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Investigation of some possible mechanisms involved in the anticonvulsant activity of *Tulbaghia violacea* Harv

By

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DECLARATION

I declare that the thesis: "Investigation of some possible mechanisms involved in the anticonvulsant activity of *Tulbaghia violacea* Harv" is my own work, that it has not been submitted before for any degree examination in any other University and that all sources I have used or quoted have been indicated and acknowledged by complete reference.

Khalid Abdussalam Ali Masoud   **December 2015**

Signed..

DEDICATION

I dedicate this thesis to my parents for their love, sacrifice and encouragement

ACKNOWLEDGEMENTS

This research was conducted in the Department of Medical Biosciences, University of the Western Cape, Bellville.

Thank you my Allah for seeing me through the completion of this degree, without You nothing is possible.

Thanks to my Supervisor and my co- supervisor, Dr. Okobi Ekpo and Prof. George Amabeoku, all my appreciation and admiration cannot be expressed in mere words for everything that you have done for me academically and personally.

Big thanks to extend appreciation to the Libyan Embassy in South Africa for funding the project.

To the rest of my family and friends, thank you for your support and love.
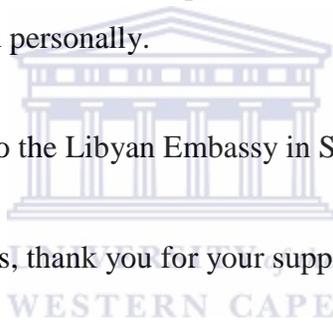
Investigation of some possible mechanisms involved in the anticonvulsant activity of *Tulbaghia violacea* Harv

KEY WORDS

Alliaceae

Anticonvulsant properties

Chromatographic techniques

Epilepsy

Gabaergic and Glutaminergic mechanism

Medicinal plants

Methanolic extract

Mice

Pharmacological evaluation

Phytochemical and HPLC analysis

Tulbaghia violacea

ABBREVIATIONS

ACh: Acetylcholine

AEDs: Antiepileptic drugs

AMPA: α-amino-3- hydroxy-5-methyl-isoxazole-4-propionic acid

BIC: Bicuculline

CNS: Central nervous system

EAAT: Excitatory amino acid transporters

EEG: Electroencephalogram

FS: Febrile seizures

GABA: Gamma aminobutyric acid

GAD: Glutamic acid dehydrogenese

GTCS: Generalized tonic-clonic seizure

ICE: International Classification of Epilepsy

iGluRs: Ionotropic glutamate receptors

LD50:   Median Lethal dose

mGlurs: Metabotropic glutamate receptors

MRI: Magnetic Resonance Imaging

nAChRs: Neuronal acetylcholine receptors

NMDLA: N-methyl-DL-aspartic acid
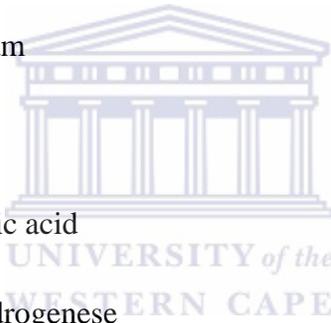
PIC: Picrotoxin

PTZ: Pentylenetetrazole

PWE: People with epilepsy

SCN: Strychnine

WHO: World Health Organisation

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ABSTRACT

Even though *Tulbaghia violacea* has been used to treat and manage epilepsy in South Africa by traditional medicine practitioners, no evidence in any literature has shown any scientific scrutiny of the effectiveness of the plant species in therapy. This project was intended, therefore, to investigate the anticonvulsant effect of the leaf methanol extract of *Tulbaghia violacea* by studying its effect against tonic convulsion induced by either pentylenetetrazole (PTZ), bicuculline, picrotoxin, strychnine or N-methyl-DL-aspartic acid (NMDLA) in mice. Qualitative phytochemical analysis, acute toxicity and HPLC studies were also carried out on the plant species.
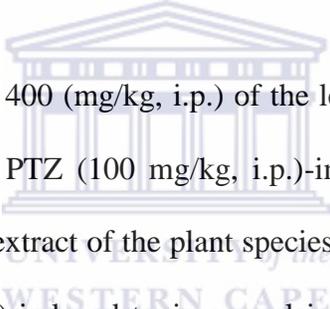
The doses of 200 (mg/kg, i.p.) and 400 (mg/kg, i.p.) of the leaf methanol extract of *T. Violacae* significantly delayed the onset of PTZ (100 mg/kg, i.p.)-induced tonic convulsion in a dose dependent manner. Leaf methanol extract of the plant species (200 mg/kg, i.p.) did not affect the incidence of PTZ (100 mg/kg, i.p.)-induced tonic convulsion while 400 mg/kg (i.p.) protected only one mouse against the tonic convulsion. Leaf methanol extract of *Tulbaghia violacea* (100mg/kg, i.p.) did not significantly affect the onset or incidence of PTZ (100 mg/kg, i.p.)-induce tonic convulsion. Phenobarbitone (12 mg/kg, i.p.), diazepam (0.5 mg/kg, i.p.) and muscimol (2mg/kg, i.p.) significantly delayed the onset of PTZ (100 mg/kg, i.p.)-induced tonic convulsion and also significantly reduced the number of animals convulsing. Muscimol (0.2 mg/kg, i.p.) did not significantly affect the onset or incidence of PTZ (100 mg/kg, i.p.)-induced tonic convulsion. However, combined therapy of sub effective doses of the leaf methanol extract of *T. Violacea* (100 mg/kg, i.p.) and muscimol (0.2 mg/kg, i.p.) significantly delayed the onset of PTZ (100mg/kg, i.p.)-induced tonic convulsion and but did not significantly reduce the number

of animals convulsing. The combined therapy of sub effective doses of the leaf methanol extract of *T. violacea* (100 mg/kg, i.p.) and muscimol (0.2 mg/kg, i.p.) protected two of the mice against the tonic convulsion. Leaf methanol extract of *Tulbaghia violacea* (100-400 mg/kg, i.p.) significantly and dose dependently delayed the onset of tonic convulsion produced by bicuculline (30 mg/kg, i.p.), picrotoxin (20 mg/kg, i.p.) or NMDLA (400 mg/kg, i.p.)-induced tonic convulsion but did not affect the incidence of any of the convulsions. Phenobarbitone (12 mg/kg, i.p.), diazepam (0.5 mg/kg, i.p.) or muscimol (2 mg/kg, i.p.) significantly delayed the onset of bicuculline (30 mg/kg, i.p.) or picrotoxin (20 mg/kg, i.p.)-induced tonic convulsion and also significantly reduced the number of animals convulsing. Phenobarbitone (12 mg/kg, i.p.) or diazepam (0.5 mg/kg, i.p.) did affect significantly affect the onset or incidence of NMDLA (400 mg/kg, i.p.)-induced tonic convulsion. LY233053 (5 mg/kg, i.p.) significantly delayed the onset of tonic convulsion produced by NMDLA (400 mg/kg, i.p.) and also significantly reduced the number of animals convulsing. Leaf methanol extract of *Tulbaghia violacea* (200 and 400 mg/kg, i.p.) significantly delayed the onset of strychnine (2 mg/kg, i.p.)-induced tonic convulsion but did not significantly affect the number of mice convulsing. The dose of 100 mg/kg (i.p.) of leaf methanol extract of *T. violacea* did not significantly affect the onset or incidence of strychnine (2 mg/kg, i.p.)-induced tonic convulsion. Phenobarbitone (12 mg/kg, i.p.) significantly delayed the onset of strychnine (2 mg/kg, i.p.)-induced tonic convulsion and also significantly reduced the number of animals convulsing. Diazepam (0.5 mg/kg, i.p.) did not significantly delay the onset of strychnine (2 mg/kg, i.p.)-induced tonic convulsion and also did not significantly affect the number of mice convulsing. Phenytoin (30 mg/kg, i.p.) or DMSO (0.25 ml, i.p.) did not significantly affect the onset or incidence of bicuculline (30 mg/kg, i.p.), picrotoxin, strychnine or NMDLA-induced tonic convulsion. The qualitative phytochemical

analysis of the plant species showed the presence of alkaloids, saponins, cardiac glycosides, flavonoids, triterpene steroids, quinones and tannins. The LD50 value obtained following oral administration of the leaf methanol extract of *Tulbaghia violacea* may be greater than 4000 mg/kg. The HPLC fingerprint of the leaf methanol extract of *Tulbaghia violacea* showed distinct peaks at the following retention times, 2.911, 3.269, 4.010, 7.597, and 15.122 min. The results obtained in this study indicate that the leaf methanol extract of *Tulbaghia violacea* has anticonvulsant activity. The results obtained also indicate that GABA, glutamic acid and glycine mechanisms may probably be involved in the anticonvulsant activity of the plant extract. The relatively high LD50 obtained for the plant species, given orally, indicate that it is safe in mice.

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Chapter 1

Introduction

Epilepsy is a chronic neurological disorder characterized by spontaneous recurrent seizures (Chang and Lowenstein, 2003). It is one of the most common neurological disorders affecting approximately 50 million people worldwide (De Boer et al., 2008; van Mierlo et al., 2014), mainly in developing countries (Perucca et al., 2001; Preux and Druet-Cabanac, 2005). People with epilepsy (PWE) usually conceal their condition due to the fear of stigmatization and discrimination (Hung, 2009). There is as yet no known cure for epilepsy but it can be controlled (Boison, 2010). Approximately one-third ofPWE have drug-resistant seizures (Kwan and Brodie, 2000) and less than 50% of affected people in developing countries receive medication (Shibre et al., 2006).Even newer anti-epileptic drugs (AEDs)are reported to have significant central nervous system (CNS) side-effects including decreased cognitive abilities and psychiatric complications (Schmidt, 2009).

In some Asian and African countries, up to 80% of the population relies on traditional (folk) medicine for their primary health care needs (Kayne, 2009), possibly due to the high cost of conventional medication. In South Africa, approximately three million people in both urban and rural areas are reported to be reliant on traditional medicine exclusively or in combination with Western medicine (Jäger et al., 2005; Govender et al., 2006). Researchers identified 122 compounds used in mainstream medicine which were derived from ethno medical plant sources, 80% of which were used in the same or related manner as the traditional ethnomedical use (Browner, 1985; Van Wyk et al., 1997). One South African study found that some PWE

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combinedWestern and traditional medicines for epilepsy treatment (Keikelame and Swartz, 2007).

Although natural products are widely used by PWE all over the world, there is currently little evidence of safety and efficacy to scientifically justify their use. Raskin (2003) acknowledged that plant-derived compounds are excellent sources for novel pharmaceutical products while Ekstein and Schachter (2010) reported that natural products with a long history of medicinal use against epilepsy should be further tested using systematic pre-clinical methods in PWE to confirm their potential as new treatments for epilepsy.

According to the World Health Organization, scientific evidence to evaluate safety and effectiveness of traditional medicine is limited (Tilburt and Kaptchuk, 2008). *Tulbaghia violacea* is a safe and potent medicinal herb widely used against various ailments (Saibu et al., 2015) but no previous study has been reported on its anti-convulsant activity. However, *Allium sativum*, a member of its family, has been shown to have potent anti-convulsant activity (Advani et al., 2011) hence one of the aims of this research is to evaluate the anti-convulsant activity and safety of *Tulbaghia violacea* in mice.

Chapter 2

Literature review

2.1. Brief epidemiology

Epilepsy is the most common neurological disorder in the world affecting approximately 50 million people worldwide (approximately 1% of world population), the majority (80-85%) of whom live in developing countries (Preux and Druet-Cabanac, 2005; Van Mierlo et al., 2014). Epidemiologic studies are generally considered important to identify the public health and healthcare priorities relating to epilepsy, including prevention, education, early detection, treatment and service needs (Jallon, 2002; Thurman et al., 2011). Seasonal variations in epileptic seizures have been reported in previous studies, with a reduction in seizures occurring during summer (Clemens et al., 2013). Similarly, seasonal variations in epilepsy births have been reported, with most births occurring in winter than in summer (Procopio et al., 1997; Procopio et al., 2006).

In developed countries the prevalence of epilepsy is 4-10 people per 1000 (Olafsson and Hauser, 1999; Õun et al., 2003). In Europe the estimated prevalence of epilepsy in 2004 was 4.3-7.8 per 1000 (Pugliatti et al., 2007) and 1.8% in Latin America (Mac et al., 2007). Country-specific prevalence rates are 0.97% in the United Kingdom (Shorvon, 2010); 0.7% in Turkey (Onal et al., 2002); 30% in Russia (Guekht et al., 2010) and 2% in Australia (Tellez-Zenteno et al., 2004). In developing countries like Panama, Tanzania, Ecuador, India, Liberia, Nigeria, Colombia and Venezuela, the prevalence of epilepsy was estimated to be more than 10% (Osuntokun et al., 1987; Leary et al., 1999) while in Africa as a whole, it is estimated at 2.2-58 per 1000 of the

population and accounts for the second or third most common reason for consultation and hospitalization (Ngugi et al., 2010). Studies in Arab and Middle East countries showed prevalence of 6.5 per 1000 in Saudi Arabia (Al Rajeh et al., 2001) and 0.9/1000 in Sudan (Younis, 1983; Perenchio et al., 2004), among others. In South Africa the lowest rate of epilepsy prevalence (2.2 per 1000 of population) is reported in urban areas while the highest rate (15 per 1000 of population) is in rural areas (WHO, 2004).

