PHARMACOGNOSTIC AND ANTIVENIN STUDIES ON LEAVES OF *SECURINEGA VIROSA* (ROXB EX WILLD) BAILL

BY

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SEPTEMBER, 2018

DECLARATION

I declare that the work in this dissertation entitled “The Pharmacognostic and Antivenin Studies on leaves of *Securinega virosa* (Roxb ex Willd.) Baill” was carried out by me in the Department of Pharmacognosy and drug development. 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 another degree or diploma at this or any other Institution.

| --- | --- | --- |
| Name of Student | Signature | Date |

CERTIFICATION

This dissertation entitled the pharmacognostic and antivenin studies on leaves of *Securinega virosa* (Rroxb. ex Willd.) Baill, meets the regulations governing the award of the degree of Masters of Science of Ahmadu Bello University, Zaria, and is approved for its scientific contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to my parents, Alhaji Alkasim Ahmad Tofa and Zainab Alkasim Ahmad for their love has been my driving force till now and forever.

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ABSTRACT

Securinega virosa (Roxb ex Willd) Baill (Euphorbiaceae) is a small, deciduous, graceful shrub that is widely distributed in Africa. It is used to treat snakebites, syphilis, rheumatism and malaria. Plant based medicines are limited in use because of inadequate standardization. Major snakebite envenomings and fatalities occur in tropical regions such as Sub-Saharan Africa. Leaf pharmacognostic standards and antivenin potentials of n-hexane (8.00g), ethyl acetate (11.22g) and methanol (46.64g) extracts of *Securinega virosa* on *Naja nigricollis* venom were studied. The pharmacognostic studies to determine the leaf microscopic features such as stomata, trichomes, vascular bundles, calcium oxalates, stomatal index, stomatal number, palisade ratio, vein islet number and vein termination number. Physical constants such as moisture constant, ash values, and water and alcohol extractive values were determined. And, chemomicroscopical studies for detection of features cell wall materials and cell inclusions were also carried out. These include; cellulose, inulin, suberin/cuticle, gum and mucilage, starch grain, aleurone grain, calcium oxalates and calcium carbonate. The preliminary and the thin layer chromatographic phytochemical screening and the fingerprinting were also conducted. The antivenin studies were divided into *in vitro* phospholipase A_2 assay, acute toxicity, *in vivo* modulatory activity which is a rodent lethality test assay in mice and histopathological study. The leaf possessed abaxial anomocytic stomata, few trichomes and prism of calcium oxalate. Stomatal number; 56 and stomatal index; 13, palisade ratio; 20, vein termination number; 6, vein islet number; 3 were determined as quantitative microscopic leaf constants. Chemo-microscopically, the leaf was found to contain cellulose, lignin, suberin, gum and mucilage (as cell wall materials). The ergastic substances observed include: starch grains, calcium oxalate and calcium carbonate, inulin and tannins, while aleurone grain was absent. Moisture, total ash and acid insoluble ash were found to be 5.6%, 11.7% and 1.2% respectively. Ethanol extractive and water extractive

values were found to be 22.00% and 34.00% respectively. Preliminary phytochemical screening of the n-hexane, ethyl acetate and methanol extracts revealed the presence of steroids/triterpenes and deoxy sugars in all the three extracts. In addition, saponins, flavonoids, tannins and alkaloids were present in ethyl acetate and methanol extracts respectively. The thin layer chromatographic fingerprints of n-hexane, ethylacetate and methanol extracts revealed eleven (11) spots in hexane: ethyl acetate (9:1), fourteen (14) spots in hexane: ethyl acetate (13:7) and eleven (11) spots in butanol: acetic acid: water (10:1:1). The thin layer phytochemical screening revealed the presence of alkaloids, phenols, saponins and triterpenes/ steroids but absence of flavonoids and anthraquinones in n-hexane and methanol extracts. Phenols, saponins and steroids/ triterpenes were present but absence of alkaloids, anthraquinones and flavonoids were noted in ethylacetate extract. N-Hexane extract produced the best protection against lethal dose (9.55mg) of *Naja nigricollis* venom which is significant at 20mg/kg at p-value < 0.05. Histopathology of brain, lungs, heart, liver and kidneys shows a near normal feature of the various organs examined after treatment of mice Important pharmacognostic characters were determined for proper identification of *Securinega virosa*, while the extracts presented a significant protection against venom of *Naja nigricollis* toxicity in mice.

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LIST OF ABBREVIATIONS

WHO - World Health Organization

USA - United States of America

WSCP - World Check list of Selected Plant

SL - Sensu Lato

SI - Stomatal Index

HIV - Human Immunodeficiency Virus

PVAC - Polyvalent Antibodies Column

ASV - Anti-Snake Venom

SEM - Scanning Electron Microscope

ANOVA - one way Analysis Of Variance

SPSS - Statistical Package of Social Sciences

HE - N-hexane Extract

EE - Ethylacetate Extract

ME - Methanol Extract

TLC - Thin Layer Chromatography

BAW - Butanol Acetate Water

HE - Hexane Ethylacetate

PLA_2 - Phospholipase A 2

IP - Intra Peritoneal

KI - Inhibition Binding Constant

LD_{50} - Lethal Dose

LH - Lymphocyte Hyperplasia

AC - Alveolar Congestion

VC - Vascular Congestion

H & E - Haematoxylin Eosin

SC - Sinusoidal Congestion

H - Hepatocyte

C - Central Vein

CI - Cellular Infiltration

N - Necrosis

I - Inflammation

G - Glomerulus

T - Tubules

HCG - Hyper Cellularity of Glomerulus

GN - Glomerular Necrosis

TN - Tubular Necrosis

C - Congestion

V - Vascular Degeneration

V - Vacoulation

IA - Inter Alveolar

A - Alveolar

EP - European Pharmacopoeia

FFA - free fatty acid

AA - arachidonic acid

ED - Effective Dose

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Traditional medicine consists of medical knowledge developed by indigenous cultures that involved the use of plant, animal, mineral based medicines, spiritual therapies and manual techniques designed to treat illness or maintain wellbeing (WHO, 2013). It was modified over hundreds to thousands of years, of which the best known include Indian traditional (Ayurveda) medicine, traditional Chinese medicine and traditional Arab (Unani) medicine. Traditional medicine or Complementary alternative medicine can be broadly grouped as natural products, mind body measures and manipulative and body based practices (Ryan, 2014).

Plants have in one way or the other provided the basic need of mankind such as food, shelter, clothing and protection from disease causing agents. The use of plants and its extracts to treat various diseases can be traced to the early days of human history. Medicinal plants contain substances that modulate the physiology of sick mammals and the knowledge of these healing properties has been passed down through generations. About 70 to 80 per cent of the world population, especially inhabitants of rural areas used medicinal plants for primary health care. This is also known as herbalism. Pharmaceutical factories also use medicinal plants as basis for production of therapeutic drugs. Medicinal plants have a promising future. And, of the approximately half a million plants around the world, a lot have their medicinal activities not yet investigated. Medicinal activities of plants could be a solution to troublesome diseases in present or future studies (Bassam, 2012) and for the production of more effective drugs (Dossou-Yovo *et al*., 2014). Furthermore, as a result of population growth, for instance, there is general increase

in demand for plant based traditional medicines. On the other hand, over exploitation and lack of standardization lead to a profound limitation to the use of plants as medicine However, the potentials of African medicinal plants are enormous (Junhua *et al.*, 2011; Ernest, 2012).

Snakebites in Nigeria are mainly associated with four families of snakes (Viperidae, Elapidae, Colubridae and Actraspididae) and three species from the first two families - carpet viper (*Echis ocellatus*), black-necked spitting cobra (*Naja nigricollis*) and puff adder (*Bitis arietans*) are the most commonly involved. The most common snakebite envenomation in Savannah region of the country results from carpet viper (*Echis ocellatus*). It accounts for at least 66% of all snakebites (Habib *et al.,* 2015). Snakebite is a common medical emergency in developing countries (Sipra *et al.,* 2016) and represents an important neglected public health problem.

For the global burden of snakebite and envenomings, an extensive literature search covering 40 years (ending 2010) and meta-analysis of the data, taking into account the heterogeneity between the studies and their respective weight was conducted. The incidence, mortality and population at risk were estimated after stratification according to the environment (urban or rural) and survey methodologies (national, hospital or community studies). The incidence of snakebite was inversely correlated with population density. The number of envenomings was estimated at over 314,000, of which 95% occurred in rural areas, while the remainder occurred in cities. The annual mortality was estimated at 7,331, of which 97% occurred in a rural environment. The annual number of amputations ranged from 5,908 to 14,614. The population most at risk was young men engaged in agricultural or pastoral labours. Household surveys indicated that actual incidence and mortality were likely 3-5 times higher. The difference may be explained by

treatment seeking behavior. However, incidences and mortalities reported here reflect the number of patients who attend modern health facilities, giving underestimated figures of the burden of snakebites in sub-Saharan Africa but realistic current requirements for antivenoms (WHO, 2015).

1.2 Statement of Research Problem

Animals such as goat and horse are the major and the only validated tool for antivenom derivation for decades. In sub-Saharan Africa, there are serious challenges associated with the availability of this validated antivenom. Some of these challenges are: lack of accessibility, ineffective products and high cost (Juan *et al.,* 2010). The abundance of folkloric knowledge on the use of medicinal plants as an alternative therapy to many troublesome diseases such as snakebite has received minimal attention. More so, snakebite envenomation is a life threatening injury that requires intensive care and may results on the death or permanent deformity of the victims. A study has shown that only 8.5% of snakebite victims in Nigeria attend hospitals and most vulnerable farmers and Cattle rearers depend on traditional healers (Ismaila and Adamu 2012; Habib, *et al* 2013; Hifumi, *et al* 2015). Also, the benefit of phytotherapy is controversial as the pharmacological and toxicological actions and standardization are not well studied and documented (Gupta and Peshin, 2012).

1.3 Justification

African traditional medicinal plants have enormous potentials to pharmaceutical industry. Herbal therapeutics for snake envenomations seems to be a viable alternative to modern medicines. Also, there is an increase on the scientific validation confirming the efficacy of the widely used

traditional medicine products across the world (WHO, 2013). Many plants existing in nature are enormous reservoir of bioactive molecules that can be developed as new chemical entities, analogs, derivatives, synthetic compounds with natural product derived pharmacophores or as natural product mimics effective for neutralization of snake envenomation (Ramar *et al*., 2012). There are a number of plants species found to be effective for the treatment of snake bites in traditional medicine whose pharmacological evaluation has been undertaken (Gupta and Peshin, 2012). Nigerian plants such as *Aristolochia albida*, *Guiera senegalensis*, *Schumaniophyton magnificum* etc. were found to relieve snake venom toxicity in experimental animals (Habib, 2013).

1.3.1 Research question

What are the characteristic/pharmacognostic features that can be used for correct identification of *Securinega virosa* leaf?

Would thin layer chromatographic finger print and phytochemical profile of *S. virosa* be useful for quality assessment of *S. virosa* leaf?

Does *Securinega virosa* leaves have any modulatory effect on the snake venom of *Naja nigricollis?*

1.4 Aim

To provide scientific rationale for the traditional use of *Securinega virosa* leaves as antivenin for snakebite and to determine the pharmacognostic characteristics of the leaves for quality assessment.

1.5 Objectives

1. To establish pharmacognostic microscopical features and physical constants useful for identification of *Securinega virosa.*

2. To establish thin layer chromatographic finger prints and phytochemical profiles of *S. virosa* leaves extracts.

3. To determine the *in vitro* antivenin activity of *S. virosa* extracts against *Naja nigricollis* Snake venom Phospholipase A_2 (PLA_2)

4. To determine the modulatory *in vivo* antivenin effects of *S. virosa* leaf extracts on *Naja nigricollis* snake venom.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The Family Euphorbiaceae

Euphorbiaceae is a family of vascular flowering plants with over 300 genera and about 9,000 species distributed within five subfamilies namely; Phyllanthoideae, Oldfieldioideae, Acalyphoideae, Crotonoideae and Euphorbioideae (Webster, 1994, Kondamudi *et al*, 2009). It is popularly known as spurge family due to the white latex they produce on injury to the plant. They are mostly herbs and sometimes shrubs or tree especially in the tropics (Abbonier, 2004).

2.1.1. Distribution of Euphorbiaceae

They are native to tropical Africa, America, Asia and Australia. Some species are found in non-tropical regions such as Mediterranean, Middle East, South Africa and Southern United States of America. They are characterized by disjunct distribution (Abbonier, 2004).

2.1.2. Description of Euphorbiaceae

The leaves are alternate rarely opposite with stipules. The leaves are simple, rarely compound and palmate but never pinnate. Fruits are succulent and non-succulent in some species. Stipules are mostly absent in succulent species. The stipules may be reduced to have glands or spines. Flowers are radially symmetrical, unisexual with male and female flower occurs on the same plant. Flowers vary structurally across the family. They are monoecious or dioecious. Stamens are one to ten or more. The female flowers are hypogymous (Abbonier, 2004).

2.1.3. Economic value of Euphorbiaceae

The members of Euphorbiaceae are valuable source of different kinds of useful products like dyes, edible tubers, oil crops, furniture, agricultural implements, ornamental plants, pharmacological products, rubber, timber and aesthetic items. They includes: castor oil (*Ricinus*

communus), cassava (*Manihot esculenta*), Para rubber tree (*Hevea brasiliensis).* . They are widely used as medicine and some have proved to be efficacious. *Securinega virosa* have proved to be efficacious against brain cancer cell lines *in vitro* (Kondamudi *et al.*, 2009; Rahman and Akter, 2013; Magaji *et al.*, 2015).

2.2 Genus *Securinega* (Bush weed)

Many researchers have merged and unmerged this genus with genus Flueggea (Webster, 1994). It is monoecious or dioecious trees, shrubs, or herbs with 14 species, widespread in the tropics and temperate eastern Asia, but with a disjunct relict distribution. Flowers in axillary glomerules (except *Richeriella*); sepals mostly 4-6, free or connate; stamens 2-15, free or connate; disk extrastaminal and usually dissected (less commonly cupular or absent); anthers introrse or extrorse; pistillode present or absent; pistillate sepals mostly 5 or 6, persistent in fruit; disk usually cupular, often lobed (sometimes dissected); carpels mostly 3 (rarely 2 or 4-10); styles free or connate, bifid or entire; ovules hemitropous; fruit usually capsular (*sometimes* baccate or drupaceous); seeds usually 2 per locule; testa smooth or sculptured; endosperm copious; cotyledons broader than and 1-2 times longer than the radicle (Webster, 1994).

2.2.1 Recent systematics modification of the genus *Securinega* and *Securinega virosa*

Now, the genus Securinega is referred to as synonym of genus Flueggea. The accepted genus name by the world check list of selected plant (WSCP) is Flueggea and currently it is recognized under the family Phyllanthaceae. Phyllanthaceae is a segregate family from Euphorbiaceae family based on congruent plastid and nuclear DNA sequence data that have recovered well-

resolved and strongly supported clades (popularly referred to as Euphorbiaceae *sensu lato* (SL) (Awomukwu *et al*, 2015; Govaerts, 2017). The name *Securinega virosa* has now been replaced with *Flueggea virosa* as the accepted specie name under genus Flueggea and family Phyllanthaceae (Govaerts, 2017).

2.3 *Securinega virosa* (Roxb ex Willd) Baill

This plant is referred to as 'cure all' due to its valuable and common application in traditional medicine for the management of many diseases (Magaji *et a*l., 2008).

2.3.1 Common names and synonyms of *Securinega virosa* (Euphorbiaceae)

English name: Dog's tentacles

Local (vernacular) names;

Hausa : Tsa

Yoruba : Iranje

Ibo : Njisi-nta

Kanuri : Shim-Shim (Komal *et al*., 2013).

Synonyms: *Flueggea virosa* (Abonnier, 2004)

2.3.2 Geographical distribution

It is widely distributed throughout tropical Africa, India, Malaya, China and Australia. In Nigeria, it is found in virtually all parts of the country. It is common, scattered and irregularly distributed (Abonnier, 2004).

2.3.3 Botanical description of *Securinega virosa*

Securinega virosa is a small, deciduous, smooth, large, graceful shrub. The leaves are extremely variable in shape, elliptic-ovate, Obovate or orbicular, 2.5 to 10 centimeters in length, glaucous beneath, and rounded, obtuse, or pointed at the top. The flowers are usually borne on axillary fascicles. The fruit is mostly small, black or white, dry, and about 3 to 5 millimeters in diameter (Burkill, 1994). Thakur and Patil (2014) further described that, the Leaves are hypostomatic. Epidermal cells are chlorophyllous, sides 4–6, straight, and mostly tetra to hexagonal. Stomata are mostly anomocytic, rarely paracytic, orientation is random and distribution is diffuse. Stomatal index (S.I.) =12. Subsidiaries mostly 4–5, rarely 3, mostly F-type, walls straight, slightly curved sides mostly 5–6, rarely 4. Guard cells have elliptical pore and narrow (Plate I).



Plate I *Securinega virosa* growing in a bush (source www.plantzafrica.com)

2.3.4 Ethnomedicinal uses of *S. 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 and one of the most commonly used medicinal plant in West Africa (Sofowora, 1993; Burkill, 1994). The roots and fruits are believed to be an effective snakebite remedy. Root of *S. virosa* is also used in some African communities as contraceptives and for the treatment of syphilis, gonorrhea, rheumatism, sterility, rashes, while an infusion of the root is taken to relieve malaria (Tshifhiwa and Thompson, 2008). The root bark is believed to provide a treatment for diarrhoea and pneumonia. The plant is used traditionally in the management of cancer and ulcer (Mike *et al*., 2010). The dried root powder of *Flueggea virosa* is used in Zimbabwe as antidote for snakebite when applied at the part bitten by snake (Maroyi, 2013).

The plant is said to have a hallucinogenic effect and the decoction of the root with other plant is used in Northern Nigeria for the treatment of mental illness (Abbonier, 2004). In North Eastern Nigeria, the root and leafy twig decoction is used for the treatment of epilepsy (Magaji *et al.*, 2007).

2.3.5 Pharmacological activities of *S. virosa*

The leaves have antioxidant (Dickson *et al*., 2006), while the methanol root bark extract of *S. virosa* was reported to contain bioactive constituents with sedative and anti-convulsant activities (Magaji *et al.*, 2007 and 2008). It also possessed anti-diabetic (Tanko *et al*., 2008), analgesic and anti-inflammatory activities (Yerima *et al.,* 2009). Wiwat and Kwantrairat (2013) reported that leaves extract of *Securinega virosa* possessed anti HIV-1 reverse transcriptase inhibitory activity

(88.2%) tested *in vitro*. Also, methanol leaf extract of *S. virosa* possess antipsychotic and sedative effects *in vitro* in mice (Magaji *et al*., 2014).

2.3.6. Chemical constituents

Reported preliminary phytochemical screening on the methanol root bark extract of *S. virosa* indicated the presence of saponins, tannins, alkaloids, flavonoids, cardiac glycosides, cyanogenic glycosides, resins, steroids/terpenoids and carbohydrates (Magaji *et al.* 2007; Aiyelero *et al.*, 2012; Wiwat and Kwantrairat, 2013). However, the methanol leaf extract of *S. virosa* contain alkaloids, tannins, saponins, flavonoids, cardiac glycol-sides, cyanogenic glycosides, resins, steroids/terpenoids and carbohydrates (Yerima *et al.*, 2009). Bergenin was isolated from the root of *S. virosa* (Magaji *et al.*, 2015). Alkaloids- securinine and triterpenes; were reported in *Flueggea virosa* (Maroyi, 2013). Zhang *et al.* (2015) isolated various types of Securinega alkaloid oligomers, fluevirosinines from *Flueggea virosa* (a synonym of *Securinega virosa* (Abonnier, 2004) with anti-HIV activity.

2.4 Snakebite; A Public Health Problem

There are over 3000 species of snakes in the world; out of which about 600 are venomous and over 200 out of this are medically important (Hider *et al.*, 1991; WHO, 2015). Snakebite is one of the neglected tropical diseases affecting a sizable number of the world population. It is a pandemic health issue occurring mostly in tropical and agricultural regions of the world. The world health organization, in 2009 added snakebite as one of the neglected tropical diseases causing a huge economic and financial burden to the population. Snakebites are associated with mortalities more than all other neglected tropical diseases (Kasturiratne *et al.*, 2008; Williams, *et*

al, 2010). The current global burden of snakebite is under estimated (Gutiérrez *et al*., 2014). The global incidence of snakebites was published by few researches, of which the data on the incidence has also been criticized with regards to the nature of data collection and type of data available that was used. In addition, regional reports on snakebites vary widely with actual incidences, especially in the developing countries (Chippaux, 2008). The global, Africa and sub-Saharan Africa incidence of snakebites are given in the table below:

Table 2.1: Global, Africa and Sub-Saharan African Burden of Snakebites

Coverage	Incidence / year	Researcher (s)
Global	Snakebite 500,000 Envenoming 500,000 Death 35,000 Disabilities -	Swaroop and Grab, 1954
	Snakebite > 5 million Envenoming > 2.5 million Death 125,000 Disabilities > 100,000	Chippaux, 1998
	Snakebite 1.2 to 5.5 million Envenoming 0.42 to 1.8 million Death 20,000 to 94,000 Disabilities > 400,000	Kasturiratne, 2008
	Snakebite up to 5 million Envenoming 2.4 million Death 94,000 to 125,000 Disabilities 400,000	WHO, 2013
Africa	Snakebite 1 million Envenoming 500,000 Death 20,000	Chippaux, 1998
	Snakebite 273,000 to 1.26 million Envenoming 91,000 to 420,000 Death 3,500 to 32,000	Kasturiratne, 2008
Sub-Saharan Africa	Snakebite 83,997 to 884,100 Envenoming 27,999 to 294,700 Death 1,599 to 18,654	Kasturiratne, 2008
Sub-Saharan Africa	Snakebite Envenoming 314,078 Death 7,331 Disabilities 5,908 - 14,614.	Chippaux, 2011

Africa reports on the incidence of snakebite reflect the scanty nature of reporting habits manned by a myriad of challenges. It is believed that a significant number of snakebite cases go

unreported, giving an under estimated figure of the real incidences and/or consequently the burden of snakebites in Africa (Kasturiratne *et al*., 2008, Habib, 2013, Gutirrez *et al*., 2014). In 2005, 45,900 deaths was attributed to snakebite envenomation in India alone while in the whole of Africa a more recent data shows 7,300 deaths, about 6000 amputations and 314,000 snakebites. Envenomings occur at 45 to 67% of the total bites. (Kasturiratne *et al*., 2008; Mohapatra *et al.*, 2011; WHO, 2015). In Nigeria the incidence of snakebite from a Benue region in the northeastern part of the country alone is 497 /100,000 persons per year and mortality of 2 to 16 /100,000 (Habib, 2013). This is almost a 10 fold than that of West African region estimate of 54/100,000 snakebites persons per year.

2.4.1. Snakebite envenomation

According to estimates, more than 5 million people in the world suffer snakebite every year with at least 2.5 million envenomings, and among those who are bitten, approximately 125 000 die and around 400 000 are left with permanent sequelae (WHO, 2015). However, more recent nationwide community based surveys in Bangladesh and India have shown that the scale of this problem is far greater than suggested by hospital based statistics and that these global figures greatly underestimate the actual incidence of snakebite envenoming and the resulting mortality and disability. An important factor that contributes to the morbidity and mortality associated with snakebites, particularly in sub-Saharan Africa and parts of Asia, is the poor availability of the only validated treatment for this disease: antivenoms (WHO, 2015). Also, among the envenomed individuals, only about 10% attend the modern health care facilities with more than 80% of the victims treated by traditional medical practitioners (Chippaux, 2008; Habib, 2013). In addition, it was found in the African continent that about 10% – 20% of the snakebite envenomed

individuals, do not receive any form of medication. Meanwhile, in Nigeria only 8.5% of the snakebite envenomed victims seek for allopathic medical care (Kasturiratne *et al*., 2008; Habib, 2013).

It is known that certain reactions are observable following a snakebite envenomation. These reactions can be local such as: blistering, swelling, redness, painful lymph node enlargement, tissue necrosis, snake fangs injury and so on, while the systemic reactions could include: shock, intravascular haemolysis, generalized haemorrhage, descending paralysis, paralyzed rhabdomyolysis and acute kidney injury (Warrell, 2015). The Savannah region of West Africa has been documented to have a high level of snakebite and its' envenoming, notably in the countries of Nigeria, Benin, Burkina-faso, Cameroon, Ghana and Togo. The most implicated species of snake in the region with the highest envenoming is carpet viper (*Echis ocellatus*) of the family Viperidae. For instance, in Nigeria in a study conducted in savanna region of Benue valley (North Central) alone, an incidence of snakebite was reported to be as high as 500 per 100,000 persons per year with mortality of up to 12% and Carpet viper is the major cause of mortality and morbidity, also it accounts to 66% of all the snakebites in that region The African cobras (Naja spp.), puff adders (*Bitis arietans*) and mambas (Dendroaspis spp.) are frequently involved in attacks on humans while Atractaspis spp. and small vipers are only occasionally involved. The main clinical features of *E. ocellatus* envenoming are systemic hemorrhage, incoagulable blood, shock, local swelling, bleeding and necrosis (Habib, 2013; Habib *et al.*, 2015; Iliyasu *et al*., 2015).

Snake venom is a complex mixture of various substances of which some are toxic while some are non-toxic and the main component implicated in envenomation following a bite are enzymes

and polypeptide toxins. More than 90% of the dried weight of venom is protein. Snake venoms composition are found to differ for both inter and intra-species. The most important and clinically significant components are haemorrhagins, pre-and post-synaptic neurotoxins, cytolytic or necrotic toxins, haemolytic and myolytic phospholipases and pro-coagulant enzymes (WHO, 2010).

2.4.2 Diagnosis of snake envenomation

No country is free from the risk of snakebite, and affects mostly rural dwellers with inadequate and unequipped health facilities. The rural and urban health workers are most at times not ready for snakebite management. This is due to lack of adequate beforehand training on snakebite treatment (WHO, 2010; Hifumi *et al*., 2015).

The venomous snakebite diagnosis and treatment is often troublesome to clinicians due to inadequate information on proper antivenom administration guideline in clinical practice (Hifumi *et al*., 2015). Diagnosis relies heavily on the specific snake species identification and careful observation of the clinical manifestation of the snake envenomation as there is no definite diagnostic markers and or kits for snake toxicity in clinical dispensation. And, it was reported that even as in most cases the snakes involved are killed, they are never taken to the health centre for identification probably due to lack of awareness for the need to do so. However, even if it was taken to the well-equipped health care facility, the health care givers lacked adequate skills to correctly identify the snake specie (WHO. 2013, Fadare and Afolabi, 2012; Hifumi *et al*., 2015).

Broadly, the diagnosis of snakebite can be classified in to two: clinical diagnosis and laboratory diagnosis. Clinical diagnosis depends on identifying local signs such as blistering, swelling and necrosis and systemic signs like: Haemorrhage, incoagulable blood and hypovolaemic shock in viper bite and ptosis, descending paralysis in elapid bite, On the other hand, laboratory diagnosis involves analysis/ determination of changes that occur in envenomed individual (WHO, 2010; David and Laing , 2014; Hifumi *et al*., 2015).

2.4.3. Treatment of snakebite

Treatment is primarily aimed at neutralizing the venom containing an array of bioactive protein molecules with specific antivenin. Unless the signs of bite or signs of envenomation are recognized, victim may not visit a health care provider and there is a strong association between snakebite-induced mortality with poverty, mistaken identity, mismanagement by untrained village based traditional therapists, poor transportation facilities, delay at arrival to medical centres and improper dosing of Anti-snake venom (Rao *et al*., 2013; Gutiérrez *et al*., 2014).

The syndromic protocol approach for management of unknown snakebite, categorizes snake bites in to three groups: painful progressive swelling, progressive weakness and bleeding syndrome. This appears to be more logical in reducing mortality. There is no specific or definite dose for antivenom therapy to victims of snakebite and same dose is given to children as in adult (WHO, 2010; Rao *et al.*, 2013). Also, in study conducted in Nepal, it has shown that; repeated administration of more antivenom has no any significance statistically in terms of mortality rate reduction determined in an independent randomized trial. Antivenoms are administered intravenously to achieve rapid onset of action. Subcutaneous or intramuscular administrations are

the most commonly used for the purpose of avoiding serious side effects. Pre-medication with an antihistamine and/or epinephrine should be used when the perceived benefit is greater than the risk of adverse effects. As for the use of hydrocortisone as pre-medication for snake antivenom, the efficacy of which has not been determined and not recommended (Hifumi *et al*., 2015; Pandey *et al.*, 2016).

Recently, polyvalent antibodies column (PVAC) was invented for use in snake venom detoxification. It is a machine that can be used alone or in combination with conventional therapy for snake envenomation. It works like a dialysis machine to perform out of body removal of venom antigens. PVAC membrane is for single use, specific and comes with a replaceable column of polyvalent antibodies. It was found to be effective and potentiates the effect of conventional antivenoms when used in combination (Shahidi, 2014).

2.4.4 First aid management of snakebite

Early recognition of the manifestation of snakebite is essential for targeted first-aid treatment. The use of tourniquet to tie the victim's snake bitten limb can results to more harm than good instead a pressure mass should be used. Prompt arrangement for transporting snakebite victims to the nearest or appropriate health facility is highly encouraged, as most often any delay may lead to longer hospital stay, more admission cost, high morbidity and mortality rate (WHO, 2010).

Symptoms and signs of snakebite vary according to the species of the snake. Local manifestations, such as bleeding and swelling, severe pain, and systemic manifestations such as

nausea and vomiting, drowsiness, weakness, and dizziness, are all common signs and symptoms (Chulin Chen, 2017).

2.4.5 Traditional methods and practices for snakebite management

Throughout Africa, traditional medicine practitioners are highly respected and known to treat illnesses of which snakebite is not an exception. In addition, as snakebites majorly occurs in the rural settings, it is not surprising that more than 80% of snakebite victims point of first visits are the traditional healers in or near the incidence site for treatment. However, none of the traditional or herbal remedies has been proved to be effective in clinical trial to date. Therefore, adequate and properly designed scientific research is highly encouraged to validate and determine the efficacy of African traditional practices or remedies (WHO, 2010; Habib, 2013 and WHO, 2015). Globally the standard antidote for snakebite victims is anti-snake venom (ASV). It is a purified immunoglobulins obtained from hyperimmunised animal in most cases horse (WHO, 2010). But, this antidote in most cases is poorly supplied or inadequately accessible especially at localities where most snakebite cases do occur (i.e. rural areas). Therefore, the most frequent point of call is the traditional healer (Kadir *et al.*, 2015).

There are various method and practices used to treat snakebite by traditional medicine practitioners. For instance, incision or excision at the bite site has been seriously discouraged by the World Health Organization. The traditional practices includes: incision at bite site, applying black stone (that is snake stone), tourniquets and herbal remedies (WHO, 2010; Hifumi *et al.*, 2015). Combinations of two or more of this practice are usually applied with herbs in common. Modes of administration are topical applications of the plant or its sap on to the bitten area,

chewing leaves or barks or drinking extracts or decoctions or injecting the extracts. Extracts from plants are the most common remedy used for snakebite treatment in countryside. Leaves and roots are the most common plant parts used in the snakebite remedy (Kunjam *et al*, 2013)

2.4.6 Plants in the traditional practice of snakebite management

Worldwide there are approximately 400,000 vascular plants 370,000 flowering plants and 18,000 medicinal plants (Kew, 2016). In addition, approximately 700 plant species are known to possess potential antivenom principles (Kadir *et al.*, 2015). The use of plants in African settings for the treatment of snakebite is often widely accepted. Africa has been one of the world's continents with high level of snakebites and envenomations incidence but also at the same time endowed richly with traditional knowledge and valuable plant resources (Kasturiratne, 2008, Gupta and Peshin, 2012). Harrison *et al., (*2009) mentioned snakebite envenoming as a disease of poverty and has established a link between it and poverty. This is supported by the fact that majority of African population rely on alternative medicine being readily available, cheap and affordable for treatment of diseases including snakebite envenoming (WHO, 2010; Gupta and Peshin, 2012).. Many plants in this region have been documented to have anti-snake venom potentials of which some have been investigated scientifically. Some of the plants are: *Hibiscus esculentus*, *Casearia sp.*, *Musa paradisiaca*, *Mucuna pruriens*, *Eclipta sp.*, *Curcuma longa*, *Bauhinia forficata*, *Annoma senegallensis*, *Mikania glomerata*, *Piper sp.*, *Cordia verbenacea*, *Pentaclethra macroloba* (Kaushik *et al.*, 2013),

In Nigeria, among the medicinal plants claimed to have been used in the folkloric management of snakebites, few have been evaluated scientifically to validate the claim and to establish their safety and efficacy (Kaushik *et al.*, 2013). See table 2.2 below;

Table 2.2: Some Plants Used in Nigeria Traditionally to Treat Snakebites

Family	Plant	Local name	Reference
Acaenthaceae	*Asysasia gangetica*	Inana	Abdel-ghani, 2016
Acanthaceae	*Barteria maderaspatensis*	Ewe-asaju	Abdel-ghani, 2016
Amaranthaceae	*Alternantheras selsilis*	Mai –kai dubu	Abdel-ghani, 2016
Asclepiadaceae	*Gymnema sylvestre*	-	Abdel-ghani, 2016
Asclepiadaceae	*Calotropis procera*	Tumfafiya	Ameen et al, 2015
Astereceae	*Ageratum conyzoides*	Akoyun	Ameen et al, 2015
Astereceae	*Aspilla africana*	Nyerki	Ameen et al, 2015
Balanitaceae	*Balanites aegyptiaca*	Tanni	Ameen et al, 2015

Combretaceae	*Chestia ferruginea*	Esise	Abdel-ghani, 2016
Combretaceae	*Guiera senegalensis*	Sabara	Ameen et al, 2015
Fabaceae	*Parkia biglobosa*	Dorawa	Ameen et al, 2015
Liliaceae	*Allium cepa*	Albasa	Ameen et al, 2015
Liliaceae	*Allium sativum*	Tafarnuwa	Ismaila et al, 2012
Moringaceae	*Moringa oleifera*	zogale	Ameen et al, 2015
Solanaceae	*Solanum sp*	Gardili (Eggon)	Ibrahim *et al* 2015

Preliminary investigations on the potentials of some plants as antidotes against snake bite were found to yield a promising result. Extract of the leaves of *Guiera senegalensis* was found to detoxify (*in vitro*) venom from two common northern Nigerian snake species, *Echis carinatus* and *Naja nigricollis* (Abubakar *et al*., 2000).

2.4.7 Bioactive principles from plants with antivenin activity

Various studies have revealed that the alkaloids (*Eclipta prostrate*, *Rauvolfia serpentina*, *Strychnos nux-vomica*, and *Mimosa pudica*), esters (*Gloriosa superba*), phenolic fraction (*Hemidesmus indicus*), terpenoids (*Aristolochia indica*, *Andrographis paniculata*), and flavonoids fractions (*Tephrosia purpurea*) neutralized the snake venom activities (Kadir *et al.*, 2015). Inhibitory activity of many medicinal plants against the snake venom enzymes has been confirmed by biological assays. Compounds found in these medicinal plants belong to chemical classes capable of interacting with macromolecular targets (enzymes or receptors). Most of the studies based on isolation of the active principle or partially purified extract revealed the presence of aristolochic acid, alkaloids, steroids, flavanoids, phenols, pterocarpanes, quinonoid xanthenes, resveratrol, glycoside and tannins. (Ismaila and Adamu, 2012; Kaushik *et al*., 2013).

2.5 Pharmacognostic Evaluation of Medicinal Plants

Most of the research in pharmacognosy has the aim of identifying controversial species of plants, authentication of commonly used traditional medicinal plants through morphological, phytochemical and physicochemical analysis. The importance of pharmacognosy has been widely felt in recent times. Unlike taxonomic identification, pharmacognostic study includes parameters which help in identifying adulteration in plant dry powder. This is again necessary because once the plant is dried and made into powder form, it loses its morphological identity and easily prone to adulteration. Pharmacognostic studies provide descriptions on plant identity and laid down standardization parameters, which will help in authentication of the plants and ensures reproducible quality of herbal products resulting to safe and effective natural products (Sumitra, 2014).

Pharmacognostic evaluation includes macroscopic, microscopic, physicochemical, fluorescence and phytochemical studies of herbal parts or powdered drugs. Herbal raw material shows a number of problems when quality and authentication aspects are considered. This is because of nature of herbal parts, ingredients and different phytochemicals present in plants (WHO, 2011). To ensure quality of herbal medicines, proper control of starting raw material is very important. The physico-chemical evaluation includes qualitative and quantitative assays and instrumentation analysis. Qualitative and quantitative chemical tests include the presence or absence, quantity, number, values and identification of various phytochemicals like flavonoids, glycosides, saponins, alkaloids etc (Evans, 2009). Macroscopic identity of medicinal plant materials is based on sensory evaluation parameters such as; shape, size, colour, texture, odour and taste while microscopy involves comparative microscopic inspection of powdered herbal drugs. Further, advances in microscopic techniques have increased the accuracy and capabilities of microscopy as a means of herbal crude material identification due to the application of improved light and scanning electron microscopes (SEM) in herbal drug standardization (Bhutani, 2003).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 List of reagents and solvents

Acetic acid (Avondale Laboratory, England)**,** Aluminium chloride**,** Anisaldehyde (Sigma-Aldrich, St. Lous, MO, USA)**,** Chloral hydrate (BDH Laboratory Chemicals Division, POOLE. England)**,** Chloroform (JHD, AR; Lobal Chem, India)**,** Dragendorff Reagent, Picric acid reagent,

Mayer's reagent, Ethyl acetate (JHD, AR; Lobal Chem, India)**,** Fast green**,** Ferric chloride**,** Glycerol (BDH Laboratory Chemicals Division, POOLE. England), Hexane (JHD, AR; Lobal Chem, India), Hydrochloric acid (BDH Laboratory Chemicals Division, POOLE. England)**,** Libermann-Buchard reagent**,** Methanol (JHD, AR; Lobal Chem, India)**,** Methylene blue**,** Phloroglucinol, Sudan Red Solution**,** Tetraoxosulphate (iv) acid (Sigma-Aldrich, St. Lous, MO, USA)**,.**

3.1.2 List of equipments

Compound microscope (Fisher Scientific, UK), Dessicator, Disposable syringes, Glass Slides and Cover slips, KERN EW Electronic Balanced, Laboratory glass wares (Funnel, Conical flask, Beakers, Measuring cylinder), Mechanical shaker (Stuart Scientific Flask Shaker, Great Britain), Metallic cages and feeding bottles for rats, Microtome (C 740527, Cambridge Instrument Company Ltd, London and Cambridge, England), Oven, Photographic camera, Plant press (local made), Slide dryer (Hospital and Lab. Supply Ltd, London, UK), Stage Micrometer and Ocular Lens (Graticules Ltd, Ton bridge, Kent. England), TLC tanks (Uni kit® TLC Chromatank®, TLC silica gel 60 F_{254} pre-coated plates (Merk-Germany)**,** Zinc chloride.

Shandon Southern Germany), UV lamp, Water bath (HHS, Mc Donald Scientific International).

3.1.3 Collection, identification and preparation of plant material

S. virosa sample comprising of leaves and fruits were collected on 20th February, 2016 from a wild field in Kakiyayi town, Zaria Local Government area of Kaduna State-Nigeria by traditional herbal practitioner. Sample of intact leaves containing fruits were authenticated by comparism on the herbarium specimen (Voucher no. 2520) at the Herbarium unit of the Department of

Biological sciences, Ahmadu Bello University Zaria-Nigeria. The sample was then cleared (dust, sand and earthy material removed) air dried under shade, coarsely powdered using mechanical grinder and stored in polythene bag for further use.

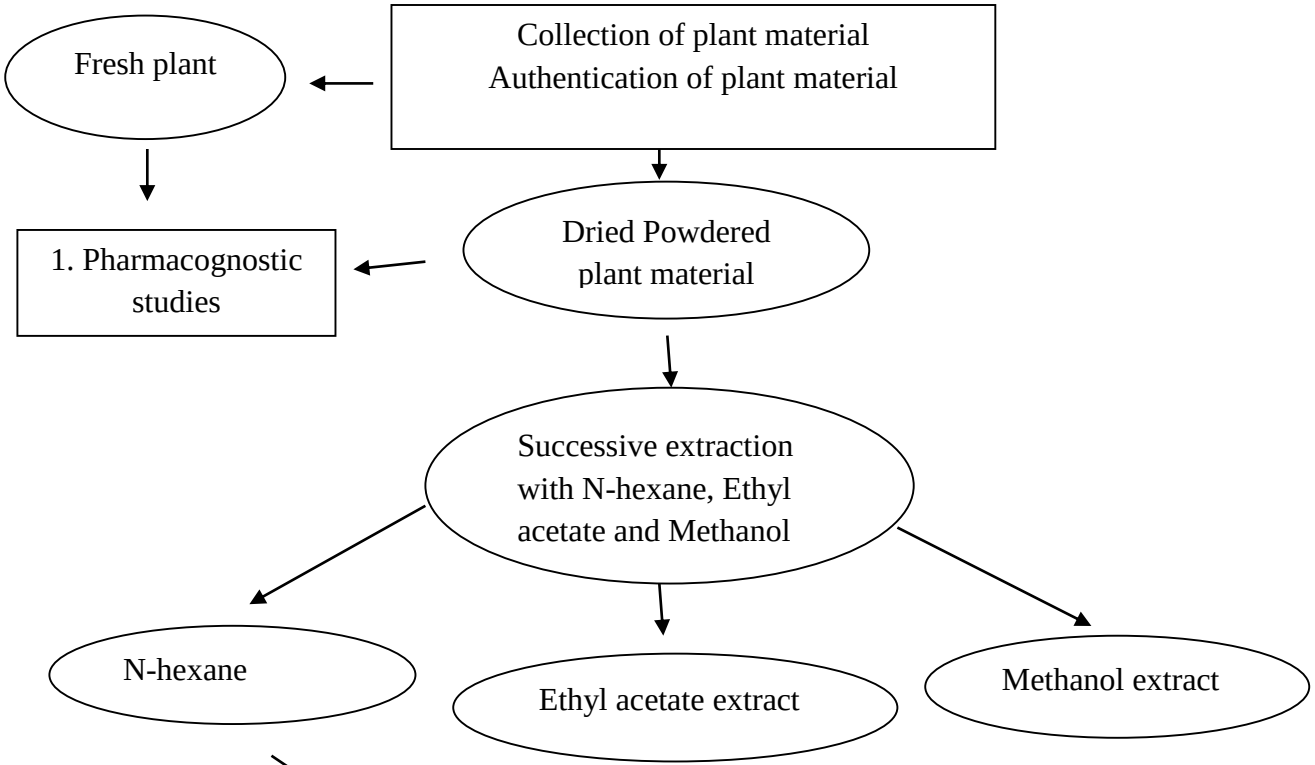
3.1.4 Extraction of plant materials

Leaf powder (300g) of *S. virosa* was extracted successively in 1 litre each of hexane, ethyl acetate and methanol using soxhlet extraction apparatus at moderate tempreture (55^{o}C). The extracts were concentrated under reduced pressure and stored in a desicator for further experiments.

3.1.5 Experimental animals

3.1.5.1 *Mice*:

A total of 80 Swiss Albino mice of both sexes, weighing 15-30 g were obtained from the Animal House facility of Department of Pharmacology and therapeutics, Faculty of Pharmaceutical Sciences, Ahmadu Bello University Zaria. Approval from ethical committee of the University was obtained before the animal experiment. The mice were acclimatized to the environment for 7 days, fed on normal rodent feed (from Vital Feed) and water served *ad libitum* at room temperature. They were divided into five groups of four mice each for the *in vivo* venom neutralization studies.


Collection of plant material
Authentication of plant material
Fresh plant
1. Pharmacognostic studies
Dried Powdered plant material
Successive extraction with N-hexane, Ethyl acetate and Methanol
N-hexane
Ethyl acetate extract
Methanol extract

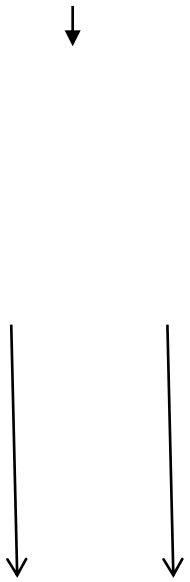


Figure 3.1: Experimental design for the Pharmacognostic and antivenin studies on the leaf of *Securinega virosa*.

3.2 Methodology

3.2.1 Microscopic studies on the leaf of *Securinega virosa*

3.2.1.1 *Surface preparation and anatomical section*

Anatomical section of the leaf sample was prepared and examined under the light microscope. The features observed were described by using the terms according to Dutta (2003) and Evans (2009).

Leaf epidermis was peeled off with a razor blade. The leaf sections were cleared using 70% chloral hydrate, mounted with dilute glycerol and observed for epidermal cells, stomata,

trichomes etc. and their types, as well as, positions. Transverse section across the midrib of the fresh leaves of the plant was prepared, cleared and observed under the microscope as described by Evans (2009).

3.2.1.2 Micrometric evaluation

It consists of measurements of dimensions (length and width) of the various diagnostic microscopic characters of the leaf namely; stomata, trichomes etc. of *S.virosa*. These activities were carried out by using a binocular microscope with the aid of graticles (Kokate, 2003).

3.2.1.3 Quantitative leaf microscopy

This involves counting the specific histological features of the leaf. The five (5) physical constants of the leaves of S. *virosa* were as follows;

(a). Palisade Ratio

It is the average number of palisade cells beneath each epidermal cell of leaf. Section from the upper epidermis of the plant was cleared with boiling 70% chloral hydrate solution and mounted on a clean microscope slide with dilute glycerol and examined with the aid of x 40 objective. A camera lucida was set up and the palisade ratio determined in groups of four and the average taken (Evans, 2009).

(b). Stomatal Number

This is the average number of stomata per square millimetre of the upper and lower epidermis of the leaves of the plant. Section from the upper and lower epidermis of the plant were cleared

with boiling 70% chloral hydrate solution and mounted on a clean microscope slide with dilute glycerol. A camera lucida was set up. With the aid of a stage micrometer a paper was divided into squares of 1mm^2 using x 10 objective. The stomata were traced and counted in the fields on a single section of the leaf of the plant and the average number of stomata per mm^2 of epidermis were recorded (Evans, 2009).

(c). Stomatal Index

This is the percentage proportion of the number of stomata formed to the total number of epidermal cells of the leaf. Sections of the epidermal portion of the leaves was mounted and examined as in stomatal number determination, except that here both stomatal and epidermal cells were counted. The stomatal index was calculated using the formula below (Evans, 2009):-

$$\text{Stomatal Index} = \frac{\text{Number of Stomata}}{\text{No.of Epidermal Cells } + \text{No.of Stomata}} \times 100$$

(d). Vein-islet number

Vein-islet number is the number of vein-islet per unit square millimetre calculated from four contiguous square millimetres in the central part of the lamina midway between the midrib and the margin. It was determined by boiling pieces of leaf of the plants in a test-tube containing 70% chloral hydrate solution, and then followed by treatment with 10% hydrochloric acid to remove calcium oxalate crystals for enhanced visibility. A camera lucida was set up and by means of a stage micrometer the paper was divided into squares of 1 mm^2 using x10 objective. The stage micrometer was replaced by the cleared preparation of the leaf and the veins traced in four contiguous squares that is a rectangle 1mm x 4mm. Each vein was traced and areas which

are completely enclosed by veins were counted and those that were not completely enclosed were excluded (Evans, 2009).

(e). Vein-islet termination number

It is the vein termination present in one square millimetre of leaf surface midway between midrib and margin. It was determined for *S. virosa* leaf using a camera Lucida set up as in vein-islet number but here the termination number in each square was counted (Evans, 2009).

3.2.2 Determination of physicochemical constants of the leaves of *S. virosa*

Physicochemical parameters for the leaf sample of the plant were determined. Five (5) physical constants were determined for the powdered leaves of *S.virosa*. They were moisture content, ash value, acid insoluble ash value, and alcohol and water extractive values. Three (3) different determinations were carried out for each parameter and the average calculated. The methods outlined in WHO (2011) Quality Control Methods for Medicinal Plant Materials was used.

3.2.2.1 Moisture content

It is the quantity of moisture present in a plant material. Moisture content of the leaves of *S. virosa* was determined by loss on drying method. About 3.0 g each of the powdered leaves eres accurately weighed and placed in to three (3) clean, dried evaporating dishes of known weights. These were placed in an oven and heated at a temperature of 105ºC for a 1 hour, then cooled in a desicator and re-weighed. Heating and weighing was repeated to a constant weight. The weight loss on drying was computed using the formula below:

$$\% \text{ Moisture content} = \frac{Weight\ of\ water\ lost}{\text{Original weight of Sample}} \times 100$$

3.2.2.2 Total ash value

This is the ash remaining after ignition of medicinal plants. 2 g of powdered plant materials was accurately weighed and placed separately in a crucible of known weight. It was then heated gently and the heat gradually increased until it is white indicating the absence of carbon. It was allowed to cool in a desicator and weighed. The heating and cooling was repeated until a constant weight was obtained and no change in colour. The total ash-value of the plant specie was determined as a percentage with the formula below:

$$\text{Total ash value} = \frac{\text{Weight of Residual Ash}}{\text{Initial Weight of Sample}} \times 100$$

3.2.2.3 Acid-insoluble ash

This is the residue that remains after boiling the total ash with dilute hydrochloric acid. This was determined for the leaves. 25 ml of dilute hydrochloric acid was added to the crucible containing each of the ash. It was covered with a watch glass and gently boiled for 5mins. The watch glass was rinsed with 5 ml of hot water and the liquid added to the crucible. The insoluble matter was collected on an ash less filter-paper and washed with hot water until the filtrate is neutral. The filter-paper containing the insoluble matter was transferred to the original crucible, dried in an oven and ignited to a constant weight. The residue was allowed to cool in a suitable desicator for 30 minutes and then weighed without delay. The acid-insoluble ash was calculated as a percentage using:-

$$\text{Acid Insoluble Ash} = \frac{\text{Weight of Residual Ash}}{\text{Initial Weight of Sample}} \times 100$$

3.2.2.4 Alcohol-Soluble Extractive Value

This is the amount of extractive in percentage of a plant sample with alcohol. 4 g of the plant material was weighed in a conical flask. 100 ml of ethanol was added and macerated for 24 hours, during which the mixture was frequently shaken within the first 6 hours using a mechanical shaker. It was filtered and 25 ml of the filtrate transferred into an evaporating dish of known weight and evaporated to dryness on a water bath. It was dried to a constant weight, the percentage of alcohol-soluble extractive value was then determined as follows:

$$\text{Alcohol Soluble Extractive Value (\%)} = \frac{\text{Weight of Residue in 25ml Extract} \times 4}{\text{Initial weight of Sample}} \times 100$$

3.2.2.5 Water-soluble extractive value

This is the amount of extractive in percentage of a plant sample with water. Same procedure as in alcohol-soluble extractive value was repeated here for the plant material, but water was used in place of alcohol and the value calculated as follows:

$$\text{Water Soluble Extractive Value (\%)} = \frac{\text{Weight of Residue in 25ml Extract} \times 4}{\text{Initial weight of Sample}} \times 100$$

3.2.3 Chemomicroscopic examination of the leaves of *S. virosa*

For the histochemical detection of cell walls and contents of the plant, the methods outlined in the updated edition of quality control methods for medicinal plant materials (WHO, 2011), was adopted.

