

Effects of *Stevia rebaudiana* Bertoni extracts in the rat model of epilepsy induced by pentylenetetrazol: Sirt-1, at the crossroads between inflammation and apoptosis

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The current study investigated the effects of stevia extracts on a PTZ-induced epileptic rat model and its potential mechanism. Thirty male Sprague-Dawley rats were equally subdivided into 3 groups; (1) normal control (NC) group, (2) PTZ-group: received PTZ (50 mg/kg, i.p. every other day) for 2 weeks, and (3) PTZ+ Stevia group: received PTZ and stevia (200 mg/kg orally daily) for 4 weeks (2 weeks before the start of PTZ treatment and 2 weeks with PTZ administration). The first jerk latency and the seizure score were assessed in rats. Also, brain tissue samples were collected by the end of the experiment, and oxidative stress markers (catalase, MDA, and total antioxidant capacity (TAC)) were measured by biochemical analysis in hippocampal brain homogenates. Also, in the hippocampus, the expression of IL6 and Bcl-2 at the mRNA level and expression of Sirt-1, P53, caspase-3, GFAP, and NF- κ B in CA3 hippocampal region by immunohistochemistry was investigated. PTZ substantially increased the seizure score and decreased the seizure latency. Also, PTZ significantly increased MDA, GFAP, IL-6, NF- κ B, caspase-3, and p53 and significantly reduced Sirt-1, TAC, and Bcl-2 in hippocampal tissues compared to the control group ($p < 0.01$). However, *Stevia Rebaudiana* Bertoni (*Stevia R.*) significantly attenuated the PTZ-induced seizures, improved oxidative stress markers, downregulated GFAP, IL-6, NF- κ B, caspase-3, and p53, and upregulated Sirt-1 and Bcl-2 in the CA3 hippocampal region ($p < 0.01$). In conclusion, *Stevia R.* exhibits neuroprotective and antiepileptic actions in PTZ-induced epilepsy due to its antioxidant, anti-apoptotic, and anti-inflammatory effects. Additionally, the Sirt-1 pathway might be involved in the antiepileptic and neuroprotective effects of stevia in PTZ-kindled epileptic rat model.

Keywords

Sirt-1; Pentylenetetrazole; Epilepsy; p53; Caspase-3; IL-6; Oxidative stress

1. Introduction

Epilepsy is a serious neurological disorder that impacts about 0.5%–2% of the world's population [1]. Intractable forms of epilepsy trouble approximately 30% of patients [2].

Moreover, associated comorbidities such as cognitive impairment burden patients with epilepsy and markedly influence the quality of life [3]. Therefore, a thorough grasp of the mechanisms behind epileptogenesis would open new avenues for novel anti-epileptic agents. Previous experimental studies have documented the role of oxidative stress, inflammatory cytokines, apoptosis, and autophagy in epileptogenesis, so therapeutic agents that target those mechanisms are considered good antiepileptic candidates [4–6].

Silent information regulator-1 (Sirt-1) is the first gene found in the mammalian sirtuins family genes. Normally, it is located in the nucleus and acts as a histone deacetylase of NAD^+ . It interacts with several protein substrates in many signaling pathways (such as Wnt, NF- κ B, p53, and Notch), so it is a crucial regulator of several biological processes, e.g., cell proliferation, differentiation, senescence, apoptosis, and metabolism [7, 8]. In the CNS, Sirt-1 is expressed in both neurons and astrocytes. Inflammation-associated diseases, including various neurodegenerative ones, exhibit a reduction in the Sirt-1 level. Also, a reduction in the Sirt-1 expression could promote microglial activation and neuroinflammation via NF- κ B, IL-6, and IL-1 β [9]. Sirt-1 exerts neuroprotective effects in many models of microglial activation-induced neurodegenerative disease [10]. Moreover, Sirt-1 mitigates glucose-induced neuronal apoptosis through p53 deacetylation [11]. In addition, it has been reported that up-regulation of Sirt-1 is essential for neuronal survival by inhibiting apoptosis, inflammation and oxidative stress via the deacetylation of p53, NF- κ B, and FoxOs respectively [12].

Nowadays, herbal supplements and alternative medicine represent a new era in the prevention and treatment of diseases. *Stevia Rebaudiana* Bertoni (*Stevia R.*) is a bush of the Asteraceae family local to South America, particularly to the

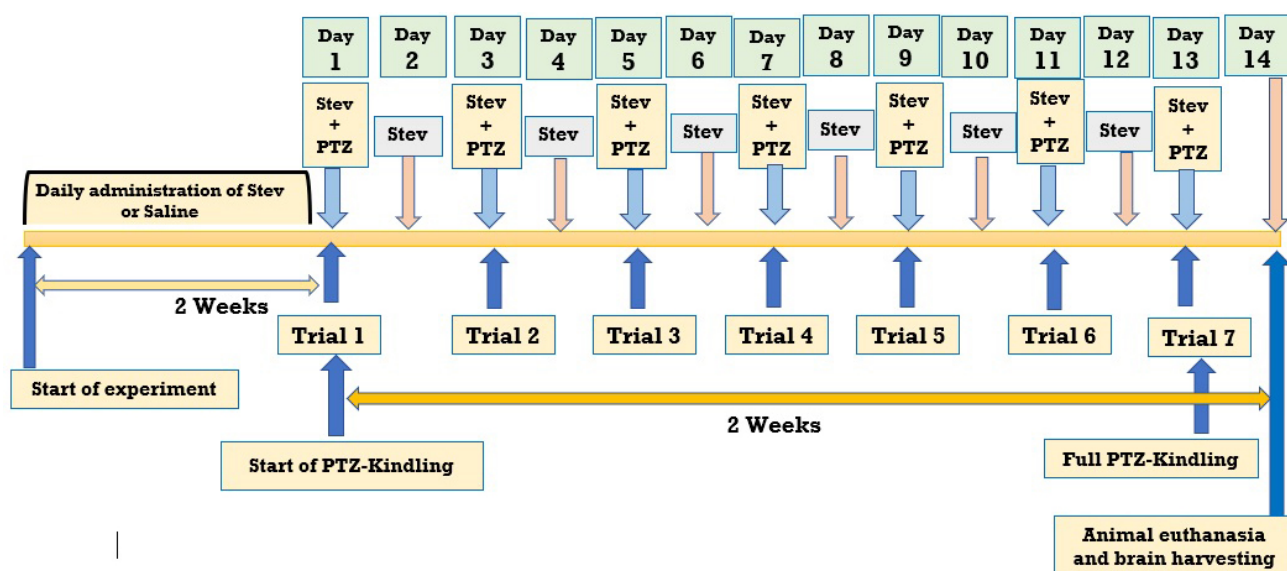


Fig. 1. Study design of the experiment. Saline 0.5 mL or stevia extracts dissolved in 0.5 mL was given daily via gastric gavage for 4 weeks (2 weeks before and 2 weeks after PTZ administration). But PTZ was given in 0.2 mL saline in PTZ and PTZ+ Stevia group every other day. On the day of PTZ injection, Stevia was given one hour before PTZ injection. Also, the rat behavior was recorded for 30 min after PTZ injection (trial or record), so we have 7 trials or records, trial 1 at day 1, trial 2 at day 3, trial 3 at day 5, trial 4 at day 7, trial 5 at day 9, trial 6 at day 11 and trial 7 at day 13.

Northeast of Paraguay. Its leaves are commonly used as a powerful alternative to artificial sweeteners [13]. Besides their sweetening properties, stevia extracts possess many potential therapeutic effects such as hypotensive, hypoglycemic, anti-inflammatory, anti-tumor, and immunomodulatory effects [14]. Also, stevia contains several phenolic compounds such as flavonoids, and phenolics which possess important antioxidant properties [15]. Few studies have investigated the neuroprotective effects of stevia extract and its derivatives against scopolamine-induced learning and memory deficits [16] and fructose-induced neuronal damage in the hippocampus, amygdala, and spinal cord [17]. Moreover, Rebaudioside A (derivative of stevia) significantly attenuated the PTZ-induced convulsive and EEG changes [18]. Mei *et al.* [19] concluded that Isosteviol sodium (a derivative of stevia) protects against oxidative stress and apoptosis in cardiomyocytes via activation of the Sirt-1/PGC-1 α pathway. However, the role of Sirt-1 in the possible anti-epileptic action of stevia was not investigated. Therefore, the current study was designed to look into the possible antiepileptic and neuroprotective effects of *Stevia R.* extracts against PTZ-induced epilepsy and the potential role for astrogliosis, oxidative stress, apoptotic proteins, inflammatory cytokines, and Sirt-1 in arbitrating its effects.

