Evaluation of the anticonvulsant activity of 80% methanol leaf extract and solvent fractions of *Buddleja polystachya Fresen. (Buddlejacea)* in Mice

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A thesis submitted to the Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, College of Health Sciences

Presented in partial fulfilment of the requirements for the Degree of Master of Science in Pharmacology

March, 2018

Addis Ababa University

Addis Ababa, Ethiopia

Addis Ababa University

School of Graduate Studies

This is to certify that the thesis prepared by Tewodros Agedew, entitled “Evaluation of the anticonvulsant activity of 80% methanol leaf extract and solvent fractions of *Buddleja polystachya Fresen. (Buddlejacea)* in Mice" and submitted in partial fulfillment of the requirements for the Degree of Master of Sciences in Pharmacology complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Abstract

Evaluation of the anticonvulsant activity of 80% methanol leaf extract and solvent fractions of *Buddleja polystachya Fresen. (Buddlejacea)* in mice.

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Addis Ababa University, 2018

Buddleja polystachya is used in Ethiopian traditional medicine for the treatment of epilepsy. Since this claim has not been investigated scientifically, this study was conducted to evaluate the anticonvulsant activity of 80% methanol leaf extract and solvent fractions of *Buddleja polystachya* in mice. The leaves of plant were extracted using maceration technique; butanol and chloroform were used for fraction. Anticonvulsant activity was evaluated by using maximal electroshock (MES) and Pentylenetetrazol (PTZ) model. Motor coordination effect was assessed by using rotarod test. Mice were randomly assigned to five groups (n=6 per group). The test groups received 100 mg/kg, 200 mg/kg and 400 mg/kg of cure extract and solvent fraction. The positive control groups received phenytoin 25mg/kg for MES test, valproate 200mg/kg for PTZ test and diazepam 5mg/kg for rotarod test. The negative control groups received distilled water or 2% Tween80, 10 ml/kg. The crude extract exhibited significant anticonvulsant effect in both MES test ($p<0.001$ for all doses) and PTZ test ($p<0.001$ for 200 mg/kg and 400 mg/kg, $p<0.05$ for 100mg/kg) compared with control. A similar effect was observed with butanol fraction in both models. Whereas, the chloroform fraction showed significant ($p<0.001$) anticonvulsant effect relative to control only in PTZ test at doses of 200mg/kg and 400mg/kg. The aqueous fraction was devoid of anticonvulsant activity in both seizure models. No significant changes in motor coordination were detected in all doses of the crude extract and solvent fractions. The plant extract contained flavonoids, phenols, tannins, steroids, terpenoids and saponins. This study indicated that the plant has a promising anticonvulsant activity, and it could be considered as a potential source to develop new anti-epileptic drug.

Key words: Epilepsy, Seizure, Anticonvulsant, *Buddleja polystachya*

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Acknowledgements

First of all, I would like to thank the almighty God who has given me the strength and patience since the start of the study.

I am so grateful to thank my advisors Dr. Teshome Nedi and Dr. Workineh Shibeshi for their encouragement, guidance and constructive comments from the beginning to the end of the project. My honest thanks also goes to Dr. Samson Salile, Mrs. Fantu Assefa and Mr. Kalkidan for their cooperation in providing chemical and materials as well as handling of laboratory animals.

I would also like to thank my family, I am grateful for their love, care, guidance, support, encouragement and for believing in me throughout my life. My sincere gratitude also goes to Mr. Ambaye Sisay for his support on medicinal plant collection. Finally, yet importantly, I am also thankful to Addis Ababa University and Arbaminch University for granting me the financial support and all the necessary inputs for the entire thesis work.

List of Abbreviations and Acronyms

AEDs	Anti-Epileptic Drugs
AMPA	α-amino-3-hydroxy-5-methyl-isoxazolopropionic acid
ANOVA	Analysis of Variance
BB	Butanol Fraction of Buddleja polystachya
CACNA1H	Calcium Voltage-gated Channel Subunit Alpha1 Human
CB	Chloroform Fraction of Buddleja polystachya
CHRNA4	Cholinergic Receptor Nicotinic Alpha 4 Subunit
EEG	Electroencephalographic
GABA	γ-aminobutyric acid
HIV	Human Immunodeficiency Virus
IL	Interleukin
ILAE	International League Against Epilepsy
MB	Methanol Extract of Buddleja polystachya
MES	Maximal Electrical Shock
mGluRs	Metabotropic Glutamate Receptors
NMDA	N-methyl-D-aspartate
OECD	Organization of Economic Cooperation and Development
PTZ	Pentylenetetrazol
SCN2A	Sodium Voltage-gated Channel Alpha Subunit 2
SEM	Standard Error of the Mean
SNNPR	Southern Nations, Nationalities, and Peoples' Region

SPSS	Statistical Package for Social Science
THLE	Tonic Hind Limb Extension
UK	United Kingdom
VNS	Vagus Nerve Stimulation

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1. Introduction

1.1. Overview of Epilepsy

Epilepsy is a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures and by the neurobiological, cognitive, psychological, and social consequences of this condition (Fisher et al., 2005). According to International League against Epilepsy (ILAE), the definition of epilepsy requires at least the occurrence of two unprovoked or reflex seizures in more than 24 hours apart or after one unprovoked seizure if the risks of recurrence are "high" (>60%) (Fisher et al., 2014). A seizure is defined as a single episode of neurological dysfunction arising from abnormal neuronal discharge, and can lead to clinically overt changes in motor control, sensory perception, behavior, or autonomic function (Fisher et al., 2005).

Based on electroencephalographic recording, clinical symptology and onset of seizure; epileptic seizures are classified into partial seizure, generalized seizure and epileptic spasms. (Berg et al., 2010). Partial seizures occur within discrete regions of the brain, result in an asymmetric motor manifestation. It is manifest as alterations in motor functions, sensory or somatosensory symptoms, or automatisms. Partial seizures with no loss of consciousness are classified as simple partial, while an alteration of consciousness is described as complex partial. EEG in patients with complex partial seizures has been often normal or may show brief epileptiform spikes (Susan & Jose, 2014).

Generalized seizures have clinical manifestations that indicate involvement of both hemispheres. Motor manifestations are bilateral, and there is a loss of consciousness. They are further classified into; tonic-clonic, absence, myoclonic, clonic, tonic and atonic seizure (Berg et al., 2010). Generalized tonic-clonic seizure results in a sudden sharp tonic contraction of muscles

followed by a period of rigidity and clonic movements. Tonic and clonic seizures can occur separately. Brief shock-like muscular contractions of the face, trunk, and extremities are known as myoclonic jerks. A sudden loss of muscle tone is known as an atonic seizure. This can be described as a head drop, the dropping of a limb, or a slumping to the ground. EEG during the tonic phase of the seizure shows polyspike discharges, while in the clonic phase shows a spike-and-wave pattern (Susan & Jose, 2014).

Generalized absence seizures are manifested by a sudden onset, interruption of ongoing activities, a blank stare, and possibly a brief upward rotation of the eyes. An EEG hallmark of absence seizures is a generalized, symmetric, 3-Hz spike-and-wave discharge (Susan & Jose, 2014). The third classification is epileptic spasms, which are manifest by a sudden flexion, extension, or mixed extension–flexion of predominantly proximal and truncal muscles that is usually more sustained than a myoclonic movement but not as sustained as a tonic seizure. The origin of epileptic spasm is unknown (Berg et al., 2010).

There are several causes epilepsy, ILAE classify these conditions into three groups; “Structural/metabolic”, “Genetic” and “Unknown cause” (Berg et al., 2010). A structural etiology refers to abnormalities visible on structural neuroimaging and it may be acquired due to stroke and brain trauma. Metabolic causes refer to a well delineated metabolic defect with manifestations or biochemical changes throughout the body such as porphyria, uremia, pyridoxine-dependent seizures (Scheffer et al., 2017). Infections were the most common etiology of epilepsy, especially in the developing country. Common infection associated with epilepsy include neurocysticercosis, cerebral malaria, cerebral toxoplasmosis, and congenital infections such as zika virus and cytomegalovirus (Godet, Druet-cabanac, Bhalla, & Druet-cabanac, 2011). Several gene mutations responsible for inherited epilepsy were identified. For instance, benign

familial neonatal-infantile seizures (SCN2A), malignant migrating partial seizures of infancy (SCN1A) and nocturnal frontal lobe epilepsy (CHRNA4) (Myers & Mefford, 2015).

