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Neuroprotective Activity of Phytoestrogens Against Scopolamine Induced Oxidative Stress in Mice

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Abstract: *Asparagus racemosus* is a potential phytoestrogenic source and has been used traditionally by Ayurvedic practitioners for neuronal disorders. The aim of the study was to examine the neuroprotective potential of methanolic root extract of *Asparagus racemosus* against scopolamine induced oxidative stress in mice model of Alzheimer's disease. Alzheimer's type dementia was induced by intraperitoneal (i.p.) injection of scopolamine (1 mg/kg) to adult Swiss albino male mice for 7 consecutive days. Methanolic root extract of *Asparagus racemosus* (100 mg/kg p. o.) was given to mice orally daily for a period of 7 days. After completion of the experimental period the animals were sacrificed, their brain tissue was collected for estimation of oxidative stress parameters. Biochemical assessment of Malondialdehyde (MDA), reduced Glutathione (GSH), Catalase (CAT), Superoxide dismutase (SOD) and AChE inhibition was done to evaluate the neuroprotective potential of *Asparagus racemosus*. Scopolamine induced mice exhibited significant increase in markers of oxidative stress as evidenced by enhanced MDA levels in whole brain homogenate. Significant decrease was observed in SOD, CAT and GSH levels in scopolamine administered mice brain homogenate. *Asparagus racemosus* methanolic root extract caused significant reduction in level of MDA in mice brain and increased antioxidant enzyme activity. Scopolamine treated mice showed significant increase in AChE activity in whole brain, AChE activity was significantly reduced by *Asparagus racemosus* methanolic root extract.

Keywords: Antioxidant, Malondialdehyde, Scopolamine hydrobromide, *Asparagus racemosus*, Oxidative stress, Neuroprotective

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Introduction

The aged brain is more vulnerable to cognitive disorder such as Alzheimer's disease (AD) and Parkinson's diseases (PD) (Gardner and Yaffe, 2015). Over accumulation of free radicals generate oxidative stress which results in neuronal disorders (Jomova *et al.*, 2010). AD is the most

common form of dementia with complex etiology, resulting in memory impairment and cognitive dysfunction with progressive neurodegeneration (Uddin *et al.*, 2016). The global prevalence of AD is estimated 46 million patients worldwide and this figure is estimated to increase to more than 130

million by 2050 (Prince *et al.*, 2015). AD is currently rank IIIrd just after heart disease and cancer, as a cause of death for older people (Adefegha *et al.*, 2016). Etiology and pathogenesis of neurodegenerative disease including AD are still a nightmare for pathologist. The pathology of AD is associated with cognitive impairment, selective death of cholinergic neurons in hippocampus, accumulation of amyloid β (A β) plaques, development of neurofibrillary tangle (NFTs), inflammation and loss of neurons in distinct area of forebrain that slowly deteriorate memory functions (Aarsland *et al.*, 2004; Oboh *et al.*, 2016). The loss of neurons in hippocampus and fore brain impairs hippocampal-dependent learning and cognitive ability (Drever *et al.*, 2011). Memory impairment is characterized to dysfunction of cholinergic system including cholinergic neurons, neurotransmitters and their receptors (Koleske, 2013). Elevated level of amyloid β is an integral part of the amyloid plaque seen in AD brain and interfere with neurotransmission in the hippocampus. In addition, a progressive decline in cognitive function in AD patient is accompanied with a decrease in the number of dendritic spines in the hippocampus and cortex (Lazecano *et al.*, 2014; Hernandez *et al.*, 2016). Although the free-radical mediated oxidative stress plays an imperative role in AD brain but the initial source of oxidative is still unclear (Buttereld *et al.*, 1997). The brain is extremely susceptible to oxidative stress due to high level of polyunsaturated fatty acids and catecholamine. Studies revealed that brains with AD have high level of H₂O₂, hydroxyl and oxygen radical that peroxidised neuronal membrane lipid and oxidize proteins resulting severe cellular damage (Stadtman, 1990; Ramezani *et al.*, 2012). Furthermore, certain regions of central nervous system (CNS), especially hippocampus and cerebellum, may be more sensitive to oxidative stress because of their low endogenous antioxidant, in relation to other brain regions (Cortes *et al.*, 2015)

