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Evaluation of Hydro-alcoholic Extract of *Clerodendrum myricoides* (Hochst. Vatke) Leaves and Its Solvent Fractionsin Pentylenetetrazole-Induced Convulsion in Mice

Teketel Eristu Kediso[1,2], Tesfaye Tolessa[2*], Fikirte Getachew[3], Eyasu Makonnen[4,5] and Daniel Seifu[6,7]

[1]Department of Biomedical Sciences, College of Health Sciences, Arbaminch University, Arbaminch, Ethiopia.
[2]Department of Physiology, College of Health Sciences, Addis Ababa University, Addis, Ababa, Ethiopia.
[3]Department of Biomedical Sciences, College of Health Sciences, Wachemo University, Hosanna, Ethiopia.
[4]Department of Pharmacology and Clinical Pharmacy, College of Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia.
[5]Center for Innovative Drug Development and Therapeutic Trials for Africa, College of Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia.
[6]Department of Biochemistry, College of Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia.
[7]Division of Biomedical Sciences, Department of Biochemistry, University of Global Health Equity, Rwanda.

Authors' contributions

This work was carried out in collaboration among all authors. Author TEK designed the study, performed the experimental procedures, statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors TT, EM and DS wrote the 2nd draft of the manuscript and supervised lab experiments. Author FG organized data, worked in the field survey, assisted plant material collection, preparation and execution of lab work. All authors read and approved the final manuscript.

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**Corresponding author: E-mail: tesfayet2002@yahoo.com;*

ABSTRACT

Introduction: Epilepsy is a chronic neurological disorder that affects people of all ages. Herbal medicines are widely used across the globe due to their wide applicability and therapeutic efficacy. The low side effects of traditional herbal medicines have encouraged many types of research into antiepileptic activity. *Clerodendrum myricoides* is a plant whose leaves extract is traditionally used as an anticonvulsant in Ethiopia.

Objective: The point of this investigation was to assess the anticonvulsant effect of the hydro-alcoholic extract and solvent fractions of *C. myricoides leaves* against pentylenetetrazole-induced seizures in mice.

Methods: Comparison of mean latency to onset of convulsion, mean duration of convulsions, and the proportion of percentage protection against seizure of the plant extract was tested against PTZ-induced seizures. Three different doses were used by giving them orally 30 minutes before subcutaneous pentylenetetrazole (80 mg/kg) administration with the positive (diazepam 2 mg/kg) and negative (physiological saline 10 mg/kg) control groups. Data were presented as the mean ± standard error of the mean and analyzed using a one-way analysis of variance (ANOVA) and followed by post-hoc Tukey's multiple comparisons test. Fisher's exact test was used for the percentage protection. $P < 0.05$ was considered statistically significant.

Results: The crude extract of *C. myricoides,* with the doses of 300, 600, 1,200 mg/kgshowed a significant delay in mean latency to onset of seizures [299.33±30.129 sec ($p< 0.05$); 387.167± 27.6 sec ($p<0.01$); 417.833±31.9 sec ($p<0.001$); respectively)]; decrease in the duration of convulsion [27.333±1.585 sec ($p<0.05$); 16.833±1.537 sec ($p<0.01$); 10.50±0.671 sec ($p<0.001$) respectively]; and a proportion of percentage protection of mice against seizure [16.33% (1/6) ($p< 0.05$); 33.33% (2/6) ($p<0.01$); 50% (3/6) ($p<0.001$) respectively] in a dose-dependent manner compared to the control group [92.833±13.006 sec; 34.167±3.683 sec, 0% respectively]. *C. myricoides* anticonvulsant activity was less than that of diazepam [1001.16± 68.430 sec, 4.500±0.619, 83.33 sec, 83.33% respectively for the doses]. Its solvent fractions, however, didn'tshow a significant anticonvulsant effect.

Conclusion: The hydroalcoholic leaves crude extract of *C. myricoides* has anticonvulsant activity but its solvent fractions do not have comparable significant effects.

Keywords: Anticonvulsant effects; hydro-alcoholic extracts; solvent fractions; Clerodendrum myricoides; pentylenetetrazole; mice.

