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Research Article

Withania somnifera Ameliorates Sodium Valproate Induced Austism in BALB/c Mice: Behavioral and Biochemical Evidences

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Abstract

Objective: The objective of the present study was to evaluate the ameliorating effect of *Withania somnifera* on VPA induced autism in BALB/c mice. **Materials and Methods:** On Post Natal Day (PND), 12 young BALB/c mice were procured from five different litters and segregated into five groups (n = 6). Group I served as control group, received saline on PND 14. Group II served as normal treated, received *Withania somnifera* (200 mg kg^{-1}) from PND 13-40. Group III served as disease control, received VPA (400 mg kg^{-1} s.c.) on PND 14 and vehicle from PND 13-40. Group IV and V served as disease treated, received valproic acid (VPA-400 mg kg^{-1} s.c.) on PND 14 and *Withania somnifera* (100 and 200 mg kg^{-1} p.o.) from PND 13-40, respectively. All the experiments were preformed in the light phase between 09:00-15:00 h. During the experimental period, various behavioral parameters were evaluated. At the end of the experimental period, all the animals were sacrificed by cervical dislocation and brains were isolated for biochemical estimations and histo-pathological examination of cerebellum for purkinje cell integrity and cerebellar damage. **Results:** Induction of autism significantly altered behavioral and oxidative stress parameters and altered histo-architecture of cerebellum (decreased number of purkinje fibers, neuronal degeneration and chromatolysis) when compared with normal control group. Pre-treatment with *Withania somnifera* significantly improved behavioral alterations, altered oxidative stress markers and restored histo-architecture of cerebellum. **Conclusion:** *Withania somnifera* ameliorates sodium valporate induced autism in BAL B/C mice and is effective due to its anti-anxiety, anti-oxidant and neuro protective activity.

Key words: Autism, *Withania somnifera*, altered behavior

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Autism or Autistic Spectral Disorders (ASD) are a combination of impaired congenital neuro-developmental disorders; it is recognized by the following behavioral patterns i.e., incapability of social interaction and communication along with monotonous behavior[1]. According to the Centre of Disease Control (CDC), autism or ASD has been prevalent in 1 of every 68 children and it is also reported to be more common in boys (1 in 42) than in girls (1 in 189)[2]. Valproic acid (VPA) induced autism in rodents, such as rats and mice are an absolute model to evaluate the pathological conditions. The mice exposed to valproic acid on Post Natal Day (PND)-14th, showed the following alteration in their behavior characterized by increased latency to reorient on an inclined plane, decrease in the midair righting reflex, improper motor coordination, increase in locomotion, decrease in the nociceptive response and an increase in anxiety. The VPA triggers the formation of reactive oxygen species, which cause damage to the neurons causing an alternation in behavior[3]. Anti-oxidants with neuroprotective activity may show beneficial effects in autism. Vitamin E[4], green tea extract[5], *Bacopa monnerf*[6] and piperine[7] were reported for protective role in sodium valproate induced autism due to their antioxidant and neuro-protective activity. For many decades, there has been no definite treatment for ASD, present treatment mainly aimed at symptomatic relief; there is an imperative need to find a therapeutic drug.

Withania somnifera (solanaceae), commonly known as Indian ginseng or ashwagandha, is an important herb in the olden medical system of ayurveda. This plant possess many pharmacological activities such as; anti-oxidant, aphrodisiac, liver tonic, anti-tumor, anti-inflammation and anti-aging effect along with neuro-protective activity[8]. This plant grows widely in India, South Africa, Pakistan, Afghanistan, Egypt, Morocco, Jordan and Sri Lanka. The plant extract of the whole plant or specific parts, such as roots, stems or leaves are used.

Nonetheless, there has been no reported study on ameliorating effect of *Withania somnifera* on sodium valproate induced autism.

MATERIALS AND METHODS

Drugs and chemicals: Sodium valproate was purchased commercially available encorate injection (100 mg mL^{-1}). *Withania somnifera* aqueous root extract was procured from Surya herbal extract, Vijaywada, Andhra Pradesh, India. The n-butanol, neutral formalin, hydrochloric acid, EDTA and other salts for preparation of buffer were purchased from SD fine chemicals, orthophthalaldehyde was purchased from HIMEDIA. Ellman's reagent, griess reagent, thiobarbituric acid, vanadium III chlorides were purchased from Sigma Aldrich, USA.

