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EFFECT OF 30-DAY ADMINISTRATION OF CELLGEVITY® ON SELECTED RAT LIVER CYP450 ENZYME ACTIVITY AND POSSIBLE INTERACTION WITH CARBAMAZEPINE

BY

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JULY, 2019

DECLARATION

DECLARATION BY THE CANDIDATE

I hereby declare that this thesis is my own research (except for references to other people's work which has been duly acknowledged), undertaken under the supervision of the underlisted below. I am wholly responsible for any flaws in the work. This thesis has not been presented in whole or in part for any degree elsewhere.

Student's signature…… Date……/……/…….

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DECLARATION BY SUPERVISORS

We hereby declare that this thesis was supervised by us in accordance with guidelines stipulated for supervision of MPhil thesis by the University of Ghana.

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(Dr. Seth Kwabena Amponsah)

DEDICATION

This thesis is dedicated to my mother, Awalisalie Adaayueba. I could never ask for a better mother.

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ABREVIATIONS

AED	Animal equivalent dose
AUC	Area under drug concentration-time curve
BSA	Bovine serum albumin
BWT	Body weight
CAM	Complementary and Alternative Medicine
C_{max}	Maximum drug concentration
CYP	Cytochrome P450
DSHEA	Dietary Supplement Health and Education Act
EX	Expected
H-D	High dose
HPLC	High Performance Liquid Chromatography
K_e	Elimination constant
kPi	Potassium phosphate
L-D	Low dose
LWT	Liver weight
M-D	Medium dose
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced)
N-C	Negative control
PAH	Polycyclic Aromatic Hydrocarbons
P.O.	Per os in Latin (oral)
P-C	Positive control
SNP	Single nucleotide polymorphism
STD	Standard
STDEV	Standard deviation
TCDD	Tetrachlorodibenzodioxin
T_{max}	Time to reach maximum drug concentration
$t_{1/2}$	Half-life
US FDA	United States Food and Drugs Authority

ABSTRACT

Background: There is considerable evidence that many patients concurrently take conventional drugs with dietary supplements or herbal products. One such supplement, with an amalgam of extracts, is Cellgevity®. There is, however, a dearth of information on the effect of this supplement on drug metabolizing enzymes, and on the pharmacokinetics of conventional drugs.

Aim: To determine the effect of Cellgevity® on the activity of rat liver CYP1A1/2, CYP3A4, CYP2B1/2, CYP2C9 and CYP2D6 enzymes, and potential effect on the pharmacokinetics of carbamazepine.

Methodology: Male and female Sprague-Dawley rats (Hsd:SD strain), weighing 150-200 g and 6-8 weeks old were put into five groups (3 males and 3 females per group). Group 1 was administered with the vehicle (distilled water), that was, solvent used in dissolving Cellgevity® (negative control). Groups 2, 3 and 4 were administered a suspension of Cellgevity® by oral gavage at a low dose (38.63 mg/kg), medium dose (77.25 mg/kg) and high dose (154.50 mg/kg) daily, respectively. Group 5 was administered phenobarbital (15 mg/kg) as positive control. After a 30-day administration period, the animals were sacrificed and livers harvested. Microsomal preparations from rat livers were made by homogenization and differential centrifugation. The substrates of each specific CYP was added to microsomal preparation in phosphate buffer (pH 7.4) and incubated at 37^{o}C and their metabolites measured using fluorometric and chromatographic assays for CYP3A4, CYP2B1/2, CYP1A1/2, CYP2C9 and CYP2D6. Modulation of enzymes by Cellgevity® treatment was determined by comparing enzyme activity of Cellgevity®-treated animals with enzyme activity of negative control animals.

The effect of Cellgevity® on the pharmacokinetics of carbamazepine in healthy male SD rats was also investigated. Rats were put into 2 groups consisting of 5 rats in each group. Group 1 was given only Cellgevity® (77.25 mg/kg, po) with normal saline and Group 2 was administered with Cellgevity® (77.25 mg/kg, po) and carbamazepine (80 mg/kg po) concurrently. Treatment was done for 14 days and blood sampled at 0.5, 1, 4, 12 and 24 h from each rat on the 15th day. Blood samples were centrifuged, and serum obtained. Analyses for the levels of carbamazepine in serum (pooled) corresponding to each time point for each group were done by Fluorescence Polarization Immunoassay. Non-compartmental pharmacokinetic analysis was used to estimate pharmacokinetic parameters for the 2 groups. The pharmacokinetic parameters: C_{max}, T_{max}, AUC, K_e and $t_{1/2}$ estimation was done by non-compartmental analysis using GraphPad Prism 8.0.

Results: Data from the current study showed that on a whole Cellgevity® increased activity of rat liver CYP3A4, CYP2B1/2, CYP1A1/2, CYP2C9 and CYP2D6. However, the increase in enzyme activity of CYP1A1/2, CYP2C9 and CYP2D6 by Cellgevity® was found to be statistically significant. Amongst treatment groups with statistically significant increase in enzyme activity, it was found that, CYP activity modulation by Cellgevity® was dose dependent for female groups of CYP1A1/2, CYP2C9 and CYP2D6, and combined sex group of CYP2C9. The pharmacokinetic parameters for SD rats administered carbamazepine with Cellgevity® *vis-a-vis* rats administered carbamazepine with normal saline were as follows: C_{max}; 20 µmol/L vrs 11 µmol/L, $AUC_{0\rightarrow24}$; 347 µmol.h/L vrs 170 µmol.h/L, K_e; 0.3 h^{-1} vrs 0.4 h^{-1}, and $t_{1/2}$; 2.3 h vrs 1.7 h, respectively.

Conclusion: A 30-day treatment period with Cellgevity® among rats led to a significant increase in the enzyme activity of CYP1A1/2, CYP2C9 and CYP2D6. From the study, it was evident that Cellgevity® altered the pharmacokinetics of carbamazepine in SD rats.

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND

A considerable number of people now resort to the use of complementary and alternative medicine (CAM) to help improve their health. CAM may include dietary supplements, megadose vitamins, herbal preparations, acupuncture, massage therapy, spiritual healing, amongst others. There are reports that suggest that a considerable number of people in developing countries use CAM (Geneva, 2002).

In Ghana, the use of herbal preparations and food supplements appears to be on the rise. Anecdotal reports suggest that a number of patients take these supplements concurrently with conventional drugs. Thus, there is a possibility of drug-food supplements interactions to occur (Bahramsoltani et al., 2017). Drug interaction results when one agent changes the concentration or biological effects of another (Baxter and Preston, 2010). This could lead to alteration in the pharmacokinetic profile of the conventional drug(s), and eventually results in either sub-therapeutic or toxic consequences. This problem of drug-supplement (herb) interaction usually does occur among geriatrics (Lavan et al., 2016). This is because of the practice of polypharmacy due to comorbidities associated with the geriatric population. Drug-diet supplement (herb) interactions can occur during absorption, distribution, metabolism or excretion of conventional drug (Palleria et al., 2013; Hu et al., 2005). The commonest are interactions that modulate levels of drug metabolizing enzymes (Zhou et al., 2003).

Drug metabolizing enzymes may catalyze Phase I or Phase II reactions. Phase I enzymes are responsible for introducing polar functional groups or unmasking polar groups in xenobiotics.

Phase II, on the other hand, are mostly involved in conjugating endogenous substrates to xenobiotics, making metabolites more polar. Among the enzymes that catalyze Phase I reactions are the Cytochrome P450 (CYP) enzymes. CYP enzymes are responsible for oxidative, peroxidative and reductive metabolism of xenobiotics (Wrighton and Stevens, 1992, Yan and Caldwell, 2001). There are 57 CYP enzymes that have been encoded in the human genome. Of the 57 CYPs, only about 15 are involved in xenobiotic metabolism, of which 5 CYPs - CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 alone account for 95% of Phase I metabolism of all marketed drugs (Guengerich, 2007).

There is substantial evidence that suggests that CYP enzyme activity can be modulated by genetic and environmental factors (Iyer, 1999; Snyder, 2000). The environmental factors include conventional drugs, herbal preparations and supplements (Michalets, 1998; Lilja et al., 1998). For example, *Ginkgo biloba* extracts was found to significantly induce levels of CYP2B1/2, CYP3A1 and CYP3A2 mRNA in rat liver after 4 weeks of treatment (Shinozuka et al. (2002). In addition Shinozuka et al. (2002) reported that the therapeutic effect of nicardipine, a CYP3A2 substrate, was significantly reduced by *Ginkgo biloba* extracts, suggesting an induction of CYP3A2. Reports also show that *Angelica sinensis*, *Citrus aurantium* and *Allium sativum* can inhibit CYP3A4 (Guo et al., 2001; Foster et al., 2001) . Thus, there is the need for studies that will identify the effect of these supplements and herbal extracts on CYP450 drug metabolizing enzymes.

A number of dietary supplements are available on the market and are believed to replenish levels of reduced glutathione, a free radical scavenger in the body. One such supplement, Cellgevity®, contains a glutathione precursor molecule riboceine (D-ribose-L-cysteine). Riboceine is known to deliver cysteine into cells and enhance reduced glutathione level in the body (Slitt et al.,

2005). The other constituents of Cellgevity® are broccoli seed extract, turmeric root extract, resveratrol, grape seed extract, quercetin, curcumin, milk thistle, vitamin C, selenomethionine, cordyceps, black pepper and aloe extract. Cellgevity®, has gained much popularity in Ghana probably due to the knowledge that the supplement has antioxidant properties (McCormick et al., 2016; Cukrov et al., 2018). Since the etiology of a number of diseases is known to be as a result of oxidative stress, Cellgevity® is used by a number of patients: diabetics, epileptic and hypertensive patients to manage their conditions (Jain et al., 2009; Kader et al., 2014). A recent study by N'guessan et al. (2018) found that Cellgevity® significantly inhibited rat liver CYP2C9, CYP2B1/2B2 and CYP3A4, emphasizing the need for studies of such nature to identify effect of supplements or herbal products on xenobiotic metabolizing enzymes.

Carbamazepine is a common drug for the treatment of epilepsy. Carbamazepine is known to induce its own metabolism (auto-induction), and has an oral bioavailability of 75 - 85%. It is highly protein bound (75 - 80%), and its absorption is not affected by food (Asai et al., 2017; Liu et al., 2015). Reports suggest that carbamazepine is a drug that is prone to a number of herb, dietary supplement and food interactions (Ren et al., 2019; Perucca, 2018; Bedada and Neerati, 2018b), because of its long-term use. Hence, the need to investigate the effect of supplements/herbal products known to be administered concurrently with carbamazepine.

1.2 PROBLEM STATEMENT

Cellgevity® is a conglomerate of extracts from herbs and the glutathione precursor riboceine. This supplement is patronized by both the healthy and sick people in Ghana. Data suggest that some of the constituents of Cellgevity® can inhibit and/ induce CYP450 enzymes. For instance resveratrol irreversibly inhibits CYP3A4 and non-competitively inhibits CYP2E1 (Chan and Delucchi, 2000; Piver et al., 2001). Another constituent of Cellgevity®, aloe vera, despite its

medicinal benefits, induces CYP450 reductase and Phase II enzyme activity (Singh et al., 2000). Furthermore, curcumin was reported by Appiah-Opong et al. (2007) to inhibit CYP1A2, CYP3A4, CYP2B6, CYP2C9 and CYP2D6. CYP2C19 inhibition by curcumin has also been reported (Liu et al., 2012).

Aside the aforementioned inhibition and induction potential of some herb or plant extracts, data also suggest alteration in the pharmacokinetics of conventional drugs concomitantly administered with these herbs/extracts. For instance, St. John's wort was found to lower the serum concentration of digoxin (Johne et al., 1999), and amitriptyline (Roots et al., 2000). Licorice, a common herb used in Chinese and Japanese herbal mixtures, has been found to increase plasma concentration of prednisolone (Chen et al., 1991).

A recent study by N'guessan et al. (2018) found that Cellgevity® significantly inhibited rat liver CYP2C9, CYP2B1/2B2 and CYP3A4. Thus there is the need for studies of such nature to identify effect of supplements or herbal products on xenobiotic metabolizing enzymes, and consequently on the pharmacokinetics of conventional drugs.

1.3 JUSTIFICATION

Clinically important modulation of CYP enzymes by supplements/herbal products could result in adverse or sub-therapeutic effects of conventional drugs. For example, St. John's wort was reported to decrease serum concentration of theophylline (a bronchodilator) as a result of CYP450 enzyme induction (Nebel et al., 1999b). This interaction between St John's wort and theophylline could lead to sub-therapeutic effect of normal doses of theophylline when there is

co-administration. For this reason, patients are often advised not to take theophylline concomitantly with St. John's wort.

Anecdotal reports suggest that a number of patients with chronic disease conditions (hypertension, epilepsy and diabetes) take Cellgevity® alongside their conventional drugs. With reports suggesting Cellgevity® inhibiting CYP2C9, CYP2B1/2B2 and CYP3A4 (N'guessan et al., 2018), it is possible that this food supplement can affect the pharmacokinetics of drugs metabolized by these CYP enzymes if there is co-administration.

In the study by N'guessan et al. (2018), using two doses of CellGevity®, rat CYP2C9, CYP2B1/2B2 and CYP3A4 were found to be significantly inhibited over a 7-day treatment period. The current follow-up study is to attempt a similar experiment with 3 doses calculated after dose scaling from humans, and CellGevity® administered over a longer period of time (30 days) to ascertain the possibility of a dose dependent effect of this supplement on the activity of CYP enzymes.

Carbamazepine is one of the most commonly prescribed drugs in the management of epilepsy. Due to the nature of epilepsy, and the fact that it is a chronic condition, patients have to take carbamazepine for a long time (lifetime in most cases). There is, thus, the potential for clinically significant interactions between carbamazepine and co-administered agents like dietary supplements, herbal products and food (Ketter et al., 1991).

Carbamazepine is known to have a narrow therapeutic index, and exhibits a wide inter-individual variability in its metabolism (Raish et al., 2017; Zhao et al., 2019), hence, identifying possible drug-supplement/herb interactions is essential. This will help avoid possible adverse reactions associated with carbamazepine, and improve its therapeutic outcome. With anecdotal reports

suggesting that concurrent administration of Cellgevity®, inhibits rat liver CYP3A4 (N'guessan et al, 2018), and carbamazepine is metabolized extensively by CYP3A4, there is the possibility of interaction between these two agents that can affect their pharmacokinetic profiles.

1.4 HYPOTHESIS

Cellgevity® inhibits or induces S-D rat CYP450 enzymes when administered for 30 days.

1.5 AIM

To determine the effect of Cellgevity® on rat CYP450 enzymes and the pharmacokinetics of carbamazepine.

1.6 SPECIFIC OBJECTIVES

1. To determine the inhibition or induction potential of Cellgevity® on rat CYP1A1/2, CYP2B1/2, CYP2C9, CYP2D6 and CYP3A4 *ex vivo*.
2. To determine the effect of Cellgevity® on the pharmacokinetic parameters (C_{max}, T_{max}, K_e $t_{1/2}$ and AUC) of carbamazepine administered to S-D rats.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 COMPLEMENTARY AND ALTERNATIVE MEDICINE (CAM)

2.1.1 Definition of CAM

Complementary and Alternative Medicine describes practices and products that people choose as adjuncts to or alternatives to Western medical approaches (Debas et al., 2006). These are practices outside conventional medicine which are seen by users as means of preventing or treating diseases and promoting health or well-being (Pal, 2002).

2.1.2 Why People Use CAM

CAM in most cases are indicated for the prevention and treatment of chronic conditions. With increasing chronic diseases due to sedentary lifestyle (Adams et al., 2019; Lemes et al., 2019), many people may resort to CAM. In addition to the increasing burden of chronic diseases, adverse effects and skyrocketing prices of conventional medicines push many economic disadvantaged individuals to use CAM. Furthermore, scientific validation of some CAM (Debas et al., 2006) has enhanced their patronage by the general public. For many others, the use of CAM is influenced by their religion, spirituality and culture (Ulrich et al., 2011).

2.1.3 Regulation of CAM

Regulation of CAM is generally lax (Debas et al., 2006). However, this regulation varies from country to country. Practice of CAM in the United Kingdom (UK) is generally unregulated, except a few such as chiropractic therapy (Pal, 2002). In Ghana much of folklore-based CAM is not regulated. However modernized CAM products are given certification by the Food and Drugs Authority (FDA). Their regulations are not as stringent as conventional drugs and medical equipment.

2.1.4 Types of CAM

CAM can be divided into (i) mind-body medicine (ii) alternative medical system (iii) lifestyle and disease prevention (iv) biologically-based therapies (v) manipulative and body-based systems (vi) biofield and (vii) bioelectromagnetic.

Mind-body medicine refers to psychological and spiritual approaches to health e.g. yoga and prayer. Alternative medical systems include traditional indigenous medicine, acupuncture, homeopathy etc. Lifestyle and disease prevention includes chirography among others. Biologically-based therapies involves the use of natural and biologically-based products, example is herbalism and use of dietary supplements. For manipulative and body-based systems, examples are chiropractic medicine and massage therapy. Biofield deals with energy balance example Reiki, and bioelectromagnetic therapy is the use of electric field for medical treatment.

2.2 DIETARY SUPPLEMENTS

2.2.1 What are Dietary Supplements?

The Dietary Supplement Health and Education Act (DSHEA), defined and formulated the category of food or agents known as dietary supplement (US Public Law 108-417, 1994). According to the law, a dietary supplement is:

"a product (other than tobacco) intended to supplement the diet that bears or contains one or more of the following dietary ingredients: (A) a vitamin; (B) a mineral; (C) a herb or other botanical; (D) an amino acid; (E) a dietary substances for use by man to supplement the diet by increasing the total dietary intake; or (F) a concentrate, metabolite, constituent, extract, or combination of any ingredient described in clause (A), (B), (C), (D) or (E)."

The US FDA interprets dietary supplement to mean substances that are already found in diets and not those that are created for the first time and added to a diet. Dietary supplements can only be ingested through the mouth and no other route of administration. Thus dietary supplement could be a tablet, capsule, liquid, powder, soft gel gelatin capsule etc.

A dietary supplement later approved as a drug could still be called a dietary supplement. However, a new drug cannot be subsequently marketed as a dietary supplement (Young et al., 2018).

2.2.2 Dietary Supplements Advertisement

According to Title 21 of the United State Code (USC) 343(r)(6), a dietary supplement could only be said to address nutritional deficiency, structure-function and general well-being of the recipient, but cannot be said to diagnose, treat, cure and prevent any disease (Young et al., 2018).

Unfortunately, there are anecdotal reports of media advertisement of claims that a number of dietary supplements can do the latter.

