UNIVERSITY OF CAPE COAST

ANTI-HEPATOCELLULAR CARCINOMA EFFECT OF AN ALKALOIDAL EXTRACT DERIVED FROM ZANTHOXYLUM ZANTHOXYLOIDES

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BY

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A thesis submitted to the Department of Biomedical Sciences of the School of Allied Health Sciences, College of Health and Allied Sciences, the University of Cape Coast in partial fulfillment of the requirements for the award of Master of Philosophy degree in Drug Discovery and Toxicology

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DECLARATION

Candidate's declaration

I hereby declare that this thesis is the result of my original research and that no part of it has been presented for another degree in this university or elsewhere.

Candidate's Signature…….. Date…….

Name: …….

Supervisor's declaration

We hereby declare that the preparation and presentation of the thesis were supervised in accordance with the guidelines on the supervision of the thesis laid down by the University of Cape Coast.

Principal Supervisor's Signature …… Date ……

Name: ……

Co-supervisor's Signature……. Date ……...

Name: …….

ABSTRACT

Background: The global burden of Hepatocellular Carcinoma (HCC) has increased. Consequently, exploring newer treatment modalities for HCC such as herbal medicines has become a necessary. **Objective:** The study investigated anti-HCC effects of an alkaloidal extract derived from leaves of *Zanthoxylum zanthoxyloides*. **Materials and methods:** Alkaloidal extract (ZZAE) from cleaned dried leaves of *Zanthoxylum zanthoxyloides* was prepared using Soxhlet extraction and liquid-liquid extraction methods and then phytochemically analyzed. CCl_4/olive oil HCC was induced in rats and subjects grouped into 6 groups 10 after the HCC was established, except for the control group with no HCC and the prophylaxis group with concurrent induction and treatment. Anti-HCC effects of ZZAE, was assessed in CCl_4/olive oil-induced HCC of rats by prophylactic treatment with ZZAE (100 mg/kg *po*) and curatively with ZZAE (50, 100, and 200 mg/kg *po*) daily for nine weeks. **Results:** Soxhlet extraction yielded 462.806 g (10.59%) of crude extract that contained terpenoids, alkaloids, saponins, tannins, flavonoids, carbohydrates, and phenol. Liquid-liquid extraction for alkaloids yielded 119.349 g (2.73%). ZZAE produced a concentration-dependent inhibition of growth of *A. cepa* root tip meristems. ZZAE also improved all liver enzyme indices and liver histology in a dose-dependent manner. ZZAE treatment restored loss in bodyweight relative to the model. ZZAE prevented progression of fibrosis into HCC. **Conclusion:** ZZAE demonstrated anti-HCC effects that needs further studies.

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DEDICATION

I dedicate this thesis to my mother, Madam Elizabeth Nkrumah.

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LIST OF ABBREVIATIONS

ALP; Alkaline Phosphatase

ALT: Alanine Transaminase

AST: Aspartate Transaminase

CCL_3: Trichloromethyl Radical

CCL_4: Carbon Tetrachloride

CSC's: Cancer Stem Cells

CYP450: Cytochrome P450

ERK: Extracellular Signal-regulatory Kinase

GGT: Gamma-glutamyl Transferase

HCC: Hepatocellular Carcinoma

HR: Hazard Ratio

5-HT: 5-hydroxytriptamin

IL-10: Interleukin-10

IL-6: Interleukin-6

IL-8: Interleukin-8

MAPK: Mitogen Activated Protein Kinase

MEK: MAPK/ERK Kinase

NF-κB: Nuclear-factor KapaB

$OOCCL_3$: Trichloromethyl Peroxyradical

PAF: Platelet Aggregation Factor

Ras: it is a single-subunit small GTPase, which is related in structure to the G_α subunit of heterotrimeric G proteins

TNF-α: Tumor Necrotic Factor-alpha

VEGF: Vascular Endothelial Growth Factor

VEGFR: Vascular Endothelial Growth Factor Receptor

WBC: White Blood Cell

ZZAE: *Zanthoxylum zanthoxyloides* Crude-alkaloidal Extract

ZZE: *Zanthoxylum zanthoxyloides* Crude-extract

RBC: Red Blood Cell

TLC: Thin Layer Chromatography

CHAPTER ONE

INTRODUCTION

Chapter one captures the introduction and background of the global burden of hepatocellular carcinoma (HCC), its geographical distribution disparities and risk factors. It further introduces the need to look fr cheaper and better treatmrnt strategies for HCC. The problem statement gives an insight into how poor prognosis of HCC contributes to its high mortality rate. The problem statement further indicates the lack of safer and more effective treatment options for HCC. The justification section under this chapter elucidates why developing countries are more at risk of HCC.

Background

Cancer is the abnormal and unrestrained growth of cells (Haugen, 2017) and is the leading cause of death among persons aged 70 years and below (Bray *et al.*, 2018). There was 18.7 million incidence of cancer with 9.6 million deaths globally in 2018 (Torre *et al.*, 2015). HCC is endemic in populations where its risk factors are high such as in Africa and Asia (McGlynn & London, 2011).

HCC is the third leading cause of death in all cancer-related mortalities globally, with about 750,000 incidences, and 700,000 deaths reported annually (Torre *et al.*, 2015). Strikingly, 80% of the incidence and deaths occur in Asia Pacific, Sub-Saharan Africa, and North America (Rawat *et al.*, 2018). In spite of this, sorafenib is the only drug approved for the management of HCC.

HCC is commonly diagnosed at its late stage, during which treatment options are limited, with poor prognosis (Ladep *et al.*, 2014). The delayed detection and poor prognosis is partly due to ability of the liver to function with remaining 40% of its mass (Ladep *et al.*, 2014). Limited treatment modalities for HCC coupled with serious adverse effects associated with chemotherapies, it has become necessary to search for safe alternative treatments particularly herbal medicine. The use of plants as medicament dates back to about the past 60,000 years but record on such medicinal application dates a few decades back (Solecki, 1975). About 80% of the world's population use traditional medicine to meet their primary healthcare needs (Martin, 2010). Reliance on natural products as the primary source of treatment is based on the assumption that they are less harmful.(Ekor, 2014).

Currently, natural therapeutic agents are made available both in drug stores, and in food stores (Ahmad, Aqil, & Owais, 2006; Bandaranayake, 2006). A practice once seen to be associated with developing countries is now widely accepted and used in the UK and other European countries. Australia and North America are gradually embracing the use of medicinal plants as alternative therapy (Anastasi, 2005; Anquez-Traxler, 2011; Committee, 2005).

Several plant-based compounds have been exploited in various studies for the treatment of liver cancers, particularly HCC (Boye, Yang, Asenso, & Wei, 2016). For instance, HCC is an inflammatory related cancer, and *Zanthoxylum Zanthoxyloides* Lam has demonstrated anti-inflammatory activity (Ferreira *et al.*, 2011; Gregor, 1989; Luo *et al.*, 2013; Ojewole, 2004). *Zanthoxylum*

Zanthoxyloides can hence be investigated for the treatment of inflammatory-related diseases such as HCC.

The study investigated the anti-HCC effect of a crude alkaloid extract from *Zanthoxylum zanthoxyloides* in Carbon tetrachloride (CCl_4)/olive oil-induced hepatocellular carcinoma (HCC) in Sprague Dawley rats.

Problem statement and justification

HCC makes up about 85% of all primary liver cancers, and it is the 5th commonly diagnosed cancer worldwide (Shariff *et al.*, 2009). Globally, it causes 700,000 deaths annually (McGlynn & London, 2011), out of which 200,000 occurs in West Africa alone (Ladep *et al.*, 2014). Despite the high burden of HCC, available therapies are associated with off-target effects or poor survival rates. For example, chemotherapies are ineffective in late-stage HCC which is the most diagnosed HCC. Surgery is unable to remove every trace of cancerous tissues and so patients would have to rely on chemotherapy after the surgery. Radiation therapy has a high probability of affecting cells and tissues which are not cancerous. Because liver donors are difficult to come by, and patient management for a potential donor is expensive, transplantation is almost left for the rich and famous. These reasons provide the rationale for an investigation into newer and better treatment options that employ different mechanisms for their anti-HCC effect. The link between inflammation and HCC has been confirmed (Ande, Nguyen, Nyomba, & Mishra, 2016), however, the possibility of anti-inflammatory agents exerting anti-cancer properties remains inconclusive as very little is known about such mecinal effects as in the case of *Zanthoxylum*

zanthoxyloides which has demonstrated anti-inflammatory effects (Folashade, Mutalib, Satyajit, & Olajide, 2017).

Aim of the study

The study assessed anti-HCC effect of an alkaloidal extract from leaves of *Zanthoxylum zanthoxyloides* in CCl_4/olive oil-induced HCC rats.

Specific objectives

To achieve the aim of the study, the study sought to:

1. prepare a crude alkaloid extract (ZZAE) from the leaves of *Zanthoxylum zanthoxyloides* and profile it phytochemically.
2. determine the cytotoxicity of ZZAE using *Allium cepa* assay.
3. establish HCC in Sprague Dawley rats using carbon tetrachloride (CCl_4) reconstituted in olive oil.
4. assess the anti-HCC efficacy of ZZAE.

Significance of the study

The study has provided a baseline knowledge for the development of new treatment for HCC. The study has provided additional knowledge on the pharmacological properties of *Zanthoxylum zanthoxyloides* and subsequently change the general impression about herbal medicine. The model of HCC research adopted in this study is novel and so will provide an additional model for HCC induction and establishment.

Limitations

1. Some methodologies could not be performed because reagents required for those procedures were too expensive and the projects had no funding.
2. The study was also limited by lack of technology such as cell culture laboratory.
3. Lack of in-house technologies to perform histology and biochemical parameters cost a lot to perform taking a lot of funds that could have been used to fund other procedures.
4. The cost of cell lines did not allow for *in vitro* studies such as the effect of the ZZAE on cell migration and cell proliferation.

Definition of terms

The meaning of the following words in the scope of this work are as defined below

Anticancer: the ability of a macromolecule to prevent the development and/or progression of a tumor

Macromolecule: compounds that can stimulate receptors and cause downstream signal transduction

Cancer Induction: the chemical manipulation of hepatocytes to stimulate nuclear instability leading to cancer development

Extraction: the application of chemical force to remove chemical compounds from the leaf of *Zathoxylum zanthoxyloides*

Phytochemical profiling: the process of qualitatively and quantitatively determining the compounds present in an extract

Anti-inflammation: the ability of a substance to inhibit and/or stop inflammation

Genotoxicity: Is the property of an agent to cause damage to the genetic information within a cell and subsequently causing mutations.

Apoptosis: Is a programmed cell death.

Necrosis: the death of most or all of the cells in an organ or tissue as a result of injury lack of blood supply or disease.

Prophylaxis: treatment given or action taken to prevent the occurrence of disease

Chapter summary

In Africa and other developing countries, *Aflatoxin* substrate foods form a bulk part of the diet. In these countries however, there is poor food handling and food processing technologies are primitive. These fundamrntal problems make developing countries more succeptible to HCC. Currently however, the cost of treatment of HCC is very high and so conscious research into the development of newer and cheaper treatment modalities is required.

CHAPTER TWO

LITERATURE REVIEW

Chapter overview

This chapter discusses cancer with specific attention to the link between inflammatory-related cancers and compounds with anti-inflammatory properties. The chapter reviews liver cancer types, staging, treatment options, and models of HCC research. Attention is also given to alternative medicine with a special focus on herbal medicine and *Zanthoxylum zanthoxyloides*. Ethnopharmacology of *Zanthoxylum zanthoxyloides*, its biological activities, and compounds that have been isolated from *Zanthoxylum zanthoxyloides* are also discussed.

Cancer

Cancer is a disease associated with wild growth and spread of abnormal cells that may cause death if not managed (Saslow *et al.*, 2012). In the United States where data on cancer incidence and mortality is regularly updated, it is reported that one in every four deaths is due to cancer (Siegel, Ma, Zou, & Jemal, 2014). Though genetic predisposition is the primary risk factor for cancers, other risk factors such as lifestyle (smoking, etc.), occupational exposures (miners, street hawkers, etc.) environmental hazards (radiation, etc.) diseases (hepatitis viral infection), food poisoning (aflatoxin B1 ingestion etc.) are essential in the initiation and progression of cancer.

Types of cancers

There are several criteria used in the classification of cancer. For instance, cancer can be classified based on the organ where the cancer is located. For instance, all cancers that affect the face, head, and neck are classified as head and neck cancers. Those that affect the kidney are classified as kidney cancer. The same applies to lung cancer, stomach cancer, liver cancer, etc. Other cancer classifications are based on whether the cancer is malignant or benign. However, the most adopted form of cancer classification is the classification based on tissue/cell type origin of cancer. Table 1 illustrates the classes of cancers based on tissue/cell origin. Among all these cancer types, carcinomas account for about 80% to 90% (Smith, Perfetti, & King, 2019). Because their tissue of origin is found throughout the body. Cancers of the epithelial tissue affect organs like the kidney, bile duct, testis and breast with the most life-threatening one occurring in the liver, particularly HCC makes up about 90% of all liver cancer epidemiology.

Table 1: Classification of cancer by tissue/cell origin

Class	Origin	Subtypes	Example
Carcinoma	Epithelial tissues	1. Adenocarcinoma 2. Squamous cell carcinoma	Breast cancer, prostate cancer, etc.
Sarcoma	Supportive and connective tissues	1. Undifferentiated 2. Differentiated	Osteosarcoma, Chondrosarcoma etc.
Myeloma	Plasma cells	N/A	Multiple myeloma
Leukemia	Bone marrow	N/A	leukemia
Lymphoma	Nodes of the Lymphatic system	Extranodal lymphoma, Non-Hodgkin lymphoma	Cancers of the spleen etc.
Mixed types	A mixture of the tissue types	N/A	Mixed mesodermal tumor

Liver cancer

Liver cancer can be defined as cancers that affect the liver. It has been reported that liver cancer is the fourth leading cause of death in all cancer-related deaths (Wang *et al.*, 2016). Among the types of liver cancers (HCC, cholangiocarcinoma, liver angiosarcoma, and hepatoblastoma) HCC is the most prevalent, and second to it is cholangiocarcinoma (Akinyemiju *et al.*, 2017). There is a vast variability in primary liver cancer burden marked by sex and geographic region (Chuang, La Vecchia, & Boffetta, 2009).

Hepatocellular carcinoma (HCC)

HCC is also referred to as hepatoma and accounts for about 75% of all primary liver cancers (Siegel, Miller, & Jemal, 2016). Development of HCC occurs in the hepatocytes which are the major liver cells. They are known to be able to spread from the liver to other organs such as the pancreas stomach and the intestines. Chronic hepatitis B virus infection (CHEN, YU, & LIAW, 1997) has been reported to be a significant risk factor for liver carcinogenesis, and it is known to account for about 55% of all HCC cases worldwide. Averagely, 80% or more of such chronic HBV infections are found in the Eastern Pacific region and Sub-Saharan Africa, and these are the areas known to have the highest HCC incidence (Chen *et al.*, 2006; Kew, 2010).

Epidemiology of HCC

HCC is the fifth most prevalent cancer (600,000 new cases each year) and the second cause of death (700,000 deaths annually) in cancer-related mortalities (Bhandari, 2015). There is a geographical variation in HCC prevalence with 80% of all cases found in Eastern Asia, North America, and Sub-Sahara-Africa (Yin *et al.*, 2010). In these areas, there is an overlap of major risk factors of HCC that corroborates its geographical distribution pattern (McCracken *et al.*, 2007; Röcken & Carl-McGrath, 2001; Ward *et al.*, 2004).

Risk factors of HCC

Usually, the development of HCC is a stepwise process occurring from chronic liver injury, inflammation and fibrosis, often caused by hepatitis B viral (HBV) infections, hepatitis C viral (HCV) infection, aflatoxin B1 (AFB1),

exposure to environmental toxins, alcoholic cirrhosis, and nonalcoholic fatty liver disease (Hernandez–Gea, Toffanin, Friedman, & Llovet, 2013). The molecular complexity and the underlying chronic liver disease from which the HCC developed make it a very difficult neoplasia to treat.

Among the several risk factors of HCC, HBV (Chen *et al.*, 2006) and HCV infections (El-Serag, Kanwal, Richardson, & Kramer, 2016), aflatoxin B1 exposure (Kaplan, Chang, & Sanyal, 2019), iron overload and extreme alcohol consumption and are the most documented (CHEN *et al.*, 1997).

Aflatoxin B1 (AFB1)

Several epidemiological studies have pointed out that AFB1 contaminated food is among the significant risk factors for human liver cancers (Madden, Finegold, & Slagle, 2002). Regions with high exposure to AFB1 have confirmed these epidemiological claims with an overwhelming prevalence of HCC (Ozakyol, 2017). In Ghana and other African countries and Asia, foods that are substrates for aflatoxin growth such as peanuts and cereals form a major part of their diet. Food is an essential part of humans, henece, relevant to elucidate AFB1-related HCC taken into full consideration of the underlining mechanism of action. This is relevant as it offers a further understanding of the risk of AFB1 in HCC development in order to minimize the incidence of AFB1-induced HCC. A better approach to achieving this will be to establish interventions geared towards adopting dietary changes and developing chemopreventive agents that can interfere with the AFB1 related HCC pathway.

AFB1 is a mycotoxin synthesized by the common *A. parasiticus and A. flavus*. Substrates for these organisms include but not limited to; corn, rice, oilseeds, dried fruits, and peanuts, which have been exposed to poor and unsanitary storage and processing conditions (Baydar, Engin, Girgin, Aydin, & Sahin, 2005). There is also evidence of secretion of AFB1 intermediate molecules into milk, egg, and meat of farm animals that had been given food contaminated with aflatoxin (Bennett, 1987). About 4.5 billion people are at risk of prolonged exposure to food contaminated by aflatoxin. It has been reported that AFB1 is a food contaminant that can only be minimized but not avoided (Williams *et al.*, 2004). There are four human and animal carcinogenic aflatoxins (aflatoxin B1, B2, G1, and G2). Among the four, aflatoxins B1 is the most hepatotoxic, and for that matter hepatocarcinogenic agent.

Among other attributes of AFB1 of pathologic concern are mutagenicity, acute toxicity, teratogenicity, and carcinogenicity (McLean & Dutton, 1995). It has been reported that AFB1 is the most hepatocarcinogenic mycotoxin that contributes to a greater proportion of aflatoxin-related HCC (Wang *et al.*, 2001). The International Agency for Research on Cancer (IARC) classifies AFB1 among the group I carcinogens for HCC (Johnson, 2019). The correlation of distribution pattern of AFB1 with socioeconomic status, explains that low-income countries have poor sanitation, and substandard ways of handling food and ineffective food regulations (Elaridi, Bassil, Kharma, Daou, & Hassan, 2017).

Mechanism of AFB1 in HCC

Close to half of all human cancers have a mutated *Tp53* gene, and this is partly because mutations affecting *p53* are varied in nature and position. For instance, in about 50% of all HCC forms, mutations like the transversion in codon 249 that exchanges guanine (G) for thymine (T) and a corresponding substitution of arginine (R) to Serine (S) is seen (Martin & Dufour, 2008). Investigations that have been conducted in areas with high AFB1 exposure have shown that AFB1-related HCC is due to mutation into the *p53* gene (Macé *et al.*, 1997). Among these studies, the majority have reported that the mutation occurs at the codon 249 hotspot in exon 7 of the *p53* gene (Bressac, Kew, Wands, & Ozturk, 1991; Egal *et al.*, 2005; Hsu *et al.*, 1991). This mutation is termed as 249^{ser}, and it occurs through the conversion of G to T, which results in R to S mutation in the p53 protein (Nogueira *et al.*, 2009).

The mutation seen is also prominent in the liver because biotransformation of the AFB1 occurs in the liver, providing a perfect site for the initiation of its mechanism of action (Lewis *et al.*, 2005). Once an AFB-contaminated food is ingested, cytochrome-P450 enzymes metabolize it to the reactive genotoxic intermediates (aflatoxin B1-8, 9-oxide, AFBO). The intermediate which is highly reactive AFBO binds to liver cell DNA to form the DNA adducts: 8, 9-dihydro-8 (N7guanyl)-9-hydroxy-AFB1 (AFB1 N7-Gua) (Lewis *et al.*, 2005; Obuseh *et al.*, 2011; Sutandyo, 2010). If this adduct progresses to DNA replication, there is an interaction between DNA adducts and the guanine that leads to mutational effects

in the *p53* tumor suppressor gene and leads to hepatocarcinogenesis (Obuseh *et al.*, 2011; Sutandyo, 2010) (Figure 1).

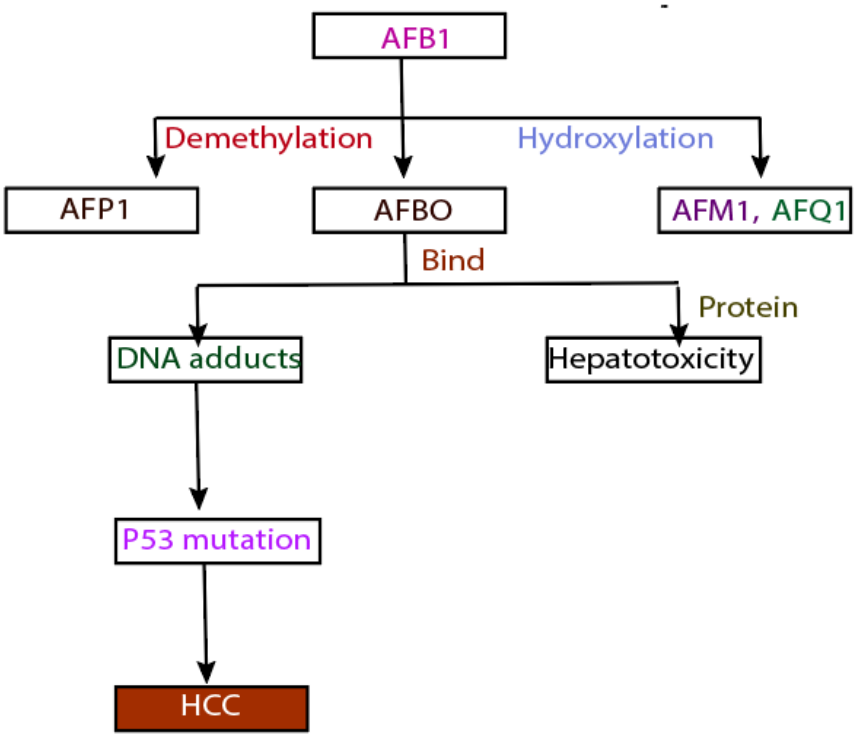

Figure 1: Aflatoxin and HCC. Ingested aflatoxin (AFB1) is biotransformed in the liver by CYP450 isoenzymes resulting in a nucleophilic genotoxic reactive intermediate, AFBO. The intermediate AFBO is then hydroxylated to AFM1 and AFQ1 or demethylated to AFP1. However, the interaction of AFBO with liver cell DNA at the N7 position of Guanine causes p53 mutation, and leads to HCC development. AFBO can also cause aflatoxicosis as it binds to proteins or amino acids. AFBI: Aflatoxin B1, AFBO: Aflatoxin B1 epoxide, AFB1-8, 9 epoxides.

Under normal physiological conditions, the body detoxifies AFBO intermediate metabolites to prevent DNA adducts formation. The detoxification of intermediate metabolites is preceded by conjugation to glutathione by glutathione S-transferase (GST) and subsequent hydrolysis to dihydrodiol by human microsomal epoxide hydrolase (mEH) (Ilic, Crawford, Egner, & Sell, 2010). Following these revelations, it is imperative to inquire about why AFB1 remains a significant risk factor to HCC. The answers to such questions lie in a study conducted using GST A3 knockout mice (Stern *et al.*, 2001), which developed the sensitivity to both the acute genotoxic and cytotoxic effects of AFB1 in the absence of the GST A3 gene (Ilic *et al.*, 2010; Stern *et al.*, 2001). In addition to that, newborn mice, humans, and other primates are sensitive to low doses of AFB1. There are as well evidence that AFB1-dihydrodiol can reorganize to form a dialdehyde conformation capable of binding to the amine groups in proteins through Schiff-based reactions under normal physiological conditions (Sabbioni, Skipper, Büchi, & Tannenbaum, 1987).

Viral Causes of HCC

Distribution of HCC reflects its risk factor endemicity. For example in eastern Asia and sub-Saharan Africa where HBV and HCV infections are endemic, they account for 70% of all risk factors of HCC occurrence (Torre *et al.*, 2015). In Europe and North America countries, HCV infection accounts for 50%-70% with alcohol steatohepatitis (ASH) contributing only 20% of all cases (Bosetti *et al.*, 2008; El–Serag & Rudolph, 2007; Llovet *et al.*, 2008). Around 80% of the time, HCC develops from a long-term chronic disease course with

primary liver cirrhosis. It is worth mentioning however that HCC can occur in non-cirrhotic liver (Alkofer, Lepennec, & Chiche, 2011).

Oncogenicity of hepatitis B virus (HBV)

Association between chronic infections of HBV and HCC development has been illustrated in Figure 2. The involvement of viral proteins in cellular damage has been demonstrated with the aid of various *in vitro* techniques and animal models with the insertion and/or modification of viral transgene (Higgs, Chouteau, & Lerat, 2014). The missing link so far is, however, the lack of understanding in the pathogenesis involved in the oncogenic initiation of hepatocarcinogenesis. In *in vitro* studies, for instance, low efficiency in viral replicon obstructs the study of the pathogenicity and cellular biology caused by these agents.

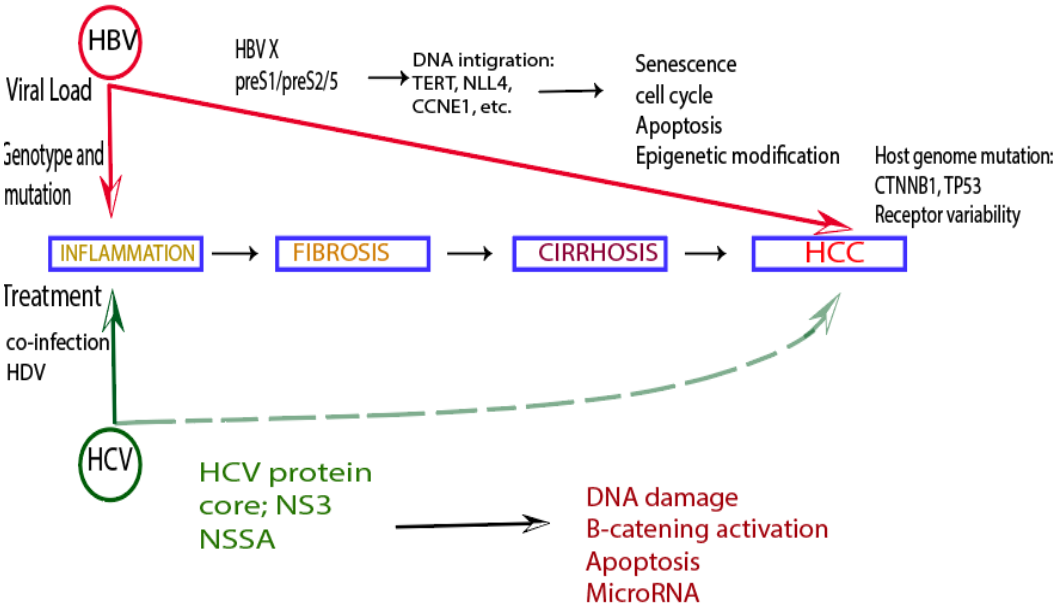

Figure 2: Role of Hepatitis B virus in HCC. HBV: hepatitis B virus, HCV: hepatitis C virus, HCC: hepatocellular carcinoma. DNA: deoxyribonucleic acid, TERT: telomerase reverse transcriptase, CCNE1: cyclin E1, CTNNB1: catenin B1.

HCV and oxidative stress

HCV RNA cannot directly be integrated into the human genome, therefore the mechanism of HCV-related HCC pathogenesis could only happen through indirect pathways and generally through the effects of chronic inflammation and oxidative stress (Liu, Cheng, Huang, Huang, & Lin, 2017). The results of such liver injury are therefore progressive, beginning from fibrosis through cirrhosis and eventually HCC.

