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Research Article
Protective Effects of Luteolin on a Rat Model of Autism: An Analysis of Luteolin Flavonoid's Effects on Rat Behaviour, Histology and Cerebellar Pathology

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Abstract

Background and Objective: Autism Spectrum Disorder (ASD) is a neurological condition characterized by impaired social communication and repetitive behaviours. Its causes are complex and involve genetic and environmental factors. Luteolin is a natural flavonoid found in plants and is known for its potential antioxidant and anti-inflammatory effects. The purpose of this study was to investigate how Luteolin affects autistic-like behaviours caused by sodium valproic acid (SVP) injection, as well as any potential contributions from oxidative stress and histopathological alterations in rat models of ASD. **Materials and Methods:** In this study, 30 newborn rat offspring (10 for each group) were examined to determine the effects of valproic acid on the rat cerebellum. Pregnant Wistar females were treated with SVP on the 12.5th gestational day and their offspring were treated with SVP on postnatal day 14. The control group was treated with saline on the same days. The experimental group received Luteolin 20 mg/kg of body weight by gastric gavage from postnatal day 14 to 45. Autism-like behaviours were evaluated using the elevated plus-maze and rotarod tests. On postnatal day 45, the cerebellum of all pups was removed and prepared for light and biochemical analysis of oxidative stress markers and the pro-inflammatory cytokine Interleukin 17 (IL-17) in cerebellar tissue homogenate. **Results:** The SVP caused a significant decrease in neurobehavioral evaluations and was associated with elevated malondialdehyde levels and impaired glutathione peroxidase in the cerebellar tissues. The expression of IL-17 in the cerebellar cortex also increased significantly after the SVP injection. Luteolin treatment significantly improved neurobehavioral alterations, oxidative stress, pro-inflammatory cytokine and cerebellar histological structures. **Conclusion:** This animal model of autism demonstrated that Luteolin has neuroprotective properties, suggesting that it may be a beneficial therapeutic agent for treating Autism Spectrum Disorders (ASDs).

Key words: Sodium valproic acid, autism, Luteolin, cerebellum, rats behaviour

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Competing Interest: The authors have declared that no competing interest exists.

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

INTRODUCTION

Autism and Autism Spectrum Disorders (ASD) are a diverse group of neurodevelopmental conditions that manifest themselves in early childhood (typically between the ages of 2 and 10 years old) and are distinguished by physical (neurological function and pathology) and behavioural (social interaction) abnormalities[1,2]. While there is some overlap in the behavioural signs of ASD, the disorder's pathophysiology is still poorly understood and the causes are highly variable. Genetic and environmental factors, as well as their interplay, raise a child's risk of developing ASD[3-5]. The maternal immune system is supposed to play a role in the aetiology of ASD by causing atypical fetal brain development and a variety of congenital malformations if exposed to stress, toxins, drugs, environmental pollutants, or maternal infections during early embryonic life or shortly after birth[6,7].

The cerebellum, a brain region that was initially associated with motor tasks, is now believed to be involved in cognitive processes as well, owing to its connections with other brain areas such as the thalamus and the cerebral cortex[8,9]. Additionally, researchers have found that the cerebellum plays a significant role in psychiatric diseases like depression[10], schizophrenia[11] and autism spectrum disorder[8,9]. Post-mortem analyses of brains of individuals with ASD have revealed cerebellar neuropathology[12]. For instance, cerebellar-related motor abnormalities[13,14] are common in children with ASD and might present at a young age, before the beginning of language or social difficulties[15,16]. The potential role of motor dysfunction in the early identification of ASD is a topic of much debate at present[17-20]. A meta-analysis found that a mother's infection during pregnancy was linked to a 12% higher incidence of ASD in her child[21]. Another meta-analysis found that if maternal infections could be avoided or treated appropriately, it would cut ASD rates by 13-17%[22]. Furthermore, maternal immune activation due to COVID-19 infection during pregnancy can increase the risk of ASD onset by altering immune responses of both mother and fetus[23,24]. Therefore, understanding the role of inflammation and oxidative stress in the development of ASD is crucial.

The treatment of Autism Spectrum Disorder (ASD) is difficult as it is often identified at a late stage and there is a widely held belief that even minor changes to the developing neurological system can have permanent effects. However, there is increasing evidence to suggest that certain substances, such as those found in *Bacopa monnieri* plant extracts[25], green tea[26] and piperine[27], which possess antioxidant and neuroprotective properties, can help to reduce the appearance of autistic-like behaviours in response to Sodium salt of valproic acid (SVP).

Luteolin, also known as 3, 4, 5,7-tetrahydroxyflavone, is a type of flavonoid that has potent antioxidant properties[28,29]. It has been labeled as a neuroprotective chemical due to its ability to exhibit antioxidant and anti-inflammatory actions[30]. Studies have shown that Luteolin has anti-inflammatory and neuroprotective properties, which make it beneficial in age-related neurodegenerative illnesses such as Alzheimer's disease, Parkinson's disease, diabetes-associated cognitive decline and traumatic brain injury[31,32]. Additionally, Luteolin is a powerful free-radical scavenger that helps reduce oxidative stress on living systems[33].

Therefore, the present study aimed to investigate the effects of Luteolin on autistic-like behaviors induced by SVP injection in rats. The study will take into account the potential roles of oxidative stress and histological abnormalities.

MATERIALS AND METHODS

Study area: The research was carried out at the Animal Facilities of the Animal House, Faculty of Medicine, King Abdul-Aziz University, Jeddah, Saudi Arabia, under the control of the King Fahd Medical Research Center. The study took place between June, 2023 and October, 2023.

Ethical consideration: Approval for this research was secured from the institution's Bioethics and Research Committee with the reference number (No.: 442-36-7263).

ASD *in vivo* rat model: From the Laboratory Animal Center in Jeddah, Saudi Arabia, 30 pregnant Wistar rats obtained aged 12 to 15 weeks weighing from 210 to 250 g. The animals had unrestricted access to regular chow and running water. Every effort was made to keep the animals' living conditions clean and normal. According to the study by Gąssowska-Dobrowolska *et al.*[34] found that the ASD model was produced by a single intraperitoneal injection (i.p.) injection of 450 mg/kg of body weight (BW) of the sodium salt of valproic acid (SVP) (Valproic acid, sodium salt, Abcam, Cat.: 120745) in 0.9% saline (100 mg/mL, pH 7.3), (ASD model and Luteolin treated groups)[34]. The control group of pregnant women was given a single intravenous injection of a solvent (sterile 0.9% NaCl). All pregnant dams were given unrestricted access to food and water and housed in a birthing room kept at a constant temperature in accordance with the LD 12/12 schedule. All mothers were free to bear and care for their young as nature intended. Postnatal day (PND) 0 was defined as the day of birth. Male and female pups stayed with their

mothers. Only males were chosen for future experimental procedures to prevent interference from the hormonal disturbances/changes. All the animals were randomly housed in groups of 5, each cage having a light/dark cycle of 12 hrs. On day one of the experiment, the newborn rats weighed between 20 and 40 g. On postnatal day 14, some pups were given subcutaneous injections of SVP dissolved in 0.9% saline at 400 mg/kg, whereas others were given saline as a control[35].

