

**ANTI-VIRAL ACTIVITIES OF SELECTED KENYAN  
MEDICINAL PLANTS AGAINST THE HEPATITIS - B  
VIRUS**

**BY**

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THE UNIVERSITY OF NAIROBI, KENYA.**

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## **LIST OF ABBREVIATIONS**

3TC	-	Lamivudine
AASLDS	-	American Association for the Study of Liver Diseases
ADV	-	Adefovir dipivoxil
AIDS	-	Acquired Immunodeficiency Syndrome
ALT	-	Alanine Transaminase
Anti – HBS	-	Antibodies to the HBsAg
API	-	Active Pharmaceutical Ingredient
AST	-	Aspartate Transaminase
ATCC	-	American Type Culture Collection
BSC	-	Biological Safety Cabinet
CC <sub>50</sub>	-	Cytotoxic Concentration killing 50% of the cell
CDC	-	Center for Disease Control and prevention
CMV	-	Cytomegalovirus
CTMDR	-	Center for Traditional Medicine and Drug Research
CQ	-	Chloroquine
DHBV	-	Duck Hepatitis B Virus
DNA	-	Deoxyribonucleic acid
EALS	-	European Association for study of the Liver (EALS)
EBV	-	Epstein Barr Virus
EC <sub>50</sub>	-	Effective Concentration giving 50% response
EDTA	-	Ethylene Diamine Tetra Acetic Acid
ETV	-	Entecavir
ELISA	-	Enzyme Linked Immunosorbent Assay
FCS	-	Fetal Calf Serum
FDA	-	US Food and Drug Administration
GSHV	-	Ground Squirrel Hepatitis Virus
HAV	-	Hepatitis A Virus
HBV	-	Hepatitis B Virus
HCV	-	Hepatitis C Virus

HDV	-	Hepatitis D Virus
HEV	-	Hepatitis E Virus
HBcAg	-	Hepatitis B core antigen
HBeAg	-	Hepatitis B e antigen
HBsAg	-	Hepatitis B surface antigen
HCV	-	Human Cytomegalovirus
HIV	-	Human Immuno- deficiency Syndrome
HRP	-	Horse Redase Peroxidase
HSV	-	Herpes Simplex Virus
HCC	-	Hepatocellular Carcinoma
IFN – $\alpha$	-	Interferon - $\alpha$
IgM	-	Immunoglobulin M
ILRI	-	International Livestock Research Institute
IU	-	International Units
Kb	-	Kilo bases
KCLB	-	Korean Cell Line Bank
Kpa	-	Kilo pascals
MEM	-	Minimum Essential Medium
ml	-	Mililiter
MTT	-	(3- (4, 5-Dimethyl thiazol-2-yl)-2, 5-diphenyltetrazoline bromide)
NRTI	-	Nucleoside Reverse Transcriptase Inhibitor
OD	-	Optical Density
ORF	-	Open Reading Frames
PBS	-	Phosphate Buffered Saline
Psi	-	Pounds per Square inch
Rpm	-	Rotations per Minute
RT	-	Reverse Transcriptase
sgHBV	-	Snow Goose Hepatitis B Virus
SNU	-	Seoul National University
Spp	-	Species
STD	-	Sexually Transmitted Disease

TDF	-	Tenofovir Disoproxil Fumarate
TMB	-	Tetramethylbenzidine
WHV	-	Wood Chuck Hepatitis Virus
WHO	-	World Health Organisation
WMHV	-	Wooley Monkey Hepatitis Virus

## ABSTRACT

### **Introduction**

Hepatitis B Virus (HBV) is a human pathogen causing serious liver disease. The virus is the major cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma worldwide. It is endemic in many parts of the world especially in Asia and Africa, and an estimated 2 billion people are infected, with over 600,000 dying each year. Kenya has been considered among high endemic areas for HBV infection with upward trends of sero-prevalence among blood donors being observed. Although immunization against HBV has been widely used, current medicines for the management of HBV infection in humans are few, limited in efficacy and relatively expensive, making them unavailable to most of the needy cases, especially those in developing countries. The resistance to these agents is also spreading fast. The search for new therapeutic agents for HBV infections is an ongoing effort and a number of researchers are now paying attention to active anti-viral compounds from natural products including plants because of their widespread use in developing countries and the large repertoire that has not been systematically investigated. The plants in this study were mainly chosen based on previous studies on their anti-viral activity. They exhibit activity against the Human Immunodeficiency Virus, Herpes Simplex Virus and Cytomegalovirus and were therefore highly postulated to have anti - HBV activity. They were also chosen based on their ethno-pharmacological use including relief of symptoms associated with viral infections.

### **Methodology**

In this study, the root barks of *Carissa edulis* (Forssk.) Vahl and *Maytenus heterophylla* (Eckl. & Zeyh.) Robson and the stem barks of *Prunus africana* (Hook.f.) Kalkman and *Acacia mellifera* (Vahl) Benth were harvested from within Kenya from among medicinal plants used widely for the management of various diseases using information obtained from literature search and their ethno - pharmacological use. Their identity was established at the School of Biological Sciences, University of Nairobi, and voucher specimens were deposited at the herbarium. These were air dried for two weeks and the water extracts of *C.edulis*, *P.africana* and *M.heterophylla*; methanol extracts of

*A.mellifera* and chloroform extracts of *P.africana* introduced into the cell cultures of an HBV producing cell line (SNU-182) at concentrations of 1000 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL, 31.25 µg/mL and 15.125 µg/mL. Lamivudine, Active Pharmaceutical Ingredient (API) was used as a reference drug for the investigations. Inhibition of the expression of the Hepatitis B Surface antigen (HBsAg) and HBV DNA released into the culture supernatant were used as the anti-viral indicator. A semi-quantitative Enzyme Linked Immuno Sorbent Assay (ELISA) technique was used for initial screening of the effects of the plant extracts on the expression of the Hepatitis B Surface antigen (HBsAg) while the quantity of HBV DNA was assayed by real time Polymerase Chain Reaction (PCR). The *in-vitro* cytotoxicity of the extracts was determined by MTT assays.

## **Results**

Of the five plant extracts examined using the ELISA technique, three exhibited some anti - HBV activity *in vitro* with a CC<sub>50</sub> of more than 100 µg/mL, suggesting the need for further investigations of their possible use in the management of HBV infections. These were the aqueous extracts from *Carissa edulis* (Forssk.) Vahl (Apocynaceae) and *Prunus africana* (Hook .f.) Kalkman (Rosaceae) and the methanol extract from *Acacia mellifera* (Vahl) Benth (Fabaceae). At a concentration of 200 µg/ml the extract of *C.edulis* exhibited the highest activity of just over 12.15 % inhibition rate relative to negative control, which was slightly below the 15 % inhibition rate of Lamivudine positive control at a concentration of 100 µg/mL. The activities of the *P.africana* and *A.mellifera* extracts of 5 % inhibition and 2.15 % inhibition respectively, relative to controls, was higher than the 2 % inhibition activity of Lamivudine positive control at a concentration of 30 µg/mL.

These results were confirmed using the quantitative real time PCR technique where the aqueous extract of *C.edulis* and the methanol extract of *A.mellifera* exhibited sustained activity over a range of plant extract concentrations above 31.25 µg/mL and 125 µg/mL. The aqueous and chloroform extracts of *P.africana* also exhibited activity using this technique. The evaluation of the EC<sub>50</sub> done on the first two plant extracts exhibiting



notable anti - HBV activity using this technique yielded considerably higher values than the corresponding  $CC_{50}$  values. The *C. edulis*'  $EC_{50}$  was 331.6  $\mu\text{g/ml}$  while that of *A.mellifera* was 295.0  $\mu\text{g/ml}$ . These values indicate a high level of toxicity at potent concentrations and signify the need for further investigations to identify and isolate the toxic components.

### **Conclusion**

The results obtained in this study provide evidence of the anti-HBV activity of *Carissa edulis* (Forssk.) Vahl , *Acacia mellifera* (Vahl) Benth and *Prunus africana* (Hook .f.) Kalkman. Further investigations are however needed to establish their possible use in the management of HBV infection and to identify and isolate toxic components with a view to improve the bio-activity.

# CHAPTER ONE

## 1.0 INTRODUCTION

During the past decade, traditional systems of medicine have become a topic of global importance. Current estimates suggest that in many developing countries, a large proportion of the population relies heavily on traditional practitioners and medicinal plants to meet their primary health care needs (WHO fact sheet No 134, 2008). Although modern medicine may be available in these countries, herbal medicines have often maintained popularity for historical, cultural and economic reasons. Concurrently, many people in developed countries have begun to turn to alternative or complementary therapies, including medicinal herbs, because of the perception that they offer a gentler means of managing chronic debilitating diseases such as heart disease, rheumatoid arthritis and cancers. (Ajazzudin and Shailendra, 2012)

The WHO defines a medicinal plant as any plant which in one or more of its organs contains substances that can be used for therapeutic purposes or which are precursors for chemo-pharmaceutical semi synthesis (WHO fact sheet No 134, 2008). Such a plant will have its parts including leaves, roots, rhizomes, stems, barks, flowers, fruits, grains or seeds employed in the control or treatment of a disease condition. Several vascular plants are known to be rich in a wide variety of non-nutrient bioactive components often referred to as phytochemicals or phytoconstituents, which constitute potential anti-infective agents. The vast majority of these are classified into various categories including terpenoids (essential oils, glycosides), nitrogen-containing compounds (alkaloids & amino acids), simple phenols, phenolics/polyphenols (flavonoids, flavones, flavonols and anthocyanins), phenolic acids (quinones, tannins and coumarins), lectin polypeptides and steroids (Doughari, 2012). A number of these compounds have exhibited various forms of activities including anti-oxidant (Jayasri *et al.*, 2009), anti-cancer (Liu, 2004), anti-microbial (Nascimento *et al.*, 2000) and anti-ulcer (Jakhetia *et al.*, 2010) activity. Among the pharmacologically potent plant derived natural products in clinical use today include quinine, a quinoline alkaloid first isolated from the plant

*Cinchona ledgeriana* in 1820 (Choong Eui Song, 2009) and artemisinin, a terpenoid isolated from the Chinese herb *Artemisia annua*. its derivatives currently providing the most potent and safe anti-malarial drugs in clinical use (Elfawal *et al.*, 2012).

Despite these significant successes, relatively few plant species that provide medicinal herbs have been scientifically evaluated for their possible medical application. There still exists the need for new sources of antimicrobial agents given the increasing prevalence of multi-drug resistant strains and the increased number of sicknesses with no specific cure (Sieradzki *et al.*, 1999). Given the large number and structural diversity of currently available plant constituents, the plant kingdom remains an exciting source for new medicinally important bioactive compounds, including antiviral agents.

### **1.1 The anti-viral activities of plants**

Viral diseases remain one area of medicine for which specific treatments are lacking owing to the general nature and morphology of viruses. Most viruses strictly depend on cellular metabolic processes for functioning because they possess only limited intrinsic enzyme systems and building blocks, which normally serve as the targets for drugs. Treatment is therefore often times of necessity symptomatic and although vaccines have been very successful in the control of many viral diseases, some are likely to be controlled only by antiviral chemotherapy, a fact which has inevitably given impetus to the search for novel antiviral agents, including those of plant origin.

This quest has however not paralleled the success of other natural anti-infectives especially those targeting bacterial infections. Only a handful of antiviral agents from natural sources have found their way to clinical use owing to their poor specificity, low potency and high levels of cytotoxicity. Colegate *et al.*, (1993) recommend that a successful plant extract with antiviral activity must inhibit the virus completely without affecting the host cell. Moreover, contrary to a bacteriostatic compound, an effective antiviral drug should not only display considerable specificity in its antiviral action but should also irreversibly block viral synthesis in order to stop cell suicide due to the viral infection, thereby restoring normal cell replication. It must also possess a broad range of activity and favourable pharmacodynamic property (Vlietinck *et al.*, 1997). Few

compounds can match this idealistic mark and at best serve as lead compounds for the semi-synthetic preparation of less toxic derivatives.

Most of the research initiatives for the discovery of novel anti-viral compounds were born after the discovery of the Human Immunodeficiency Virus (HIV) and the elucidation of the viral life cycle (Vlientick *et al.*, 1991). Such agents act by targeting any of the steps involved in viral replication, including the entry into and exit out of the host cell. Research on antivirals from plants is mainly based on their ethnomedicinal use and a number of compounds have been isolated from plants used traditionally to treat viral disease. A summary of some examples is as presented in Table 1 below.

**Table 1: Plants with established antiviral activity**

<b>Family Name</b>	<b>Species</b>	<b>Traditional Use</b>	<b>Country</b>	<b>Active Ingredient</b>	<b>Class</b>	<b>Activity</b>
Berberidaceae	<i>Podophyllum paltatum</i> L. and <i>P.emodi</i> Wall	Anogenital warts (Condylomata acuminata)	USA,Canada,India	Podophyllin	Lignans	HSV
Ephedraceae	<i>Ephedra sinica</i> Stapf	Common cold, fever, cough	China	Catechin	Flavonoids	Influenza
Hypericaceae	<i>Hypericum perforatum</i> L.	Depression and mental illness	Europe	Hypericin	Naphthodianthrones	HIV
Leguminosae	<i>Glycyrrhiza glabra</i> L	Anti-inflammatory, Anti-pyretic, and laxative activities	China	Glycyrhizin	Terpenes	Influenza, HSV, HIV
Myrsinaceae	<i>Maesa lanceolata</i> Forsk	Hepatitis and bacilliary dysentry	Rwanda	Maessaponin V 3	Terpenes	HSV

(Cos P. *et al.*, 2005)

Other examples of isolated compounds from higher plants with known anti-viral activity *in vitro* include the water soluble alkaloid castanospermine, isolated from the black bean or the Australian chest nut (*Castanospermum australe*), which is active against HIV and the dengue virus (Kevin *et al.*, 2005), the coumarin derivatives calanolide A and B, from extracts of *Calophyllum lanigerum*, which have exhibited anti-HIV activity (Kashman *et al.*, 1992) and the  $\alpha$  - glucosidase inhibitor, 1- deoxynojirimycin which has been demonstrated to block HIV envelope glycoprotein mediated membrane fusion (Papandreou *et al.*, 2002). In this category also are a number of 3-methoxyflavones whose synthetic derivatives have been shown to be promising leads for the development of anti-rhinovirus drugs (van Hoof *et al.*, 1984; Vlietinck *et al.*, 1986). Whether these compounds have any clinical potential remains to be determined.

A number of Kenyan medicinal plants have been shown to possess antiviral properties. Rukunga *et al.*, (2002) studied twenty two medicinal plants popularly used in preparing traditional remedies for activity against HIV – 1 and established that eight of them achieved at least 50 percent reverse transcriptase inhibition activities at various concentrations. These were *Prunus africana* (Hook.f.) Kalkm, *Acacia mellifera* (Vahl) Benth, *Erythrina abyssinica* DC, *Azadirachta indica* A. Juss, *Melia azedarach* L. *Myrica salicifolia* A., *Maytenus buchanii* (Leos) and *Maytenus senegalensis* (Lam). Other studies conducted by Tolo *et al.*, (2006) have established the marked antiviral effects of extracts from the root bark of *Carissa edulis* (Forssk.) and the stem bark of *Prunus africana* (Hook.f.) against HSV and CMV. These are the plants that form the basis of the present work.

## CHAPTER TWO

### 2.0 LITERATURE REVIEW

#### 2.1 HEPATITIS

##### 2.1.1 History

The term hepatitis is derived from the Greek term '*hepar*' meaning liver and the Latin term '*itis*' meaning inflammation. It refers to a series of liver diseases having both non-communicable and infectious causes, leading to the inflammation of the liver cells. Manifestations of such disease, such as jaundice, were characterized as early as in the 8<sup>th</sup> century A.D. by Hippocrates, with the first recorded reference to the contagious nature appearing to be in a letter from Pope Zacharias to St. Boniface, the Archbishop of Mainz (Zuckerman and Howard, 1979). It was however not until after the second world war, following massive vaccinations for measles and yellow fever in the United States that hepatitis was actually linked to a virus. This followed an eruption of over 200,000 cases of viral hepatitis between 1942 and 1945 caused by batches of the vaccines contaminated with the hepatitis virus, identifying the disease as a matter of prime importance and highlighting the need for effective methods to treat or prevent it (MD-IM3). The terms hepatitis A and B were subsequently introduced by Mc Callum and Bradley in 1947 in a nomenclature system that was adopted widely and underlies the current nomenclature of the human hepatotropic viruses. Accordingly, viral hepatitis type A (epidemic/infectious hepatitis) became hepatitis A virus, type B (serum hepatitis) became hepatitis B virus and so forth. Type A hepatitis was considered predominantly transmitted via the fecal-oral route while type B was believed to be primarily transmitted through percutaneous or mucosal contact with infectious blood. The terms were eventually adopted by the WHO Committee on Viral Hepatitis in 1973.

Another significant milestone in the understanding of viral hepatitis was the discovery of a previously unknown protein in the blood of an Australian aborigine by the nobel prize winner, Dr Baruch Blumberg in 1963 (Alter and Blumberg, 1966). Initially named the Australian antigen, the protein was found to be closely related to the type B hepatitis. Further research established that this antigen was only found in the serum of type B

infected patients and was designated the Hepatitis B surface antigen (HBsAg). Dane *et al.*, (1970) later found virus-like particles in the serum of patients suffering from type B hepatitis which were then designated HBV and were discovered to be the primary cause of hepatocellular carcinoma (HCC). This discovery initiated the development of the HBV vaccine, which was approved in 1981 for clinical use. Other non-related viruses were later found, but HBV has retained its name to date, with the human being remaining as the primary reservoir.

### **2.1.2 Aetiology**

Hepatitis is a common disease characterized by the inflammation of the liver and can have many causes including aflatoxins, alcohol, auto-immune disorders, toxins and infections such as Yellow fever, Leptospirosis, Epstein-Barr virus (EBV), Rubella and Cytomegalovirus (CMV). Viral hepatitis is the term normally reserved for infections of the liver by one or more of the distinct hepatitis viruses designated A, B, C, D and E. These five types are of greatest concern because of the burden of illness and death they cause and the potential for outbreaks and epidemic spread. In particular, types B and C lead to chronic disease in hundreds of millions of people and, together, are the most common cause of liver cirrhosis and cancer (WHO fact sheets No 164, 2011 & 204, 2013). It is however important to note that most liver damage is caused by the first three viruses A, B and C, with Hepatitis B causing the most serious type of viral hepatitis. Hepatitis A and E are typically caused by ingestion of contaminated food or water while hepatitis B, C and D usually occur as a result of parenteral contact with infected body fluids. A summary of the various types of viral hepatitis is as follows;

#### **2.1.2.1 Hepatitis A**

This is caused by infections of the Hepatitis A Virus (HAV). It has an incubation period of approximately 28 days (range: 15–50 days). HAV replicates in the liver and is shed in high concentrations in faeces from 2 weeks before to 1 week after the onset of clinical illness. HAV infection produces a self-limiting disease that does not result in chronic infection or chronic liver disease. Acute liver failure from Hepatitis A is also rare. HAV infection is primarily transmitted by the faecal-oral route, by either person to



person contact e.g. by anal–oral contact during sex, or consumption of contaminated food or water. Blood borne transmission of HAV is uncommon. Almost everyone who gets hepatitis A has a full recovery and to date vaccination remains the most effective means of preventing transmission (WHO fact sheet No 328, 2013).

### **2.1.2.2 Hepatitis B**

This is caused by infections by the HBV and is classified as a Sexually Transmitted Disease (STD). The incubation period from the time of exposure to onset of symptoms is about 6 weeks to 6 months. HBV is found in highest concentrations in blood and in lower concentrations in other body fluids (e.g. semen, vaginal secretions, and wound exudates). HBV infection can be self-limiting or chronic and is spread by contact with an infected person's blood, semen, or other body fluid. For this reason, blood banks test all donated blood for hepatitis B, greatly reducing the risk for getting the virus from blood transfusions or blood products. Modes of transmission are the same as those for the Human Immunodeficiency Virus (HIV), but the HBV is 50 to 100 times more infectious (WHO fact sheet No 204, 2013). Unlike HIV, HBV can survive outside the body for at least seven days. During this time, the virus can still cause infection if it enters the body of a person who is not protected by the vaccine. It is however not spread by contaminated food or water. Hepatitis B can be a serious infection that can cause liver damage, which may result in cancer. Humans are the only reservoir for the virus and some people are not able to get rid of it, making the infection chronic, or life long. Common modes of transmission for this virus include receipt of contaminated blood or blood products, invasive medical procedures using contaminated equipment, sexual contact and the transmission from mother to baby at birth. Child hood infections with HBV are normally asymptomatic and may go unrecognized for many years. In adults, only approximately half of newly acquired HBV infections are symptomatic, and approximately 1% of reported cases result in acute liver failure and death. Risk for chronic infection is inversely related to age at infection: approximately 90% of infected infants and 30% of infected children aged less than 5 years become chronically infected, compared with 2 – 6% of adults (McMahon *et al.*, 2005). Among persons with chronic HBV

infection, the risk for premature death from cirrhosis or hepatocellular carcinoma is between 15% and 25% (Lok and McMahon, 2009).

### **2.1.2.3 Hepatitis C**

This is caused by infections of the Hepatitis C Virus (HCV) and is most efficiently transmitted through large or repeated percutaneous exposure to infected blood (e.g. through transfusion of blood from unscreened donors). It is therefore routine for most blood banks to test all donated blood for hepatitis C to reduce the risk of transmission of the virus. Although much less frequent, occupational, perinatal, and sexual exposures can also result in the transmission of HCV. The average time from exposure to antibody to HCV (anti-HCV) seroconversion is between 8–9 weeks, and anti-HCV can be detected in more than 97 % of persons by 6 months after exposure. Chronic HCV infection develops in 70 – 85% of HCV infected persons while 60 – 70 % of chronically infected persons have evidence of active liver disease. (CDC factsheet). The majority of infected persons might not be aware of their infection because they are not clinically ill, and serve as a source of transmission to others as well as being at risk for chronic liver disease or other HCV related chronic diseases such as liver cirrhosis (WHO fact sheet No 164, 2011).

