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Research Article
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Effect of *Manasamitra Vatakam* Against Aluminium Induced Learning and Memory Impairment of Apoptosis in Rat's Hippocampus and Cortex

Thirunavukkarasu SV[1]*, Jayanthi M[2], Raja S[3] and Venkataraman S[4]

[1]Medical Cyclotron Facility, HCG Health Care Ltd, Kamakshi Memorial Hospital Campus, Chennai-600 100, Tamil Nadu, India
[2]Department of Chemistry, Pachaiyappa's College, Chennai-600 030, Tamilnadu, India
[3]GITAM Institute of Pharmacy, GITAM University, Visakhapatnam- Andhra Pradesh, India-530045
[4]Department of Pharmacology, C.L. Baid Metha College of Pharmacy and Research Foundation, Chennai – 600 097, Tamilnadu, India

Abstract

The present study investigates neuronal cell death and neuroprotective effect of *Manasamitra vatakam* (*MMV*) against Aluminum (Al)-induced toxicity in rats. Aluminum and its salts extensively damaged nervous system and impaired learning and memory animals in neurodegenerative disease. In this study were conducted five groups, six animals in each group. In these study reveals Al caused neuronal apoptosis and cognitive dysfunction of animals brain regions (Hippocampus and Cortex) that could be restrain by treated with *Manasamitra vatakam. MMV* is a herbo-mineral formulation and prepared on the basis of Indian system of Ayurvedic medicine. These formulations using psychiatric treatment in Indian scenario, obviously tried for alternative treatment against Aluminium induced cognitive dysfunctions. It causes synthesis, release of neurotransmitter 5-hydroxytryptamine (5-HT), antioxidants, inhibit apoptotic genes and improve cognitive functions of animals. Apoptotic, anti-apoptotic genes such as *Bcl-2, Bcl-xL* and *caspas-3* were estimated by Western blot and Reverse transcriptase polymerase chain reaction (RT-PCR). In these results clearly observed that *MMV* treatment was markedly increased 5-HT and restrain cognitive functions as well as decreased expression of pro-apoptotic genes against Al induced animals. This result indicates *MMV* could activate learning and memory function of animals due to inhibition of apoptotic genes like *Bcl-2, Bcl-xL* and *caspas-3*. The present study reveals that Ayurvedic formulation of *Manasamitra vatakam* (*MMV)* as potentially inhibited neuronal apoptosis and enhanced cognitive function leads to learning memory function against Al induced neuodegenerative disease.

Keywords: Aluminum chloride; Apoptosis; Neurodegeneration; Neurotoxicity; *Bcl-2; Bcl-xl; Caspas-3*

Introduction

Aluminum exposure causes adverse health effects, some neurodegenerative diseases like Alzheimer's disease (AD) [1]. Aluminium widely distributed metallic element in the earth's crust and it acts as a neurotoxin. Aluminium contain high levels in diet led to increase risk of central nervous system and similar to Alzheimer's disease (AD), Amyotrophic lateral sclerosis (ALS) and Parkinsonism dementia complex [2]. It promotes aggregation of synthetic ß – amyloid protein [3]. It activates neurotoxicity in central nervous, skeletal and hematopoietic systems [4]. The extensive damage of nervous system caused learning and memory impairment of animals. Yokel [5] reported that potentially damaged brain in animals and humans induced by Al toxicity [6]. Aluminum-containing chemicals are widely used in medicine, food additives, and cosmetics and are added to tap water in some areas as a flocculating agent during the water purification process, it is important to study factors that might increase the absorption of aluminum across the gastrointestinal barrier [7].

Aluminium concentration was altered essential trace elements of CNS and increased susceptibility of CNS to lipid peroxidation and induces oxidative stress of neuronal damage for formation of ROS [8,9]. The previous studies observed that *MMV* act as a potential effect against Al induced free radical generation of oxidative stress protein (HSP70) involved neuronal damage of nerve cells evidenced by histological studies [10]. Altmann et al. [11] confirmed that Al causes considerable damage of cerebral function include learning memory impairment in rats was linked to develop Alzheimer's disease (AD) [12,13]. Exacerbation of normal aging related changes and neuronal apoptosis has played key factor for impairment learning memory and neurodegenerative disorders [14-16].

Good et al. [17] reported Aluminium involved neurotoxicant of the brain system and its mechanism involved learning memory deficits to become serious damage to CNS function [18]. It induced mitochondrial changes following cytotoxic stimuli represent a primary event in apoptotic cell death due to apoptogenic factor such as cytochrome-C released into cytoplasm [19]. The cytochrome-C translated into another cytoplasmic factor binds Apaf-1 and formed a complex that initiates *Caspas-9* turn to activate effectors caspases, in which *caspas-3* is a prominent member of cell death [20]. However the exact mechanism of this study clearly indicates that Aluminium induced neurodegeneration caused by neuronal apoptosis. Hockenbery et al. [21] also reported that *Bcl-2*, gene as important gene for Al induced apoptosis and linked with learning and memory ability of CNS [22,23].

Shankhapushpine contains alkaloids (*Convolvulus pluricaulis*), volatile oil, flavonoids (kampferol derivatives), phytosterol (beta-sitosterol), carbohydrates (glucose, rhamnose, and starch), ceryl alcohol, scopoletin [24], 20-oxodotriacontanol, tetratriacontanoic acid and 29-oxodotriacontanol helpful for potent insect antifeedant constituents [25]. In dietary feeding of this plant has been found

***Corresponding author:** Dr. Thirunavukkarasu, Research officer cum Chief Radio Chemist, Medical Cyclotron Facility, Dr. Kamakshi Memorial Hospital, Pallikaranai, Chennai – 600 100, Tamilnadu, India, Tel: +91-44-66300300, 22469200; Fax: +91-44-66300500; E-mail: varasubio@rediffmail.com

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to increase protein synthesis in the hippocampus, thus enhancing memory and learning in experimental animals [26].

The present study reveals that Al induced neuronal apoptosis and cognitive dysfunction of animals brain regions (Hippocampus and Cortex) that could be restrain by the treatment of *MMV*, a traditional medicine for preparation of Ayurveda and it also potentially protected from impaired learning memory and neuronal apoptosis in Al induced neurotoxicity animals due to that *MMV* contains 20 different ingredients they are *Nagabala, Lavanga, Shankapushpi (Convolvulus pluricaulis), Manasravani, Hema, Vacha (Acorus calamus), Vidhoruma, Tulasidala, Pushkara, Malkangni (Celastrus paniculatus), Vilva and Sornavalli* are the principle ingredients were more helpful to augment cognitive functions that leads to increase learning memory of the animals from neurotoxicity. *Manasamitra vatakam* (*MMV*) is a herbo mineral formulation used for treatment psychiatric patients in India and preparation procedure followed by Indian System of Ayurveda Medicines [27]. The present study reveals that Al induced neuronal apoptosis and cognitive dysfunction of animal's brain regions (Hippocampus and Cortex) that could be restrained by treatment with *MMV*, a traditional medicine for preparation of Ayurveda and it also potentially improved neurotransmitters like 5-hydroxytryptamine (5-HT) from impaired learning memory and neuronal apoptosis in Al induced neurotoxicity animals.

Materials and Methods

Chemicals and antibodies

Aluminium chloride (Al) was purchased from Merck, Chennai, India. All chemicals were pure with an efficiency of greater than 99%. The Tritonx-100 solution, Bovine serum albumin (BSA), Na-deoxycholate, tritonx-100, acrylamide, bis-acrylamide, ammonium persulfate and N,N,N0,N0 tetramethyl ethylenediamine (TEMED) solution were purchased from Sigma-Aldrich pvt.ltd, Bangalore, India. Total RNA isolation reagent (TRIR) was purchased from Medox biotech India pvt. Ltd, India. The one-step RT-PCR Kit, Horse radish peroxidase (HRP) and conjugated with rabbit anti mouse imunoglobilin-G (IgG) antibody, polyvinylidene fluoride membrane (PVDF) millipore, (ECL kit from Pierce, Rockford, IL), primers for mouse monoclonal, *Bcl-2 and Bcl-xL* antibody (Ab) and β actin were purchased from Bangalore geni, Bangalore, India. Standard pelleted rat diet was obtained from Hindustan lever, Bangalore, India. All other chemicals were purchased from Sisco research laboratories pvt. Ltd, India.

Test Drug: Ayurvedic proprietary formulation, *Manasamitra Vatakam* (*MMV*) was obtained from Kotakkal Arya Vidya Sala, India. *MMV* (100 mg/kg bw) was weighed and dissolved with water used for treatment of animals studies.

Animals: Male healthy Swiss albino rats (200-220 gm) housed in clean polypropylene cages and maintained at the room temperature 23°C-25°C with alternating 12 h light and dark cycles were used for the study. The animals were fed with standard pelleted diet and clean drinking water. All standard procedures were carried out as per the guidelines for care and use of laboratory animals, protocols approved by the Institutional animal ethics committee of experimental animals (IAEC No. 14/18/IAEC/24/07/07) of C.L.Baid Metha college of pharmacy, Chennai, India.

Experimental design: Rats were divided into five groups, each group contains six animals. Group I served as control and received only distilled water. Group II rats received $AlCl_2$ at level of 100 mg/kilogram body weight per oral (mg/kg.b.wt.p.o) diluted and dispensed in pure drinking water; Group III rats were treated with *MMV* at dose level of 100 mg/kilogram body weight per oral (mg/kg/b.wt.p.o), along with $AlCl_2$ (100 mg/kg.b.wt.p.o). Group IV served as positive control treated with *MMV* alone at the dose of 100 mg/kg.b.wt.p.o. Group V animals were treated with standard rivastgmine at the dose of 1.0 mg/kg.b.wt.p.o. The whole experimental study was carried out for 90 days. Animals were weighed at the beginning of the experiment, and subsequently followed twice in a month up to the end of experiments. At the end of 90 days treatment schedules, the behavior studies were carried out by the techniques of [28].

Passive avoidance: Cognitive behavior study was assessed by Pole Climb apparatus. The number of times animal's escapes from shock read in 10 trials using pole climb apparatus [29].

Biochemical estimation of tissue samples: The end of experimental period, animals were sacrificed by cervical dislocation and brains removed immediately and washed thoroughly with ice cold saline and kept at -80°C. The brain samples of hippocampus and cerebral cortex were micro dissected following the technique of [30]. The cerebral cortex and hippocampus of the rats brain were homogenized using 10% homogenate with phosphate buffer (0.1M, pH 7.4) containing 1mmol ethylene diamine-tetra-acetic acid (EDTA), 0.25M sucrose, 10mM potassium chloride (KCl) and 1mM phenyl methyl sulfonyl fluoride (PMSF) with a homogenizer fitted with teflon plunger. Homogenate was centrifuged at 800 g for 5 min at 4°C in an IEC020 refrigerated centrifuge. The supernatant was again centrifuged at 12,365×g for 15 min at 4°C to obtain post-brain supernatant (PMS) which was used for determination of neurotransmitter 5-hydroxy tryphtamine and its metabolism of 5-hydroxy indole acetic acid. Another part of brain tissues were used for the quantification of *Bcl-2* and *Bcl-xL* gene expression. The cerebral cortex from each group was fixed in 10% formalin solution and used for the immunohistological study.

Assay of 5-Hydroxytryptamine (5-HT): Haubrich and Denzer [31] method were followed after isolation of the brain tissue, the wet weight of the tissue was obtained. The brain tissue was transferred to a tissue homogenizer and immediately homogenized in cold acid butanol (10%, w/v). The homogenate was centrifuged for 10 min at 2500 rpm. To the aliquot separated, 10.0 ml of heptane, 1.0 ml of 0.01 N HCl and a known amount of internal standards was added and shaken well to extract the monoamines. Samples were run without internal standard in another set of tubes. The reagent blank tube contained no standards. The tubes were then centrifuged for 10 min at 2500 rpm. The organic phase was analyzed for 5-HIAA and the acid phase was used for estimation of 5-HT level.

To 10.0 ml of organic phase, 1.5 ml of 10 mm tris– HCl buffer (pH 7.0) was added and 5-HIAA was extracted into the tris-HCl buffer by shaking the tubes for 5 min. The tubes were again centrifuged for 2500 rpm and the lower aqueous phase was aspirated and read for 5-HIAA fluorescence at 295/350 nm. To 1.0 ml of the acid phase 200 mg of alumina and 1.5 ml of 0.5 m tris–HCl buffer (pH 8.5) was added. The tubes were shaken by hand for 5 min and centrifuged later at 2500 rpm for 5 min. 2.0 ml of the supernatant was separated and transferred to 15.0-ml glass stoppered centrifuge tubes containing 6.0 ml of salt and water saturated butanol, 1.5 g of sodium chloride and 2.0 ml of 0.35 m borate buffer (pH 10.0). The mixture was shaken by hand for 5 min, then centrifuged at 2500 rpm for 5 min and 5.0 ml of the organic phase was transferred to another 15.0-ml centrifuge tube with a stopper containing 6.0 ml of heptane and 0.6 ml of 0.1 n HCl. This solution was shaken for 5 min by hand and spun at 5000 rpm for 10 min. 0.4 ml of the acid phase was aspirated to another tube and the fluorescence

was derived by adding 0.6 ml of O-phthaldialdehyde and heating for 10 min. The samples cooled and measured with fluorescence units of spectroflurometer (Hitachi 650– 10M, Japan) at 350/470 nm.

Western blot analysis: Protein concentration was measured by the method Lowry et al. [32]. The tissue homogenate was centrifuged at 10,000 × g for 10 min and supernatant was used for *Bcl-2* and *Bcl-xL* level determinations after dosage of proteins as described below. Equal amount of protein samples (50 mg) was mixed with 2X sample buffer and boiled for 5 min. The sample mixture was run on 12% SDS-PAGE gel (Sodium dodosyl sulphate) in 1x running gel buffer at 80V for 2.5 h and electro transferred to a polyvinylidene fluoride membrane (PVDF) at 100 V for 1h. The membrane was blocked in blocking buffer containing 5% skimmed milk powder overnight. After overnight, the blocked membranes were incubated with mouse monoclonal *Bcl-2* and *Bcl-xL* (1:1000), for 6 h. The membranes were washed with tris buffered saline (TBS) and were incubated with horse radish peroxidase (HRP) - conjugated with rabbit anti mouse IgG antibody (1:5000) for the appropriate primary Abs. Following two intermittent washes with 1X Tween 20-TBS (T-TBS) and TBS alternatively, the bands were developed using ECL kit (Pierce, Rockford, IL) and intensity of each band was determined using an image analyzer (Quantity One software from Bio-Rad).

Quantification of Bcl-2 and Bcl-xl gene expression

Isolation of total RNA: Total RNA was isolated by using total RNA isolation reagent (TRIR) followed by the method of Chomczynski and Sacchi [33]. To 30 mg of hippocampus and cortex of brain tissue, 1ml of TRIR was added and homogenized for 3min. To this 0.2 ml of chloroform was added, shaken vigorously for15s and placed on ice at 4°C for 5min. Then, the homogenate was centrifuged at 12,000 x g for 15min at 4°C. The lower organic phase containing DNA and protein, and the upper aqueous phase containing RNA were separate out without disturbing lower phase, the interphase (aqueous phase) was transferred to an eppendorf, equal volume of isopropanol was added and kept at 4°C for10 min. The aqueous phase and isopropanol solution were centrifuged at 12,000 x g for 15 min at 4°C to precipitate the RNA. The pellet was washed twice with 75% ethanol, vortexed and centrifuged for 5 min at 7,500 x g at 4°C. The RNA pellet was then dissolved in 100 ml of 0.2% diethylpyrocarbonate (DEPC) water and placed in water bath at 60°C for 10 min to ensure complete solubility of RNA, then vortexed and stored at -80°C. The RNA purity and concentrations were determined spectrophotometrically at A_{260}/A_{280} nm.

Further RNA was isolated with trizol solution (Sigma, St. Louis, MO, USA). One microgram of total RNA was reverse transcribed by reverse transcriptase (Sigma) at 42°C for 1 h. PCR amplification reaction mixtures contained *Bcl-2* (forward: 5'-CACCCCTGGCATCTTCTCCTT-3', reverse: 5'- AGCGTCTTCAGAGAGCCAG-3'), *Bcl-xL* (forward: 5'-AGGCTGGCGATGAGTTTGAA-3', reverse: 5'- TGAAACGCTCCTGGCCTTTC-3'), β-actin primers (forward: 5'–GTGGGGCGCCC CAGGCACCA–3', reverse: 5'- CTCCTTAATGTCACGCACGATTT3') and Tap man universal PCR master mix. Thermal cycle conditions included holding the reactions at 94°C for 3 min and cycling for 30 cycles among 94°C for 30 sec, 55°C for 1 min and 72°C for 1 min. The results were identified by gel electrophoresis. The rate of *Bcl-2* and *Bcl-xl* and inter control β-actin represented the relative expression of *Bcl-2* and *Bcl-xL* mRNA.

Reverse transcription polymerase chain reaction (RT-PCR): Total RNA (1.0 µg) was reverse transcript and amplified by Qiagen one-step RT-PCR kit according to the manufacturer's instructions. The primers used for RT-PCR were gene specific primers for *Bcl-2* and *Bcl-xL* and β actin (Zola-Morgan et al. [34]), 10 ml of each PCR product was analyzed by gel electrophoresis on 2% agarose gel. The molecular size of the amplification products was determined by comparison with a molecular weight marker (DNA ladder) run in parallel with RT-PCR products. Then the gel were subjected to densitometric scanning (Bio-Rad, USA) to find out the OD units of each band and then normalized against that of the internal control β-actin.

Immunohistopathology: The sections were collected from cerebral cortex of the animal's brain region. Free-floating coronal sections of brain were rinsed in TBS and inactivated for endogen phosphatase activity in 0.6% H_2O_2–TBS. Sections were then treated for DNA denaturation incubating in 2 M HCl at 37°C and rinsed in 0.1 M sodium borate buffer. Sections were blocked in TBS-plus containing 3% normal goat serum and 1% Triton-X in TBS for 30 min. Paraffin-embedded sections of 5-7 µm thick were mounted on gelatin coated glass slides (3% gelatin and 0.5% chrome potassium sulphate in $D.H_2O$), deparaffinized and hydrated followed by deionised H_2O washing for 1 minute. Antigen unmasking is performed at this point with 10mM sodium citrate buffer (10mM sodium citrate, 0.05% tween 20, pH 6.0) at 95°C for 5 minutes. The sections were treated with cold 0.1 -1% H_2O_2 in distilled water to quench endogenous peroxidase activity for 5 min. To suppress non specific binding of IgG, sections were incubated with 10% normal blocking serum (Normal goat serum in PBS) for 20 minutes.

The sections were incubated with anti-*caspase-3* monoclonal antibody (at a dilution of 1:200; Santa Cruz Biotech, U.S.A) overnight at 4°C, and washed with three changes of PBS phosphate buffer solution) for 5 min each. Subsequently sections were incubated 45 minutes with secondary antibody (Goat anti-rat IGg HRB conjugated, diluted to 1-5 µg/ml in PBS with 2% normal blocking serum - GeNei, India) in a dark chamber. Wash with three changes of PBS. Then sections were incubated with 0.05% DAB [3, 3'-diaminobenzidine, Sigma Cat D8001], 0.015% H_2O_2 in 0.01M PBS, pH 7.2) for 1-3 minutes at room temperature. Finally counter stain with hematoxylin. Sections were again washed, mounted, and cover slipped. Finally section were observed bright field microscope (Nikon Corporation, Tokyo, Japan) and photographed. Staining pattern of the *caspase-3* expression was dark brown and nuclei were stained with blue (Hematoxylin).

Statistical analysis: Statistical analysis was carried out using Graph pad prism software (version 4.03). One way ANOVA was used, followed by Newman-Keuls multiple comparison test. The data represent mean ± SEM. The level of significance was set at $p \leq 0.001$.

Results

Effect of *MMV* in Al intoxicated rat's behavioral changes

The Passive avoidance apparatus consists of grid chamber with shock free zone (SFZ) for used to assess short term memory of animals. A set of trials were performed on the basis of number of times taken to animal escaped from shock treatment. The results shown *MMV* acting as improved memory function against Al induced toxicity animals (Figure 1). The number of escapism was augmented ($P<0.001$) in *MMV* treated animals as compared to Al toxicity rats. Moreover *MMV* treated animals were similar to control animals. Aluminium toxicity animals found to be decreased when compared to *MMV* treated animals. This effect may be due to *MMV* contains saponin and flavonoides that could be improved approximately 65% cognitive functions as compared to Al induced animals. No significant differences were observed in positive control (rivastigmine) as well as *MMV* alone treated animals.

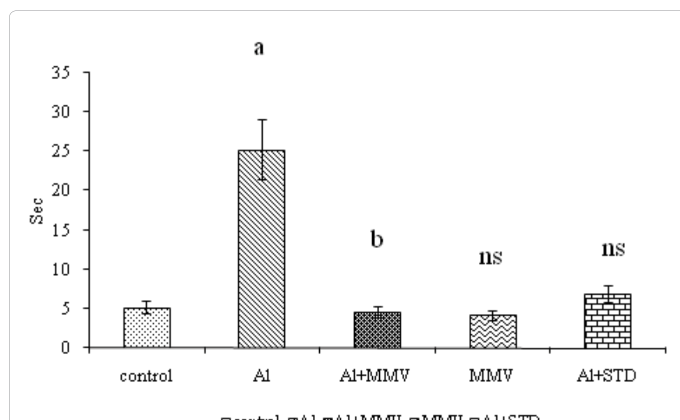


Figure 1: Effect of MMV in behavioral studies on passive avoidance on $AlCl_3$ exposed rats brain. Results were expressed as Mean ± S.E.M (n = 6), followed by One Way ANOVA – Newman-Keuls Multiple comparison test, [a] P < 0.001 by Newman-Keuls multiple comparison test $AlCl_3$ group were compared with control group. [b] P < 0.001 by Newman-Keuls multiple comparison test [a] P < 0.001 by Newman-Keuls multiple comparison test Al group were compared with control. [b] P < 0.001 by Newman-Keuls multiple comparison test *MMV*+Al were compared with Al treated group, ns– non significant.

Effect of *MMV* on Al induced 5-hydroxytryptamine (5-HT)

Effect of *MMV* against Al induced animals 5-hydroxytryptamine (5-HT) activity was shown (Figure 2). 5-HT level was found to be significantly lower in Al toxicity rat's brain regions (Hippocampus and cortex) when compared to control (p<0.001). 5-HT was significantly increased (p<0.001) in brain regions by *MMV* treated animals as compared to Al toxicity rats. *MMV* treated animals shown significantly increased 5-HT levels of Al induced rats. No significant changes were observed positive control (rivastigmine).

Effect of *MMV* and Al toxicity on *Bcl-xL* gene expression in hippocampus

An anti-apoptotic gene of *Bcl-xL* is also important regulators gene of apoptosis, their expression influenced due to Al intoxicated rats**.** A semi quantitative assay of RT-PCR revealed significantly (*P*<0.001) increased in *Bcl-xL* mRNA and protein expression in Al intoxicated rats whereas *MMV* treated animal's decreased expression (Figure 3). No significant changes were observed in *MMV* treated animals mRNA and protein expression of *Bcl-xL* when compared with positive control (rivastigmin).

Bcl-2 gene Family Expression on Al toxicity rats in hippocampus treated with *MMV*

Bcl-2 gene Family Expression on Al toxicity rats in hippocampus treated with *MMV* Pro-apoptotic gene *Bcl-2* mRNA expression is shown (Figure 4). In Al toxicity animals showed higher intensity of Bcl-2 protein and mRNA expression (P≤0.001) whereas *MMV* treated animals shown decreased protein and mRNA expression of *Bcl-2* hippocampus. There was no significant difference Bcl-2 was observed in *MMV* treated animals as compared with positive control.

Effect of *MMV* on Caspase-3 activity in Al toxicity rats

Plate 1 was shown the expression of *Caspas-3* activity against various treated groups. The activity of *Caspase 3*-like proteases was assessed by measuring the cleavage of the DAB. The result clearly indicates that neuronal apoptosis was observed on the basis of approximately three fold higher expression of *Caspase-3* in Al induced animals as compared to *MMV* treated animals. Expression of *Caspase-3* gene in cerebral cortex of brain region was found to be decreased in *MMV* treated animals and similar results were found positive control (rivastgmine) (Figure 5).

Discussion

Manasamitra vatakam is a herbo-mineral Ayurvedic formulation and inhibit neuronal apoptosis, improved cognitive functions against Aluminium induced toxicity animals. Environmental factors induced to alter the natural apoptosis and causes neurodegenerative diseases [18,35]. Aluminium and its salts hallmark of neurodegenerative dysfunction [36]. Hippocampus and cerebral cortex is the region of memory and learning ability centre, which was declined by Al induced neurodegenerative disease. The results reflect deteriorating cognitive dysfunctions of rat's brain (hippocampus) of Aluminium intoxication, While hippocampus as an indispensable integration of spatial information [37]. The passive avoidance paradigm is widely utilized for testing learning ability of rats or mice [38]. The present study indicates that *MMV* treated animals were shown to be increased pharmacological activities of animals and improve cognitive functions due to 5-HT and Acetyl Choline levels were also increased in the presence of alkaloids and flavanoids in *MMV*. Aluminium concentration of the brain regions caused poor performance passive-avoidance and visual discrimination with reversal task [39]. In these results reveals that *MMV* could inhibit impaired cognitive function and protect neurons from Al induced toxicity damages. Histopathological studies were also further supported to damage neurons. Aluminium markedly altered heavy loss of cortical neurons, lacunae spaces and vacuolated cytoplasm of the hippocampus and cerebral cortex of the brain that could be reversed by the treatment of *MMV* animals [10]. Animals escaped immediately were found under the treatment of *MMV* since it contains nutritional antioxidants such as alkaloids, flavanoids and phenolic compounds and also it improves Al induced toxicity of cognitive dysfunction rats. Aluminium induced toxicity caused neuronal apoptosis and interrupt cognitive functions to increase expression of pro and anti-apoptotic gene.

Aluminium enters into the brain region through blood brain

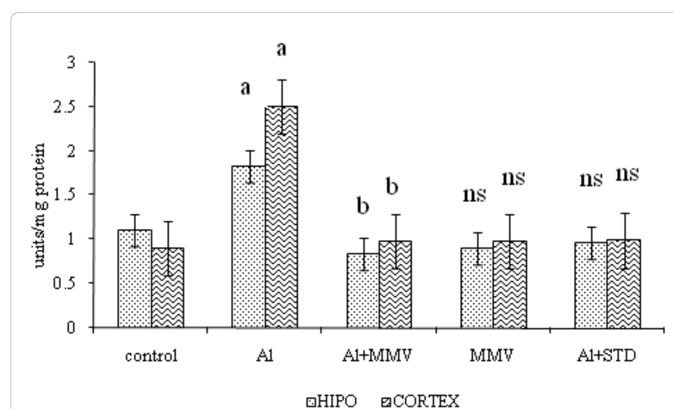
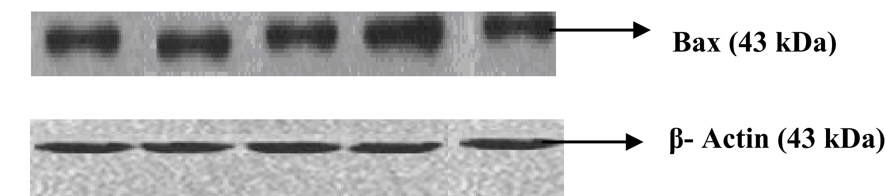
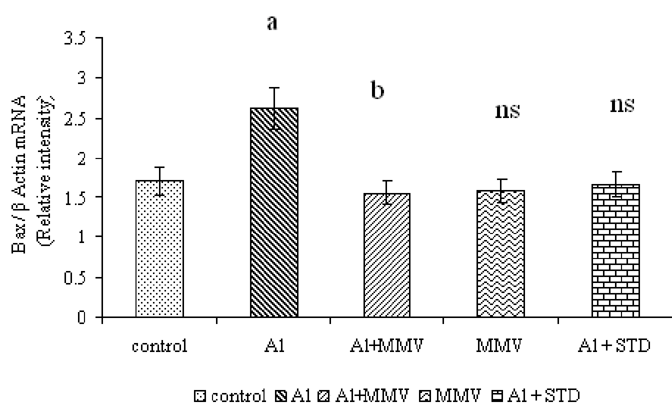
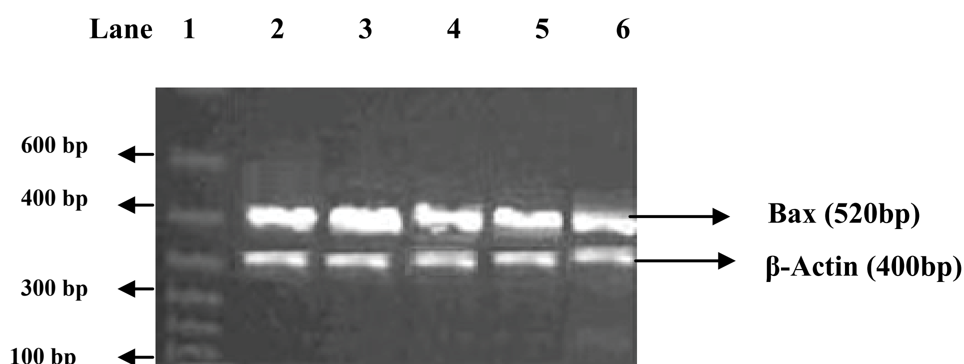


Figure 2: Effect of MMV in HT on $AlCl_3$ exposed rats brain. Shows the effect of *MMV* on 5-HT in cerebral cortex and hippocampus in different group of rats. Results were expressed as Mean ± S.E.M (n = 6), followed by One Way ANOVA – Newman-Keuls Multiple comparison test, [a] P < 0.001 by Newman-Keuls multiple comparison test $AlCl_3$ group were compared with control group. [b] P < 0.001 by Newman-Keuls multiple comparison test [a] P < 0.001 by Newman-Keuls multiple comparison test Al group were compared with control. [b] P < 0.001 by Newman-Keuls multiple comparison test *MMV*+Al were compared with Al treated group, ns– non significant.







Lane 1 Marker; Lane 2 control; Lane 3 $AlCl_3$;Lane 4 AlCl3+MMV ;Lane 5 MMV: Lane 6 Al+STD

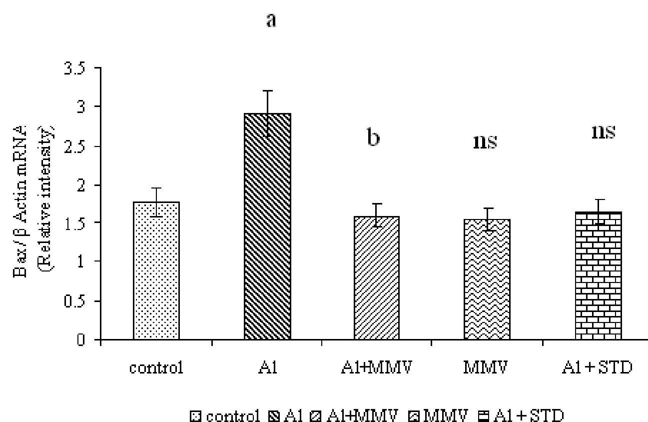
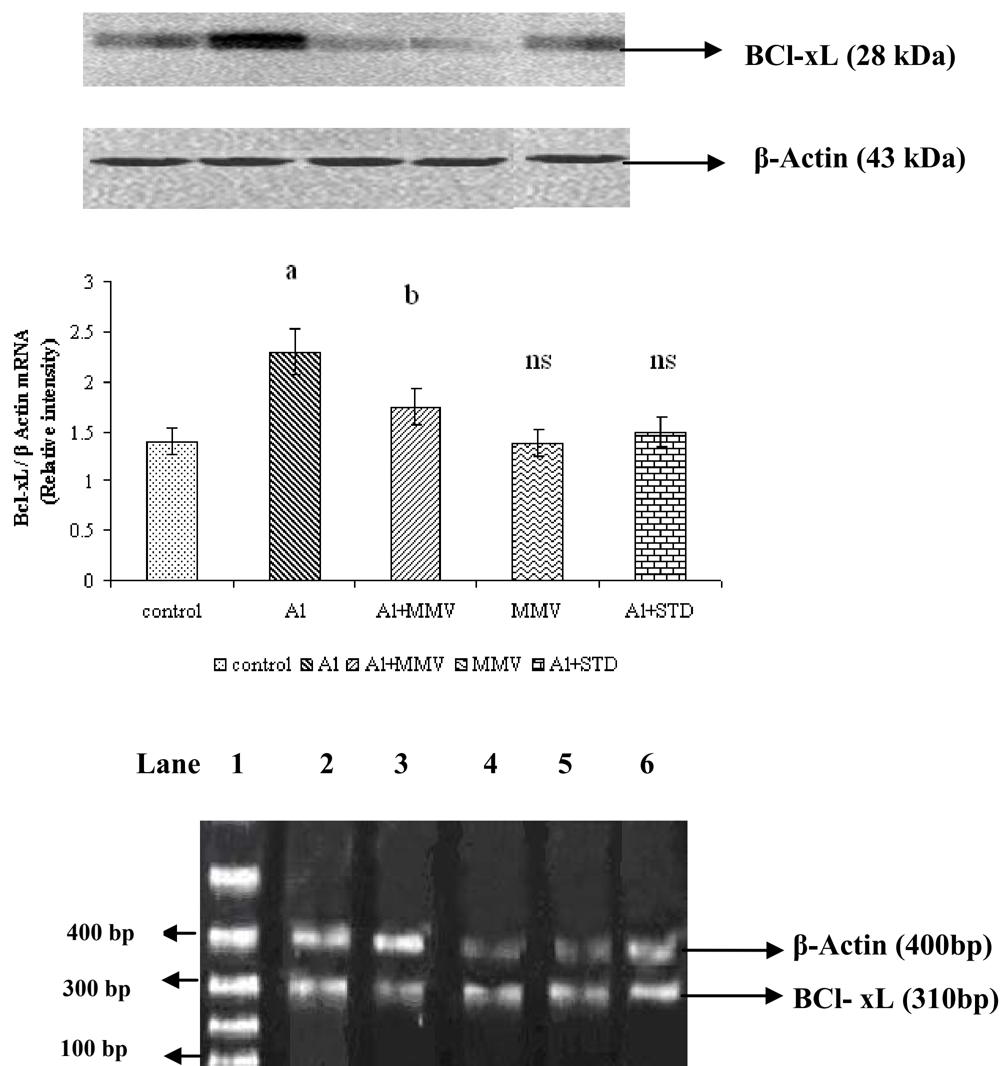


Figure 3: Effect of *MMV* on *Bax* protein in Hippocampus of in different groups of rats. *Bax* protein expression was increased in Al-exposed animals, as compared with control and *MMV* drugtreated animals. However, simultaneous supplementation of *MMV* along with Al decreased significantly, as compared with control. Each bar represents mean ± SEM of samples. Al-AlCl3, a - $P < 0.001$, by Newman-Keuls multiple comparison test was compared with control group; b-$P < 0.001$, by Newman-Keuls multiple comparison test were compared with aluminium-treated group; ns- nonsignificant compared with Al+ STD and control.



Lane 1 Marker; Lane 2 control; Lane 3 $AlCl_3$;Lane 4 AlCl3+MMV ;Lane 5 MMV: Lane 6 Al+STD

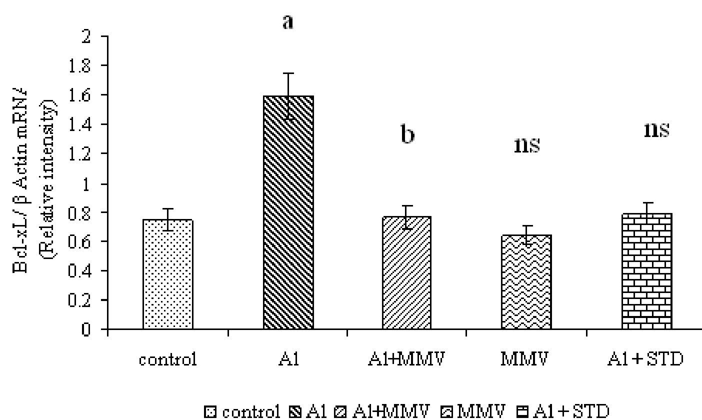


Figure 4: Effect of *MMV* on *Bcl-xL* protein in cortex of in different groups of rats. *Bcl-xL* protein expression was increased in Al-exposed animals, as compared with control and *MMV* drugtreated animals. However, simultaneous supplementation of *MMV* along with Al decreased significantly, as compared with control. Each bar represents mean ± SEM of samples. Al-AlCl3, a - $P < 0.001$, by Newman-Keuls multiple comparison test was compared with control group; b-$P < 0.001$, by Newman-Keuls multiple comparison test were compared with aluminium-treated group; ns- nonsignificant compared with Al+STD and control.

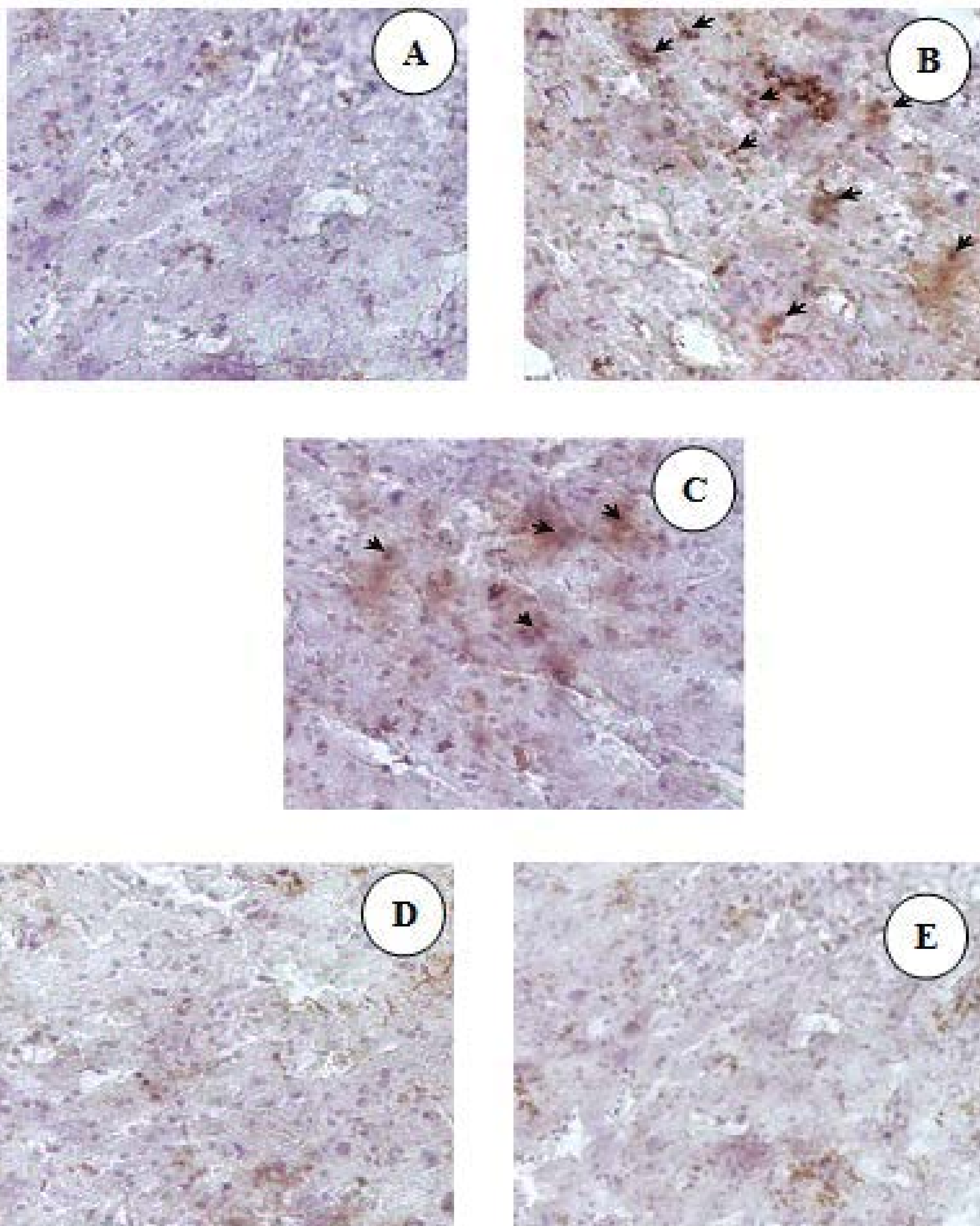
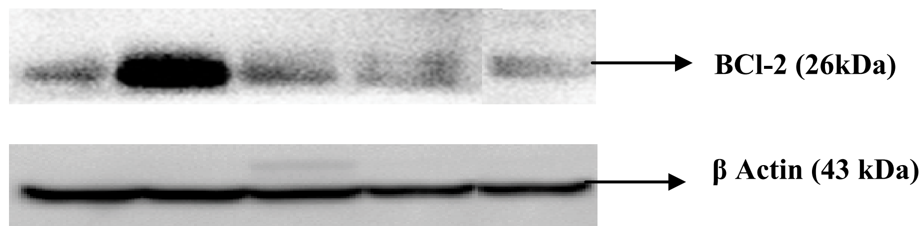
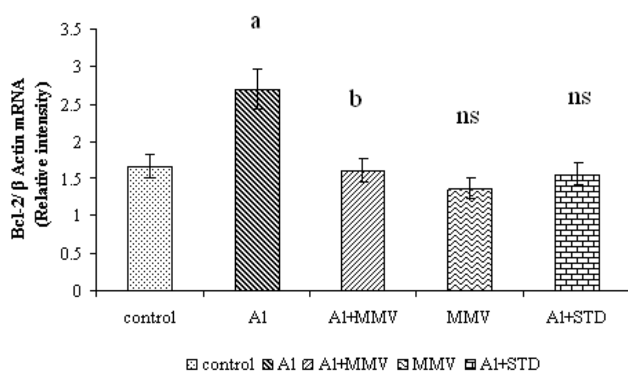
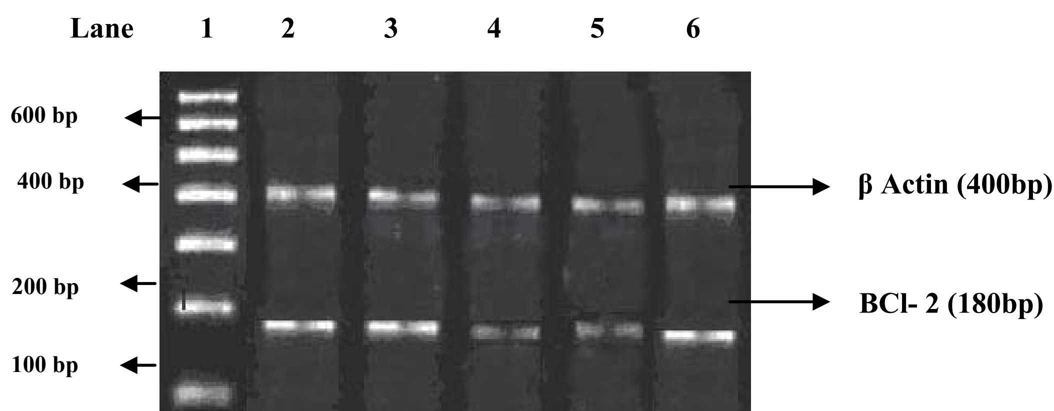


Plate 1: (A-E): photographs representing immunohistology expression of cell death gene expression *Caspas-3* the effect of *MMV* treatment along with Al damage of cerebral cortex of rat's brain under different treatment conditions. (A) Control group (tap water), (B) Aluminium chloride (100 mg/kg.b.wt.p.o) treated group, (C) Aluminium chloride (100 mg/kg.b.wt.p.o) + *MMV* drug (100 mg/kg.b.wt.p.o), (D) *MMV* alone (100 mg/kg.b.wt.p.o) treated group, (E) $AlCl_3$ (100 mg/kg/p.o) treated group + Rivastigmine (1.0 mg/kg/p.o) treated group.







