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ORIGINAL RESEARCH

Artemisia amygdalina Upregulates Nrf2 and Protects Neurons Against Oxidative Stress in Alzheimer Disease

Nasreena Sajjad[1] · Abubakar Wani[2] · Ankita Sharma[2] · Rohaya Ali[1] · Sumaya Hassan[1] · Rabia Hamid[1] · Huma Habib[4] · Bashir Ahmad Ganai[3]

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Abstract
Alzheimer disease is a complex neurodegenerative disorder. It is the common form of dementia in elderly people. The etiology of this disease is multifactorial, pathologically it is accompanied with accumulation of amyloid beta and neurofibrillary tangles. Accumulation of amyloid beta and mitochondrial dysfunction leads to oxidative stress. In this study, neuroprotective effect of *Artemisia amygdalina* against H_2O_2-induced death was studied in differentiated N2a and SH-SY5Ycells. Cells were treated with H_2O_2 to induce toxicity which was attenuated by *Artemisia amygdalina*. The nuclear factor erythroid 2-related factor 2 (Nrf2) is an emerging regulator of cellular resistance to oxidants. It controls the basal and induced expression of antioxidant response element-dependent genes. Further, we demonstrated that *Artemisia amygdalina* protects neurons through upregulation of Nrf2 pathway. Moreover, reactive oxygen species and mitochondrial membrane potential loss formed by H_2O_2 was attenuated by *Artemisia amygdalina*. Thus, *Artemisia amygdalina* may have the possibility to be a therapeutic agent for Alzheimer disease.

Keywords *Artemisia amygdalina* · Antioxidant · Oxidative stress · Neuroprotection · Nrf2

Introduction

Alzheimer disease (AD) is characterized by progressive neuronal loss and accumulation of proteins including extracellular amyloid plaques (Aβ) and intracellular neurofibrillary tangles (NFT) (Singh et al. 2015). It is the major cause of dementia affecting millions of elderly people across the world (Anand et al. 2014).Over the past decade, most of the research done on AD has focused on oxidative stress mechanisms and its importance in disease pathogenesis (Manoharan et al. 2016). The brain, as a relatively small organ mass, has a disproportionately high level of oxygen consumption due to its high ATP demand (Su et al. 2008), so various studies have linked oxidative stress to the pathogenesis and possible etiology of AD (Luca et al. 2015; Kim et al. 2015). Moreover, aging has been associated with oxidative damage and is extensive in the AD brain (Wang et al. 2014). There are ample evidences supporting the exposure of brain tissue in patients with AD to oxidative stress (e.g., protein oxidation, lipid oxidation, DNA oxidation and glycoxidation) during the course of the disease (Gella and Durany 2009).

Many studies have shown that Aβ exerts its toxic effects by generating oxidative stress (Perry et al. 2002; Carrillo-Mora et al. 2014; Ott et al. 2015). It induces peroxidation of lipids and lipoproteins which results in the generation of hydrogen peroxide (H_2O_2) and hydroxynonenal (HNE) in neurons that leads to DNA damage and inactivates transport enzymes (Gella and Durany 2009; Cheignon et al. 2018). Furthermore, mitochondrial abnormalities also contribute to oxidative stress (Meo Di et al. 2016). Mitochondria act as source of ROS production. Damaged mitochondria and

Ankita Sharma and Rohaya Ali have contributed equally to this paper.

✉ Bashir Ahmad Ganai
bbcganai@gmail.com

[1] Department of Biochemistry, University of Kashmir, Srinagar 190006, India

[2] PK-PD- Toxicology and Formulation, CSIR- Indian Institute of Integrative Medicine, Jammu 180001, India

[3] Centre of Research for Development, University of Kashmir, Srinagar 190006, India

[4] Department of Biochemistry, Islamia College of Science and Commerce, Srinagar 190002, India

formation of mitochondria-derived lysosomes and lipofuscin was evident in almost all of AD neurons (Wang et al. 2014). In AD, neurons show significantly higher number of damaged mitochondria compared to an age-matched control group (Aliev et al. 2010). Abnormality in mitochondrial morphology, membrane potential and ROS production was evident from the studies on cybrid cell lines with mitochondria DNA from AD patients (Trimmer et al. 2004). Another study in Tg2567 mice model demonstrated that mRNA level, gene expression related with mitochondrial metabolism and apoptosis were changed, suggesting mitochondrial energy metabolism is impaired by the expression of APP/Aβ (Su et al. 2008).

NF-E2-related factor 2 (Nrf2) has seen to get modulated in various neurodegenerative diseases. Its overexpression is considered as a potential therapeutic target for neurodegenerative disorders such as Amyotrophic lateral sclerosis, Alzheimer's and Parkinson's disease (Gazaryan and Thomas 2016). The endogenous antioxidant capacity of the brain is increased by either disrupting Keap1–Nrf2 interaction or genetic overexpression of Nrf2 that can render protection against oxidative stress in neurodegenerative diseases. Many studies have focused on neuroprotection role of Nrf2 against ROS generation (Wang et al. 2017; Velagapudi et al. 2018). Recent studies in aged APP/PS1 AD mouse models showed reduced Nrf2, Nqo1, GCL catalytic subunit (GCLC) and GCL modifier subunit (GCLM) mRNA and Nrf2 protein levels. Additionally, in a triple transgenic AD mouse, the GSH/GSSG ratio was reported to be reduced (Kanninen et al. 2008; Johnson and Johnson 2015).

The present time demands the need of discovering of safe, new, and effective antioxidants (Brewer 2011). To date, established treatments of AD are only symptomatic in nature, trying to counterbalance the neurotransmitter disturbance of the disease (Casey et al. 2010). Currently, approved treatments by US Food and Drug Administration (FDA), includes only five drugs that are used to treat the cognitive manifestations of AD. Acetylcholinesterase inhibitors AChEIs—rivastigmine, galantamine, tacrine, and donepzil (Aricept) and NMDA receptor antagonist—memantine (Namenda) that gives only symptomatic relief (Szeto and Lewis 2016).