2. Materials and methods

2.1 Experimental animals

Thirty male Sprague-Dawley rats weighing 170–190 g were housed in standard cages at the experimental research center, Mansoura Faculty of Medicine, Egypt. Animals were fed on a standard diet and water *ad libitum*. Animal care was done in agreement with *The Care and Use of Laboratory*

Animals (1996, distributed by the National Academy Press, 2101 Constitution Ave. NW, Washington, DC20055, USA). The IRB committee has approved all experimental procedures (code #R.20.11.1098).

2.2 Extraction and administration of *Stevia R.* leaves extracts

The leaves of *Stevia R.* plants were purchased from Stevia International Company from Agro-industry Product (SICAP), Cairo, Egypt. All steps of isolation and methanolic extraction of *S. Rebaudiana* leaves were mentioned in a previous work by our research group [20]. The methanolic extracts of *Stevia R.* were dissolved in 1.0 mL saline at a dose of 200 mg/kg orally via gastric gavage once daily for 4 weeks (2 weeks before PTZ injection and 2 weeks after PTZ injection) [21].

2.3 Study design

Rats were randomly (closed sealed envelopes) subdivided into 3 equal groups (each contains 10 rats) as follows: (a) Control group: normal animals received 0.5 mL saline via gastric gavage for 4 weeks with 0.2 mL saline via intraperitoneal injection (i.p.) for the last 2 weeks, (b) PTZ group: as the control group but rats received PTZ (50 mg/kg in 0.2 mL saline) via i.p. injection on an alternate day for the last 2 weeks of the experiment [6] and (c) *Stevia R.* extract (St)+PTZ group: as the PTZ group but rats were pretreated with Stevia in a dose of 200 mg/kg daily for 4 weeks (2 weeks before the onset of PTZ treatment and 2 weeks with PTZ administration) [21]. Stevia was given one hour before PTZ on the day of PTZ administration. Fig. 1 shows the study design.

2.4 Animal model

After each PTZ injection, each rat was placed in a transparent Plexiglas cage to record its convulsive behavior for 30 min by a video camera for 7 records or trials. The latency of seizure onset (sec) and seizure score were recorded. Scoring of seizure severity was done based on Racine's scale (0 = normal, non-epileptic activity, 1 = mouth and facial movements, hyperactivity, grooming, sniffing, scratching, wet dog shakes, 2 = head nodding, staring, tremor, 3 = forelimb clonus, forelimb extension, 4 = rearing, salivating, tonic-clonic activity and 5 = falling, status epilepticus) [22].

2.5 Harvesting of brain tissue samples

Once the study has ended, a high dose of Na⁺ thiopental (120 mg/kg) i.p. was used for rat sacrifice. Through a cardiac catheter, the brain was perfused with 100 mL heparinized saline then by 150 mL formalin (10%). Details of the collection and storing of the brain tissue are described in our preceding work [6].

2.6 Assay of oxidative stress markers (MDA, catalase activity, and total antioxidants) in hippocampal brain tissue

Hippocampal regions of the brain were homogenized in 1–2 mL of cold phosphate-buffered saline (50 mM) in EDTA (1 mM) at pH 7.5 then, centrifuged at 4000 rpm at 4 °C for 15 min. Colorimetric assay of these markers was implemented using commercially available kits (Bio-Diagnostics, Dokki, Giza, Egypt) according to the manufacturer's instructions.

2.7 Assessment of the expression of Bcl-2 and IL-6 at the mRNA level by real-time PCR

The mRNA encoding for pro-inflammatory cytokine; interleukin-6 (IL-6) and anti-apoptotic genes (Bcl-2) was identified by real-time PCR in hippocampal brain tissue samples. In agreement with the manufacturer's guidelines, total RNA from hippocampal brain tissue samples was isolated, then quantified spectrophotometrically, and its quality was determined by agarose gel electrophoresis and ethidium bromide staining. cDNA was synthesized from 1 µg total RNA and then buffered in a volume of 25 µL. Then, the 25 µL cDNA was diluted in a total volume of 100 µL. PCR was used to amplify the cDNA for IL-6 F: 5'-TCCTACCCCAACTTCCAATGCTC-3', R: 5'-TTGGATGGTCTTGGTCCTTAGCC-3', and Bcl2 F: 5'-TGTGGATGACTGACTACCTGAACC-3', R: 5'-CAGCCAGGAGAAATCAAACAGAGG-3', GAPDH, F: 5'-TATCGGACGCCTGGTTAC-3', R: 5'-CTGTGCCGTTGAACTTGC-3'. Amplification and detection were performed using a thermal cycler. The cycling parameters were as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation 95 °C for 15 s, annealing at 60 °C for 1 min, extension at 72 °C for 1 min. The cDNA was quantified using the following formula: $2^{-Ct} (2 - [(Ct \text{ of target gene} - Ct \text{ of GAPDH in treated rats}) - (Ct \text{ of target gene} - Ct \text{ of GAPDH in control rats})])$ [23].

2.8 Hematoxylin and eosin staining

Brain sections of 20 µm-thickness were made from formalin fixed brain tissues. Slides containing hippocampal regions were stained with hematoxylin for 15 min and in HCl alcohol solution for 35 sec, then, immersed with eosin for 10 min and 90% ethanol for 40 sec [6]. The hippocampal regions mainly CA3 region were examined blindly by expert pathologists under light microscope for loss of neurons and pyknotic nuclei.

2.9 Immunohistochemical examination for GFAP, NF-κB, P53, caspase-3, and Sirt-1 in the hippocampal CA3 region

Serial sagittal sections (40 µm) were obtained from brain tissue specimens and immunohistochemical staining was implemented as stated by our previous work [5]. The primary rabbit anti-glial fibrillary acidic protein (GFAP) antibody (1:200, Sigma, USA), primary rabbit polyclonal anti-NFκB-p65 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) (with final dilution 1:500), goat polyclonal anti-Sirt-1 (Santa Cruz sc-19857, Santa Cruz, CA, USA) (with final dilution 1:200), caspase-3 primary rabbit polyclonal anti-caspase-3 (Cell Signalling Technology® Cat. no. 9664). (diluted 1:50) and p53 rabbit anti-rat monoclonal antibodies anti-p53 (1:50; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) (with final dilution 1:50) were used. Briefly, the brain areas were brooded overnight at 4 °C with primary antibody taken after by a 15 min wash in phosphate-buffered saline (PBS, pH 7.4). After that the brain sections were hatched with horseradish peroxidase conjugated IgG for 60 min at room temperature. After washing for an hour, diaminobenzidine was utilized as a chromogen and counterstaining was done with hematoxylin. The negative control was performed without including the primary antibody, and the other steps were the same between the test sections and negative control. The hippocampus mainly CA3 region was examined indiscriminately utilizing Optika light microscope with Optika B-10 (OPTIKA Microscopes, Italy) (camera and the average of the number of positive cells in 5 random high-power field (HPF) in serial 5 brain slides was calculated. For the expression of Sirt-1 in CA3 region, the immunoreactivity of Sirt-1-positive cells in the region of interest (ROI) (CA3) was calculated using ImageJ software (National Institute of Mental Health Bethesda, Maryland, USA) as mentioned in our previous work [5]. The expression of p53, caspase-3, and NF-κB was nuclear, whereas the expression of Sirt-1 and GFAP was cytoplasmic and membranous in cell bodies and neurites. Previous studies by our research group found that the CA3 hippocampal region is the most affected region in PTZ-induced epilepsy, so we selected this region for investigating the expression of these proteins in this region [5, 6].

2.10 Statistical analysis

Statistical analysis was done by GraphPad Prism version 5.0 (GraphPad Software, Inc., California Corporation, USA). The statistical significance within groups for behavioral parameters was calculated using two-way ANOVA with Bon-

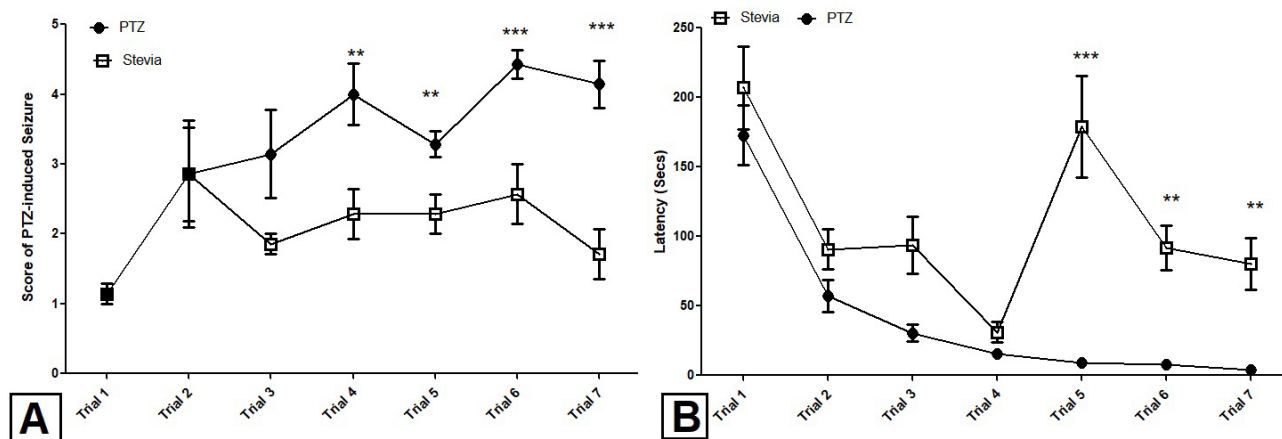


Fig. 2. Effect of Stevia R. on behavioral effects in PTZ-induced seizures. (A) Seizure score and (B) seizure latency (Sec). Data were expressed as mean \pm SEM (standard error of means) Two-way ANOVA test. *** significant vs PTZ group $p < 0.0001$, ** significant vs PTZ group $p < 0.01$ and *significant vs PTZ group $p < 0.05$. Trial 1 = record with the first PTZ dose, Trial 2 = record with the second PTZ dose, etc. and Trial 7 = record with the last or 7th dose of PTZ.

