Anticonvulsant Activity of 80% Methanol Extract and Solvent Fractions of *Ajuga integrifolia* Buch.-Ham (Lamiaceae) leaves in Mice

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Abstract

Anticonvulsant activity of 80% methanol extract and solvent fractions of *Ajuga integrifolia* Buch.-Ham (Lamiaceae) leaves in mice

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Ajuga integrifolia is one of the species in the genus *Ajuga* that is used in the Ethiopian traditional medicine for the treatment of different ailments, including epilepsy. Thus, this study was initiated to evaluate the traditional anticonvulsant use of 80% methanol leaves extract and solvent fractions of *Ajuga integrifolia.* Acute routine screening tests; the pentylenetetrazole (PTZ) and maximal electroshock (MES) models were used. The rota rod test was further performed to evaluate neurotoxic effect of the plant. Swiss albino mice of 6-8 weeks old were randomly assigned to five groups (n=6/group). The test groups received 100, 200 and 400mg/kg oral dose of crude extract and solvent fractions for both PTZ and MES tests as well as 2.5, 5 and 10mg/kg i.p. dose for rota rod test. The positive control groups received valproate 200mg/kg oral for PTZ, phenytoin 25mg/kg oral for MES, and diazepam 5mg/kg i.p for rota rod test. The negative control groups received oral dose of 10 ml/kg of distilled water or 2% tween 80. The crude extract produced dose dependent and statistically significant anticonvulsant effect on both PTZ and MES induced seizures. It was suggested by delayed latency ($p<0.001$ for 400 mg/kg and $p<0.05$ for 100mg/kg and 200mg/kg) and fast recovery ($p<0.001$ for all doses) on PTZ and reduction of duration of tonic hind limb extension ($p<0.001$ for all doses) in MES model, against control. Similarly, n-butanol and chloroform fractions displayed dose dependent activity in both models as evidenced by having significant activity against control. However, aqueous fraction at doses used did not show a significant effect on latency and duration on both models. Furthermore, all doses of the crude extract and solvent fractions did not display a significant change in motor coordination. The plant extract contained alkaloid, glycosides, flavonoids, phenols, steroids, tannins, terpenoids and saponins that might contribute to the observed anticonvulsant activity. In conclusion, the plant has anticonvulsant activity at various doses providing evidence for its traditional use.

Key words: Anticonvulsant, Epilepsy, Seizure, Pentylenetetrazole, Maximal electroshock, *Ajuga integrifolia*

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List of Abbreviations and Acronyms

AAI	Aqueous fraction of *Ajuga integrifolia*
AAU	Addis Ababa university
ANOVA	Analysis of variance
AEDs	Antiepileptic drugs
BAI	Butanol fraction of *Ajuga integrifolia*
CA	Cornu Ammonis
CAI	Chloroform fraction of *Ajuga integrifolia*
DZP	Diazepam
EEG	Electroencephalographic
FDA	Food and drug administration
GABA	γ-aminobutyric acid
KCNQ	potassium Voltage-gated Channel Q
ILAE	International league against epilepsy
LD50%	Lethal dose 50%
MAI	80% methanol extract of *Ajuga integrifolia*
MES	Maximal electroshock Induced Seizure
MRI	Magnetic resonance imaging
NMDA	N-methyl-D-aspartate
OECD	Organization for Economic Co-operation and Development
PDS	Post depolarization shift
PTZ	Pentylenetetrazole
SCN2A	Sodium Voltage-gated Channel Alpha Subunit 2
SEM	Standard error of mean
SV	Synaptic vesicles
THLE	Tonic hind limb extension
VNS	Vagus nerve stimulation

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1. Introduction

1.1 Overview of Epilepsy

The word “epilepsy” was derived from the Greek verb epilambanein, which means ‘‘to seize, possess, or afflict.” This was emanated from the consideration that epilepsy is caused by divine punishment for sinners.[1] However, this divine etiology has been changed through time and the current definition is more scientific rather than religious view. Epilepsy is a disorder of brain function characterized by the periodic, unpredictable, and spontaneous occurrence of seizures. Seizure refers to a transient alteration of behavior due to the disordered, synchronous, and rhythmic firing of populations of brain neurons.[2]

The clinical manifestations are varied among individuals depending on the brain region involved in generation of seizures and how far it spreads. Primarily, people with epilepsy experience loss of awareness followed by disturbances of movement, sensation, mood, or other cognitive functions. They may experience further physical problems such as fractures and bruising from injury.[3] Epilepsy might have convulsive features such as sudden abnormal movements including stiffening and shaking. However, some individual might experience non-convulsive features such as change in mental status.[4]

All seizures are not presumed to be an epilepsy. Provoked seizure which occur either by chemical or electrical stimulation could not considered to be an epilepsy. Hence, the seizure may not occur after the triggering agents are removed.[2] Furthermore, epilepsy imitators comprising fainting, eclampsia, meningitis, encephalitis, and migraine headaches have feature related with epilepsy and can be misdiagnosed as an epileptic seizure.[5]

The international league against epilepsy (ILAE) arranged operational definition for epilepsy in order to avoid confusion patients who could be left uncertain as to whether they have or do not have epilepsy. Accordingly, epilepsy is supposed to exist when any of the following conditions are fulfilled: “(I) two unprovoked (spontaneous) seizures happening greater than 24 hours apart; (II) one unprovoked seizure plus a likelihood of additional seizures similar with the general recurrence risk after two unprovoked seizures (at least 60%) occurring over the next 10 years; and (III) diagnosis of an epilepsy syndrome.[6]

1.2 Classification

The ILAE is an organization devoted to conduct research and development in epilepsy since 1981.[7] They arranged a new classification of epilepsy in 2017. Thus, epilepsy can be categorized according to seizure onset into focal-onset and generalized onset. Focal onset can be defined as seizure initiating within neuronal networks restricted to only one hemisphere/lobe whereas generalized onset seizures involve both hemispheres broadly from the outset. Certain seizures that cannot be determined to be focal or generalized in preliminary assessments are labeled as seizures of unknown onset. So as, it is a temporarily holding place until further evidence for re-categorization from clinical workup is obtained.[8]

A focal onset seizure can be further sub-classified into focal aware or focal impaired awareness seizure based on the person's consciousness level. A focal aware seizure occurs while the subjects are aware of self and the surrounding environment and able to recall the seizure episode after it has passed away. Focal onset impaired awareness seizure happens while the victim is conscious but unable to recognize any part of the seizure episode. Focal seizure which is subsequently changed to generalized seizure is known as focal to bilateral tonic-clonic seizure. Additionally, a focal seizure can be further sub classified directly by first prominent sign or symptom into motor onset or non-motor focal seizure as shown in the figure 1 below.[8]

Epilepsy can also be classified according to the type of seizure into focal, generalized, combined generalized and focal, and unknown. A focal epilepsy is an epilepsy with focal seizure and a generalized epilepsy is an epilepsy with generalized seizure. A combined generalized and focal epilepsy is an epilepsy which has both focal and generalized seizures. The 'unknown' epilepsy is labeled until sufficient information has been gathered to be certain about the epilepsy classification and whenever information collected relabeled it as one of the above.[8]

Furthermore, epilepsy can be classified based on Epilepsy Syndrome. An epilepsy syndrome are a group of features including seizure types, EEG (Electroencephalographic), and imaging features that are likely to happen together. This give identity to patients and more specific to define the situation that may help to match the condition with specific therapy. Hence, some epilepsy require specific medication. There are well recognized syndromes on https://www.epilepsydiagnosis.org/.[8]

