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In vitro and *In vivo* Anticonvulsant Effect of Hydroalcoholic Extract of *Moringa stenopetala* in Mice Models

Samson Sahile Salile[1,2*] and Teferra Abula[1]

[1]Department of Pharmacology, School of Pharmacy ,College of Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia.
[2]Department of Pharmacy, College of Medicine and Health Sciences, Arba Minch University, Arba Minch, Ethiopia.

Authors' contributions

This work was carried out in collaboration between both authors. Author SSS designed the study, performed the laboratory work, statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author TA supervised the work and edited the final draft. Both authors read and approved the final manuscript.

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ABSTRACT

Background: Epilepsy is a debilitating neurological disorder that directly affects approximately 65 million people worldwide. In the search of safe and effective antiepileptics traditional treatment practices are one area of research to obtain novel molecules. *Moringa stenopetala* root is claimed to be used for epilepsy treatment in Konso area, Southern Ethiopia. But there was no scientific research evidence for the claimed use of the plant.
Objective: This study was conducted to explore the anticonvulsant activity of hydro-alcoholic (80% methanol) extract of root of *Moringa stenopetala*.
Methods: The dry residues of the plant extract was used for the test. *In vitro* 0Mg^{2+} mice model at dose 0.7 mg/kg of extract, diazepam(3μM) and untreated brain slice groups were used to compare the presence of seizure like event (SLE). *In vivo* pentylenetetrazol (PTZ) model with 85 mg/kg

**Corresponding author: E-mail: samsahle@gmail.com, samsonsahile@aau.edu.et;*

subcutaneously was used to compare the seizure on set time with two extract doses and diazepam 5 mg/kg. The data was presented with mean± standard error. In maximum electric shock (MES) model 54 mA was passed for 0.2 second transauricularly in mice. The mean time of hind limb extension was recorded for doses 400 mg/kg and 800mg/kg of the extract and 10 mg/kg phenytoin. The means were compared for statistical significance using one way ANOVA post hoc LSD whereas proportions were compared using Fishers exact test with P-value < .05.

Results: *M. stenopetala* extract has shown statistically significant anticonvulsant activity *in vitro* compared to control (P<.05). A positive control, the known anticonvulsant diazepam (3μM), showed significant anticonvulsant activity (P<.05). *In vivo* MES model showed statistically significant anti-seizure activity at both doses (P<.05). But the crude extract failed to show statistically significant activity at all doses of PTZ model (P>.05).

Conclusion: The results of this study showed that crude extract of *Moringa stenopetala* exhibited anti-convulsant effect both *in vitro* and *in vivo* MES models.

Keywords: Moringa stenopetala; seizures; epilepsy; anticonvulsant; 0 Mg^{2+} model; maximal electroshock seizure model; pentylenetetrazol seizure model.

1. INTRODUCTION

Affecting 65 million individuals worldwide, epilepsy is the third leading contributor to the global burden of disease for neurological disorders [1]. An estimated 2.4 million people are diagnosed globally with epilepsy each year [2]. Even though antiepileptic drug (AED) medication is widely available, many people with active epilepsy particularly in resource-poor countries go untreated [3]. In those getting treatment refractoriness is still an important issue in epilepsy therapy despite the fact that new antiepileptic drugs have been available since late 1980s.In the search of novel antiepileptic drugs one of the approaches is investigation of naturally-occurring compounds [4].

In Ethiopia as various diseases are being treated traditionally some survey shows also the practice of treatment for epilepsy using plant extracts. *Moringa stenopetala* though its root is claimed to be used for epilepsy treatment in Konso area, Southern Ethiopia there is no scientific study conducted for the evidence of its potential value [5]. As different researches on plants used for epilepsy in different countries have shown anticonvulsant activity, these plants may also have value for the treatment of the disease [6]. Therefore scientific research should be done on these plants for their antiepileptic potential [7].

