

## Evaluation of antioxidant and cytoprotective activities of *Artemisia ciniformis* extracts on PC12 cells

Mahdi Mojarrab<sup>1</sup>, Sajjad Nasser<sup>2</sup>, Leila Hosseinzadeh<sup>1\*</sup>, Farah Farahani<sup>1</sup>

<sup>1</sup> Pharmaceutical Sciences Research Center, School of Pharmacy, Kermanshah University of Medical Sciences, Kermanshah, Iran

<sup>2</sup> Students Research Committee, Kermanshah University of Medical Sciences, Kermanshah, Iran

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### ABSTRACT

**Objective(s):** In the current study antioxidant capacities of five different extracts of *Artemisia ciniformis* aerial parts were evaluated by cell-free methods. Then seven fractions of the potent extract were selected and their antioxidant capacity was assayed by cell free and cell based methods.

**Materials and Methods:** Antioxidant ability was measured using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging test,  $\beta$ -carotene bleaching (BCB) method and ferrous ion chelating (FIC) assay. Total phenolic contents (TPC) of all the samples also were determined. The cytoprotective effect of fractions was evaluated by measuring the viability of cells after exposure to doxorubicin (DOX). The mechanism of action was studied by investigating caspase-3, mitochondrial membrane potential (MMP), the level of super-oxide dismutase (SOD) and intracellular reactive oxygen species (ROS).

**Results:** Hydroethanolic extract exhibited a notably higher antioxidant activity and phenolic content. Among the fractions (A to G) of hydroethanolic extract, the highest antioxidant capacity was observed in the Fraction E. Moreover, 24 hr pretreatment of PC12 cells with fractions B, C and D decreased DOX-induced cytotoxicity. In addition, pre-treatment of cells with fraction B resulted in significant decrease in generation of the reactive oxygen species (ROS) and increase in the activity of SOD. We were able to demonstrate remarkable reduction in the activity of caspase-3 and increase in MMP in PC12 cells following pretreatment with fraction B.

**Conclusion:** Our observations indicated that the fraction B of *A. ciniformis* hydroethanolic extract possessed protective effect on oxidative stress and apoptosis induced by DOX in PC12 cells.

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### Introduction

Reactive oxygen species (ROS) are considered as harmful products of normal aerobic metabolism of living organisms. The perpetual existence of these chemically reactive oxygen-containing molecules is believed to be in balance with natural antioxidant defense mechanisms. Whenever antioxidants and pro-oxidants can no longer maintain the defense stability, the situation is shifted toward the ROS generation and creation of oxidative stress with various implications on human health (1-4).

Recently, evidences to support plant polyphenols contribution in several important anti mutagenic (5), neuroprotective (6), anti-inflammatory and anticancer (7, 8) activities and hence the interest in plant-derived polyphenols as natural antioxidants have increased noticeably (9). Phenolic compounds commonly found in plants have shown profound antioxidant capacities (9-12). Other types of natural antioxidants such as nitrogen containing compounds

(13, 14), terpenoids (15, 16) and polysaccharide fractions (17-19) exert their antioxidant activity with rather different mechanisms including prevention of lipid peroxidation, inhibition of protease and RNase activity, scavenging of free radicals, reducing power, and metal chelating ability.

The genus *Artemisia*, Compositae (Asteraceae) is described as small herbs and shrubs belonging to the Anthemideae tribe (20). One of the species that growing widely in Iran is *Artemisia ciniformis* Krasch. & Popov ex Poljakov with the Persian names of "Dermaneye talaie" and "Dermaneye sakhreroo" (21, 22). Investigations on volatiles from aerial parts of *A. ciniformis* have resulted in the identification of some mono- and sesquiterpenoids (23, 24). Research on *A. ciniformis* extracts has revealed outstanding cytotoxicity of petroleum ether and dichloromethane extracts against a wide range of cancer cell lines (25-27). Moreover, *in vitro* leishmanicidal activity of ethanolic extract (28) and antimalarial activity of

\*Corresponding author: Leila Hosseinzadeh. Pharmaceutical Sciences Research Center, School of Pharmacy, Kermanshah University of Medical Sciences, Kermanshah, Iran. Tel: +98-83-34276480; Fax: +98-83-34276493; email: lhosseinzadeh90@yahoo.com

dichloromethane extract of the species have been demonstrated (29).

