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The Ameliorative Effect of Apigenin or Silymarin as Add-On Therapy to Risperidone on Valproic Acid Induced Autism in Albino Rats: Implication of Oxidative Stress, Apoptosis and Autophagy

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ABSTRACT

Background: Recent studies show that deficient autophagy, increased mammalian target of rapamycin (mTOR), oxidative stress and apoptosis have a critical role in the pathophysiology of autism. The objective of the current study is to evaluate and compare some possible ameliorative effects of apigenin, silymarin either alone or in combination with risperidone on autism induced by valproic acid (VPA) in albino rats concerning; oxidative stress, autophagy, apoptosis as well as histopathological examination & behavioral tests. **Methodology:** This experiment was performed on 70 male Wister albino rats divided into seven equal groups; control group; 10 rat pups from control female rats and the other 60 rat pups from female rats that received i.p VPA during pregnancy for other groups as follows untreated VPA-induced autism, risperidone treated group, apigenin treated group, silymarin treated group, apigenin and risperidone treated group, silymarin and risperidone treated group. All treatments were given from post-natal day (PND) 21st to 40$^{th.}$ behavioral tests were performed at the end of treatment. Portions of hippocampus were dissected and processed for assessment of biochemical parameters, histopathological examination and immunohistochemistry. **Results and conclusion:** The findings suggest that apigenin and silymarin produce promising effects in VPA-induced autism as they decrease oxidative stress and apoptosis; in addition, they produce modulation of autophagy with inhibition of mTOR, that all are reflected as amelioration of impaired behavior & improvement of the histopathological picture. Moreover, the use of apigenin or silymarin in combination with risperidone exhibits better and more satisfactory results than in either remedy alone.

Keywords: ASD, VPA, Risperidone, Apigenin, Silymarin, Autophagy, Tanta University.

INTRODUCTION

Autism spectrum disorder (ASD) is a severe lifelong neurodevelopmental disorder that appears within the initial three years of life. It is manifested by social interactions deficiencies, recurring forms of behavior and limited interests [(1)]. The precise etiology of ASD remains unknown; it has been regarded as a disorder with multiple etiologies that is affected by numerous factors like genetic and environmental factors. As a consequence, there is a difficulty in identifying complete curative medical therapy up till now [(2)].

As regards environmental factors, it was found that administration of valproic acid (VPA) in pregnancy leads to an increase in the prevalence of ASD between offspring. Additionally, over several animal model researches, it has been indicated that maternal therapy with VPA in rodents mimics ASD pathophysiology. As a consequence, the VPA-induced ASD-like animal model provides an excellent model for testing pharmacological agents that could be used to treat ASD [(3)].

Autophagy is a cell-protecting action that permits cells to survive when nutrients are insufficient. Neuronal autophagy is an essential determining factor of memory creation, synaptic plasticity, and structural remodeling, as well as a key regulator of protein balance [(4)]. Autophagy and associated pathways are supposed to be involved in the progress of ASD and studies showed that deficiency of autophagy leads to autism [(5)]. The mammalian target of rapamycin (mTOR) is an essential protein for dendritic plasticity and cell survival. It was discovered that mTOR might be involved in disturbed cell signaling in ASD [(6)].

Risperidone is the first FDA-approved drug for children with autism, and the most widely used. It is effective for the improvement of behavior symptoms in ASD children and adolescence. Risperidone is an atypical antipsychotic antagonist of both dopamine (D_2) and serotonin ($5HT_2A$) receptor. However, it has a lot of adverse effects that limit its long use [(7)].

Apigenin (4', 5, 7-trihydroxyflavone) is found in a variety of food. It demonstrates a number of biological actions as anti-carcinogenic, anti-inflammatory, and antioxidant properties. In addition, great evidence supports its neuroprotective effect as it has a high level of distribution in the brain [(8)].

Moreover, apigenin has antidepressant effects in chronic stress model by its ability to promote autophagy in this model through mTOR signaling pathway inhibition [(9)]. Silymarin is a naturally occurring antioxidant that is extremely safe. It is common to have anti-inflammatory, anti-apoptotic effects. Furthermore, it was found that silymarin can modulate autophagy and mTOR [(10,11)].

METHODOLOGY

VPA (2-propylpentanoic acid, sodium salt, 98%) white pure powder from Acros Organics, USA dissolved to 250 mg/ml in saline. Risperidone (Risperdal

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2mg/tablet) from Janssen-Cilag Pharmaceutica, Belgium prepared as a suspension of 0.2 mg/ml in 0.5% Carboxy methyl cellulose (CMC) vehicle. Apigenin (50mg/capsule) from Swanson Health Products, USA, prepared as a suspension in 0.5% CMC of 5 mg/ ml. Silymarin (milk thistle extract) from Now family, USA prepared as a suspension in 0.5% CMC of 400 mg/ 10 ml.