3.2.3.1 Cell wall materials

I) Cellulose cell walls

Two drops of iodinated zinc chloride were added to the powdered leaf sample and allowed to stand for few minutes and observed under a microscope. It changed the cellulose cell wall to blue colour. Cellulose is present.

ii) Lignified cell walls

The powdered plant material was moistened on a slide with a small volume of phloroglucinol and allowed to stand until almost dry. A drop of concentrated hydrochloric acid was added and viewed under a microscope. Pink stained was observed. Lignin is present in the cell wall.

iii) Suberized or Cuticular cell walls

Two drops of Sudan red was added to the powdered leaf sample and allowed to stand for a few minutes and observed under a microscope. Presence of orange red colour was observed.

iv) Gum and Mucilage

A drop of Ruthenium red was added to the dry powdered leaf sample and viewed under the microscope. Mucilage often appeared as transparent, spherically dilated fragments on a black background with pink colour on the cell wall which indicates that mucilage is present.

3.2.3.2 Cell inclusions / Cell contents

i) Starch grains

A small volume of iodine was added to the powdered leaf sample and viewed under the microscope. Observation of blue-black colour was noted. This reveals the presence of starch grains in the powdered sample of *S.virosa* leaf.

ii) Aleurone grains

A few drops of ethanol was added to the powdered leaf sample and observed under the microscope. No yellowish-brown was observed which indicated the absence of aleurone grains.

iii) Test for Calcium oxalate crystals and Calcium carbonates

To a small portion of the cleared leaf powder of the plant, 10 % HCl was added; dissolution of crystals in the powdered drug without effervescence indicated the presence of calcium oxalate and calcium carbonate.

Iv) Inulin

A drop of 1- naphthol and that of concentrated sulphuric acid was added to the powdered leaf sample and viewed under the microscope. Observation: A spherical aggregation of crystals of inulin turned brownish red and dissolved. Inulin.is present.

v) Tannins

A drop of ferric chloride was added to the powdered leaf sample and viewed under the microscope. Observation: A greenish black colour was seen. Tannins are present.

3.2.4 Preliminary phytochemical screening of *S. virosa* leaf extracts

Extracts of n-hexane, ethyl acetate and methanol were subjected to preliminary phytochemical screening using the standard method outlined below:

3.2.4.1 Test for Saponins

a) Frothing Test: About 0.5g each of the extracts was shaken with water in a test tube followed by warming on a water bath. There was a presence of frothing which persists on warming except in n-hexane extract (Sofowora, 2008).

b) Haemolysis Test: 2 ml of Sodium Chloride (1.8% solution in distilled water) were added to two test tubes A and B, followed by 2ml of distilled water and then 2ml of extract was added to test tube B alone. About 5 drops of blood were added to each tube and the tubes were inverted gently to mix the contents. Observation: Haemolysis in tube B containing the extract was noted. Saponins are present except in n-hexane (Brain and Turner, 1975).

3.2.4.2 Test for Steroids / Triterpenes

a) Lieberman-Buchard Test: a small portion of the extract was dissolved in chloroform and filtered. Equal volume of acetic anhydride was added to the filterate, followed by concentrated sulphuric acid down the side of the test tube. Observation: A Brown ring at the inter-phase was observed, which indicated the presence of steroids/triterpenes (Evans, 2009).

b) Salkowski Test: a small quantity of the extract was dissolved in 1ml of chloroform, filtered. To the filtrate, 1ml of concentrated sulphuric acid was added down the side of the test tube.

Formation of red ring at the interphase was observed which indicated the presence of sterols (Sofowora, 2008).

3.2.4.3 Test for Flavonoids

a) Shinoda Test: About 0.5g of the extract was dissolved in 5 ml of 95% ethanol, warmed and filtered. Three (3) pieces of magnesium chips were added followed by five drops of concentrated hydrochloric acid. Observation: Appearance of an orange color was noted. Flavonoids are present except in n-hexane extract (Evans, 2009).

b) Sodium hydroxide Test: About 0.5 g each of the extracts was dissolved in water and filtered. To the filtrate, 2ml of 10% aqueous sodium hydroxide solution was added. Observation: the solution turned yellow, which change to colorless on addition of dilute hydrochloric acid. This indicates the presence of flavonoids except in n-hexane extract (Evans, 2009).

3.2.4.4 Test for Tannins

a) Ferric chloride Test: About 0.5 g each of the extracts was stirred with 10 ml distilled water and filtered. Two drops of 1% ferric chloride solution was added to 2ml of the filtrate. Blue-black precipitate (ppt) was observed except in n-hexane. Hydrolysable/gallitannins are present in methanol and ethyl acetate extracts (Evans, 2009).

b) *Lead sub-acetate Test:* To 0.5 g each of the extracts, three drops of lead sub-acetate solution were added. Observation: Black green precipitate was produced except in n-hexane extract. Tannins are present (Evans, 2009).

3.2.4.5 Test for Alkaloids

About 1.0 g each of the extracts was stirred with 20 ml of 1% aqueous hydrochloric acid on a water bath and filtered. The filterate, each was basified with concentrated NH_4OH and extracted with chloroform. The chloroform layer was extracted further with 20 ml of 1% HCL. The aqueous layer was divided into three portions for the following tests:

i) To the first portion, 1 ml of freshly prepared Dragendorff's reagent was added drop-wise and observed. Rose red to brownish precipitates was formed except in n-hexane extract.

ii) To the second 1 ml of Mayer's reagent was added drop-wise and observed. Cream color precipitates were formed extract in n-hexane extract.

iii) To the third, 1 ml of Wagner's reagent was added, a reddish-brown precipitates were formed except in n-hexane extract (Evans, 2009).

3.2.4.6 Test for Anthraquinones

a) Borntrager's Test: To small portion of each of the extracts, 10 ml of benzene was added, shaken and filtered. To the filtrate, 5ml of 10 % ammonia solution was added. The mixture was shaken gently. Observation: No pink red colour in the lower part of the aqueous layer was formed. Anthraquinones are absent in all the extracts (Evans, 2009).

*b) Modified Borntrager's Test**:*** the extract was boiled with 10 ml of aqueous sulphuric acid and filtered hot, and then it was allowed to cool to room temperature. The filtrate was shaken with 5 ml benzene, the benzene layer was separated and to half of its volume, 10 % ammonium

hydroxide was added. No formation of pink-red coloration in the ammonia phase (lower phase) was produced. Combined anthraquinone or anthraquinone derivatives were absent (Evans, 2009).

3.2.4.7 Test for Cardiac Glycosides

a) Keller-Killiani Test: About 0.5 g each of the extract was dissolved in 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was then under layered with 1 ml of concentrated sulphuric acid. Observation: A brown ring was produced at the interphase. Deoxy sugars characteristic of cardenolides are present in all the extracts (Evans, 2009).

b) Kedde's Test: Few drops of 2 % solution of 3, 5- dinitrobenzoic acid in 95 % alcohol were added to small portion of each of the extracts. A solution of 5 % sodium hydroxide was added to make the solution alkaline. Observation: No purple-blue colour was observed in all the extracts. Cardenolides are absent (Evans, 2009).

3.2.5 Development of TLC fingerprint of *S. virosa* leaf extracts

3.2.5.1 Development of Solvent System

Solvent system was developed using a combination of different solvents based on polarity and the solvent systems that give reasonable separation of the components of the crude extracts were documented for phytochemical analysis. The TLC plate was spotted with portion of the extract and developed in chromatographic tank containing a mixture of solvents. It was then removed from the tank at certain point of solvent front, dried and observed in day light, in ultra violet light and by spraying with universal spray reagent P-anisaldehyde for detection of various phytochemicals such as phenols, steroids, triterpenes and so on (Wahab *et al.*, 2010)

3.2.5.2 Thin layer chromatography phytochemical screening

The TLC profiles of *S. virosa* extracts were studied on pre-coated silica gel plate by spotting on the TLC plate. It was developed in the suitable solvent system based on the preliminary studies of the methanol, ethyl acetate and n-hexane extracts. The plate was viewed under UV light/iodine vapour and sprayed with 10 % H_2SO_4 in ethanol, and heated at 110^0C (Hahn , 2007). In addition, developed plates were sprayed with specific detecting reagents such as ferric chloride for phenolic compounds, aluminium chloride for flavonoids, Liberman- Burchard for steroids/triterpenes, Borntrager's for Anthraquinones, Dragendorff's for alkaloids and Iodine vapour for saponins. The separated components were detected visually.

3.2.6 Phospholipase - A2 inhibitory assay of extracts of *S. virosa* on *Naja nigricollis* venom

3.2.6.1 *Source of venom and preparation*

The venom was obtained from *Naja nigracolis* by milking, using markflane (1967) method by a professional snake charmer at Ahmadu Bello University Veterinary Teaching Hospital Zaria. Briefly, the snake was held captive by the charmer, mouth opened and fangs placed on the edge of a glass container covered with polythene. The milking was enhanced by the pressing on and off of the snake tail. The venom drops gradually at intervals into the container. It was then lyophilized and stored in a refrigerator at 2-8^0C.

3.2.6.2. Phospholipase - A_2 inhibitory assay of S. virosa leaf extracts

The method used by Sallau *et al.,* (2005) was adopted. To a set of four (4) test tubes labeled (1-4), 100 µl, 75 µl, 50 µl and 25 µl of egg yolk suspension was added respectively. This was followed by addition of 50 µl of 1 mM $CaCl_2$, 100 µl of 20 mg /mL venom solution and 50 µl of 0.1 M phosphate buffer at pH 7.0. The mixtures were then made up to 300 µl with distilled water and incubated at 37^oC for 30 minutes then deactivated in a water bath at 100^oC for 2 minutes. Three (3) drops of an indicator (phenolphthalein) were added and the mixtures were titrated against 20 mM NaOH solution to end point. The molarity of the free fatty acid released was calculated to obtain the activity of phospholipase A_2. The experiment was repeated using phosphate buffer containing varying concentrations of the *S.virosa* crude extracts 0mg/ml, 2.5 mg/ml, 5 mg/ml and 10 mg/ml. The initial velocity data obtained was used to draw line weaver-Burk's graph to obtain intercepts for the respective plots which were used in the secondary plot (Dixon's plot). Dixon's plot (intercept against S) was` drawn to obtain the inhibition binding constant (KI).

$$Initial\ velocity = \frac{\text{Mb X Vb}}{\text{Va}}\ X\ 100$$

Ma = molarity of free fatty acid released = Initial velocity = x

Mb = molarity of base in millimole

Vb = volume of base used = titre value

Va = Total volume of the mixture

3.2.7 Studies on the modulatory effect of *S. virosa* leaf extracts on *Naja nigricollis* snake venom

3.2.7.1 Assessment of in vivo venom neutralization effect of leaf extracts of S. virosa against venom of Naja nigricollis using mice

a) Acute toxicity studies (Median Lethal Dose) of *S. virosa* leaf extracts

The median lethal dose (LD_{50}) of the extracts was estimated using the lorke's method (1983). Nine (9) mice were divided into 3 groups of 3 mice each. Mice in groups 1, 2 and 3 were given 1000, 100 and 10 mg/kg of the extract of *S. virosa* each respectively. They were observed for signs of toxicity in addition to mortality rate over a 24 hours period. While in the second phase four groups each comprising of one mouse labeled 1, 2, 3 and 4 were given 140 mg, 225 mg, 370 mg and 600 mg doses of n-hexane extract and 1200 mg, 1600 mg, 2900 mg and 5000 mg of ethyl acetate extract of *S. virosa* respectively.. Then, the LD_{50} was calculated as the square root of the product of minimum lethal dose and the maximum tolerated dose. The whole process was conducted for both ethylacetate and n-hexane extracts of *S. virosa*.

$$LD_{50} = \sqrt{\text{minimum lethal dose} \times \text{maximum tolerated dose}}$$

b) Assessment of in vivo venom neutralization on the extracts of S. virosa in mice

This was conducted according to the method previously described by Abubakar *et al.,* (2000). Also, the median lethal dose of *Naja nigricollis* which is 4.8 mg/kg determined by Abubakar *et al*. (2000) was adopted. Twenty (20) mice were divided into five groups of four mice in each group. First group 1 was given normal saline 0.2 ml and group 2 received 0.2 ml of LD_{99} of *Naja nigricollis* venom alone (control group) while groups 3, 4 and 5 (treatment groups) were given 0.2 ml of a mixture of an equivalent of LD_{99} (9.55mg/kg) containing 5, 10 and 20 mg/ml of extract incubated at 37^0C for 10 min to each mice in a group respectively. All the treatments

were given through intra-peritonial (IP) route. A sign of neurotoxicity and number of death per group was recorded over 24 hours after the injection. The result was presented as percentage death and mean time of death ± standard error of mean (SEM). The procedure was conducted for n-hexane, ethylacetate and methanol extracts of *S. virosa* leaf.

c) Histopathology of the mice control and treatment groups

The relevant tissues; brain, liver, lungs, heart and kidneys were collected from the animal and placed immediately into a fixative (10% formalin), after proper fixing for about 48 hours, the tissues were dehydrated through ascending grades of alcohol from 70% alcohol to 90 % alcohol and absolute(100%) alcohol for 16 hours. The tissues were then cleared in toluene for 2 hours after which they were impregnated in molten paraffin wax for four hours. The tissues were then embedded in paraffin wax and sectioned using a rotary microtome at five micron thickness, the sections were then stained using the heamatoxylin and eosin staining technique. The slides were examined using a light microscope and photomicrographs of the tissues were taken using a digital camera for microscope.

3.2.8 Statistical analysis

The results of pharmacognostic studies were presented as mean ± standard error of mean. The results of biological activity were analysed using One Way Analysis of Variance (one-way ANOVA). p-value of < 0.05 was considered significant. This was followed by post-hoc test using Dunnett. Statistical Package of Social Sciences (SPSS) version 16 was used.

CHAPTER FOUR

4.0 RESULTS

4.1 Plant Collection, Identification and Preparation

S virosa leaves were collected together with other aerial parts (flowers) on 20 February, 2016. The plant was identical to a herbarium sample with voucher number 2520 deposited in Department of Biological Sciences, Ahmadu Bello University, Zaria. The shade dried coarsely powdered plant of 355 g was stored in polythene bag for further use (Plate II).

a) 

b) 

Plate II *S. virosa* a) and b) growing naturally in the bush at Kakiyayi village, Zaria, Kaduna state, Nigeria

4.2 Yield of solvents Extraction of Powdered *S. virosa* leaves

Methanol extract produced the highest yield of 46.64 g (15.55%) followed by Ethyl acetate 11.22 g (3.74%) and N-hexane 8.00 g (2.67%) respectively (Table 4.1), following the successive extraction of dried *S.virosa* powder (300 g).

Table 4.1: Percentage Yield of *S. virosa* Leaf Powder (300g)

Solvent	Extract yield in grams (g)	Percentage yield
N-hexane	8.00	2.67
Ethyl acetate	11.22	3.74
Methanol	46.64	15.55

4.3 Pharmacognostic Parameters of *S. virosa* Leaf

4.3.1 Microscopic studies on the leaf of *S. virosa*

Microscopical examination of the leaves of *S. virosa* revealed the presence of some important diagnostic characters on both the upper and lower epidermal layers. These includes: Epidermal cells (Plate III) which are polygonal with straight anticlinal wall and 2-5 -8 µm in size (Table 4.2). Stomata were found only on the lower epidermis. They were anomocytic and paracytic (Plate IV) with stomatal number and index 56.00±0.55 and 12.556±0.33 (Table 4.2) respectively. Unicellular Trichomes (Plate V) are rare but present on both surfaces. Prism crystals of calcium oxalate (Plate III and VI) in a sheaths or singles were in abundance on both upper and lower epidermal surfaces. The transverse section through the midrib of the leaf showed mesophyll, vascular bundle (xylem and phloem), Calcium oxalate crystals and single layer of epidermal cell on the upper and lower leaf surfaces (Table 4.2).

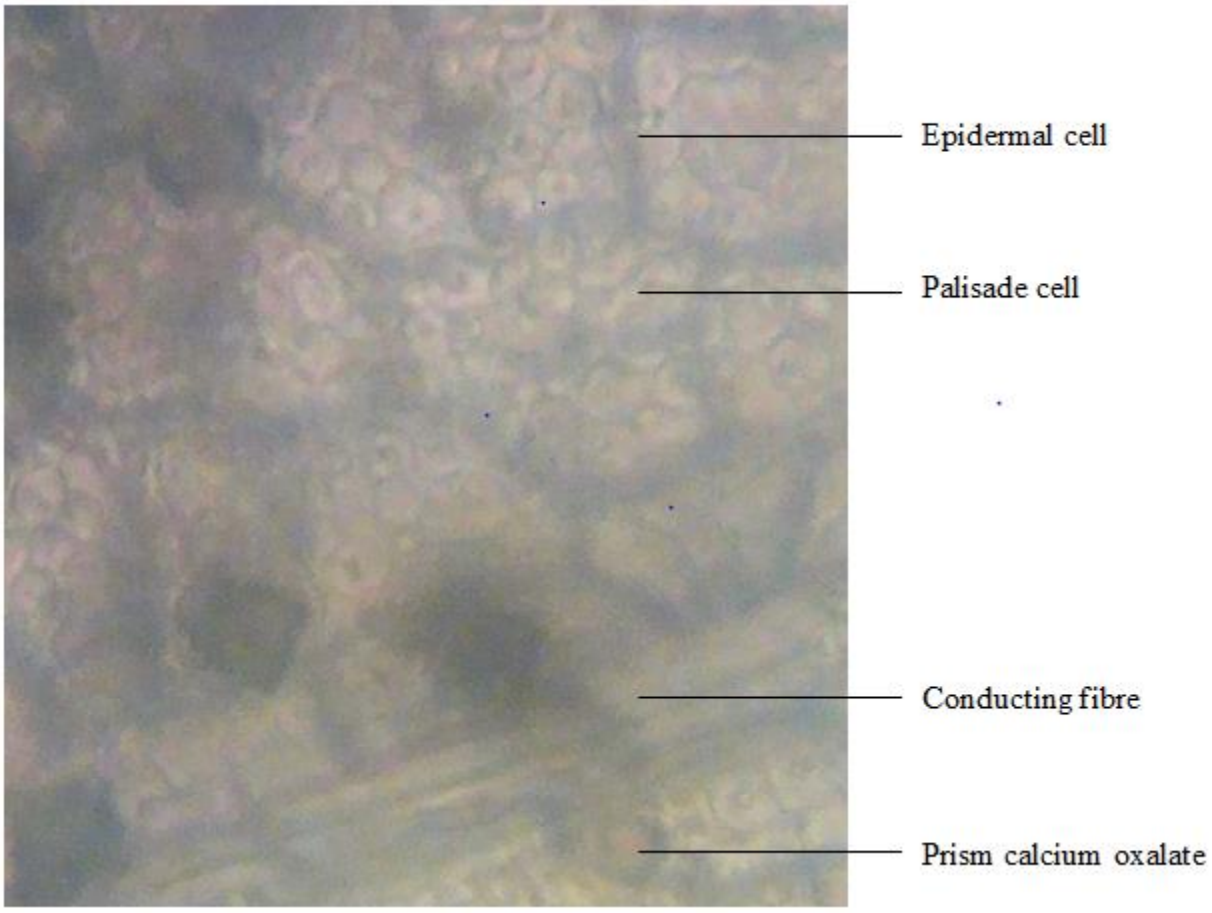


Plate III: Photomicrogaph of some microscopic features of upper epidermal layer of *S. virosa* leaf (X400)

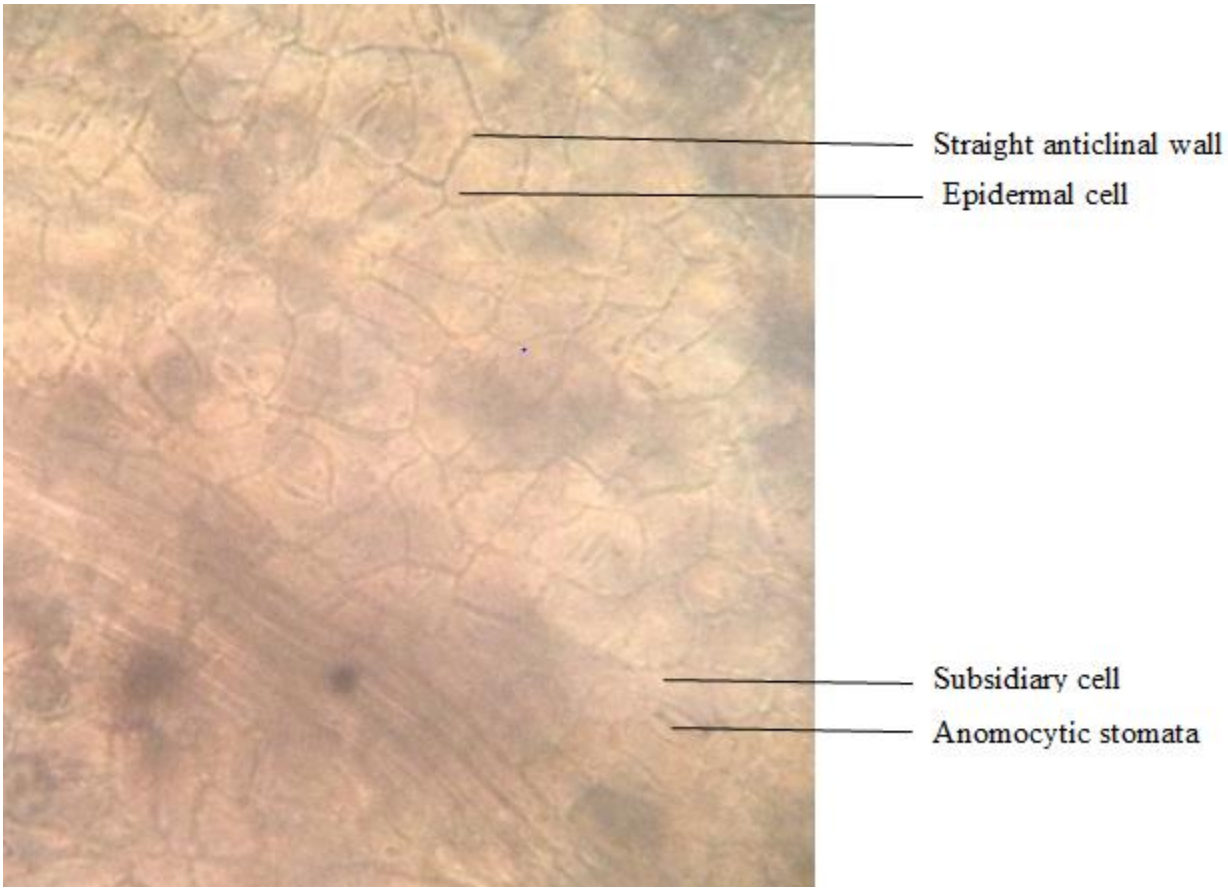


Plate IV: Stomata of the lower epidermal surface of *S. virosa* leaf (X 400)

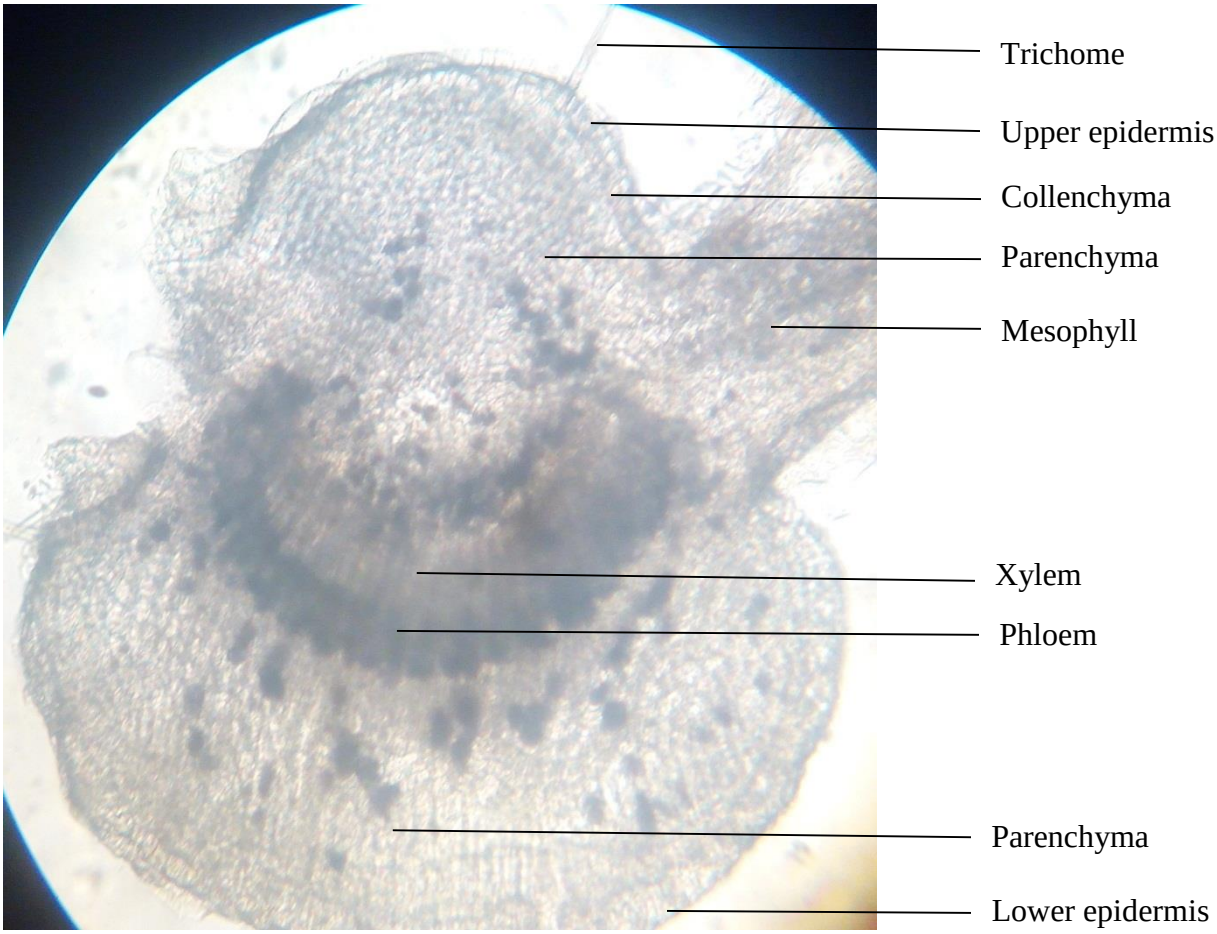


Plate V: Photomicrograph of Transverse section through the midrib of *S. virosa* leaf (X 400)

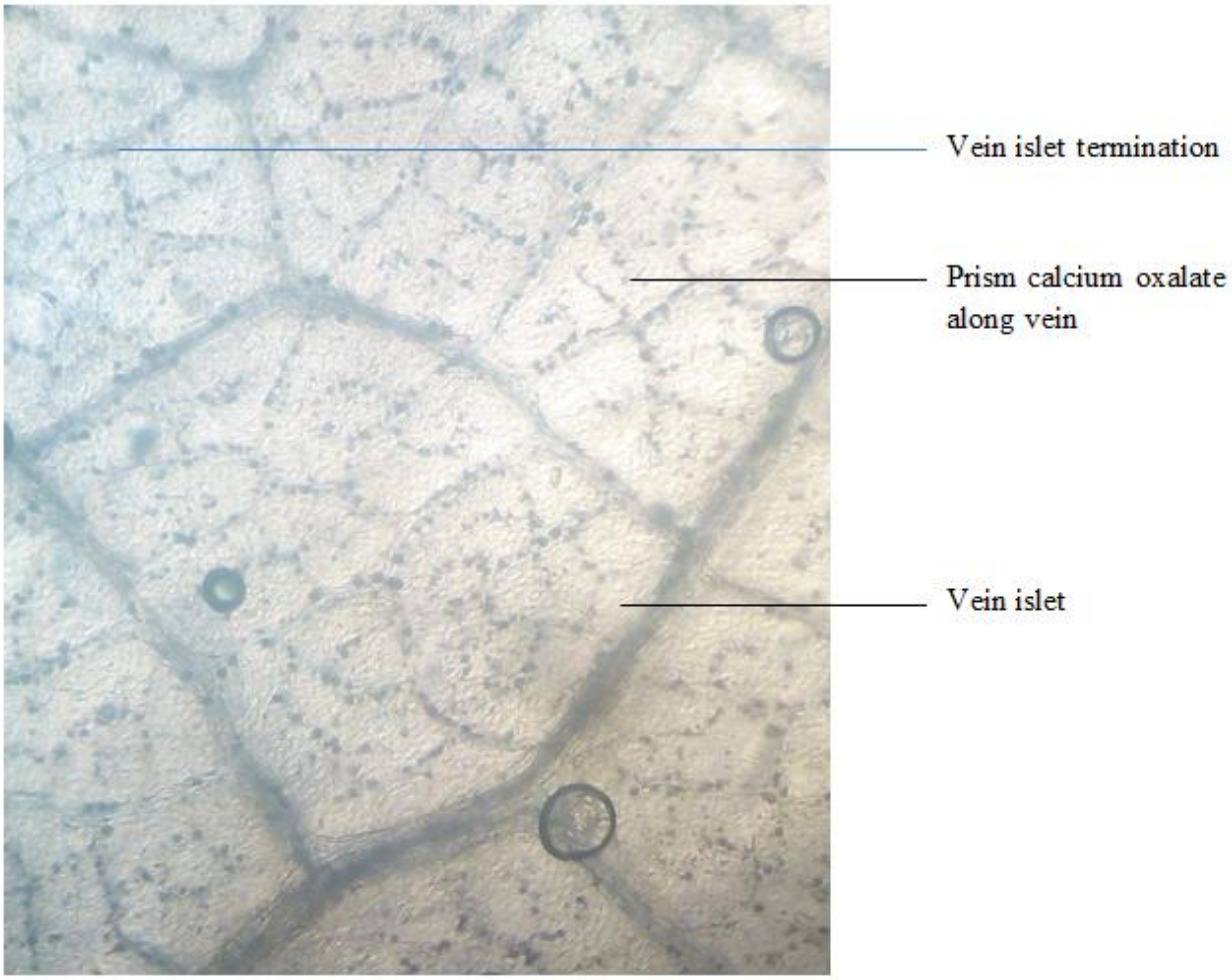


Plate VI: Photomicrograph of adaxial surface of *S.virosa* leaf (X 400)

Table 4.2 Description of the Microscopical Features of the Leaves of *S.virosa*

Characters	Observation	
	Upper Epidermis	Lower Epidermis
Epidermal cell		
Shape	Polygonal	Polygonal
Size	2-5-8 µm	2-5-8 µm
Anticlinal wall	straight	straight
Arrangement	irregular	irregular
Stomata		
Type	absent	Anomocytic
Size	absent	4-5-6 µm
Location	absent	Present
Frequency	absent	rare
Arrangement	absent	irregular
Trichomes		
Surface	smooth	-
Base	not swollen	-
Location	upper epidermis	-
Frequency	rare	-
Form	unicellular	-
Calcium oxalate Crystals		
Location	Free, mostly on veins	Free, mostly on veins
Size	1-2-3 µm	1-2-3 µm
Form	prism	prism
Distribution	Single and in sheath	single and in sheath
Frequency	Frequent	Frequent

Average of three determinations µm = Micrometre

Table 4.3 Quantitative Microscopy of the Leaves of *S.virosa*

Parameter	Mean (µm)* ± SEM
Stomatal Number	56.00 ± 0.58
Stomatal Index	12.67 ± 0.33
Palisade ratio	20.08 ± 1.12
Vein islet Termination Number	5.67 ± 0.33
Vein islet Number	3.33 ± 0.33

Average of three (3) determinations*. SEM = Standard Error of Mean

4.3.2. Physicochemical constants of the leaves of *S. virosa*

The determined physicochemical constants of *S. virosa* leaf include: Average moisture contents using loss on drying method is 5.6%, the percentage yield of total ash and acid insoluble ash were 11.7% and1.2% respectively. The extractive values determined were 22.00% and 33.00% for ethanol and water solvents respectively (Table 4.4).

Table 4.4. Physicochemical Constants of the Leaf of *S.virosa*

Parameters	Values in Percentage (%) as Mean▪ ± SEM
Moisture content	5.5533 ± 0.6190
Total Ash	11.6667 ± 0.3333
Acid insoluble Ash	1.1667 ± 0.1667
Ethanol Extractive	22.0000 ± 1.5280
Water Extractive	33.0000 ± 1.0000

▪ Average of three (3) determinations. SEM = Standard Error of Mean.

4.3.3 Chemomicroscopic characteristics of the leaf of *S. virosa*

Chemomicroscopical examination of the powdered leaf of *S. virosa* revealed the presence of cellulose cell wall, lignified cell wall, tannins, starch, calcium oxalate, suberin and mucilage but aleurone grains were absent (Table 4.5).

4.3.3.1 Cell wall Materials

i) Cellulose: Blue colour was observed on the walls of the epidermal cells which indicated the presence of cellulose.

ii) Lignin: Red stain was observed on the walls of some lignified cell in the plant which indicated the presence of lignin.

iii) Cutin: Red colour was observed on the cell wall in the plant which indicated the presence of cutin.

iv) Gums and Mucilage: Pink colour was observed in the epidermis and vascular tissues of the plant which indicated the presence of mucilage.

4.3.3.2 Cell Contents/Cell inclusions

i) Starch: Blue-black colour on some grains within the cell was observed in the plant which indicated the presence of starch.

ii) Aleurone grains: No yellowish-brown colour was observed which indicated the absence of aleurone grains in the cells.

iii) Calcium oxalate and Calcium carbonate: a clear disappearance of visible calcium oxalate crystals and later effervescence was observed. Dissolution of crystals in the powdered drug without effervescence indicated the presence of calcium oxalate while the presence of effervescence indicated calcium carbonate.

iv) Inulin: A spherical aggregation of crystals of inulin turned brownish-red and dissolved. This indicated the presence of Inulin crystals in the plant powdered sample.

v) Tannins: Greenish-black colour in some parenchyma cells was observed in the plant which indicated the presence of tannins.

Table 4.5 Chemomicroscopic Characteristics of the Powdered Leaf of *S. virosa*

Constituents	Detecting reagents	Observation	Inference
Cell wall Materials			
Cellulose	Chlor-Zinc-Iodine	Blue coloration of cell wall	Present
Lignin	Phloroglucinol	Red stain on the Lignified cell wall	Present
Cutin	Sudan red	Red color on the cell wall	Present
Gum and Mucilages	Rhuthenium red	Pink color on the cell wall	Present
Cell inclusions			
Starch grains	N50 Iodine	Blue-black colour on the grains	Present
Aleurone grains	Ethanol	No Yellowish-brown	Absent
Calcium Oxalate and Calcium carbonate	Hydrochloric acid	Dissolution of crystals occur with Effervescence	present
Inulin	Drop of 1-naphthol and sulphuric acid	Inulin crystals turns brownish-red and dissolved	Present
Tannins	Ferric Chloride	Greenish-black color observed	Present

4.4 Development of TLC Fingerprint of the Extracts of *S. virosa*

4.4.1 Preliminary phytochemical screening of extracts of *S. virosa* leaf

Preliminary phytochemical screening of the n-Hexane (HE), ethyl acetate (EE) and methanol extracts (ME) using standard methods showed that: saponins and steroids/triterpenes were present. However, anthraquinones were absent in all the extracts (Table 4.6)

4.4.1.1 Test for Saponins

a) Frothing Test: There was a presence of frothing which persists on warming except in n-hexane extract. Saponins are present in methanol and ethyl acetate extract.

b) Haemolysis Test: Haemolysis in tube B of both methanol and ethyl acetate extracts were observed. Saponins are present except in n-hexane.

4.4.1.2 Test for Steroids / Triterpenes

a) Lieberman-Buchard Test: A brown ring at the inter-phase was observed which indicated the presence of steroids/triterpenes in all the extracts.

b) Salkowski Test: A red ring at the interphase was observed. This indicated the presence of sterols in the extracts.

4.4.1.3 Test for Flavonoids

a) Shinoda Test: Orange color was observed in methanol and ethylacetate extracts. Flavonoids are present except in n-hexane extract

b) Sodium hydroxide Test: There was a formation of yellow solution of ethanol filtrate of the extracts which changed to colorless on addition of dilute hydrochloric acid. This indicates the presence of flavonoids except in n-hexane extract

4.4.1.4 Test for Tannins

a) Ferric chloride Test: A greenish black precipitate was observed which indicates the presence of condensed tannins except in n-hexane extract.

b) *Lead sub-acetate Test:* Black green precipitate was observed except in n-hexane extract. Tannins are present in methanol and ethyl acetate extracts.

4.4.1.5 Test for Alkaloids

i) Drangendoff's reagent: Rose red to brownish precipitates was observed, indicating the presence of alkaloids .except in n-hexane extract.

ii) Meyer's reagent: Creamy precipitates were observed, indicating the presence of alkaloids except in n-hexane..

iii) Wagner's reagent: A reddish-brown precipitates were observed, indicating the presence of alkaloids except in n-hexane extract.

4.4.1.6 Test for Anthraquinones

a) Borntrager's Test: There was no pink red colour. This indicates the absence of anthraquinone glycosides.

b) Modified Borntrager's: There was no pink-red colour. This indicates the absence of combined anthraquinones in all the extracts.

4.*4.1.7 Test for Cardiac Glycosides*

a) Keller-Killiani Test: A brown ring was produced at the interphase. Deoxy sugars characteristic of cardenolides are present in all the extracts.

b) Kedde's Test: There was no purple-blue colour observed. Cardenolides are absent.

Table 4.6 Preliminary Phytochemical Screenings on Extracts of *S. virosa* leaf

Tests	Hexane Extract	Ethyl acetate Extract	Methanol Extract
Saponins			
Frothing	Absent	Present	Present
Haemolysis	Absent	Present	Present
Steroids/Triterpenes			
Salkowski	Present	Present	Present
Lieberman-Burchard	Present	Present	Present
Flavonoids			
Shinoda	Absent	Present	Present
Sodium Hydroxide	Absent	Present	Present
Tannins			
Ferric chloride	Absent	Present	Present
Lead sub acetate	Absent	Present	Present
Alkaloids			
Dragendorff	Absent	Present	Present
Mayer	Absent	Present	Present
Picric acid	Absent	Present	Present
Anthraquinones			
Borntrager	Absent	Absent	Absent
Modified Borntrager	Absent	Absent	Absent
Cardiac glycosides			
Keller-kiliani	Present	Present	Present
Kedde’s	Absent	Absent	Absent

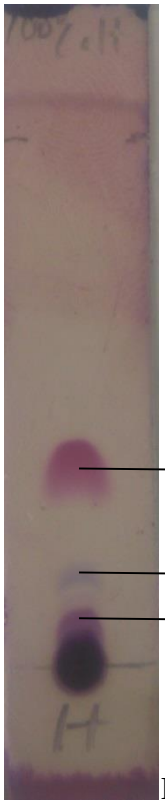
4.4.2 Development of solvent system (fingerprints) and TLC phytochemical screening of the N-hexane, ethylacetate and methanol extracts of the leaf of *S. virosa*

4.4.2.1 Development of solvent system and fingerprints of N-hexane extract (HE) of the leaf of S. virosa

HE was noted to have eleven (11) spots (Plate VII) in hexane: ethyl acetate (9:1), eight (8) spots (Plate VIII) in hexane: ethyl acetate (8:2) and three (3) spots (Plate IX) in hexane (100%) visualized with *p*-Anisaldehyde at 110ºC for 2 minutes.

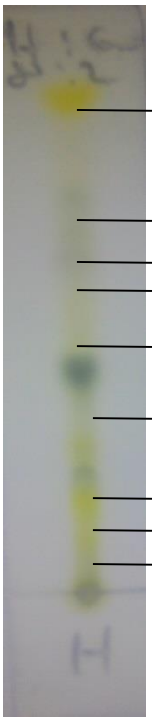


A) Day light

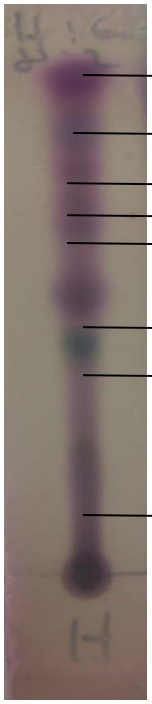


B) *P*-anisaldehyde spray reagent

Plate VII: Chromatogram of N-Hexane extract developed in 100% Hexane in daylight (A) and with spray reagent (B)

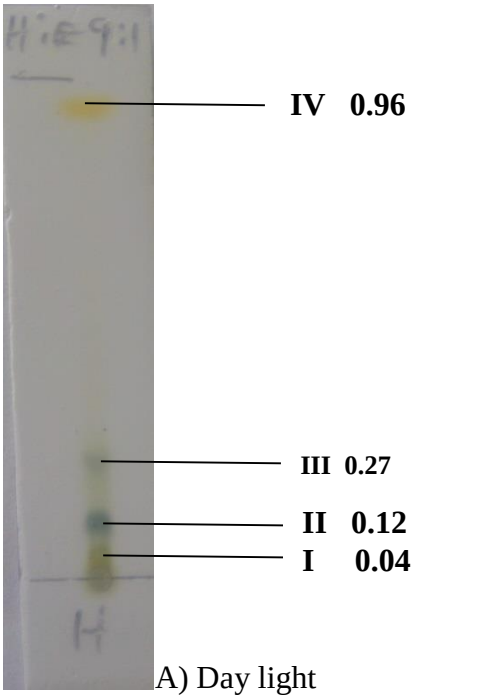


A) Day light

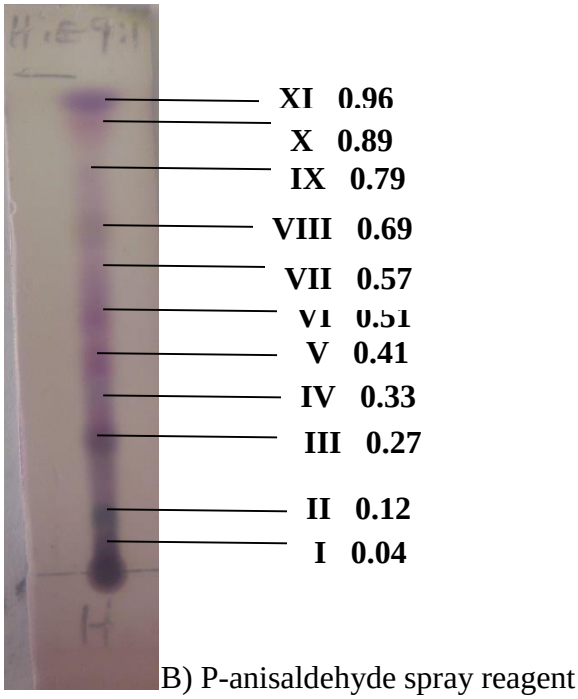


B) P-anisaldehyde Spray reagent

Plate VIII: Chromatogram of N-Hexane extract developed in Hexane: Ethyl acetate (8:2) visualized in daylight (A) and with spray reagent (B)



A) Day light



B) P-anisaldehyde spray reagent

Plate IX: Chromatogram of N-Hexane extract developed in H:E 9:1 day (A) and H:E 9:1 sprayed with P-anisaldehyde (B)

4.4.2.2 TLC Phytochemical screening of N-Hexane extract (HE)

TLC of HE developed in Hexane: Ethyl acetate (9:1) sprayed with Dragendoff (Plate X), Ferric Chloride (Plate XII), Lieberman-Buchard (Plate XV), Iodine vapour (Plate XIV), Borntrager (Plate XI) and Aluminium chloride (Plate XIII) reagents were as presented. The first four were positive results while the last two produced negative results for those phytochemicals tested.



A) Day light



B) Dragendoff's reagent

Plate X: Chromatogram of N-Hexane extract developed in H:E 9:1 day (A) and H:E 9:1 spray with Dragendoff reagent for presence of Alkaloid (B)



A) Day light



B) Borntrager's spray

Plate XI: Chromatogram of N-Hexane extract developed in H:E 9:1 day (A) and H:E 9:1 spray with Borntrager's reagent for presence of Anthraquinones (B)



A) Day light



B) $FeCl_3$

Plate XII: Chromatogram of N-Hexane extract developed in H:E 9:1 day (A) and H:E 9:1 spray with Ferric Chloride reagent for presence of Phenolic compounds (B)



A) Day light

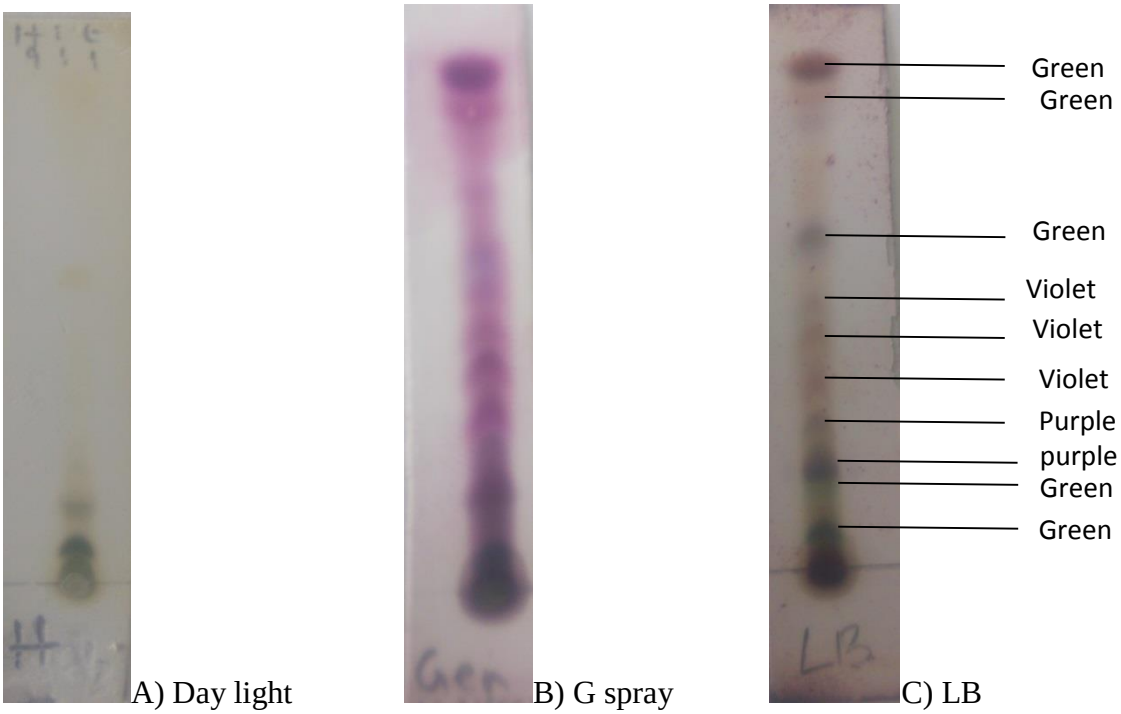


B) UV at 254nm



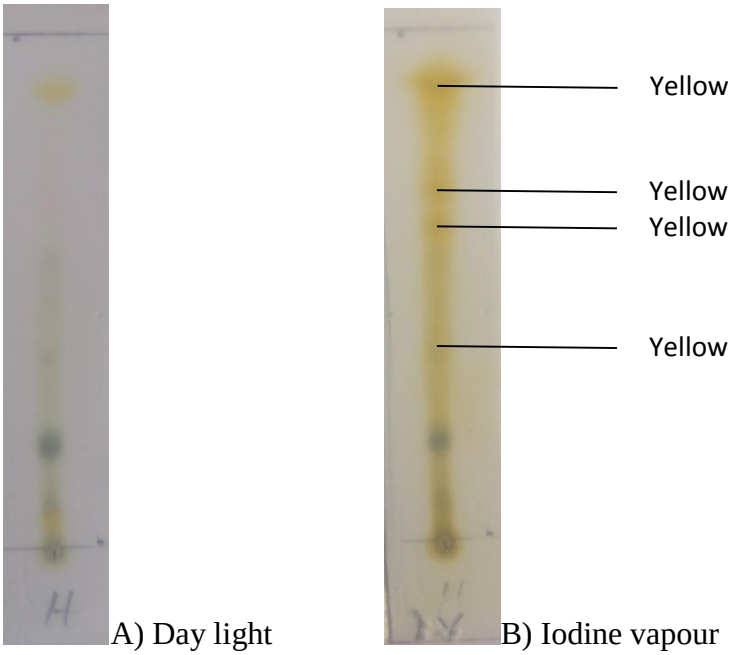
C) UV at 366nm

Plate XIII: Chromatogram of N-Hexane extract developed in H:E 9:1 day (A) and H:E 9:1 spray with Aluminium Chloride reagent viewed at 254nm (B) and 366nm (C) for presence of Flavonoids.



