KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI

EFFICACY, PHARMACOKINETICS AND SAFETY EVALUATION OF CRYPTOLEPINE-ARTEMISININ BASED COMBINATIONS IN THE MANAGEMENT OF UNCOMPLICATED MALARIA.

By

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A THESIS SUBMITTED IN PARTIAL
FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

In the

Department of Pharmacology,
Faculty of Pharmacy and Pharmaceutical Sciences
College of Health Sciences

MAY, 2016

DECLARATION

The experimental work described in this thesis was carried out at the Department of Pharmacology, KNUST; the Department of Biomedical and Forensic Sciences, University of Cape Coast; Noguchi Institute of Biomedical Research, University of Ghana, Legon, Centre for Scientific and Industrial Research (CSIR), Pretoria South Africa and the Metabolism and Pharmacokinetics (MAP)/*In Vitro* Department, Novartis Pharma AG, Basel, Switzerland. This work has not been submitted for any other degree.

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ABSTRACT

The emergence of *Plasmodium falciparum* strains (multidrug resistant) resistant to commonly used antimalarial agents has motivated the research for novel drugs and drug combinations as new strategies for the prophylaxis and treatment of malaria. *Cryptolepis sanguinolenta*, a popular West African antimalarial plant has been used for several decades for the treatment of malaria and other infections. Cryptolepine is the major alkaloid isolated from the plant with remarkable antimalarial activity and by far the most studied. This study was conducted to characterize some drug-likeness properties of cryptolepine and determine whether a safe and novel antimalarial combination could be developed in combination with the artemisinin derivatives. The *in vitro* efficacy of cryptolepine and the aqueous root extract of *Cryptolepis sanguinolenta* against the late stage gametocytes of *P. falciparum* (NF54) using the PrestoBlue® assay was also ascertained in this study. *In vivo* pharmacokinetics in rat and the *in vitro* absorption, distribution and metabolism of cryptolepine were also characterised in rat and human models. Cryptolepine hydrochloride was isolated from the root of *C. sanguinolenta* and identified by Ultra Violet (UV) spectra, Thin Layer chromatography (TLC), High

Performance Liquid chromatography (HPLC) and melting point determination studies. A SYBR Green I fluorescent-based *in vitro* drug sensitivity assay using a fixed ratio method was performed on the chloroquine-sensitive plasmodial strain 3D7 to build isobolograms from cryptolepine-based combinations with standard antimalarial drugs. Cryptolepine exhibited promising synergistic interactions *in vitro* with artesunate, artemether, dihydroartemisinin and amodiaquine. The combination of cryptolepine with chloroquine and lumefantrine showed an additive effect whereas antagonism was observed with mefloquine in the isobologram analysis. *In vivo*, the Rane's test in ICR mice infected with *Plasmodium berghei* NK-65 strains was used to build an isobologram from cryptolepine-artesunate fixed ratio combination (1:1) and fractions of their ED_{50}s. Cryptolepine

combination with artesunate again showed synergy as the Zexp was 1.02±0.02 mg/kg which was significantly lesser than the Z_{add} of 8.3±0.31 mg/kg. The aqueous root extract of *C. sanguinolenta* and its major alkaloid, cryptolepine had minimal inhibitory effects on the late stage gametocytes from *Plasmodium falciparum* strain NF54. In the *in vitro* pharmacokinetic assays, cryptolepine showed a high passive permeability, a low human P-gp efflux potential, a good metabolic stability and a moderate protein binding in rat and human plasma. The preliminary incubation in human and rat hepatocyte showed a low to moderate hepatic extraction. Nine metabolites were identified in human and rat hepatocytes, resulting from metabolic pathways involving oxidation (M2-M9) and glucuronidation (M1, M2, M4, M8 and M9). Some of the metabolites were also identified in the urine (M2, M6 and M9) and plasma of rats (M6). The enzyme phenotyping assay and the metabolites identified in the hepatocytes suggests that both cytosolic and microsomal liver enzymes may be involved in the metabolism of cryptolepine in rat and human hepatocytes and among them may be aldehyde oxidase, UDP-glucuronyltransferase and the cytochrome P450 enzyme system. The *in vivo* rat Pharmacokinetic profile of cryptolepine showed very high clearance and volume of distribution (Vss), a moderate half-life, low oral exposure, early Tmax, and a low Cmax. Elimination was faster and systemic exposure (AUC) to cryptolepine was low to moderate in rats with unchanged excretion of cryptolepine in the urine less than 0.1% of the administered dose. This indicates metabolism, unchanged drug and/or biliary excretion as the main clearance pathway(s). The haematological, biochemical, organ/body weight ratio and histopathology indices in the rats treated with cryptolepine at all doses (25, 50, 100 mg/kg *p.o*) and in combination with artesunate (4 mg/kg) or artemether (50 mg/kg) showed no significant acute toxicity compared to the control group. All treatments presented no morphological changes in the kidney, spleen, stomach, and liver tissues used in the histopathology study. These findings provide

a strong basis for the selection of cryptolepine as a potential lead compound in the development of combination therapy against malaria.

ACKNOWLEDGEMENT

I am thankful to the Almighty God, Giver of life, knowledge and wisdom for seeing me to this point of my postgraduate studies. My profound gratitude also goes to my lead supervisor, Rev. Prof. Charles Ansah of the Department of Pharmacology, KNUST for his immense contribution, fatherly advice, thorough supervision and mentorship role during the period of the programme.

I am also grateful to Caroline Rynn of the Metabolism and Pharmacokinetic (MAP/*IN VITRO*) Department, Novartis Institute of Biomedical Research (NIBR), Basel, Switzerland for her guidance and supervision during my stay in her laboratory at Novartis. Not forgetting David Pearson, Werner Gertsch, Markus Trunzer and Laurent Hoffman for their immense help and training during my stay in Novartis AG, Basel, Switzerland. I will also want to thank Professor Dr. David Obiri, the Head of the Pharmacology Department and all my Laboratory Scientific associates Sabinne Arnold, Yannick Friche, Gaelle Chenal and Thomas Ansah for their technical contribution to my research. My sincere appreciation goes to the Education, Diversity and Inclusion group of Novartis AG for funding my stay and research in Basel, Switzerland for 3 months. I appreciate greatly the help offered me by Prof. Boampong and Dr. Ameyaw of the Department of Human Biology during the *in vivo* antimalarial assay in their laboratory. Not forgetting Profrssor Ben Gyan and Dr. Michael Ofori of NMIMR, University of Ghana, Legon for their assistance and support in the *in vitro* antimalarial assays and Dr. Dalu Mancama of the Centre

for Scientific and Industrial Research in Pretoria, South Africa for his assistance with the gametocyte assay. Finally, my heartfelt gratitude goes to my family, postgraduate colleagues and staff of the Department of Pharmacology for their encouragement. Not forgetting Jennifer, Aaron, Paapa, Nana Ama, Robert, Charles, Prince, Patience, Eyram and Soudzi for their friendship.

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LIST OF ABBREVIATIONS

ADME	Absorption, Distribution, Metabolism and Excretion
ALB	Albumin
ALDH	Aldehyde dehydrogenase
ALK. PHOS	Alkaline Phosphatase
AMQ	Amodiaquine
ALT	Alanine aminotransferase
AO	Aldehyde Oxidase
AP→BL	Apical to Basolateral
ARM	Artemether

AST	Aspartate aminotransferase
BL→AP	Basolateral to Apical
BSA	Bovine Serum Albumin
CNS	Central Nervous System
CPE	Cryptolepine hydrochloride
CPS	Aqueous root extract of *Cryptolepis sanguinolenta*
CPM	Complete Parasite medium
CQ	Chloroquine
CQR	Chloroquine Resistant
CREAT.	Creatinine
CYP	Cytochrome P450
D-BIL	Direct bilirubin
DDI	Drug-drug interaction
DDT	Dichloro Diphenyl Trichloroethane
DHA	Dihydroartemisinin
DME	Drug metabolism enzyme
DMEM	Dulbecco's Modified Eagle's Medium
DMPK	Drug metabolism and pharmacokinetics
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's Phosphate Buffer solution
ED_{50}	50% Effective dose
EDTA	Ethylene diaminetetraacetic acid
FA	Fraction absorbed

FBS	Foetal Bovine serum
FIC_{5O}	Fractional 50% Inhibitory concentration
GGT	Gamma glutamyl transpeptidase
GIT	Gastrointestinal Tract
GLB	Globulin
H	Hydralazine
HBSS	Hank's balanced salt solution
HCl	Hydrochloric acid
HCT	Haematocrit
HPLC	High performance liquid chromatography
HTS	High Throughput Screening
IC_{50}	50% Inhibitory concentration
ICR	Imprinting Control Region
IND-BIL	Indirect bilirubin
i.p	Intraperitoneal
IPTs	Intermittent preventive treatment
IRS	Indoor residual spraying
IS	Internal standard
ITNs	Insecticide treated nets
Kel	Initial rate of elimination
Kg	Kilogram
L	Litre
LLINs	Long lasting insecticide nets
MAO	Monoamine Oxidase
MCH	Mean corpuscular haemoglobin

MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MDCK-MDR1	Madine Darby Canine kidney cells expressed with human multiple drug resistant P-glycoprotein
MDR	Multi-drug resistance
METH	Artemether
MetID	Metabolite Identification mL
Millilitres	
MS	Mass spectrum
NADPH	Nicotinamide adenine dinucleotide phosphate-reduced
NCE	New chemical entity
PAMPA	Parallel Artificial Membrane Permeability Assay
PAR	Peak Area ratio
P-gp	P-glycoprotein
PLT	Platelet
PK	Pharmacokinetics
R	Raloxifene
RFU	Relative Fluorescence Unit
SD	Sprague Dawley
SEM	Standard error of mean
SERCA	Sarco endoplasmic reticulum calcium
S9 fraction	Subcellular fraction prepared by collecting supernatant after centrifugation of liver tissue homogenate at 9000×g
T-PRO	Total Protein
T-BIL	Total bilirubin

TDI	Time dependent inhibition
TLC	Thin layer chromatography
UDP	Uridine diphosphate
UV	Ultraviolet radiation
WBCs	White blood cells
WHO	World Health Organisation
Z_{add}	Theoretical potency
Z_{exp}	Experimental potency
µg/ml	Microgram per millilitre
µM	Micromolar

1.0 CHAPTER ONE

INTRODUCTION

1.1 GENERAL INTRODUCTION

The discovery and development of artemisinin and its derivatives have been a major advancement in the chemotherapy of malaria. Despite their low toxicity, high effectiveness and activity on the gametocyte stage of the parasite (Price *et al*., 1996), they have their limitations. The increasing prevalence of strains of *Plasmodium falciparum* resistant to chloroquine and recently some artemisinin derivatives underscores the urgent need to develop new antimalarial compounds with novel modes of action (Peters, 1982; White, 1985; Trape *et al*., 2002).

Drug discovery and development from traditional medicines have played and continue to play an important role as drugs or as sources of lead compounds in treatment of many diseases. Quinine and artemisinin or more especially their derivatives are important examples (Phillipson *et al*., 1993). Indoloquinolines are unique natural alkaloids representing a new class of drug leads and found almost exclusively in the Central- and West-African climbing shrub *Cryptolepis sanguinolenta* (Lindl.) Schlechter (Lavrado *et al*., 2010). The aqueous extracts from the roots of the plant have been used for decades by African traditional healers mainly for the treatment of bacterial infections, fevers, hepatitis and malaria. Other uses, such as spasmolytic, antirheumatic and as a tonic have also been reported (Boakye-Yiadom, 1979; Boye and Oku-Ampofo, 1983; Boye and Oku-Ampofo, 1990).

Cryptolepine is by far the most studied alkaloid from the plant with antibacterial, antifungal, antihyperglycemic, anti-inflammatory, cytotoxic, antitumoral activity and potent activity against both

chloroquine-sensitive and chloroquine-resistant *Plasmodium falciparum* (strain K1) *in vitro* (Lavrado *et al*., 2010).

About 80% of the developing world's population as estimated by the World Health Organisation (WHO) meets their primary healthcare needs through traditional medicine (Calixto, 2000). As the patronage of this herbal medicines increase worldwide, there is an increased concern about the possible interaction of the widely used *Cryptolepis sanguinolenta* with the WHO recommended artemisinin derivatives in the West African sub-region. Many patients who use herbal medicines before reporting to the hospital do not disclose this information to their physicians and hence increase the possibility of adverse herb-drug interactions (Chen, 2006; Delgodaa *et al*., 2011).

In spite of the multiplicity of biological effects of *Cryptolepis sanguinolenta* and its major alkaloid cryptolepine, very little is known about their efficacy and/or safety with the standard antimalarial agents. This thesis seeks to collect data on the antimalarial interaction and safety of cryptolepine/cryptolepis with the artemisinin derivatives, their gametocytocidal activity as well as the *in vivo* and *in vitro* pharmacokinetic profile of cryptolepine.

1.2 *CRYPTOLEPIS SANGUINOLENTA.*

Cryptolepis sanguinolenta (Lindl.) Schlechter also known as *Cryptolepis triangularis* was originally a member of the family Asclepiadaceae and subfamily Periplocoideae and is also a member of the newly created family Periplocaceae (Paulo *et al.*, 2000).

Cryptolepis sanguinolenta is a slender, thin-stemmed climbing shrub with orange-coloured juice in the cut stem (Paulo and Houghton, 2003). The flowers are greenish-yellow, the fruit is a follicle, linear 17–31 cm long, and the seeds are 10–12 mm long with a tuft of silky hairs at the end. The leaves are glabrous, oblong-elliptic, shortly acuminate apex, rounded base. The roots are yellowish, tortuous and branched with little or no rootlets. The roots are with longitudinal ridges apparent in the dried samples and break with a short fracture exposing a smooth transverse surface (Iwu, 1993). The seeds are small (averaging, 7.4 mm in length and 1.8 mm in the middle), oblong shaped and pinkish, embedded in long silky hairs (Irvine, 1961).

The plant is distributed throughout the West coast of Africa growing very well in the rainforest and deciduous forest belts (Iwu, 1993). It is normally found in the forest and thickets but can also be cultivated.

1.3 TRADITIONAL USES OF *CRYPTOLEPIS SANGUINOLENTA*

The aqueous root extract of *Cryptolepis sanguinolenta* is a popular antimalarial agent in West African ethno medicine. The root of *Cryptolepis sanguinolenta* is noted in traditional African

medicine to treat a wide range of diseases including hepatitis, urinary tract infections, jaundice, hypertension, stomach ache and inflammatory conditions. The root extracts have also been used as a daily tonic for years without any evidence of toxicity (Juliani *et al.*, 2009).

Decoction of *C. sanguinolenta* is used by healers in many West African countries to treat a variety of conditions that could be associated with diabetes, such as vaginal *Candida albicans* infections (Luo *et al.*, 1998). The Fulani traditional healers in Guinea-Bissau use aqueous extracts of the plant to treat jaundice and hepatitis (Silva *et al*., 1996). An aqueous decoction of the root bark of cryptolepis is used in Congolese traditional medicine for the treatment of amoebiasis (Tona *et al*., 1998). There are also reports of the plant being used in the management of insomnia (Mshanna *et al*., 2000).

Today, the aqueous root extracts of *C. sanguinolenta* is widely used in most antimalarial herbal preparations in Ghana. These products on the market include Masada mixture, Fas Malacure, Nibima among others.

1.4 CRYPTOLEPINE

1.4.1 Sources

Cryptolepine is the major alkaloid of the plant *Cryptolepis sanguinolenta*. This alkaloid was first isolated in Nigeria (Gellert *et al*., 1951). Several researchers have isolated cryptolepine from the root powder of the plant (Dwuma-Badu *et al*., 1978; Cimanga *et al*., 1996; Bierer *et al*., 1998).

Cryptolepine has also been isolated from *Sida acuta* (Malvaceae) (Gunatilaka *et al*., 1980), *Microphilis guianensis* and *Cryptolepis triangularis* (Paulo *et al*., 1995; Yang *et al*., 1999). Other alkaloids that have been isolated from *Cryptolepis sanguinolenta* include quindoline (the demethylated analogue of cryptolepine), quindolinone, 11-hydroxycryptolepine, cryptosanguinolentine, cryptotackieine, isocryptolepine and isoneocryptolepine (Dwuma-Badu *et al*., 1978; Cimanga *et al*., 1997). Chemically, cryptolepine (5- methyl-10*H*-indolo [3, 2-*b*] quinoline) is an indoloquinoline compound and a weak base. The base has a deep purple colouration and calculated molecular weight of 232 g/mole, with a melting point between 175 to 179°C (Gellért *et al*., 1951; Bierer *et al*., 1998). The hydrochloride salt is yellow and has a calculated molecular weight of 268.5 g/mole and melts around 268°C (Bierer *et al*., 1998). Fichter and his co-workers were the first to report the synthesis of cryptolepine in 1906 (Fichter and Boehringer, 1906), 26 years prior to the first isolation from a natural source (Clinquart, 1929). The compound and several analogues (Figure 1.1) have since been synthesized by several researchers via different synthetic routes (Bierer *et al*., 1998; Yang *et al*., 1999; Arzel *et al*., 2001; Wright *et al*., 2001b; Jonckers *et al*., 2002; Onyeibor *et al*., 2005; Dhanabal *et al*., 2006; Seville *et al*., 2007).

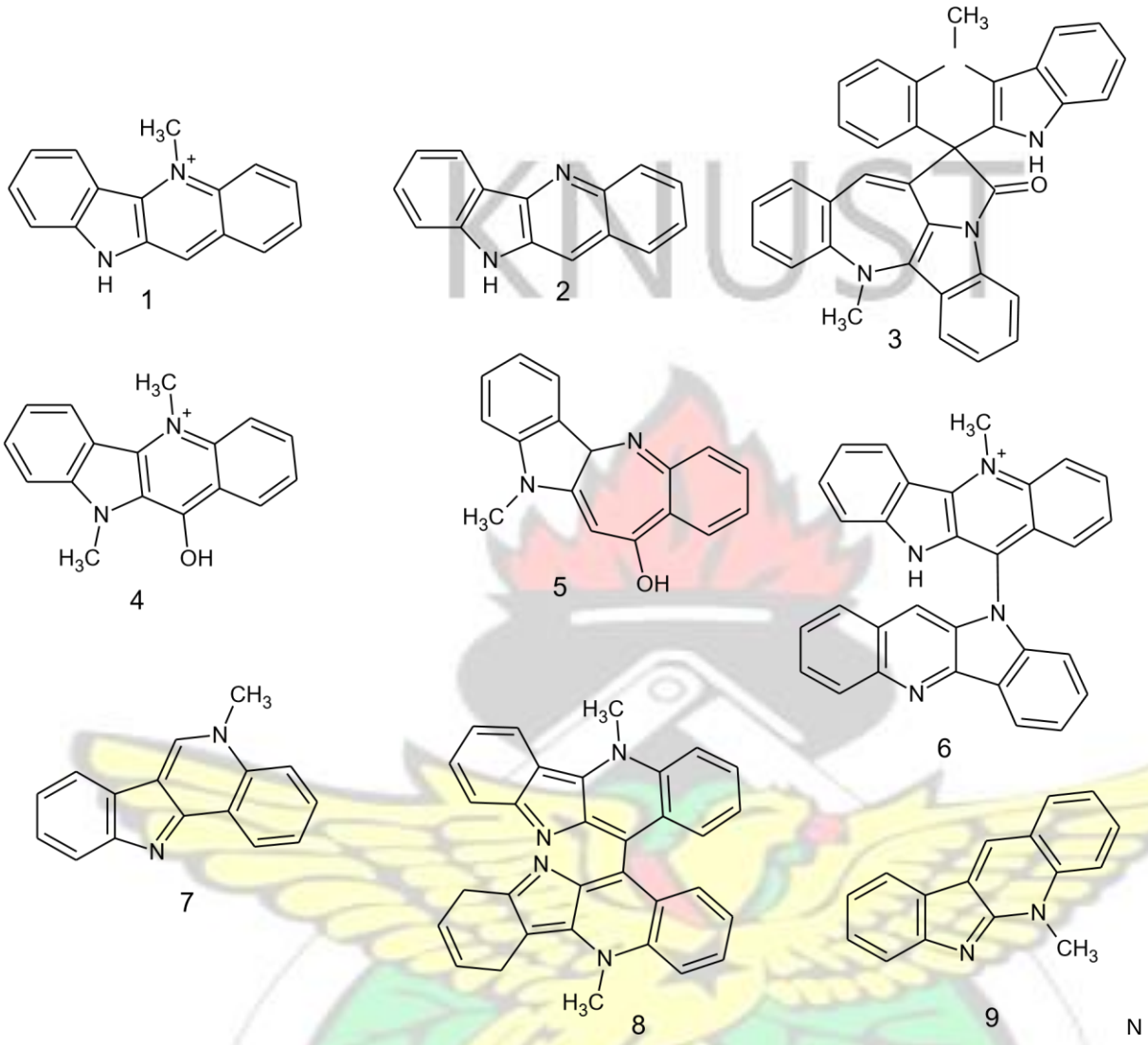


Figure 1.1: Structure of cryptolepine and some isolated compounds from *Cryptolepis sanguinolenta.*

1. Cryptolepine 2. Quindoline 3. Cryptospirolepine 4. Hydrocryptolepine 5. Cryptoheptine 6. Cryptoquindoline 7. Isocryptolepine 8. Biscryptolepine 9. Neocryptolepine

1.4.2 Biological activities of Cryptolepine

Cryptolepine and its hydrochloride salt possess several reported bioactivities, including antimicrobial (Boakye-Yiadom, 1983), anti-inflammatory (Bamgbose and Noamesi, 1981),

antihypertensive (Bamgbose and Noamesi, 1982), antipyretic, antimuscarinic (Rauwald, 1992), antibacterial (Boakye-Yiadom and Heman-Ackah, 1979; Paulo *et al.*, 1994a,b), antithrombotic (Oyekan and Okapor, 1989), noradrenergic receptor antagonistic (Noamesi and Bamgbose, 1980) and vasodilative properties (Oyekan, 1994).

Antiplasmodial Activity

The traditional use of the decoction of the roots *Cryptolepis sanguinolenta* for the treatment of malaria, with cryptolepine present in relatively large amounts in the roots (>1%), has made the compound receive tremendous interest by researchers globally. The ethanolic extracts from *Cryptolepis sanguinolenta* showed inhibition greater than 60% of the *in vitro* parasite growth at a concentration of 6 µg/ml against *P. falciparum* growth (Tona *et al*., 1999).

Cryptolepine, neocryptolepine, isocryptolepine, cryptoquindoline and biscryptolepine produced a potent antiplasmodial activity against the chloroquine-resistant *Plasmodium falciparum* strain KI (Wright *et al.*, 2001a). This selective antimalarial activity of cryptolepine and its congeners has been linked to the inhibition of beta-haematin formation (Van Miert *et al*., 2004; Wright *et al.*, 2001a). Cryptolepine and its derivatives have recently demonstrated the ability to inhibit haemozoin polymerization (Onyeibor *et al*., 2005).

Three independent researchers between 1995 and 1997 showed evidence of the antiplasmodial properties of cryptolepine *in vivo* (mice, ED_{50}<50 mg/kg i.p) and *in vitro* (IC_{50}=114 nM, SI=9,

CQR) (Cimanga *et al*., 1997; Grellier *et al*., 1996; Kirby *et al*., 1995). Cimanga *et al* (1997) investigated the antiplasmodial activity of cryptolepine, its hydrochloride salt, and analogues including 11-hydroxycryptolepine, neocryptolepine and quindoline as well as the methanol, ethanol and aqueous extracts of *Cryptolepis sanguinolenta.* These agents were tested against two *Plasmodium falciparum* chloroquine-

resistant strains K1 and W2 and the chloroquine sensitive strain D2. Cryptolepine and its analogues except quindoline were effective against all the species tested. Cryptolepine was more potent (IC_{50} values of 0.033±0.0001 µg/ml and 0.041±0.0005 µg/ml, respectively) than chloroquine (IC_{50} values of 0.072±0.0001 µg/ml and 0.068±0.0001 µg/ml, respectively) and quinine (IC_{50} values of 0.035±0.002 µg/ml and 0.102±0.0011 µg/ml,

respectively) against the K1 and W2 strains. The group also established that the total methanol extract was more potent than the ethanol and aqueous extracts. They further established that the compounds were effective *in vivo* against *Plasmodium berghei* and *yeolii* but only cryptolepine hydrochloride was considered effective against *Plasmodium berghei*.

Several interesting semi-synthetic derivatives of cryptolepine have been synthesized. Among these are: (1) 2-bromoneocryptolepine, which has less affinity towards DNA but also less active than cryptolepine (IC_{50} = 4 µM) as an antimalarial agent; (2) 1-methyl-δ-carboline, anhydronium base with IC_{50} of 1.5 µM and selectivity index (SI) higher than 100 (Arzel *et al.*, 2001); (3) both isoneocryptolepine, a synthetic derivative (IC_{50} = 40 nM), and N-methyl-isocryptolepinium iodide (IC_{50} = 17 nM) which have been shown to possess a much smaller cytotoxicity than cryptolepine (Van Mier *et al*., 2005); (4) 2, 7-dibromocryptolepine (IC_{50}= 49 nM, CQR), which has shown activity against *P. berghei* in mice (90% suppression of parasitaemia at 12.5 mg/kg *i.p*) (Onyeibor *et al*., 2005; Wright *et al*., 2001).

Anti-inflammatory activity

Bamgbose and Noamesi (1981) demonstrated the anti-inflammatory activity of cryptolepine *in vivo*. Cryptolepine showed a dose dependent reduction of the inflammatory effects in the carrageenan induced oedema in rat hind paw model at doses of 1-20 mg/kg. The potency (ID_{70} = 20 mg/kg) of cryptolepine was much less than indomethacin (ID_{70} = 2 mg/kg) and aspirin (ID_{70} =

8 mg/kg). Receptor mediator inhibition of prostaglandin E_2 was suggested to be responsible for the anti-inflammatory action seen. It also antagonized histamine and acetylcholine on the isolated guinea pig ileum (Bamgbose and Noamesi, 1981).

Anti-diabetic activity

Bierer *et al* (1998) discovered the ability of cryptolepine to stimulate glucose transport *in vitro* in 3T3-L1 adipocyte as well as its ability to lower glucose level *in vivo*. This group of researchers reported that cryptolepine and its salts (hydrochloride, hydroiodide, and hydrotriflate) stimulated glucose transport in a concentration dependent manner beginning at 3 μM in a recognised 3T3-L1 *in vitro* model. Activity of the hydroiodide salt was reportedly levelled out at concentrations beyond 10 μM due to possible toxicity. They observed that the regioisomer of cryptolepine (10methyl quindoline) with a methyl group at the N-10 position was inactive. The authors concluded that the alkylation of position N-5 was essential for the antihyperglycemic activity. In a further investigation they showed that the hydrochloride salt lowered plasma glucose levels by 16% and 47% from pre-dose glucose level at a dose of 20 and 30 mg/kg respectively in type II diabetic mice. Their findings justified the traditional use of the compound for the management of diabetes (Bierer *et al*., 1998).

Cardiovascular Activity

The sympatholytic activity of cryptolepine, resulting in hypotension, was first demonstrated *in vivo* by Raymont-Hamet in 1937 (Raymont-Hamet, 1937). The hypertensive and renal vasoconstrictive actions of adrenaline in anaesthetized and vagotomised dogs were markedly reduced by the administration of 4-24 mg/kg of cryptolepine. Cryptolepine has demonstrated sympatholytic activity at concentrations above 3 μM. In yet another study, cryptolepine showed its ability to

modulate cholinergic and noradrenergic neurotransmission. Cryptolepine acts by blocking both α_1 and α_2 adrenoceptors. It blocks the post-synaptic α_1 adrenoceptor at higher concentrations to produce its sympatholytic effect and at lower concentrations (below 0.3 µM) blocks the α_2 adrenoceptor causing extensive release of noradrenaline and hence the sympathomimetic effect seen at these lower concentrations (Bamgbose and Noamesi, 1980 and 1982). This suggest that cryptolepine has preferential pre-junctional α -adrenoceptor antagonist properties.

Antibacterial and antifungal activity

Cryptolepine is suspected to have a broad range of antibacterial activities and antifungal activity based on its many folkloric uses. Cryptolepine hydrochloride was assessed against the fast growing mycobacterial species *Mycobacterium fortuitum*, popularly used in the evaluation of antitubercular drugs. Cryptolepine showed a low minimum inhibitory concentration (MIC) (16 µg/mL) which prompted further evaluation against other fast growing mycobacteria namely, *M. bovis* BCG, *M. aurum, M. smegmatis, M. phlei* and *M. abcessus* and the MICs ranged over 2-32 µg/mL for these species. Cryptolepine was less active against some of the species compared to drugs such as ethambutol and isoniazid. The strong activity of this agent against *Mycobacterium tuberculosis*, coupled with the ethnobotanical use of *C. sanguinolenta* extracts to treat infections, highlights the potential of cryptolepine and its templates for development of antimycobacterial agents (Gibbons *et al*., 2003).

Cytotoxic, Genotoxic and Potential toxicity of Cryptolepine

Bonjean *et al.* (1998) investigated the possibility of DNA involvement in its cellular targeting as part of the numerous biological properties. Spectroscopy, circular and linear dichroism and relaxing assay using DNA topoisomerase was employed in the determination of the DNA binding capacity of cryptolepine. Cryptolepine was shown to bind tightly to DNA and behaved like a typical intercalating agent. In another assay using DNAase-1 foot printing, cryptolepine showed preferential interaction with guanine-cytosine (G-C) rich sequence and discriminated against homo-oligomeric runs of adenosine and tyrosine. Further, they discovered that cryptolepine stabilised topoisomerase II-DNA covalent complexes and stimulated the cutting of DNA at the subset of pre-existing topoisomerase II cleavage sites.

Ansah and Gooderham (2002) reported that cryptolepine (CLP) is a cytotoxic DNA intercalator that has promise as an anticancer agent. The researchers examined the *in vitro* toxicity of cryptolepine (CLP) and cryptolepis (CSE) using V79 cells, a Chinese hamster lung fibroblast frequently used to assess genetic toxicity, and a number of organ-specific human cancer cell lines. These results indicate that CSE and CLP are cytotoxic.

1.5 MALARIA

1.5.1 Disease definition, incidence and trends.

Malaria is a vector-borne infectious disease caused by protozoan parasites of the genus *Plasmodium* and is endemic in many parts of Asia, the Americas and much of Africa. High malaria transmission areas include the tropical and subtropical regions in Central and South America, subSaharan Africa, the Caribbean island of Hispaniola, parts of the Middle and South-East Asia, Oceania and Indian subcontinent (Hay and Snow, 2006). Sub-Saharan Africa represents 85– 90% of all malaria fatalities (Hay *et al*., 2004). Five of the species responsible for human malaria includes; *Plasmodium vivax, Plasmodium malariae*, *Plasmodium ovale*, *Plasmodium falciparum,*

and *Plasmodium knowlesi* (Marshall, 2000; Cox-Singh *et al.*, 2008; Daneshvar *et al.*, 2009). Other species including *P. berghei*, *P. chabaudi*, *P. vaughani*, and *P. gallinaceum* cause malaria in species other than humans. Of the species that cause disease in man, *P. falciparum* is the most deadly as it accounts for most cases of malaria and death (WHO, 2011).

A global estimation of 3.3 billion people living in 97 countries and territories are at risk of being infected with malaria and eventually developing the disease. Approximately 1.2 billion people in these group have high risk (>1 in 1000 chance of getting malaria in a year). This translates into a death from malaria every 1 minute, rendering it an eminent disease in tropical countries. WHO (2014) estimated 198 million cases of malaria globally in 2013 which translated to 584,000 deaths. Malaria has been a common disease and it continues to be one of the most widely spread health hazards in tropical and subtropical regions. Malaria deaths (90%) occur in sub-Saharan Africa with children aged under 5 years accounting for 78% of all deaths (WHO, 2014).

1.5.2 Geographical Distribution and Populations at Risk

Malaria occurs in over 97 countries worldwide (WHO, 2015). In 2015, the WHO estimates approximately 3.2 billion people (almost half of the world's population) were at risk of malaria. Sub-Saharan Africa has most of malaria cases. Some 15 countries (mainly in sub-Saharan Africa) accounts for 80% of malaria cases and 78% deaths globally (WHO, 2015). Variation in the intensity of transmission and the risk of malaria infection has been observed in malaria regions. Generally, arid areas (with less than 1000 mm rainfall per annum) and highlands (greater than 1500 m) have less malaria infections (WHO, 1996). Urban areas have at lower risk of malaria transmission compared to the rural areas however, an increasing risk has been observed in the urban areas attributed to an unplanned population growth (Knudsen and Slooff, 1992).

Malaria is described as a disease of poverty and a cause of poverty (Bourdy *et al.*, 2008) because of it tremendous economic effects aside the morbidity and mortality of the disease. The direct costs for treatment and prevention coupled with the indirect costs such as lost productivity from time spent seeking treatment and diversion of household resources (Sachs and Malaney, 2002). This heavy toll contributes to the slow economic and community development activities throughout the region. Malaria is a familiar disease in most households in sub-Saharan Africa, where it has a reputation of causing fever, teeth chattering chills and shakes.

Population at risk includes:

- Infants and children under 5 years of age in stable transmission areas without developed immunity against the disease.
- Pregnant women
- Non-immune migrants, mobile populations and travellers from non-endemic areas are at high risk of malaria and its consequences.
- Patients with HIV/AIDS are at increased risk of malaria disease when infected.

The factors above make malaria a major health problem in sub-Saharan Africa.

1.5.3 Transmission and Developmental stages of the Malaria Parasite

The transmission of malaria is mostly by the female anopheles mosquitoes (Greenwood *et al*., 2005). Only 68 of the 460 species of the *Anopheles* mosquito transmit malaria. All of the important vector species bite between dusk and dawn with the intensity of transmission depending on factors related to the environment, parasite, the vector and the human host (WHO, 2015). The African *Anopheles gambiae* is one of the best malaria vectors because it is long-lived, prefers human meal,

and lives in areas near human habitation (Cowman, 2006). The climatic conditions such as rainfall pattern, temperature and humidity affects the transmission of malaria from vector to their human host. In many regions, malaria transmission is seasonal, with peaks during and just after the rainy season.

The three distinct stages of the parasite life cycle is: the sporogonic, the exo-erythrocytic and the erythrocytic stages. The exo-erythrocytic stage occurs in the liver, the erythrocytic stage occurs in the erythrocytes and the sporogonic stage occurs in the digestive tract of the mosquito as illustrated by the A, B and C parts of Figure 1.2, respectively. The sporozoite stage of the parasite lives within the salivary gland of the vector prior to disease transmission. At the point of feeding, the mosquito introduces a small amount of saliva (containing antihemostatic and anti-inflammatory enzymes) into the skin wound to inhibit the pain reaction and disrupt the clotting process (Bruce, 1985**)**. Typically, a mosquito infected bite contains 5-200 sporozoites which proceed to cause the human infection (Gilles *et al.,* 1993). The sporozoites once introduced into the bloodstream circulate for few minutes before infecting the hepatocytes. The sporozoites in the liver multiplies into 10003000 merozoites. This stage presents no clinical symptoms (Vaughan *et al*., 2008). The sporozoites in the hepatocytes may go into dormancy in infections caused by *P. vivax* and *P. ovale* explaining the phenomenon of relapse caused by these Plasmodium species.

The erythrocytic stage follows after the merozoites in the hepatocytes rupture into the bloodstream. This stage of the parasite life cycle is responsible for most of the clinical symptoms of the disease.
The erythrocytic stage begins with the trophic stage which sees the intracellular 'ring' develop into a trophozoite. Haemoglobin metabolism into amino acid and haem results in ring multiplication. The replicative stage sees the trophozoites develop into schizonts accompanied cytokinesis. This stage is marked by red blood cell lysis and the release of merozoites that in turn infect new red

blood cells, repeating the cycle. A few of the merozoites upon invading erythrocytes differentiate into the sexual stages of the parasite called gametocytes. Gametocyte induction is stimulated especially by stressful conditions such as high carbon dioxide concentration, reduced temperature and parasite waste products (Pukrittayakamee, 2008). The gametocytes are ingested by the female anopheles mosquito during a blood meal from an infected person. The non-pathogenic gametocytes develop into sporozoites in the vector's salivary gland. The sporozoites are introduced into the host bloodstream during a blood meal and the cycle is repeated.

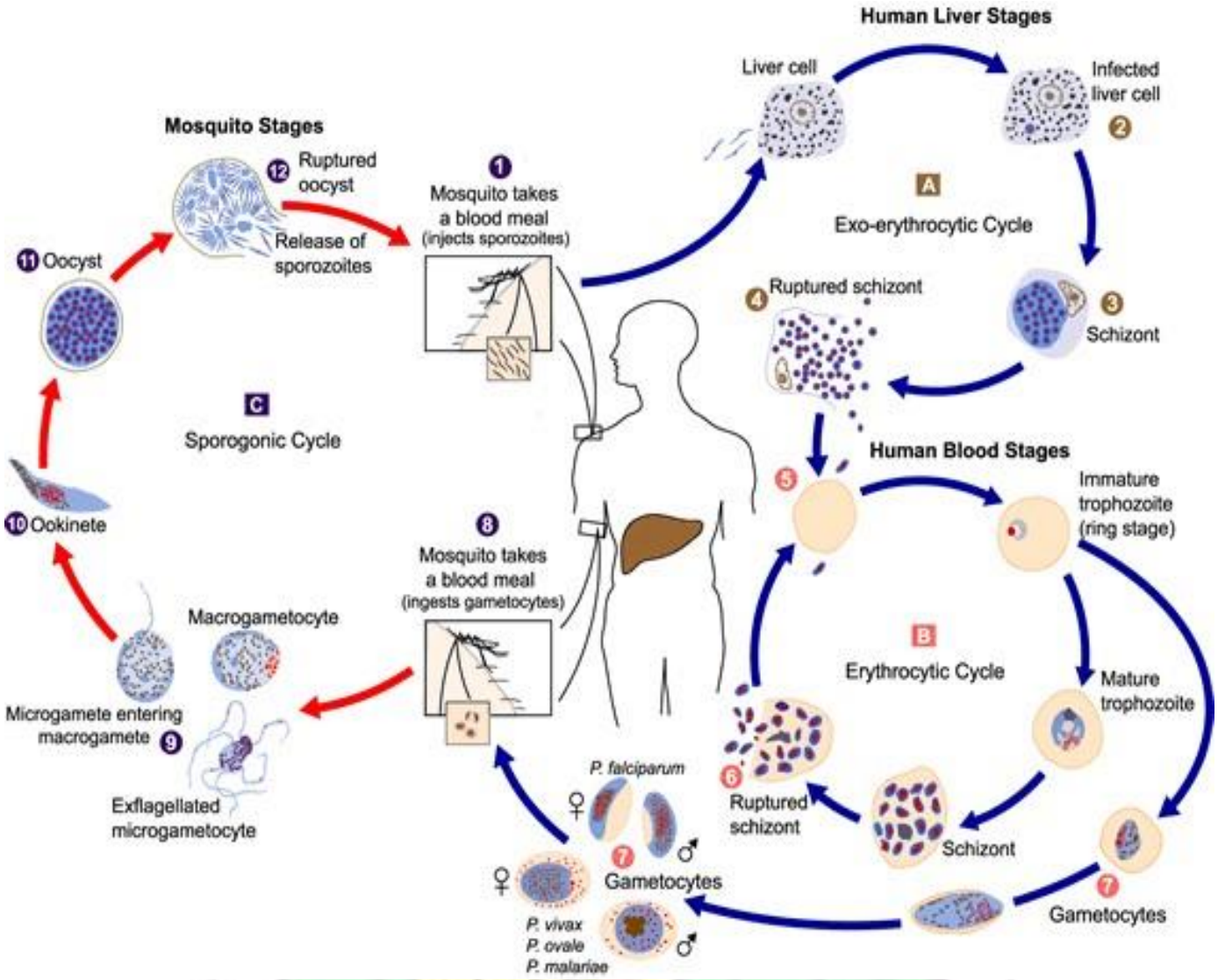


Figure 1.2: Life cycle of *Plasmodium* Species (Bruce, 1985)

1.5.4 Malaria Symptoms and Diagnosis

The plasmodium parasites multiply within the erythrocytes, causing symptoms that include fever, headache, chills, nausea, vomiting, arthralgia, bitterness in the mouth, loss of appetite, flu-like

symptoms as well as symptoms of anaemia (light headedness, shortness of breath, tachycardia etc.), convulsions and in severe cases, progression to coma and death (WHO, 1991).

These symptoms may occur in cyclical episodes of sudden feeling of coldness followed by fever and sweating at 2-3 day intervals depending on the parasite species (Boivin, 2002). *P. falciparum* can have recurrent fever every 36-48 hours or a less pronounced and almost continuous fever (Trampuz *et al.*, 2003). Children with severe malaria usually exhibit abnormal postures which may be as a result of severe brain damage (Idro *et al*., 2007). Severe malaria if untreated can lead to coma and death especially in children and pregnant women within hours or days. Hypoglycaemia, cerebral ischaemia, severe headache, splenomegaly (enlarged spleen), hepatomegaly (enlarged liver), and haemoglobinuria with renal failure may occur (Trampuz *et al.*, 2003). The renal failure is caused by the leakage of haemoglobin from lysed red blood cells into the urine (Black water fever) (Idro *et al.*, 2007).

Children who suffer episodes of severe malaria have been documented to show long term developmental impairments (Trampuz *et al*., 2003). *P. vivax* and *P. ovale* unlike *P. falciparum* cause disease relapse over month to years due to the presence of latent parasites (hypnozoites) in the liver (Kain *et al.*, 1998). *P. falciparum* infection is the deadliest form of the disease causing severe malaria and usually arises 6-14 days after infection.

1.5.5 Malaria Prevention and Treatment

Malaria prevention aims at the eradication of the mosquito vector since an important phase of the life cycle of the parasite occurs within the vector. Several approaches aimed at reducing the vector population indoors have been introduced over the years. Indoor Residual Spraying (IRS) with insecticides which targets indoor population of the *anopheles* is one of such approach to malarial control. In endemic regions, 185 million people have benefitted from IRS use (WHO, 2011).

Destruction of the vector within its natural habitat and breeding sites has been successful with insecticides such as dichlorodiphenyltrichloroethane (DDT), which has been used in some African countries as a major control tool following resistance to other insecticide agents (Coleman *et al.*, 2008). Breeding of sterile male insects aimed at reducing vector population has been used on a small scale and still undergoing development. This intervention is aimed at reducing the vector population and hence malaria transmission.

The introduction of insecticide-treated nets (ITNs), has been a positive tool in the fight against malaria. ITNs including the widely used long lasting insecticide nets (LLINs) have been recommended by the roll back malaria programme (RBM) as one of the main malaria control tools. This intervention focuses on children and pregnant women who are at the highest risk to the disease (Lengeler *et al*., 2007). The distribution of ITNs to sub-Sahara Africa is one of the most important interventions towards the elimination of malaria with about 580 million nets delivered as at 2009 (WHO, 2010). Nonetheless, the distribution of ITNs needs to be extended to the general population in endemic areas (Lengeler *et al*., 2007) in order to break the cycle of transmission.

The ineffectiveness of the indoor and outdoor residual spraying is due to the development of resistance by the vector to most of the chemicals such as pyrethroids (Munhenga *et al.*, 2008).
Despite the effectiveness of DDT, calls from environmentalists have limited its use due to its persistence in the environment and its potential to cause human toxicity through the food chain. The use of DDT was further complicated by insufficient technical capacity, public reaction to spraying and the emergence of a more resistant species of the mosquito vector (Mabaso *et al.*, 2004; Sadasivaiah *et al*., 2007).

Access to and the cost of ITNs still remain out of reach of many living in endemic areas. As at 2014, 269 million of the 840 million people at risk of malaria lived in households without a single

ITN or IRS; 15 million of the 28 million pregnant women at risk did not receive a single dose of IPTp; and between 68 and 80 million of the 92 million children with malaria did not receive ACT (WHO, 2015).

1.5.6 Antimalarial Agents-A Historical Perspective

The antifolates and the cinchona alkaloids or the quinoline-containing have been the two major classes of antimalarial drugs used for the past 50 years (Phillips, 2001). The antifolates include the biguanides, represented by proguanil (cycloguanil) and chlorproguanil; the diaminopyrimidines, such as pyrimethamine and trimethoprim; and the sulpha drugs, including the sulfones and the sulphonamides. Quinine, quinidine and the other cinchona alkaloids are classified as quinolonecontaining drugs because of the presence of the quinolone ring. There have been recent introduction of halofantrine (a 9- phenathrene methanol) and the aminoalcohol quinine analogues mefloquine (a 4-quinoline methanol) (terKuile, 1993; Philips, 2001). Primaquine is an 8aminoquinoline among the few agents with activity against the liver stage of *P. vivax* and also possesses gametocytocidal effect. Chloroquine and its derivative, amodiaquine are 4aminoquinolines (White, 1997).

Quinine and related compounds

Quinine is the oldest and most famous anti-malarial agent (Dorvault, 1982). It has a long history stretching from Peru, to the discovery of the Cinchona tree and the potential uses of its bark, to the current day and a collection of derivatives that are still frequently used in the prevention and treatment of malaria (Segurado *et al*., 1997). Quinine, an alkaloid from the Cinchona tree has potent blood schizonticidal and weak gametocytocidal activity against the *Plasmodium species.*

Quinine accumulates in the food vacuoles of *P. falciparum* and exhibits the antimalarial activity by inhibiting the haemozoin biocrystallization, thus facilitating the accumulation of cytotoxic haem. Quinine is still useful today for the treatment of acute cases of *P. falciparum* malaria despite its toxic effect compared to chloroquine (Foley and Tilley, 1998). Quinine is very useful in areas with high level of resistance to mefloquine, chloroquine and sulpha drug combinations with pyrimethamine (Foley and Tilley, 1998). It also has use as a post-exposure treatment for persons returning from malaria endemic regions (Foley and Tilley, 1997).

Quinine related antimalarial agents used in the treatment of malaria include amodiaquine, mefloquine, chloroquine, atovaquone, halofantrine, and primaquine.

Some antibiotics are currently used for the prophylaxis and treatment of malaria. Tetracycline and its derivatives such as doxycycline are very potent antimalarials used for both prophylaxis and treatment (Kremsner *et al.,* 1994). Clindamycin offers only limited advantage when compared to other antimalarial drugs as recrudescence rates are high and parasitological response is generally slow (Kremsner *et al.*, 1994).

1.5.7 Artemisinin Derivatives

The popular Chinese herb (Qinghaosu) artemisinin obtained from the plant *Artemisia annua* (Mills and Bone, 2000) has been used for fevers for over a 1,000 years (WHO, 1996). Artemisinin was first documented as a successful therapeutic agent in the treatment of malaria in 340 AD (Mueller *et al.,*

2000; 2004). The active compound was first isolated in 1971 and named Artemsinin (Mills and Bone, 2000). It is a sesquiterpene lactone with a chemically rare peroxide bridge linkage. The reactivity of the endoperoxide bridge, the common structural feature of artemisinin and all its derivatives have been shown to be responsible for the antimalarial action of these compounds

(Hien and White, 1993). The artemisinin derivatives are usually given in combination with other antimalarials and has proven to be very effective against all forms of multi-drug resistant *P. falciparum*. To prevent the development of resistance to these antimalarial agents, several strategies are employed to ensure compliance and adherence. Artesunate, artenimol (β-dihydro-artemisinin, DHA), artemether (artemotil) (Figure 1.3) and arteether are semi-synthetic sesquiterpine lactone derivatives.

Artemisinin derivatives are well known for their rapid parasite clearance from the blood and short biological half-lives. They act primarily on the trophozoite stages of the parasite and have demonstrated to be the antimalarial drug with the fastest, thus preventing disease progression. The artemisinin derivatives are converted to the active metabolite dihydroartemisinin which acts by inhibiting the Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase (SERCA) encoded by *P. falciparum* (Eckstein-Ludwig *et al.*, 2003). The use of the artemisinins as monotherapy is discouraged but preferred in combination with longer-acting drugs that have a slower onset of activity. This strategy has been largely advocated by the WHO Roll Back Malaria programme, recommending the use of artemisinin-combination therapies (ACT) in areas of emerging, high resistance to the most commonly used antimalarials (WHO, 2003). The artemisinins are the most antimalarial agents and have demonstrated an excellent tolerability and safety in humans (Price *et al*., 1999; Davis *et al*., 2005, White, 1999).

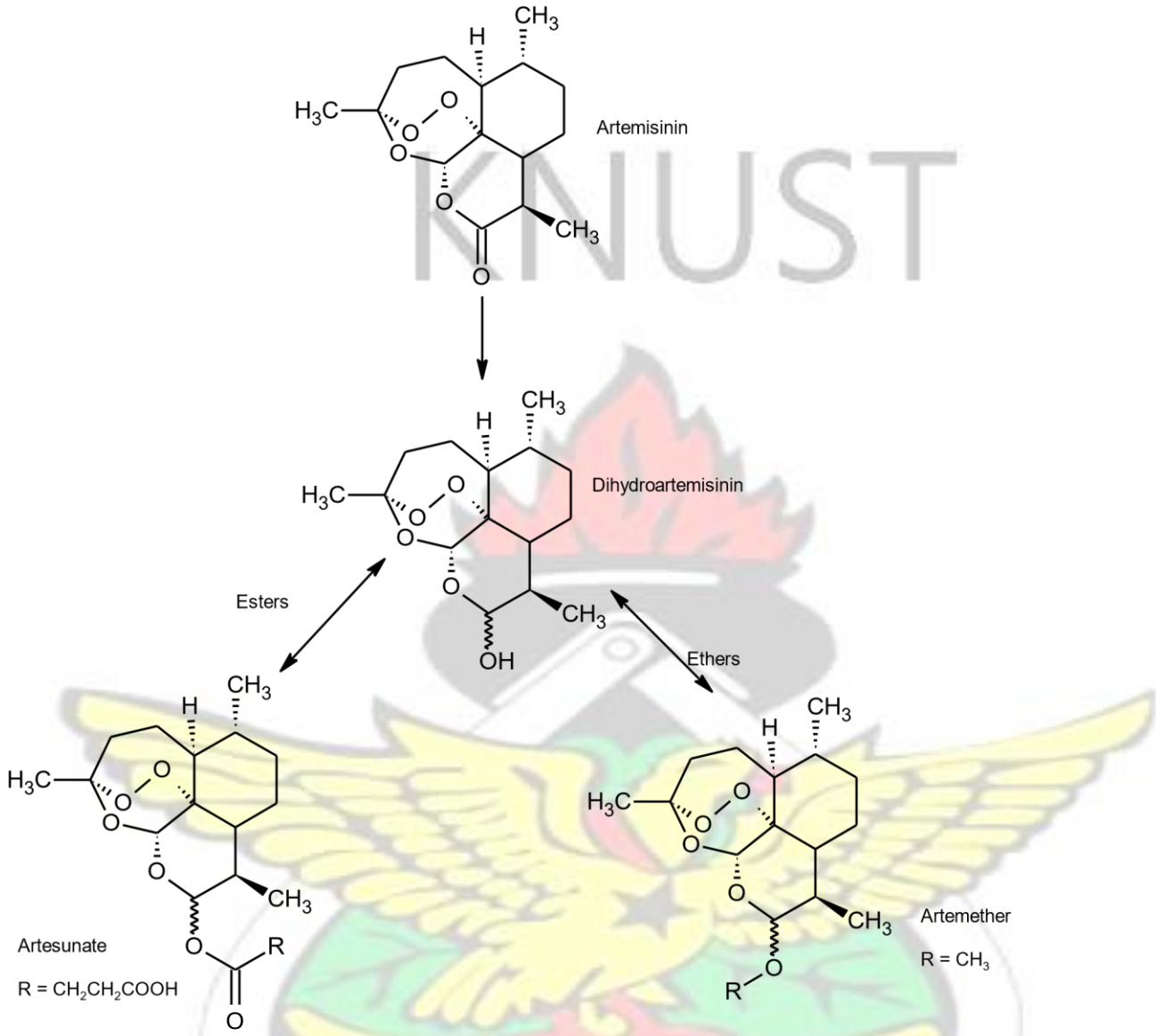


Figure 1.3: Artemisinin and its popularly used derivatives

The artemisinins have rapid parasite clearance times and faster fever resolution than quinine. Artemisinin and its derivatives possess an effective activity against multidrug resistant *P. falciparum*; have a rapid significant reduction of parasite biomass; a rapid resolution of clinical symptoms and gametocytocidal activity hence reducing disease transmission. Despite their

efficacy, the artemisinin drugs have a very short half-life allowing for multiple dose regimen of seven days to achieve an acceptable cure rate (WHO, 2003).

In some regions of South-East Asia, uncomplicated malaria can only be treated with combinations of artemisinins and mefloquine due to the development and prevalence of multidrug resistant *falciparum* malaria (White, 1999). The combination of the artemisinins with a drug with long halflife (eg. mefloquine) has been used in the South-East Asia to inhibit disease transmission as well as decreasing the intensification of drug resistance (Price *et al.*, 1996; White, 1999).

The World Health Organization recommended the use of the artemisinin-based combinations (ACT) for the treatment of *P. falciparum* malaria in Africa. Artesunate (AS) plus amodiaquine (AQ) is an example of the ACTs recommended for use in Africa (Sodiomon *et al*., 2009). Artesunate, the hemisuccinate derivative of the active metabolite dihydroartemisinin discovered in 1983 (Mills and Bone, 2000) is the most frequently used of all the artemisinin-type drugs. Artesunate has gametocytocidal properties and usually used in combination therapy for cases of uncomplicated *P. falciparum*.

Artemether, the methyl ether derivative of the active metabolite dihydroartemisinin was discovered in 1982 (Dorvault, 1982). It's mode of action is similar to artemisinin but has a reduced effectiveness as a hypnozoiticidal agent, instead acts more to decrease the carriage of gametocytes. Arteether is an ethyl ether derivative of dihydroartemisinin with no reported side effect. The metabolism of artemisinin produces the active metabolite, dihydroartemisinin which is the most effective but least stable derivative (Krettli, 2001). Dihydroartemisinin has a strong blood schizonticidal action and reduces gametocyte transmission. It is used for therapeutic treatment of cases of resistant and uncomplicated *P. falciparum*. All the derivatives just as artemisinin have

restrictions in place to reduce the development of resistance, which is these antimalarial agents are used in combination therapy for severe acute cases of drug-resistant *P. falciparum* (Krettli, 2001).

1.5.8 Antimalarial Combination Therapy

There have been recent reports on the resistance of *Plasmodium falciparum* against artemisinin in some parts of Cambodia and Thailand (WHO, 2010).

Despite the safety and effectiveness of the artemisinins, some limitations have been reported with this class of antimalarial agents. Firstly, the problem of recrudescence where an initial drug treatment appears to clear all parasite but disease recurs after few weeks. The recrudescence seen with these agents is not as a result of re-infection or drug resistance but occurs as a result of the failure of the drug to kill all the parasites. The parasites that survive the first treatment continue to multiply resulting in the recrudescence observed and this has been attributed to the short halflives of the artemisinin derivatives and the reported insensitivity of the early blood stage forms of the malaria parasite to these drugs (Wright and Warlurst, 2002)

The World Health Organisation recommended the administration of a second antimalarial agent to clear any remaining parasite after treatment with artemisinin derivatives to forestall the problem of recrudescence (Wilairatana and Looareesuwan, 2002). The key driver for combination antimalarial therapy is the need to slow development of acquired drug resistance to a New Chemical Entity (NCE) and so maintain high levels of efficacy for a longer period of time. This is best achieved by combining molecules which individually have high levels of efficacy (WHO, 2001). Simultaneous use of two antimalarial drugs with varying mechanisms of action has the potential for inhibiting the development of resistance to either of the components.

McIntosh and Greenwood (1998) demonstrated a pronounced parasite clearance when either chloroquine or amodiaquine was added to Sulphadoxine-Pyrimethamine (compared with SP alone). This study was reviewed in areas and during periods where both Chloroquine/Amodiaquine and SP retained fair amount of efficacy, and it is not clear from these studies how well such a combination would do in areas where one of the components has been significantly compromised.

Currently, two categories of combination drugs are used.

a. Artemisinin based Combinations.

b. Non Artemisinin Combinations.

Some examples of the Non artemisinin combinations are Sulfadoxime-pyrimethamine plus amodiaquine, Sulfadoxime-pyrimethamine plus mefloquine (*fansimef*®) and Tetracycline or doxycycline plus quinine.

1.5.8.1 Artemisinin Based Combinations.

Following the development of resistance by the *P. falciparum* against chloroquine, and some other anti-malaria drugs, some malaria endemic countries have adopted artemisinin based combination therapy for treatment of uncomplicated malaria (Amin *et al*., 2007; Sipilanyambe *et al*., 2008) through WHO intervention programmes such as the rollback malaria programme (WHO, 2006).

The different mechanism of action of the artemisinins from conventional antimalarial makes it particularly useful in the treatment of resistant infections (Mueller *et al*., 2000). Artemisinin-based combination therapies have the potential for transmission reductions approaching those of insecticide treated nets (Adjei *et al*., 2008; Okell *et al*., 2008). The discovery of the artemisinin derivatives has been a major advancement in malaria chemotherapy with the artemisinin combination therapy (ACT) currently adopted as first-line treatment for uncomplicated *falciparum* malaria (Nosten and White, 2007).

1.5.9 Herbal Antimalarial Remedies.

Medicinal plants have been used in virtually all cultures as a source of medicine (Hoareau and Dasilva, 1999). The earliest recorded use of herbal remedies (Greek) comes from Hippocrates, who advocated using a few simple plants, such as garlic (Rivlin, 2001).

According to the World Health Organization (WHO, 2011) about 80% of the developing world's population meet their primary healthcare needs through traditional medicine (Calixto, 2000). Plants are a rich source of novel drugs as most of the currently available antimalarial agents were either developed directly from plants (quinine and artemisinins) or served as templates for new drugs (Basco *et al*., 1994). Medicinal plants used locally to treat malaria infection include *Azadirachta indica, Acalypha fruticosa*, *Cissus rotundifolia*, *Echium rauwalfii*, *Boswellia elongate, Dendrosicyos socotrana* (Merlin, 2004; Clarkson *et al*., 2004), *Nauclea natifolia*, *Salacia Nitida,* and *Enantia chloranta* (Ogbonna *et al*., 2008).