Zero Mg^{2+} model is one of the *in vitro* models to study mechanism of seizure and antiseizure drugs. The most commonly employed *in vivo* animal models in the search for new anticonvulsant drugs are the MES test and the PTZ seizure test [8,9]. The maximal electroshock seizure test, in which tonic hindlimb seizures are induced by bilateral corneal or transauricular electrical stimulation, is thought to be predictive of anticonvulsant drug efficacy against generalized tonic-clonic seizures, while the pentylenetetrazole test, in which generalized myoclonic and clonic seizures are induced by systemic (usually s.c. or i.p.) administration of convulsant doses of PTZ, is thought to represent a valid model for generalized absence and/or myoclonic seizures in humans [8].

Hence this study was conducted with the objective to looks for the anticonvulsant potential of 80% methanol extract of *Moringa stenopetala* using *in vitro* and *in vivo* mice models.

2. MATERIALS AND METHODS

2.1 Plant Material Collection and Extraction

The plant *Moringa stenopetala*(root) was selected based on claim by the society to use for epilepsy. From the areas where it is used as traditional epilepsy therapy, the plant part used in this study was collected in April 2016. The root of *Moringa stenopetala* was collected from Arbaminch area 505 km South of Addis Ababa. The plant was identified and voucher specimen was deposited with the given herbarium code (03-S) in the national herbarium at Addis Ababa University , College of Science, Ethiopia.

The collected plant parts were then garbled in th e processing room and dried in the shade, and powdered and stored in a well-closed container at room temperature until extrac ted. The powdered, air dried materials

(500 g) of *Moringa stenopetala* (root) were then extracted by maceration with 80% methanol at room temperature for three consecutive days. The mixture filtered by gauze and then with Whatman™ filter paper 6µm pore size (125 mm GE healthcare UK limited, UK) and concentrated under vacuum in a rotary evaporator. Using this extraction technique *M. stenopetala* was extracted with dry amount of 7.35 gm(1.47%).The extract was kept in a tightly closed bottle in a refrigerator until used for anti-seizure testing [10].

2.2 Phytochemical Screening

The method used by Debella [10] was implemented to screen for the presence and/or absence of the main secondary metabolite groups in the extracts.

2.3 Acute Toxicity Study

An acute toxicity study was conducted for the extracts by acute oral toxic class method of Organization of Economic Co-operation and Development, as per 423 guidelines [11].Three female mice in a group were grouped into two groups in the test randomly. One control group was given distilled water and the other groups was treated for *Moringa stenopetala* crude extracts. The extracts were tested for a dose 2000 mg/kg and followed for acute signs in the first day and followed for 14 days.

2.4 The 0 Mg^{2+} *In vitro* Model of Seizures

Acute brain slices were prepared from 14-21 day old C57BL/6 mice. After decapitation, the mouse brain was extracted and quickly placed in a 50% sucrose cutting solution bubbled with carbogen gas (95% oxygen and 5% carbon dioxide). The cutting solution used was composed of (in mM): KCl (3); NaCl (60); NaH2PO4 (1.2); NaHCO3 (23); MgCl2 (3); CaCl2 (1) ; D-glucose (11) and sucrose (120) [12]. pH was adjusted to between 7.38 and 7.42 using 0.1mM NaOH. The mouse brain was then appropriately sectioned using a scalpel blade to ensure that the hippocampus and entorhinal cortex would be sliced in the transverse plane. 400 µm horizontal slices were cut using a vibrating VF-200 Compresstome (Precisionary Instruments, USA). This method of preparing acute brain slices is similar to that employed by [13,14]. Slice quality was confirmed by assessing the integrity of the hippocampus and its connection to the entorhinal cortex (EC). The slices were then transferred to a recovery chamber which contained a standard aCSF solution, which was again bubbled with carbogen gas. The standard aCSF solution was composed of: NaCl (120 mM); MgCl2 (2 mM); KCl (3 mM); CaCl2 (2 mM); NaHCO3 (23 mM); NaH2PO4 (1.2 mM); D-Glucose (11 mM). The slices were kept in the recovery chamber at room temperature (20-25°C) for a minimum of 40 minutes before being transferred to the interface rig for local field potential recordings.