Plant secondary metabolism is a rich source of various new bioactive compounds. Natural products and herbal remedies have been a source of many beneficial drugs. About 80% of the world's population is dependent on plant-based medicines (Rayees et al. 2013, 2015). Plants with traditional knowledge of antioxidant and anti-alzhemeric activity have been studied (Yuan et al. 2016). Extracts of plants such as, *Withania somnifera* (Singh and Ramassamy 2017), *Curcuma longa* (Turmeric) (Scapagnini et al. 2011), *Centella asiatica* (Meena et al. 2012), *Bacopa monnieri* (Simpson et al. 2015), *Ocimum sanctum* (Joshi et al. 2017), *Cassia obtusifolia* (Benjamin et al. 2008), *and Cassia obtisufolia* (Obulesu and Rao 2011). Dried ginger (Ghayur et al. 2008), Convolvulus pluricaulis (Bihaqi et al. 2011), and *Ginkgo biloba* (Ahlemeyer and Krieglstein 2003) were reported to possess antioxidant properties and neuroprotective effects. Herbal mixtures might have advantages as they have multiple target approach as compared with the single target (Tian et al. 2010; Pan et al. 2013). *Artemisia amygdalina* is the endemic species of Kashmir. The extract of this plant has been locally used in the treatment of various ailments such as nervous disorders, piles, epilepsy and pain (Rasool et al. 2012). The plant has been reported to have anti-inflammatory, antidiabetic, and hepatoprotective activity (Mubashir et al. 2013; Ghazanfar et al. 2014; Skowyra et al. 2014). In this study, *Artemisia amygdalina* was found to be neuroprotective against H_2O_2 in N2a and SH-SY5Y cells. Here, it was shown for the first time that *Artemisia amygdalina* upregulates Nrf2 pathway. Interestingly, it was observed that activation of Nrf2 leads to the inhibition of keap1 which further leads to the activation of antioxidant machinery. Furthermore, *Artemisia amygdalina* protects neurons against mitochondrial potential loss, attenuates reactive oxygen species induced by H_2O_2 thereby, and protects cells against H_2O_2-induced death. Based on this data, *Artemisia amygdalina* can be further investigated as a potent therapeutic agent for Alzheimer's disease.

Materials and Methods

Reagents and Antibodies

In this study, analytical grade chemicals were used and procured from standard commercial sources. Chloroform, ferric chloride and glacial acetic acid were purchased from Central Drug House (CDH). Picric acid, methanol, ethanol, ascorbic acid, calcium chloride, trichloroacetic acid, sodium chloride, sucrose, 2, 2 diphenyl picryl hydrazyl (DPPH) were obtained from Merk. Hydrochloric acid, potassium dihydrogen phosphate sulphuric acid, and hydrogen peroxide were purchased from Qualigens. Sodium hydroxide, potassium chloride, sodium dihydrogen monophosphate, ethylene diamine tetra acetate, ferric nitrate and dimethylsulfoxide were purchased from Himedia. Butylated hydroxytoluene (BHT) was purchased from Sisco research laboratories (SRL) India. Hexane and ethyl acetate were purchased from Rankem. Dulbecco's minimal essential medium, phosphate-buffered saline, trans-retinoic acid, BSA, Penicillin G, streptomycin sulfate, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), RIPA (Radioimmunoprecipitation assay buffer), sodium pyruvate, and rhodamine-123 were purchased from Sigma-Aldrich. DCFH-DA (20,70-dichlorofluorescein-diacetate), Rhodamine 123 were purchased from Sigma, USA.

Fetal bovine serum (FBS) was purchased from Invitrogen. Immobilon Western Chemiluminescent HRP substrate and PVDF membrane were obtained from Millipore. Anti-Nrf2, Anti-Keap, Anti HO-1 and β-actin antibodies were purchased from cell signalling technology.

Plant Material and Sample Preparation

Artemisia amygdalina was collected from Botanical garden, Department of Botany, University of Kashmir and identified in the Centre of Plant Taxonomy (COPT) under Vocher No.2701-KASH. The whole plant material was collected, dried and pulverized into coarse powder and extracted successively using hexane, ethyl acetate, methanol, ethanol respectively by soxhlet extraction. The solvents were allowed to evaporate in a rotary evaporator at 40–45 °C, and the extracts obtained were stored in a refrigerator at 4 °C. The plant extracts were solubilised in their respective solvents. The entire study was conducted using single batch of each plant extract to avoid batch-to-batch variation and maximize the product constancy.

DPPH Scavenging Assay

DPPH radical scavenging assay was conducted according to the method described by Kim et al. (2002). Different concentrations of plant extracts ranging from 100 to 700 µg/ml were incubated with 3 ml of a 0.1 mmol/l methanol solution of DPPH solution for 30 min in dark and absorbance was measured at 517 nm. BHT was used as standard. The percentage inhibition of free radical was calculated according to the following formula:

$$\% \text{ Inhibition } = \left[\left(A_c - A_s\right)/A_c\right] \times 100$$

where A_c is the absorbance of control i.e. without sample and A_s is the absorbance of sample.

Total Phenolic Content

The quantification of total phenols in different plant extracts was determined according to the Folin–Ciocalteu method with some modification. The yellow color of Folin–Ciocalteu reagent changes to blue when it detects phenolic compounds. Different plant extract concentrations were incubated with Follin's reagent in the presence of alkaline conditions followed by incubation of reaction mixtures at room temperature for 20 min and absorbance was measured at 765 nm. The content of phenols in the extracts was estimated using the standard curve of gallic acid and the results were expressed in milligrams (mg) gallic acid equivalent (GAE) per gram of plant extract.

Reducing Power Assay

The potential reducing agents convert potassium ferricyanide (Fe^{3+}) to potassium ferrocyanide (Fe^{2+}) which then reacts with ferric chloride to form blue-colored ferric ferrous complex. Different concentrations of the extracts were incubated with 0.2 M phosphate buffer (pH 6.6) and 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min followed by addition of 10% trichloroacetic acid. After that the reaction mixture was centrifuged at 3000 rpm for 10 min. Supernatant was added by equal amount of distilled water and 0.5 ml of ferric chloride. The absorbance was taken at 700 nm.

Superoxide Radical Scavenging Assay

The superoxide radical scavenging assay was performed by riboflavin/light/NBT method. The assay was based on the scavenging capability of superoxide ion generated by auto oxidation of riboflavin by light which can reduce NBT to blue-colored formazon. Different concentrations of plant extracts were incubated with 50 mM phosphate buffer (pH 7.6), 0.12 mM riboflavin, 12 mM EDTA, and 1.5 mM NBT. The reaction mixture was illuminated for a brief time of 90 s and absorbance was measured at 590 nm. BHT was used as positive control.

The percentage inhibition of superoxide anion by the extracts was calculated using the following formula:

$$\% \text{ Inhibition} = \left[\left(A_c - A_s\right)/A_c\right] \times 100$$

Hydroxyl Radical Scavenging Assay

The thiobarbituric acid reactive species are generated from the degradation of deoxyribose sugar when exposed to hydroxyl radical generated by Fenton's reaction (Fe^{3+}/ascorbate/EDTA/H_2O_2 system). Different concentrations of plant extracts were incubated with the reaction mixture containing 25 mM deoxyribose, 20 mM ferric nitrate, 10 mM ascorbic acid and 0.2 M H_2O_2. The reaction mixture was incubated at 37 °C for 30 min followed by addition of 10% TCA and then centrifuged at 5000 rpm for 5 min. 1 ml of 1% thiobarbituric was added and the mixture was heated at 100 °C till color appeared in control tube. The color intensity was measured by spectrophotometer at 532 nm and the percent inhibition of hydroxyl radical was calculated as follows:

$$\% \text{ Inhibition} = \left[\left(A_c - A_s\right)/A_c\right] \times 100$$

where A_c is the absorbance of control i.e. without sample and A_s is the absorbance of sample.

