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Der Pharmacia Sinica, 2014, 5(2):29-38

Der Pharmacia Sinica
ISSN: 0976-8688
CODEN (USA): PSHIBD

Neuroprotective activity of methanol extract of *Salvia officinalis* flowers in dementia related to Alzheimer disease

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ABSTRACT

Salvia officinalis is a Ayurvedic traditional medicinal plant in India and is considered useful in various ailments. Preliminary neuropsychopharmacological evaluation revealed cognition enhancing ability of the flower extract of the plant. Therefore, the present study aims at evaluating the potential effectiveness of the methanol extract of the flowers of this plant in dementia related to Alzheimer's disease (AD). Dementia was induced experimentally in animals by ingesting excess Copper and Aluminium in drinking water and confirmed by Radial 8 arm maze test. Differentl biochemical parameters including superoxide dismutase (SOD), glutathione-oxidized (GSSG), reduced glutathione (GSH), nitrite estimation, myeloperoxidase (MPO) activity and acetylcholinesterase (AChE) activity were also estimated. Lipid profile was also evaluated along with the histopathological observations. Results demonstrated a neuroprotective profile of the methanol extract of the flowers of Salvia officinalis as well as revealed a reversal of the dementia related to AD.

Keywords: Dementia, Alzheimer's disease, *Salvia officinalis*, acetylcholinesterase, neuroprotective, Radial 8 arm maze

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INTRODUCTION

The most prevalent form of dementia is known as Alzheimer's disease (AD). It is characterized by irreversible, progressive loss of memory [1] and has been established that cognitive impairment in AD is mainly due to the deficiency of acetylcholine (ACh) as degeneration of cholinergic neurons occurs in basal forebrain area of brain [2-5], Nevertheless, other neurotransmitters can also be responsible for the progression of the disease [6]. Current therapeutics indicated that AD patients are mostly being treated with the replacement of the deficit neurotransmitters in brain. And cholinesterase inhibitor drugs are most common drugs, being used for AD treatment. But unfortunately current drugs unable to stop the process of neurodegeneration and gastro-intestinal side effects. The present drugs can only improve memory in mild dementia and effectiveness of these agents diminishes with the increase in severity of AD.

Antioxidants are proved to be useful which can improve cognitive function [7-9]. Highly active antioxidant in diet may reverse the age-related memory declines as indicated by several studies [10-13] and this reversal effect has been claimed to be associated with the free radical scavenging activity and antioxidant activity [14].

Our literature survey suggested that extracts from different parts of this plant have been reported for their different biological activities as well as various phytoconstituents have been isolated from different parts of this plant [15], but there is no report found that has been published in reference to the flowers of this plant. Again in our preliminary research the methanol extract of the flowers of *Salvia officinalis* proved to be a potent antioxidant and lipid peroxidation inhibitor as well as a significant cognition enhancer. Therefore the present study has been initiated to evaluate the potential of this plant in reversal of dementia of Alzheimer disease (AD) in a rat model of AD.

MATERIALS AND METHODS

Animals

Adult male Sprague Dawley rats (3-4 months of age) weighing 200-250 grams are used for the study. The animals were kept under standard laboratory conditions with light and dark cycle of 12/12, temperature 23 ± 2 ^{o}c and relative humidity of 55 ± 10%. The animals were fed standard pellet diet and purified water supplied *ad libitum*. The care and use of laboratory animals were strictly in accordance with the guidelines prescribed by the Institutional Animal Ethical Committee constituted under the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

Chemicals

Vitamin - E acetate was purchased from Sigma Chemicals Company, St Louis, MO, USA. All other chemicals including acetic acid, n-butanol, thiobarbituric acid, tris buffer, chloroform, diethyl ether, acetylthiocholine, sodium hydroxide, sodium hydrogen carbonate, di-potassium hydrogen phosphate, di thionitrobenzoic acid, pyridine, sodium nitrate, sodium nitrite, sulphanilamide, napthylethylene diamine dihydrochloride, Griess reagent, hexadecyltrimethylammonium bromide (HTAB), O-dianisidine, 1,1,3,3-Tetramethoxypropane etc. were of analytical grade.

Radial 8 arm maze test

The maze consisted of eight arms extending radially from a central area (28.7 cm in diameter). The doors, 9 cm high, will be placed between each arm and the central platform. The floor of arms and central area were painted black. The apparatus was placed 40 cm above the floor, and surrounded by various extramaze cues such as a laboratory bench, posters and a clock. The extramaze cues were placed in the same positions during the study. In order to investigate the spatial memory, each arm was numbered from 1-8 outside in the training period. At the end of each arm (Nos. 1, 3, 5 and 7) there was a food cup that held a few mg of food pellet. Prior to the performance of the maze task, the animals were kept on a restricted diet. Before the actual training began, the animals were shaped for 4 days to run to the end of the arms and consume the bait, in groups of four. The bait was initially available throughout the maze, but was gradually restricted to the food cup. Following this shaping period, each animal was placed individually in the center of the maze and subjected to the maze training. The rats received five training trials every day for 5 days with a 5-min inter-trial interval. The trial was continued until all four baits in the food cups had been consumed or until 5 min has elapsed. The radial arm maze was cleaned with 70% ethanol and dried before each trial. Each animal was placed individually in the center of the maze where the same four arms (Nos. 1, 3, 5 and 7) were baited for each daily training trial. The other four arms (Nos. 2, 4, 6 and 8) were never baited. An arm entry was counted when all four limbs of the rat were within an arm. Measurements were made of the number of reference memory errors (entering an arm that does not contain food) and working memory errors (entering an arm containing food). The number of entry into the baited and non-baited arms that were previously visited was calculated as memory errors. The pattern in the arm entries until all four baits had been consumed was also recorded. After a 5-day training period (total 25 trials), the rats were maintained with one trial per day. The rats that fulfilled the criteria (no more than one error per trial and 2 or less over three consecutive trials) were used for behavioral and pharmacological experiment.

Biochemical estimations

Determination of Lipid profile

For lipid profile determination blood was collected from the rats by the retro orbital plexus method under ether anesthesia after 12 hrs of fasting and 24 hours after the last dose of drug. The blood samples were allowed to clot for 45 min at room temperature. Serum was separated from the clotted blood by centrifuging at 5000 rpm for 20 minutes. Triglycerides were analyzed by using triglyceride testing kit (God/Pod method) according to manufacturer instructions. Serum total cholesterol was determined using Total cholesterol test kit (Chod-pap, endpoint method) and HDL-Cholesterol was estimated by PTA method using HDL- Cholesterol test kit.

VLDL and LDL were calculated as:

VLDL cholesterol = TG/5

LDL cholesterol = TC – (VLDL + HDL cholesterol).

The values of lipid profile are expressed in mg/dl.

Preparation of brain homogenate
The rats brains were collected by sacrificing by decapitation, brains rinsed in ice cold normal saline followed by 0.15 M Tris-hcl (ph 7.4) blotted dry and weighed. A 10 % w/v of homogenate was prepared in 0.15 M Tris-hcl buffer and processed for the estimation of lipid peroxidation by the method described previouisly [16]. A part of homogenate after precipitating proteins with trichloroacetic acid (TCA) was used for estimation of glutathione by the method of [17]. The rest of the homogenate was centrifuged at 15000 rpm for 15 min at 4°C. The supernatant thus obtained was used for the estimation of SOD by the method described previously [18].

Estimation of SOD
SOD activity of the brain tissue was analyzed by the method described earlier [18].

Protein estimation
The protein content was measured according to the method described elsewhere [19] using bovine serum albumin as standard.

Estimation of GSSG
Foe measuring Glutathione levels in brain homogenate GSSG stock solution was prepared in 5-sulfosalicylic acid (5-SSA) 5% and used as standard for GSSG assays. Stock solution was diluted with 5-SSA 5% to give final concentrations in a range of 1.2–0.03 mM. Brain samples were successively diluted with 5-SSA 5% and the pH for both of samples and standards was adjusted to 7.4 by addition of triethanolamine solution 1:1 in water (TEAM solution). Samples and standards were kept on ice. The reaction mixture (freshly prepared) were mixed at room temperature in a reservoir: 1ml of 10 mM 5, 50-dithio-bis(2-nitrobenzoic acid), 10 ml of phosphate buffer and 30 U/mg protein of glutathione reductase. The absorbance was read at uv spectrophotometer at 505nm. Results were expressed in µmoles of GSSG/mg protein.

Estimation of GSH
To measure the reduced glutathione (GSH) level, the tissue homogenate (in 0.1 M phosphate buffer pH 7.4) was taken. The procedure was followed initially as described previously [17]**.** The homogenate was added with equal volume of 20% trichloroacetic acid (TBA) containing 1 mM EDTA to precipitate the tissue pro-teins. The mixture was allowed to stand for 5 min prior to centrifugation. The supernatant (200 µl) was then transferred to a new set of test tubes and added 1.8 ml of the Ellman's reagent (5, 5'-dithio *bis*-2-nitrobenzoic acid) (0.1mM) was prepared in 0.3M phosphate buffer with 1% of sodium citrate solution). Then all the test tubes make upto the volume of 2ml. After completion of the total reaction, solutions were measured at 412 nm against blank. Absorbance values were compared with a standard curve generated from standard curve from known GSH.

Nitrite estimation
Accumulation of nitrite was measured in cell-free supernatants from brain homogenate by spectrophotometer assay method described previously based on Greiss reaction [20]**.**

Myeloperoxidase activity
Myeloperoxidase activity was determined by modified technique described elsewhere [21].

Estimation of acetyl cholinesterase (AChE) activity
Brain Homogenates were prepared in 1.0 ml of 0.1 M phosphate buffer pH 8.0 (50 mg/ml) for 5 min in ice bath and used as enzyme source. Acetyl cholinesterase activity was measured by the method described previously [22].

Histopathology

At the end of the experiment the brain from the animals were collected by sacrificing the animals by an overdose of diethyl ether and the brains were placed in 40% v/v neutral buffered formalin. Histopathology of the brain was done by a pathologist. Hematoxylin and eosin dyes were used for the staining of tissue. The stained slides were observed by the pathologist for the pathological changes and results were interpreted.

Statistical analysis

Results were expressed as Mean ± SEM. The results were analyzed by using Graph pad prism software. Statistical analysis was carried out for One way analysis of variance (ANOVA) followed by Dunnett's test. Minimum significant value was set as $P \leq 0.05$.

RESULTS AND DISCUSSION

Radial 8 arm maze test

MESO was investigated for their effect on memory using radial 8 arm maze test model. The induction of dementia was done by Aluminum and Cupper administration as per the scheduled protocol, then the rats showed impairment in the spatial memory compared to that of the control group in which there was no change in the latency to find baited arms. The animals treated with MESO showed reversal of Al and Cu-induced dementia in terms of amnesia. After treating with MESO (200 and 400 mg/kg), there were significant reversal seen in the memory impairment, induced by Al and Cu, which demonstrated that MESO produces comparable memory enhancing effect in this model.

Lipid profile

The results obtained from the present study revealed a significant alteration in the lipid profile (Table 1). Major changes in the lipid profile were on LDL and HDL level. Administration of Al and Cu caused a significant rise (*P <0.001*) in the level of LDL and remarkable decrease (*P <0.001*) in the level of HDL as compared with control group. In the present study Vit-E caused a highly significant decrease (*P <0.001*) in the level of TG, TC and VLDL while there was highly significant increase (*P <0.001*) in the serum level of HDL as compared to positive control group. But there was significant decrease (*P <0.05*) in the level of LDL as compared with positive control group. At the 400 mg/kg dose level of MESO caused highly significant increase (*P <0.001*) in HDL and significant decrease (*P <0.01*) in VLDL, TC and LDL level as compared to PC group. But at 200 mg/kg dose of MESO, there was no any significant change observed in other parameters of lipid profile except HDL. A highly significant increase (*P <0.001*) in HDL level was observed as compare to positive control group.

GSH activity in brain

The effect of methanol extract on glutathione content in the brain is shown in table 2. In the 42 days study of Al and Cu metals (1^{st} to 35^{th} days administration) induce dementia, the GSH level of brain homogenate in positive control group was found to be lowered significantly (1.6 ± 0.11) than the GSH level in normal group (4.8 ± 0.25). After 7 days treatment with MESO (36^{th} day to 42^{nd} of study) at both selected dose levels (200 and 400 mg/kg), GSH level was found to be increased in a highly significant manner (*P≤0.001*). GSH level were found as 5.7 ± 0.24 and 7.2 ± 0.22 respectively as compared with positive control group. While, there was no significant difference with reference drug (Vit-E: 6.9 ± 0.25). Vit-E and MESO almost completely restored the glutathione level in metal treated groups to the normal level.

SOD activity in brain

The effect of methanol extract on SOD activity in brain is shown in table 2. For SOD activity the dosing scheduled followed as per GSH activity. SOD level of rat brain homogenate in positive control group (51 ± 1.5) was found to lower than in normal group (90 ± 0.73). After 7 days treatment with MESO (36^{th} day to 42^{nd} day of study) at both selected dose levels, SOD level was found to be increased in a highly significant manner (*P≤0.001*). The level of SOD were found as (63 ± 1.6) and (75 ± 1.0), respectively for the studied two dose level (200 mg/kg and 400 mg/kg). While, there was no significant difference observed in the level of SOD in brain with the reference drug (Vitamin-E: 82 ± 1.4).

MPO, S. Nitrite, MDA/ TBARS and AChE activity in brain

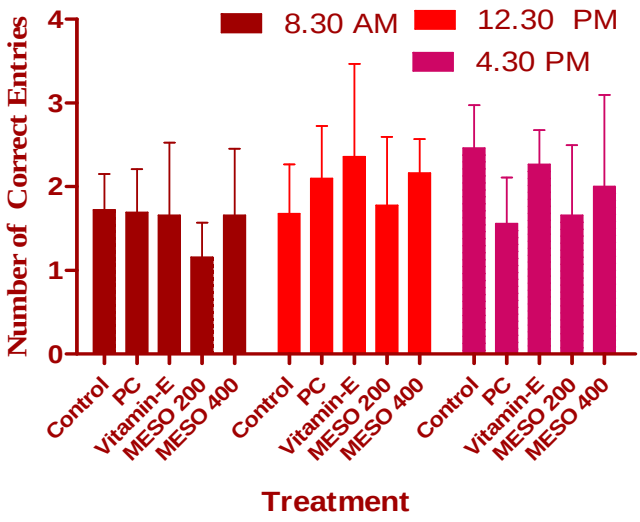
In six weeks study of Al and Cu metals (1^{st} to 35^{th} days administration) induce dementia, the MPO, S. Nitrite, MDA/ TBARS and AChE level in the rat brain homogenate in positive control group was found to be increased in a highly significant manner (*P<0.001*) compared to normal group. MPO and MDA/ TBARS levels were found to be

decreased highly significantly (*P≤0.001*) after one week treatment with MESO (sixth week) at both the selected dose levels (200 mg/kg and 400 mg/kg) in the test groups. While, effect of MESO at 400 mg/kg dose on AChE and S. Nitrite was found to be decreased significantly (*P≤0.05*) and (*P≤0.001*) respectively, as compared with positive control group. But, at the dose level of 200 mg/kg MESO significantly decreased serum nitrite level as compared with positive control group (*P≤0.01*) (Table 2).

Histopathology

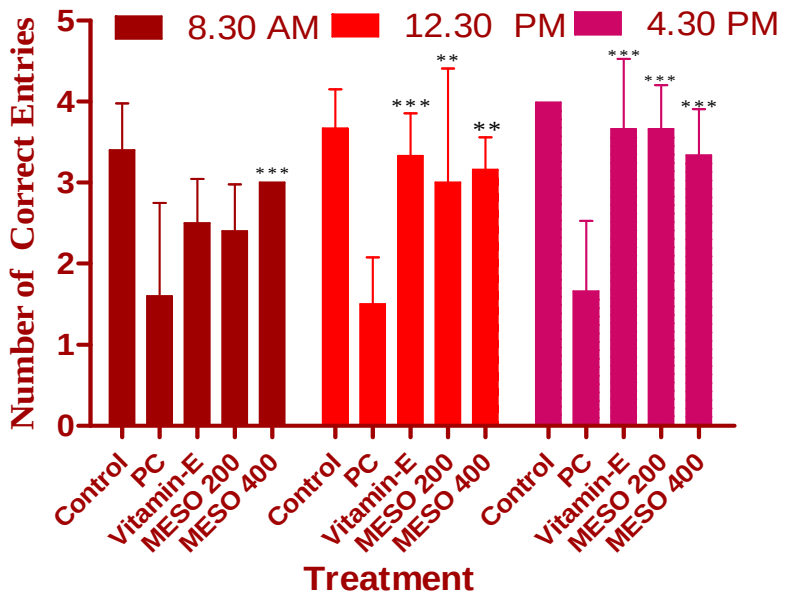
Histopathological photomicrographs of control group animals' brain tissues showed normal aging brain, with mild changes in the cellular components. Brain cells are normal showing dendrites and axons with mild (normal with aging) changes in ultrastructure. Neuronal cells (small black spots) appear to be normal in cerebral cortex (Figure 4. Slide 1, 2 & 3). Hippocampus showed normal regular integrity of neurons as well as normal ganglionic layer. Examination of photomicrograph showed no change in vascular integrity (Figure 4. Slide 4). In histopathological study of the brain tissues of positive control group animals revealed pyknosis in hippocampus along with severely disorganized cellular components. Ganglionic layer is found to be completely imprecised. Apoptotic cells can be clearly seen as well as astrogliosis due to damage of nearby neurons. Severe infiltration of inflammatory mediators is observed along with perricellular odema near the neuronal cells. Massive cellular depletion along with odema and necrosis was evident. Significant Neurofirillary degeneration is clearly observed indicating neurophagia and pericellular gliosis (Figure 4. Slide 5 & 6). In cerebral cortex, pyknotic bodies can be seen clearly. Cytoplasmic and nuclear vaculolation along with severe neuronal degeneration was evident. Necrosis, neuronal degeneration and congestion of blood vessels were also observed (Figure 4. Slide 7 & 8). In the group of animals treated with reference drug Vitamin E, brain photomicrograph showing mild neurovascular changes. Neurovascular integrity is normal and neuronal cells appeared to be effected due to treatment with Vitamin E. Necrosis is moderate and found as significantly different from positive control group (Figure 5. Slide 1). Pyknosis and mild gliosis was observed with Vitamin E treatment. Necrosis is moderate and there are no signs of inflammation. Neuronal integrity is disorganized but blood vessels are normal without any observable congestion (Figure 5. Slide 2). In the group of animals treated with MESO at 200 mg/kg dose level, photomicrograph of brain showed hippocmapus damage and moderate neurodegenerative changes. Neuronal and vascular integrity was found to be lost and post inflammatory mild pyknosis and astrogliosis was observed. There were no signs of active inflammation and necrosis was found to be present clearly (Figure 5. Slide 3 & 4). In cerebral cortex tissues necrosis was found to be present in the mid of the cerebral cortex. No signs of astrogliosis and pyknosis were observed. No active inflammatory cells were found. Neurovascular integrity (area other than necrosis) was observed as normal (Figure 5. Slide 5). In hippocampus, astrogliosis and pyknosis was present. Necrosis was found to be moderate. Associated cerebral structure was also damaged due to inflammatory conditions (Figure 5. Slide 6).

Figure 1. Radial 8 arm maze test (Day 1)



It is proposed that Alluminium (Al) induces potentiation of the activities of ATP receptors in the brain. Physiologically significant levels of Al could induce neuronal excitotoxicity at normal levels of neurotransmitter. This excitatory mechanism may act together with the disruption of other ATP-mediated signaling pathways (release of acetylcholine) as well as Ca^{+2}-mediated excitotoxicity, ultimately leading to the characteristic progress of the AD disease process [23]. The present study investigated the potential synergistic effects of exposure to Cupper (Cu), Alluminium (Al) or both metals in promoting inflammatory and oxidative events in rat brain. The design was based on the following observations: Al present in the drinking water enhanced inflammatory markers in the central nervous system (CNS). Cu is an essential metal and a component of many enzymatic reactions. However, this redox active metal can also mediate the formation of reactive oxygen species (ROS) and can have adverse consequences. Al is a trivalent cation incapable of redox changes and unlike Cu, has no known biological role. Both metals have been associated with neurological impairments. Al has been shown to play a causal role in dialysis encephalopathy and epidemiological studies suggest a possible link between exposure to this metal and a higher prevalence of AD. Various studies suggest that lipid metabolism is altered in the AD. The results obtained from our study have been shown to alter lipid profile to a significant level. Major change in the lipid profile was reflected on LDL and HDL level. Administration of Al and Cu caused a significant rise in the level of LDL and remarkable decrease in the level of HDL. In the present study Vitamin-E caused a highly significant decrease in the level of TG and VLDL while there was a highly significant increase in the serum level of HDL as compared to positive control group along with a significant decrease in the level of LDL as compared with positive control group. At the highest dose level, MESO caused highly significant increase in HDL and significant decrease in VLDL, TC and LDL level as compared to positive control group. But at low dose of MESO was found to produce any change in other parameter of lipid profile except HDL [24-26].

Figure 2. Radial 8 arm maze test (Day 2)



Cholesterol excess is also found to be linked with the amyloid excess and which ultimately cause AD [26-28]**.** This hypothesis is further confirmed by the results of this study. Experimental studies suggest that high cholesterol accelerates the production of Aβ in AD, by shifting apolipoprotein (APP) metabolism from α to β cleavage products [28]**.** Normal level of Cholesterol support elements of neural integrity as a precursor of steroid hormones (estrogens, androgens, and vitamin D), provides structural integrity and modulates fluidity of cell membranes, and is essential for basic synaptic integrity and neurotransmission. A high level of serum lipid specially TC, TG and LDL leads to production of free oxygen species, Aβ and resulting in adverse events like neuronal loss, dementia's and ultimately AD [29, 30] .

Figure 3. Radial 8 arm maze test (Day 3)

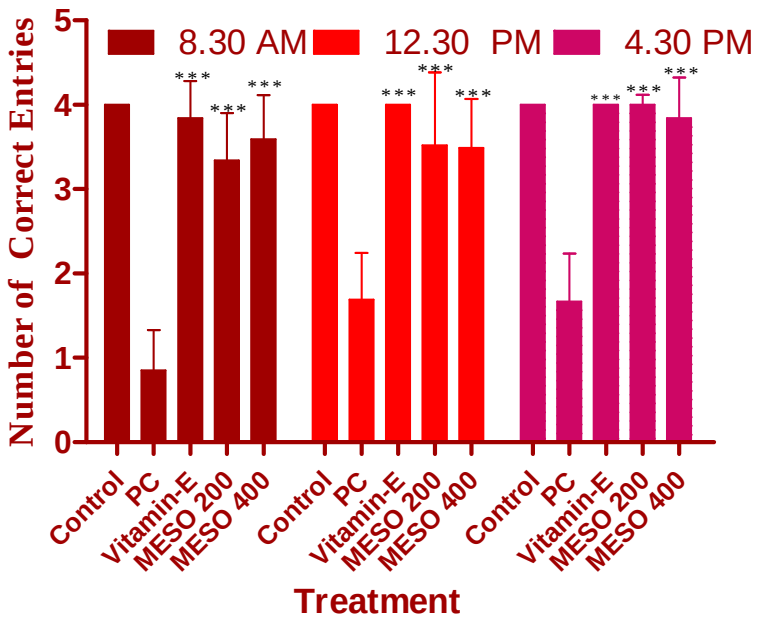
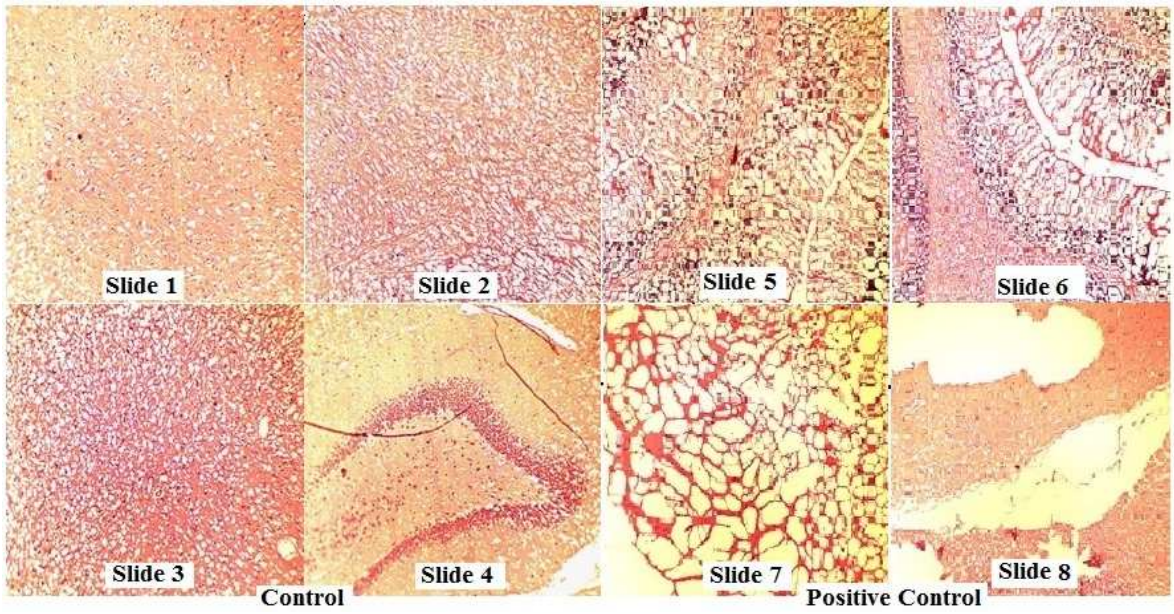


Figure 4. Histopathology of the brain for the normal control and positive control group animals



The high rate of oxygen consumption per unit mass of tissue renders the brain especially vulnerable to the deleterious effects of oxidative stress, which can arise from the overproduction of reactive oxygen species (ROS) and/or from a deficiency of the antioxidant defense systems. Oxidative stress is an important factor that may be involved in pathogenesis of neurodegenerative diseases. There are considerable evidences that oxidative stress occurs in neurodegenerative diseases [29]. Metal-catalyzed hydroxyl radicals are potent mediators of cellular injury, affecting every category of macromolecule, and are central to the oxidative injury hypothesis of Alzheimer disease (AD) pathogenesis. It is evident from various studies that the overproduction of reactive oxygen species (ROS) by

Al and Cu leads to oxidative stress. Oxidative stress represents a significant pathway that leads to the destruction of both neuronal and vascular cells. Modulation of level of SOD, TBARS, GSH, GSSG as markers of the brain antioxidant defense system in our study clearly demonstrated neuroprotection. In addition, assessment of the level of MPO and S. Nitrite in the brain tissues of positive control animals as a measure of inflammation indicated Al and Cu induced inflammatory cascade in the brain along with the production of ROS**.** At all the studied dose levels methanolic extract of flowers of *Salvia officinalis* (MESO) were found to reduce the level of MPO, S.Nitrite and TBARS as compared to positive control group in a highly significant manner. While the level of SOD, GSH and GSSG were found to be significantly raised. Results from our study furthermore confirmed the evident neuroprotection by the significant decrease in the level of MPO, S. Nitrite and AchE when compared to positive control group. The level of SOD was also found to be significantly increased further establishing the neuroprotection provided by MESO in terms of buildup of antioxidant defense enzyme system against the oxidative stress caused by Al and Cu.

Figure 5. Histopathology of the brain for the Vitamin E treated and test group (MESO) animals

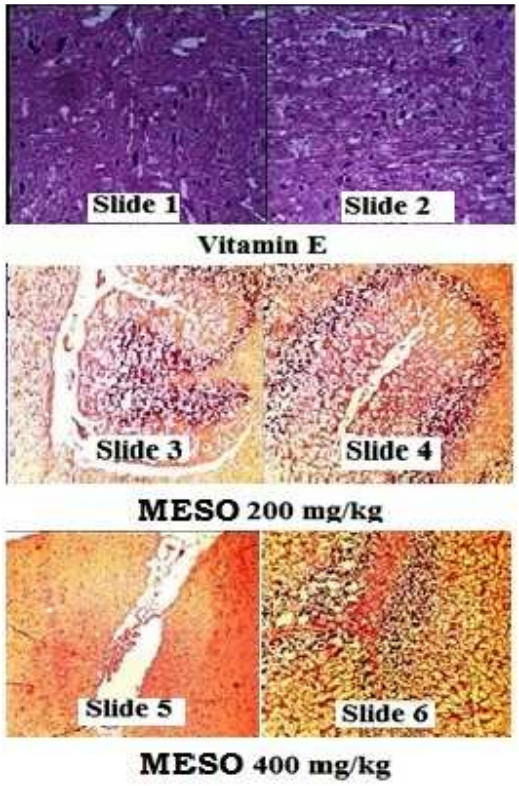


Table 1: Represent the various parameters of lipid profile. Values are in mg/dl

	Control	Positive Control	Vitamin-E	MESO 200	MESO 400
HDL	45 ± 0.65	34 ± 0.38	51 ±.60***	40 ± 1.0***	44 ± 0.89***
Triglyceride	57 ± 0.53	61 ± 0.74	56 ± 0.75***	60 ± 0.67	61 ± 1.19
Total Cholesterol	79 ± 0.30	85 ± 1.1	81 ± 0.68***	86 ± 1.8	78 ± 1.19***
VLDL	11 ± 0.10	11 ± 0.14	10 ± 0.14***	11 ± 0.20	11 ± 0.21**
LDL	79 ± 0.30	85 ± 1.1	81 ± 0.61*	84 ± 1.1	83 ± 0.69

*MESO: Methanol extract of Salvia officinalis flower. Data are expressed as mean ±SEM (n = 6). One-way ANOVA Tukey post hoc: * p < 0.05, ** p < 0.01 *** p < 0.001*

Histopathology report confirmed the hallmark signs of AD along with the neuroprotective potential of Vitamin-E. The histopathological observations confirmed the inflammation and neuronal damage in the hippocampus and cerebral cortex regions of the brain. These two structures of the brain play an important role in the memory and cognition**.** In our study these two structures of the rat brain were found to be severely affected and signs of neuronal integrity damage and inflammation were clear from the histopathological observations, clearly strengthen the

findings of Radial eight arm maze task. Microscopic examination of the stained tissue of brain reveals the significant changes in the cerebral cortex and hippocampus. Animals of the positive control group were severely affected due to oxidative stress and inflammation resulting in cerebral and hippocampus changes leading to neurodegeneration, ultimately resulting in the dementia and AD. MESO at 200 mg/kg dose level clearly demonstrated a moderate neuroprotection in terms of inflammation. At the dose level of 400 mg/kg, MESO demonstrated maximum neuroprotection as evident by the absence of signs of astrogliosis and pyknosis with no active inflammatory cells along with normal neurovascular integrity of the brain.

Table 2. Effect of Vitamin-E and MESO at both selected dose level on Biochemical markers of AD

	Control	Positive Control	Vitamin-E	MESO 200	MESO 400
AChE	16 ± 0.38	18 ± 0.20[a]	15 ± 0.24***	17 ± 0.16*	17 ± 0.15***
GSH	4.8 ± 0.25	1.6 ± 0.11	6.9 ± 0.25***	5.7 ± 0.24***	7.2 ± 0.22***
GSSG	0.28 ± .013	0.48 ± .015	0.22 ± .013***	0.49 ± 0.007	0.41 ± 0.01**
S. Nitrite	210 ± 0.88	490 ± 0.84	390 ± 2.8***	480 ± 2.3**	420 ± 4.1***
MPO	100 ± 0.00	210 ± 2.1	140 ± 2.0***	200 ± 2.3***	180 ± 2.6***
SOD	90 ± 0.73	51 ± 1.5	82 ± 1.4***	63 ± 1.6***	75 ± 1.0***
MDA/TBARS	120 ± 2.1	290 ± 0.85	200 ± 1.9***	280 ± 2.6***	240 ± 3.7***

*MESO: Methanol extract of Salvia officinalis flower. Data are expressed as mean ±SEM (n = 6). One-way ANOVA Tukey post hoc: * p < 0.05, ** p < 0.01 *** p < 0.001*

CONCLUSION

In the present study, the methanol extract of *Salvia officinalis* demonstrated significant neuroprotective activity as evident from the estimation of biochemical markers and further suggested by histopathological observation.

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