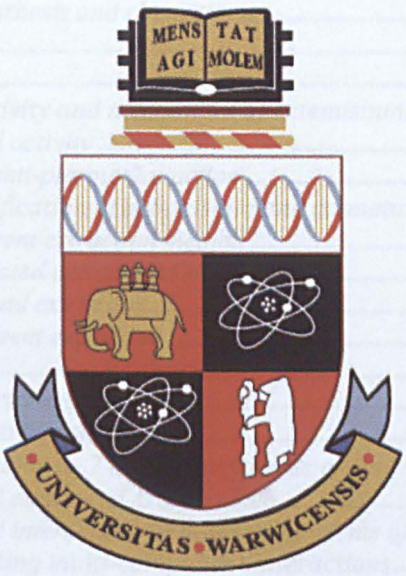
Analysis of Plant Materials for Molecules of Pharmaceutical Importance

John Oluwasegun Suberu

(BSc, MSc)

A thesis presented for the degree of Doctor of Philosophy in Biomedical Engineering



University of Warwick

School of Engineering

July 2013

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF SCHEMES

LIST OF TABLES

LIST OF APPENDED MANUSCRIPTS

1. Suberu J, Song L, Slade S, Sullivan N, Barker G. and Lapkin A. (2013) A rapid method for the determination of artemisinin and its biosynthetic precursors in Artemisia annua L. crude extracts. Journal of Pharmaceutical and Biomedical Analysis 84: 269-277. http://dx.doi.org/10.1016/j.jpba.2013.06.025

2. Suberu JO, Gorka AP, Jacobs L, Roepe PD, Sullivan N, Barker, G and Lapkin, A. (2013) Anti-plasmodial polyvalent interactions in *Artemisia annua* L. aqueous extract - possible synergistic and resistance mechanisms. PLOS ONE (accepted).

ACKNOWLEDGEMENT

My profound gratitude goes to God, who through various people and means made this PhD a possibility and for giving me health and strength to complete the project.

I am indebted to the tireless supervision given to me by my supervisory team, Prof. Alexei Lapkin, Dr. Guy Barker and Dr, Neil Sullivan. I am also thankful to SensaPharm and EPSRC for financial support for the project.

The support of the following people is thankfully acknowledged - Prof. Paul Roepe and Dr. Alex Gorka (George Town University, USA) with plasmodium assay. Dr. Isolda Romero with anti-proliferation assay, Dr Lijiang Song (both of Chemistry Dept.), Dr. Susan Slade, Dr. Krisztina Radi and Andy Jukes (School of Life Sciences) with mass spectrometry, Yamin Payman (RWTH Aachen, Germany) for help with COSMO-RS, Prof. Elizabeth Williamson (Reading University) with valuable advice on Phytocomplexes, Prof. Richard Napier, the chair of my advisory team, Matthew Mitchell for general laboratory support, my colleagues in various laboratories where my experiments were carried out including TP 108 (HRI), C105 (Gibbet Hill) and sustainable chemical processes and materials laboratory.

Special thanks to my wife, Florence for her unwavering support, prayers and understanding through many late nights, early mornings, busy weekends and cancelled holidays. My thanks also goes to my siblings and their families for their prayers and support – Dr. and Mrs. Sogbesan, Mr. and Mrs. Asuelime, Prof. and Mrs. Rotimi Suberu, Prof. and Dr. (Mrs) Ajayi and Mr. and Mrs. Gbenga Suberu. Thanks to Zac. Yisa, Adam Manis, Martin Joseph Nicholl and a host of other friends and relatives, whose support and encouragement was valuable and very much appreciated.

DEDICATION

To the memory of my mum, who with encouragement and prayers anticipated with eagerness the completion of this work but was tragically taken away from us some months before.

and

To the memory of my dad, my role model, an amazing father and friend.

DECLARATION

I declare that the work presented in this thesis was conducted by me under direct supervision of Prof. Alexei Lapkin and Dr. Guy Barker. None of the work presented has been previously submitted for any other degree.

John O. Suberu

ABSTRACT

Natural products are an important source for drug discovery. At present there is a resurgent interest in pharmacognosy as a platform for new combinations of active principles to provide highly potent and low-cost medications to treat a growing population with an increasing longevity. This project studied phytochemical interactions in *Artemisia annua* plant extracts using anti-plasmodium and anti-proliferation assays to identify interactions with potential therapeutic implications.

To enable the study a rapid tandem quadrupole mass spectrometry (TQD) method was developed for metabolites in the plant and the validation indices showed the method to be robust, quick, sensitive and adequate for a range of applications.

The plasmodium assay tests showed mild-to-strong antagonistic interactions between artemisinin, the main anti-malaria agent in the plant, and some related metabolites. Caffeoylquinic acids, artemisinic acid and arteannuin B showed additive interactions while rosmarinic acid showed synergistic interaction with artemisinin in the chloroquine-sensitive plasmodium strain (CQS). Arteannuin B potentiated the activity of artemisinin in resistant strain only. In the cytotoxic assay, an aqueous *Artemisia annua* extract showed inconsistent activity. 3-Caffeoylquinic acid (3CA) in combination with artemisinin resulted in total loss of cytotoxicity, while combination of 3CA with cisplatin, another anti-cancer drug, showed a 13% improvement in activity. A mechanistic explanation was suggested for these observations.

Metabolite interaction was also employed to solve the artemisinin purification problem. The interactions of methoxylated flavonoids, especially casticin, artemetin and retusin, with artemisinin were identified as putative causative factors for low crystallisation yields from extracts. An elevated level of artemetin was found in the East African biomass, which also demonstrated poor crystallisation yields, whilst doping experiments confirmed the negative impact of these flavonoids on crystallisation of artemisinin.

Consequently, the study showed that phytochemical interactions could be exploited and applied with benefits in pharmaceutical and chemical purification processes.

ABBREVIATIONS

AC = activated carbon
ACT = Artemisinin Combination Therapy
ADS = amorpha-diene synthase
ALDH1 = aldehyde dehydrogenase 1 enzyme
APIs = active pharmaceutical ingredients
BHT = butylated hydroxytoluene
COSMO-RS = condoctor like screening model for real solvents
CQAs = Chlorogenic or caffeoylquinic acids
CQR = chloroquine resistant
CQS = chloroquine sensitive
CYP71AV1 = cytochrome P450 enzyme
DBR2 = double bond reductase 2
DHAA = dihydroartemisinic acid
DMAPP = dimethylallyl diphosphate
DMBA = 7,12 dimethylbenz [a] anthracene
DMSO = Dimethyl-sulphoxide
DSC = Deferential Scanning Calorimetry
DXR = 1-deoxyxylulose 5-phosphate reductoisomerase
DXS = 1-deoxyxylulose 5-phosphate synthase
ECACC = European Collection of Cell Cultures
EIC = extracted Ion chromatography
ELSD = evaporative light scattering detector
ER = endoplasmic reticulum
ESI+ = positive electrospray ionisation
ETC = electron transport chain
FDP = fernesyl diphosphate
FIC = fractional inhibition concentrations
FPP = fernesyl pyrophosphate
FPS = fernesyl diphosphate synthase
Glc = glucose unit
GST = glandular secreting trichomes
Hb = hemoglobin
HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFC-134a = 1,1,1,2-tetrafluoroethane
HMEC-1 = human dermal micro-vascular endothelial cells
HMGR = 3-hdroxy-3-methylglutaryl-CoA reductase
HPLC = high pressure liquid chromatography
HRMS = high resolution mass spectrometry
HTP = high throughput analysis
IPP = isopentenyl diphosphate
IS = internal standard
LC = liquid chromatography
LLOQ = lower limit of quantification
LOD = limit of detection
LPS = Lipo-polysaccharide
MCF7 = Michigan cancer foundation 7 cancer cell line
Me = methyl group
MEP = methyl erythritol phosphate
MMP-9 = matrix metalloproteinase 9
Mpa = megapascal
MRM = multiple reaction monitoring
MS/MS = tandem mass spectrometry
MVA = mevalonic acid
NADH = nicotinamide dehydrogenase
NCI = national cancer institute
OTC =over the counter medications
PAD = photodiode array detector
PAF = platelet agregation factor
PBS = phosphate buffer saline
PfATPase6 = Ca2+ transporter pump
PfCRT = plasmodium falciparum chloroquine resistant transporter
PfMDR1 = plasmodium falciparum multi-drug resistant 1 transporter
PfMDR6 =plasmodium falciparum multi-drug resistant 6 transporter
PfTCTP = plasmodium falciparum translationally controlled tumour protein
QToF = Quardrupole time of flight
ROS = reactive oxygen species
RPMI = Roswell Park Memorial Institute medium
SPE = solid phase extraction
SRB = sulforhodamine B assay
TEA = triethylamine
TQD = tandem quardrupole detector
UBP-1 = putative de-ubiquitinating enzyme
VEGF = vascular endothelial growth factor

Units

ev = electron volt
IC_{50} = half maximal response
K = kelvin measurement for temperature
kg = kilogram
M = molar
m/z = mass to charge ratio
mg = miligram
mL = mililitre
mM = micro molar
mm = milimeter
mM = milli molar
MΩ = Megaohms
nM = nano molar
°C = degree centrigrade
RF = radio frequency
rpm = revolution per minute
V = volt
Vpp = peak to peak voltage
μCi = microcurie
μg = microgram
μL = microlitre
μm = micrometer

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

1.1 Natural products as sources of drug candidates

Natural products are an important source for drug discovery (Newman and Cragg, 2007). The Ayurvedic, Chinese and other traditional medicine systems based on millennia of experience in natural remedies provides a rich source of folk knowledge which can be exploited for discovering novel compounds of medicinal value (Verpoorte *et al.*, 2009).

Bioactive phytochemicals are a large variety of chemical compounds which are mainly secondary metabolites (Hattenschwiler and Vitousek, 2000, Ebada *et al.*, 2008). In plants, these compounds are often involved in protection against biotic and abiotic stresses and are not essential for cell maintenance or structure. Some secondary metabolites, like flavonoids and carotenoids play a major role in plant reproduction through their involvement in pigmentation of flower and seed cells (Watson *et al.*, 2001).

The chemical and pharmacological properties of some secondary metabolites are of importance to human and animal health (Raskin *et al.*, 2002, Reddy *et al.*, 2003). About 50 % of prescribed and over the counter (OTC) medications are derived directly or indirectly from plants (Newman *et al.*, 2000). Metabolites groups such as alkaloids, terpenoids and flavonoids are used as drugs or as dietary supplements in the treatment and prevention of various diseases (Raskin *et al.*, 2002).

The search for new active principles for the treatment of major diseases such as malaria, AIDS, cancers and associated multi-drug resistant challenges and the need for more effective and low cost medications resulting from increasing global life expectancy are driving the resurgent interest in pharmacognosy (Karou *et al.*, 2007). This is also in part due to the failure of the combinatorial synthetic approaches to deliver new bio-actives.

The advantage of natural product-based therapies is evident for example in oncology, where some synthetic cancer drugs cause non-specific killing of cells while natural products provide therapeutic and protective actions to all cells and are beneficial in producing nutrient replacements to people with compromised health (Reddy *et al.*, 2003). Moreover, the biological activities of plant extracts are often the results of the additive or synergistic effects of its constituents, which enhance their use in multi-component or combination therapies where resistance to single ingredient drug is anticipated (Ebel and Cosio, 1994).

Pharmacognosy has had several major successes. The isolation of paclitaxel or taxol from the bark of yew tree is an example. Paclitaxel exerts its anti-cancer effect by inhibiting mitosis and is now a drug approved by the Food and Drug Administration (FDA) for ovarian and breast cancers (Nobili *et al.*, 2009). Another pharmacognosy success is the anti-malaria compound artemisinin a sesquiterpene lactone derived from *Artemisia annua* L used in World Health Organization (WHO) backed Artemisinin Combination Therapy (ACT) for the treatment of uncomplicated *Plasmodium falciparum* malaria infection (Stringham *et al.*, 2011, WHO, 2006).

1.2 *Artemisia annua* – botany and metabolic profile

Artemisia annua known as sweet wormwood, sweet Annie and Qinghao (Chinese), is an annual herb native to Asia. The generic name *Artemisia* came from the Greek goddess Artemis (Diana) of maternity as a result of the earlier use of the plant in controlling menstrual pain and disorders (Riddle and Estes, 1992). Ancient Chinese pharamacopia mentioned the use of the plant (flower and leaves) for treatment of fevers and heats (Hsu, 2006a).

1.2.1 Botany

The plant is an aromatic annual and a vigorous weedy herb that is single stemmed and grows up to two meters (Figure 1.1). It grows in temperate conditions and high altitude in tropical areas. The seeds are very small and are sown to seedling stage before transplanting. Artemisinin, the anti-malaria agent is synthesized and stored in the glandular trichomes (GST). These are specialised cells that protrude from the epidermis of leaves, flowers and stems of the plant (Dalrymple, 2006).

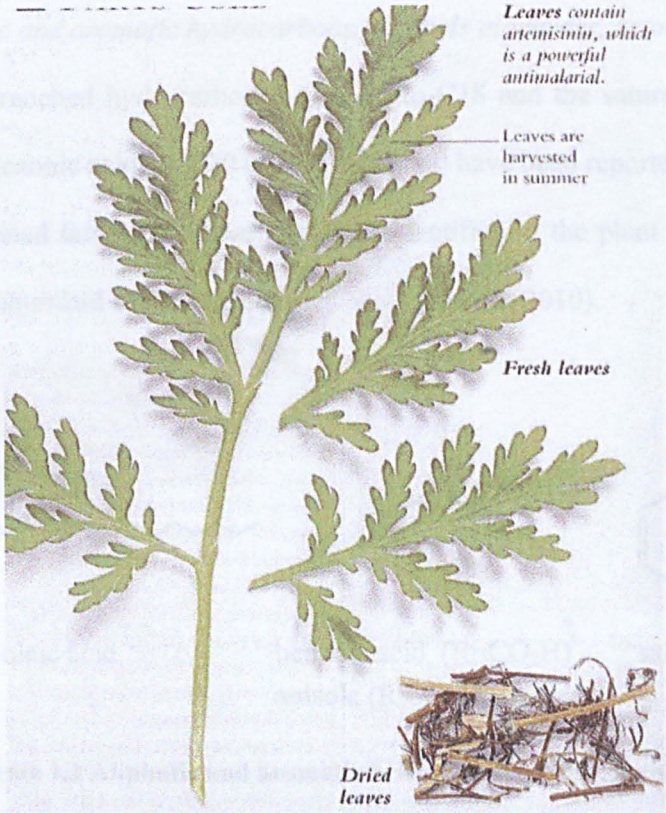


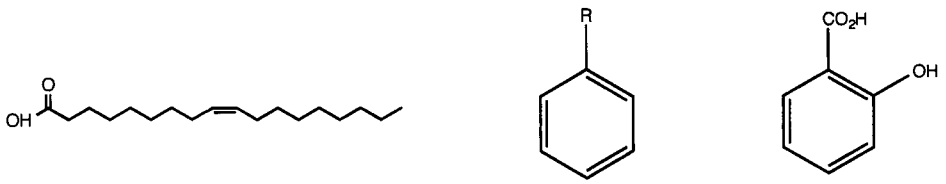
Figure 1-1 *Artemisia annua* fresh and dried leaves (Chevallier, 1996).

1.2.2 Metabolic profile

Several authors (Bhakuni *et al.*, 2002, Bhakuni *et al.*, 2001, Chen *et al.*, 2008, Brown, 2010) have reviewed the phyto-chemistry of *A. annua* and described about six hundred metabolites in the plant. Different criteria have been used to broadly categorize this vast array of plant secondary metabolites. Some more common ones are based on the recurring structural features in the compounds, increasing oxidative states of their molecule or similarity in their biosynthetic routes (biogenetic classification). In the latter, secondary metabolites are grouped starting from simple molecules – such as derivatives of aliphatic and aromatic hydrocarbons to complex steroid molecules. This classification is used in the succeeding sections as adopted in the Dictionary of Natural Products (Bruckingham, 2000, Brown, 2010).

1.2.2.1 Aliphatic and aromatic hydrocarbons, alcohols aldehydes, ketones and acids

All of the un-branched hydrocarbon from C16 to C18 and the saturated fatty acids from C12 (dodecanoic acid) to C20 (eicosanoic acid) have been reported in *A. annua.* Several unsaturated fatty acids have also been identified in the plant with oleic acid being the most abundant of the un-saturated acids (Brown, 2010).



oleic acid benzoic acid (R=CO_2H) anisole (R= OMe) salicylic acid

Scheme 1.1 Aliphatic and aromatic acids and ketone in *A. annua*.

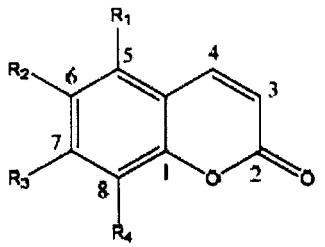
Example of aromatic ketones and acids in *Artemisia* plant are anisole, benzoic acid and its derivatives like salicylic acid (Scheme 1.1) (Brown, 2010). Oleic acid has reported antimicrobial and anti-cancer activities (Dilika *et al.*, 2000, Mizushina *et al.*, 2012). Salicylic acid and some compounds in this group (like ethylene) are known plant hormones (Cseke *et al.*, 2010). Salicylic acid is used in anti-aging treatments and is an important metabolite of the anti-inflammatory agent acetyl-salicylic acid (Yang *et al.*, 2004).

1.2.2.2 Phenylpropanoids

This group of metabolites is based on a 3-carbon substituent fused to an aromatic phenyl group. An important subgroup is hydroxycinnamic esters formed by various combinations of ferulic and cinnamic acids with the 4-hydroxyl group of quinic acid

to give compounds like chlorogenic acids (Brown, 2010). The bioactivity of chlorogenic acids is reviewed in detail in Section 1.7.2.

A number of coumarins including those shown in Scheme 1.2 have been identified in *A. annua*. Coumarins (benzopyrones) and their derivatives have a range of activities including anti-proliferative, anti-viral, anti-coagulant, anti-inflammatory, anti-microbial and anti-oxidant properties (Riveiro *et al.*, 2010).



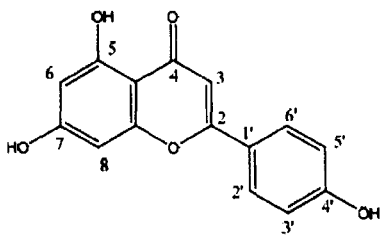
Compound	R1	R2	R3	R4
Coumarin	H	H	H	H
Scopoletin	H	OMe	OH	H
Scoparone	H	OMe	OMe	H
Scopolin	H	OMe	OGlc	H
Isofraxidin	H	OMe	OH	OMe
Tomentin	OH	OMe	OMe	H

Scheme 1.2 Coumarins in *Artemisia* plants.

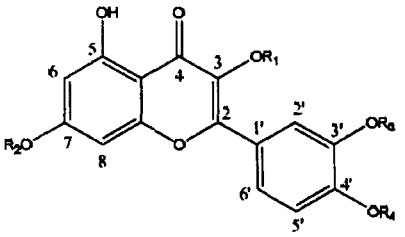
1.2.2.3 Flavonoids

Flavonoids are polyphenolic compounds based on a 15-carbon skeleton arranged in three rings (C6-C3-C6). Of the over 4,500 flavonoids found in plants, about 40 have been identified in *A. annua* (Ferreira *et al.*, 2010, Lai *et al.*, 2007). Lai *et al.* showed the main flavonoids in the plant to be rhamnetin, chrysosplenol D, quercetin

glucoside, flaviolin and pillion (Lai *et al.*, 2007). Highly significant quantities of polymethoxylated flavonoids and several long chained 5-alkyl resorcinols have also been reported (Brown, 1992). Methoxylated flavonoids have been linked to the activation *in vitro* of artemisinin (Bilia *et al.*, 2002) and they have also been shown to synergistically potentiate the anti-plasmodial activity of artemisinin but not chloroquine (Elford *et al.*, 1987).



Apigenin



	R1	R2	R3	R4
Quercetin	H	H	H	H
Quercetin 3-methyl ether	Me	H	H	H
Isoquercitrin	Glc	H	H	H
Quercetrin 3-rutinoside	Sugar	H	H	H
Isorhamnetin 3-glucoside	Glc	H	Me	H
Rhamnetin	H	Me	H	H
Quercimeritin	H	Glc	H	H
Isorhamnetin	H	H	Me	H
Quercetrin 3'-glucoside	H	H	Glc	H
Tamarixetin	H	H	H	Me

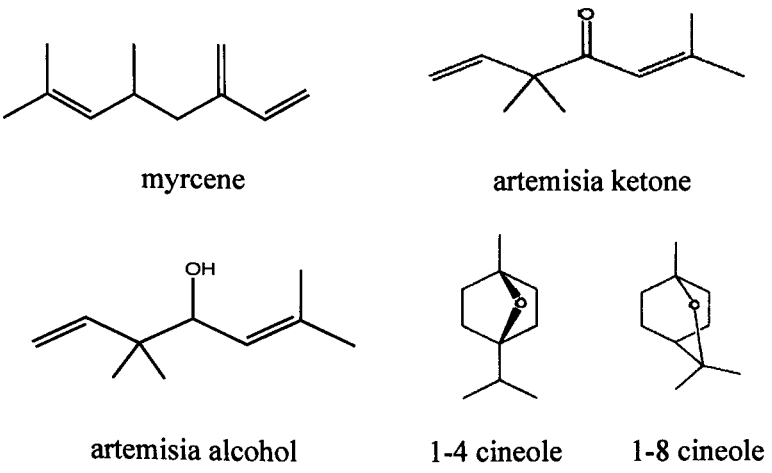
Scheme 1.3. Pentahydroxy –flavonols in *A. annua*.

Apigenin, quercetin, rhamnetin and other flavonoid shown in Scheme 1.3 have all demonstrated some level of anti-proliferative activities alone and as chemosensitisers to anti-cancer drugs (Ferreira *et al.*, 2010, Limtrakul *et al.*, 2005). The reported anti-inflammatory activities of these flavonoids would suggest their possible application in

treating pathological disturbances such as obesity, arteriosclerosis, diabetes and neurodegenerative illnesses (García-Lafuente *et al.*, 2009).

1.2.2.4 Monoterpenoids

Monoterpenoids are generally ten-carbon molecules and principal components of *A. annua* essential oils. They are believed to be localized in the trichomes (Brown, 2010) and can be extracted by steam. The oil yield in *A. annua* could be between 0.3% and 4.0% (dry weight) depending on the part and chemotype of the plant (Woerdenbag *et al.*, 1993a, Bhakuni *et al.*, 2001). The main constituents of the essential oils in *A. annua* are varied and may include artemisia ketone, artemisia alcohols, cineole, myrcene (Scheme 1.4) and camphor.



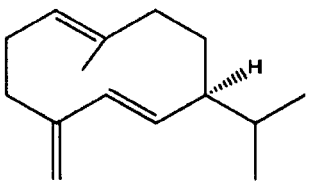
Scheme 1.4. Cyclic and acyclic monoterpenoids.

The observed differences in the bioactivity of these essential oils reflects the wide variation in their constitution (Woerdenbag *et al.*, 1993a). Several authors have reported both anti-bacterial and anti-fungi activities of *A. annua* essential oils (Juteau

et al., 2002, Ćavar *et al.*, 2012, Lopes-Lutz *et al.*, 2008). A mild antioxidant activity has also been reported.

1.2.2.5 Sesquiterpenoids

Sesquiterpenoids (C15) constitutes the most abundant and most diverse group of metabolites in *A. annua.* Structurally they range from simple acylic to tricyclic conformation (Brown, 2010). Germacrene D (Scheme 1.5), a monocyclic sesquiterepene may constitute up to 20% of the essential oils of *A. annua* (Goel *et al.*, 2008).

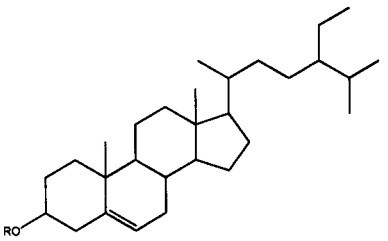


Scheme 1.5. Structure of germacrene D

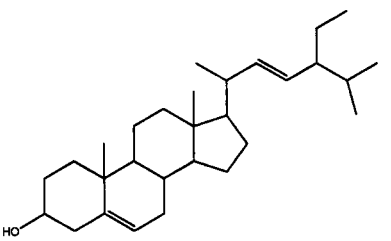
The bicyclic amorphane (cardinane) are the largest group of sesquiterpenoids found in the plant. Artemisinin, dihydroartemisinin, artemisinic acid and arteannuin B (Figure 1.2) are the most abundant of this group (You-you *et al.*, 1982, Brown, 2010, Suberu *et al.*, 2013a). A series of arteannuins (A to O with the exception of D and G) and other artemisinin-related metabolites have been identified in the plant (Brown, 2010). Apart from the anti-malaria activities associated with some of these compounds (Rydén and Kayser, 2007), their antiviral (Efferth *et al.*, 2008) and anti-cancer (Zhu *et al.*, 2013) activities have also been reported.

1.2.2.6 Other terpenoids and steroids

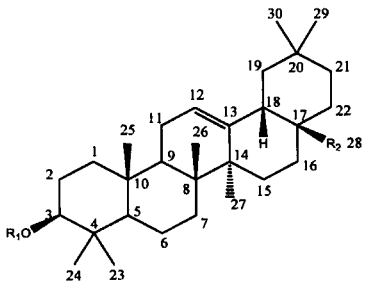
A. annua also contains diterpenoids like abscisic acids (ABA) and phytol, triterpenoids like α and β-amyrins, oleanolic acids and sterols like beta-sistosterol (Scheme 1.6). Alpha and β-amyrin have known anxiolytic (anti-anxiety) and antidepressant effects (Aragão *et al.*, 2006).



β-sitosterol (R=H)



stigmasterol



Compound	R1	R2
β-Amyrin	H	Me
β-Amyrin 3-acetate	$(C=O)CH_3$	Me
Oleanolic acid	H	CO_2H

Scheme 1.6. Some higher terpenoids and steroids in *A. annua*.

Sistosterol and stigmasterol (Scheme 1.6) are the most common of phytosterols in Chinese traditional medicine with known bioactivities to include, hypercholesterolemic (cholesterol reducing) (Chandler *et al.*, 1979), anti-inflammatory (Gabay *et al.*, 2010) and anti-proliferative (Ghosh *et al.*, 2011).

1.3 Artemisinin - biosynthesis and chemistry

1.3.1 Biosynthesis

The biosynthesis of artemisinin has been reviewed by several authors (Brown, 2010, Rydén and Kayser, 2007, Akhila *et al.*, 1987) and evidence supports that it is localized in the granular secreting trichomes (GST) (Covello *et al.*, 2007). Artemisinin, like other terpenoids, share a common biosynthetic precursor - isopentenyl diphosphate (IPP) and its isomer – dimethylallyl diphosphate (DMAPP). These two 5-carbon compounds have independent pathways to fernesyl pyrophosphate (FPP, also known as fernesyl diphosphate, FDP) and originate from the cytosol and plastid respectively, see Figure 1.2 (Weathers *et al.*, 2006, Weathers *et al.*, 2011). These two upstream branches of terpenoid biosynthesis are regulated by 1-deoxyxylulose 5-phosphate synthase (DXS) and 1-deoxyxylulose 5-phosphate reductoisomerase (DXR) or 3-hdroxy-3-methylglutaryl-CoA reductase (HMGR) respectively. One DMAPP and two IPP molecules are joined in a "head to tail" condensation by the enzyme fernesyl diphosphate synthase (FPS) to give a 15-carbon intermediate, fernesyl diphosphate (FDP).

Next, the FPP is cyclized to armorpha-4, 11-diene by amorpha-diene synthase (ADS) (Weathers *et al.*, 2006). Some authors have reported without clarity that dihydroartemisinic acid and/or artemisinic acid are intermediates in the conversion of amorpha-4, 11-diene to artemisinin (Wallaart *et al.*, 1999, Abdin *et al.*, 2003, Bertea *et al.*, 2005). However preponderant evidence seem to support the role of dihydroartemisinic acid (DHAA) as a late intermediate (Weathers *et al.*, 2011, Bertea *et al.*, 2005).

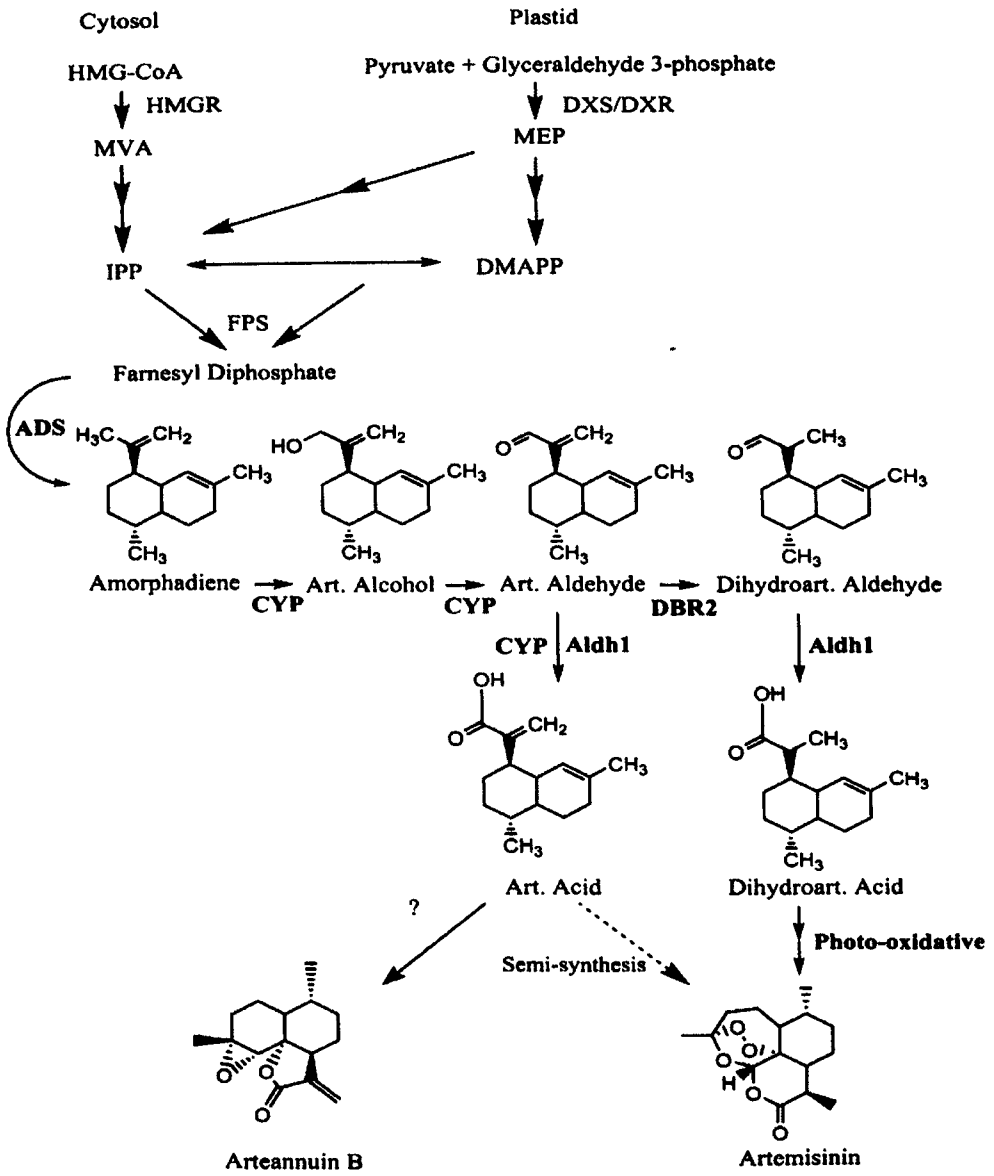
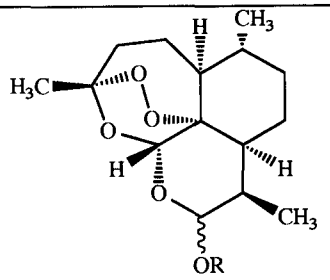


Figure 1-2 Proposed pathways by Weathers *et al.* for the biosynthesis of artemisinin with structures for artemisinic acid, dihydroartemisinic acid and arteannuin B (Weathers *et al.*, 2006). HMGR = 3-hydroxy-3-methylglutaryl-CoA reductase, DXS = 1-deoxyxylulose 5-phosphate synthase, DXR = 1-deoxyxylulose 5-phosphate reductoisomerase, ADS = amorphadiene synthase, ALDH1 = aldehyde dehydrogenase 1, CYP71AV1 = cytochrome P450 enzyme, DBR2 = double bond reductase 2, IPP = isopentenyl diphosphate, DMAPP = dimethylallyl diphosphate, MEP = methyl erythritol phosphate, MVA = mevalonic acid.

Amorpha-4, 11-diene is oxidized to DHAA via artemisinic aldehyde by the actions of a cytochrome P450 enzyme, CYP71AV1 (Ro *et al.*, 2006a), double bond reductase 2, DBR2 (Olofsson *et al.*, 2011) and most likely an aldehyde dehydrogenase 1 enzyme , ALDH1 (Teoh *et al.*, 2009) (Figure 1.2). The final step, which is the conversion of dihydroartemisinic acid to artemisinin is suggested to be non-enzymatic (Brown, 2010). The production of artemisinic acid is via a branch in the pathway at artemisinic aldehyde catalyzed by the action of CY71 AV1 and/or ALDH1. It is also suggested that arteannuin B is produced by a non-enzymatic conversion of artemisinic acid. The semi-synthetic route for artemisinin production has been developed and the main method involves the conversion of artemisinic acid in the biomass to artemisinin (Lévesque and Seeberger, 2012).

1.3.2 Chemistry

Artemisinin is a sesquiterpene lactone incorporating an endoperoxide bridge in its molecule. The artemisinin-related anti-malarial derivatives are composed of a 1,2,4-trioxane ring, which is central to the molecule's bioactivity. The compound is stable at temperatures below 100 °C (WHO, 2011). The aqueous solubility of artemisinin is poor but it is very soluble in some non-polar solvents (Rydén and Kayser, 2007). Modification of the lactone group in artemisinin has resulted in derivatives with better solubility and improved potency. The majority of these derivatives are currently used in anti-malarial therapies (Scheme 1.7).



Compound	R
Dihydroartemisinin	H (α + β)
Artemether	CH_3 (β)
Arteether	CH_2CH_3 (β)
Artelinate	$CH_2C_6H_4COONa$ (β)
Artesunate	$COCH_2CH_2COONa$ (α)

Scheme 1.7. Pharmaceutical derivatives of artemisinin (Rydén and Kayser, 2007).

1.4 Anti-plasmodial activity and mechanism of artemisinins

1.4.1 Anti-plasmodial activity

In early clinical studies, artemisinin showed fast action, low toxicity and good efficacy against both the chloroquine-resistant and sensitive strains. The drawback however was its sparing solubility in both water and oils. To combat these limitations, programs to develop more soluble and effective analogues of artemisinin were initiated, leading to the development of APIs such as the oil soluble artemether and water-soluble sodium artesunate (Scheme 1.7) (Li *et al.*, 2000). Due to the short half-life of both the parent and the derivative compounds, treatment with this class of anti-malarials is performed over 5 -7 days when used alone. A high rate of recrudescence (or re-infection) among patients administered with artemisinin based drugs has been reported (Ittarat *et al.*, 2003). World Health Organization (WHO) have therefore recommended the use of these agents in combination with longer half-life drugs to

improve efficacy and reduce the possibility of the emergence of drug resistance (Ridley, 2002).

1.4.2 Mechanism of anti-plasmodial action

The effectiveness of artemisinin compared to earlier anti-malarials is structurally due to the trioxane pharmacophore (O'Neill *et al.*, 2010a). Critical to artemisinin anti-plasmodium activity is the cleavage of the endoperoxide bridge. The mechanism of the interaction between the activated moiety and the parasite is not well understood. Four different but not mutually exclusive mechanistic models have been proposed with evidence for and against each model (Figure 1.3) (Ding *et al.*, 2011). The heme pathway model suggests that the erythrocytic *Plasmodia* in its digestive vacuole breaks down the host's haemoglobin as a source of amino acids releasing heme in the process (Ying-Zi *et al.*, 1994, O'Neill *et al.*, 2010a). The free heme is detoxified by crystallisation to haemozoin. However, in the presence of artemisinin iron from free heme catalyses cleavage of the endoperoxide bridge into free radicals, which alkylates heme and disrupts their detoxification. The build up of toxicity eventually leads to the death of the parasite.

Another model hypothesised that non-heme iron might cause the cleavage of artemisinin into reactive species, which causes the alkylation of other parasites proteins including *Plasmodium falciparum* translationally controlled tumour protein (PfTCTP) also leading to the death of the parasite (Meshnick, 2002, Asawamahasakda *et al.*, 1994).

Recently, work with mice and yeast models have lead to the postulation that the parasite mitochondrial electron transport chain (ETC) directly activates artemisinin,

resulting in a build up of reactive oxygen species (ROS) and (or) carbon centred radicals which inhibits the electron donor (NADH) dehydrogenase and in turn leads to the depolarisation of the mitochondrial membrane and apoptosis (Wang *et al.*, 2010a, Li *et al.*, 2005, Ding *et al.*, 2011).

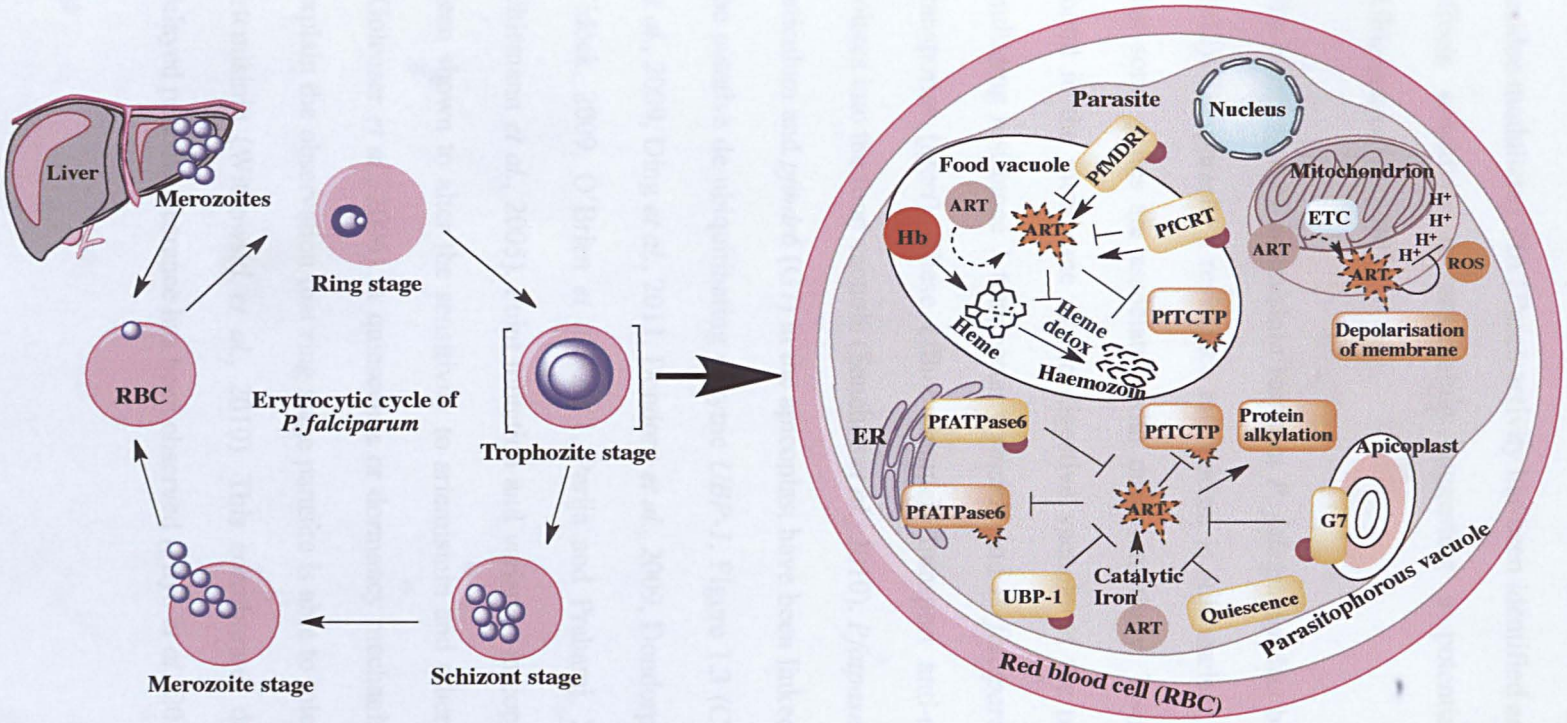


Figure 1-3 Erytrocytic life cycle of *P. falciparum* with enlargement of the trophozite stage showing possible mechanistic models for artemisinin activity and parasite resistance. Art = artemisnin. ER = endoplasmic reticulum. Hb = hemoglobin. PfCRT = *Plasmodium falciparum* chloroquine resistant transporter. PfMDR1 = *Plasmodium falciparum* multi-drug resistant 1 transporter. PfTCTP = *Plasmodium falciparum* translationally controlled tumour protein. ETC = electron transport chain. PfATPase6 = Ca^{2+} transporter pump. UBP-1 = putative de-ubiquitinating enzyme. ROS = reactive oxygen species. PfMDR6 = *Plasmodium falciparum* multi-drug resistant 6 transporter (Cui *et al.*, 2012).