Experimental design: Thirty male offspring of adult rats were used in the study and they were randomly split into three groups:

Group I (control group): Ten offspring were equally subdivided into:

- **Subgroup Ia (negative control):** Were kept without any treatment to PND 45
- **Subgroup Ib (positive control):** Received vehicle once subcutaneously at PND 14, then received Luteolin daily by gastric lavage to PND 45

Group II (ASD group): As 10 offspring rats received SVP at PND 14 and were kept without any treatment from PND 14 to 45.

Group III (treated group): The ten offspring rats received SVP at PND 14 and then were treated with Luteolin (Sigma Chemical Co., Missouri, USA, Cat.: 491-70-3) orally at a dose of 20 mg/kg b.wt., by gastric gavage from PND 14 to 45.

Neurobehavioral testing

Elevated plus-maze test: Anxiety can be a significant concern for people with autism, even though it is not the primary clinical manifestation of the condition[35,36]. To measure anxiety levels in a study, the raised plus-maze test was used. This test consisted of a maze with two open arms (50 cm in length, 12 cm in width, 30 cm in height) and two closed arms (50 cm in length, 12 cm in width, 30 cm in height). Each rat was given five minutes to explore the maze after being placed in the hub with its back to an open arm. The equipment was disinfected with 70% ethanol after each round of animal testing. Time spent in the open arm as a percentage of total time and time to enter the open arm were tracked[35].

Rotarod test: On PND 21 and 59, each animal was rotated at 40 rpm by placing it on a rod. Each animal's endurance was measured by how long it took to stay upright on the rod for 5 min[37].

Harvesting of brain tissues: At PND 59, after the behavioral evaluation, Na^+ thiopental (120 mg/kg i.p.) was administered and histological and biochemical analyses were performed. The brain was perfused with 100 mL of heparinized saline through a heart catheter, followed by 150 mL of 10% formalin and the tissues were preserved in formalin for later use. However, before collecting brain tissues for biochemical analysis, saline perfusion was necessary and the tissues were frozen at -80°C for later study.

Light microscopic examination: After being dehydrated in progressively more potent alcohol concentrations, certain specimens were fixed for 24 hrs in 10% neutral buffered formol. After that, they were cleaned up and embedded in paraffin. Finally, tissue sections were deparaffinized, microtome to a thickness of 5 microns and stained with Haematoxylin and Eosin (H&E) per usual protocol[38].

Sections of paraffin-embedded tissue were fixed in formalin and then stained with primary antibodies against Bax (rabbit polyclonal antibody, 1/50 dilution, Abcam), Bcl2 (rabbit polyclonal antibody, 1/100 dilution, Abcam) and GFAP (Glial fibrillary acidic protein) (mouse monoclonal antibody, 1/100 dilution, Thermo Scientific). Following a wash in phosphate-buffered saline (PBS), the sections were incubated with the appropriate biotinylated secondary antibody for an hour at room temperature. After 10 min at room temperature, the streptavidin peroxidase was removed by PBS washes. The chromogen 3,3'-diaminobenzidine (DAB)-hydrogen peroxide was used to locate and observe the immunoreaction. After the sections had been immunostained, Mayer's haematoxylin was used as a counterstain. Sections were used without primary antibodies as a negative control[39].

Biochemical measurements in tissue homogenates: The tissue lysate machine used PBS (100 mg tissue/mL) to mince, homogenize and chill the tissues. After 15 min of centrifugation at 1500 g (or 5000 rpm), the supernatant was collected and frozen at 80°C for further analysis. Oxidative stress markers, such as lipid peroxidation (as measured by malondialdehyde (MBS738685) and glutathione peroxidase (GBS934198) levels) and Interleukin-17 (IL-17) as a pro-inflammatory cytokine, were measured in cerebellar homogenates. These markers were assayed colorimetrically using commercially available kits (from MyBioSource, Dokki, Giza, Egypt), following the manufacturer's protocols. Data for IL-17 was given in pg/mL and data for all other oxidative stress markers were given in nmol/mg protein.

Statistical analysis: All analyses were performed using SPSS software (SPSS ver. 20.0; SPSS Inc., Chicago, Illinois, USA). The ANOVA and *post hoc* tests were used for the statistical analysis. The results were displayed as Mean±Standard Deviation (SD) and differences were considered significant when *p<0.05 and **p<0.01. The statistical figures were performed using GraphPad Prism version 9.0 (GraphPad Software, San Diego, California, USA).

RESULTS

Assessment of the effects of Luteolin on anxiety in SVP-induced autism: At PND 21 and 45, Luteolin's effect on anxiety was evaluated using the elevated plus-maze. At both PND 21 and 45, the results showed that the SVP group had significantly (p<0.0001) fewer open-arm entrances (3.60±1.07 vs 2.80±0.63) and open-arm time (4.70±1.05 vs 3.90±1.197) than the control group (10.70±1.16 vs 9.10±0.87 vs 13.50±0.70 vs 12.70±1.94). Conversely, the Luteolin group significantly (p<0.0001) outperformed the SVP group in terms of open-arm entrances (9.60±0.69 vs 8.40±0.69) and open-arm time (12.90±0.73 vs 11.40±1.35), both at PND 21 and 45. At PND 21 and 45, Luteolin-treated ASD rats showed a non-significant difference in the number of open-arm entrances compared to control group rats (p = 0.05 and p = 0.12, respectively) and a non-significant difference in the amount of time spent in open arms (p = 0.33 and p = 0.19, respectively) (Fig. 1a-d).