1.1.1. Epidemiology of Epilepsy

Epilepsy is a neurological condition with no geographic, social, or racial boundaries, occurring in men and women and affecting people of all ages (A. Singh & Trevick, 2016). Globally, at least 50-60 million people have epilepsy and nearly 80% of them are found in developing regions (Abramovici & Bagi, 2016). Studies in the developing country have reported higher prevalence rates of epilepsy, approximately 57 cases per 1000 persons (Deresse & Shaweno, 2016). In line with that, 60-70% of epileptic patients in developing country receive no modern treatment at all, largely for economic and social reasons (A. Singh & Trevick, 2016).

Epilepsy is also a common neurological disease in Ethiopia with an estimated prevalence of 5-8 per 1000 of the population (Deresse & Shaweno, 2016). Other studies even reported higher prevalence of epilepsy up to 29.5 per 1000 of the population (Almu, Tadesse, Cooper, & Hackett, 2006). Furthermore, generalized tonic–clonic seizures were the most common seizure type in Ethiopia and occurred in 69–81%. Partial seizures occurred in 18–20%, and unclassifiable seizures occurred in 11% (Worku, 2013). Worldwide, mortality among people with epilepsy is reported to be two to three times higher than that in the general population (Ba-diop et al., 2014). Studies from Ethiopia also showed that the estimated crude death rate for people with epilepsy was 31.6 per 1000 person years, which was two times higher than the general population (Tekle-Haimanot, Forsgren, & Ekstedt, 1997).

1.2. Seizure Initiation and Propagation

A seizure is a paroxysmal event due to abnormal, excessive, hypersynchronous discharges from an aggregate of central nervous system (CNS) neurons. There are two hallmark features of

seizure initiation in neurons; neuronal hyperexcitability and hypersynchronization (Engelborghs, D'Hooge, & De Deyn, 2000). The hyperexcitability caused by a relatively long-lasting depolarization of the neuronal membrane due to influx of extracellular calcium ($Ca2^{+}$), which leads to the opening of voltage-dependent sodium (Na^{+}) channels, influx of Na^{+}, and generation of repetitive action potentials. This followed by a hyperpolarizing after-potential mediated by γ-aminobutyric acid (GABA) receptors or potassium (K^{+}) channels, depending on the cell type. The synchronized bursts from a sufficient number of neurons result in a so-called spike discharge on the EEG (Ure & Perassolo, 2000; Staley, 2015).

In normal, the propagation of bursting activity is prevented by intact hyperpolarization and by inhibitory neurons (Scharfman, 2007). However, the repetitive discharges of neuron cause the recruitment of surrounding neurons by various mechanisms. These include (i) an increase in extracellular K^{+}, which blunts hyperpolarization and depolarizes neighboring neurons; (ii) accumulation of Ca2+ in presynaptic terminals, leading to enhanced neurotransmitter release; and (ii) depolarization-induced activation of the N-methyl-D-aspartate (NMDA) subtype of the excitatory amino acid receptor, which causes Ca2+ influx and neuronal activation (Engelborghs et al., 2000). Thus, the recruitment of a sufficient number of neurons leads to a loss of the surrounding inhibition and propagation of seizure activity into contiguous areas via local cortical connections, and to more distant areas via long commissural pathways such as the corpus callosum (Scharfman, 2007).

1.3. Pathophysiology of Epilepsy

The pathophysiological mechanisms of epilepsy involve various biological pathways or processes, structural and functional changes. In general, it is unclear which mechanisms are required or necessary for the genesis of epilepsy. However, several experimental studies have

provided some insights into the actual and postulated mechanisms of epileptogenesis (Kobow et al., 2012).

A) The role of neurotransmitters

i) GABA

GABA is recognized as the main inhibitory neurotransmitter in the brain. GABA exerts its action via ionotropic $GABA_A$ and the metabotropic $GABA_B$ receptors (Simeone & Rho, 2009). Activation of the $GABA_A$ receptor in the brain generates fast inhibitory postsynaptic potentials by promoting the Chlorine (Cl^-) influx thus provoking hyperpolarization with a decrease in cellular excitation state, the inhibition being postsynaptic (Avoli & Miles, 2009). Hence, antiepileptic drugs (AEDs) like benzodiazepam increases the likelihood of opening the Cl^- ionophore, while barbiturates prolong opening time (Lasoń, Dudra-Jastrzębska, Rejdak, & Czuczwar, 2011).

It has been hypothesized that the neuronal hyperexcitability in epilepsy is due to imbalance between glutamate-mediated excitation and GABA-mediated inhibition (Lasoń, Chlebicka, & Rejdak, 2013). Deficiency in GABA synthesis, loss of GABA interneurons and some changes in expression or mutations of the $GABA_A$ receptor can lead to neuronal hyper-excitability. Thus, GABAergic inhibition can be augmented by, increased $GABA_A$ and $GABA_B$ receptor function; enhanced GABA synthesis and decreased degradation; and inhibition of GABA re-uptake into neuronal and glial cells (Simeone & Rho, 2009; Lasoñ et al., 2013).

ii) Glutamate

Glutamic acid is the main excitatory amino acid neurotransmitter in the central nervous system (Jensen, 2009). It activates both inotropic receptors NMDA, AMPA (α-amino-3-hydroxy-5-

methyl-isoxazolopropionic acid), and kainate receptors, as well as metabotropic glutamate receptors (Lasoń et al., 2013). A study shows that NMDA receptor is highly involved in seizure initiation and propagation. In experimental animal's NMDA receptor antagonists, e.g., dizocilpine or ketamine, inhibit seizures evoked by PTZ, pilocarpine and maximal electroshock (MES) (Ure & Perassolo, 2000). Unfortunately, NMDA receptor antagonists are not suitable for clinical use because they showed serious undesired effects, such as motor coordination and memory impairment and disorientation (Lasoń et al., 2013).

In contrast, some allosteric modulators of NMDA receptors, especially modulators that interact with the strychnine-insensitive glycine-binding site and the polyamine-binding site, are more promising as potential AEDs (Lasoń et al., 2013). For instance, felbamate shows antagonistic activity toward the glycine-binding site on NMDA receptors (Simeone & Rho, 2009). In addition, AMPA (e.g. talampanel) receptor antagonists showed anticonvulsant effects in genetic and chemical models of seizures. Their adverse effects on memory and motor coordination in experimental animals are less profound than those evoked by the NMDA receptor antagonists (Lasoń et al., 2013). Studies also show that agonists of group I postsynaptic mGluRs (metabotropic glutamate receptors) induce seizures in experimental animals. In contrast, the stimulation of group II and III presynaptic mGluRs prevents seizures spread (Ure & Perassolo, 2000).

Glutamatergic molecular mechanisms that are involved during initiation and progression of epilepsy include up-regulation of glutamate receptors, elevation in extracellular glutamate concentration, abnormalities in glutamatergic transporters. These phenomena can be inhibited by using different mechanisms like: inhibition of glutamic acid synthesis (e.g. gabapentin & vigabatrin), decrease in synaptic release of glutamic acid (e.g. lamotrigine), enhancement of

glutamate re-uptake and attenuation of postsynaptic glutamate effects (e.g. felbamate, topiramate) (Ure & Perassolo, 2000; Simeone & Rho, 2009).

B) The voltage-gated ion channels

The voltage-gated ion channels (e.g. Na^+, $Ca^{2+,}$ K+) play a key role in the development of epilepsy (Jefferys, 2010). Voltage-gated Na^+ channels are integral membrane glycoproteins composed of α-subunit that is associated with β-subunits. Epileptic seizures are associated with modest, sustained depolarization below the inactivation threshold for action potential generating sodium channels (Simeone & Rho, 2009; Jefferys, 2010). Antagonists of voltage dependent Na+ channels decrease the maximal amplitude of sodium current and prolong the time of the channel inactivation. Channelopathies are key factors of pathogenesis in human epilepsy, Mutations in genes expressing channels of Na+ (SCN2A, SCN2A) have been reported childhood epilepsy (Myers & Mefford, 2015).

The neuronal voltage-gated Ca2+ channels are divided into L-, P/Q-, N- and T-type channels (Simeone & Rho, 2009; Jefferys, 2010). The L-type calcium channels are predominantly localized postsynaptically and mediate sustained calcium ion influx, which further augments neuronal depolarization. N-type and P/Q-type channels are expressed presynaptically and are involved in neurotransmitter exocytosis. The T-type channels open with slight depolarization, and are quickly inactivated (Lasoń et al., 2013). The low-threshold Ca2+ current regulates oscillatory activity of thalamic neurons (pacemaker) and participates in generating generalized absence seizures. Moreover, polymorphisms or mutations in the CACNA1H gene coding for the voltage gated T-type Ca^{2+} channel can be associated with childhood absence epilepsy (Myers & Mefford, 2015).

