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Original Article

In-vitro cardiovascular protective activity of a new achillinoside from *Achillea alpina*

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ABSTRACT

Achillinoside was isolated from methanol extract of *Achillea alpina* L., Asteraceae. The structure of the compound was characterized based on various spectrum data, including IR, HR-ESI-MS, 1D and 2D NMR. The cardiovascular protective effect of achillinoside was tested on H_2O_2-induced H9c2 cells. In our research, achillinoside could increase the cell viability dose-dependently in H_2O_2-induced H9c2 cells. In addition, the levels of caspase-3/9 cells were significantly decreased in H_2O_2 and achillinoside incubated H9c2 cells.

Introduction

The genus *Achillea*, Asteraceae, consists of 85 species around the world, and mainly distributed in eastern and southern Asia. Modern pharmacological research has demonstrated that this genus has anti-inflammatory, anti-oxidant, cytotoxic, and antibacterial (Küçükbay et al., 2012; Rouis et al., 2013). Previous studies have reported that numerous natural products, including volatile compounds of essential oils (Venditti et al., 2014), polyphenolic (Benedek et al., 2007) and terpenoids (Konakchiev et al., 2011), were isolated from genus *Achillea* and it is regarded as an attractive plant source of chemically and biologically intriguing secondary metabolites (Benedec et al., 2013). *Achillea alpina* L., perennial herbs, is mainly distributed in southern China and eastern Asia, and was traditionally used as dampness detoxification and blood circulation promotion in China (Chen et al., 2015). In addition, *A. alpina* has been shown to have anti-inflammatory, anti-oxidant and hepatoprotective effects. However, the cardiovascular protection of *A. alpina* has rarely been reported. In this study, we first revealed the presence of a glycosidic dihydrochalcone, trivially named as achillinoside, from the *A. alpina*, and further evaluated the cardiovascular protective effects of this compound.

Considering the anti-oxidant effect of *A. alpina*, hydrogen peroxide (H_2O_2) was selected as the induction of myocardial injury in H9c2 cells. Furthermore, H9c2 cells have been successfully used to assess the cardiovascular protective effect in previous studies, because H9c2 cell keeps the main characteristics of primary cardiomyocytes *in vivo* (Silva et al., 2010; Watkins et al., 2011). Herein we describe the isolation and structure elucidation of the compound and evaluate its cardiovascular protective effect.

Materials and methods

General experimental procedures

Infrared (IR) spectra was recorded on Nicolet^{TM} iS^{TM}50 FT-IR (Thermo Scientific, USA) with a KBr pellet. The chemical shifts (δ) in Nuclear magnetic resonance(NMR) spectra were recorded on a Bruker DRX 600 MHz NMR spectrometer. The TMS was used as internal standard. High resolution electrospray ionization mass spectro(HRESIMS) data was determined on Waters Xevo-G2-XS-Q-T of (Waters, Massachusetts, USA). Thin layer chromatography (TLC) (Silica gel GF_{254}, Shanghai Xinchushiye Lt. Co., China) was used to identify the purity of compound. Various column chromatography, including Silica gel (200–300 mesh, Shanghai Xinchushiye Co. Ltd.), Sephadex LH-20 (green herbs Co. Ltd.), ODS-C18 (50 μm, Merck), and MCI gel (Mitsubishi Chemical Corporation) were used for separation.

Plant materials

The whole aerial parts of *Achillea alpina* L., Asteraceae, were collected in Yunnan Province, China and identified by Prof. Jiawang Ding, and a voucher specimen (2016-0128) was deposited in the School of Pharmacy in China Three Gorges University.

