UNIVERSITY OF CAPE COAST

ANTIPLASMODIAL ACTIVITY AND CYTOCHROME P450 MEDIATED PHARMACOKINETIC DRUG INTERACTIONS OF XYLOPIC ACID

MARY ATTA-PANYI AGBENYEKU

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BY

MARY ATTA-PANYI AGBENYEKU

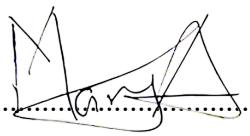
Thesis submitted to the Department of Biomedical Science of the School of Allied Health Sciences, College of Health and Allied Science, University of Cape Coast, in partial fulfillment of the requirements for the award of Master of Philosophy degree in Drug Discovery and Toxicology

JUNE 2020

DECLARATION

Candidate's Declaration

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

Candidate's Signature:  Date: .....

Name: Mary Atta-Panyi Agbenyeku

Supervisors' Declaration

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

Principal Supervisor's Signature: ..... Date: .....

Name: Dr. Elvis Ofori Ameyaw

Co-Supervisor's Signature:  Date: ... 1/06/2020.....

Name: Prof. Regina Appiah-Opong

ABSTRACT

Xylopic acid (XA), the major constituent of the fruit of *Xylopia aethiopica* has shown several pharmacological properties. Consequently, the fruit is being used in the preparation of food. Traditionally, the plant is used to treat several diseases and it has been formulated into a nasal drop despite the lack of information about its safety, food-drug interaction, and other pharmacokinetic properties. Therefore, this study investigated the antiplasmodial effect of XA on *P. falciparum*, and its effect on rat liver CYP enzymes *in vivo* and *in vitro*. To establish the effect of XA on *P. falciparum*, the parasite strain Dd2 was cultured and treated with XA. Pentobarbitone-induced sleeping time was used to investigate the effect of XA on rat liver enzymes. Inhibition or induction of some isoforms of CYP450, CYP 1A1/1A2, 1A2, 2B1/2B2, 3A4, 2D6 and 2C9 was investigated using microsomal fractions of rat liver. The *in vitro* inhibition of selected CYP (1A2, 3A4) was assessed by treating rat liver microsomes XA. Results obtained showed that Xylopic acid exhibited negligible antiplasmodial activity. The IC_{50} of XA > 20 µM. Xylopic acid induced CYP 1A1/1A2, 1A2, 2D6, 2C9, and inhibited CYP3A4, 2B1/2B2. The findings would help mitigate toxicity and therapeutic failure especially in cases of co-administration of medications with food containing XA, with metabolism altered by the latter.

KEY WORDS

Malaria

Xylopic acid

CYP450

Artemisinin

Pharmacokinetics

ACKNOWLEDGMENTS

I wish to thank God for the grace to have successfully completed this thesis. Special thanks go to my supervisors Dr. Elvis Ofori Ameyaw and Prof. Regina Appiah-Opong who invested their time and effort in making this project a success. Thanks to the staff and fellows of NMIMR (Departments of Clinical Pathology, Virology and Animal Experimentation)

DEDICATION

This work is dedicated to my Family

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LIST OF ABBREVIATIONS

UCC	University of Cape Coast
NMIMR	Noguchi Memorial Institute for Medical Research
WHO	World Health Organization
MROD	Methoxyresorufin O-demethylase
EROD	Ethoxyresorufin-O-deethylase
PROD	pentoxyresorufin O-depentylase
XA	Xylopic acid
XAE	*Xylopia aethiopica*
HPLC	High performance liquid chromatography
CYP450	Cytochrome P450

CHAPTER ONE

INTRODUCTION

Background to the study

The use of medicinal natural products is traced back to thousands of years. Traditional medicine has been practiced throughout the world even before the introduction of western medicine. WHO estimates over 75 % of the world's population to be involved in the therapeutic use of herbal remedies (Liu and Wang, 2007). This is due to the belief that herbal drugs have fewer side effects, enhance the effects of conventional agents and can be an alternative for management of several disorder (Grossberg, 2003). An example of one of such herbs is *Xylopia aethiopica.* The name Xylopia is a compression from the Greek word Xylon pikron indicating bitter wood. The second part of the plant's binomial name, *Aethiopica*, indicates the plant originates from Ethiopia (Fetse, Kofie, & Reimmel, 2016). Hence, it is commonly known as Ethiopian pepper. The fruit belongs to the family *Annonaceae* and is also known as Africa guinea pepper (Fetse, Kofie, & Reimmel, 2016) in some localities. The fruit contains substances such as zinc (Smith et al., 2000), lipids, proteins, carbohydrates, iodine, and several others (National Research Council, 1988). Study suggests the plant has anti-inflammatory, antimicrobial, analgesic, antiparasitic, antioxidant properties (Shakya, 2016).

In Ghana, the fruit is used traditionally to manage a wide range of disorders including dyspepsia, cough, pain and parasitic infections. It is equally used as a spice in the preparation of dishes such as Hausa koko, zobolo, other African cuisines (Towns & Van Andel, 2016) and soups. Some food industries use it as flavoring agent and in some cosmetic industries it is used as an agent

for fragrance. The plant is widely distributed in the West African rainforest from Senegal to Sudan in Eastern Africa, and down to Angola in Southern Africa (Irvine 1961; Booth & Wickens, 1988) where it is mostly used for local cooking, especially in the preparation of what is referred to as the African pepper soup (Booth & Wickens, 1988). The plant grows prominently in several parts of Africa including Ghana, Nigeria, Benin, Kenya, Ivory Coast, and several other places. (Fleischer, Segev, Efremidis, & Christodoulides, 2003).

Pharmacologically, the plant is being used in the treatment of bronchitis, toothache, dysentery, pain, asthma, rheumatism, antimicrobial infections, ulcerations, management of malaria and for inducing formation of protective mucus within the digestive tract (Gairola, Sharma, & Bedi, 2014; Kyere-Davies, 2017). The plant contains about 98 compounds (Konan & Haddad, 2009). Constituents of the fruit include essential oils, volatile oils, resin, arocene, rutheroside fat, bitter principles, alkaloids, glycosides, saponins, tannins, mucilage, xylopic acid and kaurenoic acid. The compounds obtained from the fruit, that are considered most relevant are xylopic acid and kaurenoic acid and xylopic acid, a kaurene diterpene, occurs as the major constituent in the fruits of *Xylopia aethiopica*.

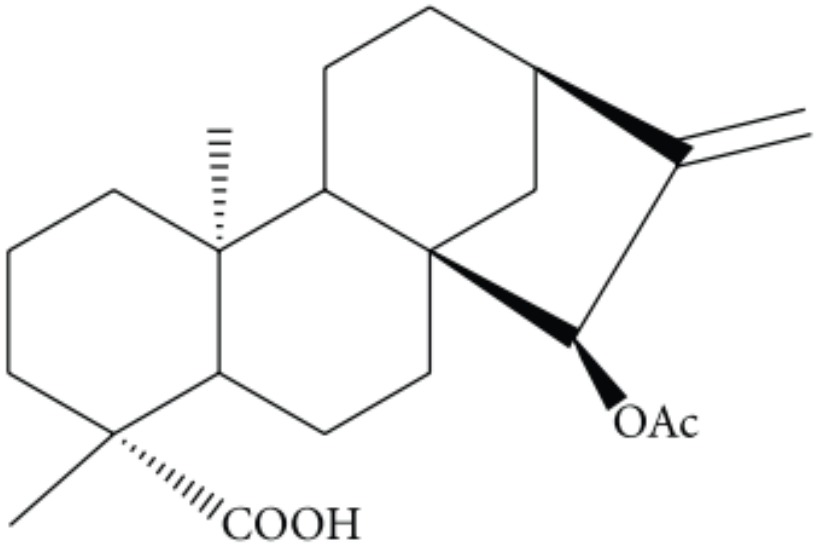

Figure 1: Chemical structure of xylopic acid (Woode et al., 2012)

Previous research reports have indicated that kaurenoic acid possesses anti-inflammatory and analgesic properties (Block, Stacey & Jones, 1998; Paiva et al., 2002; Woode, Ameyaw, Boakye-Gyasi, Abotsi, 2012). Research on xylopic acid has shown that it exhibits diuretic and vasorelaxant properties in rodents. (Somova, Shode, Moodley & Govender, 2001). Aqueous extract of *Xylopia aethiopica* was neither a miotic nor a mydriatic, but lowered the intraocular pressure, reduced the near point of convergence and increased the amplitude of accommodation (Igwe, Afonne & Ghasi, 2003). The essential oils of *Xylopia aethiopica*, Monodora myristica, Zanthoxylum xanthoxyloides and Z. leprieurii, showed antibacterial and antifungal activity (Tatsadjieu, Ngang, Ngassoum & Etoa, 2003). Betulinic acid and xylopic acid caused a substantial reduction of lung inflammation induced by carrageenan in the mice, which support the traditional use of the plant extracts of Magaritaria discoidea and *Xylopia aethiopica* as therapeutic agents in conditions associated with acute inflammation and some respiratory disorders (Ekuadzi et al. 2018). Xylopic acid

possesses curative and prophylactic properties on *P. berghei*-induced malaria in ICR mice as well as antipyretic properties (Boampong et al. 2013).