HCV direct oncogenicity

Transgenic mice models and *in vitro* studies have been used recently to demonstrate that HCV protein core, NS5A, NS3, and NS4B can induce cell transformation (Bartosch, Thimme, Blum, & Zoulim, 2009). Findings from these studies confirm that HCV viral protein can also induce HCC development devoid of inflammatory mechanisms (Lemon & McGivern, 2012) (Figure 2.2). This is mainly because, the viral protein in the cytoplasm can interact and alter the stability of host protein to favor the cellular mechanism of carcinogenesis (Lemon & McGivern, 2012). Such interaction can also progress to dysregulation of various cellular signaling, especially those involved in apoptosis, immune response, cell proliferation, and oxidative stress (Lemon & McGivern, 2012; Levrero, 2006).

Schistosomiasis

In Egypt, Ghana, and China, schistosomiasis is a major public health problem, because it is a major cause of liver disease. There are scientific reports that point to the fact that *Schistosomiasis* may modify the course of hepatitis C genotype 4 co-infection and accelerate progression to hepatitis C-associated fibrosis. This can then hasting the progression of fibrosis-related HCC, than patients without *Schistosomiasis* (Darwish, Abbas, Abdelfattah, & Darwish, 1992; El-Zayadi *et al.*, 2005; Kamel *et al.*, 1992).

Hepatocarcinogenesis

Oxidative stress is one of the implicated mechanisms of hepatocarcinogenesis induced by HCV (Arzumanyan, Reis, & Feitelson, 2013). Reactive oxygen species (ROS) is a normal by-product of several cell processes including senescence (Seeger & Mason, 2000). ROS production in hepatocytes occurs mainly in the mitochondria, and from xanthine oxidase reactions and nicotinamide adenine dinucleotide phosphate oxidase, and in Kupffer cells and in cells where inflammation has been stimulated (Koike & Miyoshi, 2006). Malondialdehyde, 8-hydroxydeoxyguanosine (8-OHdG), and thioredoxin are biomarkers that can be measured in serum and biopsy to determine the levels of oxidative stress in patients with chronic hepatitis C condition (Farinati *et al.*, 1999; Mahmood *et al.*, 2004). Higher expression of 8-OHdG is an important biomarker in DNA damage indicates that hepatic oxidative DNA damage is a feature of chronic hepatitis (Fujita *et al.*, 2008).

Role of diabetes mellitus in HCC

In the United States, a population-based study has identified diabetes as an independent risk factor for HCC (Davila, Morgan, Shaib, McGlynn, & El-Serag, 2005). This finding is regardless of whether participants have chronic HCV or HBV infection, alcoholic liver disease, or non-specific cirrhosis because 60% of the percipients had neither of these diseases at the time of the study (Davila *et al.*, 2005). Diabetes was associated with a twofold- to threefold-increase in HCC risk (Davila *et al.*, 2005).

Pathogenesis of HCC

The pathogenesis of HCC varies based on the etiology. In the preceding paragraphs, various pathogenesis have been described.

Activation of hepatic stellate cells

Several studies have highlighted the importance of cancer stem cells (CSC) in the pathophysiology of HCC (Luk *et al.*, 2018). There are several hepatic CSCs as far as the various HCC subtypes are concerned, and they have been reported as primary regulators of HCC initiation, progression, and metastasis (Oishi, Yamashita, & Kaneko, 2014; Yamashita & Kaneko, 2014). RNA-FISH for stem cell markers (OV6, CK19, and CD133) and immunostaining have been used to examine the frequency of positive stem cell markers in liver cirrhosis and HCC (Oliva, French, Qing, & French, 2010). These markers roughly correlate with the incidence of HCC that develops in the clinical setting (Oliva *et al.*, 2010).

Though several studies have demonstrated the importance of heterogeneous populations in driving carcinogenesis, knowledge about the role of viral hepatitis in the biology of hepatic CSCs is minimal. Human tissues have been histologically analyzed, and the results indicate that the levels of HBV infection occur with increasing levels of CD90 (Lu *et al.*, 2011) but with decreasing levels of CD133 (Yeh *et al.*, 2009). It is difficult to determine how HBV alters the physiology of CD90+ and CD133+ because techniques to allow co-staining of the CSC markers and the HBV proteins are non-existent. Another challenge to this lies in the several HBV genotypes that should individually be

considered necessary in assessing the role of a specific virus type with the phenotype of the cells.

Studies elsewhere have investigated the role of HBV in the generation of hepatic CSC, particularly the association between HBx expression and EpCAM+ CSC (Arzumanyan *et al.*, 2011). Other studies done elsewhere have demonstrated that stem cell transcription factors Nanog, Klf-4, Oct-4, and even EpCAM and β-catenin, are expressed in significant quantities in HBx expressing cells (Wang, Yang, *et al.*, 2012). Clinical evidence showing that high HBx expression in human HBV-related HCC is statistically associated with EpCAM+ or OV6+ tumor cells are also available (Kimura *et al.*, 2014; Wang, Yang, *et al.*, 2012). In 2015, Fan and colleagues investigated the molecular mechanism underlying how HBx induces EpCAM expression. The study suggested that DNA demethylation is a primary mechanism responsible for the re-expression of EpCAM in hepatocytes (Fan, Zhang, Pascuzzi, & Andrisani, 2016).

The role of inflammation in HCC

In the acute inflammation process, there are molecular and cellular events that efficiently minimize impending injury or infection and restores tissue homeostasis. In chronic liver injuries such as hepatitis viral infection, maladaptive reparative cell death, and regeneration, following chronic inflammation, stimulates the development of dysplastic nodules (Figure 3).

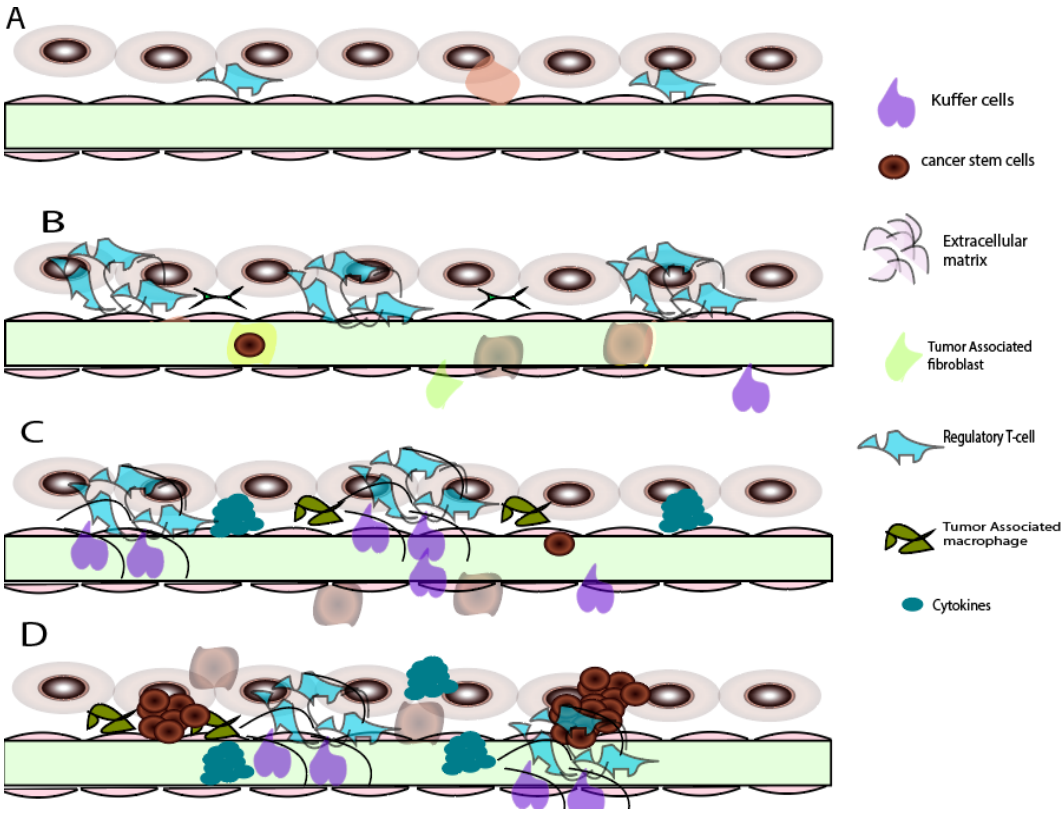

Figure 3: Inflammation and HCC. (A) Normal liver parenchyma. Hepatocytes with sinusoidal and microvilli endothelial cells with fenestrations that favor metabolic exchange. Space of Disse that has few quiescent stellate cells having lipid droplets. (B) Fibrotic liver. Once chronic liver injury ensues, hepatocytes lose their microvilli, sinusoid endothelial cells are defenestrated, and stellate cells get activated, loose lipid droplets, and secreted into the extracellular matrix (ECM). (C) HCC. Malignant transformation of hepatocytes with unrestrained growth. The intrusion of inflammatory cells and cytokines amidst extensive fibrosis and recruitment of TAFs and CSCs. (D) Development of well-differentiated HCC following chronic inflammation.

Inflammatory pathways implicated in HCC

In recent times, attention has been given to investigating inflammatory mediators and how they are implicated in sustained inflammation and immunosuppression that leads to HCC development. It has been established that the carcinogenesis of inflammation is related to its persistent cytokine production which triggers an array of unique yet, redundant interactions (Allavena, Garlanda, Borrello, Sica, & Mantovani, 2008). The expression of these cytokines in HCC appears to be localized in the tumor cells and their surrounding tissue. The constraint, however, is that the step-by-step mechanism of initiating the HCC development remains unclear.

In a more aggressive and metastatic HCC, the microenvironment is flooded with Th2-like interleukins (IL-4, IL-8, IL-10, and IL-5) concurrently with Th1-like (IL-1, IL-1β, IL-2, tumor necrosis factor) (Budhu *et al.*, 2006; Budhu & Wang, 2006). In the cirrhotic liver, the most abundant cytokine is IL-6, and they are produced by the Kupffer cells during hepatocyte damage. The IL-6 is a potent activator of STAT3, another fact that corroborates risk of HCC with poor prognosis when the levels in serum are higher (Hoshida *et al.*, 2008; Tilg *et al.*, 1992).

In women where estrogen modulates inflammatory microenvironment through the suppression of IL-6 production, HGF expression, and metastasis of HCC are significantly repressed (Wang, Xu, *et al.*, 2012). In the HCC tumor microenvironment, expression of elevated levels of IL-22 is known to promote tumor growth, inhibit apoptosis, and promotes metastasis by activating STAT3

(Jiang *et al.*, 2011). Risk of HCC progression post-resection increases when the level of IL-10 is high in the HCC tumors microenvironment (Beckebaum *et al.*, 2004; Chia *et al.*, 2002). Notwithstanding, the specific risk in the development of HCC is still not known. Other interleukins such as IL-2 and IL-15 in peritumoral liver tissue confers a decreased rate of intrahepatic tumor recurrence and prolongs overall survival (Zhou *et al.*, 2010).

The role of chemokines in the inflammatory response are mediated through the stimulation of specific receptor types (CCR, CXCR, CX3CR, and XCR). These receptors are mainly located in inflammatory, endothelial, and epithelial cells. Several scientific reports are detailing the role (immune system evasion, invasion, angiogenesis, and dissemination) of these chemokines in cancer development (Roussos, Condeelis, & Patsialou, 2011). Chemokines, CXCL12-CXCR4 axis is specifically crucial in the regulation of angiogenesis, and higher levels have been confirmed in HCC as opposed to cirrhosis (Li, Gomez, & Zhang, 2007). Once CXCL12 binds CXCR4 in endothelial cells, they synergistically work with VEGF to promote migration, proliferation, leading to the development of new vessels (Kryczek *et al.*, 2005).

In the regulation of HCC, a second principal axis is the CCL20- CCR6, which facilitates the recruitment of circulating regulatory T cells (Tregs) to the tumor microenvironment. When the levels of CCL20- CCR6 are high in a tumor microenvironment, it promotes tumor growth and intrahepatic metastasis (UCHIDA *et al.*, 2006). Nuclear factor-kappa B (NF-κB) and STAT3 are critical signaling pathways in the hepatic response to injury mediated by inflammation

and are also crucial for liver regeneration. Though NF-κB has been implicated in hepatocarcinogenesis; the role it plays, however, varies based on the mouse model and type of injury applied (Bode, Albrecht, Häussinger, Heinrich, & Schaper, 2012). In humans, hepatitis viral infections are known to be a critical proinflammatory stimulus for activation of the NF-κB pathway for hepatocarcinogenesis (Shi *et al.*, 2006).

HCC models

Over the years, models for cancer research have evolved, and currently includes both naturally occurring models and artificially induced systems (Hidalgo *et al.*, 2014; Vidal & Cagan, 2006; Xu, Farach-Carson, & Jia, 2014). These systems allow the study of the pathogenesis that leads to the discovery of new biomarkers. These systems have also played pivotal roles in drug discovery especially at the preclinical stage.

Transgenic mice

This technique was invented by Mario Capecchi in the 1980s to which he won a Nobel Award in Physiology and Medicine in 2007. The technique involves removing a single gene of interest in the mouse genome to create strains of mice that can pass the altered gene to its offspring (Table 2). This technique has been used to demonstrate the hepatocarcinogenesis resulting from chronic liver cell injury in hepatitis B viral infection (Dunsford, Sell, & Chisari, 1990). Other studies include investigation of oxidative damage in hepatocytes DNA in hepatitis B viral infection destined to develop HCC (Hagen *et al.*, 1994). Studies using transgenic mice have confirmed that the core protein of hepatitis C virus induces

HCC (Moriya *et al.*, 1998). In a separate study the molecular pathogenesis of HCC in hepatitis B virus infection has been established (Chisari *et al.*, 1989).

Table 2: Transgenic models of HCC

Technique	Method	Reference
Oncogenic transgenic mice	Ctnnb1 deletion	(Wang *et al.*, 2011)
	Platelet derived growth factor-C	(Gilbertson *et al.*, 2001)
	Transforming growth factor alpha	(Jhappan *et al.*, 1990)
	myc/tgfa and myc/e2f1	(Murakami *et al.*, 1993)
Double knockout	GNMT knockout	(Yang *et al.*, 2018)
Metabolic dysfunction transgenic mice	MDR2 deletion	(Smit *et al.*, 1993)
Inflammatory cholangitis induced transgenic mice	virus mediated Cre delivery	(He, Tian, Li, & He, 2015)
Conditional and inducible gene expression systems	HBx regulatory gene insertion	(Kim, Koike, Saito, Miyamura, & Jay, 1991)
Hepatitis virus transgenic mice		

GNMT = Glycine N-methyltransferase, MDR2 = Multidrug resistance 2, HBVx = Hepatitis B virus X protein.

In vitro models of HCC

Animal and human cell cultures have gain popularity in Life Sciences Research. Different variants of cell culture have been employed in modeling diseases, monoclonal antibody production, IVF technology, regenerative medicine therapeutic protein production, and various stages of cancer research. Credit to Hans Janssen and his son for inventing the microscope in 1590, life can now be made at the basic functional unit level. Fast-forward, 21st century, cell lines of various disease models, including HCC, are available on commercial bases (Table 3).

Table 3: In vitro models of HCC

Cell name	Cell line origin	Reference
ARL-6	Wistar rat liver hepatoma	(Qiao, Yu, Dent, & Farrell, 2005)
C2	Rat hepatoma [HGPRT-]	(Autelli *et al.*, 2009)
C2-Rev 7	Rat hepatoma [HGPRT-]	(Baumhueter, Courtois, & Crabtree, 1988)
Fao	Rat hepatoma	(Shiota, Rhoads, Wang, Nakamura, & Schmidt, 1992)
H-4-II-E	Rat hepatoma Reuber H35	(Yamamoto *et al.*, 1998)
H4-II-E-C3	Rat liver hepatoma	(Derenzini *et al.*, 2005)
H4S	Rat liver hepatoma	(Fournel, Sapieha, Beaulieu, Besterman, & MacLeod, 1999)
H5	Rat hepatoma	(Wiebel, Wolff, & Lambiotte, 1980)
Hep 3B	Human hepatoma	(Mitani, Fujita, Sassa, & Kappas, 1989)
Hep G2	Human Caucasian carcinoma	(Sells, Chen, & Acs, 1987)
Hepa 1-6	Mouse hepatoma	(Du *et al.*, 2013)
Hepa 1c1c7	Mouse hepatoma	(Sinal & Bend, 1997)
HF1	Rat hepatoma hybrid (H5xFao)	(Eray *et al.*, 1994)
HF1-5	Rat hepatoma hybrid (H5xFao)	(Deschatrette, Moore, Dubois, Cassio, & Weiss, 1979)
HTC	Rat hepatoma	(Thompson, Granner, & Tomkins, 1970)
HTC (BUdR)	Rat liver hepatoma	(O'Brien & Stellwagen, 1977)
Huh-7D12	Human hepatocellular carcinoma	(Foldes-Papp *et al.*, 2009)
MCA-RH 7777	Rat buffalo hepatoma	(Yang, Pritchard, Bhuiyan, Seccombe, & Moghadasian, 2001)
MH-22A	Mouse C3HA hepatocarcinoma	(Alexandrova & Shvemberger, 2005)

Patient-derived xenograft

This technology involves engrafting into an immunocompromised rat cancerous cells or tissues from humans. The rationale behind this model of cancer research is that the pathophysiology and/or response to any treatment is a true representative of the original tumor. The success of this technology depends on factors such as biological stability and the degree of reflection to patients' tumors with regards to histopathology, gene expression, genetic mutation, and inflammation (Kuracha, Thomas, Loggie, & Govindarajan, 2016). This technology has been employed in lymphatic leukemia (Dürig *et al.*, 2007), large B cell lymphoma (Chapuy *et al.*, 2016), pancreatic cancer (Tignanelli, Herrera Loeza, & Yeh, 2014), colorectal cancer (Seol *et al.*, 2014), high-grade serous carcinoma (Dong *et al.*, 2016) and intrahepatic cholangiocarcinoma (Cavalloni *et al.*, 2016).

In HCC, the technique has been used to investigate the role of anti-GPC3-CART in the tumor growth in HCC (Jiang *et al.*, 2017), to establish various HCC cell lines (Xin *et al.*, 2014), test the efficacy of some drugs (Wei *et al.*, 2014) and to even characterize HCC genetically (Gu *et al.*, 2015).

Chemically-induced HCC models

Perhaps the most fascinating thing about life is the high degree of organization that maintains and perpetuates itself through growth in an orderly accumulation of matter in cells. The cells' organization ensures strictly controlled and severely limited division to form tissues regulated by the action of scantily known forces embodied in each cell. In the liver, chemical induction of cancer

destabilizes this organization through molecular oxidative stress by activating Serotonin (5-HT) through its HSCs expressed receptors, especially 5-HT2A and 7 (Atallah, Elaidy, & Tawfik, 2018). In the case of CCL_4, activation of the parent compound to trichloromethyl radical ($^{\cdot}CCL_3$) and trichloromethyl peroxy radical ($^{\cdot}OOCCL_3$) by CYP450 forces the liver to stimulate the kuffer cells to produce reactive oxygen species (ROS) which initiate inflammation (Li *et al.*, 2014) (Table 4). A combination of these two hepatotoxins maintains the inflammation process and accelerates liver damage.

Table 4: Chemicals used to induce experimental HCC

Animal	Inducing agent	RoA	Duration	Reference
Rats, mice, rabbits and dogs	Diethylnitrosamine	Oral	16 weeks	(FREUND, 1937)
Rats, mice and monkeys	Carbon tetrachloride	Intraperitoneal injections	16 weeks	(Heindryckx, Colle, & Van Vlierberghe, 2009)
Rats and mice	Thioacetamide	Intraperitoneal injections	18 weeks	(Omura *et al.*, 2014)
Rats	Phenobarbital	Oral	20 weeks	(Braeuning *et al.*, 2016)

RoA = Route of administration

HCC has multifactorial etiology, hence, no single model could mimic HCC in its entirety. For instance, the genetic mutation and inflammation in the pathophysiology of Hepatitis viral-induced HCC is different from AFB1 –induced

HCC. The recommendation, therefore, is to adopt a combination of models going forward that represents various HCC forms as possible.

Treatment options for HCC

Treatment of HCC is particularly tricky because hepatocarcinogenesis is almost always accompanied by complex aberrations in developmental and oncogenic molecular signaling pathways (Bouattour & Wassermann, 2014). Various novel and promising macromolecules have been developed and their anti-HCC tested in different phases of clinical trials. In most instances, these macromolecules are either tested as a single agent or as a combined regimen (Lammers, Kiessling, Hennink, & Storm, 2012). In Asia, several plant-based small molecules are used either alone or as a combination therapy for the treatment of HCC (Boye *et al.*, 2016).

Liver re-sectioning and transplantation

The procedure involves the removal of the tumor mass from the rest of the liver tissue. Ordinarily, this procedure should work perfectly because the liver has a regenerative property, but the uncertainty of complete removal of all the tumor and its associated higher risk of relapse makes this treatment option less effective. (Albain *et al.*; Lencioni, 2010). Another significant disadvantage to this treatment option is that surgical resection and liver transplantation benefit only a selected few (25%) of HCC patients (Lencioni, 2010).

An underlying condition that needs to be met to qualify for surgical resection is a well-preserved liver function devoid of any portal hypertension

which is seen in only a quarter of patients. The treatment option also has a five-year survival of 15–39% (Takenaka *et al.*, 1996). Liver transplantation has, therefore, become the ideal treatment for early-stage HCC in cirrhotic patients mainly because its five-year survival rates are between 70–80% with a tumor recurrence of 10% (Llovet, Fuster, & Bruix, 1999).

Loco-regional treatment options (Radiation)

Radiotherapy involves the use of beams of intense energy (often X-rays, protons or other energies) to kill cancer cells (Salem, Thurston, Carr, Goin, & Geschwind, 2002). Though most treatment options available for HCC treatment are offered as palliative treatment, Radiotherapy is hardly mentioned and when employed, protons are rather used (Phillips & Murikami, 1960). More commonly, it is used to augment other treatment options (Brock & Dawson, 2010).

Chemotherapy

Because loco-regional therapy does not apply to all HCC patients, many of these patients will require palliative care alone. HCC patients who present well-preserved liver synthetic function could also benefit from systemic chemotherapy. For single-agent treatment, cytotoxic chemotherapy range from nothing up to about 20% when anthracyclines, such as doxorubicin which has the highest response rate (up to 20%) are used (Nerenstone, Ihde, & Friedman, 1988). In such a treatment regimen, no definite survival benefit could be promised to the patient (Mok *et al.*, 1999). In a randomized controlled trial, a group of 60 patients received adriamycin with 64 others receiving no treatment. Patients in both the treatment and control group had advanced HCC and the median survival rate

recorded was 10.6 as opposed to 7.5 weeks, for treatment and control respectively ($p = 0.036$). Even though the results were statistically significant, researchers could not recommend Adriamycin as an ideal drug for advanced HCC because 25% of the treated group suffered fatal septicaemia and cardiotoxicity.

Another single-agent cytotoxic chemotherapy that was tried is the novel thymidylate synthase inhibitor, nolatrexed. Results from studies showed that the drug has very limited activity in advanced HCC (Mok *et al.*, 1999; Stuart, Tessitore, Rudy, Clendennin, & Johnston, 1999). Even though nolatrexed has limited activity in advanced HCC, a phase III trial was conducted compared to the activity of nolatrexed to doxorubicin in unresectable HCC (Gish *et al.*, 2007). The study found that the median overall survival rate of nolatrexed was 22.3 weeks as against 32.3 weeks for doxorubicin ($p = 0.0068$) with a hazard ratio (HR) of 0.753 in favor of doxorubicin.

Loco-regional therapies and combination chemotherapies

Loco-regional therapy (Llovet *et al.*, 2002; Lo *et al.*, 2002), and systemic therapy (Leung & Johnson, 2001) becomes necessary in unresectable HCC. Even though transplantation is the ideal treatment option, trans-arterial embolization (TACE) has been the most employed loco-regional therapy because it demonstrates a survival advantage and remains the treatment of choice for intermediate-stage HCC (Llovet *et al.*, 1999; Llovet *et al.*, 2002). Based on this argument, TACE continued to be the standard first-line non-curative therapy for non-surgical/multifocal HCC for long a time. In practice, the embolic particles, lipoid, use, and type of chemotherapy that are used are heterogeneous, and the

same exists for the frequency of use. The type of chemotherapy used by a particular health facility and the team depends on the specific drug the physicians and radiologists are comfortable with. Doses are often extrapolated from previous experience other than being predetermined except that they remain in a certain determined safety margin.

Studies with TACE had been undertaken in intermediate-stage HCC patients with no metastasis, no main portal vein involvement, and good performance status (PS). Recently, studies with systemic therapy, as a combination therapy with sorafenib, have been undertaken in advanced HCC patients who would not have qualified on TACE only. The goal in such combination therapy studies is to establish the safety and efficacy of TACE and the novel targeted agent, sorafenib, on the bases of the five-year survival score. This study was also necessary because TACE could promote angiogenesis by increasing the levels of vascular endothelial growth factor (VEGF) (Georgiades, Hong, D'Angelo, & Geschwind, 2005). It was therefore clinically relevant that when the multi-kinase inhibitor was discovered, it be combined with TACE to determine whether it could reduce and/or alleviate this off-target effect of TACE completely.

Besides, the Raf/mitogen-activated protein kinase, the extracellular signal-related kinase (MEK), and the extracellular signal-related kinase (ERK) pathway are instrumental in HCC development (Wiesenauer, Yip-Schneider, Wang, & Schmidt, 2004). As discussed below, sorafenib is an inhibitor to both pathways. It is probably the fundamental scientific principle on which the National Cancer

Research Network coordinated the TACE-2 trials to evaluate the addition of sorafenib to TACE.

Chemotherapeutic combination therapies

Various combination chemotherapy regimens have been studied to be used in the treatment of advanced HCC. Cisplatin, doxorubicin, and 5- fluorouracil with or without interferon combination therapy have been investigated. (Leung *et al.*, 1999; Yeo *et al.*, 2005). Reports from that study are that such combinations have a modestly increased response rate (13–39%), but without any post-treatment survival enhancement than earlier known chemotherapeutic agents. Combination therapy of gemcitabine and oxaliplatin (GEMOX) for the treatment of advanced HCC have shown to have a 19% clinical response rate with a remarkable toxicity profile (Stuart *et al.*, 1999).

There are yet several other combination regimens that have not been validated in randomized trials and hence their clinical efficacy, safety profile, and survival rate are not known. It should be mentioned that all these studies were done with the knowledge that, combination therapies are often associated with relatively significant toxicity levels as opposed to single-agent therapies. For instance, a randomized controlled trial that compared doxorubicin and 5-fluorouracil, doxorubicin with cisplatin, and interferon-α-2β combination therapy in unresectable HCC, response rates were 10.5% and 20.9% respectively (Fukumoto & Ku, 2016). The disadvantage, yet, was that there was no statistically significant median survival advantage (6.83 and 8.67 months respectively) ($p = 0.83$) (Yeo *et al.*, 2005). Meanwhile, the combination regimen group reported

increased treatment-related toxicity over the single-agent regimen group. This limited the chance of clinical application of the combination regimen. In summary, though doxorubicin or doxorubicin-based combination therapies are widely used, no single of such combinations can be clearly defined as first-line treatment for advanced HCC.

The explanation that can be offered to these failures is that HCC cells are relatively well-differentiated and exist with various drug resistance mechanisms (Jiang, Lu, He, & Diasio, 1997). The presence of high levels of dihydropyrimidine dehydrogenase in HCC makes them resistant to 5-fluorouracil (Jiang *et al.*, 1997). HCC cells are also known to over-express the multidrug resistance gene (MDR1) (Chenivesse, Franco, & Bréchot, 1993) and for that matter the gene product P-glycoprotein (Soini, Virkajärvi, Raunio, & Pääkkö, 1996). This could be the basis for its total resistance to paclitaxel (Chao *et al.*, 1998), which uses P-glycoprotein as a substrate.

Alternative therapies

Apart from mainstream medicine, several other forms of treatment exist. In some jurisdictions, alternative medicine could be based on belief systems or a way of life. The National Cancer Institute defines alternative medicine as a treatment used instead of standard treatments (Institute, 2016). Angell & Kassirer defines alternative medicine as medical interventions with relatively little or no focus in U.S. medical school syllabus or generally prescribed at U.S. hospitals. (Angell & Kassirer, 1998). Treatment options that fall within this classification include; acupuncture, belief systems, imagery, therapeutic touch, magnetic field

therapy, herbal medicine, etc. One-year prevalence of alternative medicine usage is 40% and 20% in the United States and the United Kingdom respectively (Ernst, Cohen, & Stone, 2004), particularly herbal medicine. In China for instance, *Salvia miltiorrhiza* is added to food and eaten because it is believed to protect the liver (Wu *et al.*, 2019).

