Anticonvulsant activity of Soxhlet leaf extracts of *Ajuga integrifolia Buch.Ham ex D.Don* (Lamiaceae) in mice

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This is to certify that the thesis prepared by Tesfaye Desalegn entitled "Anti-convulsant activity of Soxhlet leaf extracts of *Ajuga integrifolia Buch.Ham ex D.Don* (Lamiaceae) in mice" and submitted in partial fulfillment for the requirements of Master of Science degree in Pharmacology complies with the regulation of the university and meets the respected standards with respect to originality and quality

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ABSTRACT

Epilepsy is one of the most common chronic neurological conditions that affect 70 million people in different parts of the world. The leaves of *Ajuga integrifolia Buch.ham ex D Don* have been used as anti-convulsant remedy in Ethiopian traditional medicine. However, the evidence supporting this claim 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. To this effect successive Soxhlet extraction was performed using n-hexane, ethyl acetate, methanol and water.

Anti-convulsant activity of the extracts was investigated in both acute (pentylenetetrazole, PTZ; and maximal electroshock, MES) and chronic (PTZ kindling) seizure models. For the acute model, various doses of the extract (100mg/kg, 200mg/kg and 400mg/kg) were administered. Positive controls received sodium valproate (200mg/kg) for PTZ and phenytoin (25mg/kg) for MES. Distilled water or 2% tween 80 was used for negative controls. Kindling was induced by repeated alternate day intra-peritoneal administration of sub-convulsive dose of PTZ (35 mg/kg) for 13 days and the most active extract (ethyl acetate) was tested in this model. Parameters including onset of clonus and duration of hind limb tonic extension were recorded. Moreover, total alkaloid, flavonoid and phenol contents of the most active extract were determined.

Treatment of mice with ethyl acetate extract produced a superior effect among all solvent extracts in both PTZ and MES model. The mean latency to clonic seizure was significantly increased ($p<0.01$) with all doses of ethyl acetate extract in PTZ test compared to control and n.hexane extract ranked next to ethyl acetate extract in increasing onset of clonus. It significantly increased mean onset of clonus compared to controls, with a maximum increase (12.67min, $p<0.001$) displayed by HA400 mg/kg. Methanol extract at 200mg/kg and 400 mg/kg also significantly delayed onset of clonus ($p<0.001$) in PTZ model.Once again, all doses of ethyl acetate extract of the study plant significantly reduced ($p<0.001$) the mean duration of hind limb tonic extension in MES test compared to control. Hexane extract at 200 mg/kg and 400 mg/kg also significantly reduced ($p<0.001$) duration of hind limb tonic extension. Methanol extract at 200mg/kg and 400 mg/kg also significantly reduced ($p<0.01$)

mean duration of hind limb tonic extension (HLTE) compared to control in MES test. Aqueous extract at all doses was devoid of any anti-convulsant effect in both models. A similar type of study done on the leaf crude extract and solvent fractions collected from different geographical location also reported anti-convulsant activity of the plant in acute seizure models. Treatment of mice with 200mg/kg and 400mg/kg of ethyl acetate extract along with alternate day PTZ injection significantly protected($p<0.01$ for 200mg/kg and $p<0.001$ for 400 mg/kg) against PTZ induced kindling compared to controls in chronic model. Ethyl acetate extract of the plant 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 /g of dry weight extract of flavonoids and 21.928 ±1.118 mg gallic acid equivalent / g of dry weight of extract of phenols. This study indicated that the plant has anti-convulsant activity in both acute and chronic model and it could be a potential source to develop a new anti-epileptic drug for pharmacoresistant epilepsy.

Key words: *Ajuga integrifolia,* Anti-convulsant, Epilepsy, kindling, phytoconstituents, Seizure

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LIST OF ABBREVIATIONS AND ACRONYMS

AED	Anti-epileptic Drugs
AMPA	α-Amino-3-hydroxy-5-Methyl-isoxazolopropionic Acid
ANOVA	Analysis of Variance
CA	Cornu Ammonis
CNS	Central Nervous System
DALY	Disability Adjusted Life Year
DPPH	1,1-diphenyl-2-picrylhydrazyl
EEG	Electroencephalography
GABA	Gamma Amino Butyric Acid
ICAM	Intercellular Adhesion Molecule
IL	Interleukin
ILAE	International League Against Epilepsy
IP	Intra-peritoneal
HLTE	Hind Limb Tonic Extension
MES	Maximal Electro Shock
mGluR	Metabotropic Glutamate Receptor
NMDA	N-Methyl-D-Aspartate
MRI	Magnetic Resonance Imaging
PTZ	Pentylenetetrazol
SPSS	Statistical Package for Social Sciences
TAC	Total Alkaloid content
TFC	Total Flavonoid Content
TPC	Total Phenolic Content
TLE	Temporal Lobe Epilepsy
VCAM	Vascular Cell Adhesion Molecule
VEGF	Vascular Endothelial Growth Factor
VGCCs	Voltage Gated Calcium Channels
VGSC	Voltage Gated Sodium Channel

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1. INTRODUCTION

1.1. Overview of Epilepsy

Epilepsy is one of the most common chronic neurological disorder that affects many people in different parts of the world(Thijs et al., 2019). Despite their difference, the term epilepsy and seizure are often confusing(Scharfman, 2007). As defined by the International League Against Epilepsy(ILAE), epilepsy is a disease of the brain characterized by any of the following conditions: (i) At least two unprovoked (or reflex) seizures occurring >24 h apart; (ii) one unprovoked (or reflex) 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; (iii) presence of an epilepsy syndrome even if the risk of subsequent seizure is very low (Fisher et al., 2014). Seizures are characterized by disturbed cerebral function caused by abnormal, excessive, and synchronous electrical discharges in groups of cortical neurons that may produce sub-clinical or various clinical phenomena(Fisher et al., 2017, Mosewich and So, 1996).

Epilepsy is divided into 4 categories based on etiology: idiopathic, symptomatic, provoked, and cryptogenic. Idiopathic epilepsy is epilepsy which is predominantly genetic or presumed genetic origin in which there is no gross neuro-anatomic or neuro-pathologic abnormality (epilepsy with no underlying structural brain lesion or other neurologic signs or symptoms). Symptomatic epilepsy is epilepsy of acquired or genetic cause associated with gross anatomic or pathologic abnormalities. It is the result of one or more identifiable structural lesions of the brain. On the other hand epilepsy which is predominantly caused by specific systemic or environmental factor in which there are no gross anatomic or neuro-pathologic change is known to be provoked epilepsy whereas epilepsy of presumed symptomatic nature in which the cause has not been identified is known as cryptogenic epilepsy(Engel Jr, 2001, Shorvon, 2011).

Three types of seizure can be identified on the basis of clinical phenomena as it can be evidenced by electro-encephalography (EEG). They are: generalized seizure, focal seizure and seizure with unknown onset (Fisher et al., 2017).

Generalized seizure is a type of seizure that originates simultaneously on both sides of the brain hemispheres and spreads rapidly via neuronal networks. There are a range of generalized seizure types such as tonic-clonic (grandmal), absence (non motor), myoclonic, tonic and atonic seizure. Focal seizure is a seizure originating from networks limited to one hemisphere that may be discretely localized or more widely distributed. It can present with a range of symptoms depending on site of abnormal electrical discharge in the brain. The third type is seizure with unknown onset in which the physician is occasionally certain whether it is focal or generalized. This is more common in health facilities where an access to brain imaging techniques like magnetic resonance imaging (MRI) is limited. In addition to identifying the seizure types, epilepsy can also be diagnosed by identification of an epilepsy syndrome such as EEG changes, brain imaging abnormalities, and genetic analyses (Brodie et al., 2018, Fisher et al., 2017, Richardson et al., 2015).

1.2. Epidemiology of Epilepsy

Epilepsy is one of the most common chronic neurologic disorders, affecting almost 70 million people worldwide. Despite it is a global disease, epilepsy has unequal distribution and about 80% of the affected individual live in low- and middle- income countries. The incidence and prevalence of epilepsy is higher in this area than the rest of the world and this is due to some risk factors such as head trauma, perinatal injury and CNS infection which are more common in poor regions, especially in rural areas(Espinosa-Jovel et al., 2018). Studies in developing countries have reported a prevalence rate ranging from 3.7 to 57 per 100,000 person and 80%-90% of the epileptic patients in this area do not receive treatment at all (Carpio and Hauser, 2009).

Epilepsy is also a common neurological disorder in Ethiopia with estimated prevalence of 5–8/1000 population(Deresse and Shaweno, 2016). Another study conducted in Ethiopia reported higher prevalence which is 29.5/1000(Almu et al., 2006).

1.3. Pathophysiology of Epilepsy

Epileptic seizure arises from an excessively synchronous electrical discharge from group of neurons in the brain and a persistent increase of neuronal excitability is common feature of all epileptic seizures. Although the abnormal cellular discharge can be associated with different factors such as trauma, oxygen deprivation, tumors, infection, and metabolic

derangements, specific causative factors cannot be found in about half of the epileptic patients and it is unclear which factor is responsible for the process of epileptogenesis (Engelborghs et al., 2000). However, different experimental studies have provided insights about postulated mechanisms responsible for the process of epileptogenesis (M Manchishi, 2018)

1.3.1. Neurotransmitters

Alteration in activity of different neurotransmitters in the brain plays a key role in the pathogenesis of epilepsy among which gamma amino butyric acid (GABA) and glutamate are the most common(Werner and Coveñas, 2017).