Research by Woode et al. (2012) lends support to the reports that the use of the fruit of *X. aethiopica* as an analgesic is partly due to the presence of xylopic acid (Boampong et al., 2013; Woode et al., 2012). The co-administration of cryptolepine and Xylopic acid also produced a synergistic anti-malarial effect which also resulted in minimal toxicity (Ameyaw et al., 2014; Forkuo et al., 2016). Xylopic acid and the fruit extract of *Xylopia aethiopica* were found to have significant central nervous system depressant effects in mice (Biney, Mantel, Boakye-Gyasi, Kukuia & Woodel, 2014; Ofori, Eric, Samuel, Robert & Nyarko, 2015). The use of traditional medicine has increased over time. In Ghana, traditional medicines are often used by patients in combination with prescribed drugs with the notion that it has no side effects (Appiah-Opong et al., 2018). Hence, such drugs do not pass through the appropriate approval process applied to new drug applications. Due to the lack of scrutiny, most herbal medicines result in CYP-mediated herb-drug interactions when co-administered with other orthodox medications (Appiah-Opong et al., 2018).

Interactions of traditional medicines with human CYPs have been associated with alterations in the pharmacokinetics of several drugs (Hu, Chen & Lo, 2005). They interact by either inhibiting or inducing CYPs which results in harmful side-effects. *Phyllanthus amarus* extract was reported to be a potent inhibitor of rat liver microsomal 7-ethoxyresorufin-O-deethylase (CYP1A1/1A2), 7 methoxyresorufin-O-demethylase (CYP1A2) and 7-pentoxyresorufin-O-dependentylase (CYP2B1/2) (Kaewmeesri, 2013). Studies on

Kava (Pipermethysticum) extract, a commercially available herbal anxiolytic showed a significant inhibition of human CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 *in vitro* (Etheridge, Black, Patel, So, & Mathews, 2007). Drug-drug and herb-drug interactions is an important phenomenon that should be investigated before new drugs are introduced on the market. Hence, it is useful to conduct series of investigations on the effect of traditional medicines on liver CYP drug metabolizing enzymes (Appiah-Opong et al., 2008). This study seeks to evaluate the effect of xylopic acid on CYPs and determine some pharmacokinetic and pharmacodynamic properties of the compound.

Problem statement

Despite the use of the fruit of *Xylopia aethiopica* as food in Ghana and other parts of the world, the effects of the compounds present in it on liver enzymes, including isoforms of CYPs are unknown. This poses a threat of food-drug/herb-drug interactions since it may be used alongside several orthodox medication. The fruit extract of *Xylopia aethiopica* has been formulated into a nasal drop with no details of the pharmacokinetic activity and details of its biotransformation, posing the threat of toxicity related issues. Again, XA has been reported to exhibit *in vivo* antiplasmodial activity on *P. berghei* but its activity on *P. falciparum*, the type that infects man is equally unknown. There are chances that XA may be active *in vivo* but inactive *in vitro* especially when the activity of XA is due to its active metabolites.

Aim:

The aim of the study was to investigate *in vitro* the antiplasmodial effect and CYP450 mediated pharmacokinetic properties of xylopic acid.

Specific objectives

The objectives of the study were to:

1. ascertain the *in vitro* antiplasmodial effect of xylopic acid on *Plasmodium falciparum.*
2. determine the *in vivo* effect of xylopic acid on pentobarbitone-induced sleeping time in rats.
3. determine the effect of xylopic acid on CYP1A1/1A2, 1A2, 3A4, 2B1/2B2, 2D6, and 2C9 (*in vivo*)
4. evaluate the *in vitro* effect of xylopic acid on CYP 1A2, 3A4 liver microsomal enzymes

Significance of study

The *in vitro* studies on the effect of xylopic acid on *P. falciparum* will confirm its antiplasmodial activity in humans, paving way for further studies. The study on modulation of liver CYP enzymes will help predict the interactions that may occur when food containing xylopic acid is taken with antihypertensives, antimicrobial, and other orthodox medications, which may also influence the bioavailability of these drugs if co-administered with xylopic acid. This information will also help mitigate toxicity and therapeutic failure.

Limitations

Although results obtained from an experiment conducted on rats can be extrapolated to humans, the inference may have lapses. The use of human hepatic microsomal fraction would have provided precise results on CYPs enzyme activity compared to rat microsomal fractions (Brandon, Raap, Meijerman, Beijnen & Schellens, 2003).

Ethical Statement

All animal experiment was conducted according to the guidelines of the National Institute of Health for the Care of Laboratory Animals and was approved by the Scientific and Technical Committee and Institutional Animal Care and Use Committee (IACUC), of Noguchi Memorial Institute for Medical Research, University of Ghana (UG-IACUC004/18-19) and the Department of Ethics Committee from the University of Cape Coast.

CHAPTER TWO

LITERATURE REVIEW

Introduction

The use of medicinal natural products is traced back to thousands of years. Traditional medicine has been practiced throughout the world even before the introduction of western medicine (Kapoor, 2018). Plants are the major source of medicine in most parts of the world. Especially in the tropical regions of Africa (Abdullahi, 2011) WHO estimates that eighty percent of African populations use some form of traditional herbal medicine and the worldwide annual market for these products is approximately 60 billion US dollars (Kamboj, 2000). This is due to the belief that herbal medicines have fewer side effects, enhance the effects of conventional agents and can be an alternative for management of several disorders (Grossberg & Desai, 2003). An example of one of such herbs is *Xylopia aethiopica*. The name Xylopia is a compression from the Greek word Xylon pikron indicating bitter wood.

The second part of the plant's binomial name, *Aethiopica*, indicates the plant originates from Ethiopia (Fetse et al., 2016). Hence, it is commonly known as Ethiopian pepper, although it grows most prominently in several parts of Africa such as Ghana, Nigeria, Benin, Kenya, Ivory Coast, and several other places (Fleischer, Segev, Efremidis & Christodoulides, 2003). It is used in the preparation of African cuisines and as traditional medicines (Towns & Van Andel, 2016).

Xylopia aethiopica

Xylopia aethiopica is an angiosperm belonging to the family *Annonaceae*. It is a slim, tall tree approximately sixty centimeters in diameter

and thirty meters high with smooth bark (Johnson & Murray, 2018). The fruit is commonly referred to as 'hwenteaa' by the Akans 'etso' by the Ewe's, and 'samadabile' by the Waala's in Ghana. Several studies have been performed on the fruit, and these have revealed it contains about 98 compounds (Konan & Haddad, 2009).

Constituents of the fruit include essential oils, volatile oils, resin, arocene, rutheroside fat, bitter principles, alkaloids, glycosides, saponins, tannins, mucilage, xylopic acid and kaurenoic acid. The plant possesses great nutritional and medicinal values which encompasses all parts of the plant; such as the bark, leave and fruit (Okigbo, Anuagasi & Amadi, 2009). Research has shown that the dried fruits are crushed and applied topically on the forehead to manage neuralgia (Fetse et al., 2016). Extracts obtained from the plant have been found to hold the potential of being used in adjunct therapy in the management of sickle cell (Avaligbe et al., 2012; Odugbemi, 2008).

The fruits of *Xylopia aethiopica* are crushed, added to Shea butter and used as body creams, and other forms of cosmetic products (Yapi et al., 2012). Studies reveal that essential oils obtained from the fruit can be used in the production of shampoo as it has the potential to undergo saponification (Juliani et al., 2008). Study showed that traditionalist employ the seeds in the preparation of concoctions which when administered postpartum prevents hemorrhage (Ogbonnia, Adekunle, Bosa & Enwuru, 2008). Traditionally, the seeds are administered to postpartum patients to induce the evacuation of the placenta from the uterus (Odesanmi et al., 2013). This suggests that the seed can be used in the termination of unhealthy pregnancies (abortion). In an experimental study performed on rats, the ethanolic extract of the fruits and

xylopic acid had significant analgesic activity against acetic acid-induced visceral nociception, formalin-induced paw pain, thermal pain as well as carrageenan-induced mechanical and thermal hyperalgesia (Woode et al., 2012). The hydroethanolic fruit extract was also identified to have anti-arthritic effect on Sprague-Dawley rats (Boakye, Agyare, Abotsi, Ayande & Osei, 2016). Obiri and Osafo investigated the anti-anaphylactic and anti-inflammatory effects of the aqueous ethanolic fruit extract of the plant and discovered that the extract had a significant dose-dependent anti-anaphylactic effect (Obiri & Osafo, 2013).

Ameyaw and colleagues discovered that an ethanolic fruit extract and xylopic acid from the dried fruits improved vincristine-induced tactile and cold allodynia and mechanical hyperalgesia (Ameyaw et al., 2014). In a study involving the effect of the methanolic fruit extract on human cervical cancer cell lines, it was discovered the extract inhibited the proliferation of C-33A cancer cells via cell cycle arrest at sub-G0/G1 and G2/M phases (Adaramoye et al., 2011). Another study revealed the methanolic extract inhibited proliferation of MiaPaCa-2 (prostate cancer cells), CEM/ADR5000 and CCRF-CEM (leukemia cells) (Kuete, Sandjo, Wiench & Efferth, 2013). The compounds considered most relevant from *Xylopia aethiopica* are xylopic acid and kaurenoic acid. Previous research reports indicate kaurenoic acid possesses anti-inflammatory and analgesic property (Block et al., 1998; Paiva et al., 2002; Woode et al. 2012).

