Extraction, Characterization and Evaluation of the Larvicidal, Ovicidal and Antifeedant Effects of Crude Cashew Nut Shell Liquid against *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae L.)

BY

KADANGO ZOMBE

A dissertation submitted to the University of Zambia in partial fulfillment of the requirements for the degree of Master of Science in Chemistry

THE UNIVERSITY OF ZAMBIA

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Declaration

I hereby declare that this dissertation is a product of my work and that all the sources quoted have been indicated in the references. I further declare that this work has not been previously presented elsewhere whether wholly or in part for any other study Programme at any university. It has been produced in accordance with the guidelines for the Master of Science in Chemistry dissertation for the University of Zambia.

Signed……………………… Date………………… **18/10/2023**

Kadango Zombe (Candidate)

Certificate of Approval

The University of Zambia has approved this dissertation of Kadango Zombe as partial fulfilment of the requirements for the award of the Degree of Master of Science in Chemistry.

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Abstract

Spodoptera frugiperda (J.E. Smith), commonly known as the fall armyworm (FAW), is an invasive insect pest of maize and other economically important cultivated crops globally. In the absence of proper control methods, FAW has the potential to cause 21 to 50 percent of maize crop yield losses. Currently, synthetic insecticides are widely used in the management of FAW outbreaks around the world. Nonetheless, overdependence on synthetic insecticides leads to undesirable environmental and human health issues. Botanical insecticides such as Cashew nut shell liquid (CNSL) have been shown to exhibit insecticidal properties against a broad spectrum of insect pests and are known to be less harmful to the environment and humans. Hence, this study evaluated the insecticidal properties of CNSL against FAW in order to assess its potential use as an alternative to synthetic insecticides. Crude CNSL was extracted from cashew nutshells (CNSs) by solvent extraction method. Phytochemical screening and Thin Layer Chromatography (TLC) were performed to qualitatively identify the secondary metabolites present in it. Additionally, Gas Chromatography-Mass Spectrometry (GC-MS), Fourier Transform Infrared spectroscopy (FTIR), and Ultraviolet-visible (UV-Vis) spectrometry techniques were used to characterize and quantify some of the identified compounds. Furthermore, bioassays such as topical and no-choice feeding assays were performed to evaluate the ovicidal, larvicidal, and antifeedant effects of crude CNSL against FAW. Phytochemical screening revealed the presence of various bioactive compounds including phenols, tannins, flavonoids, alkaloids, saponins, steroids, glycosides, and terpenoids. All the other characterization techniques; TLC, FTIR, and GC-MS revealed the presence of alkyl phenolic lipids such as cardol (5-pentadecyl resorcinol) and cardanol (3-pentadecyl phenol). Literature showed that these alkyl phenolic compounds are the ones responsible for the biological activity of CNSL. The toxicity of CNSL against FAW larvae was dose-dependent. The highest mortality of eggs (99.15 ± 0.0 %) and larvae (97.99 ± 0.0 %) were both recorded at 7.0 w/v %, respectively. The lethal concentrations LC_{50} (95 CI %) were 2.50 (0.22-4.05 w/v %), for egg and 1.92 (1.71-2.15 w/v %) for larval mortality, respectively. The effective concentration (EC_{50}) for the antifeedant effect was found to be 1.05 (0.81-1.29 w/v %). Results showed that CNSL has insecticidal properties and antifeedant effects against FAW. Hence, it can be exploited as a relatively cheap, safe, and eco-friendly alternative insecticide for managing FAW outbreaks in Zambia.

Keywords: Cashew, Fall armyworm, *Spodoptera frugiperda,* cashew nut shell liquid

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Dedication

This dissertation is dedicated to my father, Hanaph Zombe.

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Abbreviations and Acronyms

CNS	Cashew Nut Shell
CNSL	Cashew Nut Shell Liquid
EC	Effective Concentration
FAW	Fall Armyworm
FTIR	Fourier Transform Infrared Spectroscopy
GAE	Gallic Acid Equivalent
GC-MS	Gas Chromatography-Mass spectroscopy
HPTLC	High performance thin layer chromatography
KATC	Kasisi Agricultural Training Centre
LC	Lethal Concentration
MT	Metric Tonnes
QE	Quercetin Equivalent
TFC	Total Flavonoid Content
TIC	Total Ion Chromatogram
TLC	Thin Layer Chromatography
TPC	Total Phenolic Content
UNZA	University of Zambia
WHO	World Health Organization
ZARI	Zambia Agricultural Research Institution

Chapter 1: Introduction

1.1 Background

Spodoptera frugiperda (J. E. Smith), commonly known as the fall armyworm (FAW), is a polyphagous insect pest of economically important cultivated crops such as maize, sorghum, rice, sugar cane, wheat, and millet (Day et al., 2017). It is native to the tropical and subtropical regions of the Americas but migrated to the African continent in 2016, the Asian continent in 2018, and the Australian continent in 2020, respectively (Overton et al., 2021). The FAW has now been labelled as the most destructive agricultural insect pest globally (Yan et al., 2022), and maize which is a high-priority food crop across many Sub-Saharan countries has been reported as the most susceptible crop to FAW attack (Kamara et al., 2020). In 2018, estimates from 12 major African maize-producing countries showed that, up to 17.7 million tonnes of maize, enough to feed tens of millions of people could be lost annually due to FAW attack (Day et al., 2017). Currently, synthetic insecticides are widely used to manage FAW outbreaks in Africa and they have proved to be very effective (Young, 1979; Kansiime et al., 2019; Tambo et al., 2020). Nevertheless, overdependence on synthetic insecticides is linked to severe undesirable environmental and human health issues (Williamson et al., 2008; Kulye et al., 2021). Moreover, synthetic insecticides are nonspecific hence they also kill non-target insects and easily become ineffective over time as insects quickly develop resistance against them (Williamson et al., 2008; Kulye et al., 2021).

Botanical insecticides (also called biorational insecticides) are naturally occurring insecticides obtained from plants (Rawat et al., 2023). Several botanical insecticides have been shown to exhibit insecticidal properties against the FAW (Bullangpoti et al., 2012; Choongo et al., 2018; Parchande et al., 2021), and their efficacy has been shown to be almost comparable to that of synthetic insecticides (Sisay et al., 2019). Studies have shown that chemical compounds from plants either in crude or pure form have the ability to interfere with insect growth, feeding behaviour, and reproduction, hence influencing their survival abilities (Haouas et al., 2010). Unlike synthetic insecticides, botanicals are relatively cheap, readily available, effective, specific, and less harmful to humans, and the environment (Amoabeng et al., 2019). The cashew tree (*Anacardium occidentale)* is an evergreen plant in the *Anacardiaceae* family (Mukhopadhyay et al., 2010). The tree is

best known for producing cashew nuts, which are among the top four most consumed nuts in the world (Shahbandeh, 2023). Cashew nut shell (CNS), one of the by-products from cashew nut processing, contains a dark viscous oil called cashew nut shell liquid (CNSL) that has been shown to have numerous industrial and biological activities (Konan and Bacchi, 2007). CNSL has been reported to exhibit a broad spectrum of biological activity against a wide range of insect pests (Mukhopadhyay et al., 2010; Mahapatro, 2011; Passari et al., 2015; De Carvalho Castro et al., 2019). Nonetheless, its biological activity against the FAW has not been fully investigated in the literature. Hence, this work aimed to evaluate the larvicidal, ovicidal, and antifeedant effects of crude CNSL against FAW.

1.2 Statement of the Problem

The FAW insect destroys maize which is a staple food for Zambia and many other African countries. Some of the current synthetic insecticides that are being used to control FAW outbreaks in Zambia are very poisonous to humans and the ecosystem. For instance, Monocrotophos, Methomyl, and Profenofos are listed as highly hazardous by the World Health Organization (WHO) and have been banned in some countries (Kansiime et al., 2019; Mukherjee, 2023). Moreover, over-dependence on synthetic insecticides to control FAW in Zambia will lead to the insect pest developing resistance, which has already been seen in Brazil (Prasanna et al., 2018).

1.3 Significance of Study

Cashew nut shell liquid is a natural insecticide derived from CNSs. Unlike conventional insecticides, it is readily available, relatively cheap and effective, and less toxic to humans and the environment (Mukhopadhyay et al., 2010; Selvamuthukumar et al., 2021). CNSL is obtained from CNSs which are industrial waste; hence, developing a desired product that can be used towards the management of serious insect pests will promote value chain addition to locally produced cashew.

1.4 Aim

To extract, characterize, and evaluate the larvicidal, ovicidal, and antifeedant effects of crude CNSL against the FAW.

1.5 Specific Objectives

The specific objectives of the study were:

i.) To extract and characterize CNSL from CNSs obtained from Mongu district, Western province.

ii.) To evaluate the insecticidal properties of the extracted CNSL against FAW.

iii.) To determine the lethal dose (LD_{50}) of crude CNSL against FAW.

1.6 Research Questions

1. What is the chemical composition of CNSL extracted from CNSs obtained from Zambian-grown cashew?
2. Does CNSL possess insecticidal properties against FAWs?

Chapter 2: Literature Review

2.1 Introduction

This chapter reviews the literature on biology, distribution, economic impact, and limitations of current methods for controlling FAW outbreaks in Zambia. In addition, the chapter will highlight current knowledge on the extraction, characterization, and biological activity of CNSL. It will also highlight the gaps in the literature, which necessitated this study.

2.2 Biology and Life Cycle of the FAW

The genus *Spodoptera* consists of a number of species that are important crop pests including *S. littoralis (*Boisduval) (the Egyptian cotton leafworm), *S. exempta* (Walker) (the African armyworm), *S. litura* (Fabricius) (the tobacco caterpillar), *S. exigua* (Hübner) (the beet armyworm), *S. ornithogalli* (Guenée) (Yellow striped armyworm), and *S. frugiperda* (J. E. Smith) (the fall armyworm) (Shehzad et al., 2022). The adult FAW moth is nocturnal and is most active during warm humid evenings (Prasanna et al., 2018). It is a strong flyer; Literature showed that with the right wind pattern, it can cover a distance of 160 km in 30 hours (Prasanna et al., 2018), which explains why it spreads to new regions quickly. The life cycle of the FAW has four major stages (egg, larval, pupa, and moth) (**Figure 2-1**). It is worth noting that the life cycle of the FAW also lacks the diapause phase, which is the biological resting period for insects, thus reproduction is continuous throughout the year (Prasanna et al., 2018). The female FAW moth is very fertile (Prasanna et al., 2018). It starts laying eggs immediately after mating and can produce up to a maximum of 2000 eggs within its life span of about 10 days (Prasanna et al., 2018). The eggs are laid in batches of up to 150 - 200 and hatch within 2-3 days during warmer summer months (Prasanna et al., 2018). The larval stage undergoes six different stages called instars before going into the pupation stage (Prasanna et al., 2018). The larval stage is reported as the most destructive stage of the FAW life cycle (Overton et al., 2021). The FAW larva has over 80 host plant species (Day et al., 2017). The duration of the pupa stage is 8-9 days, but can also reach 20 to 30 days in cooler weather before the adult moths

emerge (Prasanna et al., 2018). A complete life cycle of the FAW takes about 30 days but can extend to 60-90 days in cooler temperatures (Prasanna et al., 2018).

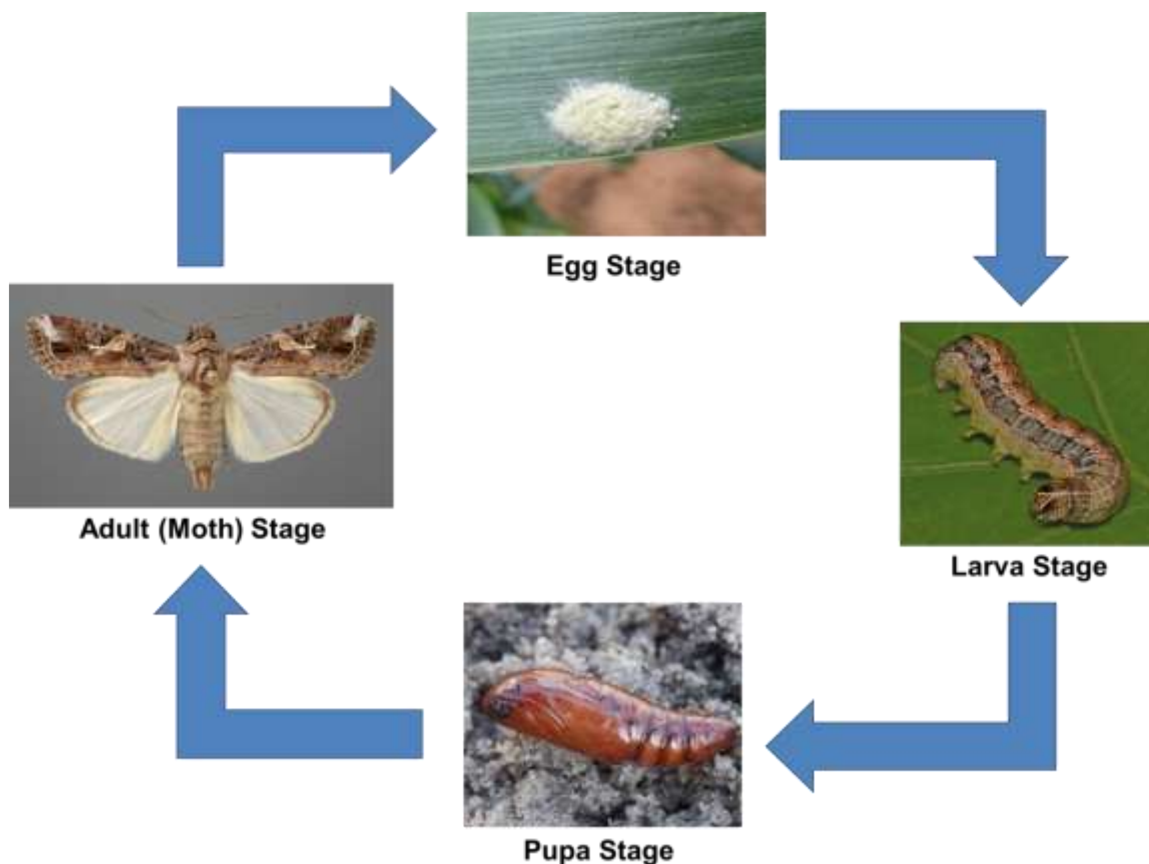

Figure 2-1. Life cycle of the FAW constructed from images from (Prasanna et al., 2018).

2.3 Origin and Distribution of the FAW

The FAW is native to the sub and tropical regions of the Americas. It first spread to West and Central Africa in 2016, then proceeded to Asia in 2018, and by 2020 it had spread to over 109 countries globally (Mudereri et al., 2023). Interestingly, the FAW insect pest is considered chronic, which means it establishes permanent colonies in every region it spreads to, provided the conditions are favourable for its survival (Flanders et al., 2017). In Zambia, the FAW was first reported in Chirundu in November 2016, and within a month it spread to all the ten provinces. Ever since, there have been sporadic outbreaks every farming season in selected areas of the country (ZARI, 2017).

2.4 Economic Impact of the FAW

The FAW was listed as a global super insect pest by the United Nations (UN), Food and Agricultural Organization (FAO) in 2017 (FAO, 2018). It prefers maize but also affects other economically important cultivated crops such as wheat, rice, sugar cane, sorghum, millet, cotton, cowpea, and other vegetable crops (Day et al., 2017; Prasanna et al., 2018). The presence of FAWs in Africa, especially in the Sub-Saharan region threatens the food security and livelihood of several people, as most countries in this region depend on maize for food and household income (Hruska, 2019; Tambo et al., 2020). In 2018, estimates from 12 African maize belt countries showed that up to 17.7 million tonnes of maize could be lost annually due to the appearance of FAW in the continent, enough to feed tens of millions of people (Day et al., 2017). In Zambia, maize has been reported as the most susceptible host crop to FAW attack (Stephen, 2018; Kasoma et al., 2021). According to Kansiime et al. (2019) the estimated maize yield losses for Zambia due to the FAW attack in the 2016/17 farming season was 28 %, but Day et al. (2017) reported losses as high as 40 % in the same farming season. According to Day et al. (2017) the following points were listed as the direct dangers FAW pose on the livelihood and the economy of countries in the Sub-Saharan regions:

- The majority of farmers in the Sub-Saharan region are smallholder farmers who entirely depend on agriculture for food and survival.
- The presence of FAW will affect natural capital, through yield losses and the ability of agricultural lands to respond to shocks.
- It will also affect financial capital, through increasing the cost of production, and income. This will also affect households' social and physical capital (the household's assets).
- Furthermore, the higher the infestation frequency, the more toxic insecticides are released into the environment. For example, in Zambia alone, more than 100,000 litres of synthetic insecticides were released into the environment due to FAWs attack during the 2016/17 farming season. This has a direct impact on human health as more people would get exposed to it during the process.

- FAWs also affects international trade as countries where the insect pest is not yet present will avoid or restrict trade with FAW-prone countries due to fear of introducing FAWs in their countries. For instance, in June 2017, the first shipment (of roses) from Africa contaminated with FAWs was intercepted in Europe.

2.5 Limitations of Current Methods Used to Control FAW Outbreaks

Currently, synthetic insecticides are commonly used to control FAWs around the world (Prasanna et al., 2018; Overton et al., 2021). They have been reported to be quick, effective and less labour intensive. They are also associated with increased crop yields of up to five times of the value the insecticide itself (Mpumi et al., 2016). Nevertheless, over-dependence on synthetic insecticides has been linked to several undesired effects both on human health and the environment, especially if not used properly (Popp et al., 2013). A report by Kansiime et al. (2019) showed that the use of chemical insecticides was common among small-scale farmers in Zambia and most of them used these chemicals inappropriately. For instance, during the 2016/17 farming season, more than 60 % of farmers in Zambia used chemical insecticides to control FAWs and in the quest to quickly eliminate the insect pest that had infested their fields, most farmers mixed more than one chemical and used dosages higher than that recommended by manufacturers as they perceived it to be more effective. Moreover, some of the chemicals that were used such as Monocrotophos, Methomyl and Profenofos are considered highly hazardous by the WHO and have been banned in some countries (Mukherjee, 2023).