### **2.1.2.4 Hepatitis D**

This is also known as the ‘delta hepatitis’ and is caused by infections with the Hepatitis D Virus (HDV). The virus was first discovered in HBV infected patients and is an incomplete RNA virus that requires the helper function of HBV to replicate. It has been proved to require the surface antigens of HBV to support its life cycle and infectivity (Rizzetto *et al.*, 1980). The virus causes a serious liver disease which can be acute or chronic in nature. Co or superinfections by HDV in HBV patients is closely correlated with the more severe symptoms of liver disease than with HBV infections alone (Chien *et al.*, 1991). The virus is transmitted through percutaneous or mucosal contact with infectious blood and can be acquired as a co-infection in persons with HBV infection. There is no vaccine for Hepatitis D, but it can be prevented in persons who are not already HBV infected by Hepatitis B vaccination. (CDC factsheet)

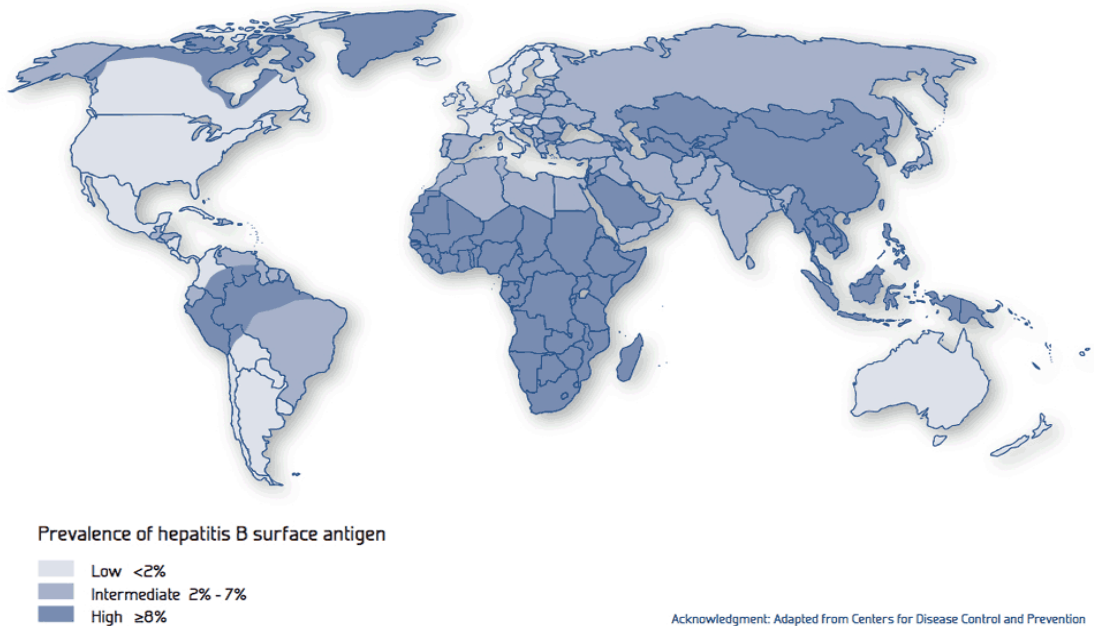
### 2.1.2.5 Hepatitis E

This is caused by the Hepatitis E Virus (HEV). A common disease throughout the world that only results in an acute infection. One gets hepatitis E by drinking water contaminated with the virus. It can also be spread through anal – oral contact. There is currently no vaccine for the prevention of the infection. (WHO fact sheet No 280, 2013)

## 2.2 Hepatitis B

### 2.2.1 Epidemiology of Hepatitis B

Prevalence of Hepatitis B Virus Chronic Infection, 2006



**Figure 1:** Prevalence of HBV chronic infection (Adopted from [http://www.nathnac.org/pro/factsheets/hep\\_b.htm](http://www.nathnac.org/pro/factsheets/hep_b.htm))

Despite effective vaccination, Hepatitis B still remains a potentially life-threatening infection. It is a major global health problem with more than 2 billion people, about one third of the world's population, having been infected at some time in their life. Of these, about 350 million remain infected throughout their life time and are at risk of suffering from chronic liver disease (Sorrell *et al.*, 2009).

The prevalence of HBV carriers varies from 0.1% to 2% in low prevalence areas (United States, Canada, Western Europe, Australia and New Zealand), to 3 - 5 % in intermediate prevalence areas (Mediterranean countries, Japan, Central Asia, Middle

East, Latin and South America), to 10 - 20% in high prevalence areas (Southeast Asia, China and Sub-Saharan Africa) (Mahoney, 1999) (Figure 1). The wide range in HBV carrier rate in different parts of the world is largely related to differences in the age at infection, which is inversely related to the risk of chronicity. Most people in the high prevalence areas become infected with HBV during childhood. The rate of progression from acute to chronic HBV infection is approximately 90 % for perinatally acquired infections, 20 to 50 % for infections between the age of 1 and 5 years, and less than 5 % for adult acquired infections (Wasley *et al.*, 2008).

In Kenya, in areas of low endemicity, 40 – 60 % of adults have HBV markers by adulthood and the average carrier rate is 2.5 – 10 % with a range of 3 – 30% (Makokha *et al.*, 2004; Okoth *et al.*, 2011).

### **2.2.2 Clinical manifestation of the Hepatitis B**

The damage of hepatitis is immune mediated and begins to appear as the host's immune system attempts to clear the virus. The spectrum of clinical manifestations of HBV infection varies in both acute and chronic disease. During the acute phase, manifestations range from anicteric hepatitis (sub-clinical, with little manifestations) to icteric hepatitis (having manifestations of jaundice) and, in some cases, fulminant hepatitis (severe hepatic impairment). During the chronic phase, manifestations range from an asymptomatic carrier state to chronic hepatitis, liver cirrhosis, and on the extreme hepatocellular carcinoma (HCC), which is among the first three causes of cancer related deaths worldwide (Ferlay *et al.*, 2013) and a common malignancy in Kenyan males (Kew, 2010). Extra hepatic manifestations of hepatitis can also occur with both acute and chronic infections.

#### **2.2.2.1 Acute hepatitis B**

The incubation period of HBV ranges from anywhere between 6 weeks and 6 months (CDC factsheet). The acute phase may occur with limited or no symptoms and approximately 70 percent of patients with acute hepatitis B have subclinical or anicteric hepatitis, while 30 percent develop icteric hepatitis (Liaw *et al.*, 1990). In the symptomatic phase the patient will initially complain of fatigue, malaise, anorexia, right

upper quadrant discomfort or flu like symptoms including coryza, photophobia, headache, and myalgia; then jaundice becomes apparent, usually within 10 days of the onset of symptoms (WHO fact sheet no 204, 2013). Low grade fever, jaundice, and mildly tender hepatomegaly are the most common signs.

Acute HBV infection may be more severe in patients with an underlying liver disease (e.g. alcoholic liver disease) or co-infections with other hepatitis viruses such as the HDV. It typically occurs in adolescents and adults who have not been vaccinated and can be life threatening due to massive liver damage from the host immune response. In this phase, Alanine Transaminase (ALT) and Aspartate Transaminase (AST) levels rise, sometimes to values above 1,000 IU/L (Heermann *et al.*, 1999) as compared to the normal ranges of 10 – 40 IU/L. Although this peak level is a reflection of hepatocellular injury, it has no reference to prognostic value. With recovery, ALT levels normalize in 1 to 4 months. This recovery phase is often marked by the loss of DNA and HBsAg in the serum and the production of HBV specific anti-bodies (Bertoletti and Gehring, 2006).

#### **2.2.2.2 Acute fulminant hepatitis B**

Fulminant hepatitis B is due to massive immune mediated lysis of infected hepatocytes to the extent that the liver fails. Many patients with fulminant hepatitis B may have no evidence of HBV replication at presentation because of early clearance of the HBsAg and low or undetected levels of the HBV DNA. Laboratory testing may however show IgM antibodies against the HBV core antigen (Wright *et al.*, 1992).

The reasons that HBV has a fulminant course in some patients are not well understood. Patients will typically present with rapidly progressive acute hepatitis characterized by signs of liver failure, such as coagulopathy, encephalopathy, and cerebral edema (Schiodt *et al.*, 1999).

#### **2.2.2.3 Chronic hepatitis B**

This is the most common presentation of HBV and is usually diagnosed as a result of a workup for abnormal liver function tests or as a result of screening patients at risk for HBV infection. The chronic nature of the HBV results in patients producing a high level

of the virus with few symptoms and usually occurs in cases where the infection has lasted for more than six months (Degertekin *et al.*, 2008). Indeed, the presence of HBsAg in serum for more than 6 months indicates a chronic HBV infection (McMahon.

, 2009). Many patients will therefore have no or non-specific symptoms such as fatigue or right upper quadrant discomfort.

Acute exacerbations due to Hepatitis B e-antigen (HBeAg) seroreversion occasionally occur in patients with chronic hepatitis B. Most of these exacerbations are asymptomatic, but occasionally an acute hepatitis like clinical picture with detectable IgM antibody against the core antigen occurs, leading to misdiagnosis of acute HBV infection in patients not previously known to have chronic HBV infection (Chu *et al.*, 1989). ALT levels may range anywhere from normal to five times higher than normal. Thrombocytopenia, hypoalbuminemia, direct hyperbilirubinemia, and prolonged prothrombin time suggest cirrhosis. Findings of chronic hepatitis B on liver biopsy range from minimal inflammation to cirrhosis. The most characteristic histologic feature of chronic HBV infection is the “ground-glass hepatocyte,” which is due to intracellular accumulation of HBsAg (Gerber *et al.*, 1974). In late cases, signs of cirrhosis such as jaundice, ascites, splenomegaly, pedal edema, encephalopathy, or variceal bleeding can be present (WHO fact sheet No 204, 2013).

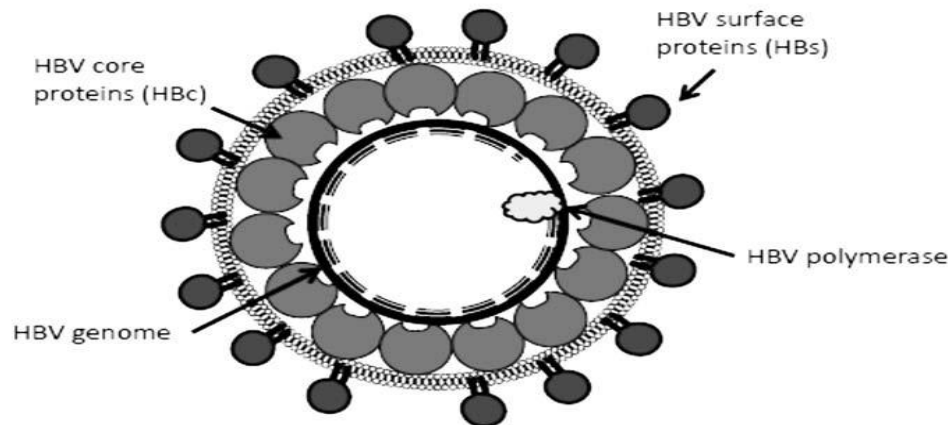
#### **2.2.2.4 Hepatocellular Carcinoma**

Hepatocellular Carcinoma (HCC) is the fifth most commonly diagnosed solid tumor (after lung, breast, colon/rectal and stomach cancers in that order) and the second leading cause of cancer related deaths worldwide after lung cancers (Ferlay *et al.*, 2013). The prognosis of HCC patients remains poor because most patients present with advanced disease and are not candidates for liver transplantation, surgical resection or regional therapy (Thomas and Abbruzzee, 2005). HCC should be suspected in cirrhotic patients with new onset right upper quadrant pain, rapidly developing ascites, a palpable liver mass or hepatic encephalopathy. Other non-specific features of HCC include watery diarrhea, hypoglycemia, and certain cutaneous manifestations such as acanthosis nigricans and the Leser Trelat (multiple pruritic seborrheic keratoses of sudden onset).

Other risk factors of HCC other than HBV infection include HCV infections, aflatoxicosis, alcoholism, smoking, high oestrogen levels, anabolic steroids and hereditary conditions such as hemochromatosis, alpha-antitrypsin deficiency and tyrosinaemia (Nagasue *et al.*, 1985).

## 2.3 Hepatitis B Virus

### 2.3.1 Virus Description

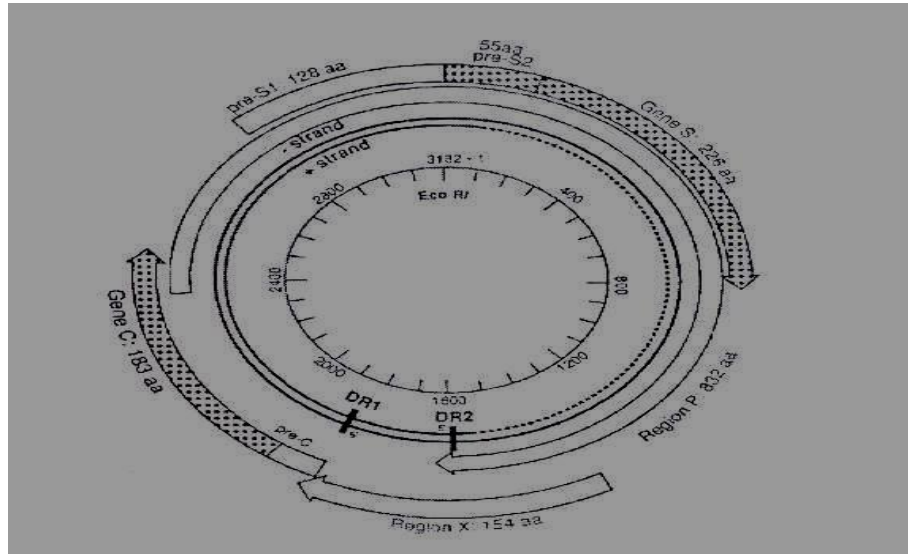


**Figure 2:** Schematic of HBV intact virion (Hovart, 2011)

HBV is a DNA virus that consists of a double shelled structure (comprising a nucleocapsid arranged into an icosahedral formation covered with surface/envelope proteins) enclosing a circular, partial double stranded DNA genome (Figure 2). The virion is about 42 nm in diameter (Chisari,1992), and belongs to the family of DNA viruses known as Hepadnaviridae, alongside other viruses such as the duck hepatitis B virus (DHBV), ground squirrel hepatitis virus (GSHV), snow goose hepatitis B virus (sg HBV), woodchuck hepatitis virus (WHV), and the wooley monkey hepatitis virus (WMHV) (Guha *et al.*, 2004). Each particular virus is species specific, with the Woodchucks responding to chronic HBV infection in much the same manner as do humans.

The highly compact double stranded HBV DNA genome is approximately 3.2 kb (Chisari, 1992) and comprises a longer strand of the DNA (-) that is a complete circle with a nick and a complementary strand (+) that is shorter. It contains genes that overlap

and are transcribed in four different Open Reading Frames (ORF's) which direct the transcription and translation of specific structural proteins by use of different start codons.



**Figure 3:** The genome organization of HBV  
(Reproduced from <http://www.ncbi.nlm.nih.gov/books/NBK7864/?report=printable>)

Consequently, the relatively small double stranded genome generates about 7 distinct proteins (Kann *et al.*, 1997) (Figure 3) namely;

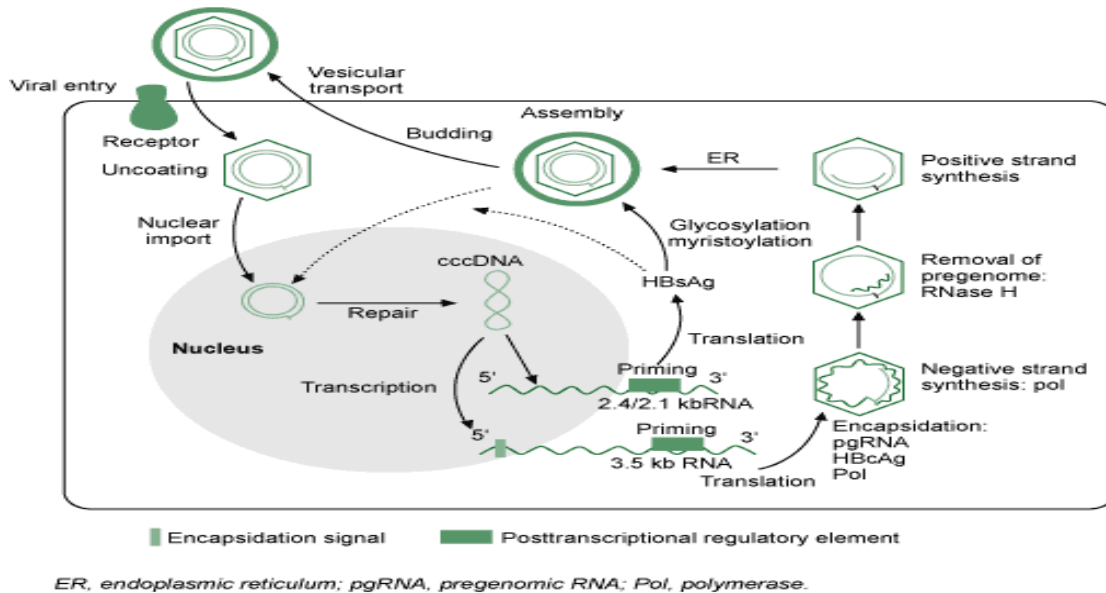
- DNA Polymerase, which is the largest HBV protein transcribed from the Pol gene.
- The nucleocapsid (core) antigen (Hepatitis B Core antigen (HBcAg)) and the pre – core antigen (Hepatitis B e antigen (HBeAg)) transcribed from the C gene.
- The viral envelope comprising the pre-S1, pre-S2 and Hepatitis B surface antigen (HBsAg) transcribed from the S gene.
- The X protein, a regulatory protein required for HBV replication transcribed from the X gene (Liang, 2009).

HBV is naturally heterogeneous with eight genotypes (labeled A though H) identified, that differ genetically by more than 8 % (Lin *et al.*, 2005). The clinical significance of these genotypes is not as clear as that of HCV, although recent data has suggested that different HBV genotypes may be associated with different rates of progression of liver



disease and different rates of response to interferon therapy (Fung and Lok, 2004). The data is however not enough to recommend routine testing for HBV genotypes in clinical practice.

### 2.3.2 Cell entry and Replication of hepadnaviridae



**Figure 4:** Replication of Hepadnaviridae viruses (Reproduced from <http://www.hepatitisannual.org>)

HBV is a DNA virus that replicates via an RNA intermediate making it unique among human viral pathogens. Replication of the virus involves its entry into the hepatocyte facilitated by the pre-S1 region, via a receptor mediated endocytosis where it sheds its envelope and the viral DNA is released into the nucleus (Figure 4). DNA polymerase completes the short (+) strand. Each strand covalently closes on its ends by the elimination of the (+) strand 5' oligoribonucleotide primer, the (-) strand 5' protein and the terminal repeat sequences of the (-) strand. The DNA then super coils to initiate transcription of (+) RNA with the cellular RNA polymerase II. Three transcripts of mRNA: 2.1, 2.4 and 3.4 kb long, are produced. The 3.4 kb or “pregenome” (+) RNA, which is longer than the DNA template due to terminal repeat sequences, is translated in cytoplasm to yield the core antigens and polymerase. The (-) DNA is transcribed with reverse transcriptase that is produced with the help of the 5' terminal protein. As transcription occurs, the (+) RNA is cleaved by RNase H until only a short 5' oligoribonucleotide that serves as a primer for the complementary (+) DNA remains.

Some of these core virus genomes are sent back to the nucleus to serve as templates for more virus production while others are packaged into nucleus virions by picking up envelope HBsAg containing proteins (L, M, and S) in the endoplasmic reticulum. Three S antigens are translated from the smaller 2.1 and 2.4 mRNA transcripts. Sometimes the (+) strand of the DNA is not given adequate time to be produced to completion when the virus leaves the cell hence it can vary in length from 1700-2800 base pairs. The virions finally exit the cell by exocytosis causing little or no damage to the cell because intracellular HBV is non-cytopathic.

### **2.3.3 Diagnostic assays of the HBV**

Two HBV specific proteins can be detected directly in the serum of an infected patient: The HBsAg and the HBeAg (Sorrell *et al.*, 2009) The HBsAg is a major viral protein inducing protective immune responses in humans. This antigen is found on the surface of the viral envelope and is also found in high concentrations in the serum of infected patients. The presence of HBsAg in serum for more than 6 months indicates a chronic HBV infection (McMahon, 2009). The presence of HBeAg indicates a high level of viral replication. Some variants of HBV do not produce the HBeAg; however, they continue to have high levels of HBsAg. These HBV variants are associated with a poor clinical outcome (Carman, 1995). Several specific antibodies can also be detected in both active and chronic HBV infections. The presence of antibody to HBsAg (anti-HBs) indicates either a past infection with HBV or an individual who has been vaccinated (Liang, 2009). In many countries the presence of anti-HBs is used to monitor for effective vaccination. The presence of Immunoglobulin M (IgM) specific antibody to Hepatitis B Core antigen (HBc) can also be detected and indicates recent HBV exposure. (Degertekin *et al.*, 2008). The anti-HBc develops first after HBV infection while anti-HBs antibody is detected later probably due to the rapid binding of the antibody to the HBsAg present at high levels in infected patients (Liang, 2009). This binding of antibody decreases the circulating anti-HBs, leading to the loss of detection in laboratory assays. The antibody to the HBeAg (anti-HBe) is detected only during acute HBV infections. It does not directly neutralize the HBV virion because intact virus particles do not contain

HBeAg. However, it has been noted that anti-HBe levels decline when viremia declines, indicating this immune response may have a protective nature (Bowden, 2006).

The presence of HBV in human serum can also be detected using several reliable molecular techniques that quantify HBV DNA levels (Valsamakis, 2007). These assays are used for both the initial evaluation of HBV infections and the monitoring of patients before and during therapy (Andersson and Chung, 2009). All of the molecular assays have wide dynamic ranges from 5 copies/mL to levels greater than 1 million copies/mL. This allows monitoring HBV DNA during infection and identifying HBV infections that become resistant to antiviral therapy (Krajden, 1998). In 2001, a high-titer HBV genotype A (code 97/746) was established as an international HBV DNA standard by the WHO. It was assigned a potency of  $10^6$  international units/milliliter (IU/mL) and now provides a reliable way to evaluate HBV DNA quantitative values. The standard also established that 1 IU of HBV is equivalent to 5 genome equivalents/copy. The available HBV DNA assays use conversion factors based on this standard material (Sorrell *et al.*, 2009)

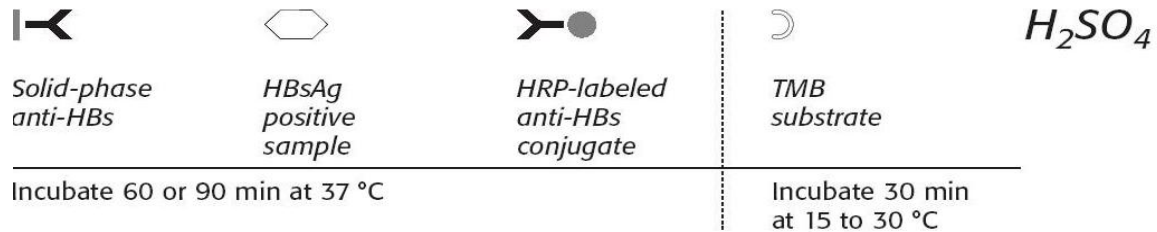
The challenge with the use of these diagnostic techniques is that the HBV has the ability to change in response to anti-viral therapy as well as to vaccinations. There is evidence that some new HBV variants evade the current diagnostic assays as well as vaccine induced immunity. (Hovart, 2011)

#### **2.3.3.1 The Hepanostika<sup>®</sup> HbsAg Ultra kit**

The Hepanostika<sup>®</sup> Kit used in the present work is an *in vitro* diagnostic medical device for the broad detection of the HBsAg. It is manufactured by BioMérieux (S.A) and is an Enzyme Linked Immunosorbent Assay (ELISA) technique based on a ‘sandwich’ principle (Figure 5). Upon completion of the assay, the development of colour indicates the presence of the HBsAg.