Herbal medicine

Records on the use of plants as medicines as seen in fossil records date back to 60,000 years ago (Solecki, 1975), even though traditional medical systems that use plants as a means of therapy were recorded a few years back. According to the records of WHO about 80% of the world's population have employed traditional medicine in their primary health care at some point (Martin, 2010). In several jurisdictions, the reliance on conventional medicine as the primary source of treatment is based on the assumption that they are less harmful and affordable. Notwithstanding, the assumed safety is a mere perception (Bussmann *et al.*, 2011; Déciga-Campos *et al.*, 2007; Ekor, 2014), natural products have played an instrumental role in drug discovery taking cues from knowledge gathered through ethnopharmacology.

The interest in herbal medicine and natural therapies has increased globally over the past ten to fifteen years (Ahmad *et al.*, 2006). Consequently, these natural therapies are available in drug stores, and food stores (Ahmad *et al.*, 2006; Bandaranayake, 2006). This therapeutic strategy was seen to be associated with only developing countries but cultural integration has increased its acceptance widely. Also, there is evidence that these practices are common in

Australia and North America (Anastasi, 2005; Anquez-Traxler, 2011; Committee, 2005).

Although this therapeutic option is gaining popularity, very few of these agents have been verified and therefore call for attention in that regard (Harvey, 2008). The authentication of such ethnopharmacology is relevant because a single herbal preparation may contain several pharmacologically active compounds which when co-administered, may be harmful to the user. The need to establish appropriate doses required to offer full therapeutic activity at tolerable toxicity parameters. The presence of particular compounds can result in drug-drug interactions, which may be antagonistic, and render a potent and safe compound inactive (Miliauskas, Venskutonis, & Van Beek, 2004). The consequences of this are that a significant number of prospective drug candidates may never be tested.

There are four fundamental reasons for which plants are used as a source of therapeutic agents. First is the possible isolation of compounds for direct use as drugs like; digoxin, digitoxin, morphine, reserpine among others (Rates, 2001). Secondly, to produce bioactive compounds, also known as scaffolds, of a novel or identified structures upon which semisynthetic and patentable entities of higher activity and/or lower toxicity are made like; metformin, nabilone, oxycodone, etc. (Gurib-Fakim, 2006). Furthermore, plants are used as pharmacologic tools, like yohimbine, mescaline, lysergic acid diethylamide, etc. (Raskin *et al.*, 2002) and finally, used as a whole or part of the plant in the preparation of a herbal remedy, e.g., echinacea, feverfew, cranberry, garlic, ginkgo biloba, etc (Raskin *et al.*, 2002) (Table 5). Drugs discovered using scaffolds from natural sources,

especially plants, are well accepted especially in developing countries (Singh, Sharma, Kanwar, & Kumar, 2016). Attributable reasons includes long time human affiliation to plants. The *Zanthoxylum zanthoxyloides* like most other plants have proven to have medicinal properties, yet very little studies have been conducted on this plant.

Table 5: Plants with hepatoprotective effects

Plant	Bioactivity	Reference
C. equisetifolia, C. cajan, G. pentaphylla, B. orellana, A. mexicana, P. minima and *C. bonduc*	Treatment Jaundice and cirrhosis	(Ahsan, Islam, Bulbul, Musaddik, & Haque, 2009)
Apium graveolens Linn	Jaundice, fibrosis and cirrhosis	(Ahmed, Alam, Varshney, & Khan, 2002; Karim & Bhatty, 1976)
Artemisia scoparia Waldst.et Kit	Anti-Fibrotic, anti-cirrhotic, and anti-HCC	(Gilani & Janbaz, 1994)
Bacopa monnieri Linn	Reverses paracetamol-induced liver damage and acute CCl_4 liver damage	(Allan, Damodaran, Deshmukh, Goudar, & Amit, 2007)
Balanites aegyptiaca Linn	Anti-cirrhotic and anti-HCC	(Jaiprakash, Karadi, Savadi, & Hukkeri, 2003)
Camellia sinensis Linn	Reverses paracetamol induced liver toxicity and acute CCl_4-induced liver injury	(Sengottuvelu, Duraisami, Nandhakumar, Duraisami, & Vasudevan, 2008)

Genus Zanthoxylum

The genus *Zanthoxyleae* belongs to the family Rutaceae, and consist of about 250 species, all deciduous and evergreen trees, and shrubs. They are native

to warm temperate and subtropical areas worldwide (Gregor, 1989). Their genus is among the subfamily Rutoideae.

Ethnobotany of the genus Zanthoxylum

The generic name *Zanthoxylum* is derived from the yellow heartwood of majority of species found in that genus (Devi, Rao, Bidalia, Wangkheirakpam, & Singh, 2015). Fruits from some species in the genus *Zanthoxylum* is used to make the spice Sichuan pepper (Devi *et al.*, 2015). About 90% of the species of this genus are known for significant economic importance. Some of them produce edible fruits, oils for various industrial and cosmetic purposes, wood for construction, and other raw materials for industries (Yang, 2008). Other ethnobotanical uses include use as herbal medicine, ornaments for aesthetic purposes, culinary applications, and in woodworking because of their satinwood (da Silva, Figueredo, & Yano, 2006). A classic example is a use of *Z. gillettii, Z. tessmannii, Z. lemairei, and Z. leprieurii* for building houses, carving drums, and construction of ship parts in Africa (Adesina, 2005). Other uses include carpentry work and use in the paper industry.

Bio-pharmacological potential of the genus Zanthoxylum

The genus *Zanthoxylum* is known for its phytochemical diversity. Through a bio-guided fractionation of ethyl acetate extract of the fruits of *Z. limonella,* xanthoxyline has been isolated and reported to have allelopathic properties (Charoenying, Teerarak, & Laosinwattana, 2010). Studies on the aqueous extract from the root bark of *Zanthoxylum zanthoxyloides* has been reported to exhibit analgesic properties through the inhibition of prostaglandin synthetase (Prempeh,

2008). In three different studies, ethanolic extracts from the root bark of *Z. fagara, Z. elephantiasis, Z. martinicense* (Villalba, Carmo, Leite, & Sousa, 2007) and *Z. coriaceum*, (Marquez *et al.*, 2005) and ethyl acetate, hexane, and ethanolic extracts from *Z. chiloperone* have shown anti-inflammatory activity (Bastos, Carvalho, de Souza, Pedrazzi, & Sarti, 2001)

Other demonstrated biological activity of species in the genus *zanthoxylum* include; antiparasitic properties (Ferreira *et al.*, 2011), antibacterial and cytotoxic activity (Misra, Wouatsa, Kumar, Kumar, & Tchoumbougnang, 2013), anti-mycobacterium property (Luo *et al.*, 2013), hepatoprotective activity (Ranawat, Bhatt, & Patel, 2010), gastroprotective activity (Freitas *et al.*, 2011), antifungal activity (Ngane, Biyiti, Zollo, & Bouchet, 2000), apoptotic induction activity (Li *et al.*, 2016), antioxidant activity (Kanwal, Arshad, Bibi, Asif, & Chaudhari, 2015), etc.

Anti-inflammatory properties of species in the genus Zanthoxylum

Various anti-inflammatory activities demonstrated with extracts and isolated compounds from species of the genus *Zanthoxylum* employed four methods. These include the carrageenan-induced paw edema in rats, arachidonic acid (AA), and 12-o-tetradecanoyl-phorbol acetate (TPA) ear edema in mice, phorbol myristate acetate (PMA), superoxide anion generation inhibition and elastase release in fMLP/CB-activated human neutrophils. Other studies have investigated the anti-inflammatory properties of extracts from the bark of *Z. elephantiasis*,(Villalba *et al.*, 2007) *Z. fagara, Z. coriaceum*, and *Z. martinicense*,

and the leaf of *Z. chiloperone* have presented promising activity (Villalba et al., 2007; Márquez et al., 2005; Bastos, 2001).

Other studies have investigated various secondary metabolites isolated from species of the genus *Zanthoxylum* for their anti-inflammatory activity. Dibenzylbutirolactonic lignan (cubebin) isolated from *Z. naranjillo* has presented potent anti-inflammatory properties (Bastos et al., 2001). Quinolone alkaloids, henzophenanthridine alkaloids, coumarins, and lignans isolated from the stem wood of *Z. nitidum* have been confirmed to have promising anti-inflammatory activity (Chen, Lin, Day, Hwang, & Chen, 2011). Phenylpropanoids and lignans isolated from the stem wood of *Z. integrifoliolum* (Chen, Wang, & Hwang, 2008) and coumarins, quinolone alkaloids, and quinoline alkaloids from *Z. avicennae* demonstrated anti-inflammatory potential (Chen, Chen, Liao, Huang, & Chen, 2007).

Lupeol is a dietary triterpene known to be present in almost all *Zanthoxylum* species and has generated global interest among medical professionals, pharmaceutical marketers, and researchers. This compound has been studied extensively and reported to have an inhibitory effect on inflammation in both *in vitro* and animal models of inflammation (Saleem, 2009).

Morphology of *Zanthoxylum zanthoxyloides*

Zanthoxylum zanthoxyloides is a shrub of about 50 ft high with 5 ft girth. It has a grey trunk covered with thick corky and woody thorns on its older branches, (Appiah, 2014). The leaves are pinnate, with 3-4 pairs of shiny aromatic

leaflets which are elliptic to oblong in shape. Each blade has a length of four and a half inches.

Geographical distribution of *Zanthoxylum zanthoxyloides*

In Ghana, the plant is commonly found in Achimota and Dodowa in the Greater Accra Region, Cape Coast in the Central Region, Afram Plains in the Eastern Region, and Wenchi in the Brong Ahafo Region. It is commonly referred to as *Fagara zanthoxyloides* or 'Candlewood'. In Ghana, it has many vernacular names such as Ɔkãntõ (Twi), Kantu (Fante), Xet, and Haatso (Ga) (CYPRIAN, 2011). *Z. Zanthoxyloides Lam* belongs to the same genus as *Z. amatum DC* whose leaf extract has been demonstrated to exhibit cytotoxicity (Ayim, Bayor, Phillips, Shnyder, & Wright, 2007). Also, Fagaroine is an alkaloid extracted from *Z. zanthoxylum* with a demonstrated anti-tumor activity (Messmer *et al.*, 1972). The root bark extract of *Zanthoxylum zanthoxyloides* has shown gastroprotective effects (Boye *et al.*, 2012) effect in Sprague-Dawley rats.

Classification of *Zanthoxylum zanthoxyloides*

Zanthoxylum zanthoxyloides (Lam.) belongs to the Kingdom Plantae. They are also members of the division Tracheophyta characteristically differentiated from other plants by the presence of vascular tissue for distribution of resources through the plant. They are dicotyledonous and so are classified among the class Magnoliopsida. It belongs to the order Sapindales consisting of 9 families, 460 genera, and about 5,700 species (Patiño, Prieto, & Cuca, 2008). They are known for their woody stems and temdril-bearing vines. Being polypetalous with hermaphrodite flowers, hypogynous, actinomorphic, and with a

disc below the ovary, it belongs to the family Rutaceae. They also have branched taproots often infected with fungus and branches are mostly thorny. They belong to the genus *Zanthoxylum* and scientifically related as *Zanthoxylum zanthoxyloides* (Lam.) (Ogwal-Okeng, Obua, & Anokbonggo, 2003)

Previous studies on *Zanthoxylum zanthoxyloides*

Zanthoxylum zanthoxyloides has many ethnomedicinal claims. Many studies have therefore been conducted to ascertain some of these claims. Below are some of the studies confirming or otherwise the medicinal claims of *Zanthoxylum zanthoxyloides*.

Anti-convulsant effects of *Zanthoxylum zanthoxyloides*

Methanol and aqueous extracts from leaves of *Z. capense* have also shown anticonvulsant activity (Amabeoku & Kinyua, 2010).

Anti-trypanosomal effects of *Zanthoxylum zanthoxyloides*

Ethanol extracts from leaves of Z. zanthoxyloides demonstrated significant anti-trypanosomal effect against *A. lumbricoides, H. contortus*, and *T. colubriformis* (Azando *et al.*, 2011)

Anti-microbial Effects of *Zanthoxylum zanthoxyloides*

Ethanol and chloroform extract of the root bark of *Zanthoxylum zanthoxyloides* have been reported to have antimicrobial activity (Ynalvez *et al.*, 2012). Essential oil from the fruit of *Zanthoxylum zanthoxyloides* has also been reported to have antimicrobial activity (Ngassoum *et al.*, 2003). Application of the root as a chewing stick has been reported regulate oral pathogen (Anne, Andrew,

& Idu, 2013). Oils from the root back of *Zanthoxylum zanthoxyloides* has also been reported to have antimicrobial activity (Tine *et al.,* 2017). Methanol extract of the stem bark of *Zanthoxylum zanthoxyloides* maintains balance in oral pathogens (Ouedraogo, Compaoré, Rouamba, Compaoré, & Kiendrebeogo, 2019).

Anti-fungal effects of *Zanthoxylum zanthoxyloides*

Ethanolic and choloform extract of the root bark of *Zanthoxylum zanthoxyloides* have been reported to have hyphal extension inhibition against *Trichoderma reesei* (Chaaib, Queiroz, Ndjoko, Diallo, & Hostettmann, 2003). Petroleum ether and 95% ethanol extracts of the root bark of *Zanthoxylum zanthoxyloides* has been confirmed to have an antifungal effect against *A. niger, Penicillium spp.,* and *C. albicans* with minimum concentration of 10 mg/ml (Boye, Koffour & Kumi-Ansah, 2013). Findings in this studies have been corroborated in an vitro studies with ethanolic extracts of leaves, stem bark and roots of *Zanthoxylum zanthoxyloides* against *C. albicans* at cocentrations raging from 4 mg/disc for the root and stem bark and 6 mg/disc for the leaves (Kosh-Komba *et al.*, 2017).

Anti-malarial effects of *Zanthoxylum zanthoxyloides*

An aqueous extract of *Zanthoxylum zanthoxyoides* has been reported to alleviate malaria symptoms in rats (Hermans, Akoègninou, & van der Maesen, 2004). In-vitro studies with the root bark and stem bark of *Zanthoxylum zanthoxyloides* have been reported to exhibit better anti-plasmodia effect than 10 µg/mL of chloroquine (Goodman *et al.,* 2019). Buesgenine and 6-

hydroxydihydrochelerythrine are alakloides isolated from the root bark of *Zanthoxylum zanthoxyloides* and have shown anti-plasmodia effect in vitro and in vivo (Wangensteen, An, Christopher, Diallo, & Karl, 2017). In an experimental malaria studies, it was found that a crude methanol extract of *Zanthoxylum zanthoxyloides* reduces parasitic load and improve the hematological and biochemical parameters of the mice (Enechi *et al.*, 2019).

Gastroprotective effects of *Zanthoxylum zanthoxyloides*

In an experimental gastroprotective study in rats, it was reported that ethanolic extract from the roor bark of *Zanthoxylum zanthoxyloides* significantly decreased the number of ulcers per stomach and the ulcerative index compared to Esomeprazole (Boye *et al.*, 2012).

Anti-inflammatory effects of *Zanthoxylum zanthoxyloides*

Anti-inflammation is common bioactivity of *Z. zanthoxyloides* (Villalba et al., 2007; Márquez et al., 2005; Bastos, 2001). Ethanol extract from the root-back of *Z. zanthoxyloides* exerts anti-inflammatory properties (Bhatt & Upadhyaya, 2010). Jao and co, conducted a study in 2014 to assess the anti-analgesic and anti-inflammatory properties of aqueous extract of *Z. zanthoxyloides* (Ojewole, 2004). Dihydroavicine, Chelerythrine, and Dihyrochelerythine are alkaloids isolated from the stem back, root bark, and leaves of *Z. zanthoxyloides* respectively, and have also demonstrated anti-inflammatory properties (Adesina, 2005). It has been confirmed that compounds with anti-inflammatory properties have also demonstrated anticancer effects especially against inflammation-related cancers such as HCC (Crusz & Balkwill, 2015).

Chapter summary

HCC is a multi-etiologic and that contributes significantly to its poor prognosis. Because the liver is able to function with only 40% of its mass, HCC is usually diagnosed in its advance stage where a significant proportion of the liver is destroyed. This makes treatment difficult and expensive. Available systemic treatments are also bedeviled with serious adverse effects that in most cases makes the treatment even more expensive. Natural products, particularly herbal sources, has played an important role in orthodox drug discovery. In China and Africa, herbal medicine remains part of the sorce of primary healthcare. The availability of the plant typre being investigated for pharmacological activity is critical and ethnopharmacological records very important. *Zanthoxylum zanthoxyloides* has ethnopharmacological records to which a majority of them have been scientifically validated. Some alkaloids that have been isolated from *Zanthoxylum zanthoxyoides* have been shown to have anticancer properties. It therefore serve a good fit to be investigated for an anti-HCC effect.

CHAPTER THREE

MATERIALS AND METHODS

Chapter overview

This chapter elaborates the various equipment, materials, and consumables that were used in this study. It further discusses the step-by-step methodologies adopted to achieve the research aim and objectives of this research.

Equipment

Equipment used in this study is listed in Appendix 1.

Chemical, reagent and drug

The chemicals and reagents used in this study were of analytical grade and are shown in Appendix 2.

Formulas

All formulas used in this study are listed in appendix 3

Preparation of solutions and reagents

All reagents that were used I this studies were of standard grades and solutions were prepared following standard procedures.

Phosphate buffered saline (PBS)

PBS was prepared from potassium chloride (KCl, 1.0 g), potassium dihydrogen phosphate (KH_2PO_4, 1.0 g), sodium chloride (NaCl, 40.0 g), hydrated

disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$, 14.4 g), and distilled water (1000 ml).

20% Tween20

This solution was made by mixing Tween20 (20 ml) and distilled water (80 ml), then stored in a refrigerator at 4 °C until use.

Harri's hematoxylin stain

Hematoxylin (1 g) was weighed and dissolved in 10 ml of ethanol and mixed with 20 gm ammonium alum dissolved in hot distilled water. The resultant mixture was boiled, 0.5 gm of mercuric oxide was added and filtered.

Eosin solution

Distilled water (80 mL) was measured and mixed with 320 ml of ethanol. 1 gm of yellow eosin was weighed and dissolved in the hydroalcoholic solution, and two drops of glacial acetic acid added.

Formaldehyde solution

Formaldehyde (70 mL) was measured and added to 630 ml of distilled water for a 10% formaldehyde solution (formalin). The solution was capped in an amber bottle until used.

Dragendorf reagent

Bismuth sub-nitrate (1.7 g) was dissolved in 20% glacial acetic acid, and 50% potassium iodide solution and mixed and store as a stock solution. 5ml of the

stock was added to 10ml glacial acetic acid and diluted to 50ml with distilled water to make the working solution.

Magnesium buffer

With the aid of a funnel, six tablets of magnesium-L-aspartate were transferred into 15 mL of 2-Amino-2-methyl-1- propanol D and Swirled gently to dissolve the tablets. The solution was aliquoted into clean analyzer bottles and store capped at 2–8°C until used.

Study design

Figure 4 shows the design for the study.

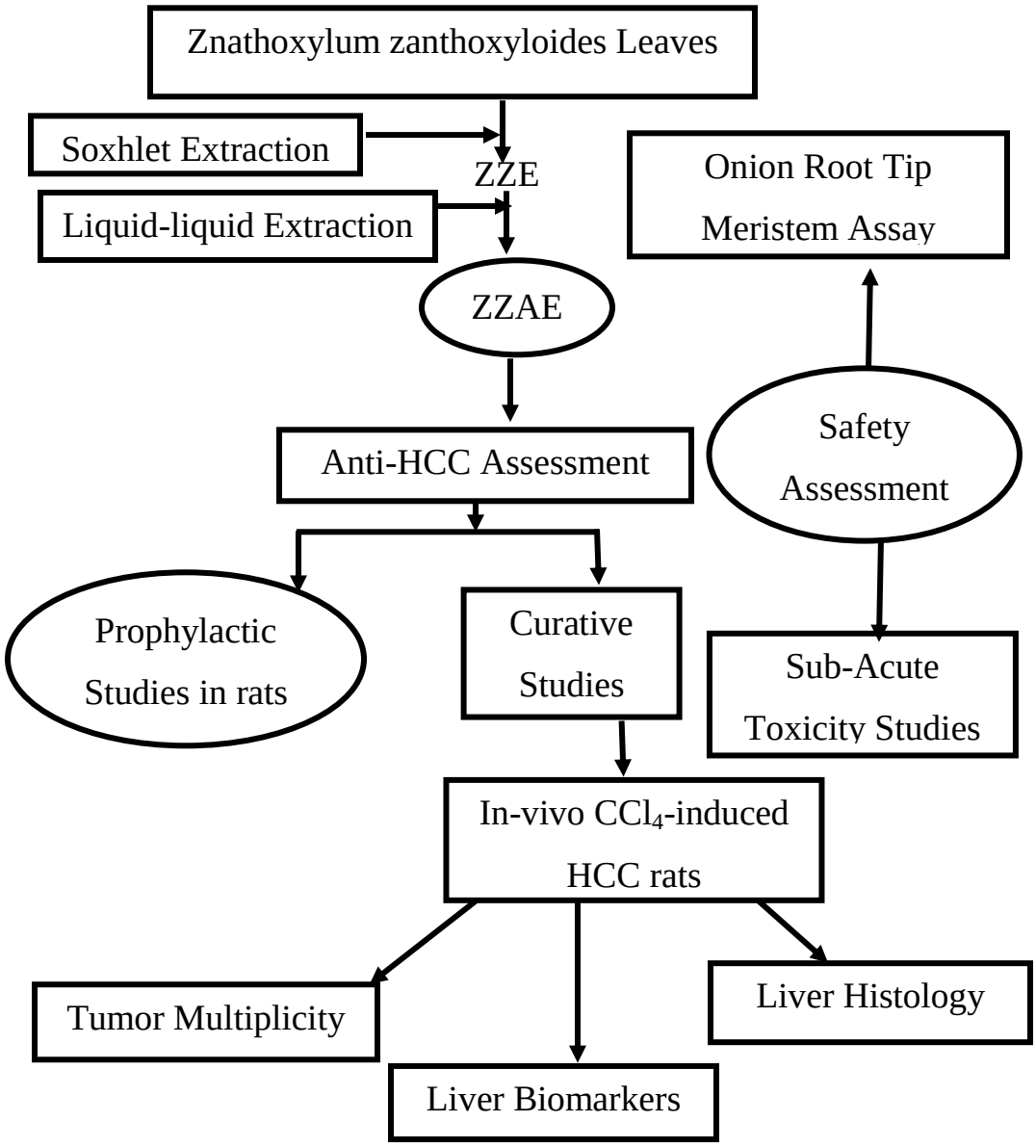

Figure 4: Flow chart of the adopted study design. ZZE = *Zanthoxylum zanthoxyloides* Extract, ZZAE = *Zanthoxylum zanthoxyloides* alkaloidal extract, HCC = Hepatocellular carcinoma, CCl_4 = Carbon Tetrachloride.

Collection, identification and authentication of *Zanthoxylum zanthoxyloides*

The aerial parts of *Zanthoxylum zanthoxyloides* were collected from Adisadel, a suburb in Cape Coast, Central Region of Ghana. The plant part was identified and authenticated by Mr. Francis Otoo, the Curator at the herbarium unit, School of Biological Sciences, University of Cape Coast, Ghana. A voucher specimen (SC/SBS/UCC/43BSH) was deposited at the herbarium for reference.

Preparation of *Zanthoxylum zanthoxyloides* extract

The collected plant material was thoroughly washed with tap water and air-dried for two weeks. The dried aerial part of the *Zanthoxylum zanthoxyloides* was pulverized using a hammer mill (Polymix Micro Hammer Cutter Mill, Glen Mills Inc, USA), and 4.37 kg of the resultant powdered leaves defatted with 7 L of petroleum ether (60- 80°C) and soxhlet extracted with 6 liters of 70% ethanol in a Soxhlet apparatus (L3 Soxhlet extractor, Ergotech Soxhlet Apparatus Co, UK). A 1,542 mL quantity of the extract was condensed in a temperature-controlled water bath (Premiere HH-4 Digital Water Bath, C & A Scientific Co Inc, USA). The crucible in a hot air oven (Oven 300 plus series, Gallenkamp, England) was maintained at a 40 °C for 24 h to yield 462.8 g (Percentage yield: 10.83%) of the dry extract which was named ZZE and will be referred to as such throughout the thesis.

Phytochemical analysis on ZZE

Qualitative phytochemical analysis was conducted on ZZE to determine the presence of the various phytoconstituents. Phytochemical analysis was carried out

by a procedure as previously reported (Evans, 2009; Tiwari, Kumar, Kaur, Kaur, & Kaur, 2011).

Test for Terpenoids

ZZE (0.5 mg) was mixed with 5 mL of chloroform and treated with concentrated sulphuric acid. A reddish-brown coloration of the interface indicated the presence of triterpenoids (Harborne, 1998).

Test for Carbohydrates

Molisch's reagent was added to 2 ml of aqueous ZZE. To this mixture, 2 mL of concentrated sulphuric acid was added and allowed to form a layer. The mixture was well shaken and made to stand for 3 mins and subsequently diluted with 5 mL of distilled water. The formation of a brown color indicated the presence of carbohydrates (Trease & Evans, 1989).

Test for Alkaloids

ZZE (1 mg) was dissolved in 5 mL diluted HCL and filtered. The solution was then treated with two drops of Dragendorff's reagent. The formation of an orange-red precipitate indicated the presence of alkaloids (Trease & Evans, 1989).

Test for Saponins

ZZE (0.5 g) was shaken with 2 mL of water in a test tube. Persistence of foam produced for ten minutes indicated the presence of saponins (Arunkumar & Muthuselvam, 2009).

Test for Tannins

To 2 mL of ZZE solution, 2 drops of 10% lead acetate were added and observed for the formation of a precipitate (Arunkumar & Muthuselvam, 2009).

Test for Phenols

ZZE (1 mg) was mixed with 5 ml Folin-Ciocalteu reagent and 4 ml of sodium carbonate. The tubes were vortexed for 15 sec and left undisturbed for 30 min at 40˚C for blue color development (Trease & Evans, 1989).

Test for Flavonoids

ZZE (0.5 g) was dissolved in water and filtered. To two (Consortium & 2) mL of the filtrate, 1 mL of lead acetate solution was added and observed for yellow-colored precipitate. (Kosalec, Bakmaz, Pepeljnjak, & Vladimir-Knezevic, 2004).

Confirmatory test for alkaloidal extract

ZZAE (0.5 mg) was dissolved in 5 mL of chloroform and 2 mL of the ZZAE solution treated with two drops of Dragendorff's reagent. The reaction endpoint color change was recorded.

Animal husbandry

Matured and healthy male Sprague-Dawley rats of body weight (180-200 g) were purchased from Noguchi memorial institute of medical research, University of Ghana.

Rats were kept in standard cages with sawdust as bedding in the School of Biological Sciences, University of Cape Coast animals' facility. Animals were

allowed two weeks to acclimatize with laboratory conditions before all experiments began. The beddings were changed three times a week. They were kept under 12 h light/dark cycle, average ambient temperature, and humidity. Animals were fed with standard pellet diet and given water *ad libitum*, and only varied to meet specific requirements of some experiments. They were humanely handled and treated by standard international guidelines as enshrined in the "Principles of laboratory animal care" (NIH publication No.85-23, revised 1985) as well as specific national and institutional requirements regarding the use of animals in scientific experimentation.

Induction, establishment and confirmation of experimental HCC

The animals were randomly divided into nine groups of 10 rats each. Group 1(control group): animals received only normal pellet died, and water, animals; Group 2-7: received CCl_4 in olive oil (1:1 *v/v*) (El Gomhorya Co, Cairo, Egypt; 2 ml/kg; *i.p*) for 14 weeks; Group 8: animals received 100 mg/kg ZZAE concurrently with CCl_4 reconstituted in olive oil (1:1 *v/v*) (El Gomhorya Co, Cairo, Egypt; 2 ml/kg; *i.p*) twice a week at 5 hours intervals for 14 weeks and group nine only 100 mg/kg of ZZAE for nine weeks. Throughout the induction period, animals were weighed once a week. One subject was randomly selected from each group 72 hours after the 14 weeks of induction, sacrificed and microscopically examined to confirm the establishment of HCC.

