EVALUATION OF THE ANTICANCER POTENTIALS OF *Senna alata* LEAF AND *Khaya senegalensis* STEM BARK EXTRACTS ON COLORECTAL CANCER CELL LINE

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MTech/SLS/2018/8290

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MINNA

JUNE, 2023

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A THESIS SUBMITTED TO THE POSTGRADUATE SCHOOL FEDERAL UNIVERSITY OF TECHNOLOGY, MINNA, NIGERIA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF TECHNOLOGY IN BIOCHEMISTRY

JUNE, 2023.

ABSTRACT

Colorectal cancer (CRC), also known as bowel cancer, colon cancer or rectal cancer, is the development of cancer from the colon or rectum (parts of the large intestine). Most colorectal cancers are due to old age and lifestyle factors, with only a small number of cases due to underlying genetic disorders. The risk of developing this cancer is related to bad alimentary habits, smoking, intestinal inflammatory disease, polyps, genetic factors, and aging. The aim of this research was to evaluate the anticancer potentials of *Senna alata* leaf and *Khaya senegalensis* stem bark extracts using colorectal cancer cell line. The quantitative phytochemical composition of the crude methanol extracts of *Senna alata* and *Khaya senegalensis* stem bark was determined using standard methods. The partitioning of the crude methanol extract of *Senna alata* was carried out using n-hexane, ethyl acetate and aqueous. Preliminary cytotoxic activity screening was carried out using the brine shrimp lethality test, while the cytotoxic activity was evaluated using CellTiter-Glo® 2.0 assay kit on Colorectal Cancer cell line. The cytotoxic activity of ethyl acetate fraction on Colorectal Cancer cell line was treated at doses 50, 100, 250, 500 and 1000 µg/ml for 24hrs and 48hrs. The phytochemical compositions of the crude methanol extract of *Senna alata* shows the presence of Phenols (397.70±1.01), Flavonoids (138.56±0.09) Tannins (43.13±0.23), Alkaloids (185.09±0.33) and Saponins (282.50±1.62) whereas the The phytochemical compositions of the crude methanol extract of *Khaya Senegalensis* shows the presence of Phenols (403.58±1.04), Flavonoids (136.7±0.06) Tannins (75.10±0.14), Alkaloids (229.84±0.59) and Saponins (166.38±1.24). The acute oral toxicity test of the plant extracts shows no toxicological effect on all the tested rats and the lethal dose (LD_{50}) was recorded to be greater than 5000mg/kg. The cytotoxic effect of the crude methanol extract of *Senna alata* on brine shrimp show higher activity with significant lethal concentration (LC_{50}) 17.87ppm than *Khaya senegalensis* with lethal concentration 30.975ppm. The Brine shrimp lethality assay revealed that, the ethyl acetate fraction with lethal concentration (LC_{50}) of 1.308ppm exhibited higher cytotoxic activity than other fractions with lethal concentration of 6.172ppm (n-hexane) and 57.805ppm (aqueous). The ethyl acetate fraction exhibited cytotoxicity towards human colorectal cancer cell line by 82% with an inhibitory concentration (IC_{50}) of 324.19µg/ml after 24hrs of exposure and 93% inhibition with an IC_{50} of 91.52µg/ml after 48hrs of exposure at doses 1000µg/ml. Results of adenosine triphosphate (ATP) assay showed high and dose-dependent inhibition of human colorectal adenocarcinoma (HT-29) cell-line growth by ethyl acetate fraction of *Senna alata* extract. The ethyl acetate fraction of *Senna alata* extract caused a significant decrease in proliferation of Colorectal Cancer cell line and thus, could serve as a source of potential candidate drug to manage Colorectal Cancer.

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

Cancer is the abnormal growth of cells that have the ability to invade or spread to other parts of the body, globally cancer is a disease which severely affects the human population (Bray *et al*., 2018**).** Colorectal cancer (CRC), also known as bowel cancer, colon cancer, or rectal cancer, is the development of cancer from the colon or rectum (parts of the large intestine). Most colorectal cancers are due to old age and lifestyle factors, with only a small number of cases due to underlying genetic disorders (Theodoratou *et al.,* 2017).

Other risk factors include diet, obesity, smoking, and lack of physical activity. Dietary factors that increase the risk include red meat, processed meat, and alcohol. Some of the inherited genetic disorders that can cause colorectal cancer include familial adenomatous polyposis and hereditary non-polyposis colon cancer; however, these represent less than 5% of cases (Jamal *et al*., 2011). It typically starts as a benign tumor, often in the form of a polyp, which over time becomes cancerous. Colorectal cancer (CRC) is the third most common cancer and the second leading cause of deaths across the world, an important strategy for combatting this deadly disease before tumors reach an invasive state is the use of a class of non-steroidal anti-inflammatory drugs (NSAIDs) (Ferlay *et al.,* 2018). According to Global Cancer Statistics 2018, estimates of cancer incidence and mortality will represent the leading cause of death throughout the world in twenty-first century (Arnold *et al.,* 2017). In 2018, there were an estimated 18.1 million new cancer cases and 9.6 million cancer deaths globally. In both sexes combined, lung cancer is the most commonly diagnosed cancer (11.6% of the total cases) and the leading cause of cancer death (18.4% of the total cancer deaths), closely followed by female breast cancer (11.6%), prostate cancer (7.1%), and colorectal cancer (6.1%) (Ferlay *et al.*, 2018).

Most of the cancer researchers have assumed that the spread of tumors rarely occurs later in the disease process. The general idea is that as tumors grow, the cancer cells accumulate more and more genetic changes. Some cells have the ability to move from primary tumor to the bloodstream or lymphatic system. Treatment depends on several factors like the size, location and the stage of the cancer and the current overall health condition of the patient. Different types of treatments are radiotherapy, chemotherapy and surgery. (Nicole, 2020). The plant kingdom produces naturally occurring secondary metabolites which are being investigated for the anticancer activities for the development of new clinical drugs and for many years herbal medicines have been used and are still used in developing countries as the primary source of medical treatment (Bray *et al*., 2018**).**

Senna alata, also known as *Cassia alata,* is an important medicinal tree, as well as an ornamental flowering plant in the subfamily Caesalpinioideae, is a widely distributed herb of the Leguminosae family. It is commonly known as candle bush, craw-craw plant or ringworm plant. The plant is commonly found in Asia and Africa, and has many local names (Kumar *et al.,* 2008). It has arrays of bioactive chemical compounds. Some of the reported chemical constituents are phenolics (rhein, chrysaphanol, kaempferol, aloeemodin, and glycosides), anthraquinones (alatinone and alatonal), fatty acids (oleic, palmitic, and linoleic acids), steroids, and terpenoids (sitosterol, stigmasterol, and campesterol) (Liu *et al.,* 2009). These secondary metabolites are reported to display numerous biological activities (Chang *et al.,* 2002). The flower, root, leaves, seed, and bark displayed diverse biological activities (Makinde *et al.,* 2007).

Khaya senegalensis commonly known as African mahogany, is a popular medicinal plant among the Nupes and Yorubas in Nigeria. It belongs to the family *Meliaceae* (mahogany). The aqueous decoction of its stem bark is traditionally used by Yoruba and Nupe tribes to treat malaria, jaundice, edema and headache (Kolawole *et al.,* 2013). Khaya

senegalensis is a tree with shiny foliage up to 25 m or more with exfoliating barks, young branches with dark, grayish-brown lenticels and leaves of 15-60 cm or more. It has pinnate leaves, glabrous with 6 to 12 alternate or opposite elliptical oblong leaflets. At the flowering, *K. senegalensis* twigs carry at their ends panicles of small white flowers consisting of successive whorls of four floral parts. Its fruits are capsules with thick and woody seed coat. The bark of this tree is very thick, scaly and dark brownish-gray color. In its natural range, *K. sengalensis* provides cattle fodder, edible and cosmetic oils, medicinal products, shade and shelter. *Khaya senegalensis* favourable habitats are those in wet soils, deep; alluvial; the edge of streams and non-flooded lowlands. It also accommodates dry or superficial or lateritic stations when rainfall is between 650-1300 mm during 4-7 months (Takin *et al*., 2013).

1.2 Statement of the Research Problem

Colorectal Cancer has been a public health concern with substantial increase in its rate and mortality. Occurrence and death rates from colorectal cancer increase with age (Simon, 2016). Standard anticancer treatments are not selective therefore they affect both tumor and normal cells, causing systemic toxicity that may lead to death.

The treatment of cancer is highly expensive accompanied with undesirable side effects that may affects the body system. Therefore there is the need to search for novel anticancer agents that are abundant, biocompatible, easily accessible and cheaper compared to conventional drugs.

1.3 Aim and Objectives

1.3.1 Aim

To evaluate the anticancer potentials of *Senna alata* leaves and *Khaya senegalensis* stem bark extracts using colorectal cancer cell lines

1.3.2 Objectives:

The objectives of this study are to determine the:

i. quantitative phytochemical composition of methanol extract of *Senna alata* leaf and *Khaya senegalensis* stem bark.

ii. lethal dose (LD_{50}) of the methanol extracts of *Senna alata* leaf and *Khaya senegalensis* stem bark in experimental rats.

iii. cytotoxicity of the methanol extracts of *Senna alata* leaf and *Khaya senegalensis* stem bark on brine shrimp and select the more active for further studies.

iv. DPPH (2, 2-Diphenyl-1-Picrylhydrazyl) radical scavenging activity of the most active fraction.

v. cytotoxic activity of the most active fraction on Colorectal Cancer cell line.

1.4 Justification of Study

The desire to produce more effective but less toxic drugs has propelled the continuous search for new bioactive compounds from plants.

For many years, herbal medicines have been used and are still used as the primary source of medical treatment (Kumar *et al.*, 2011).

Medicinal plants have been used for the treatment of various diseases over a long period of time but the dosage and their potency of most of them are yet to be ascertain. Conventional drugs as reported to possess cytotoxic effects leaving the patients to choose between living with disease or taking medications with deleterious effects. This has necessitate a search for anticancer agents from plant's extracts that are safer, easily accessible with little or no side effects compared to the conventional treatment.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Senna alata

Senna alata, also known as *Cassia alata,* is an important medicinal tree, as well as an ornamental flowering plant in the subfamily Caesalpinioideae, is a widely distributed herb of the Leguminosae family. It is commonly known as candle bush, craw-craw plant or ringworm plant. The plant is commonly found in Asia and Africa, and has many local names (Kumar *et al.,* 2008). It has arrays of bioactive chemical compounds. Some of the reported chemical constituents are phenolics (rhein, chrysaphanol, kaempferol, aloeemodin, and glycosides), anthraquinones (alatinone and alatonal), fatty acids (oleic, palmitic, and linoleic acids), steroids, and terpenoids (sitosterol, stigmasterol, and campesterol) (Liu *et al.,* 2009). These secondary metabolites are reported to display numerous biological activities (Chang *et al.,* 2002). The flower, root, leaves, seed, and bark displayed diverse biological activities (Makinde *et al,* 2007). The plant pharmacological activities include antimicrobial (Somchit *et al.,* 2003), antifungal (Donatus and Fred 2010), anticryptococcus (Ranganathan and Balajee, 2000), antibacterial (Otto *et al.,* 2014), antitumor (Olarte *et al.,* 2010), anti-inflammatory (Sagnia *et al.,* 2014), antidiabetic (Varghese *et al.,* 2013), antioxidant (Makinde *et al.,* 2007), wound healing (Midawa *et al.,* 2010), and antihelmintic activities (Kundu *et al.,* 2012). In recent times, the outbreak of drug-resistant diseases has led to several health challenges.

2.1.1 Ethnobotanical description of *Senna alata*

2.1.1.1 Description and classification.

Senna alata is a flowering shrub of the *Fabaceae* family, it has the name "candle bush" owing to the framework of its inflorescences (Abo *et al.,* 2008). It is an annual and

occasionally biannual herb, with an average height of 1 to 4 m, burgeoning in sunlit and humid zones. The leaves are oblong, with 5 to 14 leaflet sets, robust petioles (2 to 3 mm), caduceus bracts (2 × 3 by 1 × 2 cm), and dense flowers. Zygomorphic flowers have bright yellow colour with 7 stamens and a pubertal ovary. The fruit exists as a 10 to 16 ×1.5 cm tetragonal pod, thick, flattened wings, brown when ripe with many diamond- shaped brown seeds. It is propagated by seeds and dispersed to about 1,500m above the sea level (Hennebelle *et al.,* 2009). Taxonomically, *Senna alata* is classified as kingdom: *Plantae,* order: *Fabales,* family: *Fabaceae*, subfamily: *Caesalpinioideae*, tribe: *Cassieae,* subtribe: *Cassiinae*, genus: *Senna*, and species: *Senna alata*.

Figure 2.1: *Senna alata* (whole plant, flower and seeds)

Source: (Kumar *et al.*, 2008).

2.1.1.2 Geographical distribution

Senna alata is widely distributed in Ghana, Nigeria, Brazil, Australia, Egypt, India, Somalia, Sri Lanka, and all over Africa (Kumar *et al.*, 2011). Like other *Senna* species, it is cultivated in humid and tropic regions of Africa, Asia, West Indices, Mexico, Australia, South America, the Caribbean Islands, Melanesia, and different parts of India (Ross 1999). In Philippines, Thailand, and Indonesia, this shrub is widely dispersed and is cultivated for medicinal Purposes (Palanichamy and Nagarajan 1990).

2.1.2 Ethnobotanical uses

Various parts of *Senna alata* have shown diverse therapeutic activities in diseases control. In northern Nigeria, the stem, leaf, and root decoctions is used in treatment of wound, skin and respiratory tract infection, burns, diarrhoea, and constipation (El-Mahmood *et al.*, 2003). Also, in the south-western regions, leaf decoction serves as an antidote to body and abdominal pain, stress, and toothache (Benjamin and Lamikanra 2008). It also cures dermal infections, convulsion, and as purgative (Oguntoye *et al.*, 2005). Currently, seeds and roots are used in regulating uterus disorder and worms (Selvi *et al.*, 2012). The flower and leaves decoction was observed to be used in treatment of ringworms, scabies, blotch, eczema, scabies, and tine infections. The leaves are currently used in Sierra Leone to relieve abortion pain and facilitate baby delivery (Oladeji *et al.*, 2016). In China, India, Benin republic, Ghana, Nigeria, and Togo, the whole plant is used as a curative for Diabetes mellitus. Fresh leaves of the plant are used in the treatment of skin rashes, mycosis, and dermatitis (Ajibesin *et al.*, 2008). From the traditional uses, the frequent use of *Senna alata* leaves is more than that of roots, flowers, and stem-bark owing to more active metabolites reported in the leaves. Besides its pharmacological 1activities, it is processed into capsule, pellet, and tea in Nigeria for preventing diseases and maintaining sound health.