Animals grouping: The animal model of autism was performed as follows; 70 adult female Wister rats, 35 adult male Wister rats weighing 150-200g were acclimatized for seven days before the experiment. Rats were kept in animal laboratory rooms and had free admission to standard animal nutrition and water. Female rats were permitted to mate overnight. Then they were examined in the early morning for the existence of a vaginal plug. When it was found, it is deemed as gestational day (GD) 0.5. After evidence of pregnancy, on GD 12.5, pregnant rats received one dose of VPA (600 mg /kg, i.p) to develop the experimental model of autism. Control rats inoculated i.p. physiological saline. Until delivery, all pregnant rats were housed separately [12]. The experiment was performed on 70 male Wister rat pups. The rat pups were allocated randomly into equal seven groups as follows: (10 rat pups from control female rats that received i.p saline during pregnancy and 60 rat pups from female rats that received i.p VPA during pregnancy).

Group 1: rat pups of control female rats that received i.p saline during pregnancy, were received a vehicle of 0.5% of CMC orally. **Group 2**: autistic rat pups of female rats that received i.p VPA during pregnancy, were received a vehicle of 0.5% of CMC orally daily. **Group 3**: autistic rat pups were received risperidone (1mg/kg/day) [13]. **Group 4**: autistic rat pups were received apigenin (40 mg/kg/day) (9). **Group 5**: autistic rat pups were received silymarin (200mg/kg/day) [14]. **Group 6**: autistic rat pups were received apigenin and risperidone. **Group 7**: autistic rat pups were received silymarin and risperidone. The treatment with risperidone, apigenin and silymarin was administered by oral gavage daily from PND 21st to 40th

Behavioral tests: They were performed at the end of treatment.

Three-chamber social test: This is one of the most utilized tests in research of ASD in rodent models. This test evaluates sociability that means a proclivity to consume time with another animal rather than alone, and the predilection for social innovation means the ability to differentiate and select between familiar and novel animals [15]. Each chamber is 19 x 45 cm. The test is comprised of three sessions: Habituation session: lasts for 5 minutes; Sociability session (session I): lasts for 10 minutes; Social preference/novelty session (session II): lasts also for 10 minutes. The time passed with each stranger in each chamber documented in sessions I and II [16].

T-maze spontaneous alternation test: This test is used for evaluation of autistic repetitive behaviors which is one of the most characteristic symptoms of ASD. The test is established on rats' natural tendency to transition between arms in each trial over a number of trials. Normal rat behavior shows a high percentage of alternations [17]. T-maze is made up of a wooden enclosed apparatus as T-shaped with three arms at (90°) [18]. The test rat was put at the start box and the session was reset when the rat did not go in any of the free option arms after 30 seconds. Five consecutive trials were performed for each rat [17, 18]. The interchange between right and left arms was recorded. It was evaluated in proportion to the arm entered in the preceding session. The data are transformed into scores as follows: 0 = no alternations (it means that the same arm was entered repeatedly for all five sessions); 1, 2, 3 and 4 according to number of alternations [18].

Tissue sampling: After performing behavioral tests, all rats were euthanized by pentobarbital sodium (50mg/kg, i.p) then brains were removed carefully followed by dissection of hippocampus, then washed with cold saline. The right half of the hippocampus was placed in 10% formalin and processed for histopathological examination (H&E) and immunohistochemical staining. While the left part of the hippocampus was stored at - 80 ºC until processed for the biochemical parameters: Tissue reduced glutathione (GSH) (mg/gm wet tissue) & Malondialdehyde (MDA) (lipid peroxide) (nmol/gm wet tissue) were measured by spectrophotometer utilizing Biodiagnostic company's kits. Tissue mammalian target of rapamycin (mTOR) (ng/ml) were measured by ELISA kit from Cloud-Clone Corp, USA (Catalog No. E-31046Ra) following the manufacturer protocol.

Immunohistochemical expression of tissue LC3-II: Immunohistochemical appearance of LC3-II in the right half of hippocampus was done using rabbit polyclonal antibody purchased from (Sun Red Bio laboratories Catalogue No.: 201r-1501) according to manufacturer protocol. Immunohistochemical scoring was done as follows; LC3-II is positive as diffuse cytoplasmic staining with perinuclear dots. The reactivity was calculated by counting 100 cells and expressed as percentage of positive cells from the total. Immunostaining was evaluated by semi-quantitative scoring system for calculation of staining cells and concentration of staining. They were recorded on scales of 0 to 3.0; no stained cells or barely stained cells < 5% were detected 1; mild in 5- 25% of the cells.2; moderate in 25 to 75% positivity 3; more than 75% of cells showed marked positivity [19].

Immunohistochemical expression of tissue Bcl-2: Immunohistochemical manifestation of Bcl-2 in the right half of hippocampus was done using Anti- Bcl-2 Monoclonal Antibody (Clone SP66) purchased from MASTER DIAGNOSTICA, Granada, Spain. Immunohistochemical scoring was done as follows; Bcl-

2 is positive as diffuse cytoplasmic staining with or without nuclear staining. The reactivity was calculated by counting 100 cells and expressed as percentage of positive cells from the total. Immunostaining was evaluated by semi-quantitative scoring system for proportion of staining cells and intensity of staining. They were recorded on scales of 0 to 3. 0; no stained cells were detected.1; mild in < 25% of the cells, 2; moderate in 25 to 50% positivity, 3; > 50% of cells showed marked positivity [(20)].