Experimental design

After the HCC induction process, surviving rats were assigned to various treatment groups. The control group received no induction and no treatment, the

model group was given HCC induction but received no treatment. The tween group was given HCC induction and was treated with 1 mL 20% tween20. The carvedilol group received HCC induction and was treated with 25 mg/kg of carvedilol (Coreg, Beximco USA). The 50 mg/kg group received HCC induction and was treated with 50 mg/kg ZZAE, the 100 mg/kg group received HCC induction and was treated with 100 mg/kg ZZAE and the 200 mg/kg group received HCC induction and was treated with 200 mg/kg ZZAE daily. The prophylaxis group received 100 mg/kg ZZAE concurrently with CCl_4 reconstituted in olive oil (1:1 v/v) (El Gomhorya Co, Cairo, Egypt; 2 ml/kg; i.p) for 14 weeks, and the ZZAE group received 100 mg/kg ZZAE only for ten weeks. Treatment lasted for ten weeks. A 72 hour washout period was allowed after the last day of the tenth week of treatment. After the 72 hours washout period, rats were euthanized and blood is drawn from left ventricles for hematological and Biochemical analysis. One lobe of the liver from each rat in each group was harvested and stored at a temperature of - 80 °C until use. The rest of the liver and other major organs were harvested and stored in formalin until later used.

Bodyweight measurements

The weight of each rats was measured once a week and the data was used to adjust the doses and estimate the liver to bodyweight ratios of treatment groups.

Liver-to-bodyweight ratio

The liver of each rat from each group was isolated and weighed, and then the liver-to-body weight proportion calculated using the formula; L = liver weight/body weight (Wenneker & Sussman, 1951).

Tumor multiplicity

The incidence and multiplicity of tumors were determined by gross examination of tumors. Tumors in the isolated liver from each rat were macroscopically counted by two independent investigators and the mean for each group calculated.

Effect of ZZAE on full blood count (FBC)

The FBC analysis was carried out with MAXM Analyzer C23644 - DxH 900 SMS + UPS (Beckman Coulter, Breau California, USA) following a previously described procedure with few modifications (Corash, 1983). Briefly, approximately 4 ml of blood samples were collected from each rat into EDTA tubes. The blood sample was swirled and the tube inserted into the MAXM analyser sample tube and the aspirator immersed into the tube. Approximately 20 µl of the blood was aspirated by the auto analyzer when the start button was pressed and the results displayed after about 30 secs.

Effect of ZZAE on liver enzymes of CCl_4/olive oil-induced HCC rats

The kidney function test was performed per a previously described method (Dunn, Qi, Bottinger, Breyer, & Sharma, 2004). Briefly, approximately 5 ml of blood was collected into a serum-sepearator tube and centrifuged at 4000 rpm for 8 mins with Z206-A compact centrifuge (Hermle Benchmark, Seul, South Korea). The serum was aspirated into cryotubes and diluted by a factor of 8. Approximately 20 ml of the diluted serum was aspirated into the column of a Multichannel Analyzer (Hitachi Model 917, Roche Diagnostics, IN). About 10 ml

of the diluted serum was aspirated into the auto analyzer when the astart command was given and the results displayed after about 30 secs.

Effect of ZZAE on kidney biomarkers of CCl_4/olive oil-induced HCC rats

The liver function test was performed per a previously described method (Dunn, Qi, Bottinger, Breyer, & Sharma, 2004). Pre-diluted serum was loaded into the S/P Brand Accutube Flange Caps (Cat. #T1226-37) and analyzed with an automated analyzer (Hitachi Model 917, Roche Diagnostics, IN). The parameters of interest were selected and a command given. Results were automatically generated and subject to statistical analysis.

Tissue processing and histology

Livers that had been pre-fixed in formaldehyde solution (formalin) were subjected to histological assessment per a previously described method (Ross & Pawlina, 2006) as illustrated below.

Dehydration

Each organ removed from the formalin was immersed into a series of ethanol (alcohol) solutions of increasing concentration until pure, water-free alcohol concentration was reached. This process was done to remove all water from the tissue and replaced it with alcohol. This process was done with a series of increasing concentrations of alcohol and at different duration, as listed below, to avoid excessive distortion of tissue.

1. 70% ethanol for 15 min
2. 90% ethanol for 15 min

3. 100% ethanol for 15 min
4. 100% ethanol for 15 min
5. 100% ethanol for 30 min
6. 100% ethanol for 45 min

Clearing

The tissues that have been dehydrated was immersed in a clearing agent, xylene, which is fully mixable with alcohol and also mixable with paraffin wax. This step was done to displace the ethanol in the tissue, because ethanol and paraffin wax are highly immiscible and to also offer an optical to the tissue due to their relatively high refractive index. This step was also done to remove as much as possible the amount of fat from the tissue that would have otherwise presented a barrier to wax infiltration. The clearing was done in multiple changes to displace ethanol in the sequence and duration below completely:

1. xylene for 20 min
2. xylene for 20 min
3. xylene for 45 min

Waxing

Paraffin wax was heated to 60°C for it to liquefy and infiltrated into the cleared tissue. The tissues were allowed to cool to 20°C to solidify to a consistency that allowed consistent sectioned at a regular thickness in ribbons as the sections are cut on the microtome, The sequence of wax infiltration is as below:

1. wax for 30 min
2. wax for 30 min
3. wax for 45 min

Embedding

Each tissue was carefully orientated into a histological mold based on the "plane of the section". Molting wax was poured on the tissue until the mold was half filled. A cassette was placed on top of the mold, and more molten was poured onto it to fill the whole tissue and placed on a cold plate to solidify. The solidified molds were removed from the cassettes and prepared for sectioning.

Sectioning

Tissues were sectioned using a microtome. A thermoregulatory Premiere HH-4 Digital Water Bath (C & A Scientific Co Inc, USA) was filled with water and set to 37 ^{0}C. The tissue blocks were placed face down on an ice bath for 10 min. The block was inserted into the microtome chuck with the wax facing the blade and aligned in the vertical plane. The microtome dial was set to cut 10 µm sections to plane the block after which the dial was set to 5 µm sections and cut into ribbons. With the aid of forceps, the ribbons were picked and floated on the surface of the 37 ^{0}C water bath (Banchroft, Stevens, & Turner, 1996).

Staining

The sections were flamed on a burner and placed in the xylene in the same sequence as before to deparaffinized the sections. The sections were rehydrated by washing them with a decreasing concentration of alcohol (100%, 90%, 80%, 70%). The rehydrated sections were then stained in hematoxylin for 5 minutes,

washed under running water until the color of the sections changed to blue. Subsequently, the sections were differentiated in 1% acid alcohol (1% HCl reconstituted in 70% alcohol) for 5 minutes. The sections were subsequently washed under running water and dipped in an alkaline solution (e.g. ammonia water) to turn blue and washed under running water.

The sections were then stained in Eosin Y (1%, for 10 min), washed under running water for 3 min, and dehydrated in increasing concentration of alcohols and clear in xylene. The sections were then mounted in mounting media and observed under the light microscope.

Cytotoxicity assay

The antimitotic activity of ZZAE was assessed using *Allium cepa* root meristematic cells as earlier described (Hemachandra & Pathiratne, 2016; SHRIVASTAVA *et al.*, 2016), with few modifications.

***Allium cepa* bulbs**

Allium cepa bulbs of approximately the same weight (40 ± 10) g were obtained from the University of Cape Coast Science market, Cape Coast. Central Region, Ghana.

Stimulation of *Allium cepa* root tip meristems

The outer scales of the bulbs were peeled off, and the dead root tips were carefully scraped off leaving the root primordia intact. The bulbs were then grown in a 24-hour dark cycle for 48 hours with the root meristems suspended on tap water at 20^{o}C until the roots had grown to approximately 3 cm. The water was

changed every 24 hours during this period. The viable bulbs were then selected for the subsequent studies.

Exposure of *Allium cepa* root tip meristems to ZZAE

Excess water on the onion bulbs root tips was gently cleaned with tissue paper and subsequently divided into five groups, containing five bulbs each. Group I: normal control, received tap water only; Group II: received 1.0 mg/mL of carvedilol, the standard drug, in water; Groups III and IV: ZZAE treatment, received ZZAE at 0.1 and 1.0 mg/mL in water. The bulbs were placed in individual containers filled with treatment baths immediately after blotting, as described above, and incubated at (22 ± 2) °C for 72 h, away from direct sunlight and the experimental treatment baths changed every 24 hours.

Data analysis

Data were analyzed using GraphPad Prism 7.00 software (GraphPad Software, San Diego, Califonia, U.S.A.). The data were expressed as means ± standard deviation of the mean (SD) of each group. Statistical significance was then determined using analysis of variance (ANOVA). Tests with a significant difference among groups were analyzed by a multiple comparison procedure using Bonferroni's multiple comparison tests. The level of significance was set at $p < 0.05$.

Chapter summary

In this chapter, the methodologies that were used to answer the research questions have been detailed. For the purposes of the objectives of this research,

some modifications were made in the extraction technology. Newer methods of testing the safety and genotoxic activity if pharmacological samples. It is also noteworthy that a 12 weeks twice a week intraperitoneal injection of CCl_4/olive oil established experimenta HCC in rats. The statistical tools and analysis used have were also explicitly detailed.

CHAPTER FOUR

RESULTS AND DISCUSSION

Chapter overview

This chapter talks about the results obtained from the experiments performed to answer the various specific objectives. The chapter further discusses how the results obtained are related to the work and how they have answered the research questions.

Extraction yield and phytochemical profiling

The hydroalcoholic Soxhlet extraction of 4,371.173 g of powdered *Zanthoxylum zanthoxyloides* leaves yielded a crude extract of 462.806 g (10.59%) (Table 6) and was referred to as ZZE. The ZZE was subjected to phytochemical assessment (Table 7). Alkaloidal extraction of ZZE yielded 119.342 g representing 2.73% of the crude ZZE and was referred to as ZZAE. The ZZE was further subjected to

Table 6: The yield of crude ZZE

Initial sample weight (g)	Yield of crude extract (g)	% yield of crude extract*	Yield of crude alkaloid (g)	% yield of crude alkaloid#
4,371.173	462.806	10.59	119.349	2.73

* crude extract yield (g) / dry leaves (g) × 100, # crude alkaloidal extract (g) / dry leaves of (g) × 100.

Table 7: Phytochemical analysis on ZZE

Constituent	Test	Remarks
Terpenoids	Salkowski test	+
Carbohydrates	Molisch's test	+
Alkaloids	Dragendorf's test	+
Saponins	Foam test	+
Tannins	Lead acetate test	+
Flavonoids	Sodium hydroxide test	+
Phenols	Folin-Ciocalteu	-

+ = present, - = absent

In vitro cytotoxic activity of ZZAE

The *A. cepa* root tip meristem exposed to distilled water group showed significant root tip growth. There was a significant *A. cepa* root tip meristem growth inhibition in both the carvedilol and the two dose fractions of ZZAE (0.1 mg/mL and 0.01 mg/mL). The 0.1 mg/mL carvedilol treated group showed a significant *A. cepa* root tip meristem growth inhibition than the ZZAE 0.1 mg/mL. The ZZAE (0.01 mg/mL) treated group showed the highest *A. cepa* root tip meristem growth inhibition. The *A. cepa* root tip meristem growth inhibition followed a dose-dependent pattern (Figure 5).

Figure 5: Assessment of cytotoxicity effect of ZZAE. (A) shows the extent of growth of *A. cepa* root tip meristems in water; (B) is the extent of growth of *A. cepa* root meristem in 1 mg/mL carvedilol showing mild mitotic inhibition; (C) is the *A. cepa* grown in 0.1 mg/mL of ZZAE showing mild mitotic inhibition and (D) is the *A.cepa* grown in 1 mg/mL ZZAE showing significant mitotic inhibition.

Effect of ZZAE on survival of CCl_4/olive oil-induced HCC rats

No death was recorded in the control group after the treatment period. The model group and the 20% tween20 groups both recorded a 40% survival rate at the end of the 25 weeks experimental period ($p < 0.05$). The prophylaxis group and the

ZZAE (200 mg/kg) group both recorded a 90% survival rate ($p < 0.05$). The ZZAE (50 mg/kg) group had a 60% survival rate (Figure 6).

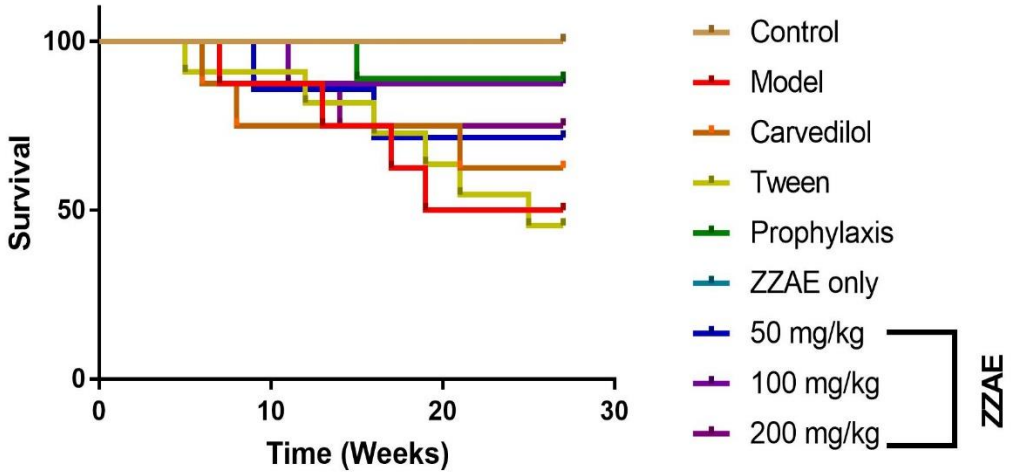

Figure 6: Effect of treatment on survival rate of CCl_4/olive oil-induced HCC rats.

Effect of ZZAE on bodyweight changes of CCl_4/olive oil-induced HCC rats

The control group had a 50% increase in mean bodyweight but the model group had a 14.7% increase in mean bodyweight at the same time period ($p < 0.05$) (Table 8**).** The ZZAE (100 mg/kg) had a 36.68% increase in mean bodyweight ($p < 0.05$), and was second to only the prophylaxis group which recorded a 40.79% increase in mean bodyweight.

Table 8: Effect of treatment on bodyweight

Treatment groups	Initial mean body weight (g)	Final mean body weight (g)	Change in mean body weight (g)	% Change in mean body weight
Control	182.851 ± 10.39	274.448 ±9.125	91.60 ± 9.002	50.1
Model	196.074 ±7.245	224.990 ± 4.93	28.92 ±8.357[a]	14.74
Carvedilol	174.803 ±5.39	227.275 ±3.224	52.472 ± 4.624[b]	30.01
20% Tween20[#]	177.005 ±3.689	209.336 ±4.579	32.331 ± 6.353	18.26
Prophylaxis*	179.717 ±6.915	253.016 ±5.921	73.299 ± 1.567[b]	40.79
ZAEE only	194.496 ±5.072	277.561 ±4.719	83.065 ± 8.261[b]	42.71
ZZAE (mg/kg)				
50	182.446 ±7.576	239.648 ±6.397	57.202 ± 7.788[b]	31.35
100	177.121 ±3.58	242.089 ±9.771	64.968 ± 9.753[b]	36.68
200	184.874 ±3.331	233.421 ±4.243	48.547 ± 3.24[b]	26.26

[#] received 20% tween20 (1 mL/rat/day) for 9 weeks after HCC induction, [*] received ZZAE (100 mg/kg) + CCl_4/olive oil (1 mg/kg/rat twice a week) for 14 weeks, [a] $p < 0.05$ (model vs control), [b] $p < 0.05$ (ZAE vs model).

Effect of treatments on liver weight of CCl_4/olive oil-induced HCC rats

In relation to the control group, the mean liver weight to bodyweight ratio of the model group was significantly high, far above the 2% to 3% range ($p < 0.05$). All ZZAE treated groups (50, 100, and 200 mg/kg) reduced the liver weight to bodyweight ratio ($p < 0.05$). The observed improvement in liver weight to bodyweight ratio was independent of the dose levels (Figure 7).

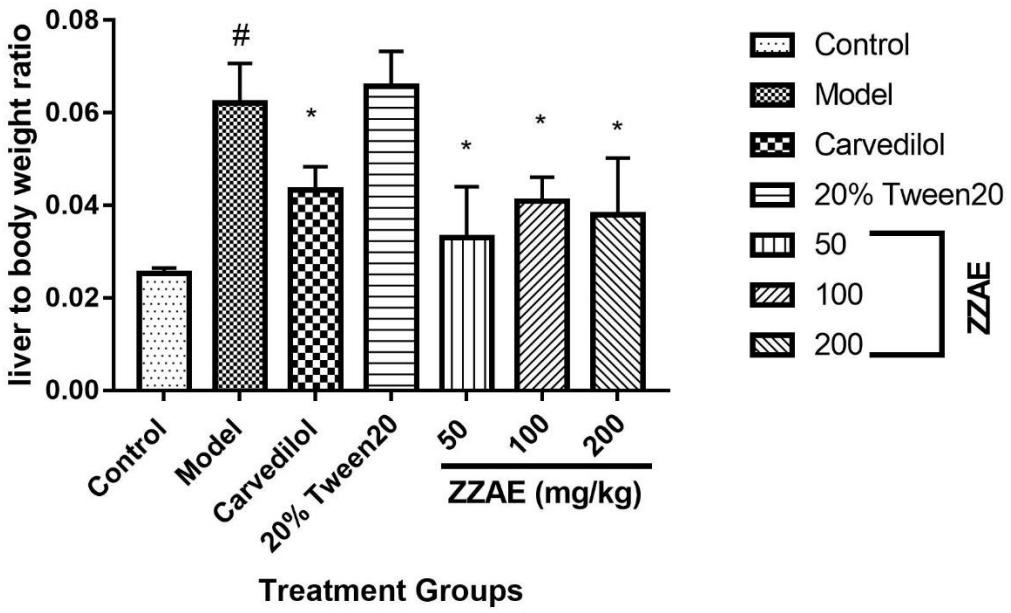

Figure 7: Effect of treatment on liver weight/bodyweight of CCl_4/olive oil-induced HCC rats. # $p < 0.05$ (model vs control), * $p < 0.05$ (ZZAE vs model). Each bar is the mean ± SD, n = 4.

Effect of treatment on tumor multiplicity of CCl_4/olive oil-induced HCC rats

The control group had normal liver morphology and size with no tumors (Figure 8). The model group and the 20% tween20 both more than 15 (± 3.5 and 2.8) respectively ($p < 0.05$) (Table 9). Treatment with ZZAE (50, 100, and 200 mg/kg) reduced the mean tumor multiplicity significantly ($p < 0.05$) with the ZZAE (200 mg/kg) being the most effective among all the treatment groups. The prophylaxis group developed fibrosis but could not progress to HCC and so recorded no tumors ($p < 0.05$)

Table 9: Effects of treatments on tumor incidence

Treatment groups	Tumor incidence[α]
Control	0 to 5 ± 0.0
Model	> 15 ± 3.5[a]
20% Tween20[#]	> 15 ± 2.8[b]
Carvedilol (6.25 mg/kg)	10 to 15 ± 2.8[b]
Prophylaxis*	0 to 5 ± 0.0[ns]
ZAEE only	0 to 5 ± 0.0[ns]
ZZAE (mg/kg)	
50	10 to 15 ± 2.3[b]
100	6 to 10 ± 0.8[b]
200	6 to 10 ± 1.7[b]

[#] received 20% tween20 (1 mL/rat/day) for 9 weeks after HCC induction, [*] received ZZAE (100 mg/kg) + CCl_4/olive oil (1 mL/kg/rat twice a week) for 14 weeks, [a] $p < 0.05$ (model vs control), [b] $p < 0.05$ (ZAE vs model). [α] was determined by finding the average of tumor counted by two independent researchers for each treatment group.

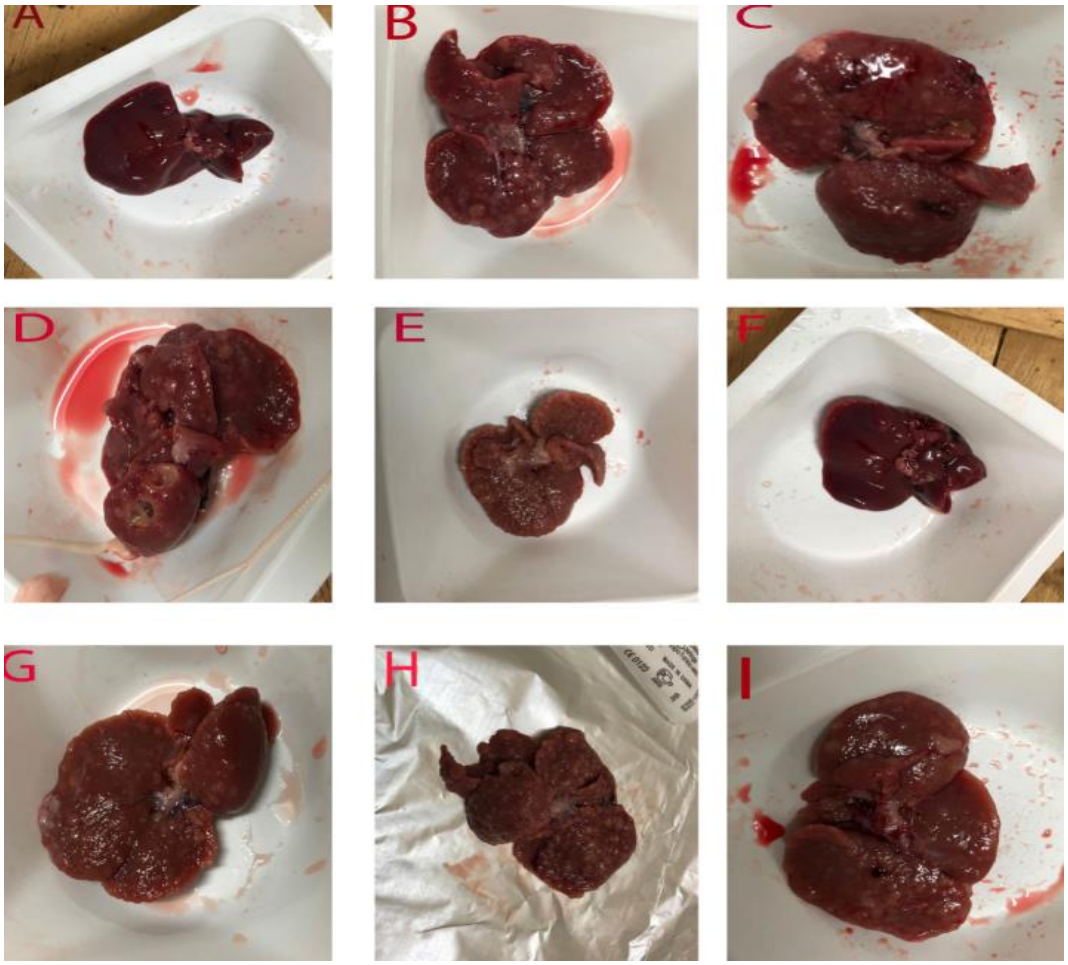

Figure 8: Effect of ZZAE on gross anatomy of livers of CCl_4-induced HCC rats. (A) Shows the control group with normal appearance, (B) the model group showed multiple tumors in the liver, (C) the carvedilol (6.25 mg/kg) group showed diffused tumors in the liver, (D) the 20% tween20 also showed multiple tumors diffused throughout the liver, (E) the prophylaxis group with no nodules but liver appeared fibrotic, (F) the 100 mg/kg ZZAE only showed normal liver appearance, (G) 50 mg/kg ZZAE showed relatively smaller tumors but diffusely distributed, (H) the 100 mg/kg ZZAE also with smaller tumors diffusely distributed, (I) is the 200 mg/kg ZZAE, showed smaller tumors in the liver.

Effect of ZZAE on liver histology of CCl_4/olive oil-induced HCC rats

In an H & E stained histological analysis, no histoarchitectural malformations were observed in the control group (Figure 9). The model group (Figure 9B), had diffused tumors surrounded by abnormal reticulin network and fatty pigments. Significant number of solitary cells in the model group showed homogeneous hypereosinophilic elements none of which were seen in the control. Histoarchitectural malformation of the 20% tween20 were virtually the same as that of the model (Figure 9D). After treatment with ZZAE (50, 100 and 200 mg/kg) there was a significant improvement in liver histology. Tumor number and sizes per field significantly reduced in a dose-dependent manner, in relation to the model (Figure 9G, Figure 9H and Figure 9I). The ZZAE (100 and 200 mg/kg) produced the best therapeutic outcome, even better than the reference drug (carvedilol 6,25 mg/kg) (Figure 9D).

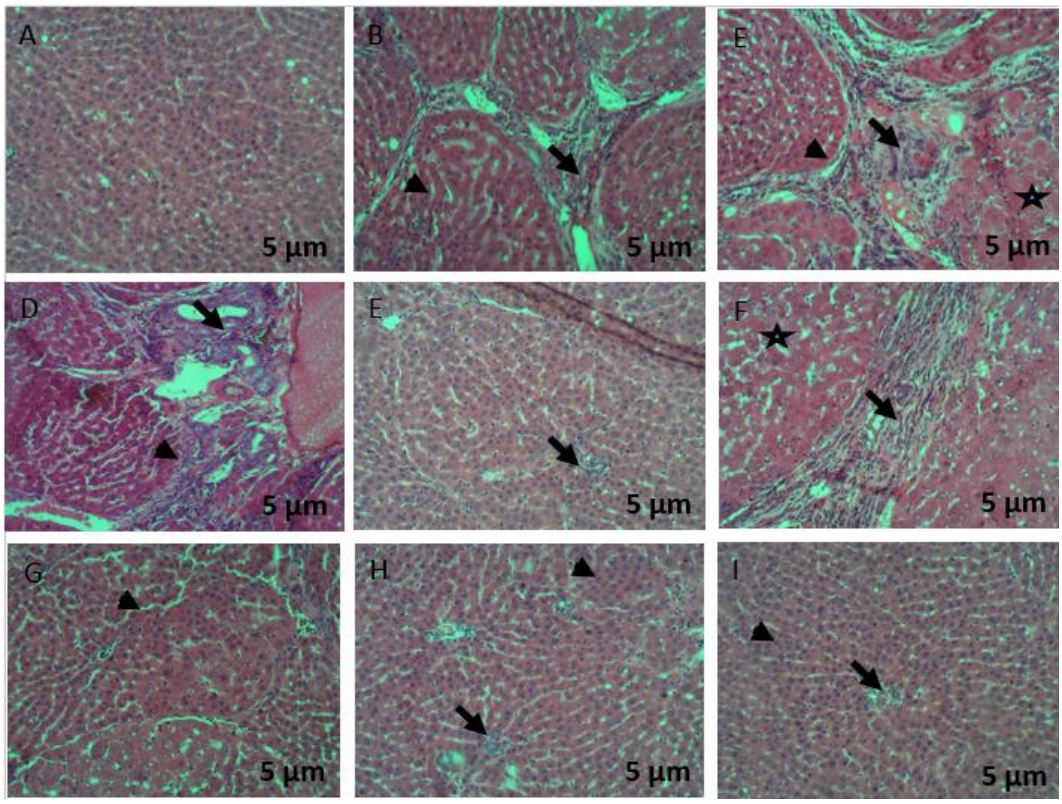

Figure 9: Effect of ZZAE CCl_4-induced HCC rats. (A) Control group, (B) Model group, (C) Carvedilol (6.25 mg/kg) group, (D) 20% tween20 group, € Prophylactic group, (F) ZZAE only group, (G) ZZAE (50 mg/kg) group, (H) ZZAE (100 mg/kg) group, (I) ZZAE (200 mg/kg) group. Arrowheads represent tumors, arrows represent necrotic cells, and stars represent fibrosis.