The Kit comprises: MicroELISA strip plates holders with 96 wells; a conjugate made up of Horseradish peroxidase (HRP) and anti-bodies (anti-Hbs); human serum, non reactive to HBsAg, as the negative control; bovine serum in sodium chloride solution containing HBsAg subtype ad, produced by a human cell line, as a positive control; Concentrated (25x) phosphate buffer with 0.05% tween 20; Tetramethylbenzidine (TMB) in citric acid;

Urea peroxide solution; Clamp and rod for closure of foil pack; Perforated and adhesive plate sealers and a sheet of labels. (Manufacturer's protocol)

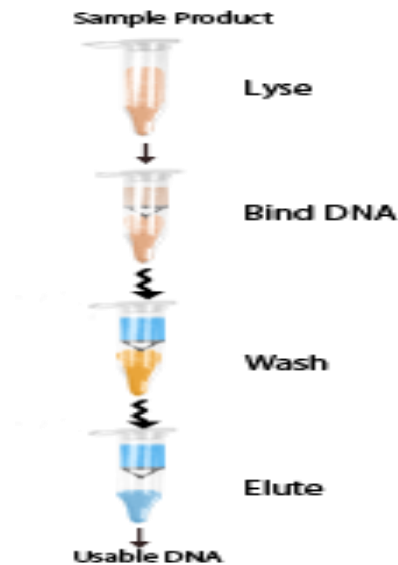


**Figure 5:** HBsAg Ultra Kit reaction schematic (Reproduced from Van Roosmalen *et al.*, 2006)

The wells of the microELISA strips contained in the kit are coated with a unique mixture of monoclonal anti-bodies comprising two murine monoclonal antibodies and a unique human monoclonal antibody combined with polyclonal antibodies to set up an assay capable of detecting all currently known HBsAg samples, even HBsAg positive samples with a severely mutated “a”- determinant which was a previously unidentified epitope outside the major immunodominant region. (Paulij *et al.*, 1999). The actual procedure entails incubating the sample diluents, test samples and appropriate controls in the micro ELISA wells and subsequently adding the HRP labeled anti – Hbs (ovine) conjugate. If HbsAg is present, a solid phase antibody/HBsAg/enzyme – labeled antibody complex is formed. Following washing and further incubation with TMB substrate, a blue colour is produced and the enzyme reaction is stopped by the addition of a 1 mol/l sulfuric acid solution which changes the colour to yellow. When HbsAg is present in the sample an intense colour develops. However if the sample is free of HBsAg no or little colour change develops after the addition of the substrate. Within limits, the amount of HBsAg in the sample is proportional to the degree of colour development (Van Roosmalen *et al.*, 2006) and is the basis of the semi - quantitative nature of this method.

### 2.3.3.2 QiAamp® minikit for the extraction of HBV DNA

The QIAamp® mini kit (Qiagen®) is a silica based extraction method designed for the rapid purification of total DNA from up to 200 µl of cultured cell media in four purification steps, summarized in figure 6 below;



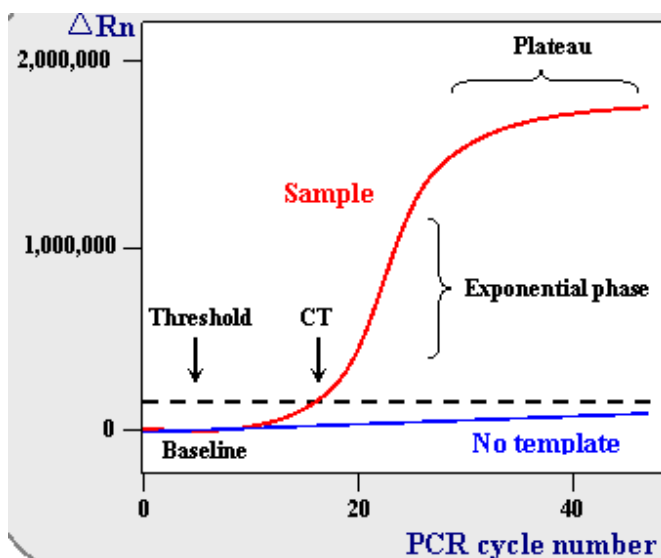
**Figure 6: Qiaamp® steps in DNA extraction.**

(Reproduced from: [http://www.nfstc.org/pdi/Subject03/pdi\\_s03\\_m04\\_02\\_a.htm](http://www.nfstc.org/pdi/Subject03/pdi_s03_m04_02_a.htm))

Lysis of the cells is achieved using the protease enzyme and lysis buffer provided in the kit. The sample lysate is then treated with alcohol and introduced into the QIAamp® mini spin columns provided and inserted into a tube. DNA from the sample lysate is purified by the removal of proteins and divalent cations using multiple buffer washes and centrifugation steps. The DNA adsorbs onto the silica membrane in the spin columns during the brief centrifugation, attracted to the silica bead under a high chaotropic salt concentration. The salt and pH conditions in the lysate ensure that the proteins and other contaminants such as  $Mg^{2+}$  which can inhibit PCR and other downstream enzymatic reactions are not retained in the QIAamp® membrane. The purified DNA is then eluted from the minispin columns in a concentrated form by use of supplied buffer AE or distilled water and is then stored at  $-20^{\circ}C$  to prevent hydrolysis. Alternatively, it can be directly loaded into the PCR thermocycler for amplification. This procedure is designed to ensure no sample – sample cross contamination and is carried out in a bio-safety level two cabinet.

### 2.3.3.3 Real time PCR technique – The light cycler<sup>®</sup> Nano SW 1.0

Real time PCR, also known as quantitative PCR, is an advancement of the conventional PCR technique that was first described in 1971 by Kleppe and Khorana (Baumforth *et al.*, 1999). It provides a simple method for determining the amount of a target sequence or gene that is present in a sample through an amplification plot of fluorescence signals versus cycle number (Figure 7).



**Figure 7: Real time PCR Plot of fluorescence against cycle number**

(Reproduced from <http://www.ncbi.nlm.nih.gov/genome/probe/doc/TechQPCR.shtml>)

A reporter dye attached to the 5' end of an oligonucleotide probe (e.g. taqman<sup>®</sup>), designed to hybridize within the target sequence, provides a fluorescence signal that indicates specific amplification. An example of such a dye is the SYBR<sup>®</sup> green 1 which binds double stranded DNA and an increase in fluorescence is also a measure of amplification. Reactions are characterized by the point in time during cycling when amplification of a PCR product is first detected. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. This is normally characterized by the cycling value or simply the C value which is defined as the intersection between an amplification curve and the threshold line. It is a relative measure of the concentration of target in the PCR reaction, although many other factors influence the absolute value of C other than the concentration of the target. These include

various environmental factors such as pH, the salt concentration, and the types of reagents used in the mix. The absolute  $C_1$  value is therefore only valuable as long as experiments are being compared using the same reaction conditions. The baseline is normally defined from the initial cycles when there is little change in the fluorescence signal.

## 2.4 Treatment of Hepatitis B

There is usually no specific treatment for acute hepatitis B. Treatment is symptomatic and largely depends on how active the virus is. It is often administered to relieve symptoms associated with the infection and would typically include painkillers, hepatotonics or anti-nausea medication. Chronic infections however may require specific anti-viral drugs, which could attenuate or stop the progression of fibrosis. Shepard *et al* (2006) noted that without such intervention, 15% to 40% of chronic HBV infected individuals would most likely develop cirrhosis, end-stage liver disease, HCC or would require liver transplantation. The primary goal of anti-viral therapy in chronic Hepatitis B is the sustained viral suppression, preferably to undetectable levels of serum HBV DNA.

Two classes of anti-viral therapy are currently in clinical use; The nucleot(s)ide analogues and the immune modulator interferon- $\alpha$  (IFN- $\alpha$ ). The nucleotide analogs includes drugs such as lamivudine (3TC), tenofovir, entecavir (ETV), adefovir – dipivoxil prodrug (ADV) and telbivudine as well as their combinations such as tenofovir/emtricitabine (*Truvada*<sup>®</sup>). The immune modulator IFN- $\alpha$  comprises either the standard or pegylated forms (Delaney *et al.*, 2006). The choice of a particular therapy for a given individual can be complex, influenced by factors including age, serum ALT, histological findings, HBeAg status, HBV viral load and genotype, previous therapy and tolerability of potential adverse effects. A full course of treatment of either therapy can cost thousands of Kenya shillings per year and is not readily available to most people in developing countries, which has led many to embrace alternative and complementary therapies including the use of herbs. Many of these seem to offer some kind of relief and are thought to confer their activities not only by inhibiting HBV secretion but also by building up immunity against the virus. These provide potential for the discovery of new

antiviral agents using modern scientific techniques and should therefore not be ignored (Vlietinck and Van den Berghe, 1991).

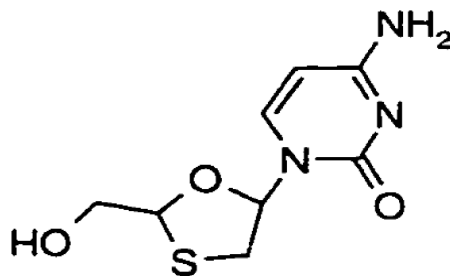
A vaccine against HBV has been available since 1982 (Shepard *et al.*, 2006) and is considered the mainstay in its prevention (Mahoney, 1999). The complete HBV vaccination series is effective in more than 95% of infants, children, and adults in preventing infection and its chronic consequences (Dentinger *et al.*, 2005) conferring protection that lasts at least up to 20 years or even possibly a lifetime with an exceptional record of safety (McMahon *et al.*, 2005). It was the first example of a successful recombinant vaccine for human infectious disease, making future preventative tactics for diseases such as HIV more of a reality. A detailed look at each of the drug groups mentioned and their modes of action is as follows;

#### **2.4.1 Nucleos(t)ide analogs**

Nucleos(t)ide analogs, also known as nucleos(t)ide inhibitors, chain terminators, or competitive inhibitors are the more commonly used option in the management of chronic HBV, often favored due to their combination of high antiviral potency, ease of use and high tolerability. Structurally, they are synthetic nucleosides which lack the 3' – OH on the sugar ring of the nucleoside essential for the elongation of the DNA strand. Termination of the DNA chain occurs during viral replication effectively stopping the process. The effectiveness of these inhibitors is not without a number of limitations including the need for long term therapy in a number of individuals, the rapid emergence of resistant viral strains and their lack of specificity for the viral reverse transcriptase (RT) enzyme, leading to the inhibition of a number of other normal cellular DNA polymerases. Examples in clinical use include;



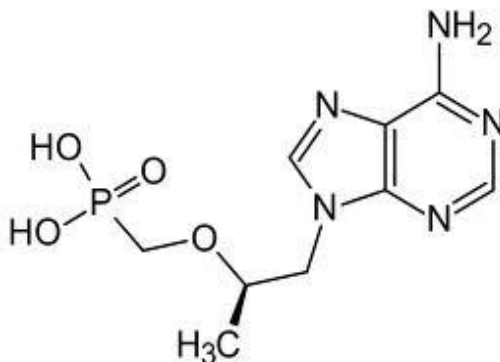
### 2.4.1.1 Lamivudine



**Figure 8: Structure of Lamivudine (2', 3'-dideoxy-3'-thiacytidine)**  
(Adopted from <http://www.rxlist.com>)

Lamivudine (Figure 8), commonly referred to as 3TC, exhibits potent anti-viral activity against HBV. It was the first oral nucleoside reverse transcriptase inhibitor (NRTI) to demonstrate viral suppression, hepatic function stabilization and morbidity reduction in HBV related decompensated cirrhosis (Kapoor *et al.*, 2000) and has been used extensively worldwide for the management of chronic hepatitis under several brand names including Epivir – HBV<sup>®</sup>. It acts by inhibiting the enzyme HBV reverse transcriptase and has been noted to improve the sero-conversion of HB e-antigen positive hepatitis B as well as the histology staging of the liver. It has the advantage of relatively low cost, favorable tolerability, and an acceptable side effect profile (Marcellin *et al.*, 2010) which includes nausea, loss of appetite and melanuria. Probably because of this wide adoption, Lamivudine is plagued by the emergence of viral resistance which has significantly reduced its clinical benefits especially in end stage liver disease (Liaw *et al.*, 2004). Genotypic resistance emerges in about 20% of patients receiving monotherapy (Lai *et al.*, 2003) and can be detected in 14% to 23% of patients during the first year of therapy (Dienstag *et al.*, 1999) increasing to between 60% and 70% after 5 years of treatment (Chang *et al.*, 2004). Because of this, it is no longer recommended as a first line therapy in the guidelines of both the American Association for The Study of Liver Diseases (AASLD) and the European Association for the study of the Liver (EASL).

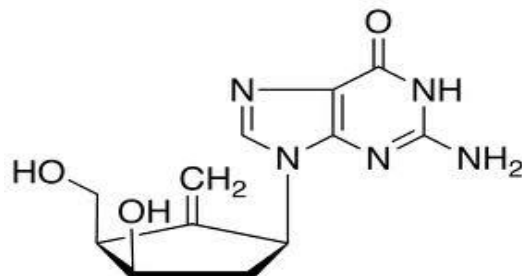
### 2.4.1.2 Tenofovir



**Figure 9: Structure of Tenofovir** (Adopted from <http://www.rxlist.com>)

Tenofovir (Figure 9) is a potent NRTI with a high genetic barrier to resistance (Marcellin *et al.*, 2008). Although the pharmacokinetics of tenofovir is quite well described in adults, very few studies have been reported in children (Hazra *et al.*, 2004). It is therefore indicated in the treatment of chronic hepatitis B in adults and pediatric patients 12 years of age and older and was approved by the FDA in 2008. It is marketed as Tenofovir disoproxil fumarate (TDF) under such brand names as Viread<sup>®</sup>, which is an oral prodrug of Tenofovir. It is rapidly absorbed and mainly eliminated unchanged in the urine by glomerular filtration and active tubular secretion (Kearney *et al.*, 2004). It is however plagued by a number of side effects including lactic acidosis, hepatomegally, steatosis and the decrease in bone marrow density. Nephrotoxicity is a well established complication of tenofovir, and patients with prevalent or incipient kidney disease may be preferentially treated with abacavir rather than tenofovir (i.e. 'channeling bias') (Horberg, *et al* 2010). In the treatment of chronic hepatitis the normal dose is normally one tablet of 300 mg taken daily. However the optimal duration of treatment is not known.

### 2.4.1.3 Entecavir

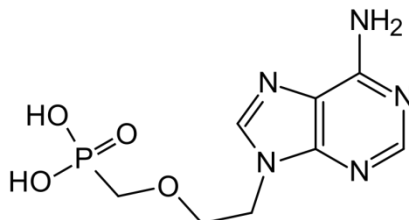


**Figure 10: Structure of Entecavir (Adopted from <http://www.rxlist.com>)**

Entecavir (ETV) (Figure 10) is the prototype for the cyclopentane class of nucleos(t)ide antiviral agents. Various studies have shown that ETV is highly potent, effectively suppressing HBV DNA in both compensated and decompensated HBV related cirrhosis patients (Shim *et al.*, 2010). Like Tenofovir, it has a high genetic barrier to resistance (Marcellin *et al.*, 2008) due to its structural formula and mechanism of action and its use is associated with the emergence of minimal resistance in the long-term treatment of nucleoside naive patients. Selection of resistant mutants is very rare with ETV when compared to Lamivudine (Chang *et al.*, 2006) and although it displays some level of cross-resistance, (Yang *et al.*, 2005) it has exhibited antiviral activity against Lamivudine resistant HBV, for which reason, it has been adopted as a rescue monotherapy treatment option for patients with resistance to both Lamivudine and Adefovir. Current guidelines recommend that the most potent drugs with optimal resistance profiles such as ETV and TDF should be used as first-line monotherapies in Chronic hepatitis (Lok and Mc Mahon, 2009).

It is sold under trade marks including Baraclude<sup>®</sup>, and is available as tablets of 0.5 mg and 1mg. Possible side effects include headache, fatigue, dizziness, insomnia, rash, vomiting and diarrhea.

#### 2.4.1.4 Adefovir

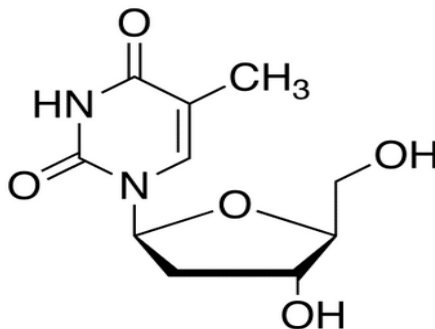


**Figure 11: Structure of Adefovir** (Adopted from <http://www.rxlist.com>)

Adefovir (ADV) (Figure 11) is an orally administered NRTI which is normally formulated as the pivoxil prodrug Adefovir dipivoxil (Hepsera<sup>®</sup>). Its use is associated with a delayed and low incidence of resistance in the treatment of NRTI naive HBV patients (Yang *et al.*, 2005) and has been found to effectively suppress some 3TC resistant HBV mutants, specifically the rtN236T and rtA181V mutants (Marcellin *et al.*, 2003). Some studies have observed that sequential ADV mono-therapy to patients infected with 3TC resistant HBV can result in a rate of ADV resistance of up to 20% after 1–2 years of therapy, (Lee *et al.*, 2006) which can develop to 30% after 5 years of use (Marcellin *et al.*, 2003). Consequently, ADV add-on therapy to 3TC is recommended for the management of resistance to 3TC (Lok *et al.*, 2009).

ADV is presented in formulations of 10 mg tablets and some side effects associated with it include lactic acidosis, indigestion, headache, steatosis, general body weakness and nephrotoxicity, especially on chronic administration. (Manolakopoulos *et al.*, 2008)

#### 2.4.1.5 Telbivudine



**Figure 12: Structure of Telbivudine**

Telbivudine (Figure 12) is also known as L - deoxythymidine and is a synthetic beta - L enantiomer of thymidine marketed under various trade marks including Sebivo<sup>®</sup> and Tyzeka<sup>®</sup>. It is an NRTI used in the treatment of chronic hepatitis B and has demonstrated greater antiviral and clinical efficacy than 3TC in patients with compensated cirrhosis (Hou *et al.*, 2008). In other clinical trials it has also been found to be slightly more effective than 3TC and ADV in suppressing HBV DNA in patients who are HBeAg positive. This benefit does not however appear to translate into a clinically important advantage for HBeAg seroconversion or histologic improvement (Lai *et al.*, 2005)

It is normally administered as a 600 mg tablet or as an oral solution of 100 mg / 5 mL given once daily. Although it is well tolerated when administered orally, it still exhibits some side effects such as lactic acidosis, severe hepatomegally and peripheral neuropathy.

#### 2.4.1.6 Emtricitabine

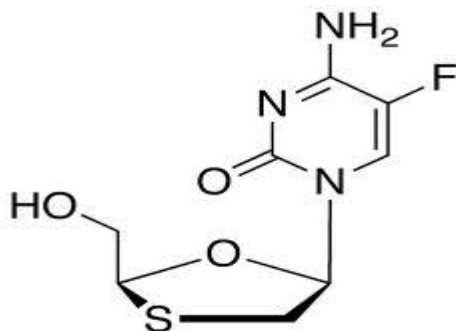


Figure 13: Structure of Emtricitabine

Emtricitabine (Figure 13) is an NRTI which is normally phosphorylated by cellular enzymes to the active form Emtricitabine 5'-triphosphate. It is used in combination with other nucleoside analogs such as TDF (Truvada<sup>®</sup>) mainly for the management of HIV - 1. It is not approved for the treatment of chronic HBV although it exhibits clinical activity against the virus. Side effects include hepatomegally, lactic acidosis and steatosis.

### **2.4.2 Interferon - $\alpha$**

Interferon -  $\alpha$  (IFN -  $\alpha$ ) is a cytokine which acts against HBV by both immunomodulatory and direct antiviral mechanisms. The molecular mechanism by which IFN -  $\alpha$  operates is mediated by its binding to receptors on the surface of the target cells, which leads to a phosphorylation cascade involving different tyrosine-kinases that ends up in the recruitment of transcription factors to the nucleus where they induce the synthesis of a number of antiviral effector proteins. IFN -  $\alpha$  alone results in 16% - 20% sustained response after 12 months of treatment (Di Besciglie, 2002). The efficacy of IFN -  $\alpha$  is limited by its undesirable side effects, low sustained response rate and high cost (Hoofnagle and Bisceglie, 1997; Levine *et al.*, 2002). A course of treatment would take about 12 – 15 weeks and is characterised by several side effects (such as fever, headache, arthralgia and myalgia) which remains a concern with regards to its clinical use. However, compared with the nucleos(t)ide analogues, it is associated with higher rates of HBeAg to anti-HBe seroconversion, greater durability of response and lack of antiviral resistance. (Lau *et al.*, 2005) which provides potential advantages over the nucleoside analogs in the treatment of chronic HBV.

Since the beginning of 2001, recombinant interferon has been replaced by newly developed pegylated IFN -  $\alpha$ 2a and pegylated IFN -  $\alpha$ 2b, with a view to improving efficacy, convenience and tolerability through its improved pharmacodynamic profile. Special precautions however still need to be taken before giving interferon to patients with decompensated cirrhosis, severe neutropenia, uncontrolled thyroid functions, thrombocytopenia, drug or alcohol abuse and past or current psychiatric illness.

### **2.4.3 The HBV vaccine**

The use of the vaccine is the mainstay for the prevention of Hepatitis B and can induce protection that lasts at least 20 years or even possibly life long, with an exceptional record of safety (Shepard *et al.*, 2006). The WHO reports that 164 countries have HBV vaccination programs for infants, and that since 1982 more than one billion doses of HBV vaccine have been administered worldwide. The vaccines used are normally based

on recombinant HBsAg produced in yeast cultures. This is based on the observation that antibodies to the HBsAg (anti – HBs) bind rapidly to the antigen which is normally present at high levels in infected patients. The presence of the antibody may therefore indicate either a past infection with HBV, or an individual who has been vaccinated. A protective antibody response to the vaccine is defined as the presence of greater than 10 IU/mL of anti-HBs (Hovart, 2011). The anti-HBs binds to a major epitope on the HBV proteins on intact virions as well as infected cells and are thus effective in preventing HBV infection. HBV mutations have however led to various vaccine failures. A number of vaccines in contemporary use also occur as combination vaccines e.g. along with hemophilus b conjugate antigen or in combination with inactivated HAV.

