Chemical characterization, antioxidant properties and anti-inflammatory activity of Chinese water chestnut extracts

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ABSTRACT: Ferulic acid, caffeic acid and *p*-coumaric acid have been identified in the *Eleocharis dulcis* (Burm.f.) Trin. ex Hensch. (Chinese water chestnut (CWC)) ethyl acetate extract. The ethyl acetate extract of CWC had the highest DPPH radical scavenging activity ($IC_{50} = 12.81 \pm 0.09$ µg/ml), which was higher than that of the positive control butyl hydroxy anisd ($IC_{50} = 14.73 \pm 0.08$ µg/ml). The reducing power of 0.5 mg/ml CWC was 1.21 at 0.5 mg/ml. The ethyl acetate extract significantly attenuated the production of reactive oxygen species in a dose-dependent manner. The anti-inflammatory activity of CWC extracts was assessed in lipopolysaccharide-induced RAW 264.7 macrophages. The highest anti-inflammatory activity was conferred by 300 µg/ml CWC ethyl acetate extract, which also significantly reduced nitric oxide production ($p < 0.05$). Treatment with the CWC ethyl acetate extract downregulated the expression of *TNF-α*, *iNOS*, and *COX-2* genes. For the first time the identification of phenolic acids from CWC and their correlation with anti-inflammatory activity were reported in this work.

KEYWORDS: antioxidant, anti-inflammatory, Chinese water chestnut, phenolic acids, peritoneal macrophages

INTRODUCTION

Biochemical intermediates in the pathways within the human body can react with oxygen to produce highly reactive molecules known as reactive oxygen species (ROS) or free radicals. If the endogenous pool of antioxidants is insufficient to compensate for the rate of ROS production and the activity of ROS, reactive nitrogen species (RNS), and free radicals, the resulting oxidative stress can enhance the pathophysiological mechanisms of several diseases such as inflammatory diseases. Inflammation is a dynamic process that occurs in vascularized tissue in response to stimuli that cause cell injury and tissue degeneration. ROS and RNS have key roles in mediating the development and progression of degenerative tissue processes [1]. Free radicals can induce lipid peroxidation, which has been linked to cancer. The high production of ROS in tumor cells has contributed to the development of sophisticated cellular responses to counterbalance the resulting oxidative stress by rapidly proliferating. Based on these data, drugs with antioxidant activities are widely used for the treatment of many disorders including inflammatory diseases, cancer and anxiety neurosis.

Plants are natural sources of a diverse array of functional metabolites and compounds. Many plant phytochemicals (secondary metabolites) have antioxidant [2], anti-inflammatory [3], anti-cancer and anti-bacterial properties. Chinese water chestnut (CWC, *Eleocharis dulcis* (Burm.f.) Trin. ex Hensch.) is a hydrophytic vegetable that grows in marshes or ponds. It is widely consumed in China because of its unique taste and high nutrient value. It is valued for its medicinal properties. CWC exhibits strong antimicrobial and antioxidant activities [4], and has been used in traditional Chinese medicine to treat pharyngitis, laryngitis, enteritis, hepatitis and hypertension [5]. To the best of our knowledge, there is little or no information on the medicinal properties of CWC extracts, which could include valuable antioxidant and anti-inflammation compounds. In this study, we investigate the medicinal properties of CWC extracts, focusing on antioxidant properties, 2,2-diphenyl-1-picrylhydrazyl (DPPH)-radical scavenging activity, reducing power, total phenolic and flavonoid contents, and anti-inflammatory activity in RAW 264.7 macrophage

cells *in vitro*. And the main antioxidants from CWC were isolated, identified and quantified.

MATERIALS AND METHODS

Samples preparation

The whole plant of Chinese water chestnut was obtained from Hezhou, Guangxi, China in December 2017. The sample was extracted with 100% methanol. The crude extract was suspended in deionized water and partitioned sequentially with n-hexane, ethyl-acetate (EtOAc) and butanol (water saturated BuOH) fractions for further analysis.

