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ORIGINAL RESEARCH

Anti-Convulsant Activity of Soxhlet Leaf Extracts of *Ajuga Integrifolia* Buch.-Ham. Ex D.Don (Lamiaceae) in Mice

Tesfaye Desalegn, Ephrem Engidawork

Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, College of Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia

Correspondence: Ephrem Engidawork, Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, College of Health Sciences, Addis Ababa University, P. O. Box 9086, Addis Ababa, Ethiopia, Email ephrem.engidawork@aau.edu.et

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Background: The leaves of *Ajuga integrifolia* Buch.-Ham. ex D.Don (Lamiaceae) have long been used as an anti-convulsant remedy in Ethiopian traditional medicine. However, the evidence supporting their use is sparse in the literature. This study was conducted to add to the existing body of knowledge about the anti-convulsant activity of the plant.

Methods: The anti-convulsant activity of the extract was investigated in both acute (pentylenetetrazol [PTZ], 80 mg/kg; and maximal electroshock [MES]) and chronic (PTZ, 35 mg/kg) kindling seizure models. For the experimental paradigms, various doses of the extract (100, 200, and 400 mg/kg) were administered. Positive controls received sodium valproate (200 mg/kg) for the PTZ model and phenytoin (25 mg/kg) for the MES model. Parameters including the onset of clonus and duration of hindlimb tonic extension were recorded and compared with controls. Moreover, the total alkaloid, flavonoid, and phenol contents of the extracts were determined.

Results: Ethyl acetate extract produced a superior effect among all solvent extracts in both the PTZ and MES models. At all doses, it significantly delayed the mean onset of clonus ($p<0.01$) in the PTZ test compared to controls. It also significantly reduced ($p<0.001$) the mean duration of hindlimb tonic extension in the MES model. Treatment of mice with 200 mg/kg ($p<0.01$) and 400 mg/kg ($p<0.001$) of ethyl acetate extract significantly protected against PTZ-induced kindling compared to controls. The leaf was found to contain 10.002±0.119 mg atropine equivalent per gram of dry extract of alkaloids, 9.045±0.8445 mg quercetin equivalent per gram of dry extract of flavonoids, and 21.928±1.118 mg gallic acid equivalent per gram of dry extract of phenols.

Conclusion: This study indicated that the plant *A. integrifolia* has anti-convulsant activity in both acute and chronic models of seizure. This plant represents a potential source for the development of a new anti-epileptic drug for pharmacoresistant epilepsy.

Keywords: *Ajuga integrifolia*, anti-convulsant, epilepsy, kindling, phytoconstituents, seizure

Background

Epilepsy is one of the most common chronic neurological disorders, affecting many people in different parts of the world.[1] Despite their differences, the terms epilepsy and seizure are often confused.[2] As defined by the International League Against Epilepsy (ILAE), epilepsy is a disease of the brain characterized by any of the following conditions: 1) at least two unprovoked (or reflex) seizures occurring >24 h apart; 2) one unprovoked seizure and a probability of further seizures similar to the general recurrence risk (at least 60%) after two unprovoked seizures, occurring over the next 10 years; and/or 3) the presence of an epilepsy syndrome, even if the risk of subsequent seizure is very low.[3] Seizures are characterized by disturbed cerebral function caused by abnormal, excessive, and synchronous electrical discharges in groups of cortical neurons that may produce subclinical or various clinical phenomena.[4,5]

The genus *Ajuga* is a medicinal plant in the Lamiaceae family comprising more than 100 species and 50 subspecies distributed around the world.[6] It is traditionally used for the treatment of distinctive sicknesses.[7–19] *Ajuga integrifolia* Buch.-Ham. ex D.Don (Lamiaceae) (synonyms: *Ajuga remota* Benth., *Ajuga bracteosa* Wall ex Benth.) is a shrub that grows broadly in East Africa, Saudi Arabia, Yemen, Afghanistan, and East Asia.[20] In Ethiopia, it grows in different parts

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of the country,[21] and is known by different vernacular names such as Armagusa (Afan Oromo),[22] Akorarach or Tut astil (Amharic),[23] and Anamuro (Guragegna and Sidamigna).[24,25]

Ethnobotanical studies conducted in different parts of the world have revealed that *A. integrifolia* is utilized for the treatment of numerous conditions, including edema, febrile conditions, gout, rheumatism, amenorrhea, malaria, and diabetes.[26–29] In Ethiopian folk medicine, the plant is used for the treatment of diabetes, retained placenta, malaria, stomach ache, wounds, amoebiasis, cancer, diarrhea, liver problems, anthrax, hypertension, pneumonia, and epilepsy.[30–45] The plant parts used for the specified activities are the leaf, stem, and root.[20]