In present medicinal system, therapies for numerous CNS abnormalities at present lack efficacy of drugs available for treatment of anxiety,

depression and other mental disorders. Conditions in general are far from being satisfied. A vast majority of currently available CNS drugs is synthetic in origin and most of them are responsible for severe side effects with developing dependency. Current pharmacological strategies for the treatment and management of AD involve the use of cholinesterase inhibitors which increase acetylcholine level in the brain (Vassar, 2014; Ali *et al.*, 2015). Cholinesterase inhibitors such as ragagline, rivastigmine and dopenzil exhibit side effect, such as nausea, vomiting, dizziness, hepatotoxicity and gastrointestinal disorders (Zhao *et al.*, 2006). Herbal remedies for many such conditions have been known since long and proved as a powerful tool. Neurodegeneration is a complex process involved in the decline of various physiological functions which may be caused by cell death. During neurodegeneration the rate of cell death is increased in post mitotic cells. Especially in the neurodegeneration of the nervous system, many of the dying neurons exhibit signs of apoptosis, which occur as a result of oxidative stress (Mayo, 1998).

The aim of this study was to explore the neuroprotective effects of phytoestrogenic plants on the apoptotic pathway in the hippocampus neurons. The increasing evidence of the neuromodulatory role of phytoestrogen on the nervous system, in particular, raises the possibilities that phytoestrogen might alter risk for neurodegeneration through its effect on apoptosis.

Asparagus racemosus (Liliaceae) or Chiavari is widely distributed in India, Shri Lanka and Himalayas. *Asparagus racemosus*, mentioned as Medhya Rasayanas in ancient Ayurveda which improve brain function, memory and intelligence. It is one of the widely used herbs in traditional system of medicine. The roots of AR are well known for its phytoestrogenic properties and useful for female rejuvenation as well as gynec problems (Hayes *et al.*, 2008). Chemical constituents of the root extract are Steroidal saponins identified as Shatavarins I to VI (Ojha *et al.*, 2010) These

saponins enhanced memory and protected scopolamine-induced amnesia in rodents (Kumeta *et al.*, 2013). Isoflavones including 8-methoxy-5-6-4' trihydroxyisoflavine and 7-O beta D-glucopyroniside, aspragamine and recemosal are known to possess estrogenic action (Buege and Aust, 1978). So, in the present study we examined the cytoprotective and the neuroprotective role of *Asparagus racemosus* as a potent phytoestrogen. In the present study, we used scopolamine induced amnesic mouse model of AD to study the neuroprotective and the neuromodulatory effect of phytoestrogen.

Materials and Methods

Experimental Animals:

Male Swiss albino male mice, weighing approximately 25-30 g were kept in polyutherine cages separately for one week to acclimatize the laboratory environment. They had free access to water and commercial standard food pellets. The mice were maintained on 12:12 h light-dark cycle. All experiments were carried out between 08.00 a.m. and 06.00 p.m. Handling and usage of animals agreed strictly with the regulation and guideline set by international norms. All protocols were made to reduce the number of animals used. Protocols was approved by Institutional animal ethical committee (IAEC Log.No.973/ac/06/CPCSEA).

Preparation of AR extract:

Asparagus racemosus roots were purchased from the local supplier. The roots were dried under shade, pulverized by a mechanical grinder and stored in airtight container for further use. The air-dried 500 g powdered material was extracted with 100% methanol in a Soxhlet extractor for 35 h. The solvent was evaporated at low temperature under reduced pressure in a Rota evaporator to obtain dry mass completely free from the solvent. The extract gave positive tests for polyphenols, flavonoids, tannins, saponins and glycosides. The sticky concentrate mass was kept in refrigerator at 4°C and dissolved in distilled water just before use.

Drugs and treatment:

All chemicals used were purchased from commercial supplier and were analytical grade. Trichloroacetic acid (TCA); thiobarbituric acid (TBA); acetyl thiocholine iodide (ATCI); 5, 5-dithiobis-2-nitrobenzoate ion (DTNB); reduced glutathione (GSH) and scopolamine hydrobromide were purchased from Sigma –Aldrich. *Asparagus racemosus* methanolic root extract was dissolved in distilled water and administered orally. Scopolamine hydrobromide was dissolved in 0.9% saline and was administered intraperitoneally (i.p.) after one hour of *Asparagus racemosus* dose.