Cell Culture and Treatments

Mouse N2a and Human SH-SY5Y cells were obtained from ATCC and were cultured in DMEM/F12 supplemented with glutamine, streptomycin, penicillin, 10% FBS at 37 °C in 5% CO_2-humidified incubator. Extracts for this study were dissolved in DMSO but the final concentration of DMSO in treated plates was kept < 0.1%. N2A and SH-SY5Y cells were differentiated with 10 µM retinoic acid supplemented by 2 and 10% FBS, respectively for 6 days. For every 2 days media was decant and replaced with fresh media. For neuroprotection assay, cells at a confluency of 70% were treated with *Artemisia amygdalina* methanol extract (AAME) followed by 100 µM H_2O_2 treatment at indicated time periods and concentrations.

Cell Viability Assay

To examine possible toxic effect, the cell viability of differentiated N2a and SH-SY5Y cells was performed by MTT assay. Briefly, N2a and SH-SY5Y cells were seeded into 96-well plates at a density of 10×10^4 cells per well and incubated with different concentrations (1 to 200 µg/ml) of AAME for 24 h. Before 4 h of termination, 20 µl MTT solution (2.5 mg/ml) was added into each well. The supernatant was discarded and 150 µl of DMSO was added to solubilize the formazan crystals. The absorbance was measured at 570 nm (Synergy Mx plate reader). The untreated wells were taken as control.

$$\text{Cell viability} = [(A_c - A_s)/A_c] \times 100$$

Neuroprotective Effects of AAME Against H_2O_2-Induced Toxicity

Briefly, differentiated N2a and SH-SY5Y cells were seeded in 96 well plates for 24 h to achieve 70% confluence and were differentiated with 10 µM retinoic acid supplemented with 5 and 10% FBS for 6 days, respectively. After 6 days, cells were treated with AAME along with 100 µM H_2O_2. Before 4 h of termination of experiment, MTT was added at a concentration of 2.5 mg/ml into each well. Supernatant was decanted and 150 µl of DMSO was added. Neuroprotection was assessed by MTT assay. Absorbance was taken on plate reader at 570 nm.

Observations of Morphological Changes in N2a cells

The cells were seeded in 6-well plates at a density of 1×10^5 and treated with different concentrations (25, 50 and 100 µg/ml) of plant extract for 24 h and H_2O_2 at a concentration of 100 µM for 24 h. The cellular morphology was observed and images were taken at phase contrast microscope (Zeiss, Germany).

Estimation of Intracellular ROS Through Flow Cytometry

The intracellular accumulation of ROS was determined using the fluorescent probe DCFH-DA. N2a were seeded in 6-well plates and pretreated with AAME at concentration of 25 µg, 50 µg, 100 µg/ml followed by H_2O_2 at a concentration of 100 µM for 24 h. DCFHDA dye (10 µM) in culture medium was used for 30 min in dark prior to the termination of experiment. The cells were washed twice with PBS followed by trypsinization and centrifuged at 400 g for 5 min. Further, cells were washed with PBS and fluorescence intensity of DCFHDA dye was measured through flow cytometer (BD FacsClibor).

Measurement of Mitochondrial Membrane Potential (MMP)

N2a cells were seeded into 6-well plates for indicative time periods and pretreated with AAME at concentration of 25 µg, 50 µg, 100 µg/ml followed by H_2O_2 at a concentration of 100 µM for 24 h. After the treatments, rhodamine 123 (10 µg/ml) was added to cells and incubated for 30 min at 37 °C. After washing twice with PBS, the cells were collected and fluorescence of rhodamine 123 was detected by flow cytometer (BD FacsCalibor).

Preparation of Cell Lysates and Western Blotting

N2a cells were treated with various concentrations of AAME for NRF2 translocation. Cells were then incubated in 400 µL of ice-cold hypotonic buffer (10 mM HEPES/KOH pH 7.9), 2 mM $MgCl_2$, 1 mM EDTA, 1 mM NaF, 1 mM $NaOV_4$, 10 mM KCl, 1 mM DTT, 0.5 mM PMSF and 1% (v/v) eukaryotic protease inhibitor cocktail for 10 min on ice. After that, cell suspension was centrifuged at 14,000×g for 30 s at 4 °C and supernatant was taken as cytosolic fraction. The remaining pellet was resuspended in 200 µL of ice-cold saline buffer (50 mM HEPES/KOH pH 7.9, 50 mM KCl, 300 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM $NaOV_4$, 10% glycerol, 1 mM DTT, 0.5 mM PMSF, 1% (v/v) eukaryotic protease inhibitor cocktail) on ice for 30 min. The suspension was again centrifuged at 14,000×g for 5 min at 4 °C and the supernatant was used as nuclear fraction. For western blot analysis of cytoplasmic and nuclear fraction for NRF 2, keap 1 and HO-1 60 µg of protein were loaded for SDS page. Sample proteins were separated by SDS–PAGE for 3 h at 75 V and were transferred to PVDF membrane for 2 h at 100 V. The protein membranes were blocked with 5% BSA at room temp for 1 h. The membranes were incubated with primary antibodies (anti-Keap, anti-HO-1, anti-Nrf2, and β-actin) overnight at 4 °C. Membranes were then washed two times with blocking buffer and incubated with

horseradish peroxidase (HRP)-conjugated secondary antibodies. Subsequently, the membrane-bound antibodies were visualized with ECL detection reagent (Millipore, Billerica, MA, USA).The X-ray films were scanned and the optical densities of the bands were analyzed by densitometry.

Statistical Analysis

All the experiments were performed in triplicates and the mean values ± standard deviations (SD) are represented. The means were analyzed statistically with the SPSS program (version 12.0) and Origin 8.1 version software. Statistical differences between control and target groups for all experiments were determined using the analyses of variance (ANOVA), followed by Bonferroni test. Statistical differences were considered significant at '*p*' value less than 0.05.

Results

DPPH Radical Scavenging Activity

The DPPH radical scavenging method is the most reliable method for determining the antioxidant potential of extracts (Proto et al. 2000). The methanol, ethanol and ethyl acetate extracts of *Artemisia amygdalina* showed dose-dependent scavenging of DPPH free radical. The maximum DPPH scavenging activity was observed in methanol extract (88.33 ± 0.268%) followed by aqueous (72.37 ± 1.142%) and ethyl acetate extract (58.77 ± 0.383%) at highest concentration of 700 µg/ml as shown in (Fig. 1). The IC_{50} values of methanol, aqueous and ethyl acetate extracts were found to be 245 ± 3.3, 360 ± 3.54 and 432 ± 3.65 µg/ml respectively (Table 1).

