SCREENING OF*SECURIDACA LONGIPEDUNCULATA*EXTRACTS FOR ACTIVITY AGAINSTNEWCASTLE DISEASE VIRUSANDBACTERIAL ISOLATES FROM UPPER RESPIRATORY TRACT OF HUMAN

BY

AMINA BALARABE ADAMU

(SPS/14/MMB/00019)

JANUARY, 2017

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A DISSERTATION SUBMITTED TO THE DEPARTMENT OF MICROBIOLOGY, BAYERO UNIVERSITY, KANO IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE DEGREE IN MICROBIOLOGY (MEDICAL)

JANUARY, 2017

DECLARATION

I hereby declare that this research work entitled **"Screening of *Securidacalongipedunculata* extracts for activity against Newcastle Disease Virus and bacterial isolates from Upper Respiratory Tract of Human"** is the product of my research efforts undertaken under the supervision of Dr. Muhammad Yusha'uand has not been presented elsewhere for the award of a degree or certificate. All sources have been duly acknowledged.

……………………………………...

(Signature and date)

Amina Balarabe Adamu

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CERTIFICATION

This is to certify that the research work for this dissertation and the subsequent write – up by Amina Balarabe Adamu with registration number SPS/14/MMB/00019 were carried out under mysupervision.

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APPROVAL

This dissertation has been examined and approved for the award of Mastersdegree in Medical Microbiology.

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DEDICATION

This dissertation is dedicated to my parents for their endless love, support and encouragement.

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In the name of Allah, the Most Merciful, the Most Compassionate, all praise be to Him, the lord of the worlds; and prayers and peace be upon Muhammad His servant and messenger.

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ABSTRACT

*Securidacalongipedunculata*Fresen (Polygalaceae) is a multi-purpose plant with a long history of use in African traditional medicine to treat various infections.This study was carried out to determine the antiviral and antibacterial activity of *Securidacalongipedunculata* root, stem bark and leaf extracts. The plant parts were extracted with soxhlet apparatus using methanol and petroleum ether as the extraction solvents. The extracts were subjected to phytochemical screening using standard procedures. Antiviral activity of the extracts was carried out using embryonated chicken eggs treated with 100EID_{50} / 0.1 ml Newcastle Disease Virus (NDV) pre-treated with *S. longipedunculata* crude extracts at concentrations of 5mg/ml to 40mg/ml based on the toxicity result and embryo survival was observed daily. The crude extracts of the plant parts were tested against upper respiratory tract bacteria using agar well diffusion method. Working concentrations of 120µg/ml, 60µg/ml, 30µg/ml and 15µg/ml of the extracts were used. Dimethylsulphoroxide served as negative control and ciprofloxacin (30µg/ml) served as positive control. The mean diameter zones of inhibition was measured and results recorded in millimeter (mm). The crude extracts were re-extracted using column chromatography analysis using standard procedures. Fractions of column chromatography for the crude extracts that showed activity were again tested using the same procedure on *S. pyogenes* and *Proteus* spp.with working concentrations of 960µg/ml, 480µg/ml, 240µg/ml, 120µg/ml, 60µg/ml, 30µg/ml and 15µg/ml. The result of phytochemical screening revealed the presence of some secondary metabolites of pharmacological significance. Antiviral result showed that embryo survival was directly proportional to increasing extract concentration, just as increase in extract concentration was directly proportional to virus death. This is similar to the antibacterial activity in which the increase in the concentrations of crude extracts and fractions is directly proportional to the increase in the zone diameter of inhibition of the bacteria. The extracts were found to contain fourteen differentfractions from column chromatography. These findings have clearly demonstrated that, *S. longipedunculata*extracts have antiviral and antibacterial activity against Newcastle disease virus and upper respiratory tract bacteria respectively.

Keywords: NDV = Newcastle Disease Virus, EID_{50} = Egg Infective Dose of virus in 50% embryonated chicken eggs, µg/ml = micro-gram per milliliter.

CHAPTER ONE

1.0 INTRODUCTION

During the last two decades, the development of drug resistance as well as the appearance of undesirable side effects of certain antibiotics (Cohen *et al.,* 1994; Poole, 2001; WHO, 2001) has led to the search for new antimicrobial agents mainly among plant extracts with the goal to discover new chemical structures which overcome the above disadvantages (Herrera *et al.,* 1996; De Smet, 1997; Sokmen *et al.,* 1999; Kelmanson *et al.,* 2000; Meng *et al.,* 2000).

Medicines are chemical substances that are used for treatment, alleviation, control, prevention of diseases, and maintenance of health(Alfred, 2013). The existence of man on earth has been accompanied by diseases, and with them, suffering and death. Man therefore, started searching for causes, prevention and treatment of diseases. Ancient management of these diseases comprised of magic, superstition and annual sacrifices (Alfred, 2013).

Fossil records date human use of plants as medicines at least to the Middle Paleolithic age some 60,000 years ago (Alfred, 2013). From that point, the development of traditional medical systems incorporating plants as a means of therapy can be traced back only as far as recorded documents (Alfred, 2013). However, the value of these systems is much more than a significant anthropologic or archeological fact. Their value is as a methodology of medicinal agents, which, according to the World Health Organization (WHO), almost 65% of the world's population has incorporated into their primary modality of health care (Alfred, 2013). Some plant-derived products are so effective and dependable that the medical fraternity cannot manage some diseases without them.There are many examples of plant-derived products that have been made into medicines and used in treatment of diseases (Alfred, 2013). Thirty percent of the

pharmaceutical agents used at present are plant derived (Alfred, 2013).Traditional methods of drug discovery and development have been influenced by the need to prevail over illness and peoples experience in witnessing and realizing the beneficial potentials of a plant to cure ailments, perhaps by trial and error (Alfred, 2013).

Plants have long provided mankind with herbal remedies for many infectious diseases and even today, they continue to play a major role in primary health care as therapeutic remedies in developing countries (Sokmen *et al.,* 1999). The search for biologically active extracts based on traditionally used plants is still relevant due to the appearance of microbial resistance of many antibiotics and the occurrence of fatal opportunistic infections. It is known that traditional healers use indigenous medicinal plants to treat many illnesses (Ndubani, 1997; 1999).

Plant materials remain an important resource to combat serious diseases in the world. Pharmacognostic investigations of plants are carried out to find novel drugs or templates for the development of new therapeutic agents (Konig, 1992). Among the more than 250 000 species of higher plants, only about 5–10% are chemically investigated (Nahrstedt, 1996). Since many drugs, like quinine and artemisinin (Wright and Phillipson, 1990), taxol and camptothecin (Debernardis *et al.,* 1996) were isolated from plants, and because of increased resistance of many microorganisms towards established drugs, investigation of the chemical compounds within traditional plants is necessary (Phillipson, 1991).

Drug discovery from medicinal plants led to the isolation of early drugs such as cocaine, codeine, digitoxin, and quinine, in addition to morphine, of which some are still in use (Newman *et al.,* 2000; Butler, 2004; Samuelsson, 2004). Isolation and characterization of

pharmacologically active compounds from medicinal plants continue today. More recently, drug discovery techniques have been applied to the standardization of herbal medicines, to elucidate analytical marker compounds (Newman *et al.,* 2000; Butler, 2004; Samuelsson, 2004).

Renewed interest in traditional pharmacopoeias has meant that researchers are concerned not only with determining the scientific rationale for the plants usage, but also with the discovery of novel compounds of pharmaceutical value. Instead of relying on trial and error, as in random screening procedures, traditional knowledge helps scientists to target plants that may be medicinally useful (Alfred, 2013).Already an estimated 122 drugs from 94 plant species have been discovered through ethnobotanical leads (Alfred, 2013).

The genus *Securidaca* comprises about 80 species, characterized by papillionaceous purplish flowers and mostly scandent shrubs and lianas, which produce compounds known as securixanthones with antimicrobial and antioxidant properties (Wallnöfer, 1998; Yang *et al*., 2001; Yang *et al*., 2003; Da Costa *et al*., 2013). Although protected under provincial and national legislation, *S.longipedunculata* stem bark and roots are still found amongst the most traded medicinal plants in Africa (Moeng, 2010; Tabuti *et al*., 2012).

Newcastle disease (ND) is an acute, rapidly spreading, contagious, nervous and respiratory disease of birds of all ages caused by the avian paramyxovirus serotype 1 (APMV-1) (Okeke and Lamorde, 1988). It is a major viral disease of economic importance in poultry (Anosa and Adene, 2007) and rated as one of the greatest constraints to the developing countries including Nigeria, causing serious threats (Oladele *et al*., 2003). The disease is caused by an enveloped, non- segmented RNA avian paramyxovirus which is transmitted through exposure to faecal,

respiratory discharges or rather discharges from infected birds and through contact with contaminated feeds, water, equipment, poultry attendants and clothing, leading to high morbidity and mortality(Oladale *et al*., 2003; Saidu *et al*., 2006).

Acute upper respiratory tract infections are a group of heterogenous diseases caused by a diverse group of organisms in which the anatomical site(s) involved consists of the airways from the nostrils to the vocal cords in the larynx, including the paranasal sinuses and the middle ear (Johnson, 2007; Simeos *et al*., 2006). These infections were grouped as syndromes involving the upper airways such as pharyngitis, sinusitis, otitis media, epiglottis and tonsillitis (Ide and Onyenegecha, 2015).

With the seriousthreatsof Newcastle disease virusin poultry and recent surge in multi – drug resistance of upper respiratory tract bacteria in human, there is an urgent need to extend the search for antimicrobials towards medicinal plants. *Securidaca longipedunculata*, which has long been used in traditional medicines by locals, and which has many different medicinal uses (Matthews, 1994), can serve as a source of new pharmaceuticals in curbing these organisms and thus the development of traditional medicines.

Statement of the Research problem

i- Over the years, Newcastle disease (ND) has defied all control measures (Shitu*et al*., 2016). The disease has remained at the forefront of infectious diseases afflicting poultry production after avian influenza. Despite the continuous global use of million doses of ND vaccine annually, the causative pathogen, avian paramyxovirus type 1 also known as Newcastle disease virus (NDV) has continued to evolve causing even

more, a threat not only to the unvaccinated but the vaccinated flocks inclusive (Shitu *et al*., 2016). However, there is currently no means of treatment existing for Newcastle disease except vaccination (Okwor and Eze, 2010). This is a serious threat to the poultry industries which is causing a huge economic loses to these industries. In view of this and economic importance of ND, the search for the treatment of this disease is reasonable. Moreover, few antiviral research (Borikini *et al*., 2013) on *Securidaca longipedunculata* were published. Hence, there is need to search for more antiviral properties of this plant.

ii- There are currently no medications or herbal remedies that have been conclusively demonstrated to shorten the duration of the respiratory tract illness (Smith *et al*., 2008). Data from National Demographic Health Survey2013 reported the prevalence of upper respiratory tract infections to be about 2% (NPC and ICF, 2014; Ide and Onyenegecha, 2015). Hence, the search for the treatment of upper respiratory tract infections became necessary.

1.2 Justification

Plant-derived products have revolutionized the medical profession, as they have commonly been used in alleviation, control, treatment and prevention of diseases in addition to improved health and life expectancy. Natural products discovered from medicinal plants (and derivatives thereof) have provided numerous clinically used medicines. Even with all the challenges facing drug discovery from medicinal plants, natural products isolated from medicinal plants can be predicted to remain an essential component in the search for new medicines(Alfred, 2013)..

Although herbal medicines have been used by traditional healers from time immemorial, chemical constituents of most of them are unknown (Chhabra *et al*., 1991). In addition, not much information is available on the antiviral screening bioassays in *Securidaca longipedunculata* plant. It is because of the numerous medicinal uses and the many chemotherapeutic compounds identified in *Securidaca longipedunculata* plant that this project was conceptualized.

1.3 Aim

To evaluate the antiviral and antibacterial activity of *Securidaca longipedunculata* extracts against Newcastle disease virus (NDV) and upper respiratory tract bacteriaof human respectively.

1.5 Objectives

The objectives of the study where to:

i- Extract root, leaf and stem bark of *Securidaca longipedunculata* with Soxhlet apparatus using methanol and petroleum ether solvents.

ii- Determine Egg Infective Dose (EID_{50}) of NDV using embryonated chicken eggs.

iii- Determine the antiviral activity of the plant extracts usingembryonated chicken eggs.

iv- Determine the antibacterial activity of the plant extracts using agar well diffusion method.

v- Identify the active compounds that may be present in *Securidaca longipedunculata*extracts.

1.5 Hypothesis

i- *Securidaca longipedunculata* extracts have antiviral effect against Newcastle disease virus.

ii- *Securidaca longipedunculata* extracts have antibacterial activity against upper respiratory tract bacteria.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Botanical description and distribution of *Securidaca longipedunculata*

Securidaca longipedunculata Fresen, (synonyms *Securidaca longipedunculata* var. *longipedunculata* or *Elsota longipedunculata*, family Polygalaceae) Figure 2.1 is a small tree up to 6 meters high with a pale grey, smooth bark and oblong, more or less hairless alternate leaves that are variable in size and shape and crowded towards the stem tips (Van Wyk *et al*., 2009). Clustered flowers are small, pink to lilac or purple in colour, sweet scented and are produced in early summer (Van Wyk *et al*., 2005). Fruits are a round nut, heavily veined, occasionally smooth, oblong, purplish green when young and possess a membranous wing of about 4 cm long (Coates-Palgrave,2005).The species is mostly distributed in various tropical African countries,including Angola, Benin, Botswana, Burundi, Cameroon, Chad, Cote d'Ivoire,Democratic Republic of Congo, Eritrea, Ethiopia, Gambia, Ghana, Guinea, Kenya,Malawi, Mali, Mozambique, Namibia, Niger, Nigeria, Rwanda, Senegal, Sierra Leone, South Africa, Sudan, Tanzania, Uganda, Zambia, Zimbabwe, Mozambique,as well as in the North West and Limpopo Provinces of South Africa (Baloyi and Tshisikhawe, 2009; Tshisikhawe *et al*., 2012).

Figure 2.1: ***Securidaca longipedunculata plant.***

Source: http//copperflora.org/eflora/photos/HQ/*Securidaca longipedunculata*/ plant&ved

Figure 2.2:*Securidaca longipedunculata plant.*

Source: http//copperflora.org/eflora/photos/HQ/*Securidaca longipedunculata*/plant&ved

2.1.1 Local names

The most common English name are; violet tree, fiber tree, or Rhodesian violet (Keshebo and Choudury, 2015), krinkhout (Afrikaans); umfufu (Ndebele); Mupesu (TshiVenda) and Mmaba in both Sotho and Tswana tribes (Orwa *et al*., 2009). In other African countries, various names in different cultural and ethnic groups have been used- Amharic (es a manahi); Arabic (saggat,alali); Lozi (mwinda) (Orwa *et al*., 2009); Lunda (mutata); Nyanja(mwinda,mpuluka); Bemba (mupapi); Luganda (lilo); Hausa (uwar magunguna,sanya); Mandinka (yodo,juto,jodo); Shona (mufufu); Swahili (muteya, mzigi, Chipvufana, mufufu, munyapunyapu, munyazvirombo, mutangeni, umfufu); Tigrigna (shotora); Tongan (njefu,bwazi,mufufuma); Wolof (fouf); Yoruba (ipeta)(Orwa *et al*., 2009).

2.1.2 Scientific classification

The plant*Securidaca longipedunculata* is classified below;

Kingdom: Plantae

Order: Fabales

Family: Polygalaceae

Genus: *Securidaca*

Species: *S. longipedunculata*

Binomial name: *Securidaca longipedunculata* Fresen.

Credit: https://en.m.wikipedia.org/wiki/*Securidaca_longipedunculata*.

2.1.3 Ethnomedicinal uses

The most commonly used plant part is the root and that the species is used in the treatment of a variety of ailments including coughs, fever, malaria, tuberculosis and sexually transmitted

diseases in different geographical areas. This provides support for a pharmacological basis of the use of the plant species in the treatment of such ailments (Mongalo *et al*., 2015).

