

# Hydro-alcoholic Extract of *Ziziphus spina christi* Attenuates Pentylentetrazole-induced Kindling in Male Mice

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## Abstract

Epilepsy is a common brain disorder that is not quite controlled via available antiepileptic drugs. It is distinguished by imbalance between excitatory neurotransmitters and that of inhibitory. Inflammatory events seem to be involved in pathophysiology of epileptic seizures and giving rise to neuronal cell death. Moreover, *Ziziphus spina christi*, is one of widely available Iraqi trees and its leaves have several effects including anti-inflammatory. Thus, this study aimed to investigate the possible neuroprotective effect of crude extract of *Ziziphus spina christi* in pentylentetrazole-induced kindling and its underlying mechanisms. Brain tissues of forty albino male mice were exposed to histopathological as well as immunohistochemical test for NMDA receptors and NLRP3 inflammasome expression. The results found that crude *Ziziphus spina christi* extract downregulates the expression of the studied markers with remarkable difference in relation to induction group. The findings of the study suggest that the oral administration of crude extract of *Ziziphus spina christi* leaves is effective in protection against in pentylentetrazole-induced kindled in male mice.

**Keywords:** epilepsy; NLRP3 inflammasome; NMDA receptor; pentylentetrazole; *Ziziphus spina christi*.

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## INTRODUCTION

Epilepsy is a brain disorder with a complex etiology. Recurrent seizures in epilepsy are caused via an imbalance between cerebral excitability and inhibition. In epilepsy, stimulation of NMDA receptors exists from the control that maintaining its excitatory activity within normal physiological limit. Also, its dysregulation contribute to neuronal excitability which have a significant role in epileptogenesis<sup>[1]</sup>.

NLRP3 (node-like receptor protein-3) inflammasome is the most characterized among inflammasome members, it is expressed primarily in immune cells<sup>[2]</sup>. In the brain, this inflammasome is mainly expressed in microglial cells. Importantly, it have been became familiar that epilepsy

results in activation of microglial cells<sup>[3]</sup> which results in aggravation of neuroinflammation and exacerbating seizures<sup>[4]</sup>.

Noteworthy, targeting of NLRP3 inflammasome is essential to limit signals that activate and produce proinflammatory cytokines IL-1 $\beta$  and IL-18<sup>[5]</sup> and this may control epileptogenesis and diminishes seizures<sup>[6-9]</sup>. Pentylentetrazole (PTZ), GABA<sub>A</sub> receptor antagonist, is used experimentally as a chemoconvulsant. Frequent injections of subconvulsant doses of PTZ can also upregulate NMDA receptors that have been occurred already in hippocampus, dentate gyrus, and somatosensory<sup>[10]</sup> and induce kindling (a model of chronic epilepsy). This model is applicable for

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investigation of the pathophysiology of epilepsy and screening of anti-epileptic drugs<sup>[11]</sup>.

*Ziziphus spina christi* is a significant species and one of few native trees around Arabic countries; it belongs to *Ziziphus* genus and Rhamnaceae family, locally is known as Sider and is named Christ's thorn in English.

On the CNS level, some of experimental studies showed that hydroalcoholic extract of *Ziziphus spina christi* leaves have antianxiety and a neuroprotective activity in rat<sup>[12, 13]</sup>. Another previous studies reported that its root bark extract has central inhibitory effect in mice<sup>[14,15]</sup>.

The present study is aimed to investigate neuroprotective effect of crude extract of *Ziziphus spina christi* leaves against pentylentetrazole-induced kindling.

### MATERIALS AND METHODS

#### Animals and experimental design

Forty albino male mice weighing (27.6-34.8) grams of body weight were involved in the current study. Mice were housed in standard cages in a room with controlled environment. Food and water were available ad libitum. The study protocol was approved and allowed by the Institutional Review Board (IRB) at College of Medicine/AL-Nahrain University. Forty male mice were utilized in this study and 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 35mg/kg dose every second day for 23 days. Thirty minutes post each PTZ injection **group III and IV** were received diazepam (1mg/kg, ip) and crude extract of *Ziziphus spina christi* leaves (50 mg/kg, orally) respectively.