A fourth model postulates that artemisinin targets the Ca^{2+} transporter pump (PfATPase6) of the parasite's endoplasmic reticulum (ER) by inhibiting the activity of PfATPase6 eventually leading to death (Eckstein-Ludwig *et al.*, 2003). A single residue modulating PfATPase6 activity has been identified and mutation in this residue affects sensitivity to artemisinin suggesting a potential resistance mechanism (Uhlemann *et al.*, 2005).

The emergence of artemisinin resistant *P. falciparum* has been reported (Phyo *et al.*, 2012). The parasite's resistance mechanism is still unclear. Current understanding is that some genes are associated with drug sensitivity. Two of these genes products found in the membrane of the digestive vacuole of the parasite are *P. falciparum* multidrug resistance 1 transporter (*pfmdr1*) and *P. falciparum* chloroquine resistance transporter (*pfcrt*). These efflux channels transport anti-malarial drugs and other solutes into the food vacuole (Sanchez *et al.*, 2010). *Pfatpase6* gene in the endoplasmic reticulum and *pfmdr6* (G7) in the apicoplast have been linked with sensitivity and so is the putative de-ubiquitinating enzyme *UBP-1*, Figure 1.3 (Cui *et al.*, 2012, Dahlström *et al.*, 2009, Ding *et al.*, 2011, Dondorp *et al.*, 2009, Dondorp *et al.*, 2010, Eastman and Fidock, 2009, O'Brien *et al.*, 2011, Parija and Praharaj, 2011, Sidhu *et al.*, 2006, Uhlemann *et al.*, 2005). Point mutation and variation in copy number of *pfmdr1* have been shown to alter the sensitivity to artemisinin and other anti-malarial compounds (Golenser *et al.*, 2006). A quiescence or dormancy mechanism has been postulated to explain the observation that ring stage parasite is able to tolerate high concentration of artemisinin (Witkowski *et al.*, 2010). This corroborates data from the field where delayed parasite clearance has been observed (Phyo *et al.*, 2012).

Several *in vitro* studies (Tucker *et al.*, 2012, Cui *et al.*, 2012, Beez *et al.*, 2011, Witkowski *et al.*, 2010) of the phenotypic and genetic mutation in resistant parasites selected by exposure to elevated drug level have shown through their varied outcomes, that plasmodium resistance is a multi-factorial trait. In one of these studies, Cul *et al.* using dihydroartemisinin showed that sensitivity was associated with increased *pfmr1* copy number and elevated anti-oxidant activity (Cui *et al.*, 2012).

1.5 Extraction and purification of artemisinin and co-metabolites

Plants are a complex matrix of metabolites. These metabolites have distinct physiochemical characteristics (e.g. solubility), which make some method of extraction for a type of compound more suited or efficient than the alternatives. The plant material is generally dried and in some cases pulverized before extraction. Below are some methods employed in the extraction and purification of artemisinin and co-metabolites in *A. annua.*

1.5.1 Traditional solvent extraction method

This method is widely used in industrial scale purification of artemisinin and co-metabolites. The traditional method of solvent extraction is characterized by the use of a large volume of petrochemical solvents and is labor and time intensive because of the longer extraction time and the multiple steps involved in obtaining a product of reasonable purity (Christen and Veuthey, 2001, Lapkin *et al.*, 2006). There is however a growing shift to more greener, faster and efficient methods with lower cost and environmental impact (Lapkin *et al.*, 2010). A variation of conventional extraction methods like maceration, Soxhlet, percolation and sonication are used in these systems.

Maceration involves leaving the pulverized biomass to soak in the extraction solvent in a closed container and may involve mechanical stirring or shaking to ensure homogeneous mixing (Sarker *et al.*, 2005). An example is the method by Elsholy *et al.* for the isolation of artemisinin, artemisinic acid and arteannuin B from *A. annua* using n-hexane as extraction solvent. Extracts were further purified by partitioning in 20% aqueous acetonitrile with hexane (1:3) and then on silica gel (ElSohly *et al.*, 1990).

In conventional Soxhlet extraction system the plant material is placed in a cellulose thimble in an extraction chamber. Below the extraction chamber is the distillation flask filled with the extraction solvent and on top is the reflux condenser (Figure 1.4).

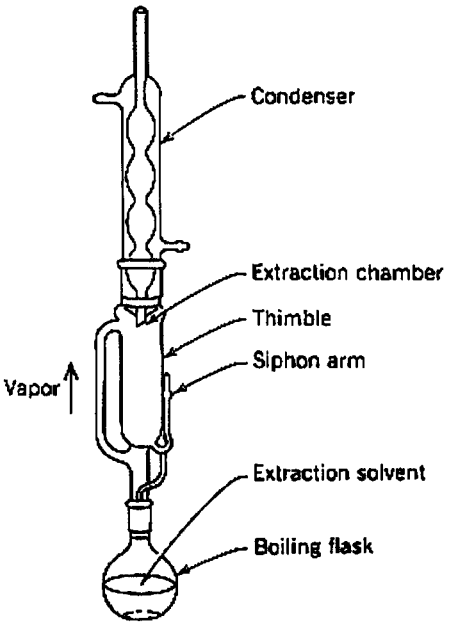


Figure 1-4. A scheme of an experimental Soxhlet extractor apparatus.

Hexane is the solvent of choice in Soxhlet extraction but there is an increasing use of alternative solvents like iso-propanol and even water due to safety and environmental concerns. The use of alternative solvents however often result in a lower recovery due

to the comparatively lower molecular affinity between solvent and solutes (Wang and Weller, 2006). Percolation is an application of the Soxhlet principle to batch extraction. This involves the circulation of solvent through a bed of biomass contained in a percolator vessel. A rich miscella is obtained which is then concentrated by external evaporators to recover solvents for recycling (Bart and Pilz, 2011). Tonk *et al.* found that Soxhlet extraction had the best performance of the alternative methods they evaluated for the extraction of the larvicidal components of *A. annua* (Tonk *et al.*, 2006).

Ultrasound assisted extraction (UAE) uses sound waves of above 20 kHz to cause mechanical vibration in biomass and solvent through expansion and compression cycles. The resulting mechanical effect allows a greater penetration of solvent into the biomass thereby improving mass transfer. Industrial application of this method, involves the use of ultrasonic baths or ultrasonic horn transducers fitted to closed extractors (Wang and Weller, 2006). Briars and Paniwnyk have suggested viable industrial scale up of their ultrasonic method for the extraction of artemisinin (Briars and Paniwnyk, 2013).

1.5.2 Microwave assisted extraction (MAE)

This method delivers microwave energy (0.3 – 300 GHz) to both solvent and plant matrix, which results in heating of the solvent and biomass efficiently and homogeneously. Cell disruption is achieved by superheated endogenous water in the plant matrix caused by the absorbed electromagnetic radiation. The resulting disruptive changes in plant tissue could lead to a comparative increase in yield of extract and migration of dissolved ions could also enhance solvent penetration into biomass matrix

and the release of target metabolites. Effective application of MAE however, depends on the dielectric susceptibility of both the extraction solvent and the plant matrix (Wang and Weller, 2006, Christen and Veuthey, 2001). Comparing MAE with commonly used extraction methods for artemisinin, Hao *et al.* reported shorter extraction time and higher extraction rate for MAE over Soxhlet and supercritical CO_2 extractions, however the later produced a cleaner extract than the other two methods (Hao *et al.*, 2002).

1.5.3 Supercritical fluid extraction

Raising the temperature and pressure of a substance above its critical value brings it to a supercritical state having the characteristic of both a gas and a liquid. The advantages of using such fluids over conventional solvents include the ability to adjust the dissolving power of the fluid through the manipulation of the pressure and/or temperature parameters. Supercritical fluids also exhibit several advantageous solvent characteristics (e.g. higher diffusing coefficient, lower viscosity and surface tension, etc) over conventional solvents and therefore better mass transfer (Wang and Weller, 2006).

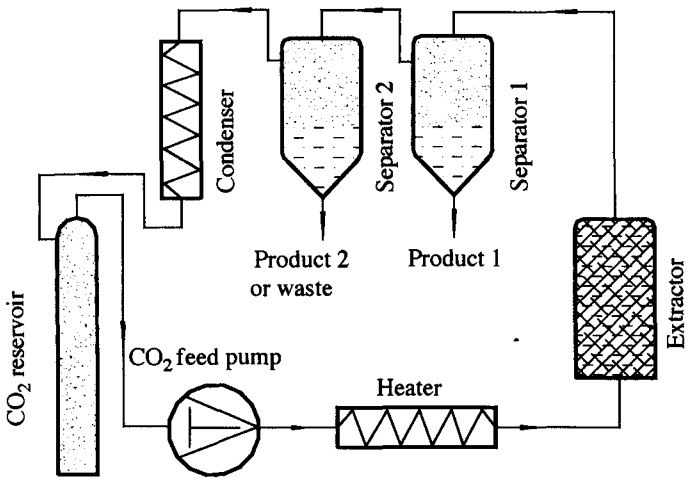


Figure 1-5. Schematics of a supercritical fluid extraction design (Wang and Weller, 2006).

The biomass is placed into the extraction vessel (extractor, Figure 1.5) maintained at desired temperature and pressure through associated control valves. Fluid is pumped into the extractor holding the biomass, which facilitates the partitioning of the analytes in the fluid. The fluid and dissolved analytes are transported to the separator chamber, (Figure 1.5) and by manipulation of the pressure and/or temperature, the solvation power of the fluid is decreased to sweep out the analytes which are then collected. The fluid is regenerated in a condenser and recycled for the next round of extraction (Christen and Veuthey, 2001, Wang and Weller, 2006).

Successful implementation of the technology requires careful selection of fluid to suit the type of analyte to be extracted. Carbon-dioxide, CO_2 is the most common of fluids used for extraction of metabolites in *A. annua* however hydro-fluorocarbon HFC-134a (1,1,1,2-tetrafluoroethane) has also been employed (Lapkin *et al.*, 2006, Kohler *et al.*, 1997a).

1.5.4 Accelerated solvent extraction

In accelerated solvent extraction, organic solvents (or pressurized hot water) are used at high pressure (10 -15 MPa) and temperature above the solvent boiling point (50 -200 °C). Although solvents are pressurized similar to SFE however, the solvents are still below their critical values in this employment. Increased temperature increases the kinetic energy of solvent molecules and interaction with the biomass, while increased pressure helps to maintain the solvent in a liquid state (Richter *et al.*, 1996, Wang and Weller, 2006).

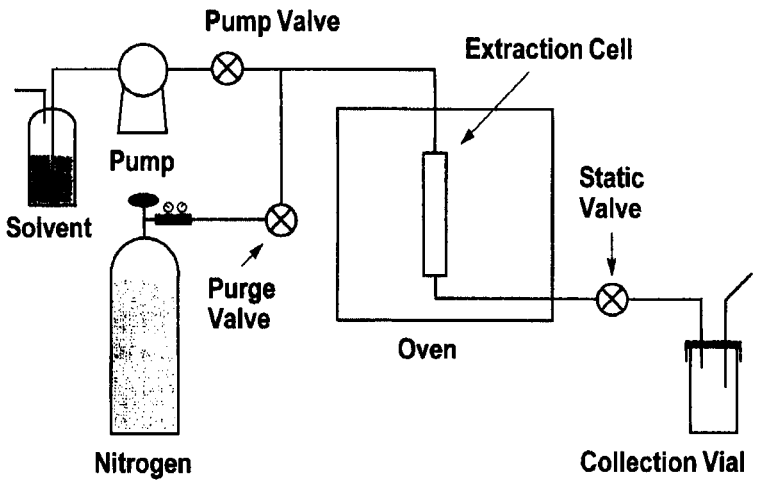


Figure 1-6. Schematic diagram of an accelerated solvent extraction design. Reproduced from Richter *et al.* (Richter *et al.*, 1996)

Figure 1.6 show a typical ASE setup. The extractor is placed in a thermostated oven. The extraction solvent is pumped into the extraction cell at an appropriate flow rate and the cell kept at the ideal temperature and pressure. The sample is collected in the collection vial. Christen and Veuthey used a similar setup to extract artemisinin and artemisinic acid from *A. annua* leaves with a favourable result (Christen and Veuthey, 2001).

The various methods discussed above are suited for both single and multi-compound extraction schemes. In the majority of *A. annua* extraction for example, the isolation of artemisinin, the main active principle is the focus. However targeted multi-component extraction paradigms are re-emerging with a range of industrial applications including, pharmaceutical, nutraceutical and biomaterials for chemical industry to name a few. Multi-component extractions for medicinal use are common in folk and herbal medicines, for example the use of *Artemisia* tea in parts of Asia and Africa as a self mediated therapy for malaria and other ailments.

1.6 *Artemisia* Tea

1.6.1 Extraction of Artemisia tea

Several methods are described in ancient Chinese texts for the extraction of Qing Hao (*A. annua*) for medicinal purposes. Two of these involve either soaking followed by wringing or pounding followed by squeezing the fresh herb (Hsu, 2006b, Wright *et al.*, 2010, Hsu, 2006a).

In their study Rath *et al.* used three methods of tea preparation using 5 and 9 grams of dried leaves for each preparation, Table 1.1 (Rath *et al.*, 2004). In a method (A), boiling water was added to leaves and left to cool to room temperature and then filtered. In another preparation (B) leaves were boiled in water for 30 min then allowed to cool to room temperature and subsequently filtered. The third method (C) had boiling water added to the leaves and the mixture briefly stirred and covered for 10 min followed by filtration and gentle squeezing of the leaves to release residual water. The efficiency of extraction, Table 1.1, was between 86 % and 30 % of the total artemisinin in biomass.

Mueller *et al.* compared two methods of *Artemisia* tea preparations (Mueller *et al.*, 2000). In one method, boiling water was added to leaves, stirred and left to cool for 15 min and in the second, the extract was kept boiling for 5 min and later filtered. The extraction efficiencies obtained were between 42 and 25 %.

Table 1-1. Efficiencies of aqueous artemisinin extraction from Rath *et al.* (Rath *et al.*, 2004).

Preparation method	Amount of *A. annua* (g)	Artemisinin concentration in Tea (mg L^{-1})	Efficiency of extraction (%)
A	5.0	57.5	83
	9.0	88.2	71
B	5.0	36.5	53
	9.0	37.8	30
C	5.0	60.0	86
	9.0	94.5	76

From these results, adding boiled water to dried leaves gave the best efficiency while cooking reduces the yield considerably. De Ridder *et al.* suggested possible reasons for the differences in results obtained in these two experiments including differences in cultivation and the harvesting of the leaves of *Artemisia* plant used in these trials (De Ridder *et al.*, 2008).

Van der Kooy and Verpoorte also quantified artemisinin in tea prepared by different methods (Van der Kooy and Verpoorte, 2011). They observed that the extraction efficiency is temperature sensitive and efficiencies of above 90 % are obtainable. They also concluded that the solubility of artemisinin is not improved by other components

in the extract and obtained aqueous solubility for pure artemisinin in the range of 50 mg L^{-1}.

1.6.2 Metabolites in Artemisia tea

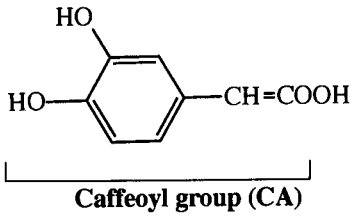
Table 1.2 shows components in *Artemisia* tea analysed by Cabonara *et al.* by HPLC-ELSD (Carbonara *et al.*, 2012). The analysed teas were prepared from *A. annua* leaves by infusion in water for 1, 24 and 48 hours. Interestingly on reconstitution of the same extracts in less polar or apolar solvents a different composition with no phenolic compounds and a much lower concentration of artemisinin resulted.

Table 1-2. Components of *Artemisia* tea. Reproduced from Cabonara *et al.* (Carbonara *et al.*, 2012).

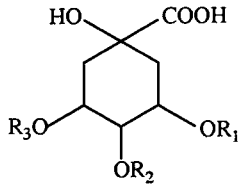
Peaks		R_t (min)	mg/g dw		
			1 h	24 h	48 h
1	3-Caffeoylquinic acid	4.23	1.14 ± 0.01	1.15 ± 0.01	1.21 ± 0.01
2	5-Caffeoylquinic acid	5.76	7.81 ± 0.02	9.11 ± 0.02	9.97 ± 0.03
3	4-Caffeoylquinic acid	6.54	1.24 ± 0.08	1.40 ± 0.02	1.58 ± 0.03
4	3-Feruloylquninic acid	7.93	0.70 ± 0.02	0.74 ± 0.01	0.86 ± 0.02
5	Tetramethoxyflavanone	9.01	0.89 ± 0.06	0.42 ± 0.01	0.29 ± 0.01
6/7	4-Feruloylquinic acid	11.95	0.28 ± 0.01	0.32 ± 0.01	0.29 ± 0.02
8	Caffeic acid	14.85	3.11 ± 0.02	3.12 ± 0.03	4.10 ± 0.06
9	5-Feruloylquininc acid	16.01	0.71 ± 0.02	0.78 ± 0.01	0.71 ± 0.04
10	6-C-arabinosyl-8-C-glucosyl apigenin	19.35	0.49 ± 0.01	0.48 ± 0.01	0.52 ± 0.02
11	6-C-glucosyl-8-C-arabinosyl apigenin	20.91	0.45 ± 0.01	0.45 ± 0.03	0.45 ± 0.02
12	Trimethoxycoumarin	37.69	1.31 ± 0.09	1.11 ± 0.09	1.68 ± 0.10
13	Chrysoeriol rutinoside	45.17	0.96 ± 0.06	0.91 ± 0.02	1.05 ± 0.05
15	Vitexin (8-C-glucosyl apigenin)	52.41	0.80 ± 0.03	0.74 ± 0.01	1.04 ± 0.02
16	Patuletinglycoside	54.79	0.69 ± 0.02	0.72 ± 0.01	0.70 ± 0.02
17	Isovitexin (6C-glucosyl apigenin)	56.00	1.14 ± 0.01	0.89 ± 0.07	0.85 ± 0.02
18	3,4-Dicaffeoylquinic acid	57.33	10.26 ± 0.02	8.98 ± 0.02	12.16 ± 0.06
19	3,5-Dicaffeoylquinic acid	58.07	3.15 ± 0.04	2.98 ± 0.03	3.11 ± 0.07
20	Luteolin-7-O-glucoside	59.00	-	-	1.88 ± 0.14
22	4,5-Dicaffeoylquinic acid	61.56	2.00 ± 0.06	1.25 ± 0.06	3.14 ± 0.06
23	3,4-Diferuloylquinic acid	65.43	0.39 ± 0.01	0.34 ± 0.01	0.60 ± 0.07
24	3,5-Diferuloylquinic acid	66.24	0.47 ± 0.02	0.44 ± 0.04	0.72 ± 0.04
28	4,5-Diferuloylquinic acid	68.48	0.58 ± 0.04	0.50 ± 0.03	0.50 ± 0.05
29	3,5-Caffeoylferuloylquinic acid	72.74	0.90 ± 0.04	0.73 ± 0.01	0.58 ± 0.06
31	4-Caffeoyl-3,5-disuccinoylquinic acid	75.96	0.78 ± 0.02	0.81 ± 0.01	0.86 ± 0.02
32	Eryodictiol	77.77	0.90 ± 0.02	0.88 ± 0.01	0.86 ± 0.02
33	Jaceidin	83.92	1.11 ± 0.02	1.15 ± 0.03	1.13 ± 0.05
34	Cirsilineol	86.68	0.06 ± 0.02	0.05 ± 0.01	0.06 ± 0.02

1.6.3 Bioactivity of major (≥ 2 mg/g) components of Artemisia tea

The major components identified by Carbonara *et al.* are a series of caffeoyl and feruloyl-quinic acids (chlorogenic acids) and some flavonoids (Carbonara *et al.*, 2012). Chlorogenic acids refer to a related family of esters of hydroxycinnamic acids (caffeic, ferulic, etc) with quinic acid (Scheme 1.8). These compounds possess a broad spectrum of pharmacological properties, including antioxidant, hepato-protectant, antibacterial, antihistiminic, chemo-preventive and other biological effects (Belkaid *et al.*, 2006, Zhang *et al.*, 2008, Feng *et al.*, 2005, Miketova *et al.*, 1999).



caffeic acid



quinic acid

	Quinic substituent		
	R_1	R_2	R_3
3-caffeoylquinic acid	CA	H	H
4-caffeoylquinic acid	H	CA	H
5-caffeoylquinic acid	H	H	CA
3,4-di-caffeoylquinic acid	CA	CA	H
3,5-di-caffeoylquinic acid	CA	H	CA
4,5-di-caffeoylquinic acid	H	CA	CA
3,4,5-tri-caffeoylquinic acid	CA	CA	CA

Scheme 1.8. Mono, di and tri-caffeoyl-quinic acids in *Artemisia* tea.

1.6.3.1 5-caffeoylquinic acid and caffeic acid

Park in his studies shows that 5-caffeoylquinic acid (Scheme 1.8) and caffeic acid orally administered are absorbed and able to suppress P-selectin expression on platelets via inhibiting COX enzymes (Park, 2009). He concluded that these compounds have beneficial effects on cardiovascular diseases by suppressing P-selectin expression on platelets. Jin *et al.* isolated 5-caffeoylquinic acid from methanol extracts prepared from stem barks of *Euonymus alatus,* which showed a strong inhibitory effect on matrix

metalloproteinase (MMP)-9 activity and therefore responsible for anti-MMP-9, known to be involved in tumor cell invasion and metastasis (Jin *et al.*, 2005).

1.6.3.2 3,4-Dicaffeoylquinic acid

Takemura *et al.* in an *in vivo* trial found that Brazilian green propolis water extract (PWE) and its chemical components, caffeoylquinic acids, such as 3,4-dicaffeoylquinic acid (Scheme 1.8) act against the influenza A virus (IAV) without influencing the viral components (Takemura *et al.*, 2012). Chikaraishi *et al.* showed both *in vitro* and *in vivo* that the chief chemical constituent of propolis, 3,4-di-caffeoylquinic acid collected from plant by honeybees, has several pharmacological actions, such as anti-tumor and anti-inflammatory effects (Chikaraishi *et al.*, 2010).

1.6.3.3 3,5-Dicaffeoylquinic acid

Kim *et al.* showed that 3,5-dicaffeoylquinic acid, (Scheme 1.8) might be a potential therapeutic agent for treating or preventing neurodegenerative diseases associated with oxidative stress (Kim *et al.*, 2005). Zha *et al.* also showed that the acid displayed anti-oxidative and anti-apoptotic activities in human dermal micro-vascular endothelial cells (HMEC-1) due to scavenging of intracellular ROS induced by Lipo-polysaccharide (LPS), and the suppression of caspase-3 activity (Zha *et al.*, 2007).

1.6.3.4 4,5-Dicaffeoylquinic acid

Robinson, *et al.* showed that 4,5-dicaffeoylquinic acid and two other analogues were potent and selective inhibitors of HIV-1 *in vitro* (Robinson Jr *et al.*, 1996). The antioxidant activity of the acid has been demonstrated by Chuda *et al.* (Chuda *et al.*, 1996).

1.6.4 Anti-plasmodial activity of Artemisia tea

Historically, *Artemisia* aqueous extracts have been used in treating fevers and associated ailments in folk Chinese medicine for millennia. Only recently however is the scientific study of the extract and its activity undertaken.

The study by Rath *et al.* was designed to evaluate artemisinin plasma concentration after oral intake of the tea preparation (Rath *et al.*, 2004). In one preparation made from 9 grams of *Artemisia* leaves, 94.5 milligrams of artemisinin was obtained which corresponded to about 19% of the recommended daily dose. Tea was absorbed quickly with maximum blood concentration reached in 30 minutes compared to pure artemisinin at 2.3 hours. Bioavailability was similar for both tea and artemisinin in capsules. They also observed that the concentration of artemisinin in the blood after intake of the tea are sufficient for clinical effects, however higher recrudescence was observed for this group than was the case for modern artemisinin based combination therapy. Similarly Mueller *et al.* investigated the efficacy and safety of traditional *Artemisia* tea preparations in the treatment of uncomplicated malaria (Mueller *et al.*, 2004). Treatment resulted in a quick resolution of parasitaemia and of clinical symptoms. After 7 day of medication, cure rates were about 74% for the tea treatment compared with 91% for quinine. They also found as did Rath *et al.* that recrudescence rates were high in the tea group (Rath *et al.*, 2004).

Hirt and Lindsey (reference from Willcox *et al.* (Willcox *et al.*, 2004)) in a trial in the Democratic Republic of Congo reported a 93% parasite clearance rate in 254 patients who had taken a 7 day medication of *Artemisia* tea, mainly for *P. falciparum* malaria.

A subset of 31 patients from the larger group was followed long-term and the recrudescence rate was 13% after 1 month.

Wright *et al.* used juice squeezed out from *Artemisia* leaves soaked in water and found that the anti-plasmodial IC_{50} values were 6 to 18 times lower than was expected based on their artemisinin content (Wright *et al.*, 2010). This juice also suppressed parasitaemia by 95% in mice infected with *Plasmodium berghei* against a 88% suppression of parasitaemia obtained when mice were administered 30 mg kg^{-1} single dose artemisinin suggesting that compounds in the juice enhanced the action of artemisinin (Rasoanaivo *et al.*, 2011).

In an *in vitro* trial using both chloroquine sensitive and resistant strains, De Donno *et al.* confirmed the improved efficacy of tea over artemisinin and suggested that because the concentration of artemisinin in tea was far too low, it could not be wholly responsible for the anti-malarial activity (De Donno *et al.*, 2012). Rather, artemisinin may be acting in synergy with other ingredients in the extract, which might have intrinsic anti-plasmodial activity or may potentiate artemisinin's activity by enhancing its solubility. They also stressed the need for targeted research to elucidate the interaction between artemisinin and co-metabolites in the extract.

1.6.5 Anti-plasmodial interactions between components of Artemisia tea

Limited work has been done to elucidate the nature of the interaction between artemisinin and other ingredients in tea. Apart from artemisinin there are other 28 sesquiterpenes identified in the plant and 36 flavonoids some of which have shown limited anti-malarial activity (Willcox *et al.*, 2004). Elford *et al.* showed that

methoxylated flavonoids - artemetin, chrysoplentin, chrysosplenol-D and cirsilineol enhanced the potency of artemisinin in a combination with each of the flavonoids, see Table 1.3 (Elford *et al.*, 1987). Interestingly they did not observe any potentiating effect with chloroquine by the flavonoids.

Table 1-3. Inhibitory effects of flavonoids alone or with artemisinin against *P. falciparum* (Elford *et al.*, 1987).

	IC_{50}	
	Flavonoid alone ($M \times 10^{-5}$)	Artemisinin ($M \times 10^{-8}$) + Flavonoid (5 μM)
Artemisinin		3.3
Artemetin	2.6	2.6
Casticin	2.4	2.6
Chrysoplenetin	2.3	2.25
Chrysosplenol-D	3.2	1.5
Cirsilineol	3.6	1.6
Eupatorin	6.5	3

Weathers *et al.* reported that the above methoxylated flavonoids are poorly extracted and are unstable in aqueous tea extract (Weathers and Towler, 2012). Therefore the improvements in potency of aqueous tea extract over equivalent artemisinin dosage will most likely be due to compounds other than these flavonoids in *Artemisia* tea.

Literature on the interactions of artemisinin with other metabolites in *A. annua* besides the polymethoxylated flavonoids is lacking. This highlights the need for further research in this area. However multi-component interactions are complex to study and appropriate methods must be chosen to elucidate these interactions and associated mechanisms.

1.7 Methods for evaluating multi-component interactions

The observed improvement in efficacy of the whole extract over an equivalent quantity of the single active ingredient underlines synergistic or multi-factorial effect of herbal medicine. Synergy defines the phenomenon where two or more components act together to produce an effect greater than the predicted effect from the sum of individual contributions. When the effect is less than would be predicted, the interaction is antagonistic (Williamson, 2001). An additive effect does not necessarily implies simply adding up the effect of individual components or an assumption of a linear dose response curve which may lead to erroneous results (Kortenkamp and Altenburger, 1998).

Because of the inherent complex nature of phyto-medicines, synergy is difficult to prove since to do so it would necessitate the testing of each individual constituent and comparing the activity with an equivalent dose in the mixture (Williamson, 2001). Several methodologies exist for the evaluation of the nature of component interactions. These methods include, isobologram, combination index and curve shift analysis to mention a few. These methods are a derivative of the Loewe additivity model (Loewe and Muischnek, 1926) that assumes that a drug cannot interact with itself. The isobole (or iso-effect) method proposed by Berenbaum (Berenbaum, 1989) is considered one of the most practical experimentally and the most demonstrative method for the proof of synergy effects (Wagner and Ulrich-Merzenich, 2009).

This method provides a graphic presentation of the nature of interaction of two drugs. The lineally arranged x and y axis reflects the dose rates of single individual components – drug (dose) A and drug (dose) B required to produce a fix effect, for

example, 50% inhibition (i.e. $IC_{50,A}$, $IC_{50,B}$ when x=50%) when used as a single agent. The line of additivity is constructed by joining point $IC_{50,A}$ to $IC_{50,B}$. The concentrations of A and B in different combinations that provides the same x=50% effect (C_{AX}, C_{BX}) are plotted on the graph as in Figure 1.7 below:

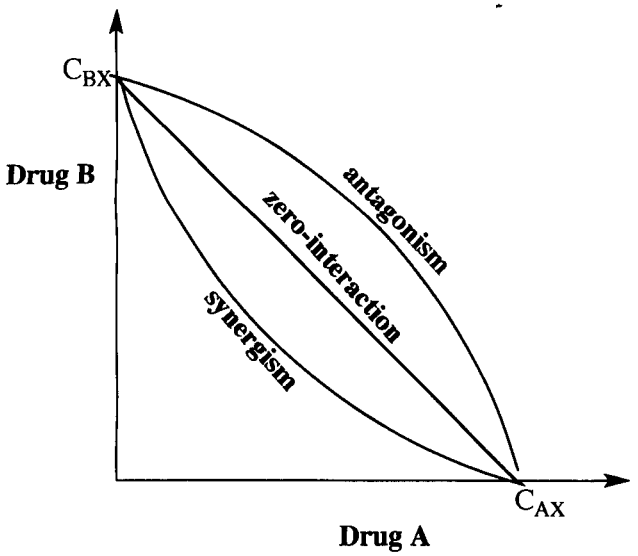


Figure 1-7. Isobologram for zero-interaction (additivity), synergism and antagonism.

In a zero or additive interaction as shown by the line in Figure 1.7 the effect of the combination of two substances is a pure summation effect (Equation 1). For interactions that are antagonistic, the overall effect is less than the summation of the individual effect (Equation 2). For synergistic interactions, the overall effect of the combination is larger than expected from the summation of separate effects of components (Equation 3). These interactions is represented equation in the following equations (Wagner and Ulrich-Merzenich, 2009):

$$E(d_a, d_b) = E(d_a) + E(d_b) \ldots\ldots\ldots\ldots (1)$$

$$E(d_a, d_b) < E(d_a) + E(d_b) \ldots\ldots\ldots\ldots (2)$$

$$E(d_a, d_b) > E(d_a) + E(d_b) \ldots\ldots\ldots\ldots\ldots\ldots (3)$$

where:

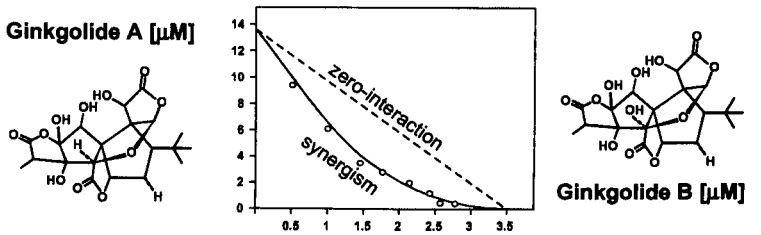
E = observed effect

d_a, = dose of component a

d_b = dose of component b

From the above, synergistic interactions necessities therefore a lower amount of the combining compounds (a and b) to achieve the desired effect.

One of the first experiments that conclusively demonstrated synergy between two natural products occurring in the same extract was conducted by Wagner and Ulrich-Merzenich using isobole method to show positive interactions between ginkgolide A and B in a thrombocyte aggregation inhibitory assay (Wagner and Ulrich-Merzenich, 2009).



GA : GB	IC50 [µg/ml]	Ginkgolide A [µM]	Ginkgolide B [µM]
3 : 1	2.40	4.41	1.42
2 : 1	2.20	3.60	1.72
1 : 1	1.80	2.21	2.12
1 : 2	1.55	1.27	2.43
1 : 3	1.40	0.88	2.57
1 : 10	1.30	0.29	2.79

Figure 1-8. Isobologram and table showing synergistic interaction between ginkgolide A and B on PAF-induced platelet aggregation. Points on graph show tested combinations that produced 50% inhibition of PAF-induced platelet aggregation. Reproduced from (Wagner and Ulrich-Merzenich, 2009).

Due to its inherent limitation the isobolographic method can only deal with the interactions between 2-3 components at a single time.

1.8 Mechanisms of interactions between components

The Isobel method is independent of the mechanism(s) behind the interactions and therefore unable to elucidate on the same for the purpose of predicting the clinical significance of such interactions. Several authors have identified some mechanistic pathways for interactions in multi-component remedies. In her review, Williamson described pharmacokinetic and pharmacodynamic effects as the two broad mechanisms for multi-component interactions (Williamson, 2011). Pharmacokinetic interactions are processes based on enhanced bioavailability, resorption rate and improved solubility. This is one component or ingredient affecting the other component's absorption, distribution, metabolism or excretion. This is the most common way of interaction in herbal medicinal. Pharmacodynamic process describes the effect of two or more compounds on the same receptor, enzyme or biological system. This describes each drug's effect on the body and will include example such as a single compound interacting with multiple targets or several compounds with a single target and multiple compounds interacting with multiple targets, see Figure 1.9. Because these interactions do not only describe synergistic but includes both additive and antagonistic effects they are therefore broadly referred to as multi-factorial (or polyvalent) effects.

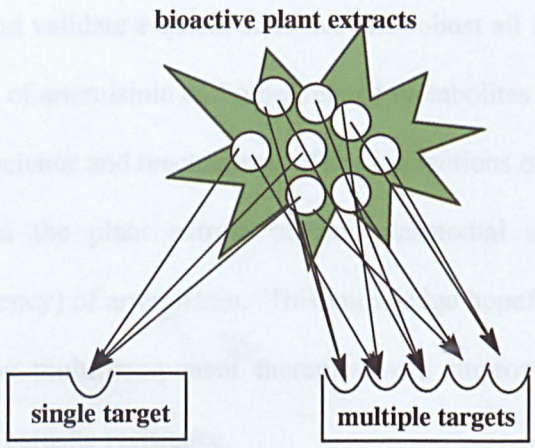


Figure 1-9. Graphical description of single and multi-target effects of agents in an extract.

Wagner and Ulrich-Merzenich suggested other mechanisms of multi-component interactions to include the inhibition of parasite resistance network by a synergist (compound) and thereby enhancing the effect of the main drug. Improvement in efficacy could also come from the elimination or neutralization of the negative or adverse side effects of a drug by agents contained in the extracts (Wagner and Ulrich-Merzenich, 2009).

1.9 Project aims and objectives

The key to successful design of multi-component medicinal extracts with improved efficacy involves identification and quantification of the components responsible for the bioactivity of the extracts. The intrinsic activity and activity in combination with other agents in specialised bioassays will lead to the elucidation of the mechanisms underpinning multi-component interactions. Using metabolites in *A. annua* as a model, this projects seeks to:

1. To develop and validate a quick, sensitive and robust all in one method for the determination of artemisinin and other related metabolites in the plant extract.
2. To study the science and mechanisms of the interactions of artemisinin with co-metabolites in the plant extract on anti-plasmodial and anti-proliferation activities (potency) of artemisinin. This knowledge hopefully could help in the design of new multi-component therapies with improved potency and less susceptible to parasite resistance.
3. To identify the cause(s) for and solution(s) to the variable and poor extraction/processing efficiencies obtained industrially in the purification of the anti-malaria agent artemisinin from *Artemisia* plant. This problem specifically relates to plant biomass obtained from East Africa.

CHAPTER 2. EXPERIMENTAL

2.1 Introduction

This chapter describes the materials and methods employed and it is organised into a general method Section (2.2) comprising methods common to most of the experiments. Sections 2.3 - 2.6 contains methods specific to major experiments in suceeding chapters bearing the chapter titles.

2.2 General methods

2.2.1 Plant samples

High-yielding *A. annua* biomasses were obtained from Mediplant (Switzerland), BIONEXX (Madagascar), REAP (Kenya), SensaPharm, (UK) CHEMO (Argentina), GSK (Australia) and ANAMED (Germany). Dried plant leaves were stored at -20 °C. Mutant (glandless) *A. annua* plant sample was kindly provided by Prof. P. Weathers (Worcester Polytechnic Institute, MA USA). *Hippophae rhamnoides* (Sea-buckthorn) used as negative control was obtained from the Centre for Alternative Land Use, CALU, (Bangor University, Wales, UK).

2.2.2 Acquity liquid chromatography method

The liquid chromatography analyses were performed using an Acquity TQD (Waters Corp., Milford, MA, USA) coupled to an Acquity tandem quadrupole detector. The high-pressure liquid chromatography (HPLC) system consisted of a binary pump, a cooling auto-sampler set at 10 °C with an injection loop of 10 µL. The column heater was set at 30 °C and a Genesis® Lightn C18 column (100 mm × 2.1 mm; 4 µm;) (Grace, IL, USA) protected by an Acquity UPLC column in-line filter unit (0.2 µm

in-line frit) was used for the separation of the metabolites. The mobile phase consisted of A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile. Chromatographic separation was achieved using a linear gradient: 0–7.0 min, 25-98% B; 7-9.5 min, 98% B; 9.5-10 min, 98-25% B; 10-15 min, 25% B; at a flow rate of 0.4 mL min^{-1}. Weak wash solvent was 10 % acetonitrile. The strong and needle wash solvents were a mixture of acetonitrile, propan-2-ol, methanol and water (30:30:30:10 v/v/v/v).

