The protective effect of different extracts of aerial parts of *Artemisia ciniformis* against H$_2$O$_2$-induced oxidative stress and apoptosis in PC12 pheochromocytoma cells

Leila Hosseinzadeh$^1$, Somayeh Mirzaei$^2$, Marziyeh Hajialyani$^1$, Farahnaz Ahmadi$^3$, Seyed Ahmad Emami$^4$, Mahdi Mojarrab$^1*$

$^1$Pharmaceutical Sciences Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran.
$^2$Students Research Committee, Faculty of Pharmacy, Kermanshah University of Medical Sciences, Kermanshah, Iran.
$^3$Department of Pharmacology and Toxicology, Faculty of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran.
$^4$Department of Pharmacognosy, Faculty of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran.

**ARTICLE INFO**

Received on: 31/12/2018  
Accepted on: 22/03/2019  
Available online: 18/04/2019

**Key words:** *Artemisia ciniformis*, neuroprotection, oxidative stress, PC12, medicinal plant.

**ABSTRACT**

The major objective of this study is to investigate whether *Artemisia ciniformis* (*A. ciniformis*) is able to rescue the PC12 neuronal cells from H$_2$O$_2$-mediated oxidative stress. Different antioxidant and apoptotic assays were conducted on *A. ciniformis*. Among different *A. ciniformis* extracts, three (petroleum ether, dichloromethane, and ethylacetate) were the most proper ones. The protective effects of these extracts against H$_2$O$_2$-induced cytotoxicity were examined. Ethyl acetate (EA) extract was fractionated and total phenolic and flavonoid contents (TPC and TFC) of fractions were estimated. EA extract was found to be the most effective extract on suppressing the toxicity of H$_2$O$_2$. It caused up to 35% reduction in H$_2$O$_2$-induced cellular death, more than 70% decrease in reactive oxygen species (ROS) accumulation, 47% increase in SOD activity, almost 175% decrease in caspase-3 activity, and more than 27% elevation in mitochondrial membrane potential level. After fractionation, the most potent fraction (F3) was found active in protecting PC12 cells from oxidative stress consequences. This fraction possessed the highest ratio of flavonoid to non-flavonoid phenolic compounds (TFC = 125.03 ± 1.31 mg quercetin equivalent/g and TPC = 127.18 ± 6.00 mg gallic acid equivalent/g), suppressed the toxicity mediated by H$_2$O$_2$ and successfully inhibited the overproduction of ROS caused by H$_2$O$_2$. These results altogether suggested *A. ciniformis* is a potential choice for preventing different neurodegenerative diseases.

**INTRODUCTION**

Evidences have shown that the oxidative stress possesses a vital role in the pathogenesis of many neurobiological diseases, such as stroke, Alzheimer’s, and Parkinson’s diseases, and also contributes to the aging process (Albarracin et al., 2012; Chen et al., 2011; Uttara et al., 2009). Several medicinal plants can target oxidative stress and mitochondrial dysfunction, and are known as good therapeutic and preventive candidates for such neural diseases. Plants, as reach sources of antioxidants, can potentially donate hydrogen to free radicals and inhibit molecular damages caused by oxidative stress (Albarracin et al., 2012). The whole plant extracts can be advantageous over individual phytochemicals because of the multitude of compounds involved in their structure, providing a mixture with variety of biological effects and much less toxicity (Nobili, 2009). Regarding the profound capacity of natural antioxidants versus the established adverse effects of the synthetic ones, it is necessary to find naturally occurring compounds protecting neuronal cells against oxidative damages.