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Extraction and isolation

Dry aerial parts of *A. alpina* (5 kg) were extracted twice with MeOH (2 × 15 l, 1.5 h each) under reflux, and concentrated under vacuum circumstance to obtain a crude extract (538 g). The water (1 l) was added to the extract to make suspension, and then the suspension was extracted with petroleum ether (2 × 1 l), ethyl acetate (3 × 1.5 l), and *n*-BuOH (3 × 1.5 l) to yield petroleum ether-fraction (39 g), ethyl acetate-fraction (85 g), and *n*-BuOH-fraction (109 g), respectively. The ethyl acetate layer (80 g) was fractioned on silica gel column chromatography eluting with a gradient of CH_2Cl_2–CH_3OH (50:1 to 0:1,v/v) to obtain five fractions F1–F5, based on TLC analysis. F3 (14 g) then was separated by MCI gel column chromatography (200 g, 8 × 100 cm) eluting with a gradient of MeOH–H_2O (3:7, 5:5, 7:3,1:0, v/v) to yield four subfractions (F3.1–F3.4). Fr. 3.2 (3.7 g) was further purified on a ODS-C18 column eluted with a gradient of H_2O–MeOH (1:9, 3:7, 5:5, 1:0, v/v) and obtained four tertiary fractions F3.2a–F3.2d–F3.2b (108 mg) then was purified by semi-preparative HPLC chromatography a gradient of H_2O–MeOH (70:30 to 100% MeOH in 15 min, 3 ml/min) to afford achillinoside (**1**, 12.1 mg).

Cell culture and treatment

H9c2 rat cardiomyocyte cells were purchased from the Cell bank of Chinese Academy of Sciences (Shanghai, China), and cultured in DMEM at 37 °C with 5% CO_2. Achillinoside (**1**) was dissolved in DMSO (50, 100, 200, and 300 μg/ml, with final DMSO <0.5%), and incubated for 24 h. H9c2 cells were incubated within creasing concentrations of achillinoside (25, 50 and 100 μg/ml, with final DMSO <0.5%) for 24 h and then treated with 50 μM H_2O_2 for another 6 h, to determine the protective effect of achillinoside. *N*-acetylcysteine (NAC) was used as positive control.

Cell viability

Cell viability was assessed using MTT method, as previously described (Ren et al., 2008). Briefly, The H9c2 cells were seeded in 24-well plates (1×10^5 cells/well). After different treatment, 100 μl fresh medium with 2 mg/ml MTT solution was added to each well, and the cells were incubated at 37 °C for 4 h. Then the medium was removed. 200 μl DMSO was added to each well to dissolve the formazan crystals after supernatants were aspirated. The absorbance was measured at 490 nm using a microplate reader (Xie et al., 2010).

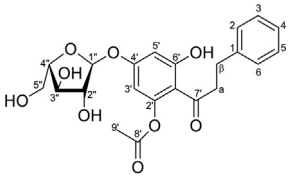
Caspase-3/9 activity assay

The caspase-3/9 activity was measured with a caspase-3/9 colorimetric assay kit according to the manufacturer's instructions. Data were expressed as the relative activity over control.

Results and discussion

Structure elucidation

Achillinoside was obtained as a yellow powder. The molecular formula was deduced as $C_{22}H_{24}O_9$ by its HRESIMS (*m/z* 433.1496 $[M+H]^+$ (calcd. 433.1313). Five aromatic protons at δ_H [7.71 (H-2/6), 7.64 (H-3/5), 7.51 (H-4)] in the ^1H NMR spectrum were assigned to the hydrogen signal on A-ring, which was combined with one singlet in the aromatic region δ_H 6.22 (brs, H-3′,5′) (supplementary Table 1) (She et al., 2011). The above data suggested the presence of a dihydrochalcone skeleton in achillinoside. In the ^1H NMR spectrum, the singlet methyl signal at δ_H 2.78 (H-9′) suggested the presence of an acetyl group, which was confirmed by the correlations from δ_H 2.78 (H-9′) to δ_C 183.2 (C-8′). The L-arabinofuranosyl was deduced from the acid hydrolysis of compound with TLC and GC analyses. A singlet resonance at 4.99 brs was observed in the ^1H NMR spectrum suggesting α-configuration of the anomeric proton of arabinofuranosyl, which was confirmed by the correlations between the 4.99 (br s, H-1″) and δ_C 98.4 in the HSQC spectrum. The sugar group was linked to C-4′ via an oxygen bond, as evidence by the HMBC (supplementary Fig. S1) correlations from H-1″ (δ_H 4.99, br s) to C-4′ (δ_C 163.2), while the acetyl group was connected to C-2′ according to the correlations from H-9′ (δ_H 2.78,s) to C-2′ (δ_C 161.6). Thus, the structure of compound **1** was elucidated as 2′-hydroxy,6′-acetyl-dihydro-chalcone-4′-*O*-α-L-arabinofuranoside, and named as achillinoside (**1**).



