UNIVERSITY OF GHANA, LEGON

SIMULTANEOUS INTERACTION OF AQUEOUS EXTRACT OF *Annona muricata* (SA-001AE) and *Croton membranaceus* (CT 0163AE) ON PHASE I AND PHASE II DRUG METABOLIZING ENZYMES USING BPH EXPERIMENTAL RAT MODELS

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THIS THESIS IS SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES, UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF PROFESSIONAL MASTERS OF SCIENCE DEGREE IN MEDICAL LABORATORY SCIENCE, (CHEMICAL PATHOLOGY OPTION)

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DECLARATION

I, Royronald Ochieng Ongong'a by this means do declare that, this project work is my actual work and idea and has neither in whole nor in part been presented for award of another degree elsewhere. The work was aptly carried out by me and the results presented herein is a true reflection of the research done under the supervision of Prof. George Asare and Mr. Richard Harry Asmah.

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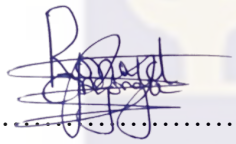

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ABSTRACT

Aqueous extracts of *Croton membranaceus* (CT 0163AE) and *Annona muricata* (SA-001AE) are known to have anti-Benign prostate hyperplasia (BPH) properties. Even though they are considered safe and natural, they may be antagonistic or synergistic, when used simultaneously due to possible interactions between the active compounds in the individual extracts that are able to induce or inhibit Cytochrome P450 enzymes (CYP450). The study strived to determine the interactive outcome of *A. muricata* and *C. membranaceus* on some Phase I and Phase II hepatic drug metabolizing enzymes. Twenty-eight (24) castrated -testosterone BPH induced and four (4) uncastrated male Albino Wister rats were used in this experimental laboratory study. The animals were organized into 7 groups consisting 4 rats each: Group I was uncastrated and used as a negative control the group was only administered distilled water. Group II was treated with 30 mg/kg b. wt extracts of *C. membranaceus*. Group III was treated with 30 mg/kg b. wt. extracts of *A. muricata* while group IV was treated with 30 mg/kg b. wt. mixed extract of both *A. muricata* and *C. membranaceus* (A60:C40), group V was treated with 30 mg/kg b. wt of both *A. muricata* and *C. membranaceus* (A40:C60), group VI was the model group while Group VII was treated with 0.5 mg/kg b. wt finasteride and it was used as the positive control. BPH was induced by administering testosterone propionate, 3mg/kg b. wt for 28 days pre-treatment.

The respective extracts were administered daily for 30 days through oral gavage. The animals were euthanized and individual liver harvested and frozen before isolating the microsomes by homogenization and differential centrifugation. The liver microsomes were assayed for, Phase I Drug Metabolizing Enzymes (DME); CYP1A2, CYP3A4, CYP2D6, CYP2C9, and phase II; Arylsulfatase G, GST-P1, and GST-M1 using Enzyme Linked Immuno-Sorbent Assay (ELISA) techniques. ANOVA Bonferroni post hoc test showed statistical significance in phase I enzymes;

CYP1A2, CYP3A4, CYP2D6, (p=0.02, 0.00, 0.01) respectively. Phase II enzymes GSTM1, GSTP1 and ARSG showed a statistical significance (p=0.01, 0.00, 0.00) respectively. CYP2C9 showed no statistical significance.

Both *A. muricata* and *C. membranaceus* had an inhibitory effect on CYP3A4, CYP2D6, GSTM1, GSTP1 ARSG and induced CYP1A2, CYP2C9 and GSTM1. The combined extract (A60:C40) inhibited CYP3A4, CYP2D6, GSTP1 and ARSG but induced CYP1A2, CYP2C9 and GSTM1.The combined extract (A40:C60) inhibited CYP3A4, CYP2D6, CYP2C9, GSTP1 and ARSG but induced CYP1A2 and GSTM1 enzymes.

DEDICATION

This work is dedicated to the Almighty God, my loving parents, George W.O. Ombonya and Pamela Akoth Njoga, my brothers and sisters. I also dedicate this work to my loving friend Selah Owino and all the patients affected with benign prostate hyperplasia (BPH).

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LIST OF ABBREVIATION

ADR	Advance Drug reaction
ANOVA	Analysis of Variance
ARSG	Arylsulfatase G
ARV	Anti-Retroviral
B. WT	Body Weight
BPH	Benign Prostate Hyperplasia
CAM	Complementary and Alternative Medicine
CM	*Croton membranaceus*
CSFP	Commonwealth Scholarship Fellowship and Plan
CVD	Cardio Vascular Disease
CYP	Cytochrome Protein
DHT	Dihydrotestosterone
DME	Drug Metabolizing Enzyme
DNA	Deoxy ribonucleic Acid
ELISA	Enzyme Linked Immuno-Sorbent Assay
Grp	Group
GSH	Glutathione
GSSG	Oxidised glutathione
GST	Glutathione S-Transferases
JNK	JUN-N-Terminal Kinase
LUTS	Lower Urinary Tract Symptoms

MAPEG	Membrane-Associated Proteins in Eicosanoid and Glutathione Metabolism
MG/KG	Milligram Per Kilogram
OD	Optical Density
OECD	Organization for Economic Co-operation and Development
ORID	Office of Research Innovation and Development
PAH	Polycyclic Aromatic Hydrocarbon
PBS	Phosphate Buffered Saline
PBS	Phosphate Buffered Solution
ROS	Reactive Oxygen Species
SME	Standard Error of mean
UGT	Uridine 5'-Diphospho-Glucuronosyltransferase
USA	United States of America
USD	United States Dollar
VEGF	Vascular Endothelial Growth factor
WHO	World Health Organization

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Benign prostate hyperplasia (BPH), is a non-cancerous and characterized by the enlargement of prostate glands with hyperplasia of both the stromal and epithelial cells, occasioned by large distinct lumps in the prostate glands that culminates to lower urinary tract symptoms (LUTS) (Cunning, 2013). The pathophysiology of the condition underscores the significant role played by genetical variation, hormonal imbalances and the action of growth factors and proliferative mediators (Rahman, 2016). The real cause is not clearly understood; however, 5-α reductase hormone is suspected to play the central role in the pathogenesis by its ability to convert testosterone to Dihydrotestosterone (DHT), which is a powerful stimulator of prostate growth (Nicholson & Ricke, 2011). Although medical therapy is widely used there is no ubiquitous therapy for all men with symptomatic BPH, a third of the patients never responds to medicinal therapy and thus would need surgery (Bechis *et al*., 2014).

Herbal medicine has been in use for the management of various types of diseases with various studies carried out globally to validate their efficacy leading to the production of plant based drugs (Sofowora *et al*., 2013). According to WHO, traditional medicine refers to the general knowledge, ability and applications considering the hypothesis, conviction and experience unique to various societies and cultures, regardless of whether it is reasonably used in the preservation of the wellbeing, as well as prophylaxis, disease detection, enhancement or treatment of physical or mental illness (WHO, 2008). Utilization of conventional medication has comprehensively increased in the last decade gaining popularity globally with a market of over USD 60 billion annually. Not only has it been used continuously as a primary choice for the poor in the

management of various diseases in developing countries, but also in countries whose health care system predominantly utilizes conventional medicine in the management of diseases (WHO, 2013).

According to the WHO fact sheet, (2003) more than fifty percent of the population in developed countries in Europe and America at one point in time have used complementary medicine, 70% in Canada and 90% in Germany. In china 30-50% of entire medicine comprises of traditional herb preparation. Herbal medicine is also used in most of the countries in sub-Saharan Africa as a first line option in the management of high fever in 60% of the children caused by communicable diseases (Mpinga, 2013).

Utilization of plant extracts as a remedy for LUTS and BPH was originally depicted in Egypt around fifteen century BC, regrettably there are so many unfilled gaps, raising a lot of questions about their use, hence the scientific rationale behind their use is not proven (Men *et al*., 2016). Around thirty compounds have been extracted from the therapeutic plants used in the management of BPH Including scopoletin and Julocrotine in *Croton membranaceus* and Alkaloids and acetogenins in *Annona muricata*. Many of the agents utilized in the treatment and management of BPH and LUTS are prepared from the roots, seeds barks or plant products. Phytosterols, fatty acids, plant oils, polysaccharides and lectins are some of proposed active compounds found either from solitary plant or from two or more plant sources (Keehn and Lowe, 2015).

Regardless of the expanding market for herbal drugs, there still exist unanswered questions related to both their use and safety. Ninety percent of herbal drugs being sold are not standardized to notable vital compounds and strict measures to ensure quality of these medications are not adhered to (Ifeoma & Oluwakanyinsola, 2013). The herbal drugs have always been considered pure and safe, nonetheless some of the contents can moderate different drug biotransformation

system in the body especially the liver which ensures metabolism and excretion. Hence drug metabolizing enzymes (DME) mediated herb-drug interactions can often arise in drug or herb based treatments (Cho & Yoon, 2015).

Reactions in drug biotransformation are categorized into two major phases, Phase I and Phase II. Phase I metabolism, normally leads to the introduction of water soluble functional groups into the molecules by either insertion of new functional group or by exchange of the existing groups through reduction, oxidation and hydrolytic reactions catalyzed by majorly cytochrome p 450 enzymes (Kebamo *et al.*, 2016). In phase II the functional groups formed in phase I are conjugated with small polar molecules to increase their polarity, the reactions are facilitated by a wide range of hydrolytic enzymes like transferases, estearases and amidases (Hoffman *et al.*, 2014).

1.2 Problem statement

Herbal medicine is used primarily in the health care system among different nations globally, (WHO, 2003). The norm is wide spread mostly in the developing countries where herbal medicine is accepted culturally and naturally as safer and most efficient way of treatment as it is the first level of contact for individuals and communities in the health care system and partly due to the low medical doctor patient ratio (WHO, 2008). In these regions and countries, the herbal drugs are used as monotherapy, in combination or drug-herb combination moreover, most countries have not laid down policies and laws that govern the administration of these herbs thus posing a danger to the patients because the majority do not understand their toxic effects that may result in case of herb-herb interactions, (Fasinu, 2012).

When herbs are used as combined therapy the outcome can be so complex to understand. Despite this, the herbalist considers the drugs less potent when used as monotherapy and regard

them as highly potent when used in combination oblivious of the herb-herb interactions (Che, 2013). Diverse interactions can occur between individual ingredients of herbs which sometimes can be fatal; the most judicious interactions are those which can lead to supplementary therapeutic benefit. Usually, this is the intention and expectation when using combined therapy; nonetheless because of the difference in composition of the herbal products, the results that arise from herb-herb or herb-drug interaction can usually be unpredicted with complications (Gurley, 2012). There have been no systematic studies exploring herb-herb combination therapy in the management of various diseases including BPH. So far, only limited scientific studies have been carried out on the compounds comprising most of the established medicinal herbs. Thus, there exists a great prospect to assess prospective therapeutic benefit from herb-herb combination in the management of BPH.

1.3 Justification

The use of herbal medicine as a remedy to various diseases is acceptable in most developed and developing countries and they are highly abundant in most developing countries especially those in the tropics. The drugs are advertised frequently in the media and internet with claimed pharmacological benefits persuading the patients to patronize them (Ekor, 2014). Most of these drugs are used to treat chronic illness such as BPH, hypertension, diabetes etc. The use of herbal medicine, including *A. muricata* and *C. membranaceus* in the management of BPH by patients has elicited interest among clinicians and research centers as their ability to cause inefficiency and systemic toxicity can be disastrous to the patients (Lahlou, 2013). Use of herbal medicine in the most developed countries is always accompanied with substantial information about the possibility of them, causing herb-drug interaction and safety instructions. However, in most African countries the information about the herbal drugs are limited mainly because of chemical complexity of the

medicinal plants which makes it hard to carry research on their pharmacokinetics (Brantley *et al*., 2014, Falodun, 2010).

In the process of drug discovery, the method applied in this study is one of the routinely used methods of determination of plant extract toxicity and development to evaluate the new chemical components that may likely cause drug interactions. Prompt screening process utilizing this method expedites attrition and averts post market recalling of drugs with prospect of drug-drug interaction (Basavaraj, 2014). Herbal drugs which are already in use for therapy by the patients contain individual compounds which can interact and need to be evaluated for their safety. Such investigations are carried out to underscore the possible risk related to these medications taken in combination (Alsanad, 2016). The study objective was to investigate the interactive outcome of *A. muricata* and *C. membranaceus* on Phase I and Phase II drug metabolizing enzymes.

1.4 Overall objective

To determine whether there is a synergistic interaction or otherwise of the aqueous root extract of *Croton membranaceus* and *Annona muricata* leaf extract on hepatic drug metabolizing enzymes.

1.4.1 Specific objectives

1. To determine the effect of aqueous root extract of *Croton membranaceus* and *Annona muricata* leaf extract on the Phase 1 DME; CYP1A2, CYP3A4, CYP2D6 and CYP2C9 independently and in combination.
2. To determine the effect of aqueous root extract of *Croton membranaceus* and *Annona muricata* leaf extract on the Phase II DME; Arylsulfatase G, GST-P1, and GST-M1 independently and in combination.

1.5 Hypothesis

Aqueous root extract of *Croton membranaceus* when administered together with the leaf extract of *Annona muricata* do not induce the DME's synergistically.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Benign Prostatic Hyperplasia (BPH)

Benign prostatic hyperplasia (BPH) and its associated lower urinary tract symptoms is known to affect 50% of the male population globally, increasing from 25% among middle aged men to more than 80% among septuagenarian men (Breyer *et al*., 2016; Sarma *et al.*, 2012). The United Nations estimates that between 2000 and 2015 there was a 48% increase of persons aged 60 and above and this number is projected to grow by 56% by the year 2030 globally (United Nations, 2015). The risk factors associated with BPH are classified into two broad categories non-modifiable risks which include age, geography and inherent factors and modifiable factors which include steroid sex hormones, diet, physical exercise and inflammation (Patel and Parsons, 2014).

Benign Prostate Hyperplasia is characterized by proliferation of both stromal and epithelial cells of the prostate in the transitional zone that surrounds the urethra (Roehrborn, 2008). Although common, BPH rarely causes death instead the disease results in compression of the urethra, causing bladder outlet obstruction (BOO) which is characterized by resistance to the flow of urine. This resistance can also result in obstruction induced changes of the bladder function, such as over reactivity or reduced contractility of the detrusor muscle, (Chughtai *et al*., 2015). BOO can present as LUTS (Lower Urinary Tract Symptoms), urinary retention, as well as other conditions such as polyuria, sleep disorders and malignant prostatic disease (Thiruchelvam, 2014).

2.2 Treatment and management

Various options of treatment in the management of BPH are in use, varying from watchful waiting, surgical interventions to use of plant extracts. The choice for the patient depends on each

individual circumstance (Cakir & Mcvary, 2014). Medical treatment of LUTS associated with BPH is usually the first line option, in countries such as American and Canada the treatment guideline policy recommends the use of adrenergic receptor blocking agents and 5α-reductase inhibitors as mono or combined therapy (Bishr *et al.*, 2016). Conventionally, drugs such as finasteride and dutasteride, have shown to be effective in the treatment of BPH, but their use is restricted due to related side effects (Bullock and Andriole 2006, Lee *et al.*, 2014). Finasteride is competitive and specific 5α-reductase inhibitor and is known to inhibit the conversion of testosterone to DHT in androgen-sensitive tissues such as the prostate and hair follicles, thus suppressing the concentration of serum and intraprostatic DHT (Steers, 2001).

2.3 Phytotherapy

Plants have been used as medicine since time immemorial, described in China and Egypt as early as 3000BC. Traditional cultures in Native America and Africa used herbs in their rituals, while in some cultures there was a developed medicinal system in which plants were used for therapies. According to WHO, 80% of people globally depends on medicine extracted from plants as their primary choice for treatment against various diseases. Phytotherapy represents a big portion of the alternative medicine market reaching a million dollars globally (WHO, 2008). The use of phytotherapy in the management of diseases is triggered by the inability of conventional medicines to treat chronic conditions, however, their effectiveness and safety is limited (Nunkoo, 2012).

Many of the commonly used phytotherapeutic agents are usually plant extracts, rather than the definite plants themselves. These extracts are a mixture of various chemical compounds (Ferreira, 2014). Mostly, the synergistic interactions exhibited can be due to the activities of several compounds present in the extracts, which are used in the management of a wide range of

diseases and not a single condition (Wink, 2015). Research has indicated that more than 15,000 compounds with therapeutic significance have been extracted from 200 plants, of which the majority are from the tropical region (Seebaluck and Mahomoodally, 2013). According to European Medicine Agency, ten years since the introduction of European registration of herbal medicine, more than 1,300 traditional medicinal herbs have been registered and more than 600 allowed into the European member state market as authorized medicine (Kraft, 2014).

2.3.1 Role of Phytotherapy in the treatment of BPH

Regardless the questionable efficacy of the phytotherapies, the use of medicinal plants as a preferred choice of treatment for BPH has been on the rise over the past few years more so in parts of Europe, where the drugs prescribed are as high as 50% (Keehn *et al.,* 2016). In the USA, sale of herbal drugs has become a very lucrative and profitable business with turnouts running close to US$6.4 billion in 2014 (Smith, 2015). Despite the increase in the use of phytotherapy in the management of BPH there has been un answered questions on the evidence approving the continual use for symptomatic BPH, with several studies still ongoing to determine their efficacy and role played in alteration of BPH course (Mcvary, 2011, Kheen *et al*., 2016, Bae, 2016).

Extract from Saw palmetto *(Serenoa repens*) has been studied widely and used for long in the management of BPH. The saw palmetto extract *(Serenoa repens*) contains over 100 known compounds notably pyresterols and fatty acids (Talpur *et al*., 2003). The fatty acids are thought to inhibit 5-Alpha reductase enzyme. The plant extract has also demonstrated an ability to reduce the action of dihydrotesterone androgen by blocking alpha -androgenic receptors (Van *et al*.,2000). It can further inhibit the growth factors such as Insulin-Growth factor, relaxation of lower urinary

tract smooth muscles by non-competitive inhibition of lipoxygenase and leukotrienes (Gordon & Shaughnessy, 2003; Abdullahi & Aji, 2016).

Pronus Africa (*Pygeum africanum*) bark extract has been proven to alleviate the symptoms presented by BPH, such as residual urine, urine retention, polyuria, nocturia, and peak flow (Nyamai *et al.*, 2016). Most of the studies that involved the use this extract showed a substantial reduction of the prostate size and manifestations. The therapeutic significance of *P. africana* bark extract is believed to be because of the synergistic action of the active compounds found in the bark extract such as pentacyclic, triterpenes, pyrosterols and ferolic acid esters of long chain unsaturated fatty acids (Nyamai *et al.*, 2015). A study undertaken to evaluate the effect of *Pygeum africanum* extract on both aromatase and 5α-reductase enzymes showed that the extract had an inhibitory activity on 5α-reductase enzymes (Hartman *et al.*, 1996). Moreover, *Pygeum africanum* extract has an antagonizing effect on production of 5-lipoxygenase in BPH (Cheetham, 2013). Furthermore, there was an indication of apoptotic activity and anti-proliferation in both prostatic myofibroblasts and fibroblasts (Kao *et al.*, 2014).

The dichlomethene, ethanol and methanol extracts of cactus flower, is known to have an inhibitory activity, it blocks aromatase and 5 alpha reductase activities, whereas the aqueous extract showed a significant antioxidant effect (Jonas *et al.*, 1998).