*Artemisia ciniformis* Krasch & Popov ex Poljakov is one of the 34 *Artemisia* species, which can be found in wide regions of Iran (Mozaffarian, 1996). The genus *Artemisia*...
Artemisia ciniformis (family Asteraceae) has about 500 species widely distributed in Europe, Asia, as well as North America (Bora and Sharma, 2011; Mucciarelli and Maffei, 2002). In the Iranian traditional medicine, different Artemisia species have long been known as a remedy for different disorders (Gorji and Ghadiri, 2001; 2002; Ghorbani, 2005; Zargari, 1995). For example, A. annua has been known as a species with hemostatic effect and a useful remedy for amelioration of constipation. Artemisia absinthium was believed to possess laxative properties and was used for parasite-induced intestinal disorders (Ghorbani, 2005). Furthermore, this species was a remedy for headache (Gorji and Ghadiri, 2002) and used for antiepileptic preparations in medieval Persia (Gorji and Ghadiri, 2001). Artemisia kopetdaghensis was believed to be effective in alleviation of parasite-induced intestinal disorders and colitis (Ghorbani, 2005). Several potentially therapeutic bioactivities, such as antiinflammatory, antioxidant, anti-inflammation, antitumor, antifungal, neuroprotective, and leishmanicidal effects, have been reported for different members of the genus Artemisia (Bora and Sharma, 2010; 2011; Emami et al., 2012; Hatami et al., 2014; Tayarani-Najaran et al., 2017). Besides, some of these species were found to have nutritive value as food additives (Kordali et al., 2005). Artemisia ciniformis has demonstrated in vitro effects, including apoptogenic and antiproliferative (Taghzadeh Rabe et al., 2011; Tayarani-Najaran et al., 2014), leishmanicidal (Emami et al., 2012), cardioprotective (Mojarrab et al., 2013), antimalarial (Mojarrab et al., 2015), free radical-scavenging and cytoprotective (Mojarrab et al., 2016), and antimicrobial effects (Taherkhani, 2016). These widespread pharmacological effects could be attributed to the variety of bioactive compounds and secondary metabolites in different parts of this species. The high content of polyphenols (Mojarrab et al., 2016) and other bioactive constituents like sesquiterpene lactones (Iranshahi et al., 2007) made this species to be of botanical and pharmaceutical interests. In addition, monoterpenoids such as camphor, myrcene, and linalool and sesquiterpenoids, such as davanone, have been reported as the main constituents in its volatile oil (Firouzni et al., 2008; Rustaiyan et al., 2007; Taherkhani et al., 2012). Recently, isochlorogenic acid isomers have been isolated from the hydroethanolic extract of the species (Nasseri et al., 2019).

Because of the notable distribution of different species of Artemisia in Iran (Naghavi et al., 2017), and with respect to neuroprotective potential of some species in the genus (Bora and Sharma, 2010; Lachenmeier, 2010) as well as the cytoprotective and antioxidant effects reported for A. ciniformis, (Mojarrab et al., 2013; 2016), it seems to be of value to examine whether different extracts of this species—in spite of lack of information on traditional use—can protect neuronal cells against oxidative stress. To the best of our knowledge, there has not been any similar cell-based study done on the neuroprotectivity of different extracts and fractions of this species and the mechanism underlying for their cytoprotective effect. As per the fact, that apoptosis is the common pathway for death of cells exposed to oxidative damage; this study has been designed to elucidate if antiapoptotic pathways are involved in inhibitory activity of this species.

MATERIALS AND METHODS

Reagents

Petroleum ether (40–60), ethyl acetate (EA), Gallic acid, sodium carbonate, Folin–Ciochette’s phenol reagent, LiChroprep® RP-18 (15–25 μm), and H2O2 were purchased from Merck, Germany. Ethanol and dichloromethane (DCM) were procured from Scharlau, Spain. Quercetin, 2′,7′-dichlorofluorescin diacetate (DCF), Triton-X100, dimethylsulphoxide (DMSO), MTT [(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), and Rhodamine 123 were purchased from Sigma-Aldrich (St Louis, MO). Trypsin-EDTA was supplied from Bon Yakteteh, Iran. Fetal bovine serum and Penicillin/Streptomycin were purchased from Gabon, USA and Gibco, USA. Dulbecco's Modified Eagle's Medium (DMEM) was supplied from ATCC, USA.