The smoke resulting from burning the root of *S. longipedunculata*, combined with that of *Zanthoxylum zanthoxyloides,* is inhaled to treat malaria and fever (Hamill*et al*., 2003). A root decoction may also be drunk to treat fever, malaria, hernias, gonorrhoea, palpitations, headaches, oedema, rheumatism, diabetes, sexualimpotence, toothache, fungal infections and malaria (Chhabra *et al*., 1991; Moshi *et al*., 2007; Ogunmefun and Gbile, 2012; Maroyi, 2013). An infusion of the soaked root bark may be drunk as an aphrodisiac or mixed with other medicines and used as an emetic (Mabogo, 1990). Alternatively, a root decoction may be drunk in beer as an aphrodisiac (Motlhanka and Nthoiwa, 2013). The root bark is pulverised in water and the resulting mixture is inhaled or used to wash the head, treating excessive headache (Nordeng *et al*., 2013). A handful of roots are combined with the roots of *Sphedamnocarpus pruriens* subsp. *pruriens* for treating people believed to be possessed by evil spirits while the powdered root is mixed with porridge and eaten to treat epilepsy and convulsions (Sobiecki, 2008).The decoction from the root is drunk or applied topically to skin treat cancer (Ashidi *et al*.,2010). Roots may also be ground into powder form, dissolved in water and taken orally for constipation, pneumonia, back ache, blood purification, sexually transmitted infections and as an aphrodisiac (Viol, 2009). Dried roots are soaked in water, along with *Citrus aurantifolia* and the resulting juice is taken orally for three days to treat constipation while the dried root is boiled in distilled water along with that of *Annonasenegalensis* and used to treat pneumonia (Mustapha, 2013a). Moreover, the dried root is ground into powder, along with that of *Parkia biglobosa* and then taken with cow’s milk as a sexual boost. The pounded root may be mixed with that of

Zanthoxylum humile and taken with soft porridge to treat erectile dysfunction (Semenya and Potgieter, 2013).

Fresh leaves are made into paste with little or no water along with the bark of *Gardenia erubescens* and applied externally twice a day for sixty-three days to treat skin cancer (Mustapha, 2013a). Moreover, fresh leaves are made into paste with little or no water along with leaves of *Jussiaea suffruticosa* and shea butter and the resulting mixture is applied externally, twice a day to treat a variety of skin infections. Dry leaves are also ground into powder and put into the fire and the resulting smoke is inhaled to treat headaches while the boiled leaves are taken orally for contraceptive purposes (Mustapha, 2013b). The leaves are either chewed fresh or both orally and nasally administered to treat epilepsy, headaches, stomach ache, infertility, snakebite, toothache and to expel the placenta (Augustino *et al*., 2011).

One cup from a whole plant decoction may be taken orally three times a day for three to four days to treat malaria (Nguta *et al*., 2010a; Nguta *et al*., 2010b). The decoction of the whole plant may either be drunk or used to wash the mouth and treat infections which include oral candidiasis, excessive coughing and other opportunistic infections associated with HIV/AIDS (Chinsembu and Hedimbi, 2010).

A spoonful of powdered stem bark is mixed with *Mondia whitei* (stem bark), *Uvaria afzelii* (root bark), *Allium ascalonicum* (bulb) and *Parkia biglobosa* (seeds) and then taken with hot porridge to treat a variety of viral infections (Borikini *et al*., 2013). The dried bark is ground into a powder and taken orally with cow's milk or porridge forfourteen days to treat dysentery (Mustapha, 2013a). A decoction from the stem barkmay be taken orally to treat stomach ache, headaches, inflammation, chestcomplaints, abortion, jaundice, ritual suicide, constipation, snake

bites, infertilityproblems, epilepsy and venereal diseases (Das, 2009; Bruschi *et al.*, 2011;Oladunmoye and Kehinde, 2011; Kadiri *et al.*, 2013). The powdered stem bark isalso mixed with hot water and taken orally to treat syphilis and gonorrhoea (Hedimbiand Chinsembu, 2012).

2.1.4 Phytochemistry

The volatile oil of *S. longipedunculata* of the roots contains large amounts of methyl salicylate as reported by Van Wyk *et al.*(2005). The report agrees with those of Jayasakara *et al.* (2002) and Lognay *et al.*(2000), which revealed that the major component (over 90%) of the volatile materialfrom the root bark is methyl-2-hydroxybenzoate (methyl salicylate). Furthermore, securinine, presenegenin, 2-hydroxybenzoate esters such as methyl 2-hydroxy-6-methoxybenzoate and its benzyl analogue were also reported (Mongalo *et al.*, 2015). In general, most classes of compounds have been isolated from the roots, using variety of solvents Cinnamic acid. Studies by Junaid *et al.* (2008), Auwal *et al.* (2012), Gbadamosi. (2012) and Haruna *et al.* (2013a) reported that, aqueous root and ethanol extracts yielded alkaloids, cardiac glycosides, flavonoids, saponins, tannins, volatile oils, terpenoids and some steroids while Adebayo and Osman (2012) reported the presence of flavonoids, saponins, coumarins, tannins and alkaloids in chloroform and ethanol extracts.

The ethyl acetate fraction of the root contained compounds such as 1,5-dihydroxy- 3,4,6,7,8-pentamethoxyxanthone,1,7-dihydroxyxanthone,5-O-prenyl-1-hydroxy-2,3,6,7,8 pentamethoxyxathanthone,2-hydroxy-1,7-dimethoxyxanthone,β-sitosterol,1,7-dihyroxy-4-methoxyxanthone, quercetin-3-O-β-galactopyranoside and 3-hydroxy-6-methoxysalicylic acid as reported by Meli *et al.* (2007). The compounds 1, 3, 6, 8- tetrahydroxy-2, 5-dimethoxyxanthone and 1, 6, 8-trihydroxy-2, 3, 4, 7-tetramethoxyxanthone were also isolated

from the acetone extract of the fresh root bark in a report of Meyer *et al*. (2008). Moreover, the hexane extract of the root indicated the presence of 1, 5-dihydroxy-2, 3, 6, 7, 8-pentamethoxyxanthone, 2-hydroxy-l, 7- dimethoxyxanthone and 1, 6-dihydroxy-xanthone as reported by Lannang *et al*.(2006).

The study of Muanda *et al*. (2010) that, water and aqueous methanol extracts from the root yielded a variety of compounds in varying amounts, including gallic acid, chlorogenic acid, caffeic acid, epicatechic acid, rutin, p-coumaric acid, cinnamic acid, apigenin, quercetin glucosyl and quercetin dehydrate. Four highly oxygenated xanthones, muchimangins A-D, with a diphenylmethyl substituent have also been isolated from the root as minor constituents as reported by Dibwe *et al*. (2012). The dichloromethane extract of the root bark yielded 4-methoxy-benzo[1,3]dioxol-5-yl-phenyl methanone and three other known compounds namely 1,7-dihydroxy-4-methoxyxanthone, benzyl-2- hydroxy-6-methoxybenzoate and methyl-2-hydroxy-6-methoxybenzoate as reported by Joseph *et al*. (2006). Dibwe *et al*. (2013) reported that, the chloroform extract of the root contained compounds such as 2- methoxy-3, 4-methylenedioxybenzophenone, benzyl 2-hydroxy-6- methoxybenzoate, 6-hydroxy-2-methoxy benzoic acid, 1,6,8-trihydroxy-2,3,4,5 tetramethoxyxanthone,1,6-dihydroxy-2,3,4,5,8-pentamethoxyxanthone, 8-hydroxy-1,4,5,6tetramethoxy-2,3- methylenedioxyxanthone,4,6,8-trihydroxy,1,2,3,5-tetramethoxyxanthone, 4,8- dihydroxy-1,2,3,5,6-pentamethoxyxanthone, benzyl 3hydroxy-2-methoxybenzoate and some other xanthones. Triterpene saponins such as 3-O-β-D-glucopyranosyl presenegenin 28-O-β-Dapiofuranosyl-(1→3)-β-D-xylopyranosyl-(1→4)-[β-D-apiofuranosyl-(1→3)]-α-Lrhamnopyranosyl-(1→2)-{4-O-[(E)-3,4,5-trimethoxycinnamoyl]}-β-D-fucopyrano ester and three other related esters have been isolated from the 70% aqueous methanol root extract as reported by Mitaine-Offer *et al*. (2010). De

Tommasi *et al*. (1993) reported that, besides sinapoic acid, caffeic acid, 4, 5-dicaffeoyl-D-quinic acid, 3, 4, 5-tricaffeoyl-Dquinic acid and a considerable number of monosaccharide and polysaccharide conjugates, the methanol extract of the stem bark revealed the two bitter principles β-D-(3,4-disinapoyl) (fructofuranosyl-α-D- (6-sinapoyl) glucopyranoside and β-D- (3-sinapoyl) (fructofuranosyl-α-D- (6-sinapoyl) glucopyranoside.

2.1.5 Toxicology

In a study by Auwal *et al*. (2012), the aqueous root bark extract has been shown to be slightly toxic to albino rats with an LD_{50} of 0.771g/kg, while Agbaje and Adekoya. (2012) reported an LD_{50} of 3.16g/kg when administered orally to rats. Moreover, acute toxicity studies of the aqueous whole root extract on mice Adeyemi *et al*. (2010), revealed LD_{50} values of 1.740g/kg and 0.020g/kg for the oral and intraperitoneal application routes respectively while Dapar *et al*. (2007) reported an LD_{50} of 0.037g/kg when aqueous root extracts were administered orally to albino rats (Sprague Dawley strain). The 80% ethanol extract of the root bark has been reported to have an LD_{50} of 0.547g/kg against albino mice (Keshebo *et al*., 2014).These findings may well suggest that the root bark extract has greater acute toxicity than the whole root extract following oral administration. In a repeated dose toxicity study by Etuk *et al*. (2006), there was no mortality observed when varying doses of 0.3, 0.9 and 2.7g/kg of aqueous root extract of *S. longipedunculats* were administered orally on a daily basis for a period of 28 days to Swiss albino mice.

However, there is no data in the literature on the administration of various isolated compounds from *S. longipedunculata* to mice or rats. In the brine shrimp bioassay, the 70% methanol extract of the root exhibited a 100% mortality rate at a concentration of 1000µg/ml (Adiele *et al*., 2013), while the 80% methanol root extract exhibited an LC_{50} of 77.1µg/ml as reported by Moshi *et al*.

(2007),suggesting that these extracts are relatively toxic. However, the brine shrimp assays have some problems as the counting of the viable larvae is performed while the live larvae are continually moving around the petri dish. This is reported by Adiele *et al*. (2013).

The aqueous root bark extract had been reported by Lawal *et al*. (2012). to be toxic to Ehrlich ascites tumor cells with a mortality rate of 82.5 % at 1000µg/ml and revealed an IC_{50} of 67µg/ml. Compounds such as 1,6,8-trihydroxy-2,3,4,5-tetramethoxyxanthone and 1,6-dihydroxy-2,3,4,5,8-pentamethoxyxanthone showed potent cytotoxicity with IC_{50} values of 22.8 and 17.4µM respectively against human pancreatic cancer cells as reported by Dibwe *et al*.(2013) while the 70% methanol extract of the root bark exhibited average inhibition of cell proliferation of 22.6 % at a concentration of 1µg/ml against HeLa cells as reported by Runyoro *et al*. (2005). However, this is not a useful result, when compared to the IC_{50} which will explain the overall average concentration at which 50% of the cells will be inhibited by the test plant extract.

2.1.6 Pharmacology

The study by Ndamitso *et al*. (2013) reported that, the aqueous leaf extract yielded zones of inhibition (ZI) of 15mm against both *Escherichia coli* and *Salmonella typhi*, while the chloroform leaf extracts exhibited a ZI of 18mm against *Pseudomonas aeruginosa* at a concentration of 7.5mg/disc. The methanol extracts and the chloroform fraction of the root bark exhibited ZI of 28mm against methicillin resistant *Staphylococcus aureus*, while hexane and ethyl acetate fractions exhibited ZI ranging from 14 to 19mm against *Streptococcus pyogenes*, *Pseudomonasfluorescens* and *Klebsiella pneumoniae* as reported by Musa *et al*.(2013). Adebayo and Osman (2012) reported a ZI of 15.10mm by the ethanol extracts of the root bark at a concentration of 100mg/ml.

Ndamitso *et al*. (2012) reported a minimum inhibitory concentration (MIC) of 0.50mg/ml against both *S. typhi* and *P.aeruginosa* in broth micro dilution assay of chloroform extracts of the leaf of *Securidaca longipedunculata*while the aqueous extracts of the leaf revealed MIC of 6.25mg/ml and minimum bactericidal concentration (MBC) of 62.5mg/ml against *S. typhi*. Besides exhibiting MIC of 0.30 and MBC of 0.60mg/ml against both *S.aureus* and *P. aeruginosa*, the acetone extract of the root had a total activity of 19200ml/g against these bacterial strains as reported by Ndamitso *et al*.(2012),suggesting that the extract may be a good source of antibacterial compounds. The extract of the acetone soluble portion of the root exhibited a potent MIC of 0.02mg/ml against *Bacillus subtilis* and*S. typhi* as reported by Ajali and Chukwurah. (2004). Moreover, similar extract exhibited MIC of 0.10mg/ml against both *E. coli* and *P. aeruginosa* (Ajali and Chukwurah, 2004).

Other reports revealed that, the essential oil from *S. longipedunculata* had MIC of 12.79mg/ml against *E. coli* (Alitonou *et al*., 2012), while the 70% methanol extract of the leaves exhibited MIC of 0.45mg/ml and 0.23mg/ml against *Serratia marcescens* and *Shigella flexneri* respectively (Karou *et al*., 2012). The *n*-hexane extract of the root exhibited MIC values ranging from 0.0312 to 0.250mg/ml against *Mycobacterium* species such as *M. smegmatis*, *M. tuberculosis*, *M.bovis* and *M. avium* (Luo *et al*., 2011; Ferreira *et al*., 2012). Water extracts of the root bark exhibited MIC of 1000 mg/ml against *S. aureus*, *E. coli* and *P. aeruginosa* using the cylinder plate technique as reported by Lino and Deogracious. (2006). Ngonda *et al*. (2012) reported MIC of 3.13mg/ml of the aqueous extract against both *S. aureus* and *P. aeruginosa*. Various extracts from *S. longipedunculata*has been reported by Ngonda *et al*. (2012) to have potent antibacterial activity against *E. coli*, *P. aeruginosa, M. smegmatis*, *M.tuberculosis, M.*

bovis and *M. avium.* These pathogens are important causative agents of various human infections (Ngonda *et al*., 2012).

It was also reported by Karou *et al*. (2012) that the 70% methanol extract of the leaf exhibited MIC of 1.2mg/ml against *Mucor rouxi*, *Fusarium oxysporum* and *Rhizopus nigricans*. Furthermore, both an acetone extract of the root and the *n*-butanol fraction exhibited MIC and minimum fungicidal concentration (MFC) of 1.25 and 2.5mg/ml respectively against *Candida albicans* as reported by Ngonda *et al*. (2012), while the 80% methanol extract of the root bark exhibited ZI of < 4mm against *C. albicans* in a report of Runyoro *et al*. (2006). Moreover, the essential oils from the root bark revealed MIC of 0.40mg/ml against *C. albicans* as reported by Alitonou *et al*. (2012). In a report by Samie and Mashau. (2013), it was revealed that, the acetone extracts of the root exhibited MIC of 3.75mg/ml against *Fusarium verticillioides* and *Fusarium oxysporum* while the hexane extract of the root exhibited MFC of 3.75mg/ml against *F. verticillioides, F. nygamai, F. proliferatum* and *F. graminearum*.

According to Fernandes *et al*. (2008), the water extract from the root exhibited a potent MIC of 0.10mg/ml against *Trichomonas vaginalis*, a causative agent of the urogenital infection known as vaginal trichomoniasis, suggesting that the plant may serve as an alternative source of treatment for sexually transmitted infections in humans. The methanol extract from the root inhibited motility of *Trypanosoma brucei brucei* and *Trypanosoma congolense* in 50 and 30 min respectively at a concentration of 0.4mg/ml as reported by Atawodi *et al*. (2003), while the petroleum ether extract from the stem bark has been shown by Atawodi. (2005) to inhibit motility of *Trypanosoma brucei* in 55 min at a concentration of 2mg/ml. The aqueous root extract caused a gradual decrease in parasitemia in rats infected with *T. brucei* for seven days at

100 and 200mg/kg according to a report by Haruna *et al*. (2013a). Moreover, 5, 10 and 20% fractions from the ethyl acetate fraction of the root revealed a LD50 of 0.14, 0.28 and 0.56mg/kg respectively against Wistar rats infected with *T. brucei* as reported by Haruna *et al*. (2013b). Water and methanol extracts of the root bark exhibited antitrypanosomal activity yielding MIC of 56µg/ml against *Trypanosoma brucei rhodesiense* in a report of Freiburghaus *et al*.(1996). In a report by Adiele *et al*. (2013), the 70% aqueous methanol extracts of the root exhibited a larvicidal effect of 75 and 70% at 1000µg/ml against *Heligmosomoides contortus* and *Heligmosomoides polygyrus* at the L3 stage.