2.1.3 Pharmacological activities

Medicinal plants belonging to the Fabaceae family have extensively been investigated for their pharmacological activities. Plants synthesised array of secondary metabolites which contribute to its therapeutic activities (Figure 2.2). Therapeutic appraisal of *Senna alata*

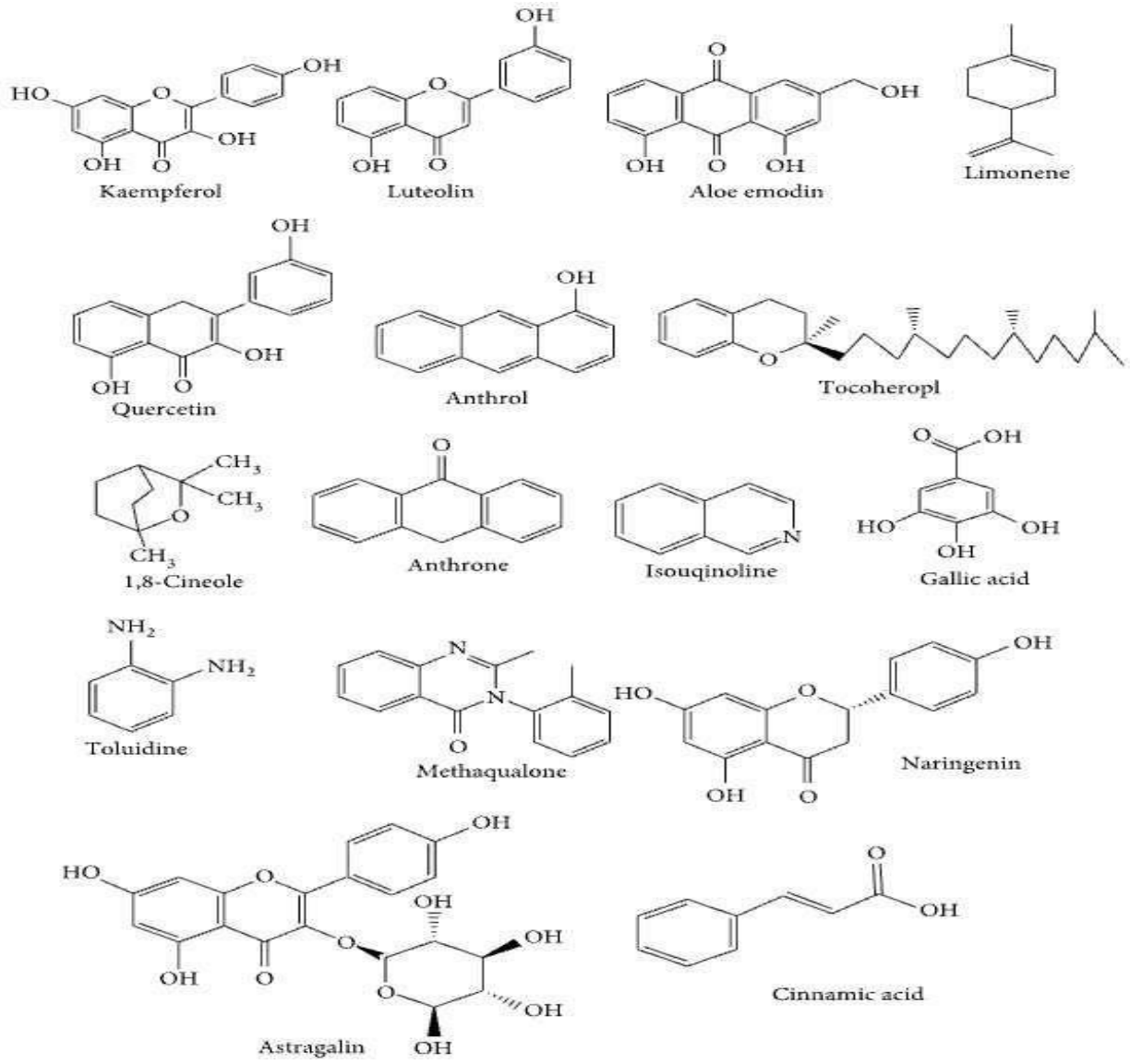

Figure 2.2: Bioactive Compounds with Therapeutic Potencies in *Senna alata*.
Source: (Oluwole *et al*., 2020)

2.1.3.1 Antibacterial activities

The antibacterial potentials of medicinal herbs are appraised using zone of inhibition (ZOI) or minimum inhibitory concentration (MIC). The *in vivo* antibacterial potential of *Senna alata* was assessed against methicillin-resistant *S. aureus* (MRSA), extended spectrum beta-lactamase, and carbapenemase-resistant. Enterobacteriaceae was isolated from infectious patients, via Mueller–Hinton broth via the microdilution technique. The

extract showed significant activities at 512mg/ml due to the flavonoids, quinones, tannins, sterols, alkaloids, and saponins analysed (Wikaningtyas and Sukandar, 2016).

2.1.3.2 Antioxidant activities

The antioxidant or scavenging activities is important in appraisal of therapeutic potential of medicinal herbs. Plants played significant role in protecting cell against oxidative stress caused by active free radicals (Zhu *et al.*, 2004). The free radical scavenging potentials of plants are appraised by antibiotic sensitivity tests (ASTs), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric-reducing antioxidant power (FRAP), 2,2′-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid (ABTS), hydroxyl radical scavenging activity (HRSA), and metal ion chelating activity (MICA). The methanolic and n-hexane leaves extracts of *Senna alata* were explored for their scavenging effects via DPPH assay. The methanolic extract displayed pronounced scavenging activities when compared to n-hexane extract. Different parts of *Senna alata*, significantly inhibited the action of free radicals causing oxidative stress (Sarkar *et al.*, 2014). The strong scavenging activities could be due to polyphenol and flavonoid analysed (Saha *et al.*, 2009).

2.1.3.3 Antifungal activities

Several bioactive compounds isolated from *Senna alata* exhibit strong *in vitro* and *in vivo* antifungal activities. *Senna alata* flowers and leaves collected from Ogbomoso, Southwest Nigeria, were examined in an attempt to justify the indigenous claims of its antifungal efficacy. *In vitro* antifungal activities of ethanolic and methanolic extracts were investigated using the disc diffusion approach. Ethanolic extracts showed pronounced inhibitory activities when compared to methanolic extracts. The IC50 value of the ethanolic extract was two folds higher than the methanolic extracts against the fungi isolates. The inhibitory activities displayed could be due to methaqualone, cinnamic acid, isoquinoline, and toluidine detected (Adelowo and Oladeji, 2017).

2.1.3.4 Dermatophytic activities

Currently, the leaves, flowers, and stem bark of *Senna alata* are used for treating various kinds of skin infections and diseases. In Thailand, the plant was mentioned as one of the 54 medicinal plants used for treating scabies, shingles, urticarial, itching, and ringworm (Neamsuvan, 2015). The dermatophytic activities displayed by *Senna alata* are linked to the bioactive compounds such as anthranols, anthrones, flavonoids, phenols, tannins, and anthracene derivatives. Leaves decoction displayed strong inhibitory activities against *S. pyogenes, S. aureus, K. pneurnoniae, E. coli, S. rnarcescens, P. cepacia,* and *P. aeruginosa* (Brown, 2011).

2.1.3.5 Antilipogenic, antidiabetic, and antihyperlipidemic activities

In Africa and Asia, *Senna alata* leaves and flowers are currently used to regulate lipid absorption, obesity, and fat levels in blood serum. Aqueous leaf extract considerably reduced blood glucose levels, serum cholesterol, triglyceride, hepatic triglyceride, serum leptin, and insulin levels in Wistar mice (Naowaboot *et al.,* 2015).

2.1.3.6 Antimalarial activities

Malaria is a global threat contributing to serious sociocultural and health issues in humid and tropical regions (Kayembe *et al.,* 2010). Chemotherapy reported the antimalarial activities of *Senna alata* could be linked to quinones, alkaloids, and terpenes. Quinones isolated from *Senna alata* significantly displayed *in vitro* antiplasmodial activity, against *Plasmodium falciparum* via the microdilution test of Desjardin (Kayembe *et al.,* 2010). Terpenes isolated from *Senna alata* leaves displayed pronounce *in vitro* antiplasmodial assays against *P. falciparum* in ethylene glycol-water fractions. Significant activity was observed at concentration below 1 μg/ml (Kayembe *et al.,* 2012). The appraisal of aqueous leaf extract justifies the ethnomedical applications of *Senna alata* as remedy for

malaria and fever. The leaf extract considerably inhibited 3D7 strain of the *P. falciparum* parasite in Wistar mice (Vigbedor *et al.,* 2015).

2.1.3.7 Anthelmintic activity

Traditionally, *Senna alata* leaf and flower decoctions are used in treatment of intestinal worm infestation and stomach disorder. The anthelmintic potency of alcoholic leaves extract of *Senna alata* and *T. angustifolia* at 10 to 100 mg/ml were assessed in clinical isolates of *Ascardia galli* and *Pheretima posthuma* by observing time of paralysis and point of death of the worms. The leaves extract significantly inhibit the worms (test organisms) more than piperazine citrate (standard anthelmintic drug) (Anbu *et al.,* 2013).

2.1.3.8 Antiviral activities

Senna alata is an indispensable bactericidal and fungicidal natural therapy. However, the justification of antiviral activities is not properly documented. The antiviral efficacy of n-hexane, ethyl acetate, butanol, and aqueous leaf extracts was assessed on dengue virus (DENV) obtained from an infected pregnant woman in Indonesia via focus assay. The extracts significantly inhibited DENV-2 with IC50 (<10 μg/ml), CC50 (645.8 μg/ml) and SI (64.5 μg/ml) (Angelina *et al.,* 2017).

2.1.3.9 Cytotoxic activities

Samy, *et al.,* (2017). Reported that Chloroform fraction of Cassia alata L leaves were evaluated for its potential antitumor properties *in-vitro*. The fraction showed remarkable cytotoxicity against HepG2 cells, this observation was confirmed by morphological investigation. Several other studies have dealt with the cytotoxic activity evaluation of C. alata leaves extracts. Hexane fraction of *Cassia alata* leaves exhibited cytotoxic activity against parental A549 (lung cancer cells) and OV2008 (ovarian cancer cells) cell lines (Levy and Carley 2012) also the hexane fraction and f6l (a mixture of polyunsaturated fatty acid esters) exerted a cytotoxic effect on MCF-7 (breast carcinoma cells), T24

(bladder carcinoma cells) and Col 2 (colorectal carcinoma cells) cell lines in a dose dependent manner, but were not effective against A549 (non-small cell lung adenocarcinoma) and SK-BR-3 (breast carcinoma cells) cell lines (Olarte *et al.,* 2013).

2.2 K*haya Senegalensis* Description, Classification and Distribution

Khaya senegalensis A. Juss (Meliaceae) commonly known as African mahogany, is a popular medicinal plant among the Nupes and Yorubas in Nigeria. It belongs to the family Meliaceae (mahogany).The stem bark aqueous extract is traditionally used by Yoruba and Nupe tribes to treat malaria, jaundice, edema and headache (Kolawole *et al.*, 2013). Khaya senegalensis is a tree with shiny foliage up to 25 m or more with exfoliating barks, young branches with dark, grayish-brown lenticels and leaves of 15-60 cm or more. It has pinnate leaves, glabrous with 6 to 12 alternate or opposite elliptical oblong leaflets. At the flowering, *K. senegalensis* twigs carry at their ends panicles of small white flowers consisting of successive whorls of four floral parts. Its fruits are capsules with thick and woody seed coat. The bark of this tree is very thick, scaly and dark brownish-gray color. In section it oozes reddish exudates. In its natural range, *K. sengalensis* provides cattle fodder, edible and cosmetic oils, medicinal products, shade and shelter. *Khaya senegalensis* favourable habitats are those in wet soils, deep; alluvial; the edge of streams and non-flooded lowlands. It also accommodates dry or superficial or lateritic stations when rainfall is between 650-1300 mm during 4-7 months (Takin *et al*., 2013).

Taxonomically, *Khaya senegalensis* is classified as kingdom: *Plantae*, phylum: *Tracheophyta,* Class: *Magnoliopsida* order: *Sapindales*, family: *Meliaceae*, genus: *Khaya*, and species: *K. sengalensis.*

It inhabits Sudanese and Sudanese-Guinean regions, It is abundant in the woodlands of most countries in the Guinea Gulf (Cameroon, Gabon, Nigeria, Benin, Togo, Ivory Coast, Guinea, Guinea Bissau, Gambia and Senegal) and where its usefulness is widely reported

(Djoueche *et al.,* 2011). But its distribution is much wider, ranging up Sudano-Sahel (Burkina Faso, Niger, Mali and Chad).

Figure 2.3: *Khaya senegalensis* plant

Source: (Kolawole *et al.,* 2013)

2.2.1. Ethnobotanical uses

The plant is used for a variety of purpose. The bitter testing back is used for a variety of medical purposes, it is taken against fever cause by malaria, stomach complains, and headaches.it is apply externally to cure skin rashes, wound or any abnormality. It is now used more locally, and is planted ornamentally as a road side tree. The therapeutic value of *Khaya senegalensis* has been recognized in different systems of traditional medicine for the treatment of various conditions. The decoction of the stem bark extract is commonly used for treating jaundice, dermatoses, malaria, fever, mucous diarrhea, and venereal diseases as well as for hookworm infection and a taeniacide remedy (Iwu. M 1993). *Khaya senegalensis* extracts have been reported to exhibit anti-inflammatory effects (Lompo *et al*,.1998) as well as anti-bacterial (Kon'e *et al*., 2004), antihelmintic (Ademola *et al*., 2004), antitumor, antioxidant (Androulakis *et al*., 2006) and antiplasmodial activities (El-Tahir *et al*,.1998). The stem bark extract and the chemical constituent profile have been the subject of extensive phytochemical and pharmacological

investigations since the 1960s (Narender *et al*., 2007). Despite the extensive research on the stem bark extract of *Khaya senegalensis*, a dearth of information on the toxicological profile of this plant still exists.This work is therefore designed to evaluate the acute and subchronic toxicity effects of the extract on the liver and kidney. There is increasing interest in the use of plant extracts as therapeutic agents, particularly the capacity for these extracts to inhibit the growth of pathogenic microorganisms (Wilkinson, 2009).

2.2.2. Chemical composition of *Khaya senegalensis*

Phytochemical screening of trunk bark allowed Lompo (Lompo, M. 1998) to highlight the main chemical groups in *K. Senegalensis*: fatty acids, carotenoids, coumarins, emodols, tannins, compounds reducers, anthracenosides, steroidal glycosides, flavonosides, carbohydrates, saponins, sterols and triterpenes, anthocyanins. Recently, (Yuan *et al*., 2010) and (Bickii *et al.,* 2000) reported the isolation of some limonoids named Khayalenoids from the stem and bark, well elucidated the structures of those molecules based on spectroscopicanalysis.