Experimental animals: Female lactating BALB/c mice with pups of 4-6 days old were obtained from National centre for laboratory animal sciences, National Institute of Nutrition, Hyderabad, India. They were housed in rooms with environmentally controlled temperature (21±1°C) with a 12 h light and dark cycle. Free access of food and water were provided. The animals were acclimatized for 7 days and were randomly selected for different experimental groups. All such procedures and tests were carried out in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines. The study was reviewed and approved by the Institutional Animal Ethics Committee (320/CPCSEA dated 03-01-2001), G. Pulla Reddy College of Pharmacy, Mehdipatnam, Hyderabad, India.

Experimental design: On PND-13 the animals were divided into five groups (n = 6); Group 1 received vehicle, Group 2 received aqueous extract of *Withania somnifera* (200 mg kg^{-1}), Group 3 received vehicle and VPA (400 mg kg^{-1}, s.c.), Group 4 and 5 received orally WS 100 and 200 mg kg^{-1}, respectively and VPA. All the groups received respective treatment from PND-13 to 40 and single dose of VPA was administered on PND-14. All the experiments were done in the time period of 9-3 pm during the experimental period, negative geotaxis, mid air righting, motor co-ordination, locomotor activity, nociception and anxiety tests were performed. At the end of the experimental period, all the animals were sacrificed by cervical dislocation and the brains were isolated for biochemical estimations and histological evaluation of cerebellum.

Behavioral tests

Negative geotaxis: This parameter was done on their post natal day (PND 14-19). The mouse was placed on a grid wire facing down with an incline of 45°C[5]. The latency to turn 180°C such that the head was faced upwards along the incline was recorded with a maximum time of 30 sec for each trial[9].

Mid-air righting: Mid-air righting is the ability to right in the mid-air; it was done on their post natal day (PND 14- 17) by holding the neck of the mouse with the paws extended up

45 cm above the padded surface[5]. If the mouse has the ability to land on its four paws, it was scored positive for the ability to right. The best two, out of three successful mid-air righting attempts was recorded as ability to right[6].

Rota rod test for motor coordination: On their post natal day (PND 24- 26), the mice were placed individually on a rotating rod at a speed of 50 rpm[5]. The time taken by each mouse to maintain its balance on the rotating rod over a 5 min period was recorded[6].

Locomotor activity: The locomotor activity was measured by placing the animal in actophotometer (INCO Pvt. Ltd.) for five minutes[5,6].

Nociception: The Eddy hot plate (INCO Pvt. Ltd.) test was carried out using the method described previously at a fixed temperature of 55°C. The animals were placed on the hot plate and the latency was recorded from time between placement and licking of hind paws or jumping response[5,6].

Elevated plus maze test for anxiety: The plus maze is made out of wood and consists of two opposite open arms (25×5 cm) and top opposite arms enclosed with 15 cm high walls. The maze was elevated 50 cm above the floor and the behavior was tested in a dimly lit room with a 40 W bulb, hung over the centre of the maze. Each mouse is placed in the maze for 5 min in a pretest arena prior to exposure to the maze. Then the exposed mouse was placed in the central square of the maze. The number of entries into the open/closed arms and time spent in each of them was recorded[5,6,10].

Biochemical parameters

Determination of malondialdehyde (MDA) concentration: The lipid peroxidation levels were estimated through the determination of MDA concentration. This test is based on the reaction of MDA with thiobarbituric acid (TBA). Tissue homogenate (2%) of 500 µL in 0.15 mol L^{-1} KCl was mixed with 200 µL 80% SDS and incubated at room temperature for 5 min. Acetic acid (20%) of 1.5 mL at pH 3.5 and 1.5 mL of 0.8% thiobarbituric acid were added. The mixture was heated at 95°C for 90 min and later cooled. Distilled water was added along with 5 mL butanol pyridine^{-1} (15:1) solution under agitation using a vortex. This solution was centrifuged for 15 min and the resultant colored layer was separated and measured at 532nm using a spectrophotometer[7].

Determination of reduced glutathione (GSH) level: Reduced GSH levels in the brain were analyzed by Ellman's method[11]. Tissue homogenate (2%) was added in equal volume of 20% trichloro-acetic acid containing 1mM EDTA. The mixture was allowed to stand for 5 min and centrifugation was done at 200 rpm for 10 min. From the above, supernatant (200 µL) was transferred in test tubes and 1.8 mL of ellman's reagents was added. Then all the test tubes were made up to 2 mL. At the end of the reaction, solutions were measured at 412 nm against blank[6,12].