A study revealed that a chloroform extract of the dried fruits of *Xylopia aethiopica* caused a dose-dependent reduction of blood glucose concentration of alloxan monohydrate-induced diabetic Wistar albino rats (Gometi, Ogugua,

Odo & Joshua, 2014; Okpashi, Bayim & Obi-Abang, 2014). In an *in vivo* experiment, the methanolic extract of the bark of *Xylopia aethiopica* caused a reduction in serum cholesterol levels in Wistar rats placed on a cholesterol rich diet (Nwozo, Orojobi, & Adaramoye, 2011). The study also revealed a reduction in LDL cholesterol in serum and liver. A study to investigate the ocular dynamics following a systemic administration of aqueous seed extracts of *Xylopia aethiopica* revealed a reduction in intraocular pressure; hence it is a candidate for management of glaucoma (Igwe et al., 2003).

A study found that the ethanolic extract of the dried fruits of *Xylopia aethiopica* and xylopic acid had a significant reduction in sperm count and motility in male albino rats with no change in morphology (Woode et al., 2012). Xylopic acid used in a study on sex hormones and spermatogenesis in male rats resulted in a reduction in the weight of the testis epididymis, seminiferous tubules of treated animals compared to the control group (Abarikwu et al., 2017). There was an increase in Follicle stimulating hormone, Luteinizing hormone, and Serum testosterone levels. After two weeks of recovery, seminiferous tubule was normal. Suggesting xylopic acid which is an active compound of the fruit can result in male infertility. Studies show that Xylopic acid, caused a significant hypotensive effect on systolic and diastolic blood pressure after an intraperitoneal administration (Somova, Shode, Moodley & Govender, 2001). Hot water extracts of *Xylopia aethiopica* dried fruits in a research conducted exhibited anti-fungal activity against *Rhizopus* sp. and *Ustilago maydis* (Ntonifor, 2011). Two epoxide derivatives were obtained by oxidation of xylopic acid (15α-acetoxy-16,17αent-epoxy-kauran-19-oic and 15α-acetoxy16,17β-epoxy-ent-kauran-19-oic acid) from *Xylopia aethiopica*.

They were found to have good trypanocidal activity against *Trypanosoma brucei* (Soh et al., 2013). Research on xylopic acid has shown that it exhibits diuretic and vasorelaxant properties in rodents (Somova et al., 2001).

Malaria

Malaria is known to affect both humans and other animals (Wolfe, Dunavan, & Diamond, 2007). It is a disease of major public health importance. The disease results from being infected by an intraerythrocytic protozoa of the genus Plasmodium (Downie, Kirk, & Mamoun, 2008). Humans can be infected with *P. falciparum, P. vivax*, *P. ovale*, and *P. malariae*, which is transmitted by the bite of an infected female Anopheles mosquito, blood transfusion and congenital transmission. The species *P. knowlesi* rarely causes disease in humans (Marchand, Culleton, Maeno, Quang, & Nakazawa, 2011).

The life cycle involves being bitten by an infected anopheline mosquito, sporozoites from the mosquito is inoculated into the bloodstream. The sporozoites invade hepatic cells dividing rapidly into exoerythrocytic merozoites also known as (tissue schizogony). The merozoites then invade erythrocytes developing into early trophozoites, which are ring shaped, vacuolated and uninucleate. Once the parasite begins to divide, the trophozoites called schizonts, consisting of many daughter merozoites also begin to divide (blood schizogony). The merozoites, lyse the erythrocytes to invade other erythrocytes, starting a new cycle of schizogony. *P. vivax* and *P. ovale*, which *P. falciparum* does not have, a dormant form called hypnozoites which remain quiescent in the liver. Each cycle in *P. falciparum* lasts for 48 hours (Le Roch et al., 2003). After several cycles, some of the merozoites develop into

gametocytes, the sexual stage of malaria, which is Asymptomatic in man but infective in mosquitoes (Bousema & Drakeley, 2011).

A Study suggests *Plasmodium falciparum* is responsible for almost all severe cases of malaria, death inclusive (Guerin et al., 2002). Complications associated with the severity of malaria include acute renal failure, cerebral malaria, severe anemia, and pulmonary edema (Murphy & Breman, 2001). Age, sex, medical conditions, and lack of or delayed treatments are risk factors associated with severe malaria (Bates et al., 2004). Reinfection with malaria parasites is likely in patients who have previously suffered from malaria (Ratcliff et al., 2007). A study suggests an individual may experience severe malaria after initial treatment response and complete clearance of parasitemia. However, this could be due to delayed release of cytokines (Trampuz, Jereb, Muzlovic, & Prabhu, 2003).

Malaria is diagnosed via microscopic examination of blood using thick and thin blood films or using an antigen-based rapid diagnostic tests (Stauffer et al., 2009). Although examination of the thick and thin blood smear is the standard, fluorescence microscopy of parasite nuclei stained with acridine orange, rapid dipstick immunoassay, and polymerase chain reaction assays are also alternative methods used in the detection of an infection (Tangpukdee, Duangdee, Wilairatana, & Krudsood, 2009). Although the dipstick tests may enhance diagnostic speed, studies have shown it occasionally tests negative in patients with high parasitemia, and sensitivity below 100 parasites per µL (Craig et al., 2002). Tests based on polymerase chain reaction for species-specific Plasmodium genome are more sensitive and specific than other tests, it has the ability to detect as low as 10 parasites per µL of blood (Demas et al., 2011).

Statistics reveal that the clinical cause of death in adults results from cerebral malaria (Newton, Hien, & White, 2000). Symptoms may vary depending on its severity. It mostly presents as a generalized convulsion, or drowsiness and confusion which may eventually lead to coma that lasts for several hours or days. Cerebral malaria is mostly caused by an infection with *Plasmodium falciparum*. The patient must be positive for this strain of the parasite, have a Glasgow Coma Scale score of 9 and test negative for viral encephalitis, bacterial meningitis and hypoglycemia (Smith et al., 2000).

The risk of being infected with malaria, either systemic or cerebral can be prevented or reduced to the barest minimum by avoiding mosquito bites (Diala, 2015) This can be achieved by consistently using mosquito nets, insect repellents and spraying with insecticides. Intake of combination medication Sulphadoxine/pyrimethamine as prophylaxis is recommended for infants and pregnant mothers in localities with high rates of malaria (Nyarango et al., 2006), as they are most pruned to death by malaria infection. The recommended treatment for malaria includes the use of artemisinins, mefloquine, lumefantrine, and Quinine alongside doxycycline (Achan et al., 2011). In Ghana, the first line drugs recommended for uncomplicated malaria are the artemisinin-based combination drugs.

Malaria parasites have developed resistance to several antimalarial medications; such as chloroquine and artemisinin (Farooq & Mahajan, 2004). Resistance to the combination of artemisinin and piperaquine was detected in 2013 in Cambodia, and has since spread through Laos, Thailand and Vietnam (Wongsrichanalai, Pickard, Wernsdorfer, & Meshnick, 2002). The disease is widespread in the tropics, subtropics, sub-Saharan Africa, Asia, and Latin

America. Malaria is associated with poverty and has a major negative effect on economic development (Black et al., 2010).

Malaria has been successfully eliminated or greatly reduced in certain areas. The disease was once common in the United States and southern Europe, but has been eliminated through vector control programs, in conjunction with the use of the pesticide DDT and environmental sanitation (Reiter, 2000). Paraguay has also been declared free of malaria, after an eradication effort that began in 1950 (Feachem et al., 2010). In November 2013, WHO and the malaria vaccine funders group set a goal to develop vaccines designed to interrupt malaria transmission with the long-term goal of malaria eradication (Cotter et al., 2013).

A vaccine against malaria called RTSs, currently undergoing pilot trials in select countries was approved by European regulators in 2015 (Gosling & von Seidlein, 2016). A study suggests one might acquire immunity against *P. falciparu*m after years of repeated infection (Weiss et al., 2010). The polymorphic nature of the parasite's protein makes development of a vaccine a challenge. Vaccine candidates that target antigens on gametes, zygotes, or ookinetes in the mosquito midgut aim to block the transmission of malaria (Stanisic, Barry, & Good, 2013). These transmission-blocking vaccines induce antibodies in the human blood; when a mosquito takes a blood meal from a protected individual, preventing the parasite from completing its developmental cycle in the mosquito. The vaccine SPf66 was tested extensively in areas where the disease is prevalent, but it proved unsuccessful. The Artemisinins are currently the most effective drugs used in the management of malaria although

parasites have proven to be resistant against them in some countries (Mutabingwa, 2005; Dondorp et al., 2009).

In the quest for a more effective antiplasmodial medication, a study conducted on rats implicated xylopic acid, as a prophylactic antimalarial agent with its activities comparable to that of Sulphadoxine/pyrimethamine and a curative antimalarial activity like Artemether/lumefantrine (Boampong, 2015; Fetse, 2016). A limitation in this study is that the study was performed using *Plasmodium berghei* (NK65) a type of Plasmodium parasite that does not affect humans. It will therefore be prudent to assess the effect of xylopic acid on *Plasmodium falciparum, vivax* and the likes which are types of the parasite that infect man. Essential oil obtained from the bark of the plant had antimalarial properties against the W2 strain of *Plasmodium falciparum* in culture (Bakarnga-Via et al., 2014). This raises the question on whether xylopic acid or its metabolite(s) could have activity against resistant strains of malaria parasites. Research by (Woode et al., 2012) lends support to the reports that the use of the fruit of *X. aethiopica* as an analgesic is partly due to the presence of xylopic acid (Boampong et al., 2013).

