SOME PHARMACOLOGICAL STUDIES OF THE METHANOLIC ROOT BARK EXTRACT OF *SECURINEGA VIROSA* (ROXB. EX WILLD) BAILL. ON LABORATORY ANIMALS

By

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DECLARATION

I declare that the work in the dissertation entitled "**Some Pharmacological studies of the methanolic root bark extract of *Securinega virosa* (Roxb. Ex Willd) Baill. on laboratory animals**" has been performed by me in the Department of Pharmacology and Therapeutics under the supervision of Prof. J. A. Anuka, Prof. I. Abdu-Aguye and Prof. I.M. Hussaini.

The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for the award of another degree or diploma at any University.

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CERTIFICATION

This dissertation titled "SOME PHARMACOLOGICAL STUDIES OF THE METHANOLIC ROOT BARK EXTRACT OF *SECURINEGA VIROSA* (ROXB. EX WILLD) BAILL. ON LABORATORY ANIMALS" meets the regulations governing the award of the degree of Doctor of Philosophy of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated

To the loving memories of Hajia Habiba Audu Gilaso and Alhaji Mudi Ahmadu

and

To my parents, Late Alhaji Garba Magaji and Hajia Fatima Mudi

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ABSTRACT

Securinega virosa is a commonly used medicinal plant in West Africa in the management of cancer, epilepsy and as a sedative in children. Previously, the crude methanolic root bark extract was found to possess anticonvulsant and sedative properties. In this work, the crude methanolic root bark extract was partitioned into chloroform, ethyl acetate and n-butanol fractions, and the fractions were evaluated for CNS depressant and anticancer activities. Purification of the n-butanol fraction led to the isolation of a compound which was subjected to chemical tests, melting point determination, nuclear magnetic resonance, mass spectroscopy and X-ray crystallography. The n-butanol (75, 150 and 300 mg/kg) and residual aqueous (125, 250 and 500 mg/kg) fractions were evaluated for anticonvulsant activities using maximal electroshock test in chicks, pentylenetetrazole, 4-Aminopyridine, strychnine and picrotoxin-induced seizures in mice, and kindling model in rats. The n-butanol and residual aqueous fractions were also evaluated for sedative and anxiolytic potentials using hole board, diazepam-induced sleeping time, elevated plus maze, staircase and open field tests in mice. The effects of these fractions on motor coordination were assessed using beam walking assay in mice. The effect of the isolated compound on sleep and motor coordination were also evaluated. The crude methanolic extract and its fractions were evaluated for anticancer activity using kill study and phosphorylation assay on glioblastoma multiforme U-1242 MG cell line. Based on the results of the analysis of the isolated compound, it was concluded to be bergenin, an isocoumarin. The n-butanol and residual aqueous fractions did not protect chicks against maximal electroshock seizure. Against seizures induced by 4- Aminopyridine, strychnine and picrotoxin, the two fractions did not produce significant activities. Conversely, the two fractions afforded 66.67% protection against pentylenetetrazole-induced seizure in mice. Both

fractions did not significantly decrease the mean seizure duration and the behavioural seizure scores in the kindled rats at the dose tested. In the hole board test, both fractions significantly ($P < 0.01$) reduced the mean number of head dips. Similarly, both fractions significantly ($P < 0.05$- $P < 0.01$) decreased the number of rearings and increased the total number of upward stairs climbed in the staircase test. In the elevated plus maze test, both fractions did not significantly increase the total number of open arm entries and total time spent in the open arms. Similarly, they did not increase the total central square crossed in the open field test. In the beam walking assay, they did not increase the mean number of foot slips. Bergenin significantly ($P < 0.05$) reduce the mean onset of sleep without affecting the total duration of sleep. However, it did not increase the mean number of foot slips. The crude methanolic root bark extract and its fractions significantly ($P < 0.001$) and concentration-dependently decreased mean viability of glioblastoma multiforme cells. The methanolic root bark extract and its residual aqueous fraction also blocked the phosphorylation of the epidermal growth factor receptor at tyrosine 1045 and 1068 sites. They also blocked the phosphorylation of platelet derived growth factor receptor. Similarly, phorbol myristate acetate and platelet derived growth factor-induced transactivations of epidermal growth factor receptor at tyrosine 1068 were also blocked. Mitogen activated protein kinase phosphorylation was also blocked by the crude methanolic root bark extract. However, the extract and its fractions did not affect the phosphorylation of protein kinase B. The findings of this study further lend credence to the ethnomedical use of the root of the *Securinega virosa* in the management of epilepsy, cancer and as a sedative. Bergenin, isolated from the root of the plant for the first time could partly be responsible for the sedative activity of the root of *Securinega virosa*.

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ABBREVIATIONS AND SYMBOLS

The following abbreviations and symbols were used in the course of writing this project report:

5HT	5-hydroxytryptamine
AEDS	Anti-epileptic drugs
AIDS	Acquired Immune deficiency syndrome
Akt	Protein kinase B
AMPA	2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl) propanoic acid
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BGN	Bergenin
BSS	Behavioural Seizure score
Ca^{2+}	Calcium ion
CCF	Chloroform fraction
COSY	Correlation spectroscopy
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DZP	Diazepam
EAF	Ethyl acetate fraction
ECL	Electrochemiluminescence
EDTA	Ethylene diamine tetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ErbB	Epidermal growth factor receptor family
GABA	Gamma aminobutyric acid
$GABA_A$	Gamma aminobutyric acid subtype A
GAD	Generalised anxiety disorder
GAT-1	Gamma amino transferase suptype 1
GBM	Glioblatoma multiforme
HER1	Epidermal growth factor receptor

HMBC	Heteronuclear multiple-bond correlation spectroscopy
HMQC	Heteronuclear single-quantum correlation spectroscopy
HVA	Homovanillic acid
IDH 1	Isocitrate dehydrogenase 1
K^+	Potassium ion
LD_{50}	Median lethal dose
LTQ	Linear Trap Quadropole
MAPK	Mitogen activated protein kinase
MCPP	*meta*-Chlorophenylpiperazine
MES	Maximal Electroshock
MRBE	Methanolic root bark extract
MS^2	Tanden Mass spectroscopy
MTIC	3-methyl-(triazen-1-yl) imidazole-4-carboxamide
Na^+	Sodium ion
NBF	n-butanol fraction
NF□b	Nuclear factor kappa-b
NMDA	N-methyl- D-aspartate
NMR	Nuclear Magnetic resonance
NOESY	Nuclear Overhauser effect spectroscopy
NR2B	N-methyl- D-aspartate subtype 2B
OCD	Obsessive compulsive disorder
PBS	Phosphate buffered saline
PD	Panic disorder
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
PF1	Pooled column fraction 1
PF2	Pooled column fraction 2
PFA	Pooled column fraction A
PFB	Pooled column fraction B
PFC	Pooled column fraction C

PHT	Phenytoin
PLWE	People Living with Epilepsy
PMA	Phorbol myristate acetate
PTEN	Phosphatase and tensin homolog
PTZ	Pentylenetetrazole
RAF	Residual Aqueous fraction
SEM	Standard error of the mean
SMEI	severe myoclonic epilepsy in infancy
SV2A	Synaptic vesicle protein 2A
TAM	Traditional African Medicine
TCA	Tricarboxylic acid
TLC	Thin layer chromatography
TP53	Tumor suppressor protein 53
TSQ	Triple stage quadrupole
VPA	Sodium valproate
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND INFORMATION

The conventional health care systems devised for the developing countries are economically unobtainable by the vast majority of the people living in these countries. A number of these countries are exploring the possibility of developing their own traditional health care system in which plants play a central role. World Health Organisation (WHO) estimated that about three-quarters of the world population living in developing countries relies upon traditional remedies (mainly herbs) for the health care of its people (Gilani and Atta-ur-Rahman, 2005). WHO attributed this high patronage of traditional medicine to its accessibility and affordability, and encourages the inclusion of herbal medicines of proven safety and efficacy in the healthcare programmes of developing countries because of the great potential they hold in combating various diseases (Amos *et al*., 2001).

Traditional medicine refers to health practices, approaches, knowledge and beliefs incorporating plant, animal and mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly, or in combination to treat, diagnose and prevent illnesses or maintain well-being (WHO, 2003). Traditional medicine, despite its limitations, is addressing the health care needs of millions of people worldwide. Countries in Africa, Asia and Latin America use traditional medicine (TM) to help meet some of their primary health care needs.

In Africa, up to 80% of the population uses traditional medicine for primary health care. In China, traditional herbal preparations account for 30%-50% of the total medicine consumption whilst in Ghana, Mali, Nigeria and Zambia, the first line of treatment for 60% of

children with high fever resulting from malaria is the use of herbal medicine at home (WHO, 2003).

African Traditional Medicine (ATM) is a socio-economic and socio-cultural heritage, servicing over 80% of the populations in Africa. Although, it has come a long way, not much significant progress on its development and utilization had taken place. This may not be unconnected to colonization, absence of Government political will, lack of the citizenry patriotism and care free attitude of African medical scientists (Eluojoba, 2005). Medicinal plants form an important aspect of the daily lives of many people, and constitute a part of African cultural heritage (Ramalivhan *et al*., 2010). They contain chemical constituents that have great potential for use in phytomedicine and as "lead" compounds for drug production by pharmaceutical companies (Sofowora, 1993; Coopoosamy and Magwa, 2006).

It has been estimated that out of the over 30,000 human diseases or disorders, only one third can be managed, to some extent, symptomatically with the existing drugs, and at a great socio-economic cost. These diseases and disorders include the infectious diseases like Acquired Immune Deficiency Syndrome (AIDS), tuberculosis and chronic disorders like cancers, auto-immune disorders and neurological disorders (including epilepsy) (Wangchuk, 2008).

Epilepsy is the second most common chronic neurological condition seen by neurologists (Sridharan, 2002). WHO estimates that eight people per 1000 worldwide are living with this disease. The prevalence of epilepsy in developing countries is usually higher than in developed countries (Mac *et al*., 2007). The estimated prevalence of epilepsy in Nigeria is 8 to 13 per thousand people (Azubuike and Nkanginieme, 1996). About 75-80% of people living with epilepsy (PLWE) may be provided with adequate seizure control with the help of conventional

antiepileptic drugs. The therapeutic failure in 20-25% of patients with refractory epilepsies has stimulated intensive research on novel antiepileptic drugs (Czapin´ski *et al*., 2005). The available antiepileptic drugs (AEDs) do not affect epileptogenesis and are associated with serious side effects, including teratogenicity, chronic toxicity and adverse effects on cognition and behaviour (Samren *et al*., 1997; Arroyo and Morena, 2001). There is a clear medical need for new AEDs with novel mechanisms of action to serve as alternate or adjunct therapy for the treatment of resistant or refractory epilepsy (Wickenden, 2002). Long term antiepileptic drug therapy is often necessary for the majority of patients diagnosed with epilepsy. For those patients who do not respond to single-agent therapy, treatment with multiple antiepileptic agents is required. Antiepileptic drugs are well known for their drug interactions; not only between each other, but also with other pharmacologic agents. These observations have led to a shift in focus to the use of herbal remedies in the management of epilepsy (Rout and Kar, 2010).

Many people living in developing countries are relying on herbal medicine for the management of epilepsy (Yaro *et al*., 2007). Medicinal plants represent a great deal of untapped reservoir of drugs and the structural diversity of their component molecules makes a valuable source of lead compounds in drug development process (Farnsworth, 1998). They can be an important source of natural and safer drugs for the treatment of epilepsy (Meldrum, 1997). Medicinal plants employed in the pharmacotherapy of epilepsy in traditional medicine have been shown to possess promising anticonvulsant activities in animal models and can be invaluable sources of new antiepileptic compounds (Arumugam *et al*., 2009).

Cancer is a leading cause of death worldwide and accounted for 7.6 million deaths (around 13% of all deaths) in 2008 (Globocan, 2008). Deaths from cancer worldwide are projected to

continue to rise to over 11 million in 2030 (Cancer, 2010). Although, the occurrence of malignant tumours of the brain is rare, accounting for approximately 2% of all cancers in adults (Mc Kinney, 2004), glioblastoma multiforme (GBM), the most common malignant primary brain tumour in adults has a dismal prognosis, with a median survival of less than 15 months (Norden and Wen, 2006).

Glioblastoma multiforme is the most aggressive of the gliomas, a collection of tumours arising from glia or their precursors within the central nervous system. Clinically, gliomas are divided into four grades; unfortunately, the most aggressive of these, grade 4 or GBM is also the most common in humans. Because most patients with GBMs die of their disease in less than a year and essentially none has long-term survival, these tumours have drawn significant attention. However, they have evaded increasingly clever and intricate attempts at therapy over the last half century (Holland, 2000). The conventional standard of care for patients with this cancer involves surgical tumour resection followed by radiation with or without chemotherapy. Most of the chemotherapeutics applied to the treatment of these tumours have been traditional cytotoxic drugs that target cellular DNA or the cytoskeleton. Unfortunately, the use of such agents has not resulted in any significant improvement in GBM response rates, and the prognosis for GBM patients has remained dismal (Rao *et al*., 2003).

The plant kingdom has been found to be a rich source of bioactive compounds which can serve as lead compounds in the production of potent agents that can be deployed in the fight against these diseases. Six out of the top 20 modern prescription drugs dispensed are plant-derived products. It is estimated that about 39% of all 520 new approved drugs in 1983-1994 were natural products and out of that 74% were discovered as a result of bio-prospecting from plants used in traditional medicines (Wangchuk, 2008). These include quinine (antimalarial agent)

from Cinchona bark, reserpine (tranquilizer) from *Rauwolfia serpentina*, vincristine and vinblastine alkaloids (antileukaemic agents) from *Catharantus roseus*, digoxin and digitoxin (cardiotonic activity) from *Digitalis purpurea*, zingiberone (antihelmintic) from *Zingiber officinalis*, bourvadine from *Bourvadin ternifolia*, sinocaculine from *Corculus tribolus* and taxol (anticancer agent) from *Taxus brevifolia* (Hardman, 1991).

The long historical use of medicinal plants in many traditional medical practices, including experience passed from generation to generation has demonstrated the safety and efficacy of traditional medicine. However, scientific research is needed to provide evidences of their safety and efficacy (WHO, 2000). One of such medicinal plants which have enjoyed wide patronage amongst traditional practitioners but with scanty scientific basis for its ethnomedicinal claims is *Securinega virosa.* It is one of the great African medicinal plants described as a true "cure all", of which all parts are used as remedies (in epilepsy, cancers, schistosomiasis, stomach ache, mental illnesses etc), particularly the root (Neuwinger, 1996).

1.2 JUSTIFICATION

WHO estimated that 80% of the people of the world living in developing countries rely on medicinal plants for primary health care needs (Farnsworth, 1998). The high cost of acquiring synthetic drugs, their inadequate supplies, the side effects associated with their uses, and the belief that plants hold cure to many disease conditions (including CNS disorders and cancers) have led to a reawakening of interest in the utilization of medicinal plants in recent years.

The validation of the folkloric claims of these medicinal plants will provide scientific basis for the deployment of the beneficial ones as phytomedicine in the primary health care of

developing countries and isolation of potential bioactive constituents which could serve as novel "lead compounds" in drug development process.

Securinega virosa (Euphorbiaceae) is one of the most commonly used medicinal plants in Africa in the management of central nervous system disorders and cancers. The root of the plant is the most commonly used either alone or in combination with other plants. Previous studies have shown that the methanolic root bark extract of the plant possesses significant anticonvulsant and sedative activities (Magaji *et al*., 2007a, c; Magaji *et al*., 2008b). This study is, therefore, directed at the investigation of fractions obtained from the methanolic root bark extract of *S. virosa* for neuropharmacological and anticancer activities.

1.3 AIM AND OBJECTIVES

1.3.1 Aim of the Study

The aim of the study is to investigate the neuropharmacological and anticancer activities of the methanolic extract and fractions obtained from the root bark of *Securinega virosa.*

1.3.2 Objectives

The objectives of the study are to;

i. Establish the sedative, anxiolytic and anticonvulsant potentials of the fractions of the methanolic root bark extract of *Securinega virosa*
ii. Establish the anticancer properties of the crude methanolic extract and fractions of root bark of *Securinega virosa*
iii. Isolate and characterize the active pronciples in the fractions of methanolic root bark extract of *Securinega virosa*

1.4 RESEARCH HYPOTHESIS

The fractions obtained from methanolic root bark extract of *Securinega virosa* contain pharmacologically active principles which account for the beneficial effect of the plant in the management of insomnia, epilepsy and cancer in traditional medicine.

CHAPTER 2

LITERATURE REVIEW

2.1 OVERVIEW OF DRUGS USED AS SEDATIVE/HYPNOTICS AND IN THE MANAGEMENT OF EPILEPSY

Drugs acting in the central nervous system (CNS) were among the first to be discovered by the primitive humans and are still the most widely used group of pharmacological agents (Nicoll, 2009). Most drugs that affect the CNS act by altering some step in the neurotransmission process. They may act presynaptically by influencing the production, storage, release, or termination of action of neurotransmitters. Other agents may activate or block postsynaptic receptors (Howland *et al*., 2006).

The mechanisms by which various drugs act in the CNS have not always been clearly understood. Since the causes of many of the conditions for which these drugs are used (schizophrenia, anxiety, etc) are themselves poorly understood. Nearly all drugs (with a very few exception such as alcohol and general anaesthetic which may have nonspecific actions on membranes) with CNS effects act on specific receptors that modulate synaptic transmission. However, even the non-receptor mediated action results in demonstrable changes in neurotransmission (Nicoll, 2009).

Agents that alter the functions of CNS achieve this action by non-selective or selective mechanisms. The non-selective agents include general anaesthetics, hypnotics, sedatives and simple aliphatic alcohols. They affect the neural axis in such a way that the highest centers involved in intellectual disintegration are dulled first. This is followed by loss of coordination at the motor and sensory levels, with the vital centers (controlling respiration and cardiovascular function) being most resistant. Selective agents produce effects upon one or

more CNS function via identifiable molecular mechanisms which are unique to target cells bearing receptors for such agents. The selective agents include; the anticonvulsants, anti-depressants antipsychotics antimanics and analgesics (Bloom, 1996). The following overview is on the anxiolytic/hypnotic and anticonvulsant agents.

2.1.1 Drugs used in the Management of Insomnia

Insomnia, the most prevalent sleep disorder is often associated with significant medical, psychological, as well as social disturbances (Attele *et al*., 2000). It is a symptom of difficulty in initiating and maintaining sleep or experiencing non-refreshing sleep and is associated with day time sequlae (Roth and Roehrs, 2003). The symptom complex can be an independent disorder (primary insomnia) or the result of another condition (secondary or co-morbid insomnia). Primary insomnia is a common form of sleep difficulty that impairs diurnal functioning, reduces quality of life and increases health care utilization/costs for millions worldwide (Edinger and Means, 2005). Despite the fact that insomnia is highly prevalent, it is not commonly considered as a significant threat to health. However, researches have shown that a strong association exists between insomnia, depression, and anxiety, where insomnia may be a risk factor (Taylor *et al*., 2003).

Benzodiazepines are the most commonly utilized drugs for insomnia, and have demonstrated efficacy in short-term treatment. They act selectively on $GABA_A$ receptors and enhance the response to GABA by facilitating the opening of GABA-activated chloride channels (Rang *et al*., 2011). However, their use is not without side effects which include anterograde amnesia and for long acting drugs, residual daytime drowsiness (Attele *et al*., 2000). They are also associated with pharmacokinetic drug interactions with drugs such as azole antifungals (ketoconazole, itraconazole), erythromycin, clarithromycin, and cimetidine which inhibit

CYP3A4. Benzodiazepines are primarily metabolized by cytochrome (CYP) P450 3A4 (except temazepam which undergoes conjugation) (Chouinard *et al*., 1999).

Newer agents with novel chemical structures have been introduced recently for the management of sleep disorders. They include zolpidem (an imidazopyridine), zaleplon (a pyrazolopyrimidine) and eszopiclone (a cyclopyrrolone). They share a similar mechanism of action with the benzodiazepines (Trevors and Way, 2009). Zaleplon and zolpidem, both having similarly degree of efficacy, are effective in relieving sleep onset insomnia without the occurrence of rebound insomnia on abrupt discontinuation (Walsh *et al*., 2000). Although the potential for tolerance development, dependence and drug abuse are less severe with these newer agents, they are not entirely devoid of side effects which include behavioural changes (Monti *et al*., 2009).

Trazodone, a triazolopyridine antidepressant has been frequently prescribed for the treatment of insomnia. However, its use is associated with cardiac rhythm disturbances and orthostatic hypotension (Mendelson, 2005). Other antidepressants used in insomnia are doxepin and amitryptiline. However, there exist a little data supporting the use antidepressants in the management of insomnia not related to depression (Walsh, 2004).

Ramelteon is the first available non prescription medication with demonstrable sleep promoting effect, with no hangover effects, rebound insomnia or withdrawal effects (Zammit *et al*., 2007). It is a melatonin receptor full agonist that is more selective for the melatonin type 1 (MT1) and type 2 (MT2) receptors in the suprachiasmatic nucleus of the hypothalamus. It is currently being used in sleep onset insomnia (Uchikawa *et al*., 2002). The MT1 receptor is localized in the hypothalamic suprachiasmatic nucleus, and it is believed to mediate circadian and

reproductive effects of melatonin. The MT2 receptor is found in the hypothalamic suprachiasmatic nucleus and the neural retina and is thought to mediate the effects of melatonin on circadian rhythms and regulate visual function (Cada, 2006).

Despite recent advances in the development of newer drugs for the management of insomnia, a significant proportion of patients with insomnia worldwide still rely on herbal hypnotics regularly (Wing, 2001).

2.1.2 Drugs used in the Management of Anxiety

Anxiety is a psychological, physiological, and behavioural state induced in animals and humans by a threat to wellbeing or survival either actual or potential. It is characterised by increased arousal, expectancy, autonomic and neuroendocrine activation, and specific behaviour patterns. The function of these changes is to facilitate coping with an adverse or unexpected situation (Steimer, 2002). It is characterized by a persistent and disproportionate fear unrelated to any genuine risk. The most common observation of anxiety is an acute stress response characterised by a state of exaggerated arousal or fear (Ninan, 2001).

Generally, anxiety is an adaptive response to supposedly dangerous stimuli, which may distort homeostasis. However, when it becomes disproportional in intensity, chronic and/or irreversible, or not genuine, it manifests as debilitating anxious state presenting itself in form of phobia, panic attacks, post-traumatic stress disorder, social anxiety disorder or generalized anxiety disorder (Gilhotra and Dhingra, 2010). Anxiety is both a state and a trait. Trait anxiety is the persistent and durable feature of the individual personality that reflects the way they interact with their physical and social environment. State anxiety, on the other hand, is that seen in a given individual at a given time. It changes over time and in response to the level of stress

and to the way that stress is perceived (Sandford *et al*., 2000). Anxiety disorders are the most common and among the most disabling of mental disorders in adults and adolescents (Oakley-Browne, 1991). Anxiety disorders affect one-eighth of the total population worldwide, and have become a very important research area in psychopharmacology (Shri, 2010). Most psychiatric patients will have two or more concurrent psychiatric diseases (co-morbidity) within their lifetime. Anxiety may be a concomitant symptom of several major psychiatric diseases.

Anxiety symptoms are extremely common in patients with mood disorders, schizophrenia, delirium, dementia, and substance use disorders (Kirkwood and Melton, 2002). A genetic predisposition to anxiety disorders is undoubted because there is increased likelihood of the disorders in siblings and children of persons with the disorder than in the relatives of someone without anxiety disorders (Goldman, 2001). The amygdala, an almond-shaped mass of nuclei located deep within the medial temporal lobes of the brain, plays a pivotal role in threat processing. It is generally regarded as fundamental for the acquisition of conditioned fear and for the expression of innate and learned fear responses. Efferent neurons emerging from the amygdala activate the sympathetic nervous system, thus driving the classic fight or flight responses in end organs, such as increased heart rate and blood pressure, and pupillary and bronchodilation (Bandelow, 2004). The spectrum of anxiety disorders includes generalized anxiety disorder, panic disorder and agoraphobia, obsessive-compulsive disorder, phobic disorder (including social phobia), and posttraumatic stress disorder (Cassano *et al*., 2002).

Generalised anxiety disorder is an ongoing state of excessive anxiety without any clear reason or focus but with chronic worry as its essential feature (Gorman, 2003). It is increasingly been recognised as an important treatable clinical disorder with an overall prevalence of about 2–5%

and twice as many women as men suffers from it. Psychotherapies have been shown to be effective in the management of generalised anxiety disorder (GAD) when used alone, and may serve to augment the beneficial effect of anxiolytics. Although benzodiazepines have been shown to be of immense benefits, especially in severe or acute situations when more immediate anxiolytic actions are required, the antidepressants (paroxetine and venlafaxine) are considered the first line of action (Gorman, 2003). Benzodiazepines, the protypical anxiolytics have been in use since 1960's and have been found to produce a rapid anxiolytic action in GAD. Their actions are mediated via the activation of Gamma aminobutyric acid (GABA) system at the $GABA_A$ receptor complex leading to reduced transmission throughout the central nervous system (Salzman *et al*., 1993).

Buspirone (an azapirone), an anxiolytic which is structurally and pharmacologically distinct from benzodiazepine is reported to be as effective as the benzodiazepine in GAD. It acts by antagonizing serotonin, presynaptically thereby reducing the firing of serotonin-affected nerve fibres (Taylor, 1990). Data have been presented demonstrating the efficacy of imipramine, a tricyclic antidepressant (TCA) in GAD, but its action is similar to buspirone in exhibiting greater benefit in cognitive dimension than the somatic symptoms of the disorder.

Panic disorder (PD) is a disabling condition which appears in late adolescence or early adulthood, affecting more frequently women than men and is frequently characterized by recurrences and sometimes by a chronic course, therefore, most patients require long-term treatments to achieve remission, to prevent relapse and to reduce the risks associated with comorbidity (Marchesi, 2008). Acute panic attacks appear to be associated with increased noradrenergic discharge in the locus coeruleus. Agents that block serotonin reuptake are therapeutic in preventing attacks. It is theorized that panic disorder patients have a heightened

sensitivity to somatic symptoms, which triggers increasing arousal, setting off the "panic attack" mechanism (Reus, 2001). Pharmacologic and metabolic probes have revealed that in panic disorder, there are alterations of serotonergic, noradrenergic, GABAergic, and brain cholecystokinin function and that there is corticosteroid dysregulation as well as increased sensitivity to the anxiety provoking effects of carbon dioxide and lactate (Malizia, 2008). GABA has been linked to anxiety by evidences that showed blockage of $GABA_A$ receptor produces severe anxiety both in man and animals (Reschke *et al*., 1995). Drugs with anxiolytic and anti-panic effects inhibit locus coerulus firing, decreasing noradrenergic sensitivity, and block the effect of anxiogenic drugs (Ninan, 1999).

Obsessive-compulsive disorder (OCD) is a chronic and severely disabling anxiety disorder with a lifetime prevalence of approximately 2–3% in the general population (Robins *et al*., 1984; Chamberlain *et al*., 2005). Obsessive-compulsive disorder is characterised by the occurrence of either obsessions, compulsive rituals or, most commonly, both. Obsessions have four essential features which are recurrent and persistent thoughts, impulses, or images that are experienced as intrusive and cause great anxiety (Abramowitz *et al*., 2009).

Neurochemically, OCD has been most associated with the serotonergic system. Results of studies of static measures of serotonergic function in obsessive-compulsive disorder have, however, been inconsistent, and other work has focused on more informative dynamic measures. For example, administration of the serotonin (5-HT) agonist m-chlorophenylpiperazine (mCPP) has been accompanied by exacerbation of obsessive-compulsive disorder symptoms and a blunted neuroendocrine response. After treatment with a serotonin reuptake inhibitor, behavioural and neuroendocrine responses to mCPP seem to be normal (Bystritsky, 2004).

In experimental models of anxiety, anxiogenic agents share in common the property of altering the binding of benzodiazepines to γ-aminobutyric acid $(GABA)_A$ receptor/chloride ion channel complex. Benzodiazepines are thought to bind two separate $GABA_A$ receptor sites: type I, which has a broad neuroanatomic distribution, and type II, which is concentrated in the hippocampus, striatum, and neocortex. The anti-anxiety effects of the various benzodiazepines and side effects such as sedation and memory impairment are influenced by their relative binding to type I and type II receptor sites. Serotonin [5-hydroxytriptamine (5HT)] also appears to have a role in anxiety. Buspirone, a partial $5HT_{1A}$ receptor agonist, and certain $5HT_{2A}$ and $5HT_{2C}$ receptor antagonists (e.g., nefazodone) may also have beneficial effects (Reus, 2001).

There are a number of medicinal plants used in complementary and alternative medicines for the treatment of various forms of anxiety. These include Kava kava (*Piper methysticum*), St. John's wort (*Hypericum perforatum*), Passion flower (*Passiflora incarnate*), Oatstraw (*Avena sativa*), Lemon balm (*Melissa officinalis*), Lavender (*Lavandula angustifolia*), Valerian (*Valeriana officinalis*) and Skullcap (*Scutellaria lateriflora*) (Volz and Kieser, 1997; Wheatley, 2005).

The central sedative properties of some medicinal plants of African origin have been evaluated and are potential sources of sedatives and anxiolytics. These include *Guirera senegalensis* (Amos *et al*., 2001), *Entandrophragma angolense* (Amos *et al*., 2002) and *Dalbergia saxatilis* (Yemitan *et al*., 2001). A number of other African medicinal plants are claimed to be beneficial traditionally in the management of anxiety and insomnia. These include *Pyllanthus muellerianus, Nauclea latifolia, Vitex doniana* (Mann *et al*., 2003), *Pericopsis laxiflora* and *Sarcocephalus latifolius* (Neuwinger, 1996).

2.1.3 Drugs used in the Management of Epilepsy

Unlike other therapeutic groups, AEDs are not usually classified into categories according to their respective modes of actions due to incomplete understanding of the molecular mechanism of actions of the agents, the approach to treatment of epilepsy and other conditions in which antiepileptic agents are deployed and lastly drug promotions in an increasing competitive markets where questionable claims of additional or innovative properties are used to support the evidence of clinical efficacy of new agents (Perucca, 2005). Antiepileptic drugs aim to inhibit abnormal neuronal discharge (Rang *et al*., 2011). In contrast to the relatively large number of antiepileptic drugs that can attenuate seizure activity, there are currently no drugs known to prevent the formation of a seizure focus following CNS injury in humans. The eventual development of such "antiepileptogenic" drugs will provide an important means of preventing the emergence of epilepsy following injuries such as head trauma, stroke, and CNS infection (Lowestein, 2001).

Currently available antiepileptic drugs appear to act primarily by blocking the initiation or spread of seizures. The three main mechanisms of action of antiepileptic agents are inhibition of sodium channel function, inhibition of calcium channel function and enhancement of GABA action (Rogawski and Loscher, 2004). Blockade of voltage-dependent sodium channels is a primary action of phenytoin (PHT), carbamazepine, oxcarbazepine, lamotrigine, topiramate, zonisamide, and felbamate (Meldrum, 1996). The voltage- and use-dependent nature of this block implies that high frequency repetitive neuronal firing is selectively prevented, thereby inhibiting spread of seizure activity without interfering with physiologic neurotransmission. Although, at therapeutic concentrations, sodium channel blockers inhibit action potential firing without directly affecting synaptic responses, blockade of neuronal firing ultimately prevents

depolarization of the nerve terminal and the consequent release of neurotransmitters, particularly glutamate (Perucca, 2005).

Drugs that are effective against absence seizures (ethosuximide, valproate, clonazepam) act by blocking T-type low voltage-activated calcium channels. The channel activity is essential in determining the rhythmic discharge of thalamic neurons associated with absence seizures (Khosravani *et al*., 2004). Drugs that enhance GABA function act by potentiation of GABA receptor function (benzodiazepines and barbiturates) or increase in the availability of GABA (valproic acid, gabapentin and tiagabine).

Phenytoin (a prototype of diphenylhydantoin) limits the repetitive firing of action potential evoked by a sustained depolarization of mouse spinal cord neuron maintained *in vitro* (McLean and Macdonald, 1986). It reduces the rate of recovery of sodium channels from inactivation, an action that is both voltage and use dependent (McNamara, 2006). As a result, phenytoin suppresses episodes of repetitive neuronal firing that are induced by passage of intracellular currents. Phenytoin delays the activation of outward K^+ currents during action potentials in nerves, leading to an increased refractory period, and can also reduce the size and duration of Ca^{2+} dependent action potentials in cultured neurons (Mclean and Macdonald, 1986; Yari *et al*., 1986). It is highly effective in reducing the intensity and duration of electrically induced convulsions in mice, although ineffective against PTZ-induced convulsions (Rang *et al*., 2011).

Like phenytoin, carbamazepine limits the repetitive firing of action potentials evoked by sustained depolarization of mouse spinal cord or cortical neurons maintained *in vitro* (McLean and Macdonald, 1986). This appears to be mediated by a slowing of the rate of recovery of voltage-activated Na^+ channels from inactivation (McNamara, 2006). Although it is particularly

effective in treating certain partial seizures e.g psychomotor epilepsy; it may aggravate absence seizure through blockade of L-type calcium channels (van Luijtelaar *et al*., 2000).

Although valproate has no effects on response to GABA, it does increase the amount of GABA that can be recovered from brain after the drug is administered to animals. *In vitro*, valproate can stimulate the activity of the GABA synthetic enzymes, glutamic acid decaboxylase, and inhibit GABA degrading enzymes, GABA transaminase and succinic semialdehyde dehydrogenase. However, it has been difficult to relate the increased in GABA levels to the anti-seizure activity of valproate (McNamara, 2006).

The anticonvulsant actions of the benzodiazepines result from their interaction with the benzodiazepine receptors (which are adjacent to the GABA receptors sites), which in turn enhances the postsynaptic GABAergic transmission (Ebadi, 1996). There may be other mechanisms by which benzodiazepines reduce the excitability of neurons. These include enhancement of Ca^{2+} and K^{+} conductance and of adenosine accumulation, neither of which requires GABA (McNamara, 2006).

Other agents used as adjunctive or back up drugs in the management of epilepsy include gabapentin, lamotrigine, levetiracetam, tiagabine, topiramate, felbamate, vigabatrin and zonisamide (Trevors *et al*., 2005). Despite the design of gabapentin as a GABA agonist; gabapentin does not mimic GABA when iontophoretically applied to neurons in primary cultures (McNamara, 2006). Gabapentin and pregabalin also exert their effects via HVA calcium channels but, rather than interacting with a traditional channel sub-type such as N- or L-type, they appear to bind to an accessory subunit termed α2δ-1, which can modulate the function of various native channels. This subunit is up-regulated in dorsal root ganglion cells of

the spinal cord in response to nerve injury, with selective calcium channel blockade via the α2δ-1 subunit explaining the efficacy of gabapentin and pregabalin in the treatment of neuropathic pain (Kwan *et al*., 2001).

Lamotrigine shares a very similar mechanism of action with conventional antiepileptic drugs, carbamazepine and phenytoin. The drug binds to the inactivated form of voltage-dependent sodium channels, thus limiting the sustained repetitive firing of neurons without any substantial effect upon normal synaptic activity and reduces calcium currents via voltage sensitive calcium channels (Macdonald and Greenfield, 1997: Stefani *et al*., 1997). The broader spectrum of activity of lamotrigine than carbamazepine and phenytoin suggests that it may have other actions in addition to regulation of recovery from inactivation of Na^+ channels. It may possibly inhibit synaptic release of glutamate by acting at Na^+ channels themselves (McNamara, 2006).

Levetiracetam does not affect voltage-dependent channels or receptors for major inhibitory or excitatory neurotransmitters (Klitgaard and Pitkänen, 2003). It is ineffective in acute models of experimental epilepsy, including maximal electroshock induced convulsions or clonic phase of pentylenetetrazole (or other chemical convulsants) seizures. In contrast, it is highly effective against amygdala-kindling in rats and a number of genetic models of epilepsy. In the amygdala-kindling model, this antiepileptic drug not only inhibits convulsions in fully kindled animals, but also retards the development of kindling as well, which may speak for its antiepileptogenic efficacy (Dooley and Plosker, 2000; Klitgaard and Pitkänen, 2003). Levetiracetam is known to bind synaptic vesicle protein 2A (SV2A). However, the precise physiological role of SV2A is still unclear (Rang *et al*., 2011).

Tiagabine inhibits the GABA transporter, GAT-1, and thereby reduces GABA uptake into neurons and glia, thus permitting more GABA to be available for receptor binding (Mycek *et al.*, 2000, McNamara, 2006). In certain areas of the brain, GABA may have a proepileptic effect (partly by suppressing activity of inhibitory pathways), which may explain the ability of GABAergic drugs to cause paradoxical seizure aggravation in certain syndromes, particularly within the group of generalized epilepsies. Vigabatrin and tiagabine, specifically, although effective against partial and secondarily generalized tonic-clonic seizures, may precipitate absence and myoclonic seizures (Perucca *et al*., 1998)

Topiramate reduces voltage-gated Na^+ currents in cerebella granule cells and may act on the inactivation state of the channel in a manner similar to that of phenytoin. It also activates a hyperpolarizing K^+ current, enhances postsynaptic $GABA_A$ receptor currents and also limits activation of AMPA-kainate subtype(s) of glutamate receptor (McNamara, 2006).

Felbamate has been documented to block voltage dependent sodium channels and NR2B subunit of NMDA receptor (Tagliatella *et al*., 1996; Harty and Rogawski, 2000). Also, felbamate presumably inhibits glutamatergic neurotransmission via AMPA/kainate receptors (DeSarro *et al*., 1994) and enhances GABA-mediated events through a barbiturate-like modulatory effect on the $GABA_A$ receptor (Rho *et al*., 1994).

Zonisamide inhibits the T-type Ca^+ currents and also inhibits the sustained, repetitive firing spinal cord neuron presumably by prolonging inactivation of voltage-gated Na^+ channels in manners similar to actions of phenytoin and carbamazepine (McNamara, 2006). At therapeutic concentrations, it enhances both dopaminergic and serotonergic neurotransmission and inhibits glutamate-induced excitation. It also reduces overproduction of nitric oxide and free radicals,

an action that may be related to its anticonvulsant and neuroprotective effects (Czapin´ski *et al*., 2005).

Lacosamide has been found to be effective against sound-induced seizures in the genetically susceptible Frings mouse, against maximal electroshock test (MES)-induced seizures in rats and mice, in the rat hippocampal kindling model of partial seizures, and in the 6 Hz model of psychomotor seizures in mice. It is may act by enhancing sodium channel inactivation (St¨ohr *et al*., 2007). However, unlike other antiepileptic, it affects slow rather than fast inactivation processes (Rang *et al*., 2011).

Rufinamide has been reported to be effective against MES and PTZ, picrotoxin and bicuculine-induced seizures in rodents (White *et al*., 2008). It appears to be acting via sodium channel inactivation and inhibition of GABA reuptake (Rang *et al*., 2011).

Stiripentol is indicated for use as adjunctive treatment with clobazam and valproate for the management of refractory generalized tonic-clonic seizures in patients with severe myoclonic epilepsy in infancy (SMEI; Dravet syndrome) (Luszczki, 2010). At clinically relevant concentrations, it enhances central GABA transmission through a barbiturate-like effect (Quilichini, 2006), and increases GABA release (Rang *et al*., 2011). At recombinant receptors, it was found to act through a unique site in a subunit dependent manner. Positive modulation by stiripentol was most effective at $GABA_A$ receptors containing a α3 subunit. The expression of the α3 subunit is developmentally regulated, with highest levels in the immature brain. The selectivity of this subunit could explain the greater clinical efficacy of stiripentol in childhood-onset epilepsies, including Dravet syndrome (Fisher, 2011).

Epilepsy is controlled completely in about 75% of patient with optimal drug therapy. However, about 10% are refractory and continue to have seizures at interval of one month or less, which severely disrupts their life and work (Rang *et al*., 2011). The existing antiepileptic agents are known to affect ictogenesis, a transient and direct event that induces seizures due to excessive discharges from groups of neurons. They do not seem to affect epileptogenesis which involves long lasting and prolonged histological /biochemical alterations of neuron networks and reorganization of neuronal matrices, with the process ranging from months to years (Weaver, 2003; Sasa, 2006).

Antiepileptic agents are frequently associated with adverse effects (Vyawahare, 2007). In addition to safety, tolerability, efficiency, expenses especially in long term therapy, serum drug monitoring etc. are other limitations with synthetic antiepileptic drugs (Sinha, 2002). Further, a large number of drug interactions associated with the use of these agents has made it difficult to attain easy control of seizures (French and Gidal, 2000). All these have led to a reawakening of interest in the use of traditonal medicine, of which medicinal plants play a central role, in the management of epilepsy, especially in developing countries. There is also intensification of research into medicinal plants with the goal of isolating bioactive principles which could serve as "lead compounds" in the search for novel anticonvulsant drugs.

Passiflora incarnata, Scutellaria lateriflora and *Valeriana officinalis* are used as herbal treatment of epilepsy in Europe and America (Murray, 1998). A number of African medicinal plants have been shown to possess active principles which make them beneficial in the management of seizure disorders. These include *Dalbergia saxatilis* (Yemitan and Adeyemi, 2006), *Schumanniophyton magnificum* (Ogbonnia *et al*., 2003) and *Glycyrrhyza glabra* (Ambawade *et al*., 2002). African traditional antiepileptic plants include *Xylopia aethiopica,*

Heliotropium indicum, Cassia occidentalis, Eleusine indica (Mann *et al*., 2003), *Disopyros physocalycina, Jatropha curcas, Securidaca longenpenduculata* and *Tetrapleura tetraptera* (Neuwinger 1996).

2.2 AN OVERVIEW ON GLIOBLASTOMA MUTIFORME AND ITS MANAGEMENT

2.2.1 Glioblastoma Multiforme

GBM (WHO grade IV) is the most common malignant brain tumour of adults, and is among the most lethal of all cancers (Mischel and Cloughesy, 2003) with an average patient life expectancy of only 15 months after diagnosis (Wheeler, 2008). It is the most malignant diffuse type astrocytic neoplasm, representing over half of all astrocytic tumours (Kleiheus and Caveness, 2000).

Genetically, GBM can be grouped into primary and secondary GBMs. Primary GBMs develop *de novo* and 40-60% harbour EGFR (epidermal growth factor receptor) amplifications and alterations of PTEN (phosphatase and tensin homolog), leading to a profound activation of the PI3-Kinase pathway resulting into enhancement of apoptotic resistance, tumour cell proliferation and angiogenesis (Chakravarti *et al*., 2004). In contrast, secondary GBMs develop out of lower grade lesions and often reveal TP53 and IDH1 (isocitrate dehydrogenase 1, affected amino acid 132) mutations (Parsons *et al*., 2008).

GBM is characterized by rapid growth, diffuse infiltration of cells into both adjacent and remote brain regions, and a generalized resistance to currently available treatment modalities (Merck *et al*., 2011). Current treatment strategy of GBM includes surgical resection, radiotherapy and chemotherapy. The introduction of temozolomide to standard treatment regimens consisting of surgical resection and radiation extended median survival time from

12.1 to 14.6 months and more than doubled overall 2-year survival from 10.4 percent to 26.5 percent (Stupp *et al*., 2005). Temozolomide is an imidazotetrazine second generation alkylating agent and the leading compound in a new class of chemotherapeutic agents that enter the cerebrospinal fluid. It is a 3-methyl derivative of mitozolomide found to be less toxic than mitozolomide and exhibited comparable antitumour activity against various murine tumours. The methylation of DNA seems to be the principal mechanism responsible for the cytotoxicity of temozolomide to malignant cells. The spontaneous conversion of temozolomide to the reactive methylating agent, MTIC (3-methyl-(triazen-1-yl) imidazole-4-carboxamide) is initiated by the effect of water at the highly electropositive C^4 position of temozolomide. This activity opens the ring, releases CO_2, and generates MTIC (Friedman, 2000).

Epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor which is overexpressed in cancer cells, and has been identified as a target of tumour therapy. 50–60% and 40% of GBMs have overexpression and amplification of the epidermal growth factor receptor (EGFR), respectively (Ohgaki *et al*., 1995). The EGFR or ErbB family belongs to subclass I of the superfamily of the receptor tyrosine kinases. Receptor tyrosine kinases represent an important subclass of these transmembrane proteins, with the EGFR being the most prominent representative. The EGFR controls a wide variety of biological processes such as cell proliferation, differentiation, and migration and modulation of apoptosis (van der Geer *et al*., 1994).

There are four members of the ErbB family: the EGFR (also termed ErbB1/HER1), ErbB2/Neu/HER2, ErbB3/HER3, and ErbB4/HER4. Members of the ErbB family are characterized by a modular structure consisting of an extracellular ligand-binding domain, a single hydrophobic transmembrane region, and the intracellular part harbouring the highly

conserved tyrosine kinase domain (Prenzel, 2001). The ErbB receptors are activated by a number of ligands known as EGF-related peptide growth factors (Riese and Stern, 2001). The EGF-related peptide growth factors bind the extracellular domain of the ErbB receptor, leading to formation of homo- and heterodimers. Dimerization consequently stimulates the intrinsic tyrosine kinase activity of the receptors and triggers autophosphorylation of specific residues within the cytoplasmic domain. These phosphorylated residues serve as docking sites for signaling molecules involved in the regulation of intracellular cascades (Ullrich and Schlessinger, 1990).