Isolation of biological compounds

In total, three compounds were isolated following the procedure described below. The EtOAc fraction (25.6 g) was separated on ion exchange chromatography using stepwise gradient elution with methanol:water (50:50–100:0) to yield 4 fractions (Fr. 1–Fr. 4). Fraction Fr. 1 (10.5 g) was chromatographed on silica gel (141.4 g, 0.3–0.2 mm) by gradient elution with dichloromethane:methanol (100:0–0:100) to yield 10 subfractions (Fr. 1-1–Fr. 1-10). Subfraction Fr. 1-8 (2.1 g) chromatographed on an octadecylsilyl (ODS) gel (25 g, 12 nm S-75 µm) by isocratic elution with methanol to give compound 1 (15.6 mg) and compound 2 (28.7 mg). Fraction Fr. 3 (8.4 g) was chromatographed on silica gel (90.4 g, 0.3–0.2 mm) by gradient elution with chloroform:methanol (50:0–0:50) then separated by Sephadex LH-20 gel by isocratic elution with methanol:water (7:3) to give compound 3. The three compounds were identified by ^{1}H and ^{13}C NMR analyses.

DPPH radical scavenging capacity

The DPPH radical scavenging activities of the extracts were determined according to the method of Blois [6] and expressed as the inhibition percentage of free radicals calculated as: (%) inhibition = (1−absorbance of sample/absorbance of control) × 100. The IC_{50} value (µg/ml) represents the concentration at which the scavenging activity is 50%.

Reducing power assay

The reducing power of the extracts was measured by the method of Oyaizu [7]. Extract or fraction was mixed with Na_3PO_4 buffer and potassium ferricyanide, after 20 min, trichloroacetic acid was added and the absorbance was measured at 700 nm.

Determination of total phenolic and flavonoid content

Total phenolic content was estimated by the Folin-Ciocalteu method [8]. The data were expressed as gallic acid equivalent (GAE) per mg of extract, based on the calibration curve of gallic acid. The total flavonoid content was determined by Park [9]. The data were expressed as mg/g quercetin.

Assay of ROS production

The evolution of reactive oxygen species (ROS) using dichlorofluorescin diacetate (DCFH-DA) assay [10]. ROS levels were measured by flow cytometry.

Nitric oxide (NO) production

RAW 264.7 cells were plated in 6-well plates and stimulated with lipopolysaccharide (LPS) in the absence or presence of various concentrations of CWC. The procedure for NO concentration was measured using the Griess reaction. The absorbance was measured at 540 nm.

Preparation of total RNA and cDNA synthesis

Total RNA and PCR analyses were described previously [11]. The primer sequences were as follows:
β-actin-F, 5′-TAC AGC TTC ACC ACC ACA GC-3′,
β-actin-R, 5′-AAG GAA GGC TGG AAA GC-3′,
iNOS-F, 5′-TTT CCA GAA GCA GAA TGT GA-3′,
iNOS-R, 5′-AAC ACC ACT TTC ACC AAG AC-3′,
COX-2-F, 5′-TGA GTA CCG CAA ACG CTT CTC-3′,
COX-2-R, 5′-TGG ACG AGG TTT TTC CAC CAG-3′,
TNF-α-F, 5′-CTA CTC CCA GGT TCT CTT CAA TG-3′,
TNF-α-R, 5′- GCA GAG AGG AGG TTG ACT TTC-3′.

Statistical Analysis

Data were statistically evaluated via Duncan's one-way analysis of variance (ANOVA). The level is considered significant when $p < 0.05$. Data were presented as means ± SD.

RESULTS AND DISCUSSION

Determination of antioxidant activity in CWC extracts

We investigated the antioxidant activity of CWC extracts by measuring total phenolic and flavonoid content, reducing power, DPPH-radical scavenging activity, and attenuation of ROS generation. We

Table 1 Total phenolic and flavonoid contents and DPPH radical scavenging activity of CWC extract and fractions.

