ANTIVIRAL POTENTIAL OF HADJOD (*CISSUS QUADRANGULARIS*) EXTRACT AGAINST NEWCASTLE DISEASE VIRUS

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A dissertation submitted to the University of Zambia in partial fulfilment for the requirements for the award of the degree of Master of Science One Health Analytical Epidemiology

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DECLARATION

I, Alberto Filipe Machamacha, do hereby declare that the contents of the thesis being submitted herein are my original work and they have not been previously submitted to any other university for the award of a degree or any other qualification.

Signature…… Date…….

CERTIFICATE OF APPROVAL

This thesis submitted by ALBERTO FILIPE MACHAMACHA is approved as fulfilling part of the requirements for the award of the degree of MASTER OF SCIENCE ONE HEALTH ANALYTICAL EPIDEMIOLOGY of the University of Zambia.

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ABSTRACT

Newcastle Disease (ND) is a highly contagious viral disease that has a devastating global impact on the poultry industry. Ethanol (EtOH) crude extract from the stem of *Cissus quadrangularis* (CQL) was prepared and tested against Newcastle Disease Virus (NDV) replication using an *in-ovo* assay in embryonic chickens. *Cissus quadrangularis* L, is a perennial plant of the Vitaceae family, with high in active ingredients that have been proved to act against a variety of diseases and disorders. The study aimed to evaluate the antiviral activity of crude extracts of *C. quadrangularis* against NDV. Nine-day-old embryonated chicken eggs were divided into ten groups (n=10) and received various treatments. Six groups were inoculated with Velogenic NDV (vNDV) isolate AF2 and used for testing antiviral efficacy. Five groups of ten (10) were treated with different concentrations of the working stock extracts solution. The phytochemicals such as alkaloids, tannins, saponins, flavonoids, phenols, steroids, and glycosides were revealed in stem crude extract in the phytochemical tests. Cytotoxicity studies, on the other hand, showed that embryonated eggs could tolerate the all-plant extract at concentrations of 100 mg/mL. Highest chick embryo mortality (73.7%) was observed in the control group (virus only) and lower (11%) in the high extract concentration (75 mg/mL). The tested extract exhibited *in ovo* antiviral activity against NDV, showing viral titre reduction at a range of 1:64 in the haemagglutination test, indicating 4-times viral load decline compared with untreated embryo (positive control; 1:512) The current findings have clearly demonstrated that the crude EtOH extract from stem of CQL has strong antiviral activity against NDV *in ovo*. The mortality was lower when the extract was applied and antiviral significant statistical efficacy was observed. In order to further validate the efficacy of the plant extract as anti-NDV, an *in vivo* study using live chickens and use of standard for fractionations to isolate individual constitutes are recommended.

Keywords : Antiviral Activity, Newcastle disease, Crude extract, *Cissus quadrangularis*

DEDICATION

To my parents, Filipe Machamacha and Prisca Sairosse, for living, for the unconditional love and dedication at all times of my life; to my brothers for the affection; to my grandparents for always being by my side and for friends for their support, love, and encouragement during this stage of my life.

In a simple way, my grandparents, Sairrosse Mhukuta and Meguia John, taught me that a person is made of character and attitude.

I dedicate.

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LIST OF ABBREVIATIONS AND ACRONYMS

APMV-1	Avian paramyxovirus type 1
cm	Centimeter
CT	Condensed tannins
PT	Proanthocyanins, and phlorotannin's
ECEs	Embryonated chicken eggs
EDTA	Ethylene-Diamine-Tetra-Acetic acid
EID50	Mean (50%) embryo-infectious dose
ELD50	Mean (50%) embryo-lethal dose
EtOH	EtOH extract
F	Fusion glycoprotein
g	Grammar
g	Acceleration due to gravity
HA	Haemagglutination or hemagglutination test /Assay
HN	Haemagglutinin neuraminidase glycoprotein on envelope of Newcastle disease virus
L	large polymerase
Log	Logarithm
M	Matrix protein
mL	Milliliter
ND	Newcastle disease
NDV	Newcastle disease virus
NP	Nucleocapsid protein
nvNDV	Neurotropic velogenic NDV
^{o}C	Degree Celsius
P	**-** Phosphoprotein
P	Probability (p-value)
PBS	Phosphate Buffered Saline
RBCs	Red blood cells
RNA	Ribonucleic Acid
RNP	Ribonucleoprotein complex

SPF	Specific pathogen free
UNZA	The University of Zambia
UTH	University Teaching Hospital
vNDV	Velogenic NDV
vvNDV	Viscerotropic velogenic NDV
w/v	Weight per volume (for instance g/mL or g/100 mL)
WHO	World Health Organization
WOAH	World Organization for Animal Health
µL	Microliter
DNA	Deoxyribonucleic acid
HT	Hydrolysable tannins

CHAPTER ONE
1.0 INTRODUCTION

1.1 Background

Poultry is a primary source of animal protein, particularly chicken, and provides a valuable supply of dietary protein and an essential source of income in rural areas of developing countries (Mubamba *et al.*, 2018). Viral diseases threaten poultry production and cause significant economic loss through mortality and reduced growth. Newcastle Disease (ND) is poultry's most prevalent zoonotic viral infection (Dimitrov *et al.*, 2016; Zhang *et al.*, 2018; Chollom *et al.*, 2022). ND is one of the most important and highly contagious, and economically significant poultry viral disease. The effects are mainly caused by a virulent strain of avian paramyxovirus serotype 1 (APMIV-1) belonging to the genus Avulavirus within the *Mononegavirales* order, the *Paramyxoviridae* family, and the *Avulavirinae* subfamily, and exists in 20 serotypes, APMV-1 to APMV-20 (Amoia *et al.*, 2021). It has three strains classified into three significant pathotypes: lentogenic, mesogenic, and velogenic, based on their pathogenicity in chickens (Talreja et al., 2017; Alemneh, 2019). The Velogenic strains of NDV cause highly virulent disease and thus are placed under the notifiable list A condition of World Organization of Animal Health (WOAH) (Omer *et al.*, 2014).

Currently, no therapeutic drug is available to treat ND. Although vaccination with live and killed vaccines is a common management practice, velogenic strains are still endemic in many countries, where they are responsible for direct and indirect economic losses (Trejo-Avila *et al.*, 2016). Failure to protect chickens from NDV via vaccine predisposes the chickens to the continuous circulation of the genetically and antigenically distinct variant strains(Bashir Bello *et al.*, 2020).

The use of vaccinations in local village settings is limited due to cost, dose format, and thermostability (require refrigeration and begin to deteriorate rapidly after 1–2 hours at room temperature (approximately 25°C). In addition, rural scavenging chickens are rarely vaccinated, and chickens remain highly susceptible to ND, with periodic outbreaks nearly destroying the flock (Ruan *et al*., 2020). As a result, many rural farmers resort to using inexpensive and readily available ethnoveterinary plants to control disease outbreaks (Nyandoro, 2017).

Plant extracts are a promising source of antiviral compounds, particularly those with high efficacy against resistant mutant viral strains and low host toxicity (Trejo-Avila *et al.*, 2016). Further, many studies have identified potential antiviral active ingredients from plants (Chollom *et al.*, 2022). Other studies have focused on the locally used medicinal plants that could carry active ingredients, (Nyandoro, 2017). Despite most of their advantages, the plants' safety and efficacy still need to be better defined (Sen and Dash, 2012; Ashraf *et al.*, 2017). The growing demand for intervention against NDV in developing economies presents an urgent need for stable plant extracts that can be produced at a relatively low cost and should be active against local virus variants. Such studies can provide information for the use of crude extracts that may lead to the development of cheaper, more accessible, and effective alternative approaches or novel drugs for therapeutic purposes to control ND.

Cissus quadrangularis (CQ), commonly known as 'Hadjod' (bone setter) in Hindi, is a fleshy perennial plant belonging to the Vitaceae (grape) family family. It is known by numerous vernacular names depending on the region. In Bangladesh, it is known as Hathisur; in Sri Lanka, it is known as Heeressa; in West Africa, it is known as Treebine, Zarnazaru, Banddiagra, Oongoonujaara. It can grow up to 500 metres above sea level in plain coastal areas, forests, and wastelands. It is widely available in Africa, Arabia and south–east Asia, India/Srilanka (Indran and Raj, 2015).

Cissus quadrangularis is an ancient medicinal plant that has been used in many traditional medicines. Traditionally it is used to treat diverse diseases like rheumatic diseases, allergies, skin diseases, piles, fracture, and bone diseases. The stem is very important part of the plant and used as raw drug in folk medicine. It possesses antioxidant, anti- inflammatory, antihelmintic, antifungal, antibacterial, antiviral, and antiul- cerogenic properties (Mukherjee, and Palbag, 2016). It has also solved the problems of weight loss and various metabolic syndromes. Phytochemical studies of Cissus quadrangularis have shown the presence of several phytochemical constituents such as alkaloids, flavonoids, (Bhujade *et al*., 2012; Sheikh *et al*., 2015; Mukherjee, and Palbag, 2016).

The aim of this study was to evaluate the antiviral activity of the Hadjod extract against the Newcastle virus, specifically, to assess the phytochemical constituent of the plant extract and to determine the antiviral potential of the plant extracts in embryonated eggs.