Activation of the EGFR in a large percentage of glioblastomas suggests an important role for this receptor in astrocytic tumour development. The downstream signaling pathways of activated EGFR include the mitogen-activated protein kinase (MAPK) pathway, the phosphatidylinositol 3 kinase pathway and the Janus kinase signaling transducer and activator of transcription pathway (Roskoski, 2004). EGFR activation may therefore contribute to tumour malignancy by promoting invasion, proliferation, and metastasis (Nishikawa *et al*., 1994).

Interference with the activation of growth factor receptor and/or with its signal transduction pathways is a promising strategy for the development of novel and selective anticancer agents (Ritter and Arteaga, 2003; Laskin and Sandler, 2004). Iressa (Gefitinib, ZD1839), a quinazoline, is a selective EGFR tyrosine kinase inhibitor (TKI) that competitively binds adenosine triphosphate (ATP) of EGFR, thereby blocking the signal transduction processes implicated in the proliferation, metastasis and angiogenesis of cancer cells (Liu *et al*., 2008). It has shown antiproliferative and anti-invasive effects in human GBM cells with amplified or transfected EGFR (Raizer, 2005).

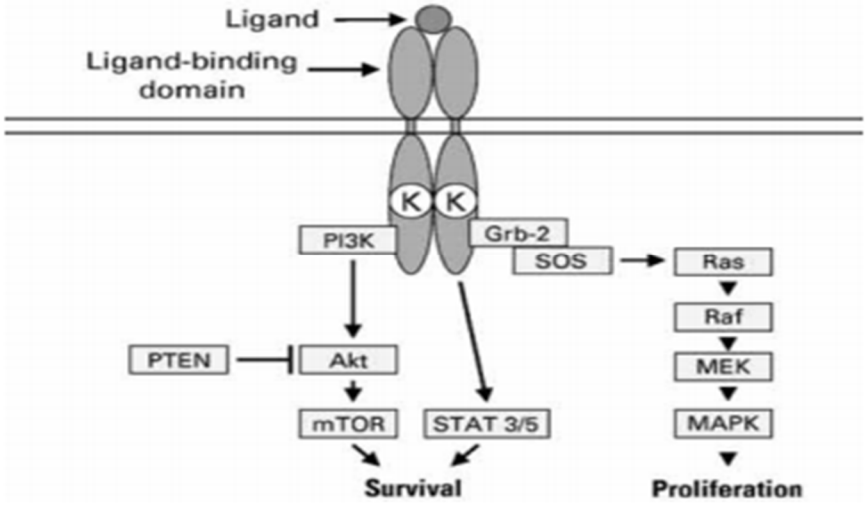


Figure 2.2.1: Epidermal growth factor receptor signaling pathway (Jesdić and Popov, 2005)

2.2.2 Medicinal Plants and Cancer

Cancer is a major public health burden in both developed and developing countries. It was estimated that there were 10.9 million new cases, 6.7 million deaths, and 24.6 million persons living with cancer around the world in 2002 (Parkin *et al*., 2005). Plants have a long history of use in the management of cancer. Many plants have been reported to be used in the management of cancer (Graham *et al*., 2000; Abubakar *et al*., 2007). The National Cancer Institute (NCI) has also screened over one hundred thousand plant extracts for anticancer activity (Shoeb, 2006). About sixty percent of the worldwide approved anticancer drugs between 1983 and 1994 are of natural origin (Cragg *et al*., 1997).

Vinca alkaloids (vincristine and vinblastine) were obtained from Madagascar periwinkle, *Catharanthus roseus* G. Don (Apocynaceae). They act by binding to tubulin, thereby inhibiting its polymerization to microtubules, preventing spindle formation in dividing cells and causing metaphase arrest (Rang *et al*., 2011). They are primarily used in combination with other cancer

chemotherapeutic drugs for the treatment of a variety of cancers, including leukaemias, lymphomas, advanced testicular cancer, breast and lung cancers, and Kaposi sarcoma (Cragg and Newman, 2005). Other principal members of the group include vindensine and vinorelbine.

Paclitaxel obtained from the bark of the Pacific Yew, *Taxus brevifolia* Nutt (Taxaceae) is reported to be significantly active against ovarian cancer, advanced breast cancer, small and non-small cell lung cancer (Rowinsky *et al*., 1992). *In vitro*, paclitaxel enhances the polymerization of tubulin to stable microtubules and also interacts directly with microtubules, stabilizing them against depolymerization by cold and calcium, which readily depolymerize normal microtubules (Horwitz, 1994). Hence, it inhibits mitosis and cancer cell growth.

Camptothecin, isolated from the Chinese ornamental tree, *Camptotheca acuminate* Decne (Nyssaceae) was advanced to Clinical trials by National Cancer Institute in the early 70's but was dropped due to severe bladder toxicity (Potmeisel, 1995). Topotecan and irinotecan are semi-synthetic derivatives of camptothecin and are indicated for the treatment of ovarian and small cell lung cancers, and colorectal cancers, respectively (Creemers *et al*., 1996; Bertino, 1997). Their molecular target has been firmly established to be human DNA topoisomerase I. Camptothecin inhibits topoisomerase I by blocking the rejoining step of the cleavage/religation reaction of topoisomerase I, resulting in accumulation of a covalent reaction intermediate, the cleavable complex (Liu *et al*., 2000).

Etoposide and teniposide are two semi-synthetic derivatives of epipodophyllotoxin which is an isomer of podophyllin (obtained from mandrake root; *Podophyllum peltatum*) and are used in the treatment of lymphomas, and bronchial and testicular cancers (Harvey, 1997; Cragg and Newman, 2005). They work via different mechanisms by inhibiting the enzyme topoisomerase II, thus preventing DNA synthesis and hence replication (Thakur, 2011).

Homoharringtonine is an alkaloid isolated from the Chinese tree, *Cephalotaxus harringtonia* var. *drupacea* (Sieb and Zucc.) (da Rocha *et al*., 2000; Itokawa *et al*., 2005). Its main mechanism of action is the inhibition of protein synthesis, blocking cell cycle progression (Zhou, 1995). It has shown efficacy against various leukaemias (Kantarjian, 1996).

Flavopiridol is a synthetic flavone derived from the plant alkaloid rohitukine, which was isolated from the leaves and stems of *Amoora rohituka* and later from *Dysoxylum binectariferum* (Maliaceae). Elliptinium is derived from ellipticine which was initially isolated from a Fijian medicinal plant, *Bleekeria vitensis* A.C. Sm. It is indicated in the treatment of breast cancer (Cragg and Newman, 2005; Shoeb, 2006).

2.3 THE PLANT, *SECURINEGA VIROSA*

Securinega virosa is one of the great African medicinal plants described as a true "cure all", of which all parts are used as remedies, particularly the root (Neuwinger, 1996). It belongs to the family, Euphorbiaceae of the order, Geraniales. *S. virosa* is a dense, low branching, many branched shrub, sometimes a small spreading tree up to about 6 meters high, although, more commonly 2 to 3 meters, evergreen or deciduous. It is widely distributed throughout tropical Africa, also in India, Malaya, China and Australia (Dalziel, 1936).

In Nigeria, it is found in virtually all parts of the country (Neuwinger, 1996). The common vernacular names of *S. virosa* include "*tsuwaawun karee*, *gussu*, *gwiiwar karee*" (Hausa), "*iranje*" (Yoruba), "*njisi nta*" (Ibo), "*shim shim*" (Kanuri), "*kartfi-kartfi*" (Shuwa Arabs) and "*camal*, *cambe*, *came*" (Fulani) (Neuwinger, 1996).

The root and leaf decoctions (separately) are drunk for fever in many parts of Africa including the south-western Nigeria, and in Ivory Coast the root is said to possess analgesic properties

and is used for labour and other pains (Neuwinger, 1996). In many parts of Africa including the north Eastern Nigeria, the root and leafy twig decoctions are used for the treatment of epilepsy. The pulp of pounded roots and fruits are rubbed into cuts to treat snake bites also in Southern Africa. The leaves have laxative properties and are taken in decoction, while the root decoction is taken for veneral disease (Dalziel, 1936), schistosomiasis, and dysmenorrhea in Tanzania (Neuwinger, 1996).

The powdered root is used for diarrhoea in Nigeria while the stem bark decoction is used for bloody diarrhoea in Ivory Coast. The whole plant is used in the treatment of cancer in Taiwan (Neuwinger (1996). The decoction of the root of *S. virosa* with some other plants is used in northern Nigeria for the treatment of mental illness.

The alcoholic extract of *Securinega virosa* root exhibited activity against *E.coli* and to a lesser extent against *Staphylococcus aureus* (Khan *et al.*, 1980) while it showed a significant antifungal activity *in vitro* (Sawhney, 1978). The alcoholic extract of the leaves shows significant cytotoxicity in tumour cells *in vitro* (Tatematsu, 1991). The hypoglycemic effect of the methanol leaf extract of the plant has been reported (Tanko *et al*., 2008).

The plant exhibited significant antimalarial activity against *Plasmodium falciparum*, *in vitro*, comparable to quinine used as standard drug (Gbeassor *et al*., 1989). The root bark caused a transient fall in arterial blood pressure in chloralised dogs, acted slightly depressive on isolated rabbit intestine and showed weak haemolytic activity (Paris *et al*., 1955). The aqueous extract of the roots exhibited hypoglycemic effect (Moshi *et al.*, 2000). The crude methanolic root bark of the plant has been reported to possess anticonvulsant (Magaji *et al*., 2007a), antidiarrhoeal (Magaji *et al*., 2007b) analgesic and anti-inflammatory (Magaji *et al*., 2008a) and sedative

(Magaji *et al*., 2007c; Magaji *et al*., 2008b) activities. A number of alkaloids have been isolated from the root bark of *S. virosa*. These include; hordenine, norsecurinine (Iketubosin and Mathieson, 1963), dihydronorsecurinine, viroallosecurinine (Saito *et al*., 1964) and 14, 15-epoxynorsecurinine (Dehmlow *et al*., 1999). Friedelin, epifriedelanol, stigmasterol and betulinic acid were isolated from the leaves and twig of the plant (Monkodkaew *et al*., 2009).



Plate I. *Securinega virosa* (Euphorbiaceae) in its natural habitat

CHAPTER 3

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Drugs and Chemicals

4-Aminopyridine (Merck-Schuchardt)

Chips of magnesium metal BDH Ltd Poole, England

Chloroform (Sigma Chemical Co.USA)

Concentrated Hydrochloric acid (BDH Ltd Poole, England)

Diazepam 10mg in 2ml (Roche Product Ltd Welnyn Garden City)

Dimethyl sulphoxide (Sigma Aldrich, USA)

Dragendorff reagent (BDH Ltd Poole, England)

Ferric chloride (BDH Ltd Poole, England)

Glacial acetic acid (Searle Essex, England)

Lead sub acetate (strong) (BDH Ltd Poole, England)

Meyer reagent (BDH Ltd Poole, England)

Pentylenetetrazole (Sigma Chemical Co. USA)

Phenobarbitone (Lab. Renaudin, France)

Phenytoin sodium capsules (Parke-Davis and Co. Ltd)

Picric acid reagent (BDH Ltd Poole, England)

Picrotoxin (Sigma Chemical Co. USA)

Sodium picrate paper (BDH Ltd Poole, England)

Sodium valproate (Sanofi Synthelabo, One Onslow st. Surrey)

Strychnine hydrochloride (Sigma Chemical Co.USA)

Sulphuric acid (BDH Ltd Poole, England)

Wagner reagent (BDH Ltd Poole, England)

3.1.2 Equipment and Apparatuses

Animal cages

Bruker AVANCE (500 MHz for ^{1}H and ^{13}C) spectrometer

Claude lyon voltage stabilizer

Crucible

Cylindrical beam (60cm, 8mm) with metal support

Digital Camcorder (JVC Everio 32G HDD)

Elevated plus maze apparatus

Gallenkamp melting point apparatus

LTQ and TSQ mass spectrometers

Metler balance P165

Open Field apparatus

Pestle and mortar

Ruler (80 cm X 3cm) with metal support

Syringes (1ml, 2ml, 5ml and 10ml)

Stair case apparatus

Tally counters

Stop watches

Ugo Basile current electroshock machine (Model 7801 with corneal electrodes)

O_2 Coulter counter instrument

3.1.3 Animals

Swiss Albino mice of either sex, weighing 18-22 g, were obtained from the Animal House Facility of the Department of Pharmacology and Therapeutics, Ahmadu Bello University Zaria, Nigeria. Day old Ranger cockrels (26-39 g) were obtained from the National Animal Production Research Institute (NAPRI), Shika, Nigeria.The Wistar rats (250-300 g) used for the kindling studies were generously donated by Professor Edward Bertram of the Department of Neurology, University of Virginia, USA.

The animals were maintained in a well-ventilated room in the Animal house. They were fed on standard laboratory animal feed and water *ad libitum.* All experiments performed on laboratory animals in this study were in accordance with Ahmadu Bello University Research policy and followed the "principles for care and use of laboratory animals" (NIH Publication, 1985).

3.1.4 Materials for Anticancer Studies

PMA (100 nM), EGF, PDGF and anti-tubulin antibody (Sigma, USA)

The phospho-specific antibodies directed against the EGFR at Tyr_{1068} and Tyr_{1045} (Cell Signaling Technology, Inc. Beverly, MA).

3.1.4.1 *Cell Cultures*

The human U-1242 MG cell line was provided by Prof Isa M Hussani. U-1242 MG is a well characterized glioblastoma cell line. The U-1242 MG cells have mutant p53 (Bostrom *et al.*, 1998). The cell lines were originally isolated from astrocytic tumours that were designated as glioblastomas, and their characteristics have been described previously by Hussaini *et al.* (2000). Cell lines were regularly determined to be free of mycoplasma with reagent from Gen-Probe Inc. (San Diego). Cells were grown in modified α-minimal essential medium with 10%

defined fetal bovine serum (Hyclone, Logan, UT) and 20 µg/ml bovine zinc insulin (25.7 IU/mg; Sigma). The cells were cultured to 100% confluence, passaged every 4-5 days from an initial concentration of 6-8 $x10^3/cm^2$ in T flasks or 6- or 24-well plates, and cultured in astrocyte growth medium 5% fetal bovine serum at 37 °C in 5% CO_2 and 90% relative humidity. Prior to assays, cultures that were 80-100% confluent was washed three times with serum-free medium.

3.2 **METHODOLOGY**

3.2.1 Preparation of the Extract

3.2.1.1 *Collection and Identification of Plant Material*

The whole plant, *Securinega virosa* was collected from Basawa town, in Sabon-Gari Local Government Area of Kaduna State, Nigeria, in November, 2008. The plant was identified and authenticated by Malam Musa Muhammed and Umar Galla of the Herbarium Section in the Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria by comparing with existing voucher specimen (NO 918).

3.2.1.2 *Preparation of crude Extract*

The root was cleaned and the bark removed. The root bark was air dried under shade until a constant weight was obtained. It was then crushed into coarse powder with a pestle and mortar. The powdered root bark (1000 g) was extracted with 2L methanol (absolute) for 72 hours using soxhlet extraction apparatus. The solvent was evaporated on a water bath at 40ºC to give a dark brownish residue (Average yield: 9.82 %w/w) which was subsequently referred to as crude methanolic root bark extract. A fresh solution of the extract was prepared for each study.

3.2.1.3 *Fractionation of Crude Extract*

About 50 g of the crude methanolic root bark extract was dissolved in distilled water and filtered. The filtrate was successively partitioned with petroleum ether, chloroform, ethyl acetate and n-butanol affording various yields as shown in the scheme (Figure 1). The extract and the fractions were stored separately in a desiccator until needed for the work. Solutions of the extract and fractions were prepared freshly for each study.

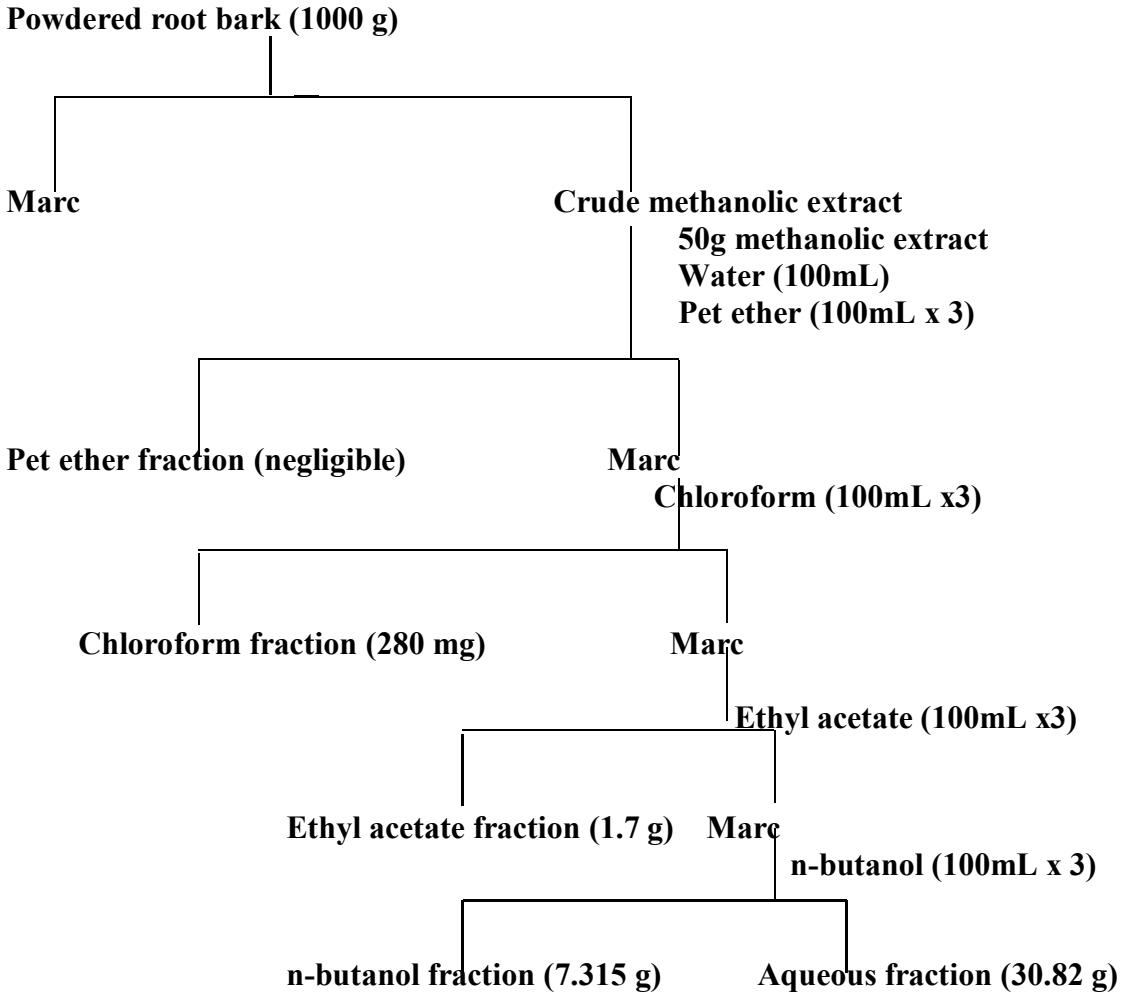


Figure 1: Successive Fractionation of Crude Methanolic Root Bark Extract of *Securinega virosa*

3.2.2 Phytochemical Screening of *Securinega Virosa*

The phytochemical screening was carried out on the crude methanolic root bark extract, its fractions and column fractions. The tests were as follows;

3.2.2.1 *Test for carbohydrates*

Molisch test:

The sample (0.5 g) was dissolved in distilled water in a test tube. Three drops of 1% α- naphthol in 80% ethanol was added followed by 5 drops of concentrated sulphuric acid without mixing. Appearance of purple ring at the interface as a result of the reaction between α- naphthol and furfural and 5-hydroxymethylfurfural aldehydes produced by dehydration of saccharides indicates the presence of carbohydrates (Silva *et al.,* 1998).

3.2.2.2 *Test for Flavonoids*

Shinoda reduction test (cyanidin test):

An alcoholic solution of the sample (3 ml) was evaporated to dryness. The residue was dissolved in 2 ml of 50% methanol with heat. Four (4) drops of concentrated hydrochloric acid was added followed by some chips of magnesium metal. Immediate appearance of orange colour denotes flavones, red – crimson colour denotes flavonols and pink – magenta colour denotes flavonones (Mahran *et al.,* 1980).

3.2.2.3 *Test for Saponins*

Froth test:

Aqueous solution of the sample (2 ml) was diluted with twice its volume of distilled water and shaken in a test tube for two (2) minutes. The occurrence of foam column, of at least 1 cm in height persisting for a minimum of 15 minutes indicates the presence of saponins (Silva *et.al.,* 1998).

3.2.2.4 *Test for Tannin*

Ferric chloride test:

Aqueous solution of the sample (1 ml) was diluted with 2 ml of distilled water. Dilute solution of ferric chloride (3 drops) was then added. The occurrence of black-blue or black-green indicates the presence of tannins (Evans, 1996).

Lead sub acetate test:

Aqueous solution of the sample (1 ml) was diluted with 2ml water. The dilute solution was mixed with lead sub acetate solution. Appearance of a light brown precipitate indicates the presence of tannins (Evans, 1996).

3.2.2.5 *Test for Steroids and Terpenoids*

Liebermann-Burchard's test:

The sample (0.5 g) was extracted with 5 ml of methanol and then filtered. The filtrate was evaporated to dryness at 45 ºC on water bath. The residue was shaken with chloroform and filtered into a clean and dry test tube. 2 ml of acetic acid anhydride was added to the filtrate and shaken. 1 ml of concentrated sulphuric acid was added carefully down the side of the tube to form a lower layer. A brownish-red or violet ring at the zone of contact of the two liquids and the upper layer turning green denotes the presence of sterols and terpenes (Cuilei, 1990).

3.2.2.6 *Test for Alkaloids*

Aqueous solution of the sample (1 ml) was treated with strong ammonia to make alkaline (pH 8) and then extracted twice with 10 ml portions of chloroform. The chloroform extracts were combined and concentrated *in vacuo* to about 5 ml. The concentrate was then tested for the presence of alkaloid using the following reagents:

Dragendorff:

Appearance of orange or orange-yellow precipitate indicates the presence of alkaloids

Meyer:

Appearance of white or creamy white precipitate indicates the presence of alkaloids

Picric acid:

Appearance of yellowish precipitate indicates the presence of alkaloids

Wagner:

Appearance of light brown to brownish precipitate indicates the presence of alkaloids

3.2.2.7 *Test for Anthraquinones and their Derivatives*

The sample (2 g) was extracted with 10 ml of chloroform and filtered. 5 ml of 10% ammonia solution was added to the filterate and shaken. The presence of a pink, red or violet colour in the ammoniacal (lower layer) phase indicates the presence of free hydroxyl anthraquinone. For the anthraquinone glycosides, 2 g of the sample was boiled with 10 ml of dilute sulphuric acid and filtered while hot. The filtrate was shaken with 5 ml chloroform. The chloroform layer was then separated and to it, 3 ml of 10% ammonia solution was added and the mixture shaken. Appearance of a pink, red or violet colouration in the lower layer indicates the presence of anthraquinone glycosides (Sofowora, 1993).

3.2.3 Chromatographic Separation

3.2.3.1 *Thin Layer Chromatography (TLC)*

This was carried out on TLC plate pre-coated with silica gel 60 PF_{254} (0.2 mm thick). Appropriate solvent systems were developed using ethylacetate: methanol in various proportions. Spots were visualized under UV light (254 and 256 nm) and sprayed with Gibbs reagent and 10% sulphuric acid, followed by heating at 110˚C for 5-10 minutes.

3.2.3.2 *Purification of the n-butanol Fraction of the Methanolic Extract of Securinega virosa*

The following column conditions were employed in running the column chromatography.

(a) *Technique* - Gradient elution

(b) *Column* - Glass column (100 cm x 4 cm) with sintered disc at the bottom of various dimensions

(c) *Stationary phase* - Silica gel, 60 – 120 mesh size

(d) *Column packing* - Wet slurry method

(e) *Sample loading* - 3 g pre-adsorbed on silica gel

(f) *Monitoring* – TLC was used to monitor fractions

The column was eluted continuously using ethyl acetate and ethyl acetate-methanol by gradient elution technique. The progress of elution was monitored using thin layer chromatography. Forty five fractions of 50 ml each were collected. The fractions were combined based on their TLC profiles to yield 6 major fractions and the column was finally washed with methanol to afford the seventh fraction. Column fraction, PFA which showed four major spots was further subjected to gel filtration**.**

3.2.3.3 *Structural Elucidation of Isolated Compound*

Purification of column fraction PFA led to the isolation of compound X. Compound X was re-crystallised in ethyl acetate and subjected to chemical test, melting point determination, mass spectroscopic analysis, 1D and 2D NMR, and X-ray crystallography.

3.2.3.4 *Purification of Residual Aqueous fraction of Methanolic Root Bark Extract of Securinega virosa*

The residual aqueous fraction (4 g) was subjected to gel filtration using sephadex LH-20 affording two major column fractions, PF1 and PF2.

3.2.4 Drug Preparation and Administration

3.2.4.1 *Preparation*

The crude methanolic extract, ethyl acetate, n-butanol and residual aqueous fractions; d-strychnine, pentylenetetrazole, picrotoxin, 4-Aminopyridine, sodium valproate, phenytoin were prepared by dissolving the powder in deionized water prior to administration. For the anticancer study, extract and fractions were dissolved in DMSO.

Diazepam and phenobarbitone were supplied in ampoules and appropriate dilutions were made with deionised water.

All drug solutions were prepared freshly for each day's experiment to maintain stability.

3.2.4.2 *Routes of Drug Administration*

The crude methanolic extract, ethyl acetate, n-butanol and residual aqueous fractions; column fractions, sodium valproate, phenytoin, diazepam and phenobarbitone were administered

intraperitoneally while pentylenetetrazole, strychnine, 4-Aminopyridine and picrotoxin were administered subcutaneously.

3.2.4.5 *Volume of Injected Drug Solutions*

The volume of drug solution administered to each mouse, rat or chick was maintained at a constant proportion irrespective of the dose.i.e 10 ml/kg for mice and chicks and 1 ml/kg for rats.

3.2.5 Toxicological Studies

3.2.5.1 *Acute Toxicity Studies in Mice*

Intraperitoneal and oral LD_{50} determinations were conducted on the methanolic root bark extract of *Securinega virosa* using the method of Lorke (1983) in mice. Briefly; the method was divided into two phases. In the initial phase, three (3) groups each containing three mice were treated with the methanolic root bark extract of the plant at doses of 10,100 and 1000 mg/kg body weight *i.p*. and observed for signs of toxicity and death for 24 hours. In the second phase, four (4) groups each containing one mouse were administered with four more specific doses of the extract based on the result of the initial phase (Phase I). The LD_{50} value was determined by calculating the geometric mean of the lowest dose that caused death and the highest dose for which the animal survived (0/1and 1/1), respectively. The same procedure was repeated for the residual aqueous, n-butanol and ethyl acetate fractions.

3.2.6 Anticonvulsant Studies

3.2.6.1 *Maximum Electroshock-induced Seizure in Chicks*

The method described by Swinyard and Kufferberg (1985) as modified by Sayyah *et al.* (2002) was employed in this study. Fifty (50) one-day old white cockerels were randomly divided into

five groups each containing ten chicks. The first group received normal saline (10 ml/kg). The second, third and fourth groups received 75, 150 and 300 mg n-butanol fraction per kg body weight, respectively. The fifth group was given 20 mg phenytoin per kg body weight. Thirty minutes after pre-treatment, maximal electroshock was administered to induce seizure in the chicks using Ugobasile electroconvulsive machine (Model 7801) connected to Claude Lyons stabilizer with corneal electrodes placed on the upper eyelids of the chicks. The shock duration, frequency and pulse width were set and maintained at 0.80 s, 200 pulse per second and 0.8 ms respectively. A current of about 90 mA, which produced tonic seizures in 90% of the control chicks, was used throughout the study. Seizures were manifested as tonic hind-limb extension (THLE) (Swinyard, 1969). The ability to prevent this feature or reduce the recovery time was considered as an indication of anticonvulsant activity (Swinyard, 1969; Sayyah *et al*., 2002). The method was repeated for ethyl acetate fraction (125, 250 and 250 mg/kg) and residual aqueous fraction (125, 250 and 500 mg/kg).

3.2.6.2 *Pentylenetetrazole-induced Seizure in Mice*

The method of Swinyard *et al.* (1989) was employed. Thirty mice were divided into five groups each containing six mice. The first group received normal saline (10 ml/kg). The second, third and fourth groups received 75, 150 and 300 mg n-butanol fraction per kg body weight. The fifth group was given 200 mg sodium valproate per kg body weight. Thirty minutes post treatment, mice in all the groups received 85 mg pentylenetetrazole per kg, *s.c.* Mice were observed over a period of 30 minutes. Absence of an episode of clonic spasm of at least 5 seconds duration indicated a compound's ability to abolish the effect of pentylenetetrazole on seizure threshold. The same method was repeated for the residual aqueous fraction (125, 250 and 500 mg/kg) and ethyl acetate fraction (125, 250 and 500 mg/kg).

3.2.6.3 *Subcutaneous Strychnine-induced Seizure in tice*

The method described by Porter *et al*. (1984) was adopted in this study. Thirty mice were divided into five groups each containing six mice. The first group received normal saline (10 ml/kg). The second, third and fourth groups received 75, 150 and 300 mg n-butanol fraction per kg body weight, respectively. The fifth group was treated with 30 mg phenobarbitone per kg body weight. Thirty minutes post-treatment, mice in all the groups received 1.5 mg strychnine per kg. The proportion of mice presenting convulsions as well as the onset of tonic convulsions was recorded. Abolition of tonic extensor jerks of the hind limbs within 30 minutes after strychnine administration was considered an indicator that the testing material could prevent strychnine-induced convulsions. The same procedure was repeated for the residual aqueous fraction (125, 250 and 500 mg/kg).

3.2.6.4 *4-Aminopyridine-induced Seizure in Mice*

The method adopted for this study was similar to those described previously (Rogawski and Porter, 1990; Yamaguchi and Rogawski, 1992). Thirty (30) mice were randomly divided into five groups each containing 6 mice. The first group received normal saline (10 ml/kg). The second, third and fourth groups received 75, 150 and 300 mg n-butanol fraction per kg body weight, respectively. The fifth group was given 30 mg phenobarbitone per kg body weight. 15 minutes post treatment; 4-Aminopyridine was administered at a dose of 15 mg per kg body weight to each mouse. The mice were observed for 30 minutes for characteristic behavioural signs, such as hyperactivity, trembling, intermittent forelimb and hind limb clonus followed by hind limb extension, tonic seizures, opisthotonus and death. Ability of the extract to protect the mice from lethality within 30 minutes observation period was considered as an indication for

anticonvulsant activity (Yamaguchi and Rogawski, 1992). The same procedure was repeated for the residual aqueous fraction (125, 250 and 500 mg/kg).

3.2.6.5 *Picrotoxin-induced Seizure in Mice*

The method previously described by Salih and Mustafa (2008) was adopted. Thirty (30) mice were grouped into five each consisting of six mice. The first group received normal saline (10 ml/kg). Mice in the second, third and fourth groups received 75, 150 and 300 mg per kg body weight of the n-butanol fraction, respectively, while mice in the fifth group received 10 mg diazepam/kg body weight. 30 minutes post treatment, 10 mg picrotoxin /kg body weight was administered to each mouse. They were then observed for hind limb tonic seizures for thirty minutes. Absence of tonic hind limb extension or prolongation of the latency of the hind limb tonic extension was considered as an indication of anticonvulsant activity (Navarro-Ruiz *et al.*, 1995). The procedure was repeated for the residual aqueous fraction (125, 250 and 500 mg/kg).

3.2.6.6 *Kindling Studies*

The kindling study was conducted in the Department of Neurology, University of Virginia, USA.

Implantation of electrodes:

Bipolar insulated stainless steel electrodes were implanted stereotaxically in the left posterior ventral hippocampus under ketamine/xylazine anesthesia according to the following coordinates (mm) from bregma: nose bar -2.5 below the interaural line, AP -5.3, L ± 4.9, H 5.0 below dura and secured to the skull with dental acrylic (Lothman *et al*., 1988).

Electroencephalographic (EEG) recordings and Behavioural seizure scale:

EEG recordings were made using bilateral cortical and hippocampal electrodes in unanaesthetized, freely moving animals using a Grass 79D polygraph. The EEG recording was continuous for at least 5 minutes after stimulation. Behavioural seizures were scored according to the modified scale of Racine (Racine, 1972) : grade 0, normal behaviour, arrest, wet dog shakes; grade 1, facial twitches; grade 2, chewing, head nodding; grade 3, forelimb clonus; grade 4, clonus with rearing; grade 5, clonus with rearing and falling on the back; grade 6, wild running.

Kindling procedure:

Kindling was started after a postoperative period of seven days, when the animals showed no behavioural signs of pain or discomfort. Each animal served as its own control. The rats were allowed to acclimatize in a Plexiglas cage and an EEG recording was made for at least 10 trains to assess the spontaneous EEG pattern. Constant current stimuli were delivered unilaterally to the ventral hippocampus through a bipolar electrode (hippocampal electrode) six times daily every other hour, on alternate days until the rats become fully kindled with consistent stage 5 behavioural seizure score. The stimulation parameters were 50-Hz, 2-ms biphasic rectangular wave pulses for 1 s. The current intensity was 400 mA. Behaviour was observed and the duration of seizure was measured in the stimulated hippocampus after each stimulation for each animal. The readings were taken for the animals two days before the experiment day to obtain the baseline readings on drug free day. On the treatment day, the animals were stimulated every other hour as described above and the BSS and EEG recording were taken. 15 minutes after the second stimulation, the animals were treated with the highest doses of the fractions [residual aqueous fraction (500 mg/kg) and n-butanol fraction (300 mg/kg)]. The animals were subjected to four more stimulations post-treatment and the EEG recordings and BSS grading were taken.

3.2.7 Behavioural Studies

3.2.7.1 *Diazepam-induced Sleep in Mice*

The method described by Beretz *et al*. (1978) and modified by Rakotonirina *et al*. (2001) was adopted in this study. Mice were randomly divided into four groups each containing 6 mice. The first group received normal saline (10 ml/kg). The second, third and fourth groups received 75, 150 and 300 mg n-butanol fraction per kg body weight. Thirty (30) minutes post-treatment, the mice were administered diazepam at a dose of 25 mg/kg body weight. The mice were placed individually in cages. The onset and the duration of sleep were determined for each animal. Loss of rightening reflex was considered as the criterion for sleep (Rolland *et al*., 1991) while the interval between the loss and the recovery of straightening was regarded as the duration of sleep (Fujimori, 1965). The same procedure was repeated for the residual aqueous fraction (125, 250 and 500 mg/kg), ethyl acetate fraction (125, 250 and 500 mg/kg), PFA, PFB and PFC (at doses of 2.5, 5 and 10 mg/kg), PF1 and PF2 (at doses of 2.5, 5 and 10 mg/kg) and compound X.

3.2.7.2 *Test for Exploratory Behaviour in Mice*

The method for the hole-board test in mice was similar to those described previously (File, 1973). The apparatus used was a white painted wooden board (60 cm x 30 cm) with 16 evenly spaced holes (1cm diameter x 2 cm depth). Mice were randomly grouped into five each consisting of six mice. The first group received normal saline (10 ml/kg); second third and fourth groups received 75, 150 and 300 mg n-butanol soluble fraction per kg body weight, respectively. The fifth group was treated with 2 mg diazepam per kg body weight. Thirty (30) minutes post-treatment, each mouse was placed at a corner of the board and the number of head dips on the hole was counted using a tally counter during a 5 minute period (Wolfman *et al*.,

1994). A head dip was considered when the mouse dipped its head into the hole to the level of the eyes. The same procedure was repeated for the residual aqueous fraction (125, 250 and 500 mg/kg).

3.2.7.3 *Mouse Beam Walking Assay in Mice*

The method previously described by Stanley *et al*. (2005) was adopted with some modification for this study. Mice were trained to walk from a start platform along a ruler (80 cm long, 3 cm wide) elevated 30 cm above the bench by metal support to a goal box. Three trials were performed for each mouse, and were designed such that the mouse tested would be aware that there was a goal box that could be reached. The goal box was a Perspex glass cage (with wood chippings beddings) with a small hole at the bottom. The mice that successfully walked along the ruler were randomly grouped into five groups each containing six mice. The first group received normal saline (10 ml/kg). The second, third and the fourth groups received the n-butanol fraction at doses of 75, 150 and 300 mg/kg body weight respectively. The fifth group received diazepam (2 mg/kg body weight). The beam was made of wood, 8 mm in diameter, 60 cm long and elevated 30 cm above the bench by metal support. Thirty (30) minutes post-treatment, each mouse was placed on the beam at one end and allowed to walk to the goal box. Mice that fell were returned to the position they fell from, with a maximum time of 60 s allowed on beam. The number of foot slips (one or both hind limb slipping from the beam) was recorded with the aid of a tally counter. The time taken to complete the task was also recorded. The same procedure was repeated for the residual aqueous fraction (125, 250 and 500 mg/kg) and compound X.

3.2.7.4 *Open Field Test in Mice*

The study was conducted according to method previously described by Brown *et al*. (1999) with some modifications. The apparatus was made up of plywood measuring 72 cm x 72 cm x 36 cm. One of the walls was made of transparent Perspex glass to ensure that the mouse under investigation was visible to the observer. The floor, made of cardboard was divided into 16

equal squares (18 cm x 18 cm) with blue marker and a central square drawn with black marker. The cardboard was covered with a transparent Plexiglas. 30 mice were divided into 5 groups (n=6). The groups were treated with normal saline (10 ml/kg), the fraction (75, 150 and 300 mg/kg) or diazepam (0.5 mg/kg). Thirty (30) minutes post-treatment, each mouse was placed individually at the corner of the arena and it behaviour monitored for 5 minutes with a video camera hung 2 m above the apparatus and connected to a monitor. The number of rearings, number of squares and number of central square crossed by each mouse was recorded. The apparatus was wiped between observations with 70 % ethyl alcohol and allowed to dry to remove any olfactory cue. The same procedure was repeated for the residual aqueous fraction (125, 250 and 500 mg/kg).

3.2.7.5 *Staircase Test in Mice*

The device used in this study consists of a wooden staircase similar to the one described by Simiand *et al.* (1984). The staircase was enclosed in transparent Perspex vertical walls (45 cm x 12 cm x 25 cm) and had 5 identical steps 2.5 cm high, 10 cm wide and 7.5 cm deep. Thirty (30) minutes before placing each mouse individually on the floor of the Perspex box (with its back to the staircase), five groups each consisting of six mice were treated with normal saline (10 ml/kg), n-butanol fractions (75, 150 and 300 mg/kg) and diazepam (0.5 mg/kg), respectively. The behaviour of each mouse was videotaped and number of upward steps climbed and rearings were recorded over a 5 minutes period. A step was considered climbed if a mouse placed all its four paws on it. Rearing was counted when a mouse rose on its hind limb both against the wall and on a step. The staircase was wiped with 70% ethyl alcohol and allowed to dry between tests to remove any olfactory cue. The same procedure was repeated for the residual aqueous fraction (125, 250 and 500 mg/kg).

3.2.7.6 *Elevated Plus Maze (EPM) Test in Mice*

The EPM test was conducted according the method previously described by Hogg (1996) and Rodgers *et al*. (1997). The maze comprised of two open arms (35 cm x 5 cm) and two closed arms (30 cm x 5 cm x 15 cm) which were connected by a common central area (5 cm x 5 cm) and elevated to a height of 60 m above the floor. The floor and the walls of each were wooden and painted black (Rabbani *et al*., 2003). The test was conducted in a room illuminated by a 60Watt red bulb. Thirty (30) mice were divided into five groups (n=6) and treated with normal saline (10 ml/kg), the fraction (75, 150 and 300 mg/kg) or diazepam (0.5 mg/kg). Thirty (30) minutes post treatment, each mouse was placed at the centre platform facing one the open arm and its behaviour videotaped over a 5 minutes period. A mouse is said to have entered an arm when it has placed all four paws over the line separating the area and the centre. The maze was wiped with 70% ethyl alcohol and dried between trials to remove olfactory cues. The numbers of open and close arm entries as well as the time spent in both open and closed arms were recorded. The same procedure was repeated for the residual aqueous fraction (125, 250 and 500 mg/kg).

3.2.8 Anticancer Studies

The anticancer studies were conducted in the Department of Pathology, University of Virginia, USA.

3.2.8.1 *Kill Study*

Cells in 6 well dishes were treated with dimethylsulphoxide, the crude methanolic root bark extract or its fractions (CCF, EAF, NBF and RAF) at concentrations of 31.5-500 µg/ml; and incubated at 37 ºC for 48 hours. Cells were subsequently washed three times with deionized water and stained with crystal violet dye. The cells were subsequently photographed. Adherent

cells were released from the plate with 0.05% w/v trypsin-EDTA (0.53 mM) for 2 min. The trypsin activity was halted by addition of equal volume of serum-free α-MEM. 30 µl of the trypsinized cells was then collected in a cuvette and the viable cell count was determined using the O_2 coulter counter instrument. The count was done in quadruplicate.

In another study, cells were treated with DMSO, the crude extract or its fractions (31.5-500 µg/ml). One hour later, EGF (25ng/µl) was added to each well. The cells were incubated for 48 hours and the same procedures for cell count were carried out as described earlier.

3.2.8.2 *Western Blot Analysis*

The method previously described by Hussaini *et al*. (2000) was adopted. Cells in 100 ml dishes were serum-starved for 24 h after reaching 80–100% confluence by replacing the medium with serum-free α-minimal essential medium. The cells were treated with the crude extract (CRE) and it fractions (CCF, EAF, NBF and RAF) at concentration of 250 ng/µl or DMSO (1µl/ml) and allowed to stand for one hour in the incubator. Thereafter, EGF (25 ng/ml), PMA (10µM) or PDGF (20 ng/µl) were added separately and allowed to stand for 30 minutes in the incubator After the different treatments, the cells were washed with phosphate-buffered saline (PBS; 137 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, and 1.5 mM KH_2PO_4, pH 7.4) containing 0.2 mM sodium orthovanadate and extracted with 1% Triton X-100 (containing 2 mg/ml sodium orthovanadate and 5 mg/ml DTT) . Cells were centrifuged at 14,000 x *g* for 1 min at 4 °C. The protein concentration of the supernatant was determined by the BCA protein assay (Bio-Rad). Proteins were boiled for 5 min in SDS-PAGE buffer. Proteins (200 µg/lane) were separated by SDS-PAGE polyacrylamide gels (10 % for EGF and PMA; and 8 % for PDGF) and then electroblotted onto nitrocellulose. Immunoblotting was carried out with mouse or rabbit antibodies specific for the EGFR, MAPK and Akt. Immunoblotted proteins were detected using

the ECL reagents (Amersham Biosciences) as described by the manufacturer with horseradish peroxidase-conjugated secondary antibodies.

3.2.9 Statistical Analysis

Results were expressed as percentages and mean ± standard error of mean. Statistical analysis was performed by analysis of variance (ANOVA); when a statistically significant result was obtained with ANOVA, a post hoc Dunnets t-test was performed for multiple comparisons. Values of $P < 0.05$ were considered significant.

CHAPTER 4

RESULTS

4.1 THE PLANT EXTRACT AND FRACTIONS

4.1.1 Fractionation of Crude Methanolic Root Bark Extract

The fractionation of the crude methanolic root bark extract of *Securinega virosa* resulted into five fractions. The residual aqueous fraction has the highest yield while the yield of the petroleum ether fraction was negligible (Table 4.1.1).

Table 4.1.1 Percentage Yield of Fractions obtained from Methanolic Root Bark Extract of *Securinega virosa*

Fractions	Yield (g)	Yield (%)
Petroleum ether fraction	-	Negligible
Chloroform fraction	0.28	0.56
Ethyl acetate fraction	1.7	3.4
n-butanol fraction	7.32	14.64
Residual Aqueous fraction	30.82	61.64

4.1.2 Chromatographic Separation of n-butanol and Residual Aqueous Fraction of Methanolic Root Bark Extract of *Securinega virosa*

Separation of the n-butanol fraction yielded seven fractions (Table 4.2.1). The three major column fractions namely PFA, PFB and PFC were subjected to further purification using gel filteration and solvent-solvent partition. The major spot of PFA was further purified resulting into a pure compound code named compound X, weighing 21.5 mg. The compound gave a characteristic greenish spot with 10% sulphuric acid and brown spot with Gibb's reagent (Plate II).

Gel filteration of the residual aqueous fraction of methanolic root bark extract of *Securinega virosa* using sephadex LH-20 eluted with methanol led to 2 major column fractions; namely PF1 and PF2.