The high epilepsy prevalence often observed in rural areas than in urban areas is generally due to a number of factors including family history, home delivery, consanguinity, increased risk of infection and prenatal insults such as asphyxia (Jallon, 2002; Rajshekhar et al., 2006). Epilepsy prevalence also varies between male and female; in Asia, available data shows that epilepsy is less common in women than in men (Fong et al., 2003; Tran et al., 2006). Some African studies done in Egypt, Libya, Tunisia, Somalia, Djiboti, Comoros, Sudan and Mauritania indicate that epilepsy prevalence is higher in males than in females, possibly because of risky behaviour among males that could lead to severe head injuries (Preux and Druet-Cabanac, 2005; Khedr et al., 2013). The age-adjusted epilepsy prevalence has also been found to range from 2.7-40 per 1000 depending on location (Reggio et al., 1996; Rocca et al., 2001).

2.2. Risk Factors and Causes of Epilepsy

Factors that predispose persons to developing seizures and epilepsy could be considered the risk factors of epilepsy and include genetic risk factor, positive family history of epilepsy, consanguinity, obstetric complications, toxemia, premature birth, low birth weight, neonatal seizures, asphyxia, pre-eclampsia, febrile convulsions, severe head trauma, maternal nicotine use, history of stroke, neurocysticercosis and excess alcohol (Casetta 2002; Nsengiyumva et al.,

2003; Edwards et al., 2008). Epilepsy symptoms can manifest at any age and may be idiopathic or symptomatic (Chokroverty, 1996).

2.3. Types of Epilepsy

Understanding the type ofseizure is a crucial step to effective management of epilepsy as this determines the treatment or surgical approaches to adopt (Rudzinski and Shih, 2011). International Classification of Epilepsy (ICE) related to seizure disorders suggests three main types (Figure 1) vizpartial seizures, generalized seizures and unclassified epileptic seizures (Chokroverty, 1996; Dekker, 2002).
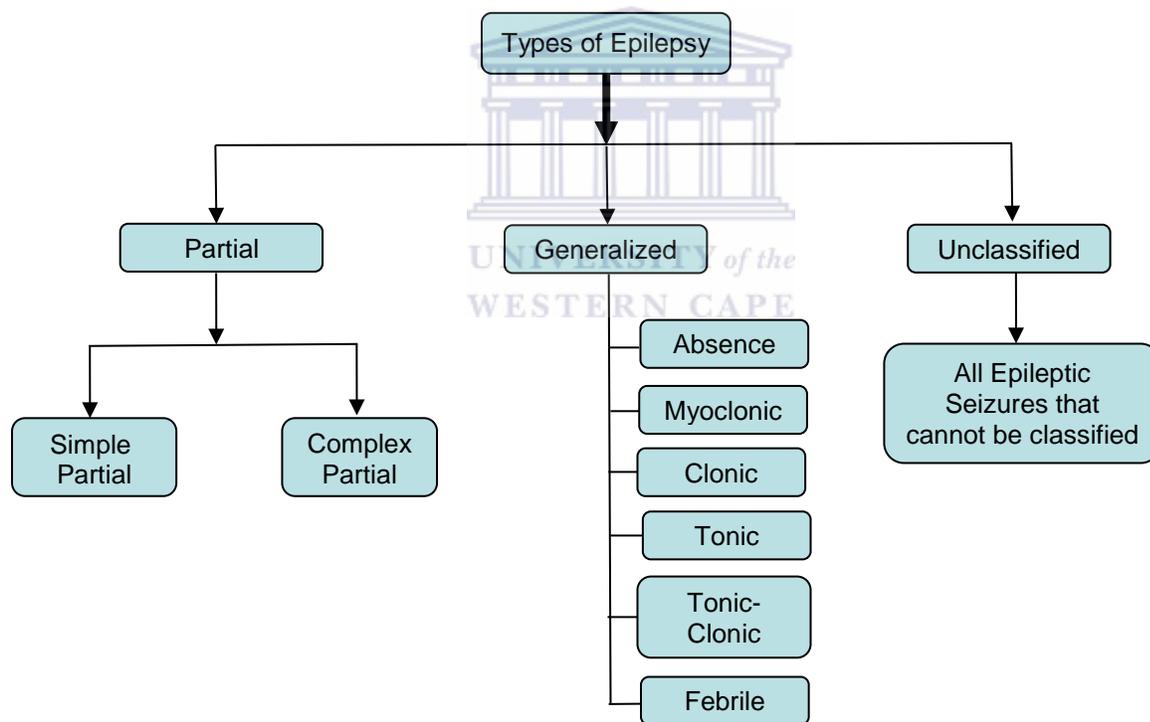
Figure 1: Classification of epilepsy

2.3.1. Partial (focal) seizures

In this type of epilepsy, seizures are caused by abnormal electrical disturbances in only a part of the brain and these disturbances may or may not spread to other parts (Figure 2). These types of seizures can be classified into simple partial seizures and complex partial seizures, both of which can lead to generalized seizures (Dekker, 2002; Rudzinski and Shih, 2011).
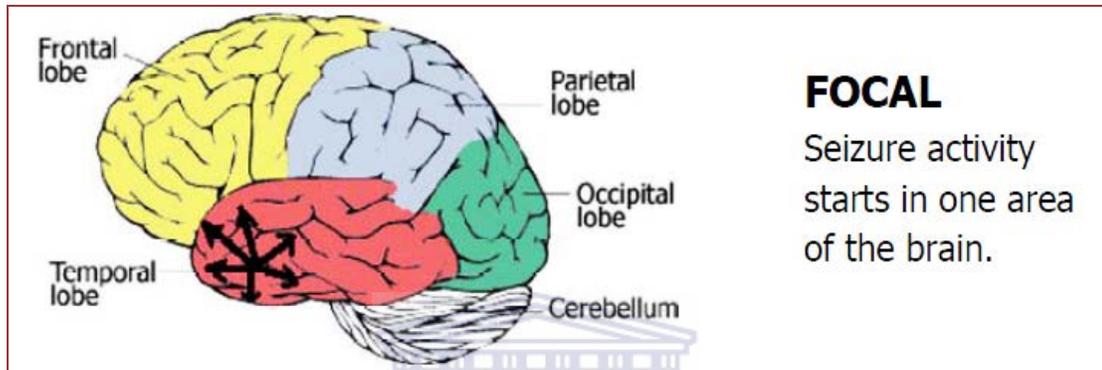
Figure 2: A partial (focal) seizure activity starts in one area of the brain (www.epilepsy.org.au)

2.3.1.1.   Simple partial seizures

In this type of epilepsy, there is no loss of consciousness and although patients may remember what happened, they may not be able to explain. The seizures emanate from the affected area in the brain such as temporal lobe (Jokeit and Schacher, 2004; Pellock and Duchowny, 2005).

2.3.1.2.   Complex partial seizures

In complex partial seizures, there is an impairment of consciousness and the condition may start as a simple partial seizure but develops into a complex partial seizure or directly as a complex seizure with impairment of consciousness at the onset of the seizure (Berendt and Gram, 1999). The clinical manifestations of complex partial seizures depend on the brain region affected by abnormal electrical activity (Chokroverty, 1996). Complex partial seizures are likely to arise

from the temporal or frontal lobes but can also occur in occipital or parietal lobes (Rudzinski and Shih, 2011).

2.3.2. Generalized Seizures

Generalized seizures are a type of epilepsy that starts from both cerebral hemispheres of the brain (Figure 3). They are characterized by a complete loss of consciousness and happen suddenly and unexpectedly (Dekker, 2002). These seizures may occur with convulsion and prominent motor activity or no convulsion at all (Friedman and Sharieff, 2006). They are often classified into six different categories of which the primary generalized tonic-clonic seizure (GTCS) type is considered the most common. Although others are less common, all seizure categories might be seen in special epilepsy clinics (Friedman and Sharieff, 2006).
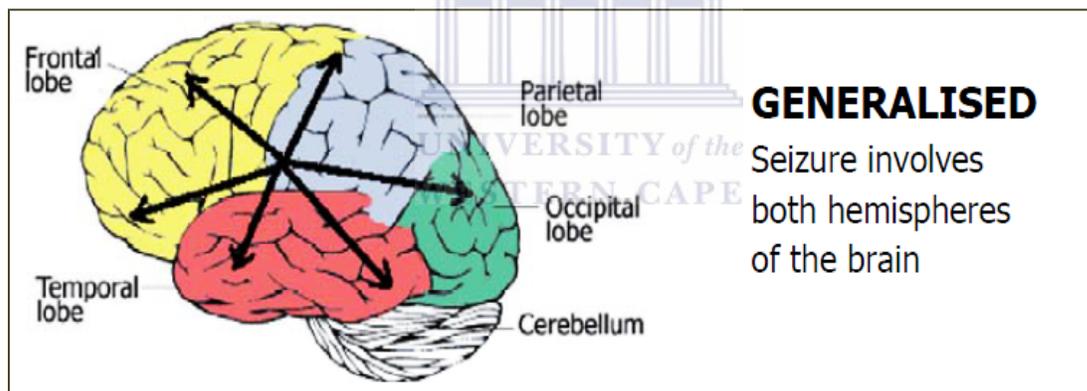
Figure 3: Generalized seizures are associated with the involvement of both cerebral hemispheres of the brain (www.epilepsy.org.au)

2.3.2.1.   Absence seizures: This seizure type may sometimes be unrecognized and are characterized by a brief loss of consciousness with minimal or no changes in muscle tone, brief upward rotation of the eyes, blank stare and an interruption of on-going activity (Dekker, 2002; Sysoeva et al., 2014).

2.3.2.2. **Myoclonic seizures:** This seizure type is often considered to occur in special epileptic syndromes (Dekker, 2002; Rudzinski and Shih, 2011) and involves sudden unilateral or bilateral shock-like muscle contractions which could manifest as single jerks or repeated jerks over longer periods (Chokroverty, 1996).

2.3.2.3. **Clonic seizures:** This type of seizures is often generalized and consists of non-tonic repetitive rhythmic flexing and stretching of the limbs (Dekker, 2002; Rudzinski and Shih, 2011).

2.3.2.4. **Tonic seizures:** These seizure types are characterized by an immediate loss of consciousness and often consist of a deviation of the eyes and head towards one side or even the rotation of the whole body (Dekker, 2002; Rudzinskiand Shih, 2011). There are often sudden sustained muscle contractions which fix the limbs in some strained position.

2.3.2.5. **Tonic-clonic seizures (GTCS):** These seizures are characterised by loss consciousness and posture and are occasionally associated with screams. They could develop into the tonic phase (a generalized stiffness) characterized by spasm in all muscles of the trunk with the patient becoming bluish in colour due to impaired breathing (Dekker, 2002).

Furthermore, the head is retracted, the arms are flexed and the legs become extended and after a while, the tonic stage may progress into a clonic phase with the patient having many jerks as a result of muscle contraction and relaxation (Dekker, 2002). The patient might bite his or her tongue, pass urine, or sometimes stool. At the end of the GTCS, the patient regains consciousness but may remain too tired and confused for a while (Chokroverty, 1996; Rudzinski and Shih, 2011).

2.3.2.6.   Febrile seizures (FS): These are the most prevalent types of convulsion that affect children aged five months to five years. Approximately 3-5% of the children under six years of age worldwide have FS (Wallace et al., 1998*)*. A study by Millar (2006) showed that up to 5% of children in North America and Western Europe experience at least one episode of febrile seizure before the age of six years. It is considered that a viral infection is the most common cause of febrile seizures (Principi and Esposito, 2013).

2.3.3.   Unclassified epileptic seizures

In this category are seizures that defy classification due to the fact that they do not fit into the descriptions for well-established seizure categories, e.g. some neonatal seizures (Chokroverty, 1996; Rudzinski and Shih, 2011).

2.3.4.   Special syndromes

This category consists of a group of signs and symptoms that can assist one identify epilepsy and epileptic syndromes, e.g. age at which seizures begin, sex, seizure type, presence of physical or learning disabilities, electroencephalogram (EEG) results (Chokroverty, 1996). The WHO estimates that there are three different types of epileptic disorders: idiopathic, symptomatic and cryptogenic disorders. Idiopathic disorders are mainly due to genetic factors with no symptoms of structural brain lesion or other neurological signs.

