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Original Research Article

Ethanol Extract of *Hymenocardia acida* Tul (Euphorbiaceae) Ameliorates Pentylenetetrazole-Induced Seizures and Neuroinflammation in Mice

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ABSTRACT

Epilepsy is a non-communicable neurological condition with recurrent seizures resulting from uncontrolled neuronal firing as its significant symptoms. Since the practice of traditional medicine and the use of herbal products is common among middle- to low-income countries, it has become imperative to investigate the probable benefit of these products in the treatment of epilepsy. This study evaluated the possible neuroprotective role of *Hymenocardia acida* leaf extract against the neuronal damage in a mouse model of chemically-induced seizures using pentylenetetrazole (PTZ). Fifty (50) mice were divided into five groups (n=10) comprising a normal control group, which received vehicle, a PTZ-only group (35 mg/kg, i.p), a 50 mg/kg *H. acida* group, a 100 mg/kg *H. acida* group and a positive control group, which received 300 mg/kg diazepam. A total of ten (10) PTZ injections were administered to mice in groups 3-5 on alternate days, one hour after treatments. The resulting seizures were graded by severity. The brain tissues were harvested and used for the determination of pro-inflammatory cytokine expression. Data was analysed using one-way ANOVA followed by Tukey's test at $p<0.05$ significance. The results showed that the ethanolic extract of *H. acida* (EEHA) significantly reduced the severity of PTZ-induced seizures as from day 5. The PTZ-induced overexpression of pro-inflammatory cytokines was significantly ameliorated by both doses of EEHA. In conclusion, the neuroprotective effect of EEHA may be due to its anti-inflammatory ability, as evidenced in the amelioration of pro-inflammatory cytokine expression.

Keywords: Epilepsy, Seizures, Natural products, Convulsions, Neuroinflammation.

Introduction

Epilepsy is a neurological condition characterised by recurrent, spontaneous epileptic seizures. [1,2] It is the fourth most common neurological disorder affecting ~50 million people of all ages around the world.[3,4] Due to its associated neurobiological and behavioural complications, individuals with epilepsy are up to three times more likely to die young than the general population.[5] These complications have most often been linked to seizure-related neurodegeneration. There is literary evidence that seizures lead to neuronal loss and brain damage.[6] Epilepsy can therefore be a comorbidity in numerous neurological conditions, such as neurodegenerative illnesses[7,8] characterised by neuroinflammation as the underlying pathology.[9,10] Furthermore, compelling evidence has shown that neuroinflammation is involved during the process of epileptogenesis, with the level of cytokine in the cerebrospinal fluid (CSF) of people living with epilepsy proportionally correlating to the duration and frequency of seizures. [11]

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In light of this fact, to properly diagnose and treat people living with epilepsy, the current management strategy of improving the symptoms will have to be expanded to include an approach from the neurodegenerative aspect by slowing down neuroinflammation among people with epilepsy, since neuroinflammation has been identified as the bedrock of neurodegeneration.[9,10]Natural substances found in fruits, vegetables, and herbs have a variety of biological characteristics, including antioxidant, anti-apoptotic, and anti-inflammatory properties.[6,12-13] They therefore play a significant role in prophylaxis and improving health.[14] The efficacy of natural products in different models of neuroinflammatory and neurodegenerative diseases has been demonstrated by numerous pharmacological research.[15-16] From time immemorial, different cultures have used herbs as a form of therapy to treat different neurological disorders, like convulsions among children. Up till today, in the middle-to-low-income countries, the use of herbs as alternative medicine in treating epilepsy is very popular among the people. *Hymenocardia acida*, the West African rubber tree, is one of such important herbs indicated for convulsions in folk medicine. Although cultural beliefs and the use of traditional medicine may be considered an impediment to proper healthcare delivery for epilepsy treatment in middle- to low-income countries, they can translate to being the first treatment option if properly investigated.[17] *Hymenocardia acida* has been used in African traditional medicine for many years to treat malaria, gastrointestinal disorders, skin diseases, diabetes, sickle cell, epilepsy, and schizophrenia.[18] The leaves, bark and roots of *H. acida* are used either in infusion or powdered form. Studies have shown that *H. acida* possesses bioactive compounds that could be useful in the management of epilepsy.[19] This study evaluated the possible neuroprotective role of *H. acida* leaf extract against the neuroinflammation and seizures in a mouse model of chemically induced seizures using pentylenetetrazole (PTZ).