Decoctions of the root of *Zanthoxylum chalybeum*, leaves of *Momordica foetida*, *Chenopodium opulifolium* and *Magnifera indica* are among herbal drugs used for the treatment of malaria in the Budiope county of Uganda (Tabuti, 2008). *Periploca linearifolia, Maytenus heterophylla, Albizia amara, Teclea simplicifolia, Olea capensis* and *Maytenus putterlickioides* are among the plants used by the indigenous people in the Meru district in Kenya for the treatment of malaria (Muthaura *et al*., 2007).

In Ghana, several plants are used in the treatment of malaria. Among them are *Afraegle paniculata, Haematostaphis barteri*, *Indigo erapulchra*, *Ozoroa insignis*, *Strychnos spinosa* and *Xeroder risstuhlmanni* (Asase *et al.,* 2005).

A decoction of the root of the climbing shrub *Cryptolepis sanguinolenta* is used in West Africa for the treatment of malaria (Boye and Ampofo, 1983). Several researches have been conducted on *C. sanguinolenta* and it remains one of the most accomplished herbs in the West African sub-region for the management of malaria (Noamesi *et al*., 1991; Kirby *et al*., 1996; Wright, 2005). On the Ghanaian market, 15 formulated herbal antimalarial drugs containing the aqueous root extract of

C. sanguinolenta include Class malacure, Nibima, Yafo, Masada bitters, among others (unpublished data).

Among these, Nibima is the only antimalarial herbal formulation permitted by the Ghana Health Service (GHS) for malaria treatment by herbal medical doctors in the Ghanaian hospital.

1.6 ASSESSMENT OF ANTIMALARIAL ACTIVITY

In general, two basic approaches are routinely used to study or measure antimalarial activity of compounds: a) *in vitro* and b) *in vivo*. These can be complementary, as data obtained from *in vitro* assays require confirmation from the use of live models.

1.6.1 In Vitro Assessment of Antimalarial Activity

Drug-sensitivity testing is aimed at measuring *P. falciparum* sensitivity to increasing drug dosages *in vitro*. Data from these assays are presented as percentages of parasite growth at each drug concentration compared to untreated cultures. *In vitro* assays help to exclude host-related interfering factors such as host immunity and host metabolism of the compound, thus offering a

direct evaluation of drug effect. Trager and Jensen (1976) first described the technique for culturing the asexual erythrocytic stages of the malaria parasite *in vitro*, it has been extensively used to provide information supplementary to that obtained by epidemiological studies (WHO, 2007). This has led to a better understanding of the malaria parasite in terms of immunology, biochemistry, molecular biology and pharmacology (Ringwald *et al.*, 1999). The short-term *in vitro* culture of freshly collected field isolates of *P. vivax* and *P. ovale* has also been established, allowing for the assessment of antimalarial activity of different drugs on these plasmodium species. Several *in vitro* methods are commonly used for the assessment of *P. falciparum* growth inhibition: 1) the gold standard method of counting schizonts on thick films with microscope, also known as the World Health Organization (WHO) Schizont maturation assay. This method is tedious and time-consuming. 2) Quantification of specific parasite proteins such as lactate dehydrogenase (LDH) and histidine rich protein 2 (HRP2) by enzyme-linked immunosorbent assay (ELISA). This method uses relatively expensive reagents and may not be preferred for large-scale drug screening efforts (Vossen *et al*., 2010). 3) Isotopic assay which involves the incorporation of a radiolabelled nuclei acid precursor (eg. radiolabelled hypoxanthine) (Noedl *et al*, 2011). 4) DNA dye intercalation assays which quantifies parasite growth after staining parasites with fluorescent DNA binding dyes such as SYBR Green I, DAPI (4,6-diamino-2-phenylindole) and Pico green, YOYO1 (Nogueira *et al*., 2010)

1.6.1.2 SYBR® Green I Assay

SYBR® Green is a cyanide dye that binds to double-stranded DNA preferring G and C base pairs. When it binds to DNA, it becomes fluorescent and absorbs light at wavelengths between 390 and 505 nm, with the highest point at 497 nm and has a secondary peak near 254 nm. It gives off light at 505 to 615 nm, with a peak at 520 nm (Sigma-Aldrich, 2008). The principle behind this assays

is the contrast between host erythrocytes, which lack DNA and RNA, and the malaria parasites, which have both DNA and RNA; thus, parasites are readily stained with dyes that show enhanced fluorescence in the presence of nucleic acids. The amount of parasite DNA detected is a measure of parasite growth and degree of inhibition by the anti-malarial agent the culture is exposed to (Vossen *et al.*, 2010). The fluorescence of the dye is measured using either fluorescence microplate readers or fluorescence activated cell sorters (FACS). The SYBR® Green assay is an inexpensive, fast and easy-to-use assay developed to monitor parasite viability *in vitro* drug sensitivity tests (Johnson *et al.*, 2007; Vossen *et al*., 2010). However it is not selective for only malaria parasite DNA and binds to all other DNA double-strands including host white blood cells and contaminant microorganisms resulting in a high background reading (Vossen *et al*, 2010).

1.6.2 In vivo assessment of antimalarial activity

An *in vivo* test is associated with animal models and their compulsory ethical issues. During *in vivo* assays, one measures mainly

- the clearance of parasites as detected by optical microscopy or other more sensitive methods (polymerase chain reaction – PCR),
- the time that elapses between last drug dose and clearance of parasitaemia, and
- the drug dosage that clears parasites in a dose response manner.

When adjusted to humans, *in vivo* studies usually represent a selected group of symptomatic and parasitaemic individuals that underwent carefully controlled treatment after which parasitological and/or clinical responses are monitored over time. The host's acquired immunity can mask a diminished therapeutic efficacy of novel compounds. When performed with human subjects, the assay reflects actual clinical or epidemiological situations; that is, the therapeutic response of the specific strain of parasite afflicting the population in which the drug will be used (Nogeuira *et al*., 2010).

In the screening for antimalarial activity of new compounds, non-human primates have provided experimental models for malaria (Meis *et al*., 1990). Nevertheless, the use of these animal models have been difficult because of the economic and ethical considerations, limited supply of monkeys, the narrow range of parasite lines that are adapted to primate infection and the different pathology that is seen in these models (Evans *et al*., 2006; Sanni *et al*., 2002). Consequently, most *in vivo* experimental studies on malaria have relied on different combinations of various murine strains and *Plasmodium* spp. of rodents (Carlton *et al*., 2001; Hernandez-Valladares *et al*., 2004); however, the biological differences between parasite species must be taken into account.

External factors, such as host immunity, variations of drug absorption and metabolism, are reduced by the use of naïve animals from well-known lines in the experiments. Also, potential misclassification of re-infection is not an issue, as assays use direct inoculation of blood stage parasites in well-protected animal house surroundings (Nogeuira *et al*., 2010).

However, antimalarial activity measured in an *in vivo* test does not always consider relevant factors, such as metabolism and host genetics, which can affect the outcome of the experiment. The rodent malaria model offers an advantage of both the parallels of their life cycle with those of the *Plasmodium* species of humans, and to practical considerations of cost and ease of manipulation. The rodent infections can serve, therefore as models for casual prophylactic, blood schizonticidal, gametocytocidal and sporonticidal drugs.

Since the identification of novel antimalarials that are effective against multidrug-resistant falciparum malaria is one of the current prime objectives, the ready availability of different drugresistant lines derived from *P. berghei, P. yoelii* and *P. chabaudi* subspecies and strains is a marked advantage for the models. The ability to use random-bred mice which are easy to handle and cheap to maintain is a distinct advantage over alternative vertebrate hosts. Avian malaria parasites which have a different life cycle have now been abandoned both for this reason and

because chicks and ducks are more difficult to handle and maintain than the mice (Nogeuira *et al*., 2010).

Different species of monkeys that are hosts to human *Plasmodium* parasites have advantages in that they provide data that may be more directly relevant to the human malaria but also have obvious disadvantages in terms of availability, cost, relative difficulty in handling and maintenance and ethical considerations. Murine models despite the numerous advantages also have some disadvantages, particularly regarding drug metabolism. For instance, the antimalarial agent mefloquine has a prolonged biological half-life in humans but very short one in the mouse. In addition, drug responses of various murine species to certain types of antimalarial drugs may differ. This will require the use of more than one rodent *Plasmodium* species for screening (Nogeuira *et al*., 2010).

1.6.2.1 Parasite Species

Several species and subspecies of rodent strains of *Plasmodium* exist. These include *Plasmodium berghei*, *P. chabaudi,* and *P. vinckei*. The rodent parasite, *P. berghei* provides a simple and satisfactory model and is used in majority of experiments on known blood schizonticides. The development of resistance to chloroquine in rodents was first seen in *P. berghei*. Several species now demonstrate resistance to chloroquine and other standard drugs. It has been shown that a synchronous infection such as that yielded by *P. vinckei* or *P. chabaudi* can give a better response to a few types of compounds. It is relatively easy, using appropriate techniques, to select lines of rodent malaria that are resistant to most antimalarial drugs (Peters, 1987).

ASSAYS SIGNIFICANT FOR *IN VIVO* ANTIMALARIAL ASSESSMENT

1.6.2.2 Suppressive Test (Peters' 4 Day test)

One of the most useful *in vivo* dose-response tests for antimalarial activity is the four-day suppression test, originally designed by Peters (Peters, 1975). Briefly, female mice are infected with rodent malaria parasites and the different treatment doses are given after infection on day 0 and repeated once daily for three days. On day 4 and day 7 post-infection, parasitaemia is assessed. After day 7, no further manipulations are performed on the mice and they are monitored for survival.

1.6.2.3 Curative or Rane Test

The Rane test was described by Ryley and Peters (1970). The Rane test evaluates the curative activity of compounds in established infection. On the first day, a standard inoculum of the rodent malaria parasites is introduced intra-peritoneally. After 72 hours, following confirmation of parasitaemia, the mice are divided into groups and treated with different concentrations of the compound on a daily basis. Blood smears are prepared daily and examined microscopically to monitor the parasitaemia levels.

1.6.2.4 Prophylactic Activity (Repository Test)

The duration of action of a compound depends, firstly on its rate of absorption from the site of administration (bioavailability) and then on other factors such as tissue binding, rate of metabolism and the rate of excretion. In this method described by Peters (Peters, 1967), the prophylactic potential of the compounds are examined. Once efficacy has been established for a novel compound, it is essential to establish the prophylactic potential. In this assay, several doses of the compound are administered to mice at -72 hr, -48 hr, -24 hr and 0 hr relative to the time of infection.

Blood smears are prepared and examined daily to assess suppression of parasitaemia and survival times are measured in days (Fidock *et al*, 2004).

For all these assays, chloroquine or the artemisinins can be used as the reference drug.

1.6.3 Gametocyte Assay

The *Plasmodium* parasites are transmitted by female Anopheles mosquitoes. After being bitten, sporozoites are introduced into the human host, causing an asymptomatic hepatic infection. The intraerythrocytic proliferation is marked by the symptoms of the disease and accompanying complications such as severe anaemia and cerebral malaria. Asexual replication occurs inside the red blood cells (RBCs) with some of them developing into the sexual forms called gametocytes. The maturation of *P. falciparum* gametocytes (male and female) occurs in five stages of development (I to V), lasting approximately 10 days. Unlike the asexual stages of the parasite, mature gametocytes remain in circulation for several days where they can be taken up by mosquitoes and mature into gametes. These gametes develop into sporozoites which are the sexual stages responsible for malaria transmission from humans to mosquitoes. This stage of parasite development represent a vital target for new antimalarial drugs to achieve malaria elimination/eradication.

Gametocyte viability and drug susceptibility can be measured by several screening methods. These methods include the parasite lactate dehydrogenase (pLDH) assay (D'Alessandro *et al*., 2013), ATP bioluminescence-based assay (Lelievre *et al*., 2012), SYBR Green I- based DNA assay (Sanders *et al*., 2014), Luciferase reporter assays (Adjalley *et al.*, 2011), colourimetric reagent assays (PrestoBlue® and AlamarBlue® assays) (Tanaka *et al.*, 2011).

1.7 DRUG METABOLISM AND PHARMACOKINETICS

Drug metabolism is an important area of study and it is understood as a set of biochemical reactions that produce changes on the chemical structure of drugs to facilitate elimination. These metabolic changes can occur in several body tissues, mainly in the liver, but also in the kidney, intestines, lungs etc. Metabolism can affect drug clearance and also produce either a desired therapeutic effect or undesired/toxic effects. Metabolism-based assays in the early stages of drug development allow researchers to improve the pharmacokinetic properties of a lead compound by either blocking or inserting sites of facile metabolism. Metabolism data obtained during drug development can be used to promote safe use of a drug in clinical and post marketing stages, including dosing adjustments or warning about drug-drug interactions. The fate of metabolism can result in the formation of an inactive, active or toxic metabolites. The inactive metabolites have no physiological effects. The second possible fate of drug metabolism is the formation of active metabolites (those having the desired activity similar to/or greater than the parent drug). These drugs are sometimes termed as prodrugs and the clearance of all active metabolites needs to be considered. The formation of toxic metabolites is a possible fate in drug metabolism. Analogues can be developed for these undesirable metabolites with sites blocked to prevent their formation. Lastly, metabolites with unrelated physiological action unrelated to the desired mechanism can be formed. These metabolic derivatives can lead to new indications of the drug (Nassar *et al.*, 2009).

1.7.1 Phase I and Phase II reactions

The routes by which drugs may be metabolised are many and varied. From a biochemical point of view, most of these changes can be grouped in two types of reactions called Phase I and Phase II reactions.

Phase I reactions are commonly described as 'functionalization' reactions and include oxidation, reduction, hydration and hydrolysis as well as isomerization. These reactions are known for modifying an existing functional group to be more polar (reductions); introducing a new polar functional group to the parent drug (oxidations); or unmasking existing polar functional groups (hydrolyses). Phase I metabolism generally prepare a compound for phase II metabolism and not to prepare the drug for excretion.

The Phase II reactions are considered the true detoxification pathways described as conjugation reactions. Phase II reactions are substitution-type reactions linking new groups including glucuronidation, sulphation, glycine, glutamine conjugates, acetylation, methylation and glutathione conjugates to either the parent drug or the phase I metabolite. These conjugates cause a dramatic increase in the polarity and generally leads to a water soluble product which can easily be excreted in urine or bile.

1.7.3 Drug Metabolizing Enzymes (DME)

Drug metabolizing enzymes (DME) play a very important role in the clearance of drugs by converting lipophilic molecules to more water-soluble metabolites to enable excretion through the kidney or the biliary system. Some DMEs are polymorphic and leads to high pharmacokinetic variability, toxicity or loss of efficacy. Metabolism by these DMEs impact clearance and therefore, half-life and oral bioavailability. It also determines how much (dose) and how often (dosing frequency) we should dose the drug (i.e. dose regimen). For oral administration, metabolism affects first-pass clearance, and therefore, oral bioavailability.

Phase I drug metabolizing enzymes include cytochrome P450 (CYP), aldehyde oxidase (AO), xanthine oxidase, flavin-containing monooxygenase, hydrolases and monoamine oxidase (MAO).

Phase II enzymes are uridine 5'-diphosphoglucuronosyltransferase/UDP-glucuronosyltransferase (UGT), sulphotransferase (SULT), N-acetyltransferase (NAT) and glutathione-S-transferase (GST). Drug molecules can be metabolised by phase I enzymes, phase II enzymes or phase I followed by a phase II enzymes. These drug metabolising enzymes are found in different sub-cellular fractions prepared from drug metabolising tissues (gut and liver). Different fractions are used in drug metabolism studies to address various questions.

Hepatocytes are the basic unit of the liver involved in drug metabolism and contains the full complement of enzymes and co-factors. The hepatocyte is important for studying both Phase I and Phase II metabolic pathways. The S9 fraction contains both microsome and cytosol and represent a complete collection of all drug metabolising enzymes. The S9 fraction is the supernatant obtained after homogenising and spinning hepatocytes at 9000×g. It is necessary to supplement the S9 fraction with the co-factors that mediate the various metabolism reaction unlike in the hepatocytes that already have these co-factors. Spinning the S9 fraction at 10,000×g separates into the cytosolic fraction and the microsomal fractions. The microsomal fraction contains membrane-bound

cytochrome P450 enzymes and primary conjugation enzymes such as UGTs. These enzymes are responsible for the metabolism of over 90% of marketed drugs, hence the most frequently utilized enzymes system. The cytosolic fraction contains the soluble enzymes namely SULT, AO, XO, ketone reductase and hydrolases (Figure 1.4). Many of these cytosolic enzymes (especially AO) are missed during drug metabolism screening with human microsomes in drug discovery (Li Di, 2014) and result in 'Crash and burn' of these drugs in the clinic.

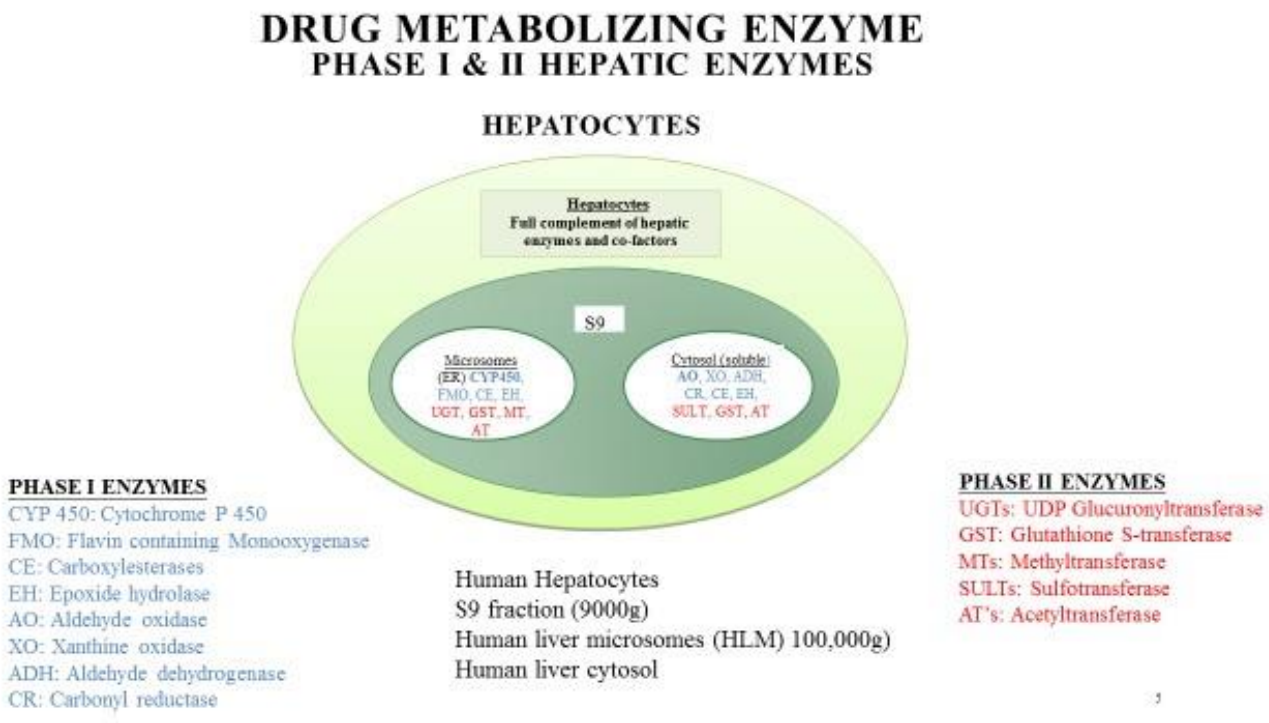


Figure 1.4: Hepatocyte fractions and the various drug metabolizing enzymes.

1.7.4 Drug-drug interaction

Drug-drug interactions (DDI) are of great concern in the process of drug development and clinical practice. Without prior knowledge of the potential DDIs, a drug can fail in clinical trials or after approval, leading to higher costs of pharmaceuticals and the lack of potentially life-saving therapeutic agents. DDIs can be divided into two major groups. The first, the pharmacodynamics effect, occurs when presence of one drug alters the response of a second administered drug through either additive or antagonist effects. The second, the pharmacokinetic effect, occurs when one drug (the "precipitant" drug) affects the absorption, distribution, metabolism, or excretion of a second drug (the "victim" drug) (Barr and Jones, 2011). While examples of the later exist, most interactions are of the former type. If the second drug has a wide therapeutic margin, the interaction may be clinically insignificant in that the increase in exposure will not cause deleterious effects. However, if the victim drug possesses a low therapeutic index, then the decrease metabolic clearance and resulting increase in exposure may manifest itself in a clinical relevant interaction.

Interactions due to the inhibition or induction of drug-metabolizing enzymes have been very frequently described in the scientific literature.

In drug-drug interaction (DDI) assessment, the cytochrome P450 enzymes are considered the most important enzyme families due to their extensive involvement in the metabolic clearance of the majority of prescribed drugs (Williams, 2004). While the CYP enzymes have been and continue to be a major focus of drug interactions, alteration in the activities of other drug-metabolizing enzymes can also be an underlying mechanism of drug-drug interactions. Non-CYP enzymes include the aldehyde oxidase (AO), monoamine oxidase (MAOs), xanthine oxidase (XO), flavin-containing monooxygenases (FMOs), aldehyde dehydrogenase (ALDH) whose involvement in the drug

metabolism and precipitation of drug-drug interaction is under evaluation (Benedetti *et al*., 2006) Among all the non-CYP mediated pathways, aldehyde oxidase mediated metabolism of substrates, and its inhibition by drugs is gaining importance recently. Aldehyde oxidase has a unique structure, distribution, and substrate recognition making the role of this enzyme important in the metabolism of drugs or substrates with different chemical structures and functionalities. This has been demonstrated by its increased role for metabolizing drugs from a proportion of 0.13 to 0.45 (Garattini and Terao, 2012).

1.7.5 Herb-drug interaction

Herbal medicines like any other medicine can interact with orthodox medicines resulting in a change in the pharmacological or toxicological effects of either component. This interaction when synergistic may complicate the dosing of long-term medications— e.g., traditional medicines locally used to decrease glucose concentrations in diabetes (Bailey and Day, 1989) could precipitate hypoglycaemia when taken together with conventional drugs. The true prevalence of drug interaction is substantial but unknown. Several reported herb-induced interactions lack crucial documentation on temporal relations and concomitant drug use.

1.8 TOXICOLOGICAL STUDIES

Toxicology is the study of the adverse effects of chemicals on living organisms as well as its symptoms, mechanisms and treatments. The extent to which an exposed tissue is damaged by a chemical substance is referred to as toxicity. Toxicity also covers the effect on a whole organism and sub-structural component of organism such as the cell (cytotoxicity) or organ (organotoxicity). Drugs in general need to be safe at the dose recommended for use. For this reason, the safety of drugs needs to be ascertained before approval for use by regulatory authorities in most countries. When found to be toxic, drugs are usually withdrawn from circulation; for example, troglitazone (Rezulin), an antidiabetic drug, was removed from the market after close to 100 of the 1.9 million patients treated with the drug suffered liver failure (Chojkier, 2005). Adequate investigation is therefore necessary to ascertain the safety dose and toxic dose of a drug.

Several factors such as the genetic composition of an individual, the time of exposure, the physical form of the toxicant (solid, liquid or gas), the pathway of administration, the frequency of exposures, and an individual's overall health among others can influence the extent of toxicity of a substance.

The toxic effects of many poisons are demonstrated only indirectly. For instance methanol's toxic effects are only manifested when it is chemically converted to formaldehyde and formic acid in the liver and these metabolites are responsible for the toxic effects seen with methanol exposure. On the other hand, idiosyncratic toxicity is a leading cause for failure of drugs in clinical testing and it is the most frequent reason for posting warnings, restricting use, or even withdrawal of the drug from the market.

Toxicity studies may be acute, sub-acute/sub-chronic and chronic.

Acute toxicity

Acute toxicological studies investigate the toxic effects produced by a single large dose exposure to a toxicant lasting no longer than twenty four (24) hours. This may result in severe biological harm or death to the organism. The results of acute toxicity are not only important in the consideration of accidental poisoning with a chemical but are also used for the planning of chronic toxicological studies (Herxhelmer, 1987).

An effect that cannot be seen in chronic exposure due to the development of tolerance is usually revealed by the acute exposure. The starting point for toxicological classification of chemicals uses the LD_{50} value, which is the dose administered in acute toxicity testing that causes death in 50% of experimental animals.

Chronic toxicity

Chronic exposure to a toxin over an extended period of time, usually measured in months or years (usually 6 to 24 months), can cause irreversible toxicity.

Periods between acute and chronic exposure could be referred to as sub-acute or sub-chronic. The results of chronic and acute toxicological studies help in the evaluation of any possible hazardous effect of a new drug or a drug which is in use with little or no documentation of its systemic toxicity.

1.8.1 Target organ Toxicity

The extent to which an organ is susceptible to toxicity varies from organ to organ. Highly vascularized organs (liver, kidney) are more exposed to toxicant than less vascularized organs (bone tissues). Changes in the body weights and relative weight of internal organs such as the liver, spleen, kidney and stomach are valuable indicators in the evaluation of the toxic effects of compounds (Grance *et al*., 2008). Direct injury to cells of an organ can lead to destructions of the normal architecture of the organ and often results in cell death. Such injuries can be as a result of genetic error, hypoxia, ischaemia, radiations, poisons or microbial invasions (Israel and Water, 1989). Depending on the stage and extent of the injury such damage may be reversible or irreversible. The ability of an organ to survive or yield to the effects of the injury agent will depend on its capacity to regenerate dead cells as well as its ability to detoxify the toxic agent (Timbrel, 2000).

1.8.1.1 Toxicity to the liver

The liver's major role of metabolism and detoxification of compounds makes it vulnerable to the toxic effects of compounds. Blood supply to the liver from the gastrointestinal tract also exposes it to relatively high concentrations of toxic substances. Drug/chemical-induced hepatotoxicity is the most common manifestation of drug toxicity (Lee, 2003) and this represents more than 50% of all cases of acute liver failure. Drug-induced hepatotoxicity is the major hurdle in drug development and remains the major reason for the withdrawal of drugs from the market (Cullen and Miller, 2006). When a compound or metabolite precipitates in the canalicular lumen, there is an interference with the production and flow of bile which may cause damage to the biliary system and surrounding hepatocytes and thus causes the leakage of liver enzymes into the blood (Timbrel, 2000).

Several methods exist for the detection of hepatic injury. The liver/body weight ratio can be measured (Raza *et al.,* 2002; Teo *et al.,* 2002). Light and electron microscopy can be employed to examine stained liver sections. Conjugated bilirubin excreted in the urine and the measurement of biochemical parameters in plasma may also be utilized. In experimental animals, the measurement of total cytochrome P450 and determination of sleeping time may also be used.

Biochemical assessments are carried out to provide information on the liver and several enzymes usually assessed include alkaline phosphatase (ALP), gamma glutamyltransferase (GGT), aspartate transaminase (AST), and alanine transaminase (ALT). Plasma levels of these enzymes are raised several folds in the first 24 hours after injury (Murray *et al.,* 1988). Abnormal levels of the liver enzymes in plasma are usually indicative of the hepatic cellular injury in experimental animals (Comporti, 1985).

The liver functions in producing most of the plasma proteins in the body. This includes albumin and globulin. A decrease in the levels of these proteins in the plasma could point to an interference with the synthetic ability of the liver.

Bilirubin is a pigment produced from the breakdown of haem in red blood cells. The bilirubin is taken to the liver for conjugation and then excreted into the bile and stored in the gall bladder. High levels of bilirubin in the plasma may be due to an increase in production, a decrease in its liver conjugation or blockade of the bile ducts resulting in a decrease in its secretion. An elevation in the unconjugated or indirect form of bilirubin in the serum due to either an increased production or decreased conjugation signifies pre-hepatic or hepatic jaundice whereas an increased conjugated bilirubin points to post hepatic jaundice. Bile duct obstruction builds up conjugated/direct bilirubin which escapes into the blood and hence increasing the plasma levels. This makes serum bilirubin levels an important marker of liver function since it reflects the liver's ability to take up, process, and secrete bilirubin into the bile.

1.8.1.2 Toxicity to the kidneys

The kidneys are involved in the excretion of urea and ammonium into urine; the re-absorption of glucose, water and amino acids as well as producing hormones such as calcitriol, renin, and erythropoietin. The kidneys also have homeostatic functions of regulating electrolytes, blood pressure and maintaining the acid-base balance.

The kidney contains some amount of cytochrome P450 enzymes which are responsible for the metabolic inactivation of some compounds. The reabsorption of many substances including salt and water after glomerular filtration leaves the concentration of the foreign substances in the tubular lumen higher than that in the blood hence predisposing the kidneys to toxic effect of foreign substances.

The role of the kidney in filtering harmful substances from the blood makes it unusually susceptible to these chemicals. Some herbal medicines have been reported to have nephrotoxic effects (Akdogan *et al*., 2003).

Kidney damage can be detected in a variety of ways ranging from simple qualitative tests to more complex biochemical assays. Urine volume, pH, kidney weight to body weight ratio (Raza *et al.*, 2002; Teo *et al.*, 2002), detection of protein or cells in the urine, and the measurement of urea or creatinine in the plasma (Timbrel, 2000) are common tests performed to ascertain injury to the kidneys.

1.8.1.3 Toxicity to the blood

The blood functions as a major transport medium for gases, nutrients, waste metabolites and xenobiotics. With almost all foreign materials distributed via the bloodstream (Timbrel, 2000), the cellular component of blood such as red blood cells, platelets, and white blood cells are at least initially exposed to significant concentrations of the toxic compounds. Olson *et al* in 2000 showed that the assessment of blood is relevant to the evaluation of risks since the haematological system carries a higher predictive value for toxicity in humans (91%) (Olson *et al*., 2000). Certain medicinal herbal preparations or conventional drugs have been reported to adversely affect various blood components (Yunis *et al*., 1980; Synder *et al*., 1977). Blood cell damage is readily detected by light microscopy and automated techniques where levels of various components of blood are determined. The automated analyser performs a full blood count after drug administration and levels of red blood cells (RBC), white blood cells (WBC), haematocrit (HCT), haemoglobin (Hb), platelets (HCT), and lymphocytes (LYMP) as well as red blood cell indices such as mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), and mean corpuscular volume (MCV) may be determined

1.9 JUSTIFICATION, AIMS AND OBJECTIVES

1.9.1 JUSTIFICATION OF STUDY

Great efforts and resources have been directed towards the eradication and elimination of malaria over decades. The World Health Organisation (WHO, 2015) reports a global fall of malaria cases from an estimated 262 million in 2000, to 214 million in 2015, a decline of 18%. However, the disease still remains a grave public health problem accounting for hundreds of thousands of deaths annually (WHO, 2014). With the research into malaria vaccines still at an advanced stage, chemotherapy remains the principal tool for the control and eradication of the disease.

The emergence of strains of *Plasmodium falciparum* and *P. vivax* resistant to current antimalarial agents (multidrug resistant or MDR) have motivated the research for potent and novel agents representing new and distinct chemical classes and mechanisms of action other than those antimalarial drugs currently in use. Antimalarial plants with bioactive compounds of novel structure represent an important source for the discovery and development of new drugs for malaria (Kaur *et al*., 2009; Schmidt *et al*., 2012a, b). Plant derived antimalarial agents such as quinine from the bark of the Cinchona tree and artemisinin isolated from *Artemisia annua* together with their synthetic or semi-synthetic derivatives have over the years made and continue to make tremendous contribution to the chemotherapy of malaria (Wright, 2011).

Cryptolepine, the major alkaloid from the West African plant *Cryptolepis sanguinolenta* (Lindl.) Schlechter (Periplocaceae) is a bioactive compound that is currently of interest as a potential antimalarial lead. Cryptolepine has been shown to have antimalarial activity similar to other quinolone compounds and acts within the acidic food vacuole of the parasites preventing the conversion of harmful by-product of haemoglobin digestion (haem) resulting in its accumulation,

which cause parasite death. The basic nitrogen in the structure of cryptolepine has been shown to be associated with the effective accumulation of the compound (Egan *et al.,* 1999).

In West Africa, a decoction of the roots of this climbing shrub is used for the treatment of malaria (Boye and Ampofo, 1983). Cryptolepine is present in relatively large amounts in the roots of *C. sanguinolenta* (>1% of the dried roots) and has been proven to have potent *in vitro* antimalarial activities against both chloroquine–sensitive and chloroquine–resistant *P. falciparum* (Wright *et al*., 2001a).

In Ghana, the decoction of the root of *C. sanguinolenta* is used both as finished packaged products (PhytoLaria®, Nibima®, Fas Malacure®, Masada mixture®, Lepiquin®, Kingdom M mixture®, Tinatett Malakare® and Yafo fever mixture®) in herbal clinics, orthodox hospitals and community pharmacies for the treatment of malaria and also used traditionally by indegines for the treatment of malaria in endemic regions.

The World Health Organization (WHO) recommended the Artemisinin-based Combination Therapy (ACT) as first– line treatment of falciparum malaria following the development *P. falciparum* resistance to Chloroquine, Sulfadoxine- pyrimethamine, mefloquine, and other antimalarial drugs. Artemisinin-based combination therapies are the most effective drugs for the treatment of *Plasmodium falciparum* malaria. ACT is the combination of an artemisinin derivative (eg. artesunate, artemether, dihydroartemisinin) and a partner drug (eg, Amodiaquine, Mefloquine, Piperaquine, lumefantrine) having a markedly longer half-life in the bloodstream than the artemisinins.

In Ghana, the Ghana Health Service adheres to the World Health Organization's directive on the discouragement of monotherapy in the malaria therapy and hence all health facilities in the country supply the ACT to patients with malaria except in first trimester of pregnancy.

Regarding antimalarial drug therapy in Ghana, there have been reports of the concurrent use of herbal antimalarial preparation mostly containing *Cryptolepis sanguinolenta* and some orthodox antimalarials (Sakyimah *et al.*, 2011). Most patients who take these herbal preparations before reporting to allopathic health facilities do not disclose this information to their healthcare providers thus increasing the chances of possible herb-drug interactions.

To date very little is known about the use of *Cryptolepis sanguinolenta* or cryptolepine and the artemisinin derivatives regarding potential toxicity, antimalarial interaction and pharmacokinetics especially when used in combination.

The research questions of interest are therefore:

1) Does cryptolepine when combined with the artemisinin derivatives possess any synergistic, antagonistic or additive antimalarial activity *in vivo* or *in vitro*?

2) Does *Cryptolepis sanguinolenta* or cryptolepine possess any gametocytocidal properties?

3) Regarding adverse effects of some artemisinin-based combinations (artesunate-amodiaquine), will a combination of cryptolepine and the artemisinins offer a better alternative?

4) Are there any toxicological implications when *Cryptolepis sanguinolenta* or its major alkaloid cryptolepine is used before, after or with the artemisinin derivatives?

5) How is cryptolepine absorbed, distributed, metabolized and excreted (Pharmacokinetic profile of cryptolepine)?

1.9.2 AIMS AND OBJECTIVES

AIMS:

The aim of the study is to investigate the potential toxicity, antimalarial profile and the pharmacokinetics *in vivo* and *in vitro* when cryptolepine or the crude extract of *Cryptolepis sanguinolenta* is used alone or in combination with the artemisinin derivatives.

SPECIFIC OBJECTIVES

- Determination of possible synergistic, additive or antagonistic antimalarial interaction of cryptolepine with the artemisinin derivatives *in vivo* and *in vitro*.
- Evaluation of the possible gametocytocidal properties of the aqueous root extract of *Cryptolepis sanguinolenta* and cryptolepine.
- Evaluate the *in vitro* permeability, plasma protein binding and plasma stability of cryptolepine.
- Determination of the *in vitro* metabolism profile of cryptolepine in different liver fractions.
- Characterize the human and rat hepatic enzymes involved in the metabolism of cryptolepine.
- Determination of *in vivo* pharmacokinetic profile of cryptolepine in Sprague Dawley rats and the identification of metabolites formed in the urine and plasma.
- Determination of the effect of the concomitant use of the aqueous root extract of cryptolepis or cryptolepine with the artemisinin bases on the serum biochemical parameters, blood parameters and target organs in Sprague Dawley rats.

2.0 CHAPTER TWO

PLANT COLLECTION, ISOLATION AND CHARACTERIZATION

2.1 PLANT COLLECTION AND EXTRACTION

2.1.1 Plant Collection

The sun-dried roots of *Cryptolepis sanguinolenta* used in this study was obtained from the Centre for Plant Medicine Research (CPMR), Mampong-Akwapim, Ghana in August 2012 and was

identified at the Plant Development Centre of the Institution. Its authenticity was confirmed by Dr. Kofi Annan of the Department of Pharmacognosy, KNUST and subsequently compared to a voucher specimen KNUST/HM1/2008/L056 at the herbarium of the Department of Pharmacognosy/ Herbal Medicine, College of Health Sciences.

2.1.2 Preparation of the aqueous extract from the roots.

The powdered roots (650 g) was boiled for 30 min with 5 litres of distilled water, decanted and filtered. The filtrate was transferred into a clean container and kept in a conical flask. The residue was further extracted successively with distilled water (3× 2 L). The filtrates were pooled together and freeze-dried to obtain a sample of the crude extract (Yield = 11.08% w/w) referred to as cryptolepis (CPS). Cryptolepis was reconstituted in distilled water prior to use.

2.2 ISOLATION AND CHARACTERIZATION OF CRYPTOLEPINE HYDROCHLORIDE

2.2.1 Chemicals/Reagent

Table 2.1: Chemicals used in the isolation and characterization of cryptolepine

DRUG / CHEMICAL	SOURCE
Methanol, chloroform, dichloromethane, hydrochloric acid, Ammonia solution	
Ethylacetate, Potassium dichromate,	BDH, Poole, England

Sodium chloride, Aluminium oxide (alumina)	
Distilled water	Department of Pharmaceutical Chemistry, Kwame Nkrumah University of Science and Technology, Kumasi.
Precoated plates-silica gel Gf 254, 0.25 mm	Merck W. (Germany)
Reference Cryptolepine hydrochloride	Kindly donated by Professor Colin Wright, University of Bradford, UK.

2.2.2 Methodology

The isolation of cryptolepine hydrochloride (CPE) from the roots of *Cryptolepis sanguinolenta* was as reported by Kuntworbe *et al.* (2012) with slight modifications incorporating some aspects of the approach by Cimanga and co-workers (Cimanga *et al*., 1996; Cimanga *et al*., 1997).

The sun-dried roots were powdered with a ball miller. Powdered root (600 g) was soaked in Petroleum ether for 24 h to remove waxes and terpenes. The powdered root was exhaustively extracted with methanol (2.5 L) by Soxhlet extraction at 50ºC for 48 h to afford a dark crude alkaloid mixture. The crude extract was concentrated *in vacuo* to about 300 mL using a rotary evaporator, (Rotavapor® R-215, Buchi, Switzerland).

The mixture was rendered alkaline (pH >11) with aqueous ammonium hydroxide and extracted with 5(100 mL) portions of chloroform in a 1 L separation funnel. The combined organic layer was again concentrated *in vacuo* to about 50 mL, adsorbed into aluminium oxide, and air-dried. The dried aluminium oxide-alkaloid mixture was dry-loaded onto a 500 mL aluminium oxide packed column and eluted with dichloromethane followed by chloroform and finally, chloroform containing 10% methanol. Fractions (10 mL) were collected and identical fractions were combined following TLC on silica gel plates (5×8 cm) using a mixture of dichloromethane, chloroform, and methanol (4:4:1) as the mobile phase. Identical fractions containing cryptolepine (deep purple solution) (Cimanga *et al*., 1997) was concentrated and acidified with aqueous HCl (200 mL, pH 4). The aqueous phase was separated and basified with aqueous ammonium hydroxide to precipitate cryptolepine, which was then extracted with 200 mL of chloroform. Finally, the chloroform layer was treated with acidified ether to precipitate cryptolepine as a yellow hydrochloride salt. The solvent was removed *in vacuo* and the material dried into a free-flowing yellow powder. The melting point was determined using an electro thermal melting point apparatus. Purity of isolated cryptolepine was confirmed with thin layer chromatography (TLC), ultra violet/visible (UV) spectra, high performance liquid chromatography (HPLC) spectra and melting point determination. The spectra were compared to both reference cryptolepine hydrochloride in literature (Dwuma-Badu *et al.*, 1978; Bierer *et al.*, 1998) as well as pure cryptolepine hydrochloride kindly donated by Professor Colin Wright of the University of Bradford, UK.

2.2.3 Determination of Ultraviolet (UV) absorption spectrum of isolated Cryptolepine.

Calibration

Potassium dichromate was dried to a constant mass at 110°C for 60 min. It was kept in a desiccator to cool. Potassium dichromate (10 g) was accurately weighed, dissolved in 0.005 M H_2S0_4 and diluted further to 1L with the same acid. The UV absorption spectrum of the solution was determined over the wavelength range 200-400 nm using 1 cm cell and the acid as reference. The A (1%, 1 cm) at λmax and λmin were compared with the British Pharmacopoeia stipulations (BP, 2013). The A (1%, 1 cm) at λmax and λmin were within maximum tolerable values.

Methodology.

Several dilutions of methanolic solution of the reference and isolated cryptolepine (0.001-0.1%) were made and scanned over wavelength range of 200-400 nm using the calibrated T90+ UVVisible spectrophotometer in a 1 cm cell with methanol as reference. The peak wavelength for all isolates and reference cryptolepine were compared with that of pure cryptolepine hydrochloride as reported by Dwuma-Badu *et al.* (1978).

2.2.4 High Performance Liquid Chromatography (HPLC) spectra of reference and isolated cryptolepine.

Reference and isolated cryptolepine samples (0.0200 g) were accurately weighed and transferred to a 100 ml volumetric flask containing about 75 mL of mobile phase (Acetonitrile 30% v/v + HPLC grade water containing 0.66% v/v Formic acid). The powder mixtures were each dissolved in the mobile phase with the aid of sonication and then made up to 100 mL with the mobile phase. The cryptolepine solutions were each filtered through Whatman filter paper (No. 5) into another 100 mL volumetric flask. From the above filtrates, 1 mL was taken in a 10 mL volumetric flask

and volume was made up with mobile phase, the solutions were then filtered using sintered glass filter. After setting the chromatographic conditions and stabilizing the instrument, 10 µL of the sample solution was injected at flow rate of 1.5 mL/min and a chromatogram was recorded using an UV absorbance detector.

2.2.4.1 Chromatographic conditions for assay

Column oven temperature	25.0 ^{0}C
Column pH	Microsorb (TM) S1 89-100-D5E61002
Injection volume	2.53
Flow rate	10 µL
Mobile Phase	1.50 mL/min
Detection method	Acetonitrile 30% v/v + HPLC grade Water containing 0.66% v/v Formic acid
Data acquisition	UV (250–1000 nm)
	ChromQuest Version 4.1

2.3 RESULTS

2.3.1 Melting Point Determination

The melting points of isolated cryptolepine and the reference were determined as a measure of purity. The melting points of both isolates (I and II) were determined with an electro thermal apparatus to be 264-266^{o}C and 265-267^{o}C, respectively. The melting point of reference cryptolepine was 264-266^{o}C. All samples therefore are within the range of 263-268^{o}C reported by (Bierer *et al.,* 1998) as the melting point of cryptolepine HCl.

2.3.2 Thin Layer Chromatography (TLC)

The isolated cryptolepine samples were run with reference cryptolepine (P) using pre-coated analytical TLC plates with a combination of mobile phases; Ethyl acetate : ethanol : Ammonia (35%) 80:15:5.

Cryptolepine fluoresces under ultraviolet light at 354 nm and hence detection of cryptolepine was monitored at this wavelength using the UV viewing system (Chromato-Vue® C- 70G).

The isolated cryptolepine showed a single spot under UV light at 254 nm and 354 nm with approximately the same R_f - value as reference cryptolepine in the solvent system used (Table 2.2 and Figure 2.1). Isolates I and II are cryptolepine hydrochloride isolated from the same roots at different times of isolation.

Solvent system = Ethyl acetate: Methanol: Ammonia (35%) (80:15:5)

Table 2.2: Retardation factor (R_f) values of reference and isolated cryptolepine

Cryptolepine (n=3)	Distance moved by cryptolepine spot(x)/mm			Distance moved by solvent front(y)/mm			R_f value= x/y		
Reference	35.00	34.00	35.00 3	64.00	63.00	65.00	0.547	0.540	0.538
Isolate I	34.00	34.50	35.00	64.00	63.00	65.00	0.531	0.548	0.538
Isolate II	35.00	35.00	34.00	64.00	63.00	65.00	0.547	0.547	0.523

Average R_f value of reference cryptolepine = **0.542±0.002**

Average R_f value of Isolate I = **0.539±0.004**

Average R_f value of Isolate II = **0.539±0.007**

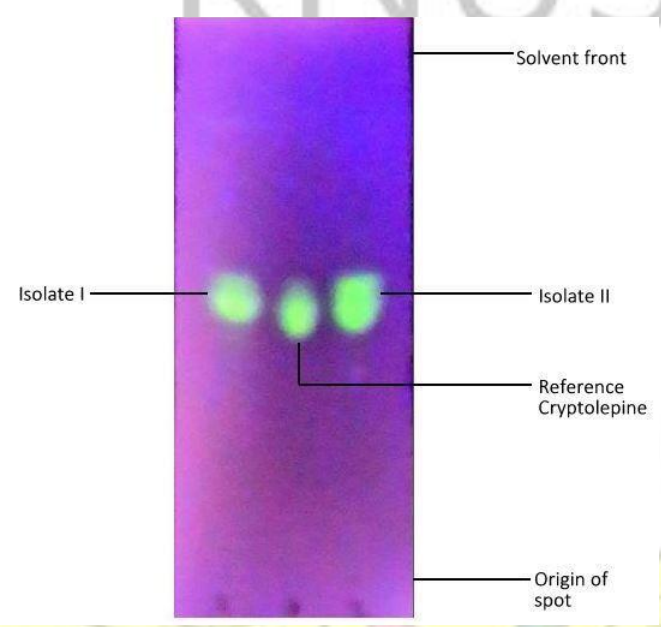
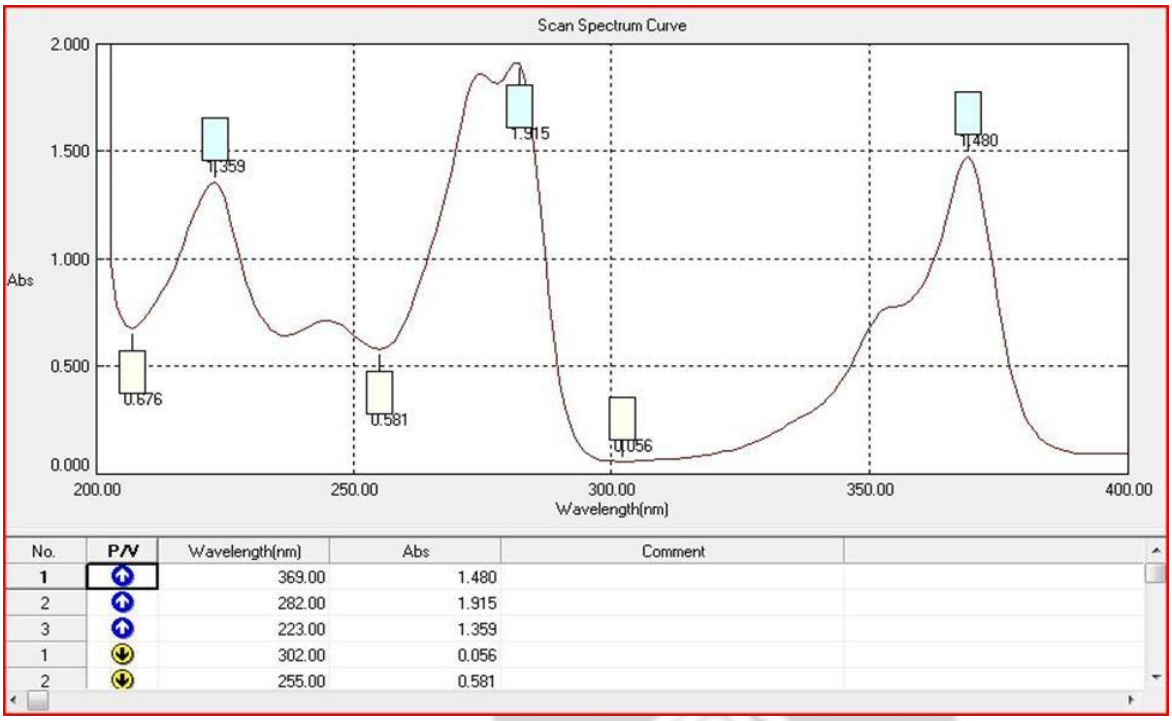


Figure 2.1: Representative TLC chromatogram of isolated cryptolepine along with reference cryptolepine.

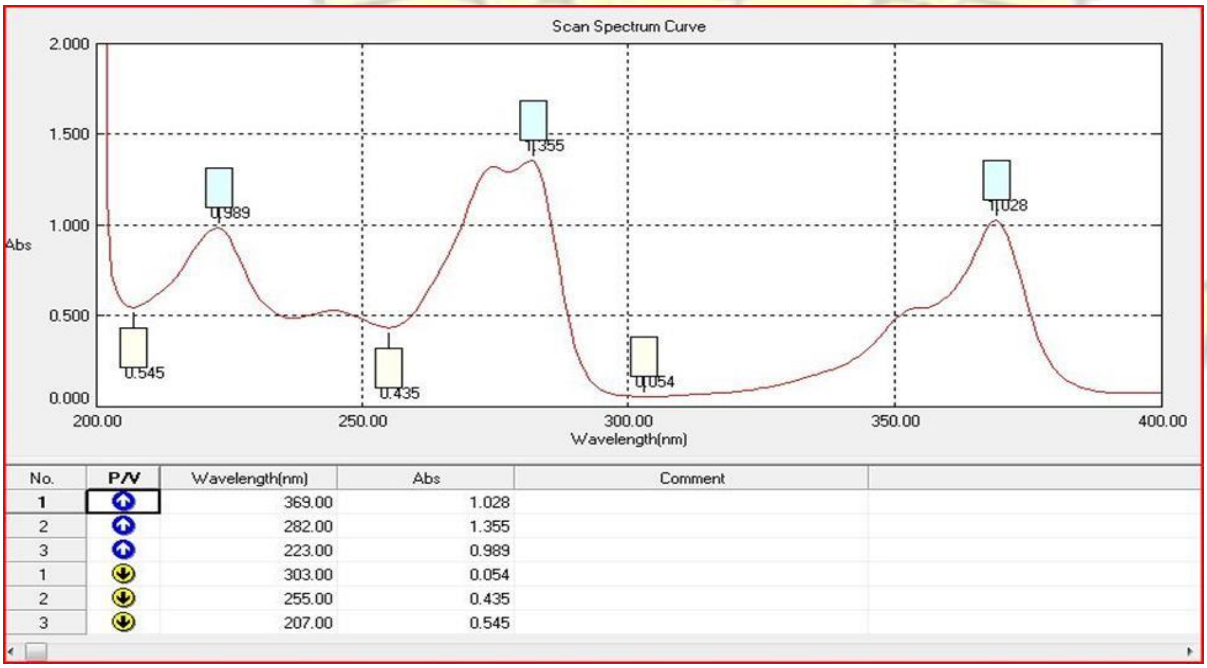
2.3.4 Ultraviolet/Visible absorption spectrum

The peak wavelengths for all samples were observed at 369, 282 and 223 nm. Figures 2.2, 2.3 and 2.4 represents the UV spectra of reference cryptolepine, and the two isolates (I and II) with their respective absorbance between the wavelengths of 200 and 400 nm.



No.	P/V	Wavelength(nm)	Abs	Comment
1	↑	369.00	1.480	
2	↑	282.00	1.915	
3	↑	223.00	1.359	
1	↓	302.00	0.056	
2	↓	255.00	0.581	

Figure 2.2: UV spectrum of reference cryptolepine (0.001%)



No.	P/V	Wavelength(nm)	Abs	Comment
1	↑	369.00	1.028	
2	↑	282.00	1.355	
3	↑	223.00	0.989	
1	↓	303.00	0.054	
2	↓	255.00	0.435	
3	↓	207.00	0.545	

Figure 2.3: UV spectrum of isolated cryptolepine (isolate I) (0.005%)

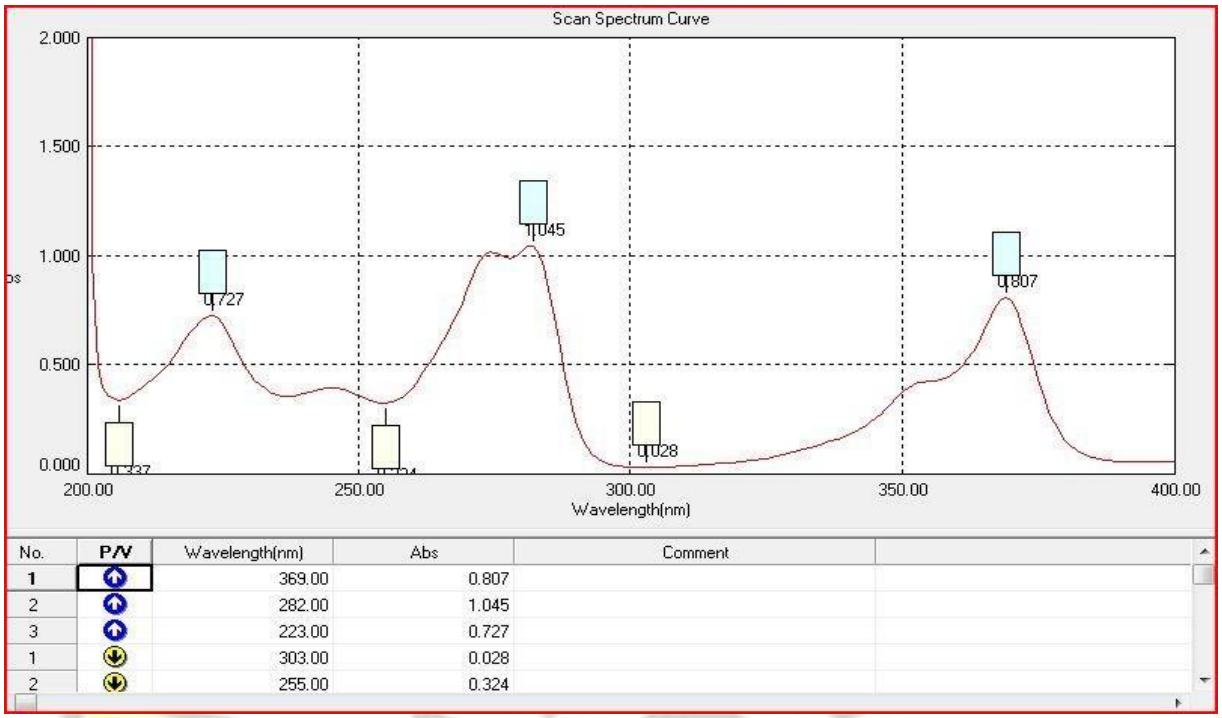


Figure 2.4: UV spectrum of isolated cryptolepine (Isolate II) (0.005%)

2.3.5 High Performance Liquid Chromatography (HPLC) spectra

The HPLC chromatogram of the reference and isolated cryptolepine gave single peaks (Fig. 2.5, 2.6 and 2.7) with average retention time of 2.558±0.04 min (n=5) for the reference sample, 2.708±0.24 min (n=5) for isolate I and 2.677±0.15 min (n=5) for isolate II (Table 2.3), respectively.

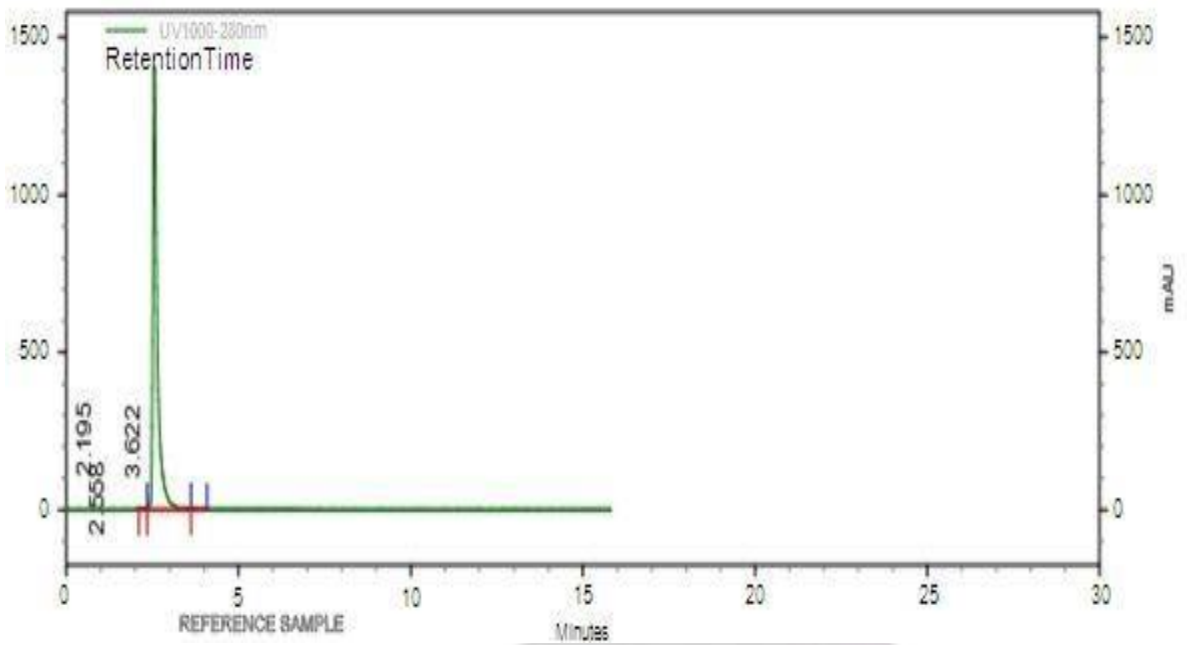


Figure 2.5: Representative HPLC chromatogram of reference cryptolepine.

