Anticonvulsant Effect of *Pterlobium stellatum* (Leaves), *Moringa stenopetala* (Root) and *Clutia abyssinica* (Leaves) Traditionally Used for Treatment of Epilepsy in Ethiopia Using Mice Model.

By: Samson Sahile Salile (DVM, MSc)

A Thesis Submitted to the Department of Pharmacology and Clinical Pharmacy, School of Pharmacy Presented in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy (PhD) in Pharmacology.

Addis Ababa University

Addis Ababa, Ethiopia

June, 2021

ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university, and that all sources of material used for this thesis have been properly recognized.

Dr. Samson Sahile **Signature.....**

PhD student

ADDIS ABABA UNIVERSITY

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This is to certify that thesis prepared by **Dr. Samson Sahile**, entitled: **Anticonvulsant Effect of *Pterlobium stellatum* (Leaves*), Moringa stenopetala* (Root) *And Clutia abyssinica* (Leaves) Traditionally Used for Treatment of Epilepsy in Ethiopia Using Mice Model** and submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Pharmacology complies with the regulation of the university and meets the accepted standards with respect to originality and quality.

Examining Board

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Chair of Department or Graduate Program Coordinator

ABSTRACT

Background : Epilepsy is the third leading contributor to the global burden of disease for neurological disorders and affects 65 million people worldwide. Although the current antiepileptic drugs achieve symptomatic seizure relief they do not prevent or reverse the pathological process that underlies human epilepsy. Drug resistant epilepsy is also one of the most important unmet needs in the daily management of epilepsy. These currently unmet needs provide a roadmap for the development of more effective antiseizure drugs, as well as for disease modifying and antiepileptogenic drugs especially from plants. This study was conducted to investigate the anticonvulsant activity of the crude hydroalcoholic extracts of 3selected plants *Pterlobium stellatum* (leaves), *Moringa stenopetala* (root) and *Clutia abyssinica* (leaves). These all are used for treatment of epilepsy by traditional healers in different parts of Ethiopia. Additionally the solvent fractions of the *Pterlobium stellatum* were tested for anticonvulsant activity as the crude extract showed positive response in all the models used.

Methods :.The dry residues of the plant extracts were used for test in different doses. Male balb c mice were used for *in vivo* study and for *in vitro* study P14-P21 of C57BL16 mice were used. *In vitro* mice model of hippocampal slice with $0Mg^{2+}$ was used and the extracts were tested at the 0.7mg/kg concentration. Diazepam 3µM was positive control and DMSO as negative control. In *in vivo* PTZ and MES mice models 400mg/kg and 800mg/kg of each test extract were used for efficacy test in as positive control Diazepam 5mg/kg and phenytoin 10mg/kg were used respectively. The negative control was 2% tween 80 .Fisher's exact test was used to analyze proportions and ANOVA with post hoc LSD to test means. The tests were conducted after the ethical clearance was obtained from the ethical committee of Addis Ababa University and university of Cape Town. Qualitative test and quantitative tests were used to determine the

secondary metabolites in the plants. And ultraperformance liquid chromatography-mass spectrometer (UPLCMS) tests were used to characterize plant constituents in *Pterlobium stellatum* crude extract and its fractions.

Results : In the *in vitro* study the hydroalcoholic extract of *P.stellatum*and *M. stenopetala*at 0.7mg/ml had a statistically significant anticonvulsant activity compared to negative control($P<0.05$). The hydroalcoholic extract of *C.abyssinica*at 0.7mg/ml didn't show statistically significant effect compared to negative control ($P>0.05$). A positive control, diazepam(3μM), showed statistically significant anticonvulsant activity compared with negative control ($P<0.05$).When we compare the *in vitro* activity of different solvent fractions of *P. stellatum*,the chloroform and water fractions at 0.7mg/ml were also shown to have significant anticonvulsant activity as compared to negative control ($P<0.05$). The petether and butanol fractions activities were not statistically significant compared to negative control ($P>0.05$). *Pterolobium stellatum* hydroalcoholic extract shown that dose dependent and statistically significant anticonvulsant activity with PTZ model($P<0.05$).Whereas the activity of *M. stenopetala* and *C. abyssinica* hydroalcoholic extracts were not statistically significant ($P>0.05$) in *in vivo* PTZ model. The *in vivo* PTZ test has also revealed the chloroform fraction and the water fraction of *Pterolobium stellatum* to have anticonvulsant effect($P<0.05$) compared with the negative control. Whereas the pet ether and butanol fractions shown activity which was not statistically significant with negative control($P>0.05$).The effect of the diazepam was statistically significant with the negative control and all test extracts ($P<0.05$). *Pterolobium stellatum* and *M. stenopetala* hydroalcoholic extracts shown statistically significant anticonvulsant activity with MES model in both lower and higher doses($P<0.05$). The *C. abyssinica* hydroalcoholic extracts activity at the

given doses were not statistically significant (P>0.05).The *in vivo* MES test has also revealed the chloroform fraction to have anticonvulsant effect at both doses (P<0.05). The water fraction at 400mg/kg dose shown anticonvulsant effect compared with the negative control(P<0.05). The pet ether and butanol fractions shown activity which was not statistically significant at the given doses (P>0.05).The effect of the phenytoin was statistically significant with the negative control as well as compared with other tested extract doses(P<0.05). The qualitative and quantitative analysis indicated the presence of different plant secondary metabolites in hydromethanolic extracts of the three plants. In *Pterolobium stellatum* the UPLCMS analysis indicated also the presence of gallic acid, ellagic acid, kaempferol, myricitrin, isoquercitrin and quercitirin in the crude extract. Of these gallic acid and ellagic acid were found in chloroform fraction. In the water fraction ellagic acid, kaempferol, myricitrin and isoquercitrin were found.

Conclusion: The results demonstrated that *Pterolobium stellatum* has anticonvulsant effect. The hydroalcoholic and chloroform and water fraction of *Pterolobium stellatum* demonstrated effect in both *in vitro* and *in vivo* MES and PTZ models of epilepsy. The crude extract of *Moringa stenopetala* has also shown to have anticonvulsant effect both in the *in vitro* and *in vivo* MES models. But was negative on PTZ model. The traditional use of both herbs for treatment of epilepsy can be supported by the finding of this study. However ,*C. abyssinica* didn't show anticonvulsant activity at the tested doses in the models used in this study and its traditional use for treatment of epilepsy is not supported according to the findings of this study.

Key words: *Pterolobium stellatum, Moringa stenopetala, Clutia abyssinica, in vitro, in vivo, anticonvulsant, mice*

ACKNOWLEDGEMENTS

Above all I thank almighty God for helping me through all my studies.

I am so grateful for my advisor Professor Teferra Abula in his unreserved guidance from proposal development , execution of the work and development of the work in this form. My sincere gratitude goes also to Dr. Joseph V. Raimondo in his kind help in my research work in university of Cape Town. I am also grateful to Dr. Gary Stafford at University of Pretoria for plant chemical analysis. I am thankful for the staff of Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, Addis Ababa University in their professional assistance and the department pharmacognosy for their cooperation in laboratory work.

I am indebted to the School of Graduate Studies, Addis Ababa University, for financial assistance and also Arba Minch University in providing me financial help in the work process. I am grateful for my families and friends in all their encouragement.

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ABBREVIATIONS

aCSF**:** artificial cerebrospinal fluid

ADD: Anticonvulsant Drug Development

AEDs : Antiepileptic drugs

4AP: 4-aminopyiridine

ALA: Alpha-linolenic acid

AMPA : α-amino-3-hydroxy-5-methylisoxazole 4-propionic acid

ANOVA: analyses of variance

AP : Action potential

ASD: Anti-seizure drugs

ATP: adenosin triphosphate

BDNF: Brain derived neurotrophic factor

BNZ: Benzodiazepine

CA : *Cornu Ammonis*

CI : Confidence Interval

CM: complementary medicine

CN : channel

CNS : Central Nervous System

DALYs : Disability-Adjusted Life Years

DHA :Docosahexaenoic acid

DG : dentate gyrus

EC: enterihinal cortex

ED : Effective dose

EEG : electroencephalography

EPA: Eicosapentaenoic acid

EPSPs : excitatory postsynaptic potential

GABA : γ-aminobutyric acid

GAD : glutamic acid decarboxylase

GALR : galanin receptor

gm: gram

HIV: human immunodeficiency virus

HPLC: High-performance liquid chromatography

hr. hour

Hz: hertz

ILAE : International League Against Epilepsy

i.p: intraperitonial

IPSPs : inhibitory postsynaptic potentials

IR :Infrared spectroscopy

KA : kainic acid

KCNQ :Potassium Channel Q

LEV: Levetiracetam

LIS: leptazole-induced seizure

MES: maximal electroshock seizure

MHD:monohydroxy

MIS: metrazole induced seizures

MRI : magnetic resonance imaging

mA: milli Amper

mV: milli volt

MTLE: mesial temporal lobe epilepsy

NINDS : National Institute of Neurological Disorders and Stroke

NMDA :*N*-methyl-D-aspartate

NMR: neuromagnetic resonance

No. : Number

PDS : paroxysmal depolarizing shift

PEMA : Phenylethylmalonamide

PIC : picrotoxin-induced convulsions

PILO : pilocarpine

PTZ: pentylenetetrazole

PUFAs :Polyunsaturated fatty acids

RMP : resting membrane potential

SB: spontaneous bursts

s.c. : subcutaneous

sec: second

SLE: seizure like event

SPSS: Statistical Package for Social Sciences

STR: strychnine

SIS: strychnine-induced seizures

SV: synaptic vesicle:

TD: Toxic dose

TG: treatment gap

TLC: Thin layer chromatography

TM: Traditional medicine

TrkB: tropomycin receptor kinase B

TTX: tetrodotoxin

UCB : Brivaracetam

UPLC-MS: ultraperformance liquid chromatography-mass spectrometer

USA : United States of America

VEEG : video–electroencephalography

VPA : valproic acid

WHO: world health organization

CHAPTER I: INTRODUCTION

1. 1. Epilepsy

1.1. 1. Definition

Epilepsy is chronic disorder of the brain characterized by an enduring disposition towards recurrent unprovoked seizures. The diagnosis requires at least 2 seizures occurring greater than 24 hours apart or one seizure with a relevant abnormal electroencephalographic (EEG) pattern or brain scan suggesting a high probability of a second seizure (Fisher *et al.*, 2014; WHO, 2019a). The current definition requires two unprovoked seizures occurring at least 24 hours apart. Conceptually, epilepsy exists after at least one unprovoked seizure, when there is high risk for another, although the actual required risk is subject to debate. After a single unprovoked seizure, risk for another is 40–52%. With two unprovoked non febrile seizures, the chance by 4 years of having another is 73%, with a 95% confidence interval (CI) of 59–87%, subsequently herein portrayed as approximately 60–90% (Fisher *et al.*, 2014). The risk level occurs with remote structural lesions, such as stroke, central nervous system (CNS) infection, certain types of traumatic brain injury, diagnosis of a specific epilepsy syndrome, or in some circumstances with the presence of other risk factors. Those with recurrent reflex seizures, for example, photosensitive seizures, are also considered to have epilepsy. This definition of epilepsy brings the term in concordance with common use by most epileptologists. Epilepsy is not necessarily life-long, and is considered to be resolved if a person has been seizure-free for the last 10 years, with at least the last 5 year off antiseizure medicines, or when that person has passed the age of an age-dependent epilepsy syndrome(Fisher *et al.*, 2014).

1.2. Epidemiology of Epilepsy

Epilepsy is a chronic disorder of the brain that affects people of all ages worldwide. It is one of the world's oldest recognized conditions (WHO, 2019a). Almost 10% of people will experience a seizure during their lives. Epilepsy is the third leading contributor to the global burden of disease for neurological disorders and affects 65 million people worldwide (Devinsky *et al.*, 2018).The estimated proportion of the general population with active epilepsy (i.e. continuing seizures or with the need for treatment) at a given time is between 4 and 10 per 1000 people. However, some studies in low and middle income countries suggest that the proportion is much higher, between 7 and 14 per 1000 people. Close to 80% of people with epilepsy live in low- and middle-income countries. Globally, an estimated 2.4 million people are diagnosed with epilepsy each year. In high-income countries, annual new cases are between 30 and 50 per 100 000 people in the general population. In low- and middle income countries, this figure can be up to two times higher. The higher figure in low- and middle-income countries is likely due to the increased risk of endemic conditions such as malaria or neurocysticercosis; the higher incidence of road traffic injuries; birth-related injuries; and variations in medical infrastructure, availability of preventive health programmes and accessible care(WHO, 2019a). Prevalence or incidence may be underestimated in areas where the condition is greatly stigmatized and cultural beliefs about the causes of epilepsy or negative attitudes toward those with epilepsy lead to the concealing of symptoms of epilepsy, or its diagnosis (Banerjee, Filippi and Allen Hauser, 2009). If we consider studies performed on population sizes up to 1000, prevalence rates of epilepsy in the African region range from 2.2 to 58 per 1000. Highest rates of are mainly detected in rural populations. They reflect poor health conditions resulting in several public health related diseases complicated by epileptogenic changes in the brain (WHO, 2004). According to the fact

sheet on epilepsy (no. 999) published by the World Health Organization(WHO) in May 2015, epilepsy accounts for 0.75% of the global burden of disease, as measured by combining years of life lost due to premature mortality and years lived in less than full health (Figure 1). In 2012 epilepsy alone was responsible for 20.6 million disability-adjusted life years (DALYs) lost (Abramovici and Bagić, 2016).

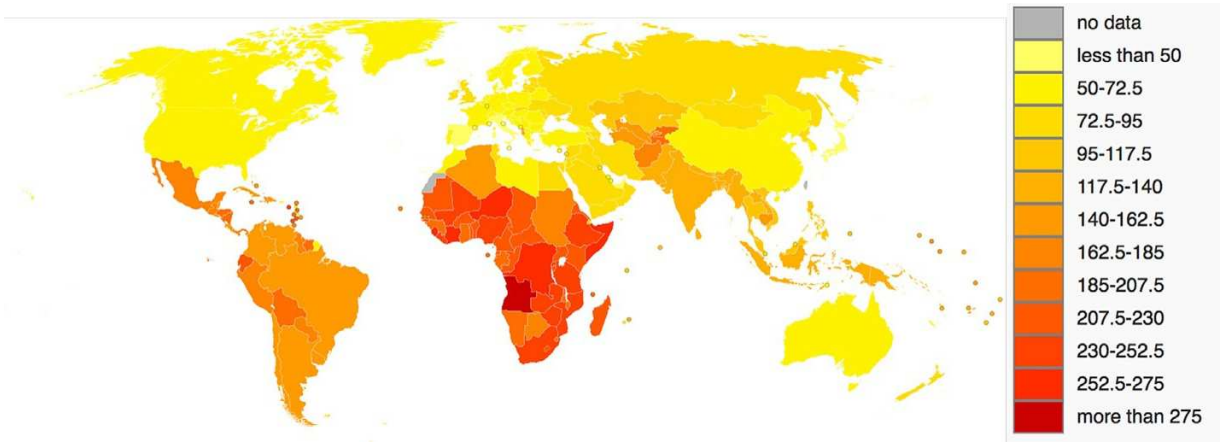


Figure 1. Disability-adjusted life-year rates from epilepsy by country (per 100 000 persons) in 2004 (Abramovici and Bagić, 2016).

1.3. Classification of Epilepsy

Determining the type of seizure that has occurred is essential for focusing the diagnostic approach on particular etiologies, selecting the appropriate therapy, and providing potentially vital information regarding prognosis(Longo *et al.*, 2012). The International League Against Epilepsy (ILAE) is the most important international organization dedicated to clinical care,

education and research in epilepsy. In 1981 it issued a classification for epilepsy that has been very widely adopted. Recognizing a number of deficiencies in this classification, the ILAE released a new seizure classification in 2017 (Liyanagedera, Williams and Bracewell, 2017; Fisher *et al.*, 2018)

This classification presents a new framework for classification of the epilepsies with three levels, as well as a major focus on looking for a cause and identifying any associated disorders, or co-morbidities, at all stages along the diagnostic process. It begins with seizure type(s) defined by their type of onset (focal; generalized; unknown), then epilepsy type and thirdly, epilepsy syndrome. Wherever possible, the aim is to classify a patient's epilepsy in a way that is recognizable across a range of individuals experiencing the same pattern of seizures, age at onset, and electroencephalographic(Michalis Koutroumanidis *et al.*, 2017)and imaging features, who often share a similar cause for their epilepsy. The revised classification also emphasizes the importance of considering the cause, or etiology, of the patient's epilepsy from the initial consultation onwards. It presents six broad headings for the etiologies (Figure 2)(Chang *et al.*, 2017; Brodie *et al.*, 2018; Falco-Walter, Scheffer and Fisher, 2018).

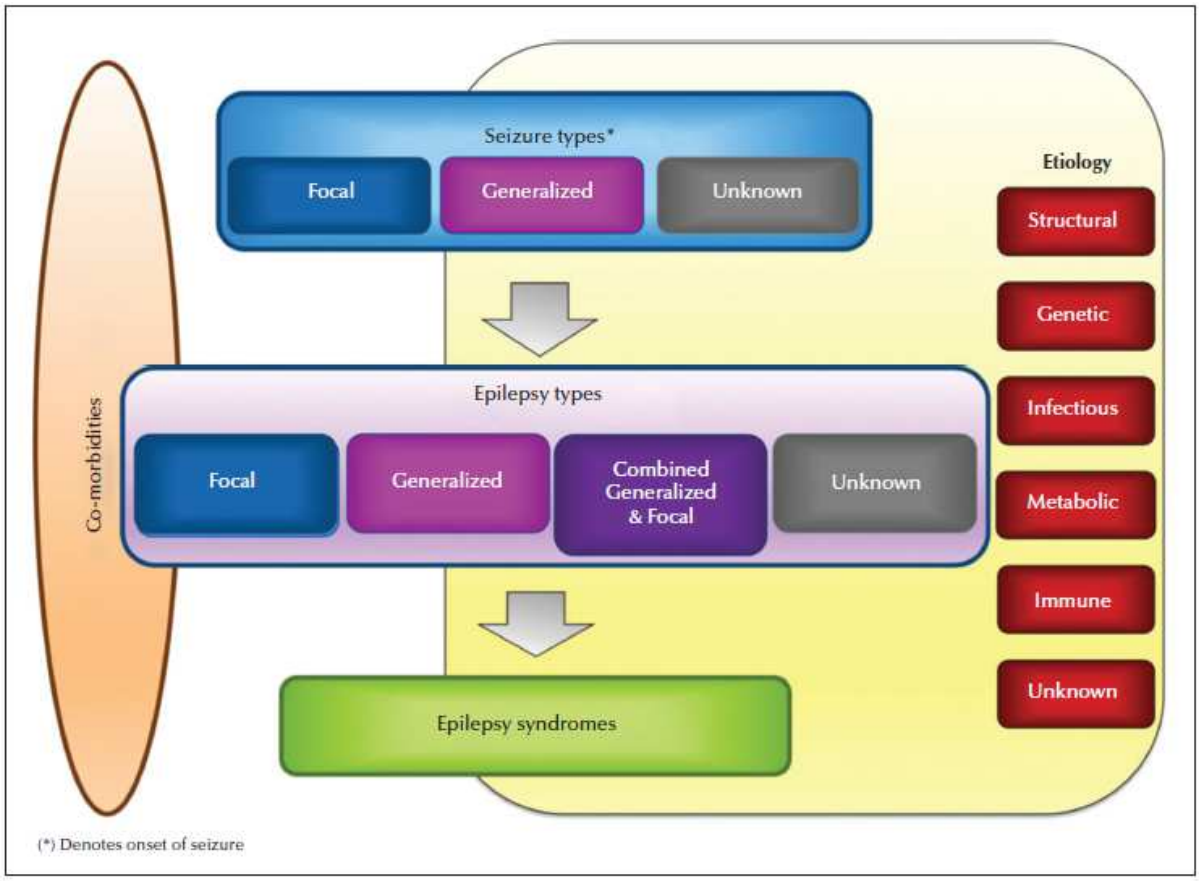


Figure 2. Framework for epilepsy classification (Brodie *et al.*, 2018).

1.3.1.Generalized Epilepsy

In these events, the abnormal electrical activity (as judged by behavior or EEG) apparently originates simultaneously on both sides of the brain and spreads rapidly via neuronal networks. Most people will recognize a generalized tonic-clonic seizure (convulsive seizure) as a typical sign of epilepsy. However, there is a range of other generalized seizures. These include absences, where the affected individual, usually a child or adolescent, loses awareness for a number of seconds resulting in a blank stare. This may be accompanied by more subtle signs, such as

flickering of the eyelids and mouth movements. Myoclonic jerks are also types of generalized seizures and occur when there is a sudden rapid contraction of a group of muscles. They can affect the head, arms, legs or whole body and can be unilateral or bilateral. The affected person may drop or spill things and, if the jerking is severe or occurs in a young child, they can fall. Because the jerk can last less than a second, there is no obvious loss of consciousness. Frequent myoclonic seizures can also occur in some severe epilepsies of infancy and early childhood. Other less common generalized seizure types include atonic (loss of muscle tone) and tonic (more prolonged increase in muscle contractions) seizures, both of which can also result in falls or drop attacks and epileptic spasms(Liyanagedera, Williams and Bracewell, 2017; Brodie *et al.*, 2018; Fisher *et al.*, 2018).

1.3.2. Focal Epilepsy

In these events, the abnormal electrical activity originates on one side of the brain, although in some situations it can spread to the other side later in the seizure. Focal seizures can present with a range of symptoms, depending on the site of origin of the abnormal electrical discharges and the extent and speed of their spread in the brain. Awareness may be present, reduced or absent(Bhasin and Sharma, 2019). Sometimes, there is jerking of one arm and/or leg. Epileptic spasms can also have a focal origin. The abnormal electrical activity can move quickly from a focal seizure to a tonic- clonic seizure, affecting both sides (bilateral), known as a focal to bilateral tonic-clonic seizure. The EEG may suggest an area in the brain from where the seizure is arising, and brain imaging may demonstrate a structural cause for the seizures, such as scarring, a developmental anatomical abnormality (brain malformation),an abscess, stroke or

tumor. In around a third of people with focal seizures, brain imaging will be reported as normal (Brodie *et al.*, 2018; Kumar and Sharma, 2018; Bhasin and Sharma, 2019).

1.3.3. Focal and generalized epilepsy

The next group consists of people who have both focal and generalized seizures. The video–electroencephalography (VEEG) (Koutroumanidis *et al.*, 2017) can be helpful in defining this category. In the severe epilepsies of infancy and childhood, the EEG is often markedly abnormal. There is often evidence of more than one type of seizure (Brodie *et al.*, 2018).

1.3.4. Unknown epilepsy

Occasionally, the doctor cannot be certain whether the epilepsy is focal or generalized. This is more common where there is limited access to VEEG studies and modern brain imaging such as magnetic resonance imaging (MRI) (Brodie *et al.*, 2018). The 'unknown' epilepsy type is the epilepsy in which the seizures are of unknown onset type or the clinician has not yet gather sufficient clinical information to be certain about the epilepsy classification. Similar to seizure of unknown onset, unknown epilepsy type may be relabeled as other types of epilepsy once adequate clinical information allows further classification. Figure 3 depicts current seizure classifications (Falco-Walter, Scheffer and Fisher, 2018).

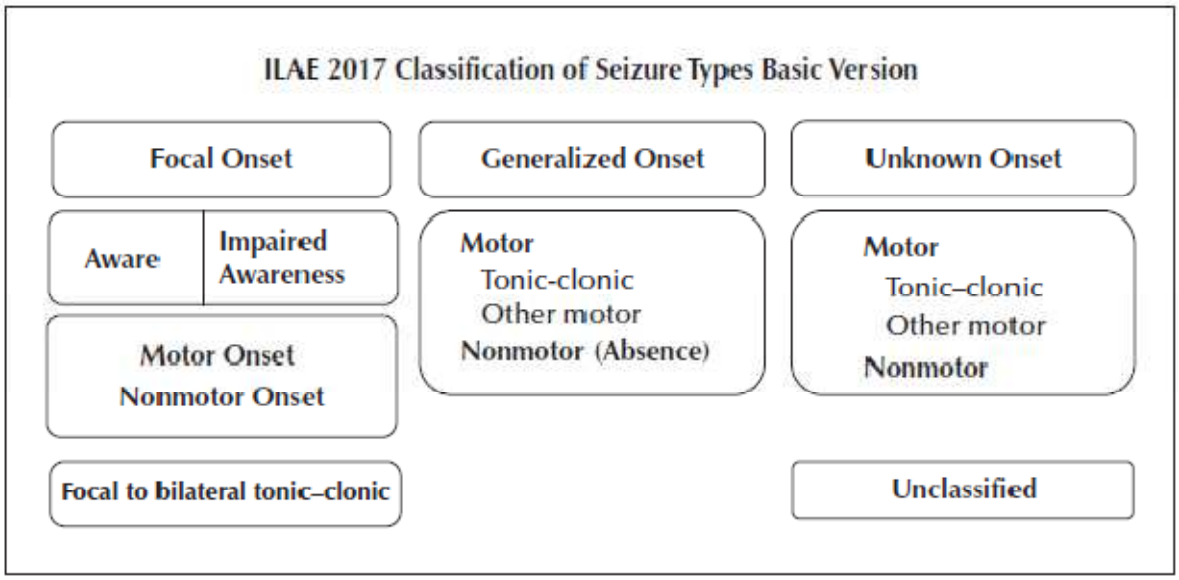


Figure 3: The basic ILAE 2017 operational classification of seizure types (Fisher *et al.*, 2018).

1.3.5. Epilepsy syndromes

The third level of diagnosis, wherever possible, is the identification of an epilepsy syndrome. This includes a cluster of features, including seizure types, EEG changes, brain imaging abnormalities, and genetic analysis that add up to a recognizable pattern (Falco-Walter, Scheffer and Fisher, 2018). Different syndromes can occur at different ages in life, and an accurate diagnosis often provides useful information on the likely outcome. Some syndromes are associated with other symptoms, such as intellectual and psychiatric problems, which may play an important part in the overall clinical picture. The recognition of a syndrome can help to determine the cause, treatment, and outcome of the epilepsy. New epilepsy syndromes are described in the emerging literature fairly often, and there is currently no "official" ILAE list of all the syndromes (Brodie *et al.*, 2018). Among the generalized epilepsies are a well recognized

group of common epilepsy syndromes: childhood absence epilepsy, juvenile absence epilepsy, juvenile myoclonic epilepsy, and generalized tonic-clonic seizures alone. They have previously been known collectively as the "idiopathic generalized epilepsies", which means that the exact cause is unknown, but the evidence is strongly in favour of a genetic basis. Less frequently, they may have an obvious hereditary component, *i.e*. run in families. In the new classification, they can be called "genetic generalised epilepsies" or "idiopathic generalised epilepsies" depending on whether the clinician finds the term "genetic" acceptable for their patient and family (Brodie *et al.*, 2018).

Another group of focal epilepsy syndromes that occur in childhood have a self-limited course. Often the diagnosis is made by the presence of spikes in a particular pattern on the EEG that relate to electrical malfunctioning in a particular part of the brain (Michalis Koutroumanidis *et al.*, 2017). These conditions may be treatment responsive and self-limiting, and usually affect the temporal, frontal or occipital lobes in the brain. Recognition of a particular syndrome can provide important information on the best approach to management and can shine a light on the likely long-term outcome.

1.4. Causes of epilepsy

As soon as a person has his/her first epileptic seizure, everyone involved wants to know the cause. A range of possibilities can be recognized, which may help with understanding the problem and point to its optimal treatment. It should be appreciated that a specific reason why seizures occur cannot always be identified. Although the cause of epilepsy in many patients is unknown, seizures can be the result of almost any insult that perturbs brain function. These

insults include acquired causes (for example, after stroke or traumatic brain injury), infectious diseases (such as neurocysticercosis), autoimmune diseases and genetic mutations (Devinsky*et al.*, 2018). As our knowledge improves and the availability of more sophisticated investigations is becoming more widespread, this "unknown" group of epilepsies is becoming smaller. The six recognized causative categories are highlighted in figure 2. It should be appreciated that the patient's epilepsy may belong to more than one group of causes. For example, a genetic disorder may cause a structural abnormality of brain development, which leads to the epilepsy. This would be termed a "structural" and "genetic" cause (Brodie *et al.*, 2018).

1.4.1. Structural causes

Structural causes of epilepsy can be recognized via a range of brain imaging investigations. The anatomical abnormality needs to relate directly to the symptoms and signs of the seizures, since many people without epilepsy have abnormal brain imaging. The past history may be a useful contributory factor, *e.g.* a previous head injury, stroke, tumor, birth injury, brain infection, *etc.*, which may be associated with the particular type of seizures under scrutiny. This association may take some time to establish. In some cases, a positive brain scan will provide the basis for subsequent epilepsy surgery, usually after treatment with appropriate antiepileptic drugs has failed. Structural abnormalities are usually acquired, although on occasion the patient may be born with an anatomical defect that may be part of a genetic syndrome. Recognizing the cause of the epilepsy can be reassuring to all concerned and can point the way to the best avenue of care. The more advanced the available technology, the more likely will a relevant anatomical abnormality be identified (Brodie *et al.*, 2018).