Doxorubicin (DOX) is one of the most effective anti-cancer drugs used for the treatment of various malignancies (30). Like any other anticancer agent, DOX is associated with numerous undesirable side effects on some organs such as the brain manifesting as decrease in hippocampal neurogenesis and volume (31). The mechanism by which DOX induces neuronal injury remains a matter of controversy. However some reports suggest that ROS may be involved in the neurotoxicity induced by DOX. Park *et al* showed that DOX generates free radicals in cultured astrocytes and induces cytotoxicity dose-dependently (32). Similarly, the findings from our previous study indicated that ROS plays an important role in DOX-induced neurotoxicity (33).

Recently, we reported the protective effect of different extracts of *A. ciniformis* on H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity in cardiomyoblast H9c2 cells (34). The present study investigated the antioxidant potential and the total phenolic contents of different extracts and fractions of *A. ciniformis*. Furthermore, the protective effect of the resulting fractions from selected extract on doxorubicin-induced oxidative stress and apoptosis in PC12 cells was also determined. PC12 cells, derived from an adrenal tumor, possess neuronal cell functions. This cell line is a good in vitro model to study the neurotoxic effects of chemotherapeutic agents (35).

## Materials and Methods

### Reagents and chemicals

All chemicals, reagents and kits that were used in this study were purchased according to the following description: Fluorescent probe 2,7-dichlorofluorescein diacetate (DCF-DA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT), Triton X-100, FBS and rhodamine-123, 1,1-diphenyl-2-picryl-hydrazyl (DPPH),  $\beta$ -carotene, doxorubicin and caspase-3 detection kit from Sigma, Aldrich (St Louis, MO, USA); DMEM-F12 from Gibco (Gibco, Grand Island, NY, USA); super oxide dismutase assay kit from Cayman; gallic acid, linoleic acid, sodium carbonate, ferrous chloride, dimethyl sulfoxide (DMSO), chloroform, EDTA, Tween® 40, Folin-Ciocalteu's phenol reagent, butylatedhydroxytoluene (BHT), LiChroprep® RP-18 (15-25  $\mu$ m) from Merck; ascorbic acid from VWR; ferrozine iron reagent from Acros; organics and all the solvents used for extraction and fractionation from Scharlau (Sentmenate, Spain).

### Preparation of extracts and fractions

Aerial parts of *A. ciniformis* Krasch. & Popov ex Poljakov were freshly collected from Tandoureh National Park, Razavi Khorasan province, Iran, during September 2010 and authenticated by Dr V Mozaffarian at Research Institute of Forest and

Rangelands, Tehran, Iran. A voucher specimen (No. 12569) is kept in the herbarium, Department of Pharmacognosy, Faculty of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran.

Air-dried aerial parts of *A. ciniformis* were grounded and used (160 g) to make the extracts with petroleum ether (40-60), dichloromethane, ethyl acetate, ethanol, and ethanol-water (1:1 v/v) respectively (Sequential maceration with ca. 3×1.6 lit of each solvent). The extracted solvents were then filtrated and dried using rotary evaporator at a temperature below 45 °C and under reduced pressure in order to obtain 8.52, 18.42, 0.64, 5.24 and 33.23 g of each extract. The amount of 18 g from the most promising sample in antioxidant assays (hydroethanolic extract) was subjected to a vacuum liquid chromatography (VLC) system (reversed-phase RP-18 (25-40  $\mu$ m), 90 g) with H<sub>2</sub>O containing increasing amounts of MeOH (5%, 10%, 20%, 40%, 60%, 80% and 100%) to yield seven fractions (A, B, C, D, E, F and G respectively) (Table 1).

### Cell-free assays

#### Measurement of total phenolic contents

The total phenolic content (TPC) of extracts was determined by applying the Folin-Ciocalteu method (32) with some modification (33). To 500  $\mu$ l of each sample, 2.5 ml of Folin-Ciocalteu reagent (0.2 N) and 2 ml of Na<sub>2</sub>CO<sub>3</sub> solution (75 g/lit) were added. The optical density was measured after 2 hr standing in the dark at 760 nm against a blank. The final results were calculated based on calibration curve of gallic acid and expressed as milligrams of gallic acid equivalent (GAE) per gram of dried samples.