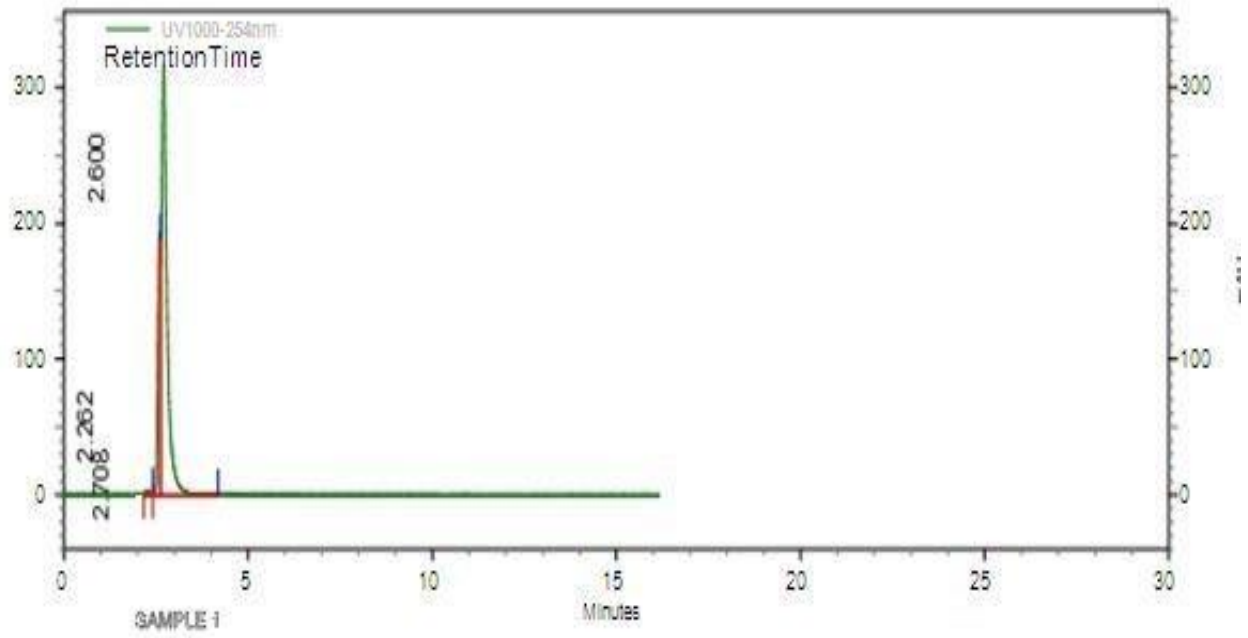


Figure 2.6: Representative HPLC chromatogram of isolated cryptolepine (Isolate I)

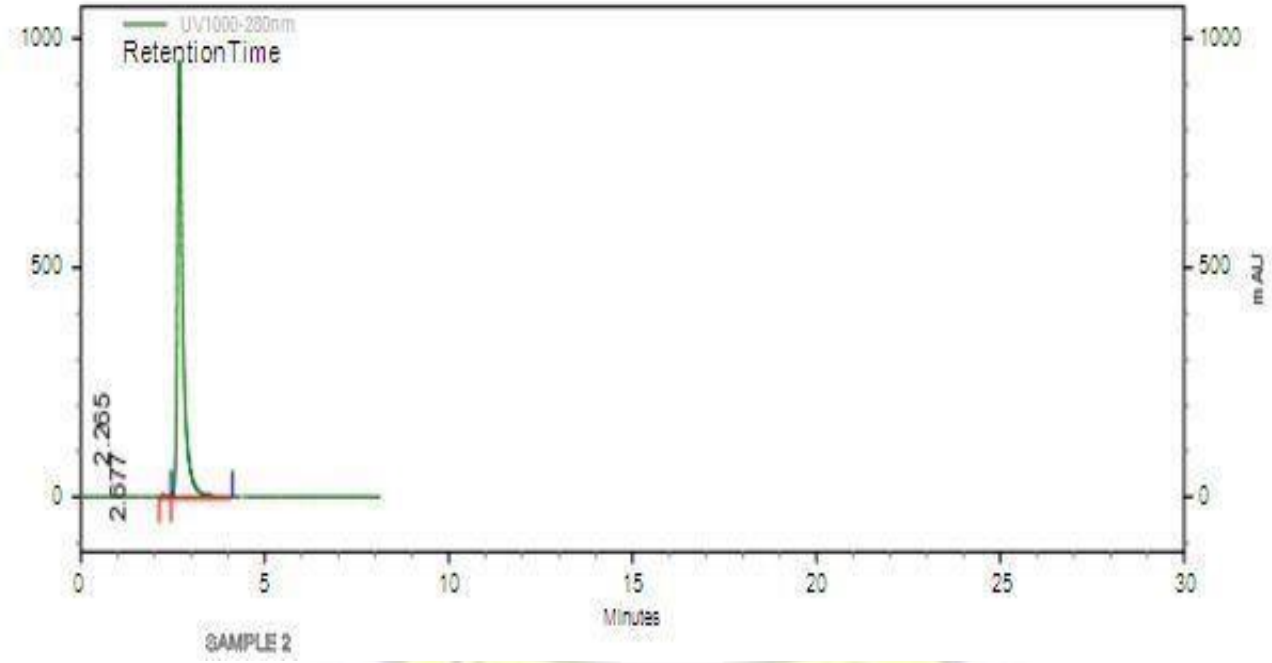


Figure 2.7: Representative HPLC chromatogram of isolated cryptolepine (Isolate II)

Table 2.3: Retention times of the reference and isolated cryptolepine

CRYPTOLEPINE HCl	RETENTION TIME(min)
REFERENCE SAMPLE	2.558±0.04
ISOLATE I	2.708±0.24
ISOLATE II	2.677±0.15

2.4 DISCUSSION

The isolation of bioactive compounds from medicinal plants, based on traditional use or ethno medical data, is a highly promising potential approach for identifying new and effective antimalarial drug candidates (Rocha *et al.*, 2011).

The chemical composition of the roots of *C. sanguinolenta* was first studied in 1929 by Clinquart and an alkaloid named cryptolepine was isolated from a sample collected in Zaire. Since then, several researchers have isolated cryptolepine from the root powder of the plant (Dwuma-Badu *et al*., 1978; Cimanga *et al*., 1996; Bierer *et al*., 1998)

In this study, cryptolepine hydrochloride was isolated from the roots of *Cryptolepis sanguinolenta* using the method described by Kuntworbe *et al.* (2012). In this method, methanol was used in the soxhlet apparatus for 48 h at 50°C to ensure exhaustive extraction of plant components. This was followed by a combination of liquid-liquid extraction and column chromatography leading to the isolation of the pure compound in high yield. Cryptolepine (Isolate I=5.79 g and Isolate II=5.82 g) were obtained by the use of this procedure. This was calculated as a yield of 0.9650 and 0.9700% w/w for isolate I and II, respectively. The melting points of isolate I and isolate II were determined with an electro thermal apparatus to be 264-266^{o}C and 265-267^{o}C, respectively. The melting point of reference cryptolepine was 264-266^{o}C and they were all within the range of 263-268^{o}C reported by Bierer *et al.* (1998) as the melting point of cryptolepine HCl.

In the thin layer Chromatography (TLC) profile, both the isolated cryptolepine (Isolate I= 0.539±0.004; Isolate II= 0.593±0.007) and the reference cryptolepine showed a single spot under UV light at 254 and 354 nm with approximately the same R_f value. This indicates the similarity between the reference and isolated cryptolepine.

UV spectra of the isolated cryptolepine showed peak wavelengths at 369, 282 and 233 nm. These peak wavelengths were similar to those of the reference cryptolepine between a wavelength ranges of 200 to 400 nm.

In the high performance liquid chromatogram, qualitative information on the samples were obtained by comparing peak positions with those of standards. The number of peaks in the chromatogram also indicates the level of complexity of the sample whereas, quantitative assessment of the relative concentration of components is obtained from peak area comparisons (Raaman, 2006). In the qualitative assessment of the isolated and reference cryptolepine, a single peak was seen with the retention times being similar in all sample spectra.

2.5 CONCLUSION

Results from the present study showed a relative high yield (0.9650 and 0.9700% w/w) of cryptolepine isolated from the roots of *Cryptolepis sanguinolenta.* The melting point, TLC, UV and the HPLC spectra in relation to the pure reference cryptolepine further verified the identity and purity of the isolated cryptolepine.

3.0 CHAPTER THREE

ANTIPLASMODIAL ASSAYS

3.1 INTRODUCTION

Traditionally acclaimed antimalarial plants serve as a major source for the search for novel antimalarials. The aqueous root extracts of *Cryptolepis sanguinolenta* have been used for decades in the treatment of malaria in the West African sub-region (Lavrado *et al.*, 2010). In Ghana, a

clinical trial using a herbal tea bag preparation (containing *Cryptolepis sanguinolenta*) studied in forty four patients with clinical features of uncomplicated malaria showed more than half of the patients cleared of *P. falciparum* parasitaemia within 72 h (mean clearance = 82.3 h). In the same study, cryptolepis also showed a mean fever clearance of 25.2 h compared to the 48 h typical of chloroquine treatment and was consistent with previously reported antipyretic activity of *C. sanguinolenta* (Bugyei *et al.,* 2010). This safety and efficacy studies of the popular antimalarial plant has brought hope to several millions of people who are affected by malaria in Ghana and other West African countries. On the Ghanaian market in 2015, there were fifteen (15) formulated herbal products containing *C. sanguinolenta* for the treatment of malaria (unpublished data).

With the scourging effect of malaria, coupled with the rising incidence of resistance and noncompliance to standard antimalarials, the practice of patients taking herbal antimalarials and artemisinin derivatives concomitantly is on the increase. The practice of concurrent administration of herbal antimalarial preparations, most of which contain *Cryptolepis sanguinolenta* and some orthodox antimalarials has also been reported in Ghana (Sakyimah *et al.*, 2011).

As the patronage of this herbal medicines increase, there is an increased concern about the possible interaction of this widely used medicinal plant and its major alkaloid, cryptolepine with the WHO recommended artemisinin derivatives.

For decades, the selection and development of most currently used antimalarial drugs have been focused on their proven activity against the symptom-causing asexual blood stage of the parasite responsible for the mortality and morbidity. Malaria eradication requires the integration of therapeutic strategies targeting both the sexual and asexual stages of the malaria parasite. To meet the current goal of malaria elimination and eventual eradication, as expressed by the MalEra initiative, discovery of antimalarial drugs with the ability to kill circulating *Plasmodium*

falciparum sexual stages (gametocytes), and therefore preventing the disease transmission will inform the basis for the development of novel therapeutic combinations for the treatment and eradication of malaria (Alonso *et al.,* 2011). A drug that significantly reduces or entirely blocks malaria transmission from human to the vector (mosquito) would serve as an important tool in the fight against this global menace.

Artemisia annua as a medicinal plant offers a perfect example of the successful development of highly effective antimalarial drugs for both asexual and sexual stages of *P. falciparum* and this has motivated the exploration of plants popularly used as antimalarial remedies for possible transmission-blocking effects. Cryptolepine is the most studied alkaloid from *Cryptolepis sanguinolenta* and its antimalarial activity has attracted particular attention as the search for effective and novel antimalarial drugs continue (Lavrado *et al*., 2010). The established efficacy against the asexual stages of *P. falciparum* (Boye, 1989; Bugyei *et al*., 2010; Wright *et al*., 2001) motivated this study to evaluate possible transmission-blocking properties of the aqueous root extract of *Cryptolepis sanguinolenta* and its major alkaloid, cryptolepine.The present study also aims at determining possible toxicity and antimalarial interaction (*in vitro* and *in vivo*) when cryptolepine is combined with standard antimalarial drugs (Artesunate, Artemether, Dihydroartemisinin, Chloroquine, Amodiaquine and Mefloquine,). The outcome of this study will provide information on the safety, activity and possible interaction when cryptolepine is combined with these standard antimalarial agents as well as the clinical significance of the combination in the treatment of malaria.

3.2 MATERIALS AND METHODS

DRUGS/CHEMICALS

Table 3.1: Chemicals used in the antiplasmodial assays.

DRUG / CHEMICAL	SOURCE
Chloroquine diphosphate, Amodiaquine hydrochloride, Artesunate, Lumefantrine, Methanol, Formalin, Immersion oil for microscopy, Giemsa stain, SYBR® Green	Guilin Pharmaceuticals, China Novartis Pharma AG. Basel, Switzerland Novartis Pharma AG. Basel, Switzerland
1, Ethanol, Saponin, Na_2HPO_4, NaCl, RPMI 1640 medium, D-glucose, Hypoxanthine, N-acetyl glucosamine (NAG) and D-Sorbitol.	Invitrogen Life Technologies Inc. (Carlsbad, CA, USA) Frosted end microscope slides Vacutainer blood collection set G23, Vacutainer tubes, 5mL
Artesunate Artemether/Lumefantrine20/120 (Coartem®) Artemether and dihydroartemisinin Gentamicin and AlbuMAX II , 0.5% Sodium bicarbonate	Gas mix (92.5% N_2, 5.5 % CO_2, 2% O_2) Gas mix (Balanced N_2 mixture, 5 % CO_2, 5% O_2) 25 cm^3 and 75 cm^3 culture flasks
Sigma-Aldrich (St. Louis, MO, USA).	96 well microtitre plate 1 mL plastic pipettes 5 and 10 mL serological pipettes 15 and 50 mL centrifuge tubes RPMI-1640 medium, L-glutamine, streptomycin/penicillin, Hypoxanthine and HEPES (4-(2-hydroxyethyl)-1piperazineethanesulfonic acid) PrestoBlue

10% human serum	
Thermo Scientific	
BD Gentest	
	Gibco BRL Life Technologies (Grand Island, NY, USA
Afrox, Johannesburg, South Africa	
	Life Technolgies
Corning Inc	Interstate Blood Bank, Chicago, USA

3.3 *IN VITRO* ANTIMALARIAL ASSAYS

3.3.1 In vitro cultivation of malaria parasite

The asexual intra-erythrocytic stage of *P. falciparum* laboratory strain (3D7) was obtained from the Department of Immunology, Noguchi Memorial Institute of Medical Research (NMIMR), University of Ghana, Legon. The parasites were continuously cultured *in vitro* according to the method of Hout *et al*. (2006) with slight modifications.

Frozen parasite vials stored in liquid nitrogen were thawed in water bath at 37°C for 15 min, spun at 2000 rpm for 10 min and the supernatant discarded. The pellets were transferred into a sterile 15 mL falcon tube and equal volume of thawing mix (3.5% NaCl in distilled water) was added, thoroughly mixed and spun at 2000 rpm for 10 min. The supernatant was removed and 1 mL of

CPM (complete parasite medium) was added and spun again for 2000 rpm for 10 min. The washing step was repeated and supernatant discarded. The complete parasite medium (pH 7.3) used consisted of filter-sterilized RPMI 1640 solution supplemented with 0.5% AlbuMAX II, hypoxanthine (0.04%), buffered with 0.4% sodium bicarbonate ($NaHCO_3$), 0.72% N-2hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) and 0.005 mg/mL gentamicin. The pellets were suspended in 25 mL culture flask (BD falcon) containing 5 mL of CPM and 200 µL freshly prepared packed RBC (sickling negative, O rhesus positive) to have a haematocrit of 4%.

The culture was flushed with a mixture of gases (2% Oxygen, 5.5% Carbon dioxide and 92.5% Nitrogen) for 30 s. The flask was closed and placed in an incubator (RS Biotech, Livingston, UK) set at 37°C. The culture medium was changed daily using fresh CPM. The parasitaemia was checked by preparing a thin smear on a microscope slide under sterile conditions in the laminar flow safety cabinet (Hitachi Clean Bench, Japan). The slides after drying were fixed in absolute methanol, stained with 10% Giemsa in phosphate buffer for 10 min. The slides were examined under the light microscope using ×100 (oil immersion) objective lens for level of parasitaemia, growth stages and viability. The culture flask was re-incubatored after gassing and adding appropriate volume of CPM.

The number of infected RBCs and total number of RBCs in a field were counted and recorded. This was done for three fields making up to at least a total of 300 RBCs. The percentage parasitaemia in each field was calculated using the formula:

$$\%\ Parasitaemia\ = \frac{Number\ of\ infected\ RBCs}{Total\ RBCs\ counted} \times 100$$

Parasitaemia levels above 4% were harvested and used for assays or diluted with appropriate volume of packed RBCs to 1% parasitaemia. The parasites were maintained in continuous culture to obtain a stable parasitaemia before they were used for the efficacy assay.

3.3.2 In vitro Drug Sensitivity Assay

The *in vitro* antimalarial activity of cryptolepine and in combination with standard antimalarial agents against *P. falciparum* (3D7, Chloroquine sensitive) was investigated using the SYBR Green I-based fluorescence assay. Cultures were synchronized by two rounds of sorbitol (5%) treatment to obtain a highly synchronous ring stage parasites used in each assay.

Stock drug solutions were prepared in 70% ethanol except for chloroquine which was dissolved in distilled water. Cryptolepine (CPE), mefloquine (MQ), chloroquine (CQ), artesunate (ART), artemether (METH) and dihydroartemisinin (DHA) stocks were prepared at 1 mM. Amodiaquine (AMQ) and lumefantrine (LUM) stocks were at 0.5 mM. Concentrations ranging from 32.5 to 2080 nM for CPE, 2 to 640 nM for LUM, 3.2 to 83 nM for CQ, 5 to 800 nM for MFQ and 1 to 128 nM for AMQ were used for the interaction assays. Cryptolepine plus a second test drug (Drug B) solutions were prepared in assay medium at ratios of 5:0, 4:1, 3:2, 2:3, 1:4 and 0:5 followed by

2fold serial dilutions in assay medium of each ratio, allowing the IC_{50} to fall approximately at the mid-point of the serial dilution of each drug alone (Fivelman *et al.,* 2004). For each combination assay, 90 µL of parasite culture were seeded into each culture well, and 10 µL of diluted drugs in complete medium solutions were dispensed into each well to obtain seven desired final concentrations. The haematocrit of cultures were adjusted to 2% by dilution with complete parasite medium and parasitaemia was stepped down to 1% with washed uninfected RBCs. The plates were arranged in a clean modular incubation chamber (Billups-Rothenberg Inc, USA) and flushed with mixed gas for 6 min. The chamber with the assay plates was placed in an incubator for 48 h and set at 37ºC. After the 48-h incubation, the plates were wrapped in aluminium foil and stored at -30ºC overnight.

The plates were thawed and mixed with 100 µl of SYBR® Green lysis buffer (0.008% w/v Saponin, 0.08% w/v Triton X-100, 5 mM EDTA and 20 mM Tris base). The plates were incubated in the dark at room temperature for about 1 h and fluorescence data were acquired using fluorescence multi-well plate reader (Tecan Infinite M200 Pro) with excitation and emission wavelength at 485 and 535 nm, respectively. The experiment was done in triplicate.

Table 3.2: Combination ratio of cryptolepine to standard antimalarial drugs (Drug B).

Combination solution	**Ratio of Cryptolepine t B**		**Volume (µL)**	
	Cryptolepine	Drug B	Cryptolepine	Drug B
1	5	0	10	0
2	4	1	8	2
3	3	2	6	4

4	2	3	4	6
5	1	4	2	8
6	0	5	0	10

Drug B refers to any of the standard antimalarial agents used in this assay (Artesunate, Artemether, Amodiaquine, Mefloquine, Dihydroartemisinin, lumefantrine and Chloroquine).

3.4 IN VIVO ANTIMALARIAL ASSAY

3.4.1 Animals

Mice infected with chloroquine-sensitive strain of *Plasmodium berghei* NK-65 were obtained from the Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Accra, Ghana and kept in the animal house of the Department of Pharmacology, KNUST, Ghana. The parasites were kept alive by continuous intraperitoneal passage in mice every 4 days (Adzu and Haruna, 2007). These infected mice were used for the study. Before the study began, one of the infected mice was kept and observed to produce disease symptoms similar to human infection (English, 1996). The use of animals were in agreement with the National Institute of Health Guidelines for Care and Use of laboratory animals (1985) and were approved by the Ethical Review Committee of the Faculty of Pharmacy and Pharmaceutical Sciences, KNUST, Ghana (PHARM/ETHIC/ET173/15).

3.4.2 Antimalarial assay of Cryptolepine hydrochloride.

The ED_{50} of cryptolepine was confirmed using the curative (Rane) test as described by Ryley and Peters (1970). In this test schizonticidal activity of cryptolepine was determined in established infection. On day 0 of this test, the percentage parasitaemia and red blood count of the donor mice were determined by using a Giemsa-stained thin blood smear of the donor mice and improved Neubaur Counting Chamber, respectively. Blood of the donor mice was collected by cardiac puncture and diluted with physiological saline to give a concentration of 1×10^6 parasitized erythrocytes per mL. The mice were injected intraperitoneally with 0.2 ml of standard inoculum of 1×10^6 *P. berghei* NK 65 infected erythrocytes on the first day (day 0). After 72 h, and following confirmation of parasitaemia, the mice were divided into six groups of five mice per group. These groups were treated with cryptolepine at doses of 3, 10, 30, and 100 mg/kg *p.o*. The positive control group was treated with Artmether+Lumefantrine (Coartem®) (4 mg/kg) and an equal volume of physiological saline was given to the negative control group. The treatment lasted for 6 days at a single dose per day after which blood smears were collected and examined microscopically to monitor the parasitaemia level.

3.4.3 In vivo antimalarial interaction assay.

The curative or Rane test was used to evaluate the antimalarial properties of the combination of cryptolepine (40 mg/kg) and artesunate (ED_{50}=6 mg/kg). The mice were injected intraperitoneally with standard inoculum of 1×10^6 *P. berghei* NK 65 infected erythrocytes on the first day (day 0). After 72 h, and following confirmation of parasitaemia, the mice were divided into seven groups of five mice per group. The first two groups were treated with cryptolepine (40 mg/kg) and artesunate (6 mg/kg), respectively. The doses selected were based on the ED_{50} of cryptolepine

obtained from previous experiment as well as the reported ED_{50} of artesunate against *P. berghei*-infected mice (Vivas *et al*., 2007). Four combinations of the two antimalarial agents of varying concentrations were used. Cryptolepine: Artesunate (40 mg/kg: 6 mg/kg), (20 mg/kg: 3 mg/kg), (10 mg/kg: 1.5 mg/kg), (5 mg/kg: 0.75 mg/kg). This was in combinations of 1:1, 1/2:1/2, 1/4:1/4, and 1/8:1/8 respectively, of a fixed dose ratio of 1:1. The negative control received equal amount of physiological saline daily for a period of six days just as the drug-treated groups.

On day 3 of inoculation, parasitaemia was confirmed and treatment with the drug combinations began. Thin blood smears of the test mice stained with 10% Giemsa solution were used to determine the percentage parasitaemia microscopically, by counting 4 fields of approximately 100 erythrocytes per field.

3.5 TRANSMISSION BLOCKING PROPERTIES OF CRYPTOLEPINE AND *CRYPTOLEPIS SANGUINOLENTA*.

3.5.1 In vitro cultivation of asexual stage Plasmodium falciparum

In vitro cultivation method of *P. falciparum* drug-sensitive strain NF54 was adapted from Reader *et al*. (2015). Parasite cultures were maintained in human erythrocytes (5% haematocrit) suspended in complete parasite medium (CPM) (RPMI 1640 medium supplemented with 25 mM HEPES, 0.2%

D-glucose, 24 µg/mL gentamicin, 0.2% sodium bicarbonate (pH=7.3), 200 µM hypoxanthine with 0.5% AlbuMAX® II and flushed with 90% N_2, 5% O_2, and 5% CO_2 in humidified modular chambers at 37°C . Parasite medium was changed daily and fresh CPM introduced. Parasitaemia of Giemsastained slides were monitored daily with light microscope.

3.5.2 Induction of Gametocytogenesis and maintenance of gametocyte cultures.

Combined conditions of low haematocrit and nutrient starvation were employed in the induction of gametocytogenesis. To trigger gametocytogenesis, asexual cultures of 6-10% parasitaemia were diluted to 0.5% parasitaemia at 6% haematocrit and then introduced into a glucose-free medium. The medium was kept at 37°C gassed with 90% N_2, 5% O_2, and 5% CO_2 without shaking. The medium was changed daily. After 72 h, the haematocrit was dropped to 3% (day 0). Gametocytogenesis was monitored daily before medium changed. Residual asexual parasites were separated on days 6-9 from the culture by continuous treatment with 50 mM N-acetyl glucosamine (NAG). The medium was then fortified with 0.2% glucose from day 10 onwards. The gametocyte levels were monitored daily by microscopy until they were predominantly stage V and were employed in the PrestoBlue™ assay.

3.5.3 Gametocytocidal activity assays

The *in vitro* gametocytocidal activity of cryptolepine and the aqueous root extract of *Cryptolepis sanguinolenta* against NF54 strain of malaria parasite were measured by assessing gametocyte survival after drug exposure using PrestoBlue™. The PrestoBlue™ assay was based on method described by Tanaka and colleagues (Tanaka *et al*., 2013). Three drug concentrations of CPE (1, 5 and 10 µM) and CPS (1, 5 and 10 mg/mL) were placed in triplicate in a transparent 96-well flat bottom plates. Parasitized RBCs are added to a final concentration of 5% haematocrit, 2% gametocytaemia in a total incubation volume of 100 µL and the plates incubated for 48 h. Dihydroartemisinin (10 µM) was used as a reference standard in the drug assay. The drug plate was placed on a mechanical shaker for 20 s before encased in an air-tight chamber and gassed for 5 min with a 5% O_2, 5% CO_2 and a balanced N_2 mixture. The plates were incubated at 37°C for 48 h after which 10 µL of PrestoBlue™ reagent was added to each well and shaken for 20 s.

The plate was left to incubate for 2 h and then centrifuged at 120×g for 1 min. The supernatant (70 µL) was transferred to a clean 96-well plate before reading in a multiwell spectrophotometer (Infinite F500, Tecan, USA) by fluorescence detection at 560 nm and 615 nm excitation and emission maxima, respectively. Percentage gametocyte survival in each well was calculated relative to control wells that received no drugs.

3.6 STATISTICAL ANALYSIS OF DATA

IN VITRO INTERACTION ASSAY

IC_{50} value (drug concentration that inhibits the parasite growth by 50%) was used as an indicator of antimalarial activity and was determined from log dose–response curves plotted using GraphPad Prism (GraphPad 6 Software, San Diego). The IC_{50} values were determined by analysis of dose–response curves.

Growth inhibition due to cryptolepine and the other antimalarial agents defined as the difference between the percentage parasitaemia of each treatment group and the corresponding control was calculated. IC_{50} values were used to calculate the fractional IC_{50s} (FIC_{50}s) for each drug ratio. ΣFIC_{50}s of cryptolepine with the standard antimalarials were calculated by the following equation and represented as isobolograms:

$$\Sigma \mathrm{FIC}_{50} = \left(\frac{IC_{50} \text{ of Cryptolepine in combination}}{IC_{50} \text{ of Cryptolepine alone}}\right) + \left(\frac{IC_{50} \text{ of Drug B in combination}}{IC_{50} \text{ of Drug B alone}}\right)$$

The FIC_{50} of cryptolepine was plotted against each of the antimalarial agent to obtain isobolograms for each of the four drug ratios, with concave curves indicating synergy, straight lines indicating addition and convex curves indicating antagonism (Fivelman *et al*., 2004). ΣFIC_{50} values indicate the nature of the interactions as follows: $\Sigma FIC_{50}<0.8$ is synergism; ΣFIC_{50} 0.8 to 1.4 is additive, $\Sigma FIC_{50}>1.4$ is antagonism. Mean ΣFIC_{50}s were used to classify the overall nature of the interaction (Snyder *et al*., 2007).

IN VIVO INTERACTION ASSAY

The antimalarial activity was determined by using the equation:

$$\%\ \text{Suppresion} = \left[\frac{Parasitaemia\ of\ negative\ control - Parasitaemia\ of\ test}{Parasitaemia\ of\ negative\ Control}\right] \times 100$$

The potency of cryptolepine and artesunate were estimated from their log-dose response curves. The negative control received physiological saline for the duration of treatment. The estimated potencies (ED_{50}'s) of cryptolepine and artesunate in both tests were also used to compute the theoretical potency (Zadd) as follows;

$$Zadd = f\ (ED_{50})\ of\ artesunate + (1\text{-}f)\ ED_{50}\ of\ cryptolepine$$

Where *f* is the fraction of each component in the mixture.

To obtain the combination potency of the co-administered drug candidates, the two agents were orally administered to mice three days post-intraperitoneal inoculation with 1×10^6 *P. berghei* NK 65 infected erythrocytes daily at doses of their respective ED_{50}'s and in fixed ratio combinations of fractions of their respective ED_{50} values of $^1/_2$, $^1/_4$, $^1/_8$. The treatment regime continued for 6 days. The experimental potency (Z_{exp}) of the co-administered test agents was determined by least

square method of regression. Data for toxicity studies were presented as Mean ± S.E.M. The presence of significance differences among means of groups was determined by one-way ANOVA using GraphPad Prism for Windows version 5.0 (GraphPad Software, San Diego, CA, USA). Significance difference between pairs of groups was calculated using the Newman-Keul's Multiple

Comparison Test.

GAMETOCYTE ASSAY

Microsoft Excel datasheet was used in the calculation of percentage inhibition in relation to the control results. Figures were made using GraphPad Prism for Windows version 6.0 (GraphPad

Software, San Diego, CA, USA).

3.7 RESULTS

3.7.1 In vitro drug interaction assay

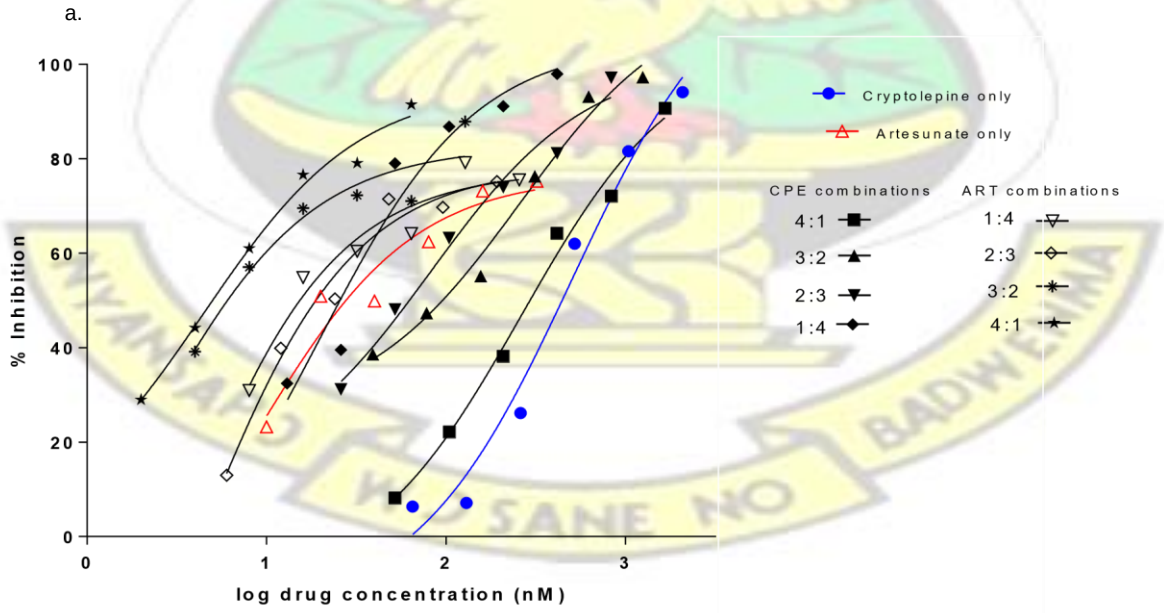
The stage specific IC_{50} of cryptolepine and standard antimalarials on *P. falciparum* blood-stages cultures are shown in Figure 3.1 and Table 3.3. Cryptolepine combination with the artemisinin derivatives showed FIC_{50} of less than 0.8 suggesting synergy. The degree of synergism was stronger

in AMQ (ΣFIC=0.235±0.15), followed by artemether (ΣFIC_{50}=0.362±0.07), DHA (ΣFIC_{50}=0.403±0.27) and finally ART (ΣFIC_{50}=0.693±0.13). Combination of cryptolepine with chloroquine and lumefantrine showed additivity (ΣFIC_{50}=1.342±0.34; 1.017±0.45) whereas antagonism (ΣFIC_{50}=4.182±0.68**)** was observed when cryptolepine was combined with mefloquine (Table 3.4). The isobolograms for the various interactions are shown in Figures 3.2 and 3.3.

Table 3.3: *In vitro* IC_{50} of Cryptolepine and standard antimalarial agents

Drug	Mean IC_{50} ± S.E.M (nM)
Cryptolepine HCl	603.82±75.57
Artesunate	6.76±1.63
Artemether	2.59±0.59
Chloroquine	11.05±1.79
Mefloquine	12.98±0.47
Dihydroartemisinin	6.02±0.17
Amodiaquine	12.63±1.66
Lumefantrine	10.80±0.97

In vitro IC_{50}s for *P. falciparum* strain 3D7. Values are means ± standard error of the means from more than 6 independent experiments.



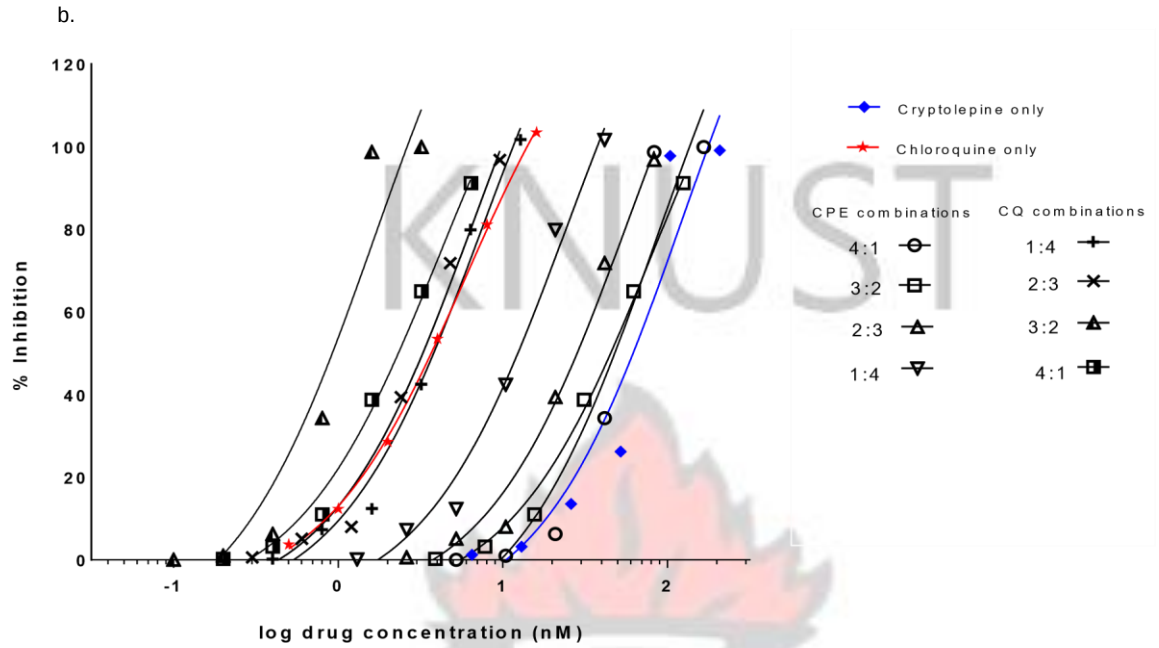


Figure 3.1: Dose-response curves produced by the fixed-ratio method of cryptolepine (CPE) combination with a) artesunate (ART) b) chloroquine (CQ)

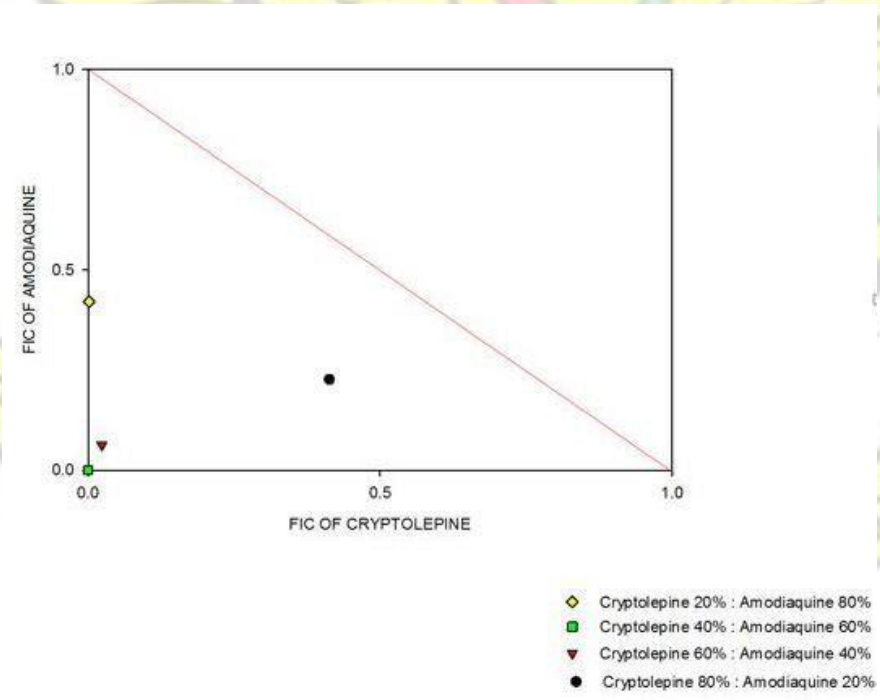
Table 3.4: *In vitro* interaction of drug combinations against 3D7 *P. falciparum* strains.

Drug combination	ΣFIC$_{50}$					
	4:1	3:2	2:3	1:4	Mean ΣFIC$_{50}$	Interaction
Cryptolepine + Chloroquine	1.95	1.775	1.66	0.475	**1.342±0.34**	Additivity
Cryptolepine + Artemether	0.4891	0.4727	0.3094	0.1765	**0.362±0.07**	Synergism
Cryptolepine + Artesunate	0.8658	0.9469	0.433	0.530	**0.693±0.13**	Synergism
Cryptolepine + Mefloquine	3.0316	3.013	5.106	5.595	**4.182±0.68**	Antagonism
Cryptolepine + Dihydroartemisinin	1.214	0.0906	0.1263	0.181	**0.403±0.27**	Synergism

Cryptolepine + Amodiaquine	0.640	0.0234	0.0631	0.421	**0.235±0.15**	Synergism
Cryptolepine + Lumefantrine	2.1108	1.6865	0.1475	0.1245	**1.017±0.45**	Additivity

The ratios 4:1, 3:2, 2:3 and 1:4 refer to fixed dosage ratios for cryptolepine to drug B. Values are the Means ±S.E.M from ≥3 experiments.

A



B

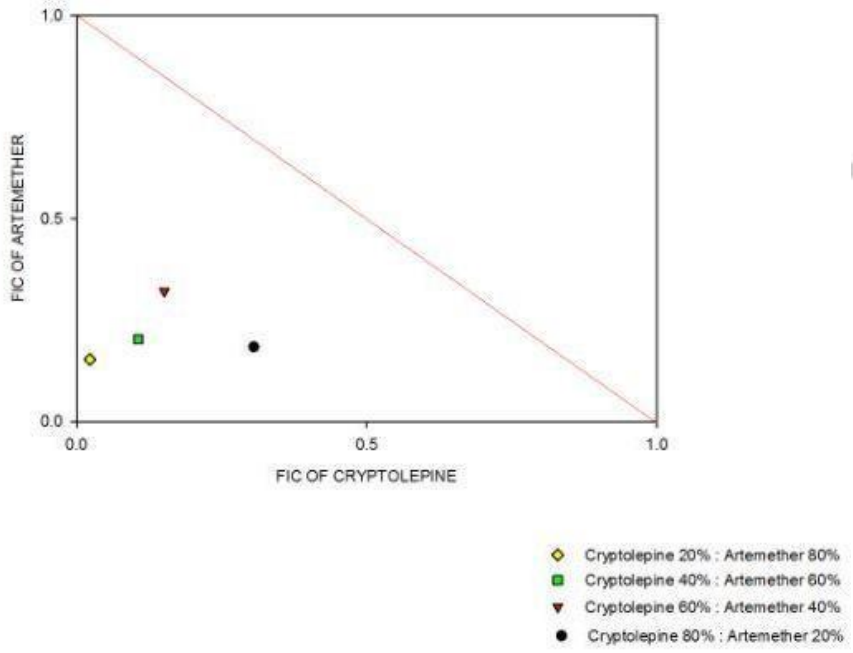
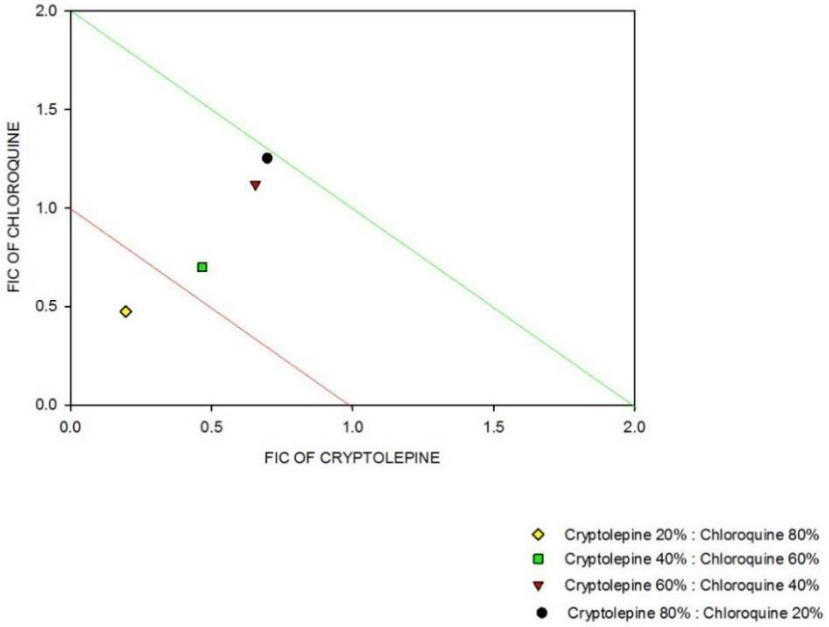


Figure 3.2: Effects of combinations of cryptolepine with standard antimalarial drugs on *P. falciparum* growth *in vitro* (3D7 strain). Isobolograms show the effect of combinations of both cryptolepine with amodiaquine (*A*) and cryptolepine with artemether (*B*). Values on both axis are FIC_{50}.

C



D

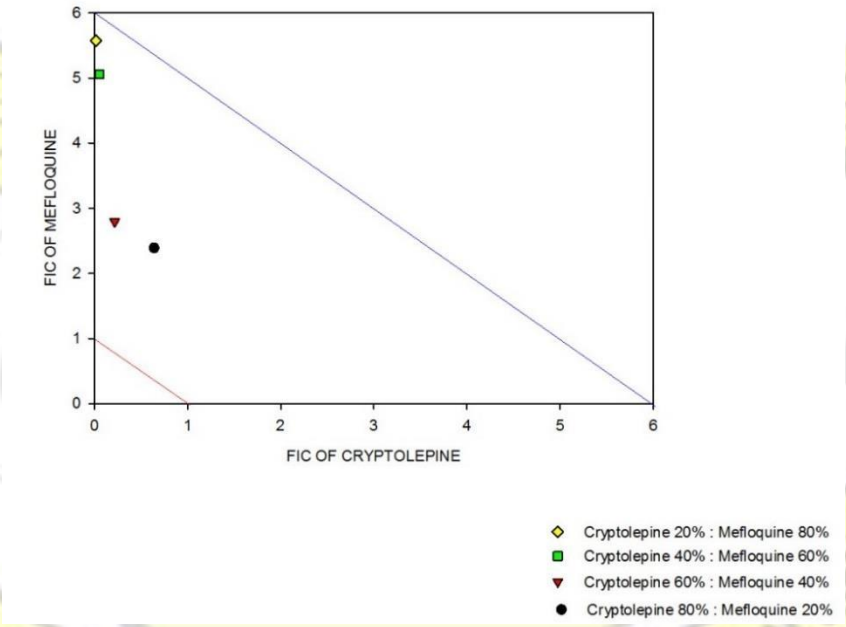


Figure 3.3: Effects of combinations of cryptolepine with standard antimalarial drugs on *P. falciparum* growth *in vitro* (3D7 strain). Isobolograms show the effect of combinations of both Cryptolepine with chloroquine (*C*) and Cryptolepine with mefloquine (*D*). Values on both axis are FIC_{50}.

3.7.2 In vivo antimalarial assay.

3.7.2.1 Determination of ED_{50} of cryptolepine in *P.berghei* infected mice.

Cryptolepine produced a dose dependent reduction in parasitaemia levels with similar reduction as in the Artemether+Lumefantrine-treated group (positive control). The ED_{50} of cryptolepine on the 5^{th} and 6^{th} days of treatment was calculated to be 10.65±0.6 mg/kg and 40 mg/kg, respectively (Figure 3.4).

Table 3.5: Curative effect of cryptolepine on *P.berghei* infected mice.

TREATMENT	% SUPPRESSION (DAY 5)	% SUPPRESSION (DAY 6)
STERILED DISTILLED WATER	-	-
CRYPTOLEPINE (mg/kg)		
3 mg/kg	4.59±0.64	35.47±3.21
10 mg/kg	53.56±0.62	73.47±1.34
30 mg/kg	90.40±1.0	95.38±0.63
100 mg/kg	95.30±0.49	96.04±0.75
Artemether+Lumefantrine	97.32±0.51	98.34±0.83

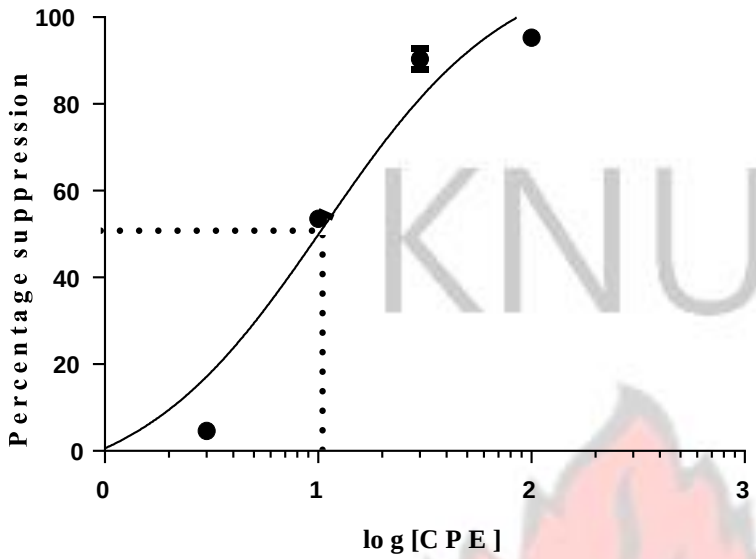


Figure 3.4: The ED_{50} (10.65±0.60 mg/kg) of cryptolepine in *Plasmodium berghei* infected mice on the fifth day of treatment.

3.7.2.2 Combination antimalarial assay of cryptolepine and artesunate

Table 3.6 shows the antimalarial activity, thus the mean percentage reduction in parasitaemia produced by the drugs alone or in combination, compared to the control, on days 1 to 6.

The combination of cryptolepine (ED_{50}=40 mg/kg) and artesunate (ED_{50}=6 mg/kg) produced a significant reduction in parasitaemia from days 1 and 6. The combination of cryptolepine and artesunate at all dose levels produced a high percentage suppression in the first three days compared to cryptolepine only. The lowest dose ratio combination (1/8:1/8) showed high parasite levels on days 5 and 6 compared to the negative control group.

Table 3.6: Percentage suppression of *P. berghei* infected mice given combination treatment

DRUGS	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6
Artesunate 6 mg/kg	40.74±10.4	89.87±2.2	97.92±1.3	90.20±2.1	87.40±6.9	90.44±2.5
Cryptolepine 40 mg/kg	53.70±13.1	57.81±4.0	65.3±7.5	77.48±15.2	82.16±5.4	83.19±6.4
ED_{50} (1:1)	73.15±9.8	95.78±9.8	93.06±1.39	68.03±12.6	78.35±9.0	88.53±4.4
ED_{50}/2 (1/2:1/2)	80.56±3.7	79.75±5.24	88.89±2.8	85.03±2.7	90.81±3.47	92.35±2.2
ED_{50}/4 (1/4:1/4)	87.96±3.1	96.84±1.1	76.39±6.1	85.03±6.8	82.94±3.5	84.70±2.2
ED_{50}/8 (1/8:1/8)	86.11±1.46	86.50±2.5	77.08±6.9	0.51±14.65	-23.0±12.4	-8.03±10.3

3.7.2.3 *In vivo* synergistic interaction of cryptolepine and artesunate (Isobologram analysis).

The theoretical ED_{50}s of cryptolepine and artesunate combination was 8.3±0.25 mg/kg. The experimental ED_{50} (Zexp) of the mixture was 1.02±0.02 mg/kg. The Zexp (open circle) lay significantly below the line of additivity as well as the Zadd (closed circles) on the isobologram indicating synergism (Figure 3.5). The degree of interaction calculated as the interaction index was

0.12 (Table 3.7).

Table 3.7: Theoretical (Zadd) and experimental (Zexp) ED_{50} ±S.E.M. values of cryptolepine and artesunate in antimalarial test.

Zadd (mg/kg)	Zexp (mg/kg)	Interaction index
8.3±0.25	1.02±0.02***	0.12

Values are presented as Mean±S.E.M. ***P<0.001compared to their respective Zadds.

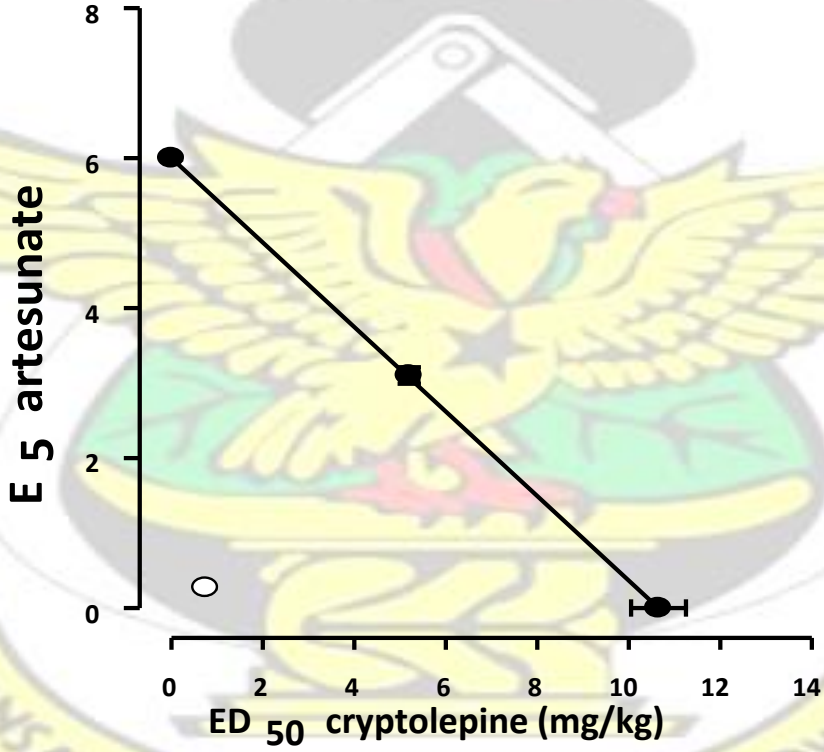


Figure 3.5: Isobologram for the combination of cryptolepine and artesunate in the Rane's antimalarial test. Filled circles (●) are the theoretical ED_{50}s ± S.E.M. and open circles (o), the experimental ED_{50}s ± S.E.M.

3.7.3 In vitro Gametocyte assay

Data from the late stage gametocyte assay, shows 20, 12 and 1 percentage inhibition for cryptolepine (CPE) at 10, 5 and 1 µM, respectively whereas 20, 10 and 0 percentage inhibition was obtained for the aqueous extract of *Cryptolepis sanguinolenta* at 10, 5 and 1 mg/mL, respectively. From the data it is clear that the assay did perform well as the reference standard (Dihydroartemisinin) gave an inhibition of 73% (Figure 3.6 and Table 3.8).

Taken together, *Cryptolepis sanguinolenta* (CPS) and its major alkaloid, cryptolepine (CPE) showed minimal to no inhibitory effect on the late stage gametocyte from *Plasmodium falciparum* strain NF54.

Table 3.8: *In vitro* gametocytocidal activity against *Plasmodium falciparum* NF54

Drug / concentrations	Blank Average (RFU)	Drug Average (RFU)	% Inhibition
Cryptolepine (CPE)			
10 µM	22640	34528	20±0.8993
5 µM	20979	34011	12±0.9045
1 µM	20806	35478	1±0.4423
Cryptolepis (CPS)			
10 mg/ml	26522	38434	20±0.7043
5 mg/ml	23631	36965	10±0.7229
1 mg/ml	21595	36512	0±0.4965
DHA 10 µM	17500	21490	73±1.9854

Percentage inhibitory values of CPE and CPS against *Plasmodium falciparum* strain NF54 expressed as means ± standard deviations of the results of at least three independent assays.

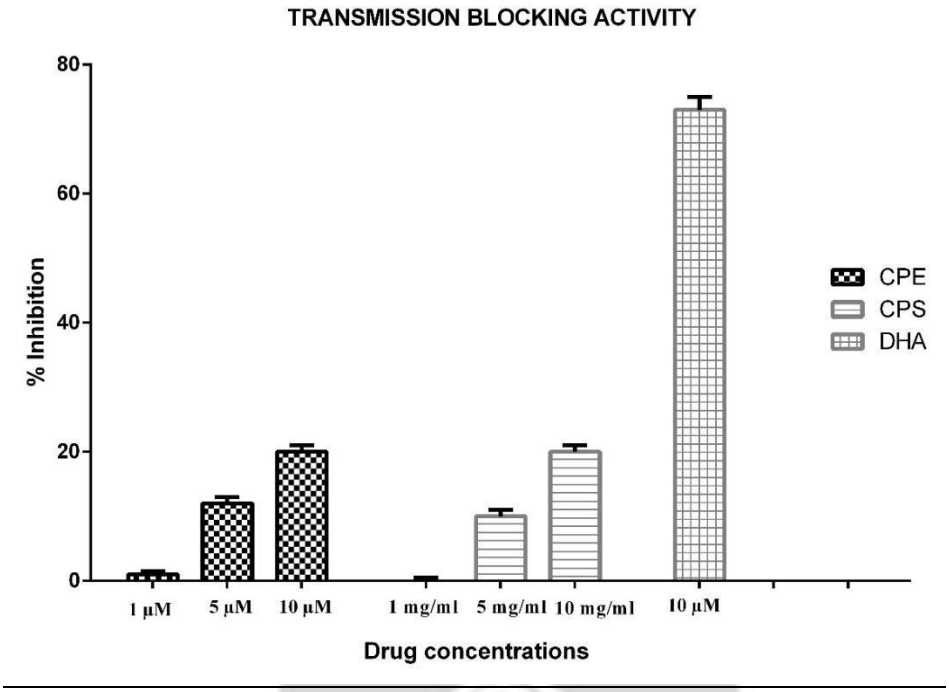


Figure 3.6: Transmission-blocking activity of CPS and CPE on late gametocyte development. Bars represent means (With standard deviation [SD]) of gametocyte stage V.

3.7.4 Haematological analysis of P. berghei infected mice treated with various drug combinations

The various treatments with cryptolepine and artesunate provided adequate information on the haematological status in the malaria condition in the mice. Figure 3.7 below show the haematological parameters after 9 days post infection and 6 days of treatment.

The white blood cell, red blood cell and haemoglobin were not significantly different from the parasite control treated group. However, a high haematocrit levels were observed with cryptolepine (40 mg/kg) compared to the parasite control group whereas no significant differences were observed with the other combinations. A significant increase ($p>0.001$) in platelet counts by all cryptolepine- treated groups compared to the vector control was observed in *P.berghei* infected mice. Lymphocyte levels were generally lowered in all groups compared to the parasite control.