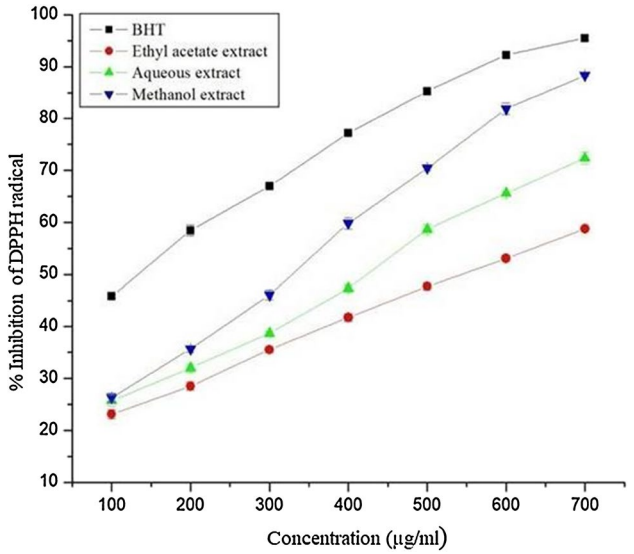

Fig. 1 Percent inhibition of DPPH free radical by different extracts of *Artemisia amygdalina* and known antioxidant BHT measured at 517 nm, with each result representing mean ± standard deviation of three separate experiments. Maximum DPPH scavenging activity was observed in AAME

Total Phenolic Content of *Artemisia amygdalina* Extracts

Phenols being antioxidants reduce the free radicals and inhibit the fast-flowing cascade of reactions, which are etiologically implicated in many diseases.

The total phenolic content analysis of different extracts of *Artemisia amygdalina* followed a decreasing trend in order of methanol > ethanol > ethyl acetate extract. The highest phenolic content of 50 mg GAE/g extract was observed in methanol extract, whereas aqueous and ethyl acetate extracts were found to contain 32 mg GAE/g and 17 mg GAE/g extract phenols, respectively.

Table 1 IC_{50} values (µg/ml) of different extracts of *Artemisia amygdalina* with reference to standard BHT

Extracts	DPPH radical	Hydroxyl radical	Superoxide radical
Methanol	245 ± 3.3	351 ± 2.72	42 ± 0.5
Aqueous	360 ± 3.54	401 ± 2.35	47.2 ± 0.42
Ethyl acetate	432 ± 3.65	487 ± 1.87	53 ± 0.26

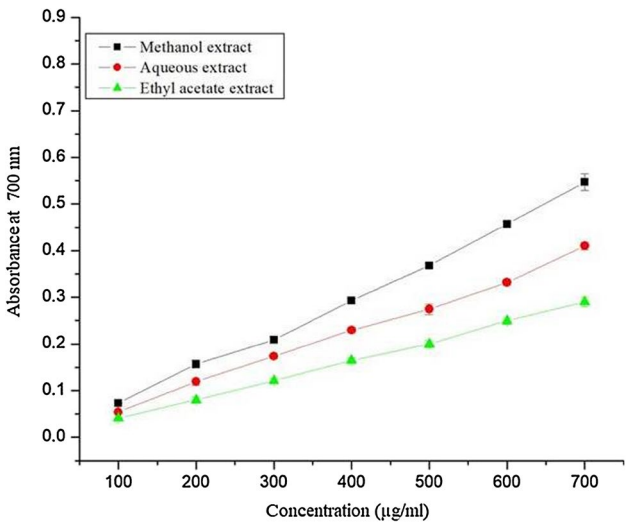

Fig. 2 Reducing power of different extracts of *Artemisia amygdalina* represented by increase in absorbance with increase in concentration. Each result representing mean ± standard deviation of three separate experiments

Reducing Power of *Artemisia amygdalina* Extracts

The reducing power was increasing in a dose-dependent manner. (Fig. 2). Higher the value of absorbance, strong is the reducing activity of the sample. The absorbance at 700 nm increased with 100–700 µg/ml from 0.073 to 0.547 of *Artemisia amygdalina* methanol extract, 0.052 to 0.41 for aqueous extract and 0.041 to 0.29 for ethyl acetate extract.

Superoxide Radical Scavenging Activity of *Artemisia amygdalina* Extracts

Methanol and aqueous extracts of *Artemisia amygdalina* were found to scavenge the superoxide radicals much efficiently than the ethyl acetate extract. Superoxide radical scavenging increased in a concentration-dependent manner. Methanol extract showed the maximum percent inhibition as compared to other extracts. The highest concentration of methanol, aqueous and ethyl acetate extracts showed 83.145 ± 1.13, 75.78 ± 0.765 and 68.24 ± 1.153 percent inhibition of superoxide radical, respectively, as shown in (Fig. 3). The IC_{50} values of the plant extracts for superoxide scavenging activity were 42 ± 0.5, 47.2 ± 0.42 and 53 ± 0.26 µg/ml, respectively.

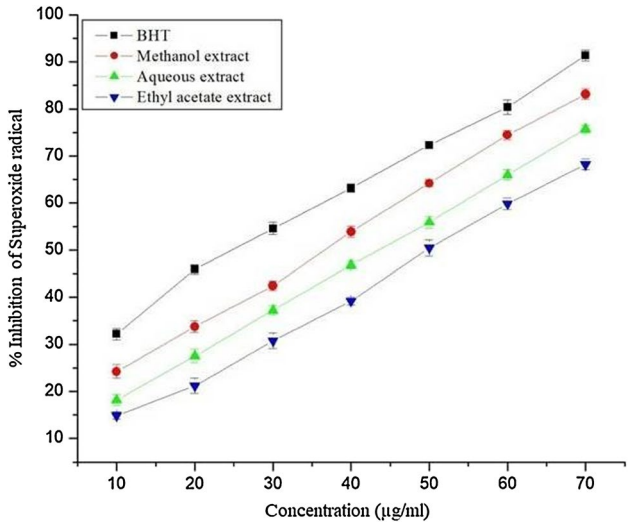

Fig. 3 Superoxide radical scavenging property of different extracts of *Artemisia amygdalina* and BHT with each result representing mean ± standard deviation of three independent experiments. Superoxide radical scavenging increased in a concentration-dependent manner. Methanol extract showed the maximum percent inhibition as compared to other extracts

Hydroxyl Radical Scavenging Activity of *Artemisia amygdalina* Extracts

Artemisia amygdalina showed significant stabilization of hydroxyl radical in a dose-dependent manner. *Artemisia amygdalina* methanol extract exhibited the maximum radical scavenging activity ($85.57 \pm 1.42\%$) followed by aqueous and ethyl acetate extracts (Fig. 4). The methanol, aqueous and ethyl acetate extracts showed IC_{50} values of 351 ± 2.72, 401 ± 2.35 and 487 ± 1.87 µg/ml, respectively.