Plant material

The aerial parts of A. ciniformis were collected from Tandooreh National Park (Razavi Khorasan province, Iran). Samples were identified by Dr. Valiollah Mozaffarian (Research Institute of Forest and Rangelands, Tehran, Iran). The voucher specimen (with the identification numbers of 12569) was deposited in the herbarium, Department of Pharmacognosy, Faculty of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran.

Preparation of extracts and fractions

The subsequent maceration method was used for the preparation of different extracts. Ninety grams of shade-dried aerial parts of A. ciniformis were milled and then were subsequently macerated in solvents with different polarities; petroleum ether (40–60), dichloromethane, EA, ethanol, and ethanol-water (1:1 v/v), respectively. After filtering the obtained extracts, all of them were dried by rotary evaporator (Heidolph, Germany) under a reduced pressure condition and at the low temperature (≤45°C) to yield 4.13, 9.66, 0.38, 2.54, and 17.01 g of corresponding extracts, respectively.

The dried extracts were kept at −20°C for further investigating. After preliminary cell-based assays, 360 mg of the most promising sample (EA extract of A. ciniformis) was fractionated by reversed-phase vacuum liquid chromatography on RP-18 (15–25 μm) with H2O containing increasing amounts (20%–100%) of MeOH to obtain six fractions (F1–F6). All the fractions were dried as described above to yield 0.071, 0.095, 0.029, 0.038, 0.066, and 0.048 g of corresponding fractions, respectively.

Estimation of total phenolic and flavonoid contents

The Folin–Ciochette method (Singleton et al., 1999) was used to determine total phenolic content (TPC). Each dried fraction of EA extract (30 mg) was mixed with 25 ml of distilled water and filtered through Whatman No. 1 paper. This solution (0.5 ml) was then mixed with 2.5 ml of 0.2-N Folin–Ciochette reagent for 5 minutes and 2 ml of 75 g/l sodium carbonate was then added. After standing in dark for 2 hours, the absorbance of the reaction mixture was measured at 760 nm. Gallic acid (0–100 mg/l) was used as a standard to produce the calibration curve. The
The mean of three readings was used and the TPC was expressed in mg of gallic acid equivalent (GAE)/g of dried fraction.

The total flavonoid content (TFC) was determined using the Dowd method as adapted by Meda et al. (2005). Briefly, 2 ml of 2% aluminum trichloride in methanol was mixed with the same volume of a methanolic solution (0.2 or 0.3 mg/ml) of each fraction. Absorption readings at 415 nm were taken after 15 minutes. TFC was determined using a standard curve with quercetin (0–50 mg/l) as the standard. The mean of three readings was used and expressed as milligrams of quercetin equivalent (QE)/g of dried fraction.

**MTT assay**

In this study, PC12 pheochromocytoma cells were supplied from Pasteur Institute of Iran (Tehran, Iran). The cell growth medium was prepared by dissolving of fetal bovine serum (10% v/v) and 1% penicillin/streptomycin (100 U/ml: 100 U/ml) in DMEM-F12 at 37°C in a humidified incubator containing 5% CO2. 15–20 × 10^5 PC12 cells were seeded in each well of a 96-well culture plate and then treated with different concentrations of each Artemisia extract in DMSO (6.25, 12.5, 25, and 50 µg/ml) and incubated for 24 hours. At appropriate time intervals, the medium was replenished with 0.5 mg/ml MTT solution and plates were further incubated for 3 hours at 37°C. Thereafter, 100 µl DMSO was added to the wells for solubilizing the crystals of formazan. The absorbance at 570 nm corresponding to different treatment groups and control was detected on Eliza micro plate reader (BioTek, H1M).

**Measurement of mitochondrial membrane potential (MMP)**

PC12 cells were treated with the selected extracts in 6-well tissue culture plates for 24 hours. After that, the IC50 concentration of H2O2 was added to the cells and incubated for another 24 hours. At the end of the treatment, cells were co-incubated with rhodamine 123 (15 µl, 20 µM) for 30 minutes at 37°C. Thereafter, 1-ml Triton-X100 was used for lysing the treated cells and the amount of their fluorescence was measured at the wavelengths in range of 488–510 nm using a fluorescence microplate reader (BioTek, H1M).