1.4.2. Genetic causes

Genetics can be considered as the part of biological sciences that is concerned with the study of genetic variation and heredity. Genetic factors are probably the most important single causative group for the epilepsies. However, we still cannot find a precise defect in most people with suspected genetic epilepsy. Epilepsies can be called “genetic” if we know that there is a strong family history, whether an implicated gene is discovered in the family or not. We know that some common types of epilepsy are largely caused by genetic factors. For instance, when epilepsy develops in identical twins, both twins will almost always be affected. Several hundred genes have now been linked to different epilepsies. The vast majority of these are associated with rare syndromes, which most often present in early childhood. Information in this area is expanding with the development of more sophisticated methodology. Identification of a potential genetic cause for the epilepsy can provide insights into what medication to choose and sometimes what not to choose for treating the seizures. Occasionally, an underlying genetic cause cannot be identified, despite the fact that a number of people in the family have a similar type of epilepsy. In addition, a range of different types of seizures can occur in some families. Although genetic syndromes are most often diagnosed in infancy or childhood, genetic disorders can also arise in adolescence or even later in adult life. Sometimes, a single gene defect is the culprit, while in other cases, multiple genes are involved in causing the individual’s epilepsy. Interestingly, the same genetic abnormality can produce different types of seizures indifferent people, and different gene defects can cause the same epilepsy syndrome. The same genetic abnormality can result in both mild and severe epilepsies. There must, therefore, be other factors that influence how the implicated gene affects the individual, *e.g*. interacts with other genes.

Often, it is thought that multiple genes contribute to the clinical picture, especially in the common situation where there is no family history of epilepsy.

It is important to appreciate that a genetic cause does not necessarily mean the person has an inherited epilepsy. An affected individual can have a new gene abnormality, or mutation, that does not occur in anyone else in the family. However, this person may have a risk of subsequently passing on the abnormal gene to his or her children. To make matters even more complicated, although around 50% of the children may inherit the mutation, this does not necessarily mean that they will all develop epilepsy, as this can depend on the presence of a range of other, as yet, unidentified factors. The next decade or two will see an acceleration in our understanding of the genetic bases of the epilepsies (Brodie *et al.*, 2018).

The most important recent progress in epilepsy research has been the identification of genetic mutations associated with a variety of epilepsy syndromes. Although all of the mutations identified to date cause rare forms of epilepsy, their discovery has led to extremely important conceptual advances. For example, it appears that many of the inherited, idiopathic epilepsies (i.e., the relatively "pure" forms of epilepsy in which seizures are the phenotypic abnormality and brain structure and function are otherwise normal) are due to mutations affecting ion channel function. These syndromes are therefore part of the larger group of channelopathies causing paroxysmal disorders such as cardiac arrhythmias, episodic ataxia, periodic weakness, and familial hemiplegic migraine. In contrast, gene mutations observed in symptomatic epilepsies (i.e., disorders in which other neurologic abnormalities, such as cognitive impairment, coexist with seizures) are proving to be associated with pathways influencing CNS development or

neuronal homeostasis. A current challenge is to identify the multiple susceptibility genes that underlie the more common forms of idiopathic epilepsies (Brodie *et al.*, 2018).

1.4.3. Infectious causes

Any infection in the brain or its lining, whether acute or chronic, can produce seizures. Much depends on the part of the world where the infection is contracted in terms of the likely culprit. The commonest infective cause of epilepsy is neurocysticercosis, a tapeworm that is found commonly in Latin America, Africa and Asia. The epilepsy is caused by ingestion of tapeworm eggs. These hatch in the stomach or intestines and the worms migrate to the brain, producing characteristic cysts. Other potential infective causes include human immunodeficiency virus (HIV),tuberculosis, malaria, bacterial meningitis, and viral encephalitis. Treatment of the infection is an essential component of the therapeutic strategy. The more widespread the brain damage, the more likely the seizures will be difficult to control. Sometimes, there is only a history of a previous infection in infancy or childhood, which is assumed to be the cause of epilepsy later in life (Brodie *et al.*, 2018; Falco-Walter, Scheffer and Fisher, 2018).

1.4.4. Metabolic causes

There are a number of unusual and complicated disorders involving the production or breakdown of natural substances in body cells that are also associated with the development of epilepsy. The biochemical changes produced can result in seizures as part of the symptoms of the condition. Thus, they may only makeup a small part of a more complex clinical picture. Many of these conditions have a genetic basis. Recognition of these rare disorders is essential to lead the clinician down the correct treatment path, as in certain cases replacing a missing chemical compound or vitamin may be indicated rather than, or in addition to, providing standard antiepileptic drug therapy (Brodie *et al.*, 2018).

1.4.5. Immune causes

Among the wide range of disorders that can be associated with the production of seizures are immune conditions, where the body attacks its own tissues by the production of antibodies (Vezzani, 2014). The epilepsy can be a consequence of inflammation in the brain and management may require specific medication to damp down the immune system, *i.e*. treat the cause of the seizures rather that the seizures themselves. These autoimmune-mediated epilepsies are unusual conditions that must be recognized promptly to ensure optimal management (Brodie *et al.*, 2018; Falco-Walter, Scheffer and Fisher, 2018).

1.4.6. Unknown causes

Some epilepsies do not have a recognizable cause. Much depends on the availability of routine, and sometimes more sophisticated, investigations. Thus, the number of people who have epilepsy for no obvious reason is higher in resource-poor countries. This often brings its own problems, since all affected individuals and their families want to know why they have developed seizures and why their treatment may be lifelong. The situation may be even more stressful for the family of an affected infant or child in the developing world (Brodie *et al.*, 2018).

In summary, it is hoped that this updated "Classification of the epilepsies" will help to improve the diagnosis, focus better on the cause, and provide a useful guide to management in as wide a range of people with epilepsy as possible. Thus, we can all understand what is happening in the affected person's brain and what is the best course of action to treat the disorder. This new classification is also an important clinical tool for communication among people with epilepsy and their doctors (Brodie *et al.*, 2018).

1.5. Basic Mechanisms of Epilepsy

1.5.1. Action potential (AP)

An AP is a very rapid change in membrane potential that occurs when a nerve cell membrane is depolarised to a sufficient degree. Specifically, the membrane potential goes from the resting membrane potential(RMP) (typically –70 mV) to some positive value (typically about +30 mV)

in a few milliseconds. The threshold for an AP is about –55 mV and is initiated at the axon hillock or initial segments of the axon (the trigger zone). This process is mediated via voltage gated ion channels (Hodgkin, A .L. and Huxley, 1952). A transient depolarizing potential, such as an excitatory postsynaptic potential (EPSP), causes some voltage gated sodium(Na^+) channels to open, and the resultant increase in membrane Na^+ permeability allows Na^+ influx to overpass the potassium (K^+) efflux. Thus, a net influx of positive charge flows across the membrane, and positive charges accumulate inside the cell, causing further depolarization. The increase in depolarization causes more voltage gated Na^+ channels to open, resulting in a greater influx of positive charge, which accelerates the depolarization still further. This positive feedback cycle develops exponentially driving the membrane potential toward the positive values. There are two processes that repolarise the membrane, terminating the AP. First, as the depolarization continues, it slowly turns off, or inactivates, the voltage gated Na^+ channels. This is so, because Na^+ channels have two types of gating mechanisms: activation, which rapidly opens that channel in response to depolarization, and inactivation, which closes the channel if the depolarization is maintained. The second repolarisation process results from the delayed opening of voltage gated K^+ channels. As K^+ channels begin to open, K^+ efflux increases. The delayed increase in K^+ efflux combined with a decrease in Na^+ influx produces a net efflux of positive charge from the cell, which continues until the cell has repolarised to its resting value. The AP propagates actively along the axon to the terminal where it causes neurotransmitter release, which triggers a postsynaptic potential in the target neuron (Wareham, 2005; Badawy, Harvey and Macdonell, 2009a; Fletcher, 2016; Feher, 2017).

1.5.2. Electrical properties of epileptic neurons

Increased excitability is the main feature of an epileptic neuron. The electrical properties of epileptic neurons have been studied with *in vitro* slice preparations of animal or human neocortex or hippocampus, *in vivo* animal models of seizures induced by proconvulsants such as penicillin, and *in vivo* human studies using depth electrical recordings in patients undergoing epilepsy surgery. These studies have shown that there are excessive discharges in epileptic neurons. When these changes occur synchronously in a population of neurons, they correlate with the interictal spike and wave discharge recorded on scalp EEG (Ebersole, 1990). As discussed later, these discharges can either arise from the epileptogenic lesion itself or the surrounding cortex.

The main intracellular component of epileptic discharges is an overt depolarisation, called a paroxysmal depolarizing shift (PDS) that occurs synchronously in a group of neurons. PDS has a slow component of sustained depolarization lasting tens of milliseconds with superadded rapid sharp depolarisations called sustained repetitive or burst firing. At a neuronal level, sustained depolarization is generated from the summation of multiple EPSPs. The rapid depolarisations occurring at the peak of this sustained depolarisation are due to Na^+ channel fluxes, which generate APs that travel down the axon to excite other neurons. During seizure activity, the concentration of extracellular K^+ is increased; resulting in reduced K^+ outflow and the net current will become inward, depolarising the neuron to the extent that calcium (Ca^{2+}) currents will be triggered. Epileptic neurons appear to have increased Ca^{2+} conductance, which may be due to the utilization of latent Ca^{2+} channels, or an increase in the number or efficacy of Ca^{2+} channels. Under normal physiologic conditions, in layer V of the cortex and in the *Cornu Ammonis* $_3$ (CA_3

) region of the hippocampus, there is a subset of pyramidal neurons that can intrinsically generate a burst firing pattern in response to a brief depolarisation. In the epileptic brain it is in these regions that the PDS tends to be seen first. Sustained burst (repetitive) firing is generated through the activation in the dendrites of slow APs mediated by Na^+ and Ca^{2+} currents, providing a sustained depolarisation of the neuron (Badawy, Harvey and Macdonell, 2009b).

The termination of the PDS and neuronal burst firing is predominantly mediated by activation of outward K^+ currents and possibly through inactivation of inward currents. The K^+ channels activated appear to be those sensitive to intracellular Ca^{2+}. The rapidly inactivating K^+ channels contribute to the switching off of the PDS, while the slowly inactivating K^+ channels contribute to the prolonged after hyperpolarisation and neuronal depression seen following spikes. An increase in inward chlorine (Cl^-) currents into the neuron also contributes to the termination of PDSs and post spike sustained hyperpolarisation. In addition, termination of the PDS and burst firing may be mediated by contributions from both $GABA_A$ and $GABA_B$ inhibitory conductances or may involve decoupling of gap-junction mediated currents, which are sensitive to changes in extracellular pH (Badawy, Harvey and Macdonell, 2009b).

1.5. 3. Mechanisms of Seizure Initiation and Propagation

Partial seizure activity can start in a very distinct area of cortex and then spread to the neighboring regions. This implies that there is seizure initiation phase and a seizure propagation phase. Characteristic two concurrent events : (1) high frequency bursts of action potentials and (2) hypersynchronization , occur in an aggregate neurons in initiation phase. The cause for the

bursting activity is a relatively long-lasting depolarization of the neuronal membrane because of influx of extracellular Ca^{2+}, which results in to the opening of voltage-dependent Na^{+} channels, influx of Na^{+}, and generation of repetitive action potentials. This is followed by a hyperpolarizing after potential mediated by γ-aminobutyric acid (GABA)receptors, K^{+} channels, depending on the cell type. The synchronized bursts from an adequate number of neurons result in a so-called spike discharge on the EEG (Shelat, 2015).

Normally, the spread of bursting activity is prevented by intact hyperpolarization and a region of surrounding inhibition created by inhibitory neurons. With sufficient activation there is a recruitment of surrounding neurons via a number of mechanisms. Repetitive discharges lead to the following: (1) an increase in extracellular K^{+}, which blunts hyperpolarization and depolarizes neighboring neurons; (2) accumulation of Ca^{2+} in presynaptic terminals, leading to enhanced neurotransmitter release; and (3) depolarization-induced activation of the *N*-methyl-D-aspartate (NMDA) subtype of the excitatory amino acid receptor, which causes Ca^{2+} influx and neuronal activation. 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 (Figure 4) (Kramer *et al.*, 2007).

Many factors control neuronal excitability, and thus there are many potential mechanisms for altering a neuron's propensity to have bursting activity. Mechanisms intrinsic to the neuron include changes in the conductance of ion channels, response characteristics of membrane receptors, cytoplasmic buffering, second-messenger systems, and protein expression as determined by gene transcription, translation, and posttranslational modification. Mechanisms

extrinsic to the neuron include changes in the amount or type of neurotransmitters present at the synapse, modulation of receptors by extracellular ions and other molecules, and temporal and spatial properties of synaptic and non-synaptic input. Non-neural cells, such as astrocytes and oligodendrocytes, have an important role in many of these mechanisms as well (Kramer *et al.*, 2007).

Certain recognized causes of seizures are explained by these mechanisms. For example, accidental ingestion of domoic acid, which is an analogue of glutamate (the principal excitatory neurotransmitter in the brain), causes profound seizures via direct activation of excitatory amino acid receptors throughout the CNS. Penicillin, which can lower the seizure threshold in humans and is a potent convulsant in experimental models, reduces inhibition by antagonizing the effects of GABA at its receptor. The basic mechanisms of other precipitating factors of seizures, such as sleep deprivation, fever, alcohol withdrawal, hypoxia, and infection, are not as well understood but presumably involve analogous perturbations in neuronal excitability. Similarly, the endogenous factors that determine an individual's seizure threshold may relate to these properties as well (Kramer *et al.*, 2007).

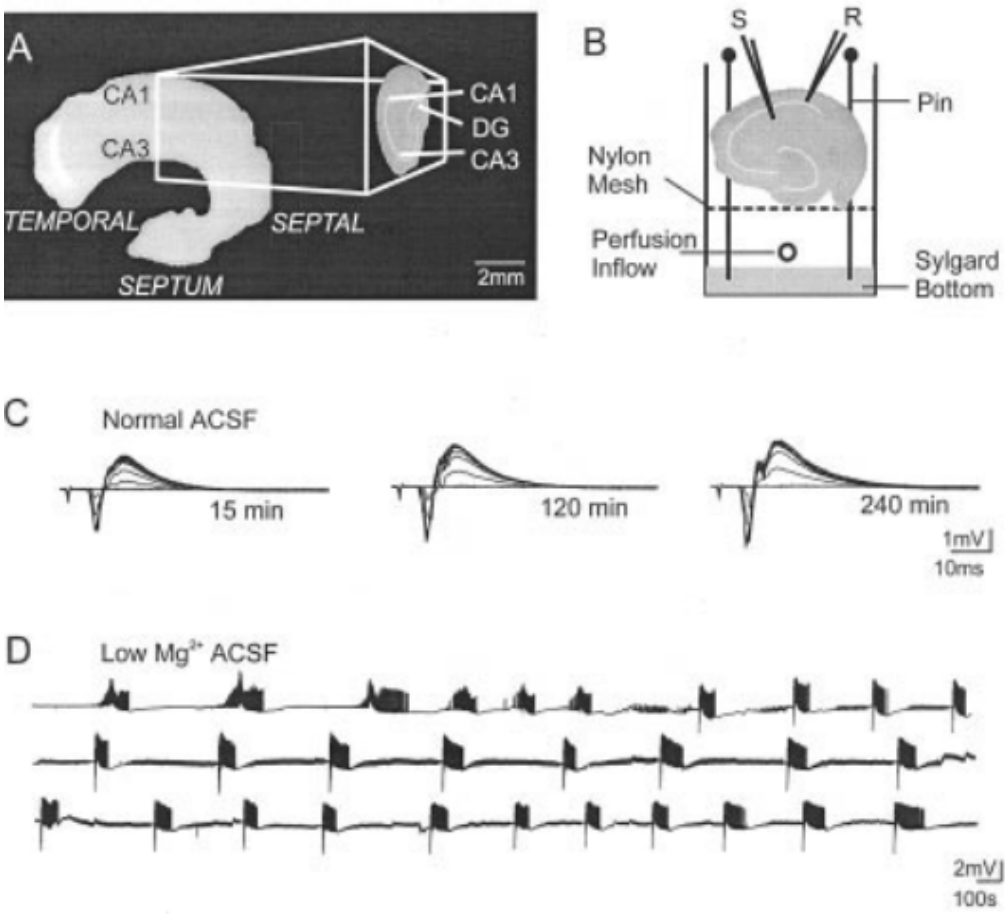


Figure 4. Intact hippocampus: stability and recurrent epileptiform activity. A: Image of the intact hippocampus and the orientation of the DG, CA1, and CA3 layers within the tissue, as visualized by a horizontal hippocampal slice. B: Schematic drawing of the experimental chamber and the orientation of the tissue. C: I/O field recordings after 15, 120, and 240 min of normal artificial cerebrospinal fluid (ACSF) perfusion show relative stability of the field response (P25). D: Representative recording of continuous recurrent epileptiform activity for over 3 h induced by low Mg2(P9**)** (Derchansky *et al*., 2004)**.**

Knowledge of the mechanisms responsible for initiation and propagation of most generalized seizures (including tonic-clonic, myoclonic, and atonic types) remains rudimentary and reflects the limited understanding of the connectivity of the brain at a systems level. Much more is understood about the origin of generalized spike-and-wave discharges in absence seizures. These

appear to be related to oscillatory rhythms normally generated during sleep by circuits connecting the thalamus and cortex. This oscillatory behavior involves an interaction between $GABA_B$ receptors, T-type Ca^{2+} channels, and K^+ channels located within the thalamus. Pharmacologic studies indicate that modulation of these receptors and channels can induce absence seizures, and there is speculation that the genetic forms of absence epilepsy may be associated with mutations of components of this system (Kramer *et al.*, 2007).

1.6. Mechanisms of Epileptogenesis

Epileptogenesis refers to the transformation of a normal neuronal network into one that is chronically hyperexcitable. There is often a delay of months to years between an initial CNS injury such as trauma, stroke, or infection and the first seizure. The injury appears to initiate a process that gradually lowers the seizure threshold in the affected region until a spontaneous seizure occurs. In many genetic and idiopathic forms of epilepsy, epileptogenesis is presumably determined by developmentally regulated events.

Pathologic studies of the hippocampus from patients with temporal lobe epilepsy have led to the suggestion that some forms of epileptogenesis are related to structural changes in neuronal networks. For example, many patients with mesial temporal lobe epilepsy (MTLE) have a highly selective loss of neurons that may contribute to inhibition of the main excitatory neurons within the dentate gyrus (DG). There is also evidence that, in response to the loss of neurons, there is reorganization or "sprouting" of surviving neurons in a way that affects the excitability of the network. Some of these changes can be seen in experimental models of prolonged electrical

seizures or traumatic brain injury (Dudek and Staley, 2012; Goldberg and Coulter, 2013; Sloviter and Bumanglag, 2013; Devinsky *et al.*, 2018). Thus, an initial injury such as head injury may lead to a very focal, confined region of structural change that causes local hyperexcitability. The local hyperexcitability leads to further structural changes that evolve over time until the focal lesion produces clinically evident seizures. Similar models have also provided strong evidence for long-term alterations in intrinsic, biochemical properties of cells within the network, such as chronic changes in glutamate or GABA receptor function (Devinsky *et al.*, 2018). Figure 5 depicts hippocampal sclerosis, the most common identified pathological feature in cases of mesial temporal-lobe epilepsy.

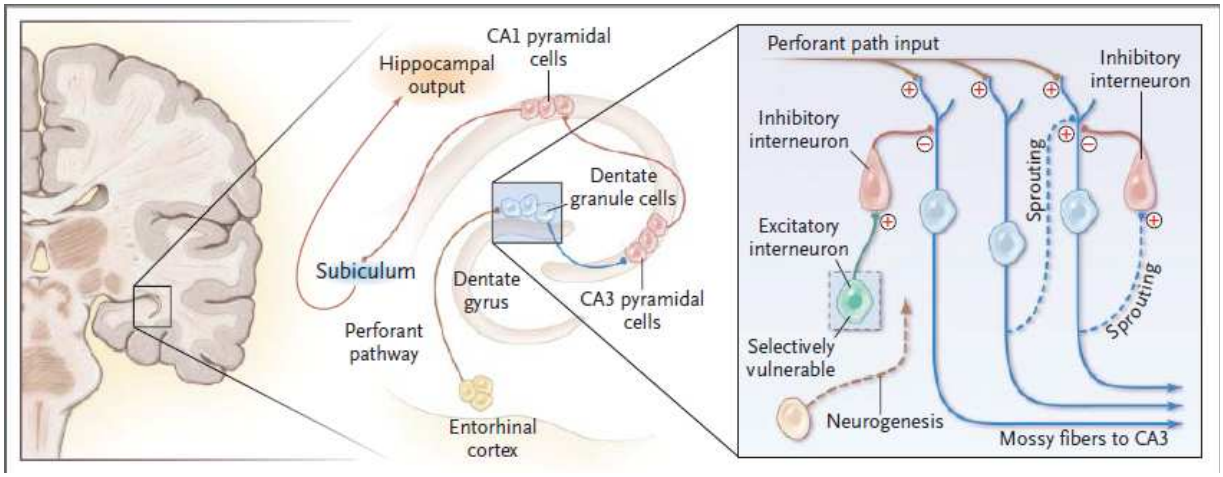


Figure 5. Hippocampal Sclerosis: Hippocampal sclerosis is the most common identified pathological feature in cases of mesial temporal-lobe epilepsy. (Chang *et al.*, 2017).

1.7. Antiepileptic drugs (AEDs)

1.7.1. History of anti-seizure drug development

Bromides were the first antiepileptic drugs to be used in epilepsy in 1857 by Sir Charles Lacock in patients of catamenial epilepsy (Rho and White, 2018). There were no formal trials and experience regarding efficacy and adverse effects were gained with their increasing usage for next 65 years or so. Bromides did reduce the seizure frequency but adverse effects were common. Then phenobarbital arrived. Phenobarbital was initially introduced as sedative and hypnotic but subsequently started to be used for epilepsy from 1919 due to pioneering efforts of Hauptman (Yasiry and Shorvon, 2012). Within next two decades, it became the mainstay of epilepsy treatment. Again, no formal clinical trials were conducted but experience of its efficacy and adverse effects gained through community use. Side effects of phenobarbital were lesser than bromides but did happen and included sedation, CNS depression, and paradoxical hyperactivity in children. By 1937, Merritt and Putnam developed an electroshock model for epilepsy using a cat and thus the drugs could be tested for their efficacy, at least experimentally (Brodie, 2010).

In 1938, they introduced phenytoin which over the next two decades became the standard drug for epilepsy treatment despite the increasing recognition of many adverse effects. With increasing knowledge of chemical nature of the AEDs and greater understanding of their mechanism of action, older AEDs were being modified chemically for greater effectiveness and lesser side effects. Modification of phenobarbital resulted in primidone in 1952 whose action however, was found to be largely due to metabolically active phenobarbital. But it had inferior

tolerability than other traditional AEDs and is rarely used now. About two decades following the introduction of phenytoin, in 1960, a drug called ethosuximide was introduced. It was found to be mainly useful for absence seizures with a fair share of side effects. 1960's also saw the development of a whole new class of drugs originally intended for psychiatry but later extensively used in epilepsy too. These were benzodiazepines (Riss *et al.*, 2008). Although very useful for emergency management, their oral long term use is limited by side effects like sedation and development of tolerance.

In 1974, carbamazepine was introduced for epilepsy in United States of America (USA) although it was already being marketed for trigeminal neuralgia since 1962. This drug proved to be one of the most efficacious, prescribed and studied AEDs produced so far. After a short time, valproate was introduced (in 1978 in USA although it was already in use in many European countries). In USA, its introduction was led by a campaign by J. Kiffin Penry who had reviewed the drug in 1975.It was soon recognized to be a broad spectrum AED and by next three and half decades of its usage, the spectrum of its adverse effects was gradually uncovered (Debashish Chowdhury, 2013).

Fuelled by exponential developments in basic epileptology, molecular biology and clinical electrophysiology, there has been a steady rise in the availability of new AEDs since early 90's, a trend that is continuing till this day. Currently, 7 traditional AEDs and at-least 15 new AEDs are available for treating epilepsy. With multiple options comes the responsibility of choosing the right drug for an individual patient. Serious considerations must be given for efficacy, tolerability, sustainability, remission after withdrawal, drug interactions, quality of life and the

cost of new AEDs (Bialer, 2012; Debashish Chowdhury, 2013). The development of new antiepileptic drugs has not changed the basic principles of the medical therapy of epilepsy , but it has substantially increased treatment choice. So far, it does not appear that the new drugs have greater anticonvulsant potency than conventional agents. However, the new drugs have a more favorable side effect profile, which may represent a significant advantage in the treatment of a chronic disorder. It remains to be demonstrated that this potential advantage outweighs the considerably greater costs of ‘‘modern’’ antiepileptic therapy(Beyenburg and Bauer, 2004).Having these choices approximately 20% to 30% of patients have epilepsy that is resistant to medical therapy despite efforts to find an effective combination of AEDs(Goldenberg, 2010; Kwan, Schachter and Brodie, 2011; Pliakou, Passos and Mironidou-Tzouveleki, 2011; Eskioglou *et al.*, 2018). AEDs introduced to the worldwide market from 1853 to 2016 are indicated in figure 6 below (Rho and White, 2018).

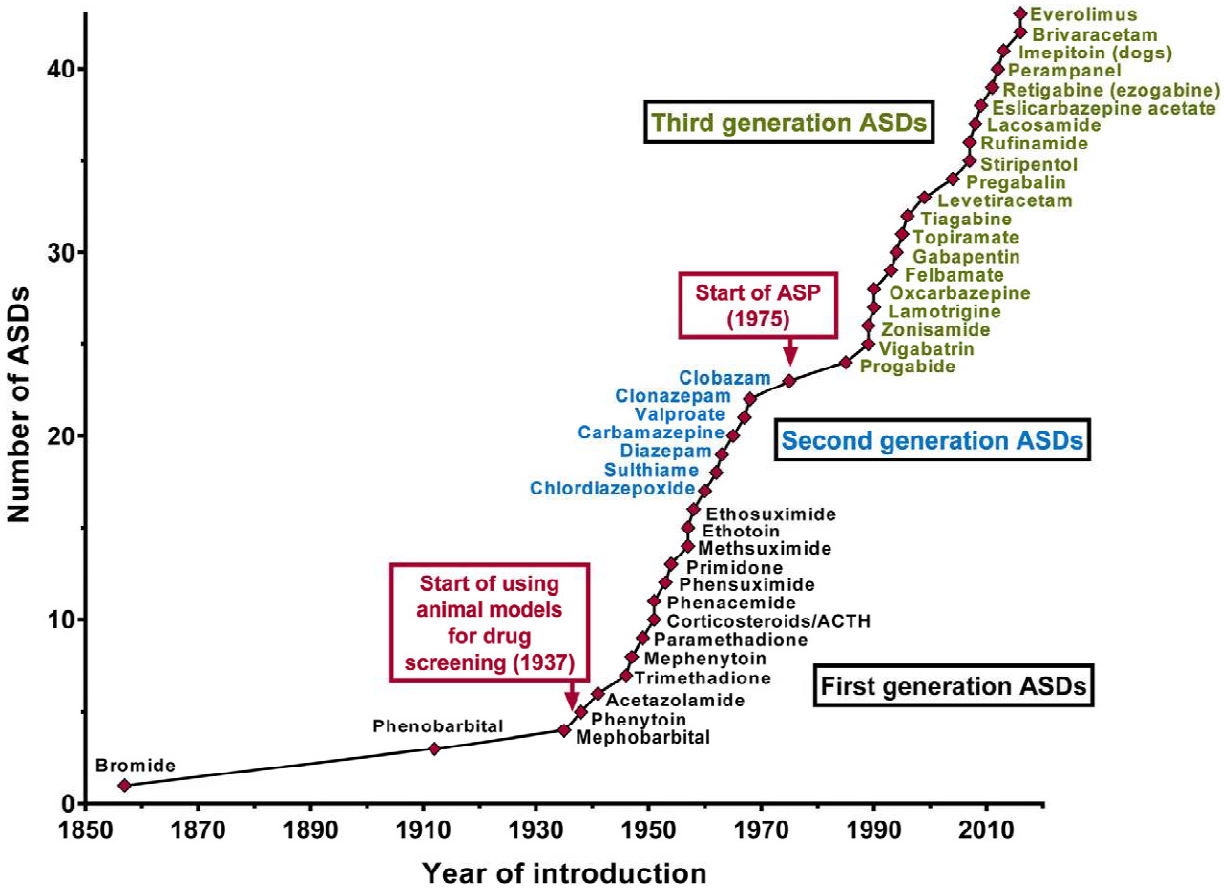


Figure 6. Introduction of ASDs to the worldwide market from 1853 to 2016. (Rho and White, 2018).