C) Changes in neuronal networks

Pathologic studies of the hippocampus of patients with temporal lobe epilepsy have led to the suggestion that some forms of epileptogenesis are related to structural changes in neuronal networks. Multiple structural alterations in the hippocampus could occur after acute seizures, including degeneration of dentate gyrus and CA1 – CA3 pyramidal neurons, aberrant sprouting and synaptogenesis of mossy fibers and loss of inhibitory GABAergic interneurons. Thus, according to this hypothesis, normal excitatory inputs from the entorhinal cortical neurons to these reorganized dentate granule cells induce excessive excitation of CA3 neurons in the setting of inadequate inhibition, to initiate sustained repetitive firing in downstream CA1 neurons as a focal seizure with the potential to propagate more widely (Goldberg & Coulter, 2014).

D) The inflammatory pathway

The inflammatory cytokines such as interleukin (IL)-1β, IL-6 and tumor necrosis factor-α have been shown to be up regulated and overexpressed in brain regions involved in generating and propagating epileptic activity. IL-1β can induce the activation of NMDA receptor, thus enhancing neuronal hyperexcitability. Similar to IL-1β, tumor necrosis factor-α can also induce neuronal excitability via up-regulation of AMPA receptors, which favors the ion calcium influx into neurons, and down-regulation of GABA receptors in which the inhibitory synapse strength decreases. Inflammatory cytokines may also contribute to apoptotic neuronal death, which is likely due to production of neurotoxic mediators and NMDA- and AMPA-mediated glutamatergic excitotoxicity (Vezzani et al., 2017).

1.4. Management of Epilepsy

The management of epilepsy is not an easy task; it comprises identification of goals of therapy, assessment of seizure type and frequency, development of a care plan, and a plan for follow-up evaluation (Susan & Jose, 2014). There are non-pharmacological, pharmacological and herbal treatments for the management of epilepsy. Thus, treatment choices should be based on seizure type and frequency, patient age, sex, comorbidities and cost of treatment (Moshe, Perucca, Ryvlin, & Tomson, 2015).

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 (VNS) (Susan & Jose, 2014). A ketogenic diet - a restrictive high-fat, low protein and very low-carbohydrate diet has been found to be effective in the treatment of intractable pediatric epilepsy (Saxena & Nadkarni, 2011). They act by modulation of neurotransmitters, levels of biogenic monoamines and protective antioxidant mechanisms of neurons. Surgical treatment to abolish seizures has been particularly recommended for patients with mesial temporal lobe epilepsy and neocortical epilepsy (Jackson, Makin, Marson, & Kerr, 2015). More importantly, surgery reduces the risk of epilepsy-associated death, and it may also improve depression and anxiety in refractory epilepsy patients (Susan & Jose, 2014). Other non-pharmacological therapeutic option for patients with epilepsy is VNS. A vagal nerve stimulator is a medical device which implanted in the left cervical vagus nerve (Krahl & Clark, 2012). It is used as an adjunctive therapy in reducing the frequency of seizures in patients older than 12 years of age with partial-onset seizures that are refractory to AEDs (Susan & Jose, 2014).

1.4.2. Pharmacological Treatment

Nowadays, there are a plenty of AEDs approved for the treatment of epilepsy. Most of conventional AEDs generally acts: by increasing GABAergic action mechanisms; by depressing excitatory glutamate neurotransmission; by acting on voltage gated ion channels (Simeone & Rho, 2009).

A. GABAergic Drugs

AEDs, that enhance GABAergic system are phenobarbital, primidone, mephobarbital, diazepam, clonazepam, tiagabine and vigabatrin (Lasoń et al., 2013). Phenobarbital inhibits seizure induced by both MES and PTZ. Benzodiazepines, on the other hand, are effective only in the inhibition of PTZ-induced clonic seizures (Lasoń et al., 2011). Barbiturates are recommended for treatment of generalized tonic-clonic seizures and focal seizures (Perucca & Tomson, 2011). The main indications for benzodiazepines are juvenile myoclonic epilepsy and status epilepticus (Moshe et al., 2015). Tiagabine and vigabatrin are also used in the management of drug-resistant focal and secondary generalized epilepsy (Ben-menachem, 2014).

B. Drugs that depress excitatory neurotransmission

Another type AEDs that inhibit excitatory neurotransmission are felbamate and talampanel. Though, felbamate has limited use in clinical practice due to their adverse effect and talampanel is still being examined in clinical trials for treatment seizure (Lasoń et al., 2013).

C. Sodium Channel blockers

Sodium channel blockers include: phenytoin, carbamazepine, lamotrigine, topiramate, oxcarbazepine and zonisamide (Simeone & Rho, 2009). Phenytoin is indicated for all types of focal and generalized tonic-clonic seizures except for generalized absence seizures (Perucca &

Tomson, 2011). It is effective in the inhibition of tonic phase of seizure in MES but not effective against PTZ-induced clonic seizures (Lasoń et al., 2011). Carbamazepine is used for the management of generalized tonic-clonic and focal seizures while its analog oxcarbazepine is mainly indicated for focal seizures (Moshe et al., 2015). Lamotrigine has a broader spectrum of anticonvulsant activity compared to phenytoin and carbamazepine, so it is indicated for focal, absence and generalized tonic-clonic seizures (Chong & Lerman, 2016). Topiramate and zonisamide are also indicated for the management of drug-resistant epilepsy (Ben-menachem, 2014).

D. Calcium Channel blockers

The inhibition of a low threshold T-calcium current in the thalamic neurons has a protective effect on absence seizure (Lasoń et al., 2013). Ethosuximide inhibits T-calcium currents and it is effective against generalized absence seizure. It is also effective against PTZ-induced clonic convulsions but not against tonic seizures induced by MES (Simeone & Rho, 2009). Valproate is another type of AED that block T calcium current. It is a broad-spectrum AED mainly used for the therapy of absences, myoclonic, partial, and tonic-clonic seizures (Perucca & Tomson, 2011).

Despite the availability of conventional AEDs, there are still significant unmet medical needs and treatment challenges. These include: drug-resistant epilepsy, adverse reactions, drug interactions, and a lack of antiepileptogenic agents (Ventola, 2014). Newer AEDs (e.g. perampanel & retigabine) with a novel mechanism of action were also developed by aiming to counteract those challenges (Moshe et al., 2015), but they have not been proven to be more effective and failed to reduce the prevalence of drug-resistant epilepsy (Dalic & Cook, 2016).

1.4.3. Herbal Treatment

In addition to pharmacological treatment, many kinds of medicinal plants have been used in folk medicines to treat epilepsy. The uses of medicinal plants to treat epilepsy were deeply rooted practice in the society of many counties like Ayur-veda in India, Kampo medicines in Japan and Chinese herbal medications in China. For example, there are more than 15 Chinese herbal plants which have been discovered with definite antiepileptic activity in experimental studies (Tyagi & Delanty, 2003; Schachter, 2009). In Africa, traditional medicinal plants still play an important role in the management of neurological diseases, mainly among populations with very low income (Ngo et al., 2011).

Many communities in Africa traditionally associate epilepsy with evil spirits and superstitions, encouraging treatment from traditional healers and religious leaders (Deresse & Shaweno, 2016). Accordingly, several medicinal plants used for treatment of epilepsy were identified by ethnopharmacological studies in various African countries (Muazu & Kaita, 2008; Ngo et al., 2011; Zhu et al., 2014). For example, medicinal plants from Cameron, Nigeria, Ghana and Tanzania (e.g. *Feretia apodanthera, Pseudospondias microcarpa, Laggera Aurita, Ricinus communis and Clausena anisata*) have shown proven anticonvulsant activity in the animal experimental studies (Muazu & Kaita, 2008; Taiwe et al., 2016; Malami, Kyari, Danjuma, Ya, & Hussaini, 2016a; Adongo et al., 2017). The uses of medicinal plants for the management of epilepsy were also reported in various ethnopharmacological study conducted in Ethiopia (Giday, Teklehaymanot, Animut, & Mekonnen, 2007; Abera, 2014; Andarge, Shonga, Agize, & Tora, 2015). However, no experimental study has been conducted in Ethiopia so far to support the traditional practice of the community.