Treatment protocol:

Adult Swiss albino male mice were used for experimental study (Table 1). Animals were divided randomly into four groups with six mice in each group. Dose was selected on the basis of a pilot study and earlier studies. All the drugs were administered in the morning session i.e. 8.00-9.00 AM each day.

Table 1: Animal groups and treatment

Group		Treatment	Duration
Group I N=6	Control	Vehicle	7 Days
Group II N=6	AR	<i>Asparagus racemosus</i> (100 mg/kg p.o.)	7 Days
Group III N=6	SCO	Scopolamine (1 mg/kg i.p.)	7 Days
Group IV N=6	SCO + AR	<i>Asparagus racemosus</i> (100mg/kg p.o.) + Scopolamine (1 mg/kg i.p.)	7 Days

Preparation of tissue homogenate:

Mice were sacrificed by rapid decapitation; brains were removed and homogenized in phosphate buffer saline (PBS pH 7.2) with ten times dilution. The homogenates were then centrifuged at 1000 rpm for 15 min. The supernatant was used for

biochemical estimation as per the following method:

Estimation of Malondialdehyde (MDA):

The whole brain MDA level was measured by the method of Buege and Aust (1978). The absorbance was measured spectrophotometrically at 535 nm.

Estimation of brain reduced glutathione (GSH):

The whole brain GSH level was measured by the method of Paglia and Valatine (1967). The absorbance was measured spectrophotometrically at 412 nm.

Estimation of Superoxide dismutase (SOD) activity:

The whole brain SOD activity was measured by the method of Winterbourne and Hawkin (1975) using a colour redox reaction. The absorbance was measured spectrophotometrically at 560 nm.

Estimation of Catalase (CAT) activity:

The whole brain CAT activity was measured by the method of Sinha (1972). The absorbance was measured spectrophotometrically at 620 nm

Estimation of brain acetyl cholinesterase (AChE) activity:

The whole brain AChE activity was measured by the method of Ellaman *et al.* (1961) using a colour redox reaction. The change in absorbance per min of sample was read spectrophotometrically at 412 nm.

Statistical Analysis:

All the results are expressed as mean $\pm$ SEM (Standard error of mean). All results and data were statically analysed using one way ANOVA, $p < 0.05$ was considered to be statistically significant.

Results

Biochemical estimation by standard method was conducted for non-enzymatic antioxidants (GSH, MDA) and enzymatic antioxidant (SOD, CAT, AChE) in brain homogenate of various groups.

Antioxidant system:

All cells have a complete set of antioxidant defence

that provide protection against the free radical generated damage. Catalase and Superoxide dismutase are the first line of defence and act as free radical scavengers. Antioxidant enzyme system play important role in brain and nerve function. Various toxicants inhibit these antioxidants enzyme system. SOD catalyses O_2 dismutation producing H_2O_2 whereas Catalase or Peroxidase removes it. Ascorbic acid also has an antioxidant activity and active detoxification property.

Effect of AR on Malondialdehyde (MDA) Levels:

Scopolamine administration increased the MDA level in whole brain homogenate (2.89 ± 0.31 , $p < 0.001$) as compared to controls (0.88 ± 0.30). AR + SCO treated mice exhibited significant decrease in MDA level in brain (1.43 ± 0.25 , $p < 0.01$) as compared to scopolamine treated mice. However, no significant difference in MDA level was observed between control (0.88 ± 0.30) and AR treated (0.75 ± 0.29) mice (Fig. 1).

Effect of AR Reduced Glutathione (GSH) Levels:

Scopolamine treated mice significantly decreased the GSH level in whole brain homogenate (2.8 ± 0.596 , $p < 0.001$) as compared to control (6.16 ± 1.169). AR + SCO treated mice exhibited significant increase in GSH level in brain (3.75 ± 0.88 , $p < 0.01$) as compared to those treated with scopolamine alone. However, no significant difference in the GSH level was observed between control (6.16 ± 1.169) and AR treated (5.91 ± 1.158) groups (Fig. 2).