2.2.3 Types of Dietary Supplements

Dietary supplements currently on the market include those that contain: vitamins, minerals, amino acids, herbs, or increase glutathione levels among others. Cellgevity® is one of the dietary supplements believed to replenish reduced glutathione levels in the body (Slitt et al., 2005).

2.3 CELLGEVITY®

Cellgevity® (Figure 2.1) is a dietary supplement that is believed to replenish glutathione in the body. Cellgevity® contains a precursor molecule, riboceine, for glutathione biosynthesis. Riboceine is a propriety substance in Cellgevity® that is formed from ribose sugar and cysteine. Riboceine enhances passage of cysteine (the rate limiting amino acid in glutathione biosynthesis) into the cells (McCormick et al., 2016).

In addition to the riboceine, Cellgevity® also contains several other constituents. They include broccoli seed extract, turmeric root extract, milk thistle, quercetin, resveratrol, grape seed extract, vitamin C, selenomethionine, cordyceps, black pepper and aloe extract.

Figure 2. 1: Cellgevity®

(Picture credit: www.amazon.com/Cellgevity-Advanced-Riboceine-Technology-Vegetable/dp)

2.3.1 Tumeric root extract/Curcumin

Curcumin is a natural polyphenol (Figure 2.2). It has a wide range of properties including antioxidant, anti-proliferative and anti-inflammation (Esmaili et al., 2011; Joshi et al., 2011). This polyphenol is isolated from rhizomes of *Curcuma longa*, also known as turmeric. Curcumin is often used in the pharmaceutical and food industries (Aggarwal et al., 2007, Niranjan and Prakash, 2008). Curcumin could be used as colouring, flavouring and preservative agent in foods (Aggarwal et al., 2007; Joshi et al., 2011). Curcumin has been shown to have antidiabetic properties (Borges et al., 2017) among others.

Reports suggests that curcumin has inhibitory effect on CYP3A enzymes (Sun et al., 2016). In vitro studies by Appiah-Opong et al. (2007) using recombinant human microsomes also showed that curcumin had inhibitory effects on CYP1A2, CYP3A4, CYP2C9, CYP2D6 and CYP2B6. Therefore despite reported pharmacological benefits of curcumin, there is the potential for pharmacokinetic interaction with conventional drugs.

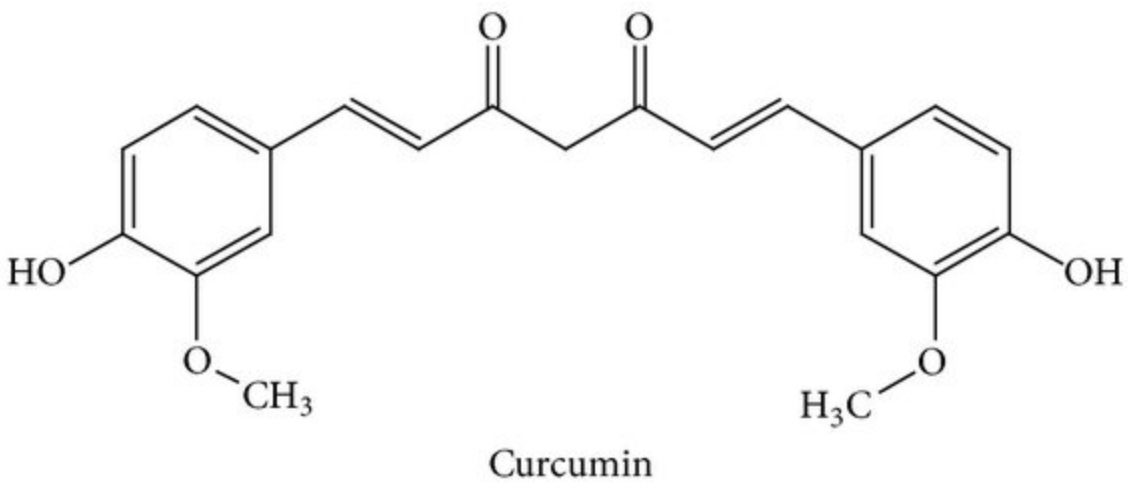

Figure 2. 2: Chemical structure of curcumin

2.3.2 Milk Thistle

Milk thistle, *Silybum marianum* (Figure 2.3) was ranked as the top sixth herbal supplement in the US in 2013 (Lindstrom et al., 2013). Crude extract of milk thistle contains a mixture of flovonolignans, called salymarin. Milk thistle has many health benefits such as antioxidant activity (Lucini et al., 2016), improvement in male fertility (Attia et al., 2017) among others.

There are reports that suggest that milk thistle extracts can modulate drug metabolizing enzymes, both *in vitro* and *in vivo*. At low concentrations (1.5 µg/mL), the crude extracts of the *Silybum marianum* plant had mild inhibitory effects on CYP450 enzymes (Doehmer et al., 2011). However at higher concentrations (15 to 150 µg/mL), significant inhibition of CYP2C8 and CYP2C9 was reported (Doehmer et al., 2011). *In vivo* studies of crude extracts of milk thistle showed that it did not affect the pharmacokinetics of probe drugs of CYP3A4/5, CYP1A2, CYP2D6 and CYP2E1 in healthy volunteers (Gurley et al., 2004). Albassam et al. (2017) found

that the major constituents of milk thistle – silybin A, silybin B, isosilybin A and isosilybin B have potent inhibitory effect on CYP2C8.

Figure 2. 3: Picture of Milk thistle plant

(Picture credit: https://www.well-beingsecrets.com/milk-thistle-benefits)

2.3.3 Quercetin

Quercetin (Figure 2.4) is a natural flavonoid that is abundantly present in most edible vegetables and fruits (Lesjak et al., 2018). It is used in the treatment and prevention of a number of disease conditions such as cardiovascular disorders (Serban et al., 2016). D'Andrea (2015) reported that an intake of quercetin-rich diet correlated positively with health promotion. (Palle and Neerati, 2017) reported that quercetin enhanced the bioavailablity of rivastigmine by inhibiting CYP3A4. Also quercetin reversed resistance to cisplatin, an anticancer drug, by inhibiting CYP1B1 (Sharma et al., 2017). All the aforementioned suggest that quercetin can modulate CYP enzymes.

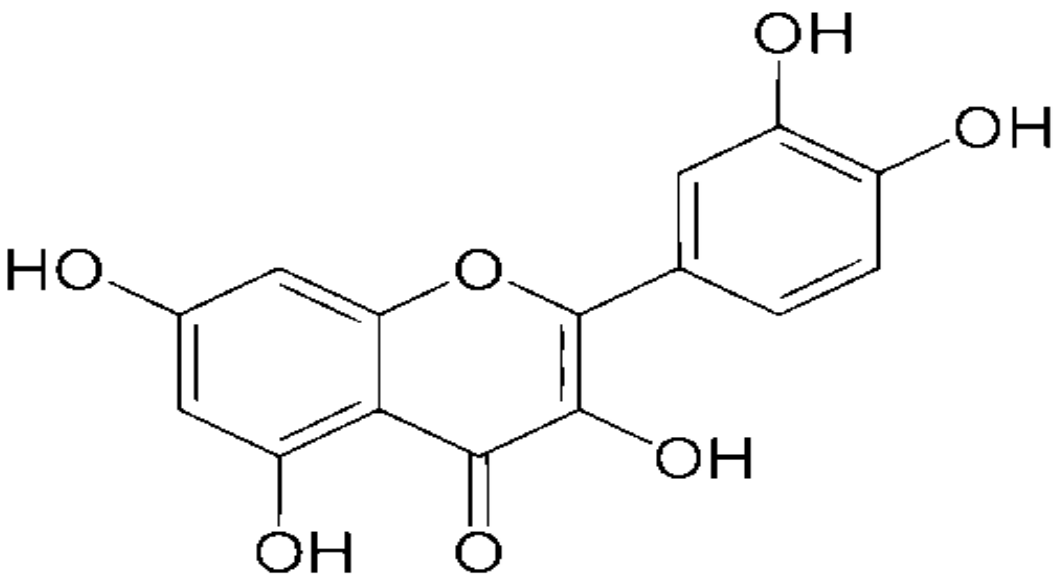

Figure 2. 4: Chemical structure of Quercetin

2.3.4 Piperine

Peperine (Figure 2.5) is an alkaloid present in *Piper nigrum* (black pepper) and *Piper longru* (Zhou et al., 2003). Piperine is reported to exhibit antioxidant (Dhivya et al., 2017, Zarai et al., 2013), anti-inflammation (Stöhr et al., 2001) and anti-dyslipidemic effects (Dhivya et al., 2017). Peperine has also been shown to have antibacterial properties (Zarai et al., 2013) and could serve as a preservative in foods.

Tsukamoto et al. (2002) reported inhibition of CYP3A4 by peperine in cDNA-expressed human microsomes. However multiples of repeated doses (500 mg/kg/day) of piperine saw about a two-fold increase in liver microsomal CYP level (Zhou et al., 2003). Peperine modulatory effect on CYP enzymes is dependent on route of administration, dose and species of organism (Zhou et al., 2003).

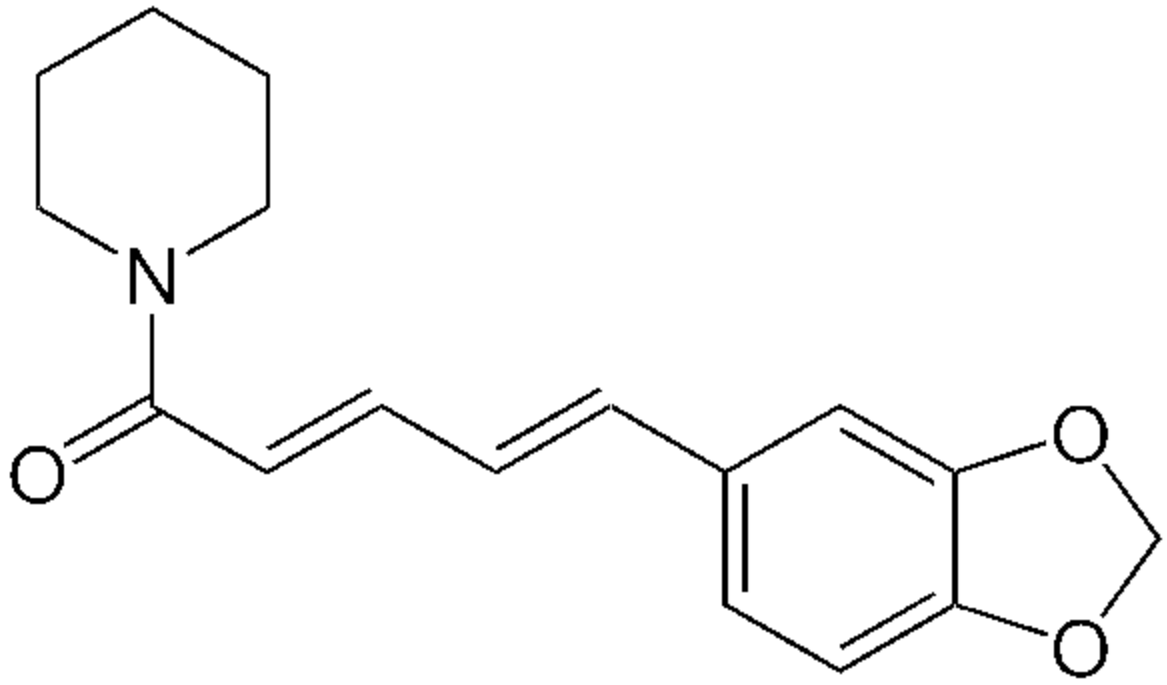

Figure 2. 5: Chemical structure of Peperine

2.4 XENOBIOTIC METABOLISM

Enzymatic metabolism of xenobiotics is relevant in the elimination of these xenobiotics from the body. The liver is the principal site of drug metabolism (Rendic and Carlo, 1997, Chen et al., 2016). The process of xenobiotic metabolism involves two phases of reactions, Phase I and Phase II.

2.4.1 Phase I Metabolism

Phase I xenobiotic metabolism involves addition or unmasking of polar groups of the parent drug. Phase I is mainly catalyzed by cytochrome P450 enzymes (Guengerich, 2001). The most important drug metabolizing enzymes of this phase are the Cytochrome P450 enzymes.

2.4.1.1 Cytochrome P450 Enzyme System

Cytochrome P450 (CYPs) were discovered by M. Klingenberg in 1954 (Omura, 2011). CYPs are made up a superfamily of hemoproteins. The term P450 is derived from the peak wavelength of 450 nm where the enzyme in its reduced state and complexed with carbon monoxide has maximum absorption (Omura, 2011). The enzymes catalyze Phase I metabolism of endogenous substrates and xenobiotics by oxidation, peroxidation and reduction (Wrighton and Stevens, 1992; Nelson et al., 1996; Yan and Caldwell, 2001). CYPs catalyze reactions by adding oxygen atom to the substrate and the other oxygen atom is reduced to water by NADPH (Parkinson, 2001). The general carbon hydroxylation reaction stoichiometry is shown below;

$$NADPH + H^+ + RH + O_2 \rightarrow NADP^+ + H_2O + ROH$$

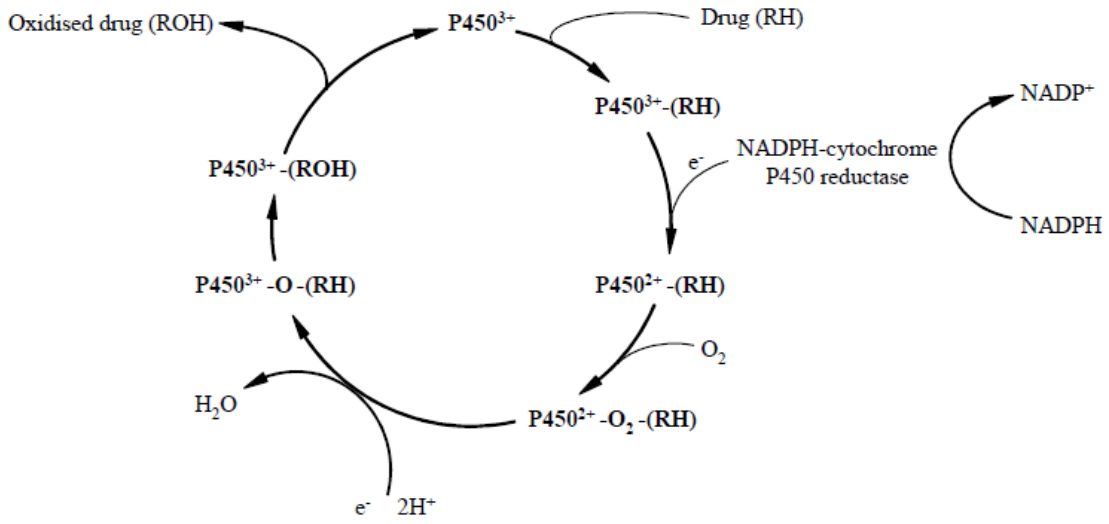

Figure 2. 6: Catalytic cycle of cytochrome P450 adopted from Turpeinen (2006)

CYPs were originally found in animal liver microsomes (Omura, 1999). Subsequently they were found to present in all eukaryotic organisms such as plants and fungi. Human CYPs are located intracellularly in the smooth endoplasmic reticulum (Lin and Lu, 1998). There are generally

lower levels of CYPs expressed in other tissues such as the intestines, kidney, lungs and brain (Venkatakrishnan et al., 2001).

Nebert et al. (1987) first developed the nomenclature of CYPs. The CYPs are classified into families, subfamilies and isoforms. CYP denotes the superfamily of the enzyme. The first number (1, 2, 3 etc.) denotes the family (enzymes with greater than 40% amino acids sequence identity within members), the letter (A, B, C, etc.) denotes the subfamily (enzymes with greater than 55% amino acids sequence identity) and the final number indicates the individual isoform or gene (Lewis, 2004; Nelson et al., 2004).

Mammalian hepatic CYPs are found in the mitochondria (for biosynthesis of steroid hormone and vitamin D metabolism), and in the endoplasmic reticulum (Parkinson, 2001). Xenobiotic metabolizing hepatic CYPs are found in the endoplasmic reticulum. These can be harvested from liver by homogenization and differential centrifugation to obtain microsomes. Human microsomal CYP isoforms involved in the metabolism of therapeutic drugs include the subfamilies CYP1A, CYP2A, CYP2B, CYP2C, CYP2D, CYP2E and CYP3A (Parkinson, 2001). The most important human CYP isoforms for drug metabolism are CYP1A2, CYP2B6, CYP2C9, CYPC19, CYP2D6 and CYP3A4/CYP3A5. CYP2A6, CPY2C8 and CYP2E1 have also been found to metabolize some important drugs (Hewitt et al., 2007; Martignoni et al., 2006; Parkinson, 2001). The hepatic microsomal CYP4A11 metabolizes endogenous substrates such as fatty acids, prostaglandins and leukotrienes (Parkinson, 2001).

2.4.1.1.1 CYP1 family

The members in this family are CYP1A1, CYP1A2 and CYP1B1. CYP1A2 is the major hepatic enzyme in this family. CYP1A1 is extrahepatic and it is a major human extrahepatic CYP isoform (Ding and Kaminsky, 2003). Hepatic expression of CYP1B1 is very low but it is been found to be expressed in almost every other tissue (Raunio et al., 1999). CYP1 family are regulated by AHR-receptor and induced by TCDD, PAHs, and smoking (Nebert et al., 2004). The CYP1 family is responsible for activation of PAHs and aromatic amines and thus plays role in chemical carcinogenesis (Ioannides et al., 2004).

CYP1A2 was thought to be only hepatic, however, studies show that it is expressed along with CYP1A1 also in the lungs (Liu et al., 2003). Although quantitatively CYP1A2 is low in the liver, it is very important in the metabolism of ethoxyresorufin (Burke et al., 1977), caffeine (Butler et al., 1989), phenacetin (Venkatakrishnan et al., 1998b), clozapine (Fang et al., 1998), melatonin (Facciolá et al., 2001), theophylline (Tjia et al., 1996), etc.

Potent inhibitors of the CYP1A2 are furafylline (Sesardic et al., 1990), fluvoxamine (Rasmussen et al., 1998), ciprofloxacin (Fuhr et al., 1993), oral hormone replacement therapy and contraceptives (Laine et al., 1999; Pollock et al., 1999). CYP1A2 is induced by cigarette smoke (Vistisen et al., 1992), cruciferous vegetable and charcoal (Murray et al., 2001; Larsen and Brøsen, 2005).