1.2 Problem Statement

Newcastle disease frequently occurs in Zambia and constitutes one of the major constraints to the development of poultry farming, since is one of the major health constraints that cause high mortality and morbidity rates which may reach up to 90 to 100% each, depending upon the virulence of the NDV and this, can affect the productivity of the village chicken system. Prevention of the disease is mainly via vaccination, however, the disease still causes severe economic losses annually, especially for those living in remote areas where conventional vaccines are inaccessible, as well as requiring cold chain conditions. Furthermore, the number of doses per package imposes limitations on its use.

1.3 Justification of the study

Newcastle disease is a global health issue, and there is no known treatment available on the market, hence the extensive use of vaccines. There is an urgent need to develop alternative antiviral products from local resource to provide cost-effective remedies. In this regard, the biological activity of Hadjod extracts as natural antiviral molecules has been described as a promising source of bioactive compounds, as it has traditionally been used to treat various diseases (Bhujade *et al.*, 2012). However, its activity against ND is yet to be studied. This study will reveal the bioactive compounds in the Hadjod plant to evaluate its antiviral potential against the NDV as an alternative therapy as it is readily available locally, easy to apply and acceptable by farmers due to their familiarity with it, the vaccine for NDV is not readily available and maintaining the cold chain is challenging in rural areas.

1.4 Study hypothesis:

The study had the following hypothesis

H_0: The plant hadjod *(C. quadrangularis)* does not have medicinal significance or antiviral activity against the Newcastle disease virus in embryonated eggs chickens.

H_a: The plant hadjod has medicinal significance and antiviral activity against the Newcastle disease virus in embryonated eggs chickens.

1.5 Objectives

1.5.1 General Objective

- To evaluate the antiviral activity of the hadjod extract against the Newcastle disease virus.

1.5.2 Specific Objectives

- To assess phytochemical constituent of the hadjod extract.
- To determine the antiviral potential of the plant extracts in embryonated eggs.

CHAPTER TWO
2.0 LITERATURE REVIEW

2.1 Historical background of Newcastle Disease

2.1.1 Etiologic of Newcastle Disease

Newcastle disease (ND) is caused by Newcastle disease virus (NDV), Avian orthoavulavirus 1, also known as avian paramyxovirus type 1 (APMV-1), and infects over 250 different bird species. It causes a wide variety of clinical diseases and has a devastating economic impact due to high morbidity and mortality rates and trade restrictions (Kapczynski *et al.,* 2013). The virus's ease of spread has allowed it to spread globally, with subjective virulence that varies depending on the virus strain, host species, dose, route of infection, age of the chicken and certain environmental conditions (Chollom *et al*., 2022).

2.1.2 The Structure of the NDV

Newcastle disease virus (NDV) is a non-segmented, single-stranded, negative-sense RNA virus that belongs to the *Paramyxoviridae* family, the *Paramyxovirinae* subfamily, and the virus genus Avula. The virus has 21 serotypes, APMV-1 to 21, and NDV is short for avian paramyxovirus type 1 (APMV-1). Each virus is a member of a virus species that are divided into three genera: metaavulaulavirus, orthoavulavirus, and paraavulavirus (World Organization for Animal Health (WOAH) (2021). Chickens are highly vulnerable to virulent NDV, which must be reported to the World Animal Health Organization. The incubation period varies depending on the virus strain, but it is typically 4–5 days (range 2–15 days) (Mubamba *et al.*, 2016). Its genome size is ~15.2 kb and contains six genes, in the 3′ to 5′ which encode for six major structural proteins arranged in the following order: nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion glycoprotein (F), hemagglutinin-neuraminidase glycoprotein (HN), and RNA-dependent RNA polymerase (L) (Glingston *et al.*, 2021). In the core of the virus particle, the NP, P, and L proteins are associated with the genome (Shahzad *et al*., 2020). In addition, the HN protein is involved in the attachment of the virions to the host-cell receptors, while the F protein mediates the fusion of the viral envelope with the host cell plasma membrane (Zhang *et al.*, 2018).

In addition, it has shown that the M protein lines the inner surface of the host-derived membrane, while F and HN are transmembrane glycoproteins that form virions' surface spike structures

(Nurzijah *et al.*, 2022). On the viral surface, approximately 8–12 nm spikes are present. The "herringbone" nucleocapsid (approximately 13–18 nm in diameter) can be seen either free or emerging from disrupted viral particles (Chollom *et al.*, 2022).

Another critical point is that the virus attachment protein, HN, binds to sialic acid-containing receptors. During virus entry, the F protein is directly responsible for the fusion of the viral and cellular membranes (Al-Hadid, 2016). The most important protein is NP, which forms the nucleocapsid helical core of NDV and causes chickens to produce antibodies. The hemagglutinin-neuraminidase (HN) protein, which is responsible for the attachment of the virus to the host cell, and the F protein, which is essential for the fusion of the virus to the host cell membrane, also play the most critical roles in virus identification and pathogenicity. F protein is essential for pathogenicity and virulence. The F and HN proteins represent the critical goals of the immune response against NDV (El-Morshidy *et al*., 2021; Hidaka *et al*., 2021).

Haemagglutinin-neuraminidase (HN) and fusion (F) glycoproteins are anchored to the surface of the virus particle envelope. Matrix (M) proteins are peripherally attached to the NDV envelope. The interior of the virus particle is composed of negative-sense single-stranded RNA, RNA-associated nucleoprotein (NP), phosphoprotein (P), and large polymerase (L) adapted from (Nurzijah *et al.*, 2022).

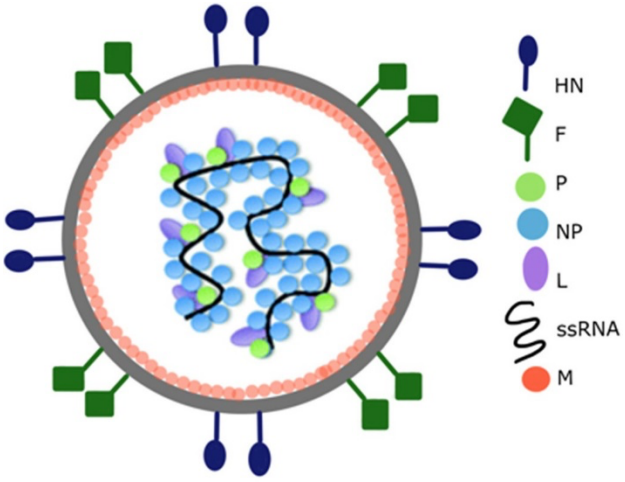

Figure 2. 1: Graphical representation of the Newcastle disease virus particle structure.

2.1.3 Classifications of APMV-1 strains

One of the most distinguishing features of different strains of APMV-1 has been their wide range of pathogenicity in chickens. APMV-1 strains have been classified into five pathotypes (or disease groups) based on clinical signs observed in infected chickens. They are classified into three pathotype groups based on their pathological index: lentogenic, mesogenic, and velogenic pathotypes (Amoia *et al.*, 2021).

Based on disease severity in chickens, NDV strains are further classified into four pathotypes:

1. Asymptomatic enteric (considered clinically non-problematic)
2. Lentogenic (causing subclinical to mild respiratory infections in younger chickens)
3. Mesogenic (causing respiratory infection with mortality rates varying according to the age of the susceptible species)
4. Velogenic (causing high morbidity and up to 100% mortality).

Velogenic viruses can be further divided into two categories: viscerotropic velogenic viruses cause acute lethal infection and occasional haemorrhagic lesions in the intestines it is (highly virulent), and neurotropic velogenic viruses cause neurological and respiratory disorders and is very highly virulent (Amoia et al., 2021; Nurzijah *et al*., 2022).

In addition, Velogenic NDV (vNDV) is a highly pathogenic strain that causes severe mortality. Neurotropic velogenic NDV (nvNDV) and viscerotropic velogenic NDV (vvNDV) strains are the two types of velogenic strains. Viscerotropic velogenic: a highly pathogenic form with frequent haemorrhagic intestinal lesions. Neurotropic velogenic: a form with a high mortality rate, usually following respiratory and nervous symptoms, and the last is the Subclinical, a form that usually consists of subclinical enteric infection (El-morshidy *et al*., 2021).

2.1.4 Epidemiology of NDV

Newcastle disease is endemic to various parts of the world (Mansour *et al*., 2021). It is a highly infectious poultry disease that severely impacts the food security and livelihoods of livestock farmers and communities throughout the world's tropical regions. Newcastle disease (ND) is the main limiting factor in rural poultry production systems. It is a persistent issue in Zambia's provinces, among the 1.5 million agricultural households in Zambia, and over 1.1 million small-scale poultry-raising households' rural poultry (Mubamba *et al.*, 2018).

The disease was first reported in native fowls in Zambia in Mazabuka, the Southern Province, in May 1952. After that, by 1957, it had spread to the country's major poultry-producing areas (Songolo and Katongo, 1952).

In addition, humans are also susceptible to NDV, which can cause conjunctivitis in individuals highly exposed to the virus for a long time. Mostly, laboratory workers and vaccine operators are infected by this virus. In humans, mild or self-limited influenza-like symptoms with fever and headache have been diagnosed (Faeji *et al.*, 2017).