Ethical approval: The work was approved by the "Research Ethics Committee, REC", Faculty of Medicine, Tanta University, Egypt (approval code: 33185/06/19).

Statistical analysis: The obtained values were statistically evaluated utilizing the graph pad prism software version 8 for Windows. Shapiro-Wilk test for normality of distribution. One Way ANOVA test (F value) followed by post-Hoc Tukey's test (parametric data). Kruskal-Wallis test then Mann-Whitney test (non-parametric).

RESULTS

Behavioral tests

Biochemical measurements

MDA and mTOR levels in hippocampus were substantially higher in the untreated VPA-induced autism group than control group. Therapy of rats with risperidone, apigenin or silymarin alone caused a significant decrease compared to the untreated VPA-induced autism group. Regarding GSH levels, the untreated VPA-induced autism group revealed significant decrease when than controls. Therapy of rats with risperidone, apigenin or silymarin alone caused a significant increase compared to the untreated VPA-induced autism group (**Table 1**).

Table (1): Comparative statistics of biochemical parameters.

Groups / Parameters	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	One-way ANOVA F value (P value)
GSH levels (mg/gm tissue)	53.72 ± 2.46	21.29 ± 2.59 **P1*** **	30.65 ± 0.804 **P2** **	39.16 ± 1.35 **P2*** P3*	43.77 ± 2.08 **P2*** P3*** P4 NS**	47.25 ± 1.21 **P2*** P5*** P6* **	52.05 ± 1.14 **P2*** P5*** P7 * P8NS**	43.14 **(P***)**
MDA levels (nmol/gm tissue)	86.85 ± 2.55	250.8 ± 10.48 **P1*** **	209.8 ± 12.41 **P2*** **	173.6 ± 4.72 **P2*** P3** **	125.2 ± 2.35 **P2*** P3*** P4*** **	155.9 ± 2.59 **P2*** P5*** P6NS **	111.3 ± 2.01 **P2*** P5*** P7NS P8*** **	74.93 **(P***)**
mTOR levels (ng/ml)	13.59 ± 0.58	24.43 ±0.69 **P1*** **	22.41 ± 0.47 **P2 NS**	16.79 ± 0.36 **P2*** P3*** **	15.89 ± 0.48 **P2*** P3*** P4 NS **	14.84 ± 0.54 **P2*** P5*** P6NS **	14.17 ± 0.52 **P2*** P5*** P7NS P8NS **	63.62 **(P***)**

P1= group 2 vs. group 1, P2= groups 3, 4, 5, 6 and 7 vs. group2, P3= groups4, 5 vs. group3, P4= group5 vs. group 4. P5= groups 6, 7 vs. group3. P6= group 6 vs. group 4. P7= group 7 vs. group 5. P8= group 6 vs. group7.*Significance: NS (non-significant) $p>0.05$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

In the three-chamber test, it was found that the untreated VPA-induced autism group showed a significantly decrease in length of time spent with stranger I and length of time spent with stranger II at session 1 and 2 respectively in comparison to the controls. Therapy of rats with risperidone, apigenin and silymarin caused a significantly increase in length of time spent with stranger I and length of time spent with stranger II in comparison to the untreated VPA-induced autism group. Autistic rats treated with a combination of risperidone and apigenin and a combination of risperidone and silymarin presented a significantly increase in length of time spent with stranger I and length of time spent with stranger II at session 1 and 2 respectively in comparison with the group treated with risperidone alone (**Figure 1**).