Table 4.1.2: Results of Column Chromatographic Separation of n-butanol Fraction

No.	Eluting solvent	Weight (g)	No of spots	TAG
1	EtoAC (100)	0.20	5	
2	EtoAC: MeOH (95:05)	0.10	1 major, 1 minor	PFA
3	EtoAC: MeOH (90:10)	0.50	3 major, 1 minor	PFB
4	EtoAC: MeOH (80:20)	0.23	2	PFC
5	EtoAC: MeOH (70:30	0.30	5	
6	EtoAC : MeOH (50:50)	0.59	6	
7	MeOH (100%)	0.80	6	



Plate II: A chromatogram of compound X developed in ethyl acetate: methanol (7:0.5)

Table 4.1.3: Results of Gel Filteration of Residual Aqueous Fraction of Methanolic Root Bark Extract of *Securinega virosa*

No	Weight (g)	No of spots	TAG
1	0.12	5 minors	
2	0.9	1 major, 4 minor	PF1
3	1.2	1 major , 5 minor	PF2
4	0.6	6	
5	0.9	5	

4.1.3 Preliminary Phytochemical Screening of Extract and Fractions of Root Bark of *Securinega virosa*

The preliminary phytochemical screening of the methanolic root bark extract, n-butanol soluble fraction and residual aqueous fraction of *Securinega virosa* revealed the presence of alkaloids, tannins, saponins, flavonoids, resin and carbohydrates. Alkaloids and saponins were found to be absent in the ethyl acetate fraction (Table 4.1.4). PFA, PFB and PFC were shown to contain flavonoids but deficient in alkaloids. Only PFC tested positive for saponins (Table 4.1.5). PF1 and PF2 were found to contain flavonoids, saponins, alkaloids and triterpenes (Table 4.1.6).

Table 4.1.4: Phytochemical Constituents of the Methanolic Root Bark Extract of *Securinega virosa* and its Fractions

Constituents	MRBE	EAF	NBF	RAF
Carbohydrate	+	+	+	+
Tannins	+	+	+	+
Saponins	+	-	+	+
Flavonoids	+	+	+	+
Alkaloids	+	-	+	+
Resins	+	+	+	+
Steroid/Terpenoids	+	+	-	+
Anthraquinone	-	-	-	-

+ Present
-Absent

Table 4.1.5: Preliminary Phytochemical Screening of Column Fractions obtained from n-butanol Fraction of *Securinega virosa*

Constituents	**PFA**	**PFB**	**PFC**
Flavonoid	+	+	+
Saponins	-	-	+
Alkaloids	-	-	-
Triterpenes	-	-	-

+ Present
-Absent

Table 4.1.6: Preliminary Phytochemical Screening on Column Fraction obtained from Residual Aqueous Fraction of *Securinega virosa*

Constituents	**PF1**	**PF2**
Flavonoid	+	+
Saponins	+	+
Alkaloids	+	+
Triterpenes	+	+

+ Present
-Absent

4.1.4 Structural Elucidation and Characterisation of Compound X

4.1.4.1 *Melting Point compound X*

Compound X was found to melt at a temperature range of 236-239 °C.

4.1.4.2. Proton NMR of Compound X

The ^{1}HNMR spectra of compound X exhibited signal for an aromatic hydrogen (δ 7.1(s) 1H), 4.98 (d), 3.42 (dd, J=9, 5; 8.5Hz, 1H and 3.80, dd J=9.5, 8.5 1H. It also revealed the presence of three hydrogens δ3.9, (s) due to methoxyl group. Other resonances were at δ 4.10, 1H, dd J=9.5; 10Hz, and at 3.7(1H) m (Table 4.1.7; Figure 4.1.1)

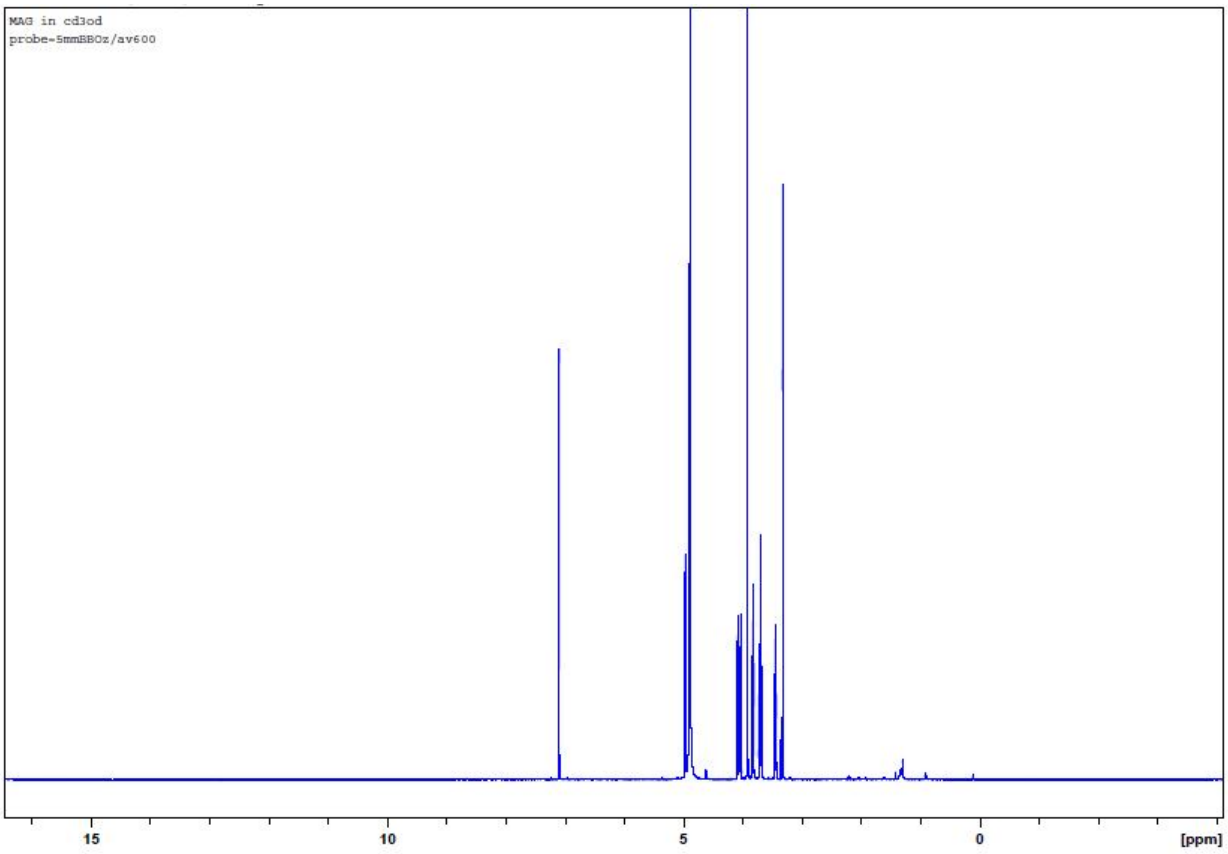


Figure 4.1.1: Full proton NMR of compound X

4.1.4.3 *Carbon-13 NMR of Compound X*

The ^{13}C and distortionless enhancement by polarization transfer (DEPT) NMR spectra of the compound disclosed one carbon singlet for methyl group, one methylenecarbon, five aliphatic methane carbons, one aromatic methine carbon and six quaternary carbon; five aromatic and one due to carbonyl carbon (Table 4.1.7; Figures 4.1.2, 4.1.3, 4.1.4)**.** The HSQC spectra of compound X were used to assign protons on their respective carbons (Table 4.1.8; Figures 4.1.5, 4.1.6).

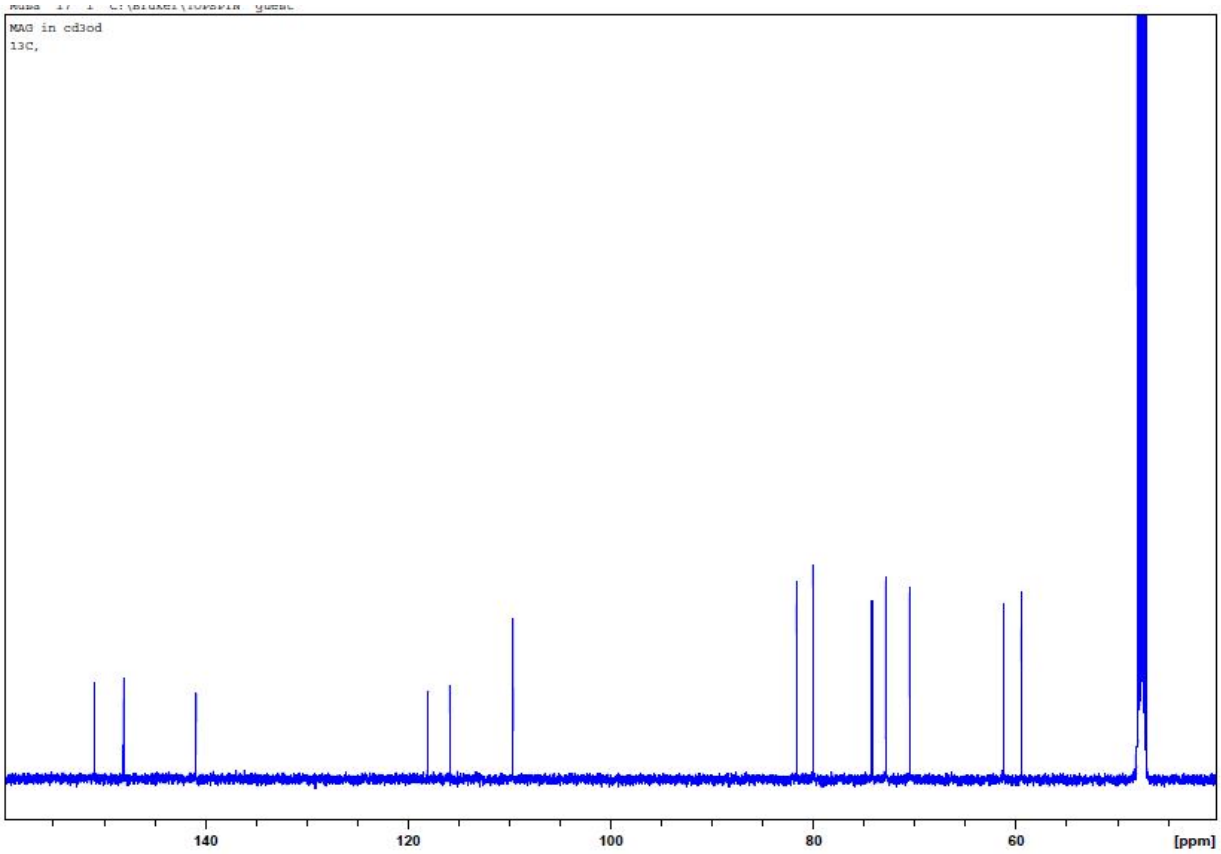


Figure 4.1.2: Carbon-13 NMR of compound X

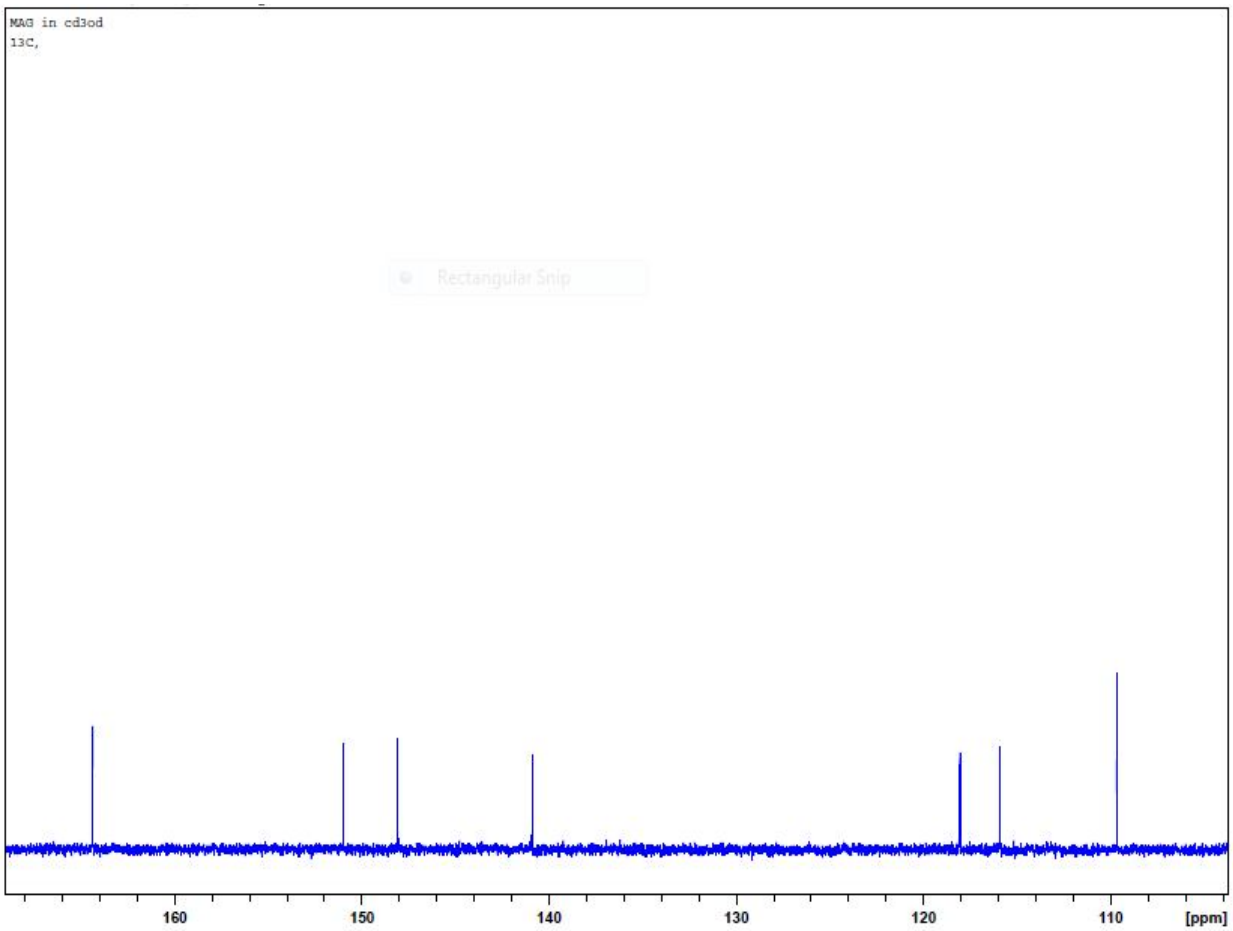
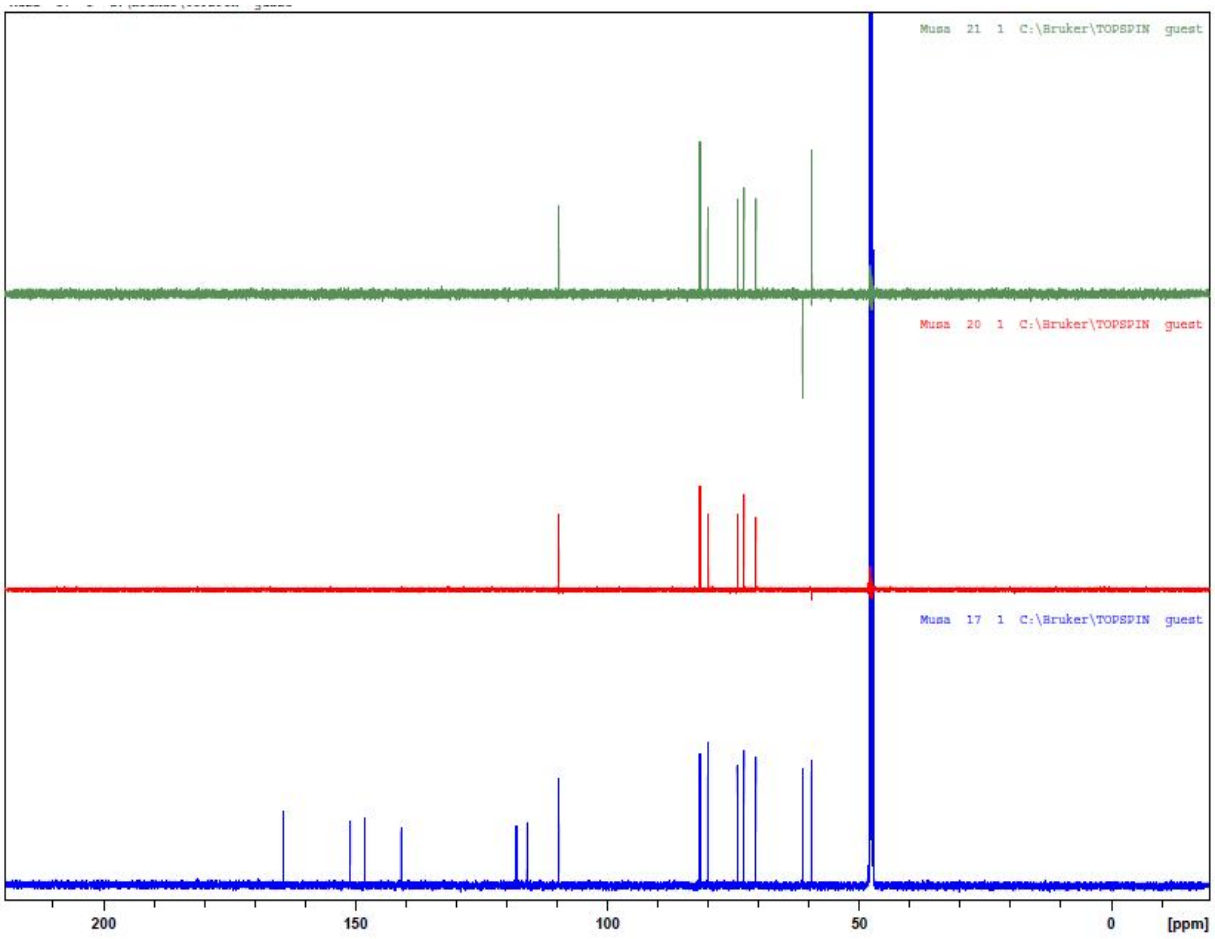


Figure 4.1.3: Carbon-13 NMR (Expanded) of compound X



4.1.4: DEPT of compound X

Table 4.1.7: NMR Data of Compound X

Position	DEPT	δC	δH, J in Hz
1	-	-	-
2	C=O	163.0	-
3	C	118.0	-
4	CH	109.0	7.1(s)
5	C	152.0	-
6	C	141.0	-
7	C	148.0	-
8	C	116.0	-
9	CH	73.0	4.98
10	-	-	-
11	CH	81.9	3.7
12	CH	71.0	3.3
13	CH	74.0	3.81
14	CH	80.0	4.04
15	CH_3	59.8	3.9
16	CH_2	61.5	4.0,3.7

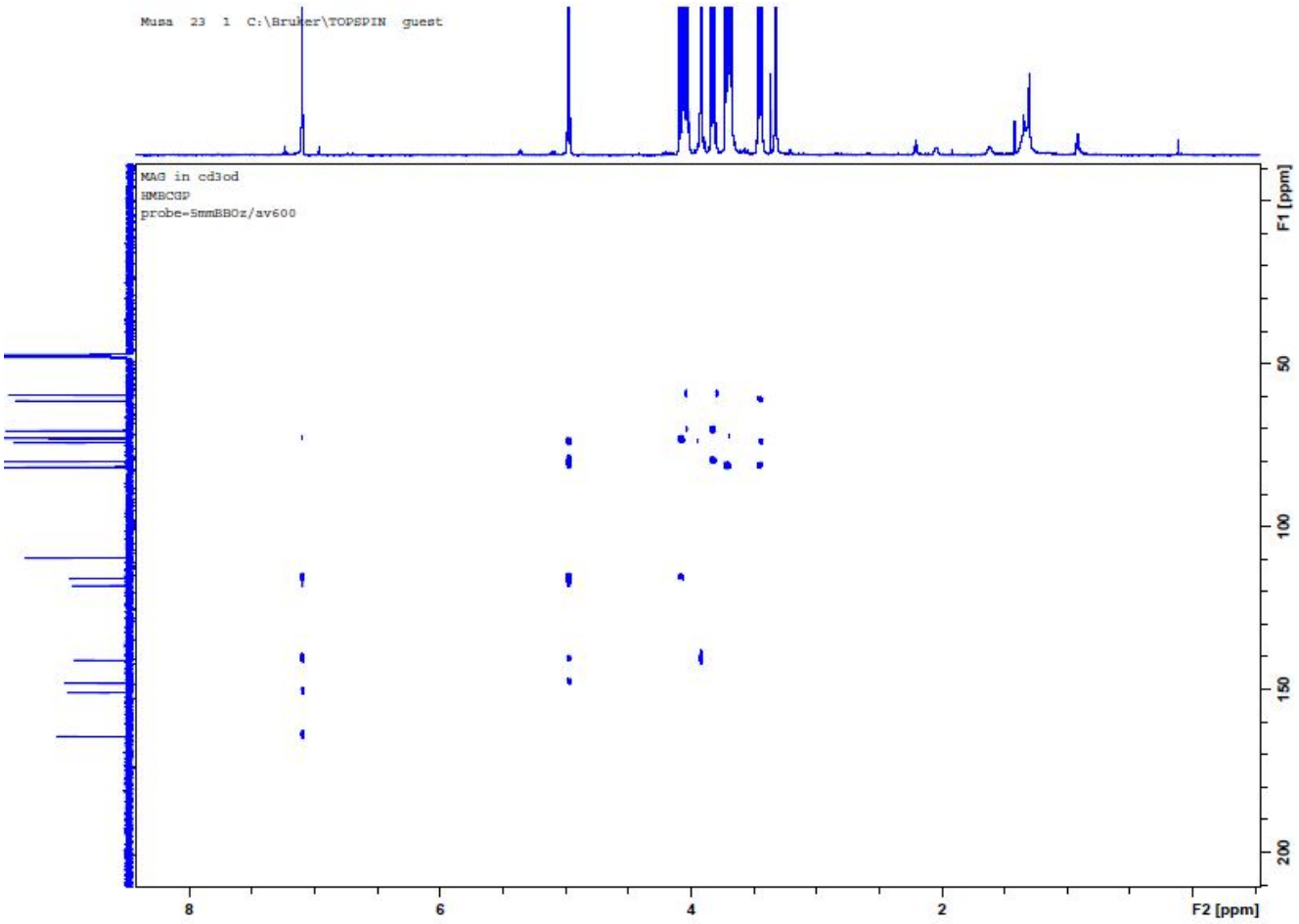


Figure 4.1.5 HMBC of Compound X

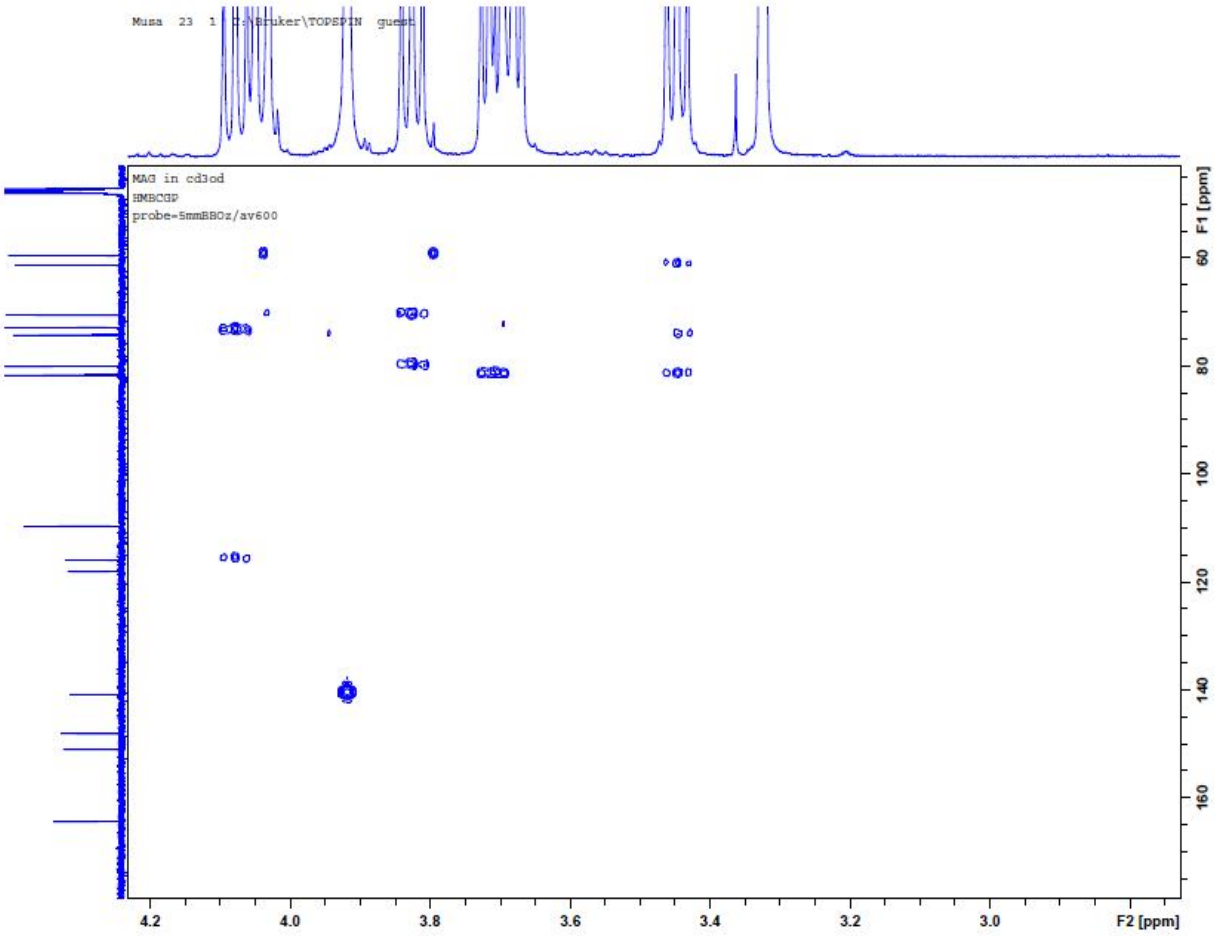


Figure 4.1.6: HMBC-2 of Compound X

Table 4.1.8: HMBC Data of Compound X

Position	δC	HMBC
1	-	-
2	163.0	-
3	118.0	9
4	109.0	5,6
5	152.0	4
6	141.0	4
7	148.0	9
8	116.0	4,9,14
9	71.0	13
10	-	-
11	81.9	11,14
12	71.0	11,13,14
13	74.0	9,12,14
14	80.0	9,11
15	59.8	14
16	61.5	12

4.1.4.4 Mass Spectroscopic and X-ray Structure Analyses of Compound X

The molecular formula of the compound X was assigned as $C_{14}H_{16}O_9$ from an $[M]^-$ peak at m/z 327 in the high-resolution EIMS with a base peak at m/z 192, also observed is a peak at 312 corresponding to M-CH_3 (Figures 4.1.7). The fragmentation pattern is in agreement with that of bergenin (Figure 4.1.8).

The title compound, 3,4,4a,10b-tetrahydro-3,4,8,10-tetrahydroxy- 2-(hydroxymethyl)-9-methoxypyrano[3,2-c][2]benzo- H)-one monohydrate, $C_{14}H_{16}O_9.H_2O$, crystallized in ethyl acetate. The data collection was made at low temperature and the results obtained agree with those reported for bergenin in the literature and database determined at room temperature (Figure 4.1.9).

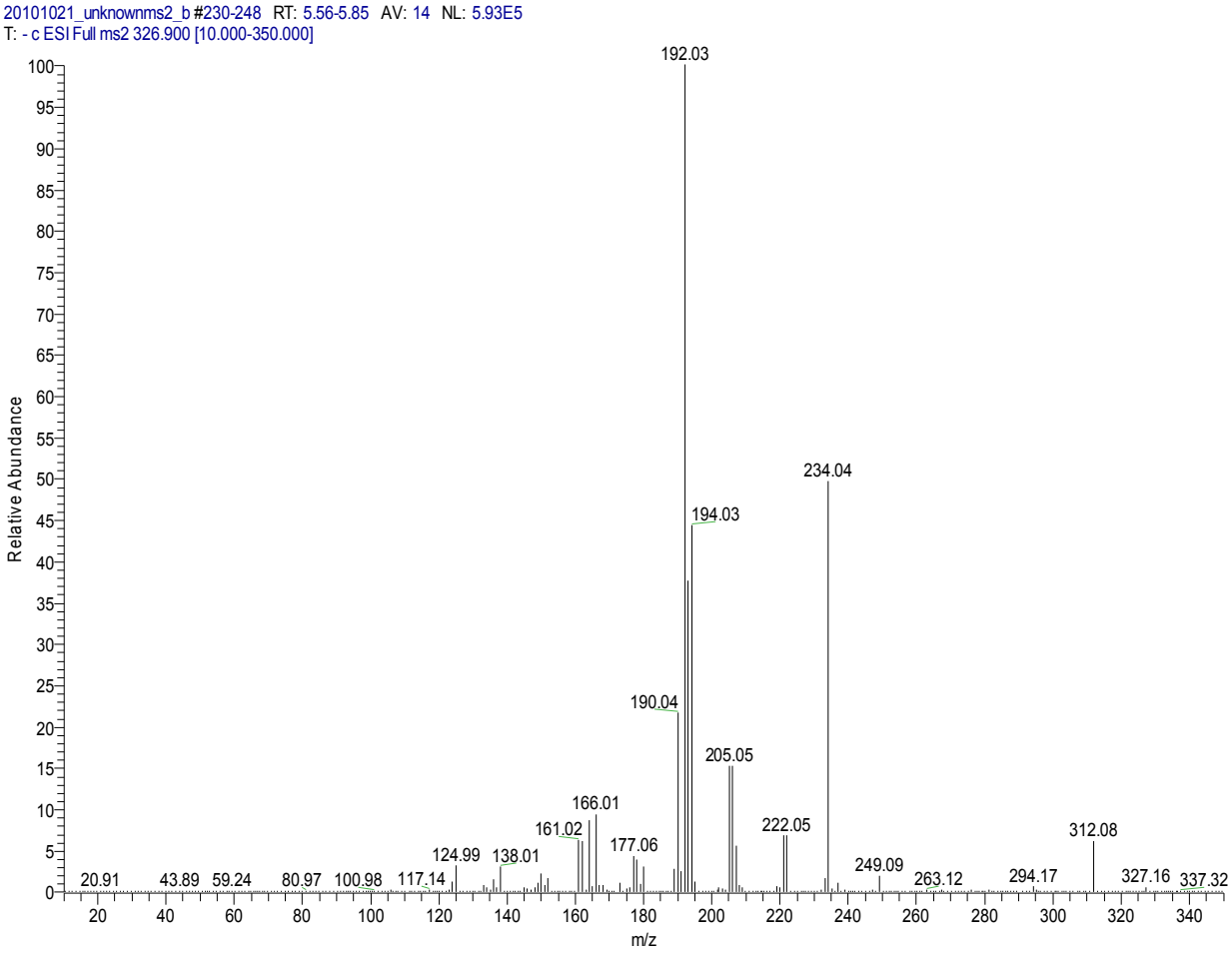


Figure 4.1.7: MS^2 Scan of Compound X

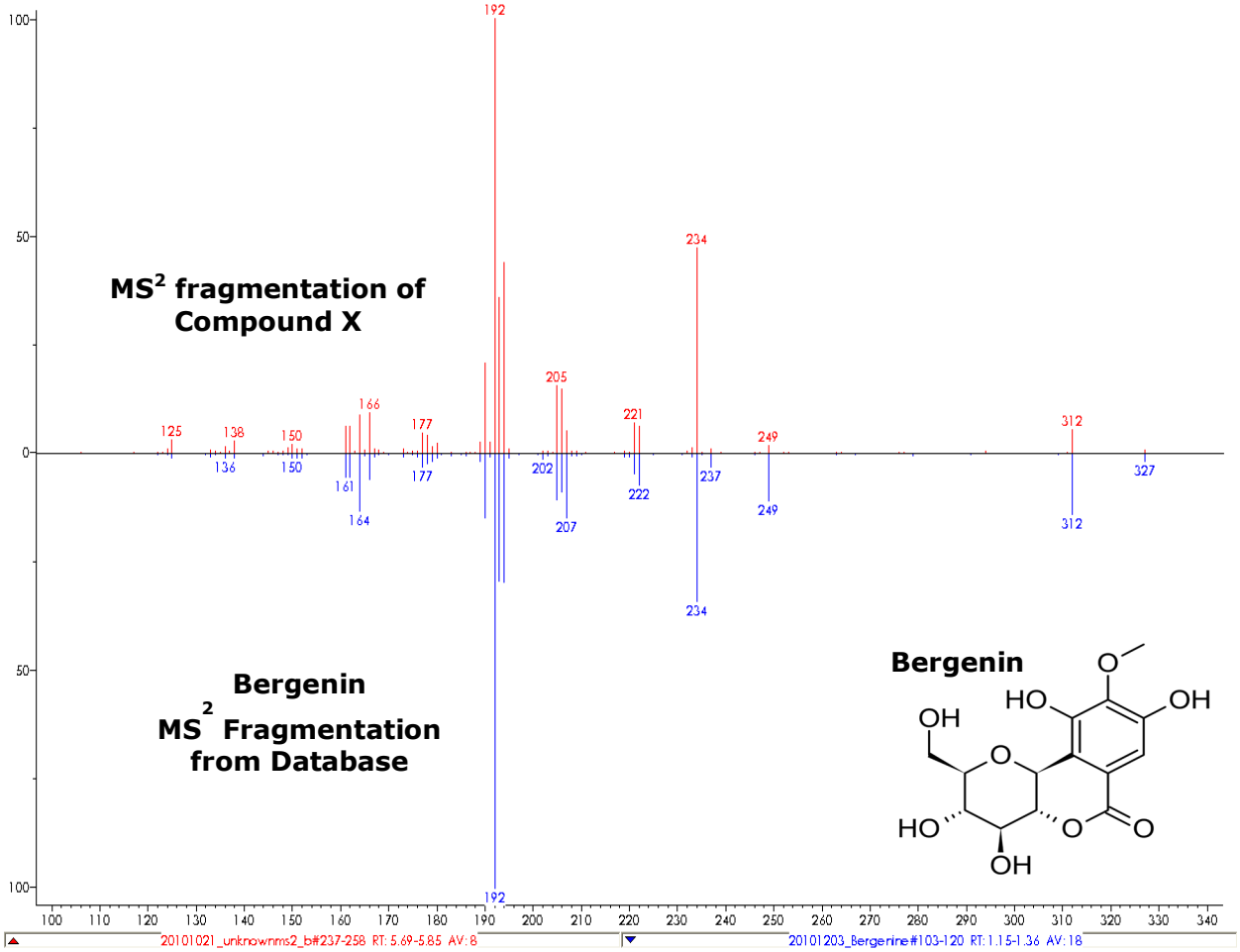


Figure 4.1.8: Comparison of MS^2 Fragmentation Patterns of Compound X and Bergenin from Database

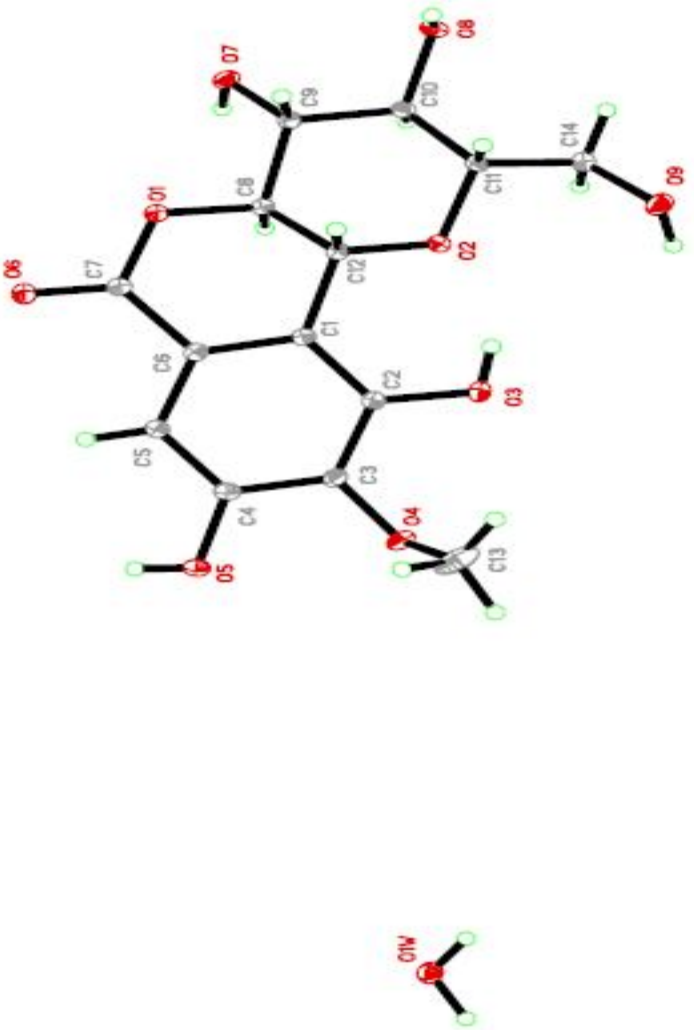


Figure 4.1.9 View of the Molecule of Bergenin showing the Atom Labelling

4.2 ACUTE TOXICITY STUDIES

The intraperitoneal median lethal doses of ethyl acetate, n-butanol and residual aqueous fractions in mice were found to be 2154, 1256.9 and 2154 mg/kg, respectively. The oral median lethal doses of the three fractions in mice were found to be greater than 5,000 mg/kg (Table 4.2.1). In rats, the intraperitoneal median lethal doses of the three fractions (ethyl acetate, n-butanol and residual aqueous fractions) were found to be 2154, 2154 and 3807.9 mg/kg, respectively. Their oral LD_{50} values in mice were estimated to be greater than 5000 mg/kg.

Table 4.2.1: Intraperitoneal and Oral Lethal Dose (LD_{50}) Values of Methanolic Root Bark Extract and Fractions of *Securinega virosa* in rodents

Fractions	LD_{50} Value (mg/kg) In Mice		LD_{50} Value (mg/kg) In rats	
	Intraperitoneal	Oral	Intraperitoneal	Oral
MRBE	774.6	>5000	1264.9	> 5000
EAF	2154	> 5000	2154	-
NBF	1256.9	> 5000	2154	> 5000
RAF	2154	> 5000	3807.9	> 5000

MRBE: Methanolic root bark extract; EAF: ethyl acetate fraction; NBF: n-butanol fraction; RAF: residual aqueous fraction

4.3 ANTICONVULSANT STUDIES

4.3.1 Effects of Fractions of Methanolic Root Bark Extract of *Securinega virosa* on Maximal Electroshock-induced Seizure in Chicks

The fractions (ethyl acetate, n-butanol and residual aqueous fractions) did not protect the chicks against tonic hind limb extension induced by maximal electroshock. There was also no significant reduction in the mean recovery time. Phenytoin protected all the chick at the dose of 20 mg/kg (Table 4.3.1).

Table 4.3.1: Effects of Fractions of Methanolic Root Bark Extract of *Securinega virosa* on Maximal Elecroshock Test in chicks

Treatment	Quantal protection	Mean Recovery time (Min)
N/Saline	0/10	5.86 ± 1.58
RAF 125	0/10	7.71 ± 1.17
RAF 250	0/10	6.88 ± 1.30
RAF 500	0/10	6.13 ± 0.48
NBF 75	0/10	4.75 ± 1.05
NBF 150	0/10	8.50 ± 1.70
NBF 300	0/10	6.86 ± 0.34
EAF 125	0/10	7.22 ± 0.88
EAF 250	0/10	7.44 ± 0.77
EAF 500	0/10	8 ± 0.85
Phenytoin 20	10/10	-

Data presented as quantal protection; EAF: ethyl acetate fraction; NBF: n-butanol fraction; RAF: residual aqueous fraction

4.3.2 Effects of Fractions of Methanolic Root Bark Extract of *Securinega virosa* on Pentylenetetrazole-induced Seizure in Mice

The residual aqueous fraction produced a dose-dependent protection with the highest dose (500 mg/kg) producing 66.67% protection (Figure 4.3.1). RAF at the dose of 250 mg/kg significantly ($P < 0.05$) increased the mean onset of seizure (Appendix 6). The column fraction PF1 obtained from the residual aqueous fraction dose-dependently protected the mice against seizure induced by PTZ with the highest protection of 66.67 % afforded at the dose of 10 mg/kg. There was also 100 % protection against mortality at the dose of 10 mg/kg (Figure 4.3.2). The column fraction PF2 obtained from the residual aqueous fraction protected 50% of the mice against both seizure and mortality induced by PTZ at the dose of 5 mg/kg (Figure 4.3.3). The column fractions of the residual aqueous fraction significantly increased the mean onset of seizure in unprotected animals (Appendix 7).

The n-butanol fraction, at the highest dose tested afforded 66.67% protection against pentylenetetrazole induced seizure in mice (Figure 4.3.4). However, there was no significant increase in the mean onset of seizure in the unprotected animals (Appendix 8). The column fraction PFA protected less than 20% of the animal against PTZ-induced seizure (Figure 4.3.5). The mean onset of seizure was increased by about 100 % at the dose of 10 mg/kg (Appendix 9). It protected 100 % of the mice against mortality at the dose of 2.5 mg/kg (Figure 4.3.5). Column fraction PFB protected 50% of the mice against PTZ-induced seizure at the dose of 10 mg/kg. There was no significant increase in the mean onset of seizure (Appendix 9). However the column fraction, PFB protected 83.33% of the mice against mortality (Figure 4.3.6). The column fraction, PFC protected 50% of the mice at the dose of 2.5 mg/kg (Figure 4.3.7) and significantly increased the mean onset of seizure at the dose of 5 mg/kg and 10 mg/kg (Appendix 4). The

column fraction, PFC protected 50% of the mice against mortality at the dose of 2.5 mg/kg (Figure 4.3.6).

The ethyl acetate fraction produced 66.67% against PTZ-induced seizure (Figure 4.3.8).

The standard agent, Sodium valproate at the dose of 200 mg/kg afforded 100 % protection against PTZ-induced seizure in mice.

