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·Research articles·

Antioxidant and cardioprotective effects of *Ilex cornuta* on myocardial ischemia injury

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[ABSTRACT] Previous studies have indicated that the *Ilex genus* exhibits antioxidant, neuroprotective, hepatoprotective, and anti-inflammatory activities. However, the pharmacologic action and mechanisms of *Ilex cornuta* against cardiac diseases have not yet been explored. The present study was designed to investigate the antioxidant and cardioprotective effects of *Ilex cornuta* root with *in vitro* and *in vivo* models. The anti-oxidative effects of the extract of *Ilex cornuta* root (ICR) were measured by 2, 2-diphenyl-1- picrylhydrazyl (DPPH) free-radical scavenging and MTT assays as well as immunoassay. Furthermore, a rat model of myocardial ischemia was established to investigate the cardioprotective effect of ICR *in vivo*. Eight compounds were isolated and identified from ICR and exhibited DPPH free-radical scavenging activities. They also could increase cell viability and inhibit morphological changes induced by H_2O_2 or $Na_2S_2O_4$ in H9c2 cardiomyocytes, followed by increasing the SOD activities and decreasing the MDA and ROS levels. In addition, it could suppress the apoptosis of cardiomyocytes. In the rat model of myocardial ischemia, ICR decreased myocardial infarct size and suppressed the activities of LDH and CK. Furthermore, ICR attenuated histopathological alterations of heart tissues and the MDA levels, while increasing SOD activities in serum. In conclusion, these results suggest that ICR has cardioprotective activity and could be developed as a new food supplement for the prevention of ischemic heart disease.

[KEY WORDS] *Ilex cornuta*; Myocardial ischemia; Antioxidant; Apoptosis

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Introduction

Ischemic heart disease, especially acute myocardial infarction, is the leading cause of morbidity and mortality in the Western world. According to the World Health Organization, it will be the major cause of death in the world [1-2]. The major causative factor of acute myocardial infarction is the increased production of reactive oxygen species (ROS) in the cytosol and mitochondria, including superoxide radical anions, hydroxyl radicals, singlet oxygen and hydrogen peroxide, which may be originated from three sources: the mitochondrial electron transport chain of myocytes [2-4], NADPH oxidase and myelope-roxidase of neutrophils [5-6], and xanthine oxidase of endothelial cells [4]. Excessively high levels of free radicals lead to oxidative stress and bring about damage to cardiac cells, as a result of peroxidation of lipids, proteins, carbohydrates and DNA [7-8]. Antioxidant intervention targeted to scavenge ROS is a useful method for delaying or preventing onset of ischemic heart disease [9].

It is reported that intake of food with antioxidant function is associated with a low risk of cardiovascular diseases [10]. In the past decades, research results have proven that many herbal products have antioxidant and protective activity against myocardial ischemia injury [11]. *Ilex* (Aquifoliaceae) species are distributed widely in China, especially in a region south of the Yangtze River, and some are used as food (tea) and folk medicines. For example, *I. cornuta* and *I. latifolia* are traditionally used for the treatment of headache, toothache, bloodshot eyes, and tinnitus [12]. The leaf of *I. cornuta* is used as Kudingcha, a popular functional tea beverage in China

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and other Southeast Asia countries, such as Singapore, Malaysia and Vietnam [13]. Previous studies on the *Ilex genus* have led to the isolation of triterpenoids, phenolic acids, flavonoids, and essential oils, some of which show antioxidant, anti-obesity, neuroprotective, antidiabetic, hepatoprotective, and anti-inflammatory activities [14-16], but little is known about the pharmacologic action andunderlying mechanisms of *Ilex cornuta* against cardiac diseases.

In our effort to develop the extract of *Ilex cornuta* as an herbal dietary supplement for prevention of coronary artery disease, the antioxidative and cardioprotective effects of the ethanol extract of *Ilex cornuta* root (ICR) were systematically evaluated with *in vitro* and *in vivo* models.

Materials and Methods

Materials

2, 3, 5-Triphenyltetrazolium chloride (TTC) and sodium pentobarbital were purchased from Beijing Chemical Reagent Company (Beijing, China). Dulbecco's modified eagle medium (DMEM), trypsin, penicillin, streptomycin, and fetal bovine serum were purchased from Invitrogen Inc. (Logan, UT, USA). 3-(4, 5-Dimethylthiazol-)2, 5-diphenyl tetrazoliumbromide (MTT), isoprenaline, hydroxyperoxide (H_2O_2), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), and sodium dithionite ($Na_2S_2O_4$) were obtained from Sigma Chemical Company (St. Louis, MO, USA). Lactate dehydrogenase (LDH), superoxide dismutase (SOD), glutathione (GSH), creatine kinase (CK), and methane dicarboxylic aldehyde (MDA) kits were purchased from Nanjing Jianchen Bioengineering Institute (Nanjing, China). Pituitrin was obtained from Shanghai First Biochemical and Pharmaceutical Company (Shanghai, China).

Animals

Healthy Sprague-Dawley rats weighing 250−300 g were obtained from the Experimental Animal Center of Soochow University (Suzhou, China). They were kept in plastic cages at (22 ± 2) ℃ with free access to pellet food and tap water under a 12-h/12-h light/dark cycle. The research was conducted in accordance with the Local Guide for the Care and Use of Laboratory Animals and approved by the Ethical Committee of Animal Care of Soochow University.