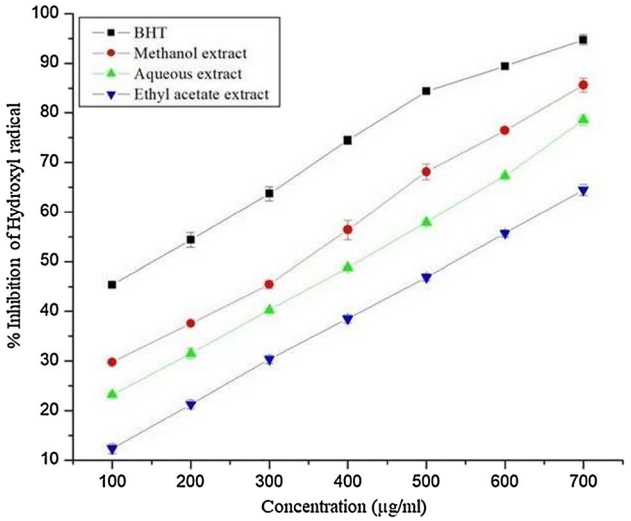

Fig. 4 Percent inhibition of hydroxyl radical by different extracts of *Artemisia amygdalina* and BHT measured at 532 nm, with each result representing mean ± standard deviation of three independent experiments

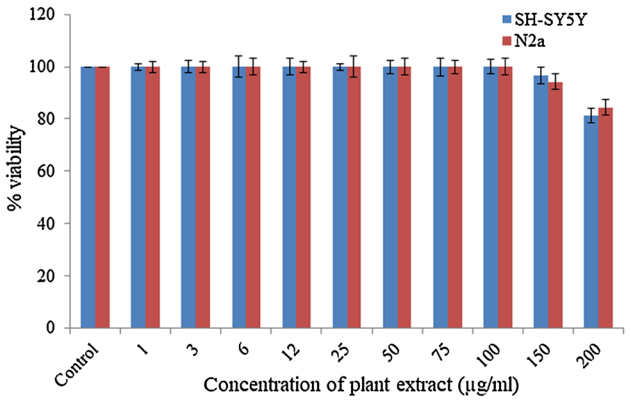

Fig. 5 Effect of AAME extract on viability of N2a and SH-SY5Y cells. Data are represented as mean ± standard deviation of three independent experiments

Cell Viability Assay

In this study, the cytotoxicity of *Artemisia amygdalina* methanol extract (AAME) on N2a and SH-SY5Y cells was carried out to determine the non-lethal concentrations. Treatment of cells with different concentrations (1 µg to 200 µg/ml) of AAME did not affect cell viability up to 200 µg/ml. These results indicate that AAME 200 µg/ml, can be used to investigate the neuroprotective effects on N2a and SH-SY5Y cells in subsequent experiments (Fig. 5).

AAME Protects SH-SY5Y and N2a Cells Against H_2O_2

To determine the neuroprotective effect of AAME on differentiated N2a and SH-SY5Y cells, they were treated with various concentrations of AAME (25, 50 and 100 µg/ml). Exposure of cells with 100 µM H_2O_2 for 24 h reduced the cell viability to half of the control group. Pretreatment of cells with various concentrations of AAME followed by exposure to H_2O_2 did not drastically affect cell viability. AAME at a concentration of 100 µg/ml neutralized H_2O_2-induced cell death ($p < 0.01$) when compared with untreated control (Fig. 6).Therefore, AAME demonstrated the potency in preventing oxidative stress in N2a cells.

Morphological Observations in N2a Cells

The protective effect of AAME was confirmed by morphological observation using phase contrast microscope. N2a cells exposed to 100 µM H_2O_2 showed cytotoxicity and altered morphology (Fig. 7). On the other hand, pretreatment of N2a cells with AAME showed intact neuritis formation and neuronal out-growth. Following pretreatment with 100 µg/ml of AAME cells showed healthy morphology.

Reactive Oxygen Species Elevated by H_2O_2 is Attenuated by AAME in N2a Cells

The exposure of N2a cells to 100 µM H_2O_2 elicited increase in ROS production as compared to control group. To assess the effect of AAME on intracellular ROS, N2a cells were treated with varying concentrations of AAME. ROS generation was attenuated significantly when cells were pretreated with AAME followed by 100 µM H_2O_2 treatment for indicated time periods. The relative fluorescence intensity of DCFHDA was increased by H_2O_2 which was further decreased by AAME as observed by flow cytometer (Fig. 8). Therefore it is clearly evident from the results that reactive oxygen species generated by H_2O_2 is attenuated by AAME.

AAME Increases Mitochondrial Membrane Potential After Exposure of H_2O_2 in N2a Cells

Integrity of mitochondrial membrane potential plays a vital role for maintenance of viability to the cells. It is also known as powerhouse of the cells, decrease in mitochondrial membrane potential leads the cells towards apoptosis. H_2O_2 decreased the mitochondrial membrane potential of N2a cells which was attenuated by AAME at a concentration of 25 µg, 50 µg and 100 µg/ml. The relative fluorescence intensity of rhodamine 123 was decreased in H_2O_2-treated cells which were further increased by AAME as evaluated by flow cytometer (Fig. 9).Therefore, these results clearly evident that mitochondrial membrane potential loss by H_2O_2 is attenuated by AAME.

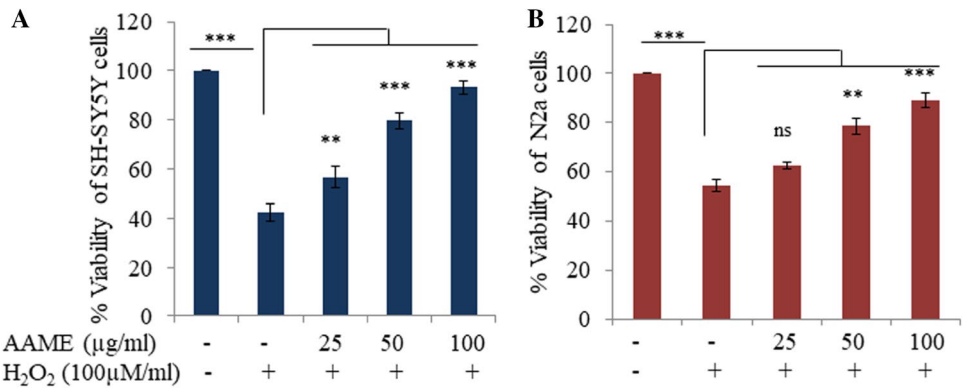

Fig. 6 Effect of AAME on SH-SY5Y (**a**) and N2a (**b**) cell viability in H_2O_2-induced oxidative stress. Differentiated N2a and SH-SY5Y cells were exposed to indicated concentration and time periods of AAME and H_2O_2, however, AAME have shown potent neuroprotective effect which was analyzed by MTT assay. Results represent mean ± SD ($n = 3$) for each concentration. $^{**}p < 0.01$ compared to control, $^{***}p < 0.01$ compared to H_2O_2 group