Symptomatic disorder etiology is usually known to be due to a structural lesion in the brain while cryptogenic disorder is thought to be a set of symptoms with unknown etiology. According to the international league against epilepsy (Dreifuss, 1985; CoCaTotILA, 1989), this classification depends on family history, clinical neurophysiology and combination of seizure semiology (Olafssonet al., 2005).

2.4.   **Epilepsy neurochemistry**

The major neurotransmitters in the brain are glutamate (GLU), gamma-amino-butyric acid (GABA), acetylcholine (ACh), nor epinephrine (NE), dopamine, serotonin, and histamine. Other molecules, such as neuropeptides and hormones, play modulatory roles that modify neurotransmission over longer time periods (Bromfield, et al., 2006). Besides its high oxygen consumption which makes it particularly vulnerable to oxidative stress, the brain also has high unsaturated fatty acid (UFA) content which makes it an easy target of lipid per oxidation (Perry et al., 2002; Nunomura et al, 2012). The metabolic rate of glucose in the cerebrum is often used as an index of brain activity (Kim, 2015) and there is currently enough evidence from human and experimental models to support the involvement of oxidative stress during seizures and in the epileptogenesis process (Cárdenas-Rodríguez etal., 2014). Thus, perturbations (an imbalance or dysfunction) in the synthesis or levels of neurochemicals in the brain will often result inillness (Pan et al., 2012). Some of the neurochemicals that may be affected in epilepsy are discussed in the sections below.

2.4.1.   **Gamma amino butyric acid (GABA)**

GABA was first synthesized in 1883 and known as a plant and microbe metabolic product. In 1950, however, GABA was discovered to be an integral part of the mammalian neurotransmitter system. Found mostly as Zwitter ion, it is synthesized *in situ* from the decarboxylation of glutamate by the enzyme glutamic acid decarboxylase (figure 4) since it does not penetrate the blood-brain barrier (Jorgensen, 2005).
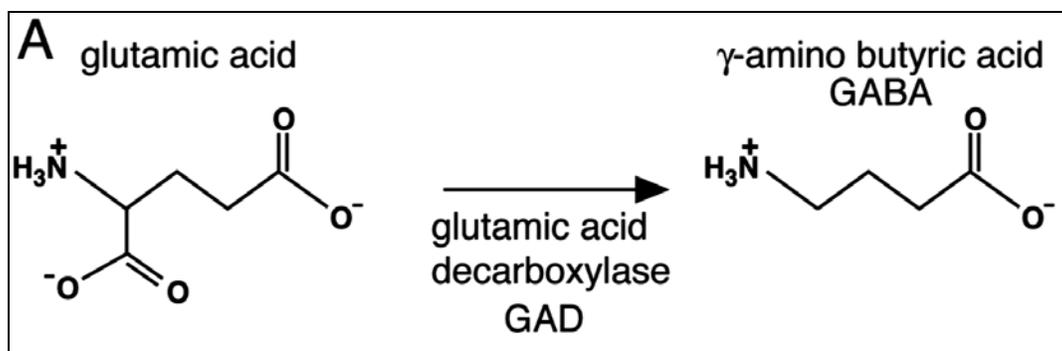
Figure 4: GABA structure (A); GABA is synthesized from glutamate by the enzyme glutamic acid decarboxylase (GAD) (Jorgensen, 2005).

The binding causes ion channels to open and allow for the flow of either negatively charged chloride ions into or positively charged potassium ions out of the cell. Depending on which ion channels open, the membrane potential is either hyperpolarized or depolarized. The GABA hypothesis suggests that epileptic patients have a low chronic seizure threshold, because they suffer from genetic or post-pathological GABA hypo-function. A corollary of the GABA hypothesis suggests that some anti-convulsant drugs act by restoring GABA function to normal states in epileptic humans or that anti-seizure drugs act by stimulating GABA activity (Rogawski, 2002). Drugs that act as agonists of GABA receptors (GABA analogues or GABAergic drugs), or drugs that increase the available amount of GABA normally have relaxing, anti-anxiety and anti-convulsant effects. The pharmacology and distribution of GABA receptors in the brain have been widely studied (Rang et al., 2012); these receptors are classified as follows:

2.4.1.1. GABA$_A$ receptors

GABA$_A$ receptors are ionotropic receptors linked to ligand-gated ion channels. These are very important and most abundant fast inhibitory neurotransmitter receptors in the mammalian brain mostly activated by GABA and play very important rolesin the mechanism

of action of many drugs (Sieghart and Sperk. 2002; Ding et al., 2014).The active site of the GABA$_A$ receptor is the binding site for GABA and several drugs such as muscimol and bicuculline (Rang et al., 2012).

2.4.1.2.   GABA$_C$ receptors

GABA$_C$ receptors are GABA-gated chloride channels considered a sub-class of GABA$_A$ receptors. Although GABA$_C$ receptors are so similar to GABA$_A$ receptors, there are many physiological and pharmacological differences which distinguish GABA$_A$ from GABA$_B$ receptors (Bormann, 2000; Johnston et al., 2003).

2.4.1.3.   GABA$_B$ receptors

GABA$_B$ receptors are G protein-coupled receptors (metabotropic receptors) widely distributed in the central and autonomic nervous systems (Bowery et al, 1981). Binding of the GABA neurotransmitter to the GABA$_B$ receptors causes inhibition of membrane excitability as K$^+$ channels open while Ca^{++} channels close (Jorgensen, 2005).

2.4.1.4.   GABA transporters

GABA transporters are divided into four types: GAT-1, GAT-2, GAT-3 and BGT-1 (Conti et al., 2004) distributed in various amounts throughout the developing human nervous system (Borden 1996). GAT-1 and GAT-3 arethe main sub-types in neurons and are considered the most pertinent to GABAergic neurotransmission (Roettger and Amara, 1999). GAT-2 and BGT-1 are fewer in the CNS and are less important for GABAergic neurotransmission (Borden 1996; Olsen et al., 1999).

2.4.2. Glutamate neurotransmitter

Glutamate is considereda principal excitatory amino acid neurotransmitter in the mammalian brain known to play an important role in neural activation (Sundaram et al., 2012). There are two types of glutamate receptors: ion tropic and metabotropic receptors (Pin and Duvosin, 1995; Pinto et al., 2014).

2.4.2.1. Ionotropic glutamate receptors

Ionotropic glutamate receptors (iGluRs) are ligand-gated ion channels which are abundant in the vertebrate central nervous system (CNS) and mediate excitatory neurotransmission (Pinto et al., 2014). There are several types of iGluRs, including N-methyl-D-aspartate (NMDA), α-amino-3- hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA) and kainite (KA) (Santiago et al., 2008). Mediation of these receptors for fast excitatory transmission in neurons occurs via the flow of the cations K^+, Na^{2+} and sometimes Ca^{2+} in response to glutamate binding leading to excitation (Pinto et al., 2014).

2.4.2.2. Metabotropic glutamate receptors

Metabotropic glutamate receptors (mGlurs) are many in the peripheral nervous system (PNS) and the CNS and show homology in different animal species (Nicoletti et al., 2011; Tharmalingam et al., 2012). The mGlurs are often divided into three sub-groups (Groups I, II and III) according to such factors as physiological activity, sequence homology and pharmacological profile. Group I receptors include the sub-types mGlu1 and mGlu5; Group II receptors include mGlu2 and mGlu3 while Group IIIreceptors include mGlu4, mGlu6, mGlu7 and mGlu8 (Niswender and Conn, 2010). The binding of glutamate neurotransmitter to glutamate receptors results in excitation (Harvey and Shahid, 2012).

2.4.2.3.   Glutamate transporters

Glutamate is the main excitatory neurotransmitter in the mammalian CNS. Chemically, glutamate is synthesized from glutamine supplied by glial cells and then packaged in the synaptic vesicles via vesicular glutamate transporters. Glutamate plays an important role in several physiological processes in the CNS including learning and memory (Yang et al., 2011; Sundaram et al., 2012). Its mechanism of action is via binding with its receptors (iGluRs and mGlurs) (Figure 5). There are five sub-types of glutamate transporters in the mammalian CNS, the excitatory amino acid transporters (EAAT 1-5). Glutamate is cleared from synapses through excitatory amino acid transporters (EAATs) on neighbouring glial cells (EAAT1 and EAAT2, and to a lesser extent, on neurons (EAAT3 and EAAT4) (Seal and Amara, 1999).
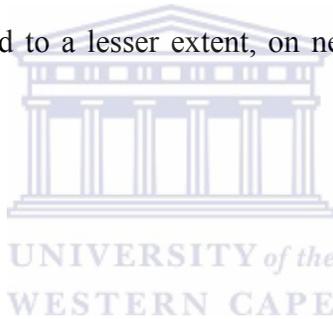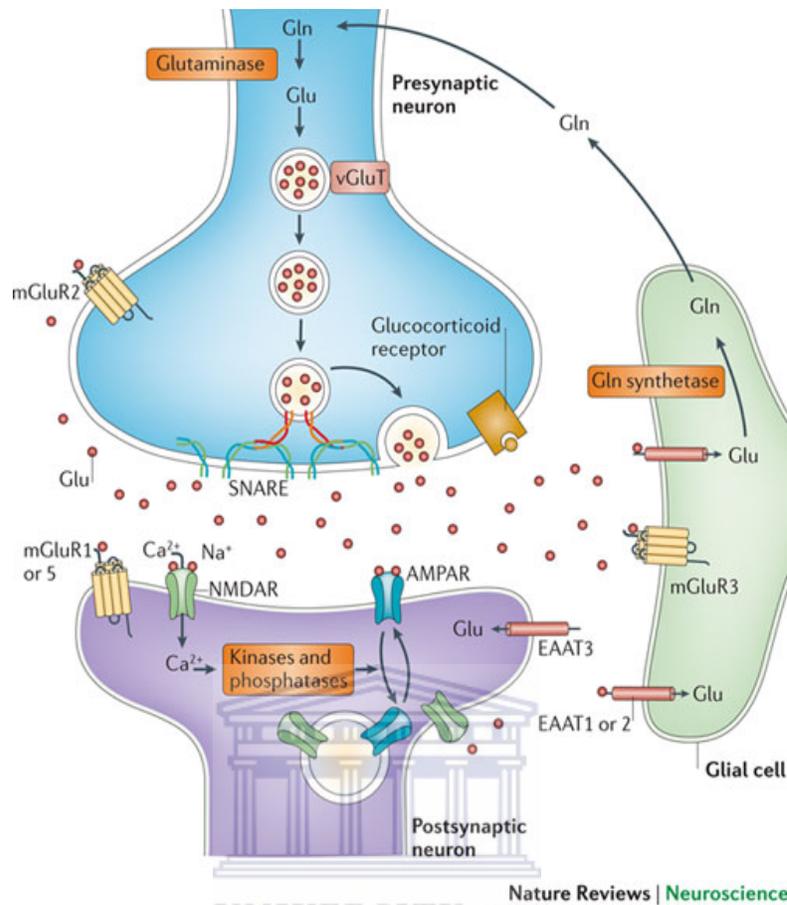
Figure 5: Schematic representation glutamate (Glu) synthesis from glutamine supplied by glial cells and packaged in the synaptic vesicles viavesicular glutamate transporters (vGluTs).After release from the vesicles, glutamate binds to the glutamate receptors such as ionotropic glutamate receptors (NMDA and AMPA receptors) and metabotropic glutamate receptors (mGluR1 to mGluR8)on the membranes of neurons and glial cells, andthe receptors initiate different responses like membrane depolarization and modulation of local protein synthesis. Glutamate is cleared from the synapse through excitatory amino acid transporters (EAATs) on neighbouring glial cells (EAAT1 and EAAT2) and, to a lesser extent, on neurons (EAAT3 and EAAT4) (Popoli et al., 2012).

2.4.3.   Acetylcholine

Although most epileptic seizures are characterized by an imbalance between GABA and glutamate i.e. excitation over inhibition, neuronal acetylcholine receptors (nAChRs) are known to play a role particularly in sleep-related epilepsy. This neurotransmitter imbalance may happen in parts of the brain (partial or focal seizures) or in the entire brain (generalized convulsive seizures).

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2.5.   Biomarkers

A biomarker is defined as an objectively measured characteristic of a normal pharmacological response to medical treatment, biologic or pathologic process (Sahu et al., 2011; Pitkanenet al., 2013). It is a term often used to refer to a protein measureable in blood or other body fluids whose concentration reflects the severity or presence of some disease state and in most cases helps to predict early stages of diseases for early diagnosis (Sahu et al., 2011). Changes in the re-organization of neuronal networks, in the number and functions of individual neurons, and in the sequential amendment of electrical properties of both the cells and the networks, could cause the firing of a large number of neurons leading to reoccurring seizures (Ben-Ari, 2001; Shukla and Prasad, 2012).