Importance of CYP450

Hepatic enzyme activity may be associated with changes in liver weight, serum clinical chemistry analytes (Maronpot et al. 2010), and pleotropic gene expression. The functionality of hepatic enzymes is compartmentalized into Phase I and Phase II (Maronpot et al. 2010). Phase I oxidative metabolism is catalyzed by various enzymes including isoforms of the P450 superfamily (Kumar & Surapaneni, 2001). The liver is generally known to maintain homeostasis and physiological functions of the body (Rushmore & Tony Kong,

2002). It is a functional system capable of responding to multiple stimuli. The size of the liver is dependent on genetic and biochemical activity to maintain optimal function (Khetani & Bhatia, 2008). Drug metabolism occurs in many sites in the body, including the liver, intestinal wall, lungs, kidneys, and plasma. As the primary site of drug metabolism, the liver functions to detoxify and facilitate excretion of harmful substances by converting lipid-soluble compounds to more water-soluble compounds (Gomez-Lechon, Castell, & Donato, 2007).

Drug metabolism is achieved through phase I reactions, phase II reactions, or both. The most common phase I reaction is oxidation, which is catalyzed by the CYP enzymes among others. The liver also readily responds to some stimuli by undergoing additive growth and function. Some of which include pregnancy and lactation, hormonal fluctuations, diet, infections that induce acute-phase proteins; and enzyme inductive responses to a variety of xenobiotics (Maronpot et al., 2010). An aspect of this thesis focuses on the induction of hepatic enzymes by xenobiotics, with CYP450 as the focus.

The term "P450" is derived from the spectrophotometric peak at the wavelength of the absorption maximum of the enzyme (450 nm) when it is in the reduced state and complexed with carbon monoxide (Schenkman & Jansson, 2006; Hannemann, Bichet, Ewen, & Bernhardt, 2007; Yan & Caldwell, 2001). The enzymes are responsible for oxidizing steroids, fatty acids, and xenobiotics, and are essential components in the clearance, synthesis and breakdown of several substances (Nebert and Russell, 2002). They are terminal oxidase enzymes in electron transfer chains, broadly categorized as P450-containing systems (Ingelman-Sundberg, 2004; Lewis and lavica, 2000). Genes encoding

CYP enzymes, and the enzymes themselves, are designated with the root symbol CYP for the superfamily, followed by a number indicating the gene family, a capital letter indicating the subfamily, and another numeral for the individual gene. In some cases, gene or enzyme names for CYPs may be different from this nomenclature, but rather represents the catalytic activity and the name of the compound used as substrate (Nebert & Wain, 2003).

In the microsomal P450 system, electrons are transferred from NADPH via cytochrome P450 reductase which could be cytochrome P450 oxidoreductase (CYPOR) (Nebert & Wain, 2003). Mitochondrial P450 systems on the other hand require the use of adrenodoxin reductase and adrenodoxin to transfer electrons from NADPH to P450 (Robin et al., 2001). Monooxygenase reaction is a common reaction catalyzed by cytochromes P450 insertion of one atom of oxygen into the aliphatic position of an organic substrate (RH) while the other oxygen atom is reduced to water (Abu-Omar, Loaiza, & Hontzeas, 2005). The active site of cytochrome P450 contains a heme-iron center tethered to the protein via a cysteine thiolate ligand (Auclair, Moënne-Loccoz, & Ortiz de Montellano, 2001; Denisov, Makris, Sligar, & Schlichting, 2005). Due to the various reactions catalyzed by CYPs, the activities and properties of most CYPs vary (Crivori & Poggesi, 2006).

Human CYPs are membrane-associated proteins located in the membrane of the mitochondria or in the endoplasmic reticulum of cells (Devi, Raghavendran, Prabhu, Avadhani, & Anandatheerthavarada, 2008) They are essentially responsible for the metabolism of endogenous and exogenous chemicals (Ding & Kaminsky, 2003). It is possible for a CYP to metabolize only one substrate mean while others metabolize multiple substrates. The

difference in characteristics could be a major factor that accounts for their central importance in drug biotransformation. Cytochrome P450 enzymes are known to play a crucial role in metabolizing of potentially toxic compounds (Markowitz et al., 2000; Zanger & Schwab, 2013). A recent study involving the Human Genome Project established the presence of 57 human genes coding for several cytochrome P450 enzymes (Guengerich, 2007; Ingelman-Sundberg, 2005). A study found that more than 75 % of known drugs are metabolized by CYPs (Zanger & Schwab, 2013). Most drugs undergo deactivation by CYPs, either directly or by facilitated excretion from the body (Yan & Caldwell, 2001).

Research suggests that most substances are bioactivated by CYPs to form their active compounds like clopidogrel (Kazui et al., 2010). It is not unusual for drugs or herbs to interact with CYPs. Most of these interactions may result in an increase or decrease in the activity of CYP isozymes either by inducing the biosynthesis of an isozyme by inhibiting the activity of CYP (Spina & De Leon, 2007). These interactions are what accounts for the adverse drug interactions observed in most treatment therapy. This in simple terms implies that a change in CYP enzyme activity may alter the metabolism and clearance of some xenobiotic. Alteration in the metabolism of one drug by another drug or food may result in an increased accumulation of the 1st drug within systemic circulation, thereby resulting in toxicity (Szakács, Váradi, Özvegy-Laczka, & Sarkadi, 2008). A study revealed that the bioactive compounds found in grapefruit juice inhibits CYP 3A4-mediated metabolism of certain medications, leading to decreased bioavailability and, hence the likelihood of an overdose (Ulbricht et al., 2008). Not all CYP-mediated drug interactions are clinically significant. The clinical significance of CYP-mediated drug interactions could

be of greater concern when drugs with narrow therapeutic window are considered. This may require dosage adjustments for one or more agents. It should be noted that drugs may still interact, despite different routes of administration.

In Ghana, traditional medicines are often used by patients in combination with prescribed drugs with the notion it has no side effects (Appiah-Opong, 2008). Hence, such drugs do not pass through the appropriate approval process applied to new drug applications. Due to the lack of scrutiny, herbal medicines may result in CYP-mediated herb-drug interactions when co-administered with other orthodox medications (Appiah-Opong et al., 2018). Interactions of traditional medicines with human CYPs have been shown to be associated with alterations in the pharmacokinetics of several drugs (Hu et al., 2005). They interact by either inhibiting or inducing CYPs which results in harmful side-effects. *Phyllanthus amarus* extract was reported to be a potent inhibitor of rat liver microsomal 7-ethoxyresorufin-O-deethylase (CYP1A1), 7 methoxyresorufin-O-demethylase (CYP1A2) and 7-pentoxyresorufin-O-dependentylase (CYP2B1/2) (Regina Appiah-Opong, Commandeur, Axson, Vermeulen, & toxicology, 2008). Studies on Kava (Pipermethysticum) extract, a commercially available herbal anxiolytic, showed significant inhibition of human CYP1A2 (56%), CYP2C9 (92%), CYP2C19 (86%), CYP2D6 (73%) and CYP3A4 (78%) *in vitro*.

Cyp450 mediated drug intractions

Drug-drug and herb-drug interactions is an important drug activity that should be investigated before new drugs are introduced on the market. Hence, it is useful to conduct *in vitro* investigations of traditional medicines on liver

CYP drug metabolizing enzymes (Appiah-Opong et al., 2008). This study seeks to evaluate the effect of xylopic acid on a resistant strain of *Plasmodium falciparum in vitro*, ascertain the effect of xylopic acid on liver enzymes using pentobarbitone sleeping time and finally identify the effect of the compound on specific cytochrome P450 isoenzymes *in vivo* and *in vitro*. Generally, it could be observed that most of the studies carried out on *Xylopia aethiopica* and its isolate xylopic acid were either *in vitro* or *in vivo* studies. Researchers should therefore consider extensive and comprehensive toxicity screening, preliminary search into understanding in precision how extracts of Xylopia aethiopica, xylopic acid and other isolates exert their therapeutic effects, their metabolic pathway and likelihood of toxicity.

CHAPTER THREE

RESEARCH METHODS

Introduction

This chapter described the techniques and procedures that were used in the process of gathering and analyzing data. It described the data collection procedure, and data analysis procedures used for the study.

Plant collection

The dried fruits of *Xylopia aethiopica* was handpicked from the botanical garden of Kwame Nkrumah University of Science and Technology (KNUST) at (06°41'6.39"N; 01°33'45.35"W) by Mr. Asare, a taxonomist, in the month of March 2019. He authenticated the fruit and kept a voucher specimen (FP/09/77) at the KNUST herbarium, before it was transported to the University of Cape Coast for further analysis.

