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Zizyphus spina christi Effect on Pentylenetetrazole-Induced Kindling

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Abstract

Background Epilepsy is a common disorder of brain that is not well controlled via drugs available for it. Gephyrin, a well-known inhibitory synaptic regulator, has a role in epilepsy. Additionally, significant development of oxidative stresses tends to be involved in epileptic seizure pathophysiology that giving rise to neuronal cell death. *Zizyphus spina christi* is a vastly available Iraqi tree and its leaves have several beneficial effects on central nervous system level.

Objective To investigate the possible neuroprotective effect of crude *Zizyphus spina christi* extract in kindling model induced.

Methods The removed brains of forty albino male mice from four separate groups were exposed to immunohistochemical test for gephyrin, glutathione, and NeuN expressions.

Results The data found that pretreatment with *Zizyphus spina christi* crude extract shows highly significant difference (P value <0.001) in immunohistochemical scores for all studied parameters as compared with those being of pentylenetetrazole-kindled group.

Conclusion These findings suggest that crude *Zizyphus spina christi* extract of leaves is effective in protection against kindling that induced via pentylenetetrazole when applied orally for male mice.

Keywords Gephyrin; glutathione; kindling; NeuN; pentylenetetrazole; *Zizyphus spina christi*

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List of abbreviations: AEDs = Antiepileptic drugs, CNS = Central nervous system, DPX = Dibutylphthalate polystyrene xylene, GABA = Gamma aminobutyric acid, GSH = Glutathione, HPLC = High performance liquid chromatography, IHC = Immunohistochemistry, NeuN = Neuronal Nuclei, NMDA = N-methyl-D-aspartate, PTZ = Pentylenetetrazole, ZSC = *Zizyphus spina christi*

Introduction

Epilepsy is a complex etiology disorder of brain that makes individual more susceptible to having unprovoked and repeated seizures. Gephyrin is the base scaffolding protein present in miscellaneous brain regions and a master regulator of neuronal activity at inhibitory sites [(1)]. Notably, one of pathological mechanisms of epileptic seizure is production of massive reactive oxygen species, which is responsible for death of neuronal cells [(2)].

The antagonist of gamma aminobutyric acid (GABA) receptor, pentylenetetrazole (PTZ), is utilized experimentally as a chemoconvulsant where its frequent sub-convulsant doses [(3)] resulting in kindling, which is an animal model applied for investigation of the pathophysiology of epilepsy and inspection of anti-epileptic drugs [(4)].

Zizyphus spina christi "Sidr" is a significant plant species and one of well-known Iraqi

trees. Different portions of this plant have been widely utilized in folk medicine.

On the central nervous system (CNS) level, some of laboratory studies have shown that the extract of *Zizyphus spina christi* has antianxiety, neuroprotective as well as central inhibitory effect on rodents [5-8].

Thus, current study aimed to investigate the neuroprotective effect of crude *Zizyphus spina christi* extract against PTZ-induced kindling.

Methods

Animals and experimental design

Forty albino male mice weighing (27.6-34.8) grams were involved in the current study. Mice were housed in standard cages in a room with controlled environment and had free access to food and water. The forty male mice utilized in this study were randomly allocated into the following four groups of ten mice each: Group I served as normal control and got only distilled water. Group II, III, and IV were exposed to induction of kindling via intraperitoneal PTZ injection of 35 mg/kg dose every second day for 23 days. Thirty minutes post each PTZ injection group III and IV were received diazepam (2 mg/kg, orally) and crude extract of *Zizyphus spina christi* leaves (50 mg/kg, orally) respectively.

PTZ induced-kindling

PTZ were purchased from Sigma-Aldrich, USA; its solution was freshly prepared at the day of administration by dissolving 2 mg of it in 1 ml of 0.9% saline [4,9]. For kindling induction, a 35 mg/kg of PTZ was applied intraperitoneally every second day. After each PTZ injection, mice were observed for thirty minutes to record the seizure score [4]. In the present study, a total of 12 injections (23 days) were needed to acquire kindling.

Preparation of brain tissues

At 24th day of study, the intact brain of mice was rapidly removed under deep anesthesia and kept in (10%) formalin containing cup for immunohistochemical evaluation.