2.2.3 Tandem mass spectrometry (MS/MS) method

The MS/MS system was operated with an ESI interface in positive ionization mode (ESI+) and acquisition was performed in MRM mode. The cone and de-solvation gas flow rates were set at 45 L h^{-1} and 800 L h^{-1}, respectively while the capillary voltage, the source and de-solvation temperatures were similar of all analytes at 28 kV, 150 °C and 350 °C respectively. MS parameters were automatically defined using Waters IntelliStart® software for the tuning and calibration of the TQD and subsequently manually optimized as shown in Table 2.1. Quantification was determined using multiple reaction-monitoring (MRM) modes for the above transitions. The dwell time was automatically set at 0.161 seconds. Data were acquired by MassLynx V4.1 software and processed for quantification with QuanLynx V4.1 (Waters Corp., Milford, MA, USA).

Table 2-1. TQD parameters for MS/MS experiments.

Metabolites	Cone Voltage (V)	Collision Voltage (V)	MRM transitions
Artemisinin	24	7	283→219+229+247+265
9-Epi-artemisinin	30	12	283 →209+219+247+ 265
Artemisitene	30	10	281→217+227+245+263
Dihydroartemisinic	32	12	237→190+200+218
Artemisinic acid	32	12	235→190+200+218
Arteannuin B	28	9	249→189+213+221+231
ß-artemether (IS)	20	5	299→221+249+267

2.2.4 Dionex-RS Liquid chromatography method

Ultra high-pressure liquid chromatography (UHPLC) was performed on a Dionex-RS 3000 instrument. The mobile phase consisted of A: water with 0.1% formic acid and B: methanol with 0.1% formic acid. The flow rate was 0.2 mL min^{-1} with a run time of 29 min. The gradient was as follows: 0-10 min, isocratic 55% B; 10-15.4 min, 55% B to 100% B; 15.4-20.4 min, isocratic 100% B; 20.4-23.4 min, 100% B to 55% B, then isocratic for 5 minutes before next run. Separation was achieved on a Zorbax RPC18 2.1x100 mm, particle size 2.2 µm with 2 µL injection volume.

2.2.5 Q-TOF high resolution – mass spectrometry

This was carried out on a Bruker MaXis UHR-Q-TOF mass spectrometer under the following conditions: ionisation mode: ESI (+); MS Scan range: 50-2500 *m/z*; end plate offset: -500 V; capillary: -3000 V. Nebulizer gas (N_2): 0.4 bar; dry gas (N_2): 4 L min^{-1}; dry temperature: 180 °C. Ion transfer conditions: funnel RF: 200 Vpp; multiple RF: 200 Vpp; Quadruple Low Mass: 55 *m/z*; Collision Energy: 5.0 ev; Collision RF: 600 Vpp; Ion Cooler RF: 50-250 Vpp ramping. Transfer time: 121 µs; Pre-Pulse Storage time: 1 µs. Calibration was achieved before each run through a loop injector of

20 μL of sodium formate (10 mM).

2.3 Methods for the determination of artemisinin and its biosynthetic precursors in raw materials and *Artemisia annua* L. crude extracts

2.3.1 Chemicals

Artemisinin reference standard (98%) was obtained from Sigma-Aldrich (Dorset UK). Samples of artemisinin were also kindly provided by Neem Biotech (Newport, UK) Chemotechnica (Argentina), Amato, (India) REAP, (Kenya) and Chengdu (Sichuan Xieli Pharmaceutical Co Ltd, China). These samples were obtained following purification of extracts from *A. annua* plants cultivated in UK, India, Kenya, China and Argentina. Dihydroartemisinic acid (> 96%) was purchased from Apin Chemicals (Oxfordshire, UK). 9-epi-artemisinin (98%) was sourced from Sensapharm Ltd (Sunderland, UK), while arteannuin B, artemisitene and artemisinic acid were kindly provided by Walter Reed Army Institute of Research (Washington USA). LC-MS grade formic acid in water, acetonitrile and HPLC grade acetonitrile were obtained from Fisher Scientific, UK. Purified water (~18 MΩ/cm) was dispensed from a Milli Q system (Millipore, UK).

2.3.2 Analytical standards

Standard stock solutions of 1 mg mL^{-1} of artemisinin, 9-epi-artemisinin, artemisitene, dihydroartemisinic acid, artemisinic acid and arteannuin B in acetonitrile were prepared. The analytical standard was a mixture of all six standards in a mobile phase spiked with glandless *Artemisia* plant matrix (section 2.1.1) in the concentration range between 0.15 - 10 μg mL^{-1} for artemisinin, 9-epi-artemisinin, artemisitene and arteannuin B. For dihydroartemisinic acid and artemisinic acid the range of 3.75 - 120

μg mL^{-1} was used. This is to provide a similar matrix for the standards as with the samples minimizing any possible effect due to ion suppression or enhancement. Glandless *A. annua* plant is devoid of artemisinin and related metabolites (Duke *et al.*, 1994). Beta-artemether was used as internal standard (IS) at 5 μg mL^{-1} to adjust for possible fluctuations in injection volumes. Based on the response from the IS, the instrument QuantLynx software automatically adjust for these fluctuations making the method more robust and accurate.

2.3.3 Sample extraction and preparation

Samples were extracted using published methods (Mannan *et al.*, 2010b, Lapkin *et al.*, 2009) with a slight modification. Briefly, 10 mL of n-hexane containing 5% v/v ethyl acetate was used to extract 1 g of biomass in a sonication bath, kept cold with ice, for 30 min. The extracts were striped of solvent *in vacuo* and the residue re-suspended in 2 mL acetonitrile. This was then filtered through a 0.2 μm syringe filter to remove waxes and other un-dissolved components. An aliquot of the filtrate was dissolved in the mobile phase and internal standard added for LC-MS/MS analysis (see section 2.1). Glanded *A. annua* (BIONEXX, REAP, Mediplant, and ANAMED), glandless *A. annua* (matrix), and *Hippophae rhamnoides* (Sea buckthorn) used as negative control were all (see section 2.1.1) extracted using the above procedure.

In several earlier studies (Arsenault *et al.*, 2010, Briars and Paniwnyk, 2013, Mannan *et al.*, 2010a) solvent extraction was combined with sonication for the extraction of artemisinin and related compounds. Sonication provides mechanical disruption of the contents of the trichomes thereby aiding in extraction. Extraction at cold temperature (<30 °C) minimizes the amount of chlorophyll and other interfering components

extracted. Briars and Paniwnyk also observed an increase in the amount of artemisinin in plant extracted using ultrasound at 25 °C compared to extraction at higher temperatures without ultra-sonication and conventional steeping at the same temperature (Briars and Paniwnyk, 2013). The use of hexane modified by 5% (v/v) ethyl acetate increases the solubility of artemisinin in the mixture by over 3500 % compared with hexane alone (Lapkin *et al.*, 2006). Initially we employed an additional sample purification step using SPE columns, however this led to significant losses for some of the metabolites. Therefore a limited extract purification procedure with acetonitrile was used in all consecutive experiments.

Treated plant extract for high resolution ESI-MS analysis (QToF, section 2.1) was prepared by a 20 minutes contact of the plant extract (above) with activated carbon (AC) and celite at a ratio of 1 g each of AC and celite to 100 mL of extract. This was filtered *in vacuo* with a 0.2 µm Millipore® filter paper.

2.4 Anti-plasmodial polyvalent interactions in *Artemisia annua* L. extracts – possible synergistic and resistance mechanisms

2.4.1 Chemicals

Reference standards of artemisinin (98%), rosmarinic acid, caffeic acid and casticin were obtained from Sigma-Aldrich (Dorset, UK). Dihydroartemisinic acid (> 96%) was purchased from Apin Chemicals (Oxfordshire, UK). 9-Epi-artemisinin (98%) was sourced from Sensapharm Ltd (Sunderland, UK). Artemisitene, artemisinic acid and arteannuin B were kindly provided by Walter Reed Army Institute of Research (Washington, DC USA). The chlorogenic acids (>99%) and isovitexin (>99%) were obtained from Biopurify, China. LC-MS grade formic acid in water, acetonitrile and

HPLC grade acetonitrile were obtained from Fisher Scientific, UK. Purified water (~18 $M\Omega\ cm^{-1}$) was dispensed from a Milli Q system (Millipore, UK).

2.4.2 Plant extracts

Artemisia tea was prepared according to published methods with slight modification (Hsu, 2006a, De Donno *et al.*, 2012). Briefly, 1 L of boiling water was added to 5 g of dried plant material (BIONEXX, section 2.1.1.), stirred and stored in the dark for 1 hour. The extract was filtered *in vacuo* and lyophilised after freezing to obtain the dried tea extract. The ethanolic extract was obtained by sonication for 30 minutes in ethanol at 1:10 (w/v) biomass to solvent ratio. The sonication bath was kept cool with ice and the extract was filtered and concentrated *in vacuo* at 30 °C, and further dried under a gentle stream of nitrogen gas. These extracts were used in the *Plasmodium* assays and metabolite profiling.

2.4.3 Fractionation of crude extracts

Hexane and acetonitrile extract:

10g of *Artemisia* biomass (Mediplant, see section 2.1.1) was first extracted with 150 mL of hexane. The extract was concentrated to about 100 mL and partitioned with 100 mL acetonitrile twice to completely remove the artemisinin, then each partition was concentrated *in vacuo* and dried in glass vial using a speed vacuum.

Methanol, chloroform and water fractions:

The marc from the hexane extraction was extracted with 150 mL 90% methanol. The extract was concentrated and suspended in water. This aqueous suspension was partitioned with chloroform. The aqueous fraction was freeze dried while the crude

chloroform was washed with 1% NaCl water to rid it of tannins. The chloroform partition was concentrated to dryness *in vacuo.*

2.4.4 Sample preparation for Plasmodium assay at Liverpool School of Tropical Medicine (LSTM)

Equi-molar artemisinin concentration of fractions and crude extracts obtained from 2.4.3 were prepared by determining the amount of artemisinin contained in the extracts and fractions and making it up to 10.00±0.5 mM with artemisinin reference standard.

Table 2-2. Controls and treatments used in *Plasmodium* assay with description and artemisinin (nM) content of each. (Art =Artemisinin)

Controls		
1	DMSO	DMSO (blank)
2	TEA50	*Artemisia* Tea 50 mg (1.0 mM art)
3	TEA25	*Artemisia* Tea 25 mg (0.5 mM art)
4	ART 10.0 mM	Art (sigma) 2.82 mg mL^{-1}
Fractions		
5	AAN	Acetonitrile fraction 11.2 mg (10.5 mM art)
6	ACL50	Art + Chloroform fraction 50.7 mg (10.0 mM art)
7	CL50	Chloroform fraction 50 mg (5.8 mM art)
8	AWR100	Art + Water fraction 100 mg (10.0 mM art)
9	AWR50	Art + Water fraction 50 mg (10.0 mM art)
10	AWR25	Art + Water fraction 25 mg (10 mM art)
11	WR100	Water fraction 100 mg (0 mM art)
12	AHX 33.3	Art + Hexane fraction 33.3 mg (10.0 mM art)
13	AHX16.7	Art + Hexane fraction 16.7 mg (10.0 mM art)
14	AHX8.3	Art + Haxane fraction 8.3 mg (10 mM art)
15	HX 33.3	Hexane fraction 33.3 mg (0.0 mM art)
Crude Extracts		
16	AEEC	Ethyl acetate Extract 35.6 mg (10.3 mM art)
17	AHXC	Hexane extract 14.7 mg (10.5 mM art)
18	AMHC	Methanol Extract 39.5 mg (10.1 mM art)

2.4.5 *Sample preparation – solubility studies*

The solubility of artemisinin, artemisitene and 9-epi-artemisinin in aqueous solvent at room temperature (22 °C) was determined by the method employed by Wang *et al.* (Wang *et al.*, 2007) with modifications. A saturated solution was prepared by dissolving excess amount of the pure (> 99.0%) standard of each material in 1 mL de-ionised water (MS grade, Brucker, UK) and vortexed. This suspension was allowed to settle and the supernatant filtered through a 0.1 μm syringe filter (Fisher Scientific, UK). An appropriate volume of the filtrate was then diluted with mobile phase for mass spectrometry analysis (see Section 2.1).

2.4.6 *HPLC analysis for artemisinin content of fractions*

Chromatographic analysis of crude extracts and fractions for their artemisinin content was performed on a Shimadzu Prominence HPLC equipped with auto-sampler, degasser, photodiode array and evaporative light scattering (ELSD) detector. The method of Stringham *et al.* was used (Stringham *et al.*, 2009b). Separation was achieved by a 50:50 acetonitrile/water mobile phase delivered at a flow of 1 mL min^{-1} on an Eclipse Zobax column (150 x 4.6 mm, 0.5 miron).

2.4.7 *HPLC method for acids and flavonoid*

Analysis of acids and flavonoid was performed on an Agilent 1100 series HPLC equipped with a quaternary pump, auto-sampler, photodiode (PDA) array and a degasser. The chromatographic method by Carbonara *et al.* was used in the analysis with slight modifications (Carbonara *et al.*, 2012). Briefly, the solvent system consisted of A (0.1% acetic acid, brought to pH 4 with NaOH) and B (0.1% acetic acid in acetonitrile) using a gradient elusion of 0-60 min: 12-25% B, 60-80 min: 25-60% B,

80-85 min: 60-100% B. The system was equilibrated back to 12% B for 5 minutes before the next run. Analytes were separated and resolved at a flow rate of 1 mL min^{-1} on a Phenomenex Luna C18 column (250 mm x 4.60 mm, 5 μm particle size) attached to a C18 guard column. Detection and quantification was at 310 nm for caffeic acid, chlorogenic acids and isovitexin. Rosmarinic acid was analysed at 330 nm.

2.4.8 Plasmodium assay (George Town University, USA)

Determination of 50% growth inhibitory concentration (IC_{50}) values of extracts, compounds and combinations against CQ-sensitive (HB3) and CQ-resistant (Dd2) strains of *P. falciparum* was performed at Georgetown University, Washington, DC, USA, using a previously reported protocol (Bennett *et al.*, 2004) with minor modifications. Typically, test samples were dissolved in DMSO to give a stock solution, followed by serial dilution using complete media (RPMI 1640 supplemented with 10% (v/v) type-O^+ human serum, 25 mM HEPES (pH 7.4), 23 mM $NaHCO_3$, 11 mM glucose, 0.75 mM hypoxanthine, and 20 mg/L gentamicin) to generate working stocks. 100 μL of these stock solutions were transferred into pre-warmed (37 °C) 96-well plates. 100 μL of asynchronous parasite culture at 2% parasitemia, 4% hematocrit was transferred into each drug pre-loaded well, for a final 1% parasitemia, 2% hematocrit. Plates were transferred to a gassed (90% N_2, 5% O_2, 5% CO_2) airtight chamber and incubated at 37 °C for 72 hours. Following this incubation, 50 μL of 10X SYBR Green I dye (diluted with complete media from a 10000X DMSO stock) was added to each well and plates incubated for an additional 1 hour at 37 °C to allow DNA intercalation. Fluorescence was measured at 530 nm (490 excitation) on a Spectra GeminiEM plate reader (Molecular Devices, USA). IC_{50} values were obtained from sigmoidal fits to % parasite growth (relative to zero drug controls) vs. concentration

curves using SigmaPlot 10.0, and are the average of three replicates. Chloroquine (CQ) was included as a positive control in the assay.

2.4.9 Plasmodium assay (LSTM)

Plasmodium falciparum 3D7 parasites were maintained in continuous culture using the method of Jensen and Trager (Trager and Jenson, 1978). Cultures were grown in flasks containing human erythrocytes (2-5%) with parasitemia in the range of 1% to 10% suspended in RPMI 1640 medium, supplemented with 25 mM HEPES and 32 mM $NaHCO_3$, and 10% human serum (complete medium). Cultures were gassed with a mixture of 3% O_2, 4% CO_2, and 93% N_2. Antimalarial activity was assessed with an adaption of the 48-h sensitivity assay of Desjardins *et al.* (Desjardins *et al.*, 1979), using 3H-hypoxanthine incorporation as an assessment of parasite growth. Stock plant extracts solutions were prepared in 100% dimethyl sulfoxide (DMSO) and diluted to the appropriate concentration using complete medium. Assays were performed in sterile 96-well microtiter plates, and each plate contained 200 µL of parasite culture (2% parasitemia, 0.5% haematocrit) with or without 10 µL drug dilutions. Triplicate drug test was carried out and parasite growth compared to control wells (which constituted 100% parasite growth). After 24-h incubation at 37 °C, 0.5 micro Curie (µCi) hypoxanthine was added to each well. Cultures were incubated for a further 24 h before they were harvested onto filter-mats, dried for 1 h at 55 °C, and counted using a Wallac 1450 Microbeta Trilux Liquid scintillation and luminescence counter. IC_{50} values were calculated by interpolation of the Probit transformation of the log dose-response curve.

2.4.10 Combination analysis

Interaction between compounds were evaluated by isobologram analysis (Bray *et al.*, 2005, Berenbaum, 1978). Briefly, a master stock solution is prepared for each compound such that its concentration following four or five twofold dilutions approximates the IC_{50}. These stock solutions were mixed at ratios of 0:4, 1:3, 1:1, 3:1 and 4:0 (v/v) to give working combination stocks. Subsequently, the combination stocks were twofold serially diluted to generate a full dose concentration range for each v/v mixture, which were then analysed under standard growth inhibitory assay conditions (see above) to provide dose response curves and an IC_{50}, for each component of each v/v mixture.

2.4.11 Data analysis for in vitro combination studies

IC_{50} values for each compound alone and in the combination were used to calculate FICs (fractional inhibition concentrations) as described elsewhere (Vivas *et al.*, 2007, Fivelman *et al.*, 2004). The FICs were summated to obtain the fractional inhibition concentration index (FIC_{index}) for the combination as in the equation below:

$$FIC_{index} = FIC_A + FIC_B$$

where:

$$FIC_A = \frac{IC_{50} \text{ of Drug A in Combination}}{IC_{50} \text{ of Drug A Alone}}$$

$$FIC_B = \frac{IC_{50} \text{ of Drug B in Combination}}{IC_{50} \text{ of Drug B Alone}}$$

The following categorization was used to determine the type of interactions between compounds evaluated: synergy (FIC_{index} <0.9), additivity (0.9<FIC_{index}<1.5) and antagonism (FIC_{index} >1.5) (Vivas *et al.*, 2007, Fivelman *et al.*, 2004).

2.5 Comparative cytotoxicity of artemisinin and cisplatin and their interactions with chlorogenic acids in MCF-7 breast cancer cells

2.5.1 Chemicals

Reference standards of artemisinin (98%), Dimethyl-sulphoxide (DMSO) and chlorogenic were obtained from Sigma-Aldrich (Dorset UK). LC-MS grade formic acid in water, acetonitrile and HPLC grade acetonitrile were obtained from Fisher Scientific, UK. Purified water (~18 MΩ cm^{-1}) was dispensed from a Milli Q system (Millipore, UK).

2.5.2 Plant extracts

Artemisia tea was prepared according to published methods with slight modification (Hsu, 2006a, De Donno *et al.*, 2012). Briefly, 1 L of boiling water was added to 5 g of dried plant material (BIONIXX, see Section 2.1.1), stirred and stored in the dark for 1 hour. The extract was filtered *in vacuo* and lyophilised after freezing to obtain the dried tea extract. The ethanolic extract was obtained by sonication for 30 minutes in ethanol at 1:10 (w/v) biomass to solvent ratio. The sonication bath was kept cool with ice and the extract was filtered and concentrated *in vacuo* at 30 °C, and further dried under a gentle stream of nitrogen gas. These extracts were analysed for their artemisinin content by mass spectrometry (see Section 2.1) used in the anti-proliferation assays.

2.5.3 Cell Culture.

MCF7 human breast carcinoma were obtained from the European Collection of Cell Cultures (ECACC) and used between passages 5 and 18. The cells were grown in Roswell Park Memorial Institute medium (RPMI-1640), supplemented with 10 % of fetal calf serum, 1 % of 2 mM glutamine and 1 % penicillin/streptomycin, as adherent monolayers at 310 K in a 5 % CO_2 humidified atmosphere and passaged at approximately 70-80 % confluence.

2.5.4 In vitro growth inhibition assay.

Briefly, 5000 cells were seeded per well in 96-well plates. The cells were pre-incubated in drug-free media at 310 K for 48 h before adding different concentrations of the compounds to be tested. Stock solutions of the compounds were firstly prepared in 5% DMSO and a mixture 0.9% saline and medium (1:1) following serial dilutions in RPMI-1640. The drug exposure period was 24 h. After this, supernatants were removed by suction and each well was washed with PBS. A further 72 h was allowed for the cells to recover in drug-free medium at 310 K. The SRB assay was used to determine cell viability (Vichai and Kirtikara, 2006). Absorbance measurements of the solubilised dye (on a BioRad iMark microplate reader using a 470 nm filter) allowed the determination of viable treated cells compared to untreated controls using the inflection point of a dose-response graph. IC_{50} values (concentrations which caused 50% of cell death) were determined as duplicates of triplicate readings in two independent sets of experiments and their standard deviations were calculated.

2.5.5 IC_{50} modulation experiments.

Experiments to investigate the effect of co-administration of artemisinin and 3CA were carried out as described above, with the following modifications: Cells were pre-incubated in drug-free medium for 48 h at 310 K, before adding artemisinin together with 3CA. In order to prepare stock solutions of the drug, the solid artemisinin was dissolved first in 5% DMSO and then diluted in a 1:1 mixture of 0.9% saline: cell culture medium. This stock was further diluted using RPMI-1640 until working concentrations were achieved. Separately, a stock solution of 3CA was prepared in a similar manner. Both solutions were added to each well independently, but within 5 min of each other. Once again the drug exposure time was 24 h and the drug-free recovery time was 72 h. The SRB assay was used to determine cell viability. IC_{50} values were determined as duplicates of triplicates in two independent sets of experiments and their standard deviations were calculated.

2.6 The effect of *O*-methylated flavonoids and other co-metabolites on the crystallisation and purification of artemisinin

2.6.1 Chemicals

Artemisinin reference standard (≥ 98%), Casticin (≥ 98%), Xanthophyll and β-carotene (≥ 97%) were obtained from Sigma-Aldrich (Dorset UK). Retusin (≥ 90%) was obtained from Extrasynthese (Genay, France). Artemetin was sources from APIN Chemicals (Oxfordshire, UK). All organic solvents used in the experiments were of HPLC grade from Fisher Scientific (UK). Purified water (~18 MΩ cm^{-1}) was dispensed from a Milli Q system (Millipore, UK).

2.6.2 Plant extraction and treatment

Plant leaves (see section 2.1.1 - BIOXEXX, REAP, SensaPharm Ltd, CHEMO and GSK) were extracted with a hexane-ethyl acetate (95:5 v/v) mixture at a ratio of 1:10 (biomass: solvent) for 1 hour, using a sonication bath, which was kept cool with ice. The extract was filtered (1.0 μm Whiteman filter paper) to give the crude extract. This was then treated with activated carbon and celite, each applied at 10% w/v for 30 min. Celite and activated carbon were kindly provided by Chemotecnica, Argentina, and Dr. Guillermo Wollace, Chemotecnica, suggested the method of treatment of *Artemisia annua* raw extracts with these adsorbents. The treated extract is obtained by filtrating over 0.45 μm Millipore filter paper. The treated extract is then concentrated in a rota-evaporator to about 10% volume.

2.6.3 Doping experiments and crystallisation

From a single batch of the concentrated treated extract prepared as described in Section 2.6.2, equal volumes (10 mL) were used in each of the treatments and replicates. Three levels of doping (0, 250 and 500 μg) were used for each of the three metabolites (casticin artemetin and retusin CAR). The dopants (CAR) due to their relatively lower solubility in extraction solvent were first dissolved in ethanol before introduction to the liquor. The control or blank was spiked with an equivalent volume of pure ethanol. The treatments and blank were together placed in 4 °C storage for 24 hours to crystallize artemisinin. Crystals were harvested from liquor in a cool room (5 °C), washed with cold hexane, filtrated *in vacuo,* and dried under a gentle stream of nitrogen gas.

2.6.4 Artemisinin determination

2.6.4.1 Sample preparation

The artemisinin content of the dried crystals were determined by LC-MS/MS analysis (see section 2.1). The artemisinin content was determined by dissolving a weighed quantity of harvested crystals or a volume of extract concentrated to dryness in a known quantity of acetonitrile. This suspension is then filtered with a syringe filter (0.25 μm Fisher, UK). The filtrate was further diluted to appropriate concentration with the mobile phase for analysis.

2.6.5 HPLC method for methoxylated flavonoids

The analyses were performed using an Agilent 1100 series HPLC instrument (Agilent Technologies, UK) equipped with a quaternary pump, auto-sampler, a degasser and a diode-array detector. The method of Bilia *et al.* was employed with some modification (Bilia *et al.*, 2006). Briefly, the isocratic mobile phase consisted of water adjusted to pH3.2 by acetic acid (eleuent A) and acetonitrile (eluent B) operated at 50% A and 50% B at a flow rate of 1mL min^{-1} for 30 minutes. Separation was on a Zobax Eclipse C18 column (150 x 4.6 mm 5 μm) protected by a Zobax C18 guard column and detection was at 280 nm with an injection volume of 20 μL.

2.6.6 Wax determination

A known amount of concentrated extract prepared as described in Section 2.3.3 was placed in a weighted 50 mL centrifuge tube. Acetonitrile was added at a ratio of 25 % v/v to extract. The mixture was thoroughly mixed together and kept in cold storage at 4 °C for 12 hours to partition. The partitioned mixture was centrifuged at 3,000 rpm, 0 °C for 5 minutes. Using a pipette, the subnatant of the partition was carefully removed

while the vial was kept in ice. The vial was spun in the centrifuge under similar conditions as before. After the supernatant was removed, the residue was carefully washed with cold acetonitrile and dried under a gentle stream of nitrogen gas.

2.6.7 Pigments determination

2.6.7.1 Xanthophylls

Crude extracts prepared as described in Section 2.3.3 were analysed for their xanthopyll content using a modification of the HPLC method by Rodriguez-Amaya and Kimura (Rodriguez-Amaya and Kimura, 2004). Briefly, a mobile phase of 5% methanol in acetonitrile (solvent A), 100% methanol (solvent B) and 0.05% triethylamine in ethyl acetate (solvent C) in three separate bottles was used in a 80:10:10 isocratic elusion. Separation was achieved on an Eclipse Zobax C18 column (150 x 4.6 mm, 5 μm) at a flow rate of 1 mL min^{-1} over a 30 min run time. Detection was at 470 nm on a photodiode array detector (PAD) attached to an Agilent 1100 series HPLC with a quart-pump, auto-sampler and a degasser.

2.6.7.2 β-carotenoid analysis

Treated extract prepared as described in Section 2.3.3 were analysed on a Shimadzu Prominence HPLC equipped with an auto-sampler, degasser, and photodiode array detector using the method by Kimura and Rodriguez-Amaya (Kimura and Rodriguez-Amaya, 2003) and modified after Morinova and Ribarova (Marinova and Ribarova, 2007). Separation was conducted on a Phasesep-Partisil C18 (250 x 4.6 mm, 5 μm) column attached to a C18 guard column maintained at 30 °C. A solvent system composed of eluent A (acetonitrile:methanol, 95:5 v/v) and eluent B (acetonitrile:methanol:ethylacetate, 60:20:20 v/v/v). The eluents were modified with

0.1% butylated hydroxytoluene (BHT) and 0.05% triethylamine (TEA) respectively. A flow rate of 1 mL min^{-1} was used on a gradient elusion which was as follow: 100% A 0-5 min, 100% B 13 min, 100% B 30 min, 100% A 45 min. Detection was done at 450 nm on the PDA.

2.6.8 Computational COSMO-RS method

COSMO-RS is an *a priori* solvation model that provides for an analytical expression of the chemical potential of a substance in the liquid phase (Klamt *et al.*, 2002, Klamt *et al.*, 2010). The implementation of COSMO-RS in COSMOtherm (C30_1201, COSMO*logic* GmbH & Co. KG) was used for the calculation of solubilities. For each substance *i* the infinite dilution solubility x_i^{∞} can be calculated using the difference between the pseudo-chemical potential of the pure substance and that of the same substance in its infinitely diluted dissolved state $\mu_i^P - \mu_i^{\infty}$, according to the following equation (Eckert, 1999):

$$\log_{10} x_i^{\infty} = \frac{\left[\mu_i^P - \mu_i^{\infty} - \max(0, \Delta G_{fus})\right]}{RT \ln 10}$$

where the term with the free energy change of fusion ΔG_{fus} is required if the state of the pure substance is solid at the examined temperature. Since this equation must also hold for any other higher concentration, an iterative process can be used to calculate the solubility limit by substituting μ_i^{∞} in every step by the chemical potential calculated at the concentration of the previous step. The concentration at the solubility limit x_i^* is thus achieved upon convergence.

Since artemisinin and the three flavonoids studied in this work are all solid at 293 K, the value of free energy change of fusion is necessary in order to carry out the solubility calculations. The free energy change of artemisinin upon melting has been recently estimated (Lapkin *et al.*, 2010) using the above equation and solubility data available from (Neau *et al.*, 1997).

The following equation was used to calculate the required free energy changes for casticin and retusin:

$$\Delta G_{fus}(T) = \Delta H_{fus}\left(1 - \frac{T}{T_m}\right) - \Delta Cp_{fus}(T_m - T) + \Delta Cp_{fus} T \ln\left(\frac{T_m}{T}\right)$$

whereby the melting temperature T_m and the enthalpy and constant pressure heat capacity change upon melting ΔH_{fus} and ΔCp_{fus} need to be known. For casticin and retusin Differential Scanning Calorimetry (DSC) was used to measure the melting point and the enthalpy change upon melting while ΔCp_{fus} was approximated using COSMOtherm with the following equation (Eckert, 1999, Neau et al., 1997):

$$\Delta Cp_{fus} = \Delta S_{fus} = \frac{\Delta H_{fus}}{T_m}$$

where ΔS_{fus} denotes the entropy change upon melting. For artemetin the melting free energy was calculated by COSMO-RS using a Quantitative Structure-Property Relationship (QSPR) (Eckert, 1999).

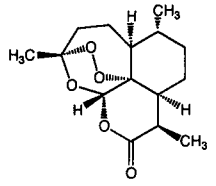
2.6.9 Deferential Scanning Calorimetry (DSC)

The thermal analysis of casticin and retusin were performed using differential scanning calorimetry (Mettler Toledo Star DSC 1). The measurements were carried out in nitrogen atmosphere. The temperature was calibrated in relation to aluminum standard (40 μL). Casticin (2.23mg) and retusin (1.99 mg) were each in turn loaded into metal pans and located on the thermoelectric disc cells. A temperature range of 25 – 210 °C was used for each scan.

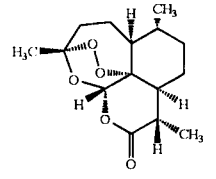
CHAPTER 3. METHODS FOR THE DETERMINATION OF ARTEMISININ AND ITS BIOSYNTHETIC PRECURSORS IN RAW MATERIALS AND *ARTEMISIA ANNUA* L. CRUDE EXTRACTS

3.1 Introduction

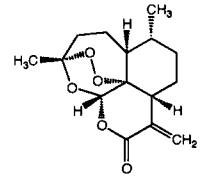
Malaria is a life threatening disease transmitted by mosquitoes with about half of the world's population at the risk of the disease (WHO, 2010). Although death from malaria has been decreasing globally since 2005 (WHO, 2011), there is the fear of a reversal due to the parasite developing resistance to traditional anti-malaria drugs. The discovery of artemisinin (**3.1**) (Scheme 3.1) three decades ago and the development of the artemisinin-based semi-synthetic drugs used in combination therapies (ACTs) backed by the World Health Organization (WHO) have provided a highly effective treatment against *falciparum*-type malaria in many countries (Qu *et al.*, 2010, Haynes, 2006, Weina, 2008).



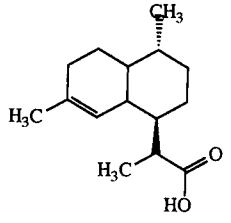
(**3.1**) artemisinin



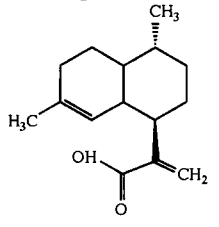
(**3.2**) 9-epi-artemisnin



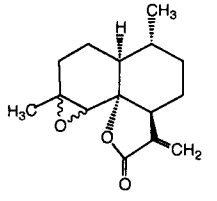
(**3.3**) artemisitene



(**3.4**) dihydroartemisinic acid



(**3.5**) artemisinic acid



(**3.6**) arteannuin B

Scheme 3.1 Chemical structures of metabolites of interest.

Currently, the worldwide demand for ACT treatments is approximately 100 million doses annually (Qu *et al.*, 2010, Haynes, 2006, Weina, 2008).

Until recently, the only commercially viable source of artemisinin was *Artemisia* (Asteraceae) plant. *Artemisia annua* L., *Artemisia apiacea* Hance, and *Artemisia lancea* Vanoit are three species in the genus *Artemisia* that have been reported to contain significant amounts of artemisinin with most of the interest focused on *A. annua* (Willcox *et al.*, 2004, Hsu, 2006a). Crystallization from extracts of dried plant biomass is the simplest method of recovery of artemisinin and with improved plant breeding methods reported yields are up to about 1.5% dry weight (Covello, 2008, Lapkin *et al.*, 2006, Larson *et al.*, 2013). Commercial interests are also focused on other biosynthetic precursors that are convertible to artemisinin analogs (Ro *et al.*, 2006b).

There are several published methods for the analysis of artemisinin and other related sesquiterpenes including 9-epi-artemisinin (**3.2**), artemisitene (**3.3**), dihydroartemisinic acid (**3.4**), artemisinic acid (**3.5**), and arteannuin B (**3.6**). However more rapid, sensitive, accurate and all-in-one methods are still needed for these metabolites. Techniques developed and validated for analysis of artemisinin include thin layer chromatography (TLC) (Quennoz *et al.*, 2010), high performance liquid chromatography with ultra violet detection (HPLC-UV) (Stringham *et al.*, 2009b, Tian *et al.*, 2012), electrochemical detection (HPLC-ECD) (Chan *et al.*, 1997), evaporative light scattering detection (HPLC-ELSD) (Lapkin *et al.*, 2009), and refractive index (HPLC-RI) (Lapkin *et al.*, 2009). HPLC-UV is the WHO recommended method and the most widely used. However because artemisinin has very weak UV adsorption

above 210 nm, the use of UV detection at the end of an HPLC separation requires very careful set-up and calibration, especially for analysis of extracts. An earlier method involved conversion of artemisinin to a UV absorbing compound Q260 to facilitate its detection (Wallaart *et al.*, 2000, Qian *et al.*, 2005, Wang and Weathers, 2007). The disadvantage of this method is that the derivatisation procedure from artemisinin to Q260 is time consuming and introduces significant experimental errors (Cheng *et al.*, 2004). Other methods include gas chromatography with flame ionization (GC-FID) (Peng *et al.*, 2006, Woerdenbag *et al.*, 1991), supercritical fluid chromatography with ELSD (Christen and Veuthey, 2001), FID (Kohler *et al.*, 1997b) and MS (Dost and Davidson, 2003) detection. NMR (Liu *et al.*, 2010) and immunoassay (He *et al.*, 2009) methods have also been reported. An excellent review of these techniques in greater detail was published by Christen *et al.* (Christen and Veuthey, 2001).

Mass and tandem mass spectrometry based methods have the advantage of high sensitivity and selectivity for metabolites in plant extracts (Wang *et al.*, 2005). Several gas chromatography and liquid chromatography methods coupled to mass spectrometry (GC-MS (Woerdenbag *et al.*, 1991), LC-MS (Wang *et al.*, 2005, Maillard *et al.*, 1993)) and tandem mass spectrometry (GC-MS/MS (Liu *et al.*, 2008), LC-MS/MS (Van Nieuwerburgh *et al.*, 2006)) have been reported for the determination of artemisinin and its derivatives in blood, plasma, serum and plant extracts. The MS/MS method developed by Van Nieuwerburgh *et al.* (Van Nieuwerburgh *et al.*, 2006) for the analysis of artemisinin and its biosynthetic precursors in *A. annua* takes about 20 minutes to analyse four metabolites.

This work evaluates the levels of impurities in artemisinin raw material and describes an MS/MS method for the analysis of six analogues of artemisinin, including 9-epi-artemisinin, in crude plant extract with minimal sample preparation, a simple binary mobile phase solvent system and a short overall analysis time, lending the method to high throughput (HTP) analysis with low consumables costs and a reduced environmental impact.

3.2 Results and Discussion

3.2.1 LC analysis of impurities in artemisinin raw material

Figure 3.1 shows the UV chromatogram for impurities in artemisinin raw material. The quantification of these impurities was done using a regression equation from the calibration curve for 9-epi-artemsinin ($y=657.24x+944.07$ with $R^2 = 1$) and for artemisitene ($y=35992x^{0.9962}$ with $R^2 = 0.99742$). The extinction coefficient for artemisitene is not linear as for 9-epi-artemisinin. A power function was used to obtain a 99.7% fit for the concentration range of 0 to 2.2mg mL^{-1}.

The term x in the equation which is the amount or concentration of impurities was calculated by substitution for y, the peak area. The percentage level of contamination was derived from the formula:

$$\mathbf{M = X/S*100}$$

where:

M = % contaminant

X = amount of contaminant (in mg)

S = amount of sample assayed (in mg)

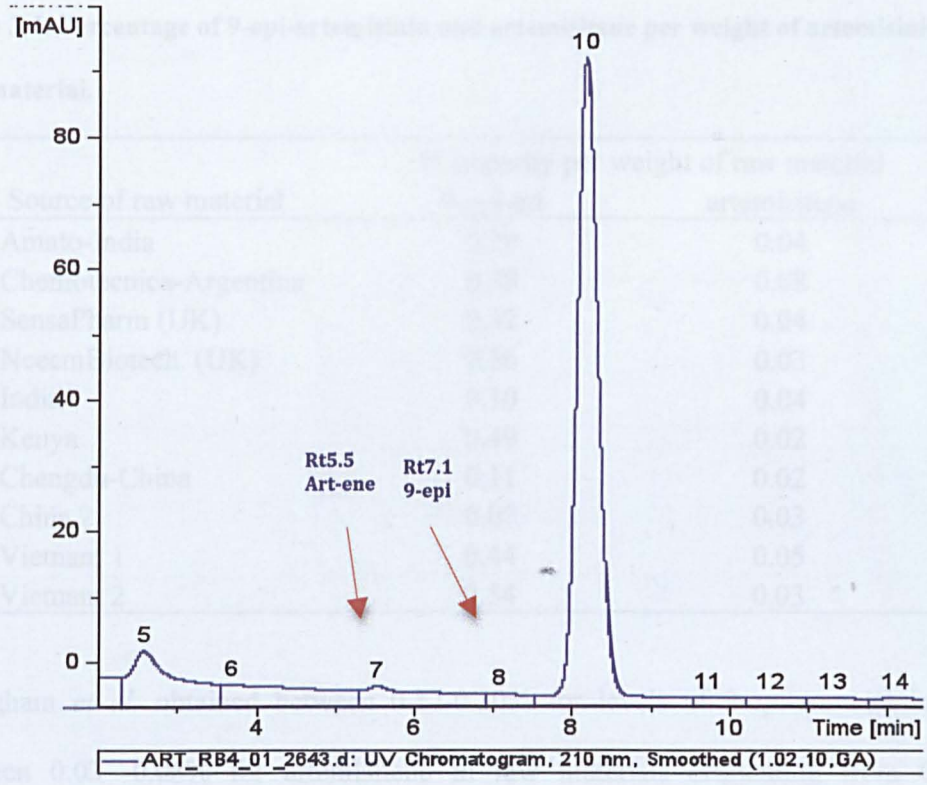


Figure 3-1. UV Chromatogram of artemisinin raw material.

Table 3.1 shows values obtained using the above calculations for estimating artemisitene and 9-epi-artemisinin in artemisinin raw material analysed. These values are similar to those obtained by Stringham *et al.* who employed a conversion factor for calculating the concentration of artemisitene. The above calculation had no need for the determination of a conversion factor; which is dependent on absorption wavelength and other variables (Stringham *et al.*, 2009a).