Due to a large amount of virus circulating, village chickens may serve as amplification hosts, increasing the likelihood that virulent NDV will spread into commercial poultry flocks. Vaccinated chickens can also serve as a reservoir for virulent strains of NDV because they can be infected with virulent strains after vaccination and shed infectious viruses even in the absence of clinical disease (Simbizi *et al*., 2021).

Newcastle disease virus (NDV) is distributed worldwide and its continual presence in multiple avian species presents a constant threat to all poultry industries and other activities that involve the raising or keeping of poultry (Absalón *et al.*, 2019).

Village chickens are reared in Southern Africa under an extensive or scavenging system and, to a lesser extent, under a semi-intensive system under subsistence farming, with few or no inputs for housing, feeding, and health care. They play an essential role in many poor rural households by providing scarce animal protein in the form of meat and eggs, which can be sold or bartered to meet essential family needs like medicine, clothing, and school fees (Simbizi *et al*., 2021).

2.1.5 Transmission

Avian paramyxovirus 1 (APMV-1), also known as Newcastle disease virus (NDV), is transmitted via inhalation or ingestion and can remain infectious for long periods if the proper environmental conditions are met. As a result, multiple potential transmission routes and the viral agent's ability to persist in the environment may facilitate interspecies transmission and spread across domestic-peri domestic-wild bird interfaces (Dimitrov *et al.*, 2016). NDVs can invade the host through inhalation, faecal-oral transmission routes, excretion of infected poultry, and contact through virus-contaminated equipment (Qosimah *et al.*, 2018; Alemneh, 2019). NDV can be transmitted to healthy birds through oropharyngeal secretions and faecal matter. Susceptible birds can be

infected by inhaling contaminated dust or aerosolised virus or ingesting the virus shed in bird droppings (Nurzijah *et al.*, 2022).

Additionally, the movement of poultry and poultry products, contaminated feed, clothing and footgear of humans, crates, feed sacks and vehicles may disseminate the infection over long distances, making the disease maintain spread (Samad *et al.*, 2022).

2.1.6 Clinical signs

The clinical signs of ND are classified as reproductive, respiratory, nervous, and enteric. Clinical signs of reproductive organ infection include a decrease in egg number, deformed eggs, rough or rough-shelled eggs, and a decrease in albumin quality. After three-four weeks, egg production may return to normal. Also, light sneezing and gasping for air are symptoms of a respiratory infection. Sneezing, coughing, nasal discharge, and difficulty breathing with relaxed breathing are more severe symptoms. A rattling noise may accompany inhalation. The breathing airways are formed by shaking the head with displaced mucus, and mucopurulent conjunctivitis may sometimes appear (Amoia *et al.*, 2021)

In addition, tremors, paralysis of the wings and legs, and twisting and circling of the neck are all symptoms of a nervous infection (Alemneh, 2019). In critical cases, death occurs suddenly and without any warning signs. The enteric infection is accompanied by greenish diarrhoea. The characteristic lesions at post-mortem may include haemorrhages in the trachea, brain, and spleen. Petechial haemorrhages, ulcers with raised borders on the mucosa of the proventriculus, caecal tonsils, and inflamed lungs are also symptoms of the disease (Mubamba *et al.*, 2016).

2.1.7 Control measures

Newcastle Disease Virus remains a constant threat to the poultry industry worldwide due to the virulent strains' ability to cause high mortality. Vaccination against NDV protects poultry from virulent virus strains (Al-Hadid, 2016). However, the problems stem from backyard-reared chicken infections, typically unvaccinated but still prevalent, allowing the virus to spread and eventually causing a community outbreak (Yasmin *et al.*, 2019).

In Zambia, medicines are administered to poultry through drinking water. Traditional methods include the following plants (in general, leaves and stalks are added to drinking water): *Agave sisalana, Aloe species, Apodytes dimidiata, Cassia obtusifolia, Cissus quadrangularis, Capanifera*

baumiana, Diplorhynchus condyocarpon, Droogmansia pteropus, Swartzia madagascariensis, Euphorbia tiru. The roots of *Droogmansia pteropus* and the bark of *Swartzia madagascariensis*, rather than the leaves, there are the two main exceptions. There has been no research into the efficacy of these remedies.

2.1.8 Pathogenicity Test of the NDV isolates

The NDV isolates are differentiated based on an *in vivo* estimate of pathogenicity. These *in vivo* tests are the meantime of death (the mean time in hours for the minimum lethal dose to kill all the inoculated embryos) mean death time [MDT]) in specific pathogen-free (SPF) embryonated chicken eggs, the intracerebral pathogenicity index (ICPI) in SPF day-old chicks, and the intravenous pathogenicity index (IVPI) in SPF 6-week-old chicks (Hidaka *et al*., 2021).

Using MDT, NDV strains are classified into the following groups: Velogenic (causes death in <60 h), mesogenic (causes death in 60-90 h), and lentogenic (causes death in more than 90 h) (Alemneh, 2019; Amoia *et al*., 2021; Hidaka *et al*., 2021).

The intracerebral pathogenicity index (ICPI) involves the inoculation of a virus derived from the fresh infective allantoic fluid into the brain of ten-day-old chicks from specific pathogen-free parents. The Intracerebral Pathogenicity Index (ICPI) is calculated after intracerebral infection of chicks at the age of one day, and a score (0: typical; 1: sick; 2: dead) is assigned to each chick daily for eight days. Each chick is examined at 24-hour intervals for eight days and graded zero if standard and one if sick, and two if dead. The index is the mean score per chicken observation over the eight days. The most virulent viruses give ICPI values approaching the maximum score of 2.0, while lentogenic viruses give values of, or close to, 0.0. This means the ICPI classifies NDV strains by giving indices ranging from 0.0 to 2.0. The maximum score of 2.0 is assigned to the most virulent strain of NDV, whereas scores close to 0.0 are assigned to lentogenic strains (Alemneh, 2019; Amoia *et al.*, 2021)

Lastly is the intravenous pathogenicity index (IVPI) in SPF 6-week-old chicks, which classifies NDV strains as lentogenic and velogenic. Slow-growing and some mesogenic strains have IVPI values of 0.0, whereas the maximum IVPI value for a virulent strain is 3.0. The Intravenous Pathogenicity Index (IVPI) is calculated similarly to the ICPI in poultry infected intravenously at six weeks of age (Amoia *et al*., 2021).

2.2 Historical background of *Cissus quadrangularis* L. (CQL)

Cissus is a genus of the Vitaceae family with 800 species divided into 13 genera spread across the globe, including Africa, Arabia, South Asia, Sri Lanka, India, and other tropical regions (Bafna *et al.*, 2021). In English, it is called an Edible-stemmed Vine, and in the Tonga language of the Southern province of Zambia, it is known as 'Namunengenenge'. Many other common names exist in different localities in the world such as "Hadjad" plant used in India.

2.2.1 Ethnobotanical information

Cissus quadrangularis L. (CQL), Vitaceae, is a dicotyledonous flowering, sprawling perennial herb characterized by a thick quadrangular fleshy twig of the grape family (Balasubramanian *et al.*, 2009). The herb is propagated using stem cuttings. The herb bears flowers from June-December (Austin, 2020).

It is a climbing herb, tendrils simple and found leafless when it ages. It is a succulent shrubby climber that reaches a height of up to 1.5 m. Leaves are 2.5–5 cm long and ±5 cm wide, sometimes 3–7 lobed, broadly ovate or kidney-shaped, dentate, serrate, rounded at the base, and glabrous. Flowers are small, bisexual, tetramerous in umbellate cymes and greenish-white in colour. The calyx is short, deciduous, and cup-shaped. Petals are 4–5 and fruits are globose or obovoid berries, very acrid, succulent, and one-seeded; seeds are ellipsoid or pyriform and found to be dark purple to black. The stem is dichotomously branched, glabrous, subangular, smooth, and fibrous and found to be buff-coloured with a greenish tinge. These stems are roughly 8–10 cm long, and 1–1.5 cm wide and are joined at nodes and internodes (Bafna *et al*., 2021).

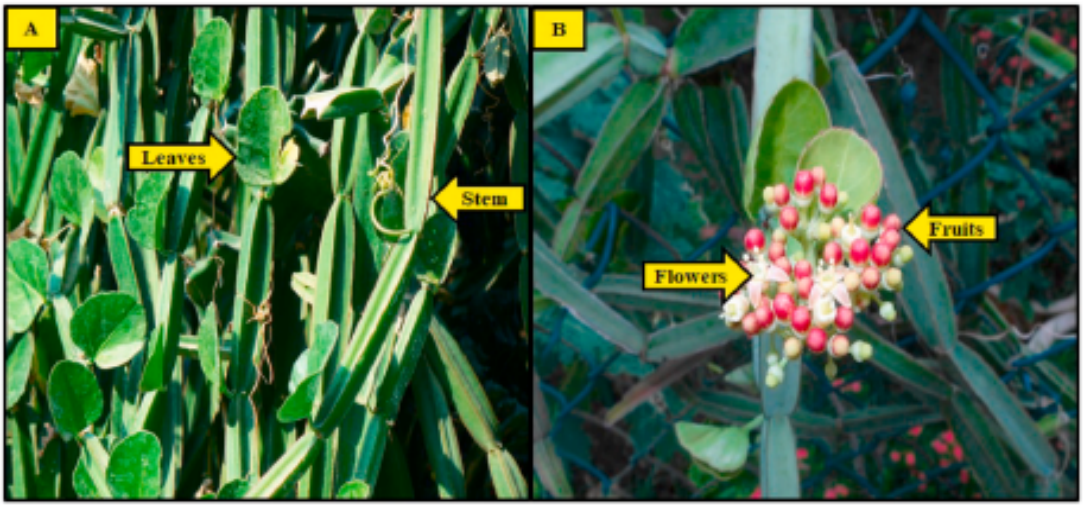

Figure 2.2: Photograph of *Cissus quadrangularis* L (A) leaves and stem; (B) flowers and fruits.