For recordings, slices were placed in an interface recording chamber perfused with aCSF using a peristaltic pump (Model 205S Watson-Marlow, UK). The temperature was adjusted to ensure the solution in the chamber surrounding the slice was kept between 33 - 35°C. Single-electrode extracellular recordings were performed using glass micropipettes, which were prepared from borosilicate glass capillaries with an outer diameter of 1.20 mm and inner diameter of 0.69 mm (Warner Instruments, USA), using a horizontal puller (Intracell Model P-1000, Sutter, USA) [15].

The tips of the micropipettes were broken under microscope visualisation using a VT-II 2147861 microscope (Olympus, Japan). Pipettes were filled with Mg2+- free aCSF and lowered onto the entorhinal cortex of brain slices under microscope guidance. Once the electrodes were satisfactorily positioned in the tissue, field potential recordings were initiated (Power lab, AD Instruments). The recordings were verified visually on the Lab Chart recording software (AD Instruments, Dunedin, New Zealand). Electrical signals were amplified by the Microelectrode AC Amplifier (A-M system, model 1800) with gain set at 10000.

To elicit *in vitro* epileptiform activity, slices were bathed in Mg^{2+}-free aCSF [16,17]. Removing extracellular Mg^{2+}reduces the voltage dependent block of Mg^{2+}on N- methyl-D-aspartic acid (NMDA) receptors. Initial interictal-like activity is observed, followed by the gradual development of seizure-like events (SLEs), which mimic what is observed in temporal lobe seizures in humans [18,19]. Seizure-like events are observable as large, high-frequency events in the local field potential recordings, which lasted more than 5s. Baseline recordings were made for 600s with standard aCSF before Mg^{2+}-free aCSF was washed in for 3000s in order to induce seizure-like activity. The 0 Mg^{2+} solutions either contained *Moringa stenopetala* extract (0.7 mg/ml), the relevant solvent dimethyl sulfoxide

(DMSO) as a negative control, or diazepam as a positive control. The presence of SLEs was compared between treated slices versus untreated control. The Fisher's exact test with P<.05 was used to determine statistical differences between groups [15,20].

2.5 *In vivo* Seizure Models

Male BALB/c mice weighing between 20-30 g were used for both the maximal electroshock seizure (MES) model and the pentylenetetrazole (PTZ) seizure model. Mice were housed under standard conditions at a temperature of 22 ± 2°C, and with a 12 hr light/ 12 hr dark cycle. The mice were provided with free access to a standard pellet laboratory diet and water. The animals were fasted for 4-8hrs prior to testing [21] and were acclimatized to the laboratory environment.

2.6 Maximal Electroshock Seizure (MES) Model

Six BALB/c mice in each group were divided into 4 groups for the test extract. Animals in control group received 0.5% twin 80 (0.3 ml), reference group received phenytoin (10 mg/kg) and test groups received test extracts (400 mg/kg and 800 mg/kg) orally. The animals in all the groups received corresponding drugs 1 hour before the application of shock. Each animal was properly held and current of 54 mA was passed for 0.2 second transauricularly through ear lobe electrodes using an electroconvulsiometer. The duration of the hind limb extension was recorded. A reduction in this duration was considered as an anti-seizure action of the agent delivered [22]. The one way analyses of variance (ANOVA) test with post hoc LSD with P<.05 was used to determine statistical differences between groups.

2.7 Pentylenetetrazole (PTZ) Model

The animals were grouped into 4 groups and administered vehicle, reference drug and extracts as described in the MES test. In this case the reference group was treated with diazepam 5 mg/kg orally. One hour after administering corresponding drugs to different groups of animals, PTZ 85 mg/kg was injected subcutaneously and mice were observed for thirty minutes for the onset of convulsive behavior if not protected by the extract. The test is thought to be predictive of the activity of anticonvulsant drugs against nonconvulsive (myo clonic or absence) seizures [22]. The onset time of convulsions was recorded. The one way ANOVA test with post hoc LSD with P<.05 was used to determine statistical difference between groups.

2.8 Statistical Analysis

Graph pad prism 5 and SPSS25 softwares were used for analysis. The percentage of protected slices were analyzed using the Fisher's Exact Test (two-tail) with Graph pad prism 5. The one way ANOVA analyzed with SPSS25 was used for *in vivo* PTZ and MES test.