Effect of AR on Superoxide Dismutase Activity (SOD) Levels:

SOD is an antioxidant enzyme, which plays an important role in detoxification of superoxide anions. Scopolamine treated mice showed significant decline in SOD activity in whole brain homogenate (42.33 ± 1.800 , $p < 0.01$) as compared to controls (67.50 ± 1.871). AR + SCO treated mice exhibited significant elevation in the activity of superoxide dismutase in brain (55 ± 1.414 , $p < 0.01$) as compared to those treated with scopolamine alone. However, no significant

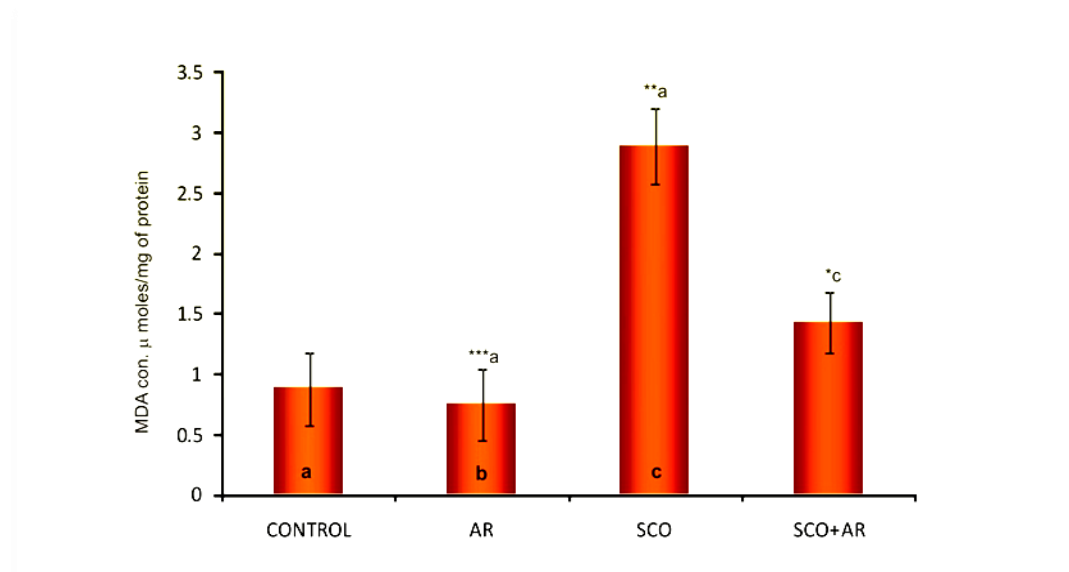


Fig. 1: Effect of AR on MDA in whole brain homogenate of SCO treated mice. Data are represented as the means SEM, N=6 in each group. ***p<0.05, **p<0.001, and *p<0.01.

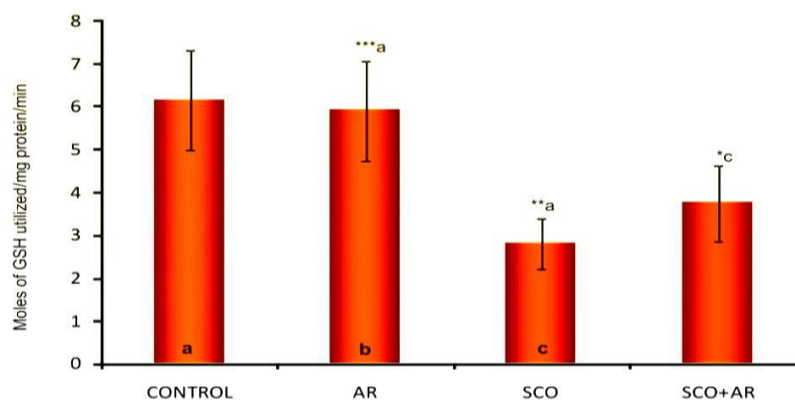


Fig. 2: Effect of AR on GSH in whole brain homogenate of SCO treated mice. Data are represented as the means SEM, N=6 in each group. ***p<0.05, **p<0.001, and *p<0.01.

difference in the activity of superoxide dismutase was observed between control (67.50 ± 1.871) and AR treated (64.33 ± 1.033) groups (Fig. 3).