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Materials and Methods

Experimental animals

A total of fifty (50) adult Swiss mice (weight: 22-25 g) were procured for this study and housed under standard conditions. The mice were allowed unrestricted access to the rodent pellet diet and water during the entire study. They were allowed to acclimate for one (1) week before the commencement of the study. The mice were handled according to the NIH Guide[20] and institutional guidelines (ABUADHREC/29/03/2025/793) on the care and use of laboratory animals.

Plant Collection and Authentication

The fresh leaves of *Hymenocardia acida* were collected in February 2020 from Chaza town in Niger state. A botanist at the National Institute for Pharmaceutical Research and Development (NIPRD), Abuja, identified and authenticated the plant, and a specimen was saved for later use with voucher number NIPRD/H/7086.

Preparation of Plant Extract

The leaves were allowed to dry in a shaded yet well-ventilated environment for about seven (7) days. Once dried, the leaves were ground into powder using an electric blender and 400 g of material was obtained. The material was divided into two (2) equal portions (200g each) and macerated by soaking in 2 L of 70% ethanol in separate flasks at room temperature. It was ensured that the materials were completely submerged in the ethanol, and the flask was shaken occasionally to ensure complete extraction. After 72 hours, the extract was slowly filtered using filter papers, evaporated to dryness using a water bath at 55°C, and monitored until the extract was properly concentrated. The concentrated ethanolic extract was weighed and stored in vials before being stored in the refrigerator.

Acute toxicity test

Using the Lorke method,[21] an acute oral toxicity test was performed. Three sets of three mice each were employed in the study's initial phase. The plant extract was given to them orally at doses of 10 mg/kg, 100 mg/kg, and 1000 mg/kg body weight, and they were monitored for a whole day. Three groups of the same animal were given plant extract orally at 1900 mg/kg, 2900 mg/kg, and 5000 mg/kg during the second phase. Within a day, the animals were monitored for mortality and indications of toxicity. For seven days, the animals that made it through were monitored for any indications of delayed toxicity.

Experimental groups and design

After acclimatisation, the mice were randomly divided into four (4) groups of ten (10) mice each (n=10).

Group 1 served as the normal control, which received vehicle only (10 mL/kg, p.o.)

Group 2 served as a negative control, which received vehicle and pentylenetetrazole (PTZ, 35 mg/kg, i.p).

Groups 3-4 were treated with graded doses of *H. acida* extract (50 mg/kg and 100 mg/kg, p.o), respectively, in addition to receiving PTZ (35 mg/kg, i.p).

Mice in groups 2-4 were injected with PTZ approximately sixty (60) minutes after respective treatments on alternate days for twenty-one (21) days. Following each PTZ injection, the mice were observed for 30 minutes. A Racine[22] grading scale adapted by Malhotra and Gupta[23] was used to rate a number of seizure grades: Stage 0 involves no response; Stage 1 involves hyperactivity and vibrissae twitching; Stage 2 involves head nodding, head clonus, and myoclonic jerk; Stage 3 involves unilateral forelimb clonus; Stage 4 involves rearing with bilateral forelimb clonus; Stage 5 involves generalised tonic-clonic seizure with loss of writhing reflex; and Stage 6 involves lethality. The total number of the different seizure types, their durations and latencies as well as mortality were reported.

Preparation of brain tissues for biochemical assays

On day 22, mice were euthanised by being injected with a high dose (200 mg/kg) of pentobarbitone. The brains of selected mice were harvested. The hippocampus was manually sectioned and homogenised in phosphate buffer solution (0.1M, pH 7.4). The homogenate was then centrifuged at 10,000 rpm at 4°C for 10 min to obtain the supernatant.

