Original Article

NEUROPROTECTIVE ACTIVITY OF FRACTIONAL FLOWER EXTRACTS OF *MIRABILIS JALAPA* AGAINST ALUMINIUM HYDROCHLORIDE INDUCED NEUROTOXICITY IN MALE WISTER RATS

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ABSTRACT

Objective: The major objective of this present study was to evaluate the neuroprotective effect of fractional flower extracts (acetone, petroleum ether, methanol and aqueous) of *Mirabilis jalapa* (MJ) against aluminium hydrochloride-induced neurotoxicity in male wister rats.

Methods: From the different fractional flower extracts of *Mirabilis jalapa* (MJ), two doses (250 and 500 mg/kg body weight) of each extract was initially selected and administered per orally 30 min prior to aluminium hydrochloride administration to the different animal groups once a day for a period of 45 d. Rat serum was collected from different animal groups on 1st, 15th, 30th and 45th days for estimation of marker enzymes, where a reduction in marker was observed. Animal was sacrificed by decapitation and the whole brain of rats was analyzed to estimate the levels of nitrite, thiobarbituric acid reactive substances (TBARS), superoxide dismutase (DOS), catalase, reduced glutathione and acetylcholinesterase (AchE).

Results: On the 9th day the Wister rats were sacrificed and cerebral cortex was removed. One-half of the cerebral cortex samples from different groups of Aluminium hydrochloride treated rats were stored in FAM mixture (40% formaldehyde, acetic acid and methanol in the ratio of 1:1:8) for histological analysis. From the study confirmed that dose of 250 and 500 mg/kg bwt of methanolic extract of *MJ* significantly (p<0.001) increases the reduced glutathione, superoxide dismutase and catalase level, whereas petroleum ether, acetone and aqueous fractional flower extracts of *MJ* significantly (p<0.01) decreases nitrite, TBARS and AchE levels of aluminium hydrochloride treated groups.

Conclusion: This result is indicating evidence for *Mirabilis jalapa* had a significant neuroprotective effect on aluminium hydrochloride-induced neurotoxicity and also supports by histopathological studies.

Keywords: *Mirabilis jalapa,* Memantine, Oxidative stress, Thiobarbituric acid reactive substances, Neuroprotective activity

INTRODUCTION

Mirabilis jalap (Four O' Clock flower or marvel of Peru) is a perennial herb or under a shrub belonging to the family Nyctaginaceae and it is the most commonly growing ornamental species of mirabilis found throughout india. The plant is decorative and a favourite garden plant with red, white, yellow, pink, purple and orange flowers. *Mirabilis jalapa and its different extract are used in* traditional medicine in various countries for the treatment of diarrhea, dysentery, conjunctivitis, edema, inflammation, swellings, abdominal colics and muscular pain, antibacterial, antiviral and antifungal etc [1]. Aqueous extract of *Mirabilis jalapa* increases the level of nitrites AchE, TBARS, reduction of glutathione (GSH), catalase and SOD (Superoxide dismutase) in rat cerebral cortex [2]. Aluminium hydrochloride metal is abundantly present in the earth crust and the environment it gets access to the human body via the gastrointestinal and the respiratory tracts. The daily intake of aluminium hydrochloride is estimated to be approximately 10-20 mg of cooking utensils, food additives, and medicine such as antacid or deodorants. Accumulation of aluminium hydrochloride in brain induces pathophysiology of neurodegenerative including Alzheimer's, dementia, amylotrophic, lateral sclerosis, Guam-Parkinson's dementia etc [3]. Since a variety of biomolecules are bound to aluminium hydrochloride so it can displace the other biological cations (calcium and magnesium) from their binding sites in every metabolic pathway and shows the adverse effect of aluminium hydrochloride of the potential target site. Therefore, aluminium hydrochloride neurotoxicity is not caused by a single alteration, but it is probably a result of adverse effects at multiple cellular levels. Aluminium hydrochloride interacts with the cholinergic projection function and intensifying the inflammation in the pathological process of Alzheimer's, diseases (AD) [4]. Aluminium hydrochloride salts in biological tissue do not have any direct pro-oxidant properties; in fact, it potentiates the ability of iron salts to promote reactive oxygen species formation (ROS) [5]. In addition, aluminium hydrochloride has been reported to enhance peroxidative damage to lipid, protein and decrease antioxidant enzymes status. Aluminium hydrochloride has a potential to get accumulated in specific brain regions, which were earlier correlated with the degenerative changes [6].