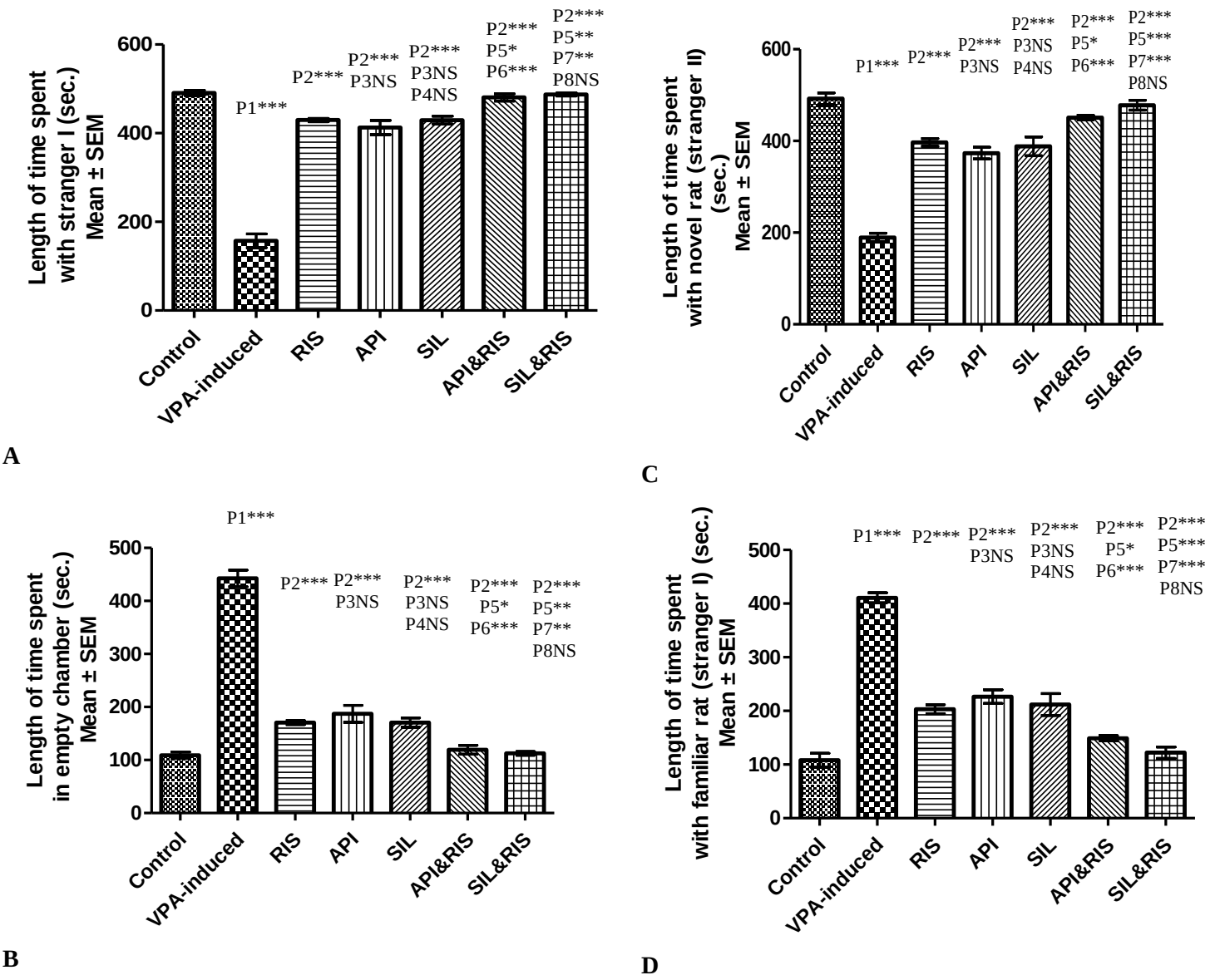

Figure 1: Three- chamber social test results.

(A): session I (difference between lengths of time spent with stranger I).
(B): session I (difference between lengths of time spent in empty chamber).
(C): session II (difference between lengths of time spent with novel rat).
(D): session II (difference between lengths of time spent with familiar rat).
P1= group 2 vs. group 1, P2= groups 3, 4, 5, 6 and 7 vs. group2, P3= groups4, 5 vs. group3, P4= group5 vs. group 4. P5= groups 6, 7 vs. group3. P6= group 6 vs. group 4. P7= group 7 vs. group 5. P8= group 6 vs. group7.*Significance: NS (non-significant) p>0.05, * p<0.05,
**** p<0.01, *** p<0.001.**
VPA-induced= valproic acid-induced autism, RIS=risperidone, API=apigenin, SIL=silymarin.

Regarding the T-maze test, the untreated VPA-induced autism group exhibited a significant decrease in alternation score than controls. Treatment of rats with risperidone, apigenin and silymarin affected a significantly increase compared with untreated VPA-induced autism group. Autistic rats received a combination of risperidone and apigenin and a combination of risperidone and silymarin indicated a significantly increase than the group received risperidone alone (**Figure 2**).

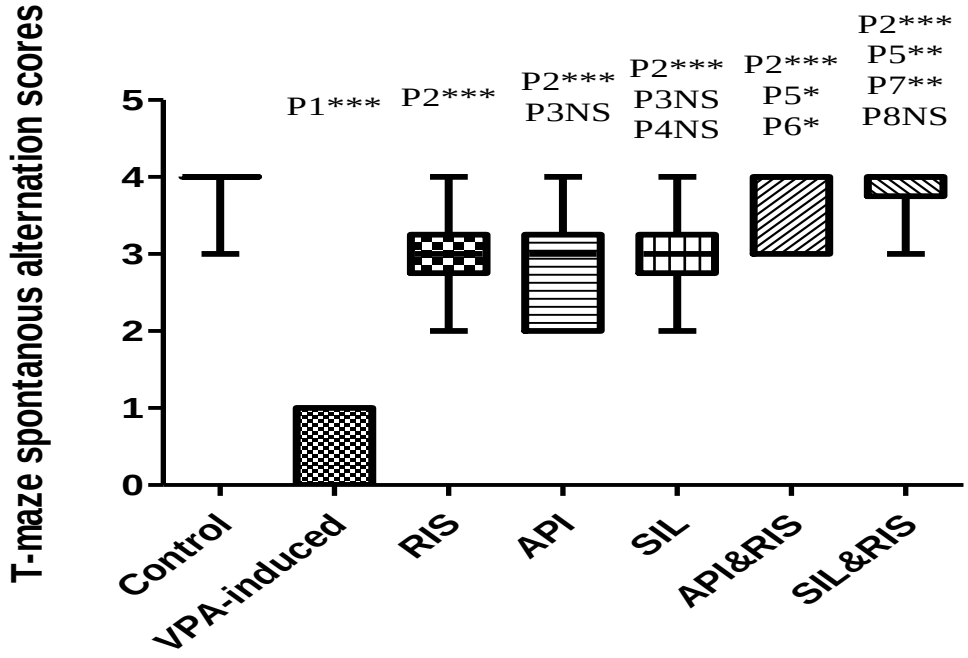

Figure 2: T-maze spontaneous alternation score.