Preparation of ICR extracts

The dried roots of *Ilex cornuta* were collected from Huanggang City, Hubei, China, in May 2007 and authenticated by Prof. LI Xiao-Ran (Soochow University). The dried plant materials (10 kg) were extracted three times with 50% EtOH (100 L) at 80 ºC under refluxing. After removing the solvent under reduced pressure, the combined extracts (365 g) were successively dissolved in distilled water, loaded onto a D101 macroporous resin column (26 cm × 200 cm; Xi'an Sunresin New Materials Co., Ltd., Xi'an, China), which was eluted with sequential mixtures of EtOH–H_2O (0, 30, 60, and 90%, 50.0 L each). The fraction eluted with 30% EtOH was lyophilized, and the residue (ICR, 175 g) was subjected to the following phytochemical and pharmacological studies.

The dried ICR powder (1.05 g) was fractioned by medium pressure liquid chromatography on a column of ODS (460 mm × 26 mm i.d., 50 μm, Büchi Corp, Flawil, Switzerland), with a MeOH/H_2O gradient elution (the ratios of MeOH/H_2O were from 45 : 55 to 65 : 35), to afford 4 fractions (fractions 1−4). Fraction 4 (62 mg) was separated by semi-preparative HPLC on a PRC-ODS column (250 mm × 20 mm i. d., 5 μm, Shimadzu Co., Ltd., Kyoto, Japan) eluted with MeOH–H_2O (85 : 15) at 2.0 mL·min^{-1} to yield compounds **1** (18.5 mg, t_R 26.5 min) and **2** (27.6 mg, t_R 28.0 min); Further purification of Fraction 3 (28 mg) through sephadex LH-20 column chromatography, eluted with MeOH, offered compound **3** (18.2 mg); Fraction 2 (85 mg) was separated by semi-preparative HPLC on a PRC-ODS column (250 mm × 20 mm i. d., 5 μm, Shimadzu Co. Ltd.), eluted with CH_3CN–H_2O (45 : 55) at 2.0 mL·min^{-1} to yield compounds **4** (32.2 mg, t_R 28.5 min), **5** (5.3 mg, t_R 37.5 min), and **6** (20.8 mg, t_R 40.6 min); Fraction 1 (668 mg) was subjected to semi-preparative HPLC purification on a Zorbax SB-ODS column (250 mm × 20 mm i. d., 5 μm, Shimadzu Co., Ltd.) eluted with MeOH–H_2O (60 : 40) at a flow rate of 2.0 mL·min^{-1}, to yield compounds **7** (253 mg, t_R 20.2 min) and **8** (232 mg, t_R 24.5 min).

Compound **1** was obtained as white powder (CH_3OH). IR (KBr) ν_{max} 3 372, 2 935, 1 730, 1 658, 1 373, 1 051 cm^{-1}; ESI-MS *m/z* 657.4 $[M + Na]^+$; ^{1}H NMR (500 MHz, C_5D_5N) δ: 0.76 (3H, s, H-25), 0.84 (3H, s, H-24), 1.02 (3H, s, H-26), 1.07 (3H, d, *J* = 7.5 Hz, H-30), 1.21 (3H, s, H-23), 1.49 (3H, s, H-29), 1.72 (3H, s, H-27), 3.21 (1H, s, H-18), 3.37 (1H, dd, *J* = 11.5, 4.5 Hz, H-3), 4.96 (1H, t, *J* = 8.5 Hz, H-1 of Glc), 5.37 (1H, br s, H-12). All the data above are in agreement with those of 19*α*-hydroxyurs-12-en-28-oic acid 28-*O*-*β*-D-glucopyranosyl ester [17].

Compound **2** was obtained as white powder (CH_3OH). IR (KBr) ν_{max} 3 410, 2 930, 1 732, 1 708, 1 655, 1 366, 1 043 cm^{-1}; ESI-MS *m/z* 685.4 $[M + Na]^+$; ^{1}H NMR (500 MHz, C_5D_5N) δ: 0.81 (3H, s, H-25), 0.95 (3H, s, H-24), 1.05 (3H, s, H-26), 1.12 (3H, d, *J* = 6.0 Hz, H-30), 1.28 (3H, s, H-23), 1.44 (3H, s, H-29), 1.74 (3H, s, H-27), 3.04 (1H, s, H-18), 3.55 (1H, dd, *J* = 10.5, 5.5 Hz, H-3), 3.73 (3H, s, H-OMe), 4.99 (1H, d, *J* = 8.0 Hz, H-1 of GlcA), 5.58 (1H, br s, H-12). All the data above are in agreement with those of 19*α*-hydroxyurs-12-en-28-oic acid 3*β*-*O*-*β*-D-glucuronopyranoside-6-*O*-methyl ester [18].

Compound **3** was obtained as white powder (CH_3OH). IR (KBr) ν_{max} 3 351, 2 938, 1 740, 1 712, 1 657, 1 358, 1 035 cm^{-1}; ESI-MS *m/z* 817.4 $[M + Na]^+$; ^{1}H NMR (500 MHz, C_5D_5N) δ: 0.87 (3H, s, H-25), 1.05 (3H, s, H-24), 1.16 (3H, s, H-26), 1.19 (3H, d, *J* = 6.0 Hz, H-30), 1.29 (3H, s, H-23), 1.49 (1H, s, H-29), 1.76 (3H, s, H-27), 3.14 (1H, s, H-18), 3.62 (1H, dd, *J* = 10.5, 4.0 Hz, H-3), 3.78 (3H, s, H-OMe), 5.01 (1H, d, *J* = 8.0 Hz, H-1 of GlcA), 5.18 (1H,