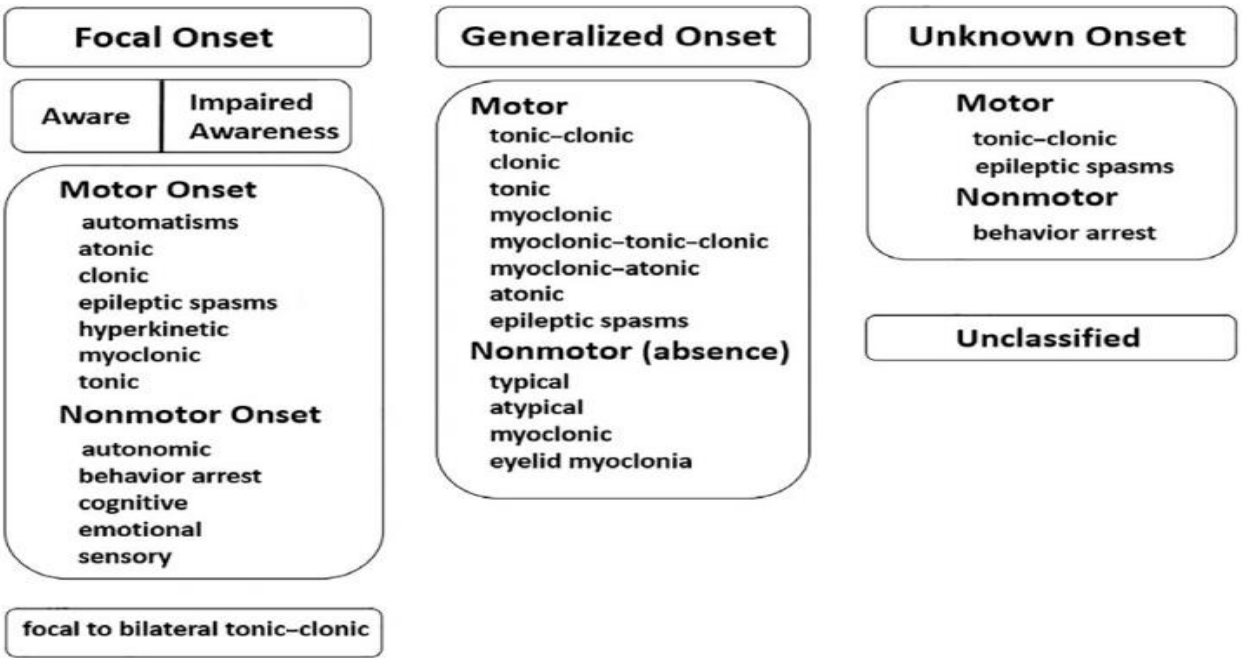

Figure 1: The expanded ILAE 2017 operational classification of seizure types.[8]

1.3 Epidemiology

Epilepsy is a neurological condition affecting all people without discriminating social or racial boundaries and happens in all age groups including both males and females. It is a public health problem accounting a significant proportion of the global burden of all disease. Today, more than 50 million people have been diagnosed with epilepsy worldwide. The estimated proportion of the condition in the general population at a given time is between 4 and 10 per 1000 people and 5 million people are diagnosed as a new case each year.[3] However, 60-70% of patients with epilepsy in developing countries received no modern treatment as a result of economic constraints and social reasons.[9]

Even though epilepsy is globally distributed condition, about 80% of the affected individuals reside in low and middle income countries.[3] This was probably due to higher rate of exposure to infectious diseases such as neurocysticercosis commonly with the pork tapeworm taenia solium,[10] and malaria; the higher incidence of road traffic injuries, birth-related injuries, and variations in medical infrastructure.[3] A community-based epidemiological study on neurological disorders performed in a rural area of Ethiopia showed epilepsy to be the most prevalent case with 5.2/1000 inhabitants were at risk. The study also revealed that 6.3% of people with epilepsy had died over a two-year period and one-third in 20 years.[11] It is one of the common causes of

disability in the world. A Systematic review on the global burden of epilepsy estimates 13 million disability adjusted life years.[12]

1.4 Etiology

Epilepsy could result from multiple etiologies. A structural brain abnormalities after stroke, trauma, infection, or genetic which are visible on neuroimaging are linked with an increased risk of epilepsy, known as structural etiology.[13] Epilepsy might arise when a known or presumed genetic mutation involved in a core symptom, is known as genetic etiology. It could be suggested based on a family history of an autosomal dominant disorder as just like the syndrome of Benign Familial Neonatal Epilepsy, where most families have mutations of one of the potassium Voltage-gated Channel Q genes (KCNQ2 or KCNQ3).[14] A genetic etiology could also be explained by clinical research in populations with the same syndrome such as Lennox's twin studies.[15]

Furthermore, identification of a molecular basis of a single gene or copy number variant of epileptic neurons could explain the involvement of a known genetic abnormality. For example, a monogenic etiology can be explained by mutation of Sodium Voltage-gated Channel Alpha Subunit 2 (SCN2A), which are associated with Dravet syndrome.[16] Genetic etiology does not equate with inheritance since a number of de novo mutations are implicated in both severe and mild kinds of epilepsy. However, responsible genes implicated in epilepsy are quite diverse and most of the cases are not yet screened and identified.[17]

Infection with neurocysticercosis, tuberculosis, HIV, cerebral malaria, sub-acute sclerosing panencephalitis, cerebral toxoplasmosis, and congenital infections such as Zika virus and cytomegalovirus is the global most common etiology of epilepsy. An infectious etiology does not mean seizures occurring in the setting of acute infection such as meningitis or encephalitis rather a known infection directly results in seizures as a core symptom of the disorder.[18,19]

Metabolic epilepsy is a metabolic abnormality which is associated with an increased risk of epilepsy development in affected individuals. A wide range of metabolic disorders like porphyria, uremia and aminoacidopathies, are found to be linked with epilepsy.[20] Immune epilepsy results directly from an immune disorder in which seizures are a core symptom of the disorder. It was evident with a greater access to antibody testing.[21] Although many underlying mechanisms were identified, the cause of the disease is still unknown in about 50% of cases globally.[3]

1.5 Pathophysiology

Normally, neurons communicate each other by maintaining the equilibrium in a network of both excitatory and inhibitory circuits. However, penicillin administration in the cortical foci of mice resulted in consistent discharge of action potentials. The pattern of interictal spike or sharp wave observed was named as "paroxysmal depolarizing shift" (PDS). It was characterized by a relatively high voltage (approximately 10-15 millivolt), and long depolarization (50-100 millisecond).[22] Similarly, application of the γ-aminobutyric acid ($GABA_A$) receptor antagonists' such as picrotoxin, and bicuculline in the mice brain slices found to produce PDS.[23,24] Epileptic seizure has two hallmark features, neuronal hyper-excitability and hypersynchronization which developed as a result of the following mechanism.

1.5.1 Neuronal network mishaps

Axonal sprouting is a common activity of the brain development and is an integral cellular process in the formation of neuronal connections and circuits. Demonstration of pathway of hippocampal formation in adult people with temporal lobe epilepsy has shown neuronal loss in the hilar polymorphic, cornu Ammonis 1 (CA1), CA3 and dentate gyrus regions. Subsequently, axons of dentate granule cells form wrong innervations or wiring with neurons of the dentate gyrus rather than CA3 and hilus. Hence, the synaptic reorganization and axonal sprouting might lead to aberrant recurrent excitation, providing a synchronizing mechanism in other parts of the hippocampal formation.[25]

1.5.2 Neuronal structure malformation

Confirmation for dendritic abnormalities was found in pathological analysis of brain samples resected for surgical treatment of intractable epilepsy. A number of dendritic abnormalities in the hippocampus was identified. The common abnormality was loss of dendritic spines followed by less common changes in length, shape, and branching patterns, as well as a focal increase of dendritic spines.[26] Dendritic spines loss manifestation in hippocampal and other relevant cortical areas also predispose individual to learning problems and other cognitive deficits. Similar clinical findings including spine loss and other dendritic changes were found both *in vivo*[27] and *in vitro* seizure models of epileptiform bursting in brain slice-cultures.[28]

The breakdown of dendritic cytoskeletal proteins such as actin was involved in the mechanistic basis of dendritic injury. Hence, injury to neural dendrites could be epileptogenic and enhance the likelihood of future seizures by disrupting the balance between excitatory and inhibitory networks in the brain, especially if inhibitory circuits are more affected.[29]