1.7. 2. Mechanisms of Action

Antiepileptic drugs (AED) act on diverse molecular targets to selectively modify the excitability of neurons so that seizure related firing can be blocked without disturbing non-epileptic activity which subserves normal signals between neurons(Stefan and Feuerstein, 2007). At the molecular level, the majority of AEDs modulate excitatory and inhibitory neural transmission. Antiepileptic drugs probably exert their anticonvulsant effects both at the cell membrane and intracellularly. The major targets of anticonvulsant drugs include the following: Sodium channels, Calcium channels, $GABA_A$ and $GABA_B$ receptors, Potassium channels, Glutamate, Glutamate receptors

including *N*-methyl-D-aspartate (NMDA) receptors, non-NMDA receptors (α-amino-3-hydroxy-5-methylisoxazole 4-propionic acid(AMPA) and kainic acid (KA)receptors), and metabotropic glutamate receptors, Synaptic vesicle proteins(D. Schmidt and Schachter, 2014; Asadi-Pooya and Sperling, 2016).

How the various drugs prevent or attenuate seizures is not fully understood, and the mechanisms listed below only summarize some of the known potential effects of these agents on the neurons and glia. In an ideal world, the specific defect(s) would be identified that underlie epilepsy in individual patients, and therapy would then be targeted to correct the dysfunctional mechanism. In reality, identification of the specific defect is rarely possible, and empiric therapy must be planned. There is presently no evidence to support the theoretical concept that use of two or more drugs that possess varying putative mechanisms of action offer clear advantage over use of two or more drugs that share similar putative mechanisms of action. The mechanisms listed below are offered to enhance fundamental understanding of the various agents, so that the reader gains further insight into the drugs(Asadi-Pooya and Sperling, 2016). Figure 7 depicts the targets and mechanism of antiseizure drugs.

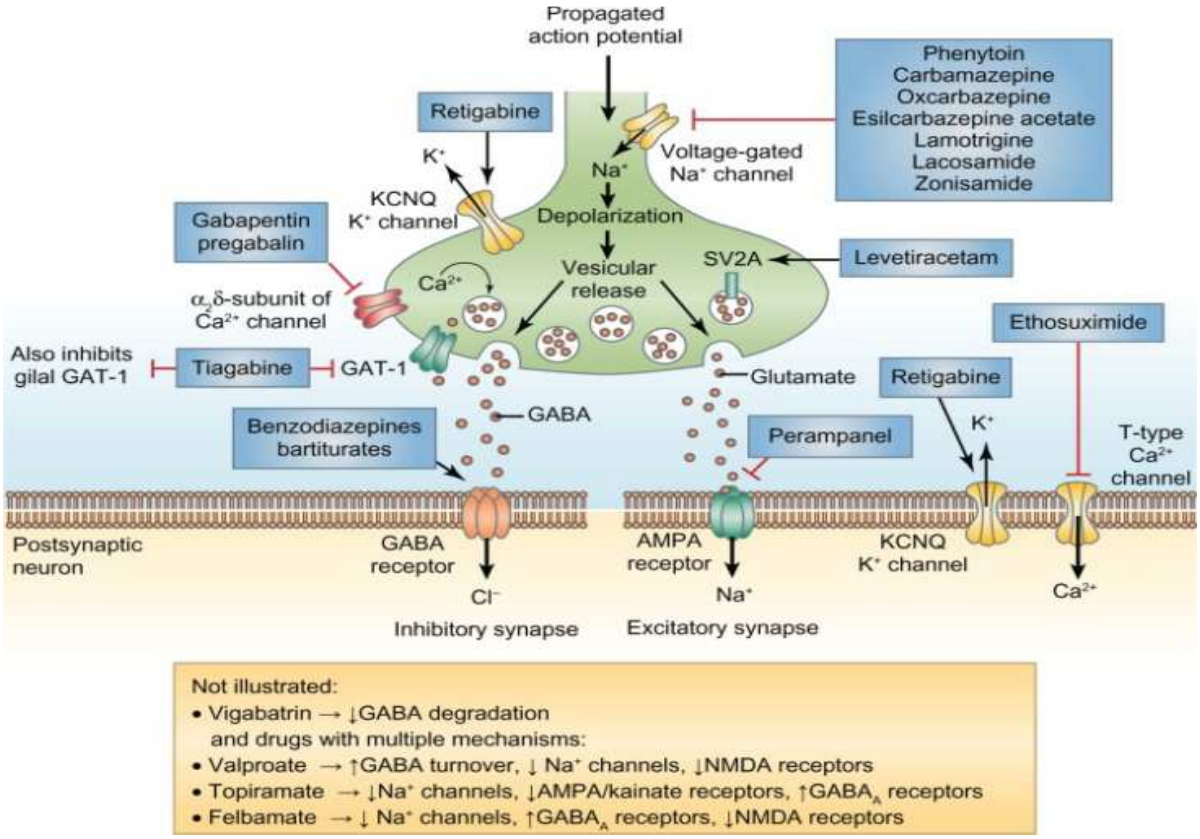


Figure 7. Mechanism of action of available antiseizure drugs

1.7. 2. 1. Sodium Channel Blocking Agents

Drugs sharing this mechanism include phenytoin (*Dilantin*), carbamazepine (*Tegretol*), oxcarbazepine (*Trileptal*), topiramate (*Topamax*), valproic acid (*Depakene*), zonisamide (*Zonegran*), and lamotrigine (*Lamictal*). All of these agents have the capacity to block sustained high-frequency repetitive firing (SRF) of action potentials. This is accomplished by reducing the amplitude of sodium-dependent action potentials through an enhancement of steady-state inactivation. The sodium channel exists in three main conformations: a resting (R) or activatable state, an open (0) or conducting state, and an inactive (I) or non-activatable state. The

anticonvulsant drugs bind preferentially to the inactive form of the channel. Because it takes time for the bound drug to dissociate from the inactive channel, there is time dependence to the block. Since the fraction of inactive channels is increased by membrane depolarization as well as by repetitive firing, the binding to the I state by antiepileptic drugs can produce voltage-, use-, and time dependent block of sodium-dependent action potentials.

The principal mechanism of action of rufinamide is considered to be suppression of neuronal hyperexcitability by prolongation of the inactive state of voltage-gated sodium channels(Cook and Bensalem-Owen, 2011). Lacosamide binds to the slow inactivated state of the voltage-gated sodium channel and evokes a long-lasting, voltage- and frequency dependent decrease in channel conductance (Curia *et al.*, 2009, Wadysaw Lasoñ and Chlebicka, 2013; Holtkamp *et al.*, 2016). Lacosamide stabilizes the slow-inactivated state in contrast to other anticonvulsants that exhibit their effects primarily on the fast-inactivation state (Abdelsayed and Sokolov, 2013). The major mechanism of action of eslicarbazepine is primarily through effects on slow inactivation voltage dependence of sodium channels (Jeklin, 2016; Holtkamp *et al.*, 2018).

1.7. 2. 2. Drugs That Primarily Act Through GABA Potentiating

Potentiation or agonism of GABA receptors and their inhibitory chloride channels is another common mechanism of action for first-generation AEDs, particularly benzodiazepines. Rather than modifying the influx of cations, GABA agonists push the neuron to hyperpolarization by opening chloride channels. Barbiturates also activate GABA receptors by binding to a different site than benzodiazepines. Valproic acid promotes the formation and inhibits the endogenous degradation of GABA, although the clinical impact of this mechanism of action is ill-defined.

Vigabatrin is believed to act as an irreversible inhibitor of GABA transaminase, the enzyme responsible for the metabolism of the inhibitory neurotransmitter GABA. This action results in increased levels of GABA in the central nervous system (Sirven *et al.*, 2012). Tiagabine also increases GABA concentrations, but, unlike vigabatrin, does so by decreasing glial and neuronal uptake of GABA . Topiramate, among its numerous mechanisms of antiepileptic activity, also appears to enhance GABA activity (Cook and Bensalem-Owen, 2011). Felbamate potentiates GABA responses via an interaction with a site on the $GABA_A$ receptor that is distinct from the benzodiazepine recognition site. This action may be of relevance to felbamate's clinical activity (Rogawski, Lö Scher and Rho, 2016). Topiramate also enhances the activity of GABA at $GABA_A$ receptors by increasing the frequency at which GABA activates $GABA_A$ receptors (White, Smith and Wilcox, 2007; Asadi-Pooya and Sperling, 2016).

1.7. 2. 3. Calcium-channel blockade

Blockade of calcium channels also confers some antiepileptic activity. Calcium channels are evident in presynaptic neurons and are involved in neuronal depolarization. Ethosuximide is a unique agent used for absence seizures, which appears to inhibit low threshold calcium channels in thalamic neurons (Davies, 1995). Valproate appears to have similar activity at these T-channels, which also makes this agent helpful for absence seizures. Some evidence suggests that phenytoin may have some activity in inhibiting calcium channel activation presynaptically. Other agents such as felbamate, which antagonizes glutamate–NMDA receptors, and barbiturates, which attenuate the response to excitatory neurotransmitters such as glutamate, inhibit calcium influx postsynaptically (Cook and Bensalem-Owen, 2011). Recent studies suggest that gabapentin has a selective inhibitory effect on voltage-gated calcium channels containing the

$\alpha_2\delta_1$subunit, reducing neurotransmitter release from neurons. It may also potentiate adenosin triphosphate (ATP)-activated inward rectifying potassium channels. Pregabalin is structurally related to gabapentin, but pregabalin has shown greater potency than gabapentin in seizure disorders (3 to 10 times more potent in animal studies). Pregabalin does not show direct GABA-mimetic effects and has no effect on GABAergic mechanisms. Pregabalin reduces neuronal calcium currents by binding to the $\alpha_2\delta$ subunit of calcium channels, and this particular mechanism may be responsible for reduced excitatory neurotransmitter release (White, Smith and Wilcox, 2007; Asadi-Pooya and Sperling, 2016).

1.7. 2. 4. SV_{2A} vesicle inhibition

Levetiracetam is the first of several agents able to inhibit the synaptic vesicle protein 2a (SV_{2A}). This protein is involved in exocytosis and neurotransmitter release. Levetiracetam also interferes with release of intracellular Ca^{2+} initiated by Gq-coupled receptor activation(Asadi-Pooya and Sperling, 2016). Brivaracetam and seletracetam are more potent inhibitors of this presynaptic protein and may provide an even broader spectrum of antiepileptic activity over levetiracetam if they ultimately garner US FDA approval (Cook and Bensalem-Owen, 2011).

1.7. 2. 5. NMDA receptor blockade

N-methyl-d-aspartate (NMDA) receptor antagonists act on membrane-associated postsynaptic calcium channels. The NMDA receptor interacts with the excitatory neurotransmitter, glutamate,

and allows calcium influx into the neuron. This represents one of the major mechanisms for neurotoxicity during traumatic brain injury, stroke and status epilepticus. Several agents can inhibit the action of glutamate on the NMDA receptor, including topiramate and zonisamide. Other older agents, such as felbamate and phenobarbital, may have some modicum of inhibition at the NMDA receptor, although it does not appear that this is the principal mechanism of action of these AEDs (Cook and Bensalem-Owen, 2011).

1.7. 2. 6. AMPA receptor blockade

Perampanel is a non-competitive AMPA receptor antagonist, which binds to a site on the extracellular domain of the channel protein distinct from the glutamate recognition site. Binding of perampanel induces a conformational change in AMPA receptor subunits that limits their ability to translate agonist (i.e. glutamate) binding into channel opening. The effect is to reduce fast excitatory neurotransmission and thereby limit the ability of seizure discharges to spread (Sills, no date; Leo *et al.*, 2018).

1.7. 2. 7. Inhibition of carbonic anhydrase

The anticonvulsant activity of acetazolamide may depend on a direct inhibition of carbonic anhydrase in the central nervous system (CNS), which reduces intracellular bicarbonate levels and may thereby reduce depolarizing GABA responses. It also may alter potassium conductance

that induces membrane hyperpolarization. Zonisamide also has weak carbonic anhydrase inhibiting activity, but this effect is not thought to be an important contributing factor in the anticonvulsant activity of zonisamide (Asadi-Pooya and Sperling, 2016).

1.7.3. New drugs in the pipeline for epilepsy

The following are new drugs in the pipeline for epilepsy: BGG492 (Novartis), a competitive AMPA/kainate receptor antagonist; Brivaracetam (UCB), a novel high-affinity SV_{2A} ligand; CPP-115 (Catalyst), a GABA transaminase inhibitor (vigabatrin derived); ICA-105665 (Pfizer), a highly selective opener of neuronal Kv7 (KCNQ) potassium channels; T2000 (Taro), a non-sedating barbiturate; Tonabersat (Upsher-Smith), which utilizes a novel mechanism of uncoupling of neuronal gap junctions; UCB0942 (UCB), a new pre- and post-synaptic inhibitor; VX-765 (Vertex), a selective inhibitor of interleukinconverting enzyme; YKP3089 (SK Life), which features a novel mechanism of action; 2-Deoxy-D-glucose (Neuro- GenomeX), a glucose analog and glycolytic inhibitor; Ganaxolone (Marinus), a synthetic neurosteroid and $GABA_A$ receptor modulator; Imepitoin, a low-affinity partial agonist at the benzodiazepine site of the $GABA_A$ receptor; NAX 810-2 (NeuroAdjuvants), a galanin receptor 1 ($GALR_1$) and $GALR_2$ agonist; and Valnoctamide (Hebrew University), a valproic acid second-generation derivative (Serrano and Kanner, 2015).

1.7. 4. Epilepsy Treatment

When a neurologist or a physician has made the diagnosis of seizures or epilepsy, the next step is to select the best form of treatment. If epilepsy is diagnosed, the neurologist usually prescribes

seizure preventing medications. If drugs are not successful, surgery, a special diet, complementary therapy, or vagus nerve stimulation may be tried. The goal of treatment is to prevent further seizures, avoid adverse effects, and enable patients to lead active lives (Goldenberg, 2010).

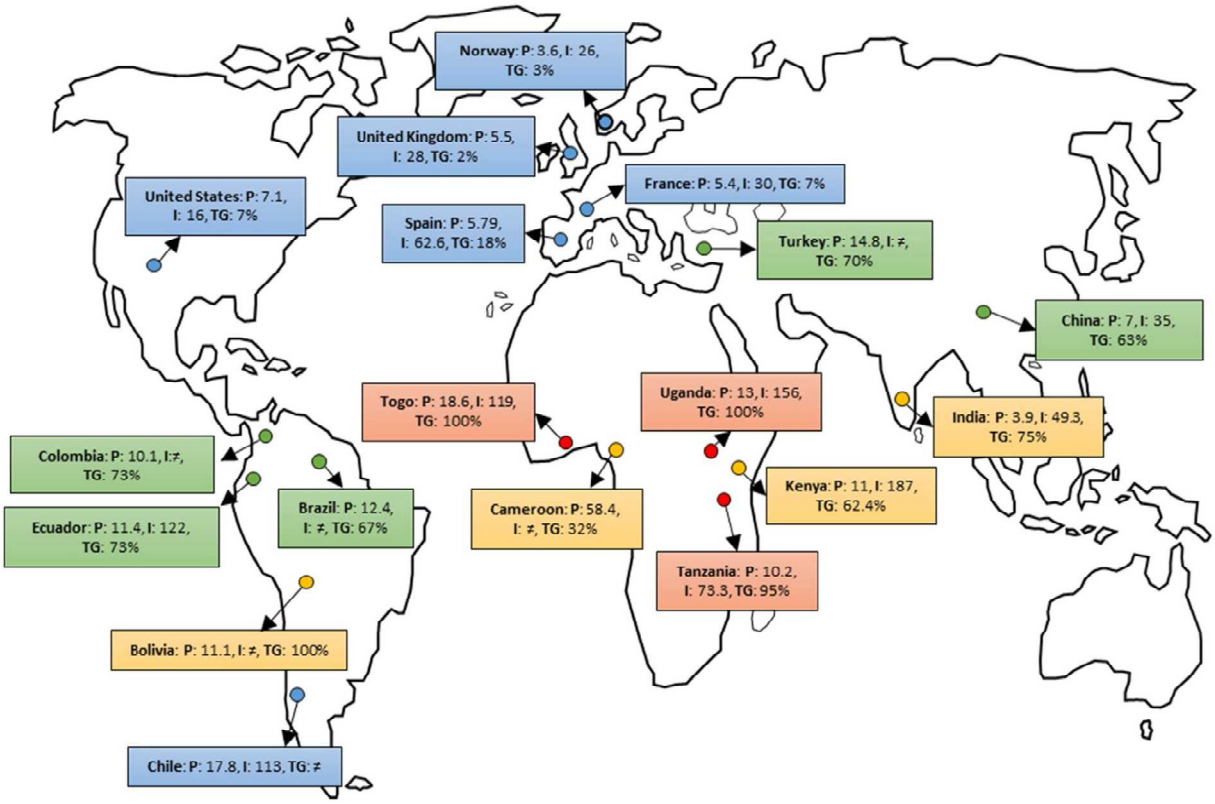


Figure 8. Differences in prevalence, incidence and treatment gap in epilepsy among several countries. The color of the circles represents the World Bank country classification according to the gross national income per capita; red = low income, yellow = lower middle income, green = upper middle income, blue = high income. P = prevalence of active epilepsy, number of cases per 1000 people. I = Incidence of epilepsy, number of new cases per 100,000 people/year. TG = treatment gap expressed as a percentage. 6¼ No data (Espinosa-Jovel *et al.*, 2018).

Since the 1990s, 15 new AEDs have been added to the pharmacologic armamentarium of epilepsy. These include felbamate, gabapentin, lamotrigine,levetiracetam, oxcarbazepine, pregabalin, tiagabine, topiramate, vigabatrin, and zonisamide. The ones included in the last 5

years include lacosamide , rufinamide, ezogabine, eslicarbazepine, and perampanel(Serrano and Kanner, 2015). Common older drugs include valproicacid, phenytoin, carbamazepine, primidone, ethosuximide, clonazepam, and phenobarbital (Goldenberg, 2010).

Despite the fact that AED therapy is widely available, many people with active epilepsy go untreated, particularly in resource-poor countries. It is estimated that 80% of the global health burden represented by epilepsy is borne by the developing world, where 80% of people with epilepsy reside and do not receive modern treatment, or are not even identified(WHO, 2004). Reasons for this treatment gap (TG) are many and in a recent systematic review of this problem in resource-poor countries, the pooled mean of the TG prevalence was 56% (95% CI 33-100). When analysed by region, the mean prevalence of TG was 64.3% (95% CI 24.3-100) in Asia, 55.4 (95% CI 39.0-78.6) in Latin America and 48.9 (95% CI 14.3-100) in Africa. The TG was higher in rural areas (73.3; 95% CI 49.5-100) compared to urban areas (46.8; 95% CI 34.1-64.2). The principal causes identified for TG were inadequate skilled manpower in the local health service (median 70%; range 64-76), cost of treatment (median 62%; range 11-90) and unavailability of drugs (median 53%: range 18-84)(Mbuba *et al.*, 2013). Some other potential causes include the level of health care development, cultural beliefs, distance from health care facilities, , and a lack of prioritization in national health policies(WHO, 2004). Misconceptions and stigma that surround the disorder is the other factor (Ngugi *et al.*, 2010). Effective strategies aimed at reducing the TG in developing countries need to be identified and implemented in order to improve the prognosis of people with epilepsy living in such countries(Mbuba *et al.*, 2013). The prevalence and incidence and treatment gap in different parts of the globe according to previous studies is depicted in figure 8 (Espinosa-Jovel *et al.*, 2018).

Antiepileptic drug (AED) therapy, the mainstay of treatment for most patients with goals: to eliminate seizures or reduce their frequency to the maximum degree possible, to evade the adverse effects associated with long-term treatment,and to aid patients in maintaining or restoring their usual psychosocial and vocational activities, and in maintaining a normal lifestyle (Goldenberg, 2010; Dan Longo, Anthony Fauci, Dennis Kasper, 2018).The decision to start AED therapy should be based on an informed analysis of the likelihood of seizure recurrence, the consequences of continuing seizures for patients, and the beneficial and adverse effects of the pharmacological agent chosen(Goldenberg, 2010).Although currentantiepileptic drugs achieve symptomatic seizure relief, which is why they are more appropriately called antiseizure drugs, they do not prevent or reverse the pathological process that underlies human epilepsy or other clinical manifestations of epilepsy, such as the comorbidity of epilepsy. They therefore do not prevent the development of epilepsy, even in patients at high risk (for example, after brain injury or craniotomy),and nor do they exert disease modifying effects that prevent or reverse drug resistant epilepsy. Also, they do not prevent or eliminate the substantial behavioral, cognitive, and somatic co-morbidities seen in many patients with epilepsy. The drugs have serious adverse effect including the recent generations which necessitates safe treatment options (Table 1) (Heo, 2012).These life limiting currently unmet needs provide a roadmap for the development of more effective antiseizure drugs, as well as for disease modifying and antiepileptogenic drugs (Dieter Schmidt and Schachter, 2014).

Drug	Common adverse events	Serious adverse events
Lacosamide	Dizziness, diplopia, blurred vision, headache, nausea	PR interval prolongation, atrial fibrillation, atrial flutter, hepatitis/nephritis
Lamotrigine	Dizziness, blurred vision, insomnia, headache, rash	Stevens-Johnson syndrome, toxic epidermal necrolysis, multiorgan failure, hepatic failure
Levetiracetam	Fatigue, dizziness, somnolence, irritability, mood swings	Psychosis
Oxcarbazepine	Dizziness, diplopia, blurred vision, headache, nausea, hyponatremia	Stevens-Johnson syndrome, toxic epidermal necrolysis
Pregabalin	Fatigue, dizziness, ataxia, diplopia, weight gain, edema	None reported
Rufinamide	Somnolence, headache, dizziness, diplopia, fatigue, nausea	Shortened QT interval (no known clinical risk), multiorgan hypersensitivity
Topiramate	Drowsiness, ataxia, word-finding difficulty, difficulty concentrating, anorexia, weight loss, paresthesias, metabolic acidosis, oligohydrosis, nephrolithiasis	Acute close angle glaucoma, heat stroke
Zonisamide	Drowsiness, ataxia, difficulty concentrating, anorexia, weight loss, nausea, nephrolithiasis, oligohydrosis, rash	Aplastic anemia, Stevens-Johnson syndrome, toxic epidermal necrolysis, heat stroke

Table 1. Side effects of the new antiepileptic drugs (commonly used)(Heo, 2012).

1.3.5. Drug resistant epilepsy

Drug resistant epilepsy is one of the most important unmet needs in the daily management of epilepsy, and it provides a challenge to our understanding of the mechanisms underlying drug resistance and how it can be overcome or avoided. Any patient in whom at least two trials of adequately selected and dosed antiepileptic drugs have not brought sustained remission fulfils the ILAE criteria for drug resistant epilepsy. Many other definitions exist for different purposes. Epilepsy may also be considered drug resistant if treatment does not stop seizures for 12 months, for whatever reason. By this wide definition, which is based on an influential hospital based observational study, and which is increasingly being used in the US, 36% of newly treated patients have drug resistant seizures. However, if the definition of frequent and severe seizures despite optimal treatment is used, with alternative treatments such as surgery being included, only 5-10% of newly diagnosed patients are estimated to have drug resistant seizures. A

diagnosis of absolute drug resistance may require failure of at least six antiepileptic drugs, because about 17% of patients become seizure free when additional antiepileptic drugs are given, even when two to five drugs have previously failed to control seizures. These data suggest that there is no room for complacency among physicians treating patients who have had persistent seizures over many years despite taking multiple antiepileptic drugs(Dieter Schmidt and Schachter, 2014).

Novel approaches to the development of new drugs are emerging. These offer hope of finding more effective antiseizure drugs to treat ongoing drug resistant epilepsy, antiepileptogenic agents to prevent symptomatic or genetic epilepsy before the first seizure, and disease modifying agents to mitigate established epilepsy. Our understanding of the mechanisms mediating the development of epilepsy, the causes of drug resistance, and the emerging role of pharmacogenetics for drug discovery have grown substantially over the past decade. Finally, new strategies are being explored, such as joint endeavors between academia and industry, identification and application of tools for new target driven and systems biology based approaches, and comparative preclinical proof-of-concept studies and innovative clinical trials designs(Dieter Schmidt and Schachter, 2014).

1.8. Animals models for testing anticonvulsant drugs (Screening)

Animal models play important role to understand the pathophysiology and pattern of disease progression (Koshal, Jamwal and Akula, 2017). The discovery and development of a new AED relies heavily on the preclinical use of animal models to establish efficacy and safety prior to first trials in humans. This approach has been very successful and crucially contributed to the development of numerous clinically effective AEDs. In the discovery and development of new AEDs, animal models of seizures or epilepsy serve a variety of purposes (Löscher, 2011).Given the highly heterogeneous nature of seizure disorders in humans, the complexity of the seizure phenotypes, and the syndromes involved, the reality is that it is highly unlikely that any one animal model will ever predict the full therapeutic potential of an investigational AED. Therefore, investigational AEDs are currently evaluated in a battery of syndrome-specific model systems. As specific models are developed (and the drugs they identify are validated clinically), they are integrated into the existing discovery process to better identify more effective antiseizure and potentially antiepileptic therapies. Moving beyond the symptomatic treatment of epilepsy, the goal of most basic and clinical scientists in epilepsy research is to identify therapies capable of preventing, delaying, or modifying the disorder (Tudur Smith *et al.*, 2007).

1.9.1. The MES and PTZ tests

The most commonly employed animal models in the search for new anticonvulsant drugs are the MES test and the PTZ seizure test (Bialer and White, 2010; Löscher, 2011). The maximal electroshock seizure test, in which tonic hind limb seizures are induced by bilateral corneal or transauricular electrical stimulation, is thought to be predictive of anticonvulsant drug efficacy

against generalized tonic-clonic seizures, while the pentylenetetrazole test, in which generalized myoclonic and clonic seizures are induced by systemic (usually s.c. or i.p.) administration of convulsant doses of PTZ, is thought to represent a valid model for generalized absence and/or myoclonic seizures in humans (Löscher, 2011). MES and PTZ tests provide some insight into the ability of a given drug to penetrate the blood-brain barrier and exert a CNS effect. Indeed, both models are nonselective with respect to mechanism and therefore are well suited for screening anticonvulsant activity, as neither model assumes that the pharmacodynamic activity of a particular drug is dependent on its molecular mechanism of action (Tudur Smith *et al.*, 2007).

After the establishment of anticonvulsant potential of new AEDs in a simple model like MES or PTZ test, different animal models like kindling model of temporal lobe can be used to investigate the anticonvulsant spectrum of novel AEDs(Koshal, Jamwal and Akula, 2017). Pilocarpine and kainate models replicate several phenomenological features of human temporal lobe epilepsy and can be used as animal preparations to understand the basic mechanisms of epileptogenesis(Lévesque, Avoli and Bernard, 2016; Nirwan, Vyas and Vohora, 2018).Figure 9. schematically illustrates initial screening steps of University of Utah Anticonvulsant Drug Development(ADD).

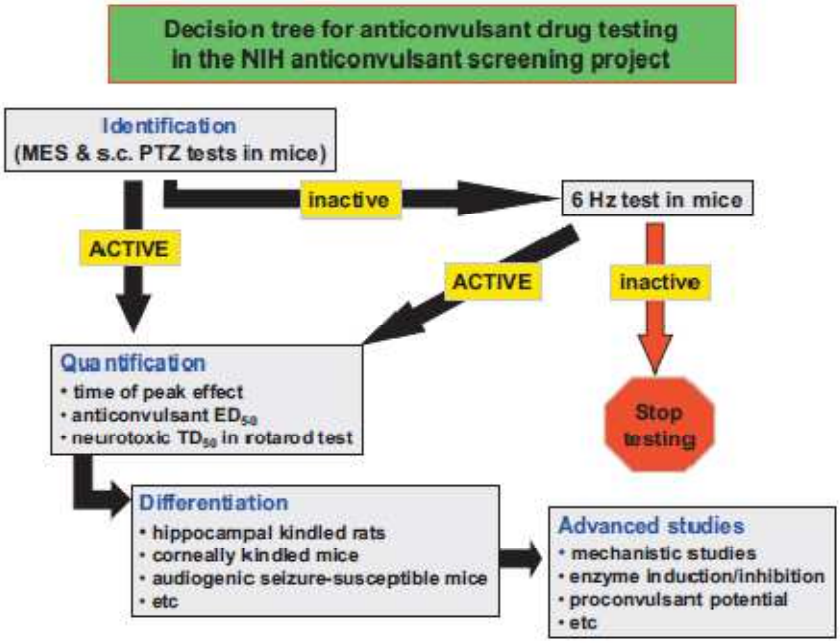


Figure 9. Schematic diagram illustrating the initial screen of the NINDS-sponsored University of Utah Anticonvulsant Drug Development (ADD) Program. An investigational compound is initially screened for efficacy in the MES and s.c. PTZ tests. Compounds found inactive in the MES and s.c. PTZ tests are evaluated in the LEV sensitive 6-Hz seizure test in mice. For those compounds that are found to be active in the 6-Hz test, their activity is quantitated at their respective time of peak effect. All compounds found to be active in one or more of these three identification screens are then differentiated on the basis of their activity in additional seizure models, including the hippocampal kindled rat model of TLE (Lo¨ scher, 2011).