#### Pentylentetrazole induced-kindling

Pentylentetrazole (PTZ) were purchased from Sigma-Aldrich (ST. Louis, MO, USA). PTZ solution was freshly prepared at the day of administration by dissolving 2 mg of pentylentetrazole in 1ml of 0.9% saline<sup>[11,18]</sup>.

For induction of kindling, a sub-convulsive dose (35 mg/kg) of PTZ was given intraperitoneally on every second day. In the present study, a total of 12 injections (23 days) were needed to acquire kindling. After each PTZ injection, the animals were observed for thirty minutes to record the seizure score<sup>[11]</sup>.

#### Preparation of brain tissues

At 24<sup>th</sup> day of study, the brain tissues of mice were rapidly removed under deep anesthesia then kept in (10%) formalin containing cup for immunohistochemical analysis.

#### Immunohistochemical expression of NLRP3 inflammasome and NMDA receptor in brain tissue

Five micrometer thicknesses tissue blocks were affixed on adhesive positively charged slide then dewaxed and rehydrated. Then, tissue section slides were put in low pH of antigen retrieval solution bath and heated in microwave at 360 watt for 15 minutes then peroxidase block was added and left for about 20 minutes. Then the slides were rinsed with buffer wash for 5 minutes followed by addition of protein block to the slide section and incubated at 25°C for 10 minutes. Then (50-75) µl of diluted primary antibody were added to the tissue sections and incubated overnight in a humid chamber. At the second day, the slides were washed and incubated with horse radish peroxidase polymer for two hours in a

humid chamber at room temperature then slides were subjected to 3, 3'-diaminobenzidine (DAB) chromogen/substrate reagent. After washing the slides with wash buffer, the slides were immersed in Meyers' hematoxylin bath for 1 minute then were washed and dehydrated in baths of ascending ethanol concentrations (50%, 70%, 90%, and absolute ethanol concentration) followed by two xylene baths for five minutes of each of the all baths. Slide tissue sections were mounted with DPX then was covered slipped and left to dry. Immunostaining results for expression of each of NMDA receptor and NLRP3 inflammasome were evaluated by microscope which equipped with digital camera at 40 X, depending on the following scores that based on the percentage of positively stained cells per section<sup>[20]</sup> score 0: less than 5%, score 1: 5-25%, score 2: 26-50%, score 3: more than 50%.

#### Statistical analysis

Data were analyzed and presented as median, 25% percentile, 75% percentile, mean as well as standard deviation using version SPSS 20.0. Comparison between each two groups was achieved via Mann-Whitney U test, Statistical significance was considered as  $P \leq 0.05$ .

### RESULTS

#### Effect of crude extract of *Ziziphus spina christi* on histopathological scores versus pentylentetrazole kindled group

As presented in table (1), the histopathological scores exhibit highly considerable decrement ( $P \leq 0.001$ ) for crude extract of *Ziziphus spina christi* leaves (1.0, 0.8 ± 0.31) in relation to those being in kindled group.

#### Effect of crude extract on immunohistochemical scores of NMDA receptors in pentylentetrazole-induced kindling

In table (2), the induction group (PTZ-kindled mice) witnessed highly significant elevation in median and mean ± SD of IHC scores of NMDA receptor (2.00, 2.43 ± 0.53); ( $P \leq 0.001$ ) when compared with corresponding apparently healthy group. IHC scores of median and mean ± SD of NMDA receptors expression indicate an extreme statistical decrement in crude extract of *Ziziphus spina christi* (1.00, 0.71 ± 0.48) ( $P \leq 0.001$ ) as compared with those being in PTZ-kindled group. Furthermore, IHC scores of expression of NMDA receptors display remarkable declining ( $P \leq 0.05$ ) in crude extract of *Ziziphus spina christi* (1.00, 0.71 ± 0.48) as compare with corresponding diazepam group.