In a study aimed at investigating, whether combination of *Ponax ginseng* and bee pollen has inhibitory effects on the development of BPH, demonstrated that the combined extract (K053) effectively reduced the size of the prostate, both epithelial and thickness. In addition, it was observed that K053 contributed to decreased expression of BTGF-B1 and VEGF (Park *et al.*, 2015).

Clinical trial, carried out in 287 patients, having been exposed to nettle (*Urtica dioica*) extract showed a substantial decrease in PSA, IPSS and prostate size (Safarinejad, 2006). A further evaluation to analyze the effectiveness of *U. dioica* on the reduction of clinical manifestation of BPH in 100 patients demonstrated that *U. dioica* had a positive outcome in alleviating the clinical manifestations of the patients with BPH equated to the placebos (Ghorbanibirgani *et al.*, 2012).

2.3.1 *Croton membranaceus*

Croton is a perennial plant with more or less woody slender stems, growing up to 1 to 2 meters tall, that belongs Euphorbiaceae family described in 1767 by Swedish botanist, Carolus Linnaeus, more than 1223 species of the genus have been established (Nath *et al*., 2013). The commonest species found in West Africa is the *Croton membranaceus*, growing mostly along the river banks and has been of interest to researchers due to its medicinal property (Aboagye, 1997). Traditionally, *C. membranaceus* root extract is known to be used to manage prostate hyperplasia and the affiliated cancers (Obu, 2015). Its various parts are commonly used to treat and manage various diseases; the leaves are significant in the treatment of digestion disorders and anorexia in Nigeria while in the Bahamas it is used to aromatize tobacco (Asare *et al*., 2011). The bark produces special oil which is used to treat farts, fever, cough and diarrhea (Aboagye *et al*., 2000). The roots have been reported to have an effect on secondary bacterial infections due to measles (Bayor *et al*., 2009). The plant is known to contain various phytochemicals, the root bark contains scopoletin, Julocrotine, sitosterol-3-d-glucoside as glutarimide alkaloid as well as calcium oxalate crystals, labdane diterpenoid, gomojoside H and dl-threitol (Aboagye *et al*., 2000, Asare *et al*., 2011). *In vivo* studies carried out by Asare *et al.* (2011), indicated that the ethanolic extract of this plant is non-toxic. In another study carried out by Afriyie *et al*. (2014), the root extract of *C.*

membranaceus was found to have a significant inhibition of proliferative glandular epithelial cells and reduction of serum PSA levels in BPH induced rats, their prostate volume was found to have significantly reduced similar to the finasteride controlled group. These marked the genesis of clinical trials in men suffering from LUTS where positive impact was noted. Further study by Asare *et al.* (2015), aimed at establishing the effect of *C. membranaceus* on cardiovascular disease markers found out that the CM alcoholic root extract had a positive effect on CVD biomarkers with hypoglycemic effect.

***Figure 2.1*:** Aerial parts (left), a branch of *Croton membranaceus* (right) and roots collected in Ghana.

Adapted from: *www. Researchgate.net*

2.3.2 *Annona muricata*

Annona muricata also referred to as soursop, graviola, guanabana, paw-paw and sirsak is an evergreen tropical plant of the *Annonaceae* family with almost 130 genera and 1300 species growing to a height of 5-6 meters with broad leaf flowering and adapted to areas of high humidity and relatively warm winters (Moghadamtousi *et al.*, 2015). The plant is traditionally found in the

tropics of North and South America, but it is now globally distributed in different tropics in the world, including Southeast Asia and some parts of West Africa, where its fruit has extensively been used for the preparation of syrups, candies beverages and shakes (Mishra, 2013). The leaves, bark, fruits and seeds of the plant have been used as remedy of choice in the management of countless conditions. The most extensively used preparation being the decoction of bark, root, seed or leaf (Badrie & Schauss, 2010).

A. muricata is reported to possess more than 212 active bio compounds prime compounds being acetogenins, alkaloids, and phenols among other compounds. Isolates from the leaves, fruits and barks of A. muricata is known to produce more than 100 annonaceous acetogenins. Most phytochemicals have been identified from organic extract, but recently focus has also been directed toward aqueous extracts (Moghadamtousi *et al*., 2015, Yang *et al*., 2015). Asare *et al*. (2015) showed that aqueous leaf extract of *A. muricata* cytotoxic anti-proliferative effect on BPH-1 cell line and is able to reduce the size of the prostate, making it a significant remedy for BPH and improvement of quality of life. The toxicity reported for the extracts is also variable depending on the plant part used, and the solvent employed (Coria-Tellez *et al*., 2016). Ioannis (2016) in both *in vitro* and *in vivo* model studies, demonstrated that *A. muricata* extract promotes necrosis in PC-3 lines, thus restraining tumor versatility and cell metabolism.

Another study by Yang *et al*. (2015) on sygenism of phytochemical among constituents of *A. muricata* leaf extracts compared to its flavonoid and acetogenin enriched fractions demonstrated antiproliferative efficacy, viability and clonogenic ability of the plant on *in vitro* and *in vivo* prostate cancer models.

Figure 2.2: *Annona muricata* plant showing stem with leaves and fruit

Adapted from: *www. commons.wikimedia.org*

2.4. Drug Interactions

Drug interaction usually occurs when a particular drug is administered together with any xenobiotics, altering the pharmacological response of the drug in terms of its strength or period of action (Davis *et al.*, 2013). At least two drugs when administered simultaneously are likely to interact and depicted as a variance in efficacy or an adverse reaction or a consummately incipient side effect that is not visually perceived with any of the drugs alone that can be sufficiently rigorous to change the outcome of the condition and warrant admission to the hospital (Rahal *et al*., 2013).

The clinically significant drug-drug interaction arises with drugs that have got low therapeutic index and observable toxicity, such that a passable change in the drug effect can significantly have adverse results (García *et al.*, 2012). The mechanism of interactions may be classified as pharmacokinetics, pharmacodynamics, synergistic or antagonistic. Adverse interactions due to simultaneous therapy are usually related to drugs that are antagonistic either physiochemically or biochemically (Barrera *et al*., 2012). In combined therapy, it is important to

ascertain the prevalence and occurrence of drug interactions with consequential results on the patients and most significantly, to find out agent responsible for adverse interaction (Kamet *et al*., 2012).

Major pathways for drug interactions are via the CYP450 enzymes. The simultaneous induction or inhibition of CYPs can alter the metabolism of either drugs administered which can result in reduced efficacy or toxicity that can be fatal. Induction for CYP enzymes often results in therapeutic failures because it lowers the plasma concentration of the drugs (Rainone *et al.*, 2015). For instance, study to investigate CYP 450 mediated drug interaction, demonstrated that St. John's wort drug is a potent inducer of CYP3A4 and consequently, the induced CYP3A4 reduces the bioavailability of other drugs, making them less effective (Wanwimolruk & Prachayasittikul, 2014). In Another *in vitro* study, aged garlic extract was found to inhibit CYP2C9, 2C19, CYP3A4, 3A5 and 3A7 activity while it did not affect the CYP2D6 activity and increased recombinant human CYP isoenzyme system (Berginc *et al.*, 2009).

2.4.1 Herb Interactions

Herbal medicine may contain compounds having antagonistic effect, this can lessen the effectiveness of another drug and lead to a probable drug failure as observed with statin therapies. Synergism or antagonism exhibited by the co-administered drugs is caused when the drugs act or compete for the same target point, for instance, the same Vitamin K epoxide reductase targeted by warfarin in the coagulation cascade is also targeted by garlic, ginger, *Medicago sativa*, *Matricaria recurtita* and danshen thus increasing the risk of bleeding in patients on warfarin therapy (Mamindla *et al*., 2016).

The inhibitory effect of the herbs may be competitive, non-competitive or mechanism based. The mechanism based is marked by NADPH-time and concentration dependent enzyme inactivation, which occurs when some compounds in the herbs are converted by the CYPs to reactive metabolites that can bind to CYPs permanently (Zhou, 2005).

Camellia sinensis (green tea) is used worldwide as a medicinal and dietary herb. Its leaves are consumed as a beverage, and its purified extract has been approved as a botanical drug. In rats, the administration of green tea extract increases the in *vivo* activity of CYP1A, CYP2B, and CYP3A (Maliakal, 2001; Nishikawa *et al.*, 2004). In human, green tea extract is known to inhibit CYP2C9, CYP2D6, and CYP3A4 activities in liver microsomes, while it induces the m-RNA and protein expression of CYP1A2 in LS- 180 cells and CYP1A1/2 in Caco-2cells (Netsch, 2006).

Herbal formulations generally are made up of various herbs, posing a great challenge in synergistic studies. A blend of (Danshen) *Salvia miltorrhiza* and (Gegen) *Pueraria lobate* is usually used to treat coronary heart disease (Sieveking *et al.*, 2005; Koon *et al.*, 2011). Cheung *et al*. (2012), in a study to establish the combined effect of Danshen and Gegen concluded that anti-atherogenic effect of the combined herbs had a synergistic, additive and antagonistic effects in anti-inflammation and anti-vascular smooth muscle cell proliferation respectively.

Yi and Wetzstein (2011), demonstrated *in vitro*, a two by two synergistic interactions and antitumor potential of a compounded herbal formulation, the study involved five commonly used medicinal herb extracts, Thyme (*Thymus vulgaris* L.), Rosemary (*Rosmarinus officinalis* L.), Sage (*Salvia officinalis* L.), Spearmint (*Mentha spicata* L.) and Peppermint (*Mentha piperita* L.).

Likewise, Adams *et al*. (2006), using five popular herbs *Rabdosia rubescens*, *Scutellaria baicalensis*, *Panax-pseudo ginseng*, *Dendranthema morifolium*, and *Sereno arepens* investigated the interactions among the herbs in prostate carcinoma cell line (22Rv1). The iso-bolographic

analysis showed that the combined effect of the five herbs was significantly higher than when the herbs are used alone or in two by two combination.

Grapefruit has been used in the management of cardiovascular diseases and as a remedy for losing weight. Both naringenin and bergamottin compounds found in the grapefruit are known to inhibit CYP3A4 enzyme in the small intestinal enterocytes, consequently increasing the blood levels of CYP3A4 substrate herbs such as black cohosh extract, or *Cimicifuga racemose*. Therefore, the action of these drugs is heightened by their increased bioavailability that can potentiate hypotension, myopathy or hepatotoxicity (Bailey, 1998). Grape fruit juice is able to increase the risk of breast cancer in post-menopausal women who take herbs that contains estrogen since the metabolism of estrogen by CYP3A4 is inhibited (Tachjian *et al.*, 2009)

Ginkgo biloba extracts used in the management of BPH have been demonstrated to contain ginkgolic acids I and II, which inhibits CYP3A4, CYP1A2, CYP2C9, CYP2D6 and CYP2C19 *in-vitro i*n human (Yale, 2005). When administered with diltiazem it increases its bioavailability through mechanism based inhibition of the CYP3A4 in rats (Ohnishi, 2003). However, the effects that *Ginkgo* might have on the bioavailability of drugs and herbs metabolized by CYP3A4 needs further studies (Nowack, 2008).

In a study, it was established that curcumin has an inhibitory reaction of ethoxyresofurin, methoxyresofurin and pentoxyresofurin, which are catalyzed by CYP1A2, CYP2B1 and CYP1ADME1 in the rat liver (Thapliyal & Maru, 2001). Evidence has also been presented showing that the activities of Phase II DME especially GST in the male mouse liver and kidney are induced by curicumin extracts (Blasius, 2004). In a different study, Wang *et al.* (2014), showed that curicumin is a strong inhibitor of CYP2C9 in human liver microsomes than in the rats through

a non-competitive inhibitory mechanism. This shows that curicumin is likely to cause a herb-herb interaction if administered with other herbs which relies on CYP2C9 for metabolism.

A study looking into the potential interaction of the seed extract of *Urtica urens* L. (Dwarf nettle), suggested that treatment with *U. urens* decreases the CYP2E1 activity, this may induce toxicity when simultaneously taken with herbs or drugs relying on CYP2E1 for metabolism. Further, it was established that *U. urens* had a selective inhibitory effect on the activities of Phase I DMEs in comparison to effect on the activities of GST enzyme (Agus, 2009).

Pharmacokinetics encompass mostly induction and inhibition interactions of the CYP450 enzymes and P-glycoproteins consequently altering the course of drug absorption, distribution, metabolism and excretion further leading to alteration of plasma drug levels (Dülger, 2012).

2.5 Xenobiotics

Xenobiotics are chemical that would not normally be found in a living organism or be expected to be produced by it. Before they get into the circulation, they have to cross intestinal epithelium, basement membrane and endothelial linings. Drugs can be classified as a subset of xenobiotics (Grace *et al*., 2008). They are usually absorbed in the intestines through, active transport, diffusions, pinocytosis, filtration through “pores” and absorption across the lymphatic system. Many xenobiotics produce a variety of biological effects, which are used, when they are characterized using bioassay (Chen., *et al*, 2007).

2.5.1 Xenobiotic Metabolism Reactions

The metabolic process of xenobiotics involves the transformation of lipophilic compounds to hydrophilic substances through the action of metabolizing enzymes, ensuring their excretion from the body system through sweats, biliary and renal systems, thus playing a key role in drug interactions and bioavailability (Rendic, 1997, Oesch *et al*., 2000).

The xenobiotic metabolism usually involves two major classes of reactions, namely, phase-I (functionalization) which includes process such as N-O dealkylation, N-S oxidation and deamination, aliphatic -aromatic hydroxylation and Phase II reactions (Conjugation) that includes glucoronidation, methylation, acetylation, sulfation, glutathione and conjugation of amino acids. The formed conjugates in phase II are highly hydrophilic than the initial compounds. Most of the xenobiotics are converted into active electrophilic metabolites, (Macherey & Dansette, 2008, Jancova, 2010).

Every compound can undergo several reactions as shown in the analysis of 6767 metabolic reactions from over 1171 substrates that included both medicinal and xenobiotic compounds. From the study, redox reaction accounted for 57% of all metabolites, estearases for 10% and conjugation for 33%. (Testa & Soine, 2003). A total of 40% and 14% of the reported metabolites were produced by the CYP and UGT enzymes respectively (Testa *et al.*, 2012). From the same study first generation metabolites accounted for 42% of all metabolites, 32% second generation and 26% accounted for third and later generation.

2.5.2 Xenobiotic Metabolizing Enzymes

Most chemical reactions in the body takes place due to the presence of enzymes. Without these enzymes, there is reduction or complete absence of metabolic processes which can lead to severe health complications (Omiecinski, 2011). A Study of the top 200 drugs prescribed showed that 73% of the drugs are cleared through metabolism, 24% through renal and only 3% trough biliary (William *et al*., 2004). The xenobiotic metabolizing enzymes, which are contained in the body tissues with highest concentration in the liver ensures safe elimination of xenobiotics through biotransformation process (Zanger *et al.*, 2014).

There is evidence that the activity and expression of DME is affected by liver hepatic diseases and injuries (Guo *et al.*, 2016). The enzymes involved in the metabolism are categorized into phase I and phase II; phase I comprises the largest family of membrane bound, non-specific mixed function, enzyme system known as the CYP450 which are the first enzymatic response towards xenobiotics (Schonborn, 2010). Phase II DMEs are usually transferases and includes; UDP-glucoronosyl transferases (UGTs), sulfotransferases (SULTs), N-acetyltransferases (NATs), glutathione-s-transferases (GSTs) and various methyltransferases (Jancova, 2010).

The elimination of products formed in Phase I metabolism is not usually undertaken in a rapid manner. However, the endogenous substrate must further combine with newly established functional groups leading to the formation of highly polar conjugates making them easily eliminated from the body system (LeBlanc & Dauterman, 2001).

2.5.2.1 Cytochrome P450) enzymes

The ability of mammalian tissues to oxidize lipid soluble xenobiotics was discovered in 1950's. However, the responsible enzyme was unknown at that time. Through spectrophotometric analysis, a single cytochrome" P-450'' was believed to exist only in the liver (Klingenberg, 1958, Garfinkel, 1958), mainly for drug and chemical metabolism (Conney, 1967). Being able to be induced implied that they would be significant in pharmacology and therapies (Gillete *et al.*, 1972, Parke 1975).

In 1964, a heme protein of the B-type class was demonstrated and it was termed Cytochrome p450 (CYP) defined as an "Iron containing protein that takes part in cell respiration as a redox catalyst" following a strong feature in its absorption spectrum (Shimizu *et al.*, 1975, Omura and Sato, 1964). Later studies using electron spin resonance spectroscopy revealed that CYP is a low spin ferric heme-protein with a thiol residue as an axil heme ligand, leading to the

emphasis of a better name for CYP 450 to be heme-thiolate monooxygenase (Bayer *et al*., 1969; Werck-Reichhart *et al*., 2000). With the heightening of molecular biology in the 1980's it was evident that the CYP 450 genes was in almost all species and alignment, inferred amino acid sequences leading to the proposition of the nomenclature system of the gene super family based on the evolutionary divergence (Nebert, 1987; Nebert *et al.*, 2013).

Currently Cytochromes P450 (CYP) is known to comprise of more than 300 different enzymes, which are classified into several families and subfamilies according to their amino acid sequences (Taxak & Bharatan, 2014). For a gene, be considered in the same family its amino acid sequences ought to be 40% or more identical, followed by a letter specifying the subfamily must have a 55% or more identical amino acid sequence and the highly-conserved gene number with the same functionality (Božina *et al.*, 2009). It is estimated that in humans, there are 57 distinctive functional genes and 58 pseudo genes which are encoded by various gene clusters distributed over most autosomal chromosomes compared to 108 functional and eighty-eight pseudo genes found in mouse (Nelson, 2004). The majority of the human genes grouped into eighteen families and forty-four subfamilies (Zanger *et al.*, 2014), have a definite endogenous function such as the biosynthesis of steroid hormones prostaglandins bile acids among others (Nebert and Russell, 2002). The main families that are involved in drug and other xenobiotic substrate metabolism belongs to 1,2 and 3 CYP families.

A survey done in the US by Wienkers and Health (2005) showed that CYP450 superfamily metabolizes 75% of clinically significant drugs prescribed. Zanger and Schwab (2013) also analyzed 248 prescribed drugs to demonstrate the activity of each drug metabolizing CYP450 enzymes to drug metabolizing pathway, in addition to numerous genetic and non-genetic factors that affect the variability in enzymatic activities, hence affecting the therapeutic outcomes as

shown in fig 2.3. Several investigations have strived to evaluate the abundance of CYP450 enzymes, these studies described the genetic and epigenetic factors that affect their expression. The abundance of data on CYP450 can be used for inferring *in vitro* data to *in vivo* pharmacokinetics parameters; hence, the simulation experiments involving implicit population for pharmacological and toxicological evaluation of drugs (Achour *et al*., 2014).