#### Measurement of DPPH radical scavenging activity

In order to measure the radical scavenging activity, Hatano *et al* method with slight modifications was used (36). Briefly, 1.5 ml of DPPH solution in methanol (0.2 mM) was added to the equal volume of all test samples. Mixtures were shaken vigorously and maintained in dark for 30 min and absorbance was then measured at 517 nm against a blank. We used BHT and ascorbic acid as standard references. The scavenging activity was calculated using this formula (1) in which Ac= absorbance of control and A= absorbance of a tested sample in 30 min:

$$I\% = (A_c - A) / A_c \times 100 \quad (1)$$

#### Determination of metal chelating activity

The chelating activity of samples with ferrous ions Fe<sup>2+</sup> was determined following the ferrous iron-ferrozine complex method (37) with some modifications. Briefly, 25  $\mu$ l of FeCl<sub>2</sub> solution (2 mM) was added to the mixtures of 1.5 ml of H<sub>2</sub>O and 2 ml of the test samples in methanol at different concentrations. The reaction initiation was by the

addition of ferrozine solution (50  $\mu$ l, 5 mM) to each test tube after 30 sec followed by shaking and incubation (10 min, room temperature). Absorbance of the solution was then measured at 562 nm. EDTA and quercetin were used as positive controls. The ability of the extracts and fractions to chelate ferrous ion were figured through adopting the formula (1).

Measurement of inhibition of  $\beta$ -carotene bleaching antioxidant potential of the samples was specified according to a slightly modified version of the  $\beta$ -carotene bleaching method (36). A solution of  $\beta$ -carotene in chloroform (5 mg/ 10 ml) was prepared and to 750  $\mu$ l of this solution, 33  $\mu$ l of linoleic acid and 225 mg of Tween 40 were added. The solvent was completely removed using a rotary evaporator. In order to obtain emulsion A, 75 ml of oxygenated distilled water was added and the mixture was then emulsified for 15 min in a sonicator. Aliquots of 3.5 ml of this emulsion were moved into a series of stopper test tubes containing 1 ml of samples dissolved in water or DMSO in different concentrations. Optical density (OD) readings were recorded at 470 nm for all samples immediately (t=0) and at the end of the assay time (t=120). A second emulsion consisting of 50 ml of oxygenated water, 22  $\mu$ l of linoleic acid and 150 mg of Tween 40 was also supplied and used as the blank to zero the spectrophotometer. The percentage inhibition was calculated according to the formula (2) where AA(120) is the absorbance of the sample at 120 min, AC(120) is the absorbance of the control at 120 min, and AC(0) is the absorbance of the control at 0 min:

$$I\% = \frac{(A_{(A(120))} - A_{(C(120))})}{(A_{(C(0))} - A_{(C(120))})} \times 100 \quad (2)$$

Cell-based assays

#### Cell culture and treatments

Rat pheochromocytoma-derived cell line, PC-12, was obtained from Pasteur Institute of Iran (Tehran, Iran). The PC-12 cells were grown in Dulbecco's modified Eagle's medium (DMEM-F12) with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin at 37 °C, 5% CO<sub>2</sub> in a humidified incubator. Stock solutions of DOX and fractions of *A. ciniformis* hydroethanolic extract were prepared in DMSO. Non-cytotoxic concentrations of fractions of *A. ciniformis* hydroethanolic extract and IC<sub>50</sub> concentration of DOX were established based on preliminary tests for cell viability using the methyl thiazol tetrazolium bromide (MTT) assay. A phase contrast inverted microscope (Motic, China) at 40 x magnifications was applied for observation of morphological changes of the cells.

#### Measurement of intracellular ROS

Intracellular ROS was monitored by using 2, 7-dichlorofluorescein diacetate (DCF-DA) as a non-fluorescent lipophilic ester which once crosses cell membrane, is oxidized to 2,7-dichlorofluorescein (DCF) fluorescent probe by unspecific esterases (38). Pretreated cells with selected fraction were treated with DOX for an additional 24 hr then washed with PBS and incubated with DCF-DA (20  $\mu$ l) at 37 °C for 30 min. The fluorescence was measured with standard Argon laser for 480-nm excitation and 530-nm band pass (FL1) filter by flow cytometry (Partec<sup>TM</sup> cytometer, Germany).

#### Determination of superoxide dismutase (SOD) activity

Superoxide dismutases are metallo enzymes that catalyze partitioning of superoxide anion to molecular oxygen and hydrogen peroxide and hence form an essential part of the cellular antioxidant defense mechanism (39). The SOD activity was measured by commercial SOD assay kit (Cayman, USA), following the manufacturer protocol. Cayman superoxide dismutase assay kit utilizes a tetrazolium salt to detect superoxide radicals made by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme required to exhibit 50% dismutation of superoxide radicals. Values were expressed as U/mg protein.