Fraction	DPPH radical scavenging activity IC_{50} (µg/ml)	TPC[1] (mg GAE/g)	TFC[2] (mg QE/g)
M	43.59 ± 0.35^b	106.27 ± 4.96^b	35.29 ± 0.64^b
H	317.34 ± 0.28^d	6.50 ± 1.00^d	0.45 ± 2.27^d
E	12.81 ± 0.09^a	271.91 ± 1.67^a	182.21 ± 2.86^a
B	281.46 ± 0.05^c	20.34 ± 1.92^c	15.59 ± 1.22^c
W	—	—	—
BHA	14.73 ± 0.08		

[1] Total phenolic contents; [2] total flavonoid contents; M, methanol extract; H, n-hexane fraction; E, ethyl acetate fraction; B, butanol fraction; W, water fraction.

* Values within the same column followed by different letters (a–e) are significantly different at $p < 0.05$.
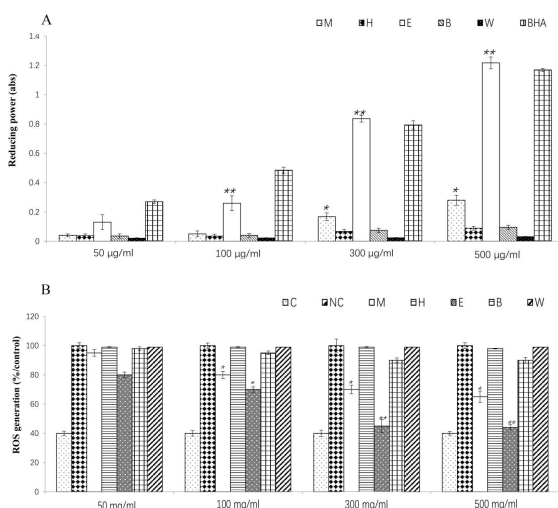

Fig. 1 Antioxidant activity of the CWC extract and fractions. (A) Reducing power of the CWC. (B) Reactive oxygen species (ROS) generation in lipopolysaccharide-induced RAW 264.7 cells following treatment with CWC. M, methanol extract; H, n-hexane fraction; E, ethyl acetate fraction; B, butanol fraction; W, water fraction. Values represent the mean ± SD. Significantly different at * $p < 0.05$ and ** $p < 0.01$.

found that CWC extract contained significant antioxidant activity. The highest DPPH-radical scavenging activity was contained in the ethyl acetate extract ($IC_{50} = 12.81 \pm 0.09$ µg/ml), which had higher activity than other extracts (n-hexane, butanol and water), and was superior to the positive control butyl hydroxy anisd (BHA, $IC_{50} = 14.73 \pm 0.08$ µg/ml) (Table 1). The ethyl acetate extract at 500 µg/ml ($OD_{700} = 1.21$) had the highest reducing power of all extracts (Fig. 1A). Consensus opinion considers that the antioxidant activity in

plant extracts is related to the total phenolic content (TPC) and total flavonoid content (TFC). This correlates with our results showing that TPC was isolated in the ethyl acetate extract (271.91 ± 1.67 mg gallic acid equivalent (GAE)/g) and was greater than that in the methanol extract (106.27 ± 4.69 mg GAE/g) or other extracts (>BuOH extract (20.34 ± 1.92 mg GAE/g) and >n-hexane extract (6.50 ± 1.00 mg GAE/g)). The ethyl acetate extract displayed higher TFC (128.21 ± 2.86 QE/g) than other extracts (Table 1).