#### Effect of crude extract on immunohistochemical scores of NLRP3 inflammasome in pentylentetrazole-induced kindling

Table (3) represents IHC scores of NLRP3 inflammasome show great increment in induction group (3.00, 2.78 ± 0.44); ( $P \leq 0.001$ ) in relation to apparently healthy group. In the opposite manner, extreme remarkable declining in median and mean ± SD of NLRP3 inflammasome (1.00, 0.89 ± 0.60) ( $P < 0.001$ ) for diazepam group in comparison with PTZ-kindled group. Likewise, marked significant diminishing ( $P \leq 0.001$ ) in crude extract scores when compared with data of induction group. Meanwhile, there is no statistical variation ( $P > 0.05$ ) comparing with median and mean ± SD of diazepam.

### DISCUSSION

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In agreement with findings of recent reports, the analysis of present study showed that crude extract of *Ziziphus spina christi* leaves contains various of bioactive compounds including alkaloids, flavonoids, steroids, phenols and saponins [21,22]. Owing to the phytoconstituents presence, the crude extract possessing different actions that may be responsible for numerous of pharmacological effects against the brain disorders such as anti-anxiety effect [12], attenuation of memory impairment induced via scopolamine [23], and neuroprotection against cerebral ischemia [13].

In the present work, the expression of NMDA receptors and NLRP3 inflammasome significantly diminished in mice pretreated with crude extract of *Ziziphus spina christi* leaf in comparison with that found in PTZ-induced kindling mice. This might be due to presence of the alkaloids, saponins and flavonoids compounds in crude extract. Indeed, epilepsy associated with NMDA receptors activation resulting in oxidative stress that is ultimately leading to damage of neurons and even death. The possible mechanism of action of these phyto-constituents could be attributed to modulation of neurotransmitter system, antagonism of NMDA receptor mediated electrical discharges from brain neurons, as well as their anti-oxidant effects [23-26].

With regard to anti-inflammatory activity, current work on *Ziziphus spina christi* leaf extract revealed a remarkable lowering in NLRP3 inflammasome expression in comparison with that of PTZ kindling group. The outcome of the current study detected anti-neuroinflammatory effect of the crude extract. Owing to the availability of valuable anti-inflammatory compounds in this plant including alkaloids, saponins, phenols as well as flavonoids [27-30]. Furthermore, both of gallic acid and epigallocatechin compounds that are available in *Ziziphus*, were known to have anti-inflammatory effects. Gallic acid; a potential inhibitor of expression of interleukin-8 (IL-8). Epigallocatechin-3-gallate (EGCG), polyphenol, resembles the structure of epigallocatechin with an additional gallate and has a good bioavailability in central nervous system (CNS). Secretion of each of TNF- $\alpha$ , IL-6, as well as IL-8 was inhibited by EGCG then can attenuate of neuroinflammation mediated via nuclear factor kappa B (NF- $\kappa$ B), a key regulator of inflammatory response [31].

This study investigated neuroprotective effect of hydroalcoholic extract of *Ziziphus spina christi* leaves confirming the previous study result [ADDIN CSL\_CITATION {"citationItems":{"id":"ITEM-1","itemData":{"DOI":"10.1016/j.sjbs.2010.05.003","ISSN":"1319562X","abstract":"In this study, anti-convulsant effect of Sidr leaf extract was examined by using pentylenetetrazol (PTZ) model on male albino rat by evaluating the changes in norepinephrine (NE), dopamine (DA) and serotonin (5-HT) contents in different brain regions (cerebellum, brainstem, striatum, cerebral cortex, hypothalamus and hippocampus). The administration of subconvulsive dose of PTZ (40. mg/kg i.p.) every other day for 9. days caused a significant decrease in monoamine content in different brain areas, this is may be due to the increase in nitric oxide levels, although antagonized the GABAA receptors which led to neurotransmitter release so the content is decreased. Administration of PTZ after treatment with Sidr (50. mg/kg i.p.) leaf extract for 3. weeks as a protective group and administration of Sidr leaf extract for 3. weeks after treatment of PTZ as a therapeutic group caused

significant increase in NE, DA, and 5-HT contents in all tested brain regions at most of the time intervals studied. This may be due to the presence of peptide and cyclopeptide alkaloids in the extract which inhibit neurotransmitter activity which led to the inhibition of neurotransmitter release. From these results, we can say that the Sidr leaf extract has neuroprotective and therapeutic roles against pentylenetetrazol convulsant effect.