Table 1. Oxidative stress markers in hippocampal brain tissues from control, PTZ and Stevia groups.

	MDA (nmol/g tissues)	CAT (U/g tissues)	TAC (mmol/g tissues)
Control group	50.76 \pm 1.77	67.67 \pm 9.97	4.647 \pm 0.41
PTZ group	81.788 \pm 1.84***	52.08 \pm 9.36*	1.907 \pm 0.40*
Stevia group	42.68 \pm 4.617###	95.25 \pm 11.21***,###	6.300 \pm 0.83***
<i>p</i> (One-Way ANOVA)	F = 45.90	F = 27.49	F = 14.55
	<i>p</i> < 0.0001	<i>p</i> < 0.0001	<i>p</i> < 0.0001

All data are expressed as mean \pm SEM, One-way Anova with Tukey's post hoc test. $p < 0.05$ was considered significant. ***significant vs control group, $p < 0.0001$, **significant vs control group, $p < 0.01$, *significant vs control group, $p < 0.05$, ###significant vs PTZ group, $p < 0.0001$, ##significant vs PTZ group, $p < 0.01$ and*significant vs PTZ group, $p < 0.05$. MDA, malondialdehyde; CAT, catalase enzyme activity; TAC, total antioxidant capacity.

ferroni post-hoc test, while in other parameters we used one-way ANOVA with Tukey's post-hoc test to find the statistical significance. Also, Pearson correlations between Sirt-1 expression and other study parameters were calculated. $p \leq 0.05$ is considered statistically significant.

3. Results

3.1 Stevia effect on seizure score and latency in PTZ-induced epilepsy

The PTZ group showed an increase in the score of PTZ-induced seizures starting from the 2nd trial compared to the 1st trial. Also, in comparison to the PTZ group, the stevia group showed a significant reduction in the PTZ-induced seizure score in trials 4 (day 7) and 5 (day 9) ($p < 0.001$) and trials 6 (day 11) and 7 (day 13) ($p < 0.0001$) (Fig. 2A). Also, the latency of epileptic seizures showed significantly longer values in the stevia group compared to the PTZ group in trials (5, 6 and 7) ($p < 0.001$) (Fig. 2B).

3.2 Stevia effect on MDA, catalase activity, and TAC in hippocampal brain tissue

The concentration of MDA was significantly higher in hippocampal tissues of the PTZ group than in the control

group ($p < 0.0001$). However, the stevia group displayed a significantly lower MDA concentration than the PTZ group ($p < 0.01$). The TAC level was significantly lower in the PTZ group compared to the control group ($p < 0.0001$). On the other hand, the stevia group showed a significant increase in the TAC compared to the PTZ group ($p < 0.0001$). The catalase enzyme activity showed a significant reduction in the PTZ group contrasted with the control group ($p < 0.0001$). However, the stevia group showed a significant increase in the CAT activity contrasted with the PTZ group (Table 1).

3.3 Stevia effect on Bcl-2 and IL-6 expression at the mRNA level in rat hippocampus

The expression of Bcl-2 at the mRNA level in the hippocampal tissue showed a significant decrease in the PTZ group compared to the control group ($p < 0.0001$). In contrast, stevia group showed a significant increase in its expression compared to the PTZ group ($p < 0.05$). Regarding the expression of IL-6 in the hippocampus, it increased significantly in the PTZ group in contrast to the control group ($p < 0.0001$). However, the stevia group displayed a significant drop in the IL-6 expression in comparison to the PTZ group ($p < 0.0001$) (Table 2).

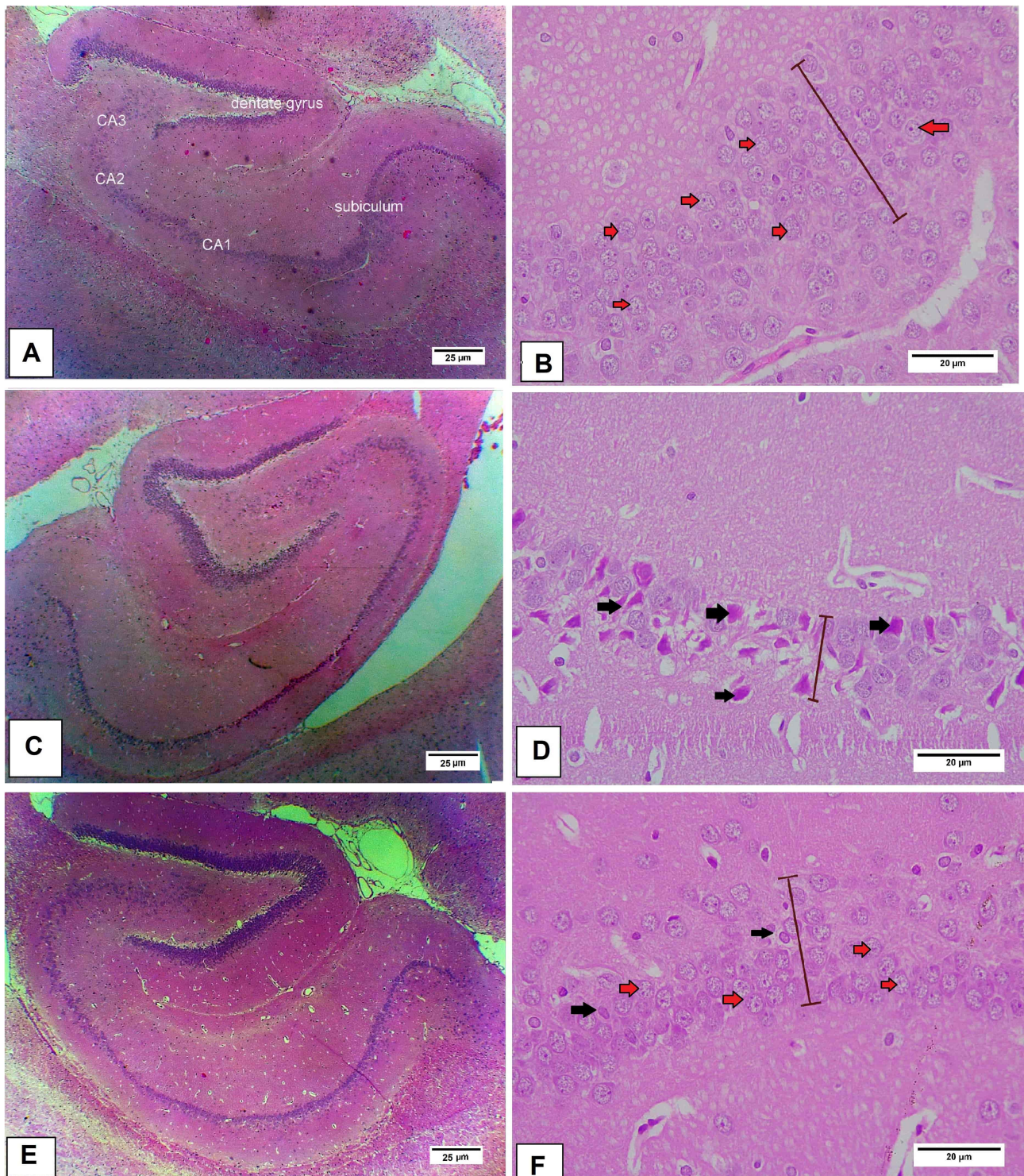


Fig. 3. Effect of *Stevia R.* on the morphology of the CA3 hippocampal region from different groups. The brain specimens from normal control group show (A) normal structure and thickness of hippocampal regions (CA3, 2, 1) (H&E, 100 \times) and (B) normal shape and distributions of neurons with round nuclei and prominent nucleoli (red arrows) in CA3 region (H&E, 400 \times), (C) while the brain specimens from PTZ group show atrophy and thinning of layers of CA3 region of hippocampus (black arrow) (H&E, 100 \times), (D) and low number of round normal cells (red arrows) with dense pyknotic nuclei in died neurons in CA3 region (black arrows) in PTZ group (H&E, 400 \times). (E) On the other hand, brain specimens from stevia group show normal structure and thickness of CA3 hippocampal region (red arrow) (H&E, 100 \times) and (F) high number of normal neurons with round nuclei and prominent nucleoli (red arrows) with few dead neurons (black arrows) in CA3 region (H&E, 400 \times). Also, the thickness of the CA3 region in figures with high power (400 \times) is represented by the blacklines.