Determination of total nitrite levels: Nitrite levels were estimated to measure the production of nitric oxide using Griess reaction[11]. Tissue homogenate (2% and 100 µL) was taken and prepared in normal saline and to this 100 µL of ferric chloride and 100 µL of Griess reagent was added, mixed and incubated at 37°C for 3 min. Later, the absorbance was measured at 540 nm. Nitrate concentration was assessed from a standard curve[6,12,13].

Statistical analysis: Data expressed as Mean±SEM. All data were analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keuls test[7] as a *post hoc* to compare more than two groups by used Graph Pad Prism software (5.0 version) except mid-air righting, for which Fisher's Exact test[14] was used. The $p<0.05$ were considered as statistically significant.

RESULTS AND DISCUSSIONS

Behavioral tests

Negative geotactic behavior: As shown in Fig. 1a, administration of VPA significantly ($^{a}p<0.001$, $^{b}p<0.01$) increased the latency to reorient on an inclined plane throughout the experimental period, when compared to normal control group. Treatment with WS (100 and 200 mg kg^{-1}) significantly ($^{\alpha}p<0.001$) reduced the latency to reorient from 16-19 PND, when compared to VPA treated group. However, on PND 18 and 19, dose dependent actions were shown and it was restored to normal level on PND 19 in 200 mg kg^{-1} treated mice.

Mid-air righting reflex: As shown in Fig. 1b, administration of VPA significantly ($^{a}p<0.001$, $^{b}p<0.01$, $^{c}p<0.05$) decreased the midair righting reflex compared with control group was observed. Treatment with WS (100 and 200 mg kg^{-1}) significantly ($^{\alpha}p<0.001$) reduced the midair righting reflex during PND 14 -17, when compared to VPA treated group. The effects were shown to have dose dependent fashion on PND 14 and 16. However, both the doses are restored to normal level on PND 17.