A good understanding of biomarkers could help in the development of interventions to prevent epileptic seizures, stop its progression, prevent recurrence and potentially cure this condition (Pitkanen et al., 2013). Biomarkers of seizures are characterized by an increase in extracellular concentrations of glutathione, taurine and phosphoethanol amine in acute hippocampal slices and in organotypic slices cultures (Wallin et al., 1999; Wallin et al., 2003) mainly as a reaction of glutamate receptor activation (Abbas, 2015).

2.6.   Epilepsy diagnosis

The determination of paroxysmal event as an epileptic seizure is considered the first step of diagnosing epilepsy (Chitre, 2013)but medical history is equally very important in diagnosing epilepsy (Babtain, 2013). However it is known that mis-diagnosis of epilepsy is common as approximately 20% to 30% of patients seen at epilepsy clinics have been incorrectly diagnosed (Smith et al., 1999; Benbadis, 2009). The conditions that are most frequently confused with epilepsy include vasovagal syncope, cardiac syncope and non-

epileptic attack disorder. Conditions that may mimic seizures include migraine, parasomnias, movement disorders, metabolic disturbances and panic disorder (Smith, 2012; Chitre, 2013). Access to complete details of the medical history is a very important step for the evaluation of seizures because there are several different causes of seizures, and there are many conditions to simulate a seizure (Friedman and Sharieff, 2006).

Besides the medical history, an eye-witness account of events during the start of patient unconsciousness is very important to give a medication of the type of seizure involved (Benbadis, 2009). Additionally, a careful review of the person's past medical history could indicate if there is a family history of cardiac arrhythmias or seizure, which may be related to such genetic disorder as idiopathic epilepsy syndrome (Karceski and Morrell, 2006).

2.7.   Laboratory testing

Laboratory blood tests are often done as part of the routine process of determining the causes of diseases including seizures. It is known that a high fever or metabolic disturbances could cause seizures to occur. For example, an electrolyte disturbance as a result of severe diarrhoea or vomiting, or a glucose imbalance cause by low blood sugar, may also result in seizures (Edmonton Epilepsy Association, 2010).

2.8.   Electroencephalography (EEG)

Electroencephalography (EEG) is the recording of brain electrical activity from the scalp which is often dependent on voltage differences between different points on the scalp, arising from excitatory and inhibitory postsynaptic potentials (Dietrich and Kanso, 2010). EEG is considered a very important test used for the diagnosis of epilepsy and is probably the most

specific method for defining the epileptogenic cortex. It has sensitivity and specificity, depending on age and recording procedures (Szilágyi et al., 2014).

2.9.   Magnetic Resonance Imaging (MRI) scans

Magnetic Resonance Imaging (MRI) is a medical imaging technique which reveals possible structural lesions underlying epilepsy. It is used to assess co-morbidities and evaluate individuals with medically refractory epilepsy for potential operation. Considering the highly sensitive and accurate images that can be obtained from MRI, it has become a standard diagnostic procedure for epilepsy, as it can provide an accurate determination of epileptogenic lesions in medically refractory epilepsy (Duncan, 2009). All patients with epilepsy are advised to do MRI, except children and young people who have idiopathic generalized epilepsy which responds to treatment with AEDs (Winston et al., 2013).

2.10.   Pharmaceutical treatment of epilepsy

The adequate treatment of epilepsy depends on the identification of the type of epilepsy and appropriate drugs. Mono-therapy has been recommended and shown to be very effective while single drug treatment is often advised as first option. If seizures remain uncontrolled, a second single drug treatment is suggested. Poly-therapy should only be contemplated after a third single drug therapy fails to control the seizures (Waller et al., 2005; SAMF, 2012).

2.10.1.   Anti-epileptic drugs (AEDs)

AEDs are a fundamental treatment for epilepsy and seizures. The choice of AEDs depends on many factors such as the type of epilepsy, the risk of recurrence, age of the patient, drug toxicity, etc. (Friedman and Sharieff, 2006). Majority of AEDs do not cure epilepsy but merely control seizures by reducing or stopping their occurrence usually through changes in

brain neuronal activity (Boison, 2010). The different types of epilepsy require different drugs with different anti-epileptic effects for their treatment (Waller et al., 2005; Rang et al., 2012). In status epilepticus, lorazepam (i.v), diazepam (i.v/ rectal) or midazolam (buccal/intranasal) should first be given. However, if seizures are not controlled or do recur after 30 minutes of treatment in adults, then phenytoin (slow i.v) followed by phenobarbitone (rapid i.v) are recommended. If there is no further response then treatment with thiopental (iv) or propofol (iv) should follow (Waller et al., 2005; SAMF, 2012).

In pregnancy, the lowest effective doses of AEDs are recommended alongside oral folic acid to prevent neural tube defect. If hepatic enzyme-inducing AEDs are used (e.g. phenytoin, carbamazepine or phenobarbitone), it is recommended that prophylactic oral vitamin K_1 should be given to the mother in the last 2 weeks of pregnancy and to the neonates, to avoid postpartum haemorrhage and neonatal bleeding respectively (Waller et al., 2005).

2.10.2.   Mechanisms of anticonvulsant drugs

AEDs are a diverse group of pharmacological agents that control epileptic seizures through different mechanisms of action (Stefan and Feuerstein, 2007). Some AEDs (e.g. carbamazepine, phenytoin, valproate, lamotrigine, zonisamide, oxcarbazepine and topiramate) act through modulation of voltage-depedent ion channels by blocking sodium channels to reduce the ability of sodium-dependent action potentials and enhance steady state inactivation. Sodium channels in mammalian brains are complex protein sub-units (Catterall, 2000) considered important targets of AEDs while others e.g pregabalin and ethosuximide, simply block calcium channels, mainly in partial and generalized seizures (Stafstrom, 2010). In addition, some AEDs (e.g. gabapentin, phenobarbital and benzodiazepines) are known

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toenhance GABA activity (Figure 6), by increasing the proportion of bound GABA receptors that are in the long-duration active state (White et al., 2000). Other AEDs (e.g. lamotrigine) are known to block glutamate-mediated excitatory neurotransmission (Hernández-Díaza and Levin, 2014); the ionotropic glutamate receptors NMDA and AMPA, known to play important roles in epilepsy treatment, are important targets for AEDs (Rogawski, 1996; Löscher, 1998).
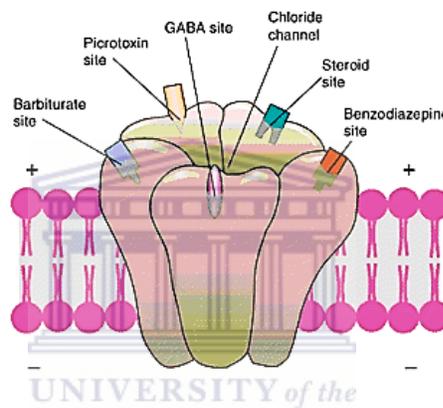
Figure 6: Pharmacological substances (anti-epileptic drugs) bind to GABA receptors to modulate GABA effects (https://www.xbrain.co.uk/alpha-brain-ingredientsgaba)

2.10.3. Non-drug treatment

Other methods that enhance treatment of epilepsy have been recommended. A number of non-drug approaches to therapy have been tried including biofeedback, hypnosis and surgery (Singer, 2001; Dewar and Pieters, 2015). Of all these, only surgery has gained widespread acceptance and for selected patients, neurosurgery offers a risky but effective cure for seizures. Surgery involves removal of an area of the brain responsible for seizures and is often attempted only when attacks appear from a 'clear cut' focal area and if seizures do not respond to medication (Günaydın et al., 2015). Evaluation for surgery is normally done by

many medical specialists: radiologists, neurologists, psychologists and neurophysiologists (Walker and Fish, 2009).

Ketogenic diets which are high in fat and low in carbohydrates and proteins have been used for epilepsy in children not responding to conventional drug treatment. The diet is said to stimulate a fasting situation that is perceived by the brain as the feeling of hunger and allows the body to produce ketones which help suppress the epileptic condition (Kossoff, 2010).

In spite of the large number of new AEDs available in the market today, there is still no cure for epilepsy (Boison, 2010). Many available drugs often have severe side effects and are highly pharmaco-resistant (Vajda, 2007). Most of these drugs do not also affect the underlying disease processes, thus there is a need to develop innovative therapeutic strategies based on knowledge of underlying molecular pathogenetic mechanisms (Boison, 2010). If the molecular pathways of epilepsy are well known, the phenotypic expression of epileptic seizures could be silenced. Gene silencing techniques aimed at over-expressing selected anticonvulsant molecules have been explored as therapeutic options for epilepsy (Riban et al, 2009).

The use of available convectional pharmaceutical products has yet to lead to an effective cure for epilepsy (Howard et al., 2011). Some of these products are unaffordable by epilepsy patients in most rural communities especially in Africa and Asia hence they resort to traditional medicines to manage their epileptic conditions (Van Wyk et al., 1997; Kayne, 2009). Many of these traditional medicines are plant-derived and are known to contain potent bioactive compounds which are effective in the control of epileptic seizures.

2.11. Epilepsy and traditional medicine

The World Health Organization (WHO) defines traditional medicine as "the knowledge, skills and practice of holistic care, recognized and accepted for its role in the maintenance of health and treatment of diseases. It is based on indigenous theories handed down from generation to generation for its beliefs and experiences (Zhang and WHO, 2000).

The use of traditional medicine for the treatmentof epilepsy is widespread in developing countries and about two-thirds of the population of these countries relies heavily on traditional medicine treatment as their basic health care system (Farnsworth and Soejarto, 1991). In developing countries like India, China and African countries, traditional medicine has been improved for the health care system and hasbecome more widespread to even developed countries (Fouche et al., 2008; Ashidi et al., 2010).

Traditional herbal medicines have been used for thousands of years by millions of peoplein Africa, for the prevention and treatment of diseases (Cragg and Newman, 2005). In South Africa, an estimated 27 million people are reported to use indigenous traditional medicines particularly in KwaZulu-Natal where households are said to spend between 4% and 6% of their annual incomes on indigenous medicine and services (Mander, 1998). Many herbal medicines are efficacious and safe (Pearl et al., 2011), have a history of use for the treatment of neurological diseases (including epilepsy) and could enhance the effects of conventional anti-depressants (Ernst 1995; Adeyemi et al., 2007).

2.12. *Tulbaghia violacea*

2.12.1. Ethnobotany and phytochemistry

Tulbaghia violacea (*T. violacea*) is a bulbous plant with hairless leaves, and a white, fleshy stalk (Van Wyk et al., 1997). It has attractive violet flowers and long, hairless green leaves. *T. violacea* is known as "itswelelomlambo" in Xhosa, "wildeknoffel" in Afrikaans, "isihaqa"in Zulu and "sweet garlic, wild garlic or society garlic" in English (Street et al., 2010; Saibu et al., 2015). *T. violacea* belongs to the *Alliaceae* family, which is the same family as onion and real garlic. Furthermore, *T. violacea* and garlic (*Allium sativum*) have the characteristic sulphur smell of garlic andthis plantis presumed to have the same biological activities and secondary (Bungu et al., 2006).

Geographically, *T. violacea* is widely spread throughout Africa and is considered an indigenous plant to the Eastern Cape Province of South Africa where it grows on rocky grasslands. *T. violacea* has been used as a substitute for garlic and also as a medicinal plant for the treatment of various diseases (Ebrahim and Pool, 2010).

2.12.2. Medicinal use of *T. violacea*

T. violacea is extensively used as a medicinal plant for various disease conditions such as fever, tuberculosis, asthma, stomach problems, oesophageal cancer, fits and rheumatoid arthritis (Ebrahim and Pool, 2010; Ncube et al., 2011). Although *T. violacea* has many benefits as a traditional medicine, several adverse effects such as abdominal pain, intestinal mucosa sloughing as well as acute inflammation have been attributed to its use (Bungu et al., 2006).

Figure 7: *TulbaghiaviolaceaHarv*alsoknown as society garlic. (A) Flowers, (B) Rhizomes and (C) Leaves (Khabbab, 2012)

2.12.3.   Experimental studies of *T. violacea*

The potential anti-cancer activities of the methanolic extracts of *T. violacea* have been investigated *in vitro* and results showed that *T. violacea* leaves and bulbs significantly inhibited the growth of four cancer cell lines namely HT29 (colon cancer), HeLa (cervical cancer), WHCO3 (oesophageal) and MCF-7 (breast cancer) respectively (Bungu et al., 2006; Bachrach, 2012).

The anti-hypertensive effects of *T. violacea* in animal models of both systolic and diastolic hypertension have been investigated (Raji et al., 2012) and *T. violacea* was found to induce significant reduction in systolic and diastolic hypertension and heart rate (HR) in spontaneously hypertensive Wistar rats (SHR) via inhibition of β1 adrenoceptors andangiotensin I converting enzyme (ACE). Mackraj et al. (2008) reported that the hypotensive effects of *T. violacea* could be secondary to a direct negative chronotropic effect on heart muscles.