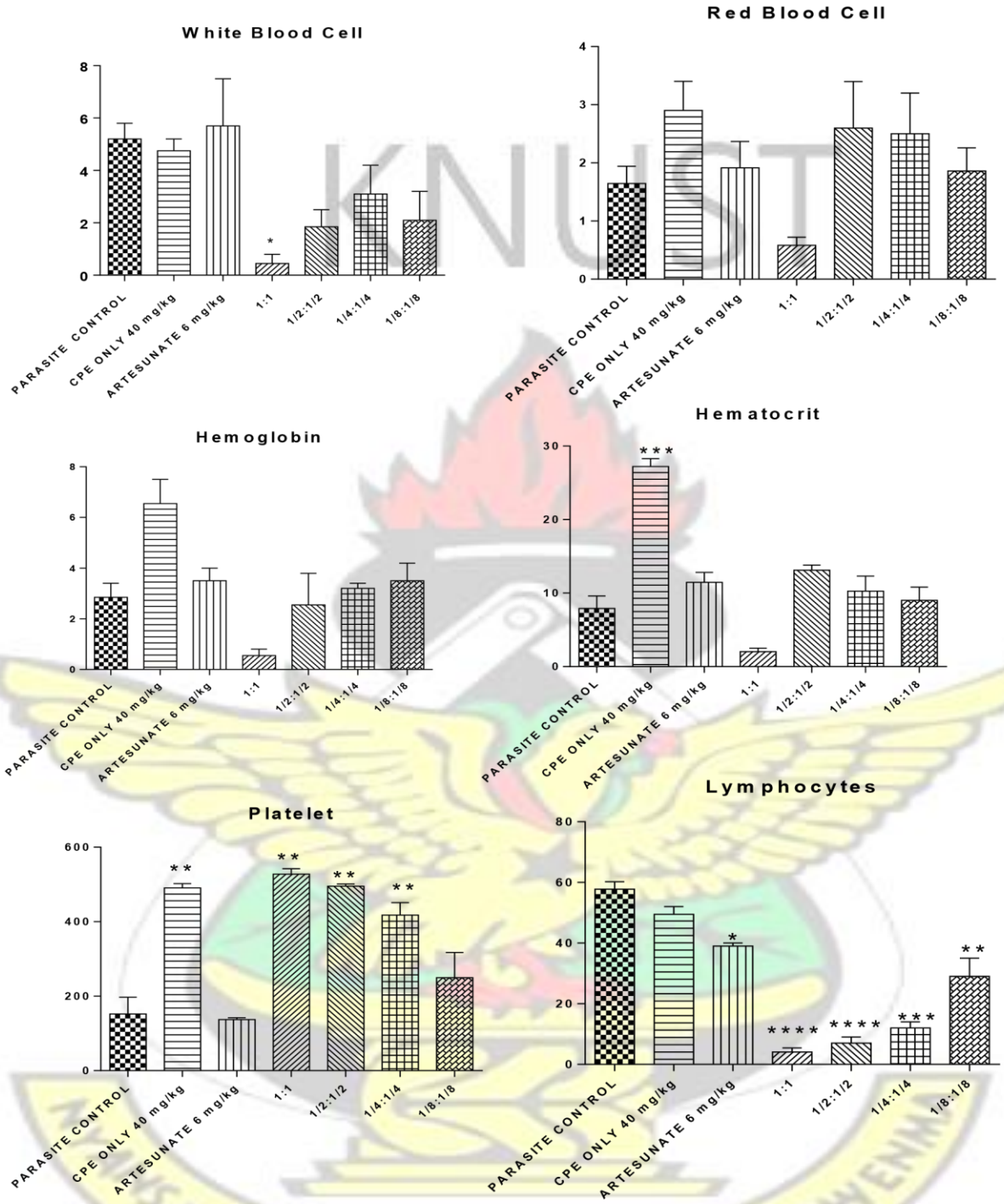


Figure 3.7: Effect of Cryptolepine and Artesunate on the haematological indices of *P. berghei* infected ICR mice treated for 6 days.

Values are expressed as means ± SEM, (*) indicates significance (P< 0.05), (**) indicates significance (P<0.01) (***) indicates significance (P<0.001), (****) indicates significance (P< 0.0001), compared to the parasite control by the Newman-Keuls.

3.7.5 Histopathology

The livers and stomachs from both the drug-treated and the distilled water-treated (control) group had normal appearance and histology. Generally, there were no observable changes in the architecture of these organs of treated animals compared to the control (Figure 3.8 and 3.9).

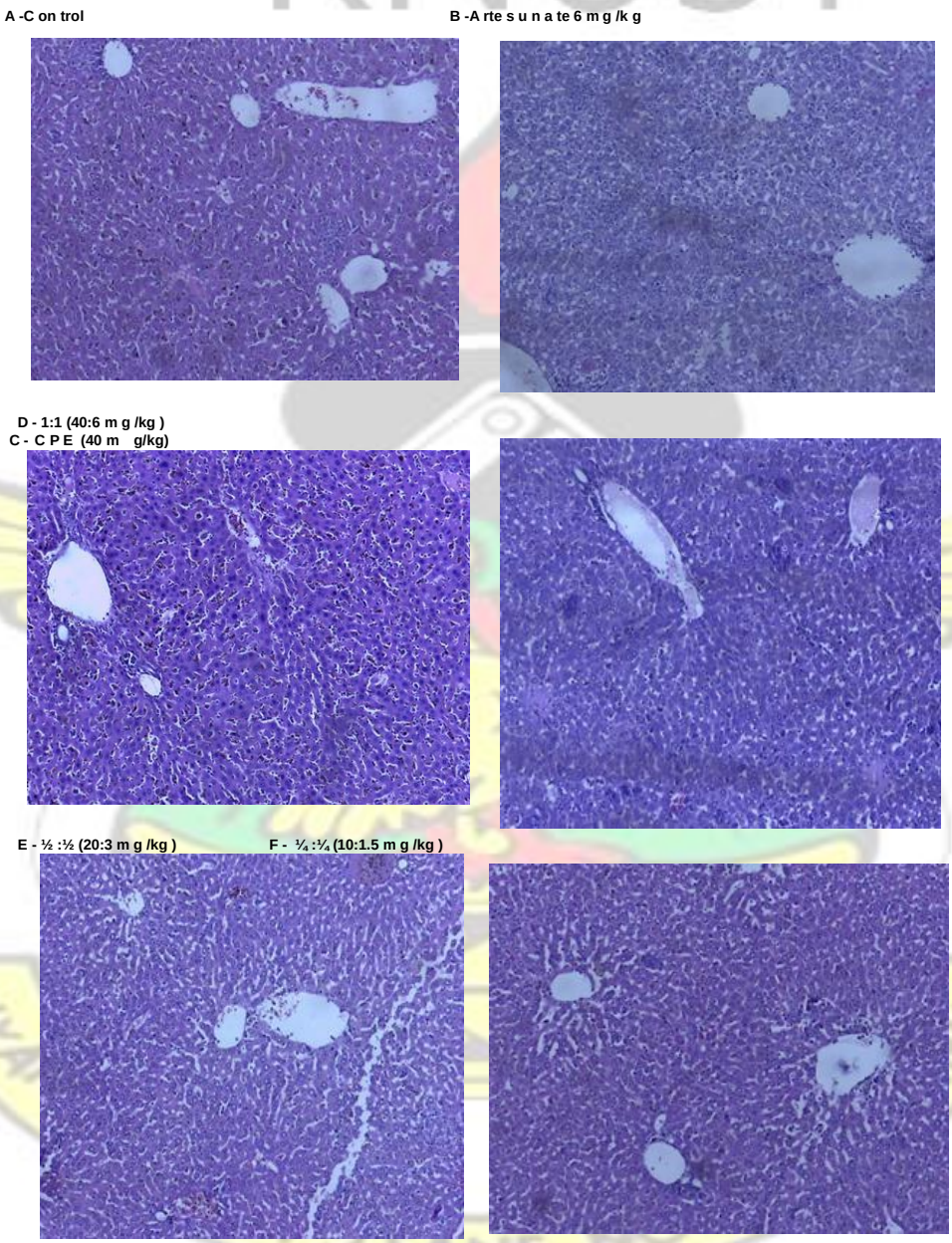


Figure 3.8: Photomicrograph (x100) showing histopathological slides of the livers of cryptolepine (CPE) and artesunate (ART, 4 mg/kg) treated *P. berghei* infected mice.

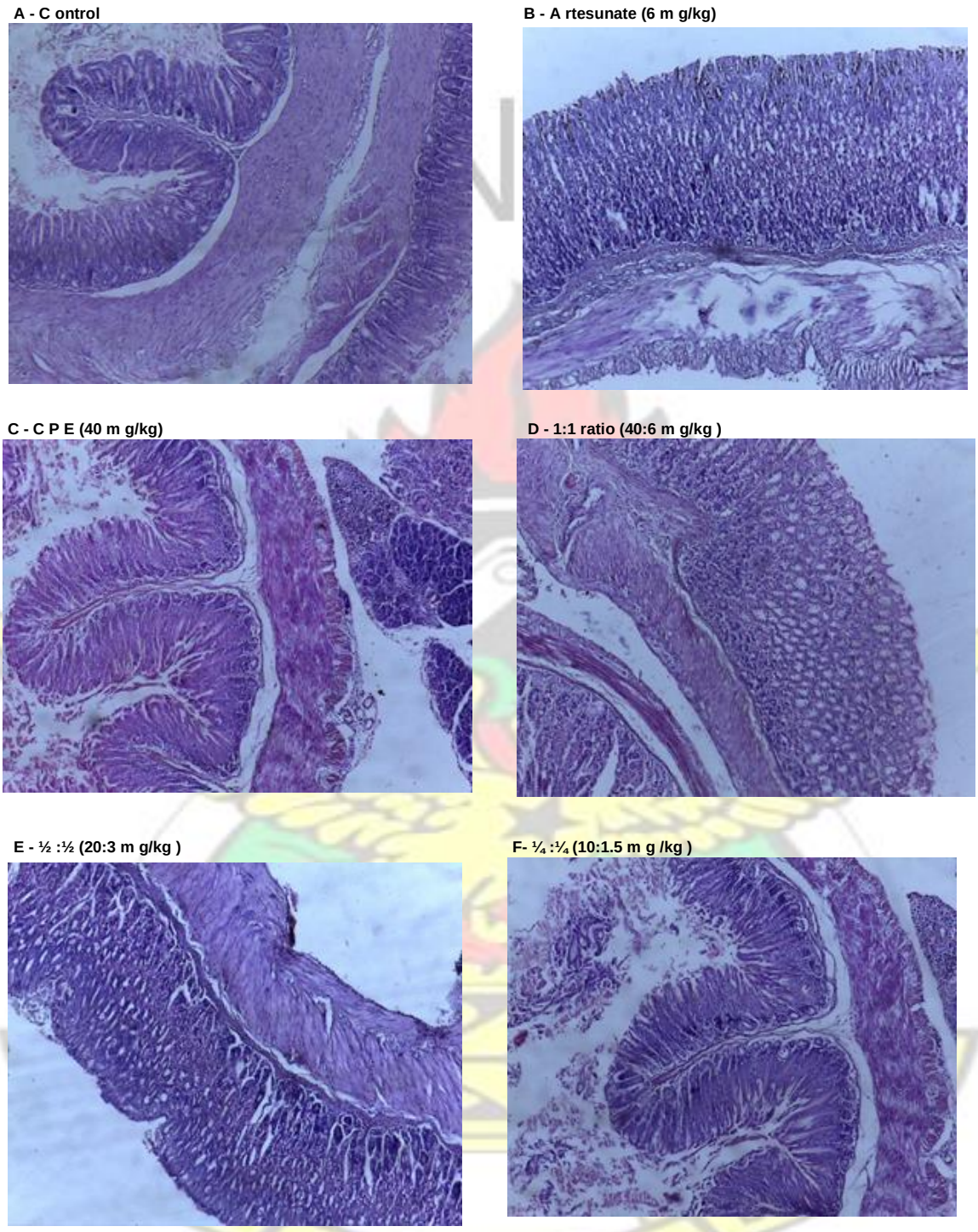


Figure 3.9: Photomicrograph (×100) showing histopathological slides of the stomachs of cryptolepine (CPE) and artesunate (ART, 4 mg/kg) treated *P. berghei* infected mice.

3.8 DISCUSSION

There are several reports on the antimalarial properties of cryptolepine and its hydrochloride salt both *in vivo* and *in vitro* (Cimanga *et al*., 1997; Grellier *et al*., 1996; Kirby *et al*., 1995). In this study, the interactions observed when this indoloquinoline is combined with some standard antimalarial drugs were demonstrated both *in vitro* and *in vivo*.

The results in this chapter confirms the therapeutic potential of cryptolepine *in vitro* in *Plasmodium falciparum* and *in vivo* in *P. berghei*. The combination of cryptolepine with the standard drugs in the present study was motivated by the recent recommendation by WHO for the use of Artemisinin Combination Therapy (ACT) to prevent the development of resistance to the artemisinins. In the *in vitro* assay, the susceptibilities of CPE and the standard drugs were close to those reported in literature (Akoachere *et al*., 2005; Lavrado *et al*., 2008).

The *in vitro* interactions of the various combinations are shown in the isobologram analysis in Figures 3.2 and 3.3. The straight line with slope and y-intercept to -1 and 1 respectively is indicative of an additive effect whatever the dose ratio. CPE combination with ART, ARM, and DHA had the mean ΣFIC always below the line of additivity. The values obtained with these three artemisinins indicates a synergistic interaction of the artemisinins with CPE. The mechanism of action of the indoloquinolones has been shown to be similar to that of chloroquine in inhibiting the conversion of poisonous haem to haemozoin (β-haematin) in the parasite food vacuole (Kumar *et al.*, 2007). The artemisinins are believed to act via the interaction of their endoperoxide group with haem in *P. falciparum* digestive vacuoles, resulting in the formation of free radicals that alkylate parasite proteins (Meshnick *et al*., 1990). The varying mechanism of actions of the two agents could contribute to the synergy observed, however further detailed mechanistic studies will shed more light on the observed effect.

In the *in vivo* assay, cryptolepine showed comparable efficacy to Artemether + Lumefantrine in the curative assay. All the combination treatment yielded a significant reduction in parasitaemia compared to the use of only cryptolepine or artesunate on the first day of treatment. This translated into a synergistic effect when the two agents were used together. The rapid onset of antimalarial activity continued through to the first three days of the combination treatment at all dose ratios. This indicates a possible rapid onset of antiplasmodial activity when cryptolepine is used in combination with artesunate compared to each of the drugs used alone. With the current three day antimalarial treatment, a combination of cryptolepine with artesunate may offer better choice for rapid clearance of parasites in the blood compared to any of the two agents used alone. Again, the long duration of action of this combination may ensure efficient parasite clearance.

Cryptolepine showed varied interaction with the 4-aminoquinolines, amodiaquine and chloroquine *in vitro*. The combination of cryptolepine with amodiaquine had a synergistic effect *in vitro* (mean ΣFIC=0.235±0.15) whereas an additive effect (mean ΣFIC=1.342±0.34) was seen with chloroquine. The 4-aminoquinolines are considered to share, in principle, the same mode of action. However, a different interactive profile was found with cryptolepine. It is therefore wrong to equate amodiaquine to chloroquine in terms of activity. The synergy observed with amodiaquine may be due to the Mannich base structure in amodiaquine. A similar synergistic effect attributed to the Mannich base side chain in pyronaridine and amodiaquine has been reported with artemisinin (Pradines *et al.*,

1998). An antagonistic effect was observed when cryptolepine was combined with mefloquine.

Mefloquine has been shown to inhibit the uptake of chloroquine as well as chloroquine's ability to cause the accumulation of undigested haemoglobin (Famin and Ginsburg, 2002). Mefloquine (and possibly quinine) has also been hypothesized to inhibit endocytosis of erythrocyte cytosol by the parasite, resulting in lowered free haem concentrations, to which chloroquine binds, in the

digestive vacuole (Famin and Ginsburg, 2002). Cryptolepine, an indoloquinoline has been shown to act similarly to chloroquine by inhibiting the biomineralization of the toxic waste material haem into an insoluble complex, haemozin leading to membrane damage and parasite death (Foley and Tilley, 1998; Kumar *et al*., 2007). On this background it is not surprising that antagonism was observed when cryptolepine was combined with mefloquine, possibly following similar pathway of antagonism demonstrated by mefloquine when combined with chloroquine. In the case of cryptolepine-lumefantrine combination at therapeutically relevant concentration ratios, an additivity effect (mean ΣFIC=1.017±0.45) was observed.

The combination of cryptolepine with amodiaquine showed a synergistic antimalarial effect *in vitro* in *P. falciparum* strain 3D7 and this combination provides a dual action with both agents inhibiting haemoglobin digestion in the asexual blood stages and amodiaquine inhibiting gametocyte maturation/gamete exflagellation by different mechanism (Delves *et al*., 2012). Such combinations with dual action are relevant in malaria endemic regions, where infections are usually asymptomatic with clinical symptoms developing late in the course of the disease, permitting the maturation of gametocytes and hence disease transmission (Greenwood, 1987; Stepniewska *et al*., 2008). The enhanced antimalarial activity of cryptolepine with the artemisinins as well as amodiaquine may possibly result in a low-dose treatment regimens and hence reduce toxicity.

The *in vitro* gametocytocidal activity of *Cryptolepis sanguinolenta* (CPS) and its major alkaloid cryptolepine (CPE) was tested against the NF54 strain of the malaria parasite, *Plasmodium falciparum.* The assay assessed gametocyte survival after drug exposure using PrestoBlue™. From the study, both CPE and CPS show very little inhibition on the late stage gametocyte of *P. falciparum* strain NF54. Given the demonstrated low gametocytocidal properties of CPS and CPE, their prominent antimalarial activity may be attributed mainly to their effect on the asexual stages

of the *Plasmodium* parasite. Cryptolepine, like chloroquine inhibits haemozoin formation in the parasite and the absence of this metabolic process in the stage IV and V gametocyte may account for the minimal activity shown (Canning and Sinden, 1975; Sinden *et al*., 1978).

Malaria infection in man and experimental animals has been shown to suppress the immune response to a variety of antigens (McGregor and Barr 1962). The haematological parameters showed no significant difference in the total white blood cell count in all treated groups compared to the parasite control except the group treated with the combination of the ED_{50} of cryptolepine and artesunate (1:1). The lymphocyte levels were significantly decreased in all combination treatments. A reduced lymphocyte count may indicate a compromised immune system associated with malaria infection (Jaeger and Hedegaard, 2002). Thrombocytopenia is frequently associated with acute malaria. In the clinical trial using *Cryptolepis sanguinolenta*, a progressive increase in platelet count after treatment was observed in the human subjects used. This was concluded as indicative of the effectiveness of the test drug against falciparum malaria (Bugyei *et al*., 2010). In the current study, the platelet count in all cryptolepine-treated groups were significantly increased ($p<0.001$) compared to the parasite control. This may be a compensatory attempt to boost or modulate the immune suppression (Reid and Lomas-Francis, 2004). Platelets assist and modulate inflammatory reactions and immune responses. The lowest dose ratio of cryptolepine with artesunate did not show any significant change in the platelet level compared to the vector control. These findings do not suggest acute toxicity of cryptolepine in the rodent models. Despite these reports of safety in the acute toxicity studies in rodents, CPE has been reported to be a DNA intercalator and also possess genotoxic properties in mammalian cells (Ansah *et al.*, 2005). While this study did not focus on these chronic toxic effects, it cannot be ruled out as possible toxic effects in the combination therapy. The administration of

Cryptolepis sanguinolenta has been shown to cause significant increase in the sizes and number of gastric parietal cells (Ajayi *et al.,* 2012). In this study, the histology of the stomach of mice treated with cryptolepine and the various combinations were examined. No observable defects were seen in the architecture of the stomach of the mice. The histology of the liver showed no deformity in the drug-treated compared to the control group. This, coupled with no reported death in the 9-day treated shows the safety of the combination of cryptolepine with artesunate in the acute toxicity studies in mice.

3.9 CONCLUSION

The results of this study confirms the potent *in vitro* and *in vivo* antimalarial properties of cryptolepine. The *in vitro* antimalarial interaction of cryptolepine and the artemisinin derivatives (artesunate, artemether and dihydroartemisinin) showed synergy in the chloroquine-sensitive *Plasmodium falciparum* strain 3D7. Additionally, amodiaquine also exhibited a synergistic action with cryptolepine *in vitro*. At therapeutically relevant concentrations, cryptolepine's combination with chloroquine and lumefantrine indicates additivity *in vitro* whereas antagonism was observed with mefloquine. *In vivo,* the combination of cryptolepine with artesunate showed a rapid onset of parasite clearance and sustained antimalarial effect. On the final day of treatment, the combined effect of cryptolepine (20 mg/kg) and artesunate (3 mg/kg) was better than any of the two drugs used independently in the curative (Rane) test translating into a synergistic interaction *in vivo*. It was interesting to observe that the synergistic interaction also existed *in vivo* in the rodent model. *Cryptolepis sanguinolenta* and it major alkaloid, cryptolepine demonstrated little transmissionblocking activity against the late-stage gametocyte of *P. falciparum* NF54. The acute toxicity evaluation of cryptolepine and in combination with the artemisinin derivative, artesunate in mice, revealed a non-toxic effect in the histopathology and on the haematological parameters.

4.0 CHAPTER FOUR

IN VITRO PHARMACOKINETIC STUDIES ON CRYPTOLEPINE

4.1 INTRODUCTION

In Drug Metabolism and Pharmacokinetics (DMPK), a new chemical entity with drug-like properties such as adequate absorption and distribution, low metabolism, complete elimination from the body and low toxicological risk is preferred (Zhang *et al.*, 2012). *In vitro* assays play a

leading role in screening these new compounds in the drug discovery stage and offers many unique advantages including (a) a simple, convenient and fast way to test the potency and drug-like properties of chemical entities (b) limited amount of test compounds are used and hence eliminating wasting of animals used in preclinical stages (c) and human-based *in vitro* assays offers a more precise estimation of human clinical outcomes compared to animal tests at the preclinical stage (Zhang *et al.*, 2012).

Several *in vitro* models exist for ascertaining absorption (apparent permeability), distribution (protein binding, blood-to-plasma partitioning), metabolism (metabolic stability, reaction phenotyping), drug-drug interaction potential (inhibition and induction of cytochrome P450), hERG inhibition, cell proliferation and cytotoxicity among others (Zhang *et al.*, 2012).

In vitro metabolism studies are performed to provide important data about the safety of a drug during the early development phases of a new drug candidate. The use of *in vitro* drug metabolism data in the understanding of *in vivo* pharmacokinetic data has recently become an area of scientific interest (Houston, 1994; Houston and Carlile, 1997).

The knowledge of the enzymes involved in the metabolism of a drug, and the effect of the drug on enzymes that metabolise other drugs, enables us to make predictions about possible interactions with other concomitant medications. Several investigators have recently described methods where preclinical drug metabolism and pharmacokinetic data can be used to predict human pharmacokinetic parameters (Obach *et al*., 1997; Lave *et al*., 1997a, b). This has directed the trend in the pharmaceutical industry to use *in vitro* drug metabolism data, using human-derived reagents, as a criterion to select compounds for further development (Rodrigues, 1997).

In addition, the comparative metabolite profile in different species in respect to man helps to determine the most suitable species for further toxicological studies, and also to provide valuable foresight on metabolic pathways in humans. To this purpose, several *in vitro* test systems from

different species and different liver fractions such as liver microsomes, S9, cytosol and hepatocytes, were used in the drug metabolism assessments.

In spite of the numerous biological activities of cryptolepine, little is known about the absorption, distribution and metabolism in humans and other mammals. The artemisinins have well-documented pharmacokinetic profiles in both preclinical and clinical studies. In the attempt to combine the artemisinins with cryptolepine, the *in vitro* absorption, distribution and metabolism of cryptolepine needed to be ascertained.

Since there are no reports on the direct comparison of metabolic stability of cryptolepine in humans, the present study was planned to generate the hitherto unreported comparison data of the interspecies variations. The absorption, plasma protein binding, plasma stability, metabolic profile, metabolite identification, and the potential species differences in the *in vitro* metabolism of cryptolepine were characterized in different biological systems. One of the objectives of this study was also to determine the extent of metabolic biotransformation of cryptolepine by hepatic metabolism enzymes. Therefore, one can decide whether it is worth modifying the chemical structure of this compound to improve its metabolism and pharmacokinetic properties.

4.2 DRUGS AND CHEMICALS

Table 4.1: Drugs and chemicals used for the *in vitro* assays.

DRUG / CHEMICAL	SOURCE
Methanol, chloroform, dichloromethane, hydrochloric acid, Ammonia solution.	BDH, Poole, England

Cryptolepine hydrate, zoniporide, carbazeran, diclofenac, NADPH, DMSO, formalin, Sodium chloride, hydralazine, raloxifene, formic acid, acetonitrile, diazepam, warfarin.	Sigma (St. Louis, MO, U.S.A)
Dulbecco's Phosphate Buffer Saline (DPBS)	Invitrogen Gibco (Paisley, UK)
Assay plates: HTS Multiwell Insert System, 24-well plates (1.0 μm-pore size, 0.3 cm^2-filter area).	Becton Dickinson, Falcon #351181
Human liver S9, Rat liver S9, Human liver cytosol and Rat liver cytosol	BD Gentest (Woburn, MA)
Culture medium: DMEM with 4.5 g/l glucose, GlutaMAX, pyruvate supplemented with 10% FBS and 1% Pen/Strep.	GIBCO BRL #61965-026
HPLC water	Millipore device
Black flat-bottom polypropylene 384 well 2 ml 96-deep well microplates	Sigma (Buchs, Switzerland)

96-well microtiter filter plates (Millipore), filter (isopore, polycarbonate) specifications: 0.4 µM pore size.	Nunc (Roskilde, Denmark)
FBS: Foetal bovine serum, Australia	GIBCO BRL #10099-141
Pen/Strep Penicillin/streptomycin	GIBCO BRL #15140-122
Trypsin: 2.5% solution (used diluted 1:10 in PBS)	GIBCO BRL #15090-046
PBS: Phosphate buffered saline with Ca^{2+} and Mg^{2+}	GIBCO BRL #14190-094
HBSS: Hank's balanced salt solution with Ca^{2+} and Mg^{2+}	GIBCO BRL #14025-050
BSA: Bovine serum albumin	SIGMA #A-7888
DMSO	HYBRI-MAX (SIGMA #D2650)
Mixed gender pooled Sprague-Dawley rat K3 EDTA plasma, mixed gender pooled Human K3 EDTA plasma	Sera Laboratories Int., ltd, Bolney, West Sussex, UK.
Rapid equilibrium dialysis (RED) device	Pierce Biotechnology, Thermo Fischer Scientific Waltham, MA.
MDR1-MDCK II cells	Prof. Anton Berns, Director of Research, The Netherlands Cancer Institute.
Culture plates: Tissue Culture Plate	Becton Dickinson, Falcon #353047

4.3 METHODOLOGY

4.3.1 Permeability Assays of cryptolepine

4.3.1.1 MDR1-MDCK II Assay (Cryptolepine assay in MDR1-MDCK cells)

DAY 1: Resuspension and Seeding of the cells.

The assay plates were first primed with culture medium before seeding the cells. A 500 µL /apicalwell and 25 mL/feeder-plate of culture medium were incubated for 1 h at 37°C and 5% CO_2. The MDR1-MDCK cells (Evers *et al*., 2000, Bakos *et al*., 1998) were routinely maintained on 10 cmculture plates. The culture medium was removed and the cell layer was rinsed once with 4 mL PBS to re-suspend the cells. Trypsin (1.5 mL 0.25%) in Phosphate buffer saline was added and incubated at 37°C and 5% CO_2 until the cells were completely detached from the culture plates (15-30 min). Culture medium (8.5 mL) was added to neutralize the trypsin and the cells gently resuspended and centrifuged for 3 min at 390 rcf in a Falcon tube at room temperature. The cells were counted with Neubauer cell counter and diluted to $2x10^5$ cells/mL in the culture medium for seeding. The culture medium used in the pre-incubation was removed from the wells and replaced with 400 µL /apical-well of the cell suspension. Cells were incubated for 3 days at 37°C and 5% CO_2 before starting the transport assay.

Day 4: Cryptolepine transport assay

The assay plates with the 3-day old MDCK cultures were removed from the incubator, the culture medium in the apical wells and in the feeder plates discharged and replaced with 500 µL / apical well and 25 mL/ feeder plate and incubated for 30 min at 37°C and 5 % CO_2. Dosing solution of cryptolepine was prepared by diluting the 10 mM stock in DMSO to a final concentration of 5 µM in HBSS containing 0.1% DMSO and 0.2% BSA. Three replicates each for apical to basolateral

(AP→BL) and basolateral to apical transport (BL→AP) were prepared.
HBSS was removed from the apical wells and the filter rack removed from the apical wells and from the feeder plate into the bottom part of a 24 well culture plate to start the transport assay. The donor compartment was filled either with 400 µL /apical well or 1.35 mL/basolateral well of the 5 µM cryptolepine solution in HBSS containing 0.1% DMSO and 0.2% BSA was added to the acceptor compartment. The transport assay was run for 2 h at 37°C and 5% CO_2. At the end of the incubation, one sample of the original 5 µM cryptolepine solution in HBSS containing 0.1% DMSO and 0.2% BSA and samples from the apical (350 µL) and basolateral wells (1 mL) were collected. All samples were stored at -20°C and delivered to the analytical laboratory for the determination of the cryptolepine concentration. An additional sample of the 10 mM stock solution in DMSO was used to establish the calibration curve.

Sample Analysis using LC-MS/MS

Extraction: Cryptolepine determinations were done using 100 µL samples from the two compartments. For the acceptor compartment, undiluted samples of cryptolepine were used and for the donor compartment 10 µL of cryptolepine diluted to 100 µL using HBSS with 0.2% BSA was used. A sample (10 µL) of the dosing solution was also included to determine the effective testing concentration and to calculate the precise recovery rate. After centrifugation for 4 min at 14000 rpm, the organic phase was transferred into another vial and the solvent evaporated under nitrogen steam at room temperature. The dried extract was then dissolved with 100 µL acetonitrile/water (50:50 v/v), centrifuged for 4 min at 14000 rpm, and 10 µL were injected into the LC-MS.

Instrumental conditions:

HPLC conditions:

Analytical column: Macherey-Nagel Nucleodur C18, 4.6x50 mm, 5 µM.

Temperature: 40°C, *HP1100 Binary pump system*: flow rate 1.0 ml/min

CTC PAL autosampler: injection volume 50 µL -cooled stack

Mobile phases: A: 50 mM ammonium acetate; *B:* methanol

Gradient program:

Time in min: %B

0.00	5
3.50	100
5.50	100
5.51	5
7.5	Stop run

MS/MS system: TSQ 7000

Ionization source and conditions: APCI ion mode. Vaporizer temperature: 550°C. Capillary temperature: 225°C. Corona discharge: 5.0 µA. Sheath gas 30

MS/MS mode: Centroid, MRM (MS/MS)

Masses test compounds and MS conditions:

Precursor ion m/z, product ion m/z, Scan time: 0.1 sec.Collision energy: ev Scan time: 0.1 sec. Collision energy: 28.8 eV

Apparent Permeability (Papp) Calculation

The permeability was expressed as apparent permeability (10^{-6} cm/sec) (Papp) and calculated with the formula below

$$P_{app=}10^{-6} \times \frac{Cf_{acceptor}}{\Delta t_{sec}} \times \frac{V_{acceptor}}{Co_{dosing}} \times \frac{1}{Area_{filter}}$$

$Cf_{acceptor}$ = final compound concentration in the acceptor compartment

Δt_{sec} = time of incubation in seconds (2 h = 7200 sec)

$V_{acceptor}$ =Volume of the test solution in the acceptor compartment (mL = cm^3)

Co $_{dosing}$ = compound concentration in the dosing solution (test concentration, 5 μM)

Area $_{filter}$ = area of the filter in square centimeters (0.3 cm^2)

The efflux ratio was estimated as the ratio of apparent permeability in basolateral to apical (B→A) to that of apical to basolateral (A→B).

The integrity and functionality of the MDCK cell layers were monitored using [^{3}H] digoxin. For this, after collecting the samples, the remaining solutions in the apical and basolateral compartments were removed completely.

4.3.1.2 Parallel Artificial Membrane Permeability (PAMPA) Assay

The parallel artificial membrane permeability assay (PAMPA) (Figure 4.1) measures a compound's ability to passively permeate across an artificial lipid membrane. This system is non-

cell based, rapid and useful for the identification of compounds whose structural properties are likely to limit their permeability. Microtiter filter plates (96-well) (Millipore), filter (isopore, polycarbonate) specifications with 0.4 µM pore size were used in this assay. Each well is impregnated with 5 µL of

15% hexadecane dissolved in hexane which served as the artificial membrane (Hexadecane Membrane-PAMPA). Acceptor (Teflon plate) and donor compartments are hydrated with 300uL of buffer (pH 4, pH 6.8 and pH 8) to mimic the pH of the gastrointestinal tract.

Cryptolepine was loaded to the donor plate at 5 µM. The incubation was done at room temperature under constant light shaking (70 rpm) for 4 h. The stability of the hexadecane membranes was tested by electrical resistance measurements. Calibrations and theoretical equilibrium concentrations were prepared independently. A Rapid Fire-LC/MSMS was used for the compound analysis. Apparent Permeability is calculated as

$$P_a = -\frac{V_D \quad V_R}{(V_D + V_R)At}.\ln(1 - r)$$

In absence of membrane retention, Pa = Pe, the effective permeability directly deduced from the PAMPA measurement.

r = amount of compound in the acceptor/ amount of compound at the theoretical equilibrium

V_R = volume in the acceptor compartment

V_D = volume in the donor compartment

A = accessible filter area (total filter area× porosity ratio)

t = incubation time

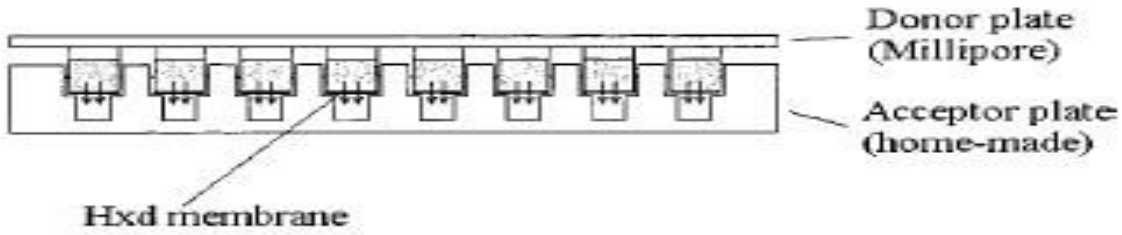


Figure 4.1: Schematic representation of a PAMPA assay (Faller, 2008).

$$FA_{calc} = \frac{100}{1 + \left(\frac{P_{C\,50}}{10^{P\tau}}\right)^{\gamma}}$$

P_{c50} = perm at 50%

γ_2= slope factor

R = 0.80

FA = Fraction absorbedF = 1646

High: FA>75% Permeability does not limit absorption.

Medium: 35 %< FA<75%

Low: FA<35% Permeability severely limits absorption

4.3.2 Distribution Assays

4.3.2.1 Plasma Protein binding of cryptolepine using the Pierce Rapid Equilibrium Dialysis (RED)

Pooled mixed gender plasma (100%, 2 mL) containing K3-EDTA anticoagulant from human and rat were thawed at 37°C, centrifuged for 15 min at 3500 rpm at 4°C. The plasma was kept on ice until needed. A 10 µL aliquot of cryptolepine stock solution (10 mM) was added to 190 µL of DMSO. Pre-warmed protein solution (1485 µL) was dispensed into a 96 deep-well plate. The pH of the dialysis buffer (100 mM KPO_4, pH 7.4) for the RED assay was checked and pre-warmed in an incubator (37°C) for 1 h. The assay was started by spiking 15 µL aliquots of the 500 µM cryptolepine solution to the rat and human plasma (final incubation concentration 5 µM, 1% DMSO).

The drug spiked plasma protein solution (300 µL) was added to the red ringed chamber in triplicate and 500 µL of the pre-warmed buffer into the buffer chamber. The plate was sealed with a gaz permeable seal and placed on the orbital shaker at 750 rpm inside the CO_2 incubator at 37°C and 5% CO_2 for 4 h. After the 4 h incubation, 50 µL aliquots were each sampled carefully from the buffer and the plasma chamber and dispensed into a 96-well plate. An equal volume of blank plasma or buffer was added to the required wells to create analytically identical sample matrices. The used inserts were discarded. To each sample 600 µL of ice cold acetonitrile containing internal standard (Diazepam 0.2 µM and Warfarin 1 µM) was added. These matrix-matched samples were stored at -20°C overnight prior to centrifugation at 3500 rpm for 15 min at 4 °C. The supernatants (50 µL) were transferred into a 384-well plate with 25 µL HPLC grade water, heat sealed, mixed and analysed with LC-MS/MS.

The percentage unbound was calculated as follows:

$$\%\ unbound = \left[\frac{Peak\ area\ of\ cryptolepine\ in\ Buffer\ fraction}{Peak\ area\ of\ 5\ \mu M\ cryptolepine}\right] \times 100$$

The percentage bound was estimated using the following equation:

$$\%\ \text{bound} = 100 - \%\ unbound$$

4.3.2.2 *In vitro* stability of cryptolepine in human and rat plasma

Cryptolepine, at a final assay concentration of 1 µM was incubated in commercially available rat plasma (mixed gender Sprague Dawley rat plasma, K3 EDTA) and human plasma (mixed gender human plasma, K3 EDTA) at 37°C with shaking (100 rpm). Serial samples of plasma incubates were taken over a pre-defined incubation time course (0, 5, 15, 30, 60, 120 min). The samples were quenched and precipitated by addition of ice cold acetonitrile containing internal standard (0.1 µM diazepam) followed by centrifugation. All assays were in triplicates.

The parent compound in the supernatants was then analysed by LC-MS/MS. Stability was determined semi-quantitatively from the peak area ratios (PAR) of analyte:internal standard which were used to determine the percentage stability at pre-defined time points in relation to zero minute time point. The percentage stability of a compound at time (t) was calculated as below:

$$\%\ Stability = \left[\frac{PAR\ at\ time\ t}{PAR\ at\ time\ t{=}0\ min}\right] \times 100$$

Percentage stability data was log transformed and plotted against time point(s) to define the initial phase of the slope also known as the initial rate of elimination, kel. The natural log of 2 (0.693) is then divided by the kel to calculate a plasma half-life (min).

$$Half - life\ (min) = \frac{0.693}{Kel}$$

4.3.3 In vitro metabolism of cryptolepine in the liver

4.3.3.1 Preliminary metabolic stability of cryptolepine in human and rat hepatocytes

Thawing and counting hepatocyte

Before the experiment, cryopreserved human and rat hepatocytes were thawed for 5 min at 37°C and transferred to a 50 mL falcon tube containing 48 mL of InVitroGro HI medium and centrifuged for 5 min at 50×g (25°C). The HI medium was decanted and gently resuspended in 2 ml Leibovitz's L15 medium (L-15). The resulting suspension (30 µL) was diluted in 470 µL of L-15 buffer. The hepatocyte number and viability in the suspension were determined using the NucleoCounter™. Cell preparation with viability greater than 80% were used.

Hepatocyte incubation (80 min)

Cryptolepine (2 µM in 50 µL incubation medium) was added to 50 µL of hepatocytes to start the metabolism assay. The 100 µL final reaction mixture was incubated on a DTS-4 shaker at 39°C at 1000 rpm. The reactions were terminated at 1, 11, 21, 41, 61 and 121 minutes by adding 150 µL of ice cold acetonitrile containing internal standards (Alprenolol 10mM and Warfarin 20mM). After the termination of the reaction, the contents were transferred into 96-well plates, cooled at -20°C and centrifuged at 5000×g at 4°C for 35 min. The supernatant (140 µL) was transferred into a 384-well plate, heat sealed and content analysed by LC-MS/MS according to the 5 min gradient LC method.

Midazolam (cytochrome P450), Ramipril (carboxylesterase), carbazeran (aldehyde oxidase), mycophenolate (UDP-glucuronyltransferase) and 7-hydroxy coumarin (sulfotransferase) were

used as specific substrates to demonstrate the presence of some hepatic enzymes in the hepatocyte fractions used.

The half-life ($t_{1/2}$) of metabolic stability was estimated using the first-order equation

$$t^{1/2} = \frac{0.693}{Kel}$$

where *K*el (elimination rate constant) is the slope of the linear portion of the log linear curve.

The microsomal intrinsic clearance (CL_{int}) was estimated using the equation

$$CL_{int} = \left[\frac{0.693}{t_{1/2}}\right] \times \left[\frac{volume\ of\ incubation}{mg\ microsomal\ protein}\right]$$

$$CL_{int\ (scaled)} = \left[\frac{CL_{int}}{1000}\right] \times (g\ liver/BW) \times (mg_{\text{of microsome/g liver}})$$

$$CL_h = \left[\frac{(CL_{int\ scaled} \times Qh)}{(CL_{int\ scaled} + Qh)}\right]$$

$$ER = \left[\frac{CL_h}{Q_h}\right]$$

Where Cl_{int} is the intrinsic clearance, Cl_{int} (scaled) is scaled intrinsic clearance, ER is the extraction ratio and CL_h is the hepatic clearance. Q_h is blood flow (55.2 ml/min/kg in the rat and 20.7 ml/min/kg in human). Number of microsomes per gram liver (Human is 120×10^6 cells and rat 120×10^6 cells) Liver scaling factor (Human =25.7, rat =40 gram liver per kilogram body weight).

4.3.3.2 *In vitro* stability of cryptolepine in human, rat and dog liver S9 and cytosol

Stock solution of cryptolepine (100 µM) was prepared in methanol by two subsequent 1:10 dilutions from 10 mM DMSO drug solutions, the final assay concentration was 1 µM. Frozen pooled rat and human liver S9 fraction (2 mg/mL) was thawed from -80°C and diluted in prewarmed phosphate buffer (with and without NADPH) at 37°C. The assay was initiated by addition of cryptolepine. Manual sample plates were incubated on a Thermo Max Q 2000 shaker placed in a temperature controlled Sartorius Certomat H hood set at 37°C. Samples were sequentially removed at designated time points (0, 5, 15, 30, 60 and 120 min) and quenched with 4 volumes of ice cold acetonitrile

(containing internal standard, diazepam), centrifuged and supernatants reconstituted in water (acetonitrile: water, 50:50% v/v). Samples were then analysed by LC-MS/MS to assess parent depletion.

The assay was repeated using rat, human and dog liver cytosol in triplicate at a protein concentration of 1 mg/ml without the cofactor, NADPH. Zoniporide and carbazeran were used as probe substrates in the assay. The parent compounds in the supernatant were then analysed by LC-MS/MS. Stability was determined semi-quantitatively from the peak area ratios (PAR) of analyte:internal standard which were used to determine the percentage stability at pre-defined time points in relation to zero minute time point. The percentage stability of a compound at time (t) was calculated as below:

$$\%\ Stability = \left[\frac{PAR\ at\ time\ t}{PAR\ at\ time\ t{=}0\ min}\right] \times 100$$

Percentage stability data was log transformed and plotted against time point(s) to define the initial phase of the slope also known as the initial rate of elimination, k_{el}. The natural log of 2 (0.693) is then divided by the k_{el} to calculate a plasma half-life (min).

$$Half - life\ (min) = \frac{0.693}{Kel}$$

A summary of the final assay conditions used are detailed in Table 4.2.

Table 4.2: Final assay conditions used to assess the half-life values of cryptolepine measured in pooled human, rat and dog liver S9 and cytosol.

Cryptolepine concentration	Nominally 1 µM
S9 concentration	2 mg/mL
Cytosol concentration	1 mg/mL
Strain/species	Sprague Dawley rat, Human, Beagle dog
Gender	Mixed gender for human, all other species were male
NADPH concentration	1 mM
Time points (n=3)	0, 5, 15, 30, 60, 120 min
Incubation condition	37°C, atmospheric air, 100 rpm
Incubation plate type	Polypropylene
Control	Diclofenac

4.3.3.3 Enzyme Phenotyping Assay

Cryptolepine was first incubated in human and rat liver S9 protein concentrations ranging from 0.5 mg/mL - 2 mg/mL to help determine the optimal protein concentration for the enzyme phenotyping assay. This was done to help reduce non-specific binding in the assay.

A final protein concentration of 2 mg/mL each of the rat and human liver S9 was used in all assays. Incubation mixtures consisted of cryptolepine, zoniporide and carbazeran (probe-substrate for aldehyde oxidase) at a final concentration of 1 µM in all assay conditions. Hydralazine (a timedependent aldehyde oxidase inhibitor [Strelevitz *et al*., 2012]) and raloxifene (an inhibitor for aldehyde oxidase and a non-specific CYP 450 enzyme inhibitor [Nirogi *et al*., 2013]) were used to evaluate the enzymes involved in the metabolism of cryptolepine. Inhibitor and drug stock solutions were made up in dimethyl sulfoxide (DMSO) and added to the incubation mixture such that the total concentration of DMSO was less than 1%v/v.

The final reaction contained DPBS, the drugs (1 µM final solution), inhibitors and 2 mg/mL of liver S9 fraction without NADPH. The reaction was initiated in a Thermo Max Q 2000 shaker with temperature controlled Santorius Certomat H hood set at 37°C by the addition of the liver S9 fraction in a 96 well plate. Sequential samples were removed after 120 min and quenched with 4 volumes of ice cold acetonitrile (containing internal standard, diazepam), centrifuged and supernatant reconstituted in water. Samples were then analysed by LC-MS/MS to assess parent depletion.

The parent compound in the supernatants was then analysed by LC-MS/MS. Stability was determined semi-quantitatively from the peak area ratios (PAR) of analyte:internal standard which

were used to determine the percentage stability at pre-defined time points in relation to zero minute time point. The percentage stability of a compound at time (t) was calculated as below:

$$\%\ Stability = \left[\frac{PAR\ at\ time\ t}{PAR\ at\ time\ t{=}0\ min}\right] \times 100$$

Percentage stability data was log transformed and plotted against time point(s) to define the initial phase of the slope also known as the initial rate of elimination, k_{el}. The natural log of 2 (0.693) is then divided by the k_{el} to calculate a plasma half-life (min).

$$Half - life\ (min) = \frac{0.693}{Kel}$$

4.3.3.4 *In Vitro* Metabolite Profiling in Hepatocytes

Cryopreserved, suspension hepatocytes were obtained from commercial sources and stored in the gas phase over liquid nitrogen until use. All experiments were carried out using Williams medium E supplemented with foetal bovine serum (10%). Stock solution of cryptolepine (2 mM) was prepared in DMSO.

Thawed hepatocytes (37°C, 2 min) were transferred to a tube containing incubation medium (40 mL, 37ºC). The suspension was centrifuged for 1 min at 50×g at room temperature, and then the supernatant was removed and discarded. The hepatocyte pellet was resuspended in William medium E by gentle agitation in a small volume (2-5 mL) of incubation medium. An aliquot of cell suspension (50 µL) was mixed with trypan blue (50 µL) for viability assessment and cell counting. An appropriate volume of incubation medium was then added to the remaining cell suspension to give a final concentration 1 x 10^6 viable cells/mL. Cell suspension (1 mL) was transferred to wells of a 12 well plate, and cryptolepine (10 µM) was added. The samples were

then incubated at 37ºC under an atmosphere of 75% O_2, 5% CO_2, 20% N_2; 98% humidity with shaking (50 rpm) in a HERAcell 240i incubator (Thermo Fischer Scientific, Waltham, MA, USA).

Incubations were initiated by the addition of cryptolepine to the hepatocytes. At each time point (0, 4, 24 h), 200 µL of incubation sample was added to 1 volume of chilled acetonitrile (0ºC), NVP-TAA501 as internal standard was added (8 µL, final concentration 5 µM), and the mixture was frozen at -80ºC.

Analytical Methods for Metabolite Profiling in hepatocytes

Samples were centrifuged at 10000×g at 4ºC for 5 min and supernatants (100 µL) were diluted with water (400 µL) and filtered (0.45 µm). Samples were analysed by Capillary HPLC-MS/MS using a Chorus 200 binary syringe pump (CS Analytics, Beckenried, Switzerland), Triart C18 column (1.9 µm particle size, 150 mm x 0.3 mm) at 40 ºC coupled to an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Electrospray ionization in positive mode was used, recording full scans (m/z 150-1500) at resolution 30000, and targeted or data dependent MS^n at unit resolution or high (30000) resolution as required for metabolite characterization. Chromatographic separation was achieved with the following mobile phases, (A): 10mM ammonium formate in MS-grade water, MS-grade water/ acetonitrile (95:5), and 0.02% Trifluoroacetic acid; (B): MS-grade water/ methanol (5:95), 10 mM ammonium formate in MSgrade water, 0.02% Trifluoroacetic acid. A linear gradient of mobile phase B from 2 to 95% was applied over 25 min on the column at a flow rate of 4.5 µL/min. Experiments for the determination of exchangeable protons were performed by exchange of H_2O by D_2O and CH_3OH by CH_3OD.

Table 4.3: Summary of LC-MS/MS condition for the metabolite identification (met ID) in human and rat hepatocytes.

Analytical method (MetID)	Capillary-HPLC/MS(-MS)
Mass Spectrometry	LTQ Orbitrap +ESI norm. coll. energy 50%/50%
HPLC column / temp.	Triart C18, 1.9 µm, 150 mm x 0.3 mm/ 40°C
Injection	4.5 µl loop injection
Mobile phase	H_2O/AcN (95:5), 10 mM $HCOONH_4$, 0.02 % TFA (A) H_2O/MeOH (5:95), 10 mM $HCOONH_4$, 0.02 % TFA (B) Experiments for the determination of exchangeable protons were performed by exchange of H_2O by D_2O and CH_3OH by CH_3OD.
Gradient	2-95/25min B, flow rate: 4.5 µL/min

4.4 RESULTS

4.4.1 Permeability assay Results

4.4.1.1 MDR1-MDCK ASSAY

P-gp-mediated transport of cryptolepine using MDCK cells stably expressing MDR1 (Madin-Darby Canine Kidney cells retro virally transduced with the human multidrug resistance gene 1; P-glycoprotein P-gp) showed a low human P-gp efflux potential (efflux ratio <5). The percent recovery were 90 and 94% with apical to basolateral (A→B) and basolateral to apical (B→A), respectively (Table 4.4).

Table 4.4: MDR1-MDCK assay results for cryptolepine

MDCK	Papp A-B [10^{-6}cm/s]	Papp B-A [10^{-6}cm/s]	Efflux ratio (B-A)/(A-B)	% RecoveryA-B	% RecoveryB-A
MDR1MDCK II	4.26	20.4	4.78	90	94

4.4.1.2 HDM-PAMPA ASSAY

PAMPA assay using pH 4, 6.8 and 8 was determined to demonstrate the acceptability of cryptolepine for oral absorption. Cryptolepine showed a high passive permeability with calculated percentage fraction absorbed (% FA) being 81%.

Table 4.5: PAMPA assay results for cryptolepine

PAMPA	Log PAMPA [cm/s]	Calc. % FA	LogPe pH 4	LogPe pH 6.8	LogPe pH 8
HDMPAMPA	-4.7	81	-5.4	-5.9	-5.8

4.4.2 Stability in Human and Rat Plasma

Cryptolepine showed good plasma stability when incubated in pooled rat and human plasma after 120 min. The measured percent plasma stability for cryptolepine was 89.2 and 91.7% in rat and human plasma, respectively after a 2 h time point.

The values obtained expressed as percent stability are presented in Table 4.6.

Table 4.6: Plasma stability of cryptolepine in human and rat

	Time points / Percentage stability						$t_{1/2}$
Cryptolepine	0	5	15	30	60	120	
Human plasma	100.0±0.12	97.4±0.21	95.3±0.31	94.1±0.24	92.1±0.14	91.7±0.21	>120
Rat plasma	100.0±0.14	100.7±0.18	98.3±0.12	96.4±0.17	93.7±0.11	89.2±0.31	>120

4.4.3 Plasma protein binding of cryptolepine in human and rat

The binding of cryptolepine to plasma proteins was shown to be moderate in human (58.7±1.7%) and rat (64.3±3.1%). The measured unbound fractions are shown in the Table 4.7 below.

In humans, 58.7±1.7% of cryptolepine exists as bound to serum proteins. Only free form of the drug exhibits its pharmacological effect, and thus 41.3±1.7% of the compound in systemic circulation will be responsible for its action. Since serum protein drug complexes are usually reversible, the moderate protein binding characteristics may contribute to an intermediate duration of action. The plasma protein binding in rat was not very different from humans.

Table 4.7: Plasma protein binding of cryptolepine in human and rat plasma

	Human (mixed gender Dawley plasma)	Sprague Rat (mixed gender human plasma)
% Plasma Protein binding, PB (Mean±SD)	58.7±1.7	64.2±3.1
% fraction unbound, f_u (Mean±SD)	41.3±1.7	35.8±3.1

4.4.4 Preliminary incubation of cryptolepine in human and rat hepatocytes

Incubation of cryptolepine in human and rat hepatocytes after 80 min showed a hepatic extraction ratio (%) of 54 and 44, respectively. The intrinsic clearance was low for rat (9 µL/min million cells) and moderate for human (8 µL/min million cells).

Table 4.8: Hepatic and intrinsic clearance of cryptolepine in human and rat hepatocytes

	Hepatocyte	
	Rat	Human
Half-life(min)	77.3	86.9
CL_{int} (µL/min million cells)	9	8
Hepatic extraction (%)	44	54
CL_h (ml min^{-1} kg^{-1})	24.2	11.2
CL_{int} rank	Low	Moderate

4.4.5 Stability in Human and Rat Liver S9

Whilst the half-life of cryptolepine in rat and human liver S9 is >120 min (Table 4.9), instability was observed in the presence and absence of NADPH. This data suggests a non-NADPH dependent enzyme(s) such as aldehyde oxidase may play a major role in driving the metabolic instability. Carbazeran and zoniporide were used to demonstrate the presence of aldehyde oxidase activity in the rat and liver S9 fractions. Carbazeran (Table 4.10) and zoniporide (Table 4.11) showed high clearance in the human and rat liver S9 fractions.

Table 4.9: Percentage stability of cryptolepine in human and rat liver S9 fraction

	Time points / Percentage stability						$t_{1/2}$
	0	5	15	30	60	120	
Human+ NADPH	100.0±0.34	94.3±0.91	85.3±2.32	85.8±0.26	73.5±0.12	58.5±0.12	>120
Human- NADPH	100.0±0.41	89.2±0.22	87.5±0.24	85.7±0.34	78.9±0.14	63.9±1.14	>120
Rat + NADPH	100.0±0.14	101.1±0.15	97.0±0.51	83.1±1.27	77.8±0.31	62.8±0.21	>120
Rat - NADPH	100.0±0.22	91.2±1.42	91.6±0.63	84.3±0.17	75.3±0.44	62.9±0.11	>120

Table 4.10: Percentage stability of carbazeran in human and rat liver S9 fraction

	Time points (min) / Percentage stability						$t_{1/2}$
	0	5	15	30	60	120	
Human+ NADPH	100.0±0.12	54.7±1.81	24.2±0.67	6.9±0.17	1.0±0.14	0.1±0.01	12.3±0.42
Human- NADPH	100.0±0.23	51.8±2.14	22.2±0.54	2.8±1.01	0.9±0.21	0.0±0.07	9.49±0.07
Rat + NADPH	100.0±0.13	76.6±1.12	53.4±0.17	32.1±3.04	11.2±0.75	4.0±0.11	25.9±1.04
Rat - NADPH	100.0±0.11	75.3±0.94	52.9±0.87	30.1±0.44	11.5±0.97	3.4±0.06	24.8±0.86

Table 4.11: Percentage stability of zoniporide in human and rat liver S9 fraction

	Time points (min) / Percentage stability						$t_{1/2}$
	0	5	15	30	60	120	
Human+NADPH	100.0±0.14	92.2±1.01	86.4±0.74	81.9±0.61	64.0±0.42	39.6±0.61	92.5±1.16
Human- NADPH	100.0±0.11	98.0±0.12	94.3±0.22	72.4±0.52	63.8±0.84	36.7±2.21	81.0±0.17
Rat + NADPH	100.0±0.06	57.9±0.54	21.1±1.04	4.1±1.21	1.3±0.57	1.8±0.05	6.6±0.31
Rat - NADPH	100.0±0.04	57.1±0.15	25.2±0.91	3.8±0.41	2.1±1.21	2.6±0.73	6.4±0.10

4.4.6 Stability in Human, Rat and Dog Liver Cytosol

Incubation of cryptolepine in human and rat cytosol showed instability even though the half-life was >120 min. Dog cytosol and S9 fraction however showed stability after the 120 min incubation with over 90% of parent compound still present.

Table 4.12: Percentage stability of cryptolepine in human, rat and dog liver S9

	Time points / Percentage stability						$t_{1/2}$
	0	5	15	30	60	120	
Human cytosol	100.0±0.14	112.5±4.16	95.6±2.10	89.5±0.46	83.6±1.01	68.3±1.04	>120
Rat cytosol	100.0±0.12	93.8±0.14	107.4±0.57	91.5±0.74	81.0±.024	70.8±0.71	>120
Dog cytosol	100.0±0.16	97.7±1.04	96.9±0.66	96.0±0.68	95.3±2.11	95.3±0.18	>120
Dog liver S9	100.0±0.01	98.2±0.81	95.7±0.24	94.8±0.45	93.9±0.84	93.2±0.11	>120

4.4.7 Enzyme Phenotyping

Raloxifene significantly reversed the metabolic instability of cryptolepine, zoniporide and carbazeran in both human and rat S9 fractions. Hydralazine did not show a significant inhibition in the metabolism zoniporide while a significant reversal of the metabolism of carbazeran and cryptolepine were seen in rat (Figure 4.2) and human (Figure 4.3) liver S9.