ROS are associated with many forms of apoptosis and can act as secondary messengers that activate the production of mediators involved in inflammatory processes. Therefore, we determined whether CWC extracts affected ROS generation. The ethyl acetate extract significantly reduced ROS production in a dose-dependent manner (Fig. 1B). A concentration of 300 µg/ml ethyl acetate significantly inhibited ROS production to 44.78% of that produced in non-treated LPS-induced cells. These results suggest that the ethyl acetate extract can inhibit ROS production by scavenging free radical. These effects were in agreement with a report by Ghosh [12], who suggest that *Aronia melanocarpa* 'Viking' extracts displayed strong radical scavenging activity and may have a crucial role in inhibiting ROS generation. Oxidative stress has been proposed as a causative factor in the toxicity of numerous chemicals, and it has a significant role in the pathogenesis of several human diseases including inflammatory conditions, certain cancers and cataracts. Antioxidants prevent the adverse effects of oxygen reactivity by capturing and neutralizing free radicals, thereby eliminating free radical damage to the human body.

Effect of CWC extracts on nitric oxide production

To examine whether the extracts attenuated the LPS-induced inflammatory response, RAW 264.7 cells were treated with different doses of CWC extract plus LPS. After 24 h, the LPS-induced inflammatory response was evident compared with controls. The CWC ethyl acetate extract significantly inhibited NO production in dose-dependent manner (Fig. 2A). Treatment of RAW 264.7 cells with 300 µg/ml ethyl acetate extract reduced NO production by approximately 60% compared with control. High concentrations of the CWC methanol extract slightly induced NO production, whereas the n-hexane, butanol and water extracts did not significantly affect NO production. These results
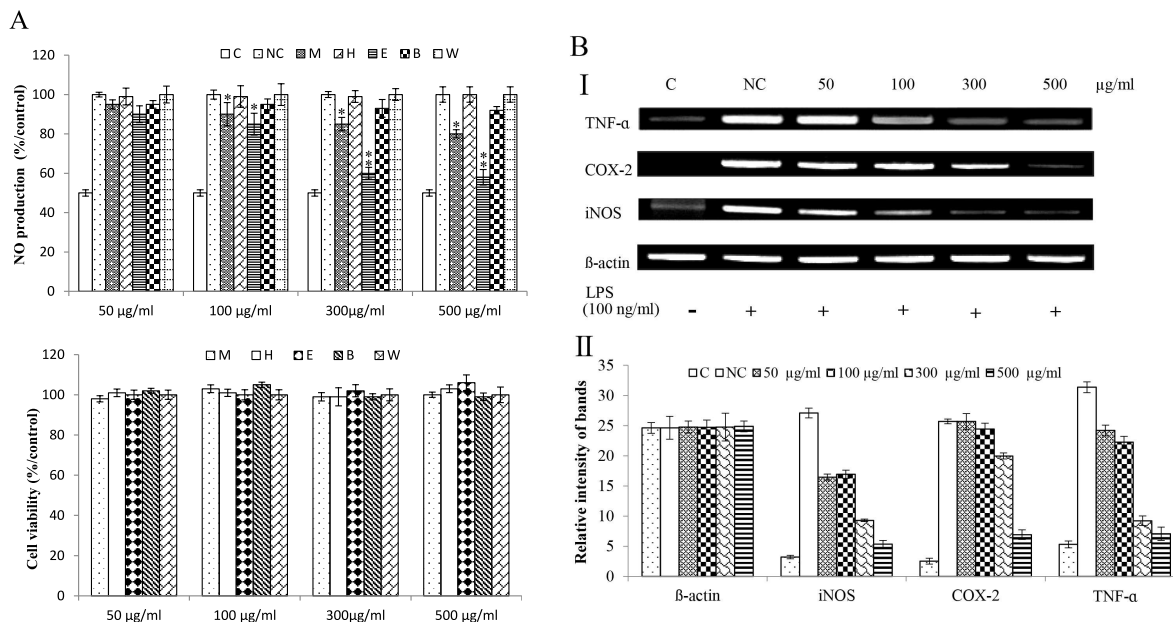
Fig. 2 The anti-inflammatory effects of CWC. (A) The effect of CWC extract on the production of NO in RAW 264.7 cells. Values represent the mean ± SD. Significantly different at * $p < 0.05$ and ** $p < 0.01$. (B) The effect of EtOAc fraction from CWC on LPS-induced *β-actin*, *iNOS*, *COX-2* and *TNF-α* expression in RAW 264.7 cells. (I) Gel electrophoresis of amplified product of *β-actin*, *iNOS*, *COX-2* and *TNF-α*; (II) quantitative data of panel (B); C, control; NC, negative control.