**Determination of intracellular ROS**

Intracellular reactive oxygen species (ROS) formation was determined using 7-dichlorofluorescein diacetate (DCF-DA), a non-florescent dye which is oxidation sensitive, and its reaction with ROS can make it fluorescent (in the form of dichlorofluorescein (DCF) molecules) (Bai et al., 2005). The intracellular ROS formation study was conducted on different groups of cells: (1) Control group, (2) Cells treated with H2O2 (62.5 µM) for 24 hours, and (3) Cells pretreated with non-toxic concentrations of different extracts (selected after MTT study) for 24 hours and subsequently treated with H2O2 (62.5 µM) for another 24 hours. After treatment period, these groups were incubated with 100 µl DCFDA at 37°C for 30 minutes. Thereafter, cells were lysed with Triton-X100 and the absorbance at wavelength of 488–510 nm was recorded using a fluorescence microplate reader (BioTek, H1M).

**Determination of SOD activity**

SOD activity was defined based on the inhibitory ability of these metallo enzymes against oxidation of hydroxylamine, which possesses a prominent role in the cellular antioxidant defense mechanism. In the present study, commercial SOD assay kit Cayman (USA) was utilized to determine SOD activity following the manufacturer’s protocol. In this method, detection of superoxide radicals (produced from the xanthine oxidase-hypoxanthine system) would be possible using a tetrazolium salt. One unit of SOD was defined as enzyme concentration required for dismutation of 50% of the superoxide radicals. The activity of SOD was expressed as U/mg protein.

**Caspase-3 activity assay**

In order to find out the mechanism in which samples exert anti-apoptogenic activity, caspase-3 activity was determined based on the instructions of sigma colorimetric caspase kit. For this purpose, PC12 cells were cultured in 6-well tissue culture plates and incubated for 24 hours. Similar to intracellular ROS formation study, three different groups of cells (control, H2O2-treated, and extracts-pretreated H2O2-treated groups) were prepared and incubated at the conditions similar to the previous section. Then, the treated cells were centrifuged for 5 minutes at 1,300 rpm and were lysed in 15 µl of the cell lysis buffer. The substrate reaction buffer containing caspase-3 substrate was added to the supernatant and incubated for 2 hours at 37°C. The absorbance was then measured at 405 nm using a plate reader (BioTek, H1M).

**Statistical analysis**

In the present study, all the experiments were conducted in triplicate and reported values represented as the mean value ± SD. The one-way analysis of variance (ANOVA) using Tukey’s test was performed to compare the results. The statistical significance of variations can be confirmed at p-values less than 0.05.

**RESULTS**

**MTT assay results**

Cell viability assay was performed to determine viability of PC12 cell after exposure of different extracts obtained from A. ciniformis. Another aim of this assay was also to investigate whether pretreatment of cells with non-toxic concentrations of these extracts can reduce the toxicity of H2O2 in PC12 cells. In order to find out the non-toxic concentration of each extract, which can suppress the H2O2-induced cytotoxicity on PC12 cells, we first examined the effect of different concentrations of each extract (0–50 µg/ml) on PC12 cell survival (Fig. 1a). A glance at Figure 1 obviously indicates that none of the A. ciniformis extracts possess cytotoxic activity toward these cells (viability percentage >75% even at the highest concentration). Accordingly, the best results could be assigned to the PE (25 µg/ml), EA (25 and 50 µg/ml), and DCM (25 µg/ml) extracts of A. ciniformis. Then, these extracts were selected for further studies. The protective study of these extracts against
Hosseinzadeh et al. / Journal of Applied Pharmaceutical Science 9 (04); 2019: 016-023

H$_2$O$_2$-induced cytotoxicity was also performed. The concentration of H$_2$O$_2$ was chosen based on a preliminary MTT assay and the IC$_{50}$ concentration (62.5 µM) was chosen for remained analyses (Fig. 1b).

