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In vitro and *In vivo* Anticonvulsant Effect of Hydroalcoholic Extracts of *Clutia abyssinica* in Mice Model

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Authors' contributions

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

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ABSTRACT

Background: Epilepsy is a chronic disorder of the brain that affects people of all ages worldwide. In the search of safe and effective antiepileptics traditional treatment practices are one area of research to obtain novel molecules. Research is also needed to validate and standardize the traditional claim. *Clutia abyssinica* leaves were one of the medicinal plants claimed for use against epilepsy and evil eye and other diseases in different parts of Ethiopia. But there was no scientific research evidence for the claimed use of the plant. Therefore this work was designed to evaluate the anticonvulsant effect of hydroalcoholic extract of *Clutia abyssinica* leaves.
Methods: The dry residue of the plant extract was used for the test. *In vitro* 0Mg2+ 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 subcutaneously was used to compare the seizure onset time with two extract doses and diazepam

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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: *In vitro* anticonvulsant tests *C. abyssinica* extract effect was not statistically significant compared to negative control (P>0.05).A positive control using the known anticonvulsant diazepam (3μM), showed significant anticonvulsant activity (P<0.05). The *in vivo* PTZ test showed no statistically significant effect in plant extract at all dose levels (P>0.05). In the *in vivo* MES test the extract of *Clutia abyssinica* both low and higher dose didn't show statistically significant effect (P>0.05) compared with the negative control. But the extract improved survival (p<0.05). The qualitative secondary metabolite test evidenced the presence of alkaloids, cardiac glycosides, flavanoids, phenols, saponins, sterols and terpeoids in *Clutia abyssinica* extract.

Conclusion: The hydroalcoholic crude extract result of the *C. abyssinica* as anticonvulsant is weak based on the models used in this study. For most of the local preparation are mixes of different plants it may have synergistic action with other plants. Or it may have action with other models of chronic epilepsy.

Keywords: Clutia abyssinica; anti-epileptic; In vitro; In vivo; mice.

1. INTRODUCTION

Epilepsy is the third leading contributor to the global burden of disease for neurological disorders and affects 65 million people worldwide [1]. In Ethiopia as various diseases are being treated traditionally some survey shows also the practice of treatment for epilepsy [2]. *Clutia abyssinica* leaves were one of the medicinal plants claimed for use against epilepsy and evil eye and other diseases in different parts of Ethiopia [3–5]. But there is no scientific evidence for the claimed use. As different researches on plants used for epilepsy in different countries have shown anticonvulsant activity, this plant may also have value for the treatment of the disease. Therefore scientific research should be done on these plants for their antiepileptic potential [6].

Zero Mg^{2+} model is one of the *in vitro* models to study mechanism of seizure and antiseizure drugs [7]. 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 *Clutia abyssinica* using *in vitro* and *in vivo* mice models. The results are of importance in validating the claimed use and in revealing its anticonvulsant potential for further scientific research.

2. MATERIALS AND METHODS

2.1 Plant Material Collection and Extraction

The plant *Clutia abyssinica* was selected based on claim by the society to use for epilepsy. The leaves of *Clutia abyssinica* were collected from Bale area, Ethiopia, in April 2016. The plant was identified and voucher specimen was deposited with the given herbarium code (01-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 wellclosed container at room temperature until extracted. The powdered, air dried materials (400 g) of *Clutia abyssinica* (leaves) were then extracted by maceration with 80% methanol at room temperature for three consecutive days. The mixture filtered by gauze and then with What man™ filter paper 6μm pore size (125 mm GE healthcare UK limited, UK) and concentrated under vacuum in a rotary

evaporator. Using this extraction technique *Clutia abyssinica* was extracted with dry amount of 44.71 gm (11.18%).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 *Clutia abyssinica* crude extracts. The extract was tested for a dose 2000mg/kg and followed for acute signs in the first day and followed for 14 days.

2.4 The 0Mg^{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 : KCl (3 mM); NaCl (60 mM); NaH2PO4 (1.2 mM); NaHCO3 (23 mM); MgCl2 (3 mM); CaCl2 (1 mM) ; D-glucose (11 mM) and sucrose (120 mM) [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. 400µm 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 Dreier [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 (120mM); MgCl2 (2mM); KCl (3mM); CaCl2 (2mM); NaHCO3 (23mM); NaH2PO4 (1.2mM); D-Glucose (11mM). 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 1.20mm and inner diameter of 0.69mm (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 Mg^{2+}- 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 (Powerlab, AD Instruments). The recordings were verified visually on the LabChart recording software (ADInstruments, 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 in terictal-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 [15,18]. 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 *Clutia abyssinica* extract (0.7mg/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 [15,18].