2.2.2 Ethnopharmacological relevance

The herb is used as a whole, especially the stem, root, and leaves. Extracts of *Cissus quadrangularis* L. (CQL) have been assessed for various preclinical as well as clinical pharmacological activities to prove its ancient folk claims such as analgesic, antipyretic, antiulcer, estrogenic, anticancer, anti-inflammatory, ant obesity, antidiabetic, antiarthritic, antiosteoporotic, antimicrobial, antiviral activity and other biological activities (Bafna *et al.*, 2021). Some studies reported the use of hadjod against some virus-like herpes simplex virus (HSV), feline immunodeficiency virus, coxsackievirus, influenza virus, parainfluenza virus, respiratory syncytial virus, and others (Balasubramanian *et al.*, 2009). Use of plant products to treat poultry diseases in African rural poultry production (Gueye, 1999). Many plant products have been reported to be in use in Africa's various regions and countries for the treatment of poultry diseases. These plant products are locally available, cheap, or free(Hartady *et al.*, 2021) .

Preparing medicinal plants for experimental purposes is an essential first step in achieving a high-quality research outcome. Before proceeding with the intended biological testing, it is necessary to extract and determine the quality of bioactive secondary metabolites (Faeji *et al.*, 2017). Further, understanding the mechanism of action of these active phytochemicals as antiviral agents would help elucidate the pathogenesis of the disease.

2.3 Solvents of extraction

The solvent used is determined by the type of plant, the part of the plant to be extracted, the nature of the bioactive compounds, and the availability of the solvent. In general, polar solvents such as water, methanol (MeOH), and ethanol (EtOH) are used in polar compound extraction, whereas nonpolar solvents such as hexane and dichloromethane are used in nonpolar compound extraction (Poojar *et al.*, 2017).

The polarity of the solvents used in extraction is classed, ranging from n-hexane, which is the least polar, to water, which is the most polar. Because the target compounds might be non-polar to polar and thermally labile, the applicability of the extraction procedures must be addressed (Abubakar and Haque, 2020).

2.3.1 Factors to consider when selecting solvents of extraction

When selecting an extraction solvent, several factors should be considered, as listed: (i) Selectivity, which refers to a solvent's ability to extract the active constituent while leaving the inert material behind; (ii) Safety, where the ideal extraction solvent should be nontoxic and non-flammable; (iii) Price which should be as low as possible; (iv) Reactivity, where the extraction solvent should not react with the extract; (v) Recovery, in which the extraction solvent should be recovered and separated from the extract as soon as possible; (vi) Viscosity should be low to allow easy penetration; (vii) Boiling temperature, where the solvent's boiling temperature should be as low as possible to prevent heat degradation (Poojar *et al.*, 2017).

2.3.2 Antiviral Effects of Flavonoids and their anti-viral mode of action

Flavonoids are widely distributed secondary metabolites produced by plants that play essential roles in plant physiology. They have many potential biological benefits, including antioxidant, anti-inflammatory, anticancer, antibacterial, antifungal, and antiviral activity (Zakaryan *et al*., 2017).

The flavonoid class of phytochemicals contains a large number of medicinally active compounds, many of which are being investigated for antiviral activity against various DNA and RNA viruses, and several of them have shown significant antiviral activity in *in vitro* and even *in vivo* studies (Zakaryan *et al*., 2017; Badshah *et al*., 2021).

Flavonoids are natural phytochemicals with antiviral properties. The flavonoids act at various stages of viral infection, including viral entry, replication, and protein translation (Badshah *et al*., 2021).

Because of the numerous health benefits reported in various epidemiological studies, there has been a surge in interest in studying plant flavonoids. Flavonoids are a group of natural compounds with variable phenolic structures and are found in plants. They are hydroxylated phenolic substances that plants produce in response to microbial infection. Among them are Flavones (e.g., flavone, apigenin, and luteolin), flavanols (e.g., quercetin, kaempferol, rutin, myricetin, and fisetin), flavanones (e.g., flavanone, hesperetin, and naringenin), Isoflavone (e.g., epigallocatechin gallate, genistein, glycitein, daidzein, puerarin, ononin), Anthocyanidin (e.g., cyanidin, peonidin, apigenidin) (Kumar and Pandey, 2013; Ninfali *et al*., 2020; Badshah *et al*., 2021).

There are several mechanisms by which flavonoid phytochemicals inhibit and act on viruses. They can obstruct virus attachment and entry into cells and interfere with various stages of viral DNA replication, protein translation, and poly-protein processing. They can also prevent viruses from being released and invading other healthy host cells (Badshah *et al*., 2021).

Another critical point is that flavonoids can attach and bind themselves to the surface proteins of viruses, preventing them from entering host cells. Some flavonoids inhibit the replication process by acting as transcription blockers, while others impede the late stages of viral assembly, packaging, and release. It can also influence the immune system and lower viral load (Badshah *et al*., 2021).

The use of medicinal plant extracts as antiviral agents against NDV in chicken embryonic systems is one such potential strategy. Moreover, this study evaluated the antiviral activity of the crude EtOH *Cissus quadrangularus* medicinal plants on the inhibition of NDV replication within SPF embryonated eggs (Al-Hadid, 2016).

Previous research has shown that virus inactivation can occur when a potent antiviral candidate is incubated with the virus under controlled conditions (Temitayo *et al*., 2020).

2.3.4 Antiviral Effects of Tannins and their antiviral mode of action

Tannins are a naturally occurring heterogeneous group of phenolic compounds with diverse structures known for their ability to bind and precipitate proteins. They are divided into three categories: hydrolysable tannins (HT), condensed tannins (CT), also known as proanthocyanins, and phlorotannin's (PT). The first two groups are found in terrestrial plants, whereas PT is only found in marine brown algae (Huang *et al.*, 2018). Furthermore, tannins' activity is primarily attributed to their ability to bind to viral structural or non-structural proteins (capsid or viral enzymes) required for viral replication or structural proteins involved in viral particle formation.

Studies have reported that plant extracts with tannins as the main component have shown promising results against the replication of various viruses. Both coated viruses (influenza viruses A/H3N2 and A/H5N3, herpes simplex virus type 1 (HSV-1), vesicular stomatitis virus, Sendai virus, and Newcastle disease viruses) and non-enveloped viruses (poliovirus, coxsackievirus, adenovirus, rotavirus, feline calicivirus, and mouse norovirus) have been affected (Vilhelmova-Ilieva, *et al*., 2020).

Tannin is found in high concentrations and has significant biological activities against various stages of viral replication, including extracellular virions, cell attachment, penetration into the cell, and the replication process in the host cell, as well as the assembly of new viral particles, transport proteins, polysaccharides, and viral enzymes (Vilhelmova-Ilieva, *et al*., 2020)

2.3.5 Antiviral activities of alkaloids and their antiviral mode of action

Alkaloids are secondary metabolite of plants that have a nitrogen atom in their ring structure. In addition, they are natural phytochemicals known for their biological activities, such as their broad spectrum of antiviral activities, antioxidant properties, scavenging abilities, DNA and RNA synthesis inhibition, and viral replication blockage. (Abookleesh *et al*., 2022)

Overall, alkaloids' molecular influence suggests a high specificity, implying that they could serve as potent and safe antiviral agents awaiting evaluation and exploitation (Swamy, 2020; Abookleesh *et al*., 2022)

2.4.0 Embryonated eggs

The term embryonated egg identifies any eggs in which an embryo is developing. It is common to refer to fertile eggs which have been incubated as being 3,5,10 days etc. This does not refer to the time exposed since the egg was laid, but to the time it has been incubated.

Moreover, certain early primitive developmental stages occur during fertilization while the shell wall forms around egg contents. This development does not continue after birds lay eggs. Newcastle disease virus (NDV) is a parasite that lives only inside cells. Only living cells can support viruses. The embryonated chicken eggs (ECEs) are one of the virus growth media that is inexpensive, simple, and accurate for detecting patterns of virus change in the host (Chinnappan, 2020).

Furthermore, embryonated chicken eggs have been used extensively in various scientific disciplines such as embryology, immunology, and virology for many decades. The embryo, particularly its extraembryonic structures, is used as a medium for virus replication in virology. Based on the former, inoculation of the embryonated egg serves a variety of purposes, including field virus isolation and propagation, preparation of attenuated vaccine virus from field virus by successive passages in chicken embryos, production of human and avian vaccine virus, titration of

virus stocks, assessment of virus virulence by mean death time, and induction of immunity by *in ovo* vaccination (Manders *et al*., 2021).

Before inoculating embryonated eggs, a few questions must be answered: which egg compartment should be inoculated/sampled, when should the needle be inserted, and how should the needle be inserted. The virus species determine the embryonic compartment to be inoculated, and inoculations are typically performed between days 5 and 12 of incubation (Manders *et al.*, 2021).