Table 3-1. Percentage of 9-epi-artemisinin and artemisitene per weight of artemisinin raw material.

Source of raw material	% impurity per weight of raw material	
	9-epi-art	artemisitene
Amato-India	0.29	0.04
Chemotecnica-Argentina	0.38	0.08
SensaPharm (UK)	0.32	0.04
NeeemBiotech (UK)	0.36	0.03
India	0.10	0.04
Kenya	0.49	0.02
Chengdu-China	0.11	0.02
China 2	0.07	0.03
Vietnam 1	0.44	0.05
Vietnam 2	0.54	0.03

Stringham *et al.* obtained between 0.17-0.30% for levels of 9-epi-artemisinin and between 0.03 -0.08% for artemisitene in raw materials originating from China (Stringham *et al.*, 2009a). The Kenyan materials they analysed had 0.07 - 0.73% for 9-epi-artemsinin and the quantity of artemisitene was between 0.04-0.06%. An Indian sample had 0.50 and 0.01% of 9-epi-artemsinin and artemisitene respectively and collaborated the tabulated values Table 3.1. The levels of 9-epi-artemsinin and artemisitene in the raw material were within the WHO guideline of not exceeding 1.00 and 0.15% respectively (WHO, 2011).

The wide range reported (Stringham *et al.*, 2009a) in the levels of these impurities between and within countries, may be due to the purification processes used and does not seem to be dependent on the geography of the growth location of the plant material.

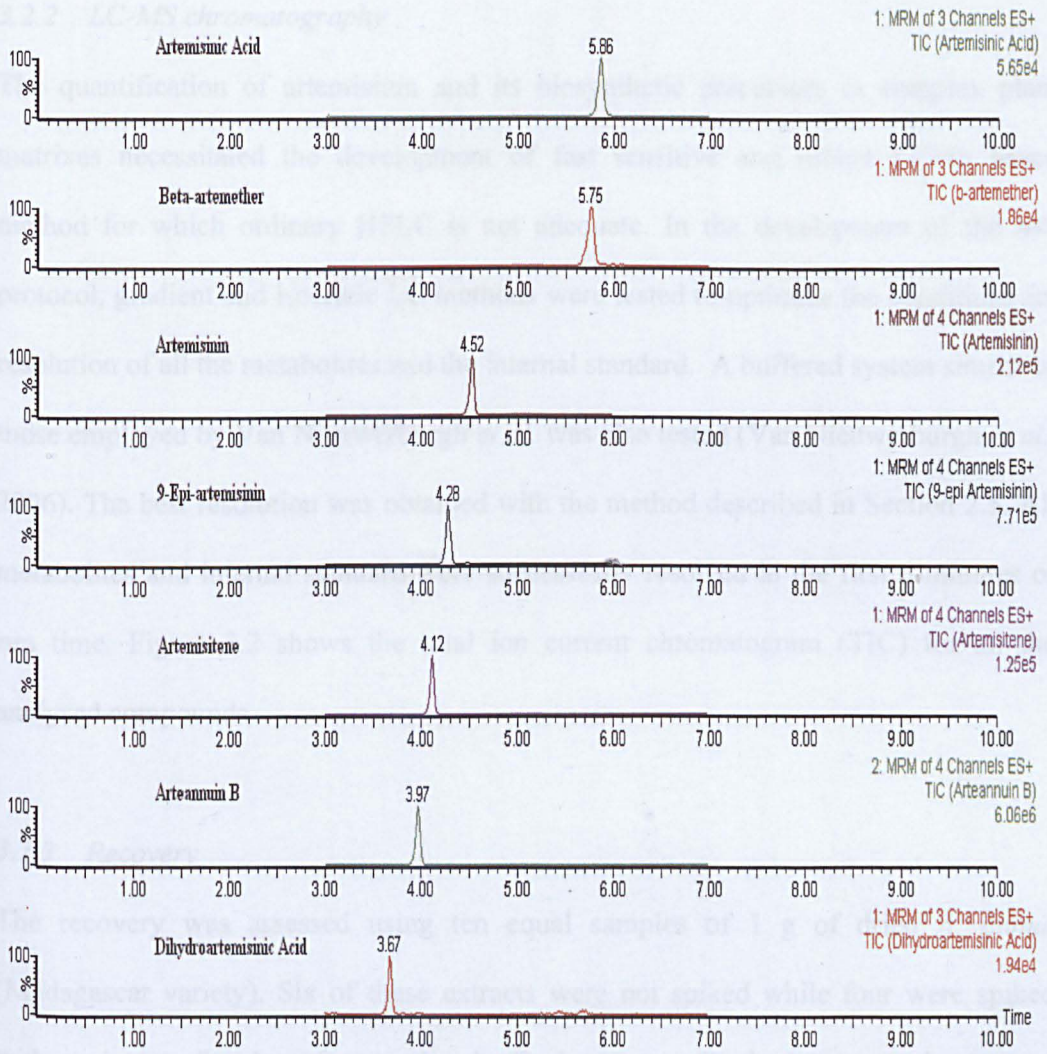


Figure 3-2. Total ion current chromatography (TIC) with retention times for all metabolites of interest and the internal standard (β-artemether). The chromatograms were acquired by multiple reaction monitoring (MRM) in positive electro-spray mode using analytical standards at a concentration of 5 μg mL^{-1} for all analytes and internal standard except for artemisinic acid and dihydroartemisinic acid which were determined at 60 μg mL^{-1}.

3.2.2 *LC-MS chromatography*

The quantification of artemisinin and its biosynthetic precursors in complex plant matrixes necessitated the development of fast sensitive and robust LCMS based method for which ordinary HPLC is not adequate. In the development of the MS protocol, gradient and isocratic LC methods were tested to optimize the conditions for resolution of all the metabolites and the internal standard. A buffered system similar to those employed by Van Nieuwerburgh *et al.* was also tested (Van Nieuwerburgh *et al.*, 2006). The best resolution was obtained with the method described in Section 2.5. All metabolites and internal standard were successfully resolved in the first 6 minutes of run time. Figure 3.2 shows the total ion current chromatogram (TIC) for all the analysed compounds.

3.2.3 *Recovery*

The recovery was assessed using ten equal samples of 1 g of dried *A. annua* (Madagascar variety). Six of these extracts were not spiked while four were spiked with a mixture of each analyte to give the final concentration in the prepared extract of 2.5 μg mL^{-1} for artemisinin, 5.0 μg mL^{-1} for 9-epi-artemisinin, artemisitene and arteannuin B, 30 μg mL^{-1} for dihydroartemisinic acid and artemisinic acid. Table 3.2 shows a recovery of between 98.44 and 105.54% was obtained for the analytes investigated.

Table 3-2. Recovery of artemisinin and analogues from A. annua.

Spiked analyte quantities μg mL^{-1}	Artemisinin*	9-Epi-artemisin*	Artemisitene	Dihydroartemisinic* acid	Artemisinic acid	Arteannuin B
Mean quantity in un-spiked sample[a]	7.49	0.13	0.05	18.15	0.00	0.39
Spiked quantity[b]	2.50	5.00	5.00	30.00	30.00	2.50
Total quantity in spiked sample[c]	9.99	5.13	5.05	48.15	30.00	2.89
Recovered quantities (μg mL^{-1})						
Spiked Sample 1[d]	10.24 (102.50%)	5.08 (99.03%)	5.08 (100.59%)	52.14 (108.28%)	28.65 (95.50%)	3.06 (105.88%)
Spikes Sample 2[d]	10 .60 (106.11%)	5.24 (102.14%)	4.97 (98.41%)	47.84 (99.36%)	30.62 (102.06%)	2.82 (97.58%)
Spiked Sample 3[d]	10.38 (103.90%)	4.92 (95.91%)	5.38 (106.53%)	51.87 (107.72%)	28.30 (94.33%)	3.09 (106.92%)
Spiked Sample 4[d]	9.97 (99.80%)	4.98 (97.08%)	5.46 (108.12%)	47.53 (98.71%)	31.31 (104.36%)	2.96 (102.42%)
Mean spiked sample (μg mL^{-1})	10.30 (103.10%)	5.05 (98.44%)	5.22 (103.37%)	49.85 (103.53%)	29.72 (99.07%)	3.05 (105.54%)
Standard deviation (μg mL^{-1})	0.23 (2.30%)	0.12 (2.34%)	0.21 (4.16%)	2.16 (4.49%)	1.27 (4.23%)	0.11 (3.79%)

Ten equal samples of 1 g dried Madagascan *A. annua* leaves were extracted and prepared for analysis. [a] Six of these extracts were un-spiked while 4 were spiked at indicated levels[b]. The total quantity[c] of analyte in samples is calculated as the sum of the mean quantities in six un-spiked sample and the spiked quantity. Analyte levels in individual spiked samples were determined[d] and absolute and percentage (in bracket) recoveries presented. * Quantitative values corrected for percentage purity

3.2.4 Specificity

Figure 3.3 shows the possible MRM transitions for artemisinin. Three or four transitions were monitored for the MS/MS experiment to identify and quantify each metabolite. The sum of combined transitions gave the total ion current (MRM) data while the signal with the highest *m/z* value was used for the quantification of each analyte.

MS/MS based assay are inherently specific. However to investigate the specificity of the method further, extracts of glandless *A. annua* and *Hippophae rhamnoides* (Sea-buckthorn) were analysed. In these negative control extracts we found no components of interest in the chromatograms from the MS/MS experiments. The result for the presence of trace level of artemisinin in the glandless biomass was not conclusive and this is being investigated further.

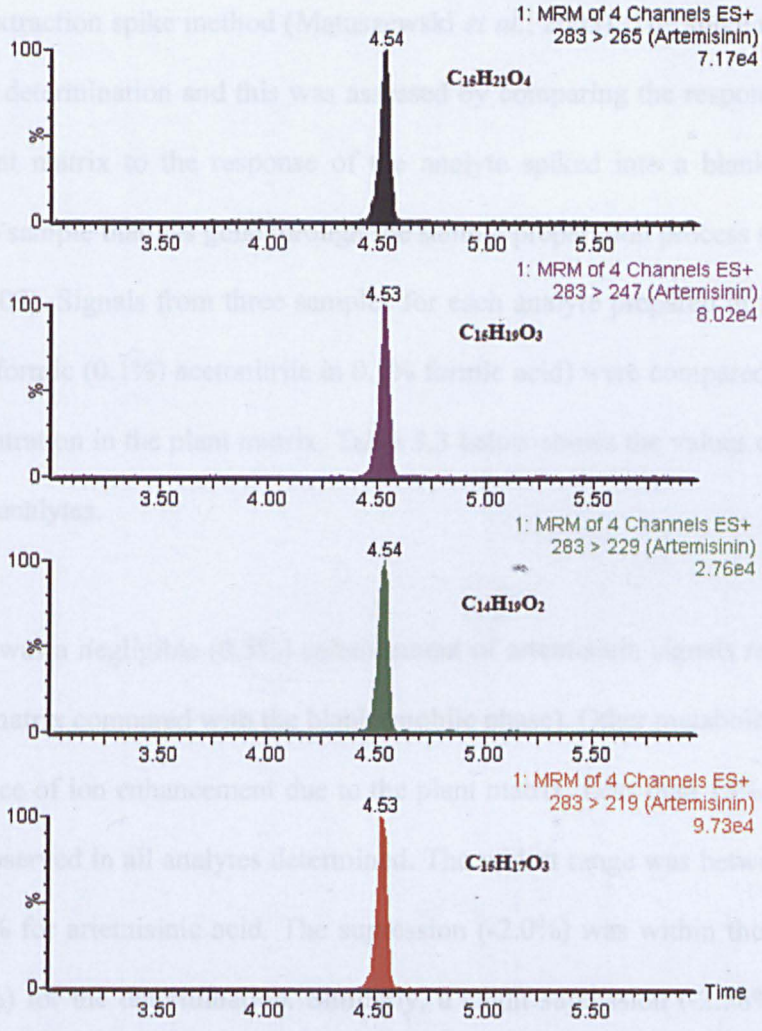


Figure 3-3. Multiple reaction monitoring (MRM) chromatogram of standard artemisinin (5 μg mL^{-1}) showing the four transitions selected, their intensities and chemical formulae.

3.2.5 Ion suppression or enhancement (matrix effect)

Quantitative analysis of plant and biological samples with positive electro-spray ionization coupled to tandem mass spectrometry is compounded by the presence of matrix components, which can interfere with the analysis hence resulting in ion suppression or enhancement effects. The common methods for the assessment of ion suppression are the post-column infusion method (Bonfiglio *et al.*, 1999) and the

post-extraction spike method (Matuszewski *et al.*, 2003). The spike method was used in our determination and this was assessed by comparing the response of the analyte in plant matrix to the response of the analyte spiked into a blank matrix (mobile phase) sample that has gone through the sample preparation process (Van Eeckhaut *et al.*, 2009). Signals from three samples for each analyte prepared in the mobile phase (25% formic (0.1%) acetonitrile in 0.1% formic acid) were compared with the similar concentration in the plant matrix. Table 3.3 below shows the values obtained for each of the analytes.

There was a negligible (0.3%) enhancement of artemisinin signals resulting from the plant matrix compared with the blank (mobile phase). Other metabolites showed some evidence of ion enhancement due to the plant matrix. Less than 15% of enhancement was observed in all analytes determined. The widest range was between about -2.0 to 14.13% for artemisinic acid. The supression (-2.0%) was within the margin of error (5.02%) for the determination. Similarly, a slight supression (-2.58%) was observed for one of the determinations of 9-epi-artemisinin; this was also within the margin of error. An average enhancement for all the determined compounds was between 0.3% for artemisinin to 8.06% for artemisinic acid.

Table 3-3. Ion suppression due to A. annua plant matrix.

	Artemisinin μg mL^{-1}	9-Epi-artemisninin μg mL^{-1}	Artemisitene μg mL^{-1}	Dihydroartemisinic acid (DHAA) μg mL^{-1}	Artemsininic acid μg mL^{-1}	Arteannuin B μg mL^{-1}
Mean quantity spiked into plant matrix[a]	2.50	5.05	5.08	32.72	30.88	2.49
Quantities in blank matrix [b]						
Sample 1	2.49(-0.22%)	4.92(-2.58%)	5.49(7.95%)	32.86(0.42%)	35.24(14.13%)	2.61(5.03%)
Sample 2	2.52(0.85%)	5.58(9.62%)	5.70(12.14%)	36.17(9.54%)	34.57(11.94%)	2.49 (0.0%)
Sample 3	2.51(0.28%)	5.54(8.67%)	5.46(7.37%)	34.20(4.32%)	30.30(-1.89%)	2.55(2.48%)
Mean	2.51(0.30%)	5.35((5.30%)	5.58(6.83%)	34.41(4.76%)	33.37(8.06%)	2.55(2.48%)
Standard error (SE)	0.04(0.24%)	0.21(3.95%)	0.08(1.50%)	0.96(2.64%)	1.54(5.02%)	0.04(1.47%)

[a] mean of three determinations of spiked standards at 2.5 μg mL^{-1} for artemisinin and arteannuin B, 5 μg mL^{-1} for 9-epi-artemisinin and artemisitene. Dihydroartemisinic acid and artemisinic acid were spiked at 30 μg mL^{-1} each. [b] 3 determination of spiked standards in blank matrix (mobile phase of 0.1% formic : 0.1% formic in acetonitrile, 75:25) at similar concentration spiked into plant matrix. Percentage suppression or enhancement shown in brackets.

3.2.6 Limit of detection (LOD), Lower limit of quantification (LLOQ), and precision

The guideline by the international conference on harmonization (ICH) (I.C.H.H.T, 2005) for bio-analytical method validation were adopted for the definition and determination of precision, LOD and LLOQ. The limit of detection is defined as the lowest amount of analyte in a sample, which can be detected but not necessarily quantitated while lower quantification limit is the lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy. Both limits were calculated from the calibration curve following Miller and Miller (Miller and Miller, 2005). Injection precision (repeatability) was calculated with at least six determination of each analyte in a single day. The coefficient of variation (CV) for these determinations is below 10 % for all metabolites investigated (Table 3.4).

Within-day precision was determined for six concentration levels covering the analyte calibration range and making a total of 12-17 analyses on a single day. Between-day precision was calculated for the same calibration range on three different days spread over a month, resulting in a total of 32-40 determinations. The range for the accuracy for both within and between day precision determinations was from 81.42 – 118.81% while the coefficient of variance in both was less than 8.5% (Table 3.4).

Table 3-4. LOD, LLOQ, injection precision, within-day and between-day precisions.

	Artemisinin	9-Epi-artemisinin	Artemisitene	DHAA	Artemisinic acid	Arteannuin B
LOD ($\mu g\ mL^{-1}$) [a]	$1.3x10^{-4}$	$1.0x10^{-3}$	$2.8x10^{-4}$	$2.0\ x10^{-1}$	$3.3x10^{-1}$	$1.2x10^{-6}$
LLOQ ($\mu g\ mL^{-1}$) [b]	$4.1x10^{-4}$	$3.0\ x10^{-3}$	$8.4x10^{-4}$	$6.0x10^{-1}$	$9.9\ x10^{-1}$	$3.5x10^{-6}$
Regression equation [c]	y=857.32x+163.1	y=344x-35.84	y =388.54x-21.01	y=13.98x+54.16	y=21.64x +71.47	y=96645x+5947.7
R^2 value	0.99396	0.99623	0.99562	0.99408	0.99803	0.99547
Injection precision [d]						
Mean ($\mu g\ mL^{-1}$)	5.67 (n=10)	0.13 (n=8)	0.24 (n=7)	19.54 (n=6)	n/a	0.32 (n=6)
CV (%)	4.48	4.54	6.24	6.20	n/a	9.89
Within and Between-day precision [e]						
Within day range (%)	93.22 – 114.20 (n=12)	83.09-106.61 (n=16)	86.04 – 116.35 (n=17)	87.26 -111.75 (n=14)	92.73 -118.81 (n=13)	82.32 -112.69 (n=15)
CV (%)	7.26	5.92	7.60	7.96	7.10	7.70
Between day range (%)	82.78-117.88 (n=36)	83.09 – 109.07 (n=37)	81.65 – 116.35 (n=40)	81.42 – 114.33 (n=38)	92.32 -118.81 (n=32)	82.32 – 119.98 (n=37)
CV (%)	7.86	6.47	7.37	8.30	6.26	8.26

[a,b,c] Calculation based on 12 point calibration graph and the following formulas $LOD=Y_B+3S_B$ and LLQD=3LOD. Where Y_B is the signal equal to the blank signal (the y intercept), S_B is standard deviation of the blank (the random error in the y-direction) (Miller and Miller, 2005). [d]Injection precision was assessed by n determination at 100% concentration. [e] Within and between-day precisions were determined over 6 concentration levels covering the calibration range for both precisions. [n/a] – undetermined values below method's LLOQ.

3.2.7 Regression indices and dynamic range

Good linearity ($r^2 > 0.99$) of the calibration curves for all the analytes of interest in both the mobile phase and the mobile phase spiked with the matrix is indicative of the robustness of the method (Table 3.4). The regression and sensitivity indices in Table 3.4 are for the standards prepared in the mobile phase spiked with extracts of the glandless plant. The method is highly sensitivity for most of the analytes. LLOQ for arteannuin B is about 3.5 pg mL^{-1}. However for dihydroartemisinic acid and artemisinic acid, the LLOQ is much higher at 0.6 and 1.0 μg mL^{-1} respectively. The dynamic ranges for these compounds reflect the same pattern with a lower range (0.15 – 10 μg mL^{-1}) for all analytes except dihydroartemisinic acid and artemisinic acid with a range of 3.75 – 120 μg mL^{-1}.

3.2.8 Artemisinin, 9-epi-artemisinin and artemisitene

Transitions used for the MS/MS analysis of artemisinin and 9-epi-artemisinin were 283→ 219 + 229 + 247 + 265 and 283 → 209 + 219 + 247 + 265 respectively (Table 2.1). The two analytes were differentiated based on their retention times as shown in Figure 3.4. We report here for the first time the detection of 9-epi-artemisinin in a plant extract. The presence of 9-epi-artemisinin and artemisitene has been reported as impurities in artemisinin raw material (Stringham *et al.*, 2011, Stringham *et al.*, 2009b, WHO, 2011).

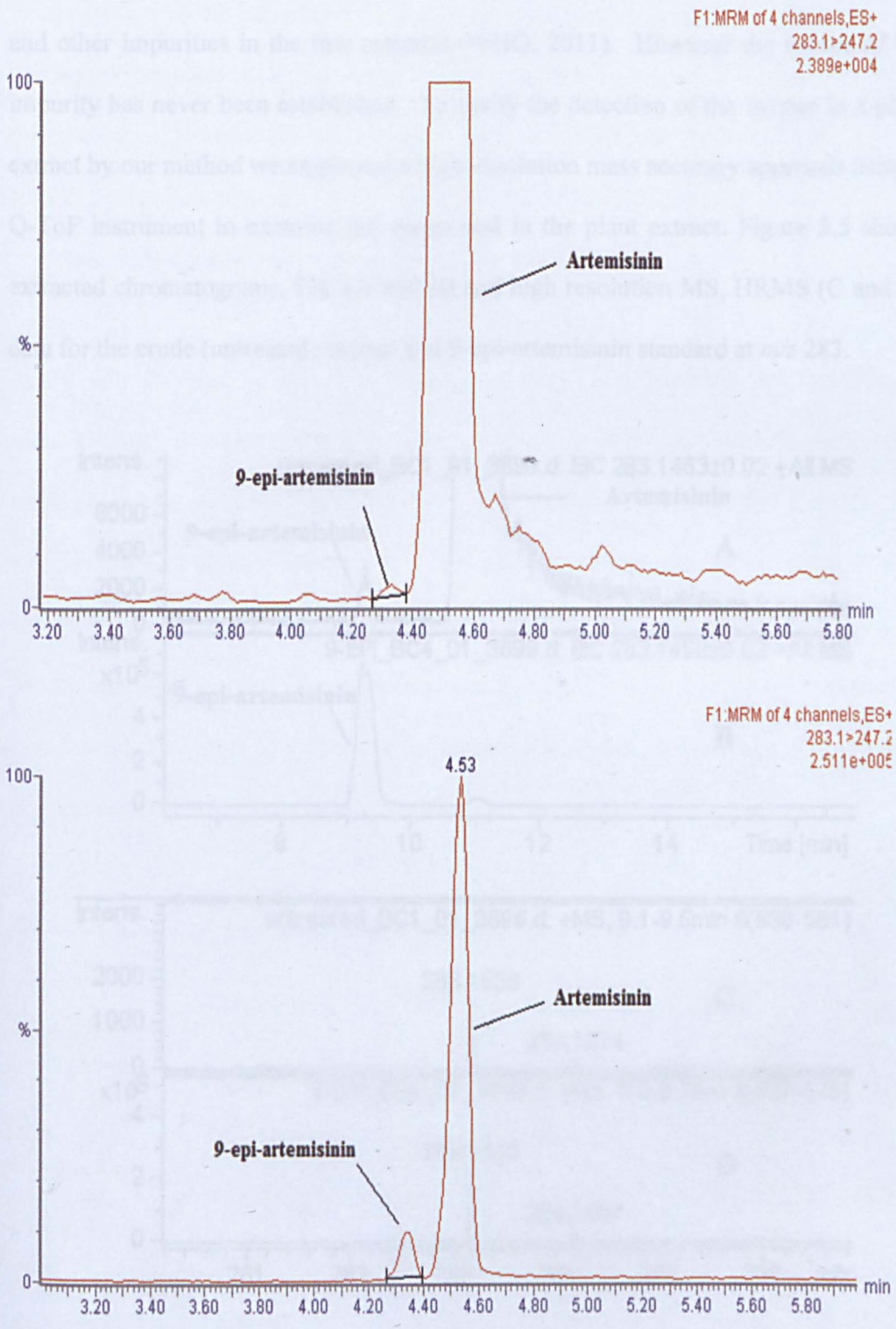


Figure 3-4. Top - MRM chromatogram for 9-epi-artemisinin in *A. annua* extract also showing artemisinin content. Bottom – chromatogram of the same extract spiked at 5 µg mL^{-1} of 9-epi-artemisinin.

WHO has guidelines and recommendations for concentration limits for this isomer and other impurities in the raw material (WHO, 2011). However the source of the impurity has never been established. To verify the detection of the isomer in a plant extract by our method we employed a high-resolution mass accuracy approach using a Q-ToF instrument to examine the compound in the plant extract. Figure 3.5 shows extracted chromatograms, EIC (A and B) and high resolution MS, HRMS (C and D) data for the crude (untreated) extract and 9-epi-artemisinin standard at *m/z* 283.

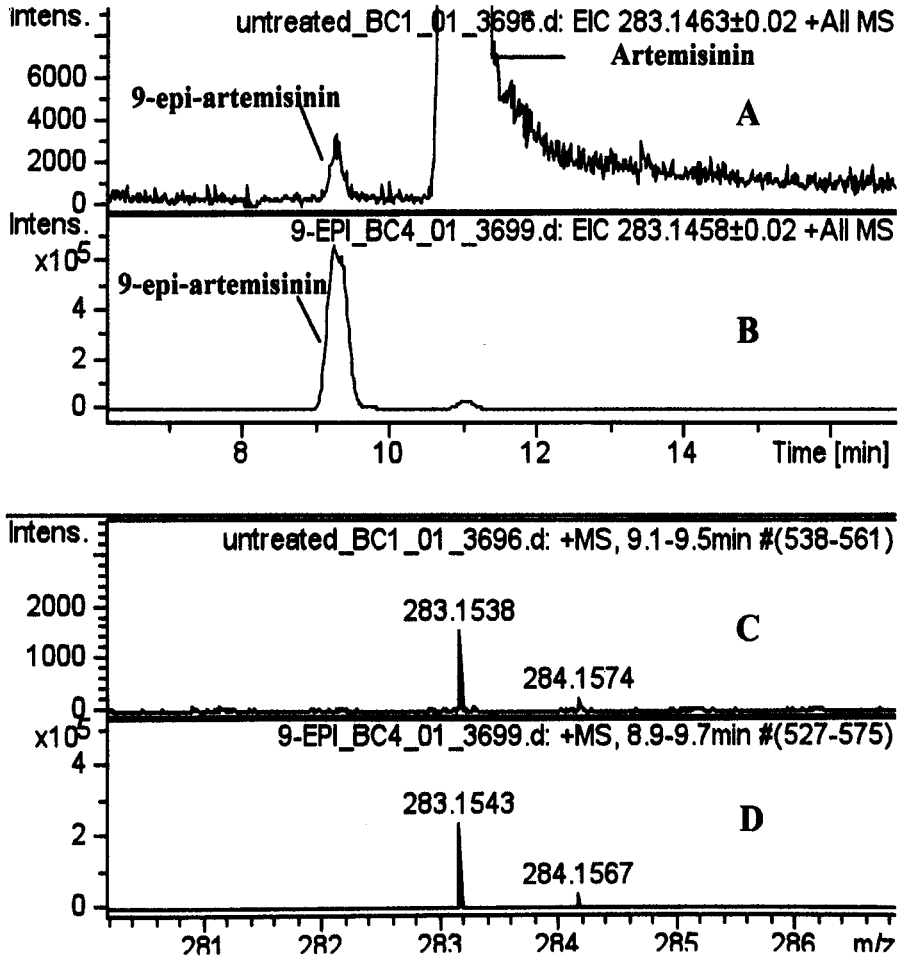


Figure 3-5. Panels (A) and (B) are EIC for extracts and 9-epi-artemisinin standard respectively. Below panels are HRMS data for extract (C) and standard (D).

A comparison of the EIC chromatograms and the HRMS simulated spectrum for both extract and standard show a peak in the extract with the identical retention time as the 9-epi-artemisinin standard and an HRMS data difference between both peaks of less than 2 ppm. This confirms and validates the MRM results and establishes the presence of 9-epi-artemisinin in *A. annua* raw extracts.

Acton and Klyman (Acton and Klayman, 1987), first reported the isolation of artemisitene from plant extracts and its possible role in the biosynthetic pathway of artemisinin has been suggested (Brown, 2010, Weathers *et al.*, 2011). Acton and Klyman have also demonstrated the conversion of artemisinin into iso-artemisitene and 9-epi-artemisinin (Acton and Klayman, 1987). Table 3.5 shows the levels of artemisinin (**3.1**) and the related metabolites in four *A. annua* biomasses analysed by our method. The level of 9-epi-artemisinin in the analysed extracts was about tenfold lower than the detected levels of artemisitene in the extracts.

Table 3-5. Levels of metabolites in four *Artemisia* biomasses.

Source of Biomass	Artemisinin ($mg\ g^{-1}$)	9-Epi-artemisinin ($\mu g\ g^{-1}$)	Artemisitene ($\mu g\ g^{-1}$)	DHAA ($mg\ g^{-1}$)	Arteannuin B ($\mu g\ g^{-1}$)
BIONEXX (Madagascar)	10.00 ±0.03	23.80±0.12	290.0±1.0	58.15±1.52	81.10±1.05
Mediplant (Switzerland)	10.63±0.11	12.70±0.07	389.0±4.0	67.38±1.22	168.60±2.11
REAP (Kenya)	10.66±0.01	12.40±0.06	110.0±1.0	68.33±2.64	20.70±0.07
ANAMED (Germany)	6.45±0.08	1.60±0.0	64.0±4.0	37.71±1.01	2.30±0.0

3.2.9 *Dihydroartemisinic and artemisinic acids*

In our method, the MRM for dihydroartemisinic acid ($[M+H]^+ = 237$) and artemisinic acid ($[M+H]^+ = 235$) are similar (237/235 → 190 + 200 + 218, see Table 2.1). Three main peaks were observed in *A. annua* plant extracts for these transitions Figure 3.6). The standard peak for dihydroartemisinic acid (DHAA) in Figure 3.6, matched one of the main peaks while an impurity in the DHAA standard matched the second peak. We were unable to confirm the composition of this impurity. The standard artemisinic acid peak at 5.86 minutes did not match any of the major peaks. A small peak in the plant extract with similar retention time to artemisinic acid standard was below the method's quantification limit for the same (Table 3.4). This is consistent with work by Brown and Sy (Brown, 2010, Brown and Sy, 2004, Dhingra and Lakshmi Narasu, 2001), who have shown that dihydroartemisinic acid rather than artemisinic acid is the true late-stage precursor to artemisinin in some *A. annua* chemotypes.

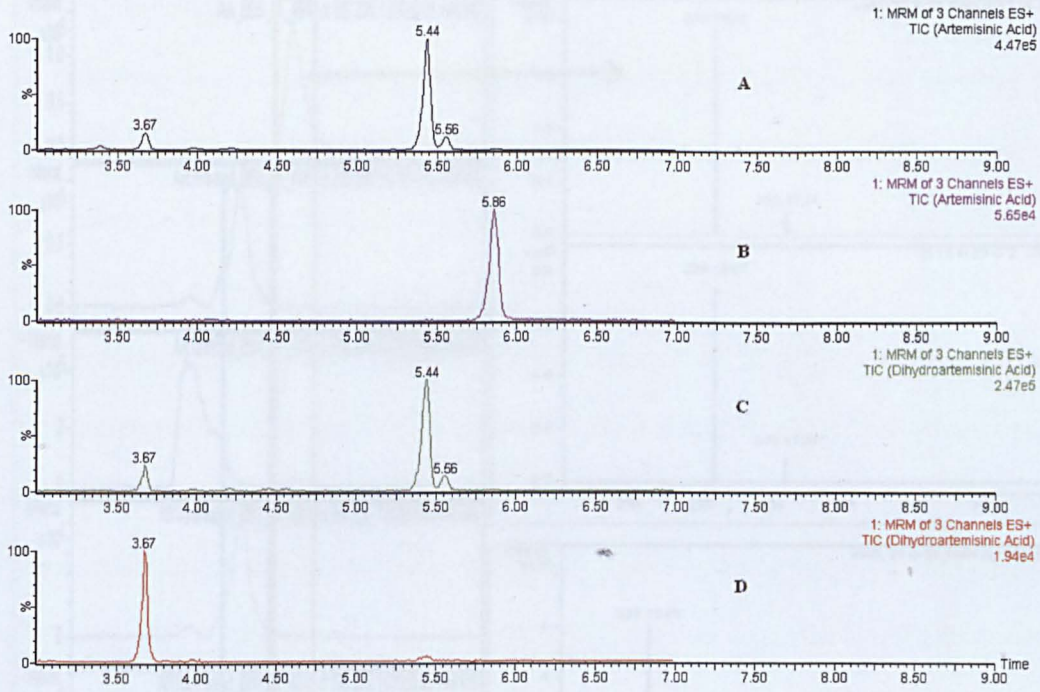


Figure 3-6. Panel (A) is MRM TIC chromatogram for *A. annua* plant extract monitored for artemisinic acid (235→190+200+218). Panel (B) is MRM chromatogram for artemisinic acid standard at 40 µg mL^{-1}. Panel (C) is MRM of plant extract monitored for DHAA (237→190+200+218) and panel (D) shows MRM of DHAA standard at 10 µg mL^{-1}.

We also verified this result on the high resolution and mass accuracy Q-ToF instrument. The extracted ion chromatograms from the Q-ToF for *A. annua* extract, artemisinic acid standard and DHAA standard at *m/z* 235 and 237 respectively are shown in Figure 3.7 (left) with corresponding high resolution MS data (right). A small peak in both treated and untreated extract matched the retention time of the standard peak for artemisinic acid and a relatively larger peak in the extracts matched the standard peak for DHAA, confirming the results from the MRM based assay.

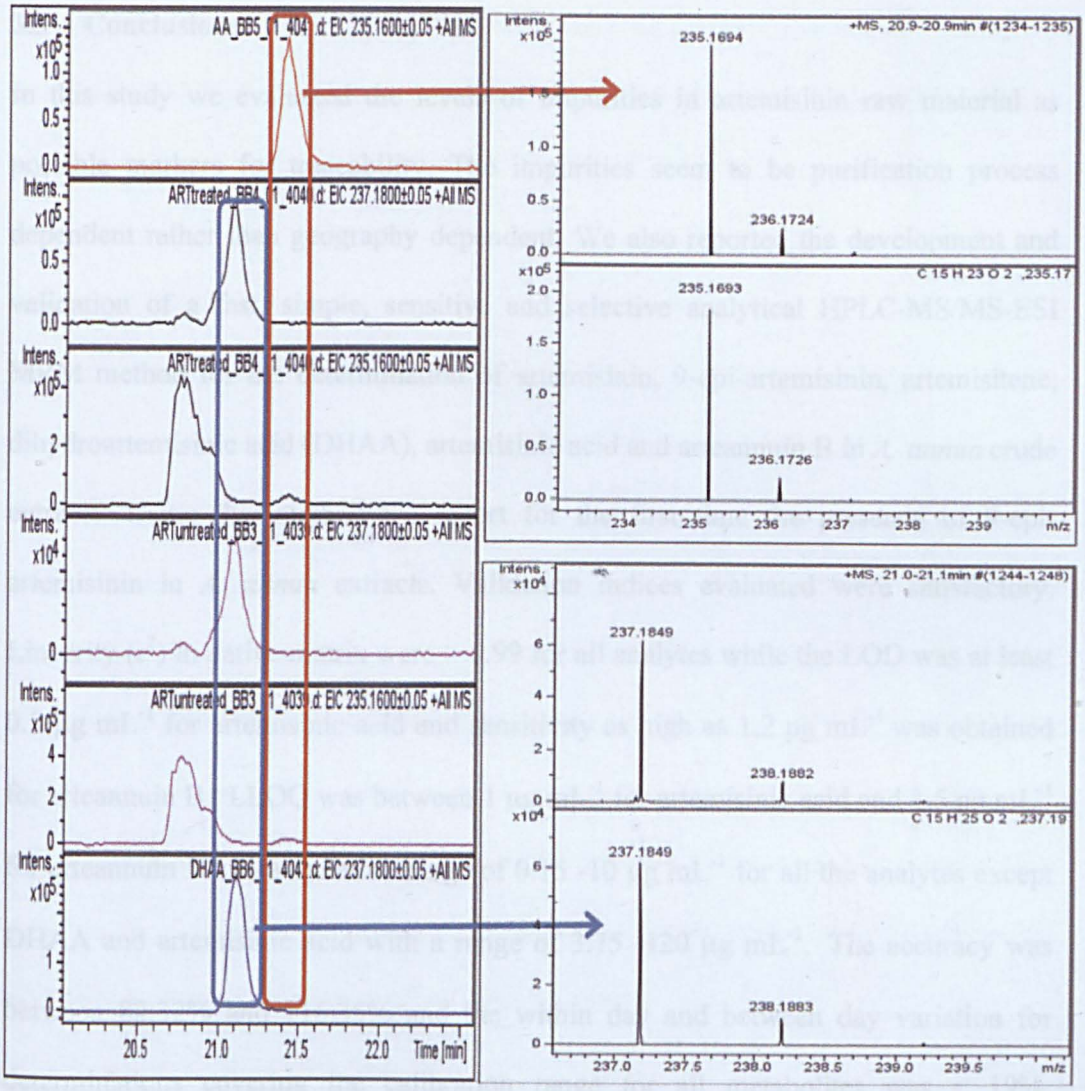


Figure 3-7. EIC at m/z 235 (artemisinic acid) and 237 (DHAA) showing EIC of extracts and standards (left) and HRMS data (right).

Figures 3.5 and 3.6 shows peaks other than dihydroartemisinic acid and artemisinic acid in the extracts. The largest peak in Figure 3.5 C and D is likely a degradation product of dihydroartemisinic acid or an intermediate in the conversion of dihydroartemisinic acid to artemisinin, which we also observed in the stressed dihydroartemisinic acid reference standard.

3.3 Conclusions

In this study we evaluated the levels of impurities in artemisinin raw material as possible markers for traceability. The impurities seem to be purification process dependent rather than geography dependent. We also reported the development and validation of a fast, simple, sensitive and selective analytical HPLC-MS/MS-ESI MRM method for the determination of artemisinin, 9-epi-artemisinin, artemisitene, dihydroartemisinic acid (DHAA), artemisinic acid and arteannuin B in *A. annua* crude extracts. Using this method we report for the first time the presence of 9-epi-artemisinin in *A. annua* extracts. Validation indices evaluated were satisfactory. Linearity (r^2) in native matrix were > 0.99 for all analytes while the LOD was at least 0.3 μg mL^{-1} for artemisinic acid and sensitivity as high as 1.2 pg mL^{-1} was obtained for arteannuin B. LLOQ was between 1 μg mL^{-1} for artemisinic acid and 3.5 pg mL^{-1} for arteannuin B and a dynamic range of 0.15 -10 μg mL^{-1} for all the analytes except DHAA and artemisinic acid with a range of 3.75 -120 μg mL^{-1}. The accuracy was between 82.32% and 116.35% and the within day and between day variation for determinations covering the calibration range for all metabolites was < 19%. Therefore showing the method to be robust, quick, sensitive and adequate for a range of applications including high throughput (HTP) analysis.

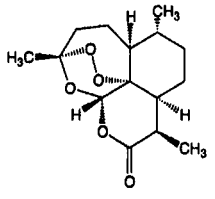
CHAPTER 4. ANTI-PLASMODIAL POLYVALENT INTERACTIONS IN *ARTEMISIA ANNUA* L. EXTRACTS – POSSIBLE SYNERGISTIC AND RESISTANCE MECHANISMS

4.1 Introduction

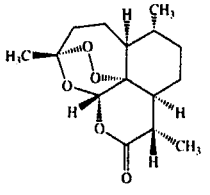
The use of *Artemisia annua* (*Qing Hao*) in traditional Chinese pharmacopeia includes the treatment of fevers and chills (Hsu, 2006a, Wright *et al.*, 2010). In the 1970s the active principle in the extract was isolated and identified as artemisinin (**4.1**, scheme 4.1), a sesquiterpene lactone. Artemisinin and its derivatives have now been established in various combination therapies (ACTs) as effective anti-malarial treatments against multidrug-resistant *P. falciparum* infection (Haynes, 2006, Weina, 2008). In some parts of Asia and Africa, a hot water infusion (tea) of the plant is used as a self-medication for malaria. The use of tea in this way has raised concern of the possible development of parasite resistance as a result of un-standardized use of artemisinin in these tea preparations (Jansen, 2006).