Extraction and purification of xylopic acid (15β-Acetoxy-(-) - kaur-16-en-19-oic Acid)

The extraction process was like that described by (Woode, Ameyaw, Boakye-Gyasi, & Abotsi, 2012) with slight modifications. An amount of the dried fruit (5 kg) was pulverized, and 50 g of the fine powder was placed in cylindrical jars. A volume of 500 ml of petroleum ether was added to each jar at room temperature. The mixture was then allowed to stand for five days with intermittent shaking to aid in the extraction of the compound. After five days, the petroleum ether extracts from each jar was pooled, filtered and concentrated at a temperature of 50 °C using a rotary evaporator. One milliliter of ethyl acetate of was added to the petroleum ether concentrate in drops to facilitate crystallization. After five days, crystals of xylopic acid had formed. The crystals

obtained were then washed with petroleum ether (40-60 °C) to eliminate impurities and essential oils stuck to the surface of the crystals. The crystals obtained were then purified by recrystallization in 96 % ethanol, the process involved placing the crystals in ethanol and then heating at a temperature of 70 ℃ to facilitate dissolution of the crystals, after which the solution was filtered hot. After 24 hours, crystals of xylopic acid were deposited but allowed to stand for five days to facilitate further crystallization. The xylopic acid obtained was purified and stored in amber colored bottles until further use. The yield was a (4.70 % w/w).

IN VITRO ANTIMALARIAL ASSAY

Parasite culture and maintenance

The asexual intra-erythrocytic stage of *Plasmodium falciparum* laboratory strain Dd2 was continuously cultured *in vitro* in erythrocytes (sickling negative; O rhesus positive) fortified in complete culture medium (pH 7.3) in a flask. The complete culture medium consisted of filter-sterilized RPMI 1640 solution supplemented with 0.5 % AlbuMAX II and hypoxanthine (0.04 %) and buffered with 0.4 % sodium bicarbonate (Na_2HCO_3) and 0.72 % HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid). Gentamicin (0.005 mg/mL) was added to the final solution. The parasite culture was kept at 1 % parasitemia, 5 % hematocrit and incubated under a gas phase of 92 % N_2, 5 % CO_2 and 3 % O_2 at 37 °C. The cultures were maintained daily by changing the media and monitoring parasite viability and growth by light microscopy. D-Sorbitol treatment (5 %) was used to generate ring stage high synchronous parasite cultures for the various assays.

In vitro antiplasmodial screening of xylopic acid

A stock solution of 25 mM XA and 10 µM ART were prepared in DMSO. Stock XA and artemisinin (ART) were diluted with RPMI 1640 to a final concentration of 40 µM and 0.2 µM, respectively. A volume of 100 µL of the drug concentrations were loaded into a 96-well plate in triplicates. Two-fold serial dilutions were made with media for the drug-wells followed by the addition of 50 µL of 2 % haematocrit (HCT) and 1 % parasitaemia to obtain a final concentration of 0.0391 µM and 0.000195 µM for XA and ART, respectively (Forkuo et al., 2016). The plates were kept in incubation chamber and a gas mixture containing 92 % N_2, 5 % CO_2, 3 % O_2) was applied for 5 minutes. The chamber with the assay plates was placed in an incubator set at 37 °C for 72 hours. Each well was mixed with 25 µL of lysis buffer containing SYBR Green. The plates were incubated at room temperature in the dark for an hour and read with fluorescence multi-well plate reader with emission and excitation wavelength at 535 and 485 nm, respectively. The percentage of growth was normalized to that of untreated control parasites.

Experimental Animals

Prior to the experiment, male Sprague Dawley rats were purchased from the Animal Experimentation Unit of Noguchi Memorial Institute for Medical Research, Accra, Ghana. The animals were fed *ad libitum* using standard animal laboratory pellet (Sankofa Flour and Feeds, Accra, Ghana) and water. They were housed under standard laboratory conditions (25±1 ℃ ambient temperature, 60-70 % relative humidity, and 12:12 hour light: dark cycle) to ensure acclimatization to the laboratory. This was done for 7 days.

***In vivo* CYP450 isoenzyme induction assay**

Male rats weighing (250-300) g were randomly assigned to one of five experimental groups, being low dose xylopic acid treatment (LDT) 30 mg/kg, high dose xylopic acid treatment (HDT) 100 mg/kg, phenobarbitone (PC) 80 mg/kg, ketoconazole (NC) 100 mg/kg and a no treatment group that received distilled water; with (n=5) animals in each group. The treatment for each group was administered orally for 7 days, after which the rats were euthanized by placing them in a chloroform chamber after which their liver samples harvested, snap-frozen in liquid nitrogen and stored at -80 °C until further analysis (Zhou, Chan, Yeung, & interactions, 2012)

Preparation of Microsomal, cytosolic fractions

The liver samples obtained were removed from the -80 ℃ freezer and thawed on ice. The liver samples were then homogenized in two volumes of 20 mM potassium phosphate buffer (pH 7.4) using a mortar and a pestle. The homogenate was centrifuged at 2,500 rpm for 50 minutes at 4 °C and the supernatant collected and stored in ultra-centrifuge tubes. The supernatant was collected and further centrifuged at 40,000 rpm for 60 minutes at 4 °C. The resultant supernatant (cytosol) was then separated from the pellet (microsomes). The microsomes obtained were further homogenized in potassium phosphate buffer 20 mM (pH 7.4) to form a solution and finally the microsomal solution stored in Eppendorf tubes and stored at -80 ℃ (Pilon, 2003).

Protein determination and standardization

The protocol for protein determination was adapted from (Harwood et al., 2003). A four-fold serial dilutions was performed on the microsomal solutions using potassium phosphate buffer. Then a two-fold serial dilution for

seven concentrations was prepared for a protein standard, bovine serum albumin (BSA). Ten microliters of the BSA and 200 μL of Bio-Rad reagent which was then added to each microsomal dilution in a 96-well plate in triplicates and incubated at room temperature for 5 minutes, after which the absorbance was read at a wavelength of 530 nm excitation and 586 nm emission.

Measurement of the effect of XA on CYP 1A1/1A2, 1A2, 3A4, and 2B1/2B2 enzymes

The effect of xylopic acid on CYP 1A1/1A2, 1A2, 2B1/2B2, and 3A4 was determined using fluorometric assays as described (Appiah-Opong, Commandeur, van Vugt-Lussenburg, & Vermeulen, 2007). This was achieved by pipetting 70 μL of potassium phosphate buffer at a pH of (7.4) in triplicate into a 96-well plate followed by addition of 10 μL of each substrate (ethoxy resorufin (EROD), methoxyresorufin (MROD), pentoxyresorufin (PROD) and benzyloxy resorufin (BROD)). Subsequently, 10 μL of the microsomal fraction obtained from each treatment group was added to each well plate and pre-incubated at 37 ^{o}C for 5 minutes. after which, 10 µL of nicotinamide adenine dinucleotide phosphate (NADPH) with a concentration of (100 μM) was added to each of the wells and incubated at 37 ^{o}C for 10, 20, and 30 minutes for CYPs 1A1/1A2, 1A2, 2B1/2B2 and 3A4, respectively. A volume of 40 μL of stopping solution (20 % 0.5 M Tris and 80 % acetonitrile) was then added to each well, and the plates were gently shaken. Fluorescence was read at specific wavelength of 586 nm emission and 530 nm excitation.

***In vitro* inhibitory activity of XA on CYP 1A2 and 3A4**

Microsomes obtained from male rats pretreated with phenobarbitone were used in this assay. The standard inhibitors used were alpha

naphthoflavone, ketoconazole for CYP 1A2 and 3A4, respectively and xylopic acid as the test compound. A cocktail was prepared for seven different concentration for each standard inhibitor. The cocktail contained 3000 µl of 0.1 M potassium phosphate buffer (pH 7.4) which was pipetted into a 50 ml eppendorf tube, followed by addition of 600 µl of the substrate (methoxyresorufin, benzyloxy resorufin) for CYP 1A2 and 3A4, respectively, followed by the addition of 600 µl of the enzyme (microsomes). A volume of 80 µl was then pipetted from the cocktail into a 96-well plate in triplicates and then preincubated at 37 °C for 5 minutes.

Afterwards 10 µL of nicotinamide adenine dinucleotide phosphate (NADPH) with a concentration of (100 μM) was added to each of the wells followed by the addition of the inhibitor alpha naphthoflavone and xylopic acid to separate plates containing the substrate MROD and the inhibitor ketoconazole, and xylopic acid was added to separate plates containing BROD. The plates were incubated at 37 °C for 10, and 30 minutes for MROD and BROD, respectively. A volume of 40 μL of stopping solution (20 % 0.5 M Tris and 80 % acetonitrile) was finally added to each well. The plate was gently shaken, and its fluorescence read at wavelengths of 586nm emission and 530 nm excitation.

Effect of XA on CYP 2D6

CYP 2D6-Dextromethorphan-O-Demethylation assay was used to determine the effect of xylopic acid on CYP 2D6. The assay was done by pipetting 350 μL of potassium phosphate buffer (pH 7.4) into eppendorf tubes in triplicate. Fifty microliters of 0.25 mM dextromethorphan were added, followed by 50 μL of microsomes obtained from each treatment group. The

mixture was preincubated at 37 °C for 5 minutes in a water bath after which 50 µL of NADPH solution (100 µM) was added. The mixture was further incubated for 45 minutes, followed by the addition of 100 µL of stopping solution (300 mM zinc sulphate heptahydrate). The mixture was centrifuged at 4,000 rpm for 15 min at room temperature, and the supernatant collected in vials. Analysis of the supernatant was done using an isocratic HPLC. The mobile phase consisted of 24 % (v/v) acetonitrile and 0.1 % (v/v) trimethylamine adjusted (pH 3.0) with perchloric acid. The carrier flow rate was 0.8 ml/min and peaks monitored at wavelengths of 280 nm excitation and 310 nm emission.