T. violacea has also been reported to have antimicrobial (antibacterial and antifungal) properties (McGaw et al., 2000; Motsei et al., 2003) especially against different microbial infections such as pulmonary tuberculosis, oral and ear infections (Lyantagaye, 2011; Aremu and Van Staden, 2013).
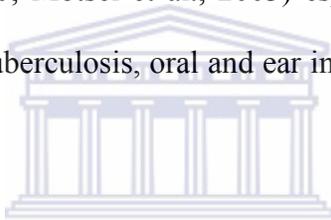
2.12.4. Experimental studies of known anticonvulsant plants

The anticonvulsant activities of some plants especially those in the *Alliaceae* family, have been reported (Advani et al., 2011) but there is no literature on the anti-convulsant effects of *T. violacea*. An understanding of the mechanisms of action of other anti-convulsant plants may help explain the mechanisms of action of *T. violacea*due to chemical similarities.

2.12.4.1. *Allium sativum (garlic)*

Allium sativum (common name: garlic) belongs to the *Alliaceae* family, originated from central Asia and spread to the rest of the world. This plant has been widely used against various ailments and physiological disorders including lowering of blood cholesterol levels, improvement of cardiovascular diseases and antimicrobial effects (Ginter and Simko, 2009;

Londhe, 2014). The oil in garlic has been shown to have anti-convulsant effects which may be mediated by an increase in brain GABA content (Advani et al., 2011).

2.12.4.2. *Rutagraveolens (R. graveolens)*

R. graveolens belongs to the *Rutaceae* family and although it is indigenous to the Mediterranean region (Watt and Breyer-Brandwijk, 1962), it is currently a global medicinal plant in many countries including South Africa, with potent anticonvulsant effects (Palmer and Pitman, 1972; Van Wyk et al., 1997). This plant appears to exert its anticonvulsant activity by enhancing GABA neurotransmission (Ahmad and Amabeoku, 2013).

2.12.4.3. *Leonotisleonurus (L. leonurus)*

L. leonurus belongs to the *Lamiaceae* family of plants and is native to South Africa with wide distribution throughout the country. It is known as "Umuuyane" in Zulu and "Wilde dagga" in Afrikaans (Hutching et al., 1996; Van Wyk and Gerick, 2000). Traditionally, *L. leonurus* has been used via smoking, to relieve epilepsy convulsions. It is known to affect both the GABAergic and glutamatergic systems by delaying seizure latency (Bienvenu et al, 2002).

2.12.4.4. *Persea Americana mill (P. Americana)*

*P. Americana*belongs to the *Lauraceae* family of plants and is known by its common name "*Avocado*" (Ojewole and Amabeoku, 2006). It is native to Mexico, but is now used worldwide (Ross, 1999). It has been used as analgesic, anti-hypertensive, anti-tussive, anti-diabetic and anti-inflammatory agents respectively (Antia et al., 2005; Owolabi et al., 2005). Avocado has also been used in African traditional medicine as an anti-convulsant

formulation to control childhood convulsions and its anti-convulsant effects are mediated by the enhancement of GABA release (Ojewole and Amabeoku, 2006).

2.12.4.5. *Cotyledon orbiculata (C. orbiculata)*

C. orbiculata belongs to the *Crassulaceae* family of plants and is found throughout South Africa. It is known as "Imphewula" in Xhosa, "Plakkie" in Afrikaans and "Seredile" in Sesotho and Tswana. In South Africa, it is used to treat several types of diseases such astoothache, earache, warts and epilepsy (Watt and Breyer-Brandwijk, 1962; Van Wyk et al., 1997). Its anti-convulsant function involves delay of the onset of tonic convulsions which may suggest glutaminergic and GABAergic mechanism of action (Amabeoku et al., 2007).

2.12.4.6. *Zanthoxylumcapense (Z. capense)*

Z. capense belongs to the Rutaceae family of plants and is native to Southern Africa; commonly distributed from Zimbabwe to the Western Cape of South Africa (Palmer and Pitman, 1972; Van Wyk et al., 1997). It is known by different names in South Africa: small knobwood (in English), "kleinperdepram" (in Afrikaans), "umnungamabele" (in isiZulu) and "monokwane" (in seSotho). *Z. capense* is a widely used as a traditional medicine against different ailments, including stomach-ache, toothache, fever and epilepsy (Van Wyk et al., 1997). This plant delays the onset of convulsions, which may suggest either or both of GABAergic and glutaminergic mechanisms (Amabeoku and Kinyua, 2010).

2.12.4.7. *Sutherlandia frutescens* (S. frutescens)

S. frutescens belongs to the *Fabaceae* family of plants and is widespread throughout Southern Africa, known by different names including Cancer bush (in English) and "Umwele" (in IsiZulu) (Van Wyk et al., 2000; Stander et al., 2007). In Southern Africa, *S.*

frutescens has been traditionally used for the treatment of childhood convulsions and epilepsy. Many studies have shown that this plant has the ability to delay the onset of convulsion either directly by acting on GABA at GABAergic receptors, or indirectly by enhancing or modulating GABAergic neurotransmission in the brain (Ojewole, 2008).

2.12.4.8. *Carissa edulis* (C. edulis)

C. edulis belongs to *Apocynaceae* family of botanicals and is known by different names including "Cizaki" in Nigeria, "Adishawel" in Somalia and "Num-num" in most Arabic countries (Hutchinson and Dalziel, 1963; Ya'u et al., 2008). *C. edulis* has been used traditionally for a wide variety of ailments including rheumatism, headache, gonorrhea, syphilis and epilepsy (Nedi et al., 2004). The anti-convulsant activity of this plant may be mediated via interaction with some substances like benzodiazepine to form GABA-benzodiazepine receptor complex (Ya'u et al., 2008).
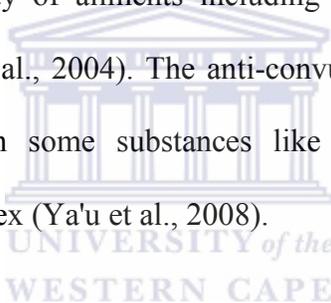
2.12.4.9. *Harpagophytumprocumbens* DC (*H.procumbens*)

H. procumbens belongs to the *Pedaliaceae* family of botanicals and is native to Southern Africa where it is known by different names such as "Wood spider" and "Devil's claw" (Van Wyk et al., 2002). *H. procumdens* has been widely used in South Africa as a traditional medicine for the treatment, management and/or control of hypertension, diabetes mellitus, gout, skin cancer, fever, infectious diseases, allergies, and osteoarthritis (Van Wyk and Gericke, 2000). This plant is used to treat epilepsy and childhood convulsions via enhancing GABAergic neurotransmission (MacDonald and Kelly, 1994; Mahomed and Ojewole, 2006).

2.12.4.10. *Crinum Zeylanicum (**C. Zeylanicum**)*

C. zeylanicum is a member of the Amaryllidaceae family of botanicals widely used for the treatment of various diseases in the Dominican Republic especially malaria (Fennell and Staden, 2001). In Nigeria, this plant is used against childhood convulsions, epilepsy, general debility and malaria (Tijani et al., 2012). The anti-epileptic effects of *C. zeylanicum* may be via enhancement of GABAergic neurotransmission (Tijani et al., 2012)

2.12.5.   Potential anticonvulsant effects of *T. violacea*

T. violacea shares similar chemical properties with most of the above-mentioned botanicals; hence it is possible that this plant also has anti-convulsant properties. However, the exact mechanisms through which *T. violacea* could exert its anti-convulsant effects are unknown, which informs the motivation for the current study. Garlic *(Allium sativum)* for instance, is known to contain volatile sulfur-containing flavour compounds which are responsible for its characteristic smell and taste. Most of these plants also contain alliinase enzymes known to play some role in stimulating the conversion of the odourless S-alk (en) yl-L-cysteine sulfoxides into the volatile thiosulfinates, with pyruvate and ammonia formed as by-products (Block, 1992; Koch and Lawson, 1996).

2.12.6. Animal models of status epilepticus and convulsion

Animal studies have long been used for scientific detection of pathogenesis, toxicity and treatment effects in various diseases (Oakley et al., 2008). The pathophysiology of seizures and epilepsy has also been widely studied using animal models which have provided useful information about the mechanisms of action of the various treatment methods currently in use against epilepsy (Epps and Weinshenker, 2013). The basic mechanisms underlying ictogenesis and epileptogenesis have been elucidated with animal studies, leading to the

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discovery and pre-clinical development of novel AEDs (Löscher, 2011). White et al. (2006) suggested thatanimal studies are a more reliable way of establishing the efficacy and safety of AEDs before prescription for humanuse.

2.12.6.1. Genetic animal models

Epilepsy in humans is characterized by chronically recurring and spontaneous seizures with symptoms that have been induced in some animal models. Genetic factors contribute largely to the etiology of seizure disorders (Schauwecker, 2011), thus most of the animal models currently in use have inborn susceptibility to seizures (Löscher, 1984, 1992; Buchhalter, 1993). A non-epileptic animal will not be a suitable chronic epilepsy model (Löscher, 2011).

2.12.6.2. Non-epileptic animal models

This type of model is slightly different to the epileptic animal models as it involves the use of convulsion-inducing agents e.g. pentylenetetazole (PTZ) to induce seizures. These models are also used to determine the mechanisms of action of new AEDs (Löscher, 2002; 2011); normal (non-epileptic) animals are usually administered a chemical such as PTZ, *N*-Methyl-D-aspartic acid (NMDA) and pilocarpine, toinduce clonic or tonic–clonic seizures in animals similar to the condition in humans.

2.12.6.3. BALB/c mice

BALB/c mice are in-bred albino mice which have peculiar features, such as easy breeding and minimal weight difference between males and females. These mice have also been used for monoclonal antibody production and have also played an important role in oncology research (Oakley et al., 2008).

2.12.7.   Objectives of the study

Despite the wide use of *T. violacea* in traditional medicine for the treatment and management of many disease conditions (Saibu et al., 2015), little or no information exists in literature about the anti-convulsant potential of this plant. The present study therefore aims to investigate the mechanism of action by which the methanolic leaf extracts of *T. violacea* exerts its anti-convulsant effects in BALB/c mice. Animals will be injected with compounds known to inhibit GABA neurotransmission (pentylenetetrazole, bicuculline and picrotoxin-PCN) as well aswith other compounds known to enhance glutamic acid neurotransmission (NMDLA) to induce convulsions. Treatment of the induced convulsions will then be done with different doses of the methanolic leaf extract of *T. violacea*.
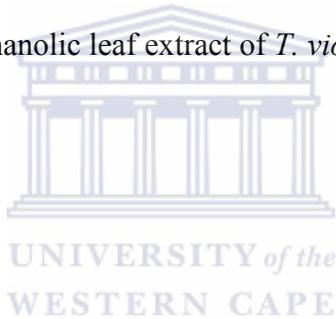
Chapter 3

Materials and Methods

3.1 Plant Material

3.1.1. Collection and identification of the plant

Fresh leaves of *Tulbaghia violacea* were collected from Kirsten Bosch National Botanical Garden, South Africa, in March 2014. The identity of the leaves was confirmed by the curator of the Garden and a taxonomist, Mr F. Weitz, in the Department of Biodiversity and Conservation Biology, University of the Western Cape. A voucher specimen of *T. volacea* (Amebeoku 007) was then deposited in the Herbarium at the University of the Western Cape.

3.1.2. Preparation of the leaf methanol extract of *T. violacea*

The fresh leaves of the plant species were separated from branches of the plant, weighed (1.7 kg) and then washed with distilled water. They were dried in a ventilated oven at 35°C for 4 days. The dried leaves (818.1 g) were ground into fine powder (486 g) using the Waring Commercial laboratory blender. To prepare the methanolic leaf extract of *T. violacea*, sixty grams (60 g) of thedried powder was extracted in a Soxhlet extractor with 500 ml of methanol for 4 hours. The methanol filtrate obtained was evaporated to dryness using a Buchi RE11 rotavapor and Buchi 461 water-bath and 5.83 g of crude methanol extract was obtained. This was then preserved in desiccators for further studies. Fresh solution of the crude leaf methanol extract of *T. violacea* was preparedon each day of the experiment by dissolving a weighed quantity of the crude methanol extract inaminimum volume of dimethylsulfoxide (DMSO) and then made up to the appropriate volume with physiological saline. The solution was injected intraperitoneally (i.p.) to mice in a volume of 1 ml/100 g of body weight of animal.

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3.2.   Animals

Male and female albino mice were used for this study. The animals were bred in the Discipline of Pharmacology, School of Pharmacy, University of the Western Cape, South Africa and weighed between 18 g and 30 g. The mice were used in groups of eight for each dose of plant extract or drug and had access to food and water *ad libitum*. All animals were fasted for 16 hours during which they had access to water before the commencement of the experiments. Laboratory conditions were maintained at ambient temperature of 22 ± 1°C, relative humidity of $55 \pm 15\%$ and 12 hours light/12 hours dark cycle atall times during the experiments. Each animal was used only once in the experiments.