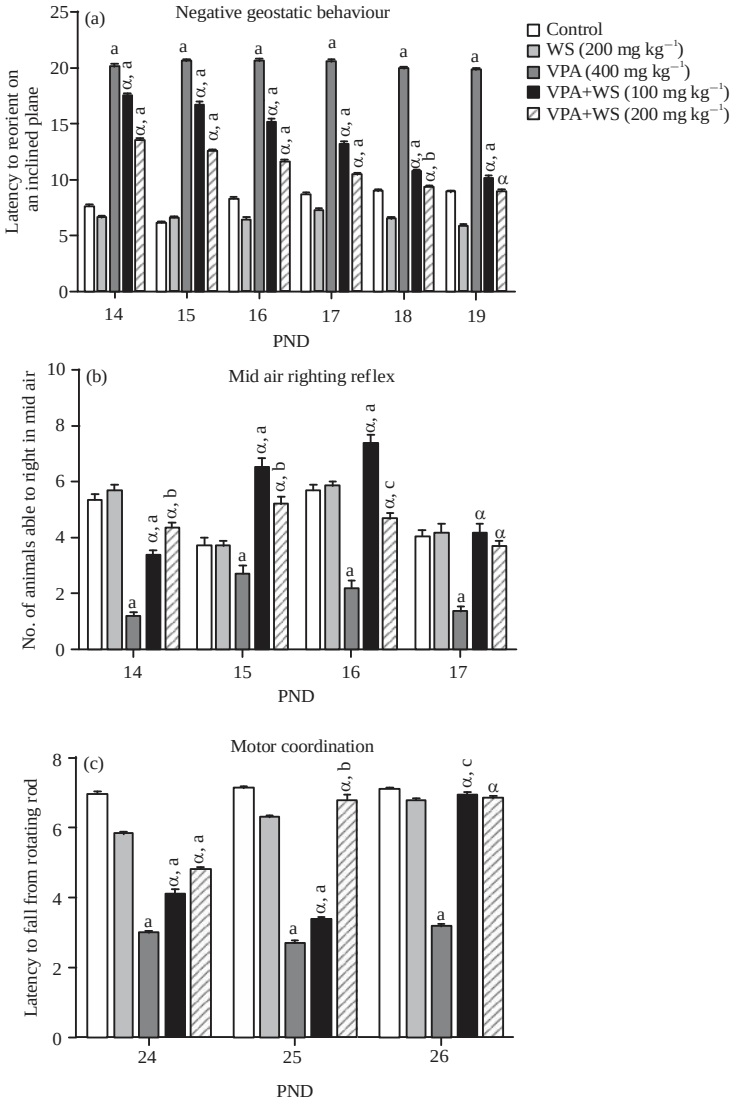

Fig. 1(a-c): Effect of *Withania somnifera* on sodium valproate induced, (a) Altered negative geotaxis, (b) Mid-air writing and (c) Motor coordination in BALB/c mice. Values are expressed as Mean±SEM. Newman-keuls (*post hoc*) test for negative geotaxis and motor coordination and Fisher's test for mid air writing were used to compare between groups. The $^{a}p<0.001$ and $^{b}p<0.01$ compared to VPA and $^{a}p<0.001$ and $^{b}p<0.01$ compared to control

Motor co-ordination: In Fig. 1c, administration of VPA significantly ($^{a}p<0.001$, $^{b}p<0.01$ and $^{c}p<0.05$) increased in the latency to fall from Rota rod compared with the control group was observed. Treatment with WS (100 and 200 mg kg^{-1}) significantly ($^{\alpha}p<0.001$) reduced the latency to restored to normal levels in higher dose on PND 26.

Locomotor activity: Figure 2a shows that administration of VPA, significantly ($^{a}p<0.001$, $^{b}p<0.01$ and $^{c}p<0.05$) increased in the locomotion in compared with control group was observed. Pre-treatment with WS (100 and 200 mg kg^{-1}) significantly ($^{\alpha}p<0.001$) reduced the locomotion from PND 34-37 when compared to VPA treated group. However, it was restored to normal levels in higher dose on PND 36 and 37.

Nociceptive response: The decreased nociceptive response is mainly due to the damage caused to sensory pathways by VPA. Figure 2b shows that administration of VPA significantly ($^{a}p<0.001$, $^{b}p<0.01$ and $^{c}p<0.05$) increased the latency to jump or paw lick throughout the experimental period, when compared to normal control group. Treatment with WS (100 and 200 mg kg^{-1}) significantly ($^{\alpha}p<0.001$) reduced the