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

Mitochondrial dysfunction has been shown to participate in the induction of apoptosis (30, 40). Accumulation of rhodamine 123 fluorescent cationic dye in mitochondria as a direct function of the membrane potential was used to measure variations of MMP. Depolarization of mitochondrial membrane during cell apoptosis results in the loss of Rh123 from mitochondria and a decrease in intracellular fluorescence intensity (41). Cells were incubated with rhodamine 123 for 30 min at 37 °C. The fluorescence was measured with standard Argon laser for 480-nm excitation and 530 nm band pass (FL1) filter by flow cytometry (Partec<sup>TM</sup> cytometer, Germany).

#### Determination of caspase-3 activity

The activation of caspase-3 was determined using commercial caspase-3 assay kit (Sigma, USA) following manufacture recommendations. Briefly, cells were collected and lysed (1 $\times$ 10<sup>6</sup> /50  $\mu$ l lysis buffer) and incubated on ice for 10 min followed by 5 min centrifugation at maximum speed. The mixture of cell lysate (10  $\mu$ l) and an equal amount of substrate reaction buffer, containing a caspase-3 colorimetric substrate, was then incubated for 2 hr at 37 °C. The pNA light emission was quantified using a microplate reader at 400 or 405 nm (BioTek,

**Table 1.** Antioxidant performance and total phenolic contents of the extracts/fractions from *A. ciniformis*

Sample	Extraction/ fractionation yield (g)	EC <sub>50</sub> (µg/ ml)			TPC (mg GAE /g)
		DPPH assay	FIC assay	BCB assay	
PE	8.52	7230.74 ± 984.22	458.29 ± 40.56	214.88 ± 24.51	1.98 ± 0.22
DCM	18.42	361.69 ± 37.74	198.69 ± 41.29	198.55 ± 17.33	15.80 ± 0.48
EA	0.64	76.63 ± 3.59	100.57 ± 15.05	45.14 ± 6.51	55.94 ± 0.59
EtOH	5.24	44.29 ± 1.22	309.70 ± 58.10	42.37 ± 11.75	76.89 ± 0.92
EtOH/Wt	33.23	36.91 ± 1.58	970.19 ± 172.22	9.04 ± 0.27	134.67 ± 0.49
Fr. A	3.81	734.63 ± 223.93	389.58 ± 26.47	262.40 ± 38.29	0.78 ± 0.14
Fr. B	3.23	106.47 ± 2.45	640.97 ± 57.38	89.40 ± 10.84	12.37 ± 0.46
Fr. C	1.11	54.23 ± 1.69	163.91 ± 8.06	19.75 ± 0.28	106.86 ± 0.98
Fr. D	1.08	44.44 ± 1.81	227.71 ± 27.54	9.35 ± 0.37	221.68 ± 3.19
Fr. E	3.23	23.48 ± 1.04	330.79 ± 52.32	5.60 ± 0.38	242.13 ± 1.32
Fr. F	3.78	33.16 ± 0.30	272.80 ± 21.65	5.72 ± 0.47	175.47 ± 1.44
Fr. G	0.75	46.98 ± 0.45	731.01 ± 87.15	7.05 ± 0.26	70.14 ± 0.67
BHT	---	4.88 ± 0.57	---	0.458 ± 0.07	---
VitC	---	4.51 ± 0.07	---	---	---
EDTA	---	---	18.00 ± 3.02	---	---
Quercetin	---	---	87.24 ± 3.94	---	---

DPPH: 2,2-diphenyl-1-picrylhydrazyl; FIC: ferrous ion chelating; BCB: β-carotene bleaching; TPC: Total phenolic contents; GAE: gallic acid equivalent

H1M). Caspase-3 activity was presented as percentage of control and protein content was determined by Bradford method with bovine serum albumin being used as standard.

### Statistical analyses

All experiment were performed in triplicate and the results were presented as mean ± SEM. One-way analysis of variance (ANOVA) followed by the Tukey test was used to compare the differences between means with the value of  $P < 0.05$  considered to be statistically significant. In the cell free assessment, the Pearson's correlation coefficients ( $r$ ) between total phenolic contents of the samples and calculated EC<sub>50</sub> values were determined in each antioxidant assay.

## Results

### Cell-free assays

#### Total phenolic contents

The total phenolic contents (TPC) of *A. ciniformis* extracts and fractions calculated from the regression equation of the calibration curve ( $r^2 = 0.997$ ,  $y = 0.011x + 0.057$ ) are shown in Table 1. The total phenolic contents that are expressed in GAE as milligrams per gram of the extracts or fractions (mg GAE/g extract or fraction) showed large variations, between  $0.78 \pm 0.14$  and  $242.13 \pm 1.32$  mg GAE/g sample. The extracts contained a combination of phenolic compounds at different levels in the following order: hydroethanol > ethanol > ethyl acetate > dichloromethane > petroleum ether.

