Pharmacogn. Mag.
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ORIGINAL ARTICLE

Protective Effect of *Pluchea lanceolata* against Aluminum Chloride-induced Neurotoxicity in Swiss Albino Mice

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Submitted: 07-04-2017 Revised: 17-05-2017 Published: 11-10-2017

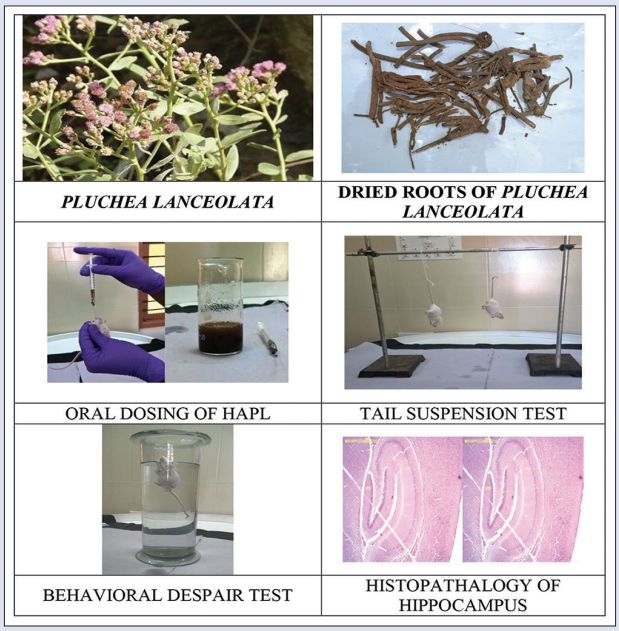
ABSTRACT

Background: Aluminum chloride ($AlCl_3$) is a known potent environmental neurotoxin causing progressive neurodegenerative changes in the brain. The herb *Pluchea lanceolata* is commonly known as "Rasana" and used as a nerve tonic in neuroinflammatory conditions in Indian system of medicine. **Objective:** To evaluate the neuroprotective activity of hydroalcoholic extract of *P. lanceolata* in chronic $AlCl_3$-induced neurotoxicity in Swiss albino mice. **Materials and Methods:** Albino mice were categorized into four different groups; Group 1served as vehicle control, Group 2 mice were administered with $AlCl_3$, 40 mg/kg body weight by intraperitoneal route for 45 consecutive days. Groups 3 and 4 mice were administered with $AlCl_3$, 40 mg/kg body weight intraperitoneal for 45 consecutive days along with hydroalcoholic extract of *P. lanceolata* at 200 and 400 mg/kg body weight. **Results:** Chronic administration of $AlCl_3$ resulted in behavioral deficits, triggered lipid peroxidation, increased acetylcholinesterase (AChE) activity, and histological alterations. Co-administration of hydroalcoholic extract of *P. lanceolata* attenuated many of the $AlCl_3$-induced alterations such as behavioral, lipid peroxidation, AChE, and histological changes of brain tissue. **Conclusion:** The results of the present study have demonstrated the protective role of hydroalcoholic extract of *P. lanceolata* against $AlCl_3$-induced neurotoxicity in Swiss albino mice. The neuroprotective efficacy of *P. lanceolata* can help reduce the symptoms caused by toxic protein aggregates in several degenerative diseases.

Key words: Acetylcholinesterase, aluminum chloride, lipid peroxidation, neurotoxicity, *Pluchea lanceolata*

SUMMARY

- The hydro alcoholic extract of *Pluchea lanceolata* showed neuroprotective activity in albino mice against $AlCl_3$ toxicity
- The benefits of *Pluchea lanceolata* against $AlCl_3$ toxicity includes reduced lipid peroxidation and acetylcholine esterase activity with improved behavioral functions
- The hydro alcoholic extract of *Pluchea lanceolata* rendered protection against $AlCl_3$ in forebrain, midbrain, cerebellum and hippocampus
- Therefore *Pluchea lanceolata* holds pharmacological potentials for treating diseases associated with neuronal toxicity.