Experimental studies carried out to evaluate acute toxicity and the claimed activity of the leaf have demonstrated that it is safe and possesses anti-hypertensive,[46] anti-malarial,[47] anti-type I and type II HIV,[48] and anti-*Mycobacterium tuberculosis* activity.[49] Reports are also available showing anti-epileptic activity of the leaf[50] and stem.[51] The root is also reported to possess anti-diabetic activity.[20,52] The leaf is reported to contain secondary metabolites such as phenols, flavonoids, alkaloids, terpenoids, carbohydrates, and steroids.[53] It also contains essential oils such as limonene, α-humulene, β-myrcene, elemol, camphene, β-caryophellene, and α-phellendrene.[54] Different diterpenes such as ajugarin I, ajugarin II, ajugarin IV, ajugarin V, and ajugapitin have been isolated using high-performance liquid chromatography from the aerial parts (leaf and stem) of the plant.[55] Triterpenes, such as ergosterol-5,8-endo-peroxide, with anti-*Mycobacterium tuberculosis* activity have also been isolated from the aerial parts.[49] The bioactive substances found in different parts of the plant may play a predominant role in the effectiveness of the plant in many conditions.[56]

Many studies have reported that herbal medicines are commonly used for the treatment of epilepsy because anti-epileptic drugs (AEDs) fail to control seizures in 30% of the epileptic patients and because of economic and cultural factors.[57] Hence, continuing the search for new therapies of plant origin with fewer side effects and better efficacy is of paramount importance. A recent experimental study reported the anti-epileptic activity of *A. integrifolia*.[50] However, the study was limited to acute models and there is a need to replicate and reproduce earlier studies. Hence, the present study was initiated to assess the efficacy of the plant in both acute and chronic models, and to quantify the major constituents thought to be responsible for the anti-convulsant effect.

Materials and Methods

Drugs and Chemicals

Pentylenetetrazol (PTZ) (Sigma Aldrich, Germany), gallic acid (Merck, Germany), Folin–Ciocalteu reagent (Loba Chemie, India), NaOH (Loba Chemie, India), $AlCl_3$ (Loba Chemie, India), chloroform (Loba Chemie, India), HCl (BDH Laboratory Supplies, UK), citric acid (Avonchem, UK), Na_2HPO_4 (BDH Laboratory Supplies, UK), atropine (BDH Chemicals, UK), quercetin dihydrate (Sigma Aldrich, Germany), Tween80 (Loba Chemie, India), BCG (Sisco Chemical Laboratories, India), n-hexane (Loba Chemie, India), ethyl acetate (Trust Chemical Laboratories, UK), methanol (Sisco Research Laboratories, India), normal saline solution (Sansheng Pharmaceuticals, Ethiopia), potassium acetate (Blulux, India), sodium valproate (Sanofi, Spain), and phenytoin (Macleods Pharmaceuticals, India) were obtained from their respective vendors. All drugs and chemicals used were of analytical grade.

Experimental Animals

Healthy male Swiss albino mice (8–10 weeks, 22–28 g) were used for the current study. The animals were obtained from the Ethiopian Public Health Institute and the animal unit of the School of Pharmacy, College of Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia. The animals were housed in groups of six and acclimatized to laboratory conditions for a week before starting the experiment. The mice were kept under standard environmental conditions (12 h light/dark cycle) and provided with commercial food pellets and water ad libitum. All procedures and techniques used in this study were conducted in accordance with the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals*.[58] The protocol was approved by the institutional review board of the School of Pharmacy, with approval number ERB/SOP/199/2019.

Collection of Plant Material

Leaves of *A. integrifolia* were collected from the plant's natural habitat in Girar Jarso district, Oromia Region, located 180 km north of Addis Ababa. Collected plant specimens were identified and authenticated by a taxonomist, Mr Melaku Wondafrash, and a voucher specimen (TD001) was deposited at the National Herbarium, College of Natural and Computational Sciences, Addis Ababa University, for future reference. The collected leaves were washed thoroughly with tap water to remove dirt and soil, and shade-dried at room temperature for 3 weeks.

Preparation of the Extracts

The air-dried leaves were subjected to size reduction using a mechanical grinder to obtain a coarse powder. The powdered plant material (500 g) was extracted by a successive Soxhlet extractor (Pyrex, Quickfit, UK) using 4 L of each of the following solvents: n-hexane (AHE), ethyl acetate (AEE), and methanol (AME). After extraction with the above solvents, the remaining residue was macerated with 1 L of distilled water three times for 72 h, with occasional shaking, to obtain the aqueous extract (AAE). Each time, before extracting with the next solvent, the powdered plant material was air-dried overnight. The resulting solution was first filtered through a cotton gauze for the aqueous extract and later through Whatman filter paper (no 1) for all solvent extracts. The non-aqueous filtrates were concentrated in a rotary evaporator (Buchi, Switzerland) under reduced pressure at 40°C and the water fraction was freeze-dried using a lyophilizer (Korea Vacuum Co, South Korea) to obtain the respective extracts. The yields (w/w) in terms of dry material for n-hexane, ethyl acetate, methanol, and water were 2.95%, 4.4%, 15.45%, and 3.29%, respectively. The dried extracts were kept in a refrigerator at −20°C until use.