© 2010 King Saud University.,"author":{"dropping-particle":"","family":"Waggas","given":"Abeer M.,"non-dropping-particle":"","parse-names":false,"suffix":""},"dropping-particle":"","family":"Al-Hasani","given":"Reem H.,"non-dropping-particle":"","parse-names":false,"suffix":""},"container-title":"Saudi Journal of Biological Sciences","id":"ITEM-1","issue":"4","issued":{"date-parts":["2010"]},"page":"269-274","publisher":"King Saud University","title":"Neurophysiological study on possible protective and therapeutic effects of Sidr (*Ziziphus spina-christi* L.) leaf extract in male albino rats treated with pentylenetetrazol","type":"article-journal","volume":"17"},"uris":["http://www.mendeley.com/documents/?uuid=47bcb9d8-1167-4e79-8326-63107be1127b"]},"mendeley":{"formattedCitation":"<sup>32</sup>","plainTextFormattedCitation":"(32)","previouslyFormattedCitation":"(32)","properties":{"noteIndex":0},"schema":"https://github.com/citation-style-language/schema/raw/master/csl-citation.json"}32] that demonstrated the anticonvulsant effect of aqueous extract of of the same plant.

### CONCLUSION

Hydro-alcoholic extract of *Ziziphus spina christi* exhibited protective effects against PTZ-induced kindling via downregulation of NMDA receptor and suppression of NLRP3 inflammasome. However, further studies are required to investigate its therapeutic effects and to detect the underlying mechanisms.

### Funding

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### Conflicts of interest

The authors declare no conflict of interest

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**Table 1.** histopathological scores of crude extract of *Ziziphus spina christi* compared with diazepam and pentylenetetrazole kindled group

Statistics	DW (N=10)	Crude extract (N=10)	Diazepam (N=10)	PTZ (N=10)
Median	0.0	1.0 <sup>NS **</sup>	1.0	3.5
25% Percentile	0.0	0	0	3
75% Percentile	0.0	2	2	4
Mean	0.0	0.8	0.9	3.5
SD	0.0	0.63	0.56	0.52

**Mann-Whitney test**

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

**\*\***, Highly significant different from corresponding PTZ score,  $P \leq 0.001$

**Table 2.** immunohistochemical scores of NMDA receptors of crude extract of *Zizyphus spina christi* compared with diazepam and pentylenetetrazole kindled group

Statistics	DW (N=10)	Crude (N=10)	Diazepam (N=10)	PTZ (N=10)
Median	1.00	1.00 <sup>* ‡</sup>	2.00	2.00
25% Percentile	0	0	1	2
75% Percentile	1	1	3	3
Mean	0.86	0.71	1.86	2.43
SD	0.37	0.48	0.69	0.53

**Mann-Whitney test**

**\***, Significantly different from corresponding diazepam score,  $P \leq 0.05$

**‡**, Highly significant different from corresponding PTZ score,  $P \leq 0.001$

**Table 3.** immunohistochemical scores of NLRP3 inflammasome of crude extract of *Zizyphus spina christi* compared with diazepam and pentylenetetrazole kindled group

Statistics	DW (N=10)	Crude extract (N=10)	Diazepam (N=10)	PTZ (N=10)
Median	1.00	1.00 <sup>NS ‡</sup>	1.00	3.00
25% Percentile	0	0	0	2
75% Percentile	1	1	2	3
Mean	0.56	0.67	0.89	2.78
SD	0.52	0.50	0.60	0.44

**Mann-Whitney test**

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

**‡**, Highly significant different from corresponding PTZ score,  $P \leq 0.01$