1.5.3 Neurotransmitter synthesis failure

The synthesis, release, reuptake, and metabolism of excitatory and inhibitory neurotransmitters glutamate and GABA respectively are tightly controlled. GABA is synthesized by decarboxylation of glutamate by making use of co-enzyme pyridoxine (vitamin B6) requiring enzyme called glutamic acid decarboxylase. The overall regulation of this enzyme specifies how much attention has given to GABA metabolism. Hence, pyridoxine plays a major role in the level of GABA synthesis in the brain. So as, pyridoxine deficiency contribute very likely to the increased seizure susceptibility related with reduction of GABA synthesis and substantial increment of brain glutamate levels.[30]

Pyridoxine dependent epilepsy is a rare autosomal recessive disorder and considered as an example of metabolic epilepsy. It has features of recurrent seizures in the prenatal, neonatal, and/or postnatal periods. The conditions can be managed with pharmacological dose of 15–30 mg/kg/day of pyridoxine divided in two to three doses and should be continued until negative biochemical test result has been confirmed. However, the condition is resistant to conventional antiepileptic drugs (AEDs).[31]

1.5.4 Inhibitory receptors dysfunction

Certain epilepsies may occur due to mutation and subsequent lack of expression of the different $GABA_A$ receptor complex making subunits. Additionally, the mutation interrupt the molecules that assist the subunits assembly and electrical properties. So that, the receptor could not be functional to be open and allow the entrance of chloride ions that normally participate in neuronal inhibitions. In patients with Angelman syndrome, hippocampal pyramidal neurons are incapable to assemble α5, β3 and γ3 receptors for the reason deletion of chromosome 15. This was further explained in the pilocarpine treated animals after decreased amount of mRNA for the α5 subunit of the surviving interneurons noted in the CA1 region of the rat hippocampus.[32]

1.5.5 Excitatory receptors dysfunction

Stimulation of N-methyl-D-aspartate (NMDA) receptors requires the presence of the agonist glutamate and the co-activator glycine. So as, cerebrospinal fluid holds enough glycine under basal conditions to bind with its binding sites on the NMDA receptor. That is why it is said activation of the NMDA receptor depends on glutamate and glycine binding with their respective sites. Furthermore, the receptor activation requires depolarization and removal of the magnesium to allow the passage of Na^+ and Ca^{2+} ions through the channel. These conditions are working for both normal neuronal activities and during seizures, which involve prolonged burst out of neuronal firing.[33]

Deficiency of glycine metabolizing enzyme due to inborn error results in buildup of large concentration of glycine in the body tissues including the brain and progress to the disorder called non-ketotic hyperglycinemia. It is an inherited autosomal recessive condition and associated with developmental abnormality and interactive epilepsy in infants.[34]

1.5.6 Synapse malformation

Both animal and human tissue studies have shown the fact that a peak of synaptogenesis occur after birth through to the first months of life. As it has been demonstrated, the inhibitory $GABA_A$ network presented delayed and lower expression in the first weeks of life. However, the delays may persist for a long period of time. These developmental changes eventually increase synaptic excitation and susceptibility to seizures in the neonatal brain.[35]

1.5.7 Ion channel abnormalities

Voltage gated ion channels are responsible for normal neurons activity where it modulate neuronal excitability. However, any abnormalities may play a vital roles in the development of human epilepsy. The pathophysiological mechanisms may be emanated from neuronal dis-inhibition or hyperexcitation induced by loss of genes encoding the expression of functional voltage gated channels. Genetic studies of patients with epilepsy have identified widespread mutations of genes that encode for the ion channels. Generalized epilepsy with febrile seizures found to be associated with SCN1A mutations, mostly missense mutations. Additionally, Dravet syndrome found associated with 40% truncation, and the remainder as splice-variant changes of SCN1A.[36]

Potassium channels are expressed ubiquitously in neuronal and glial cell membranes. More than 80 genes encoding the potassium channel pore-forming or accessory subunits were cloned. Normally, these channels transmit a particular type of signal called M-current, which prevent the neurons to not constantly active/or excitable. Mutation of KCNQ2 (more common) and KCNQ3 have been found to be linked with benign familial neonatal seizure. Without functional M-current it is well known that neurons are abnormally excited and seizures develop.[37]

Calcium activated potassium channels also play a critical role in neuronal firing and excitability. The activation and opening of these channels causes after hyperpolarization and produce potentials to be more negative than the resting membrane potential. KCNMA1 gene encoded the α-subunit of KCa1.1 channels. A missense mutation in KCNMA1 was noticed in generalized epilepsy which is associated with increased Ca^{2+} sensitivity to make the neuron more hyperexcitable likely by having faster action potential repolarization.[38]

Na^+/K^+-ATPase abnormalities found to be linked with epilepsy. Normally, GABA reuptake require a lot of energy/it is energy sensitive than glutamatergic neurons. Phosphorylation of $GABA_A$ receptors mainly relies on GAPDH, which is a product of glycolysis. Patients with drug resistant partial epilepsy have shown reduced glycolysis dependent $GABA_A$ receptor phosphorylation and subsequent GABAergic neuron dysfunctioning. In neonatal seizure Na^+/K^+-ATPase abnormalities result in post-seizure extracellular K^+ clearance which further aggravate the condition.[39]

1.6 Management of Epilepsy

1.6.1 Non-pharmacologic Management

Ketogenic diets are involved in upsurge of ketone bodies and lowering of glucose after mean and down regulation of glycolysis in human body. They are responsible for the generation of leptin[40] and consequently leptin involved in the suppression of seizure in rodents.[41] Children admitted for initiation of the ketogenic diet with blood β-hydroxybutyrate levels greater than 4 mmol/L were shown to have significantly decreased seizure frequency than those with levels less than 4 mmol/L.[42] Conversely, infusion of glucose to children who better control the seizure with

ketogenic diet develop the seizure within 45 minutes. So, it is used by some clinicians for children refractory epilepsy.[43]

Vagus nerve stimulation (VNS) is a palliative technique in which stimulating devices implanted under the skin of chest and a wire runs to the vagus nerve in the neck. The VNS device send regular and mild pulses of electrical energy to the brain via the vagus nerve and sometimes it's referred to as a "pacemaker for the brain." It is FDA approved for refractory focal onset epilepsy of patients older than 12 years.[44] Stress management and relaxation techniques will also help some people for better control of the case.[45] The last resort will be of brain surgery and it is potentially curative.[46]

1.6.2 Pharmacologic Management

Sodium Channel Blockers

Certain sodium channel blockers bind and enhance fast sodium channel inactivation.[47] Hence, limit the development of maximum seizure activity. Fast sodium channel blockers includes; phenytoin, carbamazepine, valproate, oxcarbazepine and lamotrigine. Phenytoin and carbamazepine are effective against both focal and generalized tonic-clonic seizures but not effective against myoclonic or absence seizures. However, valproate remains the most effective AED for idiopathic generalized epilepsy with generalized tonic-clonic seizures and generalized absence seizures.[48]

Lacosamide is the first AED of the group that promote slow inactivation of voltage dependent sodium channels. Unlike the older sodium channel blockers, slow inactivation occurs in repetitively discharged neurons and prolong depolarization. Lacosamide appears to be a narrow-spectrum AED against focal seizures.[49]

GABA Potentiation

Benzodiazepines act mainly on the $GABA_A$ receptors and increase the frequency of chloride channel openings. Among the benzodiazepines, only clonazepam and clobazam are used in epilepsy management typically as adjunctive therapy. Barbiturates activate GABA receptors and prolong the opening of the associated chloride channel. Typically, phenobarbital is effective

against focal seizures and generalized tonic-clonic seizures but not effective against generalized absence seizures.[2]

Vigabatrin, a structural analog of GABA irreversibly inhibits GABA transaminase, which is the enzyme used for biotransformation of GABA in to succinate semialdehyde. Thus, result in accumulation of GABA available for neuronal inhibition.[50] However, tiagabine prevent glial and neuronal reuptake of GABA by inhibiting GABA transporter.[51] Both, have narrow-spectrum of action and effective against focal seizures.[48]