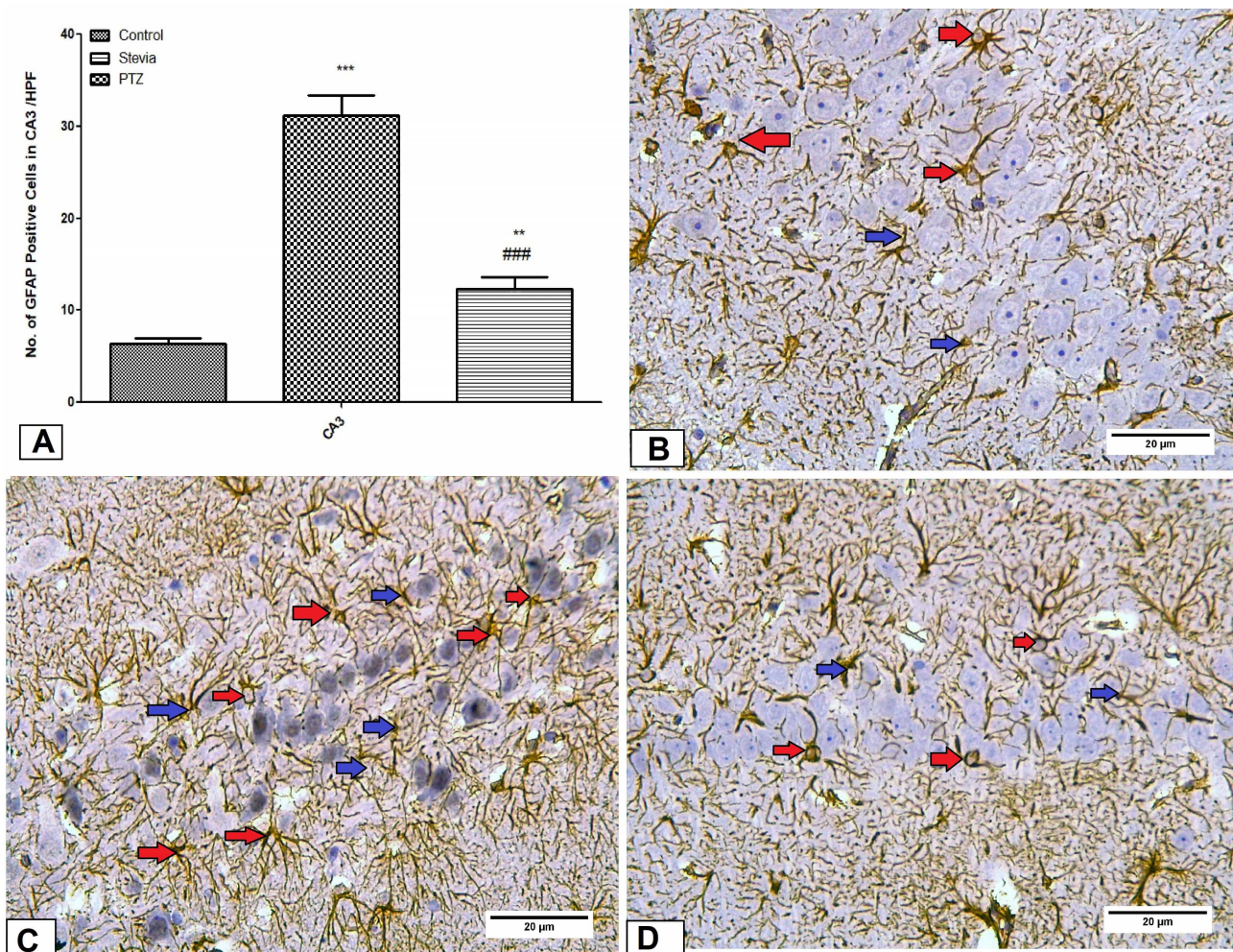


Fig. 4. Effect of Stevia on GFAP (marker of astrocytes and astrocytosis) expression in the CA3 hippocampal region. Graph (A) represents the mean of GFAP-positive cells per high power field (HPF) in the CA3 region in different groups. Brain specimens from the (B) normal control group show normal expression of GFAP in CA3 region of hippocampus (red arrows indicate cell bodies, while blue arrows indicate cell processes) (400 \times), (C) PTZ group show marked GFAP expression in CA3 hippocampal region (astrocytosis) (excess number and cell processes of astrocytes) (400 \times) and (D) stevia group show decreased GFAP expression in CA3 hippocampal region. One-way ANOVA with Tukey posthoc test. ***significant vs control group $p < 0.0001$, **significant vs control group $p < 0.01$ and ###significant vs PTZ group $p < 0.0001$.

Table 2. The relative expression of Bcl2 and IL6 (at mRNA) in hippocampal regions from control, PTZ and Stevia groups.

	Bcl2	IL6
Control group	0.963 \pm 0.043	0.96 \pm 0.1042
PTZ group	0.052 \pm 0.0067***	1.28 \pm 0.093**
Stevia group	0.896 \pm 0.16***	0.56 \pm 0.037***,###
p (One-Way ANOVA)	F = 27.95 $p < 0.0001$	F = 33.64 $p < 0.0001$

All data are expressed as mean \pm SEM, One-way Anova with Tukey's post hoc test. $p < 0.05$ was considered significant. ***significant vs control group, $p < 0.0001$, **significant vs control group, $p < 0.01$, *significant vs control group, $p < 0.05$, ###significant vs PTZ group, $p < 0.0001$, ##significant vs PTZ group, $p < 0.01$ and *significant vs PTZ group, $p < 0.05$. IL6, interleukin-6.

3.4 Stevia effect on the hippocampus histopathology

The control group exhibited normal neuronal shape and number in the hippocampal CA3 region on histopathological examination (Fig. 3A–B). However, PTZ disrupted the neuronal regular arrangement, reduced their number and resulted in pyknotic changes (darkly stained nucleus and cytoplasm) in the CA3 hippocampal region (Fig. 3C–D). Conversely, stevia displayed a significant reduction in the number of abnormal neurons in the in CA3 hippocampal region (Fig. 3E–F). Moreover, the thickness, which is represented by black lines in high power figures (400 \times) was roughly wide in control and decreased in the PTZ group and increased in the stevia group.

3.5 Stevia effect on GFAP and Sirt-1 expression by immunohistochemistry in the hippocampal CA3 region

Fig. 4A displayed a marked rise in the number of GFAP-positive cells (a marker of astrocytosis) in the hippocampal