The vaccination schedule for monovalent HBV vaccines for children and adults is three intramuscular injections, with the second and third doses administered at one and six months respectively after the first dose (Shepard *et al* 2006; Degertekin *et al.*, 2008). If this schedule is interrupted, subsequent vaccinations can be given even if they are not at the intervals stated, as long as the second and third doses are separated by at least 8 weeks. The third dose can however be given at any time if it is the only one interrupted. The CDC recommends that the vaccine be given to immune-compromised patients such as hemodialysis and HIV patients if indicated because it is still effective. In Kenya, the vaccine is administered as part of the routine immunization programme for children as well as to travellers on advisory basis.

#### **2.4.4 Anti-hepadnaviral activities of plants**

Several Herbs have been investigated for their anti-hepadnaviral effects and are known to confer their activities not only by inhibiting HBV secretion but also by building immunity against the virus. Several other plants are the current subject of various anti-hepadnaviral studies some of which have yielded promising results (Coon and Ernst, 2004).

Among all the plants that have been tested for possible antiviral use in the treatment of humans, *Phyllanthus* spp (Euphorbiaceae), and especially *Phyllanthus urinaria*, has yielded the most promising results (Martin and Ernst, 2003). Huang *et al* (2003) screened

25 compounds isolated from *Phyllanthus* spp for anti - human HBV activities *in vitro*. Four of these compounds (nirunthin, nirtetralin, hinokinin and geraniin) were found to suppress both HBsAg and HBeAg expression at non-toxic doses.

A study by Romero *et al.*, (2005), found that artemisinin derivatives of *Artemisia annua* L. induced strong inhibition of viral production at non toxic concentrations and that artesunate in conjunction with 3TC had synergistic anti- HBV effects. Huang *et al* (2006) showed that the root extract of *Boehmeria nivea* L. Gaud inhibited HBV replication in Hep. G2.2.15 cells. *Oenanthe javanica* flavone has been found to inhibit human and Duck HBV infection (Wang *et al.*, 2005), while the inhibiting effects of the root of *Mallotus apelta* on Duck HBV has been demonstrated by Xu *et al* (2006). Shuguang *et al* (2009) have reported marked activities of Astragaloside IV isolated from *Radix astragal* against HBV.

## 2.5 Medicinal plants investigated in this project

The 4 medicinal plants selected for anti – HBV activity are tabulated below (Table 2).

**Table 2: Medicinal plants investigated in this project**

<b>Name</b>	<b>Family</b>	<b>Plant part used</b>
<i>Prunus africana</i> (Hook .f.) Kalkman	<i>Rosaceae</i>	Stem bark
<i>Maytenus heterophylla</i> (Eckl. & Zeyh.) Robson	<i>Celastraceae</i>	Root bark
<i>Acacia mellifera</i> (Vahl) Benth.	<i>Leguminosae</i>	Stem bark
<i>Carissa edulis</i> (Forssk.) Vahl	<i>Apocynaceae</i>	Root bark

The plants were selected on the basis of their ethno - pharmacological reports of their use in traditional medicine (Kokwaro, 2009). The plants selected had also exhibited significant activity against the HSV, CMV and HIV in various studies conducted locally (Rukunga *et al.*, 2002; Tolo *et al.*, 2006) and therefore, their activity against the HBV is highly postulated.



### 2.5.1 *Prunus africana* (Hook.f.) Kalkman

#### Botanical description

*Prunus africana* (syn *Pygeum africanum*) is commonly referred to as the African cherry. It belongs to the Rosaceae family and occurs as a medium to large evergreen tree of between 6 and 25 m in height, with a grey black corrugated bark. The leaves are often with a red petiole, ovate and elliptic in shape with a cuneate or rounded base. The flowers are normally white or cream and produce red to purple black ellipsoid shaped fruits of up to 12 mm (Beentje , 1994) (Figure 14) It is also commonly known by the trade name Red stink wood, probably due to its characteristic colour and the fact that it gives excellent timber useful for house building and furniture. It is also known by various vernacular names including; Migambo (Matengo), Mkonde – Konde (Chagga), Mufubia (Vinza), Muiiri (Kikuyu), Ol- Kojuka (Maasai) and Ntasesa (Luganda).



(Reproduced from <http://www.ispot.org.za>)

**Figure 14: Photograph of *Prunus africana***

## Uses and Distribution

It is widely distributed in many parts of Kenya, occurring in moist ever green forests such as the Mount Kenya forest at altitudes of between 1350 m and 2750 m. (Beentje, 1994).

Traditionally, the leaves are applied as an inhalant for fever while the stem bark is pounded, water added and the resultant red liquid decoction drunk as a remedy for stomach-ache, chest pain, fever and malaria. Other uses include its use as an antidote for diarrhea, allergies and kidney diseases (Pujol, 1990; Iwu, 1993). In modern practice it is used in the management of Benign Prostate Hyperplasia (Schipmarn, 2001). The extracts have been patented in a number of countries and are sold as commercial products (Sunderland and Obama 1999) and it is estimated that *P.africana* alone has an approximate market value of \$150 million (Cunningham, 1999). The extract from the bark is also used as a purgative for cattle by the Luhya community. The leaf infusion is drunk to improve appetite or as a remedy for malaria (Kokwaro, 2009).

## Previous work done on *P.africana*

The potential use of *P.africana* for the control, treatment and management of common bacterial and fungal infections was investigated by Bii *et al.*, (2008). The methanol extracts were found to be active against *Trichophyton mentagrophyte*, *Staphylococcus aureus*, and *Streptococcus pneumoniae*. Available literature indicates that phytochemical studies have led to the isolation of triterpenic acids including derivatives of ursolic and oleanolic acids (Fourneau *et al.*, 1996). The aqueous extracts of *P.africana* have shown significant reduction in the replication of human Cytomegalovirus in human embryonic lung fibroblasts cells with an EC<sub>50</sub> of 80 µg/mL and a CC<sub>50</sub> of more than 100 µg/mL *in vitro* demonstrating its potential antiviral activities (Tolo *et al.*, 2007). The chloroform and ethanol extracts of the stem bark have demonstrated greater than 90 % inhibition rate on the HIV- 1 at concentrations of 100 µg/mL and greater than 70% inhibition rate at concentrations of 50 µg/mL (Rukunga *et al.*,2002) without causing clinical signs or pathology in rats at doses of up to 1000 mg/kg for 8 weeks (Gathumbi *et al.*, 2002)

### 2.5.2 *Maytenus heterophylla* (Eckl. & Zeyh.) Robson

#### Botanical description

*Maytenus heterophylla* occurs as an evergreen shrub or a small tree of between 1.5 – 6 m tall with spines and is therefore commonly referred to as spike thorn. It belongs to the Celastraceae family presenting with either white, cream or yellow flowers as well as yellow or red obovoid fruit. (Beentje, 1994). The leaves are petiolated, alternated or often fascicled (Figure 15). It is known locally by various names including Omuseka (Nyankore), Likunga (Luhya) and Muthuthi (Kikuyu). (Kokwaro, 2009).



(Reproduced from <http://www.jnsbm.org>)

**Figure 15: Photograph of *Maytenus heterophylla***

## Uses and distribution

It is found in dry upland and lowland forest as well as bushed grassland at altitudes of between 1 – 350 m and 1150 – 2700 m in sub humid montane regions such as the Mt Marsabit region in northern Kenya (Githae *et al.*, 2007).

Traditionally its used to treat various infections, respiratory ailments and some inflammatory diseases (da Silva *et al.*, 2010) The roots are boiled and the liquid drunk as a vegetable by the Maasai. It is also used as an antihelmintic and as a cure for hernia. The roots are used to cure syphilis. The pounded leaf paste is normally rubbed on boils as a treatment (Kokwaro, 2009).

## Previous work done on *M.heterophylla*

Hot water extracts of the root bark has moderate anti-malarial activity (33% - 49% parasitaemia suppression) *in vivo* against chloroquine resistant *Plasmodium berghei*. (Muregi *et al.*, 2007). The ethanolic extracts of the leaves have exhibited significant anti-inflammatory activity (da Silva *et al.*, 2010) with the absence of acute and sub-acute toxicity signs associated with *Maytenus senegalensis* extracts. Various studies have also demonstrated the antitumor and antiviral activities of quinone isolates from the plant (Murayama *et al.*, 2007)

### 2.5.3 *Acacia mellifera* (Vahl) Benth

#### Botanical description

It belongs to the family Leguminosae and occurs either as a shrub or a tree of between 1.5 m and 9 m tall, presenting with a brown or light grey bark, white or cream flowers and pale brown or pale yellow fruits (Figure 16). It occurs mainly in dried bushland as well as in the dried woodland (Beentje, 1994) at altitudes of between 1 and 1800 M. It is known locally by various names including Kikwata (Swahili), Muthiia (Kamba), Talamong (Pokot) and Oitipi (Maasai).





Reproduced from <http://www.southernafricanplants.net>

**Figure 16: Photograph of *Acacia mellifera***

### **Uses and distribution**

It is used in traditional African medicine against such diseases as pneumonia and malaria. Charcoal from the tree was used to smelt iron among the Embu community. The bark is boiled in water and the liquid used as a remedy for stomach trouble, cleaning primary infections of syphilis, sterility, pneumonia, malaria, coughs and chest pains among various communities in the country including the Kamba, Maasai, Pokomo and Turkana. It is also used in circumcision rites by the Maasai. (Kokwaro, 2009) while the gum it produces is also edible.

### **Previous work done on *A.mellifera***

Some studies have demonstrated the antibacterial and antifungal nature of the methanolic extracts of the stem bark (Mutai *et al.*, 2009). The extracts have also been found to be anti-plasmodial displaying selectivity for the malaria parasite *Plasmodium falciparum* as indicated by a lack of cytotoxicity against cultured KB cells (Koch *et al.*, 2005)

#### 2.5.4 *Carissa edulis* (Forssk.) Vahl

##### Botanical description

*Carissa edulis* presents as a shrub of about 1 – 6 m tall with a grey bark and simple spines (Figure 17). The leaves are ovate, elliptic and almost round, with either a rounded or cuneate base. The flowers are white inside and pink to red on the outside. The fruits are red to black and are round or ellipsoid. The fruits are edible and very tasty. It is found distributed in forest edges, bushland, thicket or bushed grassland especially in rocky places (Beentje, 1994) at altitudes of between 1 and 2550 m. It is known locally by various vernacular names including Mtanda - mboo (Swahili), Mukawa (Kamba), Mufumbwe (Hehe), Ochuoga (Luo) and Kirumba (Taita).



Figure 17: Photograph of *Carissa edulis*

##### Uses and Distribution

The root extracts are used to treat several pathological states including inflammatory disorders. The fruits are edible and have astringent properties and are sometimes taken as an antidote for dysentery. Locally it is used for indigestion and lower abdominal pains when one is pregnant. The roots are dug out, washed and boiled in a pot with water. The solution is taken warm in small quantities at any time. An infusion of roots together with

other medicinal plants is used for treating chest pains. It's also used in the treatment of headaches, chest complaints, rheumatism, gonorrhoea, syphilis, rabies, epilepsy and as a diuretic. Root decoctions are also used for treating malaria as well as a pain killer. (Kokwaro, 2009).

Known as 'mriga-riga' in Tanzania, the plant became famous between October 2010 and April 2012, after it was branded a 'miracle cure' by a retired Lutheran pastor, Ambilikile Mwasipila, locally referred to as 'Babu'. During this period, more than 7 million people from all over Tanzania and beyond travelled to a remote village in Loliondo, Tanzania, known as Samunge, to get portions of the herbal drink dispensed by Babu (Zephania, 2011). It was believed that this drink could practically cure all forms of human ailments including Cancer, Diabetes and HIV – AIDS. A massive exploitation of the plant was witnessed during the period, raising concerns from scientists and conservationists about the need to verify the claims and to institute conservation measures.

### **Previous work done on *C.edulis***

The aqueous extracts of root barks of *C.edulis* have shown significant reduction in the replication of human Cytomegalovirus in human embryonic lung fibroblasts cells with an EC<sub>50</sub> of 74 µg/mL and a CC<sub>50</sub> of more than 100 µg/mL *in vitro* demonstrating its potential antiviral activities (Tolo *et al.*, 2007). *Carissa edulis* has exhibited remarkable anti-HSV activity *in vitro* and *in vivo* for both wild type and resistant strains of HSV (Tolo *et al.*, 2006). The diuretic effects of various extracts of the *C.edulis* were investigated with the root bark soxhlet extract producing a significant increase in urine output in rats at a dose of 1000 mg/kg (Nedi *et al.*, 2004). Some studies seem to suggest its potential as an anticonvulsant (Ya'u J *et al.*, 2008)

### **2.6 *In vitro* models for the research of anti – HBV activity**

A research programme to detect and isolate anti-viral compounds from higher plants is best carried out by a multi-disciplinary team consisting of at least a pharmacognosist and a virologist (Cos *et al.*, 2005). The antiviral screening system must meet all the requirements of any good assay system including validity, lack of ambiguity, accuracy, reproducibility, simplicity, and reasonable cost. Since we are dealing with plant extracts,

the antiviral screen should also be highly selective, specific and sensitive. (Gupta *et al.*, 2006). In regard to HBV infection, this is an ideal and there still lacks a specific model that can be considered robust enough (Guha *et al.*, 2004). This is in spite of the fact that a number of both *in vitro* and *in vivo* models do exist for anti-HBV drug screening. *In vivo* models include the use of animals such as the duck and woodchuck, whose primary HBV infection closely mimicks that of the human being (Guha *et al.*, 2004) while *In vitro* models involve the use of primary cultures and cell lines.

The WHO defines a primary culture as one that has been started from cells, tissues or organs taken directly from a donor and a cell line as one arising from a primary culture at the time of the first successful subculture (WHO/IVB/04.10.). Primary cultures therefore mimic the intact tissue better than the cell line, but are both important because they provide a consistent renewable source of cell material for study. Another obvious advantage of using established cell lines is their ready availability from tissue culture banks, some of which are very well established and are considered reliable sources. Examples include the American Type Culture Collection (ATCC) and the Korean Cell Line Bank (KCLB). The later was established in 1987 at the Cancer Research Institute of Seoul National University (SNU) College (<http://www.cellbank.snu.ac.kr>. Accessed 20<sup>th</sup> March 2013) which has characterized and reported about 109 cell lines from Korean cancer patients, consisting of 12 hepatocellular cell lines which includes infections of primary hepatocytes and transient or established HBV DNA transfected cell lines designated as SNU-182, SNU-354, SNU-368, SNU-387, SNU-398, SNU-423, SNU-449, SNU-475, SNU-739, SNU-761, SNU-878 and SNU-886 (Lee J.H. *et al.*, 1995). Such *in vitro* cell culture models have become widely used in the laboratory for antiviral research because of their convenient manipulation and maintenance (Hay *et al.*, 1988) and have proven to be of value in carcinogenic studies associated with HCC and HBV (Lee S.K.*et al.*, 1994). This has led to their wide distribution to biomedical researchers worldwide through the KCLB.

The SNU – 182 cell line used in this experiment is a bio-safety level 2 adherent cell line derived from a primary HCC taken mostly from patients prior to cytotoxic therapy



(Lee *et al.*, 1995). The cells have been established at the SNU since 1982 and are HBV infected, meaning that they have HBV genome incorporated into their genome. Histologically, they are predominantly trabecular and minor acinar type (Park *et al.*, 2005). Anti-viral compounds blocking any late replication such as transcription, translation, pre-genome encapsulation, reverse transcription, particle assembly and release, can be identified and characterized using this cell line.

### **2.6.1 Handling of the cell line**

Bio-medical research using the cell line requires that it is handled according to best practice, giving careful attention to culture conditions and passage procedures. This involves the handling of only one cell line at a time in a bio-safety cabinet to avoid cross contamination while ensuring that the cell cultures are devoid of contamination from micro-organisms including bacteria, fungi, mycoplasma and viruses. Bacteria and fungi will normally kill the cells while mycoplasma will have serious effects on a cell culture without inhibiting cell growth. Viruses will typically produce a cytopathic effect on the cultures. For this reason antibiotics such as streptomycin and penicillin are used in cell cultures mainly because they are inexpensive and are readily available (WHO IVB/04.10, 2004)

A successful culture technique would also require that the cultures be maintained at incubation temperatures of 37°C and that the pH of the growth medium be maintained at between 7.2 and 7.4. The levels of glucose, L-glutamine, various inorganic ions, amino acids vitamins, oxygen and carbon dioxide are essential for cell survival and should be controlled. For reasons that are not clear, infection of primary hepatocytes and established cell lines with hepatitis virus has produced poor viral replication and low viral yields and has suffered from poor reproducibility. The addition of polyethylene glycol to the primary cultures maintained in the presence of 2% DMSO markedly increases the infection of HBV (Gripon *et al.*, 1993). Cultures will normally initially be set up in growth medium supplemented with 10%  $\frac{v}{v}$  serum, then once the cells have formed a confluent monolayer, changed to maintenance medium which is designed to maintain cultures in a healthy state for as long as possible, without stimulating growth. This is normally achieved by reducing the serum content usually to 2%  $\frac{v}{v}$ . A periodic

change of the medium and removal of dead cells is normally carried out in a process known as cell passaging (splitting) which is necessary to keep the cells alive and growing under the cultured conditions for extended periods of time. It is normally necessitated because of the production of toxic metabolites over time and is necessary to ensure the availability of healthy and quality cells. For a successful culture therefore, a number of specialized equipment will be required, the basic of which include a biological safety cabinet, CO<sub>2</sub> incubator, autoclave machine and a water purifying system.

## **2.7 Project Justification**

HBV infection kills more than 600,000 people annually (WHO fact sheet No 204, 2013) and its prevalence in Kenya is as high as 8.8% (Makokha *et al.*, 2004; Okoth *et al.*, 2011). Drugs currently available for its treatment are not very effective (Hoofnagle and Bisceglie, 1997), are too costly to the poor (Delaney *et al.*, 2006) and viral resistance to them tends to develop rapidly (Lai *et al.*, 2005). To overcome these challenges new drugs are needed.

The extracts of *Carissa edulis*, *Maytenus heterophylla*, *Prunus africana* and *Acacia mellifera* have marked activities against Herpes Simplex Virus (HSV), Human CytomegaloVirus (HCV) and Human Immunodeficiency Virus (HIV) (Rukunga *et al.*, 2002; Tolo *et al.*, 2006). They have however not been screened for anti-HBV activity and if found to have some effects on HBV, could, upon further work, turn out to be candidates for further development into products that could be an addition to the armamentarium against this virus.

## **2.8 Null Hypothesis**

The crude extracts of the selected medicinal plants do not have significant activity against HBV.

## **2.9 Objectives of the Study**

### **2.9.1 Main Objective**

To determine the anti-viral activities of the selected Kenyan medicinal plants against HBV.

### **2.9.2 Specific Objectives**

- 1) To determine the effect of the extracts of the selected plants on the expression of the HBsAg by use of ELISA.
- 2) To determine the *in vitro* cytotoxicity profiles of the extracts of the selected plants against the SNU – 182 cell line.
- 3) To determine the effect of the extracts of the selected plants on the expression of HBV DNA by use of Real time PCR.

## CHAPTER THREE

### 3.0 MATERIALS AND METHODS

#### 3.1 Collection of the medicinal plants

The medicinal plants used in this study were collected from their natural habitats in different geographical zones in Kenya. The stem barks of *Prunus africana* and roots of *Carissa edulis* were collected from Gatundu district in Kiambu county on the 20<sup>th</sup> of April 2012; the stem barks of *Acacia mellifera* from the Ngong' forest of Kajiado county on the 21<sup>st</sup> of April 2012 and the roots of *Maytenus heterophylla* from the Machakos county on the 28<sup>th</sup> of April 2012. The plants were identified on the ground before collection by the help of experienced herbalists who were part of the collection team and plant parts collected with bio-conservation aspects in mind. Voucher specimen (Ref Nos Mkwena 2012/01(*Prunus africana*), Mkwena 2012/02 (*Carissa edulis*), Mkwena 2012/03 (*Acacia mellifera*) and Mkwena 2012/04 (*Maytenus heterophylla*) were confirmed and deposited at the Chiromo School of Botany herbarium, University of Nairobi.

#### 3.2 Initial processing of the medicinal plants

The roots harvested had their barks peeled off while still fresh and cut into small pieces then air dried at room temperature (23° C - 25° C) for two weeks. When completely dry, the stem bark of the *Prunus africana* was ground using an electric mill (Christy and Norris Ltd. England) while the rest (root barks of *Carissa edulis* and *Maytenus heterophylla* and the stem barks of *Acacia mellifera*) were comminuted to fine powder using a Ramtons<sup>®</sup> blending machine. The powdered plant materials were kept separately in tightly closed plastic containers, wrapped in aluminium foil at room temperature (23°C - 25°C) and away from light.

### **3.3 Preparation of the plant extracts**

#### **3.3.1 Preparation of the aqueous extracts**

The aqueous extracts of *Carissa edulis*, *Maytenus heterophylla* and *Prunus africana* were prepared based on a technique described by Tolo *et al* (2006). These specific water extracts had exhibited anti-viral activities against the HSV, CMV and HIV in previous studies (Rukunga *et al.*, 2002; Tolo *et al.*, 2006) and were therefore considered good candidates for this study. They were also preferred because most traditional remedies use water as the choice solvent (Kokwaro, 2009).

Briefly, about 100 g of the plant material was weighed into a 1000 mL conical flask and 1000 mL of clean tap water added. The mixture was heated over a water bath at 60°C for 1½ hrs with occasional shaking. The solution was then filtered through folded cotton gauze into a clean 1000 mL beaker and the final volume measured in a measuring cylinder. The solutions were then aliquoted into 200 mL portions and introduced into 200 mL suction flasks, which were subsequently fitted onto a Modulyo freeze drier (Edwards, England) and freeze drying carried out over a period of 48 hrs. Using a spatula, the dried powder was removed from the flasks and then weighed on an analytical balance and stored in tightly sealed falcon tubes at - 4°C. The percentage yield from each plant extract was then calculated and recorded.

#### **3.3.2 Preparation of the Chloroform extract of *Prunus africana***

The chloroform extracts of *Prunus africana* have exhibited excellent anti-proliferative properties in previous studies such as those involving the management of Benign Prostate Hyperplasia (BPH). This has been attributed to lipophilic phytosterols such as the  $\beta$  – Sitosterol and Campesterol, which act mainly through an anti-inflammatory mechanism that includes the blocking of enzymatic activity (Gathumbi *et al.*, 2002). It was therefore considered a good candidate for this study given that the products are in clinical use. The extracts were obtained using the same protocol used in the BPH studies that utilizes soxhlet extraction as follows;

One thousand grammes of the powder was weighed into an extraction bag which was then introduced into an extraction chamber of the soxhlet extractor. Ten Liters of chloroform was then introduced into the siphoning chamber and the soxhlet system stabilised by letting the tap water run through the condenser for a while as well as switching on the heating mantle to temperatures of 7°C. The system was then allowed to run for 48 hrs, and the extract collected and filtered through folded medical grade cotton gauze into a clean 1000 mL beaker. The solutions were then aliquoted into 200 mL portions and introduced into 200 mL vacuum flasks, which were subsequently dried at 60 °C under reduced pressure on a rotary evaporator. Using a spatula, the dried powder was removed from the flask and then weighed on an analytical balance and stored in tightly sealed falcon tubes at room temperature and away from light. The percentage yield from the plant extract was then calculated and recorded.