The recipes in ancient Chinese texts for preparing *Qing Hao* extracts for the treatment of fevers include soaking, followed by wringing or pounding, followed by squeezing the fresh herb (Wright *et al.*, 2010, Hsu, 2006a, Hsu, 2006b). In their study, Rath *et al.* (Rath *et al.*, 2004) found that adding boiling water to the leaves, stirring briefly and leaving covered for 10 min, and filtering and gently squeezing the leaves to release residual water gave the best extraction efficiency (86%) for artemisinin in the preparation, relative to the total amount of the compound in leaves. In the literature, a range of aqueous extraction efficiencies (25-90%) has been reported for artemisinin (Rath *et al.*, 2004, De Ridder *et al.*, 2008, Van der Kooy and Verpoorte, 2011). Due to

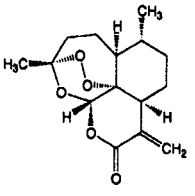
the differences in the content of artemisinin in tea preparation, Van der Kooy and Verpoorte (Van der Kooy and Verpoorte, 2011) quantified artemisinin in tea prepared by different methods.



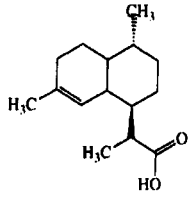
(4.1) artemisinin



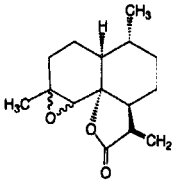
(4.2) 9-epi-artemisinin



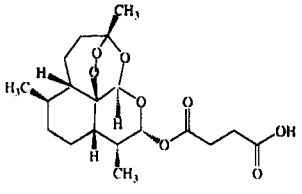
(4.3) artemisitene



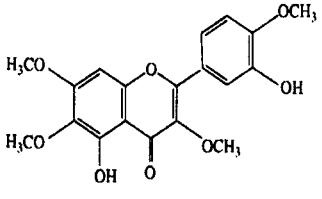
(4.4) dihydroartemisinic acid



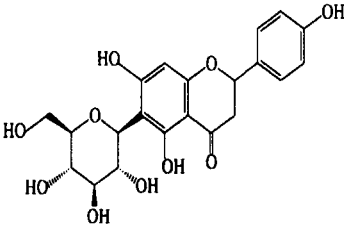
(4.5) arteannuin B



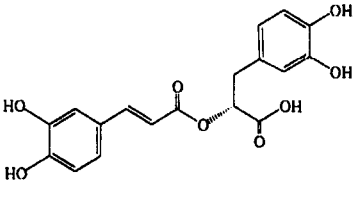
(4.6) artesunate



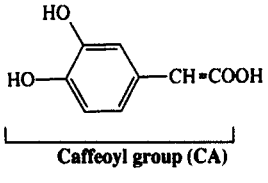
(4.7) casticin



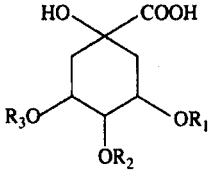
(4.8) isovitexin



(4.9) rosmarinic acid



(4.10) caffeic acid



(4.11-4.17) quinic acid

	R_1	R_2	R_3
(4.11) 3-caffeoylquinic acid	CA	H	H
(4.12) 4-caffeoylquinic acid	H	CA	H
(4.13) 5-caffeoylquinic acid	H	H	CA
(4.14) 3,4-di-caffeoylquinic acid	CA	CA	H
(4.15) 3,5-di-caffeoylquinic acid	CA	H	CA
(4.16) 4,5-di-caffeoylquinic acid	H	CA	CA
(4.17) 3,4,5-tri-caffeoylquinic acid	CA	CA	CA

Scheme 4.1. Structures of some artemisinin related compounds, flavonoids and acids identified in *A. annua* extract.

They observed that extraction efficiency was temperature-sensitive and that efficiencies of above 90% were attainable. Regardless, the amount of artemisinin in these extracts cannot fully account for its effectiveness against *Plasmodium* parasites *in vitro* and *in vivo* (Jansen, 2006, Rath *et al.*, 2004). Apart from artemisinin, there are around 30 other sesquiterpenes and over 36 flavonoids identified in the plant (Scheme 4.1), some of which have shown limited anti-malarial properties (Willcox *et al.*, 2004). Five flavonoids, including casticin (**4.7**), have been shown to potentiate the activity of artemisinin (Liu *et al.*, 1992, Elford *et al.*, 1987). Interestingly, the potentiating effect of these flavonoids was not observed with chloroquine. Billia *et al.* (Bilia *et al.*, 2002) observed that although these flavonoids have no effect on hemin (chloroferriprotoporphyrin) themselves, they do catalyze a reaction between artemisinin and hemin.

Weathers and Towler (Weathers and Towler, 2012) have shown that poly-methoxylated flavonoids like casticin are poorly extracted and unstable in the aqueous tea infusion. This suggests that compounds other than this class of flavonoids are likely responsible for the reported improvement in the potency of artemisinin in tea infusion. A recent analysis by Cabonara *et al.* (Carbonara *et al.*, 2012) of tea prepared from *A. annua* leaves by infusion in hot water for 1, 24 and 48 hours, identified a series of caffeoyl and feruloyl-quinic acids as main components of the infusion, together with some flavonoids. Chlorogenic or caffeoylquinic acids (CQAs) are esters of caffeic and quinic acids (Scheme 4.1). They possess a broad spectrum of pharmacological properties, including antioxidant, hepato-protectant, antibacterial, anti-histaminic, chemo-preventive and other biological effects (Belkaid *et al.*, 2006, Zhang *et al.*, 2008, Feng *et al.*, 2005, Miketova *et al.*, 1999).

To our knowledge, only the interactions of artemisinin with the poorly extracted poly-methoxylated flavonoids found in *Artemisia* tea have been studied. This study therefore aims at the understanding of other possible interactions and mechanisms involved in artemisinin activity in the plant extracts, and the effects of these interactions on parasite resistance to artemisinin.

4.2 Results and discussions

Two sets of in vitro experiments were carried out. The initial anti-plasmodial evaluation of *Artemisia* extracts was carried out in Liverpool School of Tropical Medicine using products of sequential extraction in various polarity of solvents. Based on the results of this a follow-on analysis was done at the Centre for Infectious Diseases, George Town University, USA.

4.2.1 *Fractionation and extraction yield*

Figure 4.1 shows the extraction and fractionation scheme employed and the corresponding percentage yield obtained. Methanol (13.4%) was the best extractant of all the three organic solvents used in crude extraction. The yield from tea preparation was 23.4%, although a comparatively larger volume of liquid (1 liter) was employed in this extraction.

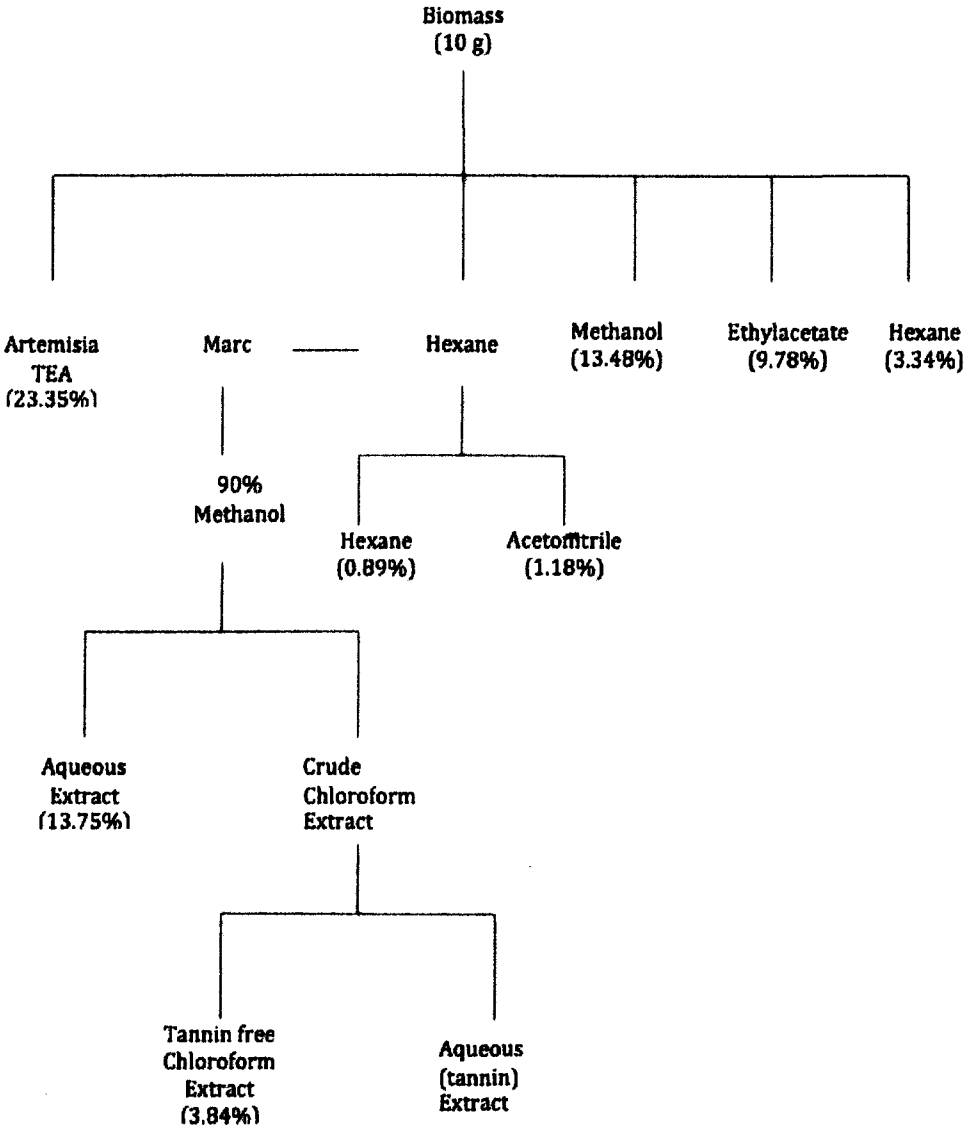


Figure 4-1. Fractionation scheme for *A. annua* plant extracts used in *P. falciparum* assay.

The artemisinin content of the fractionated and the crude extracts are shown in Table 4-1. Artemisinin was effectively partitioned into acetonitrile from the hexane extract; however de-fatting with hexane did not totally extract all the artemsinin in the biomass. The residual amount left in the marc was carried over into the methanol extraction and portioned into chloroform. The water fraction was also artemsinin free.

Methanol proved to be the best extractant for artemisinin (9.72mg g^{-1} of biomass) of all the solvents used.

Table 4-1. Artemisinin in fractionated and crude extracts.

Fractions	Amt of artemisinin mg (per 100mg)	Total artemisinin mg (per 10g biomass)
Acetonitrile	26.4	31.2
Hexane	0.0	0.0
Chloroform	3.3	12.7
Water	0.0	0.0
Crude Extracts		
Hexane	20.1	66.8
Ethyl acetate	8.2	80.2
Methanol	7.2	97.2
Water (Tea)	0.58	13.5

4.2.2 Metabolite profile of Artemisia extract

The metabolites in the aqueous extract analysed by both MS/MS and HPLC methods and their quantities in milligrams per litre of extract is shown in Table 4.2. The compounds analysed were based on the *in extenso* analysis by Carbonara *et al.* (Carbonara *et al.*, 2012), who showed them to be the major metabolites (quantitatively) in *Artemisia* tea infusions. In addition, artemisinin related compounds, which we have previously detected in such extracts, were also included. The level of artemisinin reported (Van der Kooy and Verpoorte, 2011, Rath *et al.*, 2004, Carbonara *et al.*, 2012, De Magalhaes *et al.*, 2012, Wright *et al.*, 2010) for tea extract is varied and the values obtained in this study (47.5 mg L^{-1}) are within the reported range of artemisinin content. These differences could be due to tea preparation methods and the biomass cultivar used. Carbonara *et al.* (Carbonara *et al.*, 2012) used a solvent to biomass ratio of 26:1 (v/w), while this study, as well as

others (Van der Kooy and Verpoorte, 2011, Rath *et al.*, 2004), employed the therapeutically recommended ratio (200:1, v/w or 5 g L^{-1}) (Willcox, 2009).

Table 4-2. Metabolites in the aqueous *Artemisia* extract analysed by both MS/MS and HPLC methods quantified as milligrams per litre of tea.

Compound	Amount (mg L^{-1} of tea)
Artemisinin	47.5±0.8
Arteannuin B	1.3±0.0
Dihydroartemisinic acid	70.0±0.3
Caffeic acid	0.8±0.00
3,5-Di-caffeoylquinic acid	57.0±1.7
3-Caffeoylquinic acid	72.0±1.6
4-Caffeoylquinic acid	20.4±1.6
4,5-Di-caffeoylquinic acid	31.6±4.0
5-Caffeoylquinic acid	9.0±0.7
Isovitexin	105.0±7.2
Rosmarinic acid	1.1±0.0

Dihydroartemisinic acid (**4.4**) (70 mg L^{-1}) and arteannuin B (**4.5**) (1.3 mg L^{-1}) are the only biosynthetic precursors of artemisinin detected in the tea extract using our method. Therefore artemisinin is the only compound among the metabolites we analysed in the tea with significant (IC_{50} <1 μM) anti-plasmodial activity (Table 4.3).

3-Caffeoylquinic acid (**4.11**) was found to be the most abundant (72 mg L^{-1}) of the caffeic acid derivatives (**4.11-4.17**) in the analysed extract, followed by 3,5-di-caffeoylquinic acid (**4.15**) (57 mg L^{-1}). Caffeic acid (**4.10**) was the least abundant (0.8 mg L^{-1}) of the evaluated acids. Isovitexin (**4.8**) was the only flavonoid analysed (105 mg L^{-1}), being relatively abundant in our extract. Some classes of flavonoids have

poor aqueous solubility and limited profiles of these compounds in aqueous extract have been reported (Weathers and Towler, 2012, Carbonara *et al.*, 2012). Lower level of rosmarinic acid (**4.9**) (1.1 mg L^{-1}) was detected in our samples, compared to the levels found by De Magalhaes *et al.* (De Magalhaes *et al.*, 2012). However, they reported widely different concentrations in the cultivars and samples they analysed and this acid was not detected at all in the analysis by Carbonara *et al.* (Carbonara *et al.*, 2012). Van der Kooy and Verpoorte have also shown that the method employed in preparing the hot water infusion does affect the amount of artemisinin and therefore other co-metabolites extracted (Van der Kooy and Verpoorte, 2011). These differences in profiles and concentration levels of metabolites seem to suggest that composition of prepared tea infusions differ and is significantly influenced by geography of growth area and the *Artemisia* cultivar used.

4.2.3 Activities of A. annua extract fractions in choroqine (CQ) sensitive parasites

The half-maximal response data (IC_{50}) of fractionated and crude extracts in a plasmodium assay are shown in Figures 4.2, 4.3 and 4.4. Pure artemisinin and artesunate, a more bio-available analogue of artemisinin were positive controls. A solvent control was employed and *Artemisia* tea with reported (De Donno *et al.*, 2012) synergistic anti-plasmodial effect was used both as control and as a treatment. Figure 4.2 and 4.3 shows the IC_{50} for tea infusion at 50 mg mL^{-1} (TEA50) and 25 mg mL^{-1} (TEA25) and suggests that the tea extracts were about 3.8 times and 2.2 times more potent than pure artemisinin respectively.

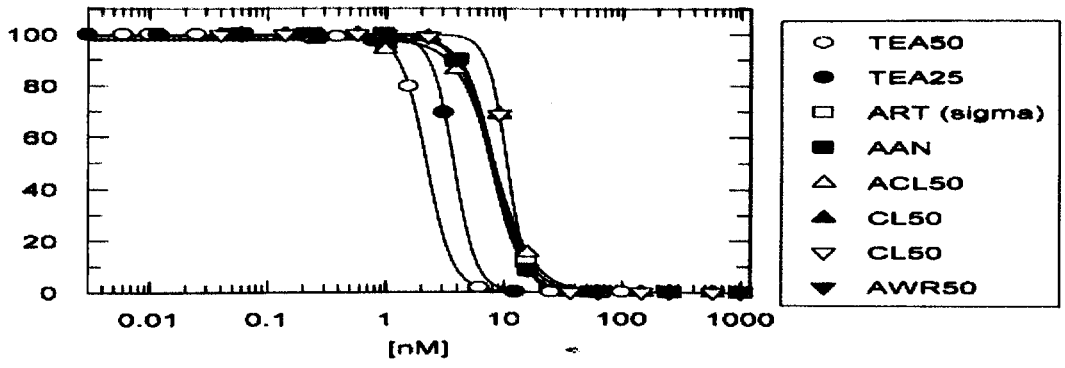


Figure 4-2. Dose-response curve for fractionated and crude extracts. TEA 25, 50 = 25 and 50mg mL^{-1} *Artemisia* tea. Art = artemisinin, AAN = artemisinin + acetonitrile fraction, ACL50 = artemisinin + 50 mg mL^{-1} chloroform fraction. CL50 = 50 mg mL^{-1} chloroform fraction. AWR50 = artemisinin + 50mg mL^{-1} water fraction.

The first set of results obtained form the plasmodium assay (Figure 4.3) shows the IC_{50} of most of the fractions was similar (7.1 - 8.5 nM) to pure artemisinin (8.03 nM), the exception being the chloroform (CL50 – IC_{50} = 10.5nM) fraction with comparatively lower level of artemisinin. Among the crude extracts, hexane (HXC) had significantly lower IC_{50} of 4.7 nM while ethyl acetate and methanol had IC_{50} comparable to that of pure artemisinin.

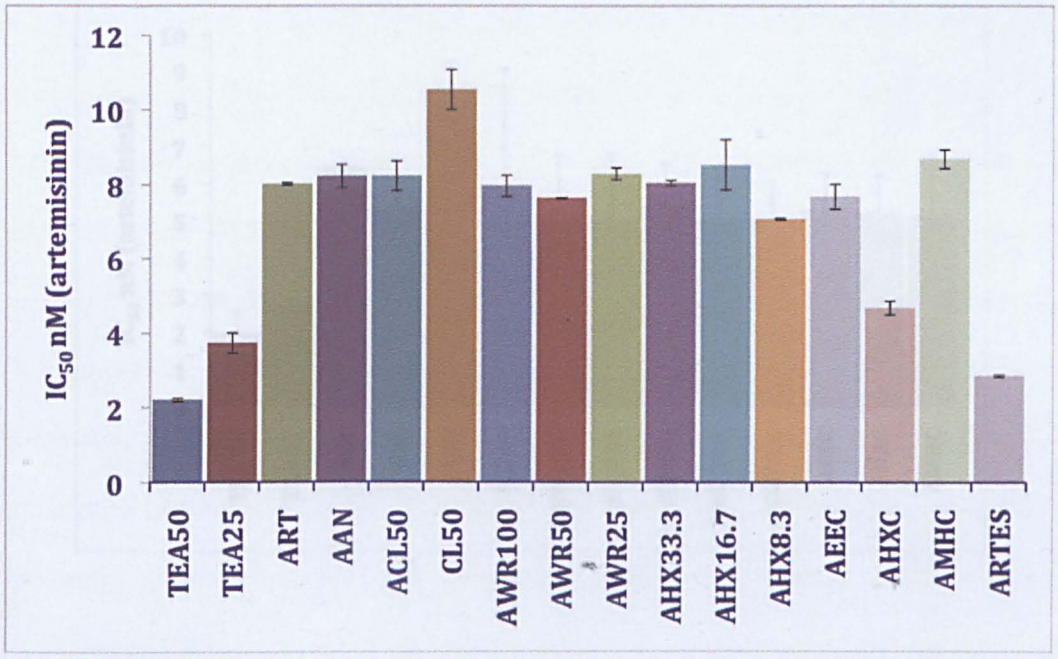


Figure 4-3. IC_{50} for artemisinin and extract fractions (first data set). TEA 25, 50 = 25 and 50 mg mL^{-1} *Artemisia* tea. Art = artemisinin, AAN = artemisinin + acetonitrile fraction, ACL50 = artemisinin + 50 mg mL^{-1} chloroform fraction. CL50 = 50 mg mL^{-1} chloroform fraction. AWR 25, 50 and100 = artemisinin + 25, 50 and 100 mg mL^{-1} water fraction. AHX 8.3, 16.7 and 33.3 = artemisinin + 8.3, 16.7 and 33.3 mg mL^{-1} hexane fraction. AEEC = artemisinin + Ethyl acetate crude extract. AHXC = artemisinin + hexane crude extract. AMHC = artemisinin + methanolic crude extract. ARTES = artesunate

A second analysis (Figure 4-4) of the same fractionated extract shows a similar pattern but with improved IC_{50} for the crude extracts (5.1 -5.3 nM) compared to pure artemisinin (6.5nM). The improvement observed for crude extracts compared to artemisinin were not significant due to the relatively large variation within treatments.

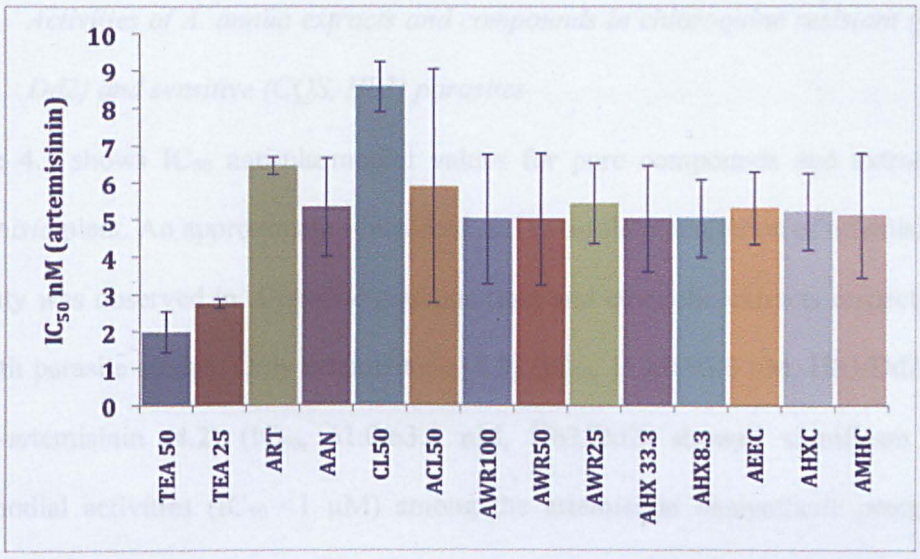


Figure 4-4. IC_{50} for artemisinin and extract fractions (second data set). TEA 25, 50 = 25 and 50 mg mL^{-1} *Artemisia* tea, Art = artemisinin, AAN = artemisinin + acetonitrile fraction, ACL50 = artemisinin + 50 mg mL^{-1} chloroform fraction, CL50 = 50 mg mL^{-1} chloroform fraction, AWR 25, 50 and100 = artemisinin + 25, 50 and 100 mg mL^{-1} water fraction, AHX 8.3, 16.7 and 33.3 = artemisinin + 8.3, 16.7 and 33.3 mg mL^{-1} hexane fraction, AEEC = artemisinin + ethyl acetate crude extract. AHXC = artemisinin + hexane crude extract. AMHC = artemisinin + methanolic crude extract, ARTES = artesunate.

4.2.4 *Activities of A. annua extracts and compounds in chloroquine resistant (CQR, Dd2) and sensitive (CQS, Hb3) parasites*

Table 4.3 shows IC_{50} anti-plasmodial values for pure compounds and extracts of *Artemisia* plant. An approximate seven-fold and two-fold potentiation of artemisinin's activity was observed in *Artemisia* aqueous (tea) and ethanolic extracts respectively, in both parasite strains. Only artemisitene (**4.3**) (IC_{50}, 114.0/95.0 nM, Hb3/Dd2) and 9-epi-artemisinin (**4.2**) (IC_{50}, 61.0/63.5 nM, Hb3/Dd2) showed significant anti-plasmodial activities (IC_{50} <1 μM) among the artemisinin biosynthetic precursors evaluated. 9-epi-artemisinin and artemisitene respectively showed about one third and one fifth of the activity of artemisinin. Acton *et al.* observed a similarly reduced activity for 9-epi-artemisinin and artemisitene, compared to artemisinin in D6 and W2 strains of *P. falciparum* (Acton and Klayman, 1987, Acton and Klayman, 2007). Artemisinin has a chiral molecular structure and the bioactivity of the molecule is influenced by its absolute configuration.

Table 4-3. IC_{50} of extracts and components of *A. annua* in CQ-sensitive (HB3) and resistant (Dd2) strains.

	IC_{50} (nM)	
Compound/extracts	Hb3 strain	Dd2 strain
Chloroquine (CQ)	23.3	205.1
Artemisinin	21.7	20.3
Artesunate	8.5	4.6
Artemisitene	114.0	95.0
9-epi-artemisinin	61.0	63.5
Artemisia aqueous extract	3.1	3.2
Artemisia ethanol extract	8.8	11.4
	IC_{50} (μM)	
Artemisinic acid	74.8	39.1
Arteannuin B	3.4	6.6
Dihydroartemisinic acid	31.3	33.6
Caffeic acid	59.4	35.6
3-Caffeoylquinic acid	102.9	124.1
4-Caffeoylquinic acid	113.2	41.6
5-Caffeoylquinic acid	151.9	93.6
3,4-Caffeoylquinic acid	33.4	36.1
4,5-Caffeoylquinic acid	34.0	36.7
3,4,5-Caffeoylquinic acid	185.6	99.6
Rosmarinic acid	84.7	50.2
Isovitexin	84.7	50.2
Casticin	10.0	13.5

To investigate if solubility of these artemisinin analogues could be partially responsible for the reduced activity, we determined the aqueous solubility of artemisinin, artemisitene and 9-epi-artemisnin.

Table 4.4 shows the solubility of these compounds at experimental conditions.

Under these conditions, 9-epi-artemisinin has a higher solubility, about twice that of artemisinin or artemisitene. The lower bioactivity could not be explained based on the solubility data alone, although the experimental data were obtained at 22 °C.

Table 4-4. Solubility in mg L^{-1} of artemisinin, artemisitene and 9-epi-artemisinin in water at 22 °C and atmospheric pressure.

Compound	Solubility / mg L^{-1} at 22 °C
Artemisinin	74.27±2.10
Artemisitene	74.21±2.99
9-Epi-artemisinin	133.08±5.44

Woerdenbag *et al.* observed that the anti-cancer activity of 11-hydroxy-11-epi-artemisinin (C11 in older and C9 in newer references for the structure) was about threefold less than the conformer (Woerdenbag *et al.*, 1993b), which is the same threefold difference we observed in anti-malarial activity for epimerisation at C9 (Table 4.4). If the threefold activity difference is consistent regardless of the differences in molecular targets and effect, this may suggest a common upstream differentiation point of molecule activation. The lower activity of 9-epi-artemsinin may therefore be due in part to a structural conformation that is relatively more difficult to activate compared to artemisinin.

4.2.5 Antagonism of artemisinin with biosynthetic precursors

Figure 4.5 shows the interaction of artemisitene and 9-epi-artemisinin with artemisinin and artesunate (**4.6**). These biosynthetic precursors of artemisinin have significant anti-malarial activities (Table 4.3). The interaction of artemisinin with 9-

epi-artemisinin and artemisitene was antagonistic, but the interaction of these compounds with artesunate was additive in both chloroquine sensitive (Hb3) and resistant (Dd2) strains.

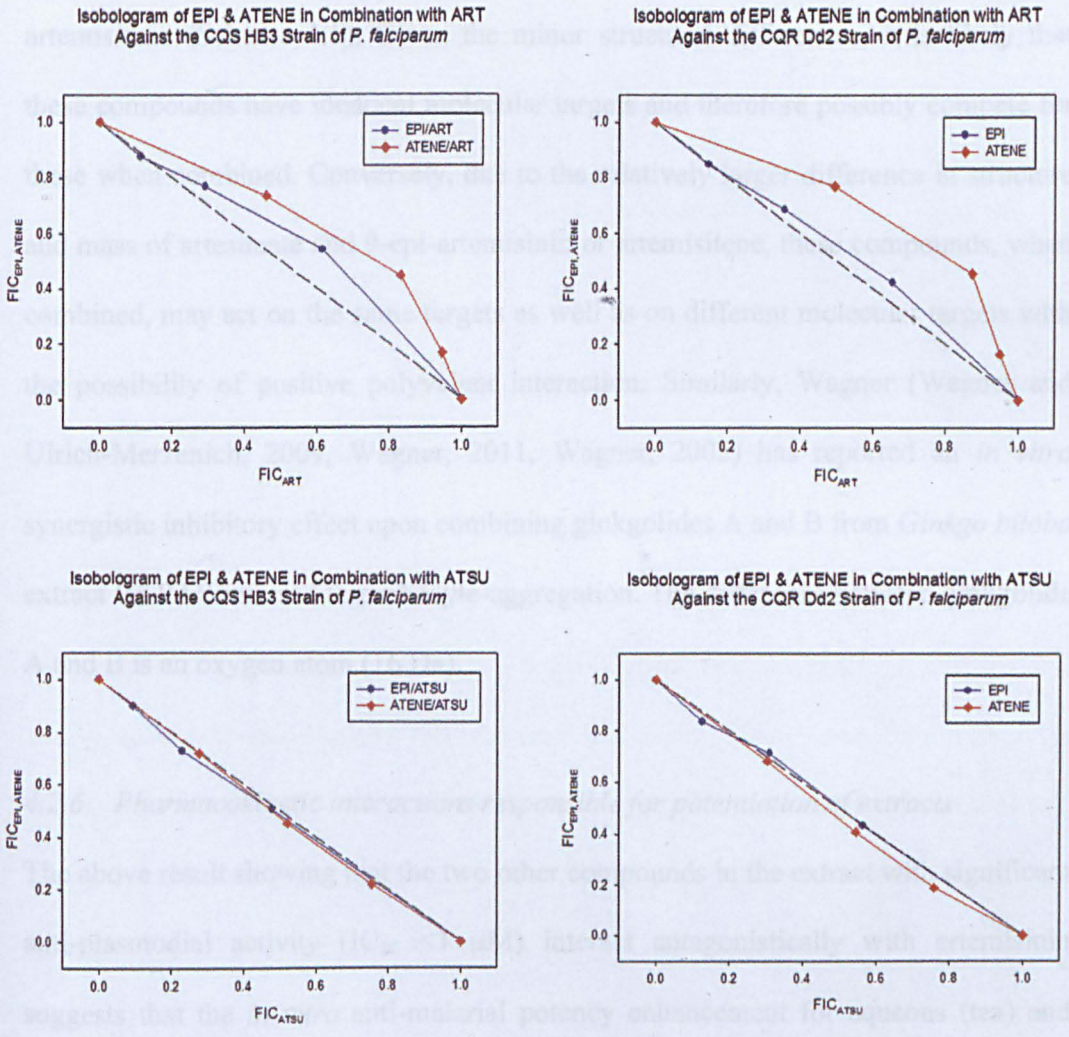


Figure 4-5. Isobologram of 9-epi-artemisinin and artemisitene with artemisinin (top) and artesunate (bottom) in CQS (HB3 (left) and CQR (Dd2) (right) strains. ART = artemisinin, ATSU = artesunate, EPI = 9-epi-artemisinin, ATENE = artemisitene.

The reason for the observed antagonistic interaction with artemisinin at the combinations investigated is unclear. Structurally, artemisinin, 9-epi-artemisinin and artemisitene are differentiated at C9. The difference from artemisinin is epimerisation of the methyl group for 9-epi-artemisinin and a methylene group attached instead for artemisitene (Scheme 4.1). Given the minor structural differences, it is likely that these compounds have identical molecular targets and therefore possibly compete for these when combined. Conversely, due to the relatively larger difference in structure and mass of artesunate and 9-epi-artemisinin or artemisitene, these compounds, when combined, may act on the same targets as well as on different molecular targets with the possibility of positive polyvalent interaction. Similarly, Wagner (Wagner and Ulrich-Merzenich, 2009, Wagner, 2011, Wagner, 2005) has reported an *in vitro* synergistic inhibitory effect upon combining ginkgolides A and B from *Ginkgo biloba* extract for PAF-induced thrombocyte-aggregation. The difference between ginkgolide A and B is an oxygen atom (16 Da).

4.2.6 Pharmacokinetic interactions responsible for potentiation of extracts

The above result showing that the two other compounds in the extract with significant anti-plasmodial activity (IC_{50} <1 μM) interact antagonistically with artemisinin suggests that the *in vitro* anti-malarial potency enhancement for aqueous (tea) and ethanolic extracts is probably due to interaction between artemisinin and some other compound(s) in the extracts. Pharmacokinetic interactions are improvements due to increased solubility, re-sorption rate and/or enhanced bioavailability (Wagner and Ulrich-Merzenich, 2009, Williamson, 2001).

4.2.7 Other combination analysis

Table 4.5 shows the interaction of co-metabolites in *Artemisia* extracts with artemisinin. In the CQ-sensitive (HB3) strain, 3-caffeoylquinic acid (3CA) showed additive interaction at 1:3 (v/v), which became synergistic at higher ratio of the acid to artemisinin (1:10, 1:100 v/v). For casticin, the interaction at 1:3 (artemisinin to casticin, v/v) is antagonistic. Synergistic interaction is however reported (Elford *et al.*, 1987, Liu *et al.*, 1992) for combination ratios at the range of 1:10-1000 (artemisinin to casticin, v/v).

Therefore, using the FIC index of casticin (1.9) as a benchmark for potential positive interactions, compounds like isovitexin, caffeic acid and dihydroartemisinic acid that show antagonistic interactions at 1:3 may also, like casticin, interact synergistically at a higher ratio. Rosmarinic acid was synergistic at a 1:3 combination with artemisinin (v/v) and some chlorogenic acids were additive at this combination also. These compounds showing positive interactions with artemisinin may collectively be responsible for the potentiation of artemisinin in the tea extract. Arteannuin B and artemisinic acid are poorly extracted in the aqueous extract, but may contribute to the synergy observed in the alcoholic extract.

Table 4-5. Anti-plasmodial interactions of co-metabolites with artemisinin in CQ-sensitive (HB3) and CQ-resistant (Dd2) strains. Art = artemisinin, CA = caffeic acid, 3CA = 3-caffeoylquinic acid, 4CA = 4-caffeoylquinic acid, 5CA = 5-caffeoylquinic acid, 3,4 CA = 3,4-di-caffeoylquinic acid, 3,5CA = 3,5-di-caffeoylquinic acid, 4,5CA = 4,5-di-caffeoylquinic acid, TCA = 3,4,5-tri-caffeoylquinic acid, ISO = siovitexin, CAS = casticin, ATCID = artemisinic acid, ARTB = arteannuin B, RA = rosmarinic acid, DHAA = dihydroartemisinic acid, ARTENE = artemisitene.

		IC_{50} (μM)		HB3				Dd2			
Combination	Compound	HB3	Dd2	FIC_{ART}	FIC_{Cpd}	FIC_{index}	Interaction	FIC_{ART}	FIC_{Cpd}	FIC_{index}	Interaction
1:3 ART:CA	ART	0.0172	0.0294	0.411	1.160	1.570	Antagonistic	0.748	3.298	4.046	Antagonistic
	CA	68.9	117.4								
1:3 ART:3CA	ART	0.0143	0.0283	0.341	0.831	1.172	Additive	0.720	1.367	2.088	Antagonistic
	3CA	85.5	169.7								
1:10 ART:3CA	ART	0.0044	0.0081	0.105	0.580	0.685	Synergistic	0.206	0.881	1.087	Additive
	3CA	59.7	109.3								
1:100 ART:3CA	ART	0.0004	0.0007	0.010	0.772	0.781	Synergistic	0.018	1.160	1.177	Additive
	3CA	79.4	143.9								
1:3 ART:4CA	ART	0.0164	0.0303	0.391	0.696	1.088	Additive	0.771	3.495	4.266	Antagonistic
	4CA	78.8	145.4								
1:3 ART:5CA	ART	0.0139	0.0259	0.332	0.596	0.928	Additive	0.659	1.801	2.460	Antagonistic
	5CA	90.5	168.6								
1:3 ART:34CA	ART	0.0269	0.0601	0.642	1.611	2.253	Antagonistic	1.529	3.332	4.862	Antagonistic
	34CA	53.8	120.3								
1:3 ART:35CA	ART	0.028	0.0594	0.668	1.644	2.312	Antagonistic	1.511	3.237	4.749	Antagonistic
	35CA	55.9	118.8								
1:3 ART:45CA	ART	0.0264	0.06	0.630	1.685	2.315	Antagonistic	1.527	3.318	4.844	Antagonistic
	45CA	52.9	120.1								
1:3 ART:TCA	ART	0.0313	0.0568	0.747	0.473	1.220	Additive	1.445	1.595	3.041	Antagonistic
	TCA	87.7	158.9								
1:3 ART:ISO	ART	0.0259	0.0567	0.618	0.916	1.534	Antagonistic	1.443	3.386	4.829	Antagonistic
	ISO	77.6	170								
1:3 ART:CAS	ART	0.0143	0.0284	0.341	1.580	1.921	Antagonistic	0.723	2.311	3.034	Antagonistic
	CAS	15.8	31.2								
1:3 ART:ATCID	ART	0.0162	0.0271	0.387	1.080	1.467	Additive	0.690	3.463	4.152	Antagonistic
	ATCID	80.8	135.4								
1:3 ART:ARTB	ART	0.0143	0.0039	0.341	0.909	1.250	Additive	0.099	0.242	0.342	Synergistic
	ARTB	3.09	1.6								
1:3 ART:RA	ART	0.0149	0.0315	0.356	0.535	0.890	Synergistic	0.802	4.150	4.952	Antagonistic
	RA	74.3	157.3								
1:3 ART:DHAA	ART	0.0182	0.0392	0.434	1.366	1.801	Antagonistic	0.997	1.863	2.861	Antagonistic
	DHAA	29.1	62.6								
1:3 ART:ATENE	ART	0.0356	0.0643	0.850	2.631	3.480	Antagonistic	1.636	5.366	7.002	Antagonistic
	ATENE	0.2136	0.3858								
ART		0.0419	0.0393	1	-			1	-		

Casticin and 3-caffeoylquinic acid (3CA) are polyphenolic compounds that are natural anti-oxidants. Endogenous anti-oxidants at cellular redox sites are considered a "double edge sword" able to act either as anti-oxidant or pro-oxidant depending on conditions such as dosage levels and presence of metal ions (Yordi *et al.*, 2012, Nemeikaitė-Čėnienė *et al.*, 2005). This "double edge sword" characteristic of anti-oxidant polyphenols could help explain our observation. At a lower combination with artemisinin, casticin and 3CA were anti-oxidative towards the ROS and carbon-centred radicals formed from artemisinin activation and, as a result, countered artemisinin activity *in vitro*. Conversely, at a higher concentration ratio to artemisinin, casticin and 3CA were pro-oxidative, enhancing the oxidative stress resulting from the activation of artemisinin and leading to improvement in potency for artemisinin. A schematic isobologram to describe the interaction between an active pharmaceutical ingredient (API) like artemisinin (A) and synergists like casticin and 3CA (B, non-API) is shown in Figure 4.6.

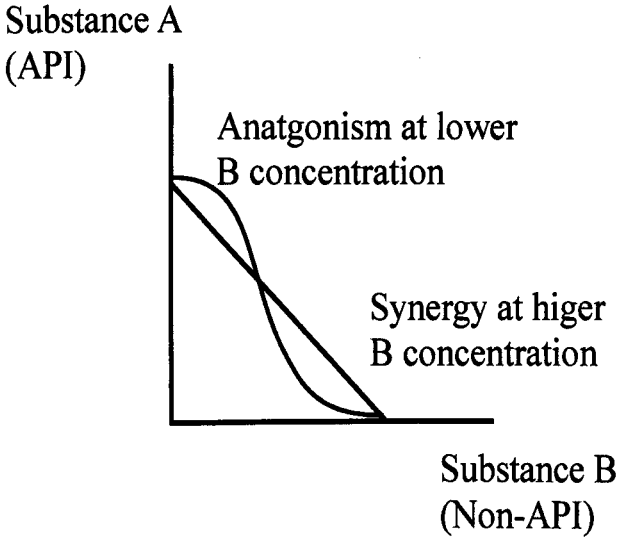


Figure 4-6. A schematic isobologram of the observed interactions.