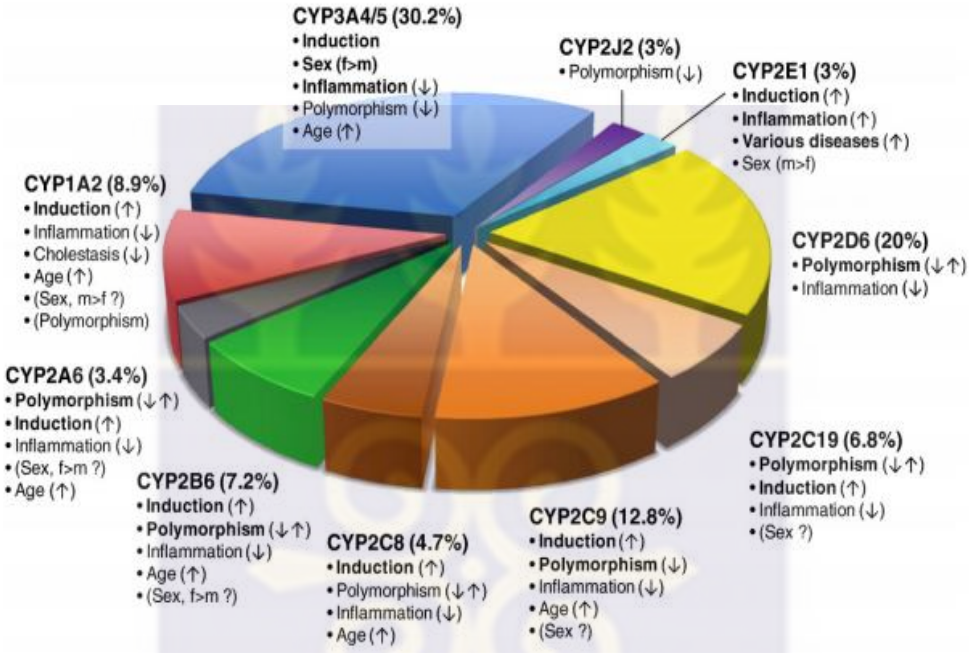

Figure 2.2: Clinical drugs metabolized by CYP450 Iso-enzymes and determinants of their variability

***Adapted from*:** Zanger and Schwab, (2013).

Figure 2.2 shows the involvement of CYP iso enzymes in the metabolism of 248 prescribed drugs. Important variable factors are shown with the probable direction of influence designated (↑, Increased activity; ↓ decreased activity; ↑↓, increased and decreased activity) Significant contentious factors are shown in brackets.

2.5.2.1.1 CYP1 Family

This family constitutes 3 subfamilies controlled by 3 functional genes, CYP1A1, CYP1A2 and CYP1B. The two highly conserved genes have seven exons and six introns, which are located on chromosomes 15q24.1 while, CYPB1 have only 3 exons located on chromosomes 2p22.2 despite encoding the largest human CYP450 enzymes in terms of the size of m RNA and number of amino acids (Nelson *et al*., 2004). There are no pseudo genes in this family (Cytochrome P450 Knowledgebase, 2006), both CYP1A1 and CYPA2 demonstrate a higher amino acid sequence homology however, they exhibit a divergent pattern of expression in the tissues. CYPA1 is primarily and mostly expressed in extra hepatic tissues like the lungs, lymphocytes and the placenta whereas the CYP1A2 is expressed in the liver (Rainone *et al.*, 2015).

On the other hand, CYP1B1 is expressed in various tissues, including placenta, brain, prostate leukocytes, ovary kidney spleen and heart (Sutter *et al*., 1994). All the three genes are controlled transcriptionally by AHR-ARNT pathway and are substrate inducible, accountable for the metabolism of several xenobiotics substrates including polycyclic aromatic hydrocarbons (PAH) like carcinogen-benzo (a) pyrene. They discretely catalyze the N-O reaction of carcinogenic aromatic and heterocyclic amines found in charred foods and cigarrate smokes (Blackburn *et al*., 2015). The Polymorphism influencing CYP1A gene transcriptional activities appear to have a restricted influence on drug metabolism, however, it can be utilized as a marker for predictive diagnosis for cancer in some people (Akillu *et al*., 2002). The cause of most cancers is interrelated to the formation of adducts between DNA and the oxidized products from CYP450 catalyzed reactions (Rainone *et al*., 2015).

2.5.2.1.1.1 The role of CYP1A2 in drug metabolism

CYP1 enzyme catalytic activities are overlying including hydroxylation and biotransformation of PAH and other aromatic substances through oxidation, although CYP1A inclines to planar aromatic hydrocarbons CYP1A2 prefers aromatic amines and heterocyclic compounds (Zanger and Schwab, 2013). Elevated amount of CYP1A2 are expressed in the liver, however it is not expressed in the extra hepatic tissues. Contrary, liver contains low or undetectable levels of CYP1A1 and CYP1B1 but these are expressed mainly in extra hepatic tissues (Shimada, 2006). Amongst the CYP1 family only CYP1A2 is significant in *in vivo* metabolism of drugs (Zhou *et al*., 2009). This includes analgesics and antipyretics such as acetaminophen and lidocaine, antipsychotics such as clozapine and olanzapine, anti-depressants, cardiovascular drugs such as propranolol, guanabenz, triamterene, hypnotic zolpidem used in the treatment of insomnia and Caffeine (Božina *et al*., 2009).

CYP1A2 has also been demonstrated to oxidize uroporphyrinogen to uroporphyrin in the heme metabolism and hydroxylate estrogen. The capability of using estrogen as substrate is significant in the role of CYP1A2 bio activation of environmental pro-estrogens (Nebert & Dalton 2006). Administration of clinically significant drugs that acts as substrates to CYP1A2, is a precursor of drug interactions, occasioned by reversible or irreversible inhibition of the enzymes by numerous minute biomolecules that attaches into the potent site or through aryl hydrocarbon receptor (Ahr) -mediated gene induction (Hiemke & Harter, 2000).

Caffeine induces CYP1A2 and 95% of the caffeine is metabolized by the enzyme. The A-C polymorphism appearing at position 163 of the CYP1A2 gene has been linked to reduced ability of the enzyme to be induced and its activity, resulting into slow caffeine metabolism (Han *et al*., 2001). A study recorded that among the breast cancer susceptibility gene, BRCA1 mutation

carriers, had a likely hood of reduced risk to breast cancer when they consumed caffeinated coffee, however, women who possessed at least one variant (c) gene at position 163, and consumed the coffee had 64% lower breast cancer risk than women who did not consume the coffee (Kotsopoulos *et al*., 2007).

Carriers of variant (c) of CYP1A2 gene have been reported to have a higher risk of getting myocardial infarction, this is suggestively attributable to prolonged presence of caffeine in the blood system among slow metabolizers due to low induction by the enzyme (Cornelis *et al.*, 2006). The described functional polymorphisms are relevant in drug effect and shows inter individual and racial variation (Guanaratna, 2000).

In vitro studies have clearly demonstrated that CYP1A2 is highly effective in catalyzing the initial step (N-hydroxylation), in the activation of numerous carcinogenic aromatic amines (4-aminobiphenyl, 2-aminonaphthalene), aromatic amides (2-acetylanminoflourine), and heterocyclic amines to mutagenic metabolites. In case of PAHs, it was seen that these carcinogens are also Ahr agonists; therefore, it was long assumed that PAHs is capable of inducing CYP1enzymes and thereby induce their own activation to carcinogenic metabolites (Shimada, 2006; Nebert and Dalton, 2006).

In contradiction to the expected, deletion of CYP1A2 protects against the toxicity and liver tumorigenicity of aromatic or heterocyclic amines. Deletion of CYP1A2 in null mice showed increased toxicity of 4-aminobiphenyl adduct formation in the liver and bladder, and occurrence of hepatocellular tumors. Deletion of CYP1A2 also increased the adduct and tumor formation by cooked food mutagens. These results highly suggest that CYP1A2 plays a protective role in aromatic, heterocyclic amines and amide metabolism, and other enzyme activates them (Ma and Lu, 2007; Shimada 2006).

2.5.2.1.2 CYP2 Family

CYP2 family is considered the largest and most diverse family of CYP 450, classified into 13 subfamilies containing 16 full length functional genes which have 9 exons and 8 introns (Nelson *et al*., 2004). Many therapeutic drugs, including anti-depressants, antipsychotic and abused drugs are metabolized into active and inactive metabolites by most enzymes in this family while others are expressed in a sex specific manner supporting hydroxylation of steroids (Paschke *et al*., 2001). The most significant enzymes in this family are highly polymorphic; CYP2A6, CYP2B6, CYP2C9, CYP2C19, & CYP2D6 (Zanger and Schwab 2013).

Enzymes in CYP2A gene family depict variables in catalytic function, for instance two of the three CYP2A enzymes expressed in rat liver, namely CYP2A1 and CYP2A2, primarily catalyzes the 7-alpha and 15- alpha hydroxylation of testosterone, respectively. Contrary the CYP2A enzyme that is expressed in human liver, CYP2A6, catalyzes the 7-hydroxylation of coumarin (Parkinson *et al*., 2013).

CYP2A sub family is composed of CYP2A6, CYP2A7 and CYP2A13; CYP2B consists of only CYP2B6 which metabolizes several drugs including benzphetamine, cinnarizine, bupropion, verapamil and lidocaine and environmental or abused toxicants including nicotine (Rainone *et al*., 2015). The CYP2C family comprises of four genes that encodes products with 80% amino acid, of the four, CYP2C9 and CYP2C19 are the main enzymes that play a key role in the metabolism of clinically prescribed drugs including the endogenous compounds such as arachidonic acid (Edwards *et al.*, 1998).

The CYP2D family has only one functional enzyme, CYP2D6 and four pseudogenes (Lee, 2002). In the evolution of rodents, many of the CYP2 subfamilies expanded extensively,

complicating the process of identifying true orthologues between human and mouse CYP450 (Nelson *et al*., 2004).

2.5.2.1.2.1 Role of CYP2C9 in Drug metabolism

The expression of CYP2C9 is highly observed in the liver tissues more than any other enzyme in the class of CYP2, followed by CYP2C8 and CYP2C19 sub families. CYP2C9 demonstrates a powerful catalytic activity towards numerous drugs, including the anticoagulant warfarin, the antidiabetic agent tolbutamide, sedatives such as hexobarbital, ibuprofen and other non-steroidal anti-inflammatory drugs, barbiturates and anticonvulsants such as phenytoin and trimethadione (Fuhr *et al*., 2007). It is highly polymorphic, 58 allelic variants of CYP2C9 have been identified, each of which lead to variability in individuals functional CYP2C9 polymorphism (Pan *et al*., 2014). The 2* and *3 variants, are recognized as the most active in drug disposition and safety. Functional CYP2C9 polymorphism is said to be approximately 0.25% in Caucasians or even less in Asians; nevertheless, the clinical ramification of the unusual polymorphic poor metabolizers can result in life threatening bleeding in patients on warfarin therapy and acute toxicity with phenytoin (Lu *et al*., 2008; Zhou *et al*., 2009).

Several studies have established the CYP2C9 polymorphism on the metabolism of first and second-generation sulfonylurea hypoglycemic drugs such as glimepinde. Further, it has been shown, they interfere with the pharmacokinetics course and partly on the therapeutic responses for instance, the secondary failure of HbA1c therapy in hypoglycemic episodes (Aquilante, 2010). Another study indicated that patients with type 2 diabetes mellitus having variant alleles of CYP2C9 responded better when treated with glibenclamide in contrast to those with normal genotype (Surendiran *et al*., 2011).

CYP2C9 catalyzes epoxiganation of arachidonic acid in endothelial cells where it is highly expressed, an indication that it plays a significant part in ischemic disease and vascular homeostasis (Chehal and Granville, 2006). Nevertheless, in three different studies with a total sample population of over 52,000 revealed that there exists no linkage between CYP2C9 *2 and CYP2C9*3 polymorphism and risk of getting atherosclerosis, ischemic heart disease or death resulting from ischemic heart attacks (Kaur-Knudsen *et al*., 2009). Apart from polymorphism the variance in the activity of CYP2C9 can be altered by other factors such as environmental factors; which is propagated by prototypical CAR, PXR, GR ligands via compounds in the promoter region or impeded by oral contraceptives (Martins *et al*., 2014).

Tienilic acid, suprofen and sylibin are all metabolism dependant of CYP2C9. Tienilic acid was withdrawn from the market because of its ability to cause hepatotoxicity. CYP2C9 converts tienilic acid to an electrophilic thiophene sulfoxide that can react with either water, giving 5-hydroxytienelic acid or with a nucleophilic amino acid in CYP2C9 to form a covalent adduct which inactivates the enzyme (Lecoeur *et al*., 1994).

2.5.2.1.2.2 Role of CYP2D6 in Drug metabolism

CYP2D6 subfamily comprises of a sole functional enzyme, the CYP2D6 and four other pseudo genes and in spite its low hepatic expression it contributes significantly in the process of drug metabolism (Lee *et al.,* 2002). CYP2D6 polymorphism is very significant in drug metabolism, the enzyme metabolizes almost quarter of the marketed drugs. CYP2D6 expression in the liver is much higher compared to expression in the duodenum and brain (Eichelbaum *et al*, 2006). The most significant CYP2D6 substrate is β-adrenergic blocking agents (*e.g.*, labetalol, timolol, propranolol, pindolol, metoprolol), antidepressants (*e.g.*,amitriptyline, paroxetine, venlafaxine, fluoxetine, Prozac, trazadone), antiarrhythmic **(***e.g.*, flecainide, mexiletine,

propafenone), antipsychotics (*e.g.*, chlorpromazine, haloperidol, thoridazine), and narcotic analgesics (*e.g.* codeine, fentanyl, meperidine, oxycodone, propoxyphene) (Rainone *et al*., 2015). Additionally, CYP2D6 utilizes amine and steroids as endogenous substrates, and seemingly it appears to significantly cause adverse drug reactions (ADR) due to its high polymorphic characteristic (Božina, *et al*., 2009). It can also have a major role in the metabolism of food constituents, particularly alkaloids (Gurley *et al*., 2008).

Genetic polymorphism of CYP2D6 have been so comprehensively studied such that the individuals have been classified into four phenotypes, namely, poor metabolizers intermediate metabolizers, extensive metabolizers, ultra-rapid metabolizers. Further, extensive metabolizers have been categorized into high active extensive metabolizers, medium-active extensive metabolizers, and low activity extensive metabolizers. More than 100 allelic variants of CYP2D6 have been identified (Zhou *et al*., 2009). Intermediate or poor metabolizers possess copies of CYP2D6 alleles with reduced activity or are non- (Hicks *et al*., 2015).

Approximately 30% of Asians and individuals of Asian descent are intermediate metabolizers and only half of CYPD6 alleles are fully functional (Gaedigk *et al*., 2010). The reduced function*10 variant being very common; about 40% compared to 2% in Caucasians, this gives a chance to Asians to have more intermediate metabolizers compared to the Caucasians. Similarly, in Africans and Afro-Americans, only half of CYP2D6 alleles are functional (Dean, 2016). Different functional CYP2D6 gene variants have been described can be classified in categories according to whether they abolish, decrease, leave normal, increase, or qualitatively alter the catalytic activity (Božina *et al.*, 2009). A study done on the selective estrogen receptor modifier tamoxifen in tamoxifen therapy and CYP2D6, established that CYP2D6 poor

metabolizers have a higher risk of developing breast cancer while CYP2D6 intermediate metabolizers have to use CYP2D6 inhibitor in the treatment of breast cancer (Dean, 2016).

Numerous compounds which are not target substrates of CYP2D6, can bind to the enzyme because its highly susceptible to inhibition, however they bind to the enzyme with high affinity, for example quinidine or methadone (Darbar *et al*., 2007). Some of these inhibitors are strong enough to change the apparent phenotype of the patient. Several opioids, drugs, including codeine, dihydrocodeine, oxycodeine and tramadol used in pain management are metabolically activated by CYP2D6 and genotypic variation has shown to affect their efficacy and safety (Madadi *et al*., 2012). The prodrug codeine is O- demethylated by CYP2D6 to pharmacologically active analgesics morphine. In a recent study investigating the chemically related selective estrogen receptor modulator clomiphene, CYP2D6 was shown to be the key enzyme in the conversion of clomiphene and (E) 4-Hydroxy desethyl-clomiphene by human liver microsomes (Ji *et al*., 2016).

2.5.2.1.3 CYP 3 Family

The CYP3 family in humans consists of a single subfamily CYP3A which has four functional genes, namely CYP3A4, CYP3A5, CYP3A7, and CYP3A43 and two pseudo genes 3A5P1 and 3A5P2 (Rainone *et al*., 2015). CYP3A4 is highly expressed in the liver. However, it has a high population variability, while CYP3A5 is found in minor quantities expressed in the lungs. CYP3A7 is largely in the human fetal liver, although it can also be found in some adult liver and other organs (Salminen *et al*., 2011). Adult Caucasians presents a much lower expression of other CYP3A4 enzymes such as CYP3A5, CYP3A7 and CYP3A43 in the liver, however CYP3A5 may contribute up to about 50% of the CYP3A pool in individuals with at least one CYP3A5∗1 allele and with low CYP3A4 expression (Klein and Zanger, 2013).

CYP3 enzymes have similar substrates specification due to the high similarity in amino acid sequence between the isoenzymes for instance CYP3A4 and CYP3A5 share more than 85% primary amino acid sequence is identical (Williams *et al.,* 2002). Even though CYP3A4 and CYP3A5 are highly identical, they exhibit some limited substrate and regio-selectivity difference has been observed for example, in the aromatic ortho-hydroxylation of atorvastatin which is 16 times more effectively catalyzed by CYP3A4 than by CYP3A5 (Feidt *et al*., 2010). Thirty percent of clinically used drugs from almost all therapeutic categories are metabolized by enzymes from this family (Liu *et al*., 2008).

The function and regulation of the CYP3A enzymes are relatively conserved among mammalian species, with some notable exceptions. For example, rifampicin is an inducer of the CYP3A enzymes in human and rabbits, but not in rats or mice, whereas the opposite is true of pregnenolone-16 alpha carbonitrile (Parkinson *et al*., 2013).

2.5.2.1.3.1 Role of CYP3A4 in Drug metabolism

The most significant drug metabolizing CYP450 enzymes in human is the CYP3A4, it is highly expressed in the liver with low expression in small intestines, prostate, breast, gut colon and brain however it has more than 100 times population variability. It is estimated that the average CYP3A4 microsomal content is 60 pmol/mg of microsomal proteins (Guengerich, 1999, Ghosh, 2011, MacKinnon *et al.,* 1995, Ohtsuki *et al.,* 2012). It has been reported that CYP3A4 metabolizes about 60% of the currently administered drugs (Zanger *et al.,* 2008).

CYP3A4 takes part in metabolism of broad scope of structurally different clinically used drugs (Zhou, 2008). CYP3A4 is also known to play a major role in the metabolism of endogenous substrates such as retinoic and bile acids and steroids such as testosterone and estrogen (Eichelbaum *et al.,* 2006, Rendic, 2002). CYP3A4 is also significant in the metabolism of dietary

and environmental chemicals such as PAH-diols, mycotoxins, aflatoxins, various food additives and flavonoids (Guengerich, 1999, Kamdem *et al.,* 2006). Recent studies by Basheer and Karem (2014) on the interaction of CYP3A4 and dietary polyphenols deduced that the polyphenols present in the herbal preparation are responsible for interaction with CYP3A4 supported with the fact that CYP3A4 is abundant in the intestines and its broad range of substrate inhibitors and cooperativity.

The enzyme can be inhibited by several drugs and chemicals such as azole antifungals agents such as ketoconazole, macrolide such as trolendomycin, Anti-retroviral (ARV) such as saquinavir and 6,7 dihydroxy bergamottin found in grape juice (Nivoix *et al.*, 2008, Božina *et al.*, 2009). The main cause of drug interactions is thought to be due to the inhibition of CYP3A4, the enzyme interactions can result into severe clinical effects of rhabdomyolysis, hypotension, ataxia and myopathy. CYP3A4 is also induced by many drugs such as rifampicin, anticonvulsants such as carbamazepine and glucorticoids. The St Johns wart contents, especially hyperforin are potent inducers (Moore, 2000).