1. INTRODUCTION

Epilepsy is a neurological condition that affects a large segment of the world population. It is a disease of the brain characterized by the erratic and intermittent incidence of a transient change of behavior due to chaotic, simultaneous, and recurrent firing of cortical neurons known as a seizure [1].

A seizure is a paroxysmal abnormal discharge at high frequency from an aggregate of neurons in the cerebral cortex; while epilepsy is a condition described by repetitive events of such seizures (2). Seizures can be "non-epileptic" when evoked in a normal brain by treatment such as electric shock or chemical convulsants or "epileptic" when occurring without evident provocation [1,2]. Epilepsy affects about 1% of the world population (3). The frequency of epilepsy in industrialized nations is roughly 50 in 100,000, while that of undeveloped nations is 100 in 100,000 (4). It has been observed that currently available antiepileptic drugs are unable to control seizures effectively [3,4].

Contemporary drug treatment of epilepsy is complicated by the incapability of medications to control seizures in certain patients and by adverse effects ranging from minimal damage of the brain to death resulting from aplastic anemia or hepatic failure [5]. Thus, the efficacy and safety of the current medication remain a challenge [5-8]. As a result, about 30% of epileptic patients continue to suffer recurrent seizures even with current antiepileptic drug therapy [9].

Cultural attitudes, lack of prioritization of epilepsy as a public health condition, poor wellbeing framework foundation, and lack of supplies of antiepileptic drugs to upset suitable treatment

[10, 11]. The point of treating an epileptic patient is not only to eliminate the event of seizures but also to help them lead a self-sustained life [9]. Hence, there is a need to look for more effective and safer alternative antiepileptic agents.

Herbal medicine continues to serve a large proportion of the population, especially those in rural and tribal areas, regardless of the advent of modern medicine due totheir traditional beliefs, accessibility, insufficient supply and low affordability of antiepileptic drugs. Evaluation of the safety and efficacy of traditional medicinal plants through experimental models provides further clues that may prove any claim on the use of plants as medicine [12].

Clerodendrum myricoides (Hochst.) Vatke (Family: Lamiaceae) commonlycalled in English Blue-flowered tinder wood, locally known as Marasissa, in Afaan Oromo and Misirich, in Amharic, is traditionally used as an antiepileptic agent in Ethiopia, especially in Bale Oromia region, and it is also a common medicinal plant in Africa [13-15].

2. MATERIALS AND METHODS

2.1 Collection and Preparation of Plant Materials

Fresh leaves of *Clerodendrum myricoides* (Family**:**Lamiaceae**)** were collected from Bale National Park, Ethiopia, 550km away from Addis Ababa, and transported in a dark plastic bag to avoid decomposition by light. The plant material was then authenticated and given a voucher number (AAU-NH 01T) by a plant Taxonomist and the plant specimen was deposited at the National Herbarium, College of Computational, and Natural Sciences, Addis Ababa University for future reference.

The fresh leaves were dried under shade at room temperature and powdered using pestle and mortar. The dried powder (800g) was macerated in 70% v/v of ethanol for three days continuously stirred using an orbital shaker at 120 rpm and then, filtered using gauze (0.1mm^{2}mesh) and Whatman filter paper (size 15cm) (Whatman® England). The filtrate was then placed in a Petri dish, and kept deep-frozen at -27°C and lyophilized for one week (-52°C, 133 x 10-3mbar, operan, Korean vacuum limited, Korea) to obtain crude extract. A total yield of 83.52g crude extract was obtained and kept in a desiccator until used for the experiment.

The crude extract of *C. myricoides* was further fractionated using petroleum ether, dichloromethane, ethanol, and water as solvents in order of decreasing polarity. Twenty grams of hydroethanolic extract of *C. myricoides* was dissolved in 100 mL of distilled water in a separate funnel. The dissolved ethanol extract was partitioned with 3x150 mL of petroleum ether at 40-60 □. The petroleum ether partitions were combined and concentrated using a rota-vapor (BÜCHI Rota-vapor R-205, Switzerland). The concentrate was labeled as petroleum ether fraction and yielded 2.03 g. The aqueous residue was then partitioned with 3x150 mL of dichloromethane. The dichloromethane filtrates were combined and evaporated to give 2.88 g of dichloromethane fraction. The aqueous residue was further partitioned with 3x100 mL ethanol. The fractionates were then combined, concentrated, and labeled as 3.98 g yield of ethanol fraction. The remaining aqueous residue was lyophilized to dryness using lyophilizer (operan, Korean vacuum limited, Korea) and labeled as 8.65 g yield of aqueous residue. All the fractions were then kept in different separate tightly closed containers with aluminum foil in a desiccator until used for the experiment.