4.2.8 Possible role of anti-oxidant defence network in resistance

Rosmarinic acid at the combination ratio evaluated had a potentiating effect (FIC_{index} 0.89) on artemisinin in CQ-sensitive (HB3) strain (Table 4.5) but this effect was not reproduced in the resistant (Dd2) strain; rather a strong antagonistic effect (FIC_{index} 4.95) was observed. The effect of rosmarinic acid on the ability artemisinin to mitigate the resistance mechanism of the parasite could be partly explained by the finding of Cul *et al* and others who observed that *in vitro* resistance in *P. falciparum* is associated with increased *pfmdr-1* copy number and anti-oxidant activity (Cui *et al.*, 2012,Sidhu *et al.*, 2006). Some experiments with rosmarinic acid have reported strong anti-oxidant activity for the compound that is over three times that of trolox (Erkan *et al.*, 2008, Petersen and Simmonds, 2003, Tepe *et al.*, 2007). In the presence of rosmarinic acid, anti-oxidant activity may further be elevated thereby promoting increased resistance. A similar trend of activity in sensitive and resistant strains in combination with artemisinin was observed for caffeic acid, 4-caffeoyl-quinic acid (**4.12**) and isovitexin with reported anti-oxidant properties (Gülçin, 2006, Xu *et al.*, 2012, Cao *et al.*, 2011). This seems to confirms the possible role of the anti-oxidant defence network in parasite resistance to artemisinin (Bozdech and Ginsburg, 2004).

4.2.9 Arteannuin B selectively potentiates the activity of artemisinin against parasite defence system

Arteannuin B at 3:1 (v/v) combination with artemisinin showed additive or no interaction (FIC_{index} 1.25) in the CQ-sensitive strain and a synergistic interaction (FIC_{index} 0.34) in the resistant parasite strain (Table 4.5). This is about a three-fold improvement in artemisinin's potency against CQ-resistant *P. falciparum*. This is not reproduced in the CQ-sensitive strain. The potentiation of artemisinin by arteannuin

B seems to be selectively directed at the parasites' chloroquine resistance mechanism. This combination could therefore help to better understand the mechanism(s) involved in parasite defence against artemisinin analogues and other anti-malarials. Reproducing this three-fold improvement in potency with other artemisinin analogues could help in the development of therapeutics effective against emerging drug-resistant strains.

Arteannuin B is an unusual α-methylene-γ-lactone, transfused via a tertiary hydroxyl group (Agrawal *et al.*, 1991). This structure could account for its easy fragmentation/ionisation observed in mass spectrometry and reported facile rearrangement in acidic conditions (Lansbury and Mojica, 1986, Suberu *et al.*, 2013a).

4.3 Conclusions

In this study we examine interactions between artemisinin and co-metabolites found in *A. annua* plant extracts for chloroquine sensitive (CQS; HB3) and resistant (CQR; Dd2*)* *P. falciparum* malarial parasites. Potentiation of artemisinin anti-malarial activity was observed in the plant extracts. The aqueous extract showed comparatively superior potentiation (about seven-fold) over the ethanolic extract (about two-fold). When pure compounds were combined, 9-epi-artemisinin and artemisitene interacted antagonistically with artemisinin at the combinations evaluated. 9-epi-artemisinin and artemisitene were the only artemisinin-related metabolites with significant anti-plasmodial activity (IC_{50} <1 μM) among those evaluated.

In CQ-sensitive parasites, caffeic acids and their chlorogenic acid derivatives showed additive interactions with artemisinin at the combination ratio evaluated. 3-Caffeoylquinic acid's interaction with artemisinin turned synergistic with the increased ratio of the former in the combination. Rosmarinic acid showed synergistic interaction with artemisinin in the drug sensitive strain but the interaction with artemisinin in the drug resistant strain was strongly antagonistic at the same level of combination. This antagonistic interaction in CQ-resistant parasites was also observed for caffeic acid and some of its derivatives known to have anti-oxidant properties. The observation seems to confirm literature evidence (Cui *et al.*, 2012, Sidhu *et al.*, 2006) for a potential role of anti-oxidants in parasite drug resistance. Therefore the effect of dietary anti-oxidants on artemisinin combination therapies used in the management of drug resistant *P. falciparium* malaria may need to be further investigated.

Arteannuin B was found to selectively potentiate the activity of artemisinin in Dd2 parasites, suggesting some interaction with the CQR mechanism, since the potentiation of artemisinin by arteannuin B was not reproduced in CQS parasites. As a result of this specificity, arteanniun B could potentially be used as a probe to better understand parasite drug resistance mechanisms and the combination might prove useful for treating CQR strains of malaria.

CHAPTER 5. COMPARATIVE CYTOTOXICITY OF ARTEMISININ AND CISPLATIN AND THEIR INTERACTIONS WITH CHLOROGENIC ACID IN MCF7 BREAST CANCER CELLS.

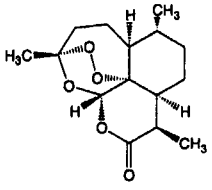
5.1 Introduction

Cancer is a major public health problem with about 7.6 million deaths in 2008 and projected to increase to over 13 million in 2030 (Ferlay *et al.*, 2010). Although a range of treatment options are available in many cases these therapies are fraught with significant levels of toxicity to healthy cells and in some treatment regimes drug-resistance is emerging (Crespo-Ortiz and Wei, 2011, Hsiao and Liu, 2010). To reduce the current cancer burden, drug discovery is being directed at developing highly effective and potent medications with considerably lower side effects.

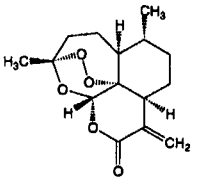
The role of natural products as a rich source of new bioactive molecules and the properties and mechanism of action of anti-malaria agent artemisinin has been discussed in Chapter 4.

The *in vitro* cytotoxic activity of artemisinin (**5.1,** Scheme 5.1) and its derivatives have been reported in different cancer cell lines including drug-resistant cell lines (Gravett *et al.*, 2011, Singh and Lai, 2004, Sadava *et al.*, 2002, Efferth *et al.*, 2004). Sigh and Lai showed that a combination of dihydroartemisinin (**5.5**) and halo-transferin effectively killed radiation resistant breast cancer cells (Singh and Lai, 2004), while artemisinin pretreated with holo-transferin was also found to be effective on both the drug sensitive and multi-drug resistant human lung carcinoma (SCLC) cells (Sadava *et al.*, 2002). Artesunate (**5.6**) inhibited the growth of highly angiogenic

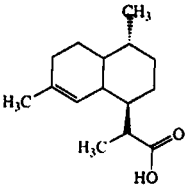
Kaposi sarcoma cells showing the anti-angiogenesis effect of artemisinins (Dell'Eva *et al.*, 2004).



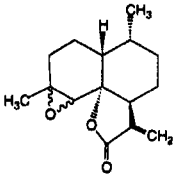
(5.1) artemisinin



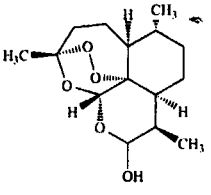
(5.2) artemisitene



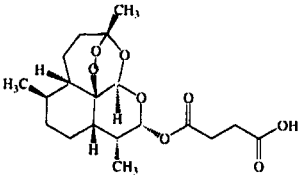
(5.3) dihyroartemisinic acid



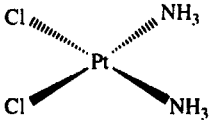
(5.4) arteannuin B



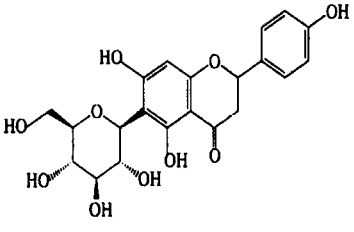
(5.5) dihydroartemisinin



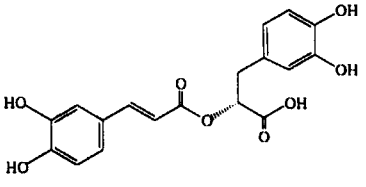
(5.6) artesunate



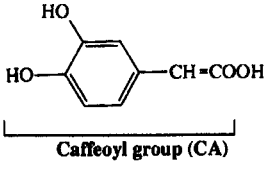
(5.7) cisplatin



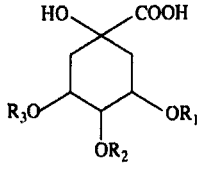
(5.8) isovitexin



(5.9) rosmarinic acid



(5.10) caffeic acid



(5.11-5.17) quinic acid

	R_1	R_2	R_3
(5.11) 3-caffeoylquinic acid	CA	H	H
(5.12) 4-caffeoylquinic acid	H	CA	H
(5.13) 5-caffeoylquinic acid	H	H	CA
(5.14) 3,4-di-caffeoylquinic acid	CA	CA	H
(5.15) 3,5-di-caffeoylquinic acid	CA	H	CA
(5.16) 4,5-di-caffeoylquinic acid	H	CA	CA
(5.17) 3,4,5-tri-caffeoylquinic acid	CA	CA	CA

Scheme 5.1. Some compounds found in *Artemisia* aqueous extract (Tea) and cisplatin.

The *in vivo* anti-proliferative bioactivities of artemisinins have also been reported. Chen *et al.* implanted nude mice with human ovarian cancer cells and found that artesunate decreased tumour growth and significantly lowered vascular endothelial growth factor (VEGF) expression in the cells (Chen *et al.*, 2004). The potential of artemisinin to prevent the development of breast cancer in rats treated with a known carcinogen (7,12 dimethylbenz [a] anthracene, DMBA) has been reported (Lai and Singh, 2006). Artesunate has also been successfully used in combination to standard chemotherapy to treat metastatic melanoma in human subjects after standard chemotherapy alone was ineffective in stopping tumour growth (Berger *et al.*, 2005).

Several workers have investigated the mechanisms of the selective cytotoxicity of artemisinin and its derivatives against neoplastic cells. Mercer *et al.* showed that selective activation of the trioxane bridge via carbon centred radicals occurs in rapidly dividing or susceptible cells (Mercer *et al.*, 2007). This then results in mitochondrial membrane depolarization leading to induction of apoptosis by the chemical stress pathway and the activation of caspase-3 and caspase-7 in HL-60 cells resulting in degraded DNA or hypodiploidy. Li *et al.* also showed that artemisinin derivatives induce apoptosis mainly in G1 phase of cell cycle. G1 phase has been associated with the increased iron intake and transferin receptor expression. Down regulation of anti-apoptotic bel-2 proteins and up regulation of pro-apoptotic bax proteins have been associated with the artesunate-treated human vein endothelial cells (Li *et al.*, 2001). Artemisinins have also been associated with lowered vascular endothelial growth factor (VEGF) expression. VEGF are potent angiogenic factors (Wu *et al.*, 2004). These studies suggest that the mechanism(s) for the cyto-toxicicty of artemisinins involves many different pathways.

In comparison, cisplatin (**5.7**) (cis-diamminedichloroplatinum, II), a platinum-based drug used in the treatment of a range of solid tumours exerts its cytotoxic effect through multiple mechanisms of which the most important and the better understood mode of action involves interaction with DNA to form DNA lesions, leading to activation of several signal transduction pathways and culminating in the induction of mitochondrial apoptosis (Siddik, 2003). Consistent rates of initial responses have been obtained by cisplatin treatment. However, this often results in the development of chemo-resistance and therapeutic failure (Galluzzi *et al.*, 2011). Combination of cisplatin with a chemosensitizer or a synergist can potentially improve efficacy and restore sensitivity to cisplatin (Chirnomas *et al.*, 2006).

Artemisinin and its derivatives have also been used as chemosensitizers to conventional treatments in drug resistant cancer cell lines (Reungpatthanaphong and Mankhetkorn, 2002, Liu *et al.*, 2011). Synergistic interaction of dihydroartemisinin with gemcitabine, a cancer drug, showed a 45% enhancement of tumour growth inhibition compared with the drug alone (Wang *et al.*, 2010b). The improved efficacy of multi-component combinations involving artemisinin in cancer treatment has encouraged some researchers to look at other compounds in the plant besides artemisinin that may exhibit cytotoxic activities and potential artemisinin synergists in the crude extract. Two artemisinin related compounds, artemisitene (**5.2**) and arteannuin B (**5.4**), and two unrelated ones, scopoletin and 1,8-cineole, showed anti-proliferative activities (Efferth *et al.*, 2011). No cross-resistance to artemisinin with any of these actives was observed, thus showing a potential for use in combination to treat drug resistant tumours.

Cabonara *et al.* observed that chorogenic acids (**5.11-5.17**) are major constituents of the *Artemisia* tea they analysed (Carbonara *et al.*, 2012). They also detected a number of feruloyl-quinic acids together with some flavonoids in the extract. Chlorogenic or caffeoylquinic acids (CQAs) are esters of caffeic (**5.10**) and quinic acids. The pharmacological properties of these catechols includes antioxidant, hepato-protectant, antibacterial, anti-histaminic, chemo-preventive and other biological effects (Belkaid *et al.*, 2006, Zhang *et al.*, 2008, Feng *et al.*, 2005, Miketova *et al.*, 1999).

Lee and Zhu have shown that chlorogenic acids and other catechols-containing dietary polyphenols can inhibit *in vitro* the methylation of synthetic DNA substrates and in human breast cancer cells inhibit the methylation of the promoter region of the RAβ gene which are normally hyper-methylated in neoplastic cells (Lee and Zhu, 2006, Sirchia *et al.*, 2000) In their study, Noratto *et al.* showed the chemo-preventive potential of dietary chlorogenic and neo-chlorogenic acids (Noratto *et al.*, 2009). These compounds exerted a relatively high growth inhibition on the estrogen-independent breast cancer cell line and low toxicity in the normal cells. Chlorogenic acid derivatives were also found to inhibit hepatocellular carcinoma cell line proliferation and induced apoptosis in leukemia cell lines (Jin *et al.*, 2005, Bandyopadhyay *et al.*, 2004).

Combinations of caffeic and chlorogenic acid with chemotherapeutic agents as chemo-sensitisers have been reported. An increased sensitivity of multidrug-resistant breast cancer cells (MCF-7/Dox) to doxorubicin was observed with caffeic acid (Ahn *et al.*, 1997). A US patent for the use of chlorogenic acid as sensitizers for

chemotherapeutic agents, reported a 30 % reduction in the viability of cancer cells sensitized by chlorogenic acids to doxorubicin compared with cells administered with doxorubicin alone (Kim, 2010).

This study therefore attempts to evaluate the *in vitro* cytotoxicity on breast cancer cells of artemisinin in combination with co-metabolites in *Artemisia* tea extract. It specifically looks at the interaction of artemisinin with chlorogenic acid (3 caffeoylquinic acid), a major metabolite in *Artemisia* tea and compares this with cisplatin's interaction with the acid in other to better understand the cyto-toxic mechanism of action of artemisinin and possible implications for the use of *Artemisia* tea in cancer therapies.

5.2 Result and discussion

5.2.1 Composition of Artemisia tea

The profile of metabolites in the aqueous extract and their quantities in milligrams per litre of extract is shown in Table 5.1. These were analysed by both MS/MS and HPLC methods. The profiling is based on the earlier *in extenso* analysis by Carbonara *et al.* who showed these compounds to be the major metabolites (quantitatively) in *Artemisia* tea infusions (Carbonara *et al.*, 2012). Based on the earlier work (Suberu *et al.*, 2013a), several artemisinin-related compounds were also analysed in the extracts.

The levels of artemisinin reported in tea extract are varied and the values obtained in this study (47.5 mg L^{-1}) are within the range (Van der Kooy and Verpoorte, 2011, Rath *et al.*, 2004, Carbonara *et al.*, 2012, De Magalhaes *et al.*, 2012, Wright *et al.*, 2010). Quantitative differences could be due to variation in biomass and tea

preparation methods especially biomass-to-solvent ratio used. Van der Kooy and Verpoorte (Van der Kooy and Verpoorte, 2011) have shown that the method employed in preparing the hot water infusion does affect the amount of artemisinin and other co-metabolites extracted. This study, as well as others (Van der Kooy and Verpoorte, 2011, Rath *et al.*, 2004), employed the therapeutically recommended ratio of 200:1, v/w or 5 g L^{-1}, (Willcox, 2009).

Table 5-1. Metabolites in the aqueous *Artemisia* extract analysed by both MS/MS and HPLC methods quantified as milligrams per litre of tea.

Compound	Amount (mg L^{-1} of tea)
Artemisinin	47.5±0.8
Arteannuin B	1.3±0.0
Dihydroartemisinic acid	70.0±0.3
Caffeic acid	0.8±0.00
3,5-Di-caffeoylquinic acid	57.0±1.7
3-Caffeoylquinic acid	72.0±1.6
4-Caffeoylquinic acid	20.4±1.6
4,5-Di-caffeoylquinic acid	31.6±4.0
5-Caffeoylquinic acid	9.0±0.7
Isovitexin	105.0±7.2
Rosmarinic acid	1.1±0.0

Dihydroartemisinic acid (**5.4**) (70 mg L^{-1}) and arteannuin B (**5.5**) (1.3 mg L^{-1}) are the only biosynthetic precursors of artemisinin detected in the tea extract using our method (Suberu *et al.*, 2013a).

The most abundant of the caffeic derivatives (**5.11-5.17**) was 3-caffeoylquinic acid (**5.11**) (72 mg L^{-1}) in the analysed extract, followed by 3,5-di-caffeoylquinic acid

(**5.15**) (57 mg L^{-1}). A comparatively lower profile (0.8 mg L^{-1}) was observed for caffeic acid (**5.10**). The only flavonoid analysed was isovitexin (**5.8**) (105 mg L^{-1}) and was relatively abundant in our extract. Rosmarinic acid (**5.9**) was lower (1.1 mg L^{-1}) in our samples compared to the levels found by De Magalhaes *et al.* (De Magalhaes *et al.*, 2012).

5.2.2 Cytotoxicity of cisplatin, artemisinin, and 3-caffeoylquinic acid (3CA)

Table 5.2 shows the 50% inhibitory concentration (IC_{50}) for artemisinin, cisplatin and 3CA in MCF7 breast cancer cells. This cell line is derived from breast adenocarcinoma tissues and are a common model employed in carcinogenesis and chemo-preventive studies (Paluszczak *et al.*, 2010).

Table 5-2. IC_{50} values for artemisinin, cisplatin and 3CA in MCF7 cells.

Compounds	Average IC_{50} (μM)
Artemisinin	38.44±0.41
Cisplatin	4.44±0.07
3-Caffeoylquinic acid (3CA)	154.27±0.82

5.2.3 Cytotoxicity of artemisinin

The cytotoxicity of artemisinin (Table 5.2 and Figure 5.1) in the MCF7 cells shows its potency against invasive breast ductal carcinoma that is oestrogen sensitive. The IC_{50} values obtained for the compound (38.44±0.41 μM) are within range of values (IC_{50}; 0.17 – 87.10 μM) reported by Efferth and Oesch for artemisinin and its derivatives determined for the tumour panel of 60 cell lines in the National Cancer Institute (NCI) screening programme (Efferth and Oesch, 2004). Artemisinin had the highest IC_{50} (least potent) of all the related derivatives reported (Efferth and Oesch, 2004).

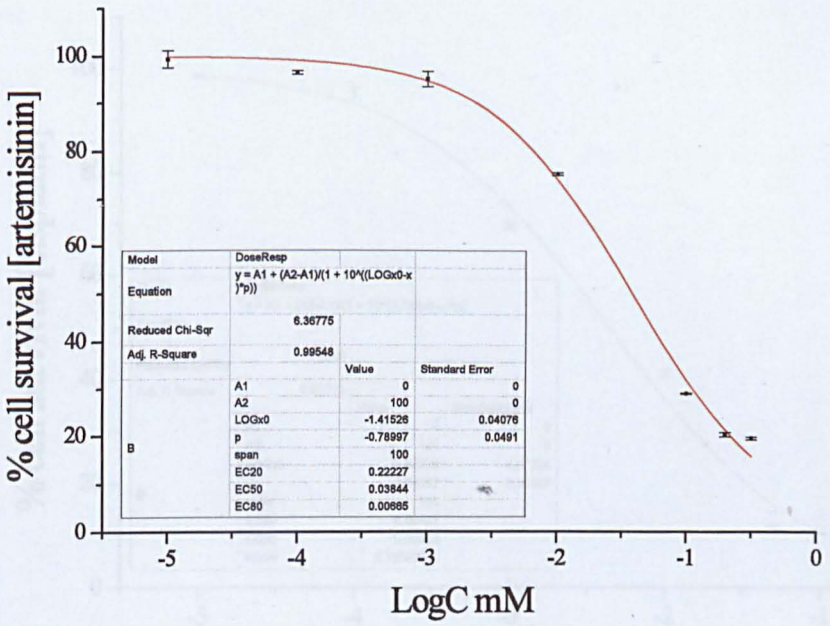


Figure 5-1. Dose response curve for artemisinin in MCF7 cells calculated based on percentage cell survival in the presence of graded concentration of the compound. Each data point was derived from duplicate determination of a triplicate measurement.

5.2.4 Activity of cisplatin

Cisplatin showed superior cytotoxicity in MCF7 cells compared to artemisinin, see Table 5.2 and Figure 5.2. The mean IC_{50} value obtained here (4.4 μM) is similar to values reported by Isikdag *et al.* (IC_{50} 8.6 μM) using the similar MCF7 cells and the same length of drug exposure (Isikdag *et al.*, 2011). Although cisplatin is very effective with solid type carcinoma, drug resistance and toxic side effects have also been reported (Tegze *et al.*, 2012, Florea and Büsselberg, 2011).

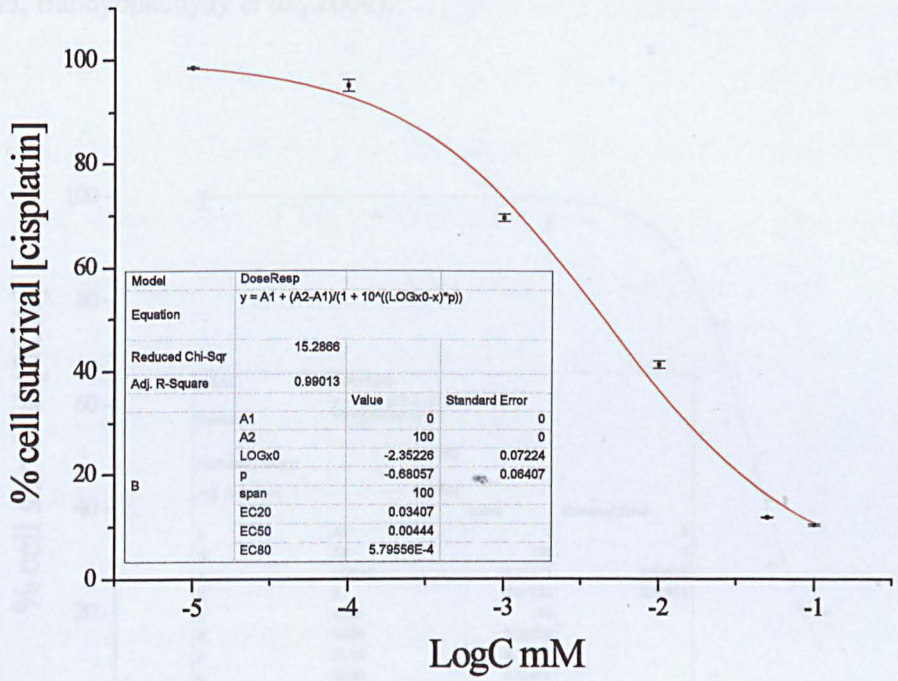


Figure 5-2. IC_{50} curve for cisplatin in MCF7 cells calculated based on percentage cell survival in the presence of graded concentration of the compound. Each data point was derived from duplicate determination of a triplicate measurement.

5.2.5 Cytotoxicity of 3-caffeoylquinic acid (3CA)

The IC_{50} (154.27 ± 0.82 μM) in MCF-7 cells for 3CA was highest among the three single agents tested (Figure 5.3). The relatively higher IC_{50} shows that 3CA's cytotoxicity occurs at relatively higher concentration. This is similar to the observation by Lee *et al.* who reported that the growth inhibition of MCF-7 cells by 3CA was insignificant up to 20 μM and only inhibited by about 15% at 50 μM concentration (Lee and Zhu, 2006). Therefore a 50% growth inhibition at about 150 μM concentration which we obtained is in the range of the reported values. The chemo-preventive and anti-proliferation effects of 3-caffeoylquinic acid along with

other dietary derivates have also been reported by others (Noratto *et al.*, 2009, Jin *et al.*, 2005, Bandyopadhyay *et al.*, 2004).

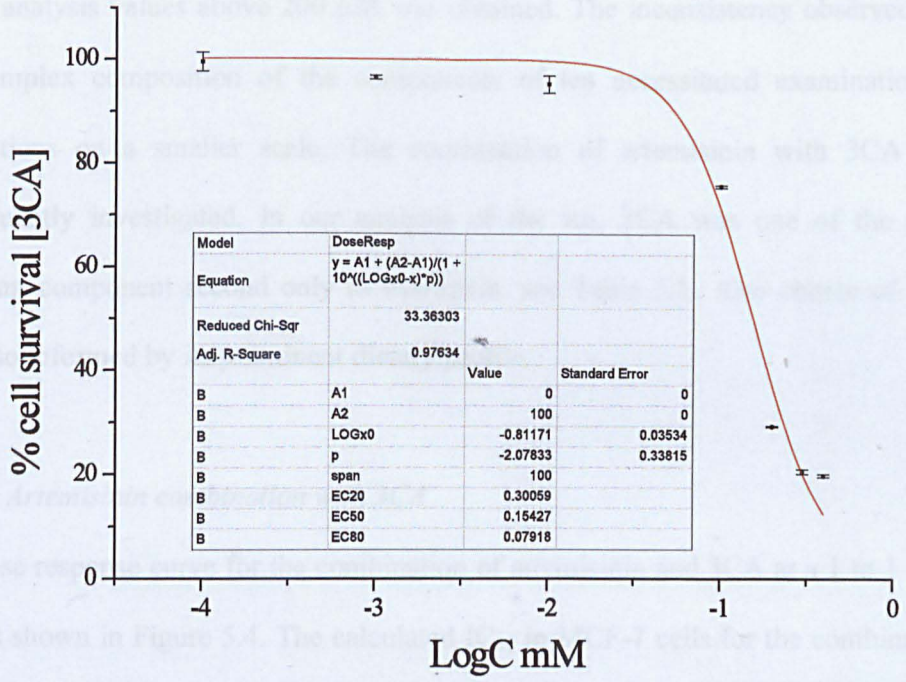


Figure 5-3. IC50 curve for 3-Caffeoylquinic acid. Data points are means of duplicate determination of triplicate measurement.

5.2.6 Cytotoxic combination studies

The cytotoxicity of *Artemisia* tea was investigated to assess its possible role in cancer therapy. Consequently the combinations of artemisinin and 3CA, a major metabolite in tea was carried out to elucidate and explain observations from the tea test. The resulting drastic modification of artemisinin's activity with 3CA, led to the investigation of the interaction of 3CA with cisplatin to see if the same effect is reproduced in another anti-cancer drug in the hope of possible elucidation of the mechanism of the interactions observed.

5.2.6.1 Cytotoxicity of Artemisia tea

The IC_{50} values obtained for *Artemisia* tea were not reproducible. In one result, the IC_{50}, was similar to that of pure artemisinin, see Table 5.2. However in a second repeat analysis values above 200 μM was obtained. The inconsistency observed and the complex composition of the components of tea necessitated examination of interactions on a smaller scale. The combination of artemisinin with 3CA was subsequently investigated. In our analysis of the tea, 3CA was one of the most abundant component second only to isovitexin, see Table 5.1. Our choice of 3CA was also informed by its prominent dietary profile.

5.2.6.2 Artemisinin combination with 3CA

The dose response curve for the combination of artemisinin and 3CA at a 1 to 1 ratio (v/v) is shown in Figure 5.4. The calculated IC_{50} in MCF-7 cells for the combination is 255.67 mM. This is about 6,500-fold deterioration in artemisinin activity and represents a complete loss of cytotoxicity for the compound in the presence of 3CA, Table 5.2. A similar loss of activity was observed in combinations involving lower 3CA concentrations with artemisinin (art to 3CA, 1 to 0.5 and 1 to 0.01). 3-Caffeoylquinic acid also lost its mild cytotoxic activity in the presence of artemisinin, Table 5.2. This suggests an antagonistic interaction between artemisinin and 3CA when equally combined (v/v) and may partly explain the high IC_{50} value (Section 5.2.6.1) observed for tea in one of the tests.

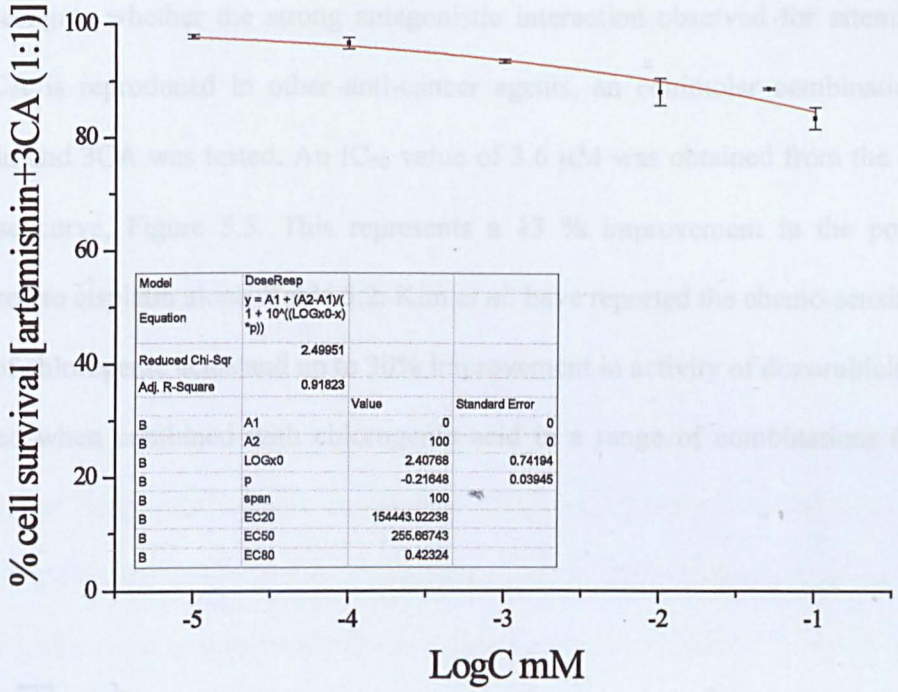


Figure 5-4. Dose-response curve for artemisinin and 3-caffeoylquinic acid. Mean values of duplicate determinations of triplicate measurements are plotted.

The cytotoxicity of 3CA like many other anti-oxidant compounds is dose (concentration) dependent and observable only above certain concentration (Lee and Zhu, 2006). Controversial and conflicting experimental results have been observed (Yordi *et al.*, 2012, Nemeikaitė-Čėnienė *et al.*, 2005) in trials involving endogenous anti-oxidants such as 3CA because of their "double edge sword" effect at cellular redox sites. Depending on the dosage level and the *in situ* matrix, these compounds can either be pro-oxidative or anti-oxidative. As a result, factors like the batch variation in crude extracts along with the dose and matrix dependent activity of the compounds such as 3CA could partly explain the conflicting and irreproducible result observed for *Artemisia* tea, see Section 5.2.6.1.

5.2.6.3 Cisplatin combination with 3CA

To investigate whether the strong antagonistic interaction observed for artemisinin and 3CA is reproduced in other anti-cancer agents, an equimolar combination of cisplatin and 3CA was tested. An IC_{50} value of 3.6 μM was obtained from the dose-response curve, Figure 5.5. This represents a 13 % improvement in the potency compared to cisplatin alone, Table 5.2. Kim *et al.* have reported the chemo-sensitizing effect of chlorogenic acids and up to 30% improvement in activity of doxorubicin was observed when combined with chlorogenic acid in a range of combinations (Kim, 2010).

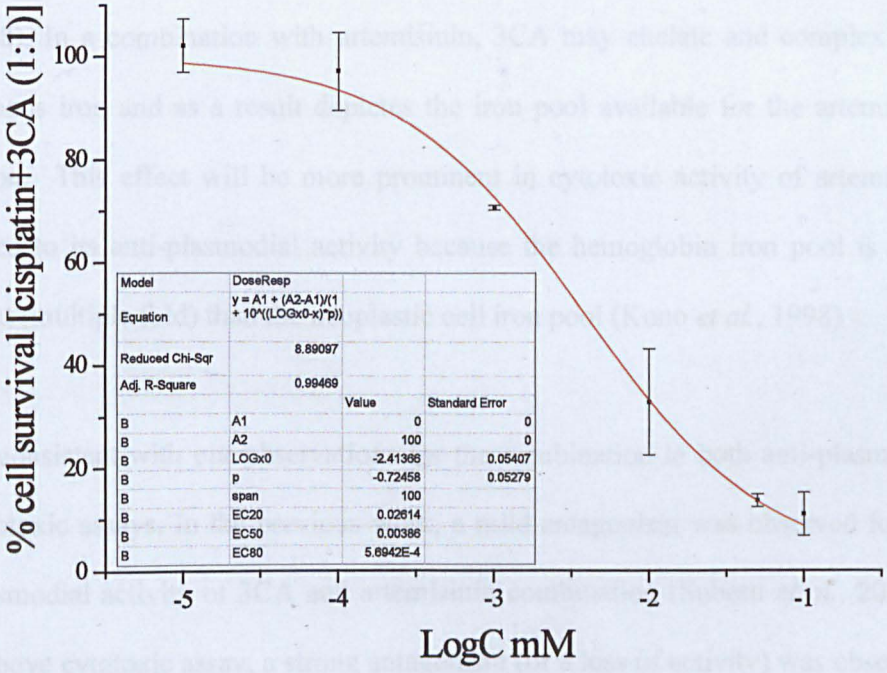


Figure 5-5. Dose-response curve for equimolar combination of cisplatin and 3CA. Plotted values are means of duplicated measurements.

The mechanism for the interaction of 3CA in MCF7 cells with artemisinin (antagonism) and cisplatin (potentiation) seem to be pharmacokinetic in nature. The

observed effect of the combination in each case seem to be a result of 3CA modification of the activities of cisplatin and artemisinin and less likely due to pharmacodynamic effect where the intrinsic cytotoxicity of 3CA is a major factor in the interaction (Williamson, 2011).

The activation of artemisinin and the cleavage of the endoperoxide bridge to form carbon centered radical and/or reactive oxygen species (ROS) is a key to the compound's cytotocity and anti-plasmodial activities. This activation has been suggested to be initiated by endogenous iron which is relatively abundant in actively dividing cells compared to normal cells (Efferth *et al.*, 2004). Efferth *et al.* reported that 3CA has iron chelating properties and forms a complex with the metal (Kono *et al.*, 1998). In a combination with artemisinin, 3CA may chelate and complex with endogenous iron and as a result depletes the iron pool available for the artemisinin activation. This effect will be more prominent in cytotoxic activity of artemisinin compared to its anti-plasmodial activity because the hemoglobin iron pool is more abundant (multiple fold) than the neoplastic cell iron pool (Kono *et al.*, 1998)

This is consistent with our observations for the combination in both anti-plasmodial and cytotoxic assays. In the previous work, a mild antagonism was observed for the anti-plasmodial activity of 3CA and artemisinin combination (Suberu *et al.*, 2013b). In the above cytotoxic assay, a strong antagonism (or a loss of activity) was observed, see Figure 5.4. An activation site for the anti-malaria activity of artemisinin is in the parasites' food vacuole, which contains ingested hemoglobin. Activation of artemisinin for cytotoxicity is suggested to take place in neoplastic or cancer cells (Kono *et al.*, 1998).

In contrast, cisplatin is activated in the cell by aquation of the molecule resulting in the loss of one or both of its chloride ions. The activation is enhanced by a lower endo-cellular chloride ion concentration compared to extracellular concentration of the ion (Florea and Büsselberg, 2011). Metal ions does not seem to play any role in cisplatin activation and thus unaffected by metal chelating properties of 3CA.

5.3 Conclusions

This study investigated *in vitro* the use of *Artemisia* tea as a chemotherapeutic agent using MCF-7 cells. The erratic and high IC_{50} observed for the tea extract, led to the investigation of the combinations of 3-caffeoylquinic acid (3CA), a major component of tea, with artemisinin, the main active ingredient in the extract. The combination showed a near total loss (strong antagonism) of cytotoxicity. This was in contrast to a 13% improvement observed when 3CA was combined with cisplatin, another anti-cancer agent. A mechanistic explanation was suggested for these observations and also a possible reason was advanced for the difference in anti-plasmodial and cytotoxicity of 3CA combination with artemisinin *via* endogenous iron-mediated activation of artemisinin molecule.

Based on these results, the use of *Artemisia* tea in cancer therapeutics seem at best unpredictable and at worst ineffective. Further *in vivo* and *in vitro* investigation of the interactions between artemisinin with 3CA and other dietary antioxidants is imperative to any recommendation for the use of artemisinin and it derivatives as anti-proliferative drugs with the possible avoidance of anti-oxidant food and drink immediately before and after intake of the drug in single or combination therapies.

CHAPTER 6. THE EFFECT OF CO-METABOLITES ON THE CRYSTALLISATION AND PURIFICATION OF ARTEMISININ

6.1 Introduction

The isolation of the anti-malaria agent artemisinin from the Chinese medicinal plant *Qinghao* (*Artemisia annua. L*) in the 1970s provided a new class of anti-malaria drugs effective against chloroquine resistant plasmodium parasites. Artemisinin is a sesquiterpene lactone with a unique endo-peroxide bridge, which is key to its bioactivity (Qu *et al.*, 2010, O'Neill *et al.*, 2010b, Meshnick *et al.*, 1996, Haynes and Krishna, 2004, Meshnick, 2002, Webster and Lehnert, 1994). In 2002, the World Health Organization (WHO) recommended the use of artemisinin in a combination therapy (ACT) as a first-line treatment for uncomplicated malaria (Qu *et al.*, 2010, Shretta and Yadav, 2012). Since then, the global delivery of ACT treatment courses to the public and private sectors has been increasing and rose sharply from 11 million in 2005 to 278 million doses in 2011 (WHO, 2012). This trend is projected to continue against the challenge of demand and supply imbalances resulting from widespread fluctuations in price and global ACT shortages in some cases (Shretta and Yadav, 2012).

In 1987, Arvey *et al.* discovered the total synthesis of artemisinin (Avery *et al.*, 1987) and seven years ago, researchers at the University of California, Berkeley, USA, successfully inserted the engineered artemisinin metabolic pathway into microbes to produce artemisinic acid, the biosynthetic precursor to artemisinin (Withers and Keasling, 2007, Ro *et al.*, 2006a). A semi-synthetic complement for the conversion of artemisinic acid to artemisinin was also recently discovered by Levesque and

Seeberger (Levesque and Seeberger, 2012). The announcement in 2012 by Sanofi of the first industrial-scale bio-engineered production of artemisinic acid that could be converted to over 40 million treatment courses via the Berkeley process will help to ease shortages but also could add to the problem of fluctuation in price for the growers of the plant (Peplow, 2013). Presently the bulk of artemisinin used in ACT and other treatments is of plant origin. East Asia (mainly China and Vietnam) cultivated approximately 80% of the global output in 2012 while 20% came from East Africa and Madagascar, Figure 6.1(Cutler, 2011).