There is a progressive decrease in CYP1A2 mRNA or protein/activity during pregnancy (Tracy et al., 2005). The decrease in CYP1A2 activity could lead to increased toxicity for CYP1A2 substrate drugs. Postnatally, CYP1A2 is the last major enzyme to be fully matured (Sonnier and Cresteil, 1998). There is no detectable levels of CYP1A2 at one month of age. However, the

CYP reaches maturity between one and two years of age (Sonnier and Cresteil, 1998, Tateishi et al., 1997).

Guessous et al. (2012) found that CYP1A2 plays a role in regulation of blood pressure. They found a relationship of CYP1A2*1F allele with an increased caffeine intake and reduced risk of hypertension among non-smokers.

2.4.1.1.2 CYP2 family

Human CYP2 family is very diverse with a good number of important drug metabolizing CYP enzymes. CYP2B6, CYP2D6 and CYP2E1 are the functional isoforms in their subfamilies. The subfamilies, CYP2A and CYP2C have two and four functional isoforms respectively. CYP2 family (CYP2C9, CYP2C19 and CYP2D6) exhibits clinically the most important polymorphism (Turpeinen, 2006).

CYP2B6 is located on the long arm of chromosome 19 within a CYP2B cluster (Nelson et al., 2004). Orthologs of human CYP2B6 can be found in species such as rats, mice and dogs with CYP2B1, CYP2B10 and CYP2B11 respectively. CYP2B6 is the only enzyme of the CYP2B subfamily with metabolic function in humans (Nelson et al., 2004; Hoffman et al., 2001). The enzyme is reported to constitute 1-10% of total hepatic CYP content (Lang et al., 2001). CYP2B6 metabolizes neutral and weakly basic molecules with one or two hydrogen bond acceptors (Lewis, 2000). The table below represents clinically utilized CYP2B6 substrates.

Table 2. 1: Clinically utilized CYP2B6-substrates, adopted from Hedrich et al. (2016)

Class	Substrate	Contribution of CYPs
Anesthetic	Ketamine	Major, CYP3A4; Minor, CYP2B6, 2C9
	Lidocaine	Major, CYP2B6, 2A6; Minor, CYP2B6
	Propofol	Major, CYP2B6; Minor, CYP2C9
Antiarrhythmic	Mexiletine	Major, CYP2A1; Minor, CYP2B6, 2E1
Anticoagulant	Coumarins	Major, CYP2B6; Minor, CYP2E1, 2C19
Anticonvulsant	Mephenytoin	Major, CYP2B6; Minor, CYP2C9
Antidepressant	Bupropion	Major, CYP2B6; Minor, CYP2D6, 3A4
Antiepileptic	Mephobarbital	Major, CYP2B6
	Valproic Acid	Major, CYP2A6; Minor, CYP2B6, 1A1
Anti-inflammatory	Aminopyrine	Major, CYP2B6, 2C19; Minor, CYP2C8, 2D6
	Antipyrine	Major, CYP3A4, 2C; Minor, CYP2B6, 1A2
	Tazofelone	Major, CYP3A4; Minor, CYP2B6
Antimalarial	Artemether	Major, CYP2B6; Minor, CYP3A4
	Artemisinin	Major, CYP2B6; Minor, CYP3A4
Antiretroviral	Efavirenz	Major, CYP2B6; Minor, CYP3A
	Nevirapine	Major, CYP2B6, 3A4; Minor, CYP2D6
Chemotherapeutic	Cyclophosphamide	Major, CYP2B6; Minor, CYP3A4, 2C9
	Ifosfamide	Major, CYP2B6, 3A4; Minor, CYP2C9, 2C19
	Tamoxifen	Major, CYP2E1, 2D6; Minor, CYP2B6, 3A4
MAOI	Selegiline	Major, CYP2B6, 2C19 ; Minor, CYP3A4, 1A2
Opioid	Methadone	Major, CYP2B6, 3A4
	Pethidine	Major, CYP2B6; Minor, CYP3A4, 2C19
Psychotropic	Clotiazepam	Major, CYP2B6, 3A4; Minor, CYP2C18, 2C19
	Diazepam	Major, CYP2B6, 2C19; Minor, CYP3A4
	Temazepam	Major, CYP2B6; Minor, CYP2C, 3A
Steroid	Testosterone	Major, CYP3A4; Minor, CYP2B6

CYP2B6 is inducible through CAR and PXR associated mechanism (Faucette et al., 2004; Faucette et al., 2006). In clinical practice, there is increased probe drug clearance after rifampicin and phenytoin treatment (Loboz et al., 2006; Robertson et al., 2005). The antiviral drugs ritonavir, and efavirenz have potent inhibitory activity on CYP2B6 (Xu and Desta, 2013, Lin et al., 2013)

CYP2C9 is a major CYP2C isoform and catalyses the metabolism of many clinically relevant drugs such as non-steroidal anti-inflammatory drugs (NSAIDS), antidepressant, anticoagulant (Aguilar et al., 2008). CYP2C9 polymorphisms are clinically important in altering the metabolism of various drugs (Zhou et al., 2010). About 33 different alleles of CYP2C9 have

been described. The most important alleles, CYP2C9*2 and CYP2C9*3, lead to decrease enzymatic activity (Zi et al., 2010; Kirchheiner and Seeringer, 2007). CYP2C9 polymorphism is emphasized in S-warfarin which uses CYP2C9 as a major pathway and has narrow therapeutic window with fatal side-effects (Kirchheiner and Brockmöller, 2005).

Inhibitors of CYP2C9 include gemfibrozil (Wen et al., 2001), tanshinones (Wang et al., 2010) and sulphaphenazole (Khojasteh et al., 2011). Multiple regulatory elements are involved in CYP2C9 and other CYP2C isoforms induction (Ferguson et al., 2005).

CYP2D6 gene is located on chromosome 22 (Stanton Jr, 2012) and metabolizes many commonly used class drugs such as antidepressants (Gressier et al., 2015) and neuroleptics (Butwicka et al., 2014). Probe substrates of CYP2D6 include dextromethorphan (Glass and Furge, 2016, Appiah-Opong et al., 2007) and debrisoquine (Varela et al., 2015). Other substrates include beta blockers such as propranolol (Zhang et al., 2016) and metoprolol (Berger et al., 2018). Antidepressants such as fluoxetine and paroxetine are strong inhibitors of CYP2D6 (Belpaire et al., 1998, Bahar et al., 2018).

A number of drugs are inhibitors of CYP2D6. Quinidine is an inhibitor of CYP2D6 (Siu et al., 2018). Unlike other CYPs CYP2D6 expression is not regulated by any known environmental agent and there are no reported CYP2D6 inducers (Turpeinen, 2006). However, physiological conditions such as pregnancy have shown to increase CYP2D6 metabolic activity (Ingelman-Sundberg, 2005a).

CYP2D6 polymorphism causes clinically important variability in its drug metabolism. The variability ranges from complete deficiency of enzyme causing high risks of ADRs to extensively increased activity resulting in non-responsiveness to treatment (Arellano et al., 2017,

Niwa et al., 2018). Individuals carrying various allelic variant of CYP2D6 gene could be described as poor metabolizers (PM), lacking the functional enzyme and ultra-rapid metabolizers (UM), having a duplicate or multiples of the gene. Close to 7% and 5.5% of Caucasians have CYP2D6 PMs and UM, respectively (Ingelman-Sundberg, 2005a). CYP2D6 genetic testing is important in dosing and monitoring in clinical practice (Eichelbaum et al., 2006).

CYP2E1 is the only isoform of CYP2E subfamily (Lewis, 2004). Substrates of CYP2E1 are hydrophobic and low molecular weight compounds (Lewis et al., 2002). Chlorzoxazone is a probe substrate (Bedada and Neerati, 2018a). CYP2E1 mediate to some extent the metabolism of acetaminophen (Hartman et al., 2015), enflurane and halothane (Kandeel et al., 2016). Toxicologically, CYP2E1 plays role in bioactivation of carcinogens, produces free radicals leading to tissue damage (Caro and Cederbaum, 2004; Gonzalez, 2005) and acetaminophen-related hepatotoxicity (Rumack, 2004). Substrates of CYP2E1 such as ethanol acts as inducing agent of the enzyme (León-Buitimea et al., 2012). The induction process include post-translational modifications (Kessova and Cederbaum, 2003).

Inhibitors of CYP2E1 are pyridine (Maksymchuk et al., 2017) and disulfiram (De Paepe et al., 2016). Disulfiram is utilised clinically in the management of alcohol (substrate of CYP2E1) dependence. Huang et al. (2018) did not find any genetic association of alcohol dependence with any of the SNPs of CYP2E1. None of the SNPs in CYP2E1 is associated with reduced CYP2E1 activity (Gonzalez, 2005).

2.4.1.1.3 CYP3 family

The human CYP3 family constitutes about 30% of total hepatic CYP content. It is the most important CYP in drug metabolism. CYP3A is the only subfamily with 3 functional isoforms. They include CYP3A4, CYP3A5 and CYP3A7 and one pseudo CYP 3A34 (Ingelman-Sundberg, 2005b). The enzymes in this family are very promiscuous and have differing tissue expression.

CYP3A5 is a minor CYP isoform in human liver (Westlind-Johnsson et al., 2003). It is extrahepatically expressed in kidney, lungs, colon and oesophagus (Ding and Kaminsky, 2003; Burk and Wojnowski, 2004). In most cases the substrates of CYP3A5 are similar to those of CYP3A4 but have lower catalytic capability (Matsumoto et al., 2019). Metabolism of Tacrolimus, an immunosuppressant used in organ transplantation was found to be affected by CYP3A5 polymorphism. CYP3A5*3*3 had reduced activity on tacrolimus than CYP3A5*1*1 and CYP3A5*1*3 (Helal et al., 2017; Deng et al., 2018b).

CYP3A4 is the most abundant enzyme in human liver at mRNA level and constitutes the major CYP isoform in the liver and in the small intestines (Paine et al., 2006; Drozdzik et al., 2018). CYP3A4 expression in adult liver is associated with expression level of hepatocyte nuclear factor 4 alpha (Deng et al., 2018b).

CYP3A4 is estimated to be involved in the metabolism of about 65% of currently used drugs (Menendez and Michel, 2016). CYP3A4 high catalytic capacity and substrate promiscuous activity could be attributed to it having a very large and flexible active site. Its substrate binding is based on hydrophobicity and some steric interaction. In addition multiple conformation of the enzyme exists both in the presence and absence of substrates (Atkins et al., 2001; Ekins et al., 2003; Scott and Halpert, 2005). Kinetic interaction between CYP3A4 and its substrate is often

atypical, thus making prediction and modeling of CYP3A4-mediated drug-drug interaction difficult (Ekins et al., 2003; Houston and Galetin, 2005).

Substrates of CYP3A4 are wide and vary substantially in size and structure. They include clinically important drugs such as tamoxifen, verapamil, midazolam (Arnold et al., 2019). Endogenous substrates of CYP3A4 include testosterone, cholesterol, progesterone, androstenedione etc. (Poon et al., 2017; Danek et al., 2017; Ducharme et al., 2019; Zanger and Schwab, 2013). CYP3A4 has been found to mediate the bioactivation of procarcinogens such as aflatoxin B_1 (Deng et al., 2018a) and PAHs (Šulc et al., 2016).

Clinically relevant inhibitors of CYP3A4 include azole antifugal (ketoconazole and itraconazole), antibacterial (clarithromycin, erythromycin), antihypertensive (verapamil, diltiazem) (Zhou et al., 2005). Herbal and food constituents that inhibit CYP3A4 include grape fruit juice, bergamottin (Fujita, 2004) among many others. Alteration of CYP3A4 activity by any of these modulating agents could lead to clinically significant drug interactions with a resultant effects of drug inactivation or adverse drug reaction. The IC_{50} values of CYP3A4 inhibitors are substrate-dependent, hence the use of multiple probe substrate is recommended in xenobiotic CYP3A4 modulation studies (Appiah-Opong et al., 2007). Although inhibition of CYP3A4 leads to adverse drug reactions, CYP3A4 inhibitors are clinically used to boost the effect of ritonavir and other protease inhibitors which are CYP3A4 substrates in HIV treatment (Echeverria et al., 2010).

A good number of substances have induction effect on CYP3A4. Examples of these are phenobarbital (Naccarato et al., 2016), rifampicin (Hawkes et al., 2017), carbamazepine (Fuchs et al., 2018), St. John's wort (Huppertz et al., 2018), and PAH (Aratsu et al., 2017).

2.4.1.2 CYP Polymorphism and Drug-drug Interaction

There are major differences in each CYP isoform. Inter-individual variability in metabolism of drugs is a concern in drug therapy. Many factors contribute to the variability of CYP drug metabolism, of which genetic factors represent an important source of variability (Kozyra et al., 2017). Gene mutations could result in enzyme variant that have higher, lower or no activity or absence of the enzyme. Clinically implicated polymorphs are those related to CYP2D6 and CYP2C19 metabolized drugs (Arellano et al., 2017; Niwa et al., 2018).

When two or more drugs or substrates are metabolized by the same enzyme, there could be significant drug-drug/herb/supplement interaction. Thus, assessment of individual CYP in a particular metabolic pathway is important in determining and predicting potential drug-drug/herb/supplement interactions (Arellano et al., 2017).

2.4.2 Phase II Metabolism

Phase II reactions are conjugation reactions. The hydrophilicity of the substrate is increased to aid elimination by the kidney. Often there is conjugation of endogenous species such as sugars and amino acids. Phase II biotransformation of xenobiotics include glutathione conjugation, glucuronidation, sulphation, methylation and acetylation. Of these reactions, glutathione conjugation and glucuronidation are the most important (Jancova et al., 2010).

2.5 CYP450 ENZYME INHIBITION

There are about seven steps in CYP enzyme catalysis: first there is the binding of the substrate to the ferric part of the enzyme. Secondly, the haem group is reduced from the ferric to the ferrous state by an electron provided by nicotinamide adenine dinucleotide phosphate (NADPH) via

NADPH CYP reductase. In the third step, molecular oxygen binds the ferrous form of the enzyme to form peroxo complex. This step is followed by a second electron transfer from CYP reductase and/or cytochrome b5. There is then O–O bond cleavage in the fifth step. There is substrate oxygenation and O–O bond cleavage in the sixth step. Lastly the product is released in the seventh step (Estabrook, 1996; Correia and Ortiz de Montellano, 1993).

Impairment of any one of these steps could lead to inhibition of a CYP, however, the first, third and sixth steps are particularly vulnerable to inhibition (Lin and Lu, 1998).

The mechanisms of CYP inhibition include: reversible, quasi-irreversible and irreversible inhibitions (Correia and de Montellano, 2005). Of these, reversible inhibition is probably the most common mechanism responsible for drug interactions (Hollenberg, 2002). Reversible interactions arise due to competition at the CYP active site: often involving only the first step of the CYP catalytic process. On the other hand, agents that act during or subsequent to the oxygen transfer step are generally irreversible or quasi-irreversible inhibitors (Hollenberg, 2002). Both irreversible and quasi-irreversible inhibition are caused by the formation of reactive metabolites (Riley et al., 2007).

2.5.1 Reversible Inhibition

Most reversible inhibitors are nitrogen-containing drugs. They include imidazoles, quinolones and pyridines. These compounds do not only bind to the prosthetic haem iron but the lipophilic region of the protein as well (Lin and Lu, 1998). The more potent inhibitors simultaneously bind to both regions. The lipophilicity and strength of the bond between the inhibitor's nitrogen lone pair electrons and the prosthetic haem iron correlates with its potency of inhibition (Wilkinson et al., 1974; Rogerson et al., 1977). For example ketoconazole exhibits strong reversible inhibition

of CYPs than cimetidine because ketoconazole is more lipophilic than cimetidine (Richardson, 1993).

There are species differences in specificity of quinidine and quinine inhibitors (Boobis et al., 1990b). Although, reason for the differences is unknown, it is thought to be due to differences in the geometry of the active site of CYP isoforms of each species (Lin and Lu, 1998).

Quinidine inhibits CYP2D6 in humans, but is metabolized by CYP3A4 and not CYP2D6 in humans. Thus an inhibitor of CYP isoform need not necessarily be a substrate of that CYP. (Von Moltke et al., 1998).

2.5.2 Quasi-Irreversible Inhibition of CYP

This type of inhibition occurs through metabolic intermediate (MI) complexation. Drugs such as methylenedioxybenzenes, alkylamines, macrolide antibiotics and hydrazines undergo metabolic transformation by CYP enzymes to form inhibitory metabolites. These metabolites can form stable complexes with the prosthetic haem of the CYP to MI complex. The CYP is sequestered in a state that makes it functionally inactive (Lin and Lu, 1998). The MI complex can be reversed by in vitro incubation of highly lipophilic compounds which displace the MI from the active site (Elcombe et al., 1975; Dickins et al., 1979). However, the complex is stable in vivo making CYP unavailable for drug metabolism. Thus require synthesis of new enzyme to restore activity. For example Piperonyl butoxide, a methylenedioxybenzene derivative, which has been used for many years as oxidative drug metabolism inhibitor act by forming an MI complex (Murray et al., 1985).

2.5.3 Irreversible Inhibition of CYP

In this type of inhibition, the enzyme is permanently inactivated. It is brought about when certain functional group of the drug is oxidized to reactive intermediates by CYP and irreversibly inactivate the enzyme before its release from the active site (Lin and Lu, 1998). Compounds that require metabolic activation to inhibit enzymes are termed as mechanism-based inhibitors (Silverman, 1988). Mechanism-based inhibition of CYP could result from irreversible inactivation of haem or protein or a combination of both. For haem, conformational change invariably inactivates the CYP. However, alternation of protein conformation only results in CYP inactivation, if essential amino acids vital for substrate binding, electron transfer and oxygen activation are altered (Ortiz de Montellano, 1988).

2.6 CYP450 ENZYME INDUCTION

Enzyme induction is an adaptive increase of a specific enzyme, brought about by an enzyme inducer compound (Barry and Feely, 1990). The inducers of CYP enzymes are usually substrates of the CYPs isoforms. Thus, these inducers facilitate their own metabolism. In addition, the inducible CYPs not only stimulate the metabolism of the inducer compound but also other substrates (Barry and Feely, 1990). Furthermore, it is observed that in enzyme induction, many inducing-compounds of CYPs also induce Phase II conjugating enzymes. For example, UDP-glucuronosyltransferase activity is induced in rat liver by phenobarbital and 3-methylcholanthrene (Bock and Bock-Hennig, 2010). The rate of CYP induction is usually greater than the rate of Phase II enzyme induction. This could potentially create an imbalance between the rate at which potentially toxic metabolites are produced and the rate at which they are inactivated by conjugation (Barry and Feely, 1990). This especially becomes very important if

the inducible CYPs belong to the CYP1A subfamilies, whose metabolic activities often mediate production of toxic (carcinogenic) metabolites (Neal, 1995).