2.2.3. Therapeutic efficacy of *Khaya senegalensis*

Various extracts of leaves, bark or seeds of *K. senegalensis* were reported to have cure properties against several human diseases and have been tested in efficacy trials. Limonoids isolated from other one species of *Khaya* (*Khaya grandifoliola*) is declared highly effective against the causative agent of malaria, *Plasmodium falciparum* (Bickii *et al.,* 2000). Bark or stem bark methanolic extract of *K. senegalensis* has Anti-proliferative and anti-inflammatory effect (Zhang *et al*., 2007).

2.2.4. Biochemical and tissue effects of *Khaya senegalensis* extracts

Studies from various backgrounds have shown that organs extracts of *Khaya senegalensis* induce significant changes in blood biochemical parameters, after a physicochemical

study, indicated that the extracted oil from the seeds of this plant is rich in bioactive substances that induce prophylactic and therapeutic effects (Ayo *et al.,* 2007).

2.2.5 General physiological and tissue effects

Ethanolic extract of dried leaves of *Khaya senegalensis* has antispasmodic and spasmolytic effects on the guinea pig ileum. These authors indicated that at the dose of 1 mg.ml-1, the extract abolishes the contraction induced by acetylcholine 0.1 p.g.ml-1. Since calcium enriched environment does not allow return of contractions the observed effect is lasting. This plant extract act as a calcium blocker relaxing the smooth muscle of guinea pig ileum. This result is in accordance with the release of gastrointestinal motility obtained by (Nwosu *et al.,* 2012) on rats at doses of 100-300 mg.kg-1 BW. Similar observation was also notified by (Elisha *et al.,* 2013) whom evaluated *in vivo* the effects of stem bark aqueous and ethanolic extracts on the digestive transit and faeces wetness in albino rats. They induced, experimentally, diarrhoea in these animals using castor oil before treated them with the herbal extracts. The authors observed then dose-dependently a decreasing of the diarrhoea without significant decrease in bowel transit. They attributed the antidiarrheal activity of *K. senegalensis* to the flavonoid and tannin compounds of the extracts. Androulakis *et al.,* (2006) used Promega's CellTiter 96 ® Aqueous One Solution Assay (Madison, WI, USA) to determine the viability of the colon cancer cells. They plated the cells (HCT-15, HT-29, HCA-7) at 10,000 cells per well in a 96-well plate. The cells were treated with a series of concentrations of *K. Senegalensis* bark extract for 3, 6 and 24 h. The controls included medium only, medium plus vehicle (<0.05% DMSO), as well as medium plus the extract at the various concentration used. At the end of each treatment time, 20 µl of One Assay Solution was added to each well, followed by incubation at 37°C for 1 h, and analysis at 490 nm with 650 nm reference wavelengths was carried out on a Beckman Coulter DU 640 spectrophotometer (Carlsbad,

CA, USA). The data-points of absorbance were converted to percent of control and were plotted on a percent *versus* concentration graph, from which the 20% and 50% inhibitory concentrations were determined. The reported result of this study is that *K. Senegalensis* bark extract displays antiproliferative, anti-inflammatory and pro-apoptotic effects on HT- 29, HCT-15 and HCA-7 cells.

2.2.6 Toxicological risks

According to (Nwosu *et al.,* 2012), the aqueous extract of leaves of *Khaya senegalensis* is not toxic. Indeed, at the end of a study conducted by them on rats in Nigeria, these authors reported that the LD50 of the extract is greater than 3000 mg.kg-1 body weight. Although other studies revealed that chronic treatment rather induces an increase of these parameters signing some hepatotoxicity. Long treatments also cause elevation of serum creatinine and blood urea which reflects renal dysfunction (Kolawole *et al*., 2012). A risk of acute ecotoxicities of aqueous and ethanolic extracts of leaves of *Khaya senegalensis* have been suspected by (Adakole and Balogun, 2011). These authors assessed the sensitivity of Chironomid larvae to aqueous and ethanolic extracts of the plant in aquatic environment. The result indicated LC_{50} of aqueous and ethanolic extracts were 1.39 g/l and 1.20 g/l respectively and a deformity of mouth parts, change in body coloration, packing of certain body segments with black particles. It's clear the extract disrupts these invertebrates ecology.

2.3 Brine Shrimp

Brine shrimp is the common name for any of the small, salinity tolerant, aquatic crustaceans comprising the genus *Artemia*, the only genus in the family *Artemiidae* of the order Anostraca (fairy shrimp). They inhabit inland salt waters, such as brine pools and other highly salty habitats, and are not found in ocean environments. Despite their small size adults reaching only about 15 millimeters

in length brine shimp have offer commercial, scientific, and educational values. They are sold as fish food for aquaculture and aquarium hobbyists, used to improve production of salt, and used in scientific and school experiments (Gzimek *et al*., 2004).

The nutritional properties of newly hatched brine shrimp make them particularly suitable to be sold as fish food, as they are high in lipids and unsaturated fatty acids (but low in calcium). Cysts of *Artemia* are harvested around salt lakes and are hatched to feed fish larvae, either for aquaculture or aquarium hobbyists (Gzimek *et al*., 2004). They have a rudimentary nervous/spinal system, which leads researchers to believe that they may be used in experiments without concern for animal ethics.

Figure 2.4: *Artemia Salina*
Source: (Aliereza *et al*., 2010)

2.3.1 Scientific classification

Kingdom: *Animalia*

Phylum: *Arthropoda*

Subphylum: *Crustacea*

Class: *Branchiopoda*

Order: *Anostraca*

Family: *Artemiidae*

Genus*: Artemia*

2.3.2 Uses of brine shrimp in anti-cancer pre-screening

One biological approach involves monitoring the cytotoxicity of the extracts of subfractions against the nauplii, *Artemia salina*. The susceptibility of *Artemia salina* (*Artemiidae*), or brine shrimp larvae to treatment with medicinal plant extracts can be used as a measure of toxicity of chemicals as well as natural products (Logarta *et al.,* 2001). It is capable of detecting a broad spectrum of bioactivity present in crude extracts. Brine shrimp has been reported to be useful in predicting biological activities such as cytotoxicity, phototoxicity, pesticidal and trypanocidal activities, enzyme inhibition and ion regulation (Mackeen *et al.,* 2000). The BSLT has also been reported to give good correlation with cytotoxicity against some tumour cell lines, including colon carcinoma cells (Wagensteen *et al.,* 2007).

In vivo lethality test of brine shrimp has been successfully employed for bioassay-guided fractionation of active cytotoxic and antitumor agents such as trilobacin from the bark of *Asimina triloba*, *cis*-annonacin from *Annona muricata* and *ent-kaur*-16-en-19-oic acid from *Elaeoselinum foetidum* (Psitthanan *et al.,* 2004).

2.4 Cancer

Cancer is a group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body. These contrast with benign tumours, which do not spread. (World cancer Report, 2014). Benign tumour Cells are confined to one area and are not able to spread to other parts of the body. This is not cancer. Malignant tumour is made up of cancerous cells, which have the ability to spread by travelling through the bloodstream or lymphatic system (lymph fluid). The cancer that first develops in a tissue or organ is called the primary cancer. A malignant tumour is usually named after the organ or type of cell affected. A malignant tumour that has not spread to other parts of the body is called localised cancer. A tumour may invade deeper into surrounding tissue

and can grow its own blood vessels (angiogenesis). If cancerous cells grow and form another tumour at a new site, it is called a secondary cancer or metastasis. A metastasis keeps the name of the original cancer. For example, lung cancer that has spread to the bones is called metastatic lung cancer, even though the person may be experiencing symptoms caused by problems in the bones. Possible signs and symptoms include a lump, abnormal bleeding, prolonged cough, unexplained weight loss, and a change in bowel movements. While these symptoms may indicate cancer, they also have other causes. Over 100 types of cancers affect humans (World cancer Report, 2014).

The most common types of cancer in males are lung cancer, prostate cancer, colorectal cancer, and stomach cancer. In females, the most common types are breast cancer, colorectal cancer, lung cancer, and cervical cancer (Ward *et al*., 2014). In children, acute lymphoblastic leukemia and brain tumors are most common, except in Africa, where non-Hodgkin lymphoma occurs more often (Dubas *et al.,* 2013). In 2012, about 165,000 children under 15 years of age were diagnosed with cancer. The risk of cancer increases significantly with age, and many cancers occur more commonly in developed countries, rates are increasing as more people live to an old age and as lifestyle changes occur (Jamal *et al.*, 2011).

2.4.1 Colorectal cancer

Colorectal Cancer (CRC), also known as bowel cancer, colon cancer, or rectal cancer, is the development of cancer from the colon or rectum (parts of the large intestine) (Bosman, 2014). Colorectal cancer may be benign, or non-cancerous, or malignant. A malignant cancer can spread to other parts of the body and damage them. Most colorectal cancers are due to old age and lifestyle factors, with only a small number of cases due to underlying genetic disorders (Bosman, 2014). Other risk factors include diet, obesity, smoking, and lack of physical activity. Dietary factors that increase the risk

include red meat, processed meat, and alcohol (Theodoratou *et al.,* 2017). Another risk factor is inflammatory bowel disease, which includes Crohn's disease and ulcerative colitis (Bosman, 2014). Some of the inherited genetic disorders that can cause colorectal cancer include familial adenomatous polyposis and hereditary non-polyposis colon cancer. It typically starts as a benign tumor, often in the form of a polyp, which over time becomes cancerous (Bosman, 2014).

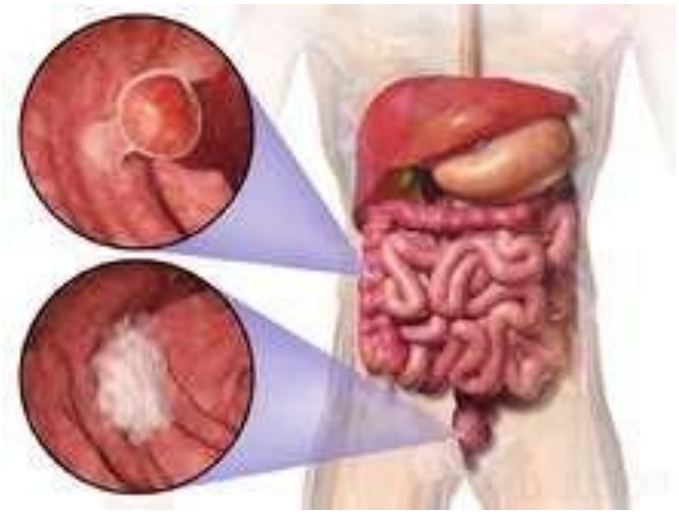

Figure 2.5: Location and Appearance of Colorectal Cancer.

Source: (Bosman, 2014).

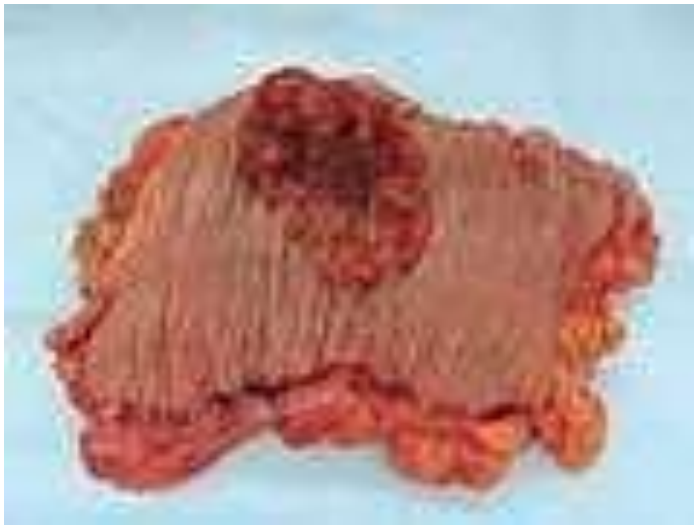

Figure 2.6: An Invasive Colorectal Carcinoma (top center) in a Colectomy Specimen.

Source: (Bosman, 2014).

2.4.1.1 Epidemiology incidence of colorectal cancer

Cancer of the colon is the fourth most incident cancer in the world, while cancer of the rectum is the eighth most incident. Together, CRCs are the third most commonly diagnosed form of cancer globally, comprising 11% of all cancer diagnoses (Ferlay *et al.,* 2018). About 1,096,000 new cases of colon cancer are estimated to be diagnosed in 2018, while about 704,000 new cases of rectal cancer are expected. Together, these comprise 1.8 million new cases of CRC. CRC is the most diagnosed cancer among men in 10 of the 191 countries worldwide. No country has CRC as the most diagnosed cancer among women (Bray *et al,* 2018). The CRC is more incident among men than women and 3–4 times more common in developed than in developing nations. Age-standardised (world) incidence rates per 100,000 of CRC in both sexes is 19.7, in males is 23.6, and in females is 16.3, While the age-standardised incidence rate among men is 30.1/100,000 in high-HDI (human development index) nations, it is 8.4 in low-HDI nations (the same statistics for women are 20.9 and 5.9, respectively) (Bray *et al,* 2018). In 2018, about 576,000 and 521,000 men and women, respectively, are projected to be diagnosed with colon cancer. This incidence constitutes a 1.51% cumulative risk of colon cancer among men age 0–74 years, and a 1.12% risk among women. About 430,000 men and 274,000 women are expected to be diagnosed with cancer of the rectum. Their cumulative, lifetime risks are 1.2% and .65%, respectively (Bray *et al,* 2018). Developed countries are at the highest risk of colon and rectal cancer. For colon cancer, Southern Europe, Australia/New Zealand, and Northern Europe are the regions of highest incidence. For rectal cancer, these regions are Eastern Europe, Australia/New Zealand, and Eastern Asia. North America also features among the highest incidence rates for both cancers. The country with the highest incidence of CRC per 100,000 populations is Hungary (70.6) among males and Norway (29.3) among females. In Japan, South Korea, Saudi Arabia, Oman,

Yemen, UAE, Bahrain, Qatar, Kuwait, and Slovakia CRC is the most diagnosed cancer among men. Meanwhile, all regions of Africa, as well as Southern Asia, have the lowest incidence rates for both cancers between both sexes (Bray *et al.,* 2018). Overall, CRC incidence is highly variable by region, with up to eight-fold variations between countries. In countries undergoing a major developmental transition, incidence rates tend to rise uniformly with increasing HDI, suggesting a causal relationship (Bray *et al.,* 2018).

Variation within a nation can also be steep. In the US, there was almost a three-fold higher incidence among those in Alaska relative to those in the Southwest. Factors that contribute to this variance are disparities in access to screenings and behaviour (Bray *et al*, 2018).