Most of the clinically significant interactions such as the drug-drug interaction, drug-herbal interaction and drug-food interaction, usually involves inhibition or induction of the CYP3A4 enzyme in the liver or in the small intestines. The three casualty drugs whose clearance entirely depends on CYP3A4, namely, terfenadine, cisapride and astemizole have been withdrawn from the market because of their ability to cause ventricular arrhythmias and heart attack when the metabolism by CYP3A4 was inhibited by drugs such as ketoconazole and erythromycin (Zhang *et al.*,2009).

2.5.2.2 Glutathione transferases

Human glutathione transferases sometimes referred to as glutathione S-transferases or GSTs are major Phase II detoxification enzymes belonging to a multigene family of dimeric multifunctioning enzymes that are found in the cytosol and are involved in the biotransformation of a wide range of exogenous and endogenous electrophilic compounds such as herbicides, drugs, insecticides and xenobiotics (Sheehan *et al.*, 2001, Higgins and Hayes, 2011). These enzymes are established in almost every tissue in the body catalyzing the transfer of GSH onto hydrophobic molecules or GSH removal from specific substrates such as reduced glutathione (GSH) or uridine diphosphate (UDP) -glucuronic acid (Hayes *et al.*, 2005).

GST activity was first discovered as the enzyme catalyzed conjugation of GSH with halogenated compounds such as chloro-nitro benzenes and bromosulphthalein (Higgins and Hayes, 2011). GSTs can catalyze numerous reactions, including nucleophilic aromatic substitutions, isomerization and reduction, conjugation numerous hydrophobic and electrophilic compounds with reduced GSH (Jancova, 2010). All compounds which are in a position to react with a thiol moiety of GSH are possible substrates for GSTs such as aliphatic and aromatic halogen compounds, peroxides and epoxides, α, β unsaturated and low molecular weight proteins (Deponte, 2013). The reaction of glutathione (GSH) with electrophilic substrates can be represented by the following general scheme:

$$GSH + R\text{-}X \rightarrow GSR + HX$$

The GST functions by bringing the substrates into proximity with GSH, joining the electrophile substrate and GSH together into the active site and to activate the sulfhydryl group of GSH, thus enabling a nucleophilic attack of GST on the substrate. GSTs can catalyze nucleophilic aromatic substitutions, Michael additions to α, β-unsaturated ketones, and epoxide ring-opening

reactions, all of which can lead to formation of GSH conjugates and the reduction of hydroperoxides, further forming oxidized glutathione (GSSG) (Armstrong, 1997).

GSTs also play a key role in the synthesis and metabolism of endogenous compounds, redox homeostasis and cellular signaling (Hayes *et al*., 2005). GST overexpression and polymorphisms are seen in several cancers as well as other diseases, and have been implicated in resistance to chemotherapy (Board and Menon, 2013). Thus, they are attractive anticancer targets. Indeed, several small-molecule GST inhibitors have been reported as anticancer agents.

GSTs comprise two well defined super families illustrated according to their basic, immunological and functional characteristic. Out of the two GSTs in human, membrane bound GSTs represent 45% of the total proteins present in the endoplasmic reticulum and the outer mitochondria while the cytosolic GSTs account for 10% of total cellular proteins (Akther *et al.*, 2016). The soluble GST superfamily is subdivided into eight separate classes assigned α (GSTA), μ (GSTM), θ (GSTT), π (GSTP), Z (GSTZ), σ (GSTS), K (GSTK), and ω (GSTO). This class of GST is chiefly depicted in the cytoplasm however, they are also found in the nucleus, mitochondrion and peroxisomes (Morel, 2004, White, 2008). Various GSTs were discovered first in the non –mammalian species, but later some were also identified in mammals. GST found in human has been classified as α (A1-A4), μ (M1-M5), π (P1), K (K1) and θ (T1, T2) with their subunit arrangement or isoenzyme assigned Arabic numerals. A class of GST shares more than 60% characteristics within the class and 30% similarity with different classes (Jancova, 2010).

The second superfamily of GSTs is known as MAPEG (Membrane-Associated Proteins in Eicosanoid and Glutathione Metabolism), it has a trimeric structure, and usually involved in the biotransformation of arachidonic acid (Sherratt and Haye, 2001). The microsomal MAPEGs are an independent group which are integral microsomal and mitochondrial membrane components.

Some GST have non-enzymatic functions such as binding of zeaxanthin in the retina and c-Jun N-terminal kinase 1 by GSTP1-1 (Parkinson *et al*., 2013). Individuals from both GST families show independent actions on selenium glutathione peroxidase (Sheehan *et al*., 2001). Curiously, an invalid variation is experienced for two individuals, GSTT1 and GSTM1, whereby the whole gene is homozygously erased in a large portion of a different population, bringing about a total lack of corresponding enzymatic activity (Bhattacharjee, 2013).

A study carried out to demonstrate the combination of exact copy numbers of variations of GSTM1 and GSTT1 genes in prostate cancer susceptibility in African decent population indicated that GSTM1 and GSTM1/GSTT1 are associated with increased risk of prostate cancer (Emiville *et al.*, 2014). However, this observation requires further evaluation in other populations. Another study among the Bolivians population demonstrated that GSTM1 is associated with risk in gall bladder cancer (Sakai *et al*., 2016).

High levels of GSTπ has been demonstrated in several studies using neoplastic nodule hepatocarcinogenesis rat models this has elicited interest in investigating the levels of GSTπ in human tumors (Kaprilian, 2015). The GSTπ class has been the most investigated form of GST in tumours affecting human beings, the matched pair of normal and tumours tissues compared showed an increased level of the enzyme in stomach, colon, bladder, cervix and lung tumors (Peter *et al.*, 1990, Peter *et al.*, 1992). The marker for hepatotoxicity in rodents has been identified to be GSTπ (Sato, 1989), and also plays an important role in carcinogen detoxification. Studies in human have also shown that GST-pi variant genotype is associated with increased numbers of tumors of the kidney, bladder, pancreas and lung. Furthermore, it was documented that GST-π polymorphisms are associated with survival in anaplastic glioma patients; an explanation is that lower activity GST genotypes will allow more prolonged exposure of tumor cells to

chemotherapeutic agents (Kilburn *et al*., 2010). Therefore, inhibition of GST activity and depletion of GSH levels might potentiate the deleterious effects of many environmental toxicants and carcinogens; overexpression was detected in 58% of the examined breast cancer tissues (Khabaz, 2014).

An expansive number of GST inhibitors are known, e.g. engineered and natural phenols, quinones or subsidiaries of vitamin C. Kulkarni *et al.* (1994), portrayed all Trans' retinoic acid as an inhibitor of human placental and hepatic glutathione transferases in the micromolar range. GSTs have been observed to be hindered by glutathione subsidiaries or substrate analogs. Ploemen *et al.* (1994), in a study showed that human GSTs are inhibited by dopamine, α-methyldopa and 5-S-glutathionyldopamine. It has also been shown that GSTP1 inhibitors are able to suppress the inhibition of JNK by disrupting the GSTP1–JNK complex (Bräutigam *et al.*, 2015).

2.5.2.3 Arylsulfatase G

Enzymes belonging to sulphatases class have a highly-conserved gene, sharing a substantial sequence homology and a high level of structural homogeneity (Ferrante *et al*., 2002). Sulphatases, hydrolyses sulphated glycosaminoglycans, glycolipids, glycoproteins and hydroxysteroids of which each has refined specificity towards its discrete substrate *in-vivo*. A subset of sulphatases, generally with each having a different characteristic substrate is active *in vitro* against a typical set of small aromatic substrates thus named arylsulphatases (Peters, 1990). There is a functional correlation which reflects a higher standard of the anticipated sequence of amino acid similarity along the whole length of the enzyme (Parrenti, 1997).

Arylsulfatase (ARSG) is one of the latest identified enzymes, it has a higher expression in human tissues with high enzymatic activity against arylsulfate pseudo substrate p-Nitro catechol sulfate (pNCS) in acidic medium (Frese, 2008). The natural substrate of ARSG has been identified

as 3-O-sulfated N-sulfoglucosamine (GlcNS3S), and despite this identification and its role in the biotransformation of heparin sulfatases explicitly described, no information has been recorded on its biochemical properties including expression in tissues, post translation changes or process of transportation to lysozymes (Kowalewski *et al.*, 2014).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Design

This study was an experimental laboratory design with twenty-eight (28) male Albino Wistar rats weighing between 150-200 grams. The rats were divided randomly into 7 groups each with four (4) rats.

3.2 Plant Material Extract

The roots of *C. membranaceus* plants were collected from Gyekiti forest reserve in the eastern region of Ghana with the assistance of Centre for scientific research in plant medicine. The roots were carefully and thoroughly washed and sundried for two weeks, crushed and packed in specimen jars, labeled and stored at room temperature (25^{O} C $\pm$ 2). The roots were squashed for 1 day with 4 liters of purified water and subjected to heat for 1 hour. The squashed root extract was filtered using sterile four-fold gauze. Second 3 liters of water was added to the sediments, soaked for an additional 24 hours and the previous process was repeated to get another root extract. The extracts were combined and freeze dried, the yielded extract was stored in cold temperatures (2-8^{O} C) until they were used.

The *A. muricata* leaves were gathered in the environs of Accra city washed under running distilled water. The leaves were subsequently dried in the sun for 72 hours and the dried leaves were pounded and soaked in 1 kg/4 liters of purified water for 1 day. The blend was then boiled for 1 hour and filtered. The resulting marc was further soaked in 3 liters of water for a day the blend was re-boiled for an hour and sieved, the sieved solutions were mixed together and dried in a freezer.

3.3 Experimental Animals

The animals were caged in stainless cages at the experimental animal unit at the university of Ghana medical school, Korle-Bu. They were maintained under controlled environment of temperature 25 $\pm$ 1^{O} C, 51$\pm$ 2 humidity and a 12 hours light-darkness cycle. The animals were acclimatized for 7 days housing condition prior to the experiment and were fed *ad libitum* standard chow diet (AIN-93G) formulation from GAFCO Ghana limited) with a constant supply of water.

Group I which was the uncastrated negative control was administered distilled water, this group acts as the native group and not disease induced and not exposed to the test extracts. Group II was treated with 30mg/kg b. wt extracts of *C. membranaceus*. Group III was treated with 30mg/kg b. wt. extracts of *A. muricata* while group IV was treated with 30mg/kg b. wt mixed extract of both *A. muricata* and *C. membranaceus* (A60:C40), group V was treated with 30mg/kg b. wt of both *A. muricata* and *C. membranaceus* (A40:C60), group VI was the model group disease induced while Group VII was treated with 0.5mg/kg b. wt finasteride as positive control. The rats were castrated to cut out the excess testosterone hormone to allow the known amount of testosterone to be administered to induce BPH and to prevent further progression of BPH to prostate cancer due to further production of testosterone (Wright *et al*., 1999). The positive control drug, finasteride, is a well-known 5a-reductase inhibitor used for BPH treatment. The effective dose of finasteride was based on previous reports (Huynh, 2002).

After elapse of 30 days before the animals were sacrificed, they were anesthetized with chloroform and afterwards sacrificed by cervical dislocation and decapitation. The blood was drained and the abdomen and thoracic cavity opened to excise the liver on a cold ice block. The excised livers were placed on tissue absorbent and rapidly weighed and frozen in a refrigerator at a temperature of -20^{O}C.

3.4 Isolation of microsomes from Rat frozen liver

Liver microsomes were separated utilizing the differential centrifugation according to the method by Pelkonen *et al*. (1974) with minor alterations.

Frozen liver of rats administered with two plant extracts were placed on a glace plate and minced to small pieces by use of a razor blade until a paste like consistency was formed. The minced livers were put in a beaker containing 1 ml of cold PBS (phosphate buffer solution). The pieces were swirled gently and allowed to settle at the bottom of the beaker before being decanted to remove blood. The livers were homogenized using dounce tissue grinder in cold PBS, for each rat 1 g of liver was used. On ice, the tissue samples were homogenized with 50 strokes in 1 ml of the phosphate buffer. Additionally, 1 ml of the buffer was added to the homogenate, and then pipetted to fully up and down, to completely suspend the homogenate, (Bio vision Inc., Cat k249-50).

The homogenate was put in a micro centrifuge tube and vortexed for 30 secs, which was followed by incubation on ice for 1 min. The homogenate, was centrifuged at 3500 RPM (revolution per minute) for 15 min at 4^{O} C.

The supernatants from the homogenates were transferred to a clean centrifuge tube and spun at 10,000 RPM for 60 minutes at 4°C. The supernatant was then poured off and the pellets washed with 1 ml of homogenization buffer, and re-suspended in homogenization buffer (1 g w/ 0.8 ml vol), and spun at 15,000 RPM for another 60 minutes at cold temperature of 4°C. The supernatants were discarded, and the remaining pellets re-suspended in homogenization buffer using dounce homogenizer (1:1, w/v). The individual rats' homogenate was pooled for each group which then was used for each cytochrome analysis.

3.5 Preparation of PBS, (pH 7.4).

Two-part buffer solution was prepared, 600 ml of Solution A mixed with 400 ml of solution B

The two-part buffer solution was prepared by stock phosphate buffer solutions A and B as shown below:

3.5.1 Stock phosphate solution A

Sodium dihydrogen phosphate ..... 27.6 g 1-hydrate (0.2M $NaH_2PO_4.H_2O$)

Distilled water..... 1000 ml

The chemical was accurately weighed as shown above and transferred to a 500 ml jar, and then the jar was half filled with water, and mixed to dissolve the chemical. Then, topped up to the 500 ml mark with distilled water, and mixed well. Then the solution was transferred to a leak-proof bottle and labeled as A.

3.5.2 Stock phosphate solution B

Di-Sodium hydrogen phosphate ..... 14.2 g anhydrous (0.2M Na_2HPO_4)

Distilled water ..... 500 ml

This solution was prepared as described above for solution A. (Cheesbrough, 2002)

3.6 Rat Cytochrome P450 Elisa Test.

The liver microsomes were assayed for, Phase I DME; CYP1A2, CYP3A4, CYP2D6, CYP2C9, and phase II; Arylsulfatase G, GST-P1, and GST-M1.

3.6.1 Principle

ELISA Kit from Sunlong Biotech Co. Ltd (Hangzhou, China) was used to assay the enzymes. The sandwich-ELISA method was used for all the enzymes. The micro-ELISA strip plates were pre-coated with CYP1A2, CYP3A4, CYP2D6, CYP2C9, Arylsulfatase G, GST-P1, and GST-M1 specific antibodies respectively. The Corresponding antigens in the plasma bonded to form antigen-antibody complex. The antigen-antibody complex bound a secondary antibody, horseradish peroxidase (HRP) - conjugated antibody specific to the enzyme, to form a stable sandwich complex. The enzyme activity of the antibody-antigen-HRP complex obtained after washing and further binding to a substrate. The chromogen developed was directly proportional to the antigen concentration of the enzyme present.

3.6.2 Procedure of the ELISA test

1. The standards were diluted in duplicates in the micro ELISA strips plate set for standards. A volume of 50 µl of each standard was into the wells labeled B1 to F1, well A1 was left to act as a blank well.
2. In the wells set for sample, a volume of 40 µl sample dilution buffer was put into each well apart from the wells with standards afterwards 10 µl of homogenized liver microsomes was added within 5 minutes after adding the dilution into the wells. Making a dilution factor of 5.
3. The plates were then incubated for 30 minutes at $37^{O}C$
4. The unbounded proteins were washed off with the wash buffer repeatedly five (5) times
5. A volume of 50 µl of the second antibody (HRP-conjugate reagent) was added to each well apart from the blank well, incubated for 30 minutes and further washed repeatedly five (5) times with the washing buffer.

6. Coloring was done by adding a volume of 50 µl chromogen A together with chromogen 50 µl chromogen B while avoiding light exposure
7. The stoppage of the reaction was achieved by adding 1N HCL stop solution, the solution developed a yellow colour from blue.
8. The absorbance of the solution in the wells was read at 450 nm within 15 minutes after stopping the solution
9. The absorbance of the standard solutions was used to construct a reference curve with known concentrations of the standards, the constructed curve was used to infer the value of the unknown enzyme concentrations.

3.7 Statistical analysis

The mean concentration and standard deviation of each enzyme in each group was calculated. Significance of difference between groups and controls was analyzed by performing a one-way analysis of variance (ANOVA) Bonferroni post hoc analysis using SPSS software version 20, P-values <0.05 was considered statistically significant.

3.8 Ethical Consideration

Consent was sought from the Ethics and Protocol review committee of the University of Ghana, School of Biomedical and Allied Health Sciences. Ethics identification number: SBAHS-MD./10556975/AA/5A/206-2017. The study complied with the Helsinki Declaration of 1964 (revised in October 2008) and OECD protocol of 1998.

CHAPTER FOUR

4.0 RESULTS

Wistar albino rats weighing between 120-150 grams were used in this experimental study. The rats were fed on standard chow diet and kept under a 12-hour light/night conditions. The Wistar albino rats were grouped according to the treatment given, they were exposed to treatment with both extracts and observed against the control group which was not administered any extracts apart from water and the normal diet.

4.1 Concentration of the Enzymes

The mean and standard error mean (SEM) for each enzyme in phase I was calculated as shown in table 4.1 CYP1A2 showed a statistical significance for group I, II and group V. CYP3A4 and CYP2D6 showed a statistical significance for all the groups while CYP2C9 showed no statistical significance for any of the groups.

Table 4.1: The mean concentration and SEM of phase I enzymes for each group

	CYP1A2		**CYP3A4**		**CYP2D6**		**CYP2C9**	
	Mean ± SEM	**P-Value**	**Mean ± SEM**	**P-Value**	**Mean ± SEM**	**P-Value**	**Mean ± SEM**	**P-Value**
C (Grp I)	2313 ± 121	0.01*	52439 ± 9157	0.00*	12 ± 1	0.00*	1323 ± 291	1.00
CT (Grp II)	3765 ± 615	0.01*	1708 ± 345	0.00*	5 ± 1	0.00*	1352 ± 80	1.00
A (Grp III)	3339 ± 202	0.16	1358 ± 54	0.00*	7 ± 1	0.00*	1364 ± 58	1.00
AC (Grp IV)	3241± 200	0.21	1567 ± 144	0.00*	5 ± 1	0.00*	1429 ± 126	1.00
CA (Grp V)	3610 ± 56	0.02*	1314 ± 14	0.00*	5 ± 0	0.00*	1304 ± 41	1.00
M (Grp VI)	2569 ± 30	1.00	1266 ± 4	0.00*	7 ± 0	0.00*	1235 ± 14	1.00
F (Grp VII)	2645 ± 83	1.00	1264 ± 1	0.00*	7 ± 0	0.00*	1680 ± 4	1.00

*. The mean difference is significant at $p \leq 0.05$.

The mean concentration and standard mean error for each enzyme in phase II was calculated and shown in table 4.2, GSTMI showed statistical significance for all the groups except the model group (group VI), GSTP1 showed a statistical significance for all the groups while ARSG showed statistical significance for group I, II, IV, V and VI

Table 4.2: The mean concentration and SEM of phase II enzymes for each group

	GSTM1		GSTP1		ARSG	
	Mean ± SEM	**P-Value**	**Mean ± SEM**	**P-Value**	**Mean ± SEM**	**P-Value**
C (Group I)	21 ± 1		1340 ± 171		505 ± 6	
CT (Group II)	27 ± 2	0.04*	66 ± 22	0.00*	553 ± 20	0.01*
A (Group III)	27 ± 1	0.02*	98 ± 11	0.00*	539 ± 7	0.18
AC (group IV)	29 ± 2	0.00*	388 ± 233	0.00*	552 ± 6	0.01*
CA (Group V)	29 ± 1	0.00*	189 ± 2	0.00*	572 ± 2	0.00*
M (Group VI)	25 ± 1	0.32	83 ± 1	0.00*	544 ± 4	0.06*
F (Group VII)	28 ± 1	0.02*	518 ± 189	0.01*	509 ± 1	1.00

*. The mean difference is significant at $p \leq 0.05$.