A comparison between the results of cell viability of H$_2$O$_2$-treated cells and those pretreated with selected A. ciniformis extracts (Fig. 2) exhibited that all of these extracts (except EA 50 µg/ml) attenuated the cytotoxicity induced by H$_2$O$_2$ on PC12 cells and significantly protected these cells against H$_2$O$_2$-induced cellular death. Although EA extract (50 µg/ml) increased the cell survival, its cytoprotective effect was not-significant ($p > 0.05$). The most protective samples were found to be DCM (25 µg/ml) and EA (25 µg/ml) extracts which caused more than 30% increase in the percentage of viable cells.

**Effect of selected extracts on the H$_2$O$_2$-induced intracellular ROS production**

The intracellular ROS accumulation was also assessed for H$_2$O$_2$-treated PC12 cells and compared to the corresponding cells pretreated with different A. ciniformis extracts. As expected, in the presence of H$_2$O$_2$, the level of intracellular ROS prominently increased (more than 40%), indicating the significant induction of free radicals by H$_2$O$_2$ in cells (Fig. 3a). In all the cell groups co-incubated with A. ciniformis extracts, overproduction of ROS was diminished. However, the highest scavenging activity could be assigned to EA (25 µg/ml) extract (more than 70% decrease) following by DCM (25 µg/ml) and EA extract (50 µg/ml). These three extracts not only attenuated the oxidative stress caused by H$_2$O$_2$, but also reduced the level of free radicals in comparison with control group, indicating their protective potential toward H$_2$O$_2$-induced oxidation.

---

**Figure 1.** The cytotoxic activity of different extracts obtained from (a) A. ciniformis and (b) H$_2$O$_2$ toward PC12 cells.

**Figure 2.** The PC12 cell viability percentage after exposure of H$_2$O$_2$ in presence and absence of selected extracts of A. ciniformis.

**Figure 3.** The effect of selected extracts of A. ciniformis on the (a) H$_2$O$_2$-induced overproduction of ROS and (b) SOD enzymatic activity in PC12 cells.
Effect of selected extracts on SOD enzymatic activity

Endogenous antioxidant enzymes, such as SOD, can promisingly eliminate or decrease the quantity of free radicals and attenuate oxidative stress. Oxidants and toxic metabolites, such as H$_2$O$_2$, are believed to cause a significant decline in expression/activity of the endogenous antioxidant enzymes like SOD. In order to examine whether co-culture of selected extracts can exert noticeable effect on the deficiency of SOD expression/activity, induced by H$_2$O$_2$, the SOD activity of co-cultured cells and corresponding H$_2$O$_2$-treated cells were compared (Fig. 3b). As expected, H$_2$O$_2$ significantly reduced the SOD enzymatic activity of PC12 cells. It is obvious from the first glance that this effect has been inhibited in the cells co-cultured with different A. ciniformis extracts. All the three extracts (dose 25 µg/ml) markedly increased the SOD activity in comparison with control cells.

Effect of selected extracts on the caspase-3 activity

As a biomarker of mitochondrial-dependent apoptotic pathway, caspase-3 activity has been determined to examine whether these extracts can inhibit or reduce apoptosis induced by oxidative stress in PC12 cells (Fig. 4a). The noticeable increase (about 140%) in caspase-3 activity of H$_2$O$_2$-treated cells is not uncommon or surprising because this oxidant causes cellular death via apoptosis by induction of oxidative stress in these cells. Upon addition of selected extracts to the cells, the apoptogenic activity of H$_2$O$_2$ was attenuated. All of them successfully reduced the caspase-3 activity. However, DCM and EA extracts were the most active ones and more than 180% reduction in caspase-3 activity (in comparison with H$_2$O$_2$-treated cells) was attributed to these extracts.