The other more benign methods that are currently used in the management of FAW around the world include cultural practices, biological control, host-plant resistance and soft chemical insecticides (Prasanna et al., 2018). Nevertheless, most of these methods are either labour-intensive (cultural practices), impractical on a larger scale (cultural and biological control), expensive (soft chemical insecticides) and sometimes ineffective (Greathead, 1995; Tambo et al., 2020). Thus, considering the risky effects of chemical insecticides on agricultural soils, water resources, human health and the environment, as well as the impracticality of other seemly sustainable and environmentally friendly methods, it is very evident that there is an urgent need to explore other better options for managing agricultural insect pests.

2.6 Alternative to Synthetic Insecticides

Botanical insecticides are a class of natural insecticides obtained from seeds, barks, flowers, roots and leaves of plants (Rawat et al., 2023). They are relatively effective, specific, cheap, available, and less harmful to humans, and the environment (Amoabeng et al., 2019). Several plants have been shown to possess insecticidal properties and have been used over the years to manage agricultural insect pests. For instance, Neem (*Azadiracta indica*) is a famous plant-based insecticide widely used in the management of all sorts of agricultural insect pests. Almost all parts of the plant (leaf, root, stem and seed) have been shown to exhibit insecticidal properties (Lokanadhan et al., 2012). The active compounds in Neem seeds and leaves are Azadirachtins; insect growth regulators and antifeedant compounds (Lokanadhan et al., 2012; Benelli et al., 2017). Another plant with interesting insecticidal properties is *Tephrosia vogelii* (Kerebba et al., 2019). Likewise, the leaf, stem and root extracts from this plant has been reported to affect various insect species. The bioactive compounds in *T. vogelii* are rotenoids, including deguelin, dehydrodeguelin, elliptone, 12a-hydroxyrotenone, rotenone and tephrosin (Ling, 2003; Stevenson and Belmain, 2017; Kerebba et al., 2019). Several other plants have been reported to display insecticidal properties against various insect species in the southern region of Africa. These include *Mucuna pruriens, Bobgunnia* (Swartzia) *madagascarensis, Euphorbia tirucali, Vernonia amygdalina, Tithonia diversifolia, Solanum panduriforme* and tobacco *(Nicotiana tabacum*) (Nyirenda et al., 2011). In Zambia, Neem, *T. vogelii*, and tobacco are the most commonly used insecticidal plants, with *T. vogelii* being the most used plant (Tavares et al., 2021). The Cashew tree is widely distributed in the subtropical and tropical regions of the world. It is popularly known for its nuts (Cashew nuts) (**Figure 2-2**) (Mwangi et al., 2013), but the medicinal and insecticidal properties of the plant have also been widely documented (Mukhopadhyay et al., 2010, Mahapatro, 2011, De Carvalho Castro et al., 2019). Similarly to Neem, all the parts of the cashew tree (leaf, bark, root, and shell) have been reported to possess insecticidal properties (Mukhopadhyay et al., 2010; Mahapatro, 2011; De Carvalho Castro et al., 2019). The shells that remain after removing the nuts produce a viscous substance called CNSL which is rich in bioactive phenolic lipid compounds that have a broad spectrum of biological activity against a wide range of insect pests (Mukhopadhyay et al.,

2010; Mahapatro, 2011; De Carvalho Castro et al., 2019). Nevertheless, there is limited literature on the application of cashew-derived insecticides in the management of agricultural insect pests in the Southern region of Africa including Zambia despite the tree being prevalent in the region for many years.

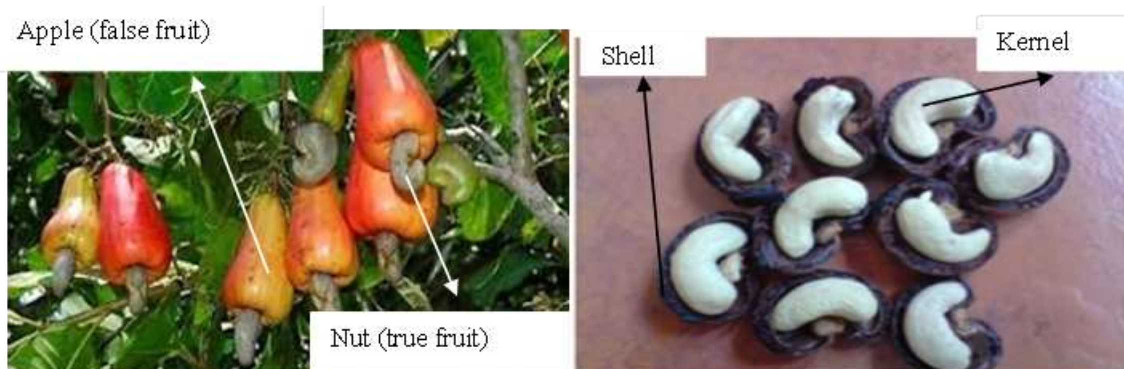

Figure 2-2. On the left, the hanging orange pear-shaped objects are the cashew apples (false fruit), while the greyish kidney shaped objects attached to the apples are the cashew nuts (true fruit). On the right, the brownish woody structure is the CNS, while the whitish curved object is cashew nut kernel (Mwangi et al., 2013).

2.7 Availability of Cashew and CNSs in Zambia

Cashew is a commercial crop grown all over the world. It is native to Brazil but popularized around the world by Portuguese sailors in the 16th century (Azam-Ali and Judge, 2001). In Zambia, cashew was first introduced in the 1940s, by Portuguese traders, in the Western province (then Barotseland) an area characterized by Kalahari sandy soil that is poor for most conventional crops (Africa Development Bank, 2015). In the year 2020, the total raw cashew nut production in Zambia stood at 850 metric tons (MT) per year from an estimated 1,700,000 cashew trees (Zambia Daily Mail, 2020). However, the production is anticipated to reach 60,000 MT by 2024, once the 6,000,000 cashew trees planted in 2015 in the Western province under the cashew infrastructure development project (CIDP) reach their full maturity (Cashew Nut Processing Project, 2020). Literature sources showed that the cashew industry in Zambia is likely to grow in the near future (Africa Development Bank, 2015, Cashew Nut Processing Project, 2020, Zambia Daily Mail, 2020). This suggests that more waste CNS will be generated, which are the sources of the proposed CNSL-based insecticide for managing FAW outbreaks in Zambia. Hence,

this study was inspired by the anticipated availability and affordability of the starting raw materials for the cashew insecticide in the near future.

2.8 Salient Features of the CNS

The CNS is the hard outer structure of the cashew nut (true fruit). It represents 40-45 % weight of the whole fruit (false and true fruit) and 70-75 % of the weight of the nut (true fruit) (Chaudhari and Thakor, 2015; Saenab et al., 2020). The CNS is a double-shelled structure with a thickness of three millimetres between the inner and outer shells (**Figure 2-3**) (Oloso and Clarke, 1993). The outermost part of the CNS is called the epicarp, while the innermost part is called the endocarp. Between the epicarp and endocarp is a soft honeycomb structure where CNSL resides called the mesocarp (Tyman, 1979). The epicarp, endocarp and mesocarp are collectively known as the pericarp (Tyman, 1979). The size and weight of the CNS vary from one region to another and from one variety to another (Akinhanmi et al., 2008; Couto et al., 2004). Akinhanmi et al. (2008) reported a variation in the size of cashew nuts from Brazil and Africa. In another study, Couto et al. (2004) reported four different varieties of CNS from Brazil classified based on sizes and weights namely small, medium, large and extra-large with weights ranging from 1.389 g, 2.603 g, 4.567 g and 5.175 g, respectively. Das and Ganesh (2003) reported that the amount of CNSL in unshelled African cashew nuts (true fruit) ranges from 15-20 %, while in the Indian nuts from 25-30 % by weight. Generally, it has been reported that the amounts of CNSL in the dry shell can range from 18-40 % (Tyman, 1979; Gandhi et al., 2013; Lubi and Thachil, 2000; Senthil Kumar et al., 2009; Garkal and Bhande, 2014).

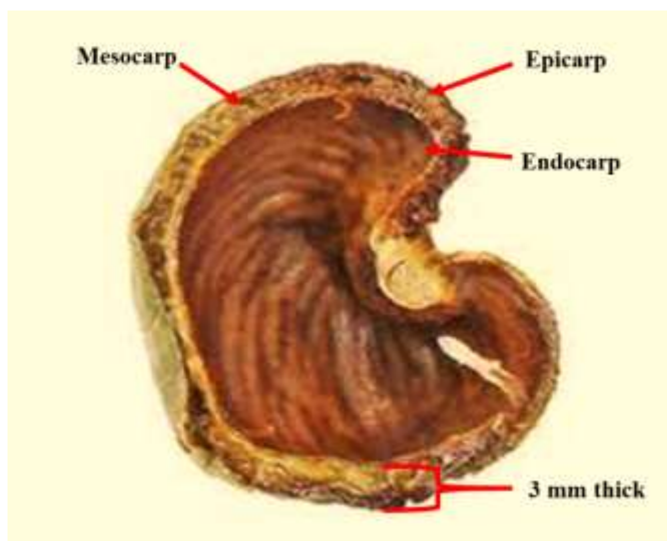

Figure 2-3. Salient features of the CNS (Tyman, 1979).

2.9 Extraction of CNSL

Several methods exist for extracting CNSL from CNS, and the choice of a method influences the amount of CNSL recovered and its chemical composition (Dendena and Corsi, 2014). Generally, there are three main methods for extracting CNSL from CNS namely mechanical pressing, thermal and solvent extraction (Gandhi et al., 2013; Subbarao et al., 2011). Mechanical pressing and solvent extraction are classified as cold extraction methods and CNSL extracted by these two methods is denoted as natural CNSL (nCNSL) (Gandhi et al., 2013). Whereas, CNSL extracted by thermal processing methods (open pan or/ hot oil bath roasting) is referred to as technical CNSL (tCNSL) (Gandhi et al., 2013).

2.9.1 Mechanical Pressing of CNSL

In mechanical pressing CNSs are pressed with a hydraulic press or screw press, the exerted pressure leads to the bursting of the oil cells in the shell and forces CNSL out of the shells (Rodrigues et al., 2011). This method is simple, straightforward and quick. However, it gives low yields of CNSL of about 20.7-21.0 % by dry weight, leaving a reasonable amount of about 10-15 % in the residues (Rodrigues et al., 2011). In addition, CNSL extracted by this method has been reported to have a lot of impurities, higher viscosity, lower thermo-oxidative stabilities and lower ebullition temperature (Rodrigues et al., 2011).

2.9.2 Thermal Extraction of CNSL

Is the most common method for commercial extraction of CNSL in practice nowadays (Subbarao et al., 2011). It involves three methods namely hot oil bath, direct pan and drum roasting. The principle is based on the expansion and contraction of oils at different temperatures. When the CNS is exposed to higher temperatures of about 180 –200 ^{0}C, the oil in the shell expands and bursts to open the oil cells, releasing CNSL (Rodrigues et al., 2011). Through this method, 85-90 % of CNSL by weight is recovered (Subbarao et al., 2011). However, due to higher temperatures, only technical CNSL can be produced by this method. Also, during processing thick toxic fumes are produced that contribute to environmental pollution and human health issues (Subbarao et al., 2011).

2.9.3 Solvent Extraction of CNSL

Solvent extraction comprises maceration, ultrasonication, soxhlet and supercritical carbon dioxide. This method is very effective such that the oil that remains in the residue is less than 1 % by weight (Garkal and Bhande, 2014). Generally, organic solvents are preferred for the extraction of CNSL from shell over aqueous solvents (Subbarao et al., 2011). The commonly used organic solvents are hexane, ethanol, methanol, petroleum ether, diethyl ether, acetone, chloroform, etc.(Tyman et al., 1989, Subbarao et al., 2011, Gandhi et al., 2013). The procedure involves soaking CNSs in an organic solvent. The contact between the solvent and the shell allows the effusion of CNSL from the shell into the solvent (Subbarao et al., 2011). To recover the extracted CNSL the solvent is usually removed by evaporation (Subbarao et al., 2011).

2.10 Characterization of CNSL

CNSL is a liquid at room temperature and insoluble in aqueous solvents, thus, it is treated and characterized like oils/fats. Unlike known oils, CNSL contains phenolic lipids rather than triglycerides (Tyman, 1979). Characterization involves determining its physicochemical properties such as specific gravity, refractive index, viscosity, moisture content, volatile matter, iodine, saponification and acid values (Akinhanmi et al., 2008; Rodrigues et al., 2011; Gandhi et al., 2013). These parameters are so important in understanding the quality and possible suitable application of a substance. **Table 2-1** shows the published physicochemical properties of CNSL. The chemical composition and

structural elucidation of crude CNSL and its components are investigated by various techniques including Thin Layer Chromatography (TLC), Column chromatography (CC), high-performance liquid chromatography (HPLC), Gas Chromatography coupled with Mass Spectrometry (GC-MS), Fourier transform infrared spectroscope (FTIR) and Nuclear Magnetic Resonance (NMR) (Tyman et al., 1989; Shobba and Ravindranath, 1991; Yuliana et al., 2012; Gandhi et al., 2013; Chaudhari and Thakor, 2015).

Table 2-1. Physicochemical properties of CNSL reported by various authors.

Physicochemical properties	Values	References
Per cent yield	38-40	(Gandhi et al., 2013)
Colour	Dark brown	(Akinhanmi et al., 2008)
Nature	Viscous liquid	(Akinhanmi et al., 2008)
Specific gravity (g/cm^3, 30 °C)	0.924	(Akinhanmi et al., 2008)
Refractive index (20 °C)	1.686	(Akinhanmi et al., 2008)
Saponification number (mg KOH/g)	47.6	(Akinhanmi et al., 2008)
Iodine number (mg I_2/g)	235	(Akinhanmi et al., 2008)
Acid number (mg KOH/g)	15.5	(Akinhanmi et al., 2008)
Free Fatty Acid %	7.8	(Akinhanmi et al., 2008)
Viscosity (centipoises, 30 °C)	41	(Akinhanmi et al., 2008)
pH	3	(Achi and Myina, 2011)

2.11 Chemical Composition of CNSL

Several studies have shown that CNS extracts are rich in bioactive compounds such as flavonoids, phenols, tannins, alkaloids, saponins terpenoids, steroids and coumarins (Tedong et al., 2006; Kannan et al., 2009; Dulo et al., 2022; Sekunowo and Obasa, 2022). In a study by Citó et al. (2011) GC-MS analysis results the revealed presence of 11 compounds in technical CNSL representing about 88.8 % of the total CNSL content (**Table 2-2**). The main constituents identified were cardanol (40.3 %), cardol (29.9 %), phytosterol (10.7 %), anacardic acid (1.97 %) and other unidentified compounds (11.2 %). In another study by Leite et al. (2015), GC-MS analysis of natural CNSL showed the

presence of anacardic acid (94.1 %) and unidentified compounds (2.6 %) respectively. Generally, CNSL is believed to contain four major heterogeneous phenolic lipids namely anacardic acid, cardol, methyl cardol and cardanol (Tyman, 1989). These phenolic lipids consist of a polar aromatic moiety and a long aliphatic hydrocarbon chain of about 15 to 17 carbon atoms that can be saturated, mono, di or tri-unsaturated (Paramashivappa et al., 2001). The chemical structures of the major CNSL phenolic lipids are shown in (**Figure 2-4**) (Telascrêa et al., 2014). Several literature sources revealed that these phenolic compounds are the ones responsible for the biological and insecticidal properties exhibited by CNSL (Buxton et al., 2017; Boongaling et al., 2008; Ferreira de Carvalho et al., 2019; Saenab et al., 2020).

Table 2-2. Chemical composition of technical CNSL

No.	Retention Time	Compounds	(%)
1	24.60	Unidentified compound	1.65
2	24.85	Monounsaturated cardanol	24.28
3	24.87	Diunsaturated cardanol	14.39
4	25.15	Saturated cardanol	1.59
5	25.51	Octacosene	I .49
6	25.65	Stigmasterol	1.46
7	25.71	Monounsaturated anacardic acid	1.79
8	25.81	B-Sitosterol	9.22
9	26.08	Monounsaturated cardol	4.23
10	26.17	Triacontane	25.72
11	26.26	Untriacontane	1.15
12	26.98	Unidentified compound	3.51
13	27.65	Unidentified compound	3.11
14	27.71	Unidentified compound	2.27
15	28.43	Unidentified compound	2.81
16	28.03	Unidentified compound	1.33

The source of this table is Citó et al. (2011).

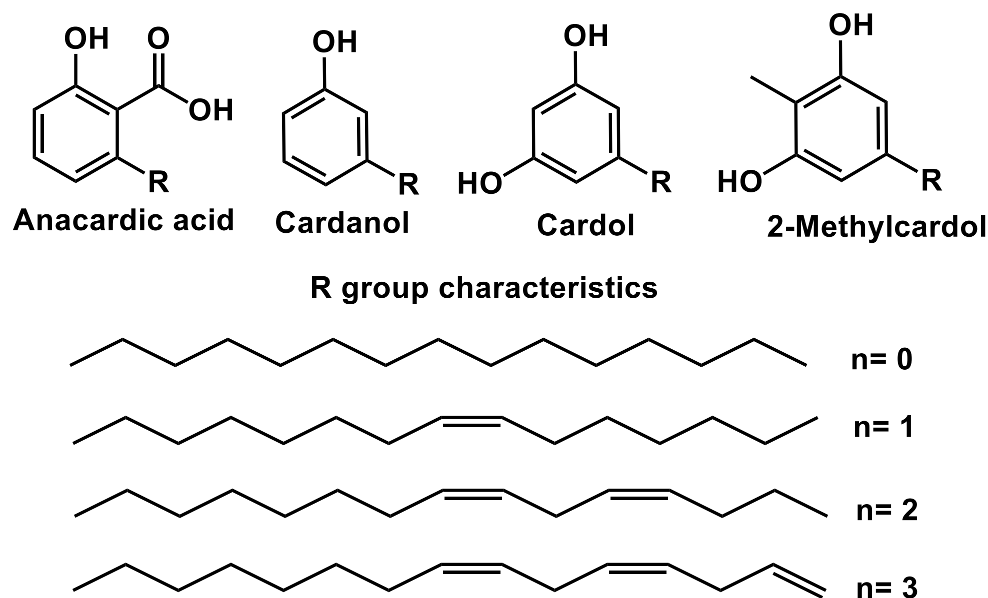

Figure 2-4. Chemical structures of major CNSL phenolic compounds (Paramashivappa et al., 2001).