1.8.2. In vitro brain preparations for studying epileptiform synchronization

1.8.2.1. Acute brain slices

Obtaining acute brain slices for electrophysiology or amperometric recordings has become a routine procedure in most labs in the field of neuroscience(Papouin and Haydon, 2018). The choice of the hippocampus stemmed from the assumption that once “sliced” its lamellar

organization could warrant interconnectivity approximating what occurring *in vivo*(Dingledine, Dodd and Kelly, 1980; Teyler, 1980; Sloviter *et al.*, 2012; Losi *et al.*, 2016). In these experiments "isolated" hippocampal slices were cut at thickness of 350–500µm employing the original McIlwain chopper, and they were maintained in an interface tissue chamber similar to that originally employed by Li and McIlwain (1957). These studies made the neuroscience community aware of the unique advantages of this preparation that included: (i) stable intracellular recordings; (ii)ability to change the extracellular milieu; and (iii) direct application of known drug concentrations to the brain tissue(LI and McILWAIN, 1957).

Overall, these experiments exploited the well-known connectivity of the hippocampus proper that was however limited to the classic circuit: dentate gyrus → CA_3 → CA_1 → subiculum. By doing so they underscored the pacing role of CA_3 networks in the generation of short lasting interictal activity that resulted from the recurrent excitatory connections that link CA_3 pyramidal cells along with their ability to produce high-threshold Ca^{2+}spikes(Schwartzkroin and Slawsky, 1977; Wong, Prince and Basbaum, 1979; Miles and Wong, 1983).The successive introduction of the vibroslicer (an instrument similar to what used by histologists) to cut brain tissue for electrophysiological experiments enlarged the application of brain slice to the study of epileptiform synchronization. In fact, besides a better preservation of those neurons that are close to the cut surface, the vibroslicer allowed the introduction in several laboratories of "large" brain slices that could contain more extensive neuronal networks including "horizontal slices" of neocortex(Fleidervish, Binshtok and Gutnick, 1998) and hippocampus (Miles, Traub and Wong, 1988; Traub and Jefferys, 1994) comprising several, and often interconnected, brain structures including those of the limbic and thalamocortical systems. These "more complex" slice

preparations have made possible to establish whether different types of epileptiform discharges were structure-specific, how they propagated from one structure to another, and how they could potentially influence each other (Avoli and Jefferys, 2016a).

1.8.2.2. Drug-induced epileptiform patterns in vitro

1.8.2.2.1. GABA receptor antagonists

During application of medium containing $GABA_A$ receptor antagonists, interictal events have been recorded in isolated hippocampal slices, neocortical slices including those obtained from epilepticpatients undergoing epilepsy surgery as well as in brain slices that included the entorhinal cortex and hippocampus. These studies firmly established that interictal events are accompanied by the reduction or blockade of $GABA_A$ receptor-mediated IPSPs. Epileptiform activity generated by hippocampal slices during reduction/blockade of $GABA_A$receptor signaling has been used to test the effects of some antiepileptic drugs. Early work has established that penicillin-induced interictal activity generated by neuronal networks in guinea pig hippocampal slices is suppressed by phenytoin, an effect that was proposed to be due to decreased excitatory synaptic transmission. Later, these experiments were extended to other antiepileptic drugs. Overall these studies have shown that epileptiform discharges induced *in vitro* by $GABA_A$ receptor antagonists are differentially sensitive to conventional and new antiepileptic drugs (Avoli and Jefferys, 2016a).

1.8.2.2.2. K+ channel blockers

K^+ channel blocker4-aminopyiridine (4AP) was known to cause seizures *in vivo* and to enhance transmitter release at both excitatory and inhibitory synapses in the hippocampal slice preparation. The *in vitro* 4AP model has been used to evaluate the effects of antiepileptic drugs. During application of 4AP, standard antiepileptic compounds can abolish ictal discharges in isolated young rat hippocampal slices (Avoli and Jefferys, 2016a). Epileptiform synchronization can also be induced by other K^+ channel blockers such as tetraethylammonium. Bath application of tetraethylammonium, like4AP, induces spontaneously occurring interictal discharges in theCA_3 area of isolated hippocampal slices obtained from adult animals; these experiments as well demonstrated that interictal discharges recorded extracellularly were associated with paroxysmal depolarizing shifts that comprised both excitatory and inhibitory currents(Rutecki, Lebeda and Johnston, 1990). As shown in the *in vitro* studies performed with 4AP, itwas later shown that ictal-like discharges occur in the CA_3 subfield of hippocampal slices obtained from young (12–18 day-old) rats(Fueta and Avoli, 1993).

1.8.2.2.3. Muscarinic agonists

In vivo, the muscarinic agonist pilocarpine represents a valuable tool for inducing status epilepticus thus establishing a chronic epileptic condition that is regarded as a useful experimental model of temporal lobe epilepsy(D'Antuono *et al.*, 2007). In line with this evidence, it has been found that bath application of pilocarpine can induce structure-specific patterns of interictal and ictal discharges in combined hippocampal-entorhinalcortex slices(Nagao, Alonso and Avoli, 1996).Prolonged periods of epileptiform synchronization have

also been reported to occur in the entorhinal cortex during application of carbachol, another muscarinic receptor agonist (Cataldi *et al.*, 2011). In one study, the antiepileptic drugs topiramate and lamotrigine were shown to be capable of reducing these carbachol-induced oscillations in the rat subiculum suggesting that muscarinic receptor mediated excitation represents a target for the action of some antiepileptic drugs (D'Antuono *et al.*, 2007; Avoli and Jefferys, 2016b).

1.8.2.2.4. Zero Mg2+ Model

It is of interest to note that in spite of the preserved inhibitory mechanisms the spontaneous epileptiform activity in low magnesium (Mg^{2+}) occurs at a much higher frequency in the hippocampus than that induced by GABA action antagonists such as bicuculline, picrotoxin and penicillin in normal medium, hence indicating that increasing excitability is probably as good a mechanism for seizure induction as depression of inhibition. Since the activity resembles seizure-like activity in the enterihinal cortex (EC) but only interictal activity in the hippocampus we conclude that the EC is the more epilepsy-prone area, providing a better model for studying temporal lobe epilepsy than the hippocampal slice preparation. Of interest is also the fact that the prolonged seizure- like events in the EC elicit only short interictal events in the dentate gyrus. This is not due to a lack of NMDA receptors since ionophoretic application of NMDA evokes large depolarizations and ionic changes in the dentate. Thus, the dentate gyrus because of its intrinsic inhibitory activity may serve as a filter which reduces the excitatory load into CA_3 and hence to CA_1 (Walther*et al.*, 1986).

1.9. Plants as treatment source

Plants have formed the basis of traditional medicine (TM) systems which have been used for thousands of years. Traditional medicine refers to health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral-based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination to treat or to diagnose and prevent illnesses or maintain well-being(WHO, 2005). Traditional medicine is an important and often underestimated part of health services. In some countries, traditional medicine or non-conventional medicine may be termed complementary medicine (CM). TM has a long history of use in health maintenance and in disease prevention and treatment, particularly for chronic disease(WHO, 2013). The use of plant-based systems continues to play an essential role in health care. It has been estimated that approximately 80% of the population in developing countries depend on TM for their primary health care (WHO, 2013; Beyene, Beyene and Deribe, 2016).

In African societies, the tradition of collecting, processing and applying plants and plant-based medications have been handed down from generation to generation. Traditional medicine, with medicinal plants as their most important component are sold in marketplaces or prescribed by traditional healers in their homes (Olajuyigbe and Afolayan, 2012; Hughes *et al.*, 2015). Because of this strong dependence on plants as medicines, ethnopharmacological studies have been conducted to determine their safety and their efficacy and on the other hand to find out new active principles from plants (Karou *et al.*, 2007; Khamkar, Motghare and Deshpande, 2016).The frequent rationale behind plant use is the need for new active principles in the treatment of many diseases. The choice of plant species that should be screened to reduce the time and the cost of

the studies is an important consideration for any ethnopharmacological investigation (Khamkar, Motghare and Deshpande, 2016; Leonti *et al.*, 2017).

Many plants were known for their anticonvulsant activity. Reviews articles (Chauhan, Dobhal and Joshi, 1988; Nsour, Lau and Wong, 2000; Srinivasan and Roy, 2017) were previously published with regards to plants with anticonvulsant properties. In fact, current world-wide interest in traditional medicine has led to rapid development and studies of many remedies employed by various ethnic groups of the world. Among those medicinal plants are found to possess anticonvulsant activity in animal models and/or folk medicine, include: *Hypericum perforatum L.Abelmoschus angulosus, Allium sativum, Artemisia* spp, *Cannabis sativa, Cinchona officinalis, Egletes viscosa, Icacina trichantha, Magnolia grandiflora, Plumbago zeylanica* and others (Ivetic *et al.*, 2002). A study with Brazilian Northeastern plants showed excellent results for the species *Bauhinia outimouta, Rauvolfia ligustrina* and *Ximenia americana Ocimum basilicum*(Siqueira *et al.*, 2008; Oliveira *et al.*, 2009). The antiepileptic activity of some medicinally important plants like *Withania somnifera, Ocimum sanctum, Brahmi grihta, Catharanthus roseus, Caesalpinia crista, Citrus sinensis, Datura stramonium, Ricinus communis, Terminalia glaucescens, Tetrapleura tetraptera, Senna singuena, Jatropha gossypiifolia, Mentha cardifolia* was screened. The role of such plants, with specific properties of their parts has been demonstrated and proved in earlier studies.In one review13 Brazilian plants were cited: *Acosmium subelegans,Artemisia verlotorum, Centella asiatica, Cymbopogon citratus, Erythrina velutina, Erythrina mulungu,Hippea strumvittatum, Lanatamicrophylla, Licariapuchury-major, Lippia alba, Nepeta cataria, Passiflora alata* and *Xylopia*spp. Among

those plants tested, a number of them (from different families) are found to possess anticonvulsant activity. While in most cases, the active constituents are yet to be found, for those where theactive components are known, they belong to different chemical classes. However, previous studies showed that some natural plant coumarins and triterpenoids exhibit anticonvulsant properties (Siqueira *et al.*, 2008).

In addition, the history of drug discovery showed that plants are highly rich sources in the search for new active compounds and they have become a challenge to modern pharmaceutical industry. Many synthetic drugs owe their origin to plant-based complementary medicine(Siqueira *et al.*, 2008).A number of animal models have demonstrated utility in the search for more efficacious and more tolerable AEDs. In fact, the models employed in the early phase of AED discovery are highly predictive of subsequent efficacy in easy-to-manage generalized and partial epilepsy. Thus, animal models more employed were leptazole-induced seizure(LIS), maximal electroshock seizure (MES), metrazole induced seizures (MIS), picrotoxin-induced convulsions(PIC), pilocarpine (PILO), pentylenetetrazole (PTZ) andstrychnine-induced seizures (SIS). However, MES, PIC and PTZ seizure models continue to represent the three most widely used animal seizure models employed in the search for new AEDs(Löscher, 2011; Barker-haliski and White, 2019). Medicinal compounds with antiepileptic/anticonvulsant activities are alkaloids, flavonoids, terpenoids, saponins and coumarins (Zhu *et al.*, 2014). .

In Ethiopia as various diseases are being treated traditionally some surveys shows also the practice of treatment for epilepsy. *Pterolobium stellatum* whole plant juice is used to treat epilepsy given orally for one month (Ragunathan and Abay, 2009). The known *Moringa olifera*

was also proved to be with anti convulsant property though there is no study done on Ethiopian species *Moringa stenopetala* though its root is claimed to be used for epilepsy in Southern Ethiopia Konso area. *Withania somnifera*is used for coughs and asthma, as a narcotic with anti-epileptic activity in Ethiopia and other traditional uses for headache. *Carissa edulis, Clerodendrum myricoides, Croton macrostachyus, Maytenus senegalensis, Sida schimperiana, Pentas schimperiana and Pluchea dioscorides ,Ajuga integrifolia Asplenium aethiopicum*(Kunth) mett, *Desmodium repandum*(V ahl) DC., *Oliniarochetiana*A. Juss, *Biophytumum braculum , Brachiaria brizontha , Buddleja polystachya , Galinirea coffeoides , Satureja abyssinica, Clutia abyssinca, Leucas abyssinica, Indigofora arrecta and Gerbera piloselloides* are some of the medicinal plants claimed for use against epilepsy. As different researches on plants used for epilepsy in different countries have shown anti seizure activity, these plants may have also value for the treatment of the disease. Therefore scientific research should be done on these plants (Andarge *et al.*, 2002; Mesfin, Demissew and Teklehaymanot, 2009; Ragunathan and Abay, 2009; Wabe, Mohammed and Raju, 2011; Abera B., 2014; Agisho, Osie and Lambore, 2014; Mesfin, Seta and Assefa, 2014; Asfaw and Helisob, 2017; Wubetu *et al.*, 2018).

1.11. Selected Plants for The Study

1.11.1. *Pterolobiumstellatum*

Pterolobium stellatum(Forsk.) Brenan. (Fabaceae) is also called *kenteffa*(Amharic). Fresh leaves and roots are chewed for medicinal purposes for tuberculosis and related respiratory diseases (Balcha *et al.*, 2014). It has been reported that the whole plant juice is given orally for one month to treat epilepsy and neuralgia in north-west Ethiopia (Ragunathan and Abay, 2009). Chemical classes present in *P. stellatum* 80%hydoalcoholic root extract are terpenoids, saponins and tannins and had antibacterial activity as reported by previous study. The result of the study on crude hydroalcoholic and fractions of the root of *P. stellatum* revealed that the plant had significant antituberculosis activity. The activity was seen with chloroform and methanol extracts of *P. stellatum*. In addition, fractions from *P. stellatum* had demonstrated promising antimycobacterial activity (Kahaliw *et al.*, 2017)**.** Figure 9 is the picture of *Pterolobium stellatum.*

Figure 10. *Pterolobium stellatum*

1.11.2. *Moringa stenopetala*

Moringa is a multipurpose tree of significant economic importance, as it has vital nutritional, industrial, and medicinal applications. *Moringa stenopetala* was domesticated in the east African lowlands and is indigenous to southern Ethiopia. Many different ecotypes and varieties of *M. stenopetala* are found in Ethiopia. *M. stenopetala* (Figure 10) is often called “cabbage tree” and is an important indigenous vegetable in south western Ethiopia where it is cultivated as a food crop. The Konso, Burji, Gamo andGofa tribes consume its leaves as a vegetable, especially during the dry season (Seifu, 2014). *M. stenopetala*is native to Ethiopia, and it is known by various vernacular names. It is called “Haleko” inGamo,Gofa and Wolaytaareas, “Shelagda” in the Konso language, and “Shiferaw” in Amharic(Engels and Goettscch, 1991; Jahn, 1991). *M. stenopetala*is particularly important as human food because the leaves, which have high nutritional value(Abuye *et al.*, 2003; Imungi *et al.*, 2011), appear towards the end of the dry season when few other sources of green vegetables are available. The leaves contain high amounts of essential amino acids and vitamins A and C (Abuye *et al.*, 2003).

The local communities residing in the biodiversity-rich areas of the southern region of Ethiopia have traditionally used and relied on plants for treating various ailments. In many cases, local knowledge of medicinal plants remains poorly documented in scientific literature. These plants have found a prime place in the indigenous system of medicine and are in focus for evaluation of their active ingredients. *Moringa stenopetala*is one of these medicinal plants which is widely used for antidiabetic purpose in the area (Toma *et al.*, 2015).The plant has several medicinal uses in areas where it is native. Local people use the plant parts to treat malaria, leishmaniasis and hypertension, stomach pain, expulsion of retained placenta during birth, asthma, epilepsy,

diarrhea, diabetes and leprosy. The root is used for epilepsy (Tesemma *et al.*, 2013a). The crude aqueous extract and n-butanol as well as chloroform fractions of the leaves of *Moringa stenopetala* have been reported to have both hypoglycemic and antihyperglycemic effect (Mussa, Eyasu Makonnen and Urga, 2008; Toma *et al.*, 2015).

The roots of *M. stenopetala* can also be used to clarify dirty water. Nomadic peoples in the Omo Valley of Ethiopia apparently use the roots of wild *M. stenopetala* to purify muddy water (Demeulenaere, 2001). The root is also used in traditional medicine to treat different aliments. The root tissues contained both 4-(R-Lrhamnopyranosyloxy)- benzylglucosinolate and benzylglucosinolate. The leaves of *M. stenopetala* contained quercetin 3-*O*-rhamnosylglucoside (rutin) and traces of quercetin 3-*O*-glucoside(Bennett *et al.*, 2003; Padayachee and Baijnath, 2012). The leaves and the root extracts of *Moringa stenopetala* were tested *in vitro* against trypomastigotes of *Trypanosoma brucei*, *Trypanosoma cruzi*and *L. donovani* amastigotes. The fresh root wood ethanol extractand the dried leaves acetone extract were found to be active against *T. brucei* (Mekonnen, 1999).The pretreatment with methanolic extract of roots of *Moringa oleifera* caused significant protection against strychnine (STR) and PTZ induced convulsions. The exact mechanism of its anticonvulsant activity is not revealed(Gupta, Mazumder and Chakrabarti, 1999; Asif, 2013). *Moringa stenopetala* root were used traditionally for epilepsy treatment in Ethiopia but not yet scientifically proven effective (Tesemma *et al.*, 2013a).

Figure 11. *Moringa stenopetala*

1.11.3. Clutia abyssinica

Clutia is a genus within a family Euphorbiaceae, having about 60 species. *Clutia abyssinica* (Figure 11) called by the Amharic name ‘fyelefej’ is herb 1-2 m high. Traditionally it is used in treatment of venereal and skin diseases, chest problems, cancer; Skin fungal infections ; yellow fever and malaria; management of ear, nose and throat diseases ,diarrhoea, gonorrhea, cough and fever, headache, toothache, menstrual pain, burns, pneumonia, enlarged spleen and kidney,

shock, abdominal problems as a laxative and to expel intestinal worms, elephantiasis, diarrhoea and tachycardia. The maceration of the crushed leaves of *C. abyssinica* given orally has been traditionally used for the treatment of animal trypanosomosis (Mergia *et al.*, 2014; Koech *et al.*, 2017).The dried root, together with other plants, is used for the treatment of dizziness. It is also used as an ascarifuge, for habitual miscarriage, convulsions, enlarged spleen and influenza(Ramathal and Ngassapa, 2001). *Clutia abyssinica* leaves and root were, one of the medicinal plants claimed for use against epilepsy and evil eye and other diseases in different parts of Ethiopia (Wereta, 2015b; Wubetu *et al.*, 2018; Tamene, Addisu and Debela, 2020) as well as in Uganda (Agrawal and Dhanasekaran, 2021). But there is no scientific evidence for the claimed use. As different researches on plants used for epilepsy in different countries have shown anticonvulsant activity, this plant may also have value for the treatment of the disease.

Figure 11. *Clutia abyssinica*

1.11. Statement of the problem

Although new antiepileptic drugs have been available since late 1980s, refractoriness to treatment is still an important issue in epilepsy care. Current available anticonvulsant drugs are able to control epileptic seizures efficiently in about 50% of the patients and lead to improvement in another 25% whereas the remainder do not benefit significantly. Furthermore, undesirable side effects of the drugs used clinically often render treatment difficult; so that a demand for new types of anticonvulsants exists. One of the approaches to search for new antiepileptic drugs is investigation of naturally-occurring compounds, which belong to new structural classes (Sayyah *et al.*, 2011). In Ethiopia as various diseases are being treated traditionally some surveys shows also the practice of treatment for epilepsy. *Pterolobium stellatum* whole plant juice is used to treat epilepsy given orally for one month. The known *Moringa olifera* was also proved to be with anti convulsant property though there is no study done on Ethiopian species *Moringa stenopetala* though its root is claimed to be used for epilepsy in Konso area. *Clutia abyssinica* was also claimed for psychiatric problems and epilepsy. As different researches on plants used for epilepsy in different countries have shown anti seizure activity, these plants may have also value for the treatment of the disease. Therefore scientific research should be done on these plants.

1.12. Significance of the study

According to the World Health Organization, more than 70% of the world's population use traditional medicine to satisfy their principal health needs (WHO, 2019b). A great number of medicinal plants used in the control of epilepsy have been reported. There are various medicinal

plants in the world, which are the potential sources of drugs. Therefore, this study could pave the way towards development of new drug from *Pterlobium stellatum, Moringa stenopetala* and *Clutia abyssinica.* Though there are some studies done on other disease treatments there is no study done for epilepsy. This study was designed to reveal the anticonvulsant potential of these plants.

CHAPTER 2. OBJECTIVES

2.1. General objective

To investigate the anticonvulsant activity of hydromethanolic extracts of selected plants *Pterlobium stellatum*, *Moringa stenopetala* and *Clutia abyssinica.*

2.2. Specific objectives

- to evaluate the *in vitro* anticonvulsant activity of the crude extracts *Pterlobium stellatum*, *Moringa stenopetala* and *Clutia abyssinica 80%* methanol on mice hippocampal brain slice

- to assess the *in vitro* anticonvulsant activity of pet ether , chloroform, butanol and aqueous fractions of *Pterlobium stellatum* in mice hippocampal brain slice

- to evaluate the *in vivo* anticonvulsant activity of the crude extracts *Pterlobium stellatum, Moringa stenopetala and Clutia abyssinica 80%* hydromethanol extract using PTZ model on mice

- to evaluate the *in vivo* anticonvulsant activity of the crude extracts *Pterlobium stellatum, Moringa stenopetala and Clutia abyssinica 80%* hydromethanol extract using MES model on mice

- to assess the *in vivo* anticonvulsant activity of pet ether , chloroform, butanol and aqueous fractions of *Pterlobium stellatum* in PTZ model in mice

- to assess the *in vivo* anticonvulsant activity of pet ether , chloroform, butanol and aqueous fractions of *Pterlobium stellatum* in MES model in mice

- to study the acute toxicity of the extracts on mice

- to evaluate the qualitative and quantitative secondary metabolite content of the crude extracts
- to characterize *Pterlobium stellatum* crude extract and fractions using ultraperformance liquid chromatography-mass spectrometer (UPLC-MS)

CHAPTER 3. MATERIALS AND METHODS

3.1. Plant Selection

The following plants were selected based on claim by the society to use for epilepsy based on literature.

Pterolobium stellatum (leaf)

It has been reported that the whole plant juice is given orally for one month to treat epilepsy and neuralgia in north-west Ethiopia (Ragunathan and Abay, 2009).

Moringa stenopetala(root)

In the Gamo, Gofa , Dherashe and Konso districts, the smoke liberated from burning Moringa is used as a treatment for epilepsy (Yisehak, Solomon and Tadelle, 2011). In the Konso district of southern Ethiopia, the same smoke is used as a treatment for epilepsy (Seifu, 2014).

Clutia abyssinca(leaf)

Clutia abyssinica leaves and root were, one of the medicinal plants claimed for use against epilepsy and evil eye and other diseases in different parts of Ethiopia (Wereta, 2015b; Wubetu *et al.*, 2018; Tamene, Addisu and Debela, 2020) as well as in Uganda (Agrawal and Dhanasekaran, 2021).

3.1.1. Plant Material Collection

The plants used in this study were collected between April and July 2016 from the areas where they are used as traditional medicine for epilepsy or from available sites.

Fresh leaves of *Pterolobium stellatum* were collected from Awash Melka area 50km South of Addis Ababa in July 2016. The root of *Moringa stenopetala* was collected from Arba Minch area 505km South of Addis Ababa in April 2016. The leaves of *Clutia abyssinica*was collected from near Dinsho town 340km South East of Addis Ababa in April 2016. The plants were identified by taxonomist at National Herbarium, College of Computational and Natural Sciences Addis Ababa University. The herbarium code was given(02-S for *Pterolobium stellatum* , 01-S for *Clutia abyssinica* and 03-S for *Moringa stenopetala*) and a sample specimen of each were deposited at the national herbarium.

3.1.2. Preparation of Crude Extracts of *Pterolobium stellatum* (leaves)

Pterolobium stellatum leaves were air dried at room temperature in the processing room under shade and were powdered to appropriate size in mortar and pestle(Figure 13 a). And the powder was kept at room temperature in a well-closed container until extracted. A total of 500 g air-dried and powdered plant materials of *Pterolobium stellatum* leaves were extracted by maceration with 80% methanol for three consecutive days (72h). The extraction process was facilitated by using an orbital shaker at 120rpm. The mixture was first filtered by gauze and then

with Whatman™ filter paper 6µm pore size (125mm GE healthcare UK limited, UK). The residue is remacerated for another 72 hr twice and filtered. The combined methanol filtrates were then concentrated under vacuum in a rotary evaporator at temperature 40°C. The residual water was removed by lyophilizer (Operon, Korea vacuum limited, Korea) at -44°C. The extract was light green gummy substance. After drying the total of 179.51g of dry extract was collected (Figure 14a). The extract was stored in tightly closed container in a refrigerator until used. This extract was used to evaluate the toxicity and efficacy of the extract in *in vitro* and *in vivo* models.

3.1.3. Preparation Of Crude Extracts of *Moringa stenopetala* (root)

Moringa stenopetala (root) were air dried at room temperature in the processing room under shade and were powdered to appropriate size in mortar and pestle(Figure 13b). And the dried powder was then kept at room temperature in a well-closed container until extracted. The air-dried and powdered plant materials (500g) of *Moringa stenopetala* (root) were extracted by maceration with 80% methanol for three consecutive days (72h). The extraction process was facilitated by using an orbital shaker at 120rpm. The mixture was first filtered by gauze and then with Whatman™ filter paper 6µm pore size (125mm GE healthcare UK limited, UK). The residue is re-macerated for another 72 h twice and filtered. The combined methanol filtrates were then concentrated under vacuum in a rotary evaporator at temperature 40°C. The residual water was removed by lyophilizer (open ,Korea vacuum limited, Korea) at -44°C. The extract was brown gummy substance. After drying the total of 7.35g (1.47%) of dry extract was collected (Figure 13b). The extract was then stored in tightly closed container in a refrigerator until used. This extract was used to evaluate the toxicity and efficacy of the extract *in vitro* and *in vivo* seizure models.

3.1.4. Preparation Of Plant Material Extracts of *Clutia abyssinica* (leaves)

Clutia abyssinica leaves were air dried at room temperature in the processing room under shade and were powdered to appropriate size in mortar and pestle(Figure 13c). And the dried powder was then kept at room temperature in a well-closed container until extracted. The air-dried and powdered plant materials (400g) of *Clutia abyssinica* leaves were extracted by maceration with 80% methanol for three consecutive days (72h). The extraction process was facilitated by using an orbital shaker at 120rpm. The residue was re-macerated for another 72 hr twice and filtered. The mixture was first filtered by gauze and then with Whatman^{TM} filter paper 6µm pore size (125mm GE healthcare UK limited, UK). The extract was then filtered and concentrated under vacuum in a rotary evaporator to yield extract of plant parts. The residual water was removed by lyophilizer (open ,Korea vacuum limited, Korea) at -44^{o}C. The extract was dark green gummy substance. After drying the total of 44.71g (11.18%) of dry extract was collected (Figure 14c). The extract was then stored in tightly closed container in a refrigerator until used. This extract was used to evaluate the toxicity and efficacy of the extract in *in vitro* and *in vivo* seizure models.

a. *Pterolobium stellatum*

b. *Moringa stenopetala* (root)

c. *Clutia abyssinica* (leaves)

Figure 13. The dried powders and crude extracts of plant materials.

a. *Pterolobium stellatum*

b. *Moringa stenopetala* (root)

c. *Clutia abyssinica* (leaf)

Figure 14: The hydroalcoholic crude extracts of the plant materials

3.1.5. Solvent fractionation of *P. stellatum*

The crude extract of *P. stellatum* was subject to further fractionation using three solvent systems (petroleum ether, chloroform and n-butanol). For *P.stellatum* crude extracts has good activity and with good yield, it was selected for further fractionation. A 50g of the powder of *P. stellatum* hydromethanolic extract was dissolved in separatory funnel in 100ml of mild hot distilled water. The dissolved extract was partitioned with 3x150ml of pet ether to obtain pet ether fraction. The pet ether partitions were combined and concentrated using a rota-vapor. Then the aqueous residues was then petitioned with 3x150ml of chloroform. The chloroform filtrates were combined and evaporated to obtain chloroform fraction . The residue again was further soaked in 3x150ml of n-butanol to get butanol fraction. Finally the residue water fraction was lyophilized (Figure 15). All the fractions were kept in tightly closed container in refrigerator at -20^0C until used for *in vitro* and *in vivo* test (Debella, 2002).

a .pet ether fraction b. chloroform fraction

c. n-butanol fraction d. water fraction

e. The four fractions labeled and sealed for storage.