Grouping and Dosing of Animals

The animals were randomly assigned into five groups for each solvent extract, each group containing six mice. In the acute model, group I served as a negative control and was treated with the vehicle used for reconstitution (2% Tween 80 for the non-aqueous extracts and distilled water for the aqueous extract). Group II was a positive control and was treated with sodium valproate 200 mg/kg (SV200) for the PTZ model and phenytoin 25 mg/kg (PHY25) for the maximum electric shock (MES) model. Groups III–IV were test groups and were administered with 100, 200, and 400 mg/kg doses of the respective extracts. The most active extract (AEE) was investigated in the chronic model of epilepsy (PTZ kindling) using the same grouping (SV200 was used as standard). All doses were administered orally and the maximum volume used was 10 mL/kg. The doses were determined in a pilot study conducted before the start of the experiment.

Anti-Convulsant Activity Test

The anti-convulsant activity of the plant was evaluated using acute and chronic models of epileptic seizure.

PTZ-Induced Seizure

For this test, the method by Salem et al,[59] with slight modification, was used. After 60 min of oral administration, as described in the Grouping and Dosing of Animals section, freshly prepared PTZ (80 mg/kg) in normal saline was administered to the scruff of the neck of each mouse. The animals were then placed in a transparent cage and observed for convulsive behavior for 30 min using a video recorder. Forelimb or hindlimb clonic seizure was taken as the endpoint. The latency to clonic convulsion and percentage protection against mortality were recorded and compared with negative controls. Percentage protection from mortality was calculated as follows:

$$\text{Percentage protection of mortality} = \frac{\text{No of death in control} - \text{No of death in test/standard} \times 100}{\text{Number of death in control}}$$

MES-Induced Seizure

For this experiment, the method described by Raza et al[60] was used. After an hour of oral administration, as described in the Grouping and Dosing of Animals section, seizure was induced by auricular stimulation (50 mA, 150 Hz, 0.2 s) using an electro-convulsometer (Rolex Ambala, India). The ear-clip electrodes were moistened with normal saline before application for better conductance. Each animal was closely followed for 2 min using a video recorder. Upon exposure to

the electric current, the animals exhibited various phases of tonic–clonic seizure, including immediate short-lived flexion of the forelimbs followed by extension of the hindlimbs. After the end of the extensor phase, they showed a stupor phase that finally led to recovery or death. The duration of hindlimb tonic extension (HLTE) (ie, outstretching of the animals 180° to the body axis) and protection against mortality were recorded. Percentage protection from mortality was calculated as follows:

$$\text{Percentage protection of mortality} = \frac{\text{No of death in control} - \text{No of death in test/standard} \times 100}{\text{Number of death in control}}$$

In this experiment, reduction in the mean duration of HLTE of MES convulsion was considered as having anti-convulsant activity.[61]

PTZ-Induced Kindling

One hour after administration, as described in the Grouping and Dosing of Animals section, mice were kindled with repeated (every 48 h) intraperitoneal administration of freshly prepared PTZ (35 mg/kg) for 13 days. On each day, animals were closely observed for 30 min after PTZ injection using a video recorder to measure the intensity of seizures. The following seizure scores were used to identify fully kindled mice: stage 0 (no response); stage 1 (hyperactivity, ear and facial twitching); stage 3 (forelimb clonic seizure); stage 4 (generalized clonic seizure with falling); and stage 5 (generalized tonic–clonic seizures). Animals that showed at least three consecutive stage 4 or stage 5 seizure scores were thought to be kindled. Animals that did not show three consecutive stage 4 or 5 seizures were considered to be protected.[62]

Quantification of Total Flavonoid Content

The total flavonoid content (TFC) was estimated as described by Chang et al,[63] with slight modification. First, 10 mg of AEE was dissolved in methanol to prepare a stock solution of 1 mg/mL. Then, 1 mL of the stock solution was transferred to a test tube and mixed with 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water. The mixture formed was allowed to stand for 30 min at ambient temperature, after which absorbance was recorded at 415 nm using a UV spectrophotometer (Jenway Model 6500, UK). Quercetin, used as a standard, was dissolved in methanol and a serial dilution was used to prepare 1.5625, 3.125, 6.25, 12.5, and 25 µg/mL standard solutions. The same procedure was followed to prepare the standard and blank solution. All experiments were performed in triplicate and the average value was recorded. A linear calibration curve was plotted with a regression coefficient (R^2) =0.9964, slope (m)=0.02432 and y-intercept=0.0159 (Figure 1A), and this curve was used to determine TFC, expressed as milligrams of quercetin equivalent per gram (mg QE/g).