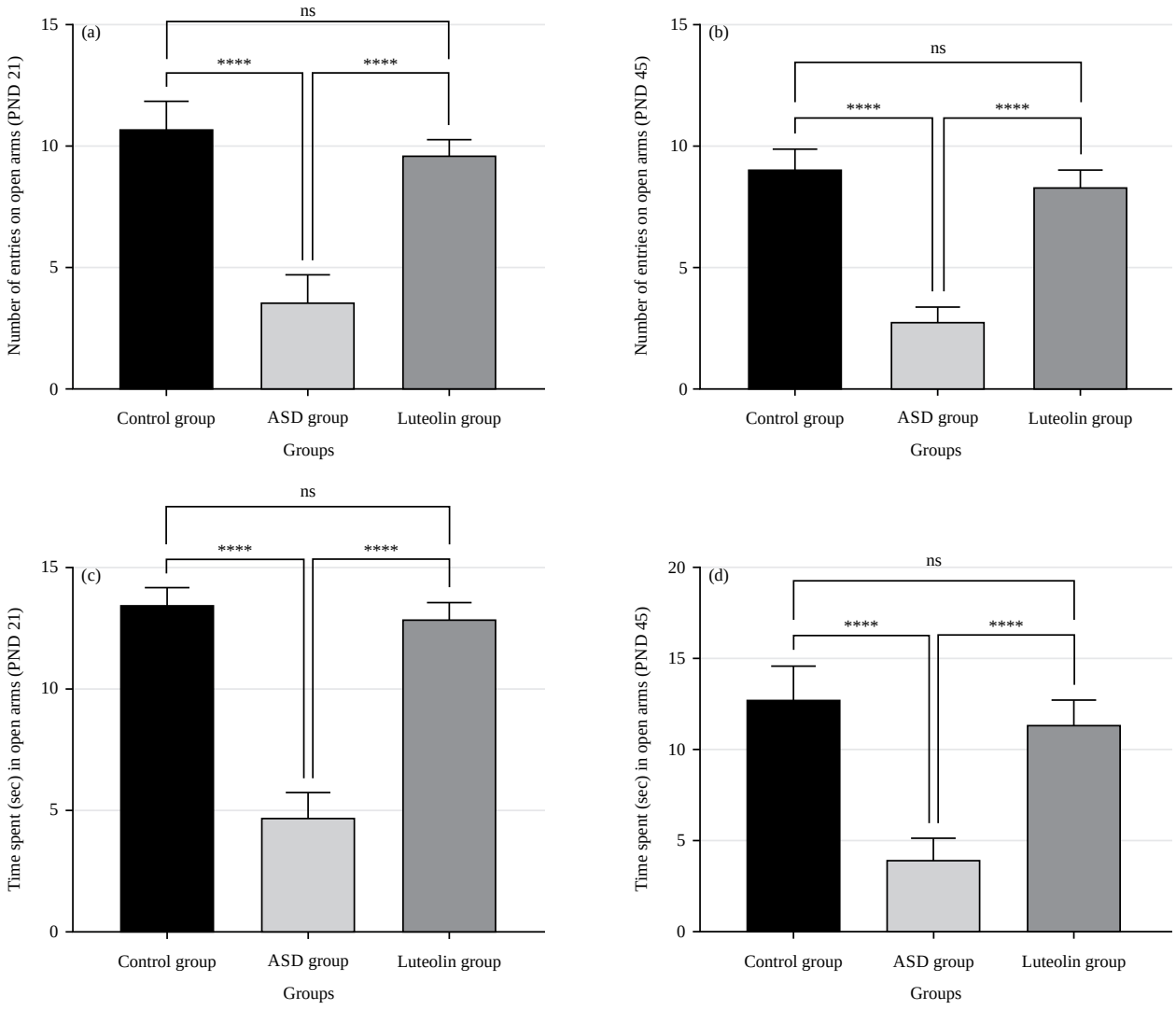

Fig. 1(a-d): Effects of Luteolin on anxiety using the elevated plus-maze in SVP-induced autism, (a) Number of open-arm entrances at PND 21, (b) Number of open-arm entrances at PND 45, (c) Amount of time spent in open arms (sec) at PND 21 and (d) Amount of time spent in open arms (sec) at PND 45

*Indicates significantly different using one way ANOVA test followed by *post hoc* test, ****p<0.0001 and ns: Non-significant

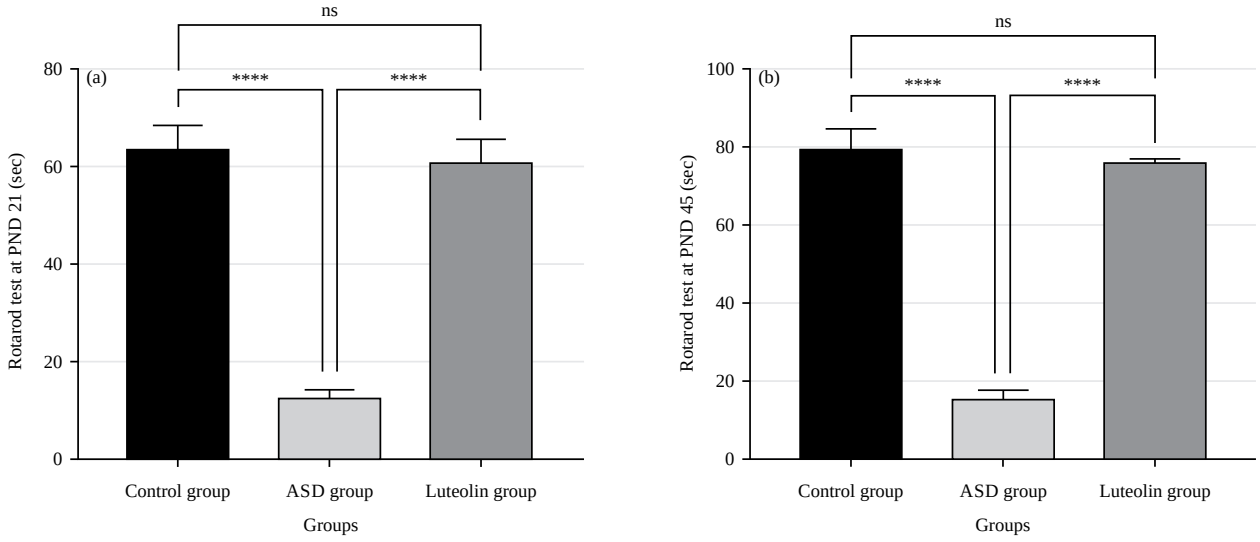

Fig. 2(a-b): Effects of Luteolin on motor coordination in SVP-induced autism, (a) Rotarod test at PND 21 and (b) Rotarod test at PND 45

*Indicates significantly different using one way ANOVA test followed by *post hoc* test, ****p<0.0001 and ns: Non-significant

Assessment of the effects of Luteolin on motor coordination in SVP-induced autism: The effects of Luteolin on motor coordination at PND 21 and 45 were analyzed using the rotarod test (Fig. 2a-b). Results showed that compared to controls, SVP-treated rats had significantly ($p<0.0001$) lower endurance times at postnatal day 21 (12.80 ± 1.596) and postnatal day 45 (15.80 ± 2.19). However, at PND 21 (60.75 ± 5.04) and PND 45 (76.25 ± 1.08), the SVP+Luteolin group's endurance time was significantly ($p<0.0001$) longer than the SVP group. Intriguingly, Luteolin-treated ASD animals spent less time in the rotarod test than the control group at postnatal day 21 ($p = 0.27$) and postnatal day 45 ($p = 0.07$).

