:

$$\text{DPPH• scavenging rate (\%)} = (A_{sample} - A_{sample\ control}) / A_{blank\ control} \times 100$$

The reducing power of AHP was performed following the method reported by Wu *et al.* (2013) with a little modification. 0.2 ml sample at different concentrations was added 1.0 ml of 0.2 mol/l phosphate buffer (pH 6.6) and 1.0 ml of 1% potassium-ferricyanide. The mixtures reacted in a water bath at 50°C for 20 min. Then, 1.0 ml of 10% trichloracetic acid was added into the mixture to terminate the reaction. 1.0 ml of the reacted mixture was mixed with 1.0 ml of ultra-purified water and 0.1 ml of 0.1% $FeCl_3$ and the absorbance at 700 nm was measured.

The ABTS assay was based on the decolorization of the blue/green $ABTS^{\bullet+}$ produced from the oxidation of ABTS by potassium persulphate for a proper time in the dark (Baltrusaityte *et al.* 2007). The prepared $ABTS^{\bullet+}$ solution was attenuated with methanol to an absorbance of 0.700 ±0.02 at 734 nm. After 100 μl of sample solution had reacted with 1.0 ml of $ABTS^{\bullet+}$ solution for 30 min at room temperature, the absorbance values at 734 nm were recorded. 1.0 ml $ABTS^{\bullet+}$ solution mixed with 100 μl water was used as blank control. The scavenging rate against $ABTS^{\bullet+}$ of tested samples was calculated following the equation below:

$$ABTS^{\bullet+}\ \text{scavenging rate (\%)} = (1 - A_{sample}/A_{blank\ control}) \times 100.$$

For the WFST, 8 weeks old male Kunming mice (Medical Laboratory Animal Center of Guangdong Province, Foshan, China) weighing 17~20 g at arrival in the laboratory, were housed at a room temperature held at 25°C with 12 hrs light/dark periods (lights from 6:00 to 18:00 hrs). Food and boiled distilled water were available casually.

The mice were randomly allocated into five groups (n = 10) by weight: Untreated control (UTC)-mice in this group were treated with saline and didn't experience the WFST; Untreated swimming group (UTS)-mice in this group were treated with saline and experienced the WFST; AHP-LDG, AHP-MDG, and AHP-HDG groups: mice in those three groups were treated with different doses of AHP (5, 10 and 20 mg/kg), respectively and experienced the WFST. Saline/AHP was intragastrically administered (8:00 a.m.) to mice for 15 days and the WFST was conducted on the last day, 30 min after compound administration.

The WFST was carried out employing the method described by Porsolt *et al.* 1978 with some modification. After the last administration, each mouse was placed in an individual glass cylinder (35 × 20 cm), containing 30 cm water (25 ± 1°C). Copper wire weighing seven per cent of its body weight was tagged to the tail root of each mouse. The mice were judged to be exhausted when they floated in the water and failed to return to the surface within 10 sec. The mice were then sacrificed for further examination.

After the mice were anesthetized mildly, blood was collected from heart and serum was prepared by centrifuging the blood at 3000 rpm at 4°C for 10 min. Values of BUN, BLA and LDH in serum were determined with commercially available kits. The content of MDA and activities of antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), were also determined using commercially available kits. Liver and muscle tissues were detached for the determination of HG and MG, respectively.

The total phenolic content of the AHP was determined by Folin-Ciocalteu method (Wu *et al.* 2013) and was expressed as mg gallic acid/g dry husk powder. AHP ingredients analysis was performed with a P/ACETM MDQ HPCE (Beckman Coulter, Fullerton, CA, USA) and a standard Beckman Coulter eCAP fused-silica capillary (50 μm inner diameter) was used for separation with the effective length was 50 cm. Detection was carried out at the cathode by on-column measurement of absorbance at 210 nm.

Phenolic standard substances were dissolved in pure CH_3OH at a concentration of 1 mg/ml. The stock standard solution was diluted with CH_3OH into different concentrations to prepare working standard solutions. 0.1 mol/l boric acid (H_3BO_3) solutions at pH 9.0 worked as the separation buffer in the HPCE analysis and the voltage was 20 kV generating 57 μA current. Temperature in capillary was 20°C. The tested samples were hydrodynamically injected into the capillary at a regular time of 5 sec with a pressure of 50 mbar. Before use, the capillary was washed orderly by 1 mol/l NaOH for 20 min and H_2O for 30 min. After each run, inner surface of the capillary was washed sequentially by 0.1 mol/l NaOH, deionized water, and the separation buffer for 5 min each.