Karou *et al*. (2012) reported an IC50 of 79.35µg/ml by 70% methanol extract of the leaf against 2, 2- diphenyl-1-picryl-hydrazyl (DPPH), a stable free radical, while the essential oil of the root bark exhibited an IC50 of 500mg/L as reported by Alitonou *et al*.(2012). The aqueous methanol extract (50%) of the root bark exhibited an IC50 of 1.351 and 9.48µg/ml against ABTS and DPPH respectively according to a report of Muanda *et al*.(2010). A variety of compounds belonging to a variety of classes have been reported to play a role in the antioxidant properties of the species (Mongalo *et al*., 2015).

According to a study by Bah *et al*. (2007), the dichloromethane extract of the leaves showed antiplasmodial activity with an IC50 of 6.9µg/ml against *Plasmodium falciparum*, while the methanol extract of the root suppressed *Plasmodium berghei* by 82% at a dose of 0.56mg/kg as Haruna *et al*. (2013c). Furthermore, Ancolio *et al*. (2002) reported that, the methanol and chloroform extracts of the root exhibited an IC50 of 250µg/ml against the chloroquinone resistant *P. falciparum* strain. Elsewhere, extracts from seeds of *S. longipedunculata* did not show any activity at 50µg/ml against *P. falciparum* FCA-2 from Ethiopia as reported by Kassa *et*

al. (1998), suggesting that the antimalarial compounds may only be present in the leaves and roots.

The extracts of these species have been reported by Muanda *et al*. (2010) to have good anti-inflammatory activity in different models. Interestingly, the water extracts, namely decoctions and infusions, are commonly applied in African indigenous medicine for treating various infections. In a study of Muanda *et al*. (2010), the 50% aqueous methanol extract has been shown to have good anti-inflammatory activity in a dose dependent manner by exhibiting reduction of production in macrophages stimulated with LPS/IFN-gamma yielding 51.3% inhibition at a concentration of 150µl. The methanol extracts, petroleum ether and methanol fractions obtained from solvent extraction of the root bark have been shown to have anti-inflammatory properties using topical edema of the mouse ear model as reported by Okoli *et al*. (2005). However, Okoli *et al*. (2005) reported the petroleum ether fraction, methanol fraction and methanol crude extract to have revealed 65.63, 53.13 and 40.63% inhibition respectively.

The plant has been shown to have activity against insects. The methanol extracts of the root were reported by Eziah *et al*. (2013) to exhibit mean repellence of 60 and 80% against *Prostephanus truncatus* and *Tribolium casteneum* respectively at concentrations of 1 and 2g/ml. Afful *et al*. (2012) reported that, methanol extract of the roots revealed mean % repellency of 70.1 and 60.3 at 0.10g/ml against *Callosobruchus maculatus* and *Sitophilus zeamais* respectively. Moreover, the extract revealed mean adult emergence of 1.0 on pupae of both C. *maculatus* and *S. zeamais* at 0.10g/ml as reported by Afful *et al*. (2012). Furthermore, the extract have been reported by Afful *et al*. (2012) to exhibit mean adult emergence ranging from 1.0 to 2.0 against both the eggs and larvae of C. *maculatus* and *S. zeamais.* In both studies, the extract inhibited the selected insects in a dose dependent manner. According to Boeke *et al*. (2004), leaf powders from *S.*

longipedunculata collected from two different geographical areas, Atacora and Borgou in Benin (West Africa), have been shown to exhibit percentage mortality rates of 18.9 and 77.2 respectively against *Callosobruchus maculatus*. The methanol extract of the root has been reported to exhibit toxicity of 95 and 100% against *Tribolium casteneum* and *Prostephanus truncatus* respectively (Eziah *et al*., 2013). The root powder of *S. longipedunculata* revealed a mean percentage mortality rate ranging from 25.1 to 75.4 against four storage insect pests, namely *Rhyzopertha dominica*, *Sitophilus zeamais*, *Callosobruchus maculatus* and *Prostephanus truncatus* as reported by Belmain *et al*.(2001). The methanol extract of the leaf has been shown to have amortality rate of 50% at 1.0, 2.0 and 3ppm, while the ethanol extracts of both the stem bark and leaf have been reported to have 70% mortality rate against juvenile snails of *Balinus globusus* (Olofintoye, 2010). Moreover, the ethanol and methanol extracts of the root, leaf and stem bark have been reported to exhibit high toxicity causing a 70-100% mortality rate at a concentration of 10.0ppm against *Bulinus globosus*(Olofintoye, 2010). According to a report by Olofintoye. (2010), the species has been shown to possess high pesticidal effects against the eggs, pupae, larvae and adult species of *Callosobruchusmaculatus* and *Sitophilus zeamais*.

The methanol extract of the root has been reported by Bangou *et al*. (2011) to have exhibited enzyme inhibition percentages of 6.95, 7.73 and 5.93% against acetylcholinesterase (AChE), carboxylesterase and xanthine oxidase (XO) respectively. Allopurinol exhibited 96.38% inhibition against XO while galanthamine and ascorbic acid exhibited 50.76 and 56.72% against AChE and CES respectively.According to Niño *et al*. (2006), AChE is an attractive target for the rational drug design and discovery of mechanism-based inhibitors because of its role in the hydrolysis of the neurotransmitter acetylcholine. Moreover, AChE inhibitors are most active in the treatment of a variety of diseases, including Alzheimer's disease, Parkinson's disease, ataxia

and senile dementia (Niño *et al*., 2006). Xanthan oxidase catalyses the oxidation of xanthine and hypoxanthine into uric acid, which may lead to a disease known as gout (Kong *et al*., 2000). Some drugs and plant-derived extracts which serve as XO inhibitors may block uric acid biosynthesis, lower the plasma uric acid concentration and are used to treat gout (Nguyen *et al*., 2004).

The aqueous extract of the root exhibited anticonvulsant, anxiolytic and sedative activities against mice in a dose dependent manner according to reports by Adeyemi *et al*. (2010) and Okomolo *et al*. (2011), suggesting that the plant extract may be used in the management of convulsion and psychosis.

Antiglycemic activity of the specie has also been reported. Onyeche and Kolawole. (2005) reported that aqueous extract of the leaves significantly lowered the blood glucose concentration from 96.3 to 71.6mg/dL after 8 h in rats treated with 2100mg/kg plant extract. This is a high concentration and the effect may not be useful in practice. The 96% ethanol extract of the root bark had no hypoglycemic effect on mice when administered at 200mg/kg as reported by Keshebo *et al*.(2014). The 200 mg/kg concentration is also relatively high, limiting the extract's practicality.

Study of Funke and Melzig. (2006) revelaed that, buffer extract (0.5g of the plant material dissolved in 2.5ml buffer at room temperature for 20min) from the root had some antidiabetic activity through exhibiting 20 to 45% inhibition of α-amylase. No information was found on the bioavailability or pharmacokinetic parameters of the extract.

The aqueous root extracts reportedly affect the tissue morphology of rats, resulting in irreversible cellular injury affecting the epithelial parenchyma and endothelial cells when administered at

2mg/kg using intra-peritoneal injection for 14 consecutive days (Dapar *et al*, 2007). The extract histopathologically resulted in acute tubular necrosis in the kidneys, diffused alveolar and alveolar capillary damage in the lungs and severe ballooning degeneration with early steatohepatitis in some foci of the liver as reported by Dapar *et al*. (2007). It is difficult to conclude the effect of *S. longipedunculata* on various tissues due to the lack of information reported.

2.2. Newcastle disease

Newcastle disease (ND) is a viral disease of birds caused by a filterable virus Newcastle Disease Virus (NDV) which belongs to the family paramyxoviridae (Alexander, 1997). It is a peracute, acute and sometimes subclinical contagious disease of poultry (Health *et al*., 1991). Newcastle disease is considered among the most important disease of poultry and outbreaks with mortality of up to 100% is common (Alders and Spreadbrow, 2001; Sa'idu and Abdu, 2008) and can also cause conjunctivitis in humans, but the condition is generally very mild and self – limiting (OIE, 2012). Newcastle infection takes place through virus inhalation or ingestion and its spread from one bird to another depends on the availability of the virus in its virulent form (Whiteman and Bickford, 1983) and its short incubation period of 5 – 6 days (Chansiripornchai and Sasipreeyajan, 2006). The disease usually affects the respiratory, gastrointestinal and nervous systems with common signs of listleness, increased respiratory rate, yellowish to greenish diarrhea and weakness followed later by prostration and death (Chansiripornchai and Sasipreeyajan, 2006).

2.2.1. History of Newcastle disease

The first outbreaks of ND were reported during the mid-1920s in Java, Indonesia and Newcastle-upon-Tyne, Englandfrom which the disease received its name (Elnagger, 2012*)*.Predate

indications of the disease have also been reported (Elnagger, 2012). The disease moved slowly through Asia to Europe and that isolated outbreaks such as in England in 1926 were chance introductions ahead of the main stream. This theory of panzootic spread of NDV would mean that the virus, which had apparently arisen in 1926, took over 30 years to spread worldwide and was still important in most countries in the early 1960s.The third one which caused by neurotropic form of NDV termed the pigeon Paramyxovirus type 1 (PPMV-1) started in the Middle East in the late 1970 and by 1981 it had reached Europe and thereafter spread rapidly throughout the world. The fourth panzootic occurred in the last of 1980 and spread in many countries (Alexander, 1991).

Accurate characterization of NDV viruses is extremely important bases on the new Office International des Epizooties (OIE) definition. Because ND, an OIE list "A" disease, requires reporting that may result in international trade restrictions (OIE,2002).Office International Des Epizootics, (2003) establishes the health standards for international trade in animals and animal products under the auspices of the World Trade Organization. The OIE considers avian influenza (AI) viruses of high pathogenicity (HP), and Newcastle disease (ND) viruses of the mesogenic and velogenic pathotype as diseases of great significance that affect poultry. So Newcastle disease considered as an enzootic in some areas of the world and is a constant threat to most birds reared domestically (Madhan *et al*., 2005). Wild water fowl is considered a natural reservoir of potentially infectious agents and a source of pathogenic viruses like avian paramyxoviruses type 1(APMV) (OIE, 2011). In free areas vaccination and biosecurity measures are actively performed to maintain this preferable sanitary condition. However, the risk of reintroduction of pathogenic viruses is always present (Flavia *et al*., 2005). Qin*et al*. (2008)stated that thirty Newcastle disease virus strains isolated from 1996 to 2005 outbreak in

china. In 2010 outbreaks were reported in Belgium, Belize, France, Germany, Honduras, Israel, Japan, Mongolia, Peru and Spain (Wahid, 2010).

In January 2011, two other outbreaks of exotic Newcastle disease in a commercial broiler operation in the Mexican state of Baja California and a breeding farm in the Mexican state of Hidalgo were reported to the OIE. AT the end of 2010 and beginning of 2011, several Newcastle disease outbreaks in unvaccinated poultry associated with pigeon Paramyxovirus type 1 (PPMV-1) infection were officially reported by two northern European countries (OIE, 2011). Israel has reported over twenty outbreaks of Newcastle Disease since December 2010 in backyard and commercial flocks in Hazafon, Hamerkaz, Haifa, Jehuda & Samaria regions. The source of infection for some outbreaks is believed to be fomites as some sites are connected epidemiologically through persons or equipment that passed from one farm to another. As a result of these outbreaks, Israel had not been able to certify disease freedom from ND for the affected regions, and therefore no imports had been consigned to the EU since then. Also Information about Newcastle outbreak received on 19/12/2011 from Mr. Hans Wyss, Chief Veterinary Officer, Schwarzenburgstrasse 161, Swiss Federal Veterinary Office, Liebefeld Berne, and Switzerland (OIE, 2012).

Forty-four Newcastle disease virus (NDV) strains, obtained between 2002 and 2007 from different poultry species in Nigeria, Niger, Burkina Faso and Cameroon were phylogenetically analysed on the basis of partial F sequence. Lineage 2 viruses were genetically identical or similar to the locally used LaSota vaccine strain and were mostly detected in commercial farms (Snoeck *et al*., 2009).

A vaccination policy has been adopted for control of the disease since 1948 (Elnagger, 2012). At first the mesogenic Mukteswar strain was used which was substituted by the Komarov strain in 1953 to immunize chicken at two months of age and older birds. In the early 1960's, the lentogenic F vaccine strain has also been used to immunize baby chicks. Outbreaks continued to occur until the ND live vaccine (Hitchner B1/47 strain) was applied in 1967 (Mase *et al*., 2002).

2.2.2History of Newcastle disease in Nigeria

The first documented outbreak of ND in Nigeria occurred between December, 1952 and February, 1953 in and around Ibadan (Hill *et al*., 1953). The disease has since this time remained a notable problem in the country (Oladele *et al*., 2002), causing huge economic loses to farmers and hampering growth of poultry industries in Nigeria, which has an estimated poultry population of 137.6 million, with backyard poultry population constituting 84% (115.8 million) and 16% (21.7 million) of exotic poultry(Saidu *et al*., 1994; Halle *et al*., 1999). Spreadbrow (1999) described Epizootic and Enzootic ND in village chickens as non-self- limiting event that can smoulder for several month, even years in a typical village housing one or thousand birds. The same event also occurs in commercial chickens in Nigeria (Ezeokoli*et al*., 1984). Spreadbrow. (1999) noted that epizootics occur when virus is introduced into a susceptible population with spectacular outbreaks and high mortalities which may cover whole village or whole area within a short time. This form comes most readily to notice.

Enzootic ND occurs when the virus transmits slowly in a partially immune population such that there are few susceptible birds to maintain an outbreak and occasional birds that die do not come to veterinary and public attention. Newcastle disease has become endemic in Nigeria in both local and commercial poultry with annual epidemics recorded in highly susceptible flocks (Halle, *et al*., 1999; Saidu and Abdu, 2008) with pockets of outbreaks occurring in between the annual

epidemic periods. The outbreaks of ND were more common in layers than in broilers (Abdu *etal*., 2005b). The disease was also reported to be more common during the dry harmattan (November- March) (Saidu *et al*., 1994; Halle *et al*., 1999; Abdu *et al*., 2005a; Sonaiya, 2009). Cold stress has been known to worsen the outcome of ND (Abdu *et al*., 1992). Commercial chickens in Nigeria are exclusively exotic chickens which are reared intensively or semi-intensively. The intensive system combines both battery cages and deep litter. In most parts of the country, the disease is seen and diagnosed throughout the year in those commercial chickens in the South Eastern derived Savannah zone of Nigeria (Saidu and Abdu, 2008).

In Nigeria, ND was reported to be the most prevalent disease of local and exotic birds (Saidu *et al*., 1994; Halle *et al*., 1999). Newcastle disease in Nigeria has age and species differences (Halle *et al*., 1999; Abdu *et al*., 2005a). Newcastle disease was reported in Nigeria in guinea fowls and a highly velogenic strain of ND virus was isolated from apparently healthy ducks (Echeonwu *et al*., 1993). In Nigeria, the virus has been isolated from natural infection in captive African grey parrot (*Psittacus erithracus*) (Onunkwo and Momoh, 1980).

Overall, seropositive rate of 32.5% was reported by Jibril *et al*. (2014) for Sokoto State, Nigeria. Chukwudi *et al*. (2012) reported a prevalence of 3.2% for NDV in clinically healthy chickens in Nsukka area, Nigeria. Manchang *et al*. (2004) reported a higher incidence rate (68.4%) of ND during the dry season against 34.6% in the rainy season and higher rate in the young (20.7%) against 12.1% in the adult.

Most research works on the prevalence of ND were centered on domestic chickens. In nature various species of poultry are kept together in households. Even in commercial poultry production other species are kept along with domestic chickens. It has been reported that

susceptibility of these species to ND varies with some serving as reservoirs for disease spread. Following the outbreak of Highly Pathogenic Avian Influenza (HPAI) in Nigeria in 2006, the poultry sector has suffered a tremendous setback. Efforts are now geared towards resuscitation of the sector. However ND outbreaks and its consequent economic losses hinder the effort to revive the poultry industry. Newcastle disease continues to decimate various poultry species which are commonly kept within the households and farms.