1.5. Overview of the Experimental Plant

The genus *Buddleja* commonly known as butterfly bush, belongs to the family Buddlejaceae and it was named in honor of the Reverend Adam Buddle (Fawzy, Gamal, & Ati, 2013). It comprises over 100 species of flowering plants. They grow in tropical and warm temperate regions of Southern Asia, East Africa and America (Pendota, Ndhlala, Aremu, Aderogba, & Van Staden, 2014). It has been reported that several *Buddleja* species have been used in traditional medicine in many parts of the world, and previous phytochemical investigations led to the isolation of alkaloids, flavonoids, iridoids, phenylpropanoids, sesquiterpenoids and saponins (P J Houghton, 1984; Peter J Houghton, Mensah, Iessa, & Hong, 2003)

In Ethiopia, the genus Buddleja is represented by *Buddleja polystachya*. It is locally known as Anfare (Amharic), Madera (Afargna) and Kanfara (Dawuro) (Andarge et al., 2015; Mohammed, Abdulwuhab, & Mohammed, 2016). *Buddleja polystachya* is a multi-branched shrub that grows to < 5 m, but can occasionally reach 12 m in favorable conditions. It has red-brown or grey, short bole deeply grooved bark. The leaves are long and narrow to 15cm with a pointed tip and are light grey-green on top (Figure 1). The flowers are bright orange on a long spike up to 20 cm (Tesfamaryam, Tsegaye, Eguale, & Wubete, 2015). It is endemic to the semi-arid highlands flanking the Red Sea in Eritrea, Ethiopia, Saudi Arabia, Somalia and Yemen (Alemu & Andualem, 2014). Often grows in secondary scrub of semi-arid upper highland forest and at forest edges in dry, moist and wet climatic zone, at elevations of between 2,200 and 3,600m above sea level (Mohamedkassm et al., 2013). Traditionally, the branches and leaves are used to wash pots, the leaves can provide fodder and many parts (mostly leaves) also have medicinal uses (Alemu & Andualem, 2014). Phytochemical studies on *Buddleja polystachya* revealed the presence of various compounds, which include terpenoids (ursolic acid, uvaol, oleanolic acid),

phenolic compounds (sakuranetin, cirsimaritin, trimethoxyflavone, linarin, herbacetin, Luteolin) and others like iridoid glycoside, saponins and tannins (P.J. Houghton, 1984; Yehya Al Ati et al., 2015; Mohammed et al., 2016).

Ethnopharmacological surveys have revealed that the roots, leaves, and flowers of various species of *Buddleja*, in several parts of the world, have been used in traditional medicine. It is used as an antiprotozoal, for liver diseases, dysentery, eye disease, as diuretic, sedative, analgesic, wound healing, anti-inflammatory, antimalarial, antimicrobial, hypoglycemic, antispasmodic, and antioxidant agent (P.J. Houghton, 1984; Abera, 2014; Gutiérrez, Chilpa, & Jaime, 2014; Seleteng, Moteetee, & Vuuren, 2015; Rehman et al., 2015). In northwest Ethiopia, for instance, the powdered leaf of *Buddleja polystachya* mixed with Tej was given orally to expel the intestinal parasite (Chekole, Asfaw, & Kelbessa, 2015). Additionally, in north and south Ethiopia fresh leaves of this plant is used for treatments of eye disease and malaria (Getaneh & Girma, 2014; Andarge et al., 2015). There are also reports that the plant used for treatments of headache and migraine (Mohammed et al., 2016). Traditional healer of Dawuro also uses this plant for treatment of epilepsy, dried leaf part of the plant is crushed, mixed with local alcoholic drink and taken as drink through the oral route (Andarge et al., 2015).

Moreover, several experimental studies have been conducted to prove the traditional medicinal uses of *Buddleja* species. For instance, experimental studies revealed that *Buddleja globosa*, *Buddleja salviifolia* and *Buddleja polystachya* have anti-inflammatory, analgesic and antioxidant properties (Backhouse et al., 2008; Pendota et al., 2014; Yehya Al Ati et al., 2015). *Buddleja polystachya* also exhibited antimicrobial, hypoglycemic, anti-cancer, antimalarial, antidiarrheal and antispasmodic activities of in various experimental studies (Fawzy et al., 2013; Alemu & Andualem, 2014; Yehya Al Ati et al., 2015; Rehman et al., 2015; Mohammed et al., 2016).

Buddleja species are known to contain some compounds with known anti-inflammatory activity, for example, kaempferol, which inhibits both cyclooxygenase and 5-lipoxygenase. Other studies also reported the presence of neuroprotective compounds such as linarin, ursolic acid and uvaol (Lou, Fan, Perez, & Lou, 2011; Zhu et al., 2014).

Figure 1. Photograph of *Buddleja polystachya.*

1.6. Rational for the Study

Epilepsy is an important cause of disability, contributing 7 million disability adjusted life years to the global burden of disease (Abramovici & Bagi, 2016). Despite the availability of AEDs, nearly one in three patients with epilepsy who have access to AEDs continue to have seizures, and a similar proportion experience unacceptable AED-related adverse effects (Ventola, 2014). Moreover, a substantial treatment gap is evident in developing countries, because human and financial resources for diagnosis and treatment are limited, misconceptions and stigma surround the disorder (World Health Organization, 2004; Berhanu, Alemu, Prevett, & Parry, 2009). Therefore, there continues to be a pressing need for new and effective treatments for epilepsy, especially for those that are accessible and affordable to patients everywhere. Medicinal plants remain the main focus by scientists and researcher as a novel source of lead compound in the search and development of new AEDs (Sucher & Carles, 2015).

The use of medicinal plants for the management of neurologic disorder is widespread practice in Ethiopia (Mesfin, Demissew, & Teklehaymanot, 2009; Avigdor, Wohlmuth, Asfaw, & Awas, 2014). *Buddleja polystachya* is one such plant that is used traditionally for treatment of epilepsy (Andarge et al., 2015). However, no experimental studies have been conducted so far to confirm the medicinal value of claimed plants. The present study was done to provide baseline information on the traditional claim of *Buddleja polystachya* for epilepsy. In addition, this study may serve as baseline information for the development of new pharmacotherapies from medicinal plants for use in epilepsy. “This allow us to isolate and identify the active compound that can be used as a potential drug or a lead compound”.

2. Objectives

2.1. General Objective

- To evaluate the anticonvulsant activity of 80% methanol leaf extract and solvent fraction of *Buddleja polystachya* in mice.

2.2. Specific Objectives

- To assess acute toxicity of the crude and solvent fractions of *Buddleja polystachya* leaf in mice.
- To assess anticonvulsant activity of the crude extract and solvent fraction of *Buddleja polystachya* leaf using PTZ test in mice.
- To evaluate anticonvulsant activity of the crude extract and solvent fraction of *Buddleja polystachya* leaf using MES test in mice.
- To determine the effect of crude extract and solvent fraction of *Buddleja polystachya* leaf on motor coordination using rotarod test in mice.
- To perform preliminary phytochemical screening on the crude extract and solvent fractions of *Buddleja polystachya* leafs.

3. Material and Methods

3.1. Drug and Chemicals

The main chemicals and drugs that were used includes: Distilled water (Ethiopian pharmaceutical manufacturer, Ethiopia), Tween 80 (Lobe Chemichals, India), Methanol (Carlo Erba Reagents, France), n-butanol (Carlo Erba Reagents, France), Chloroform (Fisher Scientific, UK), Sodium Valproate (Remedica, Cyprus), Diazepam (Remedica, Cyprus), Phenytoin (Brawn Laboratory, India) and Normal Saline (Acu Life Health Care, India) were obtained from their respective vendors. Pentylenetetrazol (Sigma Aldrich, Germany) were obtained from Department of pharmacology and clinical pharmacy, AAU. (Hydrochloric acid, Sodium hydroxide, Dragendrof's reagent, Glacial acetic acid, Sulfuric acid Ferric chloride and Potassium ferrocyanide) (Fisher Scientific, UK) and Acetic Anhydride (Park Scientific, UK) were obtained from the Department of Pharmaceutical chemistry and Pharmacognosy, AAU and were of analytical grade.

3.2. Collection of Plant Materials

The leaves of *Buddleja Polystachya* were collected from Waaka; a town in Dawuro Zone, SNNPR, which is about 600 km away from Addis Ababa. Identification & authentication of the plant specimens were done by a taxonomist at the National Herbarium, College of Natural and Computational Sciences, Addis Ababa University, where a voucher specimen was kept for future reference with voucher No. of TA001.

3.3. Experimental Animals

Healthy Swiss albino mice (weighing 30 ± 3 g) were used for the experiment. Female mice were used for the acute toxicity test while male mice were used for anticonvulsant activity testing. The

mice were obtained from the animal house unit of School of pharmacy of Addis Ababa University, Addis Ababa, Ethiopia. The animals were housed in groups of six and were acclimatized for a week to the laboratory condition before commencement of the experiment. The mice were housed under standard environmental condition (12h/12h light/dark cycle). All animals had free access to standard laboratory pellet and water ad *libtum* throughout the experiment. All procedures and techniques used in this study were in accordance with the national institute of health guidelines for the care and use of laboratory animals (Institute for Laboratory Animal Research, 2011).