Data was analyzed by Excel 2010 and SPSS v10.0 statistical analysis software and is expressed as mean ± standard error. All statistical analyses were performed. $p < 0.05$ was considered having significant difference.

Results and Discussion

The DPPH radical is easy to become a stable diamagnetic particle when it encounters electrons or hydrogen radicals and combines with them. The scavenging rate against DPPH free radical of AHP and BHT was shown in Fig. 1(A). The DPPH radical scavenging potential increased with the amount increasing of AHP in the tested range. The DPPH radical scavenging capacity of AHP at 150 μg was 45.43%, while the data of BHT was 79.78%, which meant that AHP had a considerable effect on DPPH radical scavenging with BHT as the comparison.

The presence of the antioxidant containing reducuones will result in the reduction of the Fe^{3+} ferricyanide complex will transform to Fe^{2+} insulting in a chance of absorbance reduction of the reaction mixtures (Wu *et al.* 2013). That is to say, a higher absorbance represents a stronger reducing power. As seen in Fig. 1(B), the reducing power of AHP was content-dependent and it was slightly weaker than that of BHT.

AHP had very high scavenging activity on $ABTS^{\bullet+}$ in a dose dependent manner (Fig. 1 (C)). In the experimental range, scavenging activity on $ABTS^{\bullet+}$ of AHP had no significant difference compared with that of BHT ($p < 0.05$). All the results mentioned above indicated that AHP was a potential antioxidant as it was equal to BHT in some parameter.

Among the current reported fatigue mechanisms, the ‘‘radical theory” is one of the most popular, which suggests that intense exercise can result in an imbalance of human’s oxidize-deoxidizing system. The direct results of oxidative stress are injuries on biomacromolecules and lipid peroxidation, and then, cytotoxic damage even organ lesions (Wang *et al.* 2013). The WFST has been widely used as an experimental exercise model with some related bio parameters

determined to evaluate the anti-fatigue effect of the experimental sample (Wang *et al.* 2013, Wang *et al.* 2010).