P1= group 2 vs. group 1, P2= groups 3, 4, 5, 6 and 7 vs. group2, P3= groups4, 5 vs. group3, P4= group5 vs. group 4. P5= groups 6, 7 vs. group3. P6= group 6 vs. group 4. P7= group 7 vs. group 5. P8= group 6 vs. group7.*Significance: NS (non-significant) $p>0.05$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. VPA-induced= valproic acid-induced autism, RIS=risperidone, API=apigenin, SIL=silymarin.

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Histopathological examination as shown in fig.3.

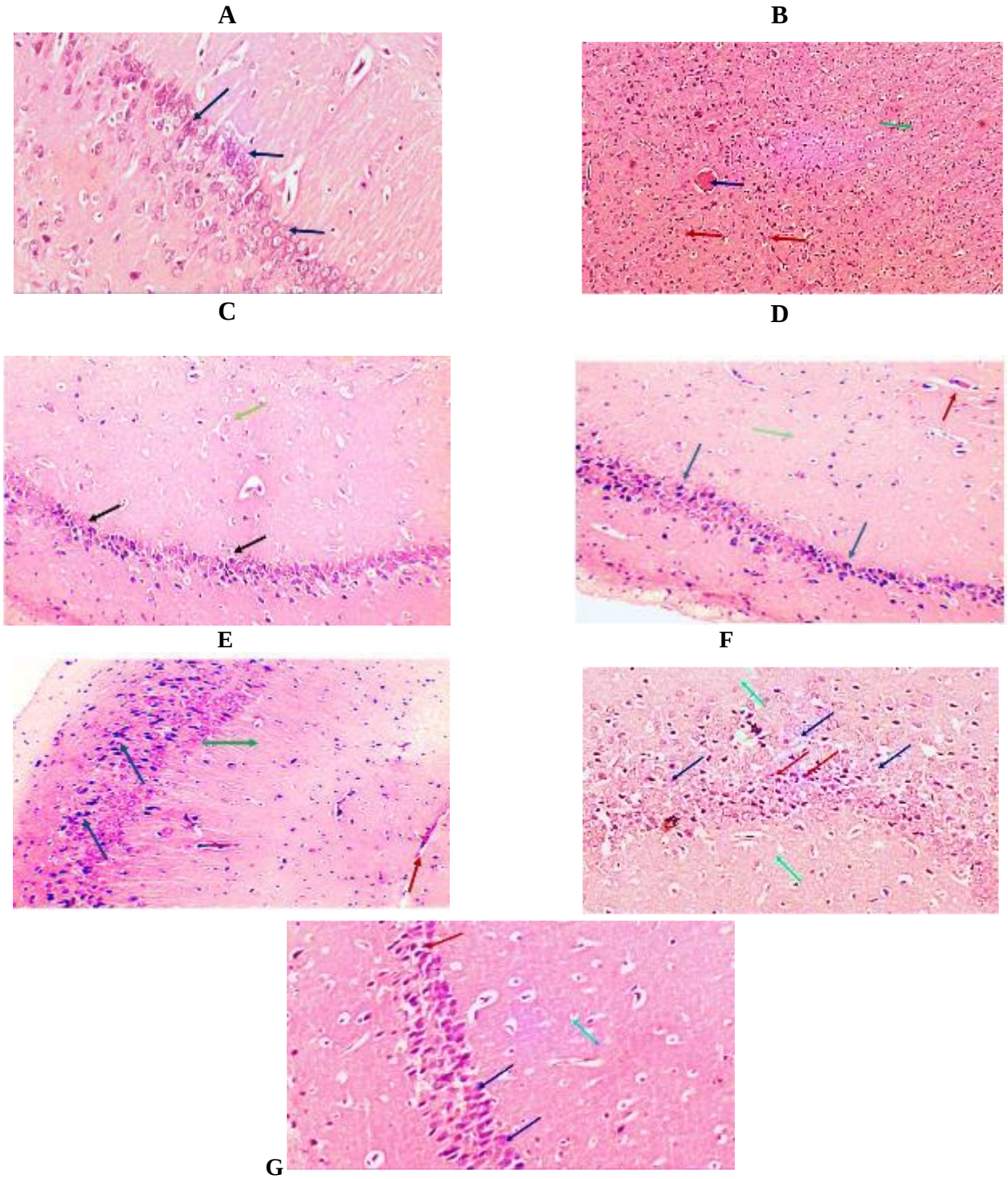

Figure 3: H&E stained hippocampus (H&E X100).