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^{o}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 [19] 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 2%twin 80 (0.3 ml), reference group received phenyto in (10mg/kg) and test groups received test extracts (400mg/kg and 800mg/kg) orally. The animals in all the groups received corresponding drugs 1hour 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 [20]. 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 5mg/kg orally. One hour after administering corresponding drugs to different groups of animals, PTZ 85mg/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 [20]. 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 Acute Toxicity Study

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

3.2 *In vitro* Anticonvulsant Tests

The *C. abyssinica* extract effect was not statistically significant compared to negative control (P>0.05). A positive control using the known anticonvulsant diazepam (3μM), showed significant anticonvulsant activity (P<0.05). The percentage of slices showing SLEs were given in Table 1.

3.3 *In vivo* Anticonvulsant Tests

The *in vivo* PTZ test showed no statistically significant effect in plant extract at all dose levels though there was dose dependent delay on seizure onset (P>0.05) (See Table 2).

In the *in vivo* MES test the extract of *Clutia abyssinica,* both low and higher dose didn't show statistically significant effect (P>0.05) compared with the negative control (See Table 3).But the extract improved survival (p<0.05).

Table 1. Anti-seizure activity of *Clutia abyssinica*extracts in the 0 Mg^{2+}*in vitro* seizure model * denotes P <0.05, Fishers exact test

Test group	SLE positive	SLE negative	Total No. slices	SLE protection percent
Control	10	6	16	37.5
Diazepam	2	10	12	83.33*
Clutia abyssinica	8	4	12	33.33

Table 2. The *Clutia abyssinica* extracts didn't shows anti-seizure activity in the PTZ seizure model. *denotes P <0.05, ANOVA test

Test group	N	Mean Latency for myoclonic seizure(s)
Control	6	239.67±33.72
C. abssinica. 400 mg/kg	6	284.00±13.93
C. abssinica. 800mg/kg	6	457.83±103.54
Diazepam 5mg/kg	6	1800.00±0,00*

Table 3. The crude extract of *Clutia abyssinica* did't show anti-seizure activity in the MES model. It improved survival compared with the negative control. *denotes P <0.05, ANOVA test and Fisher's exact test.

Treatment	N	Mean hindlimb extension time(S)	Survival
Control	6	24.33±2.45	2/6
*Clutia abyssinica*400 mg/kg	6	20.00±1.06	4/6*
*Clutia abyssinica.*800mg/kg	6	21.83 ±0.40	5/6*
Phenytoin 10mg/kg	6	00.00*	6/6*

The qualitative secondary metabolite test evidenced the presence of alkaloids, cardiac glycosides, flavanoids, phenols, saponins, sterols and terpeoids in *Clutia abyssinica* extract. The summery is depicted in Table 4.

4. DISCUSSION

This study brings scientific evidences on the therapeutic value of *Clutia abyssinica* (leaf*)* which is traditionally being used for treatment of epilepsy in Ethiopia. *Clutia abyssinica* showed little effect on the *in vitro* as well as *in vivo* models. The effect was not statistically different from the negative control. This plant was claimed by the local people for different ailments and it is used for epilepsy and evil eye [21]. Traditionally it is used in treatment of many other diseases [3,4,22]. The results in this test show it has less anticonvulsant activity on the models used to test anticonvulsant effect of the plant. The plant may probably not have anticonvulsant effect by itself but may potentiate the effect of other concomitantly administered plants.

The dichloromethanolic root extract of *C. abyssinica* demonstrated analgesic activities on acetic acid-induced pain in Swiss albino mice [23]. In other study *in vivo* antitrypanosomal activity of methanol crude leaf extracts of *C. abyssinica* against *T. congolence* field isolate was demonstrated [24]. In one study the results of serum biochemical markers and histopathological studies in the crude 80% methanol extract and n-butanol fraction pre- and post-treated group support the hepatoprotective effect of *Clutia abyssinica* leaf [25]. These studies evidence the potential of the plant as alternative treatment in the respective claimed uses.

In the current study the leaves of *Clutia abyssinica* extract showed the presence of alkaloids, cardiac glycosides, flavanoids, phenols, saponins, sterols and terpeoids. Though these are some of the components, they didn't show significant anticonvulsant effect in the current models used in this study. Further study in other seizure models as well as with higher dose in models that showed dose dependent improvement (ptz test) is needed to revalidate the claim. For most plants are given in combination it may have some potentiating effect if it is given with other plants.

Table 4. Secondary metabolites in the hydroalcolic extracts of *C. abyssinica*

Phytochemicals	C. *abyssinica*
Alkaloid	+
Cardiac glycosides	+
Flavonoids	+
Phenols	+
Saponins	+
Sterols	+
Tannins	-
Terpenoids	+

5. CONCLUSION

The hydroalcoholic crude extract result of the *C. abyssinica* as anticonvulsant is weak based on the models used in this study. For most of the local preparation are mixes of different plants it may have synergistic action with other plants. Or it may have action with other models of chronic epilepsy.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Animal Ethic committee approval has been taken to carry out this study.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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