3.3.   Drugs and Chemicals

Strychnine (STN, Sigma Chemical Co),   pentylenetetrazole (PTZ, Sigma Chemical Co.), picrotoxin (PCN, Sigma Chemical Co.), N-methyl-DL-aspartic acid (NMDLA, Sigma Chemical Co.), phenobarbitone sodium (BDH Chemicals Ltd), LY233053 (Sigma   Chemical Co.), muscimol (Sigma Chemical Co.) and5,5 di-phenylhydantoin sodium salt (Phenytoin, Sigma Chemical Co.) were dissolved in physiological saline to the required volumes. (+) Bicuculline (Sigma Chemical Co.) was suspended in a minimum amount of Tween 80 and adjusted to the appropriate volume with physiological saline. Diazepam is the generic name for Valium (Roche, South Africa) was dissolved in a minimum amount of propylene glycol and made up to the appropriate volume with physiological saline. Dimethylsulfoxide (DMSO, Sigma Chemical Co.) solution was prepared by dissolving equal volume, used to dilute the plant extract, in an appropriate volume of physiological saline. All drugs were injected intraperitoneally (i.p.) in a volume of 1 ml/100 g of animal body weight. Equal volume injections of the appropriate vehicles such as physiological saline and DMSO were given to the control animals. Fresh

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solutions of the plant extract or drugs were prepared on each day of the experiment. The doses of the methanolic leaf extract of *T. violacea* used were obtained from preliminary studies in our laboratory. The pre-treatment times following the administration of PTZ, bicuculline, PCN or NMDLA were 15 minutes (plant extract), 10 minutes (phenobarbitone), 20 minutes (diazepam), 20 minutes (phenytoin), 30 minutes (LY233053), 1 hour (muscimol) and 15 minutes (DMSO solution), as obtained from previous studies (Amabeoku and Kinyua, 2010).

3.4. Phytochemical analysis of *Tulbaghiaviolacea*

Phytochemical analysis was performed on the samples of dried powdered leaf extracts of *T. violacea* using the methods of Harbone (1984) and Ikhiri *et al*. (1992) to screen for chemical compounds including alkaloids, Saponins, Reducing sugars, Flavonoids, Cardiac glycosides, Triterpene steroids, Quinones and Tannins.

3.4.1 Alkaloids

A sample of dried powdered leaves of *T. violacea* (0.5 g) was mixed with 10 ml of dilute hydrochloric acid (alcoholic) in a test tube and boiled for 5 minutes. The mixture was cooled and the debris was allowed to settle. The supernatant was filtered and1ml of the filtrate was transferred into another test tube into which three drops of Dragendorffs' reagent was added, shaken and observed for the appearance of an orange-red spot and a precipitate which would indicate the presence of alkaloids.
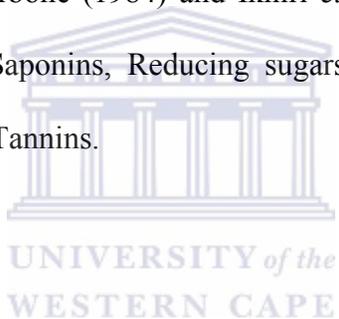
3.4.2 Saponins

A sample of driedpowdered leaves of *T. violacea* (0.4 g) was mixed with 4 ml of water in a clean test tube and shaken vigorously for 20 seconds. This was then observed for a persistent froth which would indicate the presence of Saponins.

3.4.3 Reducing sugars

A sample of dried powdered leaves of *T. violacea* (0.2 g) was boiled in 5ml of water and the mixture was cooled and filtered. An equal quantity (5ml) of Fehlings A and B solutions 1:1 was added to the filtrate (3 ml), heated in a water-bath, and then observed for a red-brownprecipitate formation which would indicate the presence of reducing sugars.

3.4.4 Flavonoids.

A sample of driedpowdered leaves of *T. violacea* (10 g) was boiled in 100 ml of water for 2 to 3 minutes in a water-bath. The mixture was allowed to cool beforefiltration. The filtrate (3 ml) was put in a test tube to which 3 ml of acid-alcohol (Ethanol: Water: concentrated hydrochloric acid in a ratio of 1:1:1), solid magnesium (1 cm) and 1 ml of t-amyl alcohol were added. The mixture was then observed for a rose-orange or violet colour which would indicate the presence of flavonoids.

3.4.5 Cardiac glycosides

A sample of dried powdered leaves of *T. violacea* (0.5 g) was boiled in 5 ml of 70% ethyl alcohol for 2 minutes. The mixture was allowed to cool and then filtered. The filtrate (3 ml) was put in a small beaker and 10 ml of water and 5 ml of chloroform were added. It was then shaken and the lower chloroform layer separated and evaporated to dryness in a water-bath. The cooled chloroform

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residue was dissolved in 3 ml of glacial acetic acid containing 0.1 ml of ferric chloride. The solution was carefully transferred to the surface of a 2 ml of sulphuric acid solution and observed for a reddish-brown layer at the interface while the upper layer may gradually acquire a bluish-green colour. These would indicate the presence of cardiac glycosides.

3.4.6 Triterpene steroids

A sample of dried powdered leaves of *T. violacea* (1 g) was extracted for 24 hours in 20 ml of ether. The resultant solution was filtered and 1 ml of the filtrate evaporated to dryness. The resultant residue was re-dissolved in several drops of acetyl anhydride to which several drops of dilute sulphuric acid were added and observed for a green colour change which would indicate the presence of triterpene steroids.

3.4.7 Quinones

A sample of dried powdered leaves of *T. violacea* (10 g) was moistened with a 10 % hydrochloric acid (HCl) solution and allowed to stand in ether: chloroform mixture (3:1, 40 ml). The mixture was filtered and 1 ml of the resultant filtrate was treated with 1 ml of 10 % sodium hydroxide (NaOH) solution. A red coloration would indicate the presence of quinones.

3.4.8 Tannins

A sample of dried powdered leaves of *T. violacea* (0.2 g) was boiled in 5 ml of water. The subsequent mixture was allowed to cool down and was filtered thereafter. The filtrate (2 ml) was put in a test-tube and 2-3 drops of 5% ferric chloride solution were added and observed for a blue-black precipitate which would indicate the presence of tannins.

3.5   High performance liquid chromatographic (HPLC) analysis

Chromatographic system: Agilent 1200 system consisting of degassing system, quaternary pump, auto loading sampler, thermos tatted column compartment, diode array detector, fluorescence detector, analyse fraction collector and Agilent Chem. Station software; column: Phenomenex Luna (C18) 5µm and dimensions (250 cm x 4.6 mm).

Chromatographic conditions: Mobile phase degassed with helium, solvent A: water-containing 0.1% formic acid; solvent B: Acetonitrile-containing 0.1% formic acid, Mode: flow rate, 0.8 ml/min; injection volume, 50 µl, detector, UV at 370 nm. The eluent was monitored at several wavelengths ranging from 210 to 370 nm, the specific wavelength of interest being 350 nm. The HPLC operating conditions were programmed to give the following: 0 minute, solvent B: 18%; 15 minutes, solvent B: 25%; 20 minutes, solvent B: 35%, 30 minutes, solvent B: 90%. The run rate was 30 minutes.
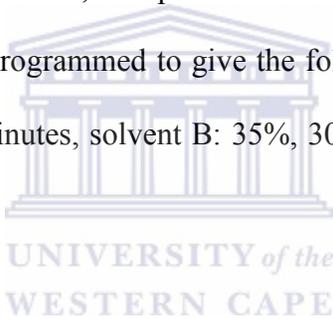
3.6 Pharmacological assessment

3.6.1 Anticonvulsant activityassessment

The method of Vellucci and Webster (1984) as modified by Amabeoku and Kinyua (2010), was used to evaluate the anti-convulsant activity of the methanolic leaf extract of *T. violacea*. The mice were acclimatized to their new environment by housing them singly in transparent perspex mouse cages 30 minutes before commencement of the experiment. Eight control animals were pre-treated for 15 minutes with physiological saline (0.25 ml, i.p.) and then administered separately with standard convulsant drugs including PCN (12 mg/kg, i.p.), PTZ (100 mg/kg, i.p.), bicuculline (40 mg/kg, i.p.) and N- methyl-DL-aspartic acid (NMDLA, 400 mg/kg, i.p.), to produce   tonic convulsion after 30 minutes. Seizures were manifested as tonic hind-limb

extensions. The onset time of tonic seizures and the proportion of animals convulsing or not convulsing were obtained during the 30-minutes observation period. Test animals (eight per group) were pre-treated with either the methanolic leaf extract of *T. violacea* (100-400 mg/kg, i.p.), phenobarbitone (12 mg/kg, i.p.), diazepam (0.5 mg/kg, i.p.), phenytoin (30 mg/kg, i.p.), muscimol (0.2-2 mg/kg, i.p), LY233053 (5 mg/kg, i.p.) or DMSO (0.25 ml, i.p.) prior to the administration of any of the convulsant agents and the animals were also observed for 30 minutes for tonic convulsion. The time of the onset of seizures and proportion of animals convulsing or not convulsing were obtained during the 30-minute observation period. The experiment was repeated with another group of eight mice pre-treated with combined sub-effective doses of leaf methanol extract of *T. violacea* (100 mg/kg, i.p.) and muscimol (0.2 mg/kg, i.p.) prior to the administration of PTZ (100 mg/kg, i.p.). The ability of the plant extract to prevent or prolong the onset of the tonic hind limb extensions was taken as an indication of anti-convulsant activity (Amabeoku and Kinyua, 2010; Amabeoku et al., 2014). All the experiments were carried out in a quiet laboratory at an ambient temperature of 22±1°C during the period between 09h00 -17h00 on each a day of the experiment.

3.7 Acute toxicity study

The method of El Hilaly et al. (2004) and the method described by Lorke (1983) modified by Ojewole (2006) were used for the acute toxicity study of *T. violacea*. The acute toxicity study was carried out to determine the median lethal dose (LD50) of the plant extract. Mice were fasted for 16 hours and then randomly divided into groups of eight mice per cage. Graded doses of the plant extract (100, 200, 400, 800, 1200, 1600, 2000, 2400, 2800, 3200, 3600 and 4000 mg/kg) were separately administered orally by means of a bulbed steel needle to mice in each test group. The control group received 0.25 ml *per os* (p.o.) of physiological saline by means of a

bulbed steel needle. The mice were then allowed free access to food and water and observed for 5 days for signs of acute toxicity including death. In the case of any death occurring at any dose range within the 5 days observation period, log dose-response curves would be constructed for the plant extract from which the median lethal dose would be calculated.

3.8   Statistical analysis:

The results obtained from this study were analysed using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test (GraphPad Prism, version 5.0, GraphPad software, Inc., San Diego Cap2130, USA). The number of animals convulsing was analysed using the Chi-squared test (Bienvenu et al., 2002). The data obtained was expressed as mean ± SEM. P-values of less than 5% ($p<0.05$) were considered statistically significant.

3.9   Ethical considerations

The experimental protocol used in this study was approved (14/7/46) by the University of the Western Cape Animal Ethics Committee, Bellville 7535, South Africa and complies with to the University's Regulations on animal experiments.
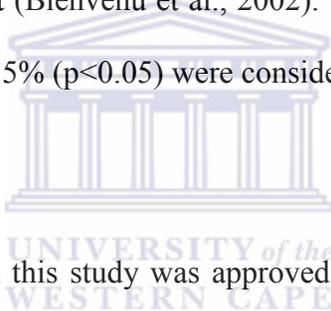
Chapter 4

Results

4.1 Phytochemical analysis

The phytochemical quantitative analysis of the dried leaf powder of *T. violacea* showed that the plant species contain the following chemical components: alkaloids, saponins, reducing sugars, flavonoids, cardiac glycosides, triterpene steroids, quinones and tannins (Table 1).

Table 1. Phytochemical analysis of the dried powdered leaf of *T. violacea*

Compounds	Results
Alkaloids	+
Cardiac glycosides	+
Flavonoids	+
Reducing sugars	+
Saponins	+
Tannins	+
Triterpene steroids	+
Quinones	+

Key: + = Positive; - = Negative

4.2 Pharmacological activity assessment

4.2.1 Anticonvulsant activity

4.2.1.1 Effects of methanolic leaf extracts of *T. violacea* on PTZ-induced tonic convulsion

PTZ (100 mg/kg, i.p.) elicited tonic convulsion in all the eight mice administered with the convulsant agent. The methanolic leaf extract of *T. violacea* (100 mg/kg, i.p.) did not significantly affect the onset or incidence of PTZ (100 mg/kg, i.p.)-induced tonic convulsion.