Abbreviations used: HAPL: Hydro alcoholic extract of *Pluchea lanceolata*; CAT: Catalase; GSH-Px: Glutathione peroxidase; SOD: Superoxide dismutase; TBARS: Thio-barbituric acid reactive substances; MDA: Malondialdehyde; AChE: Acetylcholine esterase; AOT: Acute oral toxicity; CNS: Central nervous system; H_2O_2: Hydrogen peroxide; ML: molecular layer; GL: granular layer; MC: microcytic changes; BV: blood vessels; DG: dentate gyrus; PC: pyramidal cells; LD: Lethal dose; ANOVA: Analysis of variance; SEM: Standard error of mean; PCL: Pyramidal cell layer; OCL: Outer granular layer; BV: blood vessels; PM: Pia mater.

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DOI: 10.4103/pm.pm_124_17

Access this article online
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INTRODUCTION

Aluminum is a potent environmental neurotoxin that particularly interferes with several enzymes and proteins related to neurotoxicity leading to many cognitive diseases, especially Alzheimer's disease and Parkinson's disease.[1] Chronic aluminum accumulation can induce oxidative stress and pathological changes in vital areas of the brain. There are varieties of aluminum sources from where the human beings

Cite this article as: Mundugaru R, Sivanesan S, Udaykumar P, Rao N, Chandra N. Protective effect of *Pluchea lanceolata* against aluminum chloride-induced neurotoxicity in Swiss albino mice. Phcog Mag 2017;13:S567-72.

can expose routinely, such as diet, water purification process, antacids, vaccines, and cosmetic agents. The average dietary intake of aluminum in adults ranges from 3 to 12 mg/day. The population routinely exposed to different sources of aluminum may have higher chances of neurotoxicity.[2,3]

The possible mechanism of aluminum chloride ($AlCl_3$)-induced neurotoxicity involves severe oxidative stress followed by inflammatory changes leading to neurodegeneration. $AlCl_3$ is a nonredox active metal and capable of increasing the cellular oxidation by potentiating the prooxidant properties. Chronic aluminum exposure generates reactive oxygen species which in turn causes lipid peroxidation and decreased intracellular antioxidants. $AlCl_3$ on accumulation leads to affect the slow and fast axonal transport, induces inflammatory responses, and causes synaptic structural abnormalities, which results in progressive neurodegeneration. It is also reported that $AlCl_3$ can cause degeneration of cholinergic nerve terminals in cortical and hippocampus areas, leading to cellular depletion and severe learning disability.[4]

Plants are considered as rich sources of natural antioxidants, which alleviate the oxidative stress and improve cellular antioxidant status. *Pluchea lanceolata* (family-Asteraceae) popularly known as "Rasana" in Ayurveda is a rapidly spreading perennial rhizomatous weed and distributed throughout the northwestern part of India and neighboring Asian countries.[5] In Ayurveda, it is widely used in the treatment of rheumatoid arthritis, fever, pain, and inflammation. It is also used as a nerve tonic in conditions such as neuritis, sciatica, and chronic inflammation of nervous system.[6-8] It has been reported to possess anti-inflammatory, analgesic, immunosuppressant, and antimalarial activities.[9-12] The phytochemicals such as quercetin and isorhamnetin have been identified in *P. lanceolata*.[13] It has been reported that the flavanols derived from *P. lanceolata* possess significant antioxidant properties and well attenuated the cadmium chloride-induced oxidative stress and genotoxicity.[14]

Thus, development of potential neuroprotective drugs will be the effective strategy in the management of patients with neurodegenerative cognitive impairment disorders, and hence, there is a great demand for drugs which possess potent anti-inflammatory and antioxidant properties. In the present study, we have evaluated for the first time the neuroprotective activity of hydroalcoholic extract of *P. lanceolata* in $AlCl_3$-induced neurotoxicity in Swiss albino mice.