### **3.3.3 Preparation of the methanol extract of *Acacia mellifera***

The methanol extraction of *Acacia mellifera* was selected as a follow up of studies that had demonstrated the activity of the extracts of the *Acacia spp.* on HCV and HSV (Tolo *et al.*, 2006; Sidra *et al.*, 2011). The extraction was carried out based on a protocol described by Tolo *et al* (2006) as follows;

*Acacia mellifera* powder (100g) was soaked in 1000 ml of general purpose methanol for 96 hrs at room temperature, shaking the contents occasionally. The extract was decanted, filtered through Whatman filter paper No. 1, transferred into a vacuum flask then dried at 60°C under reduced pressure on a rotary evaporator. Using a spatula, the resulting powder was removed from the flask then weighed on an analytical balance and stored in a tightly sealed falcon tube at room temperature and away from light. The percentage yield from the plant extract was then calculated and recorded.

## **3.4 Preparation of cell culture reagents**

### **3.4.1 Preparation of Phosphate Buffered Saline (PBS)**

Phosphate Buffered Saline (PBS) is a simple basic water based salt solution used in research as a buffer to maintain the pH of biological fluids. It is either used in its incomplete form (containing no calcium ( $\text{Ca}^{2+}$ ) or magnesium ( $\text{Mg}^{2+}$ ) ions) or in its

complete form. The complete form is used mainly in the preparation of specimen extracts and as a diluent for viruses since the presence of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  ions stabilizes viruses by increasing the stability of the intracellular matrix thereby making detachment of the cells from the glass/plastic difficult. For this experiment however, PBS was used to wash cells prior to cell disaggregation in its incomplete form. To prepare 500 mls of an incomplete working solution of PBS, one commercial tablet (Gibco<sup>®</sup> lot no 828468) containing; NaCl (4.00 g), KCl (0.10 g),  $\text{Na}_2\text{HPO}_4$  (anhydrous) (0.575 g) and  $\text{KH}_2\text{PO}_4$  (0.1 g) was dissolved in 500 mls of double distilled water and the solution autoclaved at 10 psi (70 kPa) for 15 minutes at 121°C. This was then stored at 4°C ready for use.

### **3.4.2 Preparation of Sodium Bicarbonate Solution 7.5 % w/v**

Sodium Hydrogen Carbonate ( $\text{NaHCO}_3$ ) is an essential metabolite and buffer used to stabilize the viral culture media together with gaseous  $\text{CO}_2$ . It was prepared by dissolving 7.5 g of  $\text{NaHCO}_3$  (Sigma-Aldrich<sup>®</sup>, USA) in 100 mL of PBS to give a final concentration of 7.5% w/v. The solution was then autoclaved at 10 psi for 15 min at 121°C and stored at 4°C ready for use.

### **3.4.3 Preparation of Versene (EDTA) 0.04% /Trypsin**

Versene and Trypsin are cell dispersing agents commonly used either separately or combined. Trypsin is a proteolytic enzyme particularly suitable for the digestion of cells from whole organs as well as for the removal of cells from glass or plastics. Versene is the disodium salt of ethylenediamine tetraacetic acid (EDTA) and is a chelating agent. Solutions of trypsin and versene were prepared in solutions of incomplete PBS. Trypsin was prepared by dissolving 1 g of Difco 1:250 trypsin in 400 ml incomplete PBS followed by gentle agitation with a magnetic stirrer for 30 minutes at 37°C. The solution was filtered through membrane filters of pore size 0.22 $\mu\text{m}$  (PVDF) and the solution stored at - 30°C. A solution of 0.04 % w/v Versene was prepared by weighing 40 mg of EDTA powder (Dojindo, Japan) in 90 mL of incomplete PBS followed by autoclaving at 15 psi and 121°C for 15 minutes. A final solution of Trypsin / versene 0.04% was then made by mixing 10 ml of trypsin with 90 mL of the Versene.

### 3.4.4 Preparation of Minimum Essential Medium Eagle (MEM)

MEM developed by Harry Eagle is one of the most widely used of all synthetic cell culture media. Historically it has been used for the cultivation of a wide variety of cells grown in monolayers (Eagle *et al.*, 1956). The Eagle's growth medium and Eagle's maintenance medium were used for culturing the SNU – 182 cell line in the presence of 5% CO<sub>2</sub> after optimization. The Growth medium differs from the Maintenance medium in the serum content. A stock solution of MEM was prepared by adding 4.7 g of powdered MEM (Sigma-Aldrich, USA) gently to 500 mls of double distilled water while stirring until dissolved. The solution was autoclaved at 121°C and 15 psi for 15 minutes and allowed to cool to room temperature. One hundred ml of the final culture media was then prepared by adding the following supplements;

	<b>Growth Medium</b>	<b>Maintenance Medium</b>
Eagle's Minimum Essential Medium	83.5 ml	90.5 ml
L-glutamine 200mM	1.0 ml	1.0 ml
Fetal Bovine Serum (FBS)	10.0 ml	2.0 ml
NaHCO <sub>3</sub> Solution 7.5% w/v.	3.5 ml	4.5 ml
HEPES 1 M	1.0 ml	1.0 ml
Penicillin/Streptomycin solution	1.0 ml	1.0 ml

### 3.4.5 Preparation of Fetal Bovine Serum (FBS)

Five hundred mL of heat inactivated neat (100x) FBS (Gibco, USA) was activated by heating it in a Memmert waterbath at 56°C with gentle agitation for 30 minutes. This was then aliquoted into 100 mls portions and topped up to 1000 mls using double distilled water to give a 10 % v/v working solution. A final concentration of 10% v/v Eagle's Growth medium or 2 % v/v Eagle's maintenance medium was then prepared by adding 10 mls or 2 mls of the respective solution to 100 mL of Minimum Essential Medium respectively.



### 3.4.6 Preparation of antibiotics

Crystalline Penicillin G ( $1 \times 10^6$  units) (Sigma, USA) and 1 g of Streptomycin sulphate (Sigma, USA) was dissolved in 100 ml of PBS. This was sterilized by filtration through membrane filters of pore size 0.22  $\mu\text{m}$  (PVDF) and distributed into 5 ml volumes then stored at  $-30^\circ\text{C}$ . One ml of this stock solution was later added to 100 mL of Minimum Essential Medium to give a final concentration of 100 units Penicillin and 100  $\mu\text{g}$  Streptomycin per mL.

### 3.5 *In vitro* Cell Culture techniques

#### 3.5.1 Culturing of the SNU – 182 cell line

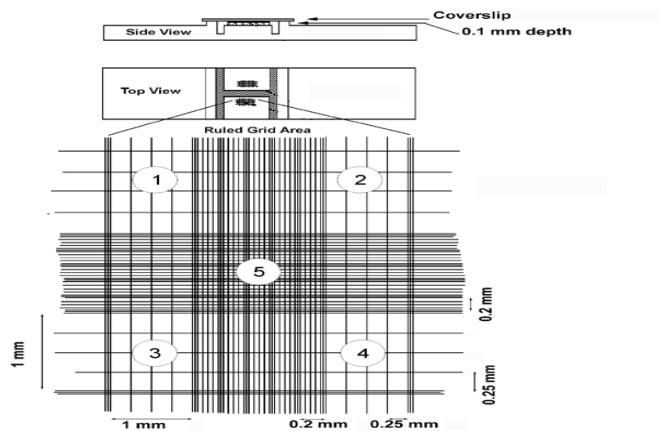
SNU – 182, an HBV transfect human hepatocellular cell line, was provided by the American Tissue Culture Collection. Upon receipt of the frozen cells, a cell bank was established in a liquid nitrogen tank by first thawing the cells in a mettler water bath at  $37^\circ\text{C}$ , resuspending them in growth medium and culturing them based on a method described by Park *et al.*, 2005.

Briefly, the cells were grown in a 150  $\text{cm}^2$  culture flask in growth medium supplemented with 10 %  $\text{v/v}$  FBS, 2 mmol/L glutamine, 100 IU/ml of penicillin and 100  $\mu\text{g}/\text{mL}$  of streptomycin, prepared as described in section 3.4 above. The cultures were incubated at  $37^\circ\text{C}$  and 5 %  $\text{CO}_2$  in a humidified incubator and cells grown to a confluent monolayer, upon which they were harvested, count determined as described in section 3.5.2 below and resuspended in preservation medium comprising 4 mL of 10%  $\text{v/v}$  DMSO, 4 ml 10%  $\text{v/v}$  FBS and 32 mL Growth medium.

This suspension was then aliquoted into thirty - 2 ml cryo-preservation vials at a density of approximately  $2 \times 10^6$  cells /mL and stored at  $4^\circ\text{C}$  for  $1\frac{1}{2}$  hrs before being transferred into a Nalgene<sup>®</sup> cryo freezing vessel containing 250 mls of isopropyl alcohol. The vessel was frozen gradually at  $1^\circ/\text{min}$  to  $-80^\circ\text{C}$  in a Brunswick freezer before being transferred into the liquid phase in the liquid nitrogen tank at  $-196^\circ\text{C}$ , in order to maintain the cells in the viable state over a long period of time.

Subsequent use of the cell line for actual experiments involved removing one of the 2 ml vials from the liquid nitrogen tank and transferring immediately into a Mettler water bath of sterile water at 37°C, where the contents were allowed to completely thaw. The vial was then wiped using 70 %  $v/v$  alcohol to reduce chances of bacterial contamination and the resulting cell suspension introduced into a 75 cm<sup>2</sup> culture vessel overlaid with 20 ml growth medium to dilute the DMSO to levels that would not rupture the cells. This was then incubated at 37°C and 5 % CO<sub>2</sub> in a humidified incubator for 24 hrs before confirming the viability of the cells under an inverted microscope. This culture was maintained at 37°C and 5 % CO<sub>2</sub> in a humidified incubator by replacing the growth medium with 20 mL of maintenance medium. Cell passaging was carried out weekly by washing off dead cells using PBS and replacing the culturing media with a fresh lot.

### 3.5.2 Cell count determination and seeding of the SNU 182 cells



**Figure 18: The ‘Improved Neubauer’ hemocytometer as observed under a microscope**  
 (Adopted from <http://www.homepages.gac.edu>)

70 %  $v/v$  Ethanol was used to disinfect a class II BSC which was used for the exercise.

The cells growing in a culture flask were first examined visually and microscopically for quality, defined by an entire monolayer of healthy cells (Park *et al.*, 2005) and the absence of contamination. The maintenance medium from the culture flask was decanted and discarded, and the confluent cell layer washed with approximately 5 mL of incomplete PBS. This was discarded and followed by a second wash and the complete

drainage of the PBS before the addition of approximately 1.5 ml of Versene 0.04 % / trypsin, which was dispersed evenly on the monolayer for the detachment of the cells.

This was incubated at 37°C for approximately fifteen minutes, until the cells detached from the surface, which was assisted by tapping the side of the flask a few times. The complete detachment of the cells was confirmed microscopically before re-suspending the cells in about 10 mL of growth medium to halt the action of the Versene 0.04 % / trypsin.

Two ml of this suspension was aliquoted into a micro-centrifuge tube and used in the Trypan Blue exclusion test for cell count determination as described by Tolo *et al* (2006). Briefly, the cells were first diluted by a factor of four by aliquoting 100µl of the suspension with 300 µl of 0.1% <sup>w/v</sup> Trypan Blue dye (Sigma-Aldrich, USA) into a 2 ml micro-centrifuge tube. The non - viable cells at this stage are stained blue while the viable are unstained.

After mixing well using a fine pasteur pipette, sufficient volume was aspirated to fill both chambers of an ‘improved neubauer’ haemocytometer (Figure 18), which was then incubated at room temperature for one minute, before counting the viable cells by aid of a cell counter and under an inverted microscope in four of the 1 x 1 mm<sup>2</sup> square divisions of one chamber. This was repeated for the second chamber and the average number of cells per square mm determined. The total number of cells in the original suspension was then calculated according to the formula below;

$$\text{Total viable cell count} = a \times \text{DF} \times b \times 10^4$$

- Where
- a = average viable cell count from hemocytometer
  - DF = dilution factor (e.g. 100µl in 300 µl is 4 as in the exp.)
  - b = Original volume of cells (e.g. 150 µl as in the exp.)
  - 10<sup>4</sup> = hemocytometer cell concentration per mL(a constant value)

The cells were then plated into 96 well micro-titre plates at a density of  $0.5 \times 10^5$  cells per well (Ma *et al.*, 2013) and maintained in 0.5 mL Growth medium supplemented with 10 %  $v/v$  FBS at 37°C and 5 % CO<sub>2</sub> in a humidified incubator.

### **3.6 Determination of the effect of the extracts of the selected plants on the expression of the HBsAg**

#### **3.6.1 Preparation of stock and working solutions of the plant extracts**

Since most plant extracts and purified compounds are readily soluble in DMSO, (Cos *et al.*, 2005) stock solutions of test samples were prepared in 100% DMSO and stored in 2 ml cryo vials at 4°C until use. An added advantage of DMSO is that it eliminates microbial contamination of the test samples, so that sterilization by autoclaving, filtration or other methods becomes unnecessary. To avoid interference in the *in vitro* cell based test systems, the test concentration of DMSO was maintained at about 1%  $v/v$ . The actual preparation of the stock and working solutions were carried out according to the Center for Traditional Medicine and Drug Research (CTMDR) protocol as follows;

Using an analytical balance approximately 20 mg of each plant extract was weighed into a 2 ml micro-centrifuge tube. To each plant extract, 1 mL of absolute DMSO (100%) was added and the mixture vortexed for 1 minute and the resulting suspension kept at 4°C under sterile conditions. A working solution of 200 µg/ml of the plant extracts and 1%  $v/v$  DMSO was prepared from the stock solution by pipetting 10 µl of the stock solution and adding 990 µl of the MEM supplemented with 2 % FBS.

#### **3.6.2 Preparation of Lamivudine Control**

Lamivudine API (USP) at a purity of 99.5%  $w/w$  on anhydrous basis was sourced from Hetero (India). A stock suspension of 10 mg/ml for *in vitro* assay was prepared by adding 300 mg of the powder directly to 30 ml of sterile double distilled water under aseptic conditions. The mixture was vortexed for 1 minute and the milky suspension stored in a 2 mL cryo vial at 4°C. For the *in vitro* assays, a solution of 1 mg/ml was achieved by diluting 100 µl stock solution with 0.9 ml (900 µl) MEM supplemented with 2 % FBS. 5 µl, 30 µl and 100 µl of the working solution was then added to 995 µl, 970 µl, and 900 µl

MEM containing 2 % FBS respectively for a standard test concentration of 5 µg/ml, 30 µg/ml and 100 µg/ml (Tolo et al., 2006; Ma *et al.*, 2013) respectively for the treatment of the cell line.

### **3.6.3 Serial dilution of HBV positive sample**

Serial dilutions (2x) of a HBV positive sample of known viral load was subjected to the Hepanostika<sup>®</sup> HBsAg technique to demonstrate its semi – quantitative nature. This was carried out according to the manufacturer’s protocol described in section 3.6.5 below.

### **3.6.4 Culturing of the cells and inoculation of the drugs**

Cell count determination and seeding of cells onto three 6-well culture plates was carried out as described in section 3.5.2 above. A total of 46 cells in all the four chambers was counted giving an average number of  $4.6 \times 10^5$ /mL in the culture flask. The cells were then seeded into the wells of a three six well culture plates at a density of  $1 \times 10^5$  cells per well by aliquoting approximately 300 µl ( $1 \times 10^5 / 3.0 \times 10^5 = 0.33$ ) into each well on the culture plates. The media was topped up to 3.0 mL by adding 2.7 ml of MEM supplemented with 10 % FBS. The plates were incubated at 37°C and 5 % CO<sub>2</sub> (Panasonic MCO – 18AC) and cells allowed to set for 48 hrs. After 48 hrs, media from the wells was aspirated off and 3 ml of the media containing the plant extracts introduced into the wells in triplicate, leaving three wells as the negative control. After five days, the media was harvested and analysed for viral load using the Hepanostika<sup>®</sup> ELISA Kit as described in Section 3.6.5 below.

### **3.6.5 Determination of viral load using the Hepanostika<sup>®</sup> ELISA Kit**

Assays using the Hepanostika<sup>®</sup> ELISA kit were conducted according to the protocol accompanying the kit.

All reagents provided in the kit and samples to be assayed were equilibrated to room temperature (25°C) before beginning the assay. The phosphate buffer concentrate (25x) provided was inspected for the presence of salt crystals and when present, resolubilization carried out by warming at 37°C in a Metler water bath till all were dissolved. 100 ml of this concentrate was then diluted by topping up to 2500 ml with

double distilled water in a conical flask. At least 25 ml of this diluted solution was used for each micro-ELISA strip.

The TetraMethylBenzidine (TMB) substrate was prepared by combining 2.5 ml of the TMB solution provided with an equal volume of urea peroxide solution in a disposable vial. This TMB substrate was protected from excessive exposure to light by working in a dimly lit room and storing the substrate in a dark area wrapped in aluminium foil. Sulfuric acid (1 mol/L) was prepared by adding 50 ml of concentrated acid (18 mol/L) slowly to 850 mL of double distilled water.

The microELISA strips were then fitted into the strip holder and 25  $\mu$ l of specimen diluents pipetted into the assigned wells. 100  $\mu$ l of the undiluted sample and controls were pipetted into the assigned wells, including three negative controls and one positive control in each strip holder. As a precaution, the controls were pipetted after the samples. The strips were then incubated at 37°C for 60 minutes.

Fifty  $\mu$ l of the conjugate solution was pipetted into each well with caution not to touch the side of the wells or the liquid surfaces with the pipette tips. This was followed by a second incubation at 37°C for 60 minutes.

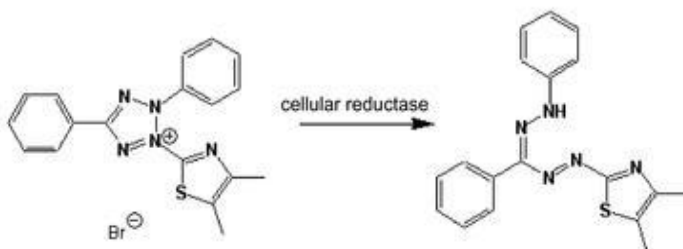
The wells were then soaked and washed six times with the diluted phosphate buffer by aspirating the well completely into a waste flask then filling the well contents completely with phosphate buffer, avoiding overflow from one well to another. This was allowed to soak for approximately 60 seconds then aspirated completely. This wash and soak procedure was repeated five times for a total of six washes using an automated Biotec<sup>®</sup> AMW washer. Any remaining fluid on the top or under the bottom of the microELISA strips and strip holder after the last aspiration was removed by carefully blotting with absorbent tissue.

TMB (100  $\mu$ l) substrate was then added to each well and the strips incubated at room temperature for 30 minutes in a dark area. The reaction was stopped by the addition of 100  $\mu$ l of 1 mol/L sulfuric acid to each well and the absorbance readings determined

using an ELISA reader within 15 mins. The absorbance readings of the test wells were then compared to controls to give a relative indication of the levels of HBsAg.

### 3.7 Determination of the *in vitro* cytotoxicity profiles of the extracts of the selected plants

#### 3.7.1 MTT Assay



**Figure 19: Reduction of MTT by cellular enzymes**  
(Reproduced from [www.pharmatutor.com](http://www.pharmatutor.com))

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a yellow coloured tetrazolium dye which is easily reduced to an insoluble purple coloured formazan in living cells (Figure 19). Based on this principle, the MTT assay is a colorimetric assay technique for measuring the activity of cellular enzymes that carry out this reduction. It is therefore a measure of cellular metabolic activity and is an indicator of cytotoxicity. (Florian *et al.*, 1999)

A fresh working solution of 5 mg/ml of MTT solution in PBS was prepared whenever the cytotoxicity experiment was to be carried out, owing to the unstable nature of MTT. (Tolo *et al.*, 2006). The experiments were carried out in duplicate in a class II BSC disinfected with 70% v/v alcohol and away from light. About 1 mL of MTT was used per 96 well plate of cultured cells by introducing 10 µL of the MTT solution per well. The cells were then incubated for 4 hours to allow for the formation of the purple precipitate. This was confirmed under an inverted microscope before removing medium from all the wells, taking care not to dislodge the cells and adding 150 µl of absolute DMSO into each well to dissolve the formazin. The Absorbance in each well was then determined at 562 nm in a microtiter plate reader. From the average values of the two readings, the cell viability was expressed as a percentage of the negative control.

### 3.7.2 CC<sub>50</sub> determination

The cytotoxic concentration which causes 50 % of cell lysis and death (CC<sub>50</sub>) for each plant extract was evaluated using the MTT Assay technique described in section 3.7.1 above using various concentrations of the plant extracts. This was carried out by introducing 100 µL of cell culture into the wells of a 96 well microtitre plate according to the technique described in Section 3.5.2 above, excluding the 3<sup>rd</sup>, 6<sup>th</sup>, 9<sup>th</sup> and 12<sup>th</sup> column (Figure 20) which would act as negative controls at later stages of the experiment.

	1	2	3	4	5	6	7	8	9	10	11	12
A	p.c	p.c	n.c	p.c	p.c	n.c	p.c	p.c	n.c	p.c	p.c	n.c
B			n.c			n.c			n.c			n.c
C			n.c			n.c			n.c			n.c
D			n.c			n.c			n.c			n.c
E			n.c			n.c			n.c			n.c
F			n.c			n.c			n.c			n.c
G			n.c			n.c			n.c			n.c
H			n.c			n.c			n.c			n.c

Figure 20: CC<sub>50</sub> Micro ELISA plate layout

**Key:** a: p.c – Positive control comprising cells not overlaid with plant extracts  
 b: n.c – Negative control comprising blank wells with no cell cultures  
 c: The rest of the wells were overlaid with plant extracts at various concentrations

A working plant extract solution of 200 µg/ml and 1% DMSO was prepared from the stock plant extract solution as described in section 3.6.1 above and 150 µL of this solution introduced into the row H of the micro-titre plate in duplicate by first aspirating off the growth medium from the row without disturbing the cells and ensuring that the cells were not left without medium for long.

A serial dilution of the specific plant extract was then achieved by the use of a multi-channel pipette by aspirating 50 µL of the extract solution overlaying the cells in the wells of row H and adding this aliquot to the wells of row G. After mixing this medium, 50 µl of the resulting solution was transferred to the wells of row F and this was mixed. This was continued up to Row B at which point the last 50 µl of this row was discarded leaving row A as a positive control comprising cells overlaid with no plant extract.



The micro titre plate was then incubated at 37°C and 5 % CO<sub>2</sub> for 48 hours after which the growth of the cells in the plate was observed under an inverted microscope to ensure normal growth after which the MTT Assay was carried out as described in section 3.7.1 above.

### **3.7.3 Calculation of the CC<sub>50</sub>**

The cytotoxic concentration (CC<sub>50</sub>) was then determined from a graph relating the percentage of cell viability to the concentration of the extracts as shown in section 4.3 below.