Effect of selected extracts of A. ciniformis on the H$_2$O$_2$-induced dissipation of MMP

It was expected that A. ciniformis extracts would potentially inhibit the depolarization of mitochondria induced by H$_2$O$_2$. To address this assumption, we further assessed the role of selected extracts on MMP level of H$_2$O$_2$-treated PC12 cells, by determining the fluorescence intensity of rhodamine 123. Upon addition of H$_2$O$_2$, a rapid reduction, about 23%, in the fluorescence intensity of rhodamine 123 was observed. Pretreating the cells with EA extract (25 and 50 µg/ml) could significantly stabilize MMP (27% and 21% increase in MMP, respectively); however, PE and DCM extracts markedly potentiated the dissipation of MMP caused by H$_2$O$_2$ (Fig. 4b).

Cytoprotective effect of fractions of EA extract

First, the cytotoxic effect of fractions was evaluated in PC-12 cells by MTT method. The MTT results of these fractions are demonstrated in Figure 5a. According to the results, none of them were able to decrease PC12 viability compared to control. When the protective effect of fractions was assayed, it was observed that F3 is strongly able to protect PC12 cells against cytotoxicity induced by H$_2$O$_2$. Pretreatment of cells with the selected fraction (F3) at concentration of 25 µg/ml significantly suppressed the cytotoxicity of H$_2$O$_2$, and about 30% increase was observed in the number of viable cells (Fig. 5b). Further investigation showed that overproduction of ROS, induced by H$_2$O$_2$, was successfully attenuated by F3 (12.5 and 25 µg/ml). As per the illustration (Fig. 5c), ROS plays a prominent role in H$_2$O$_2$-induced cellular death and F3 at both concentrations significantly overcame this alteration. Almost 85% and 170% decrease in ROS level was the result of pretreating PC12 cells with F3 (12.5 and 25 µg/ml, respectively).

Total phenolic and flavonoid contents of fractions of EA extract

TPC (mg of GAE/g of each fraction) varied from 4.36 ± 1.15 (F6) to 263.08 ± 1.23 (F2) (Table 1) using the standard curve of gallic acid ($R^2$=0.999, $y=0.016x+0.074$). Regression equation of the calibration curve of quercetin ($R^2=0.999$, $y=0.030x−0.011$) was used to calculate the TFC of fractions (mg of QE/g of each fraction). Large variations in TFC of the samples were found, ranging from 2.82 ± 0.26 (F6) to 129.64 ± 3.57 (F2) (Table 1).

DISCUSSION

Nervous system is believed to be particularly vulnerable to oxidative stress and based on its high content of polyunsaturated fatty acids; one of the most important reasons for nervous cellular damage is the over generation of oxygen species (Gitto et al., 2002; Xu et al., 2012). Oxidative stress, caused by ROS such as hydrogen peroxide, can be taken into account as one of the vital factors involved in the development of several
death caused by H$_2$O$_2$. *Artemisia ciniformis* was shown in the present study to be a promising natural source of phytochemicals, effective in inhibition of H$_2$O$_2$-mediated cellular death.

Different extracts of *A. ciniformis* (EA, DCM, and PE extracts) inhibited the cytotoxic effect of H$_2$O$_2$ toward PC12 cells. The cell viability significantly increased in the group of cells pretreated with these extracts. The H$_2$O$_2$-induced apoptotic rate of these cells was also found to be affected by these extracts. The ROS-scavenging activity and the inhibitory effect on the H$_2$O$_2$-induced reduction of SOD activity, the substantial role in suppression of caspase-3 activity, and promising effect in maintaining MMP were all exerted by these three extracts. EA extract was the most promising one, demonstrating the highest antioxidant activity against oxidative injury induced by H$_2$O$_2$.

