UNIVERSITY OF GHANA

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EFFECT OF SWEDISH BITTERS ON SELECTED RAT CYTOCHROME P450 ENZYME ACTIVITY AND HEPATIC ANTIOXIDANT LEVELS

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DECLARATION

I, Abigail Aning, hereby declare that this project, aside other cited works, is the result of research carried out by me under the supervision of Prof. Regina Appiah-Opong and Dr. Seth Kwabena Amponsah. This work has not been submitted in part or whole elsewhere for the award of any degree.

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ABSTRACT

Background: The use of herbal products has gained popularity especially in developing countries. A number of patients are known to take these herbal products concurrently with conventional drugs. Concomitant use of herbal preparations and conventional drugs may result in herb-drug interactions, often via modulation of drug metabolizing enzymes, in particular, Cytochrome P450 (CYP) enzymes. Swedish bitters is one of such herbal preparations on the market which may be concomitantly administered with other conventional drugs because of its use as a digestif.

Aims: The aims of the current study were to determine the effect of Swedish bitters on the activities of rat liver microsomal CYP1A2, CYP3A4, CYP2B6, CYP2C9 and CYP2D6 enzymes, and also its effect on hepatic antioxidant levels. The effect of Swedish bitters on rat hematological and biochemical parameters was also assessed.

Methods: Male Sprague-Dawley rats 6-8 weeks old were put into 5 groups (5 rats/group). The groups consisted of a positive control (15 mg/kg/day of phenobarbital), a negative control (distilled water), and low (5 mL/kg/day), medium (10 mL/kg/day) and high (20 mL/kg/day) doses of Swedish bitters, respectively. After a 7-day administration period of the aforementioned treatments, the rats were euthanized and blood obtained by cardiac puncture. The livers were isolated, immediately placed on ice and stored at -80^{o}C until use. Microsomal preparations were obtained from harvested livers by homogenization and differential centrifugation. The substrates of each specific CYP was added to microsomal preparations in phosphate buffer (pH 7.4) at 37^{o}C and their metabolites measured using spectrophotometric and chromatographic assays. Effect of Swedish bitters on enzyme activity was determined based on metabolite levels. Antioxidant levels/reactions such as

Glutathione (GSH), Lipid peroxidation (LPO), Superoxide Dismutase (SOD) and Catalase (CAT) were evaluated in liver cytosol using standard methods. Additionally, hematological and biochemical parameters in the various groups from blood collected were determined using automated analyzers.

Results: Results showed that Swedish bitters increased the activities of CYP2B1/2B2, CYP3A4, CYP2C9 and CYP2D6; with CYP2C9 significantly increased ($p < 0.01$). The activity of CYP1A1/1A2 did not differ significantly compared to the non-treated groups. There was a marginal increase in CYP1A2 activity in treatment groups, however, this was not statistically significant. Furthermore, with antioxidant levels/reactions assayed in liver cytosol, there was a dose dependent decrease in GSH levels and increase in SOD activity, however, both were not statistically significant. Catalase activity was found to have decreased dose-dependently, with the rats that received high dose of Swedish bitters showing a significant decrease compared to the untreated group. Lipid peroxidation did not alter significantly in treated groups compared to the untreated group. For hematological and biochemical parameters, monocytes and alkaline phosphatase (ALP) levels decreased significantly in the rats that received high doses of Swedish bitters.

Conclusion: Swedish bitters increased the activity of rat CYP2C9 significantly. Swedish bitters also altered the levels of catalase enzyme activity and reduced ALP levels, significantly. Findings suggest that Swedish bitters may interact with other drugs that are metabolized by these CYPs especially when taken over longer periods.

DEDICATION

This work is dedicated to my family, especially my mother, for their encouragement and support throughout this period of study.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

4'HD	4'Hydroxydiclofenac
A/G	Albumin/Globulin ratio
AED	Animal Equivalent Dose
ALB	Albumin
ALT	Alanine aminotransferase
AO	Aldehyde oxidase
AST	Aspartate aminotransferase
BASO	Basophil
BROD	Benzyloxyresorufin O-dealkylation
BSA	Bovine Serum Albumin
CAM	Complementary and Alternative medicine
CAT	Catalase
CBBG	Coomassie Brilliant Blue G-250
COX-1	Cyclooxygenase-1
CYP	Cytochrome P-450
DBil	Direct Bilirubin
DILI	Drug-induced Liver Injury
DN	Diabetic nephropathy

DPPH	Diphenylpicrylhydrazyl
DXM	Dextromethorphan
DXT	Dextrorphan
EDTA	Ethylenediaminetetraacetic acid
EOS	Eosinophil
EROD	Ethoxyresorufin O-deethylation
FDA	Food and Drugs Administration
FMO	Flavin-containing monooxygenases
FRAP	Ferric reducing-antioxidant power
GC	Gas chromatography
GGT	Gamma glutamyl transferase
GLB	Globulin
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GST	Glutathione S-transferase
GTE	Green Tea Extract
HCT	Hematocrit
HGB	Hemoglobin

HILI	Herb-induced Liver Injury
HL-60	Human Leukemia cell line
HPLC	High Performance Liquid Chromatography
LPO	Lipid Peroxidation
LYM	Lymphocytes
MAO	Monoamine oxidases
MCH	Mean Corpuscular Hemoglobin
MCV	Mean Corpuscular Volume
MDA	Malondialdehyde
MON	Monocytes
MPV	Mean Platelet Volume
MROD	Methoxyresorufin O-demethylation
NADPH	Reduced Nicotinamide Adenine Dinucleotide Phosphate
NAT	N-acetyl Transferase
NBT	Nitroblue tetrazolium
NEU	Neutrophils
NMIMR	Noguchi Memorial Institute for Medical Research
NSAID	Nonsteroidal Anti-inflammatory drug
OPA	O-phthalaldehyde

OPT	O-phthalaldehyde
OVCAR-3	Human Ovarian cancer cell line
P450	Cytochrome P-450
PCT	Procalcitonin
PDW	Platelet Distribution Width
P-gp	P-glycoprotein
PLT	Platelets
PROD	Pentoxyresorufin O-dealkylation
RBC	Red Blood Cells
RDW	Red Cell Distribution Width
RDW_SD	RDW expressed as a standard deviation
ROS	Reactive oxygen species
SD	Sprague-Dawley
SH	Sulfhydryl
SOD	Superoxide Dismutase
SULT	Sulfotransferase
T/CAM	Traditional Complementary and Alternative Medicine
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid Reactive Substances

TBil	Total Bilirubin
TLC	Thin-Layer Chromatography
TM	Traditional Medicine
TP	Total Protein
UDP	Uridine 5'-diphospho
UG-IACUC	University of Ghana – Institutional Animal Care and Use Committee
UGT	Uridine 5'-diphospho (UDP)–glucuronosyl transferase
WBC	White Blood Cells
WHO	World Health Organization
XME	Xenobiotic Metabolic Enzymes
XO	Xanthine oxidase

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND

Herbal preparations have been used since time immemorial for the treatment of numerous diseases (Tugume and Nyakoojo, 2019). These herbs and herbal preparations often consist of different plants or plant parts. Currently, up-to-date scientific investigations are used to elucidate purported pharmacological activities of these herbal preparations. Additionally, some of the herbal preparations that are on the market now are in processed dosage forms (Olumese and Adegbolagun, 2015). Reports suggest that a large number of individuals mostly in Africa, China and India rely on herbal medicines for treatment of various ailments (Wachtel-Galor and Benzie, 2011, Bodeker and Kronenberg, 2002).

Among the common herbal products on the market are herbal bitters. Herbal bitters are prepared using infusion or distillation methods (Awa and James, 2013). Aromatic herbs, barks, roots and/or fruits are some of the constituents used in the preparation of these bitters because of their flavor and medicinal potential (Awa and James, 2013). Some these constituents include; goldenseal rhizome, angelica root, artichoke leaf, gentian root, wormwood leaves, and bitter orange peel (Awa and James, 2013). These herbal bitters are used as digestive stimulants, antibacterial agents, and detoxifiers (Olumese and Adegbolagun, 2015). It has also been reported that these herbal bitters boost the body's immune system (Olumese and Adegbolagun, 2015). It is noteworthy that, a number of herbal bitters were once sold as medicines but are now generally considered as digestifs: drinks consumed after meals to aid digestion (Awa and James, 2013).

Swedish bitters is one of the many brands of herbal bitters currently available on the market. The bitters is a mixture of 11 herbs which include: theriaca venezian, angelica root, myrrh, saffron, rhubarb root, senna leaf, camphor, carline root manna, zedoary and aloe in fixed concentrations. Swedish bitters is known to promote biliary, pancreatic and gastric secretion; relieve bloating, flatulence and gastrointestinal cramps; improve liver function; and boost the immune system (Awa and James, 2013).

Despite the health benefits of these herbs or herbal products, it has been found that some can modulate the activity of xenobiotic metabolizing enzymes, especially the Cytochrome P450 (CYP) enzymes (Miller, 1998, Wang *et al*., 2001). CYP enzymes are a large group of heme-thiolated proteins primarily found in smooth endoplasmic reticulum of liver cells and epithelial cells of small intestines. Most of these enzymes can be found in the liver, however, the kidneys, lungs, intestines and the skin also contain amounts of these isozymes (Ogu and Maxa, 2000). CYP enzymes are essential to the synthesis of many endogenous molecules including cholesterol, thromboxane A_2, prostacyclins, and steroids (Lynch and Price, 2007). CYP enzymes are also important in the biotransformation of xenobiotics. Biotransformation makes xenobiotics water-soluble and easily excreted by the kidney. Although there are numerous isoforms of CYP enzymes exist in humans, six of them; CYP2D6, CYP3A4, CYP1A2, CYP2C9, CYP2C19, and CYP3A5 are the most important as they metabolize about 90 percent of all drugs available on the market (Lynch and Price, 2007).

Herb-drug interaction may occur when there is concomitant administration of herbal medicines and conventional drugs. The potential effect of this could be sub-therapeutic or adverse effects of the conventional drug. Clinical case reports of herb-drug interactions

involving top-selling herbs or herbal products such as *Panax ginseng* (Ginseng), *Ginkgo biloba* (Ginkgo), and *Hypericum perforatum* (St. John's wort) have been reported in the United States of America (Iwata *et al*., 2004). Co-administration of St. John's wort is known to decrease blood levels of cyclosporine, warfarin, digoxin, and many other drugs metabolized by CYP3A4 (Iwata *et al.*, 2004, Hu *et al.*, 2012). To avoid possible interactions between herbs and conventional drugs, it is necessary identify modulatory potential of commonly used herbs or herbal products (Hu *et al.*, 2012). In addition, herbs and herbal products can be beneficial or detrimental to liver function (Guan and He, 2015), hence the need to monitor liver function in the presence of herbs.

Furthermore, there is the tendency for herb-drug interactions to occur because a known majority use herbs or herbal products for various medical conditions (Wachtel-Galor and Benzie, 2011). There is, however, a paucity of scientific data on herb-drug interactions, and possible impact of these herbal preparations on hepatic and hematological parameters in many of these settings, i.e. developing countries.

1.2 JUSTIFICATION

The use of conventional and herbal formulations is a common practice particularly in patients with several disease conditions (HemaIswarya and Doble, 2006, Nadler *et al*., 2004). Many patients take herbal medicines together with conventional drugs because they believe that the combination has synergistic effect. However, herbal products can alter the absorption and/or elimination of concomitantly administered conventional drugs (Arhewoh *et al*., 2017). These herbal products often induce/inhibit drug metabolizing enzymes, the commonest being the Cytochrome P450 (CYP) enzymes (Appiah-Opong *et al*., 2008). Herb-drug interactions involving CYPs have been identified as a potential risk factor for

adverse drug effects and ultimately, therapeutic failure (Federico and Mario, 2001). For example, dihydropyridine-type of calcium-channel blockers levels in circulation are known to be elevated when there is concomitant administration of these dihydropyridine-type of calcium-channel blockers with grapefruit juice (Alabi *et al*., 2013).

Swedish bitters is used by many, both healthy and sick, mostly after meals to aid digestion. It is a blend of 11 herbs: saffron, camphor, senna leaf, theriaca venezian, rhubarb root, zedoary, manna, angelica root, carline root, aloe, and myrrh. Some constituents of Swedish bitters have been found to have modulatory effect on drug metabolizing enzymes. Aloe vera has been reported to induce CYP reductase and some Phase II biotransformation enzymes (Singh *et al*., 2000). The bioactive compounds crocin and safranal found in saffron have shown CYP modulatory activity (Dovrtelova *et al*., 2015). Crocin decreased the activity of CYP2B, CYP2A, CYP2C11 and CYP3A enzymes, whereas safranal increased the activities of CYP2C11, CYP3A and CYP2B enzymes (Dovrtelova *et al*., 2015). Rhubarb, another constituent of Swedish bitters, has shown inhibitory effect on CYP2C6 and CYP3A4 (Gao et al., 2013, Iwata et al., 2004). Furthermore, some species of Angelica have been reported to cause induction/inhibition activity against CYP2C, CYP3A, CYP2D1 and CYP2D6 (Yoo *et al*., 2007, Ishihara *et al*., 2000, Tang *et al*., 2006). Some bitters affect the levels of lipid profile measurements such as cholesterol and triglycerides and also, antioxidant parameters such as lipid peroxidation and catalase activities (Alabi *et al*., 2013, Anyasor *et al*., 2017). In addition, bitters have been found to affect hematological and chemical parameters of blood (Ekor *et al*., 2010). There was elevation in alanine and aspartate aminotransferase activities, while decreasing total protein content. There is, however, a lack of scientific information on the potential net effect of all

these extracts found in Swedish bitters on drug metabolizing enzymes. The consequence of modulation of hepatic drug metabolizing enzymes could be changes in the blood concentration of concurrently administered conventional drugs, which could lead to adverse or sub-therapeutic effect. Therefore, this study seeks to determine the effect of Swedish bitters on hepatic drug metabolizing enzymes and hepatic antioxidant levels.

1.3 HYPOTHESIS

Swedish bitters could modulate the activity of cytochrome P450 metabolizing enzymes and alter hepatic antioxidant levels.

1.4 AIM

To determine the effect of Swedish bitters on the activity of rat liver Cytochrome P450 isoforms, and potential impact on antioxidant levels.

1.5 SPECIFIC OBJECTIVES

1. To determine the inhibition or induction potential of Swedish bitters on rat CYP1A2, CYP3A4, CYP2B1/2, CYP2C9 and CYP2D6.
2. To determine the effect of Swedish bitters on lipid peroxidation (LPO), and the levels of selected hepatic antioxidants: superoxide dismutase (SOD), reduced glutathione (GSH) and catalase (CAT).
3. To perform fingerprinting of Swedish bitters using high performance liquid chromatography (HPLC).

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 COMPLEMENTARY AND ALTERNATIVE MEDICINE

Traditional medicine (TM), also referred to as "ethno-medicine, folk medicine, native healing, or complementary and alternative medicine (CAM)", is one of the oldest forms of health care (Abdullahi, 2011). Traditional medicine involves the use of health practices and beliefs that incorporate mineral and/or animal based medicines, plants, exercises, manual techniques and spiritual therapies (Zakaryan and Martin, 2012). These are often applied individually or together to diagnose, maintain good health, as well as to avoid or treat diseases (WHO, 2002). Societies have developed several indigenous ways of healing that fall within the category of TM, for example the traditional African, Chinese and Indian medicines (Abdullahi, 2011). There is well documented information on the use of these traditional/complementary and alternative medicines (T/CAM). As much as 70% of people tend to use herbs as complementary and/or alternative medicine (Fasinu *et al*., 2012). In particular, nearly half of people in developed countries frequently use some form of T/CAM (e.g. Australia, 48% and France, 49%), and a widespread use occurs in many developing countries including Colombia, 40%; China, 40%; Chile, 71%; and as much as 80% of people in Africa (Bodeker and Kronenberg, 2002). In Ghana, a percentage use of about 70% of the population has been recorded (WHO, 2001). Certain social, cultural, as well as socioeconomic factors, impact T/CAM use in industrialized societies. In developing countries, affordability, accessibility, and cultural familiarity play a role in the use of T/CAM.

The awareness of and use of CAM are multifaceted phenomena that have grown exponentially in the past few years. This apparently insatiable need for alternative approaches to medical care by a number of people seems particularly interesting as it comes at a time of extraordinary therapeutic and technological improvements. A major reason for this phenomenon undoubtedly, is the colossal rise in public access to information through the internet and widespread news media coverage. Numerous advertisements and an unending exposure through the lay media, ranging from sensationalist newspapers to magazines, medical journals, and books, have strongly encouraged the notion of disease prevention and treatment by unconventional means, striking a delicate and highly profitable chord in a truly global populace (Neldner, 2000). Another reason for the popularity of CAM is the escalating cost of current allopathic medical care. The expense and the ensuing rationing of new modalities by managed care programs have placed them out of reach of a large proportion of the population (Neldner, 2000). The resultant effect is the creation of a strong public desire for many of these complementary and alternative therapies to prevent and treat human diseases (Neldner, 2000).

It can be noted that Neldner classified CAM products into two main groups; herbal and non-herbal (Neldner, 2000). Herbal therapy as the name implies, involves the use of a variety of plant species. Non-herbal therapies on the other hand include methods such as homeopathy, relaxation techniques, aromatherapy, chiropractic, support groups, acupuncture, prayer, light therapy, massage and many others (Neldner, 2000). CAM has been further classified into five groups (Figure 1); manipulative body-based practices, mind–body interventions, whole medical systems, energy medicine, and biology-based practices (Subramani and Lakshmanaswamy, 2017).