MATERIALS AND METHODS

Chemicals

Sodium dodecyl sulfate, thiobarbituric acid, glutathione standard, n-butanol, and pyridine were purchased from Himedia-Mumbai, India. Aluminum chloride hexahydrate ($AlCl_3.6H_2O$) was obtained from Thomas Baker Pvt. Chemicals, Mumbai, India. Acetylcholinesterase (AChE) kit was purchased from Piramal Healthcare Ltd. Thane, Mumbai, India. All other chemicals and reagents used in the present work were of analytical grade.

Plant material and extract preparation

The rhizome of *P. lanceolata* were procured from Jamnagar, India, during May 2015 and authenticated in Pharmacognosy Laboratory at SDM Centre for Research in Ayurveda and Allied Sciences, Udupi, Karnataka. The voucher specimen no. 15031401-02 has been deposited for future reference. The rhizome was shade dried and powdered at SDM Pharmacy, Udupi, with the help of pulverizer. The hydroalcoholic extract was prepared by soaking 500 g of powdered rhizome of *P. lanceolata* in 2 L of 50% ethanol and 50% cold distilled water for 24 h, filtered, and concentrated by evaporating on water bath till free from water.

Experimental animals

Male Swiss albino mice weighing 30–40 g body weight were obtained from animal house attached to Pharmacology and Toxicology Laboratory at SDM Centre for research in Ayurveda and Allied Sciences Udupi, India. The animals were maintained at standard laboratory conditions such as temperature at 25°C–27°C, humidity of 50%–55%, and natural light and dark cycles. Animals were fed with commercial pellet diet (*Pranav* Agro Industry, Pune) and water *ad libitum*.

Acute oral toxicity test

It was carried out as per OECD guidelines 425, using AOT software. The hydroalcoholic extract of rhizome of *Pluchea lanceolata* [HAPL] was made into a suspension in 0.5% carboxymethyl cellulose (CMC) and dosed in the following order: 175, 550, and 2000 mg/kg body weight. The animals were observed for 14 days for mortality. The LD_{50} was determined by AOT 425 software.

Experimental design

The mice were randomized into four different groups, each comprising six animals. Group 1 mice (vehicle control) were given 0.5% CMC orally for 45 consecutive days. Group 2 ($AlCl_3$ control) were treated with 40 mg/kg $AlCl_3$ (pH 7) intraperitoneally for 45 consecutive days.[15] Group 3 and 4 mice were treated with HAPL, 200 and 400 mg/kg body weight, respectively, for 45 consecutive days. The test drug was made as suspension in 0.5% CMC and administered at 1 ml/100 g body weight orally using gavage attached with syringe. The $AlCl_3$ (40 mg/kg body weight, pH 7) was administered intraperitoneally for Groups 3 and 4 for 45 consecutive days an hour after the HAPL treatment. At the end of the experimental period, animal behavior was evaluated. Animals were anesthetized, sacrificed, and serum separated from collected blood. The brain tissues were collected from each group. Three brain samples were stored in 10% formalin and used for histopathological studies, whereas remaining three brain samples were homogenized and used for biochemical investigations.

Behavioral assessment tests

Forced swim test

Animals were subjected to forced swim test on the last day of experimentation. An hour after giving the last dose of group-specific treatment, individual mouse was put into water filled (30 cm) glass cylinder measuring about 40 cm in height and 18 cm diameter, and observations were made for 6 min. First 2 min were not considered for recording the drug effect and were taken as stabilizing time. The limb movements and the effort of the mice to get out of the cylinder in the next 4 min were noted and subtracted later from total time (6 min) to find the time of immobility. This was considered as the index of depression.[16]

Tail suspension test

The total duration of immobility induced by tail suspension was measured according to a standard method. Mice isolated both acoustically and visually were suspended 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail. Immobility time was recorded during a 6-min test and the immobility time considered as an index of CNS depression.[17]

Estimation of serum acetylcholinesterase

The AChE was estimated by the method described by recommendation of the German society of clinical chemistry.[18] The AChE hydrolyses butyrylthiocholine under release of butyric acid and thiocholine. Thiocholine reduces yellow potassium hexacyanoferrate (III) to colorless

potassium hexacyanoferrate (II). The decrease in the absorbance was measured at 405 nm.