2.12 Biological Activity of CNSL

The biological activity of CNSL and its components has been extensively published in the literature. Several authors have reported the antibacterial, fungicidal, anti-parasitic, anti-inflammatory, antioxidant, and anticancer activities of CNSL and its phenolic components (Lubi and Thachil, 2000; de Souza et al., 2018; Prakash et al., 2018; Matutino Bastos et al., 2019). In two separate studies, Dourado et al. (2015) and Lomonaco et al. (2009) demonstrated that both crude natural and technical CNSL were able to cause mortality of *Aedes aegypti*. The minimum lethal concentration of CNSL (LC_{50}) that promoted mortality of 50 % of third-instar *A. aegypti* larvae was 0.07 mg mL^{-1} for natural CNSL (Dourado et al., 2015) and 0.05 mg mL^{-1} for technical CNSL (Lomonaco et al., 2009), respectively. Buxton et al. (2017) isolated three alkyl phenolic compounds from CNSL namely (i) saturated, diene, and triene cardanol. Of the three compounds, triene cardanol exhibited strong insecticidal activity with LC_{50} of 60 mg mL^{-1} against the common rice weevil pest (*Sitophilus oryzae*). In another study by Boongaling et al. (2008), natural CNSL exhibited contact toxicity and a repellent effect against milk termites (*Coptotermes vastator* Light*)* at a concentration as low as 0.1 %. The study also reported that 10 % CNSL demonstrated 100 % mortality of termites by contact method and 66 % antifeedant effect in a no-choice feeding bioassay (Boongaling et al., 2008). In this study, it was also

observed that very low concentration CNSL (0.1 %) exhibited repellent and antifeedant effects on termites, but had no toxicity effect when ingested by termites (Boongaling et al., 2008).

2.13 Mode of Action of CNSL

The mode of action of CNSL is poorly understood (Boongaling et al., 2008). However, in a review paper by Stasiuk and Kozubek (2010), it was revealed that phenolic lipids from cashew and other plants inhibit the growth of bacteria, fungi, protozoa and parasites by interacting with their proteins or by disrupting their lipid membranes bilayer, or DNA molecules. In a study by De Lima et al. (2008), CNSL extracted from immature cashew nuts inhibited the activity of acetylcholinesterase. Stasiuk and Kozubek (2008) showed that phenolic lipids from CNSL exhibited haemolytic activity against sheep erythrocytes with EH_{50} values being dependent on the length and saturation of the hydrocarbon chain present in the molecules and/or the chemical structure of the polar heads of the molecules. Furthermore, Dourado et al. (2015), reported that CNSL at a concentration of 1 mg/ml caused degeneration of the epithelial cell lining, increased vacuoles and separation of epithelial cells from the basal membrane and disintegrations of the brush borders and damage of the peritrophic membrane in the midgut of third instar *A. aegypti* larvae.

2.14 Other uses of CNSL

Cashew nut shell liquid is a cheap and readily available renewable raw material that has great potential to replace conventional phenols in several industrial applications (Taiwo, 2015). The phenolic compounds in CNSL have the ability to undergo a series of chemical modifications such as decarboxylation, hydrogenation, nitration, methylation, epoxidation, ozonolysis, and polymerization. Hence, they can be converted into almost any desired chemical product with ease and at a low cost (Lubi and Thachil, 2000). **Figure 2-5** shows products that can be synthesized from CNSL (Lubi and Thachil, 2000; Mwangi, 2013; Taiwo, 2015).

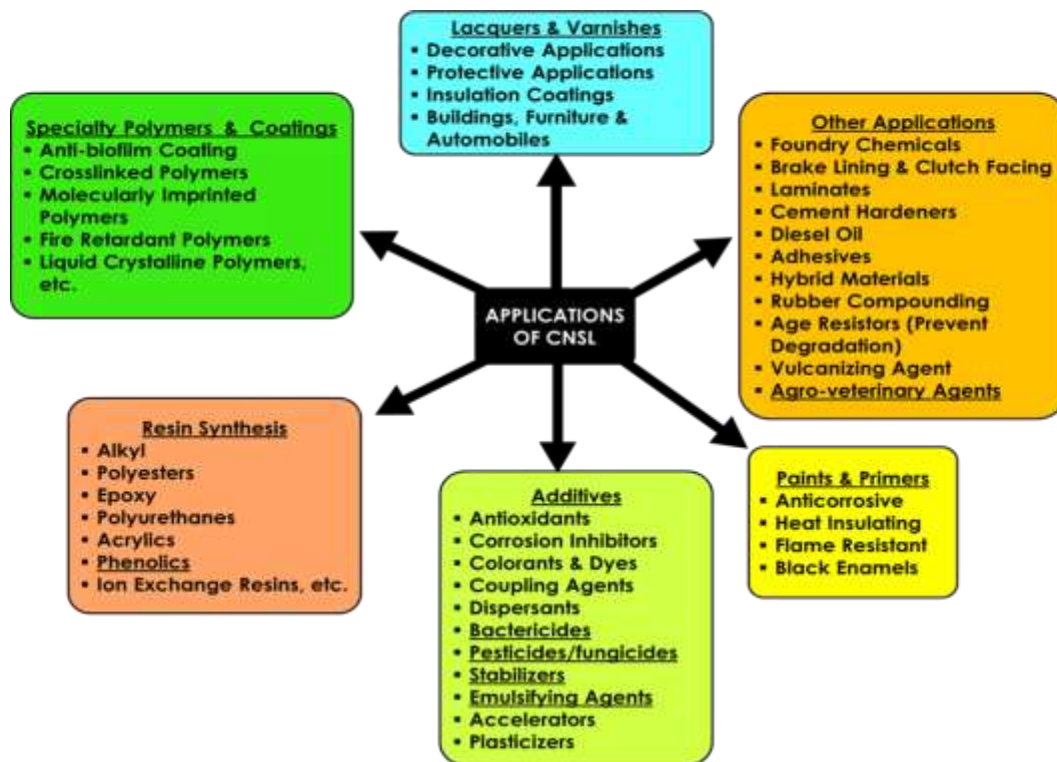

Figure 2-5. Various Applications of CNSL constructed from works by Lubi and Thachil, (2000) and Taiwo, (2015).

2.15 Gap in literature

Despite the cashew tree being prevalent in the Southern region of Africa for so many years, there is little literature on its use as an insecticide in the management of agricultural insect pests in the region, including Zambia (Tavares et al., 2021). Secondly, literature showed that the chemical composition of CNSL varies depending on the variety and regions where the plant is grown (Akinhanmi et al., 2008; Das and Ganesh, 2003). We found little information available on the chemical composition of CNSL from Zambian-grown cashew. Thirdly, although, CNSL has been widely shown to exhibit insecticidal properties against a broad spectrum of insect species, there is little information on its use against FAW. From our thorough literature search, only one article by Ferreira de Carvalho et al. (2019) was found. Therefore, the findings in this work will greatly help to fill up these scientific gaps locally and globally.

Chapter 3: Materials and Methods

3.1 Introduction

This chapter describes the materials, instruments, and methods employed to carry out experimental studies and analysis of the data obtained in this work.

3.2 Materials

All the reagents used in this study were of analytical grade. Hexane (C_6H_{10}), Chloroform ($CHCl_3$), Acetic Acid (CH_3CO_2H), Potassium Hydroxide (KOH), Potassium Iodate (KIO_3), Silica Gel 60, was purchased from Himedia. The rest of the reagents: Methanol (CH_3OH), Ethanol (CH_3CH_2OH), Acetone ($(CH_3)_2CO$), Diethyl Ether ($(C_2H_5)_2O$), Sodium Hydroxide (NaOH), Iodine (I_2), Potassium Iodide (KI), Thymolphenothlein ($CH_3(CH_2)_{16}COOH$), Aluminium Chloride ($AlCl_3$), Ammonia (NH_3) Lithium Sulfate ($LiSO_4$), Sodium Molybdate ($Na_2MoO_4.2H_2O$), Sodium Tungstate (Na_2WO_4) Sodium Carbonate ($CaCO_3$), Bromine (Br_2), Phosphoric Acid (H_3PO_4), Hydrochloric Acid (HCl), Sulphuric Acid (H_2SO_4), Formic Acid (HCO_2H), Xylene ($C_6H_4(CH_3)_2$), Haematoxylin ($C_{16}H_{14}O_6$), Eosin ($C_{20}H_6Br_4Na_2O$), Gallic Acid ($C_7H_8O_6$), Selenium (Se), Quercetin ($C_{15}H_{10}O_7$), Formaldehyde (CH_2O), were obtained from the Chemistry department at the University of Zambia. Cypermethrin EC 20% and Neemcide (0.03% ~ Azadirachtin) were purchased from reputable Agro-Vet shops in the town centre, Lusaka district.

3.3 Instrumentations

Rotavapor (BUCHI RII, Flawil, Switzerland), UV-VIS Spectrophotometer (Shimadzu UV-2600, Japan), FTIR Spectrophotometer (Shimadzu, IR spirit, Japan), HPLC (Shimadzu, LC-2030, Japan), Gas chromatography- Mass Spectrophotometer (Jas UNIS, Agilent Technologies (6890N Network GC system and 5975 inert XL Mass Selective Detector) Zurich, Switzerland), Tissue processer (Thermo Scientific, Microm, SPT 120, Japan), Bellingham + Stanley Abbe5Refractometer(xylem, Germany), Compound Microscopy (Thermo Fisher Scientific, Ecoline, USA), Electronic balance (Scout pro, OHAUS, Parsippany, NJ, USA), and Sonicator (Elmasonic S 40 H, Singen, Germany), Philips blender (Philips Electronics, HR 2061, Koninklijke, Netherlands).

3.4 Sample Collection and Preparation

In this sub-section, a description of the collection and preparation of CNSs and mass rearing of the FAWs are described.

3.4.1 Collection and Preparation of CNSs

Cashew nutshells were collected from small-scale cashew processors in Mongu District, Western Province of Zambia in October 2020. The shells were first washed with tap water to remove all the dirt and other contaminates and then rinsed with sufficient distilled water. Thereafter, the shells were shade-dried in the air for three weeks and size reduction was achieved by the use of a Philips domestic blender (Koninklijke Philips Electronics, Netherlands). The powdered shells were placed in airtight bags and stored at 4 °C to avoid biological and chemical degradation of the constituents and were only removed when required for analysis (Nyirenda et al., 2021; Zombe et al., 2022).

3.4.2 Collection and Mass Rearing of FAW

The FAW larvae, eggs and adults were collected from three different locations in Lusaka province of Zambia namely University of Zambia (UNZA) (Latitude: 15.3942” S, Longitude: 28.3350” N), Mount Makulu; Zambia Agricultural Research Institute (ZARI) (Latitude: 15.54473” S, Longitude: 28.2620” N), and Kasisi Agricultural Training Center (KATC) (Latitude: 15.2686” S, Longitude: 28.4733” N) from December 2020 to February 2021. The collected FAW insect species were identified by entomologists from ZARI and the Department of Biological Sciences, UNZA. Mass rearing of the FAW for studies was done in the Insectarium Laboratory, in the Department of Plant Science, School of Agricultural Sciences, UNZA. The FAW insects were kept under controlled laboratory conditions (temperature: 27 ± 2 ºC, relative humidity: 70 ± 5%, and photoperiod: 12 light: 12 dark). The adult moths were kept in square cages (50 cm x 50 cm) and fed on 10 % natural pure honey. Potted maize plants were provided in the cages for egg deposition by adults. The laid egg bunches on maize leaves were harvested and placed in long plastic bowls (13 cm x 21 cm), covered with a net to prevent newly hatched neonates from escaping. The newly emerged neonates were separated into groups of 20 and placed in plastic bowls (24 cm x 15 cm x 8 cm) and fed on fresh maize leaves (MRI 413). The food was changed very after 24 hours. Once the larvae reached the third-instar stage, they were

separated into groups of 10 and placed in bigger plastic bowls (30 cm x 18 cm x 9 cm) to avoid cannibalism (Prasanna et al., 2018).

3.5 Extraction of CNSL from CNS

The following sub-section describes the rationale and the experiments employed to optimisation the extraction of CNSL from CNSs.

3.5.1 Optimization experiments

3.5.1.1 Effect of Solvent Type on the Recovery of CNSL from CNS

The extraction of CNSL from CNS was based on a method described by Uslu and Özcan (2019) and adopted by Slatnar et al. (2015), with minor modifications. Two grams of powdered CNS (1.68 mm particle size) were transferred into a 50 mL centrifuge tube containing 20 mL of the respective organic solvents (methanol, ethanol, acetone, chloroform and hexane). The total solvent-to-sample ratio was fixed at 1:10 (w/v) for all the solvents. The mixture was sonicated at room temperature (27 ± 2 ^{o}C) for five minutes in an ultrasound water-bath device (Elmasonic S 40 H, 37 kHz, 140 W; Elma, Germany). Thereafter, the mixture was centrifuged at 3000 rpm for 10 min, and then the supernatant was poured into a storage container, while the residual shell was re-extracted further with another fresh solvent and treated as outlined above. The supernatant from the first extraction was combined with the second and concentrated on a Rotavapor at 40 ^{o}C until a constant mass was attained. The per cent yield of CNSL was calculated using the formula shown below. All experiments were replicated three times, and the results were expressed as M ± SEM (mean ± standard error of the mean).

$$\textbf{CNSL\%} = \frac{\textbf{Mass of extract (g)}}{\textbf{Dry mass of CNS (g)}} \textbf{ x 100 \%} \qquad \textbf{(1)}$$

3.5.1.2 Effect of Sample-To-Solvent Ratio on the Recovery of CNSL from CNS

Two grams of powdered CNS were accurately weighed and transferred into 50 mL centrifuge tubes containing different volumes of hexane to make the sample-to-solvent ratios of 1:5, 1:10, 1:15, and 1:20, respectively. The mixtures were then treated as described in the procedure under **section 3.5.1.1** above.

3.5.1.3 Effect of Extraction Time on the Recovery of Crude CNSL from CNSs

Two grams of powdered CNS were weighed and transferred into 50 mL centrifuge tubes containing 20 ml of hexane (sample/solvent ratio (1:20). The mixture was sonicated at room temperature (27 ± 2 ºC) at four different times 5, 10, 15, and 20 mins respectively in an ultrasound water-bath device. The mixtures were then treated as described in the procedure under **section 3.5.1.1** above.

3.6 Characterization of Crude CNSL

This section highlights various methods that were used to characterize the extracted CNSL.

3.6.1 Physicochemical Properties of Crude CNSL

The physicochemical properties of CNSL were determined by methods described by Hamid, (2016). These parameters are important in understanding the quality, composition and possibly the best end-use for a product.

3.6.1.1 Determination of Specific Gravity of Crude CNSL

A pre-cleaned 25 ml specific gravity bottle was weighed and then filled to the brim with distilled water. A stopper was inserted and any water that spilt out through the capillary opening on the stopper was carefully wiped off. After weighing, the bottle was dried, and then filled with CNSL and re-weighed. The procedure was replicated thrice. The specific gravity of CNSL was calculated from the following relationship.

$$SG = \frac{wc - wa}{wb - wa} \qquad (2)$$

Where:

wc = weight in grams of specific gravity bottle with CNSL at 26°C.

wa= weight in grams of specific gravity bottle at 26°C.

wb = weight in grams of specific gravity bottle with water at 26°C.

3.6.1.2 Determination of Refractive Index of Crude CNSL

The refractive index of CNSL was determined by placing two drops of the extracted oil on the prism of an Abbe refractometer model A 80251 (BS) using a syringe and then the prism was firmly closed by tightening the screw head. The instrument was allowed to stand for 5 min before taking the reading displayed on the screen. The procedure was replicated thrice.

3.6.1.3 Determination of Viscosity of Crude CNSL

The Oswald's pipette viscometer was calibrated with distilled water at room temperature. The time taken for the CNSL to fall between the two graduation marks at room temperature was recorded. The viscosity of CNSL was calculated using the formula below (Ikhuoria and Maliki, 2007).

$$\eta y = \eta w \frac{dy\ ty}{dw\ tw} \qquad (3)$$

Where:

ηy = viscosity of CNSL.

ηw = viscosity of water at 25 ^{o}C.

dy = density of CNSL.

ty = time of runoff of CNSL in seconds.

dw = density of water.

tw = time of runoff of water in seconds.

3.6.1.4 Determination of Saponification Number

An extracted CNSL sample of 1.5 g was weighed into a clean dried quick-fit conical flask (250 ml) and 25 ml of alcoholic potassium hydroxide (KOH) was added. A reflux condenser was attached and the flask was heated for 45 mins with frequent shaking. The condenser was washed down with ten millilitres of neutral alcohol, and the solution cooled to room temperature. Then one millilitre of one per cent thymolphthalein indicator was added to the solution and titrated with 0.5 M hydrochloric acid (HCl) until it reached the blue colour end-point. A blank titration was carried out at the same time and under the

same conditions. All experiments were replicated three times and the formula below was used to calculate the saponification number.

$$\mathbf{S.N} = \frac{\mathbf{56.1\ x\ (B - E)\ x\ N}}{\mathbf{dry\ mass\ of\ CNSL}} \qquad \mathbf{(4)}$$

Where:

N = Normality/concentration of the standard hydrochloric acid.

B = Volume in mL of standard hydrochloric acid required for the blank.

E = Volume in mL of standard hydrochloric acid required for the sample.