The potent radical scavenging activity of this extract in comparison with two other extracts (DCM and PE) could be probably due to the higher content of phenolic compounds (Mojarrab et al., 2016). In another study, the most potent extract of *Artemisia turanica* against H$_2$O$_2$-induced cytotoxicity was also the EA extract (Hosseinzadeh et al., 2018). The common belief is that antioxidant activity of medicinal plants is in direct correlation with their TPC, which has been examined and corroborated in several studies (Hatami et al., 2014; Ishige et al., 2001; Jing et al., 2015; Kim, 2010; Tian et al., 2017). In our previous study, we have examined the radical scavenging and ferrous iron chelating activity of different extracts obtained from *A. ciniformis* and amount of natural phenolic compounds have been estimated as well. Among the three extracts examined in this study, EA extract possessed the highest TPC (55.94 ± 0.59 mg GAE/g), followed by DCM (15.80 ± 0.48 mg GAE/g), and PE (1.98 ± 0.22 mg GAE/g) extracts, respectively (Mojarrab et al., 2016). This order was consistent with the order of cytotoxic efficacy of extracts in the present study. It is worth noting that EA extract showed the highest ferrous ion chelating ability among the samples in previous study (Mojarrab et al., 2016).

Beside the powerful antioxidant activity of EA extract, the considerable anti-apoptogenic effect was another reason making it promising for neuroprotective applications. SOD enzymatic activity is directly correlated with antioxidant and anti-apoptogenic effects. The higher endogenous SOD activity was shown to be corresponded with the higher anti-apoptotic ability (Avery, 2011). Our results suggested the prominent role of EA extract on elevation of SOD activity and decrease of ROS level, implying that this extract protects PC12 cells from apoptosis. Among the studied extracts, this extract was the only one preventing the depolarization of mitochondria induced by H$_2$O$_2$. The mitochondria possess a prominent role in activating apoptosis in mammalian cells. The rapid collapse of MMP, as a result of oxidative stress, was remarkably prevented by EA extract. The MMP results in association with the results of caspase-3 activity identified that EA extract inhibited the apoptosis of PC12 cells via a mitochondria-dependent pathway and intrinsic mitochondrial pathway can be the possible mechanism for inhibition of apoptosis. Apart from these findings, different fractions (F1–F6) were obtained from EA extract of *A. ciniformis*, while they all were safe toward PC12 cells. Fraction F3, which was estimated to be rich in flavonoids showed promising neuroprotective potential against oxidative injury caused by H$_2$O$_2$. When the protective effect of F3 in

---

**Figure 5.** The cytotoxic effect of different fractions isolated from (a) EA extract of *A. ciniformis* against PC12 cells and (b) the role of selected fraction on H$_2$O$_2$-induced toxicity, and (c) inhibitory effect of selected fraction on H$_2$O$_2$-induced generation of ROS in PC12 cells.

**Table 1.** Total phenolic and flavonoid contents of the fractions from EA extract of *A. ciniformis*.

<table>
<thead>
<tr>
<th>Sample</th>
<th>TPC (mg QE/g)</th>
<th>TPC (mg GAE/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>5.89 ± 0.15</td>
<td>89.05 ± 1.42</td>
</tr>
<tr>
<td>F2</td>
<td>129.64 ± 3.57</td>
<td>263.08 ± 1.23</td>
</tr>
<tr>
<td>F3</td>
<td>125.03 ± 1.31</td>
<td>127.18 ± 6.00</td>
</tr>
<tr>
<td>F4</td>
<td>13.01 ± 0.40</td>
<td>32.66 ± 1.47</td>
</tr>
<tr>
<td>F5</td>
<td>15.79 ± 0.63</td>
<td>18.34 ± 0.71</td>
</tr>
<tr>
<td>F6</td>
<td>2.82 ± 0.26</td>
<td>4.36 ± 1.15</td>
</tr>
</tbody>
</table>

---

neurodegenerative diseases by disrupting the cellular function and eventually mediating cellular death (Farzaei et al., 2016). The overproduction of ROS negatively modifies the organelles, such as mitochondria, and causes damaging their function (Avery, 2011). In this study, significant cellular death (almost 55% of cells) was detected during the oxidative stress, upon exposure of PC12 cells to H$_2$O$_2$. As per the fact, this oxidant mostly induces apoptosis. Apart from these findings, different fractions (F1–F6) were obtained from EA extract of *A. ciniformis*, while they all were safe toward PC12 cells. Fraction F3, which was estimated to be rich in flavonoids showed promising neuroprotective potential against oxidative injury caused by H$_2$O$_2$. When the protective effect of F3 in
PC12 cells was examined, it was observed that pretreating the cells with this fraction considerably prevented the cellular death caused by H2O2. An immense reduction in H2O2-induced overproduction of intracellular ROS indicated the promising antioxidant effect of F3 against oxidative injury in PC12 cells.