2.4.1 Viability of the embryo

Shining a light through the egg to observe embryo development is called candling. Candling the egg is the first step, where the blood vessels are examined closely. In a healthy embryo, they are well-defined. After an embryo dies, the blood vessels begin to deteriorate (Manders *et al.*, 2021). They appear as streaks under the shell when viewed through the candling lamp. Candling will reveal cracks in the eggshells as well. Eggs with cracked shells should be thrown away. Viable embryos move in response to light and have well-defined blood vessels. Candling reveals a small dark area as well as disrupted blood vessels. Deteriorating blood vessels usually show as a dark ring around the egg and are automatically discarded (Manders *et al.*, 2021; Tainika and Bayraktar, 2021).

2.4.2 Use of non-SPF (specific pathogen free) embryonated eggs

SPF eggs are used for the production of vaccines in veterinary medicine and also for the production of vaccines in human medicine. The chickens are bred under a strictly contained atmosphere and controlled periodically regarding the lack of virus infection according to relevant guidelines (Gagnieur *et al.*, 2014). Such an egg acts as a natural incubator because the embryo becomes infected with a specific strain of the virus, which will multiply there within a minimum of two days.

However, embryonated eggs from healthy chicken flocks are used in developing nations when SPF flocks are not readily available (Adamu and Abah, 2015)

Eggs from flocks vaccinated with the Newcastle disease vaccine can be used to grow the Newcastle disease virus. Eggs from these flocks will contain varying levels of antibodies in their yolks. These antibodies are derived from the hen. As the embryos develop, maternal antibodies enter the blood. Virulent strains of the Newcastle disease virus might not kill the embryos if there are high

antibodies. However, the virus will grow in the cells lining the allantoic cavity, where there are no antibodies and is then shed into the allantoic fluid. The haemagglutination test will detect the presence of the Newcastle disease virus in the allantoic fluid.

2.4.3 Inoculation of embryonated eggs by the allantoic cavity route

The most convenient method of propagating the Newcastle disease virus in the laboratory is inoculating the allantoic cavity of embryonated eggs. All strains of the Newcastle disease virus will grow in the cells lining the allantoic cavity. The virus enters these cells, where it multiplies. The virus is shed into the allantoic fluid when the cells are disrupted. Virulent strains of the virus will invade cells beyond the lining of the allantoic cavity and kill the embryo.

2.5 Hemagglutination Assay for the Newcastle disease virus

The ability of virus particles to bind to sialic acid-containing molecules on the surfaces of red blood cells is measured by hemagglutination essay. When a virus binds to RBCs, the RBCs form a lattice-like structure that coats the bottom and sides of a tube. On the other hand, RBCs that are not bound to the virus fall to the bottom of a tube, forming a very tight round button (Young *et al*., 2002).

Titration is used in the quantitative haemagglutination test to determine the amount of virus in a sample (for example, vaccine or allantoic fluid). The strength of a virus suspension is determined in titration by the amount it can be diluted before losing virus activity (in this test, haemagglutination). The quantitative haemagglutination test is also used to quantify and standardize the amount of haemagglutinin used as an antigen in the Haemagglutination Inhibition (HI) test. Twofold serial dilutions of the sample are made, and the number of haemagglutinating units in the sample is measured (Young *et al*., 2002).

CHAPTER THREE
3.0 MATERIALS AND METHODS

3.1 Research Design

This study was an experimental design in which infection-free nine-day-old embryonated chicken eggs (ECE) were used. The experiment (appendix E) was divided into groups of ten (10) treatments with each having 20 eggs. The ECE treatments groups (T1-T4) were punched and inoculated with the crude EtOH extract (at different concentrations), isolate NDV- AF2 and 5% Dimethyl sulphoxide (5% DMSO) through the allantoic route. The total volume applied in each inoculated egg in the experiment was 500 µL of which 100 µL was the isolate NDV- AF2. The ECE groups (T5) were used as positive control group whole (T6-T10) negative control. The specimen-treated embryos were then compared and contrasted with the control sets on survival and HA titre.

Initially, an egg-based cytotoxicity assay was carried out (appendix D) on varied concentrations of crude EtOH extract to determine whether it was toxic to the eggs. The 5% DMSO was used as negative control. In cases where embryo death occurred within 24 hours post-inoculation, it was considered non-specific and was not included in the study. These embryos were dropped from the study because mortality within 24 hours could have been caused by mechanical injury.

3.2 Study Area

3.2.1 Source of the plant and identification

The stem of *C. quadrangularis* L. (CQL) was collected on 15th November 2021 in Munenga village of Mazabuka District of Chief Mwanachingwala in Southern Province, Zambia. Munenga village is located at 15° 51' 0" South, 27° 34' 0" East. The morphological features of the plant were identified and collected in the field using standard descriptions and plant identification keys. Detailed species identification was done at the Department of Biological Sciences Herbarium, School of Natural Sciences, University of Zambia.

3.2.2 Source of Eggs and NDV

All commercials (non-SPF) white-shelled embryonated eggs were sourced from a local commercial hatchery flock (Ross Breeder Zambia Ltd, Chongwe). A Velogenic NDV (vNDV) isolate AF2 was obtained from UNZA- Department of Disease Control at the School of Veterinary Medicine.

3.3 Data collection procedure

3.3.1 Plant collection and storage

The collection of Hadjod (*C. quadrangularis*) plant was conducted by the botanist (UNZA – School of Biology and Natural Science) in the field and was based on morphological characteristics. Approximately, 1 Kg of fresh aerial parts (stems) from the plant was collected, transported and stored in polyethylene bags until further processing. A total of 800 g via maceration method was used for preparation, extraction and testing at Food and Drug laboratory, University Teaching Hospital at the Ministry of Health Zambia. The remaining 200 g was kept at the Museum (UNZA - Department of biological sciences) for the voucher preservation accession number: **UZL 22391** (Appendix A).

3.3.2 Assessing Phytochemical Constituent of the Hadjod Extract

3.3.2.1 Extraction of the EtOH crude extract

For the maceration process (solid-liquid extraction), the stems were cleaned using distilled water, ground and absolute EtOH solvent was added at a ratio of 1:2 (w/v). The suspension was incubated at room temperature for 72 hours, with gentle shaking twice a day. The plant suspension was filtered using Whatman paper (No. 1). The EtOH was removed by placing filtrate in oven at 37 °C for 5 days. Seven grams of crude EtOH extract was harvested as a dark green gelatinous material. A 10% w/v working solutions was prepared by dissolving 5 g crude EtOH extract in 50 mL of 5% DMSO. The extract was stored in airtight bottles at 4°C until use.

3.4 Phytochemical screening of secondary metabolites

Phytochemical tests were performed to determine the class of compounds that could be responsible for the activity and cytotoxicity in the active fractions. These were identified by distinctive colour changes based on previously described standard procedures (Jayaramu *et al.,* 2016). The screening criteria was denoted by (+) for presence and (-) for absence of the substances.

3.4.1 Qualitative Analysis

A standard protocol (Jayaramu *et al*., 2016), was used for the qualitative analysis of samples for alkaloids, cardiac glycosides, flavonoids, phenols, saponins, tannins and terpenoids.

3.4.1.1 Alkaloid

About 1 mL of extract was mixed with 1 mL of marquis reagent, 2 mL of concentrated sulphuric acid, and a few drops of 40% formaldehyde. This forms a dark orange to purple colour indicates the presence of alkaloids.

3.4.1.2 Cardiac glycosides

About 0.5 mL of glacial acetic acid and three drops of 1% aqueous ferric chloride solution was added to 1 mL of extract; the mixture forms a brown ring at the interface that indicates the presence of cardiac glycosides in the sample extract.

3.4.1.3 Terpenoids

A mixture of 1 mL of extract was obtained and 0.5 mL of chloroform was added, followed by adding a few drops of concentrated sulphuric acid after five minutes, reddish-brown precipitate indicates the presence of terpenoids in the sample extract.

3.4.1.4 Flavonoid

A total of 2 mL of extract was mixed with a few drops of 20% sodium hydroxide, and the product formed intense yellow colour. A few drops of 70% dilute hydrochloric acid were added to the mixture, which caused the yellow colour to disappear, indicating the presence of flavonoids in the sample extract.

3.4.1.5 Phenol

A total of 2 mL of 5 % aqueous ferric chloride was mixed with 2 mL of extract, formed a blue colour to indicate the presence of phenols in the sample extract.

3.4.1.6 Saponin

A total of 6 mL of distilled water was mixed with 2 mL of extract and vigorously mixed, which formed bubbles or persistent foam, indicating the presence of saponins.

3.4.1.7 Tannin

To 2 mL of extract, 10% alcoholic ferric chloride was added which formed a brownish blue or black colour to indicate the presence of tannins.

3.5. Determination of the antiviral Potential of the Plant Extracts in Embryonated Eggs

3.5 .1 Candling eggs

Candling was performed according to standard protocol of Young *et al*., (2002). This procedure was carried out before inoculation and repeated daily for 96 hours to check the viability of the embryo.