Effect of ZZAE on liver enzymes of CCl_4/olive oil-induce HCC rats

The mean serum ALP level of the control group was within physiological range. There was a significant elevation in mean serum level of ALP in the model group. ZZAE restored mean serum ALP level in a dose-dependent manner, but was statistically significant in only the ZZAE (100 and 200 mg/kg) groups (Figure 10). Mean serum albumin (g/L) was high in the model relative to the control. There was a significant restoration of mean serum albumin level in the carvedilol

(6.25 mg/kg) group and all the ZZAE (50, 100 and 200 mg/kg) groups (Figure 11). A similar results was recorded for the mean serum ALT, (Figure 12), mean serum AST, (Figure 13), and mean serum direct bilirubin, (Figure 14), as was in the mean serum total albumin. The ZZAE (50, 100, and 200 mg/kg) groups showed a dose-dependent restoration of mean serum total bilirubin (Figure 15), whilst the carvedilol (6.25 mg/kg) group did not show a significant reduction in mean serum levels of total protein. Mean serum level for GGT of the model group was substantially high compared to the control (igure 16). Treatment with ZZAE resulted in a significant reduction in mean serum levels of GGT for all dose levels of ZZAE. Reduction was also recorded in the carvedilol group. Mean serum level of total protein for the model was higher compared to the control, but was significantly reduced for all dose levels of ZZAE but not other treatment groups (Figure 17

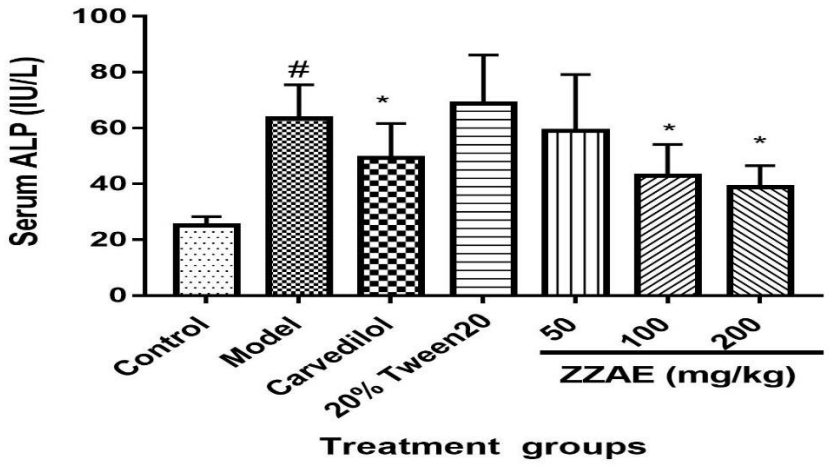

Figure 10: Effect of ZZAE on serum level of ALP of CCl_4/olive oil-induced HCC rats. # $p < 0.05$ (model vs control), * $p < 0.05$ (ZZAE vs model). Each bar is the mean ± SD, n = 4.

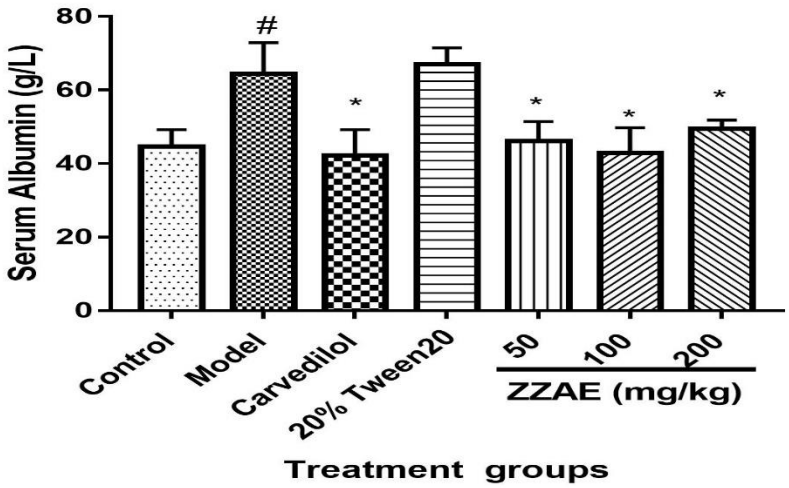

Figure 11: Effect of ZZAE serum level of albumin of CCl_4/olive oil-induced HCC rats. $^{\#}$ $p < 0.05$ (model vs control), * $p < 0.05$ (ZZAE vs model). Each bar is the mean ± SD, n = 4.

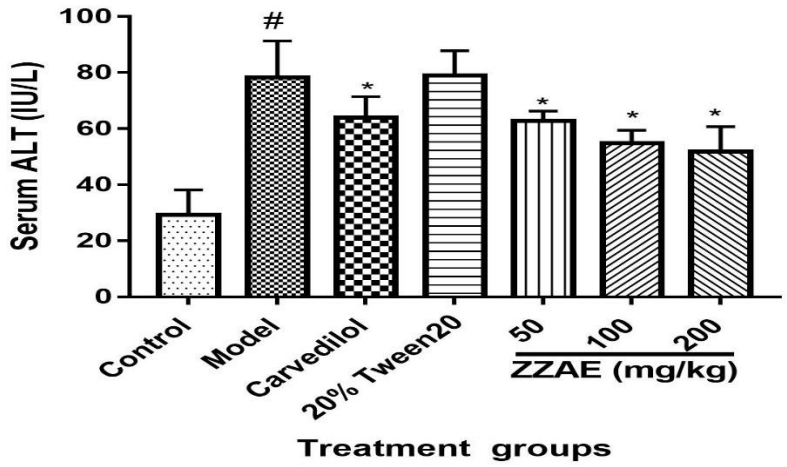

Figure 12: Effect of ZZAE on serum levels of ALT of CCl_4/olive oil-induced HCC rats. $^{\#}$ $p < 0.05$ (model vs control), * $p < 0.05$ (ZZAE vs model). Each bar is the mean ± SD, n = 4.

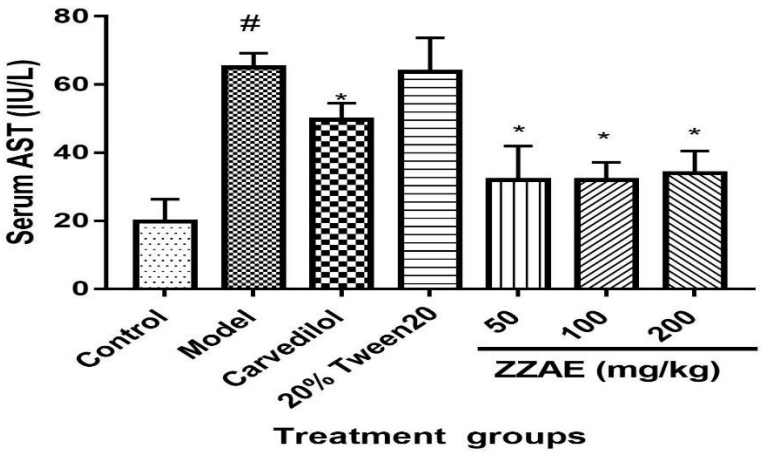

Figure 13: Effect of ZZAE on serum levels of AST of CCl_4/olive oil-induced HCC rats. # $p < 0.05$ (model vs control), * $p < 0.05$ (ZZAE vs model). Each bar is the mean ± SD, n = 4.

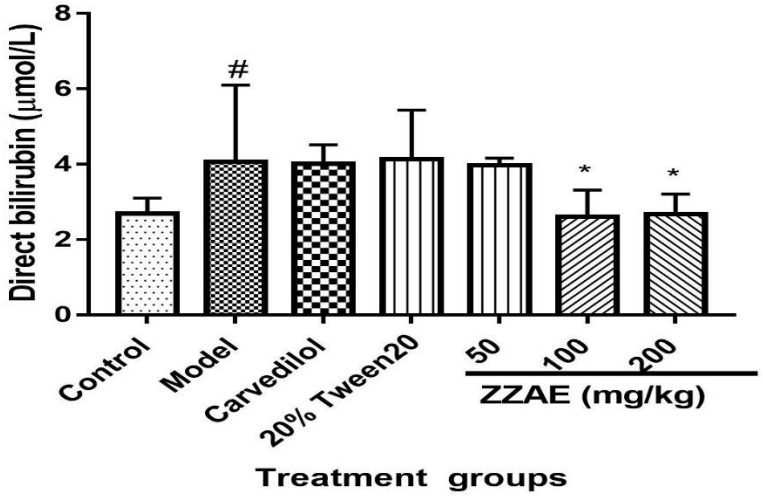

Figure 14: Effect of ZZAE on serum levels of direct bilirubin of CCl_4/olive oil-induced HCC rats. # $p < 0.05$ (model vs control), * $p < 0.05$ (ZZAE vs model). Each bar is the mean ± SD, n = 4.

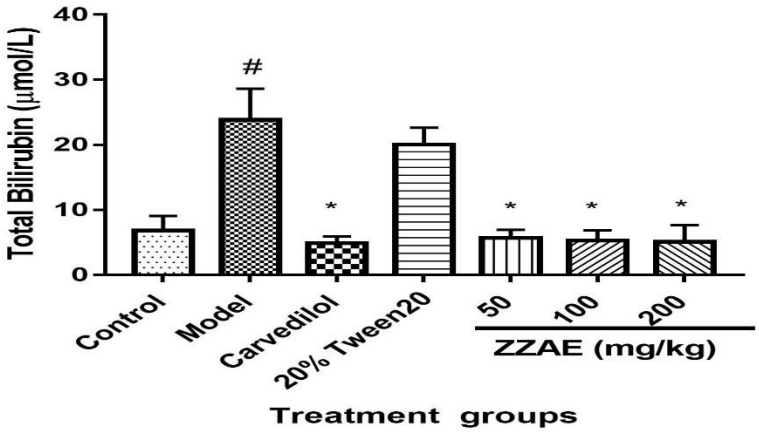

Figure 15: Effect of ZZAE on serum levels of total bilirubin of CCl_4/olive oil-induced HCC rats. # $p < 0.05$ (model vs control), * $p < 0.05$ (ZZAE vs model). Each bar is the mean ± SD, n = 4.

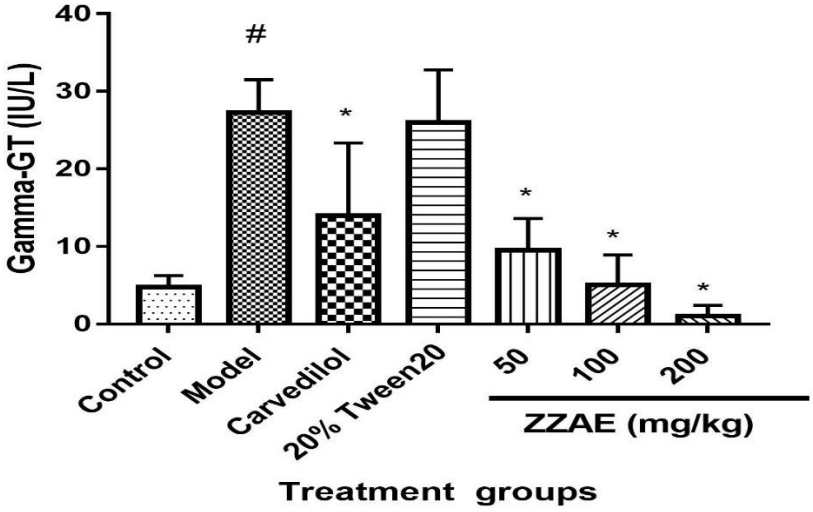

Figure 16: Effect of ZZAE on serum levels of GGT of CCl_4/olive oil-induced HCC rats. # $p < 0.05$ (model vs control), * $p < 0.05$ (ZZAE vs model). Each bar is the mean ± SD, n = 4.

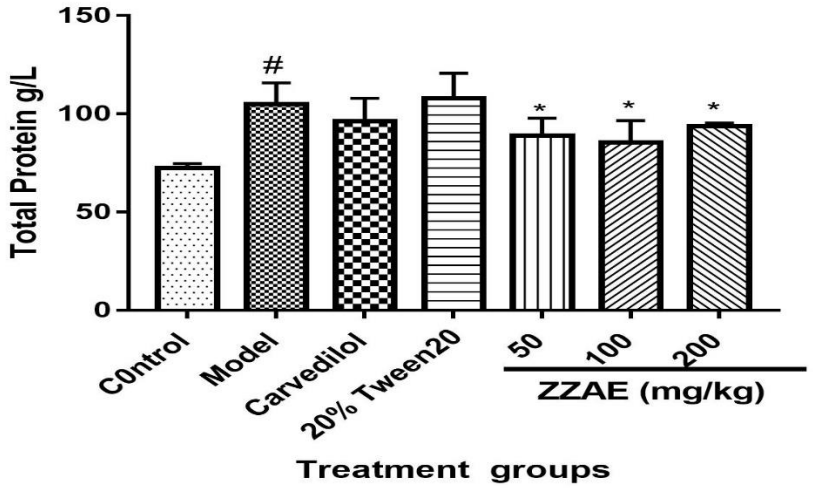

Figure 17: Effect of ZZAE on serum levels of total protein of CCl_4/olive oil-induced HCC rats. # $p < 0.05$ (model vs control), * $p < 0.05$ (ZZAE vs model). Each bar is the mean ± SD, n = 4.

Effect of ZZAE on kidney biomarkers of CCl_4/olive oil-induced HCC rats

Treatment had no effect on the mean serum chloride (Figure 18), and in the mean serum calcium (Ca^{2+}) (Figure 19) and serum potassium (K^+) (Figure 20). Treatment did nor have any siginificant difference in the mean serum sodium (Na^+) (Figure 21). Treatment had no significant difference in the mean serum pH of the various treatment groups (Figure 22). There was a significant increase in mean serum creatinine levels of the model group in relation to the control group (Figure 23) which reduced after treatment across all doses of ZZAE (50, 100, and 200 mg/kg group). The carvedilol group also had an improved mean serum creatinine levels. The model group and the 20% tween20 group recorded a very high mean serum urea levels (Figure 24). After treatment, all dose fractions of

ZZAE ZZAE (50, 100, and 200 mg/kg group) had a reduced mean serum urea levels.

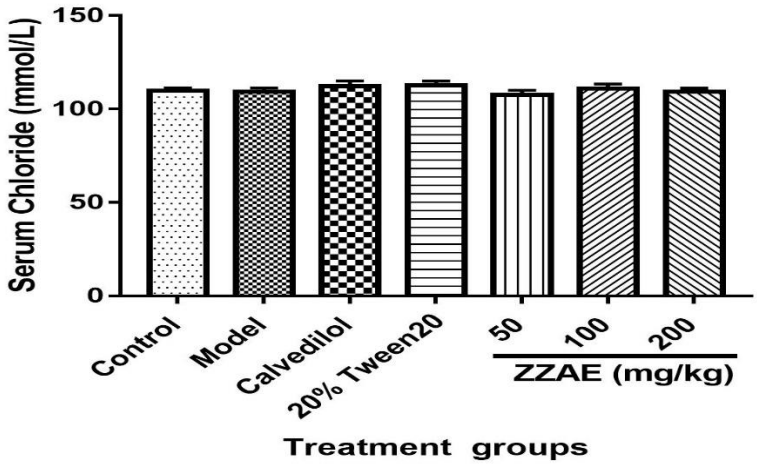

Figure 18: Effect of ZZAE on serum levels of Chloride (Cl^-) of CCl_4/olive oil-induced HCC rats. # $p < 0.05$ (model vs control), * $p < 0.05$ (ZZAE vs model). Each bar is the mean ± SD, n = 4.

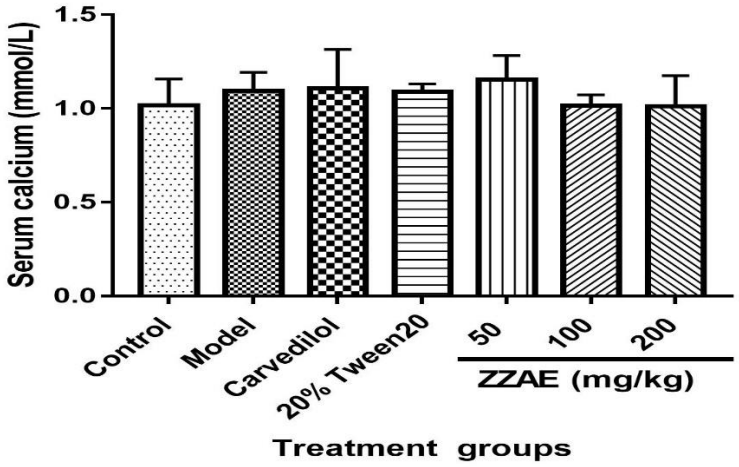

Figure 19: Effect of ZZAE on serum levels of calcium (Ca^{2+}) of CCl_4/olive oil-induced HCC rats. # $p < 0.05$ (model vs control), * $p < 0.05$ (ZZAE vs model). Each bar is the mean ± SD, n = 4.

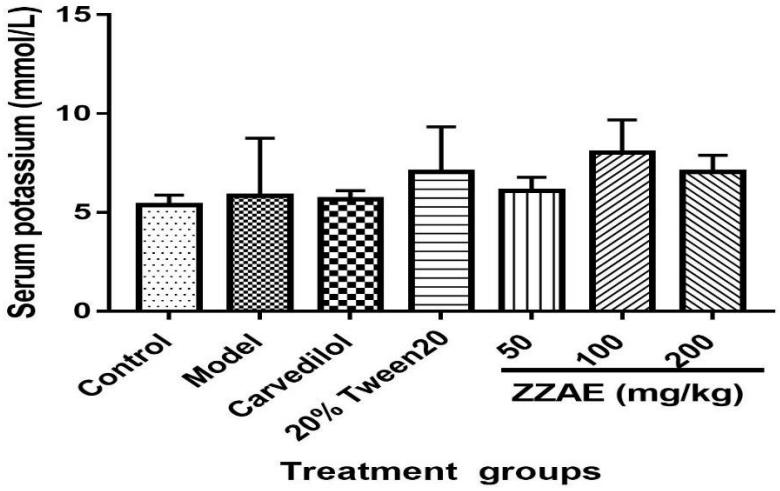

Figure 20: Effect of ZZAE on serum levels of potassium (K^+) of CCl_4/olive oil-induced HCC rats. # $p < 0.05$ (model vs control), * $p < 0.05$ (ZZAE vs model). Each bar is the mean ± SD, n = 4.

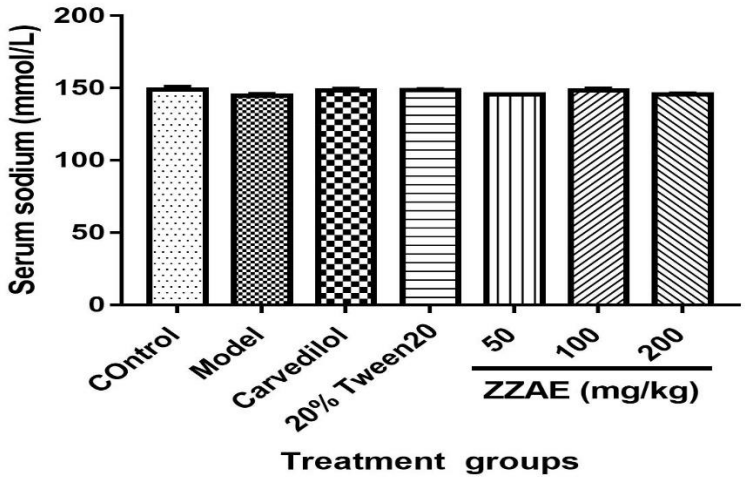

Figure 21: Effect of ZZAE on serum levels of sodium (Na^+) of CCl_4/olive oil-induced HCC rats. # $p < 0.05$ (model vs control), * $p < 0.05$ (ZZAE vs model). Each bar is the mean ± SD, n = 4.

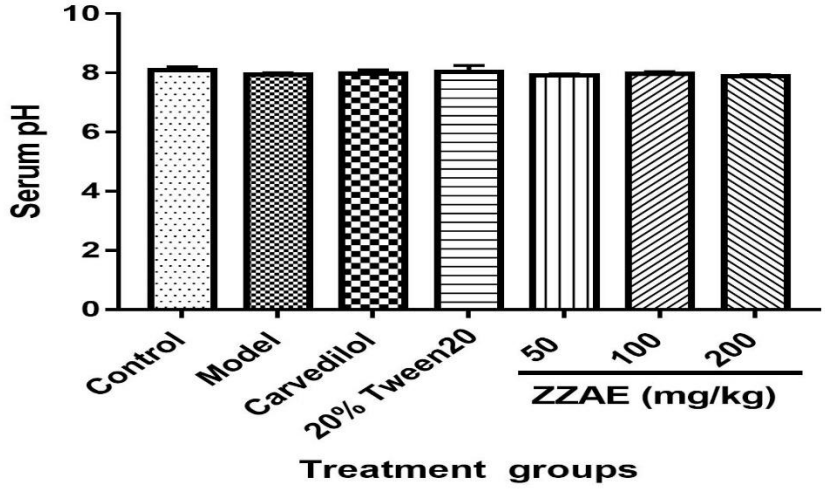

Figure 22: Effect of ZZAE on serum pH of CCl_4/olive oil-induced HCC rats. # $p < 0.05$ (model vs control), * $p < 0.05$ (ZZAE vs model). Each bar is the mean ± SD, n = 4.

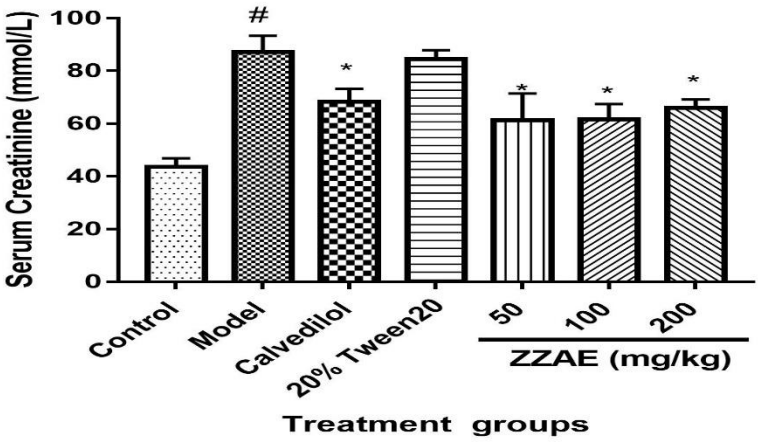

Figure 23: Effect of ZZAE on serum levels of creatinine of CCl_4/olive oil-induced HCC rats. # $p < 0.05$ (model vs control), * $p < 0.05$ (ZZAE vs model). Each bar is the mean ± SD, n = 4.

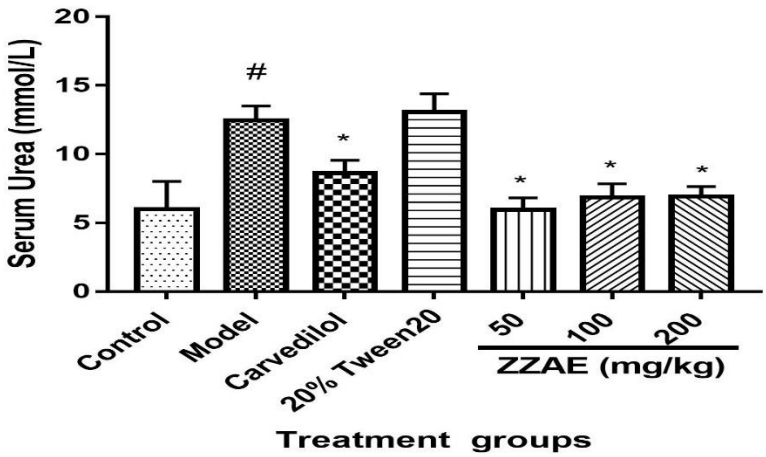

Figure 24: Effect of ZZAE on serum urea of CCl_4/olive oil-induced HCC rats. $^{\#}$ $p < 0.05$ (model vs control), * $p < 0.05$ (ZZAE vs model). Each bar is the mean ± SD, n = 4.

Effect of ZZAE on full blood count (FBC) of CCl_4/olive oil-induced HCC rats.

Treatment did not significantly affect mean serum levels of lymphocyte count (Figure 25), monocyte count (Figure 26), and RBC count (Figure 27) despite the high degree of within-group biological variations. However, the mean neutrophil count for the control group was within physiological range. In the model group, mean neutrophil count was lower than physiological range's lower limit but and was very high in the 20% tween20 group. This was the first parameters measured in the model group and the 20% tween20 were opposite of each other in this study (Figure 28). Treatment with ZZAE (50, 100, and 200 mg/kg) restored neutrophil count to within physiological range in a dose-dependent manner. The mean WBC count followed a similar pattern as that of the neutrophil count (Figure 29). The control group recorded within physiological

range of mean WBC count. The model group recorded very low mean WBC count but there was no significant difference in the mean WBC count between the control and the 20% tween20. Treatment with ZZAE (50, 100, and 200 mg/kg) restored the mean WBC count parallel to the control.

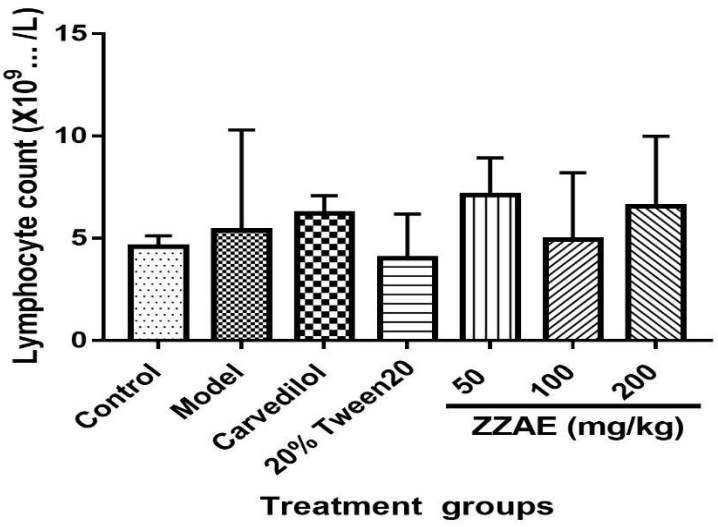

Figure 25: Effect of ZZAE on lymphocyte count of CCl_4/olive oil-induced HCC rats. $^{\#}$ $p < 0.05$ (model vs control), * $p < 0.05$ (ZZAE vs model). Each bar is the mean ± SD, n = 4.

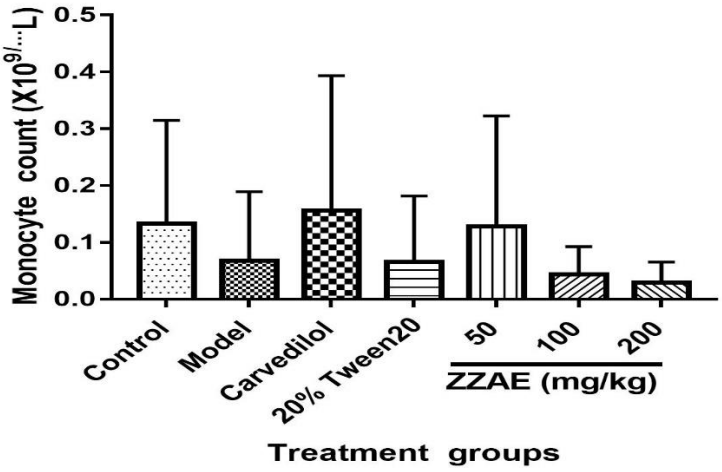

Figure 26: Effect of ZZAE on monocyte count of CCl_4/olive oil-induced HCC rats. # $p < 0.05$ (model vs control), * $p < 0.05$ (ZZAE vs model). Each bar is the mean ± SD, n = 4.

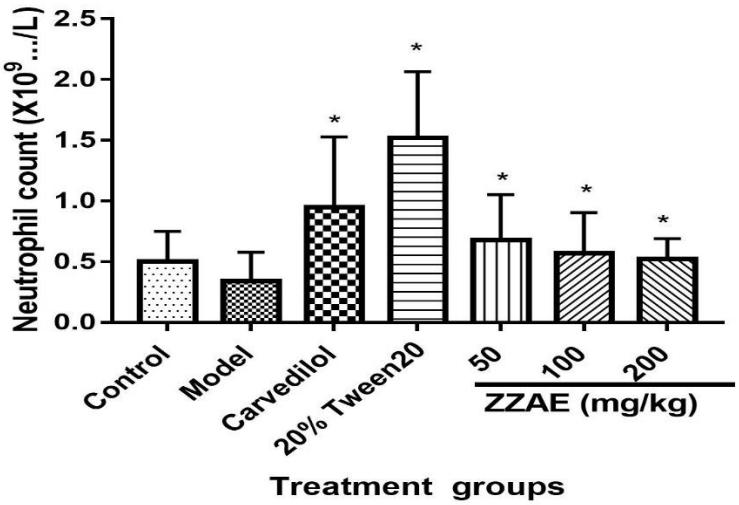

Figure 27: Effect of ZZAE on neutrophil count of CCl_4/olive oil-induced HCC rats. # $p < 0.05$ (model vs control), * $p < 0.05$ (ZZAE vs model). Each bar is the mean ± SD, n = 4.