Flavonoids can protect neuronal cells from oxidative stress (Ishige et al., 2001), as they have been isolated and identified as the major components of EA fraction with neuroprotective potential from different plant species such as Orostachys japonicus (Park et al., 2017), Hypericum afrum, Cytisus villosus (Larit et al., 2018), as well as Diospyros kaki (Jeong et al., 2018). In the current study, the highest ratio of flavonoid to non-flavonoid phenolic compounds was calculated for F3; but, based on the lack of complete phytochemical analyses on the isolated fraction; we cannot certainly discuss which bioactive components are responsible for the powerful protective effect of F3. It should be noted that although the antioxidant activity of medicinal plants is mostly attributed to their polyphenol constituents, there are several studies demonstrating the powerful antioxidative and neuroprotective activities of non-phenolic compounds, such as α-pinene and 1.8-cineole (Porres-Martinez et al., 2015), DL-3-n-butylphthalide (Li et al., 2009), panaxydol (Zhu et al., 2008), tanshinone IIA (Jia et al., 2012), and catalpol (Wang et al., 2008). Thus, the promising neuroprotective activity of EA extract of A. ciniformis cannot be certainly assigned to its phenolic constituents, and it still remains a highly possible hypothesis. The antioxidant activity-guided phytochemical investigation of A. ciniformis, based on the results of cell-free assays (Mojarrab et al., 2016) has led to isolation and identification of the known isomers of dicaffeoylquinic acid (DCQ), including 3,5-DCQ (isochlorogenic acid A), 3,4-DCQ (isochlorogenic acid B), and 4,5-DCQ (isochlorogenic acid C) (Nasseri et al., 2019), which is consistent with the previous reports on the presence of caffeoylquinic acids as the major antioxidant and neuroprotective components in the genus Artemisia (Lee et al., 2011; Kang et al., 2016; Zhang et al., 2016) Our further studies would be beneficial to elucidate the profile of active constituents of the selected fraction of EA extract of A. ciniformis to provide a definite conclusion and elucidate the neuroprotective mechanisms, unequivocally. Taken together, the results of the present study indicated the potency of different extracts of A. ciniformis as neuroprotective agents. In agreement with these results, similar studies have shown the neuroprotective potential of the extracts and phytochemicals obtained from other Artemisia species, such as A. asiatica (Kim et al., 2000), A. turanica and A. turcomanica (Hosseinzadeh et al., 2018), A. princeps (Lee et al., 2011), and A. absinthium L. (Hallal and Kharoubi, 2016), which considerably inhibited the intracellular ROS production and exerted antiapoptotic activity. These results altogether demonstrate the neuroprotective efficacy of A. ciniformis and introduces this plant species as a useful candidate to be further examined against neurodegenerative diseases.

ACKNOWLEDGMENT

The authors would like to thank the Research Council of Kermanshah University of Medical Sciences for the financial support (grant no.: 94383).

FINANCIAL SUPPORT

None.

CONFLICT OF INTEREST

The authors declared that they have no conflict of interest for this study.

REFERENCES


Hatami T, Emami SA, Miraghaee SS, Mojarrab M. Total phenolic contents and antioxidant activities of different extracts and fractions from the aerial parts of Artemisia biennis Willd. IJPR, 2014; 13(2):551.


Jeong DW, Cho CH, Lee JS, Lee SH, Kim T, Kim DO. Deastringent peel extracts of Persimmon (Diospyros kaki Thumb. cv.


Lachenmeier DW. Wormwood (Artemisia absinthium L.)—a curious plant with both neurotoxic and neuroprotective properties? J Ethnopharmacol, 2010; 131(1):227–44.


How to cite this article: