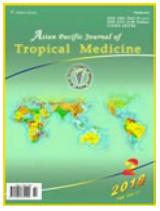

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Syzygium aromaticum ethanol extract reduces $AlCl_3$-induced neurotoxicity in mice brain through regulation of amyloid precursor protein and oxidative stress gene expression

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ABSTRACT

Objective: To investigate the neuroprotective effects of *Syzygium aromaticum* (*S. aromaticum*) extract (500 mg/kg) on $AlCl_3$ (300 mg/kg)-induced mouse model of oxidative stress and neurotoxicity. **Methods:** An ethanolic extract of *S. aromaticum* seeds was prepared and the active compounds were identified using nuclear magnetic resonance spectroscopy. BALB/c mice were divided into five groups (negative control, $AlCl_3$-treated, self-recovery, $AlCl_3$ + *S. aromaticum*, *S. aromaticum* only; n=10) and treated with $AlCl_3$ and *S. aromaticum* extract. Expression of oxidative markers [Superoxide dismutase 1 (*SOD1*) and peroxiredoxin 6 (*Prdx6*)] and amyloid precursor protein (*APP*) in the hippocampus and cortex was evaluated via PCR. Histopathological assessment was performed to investigate the extent of neurodegeneration. **Results:** It was observed that $AlCl_3$ exposure increased the expression of *APP770* while simultaneously down regulated the expression of *APP695*. $AlCl_3$ also induced a significant decrease (P<0.05) and an increase (P<0.05) in the expression level of *SOD1* and *Prdx6*, respectively. A substantial decrease substantial (P<0.05) in the density of Nissl substance was also observed in cortex of the mice treated with $AlCl_3$. Interestingly, treatment with *S. aromaticum* extract normalized the alterations in the expression level of *SOD1*, *Prdx6* and *APP* isoforms and improved the neuronal structural damage. **Conclusions:** The results showed that *S. aromaticum* is a promising antioxidant and a neuroprotective agent.

1. Introduction

Continuous exposure to heavy metals results in several deleterious effects on human health involving oxidative stress which is the root cause of various neurological disorders like Alzheimer's disease (AD) and related neurological disorders[1]. Aluminum (Al), a potent neurotoxin is widely used in utensils, foils, medicines, food additives, tooth paste and for purification of drinking water[2]. Accumulation of Al in the central nervous system causes mitochondrial functional decline which increases the formation of reactive oxygen species (ROS)[3] and ultimately leads to lipid peroxidation and oxidative damage to DNA and proteins[4].

Al also decreases antioxidant enzyme activity [e.g., catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px)][5,6]. During oxidative stress free radicals exceed the capacity of

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antioxidant defense and cause cellular dysfunction, cell membrane degradation and ultimately apoptosis[7]. Protein oxidation by free radicals may cause protein structural and functional disruptions and thereby an age-related functional brain decline[8]. Oxidative stress also precedes the deposition of β-amyloid (Aβ) plaques in AD[9]. The soluble Aβ-protein is constantly expressed in neurons and other cell types and plays a cytoprotective role. However, its over expression leads to plaque formation, cell cycle arrest and cell death[10]. It also interacts with SOD1 (potent ROS scavenger) and decreases its enzymatic activity[11]. The Aβ deposition and plaque formation are associated with elevated levels of amyloid precursor protein isoforms APP751 and APP770 as observed in the AD brain[12]. Increased protein oxidation is observed in the brain regions rich in Aβ [13]. Due to these pathological consequences there is a growing interest in search of potent antioxidants to treat and prevent heavy metals induced oxidative stress and toxicity. Potential of enzymatic and non-enzymatic anti-oxidants is limited by unsolicited effects such as toxicity profile and unwanted interaction with other cellular components. The aforementioned problem emphasizes the prerequisite for elucidation of anti-oxidant potential of natural compounds presenting minimal side effects and minimum to negligible toxicity profile. *Syzygium aromaticum* [*S. aromaticum* (Family: Myrtaceae)], commonly known as clove, is an aromatic flower bud used as a spice in Africa, Asia and many other parts of the world[14]. *S. aromaticum* oil contains a variety of chemical constituents with eugenol (79.2%) as its major chemical component. Other important components with significant biological activity include eugenol, caryophyllene, caryophyllene oxide, tanene and humulene[15]. Traditionally, it has been used for culinary purposes and to relieve abdominal discomfort. It possesses antiviral, anti-inflammatory, antidiabetic and pain relieving properties[14]. The antinociceptive potential of *S. aromaticum* is augmented when administered in combination with ketarol[15]. The pain relieving/antinociceptive effects of *S. aromaticum* are possibly mediated via modulation of γ-aminobutyric acid receptors[16].

In addition to its antinociceptive potential, antioxidant and antibacterial properties of *S. aromaticum* are also reported[17]. The antifungal activities against *Fusarium graminearum* and *Bipolaris oryzae* (commonly infect rice) are reported for clove essential oil that also makes it a potential food preservative[18].

Antiedematogenic properties of *S. aromaticum* are also evaluated using carrageenan-generated paw edema mice model. *S. aromaticum* substantially reduced the paw inflammation in carrageenan administered mice; however, exact underlying mechanism for this effect remains elucidative[19]. *S. aromaticum* extract is also rich in flavonoids, which possess anticancer potential assessed in human ovarian cancer cells (A2780)[20]. In addition, it also has the potential to reverse learning and memory deficits[21]. Based on these potential therapeutic activities associated with *S. aromaticum* extract, the present study aimed at investigating its antioxidant and neuroprotective effects on an $AlCl_3$-induced mouse model of oxidative stress and neurotoxicity.

2. Materials and methods

2.1. Chemicals and reagents

The chemicals used in the experiments include aluminum chloride hexahydrate ($AlCl_3 \cdot 6H_2O$), 2,2-diphenyl-1-picrylhydrazyl (DPPH; Product code: 101087701), electrophoresis chemicals (Sigma Aldrich, Germany), trizol (Invitrogen, USA), reverse transcriptase, deoxynucleotide triphosphate, *Taq* polymerase (Fermentas, Thermo Scientific, USA), deuterated chloroform ($CDCl_3$), deuterated methanol (CD_3OD) and deuterated dimethyl sulfoxide (DMSO-d_6).

2.2. Plant extract preparation

Fresh seeds of *S. aromaticum*, which are commonly available, were procured during spring 2014 from local spice market of Rawalpindi, Pakistan and were verified by taxonomist, Dr. Muhammad Qasim Hayat from ASAB, NUST, Islamabad. The plant material specimen was stored in the neurobiology lab for further use and record. The specimen was also submitted to and available at Pakistan Natural History Museum Islamabad (Pakistan) with a specimen voucher no. 42569. The extract was prepared using *S. aromaticum* seeds (500 g) grinded into fine powder and sieved with 80 mesh sieve. The fine powder (10 g) was loaded into the main chamber of the Soxhlet extractor with distillation flasks containing extraction solvent (100% ethanol). After 24 h, the filtrate was collected and concentrated in rotary evaporator (R200 rotavapour Buchii) with reduced pressure at 68 ˚C to afford a crude extract. The remaining solvent extract if any, was removed by incubation at 37 ˚C and later stored at 4 ˚C until further used.

2.3. In-vitro assay for DPPH free radical scavenging activity

The experimental procedure for DPPH scavenging activity was performed according to the protocol described by [22] with minor modifications. Briefly, samples of *S. aromaticum* extract and standard (ascorbic acid) were prepared in different concentrations, ranging from 100 to 0.01 μg/mL respectively. A DPPH solution (0.1 mmol/L) was added to each sample and standard. After incubation at 37 ˚C for 30 min, the absorbance was measured at 517 nm using OPTIMA SP-300 spectrophotometer. The percent antioxidant activity was calculated according to the following equation:

Percent DPPH scavenging activity = (Control absorbance – Sample absorbance)/(Control absorbance)×100.

Half maximal effective concentration for DPPH was calculated by plotting the graph using Microsoft Excel® (Microsoft Inc, USA). Concentration was plotted on x-axis whereas percent inhibition was plotted on y-axis. After that half way value of the inhibition (y) was calculated using following formula.

Half way value (y) = [Maximum inhibtion – (Maximum inhibtion-Minimum inhibtion)]/2.

Then half maximal effective concentration value was calculated using the formula: $y = mx + c$.

Where, y is half way inhibition, and m and c are constants.

2.4. Proton nuclear magnetic resonance (^{1}H-NMR) spectroscopic analysis

^{1}H-NMR spectrum of *S. aromaticum* crude extract was recorded on Bruker Avance III 500 MHz spectrometer. The chemical shifts (δ) were obtained in ppm. Tetramethylsilane was used as an internal reference standard during analysis of some samples.

2.5. Experimental animals

BALB/c mice were purchased from National Institute of Health (NIH) Islamabad, Pakistan and housed in animal house of Atta ur Rahman School of Applied Biosciences, National University of Sciences and Technology. After acclimatization period of 2 weeks animals were bred and kept in metal cages at constant temperature of (25±2) ˚C and natural light-dark cycle (12/12 h). Animals were given distilled water *ad libitum* and fed with standard diet consisting of 30% crude protein, 9% crude fat, 4% crude fiber and 10% moisture. Fifty male mice (35–45 g and 10–12 weeks of age) were used in experiments. All experiments performed were in compliance with the rulings of the Institute of Laboratory Animal Research, Division on Earth and Life Sciences, National Institute of Health, USA (NRC, 2011). The protocol was approved from the Internal Review Board, Atta-ur-Rahman School of Applied Biosciences, National University of Sciences and Technology.

2.6. Animal treatment

Fifty male BALB/c mice (35–45 g; 10–12 weeks of age) were divided into five groups (n=10). The first group (negative control) was on normal feed and distilled water while the second group (positive control) received $AlCl_3$•$6H_2O$ (300 mg/kg p.o) in distilled water with normal feed. Group three, classified as a self-recovery group, received $AlCl_3$•$6H_2O$ (300 mg/kg p.o) in distilled water with normal feed for 15 d and later left untreated for 21 d. Group four received $AlCl_3$•$6H_2O$ (300 mg/kg p.o) for 15 d followed by *S. aromaticum* extract (500 mg/kg) mixed in the normal feed for next 21 d. Group five received *S. aromaticum* extract (500 mg/kg) mixed in the normal feed for 21 d.

2.7. Gene expression studies

Animals in all groups were sacrificed after 24 h of the completion of treatment. All mice were anesthetized and euthanized by cervical dislocation. Brain was removed from the skull and placed on an ice cold plate. Initially, cerebellum and olfactory bulbs were removed. Brain was divided into two hemispheres and whole cortex was micro dissected from each hemisphere via forceps subsequently, hippocampus was also dissected out immediately and tissues were snap frozen in liquid nitrogen. Total RNA was isolated according to the manufacturer's protocol using Tri-reagent (Invitrogen). Qualitative assessment was performed by running RNA samples on 2% agarose gel and visualized by Dolphin-Doc imaging system (Wealtec Corp, USA). Extracted RNA was quantified using BioPhotometer plus (Eppendorf, Germany) and equal quantity of RNA was used to synthesize the cDNA (60 μL). PCR reactions were done using a Swift Maxi Thermal Cycler Block ESCO, Singapore. The thermo cycling conditions were; initial denaturation at 95 ˚C for 5 min, followed by denaturation at 94 ˚C for 30 s. The annealing temperature was set differently for each gene (β -actin, 55 ˚C; *APP* Common, 55 ˚C; *APP695*, 60 ˚C; *APP770*, 60 ˚C; *SOD1*, 58 ˚C and Peroxiredoxin 6, 60 ˚C) and then PCR was allowed to complete 35 cycles (30 s/cycle) at 72 ˚C. After that, extension was carried out at 72 ˚C for 10 min. β -actin gene was used as an internal control and was amplified with a specific primer pair (5'-GCC TTC CTT CTT GGG TAT GG-3'/5'-CAG CTC AGT AAC AGTC CGC-3'). The specific primer sequences of *APP* Common, *APP695*, *APP770*, *SOD1* and *Prdx6* are listed in Table 1. PCR products were separated on 2% agarose gel and visualized by Dolphin-Doc imaging system (Wealtec Corp, USA). The expression of the PCR products was densitometrically analyzed by ImageJ® analysis software.

2.8. Histology: Cresyl violet staining

Cresyl violet staining was performed to study the structural features of neurons. It is used to highlight imperative morphological features of neurons i.e. Nissl bodies or rough endoplasmic reticulum. Briefly, heart perfusion was performed in accordance with the protocol of [23]. Brain was isolated and placed in 4% paraformaldehyde for 24 h followed by paraffin embedding. After that, cortical tissue sections (4 μm) were deparaffinized in xylene for 10 min before being rehydrated by 70% isopropanol (10 min), and washed with ddH_2O (5 min). Cresyl violet stain was poured over the tissues sections and left for 4 min. The sections were then washed with ddH_2O and 70% acid alcohol (2 min) and later dried for 2 h and mounted with cover slips. The sections were visualized using an inverted microscope at 10 × and 40 × resolutions; images were captured by Pixel Pro™ image analysis software (Labomed, USA). The quantitative analysis for cell count was carried out in an area of 10 000 μm^2 (100 μm × 100 μm box) from three randomly selected sites. Following that, average values were calculated and plotted[24].

2.9. Statistical analysis

Statistical analysis of data was performed using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test using GraphPad Prism 5.0. The data are represented as mean±SEM with a 95% confidence interval. A P-value<0.05 was considered statistically significant.

Table 1
Primer sequences used for expression analysis of *SOD1*, *Prdx6*, *APP* Common, *APP770* and *APP695*.

Gene	Forward primer	Reverse primer
SOD1	5'-GAC AAA CCT GAG CCC TAA G-3'	5'-CGA CCT TGC TCC TTA TTG-3'
Prdx6	5'-TTG ATG ATA AGG GCG GGA C-3'	5'-CTA CCA TCA CGC TCT CTC CC-3'
APP Common	5'-GTG ATC TAC GAG CGC ATG AAC C-3'	5'-AAG ACA TCG TCG GAG TAG TTC TGC-3'
APP770	5'-TGC TCT GAA CAA GCC GAG ACC-3'	5'-CAT GCA GTA CTC TTC CGT GTC-3'
APP695	5'-GAT GAG GAT GTG GAG GAT GG-3'	5'-GCT GCT GTC GTG GGA ACT C-3'

3. Results

3.1. DPPH assay for free radical scavenging activity

DPPH assay was performed to assess the free radical quenching activity of *S. aromaticum* extract. Results of *in-vitro* assay indicated that *S. aromaticum* extract has strong potential to inhibit DPPH free radicals in dose dependent manner. The results were consistent with standard antioxidant (ascorbic acid) (Figure 1).

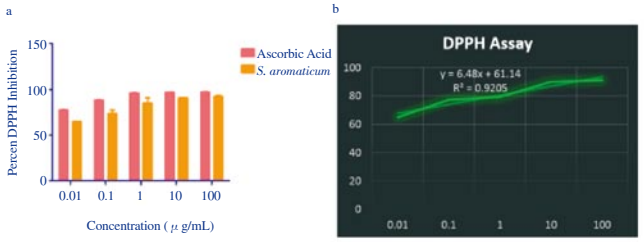

Figure 1. *In-vitro* DPPH assay of *S. aromaticum* extract.
(a) Bar diagram shows the percentage inhibition of free radicals by *S. aromaticum* extract in comparison with standard (ascorbic acid). (b) Percentage inhibition of DPPH free radicals by *S. aromaticum* extract. DPPH standard curve was plotted for five different concentrations of *S. aromaticum* extract. Concentration was plotted on the *x*-axis (independent variable) and percentage inhibition of DPPH free radicals by *S. aromaticum* extract was plotted on *y*-axis (dependent variable). Absorbance was measured at 517 nm. The obtained regression value was 980 (R^2 = 0.920).

3.2. Phytochemical analysis of S. aromaticum extract

The high-resolution ^{1}H-NMR spectra of ethanolic extract of *S. aromaticum* seeds were recorded in DMSO-d_6, revealed several polar compounds such as flavonoids (quercetin), tannins and saponins (Figure 2). Saponins consist of glucose moiety associated with triterpenoid ring at C-3 position.