A) Day light B) G spray C) LB

Plate XIV: Chromatogram of N-Hexane extract developed in H:E 9:1 day (A) and H:E 9:1 sprayed with P-anisaldehyde (general spray) reagent (B) and Liberman-Burchard (LB) for presence of Steroids and Triterpenes compound (C)

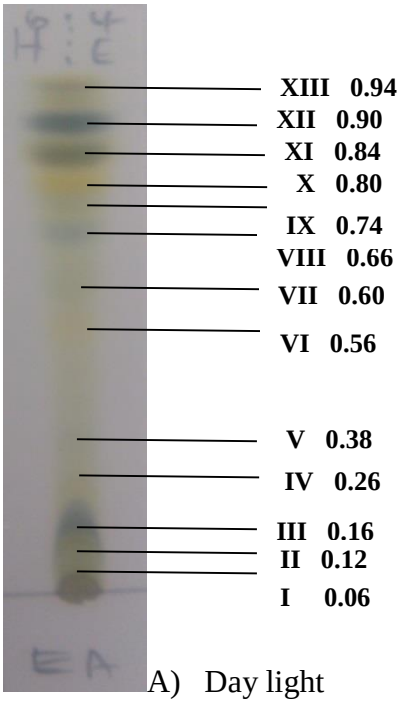


A) Day light B) Iodine vapour

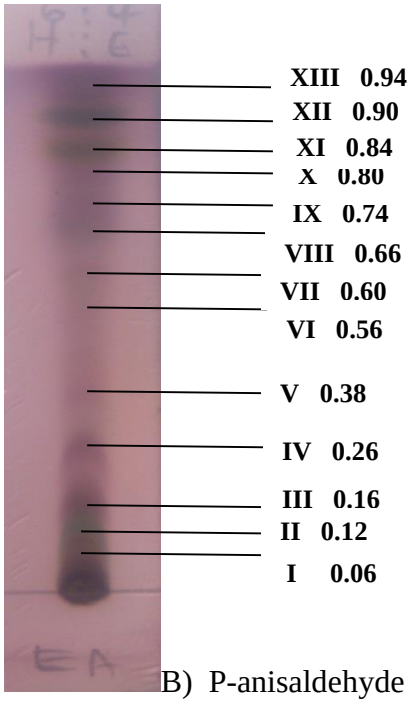
Plate XV: Chromatogram of N-Hexane extract developed in H:E 9:1 day (A) and H:E 9:1 exposed to Iodine vapour for presence of Saponins (B).

4.4.2.3 Development of solvent system for ethylacetate extract (EE) of S. virosa leaf

EE was noted to have fourteen (14) spots (Plate XVIII) in hexane: ethyl acetate (13:7), thirteen (13) spots (Plate XVI) in hexane: ethyl acetate (8: 2) and six (6) spots (Plate XVII) in hexane: ethyl acetate (7:3) visualized with *p*-Anisaldehyde at 110ºC for 2 minutes. Spots of various colours blue, grey, green and violet were revealed. The successful separation of bio-molecules by chromatographic technique depends upon suitable solvent system which needs an ideal range of partition coefficient (k) for each target compounds (Ito, 2005).

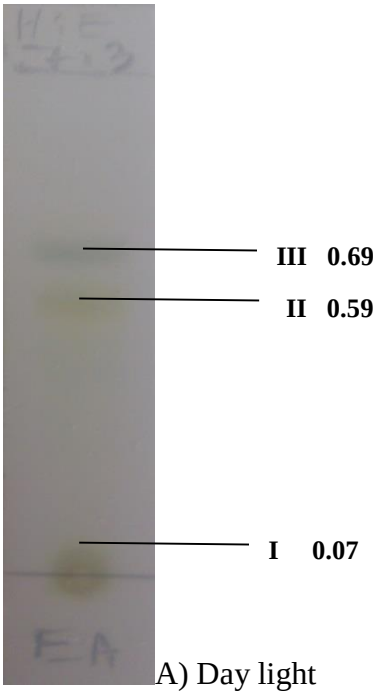


A) Day light

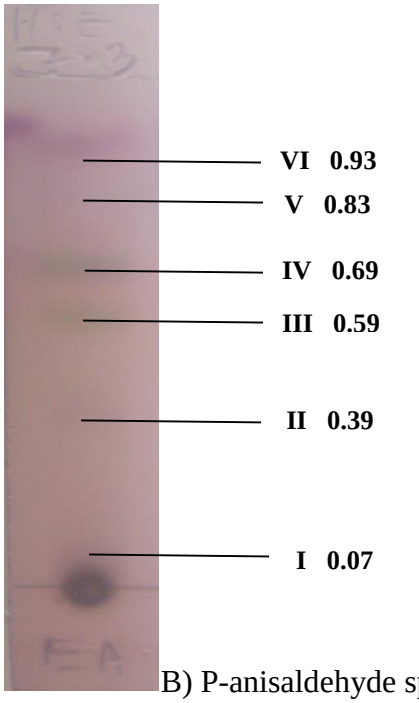


B) P-anisaldehyde spray reagent

Plate XVI: Chromatogram of Ethylacetate extract A and B developed in H:E 8:2, then B sprayed with general spray reagent (B)

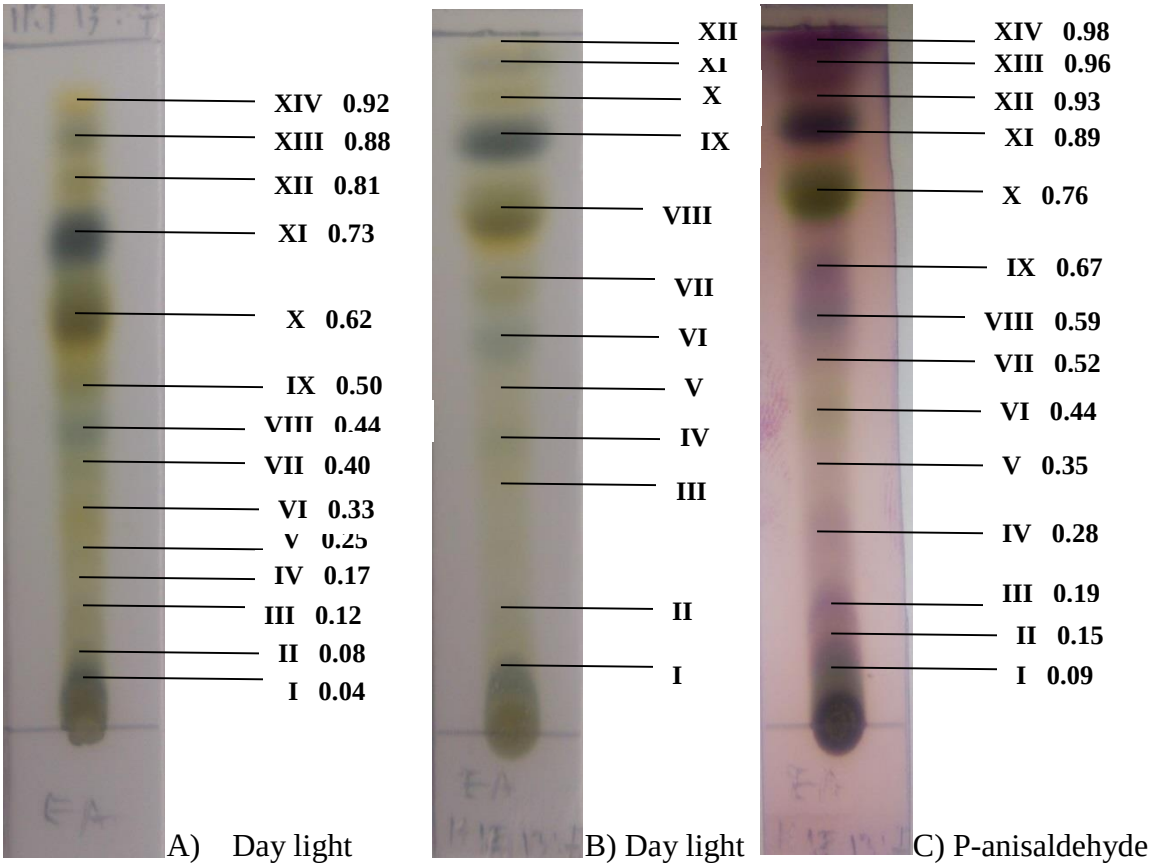


A) Day light



B) P-anisaldehyde spray eagent

Plate XVII: Chromatogram of Ethylacetate extract developed in H:E 7:3 day (A) and H:E 7:3 sprayed with general spray reagent (B).



A) Day light B) Day light C) P-anisaldehyde

Plate XVIII: Chromatogram of Ethylacetate extract developed in H:E 13:7 (A), (B) and (C) followed by (C) sprayed with general spray reagent.

4.4.2.4 TLC phytochemical screening of ethylacetate extract (EE) of S. virosa

TLC of EE developed in Hexane: Ethyl acetate (13:7) sprayed with Ferric Chloride (Plate XXI), Lieberman-Buchard (Plate XXIII), Iodine vapour (Plate XXIV), Dragendoff (Plate XIX), Borntrager (Plate XX) and Aluminium chloride (Plate XXII) reagents. The three (3) reagents gave positive results while the last three (3) produced negative results for the respective phytochemicals tested.



A) Day light



No appearance of orange colour spot

B) Dragendoff's spray

Plate XIX: Chromatogram of *S. virosa* Ethylacetate extract developed in H:E (13:7) sprayed with Dragendoff spray reagent for the detection of Alkaloids.



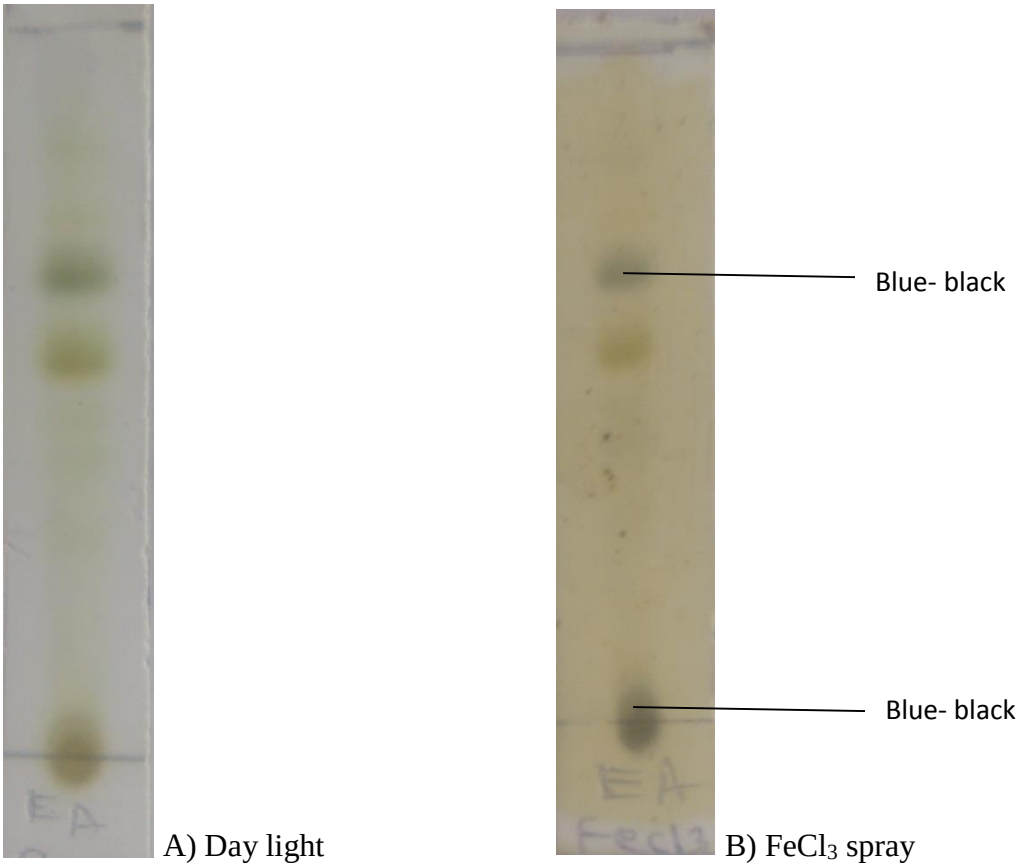
A) Day light



No appearance of yellow colour spot

B) Borntrager's spray

Plate XX: Chromatogram of *S. virosa* Ethylacetate extract developed in H:E (13:7) sprayed with Borntrager's spray reagent for the detection Anthraquinones.



A) Day light B) $FeCl_3$ spray

Plate XXI: Chromatogram of *S. virosa* Ethylacetate extract developed in H:E (13:7) sprayed with FeCl3 spray reagent for the detection of Phenolic commpounds.



A) Day light



No Yellow fluorescence

B) UV at 254nm



No Yellow fluorescence

C) UV at 366nm

Plate XXII: Chromatograms of Ethylacete extract of *S.virosa* leaf developed in H:E (13:7) sprayed with Aluminium Chloride ($AlCl_3$) and observed under UV at 254nm (B) and 366nm (C) for presence of Flavonoids



A) day light



B) LB Spray

Plate XXIII: Chromatogram of Ethylacetate extract developed in H:E 13:7 (A) and H:E 13:7 sprayed with Liberman-Burchard (LB) for presence of Steroids and Triterpenes (B)



A) Day light



B) Iodine vapour

Plate XXIV: Chromatogram of Ethylacetate extract developed in H:E (13:7) day light (A) and H:E (13:7), then exposed to Iodine vapour for presence of Saponins (B).

4.4.2.5 Development of solvent system for methanol extract (ME) of S. virosa leaf

ME was observed to have eleven (11) spots (Plate XXVII) in butanol: acetic acid: water (10:1:1) five (5) spots (plate XXVI) in Ethylacetate: methanol: water (10:3:1) and three (3) spots (Plate XXV) in Chloroform: Methanol (8:2), visualized with *p*-Anisaldehyde and heated at 110ºC for 2 minutes.



A) Day light

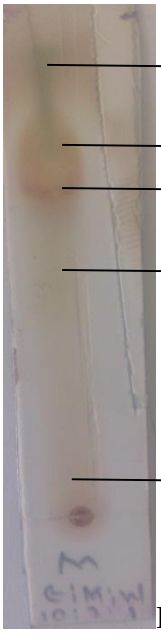


B) P-anisaldehyde spray

Plate XXV: Chromatograms of Methanol extract A and B developed in Chloroform : Methanol 8:2, then B sprayed with general spray reagent (B)

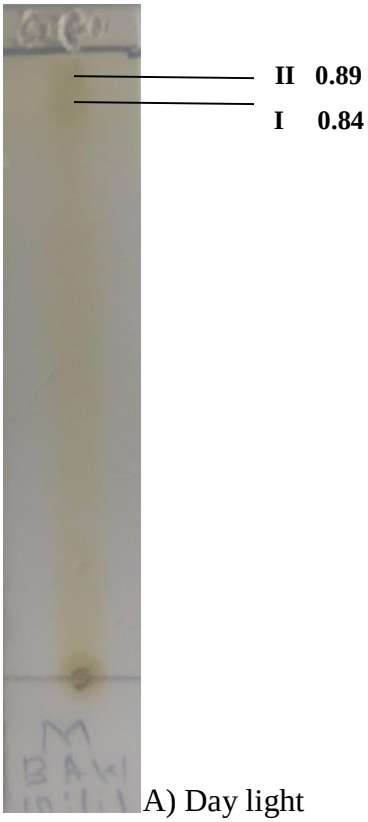


A) Day light

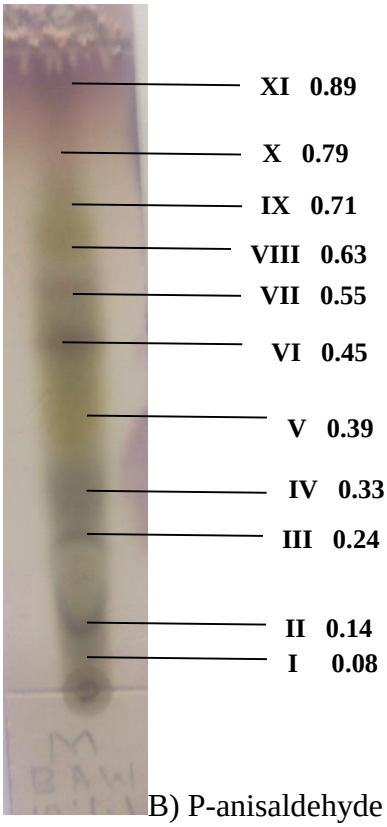


B) General spray

Plate XXVI: Chromatograms of Methanol extract A and B developed in Ethylacetate : Methanol : Water 10:3:1, then B sprayed with general spray reagent (B)



A) Day light



B) P-anisaldehyde spray

Plate XXVII: Chromatogram of Methanol extract A and B developed in N-butanol: Acetic acid: Water 10:1:1, then B sprayed with general spray reagent (B)

4.4.2.6 Phytochemical screening of methanol extract of S. virosa leaf

TLC of ME developed in butanol: acetic acid: water (10:1:1) sprayed with Dragendoff (Plate XXVIII), Ferric Chloride (Plate XXX), Lieberman-Buchard (Plate XXXII), Iodine vapour (Plate XXXIII), Borntrager (Plate XXIX) and Aluminium chloride (Plate XXXI) reagents. The first four spray reagents gave positive results while the last two spray reagents produced negative results for those phytochemicals tested.



A) Day light



B) Dragendoff's spray reagent

Plate XXVIII: Chromatogram of Methanol extract developed in B:A:W 10:1:1 (A) and B:A:W 10:1:1 sprayed with Dragendoff's reagent for presence of Alkaloid (B)



A) Day light



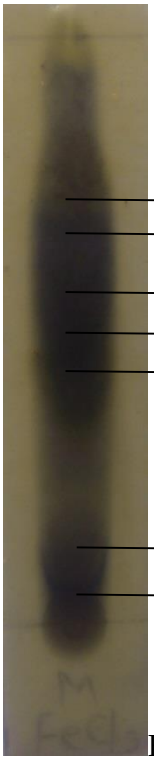
No appearance of yellow colour

B) Borntrager's spray reagent

Plate XXIX: Chromatogram of Methanol extract developed in B:A:W 10:1:1 (A) and B:A:W 10:1:1 sprayed with Borntrager's reagent for presence of Anthraquinones (B)



A) Day light

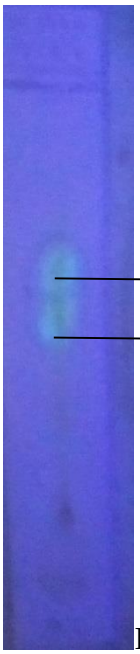


B) $FeCl_3$

Plate XXX: Chromatogram of Methanol extract developed in B:A:W 10:1:1 (A) and B:A:W 10:1:1 sprayed with Ferric Chloride spray s reagent for presence of Phenols (B)



A) AlCl3 day



B) UV at 254nm

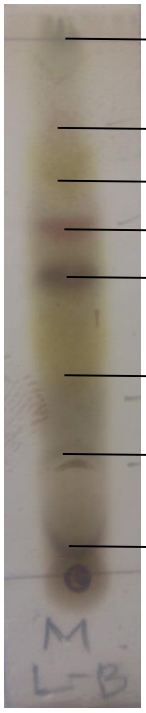


C) UV at 366nm

Plate XXXI: : Chromatogram of Methanol extract developed in B:A:W 10:1:1 (A) and B:A:W 10:1:1 sprayed with Aluminium Chloride reagent viewed at 254nm (B) and 366nm (C) for presence of Flavonoids.


A) Day light


B) LB before heating



C) LB after heating

Plate XXXII: Chromatogram of Methanol extract developed in B:A:W 10:1:1 (A, B and C), then B and C sprayed with Liberman-Burchard reagent followed by heating (C) at 110^{c}C over 2minutes for presence of Steroids (Green) and/or Triterpenes (Violet).


A) Day light



B) Iodine Vapour

Plate XXXIII: Chromatogram of Ethylacetate extract developed in B:A:W 10:1:1 (A) and B:A:W 10:1:1, then exposed to Iodine vapour for presence of Saponins (B).

4.4.2.7 Summary of the TLC phytochemical profiles/fingerprints and TLC phytochemical screening of the n-hexane, ethylacetate and methanol extracts of S. virosa leaf

The TLC fingerprints of the three extracts showed various phytochemical separation profiles of quality control significance (Table 4.7 – 4.9).

Phenols, saponins and steroids/triterpenes were present but flavonoids and anthraquinones were absent in all the three leaf extracts of *S. virosa*. Alkaloids were absent in only the ethylacetate extract (Table 4.10).

Table 4.7: Summary of TLC Profile of Hexane Extract (HE) of *S. virosa* leaf spray with *p*-Anisaldehyde

Extract	Solvent System	No. of Spots	Colour of Spots	R_f values
Hexane	Hexane 100 %	3	purple	0.35
			Green	0.33
			Purple	0.07
	Hexane:Ethyl acetate (8:2)	8	violet	0.96
			Green	0.83
			violet	0.77
			Violet	0.69
			violet	0.65
			Violet	0.52
			Green	0.44
			Purple	0.20
	Hexane:Ethyl acetate (9:1)	11	violet	0.96
			Violet	0.89
			Green	0.79
			Green	0.69
			Violet	0.57
			Violet	0.51
			Violet	0.41
			Grey	0.33
			Violet	0.27
			Green	0.12
			Purple	0.04

Table 4.8: Summary of TLC Profile of Ethyl acetate Extract (EE) of *S. virosa* leaf sprayed with *p*-Anisaldehyde

Extract	Solvent System	No. of Spots	Colour of Spots	R_f values
Ethyl acetate	Hexane:Ethyl acetate (8:2)	13	Yellow	0.94
			Violet	0.90
			Yellow	0.84
			Violet	0.80
			Green	0.74
			Yellow	0.66
			Violet	0.60
			Green	0.56
			Yellow	0.38
			Yellow	0.26
			Green	0.16
			Green	0.12
			Green	0.06
	Hexane: Ethyl acetate (7:3)	6	Green	0.93
			Yellow	0.83
			Green	0.68
			Yellow	0.59
			Green	0.39
			Yellow	0.07
	Hexane: Ethyl acetate (13:7)	14	Yellow	0.98
			Violet	0.96
			Yellow	0.93
			Violet	0.89
			Green	0.76
			Yellow	0.67

	Violet	0.59
	Green	0.52
	Yellow	0.44
	Yellow	0.39
	Green	0.28
	Green	0.19
	Green	0.15
	Green	0.09

Table 4.9: Summary of TLC Profile of Methanol Extract (ME) of *S. virosa* leaf sprayed with *p*-Anisaldehyde

Extract	Solvent System	No. of Spots	Colour of Spots	R_f values
Methanol	Chloroform: Methanol (8:2)	3	Green	0.96
			Light green	0.62
			Grey	0.29
	Ethyl acetate: Methanol: Water (10:3:1)	5	Green	0.90
			Light green	0.84
			Purple	0.74
			Light green	0.58
			Pale purple	0.08
	Butanol: Acetic acid: Water (10:1:1)	11	Violet	0.89
			Violet	0.79
			Green	0.71
			Green	0.63
			Violet	0.55
			Violet	0.45
			Light green	0.39
			Green	0.33
			Green	0.24
			Green	0.14
			Green	0.08

Table 4.10 TLC Phytochemical Screening of Extracts of *S. virosa* Leaves

Secondary Metabolites	Chromogenic Agents	N-Hexane Extract*	Ethylacetate Extract**	Methanol Extract***
Alkaloids	Dragendoff	Present	Absent	Present
Anthraquinones	Borntrager	Absent	Absent	Absent
Flavonoids	Aluminium Chloride			
	At 254nm	Absent	Absent	Absent
	366nm	Absent	Absent	Absent
Phenols	Ferric Chloride	Present	Present	Present
Saponins	Iodine Vapour	Present	Present	Present
Steroids/ Triterpenes	Liberman Buchard	Present	Present	Present

Solvent systems; *H:E 9:1 **H:E 13:7 ***B:A;W 10:1:1

4.5 *In vitro* Antivenin Activity of *S. virosa* Extracts against *Naja nigricollis* Snake Venom Phospholipase A_2 (PLA_2)

The enzyme inhibition pattern was observed to be non-competitive (figure 4.1) and the nature of the interaction (i.e. KI or dissociation constant) between enzyme, methanol extract and the substrate was determined to be weak (Figure 4.2).

Table 4.11: Inhibitory Properties of Methanol Leaf Extract of *S. virosa*

Substrate (egg yolk) concentration 1/S mg/ml	Inhibitor (S. virosa extracts) Concentration			
	0mg/ml	2.5mg/ml	5mg/ml	10mg/ml
0.010	0.188	0.250	0.450	0.300
0.013	0.378	0.281	0.463	0.346
0.020	0.332	0.500	0.375	0.375
0.040	0.450	0.563	0.500	0.750

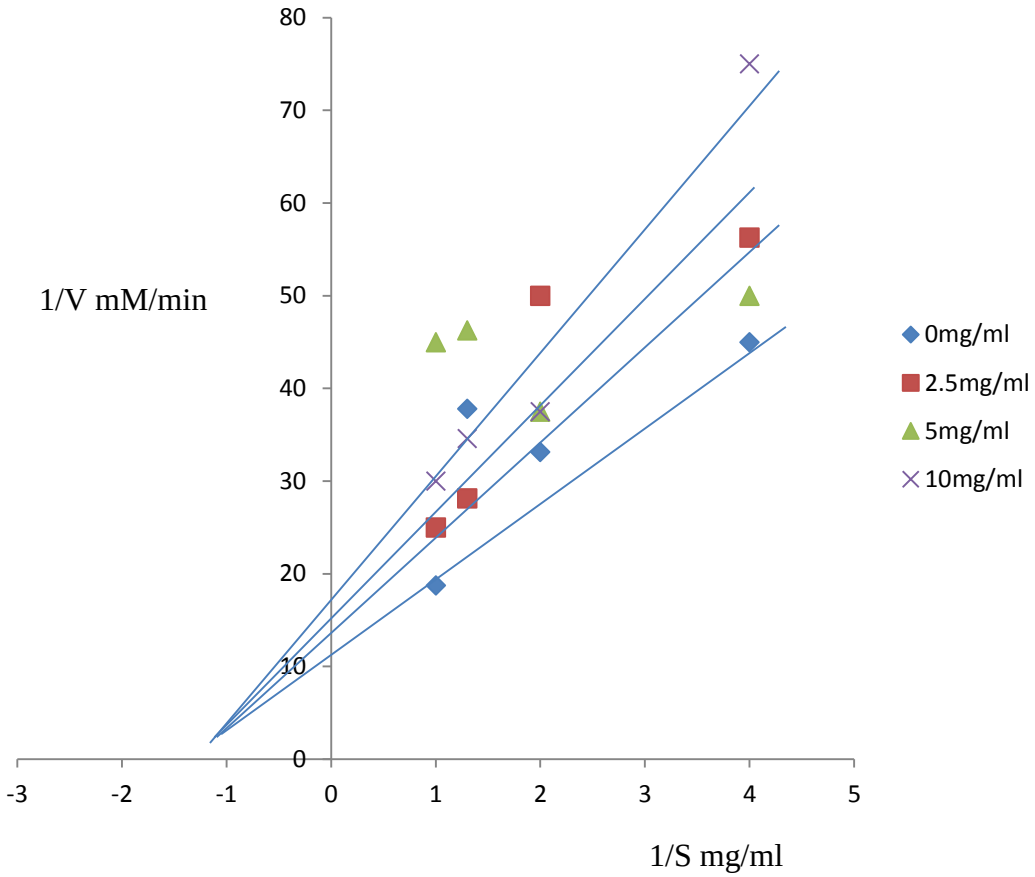


Figure 4.1: Methanol Leaf Extract of *S.virosa* Activity against Phospholipase A_2 Enzyme of *Naja nigricolis* Venom

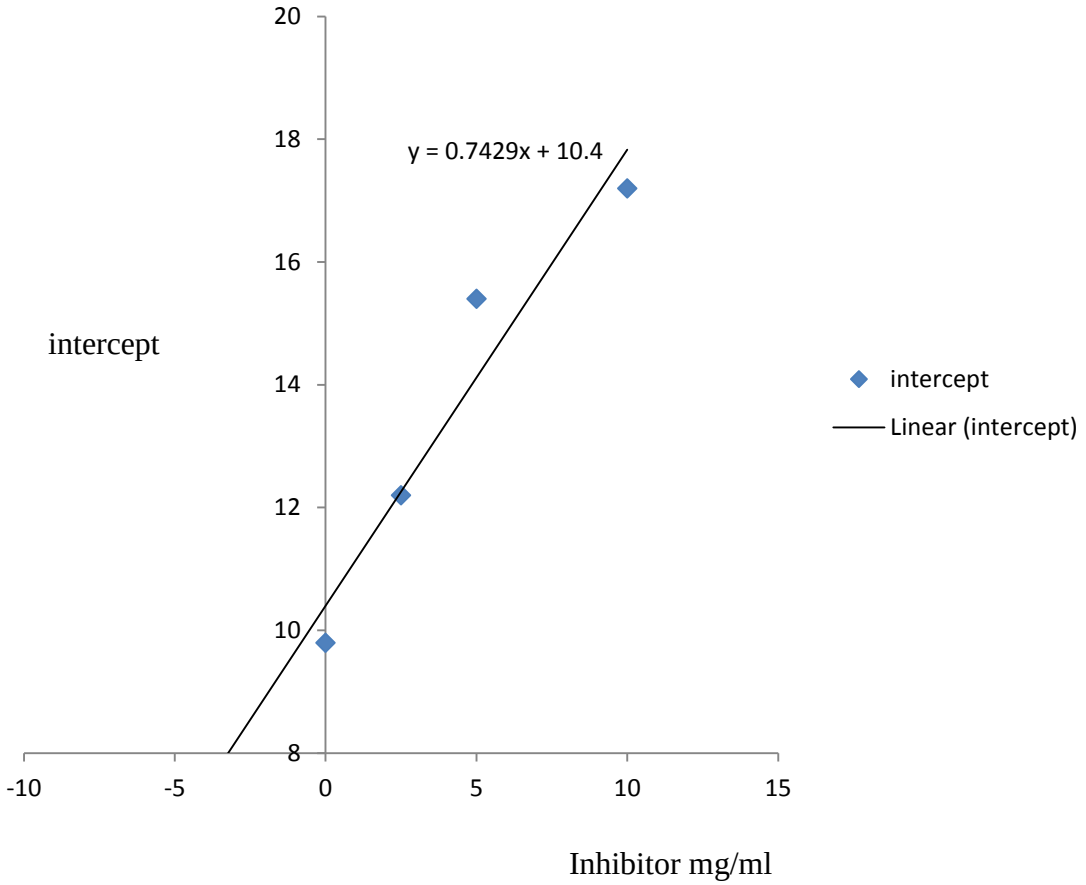


Figure 4.2: Dixon's plot (a secondary plot) that gives Inhibition Binding Constant (KI) of the Methanol Extract on the Activity of Phospholipase A_2

4.6 Modulatory effect of *S. virosa* Leaf Extracts on *Naja nigricollis* Snake Venom

4.6.1 *in vivo* venom neutralization effect of leaf extracts of *S. virosa* against venom of *Naja nigracolis* using mice

4.6.1.1 Acute toxicity study (Median Lethal Dose) of S. virosa leaf extract

The LD_{50} of N-hexane extract of *S.virosa* was found to be 774.60 mg/kg and that of Ethylacetate extract was found to be greater than 5000 mg/kg while that of methanol extract was found to be 1265 mg/kg.

4.6.1.2 Assessment of in vivo venom neutralization of S. virosa extract

The n-hexane leaf extract produced greater protection against venom induced lethal effect than both methanol and ethylacetate leaf extracts of *S. virosa* given I.P. in mice (Figure 4.3). The protections produced by n-hexane and methanol leaf extracts were significant at 20 mg/kg dose in a mixture of half LD_{99} of *Naja nigricollis* venom.

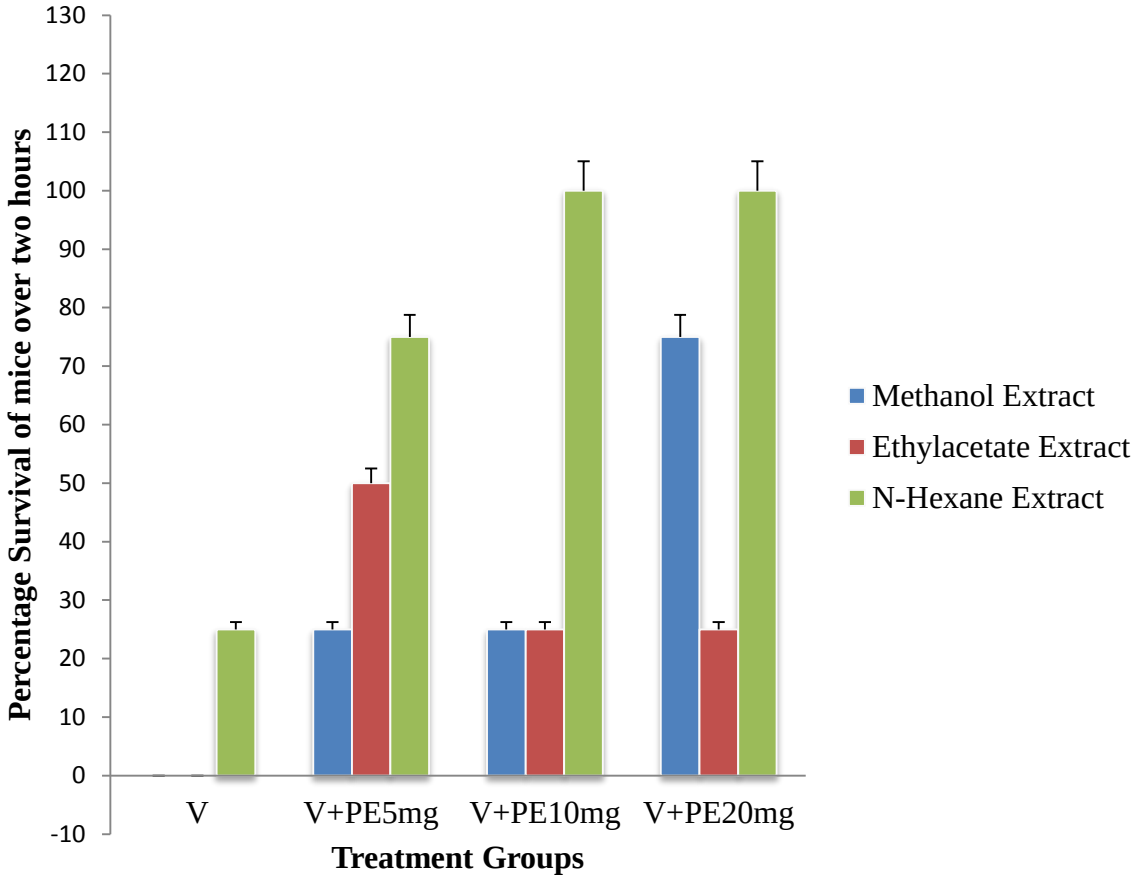


Figure 4.3: Percentage Survival for the treatments of mice with *N. nigricollis* venom and Mixtures of *N. nigricollis* Venom + Leaf Extracts of *S. virosa*.

Table 4.12: *In vivo* Response to Administration (I.P.) of Incubated Venom alone and Venom with *S. virosa* Methanol Extract in Mice

Treatment	Mortality ratio	% Survival in /2hours	Survival in 24hours
LD_{99}	4/4	0	0
LD_{99} + M5mg	3/4	25	0
LD_{99} + M10mg	3/4	25	0
LD_{99} + M20mg	1/4	75*	0

* = significant at $p < 0.05$

Table 4.13: *In vivo* Response to Administration (I.P.) of Incubated Venom alone and Venom with *S. virosa* Ethylacetate Extract in Mice

Treatment	Mortality ratio	% Survival in 2hours	% Survival %/24hours
LD_{99}	4/4	0	0
LD_{99} + E5mg	2/4	25	0
LD_{99} + E10mg	3/4`	25	0
LD_{99} + E20mg	3/4	25	25

* = significant at $p < 0.05$

Table-4.14: *In vivo* Response to Administration (I.P.) of Incubated Venom alone and Venom with *S. virosa* N-Hexane Extract in Mice

Treatment	Mortality ratio	% Survival /2hrs	% Survival in /24hrs
LD_{99}	3/4	25	25
LD_{99} + E5mg	1/4	75	0
LD_{99} + E10mg	0/4	100	25
LD_{99} + E20mg	0/4	100*	50

* = significant at $p < 0.05$

Table 4.15: Effects of Administration of *Naja nigricollis* Venom and Mixture of Venom with *S.virosa* Extracts in Mice.

Group	Survival time on mice in minutes		
	Methanol	Ethylacetate	N-Hexane
V	54.50 ± 7.90	81.00 ± 12.89	17.50 ± 13.46
V+PE5 mg	107.75 ± 18.22	99.00 ± 21.20	211.75 ± 105.04
V+PE1 0mg	71.00 ± 27.79	91.25 ± 23.64	401.50 ± 150.87
V+PE20 mg	147.25 ± 17.89*	74.00 ± 14.53	` 458.50 ± 150.87*

Results are presented as mean ± standard error of mean (SEM) *= significant at $p < 0.05$

V = venom, PE = plant extract

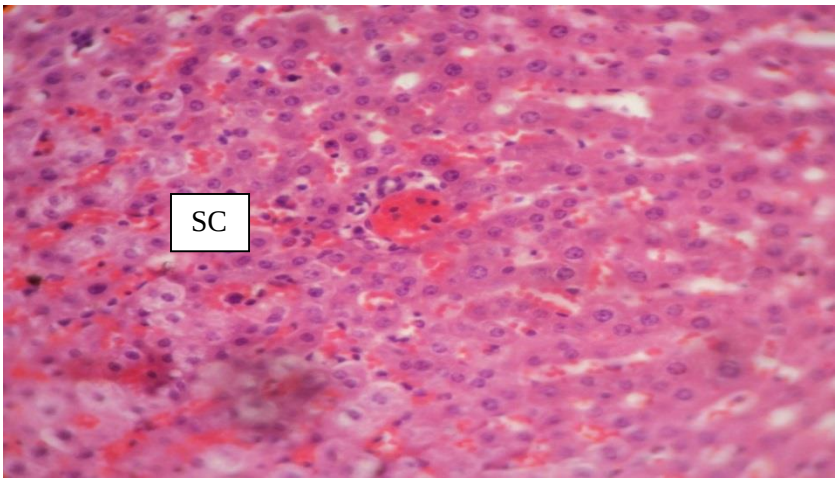
4.6.1.3 Histopathological effects of S. virosa venom-extracts mixtures treatment in mice

The histopathology of brain, heart, lungs, kidney and liver for N-hexane shows almost normal architecture of the above organs. This indicates a greater protection to the organ damage by venom of *Naja nigricollis.*

There were a normal architecture of liver, heart, kidney, lungs and brain in the control (untreated mice). The mixture of venom-methanol leaf extract treated mice shows normal heart and brain tissues, but, slight lymphocyte hyperplasia (LH) and alveoli congestion (AC) were noted in kidney and lung tissues respectively. A moderate LH and vascular congestion (VC) were observed in the liver. A mixture of venom-ethylacetate leaf extract produced normal heart, slight vacoulation in brain and slight vacoulation and necrosis in the liver were observed. However, moderate alveoli congestion in the lungs was noted. A mixture of venom-n hexane leaf extract treated mice produced normal heart, brain and lungs with slight necrosis and LH were observed in kidney and liver respectively.

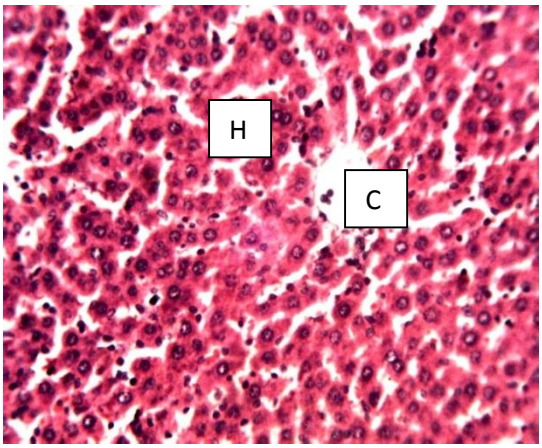
A. Liver: Control and mixture of venom- extracts (Methanol, Ethylacetate, n-Hexane) treatment groups' photomicrographs H&E

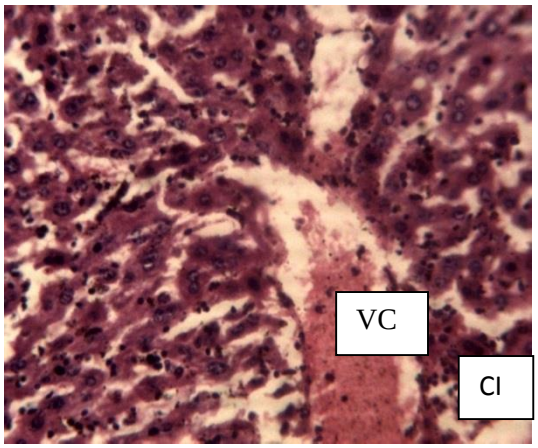
Venom of *naja nigricollis* (positive control)



Liver showing sinusoidal congestion (SC)

Normal saline **Methanol extract**

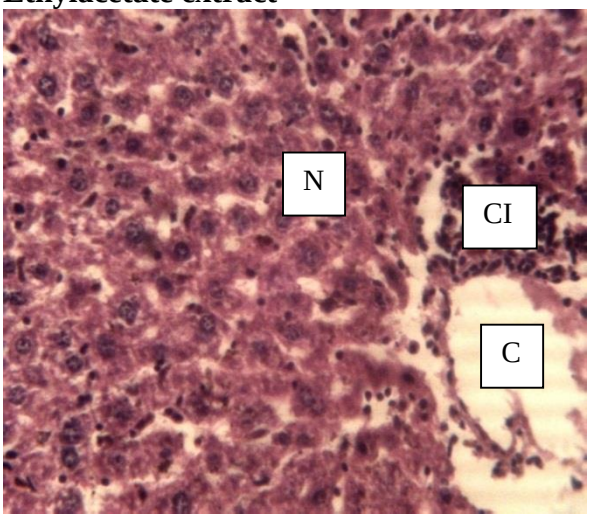




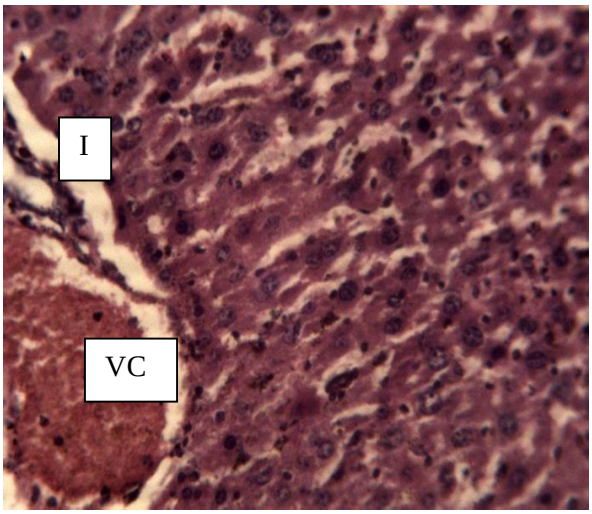
i. Liver of mice showing normal hepatocyte (H) Central vein (C) X 400

ii. Liver shows moderate vascular congestion (VC) and cellular infiltration (CI) X 400.

Ethylacetate extract **N-hexane extract**



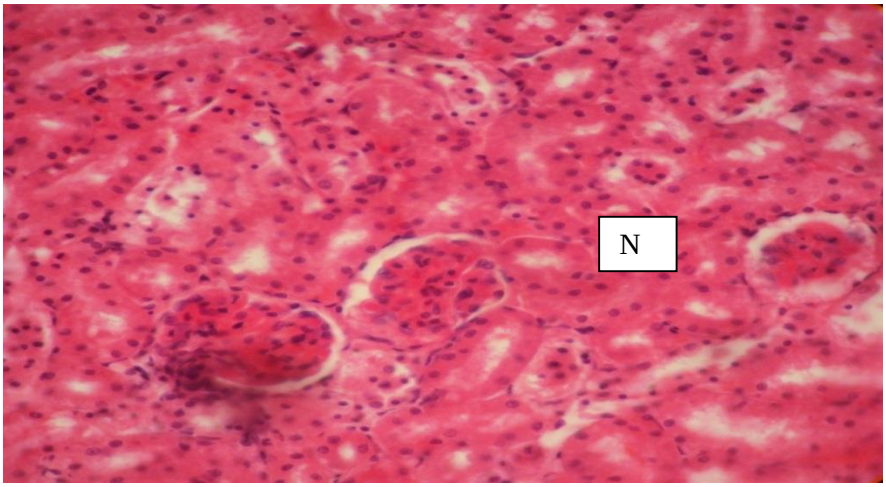
iii. Liver of mice showing necrosis (N) central vein (C) and cellular infiltration (CI) X 400



iv. Liver of mice showing vascular congestion (VC) and mild inflammation (I) X 400

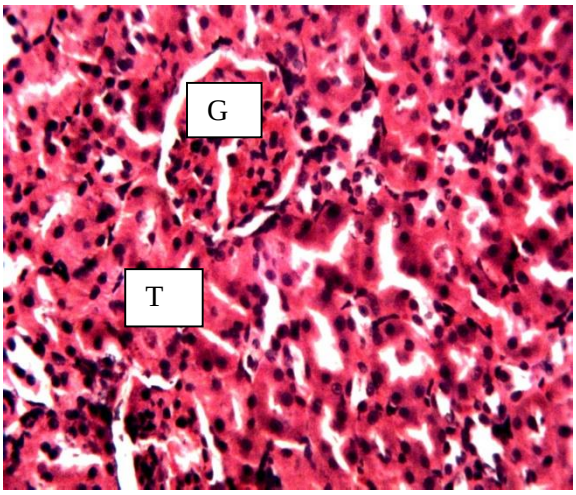
B. Kidney: Control and mixture of venom- extracts (Methanol, Ethylacetate, n-hexane) treatment groups' photomicrographs in H&E

Venom of *naja nigricollis* (positive control)



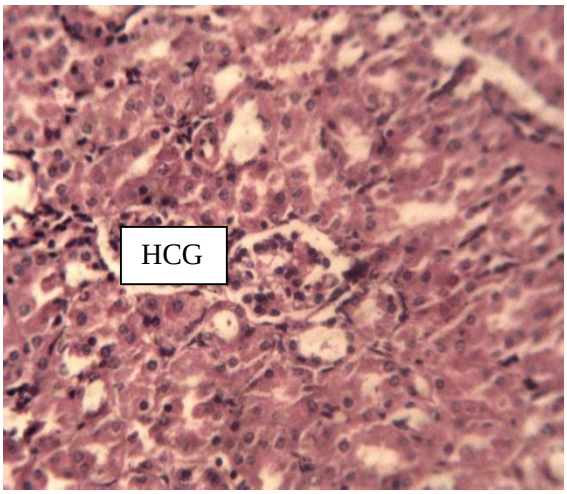
Kidney showing Necrosis (N) with intact glomeruli

Normal saline



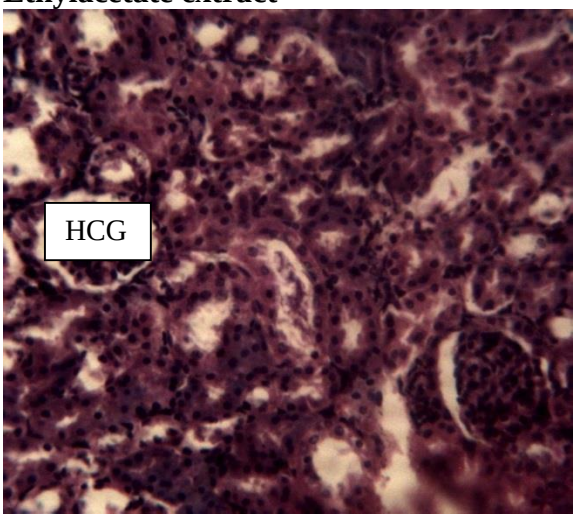
i. Kidney shows normal glomerulus (G) and tubules (T) X 400

Methanol extract



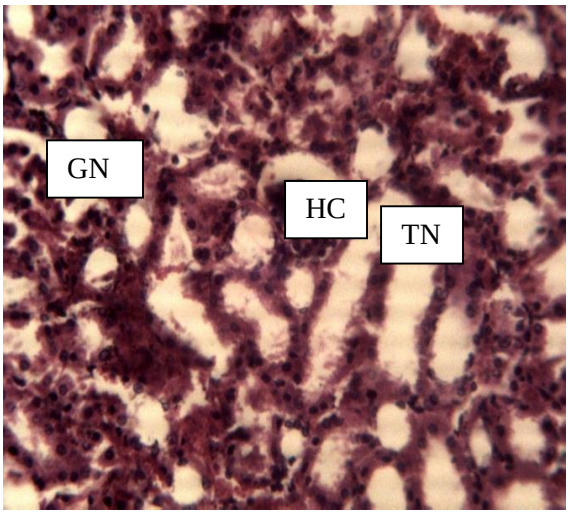
ii. Kidney of mice showing mild hyper cellularity of glomerulus (HCG) X 400

Ethylacetate extract



iii. Kidney of mice showing severe hyper cellularity of glomerulus (HCG) X 400

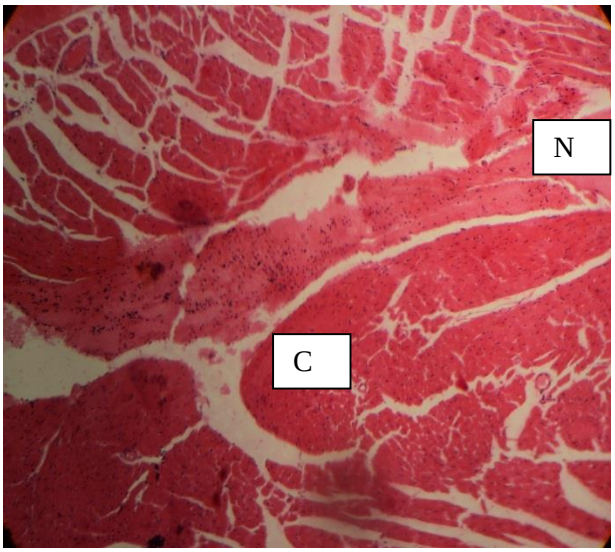
N-hexane extract



iv. Kidney shows slight tubular necrosis (TN) slight glomerular necrosis(GN) and slight hyper cellularity (HC).X 400

C Heart: Control and mixture of venom- extracts (Methanol, Ethylacetate, n-hexane) treatment groups' photomicrographs H&E

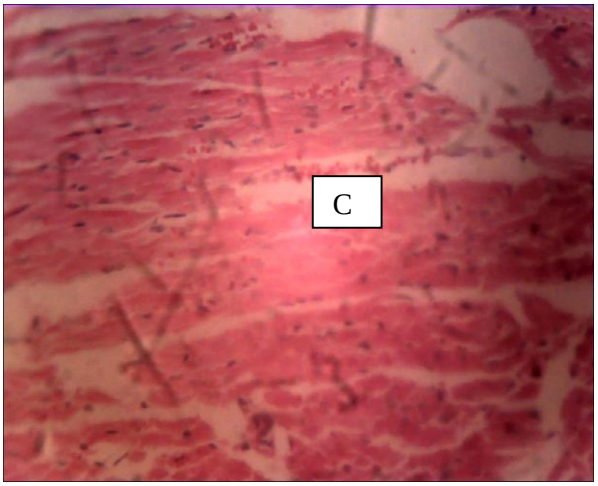
Venom of *naja nigricollis* (positive control)