Assessment of the effects of Luteolin on histological and immunohistochemical changes of cerebellum in SVP-induced autism: Cerebellum samples from the control, ASD and Luteolin groups are shown in Fig. 3-5. The cerebellar cortex of the control (negative and positive) rat was seen to be composed of the molecular layer (ML), the Purkinje cell layer (PCL) and the granular layer (GL) by H&E staining. Small stellate cells were found on the surface and basket cells were found in the ML's deeper regions, in close proximity to the Purkinje cells. The PCL was lined up in a single file at the crossroads of the ML and GL. Purkinje cells (PCs) were shaped like flasks and their nuclei were big and spherical. The GL was positioned just beneath the PCL. Ball-shaped cells with black nuclei and clear areas (cerebellar islands) where connections occur make up the GL and are composed of several tiny cells with irregular shapes (Fig. 3a-c). Shrunken, deformed PCs with condensed chromatin surrounded by vacuolated gaps and interposed with numerous neuroglial cells are seen in samples collected from the SVP-treated group. The ML had pyknotic, dispersed basket cells and vacuolations that were darkly pigmented (Fig. 4a-c). The Luteolin group, on the other hand, acted as a control group. When compared to the SVP-treated group, there was an enhancement. When compared to the ASD group, the ML thickened. The PCL was found to have a linear structure that is very typical (Fig. 5a). The typical flask shape of PCs was unaltered. However, there were still some decrepit PCs about. Also, basket cells revealed practically normal similar to those in the control group (Fig. 5b). Granular cells, both round and oval, inhabited the GL in close proximity to one another (Fig. 5c).

Control (negative and positive) rats immunostained for Glial Fibrillary Acidic Protein (GFAP) showed a moderate, positive reactivity in the cytoplasm and processes of astrocytes (Fig. 6a). Animals given SVP showed a dramatic improvement in all three levels of the cerebellum (Fig. 6b). When administered with SVP, rats showed a more subdued reactivity when treated with Luteolin (Fig. 6c).

Assessment of the effects of Luteolin on oxidative stress markers (MDA and GSH-Px) and on pro-inflammatory cytokine (IL-17) in SVP-induced autism: The effect of Luteolin on oxidative stress markers in the cerebellum is shown in Fig. 7. The SVP-treated rats (ASD group) exhibited significant changes in the MDA level (Fig. 7a) and in the GSH-Px (Fig. 7b) activities. The MDA showed a significant ($p<0.0001$) increase

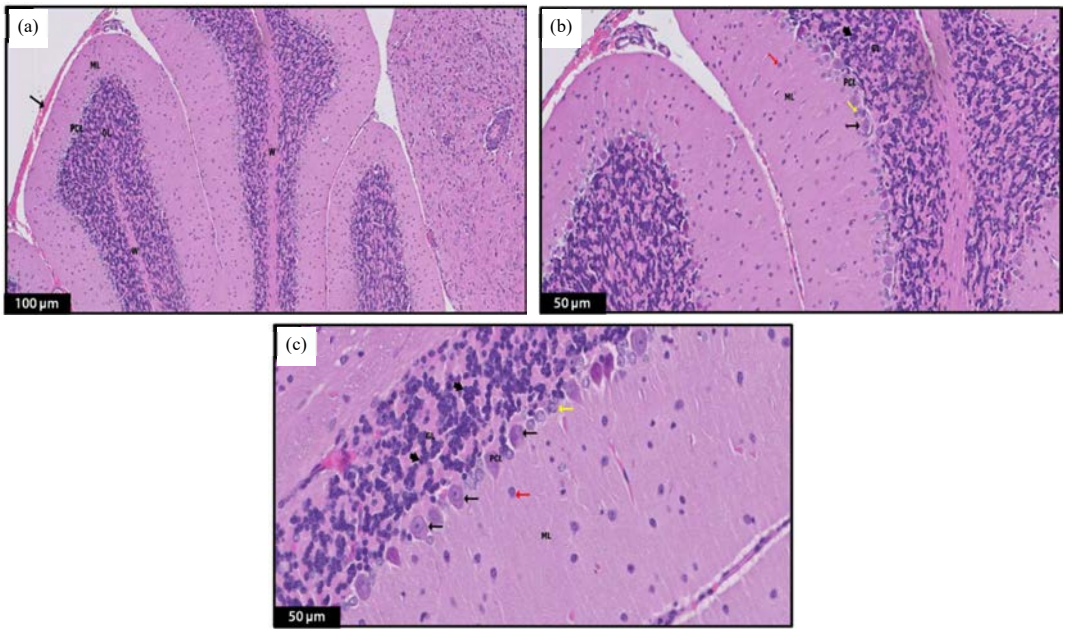

Fig. 3(a-c): A photomicrograph of the cerebellar cortex section from control rat

The section shows a typical architecture of folia of the cerebellar cortex, separated by narrow sulci. Each folium consisted of the mantle of the cerebellar cortex with a core of white matter (W). The covering pia mater (↑) can also be observed. The cerebellar cortex appears with three layers; molecular (ML), Purkinje cell (PCL) and granular cell (GL) layers. The molecular layer (ML) is consisting of superficial stellate cells (red arrow) and basket cells (yellow arrow) located deeper near Purkinje cells. The Purkinje cells appear with large pale stained nuclei and prominent nucleoli (black ↑). Notice the arrangement of Purkinje cells (↑) in one row between the other two layers. The granular layer (GL) is formed of small, rounded cells having darkly stained nuclei with cerebellar islands in between (arrowhead). H&E: AX10, BX 20, CX 40 m; Scale bar: 100, 50 and 50 µm, respectively