(A): Group 1 revealing normal pyramidal cell layer formed of densely packed rounded neurons containing large vesicular nuclei (blue arrows). (B): Group 2 showing many degenerated neurons with pyknotic nuclei (red arrows) and marked degenerated edematous stroma (green arrow) and congested blood vessels (blue arrows). (C): Group 3 showing some degenerated neurons with pyknotic nuclei (blue arrows) and moderate edematous stroma (green arrow). (D): Group 4 showing some degenerated neurons with pyknotic nuclei (blue arrows) and mild edematous stroma (green arrow) and mildly congested blood vessels (red arrows). (E): Group 5 showing some degenerated neurons with pyknotic nuclei (blue arrows) and mild edematous stroma (green arrows) and mildly congested blood vessels (red arrows). (F): Group 6 showing densely packed neurons with vesicular nuclei (blue arrows) with few degenerated neurons with pyknotic nuclei (red arrows) and mild edematous stroma (green arrows). (G): Group 7 showing densely packed neurons with vesicular nuclei (blue arrows) with few degenerated neurons with pyknotic nuclei (red arrow) and normal stroma without degeneration or edema or congestion (green arrow).

There was a significantly decreased in LC3-II scoring in the untreated VPA- induced autism group than the controls. Medication with risperidone revealed a non-significant difference than the untreated VPA-induced autism group. While treatment with apigenin or silymarin revealed a significant increase in comparison to the untreated VPA-induced autism group (**Figure 4**).

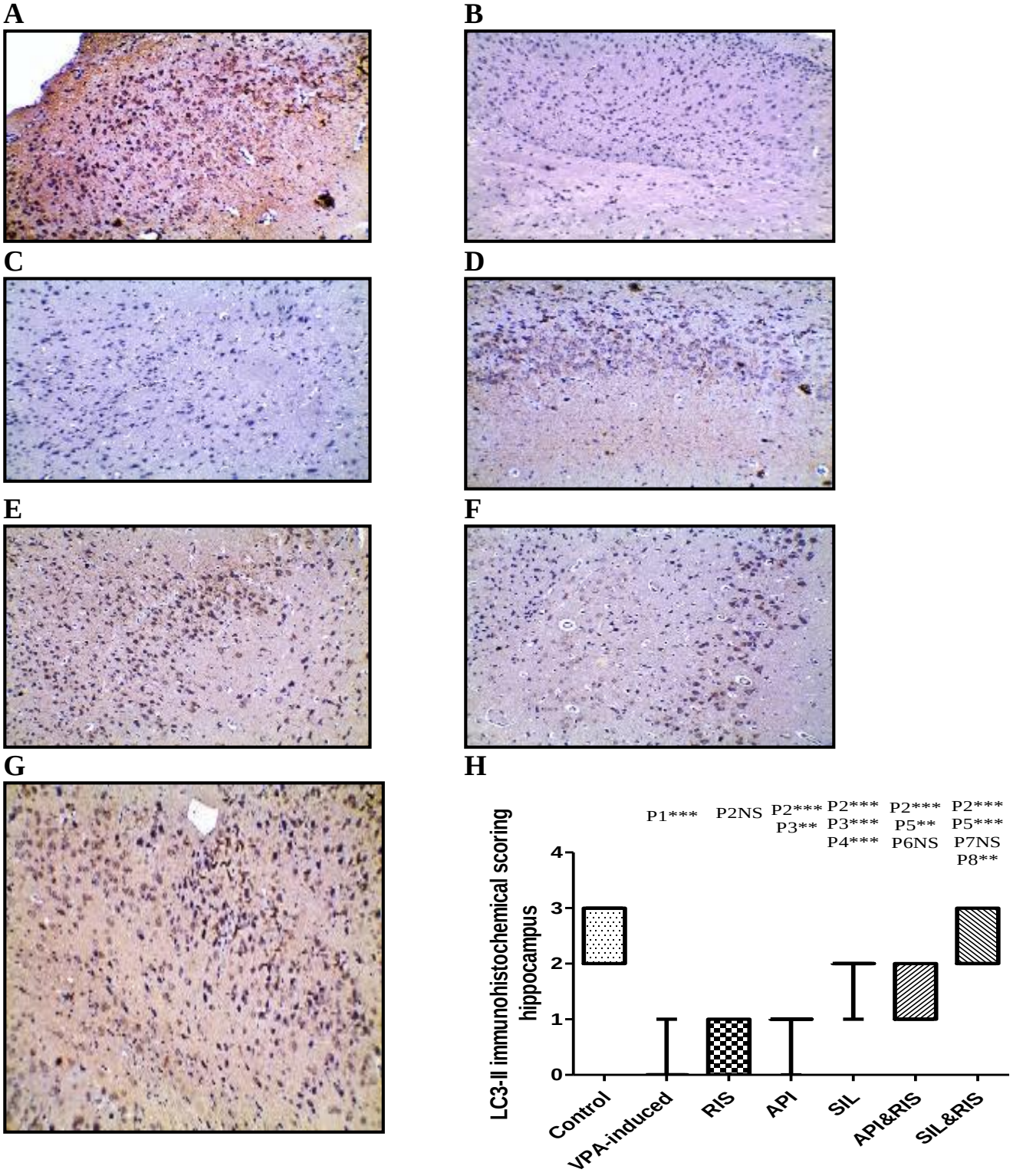

Figure 4: IHC expression of Lc3-II in hippocampus sections (X 200):
(A): Group 1 showing positive expression score 3. (B): Group 2 showing negative expression score 0. (C): Group 3 showing negative expression score 0. (D): Group 4 showing positive expression score 1. (E): Group 5 showing

positive expression score 2. (F): Group 6 showing positive expression score 1. (G): Group 7 showing positive expression score 2. (H): LC3-II scoring in hippocampus.