Figure15 :The fractions of *Pterolobium stellatum*

3.1.6. Phytochemical screening

3.1.6.1. Test for alkaloids

In 1% v/v HCL the plant extract is mixed, warmed and filtered. Now this filtered was used for following test, Mayer's test: With Mayer's reagent (Mercuric chloride + Potassium iodide in water) the filtrate was treated. The presence of alkaloids specify by the formation of yellow colored precipitates(Debella, 2002; Khalid *et al.*, 2018).

3.1.6.2. Test for flavonoids:

Alkaline reagent test: The plant extract was treated with 2-3 drops of sodium hydroxide solution. Acute yellow color formation, that indicates presence of the flavonoids, by the addition of some drops of sulphuric acid that changed to colorless (Debella, 2002; Khalid *et al.*, 2018).

3.1.6.3. Test for tannins (Braymer's test):

For qualitative test of tannins 2mls of extract was treated with 10% alcoholic ferric chloride solution and observed for formation of blue or greenish colour solution (Debella, 2002; Khalid *et al.*, 2018).

3.1.6.4. Test for Terpenoid (Salkowki's test)

For qualitative test of terpenoid 1ml of chloroform was added to 2ml of each extract followed by a few drops of concentrated sulferic acid. A reddish brown precipitate produced immediately indicated the presence of terpenoids (Debella, 2002; Khalid *et al.*, 2018)..

3.1.6.5. Test for Saponins (Foam test)

To 2ml of extract , 6ml of water was added in a test tube. The mixture was shaken vigorously and observed for the formation of honeycomb froth (Debella, 2002; Khalid *et al*., 2018).

3.1.6.6. Test for Phenols (Ferric Chloride Test)

A fraction of extracts was treated with aqueous 5% ferric chloride and observed for formation of deep blue or black colour (Debella, 2002; Khalid *et al.*, 2018).

3.1.6.7. Test for sterols (Ferric Chloride Test)

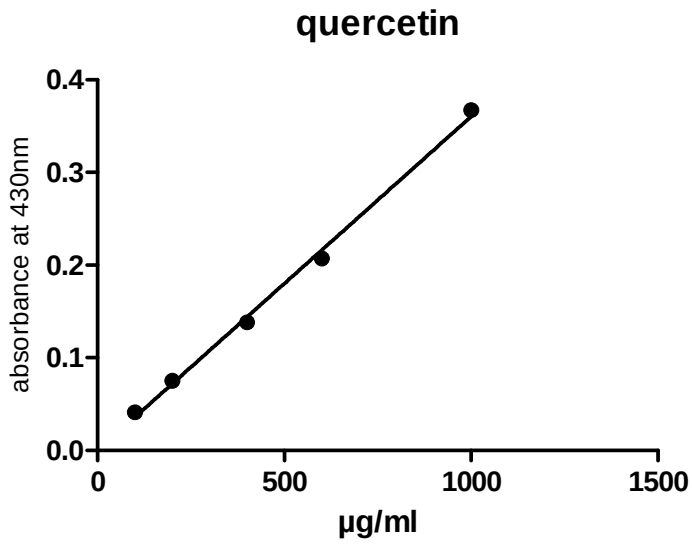
For qualitative test of sterols 1ml of extract was treated with drops of chloroform , acetic anhydride and conc. H2SO4 and observed for the formation of dark pink or red colour (Debella, 2002; Khalid *et al*., 2018)..

3.1.6.8. Test for Cardiac glycosides (Keller Kelliani's Test)

For test of sterols 5ml of each extract was treated with 2ml of glacial acetic acid in test tube and a drop of ferric chloride solution was added to it. This was carefully underlayed with 1ml concentrated sulpharic acid. A brown ring at the interface indicated the presence of deoxysugar characteristic of cardenolides. A violate ring may appear below the ring while in acetic acid layer a greenish ring may form (Debella, 2002; Khalid *et al*., 2018).

3.1.6.9. Spectromertric Quantification of Total flavonoid content

Estimation of flavonoid content in the dried extracts was done according to previosly used method (Adisakwattana *et al.*, 2012). The dried extract (0.5 mg) was dissolved in 80% ethanol (1 ml). The sample solution (50µl) was added to 10µl of $AlCl_3$ solution (10% w/v) and 10µl of 1 M sodium acetate in absolute ethanol (150µl). After incubation at 30°C for 30 min, the absorbance was measured immediately at 430 nm (Analyticjena AG, Germany).The estimation of flavonoid content was calculated from a calibration curve using quercetin as a standard (Figure 16). The results were expressed as milligram quercetin equivalent/gram dry weight of extract based on the following standard curve (Tiwari, M and Chanda, 2017).



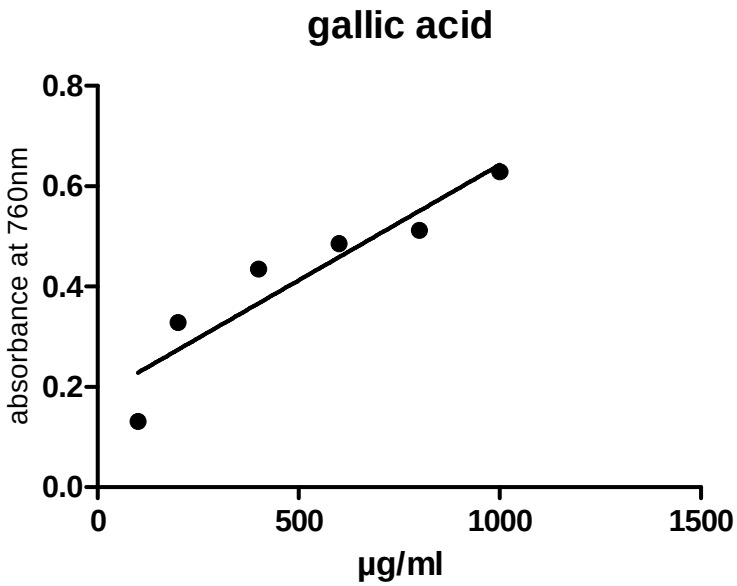
$Y = 0.0003606*X - 0.0002695$ $r^2=0.9970$

Figure 16 .Standard curve for quarcetin

3.1.6.10. Spectromertric Quantification of Total Phenolic Content

Folin Ciocalteu reagent was used to evaluate the amount of total phenolic content. Gallic acid was used as a standard for the determination of phenolic content and it was expressed as mg/g gallic acid equivalent (GAE). Concentration of 100, 200, 400, 600, 800 and 1000 µg/ml of gallic acid were prepared in methanol(Figure 17)..

Concentration of 1mg/ml of plant extract was prepared in methanol and 0.5ml of sample was placed in to test and it is mixed with 2.5ml of Folin Ciocalteu reagent. The Folin Ciocalteu reagent was previously diluted 10 fold. The mixture is added to 2ml of 7.5% sodium carbonate. The tubes were allowed to stand for 30 minutes covered with parafilm at room temperature before taking the absorbance 760 nm. Reading was taken in triplicate. Folin-Ciocalteu reagent is reactive to reducing substance including polyphenols. This is basically a colour reaction produces blue coloration which was measured spectrophotometrically (Tiwari, M and Chanda, 2017).



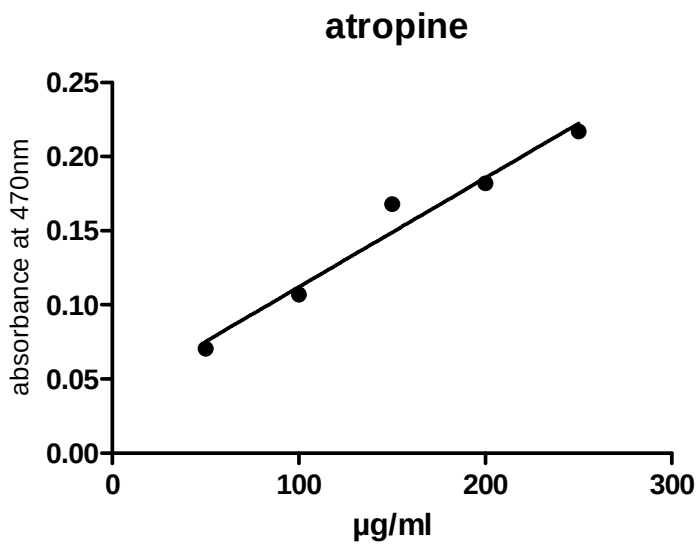
$Y = 0.0004609*X + 0.1820$ $r^2 = 0.8690$

Figure 17 .Standard curve for gallic acid

3.1.6.11. Spectrometric Quantification of Total Alkaloid Content

For the standard curve of atropine, total 5 different concentrations were used. A 100 ppm solution was initially prepared from atropine (1 mg in 10 ml of distilled water). From this stock solution, exactly 0.5, 1, 1.5, 2 and 2.5 ml of atropine solutions was transferred to five different separating funnel(Figure 18).. To each and individual funnels, 5 ml of phosphate buffer (pH4.7) and 5ml of Bromocresol green (BCG) solution was added and mixed vigorously. The formed complex mixture is extracted with chloroform. The chloroform fraction was collected in a 10ml of volumetric flask and make up the volume with chloroform. Absorption at a wavelength of 470 nm of each flask was measured and calibration graph was drawn (Tiwari, M and Chanda, 2017).

For the preparation of the sample the plant extract (1mg/ml) was dissolved in 2N HCl and then filtered. The pH of the extract was adjusted to neutral with 0.1 N NaOH. One ml of this solution transferred to a separating funnel and to that mixture 5 ml of Bromocresol green solution along with 5 ml of phosphate buffer was added and mixed properly. The formed mixture was extracted further with chloroform (5ml) and transferred to 10 ml of volumetric flask and make up the volume with the same solvent. The absorbance of the complex in chloroform was measured at 470 nm(Tiwari, M and Chanda, 2017).



$Y = 0.0007356*X + 0.03858$ $r^2 = 0.9673$

Figure 18 .Standard curve for atropine

3.1.7 Characterization of *Pterlobium stellatum* crude extract and fractions

The plant extract and respective fractions were characterized utilizing a Waters SYNAPT G2 ultraperformance liquid chromatography-mass spectrometer (UPLC-MS)(Waters Corporation, Massachusetts, USA) system. The samples were dissolved in methanol to obtain a final concentration of 1 mg/ml of which 5µl per sample was injected into the system using a Acquity autosampler (Waters Corporation, Massachusetts, USA). The compounds were separated on the LC system using a binary solvent (A: H_2O+0.1% H_2CO_2, B: MeOH+0.1% H_2CO_2) gradient at a flow rate of 0.3 ml/mincoupled to an Acquity UPLC HSS T3 Column (100Å, 1.8 µm, 2.1 mm x150 mm, Waters Corporation, Massachusetts, USA) set at 50°C. Solvent B was set to an initial concentration of 3% with a gradual increase to 100% over 14 min and held for 2 min followed by a rapid decrease to 3% over 50 s, held until the final run time of 20 min.TheMS related total ion current (TIC) chromatograms and associated mass hertz (*m/z*) fragmentation patterns were generated using positive (ES^+)- and negative (ES^-) ionisation modes at a sample infusion rate of 10 µl/min. The mass range of 50 to 1200 Da was assessed using helium as desolvation gas at a flow rate of 400 l/hr. The ion source- and desolvation temperatures were set to 120°C and 300°C, respectively. The MS collision energies (eV) and capillary voltages (kV) were set to 4eV, 2.6kV (ES^+) and 6eV, 2kV (ES^-), respectively. Data interpretation was done using MestReNovaversion 14.2 (Mnova, Mestrelab Research S.L., 2020) in combination with the National Institute of Standards and Technology (NIST) version2.2MS database (NIST 14, Agilent Technologies, USA).

3.2. Experimental Animals

Balb c mice were obtained from Ethiopian public health institute. Male mice 20g-30g were used for study 6 mice in each group with different dose for the *in vivo* efficacy study. The mice were kept at room temperature in humid environment and were exposed to 12 hours light and 12 hours darkness. The mice were given water and standard food pellets every 24hrs. The care and handling was according to international guidelines for the use and maintenance of experimental animals (OECD,2001). The procedures in the proposal got approval from Institutional Review Board of College of Health Sciences, Addis Ababa University.

For toxicity study female mice (Balb c)were obtained from AAU college of health science laboratory animal unit. The mice were kept at room temperature in humid environment and were exposed to 12 hours light and 12 hours darkness. The mice were given water and standard food pellets every 24hrs. The care and handling was according to international guidelines for the use and maintenance of experimental animals (OECD,2001).All safety rules were considered while doing all the procedures.

The *in vitro* study was conducted onP14-P21 of C57BL16 mice obtained from Cape Town college of Health sciences laboratory animal unit. The experimental protocol was approved by the Institutional Review Board (IRB) of Cape Town University. The animals house were fore told for the number of mice and age were brought to the laboratory for utilization. Animal safety rules to reduce suffer and in procedures as well as in scarifying the animals were considered while doing all the procedures.

3.3. Acute Toxicity Study

Acute toxicity study was performed for the extracts to ascertain safe dose by acute oral toxic class method of Organization of Economic Co-operation and Development, as per 423 guidelines(OECD, 2001). Three female mice were grouped randomly into four groups for the test. One control group was given distilled water and the other three groups were treated for *Pterolobiumstellatum* , *Moringa stenopetala* and *Clutia abyssinica* crude extracts. The extracts were tested with dose 2000mg/kg. The animals were observed for acute health abnormality and death in the first day. Animals were observed after dosing at least during the first 30 minutes, with close attention given during the first 4 hours, periodically during the first 24 hours, and daily thereafter, for a total of 14 days. The animals were followed for behavioral change , the change in body weight and any abnormalities as well as possible death. After14 days of observation the animals were sacrificed and post mortem examination was conducted.

3.4. *In vitro* antiseizure test

Extracellular field potential recordings were performed in coronal hippocampal slices from P14-P21 of C57BL16 mice using previously published techniques and methods. Briefly, mice were killed by quick decapitation. The brain was quickly removed and placed in ice cold (4°C) artificial cerebrospinal fluid (aCSF) that was bubbled continuously with 95% O_2 and 5% CO_2 (Carbogen). The composition of the aCSF used for dissection, storage and PS recording was (in mM) 120 NaCl, 3.3 KCl, 1.2 $MgSO_4$, 1.3 $CaCl_2$, 1.23 $NaHPO_4$, 25 $NaHCO_3$ and 10 D-glucose. Four hundred micrometers (400 µm) thick coronal slices of the forebrain containing the hip-

pocampus was cut from a block of brain tissue in ice cold (4°C) aCSF using a Leica VT 1000S (Leica Microsystems, Wetzlar, Germany) tissue slicer. Prior to recording, slices were incubated for 1 h in aCSF which was continuously bubbled with carbogen at room temperature (21–22°C). Slices were carefully trimmed of most cortical tissue and suspended on a nylon mesh in a 500 µl capacity recording chamber. Bath temperature was tightly maintained at 30–32°C to ensure that changes in responses are not due to variation in temperature. Slices were perfused at a flow rate of 2–3 mL/min with carbogenatedaCSF. An extracellular field recording glass electrode filled with 3 M NaCl (tip resistance between 5–10 MΩ) was placed in enterhnal cortex and single population spikes (PS) recorded (Qaddoumi*et al.,* 2014).

Epileptiform multiple PS and spontaneously occurring epileptiform activity (spontaneous bursts: SB), was induced chemically. The zero Mg2+model of seizures was utilized. Baseline recordings were made for 600s with normal artificial cerebrospinal fluid(aCSF) before 0Mg2+aCSF was washed in for 3000s in order to induce seizure-like activity. The 0 Mg2+ solution either contained plant extract or solvent as a control. The presence of seizure-like events was compared in treated versus untreated control. The Fisher's exact test with $P<0.05$ was used to determine statistical difference between groups (Qaddoumi*et al.,* 2014).

3.5.Experimental Design of *In vivo* antiseizure tests

After bringing the animals were randomly grouped into different groups in cages. In each group were 6 mice. The cages were also randomly allotted for respective treatment. They were kept under standard conditions (at a temperature of 22 ± 2^{o}C, and with 12 hr light/ 12 hr dark cycle) and provided with free access to standard pellet laboratory diet and water *ad libitum*. The animals were acclimatized prior to test. On the day of test they were brought to laboratory being fasted 4-8hrs food.

3.5.1.Maximal Electroshock Seizure (MES) Model:

Six albino mice in each group were divided into 16 groups. Experiments in each plant extract were designed to have the control group, reference group and test groups (with 400mg/kg and 800mg/kg doses for each plant extract). Animals in control group received 2%tween 80 (0.3 ml), the standard drug control group were allowed to take phenytoin (10mg/kg) and the two test groups were allowed to take test extracts with 400mg/kg and 800mg/kgdoseorally respectively. The animals in all the groups received corresponding drugs 1hour before the application of shock. Each animal was properly held and current of 54 mA was passed for 0.2 second transauricularly through ear lobe electrodes using an electroconvulsiometer. The reduction in duration of hind limb extension was considered as a protective action & recorded for all the animals (Insuasty *et al.*, 2014). Mice showing hind limb extension is shown in figure 19.

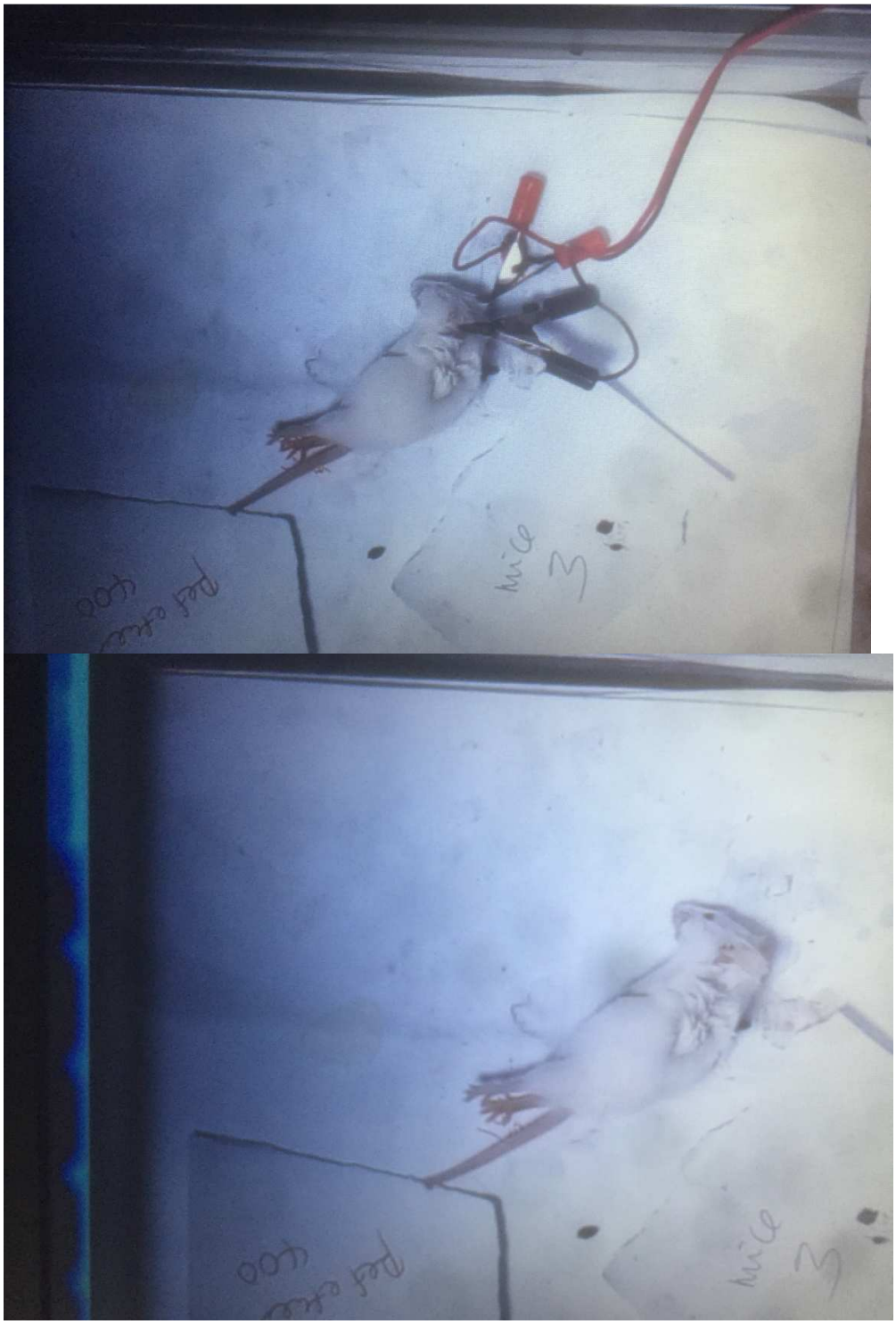


Figure 19 : Mice showing hind limb extensionin MES model

3.5.2. Pentylenetetrazole (PTZ) model

The animals were grouped and administered vehicle, reference drug and extracts as described for MES test. The reference group was treated with diazepam 5mg/kg orally. One hour after administering corresponding drugs to different groups of animals, PTZ 85mg/kg was injected subcutaneously and mice were observed for thirty minutes for the onset and duration of convulsive behavior. The number of animals convulsing or not convulsing within observation period was noted to calculate percentage of protection. The test is thought to be predictive of anticonvulsant drug activity against nonconvulsive (absence or myoclonic) seizures (Lo¨ scher, 2011).All the tests were done initially by the crude extracts and then the fractions of active crude extracts of *Pterolobium stellatum*was further were tested (Insuasty *et al*., 2014).Mice showing myoclonic seizure is shown in figure 20.

Figure 20: Mice at the onset of myoclonic seizure in PTZ model

3.6. Statistical analysis :

Graph pad prism version 5 and SPSS version 25 software were used for analysis. The percentages of protected animals were analyzed using the Fisher Exact Test (two-tail) in Graph Pad Prism 5 and the analyses of variance (ANOVA)post hoc LSD with SPSS25.

CHAPTER 4. RESULTS

After hydro alcoholic extraction of the 500 gm *P. stellatum* dry powder 179.51 gm (35.9%) was obtained. The 500gm *M. stenopetalla* was extracted with dry amount of 7.35 gm(1.47%). The yield of 400gm of *Clutia abyssinica* was 44.71 gm (11.18%).

4.1. Acute toxicity study

Acute toxicity study was conducted at 2000mg/kg dose and the animals were observed according to the procedure. There was no behavioral change on live animals on the days of follow up and no gross abnormality on postmortem examination. The mean weight difference of the treatment groups before and after treatment was not statistically significantly different from the control analyzed with ANOVA post hoc LSD (P<05). The weight gain effect of the three groups is depicted in table 2.

Test Groups	**Initial weight(g)**	**After 14 days Weiht (g)**	**Mean Difference (g)**
Negative Control	23.33±0.33	26.54±0.48	3.20±.70
***P.stelatum*2000mg/kg**	24.00±0.57	27.90± 2.05	3.90±1.58
***M. stenopetala*2000mg/kg**	24.30±0.33	26.10±0.35	1.80±.37
***C. abyssinica*2000mg/kg**	23.00±0.00	28.47±0.85	5.47±0.85

N=3 weight expressed in Mean ±SE P<0.05

Table 2: Effect of 2000mg/kg dose on weight gain

4.2.*In vitro* anticonvulsant tests

A glass electrode was placed in the pyramidal cell layer of a mouse hippocampal brain slice in order to record extracellular field potentials. Removal of Mg^{2+}(0 Mg^{2+})from the slice perfusate results in recurrent seizure-like events (red arrows in Figure 21) in control slices. Middle trace, concurrent addition of 3 μM diazepam (a known anticonvulsant) prevented SLE generation. Addition of 0.7 mg/ml of *P. stelatum*and *M. stenopetala* extract prevented the onset of SLEs in the majority of slices. Population data demonstrates the anticonvulsant efficacy of diazepam (2 of 12 slices had SLEs) and *P. stellatum* (3 of 16 slices had SLEs).The hydromethanolic extract of *P. stellatum* had a statistically significant anticonvulsant activity compared to control($P<0.05$). The *M. stenopetala* extracts also shown to have significant anticonvulsant activity as compared to negative control ($P<0.05$). The effect of *C. abyssinica* extract was not statistically significant compared to control ($P>0.05$). A positive control using the known anticonvulsant diazepam(3μM), showed significant anticonvulsant activity ($P<0.05$).The percentage of slices showing SLE were given in table 3. * denotes $P <0.05$,Fishers exact test.

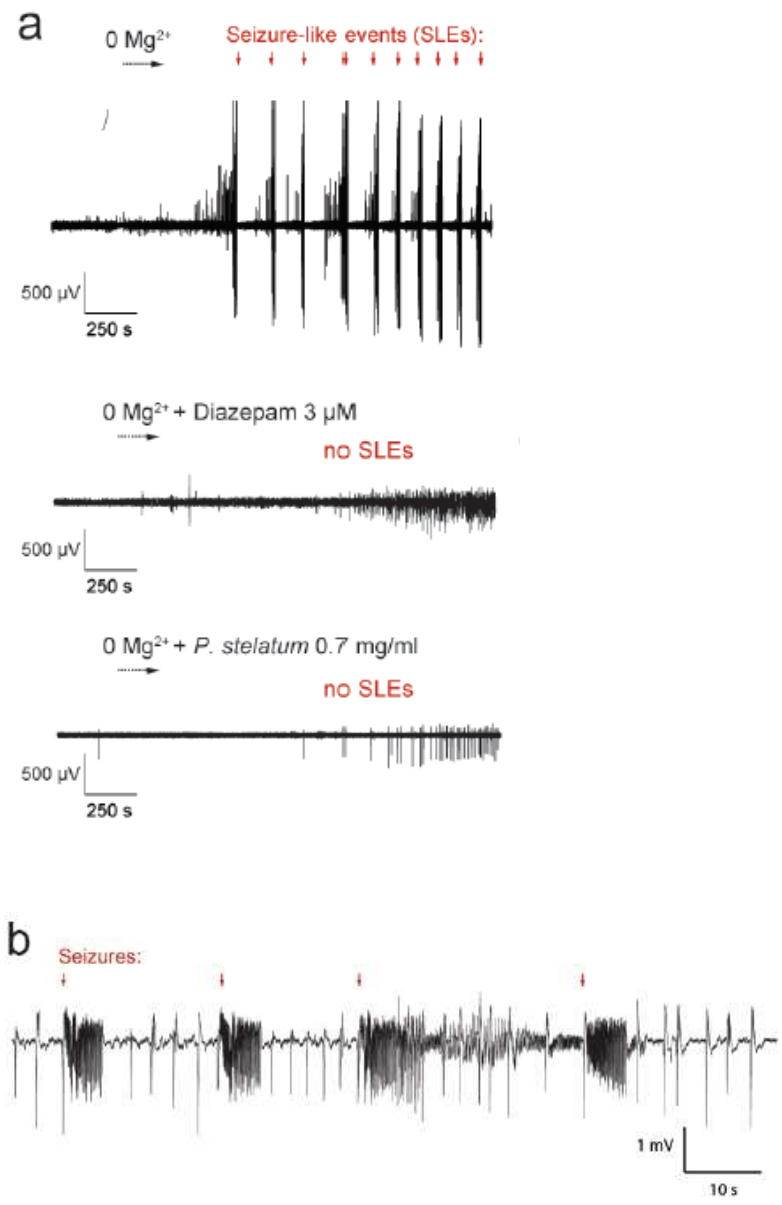


Figure 21. SLE events from field extracellular electrophysiology recording from mice hipocampal slice.a)seizure like events indicated with red arrow, seizure free slices with diazepam treatment and, seizure free slices with *P. stellatum* treatment b) control slice showing SLE.

Test groups	Number SLE positive	Number SLE negative	Number of slices	Protection percent
Negative Control (0Mg^{2+}+DMSO)	10	6	16	37.5
Diazepam (3 µM)	2	10	12	83.33*
***P.stellatum*(crude extract) 0.7 mg/ml**	3	13	16	81.25*
***M. stenopetala*0.7 mg/ml**	1	15	16	93.57*
***C. abyssinica*0.7 mg/ml**	8	4	12	33.33
***P. stellatum*0.7 mg/ml Pet ether fraction**	8	8	16	50
***P.stellatum*Chloroform fraction0.7 mg/ml**	1	15	16	93.57*
***P. stellatum*Butanol fraction0.7 mg/ml**	9	9	18	50
***P.stellatum* Water fraction(0.7 mg/ml)**	0	12	12	100*

Table 3: Percentage of SLE showing slices in acute mice brain slices. * denotes P <0.05,Fishers exact test.

The comparison of the SLE showing slices proportion of the hydromethanolic crude extracts of the 3 plants were graphically depicted in Figure 22 below.