The CYP1A2 concentration was calculated in pg/ml as shown in figure 4.1. In the figure group II showed a concentration of 3765 pg/ml, a much higher concentration compared to the control group which showed a concentration of 2313 pg/ml and whilst group VII which was treated with finasteride showed the lowest concentration of 2594 pg/ml.

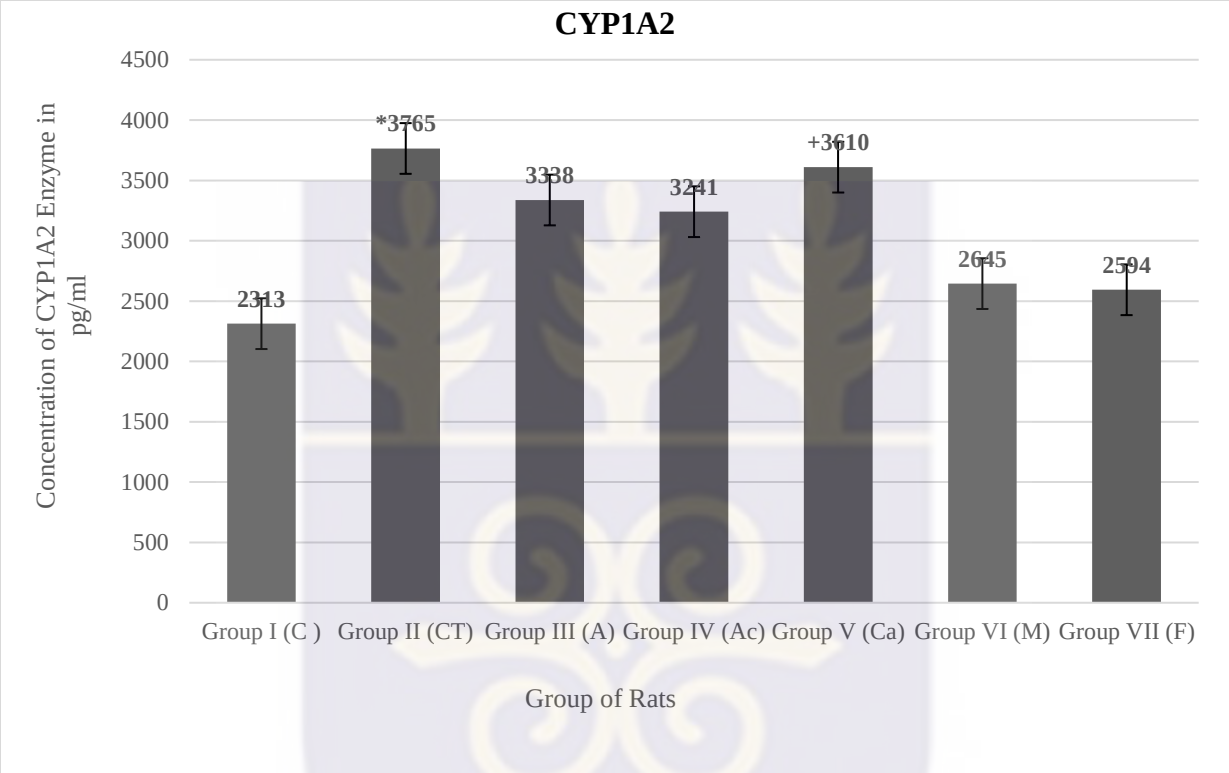

Figure 4.1: The concentration of CYP1A2 for each group. Group 1 was the control group and administered only water. All the groups showed an induction activity with Group II having the highest enzyme concentration. Group II that was administered Croton membranaceus and Group V administered mixture of higher dose of Croton membranaceus and low dose of Annona muricata were statistically significant (p =*0.01 and p=+0.02 respectively)

The concentration of CYP3A4 enzyme was also calculated in pg/ml. The control group showed the highest concentration of the enzyme of 52,432 pg/ml, while group VII which was treated with finasteride had the lowest concentration of CYP3A4 as shown in figure 4.2

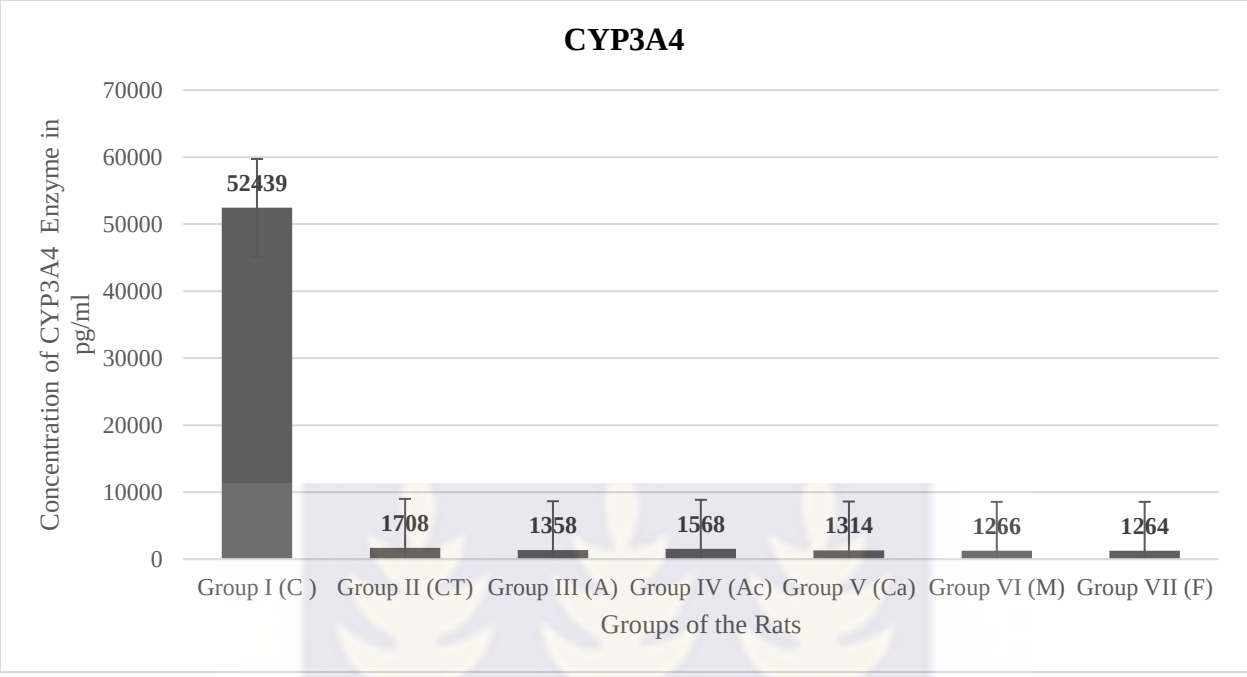

Figure 4.2: The concentration of CYP3A4 for each group showed a reduced concentration relative to the control group which had a concentration of 52,439 pg/ml. The control group was not administered any of the extracts, other groups were treated with *Annona muricata* and *Croton membranaceus* and the concentration measured after the 30th day.

The control group also showed a much higher concentration of CYP2D6 more than all the other groups. Group II and group V showed the least concentration of the enzyme. The concentration of the enzyme was calculated in ng/ml as shown in figure 4.3

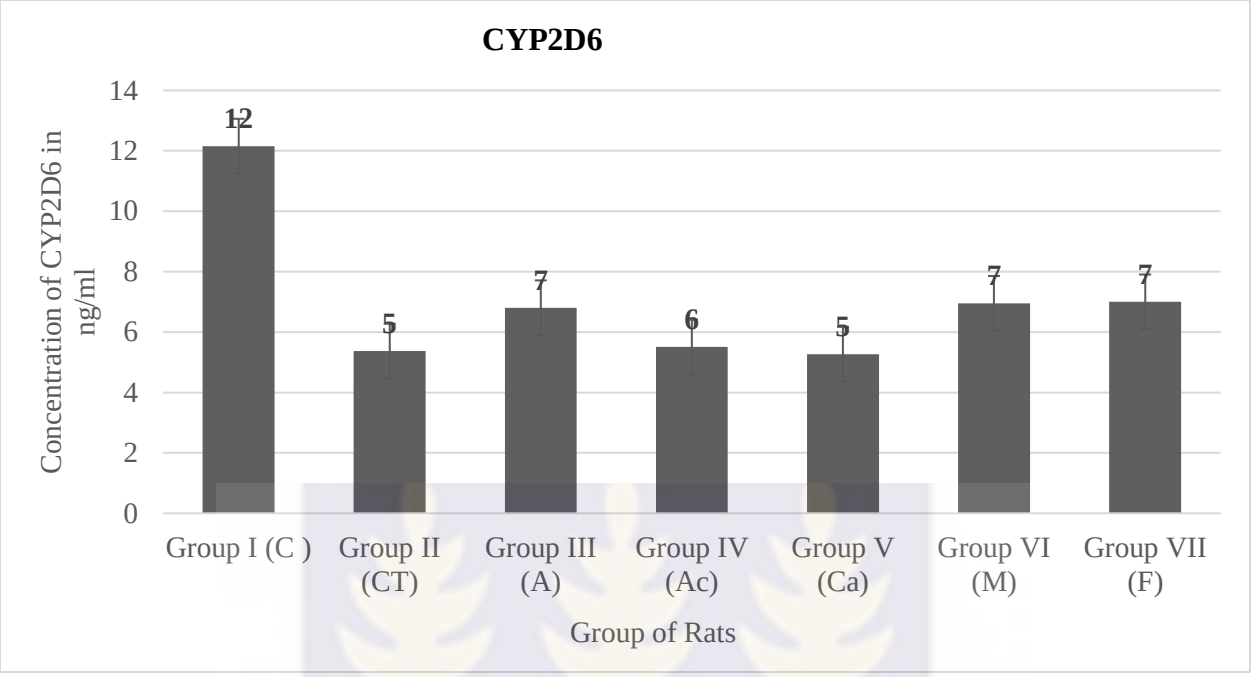

***Figure 4.3*:** A reduced concentration of CYP2D6 in comparison to the control group, the control group exhibited a higher concentration than all the groups an indication of inhibitory activity among all the groups. The control group was not administered any of the extracts, other groups were treated with *Annona muricata* and *Croton membranaceus* and the concentration measured after the 30th day.

Group IV rats showed a high concentration of enzymatic activity for CYP2C9 at a concentration of 1429 pg/ml more than the control group which showed an enzymatic activity of 1224 pg/ml. Group VII which was administered finasteride showed the least concentration of the enzyme as presented in figure 4.4.

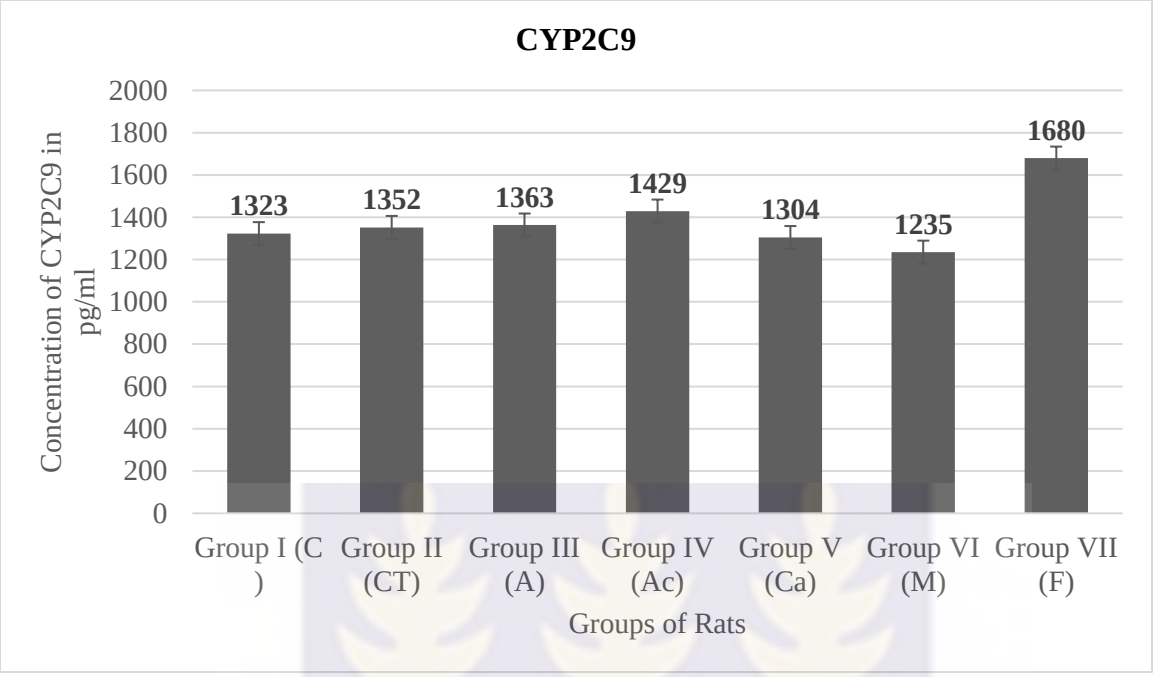

Figure 4.4: The concentration of CYP2C9 for each group. Group VII which was administered finasteride showed a higher concentration of 1680 pg/ml an indication that it highly induced the enzyme CYP2C9 as compared to other groups. The model group and Group V which was administered *Annona muricata* showed a lower concentration relative to the control group.

The phase II DME GSTMI, showed a higher concentration of 29 ng/ml in groups IV and V, respectively. The enzyme showed the least concentration in the control group with a concentration of 21 ng/ml as shown in figure 4.5.

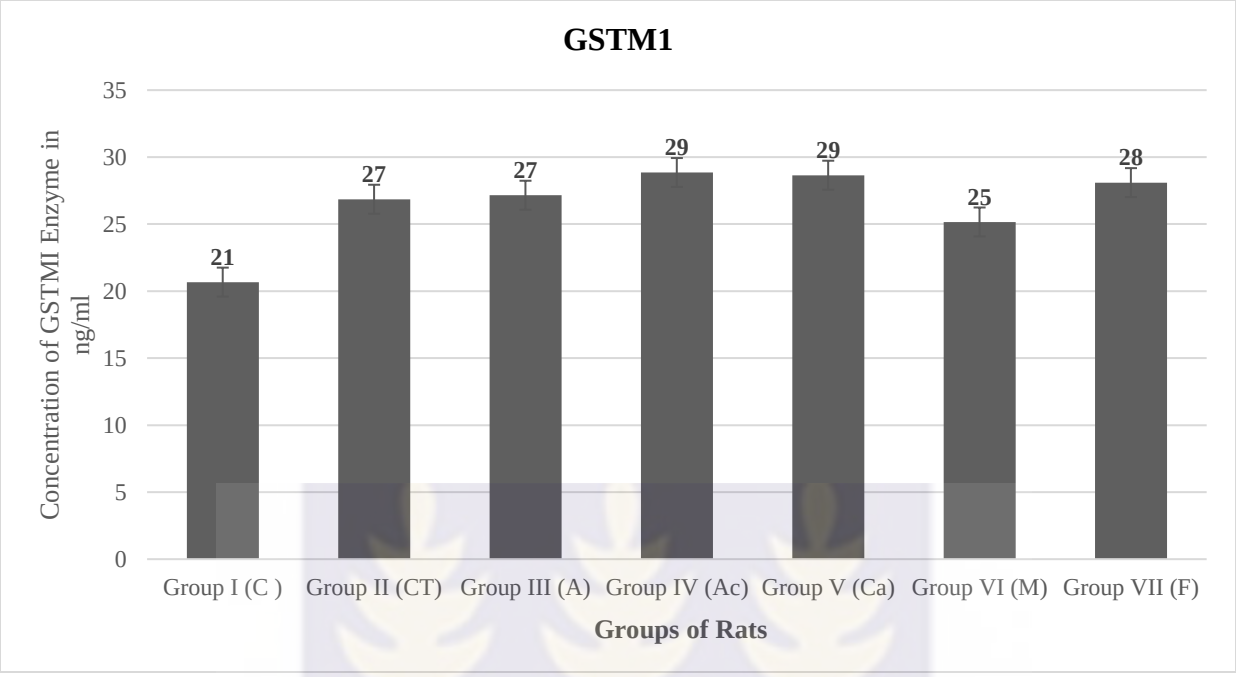

Figure 4.5: The concentration of GSTM1 for each group. Group IV and group V showed the highest concentration of 29 ng/ml relative to the control group which had 21 ng/ml. Group II, III, IV, V, and VII were significant (p=0.04, p=0.02, p=0.00, p=0.00, and p=0.00 respectively).

Figure 4.6 shows the concentration of GSTP1 enzyme for each group. From the figure, it has been shown that the control group had the highest concentration of the enzyme; 1340 pg/ml. The figure shows that group II had the lowest enzymatic concentration of 66 pg/ml.

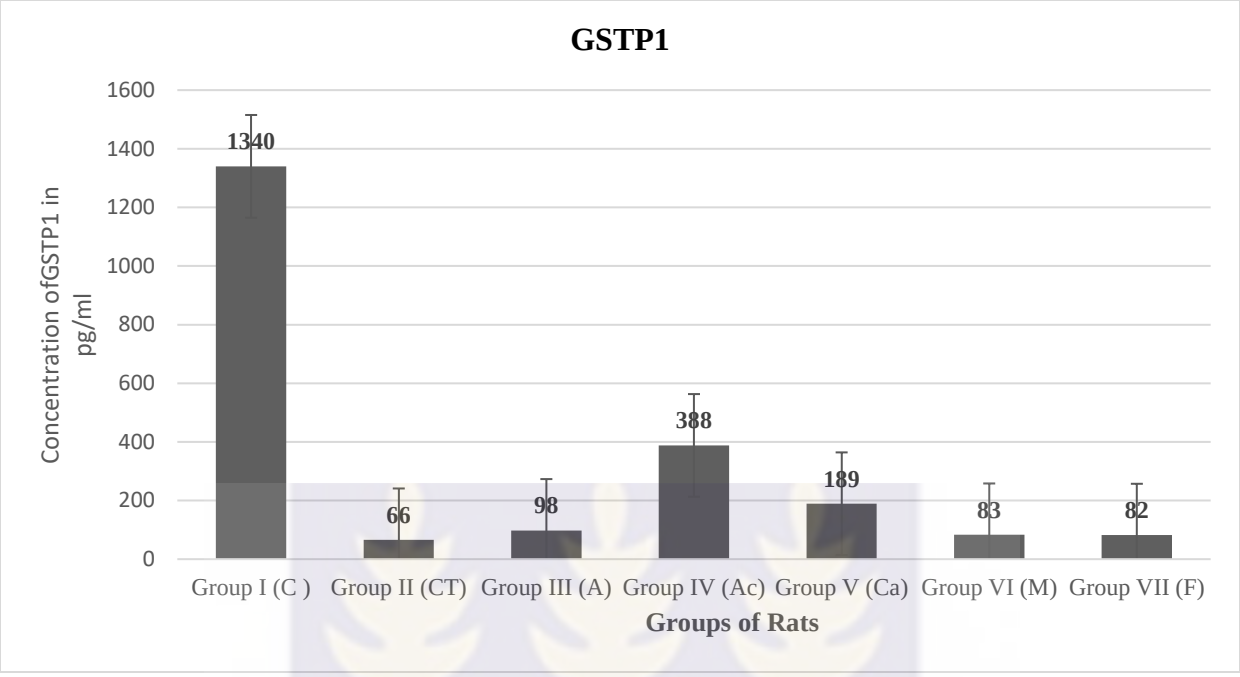

Figure 4.6: The concentration of GSTP1 for each group. All the groups showed a reduced concentration of the enzyme as compared to the control group. All the groups were significant (p=0.00) other than group VII which had a significance of (p=0.01).