correlated well with the antioxidant results, indicating that the ethyl acetate extract contained high phenolic content and substantial antioxidant activity. Antioxidants act by scavenging free radicals such as ROS, NO and hydroxyl radicals. The strong scavenging activity against ROS, which has an important role in the pathogenesis of inflammation, along with reduced NO production, explains in part the beneficial effects of CWC extracts in the treatment of inflammation. We propose that the radical-scavenging activity and anti-inflammatory activity of phenolic compounds are inseparably connected. Other studies also have reported that phenolic compounds significantly inhibit pro-inflammatory enzymes and cytokine-induced inflammatory responses in human and mouse cells via their potent antioxidant activities [13]. Inhibition of NO production may contribute to the anti-inflammatory activity of the CWC ethyl acetate extract, so it was selected for use in subsequent experiments.

Effect of the CWC ethyl acetate extract

Nitric oxide acts as a pro-inflammatory agent in inflammation. NO is synthesized by inducible nitric oxide synthase (iNOS) in response to proinflammatory agents such as lipopolysaccharides (LPS) [14].

LPS can trigger inflammation and induce the overexpression of many inflammatory mediators. Anti-inflammatory agents act by modulating the activities of pro-inflammatory enzymes and cytokines [13]. Our previous study revealed that the ethyl acetate extract of CWC significantly inhibited NO generation in a dose-dependent manner. Therefore, we investigated whether the ethyl acetate extract could inhibit the expression of proinflammatory cytokine genes such as *TNF-α*, *COX-2* and *iNOS* in RAW 264.7 cells. LPS dramatically up-regulated *TNF-α* mRNA expression (Fig. 2B). By contrast, the ethyl acetate extract inhibited *TNF-α* mRNA expression in LPS-induced RAW 264.7 cells. Similarly, *iNOS* and *COX-2* expression was markedly stimulated by LPS, whereas these expression levels declined in a dose-dependent manner after treatment with the ethyl acetate extract. Treatment with 500 μg/ml ethyl acetate extract reduced *iNOS*, *COX-2* and *TNF-α* expression to almost undetectable levels. *COX-2* is an inducible gene that converts arachidonic acid into prostaglandins, and it is implicated in pathological conditions such as inflammation, cancer and Alzheimer's disease [15]. The production of *TNF-α* also is crucial for the immune response to many inflammatory stimuli. For example, *TNF-α*
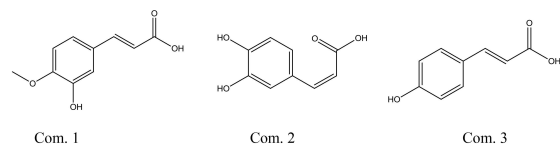
Fig. 3 Chemical structures of the compounds isolated from Chinese water chestnut.

is overexpressed in acute and chronic inflammatory disorders [13]. These combined results indicate that CWC contains anti-inflammatory activity that can reduce the expression of pro-inflammatory signals and inhibit the production of inflammatory cytokines.

Identification of the chemical structures of the isolated compounds

The following compounds were identified by comparison of its ^{1}H and ^{13}C NMR data with literature and are in agreement with the proposed structures. The structures of compounds 1–3 are presented in Fig. 3. Compound 1 was identified as ferulic acid, by NMR analysis, and comparison with its literature data [16]. Compounds 2 and 3 were identified as caffeic acid and *p*-coumaric acid, respectively, by NMR analysis, and comparison with their literature data [17].