2.2 Drugs/Chemicals/Test Substances Preparation and Administration

Pentylenetetrazol (PTZ) (Sigma Chemical Co., Sweden), diazepam (Medifarma Pharmaceuticals), and sodium chloride (BDH, England) were used in this study. All chemicals were prepared freshly just before use. Dimethyl sulfoxide (DMSO), ethanol, dichloromethane, petroleum ether (Germany). PTZ and diazepam were dissolved in saline solution (0.9% w/v). The crude extract and its fractions were dissolved in distilled water and DMSO (2:1v/v) mixture. Drug solutions were prepared according to the supplier's instructions. The maximum volume of freshly prepared herbal medicine administered at once in rodents was 1 mL/100 g body weight according to OECD guidelines [16]. The starting doses of *C. myricoides,* crude extract to be administered, was 300 mg/kg of body weight, 1/10th of LD_{50} based on the same plant previous study [14].

2.3. Experimental Procedure

Male *BALB*/*c* mice weighing between 22-30 g were obtained from Ethiopian Public Health Institute, Addis Ababa, Ethiopia and housed at

room temperature under standard nutritional and environmental conditions with relative moisture of 30-70% and 12 hours light-dark cycle. They all had access to water and food *ad libitum* and were denied food 12h before experimentation. The animals were arbitrarily assigned to 9 treatment groups consisting of 6 mice each.

Group I received the vehicle (normal saline, 10 mL/kg, i.p.) and served as a negative control; Group II received diazepam (2 mg/kg, p.o.), the standard drug and served as a positive control; Groups III, IV, and V received three different doses (300, 600 and 1,200 mg/kgp.o.) of the crude extract and Groups VI-IX received 1,200 mg/kg(p.o.)of the four solvent fractions each (petroleum ether, dichloromethane, ethanol and aqueous), 30 min before subcutaneous administration of PTZ (80 mg/kg) into the loose skin fold on the back of the neck of the animals. After PTZ administration, mice were placed in a chamber and closely observed for 30 minutes for the onset time of convulsions, and the duration of convulsion. Delayed onset and decreased duration of convulsion indicate the ability of the extract to protect animals from PTZ-induced convulsion.

2.4 Statistical Analysis

The Numbers of animals without convulsions were calculated as percentages. All results were expressed as mean ± SEM. Statistical significance between the groups was analyzed by one-way analysis of variance (SigmaPlot 14 (SigmaPlot Software, Inc., La Jolla, CA, USA) software) followed by Tukey's post hoc multiple comparison tests for comparison between groups. Fisher's exact test was used for the percentage protection. For all the tests, $p < 0.05$ was considered statistically significant.

3. RESULTS

3.1 Yields of Hydro-ethanolic Extract and its Solvent Fractions of *C. myricoides*

The yields of the crude extract and solvent fractions of *C. myricoides* leaves extract are shown in Table 1.

3.2 Effects of Hydro-alcoholic Crude Extract of *C. myricoides* on Onset of Seizure

The crude extract of *C. myricoides* at the doses of 300, 600, and 1200 mg/kg body weight showed significant delay or increase in latency [299.33±30.129 sec ($p<0.05$); 387.167±27.6 sec ($p<0.01$); 417.833± 31.9 sec ($p<0.001$), respectively] to mean onset of seizures in a dose-dependent manner against PTZ-induced seizures compared to the control group [92.833±13.006 sec]. The measured anticonvulsant activity was less than that of diazepam [1001.16± 68.430sec] as shown in Fig.1.