Calculated TPCs for two fractions (D and E) of the hydroethanolic extract were higher than 200 mg GAE/g fraction.

#### DPPH radical scavenging activity

Except for petroleum ether and dichloromethane extracts, as well as the fractions A and B, a good to

moderate inhibitory activity (calculated EC<sub>50</sub> values less than 100 µg/ ml) with respect to the DPPH radical was observed. The highest activity was obtained by the fraction E followed by the fraction F and hydroethanolic extract.

#### Ferrous ion chelating (FIC) effect

The highest ferrous ion chelating effect among the samples was observed in ethyl acetate extract followed by the fraction C and dichloromethane extract. Regardless of the records of decreases in absorbance readings, there were not any remarkable color changes in other samples.

#### Inhibition of β-carotene bleaching (BCB)

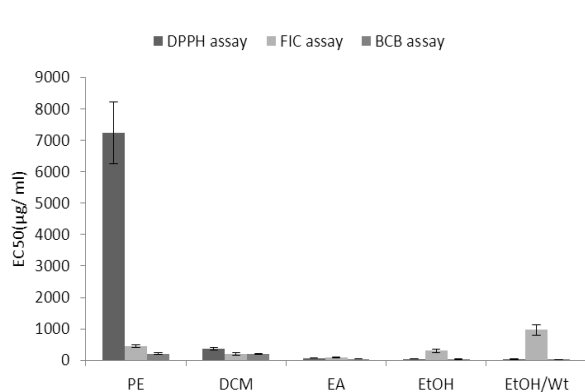
Table 1 displays the inhibitory activity of *A. ciniformis* extracts and derived fractions on β-carotene bleaching. Fraction E showed the best inhibitory performance whereas fraction A exhibited the lowest.

Pearson's correlation coefficients between TPC and calculated EC<sub>50</sub>s for DPPH, FIC and BCB assays showed the values of -0.392, -0.106, and -0.745, respectively. The lowest correlation was observed between TPC of samples and their capacity to chelate ferrous ions and there was no significant correlation between TPC of the samples and the relative DPPH radical scavenging activities. However, a significant correlation between the ability of the samples to inhibit the bleaching of β-carotene and their total phenolic contents was observed.

### Cell based assay

#### Fraction B protection against cytotoxicity and oxidative stress induced by DOX

Our results showed that fractions A-D and F were all non-cytotoxic at concentration below 50 µg/ml, while the maximum safe concentrations for fractions

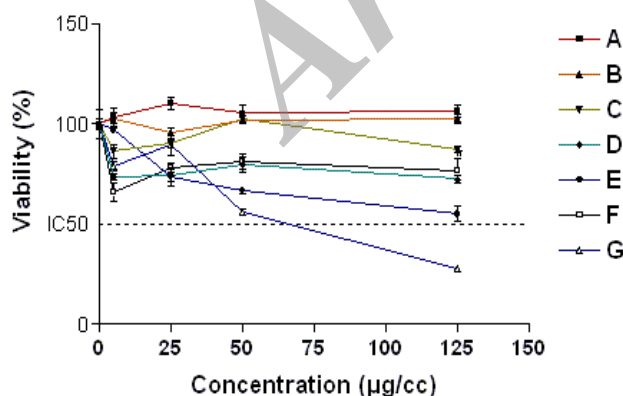


**Figure 1.** Antioxidant activity of *A. ciniformis* extracts from petroleum ether (PE), dichloromethane (DCM), ethyl acetate (EA), ethanol (EtOH) and ethanol/water (EtOH/Wt) DPPH: 2,2-diphenyl-1-picrylhydrazyl, FIC: ferrous ion chelating, BCB:  $\beta$ -carotene bleaching

