Research Article

Extracts of Artemisia ciniformis Protect Cytotoxicity Induced by Hydrogen Peroxide in H9c2 Cardiac Muscle Cells through the Inhibition of Reactive Oxygen Species

Mahdi Mojarrab,1 Maryam Jamshidi,2 Farahnaz Ahmadi,1 Ellahe Alizadeh,1 and Leila Hosseinazadeh1

1 Novel Drug Delivery Research Center, School of Pharmacy, Kermanshah University of Medical Sciences, Kermanshah 6734667149, Iran
2 Student Research Committee, Kermanshah University of Medical Sciences, Kermanshah 6734667149, Iran

Correspondence should be addressed to Leila Hosseinazadeh; lhosseinazadeh90@yahoo.com

Received 4 August 2013; Revised 19 October 2013; Accepted 19 October 2013

Academic Editor: Neal Davies

Copyright © 2013 Mahdi Mojarrab et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Objective. Artemisia ciniformis (Asteraceae) and A. biennis are two of 34 Artemisia species growing naturally in Iran. In this study we investigated whether different extracts of A. ciniformis and A. biennis have protective effect against hydrogen peroxide-induced cytotoxicity in rat cardiomyoblast cells (H9c2). Method. The dried and ground aerial parts of these two species were extracted successively using petroleum ether (40–60), dichloromethane, ethyl acetate (EA), ethanol (EtOH) and ethanol:water (1:1) by maceration method. To evaluate whether different extracts of A. ciniformis and A. biennis protect cardiomyoblast H9c2 cells from \( \text{H}_2\text{O}_2 \) cytotoxicity, we examined the direct cytotoxic effect of \( \text{H}_2\text{O}_2 \) on H9c2 cells in the presence and absence of different extracts. After then, cell viability was measured by MTT assay. Results. \( \text{H}_2\text{O}_2 \) induced cytotoxicity in a concentration dependent manner. The \( IC_{50} \) value was 62.5 \( \mu \text{M} \) for 24 h exposure. However, pretreatment of cells with various concentrations of EA, EtOH, and EtOH/wt extract of A. ciniformis protected cells from \( \text{H}_2\text{O}_2 \)-induced cytotoxicity. Moreover, pretreatment with EA, EtOH and EtOH/wt extracts of A. ciniformis lead to a decrease in the reactive oxygen species (ROS) generation. Taken together our observation indicated that nontoxic concentration of different extracts of A. ciniformis has protective effect on \( \text{H}_2\text{O}_2 \)-induced cytotoxicity in H9c2 cells.

1. Introduction

Artemisia biennis Willd. and A. ciniformis Krasch. & Popov ex Poljakov. (Compositae) grow wildly in Iran [1]. Analysis of the essential oils from the aerial parts of A. biennis growing in Iran and western Canada revealed the presence of camphor and [E] beta-farnesene as the major constituents, respectively [2, 3]. Myrcene [4] and davanone [5] have been reported as the main constituent in the aerial parts oils of A. ciniformis.

Cytotoxicity of some fractions of A. biennis and A. ciniformis as well as significant effects of ethanolic extracts of the species on in vitro leishmanicidal activity have been proved [6–8]. Iranshahi et al. [9] reported the presence of high amounts of sesquiterpene lactonesin A. ciniformis. Another study showed that antioxidant activity and total phenolic content of hydroethanolic extract of A. biennis were higher than those of other extracts [10].

Oxidative stress corresponds to an imbalance between the rate of oxidant production and degradation. It causes numerous biological effects ranging from alternation in signal transduction and gene expression to mutagenesis and finally cell death. It is well known that oxidative stress plays a significant role in the pathogenesis of heart dysfunctions [11]. In our previous study we evaluated the antioxidant activity and total phenolic content of different extracts of A. biennis using cell free systems [10]. In the current, study we aimed to examine the effects of A. biennis and A. ciniformis extracts on hydrogen peroxide (\( \text{H}_2\text{O}_2 \))-induced cytotoxicity and oxidative stress in H9c2 cardiomyoblast cells.
2. Material and Methods

2.1. Reagents and Chemicals. Hydrogen peroxide H$_2$O$_2$, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,5 dichlorofluorescin diacetate (DCF-DA) were bought from Sigma Aldrich (St Louis, MO, USA). Cell culture medium, penicillin-streptomycin, and fetal bovine serum (FBS) were purchased from Gibco (Gibco, Grand Island, NY, USA). All the solvents used for extraction were purchased from Caledon (Ontario, Canada) and Scharlau (Sentmenate, Spain).

2.2. Plant Material. Aerial parts of A. ciniformis Krasch. & Popov ex Poljakov. and A. biennis Willd. were collected from Tandoureh national park and Zoshik, respectively (Razavi Khorasan province, Iran), in September 2010. Samples were identified by Dr Vahidollah Mozaffarian (Research Institute of Forest and Rangelands, Tehran, Iran). The voucher specimens (Nos. 12569 and 12570) have been deposited in the herbarium, Department of Pharmacognosy, Faculty of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran.