2.2.3 Causative agent of Newcastle disease

Newcastle disease (ND) is caused by a filterable virus Newcastle Disease Virus (NDV) and classified below.

2.2.3.1. Classificationof Newcastle disease virus

Newcastle Disease Virus is enveloped, single-stranded, negative sense RNA virus showing helical capsid symmetry and it is the only member in the subfamily *Paramyxovirinae* that belongs to the genus Avulavirus (DeLeeuw and Peeters, 1999; Tan *et al*., 2007) in the family *Paramyxoviridae* of order Mononegavirales and is designated avian paramyxovirus serotype -1 (APMV-1) (Lamb *et al*., 2001; Alexander *etal*., 2003). Alexander *et al*. (1986) said nine serogroups of avian paramyxoviruses have been recognized which are; APMV-1 to APMV-9. Among the serogroups, Newcastle disease virus (APMV-1) remains the most important pathogen for poultry, but APMV-2, APMV-3, APMV-6, and APMV-7 are known to cause disease in poultry. The prototype viruses are the recognized natural hosts for each serogroups. Newcastle disease virus becomes infective only when the precursor glycoprotein F0 is cleaved into F1 and F2. The ability to cleave F0 varies among different strains of NDV and is the main determinant of mortality and morbidity in infected chickens (Alexander *et al*., 1986). Using mean death time (MDT) determined in chicken eggs, NDV strains can be classified as highly virulent (velogenic),

intermediate (mesogenic) or non-virulent (lentogenic) (Beard andHanson, 1984).Alexander. (2000)reported that within the highly virulent NDV strains; two types of virus can be distinguished. These strains are termed neurotropic velogenic or viscerotropic velogenic, based on the clinical signs induced.

2.2.3.2 Morphology of Newcastle disease virus

The typical NDV is a pleomorphic, enveloped particle with diameter between 100 - 300nm (Shnyrova *et al*., 2007).The envelope of NDV is derived from host cell membrane. Two glycoproteins, fusion (F) and hemagglutinin neuraminidase protein (HN) form the spike-like protrusions on the outer surface of the virions. The F protein is required for fusion of virion into the host cell membrane through pH-independent mechanism. The HN protein is responsible for attachment of the virus to host cell, this supported the idea that homologous cytoplasmic tails and matched F and HN ectodomains are important for virus replication (Shin – Hee *et al*., 2011). Under the envelope is a Matrix protein, which is thought to play a major role in mature virus budding. Inside the envelope is the ribo-nucleocapsid corestructure which is the template for virus RNA synthesis. The core structure is formed by nucleocapsid protein (NP) tightly bound to the genomic RNA, to which phosphoprotein (P) and large polymerase protein (L) are attached**.** Paramyxoviruses are rounded and 100-500 nm in diameter.Surface of virus envelope is covered with projections about 8 nm in length. In most electron micrographs ―herring bone‖ nucleocapsid and showing helical capsid symmetry may be seen either free or emerging from disrupted virus particles. The envelope contains two transmembrane glycoproteins the haemagglutinin neuraminidase (HN) and fusion (F) proteins (Alexander *et al*., 2003).

2.2.3.3. Genomic organization

Core genome of NDV is a single stranded (SS), RNA of negative sense and consisting of 15,186 nucleotides (Lamb *et al*., 2001; Czegledi*et al*., 2006).Newcastle Disease Virus genome of six genes code for six major proteins nucleoprotein (NP), phosphoprotein (P), matrix (M) that lines the inner surface of the membrane, fusion (F), haemagglutinin neuraminidase (HN) and an RNA dependent RNA polymerase (L) respectively from the 3-terminus to the 5-terminus on the genome sense RNA (Samson *et al*., 1991; Steward *et al*., 1993; Lamb *et al*., 2007).

Three genes required for NDV transcription NP, P and L proteins and the remaining three proteins M, F and HN have a role in virus entry and fusion in to the host cell (Qinshan *et al*., 2007) as in Figure 2.3.

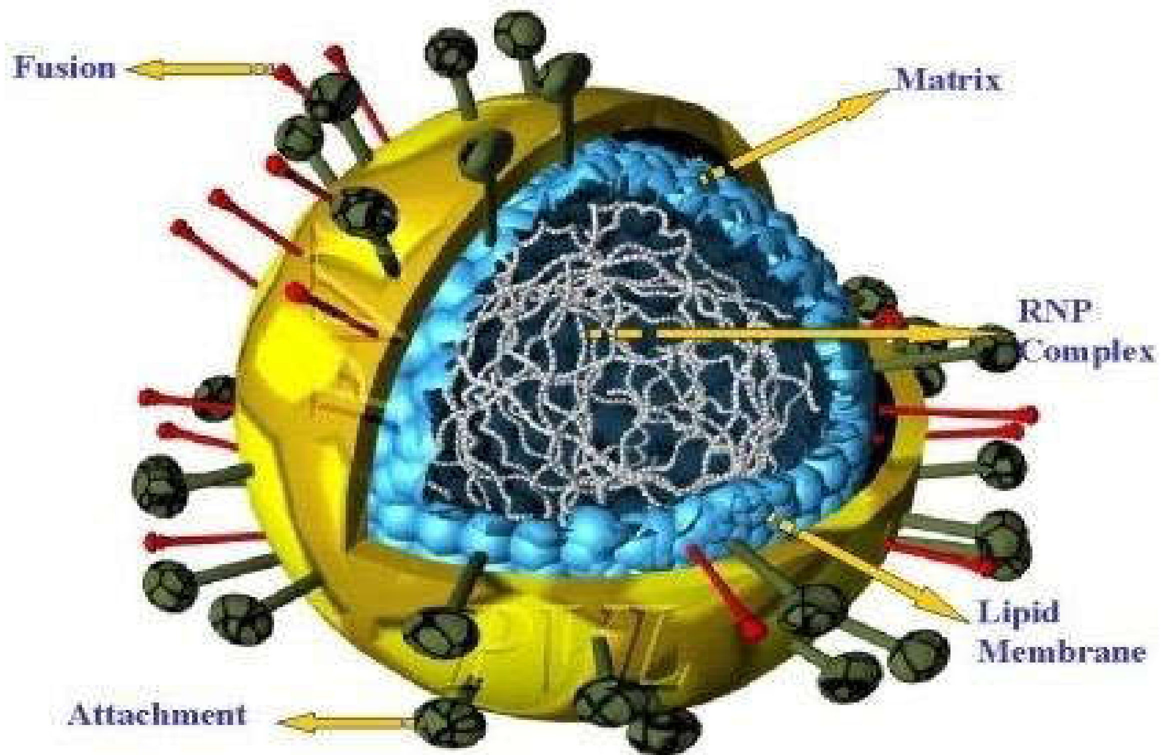

Figure 2.3 Morphology of Newcastle disease virus

Source:http//lankavet.blogspot.com/2012/12/Newcastle-disease-or-ranikhet-disease.html

2.2.3.4. Replication of NDV

The replication strategy employed by NDV is similar to that of other non-segmented negative-sense RNA viruses. The initial step of replication is the adsorption of the virus to the cell surface receptor followed by fusion with the host cellular membrane (Elnagger, 2012). The fusion causes the viral genome to be released into the cytoplasm. The transcription and replication of NDV genome occurs in the cytoplasm. At the end of the replication cycle, the viral proteins are packaged and the progeny viruses mature by budding through the plasma membrane (Elnagger, 2012).

I-Virus adsorption and entry

The initial step of virus entry requires the binding of the HN protein into the sialic acid containing receptor on the host cell surface. Following the attachment, the envelope of the virus fuses with the host cell plasma membrane. The fusion process is mediated by F protein. Upon fusion, disruption of matrix-nucleocaspid occurs and the viral nucleocapsid is released into the host cell cytoplasm. The entry of NDV into cells is believed to occur by direct fusion at the plasma membrane through a pH-independent mechanism. Recent study also suggests that NDV may infect the cells through an alternative route: caveolaedependent endocytic pathway (Cantin *et al*., 2007).

II-Transcription

The mRNA synthesis begins at the 3‘end of the genome of NDV. Since the uninfected host cell lacks the RNA dependent RNA polymerase, the viral RNA polymerase has to first transcribe the positivestranded leader RNA at the 3‘ end promoter (Elnagger, 2012). The first gene, NP, is transcribed at the NP gene start (GS) and is terminated at the NP gene end (GE) (Elnagger, 2012). This results in release of the first capped and polyadenylated mRNA, NP mRNA

(Elnagger, 2012). The transcription of paramyxovirus follows the ―start-stop‖ mechanism until the last mRNA, L mRNA, is synthesized. The polymerase stops at the upstream GE and reinitiates synthesis of the next mRNA at the next GS. Some polymerase falls down at the intergenic sequences (IGS) region, which is located between the upstream GE and downstream GS signals. Some polymerase may bypass the IGS, and the readthrough transcripts are formed(Elnagger, 2012). This starts – stopl transcription results in the gradient of mRNA abundance that reduces according to the relative distance of the location of the individual gene from the 3‘end promoter. Since the NP gene is the closest to the 3‘end promoter, the NP mRNA is produced in much quantity whereas, the L gene, which is located at the farthest to the 3‘ end promoter, produces the least amount of L mRNA. For efficient transcription and replication, most paramyxoviruses follow the ―Rule of Sixl. That is, the length of genome has to be a multiple of six. The hexamer rule is most likely that NP subunit of the nucleocapsid is associated with exactly six nucleotides (Elnagger, 2012). Apart from NDV, other paramyxoviruses, such as Sendai virus and measles virus, also follow this ―Rule of Six‖; but the members of pneumoviruses do not follow the ―Rule of Six‖ (Samal and Collins,1996).

III-Replication

Elnagger (2012) discussed that processes of replication and transcription of NDV are tightly regulated. The switch from transcription to replication is controlled by the NP protein. When free NP protein in the cytoplasm is limiting, the viral polymerase is preferentially engaged in mRNA synthesis, transcription, resulting in the increased free NP protein and other viral proteins. Once the amount of free NP protein is sufficient, the viral polymerase ignores the gene junction signal, such as GS and GE, and switches to replication. The full-length complimentary copy, known as antigenome (+), is first synthesized to serve as the template for replication of negative sense

genome RNA Both the genome and antigenome are packaged into encapsidated nucleocapsid. Leader and trailer of the genome contain the specific signals for encapsidation (Elnagger, 2012).

IV-Virus assembly and release

Recent studies suggest that the assembly and release of infectious NDV particles take place at the membrane lipid rafts (Laliberte *et al*.,2006; Dolganiuc *et al*., 2003). The first step in viral assembly is the encapsidation of genome/antigenome RNA into nucleocapsid. The nucleocapsid is thought to be assembled in the cell cytoplasm. First, the free NP proteins are tightly associated with the genome RNA to form the ribonucleoprotein (RNP) core structure; secondly, the P and L proteins are loosely bound to RNP, forming transcriptase complex (Elnagger, 2012). The assembly of viral envelope takes place at the cell surface. The membrane proteins, F and HN proteins are synthesized on the rough endoplasmic reticulum (RER) and transport to the cell surface through the secretory pathway. Before transportation, the proteins undergo stepwise conformational maturation. Folding and maturation occur inside the ER with the help of many molecular cellular chaperones. Only the correctly folded proteins are transported out of the ER to the Golgi apparatus for further post translation modifications including carbohydrate chain modification of HN protein and cleavage of F0 protein at the multiple basic cleavage sites to form functional F1 and F2 proteins (Doms *et al*., 1993). Finally, the mature F and HN proteins are transported to the cell plasma membrane through vesicles. The M Proteins are thought to play the major role in taking the assembled RNP to the plasma membrane to form budding virions. Study on NDV virus-like particle (VLP) suggests that M-HN and M-NP interactions are responsible for incorporation of HN and NP proteins into VLPs and the F protein is incorporated indirectly due to interactions with NP and HN protein,the assembled virions at the plasma membrane are then released by budding from host cell membrane (Pantua *et al*., 2006).

2.2.4 Immunity

The immunity to NDV are of two types which include Adaptive and passive.

2.2.4.1. Adaptive immunity

The adaptive immunity in avian species consists of cell- mediated,humoral and local immunity.

2.2.4.1.1. Cell-mediated immunity

In avian species, adaptive immunity involves both humoral and cell-mediated immune (CMI) Responses. Although humoral or antibody mediated immune responses are particularly effective against extracellular antigens, CMI responses are specialized in the elimination of intracellular antigens; the latter include those that have entered cells via the endocytic pathway (exogenous antigens; e.g., phagocytosed bacteria) or were produced within the cell such as viral proteins and proteins resulting from neoplastic transformation of the cell (endogenous antigens) (Arstila *et al*., 1994).

Cell- mediated immune responses, like most humoral immune responses, are tightly regulated and require ―help from T helper cells, specifically the type 1 T helper cells (Th1, hence, the name Th1 responses). Th1 cells are characterized by their production of cytokines such as interferon-γ (IFN- γ), tumor-necrosis factor-α (TNF-α), and interleukin-2 that drive CMI responses. The functional effectors of CMI responses are various immune cells including cytotoxic lymphocytes (cytotoxic T cells and natural killer cells) and macrophages (Davison,2003).

Cytotoxic lymphocytes and macrophages are specialized in the elimination of endogenous and exogenous antigens, respectively. In the past decade, substantial progress has been made in defining the role and regulation of avian CMI responses although cellular immunity is crucial in

virus infection because the viral pathogenesis includes an intracellular phase (Abbas *et al*., 2000).It is important to understand this response and therefore an estimation of the cellular immunity for viral infection such as NDV should be aligned with humoral antibody response estimation. For this purpose, an ELISA procedure was developed by Lambrecht *et al*. (2004) that utilize the presence of antigen-specific chicken interferon γ **(**ChIFN- γ**)** production as an indicator of actively acquired immunity to NDV. The CMI response measured by the ChIFN-γ ELISA carries great potential for representing the role of CMI in protection against avian ND as well as facilitating the study of the role of this cytokine in various immune mechanisms in the chicken. Other advances have addressed strategies that strengthen this arm of adaptive immunity to optimize defense as well as protection against neoplastic diseases and non-neoplastic diseases caused by intracellular pathogens (Erf, 2004).

The initial immune response to infection with NDV is cell mediated and may be detectable as early as 2—3 days after infection with live vaccine strains**,** This has been thought to explain the early protection against challenge that has been recorded in vaccinated birds before a measurable antibody response is seen.However, a later study concluded that the cell-mediated immune response to NDV by itself is not protective against challenge with virulent NDV. The importance of cell-mediated immunity in protection conferred by vaccines is, therefore, not clear, and a strong secondary response to challenge similar to the antibody response does not seem to occur (Reynolds and Maraqa, 2000). The cellular immune response was higher with the HB1 live vaccine than the killed vaccine but declines thereafter. Most but not all of the chickens vaccinated with the live NDV vaccine produce IFN-γ after recall stimulation, and this is from 2 to 4 week after vaccination, whereas the inactivated NDV vaccine produces lower IFN-γ, which is only apparent at 4 week after vaccination (Lambrecht *et al*., 2004).

2.2.4.1.2. Humoral immunity

The antibodies which are secreted at mucosal surfaces are designated IgA, while IgM and IgG circulate in blood and lymph. IgY is the bird equivalent to the IgG molecules found in mammals (Elnagger, 2012). They have the same general structure and function but some biochemical differences. They were designated IgY because they were originally isolated from egg yolk. The adult egg-laying chicken has a prodigious capacity for antibody production. It has been calculated that its weekly production is equivalent to the antibody content of 90-100ml of serum (Elnagger, 2012). The classes of antibody have varying chemical structures and numbers of attachment sites per molecule. Serological tests also vary in their ability to detect the different antibody classes (Elnagger, 2012).Once the initial challenge has been dealt with, a group of cells remain (so-called "memory" cells) which have the required genetic make-up to produce antibody against the specific antigen. Antibodies work closely with other components of the innate and acquired immune systems to help protect against pathogens. They act by a range of mechanisms, the relative importance of which can vary with the species and pathogen.Antibodies are most effective in eliminating extracellular antigens,as their interaction with the antigen will activate/enhance effectormechanisms that lead to the removal or destruction of the antigen (Abbas *et al*., 2000).