3. RESULTS

3.1 Yields of Hydromethanolic Extract

After hydro alcoholic extraction of the 500 gm *M. stenopetala ,* it was extracted with dry amount of 7.35 gm (1.47%).

3.2 Acute Toxicity Study

Acute toxicity study was conducted at 2000 mg/kg dose and the animals were observed according to the procedure for 14 days. There was no behavioral change on live animals on the days of follow up and also was no abnormality on postmortem examination with the extract.

3.3 *In vitro* Anticonvulsant Tests

Removal of Mg^{2+} (0 Mg^{2+}) from the slice perfusate results in recurrent seizure-like events in control slices. Middle trace, concurrent addition of 3 μM diazepam (a known anticonvulsant) prevented SLE generation in most slices. Addition of 0.7 mg/ml of *M. stenopetala* extract prevented the onset of SLEs in the majority of slices. Population data demonstrate the anticonvulsant efficacy of diazepam (2 of 12 slices had SLEs) and *M. stenopetala* (1 of 16 slices had SLEs). The hydroalcoholic extract of *M. stenopetala* extracts had a statistically significant anticonvulsant activity compared to control(P<.05). A positive control using the known anticonvulsant diazepam (3μM), showed significant anticonvulsant activity (P<.05). The percentage of slices showing SLE were given in Table 1.

Table 1. Anti-seizure activity of *Moringa stenopetala* extracts in the 0 Mg^{2+} *in vitro* seizure model

Test group	SLE positive	SLE negative	Total No. slices(N)	SLE Protection Percent
Control	10	6	16	37.5
Diazepam(3 µM)	2	10	12	83.33*
*M. stenopetala (*0.7 mg/ml)	1	15	16	93.57*

** denotes P <.05; Fishers exact test.*

The *in vivo* PTZ test showed no statistically significant effect with the plant extract at all dose levels (P>0.05). (See Table 2).

The *in vivo* MES test showed statistically significant effect in both low and higher dose of *Moringa stenopetala* extracts (P< .05) in mean hind limb extension time and survival. (See Table 3).

The qualitative secondary metabolite test evidenced the presence of alkaloids, cardiac glycosides, flavanoids, and saponins in *Moringa stenopetala* extracts. The summery is depicted in Table 4.

4. DISCUSSION

This study brings scientific evidences on the therapeutic value of *Moringa stenopetala (*root) which is traditionally being used for treatment of epilepsy in Ethiopia. The study provides a scientific rationale for the use of *Moringa stenopetala* root extract for the amelioration of epilepsy observed in traditional medicine in Ethiopia. The results revealed the hydoalcoholic extract of root of *Moringa stenoptela*, has shown statistically significant *in vitro* antiseizure activity. This was supported by the *in vivo* antiseizure activity in MES model. But the crude extract failed to show statistically significant activity in PTZ model.

This show the plant has antiseizure potential. The qualitative secondary metabolite test evidenced the presence of alkaloids, phenols, flavanoids, and saponins. According to the review done by Zue *et al.* medicinal compounds with antiepileptic/anticonvulsant activities are alkaloids, flavonoids, terpenoids, saponins and coumarins [6]. For the hydroalcoholic extract contains these compounds the anticonvulsant activity may be attributed to the presence of these phytochemicals. In *Moringa oleifera* alkaloids saponins, protein, flavonoids, carbohydrates, tannins, terpinoids, phenols, glycosides and phytosteroids showed presence in all the components of leaf, pod and bark and these parts showed anticancer activity [23].

Table 2. The *Moringa stenopetala* extracts didn't shows anti-seizure activity in the PTZ seizure model

Test groups	Number of mice(N)	Mean Latency for myoclonic Seizure(second)(Mean±SE)
Control	6	239.67±33.72
M.stenopetala 400 mg/kg	6	400.00±37.10
M. stenopetala 800 mg/kg	6	387.33±35.82
Diazepam 5mg/kg	6	1800.00±0,00*

**denotes P < .05; ANOVA test; N*

Table 3. The crude extract of *Moringa stenopetala* extracts shows anti-seizure activity in the MES model