There are four main classes of CYP inducing enzyme agents. They include (i) phenobarbitone and a wide variety of structurally unrelated compounds, (ii) polycyclic aromatic hydrocarbons such as 3-methylcholanthrene, (iii) steroids, including pregnenalone 16 alpha carbonitrile and (iv) ethanol. CYP inducing compounds are believed to fall within one of these agents (Barry and Feely, 1990).

2.7 MODULATORY EFFECT OF PLANT EXTRACTS/HERBS ON CYP ENZYMES

A good number of herbal products/plant extract have been investigated regarding their effect on CYP enzymes. Some have been found to exhibit modulatory effect on the expression and catalytic activities of the various CYP isoforms. For instance, when rats were treated with Huangqin (Chinese medicinal herb), a decrease of 53% hepatic CYP1A activity was recoded. However, administration of Long Dan (Chinese herb) resulted in an increase of 50% CYP2B activity (Kang et al., 1996). Immunoblot assay showed that Huangqin increased CYP1A family but decreased CYP2B protein levels. But both herbs had no effect on microsomal aniline hydroxylase (CYP2E1) activity or its protein level (Zhou et al., 2003).

Furthermore, treatment of rats with *Angelica dahurica* Radix extract resulted in an inhibition of CYP2C11, CYP2B1, CYP31/2 and CYP1A1/2 (Ishihara et al., 2000). Treatment of rats with *Ginkgo biloba* extract resulted in a reduction in the hypotensive effect of nicardipine, a substrate for CYP3A2. This suggested that the reduction in the therapeutic potency effect of nicardipine might be due to increased expression of CYP3A2 (Shinozuka et al., 2002). Lu et al. (2018) found

that Baoyuan decoction (BYD), a traditional Chinese medicine formula composed of four herbs, inhibits CYP2B6 at low doses and induce CYP2B6 at high doses. The authors also found that CYP2D6 activity was significantly increased by 56.8% after a high dose treatment of BYD. In addition, CYP3A4, a major Phase I drug metabolizing enzyme was inhibited by BYD. Investigating the modulatory effect of thymoquinone (TQ), an oxygenated monoterpene and active constituent of *Nigella sativa* volatile oils, Albassam et al. (2018) found that TQ had inhibitory effects on CYP1A2, CYP2C9, CYP2D6 and CYP3A4.

Herbs and natural compounds isolated from herbs have been identified as substrates, inhibitors and/or inducers of various CYP enzymes. The effects of herbs on CYP enzymes appear to depend on the type of herb, dose administered, route of administration, the target organ and the species of organism (Zhou et al., 2003). Given the modulatory effects of herbs and their compounds on CYP enzymes, studies must be conducted to ascertain the effect of these herbal-based products on CYP enzymes.

2.8 ASSESSMENT OF EXTRACT/HERB-CYP MODULATION

Assessing the modulatory effect of plant extracts/herbs on CYP and subsequent effect on conventional drugs can be carried out using *in vitro*, *in vivo* and *in silico* approaches.

2.8.1 *In Vitro studies*

In vitro studies to assess herb-CYP modulation confer advantages of speed, no ethical concerns on the use of live animals and reduction in cost. There are various *in vitro* systems that can be used to study herb-CYP modulation. These include whole liver, liver slices, liver cDNA

expressed enzymes, hepatocytes and liver microsomes, among others. Human liver microsomes are hotspot for *in vitro* CYP-drug studies (Venkatakrishnan et al., 2001). Liver microsomal fractions can be obtained by homogenization and differential centrifugation. Liver microsomes contain CYPs, Flavin-containing monooxygenases (FMO), UDP-glucuronyltransferases (Venkatakrishnan et al., 2001). These microsomes (containing CYPs) can be cultured with substrates, and enzyme activity assessed by colorimetric or chromatographic assays.

2.8.2 *In vivo studies*

In vivo studies usually involve the use of live animals or humans. The fate of a xenobiotic is determined by enzyme activity in the *in vivo* model. *In vivo* studies provide a good report of the likely effect of molecule in humans but interspecies variation in substrate specificity and metabolism may make it difficult to extrapolate animal data to humans (Boobis et al., 1990a). However, currently there are stringent laws and regulations on the use of laboratory animals in research (Kurosawa et al., 2018). *In vivo* studies are also time consuming and comparatively costly (Ellinger-Ziegelbauer et al., 2008).

2.8.3 *In silico studies*

In silico studies are abstract predictions of the possible effects of biological molecules (Ekins and Wrighton, 2001). *In silico* approaches could be ligand-based in which predictive studies looks at the pharmacophore and quantitative structural-activity relationship of the molecule and metabolizing enzymes (Afzelius et al., 2001). It could also be protein-based and ligand-protein interactions (de Groot and Ekins, 2002, De Graaf et al., 2005). *In silico* studies require high technical expertise and resources.

2.9 CARBAMAZEPINE

Carbamazepine has proprietary names such as Biston, Finlepsin, Stazepine, Tegretol, among others. It is a white crystalline powder (Grzesiak et al., 2003). The drug has been approved for a number of medical conditions.

2.9.1 History of Carbamazepine

Carbamazepine was discovered by Walter Schindle, a Swedish Chemist, in 1953 (Schindler and Häfliger, 1954; Tolou-Ghamari et al., 2013). It was marketed as a drug for the treatment of epilepsy in 1962, under the brand name Tegretol in Switzerland, and got approval for use in the United Kingdom in 1965 and in the United States since 1976 (Tolou-Ghamari et al., 2013; Okuma and Kishimoto, 1998).

2.9.2 Chemistry of Carbamazepine

Carbamazepine is a tricyclic compound with molecular formula ($C_{15}H_{12}N_2O$). Its chemical structure is similar to that of the tricyclic antidepressants. Carbamazepine is metabolized to carbamazepine-10, 11-epoxide ($C_{15}H_{12}N_2O_2$) which is also pharmacologically active.

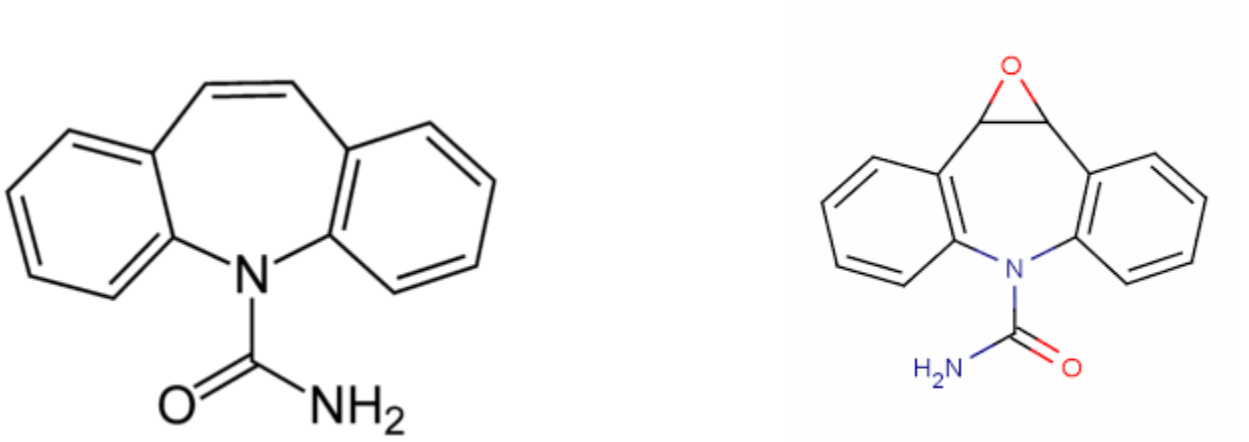

Figure 2. 7: Chemical structures of carbamazepine (left) and carbamazepine-10,11-epoxide (right)

2.9.3 Clinical Uses of Carbamazepine

Carbamazepine is used clinically for the treatment of epilepsy (partial seizures, generalized tonic-clonic seizure and mixed seizures), neuropathic pain, trigeminal neuralgia, manic, bipolar disorders and off-label treatment such as treatment of schizophrenia (Alrashood, 2016). The drug has a narrow therapeutic index, and exhibits adverse events that are related to the central nervous system (CNS) which include; fatigue, dizziness, sedation, agitation, incoordination taste changes etc. and non CNS such as dry mouth, constipation, nausea, headache muscle twitches etc. (Meador et al., 2019).

2.9.4 Pharmacodynamics (Mode of action) of Carbamazepine

Carbamazepine elicits its anti-convulsive effects by blocking sodium channels (Rogawski et al., 2016, Jo and Bean, 2014). The drug binds to voltage-gated sodium channels to inhibit convulsive episodes brought about by repetitive and sustained firing of action potential. The drug also exerts

its effect by increasing the inhibitory neurotransmitter, gamma amino butyric acid (GABA) and decreases the excitatory neurotransmitter glutamate (Tolou-Ghamari et al., 2013).

2.9.5 Pharmacokinetics of Carbamazepine

Carbamazepine is well absorbed after oral administration. Plasma levels of the drug varies with the type of formulation administered. The time to peak concentration (T_{max}) for a suspension T_{max} is 1.5 hours; conventional tablet, 4-5 hours and extended-release tablets, 3-12 hours. About 76% of the drug binds to plasma proteins.

Metabolism of the drug is mainly hepatic. CYP 3A4 is the main isoform for the metabolism of carbamazepine to carbamazepine-10, 11-epoxide. The metabolite has been found to be equipotent to the parent drug as an anticonvulsant. Metabolism of carbamazepine is slower in adults than in the young as CYP3A4 (the main isoenzyme of the drug) activity decreases with age (Tanaka, 1998). The half-life of Carbamazepine initially ranges from 25 – 65 hours and decreases to 12 – 17 hours on repeated dosing, due to induction of CYP activity. The drug is excreted in urine (72%) and in faeces (28%). About 3% of the drug is excreted as unchanged carbamazepine (Drugbank, 2019).

2.9.6 Carbamazepine interaction with Drugs/Supplements/Herbs

Carbamazepine is a potent inducer of metabolic enzymes, and thus decreases plasma concentrations of a number of drugs (Asai et al., 2017; Liu et al., 2015). The enzyme modulatory effect of carbamazepine (Ketter et al., 1991; Raish et al., 2017) makes it interact with a number of drugs when concomitantly administered.

Carbamazepine interacts with vilazodone (anti-depressive drug) by decreasing vilazodone plasma concentration through CYP3A4 induction, requiring higher than normal doses of vilazodone for therapeutic effect (Boinpally et al., 2014). Furthermore, iron supplements decreased the oral bioavailability of Carbamazepine (Perucca, 2018). Ren et al. (2019) also reports that piperine inhibits carbamazepine metabolism and suggests that high doses of piperine intake could increase carbamazepine plasma concentration with possible precipitation of adverse drug effects. In a pharmacokinetic study of the effect of *Paeonia emodi*, (herbal extract) on carbamazepine pharmacokinetics in rats, the herbal extract was found to reduce the clearance of carbamazepine through rat CYP3A2 and CYP2C11 inhibition (Raish et al., 2017). These reports of carbamazepine interaction with drugs or herbs and food supplements calls for circumspection in concomitant use of carbamazepine with other agents.

CHAPTER THREE

3.0 METHODOLOGY

3.1 Animal Care and Safety

All animal procedures and techniques used in this study were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (N.R.C., 2010).

Male and Female Sprague-Dawley rats (Hsd:SD strain), weighing 150-200 g and 6-8 weeks old, were obtained from Center for Plant Medicine Research, Mampong-Akuapim, Eastern Region, Ghana. The animals were housed in stainless steel cages. Each rat occupied a minimum space of 2 cubic feet (61 cm x 31 cm x 31 cm) with soft wood shavings as bedding for their comfort. They were fed with normal pellet diet (AGRIMAT, Kumasi), given water *ad libitum*, and maintained under standard laboratory conditions (temperature 25 ± 1°C, relative humidity 60 - 70%, and 12 h light-dark cycle). The animal feeding and water troughs were cleaned regularly to prevent contamination. Animals were acclimatized for one week under the above conditions before experiment was commenced.

3.2 CYP Enzyme Induction/Inhibition Studies

In determining the influence of Cellgevity® on liver metabolizing enzymes, animals were put into five groups (3 males and 3 females per group). The animals were administered with a single oral dose of Cellgevity® - animal equivalent of the human dose of 12.46 mg/kg *per os*, (Nair and Jacob, 2016). Group 1 was administered with the vehicle (distilled water that was used in dissolving Cellgevity®), and that served as negative control. Groups 2, 3 and 4 were administered orally with a low dose (L-D) of 38.63 mg/kg body weight (bwt), a medium dose (M-D) of 77.25 mg/kg bwt, and a high dose (H-D) of 154.50 mg/kg bwt of Cellgevity® daily,

respectively. Group 5 was administered with phenobarbital (Kinapharma Ghana, FDB/GD. 03-11149), 15 mg/kg daily, and that served as positive control. All administrations were done for 30 days. The weights of the animals were recorded before treatment and after every 7th day. Dose calculations and dilutions before treatment administration can be found at Appendices A and B. After 30-day period, the animals were sacrificed. The animals were euthanized with CO_2 administration, and death confirmed by lack of respiration and faded eye color. Livers were excised and washed in ice cold saline solution, dried with a cloth, weighed and stored at -80^{o}C.

3.3 Microsomal Preparation

The liver samples were thawed and homogenized in potassium phosphate (kPi) buffer (pH 7.4) using a mortar and pestle on ice. Homogenized samples were first centrifuged (Eppendorf 5810 R, Germany) at 4,000 rpm for 20 min at 4^{o}C. The supernatant was then ultra-centrifuged (Beckman Avanti J-25, USA) at 25,000 rpm for 2 h. The pellets of each rat liver, which constituted the microsomes were aliquoted and stored at -80^{o}C until use.

3.4 Determination of Protein Concentration of Microsomes

Two-fold serial dilution of 1mg/mL Bovine Serum Albumin (BSA), protein standard was prepared: 0.5 mg/mL, 0.125 mg/mL, 0.0625 mg/mL, 0.03125 mg/mL, 0.015625 mg/mL and 0.0078125 mg/mL. Dilutions of the microsomes, 400 and 800 times, were used for the protein concentration determination (See appendix D and E).

Aliquots of 10 µL each of the microsomal sample and the standard were pipetted in triplicates into a 96 well plate. A volume of 200 µL of protein assay dye (Biorad, USA) was added to each well. The mixtures were incubated at room temperature (25^{o}C) for 5 min. The absorbance of

mixtures was measured at the wavelength of 590 nm using spectrophotometer (Tecan Infinite M200 Pro Plate Reader, Japan).

The mean absorbance from triplicate samples was obtained from the standard curve. The equation of the line of best fit was used to estimate the corresponding protein concentrations of each sample (See appendix G).

3.5. CYP ASSAYS

The methods for the CYP assays were performed as described by Appiah-Opong et al. (2007) with few modifications.

3.5.1 CYP2C9 – Diclofenac hydroxylation and CYP2D6 – Dextromethorphan O-Demethylation Assays

The reaction mixtures for these assays consisted of a volume of 350 µL of 0.1M buffer (pH 7.4), 50 µL of 1 mM substrate (diclofenac for CYP2C9 assay and dextromethorphan for CYP2D6) and 50 µL of 2.5 mg/mL of microsome (obtained from rat livers from respective Groups) were mixed in Eppendorf tubes. The mixtures were pre-incubated at 37^{o}C for 5 min. A volume of 50 µL of 1mM nicotinamide adenine dinucleotide phosphate (NADPH) was added, mixed and incubated at 37^{o}C for 45 min. A 100 µL 60 mM stopping solution of zinc sulphate ($ZnSO_4.7H_2O$) was added and the mixture centrifuged at 4000 rpm for 5 min. The supernatant were aliquoted into High Performance Liquid Chromatography (HPLC) vials.

3.5.1.1 HPLC Analysis of Supernatant

The samples obtained as described in 3.5.1 were analyzed using a Shimadzu prominence reverse phase HPLC consisting of a binary solvent delivery system (LC - 20AB), a degasser

(DGU-20A3), an auto-sampler (SIL - 20ACHT), a column temperature controller (CTO - 10AS VP) and a photo diode array detector (SPD - M20A) for CYP2C9 metabolites and a fluorescence detector (RF - 10A $_{XL}$) for CYP2D6 metabolites. The HPLC used had a Shimadzu C18 column (diameter 5 µm, length x width, 150 mm ×4.6 mm), and the eluent for CYP2C9 consisted of 60% of 20 mM kPi (pH 7.4), 22.5% methanol and 17.5% acetonitrile, and for CYP2D6, the eluent comprised 24 % acetonitrile, 75% distilled water and 1% TEA. The flow rate was 1 mL/min. The column temperature was maintained at 40^{o}C and the injection volume was 20 µL.

3.5.2 CYP1A1/2 – Ethoxyresorufin O-Deethylase (EROD), CYP2B1/2 – Pentoxyresorufin O-dealkylase (PROD) and CYP3A4 – Benzyloxyresorufin O-Debenzylase (BROD) Assays

Assays for CYP1A1/2, CYP2B1/2 and CYP3A4 was done as described by by Appiah-Opong et al. (2007) with few modifications. Activities of the CYPs were assayed in a total volume of 100 µL. A volume of 70 µL kPi buffer (pH 7.4) was pipetted into 96-well black plate. This was followed by addition of 10 µL of 50 µM substrate (resorufin ethyl ether for CYP1A1/2, pentoresorufin for CYP2B1/2 and resorufin benzyl ether for CYP3A4). All substrates were obtained from Sigma-Aldrich, United Kingdom. The final concentration of each substrate in the assays was 5 µM in 0.25% (v/v) Dimethyl Sulfoxide (DMSO). A volume of 10 µL enzyme (microsome from each rat liver from respective Groups) corresponding to 1 mg/mL protein concentration, and vehicle was added in triplicates. The mixtures were pre-incubated at 37 ^{o}C for 5 min. A volume of 10 µL of NADPH was then added and the setup incubated for 10 min for CYP1A1/2; 20 min for CYP2B1/2 and 30 min for CYP3A4 assays, respectively. A volume of 40 µL of stopping solution (20% 0.5 M Tris: 80% acetonitrile) was added to each setup and mixed gently. Fluorescence of the metabolites was read at the wavelength of 530 nm excitation and 586

nm emission. Triplicates experiments were performed. The mean absorbance of the blank was subtracted from the mean absorbance of each sample to obtain the net absorbance.