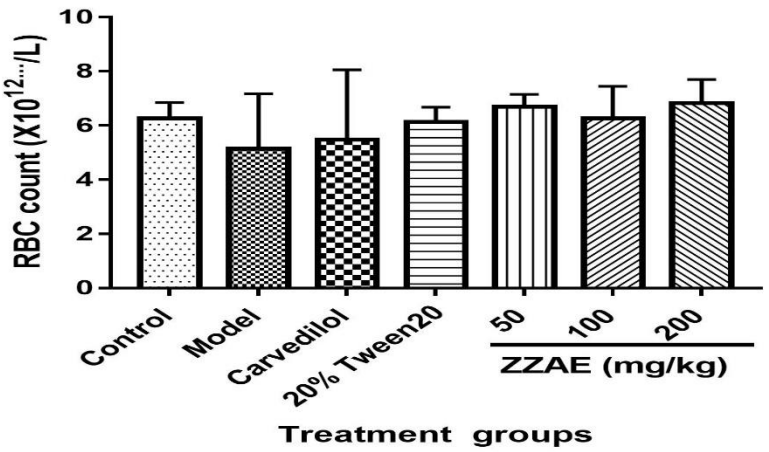

Figure 28: Effect of ZZAE on RBC count of CCl_4/olive oil-induced HCC rats. # p < 0.05 (model vs control), * p < 0.05 (ZZAE vs model). Each bar is the mean ± SD, n = 4.

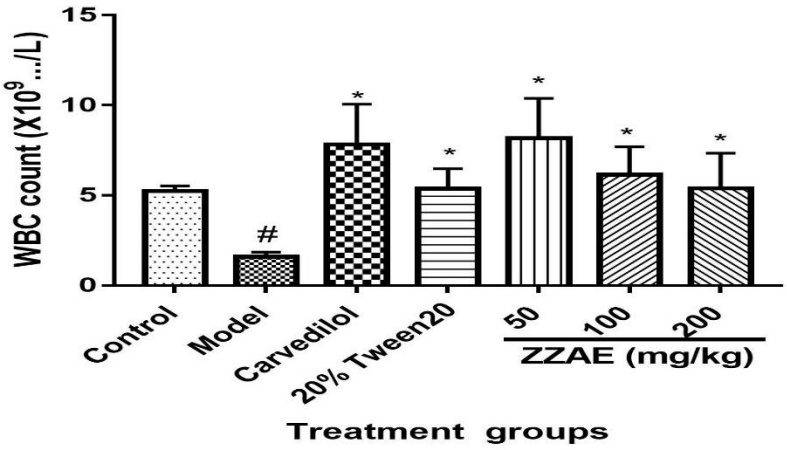

Figure 29: Effect of ZZAE on WBC count of CCl_4/olive oil-induced HCC rats. # p < 0.05 (model vs control), * p < 0.05 (ZZAE vs model). Each bar is the mean ± SD, n = 4.

Discussion

This study found that, 9 weeks daily oral treatment of ZZAE increases the survival rate, reduces tumor multiplicity, improves biochemical and hematological parameters and significantly restores liver histoarchitecture of CCl_4/olive oil-induced HCC rats. It has also been found that concurrent intraperitoneal administration 1:1 by volume of 1 mg/kg CCl_4/olive oil (twice/week) and oral administration of ZZAE (100 mg/kg, twice/week) at six hours intervals for 16 weeks prevents the establishment of CCl_4/olive oil-induced HCC in rats.

Though plants dominate in folklore medicine, much is still expected as far as contribution to the development of orthodox drugs is concerned (Mokgolodi, Hu, Shi, & Liu, 2011). For instance, out of the about 250 species in the genus *Zanthoxylum,* only a handful (including *Z. zanthoxyloides*) has ever been scientifically investigated for either their chemical components and /or therapeutic potential (Yuan *et al.*, 2015).

In this study, an hydroethanolic alkaloidal extract (ZZAE) from the leaves of *Z. zanthoxyloides* was investigated for its anti-HCC effects. Phytochemical assessment of ZZE revealed the presence of triterpenes, alkaloids, tannins, carbohydrates, phenols, saponins, and flavonoids. These phytochemicals findings were similar to those of earlier studies either on the leaves, root bark, and stem bark of the same plant (Adetuyi, Oyetayo, Popoola, & Lajide, 2016). This could also suggest that the phytochemicals of the different parts of the plant may not be entirely different. Phytochemicals are responsible for the bioactivity of plants used

in herbal medicines (Adedapo *et al*., 2008; Guzik, Korbut, & Adamek-Guzik, 2003; Kumar & Pandey, 2013).

CCl_4 is a well-known hepatotoxin widely used to induce liver injury in a wide range of laboratory animals (Polat *et al.*, 2016). CCl_4 in itself is not hepatotoxic, however, biotransformation of the parent molecule (CCl_4) yields $\bullet CCl_3$ which is highly reactive. The reactive species scavenge oxygen to produce trichloromethyl peroxy ($\bullet OOCCl_3$). This reduces oxygen partial pressure causing oxidative stress and increased production of DAG-O(O)H, leading to the PKCα-NF-kB mediated inflammation, fatty degeneration, and fibrosis in the liver (Fagone *et al.*, 2015; Zhou *et al.*, 2016). Chronic exposure to CCl_4 results in the establishment of HCC. CCl_4 induced HCC appears to by-pass cirrhosis and so reduces the duration for the establishment of HCC.

After the 9 weeks treatment, ZZAE improved general liver architecture and reduced tumor multiplicity compared to the control, vehicle, and the standard drug (6.25 mg/kg carvedilol). There was also a dose-dependent inhibition of inflammatory cells and/or inflammatory activity especially around the central vein for the 100 mg/kg and 200 mg/kg compared to the model and vehicle-treated group. A general liver biochemical biomarker improvement was also recorded in a dose-dependent manner. Interestingly, however, concurrent administration of ZZAE and CCl_4 respectively in a two-hour interval for 14 weeks prevented the establishment of experimental HCC in rats. The implication of this on this study could be that ZZAE may exert its anti-HCC effects by inhibiting the scavenging activity of $\bullet CCl_3$ to prevent the formation of $\bullet OOCCl_3$. It could also mean that ZZAE may

have acted by preventing ●$OOCCl_3$ from activating DAG and its isoform DAG-O(O)H which is the catalyst for the PKCα/NF-kB related inflammation. It is also possible that ZZAE may have acted as an antioxidant and scavenged the ●$OOCCl_3$ or DAG-O(O)H. most importantly, ZZAE may have acted through a combination of any of the mechanisms enumerated above.

Various anti-cancer drugs exhibit a cytotoxic effect through interference with cell-cycle stages or factors. The activities of such compounds are effective against fast-dividing cells by damaging the DNA during the S-phase of the cell cycle or blocks the formation of the mitotic spindle in M-phase (Gali-Muhtasib & Bakkar, 2002). Almost all plant-derived anticancer drugs exert their anticancer activity by affecting the microtubule dynamics of the cell (Amin, Gali-Muhtasib, Ocker, & Schneider-Stock, 2009). This results in the induction of persistent modification in biological processes and signaling pathways that ultimately lead to apoptotic death (Love *et al.*, 1992).

Allium cepa root tip meristems assay has been widely used for the evaluation of the cytotoxic, genotoxic, and anti-mitotic activity of various compounds (Tariq, Parmar, Qureshi, El-Feraly, & Al-Meshal, 1987). There was no cytotoxic activity recorded in the *Allium cepa* treated with water, however, there was a marginal to severe *Allium cepa* root tip meristem growth inhibition as the concentration of the ZZAE was varied from 0.01 mg/mL to 0.1 mg/mL.

Chapter summary

This research was designed to determine the anti-HCC effect of an alkaloidal extract (ZZAE). In this study, ZZAE has demonstrated a dose dependent anti-HCC effect in a 9 weeks treatment daily dose treatment period. Another significant revelation that was produced in this study is the prophylactic effect of ZZAE on CCl_4/olive oil HCC in rats. Analysis of the results showed that either ZZAE delays the establishment of CCl_4/olive oil-induced HCC or prevents its establishment altogether. It is most likely that the anti-HCC effect of ZZAE is exerted through multiple mechanisms, however, one that stands out the most is an interference withing the PKCα/NF-kB related inflammation pathway as is the mechanism CCl_4 uses to induce HCC.

CHAPTER FIVE

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

Chapter overview

This chapter is about the summary of the study, the conclusion based on the findings, and the suggestions for further studies to contribute to knowledge in the field of alternative drug discovery for cancer.

Summary

This study assessed the anti-hepatocellular carcinoma effects of an alkaloidal extract (ZZAE) derived from *Zanthoxylum zanthoxyloides* leaves. Preparation of ZZAE involved a hydro-ethanolicolic extraction to obtain a general crude extract and subsequently a liquid-liquid extraction to obtain the alkaloidal extract which was referred to as ZZAE. After a 9 weeks treatment period of experimental HCC rats with ZZAE, there was a general improvement in the liver architecture as demonstrated in liver macro anatomy and histology, as well as liver biomarkers. Concurrent treatment of ZZAE and CCl_4 reconstituted in olive oil for 15 weeks prevented the establishment of experimental HCC. CCl_4 induces HCC through the DAG/NF-κB inflammatory pathway, it was postulated that the ZZAE may have acted by targeting any of the intermediate second messengers to prevent the establishment of HCC in the prophylaxis group. Genotoxic activity of ZZAE was also performed using *Alleum cepa* root tip meristem assay. Results obtained from that study showed that ZZAE inhinbits *Alleum cepa* root tip meristem growth in a dose-dependent manner.

Conclusion

1. *Zanthoxylum zanthoxyloides* contains alkaloids that could be exploited for therapy against HCC.
2. ZZAE has cytotoxic effect towards *A. cepa* root tip meristems.
3. Exposure of rats to CCl_4 in olive oil for fourteen weeks produced experimental HCC in rats.
4. Concurrent treatment of CCl_4/olive oil and ZZAE for 16 weeks induced liver fibrosis but could not progress to experimental HCC in rats.
5. Treatment of CCl_4/olive oil-induced HCC in rats with ZZAE ameliorated HCC histologically and reduced liver injury biomarkers in a dose-dependent manner.
6. At the current doses of ZZAE, treatment with healthy rats for 9 weeks produced no observable renal or hepatic toxicity.

Recommendation

Future studies should:

1. extend the treatment period to determine whether there would be complete amelioration of CCL_4/olive oil-induced HCC with ZZAE.
2. measure some inflammatory and angiogenic biomarkers to offer a more substantive evidence for the treatment outcome.
3. should conduct the same study using commercially available cell lines to determine the treatment outcome in each case.

REFERENCE

Adesina, S. (2005). The Nigerian Zanthoxylum; chemical and biological values. *African Journal of Traditional, Complementary and Alternative Medicines, 2*(3), 282–301-282–301.

Adetuyi, A., Oyetayo, V., Popoola, A., & Lajide, L. (2016). Phytochemical and antibacterial screening of ethanol extracts of six dye plants. *Biosciences Biotechnology Research Asia, 2*(1), 41-44.

Ahmad, I., Aqil, F., & Owais, M. (2006). *Modern phytomedicine: Turning medicinal plants into drugs*: John Wiley & Sons.

Ahmed, B., Alam, T., Varshney, M., & Khan, S. A. (2002). Hepatoprotective activity of two plants belonging to the Apiaceae and the Euphorbiaceae family. *Journal of ethnopharmacology*, *79*(3), 313-316.

Ahsan, M. R., Islam, K. M., Bulbul, I. J., Musaddik, M. A., & Haque, E. (2009). Hepatoprotective activity of methanol extract of some medicinal plants against carbon tetrachloride-induced hepatotoxicity in rats. *Eur J Sci Res*, *37*(2), 302-310.

Akinyemiju, T., Abera, S., Ahmed, M., Alam, N., Alemayohu, M. A., Allen, C., . . . Artaman, A. (2017). The burden of primary liver cancer and underlying etiologies from 1990 to 2015 at the global, regional, and national level: results from the Global Burden of Disease Study 2015. *JAMA oncology*, *3*(12), 1683-1691.

Albain, K. S., Rusch, V. W., Crowley, J. J., Rice, T. W., Turrisi 3rd, A., Weick, J. K., . . . Gandara, D. R. (1995). Concurrent cisplatin/etoposide plus chest

radiotherapy followed by surgery for stages IIIA (N2) and IIIB non-small-cell lung cancer: mature results of Southwest Oncology Group phase II study 8805. *Journal of Clinical Oncology, 13*(8), 1880-1892.

Alexandrova, S., & Shvemberger, I. (2005). Genetic variability of the mouse hepatoma cells MH-22a revealed by RAPD-PCR-fingerprinting under different conditions of cultivation. *Exp. Oncol, 27*(2), 114-119.

Alkofer, B., Lepennec, V., & Chiche, L. (2011). Hepatocellular cancer in the non-cirrhotic liver. *Journal of visceral surgery, 148*(1), 3-11.

Allan, J. J., Damodaran, A., Deshmukh, N., Goudar, K., & Amit, A. (2007). Safety evaluation of a standardized phytochemical composition extracted from Bacopa monnieri in Sprague–Dawley rats. *Food and Chemical Toxicology, 45*(10), 1928-1937.

Allavena, P., Garlanda, C., Borrello, M. G., Sica, A., & Mantovani, A. (2008). Pathways connecting inflammation and cancer. *Current opinion in genetics & development, 18*(1), 3-10.

Amabeoku, G., & Kinyua, C. (2010). Evaluation of the anticonvulsant activity of Zanthoxylum capense (Thunb.) Harv.(Rutaceae) in mice. *Int. J. Pharmacol, 6*(6), 844-853.

Amin, A., Gali-Muhtasib, H., Ocker, M., & Schneider-Stock, R. (2009). Overview of major classes of plant-derived anticancer drugs. *International journal of biomedical science: IJBS, 5*(1), 1.

Anastasi, J. (2005). The use of complementary and alternative medicine by the american public.

Ande, S. R., Nguyen, K. H., Nyomba, B. G., & Mishra, S. (2016). Prohibitin-induced, obesity-associated insulin resistance and accompanying low-grade inflammation causes NASH and HCC. *Scientific reports, 6*, 23608.

Angell, M., & Kassirer, J. P. (1998). Alternative medicine—the risks of untested and unregulated remedies: Mass Medical Soc.

Anne, I. O., Andrew, O. O., & Idu, M. (2013). Phytochemistry and antimicrobial activity of Zanthoxylum zanthoxyloides root used as chewing stick in Nigeria. *The Journal of Phytopharmacology*, *2*(6), 1-7.

Anquez-Traxler, C. (2011). The legal and regulatory framework of herbal medicinal products in the European Union: a focus on the traditional herbal medicines category. *Drug information journal*, *45*(1), 15-23.

Appiah, C. O. (2014). *Dna Barcoding in Sustainable Harvesting of Tree Species and Safety of Herbal Medicines Used in The Treatment of Malaria in Southern Ghana.* University of Ghana.

Arunkumar, S., & Muthuselvam, M. (2009). Analysis of phytochemical constituents and antimicrobial activities of Aloe vera L. against clinical pathogens. *World Journal of Agricultural Sciences*, *5*(5), 572-576.

Arzumanyan, A., Friedman, T., Ng, I. O., Clayton, M. M., Lian, Z., & Feitelson, M. A. (2011). Does the hepatitis B antigen HBx promote the appearance of liver cancer stem cells? *Cancer research*, *71*(10), 3701-3708.

Arzumanyan, A., Reis, H. M., & Feitelson, M. A. (2013). Pathogenic mechanisms in HBV-and HCV-associated hepatocellular carcinoma. *Nature Reviews Cancer*, *13*(2), 123.

Atallah, M. A. A., Elaidy, S. M., & Tawfik, M. K. (2018). Assessment of the possible roles of SB-269970 versus ketanserin on carbon tetrachloride-induced liver fibrosis in rats: Oxidative stress/TGF-β1-induced HSCs activation pathway. *Pharmacological Reports, 70*(3), 509-518.

Autelli, R., Ullio, C., Prigione, E., Crepaldi, S., Schiavone, N., Brunk, U. T., . . . Bonelli, G. (2009). Divergent pathways for TNF and C2-ceramide toxicity in HTC hepatoma cells. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 1793*(7), 1182-1190.

Ayim, J., Bayor, M., Phillips, R., Shnyder, S., & Wright, C. (2007). The evaluation of selected Ghanaian medicinal plants for cytotoxic activites. *Journal of Science and Technology (Ghana), 27*(3), 16-22.

Azando, E., Hounzangbé–Adoté, M., Olounladé, P., Brunet, S., Fabre, N., Valentin, A., & Hoste, H. (2011). Involvement of tannins and flavonoids in the in vitro effects of Newbouldia laevis and Zanthoxylum zanthoxyloïdes extracts on the exsheathment of third-stage infective larvae of gastrointestinal nematodes. *Veterinary parasitology, 180*(3-4), 292-297.

Banchroft, J., Stevens, A., & Turner, D. (1996). Theory and practice of histological techniques. *Churchil Livingstone, New York, London, San Francisco, Tokyo*, 129-134.

Bandaranayake, W. M. (2006). Quality control, screening, toxicity, and regulation of herbal drugs. *Modern phytomedicine: turning medicinal plants into drugs*, 25-57.

Bartosch, B., Thimme, R., Blum, H. E., & Zoulim, F. (2009). Hepatitis C virus-induced hepatocarcinogenesis. *Journal of hepatology*, *51*(4), 810-820.

Bastos, J. K., Carvalho, J. C., de Souza, G. H., Pedrazzi, A. H., & Sarti, S. J. (2001). Anti-inflammatory activity of cubebin, a lignan from the leaves of Zanthoxyllum naranjillo Griseb. *Journal of ethnopharmacology*, *75*(2-3), 279-282.

Baumhueter, S., Courtois, G., & Crabtree, G. R. (1988). A variant nuclear protein in dedifferentiated hepatoma cells binds to the same functional sequences in the beta fibrinogen gene promoter as HNF-1. *The EMBO journal*, *7*(8), 2485-2493.

Baydar, T., Engin, A. B., Girgin, G., Aydin, S., & Sahin, G. (2005). Aflatoxin and ochratoxin in various types of commonly consumed retail ground samples in Ankara, Turkey. *Annals of Agricultural and Environmental Medicine*, *12*(2), 193-197.

Beckebaum, S., Zhang, X., Chen, X., Yu, Z., Frilling, A., Dworacki, G., . . . Cicinnati, V. R. (2004). Increased levels of interleukin-10 in serum from patients with hepatocellular carcinoma correlate with profound numerical deficiencies and immature phenotype of circulating dendritic cell subsets. *Clinical Cancer Research*, *10*(21), 7260-7269.

Bennett, J. (1987). Mycotoxins, mycotoxicoses, mycotoxicology andMycopathologia: Springer.

Bhandari, P. R. (2015). Crocus sativus L.(saffron) for cancer chemoprevention: a mini review. *Journal of traditional and complementary medicine, 5*(2), 81-87.

Bhatt, N., & Upadhyaya, K. (2010). Anti inflammatory activity of ethanolic extract of bark of Zanthoxylum armatum DC. *Pharmacology online, 2*, 123-132.

Bode, J. G., Albrecht, U., Häussinger, D., Heinrich, P. C., & Schaper, F. (2012). Hepatic acute phase proteins–regulation by IL-6-and IL-1-type cytokines involving STAT3 and its crosstalk with NF-κB-dependent signaling. *European journal of cell biology, 91*(6-7), 496-505.

Bosetti, C., Levi, F., Boffetta, P., Lucchini, F., Negri, E., & La Vecchia, C. (2008). Trends in mortality from hepatocellular carcinoma in Europe, 1980-2004. *Hepatology, 48*(1), 137-145.

Bouattour, M., & Wassermann, J. (2014). Molecular targeted drugs under investigation in hepatocellular carcinoma. *Frontiers in Anti-Cancer Drug Discovery. Bentham Science*, 39-87.

Boye, A., Koffour, G., A., Penu, D., K., A., & Kumi-Ansah, F., (201). Anti-fungal activity of an ethanolic root bark extract of zanthoxylum zanthoxyloides. *GHANA JOURNAL OF SCIENCE*, 53(1), 23-30

Boye, A., Koffuor, G. A., Boampong, J. N., Amoateng, P., Ameyaw, E. O., Owusu Ansah, E., . . . Penu, D. K. A. (2012). Gastroprotective effect and safety assessment of Zanthoxylum Zanthoxyloides (Lam) Waterm root bark extract.

Boye, A., Yang, Y., Asenso, J., & Wei, W. (2016). Anti-fibro-hepatocarcinogenic Chinese herbal medicines: a mechanistic overview. *Journal of intercultural ethnopharmacology, 5*(3), 278.

Braeuning, A., Gavrilov, A., Geissler, M., Wenz, C., Colnot, S., Templin, M. F., . . . Schwarz, M. (2016). Tumor promotion and inhibition by phenobarbital in livers of conditional Apc-deficient mice. *Archives of toxicology, 90*(6), 1481-1494.

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., & Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians, 68*(6), 394-424.

Bressac, B., Kew, M., Wands, J., & Ozturk, M. (1991). Selective G to T mutations of p53 gene in hepatocellular carcinoma from southern Africa. *Nature, 350*(6317), 429.

Brock, K. K., & Dawson, L. A. (2010). *Adaptive management of liver cancer radiotherapy.* Paper presented at the Seminars in radiation oncology.

Budhu, A., Forgues, M., Ye, Q.-H., Jia, H.-L., He, P., Zanetti, K. A., . . . Tang, Z.-Y. (2006). Prediction of venous metastases, recurrence, and prognosis in hepatocellular carcinoma based on a unique immune response signature of the liver microenvironment. *Cancer cell, 10*(2), 99-111.

Budhu, A., & Wang, X. W. (2006). The role of cytokines in hepatocellular carcinoma. *Journal of leukocyte biology, 80*(6), 1197-1213.

Bussmann, R., Malca, G., Glenn, A., Sharon, D., Nilsen, B., Parris, B., . . . Martinez, M. (2011). Toxicity of medicinal plants used in traditional medicine in Northern Peru. *Journal of ethnopharmacology, 137*(1), 121-140.

Cavalloni, G., Peraldo-Neia, C., Sassi, F., Chiorino, G., Sarotto, I., Aglietta, M., & Leone, F. (2016). Establishment of a patient-derived intrahepatic cholangiocarcinoma xenograft model with KRAS mutation. *BMC cancer, 16*(1), 90.

Chaaib, F., Queiroz, E. F., Ndjoko, K., Diallo, D., & Hostettmann, K. (2003). Antifungal and antioxidant compounds from the root bark of Fagara zanthoxyloides. *Planta Medica, 69*(04), 316-320.

Chao, Y., Chan, W., Birkhofer, M., Hu, O.-P., Wang, S., Huang, Y., . . . Lui, W. (1998). Phase II and pharmacokinetic study of paclitaxel therapy for unresectable hepatocellular carcinoma patients. *British journal of cancer, 78*(1), 34.

Chapuy, B., Cheng, H., Watahiki, A., Ducar, M. D., Tan, Y., Chen, L., . . . Zhang, L. (2016). Diffuse large B-cell lymphoma patient-derived xenograft models capture the molecular and biological heterogeneity of the disease. *Blood, 127*(18), 2203-2213.

Charoenying, P., Teerarak, M., & Laosinwattana, C. (2010). An allelopathic substance isolated from Zanthoxylum limonella Alston fruit. *Scientia horticulturae, 125*(3), 411-416.

Chen, C.-J., Yang, H.-I., Su, J., Jen, C.-L., You, S.-L., Lu, S.-N., . . . Group, R.-H. S. (2006). Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. *Jama*, *295*(1), 65-73.

CHEN, C. J., YU, M. W., & LIAW, Y. F. (1997). Epidemiological characteristics and risk factors of hepatocellular carcinoma. *Journal of gastroenterology and hepatology*, *12*(9-10), S294-S308.

Chen, J.-J., Chen, P.-H., Liao, C.-H., Huang, S.-Y., & Chen, I.-S. (2007). New phenylpropenoids, bis (1-phenylethyl) phenols, bisquinolinone alkaloid, and anti-inflammatory constituents from Zanthoxylum integrifoliolum. *Journal of natural products*, *70*(9), 1444-1448.

Chen, J.-J., Lin, Y.-H., Day, S.-H., Hwang, T.-L., & Chen, I.-S. (2011). New benzenoids and anti-inflammatory constituents from Zanthoxylum nitidum. *Food chemistry*, *125*(2), 282-287.

Chen, J.-J., Wang, T.-Y., & Hwang, T.-L. (2008). Neolignans, a coumarinolignan, lignan derivatives, and a chromene: anti-inflammatory constituents from Zanthoxylum avicennae. *Journal of natural products*, *71*(2), 212-217.

Chenivesse, X., Franco, D., & Bréchot, C. (1993). MDR1 (multidrug resistance) gene expression in human primary liver cancer and cirrhosis. *Journal of hepatology*, *18*(2), 168-172.

Chia, C. S., Ban, K., Ithnin, H., Singh, H., Krishnan, R., Mokhtar, S., . . . Seow, H. F. (2002). Expression of interleukin-18, interferon-γ and interleukin-10 in hepatocellular carcinoma. *Immunology letters*, *84*(3), 163-172.

Chisari, F. V., Klopchin, K., Moriyama, T., Pasquinelli, C., Dunsford, H. A., Sell, S., . . . Palmiter, R. D. (1989). Molecular pathogenesis of hepatocellular carcinoma in hepatitis B virus transgenic mice. *Cell, 59*(6), 1145-1156.

Chuang, S.-C., La Vecchia, C., & Boffetta, P. (2009). Liver cancer: descriptive epidemiology and risk factors other than HBV and HCV infection. *Cancer letters, 286*(1), 9-14.

Committee, I. o. M. (2005). Use of Complementary and Alternative Medicine by the American Public: National Academies Press, Washington DC.

Consortium, I. P. s. D. G., & 2, W. T. C. C. C. (2011). A two-stage meta-analysis identifies several new loci for Parkinson's disease. *PLoS genetics, 7*(6), e1002142.

Corash, L. (1983). Platelet sizing: techniques, biological significance, and clinical applications. *Current topics in hematology, 4*, 99.

Crusz, S. M., & Balkwill, F. R. (2015). Inflammation and cancer: advances and new agents. *Nature reviews Clinical oncology, 12*(10), 584.

CYPRIAN, H. G. (2011). *EVALUATION OF BIOLOGICAL ACTIVITY OF ALKALOIDS AND FLAVANOIDS IN ZANTHOXYLUM GILLETII (DE WILD WATERMAN) EXTRACTS FROM DIFFERENT GEOGRAPHICAL REGIONS IN KENYA.* SCHOOL OF PURE AND APPLIED SCIENCES, KENYATTA UNIVERSITY.

da Silva, S. L., Figueredo, P. M., & Yano, T. (2006). Antibacterial and antifungal activities of volatile oils from Zanthoxylum Rhoifolium. Leaves. *Pharmaceutical Biology, 44*(9), 657-659.

Darwish, N., Abbas, M., Abdelfattah, F., & Darwish, M. (1992). Hepatitis C virus infection in blood donors in Egypt. *The Journal of the Egyptian Public Health Association*, *67*(3-4), 223-236.

Davila, J., Morgan, R., Shaib, Y., McGlynn, K., & El-Serag, H. (2005). Diabetes increases the risk of hepatocellular carcinoma in the United States: a population based case control study. *Gut*, *54*(4), 533-539.

Déciga-Campos, M., Rivero-Cruz, I., Arriaga-Alba, M., Castañeda-Corral, G., Angeles-López, G. E., Navarrete, A., & Mata, R. (2007). Acute toxicity and mutagenic activity of Mexican plants used in traditional medicine. *Journal of ethnopharmacology*, *110*(2), 334-342.

Derenzini, M., Montanaro, L., Chillà, A., Tosti, E., Vici, M., Barbieri, S., . . . Treré, D. (2005). Key role of the achievement of an appropriate ribosomal RNA complement for G1-S phase transition in H4-II-E-C3 rat hepatoma cells. *Journal of cellular physiology*, *202*(2), 483-491.

Deschatrette, J., Moore, E. E., Dubois, M., Cassio, D., & Weiss, M. C. (1979). Dedifferentiated variants of a rat hepatoma: analysis by cell hybridization. *Somatic cell genetics*, *5*(6), 697-718.

Devi, O. Z., Rao, K. S., Bidalia, A., Wangkheirakpam, R., & Singh, O. M. (2015). GC-MS analysis of phytocomponents and antifungal activities of Zanthoxylum acanthopodium DC. Collected from Manipur, India. *European Journal of Medicinal Plants*, *10*(1), 1-9.