GABA is the major inhibitory neurotransmitter in the CNS and its inhibition results in epilepsy as evidenced by different experimental epilepsy models(Khazipov, 2016). GABA is formed within GABA ergic axon terminals, where it is discharged into neuronal connections and acts on its receptors, $GABA_A$ and $GABA_B$ located at synaptic membranes. $GABA_A$, controls chloride entry into the cell, and $GABA_B$, increases potassium conductance, decreases calcium entry, and inhibits the presynaptic release of other transmitters. Experimental and clinical studies indicated that GABA has a role in the pathogenesis and treatment of epilepsy because: (i) inhibition of GABAergic function is observed in animal models of epilepsy; (ii) GABA agonists suppress seizure and antagonists induce seizure; (iii) benzodiazepines and barbiturates employed in the treatment of epilepsy are found to enhance GABAergic function; and (iv) drugs that enhance synaptic activity like vigabatrin and tiagabin are potent anti-convulsants (Treiman, 2001).

Glutamate is the predominant excitatory neurotransmitter in the brain and applies its excitatory impact via fast ionotropic, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA),N-methyl-D-aspartate (NMDA),and kainate as well as slow metabotropic glutamate receptors (mGluR). NMDA receptors are more commonly involved in the process of epileptogenesis as proved by the role of NMDA receptor antagonists such as ketamine to suppress seizure in animal models. Currently, of all antiepileptic drugs (AEDs) approved clinically, only felbamate antagonizes NMDA receptors(Barker-Haliski and White, 2015, Huff et al., 2016).

At rest NMDA receptor is blocked by Mg+2 and it is immediately removed by post-synaptic depolarization following glutamate release rendering it permeable to both Na+ and Ca++ and this is important for neurotransmission. The balance of glutamate and GABA is also crucial for the normal functioning of neuronal cells. If excess glutamate is released as in post traumatic injury, the cell membrane remains depolarized leading to increased Ca++ entry into post –synaptic cell causing excess neuronal excitability that can lead to seizure (Guerriero et al., 2015). In addition, changes in metabotropic glutamate receptor activity can also play an important role in causing epilepsy(Engelborghs et al., 2000).

1.3.2. Voltage gated ion channels

Voltage gated ion channels (VGICs) such as sodium, potassium and calcium play a vital part in the process of epileptogenesis as proven by various currently available AEDs targeting these ion channels. Under normal physiology, these ion channels perform various activities controlling normal action potential generation or neuronal excitability. Mutation in these ion channels results in functional change causing hyper-excitability of neuronal cells leading to epileptic seizure(Sancheti and Sathaye, 2013).

Voltage gated sodium channel (VGSC) is a core in the pathogenesis of epilepsy and numerous of the commonly used AEDs like phenytoin, carbamazepine and lamotrigine block this ion channel. Diverse experimental studies demonstrated that abnormal expression or function of VGSCs has a part in the epileptogenesis of both inherited and acquired epilepsy. The presence of several hundred mutations of VGSC gene that lead to inherited epileptic syndrome is the most convincing proof indicating the ion channel plays an important role in the pathogenesis of epilepsy(Köhling, 2002, Mantegazza et al., 2010).

Voltage gated calcium channels (VGCCs) are critical for normal physiology controlling neurotransmitter release, cell excitability and gene expression. The VGCCs can be classified as P/Q, N, L, R (collectively termed high voltage activated (HVA)) and T (collectively termed low voltage activated (LVA)) based on their physical and pharmacological properties. The L-type calcium channel, which produces long-lasting inward Ca^{+2} current, has five polypeptide subunit and calcium antagonists block the $\alpha 1$ subunit. This type of channel is

documented in the area of CNS such as cortex, hippocampus, cerebellum and spinal cord. The N (high-threshold inactivating) and T (low) type calcium channel have also been documented in neurons. Rhythmic firing of neurons is contributed by T-type current where as N or L-type currents are involved in the release of neurotransmitters(Ku³ak et al., 2004).

Like VGSC, VGCCs also play a pivotal role in the process of epileptogenesis as indicated by different experimental models like: (i) kindling animal seizure model in which sub-convulsive electrical stimulation is applied to amygdala, ultimately generates intense limbic and clonic motor seizures; (ii) mutations of genes encoding VGCCs are associated with tonic-clonic and behavioral hall marks of absence seizure; (iii)ablation of VGCCs are also associated with hall marks of epilepsy; and (iv) the availability of AEDs targeted against VDCCs like ethosuximide, which is effective against absence but not partial or generalized tonic-clonic seizure by specifically blocking T-type VGCC(Jones, 2002, Weiss and Zamponi, 2019).

1.3.3. Changes in neuronal networks

Test model of chronic temporal lobe epilepsy (TLE) induced by the chemo-convulsant pilocarpine as well as finding from post-surgical or postmortem human temporal lobe specimens from patients with TLE revealed that there is a disturbance in the glial-neuronal networks which might have a role in epileptogenesis. For example investigations of specimen from patients with TLE revealed that there is an alteration in astrocytes activity(Brennan et al., 2021, Coulter and Steinhäuser, 2015). Dysfunction in the hippocampal neurons could also occur after acute seizure as proved by degeneration of neurons in the hilus of dentate gyrus and cornu ammonis (CA1-CA3) pyramidal neurons and loss of inhibitory GABAergic interneurons(Yin et al., 2013).

1.3.4. Inflammatory Mediators

Investigations using experimental models identified that inflammatory processes have a crucial role in the pathogenesis of epilepsy. In rodent models of epilepsy, pharmacological or electrical stimulation of seizures triggers the release of pro-inflammatory cytokines such as interleukins (IL) 1β, IL-6, tumor necrosis factor (TNF) alpha, and high mobility group box 1 (HMGB1) from glial cells. In addition, in these experimental models, cytokine receptor

expression is upregulated in neurons as well as astrocytes and microglia cells indicating their role in epileptogenesis. In addition to inflammatory cytokines, other inflammatory mediators such as prostaglandins (PGs) are also markedly increased following seizure (Cerri et al., 2017). Analysis of specimen from patients with TLE also indicated that there is up regulation of inflammatory mediators such as vascular endothelial growth factor (VEGF),Intercellular adhesion molecule(ICAM)-1, vascular cell adhesion molecule (VCAM)-1 and interleukins (ILs (5, 7,13, 22,25 and 27) (Kan et al., 2012).

1.4. Management of Epilepsy

Management of epilepsy is not an easy task and it requires a careful identification of goal of therapy, seizure type and frequency and epilepsy syndromes. Depending on these factors one or more of the non-pharmacological, pharmacological and herbal method of management can be employed(Brodie and French, 2000).

1.4.1. Non-pharmacological treatment

Non-pharmacological interventions are used to manage epilepsy in conjunction with or as an alternative to AEDs. The most commonly used interventions are diet, surgery and vagus nerve stimulation(Jackson et al., 2015). Ketogenic diet, a restrictive high-fat, low- protein and very low-carbohydrate has been used as an alternative to AEDS in the treatment of pharmacoresistant epilepsy in adults. In spite of the fact that the precise mechanism of ketogenic diets in controlling of seizure is obscure, serum ketone bodies alter neuronal metabolic status and thought to impact the number and work of neurotransmitters through interaction with receptors, channels and metabolic enzymes (Williams and Cervenka, 2017).

Surgical treatment to abolish seizures has been specially suggested for patients with mesial TLE and neocortical epilepsy(Jackson et al., 2015). Most patients experiencing surgery move forward uniquely when the epileptogenic tissue is resected and the surgical approach chosen depends primarily on the localization and the degree of the epileptogenic zone, MRI findings, the risk–benefit adjust of the respective surgery itself, and preoperative monitoring(Elger and Schmidt, 2008).

The other non-pharmacological strategy of epilepsy management is vagus nerve stimulation which includes discontinuous electrical stimulation of the vagus nerve through a pulse

generator device. It is utilized as an adjunct treatment for intractable partial seizures in adults and children aged 12 years or older. Although the precise mechanism of anti-epileptic effect of vagus nerve stimulation is unknown, change of synaptic activities within the thalamus, changing neurochemistry within the solitary nucleus and modulation of the reticular activating system are some of the hypothesized theories(Lim et al., 2018).

1.4.2. Pharmacological treatment

Right now, there are numerous AEDs utilized to treat epilepsy that act through distinctive mechanism and include (i) increasing GABA-ergic action; (ii) inhibiting excitatory glutamate neurotransmission; (iii) blocking sodium channels during high frequency discharges and (iv) blocking voltage-dependent calcium channels (VDCCs)(Lasoń et al., 2011).

GABAergic drugs

In a wide range of animal models of epilepsy, drugs that enhance GABA mediated inhibition are shown to have anti-convulsant activity and act through in one of the following ways; (i) as GABA agonist (progabide);(ii)enhance synthesis or release of GABA (valproate and vigabatrin)and (iii) through modulating channel opening(benzodiazepines and barbiturates) (Treiman, 2001). Among barbiturates, phenobarbitone is active in almost all preclinical tests used for screening AEDS and it inhibited tonic hind limb extension in MES test, PTZ induced clonic seizure and amygdala kindling(Lasoń et al., 2011). The main indications for barbiturates are generalized tonic-clonic seizures, partial seizures and drug resistant status epilepticus (Hogg and Goodman, 2018).