Calcium Channel Blockers

Ethosuximide is a narrow spectrum AED used exclusively for absence seizures appears to block T-type calcium currents in thalamic neurons. Valproate which has similar activity at the T type calcium channels appears to be helpful for absence seizures. Additionally, felbamate antagonizes glutamate–NMDA receptors and inhibit calcium influx postsynaptically. Felbamate is a broad-spectrum agent effective against focal seizures as well as generalized seizures in the setting of Lennox-Gastaut syndrome. Gabapentin was initially synthesized as structural analogue of GABA appears to bind the α2δ subunit of the voltage-gated calcium channel in the central nervous system. Thus, reduce the influx of calcium and associated neurotransmitter release. Pregabalin also binds with α2δ subunit and decreases the release of excitatory neurotransmitters including glutamate, noradrenaline and substance P. Both have a narrow spectrum effect and used as adjunctive treatment for focal seizures.[48]

NMDA Receptor Blockers

NMDA receptor blockers including topiramate, felbamate and zonisamide act on membrane-associated postsynaptic calcium channels. Topiramate and felbamate are FDA approved for initial monotherapy for focal seizures and generalized tonic-clonic seizures but not considered drug of first choice because of its cognitive adverse effects and serious idiosyncratic toxicity respectively. Zonisamide is another FDA approved NMDA blocker for adjunctive therapy of focal seizures.[48]

SV2a Vesicle Inhibitors

Synaptic vesicle protein 2a (SV2a) is vital to the process of neurotransmitter exocytosis into the synaptic cleft. Inhibition of this protein exhibit a broad-spectrum reduction of excitatory activity. Levetiracetam is the first agent of the group and has a broad spectrum of activity against focal seizures, generalized tonic-clonic seizures and generalized myoclonic seizures.[52] Brivaracetam is structurally related to levetiracetam but has approximately 15-30 fold higher affinity and greater selectivity for the SV2a.[53,54] Brivaracetam is FDA approved for the treatment of partial onset seizures in patients 16 years of age and older man.[55]

1.6.3 Traditional Herbal Medicine

The traditional herbal medicine is an essential method of remedy and have very long historical background that corresponds to the age of mankind.[56] Currently, about 80% of the world's population depend on traditional herbal medicine for human primary healthcare needs.[57] The WHO define herbal medicines as; herbs, herbal materials, herbal preparations and finished herbal products which contain active ingredients of plant parts, or other plant materials, or combinations intended for human therapeutic use or for other benefits in humans and sometimes animals.[58]

The use of herbal medicine for management of epilepsy predicted to be centuries old and found in diversified cultures of China, Japan, Kenya and Ethiopia.[59] Some of them have been proven to be safe and effective in pharmacological studies.[60,61] Most African communities believe in epilepsy happen as a result of evil spirits and commonly get treatments from spiritual leaders. In Ethiopia, treatment of epilepsy with local herbs, holy water and amulets are the most recognized traditional practices.[11] Though herbal medicines are extensively used for various mental illness in Ethiopia,[62] evidences on safety and efficacy are absent to support the cultural practices of the people.

1.7 Overview of the Experimental Plant

The genus *Ajuga* are evergreen, clump-forming rhizomatous annual or perennial herbaceous flowering species belong to the mint family, Lamiaceae. *Ajuga integrifolia* (synonyms: Ajuga remota, Ajuga bracteosa) among the 301 species of the genus Ajuga[63] known by several vernacular names in Ethiopia including; tut astil in Amharic[64], Armagusa in Oromiffa[65], and etse libawit in ge'ez.[66]

Ajuga integrifolia is a low herb often lying on the ground, branching usually diffusely from the base and ascend in erect manner up to 20 cm. Its leaves are oblanceolate, coarsely toothed, moderate/dense hairy grayish green and has bitter taste so as not to be eaten by animals, birds or insects.[67] The flowers are small with pale violet, light blue or white and found in small clusters in the leaves axils.[68] It is growing in the grasslands and other geographic parts of East Africa especially in Kenya and Ethiopia.[67] A lot of chemical compounds including beta-sitosterol and gamma-sitosterol have been identified from the plant leaves extract.[69,70]

The genus is traditionally used in the different regions of Africa as a remedy for various aliments including; diabetes, hypertension, fever, toothache, malaria, edema, pneumonia, liver problems and as a panacea for gastrointestinal disorders.[63,67] Certain pharmacological studies performed on *Ajuga integrifolia* confirmed activities such as; antimalarial activity[67], anti-diabetic[71], analgesic activity[72], antioxidant[72], anti-human Immunodeficiency virus[73], diuretic activity[69], anticonvulsant on stem part[74]. *Ajuga integrifolia* is among the traditional plants that are claimed to have anticonvulsant potential in Ghimbi district of Ethiopia.[75]. So as, the present study gave emphasis on the leaves part and attempted to confirm the claim of the Ethiopians traditional practice.

Hence, acute routine screening tests, including the MES and PTZ were selected and used in the present study for evaluation of the anticonvulsant activity of the plant extract and solvent fractions. The methods have high predictive values and remained as a gold standard method in early stages of many AEDs screening programs. Moreover, simplicity, time effectiveness, cost minimization and reproducibility qualified the acute test to be selected and used in this study.[76]

Figure 2: Photograph of *Ajuga integrifolia*

(Captured by Yigrem Getaneh on 12/02/2019 from Ghimbi district)

1.8 Rationale of the Study

Current advancement in the investigation of new anti-epileptic medications introduced many new therapies so as to control epilepsy.[77,78] In spite of these recent advancements and emergency of newer medications, patients don't respond to therapy otherwise continue to have seizures. Over one third of adult patients still suffer from epilepsy and respond neither to drug therapy nor to surgical treatments.[79] In addition, dose-related and chronic toxicity of AEDs which are the outcomes of long term therapy limit their therapeutic benefits and decrease adherence to treatments.[80]

Furthermore, pregnancy related complications,[81] harmful interactions with other drugs,[82] withdrawal symptoms and relapse upon discontinuation of treatment[83] clarify some people have a hard time to control their seizures with currently available medications. Thus, epilepsy remained as a significant unmet medical need for better anticonvulsants with reduced liability. In developing countries, economic constraints for treatment[84] and the disorder related stigma and discrimination are major contributor of the burden.[85] Therefore, the need for effective, safe, easily accessible and affordable treatment of epilepsy is increased.

Traditional herbal treatments are used for thousands of years in prevention and treatment of different diseases. Today it attracts the attention of researchers as a novel source of lead compound and have shown to produce promising results in the treatment of epilepsy. In Ethiopia, medicinal plants are used by most of the community especially for the management of neurologic disorder including epilepsy.[62] *Ajuga integrifolia* is one of the herbal medicines used in Ethiopia, Ghimbi district for treatment of epilepsy. The method of preparation is the leaves are crashed together and mixed with nut oil to be taken orally.[75] This Ethiopian traditional claim should be evaluated for safety and anticonvulsant effect. Consequently, the present study was conducted to confirm the anticonvulsant effect of *Ajuga integrifolia* leaves. The result of the study may serve as baseline for the development of new antiepileptic agent and to isolate and identify the active compound that can be used as lead compound.

2. Objective

2.1 General objective

- To evaluate the anticonvulsant activity of 80% methanol leaves extract and solvent fractions of *Ajuga integrifolia* Buch. -Ham in mice.

2.2 Specific Objectives

- To determine acute oral toxicity of the crude extract of *Ajuga integrifolia* leaves in mice.
- To evaluate anticonvulsant activity of the crude extract and solvent fractions of *Ajuga integrifolia* leaves using PTZ test in mice.
- To evaluate anticonvulsant activity of the crude extract and solvent fractions of *Ajuga integrifolia* leaves using MES test in mice.
- To determine the effect of crude extract and solvent fractions of *Ajuga integrifolia* leaves on motor coordination using rotary rod test in mice.
- To perform preliminary phytochemical screening of the crude extract and solvent fractions of *Ajuga integrifolia* leaves.