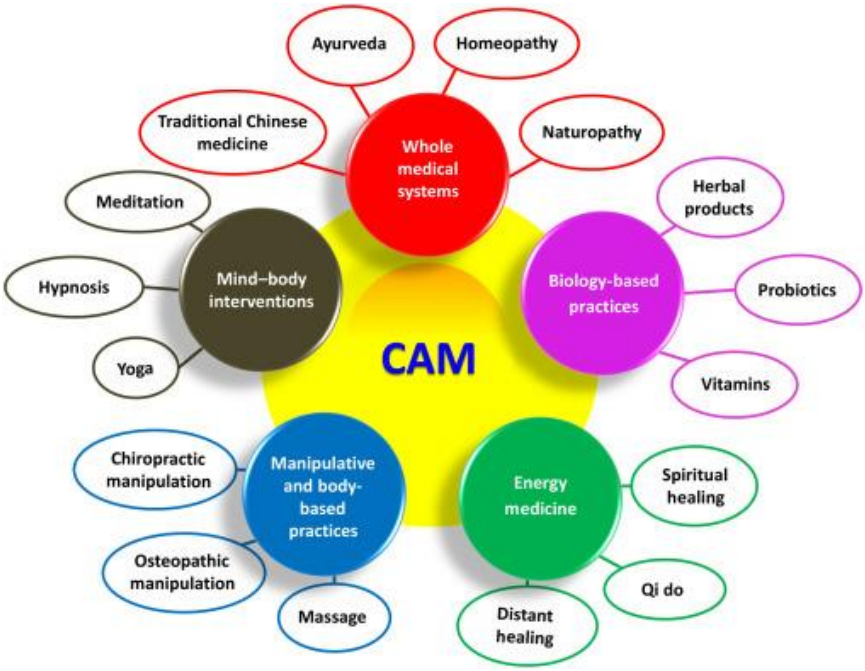

Figure 2.1. Types of Complementary and Alternative Medicine
(Subramani and Lakshmanaswamy, 2017)

2.1.1 Biology-based practices

The use of naturally occurring substances, such as herbs, vitamins, and foods to heal, maintain good health and homeostasis is considered a biologically-based practice (Bower, 2009, Romm, 2010). They are sometimes classified broadly as dietary supplements (Romm, 2010). Other examples include herbal products, fatty acids, amino acids, probiotics, prebiotics, and functional foods. Conventional drugs are subjected to intense scrutiny and should have evidence for their safety and efficacy, before obtaining approval from the Food and Drugs Administration (FDA). However, the same requirements do not apply to natural remedies, which means that claims about their efficacy are generally unverified (Tachjian *et al*., 2010). Herbs and herbal products are the most frequently used biologically-based products (Romm, 2010).

2.1.2 Herbs and herbal products

Herbs and herbal products are one of the most common forms of CAM (Patrick-Iwuanyanwu *et al*., 2012, Tachjian *et al*., 2010). Some herbal products are defined as medicines in many European countries, while in the United States they are classified as dietary supplements (Iwata *et al*., 2004). These herbs/herbal products were in use long before the introduction of modern medicine (Fasinu *et al*., 2012). They usually consist of different plants or plant parts (crude state or as plant preparations).

Herbs and herbal products are made of active phytochemicals, mostly consisting of secondary metabolites produced through pathways such as the acetate–mevalonate, acetate–malonate, and shikimate (Fasinu *et al*., 2012). These metabolites include phenolics (e.g. salicylates, lignins, quinolones, and tannins), terpenoids (e.g. saponins, sesquiterpenes, iridoids, carotenoids, and steroids) and phenolic glycosides (e.g. glucosinolates, cyanogens, and flavonoids). The herbs and herbal products may also contain other phytochemicals including peptides, alkaloids, polysaccharides (e.g. gums and mucilages), resins, and essential oils making herbs highly susceptible for drug interactions (Fasinu et al., 2012).

Secondary metabolites from plants have been effective sources of commercially important pharmaceutical compounds (HemaIswarya and Doble, 2006). Over all, plants have been a good source of novel drug compounds. About 41% of newly approved drugs within the period 1983–1994 were of natural product origin. However, within the same period, 60% of these products were found as anti-infective and anticancer compounds (Cragg *et al*., 1997). Initially, plant products were used in unmodified forms, as concentrated herbal

extracts. As time went on, and with technological advancement, plant products exist in different formulations: capsules, tablets, suspensions, etc.

A majority of herbs and herbal products have little or no toxicity assessment conducted on them, however, many exist on the market and are often self-prescribed (Arhewoh *et al*., 2017). Reports suggest that some herbs and herbal products may contain inorganic impurities such as arsenic, lead, and mercury; or intentionally added pharmaceuticals, which may be potentially harmful to humans (HemaIswarya and Doble, 2006). Thus, the need for studies to evaluate the safety of many of these herbal products.

Furthermore, herbal medicines are popular amongst many patients who have been diagnosed with chronic diseases such as hypertension, diabetes and others. Reports suggest that about 72.8% of diabetic patients use herbs/herbal products as alternative therapies (Vaghela *et al*., 2017). The use of herbs in diabetes treatment is attributed to the presence of phytoconstituents which can preserve the function of β-cells (Vaghela *et al*., 2017). Some of the very common herbal medicines include chamomile, Echinacea, *Gingko biloba*, ginger (*Zingiber officinale*), ginseng and St John's wort. Chamomile is used for its mild sedative effects but also has antispasmodic and antiseptic activity (Miller, 1998). *Ginkgo biloba* is has been shown to be very potent in dementia treatment, ginger is effective as an anti-nauseant and antispasmodic agent, while St John's wort is indicated for anxiety, sleep disorders, and depression (Miller, 1998). Indeed, recognition of the efficacy of natural products as therapeutic agents is on the rise, hence the need for extensive studies (efficacy, toxicity and interaction) on these agents (Teiten *et al*., 2013).

2.2 HERBAL BITTERS

Herbal bitters are blends of botanicals in water or alcohol (tincture) base (Olumese and Adegbolagun, 2015). The process of extracting alcohol from plants/herbs dates back to the "Hippocratic wine of the Greeks" (Tonutti and Liddle, 2010). As time went on, some of these alcohol extractions from plants were used for medicinal purposes. Herbal bitters, containing blended components in a base of alcohol (tincture) or water, were primarily sold as digestive aids on account of their capacity to increase the secretion of saliva and digestive juices (Olumese and Adegbolagun, 2015). An herbal extraction bitters containing gentian root in a distilled-alcohol base, famously known as "Stoughton's Great Cordial Elixir", became commercially available in 1690 (Johnson *et al*., 2015). This patented herbal formulation was advertised and sold for its therapeutic properties, however, it is the closest ancestor of what we know today as bitters. Currently, there are several bitters commercially available, even though most present-day manufacturers provide ingredients lists, they do not provide actual formulations (Johnson *et al*., 2015).

Herbal bitters contain several secondary constituents including phenols, flavonoids, alkaloids and polyphenols. These constituents make them good candidates for scavenging free radicals (Olumese and Adegbolagun, 2015). Herbal bitters are known to be effect immune boosters, thus, they are proposed to effectively maintain overall health status and well-being (Olumese and Adegbolagun, 2015). Bitters have been used since antiquity to treat dyspeptic disorders by stimulating the secretion of digestive juices and strengthening the smooth muscles of the gastrointestinal tract (Saller *et al*., 2001). Herbal bitters may also be used as antidiabetic agents (Jimmy and Udofia, 2014), as well as treatment against cervical cancer (Onyeaghala *et al*., 2015). Reports also suggest that some bitters induce

oxidative stress and inflammatory response (Oyewo, 2013) and affect lipid peroxidation, SOD and GSH levels (Adeyemi *et al*., 2012). Some common herbal bitters available on the market include Angostura Bitters, Hopped Grapefruit Bitters, Mole Bitters, Celery Bitters, Living Bitters and Swedish Bitters.

2.2.1 Swedish Bitters

Swedish bitters is a liquid polyherbal tonic rediscovered in the eighteenth century by Dr. Claus Samst and Dr. Urban Hiärne (Gomez-Flores *et al*., 2011). The original Swedish name, *Hiärne's Testamente* (Hiärne's Testament) is accompanied by a folklore that says the medicine is Hiärne's gift to mankind (Ahnfelt and Fors, 2016). Swedish bitters garnered universal appeal as a remedy for a wide range of disorders in the 18th century and was added to a number of pharmacopoeia and recipe collections in the 19th century (Ahnfelt and Fors, 2016). The origin of Swedish bitters however is attributed to Paracelsus, who at the beginning of the 16th century developed an "Elixir ad longam vitam" ("medicine for a long life") containing aloe, myrrh and saffron (Theiss and Theiss, 1993). It has been widely used in Europe from the 1730s until its re-popularization by Maria Treben (Theiss and Theiss, 1993). Swedish bitters as the name implies has a bitter taste, brownish-black color with a fragrant smell.

Although the recipe has changed over time, it mainly consists of *Aloe vera* (aloe), *Angelica archangelica* (angelica), *Carlina acaulis* (carline thistle), *Rheum rhabarbarum* (rhubarb), *Senna alexandrina* (senna), *Curcuma zedoaria* (zedoary), *Cinnamomum camphora* (camphor), *Fraxinus ornus* (manna), *Commiphora myrrha* (myrrh), *Sassafras albidum* (saffron) and other less defined ingredients (Teiten *et al*., 2013).

Most of the constituents of Swedish bitters have some purported medicinal uses. *Aloe vera* has been used extensively as an external burn treatment and also to help relieve constipation by oral consumption (Manvitha and Bidya, 2014). Furthermore, *Aloe vera* is known to have anti-inflammatory and wound healing properties, by accelerating the growth of epithelial cells (Eshun and He, 2004). Additionally, *Aloe vera* has been found to anticancer properties with tumour growth suppression activity (Wolfgang, 1995). In the pharmaceutical industry, Aloe vera is widely used in the production of medicines, such as ointments, burn treatments, medicated creams and lotions to combat a variety of skin conditions (Eshun and He, 2004). Furthermore, it is used for the manufacture of health drinks in the food industry (Eshun and He, 2004). *Aloe vera* reportedly induced CYP reductase and some Phase II biotransformation enzymes (Singh *et al*., 2000). Other reports have also indicated inhibitory activities against CYP3A4 and CYP2D6 enzymes (Djuv and Nilsen, 2012). Studies also indicate that *Aloe vera* gel extract significantly increased the levels of reduced glutathione, glutathione-S-transferase (GST), superoxide dismutase, lipid peroxidation and catalase in diabetic rats' kidney and liver (Rajasekaran *et al*., 2005).

The leaves, fruits and roots of *Angelica archangelica* are ingredients found amongst traditional medicine practitioners, and are recognized as one of the most esteemed medicinal plants in Nordic countries (Sigurdsson *et al*., 2013). Extensive investigation has revealed that *A. archangelica* contains quite a lot of vital bioactive compounds, including terpenes, flavonoids, polyphenols, coumarins and polysaccharides, with a number of biological effects (Sigurdsson *et al*., 2013). For example, the root extract has shown antitumor potential against breast cancer cells *in vitro* and *in vivo* (Oliveira *et al*., 2019). The root essential oil has some antibacterial property mainly against *Escherichia coli* and

Staphylococcus aureus and can be utilized as a natural preservative in foods (Acimovic *et al*., 2017). Antiviral potential of the fruit extract and five isolated compounds from the fruit have been identified against *Herpes simplex* virus-1 (Rajtar *et al*., 2017). Internally it is used in the treatment of digestive conditions, flatulence and also as a remedy for cold and other respiratory disorders (Kumar *et al*., 2011). Reports suggest that some angelica species cause induction/inhibition activity against CYP2C, CYP3A, CYP2D1 and CYP2D6 (Yoo *et al*., 2007, Ishihara *et al*., 2000, Tang *et al*., 2006). In addition, Yeh *et al*. (2003), reported the hepatoprotective effect of angelica. They indicated that this effect is a consequence of the inhibition of reactive oxygen species that cause lipid peroxidation, thus reducing oxidative stress (Yeh *et al*., 2003).

Carlina acaulis is a plant originating from the central and southern parts of Europe (Link *et al*., 2016). It is used conventionally as astringents and diuretics as well as for the treatment and management of all manner of skin disorders, pain, spasms and fevers (Jaiswal *et al*., 2011). The leaf extracts of *C. acaulis* subsp. *Caulescens* for example, has demonstrated anticancer activity against human melanoma cell lines *in vitro*, making it a potential candidate for skin cancer (Strzemski *et al*., 2017). It also has antioxidant activity, anti-inflammatory, anti-ulcer, antitrypanosomal and antimicrobial properties (Herrmann *et al*., 2011, Dordevic *et al*., 2007).

Rheum rhabarbarum, commonly known as rhubarb, is a well-known ancient Chinese traditional medicine used in the treatment of a range of ailments (Nizioł *et al*., 2017). The root extracts of rhubarb has shown antimicrobial activity against a variety of microorganisms (Canli *et al*., 2016). Other studies have also demonstrated that rhubarb has wide-ranging *in vitro* biological activities such as cathartic, anti-inflammatory, anticancer,

antibacterial, analgesic, hepatoprotective, antimutagenic, and anti-oxidative effects (Zheng *et al*., 2013). In addition, rhubarb has demonstrated good activity towards diabetic nephropathy (DN) clinically (Zheng *et al*., 2013). Rhubarb has demonstrated inhibitory effect on CYP2C6 and CYP3A4 (Gao et al., 2013, Iwata et al., 2004). Rhubarb exhibited significant enzyme lowering effect and liver protecting effect by decreasing levels of alanine aminotransaminase (ALT) and aspartate aminotransaminase (AST) (Xing *et al*., 2011).

Senna alexandrina is used in contemporary medicines as a laxative, and it is an ingredient in many herbal remedies and tonics (Pansa, 2011).

Curcuma zedoaria Roscoe, from the Zingiberaceae family and commonly referred to as zedoary is grown on a large scale usually as a vegetable, but also as a spice and perfumery material mostly in South-east Asian countries (Makabe *et al*., 2006). Conventionally, the dried rhizome of zedoary is carefully chosen to make drinks or to be processed as medicine (Lai *et al*., 2004). One notable feature of zedoary is its dark orange fleshed tubers which is similar to *Curcuma longa* (common turmeric) (Wilson *et al*., 2005). Zedoary is reported to have antimicrobial activity, anti-inflammatory activity, antioxidant activity, and anticancer activity against human ovarian cancer OVCAR-3 cells and human promyelocytic leukemia HL-60 cells (Lai *et al*., 2004, Mau *et al*., 2003, Makabe *et al*., 2006, Syu *et al*., 1998). Sun *et al.* (2010) reported a strong inhibitory effect of curcumenol, a major constituent of zedoary oil, on CYP3A4 activity (Sun *et al*., 2010).

Cinnamomum camphora, commonly referred to as camphor, is highly recommended in traditional medicinal settings for the treatment and management of a variety of disease

conditions. Among these, *C. camphora* is active as an antioxidant, anti-inflammatory, antibacterial and antifungal agent (Lee *et al*., 2006, Pragadheesh *et al*., 2013, Li *et al*., 2018, Zhou *et al*., 2017). Orally, camphor is administered for the treatment and management of hysteria, neuralgia, nervousness and also serious diarrhea. It is used effectively in the treatment of colds and chills (Lee *et al*., 2006). Camphor has been shown to affect the levels of alanine, and aspartate aminotransferase (ALT and AST respectively) liver enzymes in rats (Johari *et al*., 2015).

Fraxinus ornus, manna ash, is found growing naturally in the wild mostly in the Mediterranean region as well as in south-central Europe, most notably in Romania and the south of the Czech Republic (Kostova, 2001). The ethanolic extract of the bark has shown antioxidative activity (Marinova *et al*., 1994). The stem bark of manna ash has been used for the treatment of arthritis, dysentery, inflammation and wounds (Kostova and Iossifova, 2002).

Commiphora myrrha, a small tree or a large shrub, belongs to the Commiphora genus in the Burseraceae family, and it can be found in some African and Asian countries (Zhu *et al*., 2003). It produces a yellow non-volatile gum resin, called myrrh (Zhu *et al*., 2003). Extracts of *Commiphora myrrha* possess antioxidant, anti-inflammatory, antimicrobial, as well as analgesic effects (Su *et al*., 2011, Mohamed *et al*., 2014).

Sassafras albidum sometimes known as white sassafras, is a medium-sized aromatic tree, which grows moderately fast on moist, well-drained, sandy soils (Griggs, 1990). Isolated compounds obtained from the chloroform bark extract of *S. albidum* has shown some antileishmanial activity (Pulivarthi *et al*., 2015). It has also been used traditionally as a

blood and kidney cleanser (Cavender, 2006). However, reports indicate that the bioactive compounds crocin and safranal found in saffron alter CYP activity (Dovrtelova *et al*., 2015). Crocin significantly decreased the activity of CYP3A, CYP2A, CYP2C11 and CYP2B enzymes, on the contrary, the activity of CYP2B, CYP3A and CYP2C11 enzymes were significantly increased by safranal (Dovrtelova *et al*., 2015).

Swedish bitters is marketed as a digestif although it is claimed to have other pharmacotherapeutic properties. Some studies have shown that it detoxifies the body, aids in respiratory health, joint mobility, healthy bladder and helps with the elimination of toxins from the skin (Awa and James, 2013). Furthermore, the bitters has been shown to reduce cholesterol levels as well as regulate blood glucose level, making it a potential therapeutic candidate for diabetes and obesity (Awa and James, 2013). In addition, it has shown antimicrobial and anti-inflammatory potential (Anyasor *et al*., 2017, Gomez-Flores *et al*., 2011).