Benzodiazepines are more effective in PTZ initiated seizure test than MES test. For example clonazepam is extremely potent in PTZ test and nearly all of them have no impact in MES induced seizure test(Lasoń et al., 2011). Benzodiazepines are mostly recommended for treatment of status epilepticus(Hogg and Goodman, 2018). Vigabatrin, which is an irreversible inhibitor of GABA transaminase is indicated as monotherapy for partial epilepsy in adults whereas tiagabine which inhibits GABA transporter (GAT)-1 is indicated as add-on therapy in partial and generalized drug resistant epilepsy(Deckers et al., 2000).

Calcium channel blockers

These groups of drugs are also indicated in the treatment of diverse sorts of seizures. In general drugs that block T-type Ca+2 channels are effective in absence seizure, where as L-type calcium channel blockers are effective against partial seizure(Ku³ak et al., 2004). Drugs used for treatment of epilepsy by blocking calcium channels are ethosuximide and valproate. Valproate is a broad-spectrum AED effective in the treatment of absence, myoclonic, focal, and tonic-clonic seizures(Hogg and Goodman, 2018).

Sodium channel blockers

Sodium channel blockers have been the main pharmacological agents for management of focal and generalized tonic-clonic seizure. They include drugs like phenytoin, carbamazepine, lamotrigine, oxcarbazepine, rufinamide, lacosamide and eslicarbazepine acetate. In addition to focal and generalized tonic-clonic seizure management, lamotrigine is effective against absence seizure. Although phenytoin, carbamazepine, lamotrigine and oxcarbazepine are more likely to cause idiosyncratic reactions, all these AEDs share similar efficacy and adverse effect profile(Brodie, 2017)**.**

Drugs that inhibit excitatory neurotransmission

Another class of AEDs target excitatory neurotransmission in the brain and includes Felbamate and Falampanel. Felbamate has been shown to antagonize NMDA receptor and also potentiates GABA mediated activities in hippocampus. The therapeutic use of felbamate is limited because of its risk of aplastic anemia (Pellock et al., 2006, Pennell et al., 1995). Talampanel is an allosteric inhibitor of AMPA receptor and has no activity at NMDA receptor. In rodent models of epilepsy, the drug suppresses MES and PTZ initiated seizure (Lasoń et al., 2011).

1.4.3. Herbal treatment

In addition to pharmacological treatment, plenty of medicinal plants have been used in traditional medical practices to treat epilepsy across the globe. The use of medicinal plants to

treat epilepsy has been a common practice in the society of many countries like America, India, Iran, China and Africa (Liu et al., 2017).

Several studies in developing countries have reported that large proportions of people with epilepsy do not get treatment they need(Scott et al., 2001). Inspite of the fact that many diagnosed and start on the treatment, they cease before long because of high cost of the treatment, unavailability of AEDs and cultural beliefs(Radhakrishnan, 2009).Numerous communities in Africa moreover associate epilepsy with evil spirits and superstitions, encouraging treatment from traditional healers and religious leaders (Deresse and Shaweno, 2016). In Africa, up to 80% of the population use traditional medicine for primary health care and the global market for herbal medicine is growing steadily (Muazu and Kaita, 2008). Although numerous anti-epileptic drugs are accessible right now, they are associated with severe, life threatening side effects(Sahranavard et al., 2014). Therefore, herbal medicines are becoming the most useful approaches in the treatment of epileptic seizure in both developing and developed countries reducing the complications caused by AEDs (Liu et al., 2017).

Numerous studies have demonstrated the anti-convulsant activity of several medicinal plants. Among the many plants shown to have such activity in animal models, *Moringa concanensis* (Moringaceae)*(*Joy et al., 2013),*Prosopis cineraria Linn Druce*(Mimosaceae)(Velmurugan et al., 2012),*Astragalus* mongholicus(Leguminosae)(Aldarmaa et al., 2010), *Lantana camara* Linn(verbanaceae)(Chinnala et al., 2013),*Desmodium triflorum(*Fabaceae*)(*Bhosle, 2013*), Colebrookea oppositifolia Smith(*Lamiaceae*)*(Viswanatha et al., 2017), *Sphaeranthus indicus (*Asteraceae)(Dighe and Barve, 2019),*Punica granatum* Linn(Punicaceae)(Das and Sarma, 2014), *Indigofera tinctoria* Linn(Fabaceae)(Garbhapu et al., 2011) and *Vitex negundo* Linn (Verbenaceae)(Khokra et al., 2011) can be cited as examples.

1.5. Animal models of epilepsy

The investigation of potential helpful therapeutic agents for treatment of epilepsy requires the utilization of seizure models. To meet the need for new drug development for treatment of epilepsy, several experimental models were developed in which seizure activity is simulated and different treatments are tested. Most of these models are seizure specific. Amongst the many experimental seizure models, pentylenetetrazole (PTZ),maximal electroshock (MES),

kainic acid (KA) and strychnine are the foremost commonly used models in search for new anti-convulsant drugs (Rubio et al., 2010).

PTZ is the most commonly employed seizure model which mirrors different forms of human epilepsy. It is a chemo-convulsant which interferes with the action of GABA by specifically binding to $GABA_A$ receptor, thereby blocking chloride conductance (Sayin et al., 1993). Depending on the dose and routes of administration used, PTZ can simulate generalized tonic-clonic seizure (grandmal) and absence seizure (petitmal) forms of human epilepsy. Timed intravenous infusion test was used by scholars to determine the threshold dose of PTZ used to induce the above types of epilepsy. It was reported that intravenous administration of 50mg/kg induces clonic seizure whereas 90mg/kg is used as a threshold dose for tonic-clonic seizure (Rubio et al., 2010, De Deyn et al., 1992). Based on route of administration chosen, PTZ is administered 15-30 min (Reddy and Rogawski, 2001), 30 min (Löscher et al., 1990) or 60 min (Mandhane et al., 2007) following subcutaneous, intraperitoneal, and oral administration, respectively, of standard drug or test compound to rodents. Following PTZ administration, each animal is placed in individual plastic cage and are observed for convulsive behavior(Chowdhury et al., 2013). The dose and route of PTZ administration have been modified by different investigators (Vogel, 2002).

The major advantage of the model is its capacity to induce both petitmal and grandmal types of seizure (generalized tonic-clonic seizure) based on the dose utilized i.e., graded doses of PTZ can be administered to achieve wanted sort of seizure. Ineffectiveness of routine AEDs like phenytoin, carbamazepine and oxcarbazepine against PTZ initiated seizure is the major drawback related with the model(Ahmadiani et al., 2003, Velisek et al., 1992).

Maximal electro-shock seizure test is a test used to induce hind limb tonic extension through bilateral corneal or transauricular electrical stimulation. This method is thought to be predictive of anti-convulsant drug efficacy against grandmal (generalized tonic-clonic) seizure. In this model an electric current of 50mA for mice and 150mA for rats is applied through corneal or ear electrodes for 0.2 seconds. Upon application of stimulus, rodents display five phases of convulsion: tonic limb flexion, tonic limb extension, clonic convulsions, stupor and recovery or death. The test compound is said to possess anti-convulsant activity, if it protects against the extensor stage of MES convulsion(Afrin et al., 2017). The main advantage of the model is induction of generalized tonic-clonic seizure in animals and plays an extraordinary part in searching drugs with numerous mechanisms of activity. In addition, it is easy to perform the test and requires minimal technical expertise(Castel-Branco et al., 2009). In MES test, animals receive supramaximal electric current which is 5-10 times higher than the individual electrical seizure threshold of the animals. Therefore, anti-convulsant compounds which can increase seizure threshold but not potent enough to raise the seizure threshold above 50 mA for mice and 150mA for rats are missed by this model(Löscher et al., 1991).

KA model of epilepsy is the most extensively studied model used to simulate TLE in humans. KA is an agonist at kainate glutamate receptors and possesses potent convulsant activity. Systemic administration of 12mg/kg i.p or s.c to rats induces convulsion that originates in limbic structure, which then propagates to other brain areas. Studies revealed that KA 20-40mg/kg (i.p) can be used to initiate convulsion in mice. Following i.p administration of the convulsant, rodents display convulsive behavior such as wet-dog shakes, staring, searching and gnawing, leading to hyperactivity, forelimb clonus and tonic-clonic convulsions(Reddy and Kuruba, 2013).

KA model is very simple to be used, does not require sophisticated equipment and regardless of its routes of administration can induce status epilepticus (SE). The major impediment of the model is that, rats appear to show variable sensitivity based on strain, age, sex and weight. In addition, KA is a direct excitotoxic and this makes troublesome to distinguish the neuronal damage whether it is due to the direct excitotoxicity or seizure induced neuronal damage(Ben-Ari et al., 1981).