3.3 Effects of Hydro-alcoholic Crude Extract of *C. myricoides* on Duration of Convulsion

The crude extract of *C. myricoides* at the doses of 300, 600, and 1200mg/kg body weight significantly decreased the severity or the duration [27.333 ±1.585 sec ($p<0.05$); 16.833±1.537 sec ($p<0.01$); 10.50±0.671 sec ($p<0.001$), respectively] of convulsions in a dose-dependent manner against PTZ- induced seizures compared to the control group [34.167±3.683 sec]. The observed anticonvulsant activity was similar to that of diazepam [4.500±0.619 sec] as depicted in Fig. 2.

Table 1. *C. myricoides* 70% ethanol extract and its solvent fraction yield

Plant material	Yields obtained in gram	% of yield
*C. myricoides (*crude extract)	83.52	10.44
Petroleum ether fraction	2.03	10.15
Dichloromethane fraction	2.88	14.4
Ethanol fraction	3.98	19.9
Aqueous residue	8.65	43.25

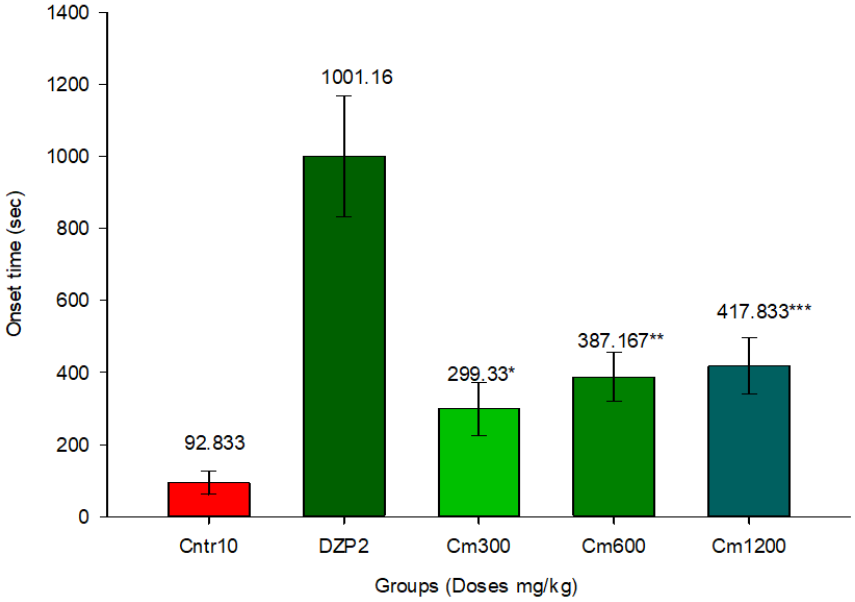

Fig. 1. Comparison of the mean latency to the onset of convulsion in groups of mice receiving hydroalcoholic *C. myricoides* crude extract (300, 600, and 1200mg/kg p.o.) 30 min before subcutaneous PTZ administration with the positive (diazepam 2mg/kg p.o.) and negative (physiological saline 10mL/kg i.p.) control groups

Values are mean ± SEM of six independent experiments (n=6). SEM=Standard error of the mean, Cm= C. myricoides hydro-alcoholic crude extract. DZP=diazepam, Ctrl=Control. P< 0.05, p<0.01**, p<0.001****