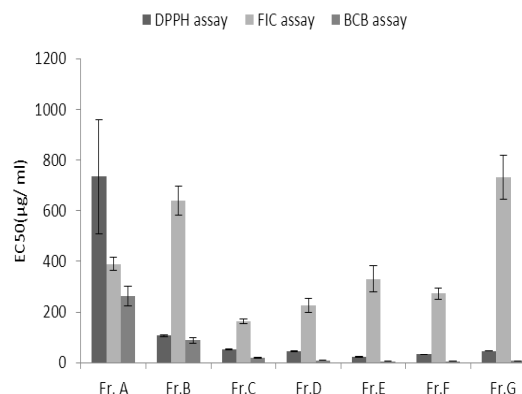
E and G were 5 and 25  $\mu\text{g}/\text{ml}$ , respectively (relative MTT activity > 80%) (Figure 3). Moreover, DOX significantly reduced PC12 cells viability as compared with control ( $\text{IC}_{50} = 2.500 \pm 0.09 \mu\text{M}$ ). When we used the nontoxic concentrations of fractions to evaluate their effects on induced cytotoxicity by DOX, fractions B, C and D proved to have outstandingly protective effects against DOX-induced toxicity in PC12 cells (Table 2). Next, we were interested in examining the ability of the selected fractions to protect PC12 cells from oxidative damage caused by DOX. As anticipated, adding DOX to PC12 cells induced a significant increase in the ROS level. Interestingly, only fraction B was able to reduce the ROS levels (Figure 4). Therefore, fraction B was used for subsequent experiments. Figure 5 shows that DOX significantly decreased SOD activity (61% of control level) but the presence of fraction B significantly increased SOD activity in PC12 cells.

#### Fraction B protection against DOX-induced apoptosis

The importance of activation of caspase cascade in initiation of apoptosis in various biological systems is



**Figure 3.** The dose-response curve of A-G fractions of hydroethanolic extract of *A. ciniformis* for 24 hr exposure time. Cell viability was determined by MTT assay as described in material and methods. Data are expressed as the mean  $\pm$  SEM of three separate experiments

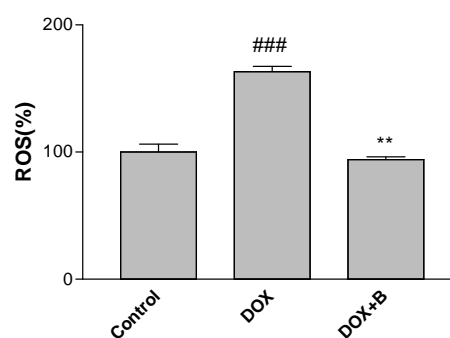
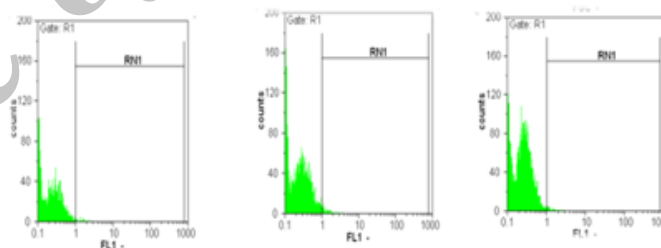


**Figure 2.** Antioxidant activity of different fractions from hydroethanolic extract of *A. ciniformis*. DPPH: 2,2-diphenyl-1-picrylhydrazyl, FIC: ferrous ion chelating, BCB:  $\beta$ -carotene bleaching, Fr: fraction

**Table 2.** Effect of fractions B, C and D of hydroethanolic extract of *A. ciniformis* pretreatment on DOX induced cytotoxicity

Group	$\text{IC}_{50} \pm \text{SEM} (\mu\text{M})^*$
DOX	$2.500 \pm 0.09$
DOX+ Fraction B	$>> 20$
DOX+ Fraction C	$9.29 \pm 1.03$
DOX+ Fraction D	$18.31 \pm 1.51$

\*Data are expressed as the mean  $\pm$  SEM of three separate experiments

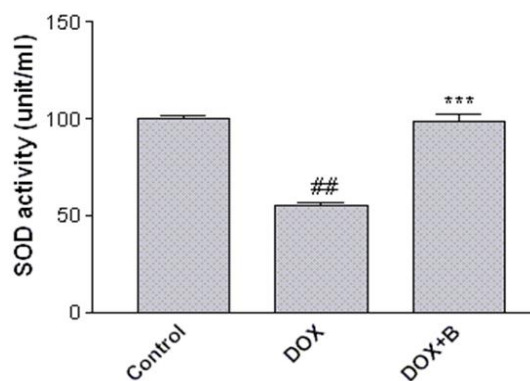


**Figure 4.** Effect of fraction B of hydroethanolic extract of *A. ciniformis* on DOX-induced ROS generation. Flow cytometry detection of ROS with DCF-DA. a) Representative of flow cytometry with DCF-DA plots of PC12 cells in different groups. b) Column bar graph of mean cell fluorescent for DCF-DA. Data are expressed as the mean  $\pm$  SEM of three separate experiments. ###  $P$ -value < 0.01 vs. Control, \*\*  $P$ -value < 0.01 vs. DOX treated cells