Enhancement of cholinergic neurotransmission represents an important target for treating adult-onset cognitive disorders memantine is uncompetitive NMDA receptor antagonist. Several studies have shown that the drug produces a dose-related effect on cognitive function that was correlated with the degree of acetylcholinesterase inhibition in the cerebrospinal fluid [7]. From reported literature P. Selvakumar *et al.,* performed a study on phytochemical screening and antioxidant activity of red flowered mirabilis jalapa leaf in different solvents. Keeping these things in mind the authors have examined the protective effect of *Mirabilis jalapa flower* extract on the impairment of cholinergic and anti-oxidant activity induced by aluminium hydrochloride in the cerebral cortex of male Wister rats. Apart from these histopathological studies is also undertaken to determine the morphological alteration in the cerebral cortex.

MATERIALS AND METHODS

Identification of plant collection

The flowers of *Mirabilis jalapa* was collected, identified and authenticated by Mr. M. R. Paul Satyakeerthi, head of the development, department of Botany of Andhra Christian College, Guntur, Andhra Pradesh, India. A voucher specimen (BSI/SC/5/23/09-10/Tech/1616) has been deposited at the Department of Botany of Andra Christian College, Guntur for species identification of *Mirabilis jalapa.*

Animals

Healthy male Wister rats strain weighing 250±20g was obtained from the Mahaveera enterprises, Hyderabad, India. Animals were allowed to acclimatize for at least 1 w prior to start experiments. The animals were housed in polypropylene cages under pathogen free at uniform conditions of light (12 h) and dark (12h) cycle at a temperature of 24±2 °C. They were fed with standard pellet diet (Hindustan Lever Limited, India). Their health status was checked frequently. All the experiment was performed in the morning and in accordance with the guidelines provided by the Institutional Animal Ethics Committee with approval number (Ethical Reg. Approval No. IAEC/SIMS 2014/001).

Chemicals and reagents

Mirabilis jalapa, memantine, aluminium hydrochloride chloride and sodium carboxymethyl cellulose (SD Fine Chemicals, Mumbai, India).

Preparation of different fractional extract of *Mirabilis jalapa* flowers

Flowers extract of *Mirabilis jalapa* was performed according to the method of the National Institute of Health and Family Welfare NIHFW, New Delhi. Flowers were collected from the plant, wash with water and dried under the shadow and made into coarse powder. The powder material was initially defatted with petroleum ether followed after 72h extractions, by the addition of acetone, petroleum ether, methanol and water respectively. The solvent eliminated by distillation under reduced pressure which produces sticky residue except for aqueous extract. The concentrated crude extracts were stored at 0-4 °C until used.

Experimental design

By using OECD guidelines [8] (organization of economic cooperation and development) for study. These are made by acute toxic class method [9]. In this, stepwise procedures with three animals of the same sex were taken. Depending on mortality and moribund status of the animals on the average 2 to 4 steps may be necessary to allow judgments on the acute toxicity of the test substance. This procedure results in the use of a minimal number of animals while allowing for acceptable data for a scientific conclusion. The method uses defined doses (100, 250, 500, 1000 and 2000 mg/kg bwt) and the results allow a substance to be ranked and classified according to the globally harmonised system (GHS) for the classification of chemical which causes acute toxicity. The methanolic extract of *Mirabilis jalapa* of different doses such as 100, 250, 500, 1000, 2000 mg/kg were given orally to mice. Body weight of rats before and after termination was noted and any change in skin, fur, eyes, mucous membrane and behaviour pattern were observed and also signs of tremors, salvations, diarrhoea, lethargy, sleep and coma were noted. The methanolic and aqueous extracts of MJ of two selected doses such as 250 and 500 mg/kg body weight were given orally to the Wister rats. Aluminium hydrochloride was run by gavage for 45 d. Gavage was performed using a syringe with a modified steel point to introduce the solution into the rat's esophagus without injuring the tissue. Rats were dividing into 11 groups such as group I [control], group II [test control, aluminium hydrochloride 100 mg/kg bwt/day], group III [standard control, received memantine (5 mg/kg bwt)] group IV [petroleum ether extract of MJ 250 mg/kg bwt], group V [petroleum ether extract of MJ 500 mg/kg bwt], group VI [acetone extract of MJ 250 mg/kg bwt], group VII [acetone extract of MJ 500 mg/kg bwt], group VIII [methanolic extract of MJ 250 mg/kg bwt], group IX [methanolic extract of MJ 500 mg/kg bwt], group X [aqueous extract of MJ 250 mg/kg bwt] and group XI [aqueous extract 500 mg/kg bwt for 45 d]. A careful record of body weight changes of all experiments groups was kept throughout the study. Rat serum was collected from different animal groups on 1st, 15th, 30th and 45th days for estimation of marker enzymes. The animal was weighed at the beginning of the experiment, then twice a week, and finally before sacrificing them.