Effect of AR on Brain Catalase Activity (CAT) Levels:

Catalase is an antioxidant enzyme involves in detoxification of oxidative free radicals. Scopolamine treated mice showed significant decline in the activity of catalase in whole brain homogenate (41.50 ± 1.871 , $p < 0.01$) as compared to controls (65.17 ± 1.169). AR+SCO treated mice

exhibited significant elevation in the activity of catalase in brain (53.83 ± 1.169 , $p < 0.01$) as compared to those treated with scopolamine alone. However, no significant difference in the activity of catalase was observed between control (65.17 ± 1.169) and AR treated (62.67 ± 1.476) groups (Fig. 4).

Effect of AR on Brain AChE Levels:

Scopolamine administered mice significantly increased the activity of AChE in whole brain

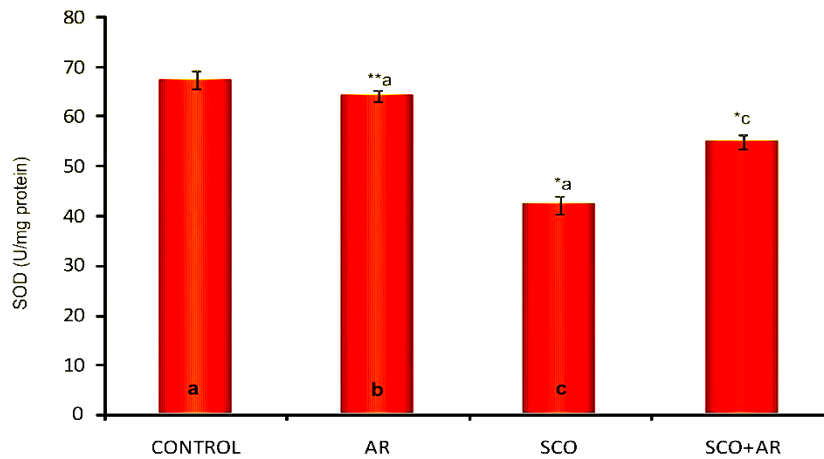


Fig. 3: Effect of AR on SOD in whole brain homogenate of SCO treated mice. Data are represented as the means SEM, N=6 in each group. **p<0.05, and *p<0.01.

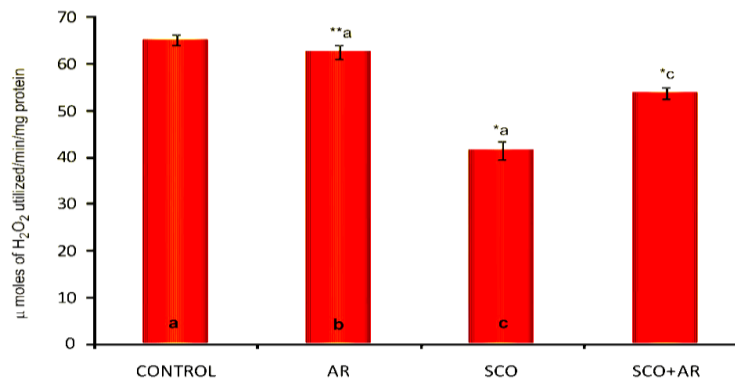


Fig. 4: Effect of AR on CAT in whole brain homogenate of SCO treated mice. Data are represented as the means SEM, N=6 in each group. **p<0.05, and *p<0.01.

homogenate (116.50 ± 1.941 , $p < 0.001$) as compared to controls (81.17 ± 1.862). AR + SCO treated mice exhibited significant decrease in the activity of AChE in brain (93.33 ± 2.503 , $p < 0.01$) as compared to those treated with scopolamine alone. However, no significant difference in the activity of AChE enzyme was observed between control (81.17 ± 1.862) and AR treated (75.67 ± 1.793) groups (Fig. 5).