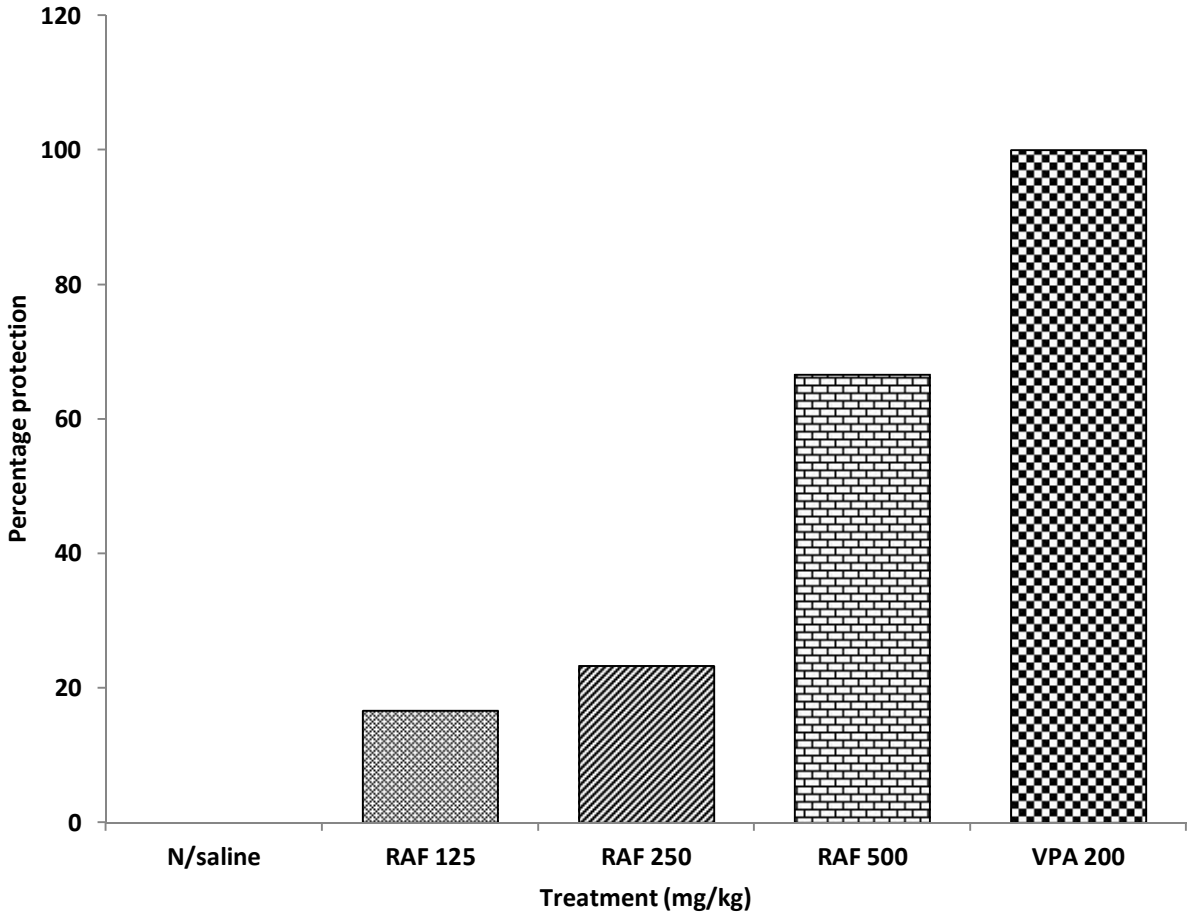
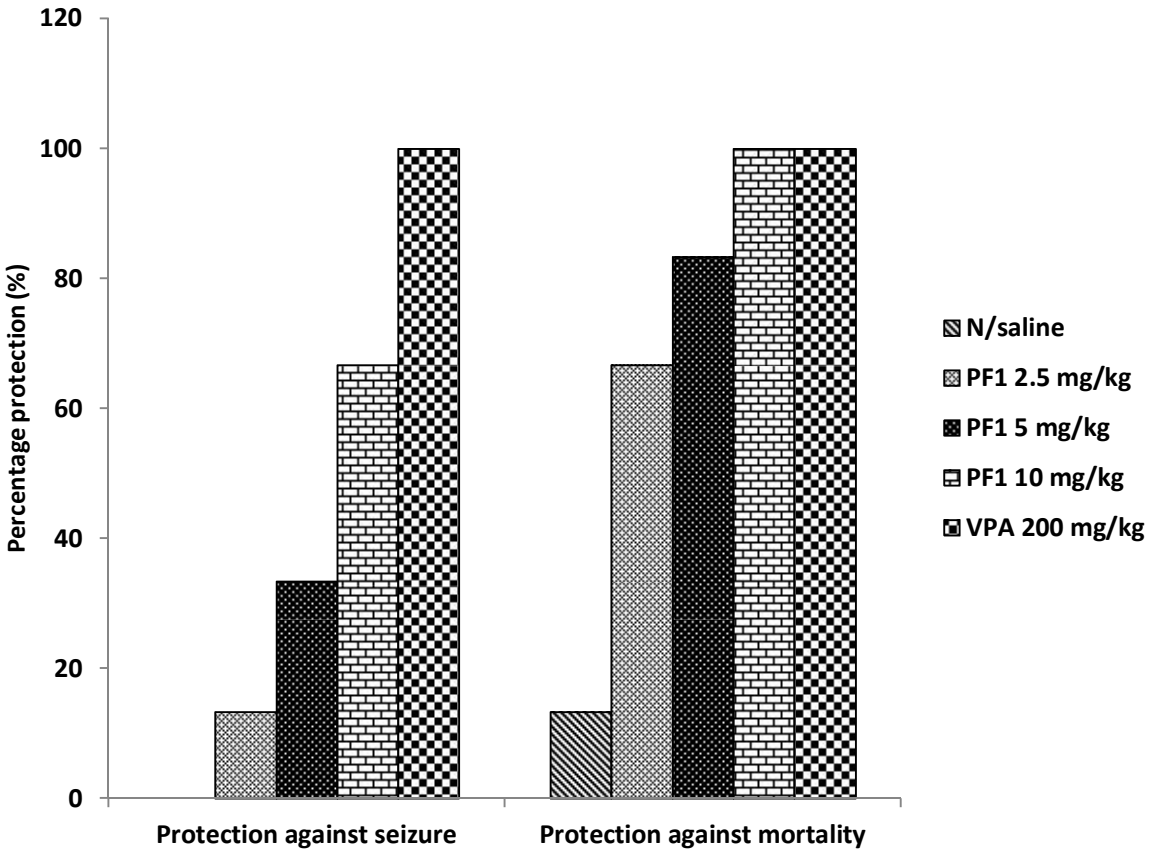
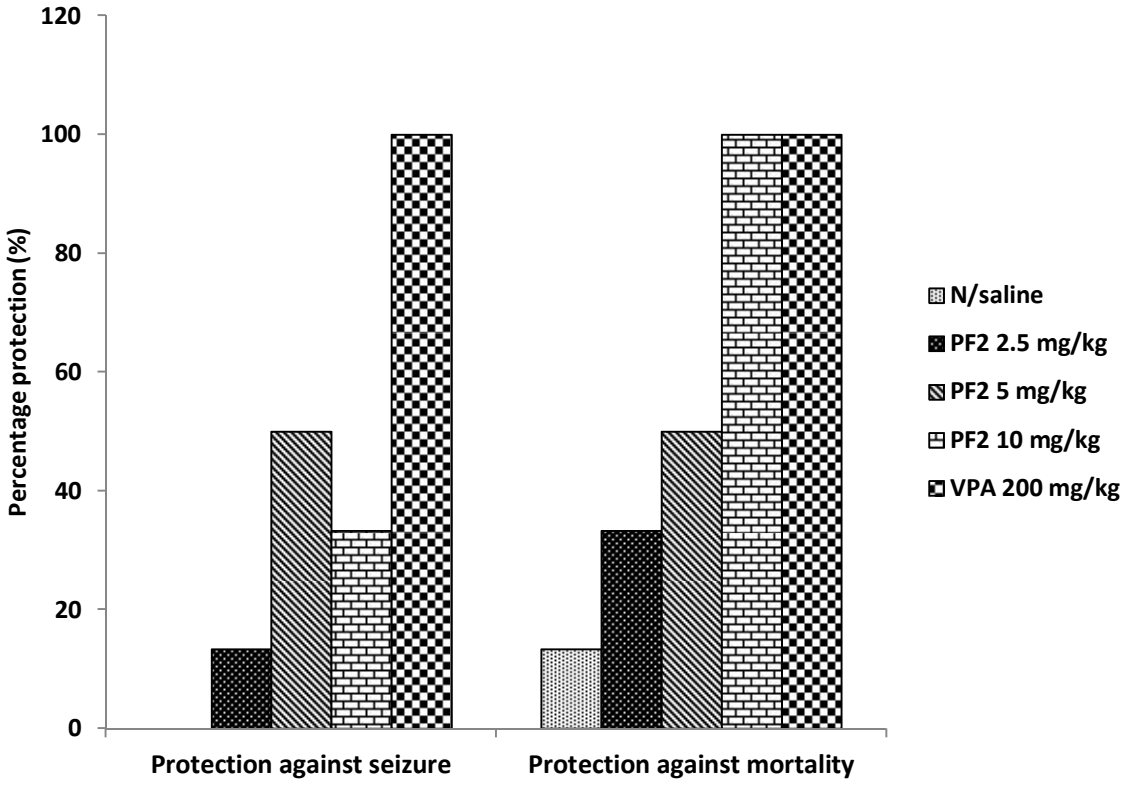


Figure 4.3.1: Effect of Residual Aqueous Fraction of Methanolic Root Bark Extract of *Securinega virosa* on Pentylenetetrazole-induced Seizure in Mice; N/Saline: Normal saline; RAF: Residual aqueous fraction; VPA: Sodium valproate; n=6



4.3.2: Effect of Column Fraction PF1 on Pentylenetetrazole-induced Seizure in Mice; N/Saline: Normal saline; VPA: Sodium valproate n=6



4.3.3: Effect of Column Fraction PF2 on Pentylenetetrazole-induced Seizure in Mice; N/Saline: Normal saline; VPA: Sodium valproate; n=6

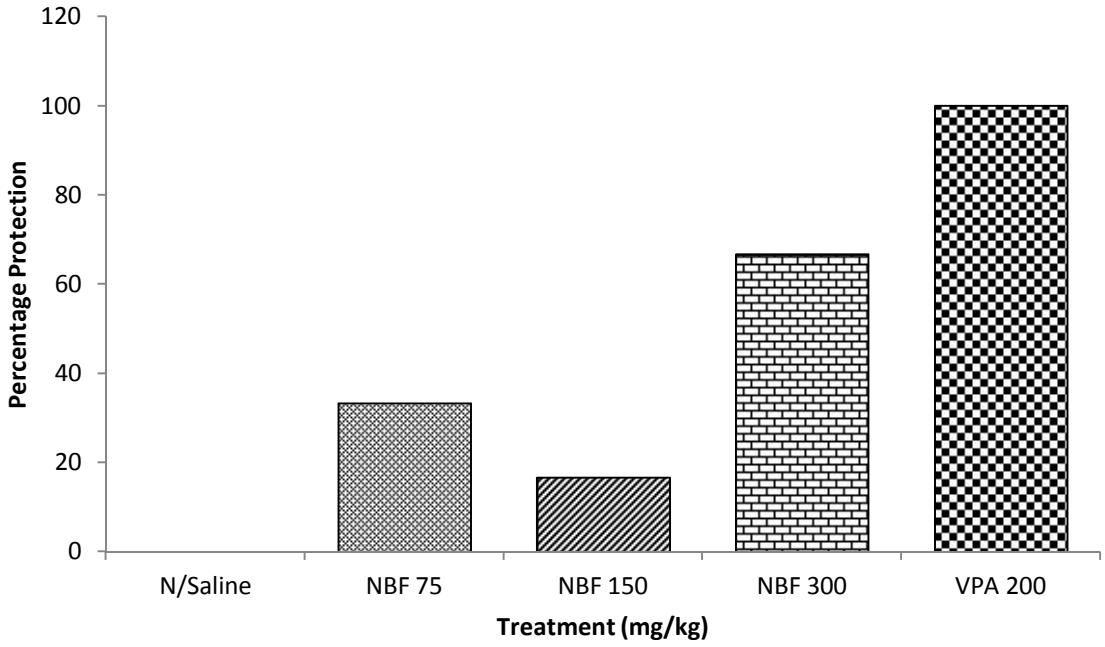


Figure 4.3.4: Effect of n-butanol Fraction of Methanolic Root Bark Extract of *Securinega virosa* on Pentylenetetrazole-induced Seizure in Mice; N/Saline: Normal saline; NBF: n-butanol fraction; VPA: Sodium valproate; n=6

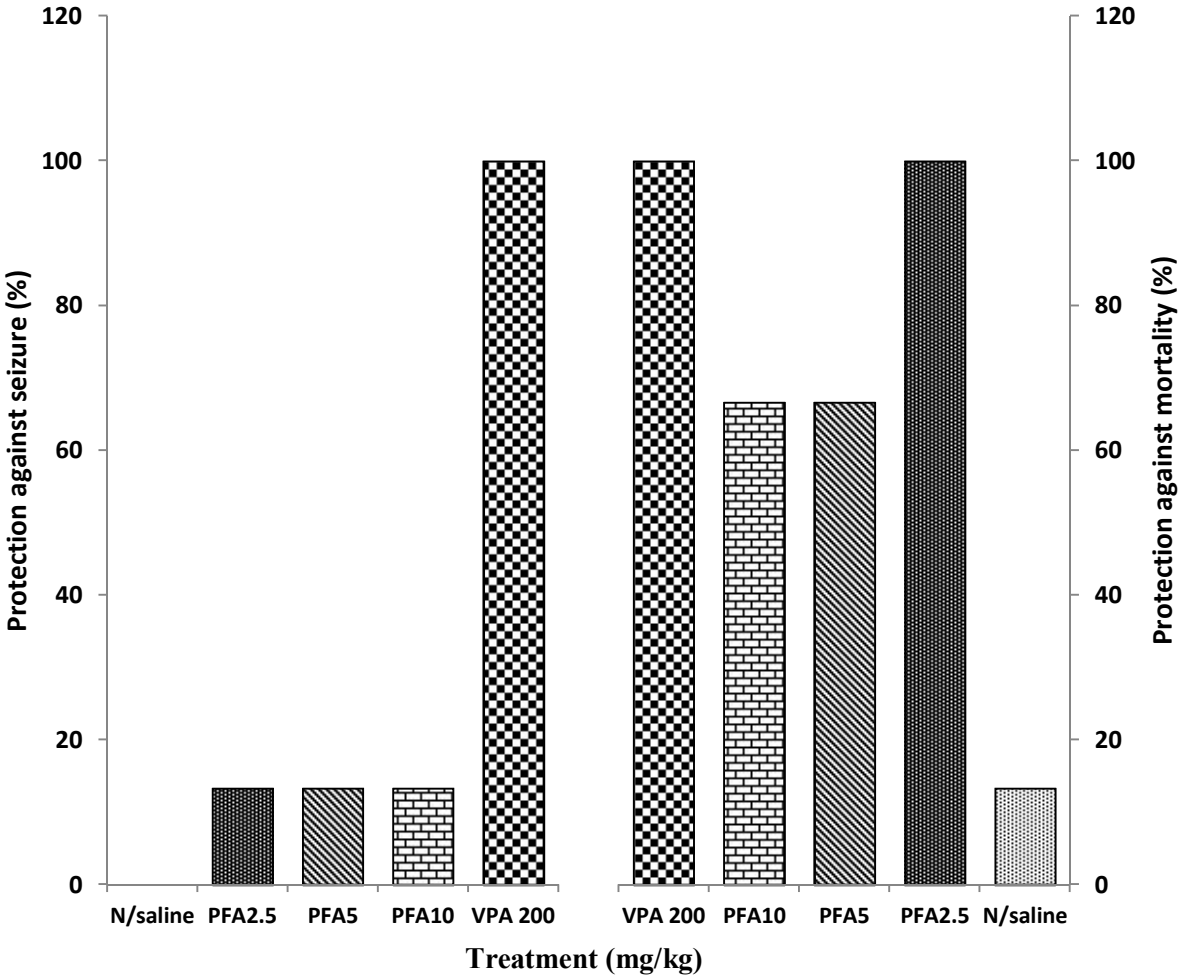


Figure 4.3.5: Effect of Column Fraction PFA on Pentylenetetrazole- induced Seizure in Mice; N/Saline: Normal saline; VPA: Sodium valproate; n=6

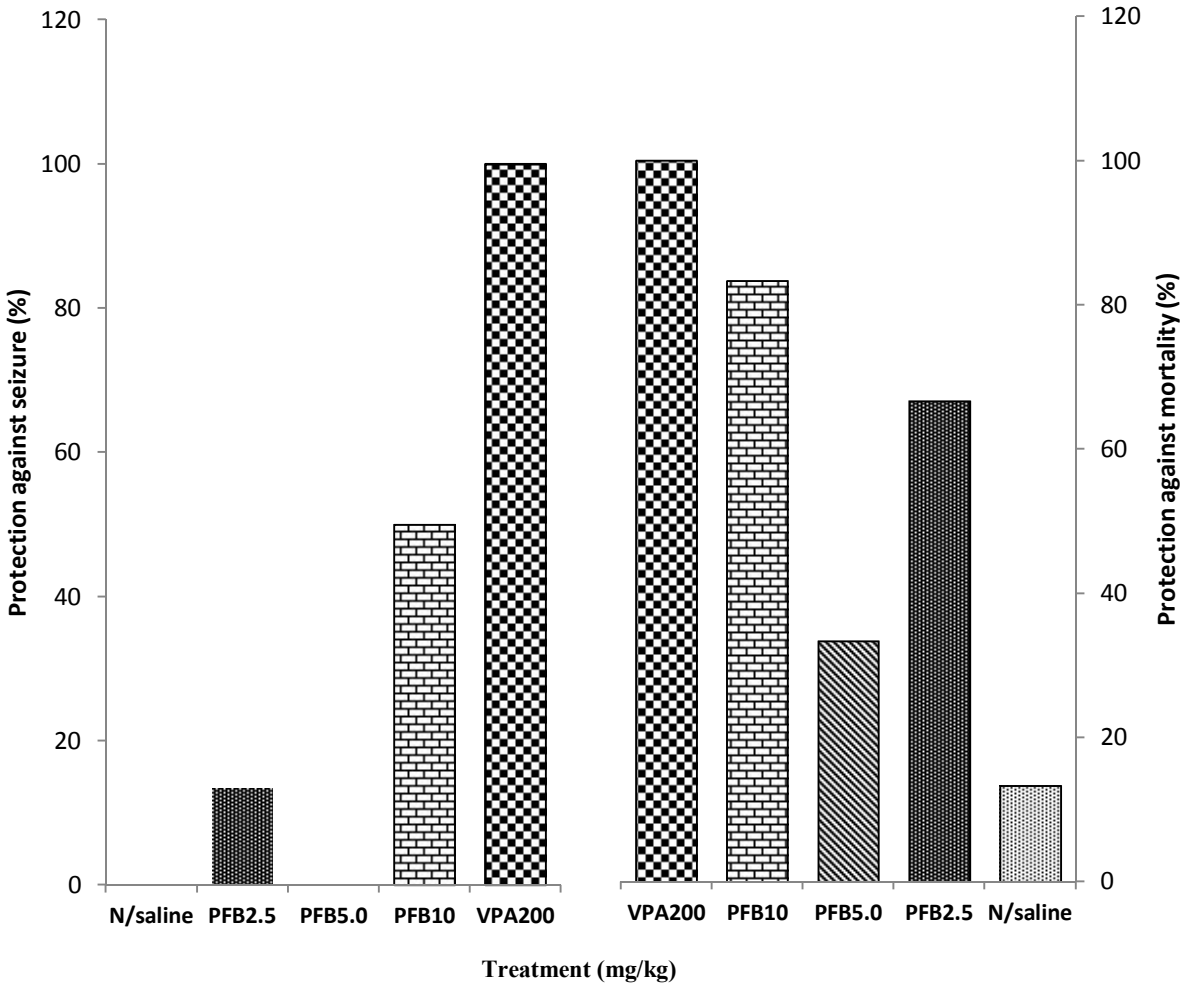


Figure 4.3.6: Effect of Column Fraction PFB on Pentylenetetrazole-induced Seizure in Mice; N/Saline: Normal saline; VPA: Sodium valproate; n=6

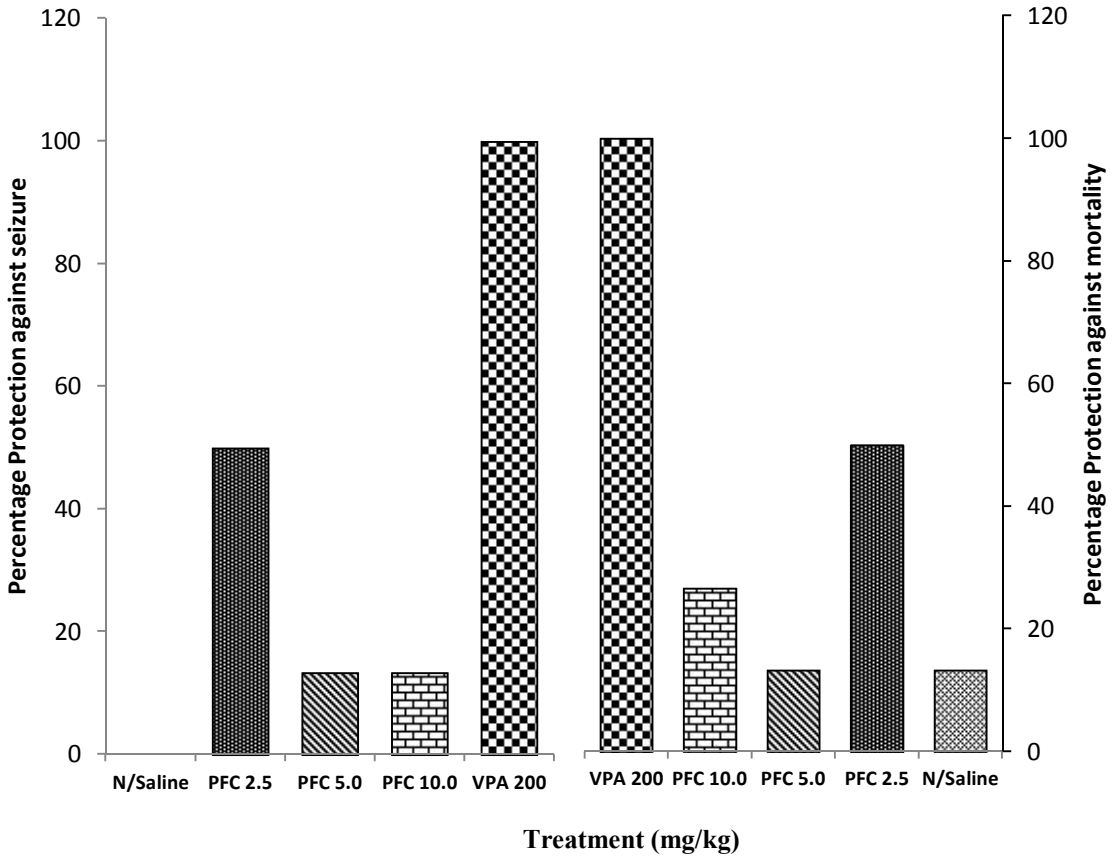


Figure 4.3.7: Effect of Column Fraction PFC on Pentylenetetrazole-induced Seizure in mice; N/Saline: Normal saline; VPA: Sodium valproate; n=6

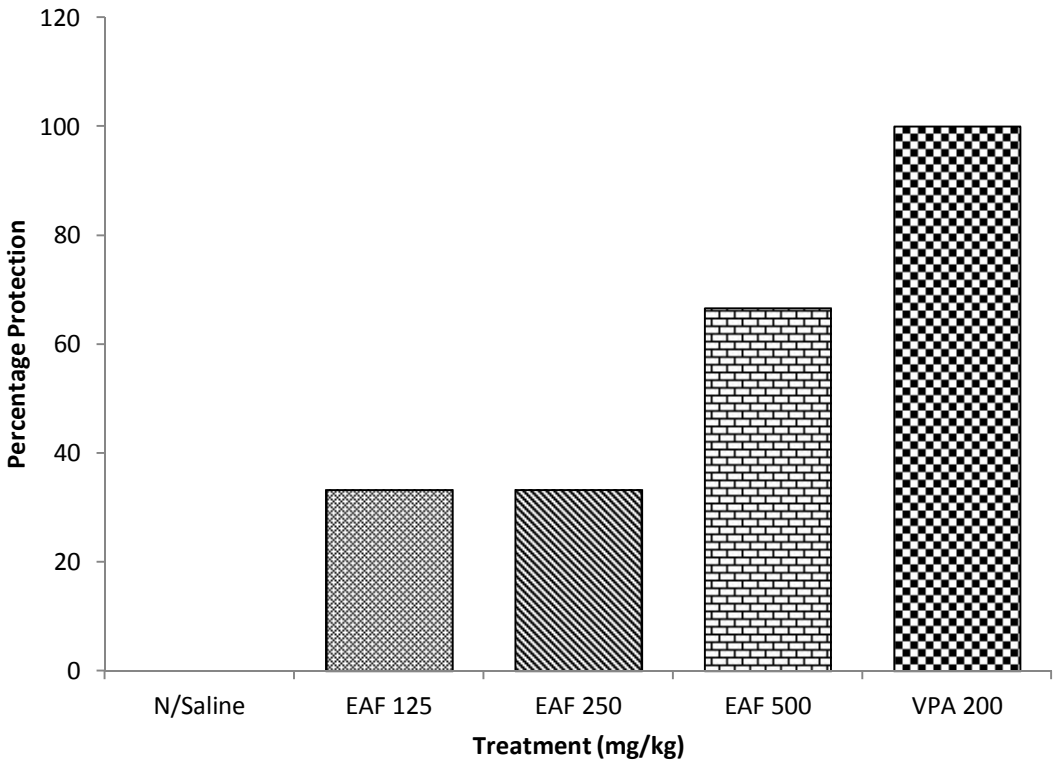


Figure 4.3.8: Effect of Ethyl Acetate Fraction of Methanolic Root Bark Extract of *Securinega virosa* on Pentylenetetrazole-induced Seizure in Mice; N/Saline: Normal saline; EAF: Ethyl acetate fraction; VPA: Sodium valproate; n=6

4.3.3 Effect of Fractions of Methanolic Root Bark Extract of *Securinega virosa* on Strychnine-induced Seizure in Mice

The two fractions (residual aqueous and n-butanol fractions) did not protect the mice against strychnine-induced seizure. There was also no significant increase in the mean onset of seizure compared with the normal saline treated group. Phenobarbitone (30 mg/kg) afforded 100% protection against strychnine-induced seizure (Table 4.3.2).

Table 4.3.2: Effect of Fractions of Methanolic Root Bark Extract of *Securinega virosa* on Strychnine-induced Seizure in Mice

Treatment	Quantal protection	Mean onset of seizure ± SEM
N/Saline	0/6	6.50 ± 0.43
RAF 125	0/6	7.83 ± 1.01
RAF 250	0/6	6.83 ± 0.40
RAF 500	0/6	7.83 ± 0.83
NBF 75	0/6	7.00 ± 0.52
NBF 150	0/6	7.50 ± 0.85
NBF 300	0/6	6.50 ± 0.50
PBT 30	6/6	-

Protection against seizure expressed as quantal protection; Mean onset of seizure (minutes) expressed as mean ± SEM; N/Saline: Normal saline; RAF: Residual aqueous fraction; NBF: n-butanol fraction; PBT: Phenobarbitone; n=6

4.3.4 Effects of Fractions of Methanolic Root Bark Extract of *Securinega virosa* on 4-Aminopyridine-induced Seizure in Mice

The fractions (n-butanol and residual aqueous fractions) did not protect the mice against 4-Aminopyridine-induced seizure. There was also no significant increase in the mean onset of seizure in the unprotected animals by both fractions compared with the control. Conversely, phenobrbitone, the standard drug used at the dose of 30 mg/kg protected 83.33% of the animals (Table 4.3.3).

Table 4.3.3: Effects of Fractions of Methanolic Root Bark Extract of *Securinega virosa* on 4-Aminopyridine-induecd Seizure in Mice

Treatment	Quantal protection	Mean onset of seizure (Minutes)
N/Saline (10 ml/kg)	0/6	14.40 ± 3.31
RAF 125	0/6	12.67 ± 1.26
RAF 250	0/6	14.67 ± 2.03
RAF 500	0/6	13.83 ± 1.08
NBF 75	0/6	9.67 ± 0.56
NBF 150	0/6	9.00 ± 0.78
NBF 300	0/6	8.50 ± 0.50
PBT 30	5/6	22.33

Protection against seizure expressed as quantal protection; Mean onset of seizure expressed as mean ± SEM; N/Saline: Normal saline; RAF: Residual aqueous fraction; NBF: n-butanol fraction; PBT: Phenobarbitone; n=6

4.3.5 Effects of Fractions of Methanolic Root Bark Extract of *Securinega virosa* on Picrotoxin-induced Seizure in Mice

The fractions (n-butanol and residual aqueous fractions) did not protect the animals against picrotoxin-induced seizure. At the dose of 125 mg/kg, the residual aqueous fraction significantly increased the mean onset of seizure. Diazepam (20 mg/kg), the standard drug used afforded 100% protection of the mice against picrotoxin-induced seizure (Table 4.3.4).

Table 4.3.4: The Effect of Fractions of Methanolic Root Bark Extract of *Securinega virosa* on Picrotoxin-induced Seizure in Mice

Treatment	Quantal protection against seizure	Mean onset of seizure (Minutes)	Quantal protection against mortality	Mean latency of Mortality (Minutes)
N/Saline	0/6	9.50 ± 0.85	0/6	13.67 ± 1.11
RAF 125	0/6	13.83 ± 0.91*	0/6	15.33 ± 0.80
RAF 250	0/6	9.33 ± 0.56	0/6	13.67 ± 0.80
RAF 500	0/6	8.67 ± 0.211	0/6	13.00 ± 0.52
NBF 75	0/6	9.00 ± 0.37	0/6	13.50 ± 0.50
NBF 150	0/6	6.50 ± 0.22*	0/6	10.83 ± 0.54*
NBF 300	0/6	6.83 ± 0.60 *	0/6	11/33 ± 0.72
DZP 20	6/6		6/6	

Protection against seizure expressed as quantal protection; Mean onset of seizure expressed as mean ± SEM; N/Saline: Normal saline; RAF: Residual aqueous fraction; NBF: n-butanol fraction; PBT: DZP: Diazepam; *P<0.05; n=6

4.3.6 Effects of Fractions of Methanolic Root Bark Extract of *Securinega virosa* on Electrically induced Kindling in Rats

The residual aqueous fraction at the dose of 500 mg/kg non-significantly reduced both the behavioural seizure scores (BSS) and mean seizure duration (MSD) at all the four hours after treatment compared with the scores before treatment (Figures 4.3.9 and 4.3.10). In the case of n-butanol fraction, there was a non-signifcant decrease in the behavioural seizure scores (Figure 4.3.11). However, there was an increase in the mean seizure duration post-treatment (Figure 4.3.12).

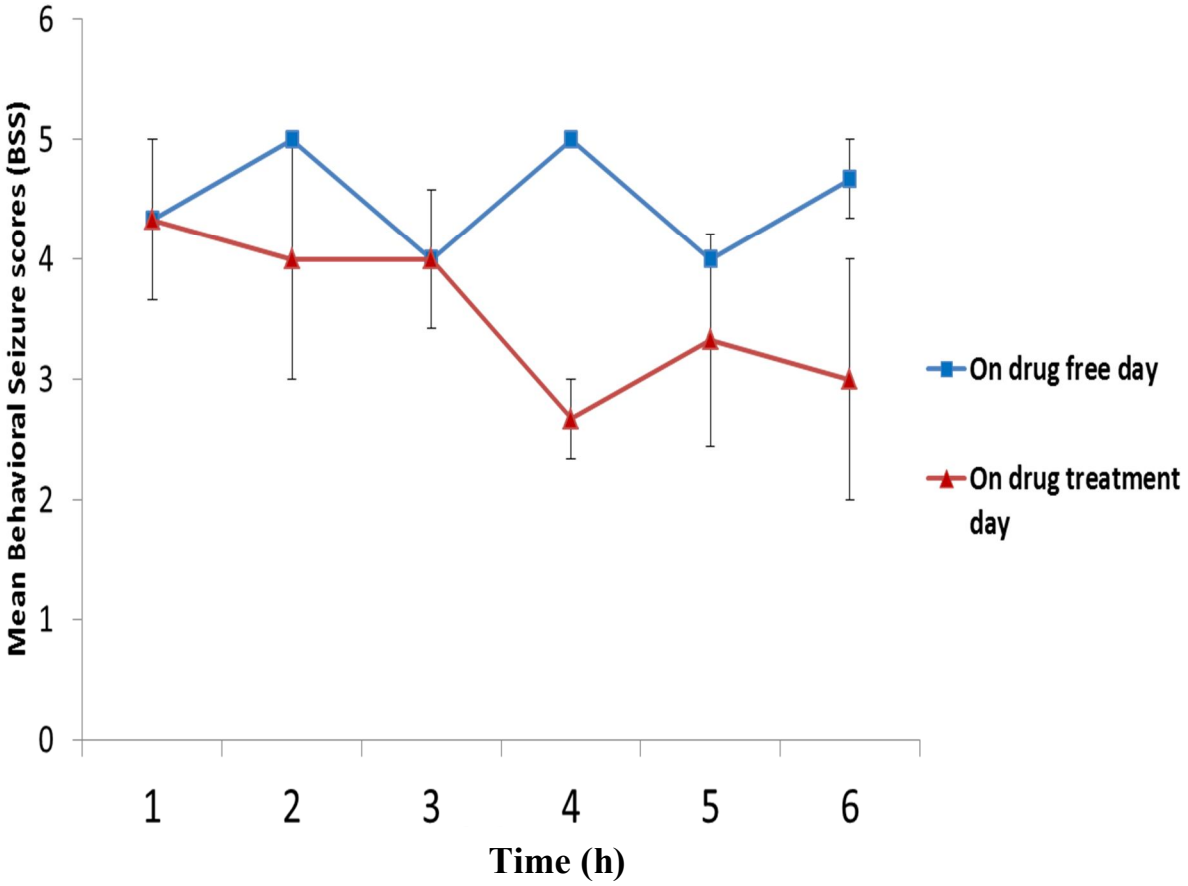


Figure 4.3.9: Effect of Residual Aqueous Fraction (500 mg/kg) of Methanolic Root Bark Extract of *Securinega virosa* on Behavioural Seizure Scores in Electrically induced Kindling in adult Rats; BSS on treatment day compared with that of drug free day; n=4

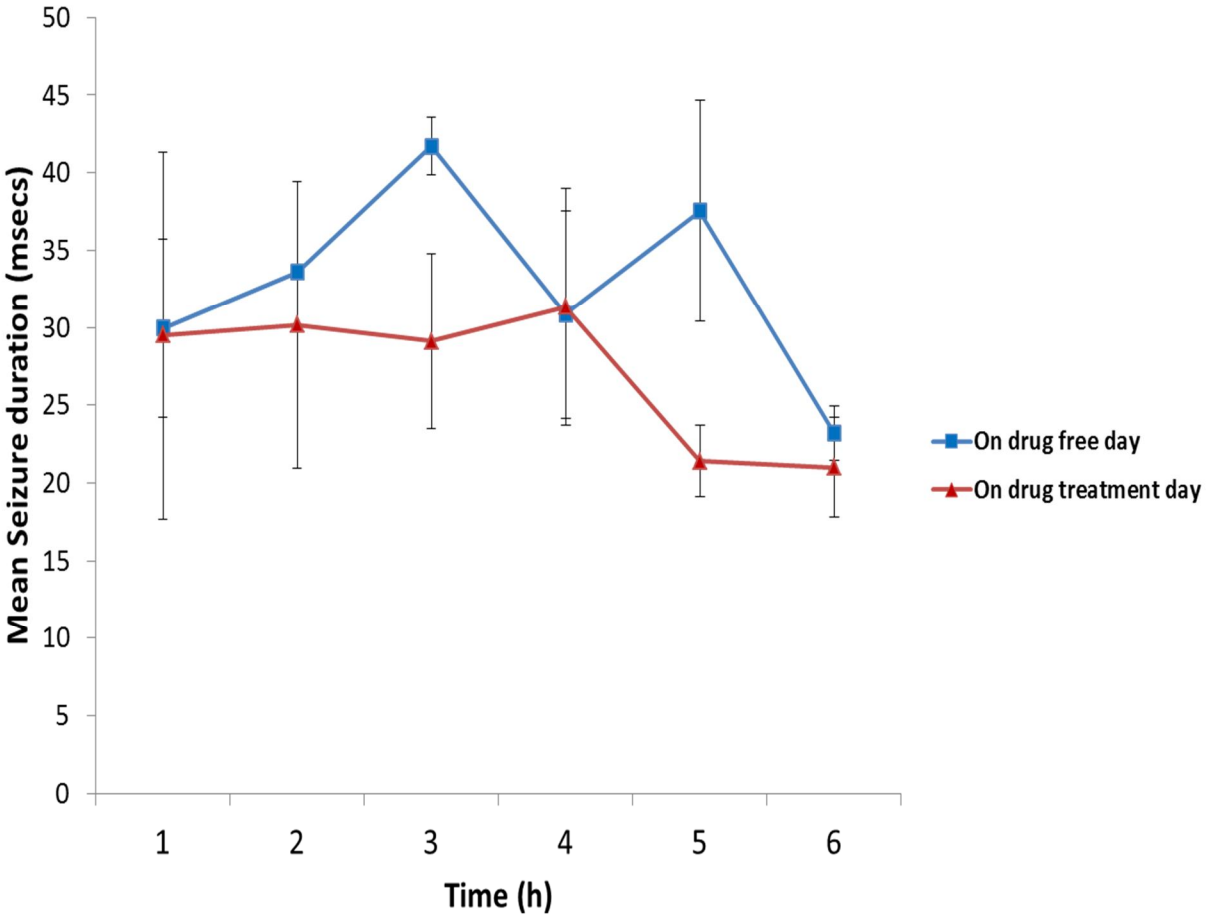
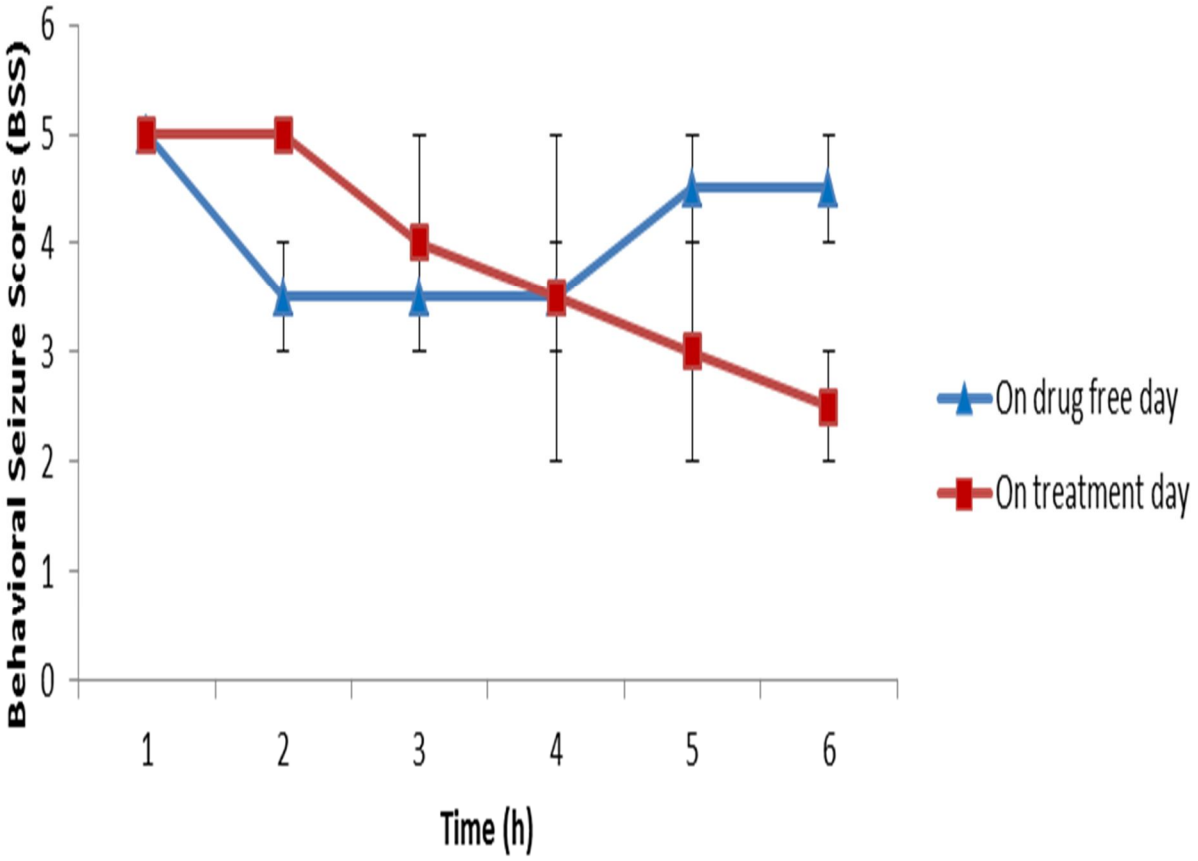
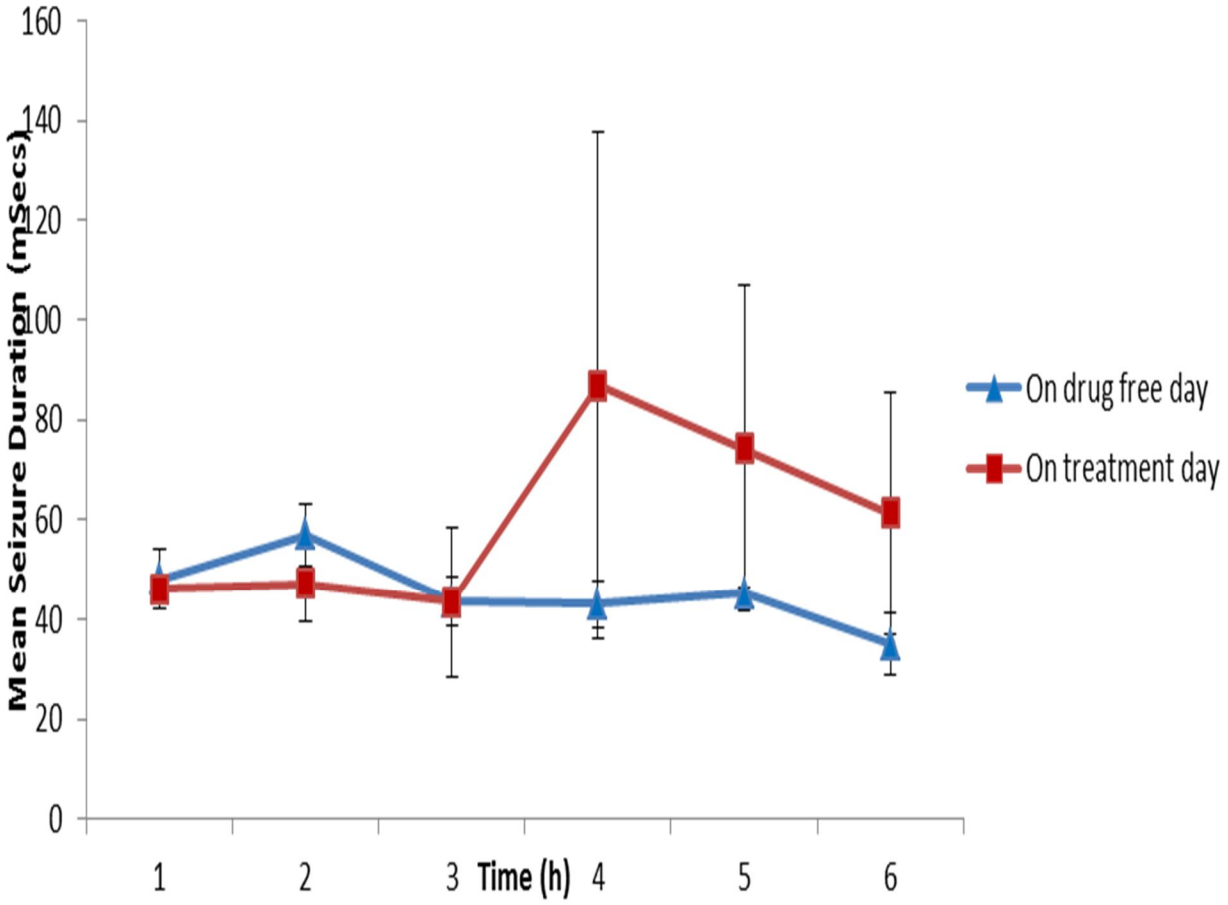


Figure 4.3.10: Effect of Residual Aqueous Fraction (500 mg/kg) of Methanolic Root Bark Extract of *Securinega virosa* on Mean Seizure Duration in Electrically induced Kindling in adult Rats; Mean seizure duration on treatment day compared with that of drug free day; n=4



4.3.11: Effect of n-butanol Fraction (300 mg/kg) of Methanolic Root Bark Extract of *Securinega virosa* on Behavioural Seizure Scores (BSS) in Electrically induced Kindling in adult Rats; BSS on treatment day compared with that of drug free day; n=4



4.3.12: Effect of n-butanol Fraction of *S. virosa* (300 mg/kg) on Mean Seizure Duration in Electrically induced Kindling in Rats; Mean seizure duration on treatment day compared with that of drug free day; n=4

4.4 BEHAVIOURAL STUDIES

4.4.1 Effects of Fractions of Methanolic Root Bark Extract of *Securinega virosa* on Diazepam-induced Sleep in Mice

The residual aqueous fraction (RAF) at the highest dose tested (500 mg/kg) significantly ($P < 0.05$) decreased the mean onset of sleep. It significantly ($P < 0.05$ - $P < 0.001$) and dose-dependently increased the mean duration of sleep (Figure 4.4.1). The column fraction PF1 obtained from residual aqueous fraction did not significantly alter the onset and duration of diazepam-induced sleep compared to the control group (Figure 4.4.2). The column fraction PF2 significantly ($P < 0.05$) reduced the mean onset of diazepam-induced sleep. However, it had no effect of the total sleep duration (Figure 4.4.3).

The n-butanol fraction significantly ($P < 0.05$ - $P < 0.01$) and dose-dependently reduced the onset of sleep but did not significantly increased the duration of sleep in mice (Figure 4.4.4). The column fraction PFA obtained from the n-butanol fraction significantly ($P < 0.01$) decreased the mean onset of sleep but affected the duration of sleep, non-significantly (Figure 4.4.5).

The column fraction, PFB significantly ($P < 0.05$) decreased the mean onset of sleep and increased the duration of sleep at the dose of 5 mg/kg (Figure 4.4.6).

The column fraction, PFC at the lowest dose tested (2.5 mg/kg) significantly ($P < 0.05$) reduced the mean onset of sleep. However, it dose-dependently increased ($P < 0.01$) the mean duration of sleep. At the highest dose tested (10 mg/kg), the duration of sleep increased by a factor of about 5 (Figure 4.4.7).

Bergenin, isolated from the PFA of the n-butanol fraction significantly ($P < 0.05$) decreased the mean onset of sleep without affecting the total duration of sleep at the dose of 10 mg/kg (Figure 4.4.8).

The ethyl acetate fraction did not significantly affect the mean onset of sleep but significantly ($P < 0.01$) increased the mean the duration of sleep at the dose of 150 mg/kg (Figure 4.4.9).