Biochemical assessment

Animal brain from different groups was removed by decapitation and rinsed with an ice-cold isotonic saline solution. Brains were subsequently homogenized with ice-cold 0.1 mmol/l of phosphate buffer (pH 7.4). The homogenates (10%w/v) were then centrifuged at speed of 1000 rpm for 15 min and the resultant supernatant was gathered and used for the estimation of selected biochemical parameters by analysis method.

Estimation of nitrite

Indicator for the production of nitric oxide means the accumulation of nitrite in the resultant supernatant and the production of nitric oxide was determined by spectrophotometer using Geris reagent [0.1%N-(1-napththyl)-ethylenediamine dihydrochloride, 1% sulfanilamide and 5% phosphoric acid]. Equal volumes of the supernatant and the Geris reagent were incubated for 10 min at room temperature in the dark room. The absorbance was then measured at λ_{max} 540 nm using a spectrophotometer. The concentration of nitrite in the supernatant was established from a sodium nitrite standard curve and expressed as a percentage of control [10].

Estimation of TBARS

The determination of lipid peroxidation was established by TBARS as per the protocol as described by Esterbauer *et al.* 1990. Estimation of TBARS by mixing of homogenate with trichloroacetic acid (10%), thiobarbituric acid (0.6%) and heated in a boiling water bath at 400Cfor 25 min. TBARS was determined from the obtained absorbance data at λmax 535 nm. Results were reported as mol of TBARS per mg of protein [11].

Estimation of reduced glutathione

Estimation of reduced glutathione was determined as described by Ellman *et al.* At first 1 ml aliquot of supernatant was precipitated with 1 ml of 4% sulfosalicylic acid and cold digested for 1 h at 4 °C. The sample were then centrifuged at 1200 rpm for 15 min at 4 °C and from the 1 ml of the obtained supernatant were added 2.7 ml of phosphate buffer (0.1 mmol/l, pH-8) and 0.2 ml of 5, 5-dithio-bis-(2-nitrobenzoic acid) (DTNB). The yellow color developed was measured at λmax 412 nm using a spectrophotometer. Results were calculated using the molar extinction coefficient of the chromophore $(1.36 \times 10^4 \text{ mol/l/cm})$ [12].

Estimation of SOD

Estimation of superoxide dismutase (SOD) was established by the reported method of Kono *et al.*, 1978. Reduction of nitrazo blue tetrazolium (NBT) was inhibited by the superoxide dismutase and is measured. The assay system consists of EDTA 0.1Mm, sodium carbonate 50 mmol and 96 mmol of nitroazo blue tetrazolium (NBT). In the cuvette, 2 ml of the above mixture, 0.05 ml of hydroxylamine and 0.05 ml of supernatant was added and auto-oxidation of hydroxylamine was measured for 2 min at 30s interval by measuring absorbance at λmax 560 nm using perkin Elmer Lambda 20 spectrophotometer [12, 13].

Estimation of catalase activity

Catalase activity was assessed by the method of Luck *et al.*, wherein the breakdown of H_2O_2 is measured. Briefly, 3 ml of H_2O_2 phosphate buffer and 0.05 ml of the supernatant of the tissue homogenate for 2 min at 30-second intervals for λ_{max} 240 nm. The results were expressed as micromoles of hydrogen peroxide decomposed/ min/mg of protein [14].

Estimation of acetylcholinesterase (AchE) activity

AchE is a marker of the loss of cholinergic neurons in the forebrain. AchE activity was assessed by the Ellaman method. The assay mixture contained 0.05 ml of supernatant, 3 ml of sodium phosphate buffer (pH-8), 0.1 ml of acetylcholine iodide and 0.1 ml of DTNB. The change in absorbance was measured for 2 min at 30s intervals of λmax 412 nm. Results were reported in micromoles of acetylcholine iodide hydrolyzed/min mg of protein [15].

Statistical analysis

The biochemical assessment was analyzed by one way ANOVA post hoc comparisons between groups were made using Tukey's test, $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

The effect of fractional extracts (acetone, petroleum ether, methanolic and aqueous) of Mirabilis jalapa on serum marker enzymes ALT and AST was estimated. The marker enzymes AST and ALT serve as indicators and suggestive for a disturbance of the cellular integrity induced by pathological conditions.