Preparation of brain homogenate

Brain was excised and cleaned with ice cold saline and stored at −20°C in freezer. Tissues were thawed and homogenized in phosphate buffered saline pH 7.4, centrifuged at 4000 rpm, and supernatant was stored at −20°C. The homogenate was subjected to determination of catalase, glutathione peroxidase activity, and lipid peroxidation.

Determination of catalase activity

The brain tissue homogenate (1 ml) was mixed with 5 ml of phosphate buffer and 4 ml of 0.2 M H_2O_2 in phosphate buffer and time was noted. Exactly after 180 s after adding H_2O_2, a set of 1 ml of reaction mixture from the above was taken in 2 ml dichromate acetic acid. It was kept in boiling water bath for 10 min, cooled all the tubes under running tap water, and finally noted the reading at 570 nm against reagent blank. Catalase activity in the tissue was expressed as micromoles H_2O_2 consumed/mg protein/min.[19]

Determination of lipid peroxidation

Lipid peroxidation activity was determined by measuring the content of the thiobarbituric acid reactive substances. The level of lipid peroxidation was expressed as millimoles of malondialdehyde formed/g wet tissue.[20]

Determination of glutathione peroxidase

Glutathione peroxidase was estimated using a standard protocol, and the glutathione peroxidase activity was expressed as micromolar glutathione utilized per mg protein per minute at 37°C.[21]

Brain histopathology

Three brain samples from each group were used, and ten slices per sample were examined for histopathological study. Immediately after the excision from mice, the brain tissue was transferred into 10% formalin. Sections of 5 µm thickness of brain tissue were prepared using microtome and stained with hematoxylin and eosin for microscopic observations.[22] All slides were then evaluated under light microscope (ZEISS Axio Lab A1 India).

Statistical analysis

The obtained data were expressed as mean ± standard error of mean and analyzed by one-way ANOVA, followed by Dunnett's multiple comparison *t*-test using GraphPad Prism 3. $P < 0.05$ was considered as statistically significant.

RESULTS

The acute toxicity study revealed no mortality with HAPL in any dose up to 2000 mg/kg body weight. The LD_{50} of HAPL is more than 2000 mg/kg. The dose taken for neuroprotective study was 1/10th and 1/5th of the higher dose of the study and found to be safe.

Effect of hydroalcoholic extract of *Pluchea lanceolata* on aluminum chloride-induced behavioral changes in behavioral despair test

In behavioral despair test, the duration of freezing time was significantly increased in $AlCl_3$ alone group as compared to vehicle control ($P < 0.01$). Co-administration of HAPL has significantly attenuated the freezing time at both dose levels as compared to $AlCl_3$ alone group ($P < 0.01$) [Table 1].

Table 1: Effect of hydroalcoholic extract of *Pluchea lanceolata* in behavioral despair test

Group	Duration of freezing time (s)
Vehicle control	23.5±3.81
$AlCl_3$ control	42.75±1.49$^{\#\#}$
HAPL (200 mg/kg)	10.6±1.9**
HAPL (400 mg/kg)	19.6±1.65**

Data expressed as mean±SEM, $^{\#\#}P<0.01$ in comparison to vehicle control group, $^{**}P<0.01$ in comparison to $AlCl_3$ group. HAPL: Hydroalcoholic extract of *Pluchea lanceolata*; $AlCl_3$: Aluminum chloride; SEM: Standard error of mean

Table 2: Effect of hydroalcoholic extract of *Pluchea lanceolata* in tail suspension test