Immunohistochemical evaluation of gephyrin, glutathione, and NeuN expression in brain tissues

The 5 µm thicknesses of tissue blocks were affixed on adhesive positively charged slide, dewaxed; rehydrated; put in retrieval solution bath; heated; and treated with peroxidase block for 20 minutes. The slides were rinsed; with wash buffer; treated with protein block; incubated; exposed to diluted antibody; and incubated again for overnight. At the second day, the slides were washed; incubated with horse radish peroxidase polymer; washed with washing buffer. Then, the slides subjected to 3, 3'-diaminobenzidine (DAB) chromogen/substrate reagent; incubated; washed with buffer wash; immersed in Meyers' hematoxylin; washed again; dehydration using ascending grades of 50%, 70%, 90%, and absolute ethanol; immersed in two changes of xylene for ten minutes; mounted with DPX and then covered slipped and left to dry. Immunostaining for expression of each of gephyrin, glutathione, and NeuN were evaluated were based on the percentage of positively stained cells per section 10: Score 0: less than 5%; score 1: 5-25%; score 2: 26-50%; score 3: more than 50%.

Statistical analysis

Data were presented as median, 25% percentile, 75% percentile, mean as well as standard deviation and analyzed using SPSS 20.0. Mann-Whitney U test utilized and the statistical significance was considered as $P \leq 0.05$.

Results

Effect of crude *Zizyphus spina christi* extract on immunohistochemical expression of gephyrin

As showed in table 1 and figure 1, IHC scores and brain expression in PTZ-kindled group declined significantly in median and mean ± SD (1.00, 0.57 ± 0.53) as compared with normal group. In contrast, IHC scores of diazepam (2.00, 2.43 ± 0.53) and crude extract of *Zizyphus spina christi* (2.00, 2.29 ± 0.48)

previewed highly significant increment (P ≤ 0.001) in relation to those being PTZ-kindled group.

Table 1: Immunohistochemical scores of gephyrin

Statistics	Normal control (N=10)	Crude extract (N=10)	Diazepam (N=10)	PTZ (N=10)
Median	2.00	2.00 NS *	2.00	1.00
25% Percentile	1	2	2	0
75% Percentile	3	3	3	1
Mean	2.00	2.29	2.43	0.57
SD	0.57	0.48	0.53	0.53

Mann-Whitney test
NS, not significantly different from corresponding diazepam score, P > 0.05
*, Highly significant different from corresponding PTZ score, P ≤ 0.001

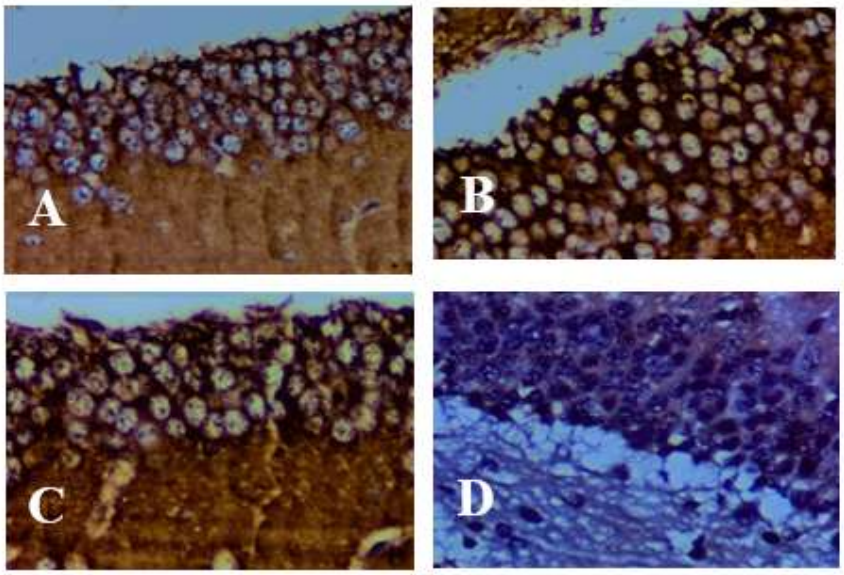

Figure 1. Immunohistochemical expression (40X) of gephyrin for (A) distilled water, (B) crude extract of *Zizyphus spina Christi*, (C) diazepam, (D) pentylentetrazole-kindled tissue

Effect of crude *Zizyphus spina christi* extract on immunohistochemical expression of glutathione

IHC scores and brain expression in PTZ-kindled group declined with great statistically significant (P ≤ 0.001) in median and mean ± SD (1.00, 0.57 ± 0.53) as compared with apparently healthy group. Contrariwise, IHC scores of diazepam (3.00, 2.71 ± 0.48) and crude *Zizyphus spina christi* extract (3.00, 2.86 ± 0.37) previewed highly marked increment (P ≤ 0.001) in median and mean ± SD of glutathione when compared with those of kindled group. All data are presented in table 2 and illustrated in figure 2.