Group V showed a much higher concentration of ARSG enzyme than other groups, group I which is the control showed the least concentrations of the enzyme. ARSG concentration in the control group was 505 pg/ml. The model group showed a concentration of 544 pg/ml which was also higher than the control group as shown in figure 4.7.

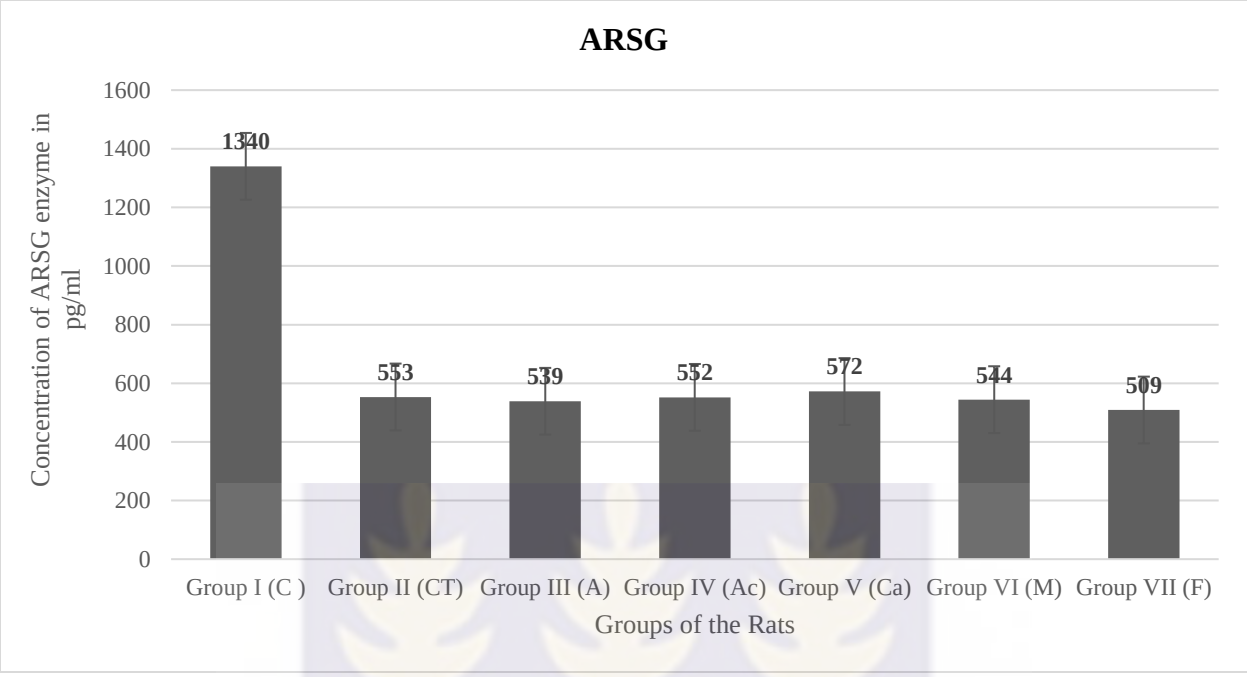

***Figure 4.7*:** The concentration of ARSG for each group. All the groups administered the extracts had a reduction in the concentration of the enzyme. Group I showed a higher concentration of the enzyme compared to the rest of the groups.

4.2 The Enzymatic Activity

The enzymatic activity of CYP1A2 calculated against the control group showed in figure 4.8 depicted an increased enzyme activity compared to the control group, group II that had been treated with *Croton membranaceus* showed a higher enzymatic activity of CYP1A2 of 62% compared to the control ,the group V, treated with only *Annona muricata* had a 56% increase in activity of the enzyme relative to the control group, the model group, group VI had a 14% increase above the control group while the group VII treated with only the finasteride showed a 12% increase in enzymatic activity compared to the control group.

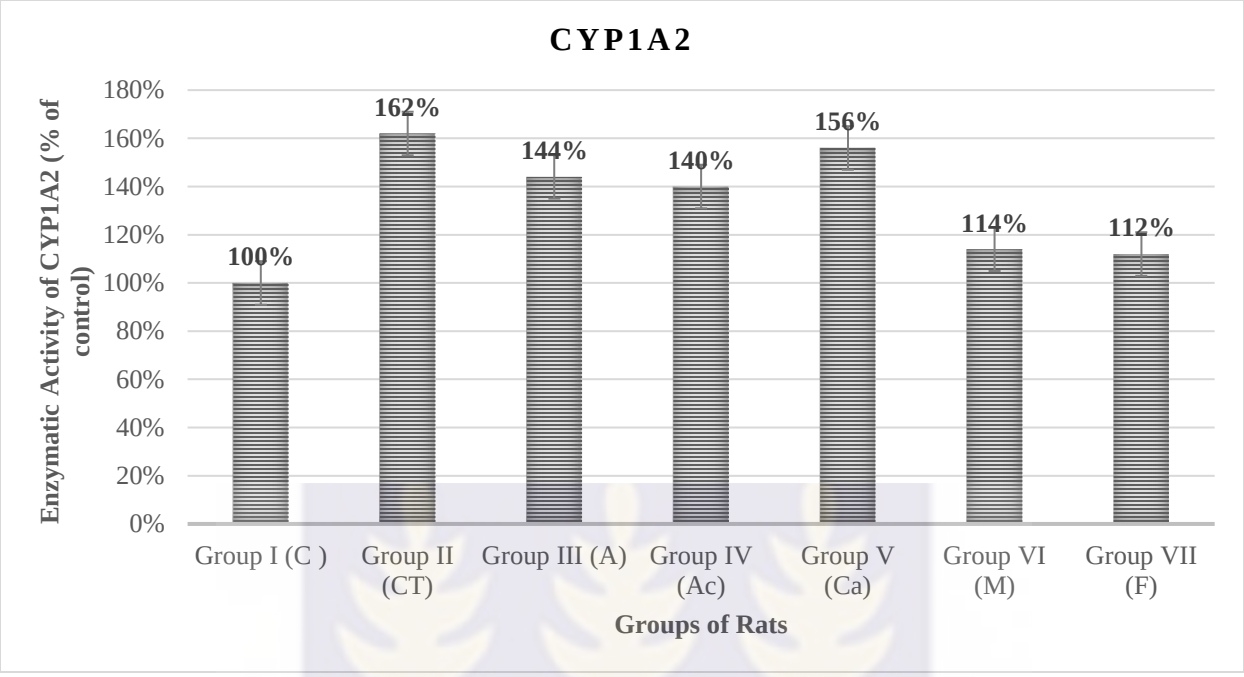

Figure 4.8 The enzymatic activity of CYP1A2 induced by each group after treatment with a mixture of *Croton membranaceus* and *Annona muricata*. The rats were fed the extract for 30 days to determine the action of the extract on the drug metabolizing enzymes both in phase I and in Phase II. Treatment with *Croton membranaceus* showed a higher enzymatic activity than all the groups compared to the control group which was only administered water and normal food diet.

The enzymatic activity of CYP3A4 enzyme as shown in figure 4.9 below compared to control group the enzymatic activity of all the groups showed a highly reduced enzymatic activity. Group II, III, IV and V showed a 97% reduced enzyme activity of CYP3A4. Group VI and Group VII showed 98% enzymatic activity below the control group.

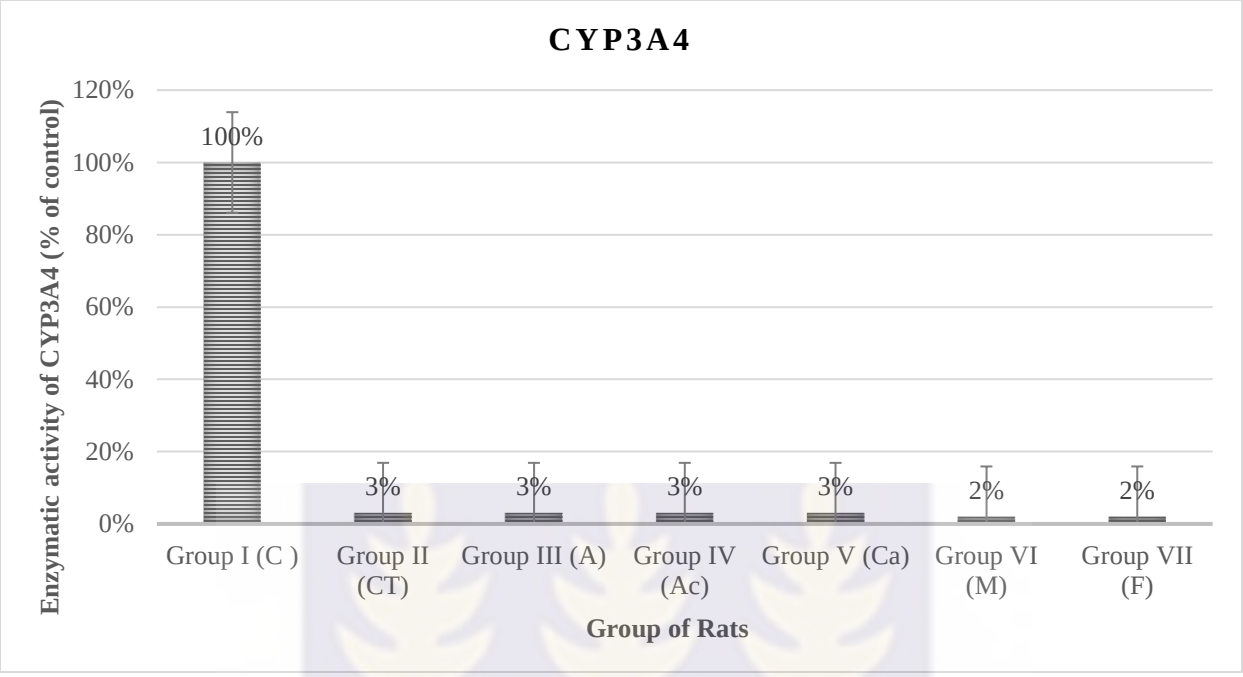

Figure 4.9: The enzymatic activity of CYP3A4 on different groups administered *Croton membranaceus* single dose or Single dose of *Annona muricata* or in mixture of both with one extract having higher concentration in the dosage. The enzymatic activity calculated against the control group which was not given any treatment.

Enzymatic activity of CYP2D6 was shown to have markedly reduced in all the groups relative to the control group, group II that was treated with only *Croton membranaceus* showed a 56% reduction of enzymatic activity as shown in figure 4.10, group III treated with only *Annona muricata* showed a 45% reduction of enzymatic activity compared to the control in the mixture with higher dose of *Annona muricata* in group IV showed a 55% reduced activity while the model group, group VI showed a 43% reduced activity. Group VII treated with finasteride showed 42% reduction on enzymatic activity.

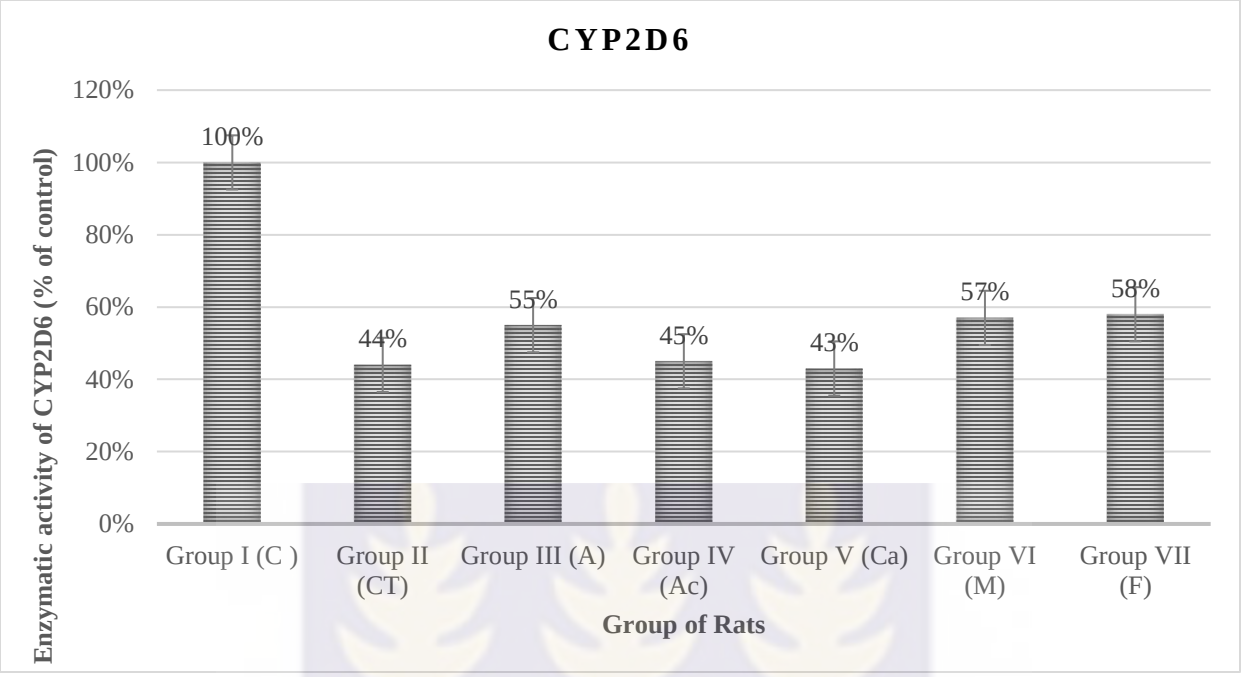

Figure 4:10 Enzymatic activity of CYP2D6 percentage of the control group, the group I administered water and normal diet, group II administered *croton membranaceus*, *group* III *Annona muricata* Group IV and V a mixture of the two extracts while group VII administered finasteride. The extracts acted as the substrate for the enzyme and observed after 30 days by use of liver microsomes.

Enzymatic activity of CYP2C9 was increased by 27% above the control group in Group VII that had been treated with finasteride and slightly increased in group II, III, IV relative to control group as shown in figure 4:11. Group V that was treated with a mixture of the extract with a higher dose of *Croton membranaceus* showed 1% reduction from the control group while group VI the model group showed a 7% reduction of the enzymatic activity compared to the control group.

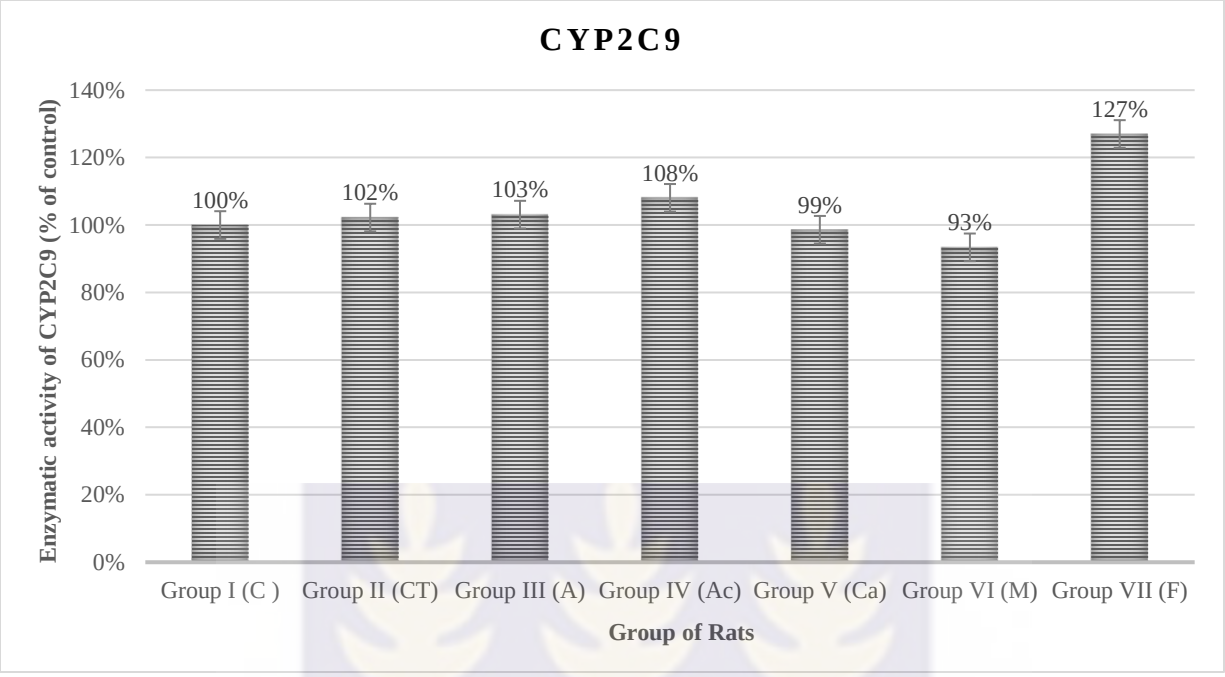

Figure 4:11: Enzymatic activity of CYP2C9 relative to control. Enzymatic activity on the extracts administered which acted as the substrate for the enzyme and measured after 30 days. The treatment involved treating the group of rats with *Annona muricata* and *Croton membranaceus* singly or in combination.

Figure 4:12 shows the activity of GSTM1 enzyme which is a phase II DME, all the groups showed an increased enzymatic activity compared to the control. Group IV treated with combined extract with higher concentration of *Annona muricata* showed the highest enzymatic activity of 140%, 40% above the control group. Group V treated with a mixture of the extracts and a higher concentration of *Croton membranaceus* showed a 39% increase in the enzymatic activity above the control group. Group II showed an enzymatic activity of 130%, 30% above the control group while the model group showed the lowest enzymatic activity of GSTM1 compared to the rest of the group at 22% above the control group as shown in the figure.