3.4. Extraction and Fractionation of Plant Material

After collection, the leaves of the plant material were thoroughly washed with distilled water to remove dirt and soil, and dried under shade with optimal ventilation for 3 weeks. After that, the dried plant leaves were cut into pieces manually and pulverized, using a mortar and pestle to get a coarse powder and subjected to extraction. The course powder of the plant was extracted by cold maceration procedure using 80% methanol in water.

Accordingly, 400g of the dried powder were weighed using electronic digital balance and then divided into two portions in two different Erlenmeyer flask (200g each for ease of extraction). The powdered plant material was soaked in each Erlenmeyer flask containing 80% methanol (1:5 (w/v)) at room temperature for 3 days with occasional shaking using orbital shaker (Bibby Scientific Limited, UK). The extract solution was then filtered, first by using cotton gauze and later by using Whatman filter paper (No. 1) and the marc was re-macerated twice using the same volume of solvent to exhaustively extract the plant material. Rotavapor (Buchi, Switzerland) was used to dry the methanol in the sample under reduced pressure at a temperature of 40^{o}C and the remaining water was freeze dried by the use of a lyophilizer (Korea vacuum limited, Korea). The

resulting dry hydro-alcoholic extract was weighed and calculated for percentage yield, which was (70gm; yield, 17.5% w/w). Finally, the concentrated extract was transferred into vials and kept at −20°C until use.

The 80% methanol extract was subjected to fractionation using solvents with differing polarity, i.e., chloroform, n-butanol and water. Accordingly, a total of 50g of 80% methanol extract of *Buddleja polystachya* was dissolved in 250 ml of distilled water using a separatory funnel. The dissolved hydro-alcoholic extract was partitioned with 250 ml chloroform and repeated until the chloroform layer becomes clear. The filtrate was concentrated in a rotary evaporator at 80 rpm and 40^0C to obtain the chloroform fraction and the yield obtained was 11.2gm (22.4%). The aqueous residue was further partitioned with 250ml n-butanol. The butanol filtrate was concentrated similarly as chloroform fraction to have butanol fraction and a yield of 17.3gm (34.6%) was obtained. The remaining aqueous residue was frozen in deep freezer overnight and then freeze dried with a lyophilizer and a total of 18.2gm (36.4%) of the aqueous fraction was obtained. All fractions were kept in tightly closed containers in the refrigerator at -20^0C until used for the experiment.

3.5. Acute Oral Toxicity Test

Acute oral toxicity was conducted according to OECD guideline (OECD, 2008). Five female albino mice of 6-8 weeks' age (each for 80% methanol extract and solvent fraction) were used for the study. According to the guideline; on day one, single mouse fasted for 3–4h was given 2000 mg/kg of the extract orally. The mouse was kept under strict observation for physical or behavioral changes for 24 h. After the results from the first mouse, another four mice were recruited then fasted for 3–4 h, administered a single dose of 2000 mg/kg, and were observed in

the same manner. These observations were continued for a further 14 days for any signs of overt toxicity.

3.6. Grouping and Dosing of Animals

The animals were randomly assigned into five groups for crude extract (MB) and solvent fractions (BB=butanol fraction, CB=chloroform fraction and AB=aqueous fraction), each group consisting of six mice for the test. The first group was assigned as negative controls and treated with the vehicles (distilled water for aqueous extract and 2% Tween 80 in water for other extracts) used for reconstitution. The second group was assigned as positive control and treated with standard drugs (Sodium Valproate (VP) 200mg/kg for PTZ test, Phenytoin (PTN) 25 mg/kg for MES test and Diazepam (DZP) 5 mg/kg for rotarod test). The rest of three groups received increasing doses of test extracts (100 mg/kg, 200 mg/kg and 400 mg/kg). Doses of the extracts were selected based on the outcome of the acute toxicity test. As per OECD 4 (60) guideline 1/10th of the limit dose 2000 mg/kg was taken as a mid-dose (200 mg/kg) after which half and doubling of the mid dose were selected as minimum and maximum doses (100 mg/kg and 400 mg/kg respectively) for the study.

3.7. Anticonvulsant Activity Tests

3.7.1. Pentylenetetrazol (PTZ) - Induced Seizure Test

This test is considered as indicative of anticonvulsant activity of drugs against absence and myoclonic seizures. PTZ produces generalized synchronized clonic movements, which are superseded by tonic convulsion characterized by flexion of limbs followed by extension. For this experiment, the method described by Ya'U et al., (2015) was used. The mice received different dose of crude extracts, solvent fractions, sodium valproate and vehicle through the oral route. After 60 min of these treatments, PTZ at 85 mg/kg in normal saline solution was injected

through subcutaneous route for each mouse. Each mouse was placed into a transparent cage and observed for convulsive behavior for 30 min by using a video recorder. The clonic seizure of forelimb and hind limb were taken as an end point in this test (Figure 2). The percentage protection of clonic seizure, percentage protection of mortality and latency to clonic convulsions (min) were noted and compared with that of vehicle control.

$$\%\ protection\ of\ clonic\ seizure = \left(\frac{no.clonic\ seizure\ in\ control - no.clonic\ seizure\ in\ test}{no.clonic\ seizure\ in\ control}\right) * 100$$

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

Figure 2. Clonic seizure exhibited after subcutaneous injection of PTZ.

3.7.2. Maximal Electroshock (MES) - Induced Seizures Test

Protection against electroshock-induced seizures in mice was used as an indication for compounds, which may prove effective in generalized seizures of the tonic–clonic (grand mal) type. For this study a protocol developed by Swinyard et al., (1952) was used. In brief, mice in different groups received varying doses of crude extract, its fractions, vehicle, and phenytoin through the oral route. After 60 min of these treatments, the mice received maximal electric shocks of 50 mA for 0.2s through ear-clip electrodes by using electroconvulsometer (Rolex

Ambala, India). Following stimulus application, the vehicle treated mice were shown an immediate severe tonic seizure with the maximal extension of the anterior and posterior legs (Figure 3.). The body becomes stiffened and lasted for 12-16 s; at the end of this tonic phase, variable phase of clonic seizures was started, characterized by paddling movements of the hind limbs and shaking of the body; 20-50 s later, the animals were maintained in their upright position and start moving around, apparently recovering their normal behavior. The mice were observed closely for 2 min by using a video recorder. The reduction in the duration of THLE compared to the control group was considered as evidence for the presence of anticonvulsant activity. The percentage reduction in duration of THLE against control was calculated according to the following formula:

$$\%reducution\ in\ dr.of\ THLE = \left(\frac{mean\ dr.of\ THLE\ in\ control - mean\ dr.of\ THLE\ in\ test}{mean\ dr.of\ THLE\ in\ control}\right) * 100$$

Where, THLE is tonic hind limb extension, and dr. is = duration

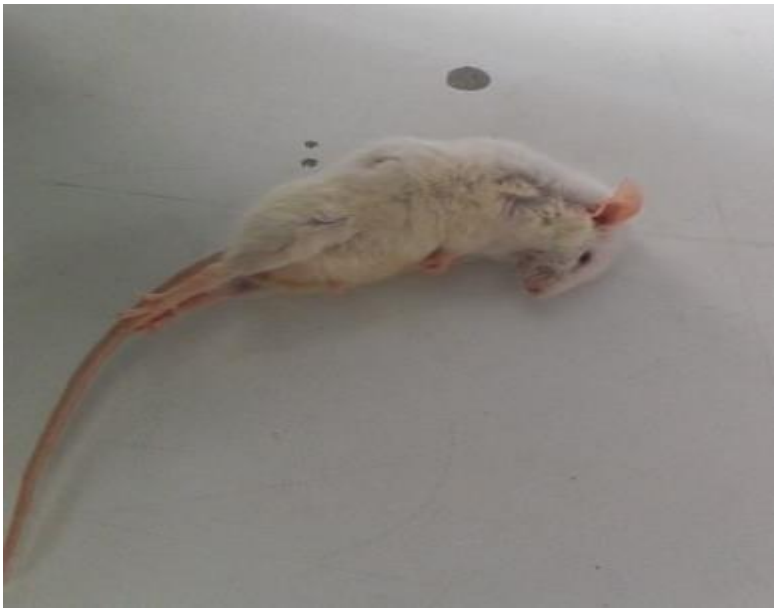

Figure 3. THLE exhibited after MES application

3.8. Rotarod Test

Rotarod test was carried out in order to rule out any motor incoordination effect the extract might possess. The test procedure described by Gawande, Druzhilovsky, Gupta, Poroikov, & Goel, (2017) was employed in this study. Briefly, the rotarod apparatus consisting of a horizontal rod with 3 cm diameters divided into four equal lanes. The mice were trained for three consecutive days on the rotarod apparatus. Accordingly, mice that were able to remain on the rod at a speed of 10 rpm for 3 min or more were selected for the study and distributed into five groups. The three groups received a crude extract and solvent fraction at doses of 100mg/kg, 200mg/kg and 400mg/kg through the oral route. The fourth and fifth groups were given diazepam (5 mg/kg i.p) and distilled water (10 mL/kg i.p.) respectively. After 60 min of the treatment with extract and 30 min of treatment with diazepam and saline, all mice were placed individually in each lane for three consecutive trials on the rotating rod and average retention time on the rod was calculated. The mouse was considered to have motor deficits, if it was unable to maintain equilibrium on the rotating rod for at least 3 min (180s).