## **3.8 Determination of the effect of the extracts of the selected plants on the expression of HBV DNA**

### **3.8.1 Preparation of working solutions of the plant extracts**

A stock solution was prepared by weighing approximately 100 mg of each plant extract into a 2 mL microcentrifuge tube followed by adding 1 mL of absolute (100%) DMSO and vortexing for 1 minute. The resulting suspension was stored at 4°C under sterile conditions. A working solution of the plant extracts of 1% v/v DMSO was then prepared from this stock solution by pipetting 10 µl of the stock solution and adding 990 µl of the MEM supplemented with 2 % FBS. A serial dilution (2 X) was then achieved by pipetting 500 µl of the working solution into a clean microcentrifuge tube and adding 500 µl of MEM supplemented with 2 % FBS. This was continued to give working plant extracts solutions of 500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL, 31.25 µg/mL and 15.125 µg/mL.

### **3.8.2 Preparation of Lamivudine positive Control**

This was done as described in section 3.6.2 above.

### **3.8.3 Culturing of the cells and inoculation of the drugs**

SNU 182 cells were plated in two 96 well microtitre plates as described in Section 3.5.2 above, by aliquoting approximately 100 µl of harvested cells into each well. The cells were allowed to set for 48 hrs after which, media from the wells was aspirated off using a multi - channel micropipette and 200 µl of media containing plant extract at

various concentrations introduced into the wells in duplicate; 200 µl of fresh media was added onto the last row of the microtiter plates to act as the negative control. The plates were then incubated at 37°C and 5% CO<sub>2</sub> for five days after which the supernatant was harvested and HBV DNA extracted as described in section 3.8.4 below.

#### **3.8.4 Extraction of viral DNA from the culture supernatant**

HBV DNA in culture supernatants after 5 days of incubation was quantified by real time PCR analysis.

Extraction of the viral DNA was carried out in a sterilised biosafety level 2 cabinet using the QIAamp<sup>®</sup> (Qiagen<sup>®</sup>, Germany) DNA mini extraction kit according to the manufacturer's protocol: Briefly, 200 µl of harvested supernatant was introduced into a 1.5 mL micro centrifuge tube and 200 µl of lysis buffer added. This was mixed by pulse vortexing for 15 sec. and introduced into a heating block where incubation was done at 56°C for 10 mins. The microcentrifuge tubes were then briefly centrifuged at 8000 g for 1 minute in an eppendorf microcentrifuge to remove drops from inside the lid.

Absolute ethanol (200 µl) was then added to the sample, followed by pulse vortexing for 15 seconds and a brief centrifugation for 1 min at 8000 rpm to remove drops from inside the lid.

The mixture was carefully applied to the QIAamp mini spin columns in a 2 ml collection tube and centrifuged at 8000 rpm for 1 min. The collection tube containing the filtrate was discarded and the QIAamp mini spin columns placed in a clean 2 mL collection tube and opened carefully to allow the addition of 500 µl of the first wash buffer. The cap was closed and column centrifuged at 8000 rpm for 1 min. The collection tube containing the filtrate was discarded and the QIAamp mini spin column placed in a clean 2 mL collection tube. The QIAamp mini spin column was carefully opened and 500 µL of the second wash buffer added without wetting the rims. The cap to the minispin column was closed and the mixture centrifuged at the full speed of 14000 rpm for 3 mins. The QIAamp mini spin column was then placed in a clean 1.5 ml microcentrifuge tube, discarding the collection tube containing the filtrate, and carefully opened to add 200µl of Buffer AE or distilled water. This was then incubated at room temperature for 1 minute

and the mixture centrifuged at 8000 rpm for 1 minute and the filtrate collected. This was the pure nucleic acid material which was then stored at -30°C to prevent hydrolysis and was used for the amplification..

### **3.8.5 Assay experiments using Real time PCR (Light cycler<sup>®</sup> 480 SYBR green 1 master kit)**

Amplification was carried out on 25 µl reaction mixture prepared under aseptic conditions in a biosafety level 2 cabinet by mixing 8 µl of the PCR grade water, 1 µl forward primer (ACT CGT GGT GGA CTT CTC TCA ATT) 1 µl reverse primer (CGC AGA CAC ATC CAG CGA TA) and 10 µl Sybergreen 1 dye with 5 µl of the DNA template harvested as described in section 3.8.4 above. The PCR primers were designed using Primer Express software (Applied Biosystems, USA) and were provided by the International Livestock Research Institute (ILRI).

The mixture was loaded into PCR tubes accompanying the Lightcycler kit and introduced into the thermocycler where amplification and detection was performed using the Lightcycler<sup>®</sup> Nano SW 1.0 system. The PCR protocol consisted a holding temperature of 94°C for 420 seconds followed by 40 cycles of a 3 step amplification comprising a denaturation at 94°C for 40 seconds, annealing at 60°C for 60 seconds and extension at 72°C for 120 seconds then a hold at 72°C for 15 seconds and melting at 60°C to 95°C at 0.1°C/s. The average threshold cycle of amplification (Cq) from two replicate determinations was used as the indicator of the levels of DNA, with a lower Cq indicating a higher level of DNA amplicon. The results were then analysed using Microsoft Excel<sup>®</sup> 2010.

## CHAPTER FOUR

### 4.0 RESULTS AND DISCUSSION

#### 4.1 Determination of the effect of the extracts of the selected plants on the expression of the HBsAg

##### 4.1.1 Results

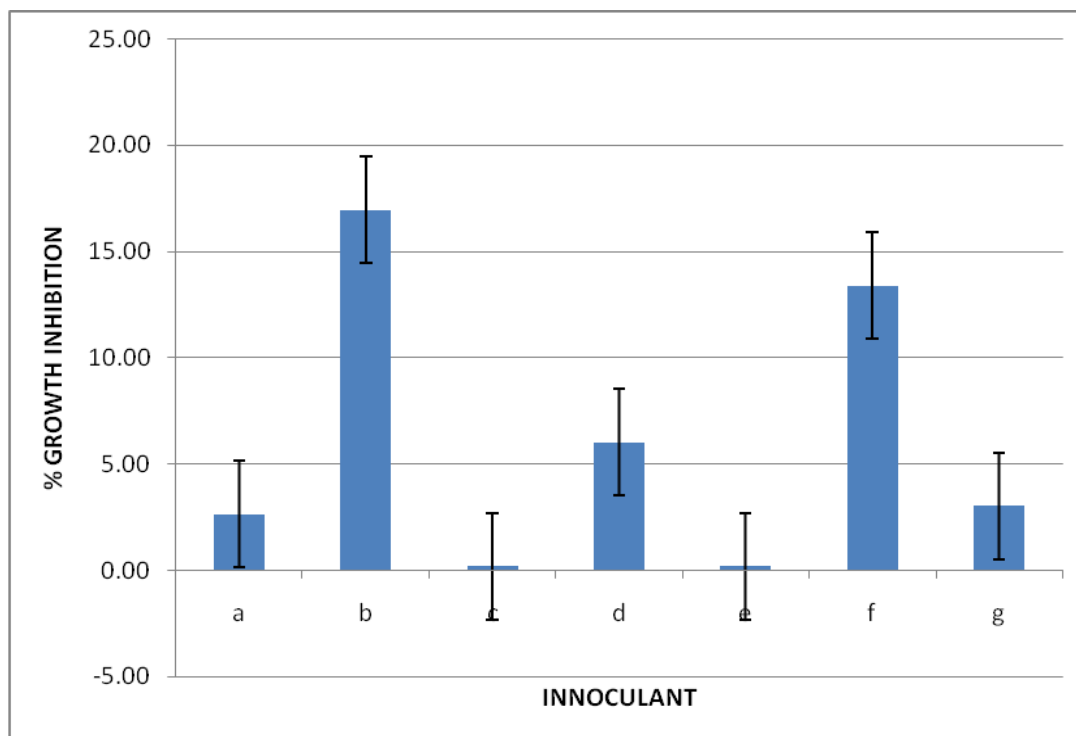
The results of the inhibitory effects of the extracts of *Carissa edulis*, *Maytenus heterophylla*, *Prunus africana* and *Acacia mellifera* at a concentration of 200 µg/ml and Lamivudine (100 µg/ml and 30 µg/ml) as determined using Hepanostika<sup>®</sup> ELISA technique are summarized in Figure 21.

The results were derived from the average of Absorbance values obtained from three replicate determinations. The percentage inhibition on the expression of the HBsAg due to each plant extract was determined using the formula:

$$\left( 1 - \frac{\text{OD (extract)}}{\text{OD (Neg. Cont.)}} \right) \times 100 \%$$

The aqueous extracts of *C. edulis* and *P. africana* and the methanol extract of *A. mellifera* exhibited notable inhibitory effects on the secretion of HBsAg by the SNU-182 cells in comparison to Lamivudine positive controls. The aqueous extract of *C. edulis* had the highest anti - HBV activity with just over 12.15 % inhibition at 200 µg/mL. This inhibition was comparable to the activity of Lamivudine at 100 µg/ml, which was just above 15% activity. The chloroform extract of *P. africana* and the aqueous extract of *M. heterophylla* did not exhibit any significant activity.

## Plant extracts effects on HBsAg expression



**Figure 21: Anti- HBV activity of the selected plant extracts**

**Key:** a = Lamivudine (30 µg/ml); b = Lamivudine (100 µg/ml); c = *P. africana* Chloroform extract (200µg/ml); d = *P. africana* aqueous extract (200 µg/ml); e = *M. heterophylla* (200 µg/ml); f = *C.edulis* aqueous extract (200 µg/ml); g = *A.mellifera* methanol extract (200 µg/ml) :

### Summary:

The aq. Ext. of *C. edulis* and *P.africana* and the methanol ext. of *A.mellifera* inhibited secretion of HBsAg. The aq. Ext. of *C.edulis* had the highest anti-HBV activity almost comparable to Lamivudine +ve control at a concentration of 100 µg/mL. The chloroform ext. of *P. africana* and the aq. ext. of *M. heterophylla* did not exhibit significant activity

### 4.1.2 Discussion

Using the Hepanostika<sup>®</sup> ELISA assay technique for the detection of HBsAg, the study established that the aqueous extracts of *C. edulis* and *P.africana* and the methanol extract of *A.mellifera* exhibited notable inhibitory effects on the secretion of HBsAg by the SNU-182 cells in comparison to Lamivudine positive controls.

Lamivudine at concentrations of 100 µg/ml (0.44 µmol/ml) had significant inhibitory effects on the secretion of the HBsAg of just above 15% while at 30 µg/ml (0.13 µmol/ml) it had a weak inhibitory effect of about 2.15 % relative to negative control. This

was consistent with findings in previous studies done on Hep G2 2.2.15 cells (Ma *et al.*, 2013)

The aqueous extract of *C.edulis* at a concentration of 200 µg/ml had the highest anti-HBV activity with slightly over 12 % growth inhibition relative to the negative control. This inhibition was almost comparable to the inhibition activity of the Lamivudine at 100 µg/ml and indicates the potential of the plant extract as an anti-HBV agent. This also is consistent with findings of previous studies on the antiviral capability of the plant extract (Tolo *et al.*, 2006). The aqueous extract of *P.africana* and the methanol extract of *A.mellifera* also exhibited notable activities of over 5 % and 2 % respectively, which is also consistent with findings in previous studies on their antiviral activity (Rukunga *et al.*, 2000; Tolo *et al.*, 2006; Sidra *et al.*, 2011). These activities were comparable to the activity of Lamivudine at 30 µg/ml.

The chloroform extract of *P. africana* and the aqueous extract of *M. heterophylla* did not exhibit any significant activity. While it would be safe to conclude that the *M. heterophylla* aqueous extract does not have any activity on the secretion of the HBsAg on the SNU -182 cells, it appears that the bio - active compounds within the *P. africana* are hydrophylic. This is because the aqueous extract of *P. africana* showed activity whereas the chloroform extract did not. The screening of the chloroform extract of *P. africana* was advised from its widespread clinical applications, including the treatment of Benign Prostate Hyperplasia (BPH), mainly due to lipophilic phytosterols such as β - Sitosterol, β - Sitosterone and Campesterol (Gathumbi *et al.*, 2002), while the aqueous extracts are commonplace in traditional settings where remedies, mainly based on boiling the extracts in water are used (Kokwaro, 2009).

## **4.2 Determination of the *in vitro* cytotoxicity profiles of the extracts of the selected plants**

### **4.2.1 Results**

The results shown in Figure 22 were obtained by subjecting the SNU 182 cell culture to various concentrations of the extracts of *P. africana*, *C.edulis*, *M. heterophylla* and *A.mellifera* and examining them using the MTT assay described in section 3.7.2 above.

The plant extracts were introduced into the cell cultures at concentrations of 200 µg/mL, 66.67 µg/mL, 22.22 µg/mL, 7.41 µg/mL, 2.47 µg/mL, 0.82 µg/mL and 0.27 µg/mL and Optical Densities (OD) determined at 562 nm.

The average absorbance value from two replicate determinations was used to express the viability of the cells as a percentage of negative controls and the CC<sub>50</sub> for each plant extract determined graphically by plotting the % growth inhibition against the plant extract concentration.

The results showed that individual plant extracts had low toxicity to the cell line with all extracts having CC<sub>50</sub> values above 100 µg/ml. The extracts of *C. edulis* and *M. heterophylla* exhibited the least toxicity with CC<sub>50</sub> values above 200 µg/ml while the extracts of *A. mellifera* and *P. africana* exhibited CC<sub>50</sub> values of 187.5 µg/ml and 195.9 µg/ml respectively.

#### Plant extracts CC<sub>50</sub> results

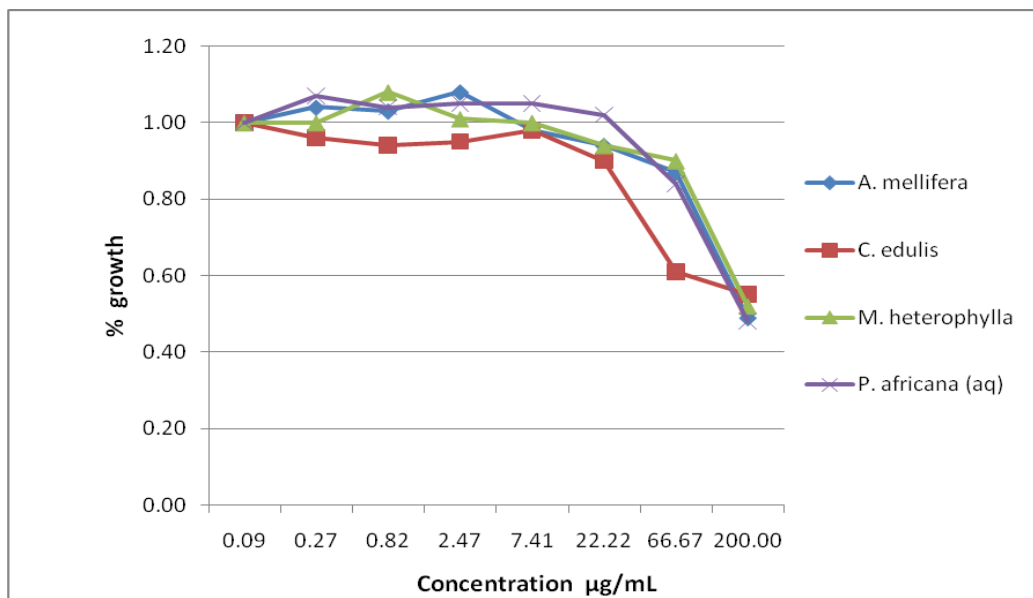


Figure 22: CC<sub>50</sub> graphical presentation

#### Summary:

The inhibition of the growth of the SNU 182 cells by the methanol ext. of *A. mellifera* and the aq. exts. of *C. edulis*, *M. heterophylla* and *P. africana* was dose dependent. By using the formula  $(ET/EC)*100$ , where ET and EC refer to the absorbance values due to the treatment and control groups respectively, the % growth inhibition was determined and plotted against plant ext. conc. to give CC<sub>50</sub> values of 187.5 µg/ml, > 200 µg/ml, >200 µg/ml and 195.9 µg/ml respectively.

## 4.2.2 Discussion

The strong claims of the antiviral capabilities of these extracts from other studies have been based in part on their low toxicity to mammalian cells, with one study in particular, establishing that the  $CC_{50}$  levels of the root barks of the *C.edulis* and stem barks of the *P.africana* on the Vero E6 cells to be greater than 100  $\mu\text{g/ml}$  (Tolo *et al.*, 2006). Such studies have generally regarded plant extract toxicity levels on mammalian cells of greater than 100  $\mu\text{g/ml}$  as generally low (Sidra *et al.*, 2011). The plant extracts examined in this study can therefore be regarded as having low toxicity towards the SNU -182 cells.

The low toxicity could be an indicator of the usefulness of the extracts as medicinal substances especially if their potency against the HBV can be achieved at much lower concentrations.

From the results in section 4.1.1 above, potency against the virus was achieved at concentrations almost or above the  $CC_{50}$ . This could be an indication that although the extracts exhibited activity, they could be too toxic at effective doses to warrant therapeutic use. Further investigations may therefore be necessary to identify and isolate the toxic components.

## 4.3 Determination of the effect of the extracts of the selected plants on the expression of HBV DNA

### 4.3.1 Results

Results shown in Figure 23 were obtained by running a quantitative Real time PCR on HBV DNA extracted from the supernatant of the SNU - 182 cell cultures that had been subjected to Lamivudine and the extracts of *C. edulis*, *M. heterophylla*, *P. africana* and *A. mellifera* at concentrations of 1000  $\mu\text{g/mL}$ , 500  $\mu\text{g/mL}$ , 250  $\mu\text{g/mL}$ , 125  $\mu\text{g/mL}$ , 62.5  $\mu\text{g/mL}$ , 31.25  $\mu\text{g/mL}$  and 15.125  $\mu\text{g/mL}$ .

The average threshold cycle of amplification (Cq) from two replicate determinations was used as the indicator of the levels of HBV DNA, with a lower Cq indicating a higher level of DNA amplicon and therefore a higher level of DNA expression. The absolute Cq value ( $\Delta\text{Cq}$ ) was calculated by correcting the Cq values due to cells treated with plant

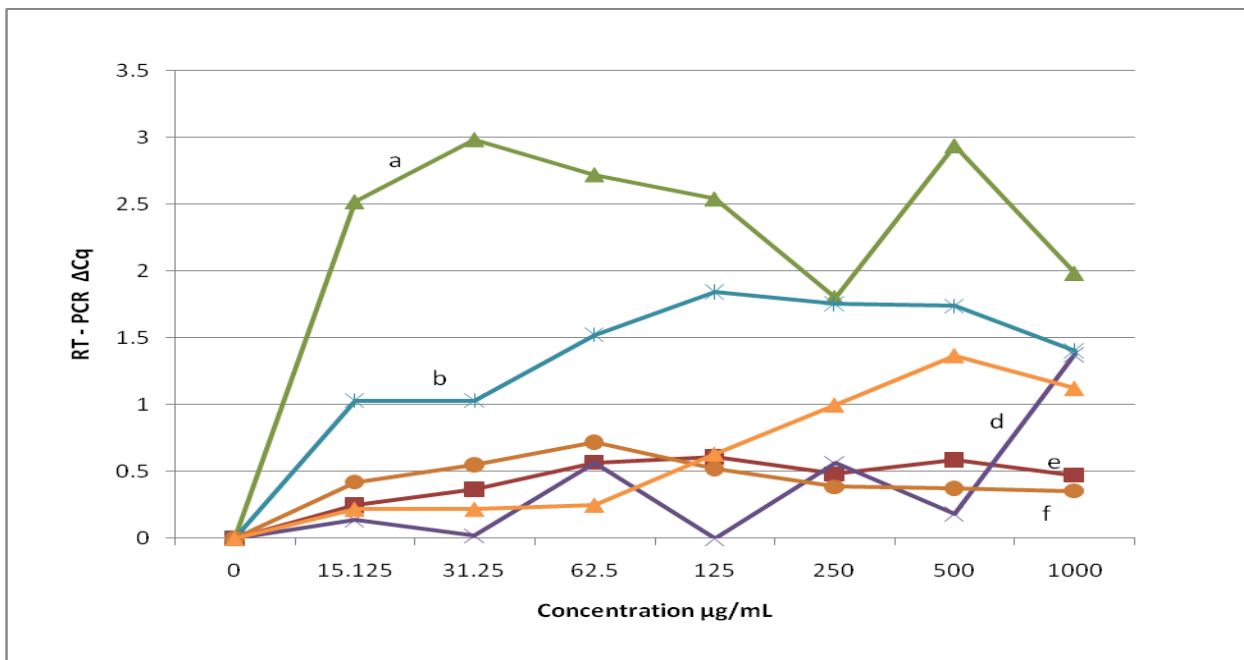


extract with the Cq values due to untreated cells. A graph relating  $\Delta Cq$  and various plant extracts was then plotted (Figure 23).

Lamivudine exhibited a reduction in Cq in a dose dependent manner, demonstrating a reduction in the inhibition of the expression of HBV DNA with increasing concentration. The aqueous extract of *C.edulis* and the methanol extract of *A. mellifera* exhibited a significant drop in the Cq at plant concentrations below 31.25  $\mu\text{g/mL}$  and 125  $\mu\text{g/mL}$  respectively indicating some level of anti-HBV activity at concentrations above these.

The aqueous and chloroform extracts of *P.africana* exhibited slight anti - HBV activity at concentrations above 62.5  $\mu\text{g/mL}$  and 125  $\mu\text{g/mL}$  respectively. The *M. heterophylla* extract did not demonstrate a meaningful drop in the Cq at the tested concentrations and could therefore indicate minimal or no anti – HBV DNA activity. Suffice to say however that the sensitivity of the real time PCR technique is highly dependent on the efficiencies of nucleic acid extractions, which is easily influenced by human and experimental errors. It is also susceptible to a number of organic and phenolic inhibitors in biological samples rendering the generation of results and the absolute gene count meaningful for the individual study in question (Martin-Laurent *et al.*, 2001). It therefore becomes difficult to replicate studies and sustain a meaningful trend, which could explain some of the erratic results observed in this study.

## Plant extracts real time PCR results



**Figure 23:** Plot of  $\Delta Cq$  against plant extracts concentration.

**Key:** a = *Carissa edulis* (Aqueous extract): b = *Acacia mellifera* (Methanol extract): c = Lamivudine: d = *Maytenus heterophylla* (Aqueous extract): e = *Prunus africana* (Chloroform extract): f = *Prunus africana* (Aqueous extract):

### Summary:

The aq. ext. of *C.edulis* and the methanol ext. of *A.mellifera* exhibited an increase in the levels of DNA expressed at concentrations below 31.25  $\mu\text{g/mL}$  and 125  $\mu\text{g/mL}$  respectively, while the aq and chloroform exts. *P.africana* exhibit a slight increase in the the levels of DNA expressed at concs below 125  $\mu\text{g/mL}$  and 62.5  $\mu\text{g/mL}$  respectively. The ext. of *M. heterophylla* did not exhibit any significant drop in the Cq at any of the test concentrations.