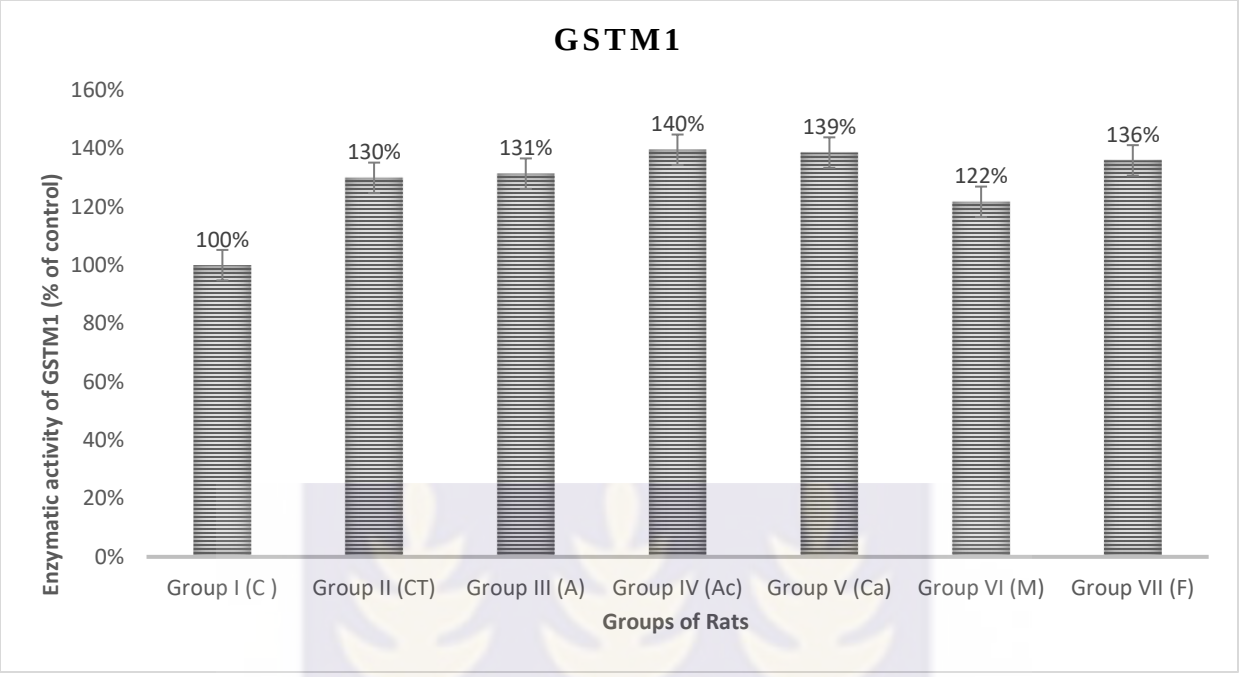

Figure 4.12: Enzymatic activity of GSTMI compared against the control group, liver microsomes was used to determine the activity of the enzyme through ELISA test. After treatment with *Annona muricata L* extract and *Croton membranaceus* root extract for 30 days. The animal slaughtered and their liver harvested and measured for the enzymatic activity on the extracts that acted as the substrate for the enzyme activity.

GSTP1 enzymatic activity showed a reduced activity across all the groups relative to control group as shown in figure 4:13. Group VII, that was treated with finasteride showed the highest activity of 39%, 61% reduction as compared to the control group IV that had been treated with a mixture of *Croton membranaceus* and *Annona muricata* with high concentration of *Croton membranaceus* showed a 71% reduction of control group, group II, III, V, VI showed a 95%, 93%, 86% and 94% enzymatic activity reduction of the control group respectively.

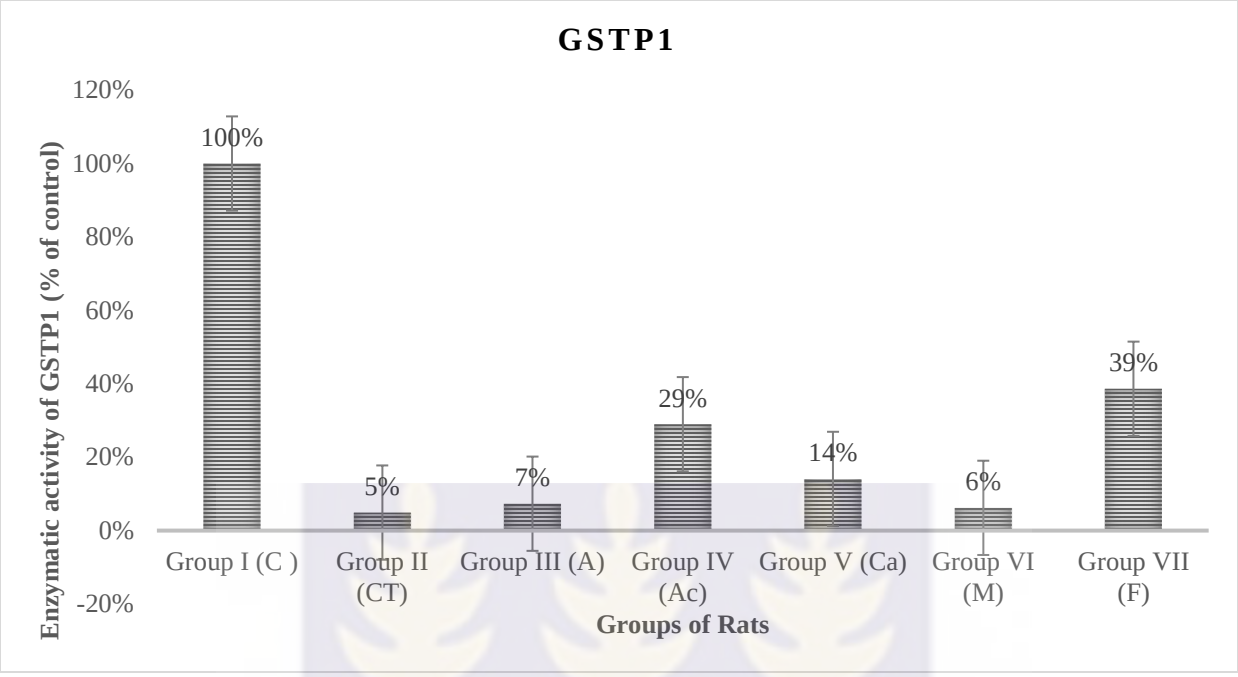

Figure 4.13: Enzymatic activity of GSTP1 a phase II DME percentage of control group. Enzymatic activity on the extracts substrates administered were measured after 30 days using homogenized liver microsomes. The treatment involved treating the group of rats with *Annona muricata* and *Croton membranaceus* singly or in combination.

Enzymatic activity of ARSG as shown in figure 4:14 showed a reduced activity across all the groups compared to the group. Group II that was treated with *Croton membranaceus* showed a 59% reduction of the enzymatic activity relative to the control group. Group III, IV, V, VI, and VII showed 60%, 59%, 57%, 59% and 62% enzymatic activity reduction respectively relative to control group

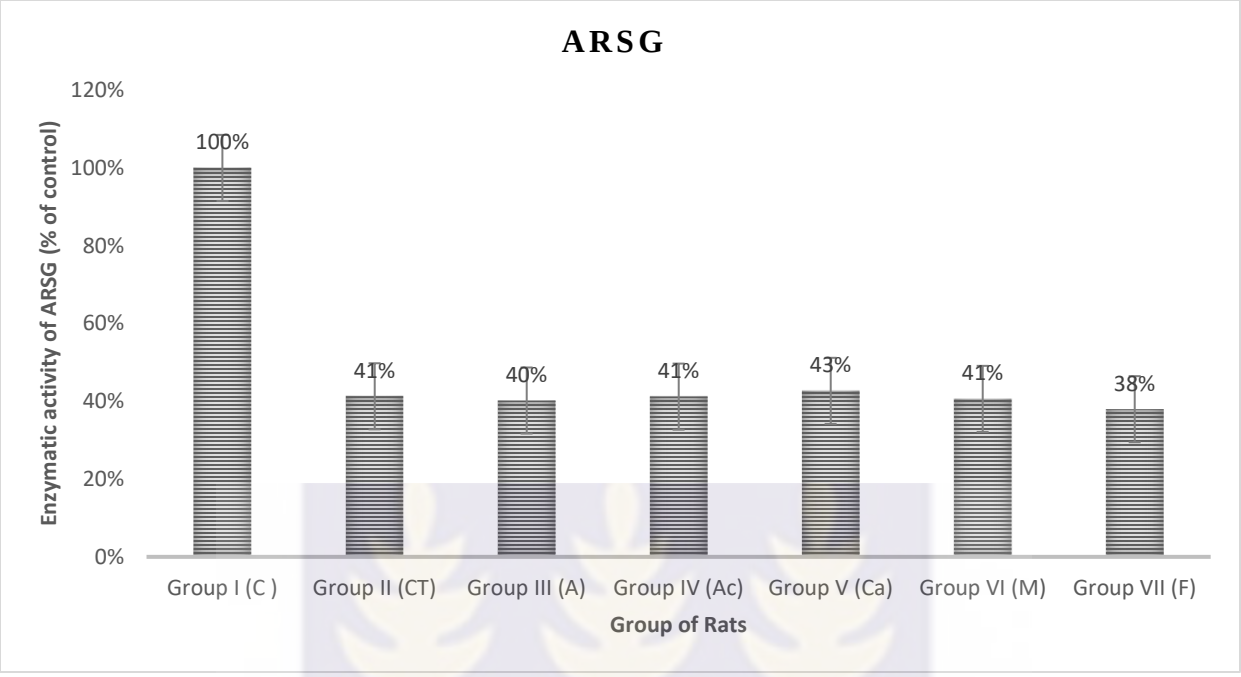

Figure 4.14: The inhibited enzymatic activity of ARSG a sulphatases, phase II DME on different groups administered with *Croton membranaceus* single dose or Single dose of *Annona muricata* or in mixture of both with one extract having higher concentration in the dosage. The enzymatic activity calculated against the control group which was not given any treatment.

4.3 The effect of Extracts on the Enzymes

All the groups showed statistical significance with ANOVA for both phase I and phase II DME except CYP2C9 a phase I enzyme. The ANOVA result showed a statistically significance value of 0.02, 0.00, 0.00 for CYP1A2, CYP3A4, CYP2D6, respectively, in phase I drug metabolizing enzymes at p value <0.05. The ANOVA result also showed significance value of 0.01, 0.00, and 0.00 for GSTM1, GSTP1 and ARSG respectively for phase II DME as shown in the table 4.1

***Table 4.3*:** Effect of extracts on Phase I drug metabolizing enzymes

	F	Sig.
CYP1A2	4.971	.002*
CYP3A4	24.76	.00*
CYP2D6	16.461	.000*
CYP2C9	.891	.518

*. The mean difference is significant at $p \leq 0.05$.

Table 4.4: Effects of the extracts on Phase II drug metabolizing enzymes

	F	Sig.
GSTM1	5.867	.001*
GSTP1	10.680	.000*
ARSG	8.365	.000*

*. The mean difference is significant at $p \leq 0.05$.

The ANOVA analysis was followed by a post hoc Bonferroni test that determined the specific difference of mean among the treatment groups administered *Croton membranaceus* extract, *Annona muricata* extract and the groups administered a mixture of both extracts on the DME.

In CYP1A2 enzyme the multiple comparison Bonferroni post hoc test showed a significant value of p=0.01 for group II rats that were administered *Croton membranaceus* extract, and p=0.02

for group V, which was administered as a mixture of both extracts with higher dose of *Croton*. The rest of the groups did not show any significance for this enzyme.

***Table 4.5*:** Effect of the extracts on CYP1A2 enzyme

Dependent Variable			Mean Difference	Std. Error	Sig.	95% Confidence Interval	
CYP1A2	C	CT (Group II)	-1452*	349.82	0.01*	-2646.05	-258.01
		A (Group III)	-1025	349.82	0.16	-2219.30	168.74
		Ac (Group IV	-927	329.81	0.21	-2053.46	198.00
		Ca (group V)	-1296*	349.82	0.02*	-2490.30	-102.26
		M (Group VI)	-255	349.82	1.00	-1449.80	938.24
		F (Group VII)	-331	349.82	1.00	-1525.55	862.49

*. The mean difference is significant at $p \leq 0.05$.

For CYP3A4 and CYP2D6 enzymes the multiple comparison Bonferroni post hoc test showed a significant level $p \leq 0.00$ in all the group

Table 4.6: Effect of extract on CYP3A4 enzyme

Dependent Variable			Mean Difference	Std. Error	Sig.	95% Confidence Interval	
CYP3A4 (pg/ml)	C	CT (Group II)	50730*	5731	0.00*	31168	70293
		A (Group III)	51080*	5731	0.00*	31517	70643
		Ac (Group IV)	50870*	5403	0.00*	32426	69314
		Ca (Group V)	51124*	5731	0.00*	31562	70687
		M (Group VI)	51172*	5731	0.00*	31609	70735
		F (Group VII)	51174*	5731	0.00*	31611	70737

*. The mean difference is significant at p≤ 0.05.

***Table 4.7*:** Effects of the extracts on CYP2D6

Dependant variable			Mean Difference	Std Error	Sig.	95% confidence interval	
CYP2D6	**C**	CT (Group II)	6.90*	0.88	0.00*	3.90	9.91
		A (Group III)	5.40*	0.88	0.00*	2.40	8.41
		Ac (Group IV)	6.75*	0.83	0.00*	3.92	9.59
		Ca (Group V)	6.90*	0.88	0.00*	3.90	9.91
		M (Group VI)	5.15*	0.88	0.00*	2.15	8.16
		F (Group VII)	5.15*	0.88	0.00*	2.15	8.16

*. The mean difference is significant at p≤ 0.05.

Post hoc analysis using the Bonferroni test for CYP2C9 enzyme activity showed no significance level in all the groups.

For the phase II DME, multiple comparison Bonferroni post hoc test showed a significant level for GSTM1 at 0.04 for group II, 0.02 for group III,0.00 for group IV, 0.00 for group V and 0.00 for group VI respectively. Group VI did not show any significance for this enzyme.

***Table 4.8*:** Effects of Extracts on GSTMI Enzyme

Dependant Variable			Mean difference	Std Error	Sig.	95% Confidence interval	
GSTM1	**C**	CT (Group II)	-6.08*	1.74	0.04*	-12.02	-0.13
		A (Group III)	-6.58*	1.74	0.02*	-12.52	-0.63
		Ac (Group IV)	-8.13*	1.64	0.00*	-13.73	-2.52
		Ca (Group V)	-8.08*	1.74	0.00*	-14.02	-2.13
		M (Group VI)	-4.58	1.74	0.32	-10.52	1.37
		F (Group VII)	-7.58*	1.74	0.00*	-13.52	-1.63

*. The mean difference is significant at p< 0.05.

For GSTP1 multiple comparison Bonferroni post hoc test showed a significant level of 0.00 for all groups except group VII which showed a significance level of 0.01.

***Table 4.9*:** Effect of the extracts on GSTP1

Dependant Variable			Mean difference	Std Error	Sig.	95% Confidence interval	
GSTP1	**C**	CT (Group II)	1274*	202	0.00*	581	1966
		A (Group III)	1242*	202	0.00*	549	1934
		Ac (Group IV)	951*	191	0.00*	299	1604
		Ca (Group V)	1151*	202	0.00*	458	1843
		M (Group VI)	1257*	202	0.00*	564	1949
		F (Group VII)	822*	202	0.01*	129	1514

*. The mean difference is significant at $p \leq 0.05$.

ARSG enzyme showed a significance level of 0.01, 0.01, 0.00 for group II, group IV and group V respectively. For multiple comparison Bonferroni post hoc test. The enzyme did not show any significance for group III, group VI and group VI.

Table 4.10: Effects of Extracts on ARSG enzyme

Dependant Variable			Mean difference	Std Error	Sig.	95% Confidence interval Dependant Variable	
ARSG	**C**	CT (Group II)	-48.49*	11.82	0.01*	-88.85	-8.13
		A (Group III)	-33.99	11.82	0.18	-74.35	6.37
		Ac (Group IV)	-47.44*	11.15	0.01*	-85.49	-9.39
		Ca (Group V)	-66.99*	11.82	0.00*	-107.35	-26.63
		M (Group VI)	-39.49	11.82	0.06	-79.85	0.87
		F (Group VII)	-3.99	11.82	1.00	-44.35	36.37

*. The mean difference is significant at $p \leq 0.05$

CHAPTER FIVE

5.0 DISCUSSION

Most population in Africa have used the medicinal plants since time immemorial and they still play an integral part of medicine in many countries and communities (Huang, 2014). Herbal products and their constituents can alter the activity of drug metabolizing enzymes and hence they can alter drug elimination, metabolic activation, and bioavailability, these outcomes may ultimately cause modification in toxicity or clinical efficiency. Medicinal plants include use of extracts derived from different parts of the plants such as the use of the leaves of *Annona muricata* and root extract of *Croton membranaceus* for the management of BPH, however there are various adverse effects that can be result from the use of these medicinal plants. The adverse effects, have been documented to also arise from combination of these medicinal plants since the CYP enzymes that are involved in the metabolism of drugs and other xenobiotics can either be inhibited or induced by these herbal products. This is usually regarded as the major mechanism of enhanced or reduced bioavailability of drugs (Yong, 2009).

In this study, the interaction between the DME's in phase I and phase II and the aqueous extract of *Annona muricata* L and aqueous root extract of *Croton membranaceus* was investigated. Sarkodie *et al.* (2014), established that ingredient compounds found in *Croton membranaceus* included crotomembranafuran, julocrotine, scopoletin among others, while according to Coria-tellez *et al.* (2016), *Annona muricata* contains acetogenins, alkaloids and phenols among other compounds. These compounds can interact with one another through the influence of the enzyme responsible for their metabolism, Rat liver microsomes from rats treated with different doses of the plant extracts individually and in mixture were used to evaluate the effect of these plant extracts

on CYP3A4, CYP2D6, CYP1A2 and CYP2C9 for Phase I metabolism and GSTP1, GSTPM1 and ARSG for phase II metabolism using ELISA test.

The result showed that CYP1A2 could be induced across all the groups, group II that was administered *Croton membranaceus* (CT alone) without combination had the highest level of induction compared to other groups, the enzymatic activity was found to be 62% above the control group and higher than the rest of the group. In the combined dose that had a higher dose of *Croton membranaceus* (combination C60:A40) also showed a high enzymatic activity of 44% above the control group. Overall ANOVA showed statistically significant difference for CYP1A2 (P=0.002). However, in post hoc analysis, the statistical significance was found to be in CT and C60:A40 which showed a statistical significance of 0.008 and 0.024 respectively. Compared to model (M) group, CT showed the highest level of statistical difference, although this difference was not statistically significance (p=0.075). This is an indication that the induction of the enzymatic activity in C60:A40 could largely be attributed to the effect of *Croton membranaceus*. There is a possibility of compounds in *Croton membranaceus* having a higher induction of CYP1A2 compared to *Annona muricata* since the results showed a reduction of enzymatic activity from the combined dose as compared to the single dose of *Croton membranaceus.* However, there is need to do further studies to establish the exact compounds in *Croton membranaceus* that is responsible for the induction of the enzyme as well as the compounds in *Annona muricata*. Other herbs have also been found to have an inducing effect on CYP1A2, a study investigating the induction of CYP enzymes by six commonly used herbs established that *Ginko giloba* extract had an inductive effect on CYP1A2 compared to the control used and inhibitory effect in higher doses (Hellum *et al.,* 2007). Aryl hydrocarbon as well as other toxins found in the environment generally influences the expression of CYP1A2 enzyme (Tritsher *et al.*,1992) including cigarrate smoke

(O'Malley *et al* 2014). CYP1A2 is also known to be strongly induced by caffeine (Han *et al*., 2001).