3.9. Preliminary Phytochemical Screening Tests

The crude extract and each fraction were screened for the presence of different phytochemical constituents following standard procedures.

a) Test for alkaloids

About 5 ml of 5% hydrochloric acid was added to 500mg of 80% crude extracts and each fraction, and heated on a water bath. When cooled, few drops of Dragendroff's reagent were added. The appearance of the reddish brown precipitate indicated the presence of alkaloids (Yadav, Kumar, Mahour, & Vihan, 2010).

b) Test for saponins

Five hundred milligrams of the sample in 10 ml of distilled water were shaken vigorously in a test tube and the formation of honeycomb froth that persists for 30 minutes was considered as positive for saponins (Jones & Kinghorn, 2012).

c) Test for flavonoids

About 500mg of crude extract and each fraction, 10 ml of distilled water was added and boiled for 5 min and filtered while hot. Few drops of 20% sodium hydroxide solution were added to 1 ml of the cooled filtrate. A change in yellow color, which in addition of acid changed to colorless solution, indicated the presence of flavonoids (Jones & Kinghorn, 2012).

d) Test for cardiac glycosides

Two ml of crude extract and each fraction was dissolved in 2 ml of glacial acetic acid containing one drop of $FeCl_3$ solution. The mixture was then poured into a test tube containing 1 ml of concentrated H_2SO_4. A brown ring at the interphase indicates the presence of a deoxysugar, characteristic of cardenolides (Mohammed et al., 2016).

e) Test for phenols

To 2 mL of filtered solutions of crude extract and each fraction, three drops of a mixture of 1 ml of 1% $FeCl_3$ and 1 ml of 1% KFe $(CN)_6$ was added and the formation of a green blue color was examined (Jones & Kinghorn, 2012).

f) **Test for steroids**

About 0.25g of each sample was weighed and placed in a test tube. This was 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 (Njoku & Obi, 2009).

g) Test for terpenoids

To 0.25g of each of the crude and solvent fractions was added by 2 ml of chloroform. Then, 3ml concentrated sulfuric acid was carefully added to form a layer. A reddish brown coloration of the interface indicates the presence of terpenoids (Mohammed et al., 2016).

h) Test for tannins

About 0.25 g of crude extract and each fraction was boiled in 10 ml of water in a test tube and then filtered. A few drops of 10% ferric chloride were added and observed for the formation of precipitates or color change. A bluish-black or brownish-green precipitate indicated the presence of tannins (Njoku & Obi, 2009).

3.10. Data Analysis

All experimental data were expressed as mean values ± SEM and were subjected to biostatistical analysis by SPSS windows version 21 statistical packages all the way through a one-way ANOVA followed by post-hoc test (Tukey Test) for multiple comparisons of the mean differences and responses of different extracts with controls. The analysis was performed with 95% confidence interval and the significance was set at $p<0.05$.

4. Result

4.1. Acute Oral Toxicity Test

The acute toxicity study revealed the non-toxic nature of extracts at a limit dose of 2000 mg/kg. This finding suggests the LD50 of the study plant to be above 2000 mg/kg as no signs of overt toxicity and mortality were observed in the crude extract and solvent fraction treated animals. Moreover, the study plant did not produce significant changes in behaviors such as alertness, restlessness, breathing, diarrhea, convulsions and coma during the observation period of two weeks.

4.2. Anticonvulsant Activity in PTZ Induced Seizure

As shown in Table 1, MB showed anticonvulsant activity against PTZ induced clonic seizure. The latency time to clonic seizure was significantly increased at all doses of MB ($p<0.001$ for MB200 and MB400, $p<0.05$ for MB100) when compared with CON. The MB400 displayed a significant increment to the latency time when compared to MB100 ($p<0.001$) but not against MB200. Maximum protection (66.67%) from mortality relative to CON was also achieved by MB400 than other doses of MB. It is worth noting that 33.33% protection from clonic seizure was observed at MB400. VP200, on the other hand, displayed 83.33% protection from clonic seizure which was greater than all doses of MB. However, no statistically significant difference was observed between the latency time produced by VP200 and MB400.

Table 1. The anticonvulsant effect of 80% methanol leaf extract of *Buddleja polystachya* in PTZ-induced seizure.

Group	Mean latency to clonic seizure (min)	% protection from clonic seizure	Percentage protection from mortality
CON	3.25 ± 0.41	-	-
MB100	6.95 ± 0.73^{a1b3d3e3}	0.00	16.67
MB200	12.87 ± 0.50^{a3b3}	0.00	50.00
MB400	16.16 ± 1.22^{a3}	33.33	66.67
VP200	19.05 ± 0.94^{a3}	83.33	100

Values are expressed as Mean ± SEM. (n = 6 mice) [a] compared to CON, [b] compared to VP, [c] compared to 100 mg/kg, [d] compared to 200 mg/kg, [e] compared to 400 mg/kg. [1] $p< 0.05$, [2] $p<0.01$, [3] $p<0.001$. MB refers to 80% methanol extract of *Buddleja polystachya*, VP: Sodium valproate, Numbers refer to dose in mg/kg, CON: group treated with 10ml/kg 2% tween 80.

Among the solvent fractions, the BB and CB exhibited anticonvulsant activity against PTZ induced clonic seizure (Table 2). BB200, BB400 and CB400 treated groups showed substantial ($p<0.001$) increment in the latency time of clonic seizure relative to CON. Significant effect had been also observed when CB200 ($p<0.01$) compared with CON, but no noteworthy effects were observed with any dose of AB as well as CB100 and BB100. The latency shown by all doses of the fractions were significantly lesser ($p< 0.001$) than that of VP200. In the BB group, BB200 and BB400 showed comparable anticonvulsant activity. Whereas, CB400 exhibited significant ($p<0.001$) effect when compared with CB200. The protection from mortality relative to CON was 50% for BB200, BB400 and CB400 even though the protection by VP200 was higher.

Table 2. Anticonvulsant Effect of solvent fraction of *Buddleja polystachya* in PTZ-induced seizure.

Group	Mean latency to clonic seizure (min)	% percentage protection from mortality
CON	3.25 ± 0.41	-
BB100	5.06 ± 0.25^{b3d3e3}	16.67
BB200	11.76 ± 0.46^{a3b3}	50.00
BB400	13.97 ± 0.29^{a3b3}	50.00
VP200	19.05 ± 0.94^{a3}	83.33
CON	3.35 ± 0.41	-
CB100	5.36 ± 0.37^{b3e3}	16.67
CB200	7.22 ± 0.46^{a2b3e3}	33.33
CB400	12.07 ± 0.46^{a3b3}	50.00
VP200	19.05 ± 0.94^{a3}	83.33
CDW	3.08 ± 0.19	-
AB100	3.68 ± 0.38^{b3}	0.00
AB200	4.23 ± 0.25^{b3}	0.00
AB400	4.66 ± 0.20^{b3}	0.00
VP200	19.05 ± 0.94^{a3}	83.33

Values are expressed as Mean ± SEM. (n = 6 mice), [a] compared to CON, [b] compared to VP, [c] compared to 100 mg/kg, [d] compared to 200 mg/kg, [e] compared to 400 mg/kg. [1] $p< 0.05$, [2] $p<0.01$, [3]$p<0.001$. CON: group treated with 2% Tween 80 in distilled water. CDW: treated with distilled water. VP: Sodium valproate (AB = aqueous fraction of *Buddleja polystachya*, BB= butanol fraction of *Buddleja polystachya*, CB= chloroform fraction of *Buddleja polystachya*), Numbers refer to dose in mg/kg.