3.6 The Effect of Cellgevity® on Carbamazepine Pharmacokinetics in Healthy SD Rats

Healthy male SD rats were put into two groups, with 5 rats in each group. Group 1 was given Cellgevity® (77.25 mg/kg, *po*) with normal saline (vehicle) and group 2 was administered with Cellgevity® (77.25 mg/kg, *po*) and carbamazepine (80 mg/kg *po*) concurrently. All treatments were done for 14 days. On the 15th day, blood samples were taken by tail snipping at 30 min, 1 h, 4 h, 12 h and 24 h post administration into microtainer gel tubes and allowed to clot. The samples were centrifuged at 2000 rpm for 5 min. The serum was collected into cryotubes with the aid of a micropipette. The serum samples were stored at -70^{o}C until assayed for carbamazepine.

3.7 Assay for Carbamazepine in Serum

Due to low sample volumes, serum samples of rats from the same group (5 animals) at each time point were pooled together, such that, for instance serum samples of Group 1 rats at time 4 hours, were pooled together to obtain a single sample. Usually, challenges with low sample volume can be circumvented by the approach of sample-pooling (Nagy et al., 2018). Analysis of carbamazepine in serum was done by Fluorescence Polarization Immunoassay (FPIA) (Cobas Integra® 400 Plus).

3.8 Statistical Analysis

CYP enzyme activity of treatment groups were compared to negative control group by calculating the percentage increase or decrease in negative control relative to treatment groups.

All values are expressed as mean ± standard deviations (SD). Differences between groups were tested for significance using One-Way and Two-Way Analysis of Variance (ANOVA). Significant differences were calculated with Bonferroni's Multiple Comparison Tests. *P*-values < 0.05 were considered statistically significant.

Pharmacokinetic (PK) parameter estimation was done by non-compartmental analysis using GraphPad Prism 8.0. Peak drug concentration (C_{max}) and time to achieve C_{max}, (T_{max}), were determined from the concentration-time curves. Area under the drug concentration-time curve (AUC) was calculated by the linear trapezoidal rule. AUC was determined till the last measurement point and was extrapolated to infinity $AUC_{0\rightarrow\infty}$. Elimination rate constant (Ke) was determined by linear regression of the terminal (linear) part of the log plasma concentration-time curve. The elimination half-life ($t_{1/2}$) was obtained using the equation, $t_{1/2} = 0.693Ke^{-1}$.

CHAPTER FOUR

4.0 RESULTS

4.1 CYP ENZYME ACTIVITY

4.1.1 CYP3A4 Activity

CYP3A4 enzyme activity in the treatment groups were estimated relative to the negative control (distilled water only). CYP3A4 activities are as shown in Figures 4.1, 4.2 and 4.3 for combined sex, female and male rats, respectively. CYP3A4 enzyme activity was found to be elevated in the phenobarbital treated and Cellgevity® treated groups in comparison with the negative control group, for the combined sex and both sexes groups. However, it was only the group treated with phenobarbital that was found to significantly differ ($p < 0.001$) compared to negative control. The Cellgevity® treated groups showed increased enzyme activity compared to negative control, but the difference was not statistically significant, in any of the groups. There was also no dose-dependent effect of Cellgevity® on rat CYP3A4 enzyme activity.

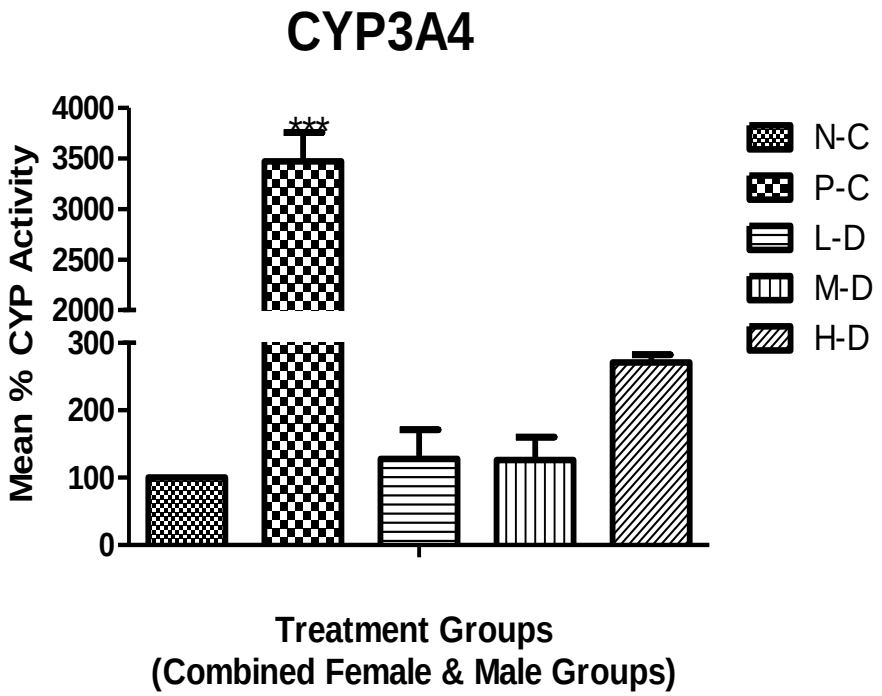

Figure 4. 1: Rat liver CYP3A4 activity for various treatment groups after 30 days administration

*N-C = Negative control; P-C = Positive control (Phenobarbital); L-D = Low dose (38.63 mg/kg) of Cellgevity®; M-D = Medium dose (77.25 mg/kg) of Cellgevity®; H-D = High dose (154.50 mg/kg) of Cellgevity®. Data represent mean ± standard deviations. *** is for values statistically different from the N-C as indicated with $p < 0.001$.*

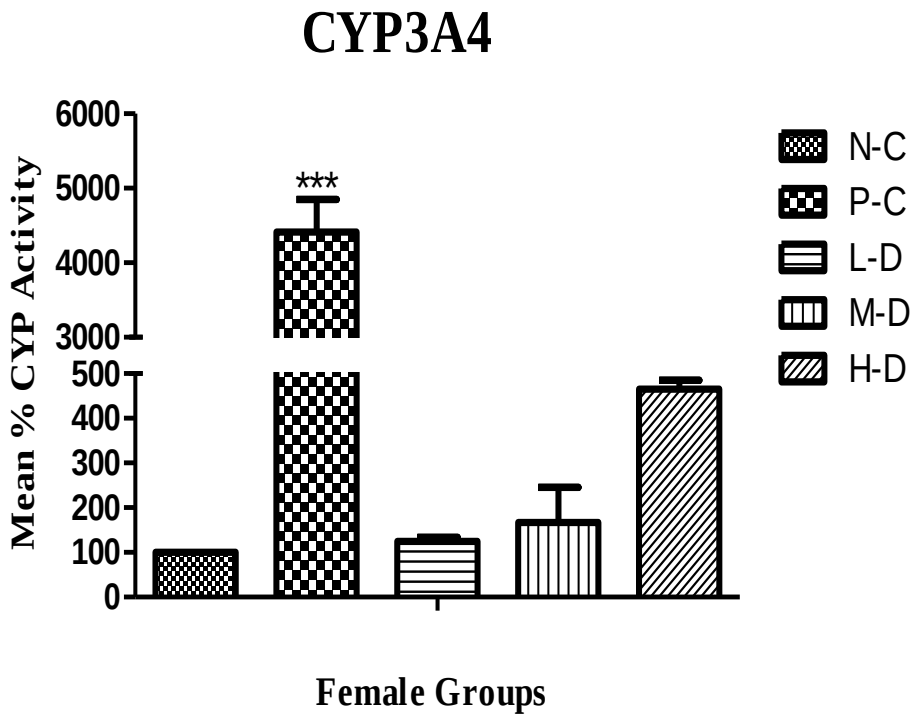

Figure 4. 2: Rat (female) liver CYP3A4 activity for various treatment groups after 30 days administration

*N-C = Negative control; P-C = Positive control (Phenobarbital); L-D = Low dose (38.63 mg/kg) of Cellgevity®; M-D = Medium dose (77.25 mg/kg) of Cellgevity®; H-D = High dose (154.50 mg/kg) of Cellgevity®. Data represent mean ± standard deviations. *** is for values statistically different from the N-C as indicated with $p < 0.001$.*

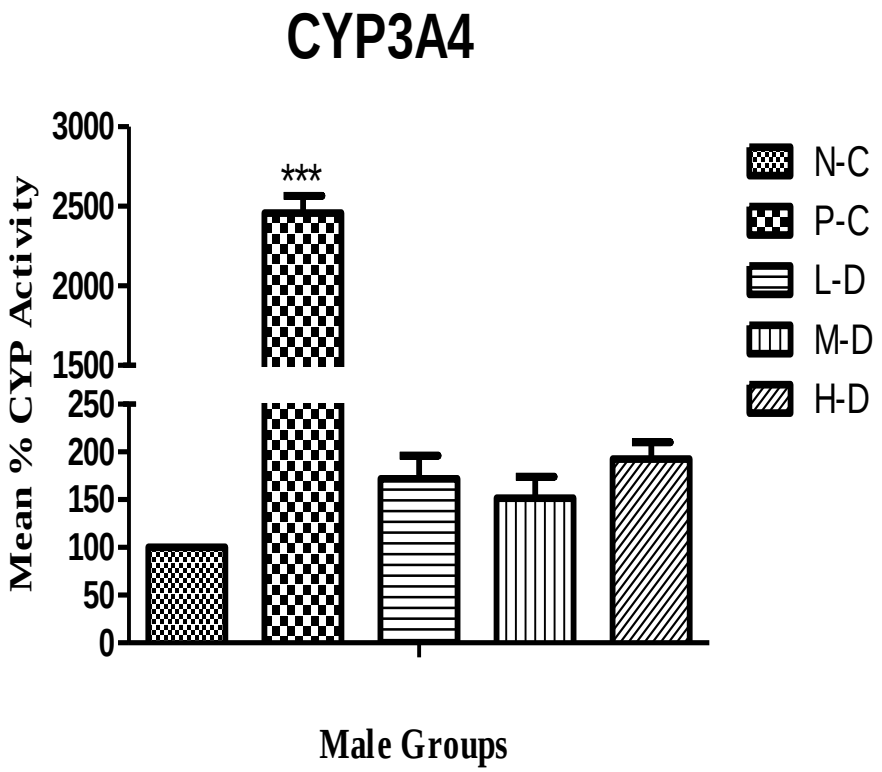

Figure 4. 3: Rat (male) liver CYP3A4 activity for various treatment groups after 30 days administration

*N-C = Negative control; P-C = Positive control (Phenobarbital); L-D = Low dose (38.63 mg/kg) of Cellgevity®; M-D = Medium dose (77.25 mg/kg) of Cellgevity®; H-D = High dose (154.50 mg/kg) of Cellgevity®. Data represent mean ± standard deviations. *** is for values statistically different from the N-C as indicated with $p < 0.001$.*

4.1.2 CYP2B1/2 Activity

CYP2B1/2 enzyme activity in the treatment groups were estimated relative to the negative control (distilled water only). Figures 4.4, 4.5 and 4.6, show CYP2B1/2 enzyme activity for combined sex, female and male rats, respectively. CYP2B1/2 enzyme activity was found to be elevated in the phenobarbital-treated and Cellgevity®-treated groups in comparison with the negative control group. However, it was only the group treated with phenobarbital that was

found to significantly differ ($p < 0.001$) compared to negative control. The Cellgevity®-treated groups showed elevated levels of CYP activity compared to the negative control, but the difference was not statistically significant, for any of the groups. There was also no dose-dependent effect of Cellgevity® on rat CYP2B1/2 enzyme activity.

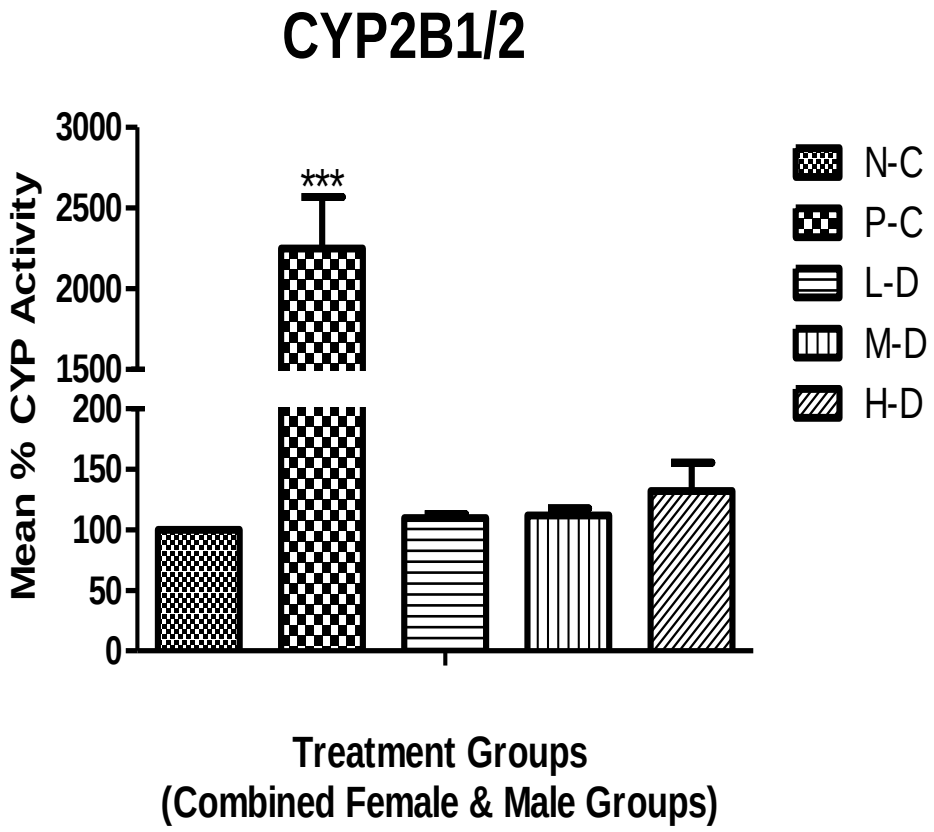

Figure 4. 4: Rat liver CYP2B1/2 activity for various treatment groups after 30 days administration

*N-C = Negative control; P-C = Positive control (Phenobarbital); L-D = Low dose (38.63 mg/kg) of Cellgevity®; M-D = Medium dose (77.25 mg/kg) of Cellgevity®; H-D = High dose (154.50 mg/kg) of Cellgevity®. Data represent mean ± standard deviations. *** is for values statistically different from the N-C as indicated with $p < 0.001$.*

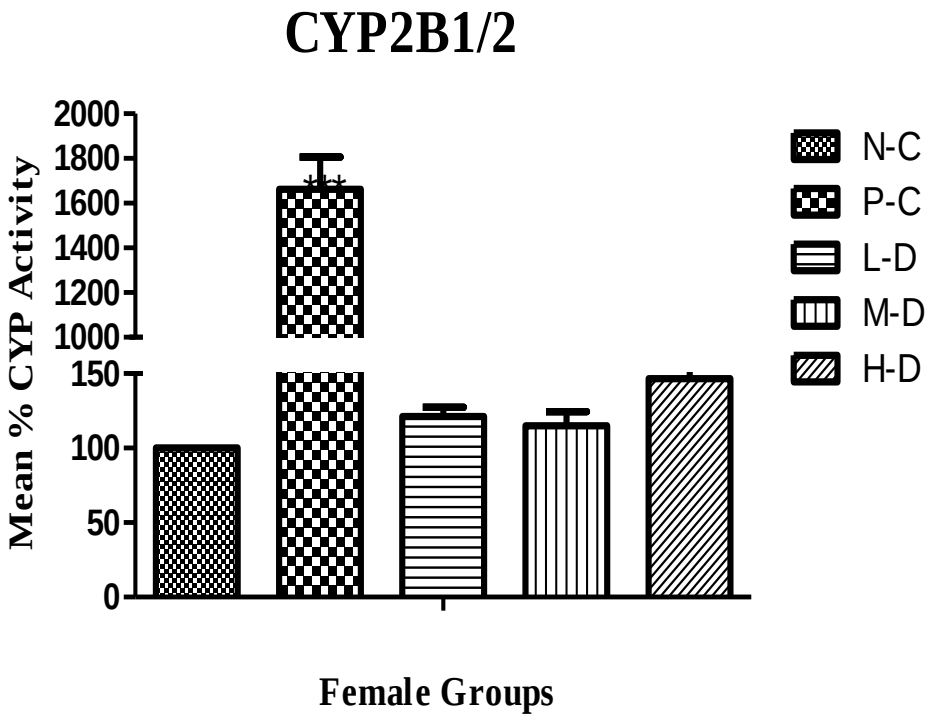

Figure 4. 5: Rat (female) liver CYP2B1/2 activity for various treatment groups after 30 days administration

N-C = Negative control; P-C = Positive control (Phenobarbital); L-D = Low dose (38.63 mg/kg) of Cellgevity®; M-D = Medium dose (77.25 mg/kg) of Cellgevity®; H-D = High dose (154.50 mg/kg) of Cellgevity®. Data represent mean ± standard deviations. ∗∗∗ is for values statistically different from the N-C as indicated with $p < 0.001$.