Treatment	Number of mice	Mean Hind limb Extension time(second) (Mean±SE)	Survival
Control	6	24.33±2.45	2/6
M. stenopetala 400 mg/kg	6	10.33±3.52*	4/6*
M. stenopetala 800 mg/kg	6	12.50±4.16*	5/6*
Phenytoin 10mg/kg	6	00.00±00*	6/6*

**denotes P < .05; ANOVA test and Fisher's exact test*

Table 4. Secondary metabolites in the hydroalcoholic extracts of *M.stenopetala*

Phytochemicals	*M.stenopetala*
Alkaloid	+
Cardiac glycosides	+
Flavonoids	+
Phenols	-
Saponins	+
Sterols	-
Tannins	-
Terpenoids	-

+ denotes positive; - denotes negative

The result of MES model is in consistent with study done in other species of Moringa, *Moringa concanensis* which abolishes both MES and PTZ seizures. The anti-convulsant activity can be due to the presence of various phytoconstituents like alkaloids, tannins, phenols, flavanoids, and carbohydrates in the plant [24]. But the PTZ effect in this species is in contrary of the finding in our study in *Moringa stenopetala.* In another study pretreatment with roots *Moringa oleifera* methanolic extract caused statistically significant protection against PTZ and strychnine (STR) induced seizures [25]. The root PTZ effect *Moringa oleifera* is also in contrary with the effect seen in *Moringa stenopetala.* This may be due to the phytochemical constituents with action on the PTZ model may be in less concentration in *Moringa stenopetala.* than with these two species.

The root is also used in traditional medicine to treat other different aliments and for which some of the chemical components were studied. In one study the acetone extract of *Moringa stenopetala* (root), which was active as antibacterial was found to contain palmitic acid , cholest-5-en-3-ol, oleic acid and n-octacosane [26]. The acetone crude extract of *Moringa stenopetala* (root bark) was subjected to column chromatography separation. Four compounds were obtained and found to be stigmastereol, ursolic acid, tasnemoxide and oleic acid based on their spectral analyses. The antibacterial activity of the compounds reveled that they show good antibacterial activities [27]. In other study *Moringa stenopetala* root extract isolates 1,3-dioleoyl-2-linolein and 1,3-dili-noleoyl-2-olein has sown anti leshmanial activity [28]. Roots of *M. stenopetala* and *M. oleifera* both had high concentrations of 4-(α-L-rhamnopyranosyloxy)-benzylglucosinolate and benzyl glucosinolate [29].

In vitro, *in vivo*, and some clinical studies evidenced the n-3 fatty acids to have anticonvulsant activity. At concentrations of up to 100 lM, Eicosapentaenoic acid (EPA), Alpha-linolenic acid (ALA), or Docosahexaenoic acid (DHA) reduced the frequency of pentylenetetrazol (PTZ) or glutamate, low Mg^{2+} or depleted of glycine induced action potentials and excitatory discharges in hippocampal slices. 161M of DHA significantly inhibited the repetitive action potentials evoked by depolarizing current pulses [30]. These data led to the hypothesis that fatty acids modulate ion channels. DHA and EPA have also been shown to stabilize the neuronal membrane in single cells by suppressing voltage-gated Na^{+} and Ca^{2+} channels, and thereby increasing the action potential firing threshold [31]. The *in vitro* seizure suppression 0-Mg^{2} model and action on the MES *in vivo* model in *Moringa stenopetala* root may be from these chemical constituents.

5. CONCLUSIONS

The hydroalcoholic crude extract of *Moringa stenopetala* root showed to have anticoncvulant effect both *in vitro* and *in vivo* models. The use of local community as an anticonvulsant is supported by the evidence obtained from this study. Having this tapping its potential as anticonvulsant is promising in identifying new antiepileptic molecules as it is widely used for treatment of other diseases with less toxic effect.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All animal handling, care and procedures were carried out in accordance with national and institutional guidelines. The tests were conducted after the ethical clearance was verified by Institutional Review Board (IRB). The experimental protocols for *in vivo* experiments were approved by the Institutional Review Board (IRB) of Addis Ababa University (AAU), College of Health Sciences. Approval for the *in vitro* experiments was granted by the University of Cape Town Animal Ethics Committee (Protocol No: AEC 014/035).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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