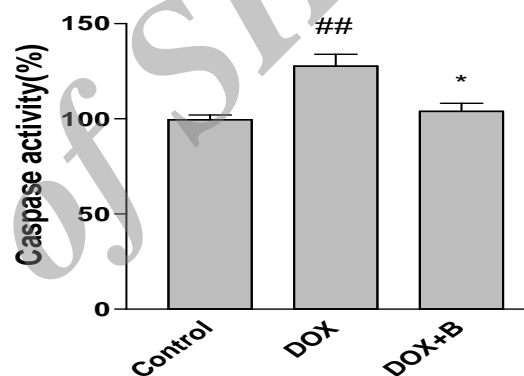
well known and caspase-3 has been shown to be a significant regulator of apoptosis (42). Caspase-3 activation can therefore be employed as a sensitive marker for apoptosis and for evaluating the ability of fraction B to inhibit DOX-induced apoptosis in PC12 cells. The obtained results showed that DOX was able to increase significantly caspase-3 activation in PC12 cells. Pretreatment with fraction B also decreased caspase-3 activation dramatically in comparison with DOX-treated cells (Figure 6). In addition, we examined MMP in the PC12 cell line. Flow cytometry analysis, showed a decrease of fluorescence indicating the rapid collapse of MMP when PC12 cells were exposed to DOX ( $2.5 \pm 0.098$ ). Pretreatment of cells with the active fraction also promoted the inhibition of MMP reduction induced by DOX (Figure 7).

## Discussion

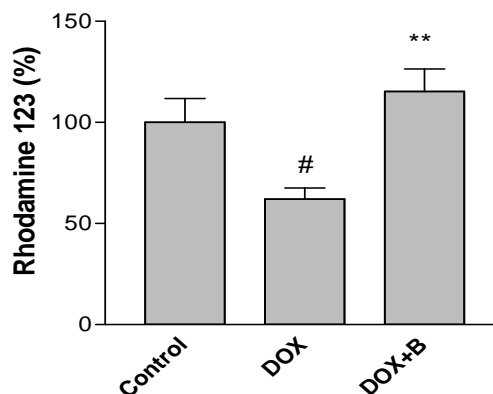
Various types of secondary metabolites of plants are known to have neuroprotective activity. Tomatine and tomatidine are examples of steroidal alkaloids with reported protective activity on glutamate-induced toxicity in SH-SY5Y neuroblastoma cells (43). Neuroprotective effect with different degrees against  $H_2O_2$ -,  $CoCl_2$ - and  $A\beta_{25-35}$ -induced cytotoxicity in SH-SY5Y cells have also been demonstrated in isolated alkaloids from the genus *Lycoris* (44). A terpenoid rich fraction from *Hygrophila auriculata* has been reported to possess neuroprotective potential against transient global cerebral ischemia induced by oxidative stress (45). Another study on *Asterias rollestoni* showed neuroprotective activities in a neurotoxicity model of Parkinson's disease for a glucan and a mannoglucan sulfate with a higher antioxidant activity for the second compound (46). Moreover, there are other compounds such as organosulfur that are considered as natural antioxidants (47) or phenolic compounds which are considered as potential neuroprotective agents (48) and include a vast variety of constituents with aromatic ring(s) bearing one or more hydroxyl substituents (49). Some of them such as the novel isolated phenolic compounds from rhizomes of *Gastrodia elata* (50), coumarin glycosides from the stems of *Hydrangea paniculata* (51) and a well-known phenyl propanoid glucoside, verbascoside, isolated from *Buddleja officinalis* (52) as well as an isolated flavone from *Dracocephalum kotschyi* (53) have shown significant protective activities in PC12 cells. However, some of the results are not in agreement with the presence of a direct and perpetual relationship between the results of cell-free antioxidant and cell-based neuroprotective assays. Jin *et al* (2014) showed that the DPPH-radical scavenging effect did not contribute to the neuroprotective effect of heteropolysaccharides from *Sargassum naozhouense* although there was a correlation between neuroprotective activity of heteropolysaccharides from



**Figure 5.** Effect of fraction B of hydroethanolic extract of *A. ciniformis* on the activity of SOD. Cells were pretreated with fraction B for 24 hr before exposure to 2.5  $\mu$ M of DOX. The activity was measured using a colorimetric assay kit, and activity was represented as the percent inhibition of the superoxide anions. ## *P*-value <0.01 vs. control, \*\*\* *P*-value <0.001 vs. DOX treated cells