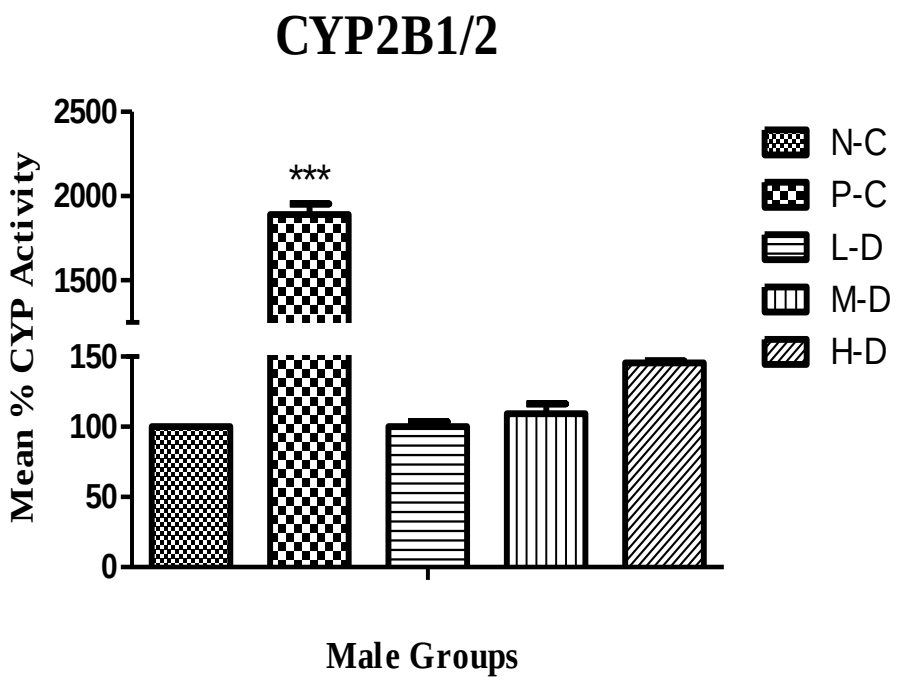

Figure 4. 6: Rat (male) liver CYP2B1/2 activity for various treatment groups after 30 days administration

*N-C = Negative control; P-C = Positive control (Phenobarbital); L-D = Low dose (38.63 mg/kg) of Cellgevity®; M-D = Medium dose (77.25 mg/kg) of Cellgevity®; H-D = High dose (154.50 mg/kg) of Cellgevity®. Data represent mean ± standard deviations; *** are values statistically different from the N-C as indicated with $p < 0.001$.*

4.1.3 CYP1A1/2 Activity

CYP1A1/2 enzyme activity in the treatment groups were estimated relative to the negative control (distilled water only). Levels of CYP1A1/2 enzyme activity are as shown in Figures 4.7, 4.8 and 4.9 for combined sex, female and male rats groups, respectively. CYP1A1/2 enzyme activity was found to be elevated in the phenobarbital-treated and Cellgevity®-treated groups in comparison with the negative control group. The phenobarbital-treated group, the M-D and H-D (combined sex) groups of Cellgevity® treatment were found to significantly differ ($p < 0.001$, p

< 0.05, p < 0.001 respectively) compared to negative control. The same results were recorded for the female rats. However, the Cellgevity®-treated L-D and M-D (male) groups showed elevated CYP activity compared to negative control, but the difference were not statistically significant. There was a dose-dependent effect of Cellgevity® on female rat CYP1A1/2 enzyme activity, but the combined sex and male groups CYP1A1/2 enzyme activity was not dose-dependent.

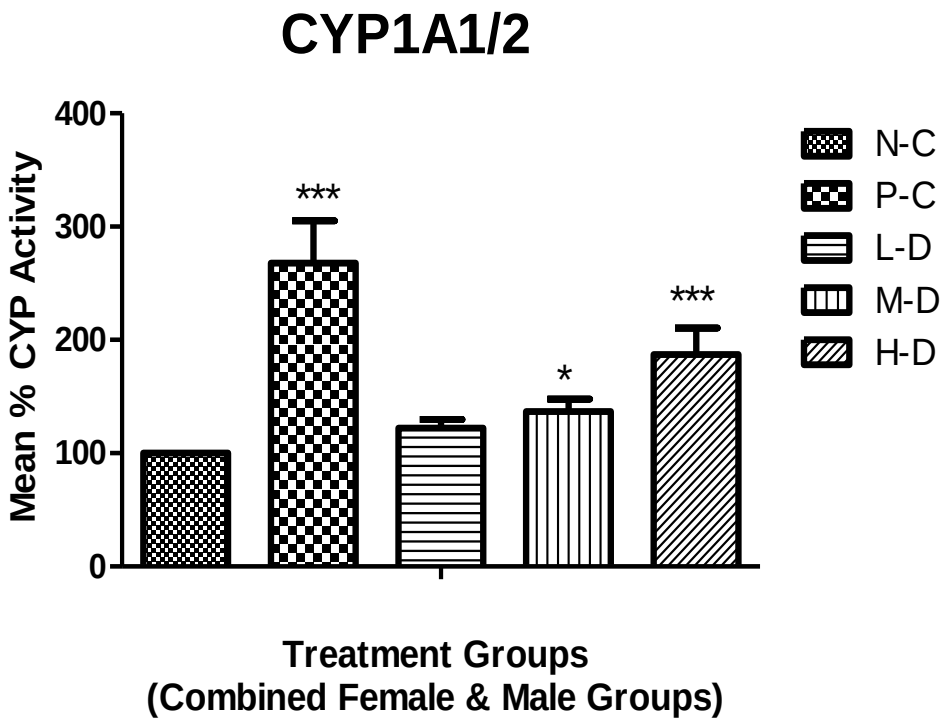

Figure 4. 7: Rat liver CYP1A1/2 activity for various treatment groups after 30 days administration

N-C = Negative control; P-C = Positive control (Phenobarbital); L-D = Low dose (38.63 mg/kg) of Cellgevity®; M-D = Medium dose (77.25 mg/kg) of Cellgevity®; H-D = High dose (154.50 mg/kg) of Cellgevity®. Data represent mean ± standard deviations. ∗ and ∗∗∗ are values statistically different from the N-C as indicated with $p < 0.05$ and $p < 0.001$, respectively.

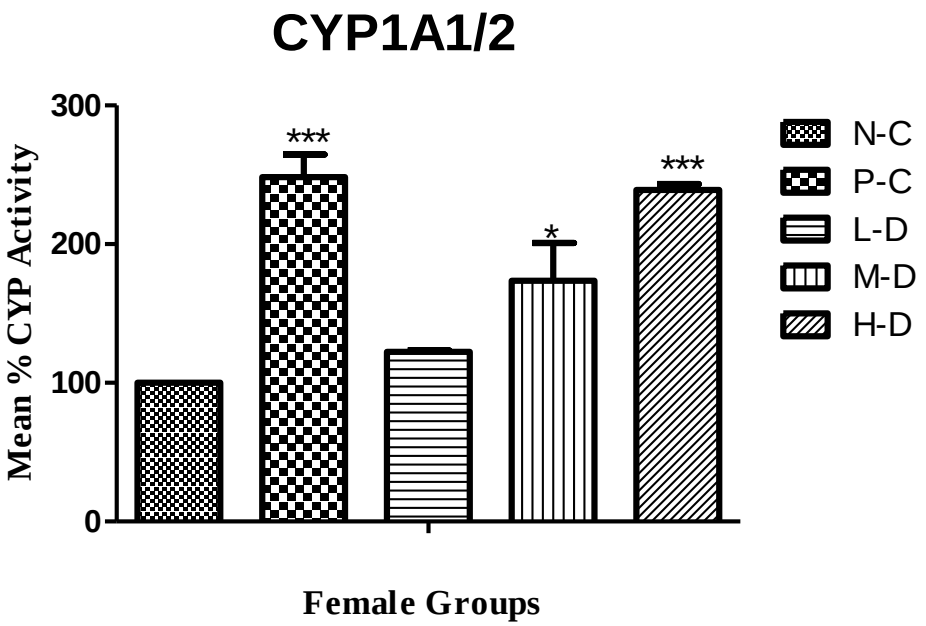

Figure 4. 8: Rat (female) liver CYP1A1/2 activity for various treatment groups after 30 days administration

N-C = Negative control; P-C = Positive control (Phenobarbital); L-D = Low dose (38.63 mg/kg) of Cellgevity®; M-D = Medium dose (77.25 mg/kg) of Cellgevity®; H-D = High dose (154.50 mg/kg) of Cellgevity®. Data represent mean ± standard deviations. ∗ and ∗∗∗ are values statistically different from the N-C as indicated with $p < 0.05$ and $p < 0.001$, respectively.

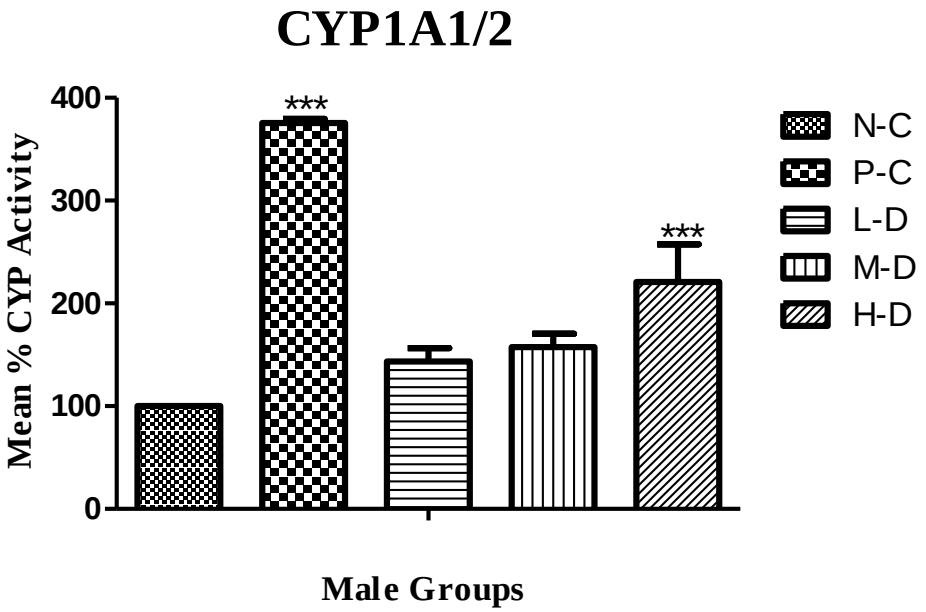

Figure 4. 9: Rat (male) liver CYP1A1/2 activity for various treatment groups after 30 days administration

*N-C = Negative control; P-C = Positive control (Phenobarbital); L-D = Low dose (38.63 mg/kg) of Cellgevity®; M-D = Medium dose (77.25 mg/kg) of Cellgevity®; H-D = High dose (154.50 mg/kg) of Cellgevity®. Data represent mean ± standard deviations.*** *is for values statistically different from the N-C as indicated with* $p < 0.001$.

4.1.4 CYP2C9 Activity

CYP2C9 enzyme activity in the treatment groups were estimated relative to the negative control (distilled water only). Levels of CYP2C9 enzyme activity are as shown in Figures 4.10, 4.11 and 4.12, for combined sex, female and male rats, respectively. CYP2C9 enzyme activity was found to be elevated in the phenobarbital-treated and Cellgevity®-treated groups in comparison with the negative control group, for both sexes. Except the combined sex and female L-D groups ($p > 0.05$), all the other treatment groups were found to significantly differ compared to the negative control. The phenobarbital-treated group, Cellgevity®-treated: L-D (male), M-D (combined sex),

M-D (female and male), and H-D groups had $p < 0.001$, $p < 0.05$, $p < 0.002$, $p < 0.001$ and $p < 0.001$ respectively. The combined sex and female groups recorded dose-dependent activity. However the male group did not show dose-dependent effect of Cellgevity® on rat CYP2C9 enzyme activity.

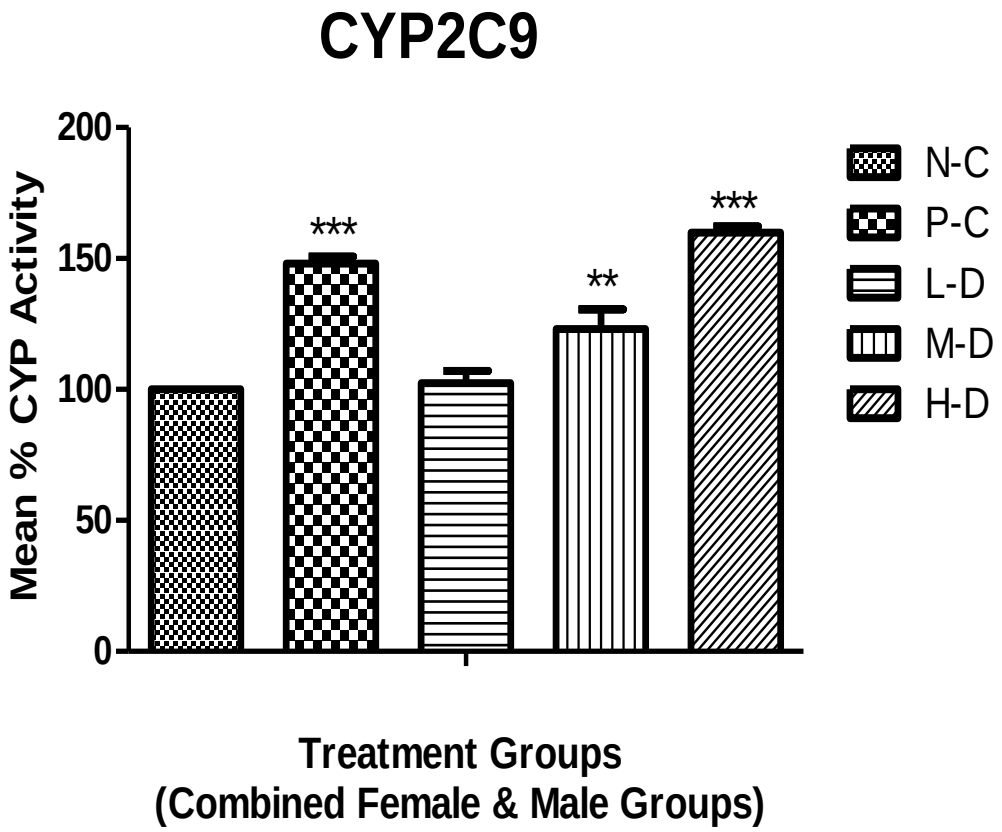

Figure 4. 10: Rat liver CYP2C9 activity for various treatment groups after 30 days administration

*N-C = Negative control; P-C = Positive control (Phenobarbital); L-D = Low dose (38.63 mg/kg) of Cellgevity®; M-D = Medium dose (77.25 mg/kg) of Cellgevity®; H-D = High dose (154.50 mg/kg) of Cellgevity®. Data represent mean ± standard deviations. ** and *** are for values statistically different from the N-C as indicated with $p < 0.01$ and $p < 0.001$, respectively.*

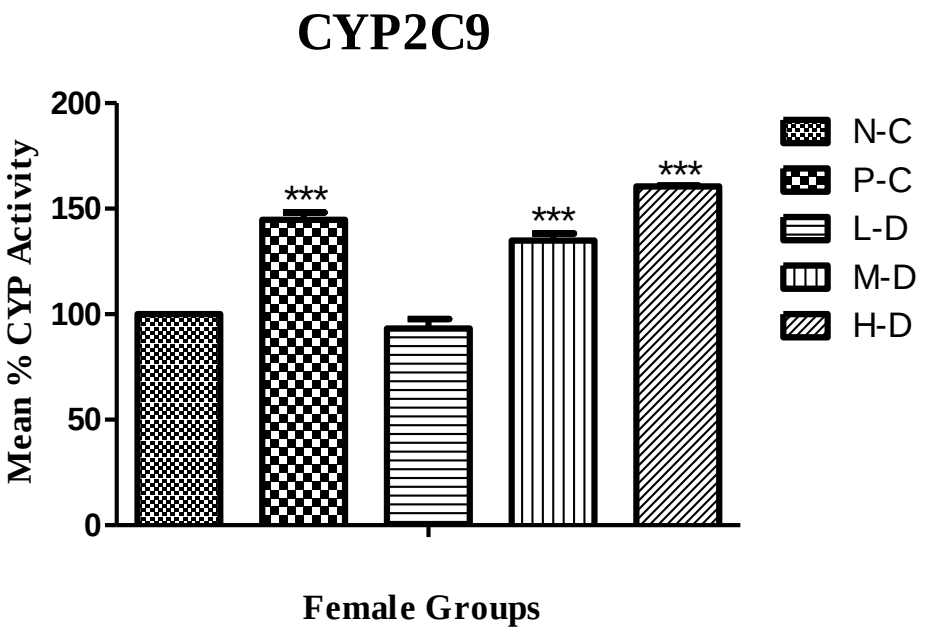

Figure 4. 11: Rat (female) liver CYP2C9 activity for various treatment groups after 30 days administration

*N-C = Negative control; P-C = Positive control (Phenobarbital); L-D = Low dose (38.63 mg/kg) of Cellgevity®; M-D = Medium dose (77.25 mg/kg) of Cellgevity®; H-D = High dose (154.50 mg/kg) of Cellgevity®. Data represent mean ± standard deviations. *** is for values statistically different from the N-C as indicated with $p < 0.001$.*

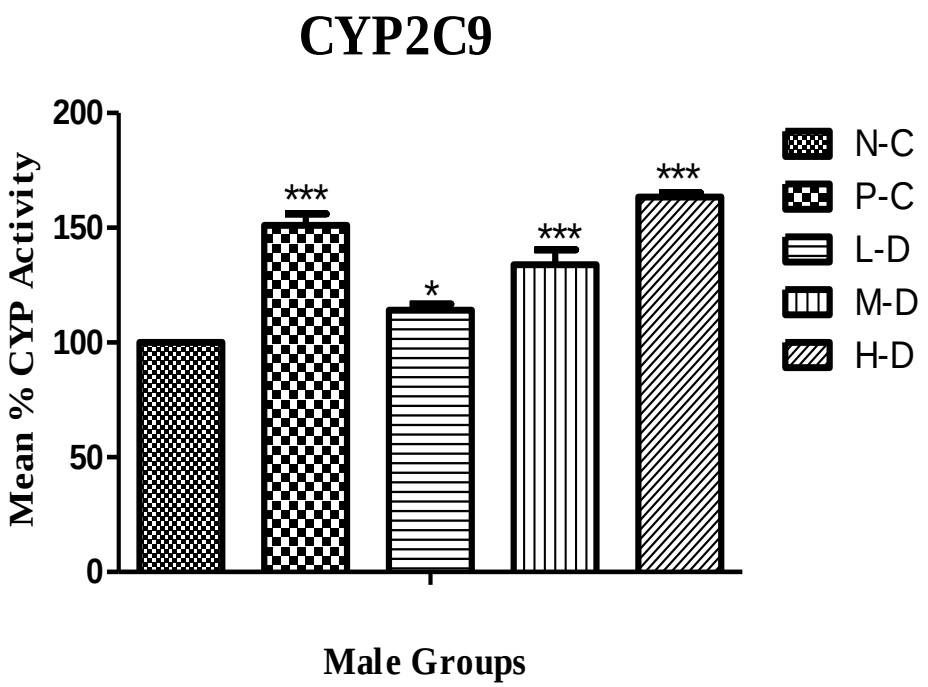

Figure 4. 12: Rat (male) liver CYP2C9 activity for various treatment groups after 30 days administration

N-C = Negative control; P-C = Positive control (Phenobarbital); L-D = Low dose (38.63 mg/kg) of Cellgevity®; M-D = Medium dose (77.25 mg/kg) of Cellgevity®; H-D = High dose (154.50 mg/kg) of Cellgevity®. Data represent mean ± standard deviations. ⁎ and ⁎⁎⁎ are values statistically different from the N-C as indicated with $p < 0.05$ and $p < 0.001$, respectively.