Effect of XA on CYP 2C9

CYP 2C9 Diclofenac Hydroxylation assay was used to determine the effect of xylopic acid on CYP 2C9 (Appiah-Opong et al., 2008). This was done by pipetting 350 µL of potassium phosphate buffer (pH 7.4) into Eppendorf tubes followed by adding 50 µL of 0.05 mM diclofenac, after which 50 µL of the microsomal fraction obtained from each treatment group was added (in triplicate) and preincubated at 37 °C for 5 minutes in a water bath. A volume of 50 µL of NADPH solution (100 µM) was added to each tube and further incubated in a water bath at 37°C for 10 minutes. The reaction was terminated by adding 200 µL of stopping solution (ice-cold methanol) to the mixture. The mixture will be centrifuged at 12,000 rpm for 8 minutes at room temperature. The supernatants were collected in vials and analyzed using high-performance liquid chromatography.

Data analysis

For the *in vivo* testing, GraphPad Prism for Windows Version 7; GraphPad Software, San Diego, CA, USA, was used for all statistical analyses

and P values <0.05 was considered statistically significant. All data obtained was expressed as a mean ± standard error of the mean. For the *in vitro* analysis, the column graphs were subjected to one-way ANOVA with Tukey's post *hoc* test. Doses for 50% of the maximal effect (ED_{50}) for each drug were determined by using an iterative computer least squares method, with the following non-linear regression (3-parameter logistic) equation. The fitted midpoints (ED_{50}s) of the curves were compared statistically using *F* test.

CHAPTER FOUR

RESULTS AND DISCUSSION

In vitro antiplasmodial Screening of XA and ART

Xylopic acid exhibited negligible antiplasmodial activity after 72 hours contrary to artemisinin. The IC_{50} of XA> 20 µM. Artemisinin on the other hand produced an IC_{50} of 0.0083±0.00008µM.

Table 1: *In vitro* effect of xylopic acid and artemisinin on chloroquine resistant Dd2 *P. falciparum*

Compound	IC_{50} µM±S.E.M.
Artemisinin	0.0083±0.00008
Xylopic acid	>20

In vivo and *in vitro* Cytochrome P450 mediated pharmacokinetics of XA

The activities of xylopic acid high dose (100 mg/kg) and xylopic acid low dose (30 mg/kg) were tested on CYP 1A2 (MROD), CYP 1A1/1A2 (EROD), CYP 2B1/2B2 (PROD), CYP 3A4 (BROD), CYP 2D6 and CYP 2C9 employing *in vivo* and *in vitro* assays. Ketoconazole, alpha naphthoflavone, sulphurfenazole diclofenac, dextromethorphan phenobarbitone were used as standard drugs in the study.

Effect of phenobarbitone, xylopic acid high and low dose on CYP 1A2

All the treatment groups produced significant ($P<0.05$) CYP 1A2 enzyme induction as shown in figure 2. Administration of xylopic acid at a high dose of 100 mg/kg to rats produced the highest activity on CYP 1A2 with an induction of 81.6 %. xylopic acid low dose 30 mg/kg pretreatment had the next

highest activity with an induction of 77.2 % and finally Phenobarbitone pretreatment produced an activity of 70.10 % increase in enzyme induction compared to the non -treatment group that served as control.

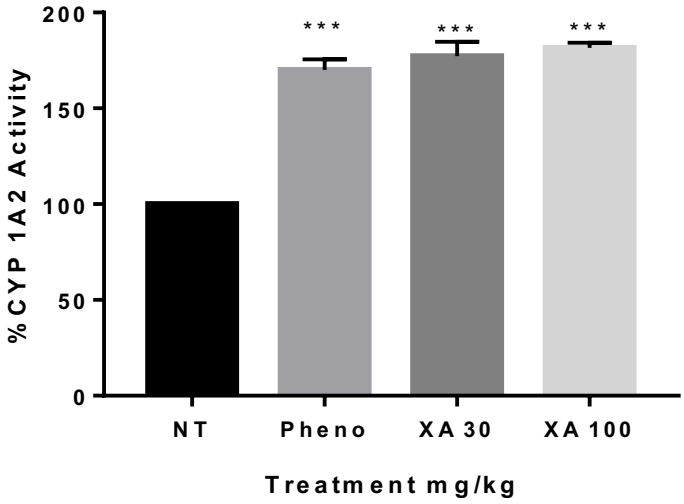

Figure 2: Effect of phenobarbitone, xylopic acid low dose (30 mg/kg), and xylopic acid high dose (100 mg/kg) on CYP1A2 (MROD) enzyme activity. Data is presented as mean±S.E.M. ***P<0.001 compared to no treatment group (One-way ANOVA followed by Tukey's post hoc test)

Effect of phenobarbitone, xylopic acid high and low doses on CYP 1A1/1A2

All the treatment groups had significant (P<0.05) effect on CYP 1A1/1A2, with phenobarbitone alone exhibiting the highest enzyme induction with a percentage activity of 139.9 % above the negative control (Figure 3). High dose of xylopic acid (100 mg/kg induced the enzyme resulting in activity of 106.9 % while xylopic acid low dose induced the enzyme with an activity of 118 % compared to the negative control. The efficacy order for the various treatment groups on CYP 1A1/1A2 was phenobarbitone > xylopic acid low dose > xylopic acid high dose.

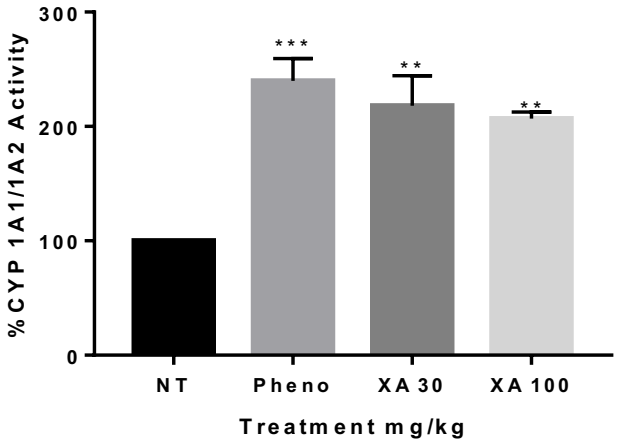

Figure 3: Effect of phenobarbitone, xylopic acid low dose (30 mg/kg) and xylopic acid high dose (100 mg/kg) on CYP 1A1/1A2 (EROD) enzyme activity. Data is presented as mean±S.E.M. **P<0.01, ***P<0.001 compared to the negative control (no treatment) group. (One-way ANOVA followed by Tukey's post *hoc* test)

Effect of phenobarbitone, xylopic acid low and high dose on CYP 2B1/2B2

From figure 4, phenobarbitone pretreatment group had the most significant (P<0.001) effect on CYP 2B1/2B2 with an activity of 455 %. Xylopic acid low and high dose had a significant (P<0.05) effect on CYP 2B1/2B2. The percentage activity for xylopic acid low and high doses were 56.8 % and 84.7 % respectively. However, Phenobarbitone was 18.5 times a more potent inducer of CYP 2B1/2B2 than xylopic acid low dose and 12.2 times more active than xylopic acid high dose.

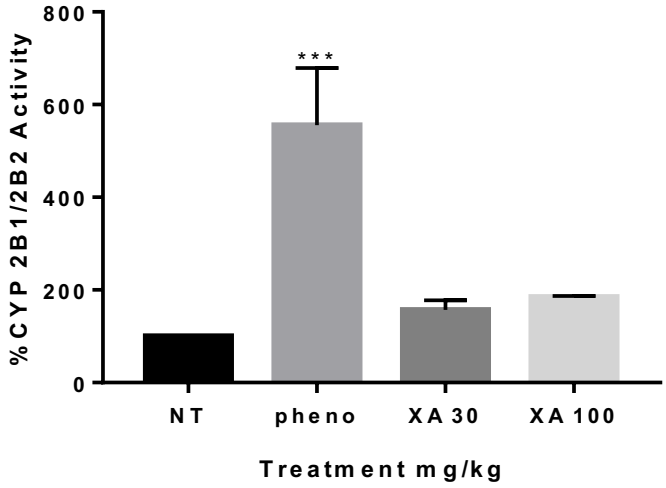

Figure 4: Effect of phenobarbitone, xylopic acid low dose (30 mg/kg) and xylopic acid high dose (100 mg/kg) on CYP 2B1/2B2 enzyme. Data is presented as mean ±S.E.M *P<0.05, ***P<0.001 compared to the no treatment group. (One-way ANOVA followed by Tukey's post hoc test)

Effects of phenobarbitone, xylopic acid low and high dose on CYP 3A4

Figure 5 illustrates the activity of Phenobarbitone, Ketoconazole, and Xylopic acid low and high dose. Phenobarbitone induced CYP3A4 with an increase in activity of 545 %, whiles ketoconazole, xylopic acid low and high dose inhibited CYP 3A4 with activities of 59.38 %, 82.11 % and 80.68 %, respectively. The inhibitory activity of xylopic acid high dose was 1.36 times more than the standard ketoconazole.