Quantification of Total Phenolic Content

Folin–Ciocalteu reagent was used for this assay. Folin reagent (1 mL of 2 N) was diluted with 20 mL of distilled water. To determine the total phenolic content (TPC), 1 mL of the prepared AEE solution (250 µg/mL) was transferred to a test tube, 0.5 mL of Folin reagent was added, and the tube was allowed to stand for 8 min. Thereafter, 2 mL of 7.5% sodium carbonate in distilled water was mixed with the solution and incubated for 30 min at ambient temperature. The absorbance was later recorded at 765 nm using a UV spectrophotometer. Gallic acid, used as standard, was prepared in different concentrations of 3.125, 6.25, 12.5, and 25 µg/mL by serial dilution to draw a standard curve. The same procedure was followed to prepare gallic acid and the blank solution. All experiments were conducted in triplicate and the average value was taken. A linear calibration curve was constructed with R^2=0.9982, slope (m)=0.009323, and y-intercept=0.005289 (Figure 1B) to determine TPC, expressed as milligrams of gallic acid equivalent per gram (mg GAE/g).[64]

Quantification of Total Alkaloids

The total alkaloid content (TAC) of the plant was determined according to the method described by Tabasum et al,[65] with slight modification. The reaction that took place between alkaloid and bromocresol green (BCG) was used to quantify TAC by a spectrophotometric method. Accordingly, 2 mL of AEE in methanol (1 mg/mL) was dissolved in 2 mL of 2

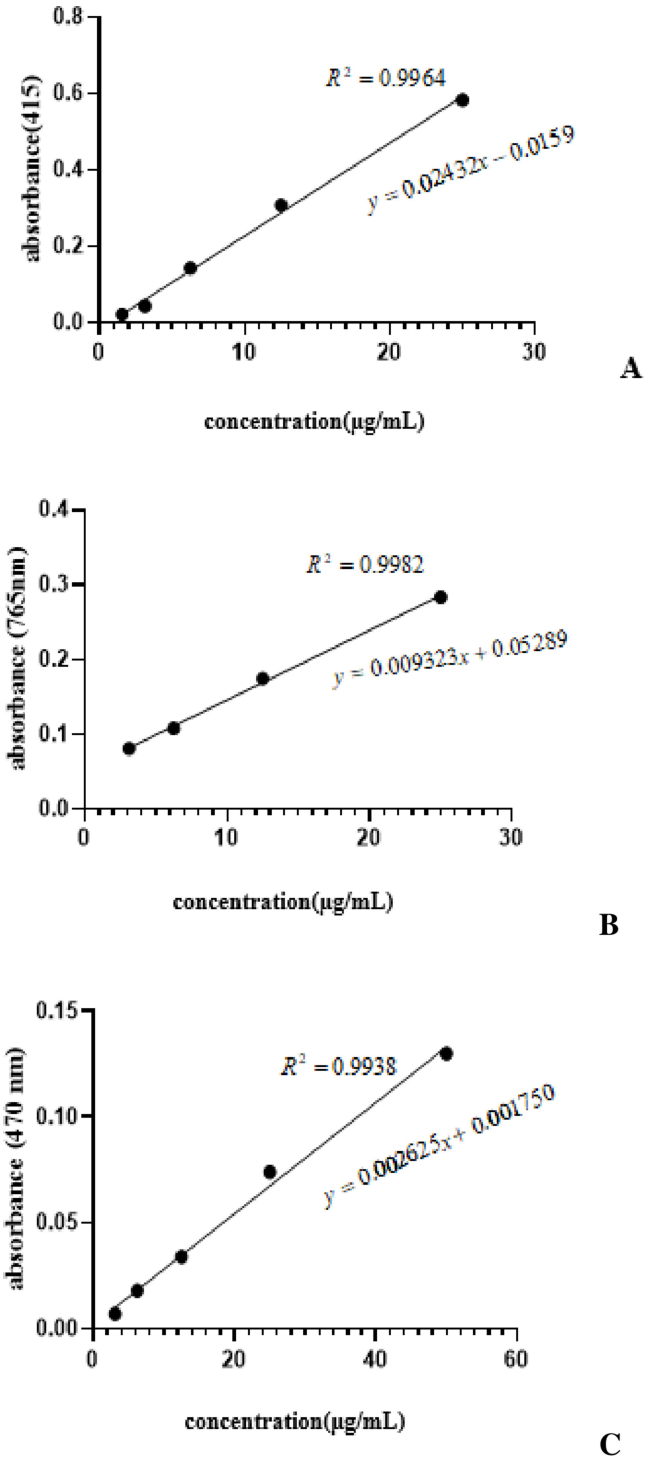

Figure 1 Standard curve constructed for the quantification of secondary metabolites: (A) quercetin; (**B**) gallic acid; and (**C**) atropine.