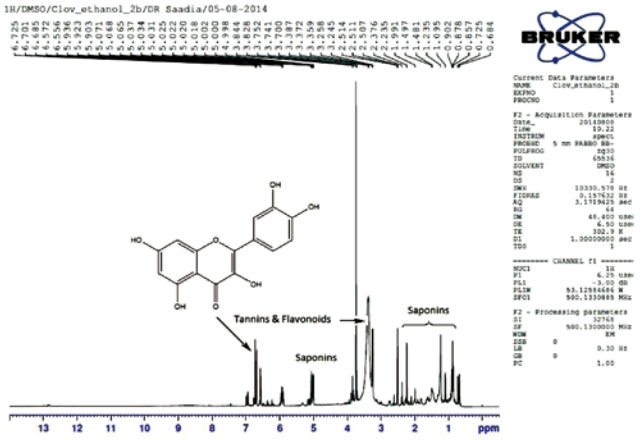

Figure 2. ^{1}H-NMR spectrum of *S. aromaticum* extract.
The peaks showed the presence of flavonoids, tannins in the aromatic region while major peaks of saponins are observed at 1–2 mg/L region.

3.3. Histopathological assessment of cresyl violet stained cortical sections

A marked reduction in Nissl substances was observed in the $AlCl_3$•$6H_2O$-treated and self-recovery group as compared to the negative control (P<0.05). *S. aromaticum* treatment post $AlCl_3$ exposure showed a significant increase in number of Nissl bodies (P<0.05). On comparison of the self-recovery group with the positive control, no significant difference was found. Shrinkage of cell bodies was evident in the $AlCl_3$•$6H_2O$-treated groups (Figure 3).

Table 2
Results of gene expression analysis of oxidative stress markers, *SOD1* and *Prdx6* (mean±SEM).

Group	*SOD1*		*Prdx6*	
	Cortex	Hippocampus	Cortex	Hippocampus
Negative control	0.98±0.10^a	0.85±0.02^a	0.90±0.20^a	0.58±0.10^a
$AlCl_3$·$6H_2O$	0.31±0.08^b	0.23±0.04^b	1.89±0.20^b	1.69±0.09^b
Self-recovery	0.35±0.03^{bc}	0.31±0.04^{bc}	1.96±0.10^{bc}	1.45±0.10^{bc}
$AlCl_3$·$6H_2O$ + *S. aromaticum*	0.80±0.03^{ac}	0.67±0.09^{ac}	2.80±0.29^{ad}	2.40±0.20^{ad}
S. aromaticum	0.94±0.06^{ad}	0.78±0.10^{ac}	0.92±0.21^{ad}	0.56±0.06^{ad}

The same letters in superscript means no statistical difference between two groups, while different letters in superscript mean statistical difference between two groups (P<0.05).

Table 3
Results of gene expression analysis of amyloid precursor protein isoforms, *APP695* and *APP770* (mean±SEM).

Group	*APP695*		*APP770*	
	Cortex	Hippocampus	Cortex	Hippocampus
Negative control	1.19±0.22^a	1.14±0.23^a	0.46±0.10^a	0.38±0.10^a
AlC_{13}.$6H_2O$	0.31±0.02^b	0.41±0.02^b	0.98±0.10^b	1.10±0.04^b
Self-recovery	0.38±0.16^{bc}	0.47±0.08^{bc}	0.93±0.14^{bc}	1.02±0.13^{bc}
AlC_{13}.$6H_2O$ + *S. aromaticum*	0.92±0.13^{ad}	0.86±0.08^{ad}	0.51±0.04^{ad}	0.51±0.09^{ad}
S. aromaticum	1.05±0.30^{acd}	1.01±0.06^{ad}	0.46±0.08^{ad}	0.43±0.17^{ad}

The same letters in superscript means no statistical difference between two groups, while different letters in superscript mean statistical difference between two groups (P<0.05).