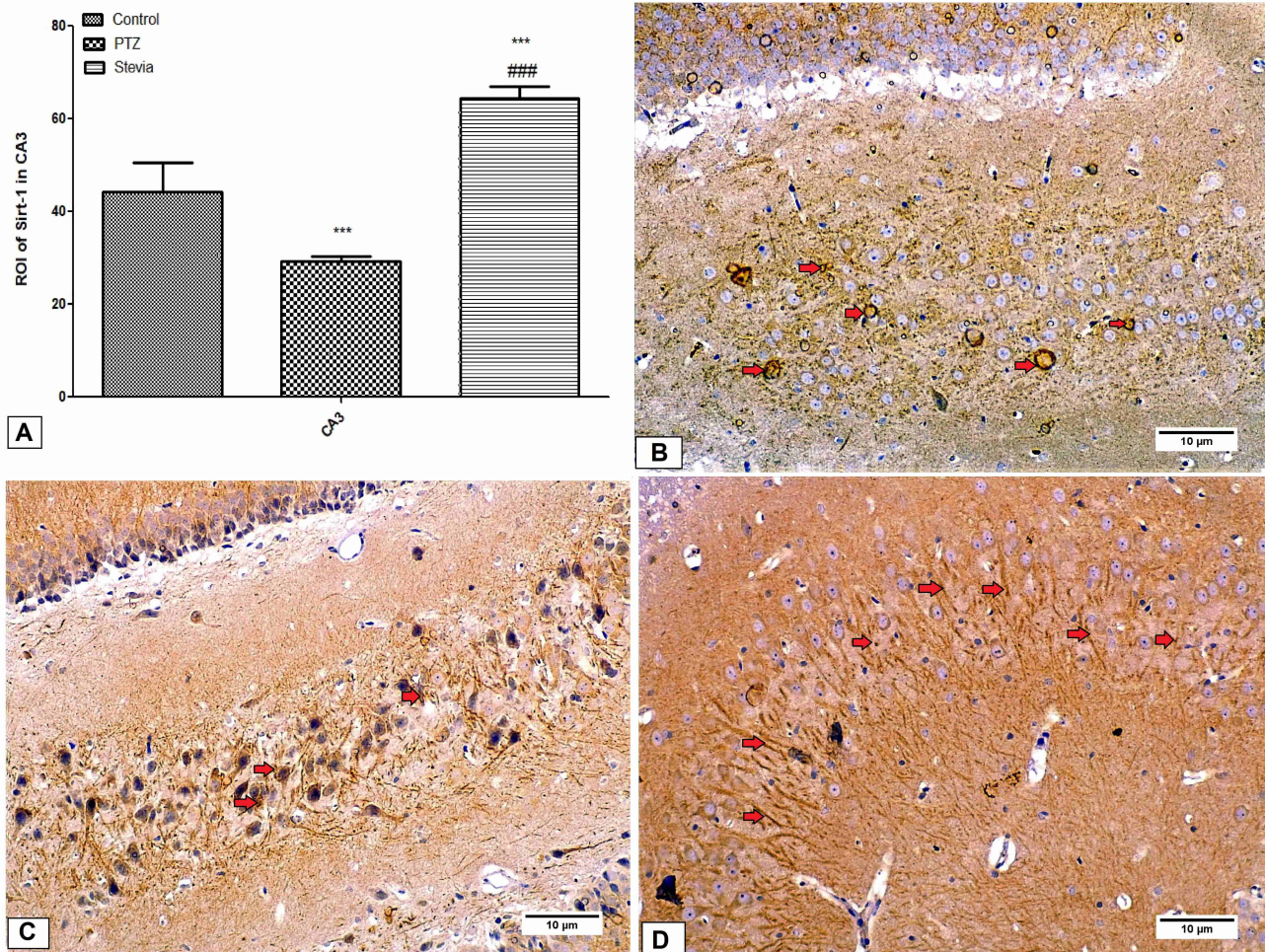


Fig. 5. Effect of Stevia on Sirt-1 expression in the CA3 hippocampal region by immunohistochemistry. Graph (A) represents the mean area of interest (ROI) of Sirt-1 expression per high power field (HPF) in different groups. Brain specimens from the (B) normal control group show moderate membranous expression of Sirt-1 (red arrows) in the CA3 region of the hippocampus (400 \times), (C) PTZ group show mild to moderate membranous (red arrows) Sirt-1 expression in the CA3 hippocampal region (400 \times) and (D) Stevia group show marked membranous (red arrows) Sirt-1 expression in the CA3 hippocampal region. One-way ANOVA with Tukey posthoc test. ***significant vs control group $p < 0.0001$ and ###significant vs PTZ group $p < 0.0001$.

CA3 region in the PTZ group in contrast to the control group ($p < 0.0001$). On the other hand, the number of positive cells was significantly reduced in the stevia group compared to the PTZ group ($p < 0.001$). The control group showed mild GFAP expression (Fig. 4B), the PTZ group showed a marked GFAP expression (Fig. 4C) suggesting astrocytosis, and the stevia group showed mild GFAP expression (Fig. 4D).

Moreover, PTZ markedly lowered the mean ROI of Sirt-1 positivity in the hippocampal CA3 region in contrast to the control group ($p < 0.001$). However, stevia significantly enhanced the Sirt-1 expression contrasted with the control group ($p < 0.01$) (Fig. 5A). Brain specimens showed moderate membranous expression of Sirt-1 in the control group (Fig. 5B), mild expression in the PTZ group (Fig. 5C), marked membranous expression in the stevia group (Fig. 5D).

3.6 Stevia effect on caspase-3, p53, and NF- κ B expression by immunohistochemistry in the hippocampal CA3 region

PTZ substantially increased the number of caspase-3 and p53-positive cells (markers of apoptosis) in the hippocampal CA3 region in contrast to the control group ($p < 0.001$). In contrast, stevia significantly reduced the expression of caspase-3 and p53 compared to the PTZ group ($p < 0.001$) (Figs. 6A,7A). The control group exhibited a mild nuclear expression of caspase-3 in the hippocampal CA3 region (Fig. 6B), the PTZ group exhibited a high nuclear expression (Fig. 6C), and the stevia group exhibited a mild nuclear expression (Fig. 6D). Also, brain sections from different groups showed mild cytoplasmic expression of p53 in the CA3 region of the hippocampus in the control group (Fig. 7B), high cytoplasmic expression in the PTZ group (Fig. 7C), and mild cytoplasmic expression in the stevia group (Fig. 7D).

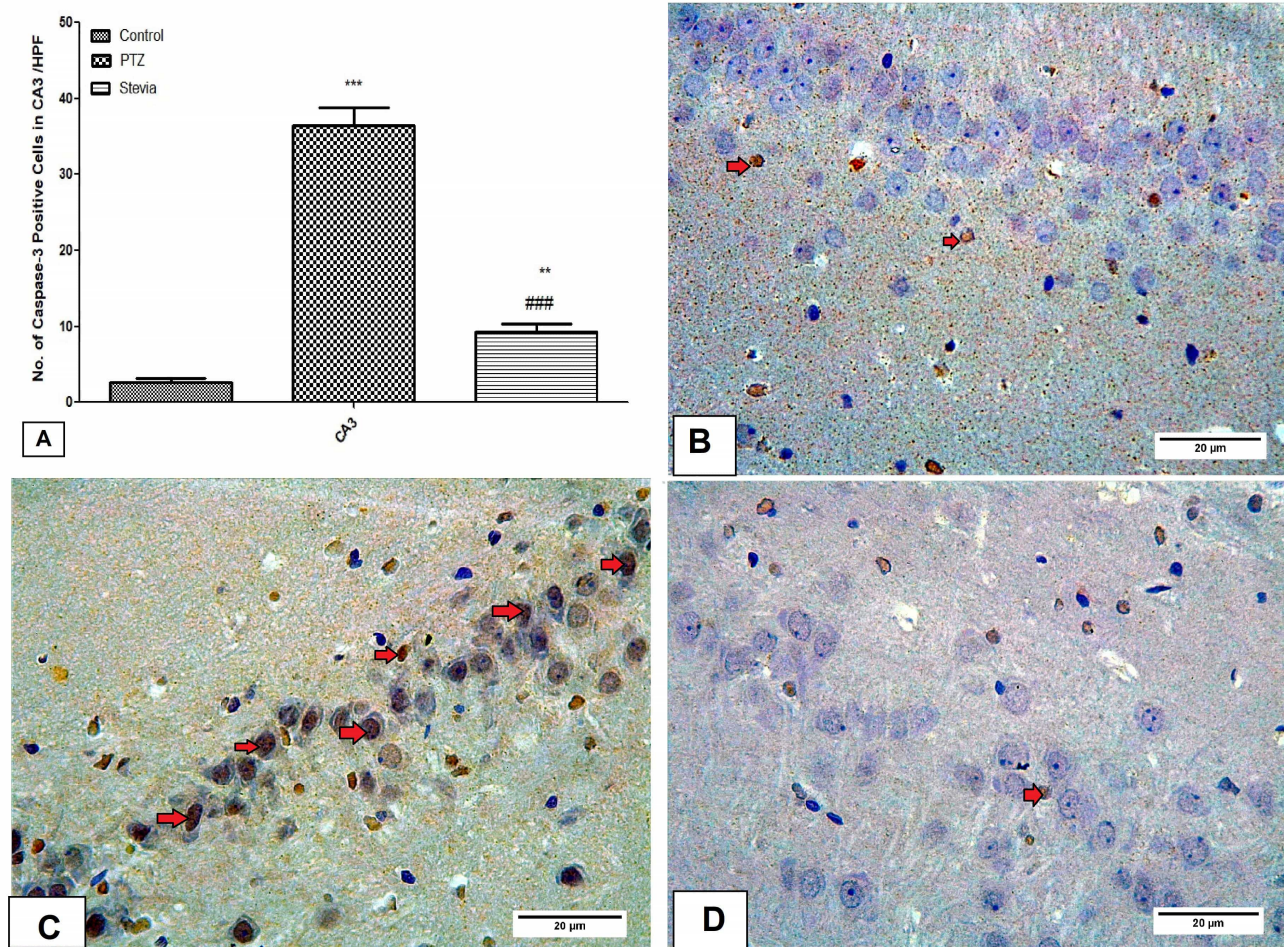


Fig. 6. Effect of Stevia on apoptotic marker (caspase-3) in the CA3 hippocampal region by immunohistochemistry. Graph (A) represents the mean of caspase-3-positive cells per high power field (HPF) in different groups. Brain specimens from the (B) normal control group show minimal nuclear expression of caspase-3 in the different parts of the hippocampus (red arrows) (400 \times), (C) PTZ group show marked nuclear caspase-3 expression in the CA3 hippocampal region (400 \times) and (D) Stevia group show minimal nuclear caspase-3 expression in the CA3 hippocampal region. One-way ANOVA with Tukey posthoc test. ***significant vs control group $p < 0.0001$, **significant vs control group $p < 0.01$, *significant vs control group $p < 0.05$ and ### significant vs PTZ group $p < 0.0001$.