Discussion

Clinically at least two abnormalities are associated

with AD patient first, the gradual loss of memory in active person with impairment of one or more of following functions: language, attention, insight, problem solving or judgment and other is neurobehavioral disturbances (Fisher, 2008). In present medicinal system, therapies for AD are not satisfactory. Therefore, alternative herbal remedies may be a powerful tool for the management of AD (Goswami *et al.*, 2011; Hajiaghache and Akhondzadeh, 2012; Doweny *et al.*, 2013; Canevelli *et al.*, 2014). The therapeutic role of phytoestrogens (which are plant originated

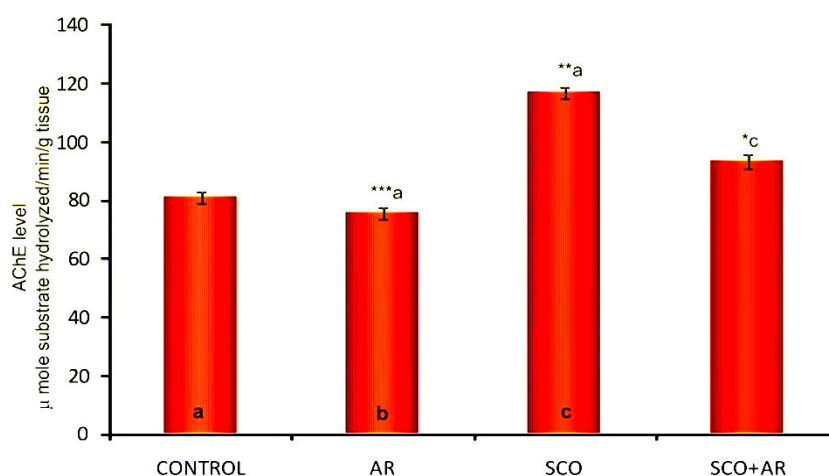


Fig. 5: Effect of AR on AChE in whole brain homogenate of SCO treated mice. Data are represented as the means SEM, N=6 in each group. ***p<0.05, **p<0.001, and *p<0.01.

xenoestrogens) in neuronal disorders has received appreciable attention in recent past and supported by clinical and laboratory studies in animals. According to current neurodegenerative studies the necrotic or apoptosis sign in the dying neuron occur as a result of oxidative stress (Zhao *et al.*, 2006). So, the plant based natural antioxidant may be advantageous in aging and neurodegenerative disorders (Di Matteo and Esposito, 2003; Zhou *et al.*, 2009).

In the present study, we used scopolamine induced amnesic mouse model to study the neuroprotective and neuromodulatory effect of phytoestrogen *Asparagus racemosus*. Scopolamine, a nonselective muscarinic antagonist blocks cholinergic transmission and causes learning and memory impairment (Liem-Moolenaar *et al.*, 2011; Kwon *et al.*, 2013). Both the long-term and short-term memory deficit is produced by scopolamine (Souza *et al.*, 2013; Kim *et al.*, 2013), and amnesic effect also produced in rat model of cognition (Yadav *et al.*, 2012; Zhong *et al.*, 2013). Scopolamine induced Alzheimer's type dementia model is well established model for screening potential cognition enhancing role of phytochemical herbal formulation (Hancianu *et al.*,

2013). Lipid peroxidation is an important indication during neurodegeneration of central nervous system. The neurolemma is more vulnerable to lipid peroxidation due to high constitute of long chain polysaturated fatty acids. The aldehyde that are produced as a consequence of lipid peroxidation are biologically active, MDA is also one of them. ROS are produced continuously in neuronal tissue during the normal metabolism and neurotransmission. The brain consumes one third part of inhaled oxygen (Attrey *et al.*, 2012). The membrane proteins and lipid components are targeted by oxidative stress in neurodegenerative diseases (Lobo *et al.*, 2010). The loss in neuronal membrane integrity is evidenced by many studies of neurodegenerative disorders. Oxidative stress has been associated with AD pathology in human (Mangialasche *et al.*, 2009; Uttara *et al.*, 2009). In the present study intraperitoneal injection of scopolamine in mice has shown significant induction of lipid peroxidation and reduced antioxidant defence indicating oxidative stress. MDA serves as a reliable marker of oxidative stress mediated lipid peroxidation and measure of free radical generation. In this study scopolamine injected mice have shown increased MDA level. In our study MDA level was measured to assess the