Figure 2.2. Swedish Bitters by NatureWorks
(Luckyvitamin, 2018)

2.3 XENOBIOTIC METABOLIZING ENZYMES

Xenobiotic metabolizing enzymes (XMEs) refers to an assorted group of proteins that breakdown a wide range of substances including drugs, food toxicants, pesticides, carcinogens, pollutants, and endogenous substances like bile acids, prostaglandins, and steroids (Penner *et al*., 2012). Metabolism of xenobiotics often leads to inactive or active and readily excretable metabolites (Brown *et al*., 2008). These metabolic reactions including oxidation, reduction, and hydrolysis are categorized as Phase I reactions while Phase II involves conjugation reactions (Wu and Lin, 2019). A third phase, Phase III, has been attributed to the function of membrane transporters on the efflux of compounds across plasma or intercellular membranes (Penner *et al*., 2012, Wu and Lin, 2019). Phase I reactions attach or reveal functional groups (e.g., - OH, - CO_2H, - NH_2, or - SH) on xenobiotics to enhance their hydrophilicity (Huang *et al*., 2018). Phase I XMEs include

flavin-containing monooxygenases (FMOs), xanthine oxidase/aldehyde oxidase (XO/AO), monoamine oxidases (MAOs) and cytochrome P450s (CYPs or P450s) (Penner *et al*., 2012). Some phase II metabolism reactions include acetylation, sulfonation, methylation glutathione (GSH) and amino acids (e.g. glutamic acid, glycine, and taurine) conjugation, as well as glucuronidation (Penner *et al*., 2012). Some Phase II XMEs include N-acetyl Transferases (NATs), Uridine 5'-diphospho (UDP)–glucuronosyl transferases (UGTs), sulfotransferases (SULTs), methyl (N-methyl - , thiomethyl - , and thiopurinemethyl -) transferases and Glutathione S-transferases (GSTs), (Wu and Lin, 2019, Penner *et al*., 2012).

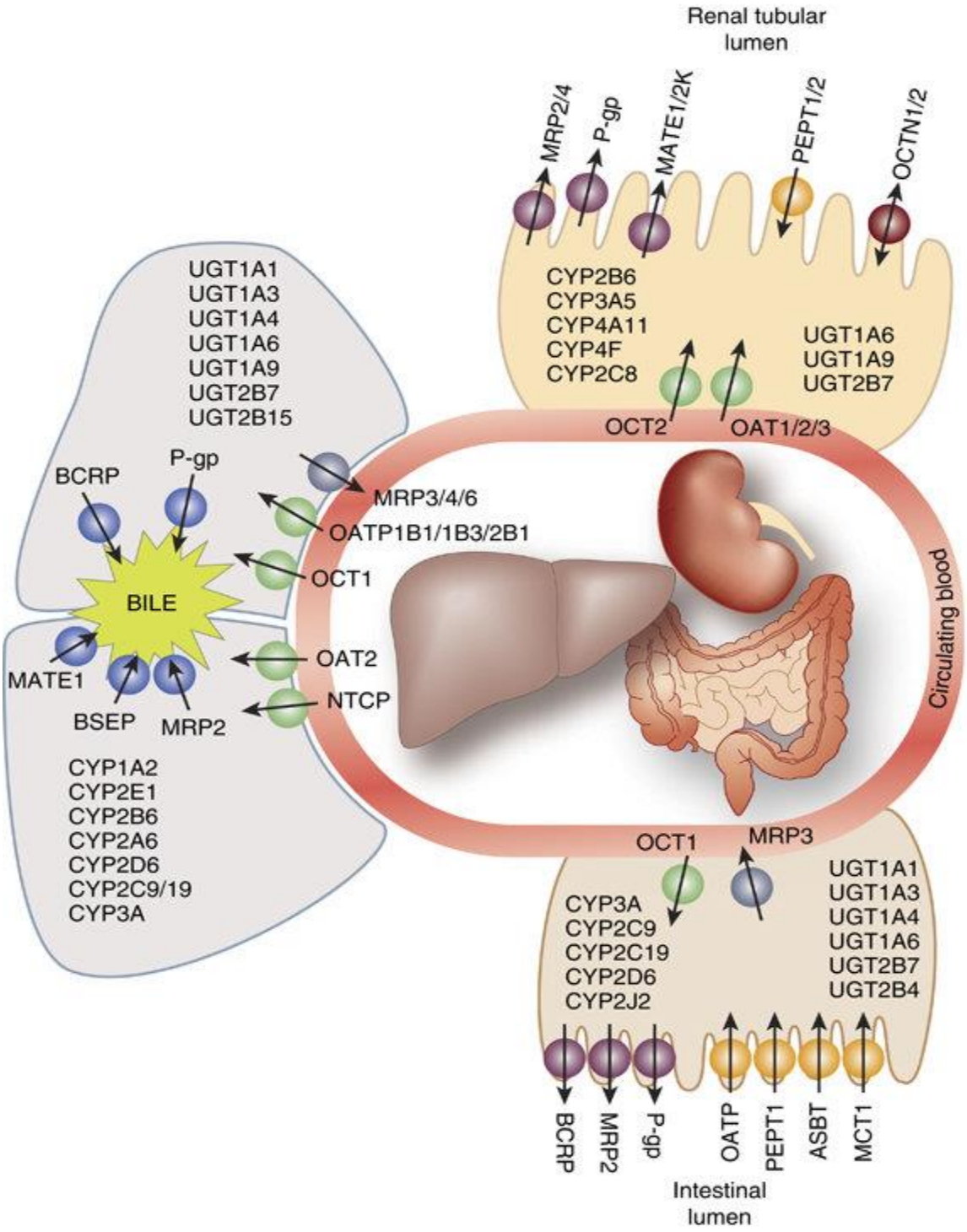

Figure 2.3. Drug transporters and metabolizing enzymes that function in the liver, intestine and kidneys.

(Yeung *et al*., 2013)

One of the major XMEs involved in Phase I reactions are the Cytochrome P450 (CYP) enzymes. The name "cytochrome P450" originated from the fact that "they are bound to membranes within a cell (cyto) and contain a heme pigment (chrome and P) that absorbs light at a wavelength of 450 nm when exposed to carbon monoxide" (Lynch and Price, 2007). The CYPs are a closely related group of enzymes that metabolize numerous drugs via oxidation (Ogu and Maxa, 2000). CYPs were first isolated by Klingenberg and Garfinkel from the liver (microsomes) of rats (Sychev *et al*., 2018). CYPs are heme-containing membrane proteins situated in the smooth endoplasmic reticulum of a number of cells. The majority of enzyme isoforms are found in the liver, however, the kidneys, lungs, intestines and the skin contain some amounts of this enzyme (Ogu and Maxa, 2000).

With time, a recommended nomenclature system for the CYPs was established whereby Roman numerals (later changed to Arabic) denoted gene families, letters denoted subfamilies and Arabic numerals denoted individual genes (Pinto and Dolan, 2011). More than 1,000 isoenzymes exist, and of these, five are very essential in metabolism of 90% of all available drugs on the market (i.e. CYP1A2, CYP2C9, CYP2D6, CYP2C19 and CYP3A4) (Pinto and Dolan, 2011).

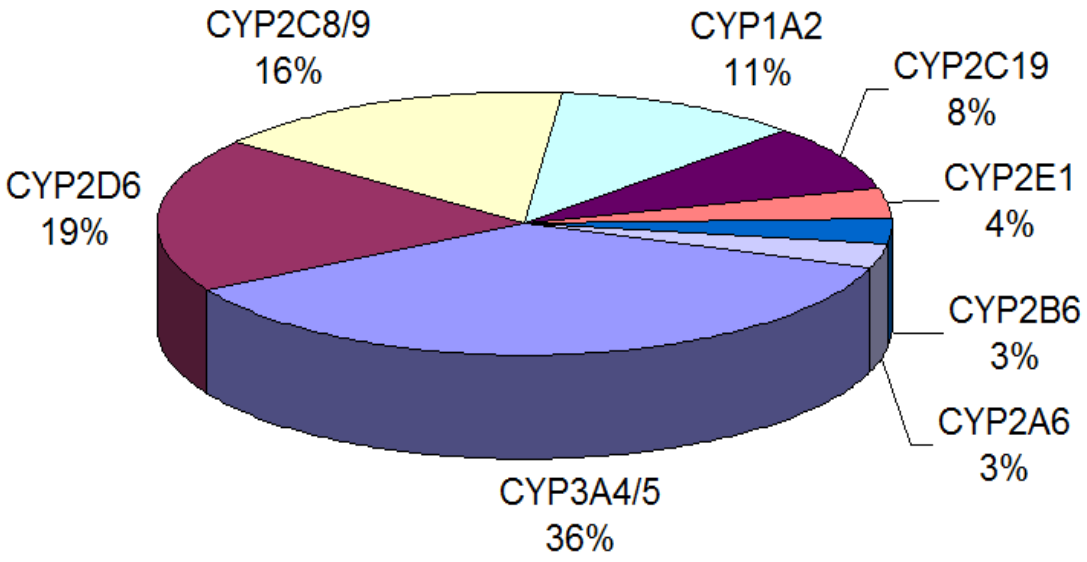

Figure 2.4. Proportion of drugs metabolized by different CYPs
(Häggström, 2014)

2.3.1 Drug Interactions

Drug-drug interactions may be defined as the effect of one drug over another (Brody, 2018). Drug interactions could result in serious undesirable effects (toxicities) or a reduction/increase in the therapeutic properties of certain medicinal agents. Multidrug therapy, which is commonly observed in aged patients, increases the possibility of drug interactions substantially (Cascorbi, 2012). Drug interactions may occur at the pharmacodynamics level or at the pharmacokinetic level (Pai and Bertino, 2015). Pharmacokinetic drug interactions are more common and predictable more than pharmacodynamic interactions (Flynn, 2007). Pharmacodynamic interactions involve one drug influencing another drug's effect directly (Cascorbi, 2012). For example, pharmacodynamic interactions can be observed where there is simultaneous administration of a nonsteroidal anti-inflammatory drug (NSAID) and phenprocoumon (an oral

anticoagulant) (Tonkin and Lindon Wing, 1988, Cascorbi, 2012). An additive interaction is observed where there is more bleeding since phenprocoumon inhibits vitamin k (Cascorbi, 2012). Another example is the simultaneous administration of aspirin and ibuprofen where ibuprofen prevents the gastrointestinal bleeding side effect of aspirin by binding to cyclooxygenase-1 (COX-1) (antagonistic interaction) (Cascorbi, 2012).

Pharmacokinetic drug interactions can take place at levels of drug absorption, elimination, metabolism (inhibition/induction of drug metabolizing enzymes) as well as drug transporters. Modulation of drug transporters and drug metabolizing enzymes are by far the most important interactions observed clinically (Pai and Bertino, 2015). The development of complexes can decrease the drugs' bioavailability and hence affect absorption of the drug (Cascorbi, 2012). For example, bisphosphonates used in osteoporosis, for instance alendronate, with already low bioavailability of only 0.5% to 2%, can be markedly reduced by calcium ions in mineral water or milk (Cascorbi, 2012). Drug transporters interactions can be caused by alterations in the transporters' expression levels or by substrates competing for binding sites (Vrbanac and Slauter, 2013). For example, verapamil and quinidine have been found to increase concentrations of digoxin, a cardiac glycoside, in plasma, because they block digoxin's biliary and/or urinary excretion via the efflux transporter P-glycoprotein (P-gp) inhibition (Vrbanac and Slauter, 2013). Alternatively, P-gp induction can speed up efflux transport and decrease the bioavailability of drugs as seen with the simultaneous administration of cyclosporine and rifampicin leading to sub-therapeutic concentrations of cyclosporine (Cascorbi, 2012).

Most metabolic interactions involve the cytochrome P450 (CYP) enzyme system, primarily expressed in the liver, and are involved in the Phase I oxidation of more than

50% of all drugs available on the market (Cascorbi, 2012). Reports suggest that a number of agents can modulate the metabolic activity of xenobiotic metabolizing enzymes (Ogu and Maxa, 2000). The most important isoforms involved in human drug metabolism include CYP1A2, CYP2C, CYP2D6, CYP2E1and CYP3A (Bertz and Granneman, 1997). CYP3A4 metabolizes over 50% of all clinically used drugs, whilst CYP2D6 metabolizes about 30% (Iwata *et al*., 2004, Pan *et al*., 2012). Clinical case reports of herbs that are known to modulate CYPs include Ginkgo, garlic, ginseng, and St John's wort. Concomitant administration of St John's wort is known to lower the blood concentration of digoxin, warfarin, cyclosporine and a host of other drugs metabolized by the CYP3A4 due to induction of CYP3A4 by St John's wort (Iwata *et al*., 2004). Naringin, a flavonoid contained in citrus fruits, most especially grapefruit, is known to inhibit CYP3A4 therefore, resulting in the increase in bioavailability of other drugs (Cascorbi, 2012). For example, co-administration of grapefruit juice is found to increase the bioavailability of midazolam, cyclosporine, terfenidine and calcium channel blockers by CYP3A4 inhibition (Iwata *et al*., 2004). Fluoxetine, which is a selective serotonin reuptake inhibitor used for the management of major depression, is a strong inhibitor of CYP2D6. CYP2D6 is known to metabolize clozapine, which is an antipsychotic in the management of severe paranoid schizophrenia. Therefore, simultaneous administration of fluoxetine and clozapine results in increased levels of clozapine in plasma and augments clozapine's therapeutic effects and possible toxicity (Ferslew *et al*., 1998). The inhibition of CYP2D6 can also affect the biotransformation of codeine into its active metabolite morphine or the formation of O-desmethyltramadol from tramadol (Cascorbi, 2012). Ciprofloxacin exhibits an inhibitory potential for CYP1A2, resulting in an inhibition of theophylline metabolism leading to an

increase in the plasma concentration of theophylline, with resultant cardiac and gastrointestinal side effects (Shakeri-Nejad and Stahlmann, 2006). Clopidogrel, which is a prodrug, requires biotransformation into its active metabolite by CYP2C19 for its antiplatelet effect (Mega *et al*., 2009). CYP2C19 is inhibited by proton-pump inhibitors (PPIs) such as pantoprazole, rabeprazole, lansoprazole, or omeprazole, therefore, simultaneous use of PPIs and clopidogrel leads to sub-therapeutic effect of clopidogrel (Mega *et al*., 2009, Ho *et al*., 2009).

Studies on herb-drug/drug-drug/drug-food interactions have become an essential part of modern research (Ogu and Maxa, 2000). However at present, herb–drug interactions are not subjected to the same scrutiny as that of drug–drug interactions (Chan *et al*., 2016). It is therefore important to determine the effect of xenobiotics on CYPs in order to predict drug interactions.

2.4 HERBS/HERBAL PRODUCT AND LIVER HEALTH

The liver is an essential organ in the body responsible for detoxification, thus any damage to it will result in the weakening of its functions (Guan and He, 2015). In the face of growing interest in the use of herbs and herbal products, issues concerning their safety is on the ascendancy (Amadi and Orisakwe, 2018). Apart from herb-drug interactions, usage of these herbs is fraught with medical problems including liver damage where patients can record abnormal liver function tests while being asymptomatic and as well, an unexpected and severe liver failure occurring (Amadi and Orisakwe, 2018). Current estimates suggest that 15% of drug-induced liver injuries (DILI) are caused by herbs i.e. herb-induced liver injuries (HILI) (Raschi and De Ponti, 2015). Determining its true occurrence remains an

uphill task, mainly because of the lack of legal controls and regulatory guidelines (Valdivia-Correa *et al*., 2016). However, there are certain factors contributing to liver toxicity by herbal medicines including misidentification or collection of wrong part of a medicinal plant, poor storage leading to modified products, adulteration during processing, as well as mislabeling of the final product (Larrey and Faure, 2011). Liver injury diagnosis commences with detailed information about the consumed herb/herbal product and the elimination of other possible causes of injury, including autoimmune diseases and viral hepatitis (Valdivia-Correa *et al*., 2016). Liver enzymes are significant biomarkers used to measure the extent of liver damage and are easily obtainable for the monitoring of individuals who have liver disease (Amadi and Orisakwe, 2018). Elevated levels of transaminase enzymes such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are important markers for chronic liver disease diagnosis (Amadi and Orisakwe, 2018). According to a database managed by the US National Library of Medicine, LiverTox, in excess of 30 herbal medicines have been reported to cause DILI (Liu *et al*., 2016). Herbal medicines with the tendency to cause major hepatotoxicity have been found to contain pyrrolizidine alkaloids (Larrey and Faure, 2011). Pyrrolizidine alkaloids are phytochemicals that are naturally produced by plants as secondary metabolites and these serve as defense mechanisms against insect herbivores and are toxic to most vertebrates (Fu *et al*., 2007). They require metabolic activation to the "pyrrolic" metabolites to exert their toxicity (Fu *et al*., 2007). Some of the known plant species having these alkaloids include *Crotalaria*, *Heliotroprium*, *Symphytum* (Comfrey), and *Senecio* as well as *Gynura segetum*. Others are germander (*Teucrium chamaedris*), *Atractylis gummifera*, kava–kava (*Piper methysticum*), *Hedeoma pulegioides*, great celandine

(*Chelidonium majus*), and *Mentha pulegium* (Larrey and Faure, 2011). Red Yeast rice (*Monascus purpureus*), black cohosh (*Actaea racemosa*), *Garcinia cambogia*, green tea extract (GTE), obtained from the leaves of the *Camellia sinensis* plant, have all been linked to herb induced liver injury (Navarro *et al*., 2017). Some constituents of Swedish bitters have shown herb-induced liver toxicities. Case reports suggest that *Aloe vera* induced hepatotoxicity indicated by high liver enzyme levels. However, liver enzyme levels were normalized after discontinuation of the aloe preparations (Yang *et al*., 2010, Rabe *et al*., 2005).