Group	Duration of freezing time (s)
Vehicle control	49.8±6.16
$AlCl_3$ control	157.25±38.09$^{\#}$
HAPL (200 mg/kg)	93.2±16.2
HAPL (400 mg/kg)	113.2±41.93

Data expressed as mean±SEM, $^{\#}P<0.05$ in comparison to vehicle control group. HAPL: Hydroalcoholic extract of *Pluchea lanceolata*; $AlCl_3$: Aluminum chloride; SEM: Standard error of mean

Table 3: Effect of hydroalcoholic extract of *Pluchea lanceolata* on serum acetylcholinesterase activity

Group	Acetylcholinesterase (IU/L)
Vehicle control	3813.3±168.16
$AlCl_3$ control	5081.9±270.56$^{\#\#}$
HAPL (200 mg/kg)	4225.28±292.76
HAPL (400 mg/kg)	4945.82±226.18

Data expressed as mean±SEM, $^{\#\#}P<0.01$ in comparison to vehicle control group. HAPL: Hydroalcoholic extract of *Pluchea lanceolata*; $AlCl_3$: Aluminum chloride; SEM: Standard error of mean

Effect of hydroalcoholic extract of *Pluchea lanceolata* on aluminum chloride-induced behavioral changes in tail suspension test

The duration of immobility time was significantly increased in $AlCl_3$-treated mice as compared to vehicle control. Whereas co-administration of HAPL considerably attenuated the freezing time, however, the observed changes were not statistically significant as compared to $AlCl_3$ alone group [Table 2].

Effect of hydroalcoholic extract of *Pluchea lanceolata* on acetylcholinesterase

A significant increase in the level of AChE activity was observed in the animals treated with $AlCl_3$ as compared to vehicle control ($P < 0.01$). The HAPL co-administration with $AlCl_3$ considerably attenuated the $AlCl_3$-induced elevation in the AChE; however, the observed changes were not statistically significant as compared to $AlCl_3$ alone group [Table 3].

Effect of hydroalcoholic extract of *Pluchea lanceolata* on antioxidant parameters

In the present study, repeated administration of $AlCl_3$ significantly increased lipid peroxidation as compared to vehicle control ($P < 0.01$). The elevated lipid peroxidation was significantly attenuated at both the dose levels of HAPL as compared to $AlCl_3$ alone group. Whereas repeated administration of $AlCl_3$ caused marked oxidative stress, which led to decrease in the antioxidant enzymes activities such as catalase and glutathione peroxidase in comparison to control group mice; while

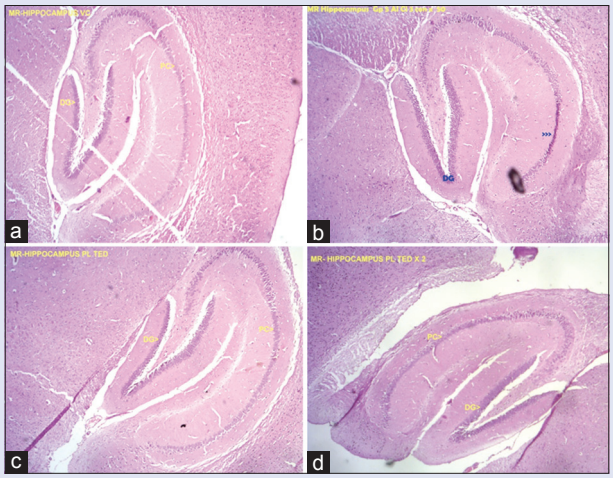

Figure 1: Photomicrograph of representative brain sections focused on hippocampus region of vehicle control (a), aluminum chloride group (b) and hydroalcoholic extract of rhizome of *Pluchea lanceolata* administered at 200 and 400 mg/kg + aluminum chloride (c and d). Aluminum chloride-administered group showed decrease in the pyramidal cells population, cellular disorganization, and distorted cells. These changes were considerably reversed by the administration of hydroalcoholic extract of rhizome of *Pluchea lanceolata* at higher dose level. DC: Dentate gyrus; PC: Pyramidal cells