Table 2: Immunohistochemical scores of glutathione

Statistics	Normal control (N=10)	Crude extract (N=10)	Diazepam (N=10)	PTZ (N=10)
Median	2.00	3.00 NS *	3.00	1.00
25% Percentile	2	2	2	0
75% Percentile	3	3	3	1
Mean	2.43	2.86	2.71	0.57
SD	0.53	0.37	0.48	0.53

Mann-Whitney test
NS, not significantly different from corresponding diazepam score, P > 0.05
*, Highly significant different from corresponding PTZ score, P ≤ 0.05

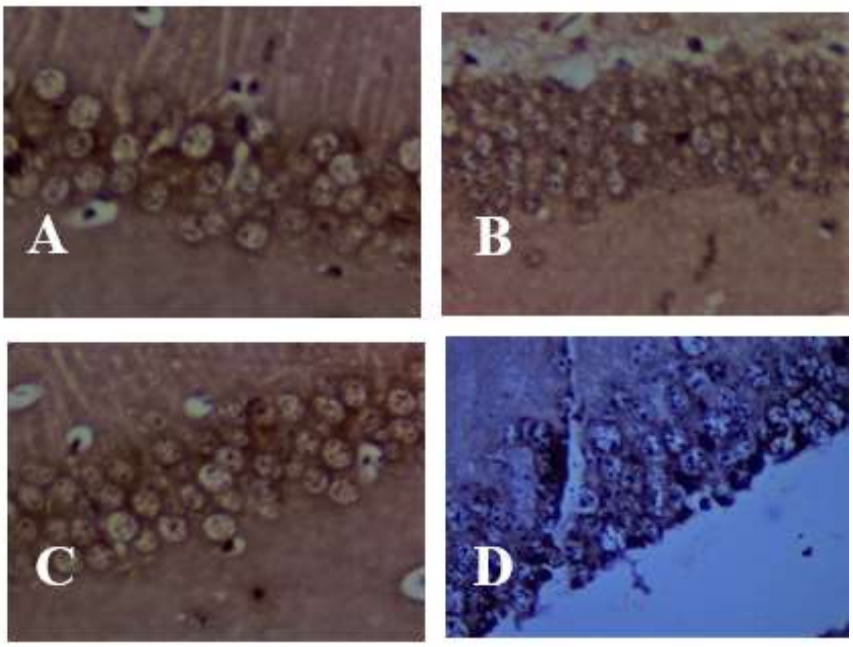

Figure 2. Immunohistochemical expression (40X) of glutathione for (A) distilled water, (B) crude extract of *Zizyphus spina Christi*, (C) diazepam, (D) pentylentetrazole-kindled tissue

Effect of crude *Zizyphus spina christi* extract on immunohistochemical expression of NeuN

IHC scores and staining are shown in table 3 and figure 3 respectively. There was highly significant suppression (P ≤ 0.001) of NeuN expression in brain tissues of PTZ-kindled group with median and mean ± SD of (1.00, 0.67 ± 0.50) in relation to normal group. In the opposite manner, the IHC scores of diazepam group (3.00, 2.57 ± 0.53) and crude *Zizyphus spina christi* extract group (2.00, 1.71 ± 0.48) were increased remarkably (P ≤ 0.001) as compared with corresponding in PTZ-kindled group.

Table 3. Immunohistochemical scores of NeuN

Statistics	Normal control (N=10)	Crude extract (N=10)	Diazepam (N=10)	PTZ (N=10)
Median	3.00	3.00 NS *	3.00	1.00
25% Percentile	2	2	2	0
75% Percentile	3	3	3	1
Mean	2.89	2.71	2.57	0.67
SD	0.33	0.48	0.53	0.5