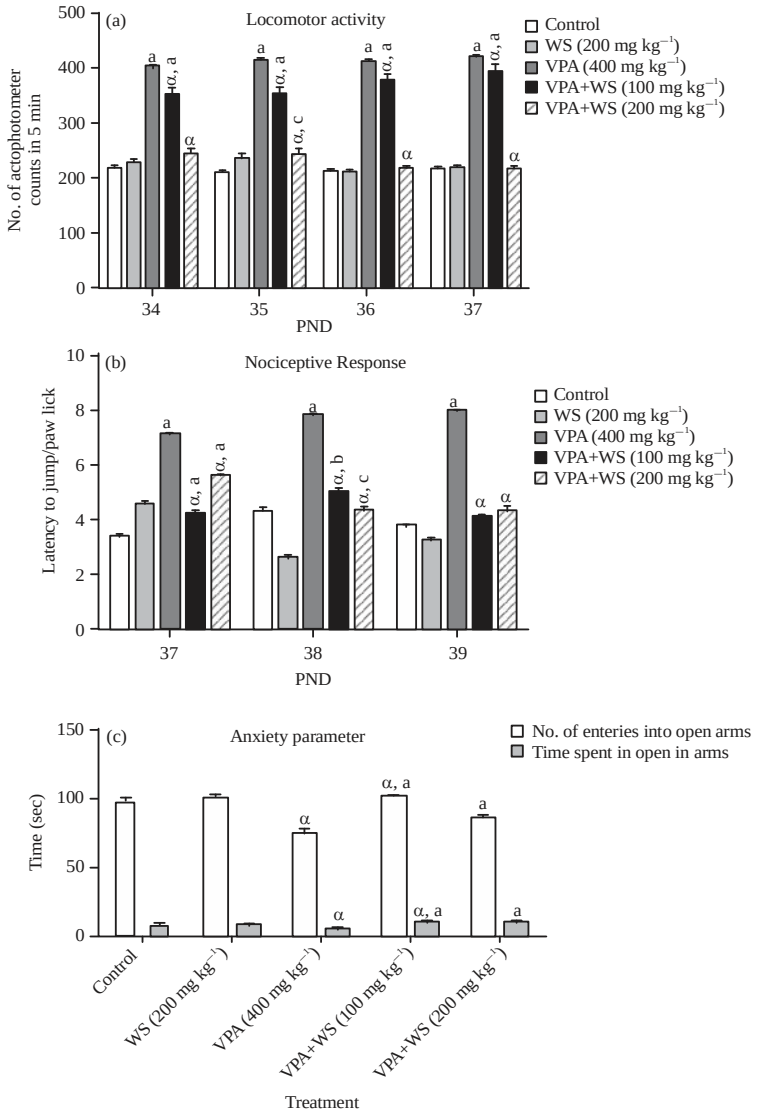

Fig. 2(a-c): Effect of *Withania somnifera* on sodium valproate induced, (a) Locomotor activity, (b) Nociceptive response and (c) Anxiety parameters in BALB/c mice. Values are expressed as Mean±SEM. Newman-keuls (*post hoc*) test was used to compare between groups. The [a]p<0.001 and[b] p<0.01 compared to VPA and [a]p<0.001 and [b]p<0.01 compared to control

latency to reorient to jump or paw lick from PND 37-39, when compared to VPA treated group. However, it was restored to normal levels in higher does on PND 38 and 39.

Anxiety on elevated plus maze test: In Fig. 2c, induction of autism significantly ([a]p<0.001, [b]p<0.01 and [c]p<0.05) decreased time spent and number of entries in open arm compared to control group. Treatment with WS (200 mg kg^{-1}) in VPA treated animals showed a moderate increase in number of entries and time spent in open arms and did not restore to normal. Whereas, treatment with WS (200 mg kg^{-1}) has shown significant ([α]p<0.001) increase in both number of entries and time spent in open arms, when compared to disease control and restored to normal.

Biochemical parameters

Effect of *Withania somnifera* on malondialdehyde (MDA) level in VPA induced autistic mice: As shown in Fig. 3a, VPA treated mice exhibited high MDA levels compared with the control ([a]p<0.001, [b]p<0.01 and [c]p<0.05). Pre-treatment with WS (200 mg kg^{-1}) has shown significantly ([α]p<0.001 and [β]p<0.01) reduced MDA levels on PND 40 compared to VPA

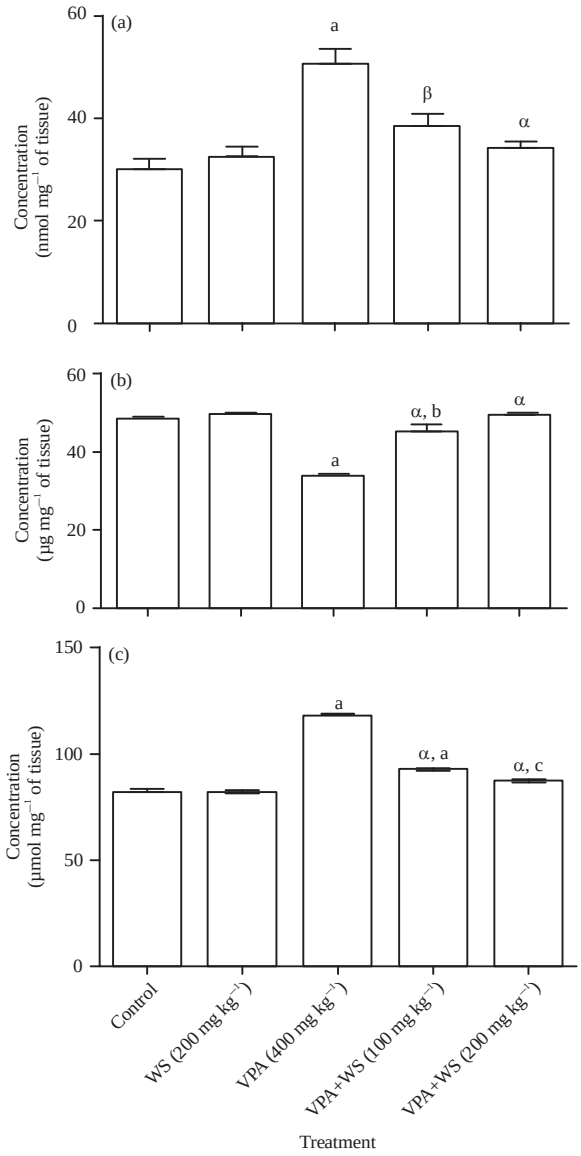

Fig. 3(a-c): Effect of *Withania somnifera* on sodium valproate induced levels of (a) MDA, (b) GSH and (c) Nitrite in BALB/c mice. Values are expressed as Mean±SEM. Newman-keuls (*post hoc*) test for negative geotaxis and motor coordination and Fisher's test for mid air writing were used to compare between groups. The $^{a}p<0.001$ and $^{b}p<0.01$ compared to VPA, $^{a}p<0.001$ and $^{b}p<0.01$ compared to control. All the values are expressed in Mean±SEM and were analyzed used one-way ANOVA and Newman-keuls *post hoc* test for comparison between groups