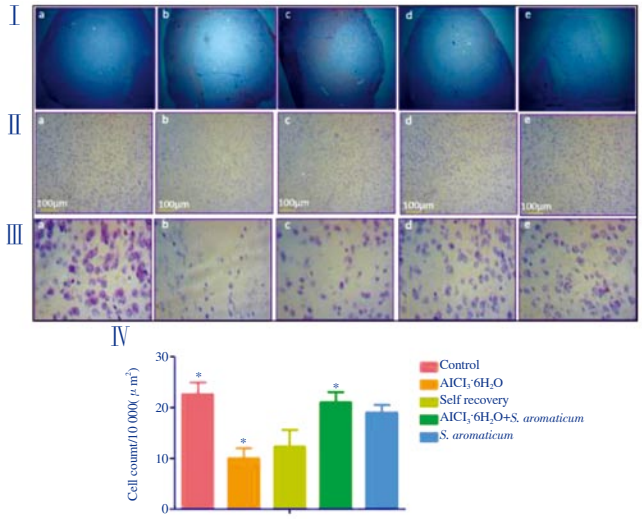

Figure 3. Nissl stained sections of mice cortex.

The stained sections of $AlCl_3$•$6H_2O$ treated cortex showed marked neurotoxicity in comparison to respective negative controls. Reduced cell count and structural deformity is also visible in the sections treated with $AlCl_3$•$6H_2O$ while restoration of cell count is evident after the administration of *S. aromaticum*. Where (a) Negative Control, (b) $AlCl_3$•$6H_2O$ (300 mg/kg)-treated, (c) Self-recovery, (d) $AlCl_3$•$6H_2O$ + *S. aromaticum* (500 mg/kg)-treated, (e) *S. aromaticum*-treated (500 mg/kg). Original magnification, I; 4 ×, II; 10 ×, III; 40 ×. (IV) Histograms representing cell count/10 000 μm^2 (at 10 × magnification) in cortex in all experimental groups.

3.4. Effect of $AlCl_3$•$6H_2O$ and S. aromaticum extract on SOD1 and Prdx6 expression

Significant differences in the expression level of oxidative stress markers, i.e., *SOD1* and *Prdx6* were observed in both the regions (Table 2). The expression level of *SOD1* significantly decreased following $AlCl_3$•$6H_2O$ as compared to the negative control (P<0.05 cortex; P<0.05 hippocampus). The group treated with $AlCl_3$•$6H_2O$ followed by *S. aromaticum* extract treatment showed significantly normalized expression of *SOD1* gene (P<0.05 cortex; P<0.05 hippocampus). There was no significant difference in the expression of *SOD1* between the negative control and the *S. aromaticum*-treated group as well between the self-recovery and the $AlCl_3$•6H2O-treated group. On the other hand, exposure with $AlCl_3$•$6H_2O$ significantly raised the expression of *Prdx6* in both the regions of the treated groups (P<0.05 cortex; P<0.05 hippocampus). Interestingly, treatment with *S. aromaticum* following $AlCl_3$•$6H_2O$ exposure further increased its expression (P<0.05 cortex; P<0.05 hippocampus) as compared to the negative control. However, no significant difference in the *Prdx6* expression was observed between the negative control and the *S. aromaticum*-treated group. Similarly, the self-recovery and the $AlCl_3$•$6H_2O$-treated groups revealed no significant differences in the expression levels of *Prdx6*.

3.5. Effect of $AlCl_3$•$6H_2O$ and S. aromaticum extract on APP isoforms

Gene expression analysis of *APP* isoforms (*APP* Common, *APP770*, *APP695*) was performed by semi quantitative PCR to determine the effect of *S. aromaticum* extract on $AlCl_3$•$6H_2O$-induced neurotoxicity. The expression level of cortical and hippocampal *APP* common gene did not show variation in expression for any of the tested groups. A significant decrease (P<0.05) in expression was observed for *APP695* in mice cortex treated with $AlCl_3$•$6H_2O$ when compared with the negative control. Whereas, in the group treated with *S. aromaticum* post $AlCl_3$•$6H_2O$ exposure, there was a significant increase in the *APP695* expression (P<0.05) which was approximately similar to the negative control (Table 3). The group treated with *S. aromaticum* showed no variation in its level of expression as compared to the negative control. The effects of $AlCl_3$•$6H_2O$ on *APP695* expression were similar in the self-recovery group and the $AlCl_3$•$6H_2O$-treated group. A similar expression pattern of *APP695* was observed in the hippocampus in all the experimental groups, indicating a potential positive effect of *S. aromaticum* treatment.