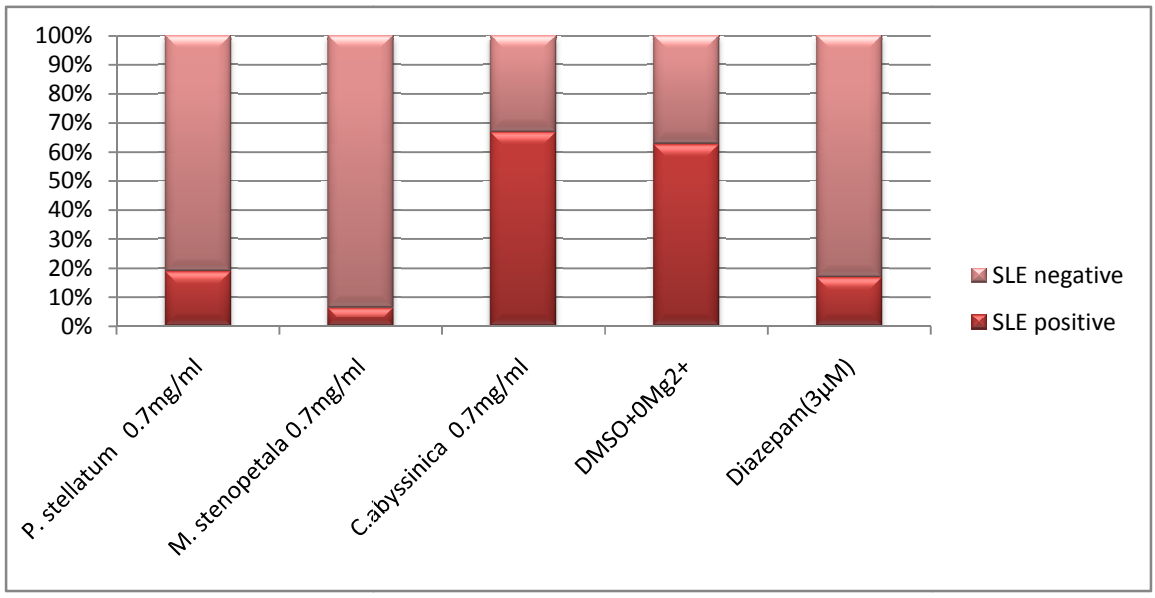


Figure 22. Bar chart depicting percentage protection of hydroalcoholic extract treatment groups(*P.stellatum* , *M. stenopetala*, *C.abyssinica*) with control 0Mg +DMSO and diazepam

When we compare the *in vitro* activity of different fraction extracts of *P. stellatum* the chloroform and water fractions were also shown to have significant anticonvulsant activity as compared to control ($P<0.05$). The pet ether and butanol extract activities were not statistically significant compared to control ($P>0.05$). Figure 23 depicts the different fraction extracts with their SLE free proportion.

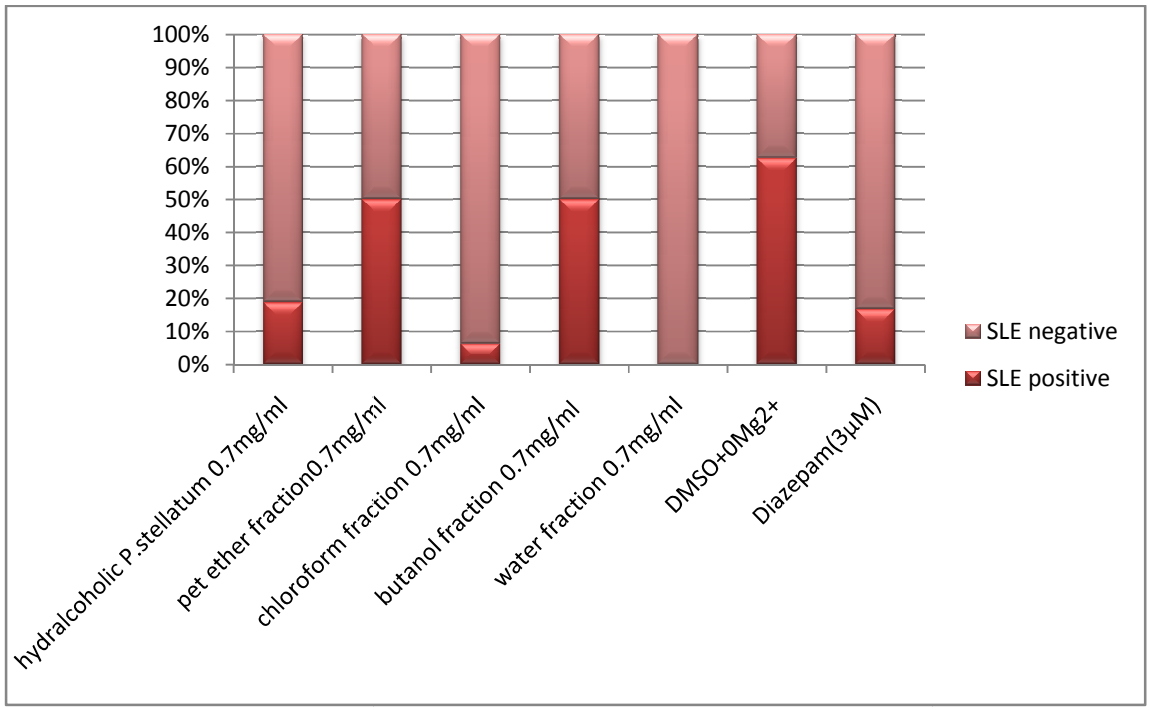


Figure 23. Bar chart depicting comparison of percentage protection of extracts of *P. stellatum* (crude hydromethanolic , pet ether , chloroform , butanol , water fractions with control 0Mg +DMSO and diazepam.

The mean time for first SLE was of untreated control was 1774.3seconds. The mean duration of seizure is 53.38seconds. The mean frequency of SLE within 3000seconds was 2. In case of hydroalcolic extract of *P. stellatum* the mean time for first SLE was 1585s ,the mean duration of seizure was 47.3seconds. The frequency of SLE on the slices that shows SLE was 3.33. *M. stenopetala* had at 1814s to show SLE in its one positive case. The duration was 33s average with two SLEs in a slice. When we see the slice of *C. abyssinica* the mean time for first SLE of untreated control was 1470.36 seconds. The mean duration of seizure is 24.03 seconds. The mean frequency of SLE was 4.25. In all observed SLEs there is no statistically significant difference on onset time of SLE (P>0.05).Table 4 shows the SLE onset time with frequency of SLEs.

Test groups	**Mean Time for First SLE(s)**	**Average duration of SLE(s)**	**Average frequency of SLE**	**Slices showing SLE**
Negative Control	1774.3±228.68	53.38±4.34	2	10/16
P. stellatum	1585±430.5	47.30±5.35	3.33	3/16
M. stenopetala	1814±00	33.00±7.00	2	1/16
C. abyssinica	1470.36±233.81	24.03±2.07	4.25	8/12

Table 4. Time of SLE onset and frequency of hydro methanolic extracts on hippocampal slices.

The butanol and petroleum ether fractions has shown high frequency of SLE. The mean time for first SLE of petroleum ether was 1096.25sec. The mean duration of seizure was 31.41sec. The frequency it shows SLE was 7.5. The mean time for first SLEof butanol extract was 1191.89.

The mean duration of seizure was 29.07. The average frequency of SLE in positive slices was 6.11. The chloroform fraction showed one SLE in one slice with mean duration of 52.4 withfrequency of 5. The onset time was not statistically different from the control (P>0.05). The water fraction didn't show any SLE (see table 5).

Test groups	**Mean Time for First SLE(s)**	**Average duration of SLE(s)**	**Average frequency of SLE**	**Slices showing SLE**
Negative Control	1774±228.69	53.38±4.34(n=20)	2	10/16
Hydromethanol extract	1585±430.5	47.30±5.35(n=10)	3.33	3/16
Pet ether fraction	1096.25±172.41	29.70±1.31(n=60)	7.5	8/16
Chloroform fraction	1814±00	52.40±9.63(n=5)	5	1/16
Butanol fraction	1191.89±201.30	31.41±2.63(n=55)	6.11	9/18
Water fraction	-	00.00± 0.00(n=0)	0	0/12

Table 5. Time of onset of SLE and frequency of SLE of different solvent fractions *P.stellatum*on hippocampal slices

4.3. *In vivo* anticonvulsant tests (PTZ and MES)

The *in vivo* result shows the *Pterolobium stellatum* hydroalcoholic extract has statistically significant anticonvulsant activity with PTZ model (P<0.05) which was dose dependent.

Whereas the activity of it was not statistically significant in delaying on set of seizure in *M. stenopetala* and *C. abyssinica* extracts (P>0.05). *In vivo* result for PTZ test of the hydroalcoholic extracts were depicted in Table 6 below.

Test group	**N**	**Latency for myoclonic seizure(s)**
Negative Control	6	239.67±33.72
***P.stellatum* 400 mg/kg**	6	542.50±94.03*
***P.stellatum* 800 mg/kg**	6	809.17±225.67*
***M.stenopetala* 400 mg/kg**	6	400.00±37.10
***M.stenopetala* 800 mg/kg**	6	387.33±35.82
***C.abssinica.* 400 mg/kg**	6	284.00±13.93
***C. abssinica.*800mg/kg**	6	457.83±103.54
Diazepam 5mg/kg	6	1800.00±0,00*

Table 6. Effect of hydroalcoholic extracts on PTZ test.Values are expressed as mean ±SEM in seconds performed with ANOVA.

The *in vivo* PTZ test has also revealed the chloroform fraction and the water fraction to have anticonvulsant effect (P<0.05). Whereas the petroleum ether and butanol fractions have shown activity which were not statistically significant (P>0.05).This goes in consistent with the *in vitro* results. The results are shown in table 7 below.

Treatment groups	N	Mean Latency for myclonic seizure(S)
Negative control	6	239.67±33.72
Pet ether 400 mg/kg	6	429.50±98.90
Pet ether 800 mg/kg	6	321.17±33.93
Chloroform 400mg/kg	6	657.00.91.62*
Chloroform 800 mg/kg	6	659.83±160.39*
Butanol 400 mg/kg	6	423.50±61.70
Butanol 800 mg/kg	6	446.17±85.93
Water 400 mg/kg	6	972.33±276.04*
Water 800 mg/kg	6	653.50±116.78*
Diazepam 5mg/kg	6	1800.0000±0.00*

Table 7. *In vivo* result for PTZ test of the fractions of *P. stellatum.*Values are expressed as mean ±SEM in seconds performed with ANOVA.

In MES test *Pterolobium stellatum, Moringa stenopetala* showed statistically significant effect in reducing the hind limb extension time which is used to detect anticonvulsant effect in this model ($P<0.05$). *Clucia abyssinica* has shown less activity which is not statistically significant ($P>0.05$)(see Table 8).

Treatment	N	Mean Hindlimb Extension time(s)	Survival rate
Negative Control	6	24.33±2.45	2/6
***P. stellatum* 400 mg/kg**	6	13.00±2.61*	5/6
***P. stellatum* 800 mg/kg**	6	11.17±4.09*	4/6
***M. stenopetala* 400 mg/kg**	6	12.50±4.16*	4/6
***M. stenopetala* 800 mg/kg**	6	10.33±3.52*	5/6
***C. abyssinica*400 mg/kg**	6	21.83 ±0.40	4/6
***C. abyssinica*.800mg/kg**	6	20.00±1.06	5/6
Phenytoin 10mg/kg	6	00.00±00*	6/6

Table 8. Effect of hydroalcoholic extracts on MES test. Values are expressed as mean ±SEM in seconds performed with ANOVA.

The *in vivo* MES test has also revealed the chloroform fraction of *P. stellatum* to have anticonvulsant effect ($P<0.05$) and also higher dose of water fraction. Whereas the other fractions showed activities which were not statistically significant ($P>0.05$)(see Table 9).

Treatment	N	Mean Hindlimb Extension time(s)	Survival rate
Negative Control	6	24.33±2.45	2/6
Pet ether 400 mg/kg	6	24.00±2.68	3/6
Pet ether 800 mg/kg	6	18.50±1.78	2/6
Chloroform 400 mg/kg	6	15.50±1.88*	5/6
Chloroform 800 mg/kg		11.50±4.29*	5/6
Butanol 400 mg/kg	6	22.50±1.23	4/6
Butanol 800 mg/kg	6	17.67±3.67	5/6
Water 400 mg/kg	6	20.17±1.85	4/6
Water 800 mg/kg	6	13.67±2.96*	5/6
Phenytoin 10mg/kg	6	00.00±00*	6/6

Table 9. Effect of *P. stellatum* fractions on MES test .Values are expressed as mean ±SEM in seconds performed with ANOVA.

4.3.Phytochemical content

The crude hydromethanolic extracts of the three plants were qualitatively tested for presence or absence of different classes of secondary metabolites. The results were shown in table 10.

Phytochemicals	*P. stellatum*	*M.stenopetala*	*C.abyssinica*
Alkaloid	+	+	+
Cardiac glycosides	-	+	+
Flavonoids	+	+	+
Phenols	+	+	+
Saponins	+	+	+
Sterols	+	-	+
Tannins	+	-	-
Terpenoids	+	-	+

Table 10: Secondary metabolites in crude extracts of *P. stellatum, M.stenopetala and C. abyssinica*

The crude hydromethanolic extracts of the three plants were quantitatively tested different classes of secondary metabolites. The results were shown in table 11.

Phytochemicals	***P. stellatum***	***M.stenopetala***	***C.abyssinica***
Alkaloid	222.64±17.40	226.69±2.39	155.54±19.81
Flavonoids	893.7±10.49	485.12±15.13	883.53±5.62
Phenols	789.76±55.15	586.53±38.35	740.58±25.68

Table 11: Secondary metabolites quantitative analysis in crude extracts of *P. stellatum, M.stenopetala* and *C. abyssinica* expressed as mean ±SE of µg/g of extract, N=3

4.4. Characterization of *P. stellatum,* extract and respective fractions

The stacked UPLC-MS total ion current (TIC) chromatograms(Fig. 2)indicate the differences in chemical constituents of the different fractions compared to the total crude hydromethanolic extract and solvent blank. The highlighted peaks are of interest as the corresponding MS fragmentation patterns correspond to that of previously identified compounds from *P. stellatum.* These compounds could be the major contributors to the *in vitro* and *in vivo* results presented, as their previously reported biological activity indicate antiseizure and anti-epileptic activity.

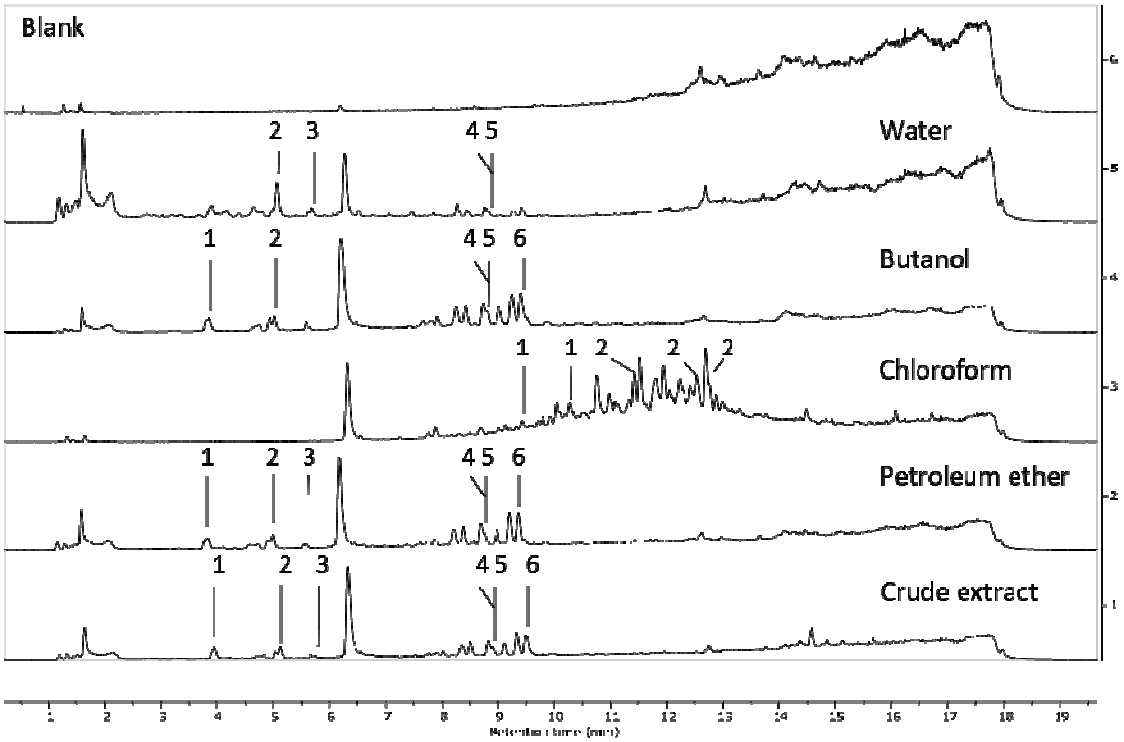


Figure 24. The negative mode ionization (ES^-)stacked UPLC-MS TICs of *P. stellatum*crude hydromethanolic extract and respective fractions. The stacked chromatograms were generated using MestReNova analytical software. The highlighted peaks represent previously identified compounds- or derivatives from *P. stellatum*which are unique to the plant extract and not found in the solvent blank. These compound peaks are described in Table 12.

The chemical properties of the identified peaks in Figure 24 are presented in Table 12. The accuracy of prediction was based onsample-to-database MS fragmentation pattern comparisons as well as correlating the relative retention time of the compounds to their solubility in the solvents used during the LC separation. Based on fragmentation pattern analyses, all the previously identified compounds were present in the extracts, however, fluctuations in qualitative concentrations were observed in the different fractions. The low MS purity values indicate that either optimal compound separation was not achieved or that these compounds

contribute as precursors of larger chemical structures, as is the case with the derivative of gallic acid, gallic acid 4-O-(6-galloylglucoside)both of which was identified in the chloroform fractions (derivative data not shown), or a combination of both these factors. The relatively low collision energies used during data acquisition allowed most compounds to be preserved with very little product ion formation (Kind *et al.*, 2017). This allowed for fast screening of the samples based on the molecular mass of the compounds adjusted according to the addition or loss of specific anions or cations, respectively. However, because of the limited fragments that can be compared between the observed fragmentation pattern and the predicted/ library patterns, care should be taken as misidentification can occur. Thus, by combining the fragmentation patterns with other chemical properties such as the solubility and polar surface area we can predict the order in which the compounds elute from the LC column, thereby providing greater confidence in the assigned compounds.

Previously identified compound from *P. stellatum* (chemical formula; monoisotopic mass)	**Retention time (min) of identified compound/ derivative in crude hydromethanolic extract***	**Observed mass (*m/z*+ $[M-H]^-$) +/- error (Da)**	**Major mass fragments- and compound*match*ion(ES^- (*m/z*))**	**MS purity &TIC Peak purity (crude hydromethanolic extract)**	**MS purity & TIC Peak purity (fractions)****
1. Gallic acid ($C_7H_6O_5$; 170.02)	Rt 3.88	169.01 + $4.0x10^{-5}$	125.04; ***169.01***; 343.07; 546.99	MS 48.40% TIC 35.63%	MS P - 45.2% C - 31.3% B - 53.2% W - ND TIC P - 28.91% C - 45.0% B - 28.57% W - ND
2. Ellagic acid ($C_{14}H_6O_8$; 302.01)	Rt 5.01	301.06 + $5.8x10^{-3}$	149.05; 169.05; ***301.06***; 603.12	MS 30.40% TIC 13.45%	MS P - 29.8% C - 9.8% B - 29.6% W - 30.2% TIC P - 15.56% C - 55.0% B - 11.85% W - 62.33%
3. Kaempferol ($C_{15}H_{10}O_6$; 286.05)	Rt 5.59	285.06 + $2.1x10^{-3}$	169.01; ***285.06***; 353.04; 495.08	MS 33.90% TIC 4.23%	MS P - 32.2% C - ND B - ND W - 38.9% TIC P - 5.11% C - ND B - ND W - 27.30%
4. Myricitrin ($C_{21}H_{20}O_{12}$; 464.09)	Rt 8.82	463.09 + $2.7x10^{-5}$	183.03; ***463.09;*** 609.15; 761.16; 927.18	MS 33.60% TIC 14.61%	MS P - 36.3% C - ND B - 36.3% W - 12.5% TIC P - 15.95% C - ND B - 15.40% W - 5.18%
5. Isoquercitrin($C_{21}H_2$	Rt 8.82	463.09 + $2.7x10^{-5}$	183.03; ***463.09;***	MS 33.60%	MS P - 36.3%

$_0O_{12}$; 464.09)			609.15; 761.16; 927.18	TIC 14.61%	C - ND B - 36.3% W - 12.5% TIC P - 15.95% C - ND B - 15.4% W - 5.18%
6. Quercitirin($C_{21}H_{20}O_{11}$; 448.10)	Rt 9.11	447.09 - $1.0x10^{-4}$	183.03; ***447.09***; 593.15; 895.19; 1041.25	MS 29.40% TIC 17.40%	MS P - 29.4% C - ND B - 29.4% W - ND TIC P - 18.51% C - ND B - 16.13% W - ND

Table 12. UPLC-MS generated chemical properties of the highlighted peaks represented in Figure 24

*The retention times presented in the table refer only to the negative ionisation mode data of the crude hydromethanolic extract.

**The fractions are described as;P- petroleum ether, C- chloroform, B- butanol, W- water

The major fragments were identified using the MestReNova MS prediction algorithm, in conjunction with the NIST 14 MS/MS spectral library database.

ND- Not Detectedand/ or Match score below 95%and/ or Rt incorrect.

CHAPTER 5. DISCUSSION

There are many classes of clinically useful antiepileptic drugs with good prognosis for controlling seizures in most patients. Despite this, many patients have seizures that are not adequately managed by the established antiepileptic drugs. Moreover, the high incidence of adverse effects from the use of established antiepileptic drugs is also a source of widespread concern in patients who use them chronically. One of the approaches to search for new antiepileptic drugs which are safe and effective is investigation of naturally occurring compounds, which belong to new structural classes (Sayyah *et al.*, 2011). Many plants were known for their anticonvulsant activity. Review articles (Chauhan, Dobhal and Joshi, 1988; Nsour, Lau and Wong, 2000; Srinivasan and Roy, 2017) were previously published with regard to plants with anticonvulsant properties. Both *in vivo* and *in vitro* models are available for the evaluation of anti epilepticactivities of drugs (Löscher, 2011; Mittal, Kaushik and Kaushik, 2011). The current study was to evaluate the anticonvulsant effect of *Pterolobium stellatum* (leaf), *Moringa stenoptela*(root), and *Clutia abyssinca* (leaf). The zero Mg^{2+} model was used for *in vitro* evaluation test while PTZ and MES for *in vivo* test. These two methods showed the anticonvulsant effect of the extracts.

Pterolobium stellatum was claimed as anticonvulsant in Ethiopia (Ragunathan and Abay, 2009). The *in vitro* result showed that the crude hydroalcoholic extract of *P. stellatum* leaves has statistically significant anticonvulsant effect compared with the untreated control with zero Mg^{2+} model used in this study. This indicates that the plant to have some molecules which act at cellular level to inhibit neuronal excitability or limit repetitive firing of neurons. The *in vitro* study shows the plant may have effect in modulating voltage-dependent sodium channels to limit

repetitive firing of neurons, decreasing voltage-gated calcium currents or inhibiting post-synaptic AMPA receptors, all potentially contributing to a decrease in neuronal excitability. Its anti-epileptic effect may also be linked to potentiation of GABAergic transmission as that of the approved antiepileptic drugs(Mittal, Kaushik and Kaushik, 2011; Taing *et al.*, 2017).

The *in vitro* results is strongly supported by the actions *in vivo* mice models in reducing MES and PTZ induced seizures. Pentylenetetrazole elicits seizures by inhibiting GABAergic mechanisms. Standard antiepileptic drugs, diazepam and phenobarbitone, are believed to produce their effects by enhancing GABA mediated inhibition in the brain (Hegde *et al.*, 2009). It is, therefore, possible that the anticonvulsant effects shown in this study by the extracts against seizures produced by PTZ might be due to the activation of GABA neurotransmission. Since the extract similarly antagonized seizures elicited by pentylenetetrazole in mice, it is probable, therefore, that it may also be exerting its anticonvulsant effects by affecting GABAergic mechanisms. Pentylenetetrazole test represents a valid model for human generalized myoclonic and also absence seizures. In general, compounds with anticonvulsant activity in the petit mal epilepsy are effective in pentylenetetrazole induced seizure model (Pai *et al.*, 2012).The hydroalcoholic extract of *Astragalus obtusifolius* which is also fabaceae inhibited clonic seizures induced by PTZ in previous studies which is in accordance with this study. Whereas *Albizzia julibrissin, Acacia juliflora* and *Acacia nubica* showed no antiseizure effect on PTZ model (Sayyah *et al.*, 2011).

Pterolobium stellatum has also shown effect in MES mice model. The maximal electroshock test is the most widely used in animal model in antiepileptic drug discovery because seizure induction is simple and the predictive value for detecting clinically effective antiepileptic is high. This method identifies the drug with activity against generalized tonic-clonic seizures and partial seizures using clinically established antiepileptic drugs. The pharmacology of acute maximal electroshock does not differ from the pharmacology of generalized tonic clonic seizures in genetic models with chronic epilepsy. It has often been stated that antiepileptic drugs that block MES induced tonic extension act by blocking seizure spread. Moreover, MES induced tonic extension can be prevented either by drugs that inhibit voltage-dependent Na+ channels, such as phenytoin, valproate, felbamate and lamotrigine, or by drugs that block glutamatergic excitation mediated by the *N*-methyldaspartate (NMDA) receptor, such as felbamate (Pai *et al.*, 2012).

The chloroform and water fractions showed also statistically significant activity *in vitro.* The Pet ether and butanol fractions have less effect which was statistically not significant compared with the untreated control. This was also supported by the activity of the chloroform and water fractions both in PTZ and MES tests having statistically significant anticonvulsant effect while the pet ether and butanol fractions were not. This could be for the active principles of the activity may be more concentrated in the chloroform or water fractions of *Pterolobium stellatum* leaf extract. These all results support the claim that the whole plant juice has been reported to be used to treat epilepsy and neuralgia in north-west Ethiopia (Ragunathan and Abay, 2009).

The phytochemical tests revealed the presence of sterols, alkaloids, phenols, flavonoides, terpenoids and saponins in the crude extract. In the crude extract of *A. obtusifolius* which showed

anticonvulsant activity in other study has the phytochemical tests result showing presence of triterpens/sterols, alkaloids, flavonoides, anthrones, and saponins. By fractionation, the entire flavonoids and saponins as well as the majority of alkaloids were transferred from the extract to the aqueous fraction while entire of terpen/sterols and negligible amount of alkaloids were transferred to the dichloromethane fraction. Anthrones were equally distributed in both fractions. The research suggested the anticonvulsant effect seems that alkaloids, flavonoids and saponins present in the extract and the aqueous fraction might be mainly responsible for the observed anticonvulsant activity(Sayyah *et al.*, 2011). These metabolites also showed anticonvulsant activity *in vitro* and *in vivo* in similar studies(Fernández *et al.*, 2006; Chindo *et al.*, 2009). In this study both qualitative and quantitative analysis shown the presence of secondary metabolites in the extracts. The LCMS analysis indicated also the presence of gallic acid, ellagic acid, kaempferol, myricitrin, isoquercitrin and quercitirin in the crude extract. Of these gallic acid and ellagic acid were found in chloroform fraction. In the water fraction ellagic acid, kaempferol, myricitrin and isoquercitrin were found. These show some of the metabolites tend to concentrate in certain fractions which may be contributed for the activity of the chloroform and water fractions.

In the other study on *P. stellatum* the aqueous acetone extracts of the species contained the high contents of phenolics as estimated by peak areas in the HPLC chromatograms (Mueller-Harvey, Hartley and Reed, 1987). Coumaric acids were one of the phenolics in leaves of *P. stellatum.* p-Coumaric acid occurred in much larger amounts than trans-ferulic acid and approximately one-third of p-coumaric acid was present as the cis-isomer. Gallic acid was identified in the acetone extracts of the species by HPLC co-chromatography and TLC. Similarly ellagic acid was

identified in *P. stellatum.* TLC also indicated the presence of ellagic acid esters and gallic acid esters in *P. stellatum.* The HPLC retention times of most of the gallic acid esters of *P. stellatum* were similar to other tannic acids (from Turkish galls and from taratannins). Isoquercitrin, quercitrin and myricitrin were identified by HPLC in the aqueous acetone extract *of P. stellatum*. Kaempferol was also detected in the same study (Mueller-Harvey, Hartley and Reed, 1987).

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Among plant derived compounds are flavonoids which recently have attracted interest because of their biological activities to human health, also because of their influence on central nervous system effects. The influence of flavonoids on anxiety, depression, nociception, learning and memory processes has been reported. Rutin and quercetin are constituents of numerous plant extracts, such as St John's wort which is used for the treatment of depression and *Ginkgo biloba* used in patients with cognitive deficits. Moreover, these flavonoids are weighty ingredients of many medical products and diet supplements which are also taken by patients with epilepsy (Gür *et al.*, 2018).