Free radicals are also implicated in the pathogenesis of toxic liver injuries which compromise the membranes of hepatocytes resulting in the seepage of enzymes and elevation in liver biomarkers (Spencer *et al*., 2011). Free radicals as well as other reactive oxygen species (ROS) are produced from the regular metabolic processes occurring in the body or from sources outside the body including industrial chemicals, air pollutants, radiation, certain drugs, and cigarette smoking (Lobo *et al*., 2010). ROS consist of both non-radical and radical oxygen species comprising of singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), hydroxyl radical (•OH) and superoxide anion ($O_2^{\bullet-}$) (Sharma *et al*., 2012). Normal levels of ROS are vital to physiological processes including cellular signaling pathways and reactions to infectious agents (Abdel lateif *et al*., 2016). On the contrary, high ROS concentrations are very dangerous to organisms, and when the amount of ROS surpasses the defense mechanisms of the organism, a cell is said to be in a state of "oxidative stress" (Sharma *et al*., 2012). Oxidative stress leads to direct or indirect ROS-mediated destruction to molecular species including lipids, proteins, and nucleic acids and has been implicated in conditions such as inflammation, certain cancers, antherosclerosis,

diabetes, and the aging process (Lobo *et al*., 2010, Ray *et al*., 2012). Cells contain several defenses against ROS induced damage that include non-enzymatic and enzymatic antioxidants (Mesa-Herrera *et al*., 2019). A balance between the levels of ROS and antioxidants favors cellular homeostasis. "Antioxidants act as radical scavenger, hydrogen donor, electron donor, peroxide decomposer, singlet oxygen quencher, enzyme inhibitor, synergist, and metal-chelating agents" (Lobo *et al*., 2010). Non-enzymatic constituents of the antioxidant defense system include glutathione, tocopherol, and carotenoids. The enzymatic antioxidants include glutathione reductase (GR), superoxide dismutase (SOD), glutathione peroxidases (GPx), catalase (CAT), and glutathione S-transferases (GSTs) (Lobo *et al*., 2010, Sharma *et al*., 2012). Several methods are employed in the evaluation of the antioxidant property of samples including *in vitro* and *in vivo* antioxidant models. Unlike the *in vitro* methods, samples to be tested for *in vivo* methods, are administered to the testing animals, and biological samples (blood and/or tissues) are frequently used for the assay (Alam et al., 2013). A number of *in vitro* methods utilize the free radical scavenging mechanisms; including diphenylpicrylhydrazyl (DPPH) radical scavenging and nitric oxide scavenging assays (Alam et al., 2013). *In vivo* methods may include but not limited to Superoxide dismutase (SOD), Ferric reducing-antioxidant power (FRAP), Glutathione-S-transferase (GST), and Catalase (CAT) activity (Alam et al., 2013).

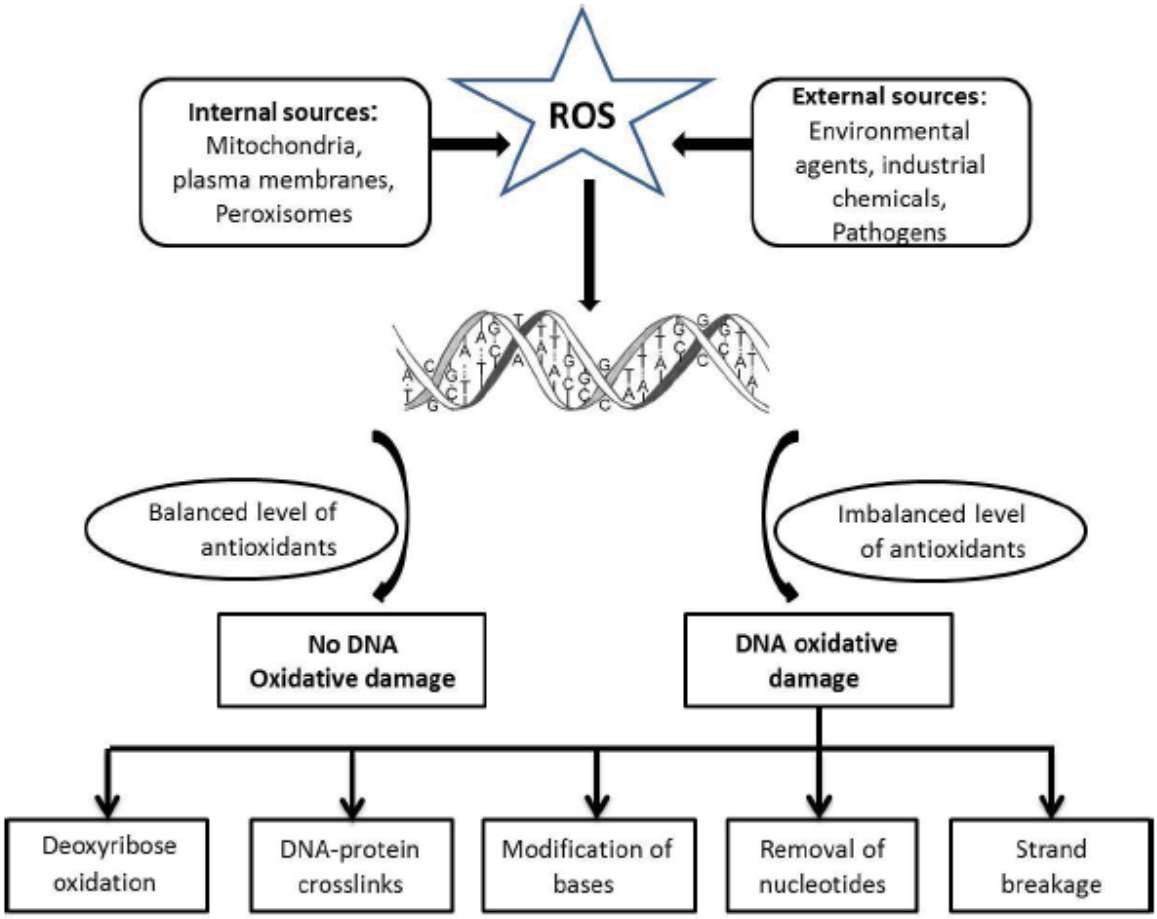

Figure 2.5. Production of ROS by internal and external sources.
(Abdel lateif *et al*., 2016)

2.5 REVIEW OF METHODS

2.5.1 Protein Determination

Several methods are used for protein content determination. In this study, the Bradford method (Bradford, 1976) was employed. This is a fast, relatively cheap and highly specific method for protein determination. It is very sensitive, detecting in the range of 1-20 µg for micro assays, and 20-200 µg for macro assays, and therefore, suitable for a wide range of substances. It is a colorimetric assay that detects the change in absorption of Bradford reagent when it binds to proteins. Bradford reagent contains Coomassie Brilliant Blue G-

250 (CBBG) dye (Figure 5) that binds to aromatic and basic amino acid residues (arginine, phenylalanine, tryptophan and proline). When the CBBG dye binds to proteins, its brown color is converted to blue and the amount of this blue form is detected at 595 nm to quantify the concentration of proteins (Figure 5). The unbound dye in solution is in its cationic form, with absorbance maximum at the wavelength of 470nm (brownish red). When it binds to protein it is found in its anionic form with absorbance maximum at the wavelength of 595nm (blue). Standards are prepared to construct a calibration curve. These standards are samples with known concentration of protein. Bovine serum albumin (BSA) dissolved in water is routinely used. BSA works effectively as a protein standard because it is extensively obtainable in high purity and relatively cheap. Dilution of the sample with unknown concentration is done to fit into the concentration range of the calibration curve. The protein concentration in the sample is determined using the calibration curve.

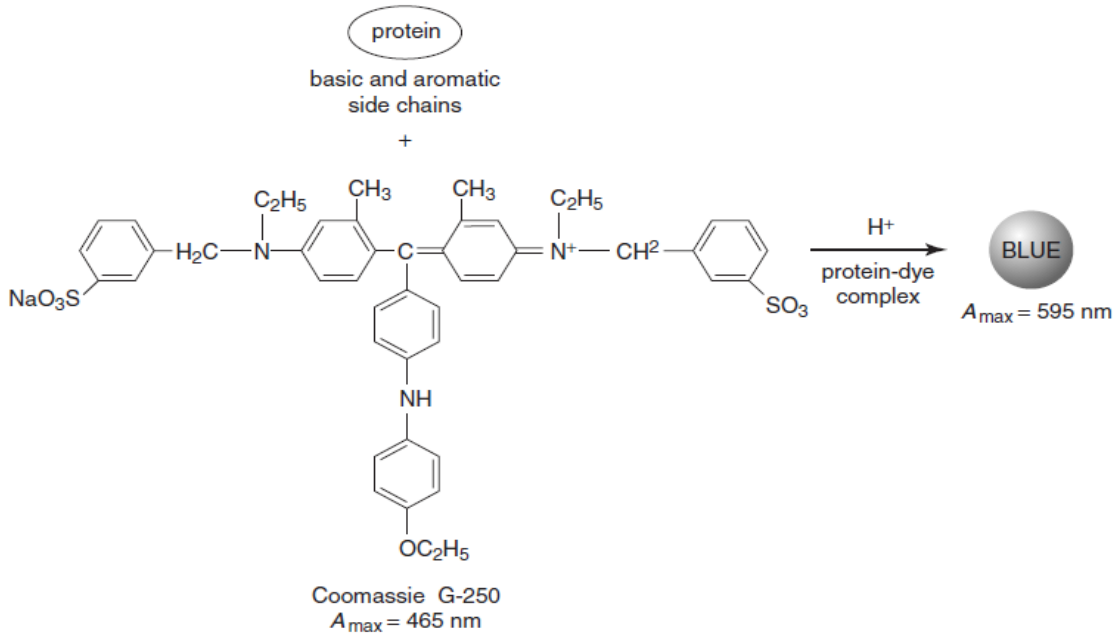

Figure 2.6. Reaction schematic for Bradford Protein Assay
(Krohn, 2005)

2.5.2 CYP Enzyme Activity

The O-dealkylations of alkylresorufins are commonly used activity probes for assessing some CYP isoforms. The CYP enzyme activity was measured using probe substrates specific for each of the enzymes in the presence of NADPH cofactor. This study employed the method as described by Appiah-Opong et.al. (2007). Ethoxyresorufin, Methoxyresorufin, Benzyloxyresorufin and Pentoxyresorufin were used as substrates for CYP1A1/1A2, CYP1A2, CYP3A4 and CYP2B2/2B2 respectively. This assay measures the formation of the metabolite (resorufin) from the substrates. In the presence of the enzymes, there is dealkylation of the substrates to form resorufin which fluoresces at 530 nm excitation and 586 nm emission (Figure 6).

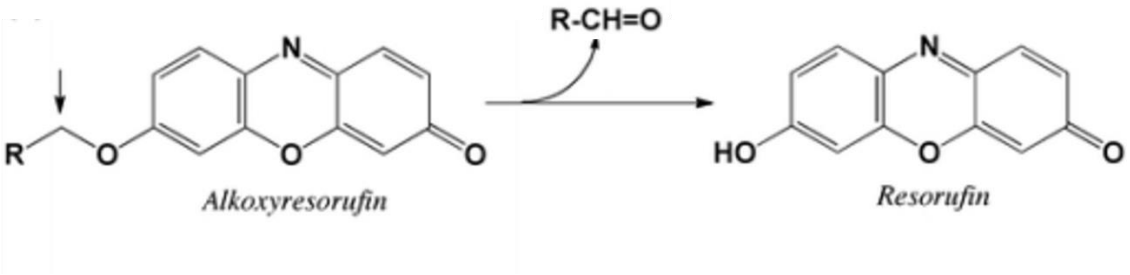

Figure 2.7. Alkoxyresorufin O-dealkylation

R is: H (methoxyresorufin), CH3 (ethoxyresorufin), C2H5 (n-propoxyresorufin), C3H7 (n-butoxyresorufin), C4H9 (n-pentoxyresorufin), C5H11 (n-hexoxyresorufin), C6H13 (n-heptoxyresorufin), C7H15 (n-octoxyresorufin), C6H5 (benzyloxyresorufin). (Vottero *et al.*, 2011)

For CYP2C9 and CYP2D6, the substrates employed were diclofenac and dextromethorphan, respectively. In the presence of the enzyme, there is hydroxylation of the diclofenac to 4-hydroxydiclofenac which is detected at a wavelength of 280 nm. Dextromethorphan (DXM) on the other hand is demethylated into dextrorphan (DXT) which is measured at 280 nm excitation and 310 nm emission.

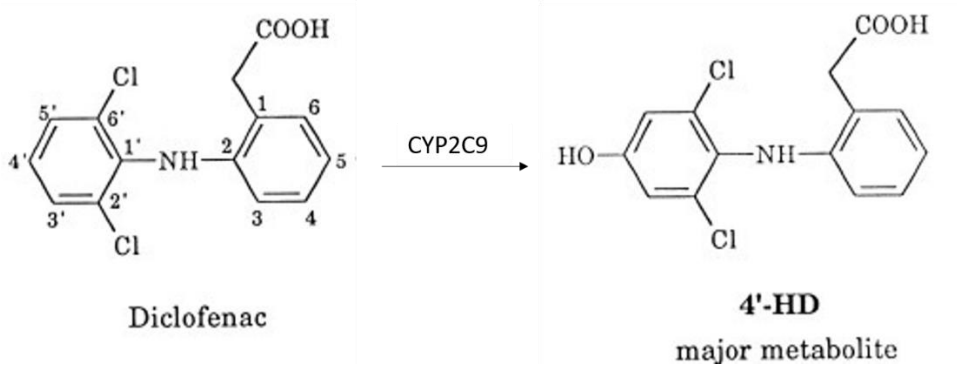

Figure 2.8. Hydroxylation of diclofenac to 4'hydroxydiclofenac
(Othman *et al*., 2000)

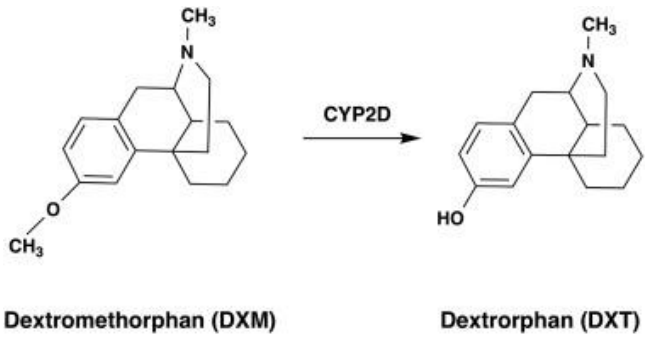

Figure 2.9. Dextromethorphan O-demethylation
(DuBois and Mehvar, 2018)

2.5.3 GSH determination

Many assays used for GSH determination are centered on the reaction of GSH with a fluorophore or chromophore such as O-phthalaldehyde (OPA or OPT) (Shetty *et al*., 2006). OPA is a dialdehyde which consists of two formyl groups bonded to adjacent carbon centers on a benzene ring. Due to the presence of a sulfhydryl (SH) group in its structure, GSH reacts freely with OPA to produce a highly stable and fluorescent iso-indole derivative (GSH-OPA) (Michaelsen et al., 2009). This reaction allows for sensitive and precise quantitative assessment of GSH in biological systems (Michaelsen *et al*., 2009).

The highly fluorescent iso-indole GSH conjugate can be measured at 340 nm (excitation) and 420nm (emission) (Singh *et al*., 2017). Healthy or normal cells produce higher fluorescence as an indication of their high GSH content. Oxidative stressed cells on the other hand, produce a lower fluorescence due to their relatively low GSH concentration.

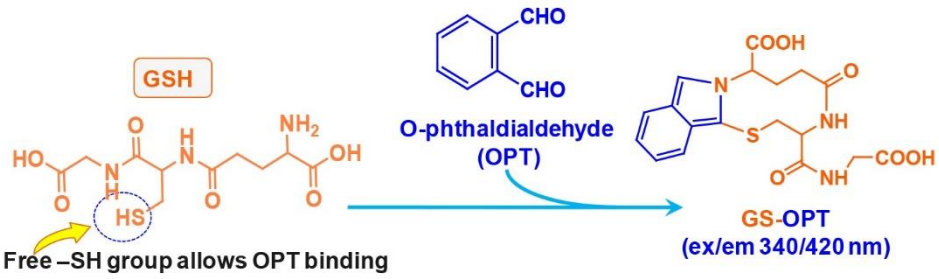

Figure 2.10. Chemical reaction between Glutathione and O-phtalaldehyde (Singh *et al*., 2017)

2.5.4 SOD Activity

Many different assays are currently in use for SOD activity measurement. SOD is responsible for the breakdown of harmful superoxide anions into hydrogen peroxide and oxygen (Spanou et al., 2011). In some assays, superoxide radicals/anions are produced by xanthine oxidase or by autoxidation of photo reduced riboflavin, and an indicator such as cytochrome c, nitroblue tetrazolium (NBT), pyrogallol, or epinephrine is monitored for a color change (Roth and Gilbert, 1984). In some of these experiments, the indicator compounds themselves produce the needed superoxide anions without the need for an added source. Autoxidation of this nature can occur with pyrogallol and epinephrine (Gao *et al*., 1998). In this study, the method by Marklund and Marklund (1974) was used with slight modifications. Pyrogallol (1,2,3-benzenetriol) has for a long time, been identified to rapidly autoxidize, particularly in alkaline solution and its autoxidation reaction has been

used for the separation of oxygen from gases (Marklund and Marklund, 1974). The oxidation of pyrogallol leads to the formation of a yellow-colored product called purpurogallin (Mesa-Herrera *et al*., 2019). SOD inhibits almost entirely the pyrogallol autoxidation by competing very proficiently for the superoxide radicals, and so SOD activity is proportional to the rate of inhibition of pyrogallol autoxidation (Semsei and Nagy, 1984).

2.5.5 Catalase Activity

Catalase (CAT) is an essential enzyme that is required to breakdown hydrogen peroxide (H_2O_2), a by-product of SOD activity, into water (H_2O) and molecular oxygen (O_2) (Shetty *et al*., 2006, Hadwan, 2018). A number of methods are available for CAT activity. In this study, CAT activity was assessed by a potassium dichromate colorimetric assay as described by Sinha (1972) with slight modifications. This method works on the principle that, in the presence of H_2O_2 and heat, dichromate in acetic acid reduces to chromic acetate with the production of perchloric acid as an unstable intermediate. The chromic acetate that is produced is measured colorimetrically at the wavelength of 570 nm (Sinha, 1972).

2.5.6 Lipid Peroxidation

Oxidation of lipids in biological systems leads to the formation of lipid peroxidation (LPO) products, and these products can function as important biomarkers for oxidative stress and antioxidant functionality (Shetty *et al*., 2006). In this study, LPO was determined by the thiobarbituric acid reactive substances (TBARS) method as described by Okhawa *et al.* (1979). The assay involves the reaction of thiobarbituric acid (TBA) with LPO products in particular, malondialdehyde (MDA) which is a secondary oxidation product of lipids. The

reaction products can be measured spectrophotometrically at wavelengths of 532-535 nm (Shetty *et al*., 2006).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 STUDY DESIGN

An experimental design using animal models, where a control group (untreated) was compared to treatment groups, and *in vitro* assays were employed. Animals were randomly selected for the various groups.