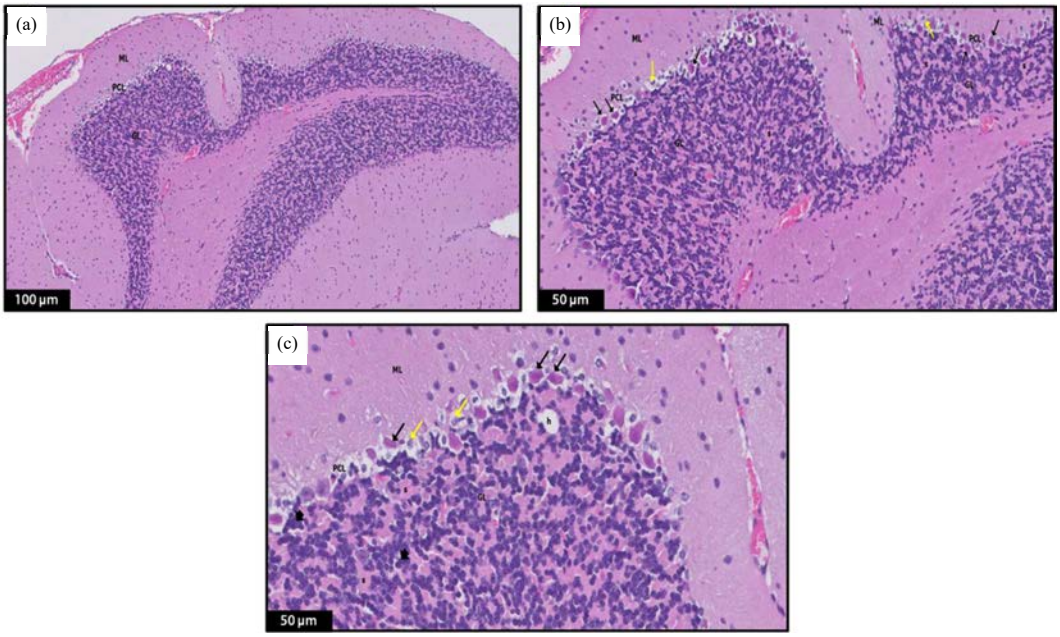

Fig. 4(a-c): A photomicrograph of the cerebellar cortex section from SVP (autism) rat

The section shows the disturbed monolayer of Purkinje cells with interposition of many neuroglial cells (yellow ↑) in between and around the Purkinje cells. Some shrunken Purkinje cells (black ↑) with homogenized deeply acidophilic cytoplasm and faint nuclei with halo of empty space (h) around it. Cells of nuclei of the granule layer appear with shrunken and deeply stained nuclei (arrowhead). Wide spaces in between them are seen (s). Notice the cerebellar cortex appears with three layers; molecular (ML), Purkinje cell (PCL) and granular cell (GL) layers, H&E: AX10, BX 20, CX 40; Scale bar = 100, 50 and 50 µm, respectively

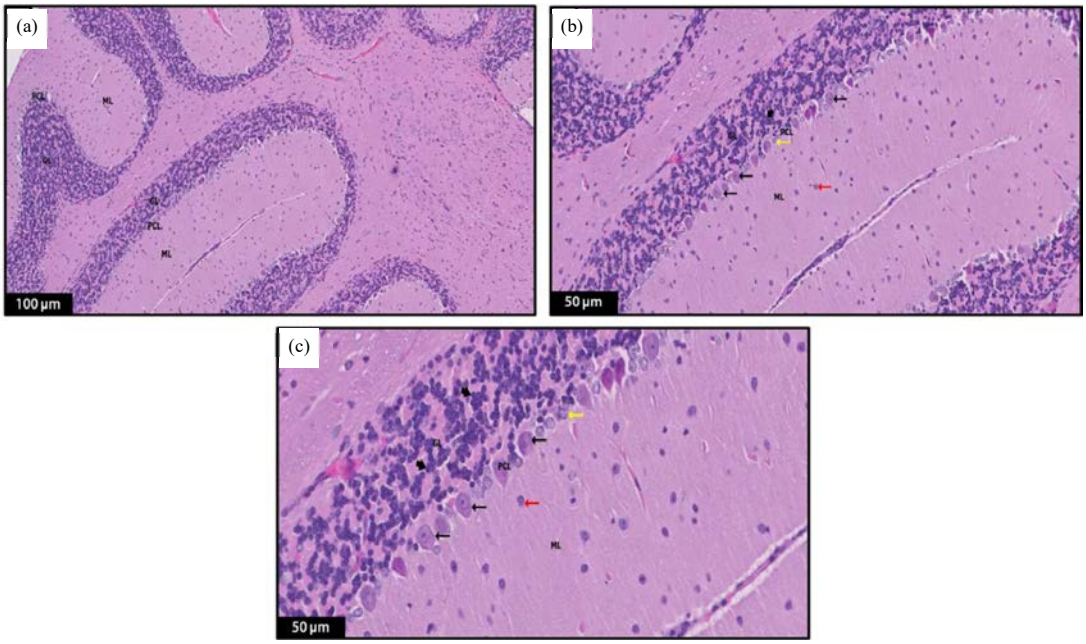

Fig. 5(a-c): A photomicrograph of the cerebellar cortex section from Luteolin treated rat

The section shows a monolayer arrangement of Purkinje cells (black ↑) in-between the other two layers. Purkinje cells, glomerular cells (arrowhead), superficial stellate cells (red arrow) and basket cells (yellow arrow) looked nearly normal like those in the control group. Notice the cerebellar cortex appears with three layers; molecular (ML), Purkinje cell (PCL) and granular cell (GL) layers. H&E: AX10, BX 20, CX 40; Scale bar: 100, 50 and 50 µm; respectively