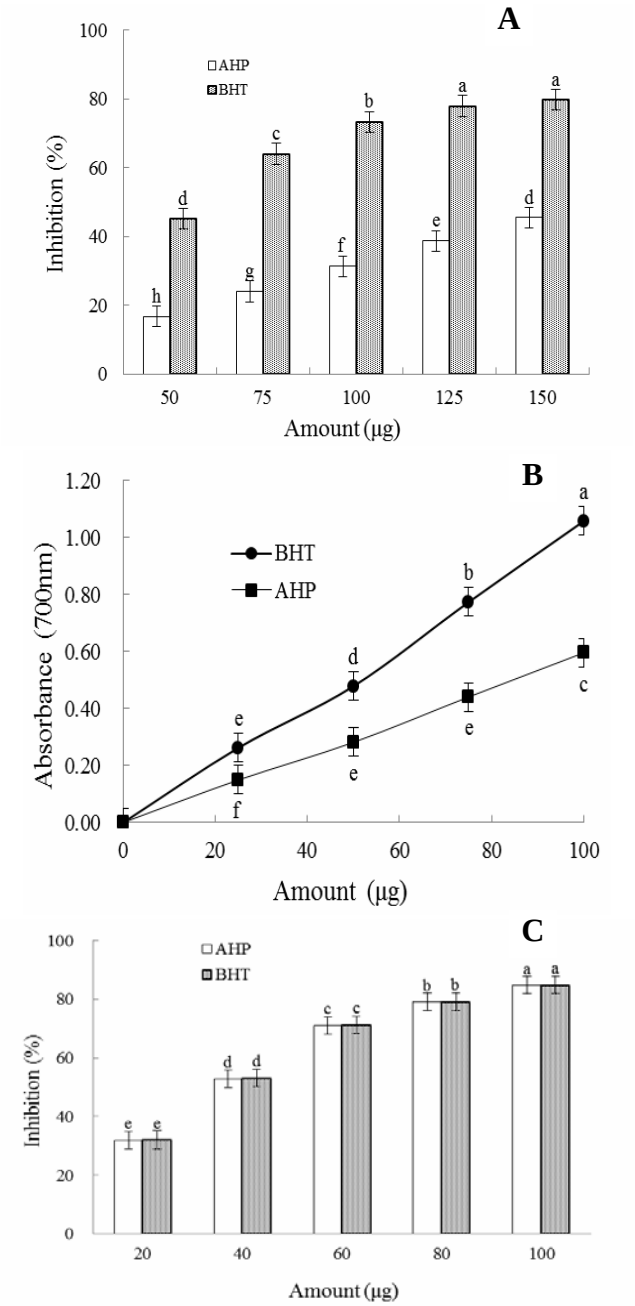

Fig. 1. Antioxidant activities of AHP: (A) DPPH radical scavenging activity of AHP and BHT; (B) Reducing power of AHP and BHT; (C) ABTS radical scavenging activity of AHP and BHT.

Means (n = 3) with different small letters represent significant differences at $p < 0.05$ and the same small letter means that there is no significantly different. Small letters have the same meaning in the following Tables and Figs.

Commonly, carbohydrates can provide energy to bodies through aerobic oxidation. If there is no enough oxygen when the body is taking some severe exercise, anaerobic glycolysis will take hold to generate adenosine triphosphate (ATP). In that procedure, BLA, which can represent the fatigue levels after exercise and restoration degree, is also generated. BLA can be further degraded via the tricarboxylic acid cycle for the production of ATP or removed to other tissue for oxidization or gluconeogenesis. So there are two dynamic balances between aerobic oxidation of glycogen and anaerobic glycolysis and between the accumulation and elimination of BLA whereas, it is unbalanced in a high-intensity exercise (Wu *et al.* 2013). As shown in Table 1, there was no significant difference of LDH values in serum among UTS, AHP-LDG and AHP-MDG, between those of UTC and AHP-HDG ($p < 0.05$). There was a dose-independent effect in the three AHP treated groups.

LDH exists in cytoplasm of all issues in human's body and plays vitally glycolytic enzyme in anaerobic glycolysis and gluconegenesis (You *et al.* 2011). The results suggested that AHP could restore LDH activity as there was no significant change on this value between UTC and AHP-HDG ($p < 0.05$, Table 1), while LDH activity of UTS, AHP-LDG and AHP-MDG reduced significantly compared with those of the former two ($p < 0.05$).

Table 1. Effects of AHP on BLA, LDH and BUN levels in serum of mice (n = 10).

Groups	BLA (mmol/l)	LDH (U/ml)	BUN (mmol/l)
UTC	23.04 ± 6.15b	757.66 ± 66.39b	8.03 ± 1.76b
UTS	35.38 ± 4.88c	890.31 ± 77.37a	12.07 ± 1.68a
AHP-LDG	30.11 ± 7.55b	804.83 ± 35.30a	10.78 ± 2.15a
AHP-MDG	20.33 ± 3.13b	774.06 ± 38.49a	9.80 ± 1.91b
AHP-HDG	12.47 ± 3.97a	714.23 ± 49.95b	7.83 ± 0.89b

BUN concentration in bodies is positively correlated with protein catabolism, and as a sequence, it is negatively correlated to exercise tolerance (LinNa *et al.* 2014). The similar change law happened to the BUN concentration of the AHP groups (Table 1). The accommodation on BLA, LDH and BUN of mice after FST was based on the AHP dose, which was consisted with the conclusion reported by Chi *et al.* 2015.

Glycogen is an important source of energy during exercise. HG can complement the exhaustion of blood glucose to maintain it at the physiologic level. MG is the storage form of sugar in the muscles, which can be automatically decomposed as an energy supply when there is a large consumption of blood glucose in strenuous exercise. Then, lactose decomposed from MG would be transferred through the blood circulation to the liver and be transformed into HG or broken down into glucose. The concentrations of HG and MG of the groups given AHP were significantly higher than those of the UTS ($p < 0.05$) respectively and they were positively related to the AHP dose (Fig. 2). The HG and MG levels of AHP-HDG were significantly higher than those of UTC. The result suggests that AHP has an anti-fatigue effect.