Expression analysis of *APP770* showed a remarkable increase in the $AlCl_3$•$6H_2O$-treated and the self-recovery group as compared to the negative control (P<0.05 cortex; P<0.05 hippocampus). The *S. aromaticum* treatment post $AlCl_3$•$6H_2O$ exposure significantly normalized the altered expression of *APP770* (P<0.05 cortex; P<0.05 hippocampus) (Table 3). No significant difference was observed between the negative control and *S. aromaticum*-treated group.

4. Discussion

A number of studies reported the neurotoxic effects of Al and its association with cognitive impairment, A β accumulation and oxidative stress in various neurological disorders[25-28]. Al impairs the integrity and permeability of blood brain barrier and facilitates iron-mediated oxidative reactions[29,30]. Our findings showed that Al exacerbates the expression levels of antioxidant enzymes and amyloid precursor protein isoforms. On the other hand, the administration of *S. aromaticum* extract (500 mg/kg) to Al fed mice showed a reversal of the deleterious effects of Al. This shows potential protective action of *S. aromaticum* against neurotoxicity. The free radical quenching activity of *S. aromaticum* extract was confirmed by applying a DPPH assay and its ^{1}H-NMR analysis showed some polar and potent chemical entities including flavonoids such as quercetin, tannins and saponins.

Flavonoids exert a protective effect by directly scavenging ROS, by activating antioxidant enzymes[31] or through metal chelating

activity[32]. In our study, a significant increase (P<0.05 cortex; P<0.05 hippocampus) in the expression level of *SOD1* following *S. aromaticum* extract treatment can be attributed to the potential antioxidant properties of the flavonoids identified in *S. aromaticum* extract.

Quercetin, a potential flavonoid, known for its iron-chelating and stabilizing properties[33], is suggested to play an important role in protection against oxidative damage. It significantly increases the activities of SOD1 and GSH-Px, and markedly reduces the level of malondialdehyde and may ameliorate brain damage. Quercetin inhibits free radical generation by enhancing the activity of endogenous antioxidant enzymes; thus providing neuroprotection[34]. Quercetin also provides resistance against neurodegeneration by reducing microglial killing of damaged neurons[35]. Based on the findings of our study, we therefore proposed that the improved expression of the antioxidant enzymes may possibly be due to the metal chelating abilities of quercetin present in the *S. aromaticum* extract.

Tannin is another major chemical constituent identified during this study. It has the potential to alleviate a sodium fluoride-induced oxidative state in the brain, and is suggested to be more effective at retrieving SOD activity than vitamin C[36]. Our results showed that there is substantial decrease in *SOD1* expression following $AlCl_3$•$6H_2O$ exposure while the *S. aromaticum* extract significantly increased *SOD1* expression in both the cortex and hippocampus (P<0.05 cortex; P<0.05 hippocampus). Self-recovery of mice against $AlCl_3$ -induced toxicity was also assessed but it was not promising, and made us conclude that although the body is no longer exposed to oxidative insult, it is still unable to cope with the consequences of the insult and an exogenous source of antioxidants is required. Saponins (steroid or triterpenoid glycosides) identified in the *S. aromaticum* ethanolic extracts are common to a large number of plants and their products, and are an important component of human and animal nutrition[37]. A group of saponins containing 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP), (an antioxidant moiety attached at C_{23}) allows saponins to scavenge super oxides thus preventing cellular damage by ROS[38].

The cerebral cortex and hippocampus are severely affected by Al as compared to other areas of the central nervous system with marked histopathological alterations, including neuronal degeneration[39-41]. The histopathological assessment of cortex of the $AlCl_3$•$6H_2O$-treated group showed aberrations in neuronal structure. The marked difference in the density of Nissl granules among different groups is suggestive of the fact that Al toxicity may led to degeneration of neurons resulting in a decreased content of Nissl bodies (P<0.05). These results are in accordance with previously published studies wherein $AlCl_3$ caused significant decrease in the number of nissl granules[24,42]. The non-significant difference found in the density of Nissl bodies between $AlCl_3$•$6H_2O$-treated group and self-recovery group indicates that the damage caused is irreversible and the body/brain itself cannot overcome the deleterious effects of Al toxicity. However, promising observations were noted for the tissue sections treated with *S. aromaticum*, which showed a marked increase in the density of Nissl substances as compared to the $AlCl_3$-treated group (P<0.05). This revealed its potential neuroprotective role. Our results vividly demonstrate that Al exposure is involved in neurodegeneration and oxidative stress while administration of *S. aromaticum* can counteract the damage inflicted by Al in the brain of mice.