3.5.2 Extract Stock solutions

Stock solutions were prepared by dissolving the extract in 5% Dimethyl sulphoxide (DMSO) solution. Different experimental concentrations of plant extracts were prepared to estimate its antiviral efficacy (Shahzad *et al.*, 2019). The stock solutions containing the test crude EtOH extract were prepared by dissolving the stock solution (extract) at 10% (5g/50mL). Briefly, 5g plant extract and 50mL in DMSO at 5%; 5g/50 mL = 100 mg/ mL = Volume 1 and the followed formula $C_1V_1=C_2V_2$ was used to calculate the total volume (500 µL) which was equal to the stock solution (extract) + DMSO 5% + virus. The Final Concentration (C_2) used was 75, 12.5, 1.95, 0.196 mg/ mL, and for the virus was 100 µL. The final DMSO concentration used *in ovo* was 5%.

3.5.3 Detection of cytotoxicity of tested EtOH crude extracts on chicken embryos

A total of one hundred and twenty (120) embryonated chicken eggs (Appendix B) were inoculated with crude extract and incubated for 4 days at 37°C with 60% humidity. Candling was carried out every 24 hours. A count of dead and live embryos at each concentration was recorded.

3.5.4 Pathogenicity index test

A Standard pathogenicity index test (SPIT) was conducted to evaluate the virulence of the virus using the *in vivo* test, the meantime of death (the mean time in hours for the minimum lethal dose to kill all the inoculated embryos) in infection-free 9-day-old chicken embryonated eggs. The highest dilution in which all embryos died was calculated as the mean lethal dose (Qosimah *et al.*, 2018).

3.5.5 Determination of median embryo infectious dose (EID50) of the virus

The fifty per cent embryo infectious dose (EID_{50}) of the virus was determined using the method of Young *et al*., (2002) and (Shahar *et al*., 2018).

Tenfold viral dilutions of 100 uL EID_{50}/0.1 mL of the virus stock were inoculated into the allantoic fluid of 9-day-old embryonated eggs. The eggs were incubated at 37 °C and 60% humidity for 5 days. The embryo mortality was recorded. Allantoic fluid was collected and tested by HA.

3.5.6 Inoculation of embryonated eggs by the allantoic cavity route

Inoculation was performed according to the (Chollom *et al*., 2022) protocol with minor modifications. The eggs were candled before and after inoculations. To avoid contamination, sets of plastic egg crates/trays were thoroughly disinfected with Virkon, and embryonated chicken eggs were thoroughly swabbed with 70% alcohol before being transferred into the disinfected trays and transferred into cleaned trays in biosafety cabinet II. The broader ends of eggs were drilled with sterile needle and inoculated with different concentrations of the crude EtOH extract with the specimen, through the allantoic route. Before inoculation, the specimen was vortexed to allow them to equilibrate. After inoculation, the hole was sealed with glue and eggs were incubated at 37°C and 65-70% humidity. The eggs were candled every 24 hours to monitor embryonic development and record mortality and survival. Eggs with dead embryos within 24 hours of injection were discarded, but the allantoic was harvested. The eggs were harvested 96 hours post-inoculation (PI) and allantoic fluids were collected and subjected to HA test to check titre of each virus. This whole procedure was done in biosafety cabinet type II.

3.6 Viral Hemagglutination (HA) test

A standard hemagglutination test was performed on the harvested allantoic fluid to calculate NDV HA titre for each sample. For the NDV assay, the allantoic fluid from the sample-treated specimens and control eggs were harvested to assess the virus for the Hemagglutination assay. From harvested allantoic 50 µL, a two-fold serial dilution was performed from the mixture of 50 µL of PBS per well of a V-bottomed 96-well plastic microplates. Then, to each well 50 µL of 0.75% RBC (freshly collected from chicken) was added, including the control (CC) wells, and mixed gently. The plate was placed on a shaker for 1 minute at a low speed to ensure thorough mixing. Thereafter, the plates were incubated at 4 °C for 30 to 60 minutes.

3.7 Data analysis instruments and procedures

Data were entered into MS-excel and analysed using R programming to draw contingency tables and for graphical analysis. Descriptive statistic was used to generate tables and multiple bar charts. To explore association between exposure to the crude extract and outcome variable (chick embryo mortality) a Fisher's exact test) at a $p \leq 0.05$ was used and Student's t-test was used to compare the HA mean differences between treatment and control at $p < 0.05$

3.8. Ethical Consideration

Ethical approval for this study was obtained from ERES CONVERGE IRB Ethics Committee (Ref. **No: 2022-Jun-013**) (Appendix B) concerns the intrinsic and extrinsic ethical principles of the sensitivity of the chicken embryo, plant collection, extraction, and safety in the laboratory.

CHAPTER FOUR

4.0 RESULTS

4.1 Assessment of the phytochemical secondary metabolites of the Hadjod extract.

4.1.1 Qualitative phytochemical analysis of the plant extracts

The phytochemical screening of secondary metabolites of *C. quadrangularus* EtOH crude extracts (Table 1) showed a highly positive result of the presence of compounds. This was carried out to determine the presence of seven classes of the secondary metabolites, (alkaloids, cardiac glycosides, terpenoids, flavonoids, phenols, saponins, and tannins) in the plant extracts. Of the seven phytochemical secondary metabolites screened from the crude extract, four showed a high presence (Table 1). Among the remaining three metabolites, one was moderately present and one was present in lower quantities while the third was not be detected (Table 1).

Table 4. 1: Outcome of Phytochemistry analysis of secondary metabolites of Hadjod Extract

Alkaloids	Cardiac glycosides	Terpenoids	Flavonoids	Phenols	Saponins	Tannins
+++	+	-	+++	+++	+++	++

Where: (+++) indicates high presence, (++) moderate presence, (+) (low presence), and (-) not detected (Jayaramu *et al*., 2016).

4.2: Cytotoxicity Assay from the EtOH crude extract on Embryonated Chicken Eggs

There was no toxicity observed in all the concentrations of the crude extracts used at 48-, 72-, and 96-hours post inoculation (Table 4. 2).

Table 4. 2:Cytotoxicity Assay from the EtOH crude extract on Embryonated Chicken Eggs

Concentration of Crude Extract	Number of eggs inoculated	Mortality recorded (Hours)		
		48	72	96
100 mg/mL	20	-	-	-
50 mg/mL	20	-	-	-
12.5 mg/mL	20	-	-	-
1.56 mg/mL	20	-	-	-
Control	20	-	-	-

Key: Control = Eggs not inoculated with extract

- = No death recorded

4.2 The antiviral potential of the *Cissus quadrangularis* crude EtOH extract in embryonated chicken eggs.

4.2.1. Determination of Protective Effect of *Cissus quadrangularis* crude EtOH extract in embryonated eggs.

The protective effect of the crude extract was determined using a contingency table (Appendix F) for mortality rate of the embryo from the highest extract concentration (75mg/mL) (the positive factor) and the virus only (the negative factor). The highest chick embryo mortality (73.7%) was observed in the control group (virus only) and low mortality (11%) in the high extract concentration (75 mg/mL). The risk of death among those embryos exposed to the high concentration of the extract compared to death in the control group was 0.158 indicating a protective effect.

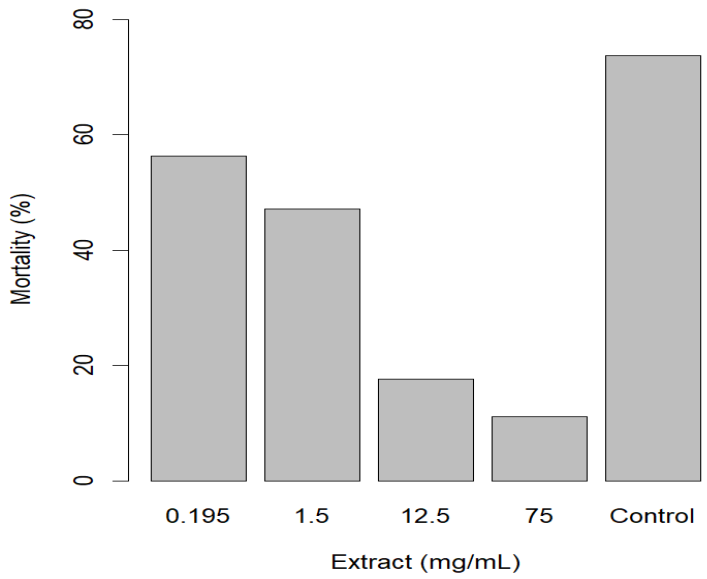

Figure 4.1: The relationship between the treatment/ risk factor and outcomes (mortality rates).

Table 4. 3:Risk of death in the exposed compared to death in the control at different concentration of exposure

Concentration of *Cissus quadrangularis* crude EtOH (mg/mL)	Mortality (risk)	Relative risk
0.195	0.5625	0.763
1.5	0.471	0.637
12.5	0.176	0.239
75	0.111	0.1506
Control (Virus only)	0.737	-

When the relationship between mortality (outcome) and exposure to the crude extract was tested, we observed that there was an association between exposure to the extract and chick embryo

mortality (**P=00026**). Hence, we reject the null hypothesis and conclude that there is an association between the mortality rate and the extract application; where by embryos exposed to the extract were less likely to die.

A quantitative assessment of the effects of crude revealed that the highest concentration of EtOH crude extracts caused a reduction in the replication of the virus, but the degree of inhibition varied with extract. At 75 mg/mL, the maximum range of the virus titre was 64, indicating a decline in the value of the HA titre. Meanwhile, the last virus titre concentration, 0.195 mg/mL, demonstrated no reduction in virus replication on allantoic fluid harvests and is precisely equivalent to the NDV-control range.