**Figure 6.** Effect of fraction B of hydroethanolic extract of *A. ciniformis* on caspase-3 activity. Cell pretreated with fraction 24 before exposure to 2.5  $\mu$ M of DOX. Caspase-3 activity was measured by colorimetric detection of p-nitroanilide and expressed as percent of control. Data are expressed as the mean  $\pm$  SEM of three separate experiments. ## *P*-value <0.01 vs. Control, \*\*\* *P*-value <0.001 vs. DOX treated cells



**Figure 7.** Effect of fraction B of hydroethanolic extract of *A. ciniformis* on DOX-induced MMP collapse. Data are expressed as the mean  $\pm$  SEM of three separate experiments. # *P*-value <0.05 vs. Control, \* *P*-value <0.05 vs. DOX treated cells

*Sargassum thunbergii*, *S. fusiforme* and *S. integerrimum* and their ability to scavenge the free radicals (54). In addition, isocampneoside II, a phenylethanoid glycoside isolated from the genus *Paulownia*, with protective effects against H<sub>2</sub>O<sub>2</sub>- induced oxidative stress and apoptosis in PC12 cells, eliminated superoxide radical by 80.75% at a concentration of 0.1 mg/ml (55) even though the superoxide-radical scavenging effect did not correlate with the neuroprotective activity of heteropolysaccharides from *Sargassum* species (54). The neuroprotective activity had also been reported in crude polysaccharide extracted by water from *Saccharina japonica* but not in its five fractions with stronger hydroxyl-radical scavenging effects and reducing power (56). In a study by Ji *et al* (2012) pigmented potatoes in general have been reported to contain higher levels of phenolic components and antioxidant activity, measured as their potency in scavenging DPPH radicals, despite the fact that the bioactive components involved in protection of cortical neurons from cell death caused by oxygen glucose deprivation were not dependent on pigmentation of potato clones (57). Some studies have shown a positive correlation exists between total phenolic content and antioxidant activity of the test samples (58).

In cell-free assays, the most promising samples in the FIC method contained relatively low amounts of total phenolic compounds whereas free radical scavenging activities of the samples displayed better correlation to their total phenolic contents as clarified by Pearson's correlation coefficients (Table 1). In a study on the aerial parts of *A. campestris* L., the extract with the highest total phenolic content showed the least amount of chelating activity (59). Due to the complexity of the compounds found in plants, it is difficult to deduce a relationship between antioxidant activity and a specific group of secondary metabolites (60). In general, notable cell-free antioxidant activities of *A. ciniformis* hydroethanolic extract and some of its derived fractions in comparison with other samples could be ascribed to their higher content of phenolic compounds.

In the present study, the obtained results from the cell-based investigation suggested that DOX could decrease the cell viability in the PC12 cell line. DOX could significantly increase the intracellular ROS levels, and inhibit the SOD activity rate, which may eventually lead to PC12 cell death. Furthermore, our data confirmed that DOX-mediated cytotoxicity is mainly executed by apoptosis in this model which all together are in accordance with our previously described findings (33). When the protective effect of seven fractions was examined, we observed that pretreatment of PC12 cells with sub-toxic concentration of fractions B markedly protected the cells from DOX-induced cytotoxicity (Table 2).

Notable increase in SOD activity as well as significant reduction of intracellular ROS indicated that fraction B may protect the PC12 cells from oxidative injury through preventing increased oxidative stress (Figure 4, 5). The results of MMP assay clearly showed that the selected fraction prevented DOX-induced collapse of mitochondrial membrane potential in PC12 cells (Figure 7). It is not surprising that in our experiments, pretreatment with potent fractions was also associated with the inhibition of downstream apoptosis signaling pathway and eventually decreasing the caspase-3 activity.

## Conclusion

Taking together, in this study we could demonstrate that the fraction B from hydroethanolic extract of *A. ciniformis* attenuates the oxidative stress injury and apoptosis induced by DOX in PC12 cells. However, in our study the cytoprotective activity of the test fractions did not correlate with their antioxidant potential measured by cell free method. This might be due to the actual antioxidant activity in physiological conditions such as specific target radicals, localization in different phases, their possible interaction and differences in cellular uptake (29). In addition, it could be concluded that there should be some other types of phytochemicals but phenolics which are in charge of acting as antioxidants in cell based assay. It looks to be necessary to isolate and elucidate the structure of the components as the following step.

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