2.3. Preparation of Extracts and Fractions. The dried powdered aerial parts (80 g) of A. biennis and A. ciniformis were extracted with petroleum ether (40–60) (PE), dichloromethane (DCM), ethyl acetate (EA), ethanol (EtOH) and ethanol-water (1:1 v/v) (EtOH/wt), respectively (Sequential maceration with ca. 3 x 0.8 L of each solvent). The extracts were filtrated with filter paper and dried using rotary evaporator at a reduced pressure at a temperature below 45°C.

2.4. Cell Culture Conditions. Cardiac H9c2 cells are a clonal heart muscle cell line originated from embryonic rat hearts that presents many cardiomyocyte traits [12]. The H9c2 cells maintained in Dubbelco modified Eagle’s medium (DMEM ATCC) with 10% (V/V) heat inactivated FBS, penicillin G (100 U/mL) and streptomycin (100 mg/mL) at 37°C in 95% CO$_2$ humified incubator. The medium was changed 2-3 days and subcultured when the cell population density reached to 70–80% confluence. Cells were seeded at an appropriate density according to each experimental design.

2.5. Cell Viability Assay. Cellular toxicities of hydrogen peroxide and different extracts of A. biennis and A. ciniformis were analysed in H9c2 cells using MTT methods. Four sets of experiments were performed at standard culture conditions: (1) untreated control cells, (2) cells were treated with different concentrations of A. biennis and A. ciniformis (10–50 μg/mL), (3) cells were treated with different concentrations of hydrogen peroxide (25–250 μM), and (4) cells were pretreated with different concentrations of extracts for 24 h. Then medium was changed and cells were treated with IC$_{50}$ concentration of hydrogen peroxide for another 24 h. Viability of cells were analyzed using MTT methods. Briefly, after treatment, 20 μL of a 5 mg/mL MTT solution was added to each well. After 2 h incubation, the medium was carefully aspirated and the purple formazan crystals were solubilized with 100 μL DMSO. Optical density was measured at 570 nm (reference wavelength 630 nm) in a microplate reader (BioTek, ELX 800, USA). The absorbance of the untreated culture was set at 100%.

2.6. Determination of Intracellular ROS. Intracellular ROS levels were examined using DCF-DA. DCF-DA is a nonfluorescent lipophilic ester that easily crosses the plasma membrane. Into the cytosol the acetate group is rapidly removed by unspecific esterases. The oxidation of this molecule to the fluorochrome DCF results in green fluorescence. The intensity of this fluorescence is generally considered to reflect the level to which ROS are present [12].

After seeding for 24 h, H9c2 cells were washed with PBS buffer (pH 7.4). The cells pretreated with test samples for 24 h were then treated with H$_2$O$_2$ for an additional 24 h. After washing with PBS, the cells were incubated with 20 μL DCF-DA at 37°C for 30 min. The percentage of DMSO insolution did not exceed from 0.5%. After incubation, cells were lysed with Triton X-100. The fluorescence was measured at an excitation wavelength of 488 nm and an emission wavelength of 528 nm using a fluorescence microplate reader (BioTek, HIM, USA).

2.7. Statistical Analysis. Each experiment was performed at least three times and the results were presented as mean ± S.E.M. One-way analysis of variance (ANOVA) followed by Turkey’s test was used to compare the differences between means. A probability value of $P < 0.05$ was considered to be statistically significant.

![Figure 1: The effect of H$_2$O$_2$ on H9c2 cell viability.](image-url)
3. Results

3.1. Cell Viability after Exposure to H2O2, A. biennis, and A. ciniformis Extracts Alone. The viability of H9c2 cardiomyoblast cells was evaluated after 24 h exposure to different concentrations of H2O2. Cell viability was evaluated by the MTT method. As shown in Figure 1, H2O2-induced cytotoxicity was dose dependent. The mean ± SEM IC50 value was 62.5 ± 0.034 μM for 24 h exposure to H2O2. In order to set extracts at concentrations which are nontoxic to cells but could prevent H2O2-induced cytotoxicity, we also examined the effects of different concentrations of A. biennis and A. ciniformis extracts on cell viability in H9c2 cells.

Figure 2 clearly revealed that 24 h treatment with PE, DCM, EA, EtOH, and EtOH/wt extracts of A. biennis had no cytotoxic effect at the concentrations up to 50 μg/mL, while 24 h exposure to DCM and PE extracts of A. ciniformis induced dose response cytotoxicity.

3.2. Effect of Pretreatment with Different Extracts of A. biennis and A. ciniformis on H2O2 Induced Cell Death. For evaluation of effect of pretreatment with different extracts on H2O2 induced cytotoxicity, H9c2 cells were pretreated for 24 h with

Figure 1: Viability of H9c2 cardiomyoblast cells after exposure to different concentrations of H2O2. Data are expressed as the mean ± SEM of three separate experiments (n = 6).

Figure 2: Cell viability of H9c2 cells after exposure to (a) A. biennis and (b) A. ciniformis. Cells were treated with different concentrations of extracts for 24 h. The cell viability was determined by MTT assay. Data are expressed as the mean ± SEM of three separate experiments (n = 6).