Heart showing necrosis (N) and congestion (C)

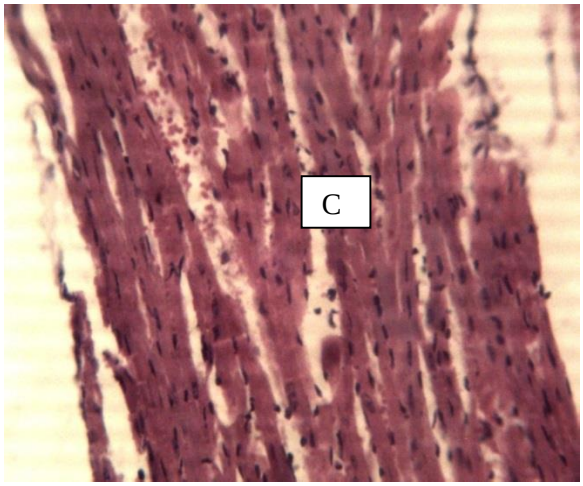
X 400

Normal saline



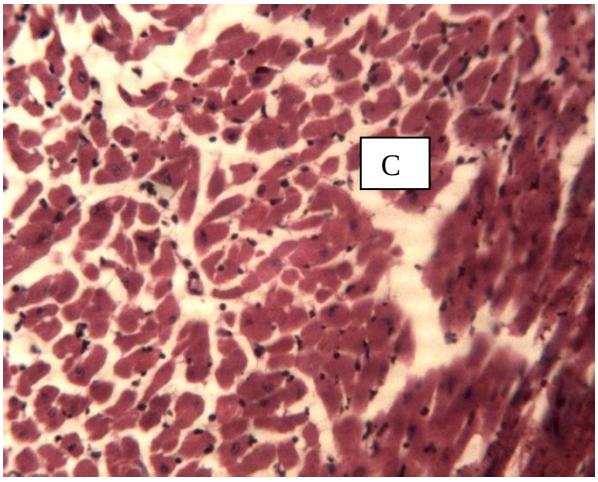
i. Heart shows normal cardiomyocytes (C)
X 400

Methanol extract



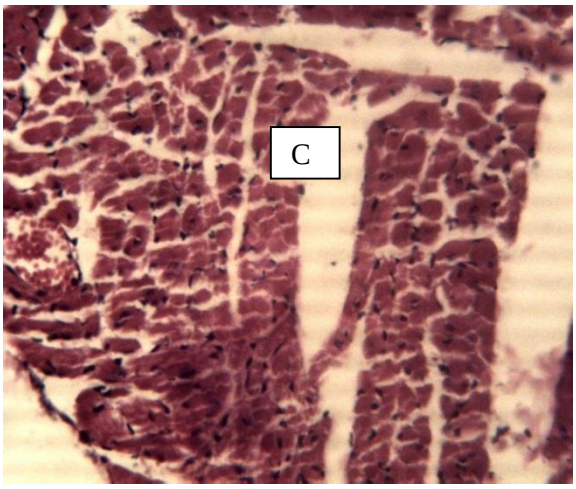
ii. Heart shows normal cardiomyocytes (C)
X 400

Ethylacetate extract



iii. Heart shows normal cardiomyocytes (C) X 400

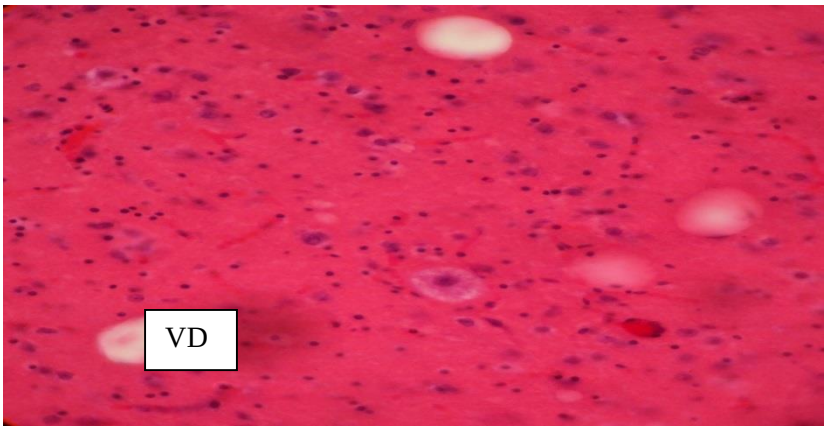
N-hexane extract



iv. Heart shows normal cardiomyocytes (C) X 400

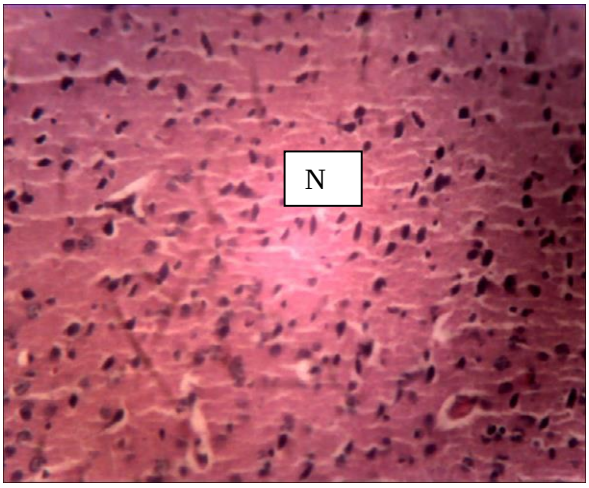
D. Brain: Control and mixture of venom- extracts (Methanol, Ethylacetate, n-hexane) treatment groups' photomicrographs H&E

Venom of *naja nigricollis* (positive control)



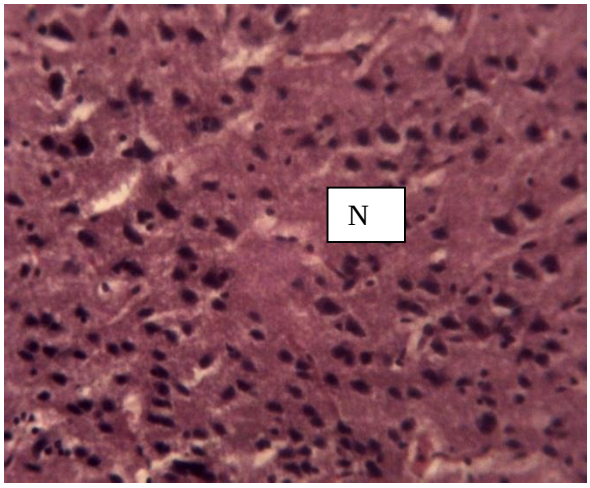
Brain showing vacoular (VD) degeneration X 400

Normal saline (negative control)



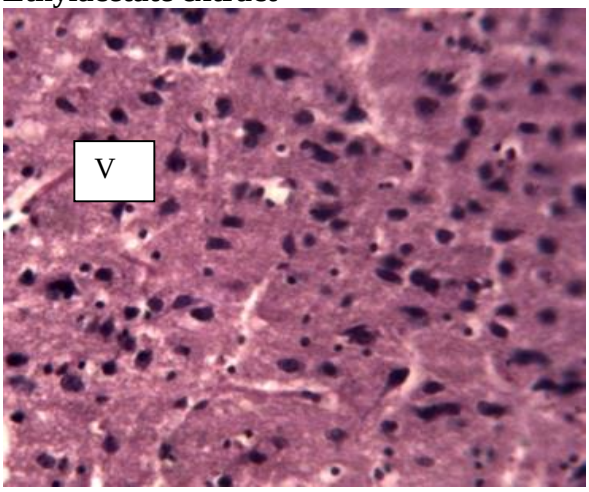
i. Brain shows normal nervous tissue (N) X 400

Methanol extract



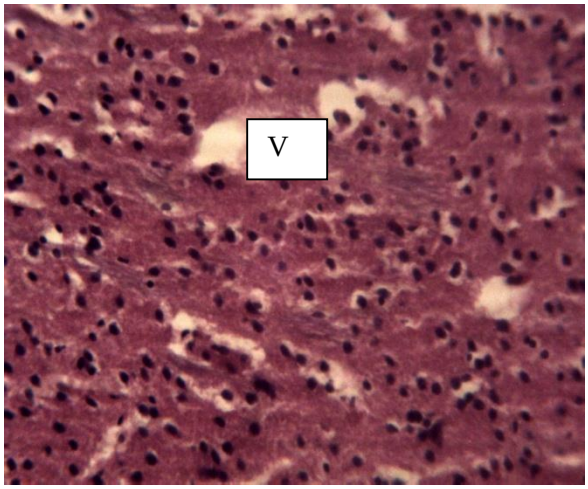
ii. Brain shows normal nervous tissue X 400

Ethylacetate extract



iii. Brain shows slight vacoulation (V) X 400

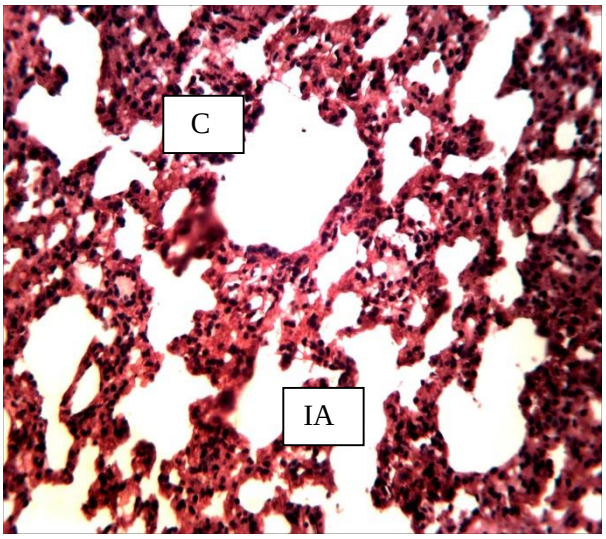
N-hexane extract



iv. Brain showing moderate vacoulation (V) X 400

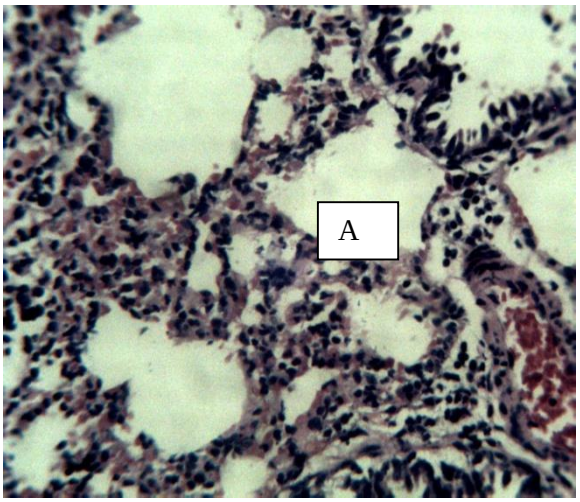
E. Lungs: Control and mixture of venom- extracts (Methanol, Ethylacetate, n-hexane) treatment groups' photomicrographs. H & E.

Venom of *naja nigricollis* (positive control)



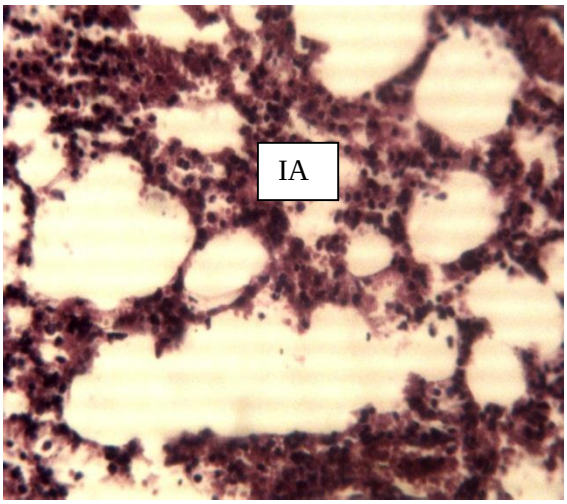
Lungs showing severe thickening and congestion (C) of the inter alveolar spaces (IA) X 400

Normal saline



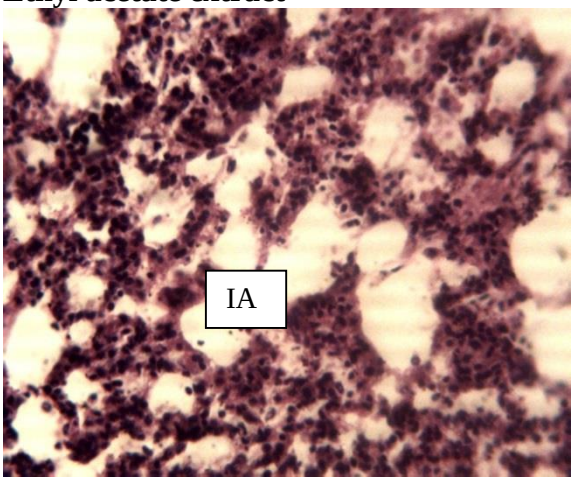
i. Lung showing normal alveoli (A), X 400

Methanol extract



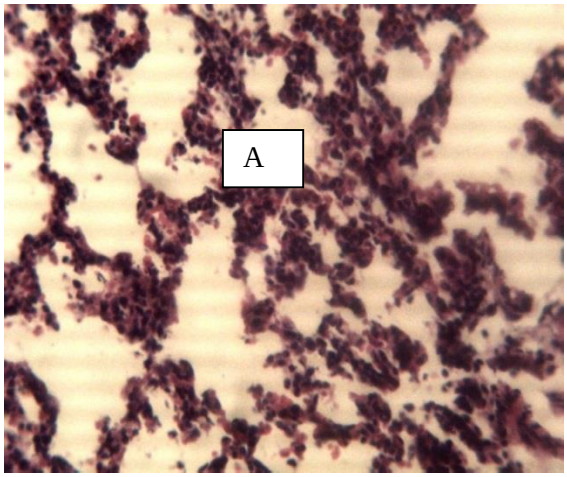
ii. Lung showing slight thickening of the inter alveolar spaces (IA) X 400

Ethyl acetate extract



iii. Lung showing slight thickening of the inter alveolar spaces (IA) X 400

N-hexane extract



iv. Lung showing normal alveolar feature X 400.

Plate XXXIV: effect of *S. virosa* extracts on various organs of mice (i.e. control and treatment groups).

CHAPTER FIVE

5.0 DISCUSSION

Approximately, about 400,000 vascular plant species, 370,000 flowering plant species and 18,000 medicinal plant species are known to science according to global plants report (Kew 2016). Africa makes a little contribution to the list, with Australia, Brazil and China being the top contributors (Paton *et al*., 2008, Kew, 2016). This shows that only 4.5% of the world plants populations have documented medicinal uses. As a result of social, economic and pressing burden of chronic diseases, herbal medicines are gaining more popularity in the global market (WHO, 2013). In Africa, India and China more than 80% of the populations rely on traditional medicine to cater for their primary health care of which herbs from plants play a major role (WHO, 2010). The World Health Organization (WHO) has therefore encouraged interaction between Western-based and indigenous-based medicines with a view to exploit and identifies compounds that could provide safe and effective remedies for ailments of both microbial and non-microbial origins (WHO, 2013).

To validate the safety and efficacy of traditional claim of medicinal plants, acute and or chronic toxicity, *in vitro* and *in vivo* studies are the mainstay to conduct. The present study was aimed at validating the traditional claim for the use of *Securinega virosa* in the traditional management of snakebite and to provide pharmacognostic standards of the leaves for quality control.

Leaf epidermis has been reported to provide important diagnostic characters, and together with characters of stomata are considered to be of first significance in the microscopical identification of leaves (Evans, 2009).

Anatomical section of the leaf of *S. virosa* revealed the presence of epidermal cells which are polygonal with straight anticlinal wall as seen in *Pilocarpus Jaborandi* (Evans, 2009). Scanty anomocytic and paracytic stomata were present only on the abaxial surface. This is in harmony with the findings in India by Thakur and Patil (2014) of which 17 species from 17 Genera of Euphorbiaceae including *S. virosa* which showed the predominance of anomocytic stomata but rarely anisocytic or paracytic. The predominance of anomocytic stomata and hypostomatic nature of the stomata were noticed in only four (4) out of the seventeen (17) studied plants.

The presence of unicellular trichomes, though rare differs with the findings of Thakur and Patil (2014) which reported the absence of trichomes on the foliar surface of *S. virosa* found in Indian specie studied but in other species of the family such as *Neoscortechinia kingii* Hook. f. The abundance of prism crystals of calcium oxalate in a sheaths or singles of mean size 2µm was noticed on both adaxial and abaxial surfaces. Earlier studies of the Euphorbiaceae had revealed the significance of anatomic characters for identification and classification throughout the family taxa (Webster, 1994) and for checking adulteration (Ghani, 1990).

A transverse section of the leaf through the midrib shows the presence of a clear vascular bundle; xylem and phloem typical of a dicot plant. The stomatal index determined which is 12.67 is nearly closer to the stomatal index of 12.00 reported by Thakur and Patil (2014).

The physicochemical constants (Table 4.3) determined were; average moisture contents using loss on drying method calculated as approximately 5.6 % appears to be lower than the value

obtained by Danlami *et al*.,(2012) which is 10.88% of same *S. virosa* leaves. This may be due to seasonal and environmental factors. In the work reported by Danlami *et al*., (2012), S. virosa leaves were collected during rainy season (August) in Abuja while in the present study; the same plant leaves were collected in dry season (March) at Zaria. It may also be due to the different method of determination where loss on drying was used in this study but Danlami used proximate analysis. Low moisture content in crude drugs suggest better stability against degradation of product (WHO, 1996) while high moisture content is considered as adulteration (Evans, 2009). Ash values, indicates the amount of care devoted to the preparation of a crude drug. The percentage yield of total ash and acid insoluble ash were 11.67% and 1.2% respectively. The total ash value was higher than that of Danlami *et al*.,(2012) which is 9.39%. The higher value suggests higher mineral elements value than that *S. virosa* leaf collected from Abuja. Also, the total ash of *S. virosa* obtained in this study; 11.67% is higher than that of *Ipomoea batatas* (Asibey-Berko and Tayle, 1999) widely use in Nigeria as food. A lower acid insoluble ash 2.5% which shows the extent of contamination with earthy material is allowed for Senna leaf while a higher value up to 12% is allowed for hyoscyamus leaf (Evans, 2009). More so, the total ash value is used as criteria to confirm the identity and purity of drugs (WHO, 1996, Prasad *et al*., 2012). The extractive values obtained were 22.00% and 33.00% for ethanol and water solvents respectively. They serve as pharmacopoeial standards for evaluating crude drugs (Evans, 2009). Extractive values provide an estimate of the possible total metabolites in a crude drug.

Plants experience changes in their basic structure overtime. These changes affect the living plant cell wall composition, shape and cell contents which can be evaluated microscopically and are

valuable for identification and detection of adulteration (Evans, 2009). For instance, on the course of these modifications certain materials (e.g. pectin, lignin, and suberin) might be added to the backbone structural material (i.e. cellulose) of the cell wall. These and other cell contents can be detected by the use of color reagents.

Chemomicroscopical examination of the powdered leaf of *S. virosa* revealed the presence of cellulose cell wall, lignified cell wall, tannins, starch, calcium oxalate, suberin and mucilage. However, aleurone grains were absent.

Phytochemical constituents in the plant samples are known to be the biologically active compounds. They are responsible for different activities such as antioxidant, antimicrobial, antifungal, anti- asthma, arthritis, anticancer and anti-snakebite (Alabri *et al.*, 2013, Hassan Bin Asad *et al*., 2014; Banu and Cathrine, 2015).

Preliminary phytochemical screening of the n-hexane (HE), ethyl acetate (EE) and methanol extracts (ME) using standard methods shows that saponins, flavonoids and alkaloids were absent in HE contrary to the findings of Danlami *et al.* (2012 and 2013) where they were all present while anthraquinones were absent in both HE, EE and ME. But, this is similar to the report of Danlami *et al*, (2012 and 2013) which indicated the absence of anthraquinone in both hexane and ethylacetate extracts of *S. virosa* leaves collected from Abuja and methanol extract of the plant collected from Zaria (Yerima *et al.*, 2009). Triterpenes and or steroids were found in all the three extracts of *S.virosa* leaves. This showed the abundance of triterpenes and or steroids in the *S. virosa* plant. Similarly, steroids have been previously reported in the methanol root bark extract

of *S. virosa* (Magaji, 2007 and 2008). The abundance of triterpenes/ steroids in *S. virosa* may support the potential anti-venin activity since triterpenoids lupeol and steroid stigmasterol, were found to possess antivenom properties (Kadir *et al.*, 2015). Furthermore, the phytoconstituents such as phenols, alkaloids and cardenolides in the extracts are known to possess medicinal properties and health promoting effect (Danlami *et al.,* 2013).

The TLC analysis is an important technique that is widely used worldwide for the study of not only phytoconstituents but also adulterants and contaminants in agricultural products, foods and beverages. The quality and purity evaluation of herbal extracts using TLC method is receiving an increased recognition in most pharmacopoeias (Sherma, 2000; Evans, 2009). In recent time, European Pharmacopoeia (EP) has emphasized the use of TLC fingerprinting as required standard for correct identification of herbal material. Finger printing, together with its schematic chromatographic presentation, provide the sequence of compounds in the herbal extract (Bauer and Franz, 2010).

HE was noted to have the best constituents separation of eleven (11) spots (Plate VII) in hexane: ethyl acetate (9:1) but EE was noted to have fourteen (14) spots (Plate XVIII) in hexane: ethyl acetate (13:7) and ME was observed to have eleven (11) spots (Plate XXVII) in butanol: acetic acid: water (10:1:1) visualized with *p*-Anisaldehyde at 110ºC for 2 minutes. Spots of various colours blue, grey, green and violet were revealed. The successful separation of bio-molecules by chromatographic technique depends upon suitable solvent system which needs an ideal range of partition coefficient (k) for each target compounds (Ito, 2005).

The presence of common classes of naturally occurring compounds in plants can be screened by the use of appropriate chromogenic reagents after separation using thin layer chromatography (Evans, 2009). The chromatogram of hexane extract was positive to Dragendoff, Ferric Chloride, Lieberman-Buchard and Iodine vapour and negative to Borntrager and Aluminium chloride reagent (which was observed under UV light at 254 nm and 366nm after spraying the plate). These confirmed the presence of alkaloids, phenols, steroids and saponins but absence of anthraquinones and flavonoids.

The chromatogram of ethyl acetate extract developed in Hexane: Ethyl acetate (13:7) was positive to Ferric Chloride, Lieberman-Buchard, Iodine vapour, and negative to Dragendoff, Borntrager and Aluminium chloride reagent (which was observed under UV light at 254 nm and 366nm after spraying the plate).. The three (3) reagents gave positive results while the last three (3) produced negative results for those phytochemicals tested. These observations showed the presence of phenols, steroids and saponins but absence of alkaloids, anthraquinones and flavonoids in ethyl acetate extract respectively. The chromatogram of methanol extract developed in butanol: acetic acid: water (10:1:1) was positive to Dragendoff, Ferric Chloride, Lieberman-Buchard, Iodine vapour but negative to Borntrager and aluminium chloride reagents. The first four gave positive results while the last two produced negative results for those phytochemicals tested. These confirmed the presence of alkaloids, phenols, steroids and saponins but absence of anthraquinones and flavonoids in methanol extract of *S. virosa* leaf from the result of preliminary phytochemical screening.

The absence and presence in all the three extracts of anthraquinone and triterpenes/steroids were confirmed by the TLC method. But, absence and presence of alkaloids in EE and HE respectively deviates from result of the preliminary phytochemical investigation conducted in this study but similar to that of Danlami *et al*., (2013). Saponins were found to be present in all the extracts but flavonoids were not detected by this method, contrary to the earlier presence in EE and ME by preliminary investigation and then might be due to limitation in the observation of yellow fluorescence. However, a colourless fluorescence was observed for ME. Thus, suggestion can be made for the use of other analytical method for the detection of flavonoids. Thin layer chromatographic analysis is a simple and cheap method for detection of plant active constituents due to its good selectivity and sensitivity of detection and providing, convincing results (Patra *et al.,* 2012). Therefore, it is considered as a reliable technique for qualitative phytochemical screening of plant active constituents.

Snake venom is a complex mixture of substances such as proteins (i.e. enzymes, peptides, etc), metal ions. By composition, it contains about 90% proteins of which majority are enzymes such as phospholipase A_2 (PLA_2), phosphodiesterase and acetylcholinesterases. Up to 40% of the 90% proteins in the snake venom are PLA_2s. PLA_2 is referred to as cellular degrading enzyme which signifies its multiple cascades of toxicities on virtually all cells in the victim's body. Phospholipase A_2 (PLA_2) is a lipolytic enzyme that hydrolyses the fatty acyl ester bonds at the 2-sn position of membrane phospholipids producing equimolar amounts of free fatty acid (FFA) and lysophospholipid; mainly arachidonic acid (AA). These products then become available for conversion to potent pro-inflammatory mediators, such as platelet-activating factor and eicosanoids respectively (Adamude *et al.,* 2016).

Animal antivenom immunotherapy is the only specific treatment against snake venom envenomation. But, it is associated with various side effects such as anaphylactic shock, pyrogen reaction and serum sickness. Alternative to this conventional therapy is antivenin from plant origin (Alam and Gomes, 1994; Meenatchisundaram *et al*, 2009, Kunjam *et al*., 2013). In this present study, *S. virosa* leaf extracts appears to exhibit a non-competitive pattern of PLA_2 enzyme inhibition of which methanol extract shows the best activity with weak interaction between the extract and the substrate (egg yolk). Indeed, polar aqueous extract of *Casearia sylvestris* showed anti PLA_2 activity (Gomes *et al.*, 2010) while the alcoholic and aqueous extracts of *Pouzolzia. indica*, significantly inhibited the PLA_2 enzyme activity possibly by preventing hydrolysis of lecithin (Ahmed *et al.*, 2010). Di-n-octyl phthalate isolate from *Ceiba pentandra* leaves extract was reported to be biologically active inhibitor of Echis ocellatus venom PLA_2 in a dose dependent manner (Ibrahim *et al*., 2011). Similarly, *Azadirachta indica, Aristolochia shimadai, Eclipta prostrate, Schummaniophyton magnificum* were found to be active inhibitors of PLA_2 (Gomes *et al.*, 2010; De and Dey, 2012). Saifullahi *et al.*, (2014) reported that Oleanyl erucoate, isolated from N-hexane extract of *Cryptolepis oblongifolia* obtained from Zaria, exhibit a non-competitive inhibition of PLA_2 *in vitro*. Polar constituents (i.e. phenols) from plants also have been reported to be potent inhibitors of PLA_2 (Kadir *et al.*, 2015).

In order to determine the safety margin of drugs and plant products for human use, toxicological evaluations are carried out in experimental animals using various methods to predict toxicity. The acute toxicity evaluation provides a guide for selecting a "safe'' dose in animals and to

estimate the therapeutic index (LD_{50}/ED_{50}) of drugs (Olson *et al.,* 2000; Rang *et al.,* 2003; Maikai *et al.,* 2008). The LD_{50} of N-hexane extract of *S.virosa* was found to be 774.60mg/kg while that of Ethylacetate extract it is practically non-toxic in mice at the dose of 5000mg/kg which suggested that the ethylacetate extract is moderately toxic. This is based on the toxicity classification which states that substances with LD_{50} values of 5000 to 15,000 mg/kg body weight are practically non-toxic (Loomis and Hayes, 1996). On the other hand, the LD_{50} of methanol extract of the leaf of *S.virosa* had already been determined by many researchers for the same plant in same study location (Magaji *et al.*, 2008, Tanko, *et al.*, 2008) which both stated LD_{50} of 1265mg/kg for the methanol extract.

Elapid such as *Naja nigricollis* envenoming is characterized by a progressive descending neuromuscular paralysis, respiratory failure and death (Alirol *et al.*, 2017). The efficacy of antivenom against particular venom is due to the ability of antivenom molecules to bind with toxins in the venom. The most widely used method for assessing antivenom efficacy is rodent lethality testing (Silva *et al.*,, 2017). In this study, the effect of the leaf extracts of *S. virosa* on ameliorating the lethal effect of venom of *Naja nigricollis* was conducted. The n-hexane extract offered better protection, followed by methanol extract at $p < 0.05$. Also, the result has shown that n-hexane and methanol extracts activities are statistically significant at 20 mg/kg dose in a mixture with half of LD_{99} of the venom given intraperitoneally to the mice.

Methanol extract of stem bark of *C. africana* produced a dose dependent in vitro detoxifying action against *Naja nigricollis* venom (Isa et al., 2015).. However, for *S. virosa*, n-hexane extract is more potent in neutralizing venom toxicity

In another study conducted in Nassarawa state, Shekins *et al.* (2014) reported that there was significant difference the time of death between *Mucuna pruriens* leaves extract treated group and those treated with *Naja hannah* venom alone. The superior activity of n-hexane extract bioactive constituents has been reported by Mathias *et al.* (2016) where a triterpene; Friedelin isolated from *Albizia chevalieri* showed a significant anti-venin activity of 64% protection. It also, showed that ethylacetate extract had the least potential antivenin effect. This is in concordance with the finding in this present study. The alleviation of toxic symptoms and survival of laboratory animals (within a short time frame) after being challenged with lethal doses of venom is in good agreement with the previous studies (Onyeama, *et al.*, 2013; Isa *et al*., 2015 and Bhole and Bhavsar, 2017) in which different classes of plant constituents have demonstrated *in vivo* activity against some snakes venom.

The extract of the leaf of *S.virosa* showed better protection to mice from *Naja nigricolis* venom induced histopathological changes in the brain, heart, lungs, liver and kidney. Bergenin from *fluggea virosa* was reported to be a potent hepatoprotective agent (Rastogi and Rawat, 2008).

Naturally occurring substances such as sitosterol, pentacyclic terpenes, nitro compounds (aristolochic acid), cinnamic acid derivatives, curcuminoids, polyphenolic compounds, and flavonoids are known compounds possessing protein-binding and enzyme-inhibiting properties. The extracts of *S. virosa* were shown to contain alkaloids, tannins, flavonoids and other secondary metabolites similar to those earlier reported with activity against snake venom

constituents. These compounds act synergistically to neutralize snake venoms *in vivo* and *in vitro*.

The findings in this work have indicated that the extract of *Securinega virosa* contain compounds which can neutralize *N. nigricollis* venom both *in vitro* and *in vivo*. This present study has explained the basis for using this plant in traditional medicine to treat snake envenomation in Nigeria. A positive correlation exists between traditional use of medicinal plants and their pharmacological investigations (Dey and De, 2012). Other parts of *S. virosa* such as root bark, fruit and phytoconstituent bergenin have not been investigated for possible potent anti-snake venom activity.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

Some pharmacognostic standard for the leaves of *Securinega virosa* obtained in Zaria were established for the first time in this study to the best of my knowledge and these data could be used as a diagnostic tool for the standardization and proper identification of this medicinal plant.

The research began with the microscopic examination of the leaf surfaces and the transverse section of the leaf of *S. virosa* which revealed some important diagnostic characters on both upper and lower epidermal layers these includes: Epidermal cells which are polygonal with

straight anticlinal wall and 2-5 -8 µm in size. Stomata were found only on the lower epidermis which are anomocytic and paracytic of 4-5-6µm in diameter.. Unicellular trichomes are rare but present on both surfaces. Prism crystals of calcium oxalate of 1-2-3 µm. it is arranged in a sheath or single which are abundance on both upper and lower epidermal surfaces. The transverse section through the midrib of the leaf shows mesophyll, vascular bundle (xylem and phloem), Calcium oxalate crystals and single layer of epidermal cell on the upper and lower leaf surfaces

The physical parameters of the powdered leaf of *S. virosa* were found to be; moisture content (5.55 %), total ash value (11.67 %), acid insoluble ash (1.16 %), ethanol extractives value (22.00 %) and water extractives value (34.00 %). Chemomicroscopical features of powdered leaf of *S. virosa* revealed the presence of cellulose cell wall, lignified cell wall, mucilage, tannins, suberin, calcium carbonate and calcium oxalate crystals. Inulin was absent.

Preliminary phytochemical analysis of the *S. virosa* leaf extracts revealed the presence of some secondary metabolites namely alkaloids, tannins, flavonoids, cardiac glycosides, saponins and steroids/ triterpenes while anthraquinones were absent. The leaf extracts of *S. virosa* possessed various chromatographic fingerprints of diagnostic and identification significance with eleven (11) spots in hexane: ethyl acetate (9:1), fourteen (14) spots in hexane: ethyl acetate (13:7) and eleven (11) spots in butanol: acetic acid: water (10:1:1) for hexane, ethylacetate and methanol extracts respectively. However, the TLC phytochemical screening of the extracts visualized with specific reagents confirmed the presence of important phytoconstituents such as alkaloids, phenols, saponins and steroids/ triterpenes. However, anthraquinones and flavonoids were absent.

The PLA_2 assay showed that leaf extracts of *S. virosa* of possessed bioactive constituents with PLA_2 inhibitory properties. Methanol leaf extract of *S.virosa* showed greater activity among the other two extracts with inhibition pattern of a non-competitive type and a weak dissociation constant. The intraperitoneal median lethal dose (LD_{50}) of the extracts was found to be 1265.5mg/kg and greater than 5000 mg/kg for hexane and ethylacetate respectively. The leaf extracts of *S. virosa* have shown potentials to possess *Naja nigricollis* snake venom neutralization activity, of which the activity of methanol and hexane extracts, were significant at the doses of 20 mg/kg in mice. The finding was correlated by the histological results where the extracts protected the mice from venom induced pathological changes in the various organs.

6.2 Conclusion

The pharmacognostic studies of the leaf of *S. virosa* yielded a set of qualitative and quantitative parameters that may be useful in ascertaining the identity of the plant and to determine the quality and purity of *S. virosa* leaf drug materials

The preliminary and thin layer chromatographic phytochemical screening, as well as, chromatographic fingerprinting conducted for leaf extracts of *S. virosa* yielded the presence of significant phytoconstituents and fingerprints that would be useful for identification and standardization of *S. virosa*.

The PLA_2 assay showed the potential of the leaf extracts of *S. virosa* possessing bioactive constituents with PLA_2 inhibitory properties. The methanol extract was found to exhibit the best PLA_2 inhibitory effect.

This work showed that n-hexane extract of *S. virosa* has antivenin properties against *Naja nigricollis* venom induced toxicity in mice. These results have provided some scientific bases for the use of the plant in traditional medicine for the management of snakebite.

6.3 Recommendation

- Further work needs to be done to identify, purify and quantify the antivenin compound(s) in *S. virosa*
- Quantification of phytochemicals in the leaf and other vegetable parts should be conducted.
- Sub-acute and chronic toxicity test should be carried out in order to determine the long-term effects of the extracts.
- Standardization of the other vegetable parts; root, stem bark, fruit and seeds traditionally used as medicine is paramount.
- Investigation into the geographical and seasonal variations of the bioactive constituents of the plant is also essential.

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APPENDIX I

Sample Calculation of percentage yield

$$percentage\ yield = \frac{-\text{final weight of extract}}{\text{initial weight of sample}}\ X\ 100$$

Weight of sample = 300g

Weight of empty container = 669.75

Weight of container + sample = 677.75g

Final weight of extract = 677.75 – 669.75 = 8.00g

$$percentage\ yield = \frac{8.00\text{g}}{300\text{g}}\ X\ 100$$

$$= 2.67\%$$

Table 1: The physical properties and percentage yield of the extraction of dried leaf powder 9300g) of *S. virosa*

S/N	Extraction solvent	Yield in grams (g)	Yield in percentage (%)
1	N-hexane	8.00	2.67
2	Ethylacetate	11.22	3.74
3	Methanol	46.67	15.65

Table 2: The physical properties of the extracts of *S. virosa*

S/N	Extracts	Colour	Odour
1	N-hexane	Dark brown	Agreeable
2	Ethylacetate	Dark green	Agreeable
3	Methanol	Dark green	Agreeable

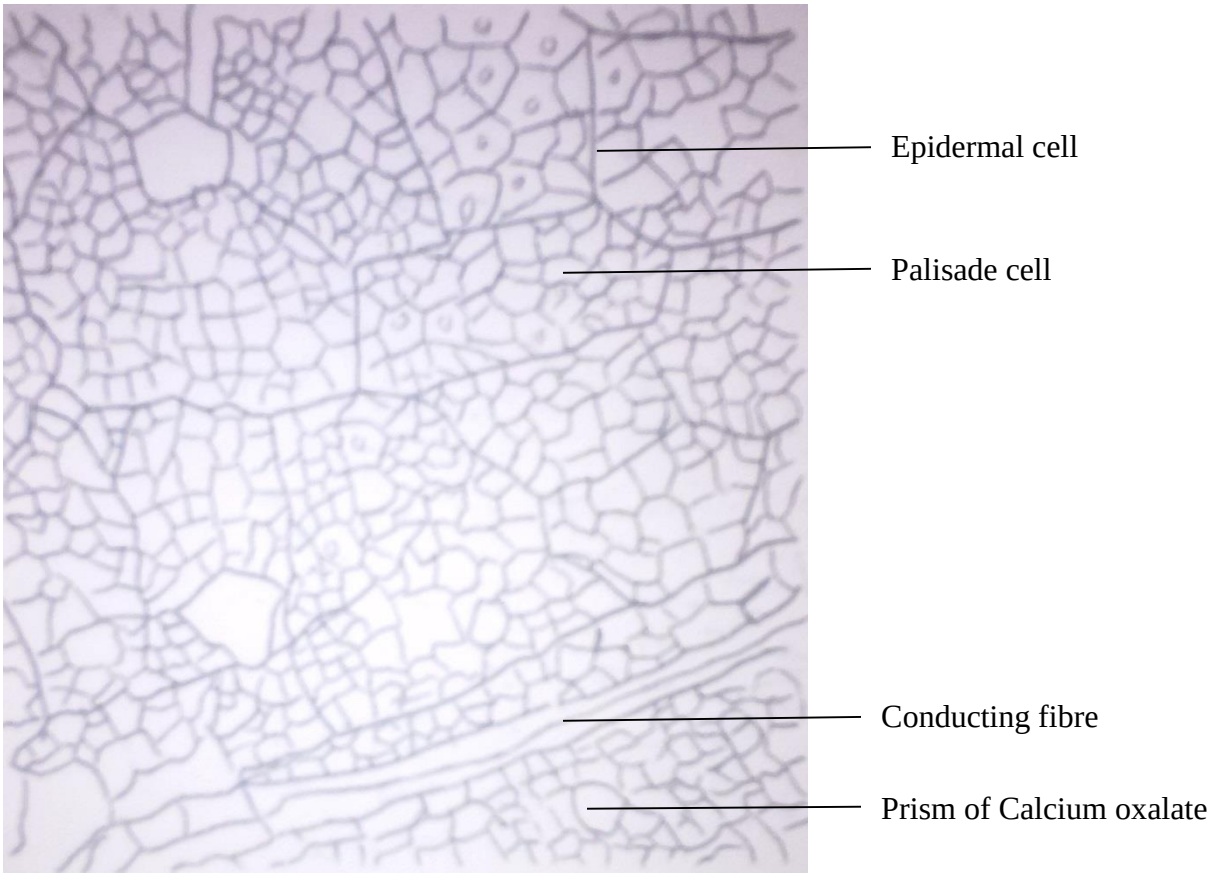


Plate III: Photomicrogaph of some microscopic features of epidermal layer of *S. virosa* leaf (X400)

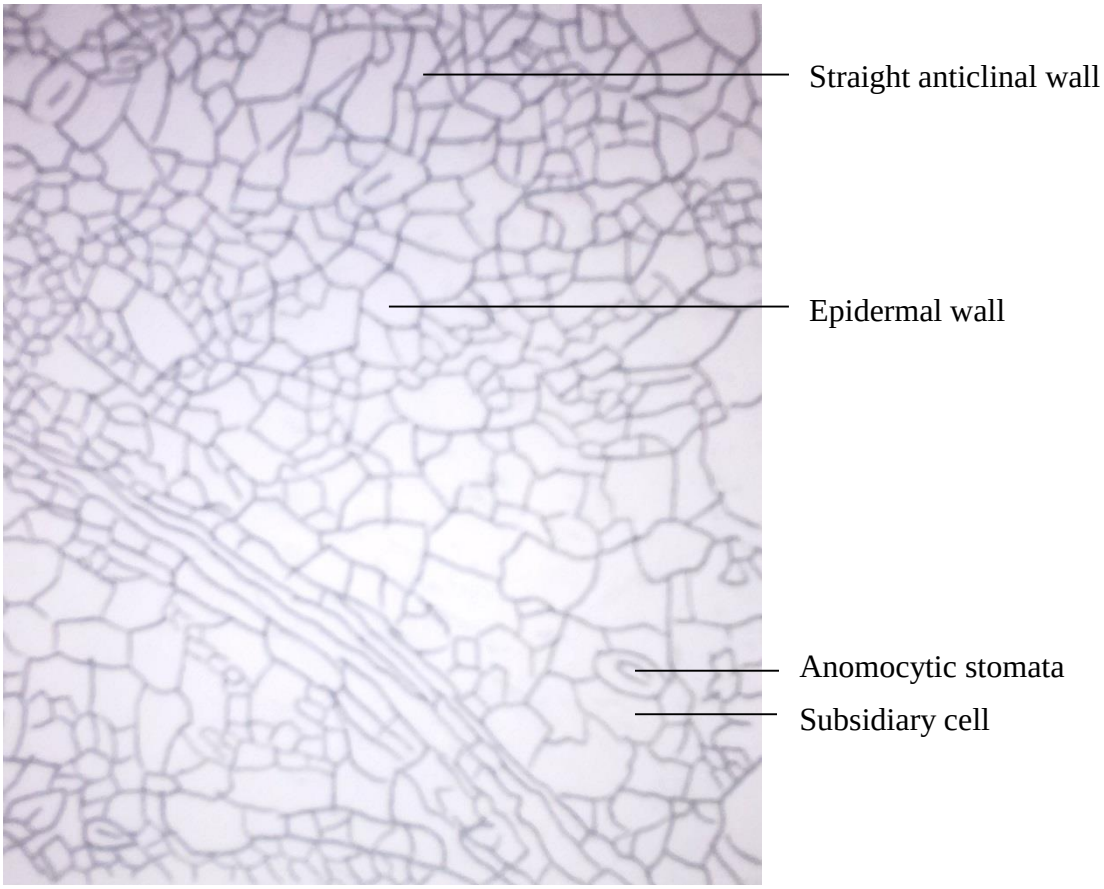


Plate IV: Stomata of the lower epidermal surface of *S. virosa* leaf (X 400)

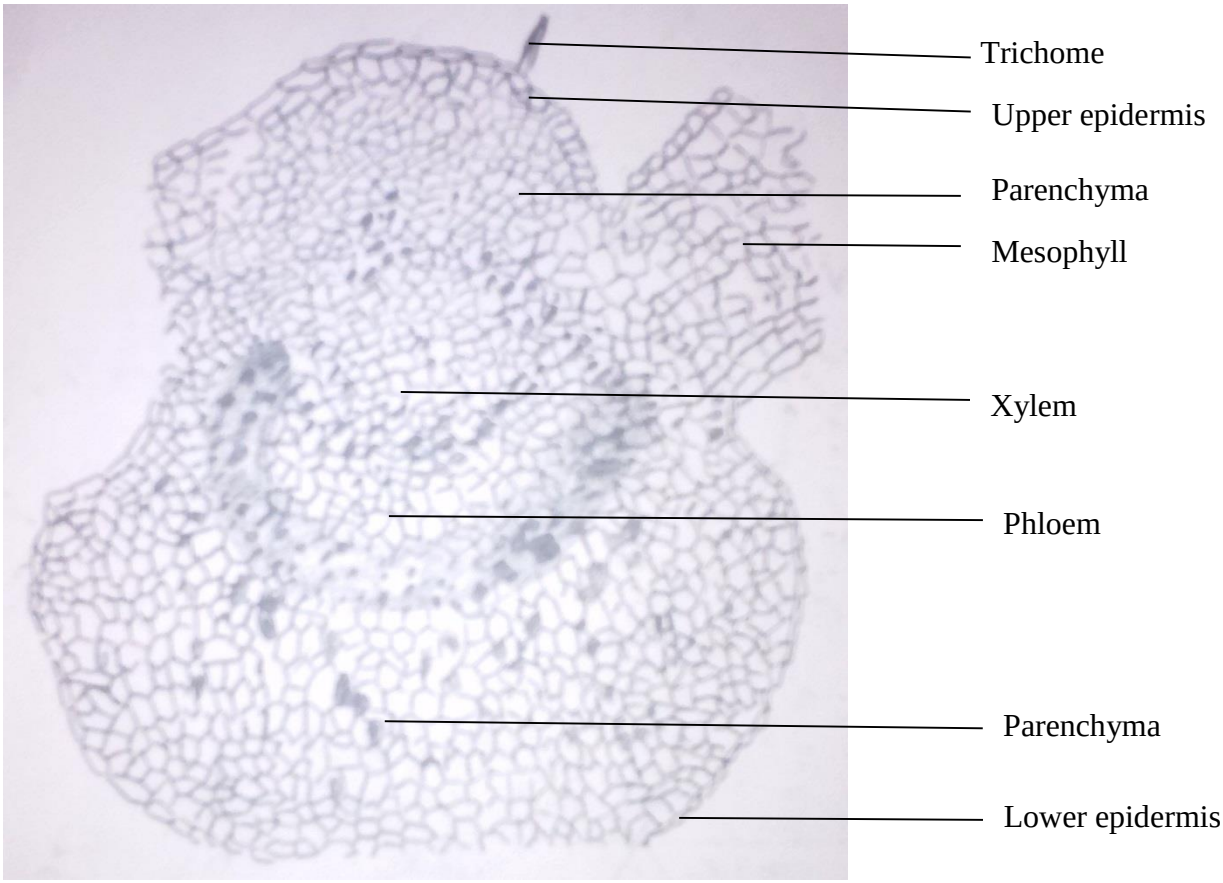


Plate V: Photomicrograph of Transverse section through the midrib of *S. virosa* leaf (X 400)

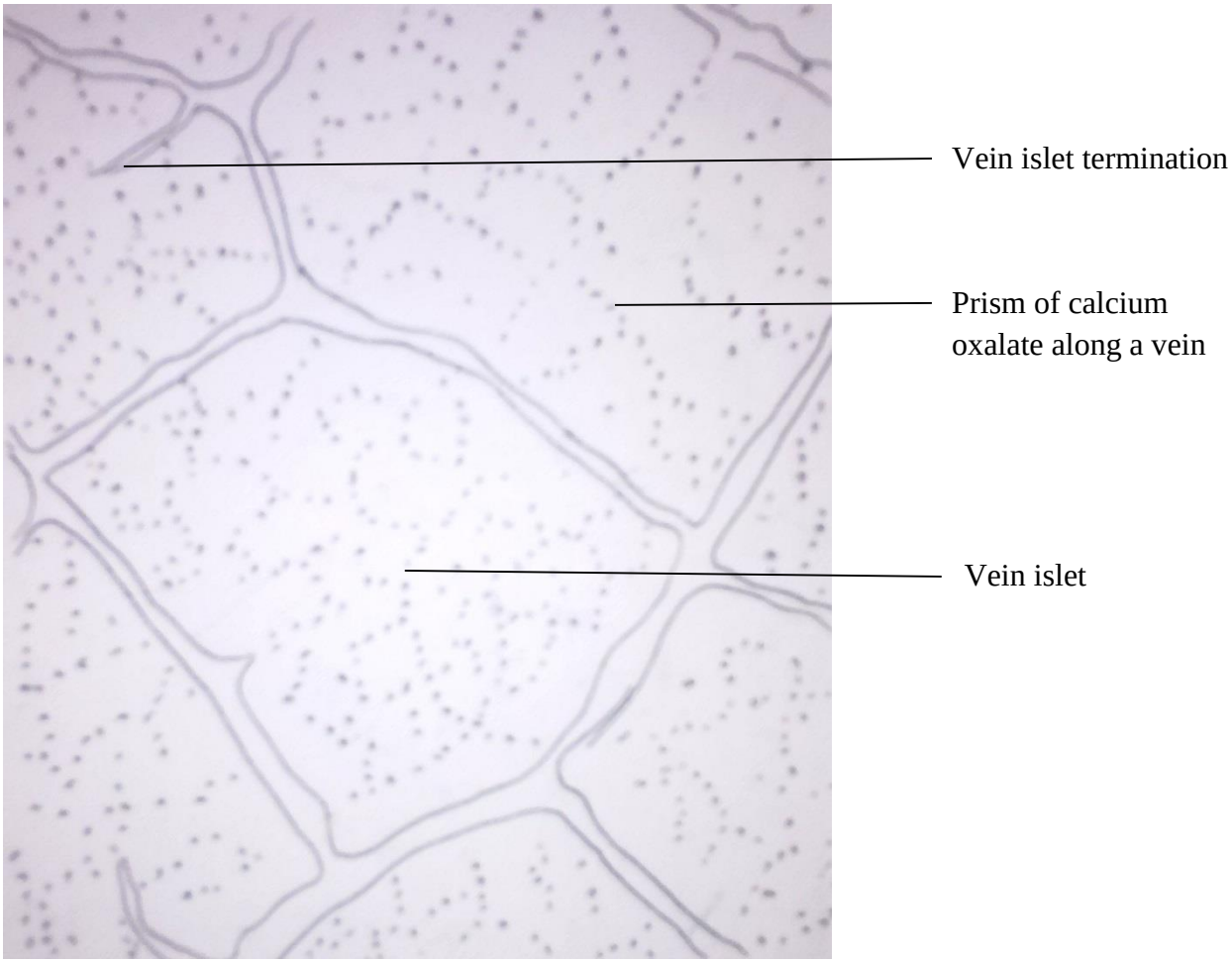


Plate VI: Photomicrograph of adaxial surface of *S.virosa* leaf (X 400)

Moisture content $= \frac{\text{weight of water loss}}{\text{initial weight of sample}} \ X \ 100$

a. Weight of sample = 3g
b. Weight of empty crucible = 30.11g
c. Weight of crucible plus sample after heating to constant weight = 32.93g
d. Weight of sample after heating = c – b =32.93g – 30.11g =2.82g
e. Weight of water lost = 3 – 2.82 = 0.18g

Moisture content $= \frac{0.18}{\text{i}3.00} \ X \ 100$ = 6%

Appendix II

Table 3: Acid – base titration values for methanol extract of S. virosa leaf extract

S/N	Initial volume (ml)	Final volume (ml)	Volume of base used (ml)
1	15.00	16.00	1.00
2	16.00	17.30	1.30
3	17.30	18.40	1.10
4	18.40	19.20	0.80

Table 4: The activity of S. virosa leaf extract on phospholipase A 2

S/n	Initial velocity (v)	1/v	Substrate Conc.	1/substrate conc.
1	66.67	0.015	25	0.040
2	86.7	0.012	50	0.020
3	73.3	0.014	75	0.013
4	53.3	0.019	100	0.010

$$Initial\ velocity = \frac{\text{Mb X Vb}}{\text{Va}}\ X\ 100$$

Mb = molarity of base in millimole = 20mM

Vb = volume of base used = titre value

Ma = molarity of free fatty acid released = Initial velocity = x

Va = Total volume of the mixture = 0.3ml

$$Initial\ velocity = \frac{20\ \text{X}\ 1}{0.3}$$

$$= 66.67\ \text{mM}$$

APPENDIX III

Table 5: the behavioural observations in mice treated with venom and mixture of venom and leaf extracts of S. virosa

Treatment groups	**methanol**	**ethylacetate**	**n-hexane**
Venom	-Ptosis - Descending flaccid paralysis	-weakness -ptosis -Descending flaccid paralysis	-Weakness -Convulsion Descending flaccid paralysis
Venom + 5mg extract	-Weakness -Respiratory distress -Ptosis	-Sweating -Paralysis	-Convulsion -Descending flaccid paralysis
Venom + 10mg extract	-Ptosis -Convulsion -Abdominal cramp	-Sweating -Paralysis	-Increase activity -Normal activity
Venom + 20mg extract	-Abdominal cramp -Ptosis	-Sweating	-Weakness Normal activity

CHAPTER ONE

2.0 INTRODUCTION

1.1 Background

Traditional medicine consists of medical knowledge developed by indigenous cultures that involved the use of plant, animal, mineral based medicines, spiritual therapies and manual techniques designed to treat illness or maintain wellbeing (WHO, 2013). It was modified over hundreds to thousands of years, of which the best known include Indian traditional (Ayurveda) medicine, traditional Chinese medicine and traditional Arab (Unani) medicine. Traditional medicine or Complementary alternative medicine can be broadly grouped as natural products, mind body measures and manipulative and body based practices (Ryan, 2014).