2.4.1.2 Signs and symptoms of colorectal cancer

The signs and symptoms of colorectal cancer depend on the location of the tumor in the bowel, and whether it has spread elsewhere in the body (metastasis). The classic warning signs include: worsening constipation, blood in the stool, decrease in stool caliber (thickness), bleeding from the rectum, abdominal pain, diarrhea, loss of appetite, loss of weight, and nausea or vomiting in someone over 50 years old (Alpers *et al.,* 2008)

2.4.1.3 Causes of colorectal cancer

Greater than 75–95% of colorectal cancer occurs in people with little or no genetic risk (Watson and Collins, 2011). Risk factors include older age, male sex and high intake of fat, sugar, alcohol, red meat, processed meats, obesity, smoking, and a lack of physical exercise (Watson and Collins, 2011). Approximately 10% of cases are linked to insufficient activity (Lee *et al.*, 2012)

2.4.1.4 Risk factors

It is estimated that more than 80% of patients with colorectal cancer were exposed to a number of risk factors, such as male gender, older age (Cunningham *et al.*, 2010) high

intake of red meat or fat, smoking and obesity (Watson and Collins 2011) Also, around 10% of these cases are related to insufficient activity (Lee *et al.*, 2012).

i. Genetics

Around 18% of all cases are patients with a family history. Thus, they have a two to three-fold risk increase than other people. Furthermore, three main genetic diseases are well associated with this type of cancer. The most common is known as the Lynch syndrome, or the hereditary nonpolyposis colorectal cancer (HNPCC) (Cunningham *et al.*, 2010). Familial adenomatous polyposis (FAP) and Gardner syndrome are both as well strongly associated with this type of cancer (Juhn and Khachemoune, 2010).

ii. Inflammatory bowel diseases

A third cause is the incidence of the Inflammatory Bowel Diseases, such as ulcerative colitis and Crohn's disease (Jawad *et al.*, 2011). The longer the onset of these diseases and the worse the inflammation will directly affect the risk of having colorectal cancer (Triantafillidis *et al.*, 2009). In these high risk groups, both prevention with aspirin and regular colonoscopies are recommended.

2.4.1.5 Pathogenesis

Colorectal cancer is a disease originating from the epithelial cells lining the colon or rectum of the gastrointestinal tract, most frequently as a result of mutations in the Wnt signaling pathway that increase signaling activity. The mutations can be inherited or acquired, and most probably occur in the intestinal stem cell (Cohen *et al.*, 2019). The most commonly mutated gene in all colorectal cancer is the APC gene, which produces the APC protein. The APC protein prevents the accumulation of β-catenin protein. Without APC, β-catenin accumulates to high levels and translocates (moves) into the nucleus, binds to DNA, and activates the transcription of proto-oncogenes. These genes are normally important for stem cell renewal and differentiation, but when inappropriately

expressed at high levels, they can cause cancer. While APC is mutated in most colon cancers, some cancers have increased β-catenin because of mutations in β-catenin (CTNNB1) that block its own breakdown, or have mutations in other genes with function similar to APC such as AXIN1, AXIN2, TCF7L2, or NKD1 **(**Tolar and Neglia, 2003).

Beyond the defects in the Wnt signaling pathway, other mutations must occur for the cell to become cancerous. The p53 protein, produced by the *TP53* gene, normally monitors cell division and induces their programmed death if they have Wnt pathway defects. Eventually, a cell line acquires a mutation in the *TP53* gene and transforms the tissue from a benign epithelial tumor into an invasive epithelial cell cancer. Somjketimes the gene encoding p53 is not mutated, but another protective protein named BAX is mutated instead **(**Tolar and Neglia, 2003).

Other proteins responsible for programmed cell death that are commonly deactivated in colorectal cancers are TGF-β and DCC (Deleted in Colorectal Cancer). TGF-β has a deactivating mutation in at least half of colorectal cancers. Sometimes TGF-β is not deactivated, but a downstream protein named SMAD is deactivated **(**Ferrara *et al.*, 2003).

2.4.1.6 Stages of cancer

The stage of a cancer defines how far it has spread. The earliest stage of colorectal cancers is called stage 0 (a very early caner), and then range from stage I (1) through IV (4). As a rule, the lower the number, the less the cancer has spread. A higher number, such as stage IV, means the cancer has spread more. Determining the stage helps chose the most appropriate treatment, cancer with similar stages tend to have a similar outlook and are often treated in much the same way (cunninghan *et al.*, 2010).

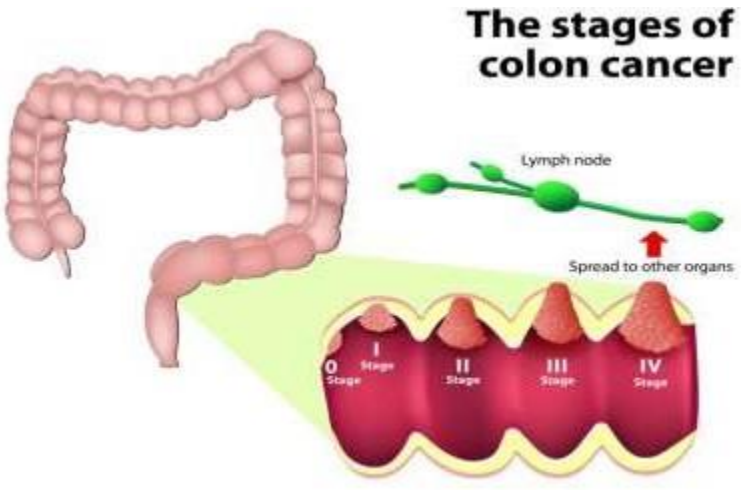

Figure 2.7: Stages of Colorectal Cancer.

Source: (Herbert *et al.,* 2004).

A commonly used system gives the stages a number from 0 to 4. The stages of colon cancer are:

I. **Stage 0**: This is the earliest stage, when the cancer is still within the mucosa, or inner layer, of the colon or rectum. It is also called carcinoma in situ.

II. **Stage 1**: The cancer has grown through the inner layer of the colon or rectum but has not yet spread beyond the wall of the rectum or colon.

III. **Stage 2**: The cancer has grown through or into the wall of the colon or rectum, but it has not yet reached the nearby lymph nodes.

IV. **Stage 3**: The cancer has invaded the nearby lymph nodes, but it has not yet affected other parts of the body.

V. **Stage 4**: The cancer has spread to other parts of the body, including other organs, such as the liver, the membrane lining the abdominal cavity, the lung, or the ovaries.

VI. **Recurrent**: The cancer has returned after treatment. It may come back and affect the rectum, colon, or another part of the body.

2.4.1.7 Diagnosis

Colorectal cancer diagnosis is performed by sampling of areas of the colon suspicious for possible tumor development, typically during colonoscopy or sigmoidoscopy, depending on the location of the lesion (Cunningham *et al.,* 2010). It is confirmed by microscopical examination of a tissue sample. The following are the most common screening and diagnostic procedures for colorectal cancer.

i. Medical imaging

A colorectal cancer is sometimes initially discovered on CT scan. Presence of metastases is determined by a CT scan of the chest, abdomen and pelvis. Other potential imaging tests such as PET and MRI may be used in certain cases (Arribas *et al.,* 2014).

ii. Fecal occult blood test (blood stool test)

This checks a sample of the patient's stool (faeces) for the presence of blood. This can be done at the doctor's office or with a kit at home. The sample is returned to the doctor's office, and it is sent to a laboratory. A blood stool test is not 100-percent accurate, because not all cancers cause a loss of blood, or they may not bleed all the time. Therefore, this test can give a false negative result. Blood may also be present because of other illnesses or conditions, such as haemorrhoids. Some foods may suggest blood in the colon, when in fact, none was present (Gelway *et al.,* 2012).

iii. Sigmoidoscopy

The doctor uses a sigmoidoscope, a flexible, slender and lighted tube, to examine the patient's rectum and sigmoid. The sigmoid colon is the last part of the colon, before the rectum.

The test takes a few minutes and is not painful, but it might be uncomfortable. There is a small risk of perforation of the colon wall. If the doctor detects polyps or colon cancer, a

colonoscopy can then be used to examine the entire colon and take out any polyps that are present. These will be examined under a microscope. A sigmoidoscopy will only detect polyps or cancer in the end third of the colon and the rectum. It will not detect a problem in any other part of the digestive tract (Wolf *et al.,* 2018).

iv. Barium enema x-ray

Barium is a contrast dye that is placed into the patient's bowel in an enema form, and it shows up on an X-ray. In a double-contrast barium enema, air is added as well. The barium fills and coats the lining of the bowel, creating a clear image of the rectum, colon, and occasionally of a small part of the patient's small intestine. A flexible sigmoidoscopy may be done to detect any small polyps the barium enema X-ray may miss. If the barium enema X-ray detects anything abnormal, the doctor may recommend a colonoscopy (Cunningham *et al.,* 2010).

v. Colonoscopy

A colonoscope is longer than a sigmoidoscope. It is a long, flexible, slender tube, attached to a video camera and monitor. Any polyps discovered during this exam can be removed during the procedure, and sometimes tissue samples, or biopsies, are taken instead. A colonoscopy is painless, but some patients are given a mild sedative to calm them down. Before the exam, they may be given laxative fluid to clean out the colon. An enema is rarely used. Bleeding and perforation of the colon wall are possible complications, but extremely rare (Tanaka *et al.,* 2018).

2.4.1.8 Prevention

The most important ways to prevent colorectal cancer are a healthy lifestyle, appropriate medication, and continuous screening.

i. Life style

Dietary recommendations include reducing the consumption of red meat and increasing the intake of fruits, vegetables, fibers and whole grains (Doyle, 2007). Physical activity like exercise also helps reducing the risk of colorectal cancer (Harriss *et al.,* 2.009).

Most studies have shown that high intake of folic acid (vitamin B_9), which is found in dark-green vegetables, reduces the risk of colorectal cancer and adenomas (Giovannucci 2002).

ii. Medication

People at high risk of having colorectal cancer are advised to take aspirin and celecoxib. They both appear to decrease the risk factor. Nevertheless, these drugs are not recommended to those at average risk (Cooper *et al.,* 2010). Vitamin D, especially its blood concentration, and Calcium intake are also associated with lower risks of colorectal cancer (Ma *et al.,* 2011).

iii. Screening

Several screening methods are applied nowadays, and they proved to be helpful in reducing death by the early detection (He and Efron, 2011). The three main tests are fecal occult blood testing of the stool, sigmoidoscopy, and colonoscopy (Qaseem *et al.,* 2012). The newest screening method is the M2-PK test to stool samples. The M2-PK enzyme biomarker is highly sensitive to colorectal cancer. It is able to detect bleeding and non-bleeding types, which a fecal occult blood test cannot do (Tonus *et al.,* 2012).

2.4.1.9 Treatment

Stage 0 cancer can be treated by removing cancer cells by colonoscopy. For stage I, II and III Cancer, it is necessary to perform surgery using radical colectomy of the segment involved with margins > 5 cm., lymphadenectomy to the root of the nutrient vessel (minimum 12N), suspicious ganglion biopsy outside the resection field. It has been

demostrated that the laparoscopic approach is as safe as the traditional open approach for colorectal cancer (Arribas *et al.,* 2014). In patients with metastases in distal organs the addition of irinotecan to Fluorouracil and Leucovorin was demonstrated prolongs survival in patients and was considered the new first-line standard therapy for this disease in patients with stage III cancer receive complementary chemotherapy after surgery for 6 to 8 months, improving symptoms and prolonging survival in people with stage IV cancer primarily (Herbert *et al.,* 2004). The 5-Fluoracil continues to be the cytostatic mostly used in the treatment of colon cancer, those who make use of it or at least approach the fulfillment of the therapeutic standard, achieve better percentages of survival. Currently, the role of an antibody variant called Bevacizumab (Avastin) approved by the US Food and Drug Administration (FDA) has been evaluated by lowering vascular endothelial growth factor (VEGF), the main angiogenesis regulator, produced by normal and neoplastic cells (Ferrara *et al.,* 2003). Preclinical trials have shown that a human monoclonal antibody against VEGF can inhibit the the growth of xenografts of human tumors (Kim *et al.,* 1993).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Apparatus

The apparatus used in this study were volumetric flask, conical flask, measuring cylinder, funnel, beaker, pipette, glass rod, s eparating funnel, dry air oven, water bath, refrigerator, filter paper, crucible, spectrophotometer, test tube, brine shrimp egg, luminometer, CellTiter-Glo assay kit, aerator, 60-watt bulb and electronic balance.

3.1.2 Chemicals and reagents

All chemicals and reagents used in this study were of analytical grade, products of Sigma Aldrich. The chemicals used include sulphuric acid, formalin, sodium carbonate and acetic acid, ethyl acetate, sodium acetate, ferrus sulphate, aluminium chloride, n-hexane among others. The reagents used were folin-Ciocalteu's reagent, deni's reagent, Folin-Denis reagent, ascorbic acid, sea water, foetal bovine serum, methanol, and normal saline among others.

3.1.3 Colorectal cancer cell line used

Human Colorectal cancer cell line (HT-29 cell line) used for this study was obtained at 3 fountain drive inchinnan, Invitrogen United Kingdom. The cell line was maintained in a standard medium at 37^0C in a tissue culture incubator with an atmosphere of 5% CO_2.

3.1.4 Sample collection and identification

Senna alata leaf used in this study was obtained from Doko, Lavun Local Government area of Niger State, Nigeria and *Khaya senegalensis* stem bark was obtained from Bida, Local Government area of Niger State, Nigeria in February 2020. The samples was transported in a polyethylene bag to Taxonomy Unit National Institute of Pharmaceutical

Research and Development (NIPRD) Abuja and identified by a taxonomist and voucher specimen number of NIPRD/H/7097 and NIPRD/H/7095 was allotted.

3.1.5 Experimental animals

A total of eighteen (18) healthy Wistar rats of both sexes weighing between 120-160 g were obtained at National Veterinary Research Institute Animal house, Vom, Plateau State, Nigeria. The animals were housed in standard cages and given access to standard pelleted feed (Vital feed) and water *ad libitum* prior to the commencement of the experiment.

3.2 Methods

3.2.1 Sample preparation and extraction

The leaf of *Senna alata* and stem bark *Khaya seneegalensis* were washed with water to remove dust and other adhering particles and air-dried under shade for 2 weeks at 25^0C. The samples were grounded into fine powder using mortar and pestle, and the powdered samples were used for the extraction process.

Two hundred grams (200 g) of the powdered leaves and stem bark were separately extracted with ethanol using heating mantle. The extracts were filtered using Whatman No 1 filter paper and extracts were collected in separate clean beakers and concentrated on water bath at 70^0C. The concentrated extracts were kept in a refrigerator at 4^0C until required for use.

3.2.2 Phytochemical screening

3.2.2.1 Total phenol determination

The method of Singleton *et al.,* (1999) was used to determine total phenol content of extracts. Briefly, 0.01 g of each extract was dissolved in 10 mL of distilled water, and 0.5 mL was oxidized by adding 2.5 mL of 10% Folin-Ciocalteu's reagent which was then neutralized by 2 mL of 7.5% sodium carbonate. The reaction mixture was incubated at

45 ^{0}C for 40 minutes. Absorbance was read at 765 nm using double beam Shimadzu UV spectrophotometer, UV-1800.