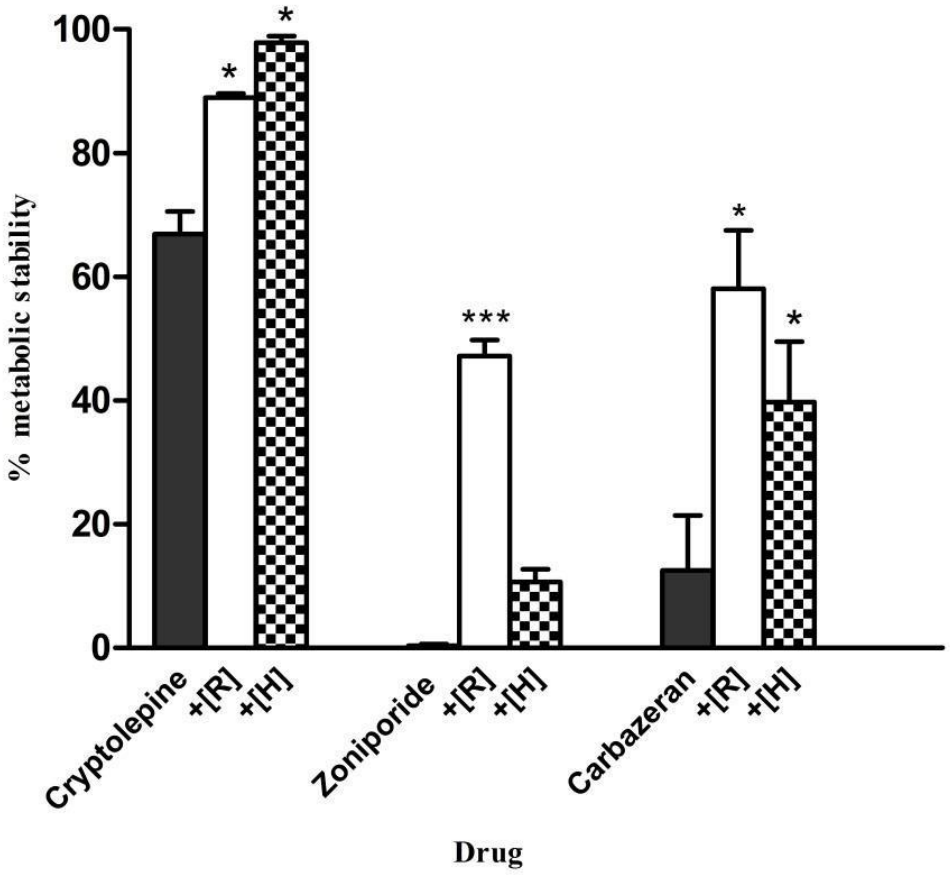


Figure 4.2: Metabolic stability of cryptolepine in rat liver S9. Cumulative values are reported mean±SEM for three replicates. *p<0.05; ***p<0.001, compared to controls (drugs) ANOVA followed by Turkey's multiple comparison test. [R]=raloxifene (5 µM), [H] = hydralazine (50µM)

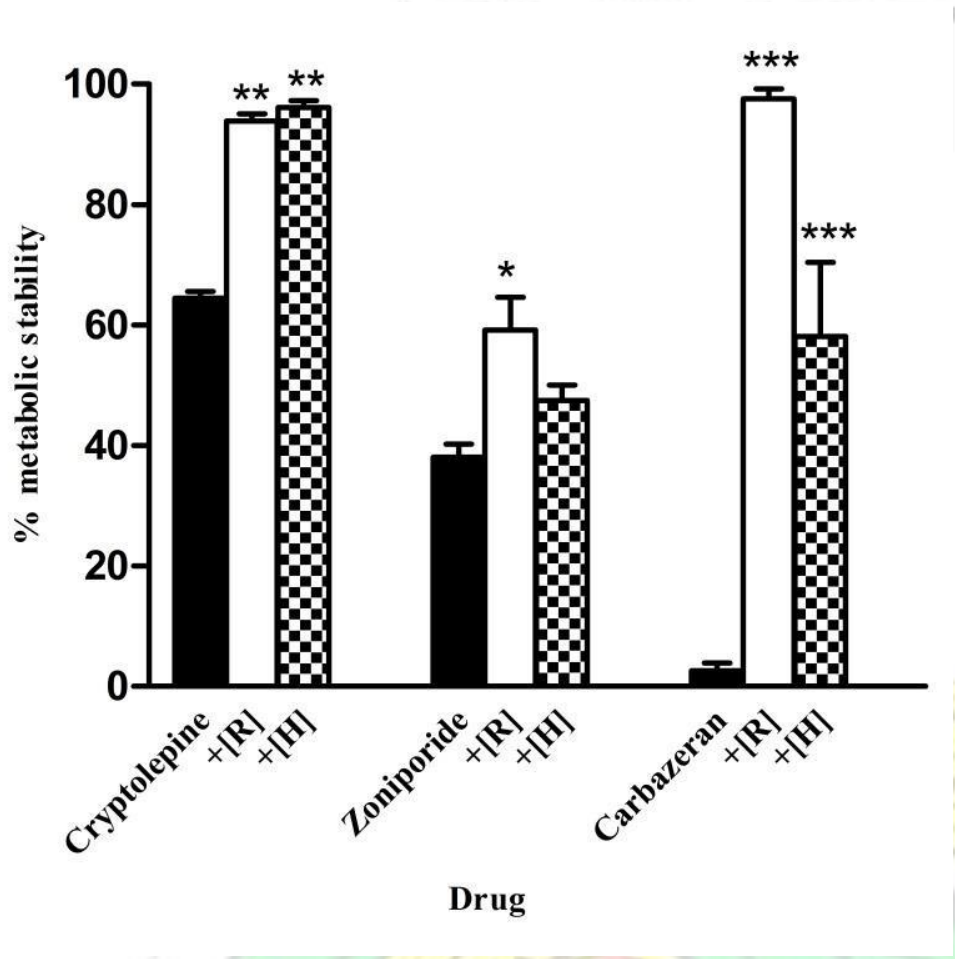


Figure 4.3: Metabolic stability of cryptolepine in human liver S9. Cumulative values are reported mean±SEM for three replicates. *p<0.05; **p<0.01; ***p<0.001, compared to controls (drugs) ANOVA followed by Turkey's multiple comparison test.
[R]=raloxifene (5 µM), [H] = hydralazine (50µM)

4.4.8 Metabolite Profiling in Human and Rat Hepatocytes

Hepatocyte incubation and metabolite identification

Nine metabolites were identified in human and rat hepatocytes resulting from metabolic pathways mainly involving hydroxylation (M2, M4, M6, M7, M8, M9), proposed dihydrodiol formation (M3, M5) and glucuronidation (M1, M2, M4, M8, M9). All metabolites were detected in hepatocyte incubations from both species except for glucuronide M1, which was only formed by human hepatocytes, and metabolites M8 and M9, which were only formed by rat hepatocytes. All proposed metabolite structures are presented in Figure 4.4. All metabolites identified after 24 h in human hepatocytes were less than 10% in relation to the parent peak area (Table 4.13).

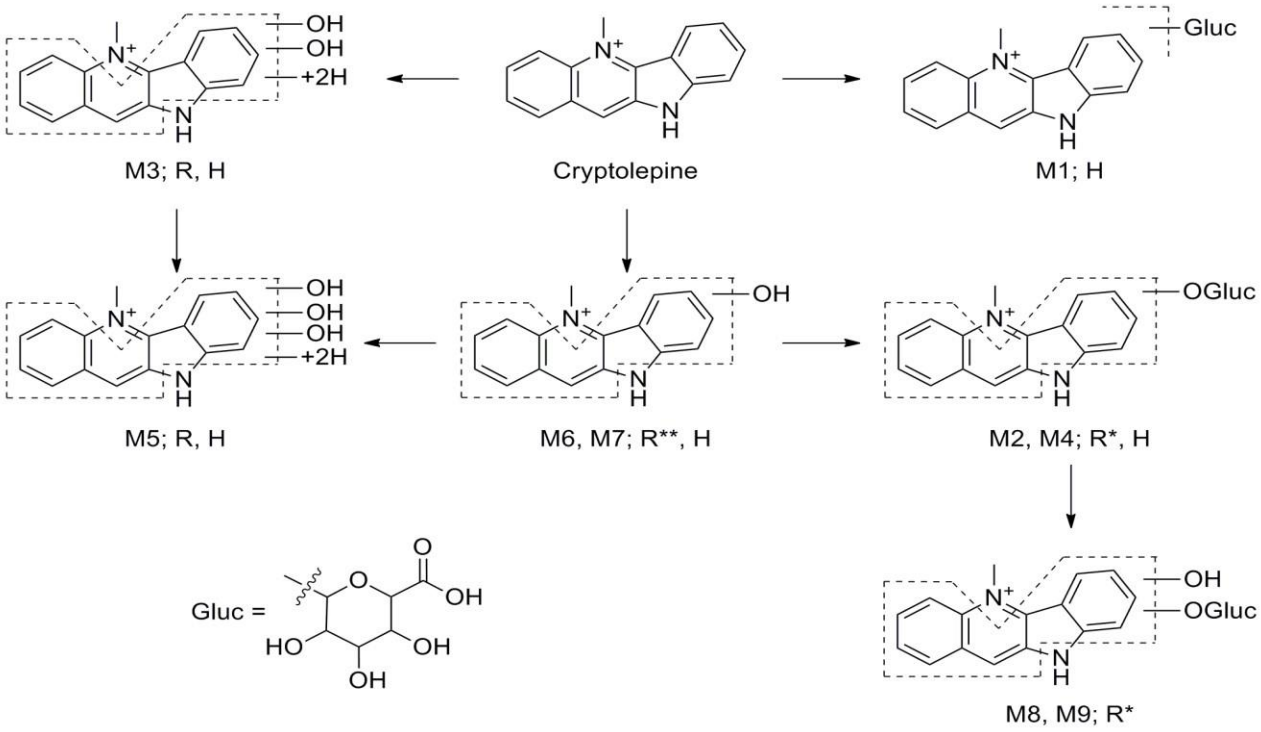


Figure 4.4: Proposed metabolite structures and metabolic pathways for cryptolepine. R: detected after rat hepatocyte incubation; H: detected after human hepatocyte incubation. * Metabolite detected in rat urine; ** metabolite detected in rat urine and plasma.

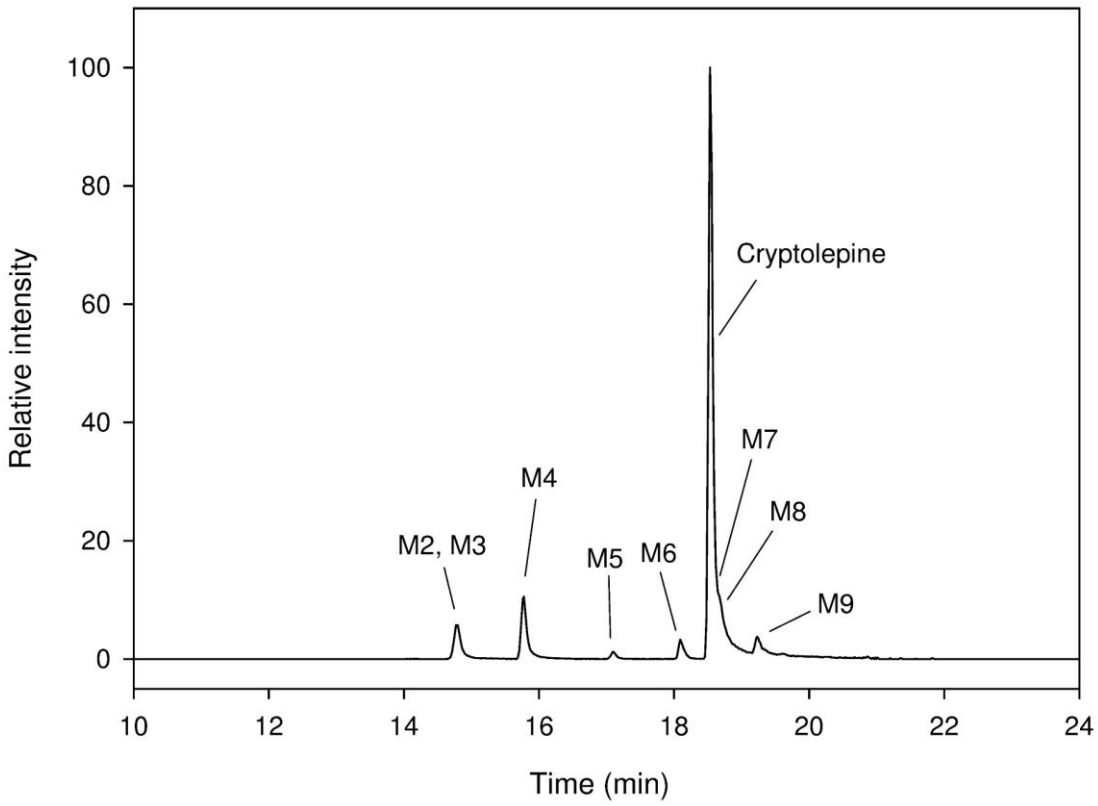


Figure 4.5: Representative extracted ion chromatogram for cryptolepine and proposed metabolites in a rat hepatocyte incubation sample. M1 is not shown as it was only detected in the human hepatocyte incubation.

Table 4.13: LC-MS data for cryptolepine and proposed metabolites.

Metabolite code	Retention time (min)	Measured m/z	Proposed MH+ formula	Product ions m/z and proposed neutral loss formula	m/z after H/D exchange	Detected in hepatocyte species	Detected in rat plasma/ urine
M1	12.9	409.1394	$C_{22}H_{21}N_2O_6$	233 (-$C_6H_9O_6$); MS3 218 (-CH_3)	413	H	
M2	14.8	425.1344	$C_{22}H_{21}N_2O_7$	249 (-$C_6H_9O_6$); MS3 221 (-CO), 234 (-CH_3)	430	R, H	Urine
M3	14.8	267.1129	$C_{16}H_{15}N_2O_2$	221 (-CH_2O_2), 239 (-CO), 249 (H_2O), 252 (-CH_3)	270	R, H	
M4	15.8	425.1343	$C_{22}H_{21}N_2O_7$	249 (-$C_6H_9O_6$); MS3 221 (-CO), 234 (-CH_3)	430	R, H	
M5	17.1	283.1078	$C_{16}H_{15}N_2O_3$	211 (-$C_3H_4O_2$), 225 (-C_3H_6O), 237 (-CH_2O_2), 239 (-C_2H_4O), 255 (-CO), 265 (-H_2O), 268 (CH_3)	287	R, H	
M6	18.1	249.1023	$C_{16}H_{13}N_2O$	221 (-CO), 231 (-H_2O), 234 (CH_3), 235 (-CH_2)	251	R, H	Urine, plasma
Cryptolepine	18.5	233.1073	$C_{16}H_{13}N_2$	218 (-CH_3), 219 (-CH_2)	234	R, H	Urine
M7	18.6	249.1022	$C_{16}H_{13}N_2O$	221 (-CO), 231 (-H_2O), 234 (CH_3), 235 (-CH_2)	251	R, H	
M8	18.7	441.1294	$C_{22}H_{21}N_2O_8$	222 (-$C_6H_9O_6$ - C_2H_3O), 237($C_6H_9O_6$ - CO), 247(-$C_6H_9O_6$ - H_2O), 250(-$C_6H_9O_6$ - CH_3), 265 (-$C_6H_9O_6$)	447	R	

M9	19.2	441.1292	$C_{22}H_{21}N_2O_8$	222 ($-C_6H_9O_6 - C_2H_3O$), 237($C_6H_9O_6 - CO$), 247($-C_6H_9O_6 - H_2O$), 250($-C_6H_9O_6 - CH_3$), 265 ($-C_6H_9O_6$)	447	R	Urine

R: rat; H: human.

4.5 DISCUSSION

In vitro assays play an important role in screening a New Chemical Entity (NCE) in the discovery stages. They help provide a simple, convenient and fast way to test the potency and drug-likeness of these new compounds (Zhang *et al*., 2012).

Firstly, the permeability of cryptolepine across the gastrointestinal tract was assessed using *in vitro* approaches that include artificial membranes such as Parallel Artificial Membrane Permeability Assay (PAMPA) which models the gastrointestinal epithelium's passive permeability (Faller, 2008) and cultured cell membrane with expressed multidrug resistance human P-gp gene (MDR1-MDCK II) to asses P-gp efflux potential of cryptolepine transport. Human P-glycoprotein (P-gp, MDR1) is known to be a determinant of drug absorption, distribution, and excretion of a number of clinically important drugs (Ambudkar *et al.,* 1999; Fromm, 2000). P-gp is widely expressed in major organs, and aside the gastrointestinal epithelium, P-gp is highly expressed in the capillaries of the blood brain barrier (BBB) and poses a barrier to brain penetration of its substrates (Schinkel, 1999).

Plant derived bioactive substances have been shown to interact with P-gp resulting in a significant variation in the pharmacokinetic and pharmacodynamics properties. Interaction with P-gp can augment toxicity and/or interfere with the drug's therapeutic outcomes. For decades now, the development and spread of resistance to chloroquine and other front-line antimalarial compounds used in the prevention and treatment of the most severe form of human malaria has been reported to be the major factor behind the high burden of malaria worldwide and has given cause for clinical concern. Development of resistance to some antimalarial agents has been attributed to the efflux mechanism of the parasite P-glycoprotein. There is compelling evidence that *P. falciparum* multidrug resistance 1 (*pfmdr1*), a gene on chromosome 5 encoding a P-glycoprotein homolog 1 (Pgh1), also contributes to chloroquine resistance (Foote *et al.,* 1990). Nevertheless, it is clear that

chloroquine resistance (CQR) cannot be conferred by Pgh1 alone and requires the presence of mutations in other (unidentified) gene(s) (Su *et al*., 1997; Foote *et al*., 1990). Two mdr genes (*pfmdr1* and *pfmdr2*) encoding P-glycoprotein homologues have been identified in *P. falciparum* and one of these (*pfmdr1*) has several alleles that have been linked to the chloroquine resistance phenotype. In addition, it's been shown that changes in Pgh1 can modulate resistance to quinine, halofantrine and mefloquine (Reed *et al*., 2000).

In this chapter, the P-gp efflux potential of cryptolepine was ascertained *in vitro* using the Mardin-Darby canine kidney epithelial cells expressed with human multiple drug resistant P-glycoprotein (MDR1-MDCK II cell lines). This study also helps to determine the involvement of human P-gp efflux pump in the transport across the gut lumen and the apical surface of endothelial cells in the capillaries of the brain. Cryptolepine (90%) was recovered in the apical to basolateral well (A→B) whereas a recovery of 94% was obtained from the basolateral to the apical well (B→A). Comparison of the efflux ratios between MDR1-MDCK II and MDCK Trans well assays can provide a measure of the specific human P-gp mediated efflux activity (Polli *et al*., 2001). An efflux ratio of 4.78 (less than 5) of cryptolepine indicates a low human P-gp efflux potential. This result shows no P-gp efflux liability. Hence the development of resistance by *P. falciparum* to cryptolepine via the P-gp efflux mechanism may not be possible. Additionally, cryptolepine can easily cross the blood brain barrier without P-gp efflux, eliciting CNS activity and possibly a high bioavailability when given orally.

The hexadecane membrane PAMPA was used to assess passive permeability of cryptolepine across different pH gradients. The varied pH ranges mimics the gastro-intestinal pH range. A calculated percentage fraction absorbed (% FA) of 81% was recorded. This shows a high passive permeability of cryptolepine and its sustainability as an oral dosage form.

The screening of the stability of a compound in early drug development provides useful information about the liabilities of the drug candidate, as certain functional groups can decompose in the bloodstream. Compounds with amide, ester, lactam and sulphonamide functional groups have the tendency to undergo hydrolysis by plasma enzymes. This accounts for their high clearance and short half-life, resulting in poor in *vivo* pharmacokinetics and disappointing pharmacological performance (Di *et al.*, 2005). Plasma degradation clearance can easily be overlooked if discovery projects focus on liver microsomal stability.

Cryptolepine was incubated in pooled plasma of human and rat to assess the stability in the plasma of these species. After 2 h of incubation, 91.7% and 89.2% of cryptolepine remained in human and rat plasma, respectively. The half-lives were also greater than 2 h indicating the non-involvement of plasma hydrolytic enzymes such as cholinesterase, aldolase, lipase, dehydropeptidase in the metabolism of cryptolepine. Data from *in vitro* plasma protein binding (PB) experiments that determine the fraction of protein-bound drug are frequently used in drug discovery to guide structure design and to prioritize compounds for *in vivo* studies. *In vitro* assays to a large extent are able to predict the concentration of a compound that is available to interact with the therapeutic target *in vivo* and produce efficacy. Plasma protein binding also influences drug clearance from the body. For drugs eliminated by renal tubular secretion or hepatic metabolism, high PB is associated with lowered drug elimination. Highly bound drugs affect glomerular filtration rate as only free unbound drugs are filtered.

The Pierce Rapid Equilibrium Dialysis (RED) device was employed in determining the plasma protein binding of cryptolepine *in vitro*. Cryptolepine showed a moderate plasma protein binding in both human (58.7±1.7) and rat (64.2±3.1). A moderate amount of free drug molecules may be available to interact with the therapeutic target to produce efficacy.

In the drug discovery stage, it is impossible to obtain metabolic transformation information from humans. Unfortunately, studies from laboratory animals do not always accurately reflect what happens in humans, partially due to the differences in drug-metabolism enzymes. Hence, the use of *in vitro* drug metabolizing systems to predict *in vivo* pharmacokinetic behaviour has become an increasingly accepted part of drug discovery (Houston, 1994; Houston and Carlile, 1997). These drug metabolizing enzymes are widely distributed amongst the different organs and tissues, such as liver, intestine, kidney, lung and brain. Since the human liver is the major organ of drug metabolism in man, human liver-derived systems are the primary models used for studying drug metabolism. In this study, whilst the half-life of cryptolepine in rat and human liver S9 was greater than 120 min, instability was observed at 60 and 120 min in the presence and absence of NADPH. This data suggests a non-NADPH dependent enzyme(s) such as aldehyde oxidase may be driving the metabolic instability. Carbazeran and zoniporide were used as probe substrates to demonstrate the presence of aldehyde oxidase activity in the rat and liver S9 fractions. Zoniporide and carbazeran showed high clearance in the human and rat liver S9 fractions.

Incubation of cryptolepine in human and rat cytosol also showed instability after 120 min despite the half-life being greater than 120 min, supporting the involvement of cytosolic enzymes in the metabolism of cryptolepine. Cryptolepine incubation in dog liver S9 and cytosol showed stability after the 120 min of incubation with over 90% of parent compound still present. Aldehyde oxidase (AO) activity among animal species may vary depending on the substrate; it generally seems to be high in monkeys and humans and low in rats, whereas dogs are to a large extent deficient in activity (Pryde *et al*., 2010). Cryptolepine has been shown to be a substrate for aldehyde oxidase in rabbit liver preparation of aldehyde oxidase (Stell *et al*., 2012). The results presented here shows the metabolic instability of cryptolepine in human and rat cytosol but stability in dog liver S9 and

cytosol confirming the possible involvement of aldehyde oxidase (AO) in the metabolism of cryptolepine in rat and human.

Further investigation in rat and human liver S9 fractions (2 mg/ml) were conducted to characterize the enzymes involved in the metabolism of cryptolepine metabolism using raloxifene (a CYP-450 and AO inhibitor) and hydralazine (a specific AO inhibitor). Raloxifene significantly reversed the metabolic instability of cryptolepine, zoniporide and carbazeran in both human and rat S9 fractions.

This indicates the possible involvement of both AO and CYP-450 in the metabolism of cryptolepine. Hydralazine did not show a significant inhibition in the metabolism of zoniporide while a significant reversal of the metabolism of cryptolepine and carbazeran was seen in human and rat liver S9 further confirming the involvement of aldehyde oxidase in the metabolism of cryptolepine.

Identification of the metabolites generated by biotransformation processes is a crucial task in various stages of drug discovery and development. In the early drug discovery stage, it is necessary to identify the metabolic soft spots of compounds that show low metabolic stability. The result can provide information for further structure modifications of lead compounds and improvement of the metabolic and pharmacokinetic properties. Metabolically active hepatocytes commercially available have been shown to contain the complete complement of drug-metabolizing enzymes and hence serves as the closest *in vitro* surrogate for *in vivo* hepatic metabolism (Fabre *et al*., 1990). To determine potential biotransformation pathways of cryptolepine, *in vitro* metabolism studies were performed using rat and human hepatocytes. In this study, the metabolic profiles in humans and rat were compared to assess whether all metabolites observed in human matrices were detected in the rodent species. Incubation of cryptolepine in rat and human hepatocytes for 24 h showed nine major metabolites resulting from metabolic pathways mainly involving hydroxylation,

dihydroxylation and glucuronidation. The proposed biotransformation scheme is summarized in Figure 4.4. All human *in vitro* metabolites were also found in rat hepatocytes, except for metabolite M1. This metabolite is an N-glucuronide and is found in human, but not rat hepatocytes, indicating the possible involvement of a human specific UDP-glucuronosyltransferase (UGT) (Li Di, 2014) in the metabolism of cryptolepine. In previous studies by Stell *et al*. (2012), the involvement of aldehyde oxidase in the metabolism of cryptolepine was proposed, leading to the formation of metabolite cryptolepine-11one. The metabolites M6/M7 identified in this study in both human and rat hepatocytes are formed by oxidative hydroxylation indicating that involvement of aldehyde oxidase is possible. Metabolite (M6) was also detected in rat plasma samples after oral administration of cryptolepine. Aldehyde oxidase metabolism has in the past caused difficulties in drug development, as it is often more active in human than in preclinical species. For example, a p38 kinase inhibitor (RO1) for the treatment of rheumatoid arthritis was terminated because of unexpected rapid clearance and short half-life in man, proposed to be due to aldehyde oxidase metabolism (Zhang *et al*., 2011). The aldehyde oxidase and N-glucuronidation pathways could potentially result in significantly different clearance and/or disposition in human compared to rat, depending on the extent of formation and stability of each metabolite in humans *in vivo*. The dihydroxylation metabolites (M3/M5) are likely to be formed via initial epoxide formation, indicating the possibility of cytochrome P450 metabolism of cryptolepine**.** The proposed dihydrodiol metabolites M3 and M5 were not found in rat *in vivo*, but may be formed in the liver and excreted in faeces.

4.6 CONCLUSION

These studies have demonstrated the drug-likeness of cryptolepine. These include the high passive permeability, low human P-gp efflux potential, good plasma stability, low to moderate metabolic

clearance and moderate plasma protein binding in rat and human. In rat and human hepatocytes, nine metabolites were observed with hydroxylation, dihydroxylation and glucuronidation proposed to be major metabolic pathways. Aldehyde oxidase, cytochrome P450 and human specific UDPglucuronosyltransferase may play major roles in the metabolism of cryptolepine.

5.0 CHAPTER FIVE

IN VIVO PHARMACOKINETIC STUDIES OF CRYPTOLEPINE IN SPRAGUE DAWLEY RATS

5.1 INTRODUCTION

Pharmacokinetics provides a mathematical basis to assess the time course of drugs and their effects on the body, enabling absorption, distribution, metabolism and excretion (ADME) of drugs to be quantified. In clinical practice, pharmacokinetic studies help provide information for dose adjustments of drugs with narrow therapeutic indices particularly when there is no immediate response to the administration of a dose.

In theoretical sense, a compound should have optimum half-life, good bioavailability and favourable metabolism to be successful in the drug development; however, these pharmacokinetic (PK) reasons alone do not dictate the go/no-go decision in drug development. Pharmacokinetics parameters in conjunction with mechanisms of efficacy and toxicity determine whether the pharmacokinetics will have significant impact on the therapeutic outcome. Various strategies are adopted accordingly to overcome suboptimal pharmacokinetics characteristics. A drug administered via the extravascular route should have good oral bioavailability but some drugs, despite having poor oral bioavailability, are clinically successful as oral drugs.

In spite of the multiplicity of biological effects of cryptolepine, very little is known about its pharmacokinetics. In previous reports by two independent researchers, cryptolepine showed a rapid disappearance from the plasma; localization in various tissues except the central nervous system and concluded that the hepatobiliary tract could be the main clearance pathway of cryptolepine (Salako *et al*., 1985; Noamesi *et al*., 1991). In contrast to earlier reports, cryptolepine hydrochloride was detected in the serum within 30 min and 10 h after oral administration of 10

mg/kg to rats. No cryptolepine metabolites were detected in these serum samples (McCurrie *et al*., 2009).

Kuntworbe *et al*. (2013), reported a superior plasma drug level, reduced distribution rate, reduced clearance and extended half-life of cryptolepine hydrochloride-loaded gelatine nanoparticles (CHN) compared to the free drug in solution in a single-dose pharmacokinetic studies in Wistar rats. This translated into a superior chemosuppressive activity of CHN. The contradictions with regard to the plasma levels after oral administration and the metabolite detection of cryptolepine in the earlier studies directed the present study. This study aims at evaluating the pharmacokinetics and to understand the mechanisms of biotransformation of cryptolepine by evaluation of the singledose pharmacokinetics of cryptolepine in male Sprague Dawley (SD) rats and identification of the metabolites formed in plasma and urine.

This chapter pays special attention to some pharmacokinetic aspects which are not clearly defined in literature.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Table 5.1: Reagents and drugs

DRUG / CHEMICAL	SOURCE
Pooled plasma (K2 EDTA) from male Sprague Dawley rat	Bioreclamation IVT (Westbury, NY)
Cryptolepine hydrate and glyburide	Sigma Aldrich (St. Louis, MO)
Acetonitrile, formic acid, and dimethyl sulfoxide	Thermo Fisher Scientific Inc. (Rockford, IL)

5.2.2 Methods

5.2.2.1 Pharmacokinetic Studies of cryptolepine in SD rat

Animals

Male Sprague Dawley rats, 320-340 g, purchased from Harlan Laboratories, Inc, Dublin, VA were used in the study. The experimental protocol was approved by NIBR-CA IACUC (approval August 2014). All rats were housed under constant environmental conditions (21 ± 2°C, 40 ± 5% humidity, and 12-hour light-dark cycles) and were allowed free access to food and water. The rats were fasted overnight (20 h) before oral dosing but thereafter allowed free access to food.

Administration of cryptolepine

To evaluate the pharmacokinetics, cryptolepine was administered intravenously and orally at doses of 1 and 5 mg/kg, respectively (n= 2 for each route of administration). Cryptolepine was dissolved in 1N NaOH/PEG300/Cremaphor EL/Solutol/phosphate buffered saline (1:30:5:5:59% volume) for intravenous administration. For oral administration, cryptolepine was suspended in Tween 80/methylcellulose/water (0.5:0.5:99.5% volume/weight/volume). Blood samples were collected from the animals at 5, 15, 30 min and 1, 2, 4, 7 and 24 h post intravenous dose and at 15 and 30 min and 1, 2, 4, 7 and 24 h post oral dose. Urine was also collected at 7 and 24 h after both routes of administration. Plasma was obtained from blood samples by centrifugation at 6,700×g for 2 min. Urine samples were diluted in 1 volume of blank rat plasma. All plasma and urine samples were then diluted in 6-fold in acetonitrile containing internal standard (glyburide) and centrifuged (5000×g at

4°C for 30 min) to precipitate proteins. The supernatant samples were then analysed by LC-MS/MS.

5.2.2.2 Quantification of Cryptolepine in rat plasma and urine

Concentrations of cryptolepine were determined using LC-MS/MS. The analytical system consisted of an API 4000 instrument mass spectrometer (AB Sciex, Foster City, CA), coupled to an Agilent 1200 system (Agilent Technologies, Inc., Santa Clara, CA) and a CTC HTS PAL autosampler (LEAP Technologies, Carrboro, NC). Analytes in plasma and urine samples were separated using an ACE C18 HPLC column (3 μm, 30 mm x 2.1 mm i.d. MAC-MOD Analytical, Inc. Chadds Ford, PA). The column was eluted using an isocratic gradient over 3.5 minutes with mobile phase consisting of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) at a flow rate of 0.7 mL/min. Cryptolepine was detected by multiple

reaction monitoring (MRM) transition of 233 → 190 under positive ion mode. The internal standard, glyburide, an MRM transition of 494 → 169 was used. Linearity of calibration curves of cryptolepine were confirmed between 0.1 and 5000 ng/ml.

Pharmacokinetic analysis

Non compartmental pharmacokinetic analysis was performed using Phoenix 6.3 (Certara, St Louis, MO, USA) to determine the following pharmacokinetic parameters; area under the plasma concentration time curve and area under the urine concentration time curve (AUC), maximum plasma concentration (C_{max}), time to reach C_{max} (T_{max}), plasma clearance (CL_p), steady state volume of distribution (V_{ss}), elimination half-life of plasma concentration ($t_{1/2}$), mean residence time of plasma concentration (MRT) and oral bioavailability (% F).

5.2.2.3 *In vivo* metabolite profiling in SD rat urine and plasma.

The plasma samples were pooled according to the Hamilton pooling method (Hamilton *et al.*, 1981). Pooled samples are extracted with 2 volumes of chilled acetonitrile with 0.1% formic acid. Thereafter, the supernatants volume was reduced to ca. 30 µL under a gentle stream of nitrogen gas. Pooled urine samples (2 mL) (approximately 20% volume of each time point for each animal) were centrifuged at 4,000 rpm for 3 min to remove any particles.

5.2.2.4 Analytical Methods for Metabolite Profiling in SD rat urine and plasma

Sample analysis and metabolite identification were carried out on a Thermo LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) interfaced with a 3 Ti high-performance LC pump and CTC PAL autosampler (LEAP Technologies, Carrboro, NC). The analytes were separated on a Waters Symmetry C18 analytical column (5µm particle size, 2.1 x 150 mm; Waters, Milford, MA) with a 35-min gradient elution method. The mobile phases consisted of (A) 10 mM ammonium formate in MS-grade water and (B) MS-grade acetonitrile. The sample aliquots were eluted at a flow rate of 0.25 mL/min with 10% B over 5 min. Mobile phase (B) was gradually increased to 90% over 24 min. The column was then returned to 10% B and held for 3 mins before the next injection.

5.3 RESULTS

5.3.1 Pharmacokinetic profile of cryptolepine in rat

The pharmacokinetics of cryptolepine in rat were investigated after intravenous and oral administration. The plasma concentration-time profiles for cryptolepine are shown in Figures 5.1

and 5.2. The pharmacokinetic parameters as determined by area under the curve (AUC) after *i.v* and *p.o* administration of cryptolepine at 1 and 5 mg/kg, respectively are shown in Tables 5.2 and 5.3.

Following intravenous administration of 1 mg/kg cryptolepine to rats, plasma concentrations were quantifiable up to and including the 7 h sampling time point and intravenous pharmacokinetics were calculated based on a Tlast of 7 h. Plasma exposure was low and the average plasma clearance ($CL_{p)}$ was high indicating very fast elimination from the systemic circulation. Cryptolepine was extensively distributed as indicated by the high volume of distribution (Vss) resulting in a moderate plasma $t_{1/2}$. Exposure of cryptolepine in urine was considerably higher than in plasma (145-fold and 173-fold higher in rats 1 and 2, respectively) indicating that renal elimination of unchanged drug is a key pathway of elimination. After oral administration of 5 mg/kg cryptolepine, plasma concentrations were quantifiable up to and including the 7h sampling time point and oral pharmacokinetics were calculated based on a Tlast of 7 h. Absorption was fast with Cmax values of 28 nM and 104 nM reached within 0.25 and 0.5 h in animal 3 and 4 respectively. Plasma exposure and oral bioavailability were low whilst exposure of cryptolepine in urine was considerably higher than in plasma (168-fold and 230-fold higher in rats 3 and 4, respectively).

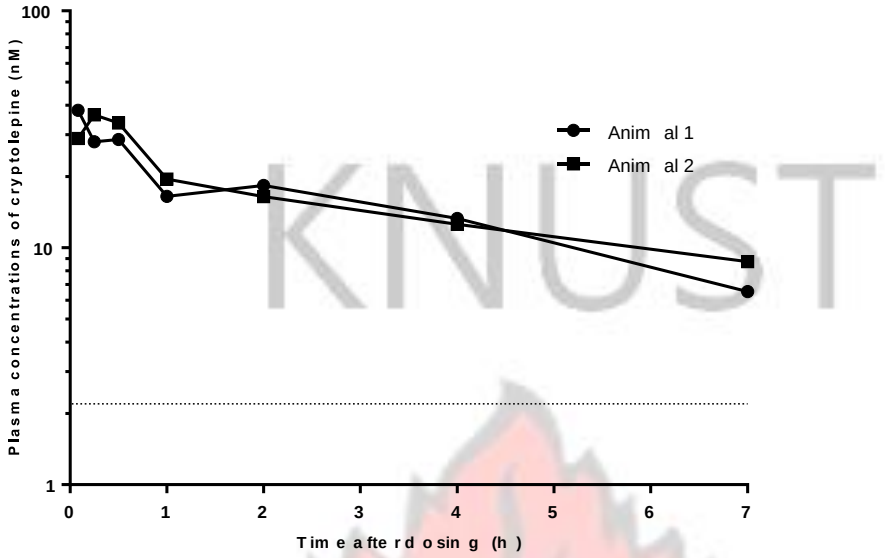


Figure 5.1: Plasma concentration-time profiles of cryptolepine after single intravenous (1 mg/kg) administrations to Sprague Dawley rats. The lower dotted line indicates the lower levels of quantification (LLOQ) (2.2 nM) of LC–MS/MS method for quantifying cryptolepine in the rat plasma.

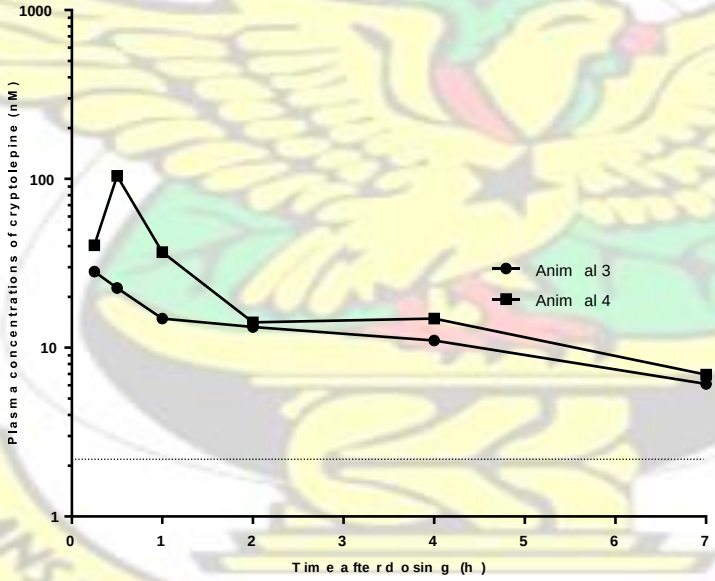


Figure 5.2: Plasma concentration-time profiles of cryptolepine after single oral (5 mg/kg) administrations to Sprague Dawley rats. The lower dotted line indicates the lower levels of quantification (LLOQ) (2.2 nM) of LC–MS/MS method for quantifying cryptolepine in the rat plasma.

Table 5.2: Pharmacokinetic parameters of cryptolepine in rats following intravenous administration at a dose of 1 mg/kg.

Animal ID	Plasma AUC_{0-7} (nM.h)	CL_p (mL/min/kg)	V (L/Kg)	$t_{1/2}$ (h)	MRT (h)	Urine AUC_{0-24} (nM.h)
1	106	521	147	3	5	15418
2	109	402	180	6	7	18940

AUC_{0-7} or AUC_{0-24}, the area under the concentration-time curve based on the last sampling time point in plasma and urine respectively; CL_p, plasma clearance; V, volume of distribution; $t_{1/2}$, elimination halflife; T_{max}; MRT, mean residence time.

Table 5.3: Pharmacokinetic parameters of cryptolepine in rats following oral administration at a dose of 5 mg/kg.

Animal ID	Plasma AUC_{0-7} (nM.h)	Cmax (nM)	Tmax (h)	F %	Urine AUC_{0-24} (nM.h)
3	83	28	0.3	16	13980
4	146	104	0.5	28	33551

AUC_{0-7} or AUC_{0-24}, the area under the concentration-time curve based on the last sampling time point in plasma and urine respectively; Cmax, maximum plasma concentration; Tmax, time to reach Cmax; F, oral bioavailability.

5.3.2 Metabolites in rat urine and plasma

Plasma and urine, pooled during 24 h after 5 mg/kg *p.o*. administration of cryptolepine were collected. The metabolites found in urine and plasma were similar to those of the *in vitro* hepatocyte incubation in the preceding chapter. A total of three metabolites were detected in SD rat urine (M2, M6, and M9) and one metabolite was detected in plasma (M6). Metabolism included; oxidation (M6), oxidation and glucuronidation (M2), and di-hydroxylation followed by glucuronidation (M9). Metabolites, M2 and M9 were observed in urine only. All proposed metabolite structures are presented in Figures 5.3 to 5.7 and Table 5.4.

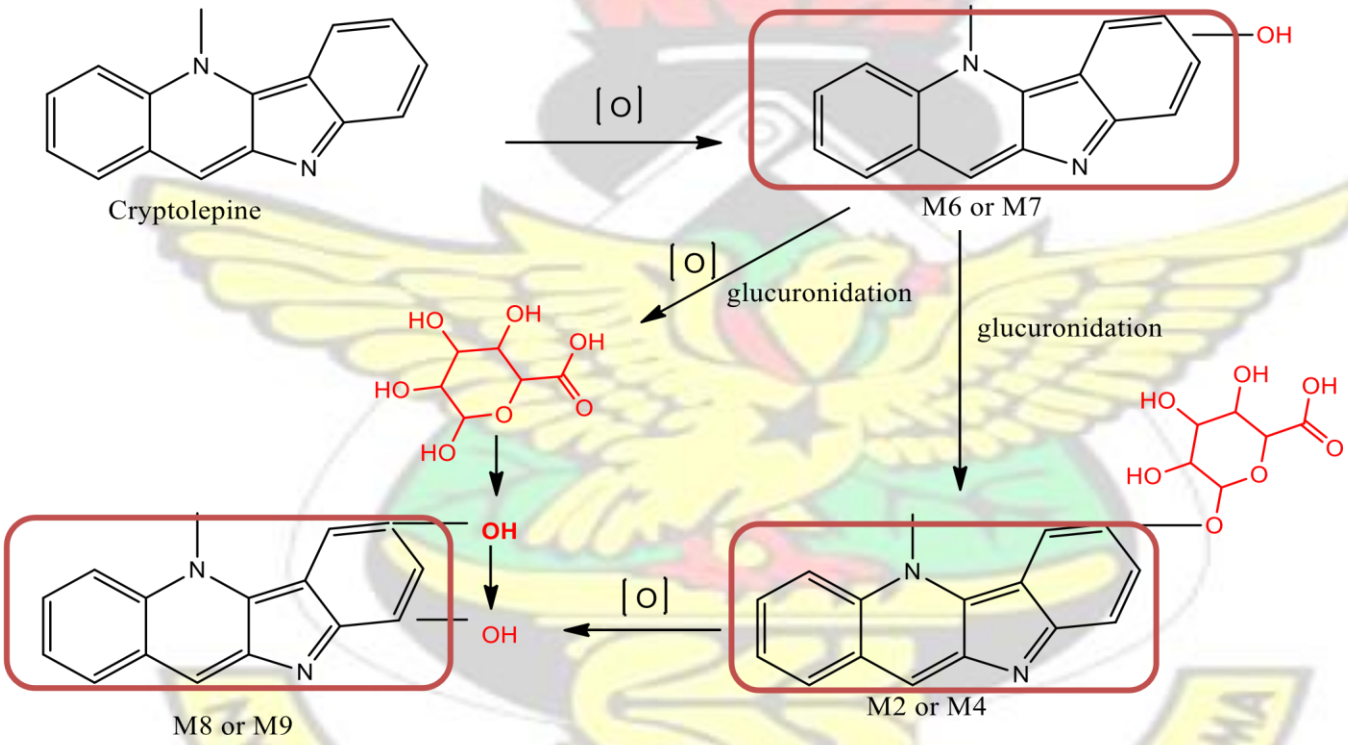
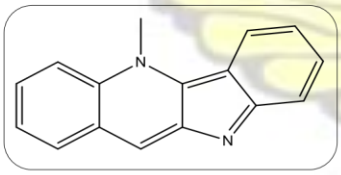


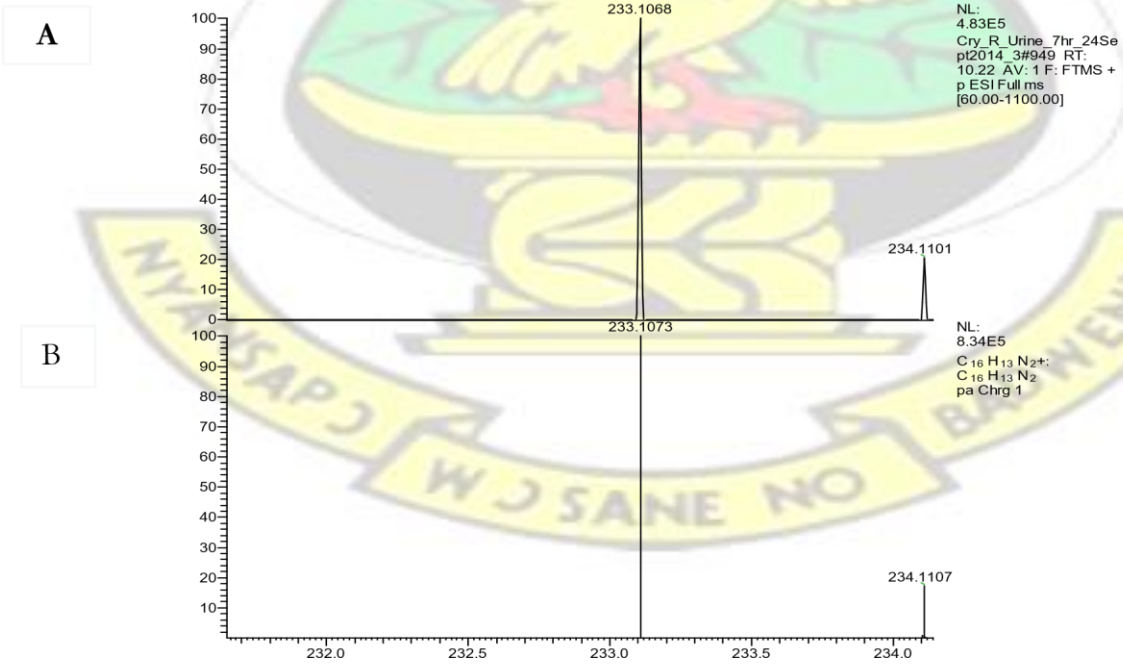
Figure 5.3: Proposed Metabolic Pathways of cryptolepine in rat urine and plasma following a 5 mg/kg *p.o* dose.

Table 5.4: Summary of Metabolites of Cryptolepine in Sprague Dawley Rat Urine and Plasma (5 mg/kg) dose.

Met ID	$[M+H]^+$	Retention time (min)	Urine	Plasma
Cryptolepine	233.1069	10.82	+	+
M2 or M4	425.1342	6.17	+	--
M6 or M7	249.1024	12.08	+	+
M8 or M9	441.1289	13.87	+	--

+ Or - indicates presence or absence in samples, respectively.





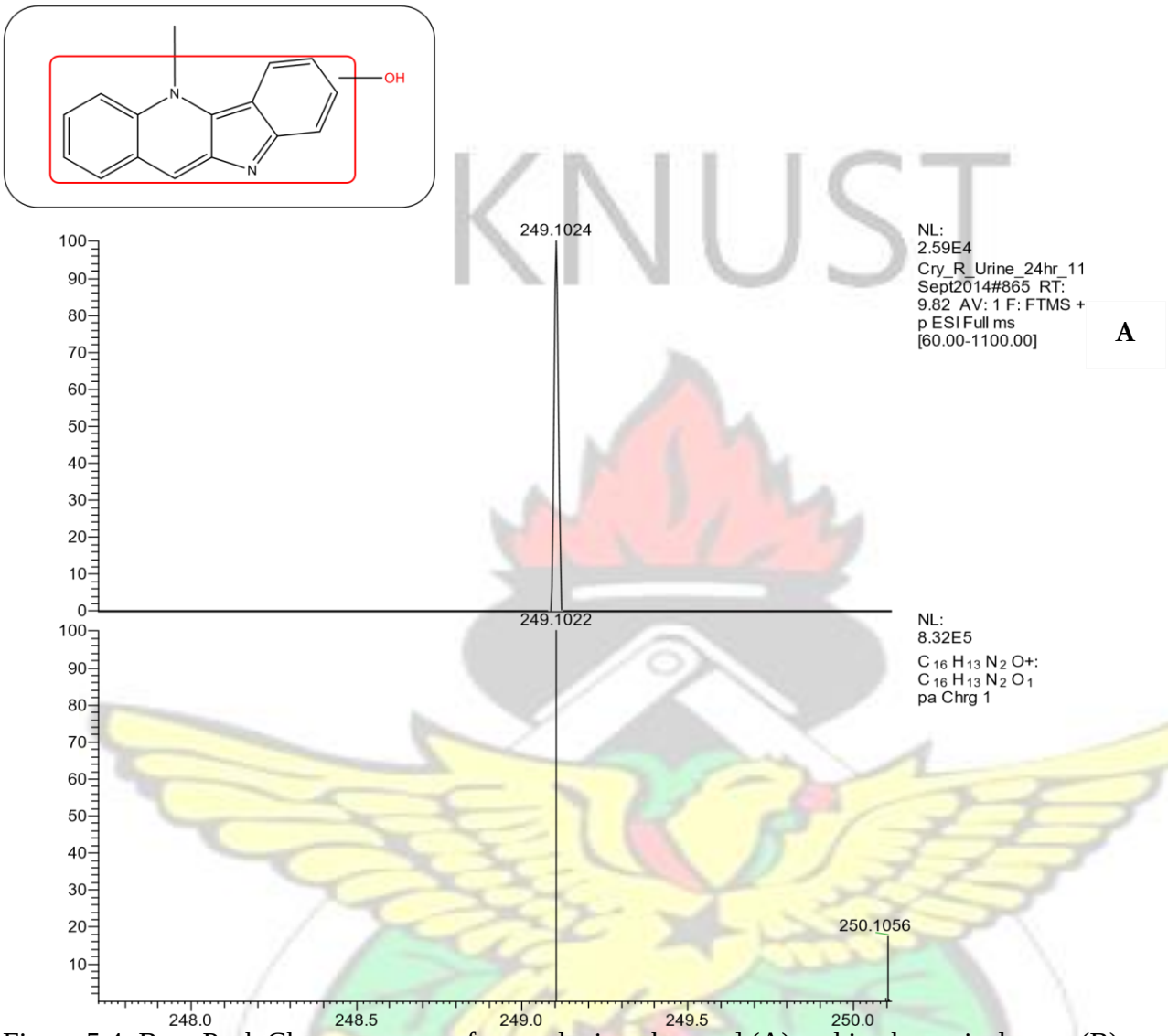
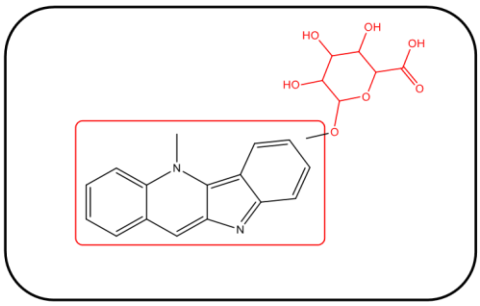


Figure 5.4: Base Peak Chromatogram of cryptolepine observed (A) and its theoretical mass (B) at m/z 233.1069 in pooled rat urine.

Figure 5.5: Base Peak Chromatogram of cryptolepine observed (A) and its theoretical mass (B) for M6 or M7 at m/z 249.1024 in pooled rat urine and plasma. Note: M6 or M7 was also observed in human and rat hepatocytes.

A



m/z

B

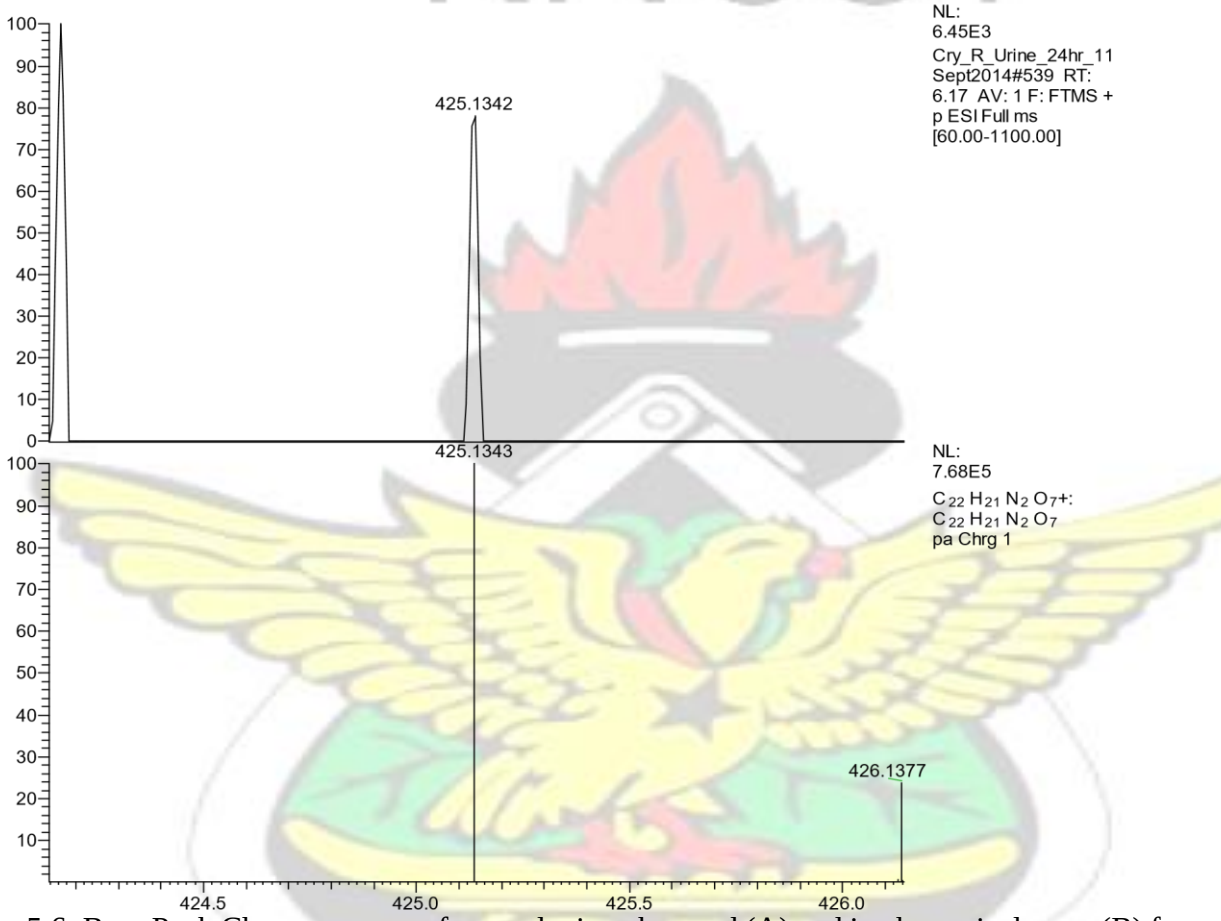


Figure 5.6: Base Peak Chromatogram of cryptolepine observed (A) and its theoretical mass (B) for M2 or M4 at m/z 425.1342 in pooled rat urine. M2 or M4 was also observed in human and rat hepatocytes.

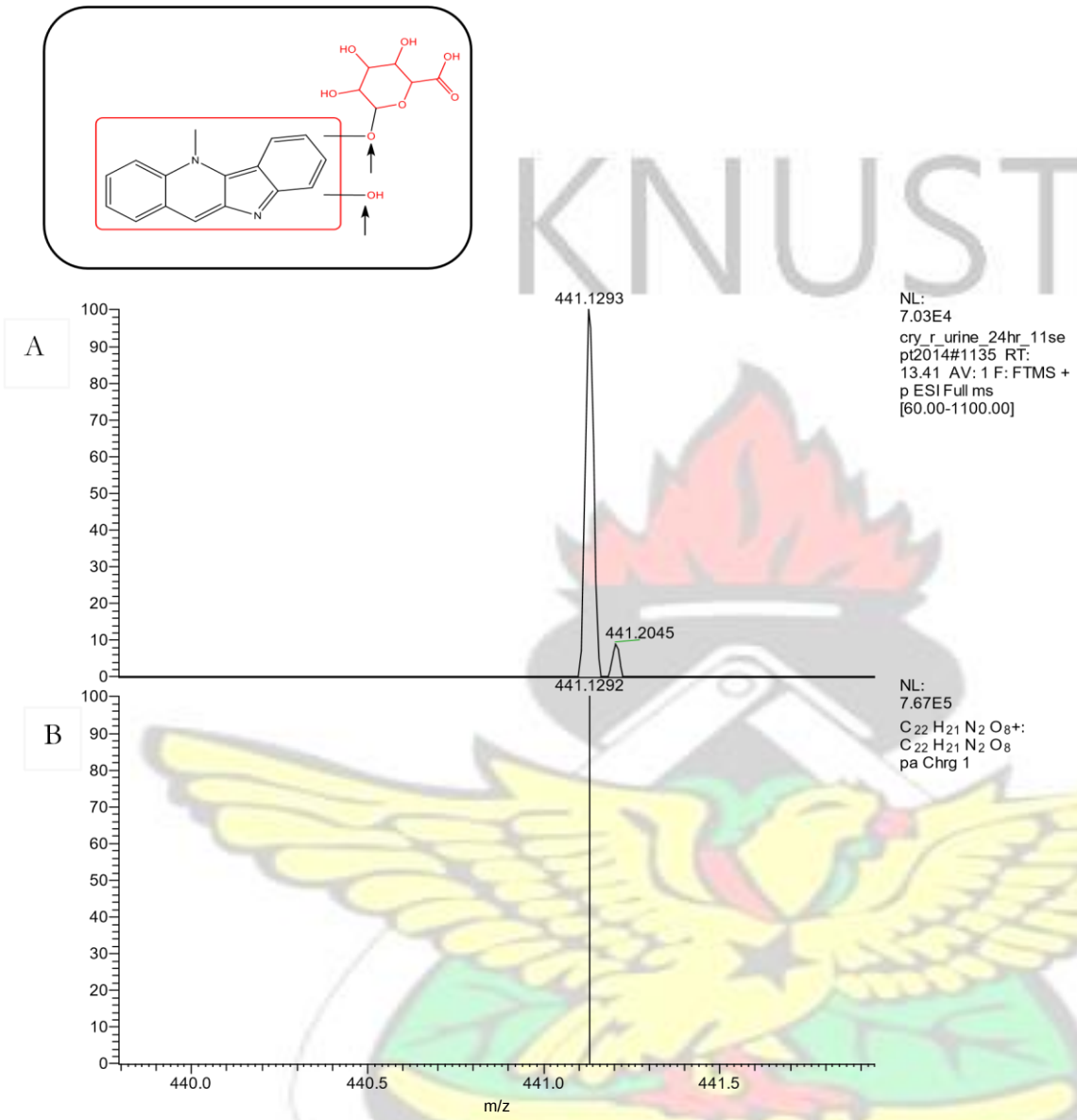


Figure 5.7: Base Peak Chromatogram of cryptolepine observed (A) and its theoretical mass (B) for M8 or M9 at m/z 441.1293 in pooled rat urine. Note: MS/MS could not be obtained (mass accuracy of 0.2 ppm). M8 or M9 was also observed in human and rat hepatocytes.