Plants have in one way or the other provided the basic need of mankind such as food, shelter, clothing and protection from disease causing agents. The use of plants and its extracts to treat various diseases can be traced to the early days of human history. Medicinal plants contain substances that modulate the physiology of sick mammals and the knowledge of these healing properties has been passed down through generations. About 70 to 80 per cent of the world population, especially inhabitants of rural areas used medicinal plants for primary health care. This is also known as herbalism. Pharmaceutical factories also use medicinal plants as basis for production of therapeutic drugs. Medicinal plants have a promising future. And, of the approximately half a million plants around the world, a lot have their medicinal activities not yet investigated. Medicinal activities of plants could be a solution to troublesome diseases in present or future studies (Bassam, 2012) and for the production of more effective drugs (Dossou-Yovo *et al*., 2014). Furthermore, as a result of population growth, for instance, there is general increase

in demand for plant based traditional medicines. On the other hand, over exploitation and lack of standardization lead to a profound limitation to the use of plants as medicine However, the potentials of African medicinal plants are enormous (Junhua *et al.*, 2011; Ernest, 2012).

Snakebites in Nigeria are mainly associated with four families of snakes (Viperidae, Elapidae, Colubridae and Actraspididae) and three species from the first two families - carpet viper (*Echis ocellatus*), black-necked spitting cobra (*Naja nigricollis*) and puff adder (*Bitis arietans*) are the most commonly involved. The most common snakebite envenomation in Savannah region of the country results from carpet viper (*Echis ocellatus*). It accounts for at least 66% of all snakebites (Habib *et al.,* 2015). Snakebite is a common medical emergency in developing countries (Sipra *et al.,* 2016) and represents an important neglected public health problem.

For the global burden of snakebite and envenomings, an extensive literature search covering 40 years (ending 2010) and meta-analysis of the data, taking into account the heterogeneity between the studies and their respective weight was conducted. The incidence, mortality and population at risk were estimated after stratification according to the environment (urban or rural) and survey methodologies (national, hospital or community studies). The incidence of snakebite was inversely correlated with population density. The number of envenomings was estimated at over 314,000, of which 95% occurred in rural areas, while the remainder occurred in cities. The annual mortality was estimated at 7,331, of which 97% occurred in a rural environment. The annual number of amputations ranged from 5,908 to 14,614. The population most at risk was young men engaged in agricultural or pastoral labours. Household surveys indicated that actual incidence and mortality were likely 3-5 times higher. The difference may be explained by

treatment seeking behavior. However, incidences and mortalities reported here reflect the number of patients who attend modern health facilities, giving underestimated figures of the burden of snakebites in sub-Saharan Africa but realistic current requirements for antivenoms (WHO, 2015).

1.2 Statement of Research Problem

Animals such as goat and horse are the major and the only validated tool for antivenom derivation for decades. In sub-Saharan Africa, there are serious challenges associated with the availability of this validated antivenom. Some of these challenges are: lack of accessibility, ineffective products and high cost (Juan *et al.,* 2010). The abundance of folkloric knowledge on the use of medicinal plants as an alternative therapy to many troublesome diseases such as snakebite has received minimal attention. More so, snakebite envenomation is a life threatening injury that requires intensive care and may results on the death or permanent deformity of the victims. A study has shown that only 8.5% of snakebite victims in Nigeria attend hospitals and most vulnerable farmers and Cattle rearers depend on traditional healers (Ismaila and Adamu 2012; Habib, *et al* 2013; Hifumi, *et al* 2015). Also, the benefit of phytotherapy is controversial as the pharmacological and toxicological actions and standardization are not well studied and documented (Gupta and Peshin, 2012).

1.3 Justification

African traditional medicinal plants have enormous potentials to pharmaceutical industry. Herbal therapeutics for snake envenomations seems to be a viable alternative to modern medicines. Also, there is an increase on the scientific validation confirming the efficacy of the widely used

traditional medicine products across the world (WHO, 2013). Many plants existing in nature are enormous reservoir of bioactive molecules that can be developed as new chemical entities, analogs, derivatives, synthetic compounds with natural product derived pharmacophores or as natural product mimics effective for neutralization of snake envenomation (Ramar *et al*., 2012). There are a number of plants species found to be effective for the treatment of snake bites in traditional medicine whose pharmacological evaluation has been undertaken (Gupta and Peshin, 2012). Nigerian plants such as *Aristolochia albida*, *Guiera senegalensis*, *Schumaniophyton magnificum* etc. were found to relieve snake venom toxicity in experimental animals (Habib, 2013).

1.3.1 Research question

What are the characteristic/pharmacognostic features that can be used for correct identification of *Securinega virosa* leaf?

Would thin layer chromatographic finger print and phytochemical profile of *S. virosa* be useful for quality assessment of *S. virosa* leaf?

Does *Securinega virosa* leaves have any modulatory effect on the snake venom of *Naja nigricollis?*

1.4 Aim

To provide scientific rationale for the traditional use of *Securinega virosa* leaves as antivenin for snakebite and to determine the pharmacognostic characteristics of the leaves for quality assessment.

1.5 Objectives

1. To establish pharmacognostic microscopical features and physical constants useful for identification of *Securinega virosa.*

2. To establish thin layer chromatographic finger prints and phytochemical profiles of *S. virosa* leaves extracts.

3. To determine the *in vitro* antivenin activity of *S. virosa* extracts against *Naja nigricollis* Snake venom Phospholipase A_2 (PLA_2)

4. To determine the modulatory *in vivo* antivenin effects of *S. virosa* leaf extracts on *Naja nigricollis* snake venom.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The Family Euphorbiaceae

Euphorbiaceae is a family of vascular flowering plants with over 300 genera and about 9,000 species distributed within five subfamilies namely; Phyllanthoideae, Oldfieldioideae, Acalyphoideae, Crotonoideae and Euphorbioideae (Webster, 1994, Kondamudi *et al*, 2009). It is popularly known as spurge family due to the white latex they produce on injury to the plant. They are mostly herbs and sometimes shrubs or tree especially in the tropics (Abbonier, 2004).

2.1.1. Distribution of Euphorbiaceae

They are native to tropical Africa, America, Asia and Australia. Some species are found in non-tropical regions such as Mediterranean, Middle East, South Africa and Southern United States of America. They are characterized by disjunct distribution (Abbonier, 2004).

2.1.2. Description of Euphorbiaceae

The leaves are alternate rarely opposite with stipules. The leaves are simple, rarely compound and palmate but never pinnate. Fruits are succulent and non-succulent in some species. Stipules are mostly absent in succulent species. The stipules may be reduced to have glands or spines. Flowers are radially symmetrical, unisexual with male and female flower occurs on the same plant. Flowers vary structurally across the family. They are monoecious or dioecious. Stamens are one to ten or more. The female flowers are hypogymous (Abbonier, 2004).

2.1.3. Economic value of Euphorbiaceae

The members of Euphorbiaceae are valuable source of different kinds of useful products like dyes, edible tubers, oil crops, furniture, agricultural implements, ornamental plants, pharmacological products, rubber, timber and aesthetic items. They includes: castor oil (*Ricinus*

communus), cassava (*Manihot esculenta*), Para rubber tree (*Hevea brasiliensis).* . They are widely used as medicine and some have proved to be efficacious. *Securinega virosa* have proved to be efficacious against brain cancer cell lines *in vitro* (Kondamudi *et al.*, 2009; Rahman and Akter, 2013; Magaji *et al.*, 2015).

2.2 Genus *Securinega* (Bush weed)

Many researchers have merged and unmerged this genus with genus Flueggea (Webster, 1994). It is monoecious or dioecious trees, shrubs, or herbs with 14 species, widespread in the tropics and temperate eastern Asia, but with a disjunct relict distribution. Flowers in axillary glomerules (except *Richeriella*); sepals mostly 4-6, free or connate; stamens 2-15, free or connate; disk extrastaminal and usually dissected (less commonly cupular or absent); anthers introrse or extrorse; pistillode present or absent; pistillate sepals mostly 5 or 6, persistent in fruit; disk usually cupular, often lobed (sometimes dissected); carpels mostly 3 (rarely 2 or 4-10); styles free or connate, bifid or entire; ovules hemitropous; fruit usually capsular (*sometimes* baccate or drupaceous); seeds usually 2 per locule; testa smooth or sculptured; endosperm copious; cotyledons broader than and 1-2 times longer than the radicle (Webster, 1994).

2.2.1 Recent systematics modification of the genus *Securinega* and *Securinega virosa*

Now, the genus Securinega is referred to as synonym of genus Flueggea. The accepted genus name by the world check list of selected plant (WSCP) is Flueggea and currently it is recognized under the family Phyllanthaceae. Phyllanthaceae is a segregate family from Euphorbiaceae family based on congruent plastid and nuclear DNA sequence data that have recovered well-

resolved and strongly supported clades (popularly referred to as Euphorbiaceae *sensu lato* (SL) (Awomukwu *et al*, 2015; Govaerts, 2017). The name *Securinega virosa* has now been replaced with *Flueggea virosa* as the accepted specie name under genus Flueggea and family Phyllanthaceae (Govaerts, 2017).

2.3 *Securinega virosa* (Roxb ex Willd) Baill

This plant is referred to as 'cure all' due to its valuable and common application in traditional medicine for the management of many diseases (Magaji *et a*l., 2008).

2.3.1 Common names and synonyms of *Securinega virosa* (Euphorbiaceae)

English name: Dog's tentacles

Local (vernacular) names;

Hausa : Tsa

Yoruba : Iranje

Ibo : Njisi-nta

Kanuri : Shim-Shim (Komal *et al*., 2013).

Synonyms: *Flueggea virosa* (Abonnier, 2004)

2.3.2 Geographical distribution

It is widely distributed throughout tropical Africa, India, Malaya, China and Australia. In Nigeria, it is found in virtually all parts of the country. It is common, scattered and irregularly distributed (Abonnier, 2004).

2.3.3 Botanical description of *Securinega virosa*

Securinega virosa is a small, deciduous, smooth, large, graceful shrub. The leaves are extremely variable in shape, elliptic-ovate, Obovate or orbicular, 2.5 to 10 centimeters in length, glaucous beneath, and rounded, obtuse, or pointed at the top. The flowers are usually borne on axillary fascicles. The fruit is mostly small, black or white, dry, and about 3 to 5 millimeters in diameter (Burkill, 1994). Thakur and Patil (2014) further described that, the Leaves are hypostomatic. Epidermal cells are chlorophyllous, sides 4–6, straight, and mostly tetra to hexagonal. Stomata are mostly anomocytic, rarely paracytic, orientation is random and distribution is diffuse. Stomatal index (S.I.) =12. Subsidiaries mostly 4–5, rarely 3, mostly F-type, walls straight, slightly curved sides mostly 5–6, rarely 4. Guard cells have elliptical pore and narrow (Plate I).



Plate I *Securinega virosa* growing in a bush (source www.plantzafrica.com)

2.3.4 Ethnomedicinal uses of *S. 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 and one of the most commonly used medicinal plant in West Africa (Sofowora, 1993; Burkill, 1994). The roots and fruits are believed to be an effective snakebite remedy. Root of *S. virosa* is also used in some African communities as contraceptives and for the treatment of syphilis, gonorrhea, rheumatism, sterility, rashes, while an infusion of the root is taken to relieve malaria (Tshifhiwa and Thompson, 2008). The root bark is believed to provide a treatment for diarrhoea and pneumonia. The plant is used traditionally in the management of cancer and ulcer (Mike *et al*., 2010). The dried root powder of *Flueggea virosa* is used in Zimbabwe as antidote for snakebite when applied at the part bitten by snake (Maroyi, 2013).

The plant is said to have a hallucinogenic effect and the decoction of the root with other plant is used in Northern Nigeria for the treatment of mental illness (Abbonier, 2004). In North Eastern Nigeria, the root and leafy twig decoction is used for the treatment of epilepsy (Magaji *et al.*, 2007).

2.3.5 Pharmacological activities of *S. virosa*

The leaves have antioxidant (Dickson *et al*., 2006), while the methanol root bark extract of *S. virosa* was reported to contain bioactive constituents with sedative and anti-convulsant activities (Magaji *et al.*, 2007 and 2008). It also possessed anti-diabetic (Tanko *et al*., 2008), analgesic and anti-inflammatory activities (Yerima *et al.,* 2009). Wiwat and Kwantrairat (2013) reported that leaves extract of *Securinega virosa* possessed anti HIV-1 reverse transcriptase inhibitory activity

(88.2%) tested *in vitro*. Also, methanol leaf extract of *S. virosa* possess antipsychotic and sedative effects *in vitro* in mice (Magaji *et al*., 2014).

2.3.6. Chemical constituents

Reported preliminary phytochemical screening on the methanol root bark extract of *S. virosa* indicated the presence of saponins, tannins, alkaloids, flavonoids, cardiac glycosides, cyanogenic glycosides, resins, steroids/terpenoids and carbohydrates (Magaji *et al.* 2007; Aiyelero *et al.*, 2012; Wiwat and Kwantrairat, 2013). However, the methanol leaf extract of *S. virosa* contain alkaloids, tannins, saponins, flavonoids, cardiac glycol-sides, cyanogenic glycosides, resins, steroids/terpenoids and carbohydrates (Yerima *et al.*, 2009). Bergenin was isolated from the root of *S. virosa* (Magaji *et al.*, 2015). Alkaloids- securinine and triterpenes; were reported in *Flueggea virosa* (Maroyi, 2013). Zhang *et al.* (2015) isolated various types of Securinega alkaloid oligomers, fluevirosinines from *Flueggea virosa* (a synonym of *Securinega virosa* (Abonnier, 2004) with anti-HIV activity.

2.4 Snakebite; A Public Health Problem

There are over 3000 species of snakes in the world; out of which about 600 are venomous and over 200 out of this are medically important (Hider *et al.*, 1991; WHO, 2015). Snakebite is one of the neglected tropical diseases affecting a sizable number of the world population. It is a pandemic health issue occurring mostly in tropical and agricultural regions of the world. The world health organization, in 2009 added snakebite as one of the neglected tropical diseases causing a huge economic and financial burden to the population. Snakebites are associated with mortalities more than all other neglected tropical diseases (Kasturiratne *et al.*, 2008; Williams, *et*

al, 2010). The current global burden of snakebite is under estimated (Gutiérrez *et al*., 2014). The global incidence of snakebites was published by few researches, of which the data on the incidence has also been criticized with regards to the nature of data collection and type of data available that was used. In addition, regional reports on snakebites vary widely with actual incidences, especially in the developing countries (Chippaux, 2008). The global, Africa and sub-Saharan Africa incidence of snakebites are given in the table below:

Table 2.1: Global, Africa and Sub-Saharan African Burden of Snakebites

Coverage	Incidence / year	Researcher (s)
Global	Snakebite 500,000 Envenoming 500,000 Death 35,000 Disabilities -	Swaroop and Grab, 1954
	Snakebite > 5 million Envenoming > 2.5 million Death 125,000 Disabilities > 100,000	Chippaux, 1998
	Snakebite 1.2 to 5.5 million Envenoming 0.42 to 1.8 million Death 20,000 to 94,000 Disabilities > 400,000	Kasturiratne, 2008
	Snakebite up to 5 million Envenoming 2.4 million Death 94,000 to 125,000 Disabilities 400,000	WHO, 2013
Africa	Snakebite 1 million Envenoming 500,000 Death 20,000	Chippaux, 1998
	Snakebite 273,000 to 1.26 million Envenoming 91,000 to 420,000 Death 3,500 to 32,000	Kasturiratne, 2008
Sub-Saharan Africa	Snakebite 83,997 to 884,100 Envenoming 27,999 to 294,700 Death 1,599 to 18,654	Kasturiratne, 2008
Sub-Saharan Africa	Snakebite Envenoming 314,078 Death 7,331 Disabilities 5,908 - 14,614.	Chippaux, 2011

Africa reports on the incidence of snakebite reflect the scanty nature of reporting habits manned by a myriad of challenges. It is believed that a significant number of snakebite cases go

unreported, giving an under estimated figure of the real incidences and/or consequently the burden of snakebites in Africa (Kasturiratne *et al*., 2008, Habib, 2013, Gutirrez *et al*., 2014). In 2005, 45,900 deaths was attributed to snakebite envenomation in India alone while in the whole of Africa a more recent data shows 7,300 deaths, about 6000 amputations and 314,000 snakebites. Envenomings occur at 45 to 67% of the total bites. (Kasturiratne *et al*., 2008; Mohapatra *et al.*, 2011; WHO, 2015). In Nigeria the incidence of snakebite from a Benue region in the northeastern part of the country alone is 497 /100,000 persons per year and mortality of 2 to 16 /100,000 (Habib, 2013). This is almost a 10 fold than that of West African region estimate of 54/100,000 snakebites persons per year.

2.4.1. Snakebite envenomation

According to estimates, more than 5 million people in the world suffer snakebite every year with at least 2.5 million envenomings, and among those who are bitten, approximately 125 000 die and around 400 000 are left with permanent sequelae (WHO, 2015). However, more recent nationwide community based surveys in Bangladesh and India have shown that the scale of this problem is far greater than suggested by hospital based statistics and that these global figures greatly underestimate the actual incidence of snakebite envenoming and the resulting mortality and disability. An important factor that contributes to the morbidity and mortality associated with snakebites, particularly in sub-Saharan Africa and parts of Asia, is the poor availability of the only validated treatment for this disease: antivenoms (WHO, 2015). Also, among the envenomed individuals, only about 10% attend the modern health care facilities with more than 80% of the victims treated by traditional medical practitioners (Chippaux, 2008; Habib, 2013). In addition, it was found in the African continent that about 10% – 20% of the snakebite envenomed

individuals, do not receive any form of medication. Meanwhile, in Nigeria only 8.5% of the snakebite envenomed victims seek for allopathic medical care (Kasturiratne *et al*., 2008; Habib, 2013).

It is known that certain reactions are observable following a snakebite envenomation. These reactions can be local such as: blistering, swelling, redness, painful lymph node enlargement, tissue necrosis, snake fangs injury and so on, while the systemic reactions could include: shock, intravascular haemolysis, generalized haemorrhage, descending paralysis, paralyzed rhabdomyolysis and acute kidney injury (Warrell, 2015). The Savannah region of West Africa has been documented to have a high level of snakebite and its' envenoming, notably in the countries of Nigeria, Benin, Burkina-faso, Cameroon, Ghana and Togo. The most implicated species of snake in the region with the highest envenoming is carpet viper (*Echis ocellatus*) of the family Viperidae. For instance, in Nigeria in a study conducted in savanna region of Benue valley (North Central) alone, an incidence of snakebite was reported to be as high as 500 per 100,000 persons per year with mortality of up to 12% and Carpet viper is the major cause of mortality and morbidity, also it accounts to 66% of all the snakebites in that region The African cobras (Naja spp.), puff adders (*Bitis arietans*) and mambas (Dendroaspis spp.) are frequently involved in attacks on humans while Atractaspis spp. and small vipers are only occasionally involved. The main clinical features of *E. ocellatus* envenoming are systemic hemorrhage, incoagulable blood, shock, local swelling, bleeding and necrosis (Habib, 2013; Habib *et al.*, 2015; Iliyasu *et al*., 2015).

Snake venom is a complex mixture of various substances of which some are toxic while some are non-toxic and the main component implicated in envenomation following a bite are enzymes

and polypeptide toxins. More than 90% of the dried weight of venom is protein. Snake venoms composition are found to differ for both inter and intra-species. The most important and clinically significant components are haemorrhagins, pre-and post-synaptic neurotoxins, cytolytic or necrotic toxins, haemolytic and myolytic phospholipases and pro-coagulant enzymes (WHO, 2010).

2.4.2 Diagnosis of snake envenomation

No country is free from the risk of snakebite, and affects mostly rural dwellers with inadequate and unequipped health facilities. The rural and urban health workers are most at times not ready for snakebite management. This is due to lack of adequate beforehand training on snakebite treatment (WHO, 2010; Hifumi *et al*., 2015).

The venomous snakebite diagnosis and treatment is often troublesome to clinicians due to inadequate information on proper antivenom administration guideline in clinical practice (Hifumi *et al*., 2015). Diagnosis relies heavily on the specific snake species identification and careful observation of the clinical manifestation of the snake envenomation as there is no definite diagnostic markers and or kits for snake toxicity in clinical dispensation. And, it was reported that even as in most cases the snakes involved are killed, they are never taken to the health centre for identification probably due to lack of awareness for the need to do so. However, even if it was taken to the well-equipped health care facility, the health care givers lacked adequate skills to correctly identify the snake specie (WHO. 2013, Fadare and Afolabi, 2012; Hifumi *et al*., 2015).

Broadly, the diagnosis of snakebite can be classified in to two: clinical diagnosis and laboratory diagnosis. Clinical diagnosis depends on identifying local signs such as blistering, swelling and necrosis and systemic signs like: Haemorrhage, incoagulable blood and hypovolaemic shock in viper bite and ptosis, descending paralysis in elapid bite, On the other hand, laboratory diagnosis involves analysis/ determination of changes that occur in envenomed individual (WHO, 2010; David and Laing , 2014; Hifumi *et al*., 2015).

2.4.3. Treatment of snakebite

Treatment is primarily aimed at neutralizing the venom containing an array of bioactive protein molecules with specific antivenin. Unless the signs of bite or signs of envenomation are recognized, victim may not visit a health care provider and there is a strong association between snakebite-induced mortality with poverty, mistaken identity, mismanagement by untrained village based traditional therapists, poor transportation facilities, delay at arrival to medical centres and improper dosing of Anti-snake venom (Rao *et al*., 2013; Gutiérrez *et al*., 2014).

The syndromic protocol approach for management of unknown snakebite, categorizes snake bites in to three groups: painful progressive swelling, progressive weakness and bleeding syndrome. This appears to be more logical in reducing mortality. There is no specific or definite dose for antivenom therapy to victims of snakebite and same dose is given to children as in adult (WHO, 2010; Rao *et al.*, 2013). Also, in study conducted in Nepal, it has shown that; repeated administration of more antivenom has no any significance statistically in terms of mortality rate reduction determined in an independent randomized trial. Antivenoms are administered intravenously to achieve rapid onset of action. Subcutaneous or intramuscular administrations are

the most commonly used for the purpose of avoiding serious side effects. Pre-medication with an antihistamine and/or epinephrine should be used when the perceived benefit is greater than the risk of adverse effects. As for the use of hydrocortisone as pre-medication for snake antivenom, the efficacy of which has not been determined and not recommended (Hifumi *et al*., 2015; Pandey *et al.*, 2016).

Recently, polyvalent antibodies column (PVAC) was invented for use in snake venom detoxification. It is a machine that can be used alone or in combination with conventional therapy for snake envenomation. It works like a dialysis machine to perform out of body removal of venom antigens. PVAC membrane is for single use, specific and comes with a replaceable column of polyvalent antibodies. It was found to be effective and potentiates the effect of conventional antivenoms when used in combination (Shahidi, 2014).

2.4.4 First aid management of snakebite

Early recognition of the manifestation of snakebite is essential for targeted first-aid treatment. The use of tourniquet to tie the victim's snake bitten limb can results to more harm than good instead a pressure mass should be used. Prompt arrangement for transporting snakebite victims to the nearest or appropriate health facility is highly encouraged, as most often any delay may lead to longer hospital stay, more admission cost, high morbidity and mortality rate (WHO, 2010).

Symptoms and signs of snakebite vary according to the species of the snake. Local manifestations, such as bleeding and swelling, severe pain, and systemic manifestations such as

nausea and vomiting, drowsiness, weakness, and dizziness, are all common signs and symptoms (Chulin Chen, 2017).

2.4.5 Traditional methods and practices for snakebite management

Throughout Africa, traditional medicine practitioners are highly respected and known to treat illnesses of which snakebite is not an exception. In addition, as snakebites majorly occurs in the rural settings, it is not surprising that more than 80% of snakebite victims point of first visits are the traditional healers in or near the incidence site for treatment. However, none of the traditional or herbal remedies has been proved to be effective in clinical trial to date. Therefore, adequate and properly designed scientific research is highly encouraged to validate and determine the efficacy of African traditional practices or remedies (WHO, 2010; Habib, 2013 and WHO, 2015). Globally the standard antidote for snakebite victims is anti-snake venom (ASV). It is a purified immunoglobulins obtained from hyperimmunised animal in most cases horse (WHO, 2010). But, this antidote in most cases is poorly supplied or inadequately accessible especially at localities where most snakebite cases do occur (i.e. rural areas). Therefore, the most frequent point of call is the traditional healer (Kadir *et al.*, 2015).

There are various method and practices used to treat snakebite by traditional medicine practitioners. For instance, incision or excision at the bite site has been seriously discouraged by the World Health Organization. The traditional practices includes: incision at bite site, applying black stone (that is snake stone), tourniquets and herbal remedies (WHO, 2010; Hifumi *et al.*, 2015). Combinations of two or more of this practice are usually applied with herbs in common. Modes of administration are topical applications of the plant or its sap on to the bitten area,

chewing leaves or barks or drinking extracts or decoctions or injecting the extracts. Extracts from plants are the most common remedy used for snakebite treatment in countryside. Leaves and roots are the most common plant parts used in the snakebite remedy (Kunjam *et al*, 2013)

2.4.6 Plants in the traditional practice of snakebite management

Worldwide there are approximately 400,000 vascular plants 370,000 flowering plants and 18,000 medicinal plants (Kew, 2016). In addition, approximately 700 plant species are known to possess potential antivenom principles (Kadir *et al.*, 2015). The use of plants in African settings for the treatment of snakebite is often widely accepted. Africa has been one of the world's continents with high level of snakebites and envenomations incidence but also at the same time endowed richly with traditional knowledge and valuable plant resources (Kasturiratne, 2008, Gupta and Peshin, 2012). Harrison *et al., (*2009) mentioned snakebite envenoming as a disease of poverty and has established a link between it and poverty. This is supported by the fact that majority of African population rely on alternative medicine being readily available, cheap and affordable for treatment of diseases including snakebite envenoming (WHO, 2010; Gupta and Peshin, 2012).. Many plants in this region have been documented to have anti-snake venom potentials of which some have been investigated scientifically. Some of the plants are: *Hibiscus esculentus*, *Casearia sp.*, *Musa paradisiaca*, *Mucuna pruriens*, *Eclipta sp.*, *Curcuma longa*, *Bauhinia forficata*, *Annoma senegallensis*, *Mikania glomerata*, *Piper sp.*, *Cordia verbenacea*, *Pentaclethra macroloba* (Kaushik *et al.*, 2013),

In Nigeria, among the medicinal plants claimed to have been used in the folkloric management of snakebites, few have been evaluated scientifically to validate the claim and to establish their safety and efficacy (Kaushik *et al.*, 2013). See table 2.2 below;

Table 2.2: Some Plants Used in Nigeria Traditionally to Treat Snakebites

Family	Plant	Local name	Reference
Acaenthaceae	*Asysasia gangetica*	Inana	Abdel-ghani, 2016
Acanthaceae	*Barteria maderaspatensis*	Ewe-asaju	Abdel-ghani, 2016
Amaranthaceae	*Alternantheras selsilis*	Mai –kai dubu	Abdel-ghani, 2016
Asclepiadaceae	*Gymnema sylvestre*	-	Abdel-ghani, 2016
Asclepiadaceae	*Calotropis procera*	Tumfafiya	Ameen et al, 2015
Astereceae	*Ageratum conyzoides*	Akoyun	Ameen et al, 2015
Astereceae	*Aspilla africana*	Nyerki	Ameen et al, 2015
Balanitaceae	*Balanites aegyptiaca*	Tanni	Ameen et al, 2015

Combretaceae	*Chestia ferruginea*	Esise	Abdel-ghani, 2016
Combretaceae	*Guiera senegalensis*	Sabara	Ameen et al, 2015
Fabaceae	*Parkia biglobosa*	Dorawa	Ameen et al, 2015
Liliaceae	*Allium cepa*	Albasa	Ameen et al, 2015
Liliaceae	*Allium sativum*	Tafarnuwa	Ismaila et al, 2012
Moringaceae	*Moringa oleifera*	zogale	Ameen et al, 2015
Solanaceae	*Solanum sp*	Gardili (Eggon)	Ibrahim *et al* 2015

Preliminary investigations on the potentials of some plants as antidotes against snake bite were found to yield a promising result. Extract of the leaves of *Guiera senegalensis* was found to detoxify (*in vitro*) venom from two common northern Nigerian snake species, *Echis carinatus* and *Naja nigricollis* (Abubakar *et al*., 2000).

2.4.7 Bioactive principles from plants with antivenin activity

Various studies have revealed that the alkaloids (*Eclipta prostrate*, *Rauvolfia serpentina*, *Strychnos nux-vomica*, and *Mimosa pudica*), esters (*Gloriosa superba*), phenolic fraction (*Hemidesmus indicus*), terpenoids (*Aristolochia indica*, *Andrographis paniculata*), and flavonoids fractions (*Tephrosia purpurea*) neutralized the snake venom activities (Kadir *et al.*, 2015). Inhibitory activity of many medicinal plants against the snake venom enzymes has been confirmed by biological assays. Compounds found in these medicinal plants belong to chemical classes capable of interacting with macromolecular targets (enzymes or receptors). Most of the studies based on isolation of the active principle or partially purified extract revealed the presence of aristolochic acid, alkaloids, steroids, flavanoids, phenols, pterocarpanes, quinonoid xanthenes, resveratrol, glycoside and tannins. (Ismaila and Adamu, 2012; Kaushik *et al*., 2013).

2.5 Pharmacognostic Evaluation of Medicinal Plants

Most of the research in pharmacognosy has the aim of identifying controversial species of plants, authentication of commonly used traditional medicinal plants through morphological, phytochemical and physicochemical analysis. The importance of pharmacognosy has been widely felt in recent times. Unlike taxonomic identification, pharmacognostic study includes parameters which help in identifying adulteration in plant dry powder. This is again necessary because once the plant is dried and made into powder form, it loses its morphological identity and easily prone to adulteration. Pharmacognostic studies provide descriptions on plant identity and laid down standardization parameters, which will help in authentication of the plants and ensures reproducible quality of herbal products resulting to safe and effective natural products (Sumitra, 2014).

Pharmacognostic evaluation includes macroscopic, microscopic, physicochemical, fluorescence and phytochemical studies of herbal parts or powdered drugs. Herbal raw material shows a number of problems when quality and authentication aspects are considered. This is because of nature of herbal parts, ingredients and different phytochemicals present in plants (WHO, 2011). To ensure quality of herbal medicines, proper control of starting raw material is very important. The physico-chemical evaluation includes qualitative and quantitative assays and instrumentation analysis. Qualitative and quantitative chemical tests include the presence or absence, quantity, number, values and identification of various phytochemicals like flavonoids, glycosides, saponins, alkaloids etc (Evans, 2009). Macroscopic identity of medicinal plant materials is based on sensory evaluation parameters such as; shape, size, colour, texture, odour and taste while microscopy involves comparative microscopic inspection of powdered herbal drugs. Further, advances in microscopic techniques have increased the accuracy and capabilities of microscopy as a means of herbal crude material identification due to the application of improved light and scanning electron microscopes (SEM) in herbal drug standardization (Bhutani, 2003).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.2 Materials

3.1.1 List of reagents and solvents

Acetic acid (Avondale Laboratory, England)**,** Aluminium chloride**,** Anisaldehyde (Sigma-Aldrich, St. Lous, MO, USA)**,** Chloral hydrate (BDH Laboratory Chemicals Division, POOLE. England)**,** Chloroform (JHD, AR; Lobal Chem, India)**,** Dragendorff Reagent, Picric acid reagent,

Mayer's reagent, Ethyl acetate (JHD, AR; Lobal Chem, India)**,** Fast green**,** Ferric chloride**,** Glycerol (BDH Laboratory Chemicals Division, POOLE. England), Hexane (JHD, AR; Lobal Chem, India), Hydrochloric acid (BDH Laboratory Chemicals Division, POOLE. England)**,** Libermann-Buchard reagent**,** Methanol (JHD, AR; Lobal Chem, India)**,** Methylene blue**,** Phloroglucinol, Sudan Red Solution**,** Tetraoxosulphate (iv) acid (Sigma-Aldrich, St. Lous, MO, USA)**,.**

3.1.2 List of equipments

Compound microscope (Fisher Scientific, UK), Dessicator, Disposable syringes, Glass Slides and Cover slips, KERN EW Electronic Balanced, Laboratory glass wares (Funnel, Conical flask, Beakers, Measuring cylinder), Mechanical shaker (Stuart Scientific Flask Shaker, Great Britain), Metallic cages and feeding bottles for rats, Microtome (C 740527, Cambridge Instrument Company Ltd, London and Cambridge, England), Oven, Photographic camera, Plant press (local made), Slide dryer (Hospital and Lab. Supply Ltd, London, UK), Stage Micrometer and Ocular Lens (Graticules Ltd, Ton bridge, Kent. England), TLC tanks (Uni kit® TLC Chromatank®, TLC silica gel 60 F_{254} pre-coated plates (Merk-Germany)**,** Zinc chloride.

Shandon Southern Germany), UV lamp, Water bath (HHS, Mc Donald Scientific International).

3.1.3 Collection, identification and preparation of plant material

S. virosa sample comprising of leaves and fruits were collected on 20th February, 2016 from a wild field in Kakiyayi town, Zaria Local Government area of Kaduna State-Nigeria by traditional herbal practitioner. Sample of intact leaves containing fruits were authenticated by comparism on the herbarium specimen (Voucher no. 2520) at the Herbarium unit of the Department of

Biological sciences, Ahmadu Bello University Zaria-Nigeria. The sample was then cleared (dust, sand and earthy material removed) air dried under shade, coarsely powdered using mechanical grinder and stored in polythene bag for further use.

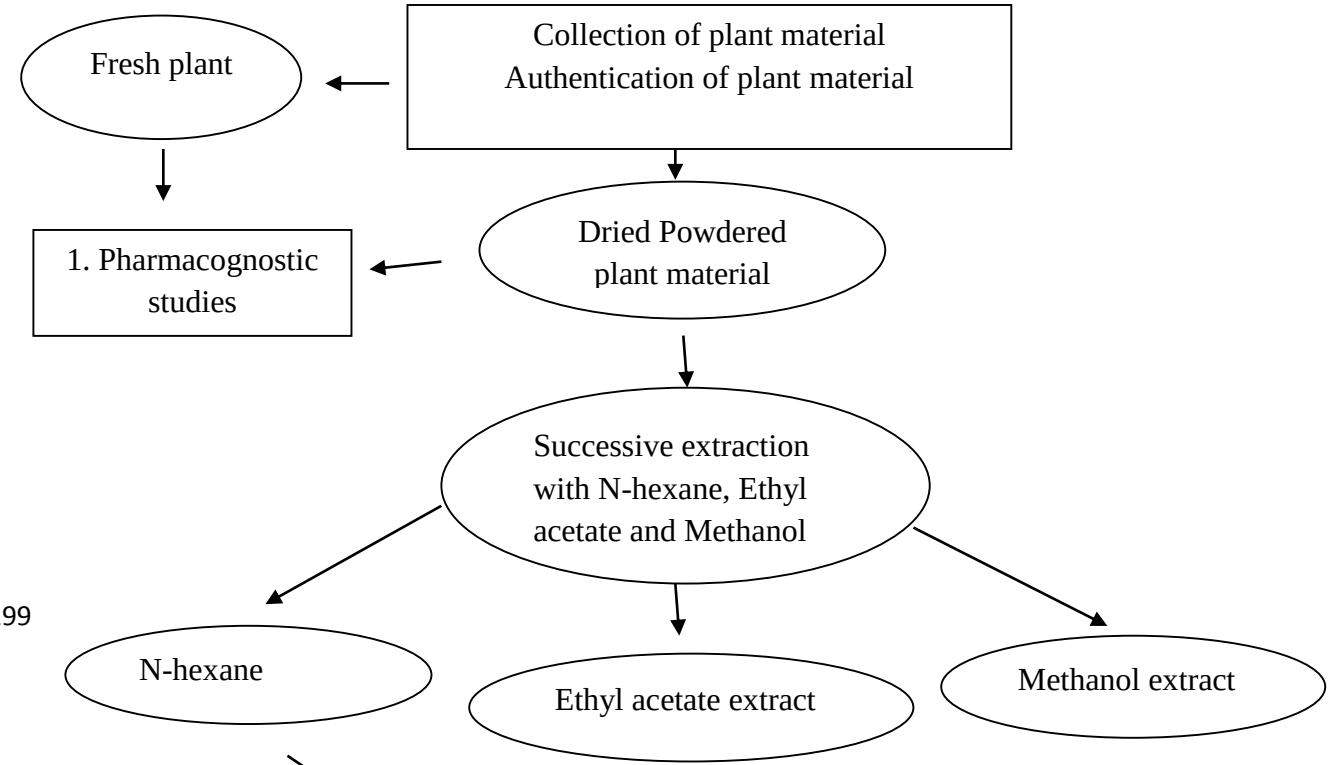
3.1.4 Extraction of plant materials

Leaf powder (300g) of *S. virosa* was extracted successively in 1 litre each of hexane, ethyl acetate and methanol using soxhlet extraction apparatus at moderate tempreture (55^{o}C). The extracts were concentrated under reduced pressure and stored in a desicator for further experiments.

3.1.5 Experimental animals

3.1.5.1 *Mice*:

A total of 80 Swiss Albino mice of both sexes, weighing 15-30 g were obtained from the Animal House facility of Department of Pharmacology and therapeutics, Faculty of Pharmaceutical Sciences, Ahmadu Bello University Zaria. Approval from ethical committee of the University was obtained before the animal experiment. The mice were acclimatized to the environment for 7 days, fed on normal rodent feed (from Vital Feed) and water served *ad libitum* at room temperature. They were divided into five groups of four mice each for the *in vivo* venom neutralization studies.


Collection of plant material
Authentication of plant material
Fresh plant
1. Pharmacognostic studies
Dried Powdered plant material
Successive extraction with N-hexane, Ethyl acetate and Methanol
N-hexane
Ethyl acetate extract
Methanol extract

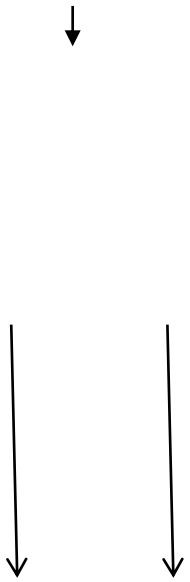


Figure 3.1: Experimental design for the Pharmacognostic and antivenin studies on the leaf of *Securinega virosa*.

3.2 Methodology

3.2.1 Microscopic studies on the leaf of *Securinega virosa*

3.2.1.1 *Surface preparation and anatomical section*

Anatomical section of the leaf sample was prepared and examined under the light microscope. The features observed were described by using the terms according to Dutta (2003) and Evans (2009).

Leaf epidermis was peeled off with a razor blade. The leaf sections were cleared using 70% chloral hydrate, mounted with dilute glycerol and observed for epidermal cells, stomata,

trichomes etc. and their types, as well as, positions. Transverse section across the midrib of the fresh leaves of the plant was prepared, cleared and observed under the microscope as described by Evans (2009).

3.2.1.2 Micrometric evaluation

It consists of measurements of dimensions (length and width) of the various diagnostic microscopic characters of the leaf namely; stomata, trichomes etc. of *S.virosa*. These activities were carried out by using a binocular microscope with the aid of graticles (Kokate, 2003).

3.2.1.3 Quantitative leaf microscopy

This involves counting the specific histological features of the leaf. The five (5) physical constants of the leaves of S. *virosa* were as follows;

(a). Palisade Ratio

It is the average number of palisade cells beneath each epidermal cell of leaf. Section from the upper epidermis of the plant was cleared with boiling 70% chloral hydrate solution and mounted on a clean microscope slide with dilute glycerol and examined with the aid of x 40 objective. A camera lucida was set up and the palisade ratio determined in groups of four and the average taken (Evans, 2009).

(b). Stomatal Number

This is the average number of stomata per square millimetre of the upper and lower epidermis of the leaves of the plant. Section from the upper and lower epidermis of the plant were cleared

with boiling 70% chloral hydrate solution and mounted on a clean microscope slide with dilute glycerol. A camera lucida was set up. With the aid of a stage micrometer a paper was divided into squares of 1mm^2 using x 10 objective. The stomata were traced and counted in the fields on a single section of the leaf of the plant and the average number of stomata per mm^2 of epidermis were recorded (Evans, 2009).

(c). Stomatal Index

This is the percentage proportion of the number of stomata formed to the total number of epidermal cells of the leaf. Sections of the epidermal portion of the leaves was mounted and examined as in stomatal number determination, except that here both stomatal and epidermal cells were counted. The stomatal index was calculated using the formula below (Evans, 2009):-

$$\text{Stomatal Index} = \frac{\text{Number of Stomata}}{\text{No.of Epidermal Cells } + \text{No.of Stomata}} \times 100$$

(d). Vein-islet number

Vein-islet number is the number of vein-islet per unit square millimetre calculated from four contiguous square millimetres in the central part of the lamina midway between the midrib and the margin. It was determined by boiling pieces of leaf of the plants in a test-tube containing 70% chloral hydrate solution, and then followed by treatment with 10% hydrochloric acid to remove calcium oxalate crystals for enhanced visibility. A camera lucida was set up and by means of a stage micrometer the paper was divided into squares of 1 mm^2 using x10 objective. The stage micrometer was replaced by the cleared preparation of the leaf and the veins traced in four contiguous squares that is a rectangle 1mm x 4mm. Each vein was traced and areas which

are completely enclosed by veins were counted and those that were not completely enclosed were excluded (Evans, 2009).

(e). Vein-islet termination number

It is the vein termination present in one square millimetre of leaf surface midway between midrib and margin. It was determined for *S. virosa* leaf using a camera Lucida set up as in vein-islet number but here the termination number in each square was counted (Evans, 2009).

3.2.2 Determination of physicochemical constants of the leaves of *S. virosa*

Physicochemical parameters for the leaf sample of the plant were determined. Five (5) physical constants were determined for the powdered leaves of *S.virosa*. They were moisture content, ash value, acid insoluble ash value, and alcohol and water extractive values. Three (3) different determinations were carried out for each parameter and the average calculated. The methods outlined in WHO (2011) Quality Control Methods for Medicinal Plant Materials was used.

3.2.2.1 Moisture content

It is the quantity of moisture present in a plant material. Moisture content of the leaves of *S. virosa* was determined by loss on drying method. About 3.0 g each of the powdered leaves eres accurately weighed and placed in to three (3) clean, dried evaporating dishes of known weights. These were placed in an oven and heated at a temperature of 105ºC for a 1 hour, then cooled in a desicator and re-weighed. Heating and weighing was repeated to a constant weight. The weight loss on drying was computed using the formula below:

$$\% \text{ Moisture content} = \frac{Weight\ of\ water\ lost}{\text{Original weight of Sample}} \times 100$$

3.2.2.2 Total ash value

This is the ash remaining after ignition of medicinal plants. 2 g of powdered plant materials was accurately weighed and placed separately in a crucible of known weight. It was then heated gently and the heat gradually increased until it is white indicating the absence of carbon. It was allowed to cool in a desicator and weighed. The heating and cooling was repeated until a constant weight was obtained and no change in colour. The total ash-value of the plant specie was determined as a percentage with the formula below:

$$\text{Total ash value} = \frac{\text{Weight of Residual Ash}}{\text{Initial Weight of Sample}} \times 100$$

3.2.2.3 Acid-insoluble ash

This is the residue that remains after boiling the total ash with dilute hydrochloric acid. This was determined for the leaves. 25 ml of dilute hydrochloric acid was added to the crucible containing each of the ash. It was covered with a watch glass and gently boiled for 5mins. The watch glass was rinsed with 5 ml of hot water and the liquid added to the crucible. The insoluble matter was collected on an ash less filter-paper and washed with hot water until the filtrate is neutral. The filter-paper containing the insoluble matter was transferred to the original crucible, dried in an oven and ignited to a constant weight. The residue was allowed to cool in a suitable desicator for 30 minutes and then weighed without delay. The acid-insoluble ash was calculated as a percentage using:-

$$\text{Acid Insoluble Ash} = \frac{\text{Weight of Residual Ash}}{\text{Initial Weight of Sample}} \times 100$$

3.2.2.4 Alcohol-Soluble Extractive Value

This is the amount of extractive in percentage of a plant sample with alcohol. 4 g of the plant material was weighed in a conical flask. 100 ml of ethanol was added and macerated for 24 hours, during which the mixture was frequently shaken within the first 6 hours using a mechanical shaker. It was filtered and 25 ml of the filtrate transferred into an evaporating dish of known weight and evaporated to dryness on a water bath. It was dried to a constant weight, the percentage of alcohol-soluble extractive value was then determined as follows:

$$\text{Alcohol Soluble Extractive Value (\%)} = \frac{\text{Weight of Residue in 25ml Extract} \times 4}{\text{Initial weight of Sample}} \times 100$$

3.2.2.5 Water-soluble extractive value

This is the amount of extractive in percentage of a plant sample with water. Same procedure as in alcohol-soluble extractive value was repeated here for the plant material, but water was used in place of alcohol and the value calculated as follows:

$$\text{Water Soluble Extractive Value (\%)} = \frac{\text{Weight of Residue in 25ml Extract} \times 4}{\text{Initial weight of Sample}} \times 100$$

3.2.3 Chemomicroscopic examination of the leaves of *S. virosa*

For the histochemical detection of cell walls and contents of the plant, the methods outlined in the updated edition of quality control methods for medicinal plant materials (WHO, 2011), was adopted.

3.2.3.1 Cell wall materials

I) Cellulose cell walls

Two drops of iodinated zinc chloride were added to the powdered leaf sample and allowed to stand for few minutes and observed under a microscope. It changed the cellulose cell wall to blue colour. Cellulose is present.

ii) Lignified cell walls

The powdered plant material was moistened on a slide with a small volume of phloroglucinol and allowed to stand until almost dry. A drop of concentrated hydrochloric acid was added and viewed under a microscope. Pink stained was observed. Lignin is present in the cell wall.

iii) Suberized or Cuticular cell walls

Two drops of Sudan red was added to the powdered leaf sample and allowed to stand for a few minutes and observed under a microscope. Presence of orange red colour was observed.

iv) Gum and Mucilage

A drop of Ruthenium red was added to the dry powdered leaf sample and viewed under the microscope. Mucilage often appeared as transparent, spherically dilated fragments on a black background with pink colour on the cell wall which indicates that mucilage is present.

3.2.3.2 Cell inclusions / Cell contents

i) Starch grains

A small volume of iodine was added to the powdered leaf sample and viewed under the microscope. Observation of blue-black colour was noted. This reveals the presence of starch grains in the powdered sample of *S.virosa* leaf.

ii) Aleurone grains

A few drops of ethanol was added to the powdered leaf sample and observed under the microscope. No yellowish-brown was observed which indicated the absence of aleurone grains.

iii) Test for Calcium oxalate crystals and Calcium carbonates

To a small portion of the cleared leaf powder of the plant, 10 % HCl was added; dissolution of crystals in the powdered drug without effervescence indicated the presence of calcium oxalate and calcium carbonate.

Iv) Inulin

A drop of 1- naphthol and that of concentrated sulphuric acid was added to the powdered leaf sample and viewed under the microscope. Observation: A spherical aggregation of crystals of inulin turned brownish red and dissolved. Inulin.is present.

v) Tannins

A drop of ferric chloride was added to the powdered leaf sample and viewed under the microscope. Observation: A greenish black colour was seen. Tannins are present.

3.2.4 Preliminary phytochemical screening of *S. virosa* leaf extracts

Extracts of n-hexane, ethyl acetate and methanol were subjected to preliminary phytochemical screening using the standard method outlined below:

3.2.4.1 Test for Saponins

a) Frothing Test: About 0.5g each of the extracts was shaken with water in a test tube followed by warming on a water bath. There was a presence of frothing which persists on warming except in n-hexane extract (Sofowora, 2008).

b) Haemolysis Test: 2 ml of Sodium Chloride (1.8% solution in distilled water) were added to two test tubes A and B, followed by 2ml of distilled water and then 2ml of extract was added to test tube B alone. About 5 drops of blood were added to each tube and the tubes were inverted gently to mix the contents. Observation: Haemolysis in tube B containing the extract was noted. Saponins are present except in n-hexane (Brain and Turner, 1975).