Figure 3: The effect of different extracts of A. biennis and A. ciniformis on H2O2-induced cytotoxicity in H9c2 cells. Cell pretreated with different extracts of A. biennis and A. ciniformis for 24 h before exposure to 62.5 μM of H2O2. Data are expressed as the mean ± SEM of three separate experiments (n = 6).

Figure 4: The effect of different extracts of A. ciniformis pretreatment on H2O2-induced ROS generation. Data are expressed as the mean ± SEM of three separate experiments (n = 4). ***P < 0.001 versus Control, and ****P < 0.001 versus H2O2-treated cells.
nontoxic concentrations of extracts, then the medium was changed and cells treated with $\text{IC}_{50}$ concentration (62.5 mM) of $\text{H}_2\text{O}_2$ for another 24 h. As shown in Figure 1, $\text{H}_2\text{O}_2$ treatment significantly decreased cell viability to $50 \pm 2.2\%$ of control. Adding EA, EtOH, and EtOH/wt extracts of *A. ciniformis* (25 $\mu$g/mL) before $\text{H}_2\text{O}_2$ treatment increased the cell viability to $76 \pm 4.53$, $72 \pm 1.25$ and $82 \pm 3.21\%$ of control, respectively (Figure 3). Other extracts were not able to protect H9c2 cells against $\text{H}_2\text{O}_2$-induced cytotoxicity.

3.3. Effect of EA, EtOH, and EtOH/wt Extracts of *A. ciniformis* on ROS Induced by $\text{H}_2\text{O}_2$ in H9c2 Cardiac Muscle Cells. In order to measure oxidative stress induced by $\text{H}_2\text{O}_2$, fluorescent dye DCF-DA was used to measure ROS generation. As anticipated adding $\text{H}_2\text{O}_2$ to H9c2 cells caused a significant increase in ROS level. Therefore, cardiomyoblast cells are probably killed due to oxidative stress, since $\text{H}_2\text{O}_2$ increases intracellular ROS levels. We investigated the inhibitory effect of different extracts on ROS production in the presence of $\text{H}_2\text{O}_2$. Pretreatment with EA, EtOH, and EtOH/wt extracts of *A. ciniformis* decreased intracellular ROS levels in H9c2 cells, significantly. These results indicate that the aforementioned extracts have potential for prevention of ROS mediated events (Figure 4).

4. Discussion

Oxidative stress is considered to be an important condition to promote cell death in response to a variety of signals and pathophysiological condition [13]. It results from increased formation of ROS and/or decreased antioxidant store. Oxidative stress can be identified in most of the key stages in the pathophysiology of atherosclerosis and the main clinical manifestations of cardiovascular disease [14, 15]. Previous reports demonstrated that anti-oxidant natural substances including herbal medicines could inhibit ROS generation [16].

In the current study we examined the protective effect of different extracts of *A. biennis* and *A. ciniformis* on the cytotoxicity induced by $\text{H}_2\text{O}_2$. The obtained results showed that only EA, EtOH, and EtOH/wt extracts of *A. ciniformis* are able to protect H9c2 cardiomyoblast cells against $\text{H}_2\text{O}_2$ cytotoxicity.

Next, it was investigated whether pretreatment with above mentioned extracts had an effect on ROS generation by $\text{H}_2\text{O}_2$. The obtained results showed that pretreatment with EA, EtOH, and EtOH/wt extracts of *A. ciniformis* leads to a decrease in the ROS generation. One possible explanation for the effect of EA, EtOH, and EtOH/wt extracts of *A. ciniformis* on the oxidative stress induced by $\text{H}_2\text{O}_2$ concerns its polyphenolic content, because it is known that plant-derived polyphenolics are potent antioxidants and free radical scavengers [17].

Despite the fact that hydroethanolic extract of *A. biennis* showed potent antioxidant effects using free radical scavenging methods it was not able to protect H9c2 cells from cytotoxicity induced by $\text{H}_2\text{O}_2$ in the current study [10]. This is due to the actual antioxidant activity in physiological conditions such as specific target radicals, localization in different phases and their possible interaction. Therefore, cell free methods may not be sufficient to assessment of antioxidant properties of phytochemicals. Taken together, our data suggested that EA, EtOH, and EtOH/wt extracts of *A. ciniformis* protected cardiomyoblasts against $\text{H}_2\text{O}_2$-induced cell death by a mechanism believed to be free radical scavenging and/or the inhibition of reactive oxygen species. Thus, EA, EtOH, and EtOH/wt extracts of *A. ciniformis* contains principals that may be useful for the prevention and treatment of cardiovascular diseases associated with ROS. Polyphenolics [18], nitrogen containing compounds [19], Polysaccharide fractions [20] and terpenoids [21] are examples of different classes of plant-derived antioxidants. Isolation and characterization of the active and/or major components as well as further studies to determine the molecular mechanisms by which the extracts exert their cardioprotective role are needed.

Conflict of Interests

The authors declares there is no conflict of interests.

Acknowledgment

This work was performed in partial fulfillment of the requirements for Pharm. D. of Maryam Jamshidi, Kermanshah University of Medical Sciences, Kermanshah, Iran.

References