Table 4. 4: Mean, mode, standard deviation, range, and sum across HA titres level

Variable	Mean (HA titres)	Mode (HA titres)	STD. Deviation (HA titres)	Range (HA titres)
HA titre of T1	60.44	64	10.35	32-64
HA titre of T2	92.00	128	38.53	32-128
HA titre of T3	101.65	128	37.92	32-128
HA titre of T4	242.00	256	187.31	32-512
HA titre of Control	328.42	512	178.76	32-512

Key: T1-4 = Treatment

Table 4.6 shows the mean HA titres results of NDV-infected chicken embryos after treatment with various concentrations of crude EtOH extract. All the concentrations of the extract tested significantly lower HA titres in a dose-dependent manner. The decrease in viral titre was statistically significant ($p<0.05$) in treatment 1 to 3 compared to the virus control, indicating the reduction of virus replication. In contrast, there was a non-significant statistical difference ($p>0.05$) in treatment 4 (at a lower dose). There was no reduction of the haemagglutination titres to confirm the efficacy of the antiviral activity. Furthermore, the antiviral assay revealed that the crude EtOH extracts have statistically significant antiviral potential between the treatments compared to the control group on reducing the HA titre against the Newcastle disease virus (NDV) infection in the ovo model.

Table 4. 5: Mean differences between sample HA titres and Control HA titres

Variable	Mean HA titre	Control HA titre	*p*-value
Treatment 1	60.44	328.42	0.001
Treatment 2	92.00	328.42	0.001
Treatment 3	101.65	328.42	0.001
Treatment 4	242.00	328.42	0.086

CHAPTER FIVE

5.0 DISCUSSION

In this study, phytochemicals of the *Cissus quadrangularis* crude EtOH extract were screened and revealed the presence of general secondary metabolites such as alkaloids, cardiac glycosides, terpenoids, flavonoids, phenols, saponins, and tannins. It is well known that such compounds have antiviral properties (Youyi *et al.*, 2022). This finding is similar to a study by Swamy (2020) reporting the presence of alkaloids, cardiac glycosides, terpenoids, flavonoids, phenols, saponins, and tannins from crude extracts of *C. Swynnertonii* and the plant inhibited NDV multiplication *in ovo*.

In the present study, most phytochemical substance classes had shown high presence, except the cardiac glycosides and terpenoids class. This could be attributed to the site and season of collection as the presence of active plant secondary metabolites is dependent on the geographical distribution, collection season, and the climatic and ecological conditions of the collection site *(Rezatofighi et al*, 2014).

The extract was safe for the embryo at the concentration studied since no egg died from subjected concentration during the 96 hours of the experiment. The diluent DMSO of 5% and the zero concentration were safe for the embryo as no mortality was recorded. It was observed that the maximum concentration of extract, in comparison with the controls, was safe for the embryo as the mortality rate was not recorded. In eggs inoculated by an experienced operator, up to 2% mortality is considered normal (Young, *et al*., 2002). The interpretation of this outcome is that the fact that embryonated chicken eggs tolerated the extract in a dose-dependent manner is consistent with biological cells' physiological disposition. When exposed to biochemical products, cells have varying degrees of tolerance (Chollom *et al*., 2022).

In this study, *in ovo* injection method was used to determine the antiviral potential since *in vitro* (using cells) and *in vivo* (chickens) is time-consuming and expensive (Rezatofighi *et al*., 2014). It was found that the extract had antiviral activity against NDV at various concentrations. A plant extract is said to have antiviral activity against NDV if it can inhibit viral replication by allowing chicken embryo growth or reducing viral titre, preventing embryonic death and viral growth (Temitayo *et al.*, 2020). These findings agree with previous research that has shown active plant chemicals such as tannins, flavonoids, and terpenes to have antiviral properties.

Previous research has shown that virus inactivation can occur when a potent antiviral candidate is incubated with the virus under controlled conditions. This study supports the findings of (Temitayo *et al.*, 2020), who found that extracts of *Commiphora swynnertonii* and *Phyllanthus amarus*, respectively, had prophylactic and therapeutic activities against Newcastle disease. It is very likely that the extract inhibits viral receptocytes and also interferes with the neuraminidase-haemagglutinin sites required for virion attachment and penetration into living cells.

Although the specific chemical constituent of *Cissus quadrangularis* mediating antiviral activity was not identified in this study, it is a topic for future research. Furthermore, the minimum concentrations of the extract and fractions inhibiting virus replication did not inhibit hemagglutination formation. However, because virus titres did not increase over time in a viable embryo, further virus replication was observed to be impaired with these concentrations.

The Hemagglutination (HA) test data, which quantified the amount of virus in the allantoic fluid of eggs, were used to demonstrate the antiviral activity of the extract further. The highly significant reductions in virus populations in embryos treated with the extract, which were also dose-dependent, suggested a potent viricidal effect (Bakari *et al.*, 2012). This result was in line with Temitayo *et al*., (2020), who considered that a plant extract is considered to possess antiviral activity against NDV if it can inhibit viral replication whilst also allowing chicken embryo growth or if it can reduce viral titre while not allowing embryonic death and viral growth.

Antiviral research aims to find antiviral agents that are specific for inhibiting viral multiplication while not interfering with normal cell division. The extract of *C. quadrangularis* used in this study demonstrated the ability to inhibit viral multiplication while not affecting the inoculated ECE. The continuation of chicken embryo growth revealed by an increase in organ formation in NDV-challenged ECE suggests that the extracts could potentially interfere with the viral replication cycle by blocking one point of propagation mechanisms inside the cells, preventing invasion, or killing the virus in the inoculate which is supports with the findings of the study of (Temitayo *et al*., 2020).

This biological activity of *C. quadrangularis* could be attributed to phytochemicals found in plant extracts. However, the varying degrees of the presence of these phytochemicals could be due to the solvents' ability to extract some of the active ingredients or substances from the plant stem bark based on its polarity. This implies that the chemical compounds in the extract are effective, and the findings of this study support the use of *C. quadrangularis* in the prevention and treatment of

viral infections in chickens. The NDV is an enveloped virus, and this plant extract presumably deactivates it by affecting the virus envelope (Vilhelmova-Ilieva, *et al*., 2020).

These phytochemicals work through various mechanisms to prevent viral replication and infection. In addition, these mechanisms can block viral binding to prevent viral attachment sites or host receptors and work either by killing the virus or interfering with viral replication. Some of these compounds, in particular, inhibit protease activity by interfering with the cleavage of the haemagglutinin neuraminidase and fusion protein, which are essential glycoproteins for NDV attachment and proliferation.

Furthermore, when phytochemicals are used *in vivo*, their effects could be modulated by the components of tissues and body fluids. Virus infections are usually accompanied by various symptoms, not necessarily due to the virus directly. Other ingredients in a plant preparation help to control the virus through additional effects, such as immune modulation, tissue healing among others.

Despite advances in modern medical science, many farmers in Africa still rely on plant parts, and herbal remedies for indigenous poultry health management due to their antiviral activity (Chollom *et al.*, 2022). In addition, *C. quadrangularis* is the medicinal plant with the most chemical and pharmacological studies in the genus. It has undergone scrutiny due to its successful use as a concoction by poultry farmers in the southern province of Zambia to cure and Prevent Newcastle in Chickens and referred to as 'Namununga' by the people.

Based on this, the antiviral study of the EtOH crude extract of *C. quadrangularis* suggests that potentials exist in plant leaf extracts. It was discovered that the leaf extracts had no acute toxicity against the embryo at concentrations of 100 mg/mL and below. No death was observed, and the embryo appeared normal, prompting further research into the extract's antiviral potencies. At the concentrations tested, the extracts were not harmful to the embryo. However, it was discovered that the various extracts have varying degrees of antiviral activity against NDV.

Finding new substances with intracellular and extracellular antiviral activities against NDV is critical today owing to the enormous loss arising from the disease. These substances must have an antiviral effect while not causing harm to the host cells.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

This study showed the potential of EtOH crude extract of *Cissus quadrangularis* having antiviral activity against Newcastle disease virus *in-ovo.* The phytochemical profile showed high levels of secondary metabolites such as alkaloids, flavonoids, phenols, saponins, tannins and moderate level of cardiac glycosides and terpenoids. The plant extract exhibited *in ovo* antiviral activity against NDV, with viral titre reduction at a range of 1:64 in the haemagglutination test, indicating 4-fold decline compared to untreated embryos. The mortality was also lower when the extract was applied. These findings demonstrate that crude extracts from stem of *Cissus quadrangularis* have potential antiviral activity against NDV *in ovo* and is safe for use.

6.2 RECOMMENDATIONS:

In order to further validate the efficacy of the plant extract as anti-NDV, an *in vivo* study using live chickens is recommended.

Other solvents such as *n*-hexane, chloroform, dichloromethane, *n*-butanol, Methanol) should be employed to increase the extraction of other secondary metabolites that could have antiviral potential and fractionations to isolate individual constitutes.

To policymakers and stockholders setting policing priorities and encouraging on use of the plant extract.