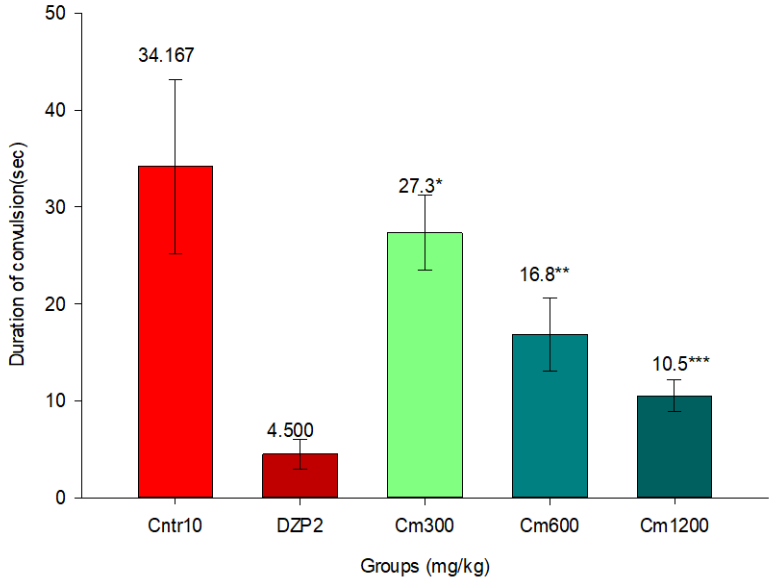

Fig. 2. Comparison of the mean duration of convulsion in groups of mice receiving hydro-alcoholic crude extracts of *C. myricoides*(300, 600, and 1200 mg/kg p.o.) 30 min before subcutaneous PTZ administration with the positive (diazepam 2mg/kg p.o.) and negative (physiological saline 10mL/kg i.p.) control groups

*Values represent the mean ± SEM of six independent experiments (n=6). SEM=Standard error of the mean, Cm= C. myricoides hydro-alcoholic crude extract. DZP=diazepam, Ctrl=Control. *P < 0.05, **p<0.01, ***p<0.001*

3.4 Protective Effect of Crude Extract of *C. Myricoides*against PTZ-induced Seizures

All the doses (300, 600 and 1200 mg/kg body weight) of the crude extract of *C. myricoides* showed significant percentage protection of the animals against seizure [16.33% (1/6) ($p<0.05$); 33.33% (2/6) ($p<0.01$); 50% (3/6) ($p<0.001$), respectively] against PTZ-induced seizure in a dose-dependent manner compared to the negative control [0%], The observed proportion of percentage protection was less than that of diazepam [83.33%,(5/6)] as indicated in Fig. 3.

3.5 Effects of Solvent Fractionation of *C. myricoides on* Onset of Seizure

The solvent fractions of *C. myricoides* did not show statistically a significant difference ($p>0.05$) in latency to onset of seizures against PTZ-induced seizures compared to the control group, as shown in Fig. 4.

3.6 Effects of Solvent Fractionation of *C. myricoides* on Duration of Convulsion

The solvent fractions of *C. myricoides* did not show a statistically significant difference in the severity of seizures or duration of convulsions against PTZ-induced seizures compared to the control group but the difference was significant to that of diazepam as indicated in Fig. 5.

4. DISCUSSION

In the present study, the anticonvulsant effect of hydroalcoholic and solvent fractions of *Clerodendrum myricoides* extract was investigated in the PTZ-induced convulsion model in mice. In this experiment, when the hydroalcoholic extract *C. myricoides* leaves were tested against PTZ-induced seizure in mice, the onset of convulsion was prolonged and the duration of convulsion was shortened significantly. This anticonvulsant effect of the extract is likely mediated through the central nervous system neurotransmitter-dependent depressant effect. This central nervous system depressant effect was assessed by prior induction of convulsion using a centrally acting pentylenetetrazole.

It is well known that pentylenetetrazole (PTZ) induction seizure is mediated through inhibition of GABAergic neurotransmission, which is through inhibition of the activity of gamma-aminobutyric acid (GABA) at $GABA_A$ receptors [17,18].