N HCl and filtered. Then, 1 mL of this solution was transferred to a separating funnel and washed with 5 mL of chloroform twice. The pH of the solution was adjusted to neutral by adding 0.1 N NaOH. After pH adjustment, 5 mL of BCG solution (prepared by heating 69.8 mg of BCG with 3 mL of 2 N NaOH and 5 mL of distilled water, and then diluted to 1000 mL with distilled water) along with 5 mL of phosphate buffer (prepared by adjusting the pH of 2 M sodium phosphate [71.6 g of Na_2HPO_4 in 1 L distilled water] to 4.7 with 0.2 M citric acid [42.02 g] citric acid in 1 L distilled water) was added. The aggregate formed was shaken and the mixture formed was extracted with 5 mL of

chloroform by vigorous shaking. The extract was collected in a 10 mL volumetric flask and the volume was made up to 10 mL with chloroform. The absorbance of the mixture in chloroform was measured at 470 nm using a UV spectrophotometer. For the construction of a standard curve (Figure 1C), atropine was dissolved in methanol to prepare different concentrations (0.5, 0.25, 0.125, 0.062, and 0.03125 mg/mL) of the standard solution. Then, 1 mL of this solution was taken and transferred to a separating funnel, 5 mL of phosphate buffer along with 5 mL BCG solution was added, followed by gentle shaking with 5 mL of chloroform twice. The mixture formed was collected in a 10 mL volumetric flask and diluted to volume with chloroform, and the absorbance was measured at 470 nm. The blank was prepared as described, but without atropine. The assay was run in triplicate and the average value was taken. TAC was determined from a calibration curve with R^2=0.9938, slope (m)=0.002625, and *y*-intercept=0.001750 (Figure 1C), and described as milligrams of atropine equivalent per gram (mg ATE/g).

Data Analysis

All experimental data are expressed as mean ± standard error of the mean (SEM) and were subjected to statistical analysis using SPSS for Windows, version 25, statistical packages. Statistical analysis of the difference among groups was performed with one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. For PTZ kindling, two-way analysis of variance and Bonferroni's post-hoc test were used for multiple comparisons of the mean difference. The analyses were performed with 95% confidence intervals and the level of significance was set at $p<0.05$.

Results

Anti-Convulsant Activity in PTZ-Induced Seizure

All of the extracts except for AAE possessed anti-convulsant activity against the PTZ model of seizure, as evidenced by an increased mean latency to clonic convulsion (Table 1). AEE was the most effective extract, as it prolonged the mean onset of clonus and decreased the percent mortality better than the other extracts. The mean latency to clonic seizure was significantly increased ($p<0.01$) with all doses of AEE compared to controls, the maximum effect (13.17 min) being achieved with 400 mg/kg (AEE400). The increment in mean onset of clonus occurred in a dose-dependent manner, as AEE400 significantly delayed the onset of clonus compared to AEE 200 mg/kg (AEE200) ($p<0.01$) and AEE 100 mg/kg (AEE100) ($p<0.001$). AEE also decreased the percent mortality in a dose-dependent manner, with maximum protection (66.67%) conferred by AEE400 (Table 1).

AHE also significantly increased the mean latency to clonic seizure compared to controls, with a maximum increase (12.67 min, $p<0.001$) displayed by AHE 400 mg/kg (AHE400). The effect produced by AHE400 was significantly greater than the effects produced by AHE 100 mg/kg (AHE100) ($p<0.001$) and AHE 200 mg/kg (AHE200) ($p<0.01$). With regard to mortality, while AHE200 and AHE400 were able to decrease death by 33.33% and 50%, respectively, AHE100 was devoid of any effect (Table 1).

AME at 100 mg/kg (AME100) was not effective on either parameter of this experimental paradigm. However, AME at doses of 200 mg/kg (AME200) and 400 mg/kg (AME400) significantly increased ($p<0.001$) mean latency and provided fairly good protection against mortality compared to controls. By contrast, AAE was devoid of any effects at any dose (Table 1).

The standard drug used (SV) was superior in both measures, as it significantly increased latency ($p<0.01$) and decreased mortality (83.33%) compared to all doses of the extracts used in this experiment.

Anti-Convulsant Activity in MES-Induced Seizure

As depicted in Table 2, all leaf solvent extracts except for AAE produced variable results in reducing the mean duration of HLTE and percent mortality in the MES test. AEE was the most effective extract, as it reduced the duration of HLTE and percent mortality better than the other extracts. The mean duration of HLTE was significantly decreased ($p<0.001$) with all doses of AEE compared to controls, with the maximum reduction (6.33 s) being conferred by AEE400. The reduction in the mean duration of HLTE occurred in a dose-dependent manner, as AEE400 significantly decreased the

Table 1 Anti-Convulsant Activity of Soxhlet Leaf Extracts of *Ajuga Integrifolia* in Pentylenetetrazol-Induced Seizure