The doses of 200 (mg/kg, i.p.) and 400 (mg/kg, i.p.) of the methanolic leaf extract of *T. Violacae* significantly delayed the onset of PTZ (100 mg/kg, i.p.)-induced tonic convulsion in a dose-dependent manner. The methanolic leaf extract of the plant species (200 mg/kg, i.p.) did not affect the incidence of PTZ (100 mg/kg, i.p.)-induced tonic convulsion while 400 mg/kg (i.p.) protected 12.5% of mice against the tonic convulsion. Phenobarbitone (12 mg/kg, i.p.), diazepam (0.5mg/kg, i.p.) and muscimol (2 mg/kg, i.p.) significantly delayed the onset of PTZ (100 mg/kg, i.p.)-induced tonic convulsion and also significantly reduced the number of animals convulsing. Both phenobarbitone (12 mg/kg, i.p.) and diazepam protected 100% of mice against the tonic convulsion respectively. Muscimol (2 mg/kg, i.p.) protected 62.5% of mice against PTZ (100mg/kg, i.p.)-induced tonic convulsion while Muscimol (0.2 mg/kg, i.p.) did not significantly affect the onset or incidence of PTZ (100 mg/kg, i.p.)-induced tonic convulsion. However, combined therapy of sub-effective doses of the methanolic leaf extract of *T. violacea* (100 mg/kg, i.p.) and muscimol (0.2 mg/kg, i.p.) significantly delayed the onset of PTZ (100mg/kg, i.p.)-induced tonic convulsion but did not significantly reduce the number of animals convulsing. The combined therapy of sub-effective doses of the methanolic leaf extract of *T. violacea* (100 mg/kg, i.p.) and muscimol (0.2 mg/kg, i.p.) protected 25% of mice against the tonic convulsion. Phenytoin (30mg/kg, i.p.) or DMSO (0.25 ml, i.p.) did not significantly affect the onset or incidence of PTZ (100 mg/kg, i.p.)-induced tonic convulsion (Table 2).

Table 2. **Effect of leaf methanol extract of *Tulbaghia violacea* (TV) on pentylenetetrazole (PTZ)-induced tonic convulsion in mice**

Dose (mg /kg)							No. convulsed / No. used	Percentage Protection	Onset of tonic convulsion (min)	
PTZ	TV	Pheno barbitone	Diazepam	Phenytoin	Muscimol	DMSO		(%)	Mean ±	SEM
100	-	-	-	-	-	-	8/8		2.50	0.33
100	100	-	-	-	-	-	8/8	0	9.0	2.89
100	200	-	-	-	-	-	8/8	0	13.63[*]	2.51
100	400	-	-	-	-	-	7/8	12.5	18.38[*]	1.99
100	-	12	-	-	-	-	0/8[++]	100	0[*]	
100	-	-	0.5	-	-	-	0/8[++]	100	0[*]	
100	-	-	-	30	-	-	8/8	0	2.41	0.66
100	-	-	-	-	2	-	3/8[+]	62.5	21.0[*]	4.42
100	-	-	-	-	0.2	-	8/8	0	2.88	0.52
100	100	-	-	-	0.2	-	6/8	25	14.50[*]	4.11
100	-	-	-	-	-	0.25ml	8/8	0	2.75	0.37

[*]$p < 0.001$ compared to PTZ (100 mg/kg, i.p.) control, ANOVA (n=8)

[+]$p < 0.05$, [++]$p < 0.001$ compared to PTZ (100 mg/kg. i.p.) control, Chi-squared test (n=8)

DMSO: Dimethylsulfoxide

4.2.1.2   Effect of leaf methanol extract of *T. violacea* on bicuculline-induced tonic convulsion

Bicuculline (30 mg/kg, i.p.) produced tonic convulsion in all eight mice used for theexperiment. Methanolic leaf extracts of *T. violacea* (100-400 mg/kg, i.p.) significantly and dose-dependently delayed the onset of tonic convulsion produced by bicuculline (30 mg/kg, i.p.) but did not affect the incidence of the convulsion. Phenobarbitone (12 mg/kg, i.p.), diazepam (0.5 mg/kg, i.p.) or muscimol (2 mg/kg, i.p.) significantly delayed the onset of bicuculline (30 mg/kg, i.p.)-induced tonic convulsion and also significantly reduced the number of animals convulsing. Phenobarbitone (12 mg/kg, i.p.), diazepam (0.5 mg/kg, i.p.) or muscimol (2 mg/kg, i.p.)

protected 87.5% of mice against bicuculline (30 mg/kg, i.p.)-induced tonic convulsion. Phenytoin (30 mg/kg, i.p.) and DMSO (0, 25 ml, i.p.) did not significantly affect the onset or incidence of bicuculline (30 mg/kg, i.p.)-induced tonic convulsion (Table 3).

Table 3:   Effect of leaf methanol extract of *Tulbaghia violacea* (TV) on bicuculline (BIC)-induced tonic convulsion in mice

Dose (mg /kg)							No. convulsed/ No. used	Percentage Protection	Onset of tonic convulsion (min)		
BIC	TV	Pheno barbitone	Diazepam	Phenytoin	Muscimol	DMSO		(%)	Mean	±	SEM
30	-	-	-	-	-	-	8/8		2.38		0.42
30	100	-	-	-	-	-	8/8	0	5.63[*]		0.38
30	200	-	-	-	-	-	8/8	0	5.88[*]		0.30
30	400	-	-	-	-	-	8/8	0	10.28[**]		1.37
30	-	12	-	-	-	-	1/8[+]	87.5	15.37[**]		0.89
30	-	-	0.5	-	-	-	1/8[+]	87.5	19.25[**]		1.12
30	-	-	-	30	-	-	8/8	0	2.44		0.51
30	-	-	-	-	2	-	1/8[+]	87.5	20.02[**]		1.27
30	-	-	-	-	-	0.25ml	8/8	0	2.34		0.60

[*]P<0.01, [**]P<0.001 compared to bicuculline (30 mg/kg, i.p.) control, ANOVA (n=8)

[+]p<0.005 compared to bicuculline (30 mg/kg, i.p.) control, Chi-squared test (n=8)

DMSO: Dimethylsulfoxide

4.2.1.3   Effect of leaf methanol extract of *T. violacea* on PCN-inducedtonic convulsion

PCN (20 mg/kg, i.p.) elicited tonic convulsion in 100% of mice used for the experiment. The methanolic leaf extract of *T. violacea* (100, 200 and 400 mg/kg, i.p.) significantly and dose-dependently delayed the onset of the PCN (20 mg/kg, i.p.)-elicited tonic convulsion but did not significantly alter the incidence of the convulsion. Phenobarbitone (12 mg/kg, i.p.), diazepam (0.5 mg/kg, i.p.) and muscimol (2 mg/kg, i.p.) significantly delayed the onset of PCN (20 mg/kg, i.p.)-induced tonic convulsion and also significantly reduced the number of animals convulsing.

Both diazepam (0.5 mg/kg, i.p.) and muscimol (2 mg/kg, i.p.) protected 87.5% of the animals against PCN (20 mg/kg, i.p.)-elicited tonic convulsion. Phenobarbitone (12 mg/kg, i.p.) protected 75% of mice against tonic convulsion while Phenytoin (30 mg/kg, i.p.) and DMSO (0.25 ml, i.p.) did not significantly affect the onset or incidence of PCN (20 mg/kg, i.p.)-induced tonic convulsion (Table 4).

Table 4: **Effect of leaf methanol extract of *Tulbaghia violacea* (TV) on picrotoxin (PCN)-induced tonic convulsion in mice**

Dose (mg /kg)							No. convulsed/ No. used	Percentage Protection	Onset of tonic convulsion (min)		
PCN	TV	Pheno barbitone	Diazepam	Phenytoin	Muscimol	DMSO		(%)	Mean	±	SEM
20	-	-	-	-	-	-	8/8		8.50		0.42
20	100	-	-	-	-	-	8/8	0	11.38[*]		1.07
20	200	-	-	-	-	-	8/8	0	13.00[*]		0.73
20	400	-	-	-	-	-	8/8	0	18.00[**]		0.91
20	-	12	-	-	-	-	2/8[+]	75	21.33[**]		1.02
20	-	-	0.5	-	-	-	1/8[++]	87.5	19.86[**]		0.95
20	-	-	-	30	-	-	8/8	0	8.64		0.82
20	-	-	-	-	2	-	1/8[++]	87.5	22.75[**]		1.16
20	-	-	-	-	-	0.25ml	8/8	0	8.73		0.59

[*]p<0.01, [**]p<0.001 compared to picrotoxin (20mg/kg, i.p.) control, ANOVA (n=8).

[+]p<0.01, [++]p<0.005, compared to picrotoxin (20mg/kg, i.p.) control, Chi-squared test (n=8)

DMSO: Dimethylsulfoxide.

4.2.1.4   Effect of leaf methanol extract of *T. violacea* on STN-induced tonic convulsion

Strychnine (2 mg/kg, i.p.) produced tonic convulsion in all the eight mice used for the experiment. The methanolic leaf extract of *T. violacea* (200 and 400 mg/kg, i.p.) significantly delayed the onset of STN (2 mg/kg, i.p.)-induced tonic convulsion but did not significantly affect the number of mice convulsing. The methanolic leaf extract of *T. violacea* (100 mg/kg, i.p.) did not significantly affect the onset or incidence of STN (2 mg/kg, i.p.)-induced tonic convulsion.

Phenobarbitone (12 mg/kg, i.p.) significantly delayed the onset of STN (2 mg/kg, i.p.)-induced tonic convulsion and also significantly protected 62.5% of the mice against STN (2 mg/kg, i.p.)-induced tonic convulsion.

Diazepam (0.5 mg/kg, i.p.), phenytoin (30 mg/kg, i.p.) or DMSO (0.25 ml, i.p.) did not significantly delay the onset of STN (2 mg/kg, i.p.)-induced tonic convulsion and did not also significantly affect the number of mice convulsing (Table 5).

Table 5: Effect of leaf methanol extract of *T. violacea* on strychnine (STN)-induced tonic convulsion in mice

STN	TV	Pheno-barbitone	Diazepam	Phenytoin	DMSO	No. convulsed/ No. used	Percentage Protection (%)	Mean ±	SEM
2	-	-	-	-	-	8/8		2.63	0.26
2	100	-	-	-	-	8/8	0	2.75	0.16
2	200	-	-	-	-	8/8	0	4.13[*]	0.23
2	400	-	-	-	-	8/8	0	3.88[*]	0.13
2	-	12	-	-	-	3/8[+]	75	16.72[**]	1.50
2	-	-	0.5	-	-	8/8	0	2.6	0.85
2	-	-	-	30	-	8/8	0	2.57	0.34
2	-	-	-	-	0.25ml	8/8	0	8.73	0.59

[*]p<0.05, [**]p<0.001 compared to strychnine (2 mg/kg, i.p.) control, ANOVA (n=8).

[+]p<0.05compared to strychnine (2 mg/kg, i.p.) control, Chi-squared test (n=8).

DMSO: Dimethylsulfoxide.

4.2.1.5 Effect of leaf methanol extract of *T. violacea* on NMDLA-induced seizures

The dose of 400 mg/kg (i.p.) of N-methyl-DL-aspartic acid (NMDLA) produced tonic convulsion in 100% of mice used. The methanolic leaf extract of *T.violacea* (100, 200 and 400 mg/kg, i.p.) significantly and dose-dependently delayed the onset of NMDLA (400 mg/kg, i.p.)-induced tonic convulsion but did not significantly affect the incidence of the tonic convulsion. LY233053 (5 mg/kg, i.p.) significantly delayed the onset of NMDLA (400 mg/kg, i.p.)-induced

tonic convulsion and significantly reduced the number of animals convulsing. The dose of 5 mg/kg (i.p.) of LY233053 protected 87.5% of mice against NMDLA (400 mg/kg, i.p.) induced tonic convulsion. Phenobarbitone (12 mg/kg, i.p.), diazepam (0.5 mg/kg, i.p.), phenytoin (30 mg/kg, i.p.) and DMSO (0.25 ml, i.p.) did not significantly affect the onset of NMDLA (400 mg/kg, i.p.)-induced tonic convulsion or the incidence of the tonic convulsion (Table 6).