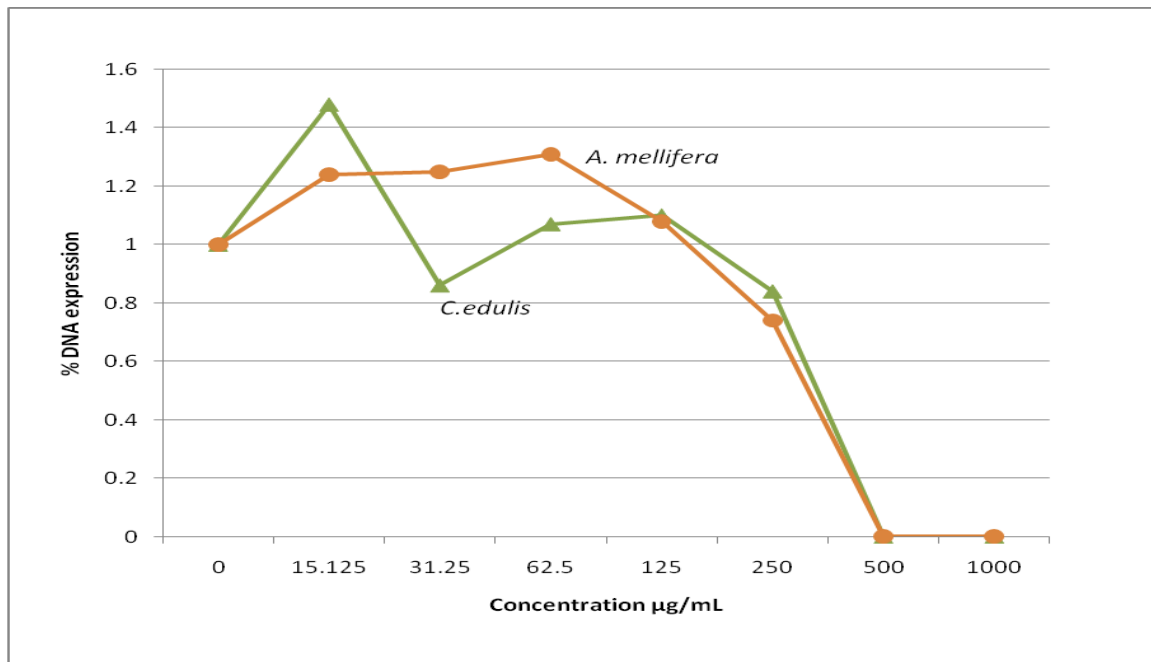
### 4.3.2 Effective Concentration ( $EC_{50}$ ) of *C.edulis* and *A. mellifera* extracts

The Effective Concentration inhibiting the expression of HBV DNA by 50% ( $EC_{50}$ ) was determined for the aqueuos extract of *C. edulis* and the methanol extract of *A. mellifera* as they had exhibited notable anti-HBV activity. This was done by calculating the percentage inhibition of DNA expression by the plant extracts at various concentrations using the formula:

$$\% \text{ inhibition} = \frac{(\text{ET})}{(\text{EC})} \times 100\%,$$

where ET and EC refer to the inhibition due to the treatment and control groups respectively. A plot of the % DNA expression against the plant concentrations was made and the EC<sub>50</sub> derived.

From the results the methanol extract of *A. mellifera* exhibited a slightly better EC<sub>50</sub> of 295.0 µg/mL as compared to 331.6 µg/mL of the aqueous extract of *C.edulis* (Figure 24 and Table 3).



**Figure 24: EC<sub>50</sub> graphical presentation**

**Summary:**

The EC<sub>50</sub> of *A.mellifera* was 295.0 µg/ml while that of *C.edulis* was 331.6 µg/ml.

**4.3.3 Discussion**

When the activity of the extracts of *C.edulis*, *M.heterophylla*, *A.mellifera* and *P.africana* were evaluated for their effects on the expression of DNA using the Molecular (PCR) technique, the extracts of *C.edulis* and *A.mellifera* still exhibited notable activity while both the aqueous and chloroform extracts of *Prunus africana* exhibited slight activity. These results that are based on a different technology platform support the findings of the previous experiment carried out using ELISA and confirms the anti-HBV activities of the plant extracts.

The *M.heterophylla* aqueous extract exhibited no anti-HBV activity *in vitro* using both techniques. It can therefore be concluded that the aqueous extract of the stem bark of *M.heterophylla* does not have any anti-HBV activity *in vitro*. Previous studies have demonstrated the anti - Cytomegalovirus activity of various quinone isolates from the plant, including Pristimerin and the Pristimerin analogue Lupeol (Murayama *et al*, 2007). Some of the active principles may not have been present in the aqueous fraction. *In vivo* tests have also demonstrated the anti-inflammatory activity of the methanolic extracts of the plant extracts (Da silva *et al.*, 2010). Such tests, by use of specific models such as the Duck Hepatitis B Virus (Guha *et al.*, 2004) may therefore be necessary to completely rule out any anti HBV activity due to the plant extract.

<b>Plant extract</b>	<b>CC<sub>50</sub></b>	<b>EC<sub>50</sub></b>
<i>C.edulis</i>	> 200 µg/mL	331.6 µg/mL
<i>A.mellifera</i>	187.5 µg/mL	295.0 µg/mL

**Table 3: Comparison of CC<sub>50</sub> and EC<sub>50</sub> for two plant extracts**

The EC<sub>50</sub> values determined were considerably higher than the CC<sub>50</sub> values obtained in the cytotoxicity experiments above (Table 3). This implies that the plant extracts at potent concentrations against the HBV would be toxic to the cells and may therefore not be useful for the treatment of HBV infections. There may be need for further investigations to possibly identify and isolate toxic components within the extracts and purify the bio-actives.

#### 4.4 General discussion

Although several studies have been conducted locally to establish the antiviral activity of local plant species (Rukunga et al., 2002; Tolo *et al.*, 2006), there are no reports on the anti-HBV activity of these species. Four of these plants, exhibiting significant antiviral activity from previous studies; *Carissa edulis* (Forssk.) Vahl, *Maytenus heterophylla* (Eckl. & Zeyh.) Robson, *Prunus africana* (Hook.f.) Kalkman and *Acacia mellifera* (Vahl) Benth, were chosen based on their ethno - pharmacological use and formed the basis of this study. The study utilized both the ELISA and the quantitative real time PCR technology platforms on each of the extracts thereby establishing the presence of anti-HBV activity in the extracts of *C. edulis*, *A. mellifera* and *P.africana*. The semi – quantitative nature of the diagnostic kit used in the ELISA experiment made it necessary to carry out real time PCR follow up studies to confirm the findings. The real time PCR technique is generally regarded as the gold standard in HBV quantification studies since it can quantify the nucleic materials at much lower levels than the former. It is therefore the preferred technique, although other considerations including cost come into play. For this particular exercise, given the limited sample size that could be run through the real time PCR technique, an initial screening using the ELISA technique was considered necessary.

The aqueous extract of *C.edulis* and the methanol extract of *A.mellifera* specifically exhibited notable anti - HBV activity using both platforms relative to controls. They consistently sustained suppression of the HBV DNA over a wide range of plant concentrations, manifested by the change of Cq at concentrations above 62.5 µg/mL and 125 µg/mL respectively. The ELISA tests conducted also revealed activities comparable to Lamivudine 100µg/ml and 30µg/ml of over 12 % and 2 % respectively for the two plant extracts. This could indicate the potential of these extracts as a source of hit compounds as have several other plants used in traditional remedies, including *Phyllanthus urinaria* L., *Salvia miltiorrhiza* Bge., *Rheum palmatum* L., *Astragalus membranaceus* (Fisch.) Bge., and their related compounds: Oxymatrine, Artemisinin, Artesunate and Wogonin. (Cui *et al.*, 2010). However, to date, no plant extract or compound has been reported to have been successfully developed into a commercial

drug. This could partly be explained by the cytotoxicity exhibited by such extracts at potent concentrations as has been demonstrated in this study where both the *C.edulis* and *A.mellifera* extracts had therapeutic indices of less than one. Further investigations may be necessary to identify and isolate the toxic components in order to improve the bioactivity of these particular plant extracts.

The activities of *C. edulis*, *A.mellifera* and *P.africana* on HIV, HSV and CMV have been well established in previous studies (Rukunga *et al.*, 2002, Tolo *et al.*, 2006). This study is therefore bound to increase this list and can be regarded as additional evidence of the significant antiviral capabilities inherent in these plant species. The plants all belong to different families and may indicate the reasoning behind the use of many different plants by herbalists in traditional practice. It has therefore set a good platform from which further investigations could be carried out, possibly leading to a commercialised product.

The study was not however without several limitations, including inconsistencies in the results of replicate tests, which could probably be due to experimental or human errors. The quantitative Real time PCR technique is especially sensitive to slight changes in the biological sample being used as well as to the method of extraction of nucleic acid material. The presence of PCR inhibitors environmental samples is also well established (Stults *et al.*, 2001). For this reason, replicate determinations using the biological systems proved challenging and considering the high costs of the reagents and kits used in the experiment it became difficult to scale up the sample size as well as the number of determinations which could be necessary in subsequent investigations to better establish the results. This could also explain the inconsistencies in some of the curves derived in the real time PCR experiment (Figure 23).

## 5.0 CONCLUSIONS AND RECOMMENDATIONS

This study has established that the aqueous extract of *C. edulis* and the methanol extract of *A.mellifera* have notable anti - HBV activity while the extracts of *P. africana* have minimal anti - HBV activity. It has also established that the aqueous extract of *M. heterophylla* has no activity against the HBV. The study however determined that the aqueous extract of *C. edulis* and the methanol extract of *A.mellifera* have high EC<sub>50</sub> values that makes them unsuitable for use in the treatment of HBV infections in their crude form.

The roles of these plant extracts in the treatment of HBV infection could be further investigated as suggested below:

- 1) Isolation and identification of the bio-active compounds present in the two plant extracts exhibiting activity through bio-assay guided fractionation and structure elucidation techniques, which could lead to the identification of a new range of compounds for the management of HBV infections.
- 2) Isolation and identification of toxic components within the two plant extracts exhibiting activity, which could significantly impact on the therapeutic index of the extracts.
- 3) *In vivo* evaluation of the anti – HBV action of the plant extracts or isolated compounds to establish if the plants can suppress an already established HBV infection.
- 4) Determination of the mechanisms of action of the active components of the two plant extracts
- 5) Investigations on the possible synergistic effects of the plant extracts or isolated compounds with standard anti-viral compounds such as Lamivudine.
- 6) Screening of the plant extracts on local HBV strains to determine their efficacy locally.

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## 7.0 APPENDICES

### Appendix 1: Extraction yield from the various plant extracts

Plant extract	Weight of powder after extraction	% Yield
<i>Carissa edulis</i> (aq)	7.07 g	8.84%
<i>Maytenus heterophylla</i> (aq)	5.67 g	5.67%
<i>Prunus africana</i> (aq)	12.654 g	12.654%
<i>Prunus africana</i> (Chloroform)	11.7g	1.157%
<i>Acacia mellifera</i> (Methanol)	23g	23%

### Appendix 2: The plant extracts



Aqueous extract of *Carissa edulis*



Aqueous extract of *Maytenus heterophylla*



Aqueous extract of *Prunus africana*







Chloroform extract of *Prunus africana*



Methanol extract of *Acacia mellifera*




### Appendix 3: Equipment used in the experiment

A: Some equipment used in the extraction of the plant powders

			
Water bath	Modulyo Freeze drier	Heidolph Rotar vapour	Soxhlet Extractor



B: Some equipment used in the extraction and amplification of the HBV DNA

		
<p><b>Phosphate buffer washer</b></p>	<p><b>Roche Real Time PCR (light cycler) Machine</b></p>	<p><b>Eppendorf Centrifuge</b></p>

C: Other equipment used in the experiment

	<b>Equipment</b>	<b>Model</b>
1	Bio Safety Cabinet II	Airtech, Japan
2	Autoclave machine	Panasonic, Japan
3	Humidified incubator	Sanyo, Japan
4	Liquid nitrogen tank	MVE series 2001, USA
5	Vortex mixer	Stuart, Germany
6	Heat block	Digisystem, Japan
7	Inverted microscope	Nikon TS 100, Japan
8	ELISA reader	Thermoscientific Multiskan EX, Japan
9	Analytical balance	Sartorius, Germany
10	Refrigerator (4°C)	Sanyo, Japan
11	Refrigerator (- 30°C)	Sanyo, Japan

## Appendix 4: SNU - 182 cell line (ATCC® CRL-2235™) ([http://www. ATCC.org](http://www.ATCC.org))

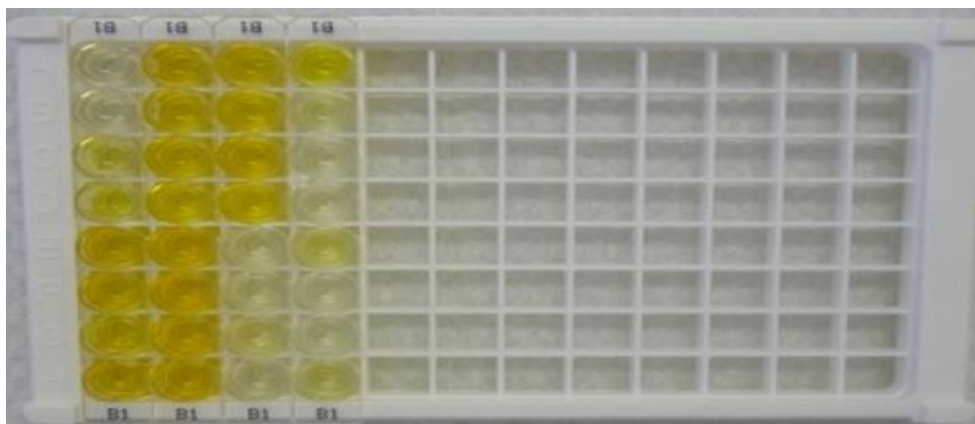
### General Information

<b>Organism</b>	<i>Homo sapiens</i> , human
<b>Tissue</b>	liver
<b>Product Format</b>	frozen
<b>Morphology</b>	epithelial
<b>Culture Properties</b>	adherent
<b>Biosafety Level</b>	2 [Cells contain Hepatitis B virus]
<b>Disease</b>	grade III/IV, hepatocellular carcinoma
<b>Age</b>	24 years
<b>Gender</b>	male
<b>Ethnicity</b>	Asian

### Characteristics

<b>Karyotype</b>	aneuploid; modal number = 70
<b>Derivation</b>	SNU-182 was derived in 1989 by J.-G. Park and associates from a primary hepatocellular carcinoma taken from a Korean patient prior to cytotoxic therapy. 24 years
<b>Clinical Data</b>	Asian male
<b>Antigen Expression</b>	Blood Type O; Rh +  Tumor cells were initially cultured in ACL-4 medium supplemented with 5% heat inactivated fetal bovine serum. After establishment, cultures were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum.
<b>Comments</b>	Grossly, the original tumor was single nodular. Histologically, it was predominantly trabecular and minor acinar type.  The cultured cells contain a single nucleus.  Hepatitis B virus (HBV) DNA was detected by Southern blot hybridization.  HBV genomic RNA was not expressed.

**Appendix 5:** Micro ELISA plate Layout and depiction of serial dilution of HBV<sup>+</sup> human sera



**Appendix 6:** Screening of plants for anti – HBV activity using the Hepanostika<sup>®</sup> kit:  
Absorbance readings (Raw data)

ATTEMPT	N.C	3 TC (30µg/ml)	3 TC (100µg/ml)	<i>P.africana</i> (CHCl <sub>3</sub> ) (200µg/ml)	<i>P.africana</i> (aq) (200µg/ml)	<i>Maytenus heterophylla</i> (200µg/ml)	<i>C.edulis</i> (200µg/ml)	<i>A.mellifera</i> ( 200µg/ml)
1	0.187	0.182	0.153	0.186	0.164	0.186	0.150	0.157
2	0.185	0.176	0.148	0.194	0.160	0.185	0.152	0.209
3	0.158	0.158	0.139	0.149	0.174	0.158	0.157	0.148
AVG	0.177	0.172	0.146	0.176	0.166	0.176	0.153	0.171

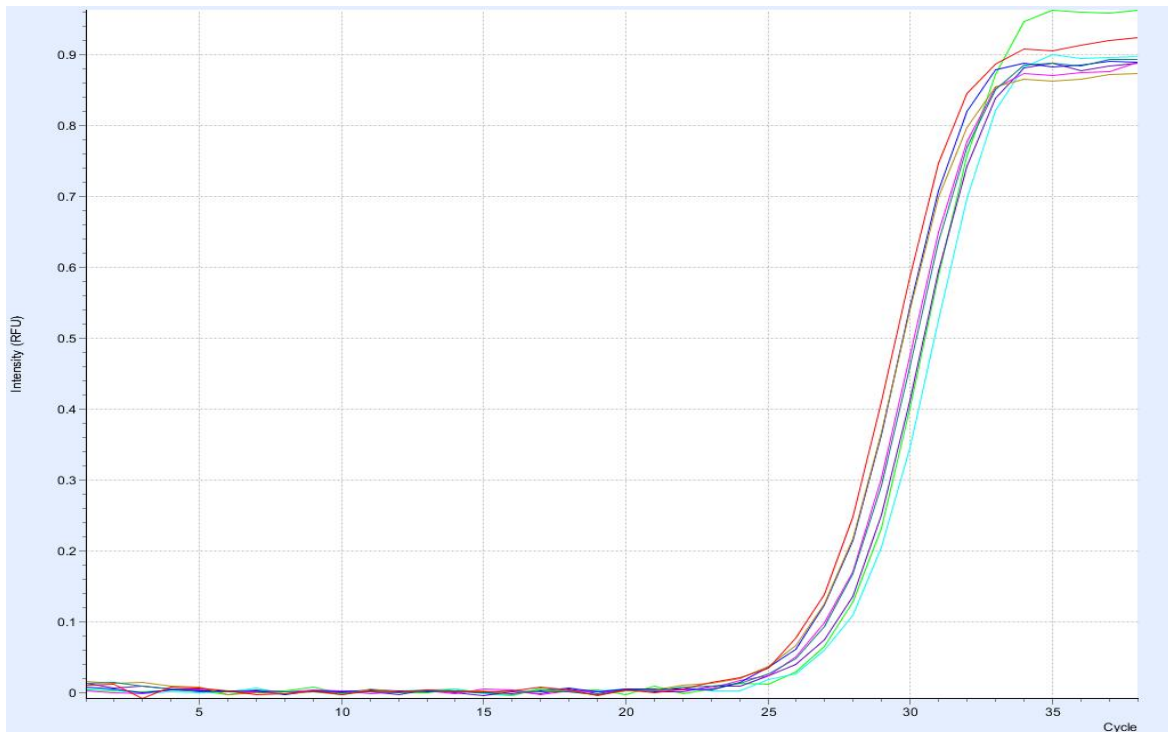
**Appendix 7:** Cytotoxicity experiments: Absorbance readings (Raw data)

	<i>P.africana</i> (aq)			<i>C.edulis</i>			<i>M.heterophylla</i>			<i>A.mellifera</i>		
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.73	0.70	<b>0.07</b>	0.76	0.79	<b>0.08</b>	0.72	0.76	<b>0.07</b>	0.79	0.66	<b>0.07</b>
B	0.73	0.78	<b>0.08</b>	0.75	0.74	<b>0.08</b>	0.73	0.77	<b>0.07</b>	0.76	0.78	<b>0.07</b>
C	0.73	0.74	<b>0.07</b>	0.75	0.73	<b>0.08</b>	0.81	0.78	<b>0.07</b>	0.74	0.76	<b>0.07</b>
D	0.78	0.77	<b>0.08</b>	0.78	0.78	<b>0.12</b>	0.77	0.77	<b>0.08</b>	0.75	0.77	<b>0.07</b>
E	0.73	0.70	<b>0.08</b>	0.79	0.74	<b>0.08</b>	0.76	0.74	<b>0.07</b>	0.76	0.77	<b>0.08</b>
F	0.74	0.61	<b>0.07</b>	0.72	0.69	<b>0.08</b>	0.67	0.76	<b>0.07</b>	0.73	0.74	<b>0.07</b>
G	0.65	0.60	<b>0.07</b>	0.53	0.47	<b>0.07</b>	0.72	0.69	<b>0.09</b>	0.61	0.61	<b>0.06</b>
H	0.40	0.39	<b>0.08</b>	0.46	0.48	<b>0.08</b>	0.40	0.45	<b>0.08</b>	0.38	0.37	<b>0.06</b>

## Appendix 8: Real Time PCR (Light Cycler) Raw data

### a) *Lamivudine*

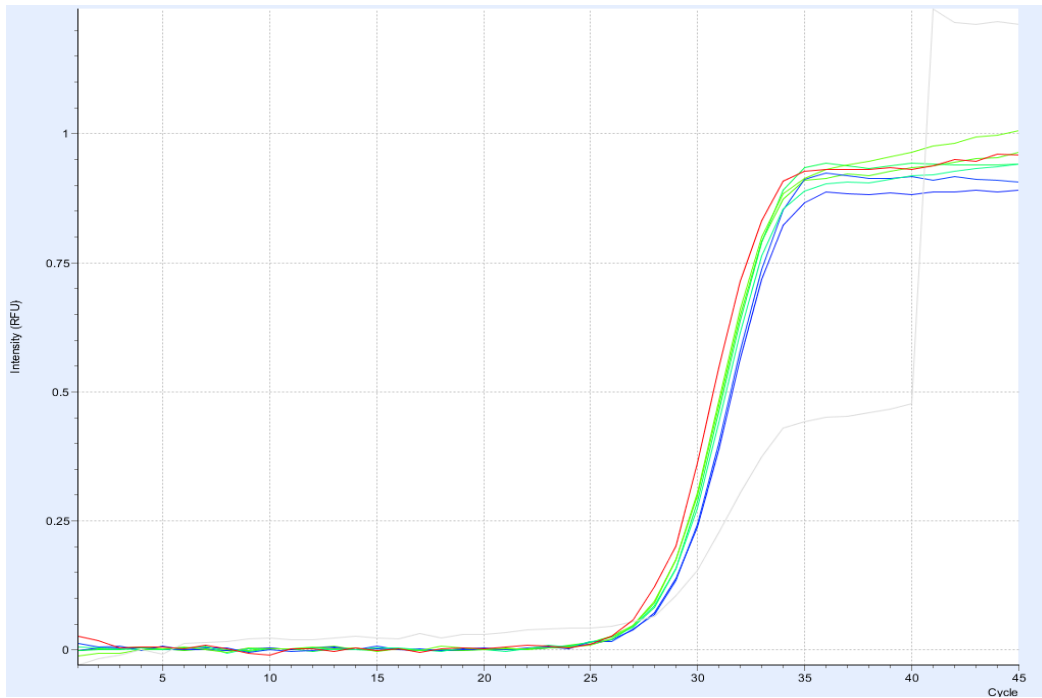
Concentration ( $\mu\text{g/mL}$ )	Cq
1000	26.83
500	27.075
250	26.706
125	26.339
62.5	25.958
31.25	25.927
15.125	26.399
0	25.708