Quercetin and isoquercitrin type of flavonoids, exhibited anticonvulsant effects in investigational epilepsy models(Alam Khan, Assad and Ali Rajput, 2018). Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is a flavonoid found in a variety of fruits and vegetables. The protective and toxic effects of quercetin depend on both timing and dose with regard to the anticonvulsant effects(Diniz *et al.*, 2015). It is suggested that it modulates $GABA_A$ receptors and antagonizes N-methyl D aspartate (NMDA) receptors(Alam Khan, Assad and Ali Rajput, 2018). They were also detected in an extract of *Galium spurium* which is used in Turkish folk medicine against epilepsy (Orhan *et al.,* 2012). In study results showed that polar extracts of *T. americana* var. mexicana

significantly prevented severity of PTZ-induced seizures and attenuated oxidative stress levels involving the presence of flavonoids such as quercetin, rutin, and isoquercitrin (Cárdenas-Rodríguez *et al.*, 2014). Antiepileptic potential of quercetin, catechin, and kaempferol studied and found of value in other study (Ahmed *et al.*, 2021). Quercetin inhibited KA-induced epilepsy by microglia cell inactivation and the production of NF-κB, TNF-α and IL-1β (Wu *et al.*, 2020).

Quercetin prevented seizures in PTZ induced seizures. It prolonged onset of seizures, reduced the seizure duration and seizure severity score in comparison with control group(Sefil *et al.*, 2014). Other study also demonstrated a dose-dependent anticonvulsant potential of quercetin and rutinin the psychomotor seizure model in mice. These compounds significantly increased the threshold for 6 Hz-induced seizures. The studied flavonoid quercetin lacks also significant interactions between quercetin and the studied AEDs, i.e., VPA and LEV, in the 6 Hz test in mice suggesting that co-administration of these drugs with the studied flavonoids included in some medical preparations or diet supplements in patients with epilepsy is entirely safe and should not produce any acute side effects in clinical practice (Nieoczym *et al.*, 2014).

Myricetin which is also component of *Pterolobium stellatum* exhibited protective effects against PTZ-induced seizures by reducing seizure severity and regulating the GAD_{65}/$GABA_A$ and BDNF-TrkB signaling. These observations suggest that myricetin may be used as an anti-epileptic drug in the future (Sun *et al.*, 2019). Being component of the plant it puts one interest in further study of the plant.

This study provides a scientific rationale for the use of the plant extract for the amelioration of epilepsy observed in traditional medicine in Ethiopia. The results revealed that treatment with the extract protected against *in vitro* seizure and *in vivo* PTZ and MES - induced convulsions. The secondary metabolites seen in*P. stellatum* were also studied extracted from other plant sources showing good anticonvulsant effect.These points suggest its potential for further study and possible active molecule search.

The hydoalcoholic extract of root of *Moringa stenopetala* has shown *in vitro* antiseizure activity. This was supported by the *in vivo* antiseizure activity in MES model. But the crude extract failed to show statistically significant activity in PTZ model. This shows the plant has antiseizure potential. The root is also used in traditional medicine to treat other different aliments and some of the chemical components were studied. The qualitative secondary metabolite test evidenced the presence of alkaloids, phenols, flavanoids, and saponins. According to the review done by Zue*et al.* medicinal compounds with antiepileptic/anticonvulsant activities are alkaloids, flavonoids, terpenoids, saponins and coumarins (Zhu *et al.*, 2014). For the hydroalcoholic extract contains these compounds the anticonvulsant activity may be attributed to the presence of these phytochemicals. In *Moringa oleifera* alkaloids saponins, protein, flavonoids, carbohydrates, tannins, terpinoids, phenols, glycosides and phytosteroids showed presence in all the components of leaf, pod and bark and these parts showed anticancer activity(Gokila Devi *et al.*, 2017).The roots of *Moringa oleifera* methanolic extract caused statistically significant protection against PTZ and strychnine (STR) induced seizures(Asif, 2013).

In one study, the acetone extract of *Moringa stenopetala*(root), which was active as antibacterial was found to contain cholest-5-en-3-ol, palmitic acid, n-octacosane and oleic acid, respectively, based on physical properties and spectroscopic (IR and NMR) data(Tesemma *et al.*, 2013b). The effects of straight chain fatty acids on seizures induced by picrotoxin and pentylenetetrazole were studied in mice. After i.p. injection capric, lauric, myristic, palmitic and stearic acid delayed the onset of picrotoxin-induced clonic convulsion in a dose-dependent manner. The survival time was also prolonged by the pretreatment with lauric, myristic, palmitic and stearic acid (Nakamura *et al.*, 1990).

Evidence related to the anticonvulsant effects of the n-3 fatty acids has come from *in vitro*, *in vivo*, and some clinical studies. *In vitro* studies involving extracellular recording first supported the idea that the n-3 polyunsaturated fatty acids (PUFAs) might have anticonvulsant properties. This was initially demonstrated in *ex vivo* studies involving hippocampal slices. These studies indicated that the extracellular application of Alpha-linolenic acid (ALA), Eicosapentaenoic acid(EPA), or Docosahexaenoic acid(DHA) to rat hippocampal slices, at concentrations of up to 100 lM, reduced the frequency of electrically or chemically induced action potentials and excitatory discharges. These studies involved slices that were: (1) treated with pentylenetetrazol (PTZ) or glutamate, (2) depleted of glycine, or (3) subjected to low Mg^{2+}.*ex vivo* studies performed with intracellular recordings further supported the idea that the n-3 PUFAs might have anticonvulsant properties, and suggested that these were due to inhibitory effects on voltage-dependent ion channels. Evidence from intracellular recordings of stratum pyramidale neurons in rat hippocampal slices, for instance, showed that 16 lM of DHA significantly inhibited the repetitive firing of action potentials elicited by depolarizing current pulses. These

findings were consistent with the results of another study, which showed that the application of DHA at concentrations of 10–100 lM to rat hippocampal slices reduced the firing of CA_3 evoked action potentials (Young *et al.*, 2000). Xiao and Li (1999) also found that DHA raised the action potential depolarization threshold and decreased the frequency of stimulus evoked action potentials. These data led to the hypothesis that fatty acids modulate ion channels.

Single cell studies involving dissociated neurons have supported this hypothesis. Studies involving EPA or DHA in dissociated cells from rat CA_1 have shown a significant shift in inactivation in the hyperpolarizing direction for both Na^+ and Ca^{2+} currents. Both EPA and DHA have also been shown to stabilize the neuronal membrane in single cells by suppressing voltage-gated Na^+ and Ca^{2+} channels, and thereby increasing the action potential firing threshold (Taha, Burnham and Auvin, 2010).

Moringa concanensis abolishes both MES and PTZ seizures, it might posses sodium channel blockade, NMDA blockade, calcium channel blockade, or GABA agonist activity. The anti-convulsant activity of *Moringa concanensis* can also be due to the antioxidant property. The anti-convulsant activity can be due to the presence of various phytoconstituents like alkaloids, tannins, phenols, flavanoids, and carbohydrates. Further studies are ongoing using different extracts of *Moringa concanensis* to elucidate the exact mechanism by which this plant acts as an anti-epileptic agent(Manikkoth, Joy and Kunhikatta, 2014). In *Moringa oleifera* pretreatment with methanolic extract of roots of *Moringa oleifera* caused significant protection against strychnine (STR) and PTZ induced convulsions which is not in accordance with the result in this

study. The overall CNS depression along with potentiation of hypnotic activity of pentobarbitone sodium as well as diazepamis also recorded (Asif, 2013).The root tissues contained both 4-(R-Lrhamnopyranosyloxy)- benzylglucosinolate and benzylglucosinolate. The leaves of *M. stenopetala* contained quercetin 3-*O*-rhamnosylglucoside (rutin) and traces of quercetin 3-*O*-glucoside (Bennett *et al*., 2003).

Clutia abyssinica leaves hydromethanolic extract anticonvulsant activity was not demonstrated on the *in vitro* as well as *in vivo* models used in the present study. This plant was claimed by the local people for different ailments and it is used for evil eye (Wereta, 2015a). We report here in the leaves of *Clutia abyssinica* extract the presence of alkaloids, cardiac glycosides, flavanoids, phenols, saponins, sterols and terpeoids. Though these are some of the components didn't show anticonvulsant effect in the current model used. The results show it has less activity and further study in other models is needed to revalidate the claim or it may have some potentiating effect if it is given with other plants.

CHAPTER 6. CONCULUSIONS

The results demonstrated that *Pterolobium stellatum* have anticonvulsant effect *in vitro* and *in vivo* in both PTZ and MES mice models. The hydromethanolic and other fractionated chloroform and water extracts demonstrated effect indicating biologically active molecules to be concentrated in this part of the extract. The UPLCMS analysis indicated also the presence of gallic acid, ellagic acid, kaempferol, myricitrin, isoquercitrin and quercitirin in the crude extract. Of these gallic acid and ellagic acid were found in chloroform fraction. In the water fraction ellagic acid, kaempferol, myricitrin and isoquercitrin were found. This result shows the claim that the plant is used by the local community as anticonvulsant is with their valid ground on their trial in combating the problem. Though this study tests the potential of anticonvulsant effect of the plant it didn't go further in identifying the specific molecule responsible for the activity.

The crude extract of *Moringa stenopetala* is also has shown to have anticoncvulant effect both *in vitro* and *in vivo* MES models. The use of local community as anticonvulsant is supported by the result obtained from this study. Having this, tapping its potential as anticonvulsant effect is promising in identifying new antiepileptic molecules as it is widely used for treatment of other diseases with less toxic effect.

The result of the *C. abyssinica* leaves as anticonvulsant is not demonstrated based on the model used in this study. For most of the local preparation are mixes of different plants it may have synergistic action with other plants. Or it may have action with other models of chronic epilepsy.

CHAPTER 7. RECOMMENDATIONS

Based on the above conclusion the following recommendations are forwarded

- Further study on dose standardization on *P. stellatum* should be conducted
- Further study on other parts of the plant should also be conducted
- isolation of the active principle should be conducted
- Further study on fraction of *Moringa stenopetala* and also isolation of the active principle should be conducted
- Study on other models chronic models should be conducted on *Clutia abyssinica*
- Study on chronic models on *Pterolobium stellatum* and *Moringa stenopetala* should also be conducted to see their antiepileptogenic potential.

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Annex I. Acute slice protocol

*A) **Preparation of solutions:***

1. 50% sucrose cutting solution:
    a. For 500ml: 50ml sucrose stock, 450ml dH20 + sucrose (20.538g), MgCl2 (1500ul), CaCl2 (500ul)
    b. Dissolve substances using magnet
    c. Bubble the solution for 5 minutes
    d. Transfer the solution into the mixer container and cover it with parafilm
    e. Place the mixer container in the -80 C freezer (level 5) for 60-75 minutes

2. aCSF
    a. prepare 500ml of aCSF solution:
        i. 50ml aCSF stock
        ii. 450ml dH20
        iii. 1ml CaCl2 stock
        iv. 500ul MgCl2

3. 0 Mg
    a. prepare 500l of 0 Mg solution
        i. 50ml aCSF stock

ii. 450ml dH20

iii. 1ml CaCl2 stock

4. PBS

    a. Prepare PBS

5. Agarose:

    a. Turn on agarose heater (should be between40-50C)

    b. Place the two “agarose” test tubes in the heater

    c. Measure out 250mg of agarose onto weigh boat and transfer into the conical flask.

    d. Use electronic pipette to transfer 12.5ml of PBS into the conical flask

    e. Swirl the conical flask over the Bunsen burner with claw-clamp until boiling (solution becomes clear)

    f. Carefully transfer the agarose solution into the “agarose” labelled test tubes

B) <u>Preparation of equipment:</u>

1. Guillotine:

    a. Make sure the sink area is clean

    b. Place guillotine next to sink

    c. Make sure handle/blade mobile

2. Brain extraction station:

a. small plate (in freezer)

b. Small, sharp tweezers

c. Blunt tweezers

d. Small scissors

e. Round surgical blade

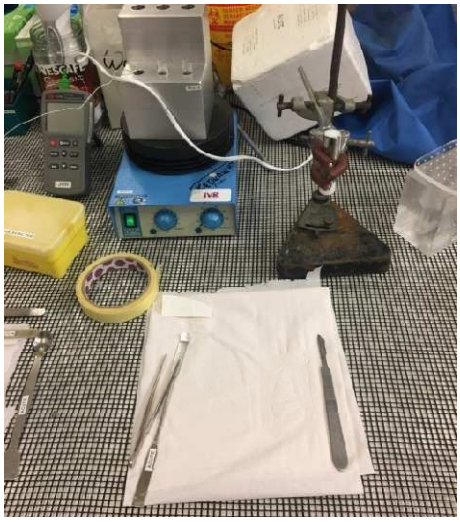
f. Scooping spatula

3. Brain sectioning station:

    a. largeplate + ice block

    b. non-round surgical blade

    c. spatula (flat surface)



4. Microtome bath
    a. Blade preparation:
        i. Fold BIC blade into half
        ii. Cut the edges of the BIC blade
        iii. Remove the blade holder from the microtome using allen key
        iv. Apply super glue (blue) onto the blade holder
        v. Carefully attach the BIC blade onto the blade holder with the cut edges aligning with the bottom edge of the blade holder.
        vi. Set blade holder back onto microtome
        vii. Switch the microtome on and press “power”?
        viii. Stop the blade 2/3 down
        ix. set the blade at an angle very close to cylinder entrance

b. equipment:

   i. fine brush

   ii. insulin syringe (normal and bent)

   iii. small spatula

   iv. small tweezers (same one from brain extraction)

5. Agarose stand/claw clamp station:

   a. 5-10 small pieces of filter paper

   b. Super glue (white)

   c. Clamp the white cylinder (in its silver casing) onto the claw-clamp

6. Cooling/set agarose:

   a. Place foam box with the metal clamp cooler and ice packs inside (just before sectioning starts)

7. Recovery chamber
    a. cut lens paper into rectangular pieces (x6)
    b. color code the pieces using 3 different color (blue, red, black) pens.
        i. To ensure laterality, make small dots in respective top corners
    c. place lens paper pieces onto netted plate
    d. place netted plate into the recovery chamber and fill the chamber with aCSF (which should be bubbled before) until the lens paper pieces are just about covered/wet (ensure no bubbles are under the lens paper pieces)
    e. connect the carbogen tube to the chamber
    f. place recovery chamber cover over the opening

C) <u>Slicing procedure:</u>

1. Mouse:
    a. Fetch mouse and place in dark, quiet area (under sink or inside cupboard)

2. Sucrose solution:
    a. Fetch the sucrose solution and use the double-edged knife to roughly break the frozen solution
    b. Use the mixer to break the solution further

3. Agarose setting:

a. Remove ice packs and metal agarose cooler clamp and place into the sponge cooler box

4. Recovery chamber:
    a. Turn on carbogen
5. Plates:
    a. Remove the two plates (big and small) from the freezer
    b. Pour sucrose solution into small plate and place next to the guillotine
    c. Pour sucrose solution into large plate and place onto ice block (sectioning station)

6. Decapitation:

    *wear gloves and lab coat prior to decapitation

    a. Carefully remove animal from the box
    b. Place head onto guillotine
    c. Decapitate quickly and allow the head to fall into the small plate
    d. Place the carcass into the sink

7. Brain extraction:
    a. Quickly transfer the small plate (now containing the head) to extraction station.
    b. Hold the head still by piercing the nostrils with the small tweezers (ensure that the head is completely submerged)
    c. Using the round blade, cut the skin down the midline (from front to back)
    d. Push the skin forward using your fingers

e. With small scissors:
    i. Cut horizontally through the orbits
    ii. Cut ventro-medially through the respective external acoustic meatuses
    iii. Cut the skull down the midline (from back to front)
f. Separate the skull from the brain (now separated into left and right) using the blunt tweezer
g. Remove the brain using the scooping spatula (front to back motion)

8. Brain sectioning:
    a. Quickly transfer the brain onto the large plate
    b. Cut off the cerebellum and part of the frontal lobe using the surgical blade (use the flat surface of the spatula for stability)
    c. Scoop up the sectioned brain using the spatula

9. Agarose-clamp:
    a. Use the filter paper to suck up the solution under the brain
    b. Place superglue onto the white cylinder
    c. Carefully place the brain onto the white cylinder (should be completely flat)
    d. Lower the white cylinder in its silver casing (make sure to support the bottom of the white cylinder always)
    e. Pour agarose solution into the sliver casing until it reaches the top.
    f. Set the agarose solution by clamping the silver casing with the metal cooler clamp

10. Microtome bath: *make note of laterality

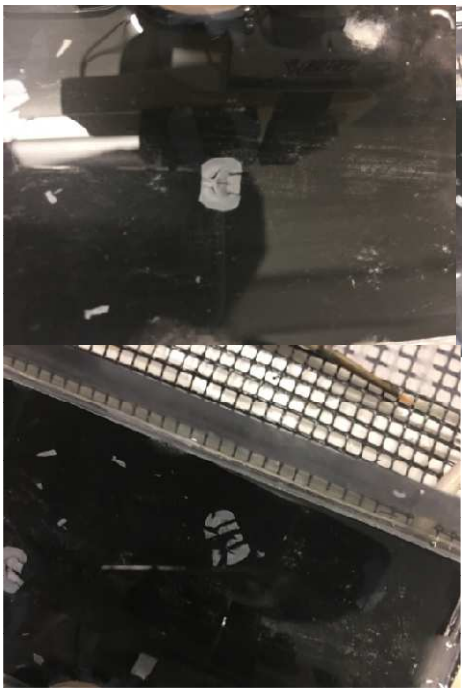
    a. Once the agarose has set, place the white cylinder-silver casing into the microtome.
    b. Pour sucrose solution into the microtome bath until it covers the whole circular entrance of the cylinder

11. Microtome slicing:

    a. Set the white cylinder into the microtome
    b. *the white cylinder is advanced by turning the “wheel” of the microtome (10 units = 100um)
    c. Cut agarose until just the top of the brain has been sectioned
    d. Cut 1200um thickness from this point
    e. Start sectioning at 400um from now on (record/observe the depth of the section for each slice)
    f. Once 400um slices have been sectioned, carefully remove the agarose using the fine brush and place the slices in the microtome bath so that it is easy to note which slices are which depth.
    g. Choose the best 3 slices to use.

12. Slice preparation:

    a. Using the bent insulin syringe and fine brush, cut the slices into hemispheres followed by further cutting into smaller pieces (as shown below)



13. Recovery chamber:

    a. Using the small spatula transfer the hemispheres (6 in total) onto the lens paper pieces in the recovery chamber. (Wet the slices on the small spatula with sucrose solution using the normal insulin syringe. This allows easier transfer of the slices)
    b. Ensure that the recover chamber is covered up in the correct way.
    c. Recovery should be minimum of 45 minutes

14. Set up the interface rig as per usual

15. Clean up all equipment

16. Replace microtome blade:

a. Remove blade (+blade holder) using “slicing blade”
b. Place in acetone
c. Detach blade from blade holder (discard blade into sharps bin)
d. Prepare new BIC blade by cutting edges with thick scissor
e. Place new blade into acetone (cleaning)
f. Dry new blade and blade holder.
g. Apply a thin layer of super glue (Loctite Brush-on super glue)
h. Carefully fit new blade onto blade holder (align the cut corners of the blade with the bottom edge of the blade holder).

17. Transferring the slices from recovery chamber to the interface:

a. Take small tweezers and grab edge of lens paper.
b. Carefully transfer them to the interface rig

*General timeline for interface of acute slices:

1. 600s calibration (aCSF)

2. wash in 0 Mg (average for 3000s)orwash in 0 Mg + other test drugs(average for 3000s)

3. Wash in for 600-900s

Annex II. Research articles

This dissertation is based on the following published papers:

1.Samson S. and TeferraA.(2020)*In vitro* and *In vivo* Anticonvulsant Effect of Hydroalcoholic Extract of *Moringa stenopetala* in Mice Models. *Journal of Complementary and Alternative Medical Research* **12**(3): 16-23.

2. Samson S. and TeferraA.(2021) *In vitro* and *In vivo* Anticonvulsant Effect of Hydroalcoholic Extracts of *Clutia abyssinica*in Mice Models. *Journal of Complementary and Alternative Medical Research* **13**(4): 8-14.

Journal of Complementary and Alternative Medical Research

12(3): 16-23, 2020; Article no.JOCAMR.64362
ISSN: 2456-6276

In vitro and *In vivo* Anticonvulsant Effect of Hydroalcoholic Extract of *Moringa stenopetala* in Mice Models

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Authors' contributions

This work was carried out in collaboration between both authors. Author SSS designed the study, performed the laboratory work, statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author TA supervised the work and edited the final draft. Both authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JOCAMR/2020/v12i330208
Editor(s):
(1) Dr. Sahdeo Prasad, Texas Tech University Health Sciences Center, USA.
Reviewers:
(1) Ambekar Abdul Wahid Abdul Aziz, Dr. Vithalrao Vikhe Patil College of Pharmacy, India.
(2) James David Adams, University of Southern California, USA.
Complete Peer review History: http://www.sdiarticle4.com/review-history/64362

Original Research Article

Received 02 November 2020
Accepted 07 January 2021
Published 19 January 2021

ABSTRACT

Background: Epilepsy is a debilitating neurological disorder that directly affects approximately 65 million people worldwide. In the search of safe and effective antiepileptics traditional treatment practices are one area of research to obtain novel molecules. *Moringa stenopetala* root is claimed to be used for epilepsy treatment in Konso area, Southern Ethiopia. But there was no scientific research evidence for the claimed use of the plant.
Objective: This study was conducted to explore the anticonvulsant activity of hydro-alcoholic (80% methanol) extract of root of *Moringa stenopetala*.
Methods: The dry residues of the plant extract was used for the test. *In vitro* 0Mg^{2+} mice model at dose 0.7 mg/kg of extract, diazepam(3µM) and untreated brain slice groups were used to compare the presence of seizure like event (SLE). *In vivo* pentylenetetrazol (PTZ) model with 85 mg/kg

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subcutaneously was used to compare the seizure on set time with two extract doses and diazepam 5 mg/kg. The data was presented with mean± standard error. In maximum electric shock (MES) model 54 mA was passed for 0.2 second transauricularly in mice. The mean time of hind limb extension was recorded for doses 400 mg/kg and 800mg/kg of the extract and 10 mg/kg phenytoin. The means were compared for statistical significance using one way ANOVA post hoc LSD whereas proportions were compared using Fishers exact test with P-value < .05.

Results: *M. stenopetala* extract has shown statistically significant anticonvulsant activity *in vitro* compared to control (P<.05). A positive control, the known anticonvulsant diazepam (3μM), showed significant anticonvulsant activity (P<.05). *In vivo* MES model showed statistically significant anti-seizure activity at both doses (P<.05). But the crude extract failed to show statistically significant activity at all doses of PTZ model (P>.05).

Conclusion: The results of this study showed that crude extract of *Moringa stenopetala* exhibited anti-convulsant effect both *in vitro* and *in vivo* MES models.

Keywords: Moringa stenopetala; seizures; epilepsy; anticonvulsant; 0 Mg^{2+} model; maximal electroshock seizure model; pentylenetetrazol seizure model.

1. INTRODUCTION

Affecting 65 million individuals worldwide, epilepsy is the third leading contributor to the global burden of disease for neurological disorders [1]. An estimated 2.4 million people are diagnosed globally with epilepsy each year [2]. Even though antiepileptic drug (AED) medication is widely available, many people with active epilepsy particularly in resource-poor countries go untreated [3]. In those getting treatment refractoriness is still an important issue in epilepsy therapy despite the fact that new antiepileptic drugs have been available since late 1980s.In the search of novel antiepileptic drugs one of the approaches is investigation of naturally-occurring compounds [4].

In Ethiopia as various diseases are being treated traditionally some survey shows also the practice of treatment for epilepsy using plant extracts. *Moringa stenopetala* though its root is claimed to be used for epilepsy treatment in Konso area, Southern Ethiopia there is no scientific study conducted for the evidence of its potential value [5]. As different researches on plants used for epilepsy in different countries have shown anticonvulsant activity, these plants may also have value for the treatment of the disease [6]. Therefore scientific research should be done on these plants for their antiepileptic potential [7].

Zero Mg^{2+} model is one of the *in vitro* models to study mechanism of seizure and antiseizure drugs. The most commonly employed *in vivo* animal models in the search for new anticonvulsant drugs are the MES test and the PTZ seizure test [8,9]. The maximal electroshock seizure test, in which tonic hindlimb seizures are induced by bilateral corneal or transauricular electrical stimulation, is thought to be predictive of anticonvulsant drug efficacy against generalized tonic-clonic seizures, while the pentylenetetrazole test, in which generalized myoclonic and clonic seizures are induced by systemic (usually s.c. or i.p.) administration of convulsant doses of PTZ, is thought to represent a valid model for generalized absence and/or myoclonic seizures in humans [8].

Hence this study was conducted with the objective to looks for the anticonvulsant potential of 80% methanol extract of *Moringa stenopetala* using *in vitro* and *in vivo* mice models.

2. MATERIALS AND METHODS

2.1 Plant Material Collection and Extraction

The plant *Moringa stenopetala*(root) was selected based on claim by the society to use for epilepsy. From the areas where it is used as traditional epilepsy therapy, the plant part used in this study was collected in April 2016. The root of *Moringa stenopetala* was collected from Arbaminch area 505 km South of Addis Ababa. The plant was identified and voucher specimen was deposited with the given herbarium code (03-S) in the national herbarium at Addis Ababa University , College of Science, Ethiopia.

The collected plant parts were then garbled in th e processing room and dried in the shade, and powdered and stored in a well-closed container at room temperature until extrac ted. The powdered, air dried materials

(500 g) of *Moringa stenopetala* (root) were then extracted by maceration with 80% methanol at room temperature for three consecutive days. The mixture filtered by gauze and then with Whatman™ filter paper 6µm pore size (125 mm GE healthcare UK limited, UK) and concentrated under vacuum in a rotary evaporator. Using this extraction technique *M. stenopetala* was extracted with dry amount of 7.35 gm(1.47%).The extract was kept in a tightly closed bottle in a refrigerator until used for anti-seizure testing [10].

2.2 Phytochemical Screening

The method used by Debella [10] was implemented to screen for the presence and/or absence of the main secondary metabolite groups in the extracts.

2.3 Acute Toxicity Study

An acute toxicity study was conducted for the extracts by acute oral toxic class method of Organization of Economic Co‐operation and Development, as per 423 guidelines [11].Three female mice in a group were grouped into two groups in the test randomly. One control group was given distilled water and the other groups was treated for *Moringa stenopetala* crude extracts. The extracts were tested for a dose 2000 mg/kg and followed for acute signs in the first day and followed for 14 days.

2.4 The 0 Mg^{2+} *In vitro* Model of Seizures

Acute brain slices were prepared from 14-21 day old C57BL/6 mice. After decapitation, the mouse brain was extracted and quickly placed in a 50% sucrose cutting solution bubbled with carbogen gas (95% oxygen and 5% carbon dioxide). The cutting solution used was composed of (in mM): KCl (3); NaCl (60); NaH2PO4 (1.2); NaHCO3 (23); MgCl2 (3); CaCl2 (1) ; D-glucose (11) and sucrose (120) [12]. pH was adjusted to between 7.38 and 7.42 using 0.1mM NaOH. The mouse brain was then appropriately sectioned using a scalpel blade to ensure that the hippocampus and entorhinal cortex would be sliced in the transverse plane. 400 µm horizontal slices were cut using a vibrating VF-200 Compresstome (Precisionary Instruments, USA). This method of preparing acute brain slices is similar to that employed by [13,14]. Slice quality was confirmed by assessing the integrity of the hippocampus and its connection to the entorhinal cortex (EC). The slices were then transferred to a recovery chamber which contained a standard aCSF solution, which was again bubbled with carbogen gas. The standard aCSF solution was composed of: NaCl (120 mM); MgCl2 (2 mM); KCl (3 mM); CaCl2 (2 mM); NaHCO3 (23 mM); NaH2PO4 (1.2 mM); D-Glucose (11 mM). The slices were kept in the recovery chamber at room temperature (20-25°C) for a minimum of 40 minutes before being transferred to the interface rig for local field potential recordings.

For recordings, slices were placed in an interface recording chamber perfused with aCSF using a peristaltic pump (Model 205S Watson-Marlow, UK). The temperature was adjusted to ensure the solution in the chamber surrounding the slice was kept between 33 - 35°C. Single-electrode extracellular recordings were performed using glass micropipettes, which were prepared from borosilicate glass capillaries with an outer diameter of 1.20 mm and inner diameter of 0.69 mm (Warner Instruments, USA), using a horizontal puller (Intracell Model P-1000, Sutter, USA) [15].

The tips of the micropipettes were broken under microscope visualisation using a VT-II 2147861 microscope (Olympus, Japan). Pipettes were filled with Mg2+- free aCSF and lowered onto the entorhinal cortex of brain slices under microscope guidance. Once the electrodes were satisfactorily positioned in the tissue, field potential recordings were initiated (Power lab, AD Instruments). The recordings were verified visually on the Lab Chart recording software (AD Instruments, Dunedin, New Zealand). Electrical signals were amplified by the Microelectrode AC Amplifier (A-M system, model 1800) with gain set at 10000.