5.4 DISCUSSION

The results in the above investigation shed light on the pharmacokinetics of cryptolepine in rats. Following intravenous and oral administration of cryptolepine, rats exhibited high plasma clearance, extensive distribution, low oral bioavailability and considerably higher exposure in urine compared to plasma suggesting that renal elimination of unchanged drug is a key pathway of elimination. This is not surprising given the physicochemical properties of cryptolepine, a small molecular weight, relatively hydrophilic and basic molecule (data not shown), properties of which may lead to a predisposition for renal elimination of parent drug unchanged. The pH of renal tubules is typically more acidic than plasma and basic drugs tend to be less extensively reabsorbed compared to acidic drugs. Additionally, charged molecules tend to have high rates of excretion in urine due to active tubular secretion (Caldwell *et al.,* 1995).

The low bioavailability could be due to the high first pass effect of cryptolepine. Kuntworbe *et al.* (2013) showed a wide distribution of cryptolepine into the spleen, heart, lungs, kidney and liver coupled with a much slower clearance from these tissues. From the rat pharmacokinetic data, it is clear that cryptolepine, the major alkaloid from the popular antimalarial plant *Cryptolepis sanguinolenta* could quickly be cleared from the plasma, possibly accumulating in vital organs and extending plasma residence time particularly important for the clearance of erythrocytic stage parasites. However, these observations in the rat cannot be extrapolated to humans. Cryptolepine is a member of the quinoline antimalarial compounds which act mainly within the acidic food vacuole of the plasmodium parasite. Effective accumulation of these compounds across the parasite membrane into the food vacuole has been attributed to the presence of basic nitrogenous groups in their molecules. This may account for the improved efficacy observed with these compounds (Egan *et al.*, 1999). The early Tmax provides an added advantage as an antimalarial

agent with rapid parasite clearance. Unchanged excretion of cryptolepine in the rats' urine was less than 0.1% of the administered dose, indicating that clearance mainly occurs by metabolism and/or biliary excretion.

Metabolite (M6) was also detected in rat plasma samples after oral administration of cryptolepine. Aldehyde oxidase metabolism has in the past caused difficulties in drug development, as it is often more active in human than in preclinical species. The aldehyde oxidase and N-glucuronidation pathways could potentially result in significantly different clearance and/or disposition in human compared to rat, depending on the extent of formation and stability of each metabolite in humans *in vivo*. The dihydroxylation metabolites (M3/M5) are likely to be formed via initial epoxide formation, indicating the possibility of cytochrome P450 metabolism of cryptolepine**.** In this study, plasma concentrations of cryptolepine were quantifiable up to 7 h and this could affect the calculated pharmacokinetics parameters. Although the present investigation provides a reasonably comprehensive review of the preliminary assessment of rat PK, further PK studies using a higher dose of cryptolepine and more rats will provide a much detail rat pharmacokinetics.

5.5 CONCLUSION

This study revealed substantial information about the pharmacokinetic profile and metabolism of cryptolepine in rat. Rat PK studies showed low oral exposure resulting from a high first pass metabolism, an early Tmax and a low Cmax. A moderate half-life, very high volume of distribution and clearance exceeding the hepatic blood flow was observed after a single intravenous administration. The metabolites found in the rat urine and plasma were similar to those found in the *in vitro* hepatocyte incubation.

6.0 CHAPTER SIX

SAFETY EVALUATION OF THE COMBINATION OF CRYPTOLEPIS OR CRYPTOLEPINE WITH SOME ARTEMISININ DERIVATIVES

6.1 INTRODUCTION

The estimated LD_{50} of *Cryptolepis sanguinolenta* (CPS) is well over 5000 mg/kg (unpublished data) suggesting a wide margin of safety. The scourging effect of malaria coupled with the high cost of conventional antimalarial drugs and perceived effectiveness of herbal preparations in the subSaharan region have motivated the frequency of exposure to these preparations. Several herbal preparations containing CPS have emerged on the Ghanaian hospitals, markets and herbal shops for the treatment of malaria. Some patients consuming these herbal preparations may not disclose this information to their physician or pharmacist when they visit allopathic health facilities thus increasing the potential of herb-drug interactions. Although cryptolepine has shown well documented extensive pharmacological activities, it is necessary to evaluate its possible toxicity when used alone or in the combination with the popular artemisinin derivatives on the market.

In previous chapters, cryptolepine and the artemisinin derivatives showed synergistic antimalarial activity *in vivo* in *P.berghei* infected mice and *in vitro* in *P. falciparum* infected red blood cells. The present study thus sought to investigate the potential acute toxicity of the aqueous root extract of *C. sanguinolenta* and the major alkaloid cryptolepine in combination with the artemisinin derivatives in rodent models. The respective combinations of the aqueous root extract of *Cryptolepis sanguinolenta* and its major alkaloid cryptolepine with the artemisinin derivatives seeks to provide a safe and affordable alternative Artemisinin-Combination Therapy (ACT). With some of the nonartemisinin components of ACT not well-tolerated by patients, toxicity testing is a matter of necessity to provide scientific validation and safety documentation for the use of these new combinations in the treatment of malaria.

Agbedahunsi *et al.* (2004) recommended that all natural products used in therapeutics must be subjected to safety tests by the same methods for new scientific drugs. For this reason, the safety

of the combination of cryptolepine/cryptolepis and the artemisinin derivatives needed to be ascertained.

In this chapter, the acute toxicity of cryptolepis /cryptolepine and their combination with artemisinin derivatives were reported. General conditions, mortality, body weight, food and water consumption, haematology, blood biochemistry and their effects on organ weight and histopathological findings were examined.

6.2 MATERIALS AND METHODS

6.2.1 Animals

Healthy Sprague-Dawley rats (180-250 g) of both sexes, aged 8 weeks old were purchased from Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, and maintained in the animal house of the Department of Pharmacology, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.

They were housed in groups of 5 in stainless cages (34×47×18 cm) with soft wood shavings as bedding, fed with normal commercial pellet diet (Agricare, Tanoso, Kumasi) and given water *ad libitum.* All animals used were pharmacologically naïve and used once only. All procedures employed were in accordance with the National Institute of Health Guidelines for Care and Use of laboratory animals and were approved by the Ethical Review Committee of the Faculty of Pharmacy and Pharmaceutical Sciences, KNUST, Ghana (PHARM/ETHIC/ET173/15).

6.2.2 Drugs/ Chemicals

Table 6.0: Reagent and Drugs

DRUG/CHEMICAL	SOURCE
Formalin and ethanol	Sigma (St. Louis, MO, U.S.A.)
Artesunate	Guilin Pharmaceutical, China
Artemether 20 mg +Lumefantrine 120 mg	Novartis Pharma AG., Basel, Switzerland
Artemether	Novartis Pharma AG., Basel, Switzerland

6.3 TOXICITY ASSESSMENT IN HEALTHY SPRAGUE-DAWLEY RATS.

6.3.1 Assessment of the effects of cryptolepis, cryptolepine, artemether, and artesunate on blood and blood cells.

Healthy male Sprague-Dawley rats (180-250 g) were divided into nine groups (n=5). The groups received 25, 50 and 100 mg/kg body weight of cryptolepine orally daily for a period of three days. Another set of groups received 250, 500 and 1000 mg/kg body weight of cryptolepis orally for 3 days. Two respective groups (n=5) also received 4 mg/kg of artesunate and 50 mg/kg of artemether orally daily for a 3 day period. The control group received equal volume of distilled water for the period of the experiment.

The drugs were prepared such that not more than 2 mL was given orally. The animals were monitored closely for signs of toxicity. At the end of the three day period the rats were sacrificed by cervical dislocation, the jugular vein was cut and blood allowed to flow freely into tubes which contained ethylenediaminetetraacetic acid (EDTA) as coagulant. Haematological parameters

including red blood cells (RBC), platelets, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), Haemoglobin, Haematocrit, White blood cells and lymphocytes were determined by an automatic analyser (Sysmex XT-2000 L CELL-DYN 1700, Abbot Diagnostics Division, Abbot Laboratories, Abbot Park, Illinois, USA).

6.3.2 Assessment of the concurrent administration of Cryptolepine/Cryptolepis with Artesunate/ Artemether on blood and blood cells.

Healthy male Sprague-Dawley rats (150-250 g) were obtained and divided into twelve groups (n=5).

The groups received either 25, 50 and 100 mg/kg body weight of cryptolepine in combination with 4 mg/kg of artesunate orally daily for a period of three days. The next 3 groups received 250, 500 and 1000 mg/kg body weight of cryptolepis in combination with 4 mg/kg of artesunate orally for 3 days.

Six other groups of five Sprague Dawley rats in each group received 25, 50 and 100 mg/kg body weight of cryptolepine each in combination with 50 mg/kg of artemether orally daily for a period of three days. The final group received 250, 500 and 1000 mg/kg body weight of cryptolepis each in combination with 50 mg/kg of artemether orally for 3 days.

The same dosage was repeated for cryptolepine and cryptolepis as above for 7 days and each group of animals treated with artesunate (4 mg/kg) for 3 days after the 7-day duration.

Two groups of five animals each also received 4 mg/kg of artesunate and 50 mg/kg of artemether orally daily for a 3 day period. The control group received equal volume of distilled water for the period of the experiment.

The drugs were prepared such that not more than 2 mL was given orally. The animals were monitored closely for signs of toxicity. At the end of the three or seven day period the rats were sacrificed by cervical dislocation, the jugular vein was cut and blood allowed to flow freely into tubes which contained ethylenediaminetetraacetic acid (EDTA) as coagulant. Blood samples were treated as described in section 6.3.1.

6.3.3 Effects on serum biochemical parameters in rats.

After the treatment in sections 6.3.1 and 6.3.2, blood was collected into tubes without anticoagulant. The serum was separated from the clotted blood by centrifugation at 750×g. Serum determination of total proteins, total albumin, direct bilirubin, indirect bilirubin, albumin, globulin, were performed by using an automated analyser ATAC 8000 Random Access Chemistry System (Elan Diagnostics, Smithfield, RI, USA). Levels of the liver enzymes alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), gamma glutamyl aminotransferase (GGT), creatinine (CREAT), urea and uric acid were also determined.

6.3.4 Evaluation of Potential Target Organ in Rats

At the end of the 3 and 10 day period of treatment in all experiments, selected organs including the spleen, liver, kidneys and stomach were excised, trimmed of fat and connective tissue, blotted dry and weighed on a balance. The relative weights of the organs were calculated and expressed as g percent of body weight.

6.3.5 Histopathological Analysis

Portions of the tissue from the liver, kidney and stomach were used for histopathological examination. Tissues were fixed in 10% buffered formalin (pH 7.2) and dehydrated through a

series of ethanol solutions (70, 90, 95 and 100%), embedded in paraffin and routinely processed for histological analysis.

Sections of 2 μm thickness were cut and stained with haemotoxylin-eosin for examination. The stained tissues were observed under an Olympus microscope (BX-51) and photographed by a charecoupled device (CCD) camera.

6.4 RESULTS

EFFECT OF TREATMENTS ON HEMATOLOGICAL PARAMETERS

6.4.1 Effects of cryptolepine and the concomitant administration of cryptolepine and artesunate/artemether on haematological parameters.

The effect of the 3-day oral administration of cryptolepine and the concomitant administration with artesunate (4 mg/kg) and artemether (50 mg/kg) on the haematological parameters is presented in Tables 6.1, 6.2 and 6.3. Haematological values of treated rats were not significantly different from those of the control group at all the doses tested (25 - 100 mg/kg) except for the mean corpuscular

volume (MCV) and the mean corpuscular haemoglobin concentration (MCHC) that was significantly decreased in the group treated concomitantly with cryptolepine (100 mg/kg) and artesunate (4 mg/kg) (Table 6.2).

The group treated with cryptolepine for 7 days showed a significant decrease in the red blood cell (RBC) and haematocrit (HCT) levels. The groups treated with artesunate for 3 days after the 7 day cryptolepine treatment also showed a significant elevation in the mean corpuscular haemoglobin concentration (MCHC) at 50 mg/kg and 100 mg/kg (Table 6.5).

Table 6.1: Effects of cryptolepine on the haematological indices of Sprague-Dawley rats treated for 3 days.

PARAMETERS	CONTROL	25 mg/kg	50 mg/kg	100 mg/kg
WBC (/µL)	9.83±1.63	10.9±0.82	7.17±0.94	6.93±0.62
RBC (/µL)	6.41±0.45	7.75±0.15	5.89±0.29	5.52±0.50
HGB (g/dL)	11.63±0.93	13.73±0.31	10.67±0.64	10.17±0.99
HCT (%)	39.27±1.68	43.17±0.71	33.87±1.56	32.1±2.62
MCV (fL)	61.7±2.19	57.77±1.51	57.53±0.15	58.3±0.98
MCH (pg)	18.13±0.69	17.73±0.30	18.07±0.26	18.4±0.52

MCHC (g/dL)	29.43±1.15	31.8±0.21	31.4±0.48	31.57±0.62
PLT (/µL)	516.33±76.50	588±133.12	603.67±149.86	681.67±114.57
LYMP (%)	72±4.18	75.53±3.93	73.83±1.34	79.37±7.54

Values are expressed as means ± SEM (n=5), compared to the control by the Newman Keuls test

Table

6.2: Effects of the concomitant administration of cryptolepine and artesunate 4 mg/kg on the haematological indices of Sprague-Dawley rats treated for 3 days. Cryptolepine was combined with a fixed dose of artesunate (4 mg/kg).

PARAMETERS	Cryptolepine (mg/kg)			
	CONTROL	25 mg/kg	50 mg/kg	100 mg/kg
WBC (/µL)	9.83±1.63	8.37±1.54	13.03±2.28	12.73±1.04
RBC (/µL)	6.41±0.45	6.86±0.26	6.58±0.24	6.47±0.29
HGB (g/dL)	11.63±0.93	12.07±0.20	11.77±0.23	11.93±0.12
HCT (%)	39.27±1.68	38.4±1.07	37.3±0.90	35.3±1.15
MCV (fL)	61.7±2.19	56.03±0.63	56.77±0.72	54.63±0.68*
MCH (pg)	18.13±0.69	17.67±0.44	17.9±0.37	18.53±0.69
MCHC (g/dL)	29.43±1.15	31.47±0.44	31.57±0.45	33.87±0.83*
PLT (/µL)	516.33±76.50	821.33±9.02	725.67±67.65	843.67±44.67
LYMP (%)	72±4.18	75.73±2.80	77.38±1.73	83.43±2.84

Values are expressed as means ± SEM (n=5), (*) indicates significance (P< 0.05), compared to the control by the Newman Keuls test

Table 6.3: Effects of the concomitant administration of cryptolepine and artemether (50 mg/kg) on the haematological indices of Sprague-Dawley rats treated for 3 days. Cryptolepine was combined with a fixed dose of artemether (50 mg/kg).

PARAMETERS	Cryptolepine (mg/kg)			
	CONTROL	25 mg/kg	50 mg/kg	100 mg/kg
WBC (/µL)	8.63±0.15	11.37±3.30	5.53±1.15	10.63±0.30
RBC (/µL)	8.32±0.25	7.36±0.78	6.52±0.15	7.24±0.59
HGB (g/dL)	14.7±0.26	13.37±0.64	11.3±0.43	12.87±0.72
HCT (%)	51.27±0.44	44.73±3.11	36.3±1.18	40.63±2.19
MCV (fL)	61.77±1.76	61.27±2.29	55.8±2.16	56.5±3.15
MCH (pg)	17.70±0.56	18.43±1.21	17.37±0.55	17.87±0.58
MCHC (g/dL)	28.7±0.26	29.97±0.94	31.17±0.58	31.7±0.74
PLT (/µL)	793.33±94.34	752±45.57	922±217.57	761±110.92
LYMP (%)	76.5±1.39	75.5±0.23	53.17±6.84	77.97±2.18

Values are expressed as means ± SEM (n=5), compared to the control by the Newman Keuls test

Table

6.4: Effects of cryptolepine on the haematological indices of Sprague-Dawley rats treated for 7days.

PARAMETERS	CONTROL	25 mg/kg	50 mg/kg	100 mg/kg
RBC (/µL)	8.12±0.48	7.88±0.23	6.99±0.51	6.32±0.21*
HGB (g/dL)	13.6±0.53	13.13±0.22	11.4±0.89	11.3±0.21
HCT (%)	46.77±3.29	45.13±1.11	38.97±2.79	36.97±1.30*
MCV (fL)	57.53±0.633	57.3±1.36	55.76±1.00	58.47±1.30
MCH (pg)	16.80±0.40	16.67±0.28	15.87±0.27	17.87±0.27
MCHC (g/dL)	29.20±1.00	29.10±0.31	28.47±0.27	30.63±0.72
PLT (/µL)	1023±161.10	532±33.71	1078±460.19	877±262.41

Table 6.5: Effects of the concomitant administration of cryptolepine (pre-treated for 7days) and a fixed dose of artesunate (4 mg/kg) (treated for 3days) on the haematological indices on Sprague-Dawley rats.

		Cryptolepine (mg/kg)		
PARAMETERS	**CONTROL**	**25 mg/kg**	**50 mg/kg**	**100 mg/kg**
RBC (/µL)	8.12±0.48	8.21±0.41	7.69±0.13	7.35±0.20
HGB (g/dL)	13.6±0.53	14.03±0.13	3.67±0.43	13.33±0.37
HCT (%)	46.77±3.29	45.5±0.71	42.83±1.50	41.97±0.93
MCV (fL)	57.53±0.633	55.57±1.85	55.7±1.15	57.1±0.27
MCH (pg)	16.8±0.40	17.2±0.92	17.8±0.32	18.13±0.03
MCHC (g/dL)	29.2±1.00	30.87±0.66	31.93±0.12*	31.73±0.17*
PLT (/µL)	1023±161.10	977.67±103.64	918±296.50	1030.33±69.09

Values are expressed as means ± SEM (n=5), (*) indicates significance (P< 0.05), compared to the control by the Newman Keuls test

6.4.2 Effects of cryptolepis (CPS) and the concomitant administration of cryptolepis and artesunate/artemether on haematological parameters

The effect of the 3-day oral administration of cryptolepis and the concomitant administration with artesunate (4 mg/kg) and artemether (50 mg/kg) on the haematological parameters is presented in Tables 6.6, 6.7 and 6.8, respectively. Haematological values of treated rats were not significantly different from those of the control group at all doses tested (250 - 1000 mg/kg) (Tables 6.9 and 6.10) except for the mean corpuscular volume and the mean corpuscular haemoglobin concentration for the group treated with cryptolepis (250 mg/kg and 500 mg/kg) for 7 days followed by artesunate (4 mg/kg). The group of animals treated with either artesunate or artemether only showed no significant difference from the group treated with distilled water (Table 6.11 and 6.12).

Table

6.6: Effects of cryptolepis on the haematological indices of Sprague-Dawley rats treated for 3 days.

PARAMETERS	CONTROL	250 mg/kg	500 mg/kg	1000 mg/kg
WBC (/µL)	9.83±1.63	11.23±1.20	7.90±1.45	13.97±2.54
RBC (/µL)	6.41±0.45	7.20±0.30	7.31±0.09	6.58±0.23
HGB (g/dL)	11.63±0.93	12.81±0.14	12.83±0.29	12.27±0.37
HCT (%)	39.27±1.68	40.53±0.61	41.47±0.75	37.57±0.84
MCV (fL)	61.72±2.19	58.13±1.63	56.73±0.42	57.13±0.77
MCH (pg)	18.13±0.69	18.36±0.60	17.57±0.18	18.67±0.35
MCHC (g/dL)	29.43±1.15	31.57±0.14	30.93±0.22	32.67±0.44
PLT (/µL)	516.33±76.50	822.33±101.74	505±82.14	783.67±95.12
LYMP (%)	72.10±4.18	76.77±0.98	77.03±0.32	73.73±4.52

Values are expressed as means ± SEM (n=5), compared to the control by the Newman Keuls test

Table

6.7: Effects of the concomitant administration of cryptolepis and artesunate (4 mg/kg) on the haematological indices on Sprague-Dawley rats treated for 3 days. Cryptolepis was combined with a fixed dose of artesunate (4 mg/kg).

PARAMETERS	Cryptolepis (mg/kg)			
	CONTROL	250 mg/kg	500 mg/kg	1000 mg/kg
WBC (/µL)	9.83±1.63	9.6±1.00	8.87±1.22	8.90±1.60
RBC (/µL)	6.41±0.45	7.05±0.66	6.72±0.28	6.62±0.31
HGB (g/dL)	11.63±0.93	13.03±0.71	12.57±0.43	11.72±0.73
HCT (%)	39.27±1.68	40.5±4.22	38.53±1.27	37.27±1.21
MCV (fL)	61.7±2.19	57.33±0.69	57.4±0.57	56.33±0.75
MCH (pg)	18.13±0.69	18.67±0.75	18.7±0.32	17.63±0.61
MCHC (g/dL)	29.43±1.15	32.53±1.70	32.63±0.37	31.37±1.26
PLT (/µL)	516.33±76.50	794.33±140.21	1034.67±228.66	808±279.20
LYMP (%)	72.40±4.18	82.97±4.29	80.63±1.07	80.53±3.45

Values are expressed as means ± SEM (n=5), compared to the control by the Newman Keuls test

Table

6.8: Effects of concomitant administration of cryptolepis and artemether 50 mg/kg on the haematological indices of Sprague-Dawley rats treated for 3 days. Cryptolepis was combined with a fixed dose of artemether (50 mg/kg).

PARAMETERS	Cryptolepis (mg/kg)			
	CONTROL	250 mg/kg	500 mg/kg	1000 mg/kg
WBC (/µL)	8.63±0.15	8.83±0.56	9.43±2.19	8.57±1.10
RBC (/µL)	8.32±0.25	6.19±0.41	7.02±0.23	7.84±0.76
HGB (g/dL)	14.70±0.26	11.73±0.18	14.43±2.19	13.63±1.09
HCT (%)	51.27±0.44	36.97±0.19	40.17±1.33	45.133±5.35
MCV (fL)	61.77±1.76	60.23±3.57	57.37±3.0	57.37±2.38
MCH (pg)	17.70±0.56	19.1±0.95	17.73±0.59	17.47±0.72
MCHC (g/dL)	28.70±0.26	31.7±0.35	31.01±0.58	30.43±1.07
PLT (/µL)	793.33±94.34	741.01±56.23	922.11±217.57	891.01±166.79
LYMP (%)	76.50±1.39	77.6±3.51	53.17±6.84	52.03±12.40

Values are expressed as means ± SEM (n=5), compared to the control by the Newman Keuls test

6.9: Effects of *Cryptolepis sanguinolenta* on the haematological indices of Sprague-Dawley rats treated for 7days.

Table

PARAMETERS	CONTROL	250 mg/kg	500 mg/kg	1000 mg/kg
RBC (/µL)	8.12±0.48	7.48±0.43	8.11±0.15	7.24±0.65
HGB (g/dL)	13.60±0.53	12.87±0.44	13.81±0.25	12.67±1.03
HCT (%)	46.77±3.29	42.75±1.51	46.77±0.07	41.37±3.47
MCV (fL)	57.53±0.633	57.57±2.27	57.80±1.14	57.21±0.91
MCH (pg)	16.81±0.40	17.27±0.49	17.07±0.42	17.53±0.38
MCHC (g/dL)	29.20±1.00	30.01±0.40	29.53±0.52	30.63±0.18
PLT (/µL)	1023.01±161.10	699.67±47.07	651.67±284.25	983.33±67.84

Table 6.10: Effects of Cryptolepis (Pre-treated for 7 days) and artesunate 4 mg/kg (Treated for 3 days) on the haematological indices on Sprague-Dawley rats.

CONTROL	250 mg/kg	500 mg/kg	Cryptolepis (mg/kg) PARAMETERS 1000 mg/kg
RBC (/µL)	8.12±0.48	6.95±0.28	7.19±0.08	6.92±0.08
HGB (g/dL)	13.60±0.53	12.63±0.49	13.03±0.47	12.53±0.32
HCT (%)	46.77±3.29	39.67±1.72	40.93±1.85	40.37±1.48
MCV (fL)	57.53±0.633	57.03±0.84	56.97±2.06	58.33±1.50
MCH (pg)	16.80±0.40	18.17±0.07	18.17±0.74	18.1±0.31
MCHC (g/dL)	29.21±1.00	31.87±0.37*	31.87±0.43*	31.07±0.55
PLT (/µL)	1023.01±161.10	811.00±173.62	765.67±153.05	991.11±71.97

Values are expressed as means ± SEM (n=5), (*) indicates significance (P< 0.05), compared to the control by the Newman Keuls test

Table

6.11: Effects of Artemether 50 mg/kg on haematological indices of Sprague-Dawley rats treated for 3 days

PARAMETERS	CONTROL	Artemether 50 mg/kg
WBC (/µL)	8.63±0.15	13.13±3.77
RBC (/µL)	8.32±0.25	6.83±0.52
HGB (g/dL)	14.70±0.26	12.9±0.95
HCT (%)	51.27±0.44	42.07±3.4
MCV (fL)	61.70±2.19	61.50±1.01
MCH (pg)	17.70±0.56	18.87±0.29
MCHC (g/dL)	28.70±0.26	30.73±0.24
PLT (/µL)	793.33±94.34	1071.33±48.48
LYMP (%)	76.50±1.39	63.67±4.23

Values are expressed as means ± SEM (n=4), compared to the control by the Newman Keuls test

Table 6.12: Effects of Artesunate 4 mg/kg on haematological indices of Sprague-Dawley rats treated for 3 days.

PARAMETERS	CONTROL	ARTESUNATE (4 mg/kg)
RBC (/µL)	8.12±0.48	7.97±0.19
HGB (g/dL)	13.6±0.53	14.07±0.21
HCT (%)	46.77±3.29	44.47±0.41
MCV (fL)	57.53±0.633	55.77±0.84
MCH (pg)	16.8±0.40	17.67±0.41
MCHC (g/dL)	29.2±1.00	30.67±0.32

Table

PLT (/µL)	1023±161.10	890.33±131.97

Values are expressed as means ± SEM (n=4), compared to the control by the Newman Keuls test.

EFFECT OF TREATMENTS ON SERUM BIOCHEMISTRY PARAMETERS

6.4.3 Effects of cryptolepine and the concomitant administration of cryptolepine and artesunate/artemether on serum biochemistry parameters.

Biochemical profiles of the animals treated with cryptolepine, and in combination with artesunate or artemether are presented in Table 6.13 to Table 6.17 below. The oral administration of cryptolepine only and with artesunate (up to a dose of 100 mg/kg body weight of cryptolepine and 4 mg/kg of artesunate) did not cause any significant changes in the serum proteins, bilirubin, liver enzymes, creatinine, urea, and uric acid. However, there was a significant decrease ($p<0.05$) in the globulin and total protein compared to the control group after 7 days of treatment with 100 mg/kg of cryptolepine (Table 6.16). The levels of AST in treated animals were significantly elevated in all combinations of cryptolepine and the artemisinins (artesunate [$p<0.01$] and artemether [$p<0.05$]) compared to the control. In the case of ALT and ALP, however, levels in rats were not significantly different from the treated groups in all doses compared to the control (Tables 6.14, 6.15 and 6.17).

Table

6.13: Effects of cryptolepine on the biochemical parameters on Sprague-Dawley rats treated for 3 days.

PARAMETERS	CONTROL	25 mg/kg	50 mg/kg	100 mg/kg
ALB (g/L)	35.77±2.54	32.80±2.38	31.14±1.37	32.66±1.19
GLB (g/L)	40.19±6.67	46.92±1.69	46.67±5.46	48.98±2.64
T. PRO (g/L)	75.96±4.2	79.73±3.76	77.81±6.65	81.65±2.76
ALK.PHOS (U/I)	288.67± 223.81	5.67±3.84	14.33±11.10	75.67±65.22
ALT (U/I)	103.00±10.01	104.57±14.8	88.17±5.22	70.27±14.28
AST (U/I)	182.47±20.75	239.47±17.88	192.87±10.52	207.87±23.49
D. BIL (µmol/L)	1.61±0.29	1.42±0.18	1.66±0.12	1.56±0.07
IND. BIL (µmol/L)	1.91±0.63	1.40±0.37	1.42±0.08	1.17±0.04
T.BIL (µmol/L)	3.52±2.48	2.82±0.55	3.08±0.19	2.73±0.11
GGT (U/I)	3.50±2.48	0.97±1.28	3.6±1.37	3.5±1.35
CREAT. (µmol/L)	55.27±4.70	61.37±3.79	56.17±6.32	56.2±9.98
UREA (mmol/L)	6.72±1.69	5.18±1.25	7.31±2.03	7.81±1.18
URIC ACID (mmol/L)	119.67±16.37	161±32.88	123.33±7.54	134.67±21.99

Values are expressed as means ± SEM (n=5), compared to the control by the Newman Keuls test

Table

6.14: Effects of 3 days concomitant treatment with cryptolepine and artesunate 4 mg/kg on the biochemical parameters on Sprague-Dawley rats. Cryptolepine was combined with a fixed dose of artesunate (4 mg/kg).

PARAMETERS	CONTROL	Cryptolepine (mg/kg) 25 mg/kg	50 mg/kg	100 mg/kg
ALB (g/L)	35.77±2.54	33.85±0.74	35.11±0.59	29.67±1.30
GLB (g/L)	40.19±6.67	50.05±3.02	51.69±3.05	49.91±1.48
T. PRO (g/L)	75.96±4.2	83.90±3.02	86.8±3.41	79.58±2.56
ALK.PHOS (U/I)	288.67± 223.81	48.67±14.26	19±14.11	-7.1±3.0
ALT (U/I)	103.00±10.01	72.10±4.50	111.93±6.80	99±9.84
AST (U/I)	182.47±20.75	191.93±16.41	212.43±20.65	212.93±20.11
D. BIL (µmol/L)	1.61±0.29	1.59±0.12	2.02±0.60	1.94±0.34
IND. BIL (µmol/L)	1.91±0.63	1.42±0.05	1.91±0.27	2.50±0.60
T.BIL (µmol/L)	3.52±0.92	3.00±0.13	3.93±0.25	4.44±0.96
GGT (U/I)	3.50±2.48	2.97±1.19	1.37±0.44	-1.97±0.98
CREAT. (µmol/L)	55.27±4.70	56.37±4.68	65.3±5.83	70.23±2.68
UREA (mmol/L)	6.72±1.69	9.56±1.73	7.09±0.49	6.10±0.91
URIC ACID (mmol/L)	119.67±16.37	161±15.50	163.67±19.34	238.33±54.57

Values are expressed as means ± SEM (n=5), compared to the control by the Newman Keuls test

Table

6.15: Effects of the concomitant administration of cryptolepine and artemether (50 mg/kg) on the biochemical parameters on Sprague-Dawley rats treated for 3 days. Cryptolepine was combined with a fixed dose of artemether (50 mg/kg).

PARAMETERS	Cryptolepine (mg/kg)			
	CONTROL	25 mg/kg	50 mg/kg	100 mg/kg
ALB (g/L)	39.21±0.53	38.04±1.88	34.81±1.37	34.56±1.21
GLB (g/L)	60.67±3.80	48.37±3.44	53.03±2.29	53.53±5.18
T. PRO (g/L)	99.88±4.32	86.40±5.32	87.84±3.60	88.09±6.07
ALK.PHOS (U/I)	39.01±14.73	84.0±35.13	45±15.18	27±1.2
ALT(U/I)	3.40±0.65	2.93±8.23	4.57±1.41	3.37±1.77
AST(U/I)	35.87±12.04	145.5±51.37*	170±15.44*	175.97±11.65*
D. BIL (µmol/L)	2.16±0.05	2.35±0.08	7.10±5.26	4.54±2.11
IND. BIL (µmol/L)	1.45±0.25	1.16±0.071	-4.39±5.05	-0.61±1.1.42
T.BIL (µmol/L)	3.61±0.24	3.51±0.14	2.72±0.21	3.93±0.75
GGT(U/I)	4.37±0.87	3.53±0.74	4.2±2.21	5.17±0.57
CREAT. (µmol/L)	61.90±1.21	54.53±4.40	40.6±8.23	56.93±5.20
UREA (mmol/L)	12.46±1.03	9.41±0.48	12.31±5.31	8.66±0.73
URIC ACID (mmol/L)	119.67±16.37	135.01±10.61	122.67±14.53	125.50±9.5

Values are expressed as means ± SEM (n=5), (*) indicates significance (P< 0.05), compared to the control by the Newman Keuls test

Table

6.16: Effects of cryptolepine on the biochemical parameters of Sprague-Dawley rats treated for 7days.

PARAMETERS	CONTROL	25 mg/kg	50 mg/kg	100 mg/kg
ALB (g/L)	36.09±1.56	35.44±0.87	35.93±0.62	32.57±1.04
GLB (g/L)	46.5±0.74	45.41±2.06	40.48±2.64	33.26±1.59*
T. PRO (g/L)	82.68±2.33	80.85±2.89	76.40±3.08	65.83±2.61**
ALK.PHOS (U/I)	51.67±6.53	59.33±1.33	56±7.0	45.33±5.63
ALT (U/I)	100.87±2.69	103.7±4.32	98.4±2.25	100.57±12.12
AST (U/I)	79.4±46.51	77.83±35.05	125.73±27.40	104.43±3.83
D. BIL (µmol/L)	1.077±0.02	1.13±0.03	1.17±0.09	1.08±0.07 **IND. BIL**
(µmol/L)	0.82±0.07	1.01±0.10	1.18±0.09	1.19±0.23
T.BIL (µmol/L)	1.89±0.05	2.15±0.08	2.35±0.09	2.27±0.22
GGT (U/I)	0.77±0.44	0.47±0.47	1.0±0.91	0.90±0.31

Values are expressed as means ± SEM (n=5), (*) indicates significance (P< 0.05), **p < 0.01; compared to the control by the Newman Keuls test

Table 6.17: Effects of cryptolepine (pre-treated for 7 days) and artesunate 4mg/kg (treated for 3 days) on the biochemical parameters on Sprague-Dawley rats.

PARAMETERS	CONTROL	Cryptolepine (mg/kg) 25 mg/kg	50 mg/kg	100 mg/kg
ALB (g/L)	36.09±1.56	34.56±1.29	36.39±1.74	35.88±1.14
GLB (g/L)	46.5±0.74	52.17±4.3	42.93±1.02	40.82±2.49
T. PRO (g/L)	82.68±2.33	86.73±3.02	79.32±1.04	76.70±2.56
ALK.PHOS (U/I)	51.67±6.53	55.67±17.16	41.33±9.13	36±12.50
ALT (U/I)	100.87±2.69	77.17±3.94	70.8±10.3	101.33±15.73
AST(U/I)	79.40±46.51	199.87±11.16**	250±10.67**	310.03±12.54**
D. BIL (µmol/L)	1.08±0.02	1.38±.12	1.38±0.07	1.51±0.03 **IND.**
BIL (µmol/L)	0.82±0.07	1.05±0.18	1.27±0.17	0.92±0.08
T.BIL (µmol/L)	1.89±0.05	2.43±0.23	2.65±0.10	2.42±0.11
GGT (U/I)	0.77±0.44	1.17±0.14	0.92±0.71	1.2±0.54

Values are expressed as means ± SEM (n=5), (**) indicates significance (P< 0.01), (***) indicates significance (P< 0.001) compared to the control by the Newman Keuls test

6.4.4 Effects of cryptolepis and the concomitant administration of cryptolepis and artesunate/artemether on biochemical parameters.

Biochemical profiles of the cryptolepis treated animals are presented in Tables 6.18 to 6.24. The levels of the liver marker enzyme, GGT, in animals concomitantly treated with cryptolepis and artesunate were significantly lower ($p>0.05$) in doses compared to groups in control (Table 6.19). The levels of liver AST in all groups treated with cryptolepis (250 to 1000 mg/kg) concurrently with artemether (50 mg/kg) were also significantly elevated ($p>0.01$) compared to the control (Table 6.20).

The 7 day treatment with cryptolepis showed a significant increase in the indirect bilirubin levels in the 1000 mg/kg dose compared to the control while groups that received a 3 additional day treatment with artesunate showed an elevated AST levels ($p>0.001$) in all treated groups. The direct and total bilirubin levels were also significantly higher ($p>0.05$) in the 1000 mg/kg treated group compared to the control group (Table 6.22).

A 3 day treated with artemether ($p>0.05$) and artesunate ($p>0.001$) showed a significant elevation in the AST level (Table 6.23 and 6.24).

Table 6.18: Effects of cryptolepis on the biochemical parameters on Sprague-Dawley rats treated for 3 days.

PARAMETERS	CONTROL	250 mg/kg	500 mg/kg	1000 mg/kg
ALB (g/L)	35.77±2.54	34.10±0.60	31.7±3.18	32.57±0.69
GLB (g/L)	40.19±6.67	51.10±2.88	55.26±4.94	50.21±4.16
T. PRO (g/L)	75.96±4.2	85.20±3.44	86.89±8.08	82.78±4.31
ALK.PHOS (U/I)	28.67± 3.81	18.33±13.08	17±6.24	34.33±14.88
ALT (U/I)	103±10.01	96.43±11.75	88.97±7.78	77.90±6.66
AST (U/I)	182.47±20.75	228.9±38.49	151.13±39.69	188.03±9.23
D. BIL (µmol/L)	1.61±0.29	1.51±0.29	1.65±0.13	1.80±0.05
IND. BIL (µmol/L)	1.91±0.63	1.71±0.21	1.29±0.19	1.49±0.08
T.BIL(µmol/L)	3.52±2.48	3.22±0.34	2.94±0.29	3.29±0.05
GGT (U/I)	3.50±2.48	0.47±0.47	6.30±1.75	3.37±0.97
CREAT. (µmol/L)	55.27±4.70	57.7±3.50	62.73±5.04	66.73±6.25
UREA (mmol/L)	6.72±1.69	7.35±1.28	5.20±0.31	5.74±0.66
URIC ACID (mmol/L)	119.67±16.37	207.67±41.60	152.33±16.83	127.67±15.41

Values are expressed as means ± SEM (n=5), compared to the control by the Newman Keuls test

Table

6.19: Effects of cryptolepis and artesunate (4 mg/kg) on the biochemical parameters on Sprague-Dawley rats treated for 3 days. Cryptolepis was combined with a fixed dose of artesunate (4 mg/kg).

PARAMETERS	Cryptolepis (mg/kg)			
	CONTROL	250 mg/kg	500 mg/kg	1000 mg/kg
ALB (g/L)	35.77±2.54	33.14±1.21	31.73±1.28	31.36±1.31
GLB (g/L)	40.19±6.67	57.44±4.67	52.07±7.74	40.69±1.20
T. PRO (g/L)	75.96±4.2	90.57±5.46	83.81±6.71	72.05±1.63
ALK.PHOS (U/I)	28.67± 3.81	17±5.39	19±2.72	19.67±3.84
ALT (U/I)	103.00±10.01	8.77±8.95	87.93±0.63	92.87±4.67
AST (U/I)	182.47±20.75	161±48.69	234.9±2.94	275.83±2.56
D. BIL (µmol/L)	1.61±0.29	3.24±2.12	1.79±0.50	2.12±0.35
IND. BIL (µmol/L)	1.91±0.63	1.3±0.77	1.44±0.35	1.73±0.22
T.BIL (µmol/L)	3.52±2.48	4.54±1.35	3.23±0.42	3.85±0.57
GGT (U/I)	3.50±2.48	-4.27±1.21*	-3.67±1.58*	-3.63±0.78*
CREAT. (µmol/L)	55.27±4.70	91.8±25.01	59.4±2.01	56.63±2.23
UREA (mmol/L)	6.72±1.69	7.91±1.68	5.41±0.62	6.94±0.30
URIC ACID (mmol/L)	119.67±16.37	366.33±125.24	218±29.67	243±67.09

Values are expressed as means ± SEM (n=5), (*) indicates significance (P< 0.05), compared to the control by the Newman Keuls test

Table

6.20: Effects of the concomitant administration of cryptolepis and artemether (50 mg/kg) on the biochemical parameters on Sprague-Dawley rats treated for 3 days. Cryptolepis was combined with a fixed dose of artemether (50 mg/kg).

PARAMETERS	Cryptolepis (mg/kg)			
	CONTROL	250 mg/kg	500 mg/kg	1000 mg/kg
ALB (g/L)	39.21±0.53	38.39±1.68	36.43±0.66	38.22±2.88
GLB (g/L)	60.67±3.80	52.6±5.11	51.79±1.06	56.57±4.25
T. PRO (g/L)	99.88±4.32	90.98±6.78	88.21±1.72	94.79±7.11
ALK.PHOS (U/I)	39±14.73	36.33±9.33	26.33±15.21	19±5.50
ALT (U/I)	34±0.65	43.13±21.40	45.5±14.89	17.0±7.17
AST (U/I)	35.87±12.04	195.23±2.03**	199.57±12.31**	173.9±19.35*
D. BIL (µmol/L)	2.16±0.05	2.12±0.09	2.09±0.46	2.52±0.28
IND. BIL (µmol/L)	1.45±0.25	1.29±0.41	1.39±0.38	1.13±0.12
T.BIL (µmol/L)	3.61±0.24	3.40±0.37	3.48±0.58	3.66±0.39
GGT (U/I)	4.37±0.87	3.23±0.50	3.27±1.21	5.03±0.38
CREAT. (µmol/L)	61.9±1.21	44.13±4.08	42.6±4.27	51.73±3.87
UREA (mmol/L)	12.46±1.03	8.55±1.26	6.60±0.32	8.10±0.35
URIC ACID (mmol/L)	247.67±63.53	138.33±35.79	171±26.56	220±47.20

Values are expressed as means ± SEM (n=5), (*) indicates significance (P< 0.05) **p < 0.01, compared to the control by the Newman Keuls test

6.21: Effects of Cryptolepis on the biochemical parameters of Sprague-Dawley rats treated for 7 days.

Table

PARAMETERS	CONTROL	250 mg/kg	500 mg/kg	1000 mg/kg
ALB (g/L)	36.09±1.56	36.89±0.73	39.34±2.85	34.63±1.84
GLB (g/L)	46.5±0.74	46.71±3.21	51.56±2.10	37.84±2.89
T. PRO (g/L)	82.68±2.33	83.60±2.55	90.91±3.84	72.47±4.27
ALK.PHOS (U/I)	51.67±6.53	55.33±9.84	54.67±27.41	51±6.84
ALT (U/I)	100.87±2.69	114.4±10.92	102.9±7.34	85.1±12.20
AST (U/I)	79.4±46.51	60.17±10.88	114.47±100.77	373.83±332.51
D. BIL (µmol/L)	1.077±0.02	1.55±0.22	1.73±0.44	1.10±0.17
IND. BIL (µmol/L)	0.82±0.07	1.3±0.20	1.69±0.24	1.57±0.65
T.BIL (µmol/L)	1.89±0.05	2.85±0.34	3.42±0.60	3.47±0.75
GGT (U/I)	0.77±0.44	1.07±0.28	1.07±1.70	1.02±0.18

Values are expressed as means ± SEM (n=5), (*) indicates significance (P< 0.05), compared to the control by the Newman Keuls test

6.22: Effects of Cryptolepis (Pre-treated for 7 days) and artesunate 4 mg/kg (Treated for 3 days) on the biochemical parameters of Sprague-Dawley rats.

Table

CONTROL	250 mg/kg	500 mg/kg	Cryptolepis (mg/kg) PARAMETERS 1000 mg/kg
ALB (g/L)	36.09±1.56	34.22±1.70	34.89±1.76	36.75±0.71
GLB (g/L)	46.50±0.74	43.60±2.05	40.99±0.13	43.63±2.33
T. PRO (g/L)	82.68±2.33	77.82±2.05	75.88±1.85	80.39±1.63
ALK.PHOS (U/I)	51.67±6.53	42.67±6.62	37.33±5.66	24.67±11.41
ALT (U/I)	100.87±2.69	86.40±1.89	87.33±11.94	81.33±6.87
AST (U/I)	79.40±46.51	1370.93±145.99***	1323.63±39.15***	1401.2±100.84***
D. BIL(µmol/L)	1.077±0.02	1.50±0.09	1.48±0.10	2.24±0.23*
IND. BIL (µmol/L)	0.82±0.07	1.09±0.05	1.28±0.10	1.44±0.15
T.BIL (µmol/L)	1.89±0.05	2.60±0.13	2.75±0.11	3.68±0.36*
GGT (U/I)	0.77±0.44	0.60±0.9	0.92±0.3	1.30±0.9

Values are expressed as means ± SEM (n=5), (*) indicates significance (P< 0.05); ***p < 0.001, compared to the control by the Newman Keuls test

6.23: Effects of artemether 50 mg/kg on biochemical parameters of Sprague-Dawley rats treated for 3 days.

Table

PARAMETERS	CONTROL	Artemether 50 mg/kg
ALB (g/L)	39.21±0.53	38.04±1.88
GLB (g/L)	60.67±3.80	48.37±3.44
T. PRO (g/L)	99.88±4.32	86.40±5.32
ALK.PHOS (U/I)	39±14.73	84.0±35.13
ALT (U/I)	34±0.65	20.93±8.23
AST (U/I)	35.87±12.04	145.5±51.37*
D. BIL (µmol/L)	2.16±0.05	2.35±0.08
IND. BIL (µmol/L)	1.45±0.25	1.16±0.07
T.BIL (µmol/L)	3.61±0.24	3.51±0.14 **GGT**
(U/I)	4.37±0.87	3.53±0.74
CREAT. (µmol/L)	61.9±1.21	54.53±4.40
UREA (mmol/L)	12.46±1.03	9.41±0.48
URIC ACID (mmol/L)	24700.67±6366.53	28421±10452.61

Values are expressed as means ± SEM (n=4), (*) indicates significance (P< 0.05), compared to the control by the Newman Keuls test

Table

6.24: Effects of artesunate 4 mg/kg on biochemical parameters of Sprague-Dawley rats treated for 3 days.

PARAMETERS	CONTROL	ARTESUNATE (4 mg/kg)
ALB (g/L)	36.09±1.56	36.7±1.71
GLB (g/L)	46.5±0.74	42.61±1.43
T. PRO (g/L)	82.68±2.33	79.31±3.11
ALK.PHOS (U/I)	51.67±6.53	67±7.62
ALT (U/I)	100.87±2.69	93.87±17.6
AST (U/I)	79.4±46.51	325.77±2.41***
D. BIL (µmol/L)	1.077±0.02	1.96±0.49
IND. BIL (µmol/L)	0.82±0.07	1.09±0.45
T.BIL (µmol/L)	1.89±0.05	2.87±0.14
GGT (U/I)	0.77±0.44	1.03±0.32

Values are expressed as means ± SEM (n=4), (***) indicates significance (P< 0.001), compared to the control by the Newman Keuls test

6.4.5 Effect of drug treatment on body weight and target organs in rats

There were no significant changes in the body weight of the rats at the end of the experiment at all levels of treatment, neither were the relative weights of selected target organs affected (Table 6.25 to 6.28 and Figure 6.1 to 6.6). The tables show relative organ weight of selected organs after 7 days treatment while the figures show the relative weight of organs after 3 days treatment.

Table 6.25: Effect of cryptolepine on organ weights of rats treated with the compound for 7 days.

Organ	**Relative organ weight**			
	Control	**25 mg/kg**	**50 mg/kg**	**100 mg/kg**
Liver	3.17±0.04	3.55±0.14	3.14±0.15	3.63±0.09
Kidneys	0.50±0.04	0.64±0.04	0.50±0.05	0.60±0.02
Spleen	0.35±0.02	0.57±0.11	0.38±0.04	0.48±0.07

Values are expressed as means ± SEM (n=3), compared to the control by the Newman Keuls test

Table 6.26: Effect of the aqueous extract of Cryptolepis on organ weights of rats treated for 7 days

Organ	Relative organ weight			
	Control	**250 mg/kg**	**500 mg/kg**	**1000 mg/kg**
Liver	3.17±0.04	3.40±0.30	3.03±0.14	3.33±0.19
Kidneys	0.50±0.04	0.61±0.04	0.62±0.02	0.64±0.02
Spleen	0.35±0.02	0.67±0.03	0.56±0.08	0.50±0.10

Table 6.27: Effect of the aqueous extract *Cryptolepis sanguinolenta* (Pre-treated for 7 days) and artesunate 4mg/kg (treated for 3 days) on organ weight of rats.

Organ	Relative organ weight			
	Control	**250 mg/kg**	**500 mg/kg**	**1000 mg/kg**
Liver	3.17±0.04	2.80±0.24	2.94±0.32	2.44±0.23
Kidneys	0.50±0.04	0.57±0.04	0.61±0.07	0.51±0.03
Spleen	0.35±0.02	0.39±0.05	0.43±0.15	0.41±0.08

Values are expressed as means ± SEM (n=3), (*) indicates significance (P< 0.05), compared to the control by the Newman Keuls test

Table 6.28: Effects of cryptolepine (Pre-treated for 7 days) and artesunate 4mg/kg (treated for 3 days) on organ weight of rats.

Organ	Relative organ weight

	Control	25 mg/kg	50 mg/kg	100 mg/kg
Liver	3.17±0.04	3.16±0.014	3.12±0.054	2.71±0.11
Kidneys	0.50±0.04	0.5±0.01	0.65±0.01	0.60±0.04
Spleen	0.35±0.02	0.33±0.01	0.66±0.01	0.52±0.01

Values are expressed as means ± SEM (n=3), compared to the control by the Newman Keuls test

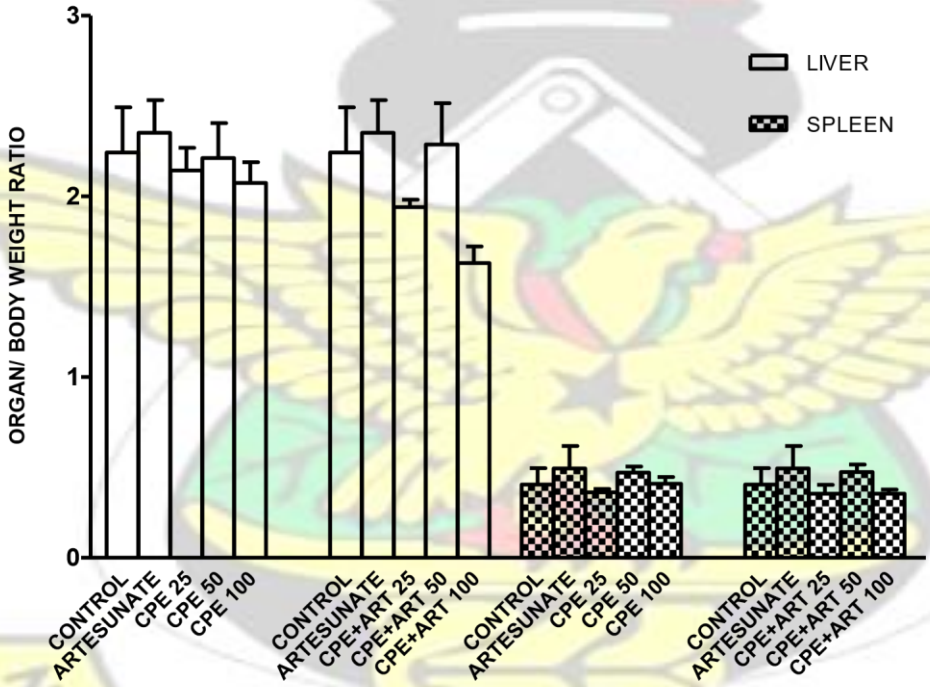


Figure 6.1: Effect of 3 day treatment of cryptolepine and artesunate on liver and spleen of SD rats.

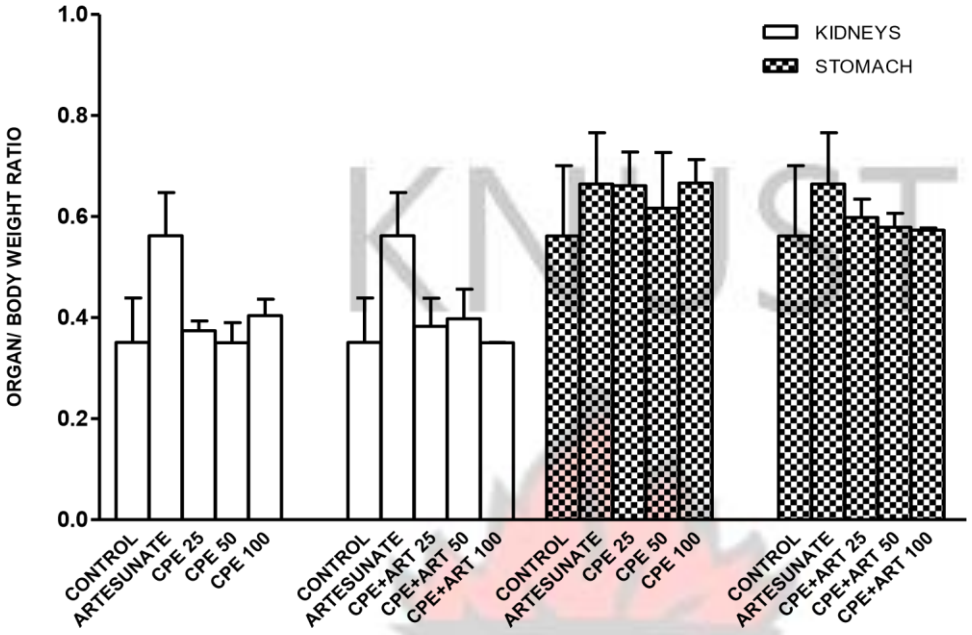


Figure 6.2: Effect of 3 day treatment of cryptolepine and artesunate on stomach and kidneys of SD rats.

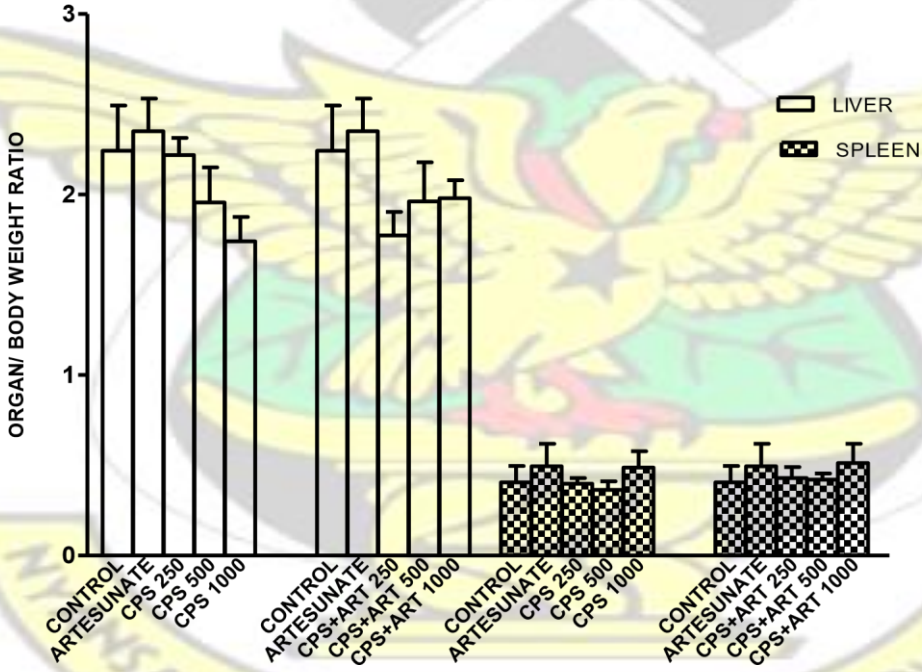


Figure 6.3: Effect of 3 days treatment of cryptolepis and artesunate on liver and spleen of SD rats.

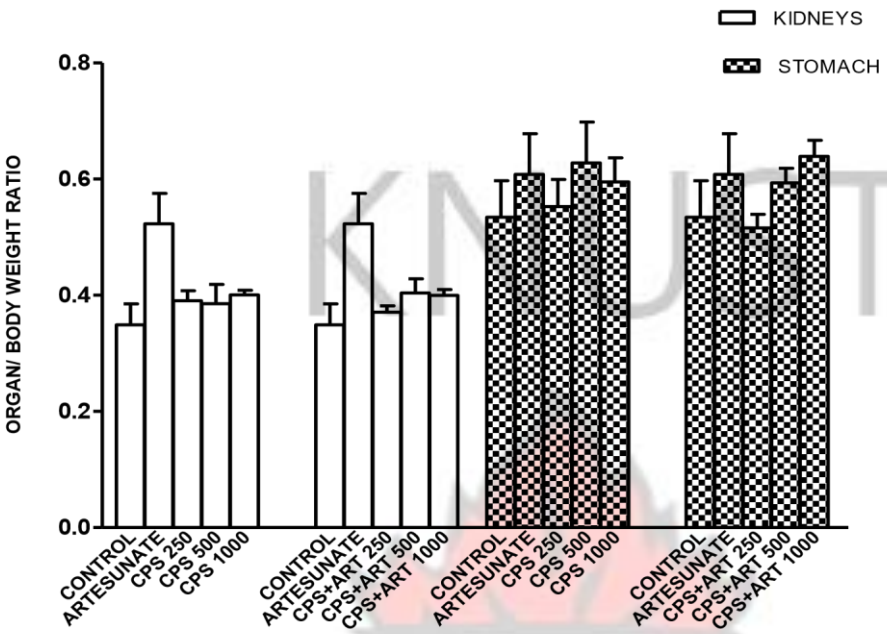


Figure 6.4: Effect of 3 days treatment of cryptolepis and artesunate on stomach and kidneys of SD rats.