These enzymes used as a sensitive marker for evaluation of the protective activity. AST and ALT level was significantly higher in aluminium hydrochloride treated rats as compared to the control group. Groups treated with petroleum ether-acetone, methanolic and aqueous flower extracts of MJ at 250 and 500 mg/kg bwt showed a decreased level of AST and ALT from the 30th day of treatment as compared to the aluminium hydrochloride treated groups respectively. This finding supports the protective effect of fractional extracts (petroleum ether, acetone, methanolic and aqueous) of Mirabilis jalapa have shown very significant neuroprotective against aluminium hydrochloride-induced neurotoxicity in rats in reducing serum AST and ALT level [16].

Effect of different fractional flower extracts of Mirabilis jalapa at doses of 250 and 500 mg/kg bwt and memantine dose (5 mg/kg) for estimation of various biochemical parameters such as reduced glutathione, catalase and superoxide dismutase etc in rat cerebral cortex are depicted in table 1.

Table 1: Effect of different fractional extracts of *Mirabilis jalapa* and memantine for estimation of various biochemical parameters in rat cerebral cortex

Treatments	Nitriteµmol/mg of protein	TBARS nano mol/mg of protein	Reduced GSH µmol/mg of protein	SODUnit/mg of protein	Catalases Unit/mg of protein	AchEµg/mg of protein
Control	125±3.2	0.12±0.07	70.6±1.28	17.01±9.8	4.28±2.4	0.65±1.02
Positive control	550.6±7.64	0.42±0.24	51.67±2.23	8.20±4.7	1.2±0.69	2.73±0.26
Memantine (5 mg/kg)	222±2.28	0.13±0.14[a]	80.12±2.82	18.14±1.6	4.71±0.3[a]	1.08±0.33
Acetone (250 mg/kg)	270±3.02	0.23±0.13	68.6±0.24	10.66±2.8	3.5±0.9	1.38±0.22
Acetone (500 mg/kg)	262.6±5.89	0.2±0.01[b]	70±0.36	11.66±1.3	3.63±1.2[b]	1.32±0.29
Petroleum ether (250 mg/kg)	285±5.78	0.32±0.18	60.0±1.12	9.2±5.8	3.21±1.8	1.47±0.54
Petroleum ether 500 mg/kg	277±7.63	0.28±0.10[b]	63.3±2.16	10.06±6.1	3.3±1.9[b]	1.4±0.05
Methanolic (250 mg/kg)	254±8.04	0.17±0.09	71.2±0.43	13.7±9.01	3.83±1.6	1.28±1.04
Methanolic (500 mg/kg)	245.6±6.67	0.15±0.05[b]	75.01±1.26	14.75±3.6	4.01±2.02[b]	1.2±0.87
Aqueous (250 mg/kg)	220.5±5.73	0.14±0.08	77±2.75	15.63±2.7	4.2±2.31	1.13±0.05
Aqueous (500 mg/kg)	221±5.02	0.133±0.07[a]	79.24±2.43	17.24±5.2	4.45±2.5[a]	1.03±0.24

Values are expressed as mean±SEM, [a]$P<0.001$, [b]$P<0.01$ compared to aluminium hydrochloride treated group (one-way ANOVA followed by turkey's test)

Treatment of aluminium hydrochloride showed a significant increase in activity of nitric oxide and TBARS levels (fig. 1 and 2), while superoxide dismutase is decreased as compared to control (fig. 3). The study has investigated the protective effect of fractional flower extracts (petroleum ether, acetone, methanol and aqueous) of *Mirabilis jalapa* (MJ) on aluminium hydrochloride-induced neuronal toxicity in the cerebral cortex of male wister rats treated for 45 d.