Group	Mean Latency to Clonic Seizure (Min)	Percentage Protection from Mortality
CON	3.00±0.447	–
EA100	6.33±0.333$^{a**}b^{***}d^{***}e^{***}$	33.33
EA200	10.17±0.543$^{a***}b^{***}c^{***}e^{**}$	50.00
EA400	13.17±0.703$^{a***}b^{**}c^{***}d^{**}$	66.67
SV200	16.67±0.494^{a***}	83.33
CON	3.00±0.447	–
HA100	5.17±0.307$^{a*}b^{***}d^{***}e^{***}$	0.00
HA200	9.33±0.494$^{a***}b^{***}c^{***}e^{***}$	33.33
HA400	12.67±0.667$^{a***}b^{***}d^{**}$	50.00
SV200	16.67±0.494^{a***}	83.33
CON	3.00±0.447	–
MA100	4.84±0.477$^{b***}d^{***}e^{***}$	0.00
MA200	7.50±0.619$^{a***}b^{***}c^{**}e^{*}$	16.67
MA400	10.00±0.577$^{a***}b^{***}c^{***}d^{**}$	33.33
SV200	16.67±0.494^{a***}	83.33
CON	2.50±0.342	–
AA100	3.00±0.365	0.00
AA200	3.33±0.333	16.67
AA400	3.50±0.428	16.67
SV200	16.67±0.494$^{a***}c^{***}d^{***}e^{***}$	83.33

Notes: Values are expressed as mean ± SEM (n=6 mice). [a]Compared to control; [b]compared to sodium valproate; [c]compared to 100 mg/kg; [d]compared to 200 mg/kg; [e]compared to 400 mg/kg; *p<0.05; **p<0.01; ***p<0.001.

Abbreviations: CON, group treated with distilled water (aqueous extract) or 2% Tween 80 (non-aqueous extract); SV, sodium valproate; EA, ethyl acetate extract of *Ajuga integrifolia*; HA, hexane extract of *Ajuga integrifolia*; MA, methanol extract of *Ajuga integrifolia*; AA, aqueous extract of *Ajuga integrifolia*; numbers refer to doses in mg/kg.

duration of HLTE compared to AEE200 (p<0.01) and AEE100 (p<0.001). AEE also decreased the percent mortality, with the maximum protection (50%) being achieved by AEE200 and AEE400 (Table 2).

AHE also significantly decreased the mean duration of HLTE compared to controls, with the maximum reduction (8.17 s, p<0.001) being obtained by AHE400. The effect displayed by AHE400 was significantly greater than the effects produced by AHE100 (p<0.05) and AHA200 (p<0.001). Concerning mortality, AHE200 and AHE400 decreased death by 16.67% and 33.33%, respectively, while AHE100 was devoid of any effect.

AME100 was not effective on either parameter used to evaluate the anti-convulsant activity of the plant. However, AME200 and AME400 significantly reduced (p<0.001) the mean duration of HLTE and resulted in a percent protection against mortality to the same extent (16.67%) compared to controls. By contrast, AAE was devoid of any effect at any dose (Table 2).

The standard drug used (PHY) was superior in both measures as it significantly reduced the occurrence of HLTE (p<0.001) and decreased mortality (83.33%) compared to all doses of the extracts used in this experiment.

Anti-Convulsant Activity in PTZ Kindling Model

The most active extract (AEE) in the acute seizure model also showed anti-convulsant activity in the chronic seizure model (Figure 2). For this test, 13 injections of PTZ (35 mg/kg ip) on alternate days produced full kindling starting from the 11th to the 13th injections in controls, while treatment of mice with different doses of AEE produced variable effects. AEE400 significantly protected (p<0.001) the animals from developing consecutive stage 4 and/or 5 seizures on the last

Table 2 Anti-Convulsant Activity of Soxhlet Leaf Extracts of *Ajuga Integrifolia* in maximal electroshock-Induced Seizure

Group	Mean Duration of HLTE (s)	Percentage Protection from Mortality
CON	18.5±0.563	–
EA100	14.33±0.964^{a***b***d***e***}	33.33
EA200	9.67±0.715^{a***b***c***e**}	50.00
EA400	6.33±0.422^{a***b***c***d**}	50.00
PHY25	0.00^{a***}	83.33
CON	18.5±0.563	–
HA100	16.67±0.558^{a*b***d**e***}	0.00
HA200	13.00±0.516^{a***b***c**e***}	16.67
HA400	8.17±0.477^{a***b***}	33.33
PHY25	0.00^{a***}	83.33
CON	18.5±0.563	–
MA100	16.50±0.671^{b***d*e***}	0.00
MA200	14.00±0.73^{a***b***e***}	16.67
MA400	10.17±0.307^{a***d***}	16.67
PHY25	0.00^{a***}	83.33
CON	19.83±0.307	–
AA100	19.33±0.615	0.00
AA200	18.33±0.422	16.67
AA400	17.83±0.477	16.67
PHY25	0.00^{a***}	83.33

Notes: Values are expressed as mean ± SEM (n=6 mice). [a]Compared to control; [b]compared to sodium valproate; [c]compared to 100 mg/kg; [d]compared to 200 mg/kg; [e]compared to 400 mg/kg; *p<0.05; **p<0.01; ***p<0.001.