3. Materials and Methods

3.1 Chemicals and Drugs

The following chemicals and drugs were used in the study: Methanol and n-butanol (Carlo Erba Reagents, France), Chloroform (Loba Chemie, India), Hydrochloric acid and Potassium ferrocyanide (BDH Ltd., England), Sodium hydroxide and Sulfuric acid (Carlo Erba Reagents, Italy), Dragendroff's reagent and Glacial acetic acid (Fisher Scientific, UK), Ferric chloride (Fisher Scientific, USA), Acetic anhydride (Techno Pharmchem, Bahadurgarm, India), Tween 80 (Atlas Chemical Industries Inc., UK), Pentylenetetrazole powder (PTZ) (Sigma Aldrich, Germany), Normal saline (Acu Life Health Care, India), and Distilled water were obtained from the Department of pharmacology and Pharmaceutical chemistry, school of pharmacy Addis Ababa University (AAU). Sodium valproate (Remedica, Cyprus), Phenytoin (Brawn Laboratory, India), and Diazepam (DZP) (Remedica, Cyprus) were obtained from their respective vendors. All chemicals and reagents used were analytical grade.

3.2 Plant Collection

Fresh leaves of *Ajuga integrifolia* were collected in early February 2019 from its natural habitat around Ghimbi district, located in Western Wollega Zone, Oromia National Regional State; 441 km southwest of Addis Ababa. The collected plant was identified and authenticated by a taxonomist at the National Herbarium, College of Natural and Computational Sciences, AAU, where a voucher specimen (collection number: YG-001) was deposited for future reference.

3.3 Experimental Animals

Experiments were carried out on healthy Swiss albino mice (20-30gm weighing) of either sex, which were bred in the animal house unit of School of pharmacy, AAU, Addis Ababa, Ethiopia. Mice were housed in plastic cage with standard wood chip bedding at standard environmental conditions (12h light/ dark cycle) and provided with the standard laboratory pellet and tap water *ad libitum*. They were acclimatized with the laboratory setting for one week before commencement of the experimental protocols.

3.4 Ethical considerations

The handling of animals and all experimental procedure were carried out in compliance with internationally accepted standard guidelines for the use of laboratory animals.[86]

3.5 Plant Extraction and Fractionation

The leaves of the plant was collected from its natural habitat, carefully washed to remove dust and dirt, shade dried for weeks, and pulverized into powder manually using a mortar and pestle. Two hundred gram of the powder were weighed using electronic balance and macerated in 1000 ml of 80% methanol for three consecutive days at room temperature with occasional shaking using orbital shaker (Bibby Scientific Limited, UK). Then after, the extracts were filtered through whatman no 1 filter paper. The marcs were re-macerated two times using the same volume of solvent to exhaustively extract the plant materials.

The hydroalcoholic extracts were combined together and methanol part was removed using Rotary evaporator (BUCHI R-200, Switzerland) at 40^{o}C under reduced pressure with rotation speed of 50rpm and then water content was removed using lyophilizer (OPERAN lyophilizer, Korea). The resulting dried hydroalcoholic extract was weighed and calculated for percentage yield, which was 47gm (23.5 w/w). The dried crude extract was transferred into amber glass container and stored in deep freezer until used for the experiment. Whenever required for the experimental purpose the dried hydroalcoholic extract was reconstituted with 2% tween 80.

For fractionation, 40gm of the crude extract was taken and dissolved in 150 ml of distilled water to be transferred into the separatory funnel, where, 150 ml of chloroform was added and thoroughly shaken until well mixed together. A while after, the two phases were come back again and the bottom chloroform layer eluted and separated. The procedure was repeated two times by adding the same volume of chloroform until its layer become clear. Then, the chloroform was removed using a rotary evaporator at 40^{o}C and the remaining residue dried in lyophilizer, 7.92gm (19.80%w/w) was obtained.

Finally, the aqueous fraction was further fractionated using n-butanol just in the same fashion with the above. Here, the bottom aqueous layer was exhaustively extracted by adding 150 ml distilled water two times until the layer appears clear. The aqueous fractions were combined together,

lyophilized and 8.82mg (22.05%w/w) was obtained. The leftover n-butanol was concentrated in water bath at 40ºC and 9.56gm (23.90%w/w) was obtained. All solvent fractions were kept in a closed container and stored in refrigerator until used for experimental purpose.

3.6 Acute Toxicity Test

Acute oral toxicity test was conducted according to OECD/OCDE 425 guideline. Female Swiss albino mice of 6-8 weeks of age were randomly selected and assigned in groups consisting of five mice. Before acute toxicity test was done, mice were allowed to fast overnight (not for water). The control groups received the vehicle (2% tween 80) and the treatment group received 2000mg/kg of crude extract orally using oral gavage and kept under strict observation for physical or behavioral changes for 2 weeks.[87]

3.7 Grouping and Dosing of Animals

The animals were randomly assigned in to five groups of six mice to test anticonvulsant activities of both the crude extract (MAI) and solvent fractions. The solvent fractions were labeled as butanol fraction (BAI), chloroform fraction (CAI), and aqueous fraction (AAI). The first group was assigned as negative control and treated with the vehicles (10mg/kg of distilled water for aqueous extracts/10mg/kg, or 2% tween 80 for non-aqueous extracts). The second group was assigned as positive control and treated with standard drugs (200mg/kg oral sodium valproate for PTZ test, 25 mg/kg oral phenytoin for maximum electroshock (MES) induced seizure test and 5 mg/kg i.p DZP for rota-rod test). Group three, group four and group five were treated with oral doses of 100mg/kg, 200mg/kg, and 400mg/kg of MAI and solvent fractions respectively.

3.8 Anticonvulsant Activity Tests

3.8.1 Pentylenetetrazole Seizure Model

Initially, mice in each group received different oral doses (100mg/kg, 200mg/kg, and 400mg/kg) of MAI and each solvent fractions, standard drug (sodium valproate 200mg/kg) and vehicles (10mg/kg of distilled water/2% tween 80) with oral route. After 60 minutes, PTZ of 85 mg/kg in normal saline solution was injected through subcutaneous route to each mouse. Finally, mice were placed inside a plastic cage separately and followed for 30 min using a hidden video recording.

Parameters like latency of clonic seizure, duration of clonic convulsion, the percentage of protection against seizure and mortality were measured.[88]

$$\%\ protection = \frac{no.of\ clonus\ in\ control - no.of\ clonus\ in\ test/standard}{no.of\ clonus\ in\ control} * 100$$

$$\%\ protection\ of\ mortality = \frac{no.of\ death\ in\ control - no.of\ death\ in\ test}{no.of\ death\ in\ control} * 100$$

3.8.2 Maximal Electroshock Induced Seizure Model

Initially, mouse in each group received different oral doses (100mg/kg, 200mg/kg, and 400mg/kg) of MAI and each solvent fractions, standard drug (phenytoin 25mg/kg) and vehicles (10mg/kg of distilled water/10mg/kg of 2% tween 80). After an hour, tonic hind limb extension (THLE) was induced in mouse by delivering electroshock of 50 mA for 0.2 seconds through trans-auricular clip of electroconvulsiometer (Rolex Ambala, India). Finally, parameters like mean duration of THLE and percentage reduction of THLE were measured using a video recorder.[88]

$$\%\ \text{reduction of THLE} = \frac{duration\ of\ THLE\ in\ control - duration\ of\ THLE\ in\ test/standard}{duration\ of\ THLE\ in\ control} * 100$$

Note: THLE stands for tonic hind limb extension

The animals which did not exhibit THLE were considered protected.[88]

3.8.3 Rota Rod Test

This test was carried out to assess both the crude extract and solvent fractions effect on motor function using protocol as described by R.L. Krall et al and D.Y. Gawande et al.[89,90] Initially, mice were trained on the rota-rod apparatus for three consecutive days and those retained on the bar at a speed of 10 rpm for 5 minutes or more were selected for the study. The experimental groups received different doses of MAI (2.5, 5 and 10 mg/kg i.p.), DZP (5 mg/kg i.p.) and distilled water/1% tween 80 (10 ml/kg i.p.). After 30 min of respective treatments, all mice were placed individually in each lane for three consecutive trials and average retention time was calculated. The mouse was considered to have motor deficits when unable to maintain equilibrium on the rotating rod within 3 min.