The cerebral cortex is chosen for a number of reasons (a) aluminium hydrochloride affects the hippocampus and cortex regions more severely than any other area of the central nervous system. (b) These brain regions are known to be particularly susceptible to Alzheimer's disease and have an important role in learning and memory functions [17]. Aluminium hydrochloride is a ubiquitous metal and has been involved in the etiology disorders and cognition dysfunction where it exacerbates brain oxidative damage causes neuronal inflammation and induces impairment in working memory. Aluminium hydrochloride was functionally altered the blood-brain barrier and produces a change in the cholinergic and noradrenergic neurotransmission [18]. It causes impaired glucose utilization, increased free-radical generation and lipid peroxidation as well as changes in phosphoinositide metabolism and protein phosphorylation, thereby causing severe neurotoxicity. The ability of aluminium hydrochloride to interfere with the downstream effect of molecules such as cyclic GMP involved in long-term potential [19].
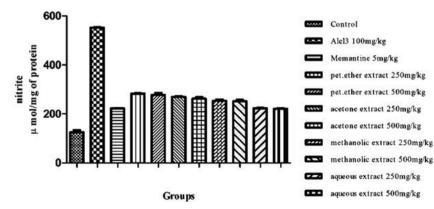

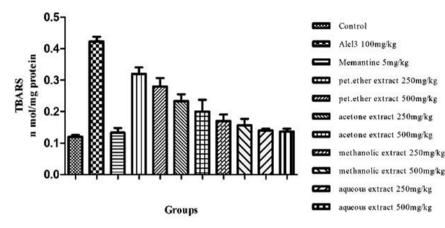**Fig. 1: Consolidated bar graph representation of MJ effects on nitric oxide**

Fig. 2: Consolidated bar graph representation of MJ effects on TBARS
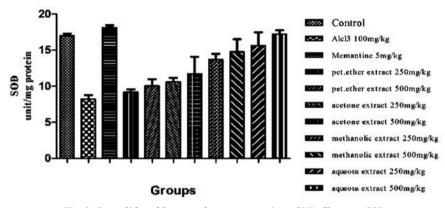

Fig. 3: Consolidated bar graph representation of MJ effects on SOD

This disruption could then explain the memory impairment and neurobehavioral deficits observed. Aluminium hydrochloride was previously considered to be a potent pro-oxidant known to enhance lipid peroxides in the cortex and hippocampus. It also targets mitochondria, causing the release of cytochrome and the activation of proapoptotic proteins like box and caspase-3, which trigger neuronal apoptotic death [20].

Memantine is a moderately, uncompetitive NMDA receptor antagonist by FDA to stabilize cognitive function and behavioural decline in several preclinical studies and in Alzheimer's disease (AD) patients with moderate to severe AD [21]. Memantine protects neurones from Aβ-induced toxicity and elicits the neuroprotective effects through multiple mechanisms and similarly NGF upregulation is one of the neuroprotective mechanisms for improving behavioural impairments and amyloidosis [22].

The study shows that the administration of memantine is able to prevent the changes in some oxidative stress parameters induced by aluminium hydrochloride in rats [23]. Under oxidative stress condition, SOD presents the first line of defence against superoxide as it dismutase the superoxide anion to H_2O_2 and O_2. Catalase protects SOD by converting H_2O_2 to water and oxygen. It is present in the peroxisomes of mammalian cells and probably serves to destroy H_2O_2 generated by oxidase enzymes located within these subcellular organelles because oxidative stress and cognitive dysfunction are strongly correlated, agents that modulate reactive oxygen species may be potentially useful as anti-dementia agents [16]. Glutathione in its reduced form is the most abundant intracellular antioxidant and it is involved in direct scavenging of free radicals or serving as a substrate for glutathione peroxides enzyme that catalyzes the detoxification of H_2O_2. It is also known that SOD and catalase are the two protective enzymes and shows function for the association of detoxification of the highly reactive free radicals [24]. Sychrounous administration of fractional extraction of (petroleum ether, acetone, methanolic, and aqueous) MJ and memantine (5 mg/kg bwt) during aluminium hydrochloride treatment prevented the decrease in the activity of catalases. Increased SOD activity associated with aluminum treatment was also significantly inhibited by MJ and memantine [25].

Intracellular GSH status is a sensitive indicator of the health of a cell or tissue and its content tends to decrease soon after aluminium hydrochloride treatment. Nitrite and TBARS level estimation play a vital role in determination of neuronal transmission. Treatment of aluminium hydrochloride chloride was found to cause a significant increase in activity of nitric oxide and TBARS levels. Studies have shown that catalases and AchE activity were altered significantly with aluminium hydrochloride treatment (fig. 4). Administration of aluminium hydrochloride caused a significant increase in the AchE activity whereas the activity of Na+/K+ATPase associated with aluminium hydrochloride treatment [26].

Another important factor which has been involved in influencing activities of choline acetyltransferase, acetylcholine (Ach) level and rate of its release and synthesis is nerve growth factor [27]. These effects may be mediated either by direct interactions of constituents of the extract with the cholinergic nerve terminal or trans-synaptically by mechanisms such as the modulation of amyloid precursor protein secretion at one side of the synaptic cleft, which in turn can result in activation of neighbouring cells and synaptic constituent.