Dong, R., Qiang, W., Guo, H., Xu, X., Kim, J. J., Mazar, A., . . . Wei, J.-J. (2016). Histologic and molecular analysis of patient derived xenografts of high-

grade serous ovarian carcinoma. *Journal of hematology & oncology*, *9*(1), 92.

Du, Q., Hu, B., An, H.-M., Shen, K.-P., Xu, L., Deng, S., & Wei, M.-M. (2013). Synergistic anticancer effects of curcumin and resveratrol in Hepa1-6 hepatocellular carcinoma cells. *Oncology reports*, *29*(5), 1851-1858.

Dunn, S. R., Qi, Z., Bottinger, E. P., Breyer, M. D., & Sharma, K. (2004). Utility of endogenous creatinine clearance as a measure of renal function in mice. *Kidney international*, *65*(5), 1959-1967.

Dunsford, H. A., Sell, S., & Chisari, F. V. (1990). Hepatocarcinogenesis due to chronic liver cell injury in hepatitis B virus transgenic mice. *Cancer research*, *50*(11), 3400-3407.

Dürig, J., Ebeling, P., Grabellus, F., Sorg, U. R., Möllmann, M., Schütt, P., . . . Flasshove, M. (2007). A novel nonobese diabetic/severe combined immunodeficient xenograft model for chronic lymphocytic leukemia reflects important clinical characteristics of the disease. *Cancer research*, *67*(18), 8653-8661.

Egal, S., Hounsa, A., Gong, Y., Turner, P., Wild, C., Hall, A., . . . Cardwell, K. (2005). Dietary exposure to aflatoxin from maize and groundnut in young children from Benin and Togo, West Africa. *International Journal of Food Microbiology*, *104*(2), 215-224.

Ekor, M. (2014). The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. *Frontiers in pharmacology*, *4*, 177.

El-Zayadi, A.-R., Badran, H. M., Barakat, E. M., Attia, M. E.-D., Shawky, S., Mohamed, M. K., . . . Saeid, A. (2005). Hepatocellular carcinoma in Egypt: a single center study over a decade. *World journal of gastroenterology: WJG, 11*(33), 5193.

El-Serag, H. B., Kanwal, F., Richardson, P., & Kramer, J. (2016). Risk of hepatocellular carcinoma after sustained virological response in Veterans with hepatitis C virus infection. *Hepatology, 64*(1), 130-137.

El–Serag, H. B., & Rudolph, K. L. (2007). Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology, 132*(7), 2557-2576.

Elaridi, J., Bassil, M., Kharma, J. A., Daou, F., & Hassan, H. F. (2017). Analysis of aflatoxin M1 in breast milk and its association with nutritional and socioeconomic status of lactating mothers in Lebanon. *Journal of food protection, 80*(10), 1737-1741.

Enechi, O. C., Amah, C. C., Okagu, I. U., Ononiwu, C. P., Azidiegwu, V. C., Ugwuoke, E. O., . . . Ndukwe, E. E. (2019). Methanol extracts of Fagara zanthoxyloides leaves possess antimalarial effects and normalizes haematological and biochemical status of Plasmodium berghei-passaged mice. *Pharmaceutical Biology, 57*(1), 577-585.

Eray, M., Tuomikoski, T., Wu, H., Nordström, T., Andersson, L. C., Knuutlla, S., & Kaartinen, M. (1994). Cross-linking of surface IgG induces apoptosis in a bcl-2 expressing human follicular lymphoma line of mature B cell phenotype. *International immunology, 6*(12), 1817-1827.

Ernst, E., Cohen, M., & Stone, J. (2004). Ethical problems arising in evidence based complementary and alternative medicine. *Journal of Medical Ethics, 30*(2), 156-159.

Fagone, P., Mangano, K., Mammana, S., Pesce, A., Pesce, A., Caltabiano, R., . . . Lombardo, G. A. (2015). Identification of novel targets for the diagnosis and treatment of liver fibrosis. *International journal of molecular medicine, 36*(3), 747-752.

Fan, H., Zhang, H., Pascuzzi, P. E., & Andrisani, O. (2016). Hepatitis B virus X protein induces EpCAM expression via active DNA demethylation directed by RelA in complex with EZH2 and TET2. *Oncogene, 35*(6), 715.

Farinati, F., Cardin, R., Degan, P., De Maria, N., Floyd, R. A., Van Thiel, D. H., & Naccarato, R. (1999). Oxidative DNA damage in circulating leukocytes occurs as an early event in chronic HCV infection. *Free Radical Biology and Medicine, 27*(11-12), 1284-1291.

Ferreira, M. E., Cebrián-Torrejón, G., Corrales, A. S., de Bilbao, N. V., Rolón, M., Gomez, C. V., . . . Torres, S. (2011). Zanthoxylum chiloperone leaves extract: first sustainable Chagas disease treatment. *Journal of ethnopharmacology, 133*(3), 986-993.

Folashade, A. O., Mutalib, A. A., Satyajit, D. S., & Olajide, O. (2017). Zanthoxylum zanthoxyoides root extract inhibits hemozoin-induced neuroinflammation in BV2 microglia. *Planta Medica International Open, 4*(S 01), Tu-PO-151.

Foldes-Papp, Z., Konig, K., Studier, H., Buckle, R., Breunig, H. G., Uchugonova, A., & Kostner, G. M. (2009). Trafficking of mature miRNA-122 into the nucleus of live liver cells. *Current pharmaceutical biotechnology, 10*(6), 569-578.

Fournel, M., Sapieha, P., Beaulieu, N., Besterman, J. M., & MacLeod, A. R. (1999). Down-regulation of Human DNA-(Cytosine-5) Methyltransferase Induces Cell Cycle Regulators p16 ink4A and p21WAF/Cip1 by Distinct Mechanisms. *Journal of Biological Chemistry, 274*(34), 24250-24256.

Freitas, F., Fernandes, H., Piauilino, C., Pereira, S., Carvalho, K., Chaves, M., . . . Oliveira, R. (2011). Gastroprotective activity of Zanthoxylum rhoifolium Lam. in animal models. *Journal of ethnopharmacology, 137*(1), 700-708.

FREUND, H. A. (1937). Clinical manifestations and studies in parenchymatous hepatitis. *Annals of internal medicine, 10*(8), 1144-1155.

Fujita, N., Sugimoto, R., Ma, N., Tanaka, H., Iwasa, M., Kobayashi, Y., . . . Takei, Y. (2008). Comparison of hepatic oxidative DNA damage in patients with chronic hepatitis B and C. *Journal of viral hepatitis, 15*(7), 498-507.

Fukumoto, T., & Ku, Y. (2016). Induction Chemotherapy for Hepatocellular Carcinoma *Induction Chemotherapy* (pp. 269-284): Springer.

Gali-Muhtasib, H., & Bakkar, N. (2002). Modulating cell cycle: current applications and prospects for future drug development. *Current cancer drug targets, 2*(4), 309-336.

Georgiades, C. S., Hong, K., D'Angelo, M., & Geschwind, J.-F. H. (2005). Safety and efficacy of transarterial chemoembolization in patients with unresectable hepatocellular carcinoma and portal vein thrombosis. *Journal of vascular and interventional radiology, 16*(12), 1653-1659.

Gilani, A.-u. H., & Janbaz, K. H. (1994). Hepatoprotective effects of Artemisia scoparia against carbon tetrachloride: an environmental contaminant. *Journal of Pakistan Medical Association, 44*(3), 65.

Gilbertson, D. G., Duff, M. E., West, J. W., Kelly, J. D., Sheppard, P. O., Hofstrand, P. D., . . . Moore, M. (2001). Platelet-derived growth factor C (PDGF-C), a novel growth factor that binds to PDGF α and β receptor. *Journal of Biological Chemistry, 276*(29), 27406-27414.

Gish, R. G., Porta, C., Lazar, L., Ruff, P., Feld, R., Croitoru, A., . . . Gallo, J. (2007). Phase III randomized controlled trial comparing the survival of patients with unresectable hepatocellular carcinoma treated with nolatrexed or doxorubicin. *Journal of Clinical Oncology, 25*(21), 3069-3075.

Goodman, C. D., Hoang, A. T., Diallo, D., Malterud, K. E., McFadden, G. I., & Wangensteen, H. (2019). Anti-plasmodial Effects of Zanthoxylum zanthoxyloides. *Planta Medica, 85*(13), 1073-1079.

Gregor, H.-J. (1989). Aspects of the fossil record and phylogeny of the family Rutaceae (Zanthoxyleae, Toddalioideae) *Woody plants—evolution and distribution since the Tertiary* (pp. 251-265): Springer.

Gu, Q., Zhang, B., Sun, H., Xu, Q., Tan, Y., Wang, G., . . . Li, J. (2015). Genomic characterization of a large panel of patient-derived hepatocellular carcinoma xenograft tumor models for preclinical development. *Oncotarget, 6*(24), 20160.

Gurib-Fakim, A. (2006). Medicinal plants: traditions of yesterday and drugs of tomorrow. *Molecular aspects of Medicine, 27*(1), 1-93.

Hagen, T. M., Huang, S., Curnutte, J., Fowler, P., Martinez, V., Wehr, C. M., . . . Chisari, F. V. (1994). Extensive oxidative DNA damage in hepatocytes of transgenic mice with chronic active hepatitis destined to develop hepatocellular carcinoma. *Proceedings of the National Academy of Sciences, 91*(26), 12808-12812.

Harborne, A. (1998). *Phytochemical methods a guide to modern techniques of plant analysis*: springer science & business media.

Harvey, A. L. (2008). Natural products in drug discovery. *Drug discovery today, 13*(19-20), 894-901.

Haugen, B. R. (2017). 2015 American Thyroid Association management guidelines for adult patients with thyroid nodules and differentiated thyroid cancer: what is new and what has changed? *Cancer, 123*(3), 372-381.

He, L., Tian, D.-A., Li, P.-Y., & He, X.-X. (2015). Mouse models of liver cancer: Progress and recommendations. *Oncotarget, 6*(27), 23306.

Heindryckx, F., Colle, I., & Van Vlierberghe, H. (2009). Experimental mouse models for hepatocellular carcinoma research. *International journal of experimental pathology, 90*(4), 367-386.

Hemachandra, C. K., & Pathiratne, A. (2016). Combination of physico-chemical analysis, Allium cepa test system and Oreochromis niloticus erythrocyte based comet assay/nuclear abnormalities tests for cyto-genotoxicity assessments of treated effluents discharged from textile industries. *Ecotoxicology and environmental safety, 131*, 54-64.

Hermans, M., Akoègninou, A., & van der Maesen, J. (2004). Medicinal plants used to treat malaria in southern Benin. *Economic Botany, 58*(1), S239-S252.

Hernandez–Gea, V., Toffanin, S., Friedman, S. L., & Llovet, J. M. (2013). Role of the microenvironment in the pathogenesis and treatment of hepatocellular carcinoma. *Gastroenterology, 144*(3), 512-527.

Hidalgo, M., Amant, F., Biankin, A. V., Budinská, E., Byrne, A. T., Caldas, C., . . . Mælandsmo, G. M. (2014). Patient-derived xenograft models: an emerging platform for translational cancer research. *Cancer discovery, 4*(9), 998-1013.

Higgs, M. R., Chouteau, P., & Lerat, H. (2014). 'Liver let die': oxidative DNA damage and hepatotropic viruses. *Journal of General Virology, 95*(5), 991-1004.

Hoshida, Y., Villanueva, A., Kobayashi, M., Peix, J., Chiang, D. Y., Camargo, A., . . . Lerner, J. (2008). Gene expression in fixed tissues and outcome in

hepatocellular carcinoma. *New England Journal of Medicine, 359*(19), 1995-2004.

Hsu, I., Metcalf, R., Sun, T., Welsh, J., Wang, N., & Harris, C. (1991). Mutational hot spot in the p53 gene in human hepatocellular carcinomas. *Nature, 350*(6317), 427.

Ilic, Z., Crawford, D., Egner, P. A., & Sell, S. (2010). Glutathione-S-transferase A3 knockout mice are sensitive to acute cytotoxic and genotoxic effects of aflatoxin B1. *Toxicology and applied pharmacology, 242*(3), 241-246.

Institute, N. C. (2016). NCI dictionary of cancer terms.

Jaiprakash, B., Karadi, R., Savadi, R., & Hukkeri, V. (2003). Hepatoprotective activity of bark of Balanites aegyptiaca Linn. *Journal of natural remedies, 3*(2), 205-207.

Jhappan, C., Stahle, C., Harkins, R. N., Fausto, N., Smith, G. H., & Merlino, G. T. (1990). TGFα overexpression in transgenic mice induces liver neoplasia and abnormal development of the mammary gland and pancreas. *Cell, 61*(6), 1137-1146.

Jiang, R., Tan, Z., Deng, L., Chen, Y., Xia, Y., Gao, Y., . . . Sun, B. (2011). Interleukin-22 promotes human hepatocellular carcinoma by activation of STAT3. *Hepatology, 54*(3), 900-909.

Jiang, W., Lu, Z., He, Y., & Diasio, R. B. (1997). Dihydropyrimidine dehydrogenase activity in hepatocellular carcinoma: implication in 5-fluorouracil-based chemotherapy. *Clinical Cancer Research, 3*(3), 395-399.

Jiang, Z., Jiang, X., Chen, S., Lai, Y., Wei, X., Li, B., . . . Liang, Q. (2017). Anti-GPC3-CAR T cells suppress the growth of tumor cells in patient-derived xenografts of hepatocellular carcinoma. *Frontiers in immunology, 7*, 690.

Johnson, P. (2019). The Epidemiology of Hepatocellular Carcinoma *Liver Cancers* (pp. 3-11): Springer.

Kamel, M., Ghaffar, Y., Wasef, M., Wright, M., Clark, L., & Miller, F. D. (1992). High HCV prevalence in Egyptian blood donors. *The Lancet, 340*(8816), 427.

Kanwal, R., Arshad, M., Bibi, Y., Asif, S., & Chaudhari, S. K. (2015). Evaluation of Ethnopharmacological and Antioxidant Potential of Zanthoxylum armatum DC. *Journal of Chemistry, 2015*.

Kaplan, D. E., Chang, K.-M., & Sanyal, A. (2019). Hepatocellular Cancer Induced by Infection *Microbiome and Cancer* (pp. 247-259): Springer.

Karim, A., & Bhatty, M. (1976). Studies on the essential oils of the Pakistani species of the family Umbelliferae. IV. Apium graveolens Linn.(celery, ajmodn) seed oil. *Pakistan Journal of Scientific and Industrial Research, 19*, 243-246.

Kew, M. (2010). Epidemiology of chronic hepatitis B virus infection, hepatocellular carcinoma, and hepatitis B virus-induced hepatocellular carcinoma. *Pathologie Biologie, 58*(4), 273-277.

Kim, C.-M., Koike, K., Saito, I., Miyamura, T., & Jay, G. (1991). HBx gene of hepatitis B virus induces liver cancer in transgenic mice. *Nature, 351*(6324), 317-320.

Kimura, O., Kondo, Y., Kogure, T., Kakazu, E., Ninomiya, M., Iwata, T., . . . Shimosegawa, T. (2014). Expression of EpCAM increases in the hepatitis B related and the treatment-resistant hepatocellular carcinoma. *BioMed research international, 2014*.

Koike, K., & Miyoshi, H. (2006). Oxidative stress and hepatitis C viral infection. *Hepatology Research, 34*(2), 65-73.

Kosalec, I., Bakmaz, M., Pepeljnjak, S., & Vladimir-Knezevic, S. (2004). Quantitative analysis of the flavonoids in raw propolis from northern Croatia. *Acta Pharmaceutica, 54*(1), 65-72.

Kosh-Komba, E., Toumnou, L. A., Zinga, I., Touckia, I., Lembo, P. N. Z. W., Mukeina, G., . . . Syssa-Magale, J. (2017). Phytochemical Screening, Antifungal and Antibacterial Effect of Zanthoxylum zanthoxyloides and Zanthoxylum macrophylum Used in Traditional Medicine in Yamboro (Central African Republic). *European Journal of Medicinal Plants*, 1-11.

Kryczek, I., Lange, A., Mottram, P., Alvarez, X., Cheng, P., Hogan, M., . . . Machelon, V. (2005). CXCL12 and vascular endothelial growth factor synergistically induce neoangiogenesis in human ovarian cancers. *Cancer research, 65*(2), 465-472.

Kuracha, M. R., Thomas, P., Loggie, B. W., & Govindarajan, V. (2016). Patient-derived xenograft mouse models of Pseudomyxoma Peritonei recapitulate the human inflammatory tumor microenvironment. *Cancer medicine, 5*(4), 711-719.

Ladep, N. G., Lesi, O. A., Mark, P., Lemoine, M., Onyekwere, C., Afihene, M., . . . Taylor-Robinson, S. D. (2014). Problem of hepatocellular carcinoma in West Africa. *World journal of hepatology*, *6*(11), 783-792. doi:10.4254/wjh.v6.i11.783

Lammers, T., Kiessling, F., Hennink, W. E., & Storm, G. (2012). Drug targeting to tumors: principles, pitfalls and (pre-) clinical progress. *Journal of controlled release*, *161*(2), 175-187.

Lemon, S. M., & McGivern, D. R. (2012). Is hepatitis C virus carcinogenic? *Gastroenterology*, *142*(6), 1274-1278.

Lencioni, R. (2010). Loco-regional treatment of hepatocellular carcinoma. *Hepatology*, *52*(2), 762-773.

Leung, T. W., & Johnson, P. J. (2001). *Systemic therapy for hepatocellular carcinoma.* Paper presented at the Seminars in oncology.

Leung, T. W., Patt, Y. Z., Lau, W.-y., Ho, S. K., Simon, C., Chan, A. T., . . . Leung, N. W. (1999). Complete pathological remission is possible with systemic combination chemotherapy for inoperable hepatocellular carcinoma. *Clinical Cancer Research*, *5*(7), 1676-1681.

Levrero, M. (2006). Viral hepatitis and liver cancer: the case of hepatitis C. *Oncogene*, *25*(27), 3834.

Lewis, L., Onsongo, M., Njapau, H., Schurz-Rogers, H., Luber, G., Kieszak, S., . . . Misore, A. (2005). Aflatoxin contamination of commercial maize products during an outbreak of acute aflatoxicosis in eastern and central Kenya. *Environmental health perspectives*, *113*(12), 1763-1767.

Li, J.-p., Gao, Y., Chu, S.-f., Zhang, Z., Xia, C.-y., Mou, Z., . . . Chen, N.-h. (2014). Nrf2 pathway activation contributes to anti-fibrosis effects of ginsenoside Rg1 in a rat model of alcohol-and CCl 4-induced hepatic fibrosis. *Acta Pharmacologica Sinica, 35*(8), 1031.

Li, K., Zhou, R., Jia, W. W., Li, Z., Li, J., Zhang, P., & Xiao, T. (2016). Zanthoxylum bungeanum essential oil induces apoptosis of HaCaT human keratinocytes. *Journal of ethnopharmacology, 186*, 351-361.

Li, W., Gomez, E., & Zhang, Z. (2007). Immunohistochemical expression of stromal cell-derived factor-1 (SDF-1) and CXCR4 ligand receptor system in hepatocellular carcinoma. *Journal of experimental & clinical cancer research: CR, 26*(4), 527-533.

Liu, H.-T., Cheng, S.-B., Huang, Y.-C., Huang, Y.-T., & Lin, P.-T. (2017). Coenzyme Q10 and oxidative stress: Inflammation status in hepatocellular carcinoma patients after surgery. *Nutrients, 9*(1), 29.

Llovet, J. M., Fuster, J., & Bruix, J. (1999). Intention-to-treat analysis of surgical treatment for early hepatocellular carcinoma: resection versus transplantation. *Hepatology, 30*(6), 1434-1440.

Llovet, J. M., Real, M. I., Montaña, X., Planas, R., Coll, S., Aponte, J., . . . Solà, R. (2002). Arterial embolisation or chemoembolisation versus symptomatic treatment in patients with unresectable hepatocellular carcinoma: a randomised controlled trial. *The Lancet, 359*(9319), 1734-1739.

Llovet, J. M., Ricci, S., Mazzaferro, V., Hilgard, P., Gane, E., Blanc, J.-F., . . . Forner, A. (2008). Sorafenib in advanced hepatocellular carcinoma. *New England Journal of Medicine, 359*(4), 378-390.

Lo, C.-M., Ngan, H., Tso, W.-K., Liu, C.-L., Lam, C.-M., Poon, R. T.-P., . . . Wong, J. (2002). Randomized controlled trial of transarterial lipiodol chemoembolization for unresectable hepatocellular carcinoma. *Hepatology, 35*(5), 1164-1171.

Love, R. R., Mazess, R. B., Barden, H. S., Epstein, S., Newcomb, P. A., Jordan, V. C., . . . DeMets, D. L. (1992). Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. *New England Journal of Medicine, 326*(13), 852-856.

Lu, J.-W., Chang, J.-G., Yeh, K.-T., Chen, R.-M., Tsai, J. J., & Hu, R.-M. (2011). Overexpression of Thy1/CD90 in human hepatocellular carcinoma is associated with HBV infection and poor prognosis. *Acta histochemica, 113*(8), 833-838.

Luk, S. T. C., Tong, M., Ng, K. Y., Yip, K. Y.-L., Guan, X. Y., & Ma, S. (2018). Identification of ZFP42/REX1 as a regulator of cancer stemness in CD133+ liver cancer stem cells by genome-wide DNA methylation analysis: AACR.

Luo, X., Pires, D., Aínsa, J. A., Gracia, B., Duarte, N., Mulhovo, S., . . . Ferreira, M.-J. U. (2013). Zanthoxylum capense constituents with antimycobacterial activity against Mycobacterium tuberculosis in vitro

and ex vivo within human macrophages. *Journal of ethnopharmacology*, *146*(1), 417-422.

Macé, K., Aguilar, F., Wang, J.-S., Vautravers, P., Gomez-Lechon, M., Gonzalez, F. J., . . . Pfeifer, A. (1997). Aflatoxin B1-induced DNA adduct formation and p53 mutations in CYP450-expressing human liver cell lines. *Carcinogenesis*, *18*(7), 1291-1297.

Madden, C. R., Finegold, M. J., & Slagle, B. L. (2002). Altered DNA mutation spectrum in aflatoxin b1-treated transgenic mice that express the hepatitis B virus x protein. *Journal of Virology*, *76*(22), 11770-11774.

Mahmood, S., Kawanaka, M., Kamei, A., Izumi, A., Nakata, K., Niiyama, G., . . . Togawa, K. (2004). Immunohistochemical evaluation of oxidative stress markers in chronic hepatitis C. *Antioxidants and Redox Signaling*, *6*(1), 19-24.

Marquez, L., Aguero, J., Hernandez, I., Garrido, G., Martinez, I., Dieguez, R., . . . Curini, M. (2005). Anti-inflammatory evaluation and phytochemical characterization of some plants of the Zanthoxylum genus. *Acta Farmaceutica Bonaerense*, *24*(3), 325.

Martin, G. J. (2010). *Ethnobotany: a methods manual*: Routledge.

Martin, J., & Dufour, J.-F. (2008). Tumor suppressor and hepatocellular carcinoma. *World journal of gastroenterology: WJG*, *14*(11), 1720.

McCracken, M., Olsen, M., Chen Jr, M. S., Jemal, A., Thun, M., Cokkinides, V., . . . Ward, E. (2007). Cancer incidence, mortality, and associated risk factors among Asian Americans of Chinese, Filipino, Vietnamese, Korean,

and Japanese ethnicities. *CA: A Cancer Journal for Clinicians, 57*(4), 190-205.

McGlynn, K. A., & London, W. T. (2011). The global epidemiology of hepatocellular carcinoma: present and future. *Clinics in liver disease, 15*(2), 223-243.

McLean, M., & Dutton, M. F. (1995). Cellular interactions and metabolism of aflatoxin: an update. *Pharmacology & therapeutics, 65*(2), 163-192.

Messmer, W., Tin-Wa, M., Fong, H., Bevelle, C., Farnsworth, N., Abraham, D., & Trojanek, J. (1972). Fagaronine, a new tumor inhibitor isolated from Fagara zanthoxyloides Lam.(Rutaceae). *Journal of pharmaceutical sciences, 61*(11), 1858-1859.

Miliauskas, G., Venskutonis, P., & Van Beek, T. (2004). Screening of radical scavenging activity of some medicinal and aromatic plant extracts. *Food chemistry, 85*(2), 231-237.

Misra, L., Wouatsa, N. V., Kumar, S., Kumar, R. V., & Tchoumbougnang, F. (2013). Antibacterial, cytotoxic activities and chemical composition of fruits of two Cameroonian Zanthoxylum species. *Journal of ethnopharmacology, 148*(1), 74-80.

Mitani, K., Fujita, H., Sassa, S., & Kappas, A. (1989). Heat shock induction of heme oxygenase mRNA in human Hep 3B hepatoma cells. *Biochemical and biophysical research communications, 165*(1), 437-441.

Mok, T. S., Leung, T. W., Lee, S.-D., Chao, Y., Chan, A. T., Huang, A., . . . Johnston, A. (1999). A multi-centre randomized phase II study of

nolatrexed versus doxorubicin in treatment of Chinese patients with advanced hepatocellular carcinoma. *Cancer chemotherapy and pharmacology, 44*(4), 307-311.

Moriya, K., Fujie, H., Shintani, Y., Yotsuyanagi, H., Tsutsumi, T., Ishibashi, K., . . . Koike, K. (1998). The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nature medicine, 4*(9), 1065.

Murakami, H., Sanderson, N. D., Nagy, P., Marino, P. A., Merlino, G., & Thorgeirsson, S. S. (1993). Transgenic mouse model for synergistic effects of nuclear oncogenes and growth factors in tumorigenesis: interaction of c-myc and transforming growth factor α in hepatic oncogenesis. *Cancer research, 53*(8), 1719-1723.

Nerenstone, S. R., Ihde, D. C., & Friedman, M. A. (1988). Clinical trials in primary hepatocellular carcinoma: current status and future directions. *Cancer treatment reviews, 15*(1), 1-31.

Ngane, A. N., Biyiti, L., Zollo, P. A., & Bouchet, P. (2000). Evaluation of antifungal activity of extracts of two Cameroonian Rutaceae: Zanthoxylum leprieurii Guill. et Perr. and Zanthoxylum xanthoxyloides Waterm. *Journal of ethnopharmacology, 70*(3), 335-342.

Ngassoum, M., Essia-Ngang, J., Tatsadjieu, L., Jirovetz, L., Buchbauer, G., & Adjoudji, O. (2003). Antimicrobial study of essential oils of Ocimum gratissimum leaves and Zanthoxylum xanthoxyloides fruits from Cameroon. *Fitoterapia, 74*(3), 284-287.

Nogueira, J. A., Ono-Nita, S. K., Nita, M. E., de Souza, M. M., do Carmo, E. P., Mello, E. S., . . . Alves, V. A. (2009). 249 TP53 mutation has high prevalence and is correlated with larger and poorly differentiated HCC in Brazilian patients. *BMC cancer, 9*(1), 204.

O'Brien, J., & Stellwagen, R. (1977). The effects of controlled substitution of 5-bromodeoxyuridine (BUdR) for thymidine in hepatoma cell DNA. *Experimental cell research, 107*(1), 119-125.

Obuseh, F. A., Jolly, P. E., Kulczycki, A., Ehiri, J., Waterbor, J., Desmond, R. A., . . . Piyathilake, C. J. (2011). Aflatoxin levels, plasma vitamins A and E concentrations, and their association with HIV and hepatitis B virus infections in Ghanaians: a cross-sectional study. *Journal of the International AIDS Society, 14*(1), 53.

Ogwal-Okeng, J. W., Obua, C., & Anokbonggo, W. W. (2003). Acute toxicity effects of the methanolic extract of Fagara zanthoxyloides (Lam.) root-bark. *African Health Sciences, 3*(3), 124-126.

Oishi, N., Yamashita, T., & Kaneko, S. (2014). Molecular biology of liver cancer stem cells. *Liver cancer, 3*(2), 71-84.

Ojewole, J. A. (2004). Evaluation of the analgesic, anti-inflammatory and anti-diabetic properties of Sclerocarya birrea (A. Rich.) Hochst. stem-bark aqueous extract in mice and rats. *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives, 18*(8), 601-608.

Oliva, J., French, B. A., Qing, X., & French, S. W. (2010). The identification of stem cells in human liver diseases and hepatocellular carcinoma. *Experimental and molecular pathology*, *88*(3), 331-340.