3.6.1.5 Determination of Iodine Number

Approximately, 0.50 g of CNSL was weighed into a 250 ml conical flask. Thereafter, 15 ml of chloroform was added and the mixture was thoroughly mixed. After complete dissolution of the sample, 25 mL of the Hanus reagent solution was added and the solution was allowed to stand in the dark for 30 min at room temperature. After the time elapsed, 10 ml of 10% potassium iodide (KI) solution was then added followed by 100 ml of freshly boiled and cooled distilled water. The resulting solution was titrated against 0.1M sodium thiosulphate ($Na_2S_2O_3$) using a starch indicator. A blank titration was carried out at the same time and under similar conditions. The procedure was replicated three times and the iodine number was calculated from the formula below:

$$\mathbf{I.N} = \frac{\mathbf{(B - E)\ x\ N\ x\ 12.69}}{\mathbf{dry\ mass\ of\ CNSL}} \qquad \mathbf{(5)}$$

Where:

N = normality of the standard sodium thiosulphate solution.

B = volume in ml of standard sodium thiosulphate solution required for the blank.

E = Volume in ml of standard sodium thiosulphate solution required for the sample.

3.6.1.6 Determination of Acid Value

Approximately, 0.5 g of CNSL was weighed into a 250 ml clean dried conical flask. Thereafter, 50 ml of freshly neutralized ethanol and one millilitre of thymolphthalein indicator were added. The solution was boiled in a water bath for 5 min and titrated while

hot against a standardized solution of potassium hydroxide (KOH) unit a blue colour end-point. The procedure was replicated three times and the acid number was calculated from the formula below:

$$\mathbf{A.V = \frac{V\ x\ N\ x\ 56.1}{dry\ mass\ of\ CNSL}} \qquad (6)$$

3.6.1.7 Free Fatty Acid

Free Fatty Acid was calculated form acid value using the formula below;

$$\mathbf{F.F.A = \frac{28.2\ x\ N\ x\ V}{dry\ mass\ of\ CNSL}} \qquad (7)$$

Where:

V = titer volume.

N = Molarity of potassium Hydroxide (KOH).

3.7 Phytochemical Analysis

The presence of secondary metabolites in CNSL was investigated by using several different methods as described by various authors Jaradat et al. (2015), Banda et al. (2017), Gul et al. (2017), Suradkar et al. (2017), Banda et al. (2018), and Zombe et al. (2022). The secondary metabolites analyzed included alkaloids, saponins, steroids, terpenoids, glycosides, flavonoids, phenols and tannins. Briefly, one gram of CNSL was dissolved in an appropriate solvent (80 % ethanol or methanol) and filtered with a Whatman filter paper and the following tests were analyzed on the filtrate.

3.7.1 Qualitative Analysis of Phytochemical Compounds in Crude CNSL Extracts

Under this section the common tests used to identify various phytochemical compounds in plant extracts are highlighted. Qualitative test analysis helps to identify the compounds that are present in a plant without quantifying them. The advantage is that the tests are simple and less time-consuming.

3.7.1.1 Test for Tannins/Phenols by Ferric Chloride Solution

Two millilitres of CNS extract solution in a test tube was mixed with three drops of five per cent Ferric Chloride. The appearance of a transient greenish to dark-bluish colour indicated the presence of phenol and tannins.

3.7.1.2 Test for flavonoids

Several tests are used to analyse flavonoids in plant extracts. The alkaline and Shinoda's tests were used in this work because they have been commonly used in literature.

3.7.1.2.1 Alkaline Test

Two millilitres of CNS extract solution were mixed with two millilitres of two per cent sodium hydroxide. The Formation of a deep yellow colour, which turned colourless after the addition of dilute hydrochloric acid, indicated the presence of flavonoids.

3.7.1.2.2 Shinoda's Test

To two millilitres of CNS extract solution, a sizeable piece of magnesium ribbon (Mg) was added, followed by one millilitre of dilute hydrochloric acid (HCl). The formation of a rose-pink colour in the solution indicated the presence of flavonoids.

3.7.1.3 Test for Alkaloids

Alkaloids in plant extracts can be tested by several reagents. Mayer's reagent is commonly used in literature.

3.7.1.3.1 Mayer's Test

Two millilitres of CNS extract solution was mixed with five drops of Mayer's reagent. The appearance of a whitish-creamy precipitate indicated the presence of alkaloids.

3.7.1.3.2 Wagner's Test

Acidified two millilitres of CNS extract solution was mixed with five drops of Wagner's reagent. The formation of a reddish-brown precipitate indicated the presence of alkaloids.

3.7.1.4 Test for Saponins (Foam Test)

Five millilitres of CNS extract solution was mixed with ten millilitres of distilled water (H_2O). The mixture was shaken vigorously and left to stand undisturbed. The formation of persistent foam (about three mins) indicated the presence of saponins.

3.7.1.5 Test for Glycosides

Two millilitres of CNS extract solution was mixed with two millilitres of glacial acetic acid. Then, three drops of five percent Ferric chloride were added, followed by two millilitres of concentrated sulphuric acid (H_2SO_4) along the sides of the test tube. The appearance of a reddish-brown colour in the lower layer indicated the presence of glycosides.

3.7.1.6 Salkowski's Test for Terpenoids

Three millilitres of CNS extract solution was mixed with two millilitres of chloroform, followed by three millilitres of concentrated sulphuric acid (H_2SO_4) carefully added along the sides of the test tube. The formation of a reddish-brown colour at the interface indicated the presence of terpenoids.

3.7.1.7 Test for Steroids

Two millilitres of CNS extract solution was mixed with three millilitres of chloroform, and then two millilitres of concentrated sulphuric acid (H_2SO_4) was added along the sides of the test tube. The formation of a reddish-brown ring at the interface indicated the presence of steroids.

3.7.2 Quantitative Determination of Secondary Metabolites in Crude CNSL

Quantitative analysis gives the actual or proximate amount of an analyte in a sample. This section describes the procedures that were used to quantify the concentration of phenols and flavonoids in this work.

3.7.3 Determination of Total Phenolic Content (TPC)

The TPC of crude CNSL was determined by Folin-Ciocalteau's method as described by Bahramsoltani et al. (2017) with modifications. Briefly, a series of working standards of Gallic Acid ranging from 2 to 6 mg/L were prepared. An aliquot of 0.5 ml (either sample

or standard) was mixed with 0.5 ml of 10 % (v/v) Folin-Ciocalteau's reagent. The mixture was mixed with a vortex and left to stand for five minutes at room temperature. Thereafter, three millilitres of distilled water was added to the mixture followed by two millilitres of 20 % Na_2CO_3. The solution was then incubated for 90 min at room temperature in the dark. The absorbance of the solution was measured with a UV-visible spectrophotometer at a wavelength of 765 nanometres (nm) against a blank. The TPC of crude CNSL was determined from the Gallic Acid standard curve and the results were expressed as mg of Gallic Acid Equivalent /gram of dry sample (GAE/g).

3.7.3.1 Determination of Total Flavonoid Content (TFC)

The TFC of crude CNSL was determined by the modified aluminium chloride colourimetric method as described by da Silva et al. (2015). A series of working standards of different concentrations of Quercetin ranging from 2 to 6 mg/L were prepared. Two millilitres of the aliquot (either the standard/or CNSL) were mixed with two millilitres ml of two per cent aluminium chloride solution and incubated at room temperature for 60 mins. The absorbance of the solutions was measured at 420 (nm) was a UV-visible spectrophotometer against the blank (80% methanol). All the experiments were repeated in triplicates and the TFC of crude CNSL was calculated from the quercetin standard curve, and the results were expressed as mg of Quercetin Equivalent (QE)/gram of dry sample (QE/g).

3.8 Chemical Composition of Crude CNSL

In order to identify the actual chemical compounds that are present in a crude plant extract, several chemical compositional analysis techniques are used such as Thin layer chromatography (TLC), Column chromatography (CC), High-Performance Liquid Chromatography (HPLC), Mass spectrometry (MS), Nuclear Magnetic Resonance (NMR) and so on. To identify the chemical compounds in our extracted CNSL the following techniques were used; TLC, CC, GC-MS, and HPLC.

3.8.1 TLC Analysis of Crude CNSL

Thin layer chromatography (TLC) was used to separate phenolic compounds in CNSL as described by Yuliana et al., (2014). Briefly, 0.5 mg of crude CNSL was dissolved in ten millilitres of hexane and spotted on a TLC plate (10 x 5 cm) (pre-coated Silica gel alumina

sheets, Kieselgel 60 F254, 0.20 mm, Merck). The plate was dried on a hot surface and placed in a developing chamber containing 20 ml of mobile phase hexane: diethyl ether: formic acid mixture (12:8:1,v/v/v). The plate was removed from the chromatogram chamber when the solvent front reached almost seven centimetres above the baseline. Identification of the spots was achieved by either iodine vapour or a developing reagent consisting of (300 mg Fast Blue B) dye dissolved in 25 ml acetone–water mixture (9:1, v/v).

3.8.2 Isolation of CNSL Alkyl Phenolic Compounds By Column Chromatography

The phenolic lipids from crude CNSL were isolated using a silica gel column (Silica Gel 60-270 mesh). A thirty centimetres long glass column with a diameter of five centimetres was filled with 15 grams of silica gel slurry. Then one gram of sample was loaded on top and eluted in a stepwise gradient with 50 ml of n-hexane–diethyl ether (10:0, 50:50, and 0:10 by volume) and finally with methanol. The fractions obtained were analysed for purity by TLC. Fractions with similar compounds were combined, while those that did not separate were put back in the column (Lomonaco et al., 2009).

3.8.3 GC-MS Analysis of CNSL

GC-MS analysis of crude CNSL was performed on a Jas UNIS, Agilent Technologies (5975 inert XL Mass Selective Detector) and (6890 network GC system) fitted with a (Crossbond®5 % diphenyl/95 % dimethylpolysiloxane) Rxi-5 MS column: 60 m x 0.25 mm ID x 0.25 µm film diameter. A one microliter aliquot of 10 mg/mL solution of CNSL in hexane was injected (Agilent Technologies, 7683B series injector) into the machine with a split ratio of 50. Helium gas (99.99 %) served as the carrier gas at 1mL/min of constant flow rate with an injector temperature of 260 ºC. The initial oven temperature was 60 ºC (hold time 4 min), which was increased to 300 ºC at the rate of 10 ºC/min (hold time 15 mins). The identification of chemical constituents of CNSL was achieved by comparing the individual mass spectra with databases of the National Institute of Standards and Technology (NIST12 or NIST62) and Wiley 229 mass spectrometry libraries (Leite et al., 2015).

3.9 Toxicity Studies

The following section highlights the procedures employed to evaluate the ovicidal, larvicidal and antifeedant effects of CNSL against FAW.

3.9.1 Ovicidal Effect of Crude CNSL against FAW Eggs

The Ovicidal effect of CNSL was conducted as described by Magrini et al. (2015) with modifications. Briefly, 30 eggs (24 h, old) of FAW were placed on a strip of filter paper (4 cm x 2 cm) that was stuck to the base of a petri dish (Diameter: 8.5 cm). Thereafter, 200 µl of different concentrations of CNSL solutions (1, 3, 5 and 7 w/v %) were applied to the eggs using a 200 µl Hamilton syringe. The excess solvent on the eggs was dried in the air for about 30 mins. A damp cotton wool was placed in the petri dish to keep the environment moist. The treated eggs were then kept under laboratory conditions (temperature 27 ± 2 ºC, relative humidity 70 ± 5 %, and photoperiod 12 light: 12 dark) until they hatched. Each treatment was replicated four times. Cypermethrin (20% EC) was used as the positive control, while acetone, as a negative control. The mortality of the eggs was monitored from the second day after treatment up to the sixth day. The eggs that did not hatch after the sixth day were considered dead. The Abbott corrected per cent mortality of the eggs was calculated from the formula below.

$$\textbf{Mortality rate} = \frac{\textbf{number of unhatched eggs}}{\textbf{intial number of eggs}} \textbf{X 100 } \% \qquad (\mathbf{8})$$

3.9.2 Larvicidal Effect of CNSL against FAW Larvae

The larvicidal effect of CNSL against FAW larvae was carried out by a contact toxicity method as described by Phambala et al. (2020) with minor modifications. Five different concentrations of CNSL were selected 0.5, 1, 3, 5, and 7 w/v %. Then five microliters of each concentration were applied to the entire body of a larva using a 200 µL Hamilton syringe. Six second-instar larvae were placed on a clean petri dish (Dia. 8.5 cm) containing a 4 x 4 cm^2 maize leaf as a natural diet. A damp cotton wool was placed in the petri dish to keep the environment moist. Cypermethrine (EC) 20% was used as the positive control while acetone as a negative control. Each treatment was replicated 20 times and mortality data was recorded after 24 hours of treatment. A larva was considered dead if it failed to

lift itself upright after being turned upside down with a soft brush. Mortality data was corrected using Abbott's formula (Tapondjou et al., 2005).

$$\% \mathbf{AC} = \frac{\mathbf{LC} - \mathbf{LT}}{\mathbf{LC}} \mathbf{X\ 100}\% \qquad (9)$$

Where:

AC = Abbott correction.

LC = live insect in control.

LT = live insect in the treatment.

3.9.3 Histology Study of the Effect of CNSL against FAW Larvae

Histological studies were performed according to the method adopted by Ruiz et al. (2004) and Khedher et al. (2017). Second-instar larvae of FAW were treated with five per cent CNSL solution in acetone and fixed in ten per cent neutral formalin for 24 hours at 4 ºC. The fixed larvae were loaded on a tissue processor (Thermo Scientific, Microm, SPT 120, Japan) for 24 hours. The processed tissue was embedded in paraffin wax and fine sections (3 µm) were obtained using a microtome. The sections were deparaffinized using 100% toluene and stained with hematoxylin-eosin. The histological sections of the treated larvae were compared with those of untreated larvae. The slides were observed using an Olympus VS 120 microscope.

3.9.4 Antifeedant Effect of Crude CNSL against FAW Larvae

The antifeedant effect of CNSL against FAW was carried out in a no-choice feeding assay as described by Huang et al (2008) with modifications. Two hundred fourth-instar larvae of FAW were starved for eight hours. Then two starved larvae were released on the petri dish (Diameter: 8.5 cm) containing a maize leaf disc of diameter five centimetres that was dipped for 30 seconds in CNSL solution of varying concentrations 0.5, 1, 3, 5, and 7 w/v %, and dried in the air for 20 min. Before the leaf disc was placed on the plate it was captured with a Canon camera. The experiment was stopped when the larvae in the control experiments had eaten at least 50 % of the leaf disk. The area consumed by the larvae was calculated by using digital image processing software (ImageJ.JS, 2016 version). Each treatment was replicated 15 times. Acetone solvent was used as a negative control, while Neemcide (Active ingredient, 0.03% Azadirachtin) was used as a positive control. The

formula below was used to calculate the feeding inhibition index (AFI) of CNSL against FAW (Huang et al., 2008).

$$\% \text{ AFI} = \frac{(C\text{-}T)}{(C+T)} \times 100\% \quad \textbf{(10)}$$

Where: C is the leaf area consumed in control and T is the leaf area consumed in treatments.

3.10 Statistical Analysis

The data obtained in this work was analysed with various tools and results were expressed as Mean ± SEM of their replications. The bar graphs were produced in GraphPad Prism 10. The relationship between the means was analysed by One-Way Analysis of variance (one-way ANOVA), and Means were separated by Tukey's test significance of $p < 0.05$. The LC_{50} and EC_{50} and their 95 % confidence limits and the Chi-squared values were calculated by Probit analysis using the Statistical Package for Social Sciences SPSS 2021 version for Windows software.

Chapter 4: Results and Discussion

4.1 Introduction

This chapter discusses in detail the results obtained from extraction, characterization and evaluation of the insecticidal properties of crude CNSL against FAW eggs and larvae.

4.2 Physical and Morphological Characteristics of CNSs

The physical and morphological characteristics of the collected CNSs were determined as described by Semporé et al. (2021). The collected CNSs were kidney-shaped with a hard smooth shiny texture. The outer surface of the shell was greyish with few dark patches probably due to roasting during the processing stage, while the inner side was dark brown (**Figure 4-1**). The average weight, length, width and thickness of an individual CNS calculated from 50 randomly selected CNSs were 1.77 ± 0.06 g, 32.00± 2.92 mm, 20.08 ± 1.85 mm and 3.08 ± 0.28 mm, respectively (**Figure 4-2**). Our physical description results of the CNS corroborate with a lot of literature sources (Mwangi, 2013; Couto et al., 2004). According to literature sources, the colour of the CNS can range from grey to brown depending on the processing method (Couto et al., 2004), while, the thickness of the CNS is generally reported to be 3 mm (Couto et al., 2004; Chaudhari and Thakor, 2015).

Figure 4-1. Physical appearance of the CNSs.

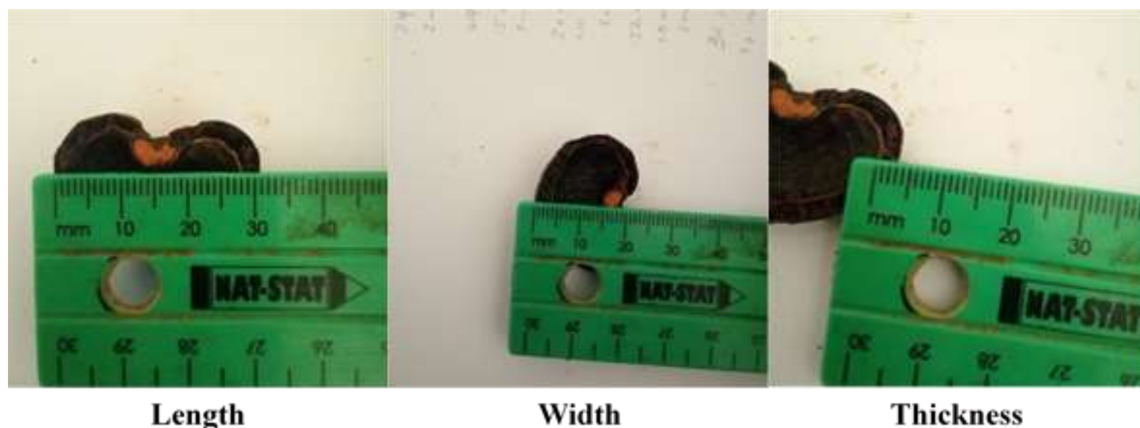

Figure 4-2. Physical measurements of the CNSs

4.3 Optimization of Crude CNSL Extraction from CNSs

In this work, the effect of extraction time, sample-to-solvent ratio and type of solvents were considered during the optimization of the extraction of crude CNSL from the CNSs.