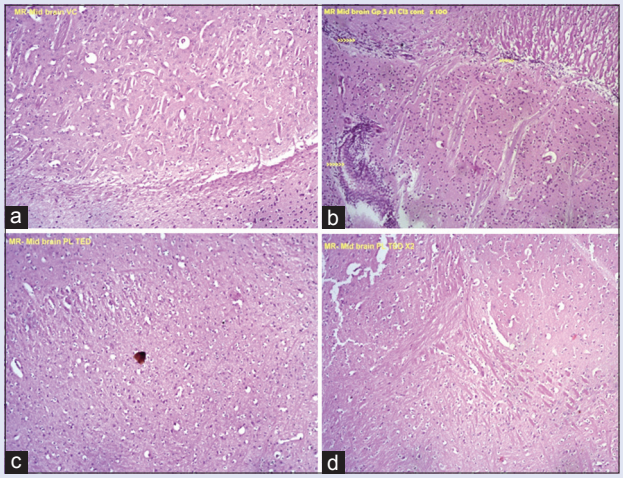

Figure 2: Photomicrograph of representative brain sections focused on midbrain region of vehicle control (a), aluminum chloride group (b) and hydroalcoholic extract of rhizome of *Pluchea lanceolata* administered at 200 and 400 mg/kg body weight + aluminum chloride (c and d). The midbrain sections from aluminum chloride group show edematous changes, cellular disorganization, sparsely cellular light staining areas, and cell distortion. The sections from hydroalcoholic extract of rhizome of *Pluchea lanceolata*-treated groups showed mild-to-moderate edematous changes and less cell infiltration

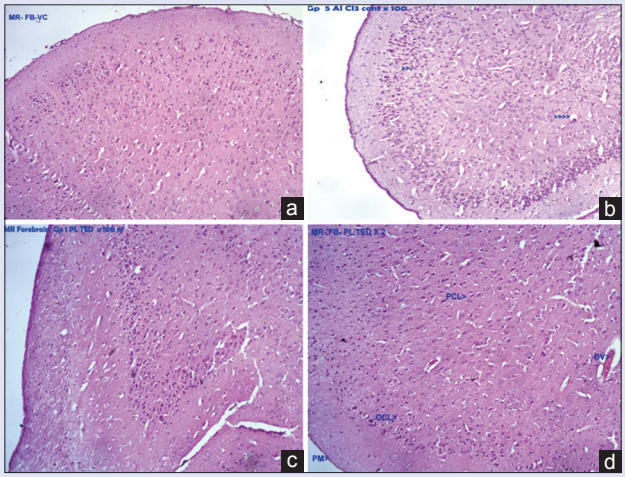

Figure 3: Photomicrograph of representative brain sections focused on forebrain region of vehicle control (a), aluminum chloride group (b), hydroalcoholic extract of rhizome of *Pluchea lanceolata* administered at 200 and 400 mg/kg body weight + aluminum chloride (c and d). The forebrain sections from aluminum chloride group showed edematous changes, cellular disorganization, and cell distortion. The sections from low-dose hydroalcoholic extract of rhizome of *Pluchea lanceolata* (200 mg/kg)-treated group exhibited edematous changes with cellular disorganization and distortion whereas high-dose hydroalcoholic extract of rhizome of *Pluchea lanceolata* (400 mg/kg)-treated group showed almost normal cytoarchitecture