Furthermore, the number of NF-kB-positive cells markedly increased in the PTZ group compared to the control group in the CA3 hippocampal region ($p < 0.001$). Alternatively, stevia significantly lowered the expression of NF-kB in the CA3 hippocampal region in comparison with the PTZ group ($p < 0.001$) (Fig. 8A). Brain sections showed minimal expression of NF-kB in the CA3 hippocampal region of the control group (Fig. 8B), high expression in the PTZ group (Fig. 8C), and minimal expression of NF-kB in the stevia group (Fig. 8D).

3.7 Correlations between Sirt-1 and other study parameters

The expression of sirt-1 showed a negative correlation with the PTZ-induced epileptic seizure score (Fig. 9A), a positive correlation with its latency (Fig. 9B), and a negative correlation with GFAP expression (Fig. 9C). Also, oxidative stress markers showed a negative correlation between Sirt-1 and MDA, and positive correlations between Sirt-1 and CAT

and TAC correlations (Fig. 9D–F, respectively). Regarding, the apoptotic markers, the Sirt-1 showed a positive correlation with Bcl-2 and negative correlations with caspase-3 and p53 (Fig. 9G–I). Finally, the inflammatory markers, there were negative correlations between Sirt-1 and IL-6, and NF-kB (Fig. 9J–K).

4. Discussion

The main findings of the current study included that: (a) administration of PTZ every alternate day for two weeks caused a significant increase in PTZ-induced seizures, which was associated with evident neuronal loss, astrogliosis, worsened oxidative stress, and a significant decrease in Sirt-1 and Bcl-2 and a significant increase in inflammatory mediators (NF-kB and IL-6) and apoptosis-related proteins (p53 and caspase-3) expressions in the hippocampal CA3 region and (b) pretreatment with stevia has neuroprotective and anti-epileptic effects accompanied by significant attenuation

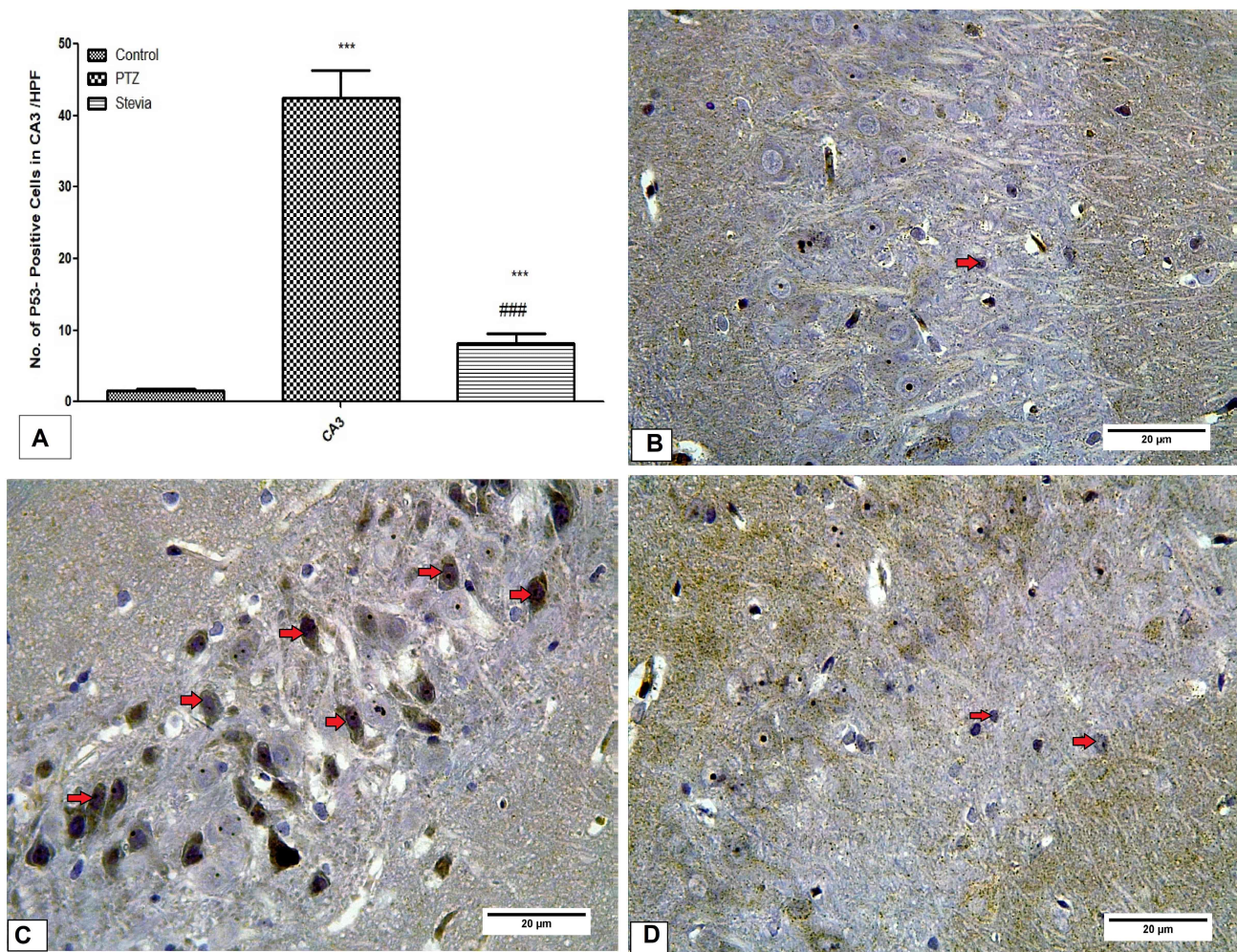


Fig. 7. Effect of Stevia on apoptotic marker (p53) in the CA3 hippocampal region by immunohistochemistry. Graph (A) represents the mean of p53-positive cells per high power field (HPF) in different groups. Brain specimens from the (B) normal control group show minimal cytoplasmic expression of p53 (red arrows) in the CA3 hippocampal region (400×), (C) PTZ group show marked cytoplasmic p53 expression (red arrows) in the CA3 hippocampal region (400×) and (D) Stevia group show low cytoplasmic p53 expression (red arrows) in the CA3 hippocampal region. One-way ANOVA with Tukey posthoc test. ***significant vs control group $p < 0.0001$ and ### significant vs PTZ group $p < 0.0001$.

in PTZ-induced neuronal loss, astrocytosis, oxidative stress, and inflammatory cytokine IL-6 and apoptotic markers (p53 and caspase-3) with the upregulation of Sirt-1 and anti-apoptotic protein (Bcl-2) in the CA3 hippocampal region.

The study at hand demonstrated, with alternate day PTZ injections for two weeks, typical epileptic seizures with a significantly shortened latency. Additionally, PTZ exhibited evident hippocampal neuronal loss and escalation in astrocytes population, which is supported by an increase in GFAP. Also, the current study demonstrates the alleged neuroprotection of stevia rebaudiana in the PTZ-induced epilepsy model through decreased neuronal loss and reactive astrocytosis with a low GFAP expression. *Stevia R.* ameliorated the high Racine's convulsion score and prolonged the latency of epileptic seizures. These findings conform to the neuroprotective actions of stevia extract and its derivatives displayed in previous studies such as scopolamine-induced memory loss [16], fructose-induced plurimetabolic changes in the hip-

pocampus and amygdala [17] and the anti-convulsive effects for Rebaudioside A (derivative of stevia) on convulsions induced by a single high dose of PTZ (70 mg/kg) [18]. However, in the current study, we demonstrated the role of *Stevia R.* during epileptogenesis induced by repeated administration of subconvulsive doses of PTZ suggesting not only anticonvulsive but also anti-epileptic effect for Stevia extracts.