4.3.1 Effect of Solvent Type on the Recovery of CNSL from CNSs

The impact of solvent type on the percent yield of CNSL from CNS is presented in **Figure 4-3**. The five different organic solvents (hexane, chloroform, acetone, ethanol, and methanol) were chosen for the extraction of CNSL based on their different polarities, and wide application in the extraction of phytochemicals from plant materials (Zhang et al., 2018; Abubakar and Haque, 2020).

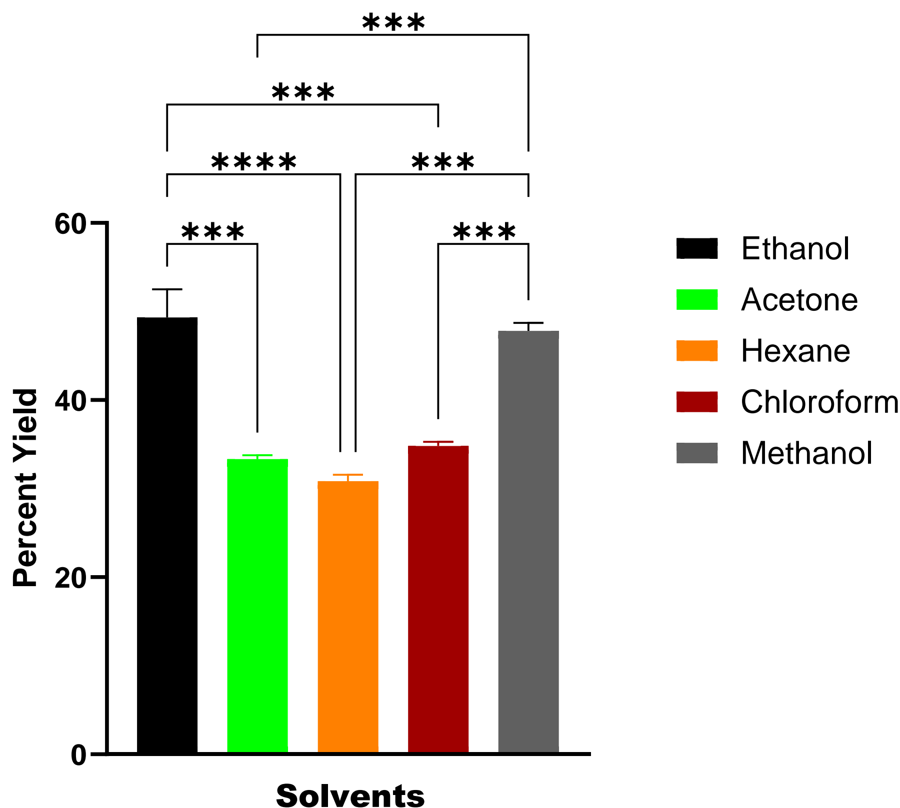

Figure 4-3. Effect of solvent type on the per cent yield of crude CNSL. Bars connected with the same line are significantly different by Tukey's test analysis ($p < 0.05$). *** ($p \leq 0.001$), and **** ($p \leq 0.0001$).

The yields of CNSL varied from 30.8 ± 0.7 to 49.3 ± 3.2 %. Ethanol (49.3 ± 3.2 %) and methanol (47.8 ± 0.9 %), recorded the highest yields, then followed by chloroform (34.8 ± 0.4 %), and acetone (33.3 ± 0.4 %), and finally, hexane (30.8 ± 0.7 %) with the least. Generally, polar solvents recorded higher yields than nonpolar solvents. This trend was also been observed by other authors (Gandhi et al., 2013; Nyirenda et al., 2021). In this work, the average per cent yield difference between polar and nonpolar solvents was 16.0 %. This huge difference suggested that polar solvents were more efficient in extracting CNSL from the CNS than nonpolar solvents. It could be because the majority of compounds in CNS are polar compounds. According to literature, about 30-35% by weight of the dry CNS is CNSL, a dark brown viscous liquid which is chiefly composed of four major polar phenolic liquids in various proportions namely anacardic acid (70 %),

cardol (10 %), cardanol (5 %), and methyl cardol (5%) (Tyman et al., 1989; Gandhi et al., 2012; Nasrollahzadeh et al., 2019). Besides having these compounds, the CNS has also been reported to contain other minor polar phenolic compounds such as tocopherols, flavonoids and naphthoquinones (Gómez-Caravaca et al., 2010). Hence, the higher yields recorded by polar solvents in this work could be because these solvents were extracting more of the aforementioned compounds. The rule of thumb is '*like dissolves like'*, thus it is expected that highly polar solvents will extract more polar compounds. The per cent yields of CNSL in this work corroborate with other literature sources. In this work, acetone, hexane and ethanol recorded yields of 33.3 ± 0.4 %, 30.8 ± 0.7 %, and 49.3 ± 3.2 %, respectively, while, other authors reported yields for acetone, hexane, and, ethanol as 33.08 %, 30.52 % and 40.1 %, respectively (Gandhi et al., 2013; Nyirenda et al., 2021). However, there was a major difference between our per cent yield for methanol 47.8 ± 0.9 %, and 5.0 % reported by Gandhi et al. (2012). Nonetheless, our result for methanol was slightly similar to the 38.36 % reported by Yuliana et al. (2012).

4.3.2 Effect of Extraction Time on the Recovery of CNSL from CNS

The effect of extraction time on the yield of CNSL from CNS is presented in **Figure 4-4**. The per cent yield ranged from 20.84 ±1.40 % to 30.10 ± 0.84 % at 5 to 20 mins. Shorter sonication times ranging from 5-20 mins were used because literature sources showed that prolonged exposure of phytochemical compounds to frequencies above 20 kHz has a deleterious effect on the active phytochemical compounds (Vinatoru, 2001; Pandey and Tripathi, 2014). The optimum extraction time in this work was found to be 15 mins. This is because there was no significant difference in the per cent yields between 15 and 20 mins ($P < 0.05$). Ultrasound-assisted-solvent extraction technique quickens the extraction process of plant compounds. It uses acoustic energy and solvent power to extract phytochemical compounds from plant materials. The mechanical effect of ultrasound enhances the extraction process by providing a greater penetration power of the solvents into the matrix and quick release of the cell content due to the break-up of plant tissues (Ebringerová and Hromádková, 2010). Hromádková et al. (2008), reported that ultrasound-assisted extraction reduced the time required to extract water-soluble polysaccharides from wheat bran from 60 minutes by classical (maceration with continuous stirring) method to five minutes.

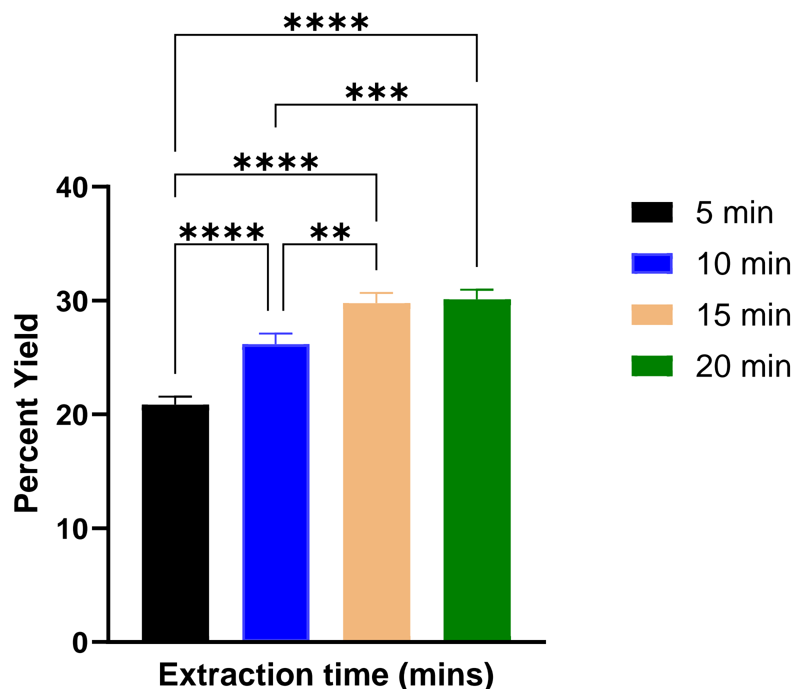

Figure 4-4. Effect of extraction time on the per cent yield of crude CNSL. Bars connected with the same line are significantly different by Tukey's test analysis ($p < 0.05$). ** ($p \leq 0.01$), *** ($p \leq 0.001$), and **** ($p \leq 0.0001$).

4.3.3 Effect of Sample –To-Solvent Ratio on the Per cent Yield of CNSL from CNS

The results for the effects of sample-to-solvent ratio on the yield of CNSL from CNS are presented in **Figure 4-5**. The per cent yields of CNSL at four different ratios, 1:5, 1:10, 1:15 and 1:20 were 24.20 ± 2.49 %, 26.28 ± 1.87 %, 27.74 ±1.61 % and 28.15 ± 1.11 %, respectively by weight of the dry CNS. A glance at the results might seem like there was an improvement in the per cent yield of CNSL from CNS when the sample-to-solvent ratio was increased. However, statistical analysis revealed no significant difference among the ratios. Hence, in this work, the ratio of 1:20 was reported as optimum. The results in this study corroborate with Martínez Aguilera (2019) who reported per cent CNSL yields of 28, 33 and 39 % from sample-to-solvent ratios of 1:7, 1:3 and 1:20, respectively. Based on these results, it was concluded that the ratio of 1:20 was the optimum.

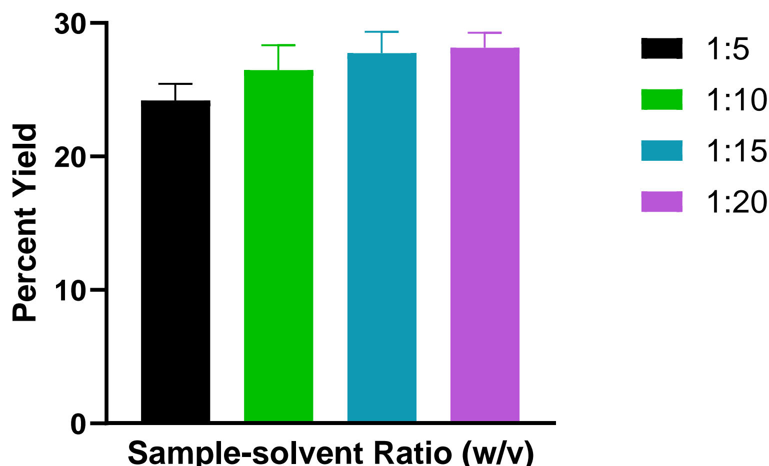

Figure 4-5. Effect of sample-solvent-ratio on the per cent yield of CNSL.

Our optimization studies showed that solvent type and extraction time have a great influence on the recovery of CNSL from the shell as compared to the sample-to-solvent ratio. Therefore, solvent type and extraction time must be carefully considered to obtain a good yield of CNSL from the shell. From our study, polar solvents such as methanol, ethanol, and acetone, and extraction time of 20 mins as well as the sample-to-solvent ratio of 1:20 are recommended.

4.4 Phytochemical Analysis of CNS Extracts

The phytochemical analysis results for CNS extracts are shown in **Table 4-1**. The results revealed that CNS extracts are rich in several phytochemical compounds including phenols, tannins, alkaloids, flavonoids, saponins, steroids, terpenoids, and glycosides. Several studies have reported that extracts from CNS exhibit numerous biological activities ranging from antimicrobial, antifungal, insecticidal, antioxidant and therapeutical properties (Dorathy et al., 2017; Prakash et al., 2018; Lubi and Thachil, 2000). Chaeib (2010) reported that saponins exhibit anti-feeding, growth regulation and mortality of harmful insects. Fowsiya and Madhumitha (2020) reported that alkaloids disturb the cellular and physiological processes of insects via redox imbalance and hormonal regulation. Acero, (2014) reported that flavonoids affect the normal functioning of insects by interfering with biochemical reactions such as mitochondrial enzymes (Vandock et al., 2012). Duke, Keeler and Tu (1991) reported that terpenoids act as

repellents, attractants, oviposition cues and antifeedants. The presence of all these phytochemical compounds in the CNS extracts suggested that it might exhibit biological activities against FAW. The results in this study, also confirmed data reported by other literature sources (Lubi and Thachil, 2000) that CNS extracts are a good source of biologically active compounds.

Table 4-1. Phytochemical compounds present in various CNS extracts.

Phytochemicals	Eth*	Met*	Chl*	Hex*	Ace*
Alkaloids	+	+	+	+	+
Phenols/Tannins	+	+	+	+	+
Flavonoids	+	+	+	+	+
Glycosides	+	+	+	+	+
Terpenoids	+	+	+	+	+
Steroids	+	+	+	-	-
Saponins	+	+	+	+	+

* Eth, met, chl, hex and ace represent ethanol, methanol, chloroform, hexane and acetone respectively. The symbol (-) represents absences of the tested secondary metabolite, while (+) represents presences of the tested secondary metabolite.

4.4.1 Total Phenolic Content

The TPC of CNSL extracts was analyzed by UV-Vis spectrophotometric method. A gallic acid standard curve was plotted and the TPC of the extracts was calculated from the standard curve using the equation ($Y = 0.0756x + 0.0083$, and $R^2 = 0.9918$). The results were expressed as mg of gallic acid Equivalent per gram of dry CNSL extract (mg GAE/g) **(Figure 4-6).** The results revealed that the nature of solvent type has a great influence on the recovery of phenolic compounds from CNS. The TPC ranged from 74.5 ± 2.7 to 101.2 ± 2.5 mg GAE/g. Acetone recorded the highest 101.2 ± 2.5 mg GAE/g, followed by methanol (99.5 ± 0.1 mg GAE/g), chloroform (95.4 ± 3.7 mg GAE/g), and then ethanol (83.3 ± 3.1 mg GAE/g) and finally hexane (74.5 ± 2.7 mg/GAE/g) with the least.

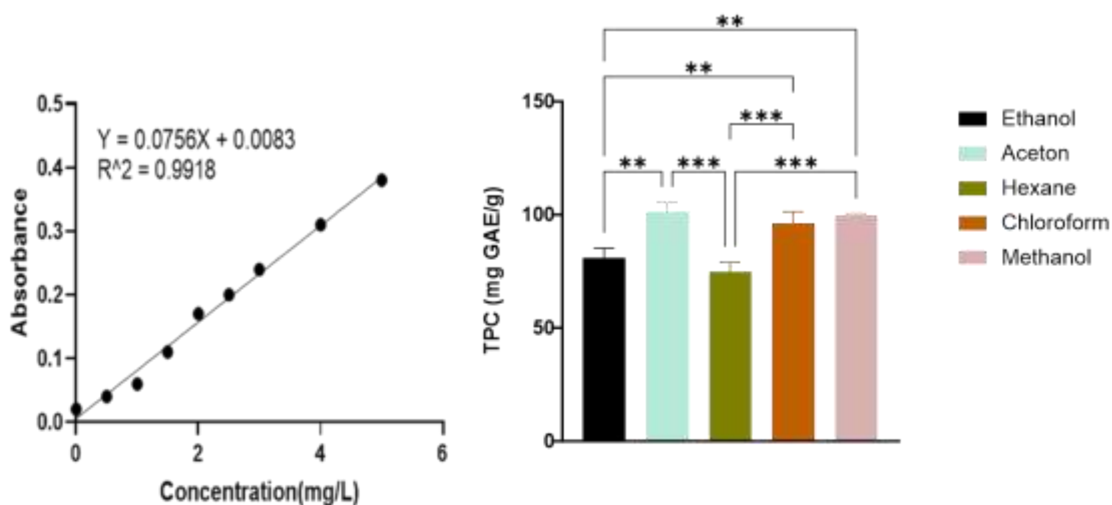

Figure 4-6. Effect of solvent type on the TPC of CNS extracts. Bars connected with the same line are significantly different as per Tukey's test analysis ($p < 0.05$). ** ($p \leq 0.001$), and *** ($p \leq 0.0001$).

Hexane recorded the lowest TPC probably because it is nonpolar. CNSL is believed to have a low composition of nonpolar compounds as compared to polar ones (Yuliana et al., 2012). The TPC of hexane was significantly different ($P < 0.05$) from other solvent extracts except for ethanol. Acetone recorded the highest content of phenol probably because it extracted both polar and minor polar compounds. Acetone has the ability to dissolve both polar and minor polar solutes (Eloff, 1998). Methanol, on the other hand, might have recorded the highest TPC because of its strong polarity. Hence, it was able to interact strongly with the major phenolic compounds in CNSL (Yuliana et al., 2012). Both acetone and methanol, either in pure forms or in aqueous mixtures have been reported to be good extracting solvents for phenolic compounds from plant materials (Zhang et al., 2018). In this work, chloroform recorded the third-highest TPC, probably because it was able to extract several minor polar flavonoid compounds (Abubakar and Haque, 2020). On the contrary, despite ethanol recording the highest per cent yield of CNSL, 49.3 ± 3.2%; it had the second lowest TPC. This is because it might have extracted a lot of non-phenolic compounds such as pigments (Yuliana et al., 2012, Nyirenda et al., 2021,) from CNSs. A fair distribution of TPC between polar and nonpolar solvents was observed in this work indicating that phenolic compounds in CNSL might have different polarities and

their extractability may be improved by mixing both polar and nonpolar solvents in various proportions.

4.4.2 Total Flavonoid Content

The TFC was determined by the aluminium chloride colourimetric method using Quercetin as a standard. A Quercetin standard curve was plotted and the TFC of CNS extracts was calculated from the standard curve using the equation ($Y = 0.606x + 0.0012$, $R^2 = 0.9945$). The TFC was expressed as milligrams of Quercetin Equivalent per gram of dry CNS extract (mg QE/g) (**Figure 4-7**). The highest TFC was recorded in hexane (33.3 ±0.7 mg QE/g), then followed by chloroform (27.2 ± 2.2 mg QE/g), ethanol (26.1 ± 1.2 mg QE/g), methanol (23.2 ±0.5 mg QE/g), and acetone (20.6 ± 2.5 mg QE/g), respectively.