Abbreviations: CON, group treated with distilled water (aqueous extract) or 2% Tween 80 (non-aqueous extract); SV, sodium valproate; EA, ethyl acetate extract of *Ajuga integrifolia*; HA, hexane extract of *Ajuga integrifolia*; MA, methanol extract of *Ajuga integrifolia*; AA, aqueous extract of *Ajuga integrifolia*; HLTE, hindlimb tonic extension; numbers refer to doses in mg/kg.

three injections compared to controls, which indicated protection of the animals. Likewise, AEE200 also produced a significant effect (p<0.01) in protection from kindling compared to controls. On the other hand, AEE100 was ineffective in the parameter used in this experimental model. The standard drug used (SV) was superior in the parameter used as it significantly prevented (p<0.001) the occurrence of kindling compared to controls.

As can be observed from Figure 2, AEE reduced the mean seizure stage in a dose-dependent manner. Statistical analysis revealed that all doses of the extract and the standard drug (SV) significantly reduced (p<0.05) the seizure stage on the first injection compared to controls. On the second injection, the seizure score observed was significant for all doses of the extract (p<0.01) and the standard (p<0.001) compared to the negative control, but no significant difference was seen between the extract- and SV-pretreated groups. A difference in the stage of seizure after PTZ injection between treatment groups was observed from the third injection onwards.

As the number of injections increased, significant differences in the mean seizure stage between AEE100 and the negative control group started to disappear. Indeed, no detectable difference in mean seizure stage was noted between the two groups from the 9th to the 13th injections. This indicated the failure of AEE100 to protect the animals from PTZ-induced kindling. The AEE400-pretreated group started to respond to the chemoconvulsant PTZ from the sixth injection, but no significant effect was seen between AEE400 and SV200 until the ninth injection.

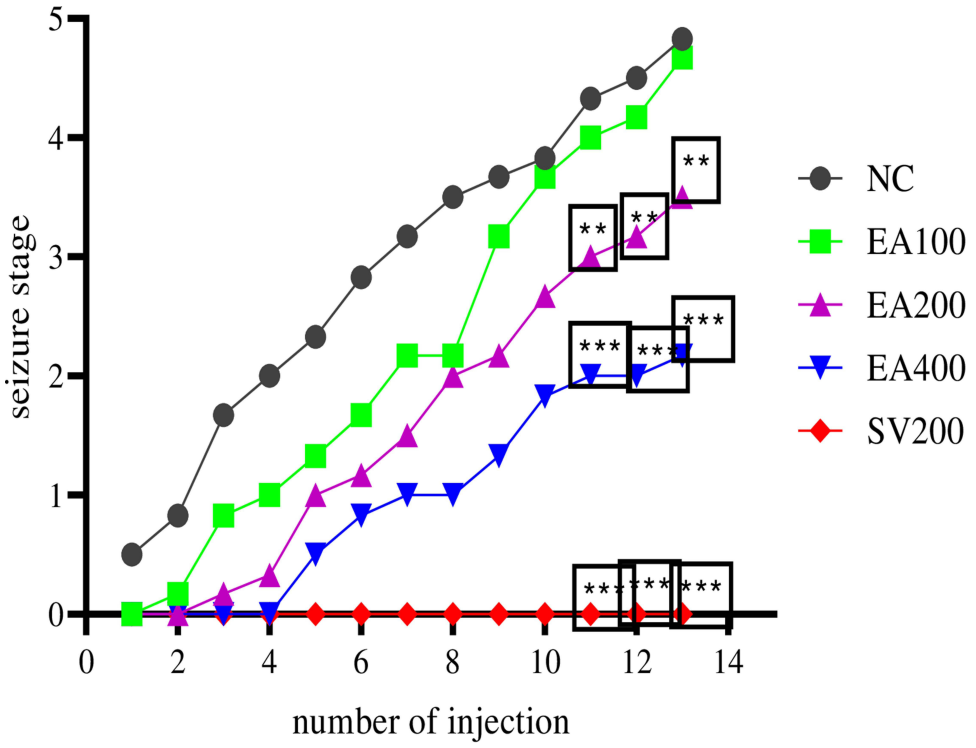

Figure 2 Effect of ethyl acetate extract on pentylenetetrazol-induced kindling in mice. Animals were treated with 100, 200, and 400 mg/kg ethyl acetate extract, and 200 mg/kg sodium valproate along with pentylenetetrazol 35 mg/kg (intraperitoneally) every other day for 13 days.
Notes: Data are expressed as mean ± SEM. NC, negative control. $^{**}p<0.01$; $^{***}p<0.001$.
Abbreviations: NC, negative control; EA, ethyl acetate; SV, sodium valproate; SEM, standard error of the mean.

Quantification of Secondary Metabolites

Quantitative phytochemical analysis revealed that AEE contained 9.045±0.8445 mg QE/g, 21.928±1.118 mg GAE/g, and 10.002±0.119 mg ATE/g of total flavonoids, phenols, and alkaloids, respectively.