When chickens survive NDV infection long enough, antibodies usually are detectable in the serum within 6—10 days. The levels largely depend on the infecting strain, but generally, peak response is at about 3—4 weeks. Decline in antibody titer varies with the titer achieved but is much slower than their development. Haemagglutination inhibition antibodies may remain detectable for up to one year in birds recovered from infection with mesogenic viruses or after a

series of immunizations. Reinfection or immunization some weeks after the titer begins to decline produces a secondary response (Elnagger, 2012).

2.2.4.1.3. Local immunity

Antibodies appear in secretions of the upper respiratory tract and intestinal tract of chickens at about the time humoral antibodies can be first detected. In the upper respiratory tract, the immunoglobulins appear to be chiefly IgA with some IgG (Parry *et al*., 1977). Similar excretions occur in the Harderian gland following ocular, but not parenteral, infection. Mucosal immune responses are an early and important line of defense against pathogens. The current understanding of the mucosal immune system allows us to consider the use of nasal immunization for induction of antigen specific immune responses at the mucosal surface and the systemic compartment. Nasal immunization is anticipated to be an optimal route of administration of vaccines against respiratory tract infections. Although oral immunization is an attractive approach to induce mucosal immunity, it has had variable success in protection against upper respiratory tract viral infections. For example, secondary nasal immunization subsequent to primary oral immunization is required for effective protection against viral host and is a major component of resistance against respiratory infections.The importance of mucosal immunity, specifically respiratory disease (Liang *et al*., 1989).

2.2.4.2. Passive immunity

Hens with antibodies to NDV will pass these on to their progeny via the egg yolk (Heller *et al*., 1977). Levels of antibody in day-old chicks will be directly related to titers in the parent. Allan *et al*., (1978) estimated that each two fold decay in maternally derived HI titer takes about 4.5 days. Maternal immunity is protective and, thus, must be taken into account when timing the primary vaccination of chicks.

2.2.5 Biological and antigenic properties of Newcastle disease virus

Newcastle disease outbreak depends largely on the relationship between antigenand biological properties of the Newcastle disease virus (Alexander *et al*., 1997).

2.2.5.1 Haemagglutinin neuraminidase and haemagglutination properties

The haemagglutinin neuraminidase and haemagglutination properties of NDV play crucial role in the process of Newcastle infection.

2.2.5.1.1 Haemagglutinin (HA)

Hemagglutinin Neuraminidase (HN) protein is responsible for virus attachment to cell surface receptors (Shengqing *et al*., 2002). Interaction between HN and F proteins for NDV fusion is needed. The globular head region of HN from 124 to 151 AA binds to the membrane HR-B region of F Protein for fusion between cell membrane and virus envelope, when HN binds to sialic acid containing receptors on the cellular membrane (Ding *et al*., 2008). So, HN plays an important role in both recognizing sialic acid containing receptors on cell surfaces and promoting fusion activity of the F protein So, HN seems to be involved in determination of virulence (Tan *et al*., 2007; DeLeeuw *etal*., 2005). HN existing as a trimer embedded in the lipid bilayer of the viral envelope. HA binds to sialic acid containing receptors during virus entry to host cell which is the main oligosaccharide on the surface of cells of fowls and humans and known as N-acetylneuraminic acid (NeuAc) in which galactose is joined in a 2, 3 or a-2, 6 configurations (Ito *et al*.,1999; Xu *et al*., 2008). It is known that NDV HN protein can stimulate cells such as dendritic cells(DCs) to produce high levels of interferon-α(IFN-α) and activate macrophages, natural killer(NK) cells and T cells. Additionally, the distinct structure of HN displayed on the tumor cell surface that can be recognized by the immune system strengthens the cytotoxicity (Li *et al*., 2006).

2.2.5.1.2 Haemagglutination properties

Haemagglutination occurs when sialic acid containing residues similar to the cellular receptors on erythrocytes bind to the receptor binding site present on the tip of the viral HA proteins (Tan *et al*., 2007). This property and the specific inhibition of agglutination by specific NDV antisera have proven to be powerful tools in the diagnosis of the disease. By adding HA recognizing antibodies before adding erythrocytes in the test, HA can be inhibited (Alexander *et al*., 2003). Chicken RBCs usually are used in HA test but NDV will cause agglutination of all amphibians, reptilian and avian cells. Human, mouse and guinea pig RBCs were agglutinated by all NDV strains tested but the ability to agglutinate cattle, goat, sheep, swine, and horse cells varied with the strain of NDV (Elnagger, 2012).

2.2.5.1.3 Neuraminidase activity

Neuraminidase (mucopolysaccharide N-acetyl neuraminyl hydrolase) is a part of HN molecule and responsible for gradual elution of agglutinated RBCs (Elnagger, 2012),the exact function of the neuraminidase in virus replication is unknown but it seems likely that neuraminidase removes virus receptors from the host cell which prevents the reattachment of released virus particles and virus clumping (preventing self-aggregation of virus particles during budding at the plasma membrane) so, it has very important role in the virus spread (Alexander *et al*., 2003).

2.2.5.1.4 Phosphodiesterase activity

A studies on a Phosphodiesterase activity associated with NDV reported the presence of a Phosphodiesterase activity in NDV (Elnagger, 2012). This activity has a different pH optimum from the Phosphodiesterase activity found in normal, uninfected cells is inhibited by NDV antiserum and is associated with the envelope polypeptides of virus.

2.2.6. Laboratory host

NDV can infect and multiply in a range of Non avian as well as avian species following laboratory infection. The chicken, however, remains the most readily available and frequently used laboratory animal, as well as the most important natural host of the disease (Elnagger, 2012).

2.2.7. Epidemiology

The epidemiology of Newcastle disease virus is based on the geographical distribution of the virus.

2.2.7.1. Geographic distribution

The almost universal use of NDV vaccines in commercial poultry throughout the world makes assessment of the true geographic distribution of virulent NDV difficult (Alexander *et al*., 2003). Even though international monitoring of ND is carried out by (FAO, 1985) and OIE, the figures produced may not represent the true distribution of VVNDV (Alexander *et al*., 2003). Four panzootics of NDV appear all over the world (Alexander, 2001),first one appear in south Asia spread to Europe (England) in 1926and then all over the world.The second one appear in middle east in1960 spread to most countries (Francis, 1973) the third one appear in thelate 1970 and spread in Europe only (Alexander, 1997) and the fourthpanzootic appear in middle east and in1980 spread to Europe and then allover all countries this form of disease in neurotropic form (Biancifiori and Fioroni, 1983). Velogenic APMV-1 is endemic in Asia, the Middle East, Africa,Central, South America and parts of Mexico. Virulent strains are endemicin wild cormorants in the U.S. and Canada but commercial poultry arefree of Velogenic isolates (OIE, 2008). Although only one NDV serotype exists, there are 2 majorsubdivisions, class I and II (Czeglédi *et al*., 2006). Class I viruses havebeen recovered from waterfowl and shore birds

(Czeglédi *et al*., 2006; Kim *et al*., 2007) and are mostly avirulent in chickens, whereas class II viruses are mainly isolated from poultry and from pet and wild birds (Aldous *et al*., 2003). The class II NDV's are further categorized into ten genotypes I to X (Liu *et al*., 2003). Genotype I consists of avirulent strains of mNDV, while viruses of genotypes II, III and IV were responsible for the first panzootic that started in the 1920s (Ballagi*et al*., 1996). Genotype V viruses are thought to be responsible for the second panzootic in the early 1970s (Wehmann *et al*., 2003). These viruses are still the major cause of outbreaks in the USA (Pedersen *et al*., 2004). The third panzootic primarily affected pigeons and was caused by genotype VI viruses (Czegledi *et al*., 2002). It started in the late 1970s in the Middle Eastand spread to Europe (Biancifiori and Fioroni,1983)where it caused major outbreaks in the poultry industry.

2.3 Upper respiratory tract infections (URTIs)

Upper respiratory tract infections are common acute infections involving the nose, paranasal sinuses, pharynx, larynx, trachea, and bronchi. It is usually identified by the community as a common cold. URTIs can be defined as an acute febrile illness with cough, coryza, sore throat, or hoarseness, which are very common in the community and are one of the major reasons for appointments to primary care physicians, particularly during the winter season (Macfarlane *et al.,* 1993; Fleming *et al*., 2001).

According to the findings of Meneghetti (2006) and Abed and Boivin (2006),URTIs are the most common acute illness found in an outpatient setting which have a wide range of clinical manifestation that may vary from the common cold (mild and self-limiting) to a life threatening disease, such as epiglottitis.

2.3.1 Etiology of upper respiratory tract infections

Both viral and bacterial pathogens are considered to play an important role in the etiology of URTIs. Fungi, other microorganisms, and chemicals (such as powder or oil that accidentally penetrate into the lungs) could also function as causative agents for URTIs (Karevold *et al.,* 2006).

2.3.1.1 Viral infections

Most URTIs are viral in origin, with an associated low morbidity rate but a tendency toward certain complications, such as otitis media, tonsillitis, and sinusitis, which can contribute to morbidity (Kavaerner *et al.,* 2000). Viruses that are commonly responsible for human respiratory tract infections include influenza virus, parainfluenza virus, respiratory syncytial virus (RSV), adenovirus, rhinoviruses and coronovirus (Smith and Sweet, 2002; Mackie, 2003).

2.3.1. 2 Bacterial infections

Bacterial pathogens are considered to be one of the causal etiological agents for URTIs. The main bacterial pathogens detected in URTIs patients are *S. pneumoniae*, *Streptococcus pyogenes*, *Haemophilus influenzae*, *S. aureus*, *Neisseria meningitidis*, *Mycobacterium tuberculosis*, *Bordetella pertussis,* as well as *P. aeruginosa* (Pfaller *et al.,* 2001; Smith and Sweet, 2002).

Some studies determined the incidence of URTIs and the type of bacteria that commonly caused URTIs. These studies found that the *H. influenzae*was the most common bacterial pathogen detected, followed by *Klebsiella pneumoniae*.

2.3.1.3 Mixed viral – bacterial infections

Viruses as a whole are considered as the most common causative organisms responsible for URTIs; however, these viruses can lead to bacterial infections, resulting in mixed viral-bacterial

infections. Mixed viral-bacterial respiratory infections are not very common, but they seem to be widespread, especially among children less than two years of age. The long period of disease associated with viral infections or antibiotic treatment failure for bacterial disease may be lead to mixed viral-bacterial infections (Jokso-Koivisto *et al.,* 2006).

2.3.2 Epidemiology of URTIs

URTIs are highly prevalent, especially in children between the ages of two and four years. Children less than six months old are relatively protected against community-based respiratory infections. The frequency of URTIs increases and becomes high during the second year of a child's life, and may increase again during child-bearing years. Parents may get an infection when exposed to their infected children who have respiratory infections. On the other hand, the frequency of respiratory infections decreases with increasing age of children (Monto, 2002; Rovers *et al*., 2006).

2.3.3 Pathophysiology of URTIs

URTIs can occur as a result of invasion by the microorganisms into mucous surfaces of the upper respiratory tract (URT), followed by penetration into the mucosal and epithelial tissues. Host defense mechanisms might be inhibited, leading to damage of host cells (Bamberger and Jackson, 2001). The ability of bacterial pathogens to reach one or more of these steps can be increased in the presence of viruses. URTIs are also increased via enhanced adherence of bacteria to the host cells resulting in infections (Smith and Sweet, 2002).

2.3.4 Seasonality of URTIs

Upper respiratory tract infections occur year round and their incidence increases especially during the rainy and winter seasons (Monto, 2002; Shek and Lee, 2003). Epidemics and mini-

epidemics are most common during cold months, with a peak incidence in late winter to early spring. Humidity may also affect the prevalence of infections, as most viral URTIs agents thrive in the low humidity conditions of winter months (Monto, 2002; Shek and Lee, 2003).

2.3.5 Routes of URTIs transmission

Viruses responsible for causing URTIs are mainly transmitted by small particle in droplets, which are usually generated by coughing. These droplets can remain suspended in the air for an hour, and might lead to infection upon inhalation (Hall and McBride, 1994). The other route of transmission can involve large particle droplets, which travel less than one meter and might infect the nasal mucosa (Hall and McBride, 1994). The spread of secretions containing bacterial or viral pathogens could also occur by direct contact. A contaminated hand could expose the pathogens to either the nose or mouth, or exposure could occur via direct inhalation of respiratory droplets from an infected person when coughing or sneezing (Bamberger and Jackson, 2001; Monto, 2002).

2.3.6 Clinical manifestations of URTIs

The clinical manifestations of URTIs are variable depending on the causative organisms. Symptoms usually begin from one to four days after infection, and range from mild (associated mainly with the common cold) to more complicated symptoms (associated with life-threatening illnesses such as epiglottitis). The duration of illness is generally one to two weeks; however, during the first week of infection, most of patients become better and can normally perform their daily activities (Porter *et al.,* 2006). URTIs symptoms can be relatively mild. They may begin with sore throat, dry cough, and runny nose. The cough may then become more severe, and can be associated with sputum. The mouth and throat may become swollen and red. However, other symptoms such as nausea and vomiting may also appear but usually associated with children

(Hall and McBride 1994; Porter, 2006). The severity of URTIs symptoms depends on the pathogens responsible for the infections. More severe symptoms such as muscle aches and fatigue are normally associated with influenza and parainfluenza infections. On the other hand, mild symptoms might be due to rhinovirus infections (Snow *et al.,* 2001; Gonzales *et al.,* 2001). Most uncomplicated URTIs cases in adults can subside spontaneously within two days, but few URTIs cases are complicated by either pneumonia or bacterial sinusitis (Gonzales *et al*., 2001).

2.3.7 Types of URTIs

Upper respiratory tract infections can be identified according to either the pathogens responsible for the infection or the characteristics of the illness. Furthermore, URTIs are a group of diseases ranging from the common cold to acute bacterial sinusitis, pharyngitis, non-specific URTIs, and influenza (Stefani, 2000; Wong *et al*., 2006). Patients with URTIs can also be classified according to the anatomic localization of the prominent clinical signs and symptoms associated with the illness (Gonzales *et al*., 2001).

2.3.7.1 Common cold

The common cold is considered to be an acute illness of the URTIs and it is caused either by respiratory syncytial viruses that are capable of repeatedly infecting an individual or rhinoviruses that initiate infection only once. These viruses are experienced by people of all age worldwide (Hendley, 1998; Monto, 2002). The common cold is characterized by malaise, sore throat, and low-grade fever, especially at the first time of onset. This illness can affect persons of all ages and are considered to represent a self-limited syndrome (Simasek and Blandino, 2007).

2.3.7.2 Acute bacterial sinusitis

Acute sinusitis is a common infection of the paranasal sinuses that is usually associated with inflammation of the nasal and sinus mucosa. Sinus disease has been shown to occur in 90% of patients with the common cold. In the first few days of infection, the symptoms are likely to be due to a viral cause that leads to upper respiratory tract infection, but this infection may later become complicated by a bacterial infection. The main pathogens responsible for bacterial infection are *Streptococcus pneumoniae, Haemophilus influenzae,* and *Moraxella catarrhalis*, although *Staphylococcus aureus* and *Streptococcus pyogenes* are isolated in rare cases (Bamberger and Jackson, 2001). Some physicians suspect acute sinusitis when cold or influenza-like illnesses persist for several days, and associated mainly with nasal congestion, sinus discomfort or tenderness, fever, headache, maxillary toothache, and facial pain. The symptoms presented may differ in children and can include irritability, lethargy, snoring, mouth breathing, feeding difficulty, and hyponasal speech (Fagnan 1998; Hirschmann, 2002).

2.3.7.3 Pharyngitis

Pharyngitis is the most common cause of sore throat, leading to an increasing number of family visits to physicians as well as ambulatory pediatric care visits (Vincent *et al.,* 2004). *Streptococcus pyogens* is considered to be the main causative agent of pharyngitis in both children and adults (Jokso-Koivisto *et al*., 2006; Wong *et al.,* 2006). The incubation period for pharyngitis ranges from one day to four days. Low-grade fever, fatigue, sore throat, coryza, and cough are the main symptoms suggesting the presence of pharyngitis.

2.3.7.4 Non-specific URTIs

These diseases are identified by a range of descriptive names, including acute infective rhinitis, acute rhinopharyngitis/nasopharyngitis, acute coryza, and acute nasal catarrh (Ressel, 2001).