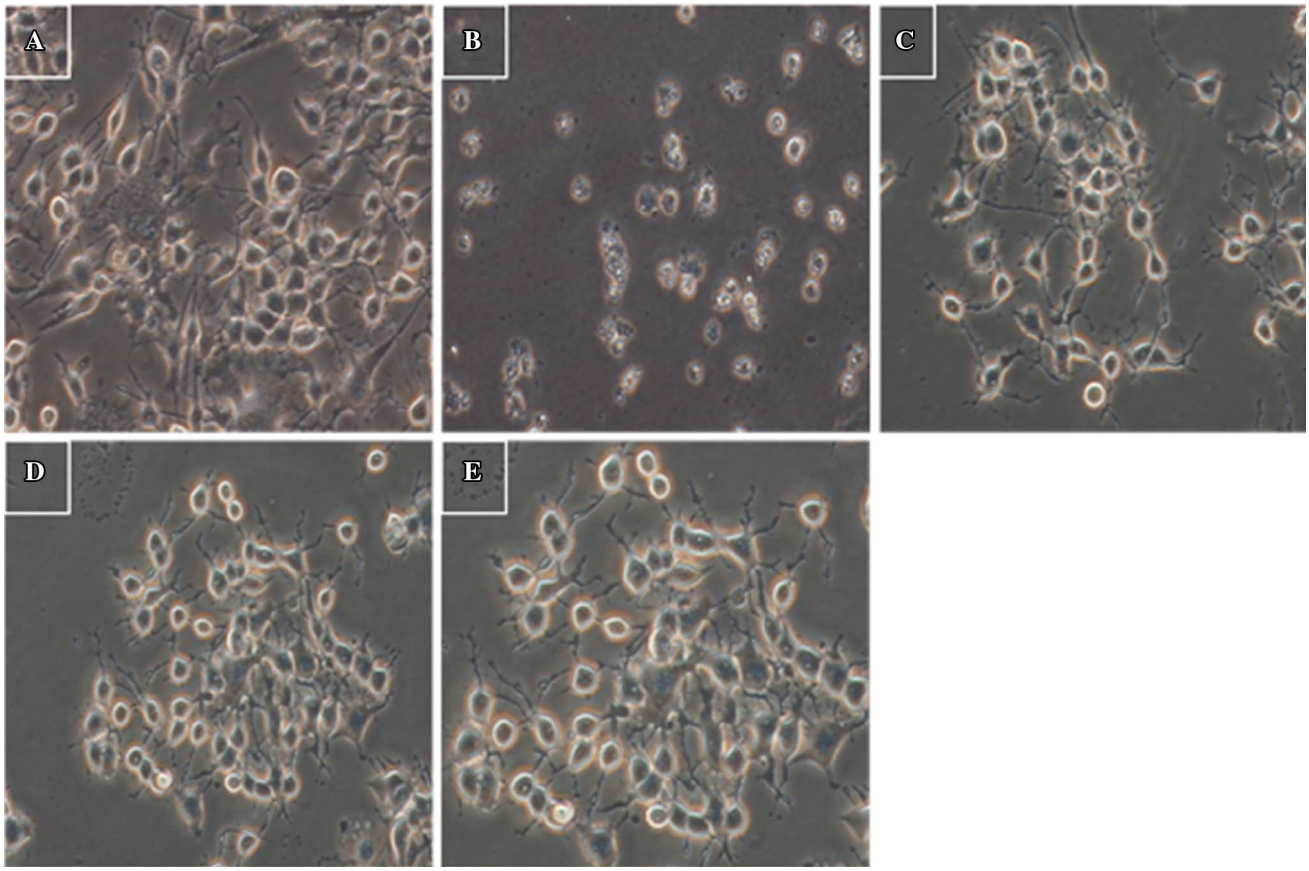

Fig. 7 AAME protects H_2O_2-induced morphological changes in N2a cells. **a** Normal **b** 100 µM H_2O_2 **c** 25 µg + 100 µM H_2O_2 **d** 50 µg + 100 µM H_2O_2 **e** 100 µg + 100 µM H_2O_2

AAME Translocate Nrf 2 from Cytoplasm to Nucleus to Activate HO-1 in N2a Cells

Under oxidative damage conditions, Nrf2 translocates to the nucleus, binds to the antioxidant response element (ARE), and enhances sequence to initiate transcription of cytoprotective genes. HO-1 plays its antioxidant role by converting heme into the powerful pro-oxidant biliverdin and finally a strong antioxidant bilirubin. The effect of AAME on the transcription of various antioxidant genes including Nrf, Keap, and HO-1 was evaluated by western blotting. AAME strongly upregulated the expression of antioxidant machinery in a dose dependent pattern in differentiated N2a cells (Fig. 10).

To elucidate the mechanism of Nrf2 activation by AAME, protein levels of Nrf2 in the nucleus and the cytosol were analyzed. It was noticed that AAME treatment increased the expression of Nrf2 in a concentration-dependent manner. As expected, the nuclear Nrf2 was also increased significantly. These observations show that AAME might disrupt the binding of Nrf2 with keap 1 making it available for translocation to nucleus. Furthermore, it was also observed that AAME-induced HO-1 upregulation via Nrf2-mediated signaling. The increase in the expression of proteins by AAME at a concentration of 100 µg/ml was statistically significant ($p < 0.001$).

Discussion

Oxidative stress is generated by pertubance of balance between reactive oxygen species (ROS) and antioxidants (Kalam et al. 2015; Szymanska et al. 2016). Damage of biomolecules—lipids, proteins and nucleic acids in response to increased ROS levels leads to oxidative stress. It plays a very crucial role in the pathogenesis of many diseases such as cardiovascular diseases, cancers, neurodegeneration, cancers, immune disorders, diabetes, aging, etc. (Chen et al. 2012). Various hypothesis have been given from time to time to explain the multifactorial nature of AD such as cholinergic hypothesis, Aβ hypothesis, tau hypothesis, oxidative stress hypothesis and inflammation hypothesis (Mohandas et al. 2009). Extensive efforts have been made to recognize both natural and artificial antioxidants and neuroprotective