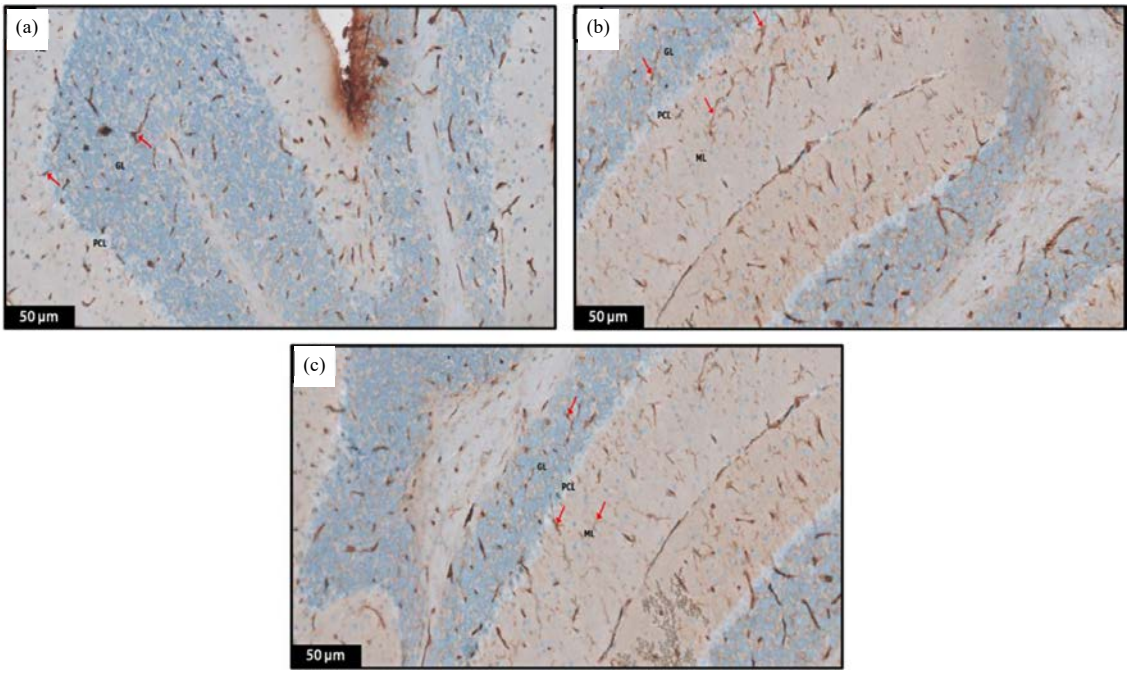

Fig. 6(a-c): A photomicrograph of a section in the cerebellar cortex

(a) Control rat showing mild GFAP immune-expression (red ↑) in the molecular, Purkinje cell (arrow heads) and granular (curved arrows) layers; (b) SVP rat showing intense GFAP immune expression (red ↑) and (c) Luteolin treated showing moderate GFAP immune-expression (red ↑) in the molecular (ML), Purkinje cell (PCL) and granular (GL) layers. GFAP immunostaining X20; Scale bar: 50 µm

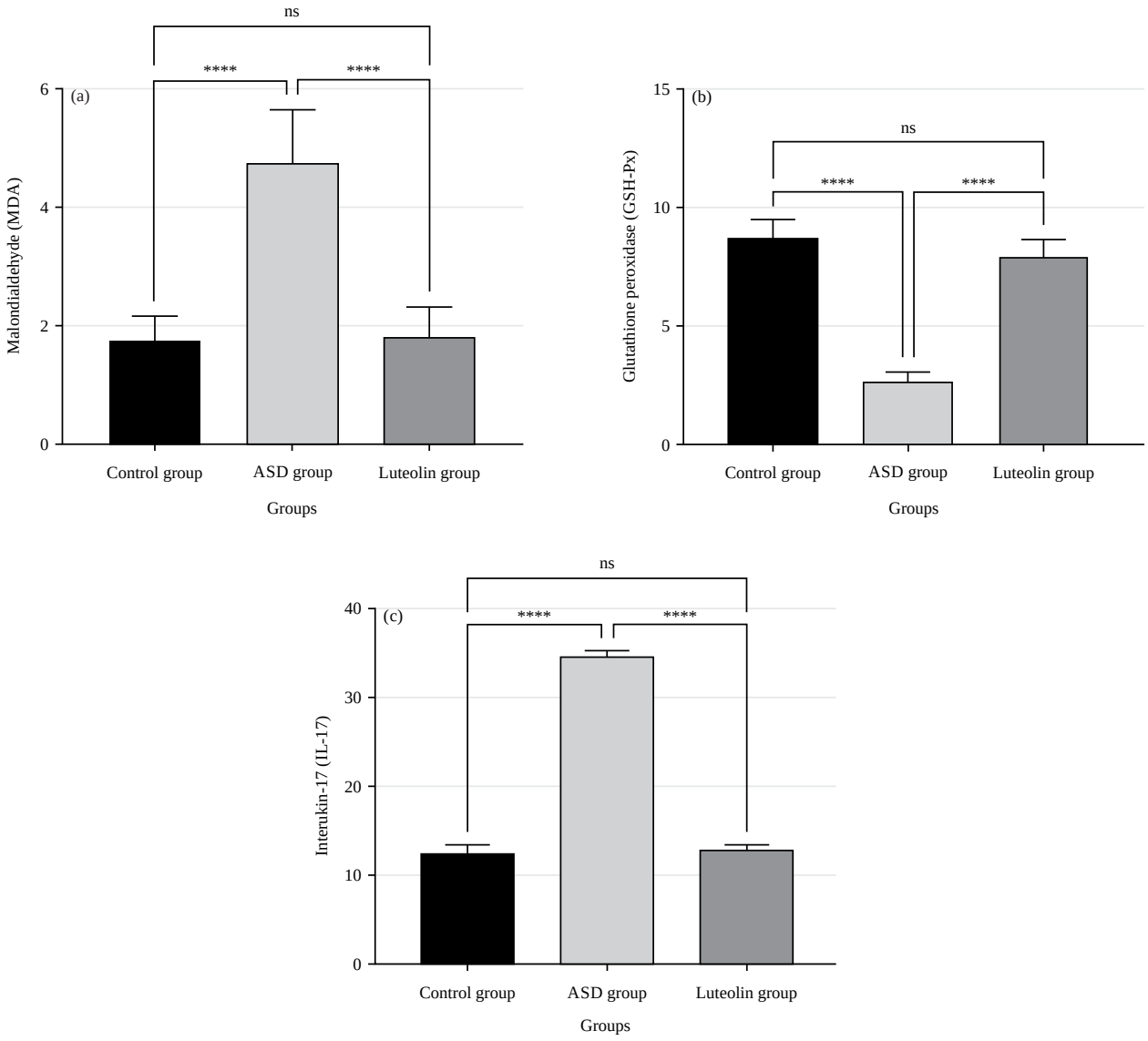

Fig. 7(a-c): Changes in oxidative stress markers and proinflammatory cytokine in Autism (ASD) postnatal male rats treated with Luteolin, (a) Malondialdehyde (MDA), (b) Glutathione peroxidase (GSH-Px) and (c) Interleukin-17 (IL-17)

*Indicates significantly different using one way ANOVA test followed by *post hoc* test, ****p<0.0001 and ns: Non-significant

in ASD group (4.727±0.8973) as compared to the control group (1.746±0.4253). Luteolin (1.820±0.5029) significantly (p<0.0001) decreased the MDA level compared to the SVP-treated rats. The GSH-Px showed a significant (p<0.0001) decrease in the ASD group (2.680±0.4315) as compared to the control group (8.737±0.8091). Whereas Luteolin significantly increased the GSH-Px (7.975±0.7615) activities compared to SVP-treated rats (ASD group). Interestingly, treated ASD rats with Luteolin showed a non-significantly (p = 0.9919) decrease in the MDA and a non-significant (p = 0.9919) increase in the GSH-Px as compared to the control group.