Omura, K., Uehara, T., Morikawa, Y., Hayashi, H., Mitsumori, K., Minami, K., . . . Urushidani, T. (2014). Detection of initiating potential of non-genotoxic carcinogens in a two-stage hepatocarcinogenesis study in rats. *The Journal of toxicological sciences*, *39*(5), 785-794.

Oriowo, M. A. (1982). Anti-inflammatory activity of piperonyl-4-acrylic isobutyl amide, an extractive from Zanthoxylum zanthoxyloides. *Planta Medica*, *44*(01), 54-56.

Ouedraogo, V., Compaoré, E., Rouamba, A., Compaoré, M., & Kiendrebeogo, M. (2019). Anti-virulence Activity of Three Medicinal Plants: Cassia occidentalis L., Crossopteryx febrifuga (Afzel ex G. Don) Benth. and Zanthoxylum zanthoxyloides (Lam) Zep. and Timl. *Microbiology Research Journal International*, 1-7.

Ozakyol, A. (2017). Global epidemiology of hepatocellular carcinoma (HCC epidemiology). *Journal of gastrointestinal cancer*, *48*(3), 238-240.

Park, J. W. (2004). Practice guideline for diagnosis and treatment of hepatocellular carcinoma. *The Korean journal of hepatology*, *10*(2), 88-98.

Patiño, L. O. J., Prieto, R. J. A., & Cuca, S. L. E. (2008). Zanthoxylum genus as potential source of bioactive compounds. *Bioactive compounds in phytomedicine*, 185-218.

Phillips, R., & Murikami, K. (1960). Primary neoplasms of the liver. Results of radiation therapy. *Cancer*, *13*(4), 714-720.

Polat, Y., Dursun, A. D., Küçük, A., Özer, A., Erer, D., & Arslan, M. (2016). Effects of Picroside II on Myocardial Ischemia-Reperfusion Injury in Streptozotocin-Induced Diabetic Rats. *Gazi Medical Journal*, *28*(1).

Prempeh, A. (2008). Analgesic activity of crude aqueous extract of the root bark of Zanthoxylum xanthoxyloides. *Ghana medical journal*, *42*(2).

Qiao, L., Yu, J., Dent, P., & Farrell, G. (2005). NF-κB protects rat ARL-6 hepatocellular carcinoma cells against hydrogen peroxide-induced apoptosis. *Cancer biology & therapy*, *4*(11), 1195-1202.

Ranawat, L., Bhatt, J., & Patel, J. (2010). Hepatoprotective activity of ethanolic extracts of bark of Zanthoxylum armatum DC in CCl4 induced hepatic damage in rats. *Journal of ethnopharmacology*, *127*(3), 777-780.

Raskin, I., Ribnicky, D. M., Komarnytsky, S., Ilic, N., Poulev, A., Borisjuk, N., . . . Yakoby, N. (2002). Plants and human health in the twenty-first century. *TRENDS in Biotechnology*, *20*(12), 522-531.

Rates, S. M. K. (2001). Plants as source of drugs. *Toxicon*, *39*(5), 603-613.

Rawat, D., Shrivastava, S., Naik, R. A., Chhonker, S. K., Mehrotra, A., & Koiri, R. K. (2018). An overview of natural plant products in the treatment of hepatocellular carcinoma. *Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents)*, *18*(13), 1838-1859.

Röcken, C., & Carl-McGrath, S. (2001). Pathology and pathogenesis of hepatocellular carcinoma. *Digestive Diseases, 19*(4), 269-278.

Ross, M. H., & Pawlina, W. (2006). *Histology*: Lippincott Williams & Wilkins.

Roussos, E. T., Condeelis, J. S., & Patsialou, A. (2011). Chemotaxis in cancer. *Nature Reviews Cancer, 11*(8), 573.

Sabbioni, G., Skipper, P. L., Büchi, G., & Tannenbaum, S. R. (1987). Isolation and characterization of the major serum albumin adduct formed by aflatoxin B 1 in vivo in rats. *Carcinogenesis, 8*(6), 819-824.

Saleem, M. (2009). Lupeol, a novel anti-inflammatory and anti-cancer dietary triterpene. *Cancer letters, 285*(2), 109-115.

Salem, R., Thurston, K. G., Carr, B. I., Goin, J. E., & Geschwind, J.-F. H. (2002). Yttrium-90 microspheres: radiation therapy for unresectable liver cancer. *Journal of vascular and interventional radiology, 13*(9), S223-S229.

Saslow, D., Solomon, D., Lawson, H. W., Killackey, M., Kulasingam, S. L., Cain, J., . . . Wilbur, D. C. (2012). American Cancer Society, American Society for Colposcopy and Cervical Pathology, and American Society for Clinical Pathology screening guidelines for the prevention and early detection of cervical cancer. *CA: A Cancer Journal for Clinicians, 62*(3), 147-172.

Seeger, C., & Mason, W. S. (2000). Hepatitis B virus biology. *Microbiol. Mol. Biol. Rev., 64*(1), 51-68.

Sells, M. A., Chen, M. L., & Acs, G. (1987). Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B virus DNA. *Proceedings of the National Academy of Sciences*, *84*(4), 1005-1009.

Sengottuvelu, S., Duraisami, S., Nandhakumar, J., Duraisami, R., & Vasudevan, M. (2008). Hepatoprotective activity of Camellia sinensis and its possible mechanism of action.

Seol, H. S., Kang, H., Lee, S.-I., Kim, N. E., Im Kim, T., Chun, S. M., . . . Singh, S. R. (2014). Development and characterization of a colon PDX model that reproduces drug responsiveness and the mutation profiles of its original tumor. *Cancer letters*, *345*(1), 56-64.

Shariff, M. I., Cox, I. J., Gomaa, A. I., Khan, S. A., Gedroyc, W., & Taylor-Robinson, S. D. (2009). Hepatocellular carcinoma: current trends in worldwide epidemiology, risk factors, diagnosis and therapeutics. *Expert review of gastroenterology & hepatology*, *3*(4), 353-367.

Shi, H., Kokoeva, M. V., Inouye, K., Tzameli, I., Yin, H., & Flier, J. S. (2006). TLR4 links innate immunity and fatty acid–induced insulin resistance. *The Journal of clinical investigation*, *116*(11), 3015-3025.

Shiota, G., Rhoads, D. B., Wang, T. C., Nakamura, T., & Schmidt, E. V. (1992). Hepatocyte growth factor inhibits growth of hepatocellular carcinoma cells. *Proceedings of the National Academy of Sciences*, *89*(1), 373-377.

SHRIVASTAVA, V., SIJORIA, R., DEY, Y. N., PANDEY, N. K., JADHAV, A., & WANJARI, M. (2016). Antimitotic and antiproliferative activity of

stem bark of Oroxylum indicum. *Journal of Basic and Applied Research International, 13*(3), 147-154.

Siegel, R., Ma, J., Zou, Z., & Jemal, A. (2014). Cancer statistics, 2014. *CA: A Cancer Journal for Clinicians, 64*(1), 9-29.

Siegel, R. L., Miller, K. D., & Jemal, A. (2016). Cancer statistics, 2016. *CA: A Cancer Journal for Clinicians, 66*(1), 7-30.

Sinal, C. J., & Bend, J. R. (1997). Aryl hydrocarbon receptor-dependent induction of cyp1a1 by bilirubin in mouse hepatoma hepa 1c1c7 cells. *Molecular pharmacology, 52*(4), 590-599.

Singh, S., Sharma, B., Kanwar, S. S., & Kumar, A. (2016). Lead phytochemicals for anticancer drug development. *Frontiers in plant science, 7*, 1667.

Smit, J., Schinkel, A. H., Elferink, R. O., Groen, A., Wagenaar, E., Van Deemter, L., . . . Van Roon, M. (1993). Homozygous disruption of the murine mdr2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. *Cell, 75*(3), 451-462.

Smith, C. J., Perfetti, T. A., & King, J. A. (2019). Rodent 2-year cancer bioassays and in vitro and in vivo genotoxicity tests insufficiently predict risk or model development of human carcinomas. *Toxicology Research and Application, 3*, 2397847319849648.

Soini, Y., Virkajärvi, N., Raunio, H., & Pääkkö, P. (1996). Expression of P-glycoprotein in hepatocellular carcinoma: a potential marker of prognosis. *Journal of clinical pathology, 49*(6), 470-473.

Solecki, R. S. (1975). Shanidar IV, a Neanderthal flower burial in northern Iraq. *Science, 190*, 880-881.

Stern, M. C., Umbach, D. M., Mimi, C. Y., London, S. J., Zhang, Z.-Q., & Taylor, J. A. (2001). Hepatitis B, aflatoxin B1, and p53 codon 249 mutation in hepatocellular carcinomas from Guangxi, People's Republic of China, and a meta-analysis of existing studies. *Cancer Epidemiology and Prevention Biomarkers, 10*(6), 617-625.

Stuart, K., Tessitore, J., Rudy, J., Clendennin, N., & Johnston, A. (1999). A Phase II trial of nolatrexed dihydrochloride in patients with advanced hepatocellular carcinoma. *Cancer, 86*(3), 410-414.

Sutandyo, N. (2010). Nutritional carcinogenesis. *Acta Med Indones, 23*.

Takenaka, K., Kawahara, N., Yamamoto, K., Kajiyama, K., Maeda, T., Itasaka, H., . . . Sugimachi, K. (1996). Results of 280 liver resections for hepatocellular carcinoma. *Archives of surgery, 131*(1), 71-76.

Tariq, M., Parmar, N., Qureshi, S., El-Feraly, F., & Al-Meshal, I. (1987). Clastogenic evaluation of cathinone and amphetamine in somatic cells of mice. *Mutation Research Letters, 190*(2), 153-157.

Thompson, E. B., Granner, D., & Tomkins, G. (1970). Superinduction of tyrosine aminotransferase by actinomycin D in rat hepatoma (HTC) cells. *Journal of molecular biology, 54*(2), 159-175.

Tignanelli, C. J., Herrera Loeza, S. G., & Yeh, J. J. (2014). KRAS and PIK3CA mutation frequencies in patient-derived xenograft models of pancreatic

and colorectal cancer are reflective of patient tumors and stable across passages. *The American Surgeon*, *80*(9), 873-877.

Tilg, H., Wilmer, A., Vogel, W., Herold, M., Nölchen, B., Judmaier, G., & Huber, C. (1992). Serum levels of cytokines in chronic liver diseases. *Gastroenterology*, *103*(1), 264-274.

Tine, Y., Diop, A., Diatta, W., Desjobert, J. M., Boye, C. S. B., Costa, J., . . . Paolini, J. (2017). Chemical diversity and antimicrobial activity of volatile compounds from Zanthoxylum zanthoxyloides Lam. according to compound classes, plant organs and Senegalese sample locations. *Chemistry & biodiversity*, *14*(1), e1600125.

Torre, L. A., Bray, F., Siegel, R. L., Ferlay, J., Lortet-Tieulent, J., & Jemal, A. (2015). Global cancer statistics, 2012. *CA: A Cancer Journal for Clinicians*, *65*(2), 87-108.

Trease, G., & Evans, W. (1989). Pharmacognsy. 11th edn. Brailliar Tiridel Can: Macmillian publishers.

UCHIDA, H., IWASHITA, Y., SASAKI, A., SHIBATA, K., MATSUMOTO, T., OHTA, M., & KITANO, S. (2006). Chemokine receptor CCR6 as a prognostic factor after hepatic resection for hepatocellular carcinoma. *Journal of gastroenterology and hepatology*, *21*(1), 161-168.

Vidal, M., & Cagan, R. L. (2006). Drosophila models for cancer research. *Current opinion in genetics & development*, *16*(1), 10-16.

Villalba, M. A., Carmo, M. I., Leite, M. N., & Sousa, O. V. (2007). Atividades farmacológicas dos extratos de Zanthoxylum chiloperone (Rutaceae). *Revista Brasileira de Farmacognosia, 17*(2), 236-241.

Wang, C., Yang, W., Yan, H. X., Luo, T., Zhang, J., Tang, L., . . . Zheng, L. Y. (2012). Hepatitis B virus X (HBx) induces tumorigenicity of hepatic progenitor cells in 3, 5-diethoxycarbonyl-1, 4-dihydrocollidine-treated HBx transgenic mice. *Hepatology, 55*(1), 108-120.

Wang, E.-Y., Yeh, S.-H., Tsai, T.-F., Huang, H.-P., Jeng, Y.-M., Lin, W.-H., . . . Chen, D.-S. (2011). Depletion of β-catenin from mature hepatocytes of mice promotes expansion of hepatic progenitor cells and tumor development. *Proceedings of the National Academy of Sciences, 108*(45), 18384-18389.

Wang, H., Naghavi, M., Allen, C., Barber, R. M., Bhutta, Z. A., Carter, A., . . . Coates, M. M. (2016). Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980–2015: a systematic analysis for the Global Burden of Disease Study 2015. *The Lancet, 388*(10053), 1459-1544.

Wang, J.-S., Huang, T., Su, J., Liang, F., Wei, Z., Liang, Y., . . . Sun, G. (2001). Hepatocellular carcinoma and aflatoxin exposure in Zhuqing village, Fusui county, People's Republic of China. *Cancer Epidemiology and Prevention Biomarkers, 10*(2), 143-146.

Wang, Y.-C., Xu, G.-L., Jia, W.-D., Han, S.-J., Ren, W.-H., Wang, W., . . . Chen, H. (2012). Estrogen suppresses metastasis in rat hepatocellular carcinoma

through decreasing interleukin-6 and hepatocyte growth factor expression. *Inflammation, 35*(1), 143-149.

Wangensteen, H., An, T. H., Christopher, D. G., Diallo, D., & Karl, E. M. (2017). Zanthoxylum zanthoxyloides–an African medicinal tree with antimalarial effects. *Planta Medica International Open, 4*(S 01), Mo-PO-50.

Ward, E., Jemal, A., Cokkinides, V., Singh, G. K., Cardinez, C., Ghafoor, A., & Thun, M. (2004). Cancer disparities by race/ethnicity and socioeconomic status. *CA: A Cancer Journal for Clinicians, 54*(2), 78-93.

Wei, W., Wu, S., Wang, X., Sun, C. K.-W., Yang, X., Yan, X., . . . So, S. (2014). Novel celastrol derivatives inhibit the growth of hepatocellular carcinoma patient-derived xenografts. *Oncotarget, 5*(14), 5819.

Wenneker, A. S., & Sussman, N. (1951). Regeneration of liver tissue following partial hepatectomy in parabiotic rats. *Proceedings of the Society for Experimental Biology and Medicine, 76*(4), 683-686.

Wiebel, F., Wolff, T., & Lambiotte, M. (1980). Presence of cytochrome P-450- and cytochrome P-448-dependent monooxygenase functions in hepatoma cell lines. *Biochemical and biophysical research communications, 94*(2), 466-472.

Wiesenauer, C. A., Yip-Schneider, M. T., Wang, Y., & Schmidt, C. M. (2004). Multiple anticancer effects of blocking MEK-ERK signaling in hepatocellular carcinoma. *Journal of the American College of Surgeons, 198*(3), 410-421.

Williams, J. H., Phillips, T. D., Jolly, P. E., Stiles, J. K., Jolly, C. M., & Aggarwal, D. (2004). Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. *The American journal of clinical nutrition, 80*(5), 1106-1122.

Wu, C., Chen, W., Fang, M., Boye, A., Tao, X., Xu, Y., . . . Yang, Y. (2019). Compound Astragalus and Salvia miltiorrhiza extract inhibits hepatocellular carcinoma progression via miR-145/miR-21 mediated Smad3 phosphorylation. *Journal of ethnopharmacology, 231*, 98-112.

Xin, H., Wang, K., Hu, G., Xie, F., Ouyang, K., Tang, X., . . . Qin, X. (2014). Establishment and characterization of 7 novel hepatocellular carcinoma cell lines from patient-derived tumor xenografts. *PLoS One, 9*(1), e85308.

Xu, X., Farach-Carson, M. C., & Jia, X. (2014). Three-dimensional in vitro tumor models for cancer research and drug evaluation. *Biotechnology advances, 32*(7), 1256-1268.

Yamamoto, A., Tagawa, Y., Yoshimori, T., Moriyama, Y., Masaki, R., & Tashiro, Y. (1998). Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes in rat hepatoma cell line, H-4-II-E cells. *Cell structure and function, 23*(1), 33-42.

Yamashita, T., & Kaneko, S. (2014). Orchestration of hepatocellular carcinoma development by diverse liver cancer stem cells. *Journal of gastroenterology, 49*(7), 1105-1110.

Yang, M.-H., Chen, W.-J., Fu, Y.-S., Huang, B., Tsai, W.-C., Chen, Y.-M. A., . . . Tyan, Y.-C. (2018). Utilizing glycine N-methyltransferasegene knockout mice as a model for identification of missing proteins in hepatocellular carcinoma. *Oncotarget, 9*(1), 442.

Yang, X. (2008). Aroma constituents and alkylamides of red and green huajiao (Zanthoxylum bungeanum and Zanthoxylum schinifolium). *Journal of Agricultural and Food Chemistry, 56*(5), 1689-1696.

Yang, Y., Pritchard, P. H., Bhuiyan, J., Seccombe, D. W., & Moghadasian, M. H. (2001). Overexpression of acyl-CoA binding protein and its effects on the flux of free fatty acids in McA-RH 7777 cells. *Lipids, 36*(6), 595-600.

Yeh, C.-T., Kuo, C.-J., Lai, M.-W., Chen, T.-C., Lin, C.-Y., Yeh, T.-S., & Lee, W.-C. (2009). CD133-positive hepatocellular carcinoma in an area endemic for hepatitis B virus infection. *BMC cancer, 9*(1), 324.

Yeo, W., Mok, T. S., Zee, B., Leung, T. W., Lai, P. B., Lau, W. Y., . . . Chan, A. T. (2005). A randomized phase III study of doxorubicin versus cisplatin/interferon α-2b/doxorubicin/fluorouracil (PIAF) combination chemotherapy for unresectable hepatocellular carcinoma. *Journal of the National Cancer Institute, 97*(20), 1532-1538.

Yin, J., Zhang, H., He, Y., Xie, J., Liu, S., Chang, W., . . . Wang, H. (2010). Distribution and hepatocellular carcinoma–related viral properties of hepatitis B virus genotypes in mainland China: A community-Based Study. *Cancer Epidemiology and Prevention Biomarkers, 19*(3), 777-786.

Ynalvez, R., Cardenas, C., Addo, J., Adukpo, G., Dadson, B., & Addo-Mensah, A. (2012). Evaluation of the antimicrobial activity of Zanthoxylum zanthoxyloides root bark extracts. *Res J Med Pl, 6*, 149-159.

Yuan, H., Qiu, L., Xie, Z., Zou, L., Zheng, J., & Fu, Q. (2015). Research progress on alkaloids constituents from Zanthoxylum and their pharmacological activities. *Zhongguo Zhong yao za zhi= Zhongguo zhongyao zazhi= China journal of Chinese materia medica*, *40*(23), 4573-4584.

Zhou, D., Wang, Y., Chen, L., Jia, L., Yuan, J., Sun, M., . . . Xu, Z. (2016). Evolving roles of circadian rhythms in liver homeostasis and pathology. *Oncotarget*, *7*(8), 8625.

Zhou, H., Huang, H., Shi, J., Zhao, Y., Dong, Q., Jia, H., . . . Zhu, X. (2010). Prognostic value of interleukin 2 and interleukin 15 in peritumoral hepatic tissues for patients with hepatitis B-related hepatocellular carcinoma after curative resection. *Gut*, *59*(12), 1699-1708.

APPENDICES

Appendix A: Equipment

- Hitachi 917 automated analyzer (Roche Diagnostics, Indianapolis, IN)
- MAXM Analyzer C23644 - DxH 900 SMS + UPS (Beckman Coulter, Breau California, USA)
- Benchtop Transilluminator, (M-26V, Single UV, Variable Intensity, 8W, 302nm - 95-0458-01 – EACH, UVP, USA)
- S/P Brand Accutube Flange Caps (Cat. #T1226-37)
- Pyriform PTFE stopcock separator funnel (DXS stores, China)
- MAXM Analyzer CX Micro Sample Tube (Part #448774, Beckman Coulter, Breau California, USA)
- DIONEX Ultimate 3000 HPLC system (Thermo-Fisher, USA)
- Hammer mill (Polymix Micro Hammer Cutter Mill, Glen Mills Inc, USA)
- WP-120 Spectrophotometer, 90-240V, 50-60Hz (Thomas Scientific, Germany)
- soxhlet apparatus (L3 Soxlet extractor, Ergotech Soxhlet Apparatus Co, UK)
- Premiere HH-4 Digital Water Bath (C & A Scientific Co Inc, USA)
- Oven 300 plus series, (Gallenkamp, England)
- Fisher hematology mixer (Fisher Scientific, Pittsburgh, PA).

Apendix B: Reagents, chemicals and drugs

- Hydrochloric acid (HCl, 0.5%)

- Dilute ammonia water (NH_4OH)
- Chloroform ($CHCl_3$)
- 2% aceto-orcin
- Ethyl acetate
- Hexane
- Diethyl ether $(C_2H_5)_2O$)
- Concentrated sulphuric acid (H_2SO_4)
- Bismuth sub-nitrate ($Bi_5(OH)_9(NO_3)$)
- Glacial acetic acid (CH_3COOH)
- Potassium iodide (KI)
- Tween20 ($C_{58}H_{114}O_{26}$)
- Potassium chloride (KCl)
- Iron chloride (0.1 M FeCl3)
- Potassium di-hydrogen phosphate (KH_2PO_4)
- Hydrated disodium hydrogen phosphate (Na_2HPO_4)
- Hematoxylin ($C_{16}H_{14}O_6$)
- Potassium ferrocyanide (0.008 M, $K_4Fe(CN)_6$)
- Ammonium alum ($(NH_4)Al(SO_4)_2$)
- Mercuric oxide (HgO)
- Formaldehyde (CH_2O)
- Ethanol (C_2H_5OH)
- Petroleum spirit (C6H14)
- Sodium chloride (N_aCl)

- Isoton III -- PN 8546733
- Beckman Coulter Clenz -- PN 8546930
- Lyse-S III diff lytic reagent- PN 8546983
- Scatter Pak -- PN 8546917
- Latron Controls - PN 7546914
- Latron Primer - PN 7546915
- 5C Cell Controls Tri Pack contains Normal, Abnormal I, Abnormal II - PN 7547001
- Calibration S-CAL - PN 7546808
- 5C Cell control normal - PN 7546923 (9 x 3.3-mL)
- Olive oil
- Carbon tetrachloride (CCl_4)
- RD Precial calibrators (RD)
- RD Precitrol normal and abnormal human assayed control serum (RD)
- Physiological saline, 0.9% (Ricca Chemical, Arlington, TX)
- Tris buffer: 125 mmol/l, pH 7.3
- L-alanine: 625 mmol/l
- LDH D 1.5 U/m
- Ketoglutarate: 94 mmol/l
- Citrate buffer: 95 mmol/l, pH 4.1
- bromcresol green: 0.66 mmol/l
- 2-Amino-2-methyl-1- propanol D 0.93 mol/l, pH 10.5
- Magnesium-L-aspartate: 1.24 mmol/l

- Hydrochloric acid;
- Zinc sulfate hepta-hydrate
- Magnesium-L-aspartate
- Tris buffer: 100 mmol/l, pH 7 and pH 8
- L-aspartate: 300 mmol/l
- NADH: 0.23 mmol/l
- MDH D 0,53 U/ml (porcine heart)
- LDH D 0,75 U/ml
- ketoglutarate: 75 mmol/l
- Bicarbonate (HCO3)
- n-Butanol ($C_4H_{10}O$)
- MgSO4: 780 µmol/l
- Preservative
- Surfactant Inhibitor
- Sodium oxamate ($NH_2COCOON_a$)a
- PEPC (plant): ≥1.24 KU/l
- MDH (porcine heart): ≥33.2 KU/l
- NADH: ≥6.45 mmol/l
- PEP: ≥21.1 mmol/l
- MgSO4: 370 µmol/l
- Buffer stabilizer
- **B**icarbonate diluent
- CAPSO buffer: 5 mmol/l, pH 9.65

- NADH ≥ 0.23 mmol/l
- BICIN buffer: 1000 mmol/l, pH 7.6
- Urease ≥ 7.2 U/ml (jack bean)
- Dextran-linked GLDH ≥ 0.90 U/ml (bovine liver)
- α-ketoglutarate ≥ 8.3 mmol/l
- Ethanolamine buffer: 1 mol/l, pH 10.6
- O-cresolphthalein complexone: 0.3 mmol/l
- 8- hydroxyquinoline: 13.8 mmol/l
- Hydrochloric acid: 122 mmol/l
- Sodium hydroxide: 0.20 mol/l
- Picric acid ($(O_2N)_3C_6H_2OH$)
- Sodium carbonate (N_aCO_3)
- Sodium nitrite (NaNO2, 5%)
- Aluminum chloride ($AlCl_3$, 10%)
- Sodium hydroxide (NaOH, 4%)
- Ferric chloride solution
- Fehling's reagent
- Folin-Ciocalteu reagent
- Ninhydrin reagent
- Ether
- Amylalcohol
- Ammonium hydroxide solution
- Dragendorff's reagent

- Molisch's reagent
- Acetic anhydride
- Tris (hydroxymethyl) aminomethane, pH 8.25; 14.3 mmol/L
- Thiourea, 140 mmol/L
- Glycylglycine
- C2H3NaO2 (sodium acetate buffer): 85 mmol/l
- H3NO3S (sulfamic acid): 110 mmol/l
- Petroleum spirit: 40/60
- Diazonium ion: 3 mmol/l

Apendix C: Supplies

- 3-mL K3 EDTA Becton Dickinson Hemogard Vacutainertube
- Tube rocker
- Capillary tubes
- Thin layer chromatography plates (Silica Gel 60 F254 type. 20x20cm plate size, 1mm (1000μm) silica layer thickness. pH 6.2-6.8, particle size 10-40 μm, pore size 60 Å)
- Clorox Bleach, 5.25% sodium hypochlorite 1-800-292-2200
- Bottled distilled water
- Three 30 mL plastic containers with lid
- Two one-liter containers with lid
- Plastic squirt bottle
- Cotton gauze pads
- Three-hole paper punch

- Notebook
- Fujitsu printer ribbon
- Flashlight
- 10-mL syringe with plastic tubing
- Precision screwdriver set
- Hydroalcoholic solution (70%)
- Distilled water bottle
- Disposable lab jacket, 48 inches long
- Pasteur's pipette
- Aluminum foil
- Sealpette variable-volume micropipets: 2-20, 20-200, and 200-1000 µL volumes (Cole Scientific, Moorpark, CA).
- Tek Pro Tek-tator V variable rotator (Baxter Healthcare, Valencia, NC).
- Pipet-aid (Drummond Scientific Co., Broomall, PA).
- 3.0-mL, class A volumetric pipets (Cole Scientific, Moorpark, CA).
- Conical-bottom 2.0-mL polystyrene autosampler cups (RD).
- Ultrapure water, Barnstead E-pure water purification system with a resistivity of >16 megohm-cm Culligan Water Systems, Alamogordo, NM).
- 3.5-mL Beral polypropylene transfer pipettes (Sarstedt, Newton, NC).
- Kim-Wipe tissues (Kimberly-Clark Corp., Roswell, GA).
- Clean-room vinyl gloves (Baxter Healthcare).
- Biohazard autoclave bags (Curtin-Matheson Scientific, Inc., Atlanta, GA).

- Bleach (10% sodium hypochlorite solution) (Cole Scientific, Moorpark, CA).
- 9½" x 11" 20-lb white computer paper (Viking Office Products, Irving, TX)

Appendix D: Formulas

1. Cx = K(Ax to Ab) + Cb where:

Cx = Concentration of Sample.

K = Concentration Factor (determined during calibration).

Ax = Mean of absorbances of Sample + R1 read during designated cycles.

Ab = Mean of absorbances of STD 1 (Blank/CALIB 1)+ R1 read during designated cycles.

Cb = Concentration of STD 1 (Blank/CALIB 1).

2. Cx = (CISS ·10) + K

Cx = concentration of test ion

K = compensation factor

CISS = concentration of Internal Standard Solution as determined during calibration

ΔEx = EMF of test sample - EMF of Internal Standard Solution (determined just prior to the sample)

3. L = liver weight/body weight.

   Where L = liver to body weight proportion

4. $V = (W^2 \times L)/2$

   Where: W = the tumor width

L = tumor length

5. Mitotic index (%) $= \frac{\text{total number of cellx}}{\text{Number of dividing celss}}\ x\ 100$

6. K = total reaction time × 100/extinction coefficient x light path (cm) x specimen volume