3.1.1 Inclusion/exclusion criteria

For animal models, only healthy male Sprague-Dawley rats were used. Co-morbid and knock-out animals were excluded from the study.

3.2 ETHICAL CONSIDERATIONS

The study was approved by the Scientific and Technical Committee of the NMIMR with approval number STC paper 3(2) 2018-19 (Appendix I) and the Institutional Animal Care and Use Committee (IACUC) with approval number UG-IACUC 004/18-19 (Appendix II)

3.3 CHEMICALS AND REAGENTS

NatureWorks® Swedish Bitters was purchased from Relish Health Foods, Osu, Accra, Ghana. Diclofenac, dextromethorphan, pentoxyresorufin and ethoxyresorufin were purchased from the Sigma-Aldrich company (St Louis, MO, USA). Other reagents include Bovine serum albumin (BSA) (Wako Pure Chemical Industries, Japan), reduced

nicotinamide adenine dinucleotide phosphate (NADPH) (Sigma-Aldrich, USA), reduced glutathione (GSH) (Sigma-Aldrich, Japan), o-phthalaldehyde (OPA) (Wako Pure Chemical Industries, Japan), zinc sulphate (Sigma-Aldrich, USA), pyrogallol (Wako Pure Chemical Industries, Japan), dichromate (BDH Chemicals Ltd, England), sodium dodecyl sulfate (SDS) (Wako Pure Chemical Industries, Japan), thiobarbituric acid (TBA) (Wako Pure Chemical Industries, Japan), pyridine (Sigma-Aldrich, USA), and butanol (Wako Pure Chemical Industries, Japan). All additional chemicals and reagents used were of analytical grade and obtained from standard suppliers.

3.4 FINGERPRINTING OF SWEDISH BITTERS USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Fingerprinting of Swedish bitters was determined using an HPLC method as follows. Analyses was performed utilizing an Agilent 1100 system (Santa Clara, CA, USA), consisting of a quaternary pump, auto sampler, diode array detector (DAD), and HP ChemStation Software. Chromatographic separation was carried out on a Tskgel ODS C18 (250 x 4.6 mm i.d., 5 µm particle size) analytical column maintained at 30°C. The injection volume was 20 µL and the eluents, water in 0.1% phosphoric acid (A) and methanol (B) as mobile phase at a flow rate of 1 mL/min. The linear gradient program used was set as follows: 0–10 min, 10–30% B; 10–15 min, 30–50% B; 15–25 min, 70–90% B; 25–35 min, 90–90% B; 35–38 min, 90–10% B; 38–40 min, 10–10% B. Ultra-violet (UV) detection was performed at 280 nm.

3.5 EXPERIMENTAL ANIMALS

Male Sprague Dawley (SD) rats, ranging from 6 – 8 weeks old, were obtained from the Department of Animal Experimentation, Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana, Legon. The rats were kept in groups of five in stainless steel cages (34 cm x 47 cm x 18 cm) and with bedding made of soft wood shavings. The SD rats were fed standard diet consisting of normal pellet (AGRIMAT, Kumasi, Ghana), given water *ad libitum*, and kept under standard laboratory conditions (temperature 25 ± 1°C, relative humidity 60-70%, and 12 h light-dark cycle). The rats were made to acclimatize with laboratory conditions for 7 days before commencing of experiment, similar to one reported by Alabi *et al.* (2013).

3.5.1 Rat grouping and treatment administration

The SD rats were put into 5 groups (5 rats/group). Group 1 was administered the vehicle (distilled water), and this was the negative control. Group 2 was administered 15 mg/kg/day phenobarbital, and this was the positive control. Groups 3 - 5 were administered Swedish bitters at a low dose (5 mL/kg/day), medium dose (10 mL/kg/day) and high dose (20 mL/kg/day), respectively. The doses of Swedish bitters were animal equivalent doses (AED) calculated as described by Shin *et al*. (2010). The dose calculations are shown at Appendix III. All administrations were done by oral gavage for a period of 7 days. After the treatment period, the rats were subjected to euthanasia in a chloroform chamber, blood obtained by cardiac puncture and their livers harvested and placed on ice immediately. The livers were stored at -80°C until use. Blood from each animal was collected into tubes with

or without ethylenediaminetetraacetic acid (EDTA), an anticoagulant, for biochemical and hematological analysis respectively.

3.6 PREPARATION OF MICROSOMAL FRACTIONS

The excised livers stored at -80°C were thawed and homogenized individually in two volumes of potassium phosphate buffer (pH 7.4) using a mortar and pestle. Microsomal fractions from the livers were obtained by the method of ultracentrifugation as described by Appiah-Opong *et al.* (2018), with slight modification. Homogenates were centrifuged at 4,500 rpm for 20 min at 4°C (Eppendorf Centrifuge 5810R, Germany) and the supernatant further centrifuged at 25,000 rpm for 2 h at 4°C with an ultra-centrifuge (Beckman Avanti J-25, USA). After ultra-centrifugation, the resultant supernatant (cytosol) was separated from the pellet (microsomes) and stored separately at -80°C. The microsomes obtained were then homogenized in potassium phosphate buffer (pH 7.4), aliquoted and stored at -80°C until use.

3.6.1 Protein Determination

The protein content of both the microsomes and cytosol was determined using the Bradford assay with Bovine Serum Albumin (BSA; St. Louis, MO, USA) as standard. Serial dilutions of the microsomes, cytosol and BSA were made. A volume of 200 µL of Biorad reagent dye (Bio-Rad Laboratories Inc., USA) was added to 10 µL of each microsomal, cytosolic and BSA dilution respectively in a 96-well plate in triplicates. The reactions were incubated for 5 min at room temperature, and absorbance read at a wavelength of 590 nm with a spectrophotometer (Tecan Infinite M200 Pro, Austria). The protein content was then calculated based on the BSA standard curve (Appendix IV).

3.7 CYP ENZYME ASSAYS

3.7.1 Methoxy- Ethoxy- Benzyloxy- and Pentoxy-resorufin O-dealkylation (MROD, EROD, BROD and PROD)

The inhibition or induction of CYP 1A1/1A2, 1A2, 3A4 and 2B1/2B2 enzymes by the Swedish bitters was carried out using fluorimetric assays as described by Appiah-Opong *et al.* (2007). Assays consisted of 0.1 M phosphate buffer (pH 7.4), the substrates ethoxyresorufin, methoxyresorufin, benzyloxyresorufin and pentoxyresorufin (5 µM), and microsomal protein (0.1 mg protein/ml). All substrates were from Sigma-Aldrich (St Louis, MO, USA). The reaction mixtures were pre-incubated at 37°C for 5 min and then initiated by adding 100 µM of nicotinamide adenine dinucleotide phosphate, NADPH (in 0.1 M phosphate buffer). The reactions were allowed to proceed for 10 min (EROD and MROD), 20 min (PROD), 30 min (BROD) at 37°C. Table 1 shows the experimental conditions for these assays. Reactions were terminated with a solution of 80% acetonitrile and 20% 0.5 M Tris. Fluorescence measurements were done using a plate reader (Tecan Infinite Pro M200) at wavelengths of 530 nm excitation and 586 nm emission. All experiments were done in triplicate.

Table 3.1. Experimental conditions for fluorescence CYP enzyme assays

CYP	Enzyme concentration (mg/mL)	Incubation time (min)	Substrate	Substrate concentration (µM)	Excitation wavelength (nm)	Emission wavelength (nm)
1A1/1A2	1	10	ERes	5	530	586
1A2	1	10	MRes	5	530	586
3A4	1	30	BRes	5	530	586
2B1/2B2	1	20	Pres	5	530	586

Abbreviations: *ERes: ethoxyresorufin; MRes: methoxyresorufin; BRes: benzyloxyresorufin; PRes: pentoxyresorufin.*

3.7.2 Diclofenac Hydroxylation

Inhibition or induction of CYP2C9 enzyme activity was assayed by measuring the formation of the metabolite 4-hydroxydiclofenac from diclofenac (Appiah-Opong *et al*., 2007). Reaction mixtures comprised of 0.1 M phosphate buffer (pH 7.4), microsomal protein (0.1 mg protein/ml) and the substrate, diclofenac (6 µM). Pre-incubation of the reaction mixture was done at 37°C for 5 min and reaction continued in the presence of 100 µM NADPH, for 10 min at 37^{o}C. The reaction was terminated by the addition of 200 µL of ice cold methanol (stopping solution), after which centrifugation was done at 12,000 rpm for 5 min at room temperature. Supernatants after centrifugation were analyzed using an isocratic HPLC method with a C18 column, and at a carrier flow rate of 0.6 ml/min. A mobile phase consisting of 60% (v/v) 20 mM potassium phosphate buffer (pH 7.4), 22.5% (v/v) methanol, and 17.5% (v/v) acetonitrile was employed. Chromatographic peaks were monitored at a wavelength of 280 nm. Duplicate experiments were performed.

3.7.3 Dextromethorphan O-demethylation

The effect of Swedish bitters on rat liver microsomal CYP2D6 levels was evaluated by measuring the formation of dextrorphan, the metabolite, from dextromethorphan, as described by Appiah-Opong *et al*.., (2007). The reaction mixtures constituted 0.1 M potassium phosphate buffer, 4.5 µM dextromethorphan and rat liver microsomal fraction (0.1 mg protein/ml). Pre-incubation was done at 37°C for 5 min before the addition of 100 µM NADPH and the reaction allowed to progress at 37°C for 45 min. Aliquots of 100 µL 300 mM zinc sulphate heptahydrate (stopping solution) were added and each reaction mixture centrifuged at 4,000 rpm for 15 min at room temperature. Consequently, the

supernatant obtained was collected into vials and analyzed using an isocratic HPLC method with a C18 column. The mobile phase consisted of 24% (v/v) acetonitrile and 0.1% (v/v) triethylamine adjusted to pH 3 with perchloric acid. A carrier flow rate of 0.6 ml/min was employed and the metabolite formed monitored at wavelengths of 280 nm excitation and 310 nm emission. Duplicate experiments were done.

3.8 BIOCHEMICAL AND HEMATOLOGICAL ANALYSIS OF BLOOD COLLECTED FROM SD RATS

Whole blood (in EDTA tubes) was analyzed for erythrocyte count (RBC), mean corpuscular volume (MCV), hemoglobin (HGB), mean corpuscular hemoglobin (MCH), platelet count (PLT), procalcitonin (PCT), hematocrit (HCT), mean platelet volume (MPV), red cell distribution width (RDW), RDW expressed as a standard deviation (RDW_SD), platelet distribution width (PDW), lymphocyte (LYM), eosinophils (EOS), monocyte (MON), neutrophils (NEU), basophils (BASO) and total white blood cells (WBCs). For biochemical analysis, blood samples obtained from the SD rats were centrifuged at 1500 rpm for 10 min to obtain serum. The serum was analyzed for the levels of alkaline phosphatase (ALP), aspartate aminotransferase (AST), gamma glutamyl transferase (GGT), alanine aminotransferase (ALT), Total bilirubin (TBil), direct bilirubin (DBil), total protein (TP), albumin (ALB), globulin (GLB), and albumin/globulin ratio (A/G). Measurements were done using a clinical chemistry analyzer (URIT-8021AVet Automatic Chemistry Analyzer) for serum and an automated hematology analyzer (URIT-5250Vet Hematology Analyzer) for whole blood.

3.9 ANTIOXIDANT ASSAYS OF CYTOSOL FROM HOMOGENIZED LIVERS OF SD RATS

3.9.1 GSH determination

Estimation of GSH levels in the cytosol was done using the method of OPA conjugation as described by Tuffour *et al.* (2018). Briefly, a stock standard GSH (1 mg/mL) was prepared and 7 concentrations obtained by serial dilution (0.125, 0.063, 0.031, 0.016, 0.008, 0.004, 0.002 mg/mL) to plot a standard curve (Appendix V). A volume of 50 µL of cytosol or GSH standard was aliquoted into a 96 well plate in triplicate. A blank of no cytosol was also set up in triplicate. A volume of 50 µL of 0.1M sodium phosphate buffer (pH 8.0) was added to each well followed by an additional 10 µL of 10 mg/mL ortho-phthalaldehyde. The plate was incubated for 15 min in the dark at room temperature after which fluorescence measurements were carried out at 340 nm (excitation) and 460 nm (emission) using a spectrophotometer (Tecan Infinite M200 Pro). The amount of GSH in the cytosol was calculated from the equation of the standard curve (Appendix V).

3.9.2 SOD Activity

SOD activity was assessed using the method as described by Marklund and Marklund (1974). An aliquot of 20 µL cytosol (10 mg/mL) was placed into a 96 well plate in triplicate. Blanks containing no cytosol were also set up in triplicate. A volume of 200 µL 75 mM Tris-HCl buffer (pH 8.2) containing 30 mM EDTA was added to each well. In addition, 30 µL of 2mM pyrogallol was added. Absorbance readings were carried out at time intervals of 0 and 5 min at the wavelength of 420 nm. The activity of SOD was expressed as percent inhibition of pyrogallol autoxidation.

3.9.3 Catalase Activity

Catalase activity was measured using the method as described by Sinha (1972). Briefly, aliquots of 50 µL cytosol (10 mg/mL) were put into 1.5 mL Eppendorf tubes. A sample blank was also set up with no cytosol. In addition 500 µL of freshly prepared 65 mM hydrogen peroxide in 50 mmol/L sodium potassium phosphate buffer (pH 7.4) was added to the samples. Negative controls were set up with no hydrogen peroxide. The mixtures were vortexed and incubated for 3 min at 37°C. A volume of 1 mL dichromate/ acetic acid was added to the mixtures and the tubes re-incubated at 100°C for 10 min. After cooling with water, the tubes were centrifuged at 2500 rpm for 5 min to remove precipitated protein. The changes in absorbance values were measured at the wavelength of 570 nm against the reagent blank.

3.9.4 Lipid Peroxidation

Lipid peroxidation was assessed using the method described by Okhawa *et al.* (1979). A volume of 20 µL cytosol (10 mg/mL) was aliquoted into Eppendorf tubes. To the cytosol, 20 µL of 8.1% sodium dodecyl sulphate was added. This was followed by the addition of 150 µL 20% acetic acid. Also, 150 µL of 8% TBA was added after which the mixture was topped up with 60 µL of distilled water. The tubes were then incubated in a water bath at 95°C for 60 min. After which the incubated tubes were allowed to cool to room temperature and the resulting mixture topped up with 100 µL of distilled water. A volume of 150 µL 1:15 pyridine: butanol mixture was finally added and the tubes were vortexed for the thorough mixing of the contents for a minute. The resultant mixture was centrifuged at 3000 rpm for 10 min. Aliquots of the supernatant were put into 96 well plates and the

absorbance read at the wavelength of 532nm using a spectrophotometer (Tecan Infinite M200 Pro). All experiments were done in triplicate.

3.10 STATISTICAL ANALYSIS

All values were stated as mean ± standard error of the mean (SEM). Group differences were tested for significance by means of a one-way analysis of variance (ANOVA) followed by post hoc analysis using the Tukey's multiple comparison test. *P*-values < 0.05 were considered to be statistically significant. All graphs and analyses were done using Microsoft Excel 2013 and GraphPad prism software version 5.01.

CHAPTER FOUR

4.0 RESULTS

4.1 CHEMICAL FINGERPRINTING

The HPLC chromatogram of Swedish bitters is shown in Figure 4.1. A total of 13 peaks were identified. The various peaks obtained are indicated in Table 4.1. Peaks 2, 6, 7, and 8 showed the largest areas under the curve and had values of 1.99083e4, 1.25567e4, 2.13501e4, and 3.29723e4, respectively.

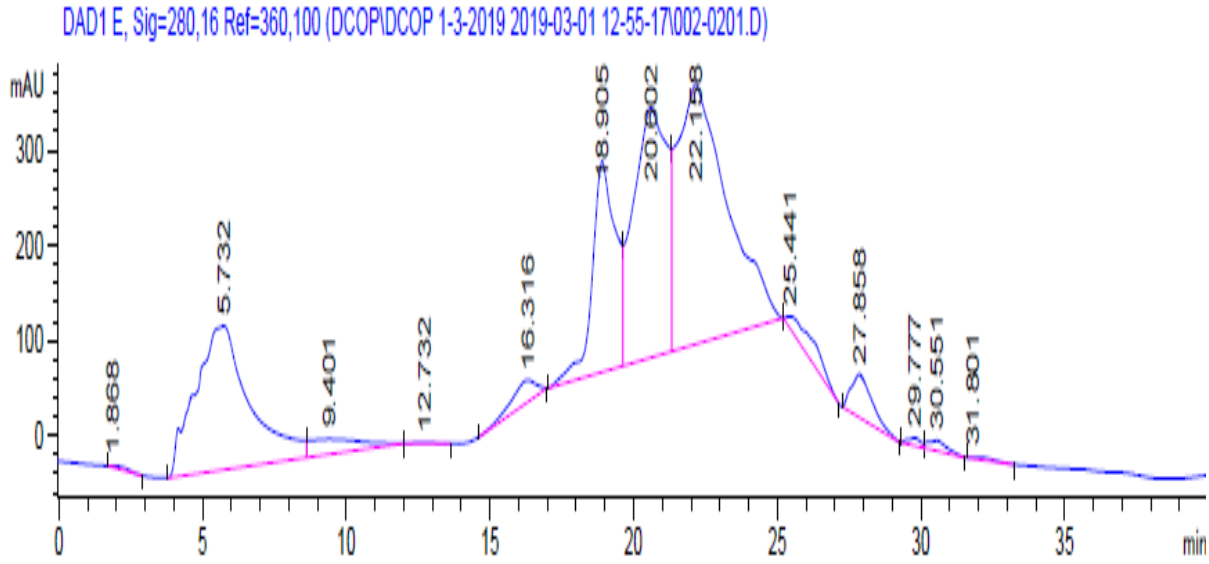

Figure 4.1. Chromatographic fingerprint of Swedish Bitters

Table 4.1. Peak retention times and area

Peak	Retention time (min)	Area (mAU)
1	1.868	111.33876
2	5.732	1.99083e4
3	9.401	1994.87793
4	12.732	55.48267
5	16.316	1451.75110
6	18.905	1.25567e4
7	20.602	2.13501e4
8	22.158	3.29723e4
9	25.441	1686.14624
10	27.858	2338.13452
11	29.777	257.78955
12	30.551	504.33902
13	31.801	109.43531

4.2 PROTEIN CONTENT OF MICROSOMES

Using the Bradford method, the microsomal protein content for each sample was calculated from the BSA standard curve (Appendix IV). The protein content ranged between 6 and 107 mg/mL. From this result, working concentrations of 1 mg/mL were prepared for the enzyme assays.