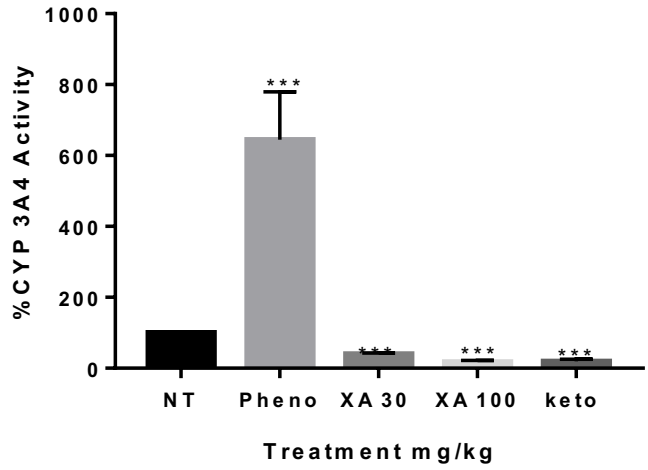

Figure 5: Effect of phenobarbitone, xylopic acid low dose (30 mg/kg), xylopic acid high dose (100 mg/kg) and ketoconazole on CYP 3A4 enzyme. Data is presented as mean ±S.E.M ***P<0.001 compared to the no treatment group. (One-way ANOVA followed by Tukey's post hoc test)

Effect of phenobarbitone, xylopic acid low and high dose on CYP 2C9

Figure 6 shows the effect of phenobarbitone, xylopic acid low and high dose on the biotransformation of diclofenac by CYP 2C9. All the treatment groups significantly induced the enzyme activity with percentage enzyme induction of 184.9 %, 129.9 % and 151.8 % for phenobarbitone, xylopic acid low dose and xylopic acid high dose, respectively, as compared to the no treatment group (P<0.001).

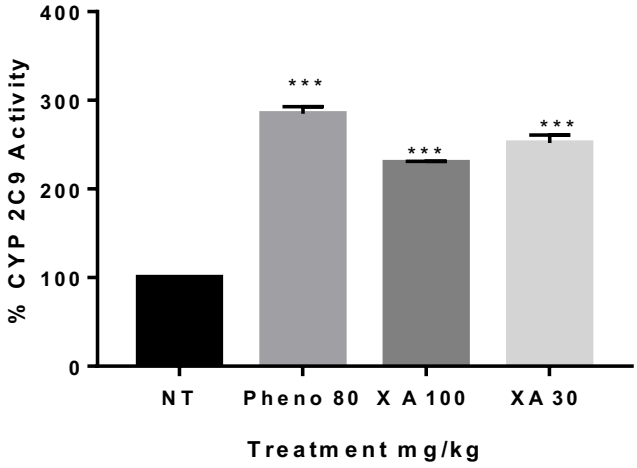

Figure 6: Effect of phenobarbitone, xylopic acid low dose (30 mg/kg), xylopic acid high dose and (100 mg/kg) on CYP 2C9 (diclofenac hydroxylation). Data is presented as mean ±S.E.M ***P<0.001 compared to the no treatment group. (One-way ANOVA followed by Turkey's hoc test)

Effect of phenobarbitone, xylopic acid high and low dose on CYP 2D6

Effects of phenobarbitone, xylopic acid low dose and xylopic acid high dose on CYP 2D6 are shown in figure 7. The figure describes a significance in difference between CYP 2D6 activity of negative control experiment and the treated (P<0.001) for phenobarbitone and xylopic acid high and a significance of (P<0.05) in the case of xylopic acid low dose. Phenobarbitone, xylopic acid low dose and xylopic acid high dose had activities of 48.6 %, 24 % and 80.5 %, respectively. Xylopic acid high dose was 1.65 times more effective than the standard phenobarbitone.

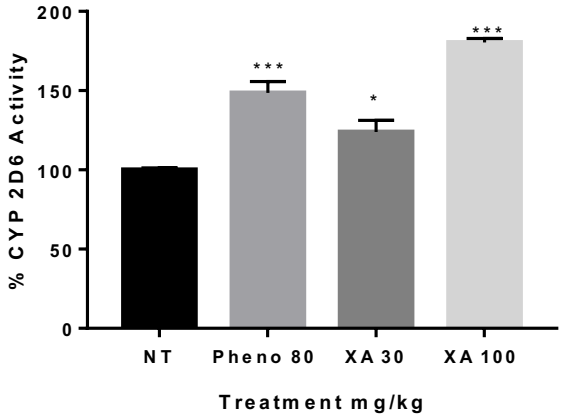

Figure 7: Effect of phenobarbitone, xylopic acid low dose (30 mg/kg), and xylopic acid high dose (100 mg/kg) on CYP 2D6 (Dextromethorphan O-Demethylation). Data is presented as mean ±S.E.M *P<0.05, ***P<0.001 compared to the no treatment group. (One-way ANOVA followed by Tukey's post hoc test)

Effect of xylopic acid on CYP 3A4 *in vitro*

Figure 8 is a dose-response curve which demonstrates the effect of various concentrations of xylopic acid on CYP 3A4 *in vitro* assay. Ketoconazole was used as positive control. The IC_{50} values for ketoconazole and xylopic acid were 0.091±0.002 µM and 1.30±0.01 µM, respectively. Thus, ketoconazole showed more potent inhibition of CYP3A4 than xylopic acid.

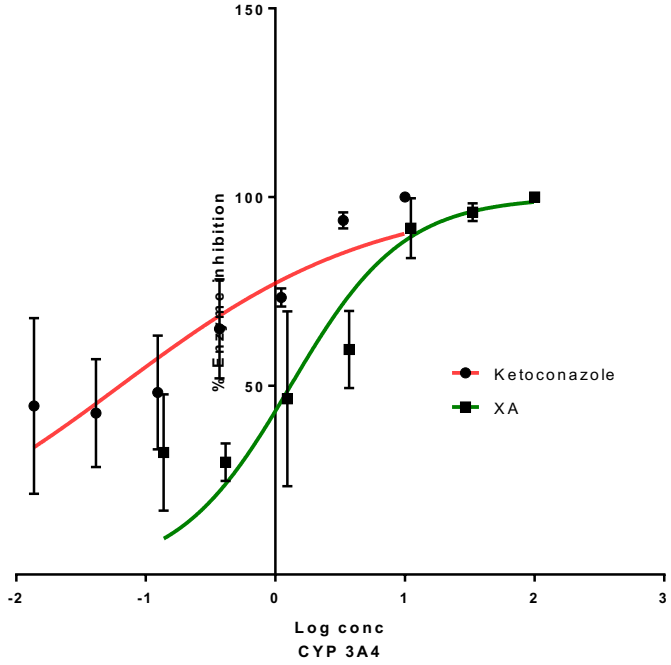

Figure 8: Dose response curves of ketoconazole and xylopic acid on the inhibition of CYP 3A4

Effect of xylopic acid on the inhibition of CYP1A2

Figure 9 represents a dose response curve, showing the effects of the standard, alpha naphthoflavone and xylopic acid at various concentrations on CYP1A2. The IC_{50} value for alpha naphthoflavone was 0.1484±2.401 µM and that for xylopic acid was 0.235±6.84 µM. Alpha naphthoflavone showed stronger inhibitory potency of CYP1A2 than xylopic acid.

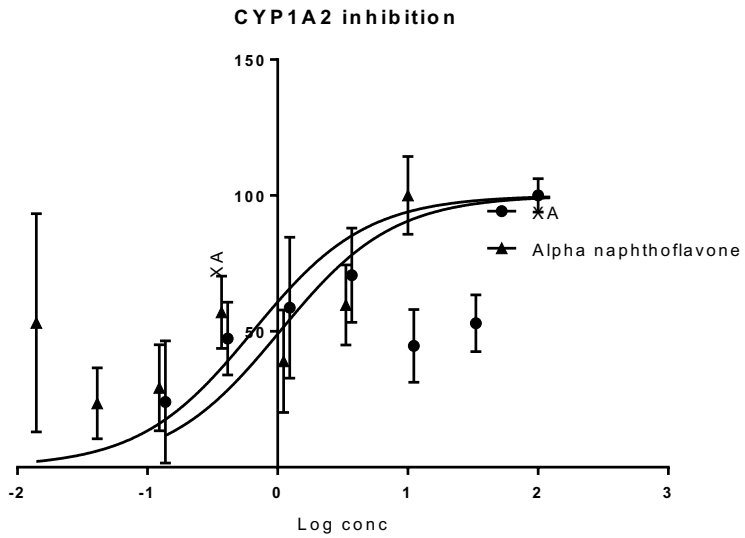

Figure 9: Dose response curves of alpha naphthoflavone and xylopic acid on the inhibition of CYP 1A2

Discussion

The study evaluated the *in vitro* effect of xylopic acid alongside artemisinin in the management of malaria. Xylopic acid exhibited negligible antiplasmodial activity (IC_{50} of XA > 20 µM) after 72 hours of administration contrary to artemisinin. Artemisinin on the other hand caused an IC_{50} value of 0.0083±0.00008 µM. However, in an *in vivo* study, xylopic acid was found to have a significant antimalarial activity (Boampong et al., 2013).