Achillinoside pretreatment decreased cell death induced by H_2O_2 stimulation in H9c2 cells

The cardiovascular activity of achillinoside was evaluated in H9c2 rat cardiomyocyte cell line. Thus, we first performed cytotoxicity assay to determine the appropriate concentration of achillinoside that would not affect the cell viability. For this, H9c2 cells were treated with various doses of achillinoside (0–300 μg/ml) and analyzed for cytotoxicity. Interestingly, H9c2 cells were found to Exhibit 100% to 90% viability up to 200 μg/ml concentration of achillinoside (Fig. 1A). The concentration of H_2O_2 was selected from 25 to 100 μM under MTT assay. As shown in Fig. 1B, H_2O_2 significantly ($p < 0.01$) reduced the cell viability in a dose-dependent manner, and 50 μM of H_2O_2 was chosen to make the cardiomyocytes injury. Therefore, in order to determine the protective effects of achillinoside on H_2O_2-induced H9c2 cells, the cells were pre-treated with achillinoside (25, 50, 100 μg/ml) for 24 h, and then co-incubated with 50 μM of H_2O_2 for another 6 h. The cell viability induced by H_2O_2 insult was improved significantly by achillinoside compared with the control ($p < 0.01$) (Fig. 1C).

Achillinoside pretreatment alters caspase activation induced by H_2O_2 stimulation in H9c2 cells

Caspases, a family of cysteine proteases, is a pivotal factor in activation of apoptosis (Danial and Korsmeyer, 2004). Caspase-3 and caspase-9 play an important role in apoptotic processes, especially the cardiomyocytes death (Miao et al., 2013). In our research, the caspase-3/9 activities were significantly increased after incubated with 50 μM H_2O_2 for 6 h compared with the control (Fig. 2). However, when the cells were pre-treated with achillinoside (25, 50, 100 μg/ml) for 24 h prior to 50 μM H_2O_2, the expressions of caspase-3/9 were decreased. Interestingly, achillinoside at 25 μM significantly decreased the level of caspase-9 ($p < 0.05$), while there is no significant difference on caspase-3 (Fig. 2). There results have suggested that achillinoside can significantly protect H9c2 cells from H_2O_2 induced apoptosis via inhibiting caspase-3/9 activation under oxidative stress.

Acid hydrolysis

Achillinoside (2.7 mg) was dissolved in 2 N aqueous HCOOH (5 ml), the mixture was refluxed at 90 °C for 2 h, then 10 ml water was added to the mixture and extracted with $CHCl_3$ (2 × 4 ml). The aqueous layer was then evaporated to afford the glycoside. The

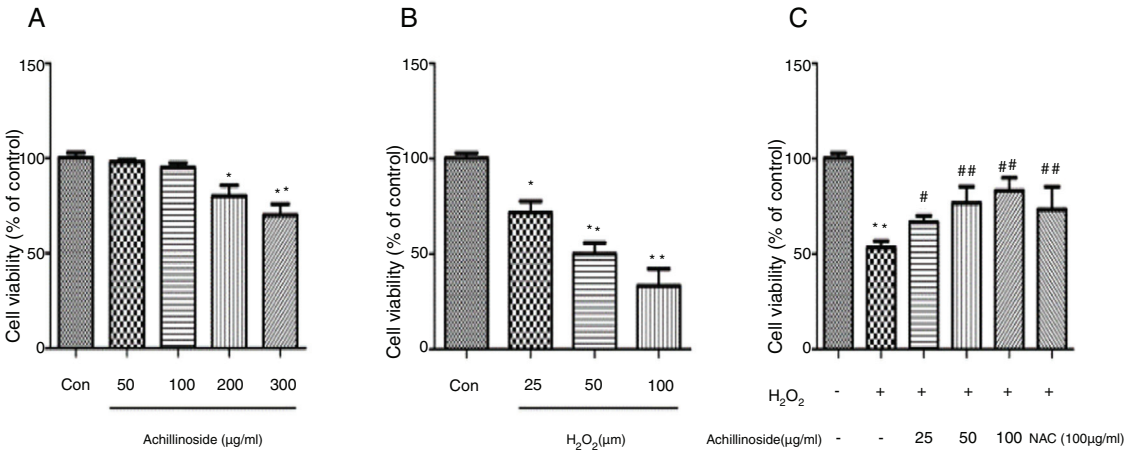


Fig. 1. Preventive effects of achillinoside (**1**) on cell viability against H_2O_2-induced injury determined by MTT assay. (A) The toxic effect of achillinoside in H9c2 cells after 24 h incubation. (B) The toxic effect of H_2O_2 in H9c2 cells after 6 h incubation. (C) Achillinoside protects H9c2 from H_2O_2-induced cytotoxicity. $^{*}p < 0.05$, $^{**}p < 0.01$ versus the normal H9c2 cells; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus the H_2O_2-induced H9c2 cells.

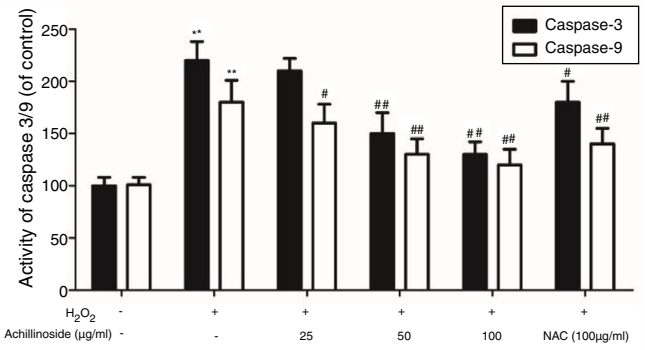


Fig. 2. Effect of achillinoside (**1**) on caspase-3/9 activity in H_2O_2-treated H9c2 cells. $^{**}p < 0.01$ versus the normal H9c2 cells; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus the H_2O_2-induced H9c2 cells.

residue was analyzed by silica gel TLC ($CHCl_3$–MeOH–H_2O, 7:3:0.5) by comparison with standard sugars, and spots were visualized by spraying with vanillin/EtOH (2:8). The R_f of arabinose by TLC was 0.56. The residue was dissolved in pyridine (2 ml) containing L-cysteine methyl ester hydrochloride (2 mg) and heated at 60 °C for 1 h to the reaction mixture was added trimethylsilyl reagent (200 μl), and stirred at 60 °C for 30 min. The standard L-arabinose was prepared as above. Then the supernatants (4 μl) were subjected to GC (Thermo Trace GC Ultra) for analysis, respectively. The configurations L-arabinose for achillinoside were determined by comparison of the retentions times of the corresponding derivatives with those of standard, and L-arabinose giving a single peak at 13.16 min (Qin et al., 2016).

Chemical characteristics

Achillinoside (**1**): yellow powder; IR (KBr): υ_{max} 3403.74, 2917.77, 1633.41, 1610.27, 1571.70, 1076.08, 827.31 cm^{-1}; ^1H NMR (600 MHz, DMSO-d_6) and ^{13}C NMR (150 MHz, DMSO-d_6), see supplementary Table; HRESIMS *m/z* 433.1496 $[M+H]^+$ (calc. for $C_{22}H_{24}O_9$ 433.1313).

Conclusions

This study describes isolation and structure elucidation of achillinoside (**1**) from *A. alpina* and the cardiovascular protective effect against H_2O_2-induced cardiotoxicity by MTT assay. Pretreatment of H9c2 cells with achillinoside prevent H9c2 cells from H_2O_2-induced cell death and significantly decreased the expression of caspase-3/9.

Authors' contributions

FZ, SL, JY (PhD student) was responsible for most experimental work (infrared spectra analyses, Cell Culture and Treatment, MTT method); CH (undergraduate student) was responsible for culture medium preparation and bacterial growth and Elisa measurements. WJD contributed to critical reading of the manuscript. LT designed the study and supervised the experiments. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2019.02.008.

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