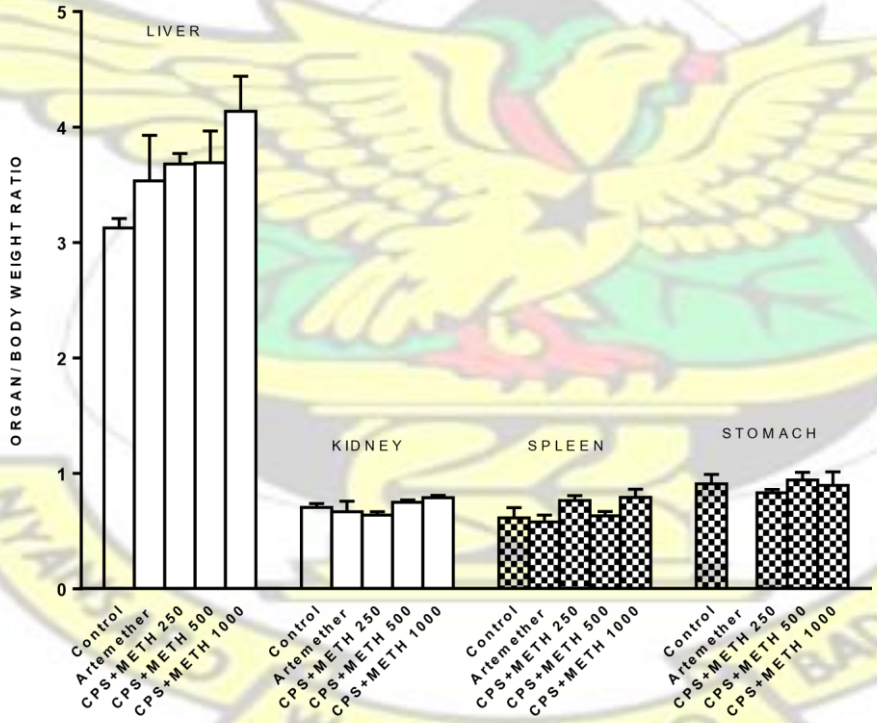


Figure 6.5: Effect of 3 day treatment of cryptolepis and artemether on organ weight

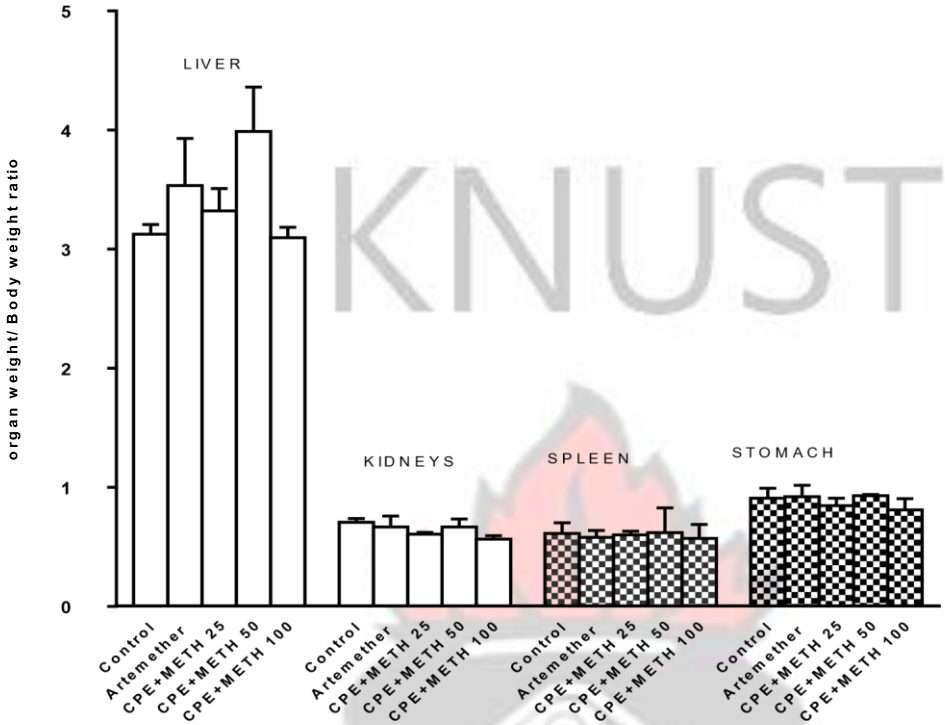


Figure 6.6: Effect of 3 day treatment of cryptolepine and artemether on organ weight.

6.4.6 Histopathology

The livers, kidneys, spleens and stomachs from the control group had normal appearance and histology. Generally, there were no observable changes in the architecture of the organs of treated animals compared to the control (Figure 6.7 to 6.10). The histology of the liver and kidney were consistent with the normal ALT, alkaline phosphate, bilirubin, creatinine and urea levels in the serum.

A - C o n tro l **B - A rtesu n ate 4 m g /kg ***

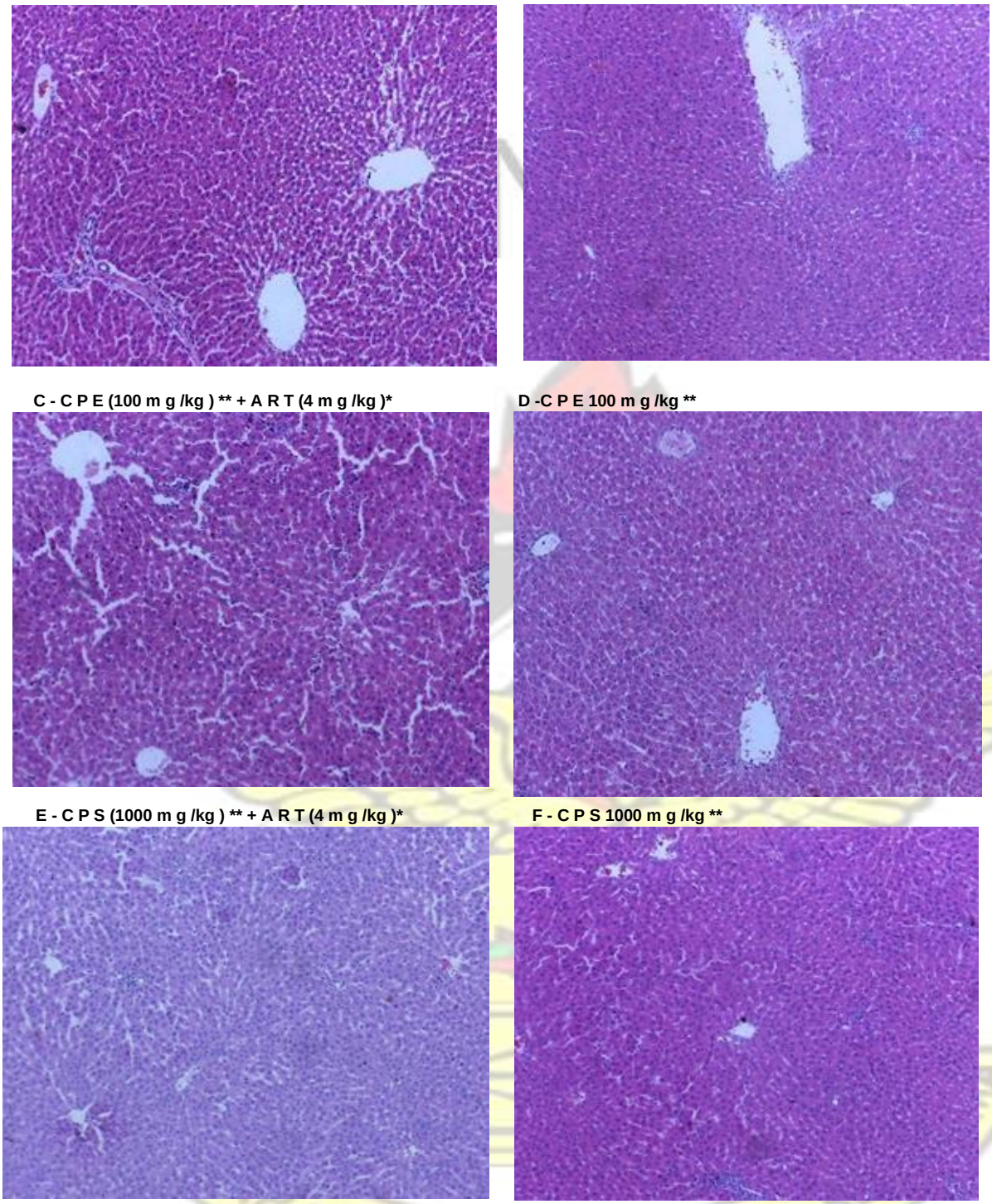


Figure 6.7: Photomicrograph (×100) showing histopathological slides of the livers of cryptolepine (CPE), *Cryptolepis (CPS)* or artesunate (ART, 4 mg/kg) treated rats.

* 3 days treatment ** 7 days treatment

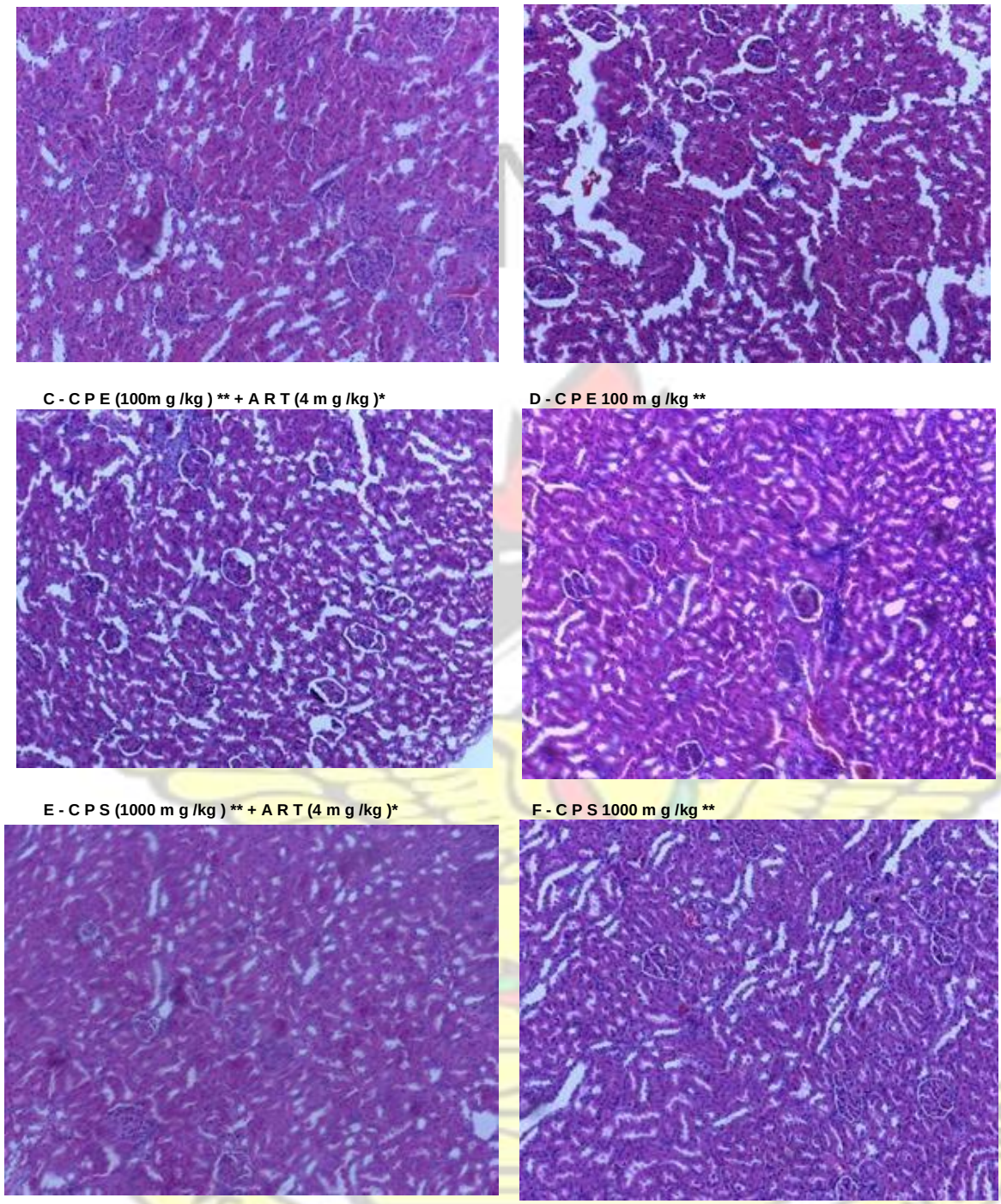


Figure 6.8: Photomicrograph (×100) showing histopathological slides of the kidneys of cryptolepine (CPE), *Cryptolepis* (CPS) or artesunate (ART, 4 mg/kg) treated rats.

* 3 days treatment ** 7 days treatment.

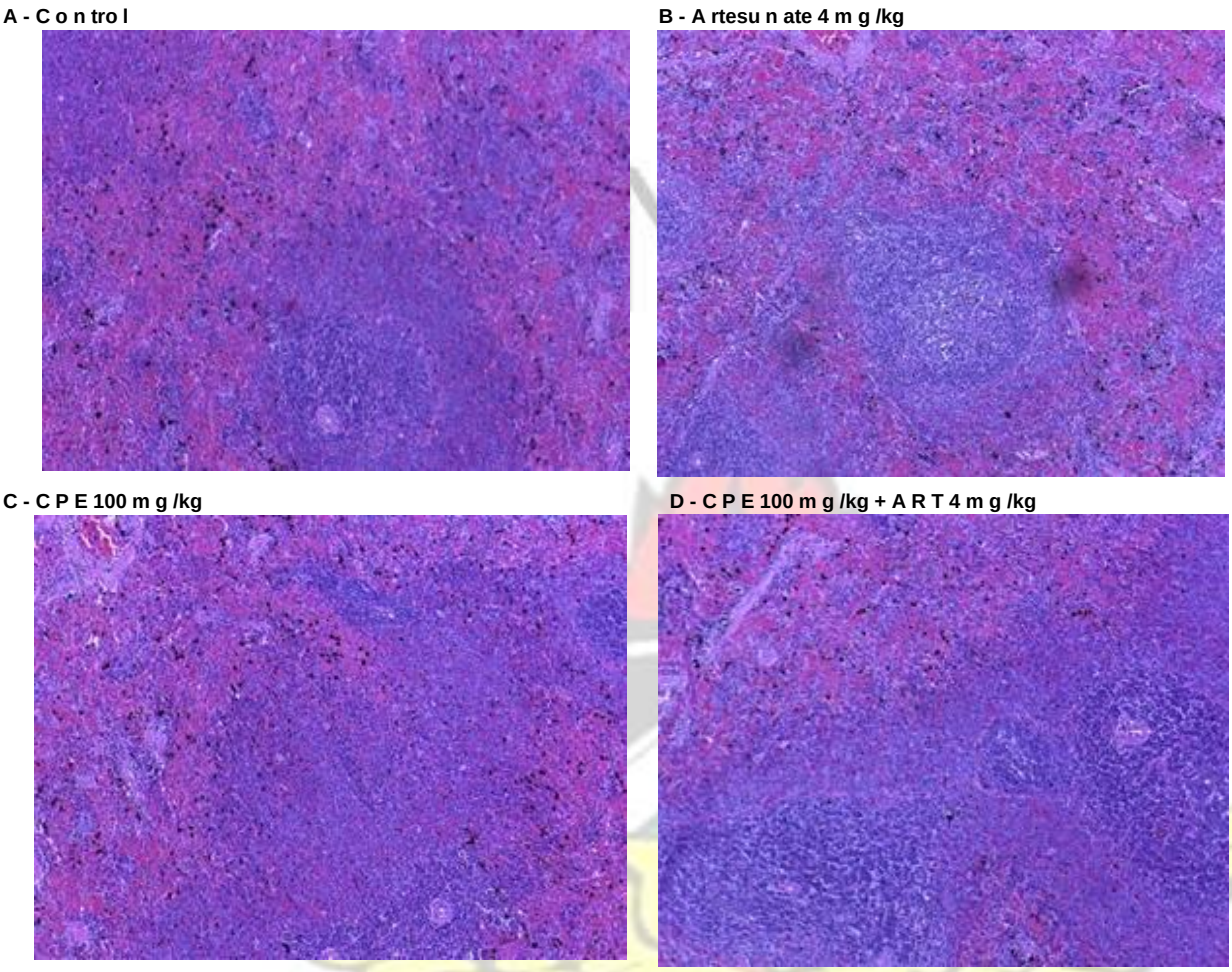


Figure 6.9: Photomicrograph (×100) showing histopathological slides of the spleen of cryptolepine (CPE), *Cryptolepis (CPS)* or artesunate (ART, 4 mg/kg) treated rats. All treatment were for three days.

A- Control

B- Artesunate 4 mg/kg

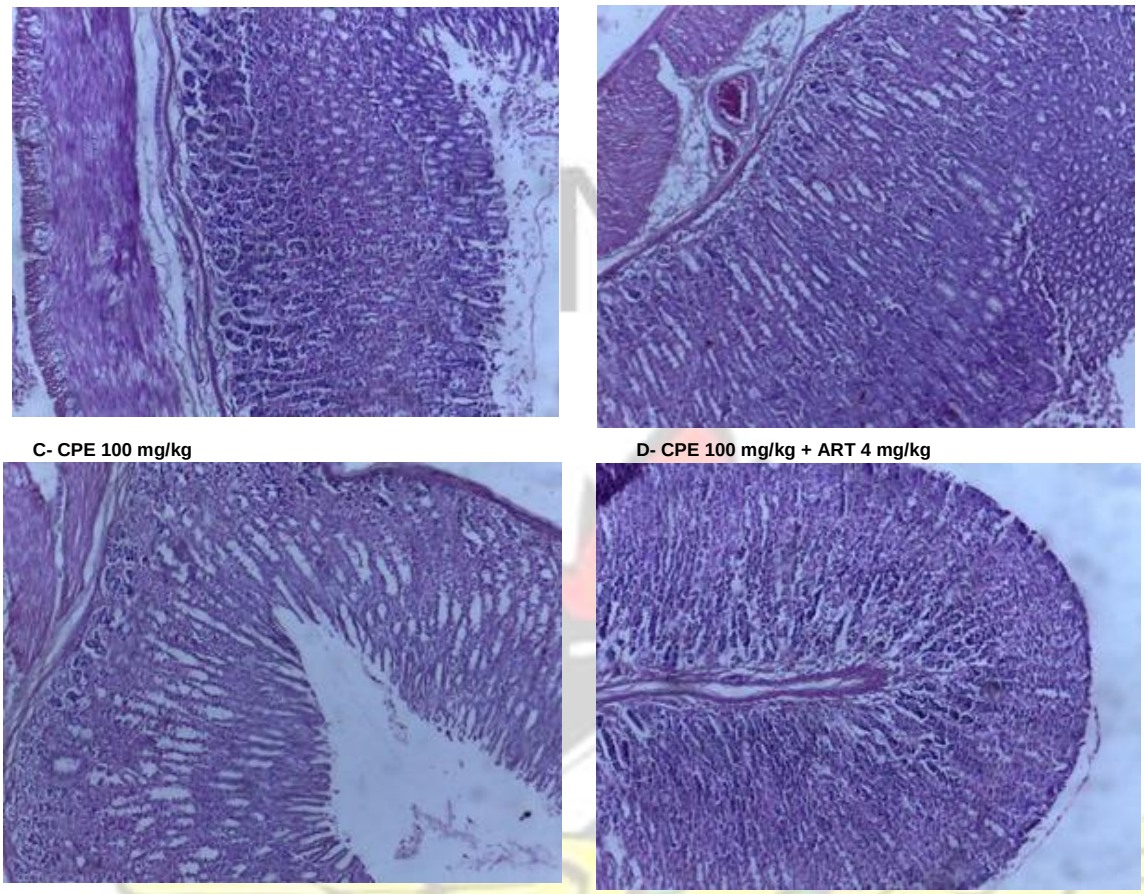


Figure 6.10: Photomicrograph (×100) showing histopathological slides of the stomachs of cryptolepine (CPE), *Cryptolepis* (CPS) or artesunate (ART, 4 mg/kg) treated rats. All treatment were for three days.

6.5 DISCUSSION

In the present chapter, the potential general toxicity of cryptolepis/cryptolepine with artesunate/artemether was studied in the rodent; Sprague-Dawley rats.

The study established that a single oral dose of 1000 mg/kg aqueous extract of *Cryptolepis sanguinolenta* administered for a period of 3 days did not cause any mortality or alter the behavioural pattern of rats. Compared with the control group, rats were alert with no motor or neurological changes, no gastrointestinal tract disorders, and respiratory distress. No significant weight differences were recorded in treated rats with reference to the weight of the control rats. The weight of target organs; the liver, stomach, spleen and kidneys were also not significantly affected compared to the control group. There were no deaths or CPE/CPS-related effects on the food and water intake at all treated doses.

The aqueous root extracts of *Cryptolepis sanguinolenta* and its major alkaloid cryptolepine had little or no effect on RBC, WBC, Hb concentration, and platelets when administered to rats for 3 or 7 days compared to the control. These findings support earlier reports by Ajayi *et al.* (2012). However there was a decrease in MCV, which measures the average volume of cells. Treatment with cryptolepis did not cause a change in the haemoglobin (Hb) concentration significantly, therefore the reduced MCV reflected in a rise in MCHC; a measure of the average concentration of haemoglobin in a red blood cell. The decrease in MCV and subsequent rise in MCHC confirmed the similar report by Ansah *et al.* (2008). Cryptolepine treatment for 7 days showed a significant decrease in RBC and HCT in the 100 mg/kg treated group. These results suggest that long term use of cryptolepine at 100 mg/kg may cause an increase in red blood cell destruction or decrease in red cell production.

To ascertain whether the defence system of the animals had been compromised, total WBC count, as well as differential count were measured. Cryptolepis and its major alkaloid cryptolepine showed no significant effect on the white blood cell count in all the rats treated compared to the control group.

Serum biochemical parameters as specific markers for damage to specific target organs (AST, ALT for liver; BUN, creatinine and electrolytes for kidney function, etc.) showed no significant differences between treated groups and control except the levels of AST which was significantly elevated in most combinations of cryptolepine with the artemisinins. The significant increase in AST was prominent when cryptolepine, at all doses, was combined with artemether (50 mg/kg) and in the treatment of cryptolepine for 7 days followed by artesunate (3 days).

Treatment of artesunate (4 mg/kg) and artemether (50 mg/kg) only showed an elevation in serum AST levels. Artesunate has been reported to show mild sinusoidal congestion with cytoplasmic vacuolation (hepatocyte edema) in wistar rats treated with 4 mg/kg for 3 days followed by 2 mg/kg for the next 4 days (Izunga *et al*., 2010). However, treatment with cryptolepine and cryptolepis did not affect the AST levels. The same results were observed for the groups treated with artemether and cryptolepis at all dose levels. This observed effect points to the possible reversal of any metabolic disorder associated with artemether when combined with cryptolepis. GGT levels were significantly decreased in groups treated with cryptolepis and artesunate. In general, no microgranuloma or focal necrosis was seen in the liver even with the highest concentrations of CPE and CPS. These findings accompanied by an unchanged serum enzyme concentration in treated rats suggest little or no hepatotoxicity in rats.

Microscopic observations revealed a normal hepatocyte architecture with a well-defined central vein. No necrosis, steatosis, chronic inflammatory infiltration, or degenerative changes were observed in any of the treated animals. Gross morphological inspection of other organs (kidney, spleen and stomach) also revealed no apparent damage. This was supported by histological studies. Generally, no changes were observed in the architecture of these organs of treated animals compared to the control. The histology of the liver and kidney were consistent with the normal

ALT, alkaline phosphate, bilirubin, creatinine and urea levels in the serum. The transient elevation of AST in the groups treated with artesunate (4 mg/kg) and artemether (50 mg/kg) may not be pathological since a normal liver architecture was observed in all treated groups.

6.6 CONCLUSION

Results from the present study found no significant toxicity in both rats treated with cryptolepis/ cryptolepine or their combination with the artemisinin derivatives (artesunate and artemether) during the 3 or 7 day treatment period. Histopathological examination revealed no toxic effects attributable to the administration of CPS/CPE and in combination with the artemisinins in any of the organs examined. It can be concluded that cryptolepis and its major alkaloid, cryptolepine is relatively safe at all doses used in combination with artesunate and artemether for the duration of treatment. Based on these findings, the NOAEL (No-observed-adverse-effects level) for cryptolepine and cryptolepis in rats were estimated to be above 100 mg/kg/day and 1000 mg/kg/day, respectively.

7.0 CHAPTER SEVEN

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

7.1 GENERAL DISCUSSION

In view of the problems associated with antimalarial drug resistance, new drugs or drug combinations are currently required for treatment of malaria. Effective combinations including artemisinin derivatives such as artesunate (David *et al*., 2004) or mixtures of older drugs such as atovaquone–proguanil combination (Malarone®) are recommended (Winter *et al*., 2006). In the absence of a functional and safe malaria vaccine, efforts to develop new antimalarial drugs continue. Plants play a dominant role in the discovery of lead compounds for the development of drugs for the treatment of malaria with quinine from *Cinchona sp* and the artemisinin derivatives

from *Artemisia annua* being classical examples. The development of a lead compound involves the evaluation of drug-like properties for further preclinical investigation and further design of more potent and less toxic compounds. A successful drug-lead candidate must have optimal drug-like properties, including selectivity to the biological target and potency, minimal toxicity, favourable stability and physicochemical profile, and desirable absorption, distribution, metabolism and excretion properties (Zhang *et al*., 2012).

In this search for new antimalarial leads and combinations to help eradicate the malaria menace, the popular West African antimalarial plant, *Cryptolepis sanguinolenta* and its major alkaloid cryptolepine were evaluated for both efficacy and toxicity when used with the artemisinin bases. With little knowledge on the pharmacokinetics of cryptolepine, the *in vivo* pharmacokinetics, metabolite identification as well as the basic *in vitro* absorption, distribution and metabolism properties were also studied.

Cryptolepine hydrochloride was extracted from *Cryptolepis sanguinolenta* using the method described by Kuntworbe *et al.* (2012) giving a high yield of 5.79 g (0.9650% w/w) and 5.82 g (0.9700%w/w) for Isolate I and Isolate II, respectively. The melting points of the reference cryptolepine and the two isolates (Isolates I and II) were within the reference range of 263-265ºC reported by (Bierer *et al.* 1998) as the melting point of cryptolepine HCl. The TLC, UV and HPLC spectra were similar to spectra of reference cryptolepine and that reported in literature (DwumaBadu *et al*., 1978) confirming the purity and authenticity of the isolated cryptolepine.

The antimalarial activity of cryptolepine was confirmed both *in vitro* and *in vivo* with the susceptibilities in both models close to those reported in literature. The *in vitro* antimalarial interaction of cryptolepine with the artemisinin derivatives in *P. falciparum* (3D7) showed a synergistic activity. Cryptolepine showed varied interaction with the 4-aminoquinolines,

amodiaquine and chloroquine. The combination of cryptolepine with amodiaquine had a synergistic effect *in vitro* (mean ΣFIC=0.235±0.15) whereas an additive effect (mean ΣFIC=1.342±0.34) was seen with chloroquine. The isobologram plot of the fixed ratio combination of cryptolepine with lumefantrine showed additivity whereas mefloquine showed an antagonistic effect. The synergistic effects observed *in vitro* with cryptolepine and artesunate were reproduced *in vivo* in the rodent model. The antimalarial activity of the artemisinins has been attributed to the reactivity of the endoperoxide bridge which is a common feature of this class of antimalarials. The indoloquinolones on other hand have their mechanism of action similar to chloroquine in inhibiting the conversion of poisonous haem to haemozoin (β-haematin) in the parasite food vacuole (Kumar *et al*., 2007). These varied mechanisms involved in the antimalarial activities may be involved in the synergy observed with the artemisinins *in vivo* and *in vitro*. This notwithstanding, it is difficult to extrapolate findings based on *in vitro* assays to *in vivo* drug interactions in humans.

In the *in vivo* assays, cryptolepine at doses 10, 30 and 100 mg/kg showed suppression greater than 70% on the 6th day of treatment. The ED_{50} of cryptolepine was determined to be 10.7 mg/kg and 40 mg/kg in *P. berghei* infected mice on the 5th and 6th days of drug treatment, respectively. The ED_{50} of cryptolepine obtained conforms to the ED_{50} of < 50 mg/kg in *P.berghei* determined by three independent scientists between 1995 and 1997 (Cimanga *et al*., 1997; Grellier *et al*., 1996; Kirby *et al*., 1995). This significant chemo-suppression produced by cryptolepine on the 5th and 6th day may account for the wide usage of the aqueous extract as an antimalarial agent. Artemether+Lumefantrine (Coartem®), the positive control used showed a higher antimalarial activity compared to all the doses of cryptolepine on the 6th day. These results indicate that cryptolepine possess blood schizonticidal activity as evidenced by early research (Kirby *et al*., 1995). All combinations of cryptolepine with artesunate showed a rapid onset of action and a high

parasite clearance rate in the first 3 days of treatment compared to each of the agents used alone. This gives the combination of cryptolepine and artesunate an advantage in the WHO recommended three days antimalarial combination therapy.

The absence of death and the general well-being observed following oral administration of cryptolepine in the mice at all doses suggests that the compound is practically non-toxic at the range of doses used. The mechanisms involved in the enhanced activity observed with cryptolepine-artemisinin combinations are yet to be elucidated. However, regarding the synergistic effect observed with the artemisinins and amodiaquine, further studies should be performed with cryptolepine alone and in combination on several *P. falciparum strains* and/or rodent malaria models to highlight the biochemical mechanisms behind its antiplasmodial interactions. On this basis, it is strongly believed that the combination of cryptolepine with the artemisinin derivatives is a potential option for an alternative antimalarial combination development.

Given the growing interest in cryptolepine and its analogues as potential antimalarial compounds, the gametocytocidal properties against late stage gametocytes was investigated. *Cryptolepis sanguinolenta* and cryptolepine showed minimal activity against *P. falciparum* late stage gametocytes proving that their prominent antimalarial activity could be attributed mainly to their effect on the asexual stages of the *Plasmodium* parasite. The absence of the metabolic process of haemozoin formation in this stage of the parasite may account for the minimal activity shown. Cryptolepis and cryptolepine possess potent anti-inflammatory activity mediated by COX-2 inhibition and in addition cryptolepine directly blocks the activity of prostaglandin E_2 (Bamgbose and Noamesi, 1981; Olajide *et al.*, 2007a; Olajide *et al.*, 2009, 2010). These reported antiinflammatory properties may offer added advantage in reducing the use of analgesics and antipyretics in the treatment of malaria when this agent is used.

The improved antimalarial activity of cryptolepine with the artemisinins observed in the preceding chapter directed further works on the drug-likeness of this lead compound in *in vitro* pharmacokinetic models. Several works have been done on the artemisinin derivatives to characterize *in vivo* and *in vitro* absorption, distribution, metabolism and excretion properties (Navaratnam, 2000). The pharmacokinetics and pharmacodynamics studies in both *in vitro* and *in vivo* are well understood for this class of antimalarial agent. Conversely, *in vivo* and *in vitro* pharmacokinetic properties of cryptolepine have not been well characterized. Consequently, in an attempt to combine the two agents in the treatment of malaria, *in vivo* and *in vitro* pharmacokinetic properties of cryptolepine needed to be established.

Human-based *in vitro* assays could provide a more accurate estimation of human clinical outcomes than could animal tests at preclinical stages especially with compounds to be used in humans (Zhang *et al*., 2012). Specific questions related to structure-activity relationship (SAR) for metabolic stability or DDI potential of the drug candidates could also be answered easily in the *in vitro* assays due to confounding factors in animal studies (Zhang *et al*., 2012).

Permeability across biological membrane is an important factor in the absorption and distribution of drugs. Poor permeability can arise due to a number of structural features and membrane based efflux mechanisms. This can bring about poor absorption across GIT mucosa or poor distribution throughout the body.

In this study, cryptolepine was incubated in MDR1-MDCK II cell lines to determine the involvement of human P-gp efflux pump in the transport across the gut lumen, the apical surface of endothelial cells in the capillaries of the brain and development of parasite resistance. Cryptolepine (90%) was recovered in the well from the apical to basolateral well whereas a recovery of 94% was obtained from the basolateral to the apical well. From the efflux ratio of 4.78 (less than 5), cryptolepine can be classified as having a low human P-gp efflux potential and hence

cryptolepine can easily cross the blood brain barrier as well as the intestinal membrane. The development of resistance by many antimalarial agents such as chloroquine has been shown to be partly mediated by the involvement of the P-glycoprotein efflux pump (Foote *et al.*, 1990) and hence cross-resistance mediated by this efflux pump may not occur with cryptolepine.

Passive permeability of the indoloquinoline was assessed across varying gastro-intestinal pH gradients using the hexadecane membrane PAMPA. The high passive permeability (percentage fraction absorbed of 81%) demonstrated makes cryptolepine a candidate drug sustainable as an oral dosage form.

Compounds with certain functional groups can decompose in the bloodstream. Unstable compounds often have high clearance and short $t_{1/2}$, resulting in poor *in vivo* pharmacokinetics and disappointing pharmacological performance. Plasma degradation clearance can easily be overlooked if discovery experiments focus on liver microsomal stability. After a 2-h incubation of cryptolepine in the rat and human pooled plasma, 91.7% and 89.2% of cryptolepine remained in human and rat plasma, respectively. The high amount of cryptolepine remaining in the plasma after 2 h indicates the possible non-involvement of plasma hydrolytic enzymes such as cholinesterase, aldolase, lipase and dehydropeptidase in the metabolism of cryptolepine. The binding of drug to plasma proteins determines the effective drug concentration at the site of the pharmacological target, because it affects both the drug volume of distribution and the free compound fraction (Craig and Welling, 1977). This information can be of great advantage during lead compound optimization and prioritization to facilitate drug development. Equilibrium dialysis is the preferred method to determine the free drug fraction, and usually serves as a reference technique against other methods such as ultrafiltration, exclusion chromatography, and ultracentrifugation (Lin *et al.*, 1987). In this study, cryptolepine showed a moderate plasma protein binding of 64.2±3.1 in rat and 58.7±1.7 in the human plasma used. These results show that a moderate amount of unbound

fraction of cryptolepine will be available to diffuse from vascular system and accumulate in tissues thereby enabling interaction with therapeutic targets and accessibility to clearance pathways.

Preliminary incubation of cryptolepine in rat and human hepatocytes showed a hepatic extraction of 44% and 54%, respectively. This low to moderate hepatic extraction suggests that other routes are involved in the elimination of cryptolepine from the body. The renal route may also play a role in cryptolepine elimination from the body.

The S9 fraction (subcellular fraction prepared by collecting supernatant after centrifugation of liver tissue homogenate at 9000×g) contains both cytosolic and microsomal enzymes. Most researchers use liver S9 fraction because of the ability to capture additional metabolism mediated by non-CYP enzymes such as aldehyde oxidase, xanthine oxidase, and aldehyde dehydrogenase.

Cryptolepine was incubated in rat and human S9 fraction in the presence and absence of NADPH (Co-factor for CYP-450 enzyme metabolism). Despite the half-life greater than 120 min in rat and human liver S9, instability was observed at 60 and 120 min in the presence and absence of the CYP- 450 enzyme co-factor NADPH. This data suggest that a non-NADPH dependent enzyme(s) such as aldehyde oxidase, xanthine oxidase and aldehyde dehydrogenase may play a role in driving the metabolic instability.

Further incubation of cryptolepine in rat and human cytosol showed instability after 120 min. This supports the involvement of cytosolic enzymes in the metabolism of cryptolepine. Despite the instability in the rat and human cytosol, cryptolepine incubation in dog liver S9 and cytosol showed high levels of stability. This effect points to the involvement of aldehyde oxidase in the metabolism of cryptolepine since aldehyde oxidase has been shown to be absent in dogs, hence the observed stability in dog liver S9 and cytosol (Pryde *et al*., 2010). Additionally, cryptolepine has been shown to be a substrate for aldehyde oxidase in rabbit liver preparation (Stell *et al*., 2012). The metabolic instability of cryptolepine in human and rat cytosol but stability in dog liver S9 and cytosol

confirms the possible involvement of aldehyde oxidase in the metabolism of cryptolepine in rat and human.

Raloxifene (a CYP-450 and AO inhibitor) and hydralazine (a specific AO inhibitor) were used to further confirm the involvement of aldehyde oxidase in the metabolism of cryptolepine in rat and human. Both AO inhibitors significantly reversed the metabolic instability of cryptolepine and the probe substrate carbazeran in both human and rat S9 fractions. These results indicate the involvement of AO and possibly CYP-450 in the metabolism of cryptolepine. Hydralazine did not show a significant inhibition in the metabolism of zoniporide in human and rat liver S9.

Metabolite profiling in rat and human hepatocytes for 24 h showed nine major metabolites resulting from metabolic pathways mainly involving hydroxylation, dihydroxylation and glucuronidation. All human *in vitro* metabolites were also found in rat hepatocytes, except for metabolite M1. This metabolite is an N-glucuronide and is found in human, but not rat hepatocytes, indicating the possible involvement of a human specific UDP-glucuronosyltransferase (UGT) (Li Di, 2014) in the metabolism of cryptolepine.

Stell *et al.* (2012) proposed the formation of metabolite cryptolepine-11-one after cryptolepine metabolism by aldehyde oxidase. The metabolites M6/M7 identified in this study in both human and rat hepatocytes are formed by oxidative hydroxylation indicating that involvement of aldehyde oxidase is possible. Metabolite (M6) was also detected in rat plasma samples after oral administration of cryptolepine. The aldehyde oxidase and N-glucuronidation pathways could potentially result in significantly different clearance and/or disposition in human compared to rat, depending on the extent of formation and stability of each metabolite in humans *in vivo*. The dihydroxylation metabolites (M3/M5) are likely to be formed via initial epoxide formation, indicating the possibility of cytochrome P450 metabolism of cryptolepine**.**

In view of this, there is the possibility of a drug-drug interaction existing between cryptolepine and zaleplon, a sedative-hypnotic known to be metabolised by AO. The antihypertensive drug, hydralazine and raloxifene, a selective oestrogen receptor modulator can inhibit the metabolism of cryptolepine thereby increasing the plasma concentration and hence its toxicity. The cytochrome P450 (CYP) enzyme system has been shown to mediate the metabolism of the artemisinin derivatives. The artemisinins also have the capacity for autoinduction of their own metabolism (Ashton *et al*., 1998a), resulting in decreasing exposure over time. This is primarily mediated by the human cytochrome P450 (CYP) isoenzymes CYP 2B6, CYP 2A6 and CYP 3A4 in individuals with low 2B6 expression (Svensson *et al*., 1999). Drug–drug interactions (DDIs) are usually increased by the coadministration of multiple drugs, many of which are pharmacokinetically based and often involve drug metabolism (Shou *et al*., 2001). The study findings suggest that aldehyde oxidase is the main enzyme in the metabolism of cryptolepine and hence the autoinduction of the CYP enzymes by the artemisinins may have very little or no effect on the metabolism of cryptolepine.

A strong relationship exists between the drug concentration at the site of action and pharmacological effect for most compounds. The concentration of a drug at its site of action depends on the size of the dose and upon the processes of absorption, distribution and clearance. Poor absorption and distribution and/or rapid elimination characteristics may result in insufficient concentrations at the site of action. Pharmacokinetic studies in early drug development helps to select an optimal dosage regimen for future clinical trial and help make informed decision to either continue or abandon clinical drug development.

The present investigation with cryptolepine also shed light on the *in vivo* pharmacokinetic properties and the metabolites in the plasma and urine of rats. In rats, cryptolepine exhibited low oral bioavailability, high plasma clearance, extensive distribution and considerably higher

exposure in urine compared to plasma suggesting that renal elimination of unchanged drug is a key pathway of elimination. From the rat pharmacokinetic data, cryptolepine showed a quick cleareance from the plasma, possibly accumulating in vital organs and extending plasma residence time particularly important for the clearance of erythrocytic stage parasites. The early Tmax observed may provide an added advantage as an antimalarial agent with rapid parasite clearance.

However, it should be noted that these observations in the rat cannot be extrapolated to humans.

The toxicity of *Cryptolepis sanguinolenta* and its major alkaloid, cryptolepine has been shown by several researchers. It has been shown that cryptolepine is a DNA intercalator and interferes with topoisomerase II activity (Bonjean *et al.*, 1998; Lisgarten *et al.*, 2001). Topoisomerase II enzyme is involved in the cleavage of DNA strands. Most anticancer agents and cell poisons that modulate the activity of this enzyme cause cell death either by apoptosis or mitotic failure once cell damage is overwhelming. Ansah *et al.* (2009a) revealed slight enlargement of liver and kidneys, with congestion and hyperaemia in the lungs and muscles of rats when the aqueous root extract was used. For these reasons, the acute toxicity of cryptolepine alone and in use with the artemisinin derivatives was determined in Sprague Dawley rats. Additional acute toxicity evaluation was performed for the aqueous root extract which already has extensive traditional use.

The haematological system has a predictive value for toxicity in human and therefore analysis of blood is relevant to risk evaluation (Oslon *et al.*, 2000). Aqueous root extracts of *Cryptolepis sanguinolenta* and its major alkaloid cryptolepine were found to have little or no effect on RBC, WBC, HB concentration, and platelets, when administered to rats for 3/7 days compared to the control. These findings support similar reports by Ajayi *et al.* (2012).

A 7-day treatment with cryptolepine showed a significant decrease in RBC and HCT in the 100 mg/kg treated group. These results show that, long term use of cryptolepine at 100 mg/kg may cause an increase in red blood cell destruction or decrease in cell production. This may not auger

well for the use 100 mg/kg cryptolepine in long term treatment of malaria. Hematinics to increase the RBC levels may be recommended for long term use of cryptolepine at 100 mg/kg.The total WBC and lymphocytes levels were not altered by the crude extract and its major alkaloid in all treated groups.

The measurement of the activities of various enzymes in tissues and body fluids play a significant and well known role in disease investigation and diagnosis in tissues and cellular damage (Malomo,

2000). AST and ALT are useful marker enzymes in assessing damage to the liver (Shahjahan *et al.*,

2004). Their presence in the serum may give information on tissue injury and organ dysfunction (Wells *et al.*, 1986). However, they are not always good indications of how well the liver is functioning as elevation of these enzymes are often unexpectedly encountered on routine blood screening test in otherwise healthy individuals (McPherson, 2007).

Serum biochemical parameters showed no significant differences between treated groups and control except in the case of the levels of AST which was significantly elevated in most combinations of cryptolepine with the artemisinins. The significant increase in AST was prominent when cryptolepine (at all doses) was combined with artemether (50 mg/kg) ($p<0.05$) and in the treatment of cryptolepine for 7 days followed by artesunate (3 days) ($p<0.01$). With the absence of elevated serum AST levels in the treatment with cryptolepis and cryptolepine only, artesunate and artemether may be responsible for this high AST serum levels. Artemisinin and artesunate have been shown to possess hepatotoxic effects manifested by an elevated serum AST levels in rats (Izunya *et al*., 2010; Udobre *et al*., 2009).

Clinically, decreases in AST, ALT and GGT levels have no documented relevance except in a patient already having a hepatic dysfunction. An increase however suggests a hepatic leakage or

acute liver damage (Chand *et al*., 2011). Seven days treatment with cryptolepis followed by a 3 day treatment with artesunate also showed a significant increase ($p<0.001$) in the AST levels at all dose levels. The total and direct bilirubin levels were also significantly increased in the group treated with 1000 mg/kg of cryptolepis (7 days) followed by artesunate (3 days). Many diseases of the liver are accompanied by jaundice, a yellowing of the eyes and skin caused by increased levels of bilirubin in the system. Bilirubin accumulates from the breakup of haemoglobin present in red blood cells. During normal function, the liver removes bilirubin from the blood and excretes it through bile. The 7 day treatment with cryptolepis 1000 mg/kg followed by a 3 day treatment with artesunate (4 mg/kg) showed an increase in total bilirubin and conjugated bilirubin compared to the control group indicating a possible compromise in the normal function of the liver in the treated rats.

Changes in the body weights and relative weight of internal organs such as the liver, spleen, kidney and stomach are valuable indicators in the evaluation of the toxic effects of compounds (Grance *et al*., 2008). Daily observations of rats treated with cryptolepis and cryptolepine did not affect the body weight. No death or significant changes in the food and water intake was observed at all treated doses. These routine observations were very important since a loss of more than 10% of initial body weight is an indicator of adverse effects (Teo *et al.,* 2002). There were no significant change in the relative weights of the selected organs. This indicates that cryptolepis and its major alkaloid cryptolepine at the doses used with or without the artemisinin derivatives will not produce any significant organ swelling, atrophy or hypertrophy in the rats for 3 and 7 days treatment.

Complete blood count provides enormous information on the haematological status in many disease conditions (Latha and Joshi, 2004) including malaria. In malaria pathophysiology, there is usually a low haemoglobin concentration (anaemia). This may result from a reduction in the red blood cell number or reduction in the concentration of haemoglobin in each erythrocyte (Latha and

Joshi, 2004). Malaria-associated anaemia represents a major public health problem and this severe anaemia probably accounts for majority of death due to malaria (Snow *et al*., 1994; Menendez *et al*., 1997). Anaemia occurs when disruption of erythrocyte is increased and/or production of erythrocyte is reduced. In the antimalarial assay in mice, the red blood cell (RBC) and haemoglobin (HGB) levels were not significantly different from the vector control. Cryptolepine, like artesunate did not provide any improvement in these haematological indices in the *P. berghei* infected mice. Cryptolepine (40 mg/kg) showed a significant increase in the haematocrit levels compared to the vector control. This high HCT level was absent in all artesunate combination with cryptolepine.

White blood cells (WBCs) are involved in the body's immune system and help fight diseases. They are increased in normal situations such as exertion and pregnancy, and abnormally in situations such as loss of blood, cancer and most infections. WBCs are generally increased in severe malaria (Modiano *et al*., 2001), however in acute malaria, WBC counts are generally observed to be low or normal (White and Breman, 2001). No significant difference was observed in the WBC levels as compared to the parasite treated group except for the 1:1 treated group of mice. The lymphocyte levels were significantly decreased in all combination treatments whereas cryptolepine (40 mg/kg) showed no significant decrease compared to the parasite control. All, but the lowest dose ratio of cryptolepine (5 mg/kg) with artesunate (0.75 mg/kg) showed a significant ($p>0.001$) elevation in the platelet count compared to the vector control. The elevated platelet count may be an attempt to boost the immune response in an attempt to fight the malaria parasite (Reid and Lomas-Francis, 2004).

Daily administration of the aqueous root extract of *Cryptolepis sanguinolenta* (250, 500 and 1000 mg/kg *p.o*) and its major alkaloid, cryptolepine (25, 50, 100 mg/kg *p.o*) with or without the artemisinin derivatives (artesunate and artemether) for 3, 7 or 10 days presented no morphology

changes in kidneys, spleen, stomach and liver tissues. The results were concurrent with the liver and kidney function test, which showed values not significantly different from the controls used. In the *P. berghei* infected mice, histopathological presentations in liver, spleen, stomach and kidney in all treatment groups were not significantly different from the control groups.

7.2 SUMMARY OF FINDINGS

- The combination of cryptolepine with the artemisinins (artesunate, artemether and dihydroartemisinin) showed a synergistic effect in *vitro*. This synergy was also seen in combination with amodiaquine *in vitro*.
- *In vivo* in mice, the combination of cryptolepine and artesunate at all doses showed a rapid onset of antiplasmodial activity compared to each of the agent used alone. This translated into a synergistic activitiy *in vivo*. This work provides the basis for the selection of cryptolepine as a potential lead compound in the development of combination therapy against malaria.
- Both cryptolepine and the aqueous root extract of *Cryptolepis sanguinolenta* showed very little inhibition on the late stage gametocyte of *P. falciparum* strain NF54 in the PrestoBlue™ assay.
- In the *in vitro* assays, cryptolepine showed a high passive permeability, good metabolic stability in the plasma, low to moderate metabolic clearance in hepatocytes and a moderate plasma protein binding in rat and human samples, hence making it a compound with druglike properties.

- Cryptolepine exhibited a low human P-glycoprotein efflux potential, signifying that cryptolepine can easily cross the blood brain barrier, the intestinal membrane as well as a lower probability of the development and spread of resistance of *Plasmodium falciparum* via the efflux pump.
- Both cytosolic and microsomal liver enzymes may be involved in the metabolism of cryptolepine in rat and humans. Reaction phenotyping studies with raloxifene and hydralazine showed a reversal of the metabolic instability in liver S9 confirming the role of aldehyde oxidase in the metabolism of cryptolepine in rat and human. This finding helps identify medications on the market that may possibly inhibit or enhance the metabolism of cryptolepine. The artemisinins therefore may have a low potential of DDI in the metabolism of cryptolepine, however further works are required in that regard.
- The metabolites formed in human and rat hepatocytes indicate the possible involvement of aldehyde oxidase, UDP-glucuronyltransferase (UGT) and the cytochrome P450 enzyme systems in the metabolism of the indoloquinoline, cryptolepine.
- Rat PK studies indicated that cryptolepine was cleared quickly from the systemic circulation, extensively distributed and eliminated unchanged in the urine. Metabolites were also detected in the plasma and urine. This data suggests that renal secretion of unchanged parent drug and metabolism are pathways of elimination of cryptolepine in rat.
- The aqueous root extract of *Cryptolepis sanguinolenta* and its major indoloquinoline alkaloid, cryptolepine, are relatively non-toxic when combined with the artemisinin derivatives (artesunate and artemether) in the acute toxicity study in rats.

7.3 CONCLUSIONS

It is concluded from these studies that

i. there exits an *in vivo* and *in vitro* synergistic antimalarial interaction between cryptolepine and the artemisinin derivatives (artesunate, artemether and dihydroartemisinin).

ii. cryptolepine and the aqueous root extract of *Cryptolepis sanguinolenta* have little activity against the late stage gametocyte involved in the transmission of malaria.

iii. cryptolepine has favourable absorption, distribution and metabolism *in vitro* and *in vivo*.

iv. aldehyde oxidase, human specific UDP-glucuronosyltransferase (UGT) and Cytochrome P450 are the main enzymes involved in the metabolism of cryptolepine in rats and humans.

v. the aqueous root extract of *Cryptolepis sanguinolenta* and cryptolepine in combination with the artemisinins is fairly nontoxic in rodent models in the acute toxicity studies.

The work undertaken in this thesis has added to the body of knowledge in the development of cryptolepine as an antimalarial agent. It also shows the safety and efficacy when cryptolepine is used with the artemisinin derivatives. These findings may guide decisions regarding further works to optimally combine cryptolepine with other antimalarial agents to provide the next generation of artemisinin combination therapies (ACT) to advance the course of malaria eradication.

7.4 FUTURE DIRECTIONS

Whilst the aims of this thesis have been achieved, many areas for future research have been highlighted, including:

1. Further studies performed with cryptolepine alone and in combination on several *P. falciparum strains* and/or rodent malaria models to highlight the biochemical mechanisms behind its antiplasmodial interactions
2. Determination the specific CYP P450 isoenzyme(s) contributing to the metabolism of cryptolepine using inhibitor studies.
3. The DNA topoisomerase II inhibition activity (cytotoxicity) of cryptolepine has been established by several researcher ((Bonjean *et al*., 1998; Lisgarten *et al*., 2001). The *in vitro* cytotoxicity of cryptolepine in combination with the artemisinin derivatives in different cell lines should be evaluated.
4. Synthesis of the metabolites of cryptolepine identified in hepatocytes, plasma and urine for toxicity and efficacy studies.
5. Designing an *in vivo* drug-drug interaction assay between cryptolepine and the artemisinins.

REFERENCES

Adjalley S.H, Johnston G.L, Li T, Eastman R.T, Ekland EH (2011). Quantitative assessment of *Plasmodium falciparum* sexual development reveals potent transmission-blocking activity by methylene blue. Proc Natl Acad Sci U S A. 108:1214–23.

Adjei, G., Kurtzhals, J., Rodrigues, O., Alifrangis, M., Hoegberg, L., Kitcher, E., Badoe, E., Lamptey, R. and Goka, B. (2008). "Amodiaquine-artesunate vs artemether-lumefantrine for uncomplicated malaria in Ghanaian children: A randomized efficacy and safety trial with one year follow-up." Malaria Journal **7**(1): 127.

Adzu, B. and Haruna, A, (2007). Studies on the use of Zizyphus spina-christi against pain in rats and mice. Afr. J. Biotechnol., 6:1317-1324.

Agbedahunsi, J. M., Fakoya, F. A., Adesanya, S. A. (2004). Studies on the anti-inflammatory and toxic effects of the stem bark of Khaya ivorensis (Meliaceae) on rats. Phytomedicine 11(6)**:** 504508.

Ajayi A.F, Akhigbe R.E, Adewumi O.M, Olaleye S.B (2012). Haematological Evaluation of *Cryptolepis sanguinolenta.* Int J Med Biomed Res*; 1(1):56-61*

Ajayi A.F, Akhigbe R.E, Iyiola T.O, Adewumi O.M, Olaleye S.B (2012). Gastric secretagogue action of *Cryptolepis sanguinolenta* in the perfused stomach of anesthetized rats. Int J Med Biomed Res;1(1):62-67

Akdogan, M, Kilinc, I, Oncu, M, Karaoz, E. and Delibas, N. (2003). Investigation of biochemical and histopathological effects of *Mentha piperita L*. and *Mentha spicata L*. on kidney tissue in rats. Human and Experimental Toxicology, 22, 213-219.

Akoachere M., Buchholz K., Fischer E., Burhenne J., Haefeli W.E., Schirmer RH, Becker K. (2005). *In Vitro* Assessment of Methylene Blue on Chloroquine-Sensitive and-Resistant *Plasmodium falciparum* Strains Reveals Synergistic Action with Artemisinins. Antimicrob. Agents Chemother. 49**:**4592-4597.

Alonso PL, Brown G, Arevalo-Herrera M, Binka F, Chitnis C, Collins F, Doumbo OK, Greenwood B, Hall BF, Levine MM, Mendis K, Newman RD, Plowe CV, Rodríguez MH, Sinden R, Slutsker L, Tanner M (2011). A research agenda to underpin malaria eradication. PLoS Med. 8:e1000406. doi:10.1371/journal.pmed.1000406.

Ambudkar SV1, Dey S, Hrycyna CA, Ramachandra M, Pastan I, Gottesman MM (1999) Biochemical, Cellular, and Pharmacological aspects of the multidrug transporter. Annu Rev Pharmacol Toxicol 39**:**361–398.

Amin, A., Zurovac, D., Kangwana, B., Greenfield, J., Otieno, D., Akhwale, W. and Snow, R. (2007). "The challenges of changing national malaria drug policy to artemisinin-based combinations in kenya." Malaria Journal **6**(1): 72.

Ansah, C. and Gooderham, N. J. (2002). "The popular herbal antimalarial, extract of cryptolepis sanguinolenta, is potently cytotoxic." Toxicological Sciences 70(2): 245-251.

Ansah, C., Khan, A. and Gooderham, N. J. (2005). "*In vitro* genotoxicity of the west african antimalarial herbal cryptolepis sanguinolenta and its major alkaloid cryptolepine." Toxicology 208(1): 141-147.

Ansah C, Mfoafo EA, Woode E, Opoku-Okrah C and Owiredu WKBA (2008). Toxicological evaluation of the anti-malarial herb *Cryptolepis sanguinolenta* in rodents J Pharmacol Toxicol.3:335-343.

Ansah, C., Otsyina, H.R., Duwiejua, M., Woode, E., Aboagye, F.A., Aning, K.G. (2009a). Toxicological Assessment of Cryptolepis sanguinolenta for possible use in Veterinary Medicine. Journal of Veterinary Medicine and Animal Health. 1(1): 011-016.

Arzel, E., Rocca, P., Grellier, P., Labaeid, M., Frappier, F., Gueritte, F., Gaspard, C., Marsais, F., Godard, A. and Queguiner, G. (2001). "New synthesis of benzo-δ-carbolines, cryptolepines, and their salts: In vitro cytotoxic, antiplasmodial, and antitrypanosomal activities of δ-carbolines, benzo-δ-carbolines, and cryptolepines." Journal of Medicinal Chemistry 44(6): 949-960.

Asase, A., Oteng-Yeboah, A. A., Odamtten, G. T. and Simmonds, M. S. J. (2005). "Ethnobotanical study of some Ghanaian anti-malarial plants." Journal of Ethnopharmacology 99(2): 273-279.

Ashton, M., Hai, T.N., Sy, N.D., Huong, D.X., Van Huong, N., Niêu, N.T., Công, L.D. (1998a). Artemisinin pharmacokinetics is time-dependent during repeated oral administration in healthy male adults. Drug Metab. Dispos. 26, 25–27.

Bailey CJ, Day C (1989). Traditional plant medicines as treatments for diabetes. Diabetes Care, 12:
553–64.

Bakos E, Evers R, Szakacs G, Tusnady GE, Welker E, Szabo K, De Haas M, Van Deemter L, Borst P, Varadi A, Sarkadi B (1998). Functional multidrug resistance protein (MRP1) lacking the N-terminal transmembrane domain. J Biol Chem; 273: 32167-32175.

Bamgbose, S.O.A.; Noamesi, B.K (1980**).** The alpha-adrenoceptor blocking properties of cryptolepine on the rat isolated vas-deferens - Studies on cryptolepine. *Planta Med.*, *39*, 51-56.

Bamgbose, S. O. A. and Noamesi, B. K. (1981). "Studies on cryptolepine." Planta Medica 41(04): 392,396.

Bamgbose, S.O.A.; Noamesi, B.K (1982) Preferential blockade of presynaptic alpha-adrenoceptors on the rat isolated vas-deferens by cryptolepine, .7.Studies on cryptolepine. Planta Med., 44, 241245.

Barr JT and Jones JP (2011). Inhibition of human liver aldehyde oxidase: implications for potential drug-drug interactions. Drug Metab Dispos 39:2381–2386.

Basco LK, Le Bras J (1994). *In vitro* susceptibility of Cambodian isolates of *Plasmodium falciparum* to halofantrine, pyronaridine and artemisinin derivatives. Ann Trop Med Parasitol.88 (2):137-44.

Beedham C (1997). The role of non-P450 enzymes in drug oxidation. Pharm World Sci 19:255–263. Review.

Benedetti MS, Whomsley R, Baltes E (2006). Involvement of enzymes other than CYPs in the oxidative metabolism of xenobiotics. Expert Opin Drug Metab Toxicol 2:895-921.

Bierer, D. E., Fort, D. M., Mendez, C. D., Luo, J., Imbach, P. A., Dubenko, L. G., Jolad, S. D., Gerber, R. E., Litvak, J., Lu, Q., Zhang, P., Reed, M. J., Waldeck, N., Bruening, R. C., Noamesi, B. K., Hector, R. F., Carlson, T. J. and King, S. R. (1998). "Ethnobotanical-directed discovery of the antihyperglycemic properties of cryptolepine: Its isolation from *cryptolepis sanguinolenta*, synthesis, and *in vitro* and *in vivo* activities." Journal of Medicinal Chemistry 41(6): 894-901.