3.2.2.2 Flavonoids determination

Flavonoids content of the extracts was determined using the method of Chang *et al.,* (2002). In this method, 0.01 g of each extract was weighed and dissolved in 10 mL of distilled water. Then 0.5 mL of each extract was added to a test tube containing 1.5 mL of absolute methanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M sodium acetate and 2.8 mL of distilled water and incubated at ambient temperature for 30 minutes. The absorbance was read at 415 nm with double beam shimadzu UV-spectrophotometer, UV-1800.

3.2.2.3. Alkaloids determination

Alkaloids content of the extracts was determined using method of Oloyede (2005). Briefly, 0.5 g of each of the extract was weighed and dissolved in 5 mL of mixture of 96% ethanol:20% H_2SO_4 (1:1) and then filtered using Whatman No 1 filter paper. 1 mL of each filtrate was then added to a test tube containing 5 mL of 60% H_2SO_4 and allowed to stand for 5 minutes. Thereafter, 5 mL of 0.5% formalin was added and allowed to stand at room temperature for 3 hours. The absorbance was read at wavelength of 565 nm.

3.2.2.4. Tannins determination

Tannin content of the extracts was determined using the method of Sofowora (1984). Briefly, 0.2 g of each of the extract was weighed into a 50 mL beaker and 20 mL of 50% methanol was added to it and covered with para film and heated in water bath at 80 ^{0}C for a period of 1 hour. The reaction mixture was shaken thoroughly to ensure uniformity. Each extract was then filtered into separate 100 mL volumetric flasks, and 20 mL of distilled water, 2.5 mL of Folin-Denis' reagent, and 10 mL of sodium carbonate were added and mixed properly. The reaction mixtures were then allowed to stand for 20

minutes at room temperature for the development of bluish-green coloration. The absorbance was recorded at 760 nm using double beam shimadzu UV-spectrophotometer, UV-1800.

3.2.2.5 *Saponins determination*

Saponins content of the extracts was determined using the method of Oloyede (2005). In this method, 0.5 g of the extract was weighed and dissolved in 20 mL of 1 N HCl and boiled in water bath at 80 ^{0}C for 4 hours. The reaction mixtures were cooled and filtered. 50 mL of petroleum ether was added and the ether layer was collected and evaporated to dryness. Thereafter, 5 mL of acetone-ethanol (1:1), 6 mL of ferrous sulphate and 2 mL of concentrated sulphuric acid were added and allowed to stand for 10 minutes. The absorbance was taken at 490 nm.

3.2.3. Determination of median lethal dose (LD_{50})

The LD_{50} of the Ethanol extracts of both *Senna alata* and *Khaya senegalensis* was determined according to method of Lorke (1983). This method consists of two distinct phases. In phase I, wistar rats were grouped into three groups of three rats each for each of the extract and administered doses of 10, 100 and 1000 mg/kg Bw. of each of the extract, respectively. In the absence of toxicological signs and more importantly mortality, phase II of this method was conducted. In phase II, rats were grouped into three groups of three rats each, for each of the extract and higher doses of 1900, 2900 and 5000 mg/kg Bw. of each of the extract was administered respectively.

3.2.4 Brine shrimp lethality assay (BSLA)

Principle

Brine shrimp lethality bioassay is a simple, high throughput cytotoxicity test of bioactive chemicals. It is based on killing ability of the test compounds on a simple zoological organism-brine shrimp (*Artemia salina*). This assay was first proposed by (Micheal *et al*., 1956) and further developed by several groups. The brine shrimp lethality bioassay is

widely used in the evaluation of toxicity of heavy metals, pesticide, medicines especially natural plant extracts (Vanhaecke *et al.*, 1981). It's a preliminary toxicity screen for further experiments on mammalian animal models.

Brine Shrimp Lethality Assay (BSLA) was carried out using the method of Meyer *et al.*, 1982. Two grams (2 g) of the Brine shrimp egg was placed in 800 mL of sea water in 1L conical flask under 60-watt bulb to provide illumination and aerator to provide oxygen. Aeration and illumination was carried out for complete 48 hours for the hatching of the Brine shrimps into nauplii. The hatched egg was left under illumination for another 48 hours prior to the assay. After two days, when the shrimp larvae are ready, 3 mL of the sea water was dispensed into calibrated 5 mL vial and 10 brine shrimps was introduced into each making a total of 30 shrimps per dilution and the volume was adjusted with sea water to 5 mL per vials. The vials were left uncovered under the lamp. The number of surviving shrimps was counted and recorded after 24 hours. Using probit analysis, the Lethality Concentration at 50 and 90 (LC_{50} and LC_{90}) was assessed at 95% confidence intervals.

3.2.5 Solvent partitioning of crude methanol extract of *Senna alata*

Fractionation by partitioning was done according to the method of Gandhi *et al.*, (2003) and Leila *et al.*, (2007) with some modification in the choice of primary solvent (water) and partitioning (separating) solvents (n-hexane, ethyl acetate and aqueous). The methanol extract residue obtained was dissolved in water (500 mL) and exhaustively extracted by consecutive liquid/liquid partition with n-hexane (500 mL), ethyl acetate (500 mL) and aqueous fractions (500 mL) using a separating funnel (1000 mL). The n-hexane, ethyl acetate, and aqueous fractions was evaporated to obtain fractions. The fractions obtained (n-hexane, ethyl acetate, and aqueous) were further subjected brine shrimp lethality assay to obtain the fraction with lowest LC_{50}.

3.2.6 DPPH (2, 2-Diphenyl-1-Picrylhydrazyl) free radical scavenging assay

The antioxidant activity of the plant extracts was estimated using the DPPH radical scavenging assay as described by Oyaizu (1986). Briefly, different concentrations of extracts and ascorbic acid (50, 100, 200 and 400 µg/mL) were prepared from stock solutions (1000 µg/mL), prepared by weighing and dissolving 0.01g of the extracts and ascorbic acid, respectively in 10 mL of methanol. Thereafter, 2 mL of 0.004% DPPH in methanol were added to 1 mL of various concentrations of plant extracts and ascorbic acid, respectively. The reaction mixtures were incubated at 25°C for 30 minutes. The absorbance of each test mixture was read against blank at 517 nm using double beam Shimadzu UV-1800 series spectrophotometer. The experiment was performed in triplicates. The percentage antioxidant activity was calculated using the formula below:

% Inhibition = I% = (1 – A sample/A blank) × 100

Where:

A blank: Is the absorbance of the control reaction

A sample: Is the absorbance of the sample.

3.2.7 Cell viability assay protocols

3.2.7.1 Measures taken for the use of cell culture

Measures such as authentication of cell line, cryopreservation, and avoidance of cross-contamination of cell lines were all taken. Furthermore, the cells were checked for microbial contamination (regular mycoplasma test), (Hall *et al*., 1998).

3.2.7.2 Cell line:

For the cell line used (HT-29), the Dulbecco's Modified Eagle's medium (DMEM) were supplemented with filter sterilised 10% foetal bovine serum (FBS), 2 mM glutamine, 1 mM sodium pyruvate, 100 µg/ml streptomycin and 100 U/mL penicillin all purchased

from Invitrogen UK. Cells were maintained at 37ºC in a tissue culture incubator with an atmosphere containing 5% CO_2.

3.2.7.3 Sub-culturing the cells

Sub-culturing of cells used in this study was performed regularly to ensure their survival, regular supply for the study and maintain proper growth conditions. It was routinely done with cells that reached 80-90% confluence either in flask or tissue culture plates. All the procedures were done using sterile techniques performed in a sterile tissue culture hood. Briefly, in the process of sub-culturing, the media, buffers and trypsin were placed in water bath at 37°C at least 30 min prior to subculturing the cells. Tissue culture lab coat and gloves were worn. The gloves were sprayed with 70% ethanol. The laminar flow in the hood was turned and the working surface of tissue culture hood was swabbed with 70% ethanol. Any apparatus, media, trypsin and buffers taken inside the hood was first swabbed by 70% ethanol except for tissue culture flasks and plates. The tissue culture flask needed to be sub-cultured (>80% cell confluence) was taken out of the incubator, observed under the microscope and placed into the hood. With the help of aspirator attached to the suction pump, the old medium was removed from the flask. 4 ml of PBS (Invitrogen) was added to the cells and the flask gently swirled to wash away the old media and dead cells. PBS was aspirated out. 1 ml of pre-warmed 0.25% trypsin (Invitrogen) was gently added on top of cells with the help of serological pipette, and the flask swirled to ensure spreading of trypsin on the entire surface area of the flask. The flask was taken into tissue culture incubator maintained at 37ºC with 5% CO_2 atmosphere and incubated for 5 min (or until the cells were detached as observed under the light microscope). After the cells detached, 4 mL of pre-warmed cell culture media was added to the flask to stop the further action of trypsin and dilute the cells. With the help of serological pipette, the cell suspension was mixed thoroughly by pipetting up and down

to ensure total detachment and to break any cell clumps formed. After this, the number of cells in the suspension was determined and either seeded into other flasks for propagation and cell culture maintenance, or cryo-frozen for future use (Hall *et al*., 1998).

3.2.7.4 Cell counting

Throughout the study, human colorectal cancer cell line (HT-29 cell line) were routinely counted for different experiments via counting chamber method. In this method, first of all, the counting chamber and the cover-slip used were cleaned with the help of lens paper and put under the light microscope. The coverslip was placed on top of the gridded area. The grid in the counting chamber was composed of squares of different areas. The cells to be counted were first trypsinized and then diluted in cell culture media. Every time micropipette is used to take 10 μL of cell suspension from the flask and put underneath the coverslip over the counting chamber where the cell suspension spreads quickly. The cells were counted in ten 0.04 mm^2 squares in the grid. Then, the number of cells per μL was calculated with the following formula:

Supposing, the number of cells counted in ten 0.04 mm^2 squares = 20 cells (supposed)

Total area in which 20 cells counted = (10 x 0.04) = 0.4 mm^2

Total volume = 0.4 mm^2 x 0.1 (height of chamber) = 0.04 mm^3

So, 20 cells in 0.04 mm^3.

1 mm^3 = 1 μL, hence, 0.04 mm^3 = 0.04 μL

If 0.04 μL has 20 cells, 1 μL has = 20 x 1 μL ÷ 0.04 μL = 500 cells.

Hence, 1 μL has 500 cells.

3.2.7.5 Cryofreezing cells and reviving frozen cells

In this case, the surplus cells were routinely cryo-frozen for future use. After the cells were trypsinized from a T75 flask, 5 mL of cell media was added to stop the action of trypsin, and the cell suspension was mixed thoroughly with the help of serological pipette

attached to the pipette buoy. The cell suspension was transferred to a sterile 15 mL centrifuge tube, capped tightly and taken to the centrifuge machine set at 37°C. The cell suspension was centrifuged at 1000 rpm for 15 min to palette the cells. In the meantime, sterile cryotubes (Thermo Fischer) were labelled with cell details and passage number. After centrifugation, the tube was taken back to the hood, the supernatant was discarded and the cell palette was gently resuspended in 1 mL of freezing media.

Following this, the cell suspension in freezing media was carefully transferred to cryotubes, capped properly and immediately taken for storage in -80°C freezer. When previously frozen cells were needed, the media was first warmed in the water bath at 37°C and 10 ml of media was transferred to the T75 flask. The cryotube with frozen cells was taken out and immediately put in the water bath at 37°C. As soon as the frozen mixture was thawed (usually after 1- 2 min), the cryotube was taken to the tissue culture hood, and with the help of pipette, very gently transferred into the T75 flask with the media. After 16-24 hrs, old media was replaced by new media to replenish the nutrients and boost cell growth and after every 3-4 days, cell media was usually changed by first aspirating the old media, washing the cells with warm PBS, and transferring fresh media into the flask to boost the healthy condition of the growing cells.

3.2.7.6 Treatment of cell lines with extracts and fractions

The extracts used in this research work involved the preparation of stock solutions of these extracts and stored as required. Working concentrations were achieved by diluting the stock in the media used and were based on prior literature. Prior to treatment with these extracts, cells to be treated were taken out of the incubator; old media removed and pre-warmed PBS added. Pre-warmed media was then transferred to falcon tubes (corning) in the tissue culture hood and the required amount of drug was added to achieve the working concentrations. The PBS over the cells was taken out and the media with the

drug carefully added. The amount of media added was dependent upon the type of tissue culture vial in which the cells were grown. For example, the cell lines were maintained in DMEM (Gibco® Invitrogen) supplemented with 10% foetal bovine serum (FBS), 2 mM glutamine, 1 mM sodium pyruvate, 100 µg/mL streptomycin and 100 U/mL penicillin in an atmosphere of 5% CO_2 and incubated at 37^0C. All the extracts were used by directly diluting the extracts in media to desired final concentrations.

3.2.7.7 Cell viability assay

The CellTiter-Glo® assay kit (Promega) was used to determine cell viability, as described crouch *et al.* (1993) and Hall *et al*. (1998) Briefly, cells were seeded in a luminometer compatible 96-well plate and allowed to adhere for 18-24 hrs. After that, the cells were treated with different concentrations of the extracts for 24hrs and 48hrs. Following treatments, the plate and its contents were equilibrated to room temperature for approximately 30 mins, a volume of CellTiter-Glo reagent equal to the volume of cell culture medium present in each well the contents were then mixed for 2 min on an orbital shaker to induce cell lysis and the plate was then incubated at room temperature for 10 min to stabilize the luminescent signal and finally the luminescence was recorded using luminometer (MODULUS, Promega). The luminescent signal is proportional to the amount of ATP in the sample, which indicates the presence of metabolically active cells (Patrick *et al.*, 2018).

3.3 Data Analysis

All numeric data generated were expressed as the mean ± standard error of mean (SEM). Comparison between different groups will be perform using analysis of variance (ANOVA Test). The significant difference between control and experimental groups will be assessed using Duncan's post hoc test using SPSS version 26.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Results

4.1.1 Phytochemical composition of the extracts

Table 4.1 shows the concentrations of phytochemical constituents present in the extract of *Senna alata* and *Khaya senegalensis.* It was observed that the amount of phenols and flavonoids present in the two extracts were not significantly different while the concentrations of tannins (75.10±0.14 mg/100 g) and alkaloids (229.84±0.59 mg/100g) were significantly ($p<0.05$) higher in *Khaya senegalensis* than *S. alata.* Saponins was significantly higher (282.50±1.62 mg/100 g) in *Senna alata* compared to that in *Khaya senegalensis* at $p<0.05$

Table 4.1: Phytochemical Constituents of *Khaya senegalensis* and *Senna alata*

Phytochemicals	*Senna alata* (mg/100 g)	*Khaya senegalensis* (mg/100 g)
Phenols	397.70±1.01[a]	403.58±1.04[a]
Flavonoids	138.56±0.09[a]	136.7±0.06[a]
Tannins	43.13±0.23[a]	75.10±0.14[b]
Alkaloids	185.09±0.33[a]	229.84±0.59[b]
Saponins	282.50±1.62[b]	166.38±1.24[a]

Values are expressed as mean ± standard error of mean. Values with different superscript on across a row are significantly different at $p<0.05$.
Footnote:
aa: Values with the same superscript across a row are not significantly different
ab: Values with different superscript across a row are significantly different

4.1.2 Acute oral toxicity test of the extracts

No mortality or any toxicological signs were observed during the acute oral toxicity testing. As a result, the LD_{50} of both ethanol and n-hexane extract was found to be greater than 5000mg/kg bw (LD_{50}> 5000 mg/kg bw.) as depicted in table 4.2. No death was recorded in all the treated groups.