Discussion

In the current study, solvent leaf extracts of *A. integrifolia* were found to possess anti-convulsant activity in both acute and chronic seizure models, which appeared to vary with dose and the nature of the extract. AEE produced the highest effect among all solvent extracts in both acute models, the rank order being AEE>AHE>AME≫AAE. Despite having anti-convulsant activity in both models, not all doses of AEE showed an equally appreciable effect in delaying the mean onset of attack and protecting the animals from death. The effect was produced in a dose-dependent manner and AEE400 considerably increased the onset and percent protection from mortality compared to the remaining doses of the extract. The anti-convulsant activity demonstrated by AEE could be attributed to the presence of secondary metabolites, such as flavonoids, phenols, alkaloids, terpenoids, and steroids, which were reported in earlier research,[53] as well as in the present study.

In line with the test result, the anti-convulsant activity displayed by AHE, in terms of the parameters used, was lower than that displayed by AEE. This may be related to the absence of major phytoconstituents such as phenols and flavonoids in the leaf hexane fraction of the plant, as reported elsewhere.[66] Likewise, the absence of steroids in the methanol extract[49] might have contributed towards the reduced anti-convulsant activity in the present study. At the doses used, AAE did not produce a considerable effect in either the PTZ or the MES model. This may indicate that semi-polar and non-polar constituents are responsible for the anti-convulsant activity of the plant.

A previous similar study on the leaf crude extract and solvent fractions of the plant collected from different geographical locations reported the anti-convulsant activity of *A. integrifolia* in PTZ and MES models.[50] The most active fraction in this study (butanol 400 mg/kg) tended to produce a better effect than AEE400 in the PTZ model (mean latency to clonic seizure 15.51 vs 13.17 min), but the reverse was true in the MES model (reduction in mean duration of

HLTE 8.33 vs 6.33 s). The present study upholds the earlier observation, and the findings of both studies strongly indicate the potential anti-convulsant activity of the plant. The subtle difference observed between the studies may be due to the variation in the geographical location as well as the method of extraction.

The MES and PTZ models for acute seizure do not simulate the chronic dysfunction of the brain often seen in epilepsy and thus cannot be used for the discovery of potential AEDs for pharmacoresistant epilepsy. Therefore, kindling is widely employed to study the process of epileptogenesis and to promote AED discovery.[67] Thus, the present study also investigated the effect of the most active extract (AEE) in a chronic model of epilepsy. AEE dose dependently prevented the occurrence of consecutive stage 4 and/or stage 5 seizures, although the lowest dose failed to do so. During the initial phases of the kindling process, the lower dose (AEE100) prevented the animals from developing the maximum stages of seizure, which only lasted for a short time. This may be due to the accumulation of PTZ in the brain following repeated administration, which could result in prolonged antagonism of GABA, making the lowest dose ineffective to overcome this antagonism. The anti-convulsant effect demonstrated by the AEE in PTZ-induced kindling might be attributed to the presence of secondary metabolites, as the leaf is reported to possess a wide range of secondary metabolites.[53] Indeed, although terpenoids were not determined in the present study, repeated administration of a triterpenoid such as oleanic acid is reported to protect animals against PTZ-induced seizures.[68]

Although the exact mechanism of the anti-convulsant activity of the study plant remains to be elucidated, it can be generalized that the active solvent extracts can act through multiple mechanisms similar to the conventional medicines. Indeed, the secondary metabolites determined in the present study have been demonstrated to produce anti-convulsant activity through a host of mechanisms, including antioxidant,[69,70] Na^+ channel blocking, and modulation of $GABA_A$ receptors.[71] The TPC and TFC determined in the present study were found to be four- to five-fold greater than those reported for the methanol extract of the aerial part of the same plant from Pakistan,[8] suggesting that the geographical location, plant part, and solvent used for extraction could contribute toward variation in the quantity of active constituents, even in the same plants.

Conclusion

The results of the present study provide support for the use of *A. integrifolia* as an anti-convulsant medicinal plant, as solvent extracts from the leaves of the plant displayed anti-convulsant activity in both acute and chronic seizure models. AEE is demonstrated to be the most effective extract in altering the parameters used in both the acute and chronic models of this experimental paradigm. The results of this study suggest that semi-polar to non-polar components are responsible for the anti-convulsant activity of the plant, while polar components are found to be devoid of anti-convulsant activity.

Abbreviations

AED, anti-epileptic drug; GABA, gamma-aminobutyric acid; HLTE, hindlimb tonic extension; MES, maximal electro-shock; PTZ, pentylenetetrazol.

Data Sharing Statement

The data sets used and/or analyzed during the current work are available and included in this article.

Ethics Approval and Informed Consent

The protocol was approved by the institutional review board of the School of Pharmacy, Addis Ababa University (reference no ERB/SOP/199/2019).

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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