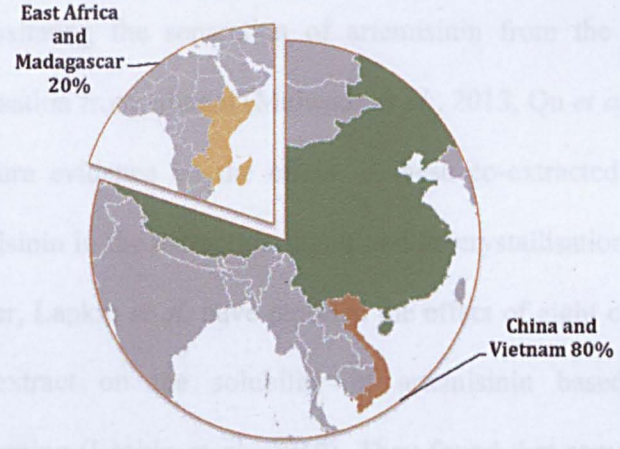


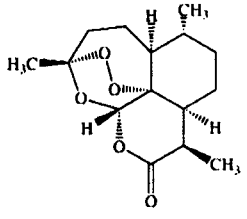
Figure 6-1 Global distribution of *A. annua* production for artemisinin extraction (Shretta and Yadav, 2012, Cutler, 2011).

This is an increase over the previous year for Madagascar and East Africa, when it contributed just only 9% or 15 metric tonnes to the global output (A2S2, 2011). This trend is significant in the light of the reported problem of variable recovery rates in the processing of the East African biomass into the active ingredient (Henfrey, 2013).

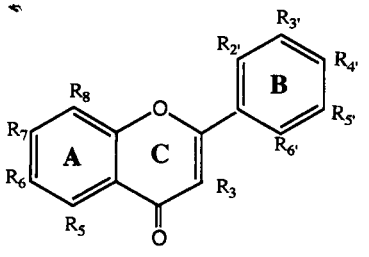
Production of artemisinin from the plant is a multi-step process starting with the drying of the plant parts, in most cases the leaves and the shoots. This is followed by extraction, which on a commercial scale involves soaking, percolation or continuous flow in warm (30 - 40 °C) organic solvents of low polarity like hexane, toluene, petroleum ether, etc (ElSohly *et al.*, 1990, Lapkin *et al.*, 2006, Haynes, 2006). To improve solubility of artemisinin in the extractant, a modifier like ethyl acetate is sometimes used. Up to three to four extraction cycles may be employed in the batch process and each cycle takes between 10 - 48 hours (Lapkin *et al.*, 2006). Simultaneously, extraction of essential oils, flavonoids, waxes and pigments occurs in the process, necessitating the separation of artemisinin from the raw extract by sequential crystallisation from ethanol (Malwade *et al.*, 2013, Qu *et al.*, 2009). To our knowledge, literature evidence on the effect of these co-extracted metabolites on solubility of artemisinin in the extraction liquor and its crystallisation and processing is lacking. However, Lapkin *et al.* have reported the effect of eight co-metabolites in *Artemisia* plant extract on the solubility of artemisinin based on *ab initio* computational screening (Lapkin *et al.*, 2010). They found that some co-metabolites increased the solubility of artemisinin in the extraction by up to 7.5%. At a given concentration, casticin and its glycosylated form had the greatest impact on the solubility of artemisinin among the metabolites tested. These data complement the anecdotal evidence from commercial extractors that recovery of artemisinin is strongly dependent on the 'quality of the plant', the latter being a technically undetermined characteristic.

Casticin is one of a group of o-methylated flavonoids found in *A. annua* (Baeva *et al.*, 1988). There are over 17-reported methoxylated flavonoids found in the plant (Shilin

et al., 1989, Bhakuni *et al.*, 2001, Ferreira *et al.*, 2010). The major ones are artemetin, casticin, chrysoplenetin, chrysosplenol-D, cirsilineol, eupatorin and retusin (Liu *et al.*, 1992). Methoxylated flavonoids are low molecular weight bioactive polyphenolics based on a C_{15} (C_6-C_3-C_6) carbon skeleton containing two aromatic rings (A and B) linked by a chroman ring (C), Scheme 6.1 (Sisa *et al.*, 2010). In plants *O*-methylated (methoxylated) flavonoids are more widely distributed than c-methylated compounds and the methoxy groups may be present on the flavone nucleus in positions 2', 3', 4', 5', 3, 5, 6, 7, and 8 (Bandyukova and Avanesov, 1971).



artemisinin



methoxylated flavonoids

Compounds	Substituent on rings									
	R_3	R_5	R_6	R_7	R_8	$R_{2'}$	$R_{3'}$	$R_{4'}$	$R_{5'}$	$R_{6'}$
Artemetin	OCH_3	OH	OCH_3	OCH_3	H	H	OCH_3	OCH_3	H	H
Casticin	OCH_3	OH	OCH_3	OCH_3	H	H	OH	OCH_3	H	H
Chrysoplenetin	OCH_3	OH	OCH_3	OCH_3	H	H	OCH_3	OH	H	H
Chrysosplenol-D	OCH_3	OH	OCH_3	OCH_3	H	H	OH	OH	H	H
Cirsilineol	H	OH	OCH_3	OCH_3	H	H	OCH_3	OH	H	H
Eupatorin	H	OH	OCH_3	OCH_3	H	H	OH	OCH_3	H	H
Retusin	OCH_3	OH	H	OCH_3	H	H	OCH_3	OCH_3	H	H

Scheme 6.1. Chemical structures of artemisinin and some methoxylated flavonoids of *A. annua* L. extract.

The maximum number of methoxy groups is seven with the molecule generally containing hydroxyl groups also. Methoxylated flavonoids are often present as *O*-glycosides or *C*-glycosides with the *O*-binding more abundant in plants (Bandyukova and Avanesov, 1971, de Rijke *et al.*, 2006). The substituent sugar (commonly arabinose, galactose, glucose or rhamnose) of the *O*-glycosides usually binds to the OH of the aglycone at position 3 or 7 while in the *C*-glycosides this is usually with the carbon of the aglycone at 6 or 8 (de Rijke *et al.*, 2006).

Bio-medical research shows that *O*-methylated flavonoids are promising cancer chemo-preventive agents in cell culture studies (Wen and Walle, 2006, Walle, 2007, Xiao *et al.*, 2009). *O*-methylated flavonoids in particular exhibited a superior anticancer activity than their corresponding hydroxylated derivatives. They are more resistant to metabolism and are better absorbed in the intestines (Bernini *et al.*, 2011, Walle, 2009). However *O*-methylation of the hydroxyl substitutions has been shown to inactivate both the antioxidant and the pro-oxidant activities of the flavonoid (Cao *et al.*, 1997). Other biological effects includes anti-inflammatory (You *et al.*, 1999) anti-viral (Conti *et al.*, 1998) and low level antibacterial (Oksuz *et al.*, 1984) activities.

In the plant, methoxylated flavonoids are considered to participate in chemical defense due to the particular structural and absorptive feature. They also participate in stress protection and as plant development regulators (Sisa *et al.*, 2010). One major role proposed for these compounds and some other flavonoids is as absorbent and as natural filters or screens for solar UV radiation. This is supported by observation that exposure to UV radiation induces higher levels of flavonoids content in plants. One

such example is the work of Cadwell which showed that Alpine plants at high altitude and tropical plants from region of intense UV radiation have higher flavonoid content than plants from other regions (Caldwell, 1971). Cuadra *et al.* (Cuadra and Harborne, 1996) obtained over a 40 % increase in the amount of UV absorbing flavonoids between control and the UV irradiated *Gnaphalium* plants.

Consequently, we suspect that the levels of flavonoids in the various *A. annua* biomass will differ according to the climatic geography of the growth location and therefore be partly or wholly responsible for the differences observed in processibility due to elevated levels of these metabolites in some plant extracts and specifically in the East African ones. The wax and pigment content in the plants have also been suggested as possible reasons for poor artemisinin crystallisation through verbal communications within the artemisinin community.

The aim of this work therefore, is to identify differences in metabolite profiles of four *A. annua* biomasses of the same genotype but grown in different parts of the world, including the problematic East African stock and attempt to isolate the possible co-metabolites of artemisinin which might exert this effect. This knowledge will allow the design of better purification technologies for the isolation of this important biomedical molecule.

6.2 Results and Discussion

6.2.1 The level of artemisinin

The level of artemisinin in the East African samples were similar to levels reported by Assured Artemisinin Supply Systems (A2S2) (A2S2, 2013), this being the highest

concentration, Table 6.1, within the group of biomass samples tested. The level of artemisinin was similar for the Australian and Argentinean biomasses. The UK biomass had the lowest content of artemisinin of the group, Table 6.1.

Table 6-1. Levels of wax, pigments and artemisinin in *A. annua* biomasses.

	Argentina	Australia	East Africa	UK
Artemisinin (% dry weight)	0.74	0.78	1.00	0.60
Wax (% dry weight)	2.16 ±0.07	1.47±0.15	2.08±0.11	1.65±0.05
Xanthophylls (μg g^{-1})	2.63±0.04	3.67±0.08	3.38±0.07	1.29±0.03
β-carotene (μg g^{-1})	4.9±0.00	150.0±1.04	30.0±0.04	4.10±0.20

6.2.2 Estimate of percentage wax

The biomass sourced from Argentina had the highest quantity (3.6 %) of precipitated waxes determined (Table 6.1), while the Australian biomass had the lowest (1.0 %) The East African and UK biomasses had 2.6 and 2.8 % waxes respectively. Based on this result, there seem to be no obvious pattern to suggest that the level of wax content in these plants is responsible for the relatively poorer crystallisation performance of the East African feedstock.

6.2.3 Levels of pigments

Suggestions from producers and processors that some of the crude extracts that process poorly for artemisinin seem to show comparatively more pigmentation, led us to undertake the analysis of the xanthophyll and carotenoid content in the samples. Table 3 shows that the Australian biomass had a marginally higher (3.67 μg g^{-1}) content of xanthophylls than the East African (3.38 μg g^{-1}) biomass. The UK biomass (1.29 μg g^{-1}) had the lowest level of the pigment and the Argentinean stock about doubled that content (2.63 μg g^{-1}). Relatively larger differences in the β-carotene

levels were observed in the group (Table 6.1), with the Australian leaves having about five times more β-carotene than the East Africa and 30 times more than the UK and the Argentina material.

6.2.4 Effect of β-carotene on artemisinin crystalisation

The effect of β-carotene was evaluated by spiking treated extracts from which pigments has been removed with graded levels of β-carotene (0, 0.018, 0.18, 1.8 and 5.4 mg) to evaluate impact on crystallisation. This represents a level of inclusion of 0 to 30 fold of the level (0.18 mg mL^{-1} extract) of the compound found in the biomass used in the experiment.

Figure 6.2 shows the amount of crystallised artemisinin and the percentage purity of the crystals obtained from each treatment. The fractions from flash chromatographic separation of *Artemisia* extract were used to spike T2 and T3. These fractions are devoid of artemisinin. The rest of the treatments apart from T1 were spiked with β-carotene reference standard. The difference in the amount of artemisinin crystallised was very small and ranged from 30.6 mg for T1, which had no β-carotene to 36.5 mg for T7 with the highest inclusion of the spike compound. This result seems to suggest that β-carotene have not negative impact on the crystallisation of artemisinin but seem rather to slightly enhance it.

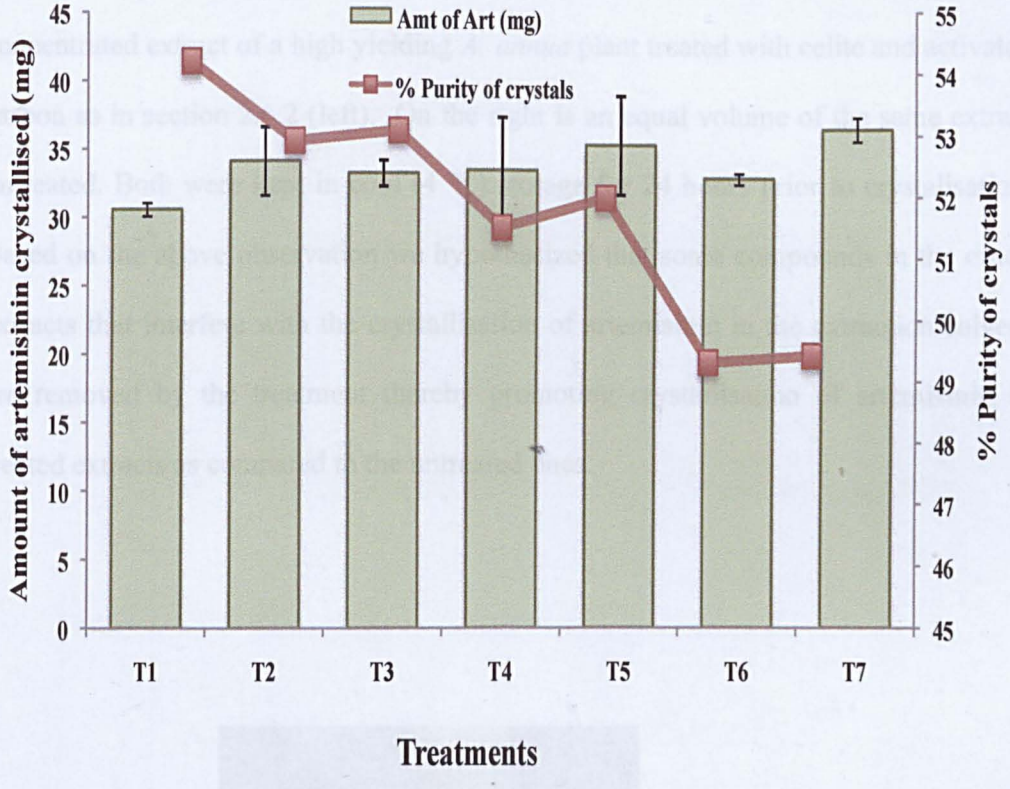


Figure 6-2. Crystallisation liquor of *Artemisia* extract spiked with various quantities of β-carotene. Treatments were spiked with 1ml of the following - T1 = hexane, T2 = fractions 1-3 from the flash chromatography of *Artemisia* extract (clear fraction containing no pigment), T3 = fractions 4-7 (the combined fractions contains an equivalent of 0.18 mg of β-carotene pigment), T4 = 0.018 mg of β-carotene standard, T5 = 0.18 β-carotene standard, T6 = 1.8 of β-carotene standard, T7 = 5.4 β-carotene standard.

However when the raw crystal (solid) were analysed before the artemisinin content was determined, the percentage purity of the harvested solids deteriorated (Figure 6.2) with the increased inclusion of β-carotene.

6.2.5 Effect of treatment with adsorbent on crystallisation

Figure 6.3 shows crystallised solids containing artemisinin obtained from the concentrated extract of a high yielding *A. annua* plant treated with celite and activated carbon as in section 2.6.2 (left). On the right is an equal volume of the same extract untreated. Both were kept in cool (4 °C) storage for 24 hours prior to crystalisation. Based on the above observation we hypothesized that some compounds in the crude extracts that interfere with the crystallisation of artemisinin in the extraction solvent are removed by the treatment thereby promoting crystallisation of artemisinin in treated extracts as compared to the untreated ones.

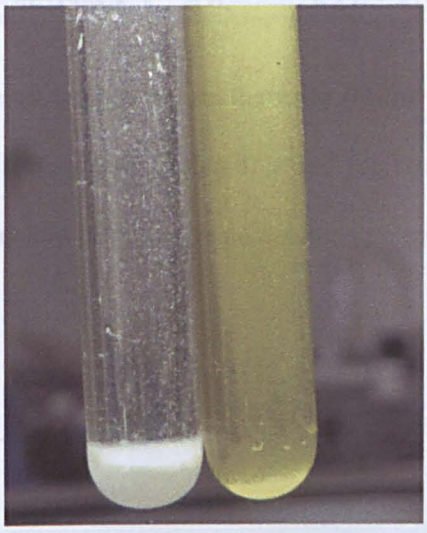


Figure 6-3. Concentrated extracts treated (left) and untreated (right) showing artemisinin crystalising out of treated extract from which metabolites have been removed.

6.2.6 The effect of treatment on metabolite profile of extract

The metabolite profiles for the treated (see section 2.6.2) and un-treated (crude) extracts were determined and the total ion count (TIC) chromatogram for both extracts showed visible differences (Figure 6-4).

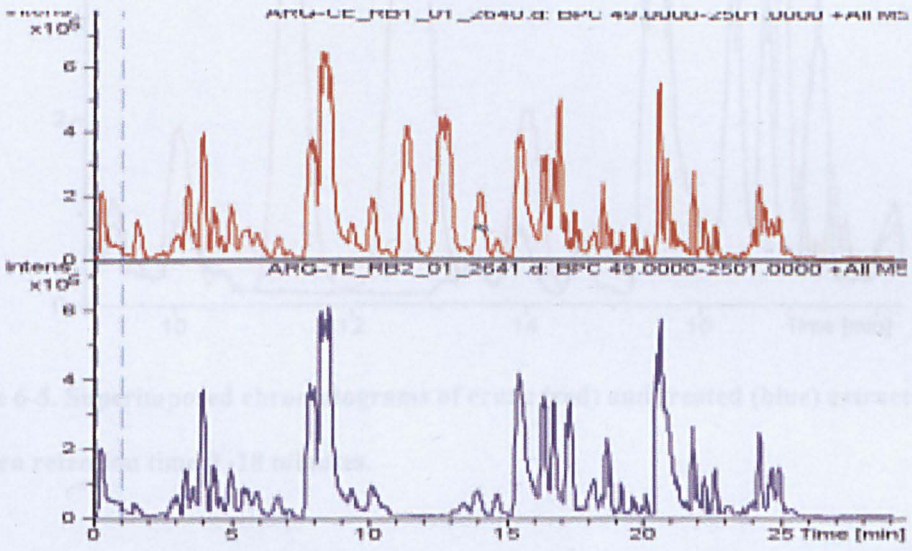


Figure 6-4 Extracted ion chromatography (EIC)of crude and treated extracts

To elucidate the difference we super-imposed the two chromatograms in Figure 6.5, to accentuate diminished or removed peaks due to treatment.

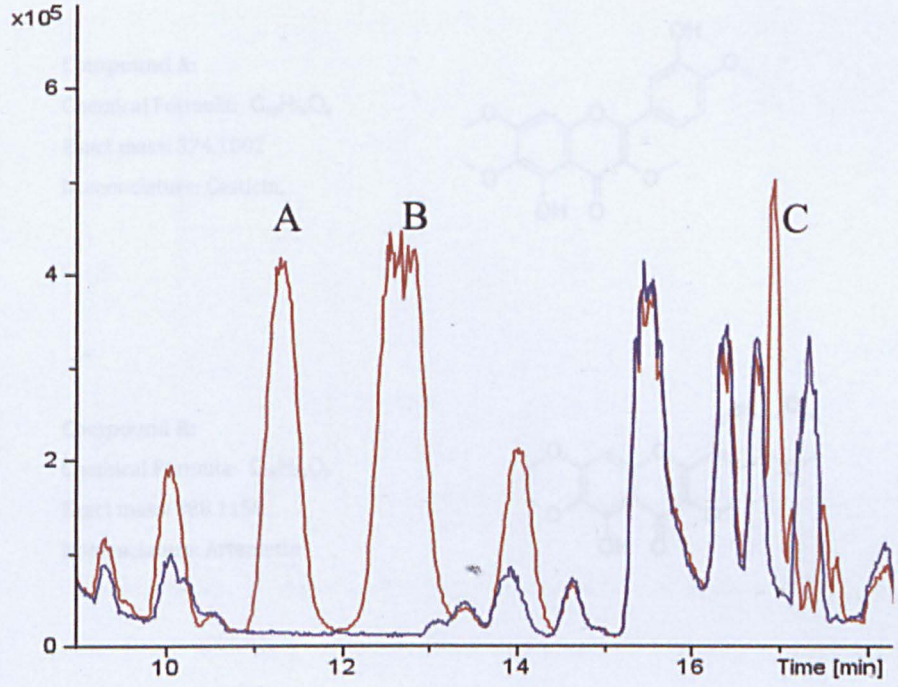


Figure 6-5. Superimposed chromatograms of crude (red) and treated (blue) extracts between retention time 9 -18 minutes.

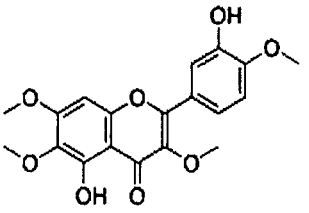
Peak A, B and C were found only in the crude extract. The high-resolution MS (HRMS) data for these peaks were determined (scheme 6.2) and coupled with literature and library searches, a positive identification for A, B and C was obtained as casticin, artemetin and retusin respectively (Scheme 6.2). These compounds are O-methylated flavonoids earlier reported in *A. annua* (Bhakuni *et al.*, 2001).

Compound A:

Chemical Formula: $C_{19}H_{18}O_8$

Exact mass: 374.1002

Nomenclature: Casticin,

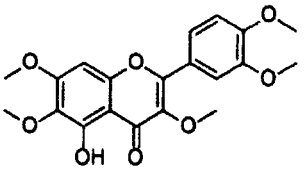


Compound B:

Chemical Formula: $C_{20}H_{20}O_8$

Exact mass: 388.1158

Nomenclature: Artemetin

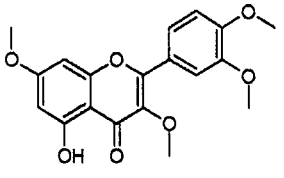


Compound C:

Chemical Formula: $C_{19}H_{18}O_7$

Exact mass: 358.1053

Nomenclature: Retusin,



Scheme 6.2. Identity of peaks A, B and C in the untreated extract.

6.2.7 Quantification of O-methylated flavonoids in extracts

Quantitative analysis of the three methoxylated flavonoids was carried out in five biomasses from different parts of the world to evaluate the effect of geography on these metabolites and to identify possible target compounds that might be responsible for the variable (poorer) crystallisation reported (Henfrey, 2013) in the East African (Kenyan) sample.

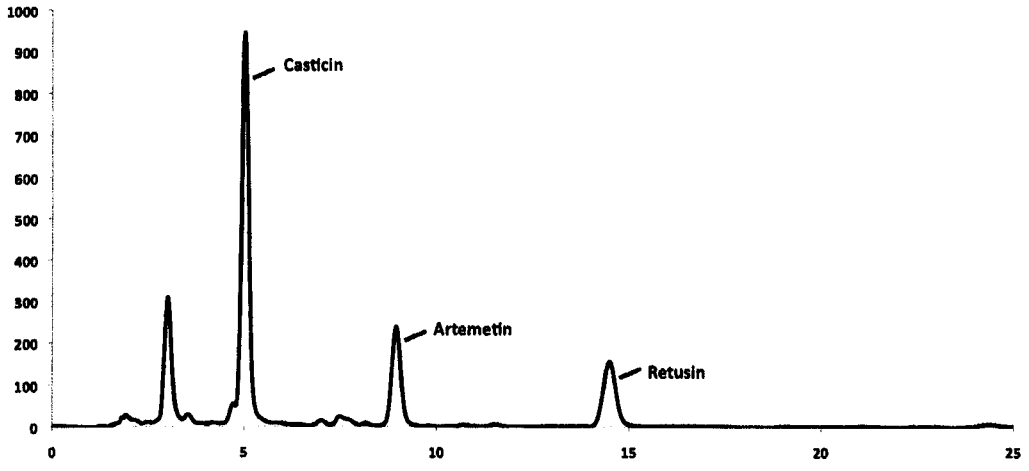


Figure 6-6. Chromatogram of the *O*-methylated flavonoids in *A. annua* crude extract.

Figure 6.5 is an HPLC chromatogram of a crude *A. annua* extract showing peaks of all flavonoids of interest while the relative quantities of the metabolites are given in Table 6.2.

Table 6-2. *O*-methylated flavonoids in *A. annua* biomasses.

Source of Biomass	Casticin mg g^{-1}	Artemetin mg g^{-1}	Retusin mg g^{-1}
Argentina	1.48 ±0.11	0.06±0.00	0.17±0.02
Australia	1.81±0.13	0.09±0.00	0.39±0.02
East Africa (Kenya)	1.45±0.08	0.22±0.01	0.49±0.02
Madagascar	1.73±0.06	0.08±0.00	0.40±0.03
United Kingdom	0.42±0.02	0.03±0.01	0.09±0.01

Mean values of triplicate determinations with ± SEM

The determined flavonoid profile for the Australian and Madagascan biomasses are very similar. This is likely to be due to their common latitudinal geography and thus receiving approximately similar intensity of the sun's ultra violet (UV) radiation. Similarly, due to its geography, United Kingdom receives comparatively lower sun

intensity and, hence, the UK-grown sample of *A. annua* has a comparatively low level of these flavonoids in the plant (Harborne and Williams, 2000). This might explain the reason for the lower values obtained for all three metabolites in the UK-grown biomass. Apart from the UK-grown biomass, the level of casticin in the plants is relatively high, up to 0.2% of dry weight, with less than 20 % difference in concentration between the different biomasses tested.

The biomass grown in Argentina had significantly lower levels of retusin compared to the other biomasses except the UK, which is consistently low. An interesting pattern was observed for artemetin levels in the biomasses. The East African (Kenyan) biomass had elevated levels (~ 60%) of the metabolite compared to any of the other biomasses. This is in contrast to the relatively smaller differences in concentration levels of casticin (~ 4%) and retusin (~ 20%) in the biomasses studied.

6.2.8 The effect of O-methylated flavonoids on artemisinin crystallisation

Figure 6.6 shows the mean from replicated treatments of crystallised artemisinin and percentage purity of crystals harvested obtained from concentrated extract of *A. annua* spiked with flavonoids at different concentrations (0, 25 and 50 μg mL^{-1}) individually and in a combination of all three. The amount of artemisinin crystallised was calculated from the percentage purity of the weighed crystallized solids.

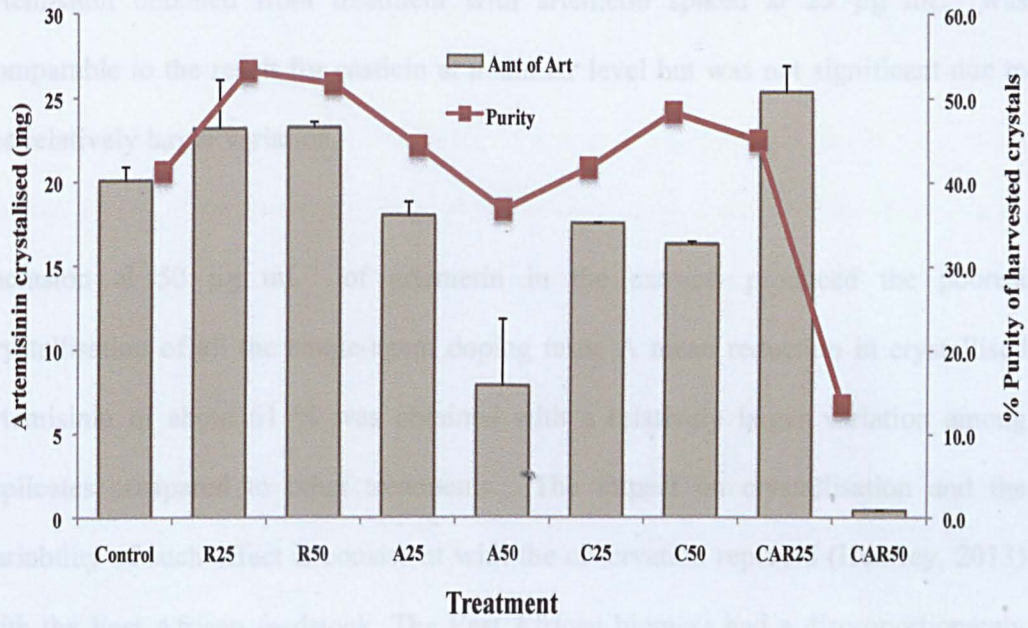


Figure 6-7. The amount of artemisinin (mg) per treatment crystallised from 10 mL of concentrated extract containing 9.5 g biomass.

The percentage purity of harvested crystals followed a pattern similar to the amount of artemisinin crystallised. Spiking with retusin at 25 and 50 μg mL^{-1} seem to have no effect on the crystallisation of artemisinin from the liquor. There was an increase in the amount of artemisinin crystallised compared to the blank un-spiked extract, however the increase was statistically insignificant ($p > 0.05$). Conversely, the extracts spiked with casticin and artemetin showed relatively poor crystallisation of artemisinin compared to control. For both flavonoids crystallisation deteriorated with increased level of spiking.

Casticin depressed (up to about 18%) the crystallisation of artemisinin from the concentrated extract, Table 6.3. The effect on crystallisation was significant ($p < 0.05$)

at both levels compared to the un-spiked samples. The mean amount of crystallised artemisinin obtained from treatment with artemetin spiked at 25 μg mL^{-1} was comparable to the result for casticin at a similar level but was not significant due to the relatively larger variation.

Inclusion at 50 μg mL^{-1} of artemetin in the extracts produced the poorest crystallisation of all the single-agent doping tests. A mean reduction in crystallised artemisinin of about 61 % was obtained with a relatively larger variation among replicates compared to other treatments. The impact on crystallisation and the variability of such effect is consistent with the observation reported (Henfrey, 2013) with the East African feedstock. The East African biomass had a disproportionately high level of artemetin of all the biomasses studied (Table 6.2).

Table 6-3. The effect of casticin, artemetin and retusin on artemisinin crystallisation.

Treatments (4 replicates per treatment)	Amount spiked (mg)	Mean % Artemisinin crystallised compared to control
Casticin		
C25	0.25	- 12.25
C50	0.50	- 18.26
Artemetin		
A25	0.25	- 10.29
A50	0.50	- 60.73
Retusin		
R25	0.25	+15.50
R50	0.50	+15.78
Combined		
CAR25	0.25 each	+ 26.55
CAR50	0.50 each	- 97.87

The combination treatment at 25 μg mL^{-1} each of the three dopants resulted in an increase (~27%) in the crystallised artemisinin. However, the effect of inclusion at 50 μg mL^{-1} of all three compounds was a near total failure (~98%) of crystallisation. This trend is difficult to interpret. However it seems the enhancing effect of retusin dominated the other two metabolites at the low level of spiking, while at the higher level of spiking the combined negative effect of artemetin and casticin reduced the crystallization significantly.

On a structural level, the number and positioning of the *O*-methylated and hydroxyl groups on the flavonoid could help explain some of our observations. Casticin has four methoxylated groups at C 3, 6, 7 and 4' with 2 hydroxyl groups at C 3' and 5 Scheme 2. Artemetin has methoxylated groups as in casticin with one extra at C5' and only one hydroxyl group at C5. Retusin is similar to artemetin but has no methoxylated group at C6 (Schemes 6.1 and 6.2).

The experimental results also seem to suggest that increasing methoxylation of the substituent on the flavonoid skeleton is associated with reduction in the amount of crystalised artemisinin from the mother liquor. An extra -OH group of casticin over retusin lead to a maginal reduction in the amount of artemisinin crystallised. However from these results, methoxylated groups seem to do this better than hydroxyl group as in artemetin compared to casticin. The mechanism(s) driving crystallisation is a complexity of physico-chemical processes (Song and Cölfen, 2011, Gou *et al.*, 2012). There is literature evidence (Lapkin *et al.*, 2010, Elford *et al.*, 1987) for the effect of flavonoids on the solubility of artemisinin. To investigate the effect of casticin, artemetin and retusin on artemisinin's solubility, we employed a predictive

computational approach using a conductor-like screening model for realistic solvation (COSMO-RS) to compare with the experimental results.

6.2.9 Predictive computational analysis using COSMO-RS

The temperature and energy values for the compounds obtained from calculations in section 2.6.8 are given in Table 6.4. Additionally, the free energy change of melting approximated by the QSPR is given for comparison.

Table 6-4. Thermodynamic data used for solubility calculations

Substance	Melting Temperature (K)	ΔH_{fus} (kJ mol^{-1})	ΔG_{fus}^{QSPR} (kJ mol^{-1})	ΔG_{fus} (kJ mol^{-1})
Artemisinin	-	-	7.16	11.83
Artemetin	-	-	5.30	5.30
Casticin	458.15	41.32	6.73	11.81
Retusin	430.15	44.56	5.34	11.65

Table 6.5 shows the predicted effect of methoxylated flavonoids on the solubility of artemisinin at 293 K in a solvent mixture of 5% ethyl acetate in n-hexane. Based on this analysis, casticin had the most impact (2.7%) on the solubility of artemisinin in the solvent mixture while artemetin had the least impact (1.1%). The combination of all three flavonoids had about 5.2%, corresponding approximately to the sum of their individual effects.

Table 6-5. The effect of co-metabolites on the solubility of artemisinin in n-hexane-ethyl acetate solvent mixture at 293 K.

Co-metabolite	Artemisinin solubility at 293K (mol fraction $x10^{-3}$)	Increase in artemisinin solubility (%)
Artemisinin	1.2145	
Artemetin	1.2277	1.1
Casticin	1.2471	2.7
Retusin	1.2333	1.6
Combination (CAR)	-	5.2

The predicted increases in solubility of artemisinin in the presence of these flavonoids by COSMO-RS were marginal compared to the experimental results in Table 6.3. This may suggest that mechanisms other than the influence on solubility of artemisinin are responsible for how these flavonoids impact the crystallisation process of artemisinin from the mother liquor containing these metabolites. Other factors that may influence crystalisation have been suggested by others (Gou *et al.*, 2012, Song and Cölfen, 2011).

6.3 Conclusions

In this study we identified co-metabolites in *A. annua* extract that potentially impact the crystallisation of artemisinin from the mother liquor. Comparison of the spectra of treated extracts that readily crystallises artemisinin with untreated extracts, which do not readily crystallize artemisinin, identified methoxylated flavonoids, casticin, artemetin and retusin as potential causes. Analysis of biomass from Argentina, Australia, East Africa, Madagascar and United Kingdom showed the East African stock had a higher level (about 60%) of artemetin compared to other biomasses.

Concentrated treated *A. annua* doped with the three *O*-methylated flavonoids at 0, 25, 50 μg mL^{-1} singularly and a combination of all three was used to investigate the effect on artemisinin crystallisation. Spiking with retusin seems to have no effect on the crystallisation of artemisinin from the liquor. A non-statistically significant ($p > 0.05$) improvement was observed compared to un-spiked samples. Casticin and artemetin inclusion showed reduction in the yield of artemisinin crystallised compared to control with the efficiency of crystallisation declining with an increasing level of spiking. A reduction of up to about 18% in the yield of artemisinin crystallised was observed for the range of inclusion of casticin in the mother liquor. Inclusion of artemetin at the investigated levels in the treated extracts led to a reduction of up to 61 % compared to blank. The combination of the three methoxylated flavonoids at the higher level reduced the yield of artemisinin crystallized from the liquor by about 98 %, representing a near total failure of crystallisation. This seems to suggest that the combined effect of methoxylated flavonoids in the un-treated concentrated extract may be responsible for holding back crystallisation of artemisinin and removal by treatment readily enhances crystallisation of artemisinin as in Figure 6.3. Artemetin seem to have the greatest singular impact among the investigated flavonoids and its elevated presence in the East African stock could account for the observed difficulty with processing and purification of artemisinin from biomass (Henfrey, 2013).

The levels of waxes and pigments were also evaluated in the different biomasses but no association was observed that suggests these may interfere with artemisinin crystallisation. A computation analysis using COSMO-RS analysis to evaluate the effect on solubility of artemisinin for the inclusion of casticin, artemetin and retusin in the extraction solvent suggests only a marginal improvement (1.1 – 2.7% for single

and 5.2% for combined agents). Because of the complexity of the crystallisation process, the effect on solubility of artemisinin by these methoxylated flavonoids may play a lesser role in the mechanism (s) by which these compounds impact artemisinin crystallisation process. This marginal difference in solubility of artemisinin does not however explain the strong effect of artemetin and the combination of methoxylated flavonoids on artemisinin crystallisation.

CHAPTER 7. GENERAL CONCLUSIONS

7.1 Introduction

The imperative for the scientific investigation of polyvalent interactions in natural product extracts stems from the need for the development of new treatments for emerging diseases and the challenge of emerging resistance, whilst delivering improved efficacy and low cost benefits. This study attempts to enhance our understanding of polyvalent interactions in plant extracts and with the knowledge tools can hopefully provide design platforms for effective therapies from natural products of low environmental and cost implications. The study also looked at the effect of interactions of plant metabolites on the processing and purification of active pharmaceutical ingredients (APIs) to address industrial purification problems. Starting with a method developed for the identification and quantification of plant metabolites, the following sections summarises and brings together the main achievements of the project.

7.2 A new method for metabolites analysis developed

A quick, robust and sensitive method for the detection and analysis of plant metabolites to support the study of the interactions between metabolites was developed. In the HPLC-MS/MS-ESI MRM method, six artemisinin-related metabolites were determined in six minutes of run time. 9-Epi-artemisinin was reported for the first time in crude extracts of *A. annua.* Plant biomasses from different geographical regions were analysed for their metabolites based on the method. The satisfactory validation indices obtained, confirmed the suitability of the

developed method for a wide range of applications including high throughput (HTP) analysis.

This method has been published: Suberu J, *et al.* (2013) A rapid method for the determination of artemisinin and its biosynthetic precursors in *Artemisia annua* L. crude extracts. Journal of Pharmaceutical and Biomedical Analysis 84: 269-277. http://dx.doi.org/10.1016/j.jpba.2013.06.025

The methods of analysis developed in this study was used to determine metabolic profiles of *Artemisia annua* grown in five different geographical regions and contributed to the paper submitted to publication: Alexei Lapkin, Eba Adou, Benhilda N. Mlambo, Smain Chemat, John Suberu Alana E. C. Collis, Andrew Clark, Guy Barker (2013) Integrating medicinal plants extraction into a high-value biorefinery: an example of *Artemisia annua* L. submitted to Comptes Rendus de Chimie.

7.3 Polyvalent interactions and the design of more potent anti-malarials

This study focused on the interactions in medicinal plants extracts and used the anti-plasmodial activities of component in *A. annua* as a model to find positive interactions and mechanisms of therapeutic value. Analysis of *Artemisia* aqueous extracts revealed a prominent profile for chlorogenic acids, flavonoids and artemisinin-related metabolites. Using the isobolographic approach, the following interactions in the extract were identified.

Antagonistic interactions occurred between artemisinin and related metabolites found in the plant having significant (IC_{50} <1μM) anti-plasmodium activity. The nature of

the interactions of artemisinin with chlorogenic acids and other metabolites with anti-oxidative properties seem to be dependent on the concentration of these anti-oxidants and was explained by the "double edge sword" principle. At lower concentration to artemisinin a general antagonistic interaction is observed. The antagonism observed for these anti-oxidants is stronger in chloroquine resistant parasite strain than for the sensitive one, which might be due in part to the anti-oxidant defence strategy suggested for resistant parasite. At a higher (greater than three fold) concentration to artemisinin, an additive or synergistic interaction develops. A 10-fold concentration above that of artemisinin produced the best synergy with 3CA for anti-malaria activity.

A synergistic interaction (of about three-fold improvement) was observed for the combination of artemisinin and arteannuin B in chloroquine resistant parasite strain. This was not reproduced in parasite strain sensitive to chloroquine, suggesting that arteannuin B potentiation of artemisinin is selectively targeted at the parasites' defence network. Based on further experimental confirmation and possible improvement, this result suggests a possible therapeutic platform for the combination of artemisinin and arteannuin B in the treatment of chloroquine and multi-drug resistant plasmodium infection.

This study has been submitted to publication. Suberu JO, Gorka AP, Jacobs L, Roepe PD, Sullivan N, Barker, G and Lapkin, A. (2013) Anti-plasmodial polyvalent interactions in *Artemisia annua* L. aqueous extract - possible synergistic and resistance mechanisms. PLOS ONE.