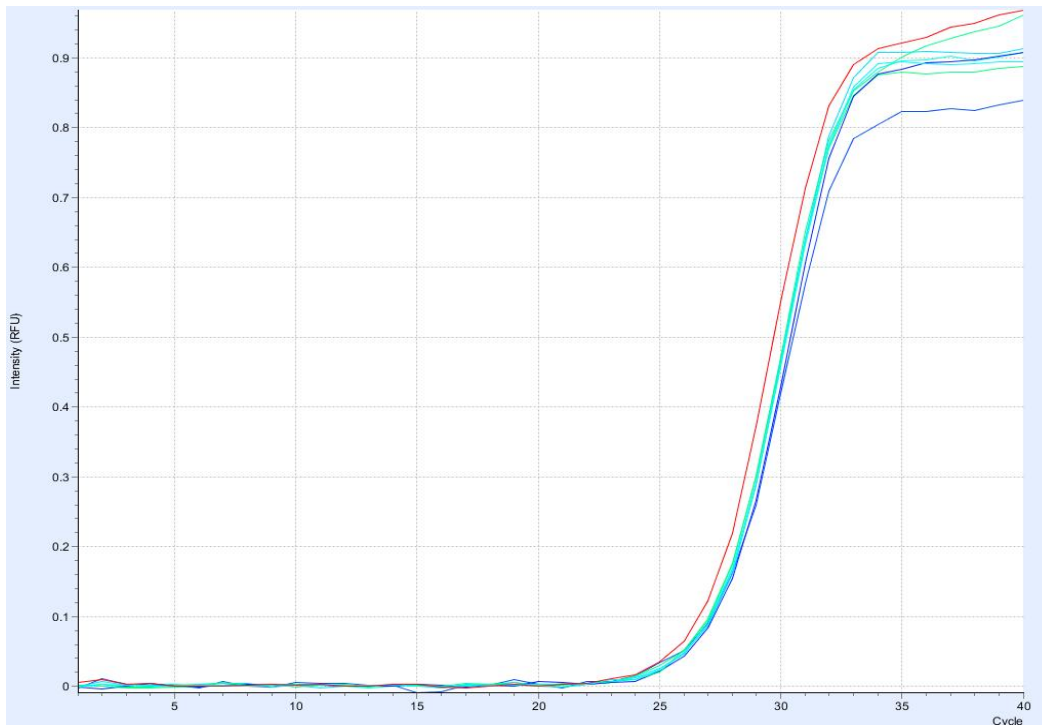
***b) Prunus africana* (aqueous extract)**

Concentration ( $\mu\text{g/mL}$ )	Cq Trial 1	Cq Trial 2	Avg
1000	27.354	26.533	26.9435
500	27.781	26.329	27.055
250	27.467	26.436	26.9515
125	27.757	26.404	27.0805
62.5	27.512	26.565	27.0385
31.25	27.334	26.343	26.8385
15.125	27.016	26.42	26.718
0	27.016	25.921	26.4685

### Trial 1



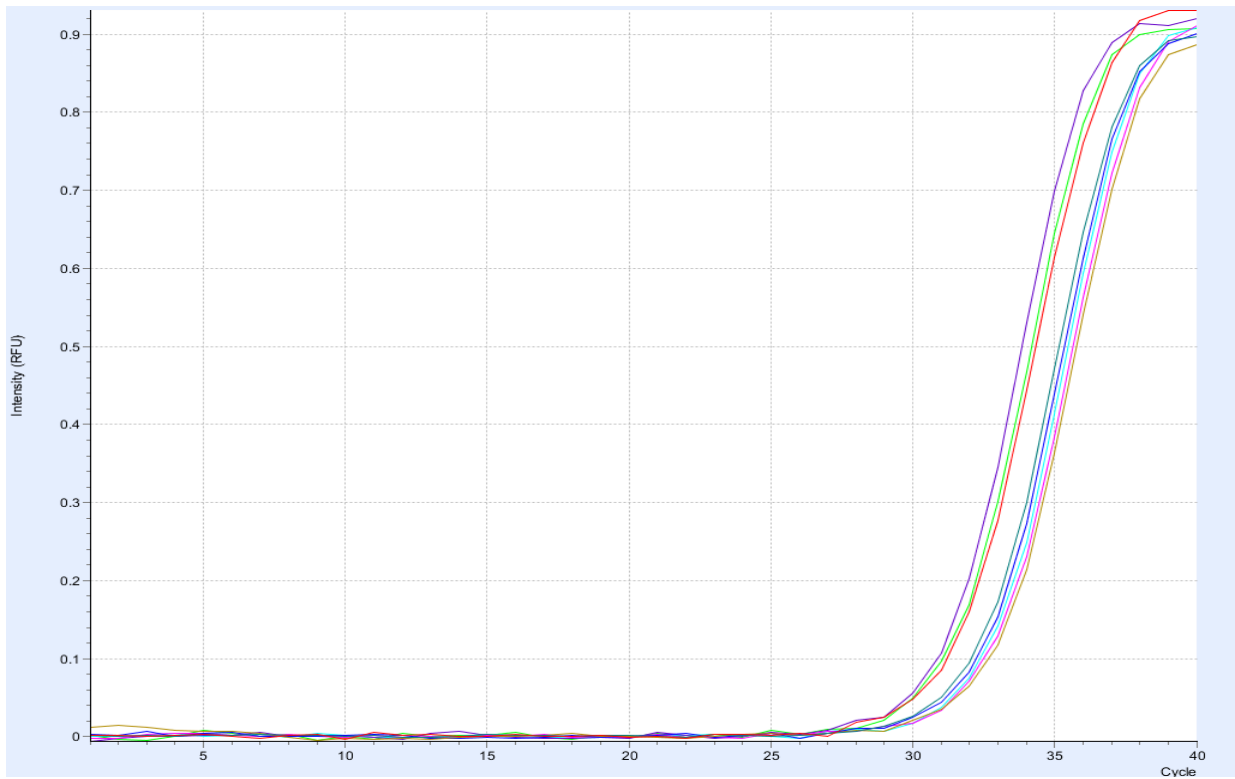
### Trial 2



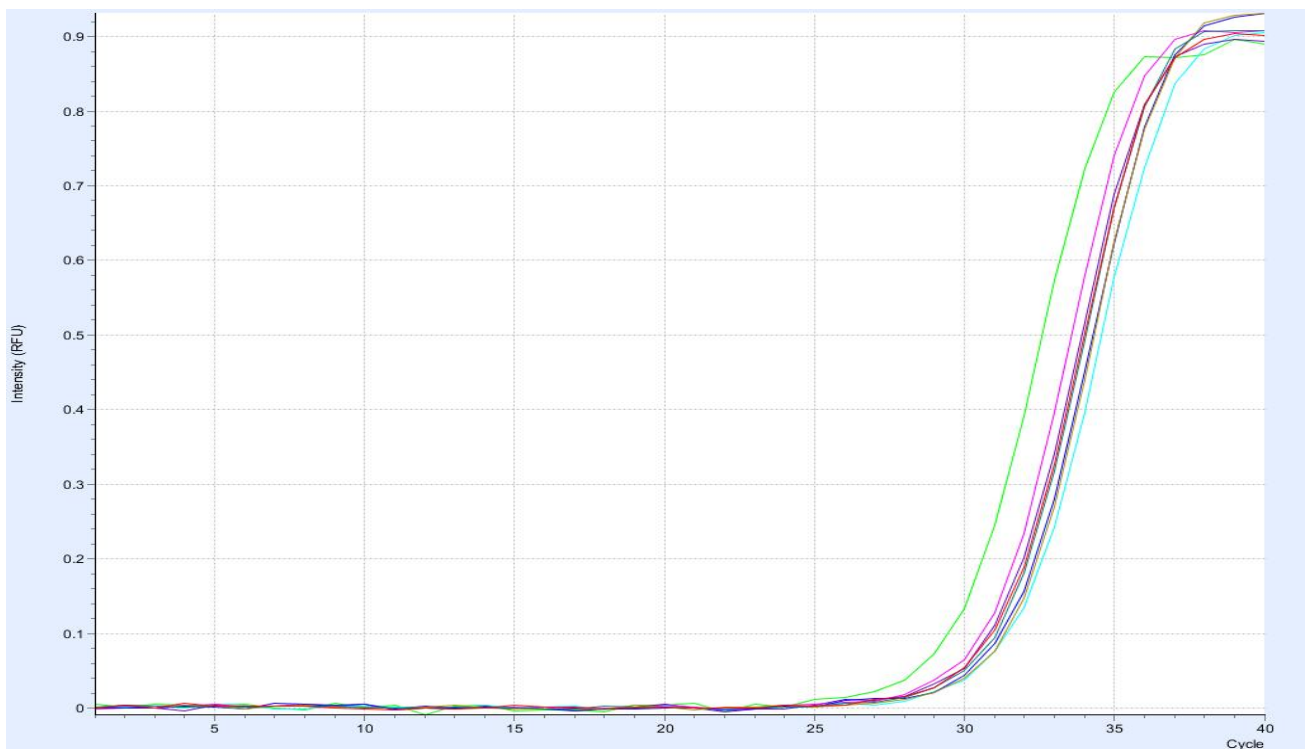
**c) *Carissa edulis* (aqueous extract)**

Concentration ( $\mu\text{g/mL}$ )	Cq Trial 1	Cq Trial 2	Avg
1000	30.186	30.354	30.27
500	30.75	31.696	31.223
250	30.093	30.086	30.0895
125	29.815	31.837	30.826
62.5	30.483	31.531	31.007
31.25	30.579	31.961	31.27
15.125	30.265	31.345	30.805
0	28.762	27.811	28.2865

***Trial 1***



***Trial 2***

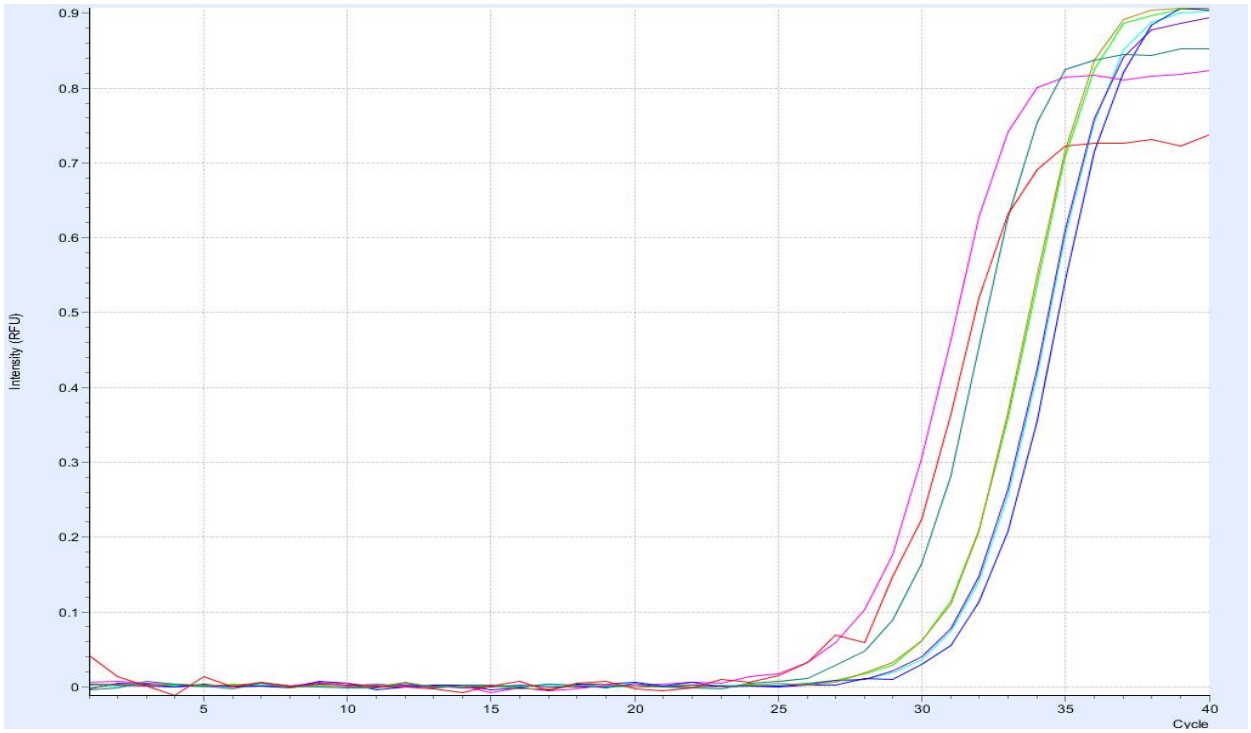




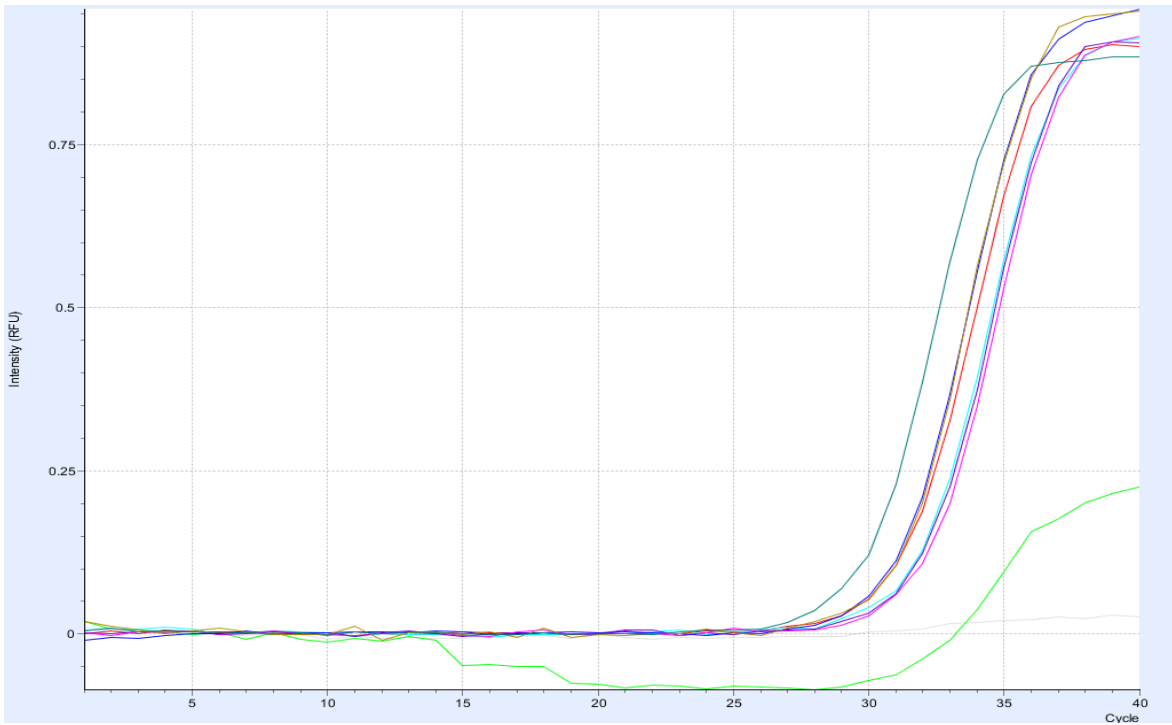
***d) Acacia mellifera (Methanol extract)***

Concentration ( $\mu\text{g/mL}$ )	Cq Trial 1	Cq Trial 2	Avg
1000	30.799	30.006	30.4025
500	30.799	30.675	30.737
250	30.904	30.606	30.755
125	31.081	30.606	30.8435
62.5	30.006	31.037	30.5215
31.25	30.063	29.999	30.031
15.125	28.86	28.436	28.648
0	30.186	27.811	28.9985

***Trial 1***



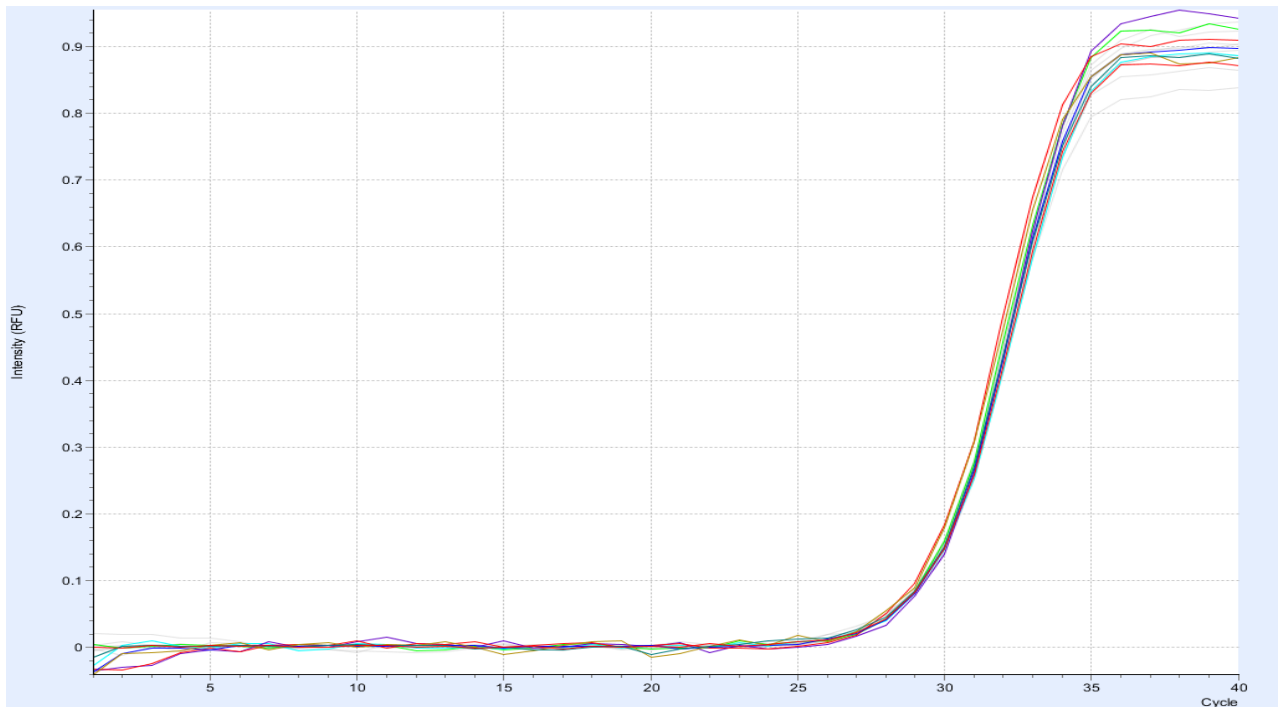
***Trial 2***



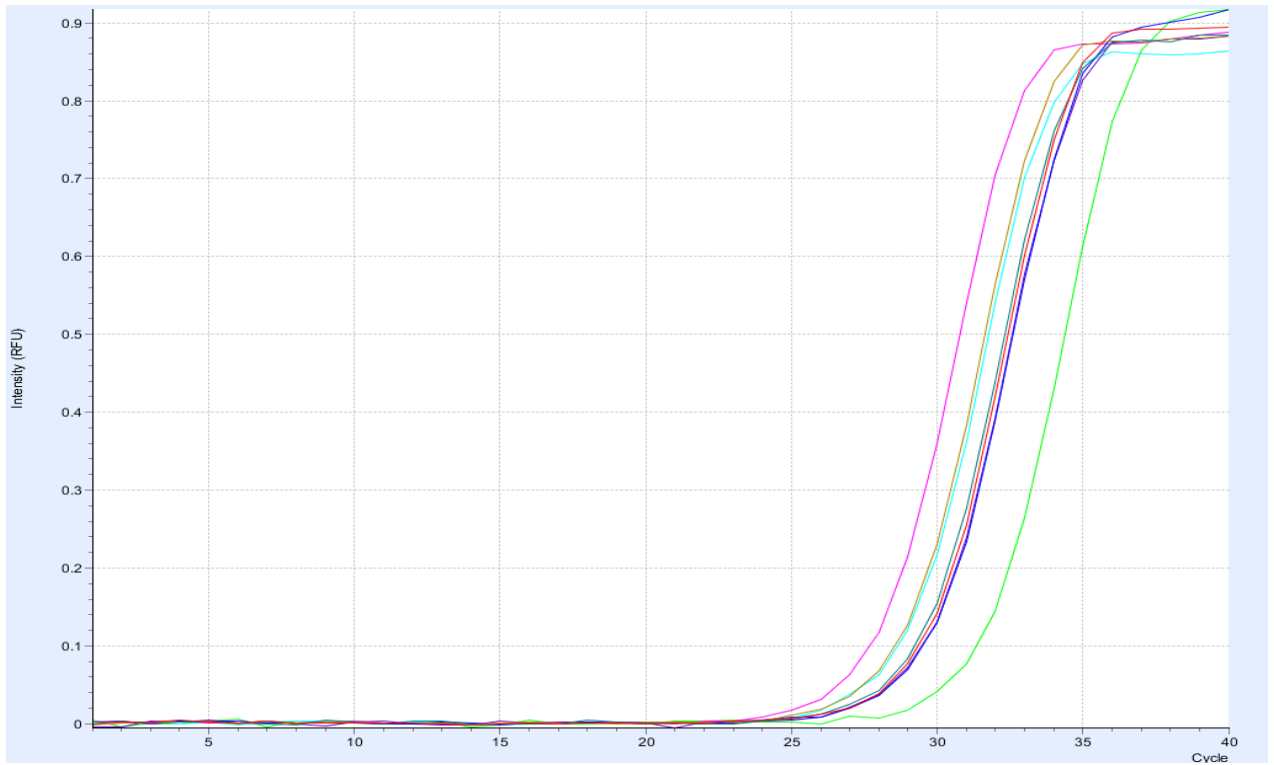
***e) Maytenus heterophylla* (aqueous extract)**

Concentration ( $\mu\text{g/mL}$ )	Cq Trial 1	Cq Trial 2	Avg
1000	26.342	30.612	28.477
500	26.613	27.957	27.285
250	26.541	28.785	27.663
125	26.337	26.982	26.6595
62.5	26.51	28.812	27.661
31.25	26.392	27.847	27.1195
15.125	25.949	28.522	27.2355
0	26.385	27.811	27.098

### Trial 1



### Trial 2



**a) *Prunus africana* (Chloroform extract)**

Concentration ( $\mu\text{g/mL}$ )	Cq Trial 1	Cq Trial 2	Avg
1000	30.027	26.89	28.4585
500	30.265	26.69	28.4775
250	29.815	27.163	28.489
125	30.214	27.037	28.6255
62.5	30.271	27.376	28.8235
31.25	30.016	27.29	28.653
15.125	29.86	27.191	28.5255
0	29.846	26.359	28.1025