Numerous viruses are considered to be causative agents for non-specific URTIs, including rhinoviruses, adenoviruses, respiratory synctial viruses, parainfluenza, and enteroviruses (Wong *et al*., 2006). Mucopurulent nasal discharge, nasal blockage, itchiness, sneezing, facial pain, and postnasal drainage with cough could be the main indications for the non-specific URTIs (Dykewicz *et al.,* 1998; Ho *et al.,* 1998).

2.3.7.5 Influenza

Influenza is caused by a virus that mainly attacks the upper respiratory tract (nose, throat, bronchi, and rarely the lungs). The infection usually lasts for about one week, and it is characterized by the sudden onset of high fever, myalgia, headache and severe malaise, non-productive cough, sore throat, and rhinitis (Taubenberger and Layne, 2001; Nicholson *et al*., 2003). Most people recover within one to two weeks with or without requiring medical treatment (Taubenberger and Layne, 2001; Nicholson *et al*., 2003). Influenza is considered as a serious condition in the very young, the elderly, and people suffering from medical conditions such as lung disease, diabetes, cancer, and kidney or heart problems. In such individuals, the infection may lead to severe complications such as pneumonia, which can result in death (Taubenberger and Layne, 2001).

Influenza can be caused by one of the three types of influenza virus: influenza A, influenza B, or influenza C (Kesson, 2007). Influenza virus type A and B mainly lead to epidemic diseases, while influenza virus type C leads to sporadic disease in human beings (Taubenberger and Layne, 2001).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study area

The viral research was carried out at the Department of Viral Research, National Veterinary Research Institute, Vom, Jos, whereas the bacterial research was conducted at the Department of Microbiology, Aminu Kano Teaching Hospital, Kano State, while chromatography analysis was carried out at Department of Chemistry, Bayero University, Kano, all in Nigeria.

3.2 Collection and processing of plant parts

Fresh leaves, roots and stem bark of *Securidaca longipedunculata* were collected directly the plant tree from Doguwa Local Government Area of Kano State, Nigeria during early morning hours. The plant parts were identified at Department of Plant Biology, Bayero University, Kano. The plant parts were then processed by washing with clean water, air dried under shade and ground into powder using an electric blender as described by Mukhtar and Tukur. (1999).

3.3 Collection of virus and 9- day old embryonated chicken eggs for the antiviral assay

A velogenic strain of NDV was obtained from department of viral research, National Veterinary Research Institute, Vom, Jos, while embryonated chicken eggs were obtained from poultry division, National Veterinary Research Institute, Vom, Jos, Nigeria.

The research was carried out asceptically in the biosafety cabinet using hand gloves, face mask and laboratory coat. Virkon® (Day – Impex limited, United Kingdom) and 70% alcohol were used as the disinfectants.

Penicillin, Streptomycin, Gentamycin and Amphotericin B were the antibiotics and antifungals used in the viral suspension to prevent contamination by bacteria and fungi.

The embryonated egss were candled and inoculation sites were marked. The eggs were then placed in egg racks with the inoculation sites uppermost.

3.4 Collection of bacteria and confirmation

Six (6) isolates of bacterial specie (*S. aureus*, *S. pneumoniae*, *S. pyogenes*, *K. pneumoniae*, *Proteus* sp. and *P. aeruginosa*) were obtained from Department of Microbiology, Aminu Kano Teaching Hospital, Kano State.

The bacteria were further subjected to confirmatory tests using biochemical procedures described by Cheesbrough. (2006).

Optochin sensitivity, catalase and bile solubility tests were done for *Streptococcus pneumoniae*. *Streptococcus pyogenes* was confirmed using catalase and bacitracin sensitivity tests. Methyl Red, indole and citrate tests were done for *Proteus* sp. *Pseudomonas aeruginosa* was confirmed by catalase, oxidase, methyl red and citrate tests. Catalase and coagulase tests were done to confirm *S. aureus* and *K. pneumoniae* was confirmed using catalase, oxidase, methyl red, indole, citrate, and urea tests and also hydrogen sulphide and gas production were observed.

3.5 Extraction of plant materials

A procedure by Redfern *et al*. (2014) was followed for extraction using Soxhlet apparatus (Pyrex Company, United Kingdom). The solvents used for the extraction were methanol and petroleum ether.One hundred grams (100g) of each of the powdered plant material was filled in filter paper.

Following this, 250ml of the solvent was added to a round bottom flask, which was attached to a Soxhlet extractor and condenser on an isomantle. The weighed plant material was placed inside the Soxhlet extractor. The side arm was lagged with glass wool. The solvent was heated using the isomantle and began to evaporate, moving through the apparatus to condenser. The condensate then dripped into the reservoir containing the plant material. As the level of solvent reached the siphon, it poured back into the flask and the circle continued, until the plant material colour turned colourless. This process was done for each of the plant part and for each of the solvent.

After the process finished, the extracts were then placed in a water bath for evaporation of the solvents, leaving behind the crude extracts of the plant material.

3.6 Phytochemical screening

Phytochemical screening was carried out using the method described by Trease and Evans (1989).

3.6.1 Test for alkaloids

To 5ml of 1% aqueous HCl, 0.5g of each *S. longipedunculata* extract was added on a steam bath. This was filtered and 1ml of the filtrate was treated with a few drops of Dragendoff's reagent and a second 1ml portion treated similarly with Wargner's reagent for the formation of precipitate.

3.6.2 Test for resins

To 0.5g of each *S. longipedunculata* extract, 5ml of boiling ethanol was added. This was filtered through Whatman No. 1 filter paper and the filtrate diluted with 4ml of 1% aqueous HCl for the formation of resinous precipitate.

3.6.3 Test for tannins

Securidaca longipedunculata extract of 0.5g was stirred with 10ml of distilled water. This was filtered and a few millilitres of 5% ferric chloride was added to the filtrate for the formation of deep green colouration. A second portion of the filtrate was treated with a few millilitres of iodine solution for formation of bluish colouration.

3.6.4 Test for glycosides

Each *S. longipedunculata* extract of 0.5g was stirred with 10ml of boiling distilled water. This was filtered and 2ml of the filtrate was hydrolyzed with a few drops of concentrated HCl and the solution rendered alkaline with a few drops of ammonia solution. Five drops of this solution was added to 2ml of Bennedicts qualitative reagent and boiled to detect the presence of glycoside by formation of reddish – brown precipitate.

3.6.5 Test for saponins

Each of *S. longipedunculata* extract of 0.5g was shaken with water in a test – tube. Frothing which persist on warming will be taken as evidence for the presence of saponin.

3.6.6 Test for flavonoids

Each *S. longipedunculata* extract of 0.5g was dissolved in 2ml diluted NaOH solution. A few drops of concentrated H_2SO_4 was then added. The presence of flavonoids was indicated when the solution became colourless.

3.6.7 Test for anthraquinones

Each *S. longipedunculata* extract of 0.5 g was taken into a dry test – tube and 5ml of chloroform was added and shaken for 5 min. The extract was filtered, and filtrate shaken with an equal volume of 100% ammonia solution. The presence of anthraquinone was indicated by the formation of a pink violet colour in the ammonical layer (lower layer).

3.6.8 Kelle Killiani test for cardiac glycosides

Each *S. longipedunculata* extract was dissolved in 2ml of glacial acetic acid containing one drop of ferric chloride solution. This was then underplayed with 1ml of concentrated sulphuric acid. A brown ring obtained at the interphase indicated the presence of a deoxysugar typical of cardenolides.

3.6.9 Salkwoski test for steroidal ring

Each *S. longipedunculata* extract of 0.5g was dissolved in 2ml of chloroform. Sulphuric acid was carefully added to form a lower layer for the formation of reddish brown colour at the interface.

3.6.10 Liberman's test for steroidal terpenes

Each *S. longipedunculata* extract of 0.5g was dissolved in 2ml of chloroform and 1ml of acetic acid anhydride was added, and then 2 drops of concentrated H_2SO_4 was added. The presence of steroidal terpenes was indicated by formation of pink colour which changes to bluish green on standing.

3.6.11 Test for phenols

Few drops (0.5 %) of neutral ferric chloride solution was added to 0.5ml of each extract of *S. longipedunculata* to check for the formation of dark green color according to the method of Poongothai *et al*. (2011).

3.7 Determination of EID_{50} (egg infective dose of NDV in 50% of embryonated chicken eggs)

The Egg infective dose (EID_{50}) of the viruses was determined according to the method of Young *et al* (2002). Sterile bijou bottles were labelled 10^{-4} to 10^{-9}. Sterile phosphate buffered saline PBS (0.9ml) was added to each bottle. A ten – fold serial dilution was carried out by adding 0.1ml of NDV to first bottle (10^{-4}) and mixed thoroughly. From the content of first bottle, 0.1ml was transferred asceptically to second bottle (10^{-5}) and mixed thoroughly. This transfer was done into the bottles until the last one (10^{-9}) when 0.1ml of the content was discarded into a discard jar. Embryonated eggs were divided into eight (8) groups of five (5) for inoculation of the dilutions. First group were inoculated via allantoic fluid with 0.2ml of contents of 10^{-4}, group two were inoculated with 0.2ml of contents of 10^{-5}. Same inoculation was done to other groups with corresponding dilutions of virus up to six (6) which was inoculated with 10^{-9} of mixture.

Uninfected control (PBS only) as well as virus control were included. Infected eggs were sealed and incubated in a humid chamber at 37°C for 24 hrs. They were candled after 24hrs for unspecific death while percentage mortalities after 48hrs and 72hrs was recorded. From this, 100 EID_{50}/ 0.1ml of the virus stock was made for the experiment which was obtained from the index calculated using Reed and Muench formula (Appendix III).

3.8 Haem agglutination test

This was done to confirm the presence of the virus in the virus suspension. A drop of 5% chicken red blood cell was mixed thoroughly with a drop of the virus stock on a slide for formation of agglutination (Young *et al*, 2002) (Appendix IV).

3.9 Inoculation of eggs

The method used by Chollom *et al* (2012) was adopted. Nine day old embryonated chicken eggs were labelled according to the extracts and concentrations that were used. A set of plastic egg trays were thoroughly cleaned with Virkon® (Day – Impex limited, United Kingdom), the eggs were swabbed with 70% alcohol in cotton wool and transferred into the cleaned trays for inoculation (Appendix IV).

3.10 Toxicity assay

This was carried out to check the toxicity of the extracts on the chicken embryos. Nine day old embryonated chicken eggs were divided into six groups of five. Group one (1) to four (4) were swabbed with 70% alcohol using cotton wool, punched with eggshell punch and immediately injected with needle attached to 1ml syringe. By keeping the needle and syringe vertical, the needle was placed through the punched hole and penetrated into the allantoic cavity

(approximately 16mm) with the extracts at concentrations of 80, 60, 40, and 20mg/ml in that order. The needle was then withdrawn from the egg and the hole was sealed with melted wax. Group five (5) were inoculated with 0.2ml phosphate buffered saline (diluent control) while group six (6) were not injected with any extract (negative control). The eggs were sealed with molten wax and incubated at 37^0C for 24hours. This was done for each of the extract. Embryo survival was observed after 24hours (Chollom *et al*., 2012).

3.11 Antiviral assay

This was carried out to check the efficacy of the extracts using the procedure of Chollom *et al*. (2012). Group one (1) to four (4) of embryonated eggs were swabbed and placed in the bio - safety cabinet where they were punched and immediately inoculated with the virus via allantoic route, sealed with molten wax and incubated at 37^0C. After 24 hours, the extracts (A, D and F) at final concentrations of 20, 15, 10 and 5mg/ml while extracts (B, C and E) at final concentrations of 10, 20, 30 and 40mg/ml were then injected in that order. Group five (5) were inoculated with 0.2ml of 100EID_{50}/ 0.1 ml standard NDV (virus control), group six (6) were inoculated with 0.2 ml extract (extract control). Group seven (7) were inoculated with 0.2ml phosphate buffered saline (diluent control) while group eight (8) were not inoculated with anything (uninoculated control). The eggs were sealed with molten wax and incubated at 37^0C for 72 hours. This was done for each extract. Embryo survival was observed daily for 3 days.

Note: Extracts A: Root methanol, B: Root petroleum ether, C: Leaf methanol, D: Leaf petroleum ether, E: Stem bark methanol, F: Stem bark petroleum ether.

3.12 Standardization of bacterial suspension

To standardize bacterial suspension, the bacterial isolates were first cultured on nutrient agar (Blood agar for *S. pneumoniae* and *S. pyogenes*) and incubated at 37^0C for 24 hrs. Enough loopful of the overnight culture was taken and emulsified in a tube containing 2ml normal saline until turbidity matched with 0.5 McFarland standard (Cheesebrough, 2006).

3.13 Antibacterial assay of the crude extracts

The antibacterial assay was done using agar well diffusion method described by Nester *et al.* (2004) was used. Blood Agar was prepared for *S. pneumoniae* and *S. pyogenes*. Mueller Hinton Agar was also prepared as specified by the manufacturer for the remaining bacteria. The media were autoclaved and poured aseptically into sterile Petri dishes and allowed to gel. A loopful of the standardized bacterial suspension was streaked evenly on each agar plate.

Stock solution (240µg/ml) of the root, leaf, and stem extracts of *S. longipedunculata* was separately prepared by dissolving 0.00024g of the extracts into 2mls dimethylsulphoxide (DMSO) to obtain the concentration of 240µg/ml. From this, the working concentrations of 120µg/ml, 60µg/ml, 30µg/ml and 15µg/ml were made. Then 0.1 ml of each crude extract was inoculated into four wells (6mm diameter) bored with a sterile cork borer in each plate. Dimethylsuphoxide (0.1ml) was inoculated in the fifth well on the plate to serve as negative control and 0.1ml of commercially prepared ciprofloxacin (30µg/ml) was inoculated in the sixth well to serve as positive control. The plates were allowed to stand for 30 minutes on the table for pre- diffusion of the extracts, after which they were incubated at 37^0C for 24 hours. The antibacterial activity of the extracts was determined after incubation by measuring the mean

diameter zones of inhibition produced by each of the extracts against the bacterial species and results was recorded in millimeter (mm).

3.14. Thin layer chromatography analysis

Thin layer chromatography (TLC) was conducted according to the method describe by Thomson and Abbott (1966) to select the best solvents for column chromatographic analysis. A spot of the extract prepared by dissolving in chloroform were placed on a coated thin layer chromatography, using capillary tube and was allowed to dry. The plates were developed using solvent ratio of n-hexane: chloroform, n- hexane: ethylacetate and chloroform: ethyl acetate in a developing tank until it reaches it solvent front, this was allowed to dry. The TLC plate was gently immersed into a beaker containing iodine crystal and brought out after some minute and chromatographic bands were observed.

3.15. Column chromatography analysis

One hundred and sixty five **(**165) grams of silica gel was mixed with n- hexane until fine slurry was obtained. The slurry was poured into a sintered- base glass column (121 x 2.5 cm).The column was packed with the slurry and the solvent allowed draining after wards. Five (5) grams of each crude extracts of root, leaf and stem bark of *S. longipedunculata* was thoroughly mixed with silica gel until it changed to a non-sticky powder. The powdered mixture of each extract was then loaded onto the bed of the silica gel in the column. Small quantity of silica gel was added on top to protect the adsorbent.

Four solvents were used for elution; n- hexane, chloroform, ethyl acetate and acetone. The column loaded was run by dispensing (440ml hexane, 720ml chloroform, 450ml ethyl acetate

and 250ml acetone) per each extract, with ration in order of increasing polarity. Starting with 100% n-hexane, then a mixture of n- Hexane/ Chloroform in the following ratio (90:10, 80:20, 60:40, 50:50, 40:60, 20:40) then 100% Chloroform, followed by Chloroform/ Ethyl acetate in the following ration (90:10, 80:20, 60:40, 50:50, 40:60, 20:80, 10:90) then Ethyl acetate 100% and finally washed with acetone 250%. A varied quantity of the solvents was eluted and the eluent was collected in fraction of 20ml. A total of eighty four (84) fractions were collected for each extract. They were allowed for complete evaporation at room temperature. Weight and Thin Layer Chromatographic analysis on pooled fraction were determined (Mudi and Muhammad, 2009).

3.16 Antibacterial assay of chromatography pooled fractions

Pooled fractions of column chromatography for the crude extracts that showed activity was again tested using the same procedure on the most susceptible isolates i.e *S. pyogenes* and *Proteus* Sp. The antibacterial activity of the fractions was determined after incubation by measuring the mean diameter zones of inhibition produced by each of the fraction against the bacterial species and results was recorded in millimeter (mm) (Nester *et al*., 2004).