4.3. Anticonvulsant Activity in MES Induced Seizure

The anticonvulsant effects demonstrated by MB in MES test are given in Table 3. The outcome of the study revealed that all doses of the MB were significantly ($p<0.001$) reduced the duration of THLE compared with CON. The percentage (50.22%) reduction in the duration of THLE by MB400 was significantly ($p<0.001$) greater than that of MB100 and MB200. MB200 treated group also noted a greater reduction ($p<0.01$) in the duration of THLE relative to MB100. It is of note that the PTN25 provided a total protection from THLE, and significant ($p<0.001$) when also compared to all doses of the MB.

Table 3. Anticonvulsant Effect of 80% methanol leaf extract of *Buddleja polystachya* in MES -induced seizure.

Group	Mean duration of THLE (sec)	% reduction in duration of THLE
CON	13.2 ± 0.28	-
MB100	9.49 ± 0.13^{a3b3d2e3}	28.11
MB200	8.21 ± 0.14^{a3b3e3}	37.80
MB400	6.57 ± 0.18^{a3b3}	50.22
PTN25	0.00^{a3}	100

Values are expressed as Mean ± SEM. (n = 6 mice) [a] compared to CON, [b] compared to PTN, [c] compared to 100 mg/kg, [d] compared to 200 mg/kg, [e] compared to 400 mg/kg.[1]$p< 0.05$, [2] $p<0.01$, [3]$p<0.001$. MB refers to 80% methanol extract of *Buddleja polystachya*, CON: group treated with 10ml/kg 2% tween 80, PTN: Phenytoin, Numbers refer to dose in mg/kg, THLE: refers to tonic hind limb extension.

The anticonvulsant activity test of the aqueous, butanol and chloroform fraction of the crude extract of *Buddleja polystachya* was further evaluated by using MES test (Table 4). All doses of BB significantly ($p<0.001$) reduced the duration of THLE when compared with CON. When doses of BB were compared with each other, BB400 displayed a significant reduction in duration

of THLE than BB200 ($p< 0.01$) and BB100 ($p< 0.001$). BB400 also showed the maximum percentage reduction (45.15%) in the duration of THLE than other fraction doses, although the reduction was not significant when compared with PTN25. In contrast to BB, all tested doses of AB and CB exhibited slight reduction in the duration of THLE, which was not significant relative to CON.

Table 4. Anticonvulsant effect of solvent fraction of *Buddleja polystachya* in MES test.

Group	Mean duration of THLE(sec)	% reduction in duration of THLE
CON	13.20 ± 0.28	-
BB100	10.46 ± 0.40^{a3b3c2e3}	20.75
BB200	8.81 ± 0.30^{a3b3e2}	33.25
BB400	7.24 ± 0.23^{a3b3}	45.15
PTN25	0.00^{a3}	100
CON	13.20 ± 0.28	-
CB100	12.83 ± 0.30^{b3}	2.80
CB200	12.49 ± 0.22^{b3}	5.37
CB400	12.43 ± 0.15^{b3}	5.83
PTN25	0.00^{a3}	100
CDW	13.05 ± 0.36	-
AB100	12.89 ± 0.29^{b3}	1.22
AB200	12.29 ± 0.66^{b3}	5.82
AB400	12.21 ± 0.97^{b3}	6.43
PTN25	0.00^{a3}	100

Values are expressed as Mean ± SEM. (n = 6 mice), [a] compared to CON, [b] compared to PTN, [c] compared to 100 mg/kg, [d] compared to 200 mg/kg, [e] compared to 400 mg/kg. [1] $p< 0.05$, [2] $p<0.01$, [3] $p<0.001$. CON: group treated with 2% Tween 80 in distilled water, CDW: group treated with distilled water. PTN: Phenytoin, THLE: refers to tonic hind limb extension. (AB = aqueous fraction of *Buddleja polystachya*, BB= butanol fraction of *Buddleja polystachya*, CB= chloroform fraction of *Buddleja polystachya*), Numbers refer to dose in mg/kg.

4.4. The Rotarod Test

The effect of *Buddleja polystachya* on motor coordination in rotarod test is presented in Table 5. Accordingly, any doses of MB, BB and CB did not show motor deficit, as all treated mice were maintained in their equilibrium on the rotating road for more than 180s, which was also comparable to CON group. Whereas, DZP5 significantly ($p< 0.001$) reduces the retention time on rotating road when compared with CON.

Table 5. Effect of *Buddleja polystachya* on motor coordination in rotarod test.

Group	Mean latency of fall (Sec)
CON	229.1 ± 3.38
DZP5	36.07 ± 3.65[a3]
MB100	213.94 ± 5.05[b3]
MB200	210.55 ± 6.40[b3]
MB400	209.15 ± 5.36[b3]
BB100	216.31 ± 7.18[b3]
BB200	212.22 ± 3.78[b3]
BB400	211.50 ± 2.30[b3]
CB100	218.61 ± 3.64[b3]
CB200	215.11 ± 4.44[b3]
CB400	212.94 ± 4.27[b3]

Values are expressed as Mean ± SEM. (n = 6 mice), [a] compared to CON, [b] compared to DZP5, [3]$p<0.001$. CON: group treated with 2% Tween 80 in distilled water, (MB refers to methanol extract of *Buddleja polystachya*, BB= butanol fraction of *Buddleja polystachya*, CB= chloroform fraction of *Buddleja polystachya*, DZP= diazepam), Numbers refer to dose in mg/kg.

4.5. Preliminary Phytochemical Screening

Phytochemical screening of the hydro-alcoholic extract and solvent fractions of *Buddleja Polystachya* revealed the presence of tannins, terpenoids, saponins, phenols, steroids, and flavonoids in crude extract while only steroids were absent from butanol fraction, and chloroform fraction was not represented by flavonoid and saponins (Table 6). Only saponins and tannins were detected in the aqueous fraction.

Table 6: Preliminary phytochemical screening of the hydro-alcoholic extract and solvent fractions of *Buddleja polystachya*.

Phytoconstituents	Crude Extract	Butanol Fraction	Chloroform Fraction	Aqueous Fraction
Alkaloids	-	-	-	-
Flavonoids	+	+	-	-
Glycosides	-	-	-	-
Phenols	+	+	+	-
Saponins	+	+	-	+
Steroids	+	-	+	-
Tannins	+	+	+	+
Terpenoids	+	+	+	-

-, absent; +, present.

5. Discussion

The present study was conducted to evaluate the anticonvulsant activity of 80% methanol leaf extract and solvent fraction of *Buddleja polystachya*, a plant claimed to be used for the management of epilepsy in the Ethiopian traditional medicine (Andarge et al., 2015). In accordance to the traditional claim, 80% methanol was used as solvent for extraction of plant material. Whereas, solvents for fractions were selected based on their polarity deference. Oral dosing of the extract and fractions were used, to replicate the traditional method of administration.

Female mice were used in the acute toxicity test due to their higher susceptibility to toxicity than male mice (OECD, 2008). In the acute toxicity study none of the animal died or showed signs of toxicity within 24 h and the next 14 days of treatment with 2000 mg/kg of the test extracts. This result was consistent with previous toxicity study done on the leaf of *Buddleja polystachya* (Mohammed et al., 2016). This might explain safe use of plant by traditional healer as well as plants leaf was considered as safe (OECD, 2008).

Male mice were used for anticonvulsant activity tests. Since, female mice have been shown to emit enhanced electroshock responses, including longer flexion phases, shorter extension phases, and lower thresholds to chemoconvulsant (Borowicz, 2009). In addition, the estrus cycle significantly alters the convulsive response of the female mice (Peterson, 1998), so it is rational to use a male mice for anticonvulsant studies to avoid such fluctuations in the seizure response.

Acute seizure models were used in this study, because they are easy to perform, time- and cost-efficient, show good reproducibility between laboratories, well validated with several AEDs and predictive of clinical activity (Castel-Branco, Alves, Figueiredo, Falcao, & Caramona, 2009).

MES and PTZ induced seizure models, which have been developed >60 years ago, remained as standard in early stages of many AED screening programs (Peterson, 1998). Since, epileptic seizures are diverse in nature (Loscher, 2011), it is worthwhile to use animal models like MES and PTZ that can induce different types of seizure.

PTZ antagonizes the $GABA_A$ receptor noncompetitively, likely through a benzodiazepine binding site and an allosteric interaction in the chloride channel, thus leading to induction of seizure in animals (Kubova, 2009). The ability of an agent to protect seizure or increase the onset of clonic seizure as well as protect from mortality induced by PTZ in animals is an indication of anticonvulsant activity. In this study, all tested doses of a crude extract showed significant anticonvulsant activity against PTZ induced clonic seizure. The latency time to clonic seizure exhibited by MB200 and MB400 was comparable but only MB400 showed protection against clonic seizure as well as higher protection from mortality. This indicates that MB400 has superior anticonvulsant effect than other dose, and there is possible localization of active ingredient in this dose.