4.3 CYP ENZYME ASSAYS

4.3.1 CYP1A1/1A2 Activity

Effect of Swedish bitters on CYP1A1/1A2 is presented in Figure 4.2. The results showed that there was a statistically significant difference between the negative control (NC) and the positive control (PC) groups ($p < 0.05$). However, no significant difference in activities was identified between the NC and the low, medium, and high dose Swedish bitters (LD, MD, and HD).

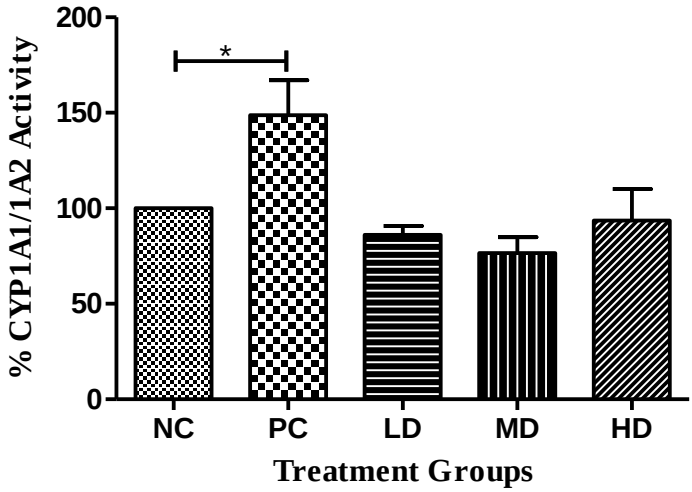

Figure 4.2. Effect of Swedish bitters on CYP1A1/1A2 activity in rat liver microsomes

Negative control (NC; distilled water), positive control (PC; phenobarbital 15mg/kg), low dose treatment (LD; 5mL/kg/day Swedish bitters), medium dose treatment (MD; 10mL/kg/day Swedish bitters), and high dose treatment (HD; 20mL/kg/day Swedish bitters). Data represents means and standard errors of the mean for triplicate experiments. ∗ represents values statistically different compared to the negative control as indicated with $p < 0.05$. One-way ANOVA followed by Tukey`s multiple comparison test.

4.3.2 CYP1A2 Activity

For CYP1A2, there was no significant difference between the untreated (NC) and the Swedish bitters treatment groups as shown in Figure 4.3. However, there was significant difference between the PC and the NC group with $p < 0.05$.

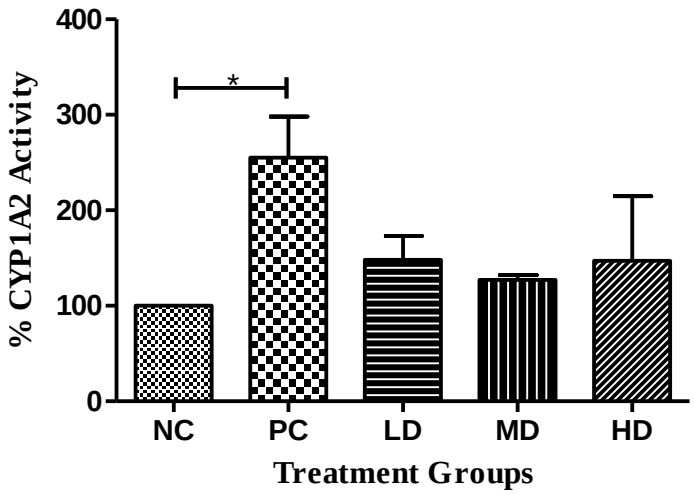

Figure 4.3. Effect of Swedish bitters on CYP1A2 activity in rat liver microsomes.

Negative control (NC; distilled water), positive control (PC; phenobarbital 15mg/kg), low dose treatment (LD; 5mL/kg/day Swedish bitters), medium dose treatment (MD; 10mL/kg/day Swedish bitters), and high dose treatment (HD; 20mL/kg/day Swedish bitters). Data represents means and standard errors of the mean for triplicate experiments. ∗ represents values statistically different compared to the control experiments as indicated with $p < 0.05$. One-way ANOVA followed by Tukey`s multiple comparison test.

4.3.3 CYP2B1/2B2 Activity

The activity of CYP2B1/2B2 is shown in Figure 4.4. There was a significant difference between the NC and the PC groups ($p < 0.001$), no significant difference was found between the NC and the Swedish bitters treatment groups.

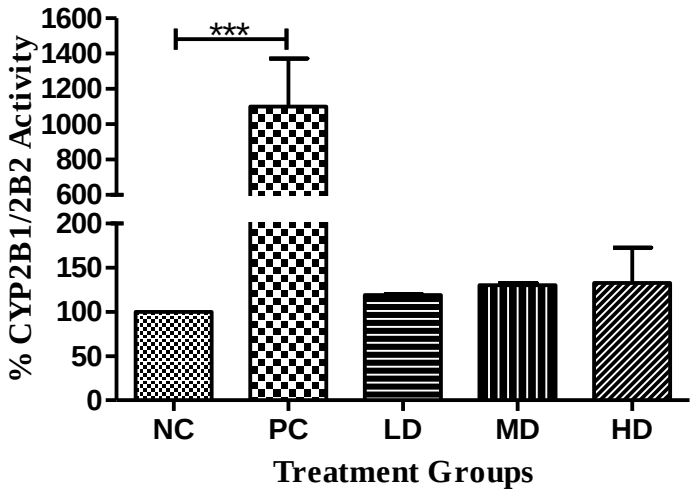

Figure 4.4. Effect of Swedish bitters on CYP2B1/2B2 activity in rat liver microsomes.

*Negative control (NC; distilled water), positive control (PC; phenobarbital 15mg/kg), low dose treatment (LD; 5mL/kg/day Swedish bitters), medium dose treatment (MD; 10mL/kg/day Swedish bitters), and high dose treatment (HD; 20mL/kg/day Swedish bitters). Data represents means and standard errors of the mean for triplicate experiments. *** represents statistical difference compared to the control experiments as indicated with $p < 0.001$. One-way ANOVA followed by Tukey`s multiple comparison test.*

4.3.4 CYP3A4 Activity

For CYP3A4 activity, no significant difference was found between the NC and treatment groups (Figure 4.5). However, there was significant difference between the NC and the PC at $p < 0.001$.

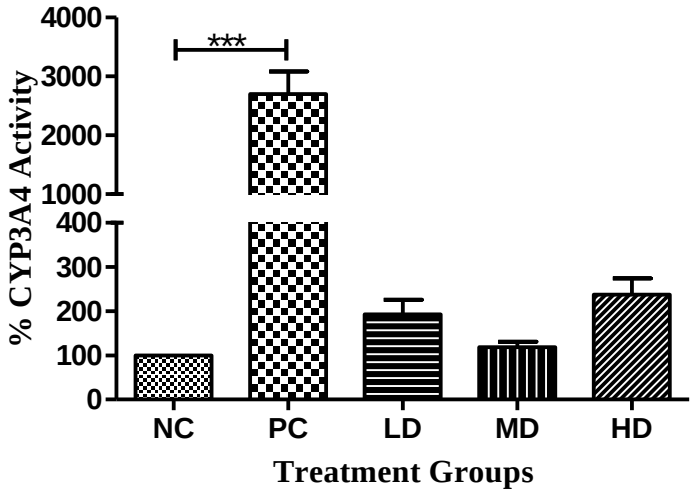

Figure 4.5. Effect of Swedish bitters on CYP3A4 activity in rat liver microsomes.

*Negative control (NC; distilled water), positive control (PC; phenobarbital 15mg/kg), low dose treatment (LD; 5mL/kg/day Swedish bitters), medium dose treatment (MD; 10mL/kg/day Swedish bitters), and high dose treatment (HD; 20mL/kg/day Swedish bitters). Data represents means and standard errors of the mean for triplicate experiments. *** represent values statistically different compared to the control experiments as indicated with $p < 0.001$. One-way ANOVA followed by Tukey`s multiple comparison test.*

4.3.5 CYP2C9 Activity

The effect of Swedish bitters on CYP2C9 activity is as shown in Figure 4.6. There was a significant ($p < 0.001$) increase in activity of CYP2C9, over 100%, for each of the given treatments compared to the NC.

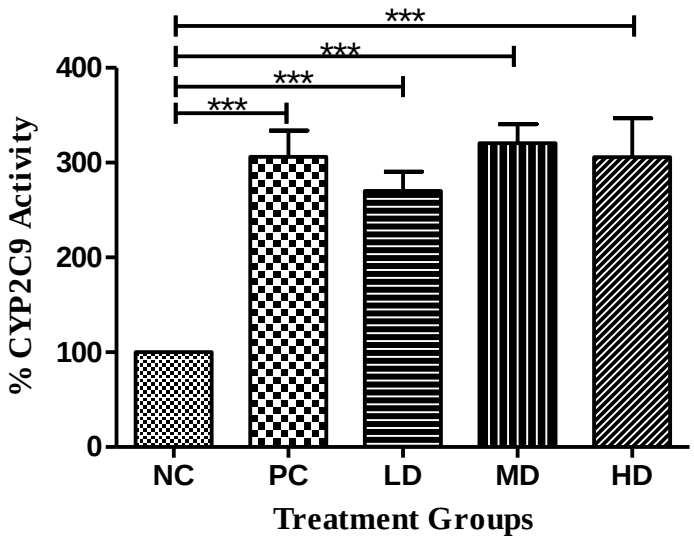

Figure 4.6. Effect of Swedish bitters on CYP2C9 activity in rat liver microsomes.

*Negative control (NC; distilled water), positive control (PC; phenobarbital 15mg/kg), low dose treatment (LD; 5mL/kg/day Swedish bitters), medium dose treatment (MD; 10mL/kg/day Swedish bitters), and high dose treatment (HD; 20mL/kg/day Swedish bitters). Data represent mean and standard error of the mean for duplicate experiments. *** represent values statistically different compared to the control experiments as indicated with $p < 0.001$. One-way ANOVA followed by Tukey`s multiple comparison test.*

4.3.6 CYP2D6 Activity

The effect of Swedish bitters on CYP2D6 activity is shown in Figures 4.7. The activity of CYP2D6 was increased for all the doses of Swedish bitters administered to SD rats, but these increases were not found to be statistically significant.

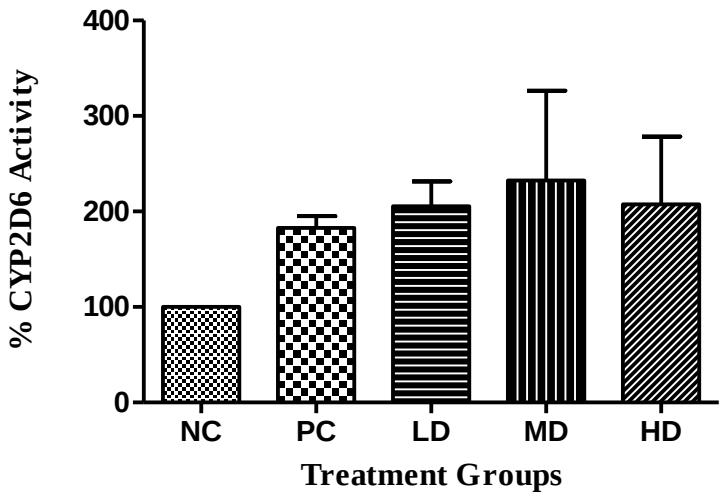

Figure 4.7. Effect of Swedish bitters on CYP2D6 activity in rat liver microsomes.

Negative control (NC; distilled water), positive control (PC; phenobarbital 15mg/kg), low dose treatment (LD; 5mL/kg/day Swedish bitters), medium dose treatment (MD; 10mL/kg/day Swedish bitters), and high dose treatment (HD; 20mL/kg/day Swedish bitters). Data represents means and standard errors of the mean for duplicate experiments.

4.3.7 Overall Effect of Swedish bitters on Rat CYP Enzyme Activity

For the Swedish bitters treated groups, the activities of CYP1A1/2, CYP3A4, and CYP2B1/2 did not differ significantly compared to the negative control group. Although there was an increase in enzyme activity for CYP2D6, it was also not statistically significant. However, CYP2C9 enzyme activity in rats treated with Swedish bitters was found to be significantly increased compared to the negative control. The overall effect of Swedish bitters on selected rat CYP enzymes is shown in Table 4.2.

Table 4.2. Summary of the effect of Swedish bitters on selected rat CYP enzyme activity

CYP ISOFORM	ASSAY	EFFECT OF SWEDISH BITTERS ON CYP ACTIVITY
CYP1A1/2	MROD	No significant effect
CYP1A2	EROD	No significant effect
CYP2B1/2	PROD	No significant effect
CYP3A4	BROD	No significant effect
CYP2C9	Diclofenac Hydroxylation	Significant increase in enzyme activity (LD/MD/HD: $p < 0.001$)
CYP2D6	Dextromethorphan O-Demethylation	No significant effect

4.4 BIOCHEMICAL AND HEMATOLOGICAL PARAMETERS

The results for the hematological and biochemical analysis are shown in Table 4.3 and Table 4.4, respectively. Generally, there were no significant differences between the negative control and treatment groups (untreated and Swedish bitters administered SD rats). However, high dose Swedish bitters showed a significant decrease (8.44%) in monocytes and ALP levels (279.75U/L) compared with untreated group (21.43% and 532.41U/L, respectively).

Table 4.3. Effect of Swedish bitters on rat hematological parameters

Parameter	Negative Control (Distilled water)	Positive Control (Phenobarb)	Low dose (5 mL/kg b.w.)	Medium dose (10 mL/kg b.w.)	High dose (20 mL/kg b.w.)
WBC ($x10^9$/L)	6.47 (0.45)	8.78 (1.32)	8.83 (0.48)	7.98 (2.39)	6.46 (1.82)
LYM (%)	31.91 (5.61)	47.18 (0.86)	37.86 (9.51)	17.71 (3.64)	26.81 (4.52)
MON (%)	21.43 (2.67)	5.03 (0.88)**	12.09 (3.10)	6.95 (1.35)*	8.44 (1.37)*
NEU (%)	51.63 (3.35)	47.95 (7.12)	46.57 (10.37)	68.44 (6.06)	61.99 (2.27)
EOS (%)	3.97 (0.51)	4.14 (0.78)	3.28 (0.34)	2.19 (0.66)	2.48 (0.74)
BASO (%)	0.22 (0.10)	0.40 (0.13)	0.21 (0.10)	0.36 (0.14)	0.30 (0.15)
LYM ($x10^9$/L)	0.41 (0.10)	0.88 (0.21)	0.60 (0.23)	0.23 (0.03)	0.30 (0.10)
MON ($x10^9$/L)	0.17 (0.06)	0.15 (0.05)	0.17 (0.07)	0.14 (0.03)	0.09 (0.00)
NEU ($x10^9$/L)	0.47 (0.09)	1.01 (0.20)	0.54 (0.10)	0.99 (0.34)	0.68 (0.08)
EOS ($x10^9$/L)	0.04 (0.01)	0.06 (0.01)	0.04 (0.01)	0.03 (0.01)	0.03 (0.00)
BASO ($x10^9$/L)	0.002 (0.001)	0.009 (0.004)	0.002 (0.001)	0.006 (0.004)	0.003 (0.001)
RBC ($x10^{12}$/L)	9.16 (0.49)	8.50 (0.23)	9.88 (0.26)	9.36 (0.14)	9.69 (0.49)
HGB (g/dL)	15.48 (0.63)	14.50 (0.51)	15.83 (0.31)	15.40 (0.25)	16.75 (1.05)
HCT (%)	46.40 (2.95)	42.28 (1.45)	47.90 (0.84)	45.93 (0.48)	50.15 (3.65)
MCV (fL)	50.66 (0.67)	49.80 (0.77)	48.63 (0.85)	49.17 (0.20)	51.75 (1.15)
MCH (pg)	16.90 (0.19)	17.02 (0.24)	16.00 (0.60)	16.40 (0.30)	17.20 (0.20)
RDW (%)	13.06 (0.99)	13.14 (0.46)	14.78 (1.37)	13.97 (1.48)	14.10 (0.30)
RDW_SD (fL)	48.02 (1.97)	48.72 (1.93)	50.55 (3.06)	51.30 (5.08)	52.90 (0.00)
PLT ($x10^9$/L)	1208.00 (162.30)	1250.40 (107.75)	1239.00 (94.62)	1462.33 (184.33)	1447.50 (117.50)
MPV (fL)	5.30 (0.56)	4.60 (0.26)	4.38 (0.38)	4.60 (0.10)	4.35 (0.15)
PDW (fL)	7.43 (0.63)	7.08 (0.45)	6.80 (0.60)	7.67 (0.23)	7.20 (0.00)
PCT (%)	0.62 (0.82)	0.57 (0.06)	0.55 (0.09)	0.67 (0.07)	0.62 (0.03)

Abbreviations: WBC, white blood cells; LYM, lymphocytes; MON, monocytes; NEU, neutrophils; EOS, eosinophils; BASO, basophils; RBC, red blood cells; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; RDW, red blood cell distribution; RDW_SD, RDW expressed as a standard deviation; PLT, platelets; MPV, mean platelet volume; PDW, platelet distribution width; PCT, platecrit.