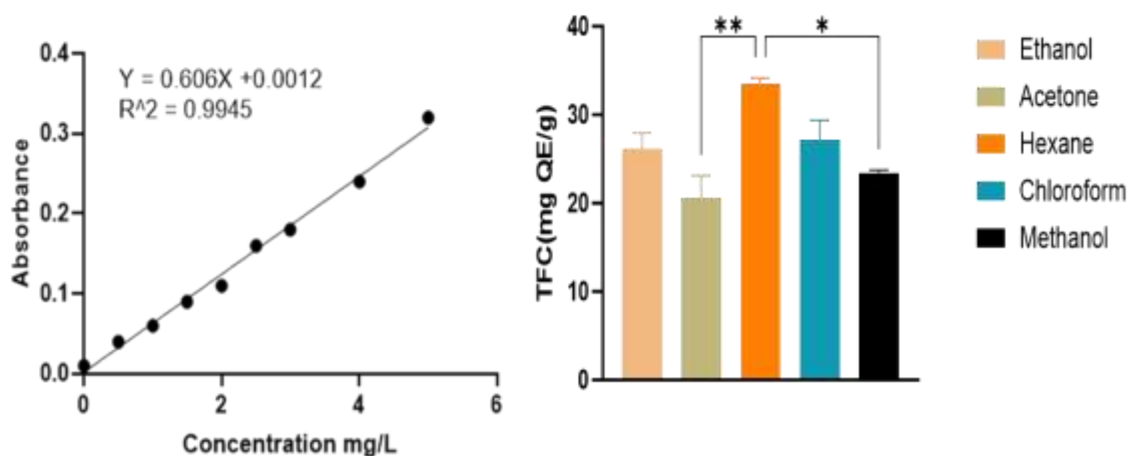

Figure 4-7. Effect of solvent type on the TFC of CNS extracts. Bars connected with the same line are significantly different by Tukey’s test analysis ($p < 0.05$). *($p \leq 0.05$), ** ($p \leq 0.01$).

In this work, nonpolar solvents, hexane and chloroform with polarity indices of 0.009 and 0.259, respectively, extracted more flavonoids than polar solvents such as methanol and acetone with polarity indices of 0.762 and 0.355, respectively (Abubakar and Haque, 2020). This suggested that the majority of flavonoids in CNS are nonpolar; hence, their extractability was enhanced within nonpolar solvents. A statistically significant difference ($p<0.05$) was found between methanol (most polar) and hexane (least polar) solvents. The

results in this study, corroborate with other literature findings that flavonoid extraction from plant materials is more favoured by nonpolar solvents than polar ones (Nawaz et al., 2020). Flavonoids are natural compounds present in plants. Studies have shown that high doses of flavonoids affect the normal functioning of insects (Acero, 2014). Flavonoids isolated from aqueous extracts of *Ammona squamosa* showed 80 % insecticidal activity against *Callosobruchus chinensis* at a concentration of 0.07 mg ml^{-1} (Kotkar et al., 2002). Vandock et al. (2012) reported that flavonoids interfere with biochemical reaction such as mitochondrial enzymes. Udebuani et al. (2015) remarked that that could be the reason why they exhibit insecticidal properties. Thus, the presence of a high concentration of flavonoids in CNSL extracted in this work suggested that it might have interesting insecticidal properties against FAW.

4.5 Physicochemical Properties of Crude CNSL

In this work, the physicochemical properties were determined on hexane-extracted CNSL. This is because its quality was better than other solvents. Polar solvents have been reported to extract other coloured pigments together with CNSL (Yuliana et al., 2012; Nyirenda et al., 2021). The physicochemical properties of CNSL are presented in **Table 4-2** below. The extracted CNSL was dark brownish and viscous in nature. Its specific gravity and refractive index values were 0.96 ± 0.00 g/cm3 and 1.52 ± 0.00, respectively. These values were higher than 0.91 g/cm3 and 1.43 reported by Gandhi et al. (2012), respectively but lower than 0.9999 g/cm3 and 1.686 reported by Akinhanmi et al. (2008) and Mohammed (2015), respectively. Specific gravity indicates whether a substance will float or sink in water, whereas, the refractive index is an optical property that gives an idea of the chemical composition of a substance (Speight, 2014). The viscosity of CNSL in this work was 104.6 ± 1.8 mPa.s. This value was lower than 160 mPa.s and 410 mPa.s reported by Mohammed (2015) and Rodrigues et al. (2011). The standard viscosity range for CNSL at 25 °C is 150–600 mPa.s. Our CNSL had viscosity slightly lower than standard. The acid and free fatty acid values for this work were 118.7 ± 9.2 mg KOH/g and 60.1 ± 4.7%, respectively. The acid value in this work was higher than most literature values 12.1, 15.5, 107 and 112 mg KOH/g reported by Mahanwar and Kale (1996) and Akinhanmi et al. (2008), but lower than 141 mg KOH/g reported by Achi and Myina (2011). The acid value measures the relative rancidity of an oil/fat as free fat acid.

Table 4-2. Physicochemical properties of hexane extracted CNSL

Physicochemical properties	This Study	Literature Values
% Yield	30.8-49.3	38 – 40 (Ghandi et al., 2013)
Colour	Dark brown	Reddish brown (Ghandi et al.,2013)
Nature	Viscous liquid	Viscous Liquid (Akinhanmi et al.,2008)
Specific gravity (g/cm^3 ,30 ^oC)	0.96 ± 0.0	0.9995 (Mohammed., 2008)
Refractive index (20 ^oC)	1.52 ± 0.0	1.686 (Mohammed., 2008)
Saponification number (mg KOH/g)	138.1 ± 3.2	161(Achi and Myina.,2011)
Iodine number (mg I_2/100g)	188.1 ± 2.3	177.7 (Achi and Myina.,2011)
Acid number (mg KOH/g)	118.7 ± 9.2	141 and 112 (Achi and Myina., 2011)
Free Fatty Acid %	60.1 ± 1.8	58 (Akinhanmi et al.,2008)
Viscosity (mPa-s ,24 ^{o}C)	104.6 ± 1.8	150-600 (Rodriques et al., 2011)
pH	3 ± 0.0	3.0 (Ach and Myina., 2011)

The values in the table represent the means of three replications expressed as (Mean ± SEM).

The saponification and iodine numbers for this work were 138.1± 3.2 mg KOH/g and 188.1 ± 2.3 mg I_2/100 g, respectively. The saponification number of oils/fats depends on the number of fatty acids present in a sample. The higher the saponification number, the higher the number of fatty acids and vice versa. It is also used to determine the average molecular weight of fatty acid chains in fats/ oils samples (Toscano et al., 2012). The iodine number indicates the unsaturation of fats/oils (Toscano et al., 2012). The higher the iodine value, the more unsaturated the fat/or oil sample is. CNSL has been reported to mainly consist of phenolic lipids with long hydrocarbon chains (C15) of varying unsaturation (Tyman, 1979). Thus, the high saponification and iodine number found in the study revealed that our extracted CNSL had a lot of phenolic lipids that were highly unsaturated. Overall, the physicochemical properties of CNSL in this work were within the range reported by other authors (Akinhanmi et al., 2008; Achi and Myina, 2011; Gandhi et al., 2012).

4.6 Chemical Composition Analysis

4.6.1 HPTLC Analysis of Crude CNSL

The extracted CNSL was subjected to thin-layer chromatography (TLC) to separate and partially identify the various components present in it. The chromatogram plates are presented in **Figure 4-8**. Visualization with UV light at 254 and 366 nm only produced one dark spot with a retention factor (R*f*) value of 0.74. Visualization with a fast-blue salt dye produced two distinctive spots with R*f* values of 0.47 (orange spot) and 0.24 (brownish spot). On the other hand, visualization with iodine vapour produced three distinctive yellow spots with R*f* values of 0.74, 0.47 and 0.24, respectively. According to Tyman et al. (1989), the spots with R*f* values of 0.74, 0.47 and 0.24 represent cardanol (3-pentadecyl phenol), anacardic acid (6-pentadecylsalicylic acid) and cardol (5-pentadecylresocinol) which are the major phenolic lipids present in CNSL (Tyman et al., 1989; Yuliana et al., 2014). Iodine vapour is a semi-destructive visualization method and is preferred for visualization of phenolic compounds and lipids because it has a high affinity for such (Meyers and Meyers, 2008). Thin-layer chromatography has been frequently used in literature to identify and determine the purity of phenolic compounds in CNSL. The results in this study, corroborate with Tyman et al. (1989) and Boongaling et al. (2008), both spotted three components in crude CNSL with slightly similar R*f* values to our findings.

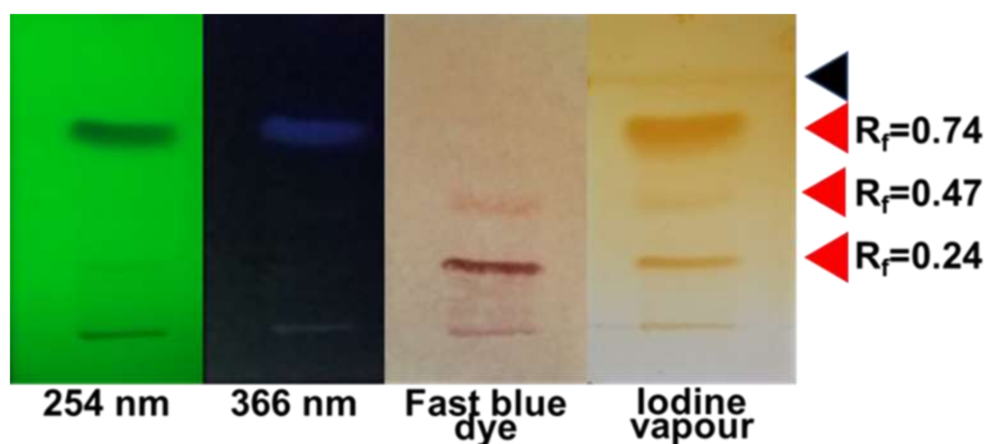

Figure 4-8. TLC chromatogram of CNSL.

4.6.2 GC-MS Chromatography Analysis of Crude CNSL

GC-MS analysis confirmed the presence of cardol and cardanol in the extracted crude CNSL**.** The retention times for cardol and cardanol are presented in **Figure 4-9**. Two

structural isomers of cardol were observed at retention times of 19.08 mins for saturated cardol (C15), and 19.79 mins for unsaturated triene. Similarly, three structural isomers of cardanol (different chain lengths) were observed at retention times of 30.35 mins (cardanol, C13), 32.76 mins (Cardanol, C15), and 35.64 mins (cardanol, C17), respectively (**Figure 4-9**). Their molecular ion peaks are presented in **Figure 4-10**. The findings in this study are similar to Patel et al. (2006), who also found three isomers of cardanol (different chain lengths) in CNSL at different retention times: cardanol C13 (40.16 mins), C15 (43.63 mins), and C17 (46.34 mins), respectively. HPLC data (**Figure A-1** in the appendix) also showed that our crude CNSL had five major components measured with a PDA at 288 nm. The composition of cardol and cardanol in crude CNSL based on peak area in GC-MS were 15.52 % and 79.51 %, respectively. The rest of the compounds were hydrocarbons 2.35 % and other unidentified compounds 2.62 %. Similar study by Kala et al. (2019), identified 32 compounds from CNSL with the majority of the components being fatty acids. Similarly, Citó et al. (2011) identified 11 compounds in technical CNSL with the main constituents being cardanol (40.26 %), cardol (29.20 %), phytosterol (10.68 %), anacardic acid (1.97 %) and other unidentified compounds (11.17%). The absence of anacardic acid in this study could be due to high temperatures set during GC-MS analysis. The injection temperature was 260 ^{o}C, while the oven temperature went up to 300 ^{o}C. Anacardic acid is a thermal labile compound that quickly decarboxylates to form cardanol at temperatures above 180 ^{o}C (Gandhi et al., 2012). This explains the high content of cardanol (79.51 %) in our work.

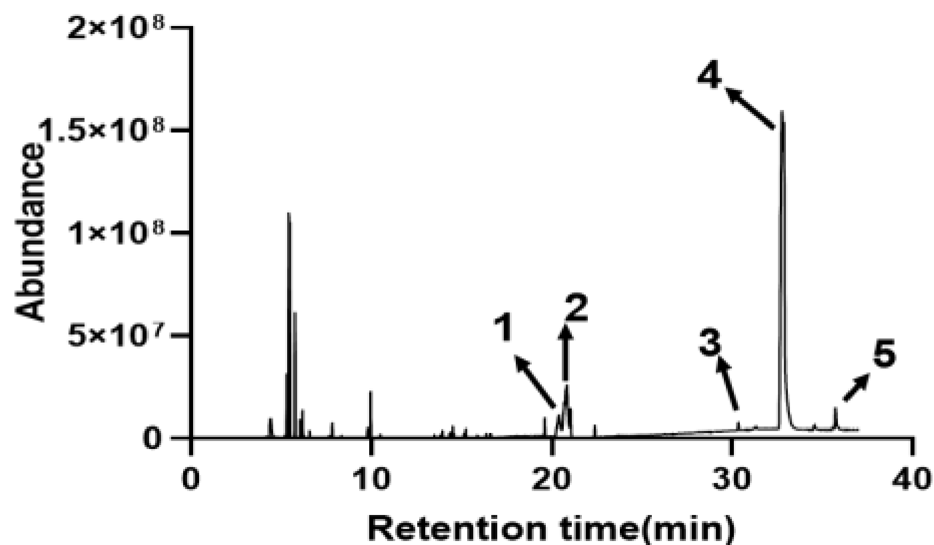

Figure 4-9. TIC chromatogram of crude CNSL. The chemical composition of CNSL were 1: cardol saturated, 2; cardol diene, 3; cardanol (C13), 4; cardanol (C15) and 5; cardanol (C17).

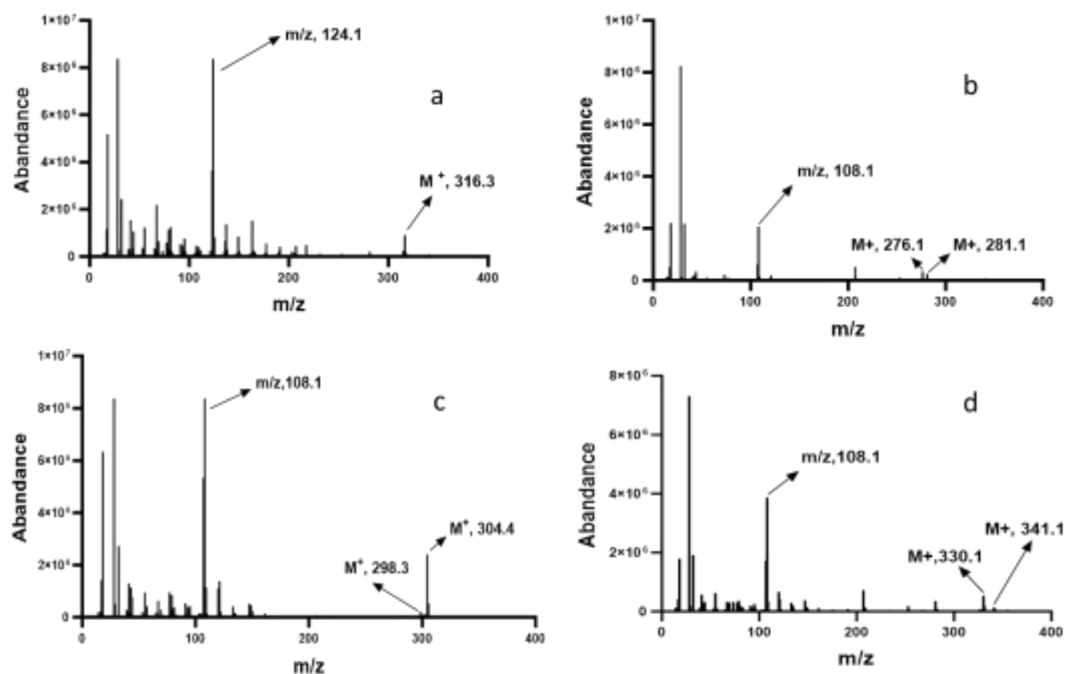

Figure 4-10. Mass spectrums showing molecular mass ions of CNSL components from GC-MS analysis. (a): cardol, (b) cardanol (C15), (c): cardanol (C15), and (d): cardanol (17).

Cardanol (C 15) has been reported to be the predominantly occurring isomer in CNSL (Yuliana et al., 2014; Balachandran et al., 2013), whereas, other isomers occur in trace amounts. GC-MS analysis results by Patel et al. (2006) showed that the composition of cardanol isomers C13, C15 and C17 in crude CNSL extracted by supercritical fluid at 200 bars were 0.69 %, 84.20 % and 2.83 %, respectively. Tyman (1979), also reported the occurrence of trace amounts of cardanol (C17) in crude CNSL. In addition, cardanol (C13) and (C17) have also been isolated from other plants such as *Gingko biloba* (Li et al., 2018).

Partial isolation of CNSL phenolic lipids by column chromatography gave pure cardanol and cardol by HPTLC analysis (**Figure 4-1).** However, GC-MS analysis of the isolated components revealed that there was still the presence of impurities in cardol (**Figure 4-12)**, thus, only cardanol was considered for further pre-structural elucidation analysis through GC-MS analysis. The mass spectrum and chemical structure of isolated cardanol (C15) is presented in **Figure 4-12**. GC-MS analysis of isolated cardanol showed the presence of three isomers as observed previously in crude CNSL above (**Figure 4-9**). The isolated cardanol (C 15) had the molecular ion of M^+, 304 for saturated and M^+ 298 for unsaturated triene isomers, respectively. The fragmentation patterns of cardanol (C15) are presented in **Figure 4-13**. The peak obtained at *m/e* 108 is characteristic of alkyl benzenes (Budzikiewicz et al., 1967). It results from β-cleavage of the aliphatic chain as shown in (**Figure 4-14**) (Strocchi and Lercker, 1979). The fragmentation at *m/e* 121 and 147 results from cleavage in the side chains of diene and triene isomers of cardanol (Madhusudhan and Murthy, 1992).