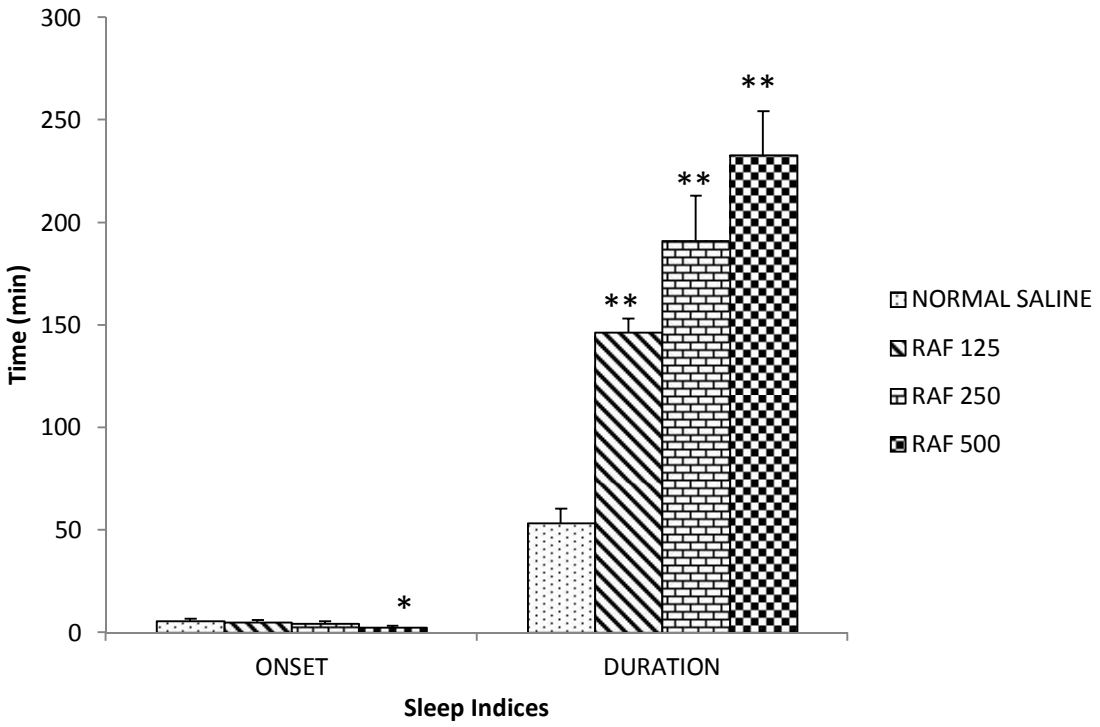


Figure 4.4.1: Effect of Residual Aqueous Fraction of Methanolic Root Bark Extract of *Securinega virosa* on Diazepam-induced Sleep in Mice; Onset and duration of sleep presented as mean ± SEM; RAF: Residual aqueous fraction; $^{*}P< 0.01$ and $^{**}P< 0.001$ compared to control; n=6

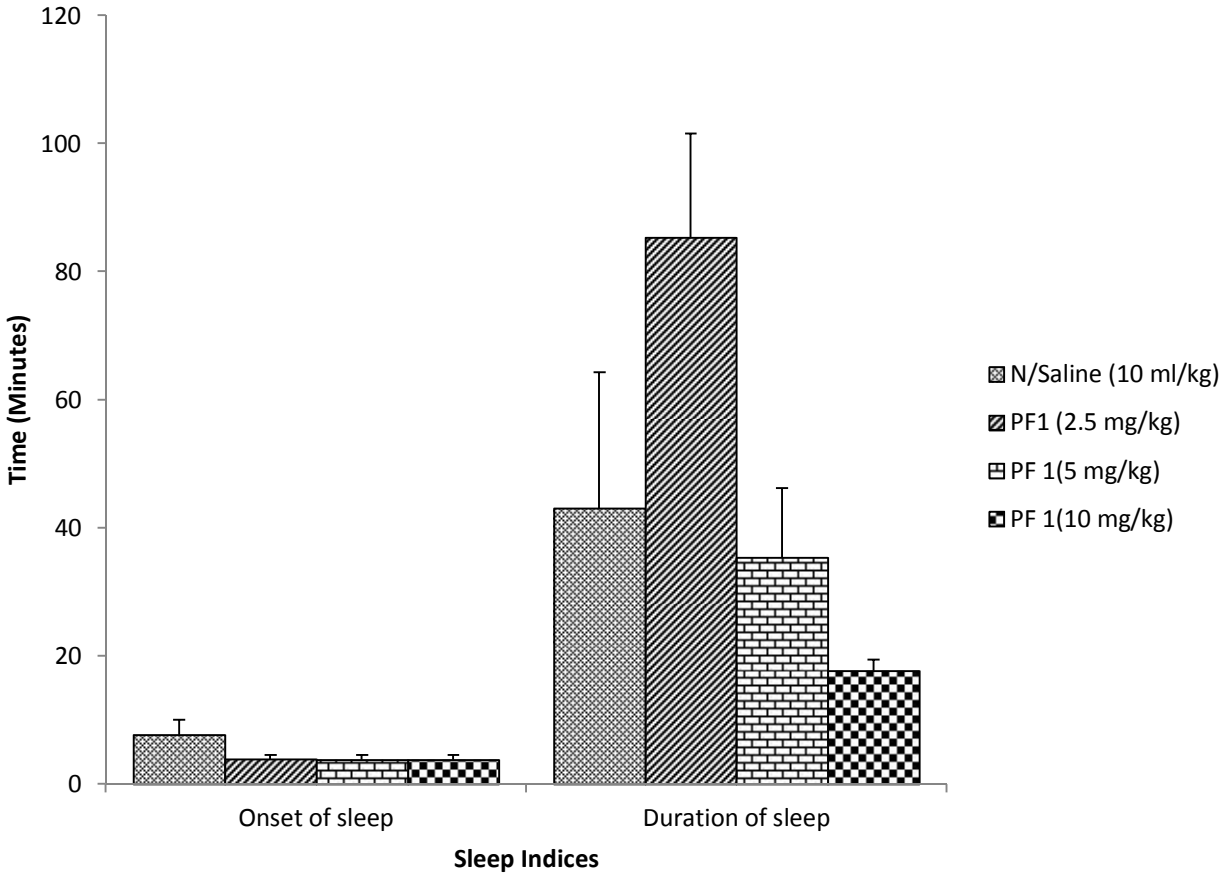


Figure 4.4.2: Effect of Column Fraction PF1 obtained from Residual Aqueous Fraction of Methanolic Root Bark Extract of *Securinega virosa* on Diazepam-induced Sleep in Mice; n=6

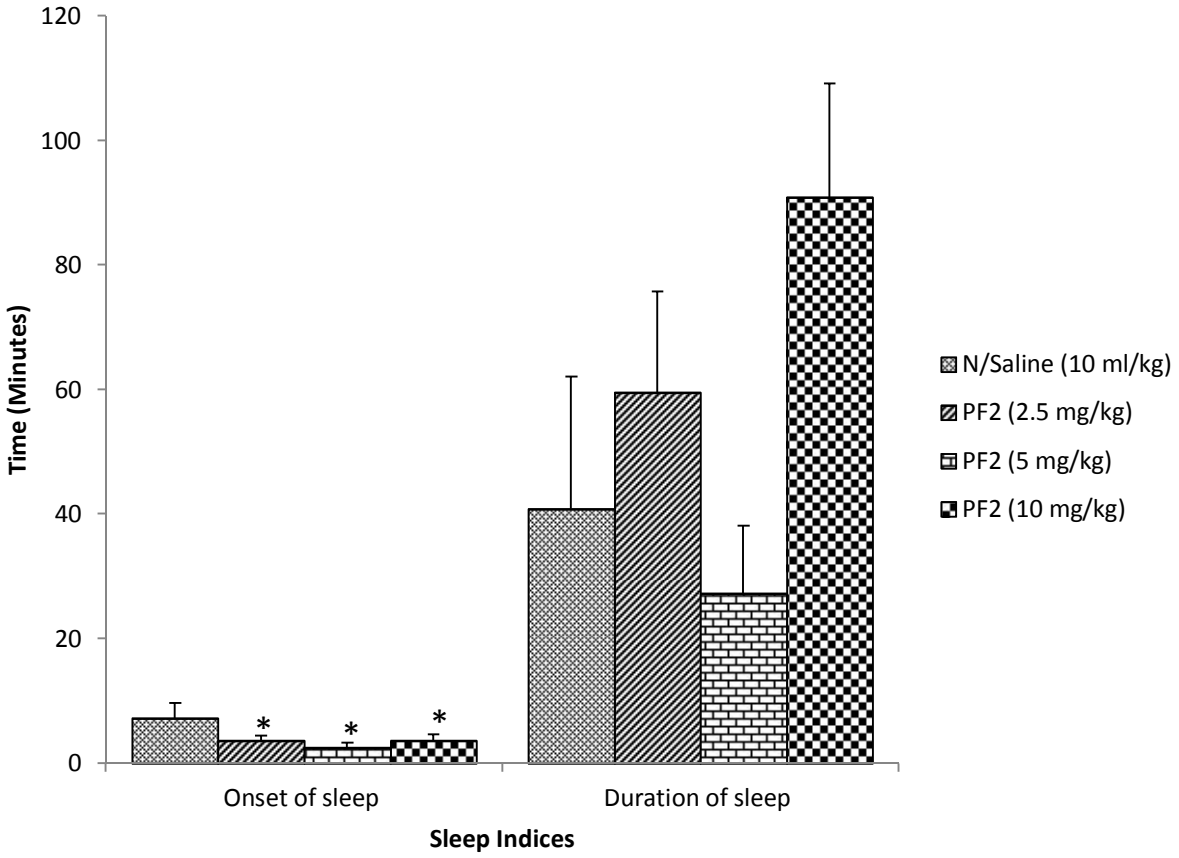


Figure 4.4.3: Effect of Column Fraction PF2 obtained from Residual Aqueous Fraction of Methanolic Root Bark Extract of *Securinega virosa* on Diazepam-induced Sleep in Mice; *P<0.05; n=6

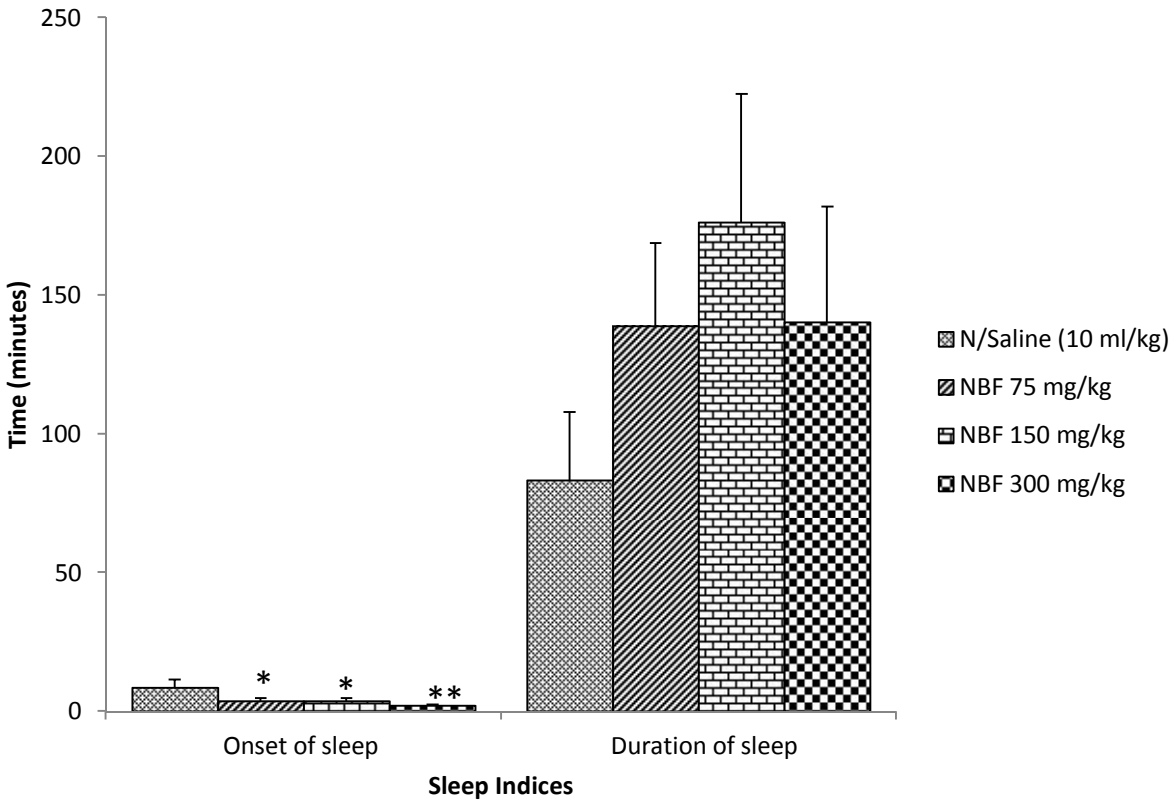


Figure 4.4.4: Effect of n-butanol Fraction of Methanolic Root Bark Extract of *Securinega virosa* on Diazepam-induced Sleep in Mice; Data presented as mean ± SEM; NBF: n-butanol fraction; $^{*}P< 0.05$ and $^{**}P< 0.001$ compared to control; n=6

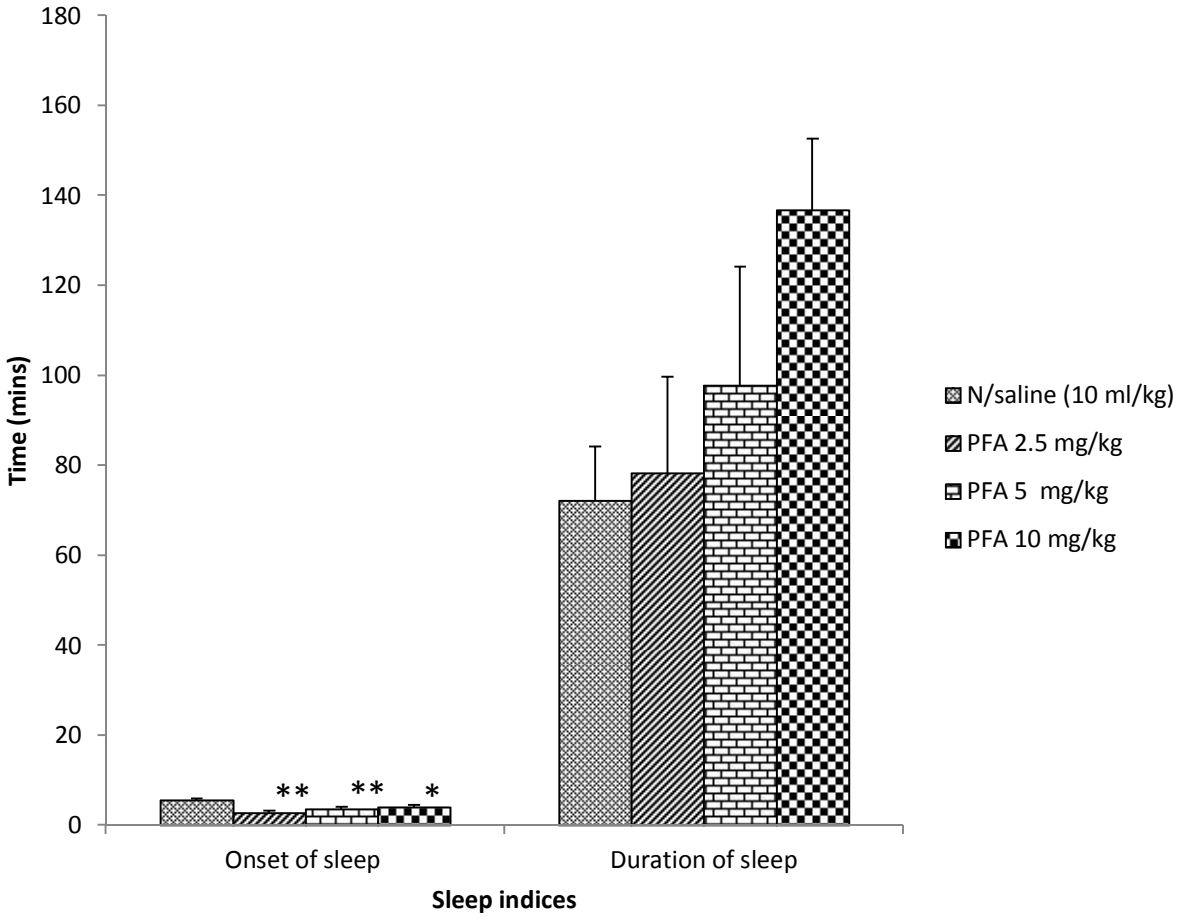


Figure 4.4.5: Effect of Column Fraction PFA of n-butanol Fraction of Methanolic Root Bark Extract of *Securinega virosa* on Diazepam-induced Sleep in Mice; Onset and Duration of sleep presented as mean ± SEM; $^{*}P< 0.05$ and $^{**}P< 0.001$ compared to control; n=6

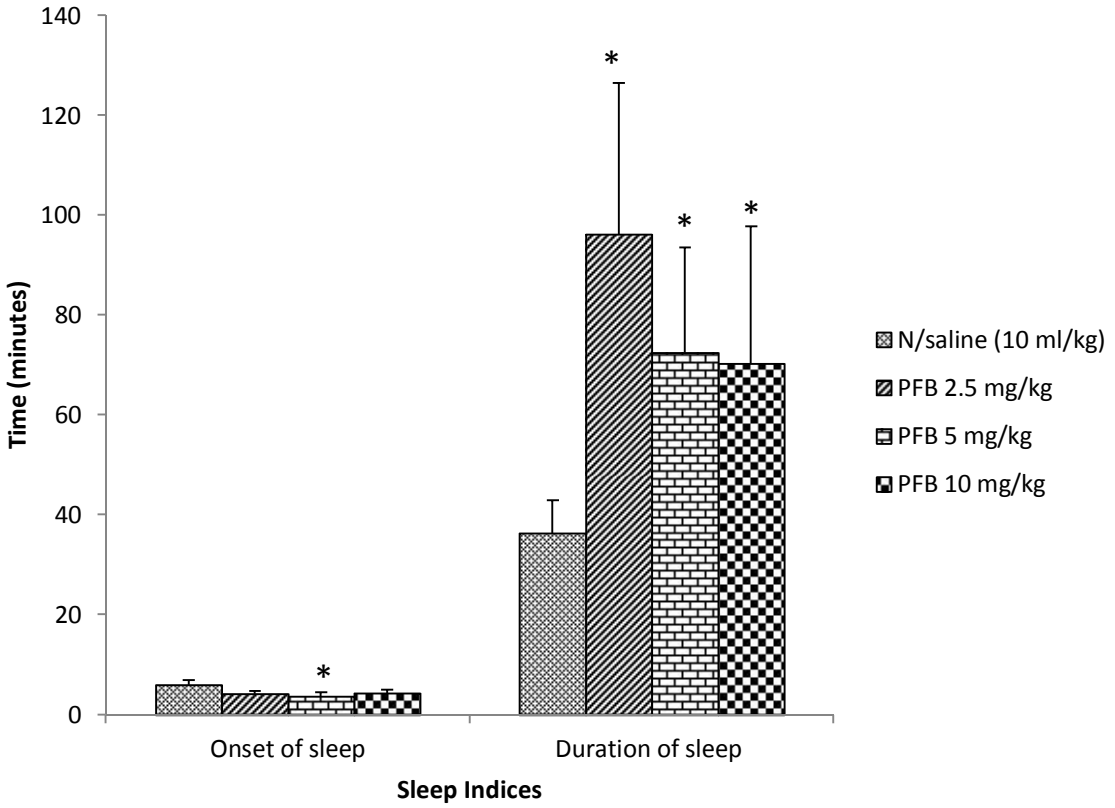


Fig. 4.4.6: Effect of Column fraction PFB of n-butanol Fraction of Methanolic Root Bark Extract of *Securinega virosa* on Diazepam-induced Sleep in Mice; Onset and duration of sleep presented as mean ± SEM; $^{*}P< 0.05$ compared to control; n=6

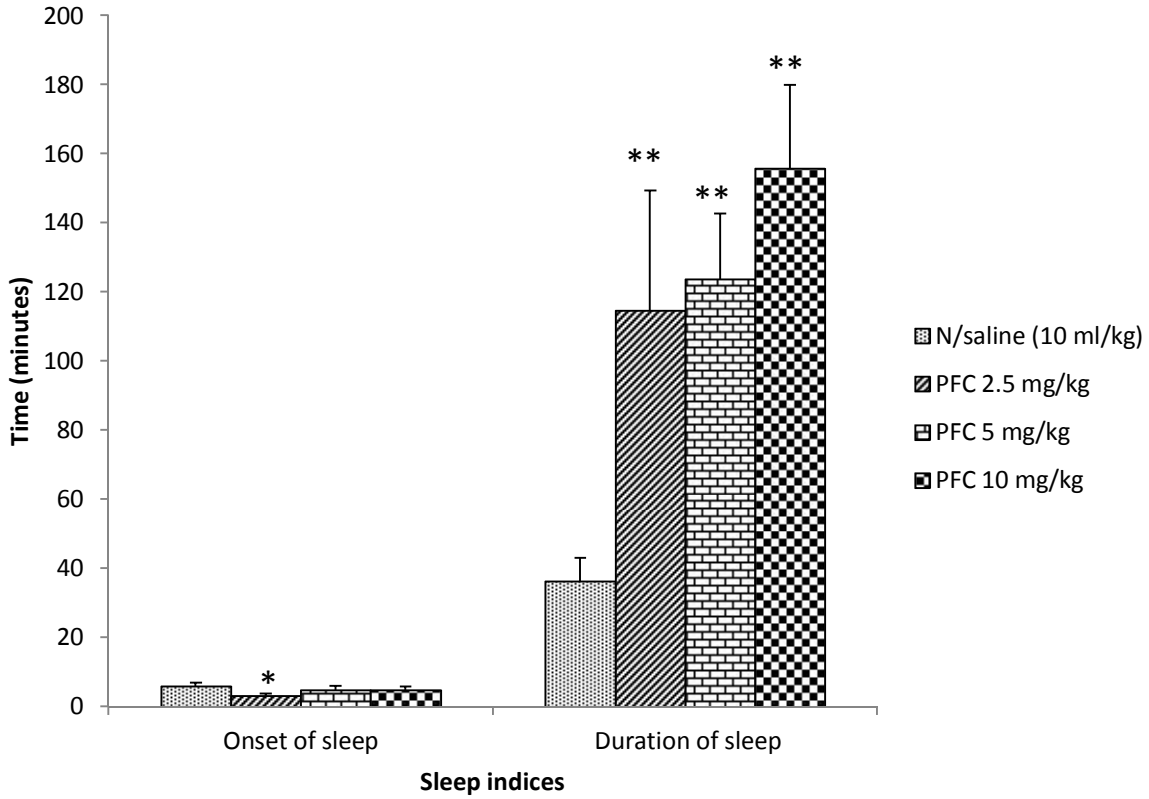


Figure 4.4.7: Effect of PFC of n-butanol Fraction of Methanolic Root Bark Extract of *Securinega virosa* on Diazepam-induced Sleep in Mice; onset and duration of sleep presented as mean ± SEM; $^{*}P< 0.05$; **P<0.001 compared to control; n=6

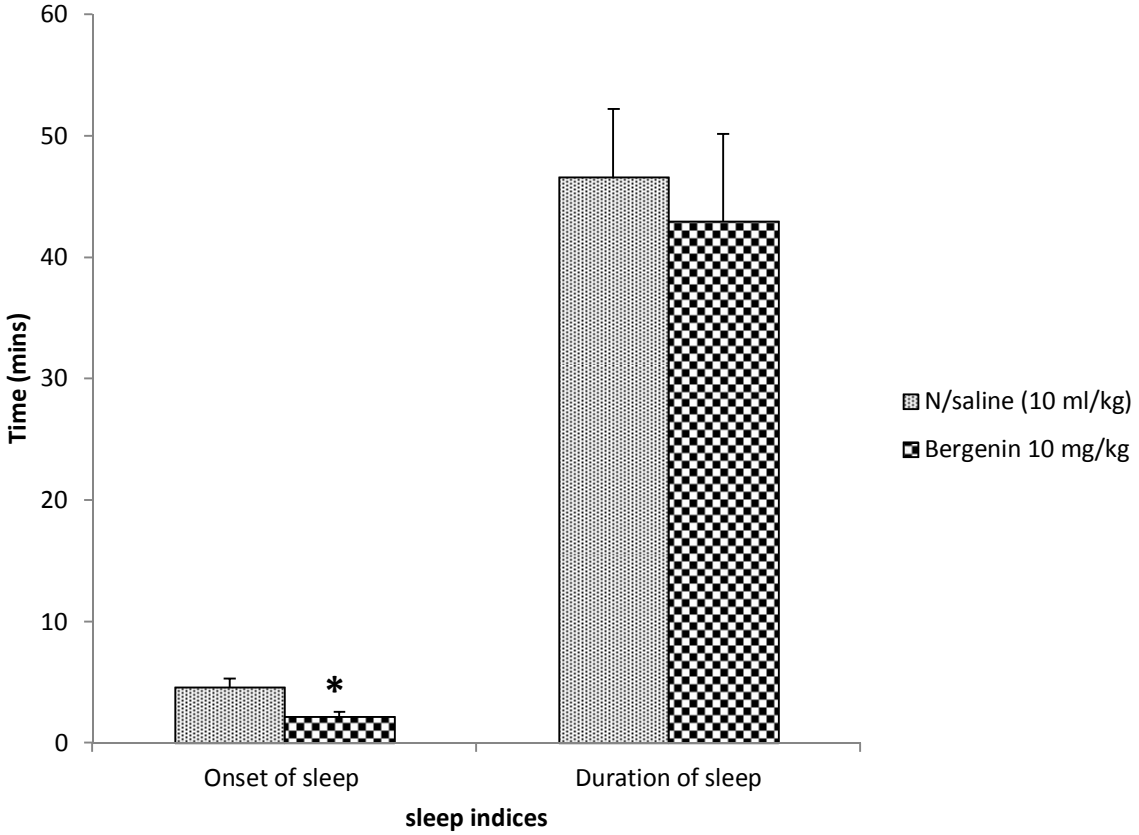


Figure 4.4.8: Effect of Bergenin obtained from n-butanol Fraction on Diazepam-induced sleep in mice; Onset and duration of Sleep presented as mean ± SEM; N/saline: Normal saline; *P<0.05; n=5

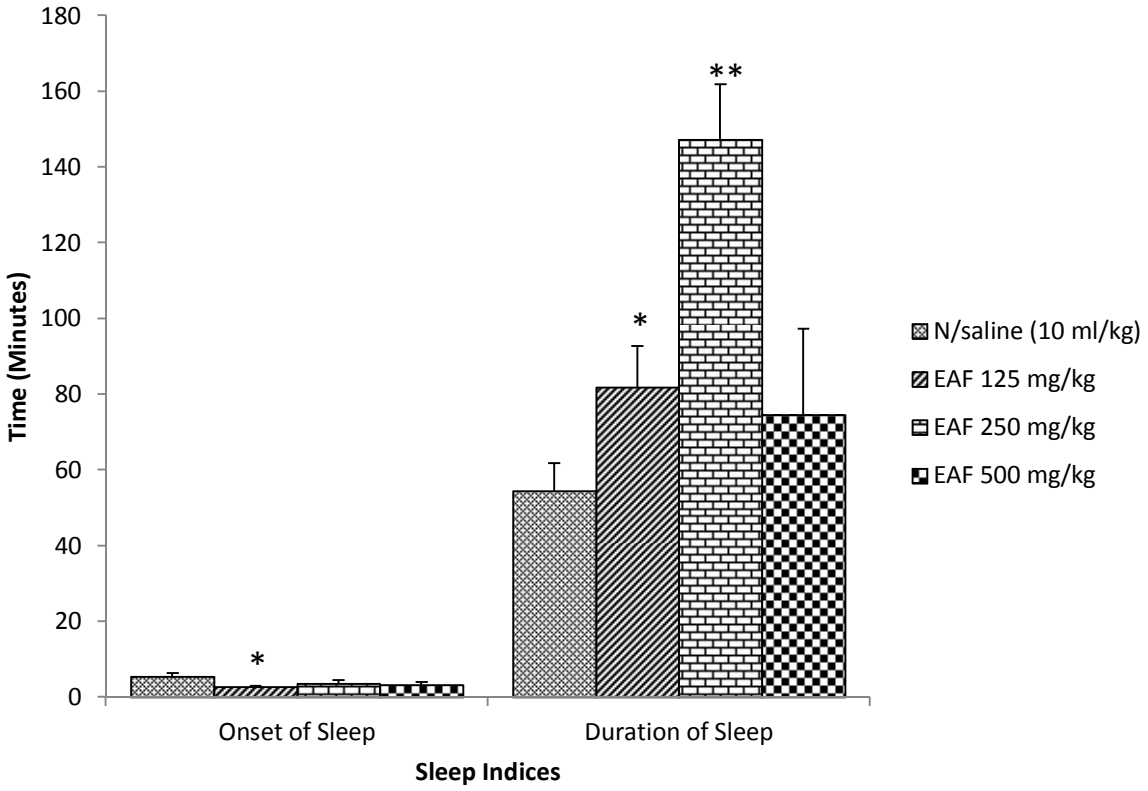


Figure 4.4.9: Effect of Ethyl acetate Fraction of Methanolic Root Bark Extract of *Securinega virosa* on Diazepam-induced Sleep in Mice; Onset and duration of sleep presented as mean ± SEM; N/Saline: Normal saline; EAF: Ethyl acetate fraction *P<0.01 and **P< 0.001 compared to control; n=6

4.4.2 Effects of Fractions of Methanolic Root Bark Extract of *Securinega virosa* on Exploratory Behaviour in Mice

The residual aqueous fraction significantly ($P<0.01$-$P<0.001$) and dose-dependently decreased the number of head dips in the hole board test (Figure 4.4.10). The n-butanol fraction significantly decreased the number of head dips in mice. However, the effect was not dose-dependent. Diazepam at the dose of 2 mg/kg, also, significantly decreased the number of head dips (Figure 4.4.11).

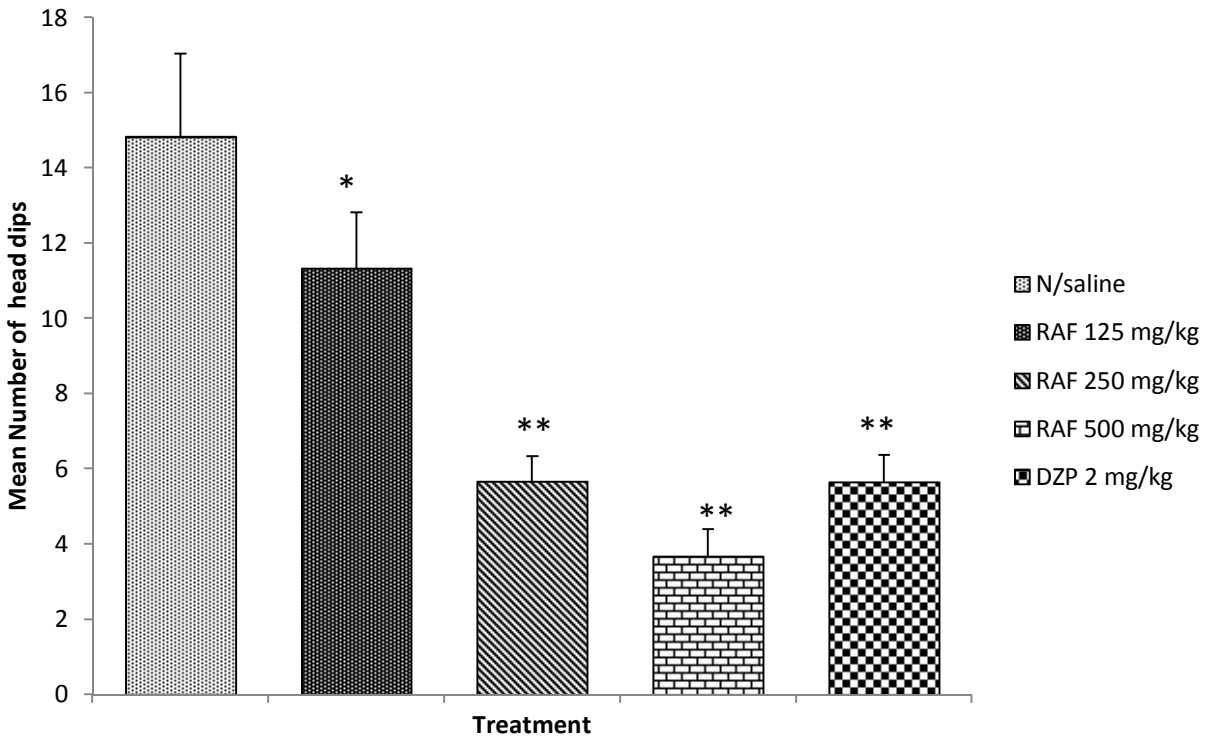


Figure 4.4.10: Effect of Residual Aqueous Fraction of Methanolic Root Bark Extract of *Securinega virosa* on Exploratory Activity of Mice in Hole board test; Number of heap dips presented as mean ± SEM; RAF: Residual aqueous fraction; DZP: Diazepam; *P<0.01; **P< 0.001; n=6

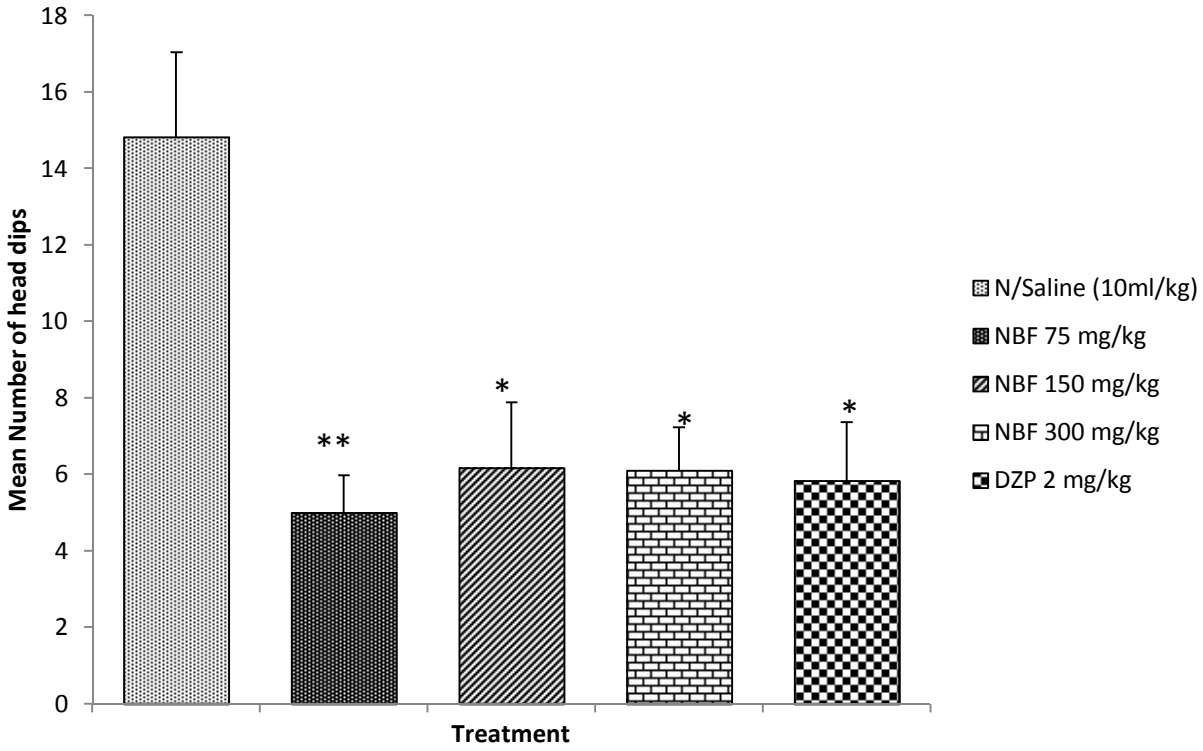


Figure 4.4.11: Effect of n-butanol Fraction of Methanolic Root Bark Extract of *Securinega virosa* on Exploratory Activity of Mice in Hole board Test; Number of head dips presented as mean ± SEM; NBF: n-butanol fraction); DZP: diazepam; *P<0.01; **P< 0.001; n=6

4.4.3 Effects of Fractions of Methanolic Root Bark Extract of *Securinega virosa* on Motor Coordination in Mice (Beam Walking Assay)

The residual aqueous (RAF) fraction did not significantly affect the time taken to complete the task on the beam and the number of toot slips (Figure 4.4.12). Similarly, the n-butanol fraction (NBF) did not alter the two parameters significantly (Figure 4.4.13). Similarly, bergenin, the isolated compound did not significantly affect both the time to complete the task and the number of foot slips (Figure 4.4.14). Conversely, diazepam at the dose of 2 mg/kg significantly ($P<0.05$) increased the number of foot slips.

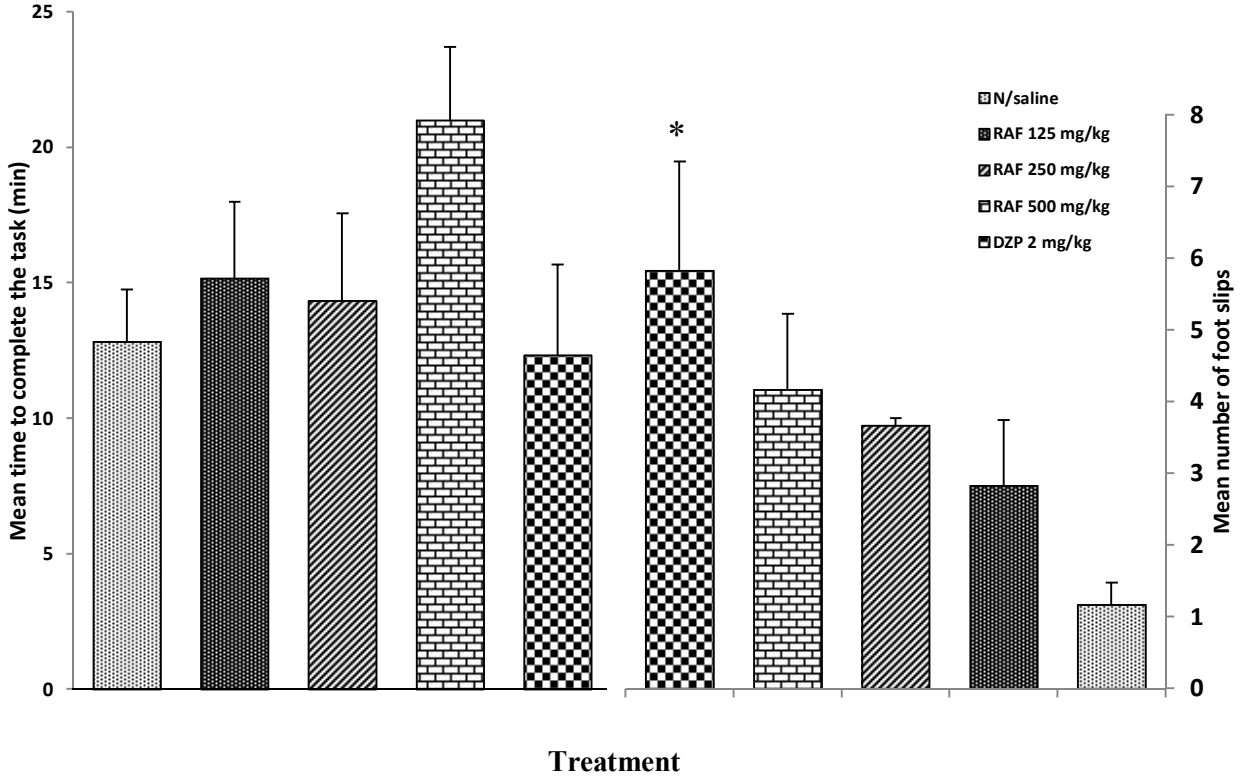


Figure 4.4.12: Effect of Residual Aqueous Fraction of Methanolic Root Bark Extract of *Securinega virosa* on Motor Coordination using Beam Walking Assay in Mice; Data presented as mean ± SEM; RAF: Residual aqueous fraction; DZP: diazepam; *P<0.01; n=6

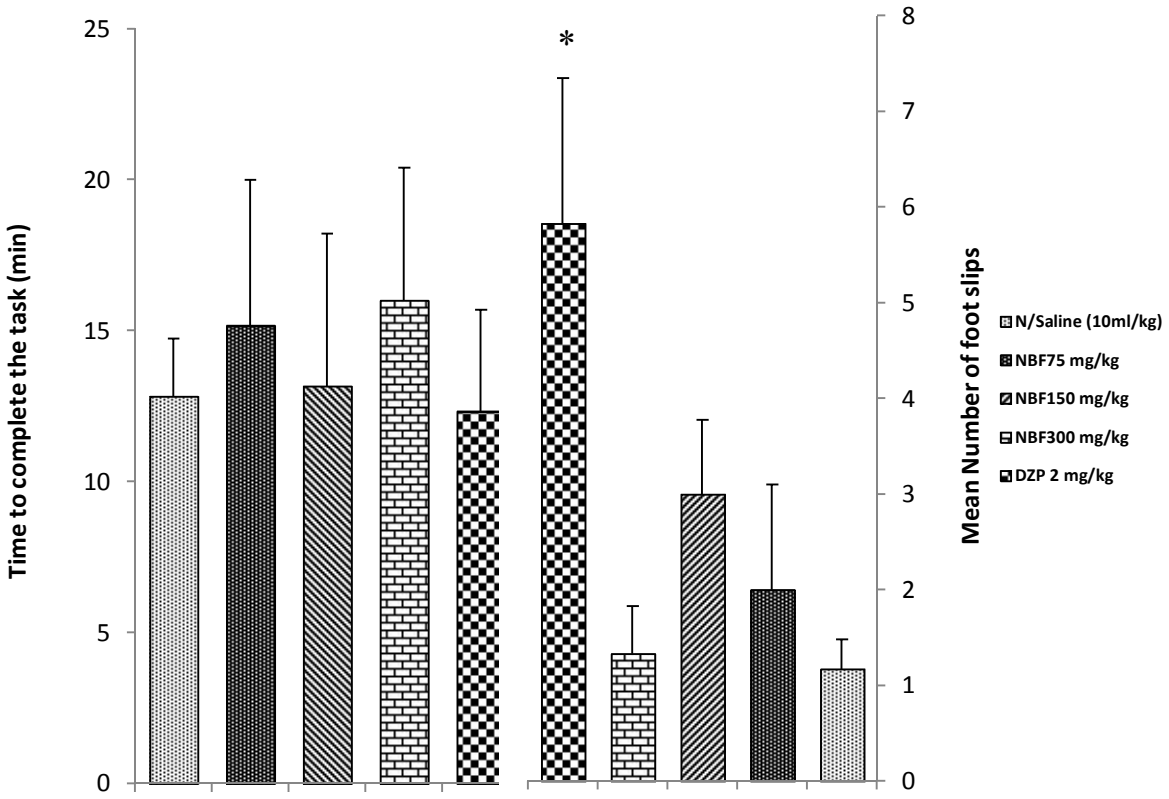


Figure 4.4.13: Effect of n-butanol Fraction of Methanolic Root Bark Extract of *Securinega virosa* on Motor Coordination using Beam Walking Assay in Mice; Data presented as mean ± SEM; NBF: n-butanol fraction; DZP: diazepam; *P<0.01; n=6

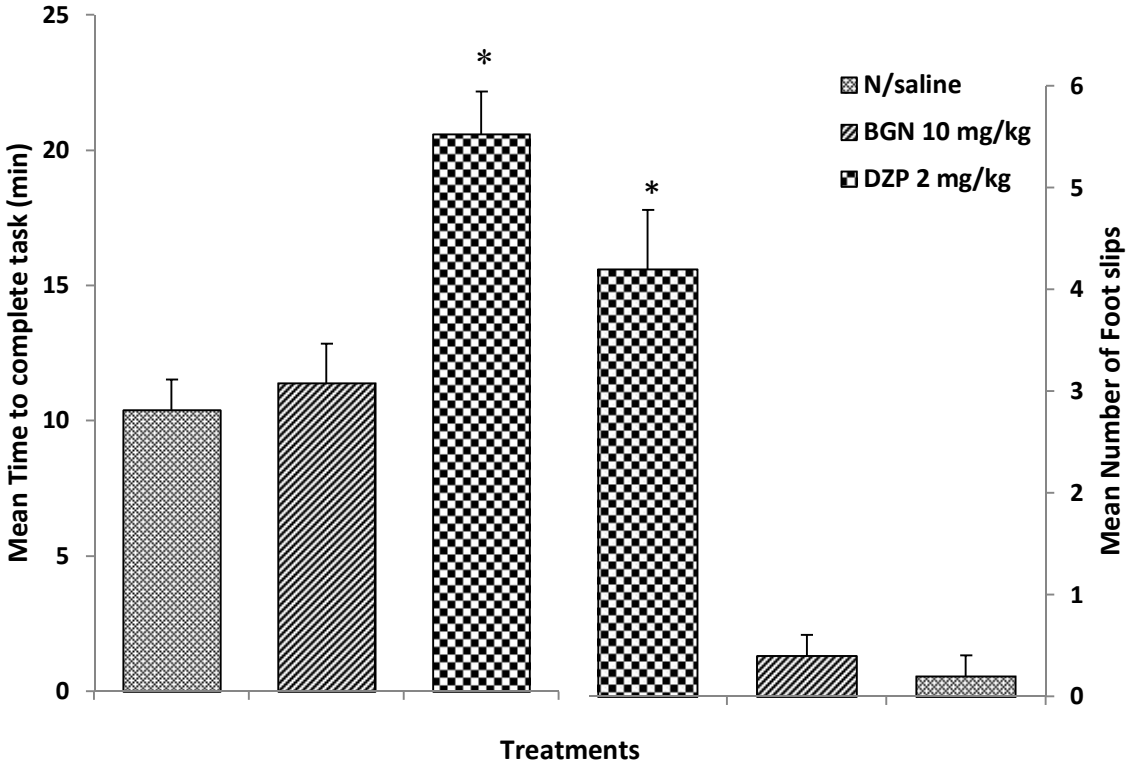


Figure 4.4.14: Effect of Bergenin on Motor Coordination using Beam Walking Assay in Mice; Data presented as mean ± SEM; BGN: Bergenin; DZP: diazepam; *P<0.001; n=5

4.4.4 Effects of Fractions of Methanolic Root Bark Extract of *Securinega virosa* on Exploratory Behaviour of Mice in Staircase Test

The residual aqueous fraction (RAF) significantly ($P < 0.01$- $P < 0.001$) and dose-dependently decreased the total number of upward stairs climbed. It also significantly decreased the total number of rearings ($P < 0.001$). However, this effect was not dose-dependent (4.4.15). The n-butanol fraction (NBF) significantly ($P < 0.05$ - $P < 0.001$) decreased both the number of rearings and the total number of upward stairs climbed. However, the effects were not dose-dependent. Diazepam, at the dose of 0.5 mg/kg, significantly decreased the total number of rearings ($P < 0.01$). It however, significantly increased the total number of stairs climbed ($P < 0.001$) (Figure 4.4.16).