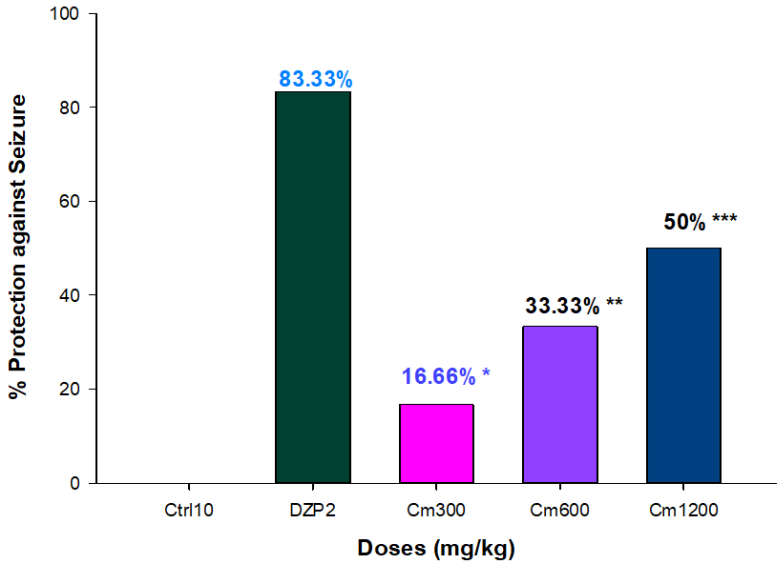

Fig. 3. The percentage protection of mice receiving hydro-ethanolic *C. myricoides*crude extract (300, 600, and 1,200) mg/kg of body weight 30 min before subcutaneous PTZ administration compared with those of the positive (diazepam 2mg/kg) and negative (physiological saline 10mg/kg) controls. Cm= *C. myricoides,* DZP=diazepam, Ctrl=Control

*Fisher's exact test was used for the percentage protection against the seizure of the animal for the statistical significance test. *P < 0.05, **p<0.01, ***p<0.001*

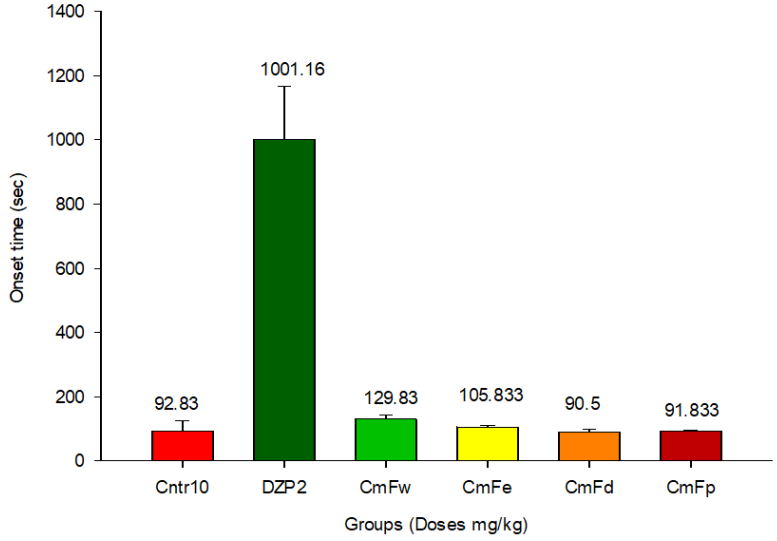

Fig. 4. Comparison of the mean latency to the onset of convulsion in groups that received 1,200mg/kg each solvent fractions (water, ethanol, dichloromethane, and Petroleum fractions) of hydro-alcoholic *C. myricoides* crude extract 30 min before subcutaneous PTZ administration with the positive (diazepam 2mg/kg) and negative (physiological saline 10mg/kg) control groups

Values are mean ± SEM of three independent experiments (n=6). SEM=Standard error of mean, CmFw= C. myricoides water fraction, CmFe= C. myricoides ethanol fraction, CmFd= C. myricoides dichloromethane fraction, CmFp= C. myricoides petroleum ether fraction, DZP=diazepam, Ctrl=Control