4.1.5 CYP2D6 Activity

CYP2D6 enzyme activity in the treatment groups were estimated relative to the negative control (distilled water only). Levels of CYP2D6 enzyme activity are as shown in Figures 4.13, 4.14 and 4.15, for combined sex, female and male rats, respectively. CYP2D6 enzyme activity was found to be elevated in the phenobarbital treated and Cellgevity® treated groups in comparison with the negative control group. All treatment groups of both phenobarbital and Cellgevity® treatment groups were found to be significantly different ($p < 0.05$) compared to the negative

control. All the female treatment groups had $p < 0.001$ in comparison to negative control. For the combined sex and male treatment groups, the M-D (combined sex), P-C and H-D groups had $p < 0.01$ and L-D and M-D (male) groups had $p < 0.05$ in comparison to negative control.

The female group showed a dose-dependent effect of Cellgevity® on rat CYP2D6 enzyme activity. However, the combined sex and male groups did not show dose-dependent effect of Cellgevity® on rat CYP2D6 enzyme activity.

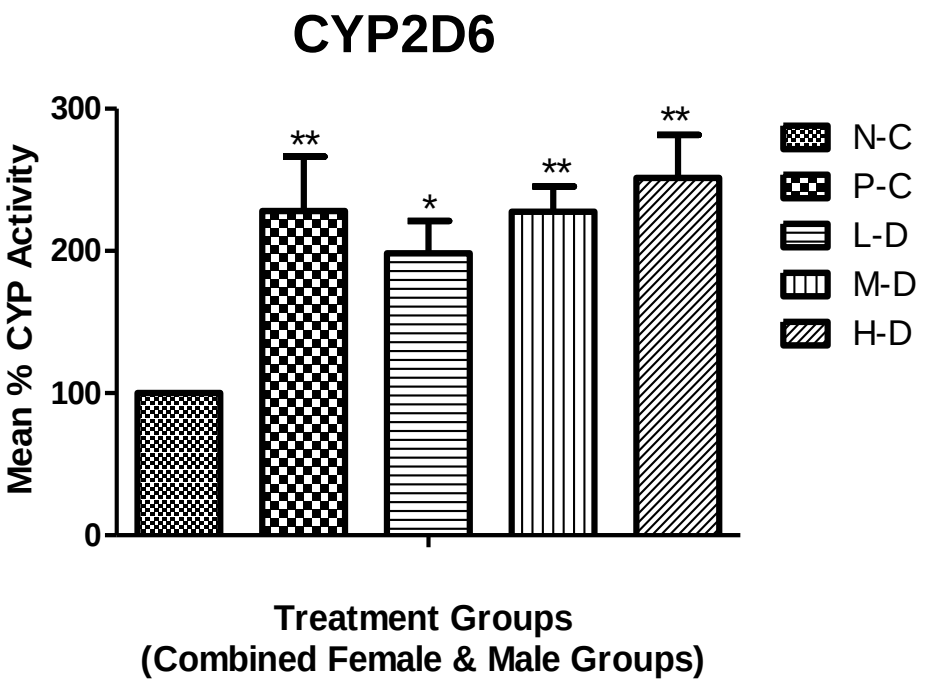

Figure 4. 13: Rat liver CYP2D6 activity for various treatment groups after 30 days administration

N-C = Negative control; P-C = Positive control (Phenobarbital); L-D = Low dose (38.63 mg/kg) of Cellgevity®; M-D = Medium dose (77.25 mg/kg) of Cellgevity®; H-D = High dose (154.50 mg/kg) of Cellgevity®. Data represent mean ± standard deviations. ⁎ and ⁎⁎ are values statistically different from the N-C as indicated with $p < 0.05$ and $p < 0.01$, respectively.

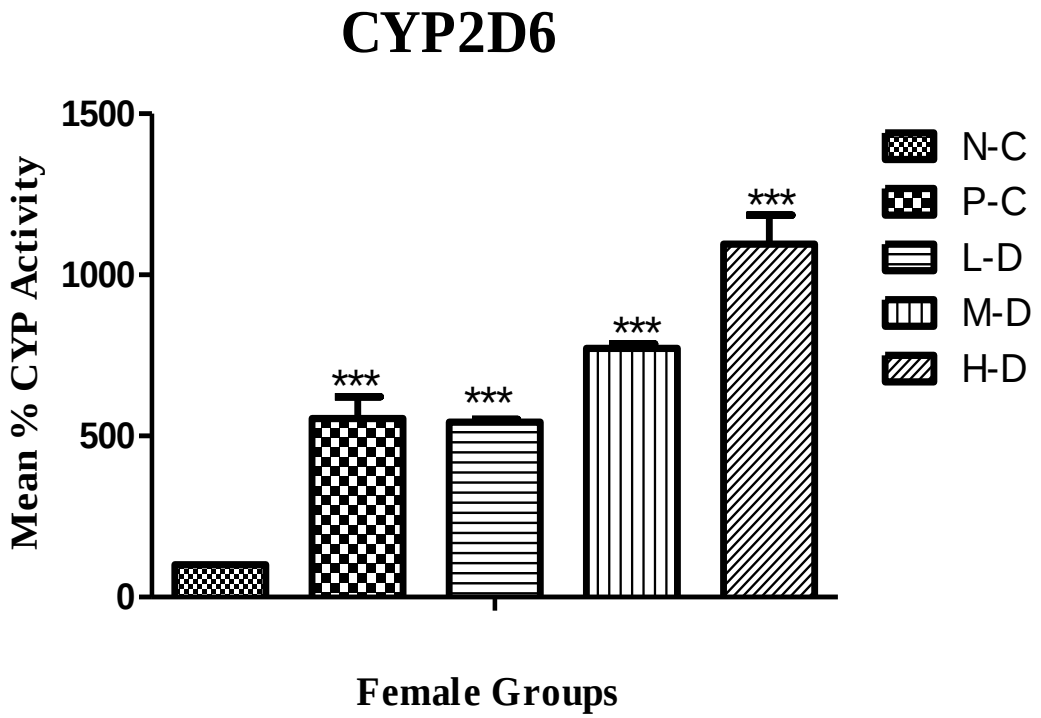

Figure 4. 14: Rat (female) liver CYP2D6 activity for various treatment groups after 30 days administration

*N-C = Negative control; P-C = Positive control (Phenobarbital); L-D = Low dose (38.63 mg/kg) of Cellgevity®; M-D = Medium dose (77.25 mg/kg) of Cellgevity®; H-D = High dose (154.50 mg/kg) of Cellgevity®. Data represent mean ± standard deviations. *** is for values statistically different from the N-C as indicated with $p < 0.001$.*

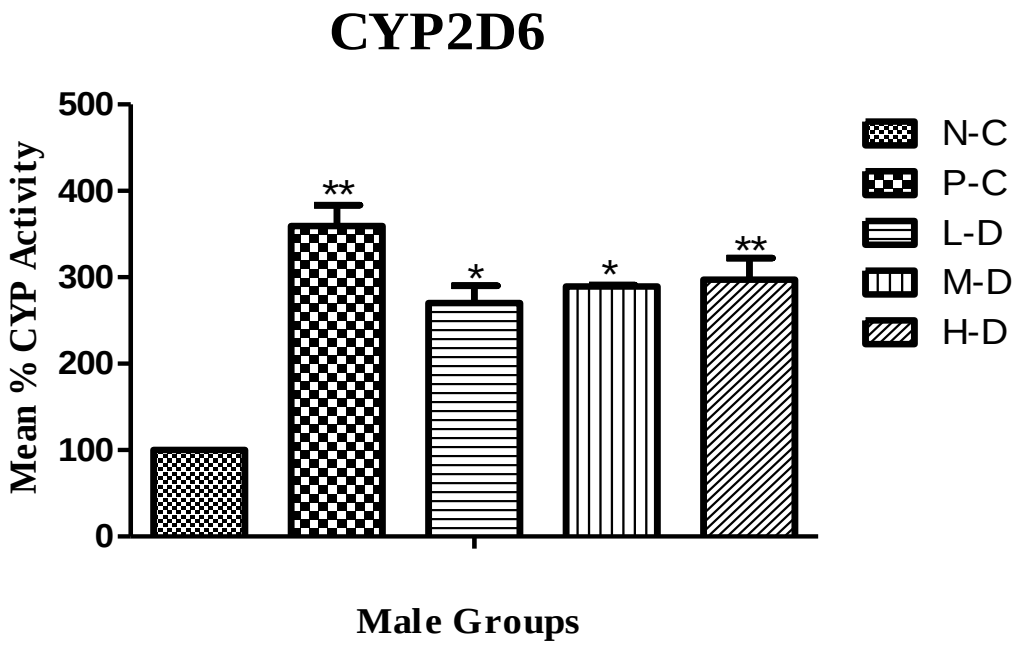

Figure 4. 15: Rat (male) liver CYP2D6 activity for various treatment groups after 30 days administration

N-C = Negative control; P-C = Positive control (Phenobarbital); L-D = Low dose (38.63 mg/kg) of Cellgevity®; M-D = Medium dose (77.25 mg/kg) of Cellgevity®; H-D = High dose (154.50 mg/kg) of Cellgevity®. Data represent mean ± standard deviations. ∗ and ∗∗ are values statistically different from the N-C as indicated with $p < 0.05$ and $p < 0.01$, respectively.

4.1.6 Overall Effect of Cellgevity® on Rat CYP Enzyme Activity

The overall effect of this supplement on selected CYP enzymes is summarized in Table 4.1. Levels of CYP3A4 and CYP2B1/2 activity were not found to differ significantly when Cellgevity®-treated groups were compared with the negative control. However, CYP1A1/2, CYP2C9 and CYP2D6 enzyme activity (both male and female rats) from rats treated with Cellgevity® (some of the groups) were found to be significantly increased compared to negative control. Increase in enzyme activity observed for CYP2C9 for the treatment groups was found to

be dose-dependent in both sexes. There was statistically significant ($p < 0.05$) differences in CYP enzyme activities for all the tested CYPs in the treatment groups across both sexes except CYP2B1/2 and CYP1A1/2 activities.

Table 4. 1: Summary of the effect of Cellgevity® on rat CYP enzyme activity

CYP Isoform	**Assay**	**Effect of Cellgevity® on CYP activity**
CYP3A4 Combined sex	BROD	No significant increase in enzyme activity
Female		No significant increase in enzyme activity
Male		No significant increase in enzyme activity
CYP2B1/2 Combined sex	PROD	No significant increase in enzyme activity
Female		No significant increase in enzyme activity
Male		No significant increase in enzyme activity
CYP1A1/2 Combined sex	MROD	Significant increase in enzyme activity (M-D: $p < 0.05$; H-D: $p < 0.001$)
Female		Significant increase in enzyme activity (M-D: $p < 0.05$; H-D: $p < 0.001$)
Male		Significant increase in enzyme activity (H-D: $p < 0.001$)
CYP2C9 Combined sex	Diclofenac Hydroxylation	Significant increase in enzyme activity (M-D: $p < 0.01$; H-D: $p < 0.001$)
Female		Significant increase in enzyme activity ($p < 0.001$; M-D and H-D)
Male		Significant increase in enzyme activity (L-D: $p < 0.05$; M-D and H-D: $p < 0.001$)
CYP2D6 Combined sex	Dextromethorphan O-Demethylation	Significant increase in enzyme activity (L-D: $p < 0.05$; M-D and H-D: $p < 0.01$)
Female		Significant increase in enzyme activity ($p < 0.001$; L-D, M-D and H-D)
Male		Significant increase in enzyme activity (L-D and M-D: $p < 0.05$; H-D: $p < 0.01$)

N-C = Negative control; P-C = Positive control (Phenobarbital); L-D = Low dose (38.63 mg/kg) of Cellgevity®; M-D = Medium dose (77.25 mg/kg) of Cellgevity®; H-D = High dose (154.50 mg/kg) of Cellgevity®.

4.2. EFFECT OF CELLGEVITY® ON PHARMACOKINETICS OF CARBAMAZEPINE

4.2.1 Concentration-time curves for two groups

Figure 4.12 shows concentration-time curves of carbamazepine in rats administered carbamazepine with Cellgevity® and carbamazepine with normal saline on Day 15 of sample collection. From visual inspection, it can be observed that the concentration-time curve of rats administered carbamazepine with Cellgevity® had a higher peak compared to the rats administered carbamazepine with normal saline.

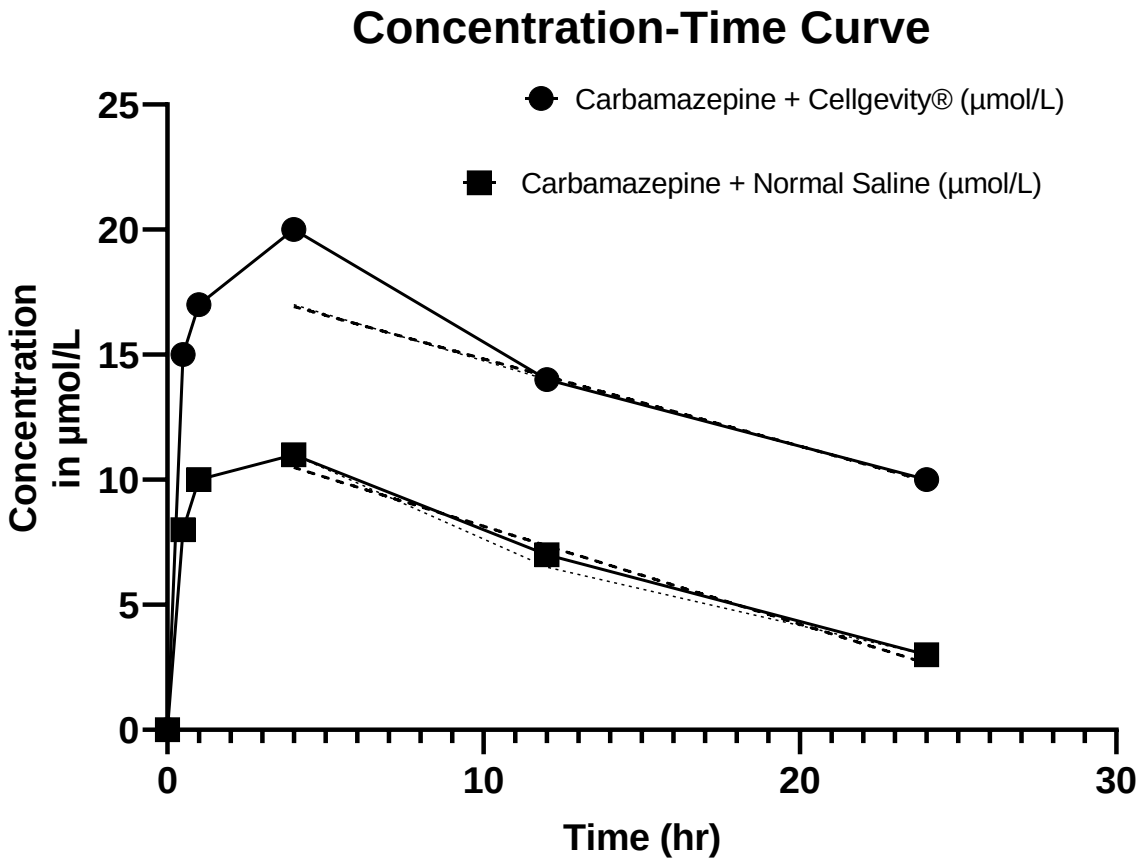

Figure 4. 16: A serum concentration-time curve (on Day 15) of carbamazepine (80 mg/kg po) administered with normal saline and Cellgevity® (77.25 mg/kg po)

4.2.2 Pharmacokinetic parameters of the two groups

Pharmacokinetic parameters obtained from the concentration-time curves for the two groups are shown in Table 4.2. The peak concentration for rats administered carbamazepine with Cellgevity® was 2-fold greater compared to carbamazepine with saline. Total drug exposure at the last sample time point ($AUC_{0\rightarrow24}$) was also about 2-fold greater in rats administered carbamazepine with Cellgevity® compared to carbamazepine with saline. The half-life was longer (2.3 hours) for rats in the carbamazepine with Cellgevity® group.

Table 4. 2: Pharmacokinetic parameters of carbamazepine for the two groups of rats

Parameter	**Carbamazepine + Cellgevity®**	**Carbamazepine + Normal Saline**
C_{max} (µmol/L)	20	11
T_{max} (h)	4	4
k_e (h^{-1})	0.3	0.4
$t_{1/2}$ (h)	2.3	1.7
$AUC_{0\rightarrow24}$ (µmol.h/L)	347	170
$AUC_{0\rightarrow\infty}$ (µmol.h/L)	375.7	177.7

CHAPTER FIVE

5.0 DISCUSSION AND CONCLUSION

5.1 DISCUSSION

The increase in chronic conditions, coupled with high cost of orthodox medicines has led to an increase in the use of CAM by patients (Shorofi and Arbon, 2017). Although there are efficacy and safety concerns of CAM (Durant, 1998), research has shown a good number of CAM are effective in preventing certain disease conditions (Moquin et al., 2009). Concerns, however, have been raised of possible interaction between these CAM, often of herbal origin, and conventional drugs (Goey et al., 2013; Bedada and Neerati, 2018b).

This study was a follow-up on an earlier one that sought to investigate the potential of Cellgevity® to modulate CYP enzymes. In the earlier study by N'guessan et al. (2018), lower doses (2 mg/kg, 4 mg/kg and 8 mg/kg) than the recommended human dose scaled to rats was used. Treatment with Cellgevity® was also for 7 days in Sprague-Dawley rats. In that study CYP3A4, CYP2C9 and CYP1A2 were significantly inhibited by Cellgevity® after the 7-day treatment period. In the current study, animal equivalent dose (77.25 mg/kg) of recommended human dose (12.46 mg/kg) per serving was used. The Sprague-Dawley rats also received treatment for a period of 30 days. Since xenobiotics are known to modulate CYP enzymes depending on factors such as dose and treatment duration (Horn et al., 2002), this study attempt to modify the study done by N'guessan et al. (2018) with 3 doses calculated after dose scaling from humans, and the agents administered over a longer period of time (30 days).

Data from the current study showed that there was some level of increase in activity for all the CYPs tested (CYP1A1/2, CYP2B1/2, CYP2C9, CYP2D6 and CYP3A4) after the 30-day

treatment period. Cellgevity® was found to have significantly increased the activities of CYP1A1/2, CYP2C9 and CYP2D6. These results are contrary to what was reported by N'guessan et al. (2018), where Cellgevity® significantly reduced the enzyme activities of rat CYP2B1/2B2 (LDT 92.4%; HDT 100%), CYP3A4 (LDT 81.2%; HDT 71.7%), and CYP2C9 (LDT 21.7%; HDT 28.5%) after a 7-day treatment period. Horn et al. (2002) showed that CYP activity is both dose and treatment duration dependent. The different results on the modulatory effect of Cellgevity® on rat liver CYP activity of the two studies could therefore be attributed to differences in the doses administered and treatment duration. Pichard-Garcia et al. (2000) reported that higher concentrations of eletriptan induced CYP3A in culture medium. However lower doses of eletriptan did not cause CYP3A induction.