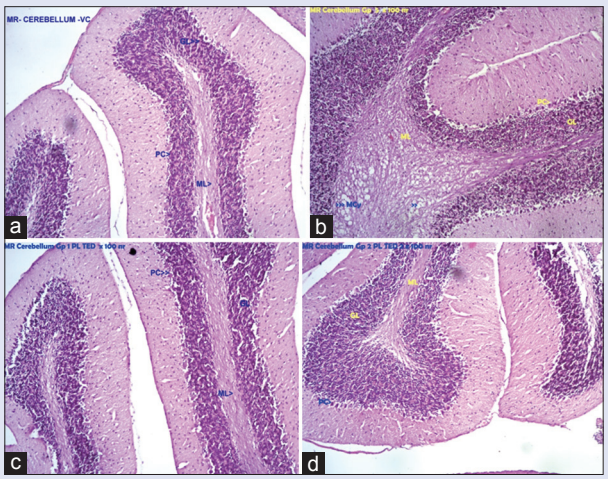

Figure 4: Photomicrograph of representative brain sections focused on cerebellum region of vehicle control (a), aluminum chloride group (b), and hydroalcoholic extract of rhizome of *Pluchea lanceolata* administered at 200 and 400 mg/kg body weight + aluminum chloride (c and d). The cerebellum sections from aluminum chloride group exhibited caused microcytic changes in the cellular layer of cerebellum. The sections from hydroalcoholic extract of rhizome of *Pluchea lanceolata* (200 and 400 mg/kg)-treated groups exhibited normal cytoarchitecture. ML: Molecular layer; GL: Granular layer; MC: Microcytic changes; BV: Blood vessels

HAPL co-administered with $AlCl_3$ has increased the activity of catalase and caused no significant changes in glutathione peroxidase levels as compared to $AlCl_3$ alone group [Table 4].

Histopathological changes

Chronic administration of $AlCl_3$ produced moderate intensity of neurodegeneration in different parts of the brain such as hippocampus, midbrain, forebrain, and cerebellum. In hippocampus, there is a decrease in the pyramidal cells population, with apparent cellular disorganization and distorted cells as compared to control group. The forebrain and midbrain sections of $AlCl_3$ alone group have shown edematous changes, cellular disorganization, and cell distortion. There are microcytic changes observed in the cellular layer of cerebellum, and these changes were attenuated to moderate extent by administration of HAPL at the higher dose level [Figures 1-4].

Table 4: Effect of hydroalcoholic extract of *Plantago lanceolata* on antioxidant parameters in aluminum chloride-induced neurotoxicity

Group	Catalase (μmoles/min/mg protein)	Glutathione peroxidase (μmoles glutathione/mg protein for 10 min)	Lipid peroxidation (mmoles of MDA formed/g wet tissue)
Vehicle control	6996.96±1078.1	3522.7±6.3	25.7621±318.2
$AlCl_3$ control	1373.31±10.7##	2325.92±788.1	404.165±39.8##
HAPL (200 mg/kg)	1256.16±129.5	1856.9±267.25	90.36±38.9**
HAPL (400 mg/kg)	8116.99±41.11**	3598.85±14.59	46.84±0.902**

Data expressed as mean±SEM, ##P<0.01 in comparison to vehicle control group, **P<0.01 in comparison to $AlCl_3$ group. HAPL: Hydroalcoholic extract of *Plantago lanceolata*; $AlCl_3$: Aluminum chloride; SEM: Standard error of mean; MDA: Malondialdehyde

DISCUSSION

Aluminum being an important environmental neurotoxin also acts as a prooxidant. On chronic exposure, it can cause severe oxidative stress in brain tissues. The oxidative stress can lead to neuroinflammation followed by neurodegenerative changes. It has been reported that the phytoconstituents such as curcumin, quercetin, naringin, and catechin have potential antioxidant properties and has shown potent neuroprotective activity in $AlCl_3$-induced neurotoxicity.[23,24]

P. lanceolata contains flavonoids such as quercetin and isorhamnetin and also chemicals such as sesquiterpenes, monoterpenes, and triterpenoids. These chemicals possess significant antioxidant and anti-inflammatory activities.[14,25,26] It has been reported that the decoction prepared from the *P. lanceolata* was used in pain and inflamed conditions such as arthritis. The taraxasterol derived from *P. lanceolata* has significant role in reducing neuroinflammation in C6 rat glial cells.[27] By extraction method, the fractions isolated from *P. lanceolata* were reported to have immunosuppressive properties by inhibiting the cytokines.[12] So far, there are no reports to substantiate the neuroprotective effect of *P. lanceolata* in $AlCl_3$ toxicity.