The butanol and chloroform fraction exerted higher anticonvulsant activity against PTZ induced clonic seizure than aqueous fraction. Although their anticonvulsant effect was lower than that of crude extract. This was evidenced as both fractions were failed to protect the mice from clonic seizure. Activity reduction in the fractions could be explained by the loss of synergistic action among the chemical compounds or differential distribution of secondary metabolites within the fractions. Among fraction, the butanol fraction had exerted superior increment in the latency time to the onset of clonic seizure. The highest effect might have been emanated from the presence of crude extract phytoconstituents in butanol fraction, but not steroid as indicated in

Table 6. From the phytoconstituents flavonoid, terpenoid and phenols were appeared to be more responsible for the observed anticonvulsant activity of this fraction.

Chloroform fraction was found to have the second highest anticonvulsant activities from fractions in PTZ test. The absence of flavonoid could probably be the major reason for this fraction to have lower activity than a butanol fraction. Unlike crude extract, the lower dose of butanol and chloroform fraction was failing to display significant increment in latency time of clonic seizure. This might probably emanate from the absence of sufficient concentration of active constituents or might be related to partial loss of active ingredients due to its insufficient uptake to physiologically active level. The aqueous fraction, on the other hand, was found not effective at all tested doses. This may possibly due to the absence of most of the secondary metabolites from this fraction that appeared to be responsible for the observed anticonvulsant activity.

The observed anticonvulsant activity in PTZ model suggests that the study plant could probably raise the seizure threshold in the brain and could be used against myoclonic and absence seizures. Indeed, PTZ test considered as a reliable predictor of a drug's ability to elevate the seizure threshold and its potential activity against myoclonic and absence seizures. Moreover, PTZ induced seizures are abolished by agents that act by reducing T-type calcium currents and/or enhance GABAergic neurotransmission such as ethosuximide, valproate and benzodiazepines, respectively (Loscher, 2011). It is possible to say that the anticonvulsant effects shown by study plant in PTZ model might be due to inhibition of T-type calcium currents or enhancement of GABAergic neurotransmission, which is not tested in this study.

In MES test, the ability of an agent to reduce the duration of THLE considers as anticonvulsant activity. One can see from the result that the crude extract significantly reduced the duration of

THLE at all tested doses in mice. The higher dose of crude extract was found to be an effective dose in both MES and PTZ model. Similar trend was also noted with BB400 in both models and CB400 in PTZ model. This indicate that the higher dose could be taken as the maximum effective dose relative to lower and middle dose and the presence of good concentrations of active compound(s).

Among the fraction, only a butanol fraction had exerted a higher reduction in the duration of THLE than aqueous fraction and chloroform fraction. It was interesting to note that only the crude extract and butanol fraction was found to be effective in both MES and PTZ model. It has been shown that drugs acting on Na+ channels (e.g., carbamazepine, phenytoin) effectively abolished TLHE induced by MES. In fact, drugs like valproate, phenobarbital and felbamate with multiple mechanism actions are effective in both animal models (Loscher, 2011; Lasoń et al., 2013). This might probably indicate that phytoconstituent(s) present in both extract exert their anticonvulsant activity through multiple mechanism of action, with broad-spectrum activity against myoclonic, absence and tonic-clonic seizures. However, the exact mechanisms underlying the anticonvulsant activity of the *Buddleja polystachya* is remain to be elucidated

In contrast to PTZ model, the chloroform fraction did not demonstrate anticonvulsant activity against MES induced seizure in mice. The absence of anticonvulsant effect was probably emanated from lack of flavonoid in this fraction. As mentioned earlier, the butanol fraction containing flavonoid showed significant anticonvulsant activity in MES. Therefore, it is possible to say that flavonoids may play a major role in anticonvulsant activity of study plant in MES model. This indicate that chloroform fraction might endow activity only against myoclonic, or absence type of seizures, but not against generalized tonic clonic seizure. In the aqueous fraction, one could somehow see a similar trend like PTZ model, there was no significant anticonvulsant

activity at all test doses in MES model. This might indicate that either the observed anticonvulsant property of the plant was not emanating from of saponins or tannins present in this fraction.

Rotarod test was used in this study to evaluate the activity of crude extract on motor incoordination (Gawande et al., 2017). The observed anticonvulsant effect of the plant could be due to spinal or peripheral toxic effects such as neuromuscular blocking. Thus, it is essential to demonstrate the rotarod test to determine the muscle coordination effect of the plant. This test was based on the assumption that a mouse with normal motor competence was able to maintain its equilibrium on a rotating rod (D. Singh, Singh, & Goel, 2012). Accordingly, all crude extract and solvent fraction received mice retained on the rotating rod for more than 180 s during the observation period, which was comparable with negative control groups. Whereas, diazepam showed significant neurotoxic potential at 5 mg/kg. This indicates that Buddleja polystachya didn't cause deficits of the motor coordination in mice. Therefore, the observed anticonvulsant effect of study plant may not be due to muscle relaxation effects and the plant was considered as good candidate for further anticonvulsant studies.

As revealed in phytochemical analysis flavonoids, saponins, tannins, steroids, phenols and terpenoids were present in the crude extract and it seems likely that the anticonvulsant activity could be attributed to one of these active constituent(s). Other phytochemical screening studies done on *Buddleja polystachya* leaves were consistent with the finding of this study (Yehya Al Ati et al., 2015; Mohammed et al., 2016).

Even though at this point, it's difficult to decide which phytoconstituent(s) are responsible for the anticonvulsant activity of *Buddleja polystachya*, it is worthwhile to mention phytoconstituents with probable anticonvulsant activity for further studies. Several studies

indicated the modulation of GABAergic functions by plant isolated terpenoids (Abbasi, Nassiri-Asl, Shafeei, & Sheikhi, 2012; Zhu et al., 2014; Citraro et al., 2016). Ursolic acid a pentacyclic triterpenoid, which also found in *Buddleja polystachya* (Yehya Al Ati et al., 2015), showed anticonvulsant activity against PTZ model with possible facilitation of GABA transmission (Zhu et al., 2014). Several studies also reported the anticonvulsant property of flavonoids isolated from different plants (Zhu et al., 2014). Linarin, a flavonoid derived from *Buddleja davidii*, which also found in *Buddleja polystachya* (Yehya Al Ati et al., 2015), exhibited neuroprotective effect in experimental studies (Lou et al., 2011). In other study, *Buddleja polystachya* exhibited antidiarrheal and antispasmodic activities through inhibition of voltage gated calcium channel (Rehman et al., 2015), which might indicate possible effect on voltage gated calcium channels. Those all observations further strengthen the notion that the study plant had anticonvulsant activity probably through multiple mechanism of action.

5.1. Limitation of the study

The major limitation of this study arises from the very nature of the anticonvulsant screening tests. Even though acute seizure models have high reproducibility in the screening of AED, they are failed to generate spontaneous recurrent seizures and neuropathological hallmarks which are characters of a human epilepsy. The other limitation is the absence of measurement of plant effect on EEG component of seizures. In addition, lack of measurement in neurochemical changes that occur with plant extract administration, which would have been helpful to elucidate the mechanism of action involved. The scarcity of resources has also restricted the scope of the study. As a final point, anticonvulsant activity recording was also carried out manually which can also be a limiting factor as it can be source of bias.

6. Conclusion

The present study provides support for the traditional use of *Buddleja polystachya* as an anticonvulsant agent, and the crude as well as the fractions displayed varying degrees of anticonvulsant activity. The anticonvulsant activity of crude extract and butanol fraction was remarkable in both PTZ and MES model. While, chloroform fraction was found to be effective only on PTZ induced seizure, and the aqueous fraction was devoid of anticonvulsant activity in both seizure models. This indicates that the semi-polar to non-polar ingredients are possibly the ones responsible for the anticonvulsant activity. Additionally, the outcome of the rotarod test indicates the plant had no effect on motor coordination suggesting that the observed anticonvulsant activity is not caused by a muscle relaxation.

7. Recommendations

The plant has promising anticonvulsant activity; therefore, the following works are recommended to be done in future;

- Additional anticonvulsant activity studies should be done for other type's seizure phenotypes, including a chronic model of seizure.
- The effects of plant on EEG component of seizures need to be studied.
- Further studies should be done to isolate, purify and identify pharmacologically active principle (s) responsible for anticonvulsant activity displayed by the plant.
- In-vitro receptor binding studies should be performed in order to elucidate the mechanism of action(s) involved.
- Further toxicological studies need to be done to better establish the safety status of the plant.

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