Glial cells have been considered just a brain glue for neurons, however, a crucial role has been reported for glial cells especially astrocytes in the development of neurodegenerative diseases, including epilepsy. Upon brain insult, astrocytes become reactive and participate in hyper-excitation, neurotoxicity, and seizure spreading. After activation, astrocytes secrete pro-inflammatory mediators to initially protect, adapt, and return the central nervous system to its regular function. However, if the insult is maintained, persistent activation of inflammatory pathways contributes to the generation of seizures, activation of neuronal death pathways, and

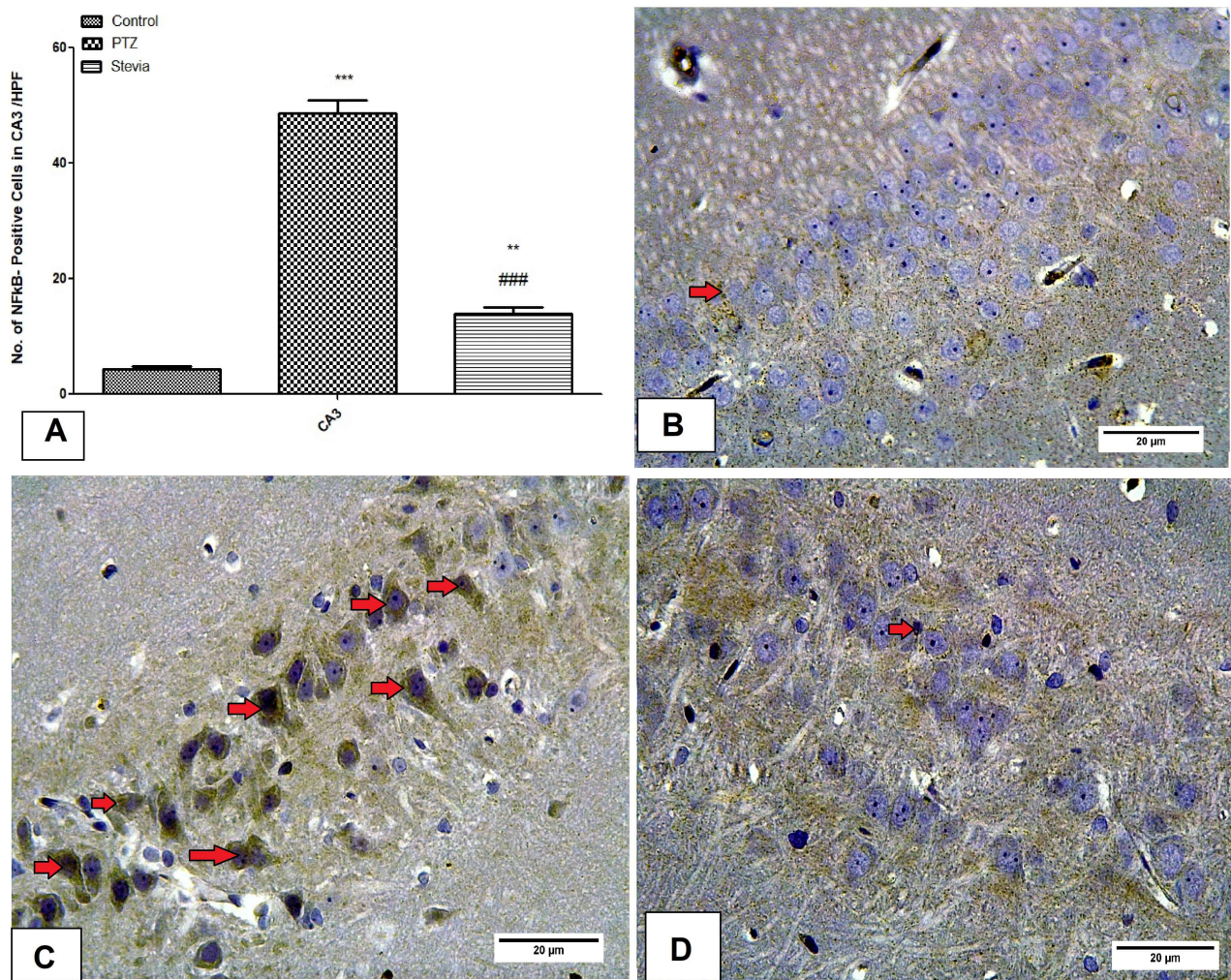


Fig. 8. Effect of Stevia on NF-kB in the CA3 hippocampal region by immunohistochemistry. Graph (A) represents the mean of NF-kB-positive cells per high power field (HPF) in different groups. Brain specimens from the (B) normal control group show minimal nuclear expression (red arrows) of NF-kB in the CA3 hippocampal region (400 \times), (C) PTZ group show marked NF-kB nuclear expression (red arrows) in the CA3 hippocampal region (400 \times) and (D) Stevia group show low NF-kB nuclear expression (red arrows) in the CA3 hippocampal region. One-way ANOVA with Tukey posthoc test. ***significant vs control group $p < 0.0001$, **significant vs control group $p < 0.01$ and ###significant vs PTZ group $p < 0.0001$.

compromising the oxidative state [24]. GFAP is an intermediate filament found specifically in astrocytes, and its accumulation indicates astrocyte activation, which is termed astrogliosis. The current study displays GFAP up-regulation with PTZ indicating astrogliosis alongside its ensuing detrimental effects, which is consistent with previous studies [25–27]. Moreover, the current work demonstrated down-regulation of GFAP with *Stevia rebaudiana* treatment indicating regression of astrogliosis with a potential decline in its repercussions, inflammation, apoptosis, and oxidative stress. To the best of our knowledge, our study is the first study to demonstrate the effect of *Stevia R.* on astrogliosis in drug-induced epileptogenesis.

Inflammation stands as one of the three pillars of epileptogenesis along with apoptosis and oxidative stress. Various studies have tied hippocampal neuronal damage with an ongoing process of inflammation. IL-6, a pro-inflammatory cy-

tokine, is normally concentrated in low quantities within the brain and is up-regulated with seizures. IL-6 up-regulation with PTZ decreases hippocampal neurogenesis and increases neuro-degeneration and astrogliosis [28]. The activation of nuclear transcription factor NF-kB can efficiently induce extensive gene expression and regulate the transcription of cytokine target genes, playing a regulatory role in inflammation and immune responses. According to Bertoglat *et al.* [29] high hippocampal expression of NF-kB was reported to induce neuronal death and activation of glial cells, which promotes the pathological changes with PTZ-induced seizures. Consistent with these studies, in this study, we found an increase in IL-6 expression and NF-kB in hippocampal neurons from the PTZ group. On the other hand, *Stevia R.* down-regulated the hippocampal expression of both NF-kB and IL-6, implying an anti-inflammatory role for stevia in epilepsy. In consistence with these findings, Latha *et al.* [30]