Bloland BP (2001). Drug Resistance in Malaria. WHO/CDS/CSR/DRS/2001.4:1

Boakye-Yiadom, K., (1983). In *Proceeding of the First International Symposium on Cryptolepine*; Boakye-Yiadom, K., Bamgbose, S.O. A., Eds.; University of Science and Technology: Ghana, pp 57-64.

Boakye-Yiadom, K. and Heman-Ackah, S. M. (1979). "Cryptolepine hydrochloride effect on *staphylococcus aureus*." Journal of Pharmaceutical Sciences 68(12): 1510-1514.

Boakye-Yiadom, K. (1979). The antimicrobial activity of some West African medicinal plants. *0.* J. Crude Drug Res. 2, 78-80.

Boivin MJ (2002). "Effects of early cerebral malaria on cognitive ability in Senegalese children," Journal of Developmental and Behavioral Pediatrics 23 (5):353–364.

Bonjean, K., De Pauw-Gillet, M. C., Defresne, M. P., Colson, P., Houssier, C., Dassonneville, L., Bailly, C., Greimers, R., Wright, C., Quetin-Leclercq, J., Tits, M. and Angenot, L. (1998). "The DNA intercalating alkaloid cryptolepine interferes with topoisomerase II and inhibits primarily DNA synthesis in b16 melanoma cells†." Biochemistry **37**(15): 5136-5146.

Bourdy M, Willcox L, Ginsburg H, Rasoanaivo P, Graz B, and Deharo E (2008). Ethnopharmacology and malaria: New hypothetical leads or old efficient antimalarials? International Journal for Parasitology **38**: 33–41.

Boye, G. L., and Oku-Ampofo (1983). Clinical uses of *Cryptolepis sanguinolenta*. Proceedings of the First International Seminar on Cryptolepine, *27-30* July *1983,* University of Science and Technology, Kurnasi, Ghana, pp. 37-40.

Boye, G. L., and Oku-Ampofo (1990). The role of plants and traditional medicine in primary health care in Ghana. In Economic and Medicinal Plant Research, Vol. 4 ed. by H. Wagner and N. R. Farnsworth, Academic Press, London.

Breman J (2001). "The ears of the hippopotamus: manifestations, determinants, and estimates of the malaria burden." American Journal of Tropical Medicine and Hygiene 64: 1-11.

British Pharmacopoeia, (2013) software. Volume I and II, Appendix II B, page 842.

Bruce Chwatt LJ (1985). Essential Malariology. 2nd Ed. London, Heinemanna. 61-270 Chanda P, Hamainza B, Mulenga S, Chalwe V, Msiska C and Chizema-Kawesha E (2009).Early results of integrated malaria control and implications for the management of fever in under-five children at a peripheral health facility: A case study of Chongwe rural health centre in Zambia. Malaria Journal 8:49

Bugyei KA, Boye GL, and Addy ME. Clinical efficacy of a tea-bag formulation of *cryptolepis sanguinolenta* root in the treatment of acute uncomplicated falciparum malaria. Ghana Med J 2010; 44: 3-9.

Caldwell J, Gardner I, and Swales N (1995). An Introduction to Drug Disposition: The Basic Principles of Absorption, Distribution, Metabolism, and Excretion. Toxicologic Pathology; 23(2).

Calixto, J. B. (2000). Efficacy, safety, quality control, marketing and regulatory guidelines for herbal medicines (phytotherapeutic agents). Braz J Med Biol Res 33: 179-189.

Canning EU, Sinden RE (1975). Nuclear organisation in gametocytes of Plasmodium and hepatocystis: a cytochemical study. Z Parasitenkd 46:297–9.

Carlton JM, Muller R, Yowell CA, Fluegge MR, Sturrock KA, Pritt JR, Vargas-Serrato E, Galinski MR, Barnwell JW, Mulder N, Kanapin A, Cawley SE, Hide WA, Dame JB (2001).

Profiling the malaria genome: a gene survey of three species of malaria parasite with comparison to other apicomplexan species. Mol Biochem Parasitol. 118(2): 201-10.

Chand, N., Durrani, F. R., Ahmad, S., Khan, A. (2011). Immunomodulatory and hepatoprotective role of feed-added Berberis lycium in broiler chicks. J Sci Food Agric 91(10)**:** 1737-1745.
Chen J. (2006) Recognition and Prevention of Herb-Drug Interactions: Pharmacokinetic Interactions. Acupuncture Today. 7(11).

Chojkier, M. (2005). Troglitazone and liver injury: in search of answers. Hepatology 41(2)**:** 237246.

Cimanga, K., De Bruyne, T., Lasure, A., Van Poel, B., Pieters, L., Claeys, M., Berghe, D. V., Kambu, K., Tona, L. and Vlietinck, A. J. (1996). "*In vitro* biological activities of alkaloids from *cryptolepis sanguinolenta*." Planta Medica 62(1): 22-27.

Cimanga, K., De Bruyne, T., Pieters, L., Vlietinck, A. J. and Turger, C. A. (1997). "*In vitro* and *in vivo* antiplasmodial activity of cryptolepine and related alkaloids from *cryptolepis sanguinolenta*." Journal of Natural Products 60(7): 688-691.

Clarkson C, Maharaj VJ, Crouch NR, Grace OM, Pillay P, Matsabisa MG (2004). *In vitro* antiplasmodial activity of medicinal plants native to or naturalised in South Africa. Journal of Ethnopharmacology 92: 177–191.

Clinquart, E. (1929). "The chemical composition of cryptolepis triangularis-a plant in Belgium congo." Bulletin academie royale de medecine de Belgique 9: 627-635.

Comporti, M (1985). Biology of diseases: lipid peroxidation and cellular damage in toxic liver injury. Lab Invest 53:599-623.

Coleman, M., Casimiro, S., Hemingway, J. and Sharp, B. (2008). "Operational impact of ddt reintroduction for malaria control on anopheles arabiensis in mozambique." Journal of Medical Entomology 45: 885-890.

Cowman AF (2006). "Invasion of Red Blood Cells by Malaria Parasites". *Cell* 124: 755–766.

Cox-Singh, J., Davis, T. M. E., Lee, K. S., Shamsul, S. S. G., Matusop, A., Ratnam, S., Rahman, H. A., Conway, D. J. and Singh, B (2008). "Plasmodium knowlesi malaria in humans is widely distributed and potentially life threatening." Clinical Infectious Diseases 46(2): 165-171.

Craig WA, Welling PG (1977). Protein binding of antimicrobials: Clinical pharmacokinetic and therapeutic implications. Clin Pharmacokinet 2:252-268.

Cullen, JM, Miller, RT (2006). The role of pathology in the identification of drug-induced hepatic toxicity. Expert opinion on Drug Metabolism and Toxicology 2:241-247

D'Alessandro S, Silvestrini F, Dechering K, Corbett Y, Parapini S, Timmerman M, Galastri L, Basilico N, Sauerwein R, Alano P, Taramelli D (2013). A Plasmodium falciparum screening assay for anti-gametocyte drugs based on parasite lactate dehydrogenase detection. J Antimicrob Chemother. 68:2048–58.

Daneshvar, C., Davis, T. M. E., Cox-Singh, J., Rafa'ee, M. Z., Zakaria, S. K., Divis, P. C. S. and Singh, B (2009). "Clinical and laboratory features of human plasmodium knowlesi infection." Clinical Infectious Diseases 49(6): 852-860.

Davis TME, Karunajeewa HA, Illett KF (2005). Artemisinin-based combination therapies for uncomplicated malaria. Med. J. Aust. 182 (4), 181-185

Fidock D.A, Rosenthal J.P, Croft S.L, Reto B. and Nwaka S (2004). Antimalarial drug discovery: Efficacy models for compound screening. Nature Rev., 3:509-520.

Delgodaa R, Youngerb N, Barretc C, Braithwaitec J, Davisc D (2011). The prevalence of herbs use in conjunction with conventional medicines in Jamaica. Complementary therapies in Medicine; 18(1):13-20.

Delves M, Plouffe D, Scheurer C, Meister S, Wittlin S, Winzeler EA, Sinden RE, Leroy D (2012). The Activities of Current Antimalarial Drugs on the Life Cycle Stages of *Plasmodium*: A Comparative Study with Human and Rodent Parasites. PLoS Med 9(2): e1001169. doi:10.1371/journal.pmed.1001169

Di L, Kerns EH, Hong Y, Chen H (2005). Development and application of high throughput plasma stability assay for drug discovery. Int. J. Pharm, 297 (1–2):110–119. [PubMed: 15876500]

Dhanabal, T., Sangeetha, R. and Mohan, P. S (2006). "Heteroatom directed photoannulation: Synthesis of indoloquinoline alkaloids: Cryptolepine, cryptotackieine, cryptosanguinolentine, and their methyl derivatives." Tetrahedron 62(26): 6258-6263.

Dorvault F (1982). L'officine, xxi ed. Vigot. Paris. Pp 110.

Dwuma-Badu, D., Ayim, J. S. K., Fiagbe, N. I. Y., Knapp, J. E., Schiff, P. L. and Slatkin, D. J (1978). "Constituents of West African medicinal plants xx: Quindoline from cryptolepis sanguinolenta." Journal of Pharmaceutical Sciences 67(3): 433-434.

Eckstein-Ludwig, U., Webb, R. J., van Goethem, I. D. A., East, J. M., Lee, A. G., Kimura, M., O'Neill, P. M., Bray, P. G., Ward, S. A. and Krishna, S (2003). "Artemisinins target the serca of *plasmodium falciparum*." Nature 424(6951): 957-961.

Egan, T. J., Hunter, R., Kaschula, C. H., Marques, H. M., Misplon, A. and Walden, J (1999). "Structure−function relationships in aminoquinolines: Effect of amino and chloro groups on

quinoline−hematin complex formation, inhibition of β-hematin formation, and antiplasmodial activity." Journal of Medicinal Chemistry 43(2): 283-291.

English, M (1996). Late-threatening severe malarial anemia trans. R.Soc Trop. Med. Hyg., 94: 585-588.

Evans KJ, Hansen DS, van Rooijen N, Buckingham LA, Schofield L (2006). Severe malarial anemia of low parasite burden in rodent models results from accelerated clearance of uninfected erythrocytes. Blood. 107(3):1192-9.

Evers R., Kool M., Smith A. J., van D. L., de H. M. and Borst P (2000). Inhibitory effect of the reversal agents V-104, GF120918 and Pluronic L61 on MDR1 Pgp-, MRP1- and MRP2-mediated transport. British J Cancer; 83: 366-374.

Fabre G, Combalbert J, Berger Y, and Cano JP (1990). Human hepatocytes as a key *in vitro* model to improve preclinical drug development. Eur J Drug Metab Pharmacokinet 15:165–171.

Faller B. (2008). Artificial Membrane Assays to Assess Permeability.Current Drug Metabolism, 9, 886-892.

Famin, O. and Ginsburg, H. (2002). Differential effects of 4-aminoquinoline-containing antimalarial drugs on haemoglobin digestion in *Plasmodium falciparum*-infected erythrocytes. Biochem. Pharmacol. 63, 393–398.

Fromm MF (2000). P-glycoprotein; a defense mechanism limiting oral bioavailability and CNS accumulation of drugs. Int J Clin Pharmacol Ther 38**:**69–74.

Fidock DA, Rosenthal PJ, Croft SL, Brun R, Nwaka S (2004). Antimalarial drug discovery: efficacy models for compound screening. Nat Rev Drug Discov.3 (6):509-20.

Fivelman QL, Adagu IS, Warhurst DC (2004). Modified Fixed-Ratio Isobologram Method for Studying *In Vitro* Interactions between Atovaquone and Proguanil or Dihydroartemisinin against Drug-Resistant Strains of *Plasmodium falciparum*. Antimicrobial Agents and Chemotherapy, 48 (11), 4097-4102

Foley M and Tilley L (1997). Quinoline antimalarials: mechanisms of action and resistance. International Journal for Parasitology 27:231–240.

Foley M and Tilley L (1998). Quinoline antimalarials: mechanisms of action and resistance and prospects for new agents. Pharmacology and Therapeutics 79(1): 55-87.

Foote SJ , Kyle DE , Martin RK , Oduola AM , Forsyth K , Kemp DJ , Cowman AF , (1990). Several alleles of the multidrug-resistance gene are closely linked to chloroquine resistance in *Plasmodium falciparum*. Nature 345, 255-258.

Fr. Fichter, R. B. (1906). "Ueber chindolin." Berichte der deutschen chemischen Gesellschaft. 39(4): 3932-3942.

Garattini E and Terao M (2012). The role of aldehyde oxidase in drug metabolism. Expert Opin Drug Metab Toxicol 8:487–503.

Gellért, E., Raymond, H. and Schlittler, E. (1951). "Die konstitution des alkaloids cryptolepine." Helvetica Chimica Acta **34**(2): 642-651.

Gibbons, S., Fallah, F. and Wright, C. W. (2003). "Cryptolepine hydrochloride: A potent antimycobacterial alkaloid derived from cryptolepis sanguinolenta." Phytotherapy Research **17**(4): 434-436.

Gilles HM, Warrell DA and Edward A (1993). Diagnostic Methods in Malaria. Bruce Chwatt's Essential Malariology 3rd Ed London 78-95.

Grance, SRM, Maria, AT, Roseana, SL, Eur'ipedes. BG, Jõ ao, MS, Wander, FOF, Simon, BSV, Maria, CV (2008). Baccharis trimera: Effect on hematological and biochemical parameters and hepatorenal evaluation in pregnant rats. Journal of ethnopharmacology 177:28-33

Greenwood BM, Bojang K, Whitty CJ, Targett GA (2005). "Malaria". Lancet 365: 1487-1498.

Greenwood BM (1987). Asymptomatic malaria infections—do they matter? Parasitol Today 3:206–214.

Grellier, P., Ramiaramanana, L., Millerioux, V., Deharo, E., Schrével, J., Frappier, F., Trigalo, F., Bodo, B. and Pousset, J.-L. (1996). "Antimalarial activity of cryptolepine and isocryptolepine, alkaloids isolated from *cryptolepis sanguinolenta*." Phytotherapy Research **10**(4): 317-321.

Gunatilaka, A.A.L.; Sotheeswaran, S.; Balasubramaniam, S.; Chandrasekara, A.I.; Badrasriyani, HT (1980). Studies on medicinal plants of Sri-Lanka .3. Pharmacologically important alkaloids of some Sida species. Planta Med., *39*, 66-72.

Hamilton RA, Garnett WR, and Kline BJ (1981). Determination of mean valproic acid serum level by assay of a single pooled sample. Clin Pharmacol Ther 29**:**408–413.

Hay S, Guerra C, Tatem A, Noor A and Snow R (2004). "The global distribution and population at risk of malaria: past, present, and future." Lancet 4(6): 327-336.

Hay SI and Snow RW (2006). "The Malaria Atlas Project: Developing Global Maps of Malaria Risk." PLoS Medicine 3*(*12): e473.

Hernandez-Valladares M, Rihet P, ole-MoiYoi OK, Iraqi FA (2004). Mapping of a new quantitative trait locus for resistance to malaria in mice by a comparative mapping approach with human Chromosome 5q31- q33. Immunogenetics. 56(2):115-7.

Herxhelmer, H. (1987). Basic information that prescribers are not getting about drugs. Lancet 1:32.

Hien TT and White NJ (1993). Qinghaosu. Lancet 341:603-608.

Hoareau L and Dasilva EJ (1999). Medicinal plants: a re-emerging health aid. Electronic Journal of Biotechnology 2(2): 56-69.

Houston JB (1994) Utility of *in vitro* drug metabolism data in predicting *in vivo* metabolic clearance. Biochem Pharmacol 47**:**1469–1479.

Houston JB and Carlile DJ (1997) Prediction of hepatic clearance from microsomes, hepatocytes, and liver slices. Drug Metab Rev 29**:**891–922.

Hout, S., Chea, A., Bun, S.Y., Elias, R. Gasquet, M. Timon-David, P., Balansard, G. and Azas, N. (2006). Screening of selected indigenous plants of Cambodia for antiplasmodial activity, J. Ethnopharmacol., 107(1): 12-18

Idro R, Otieno G, White S, Kahindi A, Fegan G, Ogutu B, Mithwani S, Maitland K, Neville BG and Newton CR (2007). "Decorticate, decerebrate and opisthotonic posturing and seizures in Kenyan children with cerebral malaria". Malaria Journal 4:57.

Irvine, FR (1961) Woody Plants of Ghana. Oxford University Press: London. 878

Israel, DM, Water, JB (1989) Cell response to injury. In: General Pathology 6th ed.:57-67.

Iwu, M.M. (1993). Handbook of African medicinal plants. CRC Press, LLC, 2000 N.W corporate Blvd., and Boca Raton, Florida 33431. Pp163-4

Izunga A.M, Nwaopara A.O, Aigbiremolen, Odike M.A.C, Oaikhena G.A and Bankole J.K (2010). Histological effects of oral administration of artesunate on the liver in Wista rats. Res. J. Appl. Sci. Eng. Technol., 2(4): 314-318

Jaeger, J. J., & Hedegaard, H. (2002). Liver function tests and blood tests: In the Danish Hepatitis C website. Retrieved from http://home3 .inet.tele.dk/omni/ alttest.html.

Jonckers, T.H.; Maes, B.U.W.; Lemiere, G.L.F.; Rombouts, G.; Pieters, L.; Haemers, A.; Dommisse, R.A. (2003) Synthesis of isocryptolepine *via* a Pd-catalyzed 'Amination-Arylation' approach. Synlett, 615-618.

Johnson, J. D., Dennull, R. A., Gerena, L., Lopez-Sanchez, M., Roncal, N. E. and Waters, N. C. (2007). Assessment and continued validation of the malaria SYBR green I-based fluorescence assay for use in malaria drug screening. Antimicrob Agents Chemother 2007, 51**:** Pp. 1926-1933.

Juliani, H. R, Simon, J. E., Chi-Tang H (2009**).** African Natural Plant Products: New Discoveries and Challenges in Chemistry and Quality. American Chemical Society 1021 (13):231–239.

Kain K, Harrington M, Tennyson S and Keystone J (1998). "Imported malaria: prospective analysis of problems in diagnosis and management." Clinical Infectious Diseases 27 (1): 142-149.

Kaur, K.; Jain, M.; Kaur, T.; Jain, R. (2009**)** Antimalarials from nature. Bioorg. Med. Chem., *17*, 3229-3256.

Kirby, G. C. (1996). "Medicinal plants and the control of protozoal disease, with particular reference to malaria." Transactions of the Royal Society of Tropical Medicine and Hygiene 90(6): 605-609.

Kirby, G. C., Paine, A. and Warhurst, D. C. (1995a). "*In vitro* and *in vivo* antimalarial activity of cryptolepine, a plant-derived indoloquinoline." Phytotherapy Research **9**: 359-363.

Kirby, G. C., Paine, A., Warhurst, D. C., Noamese, B. K. and Phillipson, J. D. (1995b). "*In vitro* and *in vivo* antimalarial activity of cryptolepine, a plant-derived indoloquinoline." Phytotherapy Research 9(5): 359-363.

Knook. DL, Bosma, A, Seifert, WF (1995) Role of vitamin A in liver fibrosis. J Gastroenterol Hepatol 10:47-49.

Knudsen AB and Slooff R (1992). Vector-borne disease problems in rapid urbanization: new approaches to vector control. Bulletin of the World Health Organization 70:1–6.

Kremsner PG, Winkler GS, Brandts C, Neifer S, Bienzle U, and Graninger W. (1994). Clindamycin in combination with chloroquine or quinine is an effective therapy for uncomplicated *Plasmodium falciparum* malaria in children from Gabon. The Journal of Infectious Diseases 169:467–470.

Krettli AU (2001). Antimalarial chemotherapy. Mechanisms of action, resistance and new directions in drug discovery. Memorias do Instituto Oswaldo Cruz 96(8): 1185-1186.

Kumar S., Guha M., Choubey V., Maity P., Bandyopadhyay U., (2007) Antimalarial drugs inhibiting hemozoin (β-hematin) formation: A mechanistic update: Life Sciences 80,813–828.

Kuntworbe, N., Martini, N., Brimble, M., Alany, G.R., Al-Kassas, R., (2012). Isolation and development of an HPLC method for the detection and quantification of cryptolepine and its application in the determination of the total cryptolepine content in the root powder of *Cryptolepis sanguinolenta*. Pharmacol. Pharm. 3, 263-270

Kuntworbe, N., Ofori, M., Addo P., Tingle M., Al-kassas R. (2013). Pharmacokinetics and in vivo chemosuppressive activity studies on cryptolepine hydrochloride and cryptolepine-loaded gelatine

nanoformulation designed for parenteral administration for the treatment of malaria. Acta tropica 127: 165-173.

Lathia, T. B., and Joshi, R. (2004). Can hematological parameters discriminate malaria from nonmalarious acute febrile illness in the tropics? Indian Journal of Medical Sciences, *58*, 239-244.

Lave T, Dupin S, Schmitt C, Chou RC, Jaeck D and Coassolo P (1997a) Integration of *in vitro* data into allometric scaling to predict hepatic metabolic clearance in man: Application to 10 extensively metabolized drugs. J Pharm Sci 86**:**584–590.

Lave T, Dupin S, Schmitt C, Valles B, Ubeaud G, Chou RC, Jaeck D and Coassolo P (1997b). The use of human hepatocytes to select compounds based on their expected hepatic extraction ratios in humans. Pharm Res 14**:**152–155.

Lavrado, J., Paulo, A., Gut, J., Rosenthal, P. J. and Moreira, R. (2008). "Cryptolepine analogues containing basic aminoalkyl side-chains at c-11: Synthesis, antiplasmodial activity, and cytotoxicity." Bioorganic & Medicinal Chemistry Letters 18(4): 1378-1381.

Lavrado, J., Reszka, A. P., Moreira, R., Neidle, S. and Paulo, A. (2010). "C-11 diamino cryptolepine derivatives nsc748392, nsc748393, and nsc748394: Anticancer profile and gquadruplex stabilization." Bioorganic and Medicinal Chemistry Letters 20(23): 7042-7045.

Lee, WM (2003). Acute liver failure in the United States. Semin. Liver Dis. 23:217-226.

Lengeler, C., Grabowsky, M., McGuire, D. and deSavigny, D. (2007). "Quick wins versus sustainability: Options for the upscaling of insecticide-treated nets." American Journal of Tropical Medicine and Hygiene **77**(6_Suppl): 222-226.

Lelievre J, Almela MJ, Lozano S, Miguel C, Franco V, Leroy D, Herreros E *(*2012*)*. Activity of clinically relevant antimalarial drugs on *Plasmodium falciparum* mature gametocytes in an ATP bioluminescence “transmission blocking” assay. PLoSOne. 7:35019.

Li Di (2014). The role of drug metabolism enzymes in clearance. Expert Opin. Drug Metab. Toxicol.10 (3):379-393

Lin JH, Cochetto DM, Duggan DE. (1987). Protein binding as a primary determinant of clinical pharmacokinetic properties of non-steroidal anti-inflammatory drugs. Clin Pharmacokinet 12:402432.

Lisgarten, J. N., Coll, M., Portugal, J., Wright, C., W., and Aymami, J. (2001). "The antimalarial and cytotoxic drug cryptolepine intercalates into DNA at cytosine-cytosine sites." Nature Structural and Molecular Biolology **9**(1): 57-60.

Luo, J., Fort, D. M., Carlson, T. J., Noamesi, B. K., nii-Amon-Kotei, D., King, S. R., Tsai, J., Quan, J., Hobensack, C., Lapresca, P., Waldeck, N., Mendez, C. D., Jolad, S. D., Bierer, D. E., and Reaven, G. M. (1998). *Cryptolepis sanguinolenta*: an ethnobotanical approach to drug discovery and the isolation of a potentially useful new antihyperglycaemic agent. Diabet Med 15, 367-374.

Mabaso, M. L. H., Sharp, B. and Lengeler, C. (2004). "Historical review of malarial control in southern african with emphasis on the use of indoor residual house-spraying." Tropical Medicine & International Health 9(8): 846-856.

Malik, E., Mohamed, T., Elmardi, K., Mowien, R., Elhassan, A., Elamin, S., Mannan, A. and Ahmed, E. (2006). "From chloroquine to artemisinin-based combination therapy: The Sudanese experience." Malaria Journal **5**(1): 65.

Malomo, S.O., 2000. Toxicological implication of triaxone administration in rats. Nigerian Journal of Biochemistry and Molecular Biology. 15(1): 33-38.

Marshall, E. (2000). "Drugs: Reinventing an ancient cure for malaria." Science **290**(5491): 437-439.

McCurrie J., Albalawi, S., Wright, C., Kuntworbe, N. (2009). Investigation of the absorption and vascular effects of the indoloquinoline alkaloid, cryptolepine. J Pharm Pharmacol; 61(Suppl. 1): A67.

McGregor, I.A. and Barr, M. (1962) Antibody response to tetanus toxoid inoculation in malarious and nonmalarious Gambian children. Trans. Roy. Soc. trop. Med.Hyg.56, 364.

McIntosh HM & Greenwood BM (1998) Chloroquine or amodiaquine combined with sulfadoxine pyrimethamine as a treatment for uncomplicated malaria – a systematic review. Annals of Tropical Medicine and Parasitology 93**,** 265–270.

McPherson K, Marsh T, Brown M (2007). Tackling obesities: future choices -modeling future trends in obesity and their impact on health. Report for Foresight. Government Office of the Chief Scientist.

Menendez C, Kahigwa E, Hirt R, Vounatsou P, Font F, Aponte JJ, Acosta CJ, Schellenberg DM, Galindo CM, Kimario J, Urassa H, Brabin B, Smith TA, Kitua AY, Tanner M, Alonso PL (1997). Randomised placebo-controlled trial of iron supplementation and malaria chemoprophylaxis for prevention of severe anaemia and malaria in Tanzanian infants. Lancet 350, 844-50

Meis JF, Ponnudurai T, Mons B, van Belkum A, van Eerd PM, Druilhe P, Schellekens H (1990). *Plasmodium falciparum*: studies on mature exoerythrocytic forms in the liver of the chimpanzee, *Pan troglodytes*. Exp Parasitol. 70(1):1-11.

Merlin LW (2004). Traditional Medicinal Plants and Malaria. Buckingham, Gerard Bodeker, University of Oxford England, UK: University of Oxford.

Meshnick, S.R., Scott, M.D., Lubin, B., Ranz, A. and Eaton, J.W. (1990) Antimalarial activity of diethyldithiocarbamate. Potentiation by copper. Biochem. Pharmacol. 40, 213–216.

Mills S and Bone K (2000). Principles and practice of phytotherapy. Churchill Livingstone, Edinburgh, 91-108.

Modiano, D., Sirima, B. S., Konate, A., Sano, I., & Sawadogo, A. (2001). Leucocytosis in severe malaria.Transactions of the Royal Society of Tropical Medidicne and Hygiene, 95(2), 175-176. http://dx.doi.org/10.1016/S0035-9203 (01)90152-X

Mshana, N. R., Abbiw.K, Addae-Mensah, I., Adjanohoun, E., Ahyi, M. R. A., Ekpere, J. A., Enow-Orock, E. G., Gbile, Z. O., Noamesi, G. K., Odei, M. A., Odunlami, H., Oteng-Yeboah, A. A., Sarpong, K., Soforowa, A. and Tackie, A. N. (2000). Traditional medicine and pharmacopoeia. Contribution to the Revision of Ethnobotanical and Floristic Studies in Ghana Organization of African Unity/ Scientific, Technical and Research Committee, pp: 473.

Mueller MS, Runyambo N, Wagner I, Bormann S, Dietz K and Heide L (2004). Randomized controlled trial of a traditional preparation of Artemisia annua L. (Annual Wormwood) in the treatment malaria. Transactions of the Royal Society of Tropical Medicine & Hygiene 98:318-321

Mueller, M.S., Karhagomba, I.B., Hirt, H.M., Wemarkor, E., Li, S.M., and Heide L (2000). The potential of *Artemisia annua* L. as a locally produced remedy for malaria in the tropics: agricultural, chemical and clinical aspects. Journal of Ethnopharmacology 74:487-493.

Munhenga, G., Masendu, H., Brooke, B., Hunt, R. and Koekemoer, L. (2008). "Pyrethroid resistance in the major malaria vector anopheles arabiensis from gwave, a malaria-endemic area in zimbabwe." Malaria Journal **7**(1): 247.

Murray, RK. Granner, DK, Mayes, PA, and Rodwell, VW. (1998). Harper's Biochemistry (a Lange Medical book), international ed.: Appleton and Lang Lange Medical Publication, p.574-6.

Muthaura, C.N.; Rukunga, G.M.; Chhabra, S.C.; Omar, S.A.; Guantai, A.N.; Gathirwa, J.W.; Tolo, F.M.; Mwitari, P.G.; Keter, L.K.; Kirira, P.G.; Kimani, C.W.; Mungai, G.M.; Njagi, E.N (2007).Antimalarial activity of some plants traditionally used in treatment of malaria in Kwale district of Kenya. J. Ethnopharmacol. *112*, 545-551.

Nassar AF, Hollenberg PF and Scatina JA (2009). Drug Metabolism Handbook: Concepts and Applications, Wiley, New Jersey, USA.

Navaratnam, V, Mansor, SM, Sit, NW, Grace, J, Li, Q, Olliaro, P (2000). Pharmacokinetics of artemisinin-type compounds. Clin Pharmacokinet., 39 (4); 255-70.

Nirogi R., Kandikere V., Choudary P., Bhyrapuneni G., Kanamarlapudi VB., Ponnamaneni RK., Manoharan AR (2013). Identification of a suitable and selective inhibitor towards aldehyde oxidase catalyzed reactions. Xenobiotica, 44(3):197-204.

Noamesi, B. K., Larsson, B. S., Laryea, D. L. and Ullberg, S. (1991). "Whole-body autoradiographic study on the distribution of 3h-cryptolepine in mice." Archives Internationales de Pharmacodynamie et de Thérapie 313:5-14.

Noedl H, WernsdorferWH, Miller RS, Wongsrichanalai C (2002). Histidine-rich protein II: a novel approach to malaria drug sensitivity testing. Antimicrob Agents Chemother. 46(6):1658-64.

Nogueira F, Estólio do Rosário V. (2010) Methods for assessment of antimalarial activity in the different phases of the Plasmodium life cycle. Rev Pan-Amaz Saude; 1(3):109-124

Nosten F and White NJ (2007). Artemisinin-based combination treatment of falciparum malaria. American Journal of Tropical Medicine and Hygiene **77**:181-192.

Obach RS (1997) Nonspecific binding to microsomes: Impact on scale-up of in vitro intrinsic clearance to hepatic clearance as assessed through examination of warfarin, imipramine, and propranolol. Drug Metab Dispos 25:1359–1369.

Ogbonna DN, Sokari TG, and Agomuoh AA (2008). Antimalarial activities of some selected traditional herbs from Southeastern Nigeria against *Plasmodium* species. Research Journal of Parasitology **3**(1):25-31

Okell, L., Drakeley, C., Ghani, A., Bousema, T. and Sutherland, C. (2008). "Reduction of transmission from malaria patients by artemisinin combination therapies: A pooled analysis of six randomized trials." Malaria Journal**7** (1): 125.

Olajide, O.A., Ajayi, A.M., and Wright, C.W. (2009). Anti-inflammatory Properties of Cryptolepine. Phytother Res 23**,** 1421-1425.

Olajide, O.A., Heiss, E.H., Schachner, D., Wright, C.W., Vollmar, A.M., and Dirsch, V.M. (2007b). Synthetic cryptolepine inhibits DNA binding of NF-kappaB. Bioorg Med Chem 15, 43-49.

Olajide, O.A., Pinheiro de Oliveira, A., Unekwe, J., Wright, C., and Fiebich, B. (2010). Cryptolepis sanguinolenta (Lindl.) Schltr. root extract inhibits prostaglandin production in IL-1b stimulated SKN-SH neuronal cells. Planta Med 76, 601.

Olson, H., Betton, G., Robinson, D., Thomas, K., Monro, A., Kolaja, G., Lilly, P., Sanders, J., Sipes, G., Bracken, W., Dorato, M., Van Deun, K., Smith, P., Berger, B., Heller, A. (2000). Concordance of the toxicity of pharmaceuticals in humans and in animals. Regul Toxicol Pharmacol 32(1): 56-67.

Onyeibor, O., Croft, S. L., Dodson, H. I., Feiz-Haddad, M., Kendrick, H., Millington, N. J., Parapini, S., Phillips, R. M., Seville, S., Shnyder, S. D., Taramelli, D. and Wright, C. W. (2005). "Synthesis of some cryptolepine analogues, assessment of their antimalarial and cytotoxic activities, and consideration of their antimalarial mode of action." Journal of Medicinal Chemistry 48(7): 2701-2709.

Oyekan, A. O. (1994). "Role of the endothelium and cyclic GMP in renal vasodilator responses to cryptolepine in rats." Journal of Cardiovascular Pharmacology **23**(4): 602-611.

Oyekan, A.O. and J.P.O. Okafor (1989). Effects of cryptolepine alone and in combination with dipyridamole on a mouse model of arterial thrombosis. J. Ethnopharmacol., 27: 141-148.

Paulo, A., Duarte, A. and Gomes, E. T. (1994a). "*In vitro* antibacterial screening of *Cryptolepis sanguinolenta* alkaloids." Journal of Ethnopharmacology 44(2): 127-130.

Paulo, A., Gomes, E. T. and Houghton, P. J. (1995). "New alkaloids from *Cryptolepis sanguinolenta*." Journal of Natural Products 58(10): 1485-1491.

Paulo A. Houghton PJ (2003). Chemotaxonomic analysis of the genus *Cryptolepis.*Biochem. Syst. Ecol. 31:155-166.

Paulo, A., Pimentel, M., Viegas, S., Pires, I., Duarte, A., Cabrita, J. and Gomes, E. T. (1994b). "*Cryptolepis sanguinolenta* activity against diarrhoeal bacteria." Journal of Ethnopharmacology 44(2): 73-77.

Paulo, A.; Gomes, E.T.; Steele, J.; Warhurst, D.C.; Houghton, P.J.(2000) Antiplasmodial activity of *Cryptolepis sanguinolenta* alkaloids from leaves and roots. Planta Med., 66, 30-34.

Peters, W. (1967). Rational methods in the search for antimalarial drugs. Trans. R. Soc. Trop. Med. Hyg., 61, 400-410.

Peters, W. (1975). "The chemotherapy of rodent malaria, xxii. The value of drug-resistant strains of *P. berghei* in screening for blood schizontocidal activity." Annals of Tropical Medicine and Parasitology 69(2): 155-171.

Peters, W. (1982). Antimalarial drug resistance: an increasing world problem. Br. Med. Bull. 38, 187-192.

Peters W. (1987). Chemotherapy and Drug Resistance in Malaria, 2nd ed. London: Academic Press.

Phillips RS (2001). Current Status of Malaria and Potential for Control. Clinical Microbiology Reviews 14(1):208-226.

Phillipson, J. D., Wright, C. W., Kirby, G. C., and Warhurst, D. C. (1993). Tropical plants as sources of antiprotozoal agents. In Recent Advances in Phytochemistry, Vol. 27, Phytochemical Potential of Tropical Plants, ed. by K. R. Downum, J. T. Romeo and H. A. Stafford, pp. 1-40. Plenum Press, New York.

Polli JW, Wring SA, Humphreys JE, Huang L, Morgan JB, Webster LO, Serabjit-Singh CS *(*2001). Rational use of *in vitro* P-glycoprotein assays in drug discovery. Pharmacol Exp Ther; 299: 620-628.

Pradines B, Tall A, Parzy D, Spiegel A, Fusai T, Hienne R, Trape JF, Doury JC (1998). *In vitro* activity of pyronaridine and amodiaquine against African isolates (Senegal) of *Plasmodium falciparum* in comparison with standard antimalarial drugs—Mannich bases: promising drugs for Africa? *J Antimicrob Chemother 42:* 333–339.

Price RN, Nosten F, Luxemburger C, ter Kuile FO, Paiphun L, Chongsuphajaisiddhi T and White NJ (1996). Effects of artemisinin derivatives on malaria transmissibility. Lancet 347:1654-1658.

Price R, Van Vugt M, Phaipun L, Luxemburger C, Simpson J, McGready R, Kuile FT, Khan A, Chongsuphajasiddhi T, White NJ, Nosten F (1999). Adverse effects in patients with acute falciparum malaria treated with artemisinin derivatives. Am. J. Trop. Med. Hyg., 60 (4), 547-555

Pryde DC, Dalvie D, Hu Q, Jones P, Obach RS, and Tran T-D (2010) Aldehyde oxidase an enzyme of emerging importance in drug discovery. J Med Chem 53:8441–8460.

Pukrittayakamee, S., Chotivanich, K., Chantra, A., Clemens, R., Looareesuwan, S. and White, N. J. (2004). Activities of artesunate and primaquine against asexual and sexual stage parasites in *falciparum* malaria. Antimicrob Agents. 48: Pp. 1329-1334.

Raaman, N., (2006). Phytochemical Techniques, New India Publishing Agency, 101, Vikas Surya Plaza, CU Block, L.S.C Mkt., Pitam Pura, New Delhi-110 088, pp 9-10, 25-27.

Rauwald, H.W.,Kober, Mutschler, E. and Lambrecht, G. (1992). *Cryptolepis sanguinolenta*: antimuscarinic properties of of cryptolepine and the alkaloid fraction at M1, M2 and M3 receptors. Planta Med, 58, 486-8.

Raymont-Hamet (1937). Sur quelque propriétés physiologiques des alcaloides du *Cryptolepis sanguinolenta* Schlechter. Compt. Rend. Soc. Biol., *126*, 768-770.

Raza, M., Al-Shabanah, O.A., El-Hadiyah, T.M and Al-Majed, A.A. (2002). Effect of prolonged vigabatrin treatment on hematological and biochemical parameters in plasma, liver and kidney of Swiss albino mice. Scientia Pharmaceutica 70, 135-145.

Reader, J, Botha M, Theron A, Lauterbach SB, Rossouw C, Engelbrecht D, Wepener M, Smit A, Leroy D, Mancama D, Coetzer TL and Birkholtz L (2015). Nowhere to hide: interrogating different metabolic parameters of *Plasmodium falciparum* gametocytes in a transmission blocking drug discovery pipeline towards malaria elimination. Malaria Journal 14:213

Reed, M. B., K. J. Saliba, S. R. Caruana, K. Kirk, and A. F. Cowman (2000). Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. Nature 403:906–909.

Reid, M.E. and C. Lomas-Francis, (2004). The Blood Group Antigen Facts Book. Second ed., New York: Elsevier Academic Press.

Ringwald, P., Meche, F. S., Bickii, J. and Basco, L. K. (1999). In Vitro Culture and Drug Sensitivity Assay of Plasmodium falciparum with Nonserum Substitute and Acute-Phase Sera. J Clin Microbiol. 37 (3): Pp. 700-705

Rivlin, R. S. (2001). Historical perspective on the use of garlic. J Nutr 131(3s): 951-954.

Rocha de A Silva J, Aline de S Ramos, Marta Machado, Dominique F de Moura1, Zoraima Neto, Marilene M Canto-Cavalheiro, Paula F, Virgilio E do Rosário, Ana Claudia A, Dinora L (2011) A review of antimalarial plants used in traditional medicine in communities in Portuguese-Speaking countries: Brazil, Mozambique, Cape Verde, Guinea-Bissau, São Tomé and Príncipe and Angola. Mem Inst Oswaldo Cruz, Rio de Janeiro, Vol. *106*(Suppl. I): 142-158.

Rodrigues AD (1997) Preclinical drug metabolism in the age of high-throughput screening: An industrial perspective. Pharm Res 14:1504–1510.

Ryley, J.F and Peters W. (1970). The antimalarial activity of some quinolone esters. Am. Trop. Med. Parasitol., 84: 209-222.

Sachs J and Malaney P (2002). The economic and social burden of malaria. Nature 415(7): 680-685.

Sadasivaiah, S., Tozan, Y. and Breman, J. G. (2007). "Dichlorodiphenyltrichloroethane (ddt) for indoor residual spraying in Africa: How can it be used for malaria control?" The American Journal of Tropical Medicine and Hygiene **77**(6 Suppl): 249-263.

Sakyiamah, MM, Ocloo, A, Adjimani, JP, Sittie, A. (2011). Effect of Aqueous Extract of *Cryptolepis Sanguinolenta* on the Pharmacokinetics of Artesunate. Int J Pharm Sci Drug Res. 3(4): 313-318

Salako, Q., Ablordeppey, S.Y., Dwuma-Badu, D., Thornback, F.R., (1985). Radioiodination and preliminary in vivo investigation of the alkaloid cryptolepine. Int. J. APPL. Radiat. Isot. 36, 10031004

Sanders NG, Sullivan DJ, Mlambo G, Dimopoulos G, Tripathi AK (2014). Gametocytocidal Screen Identifies Novel Chemical Classes with *Plasmodium falciparum* Transmission Blocking Activity. PLoS ONE 9(8): e105817. doi:10.1371/journal.pone.0105817

Sanni LA, Fonseca LF, Langhorne J (2002). Mouse models for erythrocytic-stage malaria. Methods Mol Med. 72:57-76.

Schinkel AH (1999). P-glycoprotein, a gatekeeper in the blood-brain barrier. Adv Drug Deliv Rev 36:179–194.

Schmidt, T.J., Khalid, S.A., Romanha, A.J., Alves, T.M.A., Biavatti, M.W., Brun, R., Costa, F.B., Castro, S.L., Ferreira, V.F., Lacerda, M.V.G., Lago, J.H.G., Leon, L.L., Lopes, N.P., Amorim, R.C.N., Niehues, M., Ogungbe, I.V., Pohlit, A.M., Scotti, M.T., Setzer, W.N., Soeiro, M.N.C., Steindel, M., Tempone, A.G., (2012a). The potential of secondary metabolites from plants as drugs or leads against protozoan neglected diseases.
Part I. Current Medicinal Chemistry 19, 2128–2175.

Schmidt, T.J., Khalid, S.A., Romanha, A.J., Alves, T.M.A., Biavatti, M.W., Brun, R., Andrade Neto, V.F., Costa, F.B., Castro, S.L., Ferreira, V.F., Lacerda, M.V.G., Lago, J.H.G., Leon, L.L., Lopes, N.P., Amorim, R.C.N., Niehues, M., Ogungbe, I.V., Pohlit, A.M., Scotti, M.T., Setzer, W.N., Soeiro, M.N.C., Steindel, M., Tempone, A.G., (2012b).
The potential of secondary metabolites from plants as drugs or leads against protozoan neglected diseases – Part II. Current Medicinal Chemistry 19, 2176–2228.

Segurado AA, di Santi SM and Shiroma M (1997). *In vivo* and *in vitro Plasmodium falciparum* resistance to chloroquine, amodiaquine and quinine in the Brazilian Amazon. Revista do Instituto de Medicina Tropical de São Paulo 39:85–90

Seville, S.; Phillips, R.M.; Shnyder, S.D.; Wright, C.W. (2007). Synthesis of cryptolepine analogues as potential bioreducible anticancer agents. Bioorg. Med. Chem., 15, 6353-6360.

Shahjahan, M., K.E. Sabitha, J. Mallika, C.S. Shyamala-Devi, (2004). Effect of *Solanum trilobatum* against carbon tetrachloride induced hepatic damage in albino rats. Indian J. Med Res., 120: 194198.

Shou M, Lin Y, Lu P, Tang C, Mei Q, Cui D, Tang W, Ngui JS, Lin CC, Singh R, Wong BK, Yergey JA, Lin JH, Pearson PG, Baillie TA, Rodrigues AD, Rushmore TH (2001). Enzyme kinetics of cytochrome P450-mediated reactions. Curr Drug Metab 2(1):17–36.

Silva O, Duarte A, Cabrita J, Pimentel M, Diniz A, Gomes E (1996). Antimicrobial activity of Guinea-Bissau traditional remedies. J .Ethnopharmacol., 50: 55-59.

Sigma Aldrich SYBR® Green I product information (2008). http://www.sigmaaldrich.com /sigmaaldrich/datasheet/s9430dat.pd. Date accessed: 12/11/2012.

Sinden RE, Canning EU, Bray RS, Smalley ME (1978). Gametocyte and gamete development in Plasmodium falciparum. Proc R Soc Lond B Biol Sci.201:375–99.

Sipilanyambe, N., Simon, J., Chanda, P., Olumese, P., Snow, R. and Hamer, D. (2008). "From chloroquine to artemether-lumefantrine: The process of drug policy change in Zambia." Malaria Journal **7**(1): 1-7.

Snow RW, Mung'ala VO, Foster D, Marsh K (1994). The role of the district hospital in child survival at the Kenyan Coast. Afr J Health Sci; 1:71–75

Sodiomon BS, Alfred BT, Adama G, Amidou D, Amidou O, Amadou TK, Jean-Rene K, Morgan CC, Olliaro PL and Walter RJT (2009). The efficacy and safety of a new fixed-dose combination of amodiaquine and artesunate in young African children with acute uncomplicated *Plasmodium falciparum* Malaria Journal 8:48

Stepniewska K, Price NC, Sutherland CJ, Drakeley CJ, von Seidlein L, Nosten F, White NJ (2008). *Plasmodium falciparum* gametocyte dynamics in areas of different malaria endemicity. Malar J 7:249. doi: 10.1186/1475-2875-7-249

Stell JGP, Wheelhouse RT, Wright CW (2012). Metabolism of Cryptolepine and 2Fluorocryptolepine by aldehyde oxidase. Journal of Pharm. And Pharmacol, 64,237-243.

Strelevitz TJ, Orozco CC, and Obach RS (2012). Hydralazine as a selective probe inactivator of aldehyde oxidase in human hepatocytes: estimation of the contribution of aldehyde oxidase to metabolic clearance. Drug Metab Dispos 40:1441–1448.

Snyder C, Chollet J, Wna Santo-Tomas J, Scheurer C, Wittlin S (2007). *In vitro* and *in vivo* interaction of synthetic peroxide RBx11160 (OZ277) with piperaquine in *Plasmodium* models. Experimental Parasitology; 115:296–300

Su, X. Z., Kirkman, L. A., Fujioka, H. & Wellems, T. E (1997). Complex polymorphisms in an approximately 330 kDa protein are linked to choroquine-resistant *P. falciparum* in Southeast Asia and Africa. Cell 91(5), 593-603.

Svensson USH, Ashton M (1999). Identification of the human cytochrome P450 enzymes involved in the *in vitro* metabolism of artemisinin. Br J Clin Pharmacol; 48(4): 528–535.

Synder, R., Lea, G.W., Kocsis, J.J. and Witner, C.M. (1977). Bone marrow depressant and leuconogenic actions of benzene. Life Science 21, 1709-1722.

Tabuti, J. R. S. (2008). "Herbal medicines used in the treatment of malaria in Budiope County, Uganda." Journal of Ethnopharmacology 116(1): 33-42.

Tanaka TQ, Dehdashti SJ, Nguyen DT, McKew JC, Zheng W, Williamson KC (2013). A quantitative high throughput assay for identifying gametocytocidal compounds. Mol Biochem Parasitol. 188:20–5.

Tanaka TQ, Williamson KC. **(**2011). A malaria gametocytocidal assay using oxidoreduction indicator, alamarBlue. Mol. Biochem. Parasitol.177:160 –163.

Teo, S, Stirling, D, Thomas, S, Hoberman, A, Kiorpes, A, Khetani, V (2002). A 90-day oral gavage toxicity study of d-methylphenidate and d,I-methylphenidate in Sprague Dawley rats. Toxicology 179: 183-196.

ter Kuile F, White NJ, Holloway P, Pasvol G, Krishna S (1993). Plasmodium falciparum: *in vitro* studies of the pharmacodynamic properties of drugs used for the treatment of severe malaria. Experimental Parasitology 76:85–95.

Timbrel, J (2000). Principles of Biochemical Toxicology. Taylor & Francis Ltd. 261-277.

Tona L, Kambu K, Ngimbi N, Cimanga K, Vlietinck AJ (1998). Antiamoebic and phytochem. Screening of some Congolese med. plants. J. Ethnopharmacol., 61**:** 57-65.

Tona L, Ngimbi NP, Tsakala M, Mesia K, Cimanga K, Apers S, De Bruyne T, Pieters L, Totté J, Vlietinck AJ (1999). Antimalarial activity of 20 crude extracts from nine African medicinal plants used in Kinshasa, Congo. J Ethnopharmacol 68: 193-203.

Trager, W and Jensen JB (1976). Human malaria parasites in continuous culture. Science 193:673–675.

Trampuz A, Jereb M, Muzlovic I, Prabhu R (2003). "Clinical review: Severe malaria". Critical Care **7** (4): 315–23.

Trape, J.F., Pison, G., Spiegel, A., Enel, C., Rogier, C. (2002). Combating malaria in Africa. Trends in Parasitology 18, 224–230.

Udobre A., Edoho J.E., Eseyi O. and Etim I.E (2009). Effects of Artemisinin with Folic acid on the activities of Aspartate Amino Transferase, Alanine Amino Transferase and Alkaline Phosphatase in Rats. Asian J. Biochem., 4(2): 55-59

Van Miert, S.; Hostyn, S.; Maes, B.U.W.; Cimanga, K.; Brun, R.; Kaiser, M.; Matyus, P.; Dommisse, R.; Lemiere, G.; Vlietinck, A.; Pieters, L (2005). Isoneocryptolepine, a synthetic indoloquinoline alkaloid, as an antiplasmodial lead compound. J. Nat. Prod., 68, 674-677.

Van Miert, S.; Jonckers, T.; Cimanga, K.; Maes, L.; Maes, B.; Lemiere, G.; Dommisse, R.; Vlietinck, A.; Pieters, L (2004**)**. *In vitro* inhibition of beta-haematin formation, DNA interactions, antiplasmodial activity, and cytotoxicity of synthetic neocryptolepine derivatives. Exp. Parasit., 108, 163-168.

Vaughan, A. M., Aly, A. S. I. and Kappe, S. H. I. (2008). Malaria parasite pre-erythrocytic stage infection: Gliding and Hiding. Cell Host Microbe. 4(3): Pp. 209-218.

Vivas L, Rattray L, Stewart LB, Robinson BL, Fugmann B, Haynes RK, Peters W, Croft SL *(*2007). Antimalarial efficacy and drug interactions of the novel semi-synthetic endoperoxide artemisone *in vitro* and *in vivo.* J Antimicrob Chemother; *59*(4): 658–65.

Vossen, M. G., Pferschy, S., Chiba, P. and Noedl, H. (2010). The SYBR Green I malaria drug sensitivity assay: performance in low parasitemia samples. Am J Trop Med Hyg. 82(3): Pp. 398401.

Wells, R.M., A.K. Mcintyre and P.S. Davie (1986). Physiological stress responses in big game fish after exposure: Observation in plasma chemistry and blood fractions. Comparative Biochemistry and Physiology, 64A: 565-571.

White NJ (1997). Assessment of the pharmacodynamic properties of antimalarial drugs *in vivo*. Antimicrobial Agents and Chemotherapy 41:1413–1422.

White, N. J. (1985). Clinical pharmacokinetics of antimalarial drugs. Clin. Pharmacokinetics 10, 187-215.

White NJ (1999). Delaying antimalarial drug resistance with combination chemotherapy. Parasitologia 41:301-308.

White, N. J., & Breman, J. G. (2001). Malaria and Babesiosis: Diseases caused by red blood cell parasites. In Eugene Braunwald (Ed.), Harrison's Principles of Internal Medicine (pp. 12031212). New York: Mcgraw-Hill.

WHO (1990). Mefloquine therapy for *Plasmodium falciparum* malaria in children under 5 years of age in Malawi: *in vivo*/*in vitro* efficacy and correlation of drug concentration with parasitological outcome. Bulletin of the World Health Organization 68:53–59.

WHO (1991). Basic Laboratory Methods in Medical Parasitology. World Health Organization Geneva 12.

WHO (1996). World malaria situation in part I. Weekly Epidemiological Record 71:17– 22.

WHO (1996a). A rapid dipstick antigen capture assay for the diagnosis of falciparum malaria. WHO informal consultation on recent advances in diagnostic techniques and vaccines for malaria. Bulletin of the World Health Organization 74:47–54.

WHO (1996b). Assessment of therapeutic efficacy of antimalarial drugs for uncomplicated *falciparum* malaria in areas with intense transmission.

WHO (2001). *In vitro* micro-test (Mark III) for the assessment of *P. falciparum* to chloroquine, mefloquine, quinine, amodiaquine, sulfadoxine/pyrimethamine and artemisinin. CTD/MAL/9720 Rev 2. Geneva, Switzerland.

WHO (2001). WHO recommended strategies for the prevention and control of communicable disease. WHO/CDLS/CPE/ SMT/ 13:107-110.

WHO (2001). Antimalarial drug combination therapy: Report of a WHO Technical Consultation 35.

WHO (2003). Global defense against the infectious disease threat. WHO/CDS/2003/15. 18:178181.

WHO (2003). Monitoring antimalarial drug resistance: a report of a consultation. WHO/CDS/CSR/EPH/2002.17. WHO/CDS/RBM/2002.39. Geneva, Switzerland.

WHO. (2005). "World malaria report." 20/05/2010, from http://www.rollbackmalaria.org/wmr2005/.

WHO. (2006). "Roll back malaria partnership." Facts on ACTs Retrieved 23rd August, 2010.

WHO. (2007). Field application of *in vitro* assays for the sensitivity of human malaria parasites to antimalarial drugs.From http://apps.who.int/iris/bitstream/10665/43610/1/9789241595155_eng.pdf Acessed 10/07/2017.

WHO. (2010). "World malaria report." From http://www.who.int. /malaria/world_malaria_report_2010/en/index.html. Access 23th August, 2013.

WHO. (2011).Traditional medicine fact sheets N134, 2008

WHO. (2011). "World malaria report." 09/09/2013, from http://www.who.int/malaria/world_malaria_report_2011/en/.

WHO (2014). World Malaria Report. Geneva, Switzerland: World Health Organization.

WHO (2015). Fact Sheet: World Malaria Report 2015. WHO, Geneva, Switzerland. http://www.who.int/malaria/media/world-malaria-report-2015/en/

Wilairatana P. and Looareesuwan S. (2002). The clinical use of artemisinin and its derivatives in the treatment of malaria, In: Wright CW (ed), Artemisia (pp. 289–307). London: Taylor and Francis

Willcox, M. L., Graz, B., Falquet, J., Sidibé, O., Forster, M. and Diallo, D. (2007). "Argemone mexicana decoction for the treatment of uncomplicated falciparum malaria." Transactions of the Royal Society of Tropical Medicine and Hygiene **101**(12): 1190-1198.

Williams JA, Hyland R, Jones BC, Smith DA, Hurst S, Goosen TC, Peterkin V, Koup JR, Ball SE. (2004). Drug-drug interactions for UDP-glucuronosyltransferase substrates: a pharmacokinetic explanation for typically observed low exposure (AUCi/AUC) ratios. Drug Metab Dispos; 32(11):1201-8

Winter RW, Kelly JX, Smilkstein MJ, Dodean R, Bagby GC, Rathbun RK, Levin JI, Hinrichs D, Riscoe MK (2006). Evaluation and lead optimization of antimalarial acridones. Exp. Parasitol., 114:47-56.

Wright, C. W. (2005). Plant derived antimalarial agents: leads and challemges. Phytochemistry Reviews 4:55-6

Wright, C. W. (2005). "Traditional antimalarials and the development of novel antimalarial drugs." Journal of Ethnopharmacology 100(1-2): 67-71.

Wright, C. W., Addae-Kyereme, J., Breen, A. G., Brown, J. E., Cox, M. F., Croft, S. L., Gokcek, K. Y., Kendrick, H., Phillips, R. M. and Pollet, P. L. (2001a). "Synthesis and evaluation of cryptolepine analogues for their potential as new antimalarial agents." Journal of Medicinal Chemistry 44(19): 3187-3194.

Wright, C. W., Addae-Kyereme, J., Breen, A. G., Brown, J. E., Cox, M. F., Croft, S. L., Gökçek, Y., Kendrick, H., Phillips, R. M. and Pollet, P. L. (2001b). "Synthesis and evaluation of cryptolepine analogues for their potential as new antimalarial agents." Journal of Medicinal Chemistry 44(19): 3187-3194.

Wright, C. W., Phillipson, J. D., Awe, S. O., Kirby, G. C., Warhurst, D. C., Quetin-Leclercq, J. and Angenot, L. (1996). "Antimalarial activity of cryptolepine and some other anhydronium bases." Phytotherapy Research **10**(4): 361-363.

Wright CW and Warhurst DC (2002). The mode of action of artemisinin and its derivatives, In: Wright CW (ed), Artemisia (pp. 249–288). London: Taylor and Francis.

Yang, S.W.; Abdel-Kader, M.; Malone, S.; Werkhoven, M.C.M.; Wisse, J.H.; Bursuker, I.;

Neddermann, K.; Fairchild, C.; Raventos- Suarez, C.; Menendez, A.T.; Lane, K.; Kingston, D.G.I. (1999). Synthesis and biological evaluation of analogues of cryptolepine, an alkaloid isolated from the Suriname rainforest. J. Nat. Prod., 62, 976-983.

Yunnis, A.A., Miller, A.M., Sallen, Z. and Arimura, G.K. (1980). Chloramphenicol toxicity: pathogenic mechanisms and the role of pNO_2 in aplastic anemia. Clinical Toxicology, 17, 359-373.

Zhang D, Luo G, Ding X, Lu C (2012). Preclinical experimental models of drug metabolism and disposition in drug discovery and development. Acta Pharmaceutica Sinica B; 2(6):549-561.

Zhang X, Liu HH, Weller P, Zheng M, Tao W, Wang J, Liao G, Monshouwer M, and Peltz G (2011). *In silico* and *in vitro* pharmacogenetics: aldehyde oxidase rapidly metabolizes a p38 kinase inhibitor. Pharmacogenomics J 11:15–24.