treated group. There was no significant difference observed in MDA levels among normal mice treated with WS compared to control.

Effect of *Withania somnifera* on reduced glutathione levels (GSH) in VPA induced autistic mice: As shown in Fig. 3b, VPA treated mice exhibited significantly ($^{a}p<0.001$, $^{b}p<0.01$ and $^{c}p<0.05$) decreased GSH levels compared to control group. Treatment with WS (100 mg kg^{-1}) has shown moderate protective effect, whereas, treatment with WS (200 mg kg^{-1}), significantly increased ($^{\alpha}p<0.001$, $^{\beta}p<0.01$) glutathione levels compared to VPA mice and it was restored to normal level. There was no significant difference observed in reduced glutathione levels among WS alone treated group and control group.

Effect of *Withania somnifera* on total nitrite levels in VPA induced autistic mice: As shown in Fig. 3c, VPA treated mice

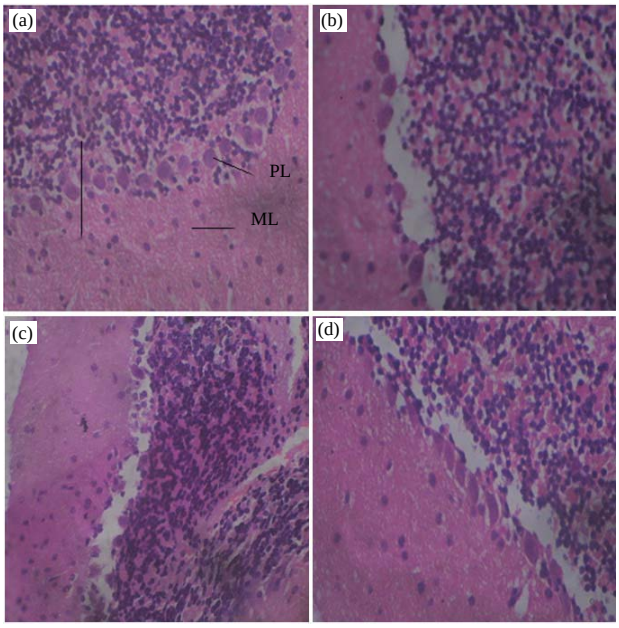

Fig. 4(a-d): Microscopic examination of sections of the cerebellum using H and E 200x (a) NC, (b) VPA, (c) VPA+WS (100mg kg^{-1}) and (d) VPA+WS (200 mg kg^{-1}). The GL depicts the granular cell layer of cerebellum, PL indicates the purkinje cell layer of the cerebellum and ML depicts the molecular layer of cerebellum

has shown increased ($^{a}p<0.001$, $^{b}p<0.01$ and $^{c}p<0.05$) total nitrite levels compared to control animals. Treatment with WS (100 and 200 mg kg^{-1}) has shown significantly ($^{\alpha}p<0.001$ and $^{\beta}p<0.01$) decreased total nitrite levels, when compared to VPA treated mice and these changes where shown dose dependent fashion.

Histopathology: Histo-pathological findings of cerebellum revealed intact purkinjee layers and cells in normal group as shown in Fig. 4a. The VPA treated rats had shown pathological findings like diminished number of purkinje cells and altered structure (Fig. 4b). Treatment with *Withania somnifera* ameliorated VPA induced histopathological alterations (Fig. 4c, d).

DISCUSSION

Autism or autistic spectral disorders currently are the most ignorant and highly misunderstood CNS disorder in underdeveloped countries. Research work has been carried on the etiology and finding a permanent cure for ASD in developed countries like USA, UK, Australia etc., which have shown positive results and stood as an encouragement to most of the budding researchers.