Strychnine is a competitive inhibitor at all glycine receptors, which is an important inhibitory neurotransmitter in the spinal cord. In this model, test compound or standard drug (diazepam 5 mg/kg) is administered to rodents 1h before administration of strychnine at a dose of 1.75-2.5mg/kg i.p. Occurrence of tonic extensor convulsions and death is observed during a 1 h period(Hunter et al., 1989, Vogel, 2002). An advantage of strychnine as a convulsant chemical is its capacity to be applied either topically or systemically (Zabara, 1992). Invasiveness (application of surgical procedures) , potential impact of anesthetic agents used on behavioral analysis of the animals amid the experiment and difficulty of exact application of the systemically managed convulsant to particular location of the brain is its major confinements (McCandless and FineSmith, 1992).

In addition to the most commonly used seizure models described above, amygdala kindling(Löscher, 2017),pilocarpine(Reddy and Kuruba, 2013), picrotoxin(Kesim et al., 2012) and penicillin(Bostanci and Bağirici, 2007) can also be used to initiate seizure induction in laboratory animals during search for potential anti-convulsant agents.

1.6. Overview of the experimental plant

The genus *Ajuga* is a member of the Lamiaceae family that contains more than 100 species and 50 sub-species distributed over the world(Topçu et al., 2004).It is traditionally used for treatment of distinctive sicknesses and some of them were tested for the specified activity. The most common ailments treated by the genus *Ajuga* include cancer(Pal et al., 2014),depression(Kayani et al., 2016),inflammation(Gautam et al., 2011),diabetes and hypertension(Tahraoui et al., 2007, Boudjelal et al., 2015, El-Hilaly et al., 2021),Alzheimer's disease(AD)(Movahhedin et al., 2016),wound healing(Khalil et al., 2007),pain(anti-nociceptive)(Khanavi et al., 2014),bacterial infections (Rahman et al., 2013, Setif, 2011),helminths infestation and dental problems(Rahman et al., 2016).

Ajuga integrifolia Buch.Ham ex D.Don (synonyms: Ajuga remota Benth, Ajuga bracteosa Wall ex Benth.) (Figure-1) is a shrub that grows broadly in East Africa at an altitude of 1500-3400 m above sea level. It also grows in Saudi Arabia, Yemen , Afghanistan and East Asia (Tafesse et al., 2017) . In Ethiopia, it grows in different parts of the country including

Bale, Gojam, Gondar, Hararghe, Kefa, Shoa, Sidamo, Tigary, Wollo and Dawuro (Seifu, 2017).

A.integrifolia is a herb covered with short hairs and its stem can grow up to 40 cm high. Its leaves are oblanceolate and coarsely toothed and it flowers from late August to October (Seifu, 2017). In Ethiopia, *A.integrifolia* is known by different vernacular names like Armagusa (Afan Oromo) (Kefalew et al., 2015), Akorarach or Tut astil (Amharic) (Degu et al., 2020), Anamuro (Guragegna) (Teka et al., 2020a) and Anamuro (Sidamigna) (Tefera and Kim, 2019).

Ethno-botanical studies conducted in different parts of the world revealed that *A. integrifolia* is utilized for treatment of numerous conditions. For example, in Himalaya's folk medicine, the leaves are used for edema, febrile conditions ,gout, rheumatism and amenorrhea(Singh and Thakur, 2014). In India, one cup of decocted root assorted with honey is administered orally after breakfast to treat malaria (Wangpan et al., 2016). In Kenyan traditional medicine it is utilized for treatment of diabetes (Keter and Mutiso, 2012) and malaria (Njoroge and Bussmann, 2006).

In Ethiopian folk medicine, the plant is used for the treatment of diabetes(Meresa et al., 2017),retained placenta(Giday et al., 2009), malaria(Asnake et al., 2016, Gedif and Hahn, 2003), stomachache(Regassa, 2013, Tilahun, 2018),wound and ameobiasis(Giday et al., 2010),cancer(Tesfaye et al., 2020), diarrhea(Parvez and Yadav, 2010) , liver problem(Teka et al., 2020b), anthrax(Mesfin et al., 2014),hypertension(Getahun, 1976, Teshome et al., 2019), pneumonia(Regassa et al., 2017) and epilepsy(Abera, 2014, Atnafu et al., 2018). The plant parts used for the specified activities are leaves, stem and root (Tafesse et al., 2017).

Some of these traditional uses and the lethal dose (LD50) of the plant have been investigated by different scholars. The acute toxicity study conducted on the aqueous and hydro-alcoholic leaf extract of *A. integrifolia* demonstrated that the LD50 of the plant is greater than 5000mg/kg as the animals experienced no signs of overt toxicity at this dose. It was also reported that leaf extracts have anti-hypertensive(Hailu and Engidawork, 2014), anti-malarial(Nardos and Makonnen, 2017), anti-type I and II HIV(Asres et al., 2001) and anti-Mycobacterium tuberculosis activity (Cantrell et al., 1999). There are also reports that

confirmed anti-epileptic activity of the leaf(Getaneh, 2020) and stem(Qasim et al., 2017) extracts. In addition to the leaf, different extracts of the root of the plant was also reported to possess anti-diabetic activity (Alene et al., 2020, Tafesse et al., 2017).

The leaf of the plant was found to contain different secondary metabolites such as phenols ,flavonoids ,alkaloids ,terpenoids ,carbohydrates and steroids(Tebeje, 2019) .

In addition to the secondary metabolites listed above, the leaf also contains essential oil such as limonene, α-humulene, β-myrcene, elemol, camphene, β-caryophellene, and α-phellendrene(Vohra and Kaur, 2011). From aerial parts (leaf and stem) of the plant different diterpenes such as ajugarin I, ajugarin II, ajugarin IV, ajugarin V and ajugapitin were isolated using high performance liquid chromatography(Coll and Tandrón, 2005). Triterpene, such as, ergosterol-5,8-endoperoxide, with anti-mycobacterium tuberculosis activity was also isolated from the aerial parts (Cantrell et al., 1999). The root of the plant was also investigated and found to contain secondary metabolites such as alkaloids, flavonoids, phenols, glycosides, terpenoids, tannins, saponins and steroids. The bio-active substances found in different parts of the plant might play a predominant role in the effectiveness of the plant in many conditions (Bekeri et al., 2018).

Figure 1: Photograph of *Ajuga integrifolia Buch.Ham ex D.Don* (Photo Credit: Tesfaye Desalegn)

1.7. Rationale of the study

Epilepsy is one of the foremost common neurological disorders, especially in poor zones of the world, and can have a devastating impact on individuals with the disorder and their families. Its burden in low income countries is two-fold more than observed in high income countries(Newton and Garcia, 2012). Epilepsy represents around 0.7% of the overall global burden of diseases measured in Disability adjusted life years (DALYs) and ranks as the 36th leading cause of DALYs globally. Pharmacological treatment is the mainstay of therapy in the management of epilepsy and about 70% of the cases are controlled by currently available AEDs(Espinosa-Jovel et al., 2018). However, poor availability and high cost of the medications are the major problems associated with the conventional AEDs (Randrianarivo et al., 2016) and about half of epileptic patients treated with these drugs develop at least one adverse effect during their treatment with AEDs(de Biase et al., 2019). In addition, although the AEDs can be used for controlling epilepsy in larger percent of the patients, most of them do not prevent or reverse the pathological processes that underlie the disease and 30–40% of patients typically develop pharmacoresistant epilepsy(M Manchishi, 2018, Prunetti and Perucca, 2011).

Many studies have reported that herbal medicines are commonly used for treatment of epilepsy because AEDs fail to control seizure in 30% of the epileptic patients. In addition, patients also take herbal medicine because of economic and cultural factors(Liu et al., 2017). Hence, continuing search for new therapies of plant origin with fewer side effects and better efficacy is important.

Ethno-Botanical studies conducted in many parts of Ethiopia showed that use of herbal medicine for management of epilepsy is also a common practice(Abera, 2014, Atnafu et al., 2018). Although a recent experimental study reported anti-epileptic activity of *A. integrifolia*(Getaneh, 2020);it was limited to acute models and did not quantify the major phytoconstituents. Thus, further studies that bridge theses gaps are required. Hence the present study was initiated to assess the efficacy of the plant in both acute and chronic models as well as quantify major constituents thought to be responsible for the anti-convulsant effect. On this regard, the results of this study could serve as baseline

information for the development of new anti-epileptic drug for pharmacoresistant epilepsy and can give a clue for isolation and identification of active principal that can be used as a lead compound.

2. OBJECTIVES

2.1. General objective

- To verify the anti-convulsant activity of the leaf Soxhlet extracts of *A.integrifolia* in mice and quantify the major secondary metabolites.

2.2. Specific objectives

- ✓ To assess anti- convulsant activity of the extracts in PTZ- induced seizure test.
- ✓ To determine the effect of the extract in MES- induced seizure test.
- ✓ To evaluate the effect of the extract in PTZ- induced kindling model
- ✓ To quantify the major secondary metabolites (flavonoids, phenols and alkaloids) content of the plant.

3. EXPERIMENTAL METHODS

3.1. Drugs and chemicals

Pentylenetetrazole(Sigma Aldrich, Germany), gallic acid(Merck, Germany)and Folin Ciocalteu reagent was obtained from the Department of Pharmacology and Clinical Pharmacy. NaOH(Loba Chemie, India),AlCl3(Loba Chemie, India),Chloroform(Loba Chemie, India), HCl (BDH laboratory supplies, England),citric acid(Avonchem,UK),Na2HPO4(BDH laboratory supplies, England), Atropine(BDH chemicals, England),Quercetin Dihydrate (Sigma Aldrich, Germany) were obtained from the Department of Pharmacognosy and Pharmaceutical chemistry.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 the drugs and chemicals used were of analytical grade.