Lane 1 Marker; Lane 2 control; Lane 3 $AlCl_3$;Lane 4 AlCl3+MMV ;Lane 5 MMV: Lane 6 Al+STD

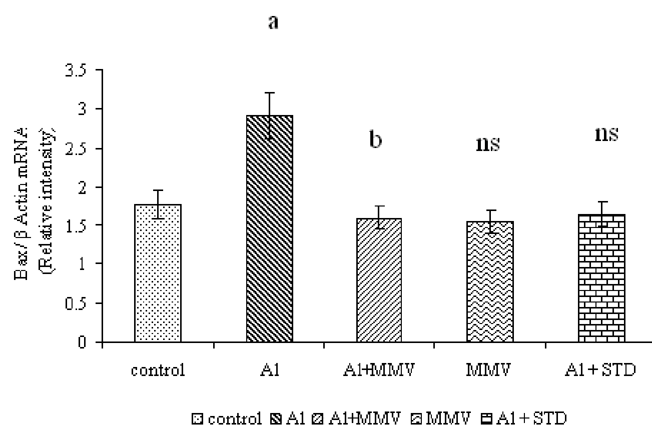


Figure 5: Effect of *MMV* on *Bcl-2* protein in cortex of in different groups of rats. *Bcl-2* protein expression was increased in Al-exposed animals, as compared with control and *MMV* drugtreated animals. However, simultaneous supplementation of *MMV* along with Al decreased significantly, as compared with control. Each bar represents mean ± SEM of samples. Al-AlCl3, a - $P < 0.001$, by Newman-Keuls multiple comparison test was compared with control group; b-$P < 0.001$, by Newman-Keuls multiple comparison test were compared with aluminium-treated group; ns- nonsignificant compared with control.

barrier (BBB) by following mechanism, Al penetrates brain effortless with complex formation of transferin through receptor mediated endocytosis [40]. Phytoconstituents like flavanoids, phenolic compounds and Saponins present in *MMV* that could be inhibited complex formation between Al and transferin. These results confirmed that *MMV* increased cognitive functions and inactivate interruption of BBB system (Blood Brain Barrier) of neurons by Al concentration in brain regions.

Serotonin or 5-hydroxytryptamine (5-HT) involved in eating, sleeping, behavior and neuroendocrine functions. Serotonin helps to balance normal physiological functioning of animals and behavioral activities of brain that could be altered by Al induced toxicity rats [41]. Increased Aluminium levels in brain might change in behavior, long term memory and intellectual animals due to endorsed aggregation of ß-amyloid protein results in neurotoxicity [3,42]. Kumar [43] reported Al induces to decrease serotonin level on 60 days treatment. In the present study found that Al significantly decreased serotonin on 90 days that could be improved serotonin under treatment with *MMV*. Al disrupts tryptophan metabolism by decreasing 5-HT. *MMV* was shown significantly increased serotonin (5-HT) due to the presence of alkaloids and Saponin. These results attributed *MMV* could help to improve the coordination of cognitive functioning of animals.

Brain contains large amount of lipids that are rich in polyunsaturated fatty acids. Aluminium involved catalytic reaction of free radicals resulted peroxidation of lipids [44,45]. These salts of biological tissues do not have any direct pro-oxidant properties but they may potentiate Fe to promote ROS formation and enhanced peroxidative damage of lipids and protein. Mariani et al. [46] reported that Al has increased oxidation significantly decreased compared to control. The SOD and GSH are important antioxidants in the brain which protect it from H_2O_2 mediated neuronal damage and therefore decreased level of these biomolecules, which may lead to increase severity of Al toxicosis in rat's brain. Usually decreased antioxidant versus oxidant ratio plays a crucial role in generating a condition of oxidative stress [47]. The decreased activity might have resulted from oxidative modification of proteins. *MMV* treated animals shown significant decreased oxidation of cells. These antioxidants properties of *MMV* could prevent both free radical damage and generating oxidative stress of brain cells [10].

Heat shock protein (HSP70) plays a key role protection of cells against oxidative damage and it also restored proteins and resulted maintain original structure and function of the cells [48,49]. In brain, HSP70 mediated cell survival is affected by cell type and age-dependent on the course of hyperthermic responses [50]. Aluminium induces stress protein of HSP70 and it has been involved damage wide range of cells and especially brain cells. In previous studies reported that *MMV* potentially protect brain cells from adverse effect of HSP70 protein expression in oxidative stress conditions which is induced by Al toxicity [10]. The present study indicated that *MMV* declined neuronal apoptotic genes which indirectly involved inhibiting the oxidative stress conditions of Al toxicity. The previous studies also further supported, *MMV* could prevent neuronal cell death and possible mechanism interlinked to decrease pro-apoptotic genes and HSP70 gene, which is responsible for neuronal cell death in Al induced rats.

Aluminium is a potent neurotoxins and it involves regulating cell inflammation and apoptosis. Accumulation of aluminium in neurons that affects mitochondria integrity and functionality of neuron cells [51]. Apoptosis is a prominent form of cell death in many human neurodegenerative diseases like AD and Parkinson [52]. The role of environmental factors like Al induced apoptosis processes in vital etiology of neurodegenerative diseases. A recent study shows that Al potentially induces apoptosis of neurodegeneration in brain [53]. Generally Aluminium an intoxicated rat causes increased apoptotic cells and *Bcl-2* positive cells in the brain cells. Aluminium induces cytochrome-C translocation and increased expressions of pro-apoptotic and antiapoptotic protein like *Bcl-2* were responsible for cell apoptosis [54,55]. The regulation of cell apoptosis may change due to over expression of *Bcl-xL* and *Bcl-2*. The level of *Bcl-2* protein increases responsible for dementia in Al induced toxicity animals [56]. The present study first time to report that *MMV* could protect brain cells by inhibiting neuronal apoptosis due to presence of Saponin and flavanoids decreased the expression of *Bcl-2* and *Bcl-xL* protein, mRNA in cerebral cortex, hippocampus of brain regions. In this result clearly indicates that *MMV* potentially inhibit neuronal cell death which improved cognitive functions against A1 induced animals. *Caspases* are important mediators of apoptosis and activate Al induced toxicity of neurodegenerative disease [54]. The regulation of *Caspas-3* act as a new pro-*Caspas-3* synthesis and synthesis of other proteins required for *Caspase-3* activation [54]. In this result further supported immunological expression of *Caspase-3* (plates) has shown that *MMV* treated rats to diminish the activation of *Caspas-3* in Aluminium induced toxicity animals. These results indicate that *MMV* could protect from interruption of cognitive functions of neurobehavioral changes in Al intoxicated animals.

Conclusion

The present study suggested *MMV* has a pivotal role of protection of primary neuronal apoptosis against Al intoxicated rats and profound alteration of *Bcl-xL, Bcl-2* and *Caspas-3* protein and mRNA expressions of hippocampus and cerebral cortex. Further target gene studies needed to determine possible role of *MMV* in cell survival.

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