Measurement of brain levels of pro-inflammatory cytokines

Using Enzyme-Linked Immunosorbent Assay (ELISA) kits, the levels of interferon gamma (IFN-γ), interleukin-12 (IL-12), and tumour necrosis factor-α (TNF-α) in mouse brain tissues were assessed in accordance with the manufacturer's instructions. In this protocol, precoated antibody microtitre well plates were used. The samples were added to the wells, incubated to ensure binding, before being subjected to washing. Afterwards, an enzyme-conjugated molecule was added before a second round of washing. At the addition of the substrate, the resulting-coloured product was measured using a microplate reader at 450 nm, allowing for quantitative analysis of the cytokine concentration.[24]

Statistical Analysis

Data were presented as mean ± standard deviation (SD). Analysis was done using one-way ANOVA followed by Tukey's multiple comparison test. The analysis and comparisons across groups were performed using GraphPad Biostatistics software version 8. Statistical significance was set at $p < 0.05$.

Results and Discussion

Before this study, there has been literature on the ethnomedicinal and therapeutic value of *Hymenocardia acida;* traditional healers used the different parts of the plant to treat diabetes,[25] cancer,[26] gastrointestinal disturbances, menstrual pain, tetanus, ulcer, asthma, cough, and epilepsy.[27,28] Considering these benefits, this research was designed to investigate the neuroprotective effect of the ethanol extract of *Hymenocardia acida* (EEHA) against pentylenetetrazole-induced seizures in mice.

In this study, following the Singh et al[29] approach, mice were kindled with multiple low doses of pentylenetetrazole (35 mg/kg) administered intraperitoneally, and there was a significant ($p<0.05$) increase in the mean seizure scores (indicating kindling) of the group of animals exposed to PTZ treatment alone compared to control. This correlates with a recent study, which demonstrated that the severity score of seizures after PTZ kindling in rats was significantly higher than that in the single seizure model.[2] This further corroborates the studies of Adeoluwa et al.,[30] and Aleshin et al.,[2] in which animals exposed to multiple low doses of PTZ demonstrated a significant increase in seizures. However, treatment with the two (2) doses of the ethanol extract (50 and 100 mg/kg) of *H. acida* significantly ($p<0.05$) ameliorated the PTZ-induced kindling compared to the PTZ-only group (Figure 1).

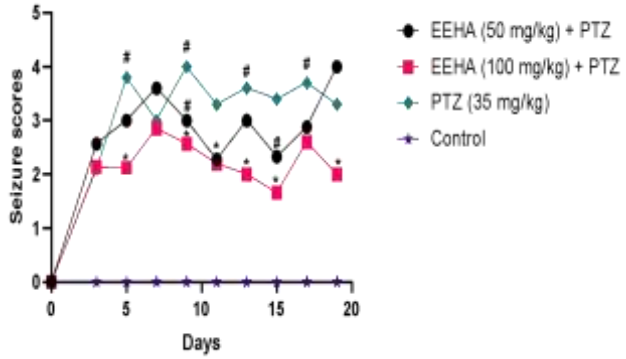

Figure 1: Effect of ethanolic extract of *H. acida* on intensity of PTZ-induced seizures in mice

Data are expressed as mean ± SD (n=10). Analysed by one-way ANOVA and Tukey's test. #p=0.0001 when compared to normal control, *p=0.0088 when compared to the PTZ-only group. EEHA, ethanolic extract of *Hymenocardia acida*; PTZ, pentylenetetrazole

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Figure 1 shows that PTZ-treated animals were kindled on day 3 (2nd injection) and were fully kindled on day 7 (4th injection) and it continued till the last day. Similarly, mice treated with 50 and 100 mg/kg EEHA were kindled on day 5 (3rd injection), compared to the control. However, the intensity of kindling significantly reduced from 5th through 9th injections compared to the PTZ-treated group of animals, thus suggesting its antiepileptic property and validating its use in folk medicine for convulsions.[28] This finding is consistent with and corroborates the outcomes of the earlier studies in which the extract of *H. acida* demonstrated anticonvulsant effects in mice and chicks exposed to an acute dose of PTZ.[19,27]