3.2. Experimental Animals

Healthy male Swiss albino mice (8-10 weeks, 22-28g) were used for the current study. The animals were obtained from the Ethiopian Public Health Institute as well as, the animal unit of School of Pharmacy, College of Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia. The animals were housed in group of six and acclimatized to laboratory conditions for a week before starting of the experiment. The mice were kept under standard environmental condition (12h light/ dark cycle) and provided with a commercial food pellet and water *ad libtum*. All procedures and techniques used in this study were conducted in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (Council, 2010). The protocol was approved by IRB of the School of Pharmacy on December 03, 2019 with approval number of RRB/SOP/199/2019.

3.3. Collection of plant material

Leaves of *A.integrifolia* were collected from its natural habitat in Girar Jarso district located 180 km north of Addis Ababa, Oromia region, North Ethiopia. Collected plant specimen was identified & authenticated by a taxonomist, Mr. Melaku Wondafrash at the National

Herbarium, College of Natural and Computational Sciences, Addis Ababa University and a voucher specimen (TD001) was deposited for future reference.

3.4. Preparation of the extracts

The collected leaves were washed thoroughly with tap water to remove dirt and soil and shade dried at room temperature for three weeks. The air dried leaves were subjected to size reduction using mechanical grinder to get a coarse powder. The powdered plant material (500g) was extracted by successive Soxhlet extractor (Pyrexquickfit, UK) using 4 liters of each of the following solvents; n-hexane, ethyl acetate and methanol. After extracting with the above solvents, the remaining residue was macerated with one liter of distilled water three times for 72 h with occasional shaking to get the residual aqueous extract. Each time before extracting with the next solvent, the powdered plant material was air dried over night. The resulting solution was first filtered by cotton gauze for aqueous extract and later by 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 extract was freeze dried by using a lyophilizer(Korea vacuum limited, Korea) to obtain the respective extracts. The yield (w/w) in terms of dry material for n-hexane, ethyl acetate, methanol and water was 2.95%, 4.4%, 15.45%, and 3.29% respectively. The dried extracts were kept in a refrigerator at -20°C until use.

3.5. Grouping and dosing of animals

The animals were randomly assigned in to five groups for each solvent extracts, each group containing six mice. In the acute model: group I served as negative control and treated with the vehicle used for reconstitution (2% Tween80 for the non-aqueous extracts and distilled water for the aqueous extract). Group II was a positive control and treated with sodium valproate 200 mg/kg (SV200) for PTZ model and Phenytoin 25 mg/kg (PHY25) for MES. Group III-IV were test groups and administered with 100mg/kg, 200mg/kg and 400mg/kg doses of the respective extracts. The most active extract (ethyl acetate extract) was investigated in the chronic model of epilepsy (PTZ kindling) using the same grouping (SV200 was used as standard). All animals were administered orally and the maximum volume used was 10ml/kg. The doses were determined by a pilot study conducted before commencement of the experiment.

3.6. Anti-convulsant activity test

Anti-convulsant activity of the plant was evaluated using acute and chronic models of epileptic seizures.

3.6.1. PTZ induced seizure

For this test, a method modified by (Salem et al., 2019) was used. The mice in each group received different doses of extracts, standard drug and vehicle. After 60 min of oral administration as described in section 3.5, freshly prepared PTZ in normal saline (80mg/kg) 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 recording. Fore limb or hind limb clonic seizure (Figure 2) was taken as endpoint. The latency to clonic convulsion and percentage protection of mortality were recorded and compared with negative controls. Percentage protection of mortality was calculated as follows.

$$\text{Percentage protection of mortality} = \frac{\text{No of death in control} - \text{No of death in test/standard x100}}{\text{Number of death in control}}$$

Figure 2: Clonic seizure exhibited after 80mg/kg subcutaneous injection of PTZ.

3.6.2. MES induced seizure

For this experiment a method described by (Raza et al., 2001) was used. After an hour of oral administration as described in section 3.5, seizure was induced by auricular stimulation (50mA, 150 Hz, 0.2 sec) 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 electric current, the animals exhibited various phases of tonic-clonic seizure that include immediate short-lived flexion of fore limbs followed by extension of hind limbs. After the end of the extensor phase, they showed stupor phase that finally led to recovery or death. Duration of HLTE (i.e. outstretching of the animals 180 ° to the body axis) and protection against mortality were recorded.

Percentage protection of mortality was calculated as follows:

$$\text{Percentage protection of mortality} = \frac{\text{No of death in control} - \text{No of death in test/standard x100}}{\text{Number of death in control}}$$

In this experiment reduction in the mean duration of HLTE of MES convulsion was considered as having anti-convulsant activity(Mahendran et al., 2011).

3.6.3. PTZ induced Kindling

One hour after administration as described in section 3.5, mice were kindled with repeated (every 48 h) intraperitoneal administration of freshly prepared PTZ (35mg/kg) for 13 days. On each day animals were closely observed for 30 min using a video recorder after PTZ injection to measure intensity of seizure. The following seizure scores were used to identify fully kindled mice: Stage 0(no response); stage 1(hyperactivity, ear and facial twitching); Stage 2(head nodding and myoclonic body jerks); stage 3(fore- limb 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 score were thought to be kindled. Animals that did not show three consecutive stage 4 or 5 were considered to be protected (Abdel-Zaher et al., 2017).

3.7. Quantification of phytochemical constituents

3.7.1. Quantification of total flavonoids content

Total flavonoids content (TFC) was estimated as described by(Chang et al., 2002) with slight modification. Ten mg of the ethyl acetate extract (EA) was dissolved in methanol to prepare a stock solution of 1mg/ml. One ml of the stock solution was transferred to a test tube and mixed with 0.1ml of 10% aluminum chloride, 0.1ml of 1M potassium acetate and 2.8ml 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, England). Quercetin was used to construct the standard curve. It was dissolved in methanol and a serial dilution was used to prepare 1.5625, 3.125, 6.25, 12.5 and 25µg/ml standard solution. The same procedure was followed to prepare the blank solution. All experiments were performed in triplicate. The TFC of the extract was expressed as mg of quercetin equivalent per gram of the dry weight of the extract and calculated as follows;

$$TFC = \mathrm{CV/M}$$

Where C is the sample concentration (µg/ml) from the calibration curve, V is the volume of methanol used to dissolve the extract and M represents the mass of the dry extract used in gram.

3.7.2. Quantification of total phenolic content

Folin-Ciocalteu reagent was utilized for this assay. Folin reagent (1ml of 2N) was diluted with 20ml of distilled water. To determine the total phenolic content (TPC), 1ml of the prepared EA solution (250µg/ml) was transferred to a test tube and 0.5ml of Folin reagent was added and allowed to stand for 8 minutes. Thereafter 2ml of 7.5% sodium carbonate in distilled water was mixed with the solution and incubated for 30 min at ambient temperature. An absorbance was later recorded at 765nm by a UV-spectrophotometer and compared with gallic acid calibration curve. Gallic acid was prepared in different concentration of 3.125, 6.25, 12.5, and 25µg/ml by serial dilution to draw the standard curve. The same procedure was followed to prepare gallic acid and the blank solution. All experiments were conducted in triplicates and the average value was taken. The TPC was described as gallic acid equivalent per gram of sample(Maria et al., 2018).

3.6.3. Quantification of total alkaloids

Total alkaloid content (TAC) of the plant was determined according to a method described by (Tabasum et al., 2016) with slight modification. The reaction that took place between alkaloid and bromocresol green (BCG) was used to quantify the total alkaloid content by spectrophotometric method. Accordingly, 2ml of plant extract in methanol (1mg/ml) was dissolved in 2ml of 2N HCl and filtered. One ml of this solution was transferred to a separating funnel and washed with 5ml of chloroform twice. pH of the solution was adjusted to neutral by adding 0.1N NaOH. After pH adjustment, 5ml of BCG solution (prepared by heating 69.8 mg of BCG with 3ml of 2 N NaOH and 5ml of distilled water and then diluted to 1000ml with distilled water) along with 5ml of phosphate buffer (prepared by adjusting the PH of 2M sodium phosphate (71.6 gm of Na_2HPO_4 in 1L distilled water) to 4.7 with 0.2 M citric acid (42.02 gm) citric acid in 1 L distilled water) was added. The aggregate formed was shaken and the mixture formed was extracted with 5ml of chloroform twice by vigorous shaking. The extract was collected in 10ml volumetric flask and the volume was made up to 10ml with chloroform. The absorbance of the mixture in chloroform was measured at 470nm by using a UV-spectrophotometer. For standard curve construction, 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. One ml of this solution was taken and transferred to a separating funnel and 5ml of phosphate buffer along with 5ml BCG solution was added and shaken gently with 5ml of chloroform twice. The mixtures formed was collected in 10ml volumetric flask and diluted to the volume with chloroform and absorbance was measured at 470nm. The blank was prepared as described without atropine. The assay was run in triplicates and the average value was taken.