Hence, suggesting another possible mechanism of protective action of MJ against aluminium hydrochloride-induced neurotoxicity. It is worth mentioning that the present work was performed based on the results obtained by jyoti *et al.*, keeping the dose of aluminium hydrochloride (per oral administration of 100 mg/kg bwt) and enhancing the time of exposure to 45 d [28].
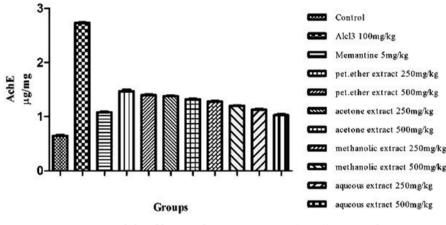
Fig. 4: Consolidated bar graph representation of MJ effects on AchE

Another reason for restricting two doses of Mirabilis jalapa and a single dose of aluminium hydrochloride is because we have to abide by the rules of the animal ethical committee and hence place a restriction on the number of animals used. The dosage of Mirabilis jalapa used in the present study has been standardised in our laboratory. The result of the present study suggests that MJ prevented aluminum-induced neurotoxicity in the cerebral cortex. Besides, inhibiting oxidative stress and histopathological alteration MJ showed promising results in normalising the altered activity/levels of protein at a cholinergic synapse induced by aluminium. From the histopathological studies, it is seen that control rat brain showing intact neuron, without any spongiosis (fig. 5A). Rat is exposed to aluminium hydrochloride 100 mg/kg showing perinuclear spaces, lipofusion and congestion in the blood vessel and prominently degenerated neuron (fig. 5B). Rat administrator to memantine 5 mg/kg showing no degenerated neuron and without spongiosis (fig. 5C). Petroleum ether extract of MJ showing the existence of degenerated neuron (fig. 5D). Acetone extract of MJ showing perinuclear spaces in the blood vessel. Methanolic extract of MJ showing no spongiosis with normal neuron and aqueous extract of MJ showing normalization of brain microstructural elements, normal neuron, intact nuclei, no spongiosis and degenerated neuron could be observed (fig. 5E). Thus, the selected extract is a possibility formulation for treating aluminium hydrochloride-induced neurotoxicity. Further studies are warranted to determine the active principle of these extracts and also determine the extract mechanism by which the phytochemicals exerts a neuroprotective effect.
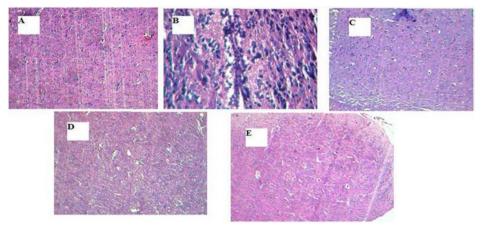

Fig. 5: Histological section of the brain was stained with haematoxylin and eosin. (A) Control rat brain showing intact neurons, without any spongiosis. (B) Rat exposed to 100 mg/kg/day AlCl$_3$ for 6 w with perinuclear spaces, lipofuscin deposits, congestion in the blood vessels and degenerated neurons and disrupted nuclei and oedema. (C) Rats administered memantine showed the existence of the perinuclear spaces. (D-E) administered TSEE showed normalization of the brain microstructural elements, with normal neurons and intact nucleus and astrocytes

CONCLUSION

On the basis of our result, it may conclude that higher doses (500 mg/kg bwt) of methanolic extraction of Mirabilis jalapa showed significant protection against aluminium hydrochloride-induced neurotoxicity. It may be concluded that neuroprotective effects of *Mirabilis jalapa* due to the presence of alkaloids, tannins and flavonoids in the flower extracts.

AUTHORS CONTRIBUTION

All the authors are equally contributed for this above research outcomes in the form of manuscript preparation by conducting research related to the animal work, acute toxicity, compilation, writing, copy editing of text etc.

LIST OF ABBREVIATION

AchE: Acetylcholinesterase, AD: Alzheimer's disease, ALT: Alanine transaminase, AST: Aspartate transaminase, MJ: *Mirabilis jalapa,* NBT: Nitroazo blue tetrazolium, SOD: Superoxide dismutase, TBARS: Thiobarbituric acid reactive substances.

CONFLICT OF INTERESTS

The author(s) declare(s) that they have no conflicts of interest

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