As shown in Fig. 7c, IL-17 exhibited a significant (p<0.0001) increase in the ASD group (34.75±0.7807) as compared to the control group (12.57±0.9346). Moreover, the results showed a significant decrease (p<0.0001) of IL-17 in treated ASD rats with Luteolin (13.05±0.5257) as compared to the ASD group. Interestingly, treated ASD rats with Luteolin showed a non-significantly (p = 0.4385) increase in the IL-17 as compared to the control group.

DISCUSSION

Early impairment in social cognition and social perception, executive dysfunction, atypical perceptual and information processing problems in social communication and unusually limited, repetitive behavioral and interests are all hallmarks of the spectrum of neurodevelopmental disorders known as ASD. Although autism is commonly thought to be present at birth, up to 40% of children who are diagnosed with autism appear to develop normally until the ages of 18-30 months, after which they have a regression during which their controlled abilities fail to mature along a normal trajectory[40]. The primary goal of pharmacological treatments has been symptom management rather than addressing the underlying aetiology. This is the first study to use the SVP animal model of ASD and show that a Luteolin formulation can reverse neural behaviour and biochemical and histological cerebellar alterations in SVP-induced autistic-like rats.

Animal models could be useful for studying autism's neurological structure and defining the neural systems that make up the social brain and mediate repetitive behaviours because of the scarcity of post-mortem brain tissue available for studying these changes. The safety and efficacy of possible medicinal substances can also be evaluated using these preclinical models[41]. Animal models of autism are widespread; causes range from single-gene mutations to epigenetic variables and prenatal chemical exposure to neonatal injuries of specific brain areas[42,43]. Epilepsy, major depressive disorder and bipolar disorder are commonly treated with SVP since it is a Gamma-Aminobutyric Acid (GABA) agonist anti-epileptic medication. Deficits in language and communication, stereotypic behaviour, hyperexcitability and delays in behavioural development are some of the symptoms shown in persons exposed to SVP in utero comparable to those seen in those with autism[44]. Based on these clinical similarities, prenatal exposure to SVP in rodents has been proposed as a potential animal model of autism[45]. Based on pharmaco-epidemiological findings that maternal use of SVP during pregnancy (for example, to treat migraines, mania or epilepsy) is strongly associated with the child's subsequent development of ASD, this animal model of ASD was created[46-48]. Treatment of rats with a single dose of SVP on embryonic day 12.5 before neural tube closure and the development of the brain stem nuclei in rats results in offspring with neuroanatomical and behavioural characteristics similar to those seen in people with autism[9,10]. Previous experiments have shown that prenatal injections of less than 500 mg/kg SVP create a severe autism-like state in animals without causing death[49-51].

In addition, SVP exposure in the neonatal period caused motor and cognitive abnormalities that mirrored those reported in individuals with autism. Important behaviours mediated by the cerebellum were first documented in PND 14[40]. In the current investigation, motor and cognitive deficits similar to autistic regression were caused by a single subcutaneous injection of 400 mg/kg SVP on PND14. The PND 14 roughly corresponds to the third trimester of human development, when granule cells migrate to the hippocampus and the cerebellum[52].

Evidence from several human and animal studies suggests a role for the cerebellum in the pathophysiology of ASD[53-55]. Significant cerebellar pathology, including deteriorated Purkinje cells, was observed in individuals with ASD[53]. Present study findings corroborate and extend previous research on the neurotoxic effect of SVP exposure throughout both the fetal and postnatal stages on the cerebellar cortex[54].

Flavonoids are a class of polyphenolic substances found in foods like fruits, vegetables and beverages made from plants. The biological effects of these chemicals on conditions as diverse as neurodevelopmental delay and mood disorders are extensive[56]. Many plant foods, such as fruits, vegetables and herbs, contain the flavonoid Luteolin. Anti-inflammatory, antioxidant and neuroprotective qualities are just a few of the many biological effects attributed to Luteolin, making it one of the most potent and effective flavonoids[32]. Inhibition of NF-κB and elevation of redox-sensitive transcription factors involved in activating antioxidant defense systems have been linked to Luteolin's anti-inflammatory and antioxidant actions, according to several studies[57,58]. Similar results have been reported with quercetin, which shares structural chemistry characteristics with Luteolin. In an animal model of autism, quercetin was found to have neuroprotective effects via antioxidant and anti-inflammatory pathways, suggesting that this natural chemical could be an effective therapeutic agent for treating ASDs[59]. It is hypothesize that Luteolin may have a positive effect on cerebellar affection in ASD-animal models, similar to the effect quercetin has on these models.

The current study found that prenatal and postnatal SVP substantially damaged rats' cognitive and motor capacities by testing them on PND 21 and 45 using neurobehavioral tests. Morakotsriwan *et al.*[35], Schneider *et al.*[60] and Hussein *et al.*[61] all reported similar findings. Morakotsriwan *et al.*[35], report injecting SVP on postnatal day 14, then feeding the animals' silkworm pupae at 50, 100 and 200 mg/kg b.wt., from postnatal day 14 to postnatal day 40. An array of tests, including negative geotaxis, hot-plate, rotarod, open-field, elevated plus-maze, Morris water maze and social behaviour

tests, were used to characterize the behaviour of the experimental animals[35]. Motor dysfunctions on the rotarod test at PND 24, 25 and 26 and anxiety on the elevated plus-maze are among the autistic-like symptoms reported by Schneider *et al.*[60] after injection of SVP (400 mg/kg i.p.) on E 12.5th day (at PND 30, 35 and 40). Histopathological analysis of the brain showed that the SVP-treated group had a considerably lower number of Purkinje cells than the control group, confirming the presence of cerebellar cortex injury. The cerebellum has been linked to the regulation of cognitive function alongside the prefrontal cortex, the posterior parietal cortex and the cortical motor region[60]. Neurobehavioral tests performed on the rats at PND 21 and 45 revealed that a single dosage of VPA (500 mg/kg on E 12.5th day) severely impacted their learning, memory and motor skills, as reported by Hussein *et al.*[61]. To investigate the potential neuroprotective impact of Luteolin in this rat model of autism, we discovered that therapy with Luteolin dramatically reduced motor impairments and anxiety in the offspring of mothers treated with VPA. Several previous research corroborated the current study's findings, showing that Luteolin has favorable preventive and therapeutic benefits on neurological illnesses such as Alzheimer's disease[62], Parkinson's disease[63] and epilepsy[64]. This is the first study to our knowledge to indicate that Luteolin can treat SVP-induced autism. The neuroprotective properties of Luteolin may stem from the fact that it reduces oxidative stress, lowers inflammation and prevents cell death[65].