Table 6: Effect of leaf methanol extract of *Tulbaghia violacea* (TV) On N-methyl-DL aspartic acid (NMDLA)-induced tonic convulsion in mice

Dose (mg /kg)							No. convulsed/ No. used	Percentage Protection	Onset of tonic convulsion (min)	
NMDLA	TV	Pheno barbitone	Diazepam	Phenyt oin	LY 233053	DMSO		(%)	Mean	± SEM
400	-	-	-	-	-	-	8/8		4.38	0.32
400	100	-	-	-	-	-	8/8	0	7.75*	0.37
400	200	-	-	-	-	-	8/8	0	9.75**	0.70
400	400	-	-	-	-	-	8/8	0	14.13***	0.41
400	-	12	-	-	-	-	8/8	0	4.22	0.75
400	-	-	0.5	-	-	-	8/8	0	4.51	0.37
400	-	-	-	30	-	-	8/8	0	4.57	0.42
400	-	-	-	-	5	-	1/8+	87.5	20.08***	0.94
400	-	-	-	-	-	0.25ml	8/8	0	4.33	0.29

*$P < 0.01$, **$p < 0.005$, ***$p < 0.001$ compared to NMDLA (400mg/kg, i.p.) control, ANOVA (n=8)

+$p < 0.005$ compared to NMDLA (400mg/kg, i.p.) control, Chi-squared test (n=8)

DMSO: Dimethylsulfoxide

4.3   Acute Toxicity

The methanolic leaf extract of *T. violacea* **(**100–4000 mg/kg, p.o.) produced no signs of acute toxicity or death in mice after 5 days of observation. The highest dose tested, 4000 mg/kg (p.o.), is taken as the no-adverse-effect-level (NOAEL) since no signs of acute toxicity were observed at this dose level. The LD50 obtained for *T. violacea* following oral administration may probably be greater than 4000 mg/kg.

4.4   HPLC Analysis.

The HPLC fingerprint of the leaf methanol extract of *Tulbaghia violacea* showed distinct peaks

at the following retention times, 2.911, 3.269, 4.010, 7.597, and 15.122 minutes (Figure 8).
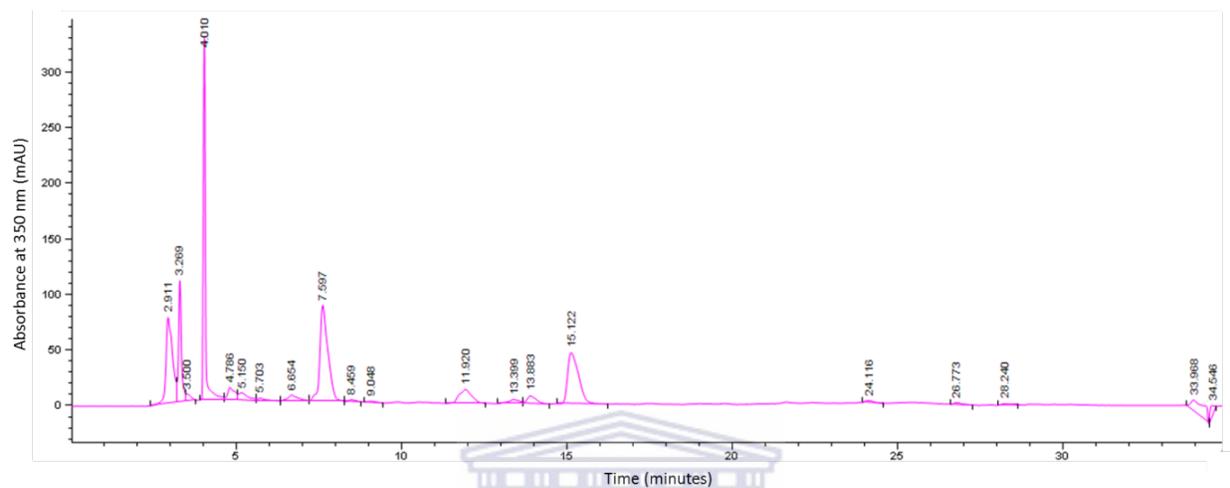
Figure 8: HPLC fingerprint of methanol extract of *Tulbaghia violacea*
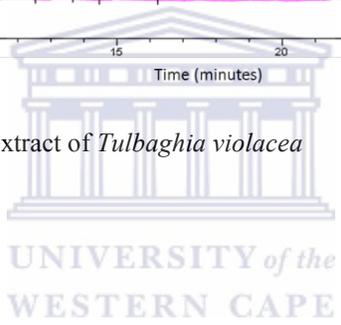
Chapter 5

Discussion

The study evaluated the anti-convulsant activity of the leaf methanol extract of *Tulbaghia violacea* with a view to scientifically scrutinize the effectiveness of the plant species in the treatment of epilepsy as claimed by traditional medicine practitioners. The study also investigated possible mechanism(s) of the plant species' anticonvulsant activity by using convulsant agents known to affect certain neurotransmitters in the central nervous system (CNS) implicated in epilepsy. The present study shows that PTZ (100 mg/kg, i.p.), bicuculline (30 mg/kg, i.p.), PCN (12 mg/kg, i.p.), STN or NMDLA (400 mg/kg, i.p.) produced tonic convulsion in all the mice used. The methanolic leaf extract of *T. violacea* (200-400 mg/kg, i.p.) was found to attenuate the tonic convulsions produced by PTZ and STN. Similarly, the 100-400 mg/kg (i.p.) dose also attenuated tonic convulsions produced by bicuculline, PCN and NMDLA-induced tonic convulsion. Phenobarbitone (12 mg/kg, i.p.) and diazepam (0.5 mg/kg, i.p.) attenuated the tonic convulsions produced by PTZ, bicuculline and PCN but did not affect NMDLA-induced tonic convulsion. Muscimol (2 mg/kg, i.p.) attenuated the tonic convulsions produced by PTZ, bicuculline and PCN. Phenytoin (30 mg/kg, i.p.) did not affect the tonic convulsions produced by PTZ, bicuculline, PCN, STN or NMDLA whereas LY23350 (5 mg/kg, i.p.) attenuated NMDLA-induced tonic convulsion and Phenobarbitone (12 mg/kg, i.p.) also attenuated the tonic convulsions produced by STN.

An imbalance between gamma amino butyric acid (GABA), a major inhibitory neurotransmitter and glutamic acid, an excitatory neurotransmitter in the brain underpins epilepsy. The inhibition of GABA mediated inhibition at $GABA_A$ receptors and the enhancement of glutamic acid

neurotransmission at NMDA receptors in the brain may cause convulsion (Olsen 1981, Waller et al., 2005, Rang etal., 2015). PTZ is thought to act by blocking $GABA_A$ receptors thus inhibiting GABA neurotransmitter (De Sarro et al., 1999, Rang et al., 2015). According to Waller et al., 2005 and Rang et al., 2015, the standard AEDs, phenobarbitone and diazepam are known to act by enhancing GABA neurotransmission in the brain by respectively increasing the duration and frequency of the opening of $GABA_A$ receptor-linked chloride channels in the $GABA_A$ receptor-chloride ionophore complex to facilitate chloride ion conductance into the brain. It is not surprising therefore that phenobarbitone and diazepam attenuated PTZ-induced tonic convulsion in the present study. Phenytoin, another standard AED, acts by blocking the entry of sodium ions into brain cells and thus, inhibiting the generation of repetitive action potential (Waller et al., 2005 and Rang et al., 2015) and therefore, did not affect PTZ-inducedtonic convulsion in this study. According to Lança (1998) and Rang et al. (2015), muscimol, a selective and powerful $GABA_A$ receptor agonist, acts by interacting with $GABA_A$ receptors in the brain to mimic the effects of GABA. Accordingly, muscimol significantly attenuated PTZ-induced tonic convulsion in mice.

In this study, the methanolic leaf extracts of *T. violacea* appeared to attenuate PTZ-induced tonic convulsion suggesting that *T. violacea* could be affecting with the anti-convulsant activity of GABA. The combined therapy of sub-effective doses of *T. violacea* and muscimol, significantly attenuated PTZ-induced tonic convulsion, suggesting that GABA mechanism may be involved in the anti-convulsant activity of *T. violacea*. According to Rang et al. (2015), bicuculline, a $GABA_A$ receptor antagonist, produces its convulsant activity by blocking $GABA_A$ receptors thus inhibiting GABA neurotransmission in the brain. In this study, bicuculline produced tonic convulsion which was attenuated by phenobarbitone and diazepam, both of which are known to

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enhance GABA neurotransmission. Furthermore, muscimol, a specific GABA$_A$ receptor agonist, known to mimic the effect of GABA at GABA$_A$ receptors in the brain was shown to attenuate bicuculline-induced tonic convulsion. The methanolic leaf extract of *T. violacea* also attenuated bicuculline-induced tonic convulsion while Phenytoin (known to exert its anti-epileptic effects by blocking sodium ion entry into the brain), did not attenuate bicuculline-induced tonic convulsion. These findings further support the suggestion that GABA mechanism may be involved in the anti-convulsant activity of *Tulbaghia violacea*.

PCN is known to produce convulsion by blocking GABA$_A$ receptor-linked chloride ion channels to prevent the entry of chloride ions into the brain to inhibit GABA-mediated inhibition. The present study shows that PCN-induced tonic convulsion in mice was attenuated by phenobarbitone and diazepam, both of which are known to enhance GABA neurotransmission in the brain. Muscimol, a specific GABA$_A$ receptor agonist, known to mimic the effect of GABA at GABA$_A$ receptors, also attenuated PCN-induced tonic convulsion. Phenytoin, on the other hand, did not affect PCN-induced tonic convulsion. This drug is known to produce its anti-convulsant effects by blocking sodium ion entry into the brain (Rang et al., 2015). The methanolic leaf extract of *T. violacea* also attenuated PCN-induced tonic convulsion further indicating that GABA mechanism may be involved in the anti-convulsant activity of *T. violacea*.

Rang et al. (2015), suggested that STN produces convulsion by blocking receptors for glycine in the brain. In this study, phenobarbitone and the methanolic leaf extract of *T. violacea* attenuated STN-induced tonic convulsion, suggesting that glycine mechanism may also be involved in the anti-convulsant activity of *T. violacea*. Rang etal. (2015) have reported that phenobarbitone may produce its anticonvulsant activity by different mechanisms. It is possible that phenobarbitone

may be producing its anti-convulsant activity by enhancing glycinergic neurotransmission in the brain. Benzodiazepines have been shown not to alter STN-induced convulsion in experimental animals (Rang et al., 2015). However, of moderate to high doses of phenobarbitone (i.v), diazepam (i.v) and phenytoin (i.v) have been used historically to prevent strychnine convulsion in strychnine poisoning in humans (Lambert et al., 1981, Boyd et al., 1983).

According to Chapman and Meldrum (1993), Besancon et al. (2008) and Rang et al. (2012), N-Methyl-DL-aspartic acid (NMDLA) produces its anti-convulsant effects by specifically stimulating NMDA receptors to mimic the action of glutamate, the excitatory neurotransmitter in the brain. The present study shows that phenobarbitone and diazepam (both of which are known to enhance GABA neurotransmission in the brain) did not affect NMDLA-induced tonic convulsion in mice whereas LY233053 attenuated NMDLA-induced tonic convulsion. On the other hand, Phenytoin known to exert its anticonvulsant effect by blocking sodium ion entry into the brain also did not alter NMDLA-induced tonic convulsion.According to Madden et al. (1992) and Borowicz et al. (1996), LY233053, is a competitive NMDA receptor antagonist, which acts by blocking the effects of glutamic acid at NMDA receptors. In this study, the methanolic leaf extract of *T. violacea* was found to attenuate NMDLA-induced tonic convulsion. These findings suggest the possible involvement of glutamic acid mechanism(s) in the anticonvulsant activity of *T. violacea*.

In this study, the phytochemical qualitative analysis of the dried leaf extract of *T. violacea* revealed the presence of alkaloids, saponins, reducing sugars, flavonoids cardiac glycosides, triterpene steroids, quinones and tannins. Alkaloids, flavonoids, saponins triterpene steroids have been shown in different studies to possess anti-convulsant activities (Chauhan et al. 1988,

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Mimaki et al., 1997, Van Heerden et al., 2002, Muazu and Kaita 2008, Ibrahim et al., 2008, Singh et al., 2012). It is therefore possible that these secondary metabolites may be contributing to the anti-convulsant activity of *T. violacea*. The results obtained from the acute toxicity study carried out showed that following oral administration of the methanolic leaf extract to mice, the LD50 may be greater than 4000 mg/kg which suggests that *T. violacea* is safe and non-toxic to mice. The HPLC finger print obtained for *T. violacea* revealed the presence of characteristic peaks at 350 nm which may be used to identify the exact species of *T. violacea*.

5.1 Conclusion

The results obtained from this study indicate that the methanolic leaf extract of *T. violacea* has anti-convulsant activity which may involve GABA, glutamic acid and glycine mechanisms. Secondary metabolites such as saponins, triterpene steroids, alkaloids and flavonoids found in the leaves of the plant species may also in part be contributing to the observed anti-convulsant activity. The relatively high LD_{50} value obtained for the plant species following oral administration shows that it is safe in mice. These findings justify the use of *T. violacea* by traditional practitioners in the management and treatment of epilepsy. However, further studies are needed to fully elucidate the mechanism(s) of its anti-convulsant activity as well as establish its toxicity and safety profile.

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