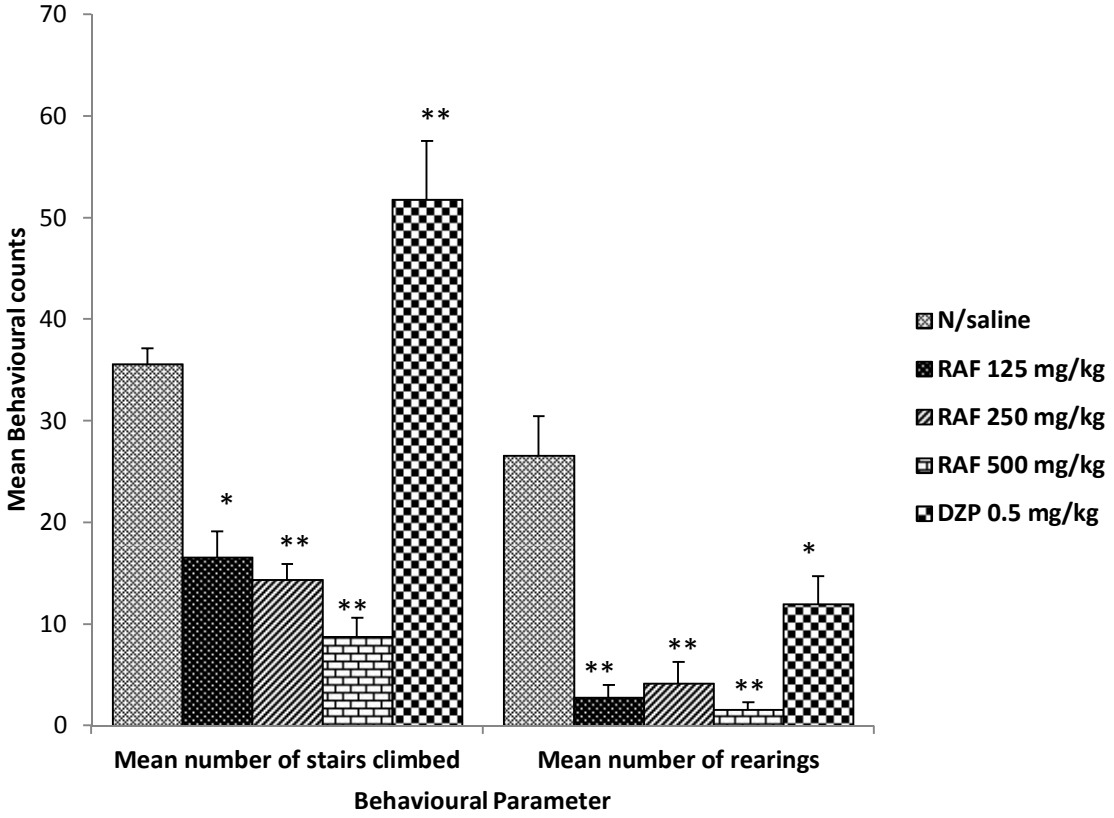


Figure 4.4.15: Effect of Residual Aqueous Fraction of Methanolic Root Bark Extract of *Securinega virosa* on Behaviour of Mice in Staircase Test; Data presented as mean ± SEM; N/saline; RAF: Residual aqueous fraction; DZP: Diazepam; *P<0.01, **P<0.001; n=6;

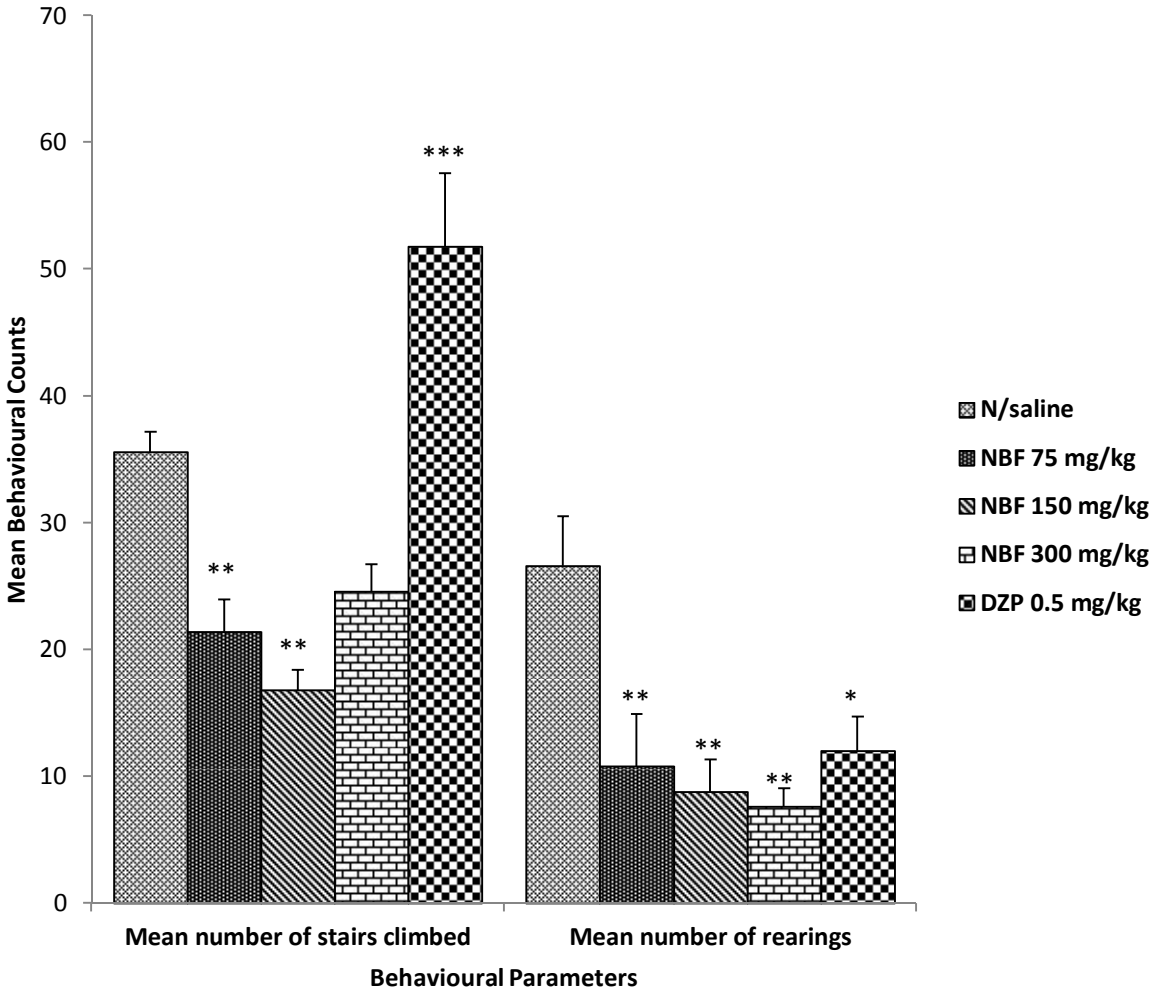


Figure 4.4.16: Effect of n-butanol Fraction (NBF) of Methanolic Root Bark Extract of *Securinega virosa* on Behaviour of Mice in Staircase Test; Data presented as mean ± SEM; N/saline: Normal saline; NBF: n-butanol fraction; DZP: diazepam; ; *P < 0.05, **P<0.01, **P<0.001; n=6

4.4.5 Effects of Fractions of Methanolic Root Bark Extract of *Securinega virosa* on Behaviour of Mice in Elevated Plus Maze

Both the residual aqueous and n-butanol fractions did not significantly change the number open and close arm entries compared with the control. There were also no significant changes in the total time spent in the open or closed arms. Diazepam, the standard agent used, at the dose of 0.5 mg/kg, significantly ($P < 0.05$) increased the number of open arm entries and total time spent in the open arm (Table 4.4.1).

Table 4.4.1: Effects of Fractions of Methanolic Root Bark Extract of *Securinega virosa* on Behaviour of Mice in Elevated Plus Maze

Treatments (mg/kg)	OAE	CAE	TTOA (S)	TTCA (S)
Normal saline (10 ml/kg)	25.9 ± 4.36	74.1 ± 4.36	23.93 ± 5.63	69.32 ± 8.53
NBF 75	13.79 ± 5.24	86.21 ± 2.24	8.89 ± 4.65	91.11 ± 4.65
NBF 150	18.78 ± 6.76	81.22 ± 6.76	16.19 ± 7.1	83.81 ± 7.1
NBF 300	25.98 ± 7.64	74.02 ± 7.64	30.69 ± 8.53	69.31 ± 8.53
RAF 125	9.15 ± 4.74	90.85 ± 4.74	4.59 ± 2.13	95.42 ± 2.13
RAF 250	24.37 ± 10.56	75.62 ± 10.55	15.71 ± 7.64	84.27 ± 7.64
RAF 500	30.23 ± 7.92	69.77 ± 7.92	22.05 ± 9.03	77.36 ± 8.81
DZP 0.5	52.85 ± 2.41*	47.03 ± 2.41*	52.03 ± 3.34*	47.97 ± 3.34*

OAE: number of open arm entries; CAE: number of closed arm entries; TTOA: Total time spent in the open arm; TTCA: Total time spent in the closed arm; Data presented as mean ± SEM; RAF: Residual aqueous fraction; NBF: n-butanol fraction; DZP: diazepam; *P<0.01; n=6

4.4.6 Effects of Fractions of Methanolic Root Bark Extract of *Securinega virosa* on Behaviour of Mice in Open Field Test

The residual aqueous fraction (RAF) of *Securinega virosa* significantly ($P < 0.05$- $P < 0.001$) reduced the number of rearings and number of square crossed (Figure 4.4.17). Similarly, the n-butanol fraction (NBF) significantly reduced both the number of rearings and total number of square crossed (Figure 4.4.18). However, both fractions did not significantly alter the number of central square crossed. Diazepam (DZP), the standard agent used increased both the number of rearings and total number of central square crossed (Appendices 11 and 12).

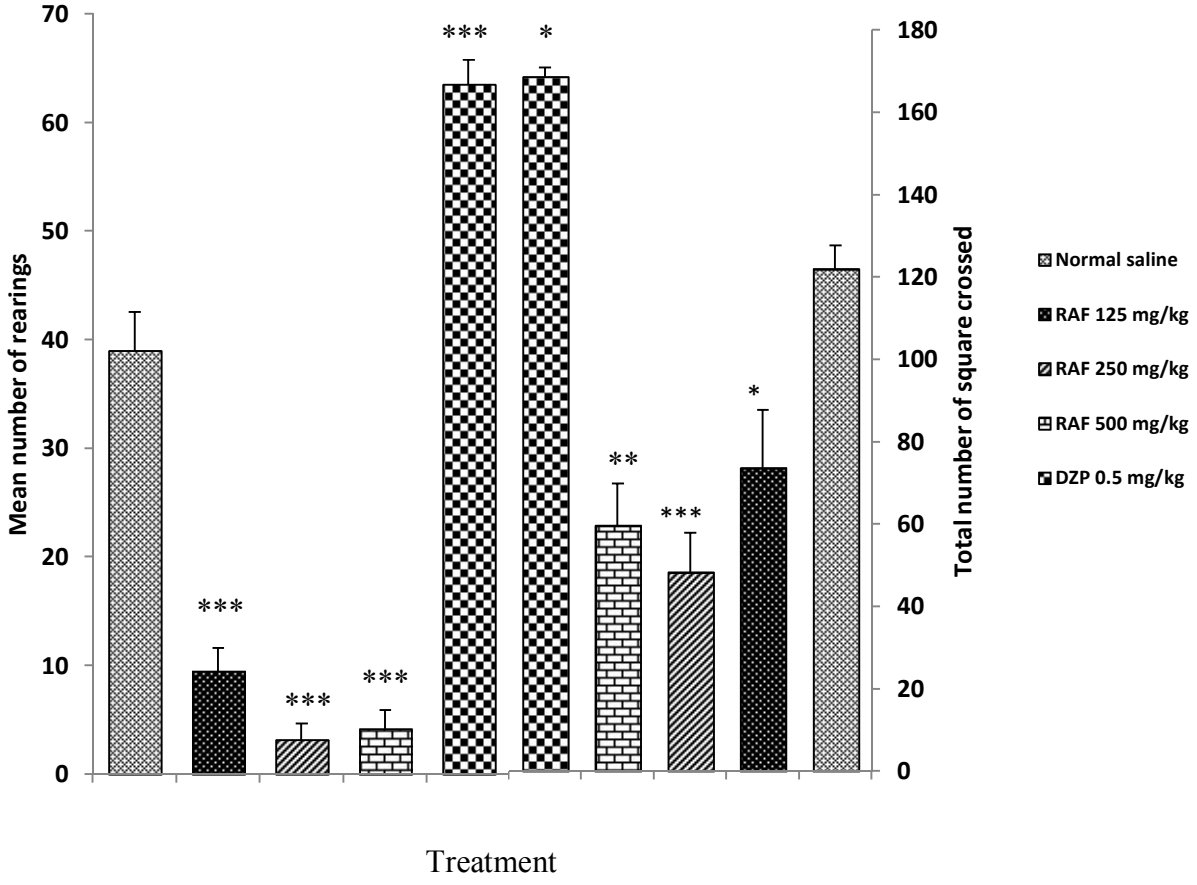


Figure 4.4.17: Effect Residual Aqueous Fraction of Methanolic Root Bark Extract of *Securinega virosa* on Behaviour of Mice in Open Field Test; Data presented as Mean ± SEM; RAF: Residual aqueous fraction; DZP: diazepam; *P<0.05; **P<0.01; ***P<0.001; n=6

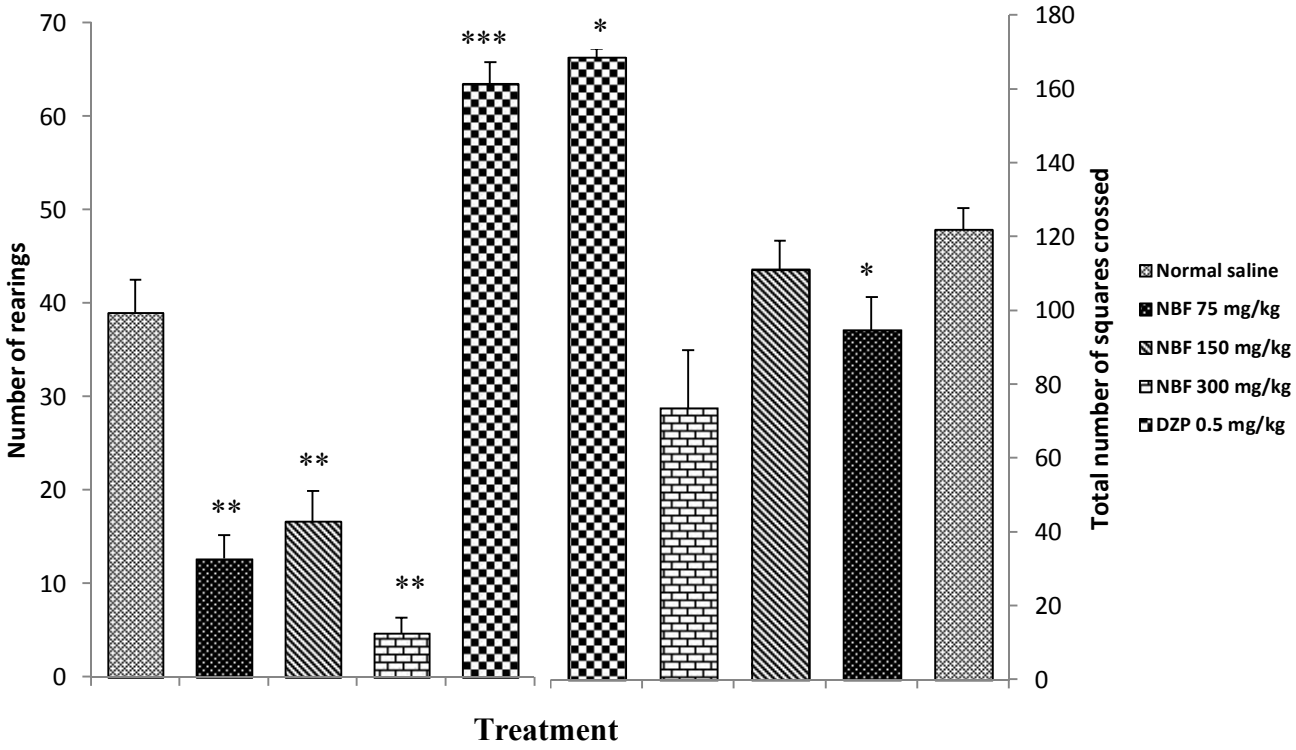


Figure 4.4.18: Effect of n-butanol Fraction of Methanolic Root Bark Extract of *Securinega virosa* on Behaviour of Mice in Open Field Test; Data presented as mean ± SEM; NBF: n-butanol fraction; DZP: diazepam; *P<0.05; **P<0.01; ***P<0.001; n=6

4.5. ANTICANCER STUDIES

4.5.1: Effects of Methanolic Root Bark Extract of *Securinega virosa* and its Fractions on Glioblastoma Multiforme Cells Viability

The crude methanolic root bark extract of *Securinega virosa* (CRE) significantly ($P< 0.001$) and dose-dependently decreased the cell count of the U-1242 MG GBM cells. At the concentration of 250 μg/ml, the cell viability was reduced from 17×10^4 cells to 1.7×10^4 cells (Figure 4.5.1). The residual aqueous fraction (RAF) similarly decreased the total cell count in a dose-dependent manner (Figure 4.5.2). The n-butanol fraction (NBF) significantly ($P < 0.001$) and dose-dependently decreased cell viability (Figure 4.5.3). The most remarkable activity was produced by the chloroform fraction (CCF) which reduced the mean cell viability by more than 95% at the concentration of 250 μg /ml (Figure 4.5.4).

The column fractions obtained from the n-butanol fraction variously inhibited cell growth. PFA and PFC reduced the mean cell count by 27% and 47%, respectively; while PFB reduced the cell viability by 79% (Figure 4.5.5). Both PF1 and PF2 obtained from the residual aqueous fraction afforded more than 90% reduction of cell viability (Figure 4.5.6). Bergenin, isolated from PFA did not produce any appreciable reduction in mean cell growth of the GBM cells (Figure 4.5.7).

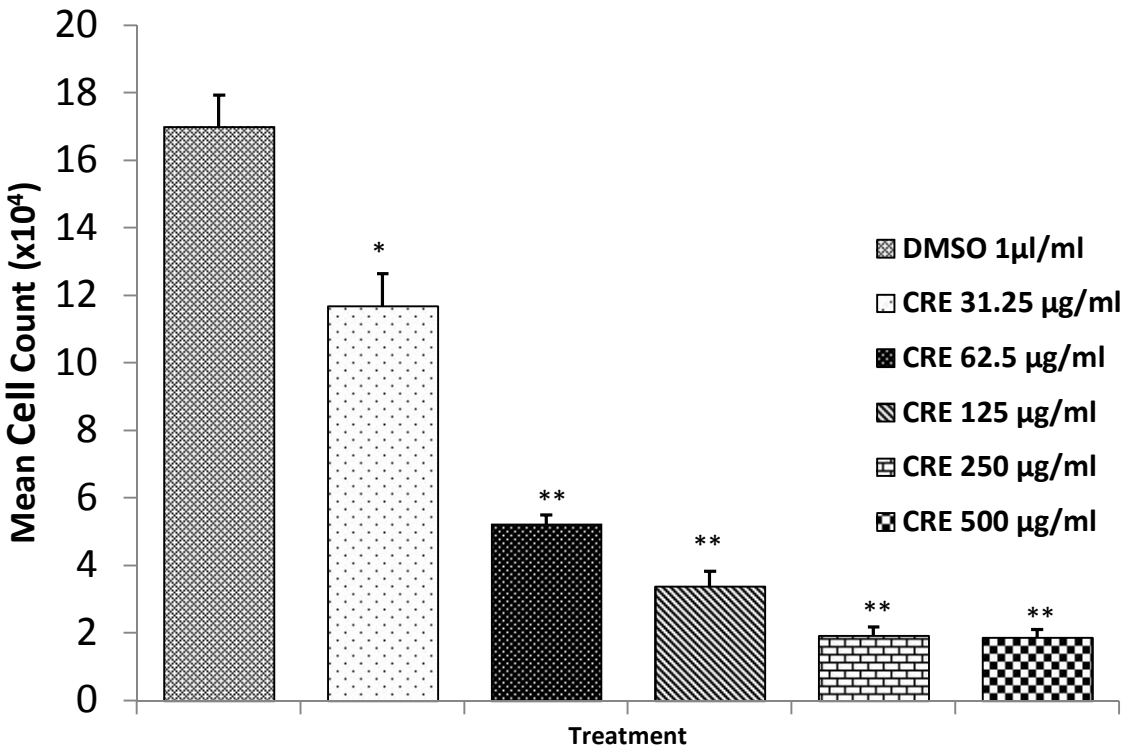


Figure 4.5.1: Effect of Crude Methanolic Root Bark Extract of *Securinega virosa* on Cell Viability of GBM cells; Data presented as mean ± SEM; CRE: crude methanolic root bark extract; DMSO: Dimethyl sulphoxide; *P < 0.01; **P < 0.001; n=4

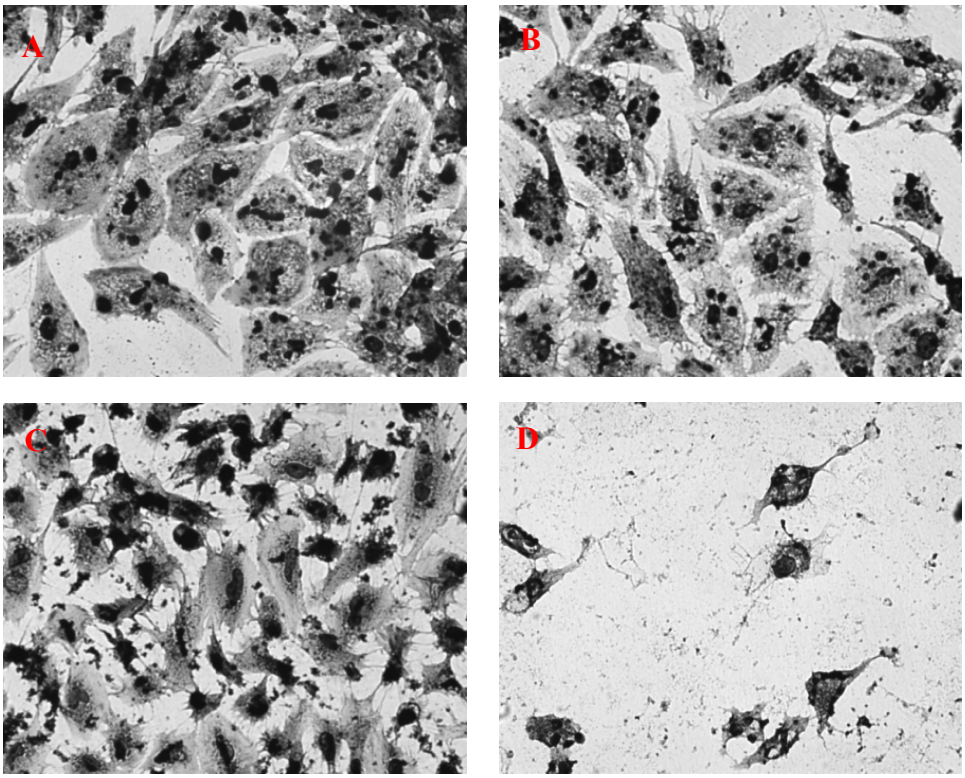
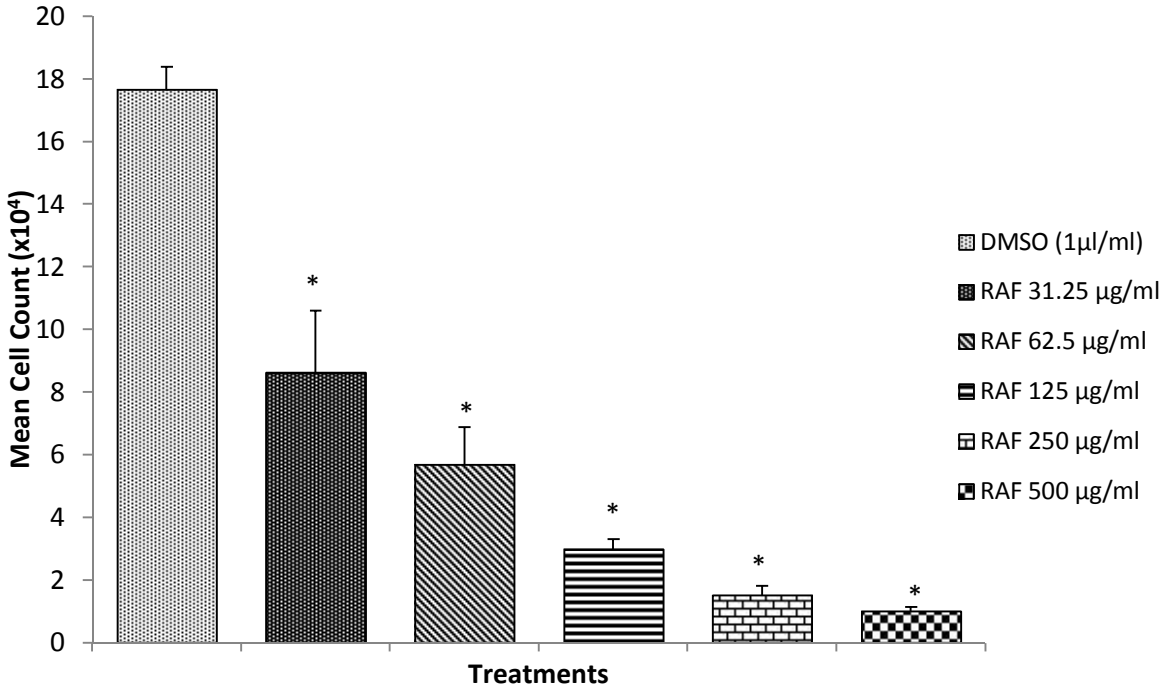


Plate III: GBM U-1242 MG cells treated with either DMSO or the crude extract (CRE). After 48 hrs cells were stained with crystal violet dye and observed under microscope (X20) A: DMSO; B: CRE 62.5 µg/ml; C: CRE 125 µg/ml; D: CRE 250 µg/ml



4.5.2: Effect of Residual Aqueous Fraction of Methanolic Root Bark Extract of *Securinega virosa* on Cell Viability of GBM cells; Data presented as mean ± SEM; RAF: Residual aqueous fraction; DMSO: Dimethyl sulphoxide; *P < 0.001; n=4

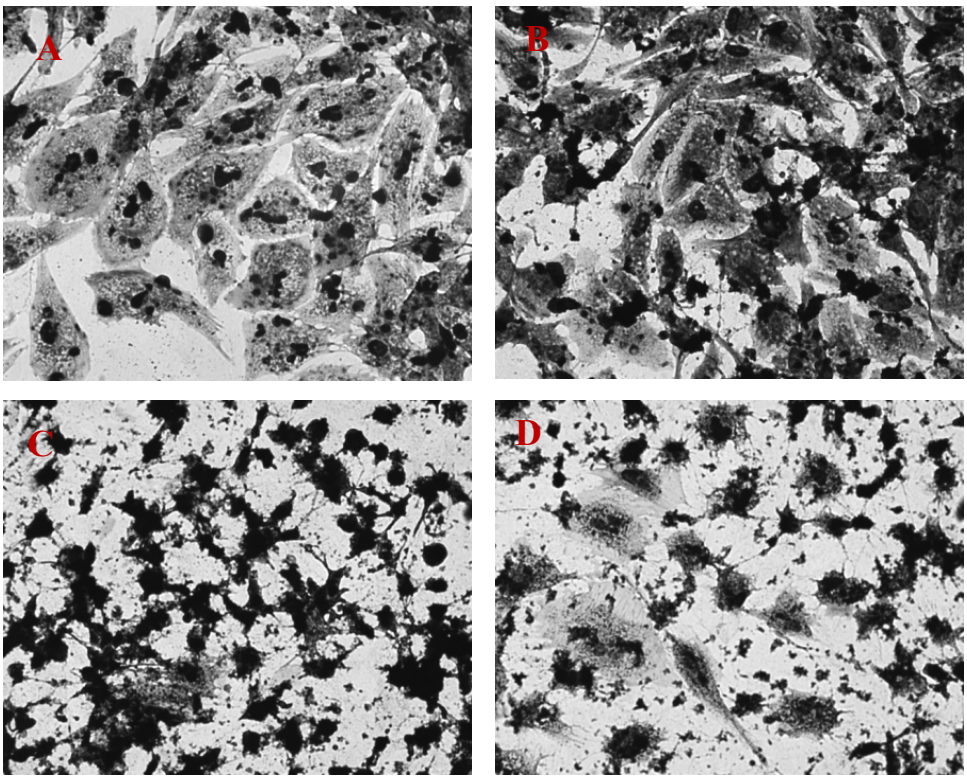


Plate IV: GBM U-1242 MG cells treated with either DMSO or the Residual aqueous fraction (RAF). After 48 hrs cells were stained with crystal violet dye and observed under microscope (X20) A: DMSO (1µl/ml); B: RAF 62.5 µg/ml; C: RAF 125 µg/ml; D: RAF 250 µg/ml

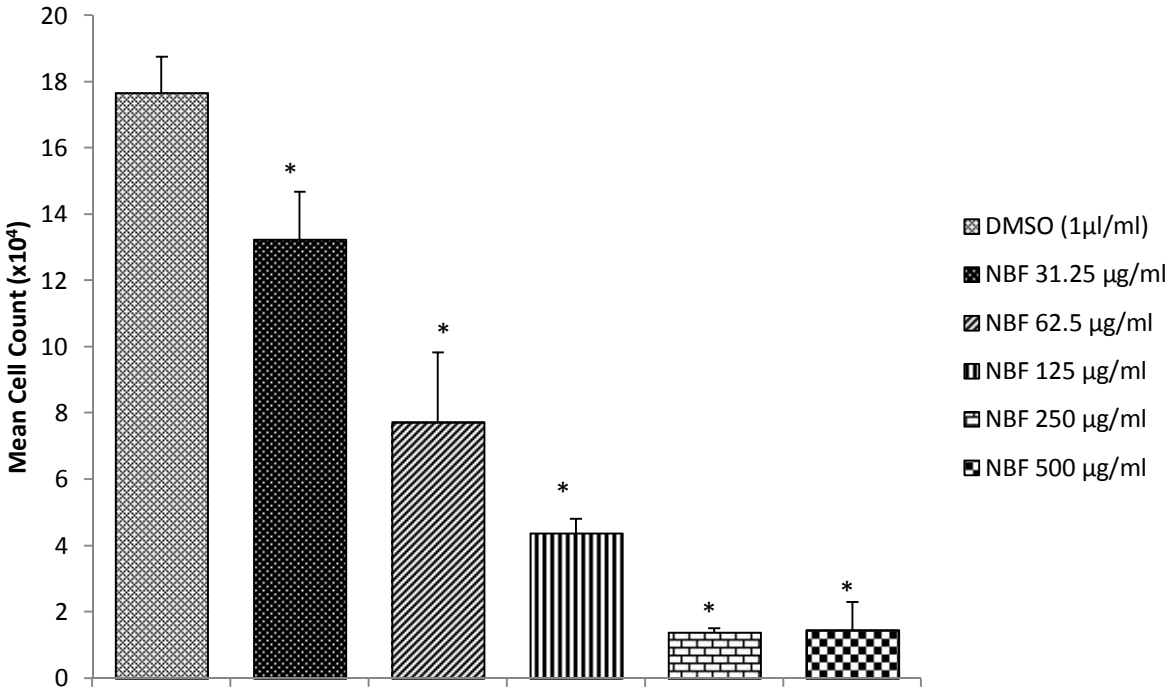


Figure 4.5.3: Effect of n-butanol Fraction of Methanolic Root Bark Extract of *Securinega virosa* on Cell viability of GBM cells; Data presented as mean ± SEM; NBF: n-butanol fraction; DMSO: Dimethyl suphoxide; *P < 0.001; n=4

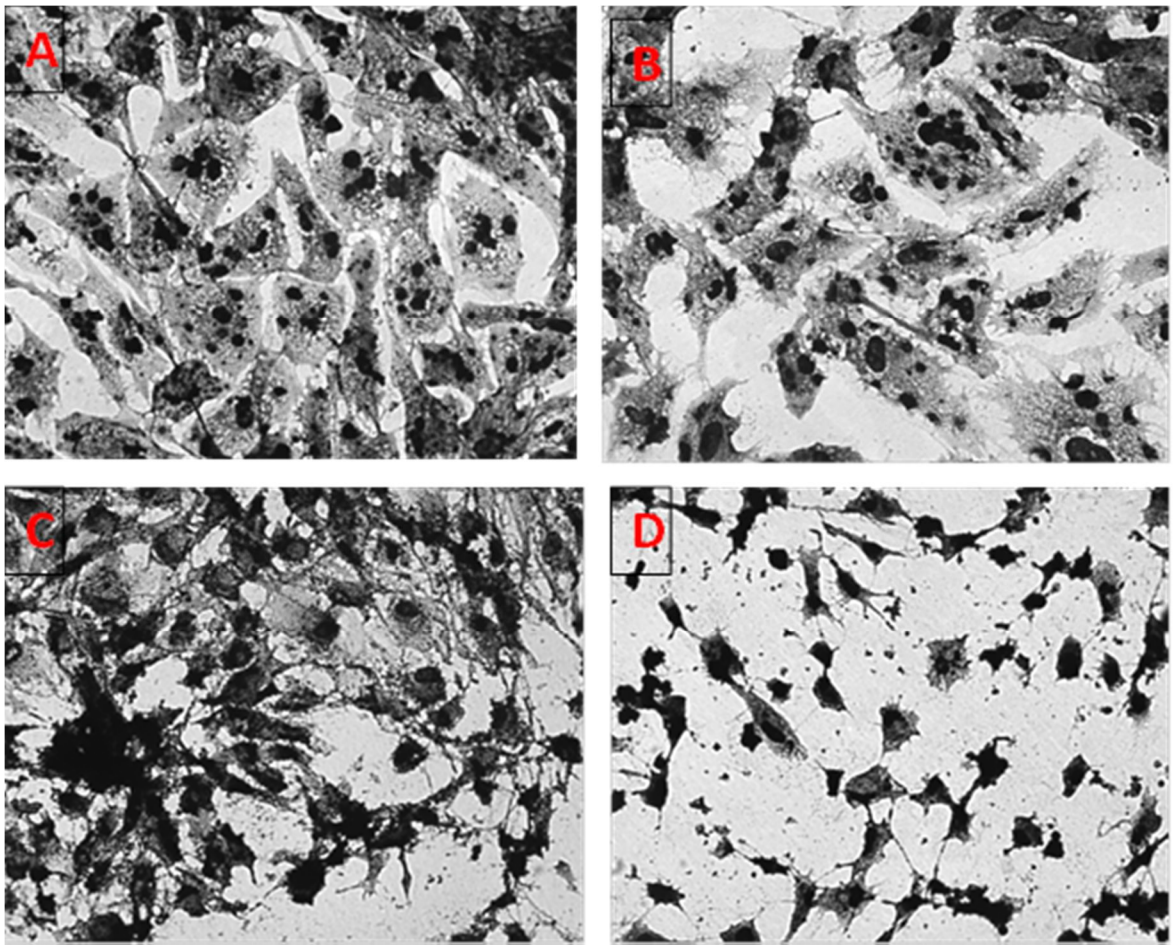


Plate V: GBM U-1242 MG cells treated with either DMSO or the n-butanol fraction (NBF). After 48 hrs cells were stained with crystal violet dye and observed under microscope (X20) A: DMSO (1µl/ml); B: NBF 62.5 µg/ml; C: NBF 125 µg/ml; D: NBF 250 µg/ml

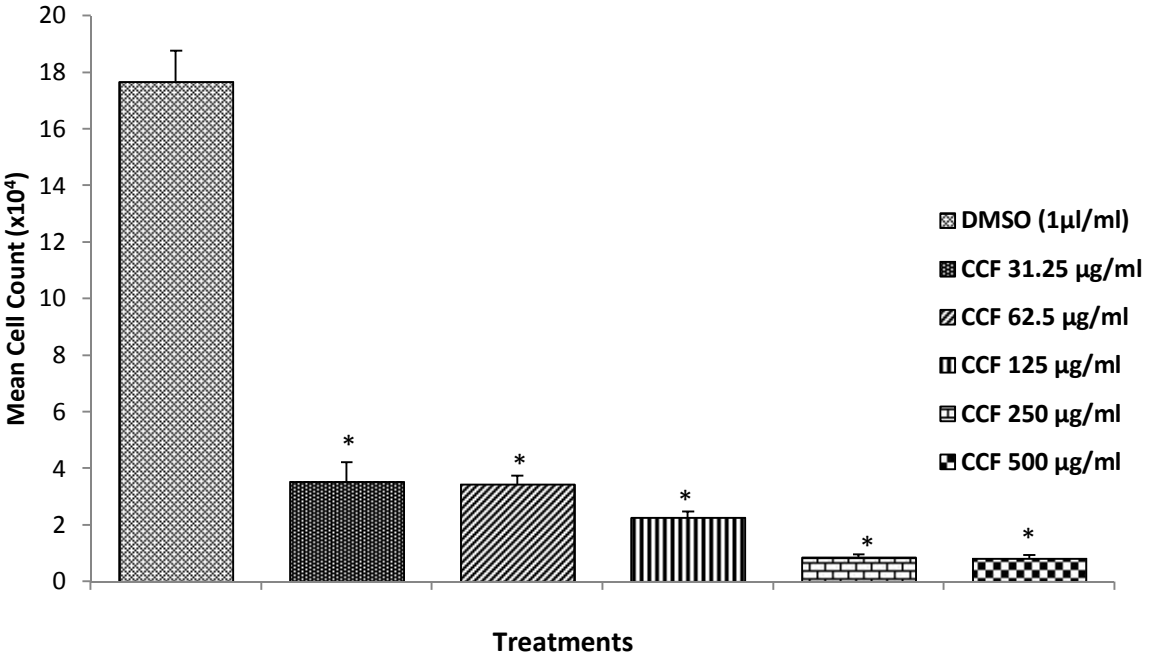


Figure 4.5.4: Effect of Chloroform Fraction of Methanolic Root Bark Extract of *Securinega virosa* on cell viability of GBM cells; Data presented as mean ± SEM; CCF: Chloroform fraction; DMSO: Dimethyl suphoxide; *P < 0.001; n=4

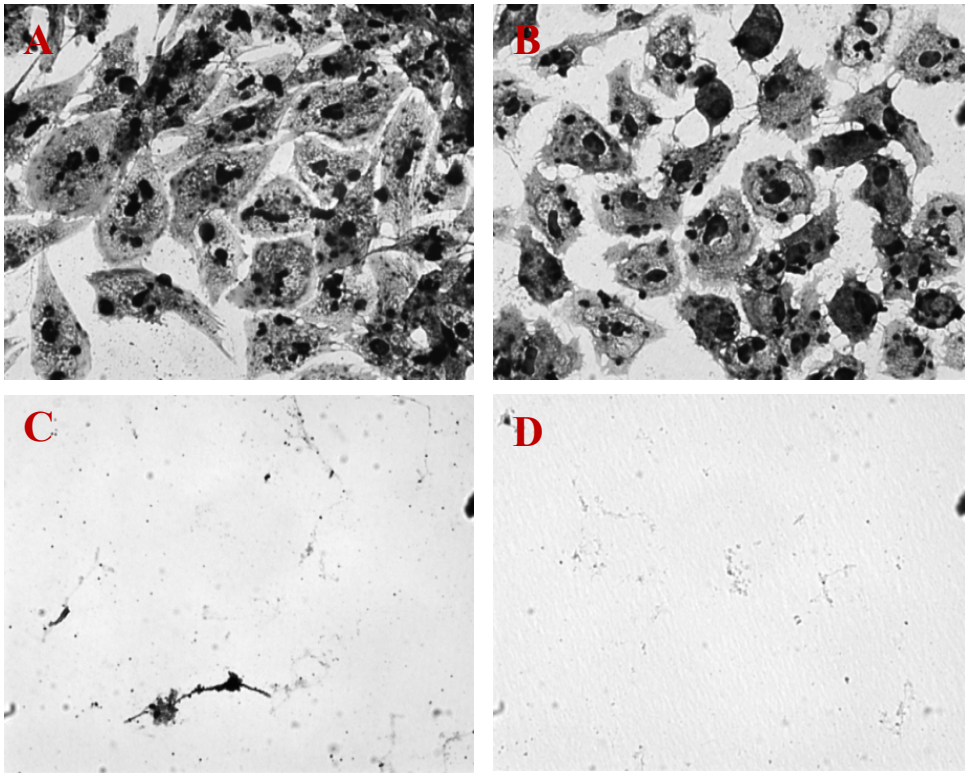


Plate VI: GBM U-1242 MG cells treated with either DMSO or the Chloroform fraction (CCF). After 48 hrs, cells were stained with crystal violet dye and observed under microscope (X20) A: DMSO (1µsl/ml); B: CCF 62.5 µg/ml; C: CCF 125 µg/ml; D: CCF 250 µg/ml