3.2.4.2 Test for Steroids / Triterpenes

a) Lieberman-Buchard Test: a small portion of the extract was dissolved in chloroform and filtered. Equal volume of acetic anhydride was added to the filterate, followed by concentrated sulphuric acid down the side of the test tube. Observation: A Brown ring at the inter-phase was observed, which indicated the presence of steroids/triterpenes (Evans, 2009).

b) Salkowski Test: a small quantity of the extract was dissolved in 1ml of chloroform, filtered. To the filtrate, 1ml of concentrated sulphuric acid was added down the side of the test tube.

Formation of red ring at the interphase was observed which indicated the presence of sterols (Sofowora, 2008).

3.2.4.3 Test for Flavonoids

a) Shinoda Test: About 0.5g of the extract was dissolved in 5 ml of 95% ethanol, warmed and filtered. Three (3) pieces of magnesium chips were added followed by five drops of concentrated hydrochloric acid. Observation: Appearance of an orange color was noted. Flavonoids are present except in n-hexane extract (Evans, 2009).

b) Sodium hydroxide Test: About 0.5 g each of the extracts was dissolved in water and filtered. To the filtrate, 2ml of 10% aqueous sodium hydroxide solution was added. Observation: the solution turned yellow, which change to colorless on addition of dilute hydrochloric acid. This indicates the presence of flavonoids except in n-hexane extract (Evans, 2009).

3.2.4.4 Test for Tannins

a) Ferric chloride Test: About 0.5 g each of the extracts was stirred with 10 ml distilled water and filtered. Two drops of 1% ferric chloride solution was added to 2ml of the filtrate. Blue-black precipitate (ppt) was observed except in n-hexane. Hydrolysable/gallitannins are present in methanol and ethyl acetate extracts (Evans, 2009).

b) *Lead sub-acetate Test:* To 0.5 g each of the extracts, three drops of lead sub-acetate solution were added. Observation: Black green precipitate was produced except in n-hexane extract. Tannins are present (Evans, 2009).

3.2.4.5 Test for Alkaloids

About 1.0 g each of the extracts was stirred with 20 ml of 1% aqueous hydrochloric acid on a water bath and filtered. The filterate, each was basified with concentrated NH_4OH and extracted with chloroform. The chloroform layer was extracted further with 20 ml of 1% HCL. The aqueous layer was divided into three portions for the following tests:

i) To the first portion, 1 ml of freshly prepared Dragendorff's reagent was added drop-wise and observed. Rose red to brownish precipitates was formed except in n-hexane extract.

ii) To the second 1 ml of Mayer's reagent was added drop-wise and observed. Cream color precipitates were formed extract in n-hexane extract.

iii) To the third, 1 ml of Wagner's reagent was added, a reddish-brown precipitates were formed except in n-hexane extract (Evans, 2009).

3.2.4.6 Test for Anthraquinones

a) Borntrager's Test: To small portion of each of the extracts, 10 ml of benzene was added, shaken and filtered. To the filtrate, 5ml of 10 % ammonia solution was added. The mixture was shaken gently. Observation: No pink red colour in the lower part of the aqueous layer was formed. Anthraquinones are absent in all the extracts (Evans, 2009).

b) Modified Borntrager's Test: the extract was boiled with 10 ml of aqueous sulphuric acid and filtered hot, and then it was allowed to cool to room temperature. The filtrate was shaken with 5 ml benzene, the benzene layer was separated and to half of its volume, 10 % ammonium

hydroxide was added. No formation of pink-red coloration in the ammonia phase (lower phase) was produced. Combined anthraquinone or anthraquinone derivatives were absent (Evans, 2009).

3.2.4.7 Test for Cardiac Glycosides

a) Keller-Killiani Test: About 0.5 g each of the extract was dissolved in 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was then under layered with 1 ml of concentrated sulphuric acid. Observation: A brown ring was produced at the interphase. Deoxy sugars characteristic of cardenolides are present in all the extracts (Evans, 2009).

b) Kedde's Test: Few drops of 2 % solution of 3, 5- dinitrobenzoic acid in 95 % alcohol were added to small portion of each of the extracts. A solution of 5 % sodium hydroxide was added to make the solution alkaline. Observation: No purple-blue colour was observed in all the extracts. Cardenolides are absent (Evans, 2009).

3.2.5 Development of TLC fingerprint of *S. virosa* leaf extracts

3.2.5.1 Development of Solvent System

Solvent system was developed using a combination of different solvents based on polarity and the solvent systems that give reasonable separation of the components of the crude extracts were documented for phytochemical analysis. The TLC plate was spotted with portion of the extract and developed in chromatographic tank containing a mixture of solvents. It was then removed from the tank at certain point of solvent front, dried and observed in day light, in ultra violet light and by spraying with universal spray reagent P-anisaldehyde for detection of various phytochemicals such as phenols, steroids, triterpenes and so on (Wahab *et al.*, 2010)

3.2.5.2 Thin layer chromatography phytochemical screening

The TLC profiles of *S. virosa* extracts were studied on pre-coated silica gel plate by spotting on the TLC plate. It was developed in the suitable solvent system based on the preliminary studies of the methanol, ethyl acetate and n-hexane extracts. The plate was viewed under UV light/iodine vapour and sprayed with 10 % H_2SO_4 in ethanol, and heated at 110^0C (Hahn , 2007). In addition, developed plates were sprayed with specific detecting reagents such as ferric chloride for phenolic compounds, aluminium chloride for flavonoids, Liberman- Burchard for steroids/triterpenes, Borntrager's for Anthraquinones, Dragendorff's for alkaloids and Iodine vapour for saponins. The separated components were detected visually.

3.2.6 Phospholipase - A2 inhibitory assay of extracts of *S. virosa* on *Naja nigricollis* venom

3.2.6.1 *Source of venom and preparation*

The venom was obtained from *Naja nigracolis* by milking, using markflane (1967) method by a professional snake charmer at Ahmadu Bello University Veterinary Teaching Hospital Zaria. Briefly, the snake was held captive by the charmer, mouth opened and fangs placed on the edge of a glass container covered with polythene. The milking was enhanced by the pressing on and off of the snake tail. The venom drops gradually at intervals into the container. It was then lyophilized and stored in a refrigerator at 2-8^0C.

3.2.6.2. Phospholipase - A_2 inhibitory assay of S. virosa leaf extracts

The method used by Sallau *et al.,* (2005) was adopted. To a set of four (4) test tubes labeled (1-4), 100 µl, 75 µl, 50 µl and 25 µl of egg yolk suspension was added respectively. This was followed by addition of 50 µl of 1 mM $CaCl_2$, 100 µl of 20 mg /mL venom solution and 50 µl of 0.1 M phosphate buffer at pH 7.0. The mixtures were then made up to 300 µl with distilled water and incubated at 37^oC for 30 minutes then deactivated in a water bath at 100^oC for 2 minutes. Three (3) drops of an indicator (phenolphthalein) were added and the mixtures were titrated against 20 mM NaOH solution to end point. The molarity of the free fatty acid released was calculated to obtain the activity of phospholipase A_2. The experiment was repeated using phosphate buffer containing varying concentrations of the *S.virosa* crude extracts 0mg/ml, 2.5 mg/ml, 5 mg/ml and 10 mg/ml. The initial velocity data obtained was used to draw line weaver-Burk's graph to obtain intercepts for the respective plots which were used in the secondary plot (Dixon's plot). Dixon's plot (intercept against S) was` drawn to obtain the inhibition binding constant (KI).

$$Initial\ velocity = \frac{\text{Mb X Vb}}{\text{Va}}\ X\ 100$$

Ma = molarity of free fatty acid released = Initial velocity = x

Mb = molarity of base in millimole

Vb = volume of base used = titre value

Va = Total volume of the mixture

3.2.7 Studies on the modulatory effect of *S. virosa* leaf extracts on *Naja nigricollis* snake venom

3.2.7.1 Assessment of in vivo venom neutralization effect of leaf extracts of S. virosa against venom of Naja nigricollis using mice

a) Acute toxicity studies (Median Lethal Dose) of *S. virosa* leaf extracts

The median lethal dose (LD_{50}) of the extracts was estimated using the lorke's method (1983). Nine (9) mice were divided into 3 groups of 3 mice each. Mice in groups 1, 2 and 3 were given 1000, 100 and 10 mg/kg of the extract of *S. virosa* each respectively. They were observed for signs of toxicity in addition to mortality rate over a 24 hours period. While in the second phase four groups each comprising of one mouse labeled 1, 2, 3 and 4 were given 140 mg, 225 mg, 370 mg and 600 mg doses of n-hexane extract and 1200 mg, 1600 mg, 2900 mg and 5000 mg of ethyl acetate extract of *S. virosa* respectively.. Then, the LD_{50} was calculated as the square root of the product of minimum lethal dose and the maximum tolerated dose. The whole process was conducted for both ethylacetate and n-hexane extracts of *S. virosa*.

$$LD_{50} = \sqrt{\text{minimum lethal dose} \times \text{maximum tolerated dose}}$$

b) Assessment of in vivo venom neutralization on the extracts of S. virosa in mice

This was conducted according to the method previously described by Abubakar *et al.,* (2000). Also, the median lethal dose of *Naja nigricollis* which is 4.8 mg/kg determined by Abubakar *et al*. (2000) was adopted. Twenty (20) mice were divided into five groups of four mice in each group. First group 1 was given normal saline 0.2 ml and group 2 received 0.2 ml of LD_{99} of *Naja nigricollis* venom alone (control group) while groups 3, 4 and 5 (treatment groups) were given 0.2 ml of a mixture of an equivalent of LD_{99} (9.55mg/kg) containing 5, 10 and 20 mg/ml of extract incubated at 37^0C for 10 min to each mice in a group respectively. All the treatments

were given through intra-peritonial (IP) route. A sign of neurotoxicity and number of death per group was recorded over 24 hours after the injection. The result was presented as percentage death and mean time of death ± standard error of mean (SEM). The procedure was conducted for n-hexane, ethylacetate and methanol extracts of *S. virosa* leaf.

c) Histopathology of the mice control and treatment groups

The relevant tissues; brain, liver, lungs, heart and kidneys were collected from the animal and placed immediately into a fixative (10% formalin), after proper fixing for about 48 hours, the tissues were dehydrated through ascending grades of alcohol from 70% alcohol to 90 % alcohol and absolute(100%) alcohol for 16 hours. The tissues were then cleared in toluene for 2 hours after which they were impregnated in molten paraffin wax for four hours. The tissues were then embedded in paraffin wax and sectioned using a rotary microtome at five micron thickness, the sections were then stained using the heamatoxylin and eosin staining technique. The slides were examined using a light microscope and photomicrographs of the tissues were taken using a digital camera for microscope.

3.2.8 Statistical analysis

The results of pharmacognostic studies were presented as mean ± standard error of mean. The results of biological activity were analysed using One Way Analysis of Variance (one-way ANOVA). p-value of < 0.05 was considered significant. This was followed by post-hoc test using Dunnett. Statistical Package of Social Sciences (SPSS) version 16 was used.

CHAPTER FOUR

4.0 RESULTS

4.1 Plant Collection, Identification and Preparation

S virosa leaves were collected together with other aerial parts (flowers) on 20 February, 2016. The plant was identical to a herbarium sample with voucher number 2520 deposited in Department of Biological Sciences, Ahmadu Bello University, Zaria. The shade dried coarsely powdered plant of 355 g was stored in polythene bag for further use (Plate II).

a) 


b)

Plate II *S. virosa* a) and b) growing naturally in the bush at Kakiyayi village, Zaria, Kaduna state, Nigeria

4.2 Yield of solvents Extraction of Powdered *S. virosa* leaves

Methanol extract produced the highest yield of 46.64 g (15.55%) followed by Ethyl acetate 11.22 g (3.74%) and N-hexane 8.00 g (2.67%) respectively (Table 4.1), following the successive extraction of dried *S.virosa* powder (300 g).

Table 4.1: Percentage Yield of *S. virosa* Leaf Powder (300g)

Solvent	Extract yield in grams (g)	Percentage yield
N-hexane	8.00	2.67
Ethyl acetate	11.22	3.74
Methanol	46.64	15.55

4.3 Pharmacognostic Parameters of *S. virosa* Leaf

4.3.1 Microscopic studies on the leaf of *S. virosa*

Microscopical examination of the leaves of *S. virosa* revealed the presence of some important diagnostic characters on both the upper and lower epidermal layers. These includes: Epidermal cells (Plate III) which are polygonal with straight anticlinal wall and 2-5 -8 µm in size (Table 4.2). Stomata were found only on the lower epidermis. They were anomocytic and paracytic (Plate IV) with stomatal number and index 56.00±0.55 and 12.556±0.33 (Table 4.2) respectively. Unicellular Trichomes (Plate V) are rare but present on both surfaces. Prism crystals of calcium oxalate (Plate III and VI) in a sheaths or singles were in abundance on both upper and lower epidermal surfaces. The transverse section through the midrib of the leaf showed mesophyll, vascular bundle (xylem and phloem), Calcium oxalate crystals and single layer of epidermal cell on the upper and lower leaf surfaces (Table 4.2).

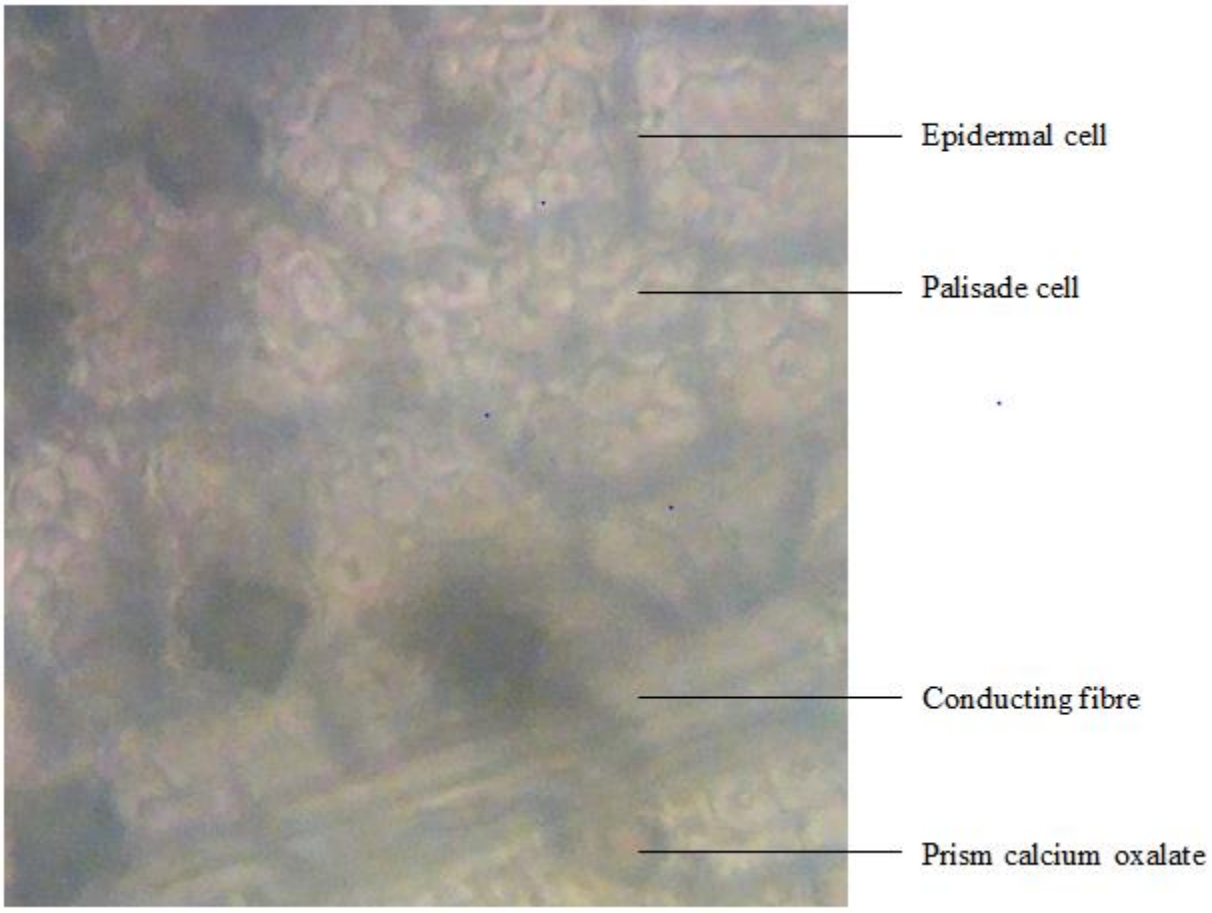


Plate III: Photomicrogaph of some microscopic features of upper epidermal layer of *S. virosa* leaf (X400)

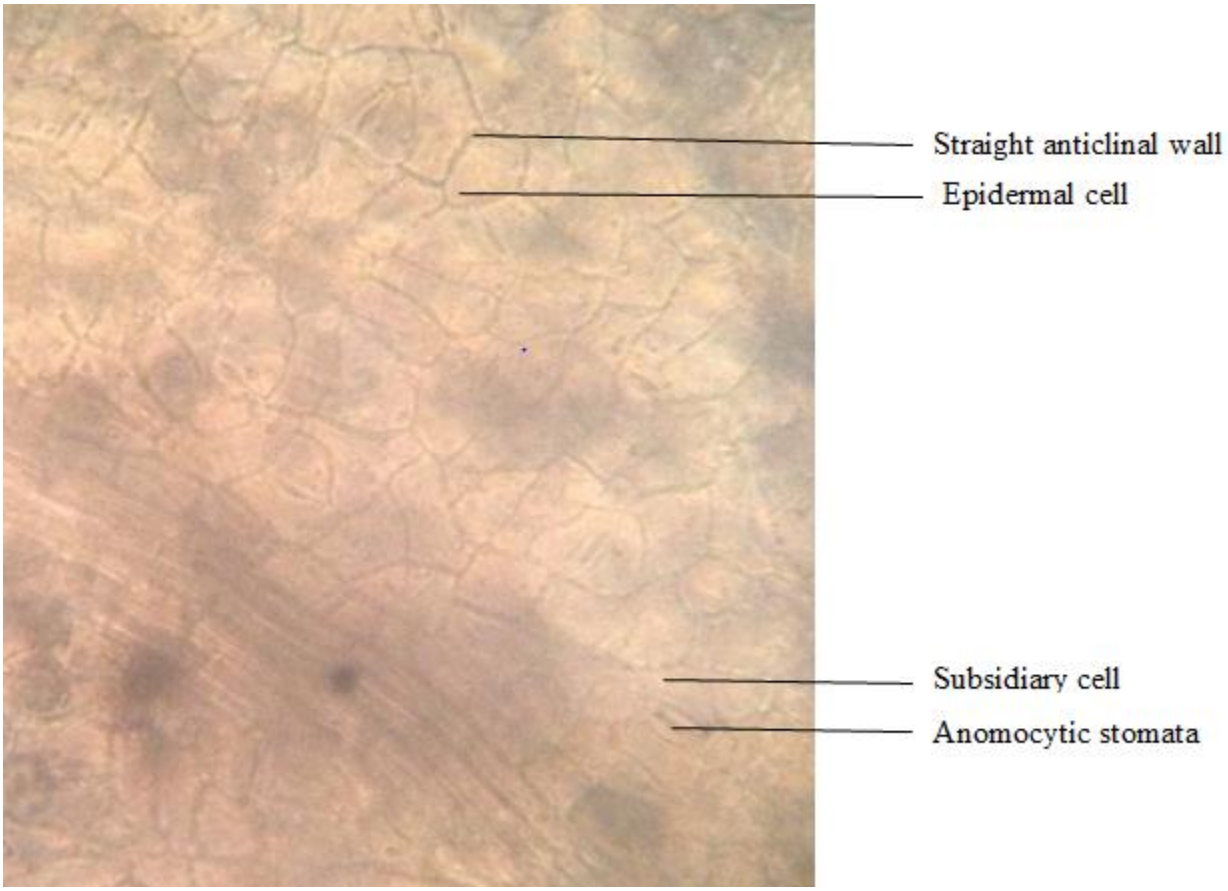


Plate IV: Stomata of the lower epidermal surface of *S. virosa* leaf (X 400)

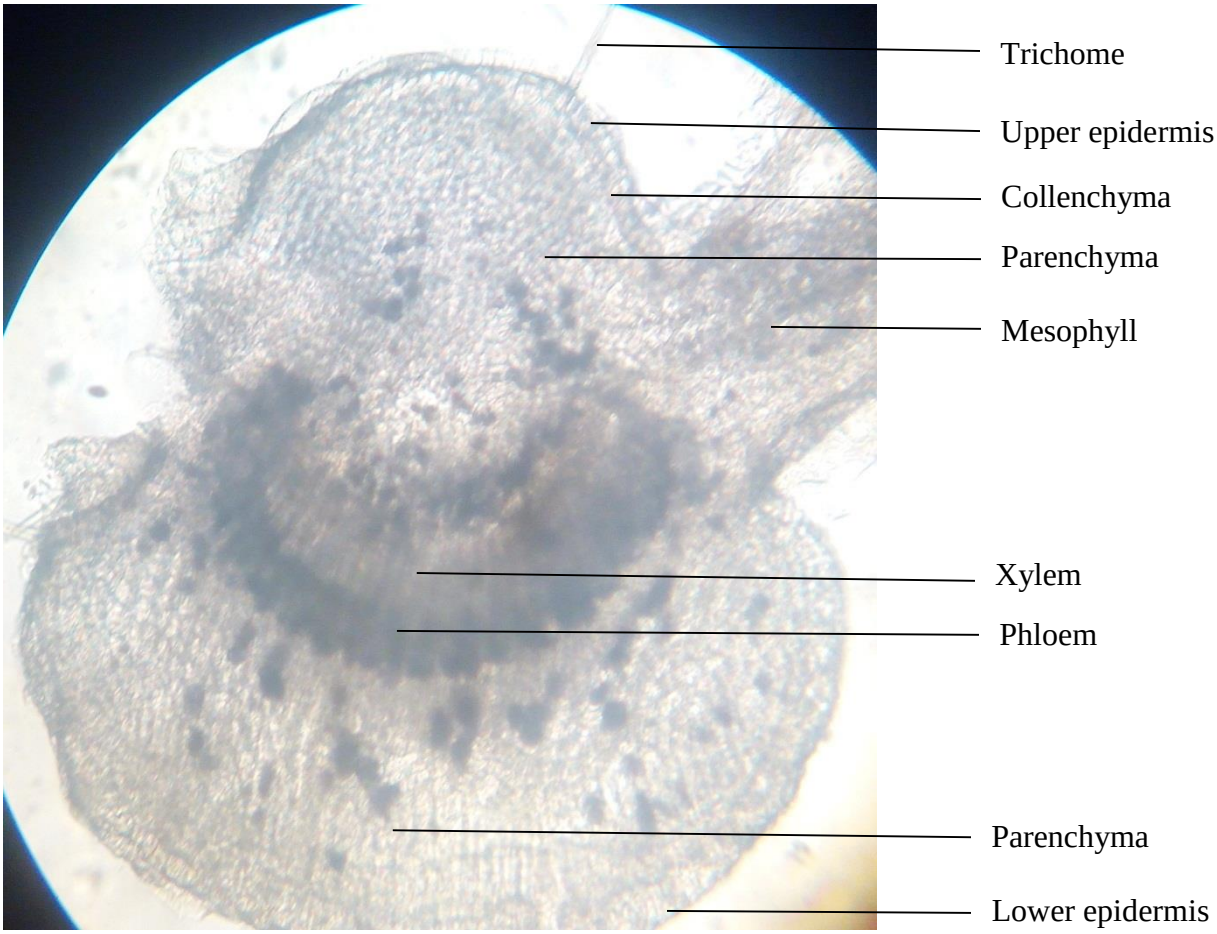


Plate V: Photomicrograph of Transverse section through the midrib of *S. virosa* leaf (X 400)

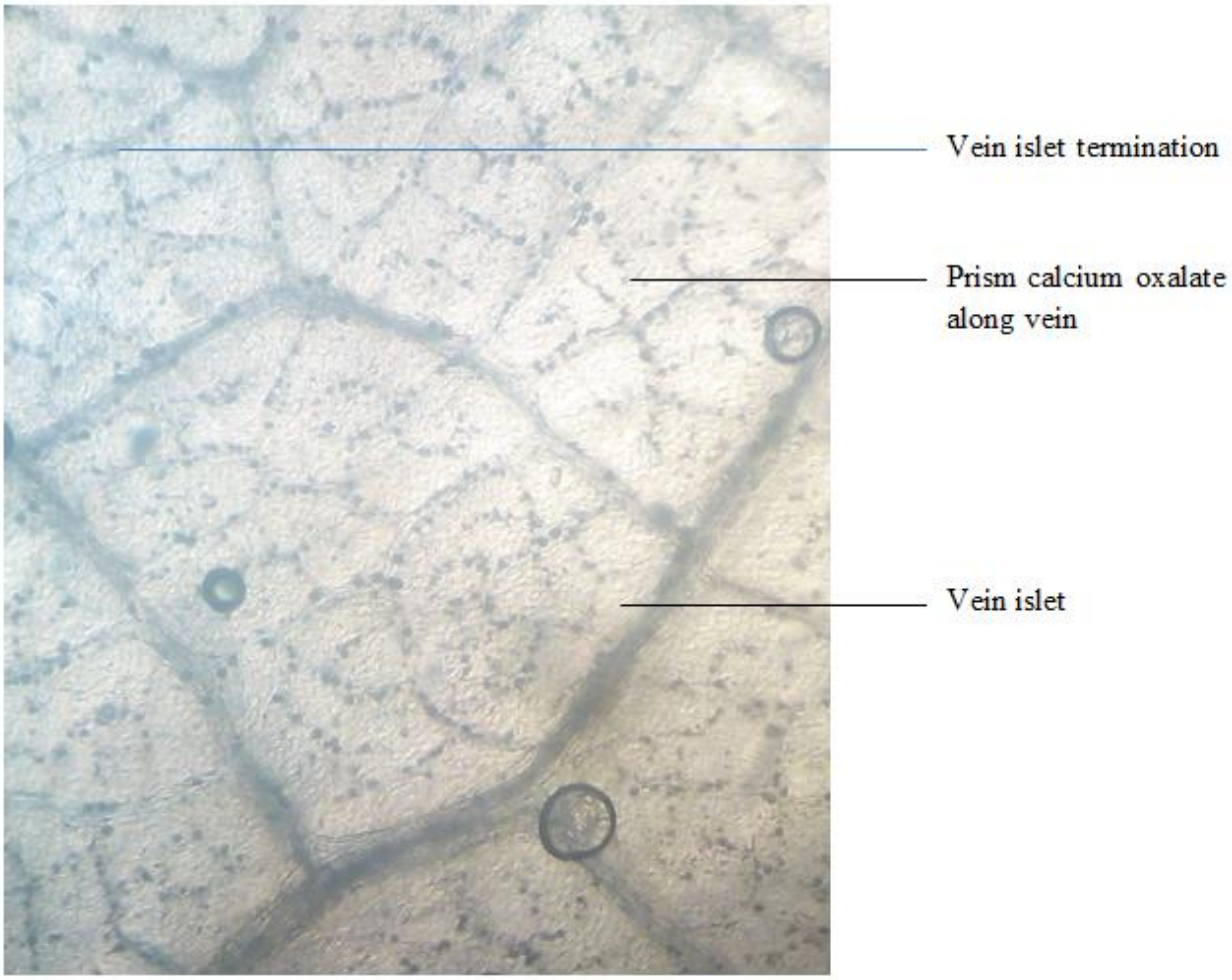


Plate VI: Photomicrograph of adaxial surface of *S.virosa* leaf (X 400)

Table 4.2 Description of the Microscopical Features of the Leaves of *S.virosa*

Characters	Observation	
	Upper Epidermis	Lower Epidermis
Epidermal cell		
Shape	Polygonal	Polygonal
Size	2-5-8 µm	2-5-8 µm
Anticlinal wall	straight	straight
Arrangement	irregular	irregular
Stomata		
Type	absent	Anomocytic
Size	absent	4-5-6 µm
Location	absent	Present
Frequency	absent	rare
Arrangement	absent	irregular
Trichomes		
Surface	smooth	-
Base	not swollen	-
Location	upper epidermis	-
Frequency	rare	-
Form	unicellular	-
Calcium oxalate Crystals		
Location	Free, mostly on veins	Free, mostly on veins
Size	1-2-3 µm	1-2-3 µm
Form	prism	prism
Distribution	Single and in sheath	single and in sheath
Frequency	Frequent	Frequent

Average of three determinations µm = Micrometre

Table 4.3 Quantitative Microscopy of the Leaves of *S.virosa*

Parameter	Mean (µm)* ± SEM
Stomatal Number	56.00 ± 0.58
Stomatal Index	12.67 ± 0.33
Palisade ratio	20.08 ± 1.12
Vein islet Termination Number	5.67 ± 0.33
Vein islet Number	3.33 ± 0.33

*Average of three (3) determinations. SEM = Standard Error of Mean

4.3.2. Physicochemical constants of the leaves of *S. virosa*

The determined physicochemical constants of *S. virosa* leaf include: Average moisture contents using loss on drying method is 5.6%, the percentage yield of total ash and acid insoluble ash were 11.7% and1.2% respectively. The extractive values determined were 22.00% and 33.00% for ethanol and water solvents respectively (Table 4.4).

Table 4.4. Physicochemical Constants of the Leaf of *S.virosa*

Parameters	Values in Percentage (%) as Mean▪ ± SEM
Moisture content	5.5533 ± 0.6190
Total Ash	11.6667 ± 0.3333
Acid insoluble Ash	1.1667 ± 0.1667
Ethanol Extractive	22.0000 ± 1.5280
Water Extractive	33.0000 ± 1.0000

▪ Average of three (3) determinations. SEM = Standard Error of Mean.

4.3.3 Chemomicroscopic characteristics of the leaf of *S. virosa*

Chemomicroscopical examination of the powdered leaf of *S. virosa* revealed the presence of cellulose cell wall, lignified cell wall, tannins, starch, calcium oxalate, suberin and mucilage but aleurone grains were absent (Table 4.5).

4.3.3.1 Cell wall Materials

i) Cellulose: Blue colour was observed on the walls of the epidermal cells which indicated the presence of cellulose.

ii) Lignin: Red stain was observed on the walls of some lignified cell in the plant which indicated the presence of lignin.

iii) Cutin: Red colour was observed on the cell wall in the plant which indicated the presence of cutin.

iv) Gums and Mucilage: Pink colour was observed in the epidermis and vascular tissues of the plant which indicated the presence of mucilage.

4.3.3.2 Cell Contents/Cell inclusions

i) Starch: Blue-black colour on some grains within the cell was observed in the plant which indicated the presence of starch.

ii) Aleurone grains: No yellowish-brown colour was observed which indicated the absence of aleurone grains in the cells.

iii) Calcium oxalate and Calcium carbonate: a clear disappearance of visible calcium oxalate crystals and later effervescence was observed. Dissolution of crystals in the powdered drug without effervescence indicated the presence of calcium oxalate while the presence of effervescence indicated calcium carbonate.

iv) Inulin: A spherical aggregation of crystals of inulin turned brownish-red and dissolved. This indicated the presence of Inulin crystals in the plant powdered sample.

v) Tannins: Greenish-black colour in some parenchyma cells was observed in the plant which indicated the presence of tannins.

Table 4.5 Chemomicroscopic Characteristics of the Powdered Leaf of *S. virosa*

Constituents	Detecting reagents	Observation	Inference
Cell wall Materials			
Cellulose	Chlor-Zinc-Iodine	Blue coloration of cell wall	Present
Lignin	Phloroglucinol	Red stain on the Lignified cell wall	Present
Cutin	Sudan red	Red color on the cell wall	Present
Gum and Mucilages	Rhuthenium red	Pink color on the cell wall	Present
Cell inclusions			
Starch grains	N50 Iodine	Blue-black colour on the grains	Present
Aleurone grains	Ethanol	No Yellowish-brown	Absent
Calcium Oxalate and Calcium carbonate	Hydrochloric acid	Dissolution of crystals occur with Effervescence	present
Inulin	Drop of 1-naphthol and sulphuric acid	Inulin crystals turns brownish-red and dissolved	Present
Tannins	Ferric Chloride	Greenish-black color observed	Present

4.4 Development of TLC Fingerprint of the Extracts of *S. virosa*

4.4.1 Preliminary phytochemical screening of extracts of *S. virosa* leaf

Preliminary phytochemical screening of the n-Hexane (HE), ethyl acetate (EE) and methanol extracts (ME) using standard methods showed that: saponins and steroids/triterpenes were present. However, anthraquinones were absent in all the extracts (Table 4.6)

4.4.1.1 Test for Saponins

a) Frothing Test: There was a presence of frothing which persists on warming except in n-hexane extract. Saponins are present in methanol and ethyl acetate extract.

b) Haemolysis Test: Haemolysis in tube B of both methanol and ethyl acetate extracts were observed. Saponins are present except in n-hexane.

4.4.1.2 Test for Steroids / Triterpenes

a) Lieberman-Buchard Test: A brown ring at the inter-phase was observed which indicated the presence of steroids/triterpenes in all the extracts.

b) Salkowski Test: A red ring at the interphase was observed. This indicated the presence of sterols in the extracts.

4.4.1.3 Test for Flavonoids

a) Shinoda Test: Orange color was observed in methanol and ethylacetate extracts. Flavonoids are present except in n-hexane extract

b) Sodium hydroxide Test: There was a formation of yellow solution of ethanol filtrate of the extracts which changed to colorless on addition of dilute hydrochloric acid. This indicates the presence of flavonoids except in n-hexane extract

4.4.1.4 Test for Tannins

a) Ferric chloride Test: A greenish black precipitate was observed which indicates the presence of condensed tannins except in n-hexane extract.

b) *Lead sub-acetate Test:* Black green precipitate was observed except in n-hexane extract. Tannins are present in methanol and ethyl acetate extracts.

4.4.1.5 Test for Alkaloids

i) Drangendoff's reagent: Rose red to brownish precipitates was observed, indicating the presence of alkaloids .except in n-hexane extract.

ii) Meyer's reagent: Creamy precipitates were observed, indicating the presence of alkaloids except in n-hexane..

iii) Wagner's reagent: A reddish-brown precipitates were observed, indicating the presence of alkaloids except in n-hexane extract.

4.4.1.6 Test for Anthraquinones

a) Borntrager's Test: There was no pink red colour. This indicates the absence of anthraquinone glycosides.

b) Modified Borntrager's: There was no pink-red colour. This indicates the absence of combined anthraquinones in all the extracts.

4.*4.1.7 Test for Cardiac Glycosides*

a) Keller-Killiani Test: A brown ring was produced at the interphase. Deoxy sugars characteristic of cardenolides are present in all the extracts.

b) Kedde's Test: There was no purple-blue colour observed. Cardenolides are absent.

Table 4.6 Preliminary Phytochemical Screenings on Extracts of *S. virosa* leaf

Tests	Hexane Extract	Ethyl acetate Extract	Methanol Extract
Saponins			
Frothing	Absent	Present	Present
Haemolysis	Absent	Present	Present
Steroids/Triterpenes			
Salkowski	Present	Present	Present
Lieberman-Burchard	Present	Present	Present
Flavonoids			
Shinoda	Absent	Present	Present
Sodium Hydroxide	Absent	Present	Present
Tannins			
Ferric chloride	Absent	Present	Present
Lead sub acetate	Absent	Present	Present
Alkaloids			
Dragendorff	Absent	Present	Present
Mayer	Absent	Present	Present
Picric acid	Absent	Present	Present
Anthraquinones			
Borntrager	Absent	Absent	Absent
Modified Borntrager	Absent	Absent	Absent
Cardiac glycosides			
Keller-kiliani	Present	Present	Present
Kedde’s	Absent	Absent	Absent

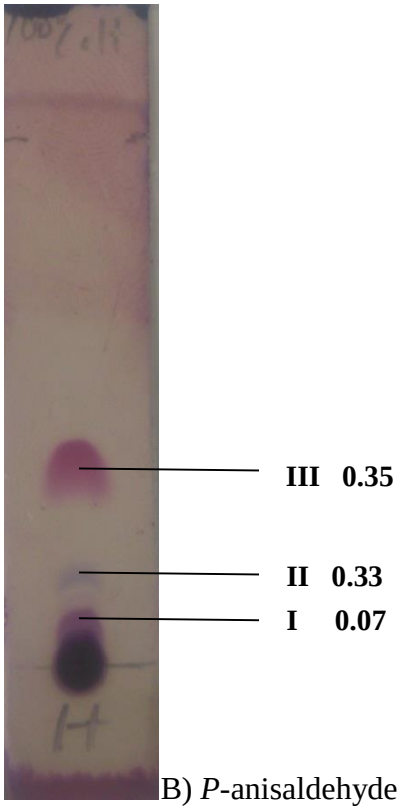
4.4.2 Development of solvent system (fingerprints) and TLC phytochemical screening of the N-hexane, ethylacetate and methanol extracts of the leaf of *S. virosa*

4.4.2.1 Development of solvent system and fingerprints of N-hexane extract (HE) of the leaf of S. virosa

HE was noted to have eleven (11) spots (Plate VII) in hexane: ethyl acetate (9:1), eight (8) spots (Plate VIII) in hexane: ethyl acetate (8:2) and three (3) spots (Plate IX) in hexane (100%) visualized with *p*-Anisaldehyde at 110ºC for 2 minutes.

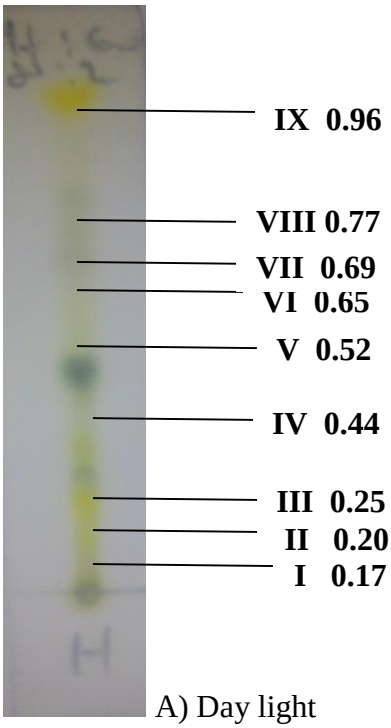


A) Day light

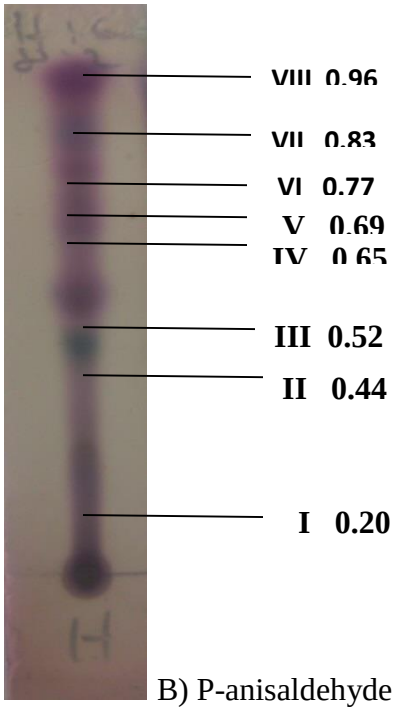


B) *P*-anisaldehyde spray reagent

Plate VII: Chromatogram of N-Hexane extract developed in 100% Hexane in daylight (A) and with spray reagent (B)

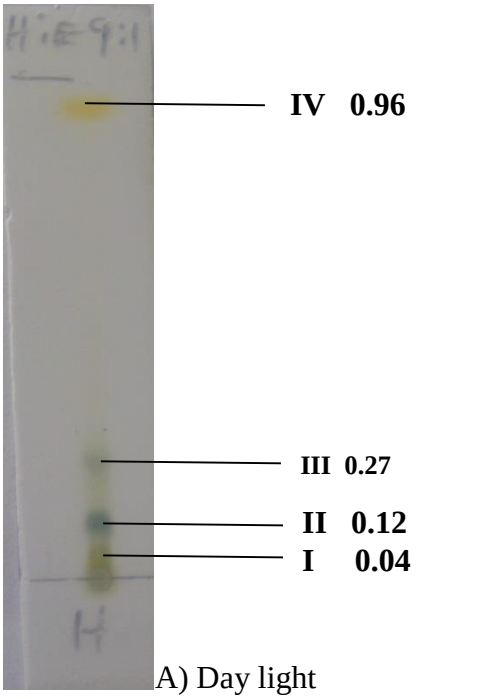


A) Day light

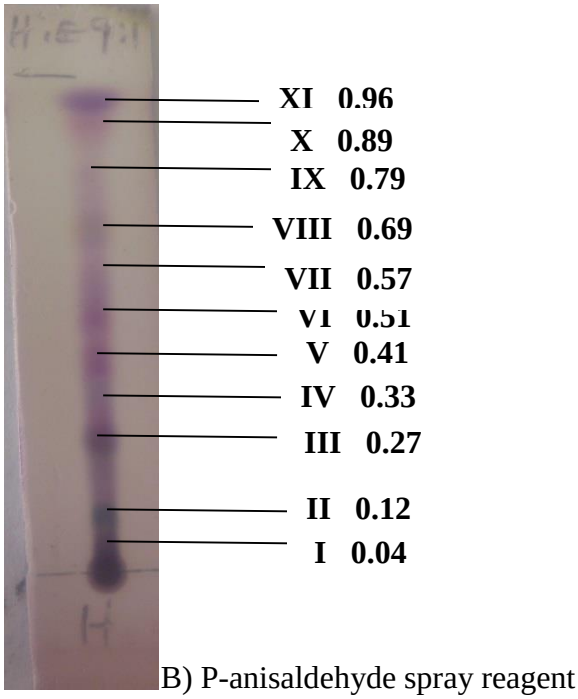


B) P-anisaldehyde Spray reagent

Plate VIII: Chromatogram of N-Hexane extract developed in Hexane: Ethyl acetate (8:2) visualized in daylight (A) and with spray reagent (B)



A) Day light



B) P-anisaldehyde spray reagent

Plate IX: Chromatogram of N-Hexane extract developed in H:E 9:1 day (A) and H:E 9:1 sprayed with P-anisaldehyde (B)

4.4.2.2 TLC Phytochemical screening of N-Hexane extract (HE)

TLC of HE developed in Hexane: Ethyl acetate (9:1) sprayed with Dragendoff (Plate X), Ferric Chloride (Plate XII), Lieberman-Buchard (Plate XV), Iodine vapour (Plate XIV), Borntrager (Plate XI) and Aluminium chloride (Plate XIII) reagents were as presented. The first four were positive results while the last two produced negative results for those phytochemicals tested.



A) Day light



B) Dragendoff's reagent

Plate X: Chromatogram of N-Hexane extract developed in H:E 9:1 day (A) and H:E 9:1 spray with Dragendoff reagent for presence of Alkaloid (B)



A) Day light



B) Borntrager's spray

Plate XI: Chromatogram of N-Hexane extract developed in H:E 9:1 day (A) and H:E 9:1 spray with Borntrager's reagent for presence of Anthraquinones (B)



A) Day light



B) $FeCl_3$

Plate XII: Chromatogram of N-Hexane extract developed in H:E 9:1 day (A) and H:E 9:1 spray with Ferric Chloride reagent for presence of Phenolic compounds (B)



A) Day light

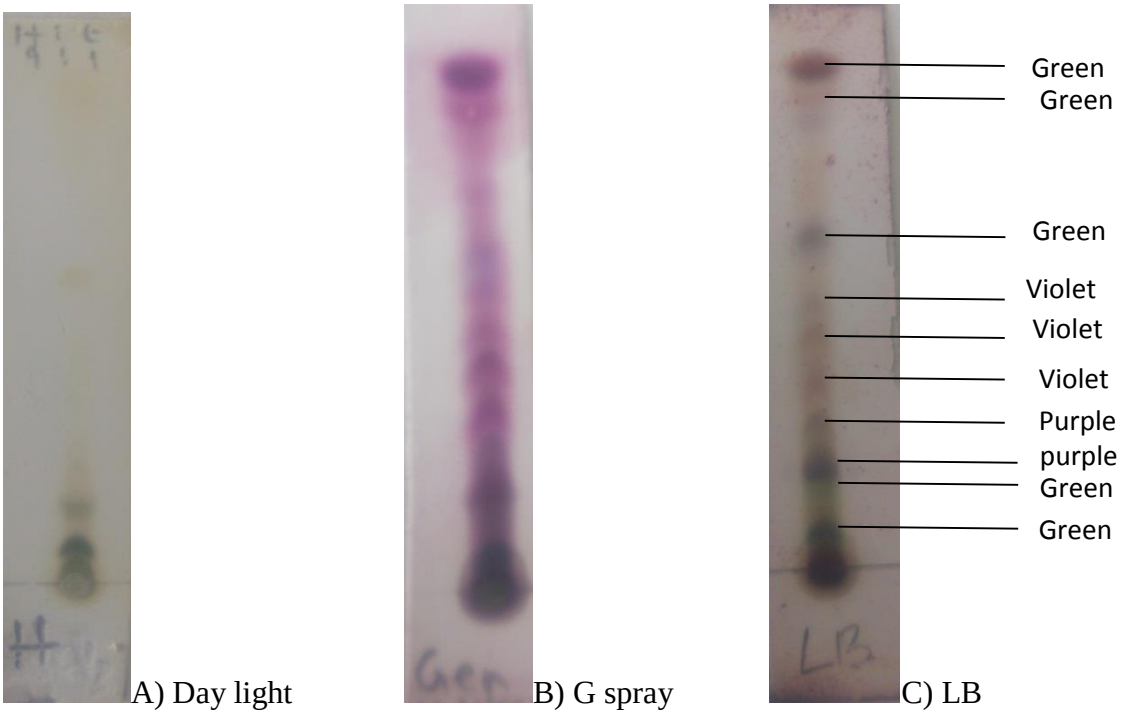


B) UV at 254nm



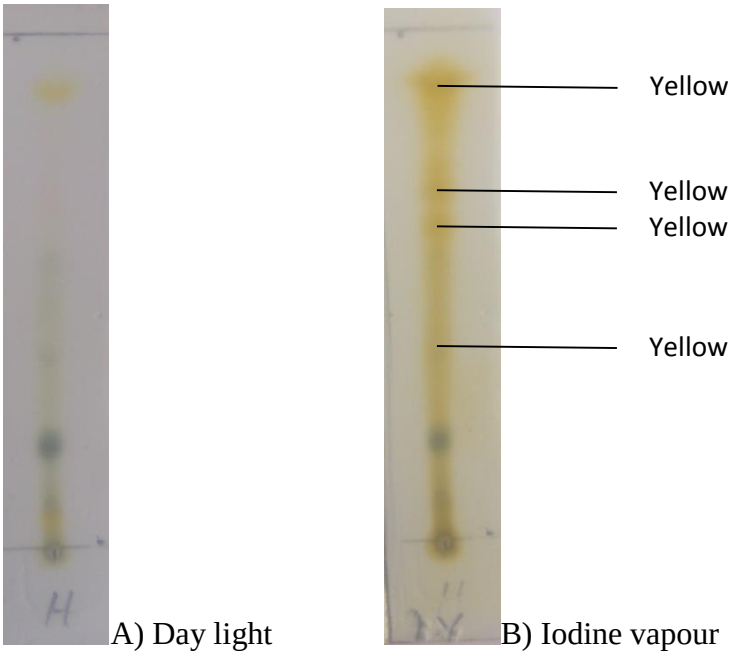
C) UV at 366nm

Plate XIII: Chromatogram of N-Hexane extract developed in H:E 9:1 day (A) and H:E 9:1 spray with Aluminium Chloride reagent viewed at 254nm (B) and 366nm (C) for presence of Flavonoids.



A) Day light B) G spray C) LB

Plate XIV: Chromatogram of N-Hexane extract developed in H:E 9:1 day (A) and H:E 9:1 sprayed with P-anisaldehyde (general spray) reagent (B) and Liberman-Burchard (LB) for presence of Steroids and Triterpenes compound (C)

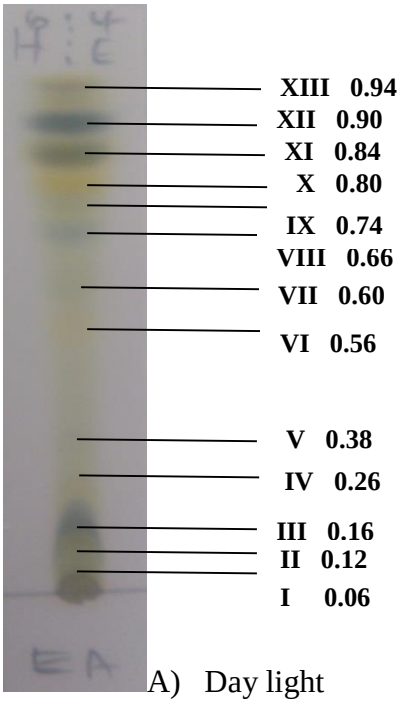


A) Day light B) Iodine vapour

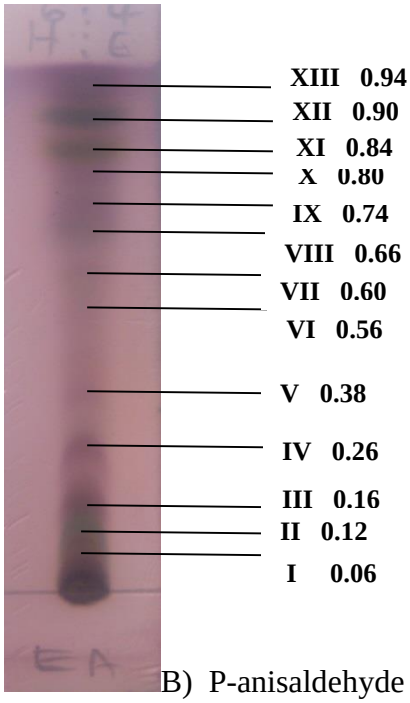
Plate XV: Chromatogram of N-Hexane extract developed in H:E 9:1 day (A) and H:E 9:1 exposed to Iodine vapour for presence of Saponins (B).

4.4.2.3 Development of solvent system for ethylacetate extract (EE) of S. virosa leaf

EE was noted to have fourteen (14) spots (Plate XVIII) in hexane: ethyl acetate (13:7), thirteen (13) spots (Plate XVI) in hexane: ethyl acetate (8: 2) and six (6) spots (Plate XVII) in hexane: ethyl acetate (7:3) visualized with *p*-Anisaldehyde at 110ºC for 2 minutes. Spots of various colours blue, grey, green and violet were revealed. The successful separation of bio-molecules by chromatographic technique depends upon suitable solvent system which needs an ideal range of partition coefficient (k) for each target compounds (Ito, 2005).