3.8. Data Analysis

All experimental data are expressed as mean ± SEM and subjected to statistical analysis by SPSS 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 Benferroni's post hoc test was used for multiple comparison of the mean difference between the groups. The analyses were performed with 95% confidence interval and the significance was set at $p<0.05$.

4. RESULTS

4.1. Anti-convulsant activity in PTZ induced seizure

All *A.integrifolia* leaf solvent extracts but the aqueous extract (AA) possessed anti-convulsant activity against absence (petitmal) seizure model of epilepsy as evidenced by an increased mean latency to clonic convulsion (Table 1). EA was the most effective extract, as it prolonged the mean onset of clonus and decreased percent mortality better than the other extracts. The mean latency to clonic seizure was significantly increased ($p<0.01$) with all doses of EA compared to controls, with maximum effect (13.17min) achieved by EA 400mg/kg (EA400). The increment in mean onset of clonus was in a dose dependent manner as EA400 significantly delayed onset of clonus compared to EA200 mg/kg (EA200) ($p<0.01$) and EA100 mg/kg (EA100) ($p<0.001$). EA also decreased percent mortality in a dose- dependent manner, with maximum protection (66.67%) conferred by EA 400 (Table 1)

The hexane extract (HA) also significantly increased mean latency to clonic seizure compared to control, with a maximum increase (12.67min, $p<0.001$) displayed by HA400 mg/kg (HA400). The effect produced by HA400 was significantly greater than the one produced by HA 100 mg/kg (HA100) ($p<0.001$) and HA200mg/kg (HA200) ($p<0.01$). As regards to mortality, whilst HA200 and HA400 were able to decrease death by 33.33% and 50%, respectively, HA100 was devoid of any effect (Table 1).

The methanol extract (MA) at 100 mg/kg (M100) was not effective in both parameters of this experimental paradigm. However, the MA, at dose of 200mg/kg (MA200) and 400mg/kg (MA400) significantly increased ($p<0.001$) mean latency and fairly well protected mortality compared to controls. By contrast, the AA was devoid of any effect at all doses (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.

Table 1. Anti-convulsant activity of the Soxhlet leaf extracts of *Ajuga integrifolia* in pentylenetetrazole induced seizure

Group	**Mean latency to clonic seizure (min)**	**Percentage protection of mortality**
CTN	3.00±0.447	-
EA100	6.33±0.333^{a2b3d3e3}	33.33
EA200	10.17±0.543^{a3b3c3e2}	50.00
EA400	13.17±0.703^{a3b2c3d2}	66.67
SV200	16.67±0.494^{a3}	83.33
CTN	3.00±0.447	-
HA100	5.17±0.307^{a1b3d3e3}	0.00
HA200	9.33±0.494^{a3b3c3e2}	33.33
HA400	12.67±0.667^{a3b3d2}	50.00
SVP200	16.67±0.494^{a3}	83.3
CTN	3.00±0.447	-
MA100	4.84±0.477^{b3d3e3}	0.00
MA200	7.50±0.619^{a3b3c2e2}	16.67
MA400	10.00±0.577^{a3b3c3d2}	33.33
SV200	16.67±0.494^{a3}	83.33
DWC	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^{a3c3d3e3}	83.33

Values are expressed as mean ±SEM. (n=6 mice) [a]compared to control, [b] compared to sodium valproate, [c] compared to 100mg/kg, [d] compared to 200mg/kg, [e] compared to 400mg/kg. [1] $p<0.05$, [2] $p<0.01$, [3] $p<0.001$. CTN: group treated with 2% tween80, CDW: group treated with distilled water, 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.

4.2. Anti-convulsant activity in MES induced seizure

As it can be observed from Table 2, all leaf solvent extracts of *A.integrifolia* except the aqueous extract (AA) produced variable results in reducing the mean duration of HLTE and percent mortality in MES test. The EA extract 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 EA compared to controls, with maximum reduction (6.33sec) achieved by EA400. The reduction in mean duration of HLTE was in a dose dependent manner as EA400 significantly decreased the duration of HLTE compared to EA200 ($p<0.01$) and EA100 ($p<0.001$). EA also decreased percent mortality, with maximum protection (50%) achieved by EA200 and EA400 (Table 2).

The hexane extract (HA) also significantly decreased mean duration of HLTE compared to control, with maximum reduction (8.17 sec, $p<0.001$) obtained by HA400. The effect displayed by HA400 was significantly greater than the one produced by HA100 ($p<0.05$) and HA 200mg/kg (HA200) ($p<0.001$). Concerning mortality, HA200 and HA400 decreased death by 16.67% and 33.33%, respectively, while HA100 was devoid of any effect.

MA100 was not effective in both parameters used to evaluate anti-convulsant activity of the plant in MEs test. However, MA200 and MA400 significantly reduced ($p<0.001$) the mean duration of HLTE and equally resulted in percent protection of mortality (16.67%) compared to controls. By contrast, the AA was devoid of any effect at all doses (Table 2).

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

Table 2. Anti-convulsant activity of the Soxhlet leaf extracts of *Ajuga integrifolia* in MES induced seizure.

Group	**Mean duration of HLTE(sec)**	**Percentage protection from mortality**
CTN	18.50±0.563	-
EA100	14.33±0.964^{a3b3d3e3}	33.33
EA200	9.67±0.715^{a3b3c3e2}	50.00
EA400	6.33±0.422^{a3b3c3d2}	50.00
PHY25	0.00^{a3}	83.33
CTN	18.50±0.563	-
HA100	16.67±0.558^{a1b3d2e3}	0.00
HA200	13.00±0.516^{a3b3c2e3}	16.67
HA400	8.17±0.477^{a3b3}	33.33
PHY25	0.00^{a3}	83.33
CTN	18.50±0.563	-
MA100	16.50±0.671^{b3d1e3}	0.00
MA200	14.00±0.73^{a3b3e3}	16.67
MA400	10.17±0.307^{a3d3}	16.67
PHY25	0.00^{a3}	83.33
DWC	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^{a3}	83.33

Values are expressed as mean ±SEM. (n=6 mice), [a] compared to control, [b] compared to Phenytoin, [c] compared to 100mg/kg, [d] compared to 200mg/kg, [e] compared to 400mg/kg. [1] $p<0.05$, [2] $p<0.01$, [3] $p<0.001$. CTN: group treated with 2% tween80, CDW: group treated with distilled water, PHY: phenytoin, 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.

4.3. Anti-convulsant activity in PTZ kindling Model

The most active extract (EA) in the acute seizure model also showed anti-convulsant activity in the chronic seizure model (Figure 3). For this test, 13 injections of PTZ (35 mg/kg i.p) on alternate day basis produced full kindling starting from the 11^{th} -13^{th} injections in controls, while treatment of mice with different doses of EA extract produced variable effects. EA400 significantly protected ($p<0.001$) the animals from developing consecutive stage 4 and/ or 5 seizure on the last three injections compared to controls, which indicated protection of the animals. Likewise, EA200 also produced significant effect ($p<0.01$) in protection of kindling compared to controls. On the other hand, EA100 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 ($p<0.001$) occurrence of kindling compared to controls.

As it can be observed from Figure 3, the EA extract reduced 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 still seen between extract and SV pretreated group. Difference in stage of seizure after PTZ injection between treatment groups was observed from the third injection onwards.

As the number of injection increased, significant difference in mean seizure stage between EA100 and negative control group started to disappear. Indeed no detectable difference in mean seizure stage was noted between the two groups from the 9^{th} -13^{th} injections. This indicated failure of EA100 to protect the animals from PTZ induced kindling. EA400 pretreated group started to respond to the chemoconvulsant PTZ from the 6^{th} injection but no significant effect was seen between EA400 and SV200 until the 9^{th} injection.

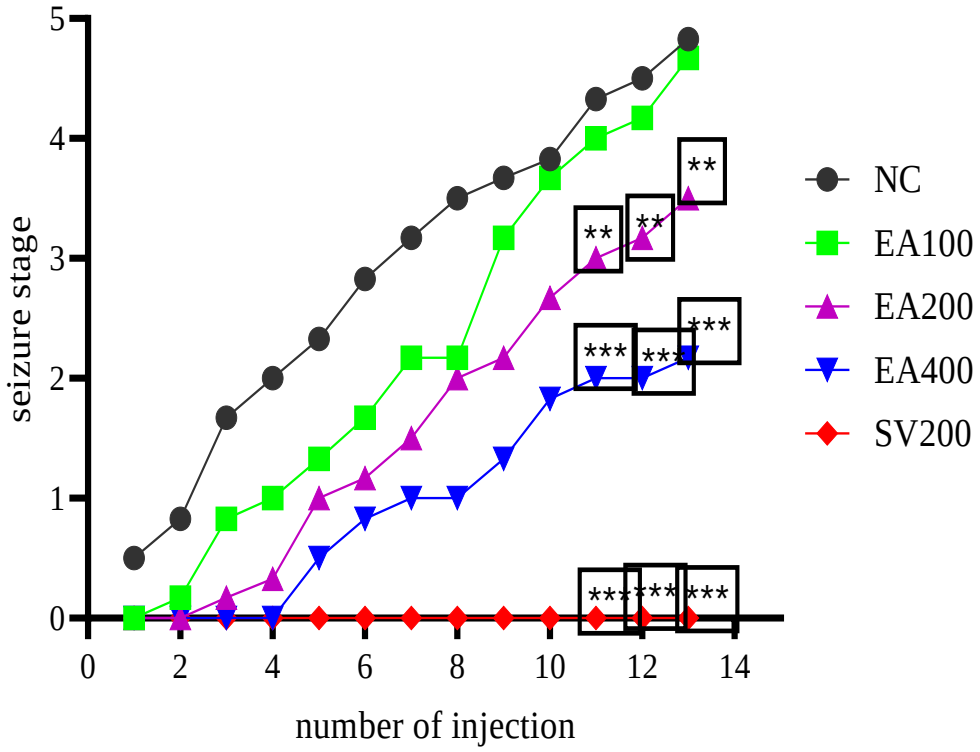

Figure 3: Effect of Ethyl acetate extract on Pentylenetetrazole induced kindling in mice. Animals were treated with 100mg/kg, 200mg/kg and 400mg/kg ethyl acetate extract and 200mg/kg sodium valproate along with PTZ 35mg/kg i.p every other day for 13 days. Data expressed as mean± SEM of 22 days observations. ** $p<0.01$, *** $p<0.001$.