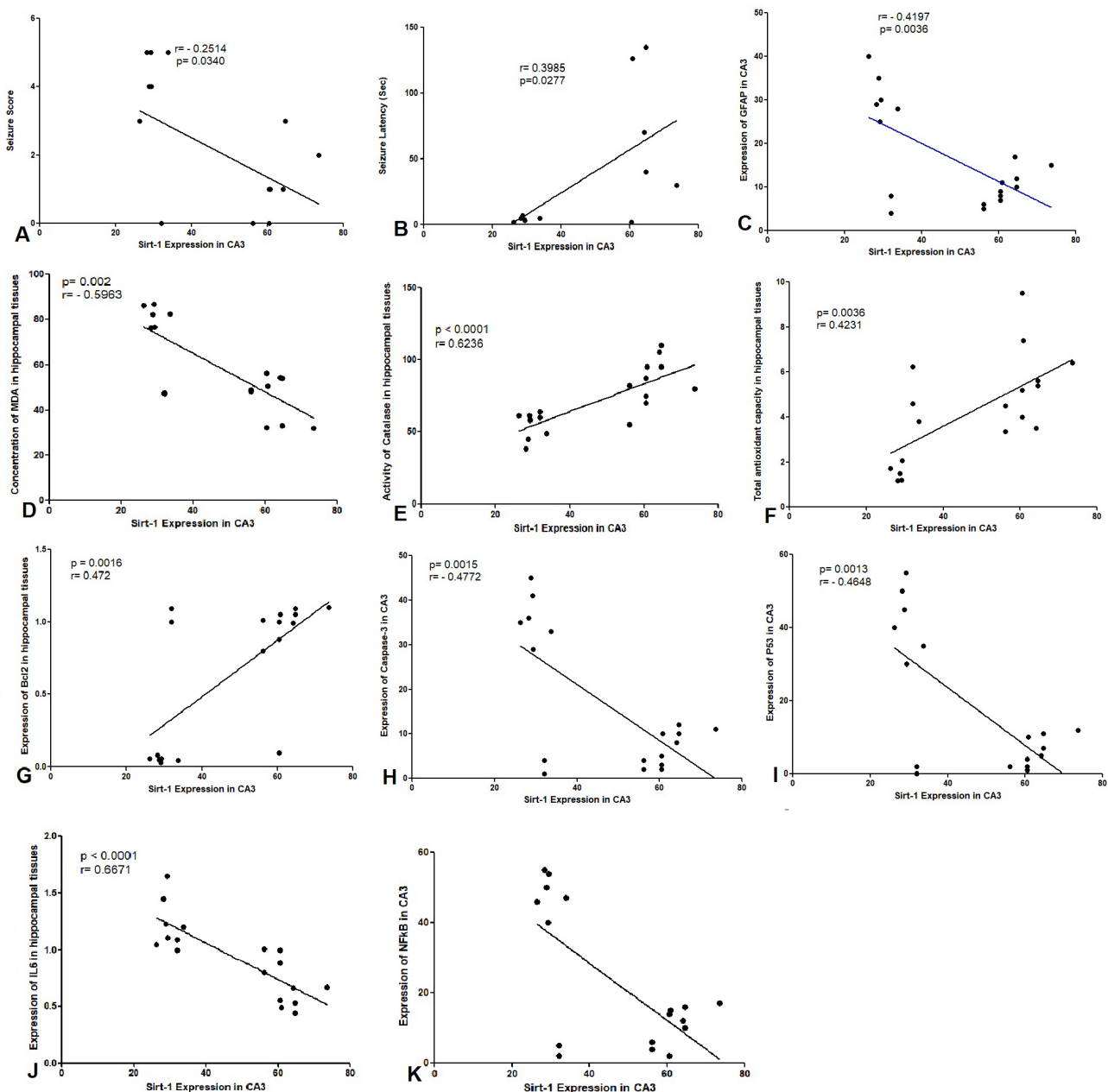


Fig. 9. Correlations of Sirt-1 expression with different studied parameters including; seizure score (A), seizure latency (B), GFAP expression (C), MDA concentration (D), catalase enzyme activity (E), total antioxidant capacity (F), Bcl-2 expression (G), caspase-3 expression (H), p53 expression (I), IL-6 expression (J), and NF- κ B expression (K). Pearson correlations, r = correlation coefficient and p = probability significance.

demonstrated that hydro-alcoholic extract of stevia leaves (500 mg/kg) attenuates the inflammatory process and oxidative stress in the lipopolysaccharides-induced acute liver injury mainly via the reduction of pro-inflammatory cytokines, e.g., TNF- α , IL-1 β , and IL-6. Also, Jiao and Gong [9] demonstrated that stevioside attenuated lipopolysaccharide (LPS)-induced inflammation in RAW264.7 cells via down-regulation of mitogen-activated protein kinase (MAPK) and NF- κ B signaling pathways.

Apoptotic hippocampal neuronal cell death is implicated in the process of epileptogenesis. PTZ-induced apoptosis is

mediated by the activation of enzymes such as caspase-3, increased expression of apoptotic proteins such as p53, and decreased levels of anti-apoptotic Bcl-2 protein. The current study demonstrated a significant increase in caspase-3, p53 with a significant reduction in Bcl-2 in brain tissue of PTZ-treated rats, which is in agreement with previous studies by Hussein *et al.* [31] and Hamdy *et al.* [32]. Moreover, *Stevia R.* exerted an anti-apoptotic action, as evidenced significant decline in caspase-3 and p53 with high expression of Bcl-2 with stevia-treated rats. These findings agree with the study of Hussein *et al.* [33] that reported anti-apoptotic effects for

Stevia R. in diabetic cardiomyopathy by downregulating the p53 and caspase-3. Oxidative stress contributes to neuronal hyperexcitability and degeneration. Oxidative stress is intertwined with apoptosis in the pathogenesis of epilepsy since it induces the loss of the mitochondrial membrane potential and releases pro-apoptotic proteins causing downstream activation of caspase-dependent and caspase-independent cell death [34]. Our findings showed that PTZ worsened the oxidative state, increasing the MDA level marking high lipid peroxidation along decreasing the total antioxidant capacity (TAC). These findings conform to our previous studies [6, 31, 33]. Alternatively, *Stevia R.* improved the oxidative stress state by increasing the activity of the catalase enzyme and the concentration of TAC and decreasing the MDA level, which confirms its antioxidant effects in the PTZ-induced epileptic rat model. The anti-oxidant effect of *Stevia R.* was demonstrated by our research group in diabetic cardiomyopathy [33].

The main objective in the current study was to test the role of Sirt-1 in the pathogenesis of epilepsy owing to its presumed neuroprotective role. Sirt-1 is highly expressed in both neuroglial and nerve cells in the brain. Kaewmool *et al.* [10] demonstrated that Sirt-1 exerts neuroprotective effects in rat models of microglial activation-induced neurodegenerative disease and Romeo-Guitart *et al.* [34] demonstrated that activation of Sirt-1 significantly improved motor nerve regeneration and recovery. Moreover, Dabke and Das [35] demonstrated that ketogenic diet metabolites protect the cultured cells via upregulation of Sirt-1 expression. In the current study, we found significant down-regulation of the expression of Sirt-1 expression in the PTZ group. It has been demonstrated that Sirt-1 was found in the cytoplasm of embryonic and adult neural precursor cells (NPCs) which was transiently translocated within 10 min to the nucleus in response to differentiation stimulus, then gradually retranslocated to the cytoplasm after several hours (~3 hrs) [36]. Also, Teertam *et al.* [37] found a significant reduction in nuclear expression of Sirt-1 with a significant increase in immunopositivity the cytoplasm and cell processes of neurons in both young and aged experimental rats after ischemic insults. In the current study, the rats were euthanized 16 hrs. after the last stevia and PTZ injection, which might explain the membranous and cytoplasmic not nuclear expression of Sirt-1 in CA3 neurons in the current study. Also, we found that the expression of Sirt-1 showed negative correlations with seizure score, GFAP expression, apoptotic markers (p53, caspase-3), a marker of lipid peroxidation (MDA) and inflammatory mediators IL-6 and NF-kB, and positive correlations with Bcl-2 and seizure latency suggesting that the downregulation of Sirt-1 is linked to the process of the PTZ-induced astrocytosis, apoptosis, inflammation, and oxidative stress. In agreement with these findings, previous studies reported down-regulation of Sirt-1 during epileptogenesis [10, 11, 38]. Moreover, Zhao *et al.* [39] demonstrated up-regulation of Sirt-1 in a rodent model of traumatic

brain injury (TBI), which protects against neuronal apoptosis in a rodent model of focal brain ischemia and Zhou *et al.* [40] demonstrated that suppression of Sirt-1 expression by sirtinol in a rat model of subarachnoid hemorrhage (SAH) worsened neurological deficits, brain edema, disruption of the blood-brain barrier (BBB) and endothelial cell apoptosis suggesting a neuroprotective role for Sirt-1. Also, Chen *et al.* [41] found that Sirt-1 upregulates the expression of anti-apoptotic protein, Bcl-2 and inhibits caspase-3 and Bax.

Moreover, we found that treatment of rats with *Stevia R.* upregulates the expression of Sirt-1 in CA3 hippocampal region, suggesting that Sirt-1 could be a mechanism for the neuroprotective effects of *Stevia R.* against PTZ-induced epilepsy. This hypothesis was supported by the results of Mei *et al.* [19], who reported that *Stevia R.* exhibited antioxidant and anti-apoptotic actions in cardiomyocytes through up-regulation of Sirt-1. Also, in agreement with these findings, Fu *et al.* [42] demonstrated that an enhancement of Sirt-1 expression by alpha-lipoic acid (ALA) protects against focal ischemia via up-regulation of the expression of PGC-1 α . Although, these findings are novel and not tackled by previous studies, they are considered preliminary findings that need further investigations to clarify the role of Sirt-1 in the neuroprotective effect of *Stevia* and open new perspectives for understanding its role in the process of epileptogenesis. This point is considered a limitation of the current study.

5. Conclusions

In conclusion, *Stevia R.* exhibited neuroprotective and anti-epileptic effects on PTZ-induced epilepsy. This effect might be due to the suppression of PTZ-induced astrocytosis, inflammation (downregulating IL-6 and NF-kB), apoptosis (upregulating Bcl-2 and downregulating p53 and caspase-3), and oxidative stress (decreasing MDA and increasing the catalase activity and TAC). Moreover, the up-regulation of sirt-1 takes part in the neuroprotective and anti-epileptic effects of *Stevia R.* in PTZ-kindled rat model.

Author contributions

EMEN, AMH and MAA, WO conceived and designed the study. WO, SS, AY, AM and NAK performed all behavioral experiments and conducted biochemical and molecular experiments. AMH, EMEN, MAA, and WAA collected the data and performed statistical analysis. AMH, EMEN, WO, AY, SS, MAA wrote the manuscript. All authors reviewed and approved the final draft.

Ethics approval and consent to participate

The IRB-committee approved all experimental procedures (code #R.20.11.1098).

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Conflict of interest

The authors declare no conflict of interest.

Data availability statement

The data will be available on reasonable request.

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