3.9 Phytochemical Analysis

The crude extract and all fractions of *Ajuga integrifolia* were screened for the existence of various phytochemical constituents using standard procedures as described by Tewodros et al.[91]

Test for Alkaloids

About 5 ml of 5% hydrochloric acid was added to 0.5mg of MAI and each fraction, and heated on a water bath. After cooled down, few drops of dragendroff's reagent were added. The appearance of the reddish brown precipitate indicated the presence of alkaloids

Test for Saponins

About 0.5gm of MAI and each fraction were mixed with 10ml of distilled water in a test tube and shaken vigorously. The formation of stable foam was considered as an indicator for the presence of saponins.

Test for Flavonoids

About 0.5gm of MAI and each fraction mixed with 10 ml of distilled water, boiled for 5 min and filtered while hot. Few drops of 20% sodium hydroxide solution were added to 1 ml of the cooled filtrate. Formation of a yellow color which changed to colorless solution while treated with acid indicated the presence of flavonoids.

Test for Cardiac Glycosides

Two ml of MAI and each fraction were dissolved in 2.0 ml of glacial acetic acid containing one drop of ferric chloride ($FeCl_3$) Solution. This mixture was then added into a test tube containing 1 ml of concentrated H_2SO_4. A brown ring at the interphase indicates the presence of deoxysugar, characteristic of cardenolides.

Test for Phenols

About 0.5gm of MAI and each fraction were treated with 3 drops of freshly prepared mixture of 1% ferric chloride solution and 1% potassium ferrocyanide. Formation of a green blue color indicates the presence of phenols.

Test for Steroids

About 0.5gm of each sample were dissolved in 2 ml of acetic anhydride, followed by the addition of 4 drops of chloroform. Two drops of concentrated sulphuric acid were then added at the side of the test tube. The development of a brownish ring at the interface of the two liquids and the appearance of violet color in the supernatant layer were indicative of the presence of steroids.

Test for Terpenoids

About 0.5gm of MAI and each fraction were dissolved in 2ml of chloroform and evaporated to dry. Then, 2ml of concentrated H_2SO_4 was added and heated for about 2 minutes. A grayish color indicated the presence of terpenoids

Test for Tannins

About 0.5gm of MAI and each fraction were boiled in 10 ml of water in a test tube and filtered. A few drops of 10% ferric chloride were added and a bluish-black or brownish-green precipitate indicated the presence of tannins

3.10 Data Analysis

Data analysis was performed using the statistical package for the social sciences (SPSS), version 25.0. The results were expressed as mean ± SEM and analyzed statistically using One-way ANOVA followed by Dunnett post-hoc test to find out significant difference between control groups against each test groups separately. The results were considered statistically significant at 95% confidence interval with p value <0.05.

4. Results

4.1 Acute Oral Toxicity Test

Mice did not show any observable sign of toxicities upon oral administration of 80% methanol leaves extract and solvent fractions of *Ajuga integrifolia* at the limit dose of 2000mg/kg. This was proven by the absence of significant changes in behaviors such as alertness, motor activity, breathing, diarrhea, convulsions, and coma. Moreover, mortality was not recorded during the observation period.

4.2. Anticonvulsant Activity in PTZ Induced Seizure

The treatment of mice with crude extract showed statistically significant increment on the latency of clonic seizure with $p<0.001$ for MAI400 as well as $p<0.05$ for MAI100 and MAI200 when compared with control (Table 1). Concerning the duration, all doses of MAI showed statistically significant difference with $p<0.001$ as compared with control. The latency and duration exhibited by MAI400 was greater than MAI200 and MAI100. All doses of MAI displayed statistically significant difference in latency and duration as compared with the standard (valproate). Both the increment of latency and decrement of duration were dose dependent.

Treatment of mice with MAI400 and MAI200 exhibited protection against clonic seizure, 33.33% and 16.67% respectively (Table 1). However, valproate displayed maximum protection of 83.33% against clonic seizure which was greater than all the crude extract doses. But, neither of the solvent fractions had protection against clonic seizure. The maximum protection against mortality was achieved by MAI400 (83.33%) followed by MAI200 (66.67%) and MAI100 (33.33%). Nevertheless, none of mice died as result of treatment with valproate (Table 1).

The solvent fractions also displayed statistically significant difference against PTZ induced seizure with the exception of AAI (Table 2). Among the solvent fractions; BAI400, BAI200 and CAI400 showed substantial increment in the latency time of clonic seizure with $p<0.001$ relative to control. Additionally, statistically significant latency was exhibited by CAI200 and BAI100 with $p<0.05$ when compared with control.

Table 1: The anticonvulsant effect of 80% methanol leaves extract of *Ajuga integrifolia* in PTZ induced seizure.

Groups	Mean latency of clonic seizure (sec)	Mean duration of clonic seizure (sec)	% protection of clonic seizure	% reduction of mortality
Control	163.00 ± 13.72	49.67 ± 2.15	-	-
MAI100	479.67 ± 26.96^{a1b3}	19.17 ± 1.22^{a3b3}	-	33.33
MAI200	625.00 ± 23.98^{a1b3}	11.17 ± 0.95^{a3b1}	16.67	66.67
MAI400	1139.00 ± 209.56^{a3b3}	8.67 ± 2.77^{a3b1}	33.33	83.33
Valproate200	1623 ± 177^{a3}	1.83 ± 1.83^{a3}	83.33	100

Values expressed as Mean ± SEM. (n = 6 mice). a=compared to negative control, b=compared to positive control (valproate), ^{1}=p<0.05, ^{2}= p<0.01, ^{3}= p<0.001. MAI=80% methanol leaves extract of *Ajuga integrifolia*. 100, 200, and 400 doses mg/kg. Control=group treated with 10ml/kg of 2% tween 80, - indicates zero.

All doses of BAI, CAI400 and CAI200 showed significantly shorter duration of clonic seizure with p<0.001 when compared with control. Similarly, CAI100 displayed statistically significant difference in the duration of clonic seizure with p<0.05 when compared with control. However, the duration of clonic seizure displayed by all doses of the fractions were statistically different from the valproate. The highest % of protection against mortality was displayed by BAI400 (66.67%) followed by BAI200 (50%) and CAI400 (50%) (Table 2). The latency as well as the duration displayed by all doses of the solvent fractions were dose dependent and statistically different from the standard (valproate).

Table 2: Anticonvulsant Effect of solvent fractions of *Ajuga integrifolia* in PTZ induced seizure.

Groups	Mean latency of clonic seizure (sec)	Mean duration of clonic seizure (sec)	% protection of mortality
Control 1	163.00 ± 13.72	53.17 ± 3.74	-
BAI100	414.00 ± 13.53^{a1b3}	27.17 ± 1.07^{a3b3}	16.67
BAI200	662.50 ± 17.75^{a3b3}	20.17 ± 1.14^{a3b3}	50.00
BAI400	931.17 ± 16.96^{a3b3}	12.5 ± 0.92^{a3b3}	66.67
Control 1	163.00 ± 13.72	53.17 ± 3.74	-
CAI100	373.17 ± 12.47^{b3}	41.00 ± 1.98^{a1b3}	16.67
CAI200	477.17 ± 11.56^{a1b3}	28.17 ± 1.47^{a3b3}	33.33
CAI400	633.67 ± 18.66^{a3b3}	20.67 ± 1.60^{a3b3}	50
Control 2	155.67 ± 9.98	51.33 ± 1.83	-
AAI100	248.17 ± 15.61^{b3}	47.50 ± 1.72^{b3}	-
AAI200	317.50 ± 14.54^{b3}	46.67 ± 1.72^{b3}	-
AAI400	358.5± 14.65^{b3}	45.00± 1.63^{b3}	
Valproate200	1623 ± 177^{a3}	1.83 ± 1.83^{a3}	83.33

Values expressed as Mean ± SEM. (n = 6 mice). a=compared to negative control, b= compared to positive control (valproate), ^{1}=p < 0.05, ^{2}= p<0.01, ^{3}= p < 0.001. BAI=Butanol fraction, CAI=Chloroform fraction, AAI=Aqueous fraction. 100, 200, and 400 doses mg/kg. Control 1=group treated with 10ml/kg of 2% tween 80, Control 2=10ml/kg of distilled water, - indicates zero.