To elicit *in vitro* epileptiform activity, slices were bathed in Mg^{2+}-free aCSF [16,17]. Removing extracellular Mg^{2+}reduces the voltage dependent block of Mg^{2+}on N- methyl-D-aspartic acid (NMDA) receptors. Initial interictal-like activity is observed, followed by the gradual development of seizure-like events (SLEs), which mimic what is observed in temporal lobe seizures in humans [18,19]. Seizure-like events are observable as large, high-frequency events in the local field potential recordings, which lasted more than 5s. Baseline recordings were made for 600s with standard aCSF before Mg^{2+}-free aCSF was washed in for 3000s in order to induce seizure-like activity. The 0 Mg^{2+} solutions either contained *Moringa stenopetala* extract (0.7 mg/ml), the relevant solvent dimethyl sulfoxide

(DMSO) as a negative control, or diazepam as a positive control. The presence of SLEs was compared between treated slices versus untreated control. The Fisher's exact test with P<.05 was used to determine statistical differences between groups [15,20].

2.5 *In vivo* Seizure Models

Male BALB/c mice weighing between 20-30 g were used for both the maximal electroshock seizure (MES) model and the pentylenetetrazole (PTZ) seizure model. Mice were housed under standard conditions at a temperature of 22 ± 2°C, and with a 12 hr light/ 12 hr dark cycle. The mice were provided with free access to a standard pellet laboratory diet and water. The animals were fasted for 4-8hrs prior to testing [21] and were acclimatized to the laboratory environment.

2.6 Maximal Electroshock Seizure (MES) Model

Six BALB/c mice in each group were divided into 4 groups for the test extract. Animals in control group received 0.5% twin 80 (0.3 ml), reference group received phenytoin (10 mg/kg) and test groups received test extracts (400 mg/kg and 800 mg/kg) orally. The animals in all the groups received corresponding drugs 1 hour before the application of shock. Each animal was properly held and current of 54 mA was passed for 0.2 second transauricularly through ear lobe electrodes using an electroconvulsiometer. The duration of the hind limb extension was recorded. A reduction in this duration was considered as an anti-seizure action of the agent delivered [22]. The one way analyses of variance (ANOVA) test with post hoc LSD with P<.05 was used to determine statistical differences between groups.

2.7 Pentylenetetrazole (PTZ) Model

The animals were grouped into 4 groups and administered vehicle, reference drug and extracts as described in the MES test. In this case the reference group was treated with diazepam 5 mg/kg orally. One hour after administering corresponding drugs to different groups of animals, PTZ 85 mg/kg was injected subcutaneously and mice were observed for thirty minutes for the onset of convulsive behavior if not protected by the extract. The test is thought to be predictive of the activity of anticonvulsant drugs against nonconvulsive (myo clonic or absence) seizures [22]. The onset time of convulsions was recorded. The one way ANOVA test with post hoc LSD with P<.05 was used to determine statistical difference between groups.

2.8 Statistical Analysis

Graph pad prism 5 and SPSS25 softwares were used for analysis. The percentage of protected slices were analyzed using the Fisher's Exact Test (two-tail) with Graph pad prism 5. The one way ANOVA analyzed with SPSS25 was used for *in vivo* PTZ and MES test.

3. RESULTS

3.1 Yields of Hydromethanolic Extract

After hydro alcoholic extraction of the 500 gm *M. stenopetala* , it was extracted with dry amount of 7.35 gm (1.47%).

3.2 Acute Toxicity Study

Acute toxicity study was conducted at 2000 mg/kg dose and the animals were observed according to the procedure for 14 days. There was no behavioral change on live animals on the days of follow up and also was no abnormality on postmortem examination with the extract.

3.3 *In vitro* Anticonvulsant Tests

Removal of Mg^{2+} (0 Mg^{2+}) from the slice perfusate results in recurrent seizure-like events in control slices. Middle trace, concurrent addition of 3 μM diazepam (a known anticonvulsant) prevented SLE generation in most slices. Addition of 0.7 mg/ml of *M. stenopetala* extract prevented the onset of SLEs in the majority of slices. Population data demonstrate the anticonvulsant efficacy of diazepam (2 of 12 slices had SLEs) and *M. stenopetala* (1 of 16 slices had SLEs). The hydroalcoholic extract of *M. stenopetala* extracts had a statistically significant anticonvulsant activity compared to control(P<.05). A positive control using the known anticonvulsant diazepam (3μM), showed significant anticonvulsant activity (P<.05). The percentage of slices showing SLE were given in Table 1.

Table 1. Anti-seizure activity of *Moringa stenopetala* extracts in the 0 Mg^{2+} *in vitro* seizure model

Test group	SLE positive	SLE negative	Total No. slices(N)	SLE Protection Percent
Control	10	6	16	37.5
Diazepam(3 µM)	2	10	12	83.33*
*M. stenopetala (*0.7 mg/ml)	1	15	16	93.57*

** denotes P <.05; Fishers exact test.*

The *in vivo* PTZ test showed no statistically significant effect with the plant extract at all dose levels (P>0.05). (See Table 2).

The *in vivo* MES test showed statistically significant effect in both low and higher dose of *Moringa stenopetala* extracts (P< .05) in mean hind limb extension time and survival. (See Table 3).

The qualitative secondary metabolite test evidenced the presence of alkaloids, cardiac glycosides, flavanoids, and saponins in *Moringa stenopetala* extracts. The summery is depicted in Table 4.

4. DISCUSSION

This study brings scientific evidences on the therapeutic value of *Moringa stenopetala (*root) which is traditionally being used for treatment of epilepsy in Ethiopia. The study provides a scientific rationale for the use of *Moringa stenopetala* root extract for the amelioration of epilepsy observed in traditional medicine in Ethiopia. The results revealed the hydoalcoholic extract of root of *Moringa stenoptela*, has shown statistically significant *in vitro* antiseizure activity. This was supported by the *in vivo* antiseizure activity in MES model. But the crude extract failed to show statistically significant activity in PTZ model.

This show the plant has antiseizure potential. The qualitative secondary metabolite test evidenced the presence of alkaloids, phenols, flavanoids, and saponins. According to the review done by Zue *et al.* medicinal compounds with antiepileptic/anticonvulsant activities are alkaloids, flavonoids, terpenoids, saponins and coumarins [6]. For the hydroalcoholic extract contains these compounds the anticonvulsant activity may be attributed to the presence of these phytochemicals. In *Moringa oleifera* alkaloids saponins, protein, flavonoids, carbohydrates, tannins, terpinoids, phenols, glycosides and phytosteroids showed presence in all the components of leaf, pod and bark and these parts showed anticancer activity [23].

Table 2. The *Moringa stenopetala* extracts didn't shows anti-seizure activity in the PTZ seizure model

Test groups	Number of mice(N)	Mean Latency for myoclonic Seizure(second)(Mean±SE)
Control	6	239.67±33.72
M.stenopetala 400 mg/kg	6	400.00±37.10
M. stenopetala 800 mg/kg	6	387.33±35.82
Diazepam 5mg/kg	6	1800.00±0,00*

**denotes P < .05; ANOVA test; N*

Table 3. The crude extract of *Moringa stenopetala* extracts shows anti-seizure activity in the MES model

Treatment	Number of mice	Mean Hind limb Extension time(second) (Mean±SE)	Survival
Control	6	24.33±2.45	2/6
M. stenopetala 400 mg/kg	6	10.33±3.52*	4/6*
M. stenopetala 800 mg/kg	6	12.50±4.16*	5/6*
Phenytoin 10mg/kg	6	00.00±00*	6/6*

**denotes P < .05; ANOVA test and Fisher's exact test*

Table 4. Secondary metabolites in the hydroalcoholic extracts of *M.stenopetala*

Phytochemicals	*M.stenopetala*
Alkaloid	+
Cardiac glycosides	+
Flavonoids	+
Phenols	-
Saponins	+
Sterols	-
Tannins	-
Terpenoids	-

+ denotes positive; - denotes negative

The result of MES model is in consistent with study done in other species of Moringa, *Moringa concanensis* which abolishes both MES and PTZ seizures. The anti-convulsant activity can be due to the presence of various phytoconstituents like alkaloids, tannins, phenols, flavanoids, and carbohydrates in the plant [24]. But the PTZ effect in this species is in contrary of the finding in our study in *Moringa stenopetala.* In another study pretreatment with roots *Moringa oleifera* methanolic extract caused statistically significant protection against PTZ and strychnine (STR) induced seizures [25]. The root PTZ effect *Moringa oleifera* is also in contrary with the effect seen in *Moringa stenopetala.* This may be due to the phytochemical constituents with action on the PTZ model may be in less concentration in *Moringa stenopetala.* than with these two species.

The root is also used in traditional medicine to treat other different aliments and for which some of the chemical components were studied. In one study the acetone extract of *Moringa stenopetala* (root), which was active as antibacterial was found to contain palmitic acid , cholest-5-en-3-ol, oleic acid and n-octacosane [26]. The acetone crude extract of *Moringa stenopetala* (root bark) was subjected to column chromatography separation. Four compounds were obtained and found to be stigmastereol, ursolic acid, tasnemoxide and oleic acid based on their spectral analyses. The antibacterial activity of the compounds reveled that they show good antibacterial activities [27]. In other study *Moringa stenopetala* root extract isolates 1,3-dioleoyl-2-linolein and 1,3-dili-noleoyl-2-olein has sown anti leshmanial activity [28]. Roots of *M. stenopetala* and *M. oleifera* both had high concentrations of 4-(α-L-rhamnopyranosyloxy)-benzylglucosinolate and benzyl glucosinolate [29].

In vitro, *in vivo*, and some clinical studies evidenced the n-3 fatty acids to have anticonvulsant activity. At concentrations of up to 100 IM, Eicosapentaenoic acid (EPA), Alpha-linolenic acid (ALA), or Docosahexaenoic acid (DHA) reduced the frequency of pentylenetetrazol (PTZ) or glutamate, low Mg^{2+} or depleted of glycine induced action potentials and excitatory discharges in hippocampal slices. 161M of DHA significantly inhibited the repetitive action potentials evoked by depolarizing current pulses [30]. These data led to the hypothesis that fatty acids modulate ion channels. DHA and EPA have also been shown to stabilize the neuronal membrane in single cells by suppressing voltage-gated Na^{+} and Ca^{2+} channels, and thereby increasing the action potential firing threshold [31]. The *in vitro* seizure suppression 0-Mg^{2} model and action on the MES *in vivo* model in *Moringa stenopetala* root may be from these chemical constituents.

5. CONCLUSIONS

The hydroalcoholic crude extract of *Moringa stenopetala* root showed to have anticoncvulant effect both *in vitro* and *in vivo* models. The use of local community as an anticonvulsant is supported by the evidence obtained from this study. Having this tapping its potential as anticonvulsant is promising in identifying new antiepileptic molecules as it is widely used for treatment of other diseases with less toxic effect.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All animal handling, care and procedures were carried out in accordance with national and institutional guidelines. The tests were conducted after the ethical clearance was verified by Institutional Review Board (IRB). The experimental protocols for *in vivo* experiments were approved by the Institutional Review Board (IRB) of Addis Ababa University (AAU), College of Health Sciences. Approval for the *in vitro* experiments was granted by the University of Cape Town Animal Ethics Committee (Protocol No: AEC 014/035).

ACKNOWLEDGEMENTS

Funding for this research was received from Addis Ababa University graduate studies and from Arba Minch University. Funding was also

received from the South African Medical Research Council Self-Initiated Research (SIR) grant “Validating the anticonvulsant action of African plant extracts”. I am grateful to Dr. Joseph Valentino Raimondo and Mr. John Hamin Lee in the University of Cape Town for facilitation and help in the *in vitro* laboratory study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/64362

Journal of Complementary and Alternative Medical Research

13(4): 8-14, 2021; Article no.JOCAMR.67277
ISSN: 2456-6276

In vitro and *In vivo* Anticonvulsant Effect of Hydroalcoholic Extracts of *Clutia abyssinica* in Mice Model

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Authors' contributions

This work was carried out in collaboration among all authors. Author SSS designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author TA supervised the study and read, edited and approved the final manuscript.

Article Information

DOI: 10.9734/JOCAMR/2021/v13i430230
Editor(s):
(1) Dr. Sahdeo Prasad, Texas Tech University Health Sciences Center, USA.
Reviewers:
(1) Maria Gabriella Conceição, United Metropolitan University (FMU), Brazil.
(2) Gloria Esther Castillo Lara, University of Medical Sciences of Havana (UCMH), Cuba.
Complete Peer review History: http://www.sdiarticle4.com/review-history/67277

Original Research Article

Received 25 January 2021
Accepted 02 April 2021
Published 03 April 2021

ABSTRACT

Background: Epilepsy is a chronic disorder of the brain that affects people of all ages worldwide. In the search of safe and effective antiepileptics traditional treatment practices are one area of research to obtain novel molecules. Research is also needed to validate and standardize the traditional claim. *Clutia abyssinica* leaves were one of the medicinal plants claimed for use against epilepsy and evil eye and other diseases in different parts of Ethiopia. But there was no scientific research evidence for the claimed use of the plant. Therefore this work was designed to evaluate the anticonvulsant effect of hydroalcoholic extract of *Clutia abyssinica* leaves.
Methods: The dry residue of the plant extract was used for the test. *In vitro* 0Mg2+ mice model at dose 0.7 mg/kg of extract, diazepam (3μM) and untreated brain slice groups were used to compare the presence of seizure like event (SLE). *In vivo* pentylenetetrazol (PTZ) model with 85 mg/kg subcutaneously was used to compare the seizure onset time with two extract doses and diazepam

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5 mg/kg. The data was presented with mean± standard error. In maximum electric shock (MES) model 54 mA was passed for 0.2 second transauricularly in mice. The mean time of hind limb extension was recorded for doses 400 mg/kg and 800mg/kg of the extract and 10 mg/kg phenytoin. The means were compared for statistical significance using one way ANOVA post hoc LSD whereas proportions were compared using Fishers exact test with P-value < .05.

Results: *In vitro* anticonvulsant tests *C. abyssinica* extract effect was not statistically significant compared to negative control (P>0.05).A positive control using the known anticonvulsant diazepam (3μM), showed significant anticonvulsant activity (P<0.05). The *in vivo* PTZ test showed no statistically significant effect in plant extract at all dose levels (P>0.05). In the *in vivo* MES test the extract of *Clutia abyssinica* both low and higher dose didn't show statistically significant effect (P>0.05) compared with the negative control. But the extract improved survival (p<0.05). The qualitative secondary metabolite test evidenced the presence of alkaloids, cardiac glycosides, flavanoids, phenols, saponins, sterols and terpeoids in *Clutia abyssinica* extract.

Conclusion: The hydroalcoholic crude extract result of the *C. abyssinica* as anticonvulsant is weak based on the models used in this study. For most of the local preparation are mixes of different plants it may have synergistic action with other plants. Or it may have action with other models of chronic epilepsy.

Keywords: Clutia abyssinica; anti-epileptic; In vitro; In vivo; mice.

1. INTRODUCTION

Epilepsy is the third leading contributor to the global burden of disease for neurological disorders and affects 65 million people worldwide [1]. In Ethiopia as various diseases are being treated traditionally some survey shows also the practice of treatment for epilepsy [2]. *Clutia abyssinica* leaves were one of the medicinal plants claimed for use against epilepsy and evil eye and other diseases in different parts of Ethiopia [3–5]. But there is no scientific evidence for the claimed use. As different researches on plants used for epilepsy in different countries have shown anticonvulsant activity, this plant may also have value for the treatment of the disease. Therefore scientific research should be done on these plants for their antiepileptic potential [6].

Zero Mg^{2+} model is one of the *in vitro* models to study mechanism of seizure and antiseizure drugs [7]. The most commonly employed *in vivo* animal models in the search for new anticonvulsant drugs are the MES test and the PTZ seizure test [8,9]. The maximal electroshock seizure test, in which tonic hindlimb seizures are induced by bilateral corneal or transauricular electrical stimulation, is thought to be predictive of anticonvulsant drug efficacy against generalized tonic-clonic seizures, while the pentylenetetrazole test, in which generalized myoclonic and clonic seizures are induced by systemic (usually s.c. or i.p.) administration of convulsant doses of PTZ, is thought to represent a valid model for generalized absence and/or myoclonic seizures in humans [8].

Hence this study was conducted with the objective to looks for the anticonvulsant potential of 80% methanol extract of *Clutia abyssinica* using *in vitro* and *in vivo* mice models. The results are of importance in validating the claimed use and in revealing its anticonvulsant potential for further scientific research.

2. MATERIALS AND METHODS

2.1 Plant Material Collection and Extraction

The plant *Clutia abyssinica* was selected based on claim by the society to use for epilepsy. The leaves of *Clutia abyssinica* were collected from Bale area, Ethiopia, in April 2016. The plant was identified and voucher specimen was deposited with the given herbarium code (01-S) in the national herbarium at Addis Ababa University, College of Science, Ethiopia.

The collected plant parts were then garbled in th e processing room and dried in the shade, and powdered and stored in a wellclosed container at room temperature until extracted. The powdered, air dried materials (400 g) of *Clutia abyssinica* (leaves) were then extracted by maceration with 80% methanol at room temperature for three consecutive days. The mixture filtered by gauze and then with What man™ filter paper 6μm pore size (125 mm GE healthcare UK limited, UK) and concentrated under vacuum in a rotary

evaporator. Using this extraction technique *Clutia abyssinica* was extracted with dry amount of 44.71 gm (11.18%).The extract was kept in a tightly closed bottle in a refrigerator until used for anti-seizure testing [10].

2.2 Phytochemical Screening

The method used by Debella [10] was implemented to screen for the presence and/or absence of the main secondary metabolite groups in the extracts.

2.3 Acute Toxicity Study

An acute toxicity study was conducted for the extracts by acute oral toxic class method of Organization of Economic Co-operation and Development, as per 423 guidelines [11].Three female mice in a group were grouped into two groups in the test randomly. One control group was given distilled water and the other groups was treated for *Clutia abyssinica* crude extracts. The extract was tested for a dose 2000mg/kg and followed for acute signs in the first day and followed for 14 days.

2.4 The 0Mg^{2+} *In vitro* Model of Seizures

Acute brain slices were prepared from 14-21 day old C57BL/6 mice. After decapitation, the mouse brain was extracted and quickly placed in a 50% sucrose cutting solution bubbled with carbogen gas (95% oxygen and 5% carbon dioxide). The cutting solution used was composed of : KCl (3 mM); NaCl (60 mM); NaH2PO4 (1.2 mM); NaHCO3 (23 mM); MgCl2 (3 mM); CaCl2 (1 mM) ; D-glucose (11 mM) and sucrose (120 mM) [12]. pH was adjusted to between 7.38 and 7.42 using 0.1mM NaOH. The mouse brain was then appropriately sectioned using a scalpel blade to ensure that the hippocampus and entorhinal cortex would be sliced in the transverse plane. 400µm horizontal slices were cut using a vibrating VF-200 Compresstome (Precisionary Instruments, USA). This method of preparing acute brain slices is similar to that employed by Dreier [13,14]. Slice quality was confirmed by assessing the integrity of the hippocampus and its connection to the entorhinal cortex (EC). The slices were then transferred to a recovery chamber which contained a standard aCSF solution, which was again bubbled with carbogen gas. The standard aCSF solution was composed of: NaCl (120mM); MgCl2 (2mM); KCl (3mM); CaCl2 (2mM); NaHCO3 (23mM); NaH2PO4 (1.2mM); D-Glucose (11mM). The slices were kept in the recovery chamber at room temperature (20-25°C) for a minimum of 40 minutes before being transferred to the interface rig for local field potential recordings.

For recordings, slices were placed in an interface recording chamber perfused with aCSF using a peristaltic pump (Model 205S Watson-Marlow, UK). The temperature was adjusted to ensure the solution in the chamber surrounding the slice was kept between 33 - 35°C. Single-electrode extracellular recordings were performed using glass micropipettes, which were prepared from borosilicate glass capillaries with an outer diameter of 1.20mm and inner diameter of 0.69mm (Warner Instruments, USA), using a horizontal puller (Intracell Model P-1000, Sutter, USA) [15].

The tips of the micropipettes were broken under microscope visualisation using a VT-II 2147861 microscope (Olympus, Japan). Pipettes were filled with Mg^{2+}- free aCSF and lowered onto the entorhinal cortex of brain slices under microscope guidance. Once the electrodes were satisfactorily positioned in the tissue, field potential recordings were initiated (Powerlab, AD Instruments). The recordings were verified visually on the LabChart recording software (ADInstruments, Dunedin, New Zealand). Electrical signals were amplified by the Microelectrode AC Amplifier (A-M system, model 1800) with gain set at 10000.

To elicit *in vitro* epileptiform activity, slices were bathed in Mg^{2+}-free aCSF [16,17]. Removing extracellular Mg^{2+}reduces the voltage dependent block of Mg^{2+}on N- methyl-D-aspartic acid (NMDA) receptors. Initial in terictal-like activity is observed, followed by the gradual development of seizure-like events (SLEs), which mimic what is observed in temporal lobe seizures in humans [15,18]. Seizure-like events are observable as large, high-frequency events in the local field potential recordings, which lasted more than 5s. Baseline recordings were made for 600s with standard aCSF before Mg^{2+}-free aCSF was washed in for 3000s in order to induce seizure-like activity. The 0 Mg^{2+} solutions either contained *Clutia abyssinica* extract (0.7mg/ml), the relevant solvent dimethyl sulfoxide (DMSO) as a negative control, or diazepam as a positive control. The presence of SLEs was compared between treated slices versus untreated control. The Fisher's exact test with $P<.05$ was used to determine statistical differences between groups [15,18].

2.5 *In vivo* Seizure Models

Male BALB/c mice weighing between 20-30 g were used for both the maximal electroshock seizure (MES) model and the pentylenetetrazole (PTZ) seizure model. Mice were housed under standard conditions at a temperature of 22 ± 2^{o}C, and with a 12 hr light/ 12 hr dark cycle. The mice were provided with free access to a standard pellet laboratory diet and water. The animals were fasted for 4-8hrs prior to testing [19] and were acclimatized to the laboratory environment.

2.6 Maximal Electroshock Seizure (MES) Model

Six BALB/c mice in each group were divided into 4 groups for the test extract. Animals in control group received 2%twin 80 (0.3 ml), reference group received phenyto in (10mg/kg) and test groups received test extracts (400mg/kg and 800mg/kg) orally. The animals in all the groups received corresponding drugs 1hour before the application of shock. Each animal was properly held and current of 54 mA was passed for 0.2 second transauricularly through ear lobe electrodes using an electroconvulsiometer. The duration of the hind limb extension was recorded. A reduction in this duration was considered as an anti-seizure action of the agent delivered [20]. The one way analyses of variance (ANOVA) test with post hoc LSD with P<.05 was used to determine statistical differences between groups.

2.7 Pentylenetetrazole (PTZ) Model

The animals were grouped into 4 groups and administered vehicle, reference drug and extracts as described in the MES test. In this case the reference group was treated with diazepam 5mg/kg orally. One hour after administering corresponding drugs to different groups of animals, PTZ 85mg/kg was injected subcutaneously and mice were observed for thirty minutes for the onset of convulsive behavior if not protected by the extract. The test is thought to be predictive of the activity of anticonvulsant drugs against nonconvulsive (myo clonic or absence) seizures [20]. The onset time of convulsions was recorded. The one way ANOVA test with post hoc LSD with P<.05 was used to determine statistical difference between groups.

2.8 Statistical Analysis

Graph pad prism 5 and SPSS25 softwares were used for analysis. The percentage of protected slices were analyzed using the Fisher's Exact Test (two-tail) with Graph pad prism 5. The one way ANOVA analyzed with SPSS25 was used for in vivo PTZ and MES test.

3. RESULTS

3.1 Acute Toxicity Study

Acute toxicity study was conducted at 2000mg/kg dose and the animals were observed according to the procedure. There was no behavioral change on live animals on the days of follow up and no abnormality on postmortem examination.

3.2 *In vitro* Anticonvulsant Tests

The *C. abyssinica* extract effect was not statistically significant compared to negative control (P>0.05). A positive control using the known anticonvulsant diazepam (3μM), showed significant anticonvulsant activity (P<0.05). The percentage of slices showing SLEs were given in Table 1.

3.3 *In vivo* Anticonvulsant Tests

The *in vivo* PTZ test showed no statistically significant effect in plant extract at all dose levels though there was dose dependent delay on seizure onset (P>0.05) (See Table 2).

In the *in vivo* MES test the extract of *Clutia abyssinica,* both low and higher dose didn't show statistically significant effect (P>0.05) compared with the negative control (See Table 3).But the extract improved survival (p<0.05).

Table 1. Anti-seizure activity of *Clutia abyssinica*extracts in the 0 Mg^{2+}*in vitro* seizure model * denotes P <0.05, Fishers exact test

Test group	SLE positive	SLE negative	Total No. slices	SLE protection percent
Control	10	6	16	37.5
Diazepam	2	10	12	83.33*
Clutia abyssinica	8	4	12	33.33

Table 2. The *Clutia abyssinica* extracts didn't shows anti-seizure activity in the PTZ seizure model. *denotes P <0.05, ANOVA test

Test group	N	Mean Latency for myoclonic seizure(s)
Control	6	239.67±33.72
C. abssinica. 400 mg/kg	6	284.00±13.93
C. abssinica. 800mg/kg	6	457.83±103.54
Diazepam 5mg/kg	6	1800.00±0,00*

Table 3. The crude extract of *Clutia abyssinica* did't show anti-seizure activity in the MES model. It improved survival compared with the negative control. *denotes P <0.05, ANOVA test and Fisher's exact test.

Treatment	N	Mean hindlimb extension time(S)	Survival
Control	6	24.33±2.45	2/6
*Clutia abyssinica*400 mg/kg	6	20.00±1.06	4/6*
*Clutia abyssinica.*800mg/kg	6	21.83 ±0.40	5/6*
Phenytoin 10mg/kg	6	00.00*	6/6*

The qualitative secondary metabolite test evidenced the presence of alkaloids, cardiac glycosides, flavanoids, phenols, saponins, sterols and terpeoids in *Clutia abyssinica* extract. The summery is depicted in Table 4.

4. DISCUSSION

This study brings scientific evidences on the therapeutic value of *Clutia abyssinica* (leaf*)* which is traditionally being used for treatment of epilepsy in Ethiopia. *Clutia abyssinica* showed little effect on the *in vitro* as well as *in vivo* models. The effect was not statistically different from the negative control. This plant was claimed by the local people for different ailments and it is used for epilepsy and evil eye [21]. Traditionally it is used in treatment of many other diseases [3,4,22]. The results in this test show it has less anticonvulsant activity on the models used to test anticonvulsant effect of the plant. The plant may probably not have anticonvulsant effect by itself but may potentiate the effect of other concomitantly administered plants.

The dichloromethanolic root extract of *C. abyssinica* demonstrated analgesic activities on acetic acid-induced pain in Swiss albino mice [23]. In other study *in vivo* antitrypanosomal activity of methanol crude leaf extracts of *C. abyssinica* against *T. congolence* field isolate was demonstrated [24]. In one study the results of serum biochemical markers and histopathological studies in the crude 80% methanol extract and n-butanol fraction pre- and post-treated group support the hepatoprotective effect of *Clutia abyssinica* leaf [25]. These studies evidence the potential of the plant as alternative treatment in the respective claimed uses.

In the current study the leaves of *Clutia abyssinica* extract showed the presence of alkaloids, cardiac glycosides, flavanoids, phenols, saponins, sterols and terpeoids. Though these are some of the components, they didn't show significant anticonvulsant effect in the current models used in this study. Further study in other seizure models as well as with higher dose in models that showed dose dependent improvement (ptz test) is needed to revalidate the claim. For most plants are given in combination it may have some potentiating effect if it is given with other plants.

Table 4. Secondary metabolites in the hydroalcolic extracts of *C. abyssinica*

Phytochemicals	C. *abyssinica*
Alkaloid	+
Cardiac glycosides	+
Flavonoids	+
Phenols	+
Saponins	+
Sterols	+
Tannins	-
Terpenoids	+

5. CONCLUSION

The hydroalcoholic crude extract result of the *C. abyssinica* as anticonvulsant is weak based on the models used in this study. For most of the local preparation are mixes of different plants it may have synergistic action with other plants. Or it may have action with other models of chronic epilepsy.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Animal Ethic committee approval has been taken to carry out this study.

ACKNOWLEDGEMENTS

Funding for this research was received from Addis Ababa University graduate studies and from Arba Minch University. Funding was also received from the South African Medical Research Council Self-Initiated Research (SIR) grant “Validating the anticonvulsant action of African plant extracts”. I am grateful to Dr. Joseph Valentino Raimondo and Mr. John Hamin Lee in the University of Cape Town for facilitation and help in the *in vitro* laboratory study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/67277