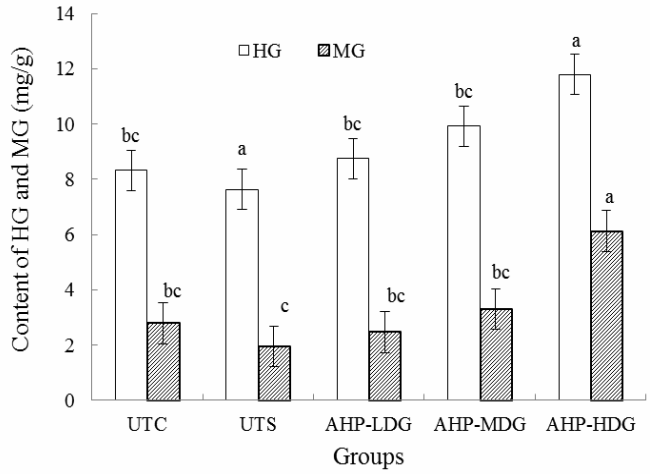

Fig. 2. Effects of AHP on HG and MG in mice (n = 10).

Both the antioxidant enzymatic and noenzymatic systems work together to protect the cells from inner and external oxidative damage. The enzymatic antioxidants include SOD, GSH-Px, and CAT. Each of these enzymes is exclusively responsible for the reduction of one or several different ROS. SOD can scavenge superoxide anion free radical by converting it to O_2 and H_2O_2. GSH-Px is responsible for the elimination of various hydroperoxides. The main function of CAT is to catalyses H_2O_2 converting to H_2O and O_2. MDA is produced from the attack of ROS on unsaturated fatty acids and its content can reflect the damage to the cell membrane (Gomes *et al.* 2012). So, promoting the potential of these defense mechanisms is an effective path to enhance exercise performance. As seen in Table 2, all the three antioxidant enzymes activities were higher than those values of the UTS while the MDA contents were lower. The SOD activities of the AHP-HDG and AHP-MDG were significantly higher than those of the UTC and the UTS ($p < 0.05$), as there was no statistical difference between the AHP-LDG and the UTC. GSH-Px and CAT activities of the AHP-HDG were significantly higher than the other four groups except the ($p < 0.05$). Meanwhile there were no statistical significance on those for the low and middle doses of AHP treatment ($p > 0.05$), compared to the UTC.

Table 2. Effects of AHP on antioxidant enzymes activities and MDA content in serum (n = 10).

Groups	SOD	CAT	GSH-Px	MDA
UTC	93.56 ± 18.84b	32.82 ± 4.89b	560.00 ± 46.65b	16.65 ± 3.06b
UTS	70.37 ± 13.33c	19.70 ± 5.75b	504.67 ± 53.57b	23.85 ± 4.14a
AHP-LDG	83.94 ± 15.75b	27.55 ± 7.49b	518.22 ± 90.28b	17.83 ± 3.08b
AHP-MDG	102.12 ± 24.80a	37.94 ± 17.10b	578.69 ± 115.85b	14.44 ± 1.79b
AHP-HDG	113.17 ± 27.66a	46.20 ± 16.06a	723.11 ± 97.78a	11.39 ± 4.43c

Results in Table 2 indicated that AHP treatment could reduce the MDA content as the data of the AHP-LDG and AHP-MDG were significantly lower than that of the UTS and were without statistically differences with that of the UTC. MDA content of the AHP-HDG was significantly

lower than that of the UTC. The results show that AHP can accelerate increase of those antioxidant enzymes activities and decrease the MDA content, again supporting that AHP accommodated the fatigue of mice after exercise and might become another potential resource as an exhilarant.

Components of AHP after purification, the total phenolic content of AHP hit 168.4 ± 2.4 mg/g gallic acid equivalents in per gram of dry weight, which was quite higher than that reported previously (Chen *et al.* 2014). According to the HPCE analysis, AHP was mainly composed of (–)-epicatechin, (+)-catechin, keampferol, naringenin, ferulic acid, and chlorogenic acid, which generally coincide with the results obtained from HPLC that catechin and epicatechin were two main components of the noncondensed tannin phenol extraction from areca nut (Wang *et al.* 1996). Performance of the HPCE method was evaluated by precision and stability. The R.S.D. values of run-to-run and day-to-day of the peak area were less than 4 and 7%, respectively. The relative values for migration time were less than 3 and 5%, respectively.

In conclusion, the current study suggests that AHP could be a potential and readily accessible source of natural antioxidants and might become a new functional substance or medicine for fatigue resistance. To the utilization commercially of this product as an antioxidant and functional food further work is still needed for the chemical compounds identification and safety evaluation of the AHP.

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