The most highly expressed CYP iso enzyme in the human liver and small intestines is the CYP3A4, however, the amount of CYP3A4 found in small intestines is much lower than the one in the liver (Zanger *et al.,* 2014). CYP3A4 metabolizes a wide range of compounds that have a varying molecular weight. It is prescribed that CYP3A4 has a large hydrophobic active site which can accommodate diverse range of compounds thus having an indiscriminating selectivity on its substrates (Zhou *et al*., 2005). CYP3A4 is known to be induced by some herbal medicines such as John wart which is a strong inducer of the enzyme CYP3A4, however in the this study the aqueous extract of *Annona muricata* L and root extract of *Croton membranaceus* in single dose and in combination had an inhibitory effect on CYP3A4 relative to the control group. The inhibitory effect of each group administered the extracts, compared to the control was highly significant ($p=0.001$), the inhibitory activity recorded for all groups were between 97% to 98%. Inhibitory effect depicted by both extracts is suggestive of a competitive mechanism. Irreversible mechanism inhibition of CYP3A4 inactivates the enzyme by forming a metabolic intermediate by binding tightly and irreversibly forming a covalent bond with the enzyme (Polasek & Miners 2007). Studies have shown that CYP3A4 is inhibited by flavonoids but induced by organic acids (Ho *et al*., 2001; Hu *et al*., 2005; Fasinu *et al*., 2013). The presence of flavonoid reported in *Annona muricata* and *Croton membranaceus* could account for the high inhibitory effect of these extracts on CYP3A4 (Yang *et al*., 2015; Bayor *et al*., 2009). However, some studies looking into the interaction of herbs drugs established that *Panox griseng* and *Panox quinquifolius* inhibits CYP3A4 due to the presence of polysaccharides (Chen and Raymond 2006).

CYP3A4 inhibitory activity has also been attributed to the secondary metabolites in the various medicinal plants. Ashour *et al.* (2017), while studying the inhibitory effect of extracts from 57chinese medicinal herbs on CYP3A4 iso enzyme observed that the inhibitory effect was due to polyphenolic class of compounds which forms hydrogen and ionic bonds at the active site of the enzyme due to the existence of several reactive phenolic hydroxyl groups. The phenolic hydroxyl groups can sparingly dissociate in physiological conditions eliciting an interaction between oxide anions and the positively charged amino acids, such as arginine and lysine. The charged and polar polyphenols interact with proteins by forming ionic bonds. In addition to hydrogen bonds with a variety of amino acids at the active site which might result into enzyme inhibition and loss of function (Wink, 2008). In this manner, inhibitory effect from both extracts of *Annona muricata* and *Croton membranaceus* could be attributed to their content of phenol and threitol as polyphenols have been shown to have the ability to bind the enzyme directly making them inactive.

CYP2D6 metabolizes approximately 25% of the marketed drugs with its expression being polymorphic (Zhou *et al*., 2009). In all the groups, there was a reduction in CYP2D6 activity with the highest group having a 58% activity change relative to the control group. Inhibition shown was statistically significant (p=0.000) between all the treatment groups compared to the negative control, the highest change of inhibition was observed in C60:A40. It is likely that drug-drug interaction between the compounds from *Annona muricata* and *Croton membranaceus* accounted for the high inhibition observed (Kumar *et al.*, 2011). There are some minimal reports about induction of CYP2D6, while some studies have described it as a non-inducible enzyme hence this study is tandem to other studies on the activity of this enzyme (Zhou 2009; Rodríguez-Antona *et al.*, 2000; Parkinson *et al*., 2013). Reduced CYP2D6 activity have been linked to hepatotoxicity, this is based on the observation of poor metabolism of Kava herbs which postulated that CYP2D6

deficiency is a risk factor associated with herbal hepatoxicity (Mathews *et al.*, 2002). Another study considered the inhibitory activities of CYP3A4 and CYP2D6 of Indonesian medicinal plants, CYP2D6 demonstrated a 70% inhibitory activity and a 90% in 7 of the herbs included in the study (Usia *et al*., 2006).

In this study, it would be suggestive that the inhibition observed on CYP2D6 enzyme by both extracts would be due to the normal low levels of CYP2D6 expression in general. Another reason may be attributed to the presence of Alkaloids in *Annona muricata* and *Croton membranaceus.* Usia *et al*. (2005), in their study demonstrated that alkaloids have an inhibitory effect on the enzyme CYP2D6.

CYP2C9 is actively and significantly involved in metabolism of most of the prescribed drugs; non-steroidal anti-inflammatory drugs such as ibuprofen, diclofenac and typically substrates that contain anionic site and a hydrophobic site. None the less it is also possible for the enzyme to metabolize neutral and positively charged compounds. The enzyme also is capable of metabolizing natural products that can form toxic metabolites (Mo *et al.*, 2009). This enzyme metabolizes endogenous bioactive substrates such as steroids, melatonin, retinoids and arachidonic acids (Mazarin and Prieto, 2014). In this study, it was clearly shown that finasteride induced CYP2C9, although the level of induction was not found to be statistically significant, an induction that was statistically significant was however seen in one study by Lundahl, (2010) which showed that finasteride could actually induce the activity of CYP2C9. All other treatments did not show any statistical significance induction.

It has been shown that CYP2C9 activity is inhibited by extracts from some plants, such as pineapple juice, green tea and cranberry juice. The mechanism is thought to be due to bromelain found in this plant and is known to be a cysteine protease. Bromelain has a very strong inhibitory

property on CYP2C9, it degrades the enzyme thus reducing its enzymatic activity (Hidaka *et al*., 2008). From this study, the results indicated an inhibitory effect across all the groups, the enzymatic activity was not observed in all the groups other than the positive control group. There is not much literature describing the effect of finasteride on CYP2C9.

CYP2C9 act on the long chain polysaturated fatty acids at the double bond forming epoxide products which have been implicated in regulating inflammatory diseases and growth of various cancers (Spector and Kim 2015). An in vitro study by Foti and Wahlstrom on the role of supplements in CYP450 mediated drugs, showed that extracts from crowberry, devil's claw, Echinacea, Eucalyptus, garlic, ginger, green tea, kava, milk thistle, St. John warts Ginkgo and turmeric inhibit CYP2C9 enzyme.

Metabolic processes of drugs in Phase II involves enzymatic conjugation of the drugs metabolites from Phase I pathway with aid of transferase enzymes they transform endogenous compounds to more easily excretable forms as well as metabolically inactivate the pharmacologically active compounds (Jancova *et al*., 2010). When the metabolizing capacity of phase II enzymes are reduced there is increased risk of toxic effects from the drugs. Most of phase II enzymes catalyze conjugation reactions that involves UDP glucoronyl transferases (UGTS), Sulfatases or glutathione transferases (GSTs). The metabolites generated in phase I and phase II are excreted through the membrane efflux pumps (Iyanagi, 2007).

GST enzymes are classified into different isoforms and they differ in their substrate specificity. GSTM1 is one of the isoforms of GST enzyme and detoxifies the reactive oxygen species (ROS) produced via oxidative metabolism from the phase I enzymes. The mμ class have a loop between the β-2 strand and helix α-3 structure (Parkinson *et al*., 2013). This study showed an increased activity of GSTM1 enzyme across all groups. Group VI that was treated with a

mixture of the two extracts with a higher dose of *Annona muricata* showed a much higher concentration and activity of the enzyme at 40% above the control group. The GST is involved in the biotransformation of endogenous and exogenous electrophilic compounds found in insecticides and xenobiotics. *Annona muricata* is known to possess insecticidal, bacteriocidal, fungicidal and anti-parasitic properties (Radji *et al*., 2015; Ferreira *et al*., 2013). The presence of such compounds in *Annona muricata* and potentially in *Croton membranaceus* could cause the availability of substrate for activities of GST. This results in net inhibition of enzyme in all the treatment group relative to the control group($p=0.001$). Overall ANOVA showed statistical significance ($p=0.001$). This might be attributed to the high concentration and increased activity of CYP1A2 in phase I, which could have produced metabolites that acts as the substrate for GSTM1 in phase II however the study could not establish the metabolite involved but could suggest that this could be due to mutagens and polycyclic aromatic hydrocarbons compounds that could be present in *Annona muricata* and *Croton membranaceus*. A study conducted by Mazarin and Prieto (2014) on the pharmacokinetic profile and possible interaction of the herbs and drugs identified that GSTM1 is involved in the detoxification of polycyclic aromatic hydrocarbons and some mutagens. Kuo *et al*. (2003), in their study to investigate the differential induction of the GST subunit expression by *Gardenia jasminodes* fruits found out that the extract induces GSTM1 via the MEK-1 pathway.

GSTP1 is also involved in protection of the cells from cytotoxic and carcinogenic agents and it is expressed in normal tissues. Most tumours have reported an altered activity of GSTP1-DNA hyper methylation at CPG island in the promoter 5 . The GSTP1 enzyme inactivates electrophilic carcinogens by conjugation (Jeronimo *et al.,* 2002). The activity across all the groups in this study indicated an inhibitory characteristic of GSTP1. Most of the studies done and the ongoing have confirmed that GSTP1 inhibition as a principle for antitumor therapy. In addition to

its catalytic role in the cellular detoxification of antitumor drugs GSTP1 is also able to promote tumor progression through a non-catalytic mechanism which results to inhibition of apoptosis (Wu *et al*., 2006).

Stress and cytokines are known to activate the JUN-N-Terminal Kinase (JNK) signaling pathway, this pathway is modulated by GSTP1 enzyme. Most of the transcription factors such as the P53 may regulate JNK thus resulting to induction of cell proliferation, cell survival and consequent promotion of apoptosis (Tournier, 2013). Increased proliferation of monomeric GSTP1directly inhibits JNK thus, the cancerous cells are protected from apoptosis. The GSTP1 inhibitors suppresses the JNK inhibitors by interfering with the complex formed by GSTP1 and JNK (Adler *et al.,* 1999). Additionally, the GSTP1 is involved in the regulation of tumor necrosis factors alpha (TNFα) signals by forming a complex with the receptor associated factor 2 (TRAF-2) which is a signal transducer for TNF receptor, this leads to down regulation of apoptosis (Wu *et al*., 2006). Excessive expression of GSTP1 is suspected to be responsible for poor prognosis of cancer (Ekhart, 2009).

Asare *et al. (*2015), in their quest to establish the antiproliferative property of *Annona muricata* established that *Annona muricata* has an anti-proliferative effect on BPH-1 cell lines and reduction of cell size through apoptosis. Another study by Abbah *et al*. (2016) reported that *Annona muricata* has an antitumor potential on prostate. Further study to investigate the anti-proliferative property and capability of inducing apoptosis using human cancer cells, concluded that *Annona muricata* has a strong anti-proliferative potential and can induce apoptosis through the loss of membrane mitochondrial potential and Go/G1 phase cell arrest (Pieme *et al.,* 2014). This might be attributed to the to the presence of flavonoids and acetogenins compounds found in *Annona muricata*. However further studies are required to confirm the exact extract compound

that is responsible for this. *Croton membranaceus* has also been shown to have a markedly higher cytotoxicity activity against cancer cell lines. Afriyie *et al.,* 2014 confirmed that there is a possibility of *Croton membranaceus* aq root extract to have the ability to induce mitochondrion dependant apoptosis of BPH-1 cell lines. The presence of croton membranafuran compound has been found to account for the induction of the apoptotic activity (Asare *et al.,* 2015). With the studies supporting the anti-apoptotic activity of both *Annona muricata* and *Croton membranaceus,* this elucidates the reason why there was an inhibitory effect on GSTP1.

Aryl sulfatases are key in control of cellular degradation, cell signaling and hormone regulation. The loss of sulfatase enzymatic activity may result in development of various disorders for example lysosomal storage disorder and some cancers. The mannose-6-phosphate receptors binds only to ARSG a reason explaining its confinement to the lysosomes (Frese *et al.*, 2008). In this study, there was inhibitory activity across all the groups compared to the control group. A novel study of ARSG revealed that inhibition of ASG by sulfate is probably of no relevance, since the physiological sulfate concentration do not result in significant activity of ARSG (Ferrante *et al.*, 2002). However, not much study has been done to explain the role of this enzyme in disease and in the metabolism of medicinal plants, further studies are needed so as to explain its involvement in drug and xenobiotics metabolism.

5.1 Conclusion

In phase I, *Annona muricata* extract alone was shown to have inhibitory effect on CYP3A4, CYP2D6, GSTP1 and induced CYP1A2, CYP2C9 while *Croton membranaceus* alone treatment had an inhibitory effect on CYP3A4, CYP2D6, and CYP2C9. The combination treatment of *Annona muricata* and *Croton membranaceus* (A60:C40) showed an inhibitory effect on CYP3A4,

CYP2D6, and inducing CYP1A2, CYP2C9. The combined treatment of *Annona muricata* and *Croton membranaceus* (A40:C60) had the potential to inhibit CYP3A4, CYP2D6, CYP2C9, while inducing CYP1A2. All the treatments showed almost a similar pattern in the inhibitory and induction effects on the drug metabolizing enzymes except in CYP2C9 where the combined treatment of A60:C40 induced CYP2C9 while the combined treatment of A40:C60 slightly inhibited CYP2C9.

In phase II, *Annona muricata* extract alone was shown to have inhibitory effect on GSTP1 and ARSG, but induced GSTM1.Treatment with *Croton membranaceus* alone had an inhibitory effect on GSTP1 and ARSG while inducing GSTM1. The combination treatment of *Annona muricata* and *Croton membranaceus* (A60:C40) showed an inhibitory effect on GSTP1 and ARSG but induced GSTM1. The combined treatment of *Annona muricata* and *Croton membranaceus* (A40:C60) had the potential to inhibit GSTP1 and ARSG while inducing GSTM1 enzymes.

Combination of aqueous extract *Annona muricata* L and aqueous extract of *Croton membranaceus* roots exhibited a synergistic interaction and having shown the ability to induce CYP450 and phase II enzymes according to this study they may be safe to be taken in combination. Finally, Drugs that rely on the enzymes that are inhibited by the two plant extracts may not be safe to be taken in combination with them since they may trigger an adverse effect from the combination.

5.2 Recommendation

1. For the treatment that showed slight enzymatic activity difference from the control should be subjected to longer period than the 30 days to see whether there will be a change in longer exposure.

2. Further studies should be done to identify the exact compounds in *Annona muricata* and *Croton membranaceus* responsible for the induction and inhibition of the drug metabolizing enzymes.

3. More studies are needed to establish other aspects of toxicity on other organ functions of the combination treatment of the two plant extracts.

5.3 Limitations

1. The individual enzymatic activity of the enzymes was not measurable using the kit.
2. Confirmation of the BPH in the model group was not possible apart from using the PSA level.

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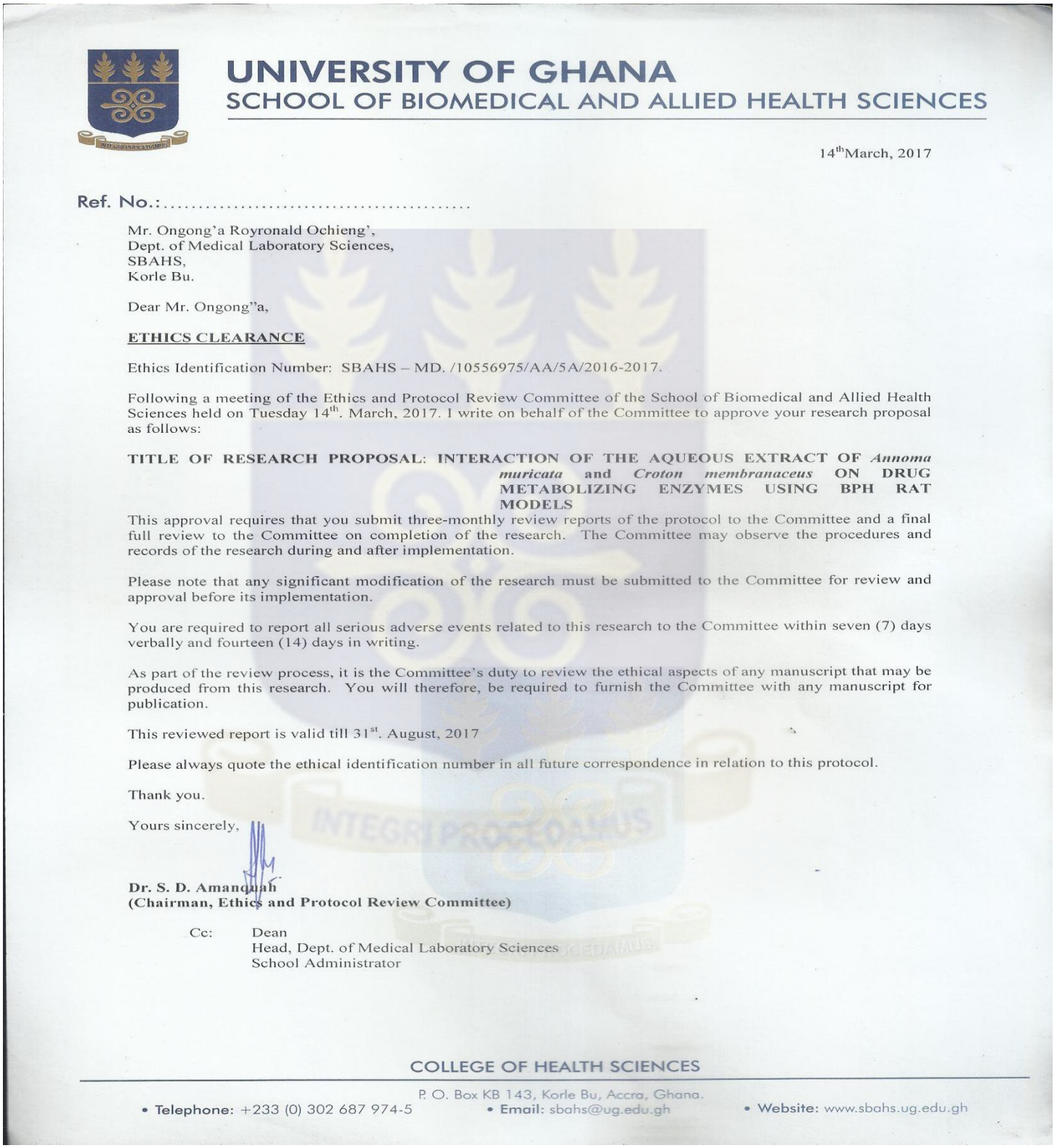
APPENDIX

ETHICS CLEARENCE



UNIVERSITY OF GHANA
SCHOOL OF BIOMEDICAL AND ALLIED HEALTH SCIENCES

14th March, 2017

Ref. No.:.....

Mr. Ongong'a Royronald Ochieng',
Dept. of Medical Laboratory Sciences,
SBAHS,
Korle Bu.

Dear Mr. Ongong"a,

ETHICS CLEARANCE

Ethics Identification Number: SBAHS – MD. /10556975/AA/5A/2016-2017.

Following a meeting of the Ethics and Protocol Review Committee of the School of Biomedical and Allied Health Sciences held on Tuesday 14th. March, 2017. I write on behalf of the Committee to approve your research proposal as follows:

TITLE OF RESEARCH PROPOSAL: INTERACTION OF THE AQUEOUS EXTRACT OF *Annoma muricata* and *Croton membranaceus* ON DRUG METABOLIZING ENZYMES USING BPH RAT MODELS

This approval requires that you submit three-monthly review reports of the protocol to the Committee and a final full review to the Committee on completion of the research. The Committee may observe the procedures and records of the research during and after implementation.

Please note that any significant modification of the research must be submitted to the Committee for review and approval before its implementation.

You are required to report all serious adverse events related to this research to the Committee within seven (7) days verbally and fourteen (14) days in writing.

As part of the review process, it is the Committee's duty to review the ethical aspects of any manuscript that may be produced from this research. You will therefore, be required to furnish the Committee with any manuscript for publication.

This reviewed report is valid till 31st. August, 2017

Please always quote the ethical identification number in all future correspondence in relation to this protocol.

Thank you.

Yours sincerely,

Dr. S. D. Amanquah
(Chairman, Ethics and Protocol Review Committee)

Cc: Dean
Head, Dept. of Medical Laboratory Sciences
School Administrator

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