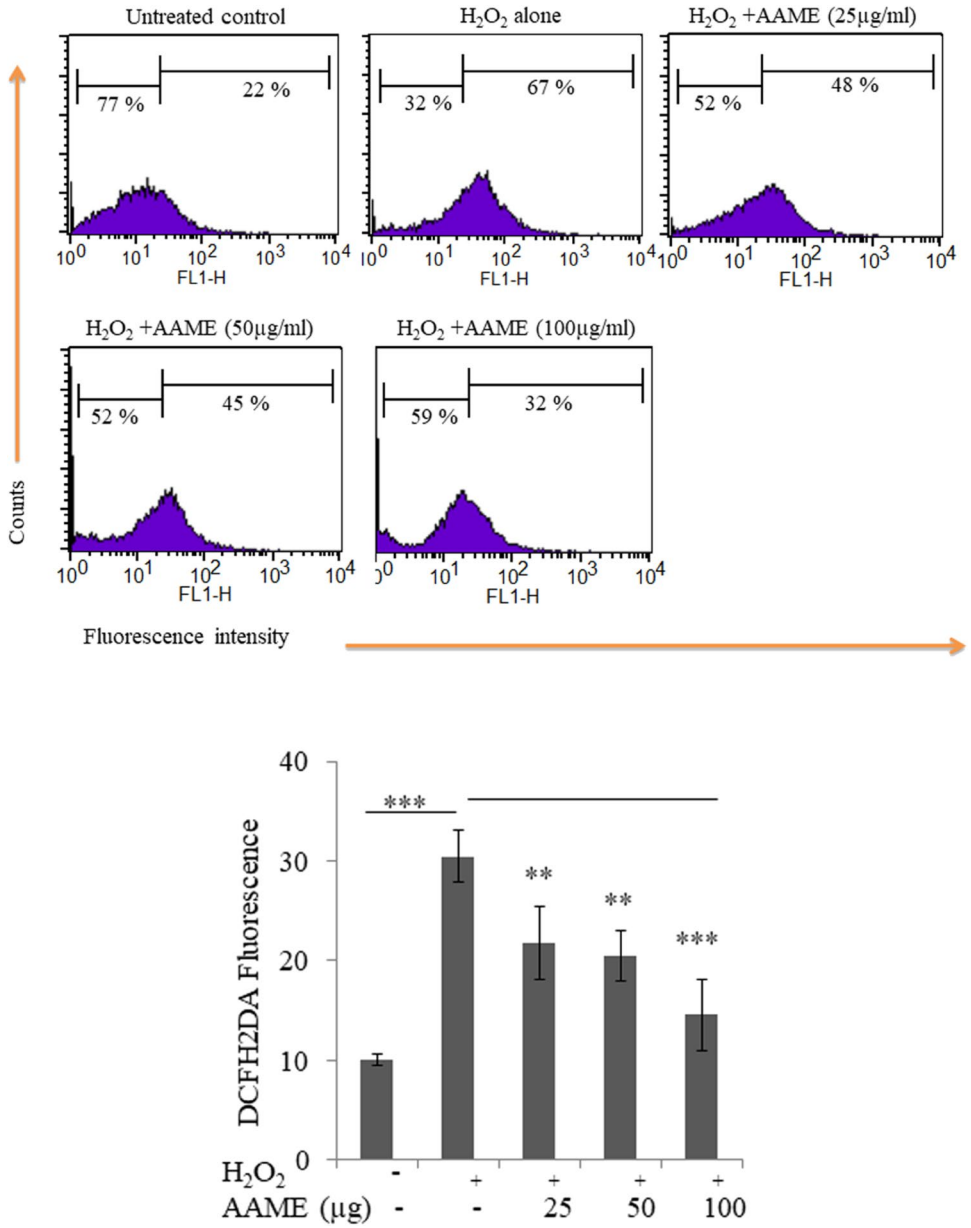

Fig. 8 AAME attenuates ROS generated by H_2O_2 induced-AAME and H_2O_2-treated N2a cells were incubated with 10 µM DCFH-DA for 30 min. The relative fluorescence intensity of fluorophore DCFH-DA was detected by flowcytometer. ***$p < 0.001$ versus control, **$p < 0.001$ versus H_2O_2 alone

agents in recent years. It has been reported that the nuclear factor erythroid 2-related factor 2 (Nrf2) is a key regulator of endogenous inducible defense systems in the body and increase the level of many antioxidants (Nguyen et al. 2009).Under oxidative damage conditions, Nrf2 translocates to the nucleus, binds to the antioxidant response element (ARE), and enhances sequence to initiate transcription of cytoprotective genes. In general, Nrf2-ARE activation is a novel neuroprotective pathway that can be considered as a promising therapeutic strategy for the treatment of neurodegenerative disorders, such as Alzhemier's disease (Niture et al. 2014).

Artemisia amygdalina is an erect, upto 1.5 m tall perennial herb, belonging to family Asteraceae. The plant extract has been used locally in the treatment of various ailments such as piles, nervous disorders, epilepsy and pain. In India, *Artemisia* species are mainly used in traditional system of medicine, *Unani-tibb* and *Ayurveda* for the management of various ailments (Lone et al. 2013).

Scavenging property of antioxidants is determined by DPPH radical scavenging assay. It is one of the most reliable and quick methods for determining the radical scavenging property of antioxidant as it is accepted as model free radical originating from lipids (Proto et al. 2000). The stable purple-colored DPPH free radical was scavenged by antioxidants present in the extracts changing the color of solution from purple to yellow. More the antioxidant potential of the extract less is the intensity of purple color in the solution.

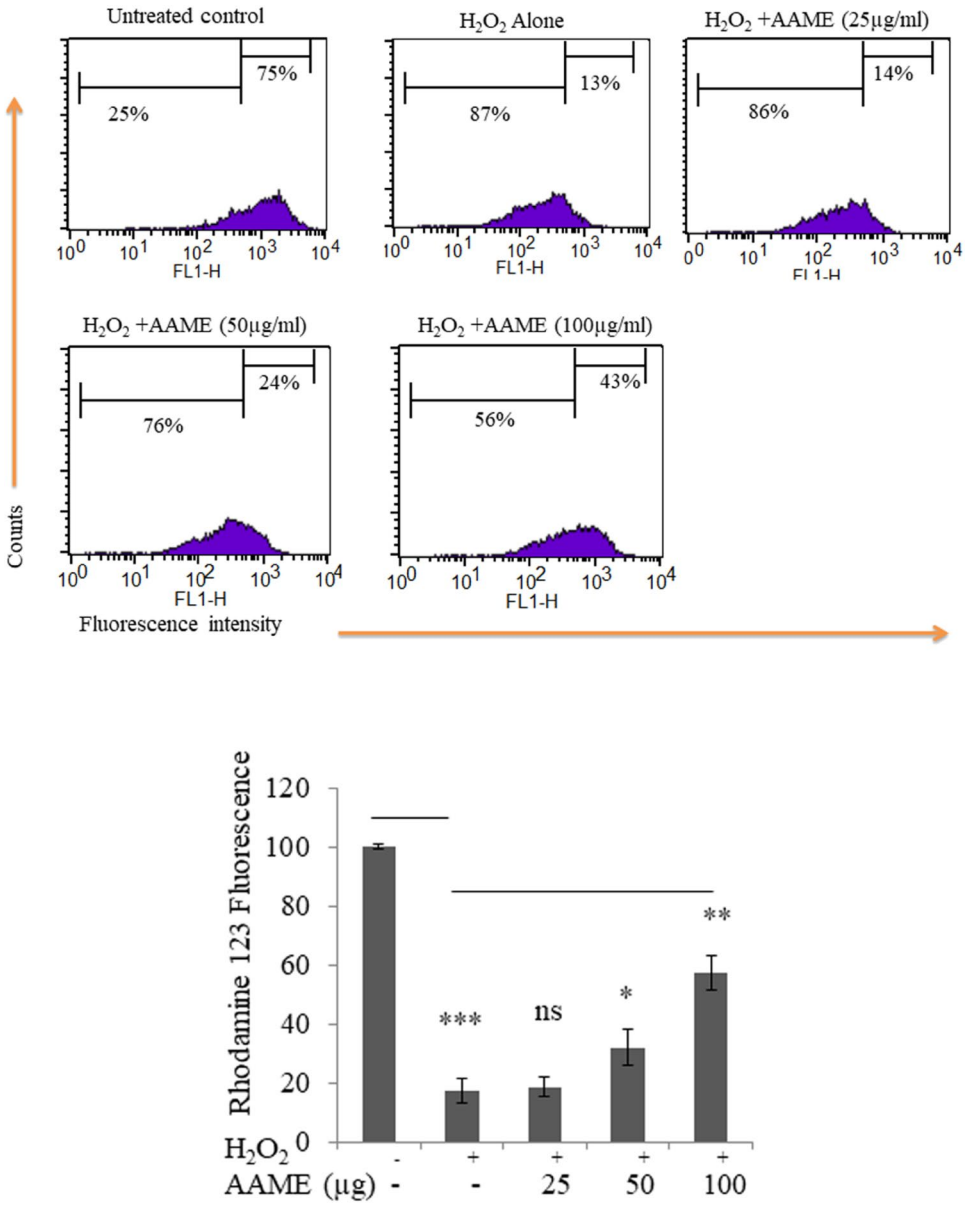

Fig. 9 Effect of AAME extract on H_2O_2-induced depolarization of MMP: AAME and H_2O_2-treated N2a cells were incubated with Rhodamine 123 for 30 min. The relative fluorescence intensity of fluorophore Rhodamine 123 was detected by flowcytometer. ***$p < 0.001$ versus control, **$p < 0.001$ versus H_2O_2 alone

In the current study, AAME was more potent in neutralizing the DPPH free radical.