There is a strong connection between inflammation and oxidative stress. Neurodevelopmental disorders like ASD and ADHD start with inflammation which results in oxidative stress and mitochondrial dysfunction. This can exacerbate oxidative stress and release negative feedback, leading to downstream abnormalities and dysfunction in brain development[66]. Inflammation and oxidative stress are essential for normal physiological processes, including immunity. Understanding their role in autism spectrum disorder is crucial for finding effective treatments[67-71].

The SVP-induced autistic rats were shown to have increased oxidative stress in the brain, as evidenced by an increase in cerebellar MDA (lipid peroxidation marker) concentration and a decrease in GSH-Px (antioxidant) enzyme activity. Glutathione redox imbalance and high indicators of oxidative stress have been identified in the cerebellum and Brodmann area 22 (BA22) in children with ASD, according to post-mortem analyses[72]. Furthermore, it discovered a significant reduction in oxidative stress in the brains of Luteolin-treated autistic rats, indicating an antioxidant impact for Luteolin in this model system. The current findings were consistent with those of a previous study by Hussein *et al.*[61] which found that Luteolin reduced oxidative stress in various neurological illness models, including neurodegeneration and movement abnormalities. Treatment with flavonoids such as Luteolin, quercetin, hesperetin and catechin has been shown in animal experiments to boost the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GRx) and peroxidase ($PerO_2$) (GPx). Flavonoids may also lower levels of reactive oxygen species (ROS), nitrites and malondialdehyde (MDA) in ASD model organisms[73-75]. This is the first study we're aware of to show the antioxidant impact of Luteolin in a rat model of SVP-induced autism.

In addition, the current study also found that the expression of IL-17 was significantly upregulated in the cerebellum of SVP-treated rats, suggesting a potential function for this cytokine in the pathogenesis of this disorder. Previous research has revealed similar results; for example, Suzuki *et al.*[76] described increased IL-17 levels in the serum of children with ASD. Determinations in preclinical models linked the increase in IL-17 levels during pregnancy to ASD-like behaviors, providing further evidence that IL-17 is involved in the development of ASD[77]. Experimental evidence from animal models has shed new light on the possible link between ASD-related characteristics and altered IL-17 levels. Increased levels of IL-17 in maternal serum were followed by upregulation of IL17Ra in fetal brains in inducible models such as maternal intrauterine infection caused by the injection of polyinosinic:Polycytidylic acid (poly(I:C) or lipopolysaccharide[77,78]. However, there is a lack of consistency in the findings on IL-17 levels in the fetus, postnatally and in adult offspring of inducible or genetic models of ASD[79,80]. The results of the current investigation reveal that Luteolin has an anti-inflammatory impact in autistic rats by significantly decreasing the levels of the pro-inflammatory cytokine IL-17 in the cerebellum tissue of these animals. Although Luteolin has been shown to have anti-inflammatory effects in other models of disease, to the best of our knowledge, this is the first study to reveal such an impact in the SVP-induced autism rat model.

Degenerative alterations and death in Purkinje cells of the cerebellar cortex were observed in animal models of autism following SVP injection in rats, as shown by our histological and immunohistochemical analyses. These findings matched those of prior studies showing that SVP is neurotoxic[34,35,61]. Neurotoxins are widely regarded as a critical risk factor in the etiology of progressive neurodegenerative diseases. This neurodegeneration has been linked to inflammatory processes, oxidative stress and apoptosis. The Purkinje cell layer of the cerebellar cortex was most vulnerable to SVP-induced changes in this study. The ML displayed highly stained pyknotic scattered basket cells, while the PC shrank and became misshapen, displaying condensed chromatin surrounded by vacuolated gaps.

Additionally, some authors have interpreted the observation of shrunken PCs with dark-stained cytoplasm and unidentifiable nuclei as evidence for chromatolysis and gliosis. Neuronal soluble mediators and cell-cell interaction may regulate the rapid response of gliosis or increased neuroglial cells to brain injury. Researchers disagreed, with some pointing to the darkened, shrunken neurons as evidence of a specific apoptotic phase characterized by notably constricted cytoplasm and nucleoplasm[81]. Several authors have used "spongiform alterations" to characterize the vacuolation that developed within the Purkinje cell layer. Others have hypothesized that cellular component loss in the cerebellar cortex is to blame for this heightened vacuolation[82]. When comparing the SVP-treated animals (Autistic rat model) to the control group, it is found that cytoplasmic immune expression of GFAP in the cell body and processes of astrocytes was significantly increased. Since GFAP is the primary, intermediate filament protein in mature astrocytes, it is considered a hallmark unique to these cells. According to previous studies, a severe form of astrogliosis is triggered by degenerative brain trauma, leading to astrocyte proliferation, hypertrophy and increased GFAP production. The emergence of these reactive astrocytes after neurodegeneration due to neuronal injury may be seen as a compensatory process[83]. Present findings showed that Luteolin protected the cerebellum from the damage caused by SVP. This finding was consistent with earlier studies that have shown Luteolin's anti-inflammatory and antioxidant properties provide neuroprotection[32,84,85].

CONCLUSION

Overall, the findings of this study suggested that Luteolin may hold promise as a natural treatment option for individuals with ASD, although more research is needed to confirm these results and determine its safety and efficacy in humans. Based on the results of this study, it is recommended that further research be conducted to determine the potential therapeutic effects of Luteolin in treating ASD symptoms. Additionally, it may be beneficial to investigate the optimal dosage and administration route for Luteolin in order to maximize its efficacy and minimize potential adverse effects.

SIGNIFICANCE STATEMENT

The study aimed to evaluate the therapeutic effects of Luteolin in a rat model of Autism Spectrum Disorder (ASD) induced by sodium valproic acid (SVP) injection. This study showed sodium valproic acid (SVP) injection induced ASD-like behaviours in rats, which was accompanied by increased oxidative stress and pro-inflammatory cytokine IL-17 levels in the cerebellar tissues. Luteolin treatment was found to improve neurobehavioral alterations, reduce oxidative stress, normalize cytokine levels and restore cerebellar histology. These results indicate that Luteolin has potential neuroprotective effects and could be used as a therapeutic agent for ASD. Further research is suggested to investigate the specific mechanisms and long-term effects of Luteolin treatment, as well as its potential therapeutic application in human patients with autism.

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