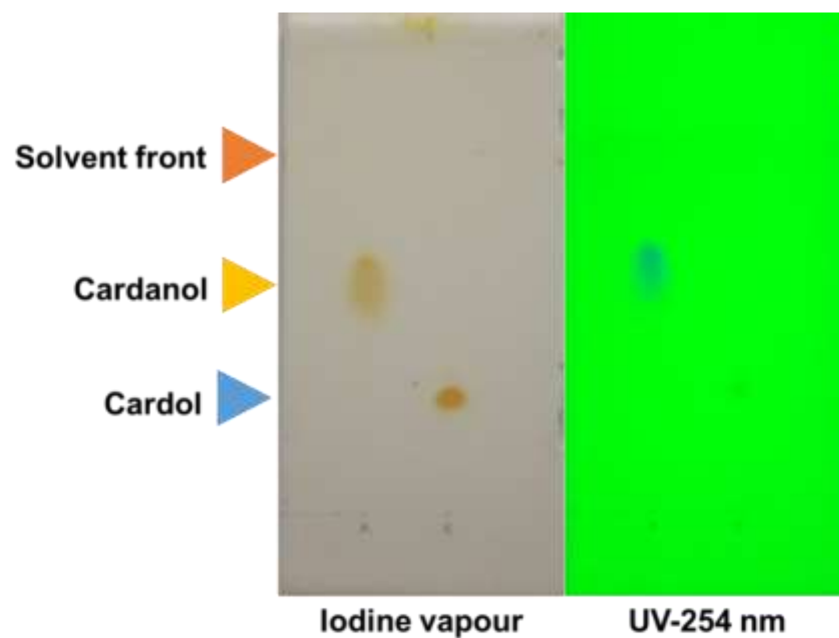

Figure 4-11. HPTLC plates of isolated cardanol and cardol.

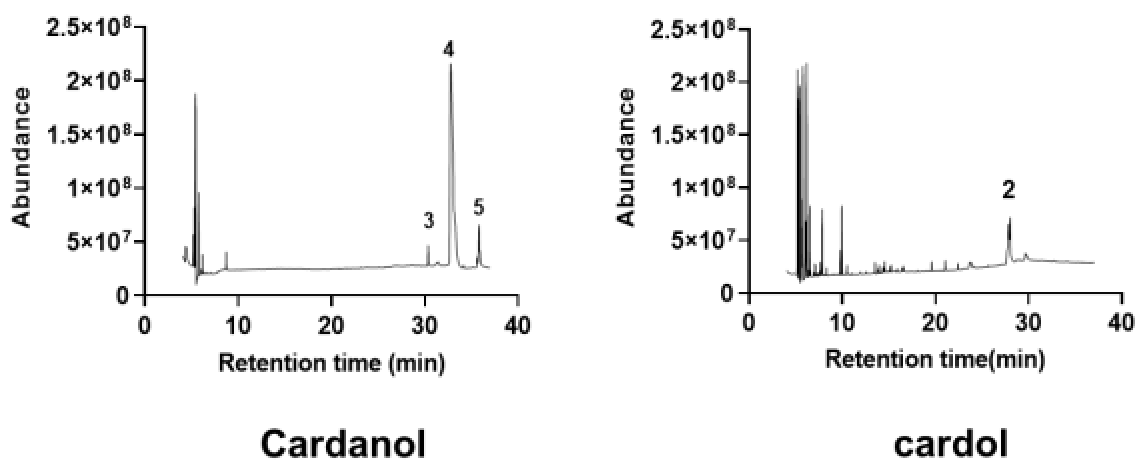

Figure 4-12. TIC of isolated cardanol and cardol. 2; cardol (C17) triene, 3, 4 and 5 are cardanol (C13), 4; (C15), and 5 (C17), respectively.

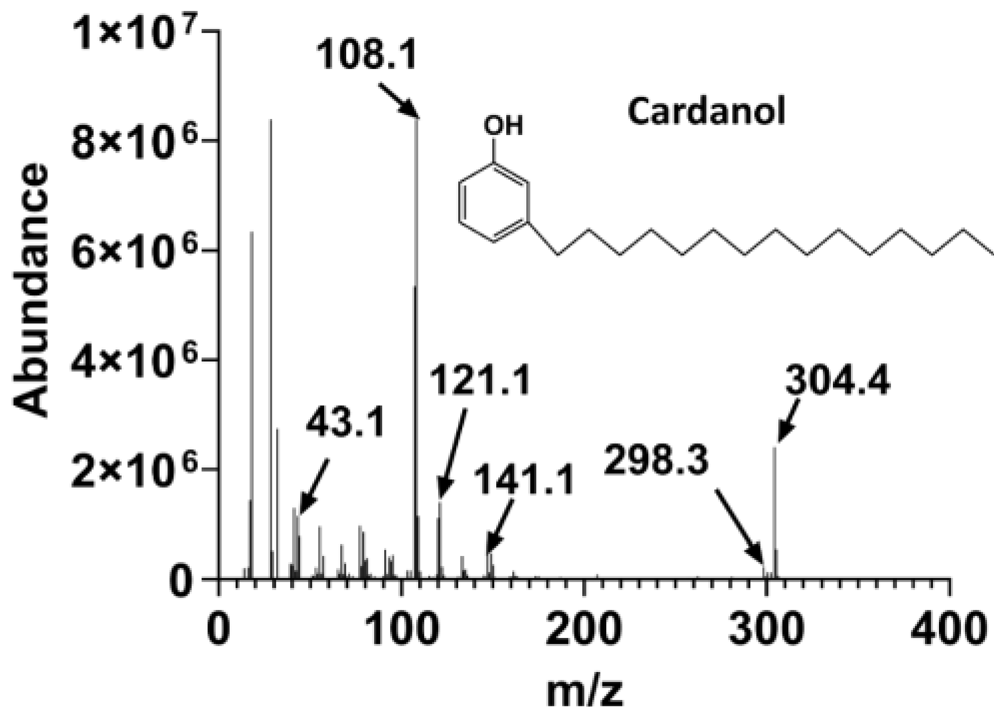

Figure 4-13. Mass spectrum of cardanol (C15) a component of CNSL.

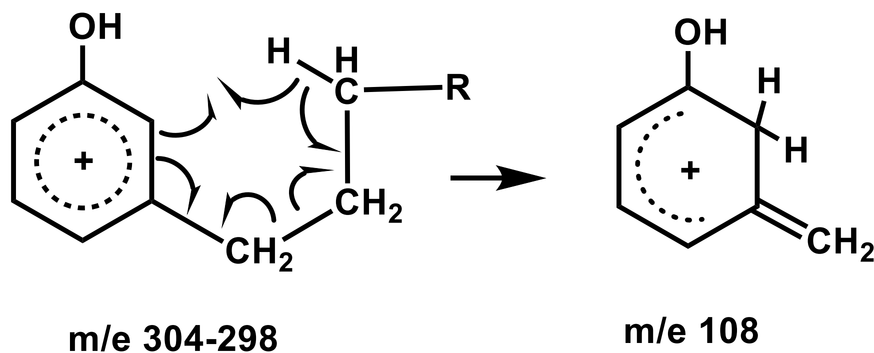

Figure 4-14. β-cleavage of alkyl benzenes. Adpated and modified from Strocchi and Lerker (1979).

4.7 Toxicity Study analysis

4.7.1 Ovicidal Effect of Crude CNSL against FAW

The toxicity of CNSL against FAW eggs is shown in **Figure 4-15**. The mortalities of eggs at different concentrations of CNSL were 99.17 ± 0.00 at 7 w/v %, 86.65 ± 4.8 at 5 w/v %, 72.5 ± 10.23 at 3 w/v %, and 0.0 ± 0.5 % at 1 w/v %, respectively. The mortality of

FAW eggs was dose-dependent; increasing the concentration of CNSL resulted in increased mortality. This has also been reported by other authors in literature sources (Pandiyan et al., 2020; Parchande et al., 2021). The calculated LC_{50} (95 CI %) value for CNSL against FAW egg in this work was 2.50 (0.22-4.02 w/v %) or 25 mg/ml (**Table 4-3**). This value was higher than the 11.72 mg/ml and 13.75 mg/ml reported by Parchande et al (2021), for the *Argenome mexican* and *Clerodendium viscosum* ethanolic leaf extracts against FAW eggs. But it was almost similar to 23.48 mg/ml, 21.46 mg/ml and 20.82 mg/ml reported for *Clerodendium viscosum, Argenome mexican* and *Vitex negundo* ethanolic leaf extracts against *S. litura* eggs (Parchande et al., 2021). Generally, it is widely accepted that phenolic compounds in CNSL are responsible for its biological activity (De Lima et al., 2008; Buxton et al., 2017). However, the mechanism of action has not yet been fully understood. Few literature sources that have reported on the subject such as the work by Stasiuk and Kozubek (2010), revealed that phenolic lipids from cashew and other plants inhibit the growth of bacteria, fungi, protozoa and parasites by interacting with their proteins or by disrupting their membranes or DNA molecules. The mode of action of CNSL in this work was not determined, but could be due to one of the aforementioned mechanisms; hence, a study needs to be conducted to confirm this.

Table 4-3. LC_{50} (95 CI %), Chi-squared and P-values of egg mortality.

Treatment	N	LC_{50} (95 CI %)	Chi-Square	P-value
Eggs	600	2.50 (0.22-4.05)	13.06	0.001[a]

CI: Confidence interval and N: total number of eggs.

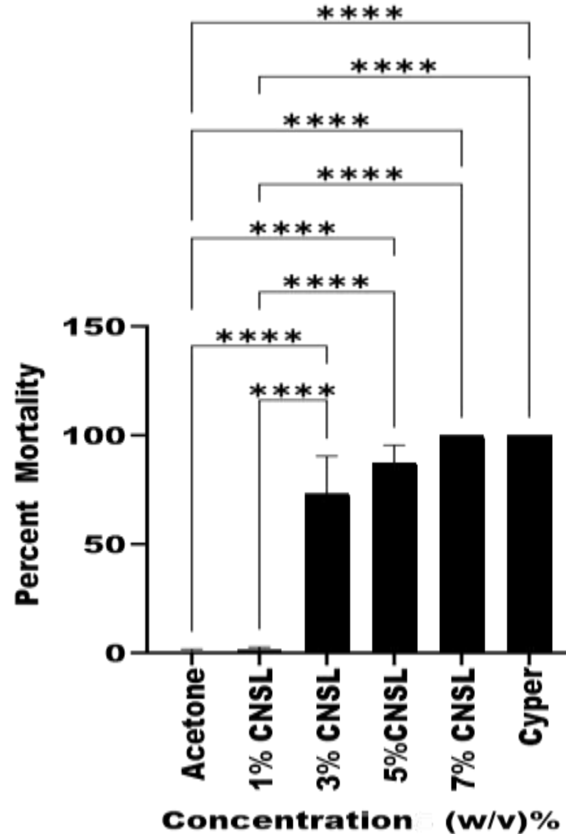

Figure 4-15. Percent mortality of FAW eggs. Bars connected with the same line are significantly different by Tukey's test analysis ($p < 0.05$), **** ($p \leq 0.0001$).

4.7.2 Larvicidal Effect of Crude CNSL against FAW

The results in **Table 4-4** and **Figure 4-16** show the larvicidal effect of CNSL against FAW larvae by topical contact toxicity. The toxicity of CNSL against FAW larvae was dose-dependent. The lowest mortality 3.33 % was recorded at 0.5 % w/v (5 mg/ml), while the highest mortality 100 % was recorded at 7 % w/v (70 mg/ml), after 2 hrs of treatment. The calculated LC_{50} (95 CI %) value was 1.92 (1.71-2.15 w/v %) after 2 hrs of treatment. Although the biological activity of CNSL against various insect species has been widely documented in literature, our thorough literature search only found one paper by Ferreira de Carvalho et al. (2019) where they tested the larvicidal effect of CNSL against FAW. In this study, it was shown that CNSL was toxic to first and second-instar larvae of FAW by systemic poisoning (ingestion). The LC_{50} values after 72 hrs of treatment were 353.4 & 366.0 mg/L and 480.3 & 492.2 mg/L for natural and technical CNSL on the first and

second-instar larvae, respectively. Our LC_{50} value of 19.2 mg/ml was far much higher than 0.4803 and 0.4822 mg/ml recorded in this paper for natural and technical CNSL, respectively. However, our treatment time of 2 hrs was much shorter than the 72 hrs reported in this paper. The huge difference in the LC_{50} values could be due to the methods of administration used. While theirs was by ingestion, ours was topical. This can suggest that CNSL is more effective as a systemic poison than as a topical larvicide. Nevertheless, in other literatures, some insect pests were shown to respond to higher doses of CNSL by systemic poisoning. For instance, both the works by Andayanie and Ermawati (2019) and e Carvalho Castro et al. (2019) separately showed that 6 w/v % and 5 w/v % concentrations of CNSL were required to cause 99.5 % and 99.1 % mortality of *Bemisia tabaci* first-instar nymphs and *Rhipicephalus microplus* third-instar larvae, respectively.

Table 4-4. LC_{50} (95 CI %), Chi-squared and P-values for larvae mortality.

Treatment (hours)	N	LC_{50} (95 CI %) w/v %	Chi-Square	P-value
Larvae	630	1.92 (1.71 - 2.15)	1.80	0.0408[a]

LC: lethal concentration, N: total number of insects, CI: confidence interval.

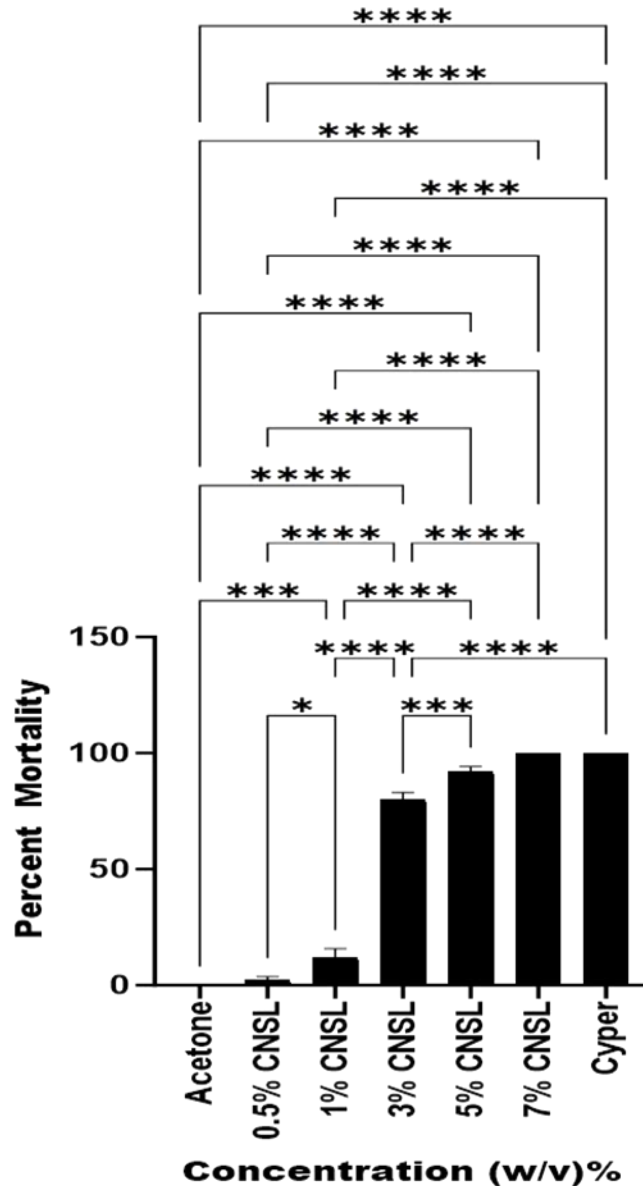

Figure 4-16. Effect of concentration and time on percent mortality of FAW larvae. Bars connected with the same line are significantly different by Tukey's test analysis ($p < 0.05$). * ($p \leq 0.05$), *** ($p \leq 0.001$), and **** ($p \leq 0.0001$).

4.7.3 Histology Analysis

The histology analysis results of the effect of CNSL on FAW larvae are presented in **Figure 4-17**. The results revealed that CNSL might have caused the death of FAW larvae by injuring their cuticle. The physical inspection of the untreated live larva and treated dead larva showed that the cuticle of the treated larva was dark, swollen and shiny

probably due to oxidation (**Figure 4-17; a & b**). Histology analysis showed that the cuticle of the larva in the control remained intact, as can be seen in **Figure 4-17 c**. The cuticle and epidermal cells are neatly joined together. Whereas, in the treated the cuticle and epidermal cells have detached (**Figure 4-17 d**). This similar pattern was also observed by Zahed et al. (2021); essential oils from *Thymus vulgaris (L)* and *Lavandula angustifolia* caused thickening and detachment of the cuticle from the epidermal cells in third and fifth-instar larvae of *Thaumetopoeapity ocampa,* respectively. Dourado et al. (2015) reported that CNSL caused irreversible degeneration of the epithelial cell lining, increased vacuoles and separation of epithelial cells from the basal membrane and disintegrations of the brush borders and damage of the peritrophic membrane in the midgut of third-instar *A. aegypti* larvae. These deleterious changes were attributed to phenolic lipids found in CNSL. According to Dourado et al. (2019) the hydrophobic part of the phenolic lipids (aliphatic chains) allows them to permeate into the cell membrane of the cell, affecting its permeability, whereas the polar groups (hydroxyl and carboxylic groups) act on the protein amino acid residues of the organism disabling them. This leads to the loss of control of ionic balance and water intake in the organism, which eventually leads to death.