Furthermore, hydroxyl radical is generated by Fenton's reaction which on reacting with TBA produces TBA reactive species (TBARS), predominantly malondialdehyde. Hydroxyl radicals cause damage to lipids, proteins and DNA (Ayala et al. 2014). The amount of Malondialdehyde produced is directly proportional to the pink color intensity which is measured at 532 nm. AAME was able to scavenge the hydroxyl radical more effectively than other extracts. These extracts might be either scavenging the free radical or chelating the Fe^{2+} ion, hence making it unavailable for the Fenton's reaction.

Phenolic compounds found in herbs are able to scavenge free radicals thereby inhibiting lipid peroxidation (Lobo et al. 2010). Methanol extracts of *Artemisia amygdalina* was found to possess maximum phenolic compounds. Moreover, the antioxidant activity and reducing power are correlated to each other (Irshad et al. 2012). The reduction of Fe^{3+} to Fe^{2+} is carried out by potent reducing agents which lead to the development of bluish color in the solution. High reducing power is indicated by high absorbance at 700 nm. AAME was found to be more potent reducing agents when compared to other extracts of the respective plants.

Superoxide anion free radical is involved in initiating redox reactions related to aging (Wickens 2001). AAME with IC_{50} values of 53 µg/ml were more potent of all other extracts. The decrease in the absorbance with increasing concentration of extracts was due to the less formation of formazon which is produced by the reduction of NBT by

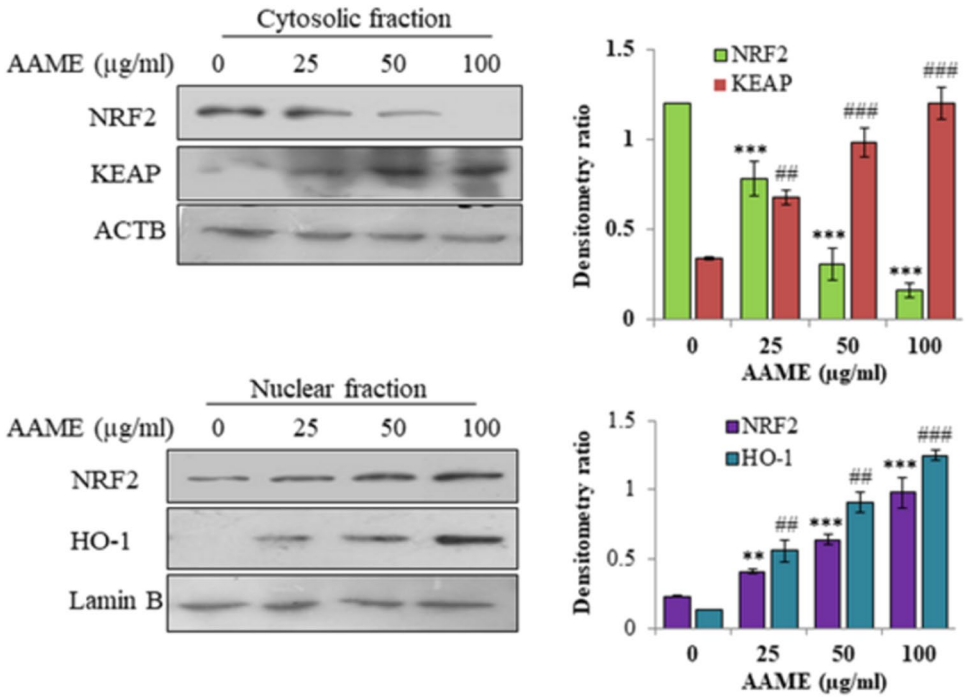

Fig. 10 Effect of AAME on Nrf2 translocation: Western blot analysis for Nrf2 translocation and enhanced protein levels of HO-1 in concentration dependent manner in differentiated N2a cells, for 24 h. Comparison was done between control and treated cells. Statistical significance was assessed by one-way ANOVA. ###$p < 0.001$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

superoxide radical. The scavenging potential of the extracts was found to be concentration dependent.

Many plant species in different regions of the world have been screened for their neuroprotective activity (Ghaffari et al. 2014).The neuroprotective effect of AAME was studied in oxidative stress-induced N2a and SH-SY5Y cells. For the measurement of neuroprotective activity hydrogen peroxide cytotoxicity was employed. The differentiated N2a and SH-SY5Y cells treated with various concentrations of AAME were exposed to 100 µM of H_2O_2. The H_2O_2-induced cell death was significantly attenuated by AAME pretreatment.

H_2O_2 the major inducer of oxidative stress penetrates into the cells and produce radicals i.e., hydroxyl radicals which are very reactive. They target cellular components, such as proteins, lipids and DNA-inducing oxidative damages (Chow et al. 2005). The production of ROS was estimated through flow cytometer using fluorescent probe 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA).The fluorescence is directly proportional to the oxidative stress induced by H_2O_2. However, in the cells pretreated with AAME followed by H_2O_2 treatment, we observed a significant decrease in ROS indicating the ROS inhibitory effect of AAME.

Furthermore, mitochondrial dysfunction has been shown in the pathogenesis of many diseases therefore, the capability to determine mitochondrial membrane potential. Rhodamine 123 (RH-123) is a mitochondrial specific, cationic, and lipophilic dye, was used to evaluate the membrane potential of mitochondria. Quenching of RH-123 fluorescence occur by mitochondrial energization, therefore, the rate of fluorescence decay is proportional to the mitochondrial membrane potential (Joshi and Bakowska 2011). The change in fluorescence intensity of rhodamine 123 reflects the change in relative levels of MMP. Decrease in MMP can be related with apoptosis and its estimation help in to evaluating stress-induced apoptotic cell damage (Nirmaladevi et al. 2014). In the present study, cells treated with 100 µM H_2O_2 exhibited decrease of MMP compared to that of control indicating the depolarization of MMP. However, we observed the restoration of MMP. By AAME pretreatment, which was dissipated with H_2O_2 treatment. Furthermore, we tried to elucidate the underlying mechanism of action. It was shown that Nrf2 expression was upregulated by AAME in a dose-dependent manner. In conclusion, our results demonstrated that *Artemisia amygdalina* can be further investigated as a potent therapeutic target in Alzheimer's disease.

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Author Contributions NS has designed and performed the experiments and wrote the manuscript. ABW performed few experiments. RA helped with some experiments. SH carried few in vitro assays. AS and HH edited revised manuscript. RH and BAG supervised the findings of this work and reviewed the final manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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