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APPENDENCES

Appendix (A): Plant identification report

UNIVERSITY OF ZAMBIA
DEPARTMENT OF BIOLOGICAL SCIENCES
PLANT IDENTIFICATION REPORT

Date: 16th November 2021

NAME OF CLIENT: Mr. Alberto Machamacha (MSc Student)

ADDRESS: School of Veterinary Medicine, University of Zambia

IDENTIFICATION:
Plant materials consisting of stems, leaves and fruits were submitted for taxonomic identification. Below are the details of the identification:

BOTANICAL NAME : *Cissus quadrangularis* L.

FAMILY : Vitaceae

COMMON NAME: Namununga (Tonga), Veldt grape, winged treebine, Devil's backbone, adamant creeper, climbing cactus, kangaroo vine, succulent-stemmed wild grape. Note that many other common names exist in different localities.

ACCESSION NUMBER : UZL 22391

REMARKS:

Brief description
The plant is a perennial herbaceous climber. It has thick, succulent, quadrangular stems that have wings at the angles. The stems are constricted at the nodes and either glabrous or slightly covered with fine soft hair. They have long, slender and simple tendrils. The leaves are petiolate and simple. The specimen had no flowers, however when present inflorescences are compound umbelliform cymes with pink and white flowers. Fruits are red when ripe.

Habitat
In Zambia, the species is usually found low rainfall habitats of Mopane and Brachystegia woodlands.

Distribution
The plant has a wide spread distribution in drier parts of tropical Africa and Madagascar, in Arabia, India, Sri Lanka, Malesia and the Philippines.

In the Flora Zambesiaca region, the plants have been recorded in Mozambique, Malawi, Zimbabwe and Zambia (Copperbelt, Eastern and Southern Provinces).

IDENTIFIED BY: Dr. David Chuba Signature

UNZA, Dept. of Biological Sciences, P.O. Box 32379, Lusaka, Zambia 10101
Phone +260974002051 Email: David.chuba@unza.zm

Appendix (B): Ethical Clearance from ERES CONVERGE IRB

Plot No. 272, Cnr Olive Tree Meanwood Road,
Meanwood Ibex
Lusaka - Zambia
Tel: +260 955 155 633
+260 955 155 634
Cell: +260 977 493 220
Email: eresconverge@yahoo.co.uk

I.R.B. No. 00005948
F.W.A. No. 00011697

26th September, 2022.

Ref. No. 2022-Jun-013

The Principal Investigator
Mr. Alberto Filipe Machamacha
The University of Zambia
School of Veterinary Medicine
Lusaka, Zambia

Dear Mr. Machamacha

RE: ANTIVIRAL POTENTIAL OF HADJOD (CISSUS QUADRANGULARIS) EXTRACT AGAINST NEWCASTLE DISEASE VIRUS

Reference is made to your protocol submission. The IRB resolved to approve this study and your participation as Principal Investigator for a period of one year.

Review Type	**Ordinary**	Approval No. **2022-Jun-013**
Approval and Expiry Date	Approval Date: 26th September,2022	Expiry Date: 25th September, 2023
Protocol Version and Date	Version - Nil.	25th September, 2023
Information Sheet, Consent Forms and Dates	• English.	25th September, 2023
Consent form ID and Date	Version - Nil	25th September, 2023
Recruitment Materials	Nil	25th September, 2023
Other Study Documents		25th September, 2023
Number of participants approved for study	-	25th September, 2023

Specific conditions will apply to this approval. As Principal Investigator it is your responsibility to ensure that the contents of this letter are adhered to. If these are not adhered to, the approval may be suspended. Should the study be suspended, study sponsors and other regulatory authorities will be informed.

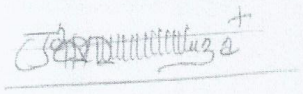
Conditions of Approval

- No participant may be involved in any study procedure prior to the study approval or after the expiration date.
- All unanticipated or Serious Adverse Events (SAEs) must be reported to the IRB within 5 days.
- All protocol modifications must be IRB approved prior to implementation unless they are intended to reduce risk (but must still be reported for approval). Modifications will include any change of investigator/s or site address.
- All protocol deviations must be reported to the IRB within 5 working days.
- All recruitment materials must be approved by the IRB prior to being used.
- Principal investigators are responsible for initiating Continuing Review proceedings. Documents must be received by the IRB at least 30 days before the expiry date. This is for the purpose of facilitating the review process. Any documents received less than 30 days before expiry will be labelled "late submissions" and will incur a penalty.
- Every 6 (six) months a progress report form supplied by ERES IRB must be filled in and submitted to us.
- A reprint of this letter shall be done at a fee.

Should you have any questions regarding anything indicated in this letter, please do not hesitate to get in touch with us at the above indicated address.

On behalf of ERES Converge IRB, we would like to wish you all the success as you carry out your study.

Yours faithfully,
ERES CONVERGE IRB



Dr. Jason Mwanza
Dip. Clin. Med. Sc., BA., M.Sc., PhD
CHAIRPERSON

Appendix (C): Namununga Plant (Cissus *quadrangularis*)

From: *Flora of Zambia: Species information: individual images: Cissus quadrangularis (zambiaflora.com)*

Appendix (D): Plant extract Cytotoxicity Assay layout

Treatment	Final Concentration -C2	DMSO	Extract	Total volume (µl) -V2 = (DMSO 5% + Extract)	Number of eggs inoculated	Total Extract needed	Total DMSO needed
1	100 mg/ mL	-	-	500 µL	20	0 mL	0 mL
2	50 mg/mL	250 µL	250 µL	500 µL	20	5 mL	5 mL
3	12.5 mg/mL	62.5 µL	437.5 µL	500 µL	20	1.25 mL	8.75 mL
4	1.56 mg/mL	7.8 µL	492.2 µL	500 µL	20	0.156 mL	9.84 mL
5. DMSO only	5%	500 µL	0 µL	500 µL	20	0 µL	10 mL
6. Normal eggs without any inoculation	500 µL	500 µL	0 µL	500 µL	20	0 mL	10 mL

Appendix (E): Final concentration of crude extract and total volume Assay layout

Treatment	**Final Concentration -C2**	**DMSO**	**Virus**	**Extract**	**Total volume (µl) -V2 =** (DMSO 5% + Virus+ Extract)	Number of eggs inoculated	Extract needed	Total DMSO needed	Virus needed
1	75 mg/mL	25µL	100 µL	375µL	500 µL	20	7.5 mL	0.5 mL	2 mL
2	12.5 mg/mL	337.5 µL	100 µL	62.5µL	500 µL	20	1.25 mL	6.75 mL	2 mL
3	1.56 mg/mL	392.187 µL	100 µL	7.813 µL	500 µL	20	0.15626 mL	7.84 mL	2 mL
4	0.195 mg/mL	399.023 µL	100 µL	0.9765 µL	500 µL	20	0.0196 mL	7.98 mL	2 mL
5. Virus only	10^{-2}	0 µL	100 µL	0 µL	0 µL	20	0 mL	0 mL	2 mL
6. Extract only	100 mg/ mL	0 µL	0 µL	500 µL	0 µL	20	10 mL	0 mL	0 mL
7. DMSO only	5%	500 µL	0 µL	0 µL	500 µL	20	0 µL	10 mL	0 mL
8. pricked eggs with PBS only	--	500 µL (PBS)	0 µL	0 µL	0 µL	20	0 µL	10 mL (PBS)	0 mL
9. DMSO 5% + Virus	5%	400 µL	100 µL	0 µL	500 µL	20	0 mL	8 mL	2 mL
10. Normal eggs without any inoculation	500 µL	500 µL	0 µL	0 µL	500 µL	20	0 mL	5 mL	0 mL

Appendix (F): Two x Two tables for Relative Risk

	Mortality		
	D+	D-	Total
Factor + (0.195 mg/mL)	9	7	16
Factor - (control NDV -AF2 virus only)	14	5	19

Risk of death = factor + (plant extract = 0.5625= 56.25%

Risk of death =factor - (virus) = 0.737=73.7%

$$\text{Relative Risk} = \frac{\text{Risk factor positive/}\quad 0.5625}{\text{Risk factor negative}\quad 0.737} \quad 0.763$$

	Mortality		
	D+	D-	Total
Factor + (1.56 mg/mL)	8	9	17
Factor - (control NDV -AF2 virus only)	14	5	19

Risk of death = factor + (plant extract = 0.47= 47%

Risk of death =factor - (virus) = 0.737=73.7%

$$\text{Relative Risk} = \frac{\text{Risk factor positive/}\quad 0.47}{\text{Risk factor negative}\quad 0.737} \quad 0.637$$

	Mortality		
	D+	D-	Total
Factor + (12.5 mg/mL)	3	14	17
Factor - (control NDV -AF2 virus only)	14	5	19

Risk of death = factor + (plant extract = 0.176= 16.6%
Risk of death =factor - (virus) = 0.737=73.7%

$$\text{Relative Risk} = \frac{\text{Risk factor positive/} \quad 0.176}{\text{Risk factor negative} \quad 0.737} \quad 0.239$$

	Mortality		
	D+	D-	Total
Factor + (75 mg/mL)	2	16	18
Factor - (control virus only)	14	5	19

Risk of death = factor + (plant extract = 0.11= 11%
Risk of death =factor - (virus) = 0.7368 = 73.7%

$$\text{Relative Risk} = \frac{\text{Risk factor positive} \quad 0.111}{\text{Risk factor negative} \quad 0.737} \quad 0.1506$$