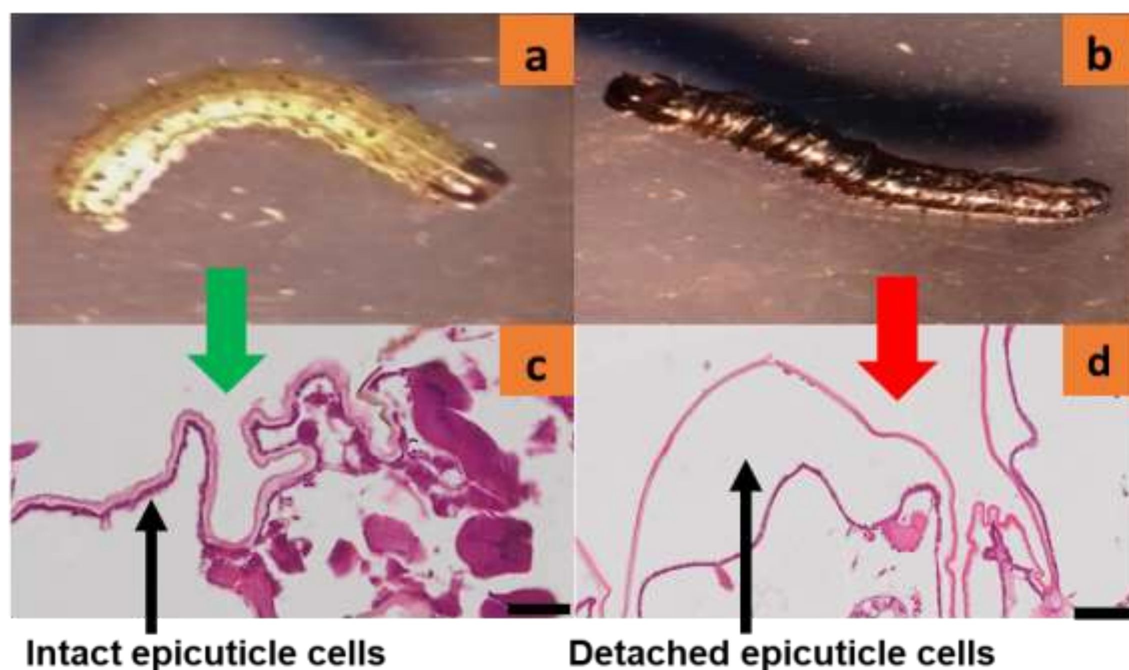

Figure 4-17. Histology of FAW larvae. (a): untreated larva, (b) dead treated larva, (c): intact epicuticle cells of untreated larva, and (d): damaged epicuticle cells of treated larva. The black horizontal bars represent 50 µm at a magnification of 200x.

4.7.4 Antifeedant Effect of CNSL against FAW

As shown in **Table 4-5** and **Figure 4-18** CNSL exhibited antifeedant effect against FAW larvae. The antifeedant effect was dose-dependent implying that it increased with an increase in CNSL concentration. The antifeedant index (AFI %) for CNSL ranged from 45.02 ± 6.90 to 98.56 ± 2.38 % respectively, whereas the EC_{50} was 0.74 (0.28-1.21 w/v%). The antifeedant effect of CNSL was compared with Neemcide (commercial insecticide) and results are shown in **Table 4-5 and Figure 4-18**. The AFI % of Neemcide ranged from 32.18 ± 10.5 to 93.66 ± 1.90 %, respectively, while the EC_{50} was 1.05 (0.81-1.29 w/v %). As can be seen from the two EC_{50} values, CNSL exhibited a stronger antifeedant effect than Neemcide, suggesting that it can be exploited as an alternative insecticide in the management of FAW outbreaks. The lower antifeedant EC_{50} (0.74 w/v %) for CNSL against FAW also suggested that it would be better suited as an antifeedant insecticide than as an ovicide (LC_{50} = 2.50 w/v %) or larvicide (LC_{50} = 1.92 w/v %). Insecticides with antifeedant properties might be better than conventional insecticides, which are broad spectrum because they are specific to chewing insect pests, leaving beneficial insects unaffected (Gaharwar et al., 2021).

Table 4-5. EC_{50}, (95 CI %), Chi-squared and P-values for antifeedant effects.

Compounds	N	LC_{50} (95 CI %) w/v %	Chi-Squared	P-values
CNSL	140	0.74 (0.28-1.21)	11.21	0.01
Neemcide	140	1.05 (0.810-1.29)	5.30	0.15

EC: effective concentration, N: total number of insects, CI: confidence interval.

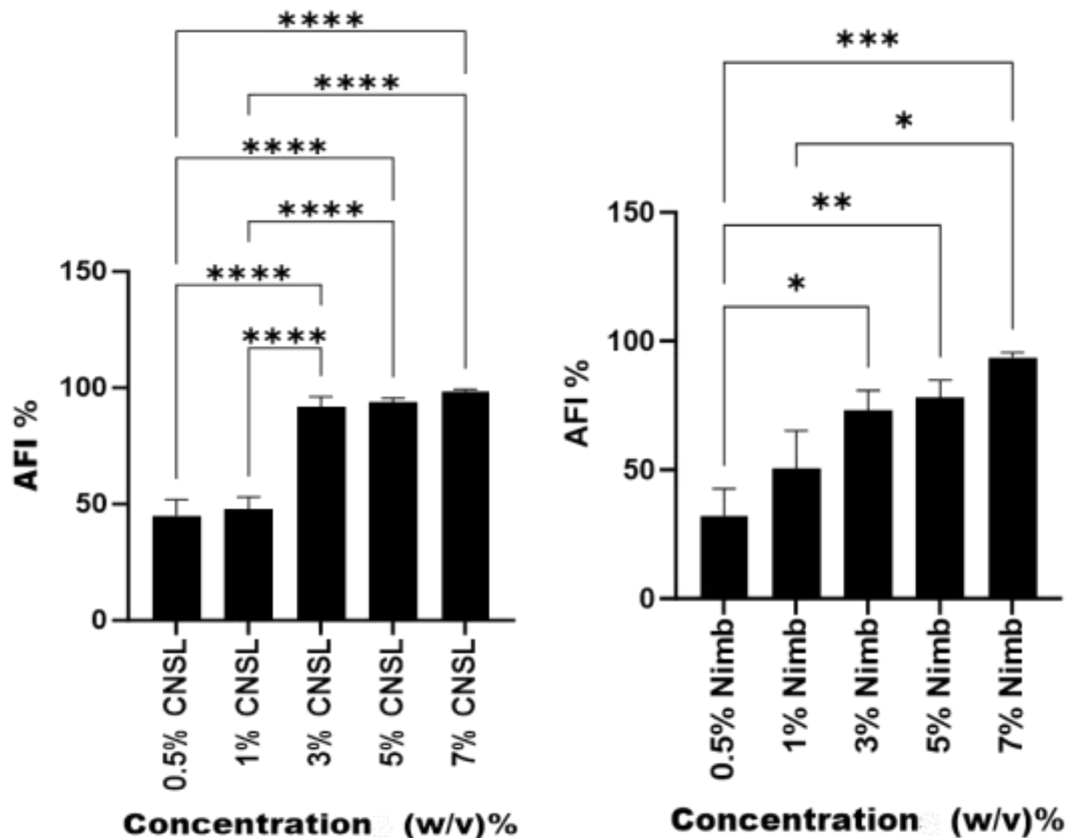

Figure 4-18. Percent feeding inhibition of CNSL against FAW larvae. Bars connected with the same line are significantly different by Tukey's test analysis ($p < 0.05$). * ($p \leq 0.05$) ** ($p \leq 0.01$), *** ($p \leq 0.001$), and **** ($p \leq 0.0001$).

Several plant oils have been reported to exhibit antifeedant effects against various Lepidopteran insect larvae. Eucalyptus and gaultheria oils at 2 w/v % concentrations were reported to exhibit 96.2 % and 87.2 % antifeedant effects against black cutworm, *Agrotis ipsilon* (Jeyasankar, 2012). Similarly, garlic and lemon oils were reported to exhibit a strong antifeedant effect against *S. littoralis* fourth-instar larvae (Ali et al., 2017). Literature sources revealed that antifeedants such as Azadirachtin compounds inhibit insect feeding by either blocking the input from chemoreceptors that normally respond to phagostimulants or/ by stimulating specific "deterrent" cells or broad spectrum receptors, or through both of these mechanisms (Garcia et al., 1984). Lepidopterous larvae (e.g. *S. frugiperda*) are believed to have deterrent-sensitive cell (s) that are stimulated by a number of secondary metabolites from plants which act as deterrents (Schoonhoven, 1972, Schoonhoven, 1981). The mode of action for the antifeedant effect of CNSL against FAW

larvae was determined but, we believe the phenolic lipids in CNSL might have interacted with some of these receptors.

The use of plant-based insecticides has many advantages over synthetics because they are relatively cheaper, specific, bio-degradable and rarely induce insect resistance (Amoabeng et al., 2019). CNSL might have an advantage over other botanical insects in that it is obtained from waste materials and the tree is widely distributed around the world, and is already commercialized, hence raw materials are widely and readily available. Moreover, the cashew tree does not only produce CNSL, but also many other valuable products such as cashew nut kernels, apples, and gum which are in demand around the world (Dendena and Corsi, 2014, Shahbandeh, 2023).

Chapter 5: Conclusion and Recommendations

5.1 Conclusion

In this study, CNSL was extracted and characterized and its biological activity was evaluated against FAW. Solvent type and extraction time were found to strongly influence the recovery of CNSL from CNS ($p < 0.05$). Increasing the extraction time and polarity of solvents resulted in an increased yield of CNSL. Phytochemical screening of the extracted CNSL revealed that it was rich in bioactive compounds such as phenols (74.5 ± 2.7 to 101.2 ± 2.5 mg GAE/g), and flavonoids (20.6 ± 2.5 to 33.3 ± 0.7 QE/g). TLC and GC-MS analysis of crude CNSL revealed presence of cardol (15.55 %) and cardanol (79.51 %) in the extracted CNSL. These two phenolic lipid compounds and anacardic acid are characteristic of CNSL and literature showed that they are responsible for the biological activity that is observed (Stasiuk et al., 2014; Passari et al., 2015). Evaluation of the biological activity of CNSL against FAW showed that it was active against eggs and larvae, and demonstrated antifeedant effect. The LC_{50} for eggs and larvae mortality were 2.50 (0.22-4.05) and 1.92 (1.71 - 2.15 w/v %), respectively, while the antifeedant EC_{50} was 0.74 (0.28-1.21 w/v %.) This study showed that CNSL is effective against FAWs, and can be exploited as a relatively cheap, readily available and eco-friendly alternative insecticide for managing FAW outbreaks in Zambia.

5.2 Recommendations

The following recommendations are suggested for future works:

i. Quantification and isolation of CNSL phenolic lipids in Mongu CNSs.

ii. Structure elucidation of CNSL phenolic lipids by Nuclear Magnetic Resonance (NMR).

iii. Study the phytotoxicity of CNSL against maize and other economically important crops. In one study, 6% w/v CNSL was reported to show phytotoxic symptoms in soybeans leaves (Andayanie et al., 2019).

iv. Study the toxicity of CNSL against FAW and other agricultural insect pests in the field.

v. Study the toxicity of CNSL on beneficial insects such as pollinators, parasitoids and predators.

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Appendices

Appendix A: HPLC Data

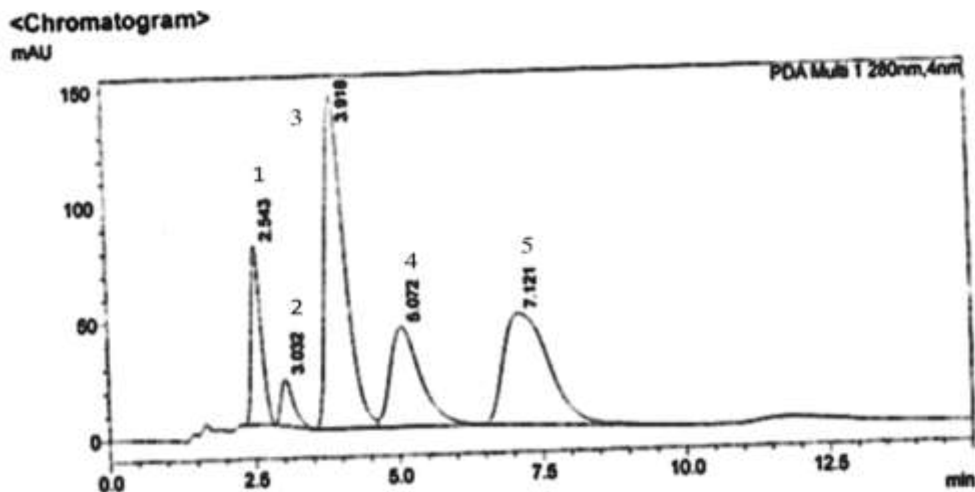

Figure A-1 HPLC-PDA chromatogram of Crude CNSL: 2: cardol, 3 and 4: anacardic acid, and 5: cardanol.

Table A-1 Retention time and approximate composition of CNSL components in Crude CNSL.

Peak No.	Ret. time	Area	Height	Conc.
1	2.543	907358	76842	10.345
2	3.032	292806	19882	3.338
3	3.916	3367637	143757	38.395
4	5.072	1575546	42860	17.963
5	7.121	2627737	47855	29.956
Total		8771085	331196	

Identification of the peaks was based on the work by Oiram Filho et al., (2018). In this sudy, the retention times at 3.978, 5.492 and 7.681 mins were identified as cardol, anacardic acid and cardanol respectively. Hence, the peaks at retention times of 3.918 (2), 5.072 (3) and 7.121 mins (5) were identified as cardol, anacardic acid and cardanol respectively. Although, the retention times are almost similar we cannot be certain if the compounds are the same. Thus there is need to analyse the compounds in the presence of standards.

Appendix B: Experimental Photos

Figure B-1 Commercial insecticides (Cypermethrin and Neemcide) Positive controls.

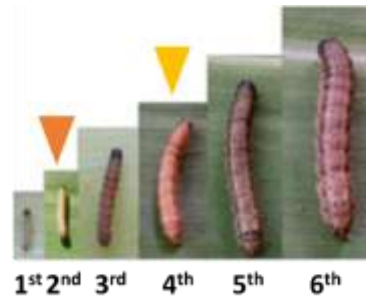

Figure B-2 Instars of FAW used in this study. The highlighted instars are the ones that were used in this study.

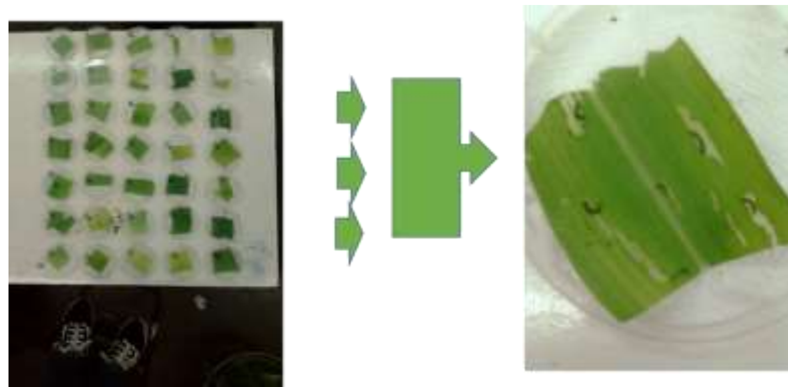

Figure B-3 Topical toxicity bioassay. Six second instar larvae were treated with CNSL extract and placed on a petri dish containing a 5 x 5 cm^2 leaf as food.

***S. Frugiperda* collection. UNZA field station. Sch. of Agricultural Sciences**

Mass rearing of *S. frugiperda* larvae in lunch boxes

Mass rearing of *S. Frugiperda* moths in cages

Figure B-4 Sample collection and Mass rearing of FAW in the Insectarium Laboratory, Department of Plant Science, School of Agricultural Sciences, UNZA

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Appendix C: Ethical Approval Letter

THE UNIVERSITY OF ZAMBIA
DIRECTORATE OF RESEARCH AND GRADUATE STUDIES

Great East Road Campus | P.O. Box 32379 | Lusaka10101 | Tel: +260-211-290 258/291 777
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APPROVAL OF STUDY

***IORG** No. 0005376/*
***NASRECREC IRB** No. 00006465*

16th May, 2023

REF NO. NASREC-2023 – APR - 004

Mr. Kadongo Zombe,
The University of Zambia,
School of Natural Sciences,
P.O. Box 32379,
LUSAKA.

Dear, Mr. Zombe,

RE: "INSECTICIDAL AND FEEDING INHIBITION EFFECTS OF CASHEW NUT SHELL LIQUID EXTRACT AGAINST *SPODOPTERA FRUGIPERDA*"

Reference is made to your protocol dated as captioned above. NASREC resolved to approve this study and your participation as Principal Investigator for a period of one year.

REVIEW TYPE	ORDINARY REVIEW	APPROVAL NO. NASREC-2023 - APR - 004
Approval and Expiry Date	Approval Date: 16th May, 2023	Expiry Date: 15th May, 2024
Protocol Version and Date	Version - Nil.	15th May, 2024
Information Sheet, Consent Forms and Dates	• English.	To be provided
Consent form ID and Date	Version - Nil	To be provided
Recruitment Materials	Nil	Nil
Other Study Documents	Questionnaire.	

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 NASREC, we would like to wish you all the success as you carry out your study.

Yours faithfully,

Dr. Mususu Kaonda
VICE-CHAIRPERSON
THE UNIVERSITY OF ZAMBIA NATURAL AND APPLIED SCIENCES RESEARCH ETHICS COMMITTEE - IRB

cc: Director, Directorate of Research and Graduate Studies
Assistant Director (Research), Directorate of Research and Graduate Studies
Assistant Registrar (Research), Directorate of Research and Graduate Studies

Figure C-1 Excerpt of the Ethical Approval of the research project

Appendix D: Prior Publications

Part of this work has been published in the following journals

1. Nyirenda, J., Kadango, Z., Funjika, E. and Chipabika, G., 2024. Larvicidal, ovicidal and antifeedant activity of crude cashew nutshell liquid against fall armyworm, Spodoptera frugiperda (JE smith), (Lepidoptera, Noctuidae). *Crop Protection*, p.106619.
2. Zombe, K., Nyirenda, J., Lumai, A. and Phiri, H., 2022. Impact of Solvent Type on Total Phenol and Flavonoid Content and Sun Protection Factor of Crude Cashew Nutshell Liquid. Sustainable Chemistry, 3(3), pp.334-344.
3. Nyirenda, J., Zombe, K., Kalaba, G., Siabbamba, C. and Mukela, I., 2021. Exhaustive valorisation of cashew nut shell waste as a potential bioresource material. Scientific Reports, 11(1), p.11986.