There was a significantly decreased in Bcl-2 scoring in the untreated VPA- induced autism group than the controls. Treatment with risperidone, apigenin or silymarin revealed a significant increase than the untreated VPA-induced autism group (**Figure 5**).

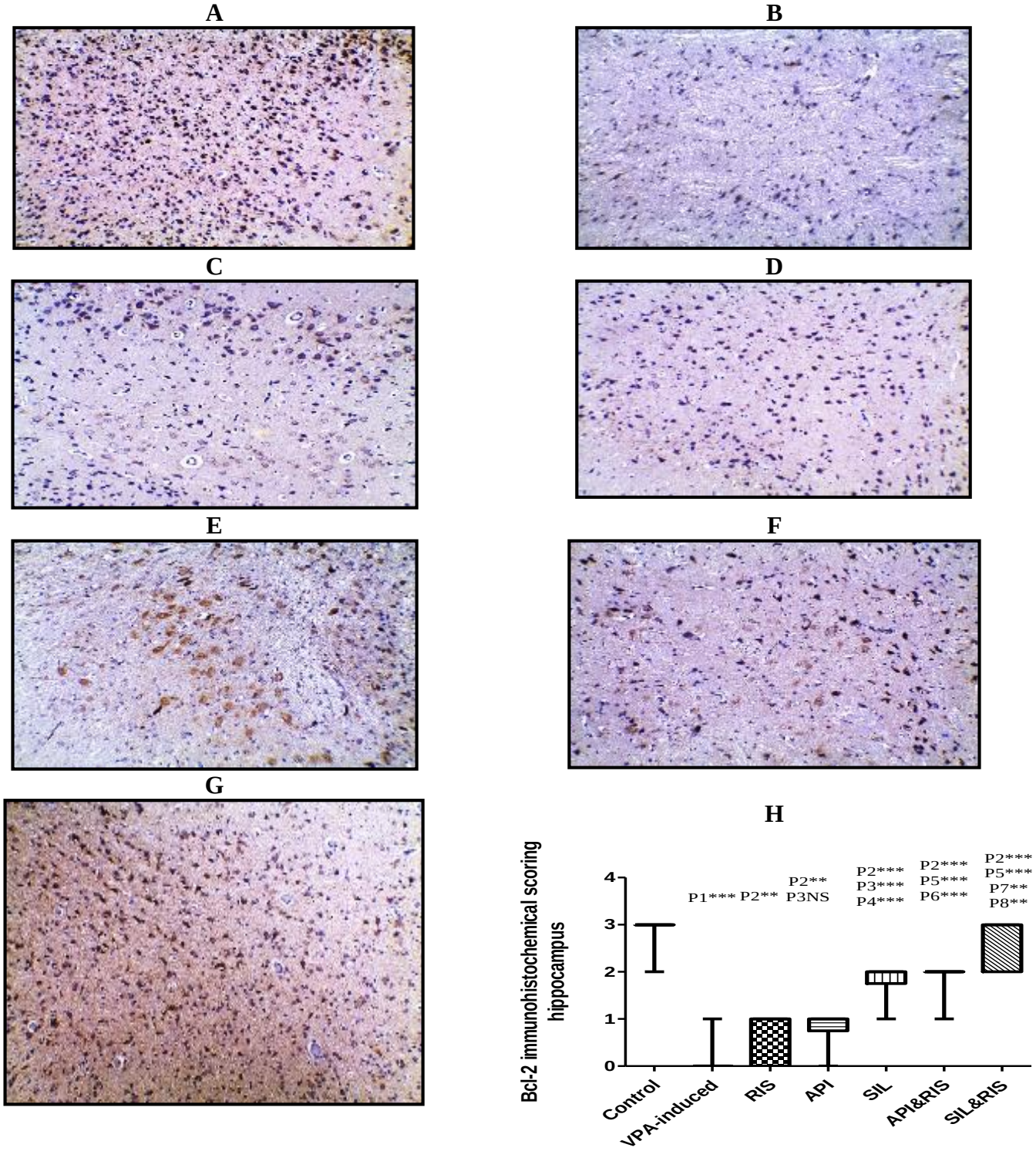

Figure 5: IHC expression of Bcl-2 in hippocampus sections (X 200):
(A): Group 1 showing positive expression score 3. (B): Group 2 showing negative expression score 0. (C): Group 3 showing positive expression score 1. (D): Group 4 showing positive expression score 1. (E): Group 5 showing positive expression score 2. (F): Group 6 showing positive expression score 2. (G): Group 7 showing positive expression score 3. (H): Bcl-2 scoring in hippocampus.