Table 4.2: Acute Oral Toxicity Test of Methanol Extracts of *Khaya senegalensis* and *Senna alata* in Wister Rats

Treatment	*Senna alata* leaf extract			*Khaya senegalensis* Stem bark extract		
Phase I						
No of rats used	3	3	3	3	3	3
Dosage (mg/kg bw.)	10	100	1000	10	100	1000
Mortality	0/3	0/3	0/3	0/3	0/3	0/3
Sign of toxicity	None	None	None	None	None	None
Phase II						
No of rats used	3	3	3	3	3	3
Dosage (mg/kg BW)	1900	2900	5000	1900	2900	5000
Mortality	0/3	0/3	0/3	0/3	0/3	0/3
Sign of toxicity	None	None	None	None	None	None

4.1.3 Brine shrimp lethality assay

The cytotoxic effect of crude extracts as well as fractions of *Senna alata* and *Khaya senegalensis* to brine shrimp is presented in the Table 4.3. The results showed that the ethyl acetate fraction of *Senna alata* was more lethal against brine shrimp than other extracts and fractions. It had an LC_{50} of 1.308 ppm while the LC_{90} was 249.925 ppm. Next to this sample in lethality was the n-hexane fraction of *Senna alata* with an LC_{50} and LC_{90} of 6.172 and 1024.843 ppm. The crude extract of *Senna alata* had LC_{50} of 17.87 ppm and LC_{90} of 7,951,036 ppm while the crude extract of *Khaya senegalensis* had LC_{50} of 30.97 ppm and LC_{90} of 9,683,402 ppm. Lastly is the aqueous fraction of *Senna alata* with an LC_{50} of 57.805 ppm and LC_{90} of 2,393,551 ppm respectively.

Table 4.3: Cytotoxicity of *Senna alata* and *Khaya senegalensis* to Brine Shrimp

Extracts	LC_{50} (ppm)	LC_{90} (ppm)
CruSA	17.87^{c}	$7,951,036^{d}$
CrKS	30.975^{d}	$9,683,402^{e}$
EtAcSA	1.308^{a}	$249,925^{a}$
nHexSA	6.172^{b}	$1,024,843^{b}$
AqSA	57.805^{e}	$2,393,551^{c}$

Key:
CruSA = Crude extract of *Senna alata*
CrKS = Crude extract of *Khaya senegalensis*
EtAcSA = Ethyl Acetate fraction *Senna alata*
nHexSA = N-hexane fraction of *Senna alata*
AqSA = Aqueous fraction of *Senna alata*

4.1.4 DPPH (2, 2-Diphenyl-1-Picrylhydrazyl) free radical scavenging assay

The Figure 4.4 shows the DPPH antioxidant scavenging ability of ethyl acetate fraction of *Sanna alata*. A dose dependent increase in scavenging ability was observed for the standard ascorbic acid as well as the ethyl acetate fraction of *Sanna alata*. However, the standard ascorbic acid had higher scavenging ability (%) than in the ethyl acetate fraction of *Sanna alata* at all the concentrations. At the concentration of 100 µg/mL, the % scavenging ability obtained for both standard and the fraction were 88.51 and 58.37 % respectively. The IC_{50} of the standard ascorbic acid was 12.32 µg/mL while that of ethyl acetate fraction of *Sanna alata* was 75.56 µg/mL.

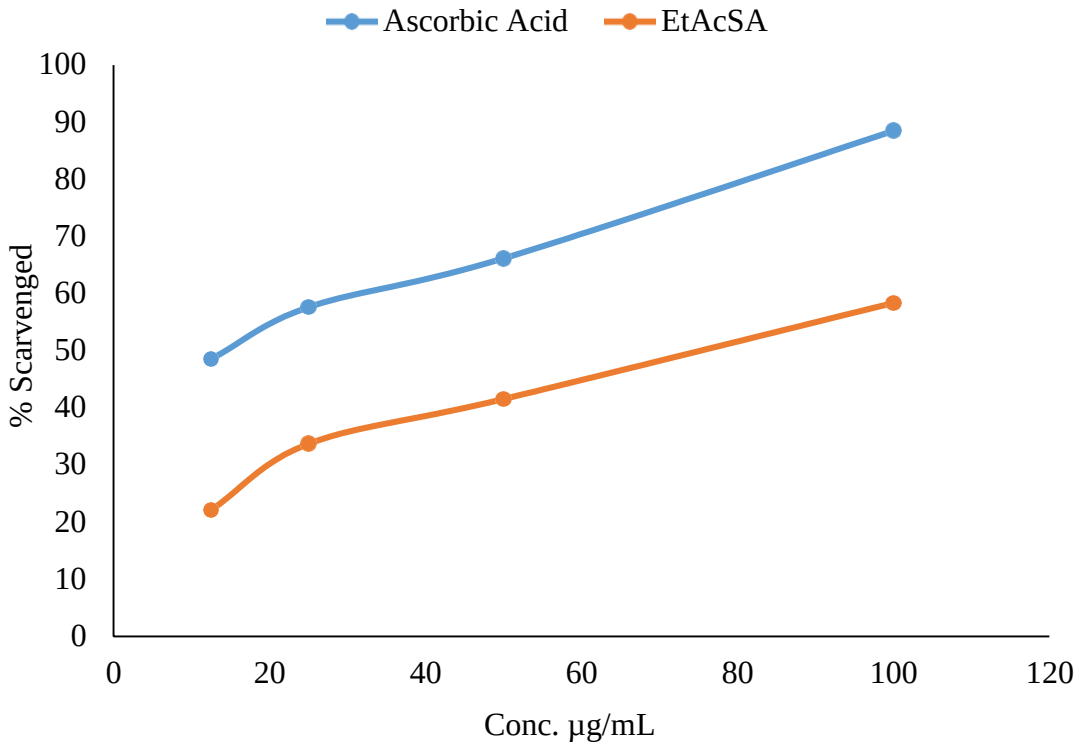

Figure 4.1: DPPH Antioxidant Assay for Ethyl Acetate Fraction of *Sanna alata* (EtAcSA)

Ascorbic: y = 0.4405x + 44.573 (R^2 = 0.9923) X = 12.32 µg/mL
EtAcSA: y = 0.386x + 20.835 (R^2 = 0.9624) X = 75.56 µg/mL

4.1.5 Inhibition of HT-29 cells proliferation after 24 hours

Figures 4.5 present the percentage inhibition after exposing the cancer cell lines for 24 hours to the ethyl acetate fraction of *Senna alata* and the standard doxorubicin drug respectively. At an exposure of 24 hours, increase in concentration of both the extract (50-1000 µg/mL) as well as standard drug (0.5-10 µg/mL) resulted to higher percentage of inhibition. The percentage inhibition displayed by the ethyl acetate fraction of *Senna alata* ranged from 28.51 to 82.72 % at 50 and 1000 µg/mL respectively while the standard drug, doxorubicin had % inhibition ranged for 29.55 to 84.00 µg/mL at a concentration of 0.5 and 10 µg/mL respectively. The IC_{50} of the extract (324.19 µg/mL) was higher than that of the standard drug (2.91 µg/mL) at 24 hours exposure.

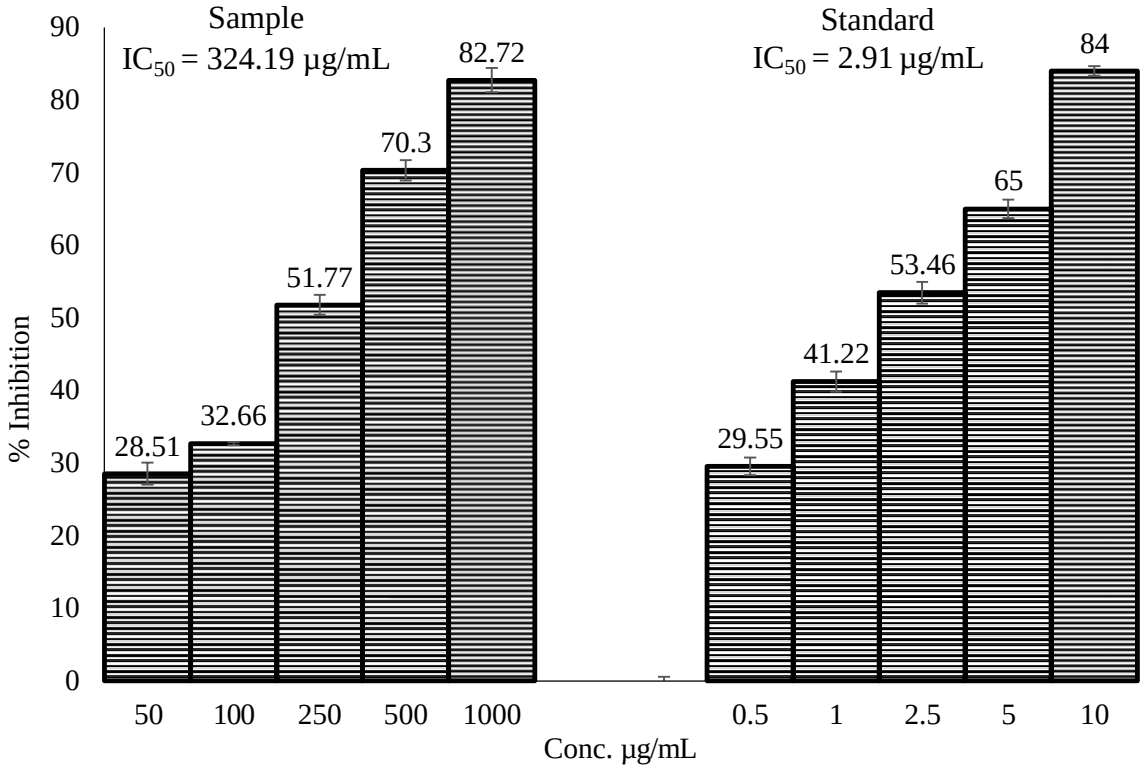

Figure 4.2: Percentage Inhibition of HT-29 Cancer Cells Treated with Ethyl Acetate Fraction of *Sanna alata* and Doxorubicin Drug for 24 hours

4.1.6 Inhibition of HT-29 cells proliferation after 48 hours

The percentage inhibition of HT-29 Cancer cells by ethyl acetate fraction of *Senna alata* and the standard doxorubicin drug after an exposure of 48 hours is shown in Figure 4.6. Similar trend was observed as was shown in Figure 4.5 for 24 hours exposure. Although the percentage inhibition of the cancer cell was higher for both the sample and standard drug on exposure at 48 hours than 24 hours. The percentage inhibition of the HT29 Cancer Cells by ethyl acetate fraction of *Senna alata* ranged from 38.00 to 93.04 % at 50 and 1000 µg/mL respectively while the standard drug, doxorubicin had % inhibition which ranged from 37.91 to 95.00 µg/mL at a concentration of 0.5 and 10 µg/mL respectively. The IC_{50} of ethyl acetate fraction of *Senna alata* (91.52 µg/mL) was higher than that of the standard drug (1.18 µg/mL) at 48 hours exposure.

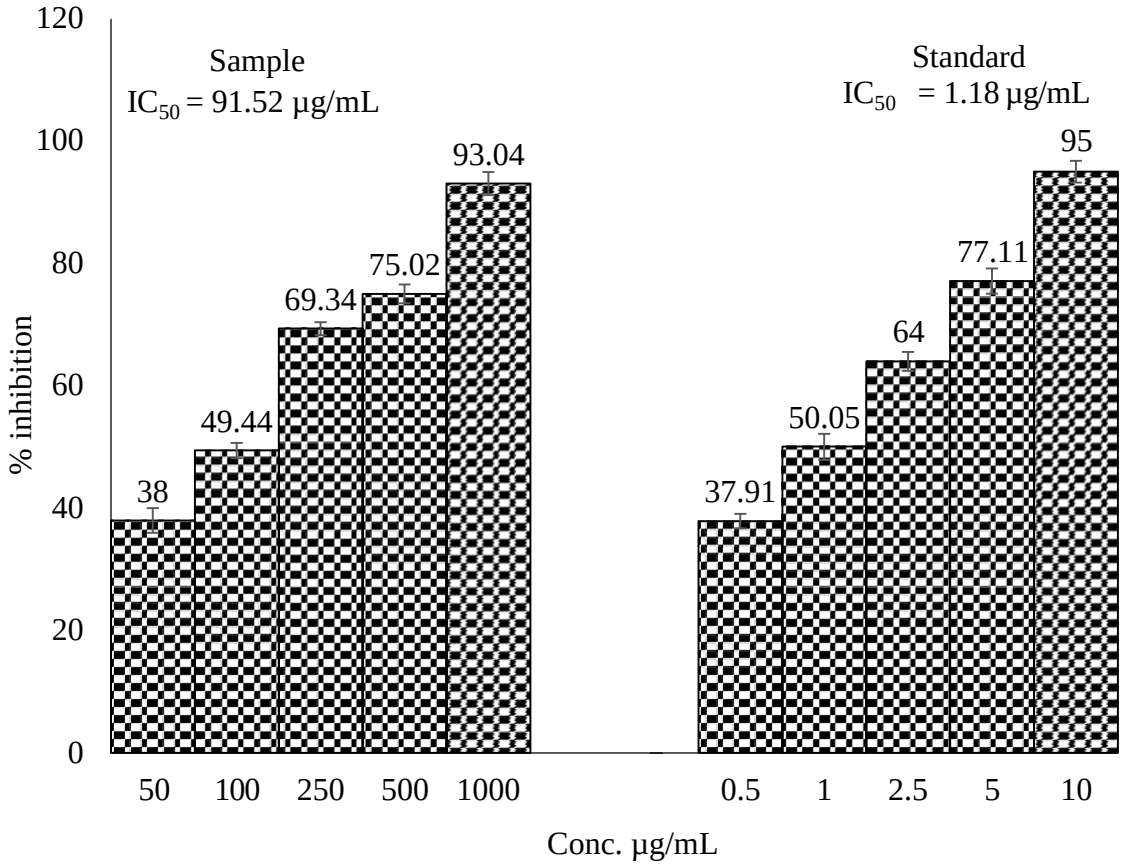

Figure 4.3: Percentage Inhibition of HT-29 Cancer Cells Treated with Ethyl Acetate Fraction of *Sanna alata* and Doxorubicin Drug for 48hours

4.2 Discussion

4.2.1 Quantitative phytochemical screening

Quantitative preliminary phytochemical screening of the leaves of *Senna alata* and *Khaya senegalensis* showed appreciable amounts of Phenols, Flavonoids, Tannins, Alkaloids and Saponins. The valuable pharmaceutical properties of these plants may be attributed to the presence of phenols and othser bioactive compounds such as Flavonoids. The presence of phenolic compounds has been extensively used in disinfection and remain the standard with which other infections are compared. Phenolic compounds are electron donors which are readily oxidized to form phenolate ion, and electron acceptor giving rise to practical use of protonated phenols as a cleaning agent (Okwu, 2004). Flavoniods were presence in both extract of the plants. Flavonoids are potent water soluble super antioxidants and free radical scavengers which prevent oxidative cell damage, have strong anticancer activity and inhibit tumor growth (Okoli and Okere 2010). Saponins are very important as they are shown to have hypolipidemic and anti-cancer activity. The natural anti-cancer agent saponin reacts with cholesterol rich plasma membrane of various cancer cells and arrests their proliferation (Rao *et al.,* 1995). Alkaloids contribute various medicinal properties such as analgesic, anti-oxidant and astringent activity (Chung *et al.*, 1998). It is reported that the presence of tannins serves as an anti-inflammatory, antiseptic, antioxidant agent (De Bruyne *et al.,* 1999). However, tannin-containing plant extracts have been reported to be used as astringents, against diarrhoea, as diuretics, against stomach and duodenal tumours and as anti-inflammatory, antiseptic, antioxidant and haemostatic pharmaceuticals (Dolara *et al.,* 2005). Saxena *et al.,* (2013) pointed out that recently, tannins have attracted scientific interest, especially due to the increased incidence of deadly illnesses such as acquired immune deficiency syndrome (AIDS) and

various cancers (Blytt *et al*., 1988). This implies that these plants contain secondary metabolites that can serve as tools in medicinal plant research.