4.4. Quantification of Secondary Metabolites

4.4.1. Determination of Total flavonoids content

The assay performed to determine TFC using the standard produced a curve with a regression coefficient ($R^{2)} =0.9964$, slope (m) =0.02432 and y-intercept= 0.0159 (Figure 4). The extract was found to contain 9.045 ±0.8445 mg of quercetin equivalent (QE) of flavonoids per gram of dry extract used.

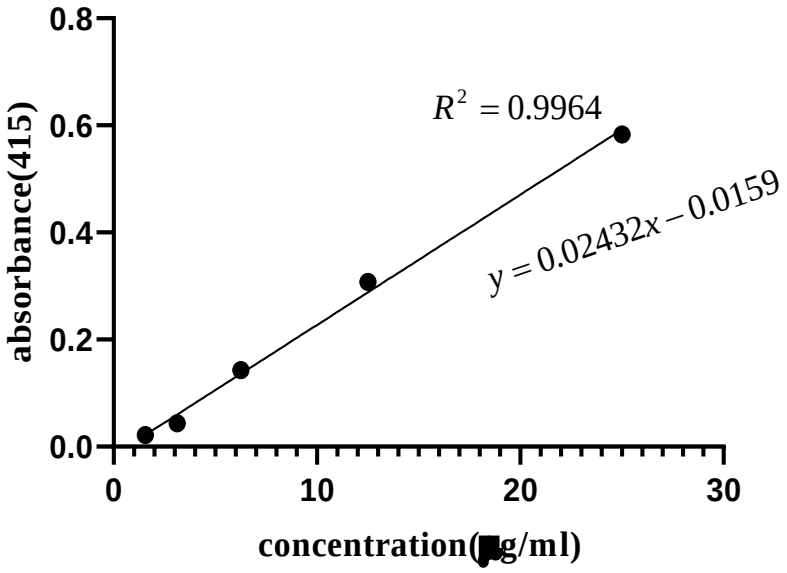

Figure 4: Standard curve constructed for quercetin based on absorbance measured at 415 nm. Data are expressed as mean ±SEM, n=3.

4.4.2. Determination of total phenolic content

The experimental study conducted to determine the TPC using gallic acid produced a standard curve with (R^2)= 0.9982, slope(m)=0.009323 and y-intercept=0.005289 (Figure 5). The EA extract was found to contain 21.928 ±1.118 mg of gallic acid equivalent (GAE) per gram of dry extract utilized.

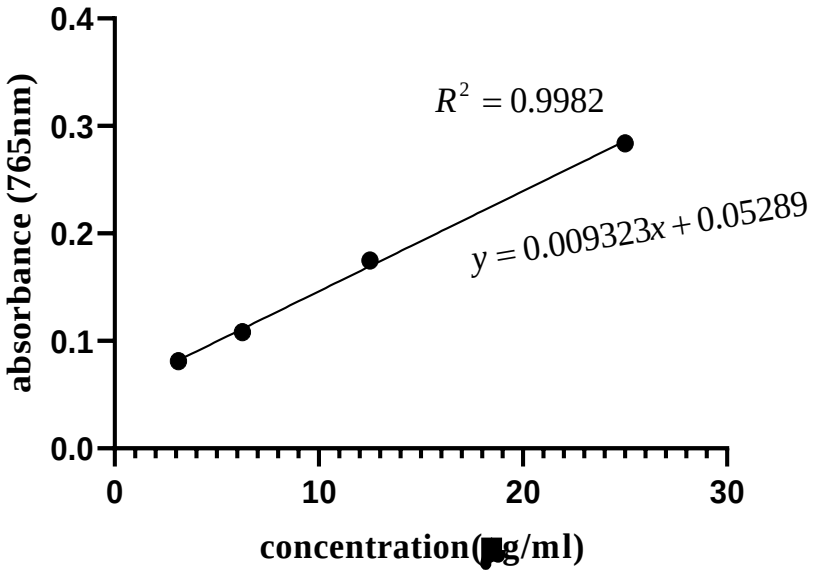

Figure 5: Standard curve constructed for gallic acid based on absorbance measured at 765 nm. Data are expressed as mean ±SEM, n=3.

4.4. 3. Quantification of Total alkaloid content

The assay conducted to determine TAC of the EA extract using atropine produced a standard curve with (R^2) =0.9938, slope (m) =0.002625 and y-intercept=0.001750 (Figure 6). The extract was found to contain 10.002±0.119 mg atropine equivalent per gram of dray extract used.

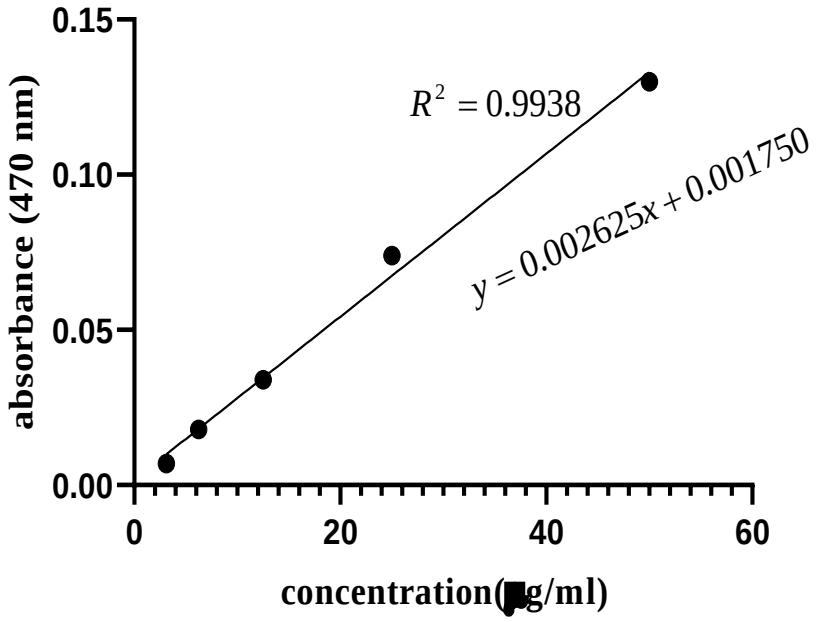

Figure 6: Standard curve constructed for atropine based on absorbance measured at 470 nm. Data are expressed as mean ±SEM, n=3.

5. DISCUSSION

In Ethiopian traditional medicine, the dried leaf of *A.integrifolia* is pounded and mixed with nut oil and the patient is informed to eat it to treat epilepsy. The dried leaves of the plant were therefore extracted with organic solvents of different polarity and oral dosing of the extract was used to replicate the traditional use.

Male mice were used for the anti-convulsant activity testing because female mice are less sensitive to the convulsive activity of MES and PTZ due to the effect of estrus cycle on seizure threshold (Medina et al., 2001, Dai et al., 2014, Tandon and Gupta, 2005, Kaboutari et al., 2012).

MES and PTZ which were developed long years ago have been used as a cornerstone in the early search of potential anti-convulsant drugs. Since their development, many compounds have been tested against MES and PTZ in laboratory animals for anti-epileptic activity. These two models are the most commonly employed seizure models because they are rapid and easy to perform for the preliminary screening of potential anti-convulsant drugs (Swinyard, 1949).

In the present study, solvent leaf extracts of *A.integrifolia* were found to possess anti-convulsant activity in both acute and chronic seizure models that appeared to vary with dose and nature of the extract. EA extract produced the highest effect in both parameters used in the acute PTZ experimental paradigm among all solvent extracts. The anti-convulsant activity demonstrated by the EA extract could be attributed to the presence of secondary metabolites such as flavonoids, phenols, alkaloids, terpenoids and steroids, which were reported by earlier studies(Tebeje, 2019) as well as by the present study. A similar type of study done on crude stem extract and solvent fractions of *A.integrifolia* reported anti-convulsant activity of the plant in PTZ model of seizure. Although ethyl acetate fraction was the most effective among all fractions, the effect produced in terms of increasing mean onset of clonic seizure by the higher dose of the fraction (EA 1000mg/kg,7.875 min)(Qasim et al., 2017) was lower than the one produced by the most effective extract(EA400mg/kg, 13.17min) of the current study. The better anti-convulsant activity obtained by the present

study could be because of better accumulation of secondary metabolites such as terpenoids in leaves than stem and the difference in geographical location(Li et al., 2020)

The hexane extract ranked next to EA extract in increasing the mean latency to clonic seizure and percent protection of mortality in PTZ induced seizure test. In line with the test result, the anti-convulsant activity displayed by HA in terms of the parameters used, was lower than the one displayed by the EA extract. It was reported that the leaf hexane fraction of *A.integrifolia* did not contain the major phytoconstituents such as phenols and flavonoids(Dechasa, 2019). Therefore, the reduced anti-convulsant activity of the extract could be because of the absence of the indicated phytoconstituents.