4.3 Anticonvulsant Activity in MES Induced Seizure

The anticonvulsant effects of the crude extract and solvent fractions of the plant was further evaluated using MES test (Table 3 and 4). According to the test result, all doses of the MAI significantly reduced the duration of THLE compared with control (p<0.001). The percentage reduction in the duration of THLE exhibited by MAI400 (56.03%) was greater than that of MAI200 (39.55%) and MAI100 (29.66%). Nevertheless, all doses of MAI displayed significantly longer mean duration of THLE when compared with standard (Phenytoin) (p<0.001). Rather, the standard displayed full protection against THLE.

Table 3. Anticonvulsant Effect of 80% methanol leaves extract of *Ajuga integrifolia* in MES induced seizure.

Groups	Mean duration of THLE (sec)	% reduction of THLE
Control	15.17 ± 0.83	-
MAI100	10.67 ± 0.8^{a3b3}	29.66
MAI200	9.17 ± 0.6^{a3b3}	39.55
MAI400	6.67 ± 0.49^{a3b3}	56.03
Phenytoin25	0.00^{a3}	100

Values expressed as Mean ± SEM. (n = 6 mice). a=compared to negative control, b=compared to positive control (Phenytoin), [1]=p<0.05, [2]= p<0.01, [3]= p<0.001. MAI: 80% methanol leaves extract of *Ajuga integrifolia*. 25, 100, 200, and 400 doses mg/kg. Control: group treated with 10ml/kg of 2% tween 80, - indicates zero, THLE=tonic hind limb extension

BAI400, BAI200 and BAI100 displayed statistically significant reduction in the duration of THLE with p<0.001, p<0.001 and p<0.05 respectively when compared with control. Similarly, treatment with CAI400 and CAI200 presented statistically significant reduction with p<0.001 and p<0.05 respectively. Nevertheless, all doses of AAI did not display a significant reduction compared to control. The maximum % reduction in the duration of THLE among the fractions were exhibited by BAI400, 45.08%. However, all doses of fractions showed significantly longer duration of THLE compared to phenytoin (p<0.001).

Table 4. Anticonvulsant effect of solvent fractions of *Ajuga integrifolia* in MES test.

Group	Mean duration of THLE (sec)	% reduction in the duration of THLE (sec)
Control 1	15.17 ± 0.83	-
BAI100	11.83 ± 0.79^{a1b3}	22.01
BAI200	10.67 ± 0.71^{a3b3}	29.66
BAI400	8.33 ± 0.84^{a3b3}	45.08
Control 1	15.17 ± 0.83	-
CAI100	13.83 ± 0.70^{b3}	8.83
CAI200	12.17 ± 0.60^{a1b3}	19.77
CAI400	11.67 ± 0.76^{a2b3}	23.07
Control 2	15.50 ± 1.54	-
AAI100	14.83 ± 0.70^{b3}	4.32
AAI200	13.83 ± 0.79^{b3}	10.77
AAI400	12.67 ± 0.84^{b3}	18.26
Phenytoin 25	0.00^{a3}	100

Values expressed as Mean ± SEM. (n = 6 mice). a=compared to negative control, b= compared to positive control (phenytoin), [1]=p<0.05, [2]= p<0.01, [3]= p<0.001. BAI=Butanol fraction, CAI=Chloroform fraction, AAI=Aqueous fraction. 25, 100, 200, and 400 doses mg/kg. Control 1=group treated with 10ml/kg of 2% tween 80, Control 2=10ml/kg of distilled water, - indicates zero.

4.4 Motor Coordination Test

All doses of MAI along with all solvent fractions were further evaluated for effect on motor coordination using rota rod test (Table 5). Accordingly, all doses of the crude extract and solvent fractions did not show motor incoordination, as all treated mice were retained on the rotating rod for more than 180sec. All doses of the MAI and solvent fractions displayed statistically significant difference compared to standard (DZP) ($p<0.001$) but not compared to the negative control.

Table 5. Effect of *Ajuga integrifolia* and solvent fractions on motor coordination using Rota rod test

Groups	Mean Duration of Retention on the Rod (sec)
Control 1	281.00 ± 14.45
MAI 2.5	248.67 ± 17.58
MAI 5	232.50 ± 14.79
MAI 10	232.83 ± 18.86
Control 1	281.00 ± 14.45
BAI	260.00 ± 12.24
BAI 5	213.00 ± 5.94
BAI 10	263.50± 14.23
Control 1	281.00 ± 14.45
CAI 2.5	242.17 ± 16.83
CAI 5	275.17 ± 9.90
CAI 10	227.17 ± 8.66
Control 2	272.67 ± 19.63
AAI 2.5	236.83 ± 10.89
AAI 5	243.17 ± 21.28
AAI 10	230.00 ± 6.16
DZP 5	25.83 ± 7.14

Values expressed as Mean ± SEM. (n=6 mice). MAI=80% Methanol leaves extract of *Ajuga Integrifolia*, BAI=Butanol fraction, CAI=Chloroform fraction, AAI=Aqueous fraction, 2.5, 5, 10 doses mg/kg, Control 1=group treated with 10ml/kg of 2% tween 80, Control 2=10ml/kg of distilled water, DZP=Diazepam

4.5 Preliminary Phytochemical Screening

Phytochemical screening of the hydro-alcoholic extract and solvent fractions of *Ajuga integrifolia* showed the presence of alkaloids, flavonoids, cardiac glycoside, phenols, saponins, steroids, tannins and terpenoids in crude extract. Alkaloids and steroids were absent from aqueous fraction while only saponins were absent from both n-butanol and chloroform fraction (Table 6).

Table 6. Preliminary Phytochemical Screening of the 80% Methanol Extract and Solvent Fractions of the Leaves of *Ajuga integrifolia*

Phytoconstituents	**Crude extract**	**n-butanol fraction**	**Chloroform fraction**	**Aqueous fraction**
Alkaloids	+	+	+	–
Flavonoids	+	+	+	+
Cardiac glycosides	+	+	+	+
Phenols	+	+	+	+
Saponins	+	–	–	+
Steroids	+	+	+	–
Tannins	+	+	+	+
Terpenoids	+	+	+	+

- Absent **+** Present

5. Discussion

The present study was carried out to evaluate the anticonvulsant activity of 80% methanol extract and solvent fractions of *Ajuga integrifolia* in experimental animals based on the claim of the traditional practice of Ghimbi district, Ethiopia. The claimed property of the plant was confirmed to have a statistically significant and dose-dependent anticonvulsant activities both in PTZ and MES models. In PTZ model, the plant crude extract along with its solvent fractions have found to exert anticonvulsant activity owing to their ability to increase the latency and decrease the duration of clonic seizure caused by administration of PTZ. Similarly, statistically significant reduction in the mean duration of THLE against control was displayed in MES model.

The PTZ induced seizure model was a valid test that represent human generalized myoclonic and absence seizures.[92] Hence, this model was used to investigate the potential of the extract in controlling generalized myoclonic and absence seizure. So as, the plant extracts might have the potential against PTZ induced clonic seizure[93] since which either delays/shorten the duration or completely abolish the clonic convulsions. Pentylenetetrazole is a non-competitive $GABA_A$ receptor antagonist act through benzodiazepine binding site and used to create seizure. The neurotransmitter GABA is a major inhibitory neurotransmitter in the brain; its inhibition has been supposed to be the principal feature in epilepsy. Nevertheless, enhancement of its effect is reported to antagonize seizure generation with standard drugs like valproate and benzodiazepines. Moreover, this type of seizure is attenuated by agents that act through reduction of T-type calcium currents, ethosuximide and valproate.[76]

Inhibition of PTZ-induced seizures with the crude extract/solvent fractions probably suggest its effects on GABAergic neurotransmission or inhibition of T-type calcium current.[93] This was supported by similar study done on stem extract of *Ajuga integrifolia* and solvent fractions which revealed an increment of the GABA level in the serum of treated animals, mainly with methanol (crude) extract.[74] Moreover, the leaves of the plant extract was identified to have specific sterols such as β-sitosterol and stigmasterol.[94] These sterols have a base structure similar to the neuroactive steroids such as progesterone and allopregnanolone so as to exert anticonvulsant activity probably through the activation of a $GABA_A$ receptor.[95,96] Furthermore, an antidiarrheal

activity of the study plant was discoursed in relation with reduction of peristalsis and secretion by reducing the intracellular Ca^{2+} inward current.[70]

According to the test result; the latency, duration, % protection against clonic seizure and mortality displayed by MAI400 in PTZ induced seizure was greater than all the other doses of the crude extract and solvent fractions. This magnificent activity were recorded possibly related with accumulation of phytochemical constitutes in this dose. In a contrary, the solvent fractions had lesser activity against the above parameters probably colligated with lack of synergetic activity as the secondary metabolites were preferentially distributed among them.