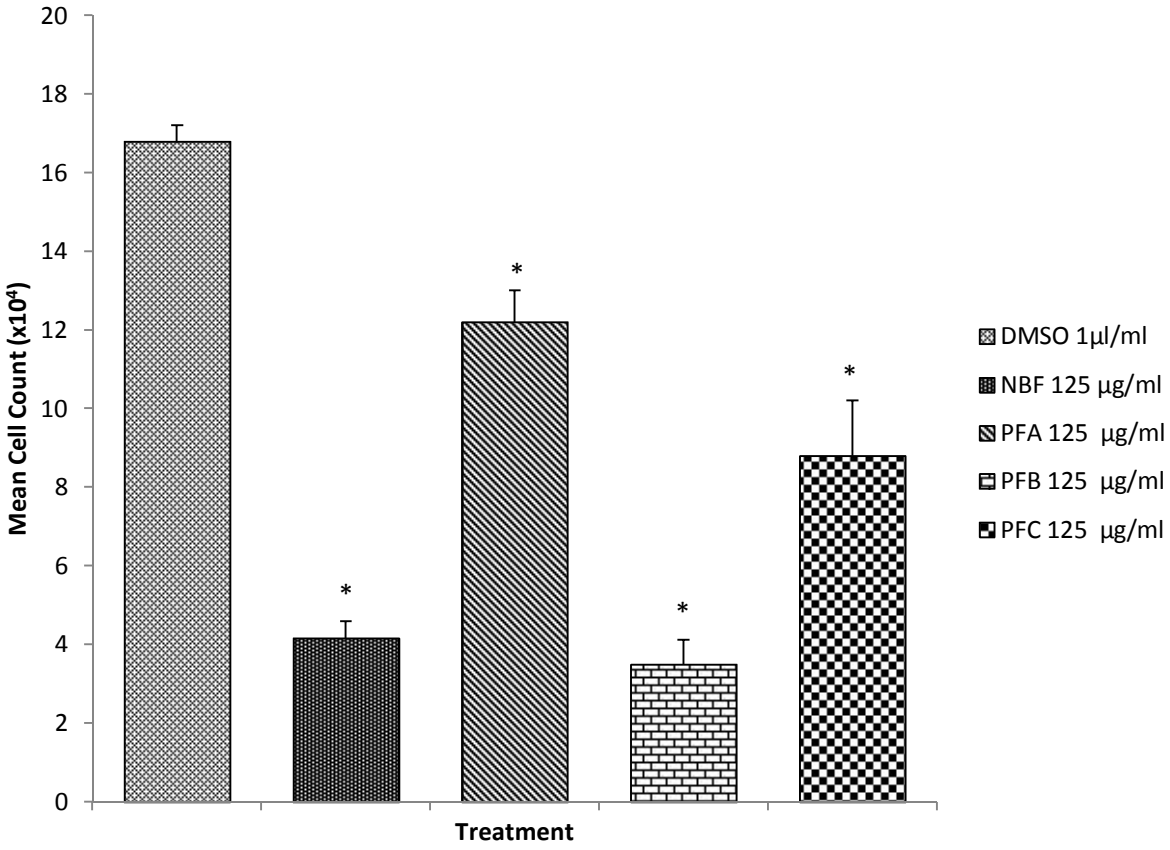


Figure 4.5.5: Effects of Column Fractions obtained from n-butanol Fraction (NBF) of Methanolic Root Bark Extract of *Securinega virosa* on Cell Viability of GBM cells; Data presented as mean ± SEM; DMSO: Dimethyl sulphoxide; *P < 0.001; n=4

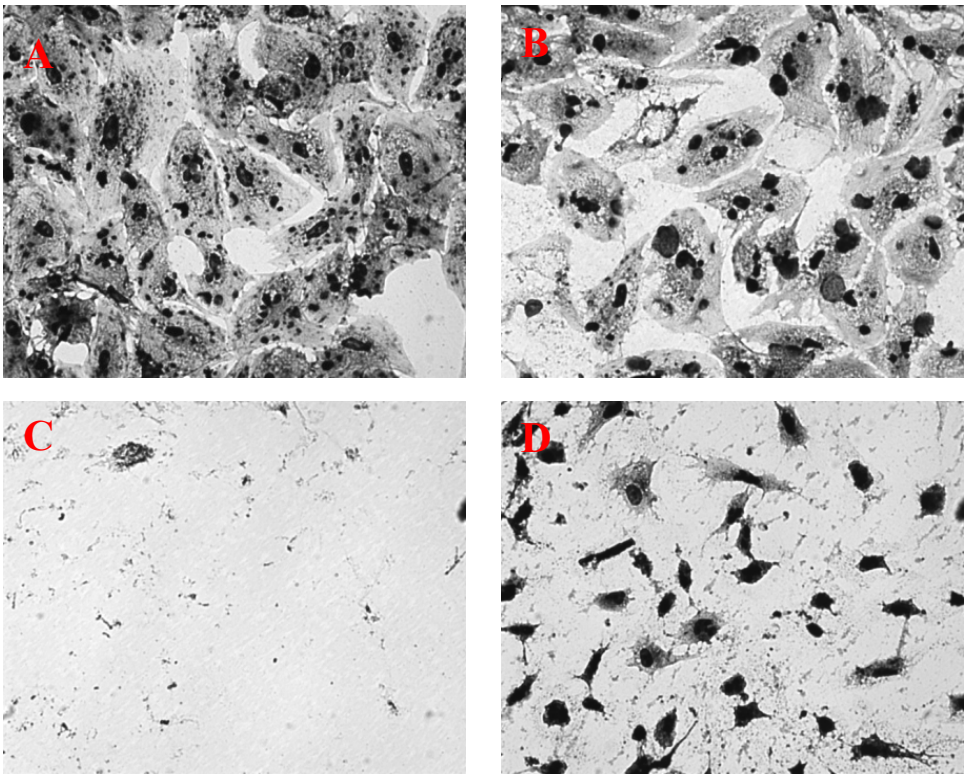


Plate 6: GBM U-1242 MG cells treated with either DMSO, the column fractions of n-butanol fraction. After 48 hrs cells were stained with crystal violet dye and observed under microscope (X20) A: DMSO (1µl/ml); B: PFA: 125 µg/ml; C: PFB (125 µg/ml); D: PFC (125 µg/ml)

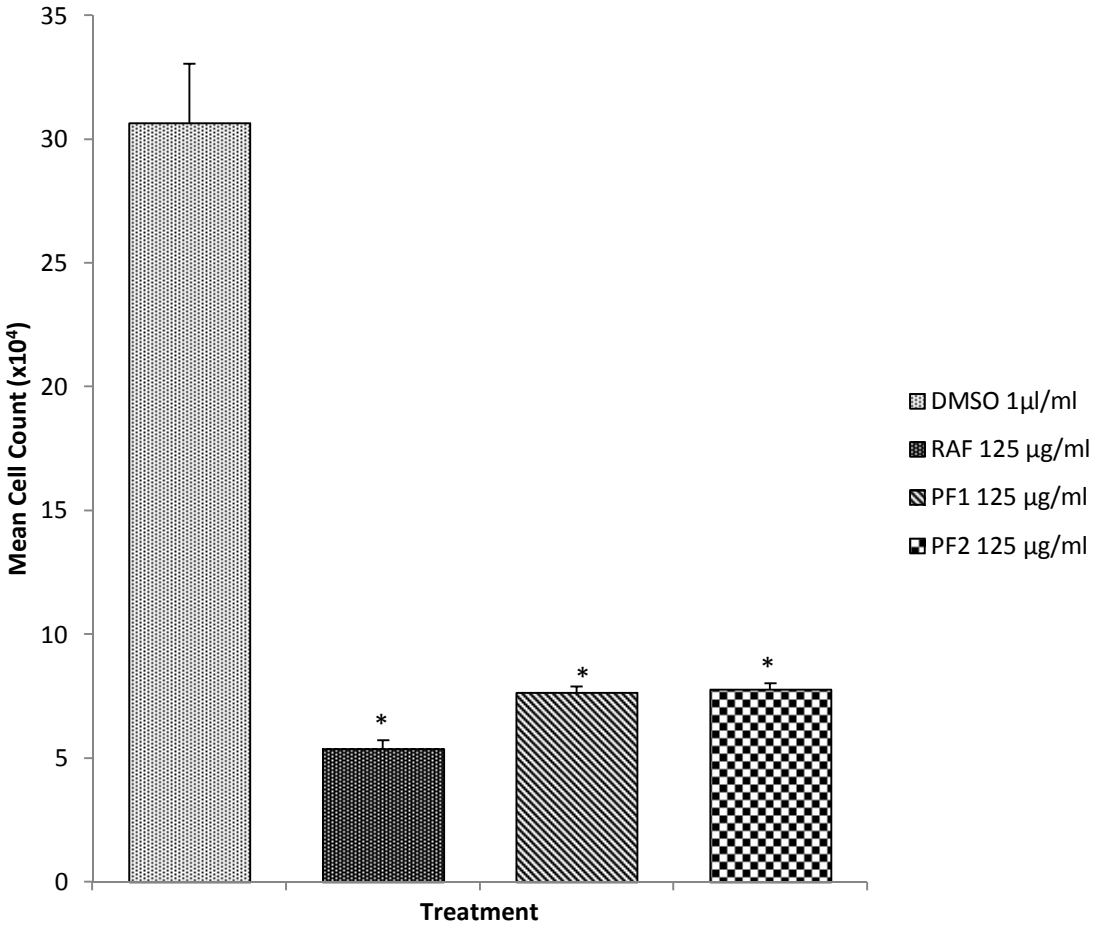


Figure 4.5.6: Effects of Column Fractions obtained from Residual Aqueous Fraction of Methanolic Root Bark Extract of *Securinega virosa* on Cell Viability of GBM cells; Data presented as mean ± SEM; RAF: Residual aqueous fraction; DMSO: Dimethyl suphoxide; *P < 0.001; n=4

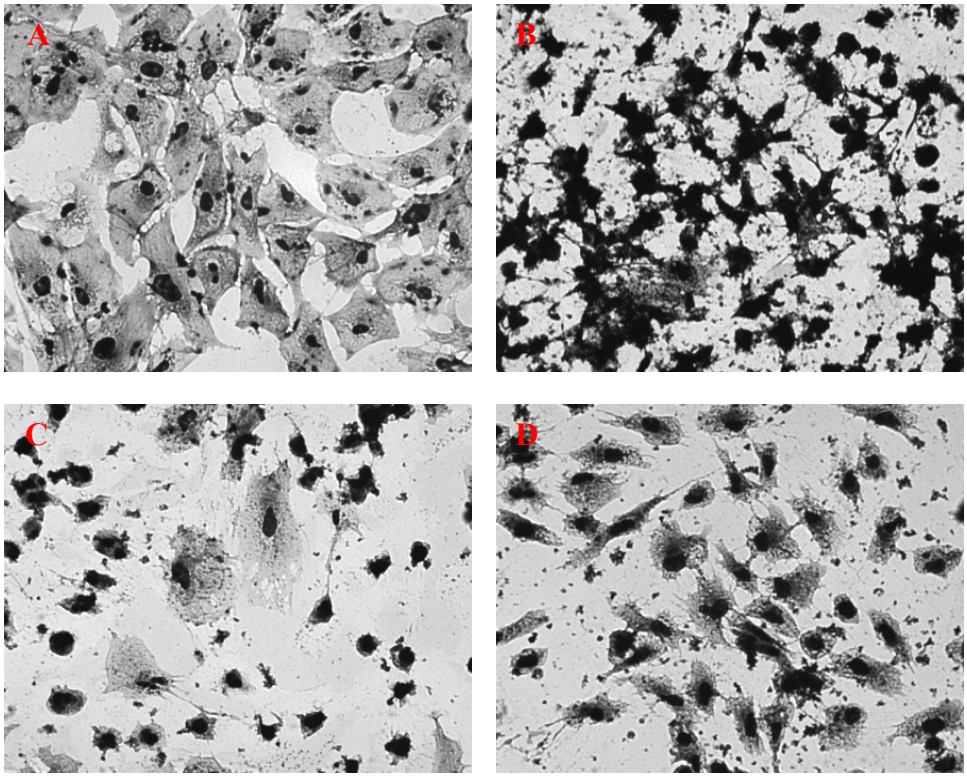


Plate 7: GBM U-1242 MG cells treated with either DMSO, the Residual Aqueous fraction (125 µg/ml) or its fractions. After 48 hrs cells were stained with crystal violet dye and observed under microscope (X20) A: DMSO; B: RAF: 125 mg/ml; C: PF1 (125 µg/ml); D: PF2 (125 µg/ml)

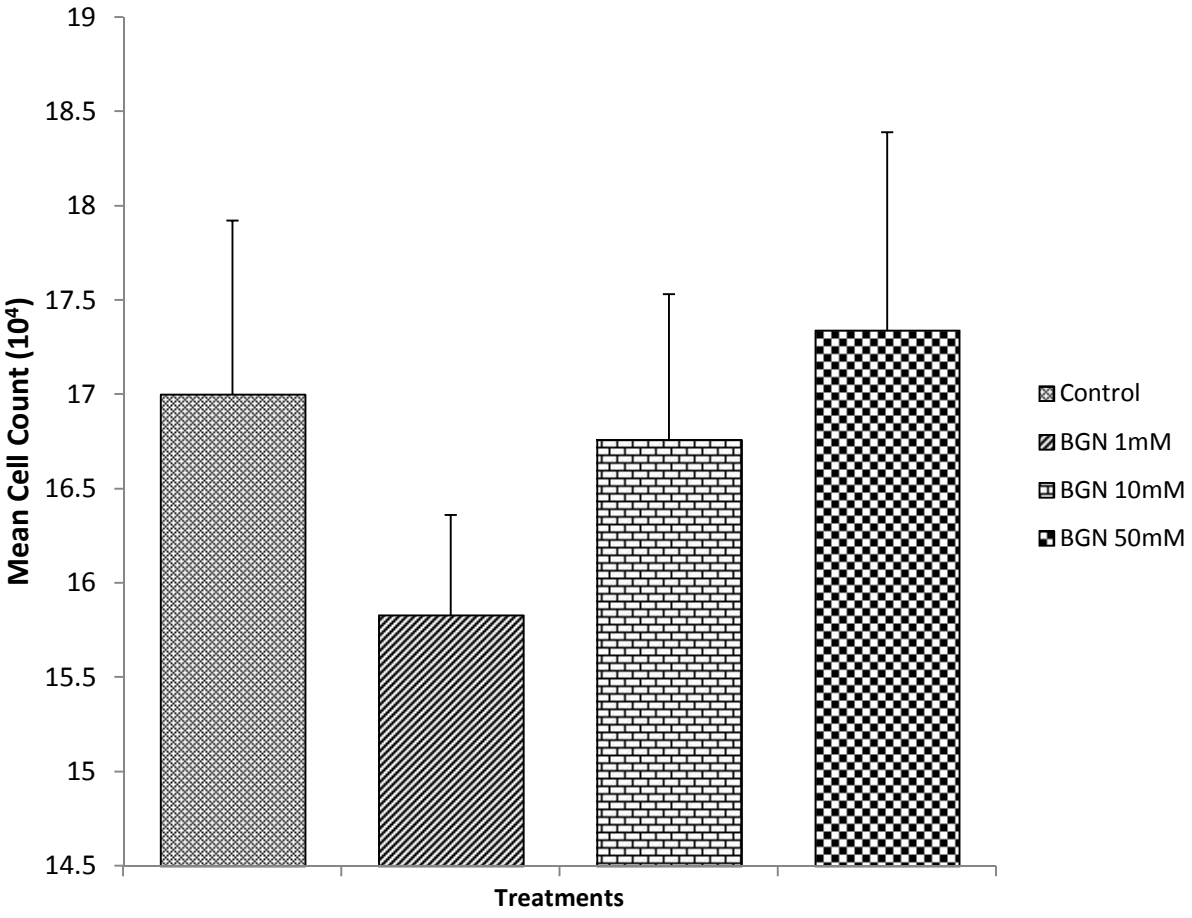


Figure 4.5.7: Effect of Bergenin isolated from n-butanol fraction of Methanolic Root Bark Extract of *Securinega virosa* on Cell Viability of GBM Cells; Data presented as mean ± SEM; BGN: Bergenin; *P < 0.001; n=4

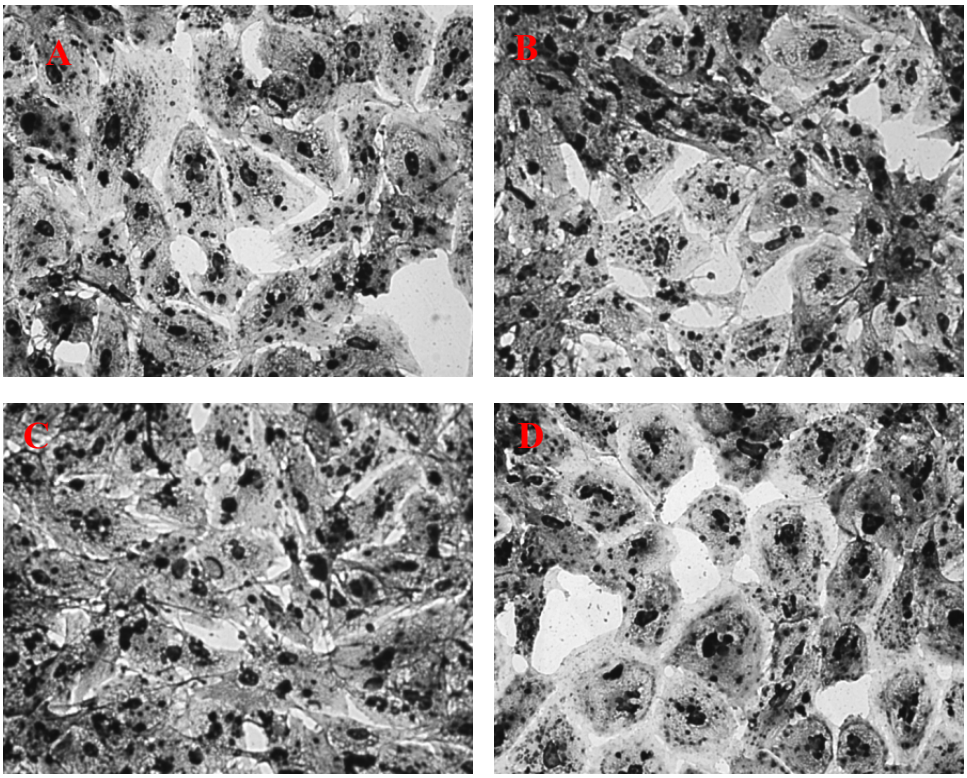


Plate 8: GBM U-1242 MG cells treated with either DMSO or bergenin (1, 10 and 50 mM). After 48 hrs cells were stained with crystal violet dye and observed under microscope (X20) A: DMSO; B: BGN 1mM; C: BGN 10 mM; D: BGN 50 mM

4.5.2 Effects of Methanolic Extract and Fractions of Root Bark of *Securinega virosa* on EGF-driven Cell Growth

Treatment of the cells with EGF resulted in 38% increase in cell count compared with DMSO treated cells. The crude methanolic extract (CRE) significantly ($P < 0.001$) and dose-dependently decreased cell viability of the U-1242 MG cells treated with EGF. At the highest concentration tested (250 µg/ml), CRE reduced the mean cell count by 92% (Figure 4.5.8). The residual aqueous fraction (RAF) reduced the cell viability by a factor of 10 at the concentration of 125 µg/ml (Figure 4.5.9). Similarly, the n-butanol fraction (NBF) exhibited significant ($P < 0.001$) and concentration-dependent cells kill (Figure 4.5.10). The highest reduction in cell viability was observed with the chloroform fraction which reduced the viable cell count by 94 % at the concentration of 250 µg/ml (Figure 4.5.11).

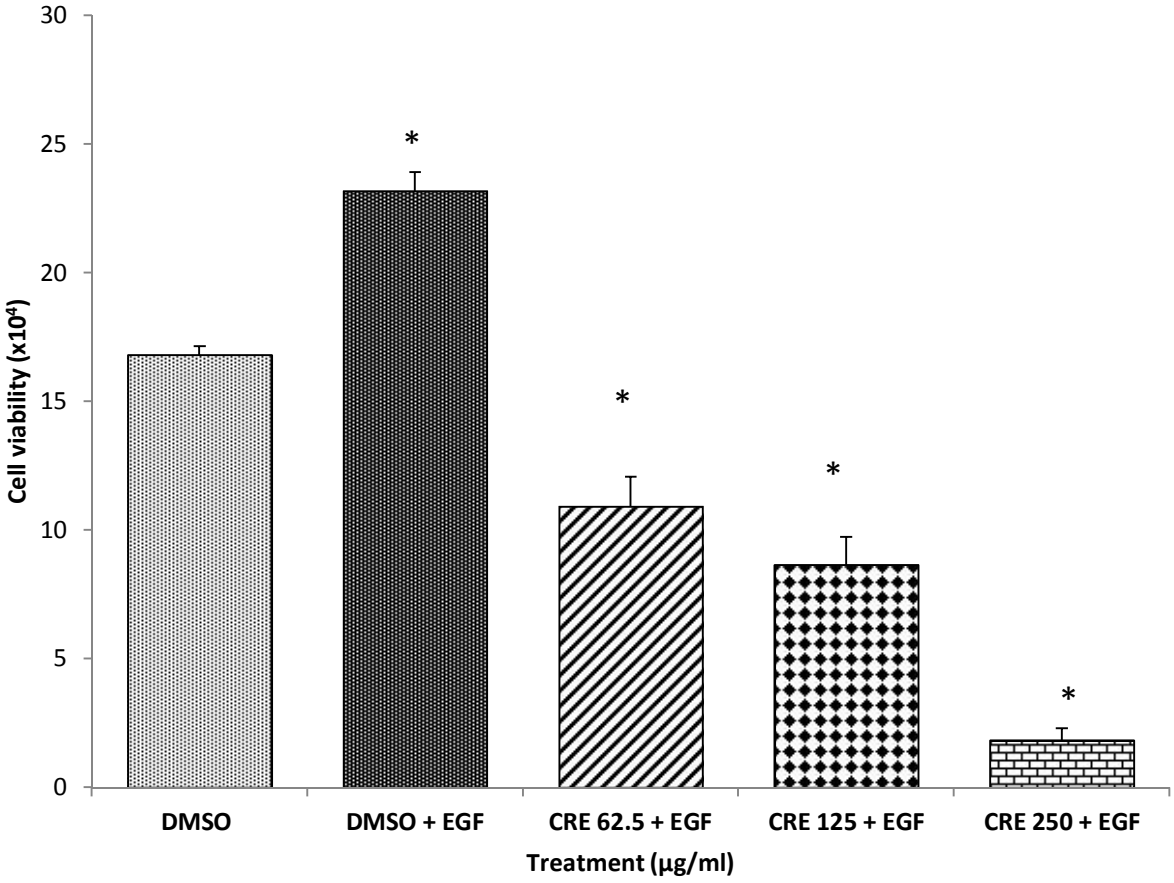
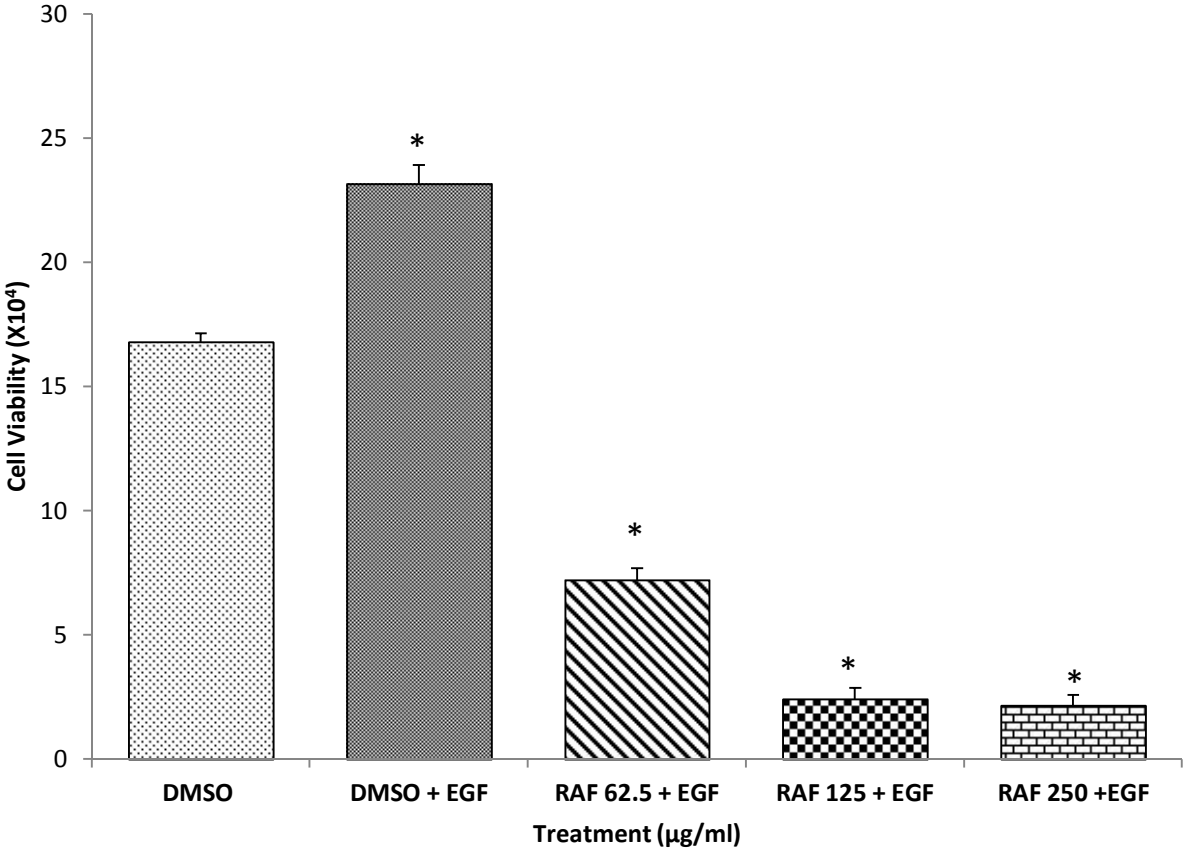


Figure 4.5.8: Effect Crude Methanolic Root Bark Extract of *Securinega virosa* on Viability of U-1242 MG GBM cells treated with EGF. Cell count was carried out 48 hours after the treatment; Data presented as mean ± SEM; CRE: Crude Methanolic Root Bark Extract; DMSO: Dimethyl sulphoxide; EGF: Epidermal growth factor; n=4



4.5.9: Effect of Residual Aqueous Fraction of Methanolic Root Bark Extract of *Securinega virosa* on Viability of U-1242 MG GBM cells treated with EGF. Cell counts were determined 48 hours after the treatment; Data presented as mean ± SEM; RAF: Residual aqueous fraction; DMSO: dimethyl sulphoxide; EGF: Epidermal growth factor; *P < 0.001; n=4

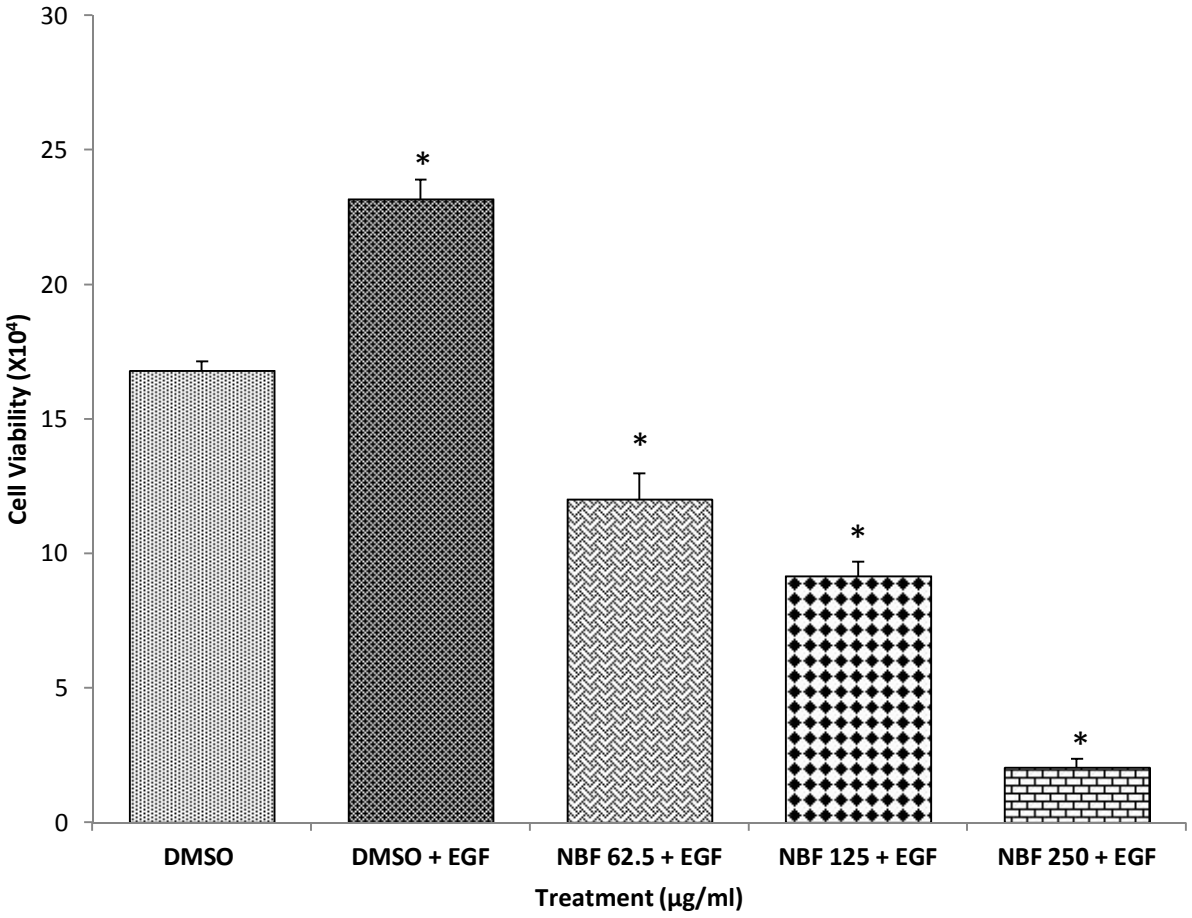


Figure 4.5.10: Effect of n-butanol Fraction of Methanolic Root Bark Extract of *Securinega virosa* on Viability of U-1242 MG GBM cells treated with EGF. Cell count was carried out 48 hours after the treatment; Data presented as mean ± SEM; NBF: n-butanol fraction; DMSO: Dimethyl sulphoxide; EGF: Epidermal growth factor; *P < 0.001; n=4

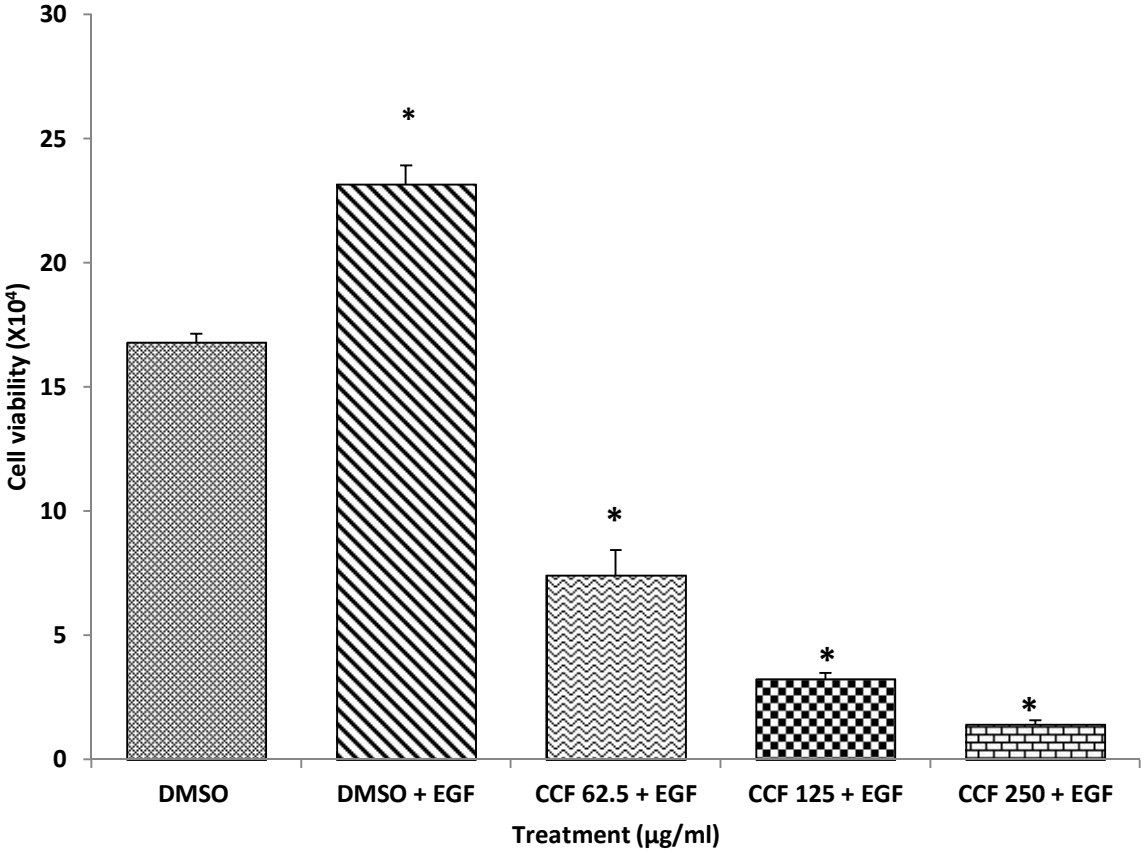


Figure 4.5.11: Effect of Chloroform Fraction of Methanolic Root Bark Extract of *Securinega virosa* on Viability of U-1242 MG GBM cells treated with EGF. Cell count was carried out 48 hours after the treatment; Data presented as mean ± SEM; CCF: Chloroform fraction; DMSO: Dimethyl sulphoxide; EGF: Epidermal growth factor;; *P < 0.001; n=4

4.5.3 Results of Western Blot Analysis

The crude methanolic root bark extract (CRE) of *Securinega virosa* blocked the phosphorylation of EGFR at tyr 1068 site. It also blocked the phosphorylation of MAPK (Figure 4.5.12). The crude extract, residual aqueous fraction and n-butanol fraction blocked the phosphorylation of EGFR at tyr 1045. However, the activity was more remarkable with the crude extract and residual aqueous fraction. Only the crude extract and the residual aqueous fraction remarkably blocked EGFR phosphorylation at Tyr 1068 site (Figure 4.5.13). The crude extract significantly blocked PMA-induced transactivation of EGFR at Tyr 1068 site. The effects produced by the residual aqueous and n-butanol fractions were less remarkable (Figure 4.5.14). The crude extract significantly blocked the phosphorylation of PDGFR. The extract and the residual aqueous fraction remarkably blocked PDGF-induced transactivation of EGFR at tyr 1068. Conversely, the extract as well as its fractions produced no effect on the phosphorylation of Akt (Figure 4.5.15).

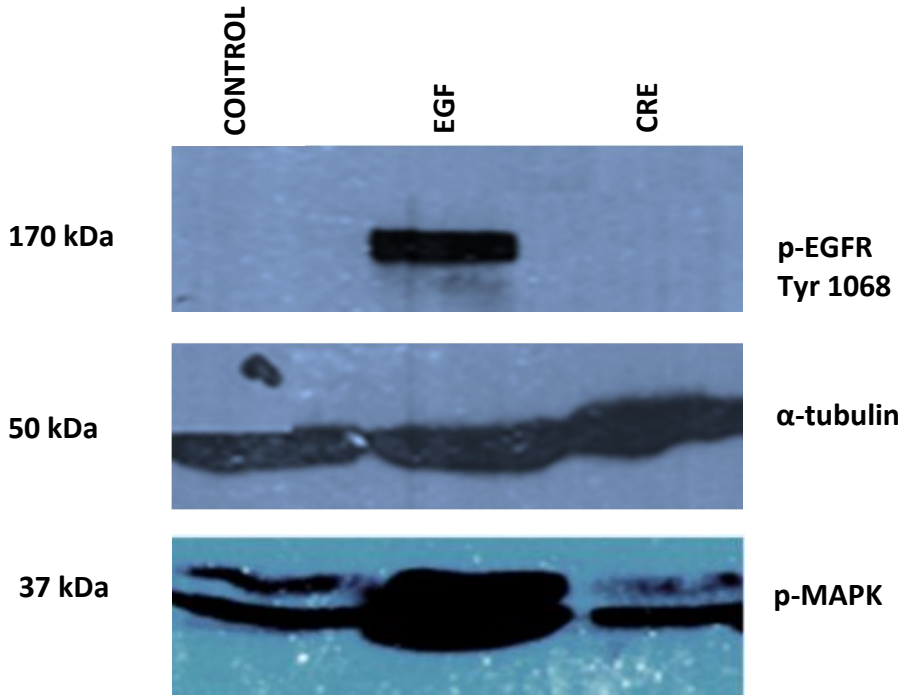


Figure 4.5.12: Effect of Methanolic Root Bark Extract (CRE) of *Securinega virosa* on Phosphorylation of EGFR receptor at tyr 1068 site and Mitogen protein kinase (MAPK)

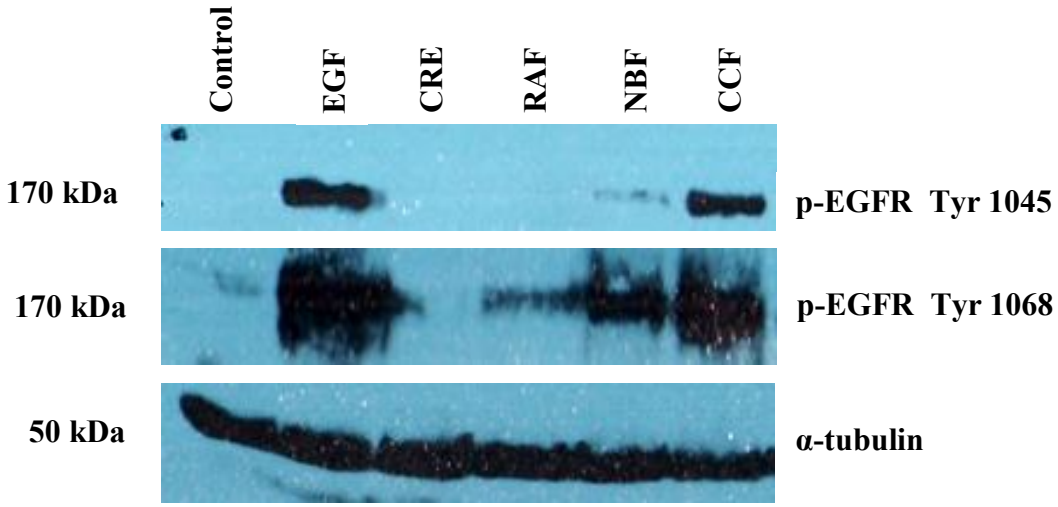


Figure 4.5.13: Effect of Crude Methanolic Root Bark Extract of *Securinega virosa* and its Fractions on EGF-induced Phosphorylation of EGFR at tyr 1045 and 1068 sites

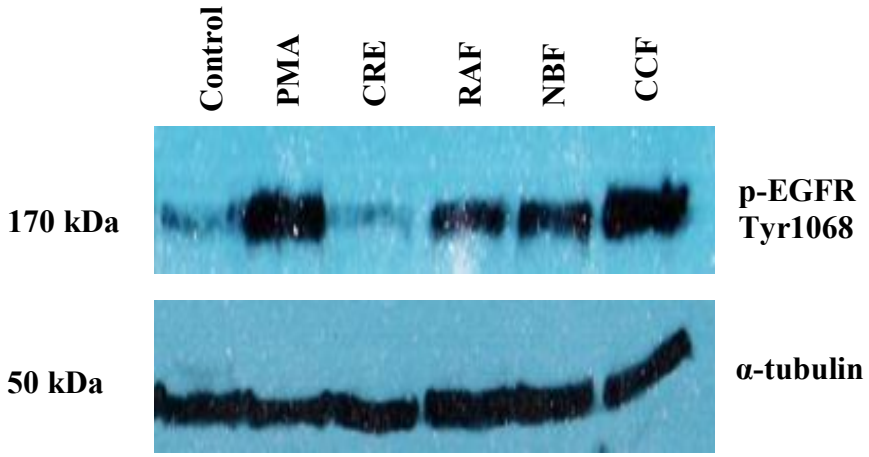


Figure 4.5.14: Effect of Methanolic Root Bark Extract of *Securinega virosa* and its Fractions on PMA- induced Transactivation of EGFR at tyr 1068 site

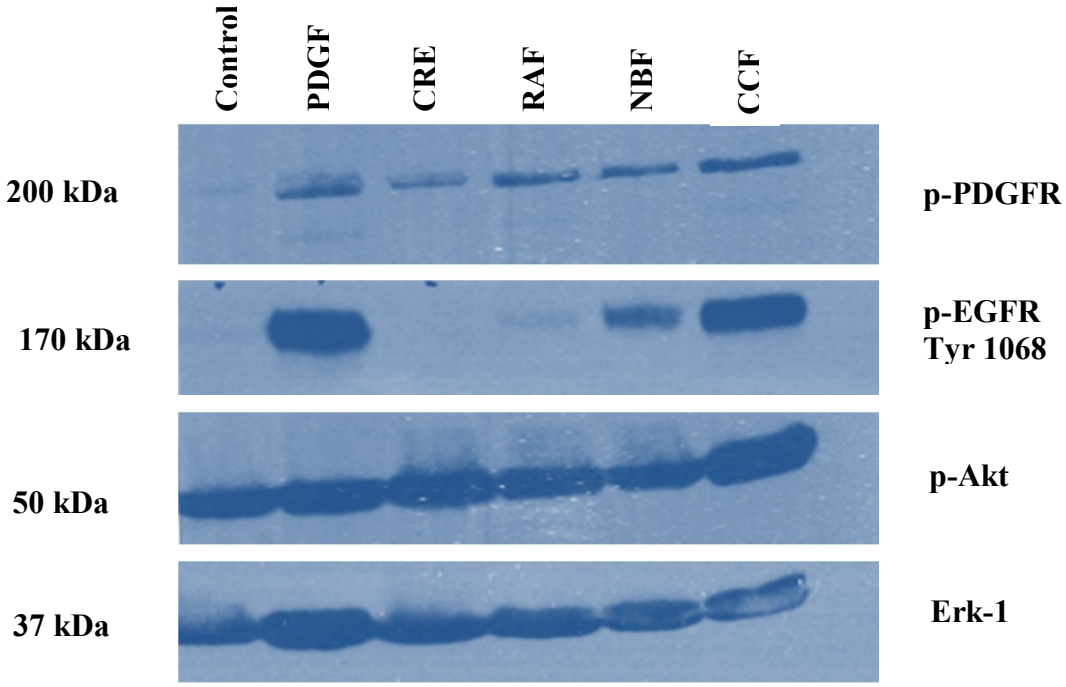


Figure 4.5.15: Effect of Methanolic Root Bark Extract of *Securinega virosa* and its Fraction on PDFR-induced Phosphorylation of PDGFR, Transactivation of EGFR at tyr 1068 Receptor site and Phosphorylation of Akt

CHAPTER 5

DISCUSSION

The results obtained from this study suggest that the fractions of methanolic root bark extract of *Securinega virosa* contain bioactive constituents that may be beneficial in the management of neuropsychiatric disorders such as epilepsy and sleep disorders; and glioblastoma multiforme. Also, the isolated compound, bergenin possesses sleep inducing property.

The phytochemical constituents (flavonoids, tannins, saponins, alkaloids and steroids) found to be present in the crude extract and its various fractions might be responsible for the observed pharmacological activities of the extract.

The fractionation of the crude methanolic root bark extract yielded four (4) fractions namely: chloroform, ethyl acetate, n-butanol and residual aqueous fractions. The yields of these fractions were found to be 0.56%, 3.4%, 14.63% and 61.64%, respectively. The high yields of n-butanol and the residual aqueous fractions suggest that the bioactive constituents are mostly polar.

Column chromatography of the n-butanol fraction followed by gel filtration led to the isolation of compound X. The positive chemical reaction shown by the compound when subjected to ferric chloride test gave an indication that compound X has phenolic hydroxyl group (Silva *et al.,* 1998). The greenish coloured spot observed when the compound was subjected to thin layer chromatography and sprayed with Gibbs reagent further suggests that the compound has phenolic nucleus. The molecular formula of compound X was assigned as $C_{14}H_{16}O_9$ from an $[M]^-$ peak at m/z 327 in the high-resolution EIMS with a base peak at m/z 192, also observed is a peak at 312 corresponding to $M\text{-}CH_3$.

The ^{1}HNMR spectrum of compound X exhibited signals for one aromatic proton at 7.1 singlet, this is an indication of a pentasubstituted aromatic ring. It also revealed the presence of three hydrogens singlet δ 3.9, this is an indication of the presence of methoxyl group on benzene nucleus. The resonances observed at δ 3.3, 3.4, 3.7, 3.8 and 4.0 and 4.98 suggest presence of sugar probably glucose in the molecule. The ^{13}C and DEPT NMR spectra of compound X revealed one tertiary(δ 109) and five quaternary aromatic methine signals (δ 118,152,141,148 and 116) confirming the presence of a pentasubstituted benzene nucleus. The methyl signal at δ 59.8 further confirmed the presence of methoxyl group. Resonances at δ 61.5 due to oxymethylene group and methine carbons at δ 71, 73, 74, 80.5 and 82 were also observed. These carbon signals are typical of sugar molecule. Also observed is a signal at 163.0 which is due to carbonyl carbon of carboxylic acid derivative. The assignment of carbons and the placement of the sugar moiety were achieved using two dimensional NMR experiment. In the HMBC spectrum, a common ^{3}J correlations between hydrogen (H4) at δ 7.10 and that at δ 4.98 (H9) to a quaternary carbon δ 116(C8) confirmed that the sugar moiety is attached to C8.

Based on the results of chemical tests, 1D, 2D NMR; MS and X-ray crystallography and comparing the spectral and crystallographic data with literature and database, compound X was concluded to be bergenin, an isocoumarin. The melting point range of the isolated compound found to be 236-239 °C was consistent with published melting point of bergenin (Madusolumuo and Okoye, 1995).

This is the first report of the isolation of this compound from the root of *Securinega virosa*. Bergenin is a C-glucoside of 4-O-methylgallic acid which has been reported to occur naturally in several genera. It has been reported to possess antiulcer, hepatoprotective, antiviral,

antidiabetic/antiobesity, anti-arrhythmic, antioxidant, anti-arthritic, wound healing and trypanocidal activities (Rastogi and Rawat, 2008).