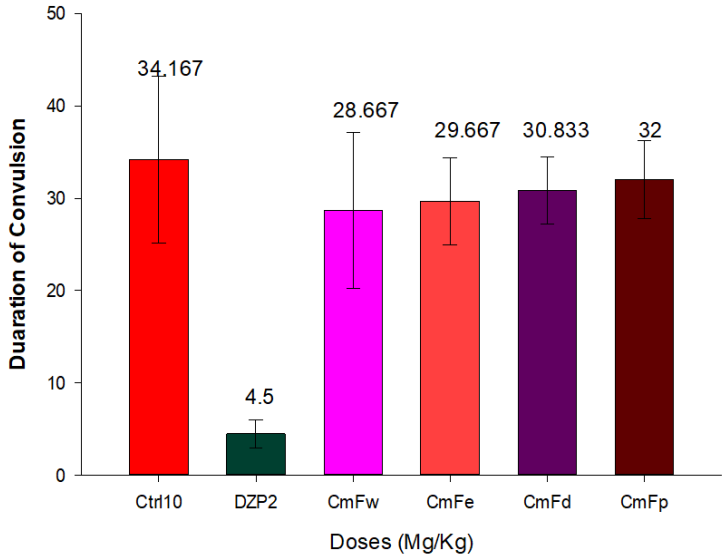

Fig. 5. Comparison of the mean duration of convulsion in those mice group which received solvent fractions of hydro-alcoholic *C. myricoides* crude extract 1,200mg/kg each (water, ethanol, dichloromethane, and petroleum ether fractions) 30 min before subcutaneous PTZ administration with the positive (diazepam 2mg/kg) and negative (physiological saline 10mg/kg) control groups

Values represent the mean ± SEM of three independent experiments (n=6). SEM=Standard error of the mean, CmFw= C. myricoides water fraction, CmFe= C. myricoides ethanol fraction, CmFd= C. myricoides dichloromethane fraction, CmFp= C. myricoides petroleum ether fraction, DZP=diazepam, Ctrl=Control

Enhancement of GABA neurotransmission attenuates convulsions; while inhibition of neurotransmission of GABA heightens convulsions [19,20]. GABA is an endogenous agonist of the $GABA_A$ receptor boosting opening of the channels to Chloride ions in the neuronal plasma membrane inducing hyperpolarization bringing. This effect blocks nerve impulse transmission and henceforth responsible for the counter epileptic actions [21]. It has been found that PTZ also increases the neurotoxic free radicals in the brain leading to neuronal damage-induced epilepsy [22, 23]. The PTZ test in an experimental animal can be taken as a model for human generalized and absence seizures [24,25]. Diazepam is a known standard antiepileptic drug that generally inhibits sodium currents and enhances GABA transmission in the brain, consequently blocking PTZ-induced seizure [20].

The observation that the crude hydroalcoholic extract of *clerodendrum myricoides* significantly increased latency to onset of seizures, decreased the severity of seizures and showed a proportion of percentage protection against PTZ-induced seizure in a dose-dependent manner clearly shows the anticonvulsant activity of the extract. This observed anticonvulsant effect of the extract might well be attributed to the direct and/or indirect enhancement action on GABAergic neurotransmission in the brain [17,18].

The findings of this study are in agreement with those of the study done on the anticonvulsant effect of *Antiaris toxicaria* (Pers.) and pycnogenol extract from maritime pine bark (*Pinus pinaster*) containing flavonoids [26,27] and on the root extract of *Carissa carandas* Linn and fruit extract *Phoenix dactylifera* [20,28].

Neither of the fractions, i.e., petroleum ether, dichloromethane, ethanol, and an aqueous fraction, however, showed a significant protective effect against PTZ-induced seizures. This negative effect of the solvent extract is probably related to the presence of low concentrations of the active ingredients in each of such solvent fractions. Therefore, the observation in the present study shows that the anticonvulsant effect of crude extract as compared to its separate solvent extract might be related to an additive and/or synergistic effect of the active ingredients present in the crude extract. These findings agree with other similar studies in which solvent fractions were reported to be ineffective against PTZ-induced seizures [15,29,30].

5. CONCLUSION

From the present findings, it can be concluded that hydro-alcoholic crude extract of *Clerodendrum myricoides* has an anticonvulsant effect against PTZ-induced seizures. The effect is likely attributed to the presence of different active ingredients in the extract that exert a synergistic effect. The solvent fractions of *C. myricoides* do not have the same anticonvulsant activity. This finding also justifies the traditional claim of the use of the plant in the treatment of epilepsy.

DISCLAIMER

The plant (product) used for this research is common in the country and predominantly used locally to treat many ailments and also in research in Ethiopian higher institutions. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company, rather it was funded by Addis Ababa University and Arbaminch University in an investigation that offers a tertiary degree for the first author.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The protocol was approved by the Institutional Review Board, College of health sciences, Addis Ababa University (Protocol number; 053/15/Physio). Before, during, and after the study animals were handled according to OECD guidelines [15].

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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