DISCUSSION

In the present work the untreated VPA induced autism group showed diminished social behavior and diminished social preference / novelty. These findings were in harmony with several preceding researches of VPA-induced autism in rodent models [21]. Risperidone, apigenin and silymarin groups showed an increase in social interaction and social preference. The results of T-maze in the untreated VPA induced autism group were in accordance with **Cezar *et al.*** [17] and **Kirsten *et al.*** [18]. Groups treated with risperidone or apigenin or silymarin showed significant increases in alternation score. This impact is agreed with preceding research [22-24] that observed that risperidone, apigenin and silymarin enhanced the behavior in T-maze.

In the present study, the hippocampus is used as it has an essential role in the spatial learning and memory processing, and it is a significant locus that is disrupted in autism [25].

Several evidences point to oxidative stress as a possible cause of neuronal injury in autism. Oxidative stress results in cell damage and may cause cell loss. In line with the present study **Zhang *et al.*** [26] showed that prenatal VPA-treated rats demonstrated an increase in MDA and reduced glutathione level. These findings clearly show an increased degree of oxidative stress in the prenatal VPA treated rats, which disrupts the early stages of brain development.

Several studies have discovered that the BDNF / Bcl-2 anti-apoptotic signaling pathway is impaired in autistic people [27].

The antioxidant effects of risperidone are in line with **Tendilla-Beltrán et al.** [28] who described the ability of risperidone to decrease oxidative stress in various brain regions. The anti-apoptotic effects of risperidone were reported also in several studies as **Abekawa *et al.*** [29] who stated the anti-apoptotic effect of risperidone in schizophrenia and suggested that risperidone can ameliorate the behavioral abnormalities and accompanied apoptosis. On the contrary with the results of the present study **da Cruz Jung *et al.*** [30] suggested that high concentrations of risperidone showed elevation of caspase-3 levels and downregulation of Bcl-2 in macrophage cells and they showed that the apoptotic effect of risperidone is cell type-dependent, and it may contribute to obesity occurrence and other endocrinal disorders caused by risperidone use.

The antioxidant effects of apigenin in nervous tissue were reported in several studies [31] that showed the ability of apigenin treatment to reverse the decreased GSH levels, and the increase of MDA level. In line with the present study, previous studies also showed that apigenin has an anti-apoptotic effect on different tissues including nervous tissues [32].

In this study, silymarin was preferred because of its natural source and its low toxicity even at high doses, as well as its entry into the brain. The antioxidant effects of silymarin are owing to its potential effects in reduction of lipid peroxidation, enhancing the GSH levels which protecting nervous tissue from oxidative stress and injury [33]. In addition, the anti-apoptotic effects of silymarin were reported as it was found that the anti-apoptotic effects of silymarin and silibinin lead to decrease neuronal degeneration and leads to improvement in cognitive deficits and memory impairment in rat models [34].

Neuronal autophagy is essential for neuron interaction and development, and changes in autophagy have a negative impact on neuron growth and function [6]. In the present work, there was a significant decrease in LC3-II immunohistochemical scoring and a significant increase in mTOR levels when compared to the control group which reflects deficient autophagy with increased mTOR signaling pathway. In agreement with the present study, **Zhang *et al.*** [5] showed that the VPA-induced autism group showed no autophagosome by electron microscope which increased by mTOR inhibitors. This study concluded that using mTOR inhibitors in VPA-exposed rat pups inhibits the mTOR signaling pathway thus increasing autophagy, inhibiting apoptosis, and improving social interaction, providing a new target and path for ASD treatment Furthermore, **Kirsten *et al.*** [18] showed that mTOR were increased in the striatum of autistic rats and their levels were decreased by postnatal zinc treatment which prevented cognitive and social impairments.

In line with the present study, several studies reported the effect of apigenin on autophagy as **Lu *et al.*** [35] who showed that apigenin increases autophagosomes formation and increases LC3-II expression while decreasing mTOR protein levels and P62 protein expression. They concluded that the hypolipidemic effect of apigenin is related to autophagic lipid degradation.

Furthermore, **Zhang *et al.*** [9] reported that apigenin stimulates autophagy by regulating mTOR / AMPK/ ULK1 signaling as it leads to decrease elevated mTOR

levels, enhance LC3-II levels, and downregulate the expression of P62 in hippocampal samples.

Regarding silymarin, it was found that silymarin and its active component silibinin can modulate autophagy in many studies [(10)]. **Song *et al.*** [(10)] showed that silibinin exerts neuroprotective effects in Alzheimer's disease by increasing the level of autophagy. They showed that silibinin increases the levels of LC3-II& increases autolysomses and autophagic vacuoles & they concluded that silibinin exerts its neuroprotective effects by modulating the impaired autophagy in this model. Additionally, several studies showed that silymarin inhibit the mTOR signaling pathway [(11)].

CONCLUSION

These findings suggest that apigenin and silymarin produce promising effects in VPA-induced autism as they decrease oxidative stress and apoptosis and produce promising effects regarding modulation of autophagy with inhibition of mTOR, that are all reflected as amelioration of impaired behavior and improvement of the histopathological picture in both the hippocampus and the cerebellum. Moreover, the use of apigenin or silymarin in combination with risperidone exhibits better and more satisfactory results than in either remedy alone. These results encourage the use of apigenin or silymarin as an adjuvant therapy to risperidone. However, this would be confirmed in further clinical studies.

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Author contribution: Authors contributed equally in the study.

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