Antioxidant capacities of compounds

The compounds isolated from CWC were examined for their inhibitory activity against the DPPH radical. Compound 2 (at a concentration of 13.27 ± 0.33 µmol/ml) showed higher radical scavenging activity than compound 1 ($IC_{50} = 40.36 \pm 0.98$ µmol/ml). Compound 3 possessed weak activity ($IC_{50} = 2307.15 \pm 1.21$ µmol/ml). ROS production can be inhibited by antioxidants capable of entering live cells and can be further inhibited by anti-inflammatory compounds in a complex product. The pretreatment of RAW 264.7 cells with samples of compounds 1, 2 and 3 resulted in inhibition of the formation of ROS by the RAW 264.7 cells (Fig. 4). Untreated RAW 264.7 cells (no sample, no LPS) served as a baseline, and RAW 264.7 cells treated with LPS in the absence of sample served to show maximum ROS production. RAW 264.7 cells treated with the sample produced fewer ROS than cells treated with LPS in the absence of sample. The inhibition of ROS formation was dose-dependent and, at the highest dose of compound 2 tested (100 µmol/ml), the inhibition was highly
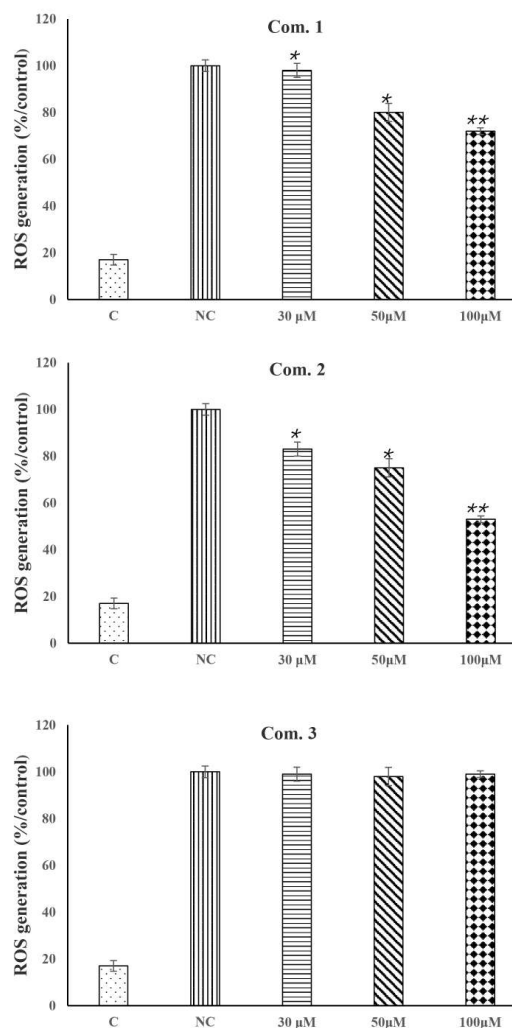

Fig. 4 Antioxidant activity of compounds from CWC. Reactive oxygen species (ROS) generation in lipopolysaccharide-induced RAW 264.7 cells following treatment with compounds from CWC.

significant. These results confirm that compounds 1 and 2 scavenged free radicals and inhibited radical-mediated oxidation in RAW 264.7 cells.

CONCLUSION

This study investigated the antioxidant and anti-inflammatory activities of Chinese water chestnut (CWC) extract using the DPPH assay to test for radical scavenging activity and RT-PCR analysis of pro-inflammatory signal expression in LPS-stimulated RAW 264.7 cells, respectively. The results showed that CWC extract contained high DPPH radical scavenging activity and high phenolic and flavonoid

contents. CWC extract significantly reduced ROS production in a dose-dependent manner. Our results clearly demonstrate that CWC extract inhibited NO production and down regulated *iNOS*, *COX-2* and *TNF-α* expression. In addition, we isolated ferulic acid (compound 1), caffeic acid (compound 2) and p-coumaric acid (compound 3) from the ethyl acetate fraction of the CWC and evaluated for the antioxidant properties. These data indicate that CWC could be developed as a herbal remedy for preventing and treating inflammatory diseases.

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