Methanol extract showed the least anti-convulsant activity among all solvent extracts. The lowest dose of the extract was unsuccessful in delaying the mean onset of clonus and protecting the animals from death though the medium and highest dose displayed anti-convulsant activity. The absence of steroids in the methanol extract, as it was reported, might contribute for the reduced anti-convulsant activity(Tebeje, 2019). At any dose the aqueous extract did not produce a considerable effect in delaying onset of clonic seizure and increasing percent protection of mortality. This might because of reduced concentration of secondary metabolites in aqueous extract as studies showed the yield of secondary metabolites such as alkaloids, phenols and flavonoids increases with non-polar solvents than polar solvents(Bouterfas et al., 2016).

A previous similar type of study done on the leaf crude extract and solvent fractions of the plant collected from different geographical location, Ghimbi district, reported anti-convulsant activity of *A.integrifolia* in PTZ model(Getaneh, 2020). In this study, the mean latency to clonic seizure exhibited by the most active solvent fraction (butanol 400 mg/kg) was greater (15.51min) than the most active extract (EA 400 mg/kg, 13.17min) in the current study. The subtle difference might have emanated from variation in the geographical location(Borges et al., 2017).

The present study also evaluated anti-convulsant activity of the plant in MES model. Once again, EA extract displayed the highest reduction in the mean duration of HLTE than HA and MA extracts which might be because of accumulation of sufficient concentration of the phytoconstituents in the EA extract. Like in the PTZ test, the AA extract did not produce an appreciable effect in reducing the mean duration of HLTE may be because of absence of active metabolites.

It was reported that crude leaf extract and solvent fractions *A.integrifolia* possessed anti-convulsant activity in MES test(Getaneh, 2020)**.** However, the highest dose of the most active extract of the current study (EA400 mg/kg) produced a significant effect in reducing the mean duration of HLTE (6.33 sec) compared to the one displayed by its corresponding reported butanol fraction (400 mg/kg, 8.33 sec). The difference in the effect produced could emanate from the type of extract used indicating less non-polar components were responsible for the anti-convulsant activity of the plant in MES test.

MES and PTZ, which are only models for acute seizure, cannot simulate chronic dysfunction of brain which is seen in epilepsy and cannot be used for discovery of potential AEDs for pharmacoresistant epilepsy. Therefore, kindling is widely employed to study the process of epileptogenesis and anti-epileptic drug discovery(Löscher, 2002). Thus the present study also investigated the effect of the most active extract (EA) of *A.integrifolia* in chronic model of epilepsy and it dose dependently prevented the occurrence of consecutive stage 4 and/ or stage 5 seizure though the lowest dose failed to do so. During the initial phases of the kindling process the lowest dose prevented the animals from developing maximum stages of seizure which only lasted for a short time. This might be because of repeated administration of PTZ caused its accumulation in the brain resulting in prolonged antagonism of GABA making the lowest dose ineffective to restore the activity of GABA(Corda et al., 1990).

Though 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 because of their activity against PTZ and MES models. In fact, some of the

conventional anti-epileptic drugs like valproate, felbamate, topiramate and benzodiazepines have been effective against both seizure models with broad spectrum anti-epileptic activity(Rogawski and Löscher, 2004, Swinyard et al., 1986). This might indicate that the study plant contains different phytoconstituents acting through different mechanisms of action. Even if at this point we are uncertain to identify specific phytoconstituents responsible for the anti-convulsant activity of the plant, the presence of plant derived terpenoids, as reported in India and Iran(Kasture et al., 2002, Sayyah et al., 2002), might have played a major role. Therefore, the anti-convulsant activity of the plant could emanate from different types of terpenoids it contained(Kuria et al., 2002).

In addition to terpenoids, the presence of phenols perhaps contributed for the anti-convulsant effect produced by the different solvent extracts. As reactive oxygen species play a major role in the pathogenesis of epilepsy, the anti-oxidant activity of phenolic compounds as reported by different investigators(Foti, 2007, Nugroho et al., 2012) could have played a major role for the anti-convulsant effect exhibited by the study plant. In expansion to anti-oxidant activity, studies also identified that, plant derived polyphenolic compounds have anti-convulsant effect against PTZ induced seizure by increasing brain level of GABA (Dhingra and Jangra, 2014). Different studies also indicated the role of alkaloids in the treatment of epileptic seizure. Plant derived alkaloids have been found to possess anti-convulsant activity by blocking Na^+ channels. As a result, the presence of these alkaloids in the leaf extracts of the plant(Ameri et al., 1997) could also be a major contributors for the produced anti-convulsant effect. It was reported that a steroidal compound known as Ergosterol-5,8-endoperoxide was isolated from the leaf part of *A.integrifolia (*Cantrell et al., 1999) and studies revealed that steroidal compounds have anti-convulsant effect against MES (Li et al., 2016) as well as PTZ induced seizure(Aliyu et al., 2014). Therefore, the anti-convulsant activity demonstrated by the study plant could be because of this steroidal compound present in the different solvent extracts.

In addition to the above phytoconstituents, flavonoids identified in the leaves of the plant possibly contributed for the anti-convulsant activity obtained in different seizure models; because studies showed that flavonoids possess anti-convulsant activity(Citraro et al., 2016). The ubiquitous plant derived polyphenolic flavonoids were identified as one of compounds

with $GABA_A$ modulatory activity(Hanrahan et al., 2011). Therefore, flavonoids present in the different extracts could be responsible for the anti-convulsant activity obtained by enhancing $GABA_A$ mediated activity.

Studies indicated that chronic administration of PTZ increases production of reactive oxygen species in different regions of the brain, which could be a mechanism for development of epileptic seizure(Samokhina and Samokhin, 2018). Investigation of the leaf of *A.integrifolia* for its anti-oxidant activity by using 1,1-diphenyl-2-picrylhydrazyl (DPPH) revealed the plant's ability to scavenge free radicals by decreasing the concentration of DPPH by 50%(Nasser et al., 2010). Therefore, this anti-oxidant activity could be a potential mechanism to protect the animals against PTZ induced kindling.

In addition to the anti-convulsant activity testing, determination of the major secondary metabolites of the most active extract, ethyl acetate, revealed that it contained 21.928 ±1.118 mg GAE/ g, 9.045 ±0.8445 mg QE/g and10.002±0.119 mg atropine equivalent (AE) per gram of dry weight of extract of phenols, flavonoids and alkaloids respectively. Methanolic extract of aerial parts of the plant is reported to contain 5.94±1.98mg GAE/gram and 1.98 ±0.06mg QE/ gram of dry extract of phenols and flavonoids, respectively(Kayani et al., 2016). The total phenolic and flavonoid content determined in the current study was greater than the one reported by previous study done in Pakistan. The difference in the total content of these major phytoconstituents could be because of difference in the geographical factors and type of solvent used as studies indicated that the use of non-polar solvent increases the yield of total phenols and flavonoids(Bouterfas et al., 2016).

6. LIMITATION OF THE STUDY

The major limitation of the current study is lack of measurement of the plant's solvent extracts effect on EEG component of seizure, which is used to measure change in electrical activity that occur in the brain during epileptic seizure. The other limitation is lack of measurement of neurochemical changes (for example enzyme levels like superoxide dismutase (SOD)) that could take place in the brain of the animals after administration of plant extracts that would have been useful in indicating mechanism of action of the plant's anticonvulsant activity. In addition, inability to perform behavioral analysis after kindling process because of scarcity of resources is another limitation of the study as PTZ kindling can result in cognitive impairment, hippocampal neuronal degeneration and oxidative stress. Finally, parameter recording during anti-convulsant activity testing which was carried out manually could also be a limiting factor as it can be a source of bias.

7. CONCLUSION

The outcome of the present study provides support for the traditional use of Ajuga integrifolia as anti-convulsant medicinal plant as the leaves of the plant's solvent extracts displayed anti-convulsant activity in both acute and chronic seizure models. The results of this study suggest that semi-polar to non-polar components are responsible for anti-convulsant activity of the plant while polar components are found to be devoid of anti-convulsant activity.

8. SUGGESTION FOR FUTURE WORK

Anti-convulsant activity of the plant is promising; therefore, the following works are recommended to be done in the future to make it clinically useful;

- ✓ The effect of plant extracts on EEG component of epileptic seizure needs to be evaluated
- ✓ Isolation of pharmacologically active principles responsible for the displayed anti-convulsant activity should be done
- ✓ Further studies should be done to elucidate the mechanism of action involved.
- ✓ More studies need to be done to analyze behavioral changes that can be seen after PTZ kindling.

It is also good if another study is conducted to test anti-convulsant activity of roots of the plant.

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