4.2.2 Toxicity test

The acute toxicological evaluation of drugs is essential for determining safety level of such drugs. Therefore, the absence of mortality or toxic signs upon oral administration of dose of 5000 mg/kg bw of both extracts, implies that the LD_{50} of the two extracts is greater than 5000 mg/kg bw (LD_{50} > 5000 mg/kg bw) which rendered the extracts safe. This is justified by the use of the plant extracts in the folklore medicine with no report of acute toxicological signs (OECD, 2000).

Brine shrimp lethality assay is a safe, economical method for determination of the cytotoxicity of synthetic and plant products (Almeida *et al* 2002). The toxicity of plant extracts can be evaluated by their LC_{50} values. If the LC_{50} values are lower than 1000 µg/ml is considered as cytotoxic (Meyer *et al* 1982). It is a guide for active cytotoxic and antitumor agents. This simple and effective procedure permits convenient standardization.

The cytotoxic effect of crude extracts as well as fractions of *Senna alata* and *Khaya senegalensis* to brine shrimp as presented that Ethyl acetate fraction of *Senna alata* were more toxic to the brine shrimps nauplii, (it exhibit potent toxicity) compared to other fractions of *Senna alata* and crude extract of *Khaya senegalensis*. The relation of toxicity of the extract to the nauplii was found to be directly proportional from low to high (0.1 µg/ml to 1000 µg/ml). Concentrations of the extracts ranging from lowest concentration (0.1 µg/ml) to highest concentration (1000 µg/ml) (Isaac *et al.,* 2018). The presences of components like alkaloids, flavonoids, saponins, and tannins which are already reported to have the cytotoxic property may be responsible for the reported activity in a dose dependant manner (Isaac *et al.*, 2018).

Researchers have shown their much interest on natural antioxidants because of their ability to scavenge free radicals without exerting toxic side effects. The presence of antioxidants such as phenols, flavonoids, tannins saponins and alkalods in plants may provide protection against a number of diseases; for example, ingestion of natural antioxidants has been inversely associated with morbidity and mortality from degenerative disorders (Gulcin, 2012).

4.2.3 Antioxidant activity

The DPPH antioxidant scavenging ability of ethyl acetate fraction of *Sanna alata* revealed that at the concentration of 100 µg/mL, the % scavenging ability of ethyl acetate fraction of *Senna alata* was 58.37% with an inhibitory concentration (IC_{50}) of 75.56 µg/ml. The ethyl acetate fraction of *Sanna alata* contains substantial amount of phenolics. This may be responsible for its marked antioxidant activity as assayed because of its ability to scavenge oxygen derived free radicals through protonation (Robert *et al.,* 2007). Flavonoids suppress reactive oxygen formation, chelate trace elements involved in free radical production, scavenge reactive species and up-regulate and protect antioxidant defenses. (Agati *et al.,* 2012) The free radical scavenging property was evaluated using neutralizing capacity towards DPPH free radical. The results showed that ethyl acetate fraction of *Sanna alata* has significant free radical scavenging property correlating with the previous study where strong antioxidant capacity was reported from root part (Mahmud *et al.,* 2017).

4.2.4 Colorectal cancer cell viability test

Result obtained from ATP cell viability assay showed a remarkable reduction in the total number of proliferating cells and an increase in the total number of non-viable cells over 24 and 48hours post treatment with the fraction of *Senna alata* in a dose dependent manner. The decrease in the number of proliferating cells is as a result of cytotoxicity

induced by varying concentrations of the fractions used. The result of ATP cell viability assay of ethyl acetate fraction of *Senna alata* correlates with the report of (Levy and Carley 2012). The decrease in the number of proliferating cells is as a result of cytotoxicity induced by varying concentrations of the fraction. Flavonoids such as Quercetin, kaempferol, Luteolin and a phenolic acid - Caffeic acid are very common dietary phytochemicals and are known for their cytotoxic properties against various cell lines (Lee *et al.,* 2017). This result suggests that the cytotoxic effect of ethyl acetate fraction of *Senna alata* on HT-29 cancer cell lines may be derived from the synergistic effects of these phytochemical constituents present in the leaves of the plant.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The results from the phytochemical composition of *Senna alata* and *Khaya senegalensis* revealed the high amount of phenol of (397.70±1.01) and (403.58±1.04), this may be responsible for its marked antioxidant activity because of its ability to scavenge oxygen derived free radicals through protonation.

The results obtained from the lethal dose (LD_{50}) of *Senna alata* and *Khaya senegalensis* extract did not cause any apparent sign of toxicity in the rats administered 5000 mg/kg body weight. The extract is therefore assumed to be safe.

The cytotoxic effects of the crude extract and fractions of *Senna alata* and *Khaya senegalensis* revealed that the ethyl acetate fraction of *Senna alata* with the lowest LC_{50} of 1.308 ppm and LC_{90} of 249.925 ppm was more toxic to the brine shrimp nauplii, because of the presence of component like Alkaloids, Flavonoids, Saponins and Tannins which have been reported to have cytotoxic property may be responsible for the reported activity.

The antioxidant scavenging ability of ethyl acetate fraction revealed that at the concentration of 100 µg/ml, the % scavenging ability obtained from ethyl acetate fraction of *Senna alata* was 58.37% with an IC_{50} of 75.56 µg/ml, the ethyl acetate fraction of *Senna alata* may contain substantial amount of phenolic that is responsible to scavenge oxygen derived free radicals.

The study also provided knowledge on the *in-vitro* cytotoxic potential of ethyl acetate fraction of *Senna alata* on HT-29 cell line with 93% inhibition (IC_{50} of 91.52 µg/ml) suggesting the possible use of this natural plant product as a promising anticancer agent.

5.2 Recommendations

i. Further studies on the efficacious of other non-polar solvent and their possible mechanism of action are needed.

5.3 Contribution of Research to Knowledge

The cytotoxic effects exerted by the ethyl acetate fraction of *Senna alata* to brine shrimp nauplii causes an increase in the number of non-viable cells there by decreasing the proliferating cells. The result obtained for ATP cell viability assay cause a remarkable reduction in the number of proliferating cells with 93% inhibition, and an increase in the number of non-viable cells over 48 hours post treatment with the fraction of *Senna alata* in a dose dependent manner. Ethyl acetate fraction of *Senna alata* could be useful in the management of Colorectal Cancer.

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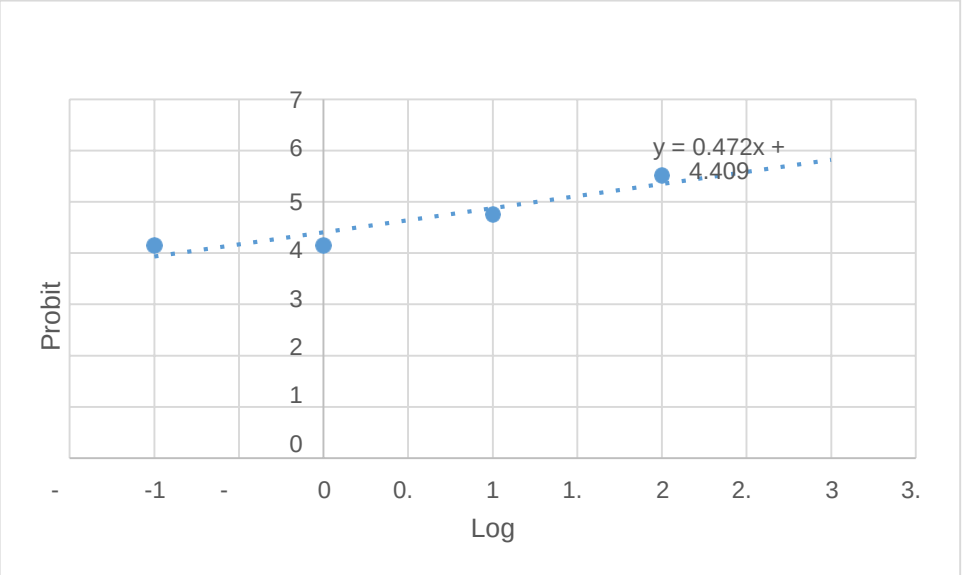
Appendix i: Phytochemical Composition of the Plant Sample

Sample	Phytochemical (mg/100g)				
	Phenols	Flavonoids	Tannins	Alkaloids	Saponins
Khaya Senegalensis Stem Bark	402.54 404.62	136.77 136.65	74.96 75.24	229.25 230.43	165.14 167.62
Senna alata Leaves	396.69 398.71	138.65 138.47	42.83 43.42	184.76 185.41	280.91 284.09

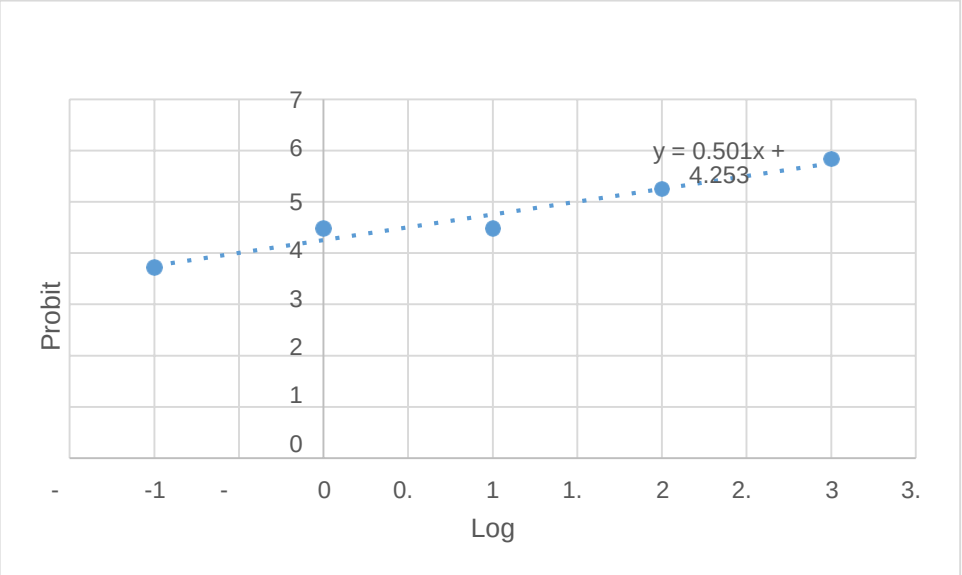
Appendix ii: Cytotoxicity of crude Extract of *Senna alata* to Brine Shrimp

C	Log (C)	No of dead nauplii	% Mortality	Probit Value	LC50	LC90
0						
0.1	-1	2	20	4.15		
1	0	2	20	4.15	17.87	7,951.036
10	1	4	40	4.76		
100	2	7	70	5.52		
1000	3	10	100			



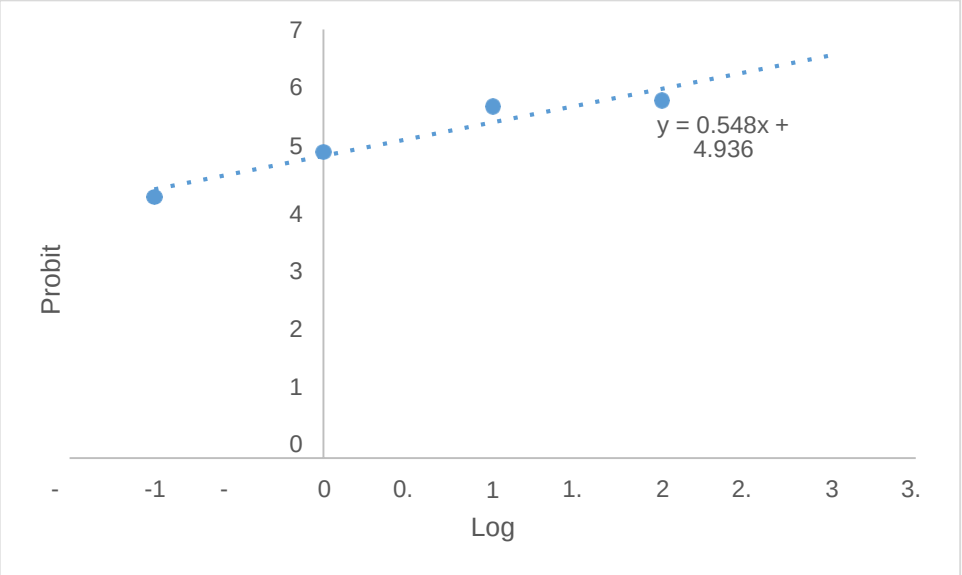
Appendix iii: Cytotoxicity of Crude Extract of *Khaya senegalensis* to Brine Shrimp

C	Log (C)	No of dead nauplii	% Mortality	Probit Value	LC50	LC90
0						
0.1	-1	1	10	3.72		
1	0	3	30	4.48	30.975	9,683.402
10	1	3	30	4.48		
100	2	6	60	5.25		
1000	3	8	80	5.84		