The intraperitoneal LD_{50} values of the n-butanol fraction found to be 1256.9 mg/kg suggests that it is relatively toxic. The ethyl acetate and residual aqueous fractions, with LD_{50} values of 2154 mg/kg are considered relatively safe (Matsumura, 1985). The oral LD_{50} values of the fractions in both mice and rats, found to be greater than 5000 mg/kg body weight, suggest that they are relatively nontoxic orally. However, the doses selected and used in this work were lower than 30% of the LD_{50} which have been shown to be relatively safe for ethnopharmacological research (Vongtau *et al*., 2004).

The ethyl acetate fraction (EAF), n-butanol fraction (NBF) and residual aqueous fraction (RAF) did not protect the chicks against maximal electroshock-induced seizure (MES). They did not significantly alter the mean recovery time in the convulsed animals. This finding is in contrast with what was obtained with the crude methanolic root bark extract which offered up to 60% protection at the dose 12.5 mg/kg against MES (Magaji *et al*., 2007a). The loss of activity with the various fractions against MES may be due to fractionation which might have separated constituents that act synergistically in the crude extract. The MES is a model with high reproducibility and consistent end point. Inhibition of the MES seizure predicts activity against generalized tonic-clonic and cortical focal seizures (Ambawade *et al*., 2002).

There is no false negative in MES test and all currently available AEDs that are clinically effective in the management of generalized tonic-clonic and partial seizures such as phenytoin, carbamazepine and lamotrigine all suppress tonic hind limb extension (THLE) in MES test (Browning, 1992; Rho and Sankar, 1999). The behavioural and electrographic seizures generated in the MES model are consistent with the human disorder (Swinyard *et al*., 1989). MES induced

tonic seizure can be prevented by agents that either inhibits sodium channel such as phenytoin, valproate, felbamate and lamotrigine (Rogawski and Porter, 1990; White, 1997 and Sayyah, 2004) or agents that block glutamatergic excitation mediated by N-methyl-D-Aspartate (NMDA) receptors (Subramaniam *et al*., 1995 and Sayyah, 2004). The inability of the fractions to inhibit the THLE suggests they may not be beneficial in the management of generalized tonic-clonic and partial seizures.

Pentylenetetrazole (PTZ) is a known convulsant and anticonvulsant effect against PTZ test identifies compounds that can raise seizure threshold in the brain (White *et al.*, 1998). The PTZ-induced seizures are similar to the symptoms observed in the absence seizures and drugs useful in treatment of absence seizures suppress PTZ-induced seizures (McNamara, 2006). Drugs that reduce T-type Ca^{2+} currents, such as ethosuximide can prevent seizures induced by PTZ. Similarly, PTZ has been shown to interact with GABA neurotransmitters and the GABA receptor complex (Loscher and Schmidt, 1988; De Deyn *et al.*, 1992). Therefore, agents that enhance $GABA_A$ receptor mediated inhibitory neurotransmission such as benzodiazepines and barbiturates prevent PTZ-induced seizure. A dopaminergic mechanism has also been implicated in PTZ-induced seizures. Dopamine has been found to reduce the threshold of PTZ convulsions in mice and specific receptor antagonists of dopamine such as pimozide protected experimental animals against PTZ-induced seizures (Dadkar *et al*., 1979; Amabeoku, 1989). Drugs such as felbamate, which block glutamatergic excitation mediated by NMDA receptors, have demonstrated activity against PTZ-induced seizures suggesting the involvement of N-methyl-D-aspartate (NMDA) system in the initiation and propagation of PTZ-induced seizures (MacDonald and Kelly, 1993).

Moderate anticonvulsant activity of the fractions (RAF, NBF and EAF) against PTZ-induced seizures suggests the presence of bioactive compounds effective in the therapy of absence or myoclonic seizures. It is plausible to suggest, therefore, that the anticonvulsant effects of the fractions and the column fractions may be due to either reduction in T-type Ca^{2+} current, activation of GABA-ergic neurotransmission, blockade of glutamatergic neurotransmission mediated by NMDA receptor or blockade of dopaminergic system in specific areas of the CNS.

Strychnine is a competitive antagonist of the inhibitory amino acid, glycine (Larson, 1969). It blocks the inhibitory effects of glycine at all glycine receptors (Parmar and Shiv Prakash, 2006). Ability of compounds to protect against strychnine-induced seizure suggests interference with strychnine-sensitive channels (Ogbonnia *et al.,* 2003). However, the fractions (NBF and RAF) did not retain the activity found with the crude extract (Magaji *et al*., 2007a).

K^+ channels play a major role in the control of all aspect of neuronal excitability, including resting membrane potential, responsiveness to synaptic inputs, frequency adaptation and neurotransmitter release (Wickenden, 2002). Drugs like phenytoin which block seizure spread are effective antagonists of seizures induced by K^+ channel blockade while those with specific actions on other cellular targets may be weak or inactive, presumably because they are unable to attenuate the spread of intense (non-NMDA receptor mediated) excitation evoked by 4-Aminopyridine (Yamaguchi and Rogawski, 1992). The inability of the fractions to produce significant activities against 4-AP induced seizure suggests that they may likely not be interacting with K^+ channel in producing their anticonvulsant activities.

Picrotoxin-induced seizure model is a model for elucidating the probable mechanism of action of an anticonvulsant agent on GABA receptor. Picrotoxin is a non-competitive $GABA_A$ receptor

antagonist which selectively blocks chloride channels. Agents such as barbiturates, benzodiazepines, valproic acid, vigabatrin, gabapentin and tiagabine which protect against picrotoxin-induced seizure may interfere with the GABAergic pathway thereby enhancing the GABA mediated neurotransmission (Loscher and Schmidt, 1994; Raza *et al*., 2010; Rang *et al*., 2011). The inability of the fractions to protect mice against picrotoxin-induced seizure suggests that their anticonvulsant action may not involve interaction with picrotoxin sites on $GABA_A$-chloride ion channel complex.

The kindling phenomenon has been employed as a chronic model of temporal lobe epilepsy. It initially presents itself as a simple phenomenon whereby provocation of focal seizure using focal electrical brain stimulation induces a clear progressive change in response over daily repetition (Mcintyre, 2006). The kindling model may approximate the human conditions more closely than directly evoked seizure models (Rang *et al*., 2011). Once produced, kindled state persists. The change which may involve processes similar to those that cause long term potentiation of synaptic transmission in the hippocampus is prevented by NMDA receptor antagonists (Rang *et al*., 2011). Although, the fractions did not significantly alter the behavioural seizure scores (change from convulsive to non-convulsive) and the seizure duration, the slight reduction of the behavioural seizure scores (BSS) and the mean seizure duration post-treatment suggests that they possess mild activities in the kindling model.

Anticonvulsant activities have been reported for saponins and flavonoids (Shibata, 2001; Kavvadias *et al*., 2004). These constituents, present in these fractions (NBF and RAF) and column fractions may be responsible, in part or in combination for the observed anticonvulsant effect against pentylenetetrazole-induced seizure.

Potentiation of sleep induced by CNS depressant such as the barbiturates and benzodiazepines has been used as an effective model in screening CNS active agents (Danjuma, 2007). Neurotransmitters in the brain have been shown to play a vital role in the process of sleep and wakefulness (Gottesmann, 2002; Chindo, 2006). It is known that drugs such as amphetamine and cocaine, which enhance the release or synaptic concentrations of dopamine and norepinephrine, enhance and prolong wakefulness (Osuide and Wambebe 1980).

The nigrostriatal dopamine (DA) pathway mediates activation of motor activity, including exploration, which may promote waking and inhibit sleep, although the discharge of nigral neurons is not dependent on vigilance states. It has been postulated that there are two antagonizing cholinergic sleep-regulatory systems: a rostral waking system, and a caudal sleep system (Stenberg, 2007). The release of acetylcholine in the cortex was increased during waking while injection of acetylcholine or muscarinic agonists like carbachol into the pons has been found to induce a REMS-like state with muscle atonia (George *et al*., 1964; Gadea-Ciria *et al*., 1973). A decrease in serotonin concentration or destruction of the dorsal raphe nucleus, which houses most the brain's serotonergic cell bodies, is associated with sleep reduction (Curtis and Jermaine, 2002). Glutamate is the most common excitatory transmitter in the brain, and is likely to have the greatest direct impact on neuronal activity. The ascending activating reticular system is probably glutamatergic. Glutamate is the transmitter in pontine networks mediating motor and autonomic activation in the thalamocortical projection responsible for cortical activation, in intracortical networks and in corticofugal pathways. The role of other transmitters considered in the regulation of vigilance is primarily to modulate the excitability of neurons (Stenberg, 2007). It is a well-established fact that activation of $GABA_A$ receptor by GABA, the major inhibitory neurotransmitter in the brain favours sleep (Gottesmann, 2002).

The fractions and the column fractions significantly influenced the onset and duration of sleep induced by diazepam in varying capacities. The column fractions behaved in a similar manner to the fractions from where they were derived. Bergenin isolated from the column fraction PFA of the n-butanol fraction decreased the onset of sleep by 50%. However, it did significantly alter the duration of sleep. It may, therefore, contribute to the sleep inducing property observed with the n-butanol fraction. Compounds that decrease the onset of sleep are considered to be beneficial in initiation of sleep while agents that prolong the duration of sleep may be beneficial in sleep maintenance. By potentiating the diazepam-induced sleep, the extract seems to possess sleep-inducing properties (Rakotonirina *et al*., 2001).

The Hole-board test is a simple model for measuring exploratory activity in rodents (File and Wardill, 1975). The behavioural head dipping in hole-board assay is sensitive to changes in the emotional state of the animals and increase in head dipping behaviour is a reflection of anxiolytic activity (Takeda *et al*., 1998) whereas a decrease in the parameter reveals a sedative behaviour (File and Pellow, 1985). In this test, both fractions (NBF and RAF) significantly produced decrease in exploratory behaviour as indicated by decrease in the number of head dip. The finding supports the sedative property of the fractions. Diazepam produced a decrease in the number of head dips at the dose of 2 mg/kg in this study which is in agreement with the biphasic profiles of anxiolytic showing a facilitation of exploratory behaviour at low doses and an inhibition at high doses (Treit, 1984).

The staircase test is a simple, quick and differential test based on the inherent tendency of rodents to explore a novel environment which gives a general idea on the level of emotivity of the rodent (Lepicard, 2000). Compounds that reduce rearing activity are said to possess anxiolytic activity (Abid *et al*., 2006). Non-benzodiazepine compounds, such as neuroleptics,

tricyclic antidepressants, and buspirone have been found to suppress both rearing and climbing behaviour in the staircase test. However, agents which activate the $GABA_A$-benzodiazepine receptor-chloride channel complex are known to also suppress the rearing behaviour without significantly affecting climbing (i.e., the separation of the two behaviours) (Simiand *et al*. 1984). It is therefore plausible to suggest that since both fractions decreased both parameters (rearing and step climbed), non-preferentially, they possess central depressant activity.

To further ascertain that the fractions did not possess anxiolytic activity, Elevated Plus Maze (EPM) test was conducted. The EPM test is one of the most widely validated tests for identifying new anxiolytic agents. In this test, it is assumed that animals felt safe in the closed arms but exhibited fear and anxiety during exploration of the open arms (Saiyudthong and Marsden, 2011). An anxiolytic agent increases the frequency of entries into the open arms and increases the time spent in open arms of the EPM (Grundmann, 2007). The present study shows that both the residual aqueous and n-butanol fractions failed to exert anxiolytic activity, being unable to modify the behaviour of mice subjected to elevated plus maze test.

Agents that alter general motor activity are reported to give false positive or negative result in the elevated plus maze. It is, therefore, worthwhile to evaluate the effect of the fractions on general locomotor activity in rodents using open field test, a classical model for evaluating the autonomic effect of compounds (Sousa *et al*., 2005). The CNS depressant activity of the fractions was further confirmed by the significant decrease in the number of rearings as well as number of square crossed in the open field test (Morais *et al*., 1998). The inability of the fractions to increase the number of central square crossed further confirms their lack of anxiolytic potential. Diazepam, at the dose of 0.5 mg/kg significantly increased the number of central square crossed.

The mouse beam walking assay was used to evaluate the effect of the fraction on motor coordination. It is a more sensitive model than rota rod in predicting clinical sedation in humans caused by novel drugs (Stanley *et al*., 2005). The fractions (n-butanol and residual aqueous fractions) and bergenin did not significantly increase the number of foot slips, an index of motor coordination deficit, thus suggesting that the depressant effect of the fractions and bergenin might be centrally mediated and not due to peripheral muscular blockade (Perez *et al*., 1998).

The phytochemical screening showed that the fractions contains alkaloids, flavonoids, saponins and steroids which have been reported to possess CNS depressant activity (Soulimani *et al*., 1997; Yao, *et al*., 2010; Hanrahan *et al*., 2010). These constituents may be responsible for the observed activity of the residual aqueous and n-butanol fractions.

GBM cells are highly proliferative and diffusely invade surrounding brain structures, thereby making complete surgical resection practically impossible (Barcellos-Hoff *et al*., 2009). Furthermore, the majority of GBMs are intrinsically resistant to most forms of radiation and chemotherapy, thus rendering the standard arsenal of anticancer treatments rather ineffective (Reardon and Wen, 2006). GBMs commonly overexpress oncogenes such as Epidermal growth factor receptor (EGFR) and Platelet derived growth factor receptor (PDGFR) and contain mutations and deletions of tumor suppressor genes such as phosphatase and tensin homolog (PTEN) and tumour suppressor protein 53 (TP53), all of which can have an impact on the activation state of signal transduction pathways that influence their biological behaviour (Mischel and Cloughesy, 2003).

The methanolic root bark extract, its fractions and their column fractions significantly ($P < 0.001$) reduced cell viability of the GBM cells. Similarly, EGF-driven cell proliferation was also

significantly ($P < 0.001$) reduced by the extract and its fractions. The methanolic root bark extract and its residual aqueous fraction also blocked the phosphorylation of the EGFR at tyrosine 1045 and 1068 sites. They also blocked the phosphorylation of PDGFR. Similarly, PMA and PDGFR induced transactivation of EGFR at tyr 1068 were also blocked. MAPK phosphorylation was also blocked by the crude methanolic root bark extract. However, the extract and its fractions did not affect the phosphorylation of Akt. Although the chloroform fraction gave the highest activity in terms of reduction in cell viability, its mechanism of action did not involve EGFR, PDGFR, MAPK or Akt. In addition, bergenin, isolated from the n-butanol fraction did not affect cell viability at concentrations tested.

The ability of the extract and fractions to block the phosphorylation of EGFR and PDGFR suggests that they may be acting via tyrosine kinase receptors. However, their more pronounced activity on EGFR and blockade of PMA and PGDFR mediated transactivation of EGFR at tyr 1068 suggest that they may be more specific for the EGFR. The blockade of MAPK phosphorylation by the crude methanolic extract suggests that its activity may involve some downstream mediators. The inability of the extract and its various fractions to block the phosphorylation of Akt suggests that their activity may not involve PI3k/Akt pathway.

Although, bergenin did not produce remarkable anti-GBM activity, the finding was not unexpected because it was isolated from column fraction PFA which showed the least activity among the column fractions obtained from the n-butanol fraction. The phytochemical screening conducted on these column fractions showed that the column fraction PFA has more of flavonoids while column fractions PFB and PFC are richer in alkaloidal content. Similarly, the column fractions obtained from the residual aqueous fraction namely, PF1 and PF2 which exhibited significant activity were found to be rich in alkaloids. Previously, it was reported that

alkaloids, virosecurinine and viroalloscurinine isolated from the leaves of *Securinega virosa* possess some cytotoxic principles (Tatematsu *et al*., 1991). It may therefore, be suggested that alkaloids present in the extract may be highly contributory to the anticancer potentials of the extract.

CHAPTER 6

SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 SUMMARY

Securinega virosa is a commonly used medicinal plant in West Africa. It is reputed to be used in the management of cancer, epilepsy and as a sedative in children. Previously, the crude methanolic root bark extract was found to possess anticonvulsant, sedative and antipsychotic properties. In an attempt to isolate and characterize the bioactive principle, the crude methanolic root bark extract was subjected to fractionation to obtain chloroform, ethyl acetate, n-butanol and residual aqueous fractions.

Due to poor yield (less than 1 %), the chloroform fraction was only subjected to anticancer study. The intraperitoneal median lethal dose values for the ethyl acetate, n-butanol and residual aqueous fractions in mice were found to be 2154 mg/kg, 1257 mg/kg and 2154 mg/kg, respectively. In rats, their LD_{50} were found to be 2154, 2154 and 3807.9 mg/kg, respectively.The oral median lethal dose values of the crude methanolic extract and its fractions in both mice and rats were found to be greater than 5000 mg/kg.

The preliminary phytochemical screening conducted on the three fractions suggested that they contain alkaloids, saponins, flavonoid and tannins. Preliminary neuropharmacological screenings (diazepam-induced sleep, maximal electroshock and pentylenetetrazole-induced seizure tests in mice) conducted on the ethyl acetate, n-butanol and residual aqueous fractions showed that they have comparable pharmacological activities. As such; the ethyl acetate fraction (with a yield of 3.4%) was set aside.

The other two fractions namely; n-butanol and residual aqueous fraction were subjected to anticonvulsant and behavioural studies. The fractions did not protect chicks against maximal electroshock seizure; neither did they shorten the mean recovery time. In the seizures induced by chemoconvulsant such as 4- Aminopyridine, strychnine and picrotoxin, there were no significant activities by the two fractions. Similarly, the fractions did not significantly protect the animals against seizure due to electrical kindling. Conversely, the two fractions afforded 66.67% protection against pentylenetetrazole-induced seizure in mice.

In the hole board test for exploratory behaviour, both fractions significantly reduced the mean number of head dips; an indication of sedative potential. Similarly, both fraction decreased the number of rearings and increased the total number of upward stairs climbed in the staircase test. This finding supports sedative activity.

In the Elevated Plus Maze test for anxiolysis, both fractions did not significantly increase the total number of open arm entries and total time spent in the open arms. Similarly, the two fractions did not increase the total central square crossed in the open field test but they both decreased total ambulation and rearings. These findings further support CNS depressant activities of the two fractions. In the beam assay for evaluation of motor coordination deficit, both fractions did not increase the mean number of foot slips, neither did they increase the total time taken to complete the task. This finding suggests that the activities of the fractions may be centrally mediated.

The n-butanol fraction was subjected to column chromatographic separation using silica gel affording three major column fractions after pooling similar fraction based on TLC profile namely; PFA, PFB and PFC. Similarly, the residual aqueous fraction was subjected to gel

filtration using sephadex LH-20 affording two major column fractions namely PF1 and PF2; after pooling based on TLC profiling. The three column fractions obtained from NBF and two obtained from RAF were subjected to preliminary phytochemical screening, diazepam-induced sleeping time and pentylenetetrazole in mice.

PFA was found to be devoid of alkaloids but rich in flavonoids and saponins. PFB and PFC were found to be rich in alkaloids in addition to the saponins and flavonoid present. PF1 and PF2 contained alkaloids, saponins and flavonoids. PFA significantly decreased the mean onset of sleep without offering significant protection against PTZ-induced seizure. Conversely, PFB and PFC had little influence on the diazepam-induced sleep but significantly protected mice against PTZ-induced seizure in mice. Both PF1 and PF2 significantly protected mice against pentylenetetrazole-induced seizure. However, only PF2 significantly reduced the mean onset of diazepam-induced sleep in mice.

Further purification of PFA led to the isolation of compound X. Based on the result of chemical tests, melting point determination, 1D, 2D NMR, MS and X-ray crystallography; and comparing the spectral and crystallographic data with literature and database, compound X was concluded to be bergenin, an isocoumarin. Bergenin was subjected to diazepam-induced sleep and beam walking assay in mice. It was found to significantly reduce the mean onset of sleep without affecting the total duration of sleep. It also did not induce a significant motor coordination deficit.

The crude methanolic root bark extract, its fractions (RAF, NBF and EAF) and their column fractions were subjected to anti-GBM screening using U-1242 MG cell line. They significantly and concentration-dependently decreased mean cell viability. In the presence of epidermal

growth factor (EGF), they produced a remarkable and concentration-dependent reduction of the mean cell count. Bergenin, isolated from the n-butanol fraction did not affect cell viability at concentrations tested.

The methanolic root bark extract and its residual aqueous fraction also blocked the phosphorylation of the EGFR at tyrosine 1045 and 1068 sites. Similarly, PMA and PDGFR-induced transactivation of EGFR at tyr 1068 were also blocked. MAPK phosphorylation was also blocked by the crude methanolic root bark extract. However, the extract and its fractions did not affect the phosphorylation of Akt.

6.2 CONCLUSION

It may therefore be concluded, based on the data presented here, that the use of *Securinega virosa* in traditional medicine in Nigeria and other African countries in the management of neuropsychiatric disorders and cancer has some scientific validity. The residual aqueous and n-butanol fractions possess significant activity against pentylenetetrazole-induced seizure, indicating that they may be beneficial in absence seizure. Furthermore, the fractions also possess significant sedative activities which may be useful in the management of insomnia. The crude methanolic root bark extract and its fractions exhibit significant activities against glioblastoma mutiforme cells. This activity may be mediated through EGFR.

Bergenin, isolated from the the root of the plant for the first time could be responsible for the sedative activity of the root bark extract of *Securinega virosa*.

6.3 RECOMMENDATION

Based on the findings in this work and the limitations, the following recommendations are made: Further studies should be conducted on the isolated compound, bergenin; vis anticonvulsant and anxiolytic studies.

Further purification and biological evaluation of PFB, PFC, PF1 and PF2 should be carried out. The effects of crude methanolic root bark extract and its fractions on downstream players of cell proliferation such as NF-□B, caspases and polyADP-ribose polymerase (PARP) should be evaluated. Also, the effect of the extract and its fractions on tumour xenograft models should be evaluated.

Finally, the toxicological profiling of the crude methanolic root bark extract using sub-acute and chronic toxicity models should be carried out.

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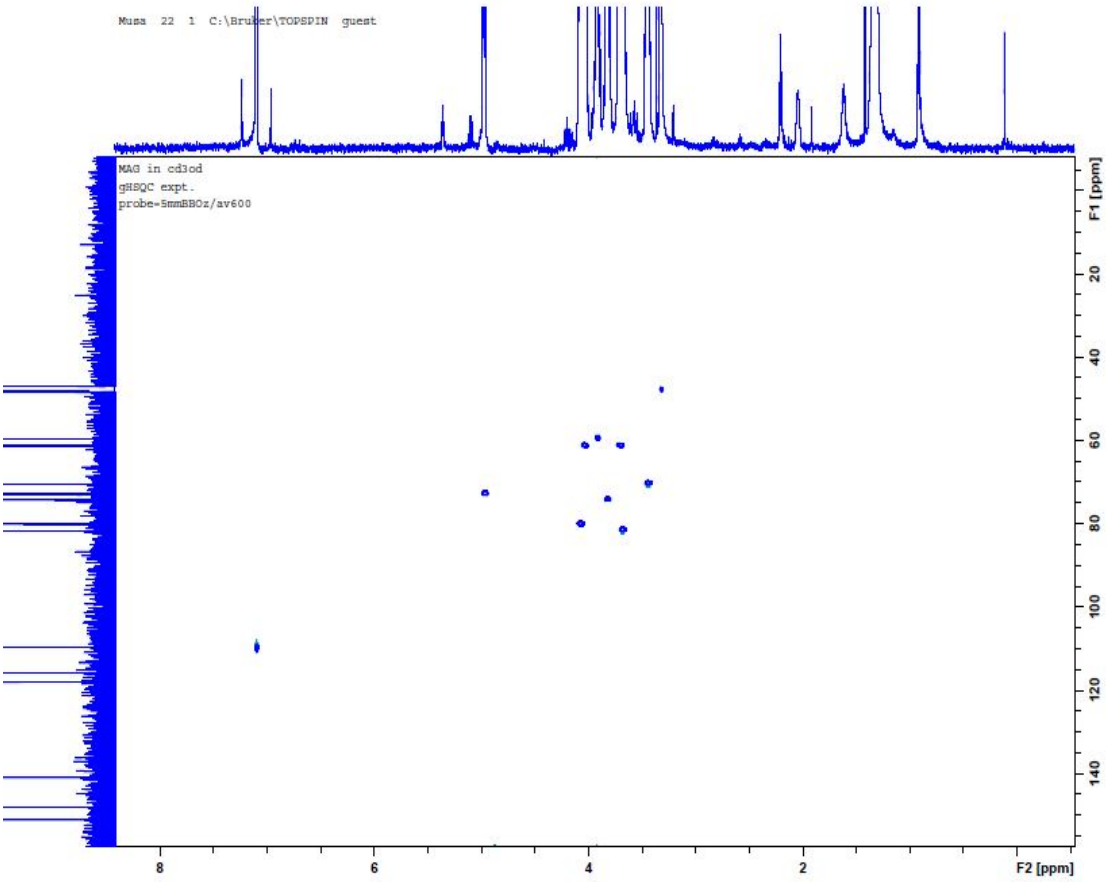
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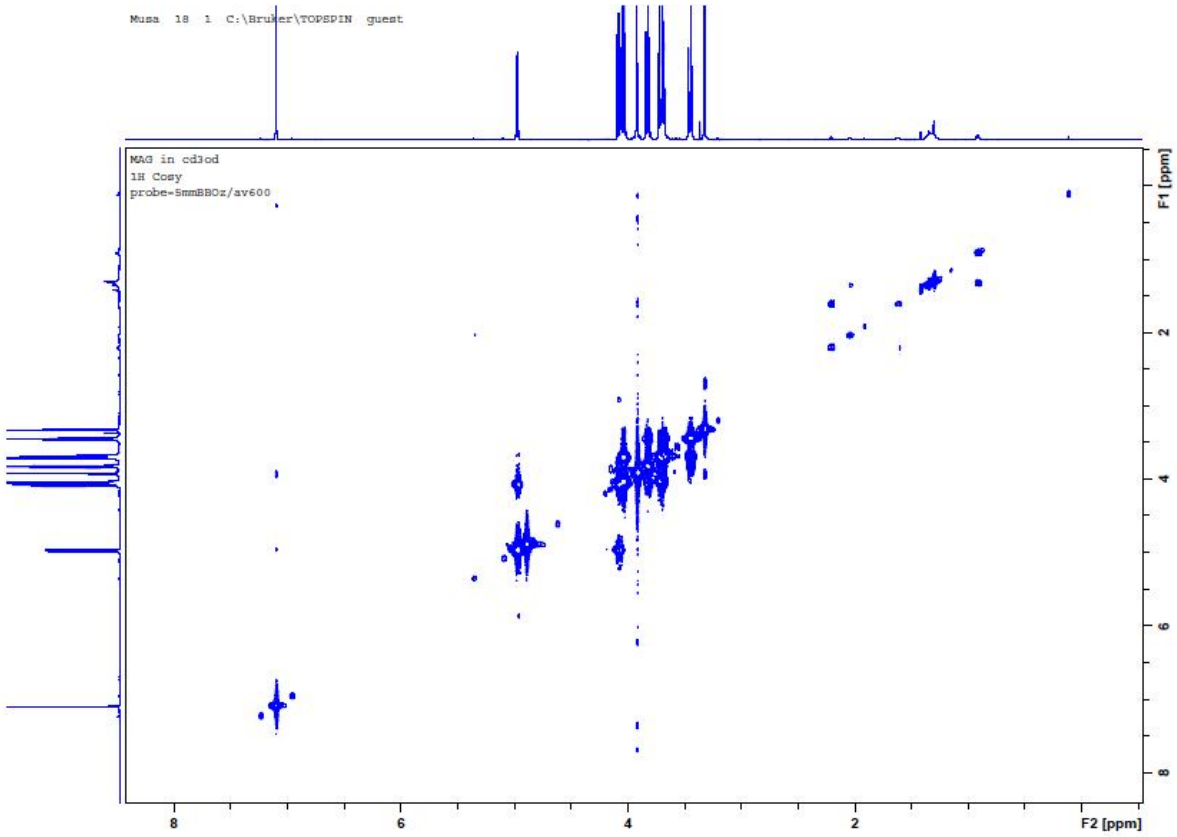
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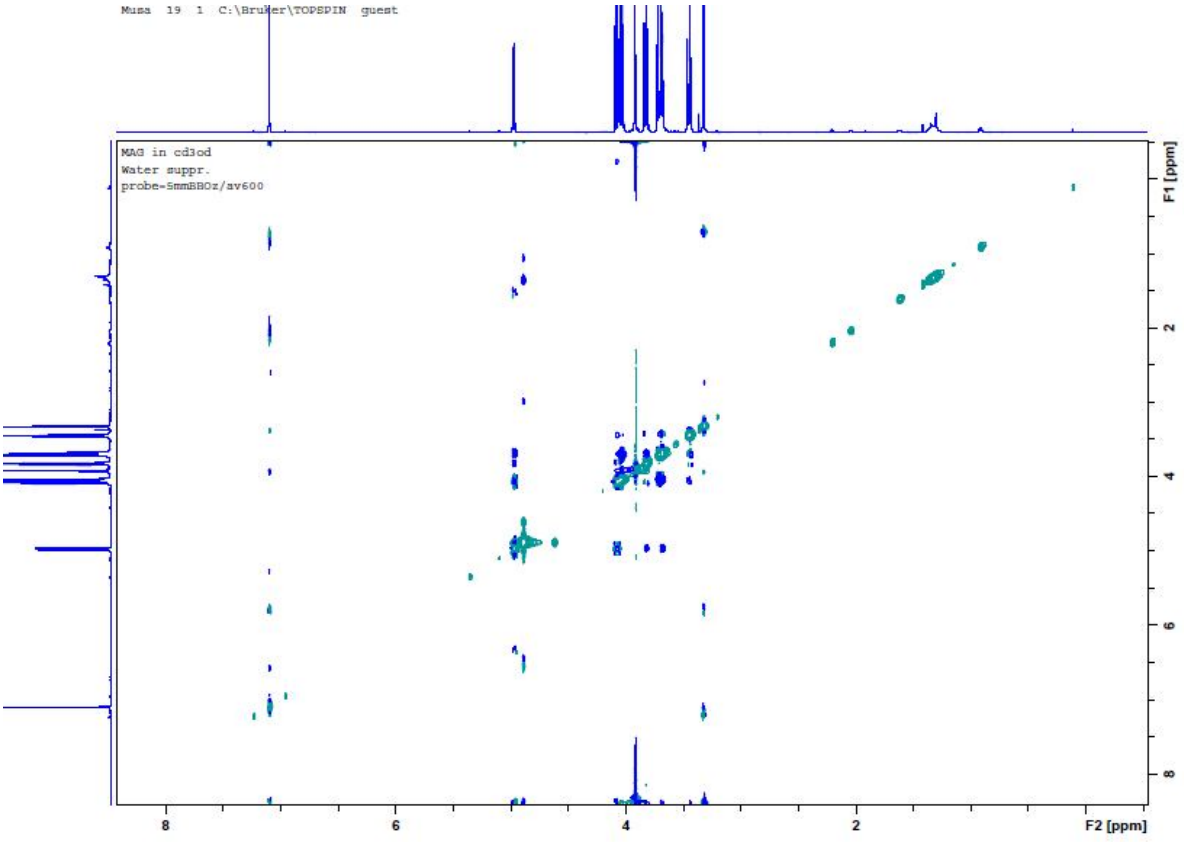
Appendices



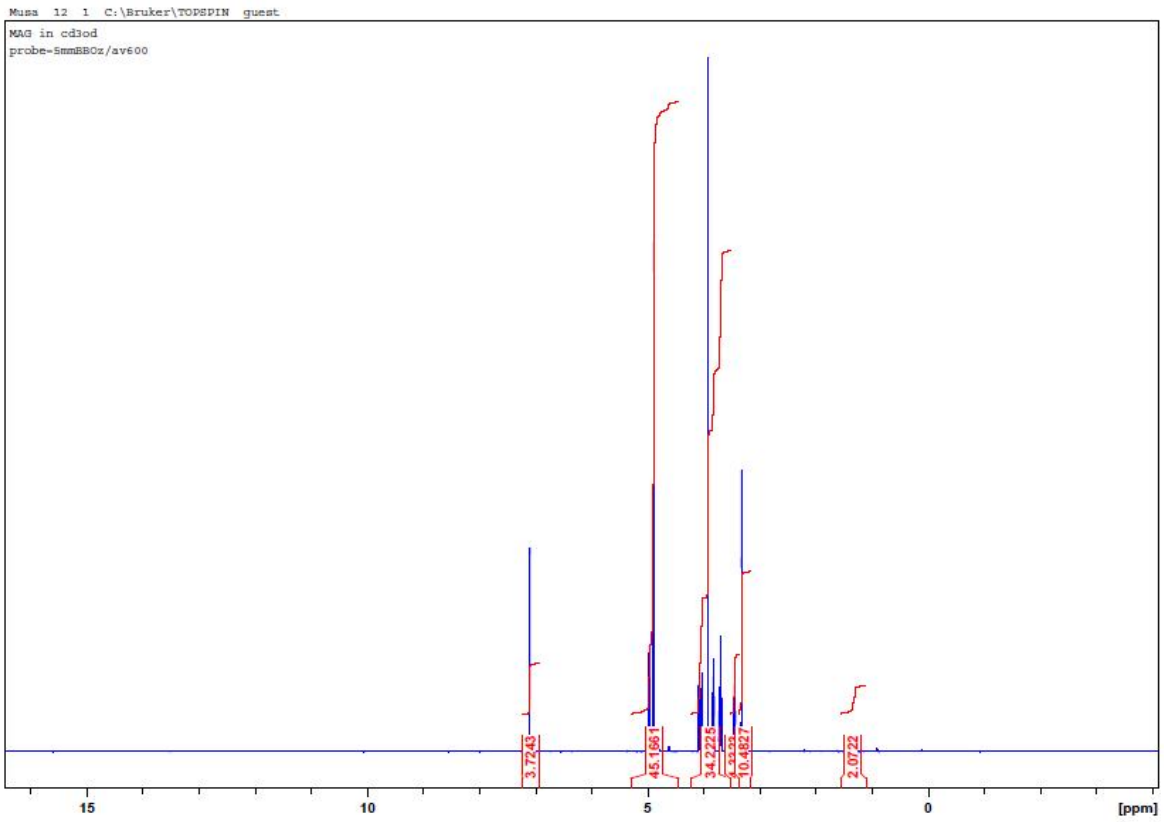
Appendix 1: HSQC of Compound X



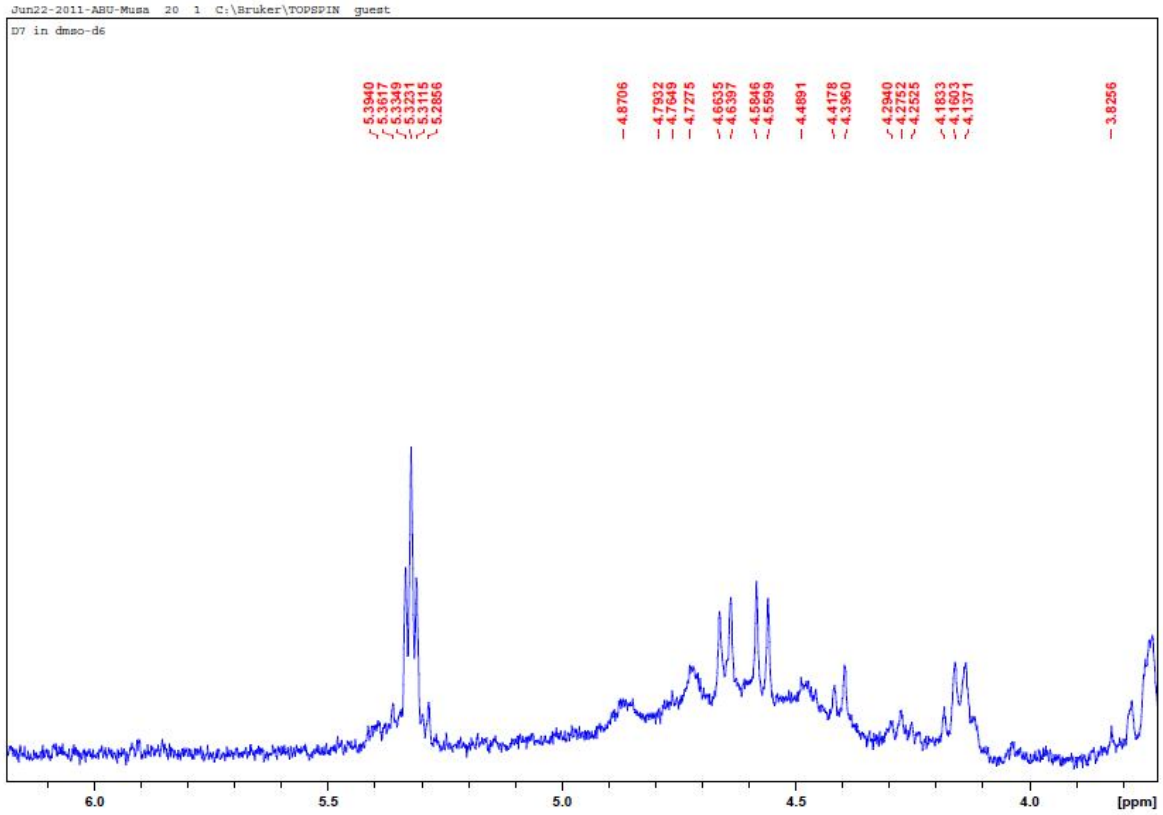
Appendix 2: COSY of Compound X



Appendix 3: NOESY OF Compound X



Appendix 4: Proton Integration of Compound X



Appendix 5: D7 Proton Sugar of Compound X

Appendix 6: Effect of Residual Aqueous Fraction of Methanolic Root Bark Extract of *Securinega virosa* on Pentylenetetrazole-Induced Seizure in Mice

Treatments	Quantal protection against seizure	Mean onset of seizure	Quantal protection against mortality	Mean time of death
N/Saline	0/6	3.5 ± 0.43	4/6	4.8 ± 0.324
RAF 125	1/6	6.00 ± 1.00	5/6	4
RAF 250	2/6	7.25 ± 1.60*	6/6	-
RAF 500	4/6	4.0 ± 0.00	5/6	8
VPA 200	6/6	-	-	-

Protection against seizure and mortality presented as quantal protection; onset of seizure and time of death presented as Mean ± SEM; RAF: Residual Aqueous fraction; *P < 0.05; n=6

Appendix 7: Effect of Column Fractions of Residual Aqueous fraction on Pentylenetetrazole-Induced Seizure in Mice

Treatment	Dose (mg/kg)	Quantal protection against seizure	Mean onset of seizure (min)	Quantal protection against mortality	Mean time of death (min)
N/Saline	10ml/kg	0/6	2.72 ± 0.34	1/6	11.21 ± 3.1
PF1	2.5	1/6	7.4 ± 0.93*	4/6	12.32 ± 0.9
	5.0	2/6	7.38 ± 1.22	5/6	12.8
	10.0	4/6	7.12 ± 2.11	6/6	-
PF2	2.5	1/6	4.4 ± 1.93	2/6	12.32 ± 0.81
	5.0	3/6	6.37 ± 0.22*	3/6	13.81 ± 3.44
	10	2/6	5.53 ± 2.61	6/6	-
VPA	200	6/6	-	6/6	-

Protection against seizure and mortality presented as quantal protection; onset of seizure and time of death presented as Mean ± SEM; PF1: Pooled fraction 1; PF2: Poooled fraction 2; *P < 0.05; n=6

Appendix 8: Effect of n-butanol Fraction of Methanolic Root Bark Extract of *Securinega virosa* on Pentylenetetrazole-Induced Seizure in Mice

Treatments	Quantal protection against seizure	Mean onset of seizure	Quantal protection against mortality	Mean time of death
N/Saline	0/6	3.5 ± 0.428	4/6	4.8 ± 0.324
NBF 75	2/6	5.5 ± 0.866	5/6	10
NBF 150	1/6	4.4 ± 0.51	4/6	8.5 ± 0.5
NBF 300	4/6	3.0	5/6	3
VPA 200	6/6	-	-	-

Protection against seizure and mortality presented as quantal protection; onset of seizure and time of death presented as Mean ± SEM; NBF: N-butanol fraction; VPA: Sodium valproate; *P < 0.05; n=6

Appendix 9: Effect of Column Fractions of n-butanol Fraction on Pentylenetetrazole-Induced Seizure in Mice

Treatment	Dose (mg/kg)	Quantal protection against seizure	Mean onset of seizure (min)	Quantal protection against mortality	Mean time of death (min)
N/Saline	(10 ml/kg)	0/6	2.67 ± 0.21	1/6	12.40 ± 2.46
PFA	2.5	1/6	6.40 ± 1.03	6/6	-
	5.0	1/6	4.00 ± 0.89	4/6	9.00
	10	1/6	5.40 ± 1.12	4/6	9.50 ± 0.50
PFB	2.5	1/6	5.40 ± 1.12	4/6	9.50
	5.0	0/6	5.83 ± 1.54	2/6	12.75 ± 1.53
	10	3/6	3.67 ± 1.22	5/6	2.00
PFC	2.5	3/6	1.67 ± 0.33	3/6	8.67 ± 0.88
	5.0	1/6	7.00 ± 1.7*	1/6	12.00 ± 0.95
	10.0	1/6	11.00 ± 1.3**	2/6	11.50 ± 1.56
VPA	200	6/6	-	-	-

Protection against seizure and mortality presented as quantal protection; onset of seizure and time of death presented as Mean ± SEM; PFAPPooled fraction A; PFB: Pooled fraction B; PFC: Pooled fraction C; VPA: Sodium valproate *P < 0.05;**P < 0.01; n=6

Appendix 10: Effect of Ethyl Acetate Fraction of Methanolic Root Bark Extract of *Securinega virosa* on Pentylenetetrazole-Induced Seizure in Mice

Treatments	Quantal protection against seizure	Mean onset of seizure	Quantal protection against mortality	Mean time of death
N/Saline	0/6	3.5 ± 0.43	4/6	4.8 ± 0.32
EAF 62.5	2/6	8.99 ± 2.85*	6/6	-
EAF 125	2/6	6.93 ± 2.01*	6/6	-
EAF 250	4/6	7.12 ± 1.87	5/6	12
VPA 200	6/6	-	-	-

Protection against seizure and mortality presented as quantal protection; onset of seizure and time of death presented as Mean ± SEM; EAF: Ethyl acetate fraction; *P < 0.05; n=6

Appendix 11: Effect of Residual Aqueous Fraction of Methanolic Root Bark Extract of *Securinega virosa* on Behaviour of Mice in Open Field Test

Treatment	No of Rearings (± SEM)	No of Square crossed (± SEM)	No of central Square crossed (± SEM)
Normal Saline	39 ± 3.51	122 ± 5.64	3 ± 0.37
RAF 125	9.5 ± 2.08***	73.67 ± 14 *	1.5 ± 0.5
RAF 250	3.17 ± 1.45***	48.33 ± 9.6***	0.83 ± 0.4
RAF 500	4.17 ± 1.74***	59.67 ± 10.17**	1 ± 0.37
DZP 0.5	63.5 ± 2.26***	168 .67 ± 2.26*	6.33 ± 0.21*

DZP: Diazepam; *P<0.05;**P<0.01;***P<0.001

Appendix 12: Effect of n-butanol Fraction of Methanolic Root Bark Extract of *Securinega virosa* on Behaviour of Mice in Open Field Test

Treatment	No of Rearings (± SEM)	No of Square crossed (± SEM)	No of central Square crossed (± SEM)
Normal Saline	39 ± 3.51	122 ± 5.64	3 ± 0.37
NBF 75	12.67 ± 2.5***	94.83 ± 8.76*	1.33 ± 0.49
NBF 150	16.67 ± 3.19***	111.17 ± 7.75	3.83 ± 1.3
NBF 300	4.67 ± 1.67***	73.67 ± 15.54	3 ± 1.9
DZP 0.5	63.5 ± 2.26***	168 .67 ± 2.26*	6.33 ± 0.21*

DZP: Diazepam; *P<0.05;**P<0.01;***P<0.001

Published Articles from the Dissertation

1. **Magaji, MG**, Yaro, AH, Musa, AM, Anuka, JA, Abdu-Aguye, I and Hussaini, IM (2011). Residual Aqueous fraction of *Securinega virosa* methanol root bark offers protection against pentylenetetrazole-induced seizure in laboratory animals. *Journal of Pharmacology and Tropical Therapeutics,*1(2):1-6

2. **Magaji, MG**, Yaro, AH, Musa, AM, Anuka, JA, Abdu-Aguye, I and Hussaini, IM (2011) Sedative activity of residual aqueous fraction of *Securinega virosa* (Roxb. ex Willd) Baill. Root bark extract in mice. *Nigerian Journal of Pharmaceutical Sciences*,10 (2):34-44

3. **Magaji, MG**, Yaro, AH, Musa, AM, Anuka, JA, Abdu-Aguye, I and Hussaini, IM (2012). Central Depressant Activity of butanol fraction of *Securinega virosa* root bark in mice. *Journal of Ethnopharmacology*,141:128-133