effect of methanolic root extract of *Asparagus racemosus*. The elevated level of MDA produced by scopolamine was decreased by *Asparagus racemosus* which shows the reduced lipid peroxidation. Glutathione, a tri-peptide an endogenous antioxidant present mainly in the reduced form within the cells provides the major intracellular defence against ROS. Lipid peroxidation may enhance depletion of glutathione contents in the brain. Agents which deplete glutathione will indirectly increase ROS formation and induce cell death. It has been observed that defence against H₂O₂ in neurons is mediated primarily by the glutathione (Drigen *et al.*, 2005). The decreased level of reduced glutathione in scopolamine treated mice observed in our study is clear indication of increased free radical generation. *Asparagus racemosus* attenuated the scopolamine affects and raised the GSH at significant level which further indicates an antioxidant mechanism against scopolamine induced amnesia.

This study revealed that the oxidative stress induced by scopolamine administration in mice significantly depletes the most important antioxidant enzyme activity SOD and CAT. SOD protects the cell membrane and bio-molecules from possible threats of superoxide anions, which may cause damage. Catalase efficiently scavenges potent ROS hydrogen peroxide. Release of hydrogen peroxide induces the production of many other oxidant species that produce oxidative stress leading AD pathogenesis (Balu *et al.*, 2005). Many clinical studies revealed that oxidative stress is involved in the pathogenesis of AD (Stoker, 1994; Marcus *et al.*, 1998) and antioxidant can delay the progression of AD (Bhatnagar *et al.*, 2005; Karmakar *et al.*, 2011). *Asparagus racemosus* has antioxidant activities associated with free radical scavenging. The present study revealed that scopolamine administration depleted activity of antioxidant enzymes SOD and CAT. *Asparagus racemosus* could retain the activity of SOD and CAT antioxidant enzymes and possibly reduce generation of free radicals. The role of acetylcholine as neurotransmitter has been well

documented in the cholinergic transmission (Anisuzzaman *et al.*, 2013; Prado *et al.*, 2013). Decrease in the cholinergic tone is associated with cognitive dysfunction and are reported in neurodegenerative diseases such as AD (Lee *et al.*, 2009; Jager and Saaby, 2011). Alteration in acetylcholine or AChE activity may affect the cholinergic transmission as well as cognition (Musial *et al.*, 2007; Micheau and Marighetto, 2011). In the present study a significant increase in AChE activity was observed in the scopolamine treated mice which are responsible for generation of oxidative stress and cytokines (Jeog *et al.*, 2008; Kwon *et al.*, 2013; Hancianu *et al.*, 2013). A significant inhibition of AChE activity was observed in *Asparagus racemosus* treated mice. Intraperitoneal administration of scopolamine desperately impaired memory acquisition and retention, similarly Alzheimer's dementia (Kwon *et al.*, 2013). The similar effect has been observed by pre-treatment with herbal formulation and extracts, and phytochemicals (Hajiaghvaei and Akhondzadeh, 2012; Downey *et al.*, 2013; Canevelli *et al.*, 2014).

Asparagus racemosus root contains phytoconstituents mainly flavonoids, tannins and terpenoids which might be responsible for exhibiting anti-amnesic activity. The use of Phyto remedies to scavenge free radicals and to treat disorders leading to oxidative stress has proven to be clinically very effective and relatively less toxic than existing synthetic drugs. The root of *Asparagus racemosus* has antioxidant activity and improve cognition in mice. In this study methanolic root extract of *Asparagus racemosus* decreased AChE activity. Improvement in cholinergic neurotransmission may be significant in neurodegeneration that associated with cholinergic deficiencies.

Conclusion

In conclusion, Scopolamine is an anti-cholinergic nonselective receptor antagonist drug that causes Alzheimer's type dementia via disruption of antioxidant defence system in neuron and increase AChE activity. The results of this research work

signify the use of herbal medication to prevent cholinergic dysfunctions and oxidative stress related with Alzheimer's type dementia. The present study indicated that *Asparagus racemosus* has a potential phytoestrogen source and could be useful for control of dementia and Alzheimer's disease.

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