In the present study, pre-treatment with *Withania somnifera* was demonstrated to combat the VPA induced behavioral alterations and oxidative parameters. In accordance with the previous reports, administration of VPA on PND 14 developed autism characterized by behavioral aberrations like decreased sensitivity to pain, delayed negative geotaxis response, increased locomotor activity, increased anxiety behavior[15,16,17], alter oxidative stress parameters and alter integrity of purkinje cells in cerebellum[18].

Negative geotaxis is one of the most widely exploited tests to evaluate the ability of the infant mice to perform the reflexive negative geotaxis response. It is established that negative geotaxis determines the neuro-motor coordination and vestibular function, controlled by cerebellum, which are developed in the first three weeks of life. Increase in time taken to reorient on the inclined plane in the VPA treated mice maybe due to the deleterious effects of VPA on cerebellar development[9]. Pre-treatment with *Withania somnifera* has decreased the reorientation time from PND 17 onwards. This would be related to neuro-protective of *Withania somnifera* to combat the cerebellar damage induced by VPA[8].

Mid air righting reflex appears on PND 13 and completely matured on PND 14 indicating the cerebellar and general muscular maturation[4]. In consistent with the previous reports, VPA treated mice showed a regression in mid air righting from PND 15 indicates cerebellar damage[17]. However, pre-treatment with *Withania somnifera* eliminated the regression to right in mid air due to it neuro-protective effect[8,19].

Motor clumsiness is one of the variable or associated symptoms in autism, which can be tested by using the rota rod motor coordination and balance. In agreement with the previous results, VPA mice have decreased latency to fall from rotating rod, which can be attributed to cerebellar damage caused due to post natal exposure to VPA. Performances of these symptoms in present study were observed using rotating rod for motor clumsiness[4]. Upon pre-treatment with *Withania somnifera*, there was an increase in latency, indicating the protecting of *Withania somnifera* towards the cerebellar tissue from injury[19].

An increase in locomotor behavior in novel environment is normally observed in VPA induced mice. Increased motor activity in actophotometer is mainly due to the hyperactivity caused by the increase in the glutaminergic transmission[20]. Pretreatment with *Withania somnifera* restored the animals to normal locomotion due to its protective effect against glutamate toxicity[19].

Idiosyncratic responses to sensory stimuli are associated symptom in autism and the hypothesized analogous test for mice is Eddy's hot plate test. Perception of pain is due to activation and transmission through afferent and sensory networks[21]. In accordance with previous reports, there was an increase of pain threshold in VPA treated mice. Decreased nociceptive response is mainly due to the damage caused to sensory pathway by VPA[16,10]. Pre-treatment with *Withania somnifera* significantly decreases the pain threshold in VPA treated mice.

It has been well established that anxiety is one for the variable clinical symptom in autistic subjects and the screening of this behavioral challenge was done by subjecting the animals to elevated plus maze test. It is predicted that overactive and plastic amygdala producing enhanced anxiety and fear leading to social withdrawal may be due to impairments in the inhibitory system of amygdale or due to increase in glutaminergic transmission leading to neural hyper-excitability in certain brain region[18]. Pre-treatment with *Withania somnifera* to VPA mice decreases anxiety due to its protective role on amygdale and inhibition of glutamate in neuro-terminals.

Under condition of severe oxidative stress, free radical generation lead to elevated levels of MDA and nitrite and decreased levels of GSH. This results in the interference with the neuro-developmental process, in other cases it can cause neural damage resulting in abnormal behavioral process[6,22]. In the current study, pre-treatment with *Withania somnifera* significantly alters the oxidative stress marker (MDA, NO, GSH etc.,) hence, proving its antioxidant activity[8].

Histo-pathological studies showed that VPA administration damaged purkinje cell layer and also caused granule cell death in cerebellum[23]. Purkinje cell layer integrity was restored with *Withania somnifera* pre-treatment indicating neuro-protective action.

CONCLUSION

In conclusion, the aqueous extract of *Withania somnifera* has potential activity for ameliorating the VPA induced behavioral deficits. In a way, the active constituents responsible for this activity are alkaloids (isopelletierine and anferine), steroidal lactones (withanolides and withaferins) and saponins[23]. The above constituents are responsible for the ameliorating effect for the abnormal behavior along with the altered oxidative stress markers. Evidence obtained from this study is encouraging enough to warrant further study on the aqueous extract of *Withania somnifera* to find out the mechanism of action of the plant's chemical constituents and also to carry out research for the definite etiology for ASD.

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