*Values are group means. Numbers in bracket ( ) represent SEM. * represents statistically significant difference from negative control: ** P < 0.001; * P < 0.05 (One-way ANOVA followed by Tukey`s multiple comparison test).*

Table 4.4. Effect of Swedish bitters on rat serum biochemical parameters

Parameter	Control (Distilled water)	Positive Control (Phenobarb)	Low dose (5 mL/kg b.w.)	Medium dose (10 mL/kg b.w.)	High dose (20 mL/kg b.w.)
TBil (umol/L)	2.22(0.12)	1.67(0.32)	2.25(0.30)	2.03(0.23)	2.80(0.20)
DBil (umol/L)	0.86(0.08)	0.80(0.20)	0.93(0.22)	0.97(0.12)	0.65(0.35)
ALT (U/L)	70.67(4.66)	110.78(20.08)	97.81(9.45)	54.31(4.62)	67.90(3.11)
AST (U/L)	439.37(37.84)	351.71(74.50)	397.52(32.13)	401.81(31.43)	355.40(7.87)
ALP (U/L)	532.41(40.83)	284.52(69.25)*	420.75(51.08)	471.17(42.13)	279.75(8.17)*
GGT (U/L)	5.50(0.65)	5.00(0.58)	6.50(0.65)	6.50(2.50)	5.00(1.00)
TP (g/L)	66.23(1.12)	66.27(0.26)	68.25(2.13)	68.23(3.15)	65.17(1.26)
ALB (g/L)	31.00(0.94)	31.03(0.44)	29.80(1.32)	30.07(0.84)	30.03(0.68)
GLB (g/L)	35.83(0.75)	35.23(0.67)	38.45(0.97)	38.17(2.31)	35.13(0.83)
A/G	0.85(0.03)	0.88(0.03)	0.78(0.02)	0.79(0.03)	0.85(0.02)

Abbreviations: TBil, total bilirubin; DBil, direct bilirubin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, gamma glutamyl transferase; TP, total protein; ALB, albumin; GLB, globulin; A/G, albumin/globulin ratio.

*Values are group means. Numbers in bracket ( ) represent SEM. * represents statistically significant difference from negative control: * P < 0.05 (One-way ANOVA followed by Tukey`s multiple comparison test).*

4.5 ANTIOXIDANT ASSAYS OF CYTOSOL FROM HOMOGENIZED LIVERS OF SD RATS

4.5.1 GSH content

The levels of GSH are presented in Figure 4.8. The treatment groups showed slight decrease in GSH levels (0.08 mg/mL for untreated, and approximately 0.05 mg/mL for treated groups) however, these decreases were not statistically significant.

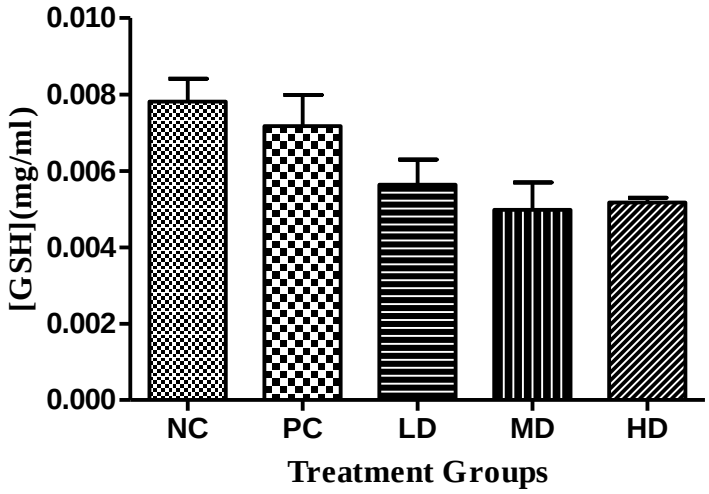

Figure 4.8. GSH levels in Swedish bitters treated rat groups compared with untreated.

Negative control (NC; distilled water), positive control (PC; phenobarbital 15mg/kg), low dose treatment (LD; 5mL/kg/day Swedish bitters), medium dose treatment (MD; 10mL/kg/day Swedish bitters), and high dose treatment (HD; 20mL/kg/day Swedish bitters). Data represent means and standard errors of the mean for triplicate experiments.

4.5.2 SOD Activity

The percentage SOD activity is shown in Figure 4.9. The positive control exhibited a significantly high SOD activity (60% more activity) compared to the negative control (untreated group). There was a dose dependent increment in SOD activity in SD rats administered Swedish bitters. This increase in SOD activity among Swedish bitters-treated groups were not statistically significant compared to negative control.

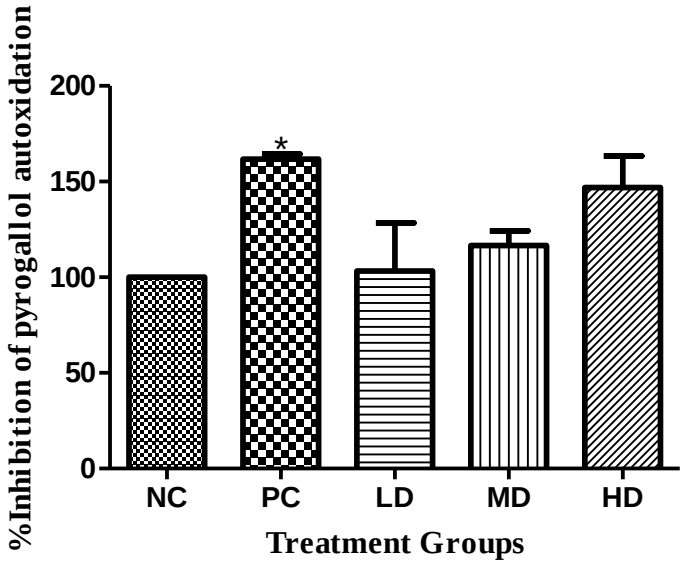

Figure 4.9. SOD activity in Swedish bitters treated groups compared with untreated.

Negative control (NC; distilled water), positive control (PC; phenobarbital 15mg/kg), low dose treatment (LD; 5mL/kg/day Swedish bitters), medium dose treatment (MD; 10mL/kg/day Swedish bitters), and high dose treatment (HD; 20mL/kg/day Swedish bitters). Data represent means and standard errors of the mean for triplicate experiments. ∗ are values statistically different compared to the negative control as indicated with $p < 0.05$. One-way ANOVA followed by Tukey`s multiple comparison test.

4.5.3 Catalase Activity

Effect of Swedish bitters on catalase activity is shown in Figure 4.10. There was a dose dependent decrease in catalase activity, with the high dose treatment group statistically significant ($p < 0.05$) compared to untreated group. Catalase activity decreased from about 26kU (untreated) to about 12kU (high dose Swedish bitters treatment group).

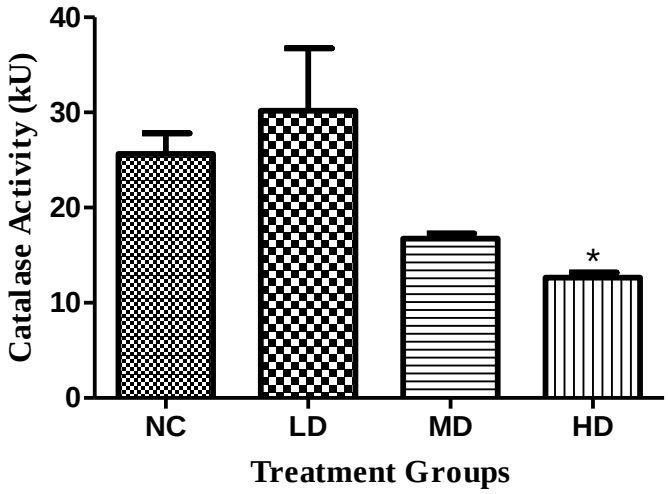

Figure 4.10. Catalase activity of Swedish bitters treatment groups compared with untreated.

Negative control (NC; distilled water), low dose treatment (LD; 5mL/kg/day Swedish bitters), medium dose treatment (MD; 10mL/kg/day Swedish bitters), and high dose treatment (HD; 20mL/kg/day Swedish bitters). Data represent means and standard errors of the mean for triplicate experiments. ∗ are values statistically different compared to the negative control as indicated with $p < 0.05$. One-way ANOVA followed by Tukey`s multiple comparison test

4.5.4 Lipid Peroxidation

Effect of Swedish bitters on lipid peroxidation is shown in Figure 4.11. Thiobarbituric acid reactive substances (TBARS) was measured as a by-product of lipid peroxidation, and was expressed as moles/mg protein. The present results indicated no significant differences between the untreated and Swedish bitters treatment groups (with values between 350 and 400 n moles/mg protein).

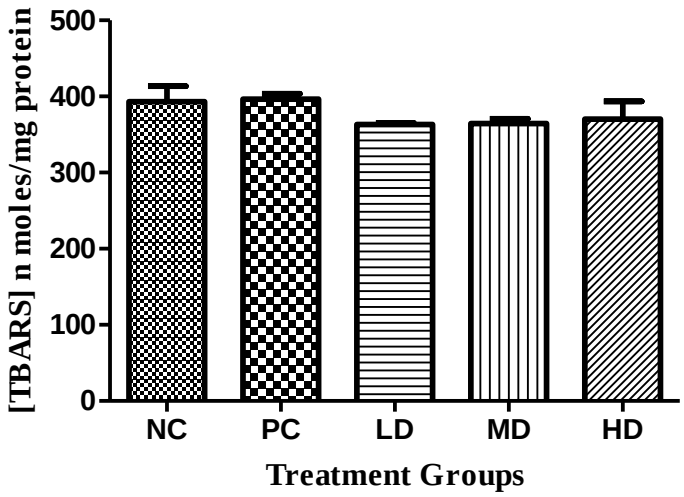

Figure 4.11. Lipid peroxidation of Swedish bitters treatment groups compared with untreated

Negative control (NC; distilled water), positive control (PC; 15mg/kg phenobarbital), low dose treatment (LD; 5mL/kg/day Swedish bitters), medium dose treatment (MD; 10mL/kg/day Swedish bitters), and high dose treatment (HD; 20mL/kg/day Swedish bitters). Data represent means and standard errors of the mean (SEM) for triplicate experiments.

CHAPTER FIVE

5.0 DISCUSSION

The safety of herbal products has turned out to be a major worry in public health as their acceptance and global market growth continues to rise (Kosalec *et al*., 2009). Herbal medicines, single or poly herbal formulations, contain a number of different compounds wherein no particular active component is accountable for the total efficacy. Quality control and quality assurance of herbal drugs remain a challenge for the reason that a high variability of chemical components are involved (Mohammad *et al*., 2010). Chromatographic fingerprinting is often used in the quality assessment of herbal preparations (Gunalan *et al*., 2012), and WHO has accepted fingerprint analysis as a suitable measure for the quality control of herbal preparations (Cieśla, 2012). A herbal sample fingerprint is defined as "a set of characteristic chromatographic or spectroscopic signals, whose comparison leads to an unambiguous sample recognition" (Cieśla, 2012). Some chromatographic methods for fingerprinting include gas chromatography (GC), thin layer chromatography (TLC), as well as high performance liquid chromatography (HPLC) which was utilized in this study. The HPLC chromatogram of Swedish bitters in this study recorded 13 peaks at the wavelength of 280 nm. The HPLC fingerprint of saffron, a constituent of Swedish bitters, has been established (Ahnfelt and Fors, 2016). However, no HPLC chromatogram of the bitters exists in literature. This chromatogram can therefore serve as a reference pattern for Swedish bitters. Generally, samples with comparable chromatographic fingerprints may show some similar properties (Fan *et al*., 2006).

Cytochrome P450 enzymes are key Phase I drug metabolizing enzymes that catalyze biotransformation of lipophilic xenobiotics into polar forms that can be easily excreted via

the kidneys. Modulation in the activities of these enzymes, particularly via drug/herb interactions may consequently affect levels of conventional drugs in circulation (Doligalski *et al*., 2012). A typical example of a drug/herb interaction is what is observed between grapefruit juice and drugs such as midazolam, cyclosporine, terfenidine and calcium channel blockers metabolized by CYP3A4. The grapefruit inhibits CYP3A4 enzyme activity leading to the increase in bioavailability of these drugs. In the present study, the activities of six CYPs including CYP1A1/1A2, CYP1A2, CYP2B1/2B2 and CYP3A4 were determined after a 7-day treatment with Swedish bitters. From the results obtained, there were no significant differences between the activities of rat CYP1A1/1A2 and CYP1A2 when compared to the negative control groups; suggesting that Swedish bitters did not modulate these enzymes over the treatment period. In addition, CYP2B1/2B2 and CYP3A4 activity in rats administered Swedish bitters did not show an increase in enzyme activity compared to the negative control, however, these were not statistically significant when compared to the negative control group. There have been reports of some of the constituents of Swedish bitters having modulatory effect on CYP enzymes. For example, *Aloe vera* juice has been found to have inhibitory effect against CYP3A4 and CYP2D6 (Djuv and Nilsen, 2012). Zedoary turmeric oil also has also been shown to inhibit CYP2C9 and CYP2D6 (Cheng *et al*., 2014). In addition, safranal, an active component of saffrom, has been shown to increase the activity of CYP2B, CYP3A and CYP2C11 enzymes (Dovrtelova *et al*., 2015). In the current study however, Swedish bitters was found to increase the activities of CYP2C9 and CYP2D6, with activity of CYP2C9 being significantly different compared to the untreated. So far CYP2D6 is known to be a non-inducible enzyme, therefore it is possible that Swedish bitters enhanced the activity of the

enzyme. Since protein expressions of these enzymes were not assessed, it cannot be confirmed that CYP induction occurred due to the treatment with Swedish bitters. Studies on some constituents of Swedish bitters have shown modulatory activities however no reports have been made on the combined effect the components of Swedish bitters have on CYP enzymes in rats. The increased activity of CYP2C9 by Swedish Bitters may cause herb-drug interactions involving this enzyme. Drugs that are metabolized by this enzyme may be metabolized faster in the presence of Swedish bitters, and this may lead to sub-therapeutic effect of drugs. From these results, administration of Swedish bitters especially over long periods, may alter the pharmacokinetics of xenobiotics that are metabolized by CYP2C9. Furthermore, results from this study serves as a basis for human studies to ascertain effect of Swedish bitters on concomitantly administered conventional drugs (drug-herb interaction).

The liver plays a vital role in storage, secretion, and metabolism, and is a major detoxification organ. Therefore, any injury or pathological condition to the liver (liver cirrhosis and hepatic failure) can be life threatening (Girish *et al*., 2009). Some common implicated agents of liver injuries include therapeutic drugs (e.g., antibiotics and anti-tubercular drugs), toxic compounds (e.g., aflatoxin and CCl_4), alcohol, and parasites (e.g., leptospira, hepatitis virus and malarial parasites) (Subramoniam and Pushpangadan, 1999). In the current study, biochemical and hematological investigation on blood samples were performed to evaluate the toxicity of Swedish bitters on the liver. From the results, there appeared to be no significantly difference between the biochemical and hematological parameters of the Swedish bitters-treated groups and the negative control group (untreated). However, the high dose Swedish bitters treated group had a significant

decrease in ALP as well as monocyte levels. Previous reports have indicated that Swedish bitters decreased total protein level and elevated white blood cell count (Ekor *et al*., 2010). In addition, Ekor *et al.* (2010) also showed that there was increase in alanine and aspartate aminotransferase activities and significant decrease in total protein. Awa and James (2013) also reported an increase in the level of total proteins when Swedish bitters was administered to rats. Contrary to the previous reports, findings from this study indicate that Swedish bitters decreased the monocyte count, although other white blood cell counts were not affected. Also, alanine and aspartate aminotransferases were not altered significantly, however, ALP levels were significantly lowered in Swedish bitters treated groups compared to the untreated group. In the study carried out by Ekor *et al.* (2010), administration of the bitters was done for a period of 30 days, however, in the present study treatment was done for 7 days. The duration of administration may have had a role in the difference in hematological and biochemical levels as stipulated by the principles of toxicity. In the current study, high dose Swedish bitters decreased ALP and monocytes in treatment rats after 7 days. It may, however, be prudent to ascertain this observed effect over a longer period (30 days).

Glutathione is a major antioxidant of the body, which protects cells from oxidative stress/damage. Glutathione works as a scavenger of radicals, directly or indirectly using GSH-dependent enzymes (Kanďár *et al*., 2014). A decrease in GSH levels below normal may cause oxidative stress leading to cell damage. The concentration of GSH present in the cytosol of cells is usually in region of 1–10 mM (Meister, 1988). The GSH concentration in many cells is found to be 1–2 mM, and in hepatocytes, which transports GSH, the concentration can be as high as 10 mM (3.0733 mg/mL) (Forman *et al*., 2009).

This study showed GSH concentrations in the range of 0.0049 mg/mL to 0.00789 mg/mL across treatment groups. There was a decrease in GSH concentrations for Swedish bitters treatment groups although not statistically significant compared to the negative control group. A study carried out by Awa and James (2013) showed that there was a slight reduction in the level of GSH in rats administered with Swedish bitters over a period of three weeks compared to the control, which was also shown in this study. Given that the rats were treated for only 7 days, it is not entirely clear what will happen to GSH levels after prolonged intake of the bitters.

The SOD enzyme protects living cells against superoxide radicals. (Semsei and Nagy, 1984). SOD is responsible for the breakdown of the harmful superoxide radical ($O_2^{\bullet-}$) into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) (Spanou *et al*., 2011). The SOD activity was reported as the percentage inhibition of pyrogallol autoxidation. The enzyme inhibits almost entirely the pyrogallol autoxidation by competing effectively for the superoxide radicals, as a result, SOD activity is measured by the rate of inhibition of pyrogallol autoxidation. There was dose dependent increment in SOD activity in the Swedish bitters treatment groups compared to the untreated group. A study carried out by Awa and James (2013) showed that Swedish bitters reduced the levels of SOD in the test group compared to the negative control group. In this study, there was dose dependent increase in SOD activity. However, this increment was not statistically significant and so may not be clinically significant.