The male rats exhibited, in most cases higher CYP enzyme activity than female rats. There was a statistically significant ($p < 0.05$) differences in CYP enzyme activities for all the tested CYPs per treatment across both sexes except CYP2B1/2 and CYP1A1/2 activities. It thus emphasizes the need for sex selectivity (male or female) for experimental animal studies (Mairinger et al., 2020). CYP activity, ie CYP1A1/2, CYP2C9 and CYP2D6, in rats treated with Cellgevity® were found to have increased significantly compared to the negative control CYP activity (Table 4.7 and Figure 4.5 – 4.15). These CYPs are important drug metabolizing enzymes (Hewitt et al., 2007). It can be speculated that Cellgevity® may affect the metabolism of drugs that are metabolized by these isoforms if concurrently administered with Cellgevity®. Metabolism of endogenous substrates of these CYPs could also be affected with continued use of the supplement.

There are documented reports of drug-drug and herb-drug interaction due to modulation of CYP enzyme activity by drugs or herbs. The pharmacological effect of warfarin was significantly

reduced by co-administration of warfarin and *Hypericum perforatum* (St. John's wort) due to CYP1A2 induction caused by the herb (Jiang et al., 2004). Also Nebel et al. (1999a) found that CYP1A2 induction caused reduction in plasma concentration of theophylline requiring increased doses of theophylline to attain therapeutic concentration of the drug. In addition, iron supplement decreased the bioavailability of carbamazepine, and piperine also inhibited the metabolism of carbamazepine (Perucca, 2018; Ren et al., 2019).

The increase in the catalytic activities of the CYPs by Cellgevity® as reported in the current study was dose-dependent especially for female CYP1A1/2, CYP2C9 and CYP2D6. If this dose-dependent increase in CYP activity observed is clinically relevant, then emphasis should be made on dose modification, if need be, for daily doses of Cellgevity® in humans.

Based on this study and that reported by N'guessan et al. (2018), there is the potential that Cellgevity® could modulate CYP enzymes in humans. Indeed, it may not be entirely correct to extrapolate animal studies to humans, but these data give evidence of possible CYP enzyme modulation. However, it is not a sure proxy that any clinically relevant supplement-drug interaction may occur in humans. There are reports of marked differences in CYP activities between species (Horn et al., 2002). Furthermore, a CYP that metabolizes a particular substrate in humans may not be the same CYP that metabolizes that substrate in rats. For example, in humans, CYP3A4 and CYP2C9 are the major CYP isoforms that metabolize carbamazepine (Pearce et al., 2008). However in rats, carbamazepine is metabolized by CYP3A2 and CYP2C11 (Tateishi et al., 1999). It is noteworthy that, these enzymes in rat and humans that metabolize carbamazepine all do belong to same sub-family classification of CYP enzymes.

In view of the modulatory effect of Cellgevity® on rat CYP450 enzymes as reported in this study and N'guessan et al. (2018), and carbamazepine being extensively metabolized by

CYP3As and CYP2Cs (Pearce et al., 2008, Tateishi et al., 1999), the effect of Cellgevity® on carbamazepine pharmacokinetics was investigated using an animal model.

The time to achieve peak carbamazepine concentrations (T_{max}) was 4 h for S-D rats administered carbamazepine with Cellgevity®, and among S-D rats administered carbamazepine with normal saline. Peak carbamazepine concentration (C_{max}) was higher (20 µmol/L) for the group administered carbamazepine with Cellgevity® compared to the group administered carbamazepine with normal saline (11 µmol/L). Since rate of absorption was not estimated in this study, it may be speculative to say absorption was faster for S-D rats administered carbamazepine with Cellgevity®, hence that group having a higher C_{max}. In the current study, the total drug (carbamazepine) exposure, represented by AUC obtained for the carbamazepine with normal saline was 347 µmol.h/L, as against 170 µmol.h/L for the carbamazepine with Cellgevity® group. This was as expected, as elimination rates were 0.3 h^{-1} for carbamazepine with Cellgevity® and 0.4 h^{-1} for the carbamazepine with normal saline. The aforementioned elimination rates indicate that there was a slower carbamazepine clearance in rats administered carbamazepine with Cellgevity®. This ultimately led to a longer half-life (2.3 hours) among rats administered carbamazepine with Cellgevity®. It had been previously reported that Cellgevity® inhibited CYP3A4, CYP2C9 and CYP2B1/2B2 in rats (N'guessan et al, 2018). Carbamazepine is also known to be metabolized extensively by CYP3A4, thus, there is the potential for Cellgevity® to inhibit metabolism of carbamazepine. It can, therefore, be inferred from the current study that Cellgevity® had some level of interaction with carbamazepine, possibly through inhibition of CYP3A in the rats.

Anecdotal reports which suggested that epileptic patients were taking diosmin (a widely used flavonoid in the treatment of varicose veins and hemorrhoids) along with carbamazepine, led to a

study to ascertain possible interaction between these two agents in an animal model (Bedada and Neerati, 2018b). In that study, diosmin significantly enhanced maximum plasma concentration (C_{max}), area under the curve (AUC), and half-life ($t_{1/2}$) of carbamazepine as compared to control rats. Diosmin also significantly decreased elimination rate constant (k_e) and apparent oral clearance (CL/F) of carbamazepine as compared to control rats (Bedada and Neerati, 2018b). This therefore corroborates findings from the current study, that there is a potential for herbal medicines, dietary supplements, and food to interact with conventional drugs *in vivo*.

Indeed, herbal medicines, dietary supplements, and food may interact with drugs pharmacokinetically and/or pharmacodynamically (Fong et al., 2013). In patients taking conventional medicines for a long period, there could be clinically significant interactions that may occur with herbal medicines, dietary supplements, and food. Studies reveal that there is a surge in concomitant use of dietary supplements and antiepileptic medications in both developed and developing countries (Fong et al, 2013), hence, the need for studies like the current one being reported.

5.2 CONCLUSION

In the current study, Cellgevity® was found to cause an appreciable increase in the catalytic activities of CYP1A1/2, CYP2B1/2, CYP2C9, CYP2D6 and CYP3A4 at the end of a 30-day treatment period. The increase in CYP activity was, however, statistically significant for CYP1A1/2, CYP2C9 and CYP2D6. Increase in enzyme activity was found to occur in both sexes and was especially dose dependent for female CYP1A1/2, CYP2C9 and CYP2D6. Additionally, Cellgevity® was found to alter the pharmacokinetics of carbamazepine in Sprague-Dawley rats.

Although this study was conducted in an animal model, this finding is noteworthy, as this may serve as a basis for future studies in humans.

5.3 LIMITATIONS

1. The chemical reagents for the CYP assays had to be imported and as such delayed aspects of this study.

2. A High Performance Liquid Chromatography (HPLC) was not available for the assay of serum carbamazepine for the pharmacokinetic study. The assay was thus done with Fluorescence Polarization Immunoassay (FPIA), which required that serum samples had to be pooled together, thus mean (average) pharmacokinetic parameters could not be determined.

5.4 RECOMMENDATION

Further research should be conducted using human recombinant CYPs or human participants to ascertain the effect Cellgevity® has on drug metabolizing enzymes.

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APPENDICES

APPENDIX A

Calculation of Animal Equivalent Dose of Cellgevity (AED)

Human dose = 2 capsules = 747.50 mg

Average human weight = 60 kg

Dose = 747.50 mg/60 kg = 12.46 mg/kg

AED = Human dose x Km ratio

= 12.46 mg/kg x 6.2 = 77.25 mg/kg

Km = correction factor = body weight of species/body surface area

Animal equivalent dose = 77.25 mg/kg

Low animal dose = 77.25 mg/kg /2 = 38.63 mg/kg

High animal dose = 77.25 mg/kg x 2 = 154.50 mg/kg

APPENDIX B

Calculation of Mass of Cellgevity and Phenobarbital to Weigh According to Animal Weight

Positive control (Phenobarbital)

Week-1

Weight (w)/g	Frequency (f)	wf/g	(wf/1000)/kg	((wf/1000)/kg x 15 mg/kg)/ mg
185.00	2	370	0.37	5.55
218.00	1	218	0.22	3.27
254.50	2	509	0.51	7.64
231.00	1	231	0.23	3.47

Week-2

Weight (w)/g	Frequency (f)	wf/g	(wf/1000)/kg	((wf/1000)/kg x 15 mg/kg)/ mg
190.50	2	381	0.38	5.72
223.00	1	223	0.22	3.35
269.50	2	539	0.54	8.09
246.00	1	246	0.25	3.69

Week-3

Weight (w)/g	Frequency (f)	wf/g	(wf/1000)/kg	((wf/1000)/kg x 15 mg/kg)/ mg
203.00	2	406	0.41	6.09
227.00	1	227	0.23	3.41
282.00	2	564	0.56	8.46
258.00	1	258	0.26	3.87

Week-4

Weight (w)/g	Frequency (f)	wf/g	(wf/1000)/kg	((wf/1000)/kg x 15 mg/kg)/ mg
216.50	2	433	0.43	6.50
229.00	1	229	0.23	3.44
288.50	2	577	0.58	8.66
264.00	1	264	0.26	3.96

Low Dose

(Cellgevity®)

Week-1

Weight (w)/g	Frequency (f)	wf/g	(wf/1000)/kg	((wf/1000)/kg x 38.63 mg/kg)/ mg
185.33	3	556	0.56	21.48
185.00	2	370	0.37	14.29
252.00	1	252	0.25	9.73

Week-2

Weight (w)/g	Frequency (f)	wf/g	(wf/1000)/kg	((wf/1000)/kg x 38.63 mg/kg)/ mg
197.00	3	591	0.59	22.83
181.00	1	181	0.18	6.99
212.00	1	212	0.21	8.19
258.00	1	258	0.26	9.97

Week-3

Weight (w)/g	Frequency (f)	wf/g	(wf/1000)/kg	((wf/1000)/kg x 38.63 mg/kg)/ mg
209.50	4	838	0.84	32.37
185.00	1	185	0.19	7.15
278.00	1	278	0.28	10.74

Week-4

Weight (w)/g	Frequency (f)	wf/g	(wf/1000)/kg	((wf/1000)/kg x 38.63 mg/kg)/ mg
201.67	3	605	0.61	23.37
184.00	1	184	0.18	7.11
251.00	1	251	0.25	9.70
287.00	1	287	0.29	11.09

Medium Dose
(Cellgevity®)

Week-1

Weight (w)/g	Frequency (f)	wf/g	(wf/1000)/kg	((wf/1000)/kg x 77.25 mg/kg)/ mg
223.00	1	223	0.22	17.23
192.50	2	385	0.39	29.74
290.00	1	290	0.29	22.40
238.00	1	238	0.24	18.39

273.00	1	273	0.27	21.09

Week-2

Weight (w)/g	Frequency (f)	wf/g	(wf/1000)/kg	((wf/1000)/kg x 77.25 mg/kg)/ mg
216.00	1	216	0.22	16.69
197.50	2	395	0.40	30.51
301.00	1	301	0.30	23.25
239.00	1	239	0.24	18.46
274.00	1	274	0.27	21.17

Week-3

Weight (w)/g	Frequency (f)	wf/g	(wf/1000)/kg	((wf/1000)/kg x 77.25 mg/kg)/ mg
226.00	1	226	0.23	17.46
212.00	2	424	0.42	32.75
311.00	1	311	0.31	24.02
255.00	1	255	0.26	19.70
277.00	1	277	0.28	21.40

Week-4

Weight (w)/g	Frequency (f)	wf/g	(wf/1000)/kg	((wf/1000)/kg x 77.25 mg/kg)/ mg
228.00	1	228	0.23	17.61
217.00	2	434	0.43	33.53
315.00	1	315	0.32	24.33
269.00	1	269	0.27	20.78
282.00	1	282	0.28	21.78

High Dose
(Cellgevity®)
Week -1

Weight (w)/g	Frequency (f)	wf/g	(wf/1000)/kg	((wf/1000)/kg x 154.50 mg/kg)/ mg
191.50	2	383	0.38	59.17
224.00	1	224	0.22	34.61
244.00	2	488	0.49	75.40
345.00	1	345	0.35	53.30

Week-2

Weight (w)/g	Frequency (f)	wf/g	(wf/1000)/kg	((wf/1000)/kg x 154.50 mg/kg)/ mg
197.50	2	395	0.40	61.03
227.00	1	227	0.23	35.07
242.00	2	484	0.48	74.78
363.00	1	363	0.36	56.08

Week-3

Weight (w)/g	Frequency (f)	wf/g	(wf/1000)/kg	((wf/1000)/kg x 154.50 mg/kg)/ mg
202.50	2	405	0.41	62.57
247.00	3	741	0.74	114.48
372.00	1	372	0.37	57.47

Week-4

Weight (w)/g	Frequency (f)	wf/g	(wf/1000)/kg	((wf/1000)/kg x 154.50 mg/kg)/ mg
200.00	2	400	0.40	61.80
231.00	1	231	0.23	35.69
258.50	2	517	0.52	79.88
376.00	1	376	0.38	58.09

APPENDIX C

Preparation of 0.1M Potassium Phosphate Buffer solution (pH = 7.4)

200 mL 0.1M monobasic (KH_2PO_4)

Molar mass (KH_2PO_4) = 139.09 $gmol^{-1}$

Mass (KH_2PO_4) = 0.1M x 0.2 dm^3 x 139.09 $gmol^{-1}$ = **2.78 g**

400 mL 0.1M dibasic (K_2HPO_4)

Molar mass (K_2HPO_4) = 174.18 $gmol^{-1}$

Mass (K_2HPO_4) = 0.1M x 0.4 dm^3 x 174.18 $gmol^{-1}$ = **6.967g**

APPENDIX D

Preparation of concentrations of Standard Bovine Serum Albumin (BSA)

1mg BSA in 1 mL buffer = 1mg/mL of 1mg/mL

Pick 100 µL of 0.5 mg/ml into 100 µL of buffer = 0.5 mg/mL

Pick 100 µL of 0.5 mg/ml into 100 µL of buffer = 0.25 mg/mL

Pick 100 µL of 0.25 mg/ml into 100 µL of buffer = 0.125 mg/mL

Pick 100 µL of 0.125 mg/ml into 100 µL of buffer = 0.0625 mg/mL

Pick 100 µL of 0.0625 mg/ml into 100 µL of buffer = 0.03125 mg/mL

Pick 100 µL of 0.03125 mg/ml into 100 µL of buffer = 0.015625 mg/mL

Pick 100 µL of 0.015625 mg/ml into 100 µL of buffer = 0.00781255 mg/mL

APPENDIX E

Sample Dilution

50x dilution → 100x dilution → 200x dilution → 400x dilution → 800x dilution

5 µL microsomal sample in 245 µL buffer = 50x dilution

Pick 100 µL of 50x dilution into 100 µL of buffer = 100x dilution

Pick 100 µL of 100x dilution into 100 µL of buffer = 200x dilution

Pick 100 µL of 200x dilution into 100 µL of buffer = 400x dilution

Pick 100 µL of 400x dilution into 100 µL of buffer = 800x dilution

APPENDIX F

Preparation of CYP Assay Reagents

0.1 M Potassium Phosphate buffer

2.78 g (KH_2PO_4) in 200 ml of distilled water

6.967 g (K_2HPO_4) in 400 ml of distilled water

20 mM Potassium Phosphate buffer

20 mM K_2HPO_4

Molar masss (K_2HPO_4) = 174.17 g/mol^{-1}

1 M = 174.17 g/dm^3

1 mM = 0.17417 mg/mL

20 mM = 3.4834 mg/mL; 1 mL (20 mM) = **3.4834 mg**

20 mM KH_2PO_4

Molar mass (KH_2PO_4) = 136.08 g/mol^{-1}

1 M = 136.08 g/dm^3

1 mM = 0.13608 mg/mL

20 mM = 2.7216 mg/mL; 1 mL (20 mM) = **2.7216 mg**

Add K_2HPO_4 solution to KH_2PO_4 solution to get pH = 7.4

1 mM NADPH

Molar mass = 1142.12 g/mol^{-1}

1 M = 1142.12 g/dm^3

1 mM = 1.14212 mg/mL

1 mL = **1.14212 mg**

Stopping solution ($ZnSO_4.7H_2O$) for CYP2D6 Assay

Molar masss = 287.55 g/mol^{-1}

1 M = 287.55 g/dm^3

1 mM = 0.28755 mg/mL

1 mL = **0.28755 mg**

1 mM Dextromethorphan

Molar mass = 370.32 g/dm^3

1 M = 370.32 g/dm^3

1 mM = 0.37032 mg/mL

1 mL = 0.37032 mg

10 mL = **3.7032 mg**

1mM Diclofenac

Molar mass = 308 g/dm^3

1 M = 308 g/dm^3

1 mM = 0.308 mg/mL

1 mL = 0.308 mg

10 mL = **3.08 mg**

2 mM Resorufin ethyl ether

Molar mass = 241.24 g/dm^3

1 M = 241.24 g/dm^3

1 mM = 0.24124 mg/mL

1 mL = 0.24124 mg

0.24124 mg in 500 µL DMSO = **2 mM** (Stock solution)

2 mM Resorufin benzyl ether

Molar mass = 303.31 g/dm^3

1 M = 303.31 g/dm^3

1 mM = 0.30331 mg/mL

1 mL = 0.30331 mg

0.30331 mg in 500 µL DMSO = **2 mM** (Stock solution)

2mM Pertoxyresorufin

Molar mass = 283.327 g/dm^3

1 M = 283.327 g/dm^3

1 mM = 0.283327 mg/mL

1 mL = 0.283327 mg

0.283327 mg in 500 µL DMSO = **2 mM** (Stock solution)

Stock solution = 2 mM (2000 µM) substrate; 100% DMSO

40x dilution

50 µM substrate; 2.5% DMSO (Working solution)

Mobile phase of CYP2D6 HPLC analysis (24% ACN: 75%DH_2O: 1% TEA)

24% Acetonitrile = 24/100 x 500 = 120 mL

75% Distilled water = 75/100 x 500 = 375 mL

1% TEA = 1/100 X 500 = 5 mL

Mobile phase of CYP2C9 HPLC analysis (60% 20mM kPi:22.5%MeOH:17.5%ACN)

60% kPi = 60/100 x 500 = 300 mL

22.5% MeOH = 22.5/100 x 500 = 112.50 mL

17.5% ACN = 17.5/100 x 500 = 87.50 mL

APPENDIX G

Calibration curve for microsomal protein determination