Mann-Whitney test

NS, not significantly different from corresponding diazepam score, P > 0.05

*, Highly significant different from corresponding PTZ score, P ≤ 0.001

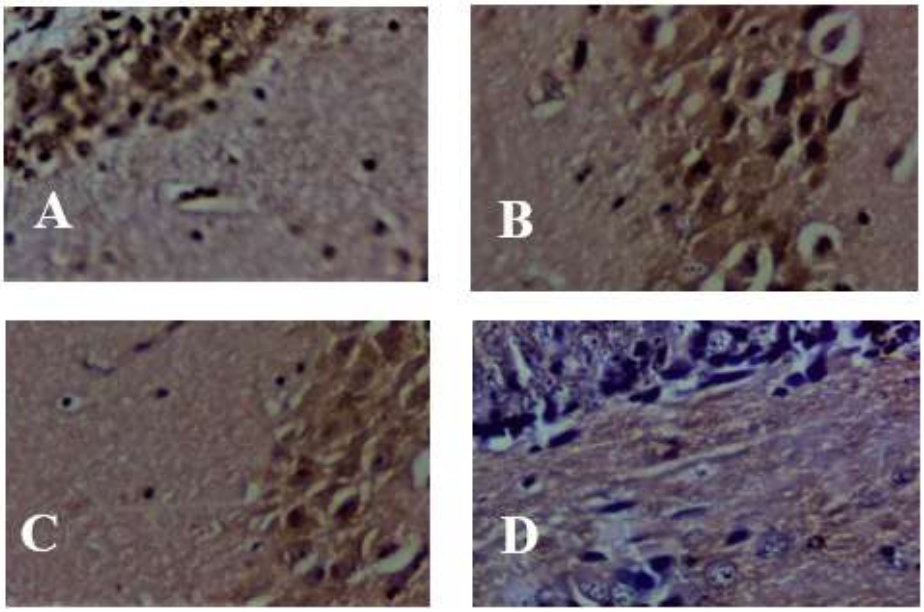

Figure 3. Immunohistochemical expression (40X) of NeuN for (A) distilled water, (B) crude extract of *Zizyphus spina Christi*, (C) diazepam, (D) pentylentetrazole-kindled tissue

Discussion

The kindling models are more look alike human epilepsy syndromes and have been widely utilized to reveal the disease modifying agents [11]. In PTZ-induced kindling, repeated administration of sub-convulsive doses of PTZ, a pro-convulsant, resulting in development of seizure activity and ultimately leads to occurrence of primary generalized tonic-clonic seizures [12,13]. PTZ originally acts as an antagonist on GABA receptor. Owing to presence of miscellaneous bioactive compounds such as alkaloids, flavonoids, steroids, phenols and saponins in *Zizyphus spina christi* [14,15], it was disclosed that its extract possess diverse pharmacological effects against the brain disorders such as anti-anxiety effect [5], attenuation of memory impairment induced via scopolamine [16], and neuroprotection against cerebral ischemia [6].

The present work found that the effects of pretreatment with crude extract exhibited a remarkable gephyrin expression in brain tissues similar to those of diazepam group as compared to those of PTZ-kindled mice. The finding reinforced the sedative effect of *Zizyphus spina christi* leaf extract and it is in harmony with that of previous study [5]. This

sedative nature of *Zizyphus crude extract* may be related to its peptide, cyclopeptide alkaloids, and saponin contents. The possible mechanism of alkaloids action may be through modulation of systems of neurotransmitter involving GABA receptors [17]. Meanwhile, the underlying mechanism of saponins might be related to its targeting GABAA receptors [18]. In the present work, the glutathione and NeuN expression witnessed a remarkable increment in mice pretreated with crude extract of *Zizyphus spina christi* leaf in comparison with that being in PTZ-induced kindled mice. This might be attributed to the presence of the alkaloids, saponins and flavonoids compounds in crude extract. In fact, epilepsy associated with N-methyl-D-aspartate (NMDA) receptors activation that giving rise to oxidative stress production and consequently damage of neurons and even death. The possible mechanism of action of phyto-constituents of plant extract could be due to modulation of neurotransmitter system as well as their anti-oxidant effects [16,17,19,20].

In conclusion, hydro-alcoholic extract of *Zizyphus spina christi* exhibited protective effects against PTZ-induced kindling via upregulation of gephyrin, glutathione as well as NeuN expression. However, further studies are required to investigate its therapeutic effects and to detect its underlying mechanisms.

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Author contribution

Dr. Al-Humaidhi: designed and conducted this research; Dr. Abd and Dr. Ghazi supervised the study and participated in its interpretation.

Conflict of interest

Authors declare no conflict of interest.

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