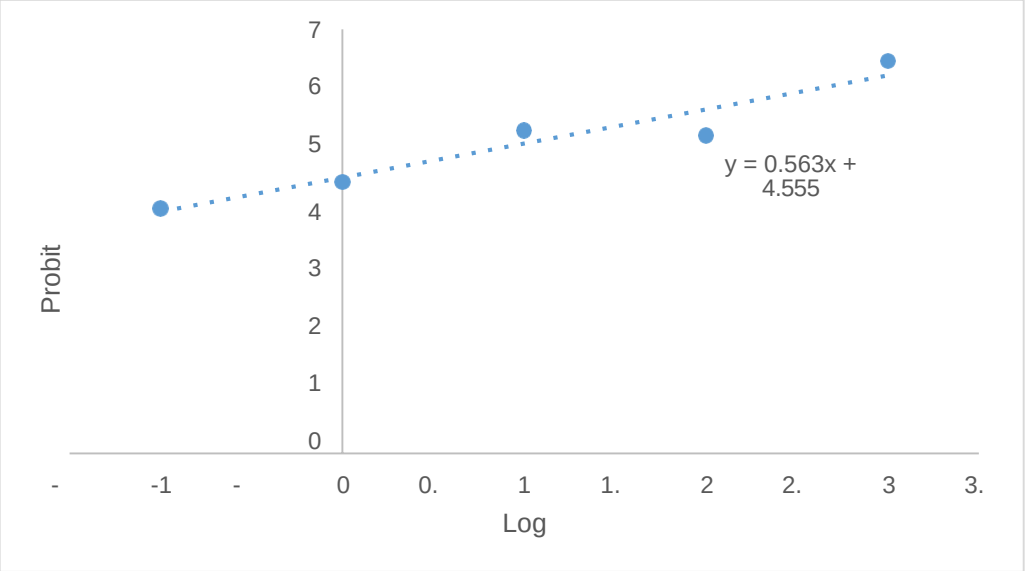
Appendix iv: Cytotoxicity of Ethyl Acetate Fraction of *Senna alata* to Brine Shrimp

C	Log (C)	No of dead nauplii	% Mortality	Probit Value	LC50	LC90
0		0				
0.1	-1	2.3	23	4.26		
1	0	5	50	5	1.308	249.925
10	1	7.7	77	5.74		
100	2	8	80	5.84		
1000	3	10	100			



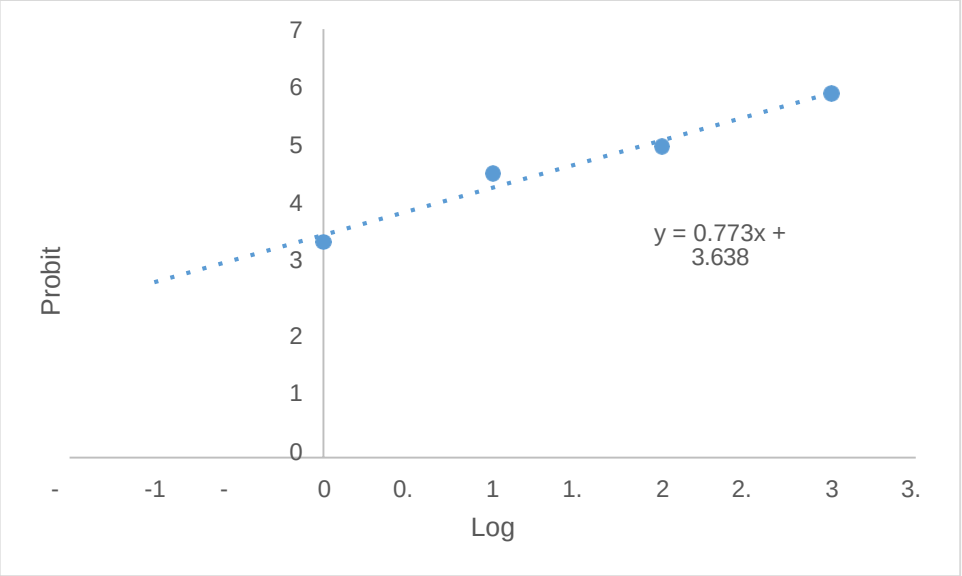
Appendix v: Cytotoxicity of n-hexane Fraction of *Senna alata* to Brine Shrimp

C	Log (C)	No of dead nauplii	% Mortality	Probit Value	LC50	LC90
0						
0.1	-1	1.7	17	4.05		
1	0	3	30	4.48	6.172	1,024.843
10	1	6.3	63	5.33		
100	2	6	60	5.25		
1000	3	9.3	93	6.48		

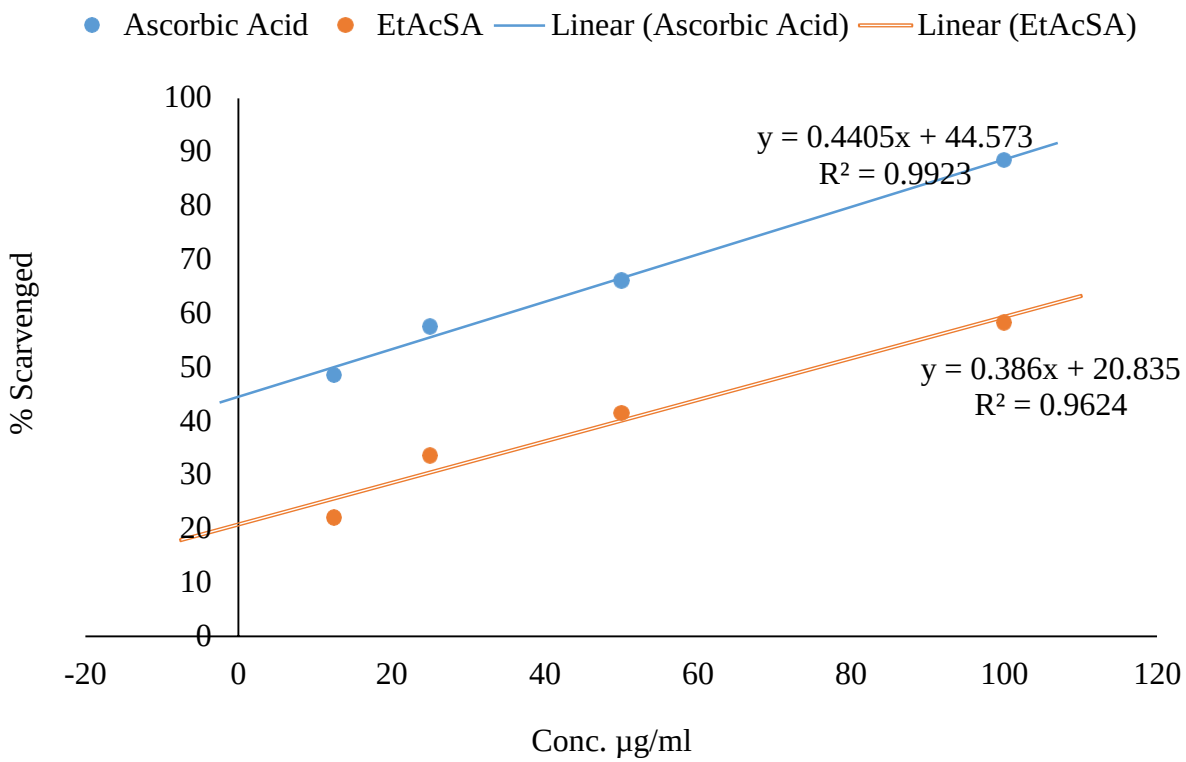


Appendix vi: Cytotoxicity of Aqueous Fraction of *Senna alata* to Brine Shrimp

C	Log (C)	No of dead nauplii	% Mortality	Probit Value	LC50	LC90
0						
0.1	-1	0	0			
1	0	0.7	7	3.52	57.805	2,393.551
10	1	3.7	37	4.64		
100	2	5.3	53	5.08		
1000	3	8.3	83	5.95		



Appendix vii: DPPH (2, 2-Diphenyl-1-Picrylhydrazyl) Free Radical Scavenging Assay

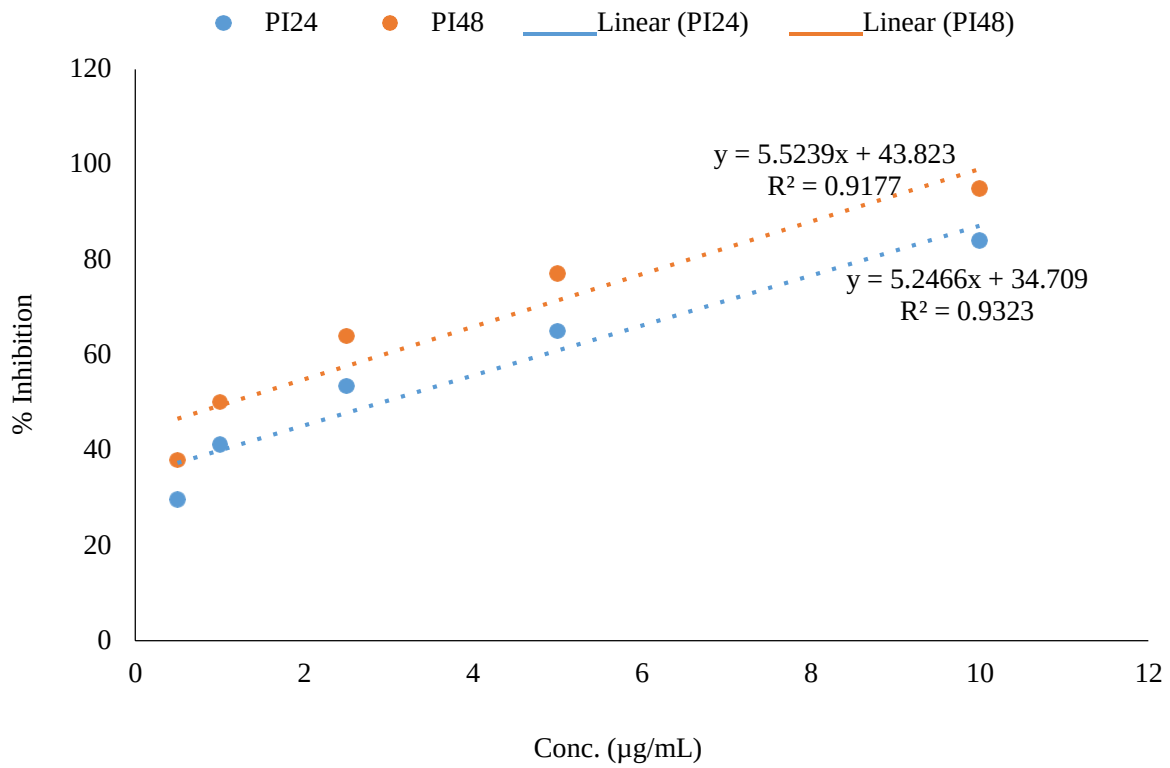


DPPH Antioxidant Assay for Ethyl Acetate Fraction of *Sanna alata*

Ascorbic: y = 0.4405x + 44.573 (R^2 = 0.9923) X = **12.32 µg/ml**

EtAcSA: y = 0.386x + 20.835 (R^2 = 0.9624) X = **75.56 µg/ml**

Appendix viii: Inhibition of HT-29 Cells Proliferation after 24 and 48hours with the standard

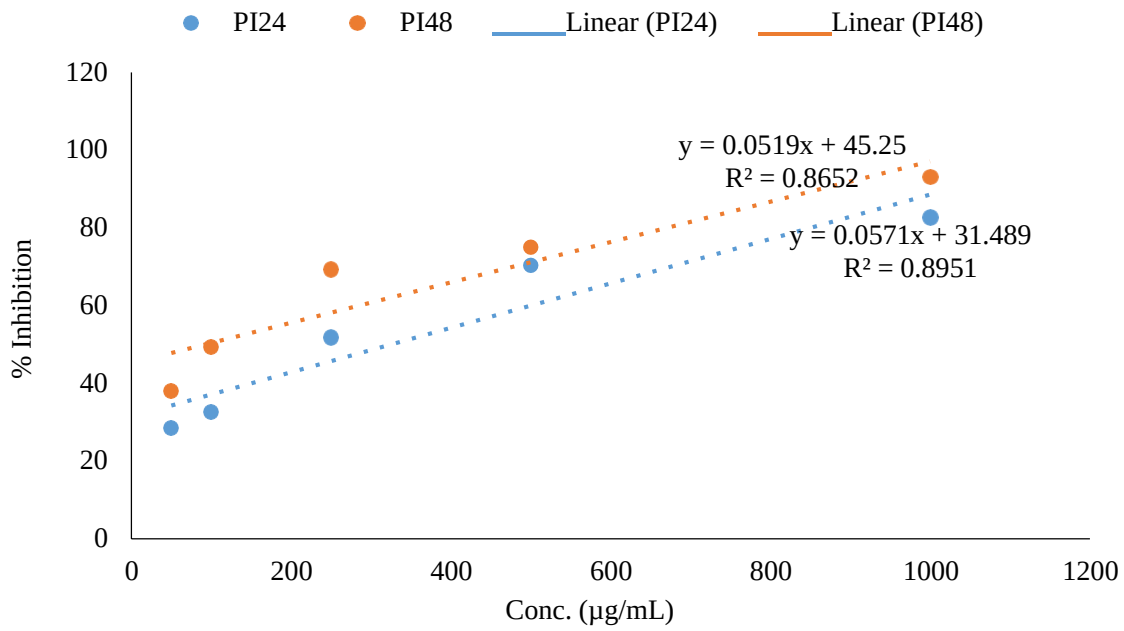


Percentage Inhibition of HT-29 Cancer Cells treated with Doxorubicin Drug for 24 and 48hrs

IC_{50} at 24hrs: y = 5.2466x + 34.709 (R^2 = 0.9323) X = **2.91 µg/ml**

IC_{50} at 48hrs: y = 5.5239x + 43.823 (R^2 = 0.9177) X = **1.118 µg/ml**

Appendix ix: Inhibition of H-T29 Cells Proliferation after 24 and 48hours with the Fraction



Percentage Inhibition of HT29 Cancer Cells treated with Ethyl Acetate Fraction of *Sanna alata* and for 24 and 48hrs

IC_{50} at 24hrs: y = 0.0571x + 31.489 (R^2 = 0.8951) X = **324.19 µg/ml**

IC_{50} at 48hrs: y = 0.0519x + 45.25 (R^2 = 0.8652) X = **91.52 µg/ml**

Percentage inhibition is calculated as

PI = 100-cell viability

Percentage Yields is calculated below

% Yield of *Khaya Senegalensis* stem back

$\frac{127}{300} \times 100 = 42\%$

% Yield of *Senna alata* leave

$\frac{110}{300} \times 100 = 36\%$

% Yield of ethyl acetate fraction of *Senna alata*

$\frac{19}{300} \times 100 = 6\%$

% Yield of n-hexane fraction of *Senna alata*

$\frac{15}{300} \times 100 = 5\%$

% Yield of aqueous fraction of *Senna alata*

$\frac{21}{300} \times 100 = 7\%$