Catalase enzyme is responsible for the decomposition of harmful H_2O_2 into water (H_2O) and molecular oxygen (O_2) (Spanou *et al*., 2011). A study by Alabi *et al.* (2013) showed significant increase in catalase activity after treatment with Swedish bitters over a period

of 32 days. Contrary to this finding, the study carried out by Awa and James (2013) showed that there was a decrease in the levels of catalase enzyme in the Swedish bitters treatment group compared to the control. Results from the present study revealed a dose dependent decrease in catalase activity compared to the untreated group. These findings are in line with findings from studies conducted by Awa and James (2013) where treatment was for 3 weeks. From the current study, it can be postulated that administration of Swedish bitters over a long period may cause an accumulation of harmful H_2O_2, and this may lead to oxidative stress.

The oxidation of lipids leads to the production of products which further propagates free radical reactions (Shetty *et al*., 2006). A study by Alabi *et al.* (2013) showed a significant decrease in lipid peroxidation in Swedish bitters treated rats. Another study by Awa and James (2013) indicated that the level of malondialdehyde (MDA) in their test group (Swedish bitters treated rats) was elevated when compared to the control, suggesting elevated levels of lipid peroxidation. These results were however, not statistically significant. Similarly, in this study, there was no significant difference found between the untreated and the Swedish bitters treatment groups of rats indicating that Swedish bitters did not affect lipid peroxidation. These findings differ from studies conducted by Alabi *et al.* (2013) as well as Awa and James (2013) as described previously. The differences may be due to the differences in strain of rats used as well as duration of administration. Alabi *et al.* used albino rats for a period of 32 days whilst Awa and James used albino rats for a period of 3 weeks. In addition, there are several brands of the bitters with possible differences in their constituents. Even though these studies did not state the brand of

Swedish bitters used, they were sourced from different locations, which could account for the differences observed.

CHAPTER SIX

6.0 CONCLUSION, LIMITATIONS AND RECOMMENDATION

This study showed that Swedish bitters increased the activities of rat liver CYP2B1/2B2, CYP3A4, CYP2C9 and CYP2D6 however, the increase in activity was found to be significant for CYP2C9 compared to the negative control. No significant changes were found in rat CYP1A1/1A2, and 1A2 activities after treatment with the bitters. The bitters did not cause any significant changes in GSH level and lipid peroxidation activity. There was a statistically significant decrease in catalase activity in the high dose treatment group, and an increase in SOD activity across treatment groups. Generally, the bitters did not alter hematology and liver function as assessed by full blood count and the biochemistries, except for monocytes and ALP levels which decreased significantly at high doses of Swedish bitters.

Limitations of the current study included; the lack of HPLC fingerprint of Swedish bitters from literature, making it difficult to have a comparison. In addition limited resources did not enable the acquisition of pure compounds for targeted analysis of the chemical constituents of the bitters. Animal studies cannot always be extrapolated to man and so some human recombinant CYPS could have been used.

It is recommended that Swedish bitters be administered to rats over an extended period of times and the effect of this duration on CYP enzymes assessed. Furthermore, hematology and biochemistry parameters ought to be assayed over this extended period of administration of Swedish bitters. Further research should also be conducted to ascertain the effect of Swedish bitters on human recombinant CYPs, Phase II drug metabolizing enzymes or studies with human subjects.

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APPENDICES

APPENDIX I

STC approval letter

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Post Office Box LG 581
Legon, Accra
GHANA

My Reference:

Your Reference:

5th April, 2019

Ms. Abigail Aning
Department of Clinical Pathology
NMIMR
Legon

Dear Madam,

APPROVAL OF PROTOCOL

The Scientific and Technical Committee of the Noguchi Memorial Institute at its meeting on 9th October, 2018, reviewed the protocol entitled: "Effect of Swedish Bitters on selected Rat Cytochrome P450 Enzyme Activity", STC Paper 3(2) 2018-19, which was submitted by Ms. Abigail Aning.

The Committee after the review approved the protocol on Friday, 5th April 2019, and recommended that the study should be carried out.

The Scientific and Technical Committee avails to you the assurances of its highest consideration.

Thank you.

Yours faithfully,

Professor Abraham Kwabena Anang, PhD
DIRECTOR

APPENDIX II

Ethical clearance from UG-IACUC

UNIVERSITY OF GHANA

University of Ghana Institutional Animal Care and Use Committee
(UG-IACUC)

Phone:
Email: UG-IACUC@ug.edu.gh

P.O. Box LG 581
Legon, Accra
Ghana

Office Location: Department of Animal Experimentation Building, Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana

13/05/2019

ETHICAL CLEARANCE
(UG-IACUC 004/18-19)

On February 13th, 2019 the University of Ghana – Institutional Animal Care and Use Committee (UG-IACUC) at a full committee meeting reviewed and approved your protocol as follows:

TITLE OF PROTOCOL: Effects of Swedish bitters on selected rat cytochrome P_{450} Enzyme activity

STUDENT INVESTIGATOR: Miss. Abigail Aning

Please note that the final review report must be submitted to the Committee at the completion of the study. Your research records may be audited at any time during or after the implementation.

Any modification of this research project must be submitted to UG-IACUC for review and approval prior to implementation.

Please report all serious adverse events related to this study to UG-IACUC within seven days verbally and in writing within fourteen days.

This certificate is valid till 13th May, 2020. You are to submit annual reports for continuing review.

Prof. Major (Rtd.) George A. Asare
Chairperson

APPENDIX III

Calculation of animal equivalent dose (AED) of Swedish bitters

Pharmacologically active dose (PAD) = 10 mL

PAD = 10 mL/day for an adult

Human dose = 10 mL/60 kg = 0.167 mL/kg/day

AED = PAD × K_m ratio = 0.167 mL/kg/day × 6.2 = 1.03 mL/kg/day

Multiply by safety factor (10)

AED = 1.03 mL/kg/day x10 = 10.3 mL/kg/day

Medium dose = 10 mL/kg/day

Low dose = (10 mL/kg/day) /2 = 5 mL/kg/day

High dose = (10 mL/kg/day) × 2 = 10 mL/kg/day

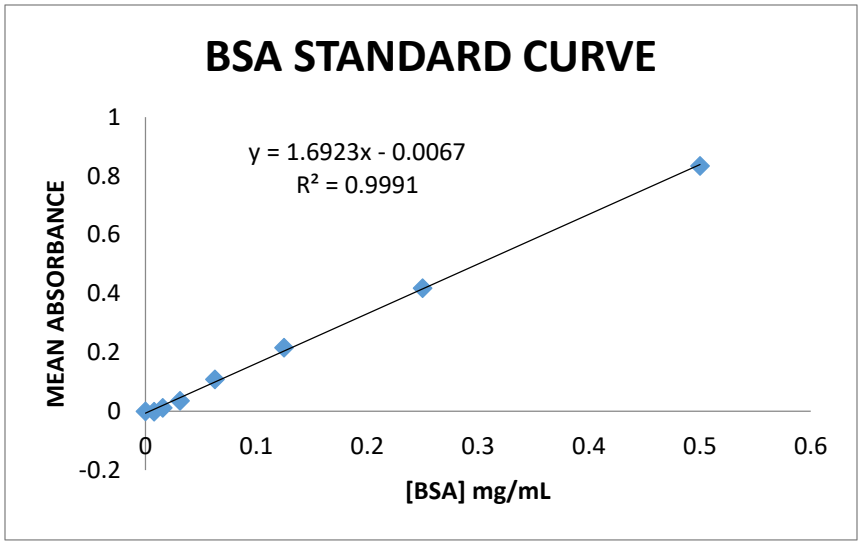
Km: Correction factor estimated by dividing body weight of species to its body surface area.

Km ratio = Human Km/Rat Km = 37/6

Reference: (Shin *et al.*, 2010)

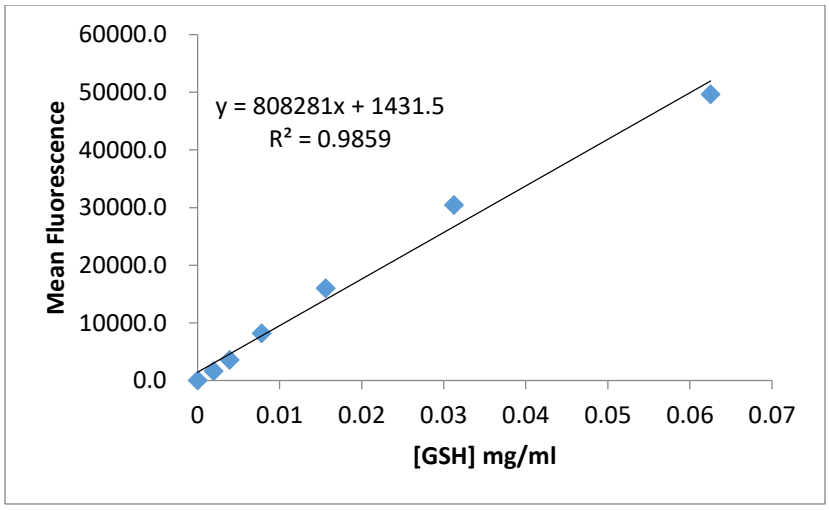
APPENDIX IV

Protein standard curve



APPENDIX V

GSH standard curve



APPENDIX VI

Representative HPLC results for CYP2C9 and CYP2D6

CYP2C9 ASSAY

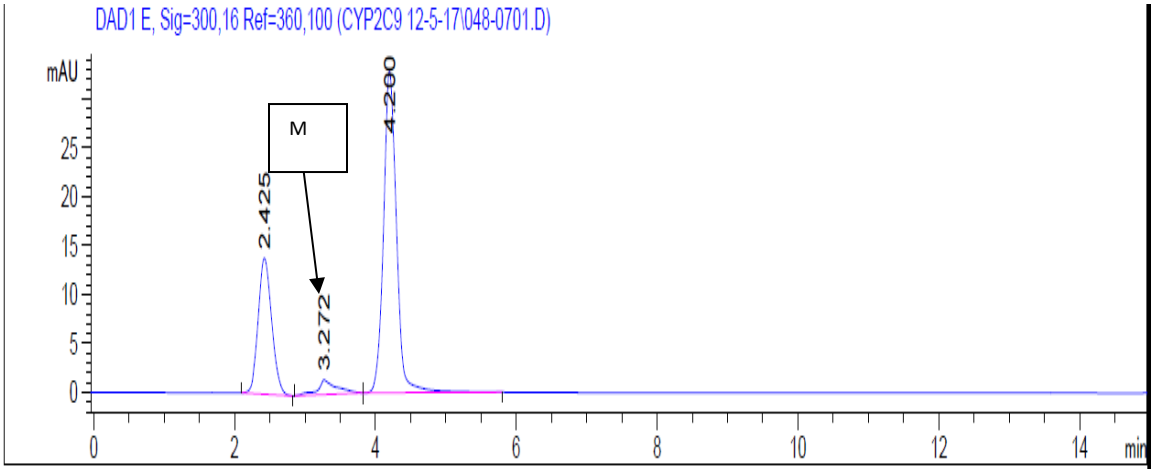
Flow rate = 1 mL/min

Injection Volume = 50 µL

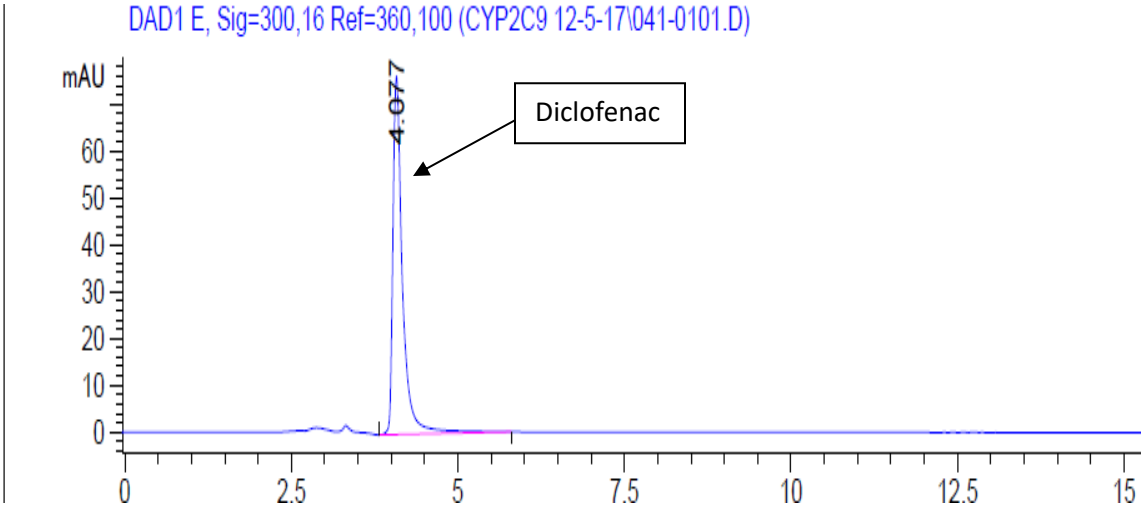
Wave length = 300 nm

Mobile Phase = 20mM Kpi (60%): Methanol (25.5%): Acetonitrile (14.5%)

Column TSKgel ODS 100v 5µm; 250mm x4.6mm



M= Metabolite (RT = 3.272 min)



CYP2D6

Flow rate = 0.7 mL/min

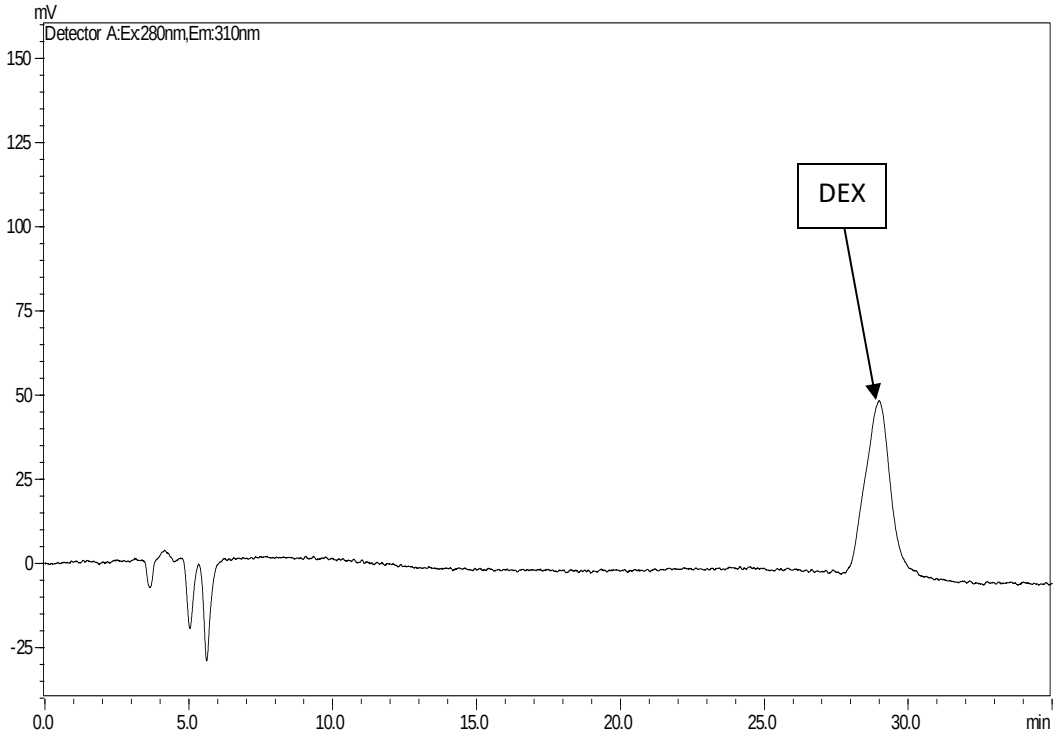
Injection Volume = 20 µL

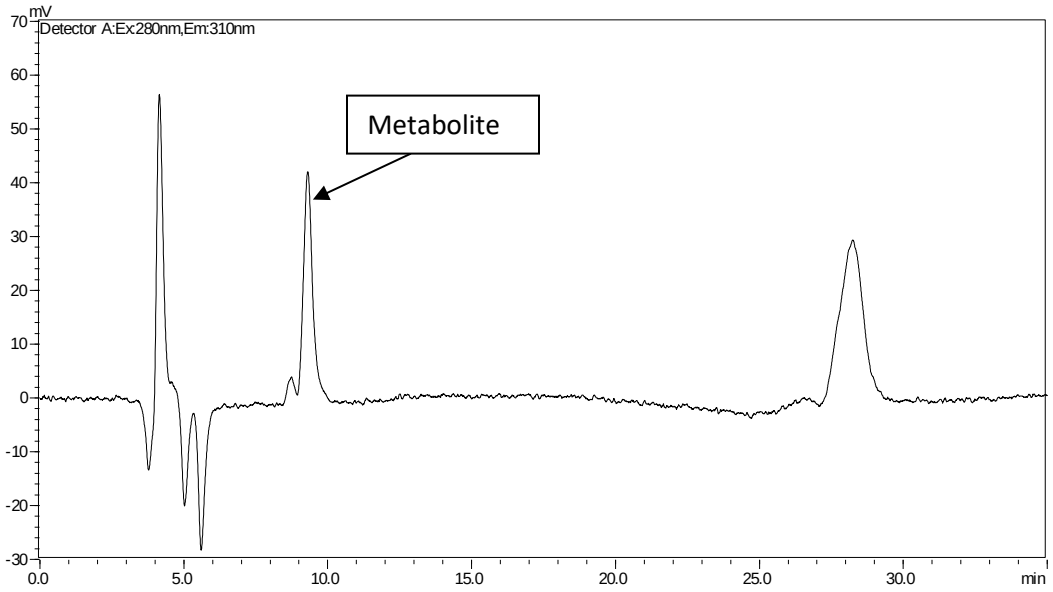
Detection 280 Excitation; 310 Emmission

Mobile Phase = Water (75%): Acetonitrile (24%): TEA (1%)

Column TSKgel ODS 100v 5 µm; 250mm x4.6mm

RT of Metabolite = 9.3min





Data Comparison