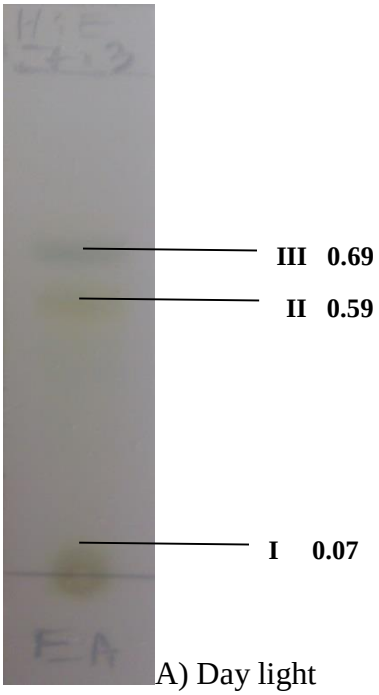


A) Day light

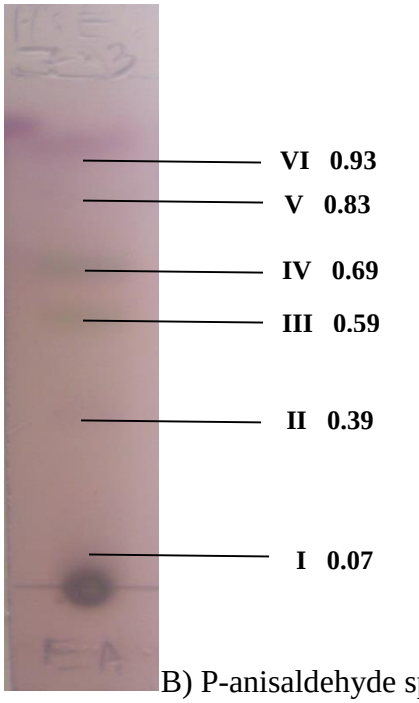


B) P-anisaldehyde spray reagent

Plate XVI: Chromatogram of Ethylacetate extract A and B developed in H:E 8:2, then B sprayed with general spray reagent (B)

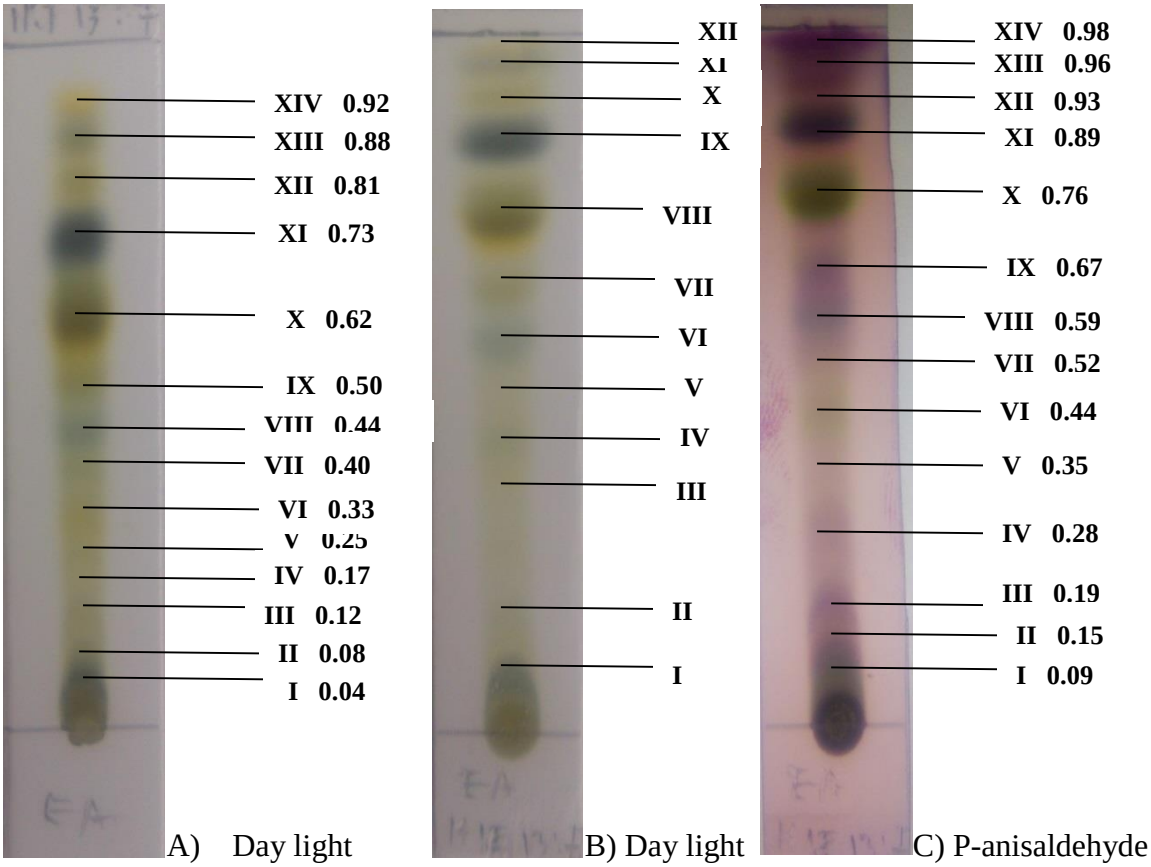


A) Day light



B) P-anisaldehyde spray eagent

Plate XVII: Chromatogram of Ethylacetate extract developed in H:E 7:3 day (A) and H:E 7:3 sprayed with general spray reagent (B).



A) Day light B) Day light C) P-anisaldehyde

Plate XVIII: Chromatogram of Ethylacetate extract developed in H:E 13:7 (A), (B) and (C) followed by (C) sprayed with general spray reagent.

4.4.2.4 TLC phytochemical screening of ethylacetate extract (EE) of S. virosa

TLC of EE developed in Hexane: Ethyl acetate (13:7) sprayed with Ferric Chloride (Plate XXI), Lieberman-Buchard (Plate XXIII), Iodine vapour (Plate XXIV), Dragendoff (Plate XIX), Borntrager (Plate XX) and Aluminium chloride (Plate XXII) reagents. The three (3) reagents gave positive results while the last three (3) produced negative results for the respective phytochemicals tested.



A) Day light



No appearance of orange colour spot

B) Dragendoff's spray

Plate XIX: Chromatogram of *S. virosa* Ethylacetate extract developed in H:E (13:7) sprayed with Dragendoff spray reagent for the detection of Alkaloids.



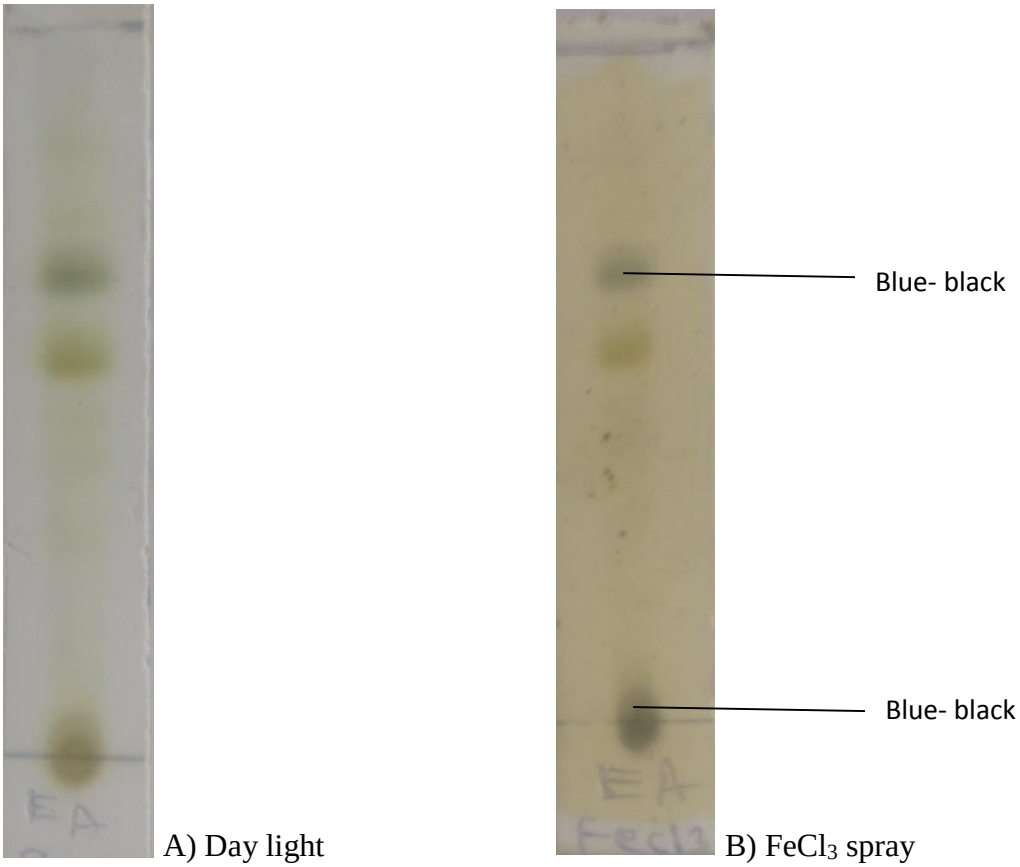
A) Day light



No appearance of yellow colour spot

B) Borntrager's spray

Plate XX: Chromatogram of *S. virosa* Ethylacetate extract developed in H:E (13:7) sprayed with Borntrager's spray reagent for the detection Anthraquinones.



A) Day light B) $FeCl_3$ spray

Plate XXI: Chromatogram of *S. virosa* Ethylacetate extract developed in H:E (13:7) sprayed with FeCl3 spray reagent for the detection of Phenolic commpounds.



A) Day light



No Yellow fluorescence

B) UV at 254nm



No Yellow fluorescence

C) UV at 366nm

Plate XXII: Chromatograms of Ethylacete extract of *S.virosa* leaf developed in H:E (13:7) sprayed with Aluminium Chloride ($AlCl_3$) and observed under UV at 254nm (B) and 366nm (C) for presence of Flavonoids



A) day light



B) LB Spray

Plate XXIII: Chromatogram of Ethylacetate extract developed in H:E 13:7 (A) and H:E 13:7 sprayed with Liberman-Burchard (LB) for presence of Steroids and Triterpenes (B)



A) Day light



B) Iodine vapour

Plate XXIV: Chromatogram of Ethylacetate extract developed in H:E (13:7) day light (A) and H:E (13:7), then exposed to Iodine vapour for presence of Saponins (B).

4.4.2.5 Development of solvent system for methanol extract (ME) of S. virosa leaf

ME was observed to have eleven (11) spots (Plate XXVII) in butanol: acetic acid: water (10:1:1) five (5) spots (plate XXVI) in Ethylacetate: methanol: water (10:3:1) and three (3) spots (Plate XXV) in Chloroform: Methanol (8:2), visualized with *p*-Anisaldehyde and heated at 110ºC for 2 minutes.



A) Day light

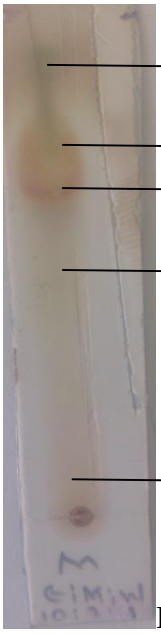


B) P-anisaldehyde spray

Plate XXV: Chromatograms of Methanol extract A and B developed in Chloroform : Methanol 8:2, then B sprayed with general spray reagent (B)

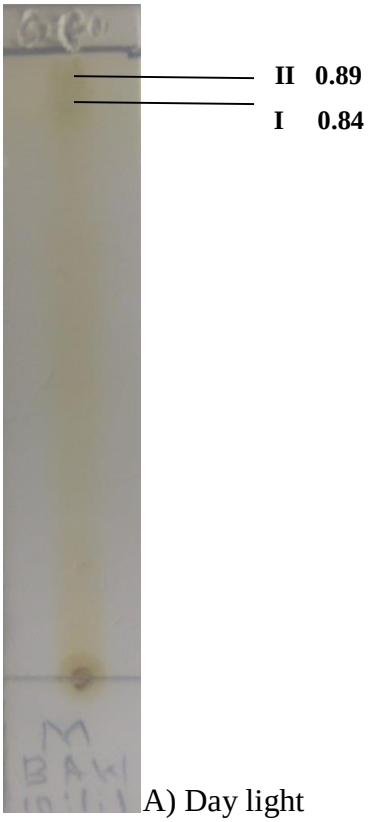


A) Day light

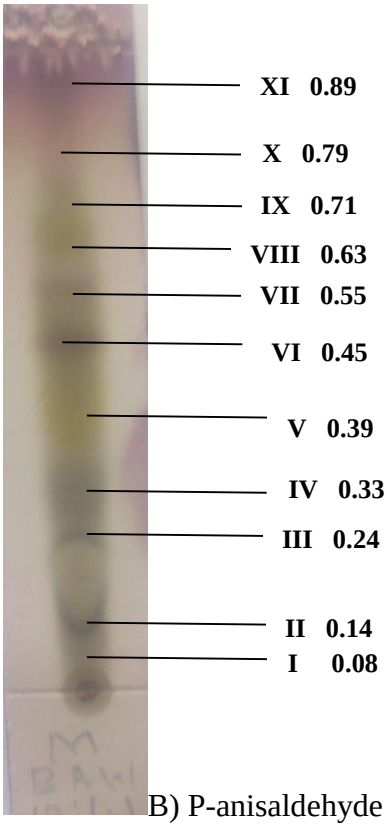


B) General spray

Plate XXVI: Chromatograms of Methanol extract A and B developed in Ethylacetate : Methanol : Water 10:3:1, then B sprayed with general spray reagent (B)



A) Day light



B) P-anisaldehyde spray

Plate XXVII: Chromatogram of Methanol extract A and B developed in N-butanol: Acetic acid: Water 10:1:1, then B sprayed with general spray reagent (B)

4.4.2.6 Phytochemical screening of methanol extract of S. virosa leaf

TLC of ME developed in butanol: acetic acid: water (10:1:1) sprayed with Dragendoff (Plate XXVIII), Ferric Chloride (Plate XXX), Lieberman-Buchard (Plate XXXII), Iodine vapour (Plate XXXIII), Borntrager (Plate XXIX) and Aluminium chloride (Plate XXXI) reagents. The first four spray reagents gave positive results while the last two spray reagents produced negative results for those phytochemicals tested.



A) Day light



B) Dragendoff's spray reagent

Plate XXVIII: Chromatogram of Methanol extract developed in B:A:W 10:1:1 (A) and B:A:W 10:1:1 sprayed with Dragendoff's reagent for presence of Alkaloid (B)



A) Day light



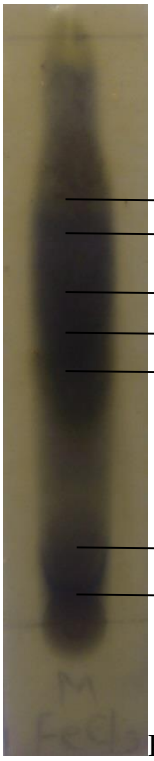
No appearance of yellow colour

B) Borntrager's spray reagent

Plate XXIX: Chromatogram of Methanol extract developed in B:A:W 10:1:1 (A) and B:A:W 10:1:1 sprayed with Borntrager's reagent for presence of Anthraquinones (B)



A) Day light

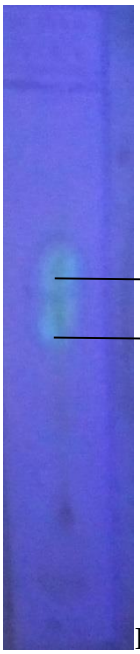


B) $FeCl_3$

Plate XXX: Chromatogram of Methanol extract developed in B:A:W 10:1:1 (A) and B:A:W 10:1:1 sprayed with Ferric Chloride spray s reagent for presence of Phenols (B)



A) AlCl3 day



B) UV at 254nm

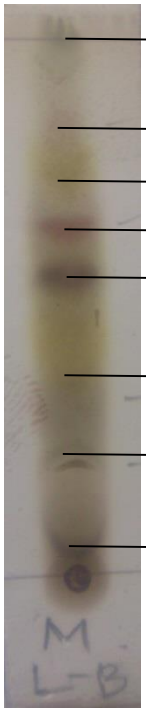


C) UV at 366nm

Plate XXXI: : Chromatogram of Methanol extract developed in B:A:W 10:1:1 (A) and B:A:W 10:1:1 sprayed with Aluminium Chloride reagent viewed at 254nm (B) and 366nm (C) for presence of Flavonoids.


A) Day light


B) LB before heating



C) LB after heating

Plate XXXII: Chromatogram of Methanol extract developed in B:A:W 10:1:1 (A, B and C), then B and C sprayed with Liberman-Burchard reagent followed by heating (C) at 110^{c}C over 2minutes for presence of Steroids (Green) and/or Triterpenes (Violet).


A) Day light



B) Iodine Vapour

Plate XXXIII: Chromatogram of Ethylacetate extract developed in B:A:W 10:1:1 (A) and B:A:W 10:1:1, then exposed to Iodine vapour for presence of Saponins (B).

4.4.2.7 Summary of the TLC phytochemical profiles/fingerprints and TLC phytochemical screening of the n-hexane, ethylacetate and methanol extracts of S. virosa leaf

The TLC fingerprints of the three extracts showed various phytochemical separation profiles of quality control significance (Table 4.7 – 4.9).

Phenols, saponins and steroids/triterpenes were present but flavonoids and anthraquinones were absent in all the three leaf extracts of *S. virosa*. Alkaloids were absent in only the ethylacetate extract (Table 4.10).

Table 4.7: Summary of TLC Profile of Hexane Extract (HE) of *S. virosa* leaf spray with *p*-Anisaldehyde

Extract	Solvent System	No. of Spots	Colour of Spots	R_f values
Hexane	Hexane 100 %	3	purple	0.35
			Green	0.33
			Purple	0.07
	Hexane:Ethyl acetate (8:2)	8	violet	0.96
			Green	0.83
			violet	0.77
			Violet	0.69
			violet	0.65
			Violet	0.52
			Green	0.44
			Purple	0.20
	Hexane:Ethyl acetate (9:1)	11	violet	0.96
			Violet	0.89
			Green	0.79
			Green	0.69
			Violet	0.57
			Violet	0.51
			Violet	0.41
			Grey	0.33
			Violet	0.27
			Green	0.12
			Purple	0.04

Table 4.8: Summary of TLC Profile of Ethyl acetate Extract (EE) of *S. virosa* leaf sprayed with *p*-Anisaldehyde

Extract	Solvent System	No. of Spots	Colour of Spots	R_f values
Ethyl acetate	Hexane:Ethyl acetate (8:2)	13	Yellow	0.94
			Violet	0.90
			Yellow	0.84
			Violet	0.80
			Green	0.74
			Yellow	0.66
			Violet	0.60
			Green	0.56
			Yellow	0.38
			Yellow	0.26
			Green	0.16
			Green	0.12
			Green	0.06
	Hexane: Ethyl acetate (7:3)	6	Green	0.93
			Yellow	0.83
			Green	0.68
			Yellow	0.59
			Green	0.39
			Yellow	0.07
	Hexane: Ethyl acetate (13:7)	14	Yellow	0.98
			Violet	0.96
			Yellow	0.93
			Violet	0.89
			Green	0.76
			Yellow	0.67

	Violet	0.59
	Green	0.52
	Yellow	0.44
	Yellow	0.39
	Green	0.28
	Green	0.19
	Green	0.15
	Green	0.09

Table 4.9: Summary of TLC Profile of Methanol Extract (ME) of *S. virosa* leaf sprayed with *p*-Anisaldehyde

Extract	Solvent System	No. of Spots	Colour of Spots	R_f values
Methanol	Chloroform: Methanol (8:2)	3	Green	0.96
			Light green	0.62
			Grey	0.29
	Ethyl acetate: Methanol: Water (10:3:1)	5	Green	0.90
			Light green	0.84
			Purple	0.74
			Light green	0.58
			Pale purple	0.08
	Butanol: Acetic acid: Water (10:1:1)	11	Violet	0.89
			Violet	0.79
			Green	0.71
			Green	0.63
			Violet	0.55
			Violet	0.45
			Light green	0.39
			Green	0.33
			Green	0.24
			Green	0.14
			Green	0.08

Table 4.10 TLC Phytochemical Screening of Extracts of *S. virosa* Leaves

Secondary Metabolites	Chromogenic Agents	N-Hexane Extract*	Ethylacetate Extract**	Methanol Extract***
Alkaloids	Dragendoff	Present	Absent	Present
Anthraquinones	Borntrager	Absent	Absent	Absent
Flavonoids	Aluminium Chloride			
	At 254nm	Absent	Absent	Absent
	366nm	Absent	Absent	Absent
Phenols	Ferric Chloride	Present	Present	Present
Saponins	Iodine Vapour	Present	Present	Present
Steroids/ Triterpenes	Liberman Buchard	Present	Present	Present

Solvent systems; *H:E 9:1 **H:E 13:7 ***B:A;W 10:1:1

4.5 *In vitro* Antivenin Activity of *S. virosa* Extracts against *Naja nigricollis* Snake Venom Phospholipase A_2 (PLA_2)

The enzyme inhibition pattern was observed to be non-competitive (figure 4.1) and the nature of the interaction (i.e. KI or dissociation constant) between enzyme, methanol extract and the substrate was determined to be weak (Figure 4.2).

Table 4.11: Inhibitory Properties of Methanol Leaf Extract of *S. virosa*

Substrate (egg yolk) concentration 1/S mg/ml	Inhibitor (S. virosa extracts) Concentration			
	0mg/ml	2.5mg/ml	5mg/ml	10mg/ml
0.010	0.188	0.250	0.450	0.300
0.013	0.378	0.281	0.463	0.346
0.020	0.332	0.500	0.375	0.375
0.040	0.450	0.563	0.500	0.750

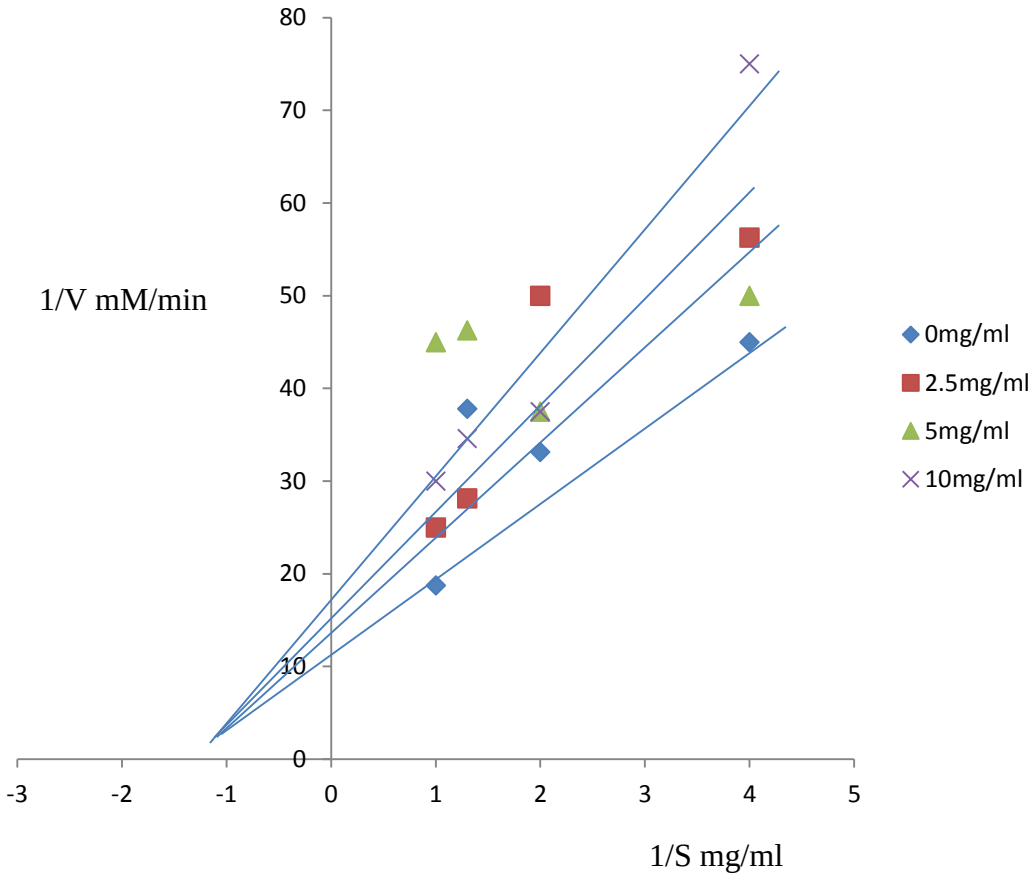


Figure 4.1: Methanol Leaf Extract of *S.virosa* Activity against Phospholipase A_2 Enzyme of *Naja nigricolis* Venom

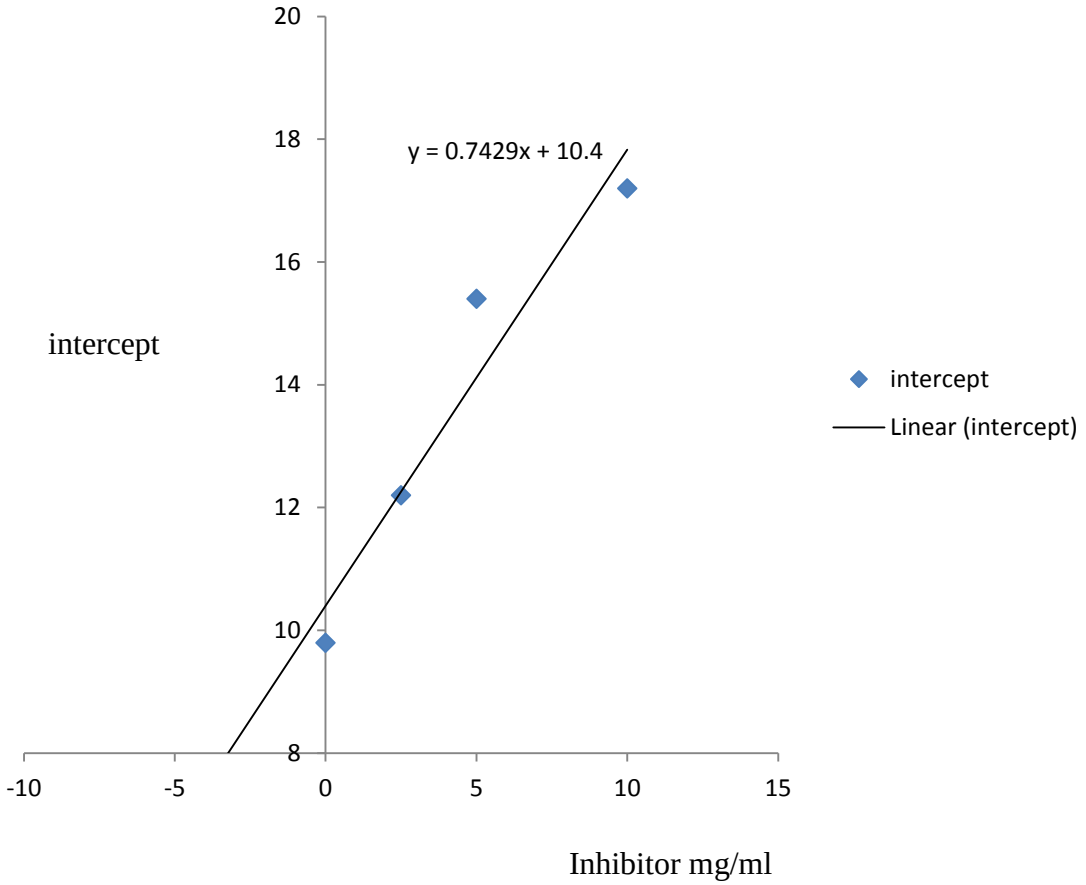


Figure 4.2: Dixon's plot (a secondary plot) that gives Inhibition Binding Constant (KI) of the Methanol Extract on the Activity of Phospholipase A_2

4.6 Modulatory effect of *S. virosa* Leaf Extracts on *Naja nigricollis* Snake Venom

4.6.1 *in vivo* venom neutralization effect of leaf extracts of *S. virosa* against venom of *Naja nigracolis* using mice

4.6.1.1 Acute toxicity study (Median Lethal Dose) of S. virosa leaf extract

The LD_{50} of N-hexane extract of *S.virosa* was found to be 774.60 mg/kg and that of Ethylacetate extract was found to be greater than 5000 mg/kg while that of methanol extract was found to be 1265 mg/kg.

4.6.1.2 Assessment of in vivo venom neutralization of S. virosa extract

The n-hexane leaf extract produced greater protection against venom induced lethal effect than both methanol and ethylacetate leaf extracts of *S. virosa* given I.P. in mice (Figure 4.3). The protections produced by n-hexane and methanol leaf extracts were significant at 20 mg/kg dose in a mixture of half LD_{99} of *Naja nigricollis* venom.

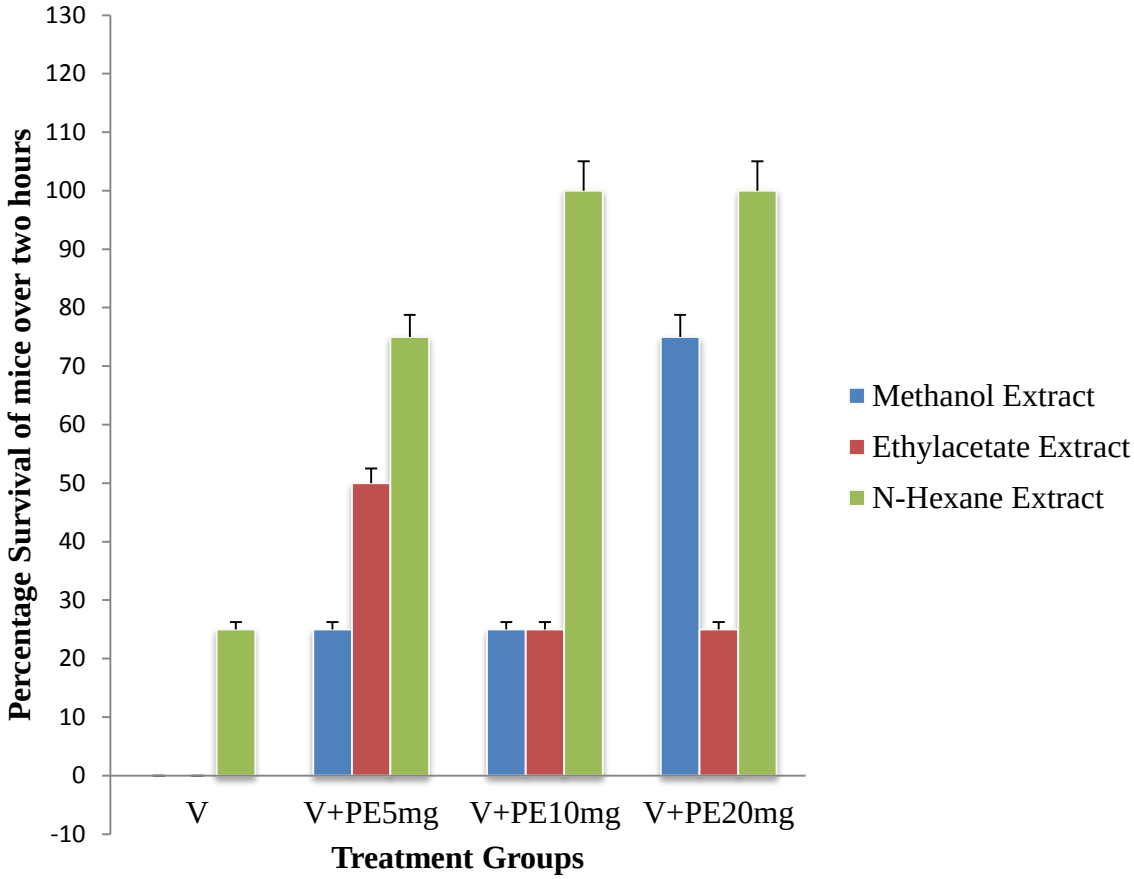


Figure 4.3: Percentage Survival for the treatments of mice with *N. nigricollis* venom and Mixtures of *N. nigricollis* Venom + Leaf Extracts of *S. virosa*.

Table 4.12: *In vivo* Response to Administration (I.P.) of Incubated Venom alone and Venom with *S. virosa* Methanol Extract in Mice

Treatment	Mortality ratio	% Survival in /2hours	Survival in 24hours
LD_{99}	4/4	0	0
LD_{99} + M5mg	3/4	25	0
LD_{99} + M10mg	3/4	25	0
LD_{99} + M20mg	1/4	75*	0

* = significant at $p < 0.05$

Table 4.13: *In vivo* Response to Administration (I.P.) of Incubated Venom alone and Venom with *S. virosa* Ethylacetate Extract in Mice

Treatment	Mortality ratio	% Survival in 2hours	% Survival %/24hours
LD_{99}	4/4	0	0
LD_{99} + E5mg	2/4	25	0
LD_{99} + E10mg	3/4`	25	0
LD_{99} + E20mg	3/4	25	25

* = significant at $p < 0.05$

Table-4.14: *In vivo* Response to Administration (I.P.) of Incubated Venom alone and Venom with *S. virosa* N-Hexane Extract in Mice

Treatment	Mortality ratio	% Survival /2hrs	% Survival in /24hrs
LD_{99}	3/4	25	25
LD_{99} + E5mg	1/4	75	0
LD_{99} + E10mg	0/4	100	25
LD_{99} + E20mg	0/4	100*	50

* = significant at $p < 0.05$

Table 4.15: Effects of Administration of *Naja nigricollis* Venom and Mixture of Venom with *S.virosa* Extracts in Mice.

Group	Survival time on mice in minutes		
	Methanol	Ethylacetate	N-Hexane
V	54.50 ± 7.90	81.00 ± 12.89	17.50 ± 13.46
V+PE5 mg	107.75 ± 18.22	99.00 ± 21.20	211.75 ± 105.04
V+PE1 0mg	71.00 ± 27.79	91.25 ± 23.64	401.50 ± 150.87
V+PE20 mg	147.25 ± 17.89*	74.00 ± 14.53	` 458.50 ± 150.87*

Results are presented as mean ± standard error of mean (SEM) *= significant at $p < 0.05$

V = venom, PE = plant extract

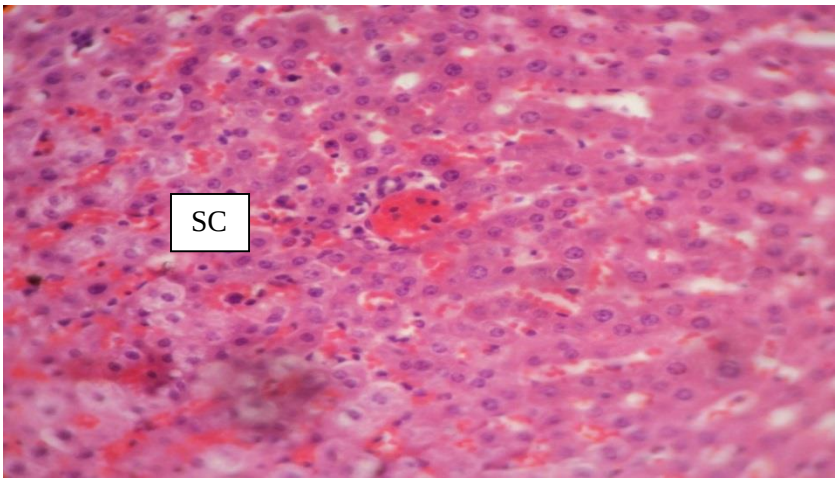
4.6.1.3 Histopathological effects of S. virosa venom-extracts mixtures treatment in mice

The histopathology of brain, heart, lungs, kidney and liver for N-hexane shows almost normal architecture of the above organs. This indicates a greater protection to the organ damage by venom of *Naja nigricollis.*

There were a normal architecture of liver, heart, kidney, lungs and brain in the control (untreated mice). The mixture of venom-methanol leaf extract treated mice shows normal heart and brain tissues, but, slight lymphocyte hyperplasia (LH) and alveoli congestion (AC) were noted in kidney and lung tissues respectively. A moderate LH and vascular congestion (VC) were observed in the liver. A mixture of venom-ethylacetate leaf extract produced normal heart, slight vacoulation in brain and slight vacoulation and necrosis in the liver were observed. However, moderate alveoli congestion in the lungs was noted. A mixture of venom-n hexane leaf extract treated mice produced normal heart, brain and lungs with slight necrosis and LH were observed in kidney and liver respectively.

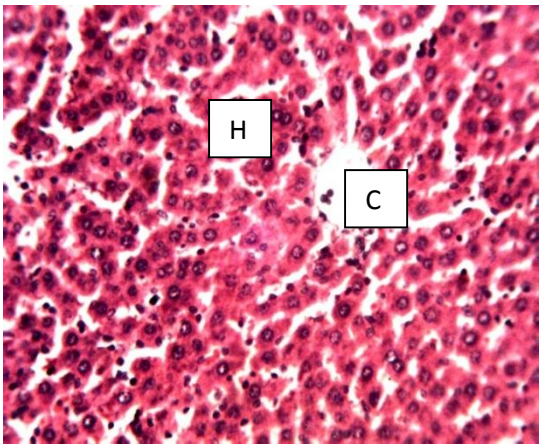
A. Liver: Control and mixture of venom- extracts (Methanol, Ethylacetate, n-Hexane) treatment groups' photomicrographs H&E

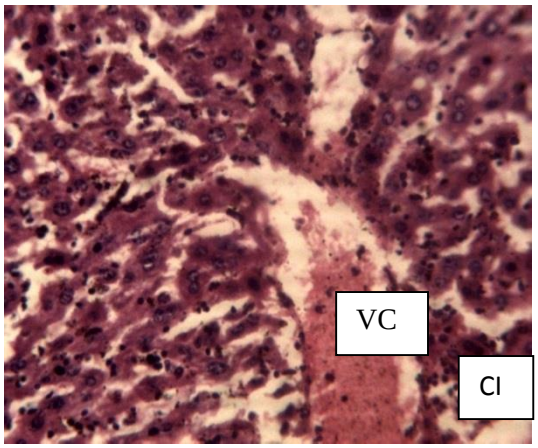
Venom of *naja nigricollis* (positive control)



Liver showing sinusoidal congestion (SC)

Normal saline **Methanol extract**

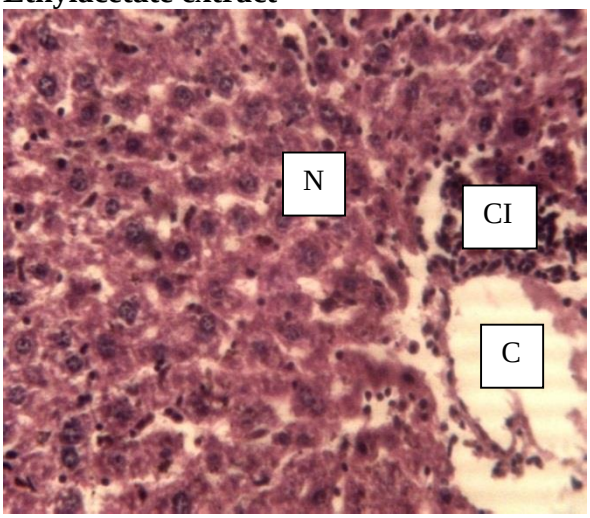




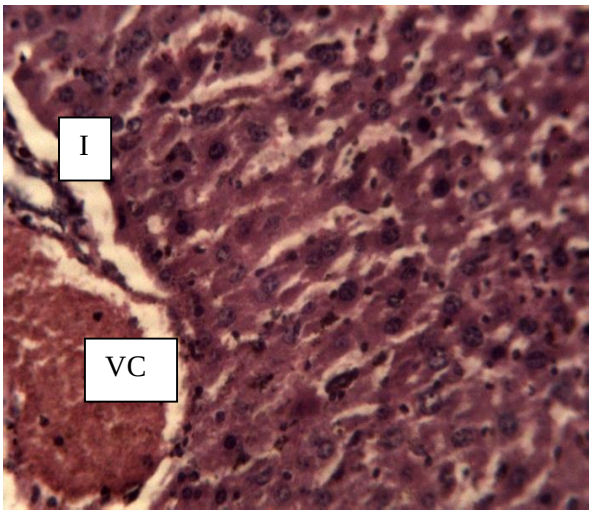
i. Liver of mice showing normal hepatocyte (H) Central vein (C) X 400

ii. Liver shows moderate vascular congestion (VC) and cellular infiltration (CI) X 400.

Ethylacetate extract **N-hexane extract**



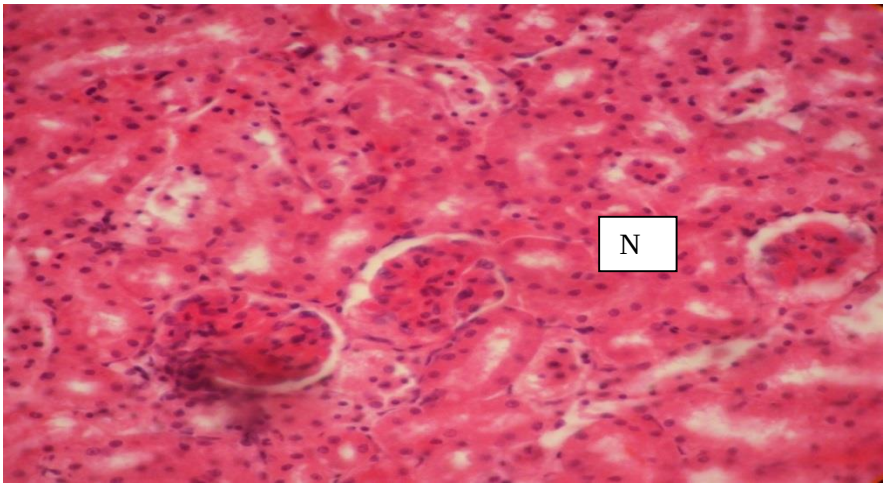
iii. Liver of mice showing necrosis (N) central vein (C) and cellular infiltration (CI) X 400



iv. Liver of mice showing vascular congestion (VC) and mild inflammation (I) X 400

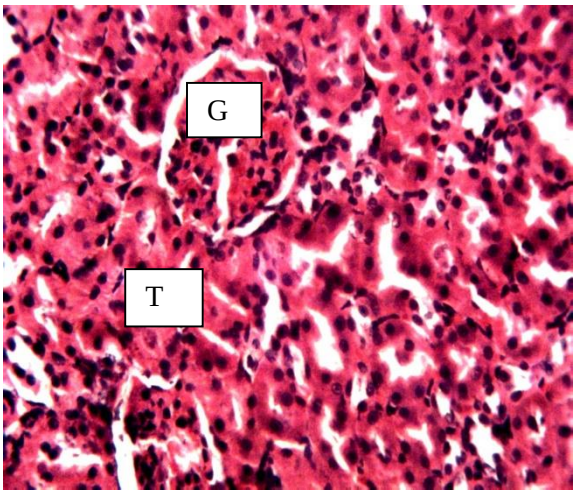
B. Kidney: Control and mixture of venom- extracts (Methanol, Ethylacetate, n-hexane) treatment groups' photomicrographs in H&E

Venom of *naja nigricollis* (positive control)



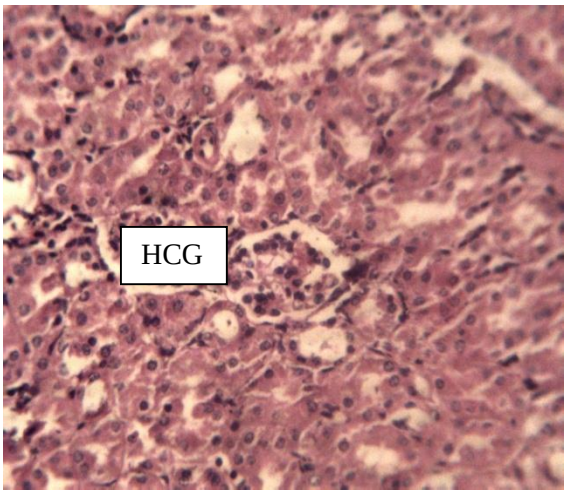
Kidney showing Necrosis (N) with intact glomeruli

Normal saline



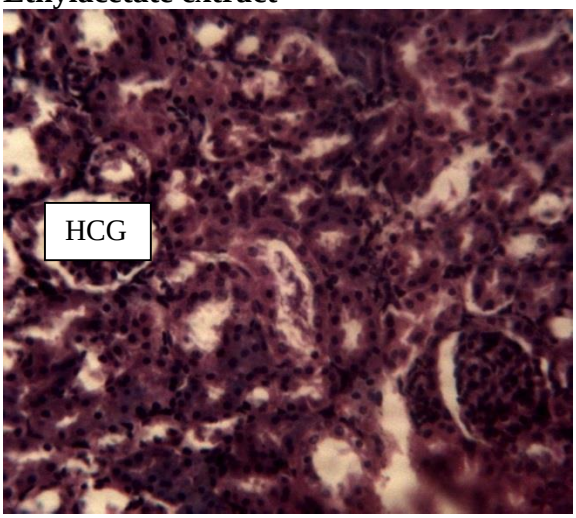
i. Kidney shows normal glomerulus (G) and tubules (T) X 400

Methanol extract



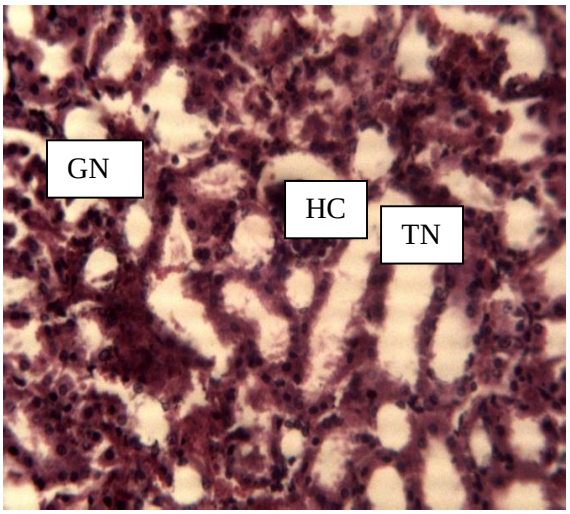
ii. Kidney of mice showing mild hyper cellularity of glomerulus (HCG) X 400

Ethylacetate extract



iii. Kidney of mice showing severe hyper cellularity of glomerulus (HCG) X 400

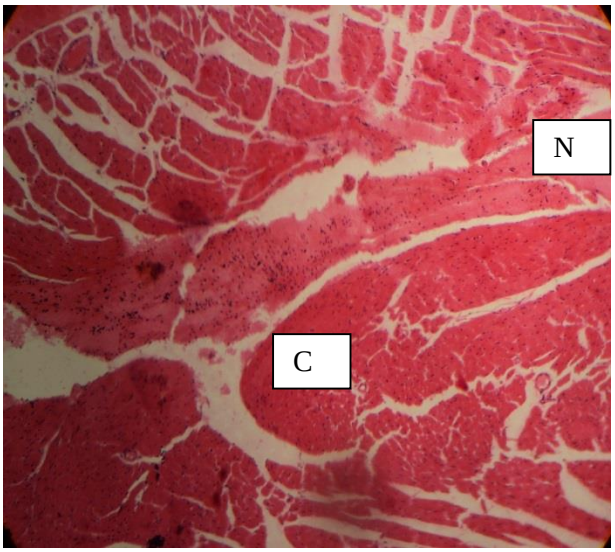
N-hexane extract



iv. Kidney shows slight tubular necrosis (TN) slight glomerular necrosis(GN) and slight hyper cellularity (HC).X 400

C Heart: Control and mixture of venom- extracts (Methanol, Ethylacetate, n-hexane) treatment groups' photomicrographs H&E

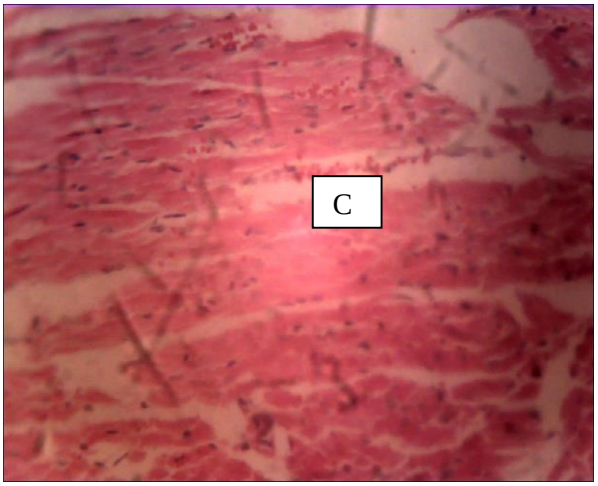
Venom of *naja nigricollis* (positive control)



Heart showing necrosis (N) and congestion (C)

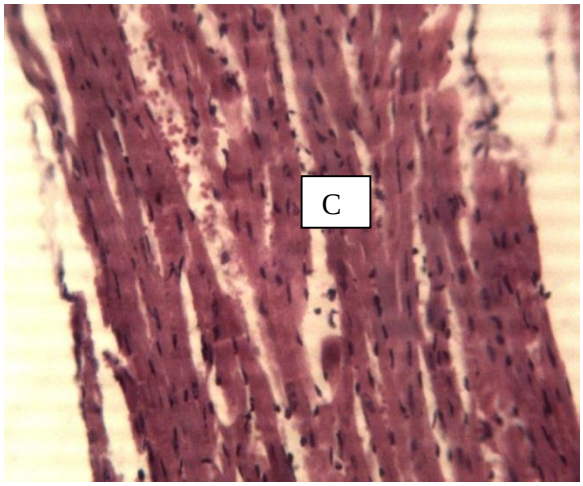
X 400

Normal saline



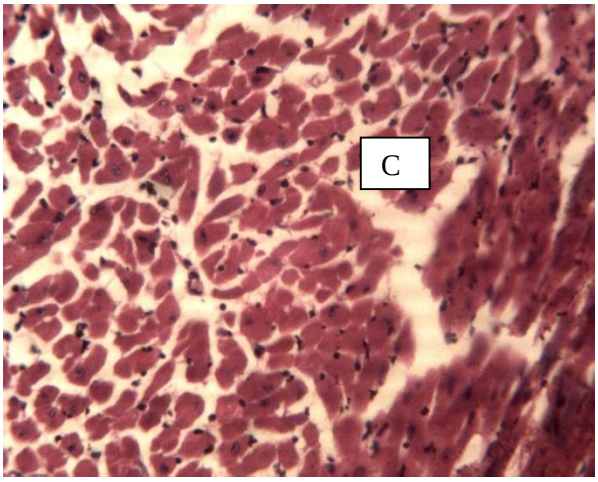
i. Heart shows normal cardiomyocytes (C)
X 400