In addition, inflammation is a major factor underlying the pathogenesis of several neurological disorders.[9,10] Therefore, the study investigated and measured the levels of central proinflammatory cytokines such as IL-12, IFN-γ, and TNF-α. The findings showed that all proinflammatory markers were significantly ($p<0.05$) increased following exposure to multiple low doses of PTZ alone compared to the control, thus indicating neuroinflammation. Similarly, Nader et al.,[31] reported that PTZ-induced seizures resulted in increased NF-**κ**B activity, which is a trigger for various inflammatory mediators. In another study, the brain levels of key inflammatory mediators such as NF-**κ**B and TNF-α were significantly elevated after a single mouse exposure to PTZ, as well as increased generation of reactive oxygen species, which all contribute to neuroinflammation.[34] While in clinical cases of epilepsy, the level of cytokine in the cerebrospinal fluid of people living with epilepsy has been proportionally correlated to the frequency and duration of seizures.[11,33] Conversely, the group of animals pretreated with the ethanol extract of *H. acida* before exposure to PTZ showed a significant ($p<0.05$) decrease in the brain level of IFN-γ (Figure 2A), TNF-α (Figure 2B) and IL-12 (Figure 2C) compared to PTZ-treated mice. This shows that the plant possesses compound(s) having anti-inflammatory activity.

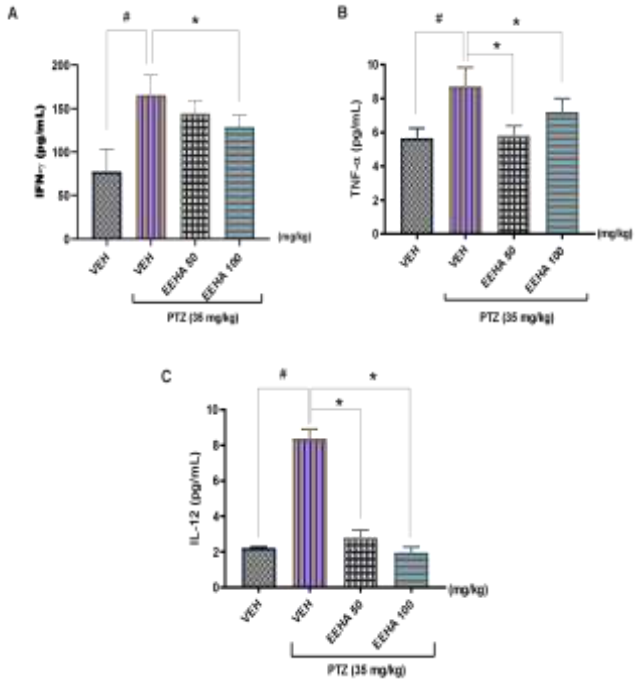

Figure 2: Effect of ethanolic extract of *H. acida* on central expression of proinflammatory cytokines in PTZ-treated mice. Data are expressed as mean ± SD (n=6). Analysed by one-way ANOVA and Tukey's test. 2A): #$p<0.0001$ when compared to normal control, *$p=0.0221$ when compared to the PTZ-only group. 2B): #$p=0.0337$ when compared to normal control, *$p<0.02$ when compared to the PTZ-only group. 2C): #$p<0.0001$ when compared to normal control, *$p<0.0001$ when compared to the PTZ-only group. EEHA, ethanolic extract of *Hymenocardia acida*; PTZ, pentylenetetrazole

Oral acute toxicity determination of H. acida

To determine the safety of the ethanol extract of *H. acida*, the oral median lethal dose (LD50) of *H. acida* was estimated to be greater than 5000 mg/kg body weight (Table 1). This demonstrates the relative safety of *H. acida.*[27] The ethanol extract of *H. acida* is considered relatively safe for oral consumption since there was no record of death even at 5000 mg/kg.

Table 1: Oral acute toxicity determination of *H. acida*

Dose (mg/kg)	Number of dead animals/Number of animals used (n)
PHASE I (n=3)	
10	0/3
100	0/3
1000	0/3
PHASE II (n=1)	
1900	0/1
2900	0/1
5000	0/1

Conclusion

The ethanol extract of *Hymenocardia acida* attenuated PTZ-induced seizure and inhibited the accompanying neuroinflammation, thus supporting its potential benefit in the treatment of neurological diseases like epilepsy in traditional medicine. However, further studies are required to elucidate the lead compound and the actual mechanisms of its action.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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