Although BAI and CAI investigated to own similar kind of secondary metabolites, BAI displayed surplus anticonvulsant activity. This might be related with the presence of greater concentration of secondary metabolites in BAI. CAI ranked next to BAI in anticonvulsant activity perhaps associated with the presence of lesser quantity of phytochemical constitutes that unable to be absorbed in sufficient concentration and exert its pharmacological activity. However, it requires further investigation to come across with the exact amount of secondary metabolite present in each fraction.

Mice treated with any doses of the solvent fractions did not protect against clonic seizure possibly associated with preferential distribution of secondary metabolites between solvent fractions and loss of synergetic effect. In addition, absence of saponins in butanol and chloroform fractions might played role in deterioration of such parameter. The aqueous fraction did not display statistically significant anticonvulsant activity which might be associated with absence of major contributors such as steroids and alkaloids. Additionally, extracts of aqueous fraction may not cross the biological membrane because of its polar nature in order to exert anticonvulsant activity.

A previous similar type of study done on the stem part of *Ajuga integrifolia* but growing at different geographical place, Pakistan, showed that the stem of the plant attenuated PTZ induce seizures in mice.[74] In comparison, the mean latency to clonic seizure exhibited by the crude extract of the present study plant was greater than the stem (MAI400, 1139.00 ± 209.56 vs. MAI500, 378.66 ± 8.21). Regarding the duration, the leaves extract displayed maximum reduction (MAI400, 8.67 ± 2.77 vs. MAI 500, 440±8.26). The difference may be emanated from low percentage yield of the stem extract (23.5% vs. 6.3%). Additionally, geographical variation may contribute to

dissimilarities in phytochemical constitutes present in these plants, which might be responsible for variation in their anticonvulsant activities.

The MES test was done by trans-auricular electrical stimulation of mice in which seizure spread when all neuronal circuits in the brain are maximally active. Drugs effective in MES model are considered as possible antiepileptic agent against generalized tonic–clonic (grand mal) seizures. Effective standard drugs in MES model act against generalized tonic–clonic seizures by blocking the seizure spread. For example, phenytoin mainly acts by blocking the voltage-dependent Na^+ channels. Additionally, drugs act against glutamatergic excitation such as felbamate can also be used for the prevention of this type of seizure.[97]

Any test drug which decrease the duration of THLE displays its ability to slow down the spread of seizure. Whereas, animals were considered "protected" upon abolition of the MES induced THLE.[97] Therefore, the study plant extract probably showed anticonvulsant activity against MES induced seizure hence it found to significantly reduce the duration of THLE when compared to control. The probable working mechanism of the plant extract against MES induced THLE might be associated with blockade of voltage-dependent Na^+ channels as well as the spread of seizure and perhaps it is effective against generalized tonic–clonic (grand mal) seizures. The highest % reduction of THLE was recorded by MAI400, since maximum of the secondary metabolites were accumulated in the specified dose.

Just like phenobarbital and sodium valproate, MAI400 had highest activities in both PTZ and MES models. These standard agents had multiple of working mechanisms against seizure as described above. Similarly, MAI400 had highest activities against both PTZ and MES induced seizure might be associated with the phytochemical constitute present comprising multiple of working mechanisms in epilepsy. For example: the anticonvulsant activity attributed to alkaloids is reported via its antioxidant activity[98], GABA modulation[99], and inhibition of NMDA receptor mediated current in brain[100]. Additionally, flavonoids had multiple of working mechanisms of anticonvulsant activities such as modulatory effect on voltage gated $Na^+/Ca^{2+}\cdot K^+$[101], GABAergic system[102], opioid receptors[103], NMDA receptors[104] etc.

Once again, BAI displayed a highest reduction in the duration of THLE than CAI which might be associated with deferential accumulation of higher quantity of secondary metabolites. AAI did not

display statistically significant anticonvulsant activity against both PTZ and MES induced seizure models possibly associated with absence of major contributors, alkaloids and steroids. Moreover, the concentration of phytochemical constitutes present in AAI is probably low in order to absorb and inhibit both PTZ and MES induced seizures. This indicates that AAI mayn't have activity against both myoclonic/absence type of seizures and generalized tonic clonic seizure.

The rota rod test was used to evaluate the activity of crude extract and solvent fractions on motor coordination. The anticonvulsant effect of the plant extract might be exerted because of muscle relaxation rather than having certain anti-epileptogenic activity that must be ruled out. Thus, it is important to evaluate and determine the effect of the plant extract on muscle coordination using the rota-rod test. A mouse with normal motor competence was able to maintain equilibrium on a rotating rod for more than 180 s. However, agents are considered neurotoxic when animals fall off from rotating rod within 3 min period.[90]

According to the test result, all mice that received crude extract and solvent fractions did not fall off from the rotating bar within 180s. However, mice received DZP 5mg/kg showed a sign of neurotoxic effect evidenced by mice did not remain on the rotating bar for the given period. This indicates that *Ajuga integrifolia* didn't affect motor coordination in mice and the observed anticonvulsant activity was probably associated with having certain anti-epileptogenic effect that require further investigation rather than muscle relaxation. Generally, it can be guessed that the anticonvulsant activities of the plant might be exerted as a result of non-polar phytochemical agents. Hence, the n-butanol fraction got the highest anticonvulsant activity followed by chloroform and finally aqueous fraction.

The acute oral toxicity of the study plant was done only on female mice associated with particular vulnerability to injury than males.[87] Accordingly, neither death nor any signs of toxicities were observed at limit dose of 2000 mg/kg of the crude test extract. So as, the lethal dose 50% (LD50%) of the plant extract estimated to be greater than 2000mg/kg in mice. The LD50% was similar with other previous studies done on the leaves of *Ajuga integrifolia*.[69–71] Hence, the traditional use of the leave of *Ajuga integrifolia* for various illness is safe.

However, female mice were excluded from the actual anticonvulsant study. Females mice have cyclic excitability and seizure susceptibility in relation to the menstrual cycle.[105] Progesterone

and its metabolites allopregnanolone being anticonvulsive[106] whereas estrogens being mainly pro-convulsive[107] and the monthly fluctuations in the level of estrogen and progesterone are thought to be the basis for alteration in the convulsive response of the female mice in PTZ induced seizure model. Additionally, female mice have shown to produce enhanced electroshock responses.[91] So as to avoid such fluctuation in seizure response with female mice, only male mice were used for anticonvulsant activity tests.

6. Conclusion

The present study provided evidence that, the crude extract along with the fractions displayed varying degrees of anticonvulsant activity. The anticonvulsant activity of crude extract, butanol and chloroform fraction were effective in both PTZ and MES models. However, aqueous fraction was shown to be ineffective in both seizure models. Furthermore, the rota rod test validated the plant had no neurotoxic effect as evidenced by mice kept on rotating rod and the observed anticonvulsant activity was not cause by muscle relaxation. All over, the results from the present study provide a scientific evidence to support the safe traditional use of *Ajuga integrifolia* in the treatment of epilepsy.

7. Recommendations

The results obtained from the present study clarified that crude extract and fractions of leaves of *Ajuga integrifolia* has anticonvulsant activity. However, further researches will be required to;

- Confirm the anticonvulsant effect of the plant on chronic seizure models,
- Investigate the chronic toxicity of the plant,
- Isolate, identify and characterize pharmacologically active phytochemical compounds of the extract that are responsible for the anticonvulsant activity.
- Determine the molecular mechanism of action of the plant.
- Evaluate the effects of plant on EEG component of seizures

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