In the present study, two neurobehavioral tests were carried out to explore the degree of cognitive impairment and depressant component of the central nervous system. The chronic exposure to $AlCl_3$ significantly increased the freezing time in behavioral despair test and increased immobility time in tail suspension test. This indicates on repeated dose of $AlCl_3$ decreased motor activity, and CNS depression is evident. On the other hand, the co-administration of HAPL significantly attenuated $AlCl_3$-induced CNS depression.

Normal brain cells maintain intact antioxidant milieu of enzymatic and nonenzymatic mediators. The enzymatic components such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) and the nonenzymatic components such as glutathione, thioredoxin, and thiol-containing molecules play a major role in maintaining cellular integrity. SOD converts superoxide anions into H_2O_2 and oxygen, and it is neutralized by CAT and GSH-Px. Thus, the enzymes and indigenous antioxidant molecules protect the cells from damaging aggressive hydroxyl radicals.[28] In the present study, chronic exposure to $AlCl_3$ significantly increased the lipid peroxidation levels ($P < 0.01$) and significantly decreased the activity of CAT and moderately the GSH-Px, whereas these adverse effects were significantly ($P < 0.01$)attenuated by co-administration of HAPL, except for GSH-Px.

Acetylcholine is an important neurotransmitter in the cholinergic system, and AChE is an important enzyme involved in the metabolism of acetylcholine neurotransmitter. The increase in the AChE level in turn increases the metabolism of acetylcholine and leads to oxidative stress causing neurobehavioral changes, especially memory and cognitive failure. However, recently, it has been shown that circulating AChE activity reflects inflammatory response since acetylcholine suppresses inflammation. Based on this contention, donepezil, an AChE inhibitor, is being investigated for neuroprotective activity. In the present study, significant elevation in serum AChE activity was observed in $AlCl_3$-administered group in comparison to the control. This elevation was found to be moderately attenuated by HAPL drug treatment. This observation can be considered as an additional evidence for the neuroprotective activity indicating the role of *P. lanceolata* in the regulation of cholinergic function.[29]

The histological investigation also supports the pathological changes induced by chronic exposure of $AlCl_3$. The high-dose HAPL drug has shown considerable protective effect. $AlCl_3$-induced microcytic changes in the cellular layer of cerebellum and in forebrain sections with evidence of edematous changes, cellular disorganization, and cell distortion in few mice could be visualized. In the hippocampus, there was a decrease in the pyramidal cells population, cellular disorganization, and distorted cells. Thus, chronic administration of $AlCl_3$ produced moderate intensity of neurodegeneration in different parts of the brain such as forebrain, hippocampus, and cerebellum. The test drug administered at higher dose level attenuated the $AlCl_3$-induced histopathological changes from mild to moderate extent. These results support the protective role of HAPL in $AlCl_3$-induced neurotoxicity and improved functional outcome.

CONCLUSIONS

Based on the findings of the present work, it can be concluded that the hydroalcoholic extract of *P. lanceolata* exhibited neuroprotection in mice against $AlCl_3$ neurotoxicity. Our preliminary experiments using histological, behavioral, and biochemical analysis support this proof of concept and warrants deeper investigations in future using HAPL for gaining better pharmacological information and intervention.

Acknowledgements

The authors are highly grateful to Research Director and Dean, Saveetha University, Chennai, for their constant motivation to carry out this research work. The authors thank revered President Dr. D. Veerendra Heggade for his constant support, and Dr. B. Yashoverma, Secretary, SDM Educational Society and Dr. B. Ravishankar, Director, SDM Research Centre, Udupi, for their guidance.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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