3.17 Data analysis

Data for the screening of activity of *Securidaca longipedunculata* extracts against Newcastle disease virus and upper respiratory tract bacterial were determined by the analysis of variance using the GraphPad Instat3.0 statistical software for windows 2006. Values were considered significant when $p < 0.05$.

CHAPTER FOUR

4.0 RESULTS

Methanol extract of the leaf of *Securidaca longipedunculata* yielded the highest extracts with weight of 18.5grams whereas, extract from stem bark petroleum ether yielded the least extract with 9.0grams. All extracts showed slight difference in their colour before and after evaporation of the respective solvents. The extracts also varied in their consistency after evaporation. The consistency nature of the extracts includes hard solid, gummy, powdered and sticky. This is shown in Table 4.1.

The egg infective dose in 50% of the embryonated eggs (EID_{50}) of Newcastle disease virus was found to be at dilution 10^{-9}. This is shown in Table 4.2.

Table 4.1: Physical appearance and weights of *S. longipedunculata* crude extracts

	Physical appearance		
Extract	**Before evaporation of extract**	**After evaporation of extract**	**Weight/grams**
A	Yellow Liquid	Yellowish brown hard solid	10.3
B	Yellow Liquid	Yellow Powdered solid	9.2
C	Green liquid	Greenish brown gummy Solid	18.5
D	Green liquid	Greenish gummy solid	15.1
E	Green liquid	Greenish brown sticky solid	16.4
F	Pale yellow liquid	Yellow powdered solid	9.0

Key: A = *S. longipedunculata* root methanol extract, B = *S. longipedunculata* root petroleum ether extract, C = *S. longipedunculata* leaf methanol extract, D = *S. longipedunculata* leaf petroleum ether extract, E = *S. longipedunculata* stem bark methanol extract, F = *S. longipedunculata* stem bark petroleum ether extract.

Table 4.2: EID_{50} of Newcastle disease virus after 72 hours

Dilution	No. of eggs with live embryo	No. of eggs with dead embryo	% Mortality (%)
10^{-4}	1	4	80
10^{-5}	1	4	80
10^{-6}	1	4	80
10^{-7}	1	4	80
10^{-8}	2	3	60
10^{-9}	3	2	40

In the antiviral assay, the toxicity result showed that all the extracts were relatively toxic. Root methanol, leaf methanol and stem bark petroleum ether extracts were more toxic in which the highest concentrations of these extracts the embryonated eggs survived was 20mg/ml. Meanwhile, 40mg/ml concentrations of the root petroleum ether, leaf methanol and stem bark extracts were the highest concentrations of these extracts the embryonated eggs survived. At 80mg/ml concentration of extract A, the embryonated eggs were not survived after 24hours. Also, only one embryonated egg survived the concentrations of extracts B, C, D and E while extract F did not kill the embryos of the eggs at concentrations of 60mg/ml and 80mg/ml. This is shown in Table 4.3.

Table 4.3 Toxicity assay of *S. longipedunculata* crude extracts on Newcastle disease virus

(No. of eggs inoculated = 5)

Extract(s)	Conc. (mg/ml)	No. of eggs with dead embryo after 24h	No. of eggs with live embryo after24h
A	20	0	5
	40	2	3
	60	3	2
	80	5	0
B	20	0	5
	40	0	5
	60	3	2
	80	4	1
C	20	0	5
	40	0	5
	60	2	3
	80	4	1
D	20	1	4
	40	3	2
	60	4	1
	80	4	1
E	20	0	5
	40	0	5
	60	3	2
	80	4	1
F	20	0	5
	40	2	3
	60	3	2
	80	3	2
Dc		0	5
Nc		0	5

Key: A = *S. longipedunculata* root methanol extract, B = *S. longipedunculata* root petroleum ether extract, C = *S. longipedunculata* leaf methanol extract, D = *S. longipedunculata* leaf petroleum ether extract, E = *S. longipedunculata* stem bark methanol extract, F = *S. longipedunculata* stem bark petroleum ether extract, Dc: Diluent control, Nc: Negative control, h = Hour.

The result of the antiviral activity of *Securidaca longipedunculata* root, stem bark and leaf extracts revealed that, stem bark methanol extract exhibited the highest activity with mortality rates of embryonated eggs of only 10 % at 40mg/ml concentration of the extract. Petroleum ether extracts of root and leaf revealed the least activity with 100% mortality rate of embryonated eggs at the highest working concentrations of the extracts (40mg/ml and 20mg/ml respectively) as shown in Table 4.4.

The outcome of the antibacterial assay of the crude extracts showed that, methanol and petroleum ether extracts of the root revealed activity against *Streptococcus pyogenes*, and *Proteus* sp. with highest activity exhibited by root petroleum ether extract at 120µg/ml concentration. At this concentration, the zone of inhibition was 30mm which is similar to the zone of inhibition exhibited by ciprofloxacin (positive control) at 30 µg/ml. Leaf methanol extract revealed activity on *Streptococcus pyogenes* only and exhibited the least zone of inhibition of 30mm at 120µg/ml concentration of the extract. This is shown in Table 4.5.

Table 4.4: Antiviral activity of *S. longipedunculata* crude extracts against NDV

Extract	Conc. (mg/ml)	No. of eggs	Mortality 24h	48	72h	% Mortality after 72h
A	5	5	0	5	0	100
	10	5	0	5	0	100
	15	5	0	3	2	100
	20	5	0	0	5	60
Ec		5	0	0	5	0
B	10	5	0	5	0	100
	20	5	0	5	0	100
	30	5	0	5	0	100
	40	5	0	3	2	100
Ec		5	0	0	0	0
C	10	5	0	5	0	100
	20	5	0	4	1	100
	30	5	0	3	2	100
	40	5	0	2	2	80
Ec		5	0	0	0	0
D	5	5	0	5	0	100
	10	5	0	5	0	100
	15	5	0	3	2	100
	20	5	0	4	1	100
Ec		5	0	0	0	0
E	10	5	0	5	0	100
	20	5	0	3	1	80
	30	5	0	1	1	20
	40	5	0	1	0	10
Ec		5	0	0	0	0
F	5	5	0	5	0	100
	10	5	0	5	0	100
	15	5	0	3	2	100
	20	5	0	0	3	60
		5	0	0	0	0
Ec		5	0	0	0	0
Vc		5	5	0	0	100
Dc		5	0	0	0	0
Nc		5	0	0	0	0

Key: Vc: virus control, Ec: extract control, Dc: diluent control, Nc> negative control, h=Hours

Table 4.5: Antibacterial activity of *S.longipedunculata* crude extracts upper respiratory bacteria

Concentrations (µg/ml) / Zone diameter (mm)																										
Organisms	**A**				**B**				**C**				**D**				**E**				**F**				**+c**	**-c**
	120	**60**	**30**	**15**	**120**	**60**	**30**	**15**	**120**	**60**	**30**	**15**	**120**	**60**	**30**	**15**	**120**	**60**	**30**	**15**	**120**	**60**	**30**	**15**	**30**	
S. aureus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	45	0
S. pyogenes	19	0	0	0	17	0	0	0	0	0	0	0	15	0	0	0	0	0	0	0	0	0	0	0	17	0
S. pneumoniae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	18	0
K. pneumoniae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	45	0
P. aeruginosa	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	18	0
Proteus sp.	0	0	0	0	30	26	21	18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	30	0

Key: A = *S. longipedunculata* root methanol extract, B = *S. longipedunculata* root petroleum ether extract, C = *S. longipedunculata* leaf methanol extract, D = *S. longipedunculata* leaf petroleum ether extract, E = *S. longipedunculata* stem bark methanol extract, F = *S. longipedunculata* stem bark petroleum ether extract, +c = Positive control, -c = Negative control.

Plate 4.1: Antibacterial sensitivity of *S. longipedunculata* crude extract against *Proteus* sp.

Plate 4.2: Antibacterial sensitivity of *S. longipedunculata* crude extract against *S. pyogenes*.

Antibacterial assay of chromatography fractions of fraction one of root methanol and fraction three of leaf methanol crude extracts revealed that fraction one of root methanol (AA1) showed highest activity on *Streptococcus pyogenes* with 28mm at 120µg/ml while fraction three of the leaf methanol did not show any activity as shown in Table 4.6.

Antibacterial assay of fraction one of root petroleum ether crude extract exhibited highest activity at 120µg/ml on *Proteus* sp. (zone of inhibition of 19mm) than on S. *pyogenes* (zone of inhibition of 8m) as shown in Table 4.7.

Table 4.6: Antibacterial activity of *S. longipedunculata* pooled fractions against *S. pyogenes*

	Concentrations (µg/ml) / zone diameter (mm)								
Extract	**960**	**480**	**240**	**120**	**60**	**30**	**15**	**+c(30 µg)**	**-c**
AAA1	43	39	30	28	24	23	22	61	0
AAA2	25	20	19	16	15	13	11	61	0
BBB1	14	12	11	8	6	4	3	61	0
DDD1	24	23	21	15	13	12	10	61	0
DDD2	0	0	0	0	0	0	0	61	0
DDD3	6	6	10	6	7	9	12	61	0

KEY: AAA1 = *S. longipedunculata* root methanol pooled fraction 1, AAA2 = *S. longipedunculata* root methanol pooled fraction 2, BBB1 = *S. longipedunculata* root petroleum ether pooled fraction 1, DDD2 = *S. longipedunculata* leaf petroleum ether pooled fraction 2, DDD3 = *S. longipedunculata* leaf petroleum ether pooled fraction 3, DDD4 = *S. longipedunculata* leaf petroleum ether pooled fraction 4, +c = Positive control, -c =Negative control.

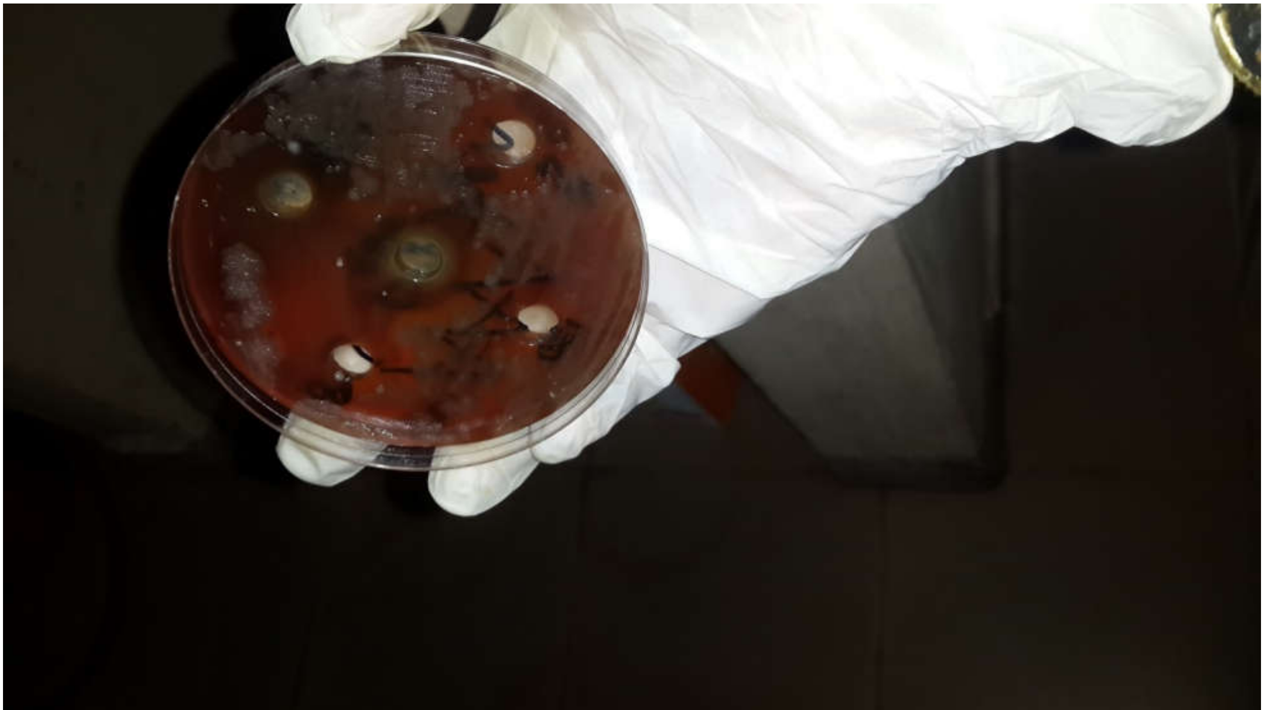

Plate 4.3: Antibacterial sensitivity of *S. longipedunculata* pooled fractions against *S. pyogenes*.

Table 4.7: Antibacterial activity of *S. longipedunculata* pooled fractions against *Proteus* sp.

Extract	Concentrations (µg/ml) / zone diameter (mm)						
	240	**120**	**60**	**30**	**15**	**+c**	**-c**
BBB1	18	17	15	14	14	42	0

Key: BBB1 = *S. longipedunculata* root petroleum ether pooled fraction 1, +c = Positive control, -c = Negative control.

Plate 4.4: Antibacterial sensitivity of *S. longipedunculata* pooled fractions against *Proteus* sp.

The result of phytochemical screening revealed the presence of bioactive components tested. Cardiac glycoside was present in larger quantity in all the extracts. Saponin was absent in the leaf extracts, also, glycoside was absent in the stem bark extracts and root petroleum ether extract. Anthraquinone was also absent in the stem bark petroleum ether extract (Table 4.8).

The outcome of column chromatography revealed various fractions from all the plant parts. Leaf methanol crude extract recovered the highest amount in fraction three (0.4g) while the least fraction was obtained in fraction one and three of root methanol and fraction three of stem bark petroleum ether extracts. The result is shown in Table 4.9.

Table 4.8: Phytochemical constituents of *S.Longipedunculata* extracts

	Extracts					
Compounds	**A**	**B**	**C**	**D**	**E**	**F**
Alkaloids	+	+	+	+	+	+
Resins	+	+	+	+	+	+
Tannin	+	+	+	+	+	+
Glycoside	+	-	+	+	-	-
Saponin	+	+	-	-	+	+
Flavonoids	+	+	+	+	+	+
Anthraquinone	+++	+	+	+	+	-
Cardiacglycoside	+++	+++	+++	++	+++	+++
Steroidal ring	+++	+++	+++	+++	+++	+
Steroidalterpenes	++	+	+++	+++	+++	+
Phenols	+	+	+++	+	+	+
Reducing sugar	+	+	+	+++	+	+

Key: A = *S. longipedunculata* root methanol extract, B = *S. longipedunculata* root petroleum ether extract, C = *S. longipedunculata* leaf methanol extract, D = *S. longipedunculata* leaf petroleum ether extract, **E = *S. longipedunculata* stem bark methanol extract, F = *S. longipedunculata* stem bark petroleum ether extract, + = Present, - = Absent**

Table 4.9: Physical properties of pooled fractions of *S. longipedunculata* crude extracts

		Physical appearance			
Fraction	**S/no.**	**Before evaporation of eluent**	**After evaporation of elluent**	**Pooled fraction code**	**Weight /g**
	1-43	Yellow liquid	Deep yellow oil	AAA1	0.002
AA	44-52	Yellow liquid	Yellow oil	AAA2	0.002
	53-82	Yellow liquid with a hint of some green	White crystals	AAA3	0.003
	1-82	Yellow liquid	Yellow oil	BBB1	0.050
CC	1-65	Green liquid	Green oil	CCC1	0.070
	66-84	Green liquid	Greenish yellow oil with green crystals	CCC2	0.200
DD	1-40	Greenish yellow liquid	Green crystals	DDD1	0.210
	41-69	Pale green liquid	Yellow crystal oil	DDD2	0.300
	70-84	Green liquid	Sticky green solid	DDD3	0.400
EE	1-64	White liquid	White oil	EEE1	0.004
	65-84	White liquid	White solid	EEE2	0.100
FF	1-32	White liquid	White crystals	FFF1	0.070
	33-43	White liquid	White oil	FFF2	0.040
	44-80	White liquid	White solid	FFF3	0.020
Total weight of fractions pooled from column chromatography :			**A= 0.007, B= 0.05, C= 0.27, D=0.91, E=0.104, F= 0.13**		

KEY: AA = First fraction of *S. longipedunculata* root methanol crude extract, BB = First fraction of *S. longipedunculata* root pet ether crude extract, CC = First fraction of *S. longipedunculata* leaf methanol crude extract, D = First fraction of *S. longipedunculata* leaf pet ether crude extract, E = *S. longipedunculata* stem bark methanol crude extract, F = *S. longipedunculata* stem bark pet ether crude extract, S/no. = Serial number, g = Grams.