The lack of activity of xylopic acid observed in the *in vitro* studies could be explained by the fact that xylopic acid has activity against *Plasmodium berghei* but not *Plasmodium falciparum* as seen in this study. There is also evidence supporting the traditional use of the fruit of *Xylopia aethiopica* from which xylopic acid is obtained (Fetse, Kofie, & Reimmel, 2016). There is the possibility that other phytochemicals within the extract of the fruit of *Xylopia aethiopica* could be responsible for the antimalarial activity observed. One other compound identified from the fruit of *Xylopia aethiopica* is kaurenoic acid

which is parent moiety of xylopic acid. An *in vitro* study implicated kaurenoic acid as a potent compound with activity against malaria. Similarly, xylopic acid could be acting as a pro-drug hence an active metabolite could be responsible for the antimalarial activity observed *in vivo*. Therefore, the contribution of CYP450 iso enzymes on the antiplasmodial activity of xylopic acid cannot be ruled out. This must be investigated.

Interestingly, a recent study suggested that xylopic acid has a biphasic effect on hepatic CYP450 enzymes at low and high doses (Biney, Mantel, Boakye-Gyasi, Kukuia, & Woode, 2014) but no specific enzymes were implicated in the biotransformation of the compound. It was therefore imperative to identify the specific isoenzymes induced and inhibited on administration of xylopic acid with focus on CYP 1A1/1A2, 1A2, 2B1/2B2, 3A4, 2D6, and 2C9.

Xylopic acid induced CYP 1A1/1A2 at high and low doses. Other substances that induce CYP 1A1/1A2 are barbiturates, tobacco, and rifampin among others (Martignoni, Groothuis & de Kanter, 2006). A study revealed that Polycyclic aromatic hydrocarbons (PAHs) are some of the major lung carcinogens found in tobacco smoke. PAHs are potent inducers of isoenzymes 1A1 and 1A2 (Luckert et al., 2013). This suggests that an interaction exists between tobacco and other inducers of CYP 1A1/1A2. Therefore, smokers taking xylopic acid may require higher dosages than nonsmokers.

Xylopic acid induced CYP 1A2 at low and high doses, like the effect seen in phenobarbitone. Another inducer of CYP 1A2 is tobacco, studies has shown that CYP1A2 is responsible for the primary metabolism of theophylline (Orlando et al., 2006), and propranolol amongst others. A study implicated

cimetidine which is a Histamine H2 – receptor antagonist as an inhibitor of CYP 1A2 (Martínez et al., 1999). This suggested that administration of xylopic acid with H2 – receptor antagonists may result in drug-drug interaction.

CYP 2B1/2B2 is a Phenobarbitol-inducible member of the P450 xenobiotic-inducible superfamily involved in hydroxylation of decanoic and other fatty acids (Meunier, de Visser, & Shaik, 2004). The current study revealed xylopic acid had no activity (induction/inhibition) on CYP 2B1/2B2. A recent study suggested that CYP 2B1/2B2 has a higher expression in the lung compared to the liver of rats (Hurst, Loi, Brodfuehrer, & El-Kattan, 2007). This could be a reason for the lack of activity observed. There is evidence that rat CYP450 enzymes are closely related to the CYP450 enzymes found in humans (Martignoni, Groothuis & de Kanter, 2006). CYP450 enzymes are responsible for the biotransformation of majority of drugs (Tari, Anwar, Liang, Cai, & Baral, 2010).

CYP3A4 was inhibited by low and high doses of xylopic acid. The inhibition observed is 14 times weaker than that expressed by ketoconazole a standard inhibitor of 3A4. CYP 3A4, accounts for 30-50% of drugs metabolized through type I enzymes (Pirmohamed &Park, 2003) therefore it was paramount to investigate the effect of xylopic acid on CYP 3A4. Xylopic acid at low and high doses induced CYP 2C9. It is primarily expressed in the liver; a study suggests expression level is the second highest among CYP isoforms. It is responsible for the metabolic clearance of up to 15-20% of all drugs (Läpple et al., 2003). Some other substrates metabolized by this CYP aside diclofenac include alkylating agents such as cyclophosphamide (Gonzalez, Tukey, & McGraw-Hill, 2006), anti-estrogenic drugs such as tamoxifen (Pan et al., 2014)

and some angiotensin II blockers such as Irbesartan (Van Booven et al., 2010). Treatment with rifampicin has been implicated in increased clearance of drugs metabolized(M. Farooq, Kelly, & Unadkat, 2016) by CYP2C9 (Chen & Raymond, 2006).

Drugs that inhibits this enzyme include fluconazole and sulphaphenazole. There is evidence supporting the danger of drug-drug interaction when sulphaphenazole is added to a therapeutic regime that includes drugs with a low therapeutic index , such as warfarin (Van Booven et al., 2010). This suggests the use of xylopic acid with these inhibitors should be carefully considered. Other inducers of this enzyme include corticosteroids an example of which is prednisone among others (Farooq, Kelly & Unadkat, 2016). Patients vary widely in their response to drugs (Belle & Singh, 2008). They include individuals with no CYP2D6 activity and individuals with genetically elevated CYP2D6 activity (Kawanishi, Lundgren, Ågren, & Bertilsson, 2004). Suggesting an altered response to drugs that are CYP2D6 substrates. Drugs that inhibit CYP2D6 activity are likely to increase the plasma concentrations of other medications, such as fluoxetine, paroxetine leading to drug toxicity.

Xylopic acid significantly inhibited CYP1A2 activity IC_{50} value like that exhibited by the known inhibitor, alpha naphthoflavone. This enzyme is known to catalyze the metabolism of imipramine (Lemoine et al., 1993) clozapine and theophylline (Sarkar, Hunt, Guzelian, & Karnes, 1992). A study showed that these drugs are altered after the administration or exposure to inducers or inhibitors of CYP1A2 (Kelly & Sussman, 2000), and fluvoxamine (Orlando et al., 2006), which inhibits the enzyme. Thus, if co-administered with known substrates of CYP1A2 there will be increases in plasma concentrations

of drug substrates of the enzyme such as imipramine, clozapine and theophylline.

Xylopic acid in an *in vitro* assay inhibited CYP 3A4 activity “figure 8”. This finding supports the data represented in figure 5. Other inhibitors of CYP3A4 aside ketoconazole include itraconazole,(Varis, Kivisto, Backman, & Neuvonen, 2000) erythromycin (Paine, Wagner, Hoffmaster, Watkins, & Therapeutics, 2002) among others. Studies indicate co-administration of 3A4 inhibitors with dihydropyridine calcium antagonists, phosphodiesterase inhibitor sildenafil may result in hypotension (Dresser, Spence, & Bailey, 2000).

CHAPTER FIVE

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

Introduction

In this final chapter, the summary, conclusions and recommendations of the study are herein presented. The chapter places emphasis on the overview of the research process, the key results, conclusions drawn, recommendation made and the suggestions put forward for further studies

Summary

Xylopic acid (XA), the major constituent of the fruit of *Xylopia aethiopica* has shown several pharmacological properties. Consequently, the fruit is being used in the preparation of food, used traditionally to treat several diseases and it has been formulated into a nasal drop despite the lack of information about its food-drug interaction, and other pharmacokinetic properties. Although it had significant activity against rats infected with plasmodium berghei, the results obtained cannot be extrapolated to humans as plasmodium berghei does not infect man. Therefore, this study investigated the antiplasmodial effect of XA on *P. falciparum*, its effect on rat liver enzymes (*in vivo*), its and inhibitory effect on selected rat liver CYP enzymes (*in vivo*) and induction its inhibitory effect at various concentration on selected CYP enzymes (*in vitro*). To establish the effect of XA on *P. falciparum*, the asexual intra-erythrocytic stage of the parasite strain Dd2 was cultured and treated with XA.

Pentobarbitone-induced sleeping time was used to investigate the effect of XA on rat liver enzymes. Inhibition or induction of some isoforms of CYP450 such as (CYP 1A1/1A2, 1A2, 2B1/2B2, 3A4, 2D6,2C9) was investigated using microsomal fractions of rat liver. The *in vitro* inhibition of

selected CYP (1A2, 3A4) was established by administering XA on rat liver microsomes. Xylopic acid exhibited negligible antiplasmodial activity. The IC_{50} of XA> 20 µM. Artemisinin had a significant IC_{50} of 0.0083±0.00008 µM. Xylopic acid induced CYP 1A1/1A2, 1A2, 2D6, 2C9, and inhibited CYP3A4, 2B1/2B2. The findings would help mitigate toxicity and therapeutic failure especially in cases of co-administration of medications with food containing XA, with metabolism altered by the latter.

Conclusions

Xylopic acid induced rat liver microsomal CYP 1A1/1A2, 1A2, 2D6, 2C9, and inhibited CYP3A4, 2B1/2B2 *in vivo*. Xylopic acid also has inhibited the activities of CYP1A2 and 3A4 *in vitro*. Animal findings cannot be extrapolated to humans without further verification and testing. Therefore, further work using human microsomal and cytosolic fractions will help mitigate toxicity and therapeutic failure especially in cases of co-administration of medications with food containing XA, with metabolism altered by the latter.

Recommendations

The active metabolites of xylopic acid should be identified, synthesized and tested for pharmacological properties. Other pharmacokinetic properties (peak plasma concentration, AUC, rate of excretion) should also be worked on. Generally, it could be observed that most of the studies carried out on the plant extracts and xylopic acid are either *in vitro* or *in vivo* studies. Research should therefore include preliminary studies involved in understanding in precision the mechanism of action and metabolic pathway utilized by the compound.

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