Methanol extract



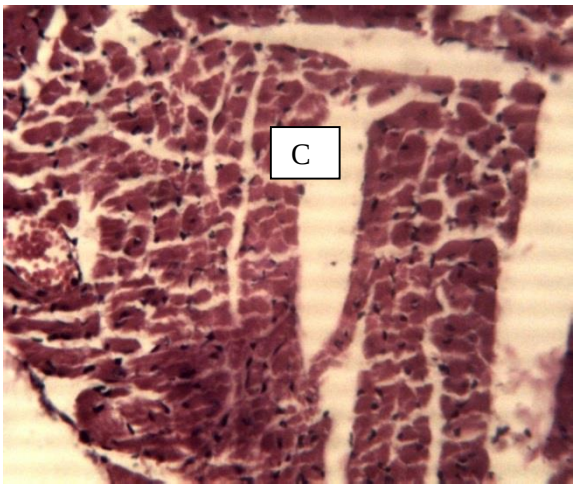
ii. Heart shows normal cardiomyocytes (C)
X 400

Ethylacetate extract



iii. Heart shows normal cardiomyocytes (C) X 400

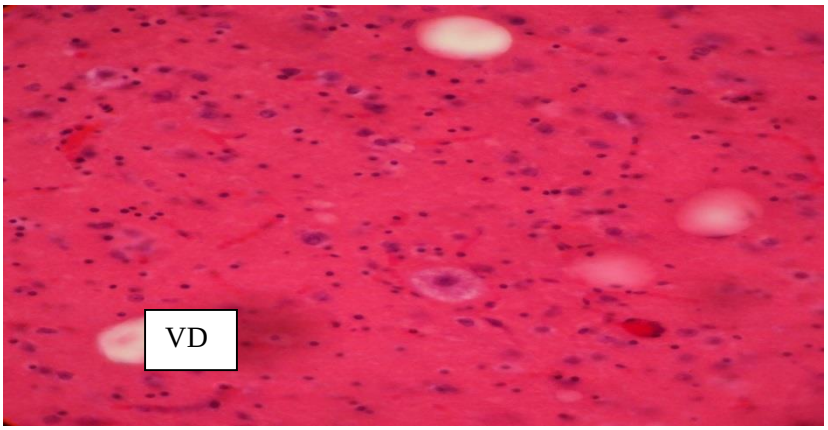
N-hexane extract



iv. Heart shows normal cardiomyocytes (C) X 400

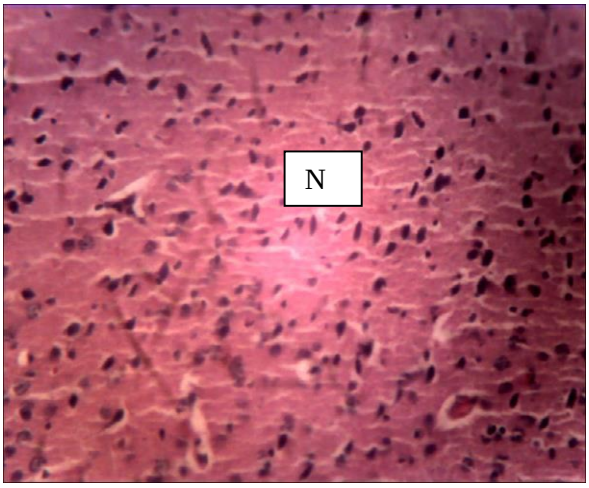
D. Brain: Control and mixture of venom- extracts (Methanol, Ethylacetate, n-hexane) treatment groups' photomicrographs H&E

Venom of *naja nigricollis* (positive control)



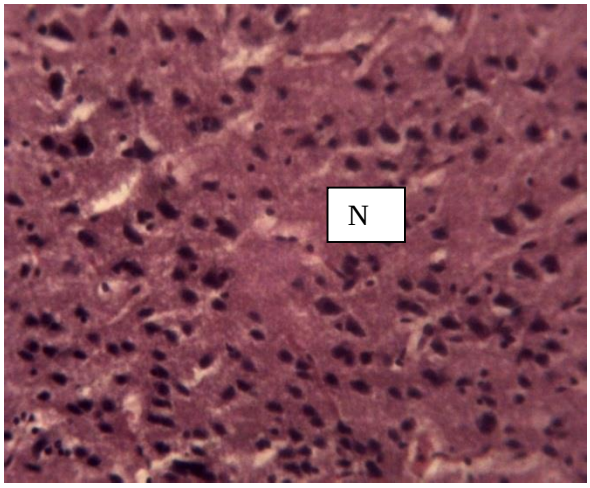
Brain showing vacoular (VD) degeneration X 400

Normal saline (negative control)



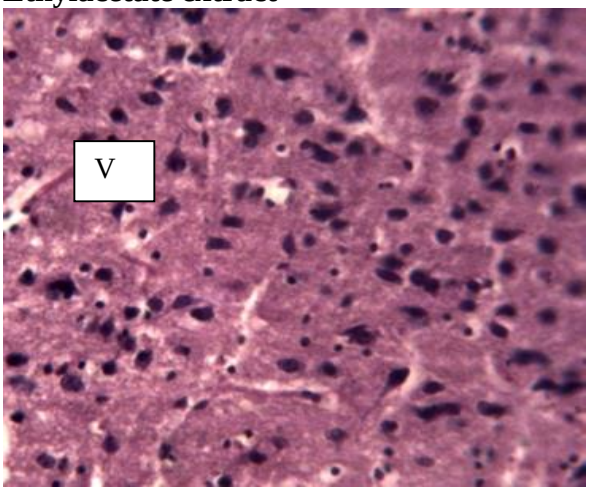
i. Brain shows normal nervous tissue (N) X 400

Methanol extract



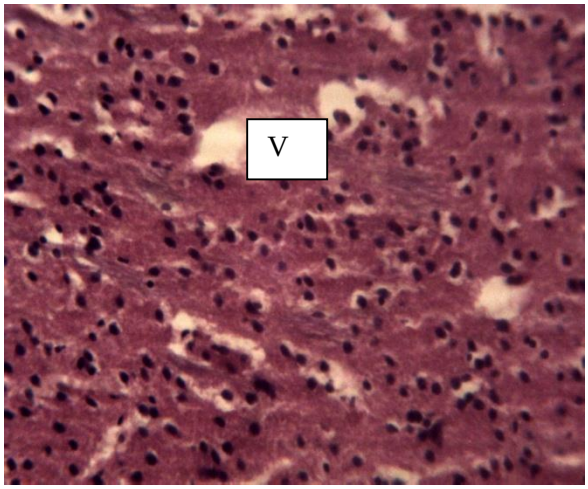
ii. Brain shows normal nervous tissue X 400

Ethylacetate extract



iii. Brain shows slight vacoulation (V) X 400

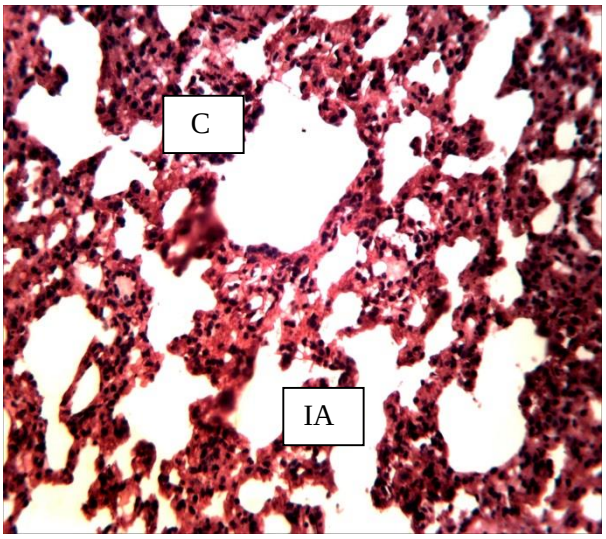
N-hexane extract



iv. Brain showing moderate vacoulation (V) X 400

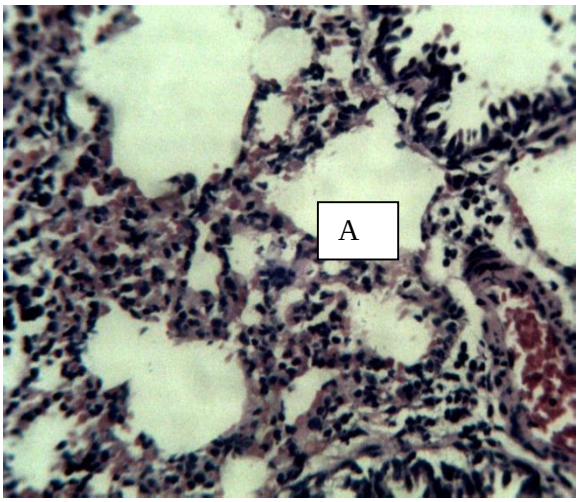
E. Lungs: Control and mixture of venom- extracts (Methanol, Ethylacetate, n-hexane) treatment groups' photomicrographs. H & E.

Venom of *naja nigricollis* (positive control)



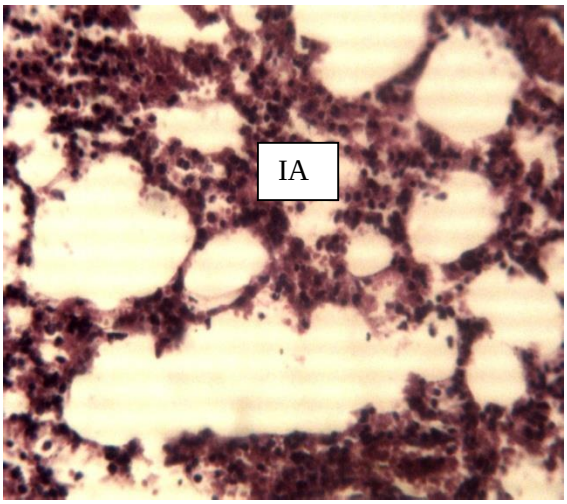
Lungs showing severe thickening and congestion (C) of the inter alveolar spaces (IA) X 400

Normal saline



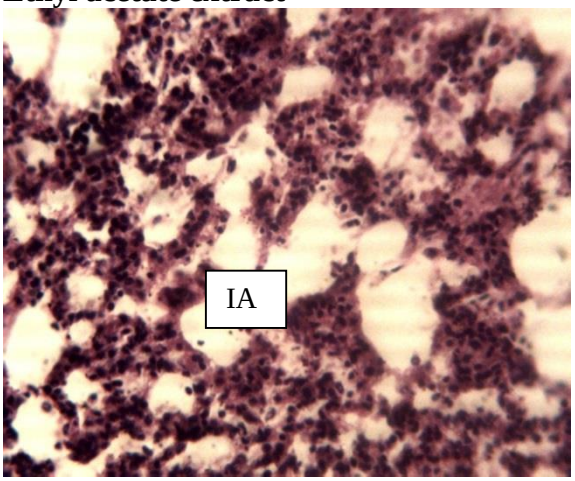
i. Lung showing normal alveoli (A), X 400

Methanol extract



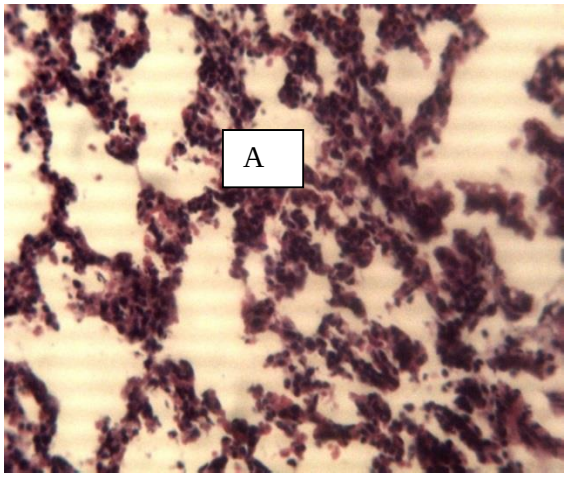
ii. Lung showing slight thickening of the inter alveolar spaces (IA) X 400

Ethyl acetate extract



iii. Lung showing slight thickening of the inter alveolar spaces (IA) X 400

N-hexane extract



iv. Lung showing normal alveolar feature X 400.

Plate XXXIV: effect of *S. virosa* extracts on various organs of mice (i.e. control and treatment groups).

CHAPTER FIVE

5.0 DISCUSSION

Approximately, about 400,000 vascular plant species, 370,000 flowering plant species and 18,000 medicinal plant species are known to science according to global plants report (Kew 2016). Africa makes a little contribution to the list, with Australia, Brazil and China being the top contributors (Paton *et al*., 2008, Kew, 2016). This shows that only 4.5% of the world plants populations have documented medicinal uses. As a result of social, economic and pressing burden of chronic diseases, herbal medicines are gaining more popularity in the global market (WHO, 2013). In Africa, India and China more than 80% of the populations rely on traditional medicine to cater for their primary health care of which herbs from plants play a major role (WHO, 2010). The World Health Organization (WHO) has therefore encouraged interaction between Western-based and indigenous-based medicines with a view to exploit and identifies compounds that could provide safe and effective remedies for ailments of both microbial and non-microbial origins (WHO, 2013).

To validate the safety and efficacy of traditional claim of medicinal plants, acute and or chronic toxicity, *in vitro* and *in vivo* studies are the mainstay to conduct. The present study was aimed at validating the traditional claim for the use of *Securinega virosa* in the traditional management of snakebite and to provide pharmacognostic standards of the leaves for quality control.

Leaf epidermis has been reported to provide important diagnostic characters, and together with characters of stomata are considered to be of first significance in the microscopical identification of leaves (Evans, 2009).

Anatomical section of the leaf of *S. virosa* revealed the presence of epidermal cells which are polygonal with straight anticlinal wall as seen in *Pilocarpus Jaborandi* (Evans, 2009). Scanty anomocytic and paracytic stomata were present only on the abaxial surface. This is in harmony with the findings in India by Thakur and Patil (2014) of which 17 species from 17 Genera of Euphorbiaceae including *S. virosa* which showed the predominance of anomocytic stomata but rarely anisocytic or paracytic. The predominance of anomocytic stomata and hypostomatic nature of the stomata were noticed in only four (4) out of the seventeen (17) studied plants.

The presence of unicellular trichomes, though rare differs with the findings of Thakur and Patil (2014) which reported the absence of trichomes on the foliar surface of *S. virosa* found in Indian specie studied but in other species of the family such as *Neoscortechinia kingii* Hook. f. The abundance of prism crystals of calcium oxalate in a sheaths or singles of mean size 2µm was noticed on both adaxial and abaxial surfaces. Earlier studies of the Euphorbiaceae had revealed the significance of anatomic characters for identification and classification throughout the family taxa (Webster, 1994) and for checking adulteration (Ghani, 1990).

A transverse section of the leaf through the midrib shows the presence of a clear vascular bundle; xylem and phloem typical of a dicot plant. The stomatal index determined which is 12.67 is nearly closer to the stomatal index of 12.00 reported by Thakur and Patil (2014).

The physicochemical constants (Table 4.3) determined were; average moisture contents using loss on drying method calculated as approximately 5.6 % appears to be lower than the value

obtained by Danlami *et al*.,(2012) which is 10.88% of same *S. virosa* leaves. This may be due to seasonal and environmental factors. In the work reported by Danlami *et al*., (2012), S. virosa leaves were collected during rainy season (August) in Abuja while in the present study; the same plant leaves were collected in dry season (March) at Zaria. It may also be due to the different method of determination where loss on drying was used in this study but Danlami used proximate analysis. Low moisture content in crude drugs suggest better stability against degradation of product (WHO, 1996) while high moisture content is considered as adulteration (Evans, 2009). Ash values, indicates the amount of care devoted to the preparation of a crude drug. The percentage yield of total ash and acid insoluble ash were 11.67% and 1.2% respectively. The total ash value was higher than that of Danlami *et al*.,(2012) which is 9.39%. The higher value suggests higher mineral elements value than that *S. virosa* leaf collected from Abuja. Also, the total ash of *S. virosa* obtained in this study; 11.67% is higher than that of *Ipomoea batatas* (Asibey-Berko and Tayle, 1999) widely use in Nigeria as food. A lower acid insoluble ash 2.5% which shows the extent of contamination with earthy material is allowed for Senna leaf while a higher value up to 12% is allowed for hyoscyamus leaf (Evans, 2009). More so, the total ash value is used as criteria to confirm the identity and purity of drugs (WHO, 1996, Prasad *et al*., 2012). The extractive values obtained were 22.00% and 33.00% for ethanol and water solvents respectively. They serve as pharmacopoeial standards for evaluating crude drugs (Evans, 2009). Extractive values provide an estimate of the possible total metabolites in a crude drug.

Plants experience changes in their basic structure overtime. These changes affect the living plant cell wall composition, shape and cell contents which can be evaluated microscopically and are

valuable for identification and detection of adulteration (Evans, 2009). For instance, on the course of these modifications certain materials (e.g. pectin, lignin, and suberin) might be added to the backbone structural material (i.e. cellulose) of the cell wall. These and other cell contents can be detected by the use of color reagents.

Chemomicroscopical examination of the powdered leaf of *S. virosa* revealed the presence of cellulose cell wall, lignified cell wall, tannins, starch, calcium oxalate, suberin and mucilage. However, aleurone grains were absent.

Phytochemical constituents in the plant samples are known to be the biologically active compounds. They are responsible for different activities such as antioxidant, antimicrobial, antifungal, anti- asthma, arthritis, anticancer and anti-snakebite (Alabri *et al.*, 2013, Hassan Bin Asad *et al*., 2014; Banu and Cathrine, 2015).

Preliminary phytochemical screening of the n-hexane (HE), ethyl acetate (EE) and methanol extracts (ME) using standard methods shows that saponins, flavonoids and alkaloids were absent in HE contrary to the findings of Danlami *et al.* (2012 and 2013) where they were all present while anthraquinones were absent in both HE, EE and ME. But, this is similar to the report of Danlami *et al*, (2012 and 2013) which indicated the absence of anthraquinone in both hexane and ethylacetate extracts of *S. virosa* leaves collected from Abuja and methanol extract of the plant collected from Zaria (Yerima *et al.*, 2009). Triterpenes and or steroids were found in all the three extracts of *S.virosa* leaves. This showed the abundance of triterpenes and or steroids in the *S. virosa* plant. Similarly, steroids have been previously reported in the methanol root bark extract

of *S. virosa* (Magaji, 2007 and 2008). The abundance of triterpenes/ steroids in *S. virosa* may support the potential anti-venin activity since triterpenoids lupeol and steroid stigmasterol, were found to possess antivenom properties (Kadir *et al.*, 2015). Furthermore, the phytoconstituents such as phenols, alkaloids and cardenolides in the extracts are known to possess medicinal properties and health promoting effect (Danlami *et al.,* 2013).

The TLC analysis is an important technique that is widely used worldwide for the study of not only phytoconstituents but also adulterants and contaminants in agricultural products, foods and beverages. The quality and purity evaluation of herbal extracts using TLC method is receiving an increased recognition in most pharmacopoeias (Sherma, 2000; Evans, 2009). In recent time, European Pharmacopoeia (EP) has emphasized the use of TLC fingerprinting as required standard for correct identification of herbal material. Finger printing, together with its schematic chromatographic presentation, provide the sequence of compounds in the herbal extract (Bauer and Franz, 2010).

HE was noted to have the best constituents separation of eleven (11) spots (Plate VII) in hexane: ethyl acetate (9:1) but EE was noted to have fourteen (14) spots (Plate XVIII) in hexane: ethyl acetate (13:7) and ME was observed to have eleven (11) spots (Plate XXVII) in butanol: acetic acid: water (10:1:1) visualized with *p*-Anisaldehyde at 110ºC for 2 minutes. Spots of various colours blue, grey, green and violet were revealed. The successful separation of bio-molecules by chromatographic technique depends upon suitable solvent system which needs an ideal range of partition coefficient (k) for each target compounds (Ito, 2005).

The presence of common classes of naturally occurring compounds in plants can be screened by the use of appropriate chromogenic reagents after separation using thin layer chromatography (Evans, 2009). The chromatogram of hexane extract was positive to Dragendoff, Ferric Chloride, Lieberman-Buchard and Iodine vapour and negative to Borntrager and Aluminium chloride reagent (which was observed under UV light at 254 nm and 366nm after spraying the plate). These confirmed the presence of alkaloids, phenols, steroids and saponins but absence of anthraquinones and flavonoids.

The chromatogram of ethyl acetate extract developed in Hexane: Ethyl acetate (13:7) was positive to Ferric Chloride, Lieberman-Buchard, Iodine vapour, and negative to Dragendoff, Borntrager and Aluminium chloride reagent (which was observed under UV light at 254 nm and 366nm after spraying the plate).. The three (3) reagents gave positive results while the last three (3) produced negative results for those phytochemicals tested. These observations showed the presence of phenols, steroids and saponins but absence of alkaloids, anthraquinones and flavonoids in ethyl acetate extract respectively. The chromatogram of methanol extract developed in butanol: acetic acid: water (10:1:1) was positive to Dragendoff, Ferric Chloride, Lieberman-Buchard, Iodine vapour but negative to Borntrager and aluminium chloride reagents. The first four gave positive results while the last two produced negative results for those phytochemicals tested. These confirmed the presence of alkaloids, phenols, steroids and saponins but absence of anthraquinones and flavonoids in methanol extract of *S. virosa* leaf from the result of preliminary phytochemical screening.

The absence and presence in all the three extracts of anthraquinone and triterpenes/steroids were confirmed by the TLC method. But, absence and presence of alkaloids in EE and HE respectively deviates from result of the preliminary phytochemical investigation conducted in this study but similar to that of Danlami *et al*., (2013). Saponins were found to be present in all the extracts but flavonoids were not detected by this method, contrary to the earlier presence in EE and ME by preliminary investigation and then might be due to limitation in the observation of yellow fluorescence. However, a colourless fluorescence was observed for ME. Thus, suggestion can be made for the use of other analytical method for the detection of flavonoids. Thin layer chromatographic analysis is a simple and cheap method for detection of plant active constituents due to its good selectivity and sensitivity of detection and providing, convincing results (Patra *et al.,* 2012). Therefore, it is considered as a reliable technique for qualitative phytochemical screening of plant active constituents.

Snake venom is a complex mixture of substances such as proteins (i.e. enzymes, peptides, etc), metal ions. By composition, it contains about 90% proteins of which majority are enzymes such as phospholipase A_2 (PLA_2), phosphodiesterase and acetylcholinesterases. Up to 40% of the 90% proteins in the snake venom are PLA_2s. PLA_2 is referred to as cellular degrading enzyme which signifies its multiple cascades of toxicities on virtually all cells in the victim's body. Phospholipase A_2 (PLA_2) is a lipolytic enzyme that hydrolyses the fatty acyl ester bonds at the 2-sn position of membrane phospholipids producing equimolar amounts of free fatty acid (FFA) and lysophospholipid; mainly arachidonic acid (AA). These products then become available for conversion to potent pro-inflammatory mediators, such as platelet-activating factor and eicosanoids respectively (Adamude *et al.,* 2016).

Animal antivenom immunotherapy is the only specific treatment against snake venom envenomation. But, it is associated with various side effects such as anaphylactic shock, pyrogen reaction and serum sickness. Alternative to this conventional therapy is antivenin from plant origin (Alam and Gomes, 1994; Meenatchisundaram *et al*, 2009, Kunjam *et al*., 2013). In this present study, *S. virosa* leaf extracts appears to exhibit a non-competitive pattern of PLA_2 enzyme inhibition of which methanol extract shows the best activity with weak interaction between the extract and the substrate (egg yolk). Indeed, polar aqueous extract of *Casearia sylvestris* showed anti PLA_2 activity (Gomes *et al.*, 2010) while the alcoholic and aqueous extracts of *Pouzolzia. indica*, significantly inhibited the PLA_2 enzyme activity possibly by preventing hydrolysis of lecithin (Ahmed *et al.*, 2010). Di-n-octyl phthalate isolate from *Ceiba pentandra* leaves extract was reported to be biologically active inhibitor of Echis ocellatus venom PLA_2 in a dose dependent manner (Ibrahim *et al*., 2011). Similarly, *Azadirachta indica, Aristolochia shimadai, Eclipta prostrate, Schummaniophyton magnificum* were found to be active inhibitors of PLA_2 (Gomes *et al.*, 2010; De and Dey, 2012). Saifullahi *et al.*, (2014) reported that Oleanyl erucoate, isolated from N-hexane extract of *Cryptolepis oblongifolia* obtained from Zaria, exhibit a non-competitive inhibition of PLA_2 *in vitro*. Polar constituents (i.e. phenols) from plants also have been reported to be potent inhibitors of PLA_2 (Kadir *et al.*, 2015).

In order to determine the safety margin of drugs and plant products for human use, toxicological evaluations are carried out in experimental animals using various methods to predict toxicity. The acute toxicity evaluation provides a guide for selecting a "safe'' dose in animals and to

estimate the therapeutic index (LD_{50}/ED_{50}) of drugs (Olson *et al.,* 2000; Rang *et al.,* 2003; Maikai *et al.,* 2008). The LD_{50} of N-hexane extract of *S.virosa* was found to be 774.60mg/kg while that of Ethylacetate extract it is practically non-toxic in mice at the dose of 5000mg/kg which suggested that the ethylacetate extract is moderately toxic. This is based on the toxicity classification which states that substances with LD_{50} values of 5000 to 15,000 mg/kg body weight are practically non-toxic (Loomis and Hayes, 1996). On the other hand, the LD_{50} of methanol extract of the leaf of *S.virosa* had already been determined by many researchers for the same plant in same study location (Magaji *et al.*, 2008, Tanko, *et al.*, 2008) which both stated LD_{50} of 1265mg/kg for the methanol extract.

Elapid such as *Naja nigricollis* envenoming is characterized by a progressive descending neuromuscular paralysis, respiratory failure and death (Alirol *et al.*, 2017). The efficacy of antivenom against particular venom is due to the ability of antivenom molecules to bind with toxins in the venom. The most widely used method for assessing antivenom efficacy is rodent lethality testing (Silva *et al.*,, 2017). In this study, the effect of the leaf extracts of *S. virosa* on ameliorating the lethal effect of venom of *Naja nigricollis* was conducted. The n-hexane extract offered better protection, followed by methanol extract at $p < 0.05$. Also, the result has shown that n-hexane and methanol extracts activities are statistically significant at 20 mg/kg dose in a mixture with half of LD_{99} of the venom given intraperitoneally to the mice.

Methanol extract of stem bark of *C. africana* produced a dose dependent in vitro detoxifying action against *Naja nigricollis* venom (Isa et al., 2015).. However, for *S. virosa*, n-hexane extract is more potent in neutralizing venom toxicity

In another study conducted in Nassarawa state, Shekins *et al.* (2014) reported that there was significant difference the time of death between *Mucuna pruriens* leaves extract treated group and those treated with *Naja hannah* venom alone. The superior activity of n-hexane extract bioactive constituents has been reported by Mathias *et al.* (2016) where a triterpene; Friedelin isolated from *Albizia chevalieri* showed a significant anti-venin activity of 64% protection. It also, showed that ethylacetate extract had the least potential antivenin effect. This is in concordance with the finding in this present study. The alleviation of toxic symptoms and survival of laboratory animals (within a short time frame) after being challenged with lethal doses of venom is in good agreement with the previous studies (Onyeama, *et al.*, 2013; Isa *et al*., 2015 and Bhole and Bhavsar, 2017) in which different classes of plant constituents have demonstrated *in vivo* activity against some snakes venom.

The extract of the leaf of *S.virosa* showed better protection to mice from *Naja nigricolis* venom induced histopathological changes in the brain, heart, lungs, liver and kidney. Bergenin from *fluggea virosa* was reported to be a potent hepatoprotective agent (Rastogi and Rawat, 2008).

Naturally occurring substances such as sitosterol, pentacyclic terpenes, nitro compounds (aristolochic acid), cinnamic acid derivatives, curcuminoids, polyphenolic compounds, and flavonoids are known compounds possessing protein-binding and enzyme-inhibiting properties. The extracts of *S. virosa* were shown to contain alkaloids, tannins, flavonoids and other secondary metabolites similar to those earlier reported with activity against snake venom

constituents. These compounds act synergistically to neutralize snake venoms *in vivo* and *in vitro*.

The findings in this work have indicated that the extract of *Securinega virosa* contain compounds which can neutralize *N. nigricollis* venom both *in vitro* and *in vivo*. This present study has explained the basis for using this plant in traditional medicine to treat snake envenomation in Nigeria. A positive correlation exists between traditional use of medicinal plants and their pharmacological investigations (Dey and De, 2012). Other parts of *S. virosa* such as root bark, fruit and phytoconstituent bergenin have not been investigated for possible potent anti-snake venom activity.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

Some pharmacognostic standard for the leaves of *Securinega virosa* obtained in Zaria were established for the first time in this study to the best of my knowledge and these data could be used as a diagnostic tool for the standardization and proper identification of this medicinal plant.

The research began with the microscopic examination of the leaf surfaces and the transverse section of the leaf of *S. virosa* which revealed some important diagnostic characters on both upper and lower epidermal layers these includes: Epidermal cells which are polygonal with

straight anticlinal wall and 2-5 -8 µm in size. Stomata were found only on the lower epidermis which are anomocytic and paracytic of 4-5-6µm in diameter.. Unicellular trichomes are rare but present on both surfaces. Prism crystals of calcium oxalate of 1-2-3 µm. it is arranged in a sheath or single which are abundance on both upper and lower epidermal surfaces. The transverse section through the midrib of the leaf shows mesophyll, vascular bundle (xylem and phloem), Calcium oxalate crystals and single layer of epidermal cell on the upper and lower leaf surfaces

The physical parameters of the powdered leaf of *S. virosa* were found to be; moisture content (5.55 %), total ash value (11.67 %), acid insoluble ash (1.16 %), ethanol extractives value (22.00 %) and water extractives value (34.00 %). Chemomicroscopical features of powdered leaf of *S. virosa* revealed the presence of cellulose cell wall, lignified cell wall, mucilage, tannins, suberin, calcium carbonate and calcium oxalate crystals. Inulin was absent.

Preliminary phytochemical analysis of the *S. virosa* leaf extracts revealed the presence of some secondary metabolites namely alkaloids, tannins, flavonoids, cardiac glycosides, saponins and steroids/ triterpenes while anthraquinones were absent. The leaf extracts of *S. virosa* possessed various chromatographic fingerprints of diagnostic and identification significance with eleven (11) spots in hexane: ethyl acetate (9:1), fourteen (14) spots in hexane: ethyl acetate (13:7) and eleven (11) spots in butanol: acetic acid: water (10:1:1) for hexane, ethylacetate and methanol extracts respectively. However, the TLC phytochemical screening of the extracts visualized with specific reagents confirmed the presence of important phytoconstituents such as alkaloids, phenols, saponins and steroids/ triterpenes. However, anthraquinones and flavonoids were absent.

The PLA_2 assay showed that leaf extracts of *S. virosa* of possessed bioactive constituents with PLA_2 inhibitory properties. Methanol leaf extract of *S.virosa* showed greater activity among the other two extracts with inhibition pattern of a non-competitive type and a weak dissociation constant. The intraperitoneal median lethal dose (LD_{50}) of the extracts was found to be 1265.5mg/kg and greater than 5000 mg/kg for hexane and ethylacetate respectively. The leaf extracts of *S. virosa* have shown potentials to possess *Naja nigricollis* snake venom neutralization activity, of which the activity of methanol and hexane extracts, were significant at the doses of 20 mg/kg in mice. The finding was correlated by the histological results where the extracts protected the mice from venom induced pathological changes in the various organs.

6.2 Conclusion

The pharmacognostic studies of the leaf of *S. virosa* yielded a set of qualitative and quantitative parameters that may be useful in ascertaining the identity of the plant and to determine the quality and purity of *S. virosa* leaf drug materials

The preliminary and thin layer chromatographic phytochemical screening, as well as, chromatographic fingerprinting conducted for leaf extracts of *S. virosa* yielded the presence of significant phytoconstituents and fingerprints that would be useful for identification and standardization of *S. virosa*.

The PLA_2 assay showed the potential of the leaf extracts of *S. virosa* possessing bioactive constituents with PLA_2 inhibitory properties. The methanol extract was found to exhibit the best PLA_2 inhibitory effect.

This work showed that n-hexane extract of *S. virosa* has antivenin properties against *Naja nigricollis* venom induced toxicity in mice. These results have provided some scientific bases for the use of the plant in traditional medicine for the management of snakebite.

6.3 Recommendation

- Further work needs to be done to identify, purify and quantify the antivenin compound(s) in *S. virosa*
- Quantification of phytochemicals in the leaf and other vegetable parts should be conducted.
- Sub-acute and chronic toxicity test should be carried out in order to determine the long-term effects of the extracts.
- Standardization of the other vegetable parts; root, stem bark, fruit and seeds traditionally used as medicine is paramount.
- Investigation into the geographical and seasonal variations of the bioactive constituents of the plant is also essential.

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APPENDIX I

Sample Calculation of percentage yield

$$percentage\ yield = \frac{-\text{final weight of extract}}{\text{initial weight of sample}}\ X\ 100$$

Weight of sample = 300g

Weight of empty container = 669.75

Weight of container + sample = 677.75g

Final weight of extract = 677.75 – 669.75 = 8.00g

$$percentage\ yield = \frac{8.00\text{g}}{300\text{g}}\ X\ 100$$

$$= 2.67\%$$

Table 1: The physical properties and percentage yield of the extraction of dried leaf powder 9300g) of *S. virosa*

S/N	Extraction solvent	Yield in grams (g)	Yield in percentage (%)
1	N-hexane	8.00	2.67
2	Ethylacetate	11.22	3.74
3	Methanol	46.67	15.65

Table 2: The physical properties of the extracts of *S. virosa*

S/N	Extracts	Colour	Odour
1	N-hexane	Dark brown	Agreeable
2	Ethylacetate	Dark green	Agreeable
3	Methanol	Dark green	Agreeable

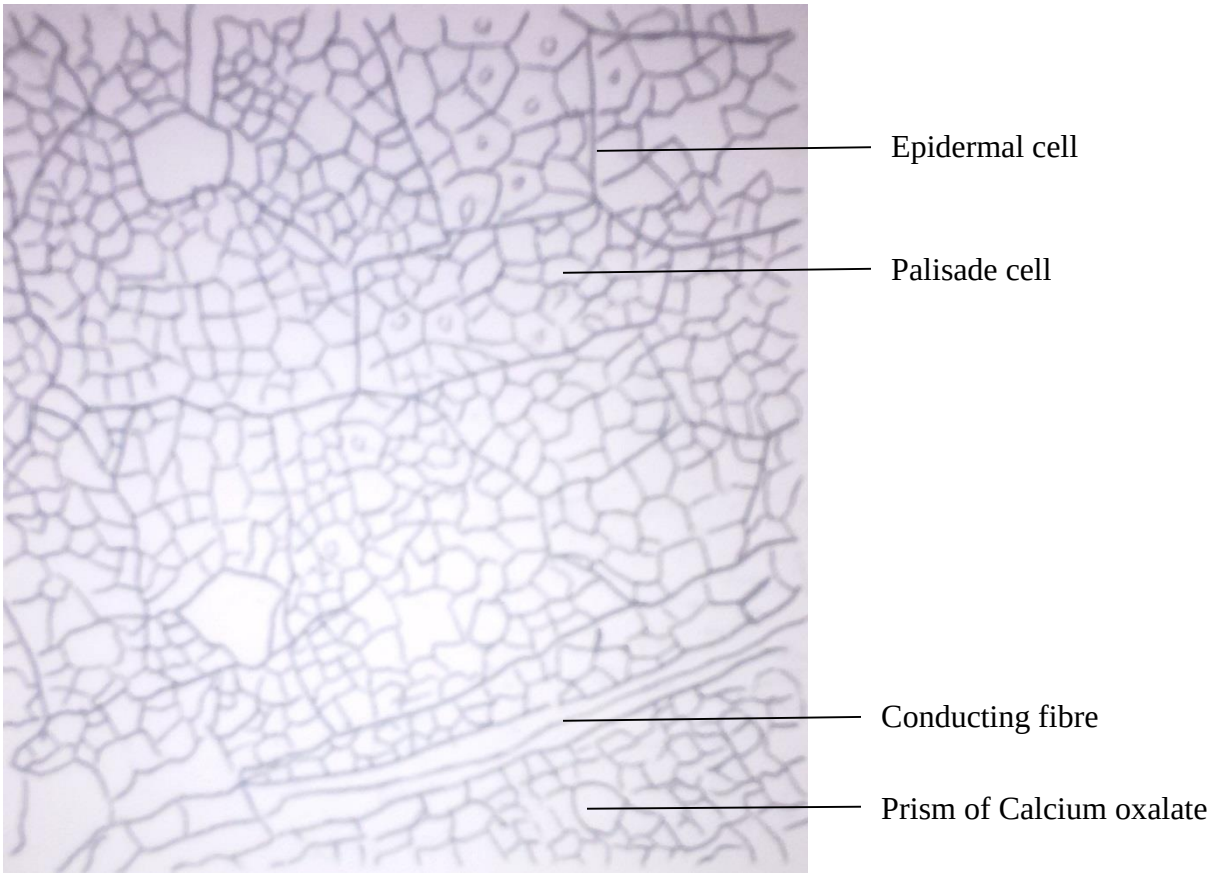


Plate III: Photomicrogaph of some microscopic features of epidermal layer of *S. virosa* leaf (X400)

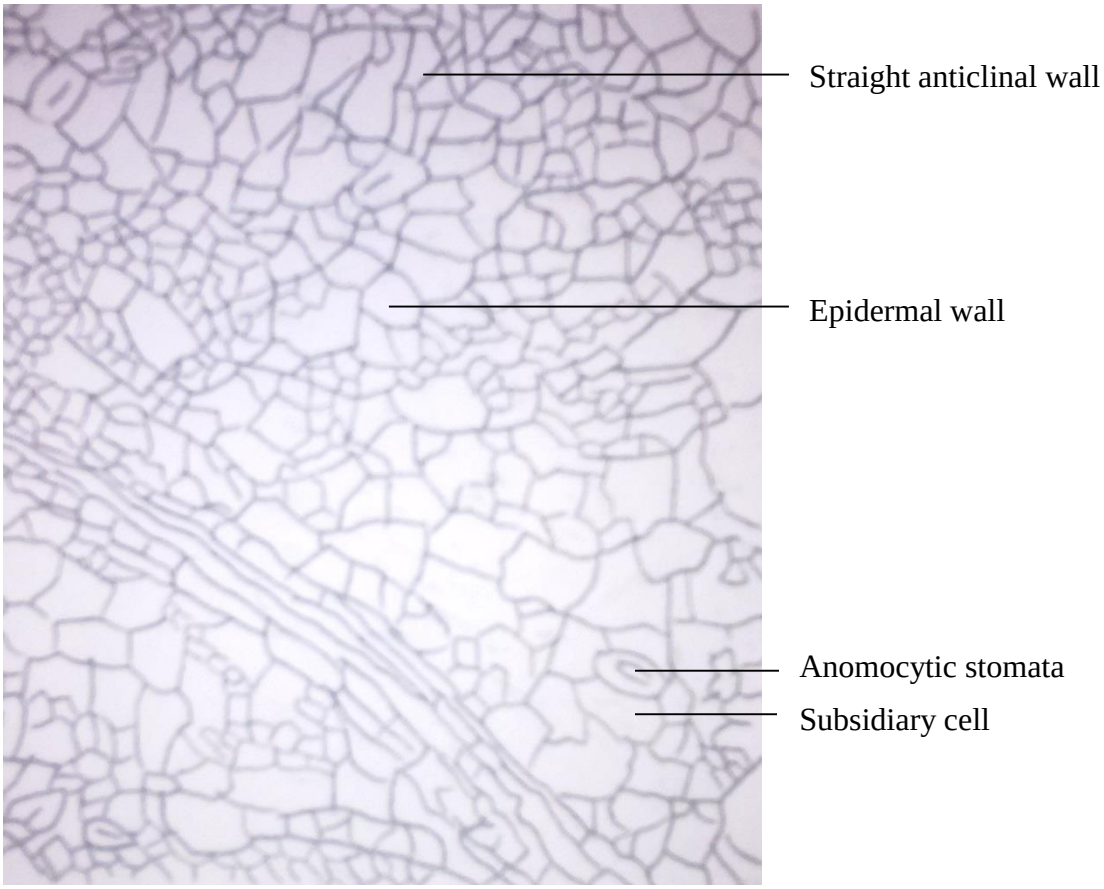


Plate IV: Stomata of the lower epidermal surface of *S. virosa* leaf (X 400)

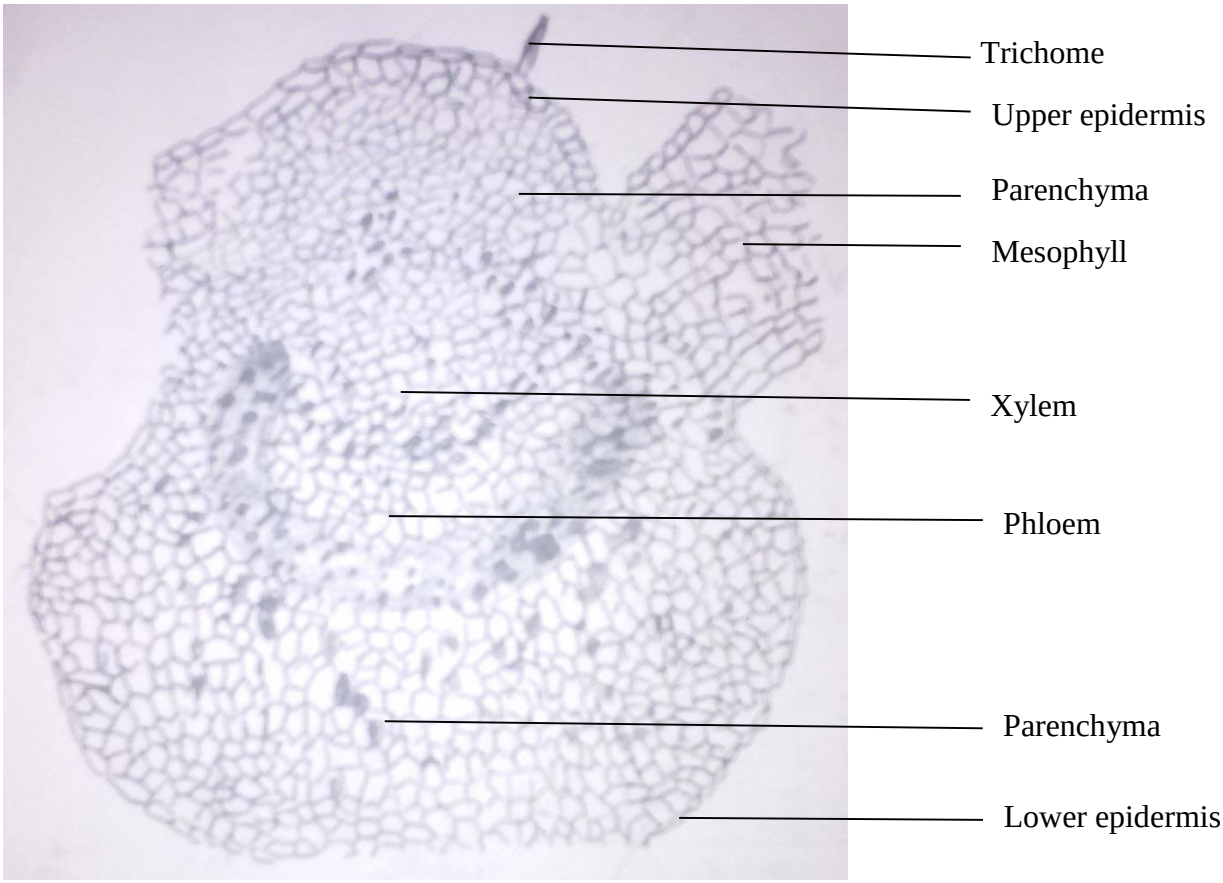


Plate V: Photomicrograph of Transverse section through the midrib of *S. virosa* leaf (X 400)

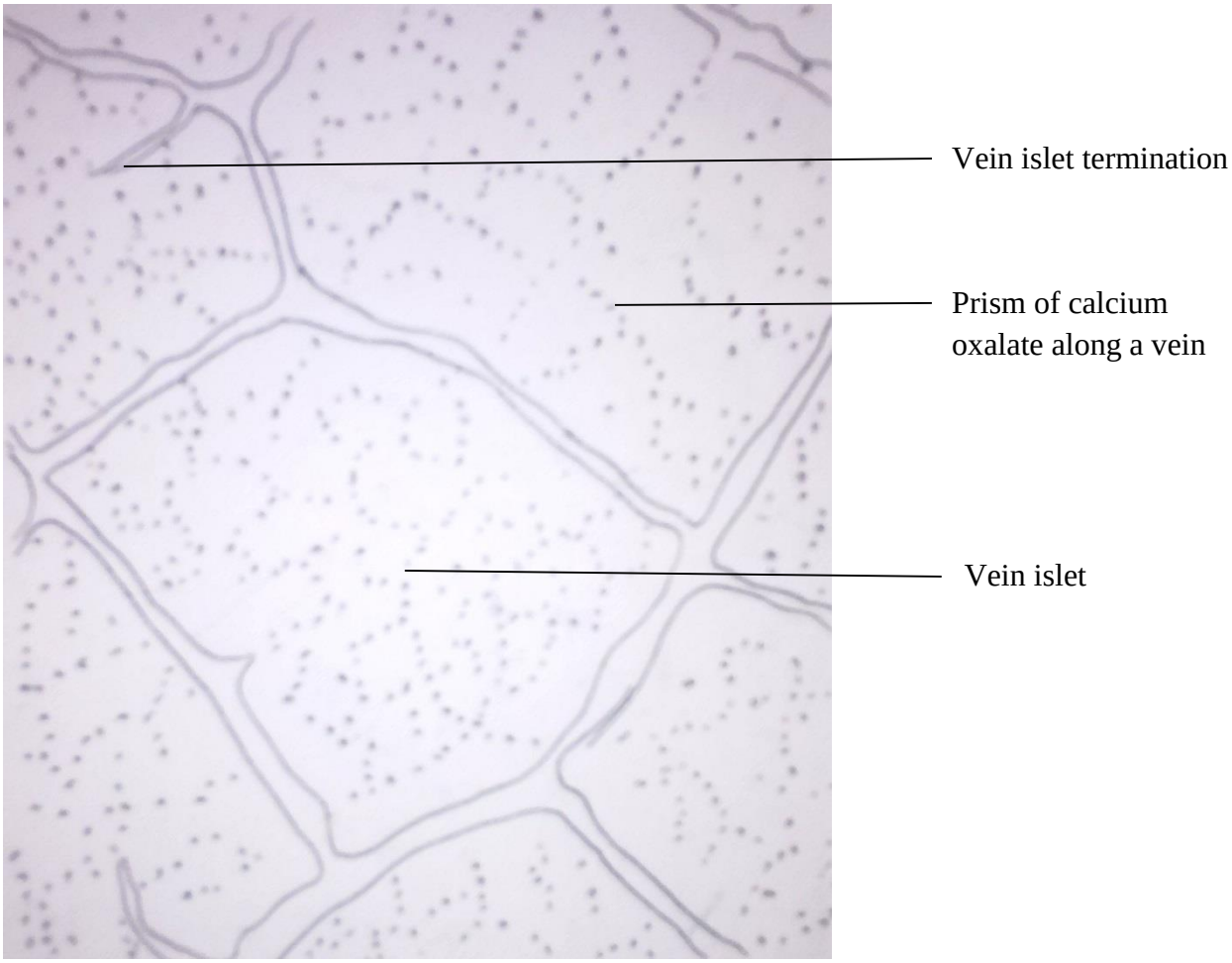


Plate VI: Photomicrograph of adaxial surface of *S.virosa* leaf (X 400)

Moisture content $= \frac{\text{weight of water loss}}{\text{initial weight of sample}} \ X \ 100$

f. Weight of sample = 3g
g. Weight of empty crucible = 30.11g
h. Weight of crucible plus sample after heating to constant weight = 32.93g
i. Weight of sample after heating = c – b =32.93g – 30.11g =2.82g
j. Weight of water lost = 3 – 2.82 = 0.18g

Moisture content $= \frac{0.18}{\text{i}3.00} \ X \ 100$ = 6%

Appendix II

Table 3: Acid – base titration values for methanol extract of S. virosa leaf extract

S/N	Initial volume (ml)	Final volume (ml)	Volume of base used (ml)
1	15.00	16.00	1.00
2	16.00	17.30	1.30
3	17.30	18.40	1.10
4	18.40	19.20	0.80

Table 4: The activity of S. virosa leaf extract on phospholipase A 2

S/n	Initial velocity (v)	1/v	Substrate Conc.	1/substrate conc.
1	66.67	0.015	25	0.040
2	86.7	0.012	50	0.020
3	73.3	0.014	75	0.013
4	53.3	0.019	100	0.010

$$Initial\ velocity = \frac{\text{Mb X Vb}}{\text{Va}}\ X\ 100$$

Mb = molarity of base in millimole = 20mM

Vb = volume of base used = titre value

Ma = molarity of free fatty acid released = Initial velocity = x

Va = Total volume of the mixture = 0.3ml

$$Initial\ velocity = \frac{20\ \text{X}\ 1}{0.3}$$

$$= 66.67\ \text{mM}$$

APPENDIX III

Table 5: the behavioural observations in mice treated with venom and mixture of venom and leaf extracts of S. virosa

Treatment groups	**methanol**	**ethylacetate**	**n-hexane**
Venom	-Ptosis - Descending flaccid paralysis	-weakness -ptosis -Descending flaccid paralysis	-Weakness -Convulsion Descending flaccid paralysis
Venom + 5mg extract	-Weakness -Respiratory distress -Ptosis	-Sweating -Paralysis	-Convulsion -Descending flaccid paralysis
Venom + 10mg extract	-Ptosis -Convulsion -Abdominal cramp	-Sweating -Paralysis	-Increase activity -Normal activity
Venom + 20mg extract	-Abdominal cramp -Ptosis	-Sweating	-Weakness Normal activity