Plate 4.5: Pooled fractions of *S. longipedunculata* extracts.

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

The leaf methanol extract yielded more extract while the petroleum ether of the stem bark yielded the least extract. All the extracts yielded a variety of compounds in varying amounts but the methanol extract of the root contained all the compounds tested which implies that the solvent had the highest extraction capacity. This is in agreement with the study done by Muanda *et al*. (2010).

The result of the EID_{50} indicated that, the titre of Newcastle disease virus that was able to infect fifty percent of the experimental eggs was found to be at dilution 10^{-9} which is similar to the work of Chollom *et al*. (2012).

In the antiviral assay, toxicity result showed that, petroleum ether extracts were more toxic than the methanol extracts which corresponded with the study of Auwal *et al*. (2012) on albino rats. Acute toxicity evaluation of the extracts showed that, the extracts were toxic to the experimental eggs at concentration above 40mg/ml.

However, antiviral assay of *S. longipedunculata* confirmed that the stem bark methanol crude extract had the highest antiviral properties against ND virus. This was revealed by the inhibition of virus growth at 20, 30 and 40mg/ml. At these concentrations, all the inoculated eggs had live embryos. The petroleum ether extracts of the root and leaf revealed 100% mortality. The root

and leaf methanol extracts showed slight activity at 20mg/ml and 40mg/ml respectively. However, petroleum ether extract of the stem bark revealed slight activity at 20mg/ml. The methanol extract of the stem bark exhibited a greater activity at 40mg/ml and 30mg/ml with 10 and 20 percentage mortality respectively and also slight activity at 20mg/ml with percentage mortality of 80%. This is unusual considering the phytochemical composition of the plant and a previous citation of the antiviral potentials of the plant against viruses which according to Borikini *et al*. (2013), the stem bark is mixed with *Mondia whitei* (stem bark), *Uvaria afzelii* (root bark), *Allium ascalonicum* (bulb) and *Parkia biglobosa* (seeds) and then taken with hot porridge to treat a variety of viral infections. These findings are scientific and relevant judging from the performance of the control groups. The virus control was potent enough to cause embryo death within 24h post inoculation just as extract, diluents and uninoculated controls did not interfere with embryo survival signifying the acceptability of the outcome of the tests groups. To the best of my knowledge, this is the first documented animal virus to be confirmed susceptible to the plant.

The bacterial assay revealed activity of the crude extracts against Gram positive (*S. pyogenes*) and Gram negative (*Proteus* sp.) bacteria with high degree of activity in the column chromatography fractions. The petroleum ether crude extract of the root yielded the highest zone of inhibition (ZI) of 30mm at concentration of 120µg/ml against *S. pyogenes* which is similar to the ZI of the control drug (ciprofloxacin) at concentration of 30µg/ml. Furthermore, the methanol and petroleum ether extracts of the root and also leaf petroleum ether extract exhibited ZI of 19mm, 17mm and 15mm respectively all at concentrations of 120µg/ml. These findings correspond to earlier reports by Ndamitso *et al*. (2013) and Musa *et al*. (2013) but in contrast

with the report of Adebayo and Osman. (2012) where they recorded ZI of 15mm by the ethanol extract of the root bark of *S. longipedunculata* at a concentration of 100mg/ml.

The root methanol pooled fraction one (1) and two (2) from the column chromatography revealed greater ZI more than the petroleum ether pooled fraction. The leaf petroleum ether fraction one (1) showed greater activity while fraction two (2) showed no ZI, fraction three (3) of the same extract exhibited average zone of inhibition.

The presence of the phytochemical constituents from the phytochemical screening and column chromatography in the root, stem bark and leaf extracts of *S. longipedunculata* is an indication that this plant would yield drugs of plant origin with pharmacological significance. This is better supported by the fact that, the plant family (*Polygalaceae*) to which *S.longipedunculata* belongs, is known to be involved in ethnomedicine in the management of some ailments notably epilepsy (Mathias, 1982). They are also used as anti-snake venom and as purgative (Chhabra *et al*., 1991). Moreover, the presence of alkaloids, cardiac glycosides, flavonoids, saponins and tannins in this plant is in compliance with an already documented literature which stated that, plants belonging to families *Polygalaceae*, *Moraceae* and *Cannabinaceae* are known to contain glycosides, triterpenes, resins and higher fatty acids in their stem and root bark (Evans, 1996).

5.2 Conclusion

From the result obtained in this study, it showed that;

1. *Securidaca longipedunculata* had more extracts in methanol than in petroleum ether.
2. Egg infective dose (EID_{50}) of Newcastle disease virus was determined at dilution 10^{-9}.

3. *Securidaca longipedunculata* showed antiviral activity against Newcastle disease virus especially the stem bark methanol extract.
4. Root petroleum ether extract of *Securidaca longipedunculata* was active against *Streptococcus pyogenes* and *Proteus* sp. isolated from upper respiratory tract of human. Moreover, root methanol extract was active against *Streptococcus pyogenes* from upper respiratory tract of human.
5. *Securidaca longipedunculata* had various bioactive compounds in appreciable quantities.

5. 3 Recommendations

In view of the results observed in this study, it is recommended that;

1. More solvents needs to be used to extract various bioactive compounds from this plant.
2. More antiviral activity from this plant especially the stem bark needs to be explored against Newcastle disease virus and other animal and human viruses of economic and medical importance.
3. There is the need to explore the biological activity of various extracts from the plant against microorganisms of the upper respiratory tracts of human and other bacteria of medical importance.
4. There is also a need to investigate the biological activity of the other compounds occurring in the plant extracts. Also, further identification of the fractions obtained from column chromatography needs to be undertaken to confirm the active compound responsible for the plant activity.

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APPENDIX I

One-way Analysis of Variance (ANOVA)

The P value is 0.1598, considered not significant.
Variation among column means is not significantly greater than expected by chance.

T-8

One-way Analysis of Variance (ANOVA)

The P value is 0.7087, considered not significant. Variation among column means is not significantly greater than expected by chance.

Post tests were not calculated because the P value was greater than 0.05.

Assumption test: Are the standard deviations of the groups equal?

ANOVA assumes that the data are sampled from populations with identical SDs. This assumption is tested using the method of Bartlett.

Bartlett statistic (corrected) = 4.381
The P value is 0.6252.
Bartlett's test suggests that the differences among the SDs is not significant.

Assumption test: Are the data sampled from Gaussian distributions?

ANOVA assumes that the data are sampled from populations that follow Gaussian distributions. This assumption is tested using the method Kolmogorov and Smirnov:

Group	KS	P Value	Passed normality test?
960	0.1741	>0.10	Yes
480	0.1574	>0.10	Yes
240	0.1554	>0.10	Yes
120	0.1807	>0.10	Yes
60	0.1764	>0.10	Yes

30	0.1947	>0.10	Yes
15	0.2144	>0.10	Yes

Intermediate calculations. ANOVA table

Source of Variation	Degrees of freedom	Sum of squares	Mean square
Treatments (between columns)	6	444.00	74.000
Residuals (within columns)	35	4141.3	118.32
Total	41	4585.3	

F = 0.6254 = (MStreatment/MSresidual)

Summary of Data

Group	Number of Points	Mean	Standard Deviation	Standard Error of Mean	Median
960	6	18.667	15.436	6.302	19.000
480	6	16.667	13.880	5.667	16.000
240	6	15.167	10.420	4.254	15.000
120	6	12.167	9.766	3.987	11.500
60	6	10.833	8.377	3.420	10.000
30	6	10.167	7.985	3.260	10.500
15	6	9.667	7.711	3.148	10.500

Group	Minimum	Maximum	95% Confidence Interval From	To
960	0.000	43.000	2.465	34.868
480	0.000	39.000	2.098	31.236
240	0.000	30.000	4.230	26.103
120	0.000	28.000	1.917	22.417
60	0.000	24.000	2.041	19.625
30	0.000	23.000	1.785	18.548
15	0.000	22.000	1.573	17.761

APPENDIX II

Preparation of Mcfarland standard solution

For 0.5 McFarland standard solution,

0.05 of 1% Bariumchloride

9.95 of 1% Sulfuric acid (H_2SO_4)

Method

To 0.05mL of 1% bariumchloridediihydrate($BaCl.2H_2O$),9.9mL of 1% sulphuric acid(H_2SO_4) was added.

APPENDIX III

Reed and Muench formular for calculation of 100EID$_{50}$.

$$Index = \frac{(\%\ \text{infected at dilution immediately above } 50\%) \quad 50\%}{(\%\ \text{infected at dilution immediately above } 50\%) \quad (\%\ \text{infected at dilution immediately below } 50\%)}$$

Dilution 10^{-8} is the dilution that produced % of infected at dilution immediately above 50% and dilution 10^{-9} is the dilution that produced % of infected at dilution immediately below 50%.

$$= \frac{60\%-50\%}{60\%-40\%} = 0.5$$

The index was then applied to the dilution that produced the percentage immediately above 50% as shown below;

Dilution $10^{-8} + 0.5 = 10^{-8.5}$

The reciprocal of this dilution is the amount of virus contained in the 0.1ml of the original suspension = $10^{-8.5}$ EID$_{50}$/ 0.1ml

100EID$_{50}$/ 0.1ml = $10^{-9.5}$

Therefore, the infective titre of virus has been calculated to be in dilution 10^{-9}.

APPENDIX IV

Pictures of the research work

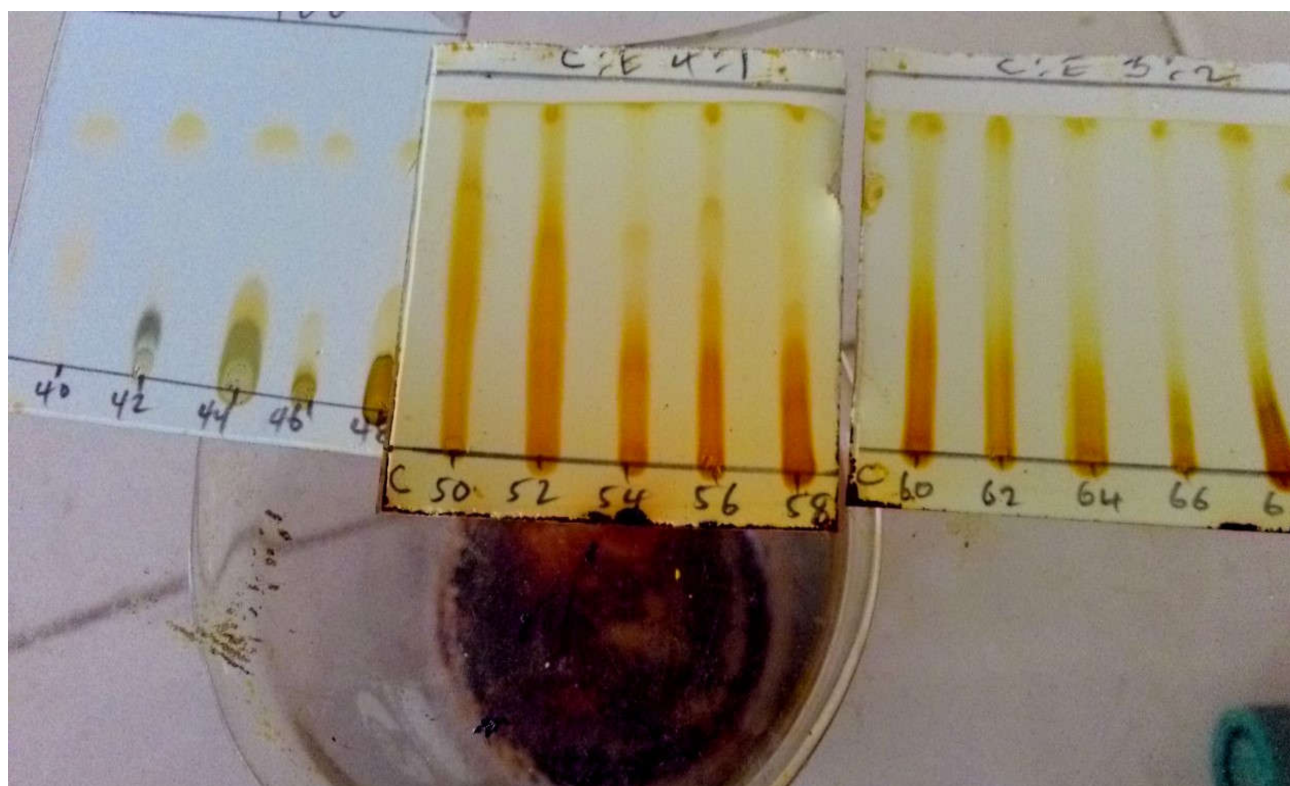

Plate 4.6: TLC analysis of *S. longipedunculata* fractions.

Plate 4.7: Viral suspension, antibiotics and PBS.

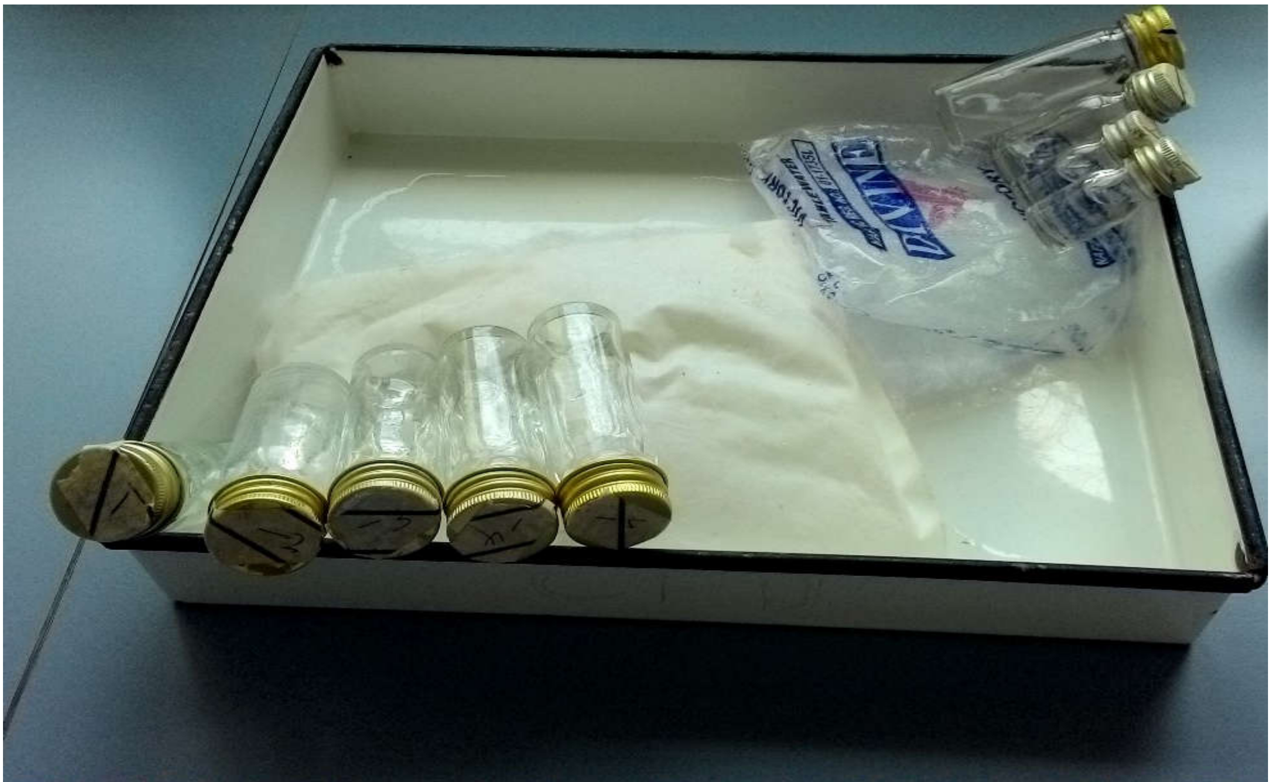

Plate 4.8: Serial dilution for EID_{50}.

Plate 4.9: Candling of embryonated eggs.

Plate 4.10: Inoculation of embryonated eggs.

Plate 4.11: Sealing of embryonated eggs.

Plate 4.12: Haemagglutination test.