The expression level of potent antioxidant enzyme Prdx6 was substantially higher in the $AlCl_3$•$6H_2O$-treated group as compared to the negative control group (P<0.05 cortex; P<0.05 hippocampus). This over expression could be due to the resistance produced against membrane damage associated with phospholipid peroxidation during the early stages of oxidative stress[43]. Interestingly, treatment with *S. aromaticum* further increases the concentration of enzyme which may help it to cope with oxidative stress (P<0.05 cortex; P<0.05 hippocampus). While the group of mice treated with *S. aromaticum* (without prior treatment to Al) did not show any significant difference in the expression of *Prdx6* as compared to the negative control group.

We have also examined the expression of *APP* common following Al and *S. aromaticum* extract administration; results indicated that the *APP* common expression level did not vary significantly among the groups in both the cortex and hippocampus. This shows that although the concentration of the different isoforms varies among the groups, overall expression of *APP* remains the same in accordance with an earlier study[44]. APP isoforms plays a critical role in memory formation, synaptic plasticity and brain development. Among the various APP isoforms, *APP695* is abundantly present in cerebral cortex and some other brain regions[45]. Increased expression of *APP695* was observed in negative control which is suggestive of its neuroprotective effects. However, the expression of *APP695* was significantly decreased in the $AlCl_3$•$6H_2O$-treated group (P<0.05 cortex; P<0.05 hippocampus). This decreased *APP695* expression in the $AlCl_3$-treated group is concomitant with neuronal loss in very same group as APP695 resides inside the neurons[46]. The *S. aromaticum* extract significantly increased the expression of *APP695*, which indicates its neuroprotective features (P<0.05 cortex; P<0.05 hippocampus).

Moreover, there was a high level of *APP770* expression in the cortex and hippocampus of $AlCl_3$•$6H_2O$-induced oxidative stress mice as compared to the negative control (P<0.05 cortex; P<0.05 hippocampus). Previously, Iqbal *et al*. has reported similar effects of $AlCl_3$•$6H_2O$ administration on *APP770* expression levels in mice brain cortex, hippocampus and amygdala[46]. These results demonstrate that Al exerts neurotoxic effects in brain via up regulation of amyloidogenic *APP770* gene expression. Decreased expression of *APP770* gene expression is observed in *S. aromaticum*-treated mice. It has been reported that quercetin exhibits protective

effects against A β -induced toxicity on both neurons and endothelial cells by reducing the production of prostaglandin E2 in response to amyloid- β_{1-42}[47]. Tannins have potent antioxidant motifs that could bind specifically to A β fibrils and inhibit fibril formation and/or destabilize preformed A β fibrils through mechanisms yet unknown[48]. Based on the findings from our study it is proposed that the decreased expression of *APP770* in *S. aromaticum*-treated mice bodes well for the capacity of active phytochemicals like flavonoids, tannins and saponins to combat A β related neurotoxicity. This proves its therapeutic potential to intervene against neurodegenerative disorders.

This study reveals that Al has strong potential to exert oxidative stress in the brain as well as having deleterious effects on the neurons leading to neurodegeneration. The neuroprotective role of *S. aromaticum*, by preventing $AlCl_3$-induced neurotoxicity in the studied brain regions, is an important finding of the present work. In addition to stabilizing the histopathological alterations, *S. aromaticum* also showed promising results in normalizing the altered levels of oxidative stress markers and amyloid precursor protein isoforms. However, further characterization of the compounds of *S. aromaticum* extract is required to determine the active principle and to determine the exact mechanism by which the phytochemicals exert pharmacological and neuroprotective effects.

Author contribution

S.Z., substantial contribution to conception and design of the study and finalization of the manuscript; S.A.A.S., substantial contribution of acquisition and interpretation of ^{1}H NMR data; T.A., analysis of gene expression data, histological assessment; S.A., all experimental work, analysis and interpretation of data, drafting the article. All authors read and approved the final manuscript.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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