7.4 Interactions in tea and the implications for use in anti-cancer therapy

The cytotoxicity of artemisinin and combinations with co-metabolites was investigated and the therapeutic use of *Artemisia* tea for malignancy was also examined. The observed cytotoxicity of tea in breast MCF-7 cancer cells was contradictory and irreproducible. Initial analysis gave an IC_{50} similar to that of artemisinin, however a repeat analysis gave results of above 200 μM. The interactions in tea were investigated further on a smaller scale of two component metabolites, artemisinin and 3CA. The result showed a strong antagonistic interaction, resulting in loss of artemisinin's cytotoxicity.

The metal and iron-chelating characteristic of 3CA was proposed for the loss in cytotoxicity of artemisinin. Artemisinin is suggested to be activated by endogenous iron. This "depletion of iron" hypothesis, seem to be re-enforced by the observation that the antagonism is not reproduced in cisplatin another anti-cancer drug, activated by a route that is not dependent on cellular iron or metal pool. The strong negative interactions observed in components of tea would suggest that the extract is not suitable for use in cancer treatments, however further investigations both *in vitro* and *in vivo* are needed to establish this assertion.

This study is being drafted for publication: Suberu, J; Romero, I; Saddler, P; Sulluvan, N; Lapkin, A. and Barker, G. - Comparative cytotoxicity of artemisinin and cisplatin and their interactions with chlorogenic acids in MCF7 breast cancer cells. Suggested Journal – Journal of Medicinal Chemistry.

7.5 Application of polyvalent interactions to industrial purification problem

The knowledge of plant metabolite interactions is applicable also to extraction and purification problems. Spectra subtraction of purified extracts that crystallises artemisinin from those of untreated extract that does not, revealed the absence of some methoxylated flavonoids' peaks in the treated extract. The doping of treated *Artemisia* extract with these flavonoids confirmed the negative impact on crystallisation of some of these compounds. Artemetin had the greatest effect and doping a combination of artemetin, casticin and retusin into treated extracts caused a near total failure of crystallisation of artemisinin from the crystallisation liquor. Metabolic profile analysis of biomass from different region showed that the levels of methoxylated flavonoids in *A. annua* biomass seem geography dependent and interestingly artemetin had an elevated profile in the problematic East African biomass compared to other regions. The use of solid adsorbents to treat and remove these methoxylated flavonoids from the crude extract prior to crystallisation was employed in the investigation with success.

This study has been submitted for publication: Suberu J.O; Yamin, P; Leonhard, K; Song, L; Sullivan, L; Barker, G and Lapkin, A. (2013) The effect of *O*-methylated flavonoids and other co-metabolites on the crystallisation and purification of artemisinin, Separation and Purification Technology.

7.6 Technology transfer and industrial application of project outcomes:

The application of the developed purification strategy utilising low-cost technology method of activated carbon and celite as solid adsorbents in the purification of artemisinin raw material from the plant extract is easily transferable to developing

countries and of relative economic and environmental benefit, compared to alternative purification strategies. In fact, we believe that Botanical Extracts Ltd in Kenya is presently using the method. The present system of bulk purchased biomass at fluctuating prices from growers in developing countries being transported over long distances to extraction and purification sites in China, Europe and India is inefficient, unstable for growers and represents considerable environmental impact. The implementation of low-cost and simple purification methods, such as employed here, has many benefits for local purification of artemisinin by ensuring more stable pricing, technological leapfrogging for artemisinin grower communities and a more environmentally friendly artemisinin supply chain.

The adoption of new analytical protocols developed in this study requires significant investment in advanced instrumentation, which is only viable for well-funded laboratories and larger companies. However, the high-throughput nature of the methodology should allow central laboratories to support a large number of end-users, which justifies the use of expensive instrumentation.

7.7 Suggestions for further work

The following are suggested areas for further research.

7.7.1 Identification of other artemisinin related metabolites in A. annua extracts

The analysis of biosynthetic precursor in *A. annua* crude extract revealed the presence of other artemisinin related metabolites of prominent profile quantitatively. The identification of these metabolites will help in furthering current understanding in the biosynthesis of artemisinin especially at the final stages and these metabolites may also provide a bio-refinery platform for increased revenue from the biomass.

7.7.2 Is the production of artemisinin limited to GST alone?

In the analysis of glandless (mutant) *A. annua* biomass using the method we developed, low levels of artemisinin were detected in the sample but the finding was not conclusive as biomass might have been contaminated from source. The glandless biomass have been reported to be artemisinin-free due to the lack of glandular secreting trichomes (GST) where artemisinin is believed to be synthesized and stored (Duke *et al.*, 1994). Further research with glandless biomass for artemisinin content will help to clarify if other sites for artemisinin production in the plant exist outside the GST.

7.7.3 In vivo anti-plasmodium trials for artemisinin combination with co-metabolites

The inherent limitation of *in vitro* trial makes it a relatively poor mimic of normal environment for the study of synergistic interactions. An *in vivo trial* opens up other possible route for metabolite interactions. The investigation of artemisinin combination with co-metabolites in animal models (*P. berghei*) will help to better qualify the nature of these interactions and to delineate those interactions with possible therapeutic applications.

7.7.4 In vivo and in vitro cyto-toxic activity of artemisinin combination with other metabolites

The *in vitro* investigation of artemisinin combinations with co-metabolites was limited to 3CA alone due to time constraint. Interactions with other major metabolites like isovitexin, rosmarinic acid etc, in the extract both *in vitro* and *in vivo*

will further our understanding of cytotoxic polyvalent interactions in herbal medications and specifically help to assess the nutritional and pharmacological impact of these interactions. This will have a wider implication on drug design including increased efficacy and reduced cost.

References

A2S2 2011. A2S2 Newsletter 2, October 2011. *http://www.a2s2.org/news-and-events/a2s2-newsletter-2,-october-2011.html*.

A2S2 2013. Artemisia Data. *A2S2 homepage* *http://www.a2s2.org/*.

ABDIN, M., ISRAR, M., REHMAN, R. & JAIN, S. 2003. Artemisinin, a novel antimalarial drug: biochemical and molecular approaches for enhanced production. *Planta Medica,* 69, 289-299.

ACTON, N. & KLAYMAN, D. L. 1987. Conversion of artemisinin (qinghaosu) to iso-artemisitene and to 9-epi-artemisinin1. *Planta medica,* 53, 266.

ACTON, N. & KLAYMAN, D. L. 2007. Conversion of Artemisinin (Qinghaosu) to Iso-Artemisitene and to 9-Epi-Artemisinin1. *Planta medica,* 53, 266-268.

AGRAWAL, P. K., VISHWAKARMA, R. A., JAIN, D. C. & ROY, R. 1991. High field NMR spectroscopic studies of arteannuin B and a reappraisal of the structure of arteannuin C. *Phytochemistry,* 30, 3469-3471.

AHN, C., CHOI, W. & KONG, J. 1997. Chemosensitizing activity of caffeic acid in multidrug-resistant MCF-7/Dox human breast carcinoma cells. *Anticancer Research,* 17, 1913.

AKHILA, A., THAKUR, R. S. & POPLI, S. P. 1987. Biosynthesis of artemisinin in *Artemisia annua. Phytochemistry,* 26, 1927-1930.

ARAGÃO, G. F., CARNEIRO, L. M. V., JUNIOR, A. P. F., VIEIRA, L. C., BANDEIRA, P. N., LEMOS, T. L. G. & VIANA, G. S. D. B. 2006. A possible mechanism for anxiolytic and antidepressant effects of alpha- and beta-amyrin from *Protium heptaphyllum* (Aubl.) March. *Pharmacology Biochemistry and Behavior,* 85, 827-834.

ARSENAULT, P. R., VAIL, D., WOBBE, K. K., ERICKSON, K. & WEATHERS, P. J. 2010. Reproductive development modulates gene expression and metabolite levels with possible feedback inhibition of artemisinin in *Artemisia annua. Plant physiology,* 154, 958-968.

ASAWAMAHASAKDA, W., ITTARAT, I., PU, Y.-M., ZIFFER, H. & MESHNICK, S. R. 1994. Reaction of antimalarial endoperoxides with specific parasite proteins. *Antimicrobial agents and chemotherapy,* 38, 1854-1858.

AVERY, M. A., JENNINGS-WHITE, C. & CHONG, W. K. M. 1987. The Total synthesis of (+)-artemisinin and (+)-9-desmethyltemesinin. *Tetrahedron letters,* 28, 4629-4632.

BAEVA, R., NABIZADE, L., ZAPESOCHNAYA, G. & KARRYEV, M. 1988. Flavonoids of *Artemisia annua. Chemistry of Natural Compounds,* 24, 256-257.

BANDYOPADHYAY, G., BISWAS, T., ROY, K. C., MANDAL, S., MANDAL, C., PAL, B. C., BHATTACHARYA, S., RAKSHIT, S., BHATTACHARYA, D. K. & CHAUDHURI, U. 2004. Chlorogenic acid inhibits Bcr-Abl tyrosine kinase and triggers p38 mitogen-activated protein kinase–dependent apoptosis in chronic myelogenous leukemic cells. *Blood,* 104, 2514-2522.

BANDYUKOVA, V. & AVANESOV, Â. 1971. The structure of methoxylated flavonoids. *Chemistry of Natural Compounds,* 7, 250-253.

BART, H.-J. & PILZ, S. 2011. *Industrial Scale Natural Products Extraction,* John Wiley & Sons.

BEEZ, D., SANCHEZ, C. P., STEIN, W. D. & LANZER, M. 2011. Genetic predisposition favors the acquisition of stable artemisinin resistance in malaria parasites. *Antimicrobial agents and chemotherapy,* 55, 50-55.

BELKAID, A., CURRIE, J.-C., DESGAGNÉS, J. & ANNABI, B. 2006. The chemopreventive properties of chlorogenic acid reveal a potential new role for the microsomal glucose-6-phosphate translocase in brain tumor progression. *Cancer cell international,* 6, 7.

BENNETT, T. N., PAGUIO, M., GLIGORIJEVIC, B., SEUDIEU, C., KOSAR, A. D., DAVIDSON, E. & ROEPE, P. D. 2004. Novel, rapid, and inexpensive cell-based quantification of antimalarial drug efficacy. *Antimicrobial agents and chemotherapy,* 48, 1807-1810.

BERENBAUM, M. 1978. A method for testing for synergy with any number of agents. *Journal of Infectious Diseases,* 137, 122-130.

BERENBAUM, M. C. 1989. What is synergy? *Pharmacological Reviews,* 41, 93-141.

BERGER, T. G., DIECKMANN, D., EFFERTH, T., SCHULTZ, E. S., FUNK, J.-O., BAUR, A. & SCHULER, G. 2005. Artesunate in the treatment of metastatic uveal melanoma-first experiences. *Oncology Reports,* 14, 1599-1604.

BERNINI, R., CRISANTE, F. & GINNASI, M. C. 2011. A convenient and safe O-methylation of flavonoids with dimethyl carbonate (DMC). *Molecules,* 16, 1418-1425.

BERTEA, C., FREIJE, J., VAN DER WOUDE, H., VERSTAPPEN, F., PERK, L., MARQUEZ, V., DE KRAKER, J.-W., POSTHUMUS, M., JANSEN, B. & DE GROOT, A. 2005. Identification of intermediates and enzymes involved in the early steps of artemisinin biosynthesis in *Artemisia annua. Planta Medica,* 71, 40-47.

BHAKUNI, R., JAIN, D., SHARMA, R. & KUMAR, S. 2001. Secondary metabolites of *Artemisia annua* and their biological activity. *Curr. Sci,* 80, 35-48.

BHAKUNI, R. S., JAIN, D. C., SHARMA, R. & WRIGHT, C. 2002. *Phytochemistry of Artemisia annua and the development of artemisinin-derived antimalarial agents,* Taylor & Francis, London.

BILIA, A., MELILLO DE MALGALHAES, P., BERGONZI, M. & VINCIERI, F. 2006. Simultaneous analysis of artemisinin and flavonoids of several extracts of *Artemisia annua* L. obtained from a commercial sample and a selected cultivar. *Phytomedicine,* 13, 487-493.

BILIA, A. R., LAZARI, D., MESSORI, L., TAGLIOLI, V., TEMPERINI, C. & VINCIERI, F. F. 2002. Simple and rapid physico-chemical methods to examine action of antimalarial drugs with hemin: its application to *Artemisia annua* constituents. *Life Sciences,* 70, 769-78.

BONFIGLIO, R., KING, R. C., OLAH, T. V. & MERKLE, K. 1999. The effects of sample preparation methods on the variability of the electrospray ionization response for model drug compounds. *Rapid Communications in Mass Spectrometry,* 13, 1175-1185.

BOZDECH, Z. & GINSBURG, H. 2004. Antioxidant defense in *Plasmodium falciparum*–data mining of the transcriptome. *Malaria journal,* 3, 23.

BRAY, P. G., DEED, S., FOX, E., KALKANIDIS, M., MUNGTHIN, M., DEADY, L. W. & TILLEY, L. 2005. Primaquine synergises the activity of chloroquine against chloroquine-resistant *P. falciparum. Biochemical Pharmacology,* 70, 1158-1166.

BRIARS, R. & PANIWNYK, L. 2013. Effect of ultrasound on the extraction of artemisinin from *Artemisia annua. Industrial Crops and Products,* 42, 595-600.

BROWN, G. 1992. Two new compounds from *Artemisia annua. Journal of Natural Products,* 55, 1756-1760.

BROWN, G. D. 2010. The biosynthesis of artemisinin (Qinghaosu) and the phytochemistry of *Artemisia annua L.*(Qinghao). *Molecules,* 15, 7603-7698.

BROWN, G. D. & SY, L.-K. 2004. Synthesis of labelled dihydroartemisinic acid. *Tetrahedron,* 60, 1125-1138.

BRUCKINGHAM, J. 2000. Dictionary of natural products on CD-ROM. *New York: Champman and Hall.*

CALDWELL, M. M. 1971. Solar UV irradiation and the growth and development of higher pants. *Photophysiology,* 6, 131-177.

CAO, D., LI, H., YI, J., ZHANG, J., CHE, H., CAO, J., YANG, L., ZHU, C. & JIANG, W. 2011. Antioxidant Properties of the Mung Bean Flavonoids on Alleviating Heat Stress. *PLoS ONE,* 6, e21071.

CAO, G., SOFIC, E. & PRIOR, R. L. 1997. Antioxidant and Prooxidant Behavior of Flavonoids: Structure-Activity Relationships. *Free Radical Biology and Medicine,* 22, 749-760.

CARBONARA, T., PASCALE, R., ARGENTIERI, M. P., PAPADIA, P., FANIZZI, F. P., VILLANOVA, L. & AVATO, P. 2012. Phytochemical analysis of a herbal tea from *Artemisia annua* L. *Journal of Pharmaceutical and Biomedical Analysis,* 62, 79-86.

ĆAVAR, S., MAKSIMOVIĆ, M., VIDIC, D. & PARIĆ, A. 2012. Chemical composition and antioxidant and antimicrobial activity of essential oil of *Artemisia annua* L. from Bosnia. *Industrial Crops and Products,* 37, 479-485.

CHAN, K. L., YUEN, K. H., JINADASA, S., PEH, K. K. & TOH, W. T. 1997. A high-performance liquid chromatography analysis of plasma artemisinin using a glassy carbon electrode for reductive electrochemical detection. *Planta Medica-Natural Products and Medicinal Plant Research,* 63, 66-69.

CHANDLER, R. F., HOOPER, S. N. & ISMAIL, H. A. 1979. Antihypercholesterolemic studies with sterols: β-sitosterol and stigmasterol. *Journal of Pharmaceutical Sciences,* 68, 245-247.

CHEN, H.-H., ZHOU, H.-J., WU, G.-D. & LOU, X.-E. 2004. Inhibitory effects of artesunate on angiogenesis and on expressions of vascular endothelial growth factor and VEGF receptor KDR/flk-1. *Pharmacology,* 71, 1-9.

CHEN, J., ZHOU, Y.-B., ZHANG, X., HUANG, L., SUN, W. & WANG, J.-H. 2008. Chemical constituents of *Artemisia annua* L. *Journal of Shenyang Pharmaceutical University,* 11, 006.

CHENG, Y. Q., CHEN, H. L., FAN, L. Y., CHEN, X. G. & HU, Z. D. 2004. On-line conversion and determination of artemisinin and its kinetic parameters using orthogonal design by coupling of flow injection with capillary electrophoresis. *Analytica chimica acta,* 525, 239-245.

CHEVALLIER, A. 1996. The encyclopedia of medicinal plants. *London: Dorling Kindersley 336p. ISBN,* 751303143.

CHIKARAISHI, Y., IZUTA, H., SHIMAZAWA, M., MISHIMA, S. & HARA, H. 2010. Angiostatic effects of Brazilian green propolis and its chemical constituents. *Molecular Nutrition & Food Research,* 54, 566-575.

CHIRNOMAS, D., TANIGUCHI, T., DE LA VEGA, M., VAIDYA, A. P., VASSERMAN, M., HARTMAN, A.-R., KENNEDY, R., FOSTER, R., MAHONEY, J. & SEIDEN, M. V. 2006. Chemosensitization to cisplatin by inhibitors of the Fanconi anemia/BRCA pathway. *Molecular cancer therapeutics,* 5, 952-961.

CHRISTEN, P. & VEUTHEY, J. 2001. New trends in extraction, identification and quantification of artemisinin and its derivatives. *Current medicinal chemistry,* 8, 1827-1839.

CHUDA, Y., ONO, H., OHNISHI-KAMEYAMA, M., NAGATA, T. & TSUSHIDA, T. 1996. Structural Identification of Two Antioxidant Quinic Acid Derivatives from Garland (*Chrysanthemum coronarium* L.). *Journal of Agricultural and Food Chemistry,* 44, 2037-2039.

CONTI, C., MASTROMARINO, P., SGRO, R. & DESIDERI, N. 1998. Anti-picornavirus activity of synthetic flavon-3-yl esters. *Antiviral chemistry & chemotherapy,* 9, 511-515.

COVELLO, P. S. 2008. Making artemisinin. *Phytochemistry,* 69, 2881-2885.

COVELLO, P. S., TEOH, K. H., POLICHUK, D. R., REED, D. W. & NOWAK, G. 2007. Functional genomics and the biosynthesis of artemisinin. *Phytochemistry,* 68, 1864-1871.

CRESPO-ORTIZ, M. P. & WEI, M. Q. 2011. Antitumor activity of artemisinin and its derivatives: from a well-known antimalarial agent to a potential anticancer drug. *Journal of Biomedicine and Biotechnology,* 2012.

CSEKE, L. J., KIRAKOSYAN, A., KAUFMAN, P. B., WARBER, S., DUKE, J. A. & BRIELMANN, H. L. 2010. *Natural products from plants,* CRC Press Inc..

CUADRA, P. & HARBORNE, J. 1996. Changes in epicuticular flavonoids and photosynthetic pigments as a plant response to UV-B radiation. *Zeitschrift fur Naturforschung. C. A journal of biosciences,* 51, 671-680.

CUI, L., WANG, Z. L., MIAO, J., MIAO, M., CHANDRA, R., JIANG, H. Y., SU, X. Z. & CUI, L. W. 2012. Mechanisms of in vitro resistance to dihydroartemisinin in *Plasmodium falciparum. Molecular Microbiology,* 86, 111-128.

CUTLER, M. 2011. RBM/UNITAID/WHO Artemisinin conference 2011 Final Report. 1-19.

DAHLSTRÖM, S., FERREIRA, P. E., VEIGA, M. I., SEDIGHI, N., WIKLUND, L., MÅRTENSSON, A., FÄRNERT, A., SISOWATH, C., OSÓRIO, L. & DARBAN, H. 2009. *Plasmodium falciparum* multidrug resistance protein 1 and artemisinin-based combination therapy in Africa. *Journal of Infectious Diseases,* 200, 1456-1464.

DALRYMPLE, D. G. 2006. Artemisia, agriculture and malaria in Africa: The interplay of tradition, science and public policy. *http://www.mmv.org/sites/default/files/uploads/docs/artemisinin/2007_event/11_ArtemisiaAgricultureMalaria-Dana_Dalrymple.pdf (accessed May 1, 2013).*

DE DONNO, A., GRASSI, T., IDOLO, A., GUIDO, M., PAPADIA, P., CACCIOPPOLA, A., VILLANOVA, L., MERENDINO, A., BAGORDO, F. & FANIZZI, F. P. 2012. First-time comparison of the in vitro antimalarial activity of *Artemisia annua* herbal tea and artemisinin. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 106, 696-700.

DE MAGALHAES, P. M., DUPONT, I., HENDRICKX, A., JOLY, A., RAAS, T., DESSY, S., SERGENT, T. & SCHNEIDER, Y.-J. 2012. Anti-inflammatory effect and modulation of cytochrome P450 activities by *Artemisia annua* tea infusions in human intestinal Caco-2 cells. *Food Chemistry,* 134, 864-871.

DE RIDDER, S., VAN DER KOOY, F. & VERPOORTE, R. 2008. *Artemisia annua* as a self-reliant treatment for malaria in developing countries. *Journal of Ethnopharmacology,* 120, 302-314.

DE RIJKE, E., OUT, P., NIESSEN, W. M., ARIESE, F., GOOIJER, C. & BRINKMAN, U. A. T. 2006. Analytical separation and detection methods for flavonoids. *Journal of chromatography. A,* 1112, 31-63.

DELL'EVA, R., PFEFFER, U., VENÉ, R., ANFOSSO, L., FORLANI, A., ALBINI, A. & EFFERTH, T. 2004. Inhibition of angiogenesis in vivo and growth of Kaposi's sarcoma xenograft tumors by the anti-malarial artesunate. *Biochemical Pharmacology,* 68, 2359-2366.

DESJARDINS, R. E., CANFIELD, C., HAYNES, J. & CHULAY, J. 1979. Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique. *Antimicrobial agents and chemotherapy,* 16, 710-718.

DHINGRA, V. & LAKSHMI NARASU, M. 2001. Purification and Characterization of an Enzyme Involved in Biochemical Transformation of Arteannuin B to Artemisinin from *Artemisia annua. Biochemical and biophysical research communications,* 281, 558-561.

DILIKA, F., BREMNER, P. D. & MEYER, J. J. M. 2000. Antibacterial activity of linoleic and oleic acids isolated from *Helichrysum pedunculatum*: a plant used during circumcision rites. *Fitoterapia,* 71, 450-452.

DING, X. C., BECK, H.-P. & RASO, G. 2011. Plasmodium sensitivity to artemisinins: magic bullets hit elusive targets. *Trends in parasitology,* 27, 73-81.

DONDORP, A. M., NOSTEN, F., YI, P., DAS, D., PHYO, A. P., TARNING, J., LWIN, K. M., ARIEY, F., HANPITHAKPONG, W. & LEE, S. J. 2009. Artemisinin resistance in Plasmodium falciparum malaria. *New England Journal of Medicine,* 361, 455-467.

DONDORP, A. M., YEUNG, S., WHITE, L., NGUON, C., DAY, N. P., SOCHEAT, D. & VON SEIDLEIN, L. 2010. Artemisinin resistance: current status and scenarios for containment. *Nature Reviews Microbiology,* 8, 272-280.

DOST, K. & DAVIDSON, G. 2003. Analysis of artemisinin by a packed-column supercritical fluid chromatography-atmospheric pressure chemical ionisation mass spectrometry technique. *Analyst,* 128, 1037-1042.

DUKE, M. V., PAUL, R. N., ELSOHLY, H. N., STURTZ, G. & DUKE, S. O. 1994. Localization of artemisinin and artemisitene in foliar tissues of glanded and glandless biotypes of *Artemisia annua* L. *International Journal of Plant Sciences,* 365-372.

EASTMAN, R. T. & FIDOCK, D. A. 2009. Artemisinin-based combination therapies: a vital tool in efforts to eliminate malaria. *Nature Reviews Microbiology,* 7, 864-874.

EBADA, S. S., EDRADA, R. A., LIN, W. & PROKSCH, P. 2008. Methods for isolation, purification and structural elucidation of bioactive secondary metabolites from marine invertebrates. *Nature Protocols* 3 1820 - 1831.

EBEL, J. & COSIO, E. G. 1994 Elicitors of plant defense responses. *Int. Rev. Cytol.*, 148, 1-36.

ECKERT, F. 1999. COSMOtherm Users Manual, Version C3.0 Release 12.01©1999-2012, Frank Eckert, COSMOlogic GmbH & Co KG.

ECKSTEIN-LUDWIG, U., WEBB, R., VAN GOETHEM, I., EAST, J., LEE, A., KIMURA, M., O'NEILL, P., BRAY, P., WARD, S. & KRISHNA, S. 2003. Artemisinins target the SERCA of *Plasmodium falciparum. Nature,* 424, 957-961.

EFFERTH, T., BENAKIS, A., ROMERO, M. R., TOMICIC, M., RAUH, R., STEINBACH, D., HÄFER, R., STAMMINGER, T., OESCH, F. & KAINA, B. 2004. Enhancement of cytotoxicity of artemisinins toward cancer cells by ferrous iron. *Free Radical Biology and Medicine,* 37, 998-1009.

EFFERTH, T., HERRMANN, F., TAHRANI, A. & WINK, M. 2011. Cytotoxic activity of secondary metabolites derived from *Artemisia annua* L. towards cancer cells in comparison to its designated active constituent artemisinin. *Phytomedicine,* 18, 959-969.

EFFERTH, T. & OESCH, F. 2004. Oxidative stress response of tumor cells: microarray-based comparison between artemisinins and anthracyclines. *Biochemical Pharmacology,* 68, 3-10.

EFFERTH, T., ROMERO, M. R., WOLF, D. G., STAMMINGER, T., MARIN, J. J. & MARSCHALL, M. 2008. The antiviral activities of artemisinin and artesunate. *Clinical Infectious Diseases,* 47, 804-811.

ELFORD, B. C., ROBERTS, M. F., PHILLIPSON, J. D. & WILSON, R. J. M. 1987. Potentiation of the antimalarial activity of qinghaosu by methoxylated flavones. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 81, 434.

ELSOHLY, H. N., CROOM, E. M., EL-FERALY, F. S. & EL-SHEREI, M. M. 1990. A Large-Scale Extraction Technique of Artemisinin from *Artemisia annua. Journal of Natural Products,* 53, 1560-1564.

ERKAN, N., AYRANCI, G. & AYRANCI, E. 2008. Antioxidant activities of rosemary (Rosmarinus Officinalis L.) extract, blackseed (Nigella sativa L.) essential oil, carnosic acid, rosmarinic acid and sesamol. *Food Chemistry,* 110, 76-82.

FENG, R., LU, Y., BOWMAN, L. L., QIAN, Y., CASTRANOVA, V. & DING, M. 2005. Inhibition of activator protein-1, NF-kappaB, and MAPKs and induction of phase 2 detoxifying enzyme activity by chlorogenic acid. *Journal of Biological Chemistry,* 280, 27888-27895.

FERLAY, J., SHIN, H. R., BRAY, F., FORMAN, D., MATHERS, C. & PARKIN, D. M. 2010. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *International journal of cancer,* 127, 2893-2917.

FERREIRA, J. F., LUTHRIA, D. L., SASAKI, T. & HEYERICK, A. 2010. Flavonoids from *Artemisia annua* L. as antioxidants and their potential synergism with artemisinin against malaria and cancer. *Molecules,* 15, 3135-3170.

FIVELMAN, Q. L., ADAGU, I. S. & WARHURST, D. C. 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, 4097-4102.

FLOREA, A. & BÜSSELBERG, D. 2011. Cisplatin as an anti-tumor drug: cellular mechanisms of activity, drug resistance and induced side effects. *Cancers,* 3, 1351-1371.

GABAY, O., SANCHEZ, C., SALVAT, C., CHEVY, F., BRETON, M., NOURISSAT, G., WOLF, C., JACQUES, C. & BERENBAUM, F. 2010. Stigmasterol: a phytosterol with potential anti-osteoarthritic properties. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society,* 18, 106-116.

GALLUZZI, L., SENOVILLA, L., VITALE, I., MICHELS, J., MARTINS, I., KEPP, O., CASTEDO, M. & KROEMER, G. 2011. Molecular mechanisms of cisplatin resistance. *Oncogene,* 31, 1869-1883.

GARCÍA-LAFUENTE, A., GUILLAMÓN, E., VILLARES, A., ROSTAGNO, M. A. & MARTÍNEZ, J. A. 2009. Flavonoids as anti-inflammatory agents: implications in cancer and cardiovascular disease. *Inflammation Research,* 58, 537-552.

GHOSH, T., MAITY, T. K. & SINGH, J. 2011. Evaluation of antitumor activity of stigmasterol, a constituent isolated from Bacopa monnieri Linn aerial parts against Ehrlich Ascites Carcinoma in mice. *Oriental Pharmacy & Experimental Medicine,* 11, 41-49.

GOEL, D., MALLAVARUPU, G. R., KUMAR, S., SINGH, V. & ALI, M. 2008. Volatile Metabolite Compositions of the Essential Oil from Aerial Parts of Ornamental and Artemisinin Rich Cultivars of Artemisia annua. *Journal of Essential Oil Research,* 20, 147-152.

GOLENSER, J., WAKNINE, J. H., KRUGLIAK, M., HUNT, N. H. & GRAU, G. E. 2006. Current perspectives on the mechanism of action of artemisinins. *International Journal for Parasitology,* 36, 1427-1441.

GOU, L., LORENZ, H. & SEIDEL-MORGENSTERN, A. 2012. Investigation of a Chiral Additive Used in Preferential Crystallization. *Crystal Growth & Design,* 12, 5197-5202.

GRAVETT, A. M., LIU, W. M., KRISHNA, S., CHAN, W.-C., HAYNES, R. K., WILSON, N. L. & DALGLEISH, A. G. 2011. In vitro study of the anti-cancer effects of artemisone alone or in combination with other chemotherapeutic agents. *Cancer chemotherapy and pharmacology,* 67, 569-577.

GÜLÇIN, İ. 2006. Antioxidant activity of caffeic acid (3,4-dihydroxycinnamic acid). *Toxicology,* 217, 213-220.

HAO, J.-Y., HAN, W., HUANG, S.-D., XUE, B.-Y. & DENG, X. 2002. Microwave-assisted extraction of artemisinin from *Artemisia annua* L. *Separation and purification technology,* 28, 191-196.

HARBORNE, J. B. & WILLIAMS, C. A. 2000. Advances in flavonoid research since 1992. *Phytochemistry,* 55, 481-504.

HATTENSCHWILER, S. & VITOUSEK, P. 2000. The role of Phenols in terrestrial ecosystem nutrient cycling. *Trends in Ecology and Evolution* 15, 238-243.

HAYNES, R. K. 2006. From artemisinin to new artemisinin antimalarials: biosynthesis, extraction, old and new derivatives, stereochemistry and medicinal chemistry requirements. *Current topics in medicinal chemistry,* 6, 509-537.

HAYNES, R. K. & KRISHNA, S. 2004. Artemisinins: activities and actions. *Microbes and Infection,* 6, 1339-1346.

HE, S. P., TAN, G. Y., LI, G., TAN, W. M., NAN, T. G., WANG, B. M., LI, Z. H. & LI, Q. X. 2009. Development of a sensitive monoclonalantibody-based enzyme-linked immunosorbent assay for the antimalaria active ingredient artemisinin in the Chinese herb *Artemisia annua* L. *Analytical and bioanalytical chemistry,* 393, 1297-1303.

HENFREY, P. 2013. Artemisinin Production in East Africa - 2013 Artemisinin Conference Presentation. *A2S2-UNITAID* http://www.a2s2.org/upload/5.ArtemisininConferences/1.2013Kenya/Presentations/Day1/7.EastAfricaBE.pdf, 1-5.

HSIAO, W. W. & LIU, L. 2010. The role of traditional Chinese herbal medicines in cancer therapy-from TCM theory to mechanistic insights. *Planta medica,* 76, 1118-1131.

HSU, E. 2006a. The history of qing hao in the Chinese materia medica. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 100, 505-508.

HSU, E. 2006b. Reflections on the discovery of the antimalarial qinghao. *British journal of clinical pharmacology,* 61, 666-670.

I.C.H.H.T, G. 2005. Validation of analytical procedures: text and methodology Q2 (R1). *IFPMA: Geneva.*

ISIKDAG, I., OZKAY, Y. & INCESU, Z. 2011. Synthesis and anti-cancer activity of some bisquinoxaline derivatives *Turkish Journal of Pharmaceutical Science* 8, 178-188.

ITTARAT, W., PICKARD, A. L., RATTANASINGANCHAN, P., WILAIRATANA, P., LOOAREESUWAN, S., EMERY, K., LOW, J., UDOMSANGPETCH, R. & MESHNICK, S. R. 2003. Recrudescence in artesunate-treated patients with falciparum malaria is dependent on parasite burden not on parasite factors. *The American journal of tropical medicine and hygiene,* 68, 147-152.

JANSEN, F. 2006. The herbal tea approach for artemisinin as a therapy for malaria? *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 100, 285.

JIN, U. H., LEE, J. K., KANG, S. K., KIM, J. K., PARK, W. H., KIM, J. G., MOON, S. K. & KIM, C. H. 2005. A phenolic compound, 5-caffeoylquinic acid (chlorogenic acid), is a new type and strong matrix metalloproteinase-9 inhibitor: Isolation and identification from methanol extract of *Euonymus alatus. Life Sciences,* 77, 2760-2769.

JUTEAU, F., MASOTTI, V., BESSIÈRE, J. M., DHERBOMEZ, M. & VIANO, J. 2002. Antibacterial and antioxidant activities of *Artemisia annua* essential oil. *Fitoterapia,* 73, 532-535.

KAROU, D., NADEMBEGA, W. M. C., OUATTARA, L., ILBOUDO, D. N. P., CANINI, A., NIKIÉMA, J. B., SIMPORE, J., COLIZZI, V. & TRAORE, A. S. 2007. African Ethnopharmacology and New Drug Discovery. *Medicinal and Aromatic Plant Science and Biotechnology* 1, 61-69.

KIM, S.-S., PARK, R.-Y., JEON, H.-J., KWON, Y.-S. & CHUN, W. 2005. Neuroprotective effects of 3,5-dicaffeoylquinic acid on hydrogen peroxide-induced cell death in SH-SY5Y cells. *Phytotherapy Research,* 19, 243-245.

KIM, S. Y. 2010. A cancer sensitizer comprising chlorogenic acid EP Patent 2,211,853.

KIMURA, M. & RODRIGUEZ-AMAYA, D. B. 2003. Carotenoid Composition of Hydroponic Leafy Vegetables. *Journal of Agricultural and Food Chemistry,* 51, 2603-2607.

KLAMT, A., ECKERT, F. & ARLT, W. 2010. COSMO-RS: an alternative to simulation for calculating thermodynamic properties of liquid mixtures. *Annual review of chemical and biomolecular engineering,* 1, 101-122.

KLAMT, A., KROOSHOF, G. J. & TAYLOR, R. 2002. COSMOSPACE: Alternative to conventional activity-coefficient models. *AIChE journal,* 48, 2332-2349.

KOHLER, M., HAERDI, W., CHRISTEN, P. & VEUTHEY, J.-L. 1997a. Extraction of artemisinin and artemisinic acid from *Artemisia annua* L. using supercritical carbon dioxide. *Journal of Chromatography A,* 785, 353-360.

KOHLER, M., HAERDI, W., CHRISTEN, P. & VEUTHEY, J. L. 1997b. Extraction of artemisinin and artemisinic acid from *Artemisia annua* L. using supercritical carbon dioxide. *Journal of Chromatography A,* 785, 353-360.

KONO, Y., KASHINE, S., YONEYAMA, T., SAKAMOTO, Y., MATSUI, Y. & SHIBATA, H. 1998. Iron chelation by chlorogenic acid as a natural antioxidant. *Bioscience, Biotechnology, and Biochemistry,* 62, 22-27.

KORTENKAMP, A. & ALTENBURGER, R. 1998. Synergisms with mixtures of xenoestrogens: a reevaluation using the method of isoboles. *Science of the Total Environment,* 221, 59-73.

LAI, H. & SINGH, N. P. 2006. Oral artemisinin prevents and delays the development of 7, 12-dimethylbenz [a] anthracene (DMBA)-induced breast cancer in the rat. *Cancer Letters,* 231, 43-48.

LAI, J.-P., LIM, Y. H., SU, J., SHEN, H.-M. & ONG, C. N. 2007. Identification and characterization of major flavonoids and caffeoylquinic acids in three Compositae plants by LC/DAD-APCI/MS. *Journal of Chromatography B,* 848, 215-225.

LANSBURY, P. T. & MOJICA, C. A. 1986. Total synthesis of (±)-arteannuin B. *Tetrahedron letters,* 27, 3967-3970.

LAPKIN, A. A., PETERS, M., GREINER, L., CHEMAT, S., LEONHARD, K., LIAUW, M. A. & LEITNER, W. 2010. Screening of new solvents for artemisinin extraction process using ab initio methodology. *Green Chemistry,* 12, 241-251.

LAPKIN, A. A., PLUCINSKI, P. K. & CUTLER, M. 2006. Comparative assessment of technologies for extraction of artemisinin. *Journal of Natural Products,* 69, 1653-1664.

LAPKIN, A. A., WALKER, A., SULLIVAN, N., KHAMBAY, B., MLAMBO, B. & CHEMAT, S. 2009. Development of HPLC analytical protocols for quantification of artemisinin in biomass and extracts. *Journal of Pharmaceutical and Biomedical Analysis,* 49, 908-915.

LARSON, T. R., BRANIGAN, C., HARVEY, D., PENFIELD, T., BOWLES, D. & GRAHAM, I. A. 2013. A survey of artemisinic and dihydroartemisinic acid contents in glasshouse and global field-grown populations of the artemisinin-producing plant *Artemisia annua* L. *Industrial Crops and Products,* 45, 1-6.

LEE, W. J. & ZHU, B. T. 2006. Inhibition of DNA methylation by caffeic acid and chlorogenic acid, two common catechol-containing coffee polyphenols. *Carcinogenesis,* 27, 269-277.

LEVESQUE, F. & SEEBERGER, P. H. 2012. Continuous-Flow Synthesis of the Anti-Malaria Drug Artemisinin. *Angewandte Chemie International Edition,* 51, 1706-1709.

LÉVESQUE, F. & SEEBERGER, P. H. 2012. Continuous-Flow Synthesis of the Anti-Malaria Drug Artemisinin. *Angewandte Chemie International Edition,* 51, 1706-1709.

LI, W., MO, W., SHEN, D., SUN, L., WANG, J., LU, S., GITSCHIER, J. M. & ZHOU, B. 2005. Yeast Model Uncovers Dual Roles of Mitochondria in the Action of Artemisinin. *PLoS Genet,* 1, e36.

LI, Y., SHAN, F., WU, J.-M., WU, G.-S., DING, J., XIAO, D., YANG, W.-Y., ATASSI, G., LÉONCE, S. & CAIGNARD, D.-H. 2001. Novel antitumor artemisinin derivatives targeting G1 phase of the cell cycle. *Bioorganic and Medicinal Chemistry Letters,* 11, 5-8.

LI, Y., ZHU, Y.-M., JIANG, H.-J., PAN, J.-P., WU, G.-S., WU, J.-M., SHI, Y.-L., YANG, J.-D. & WU, B.-A. 2000. Synthesis and antimalarial activity of artemisinin derivatives containing an amino group. *Journal of Medicinal Chemistry,* 43, 1635-1640.

LIMTRAKUL, P., KHANTAMAT, O. & PINTHA, K. 2005. Inhibition of P-glycoprotein function and expression by kaempferol and quercetin. *Journal of Chemotherapy,* 17, 86-95.

LIU, K., YANG, S.-L., ROBERTS, M., ELFORD, B. & PHILLIPSON, J. 1992. Antimalarial activity of *Artemisia annua* flavonoids from whole plants and cell cultures. *Plant Cell Reports,* 11, 637-640.

LIU, N. Q., CHOI, Y. H., VERPOORTE, R. & VAN DER KOOY, F. 2010. Comparative quantitative analysis of artemisinin by chromatography and qNMR. *Phytochemical Analysis,* 21, 451-456.

LIU, S., TIAN, N., LIU, Z., HUANG, J., LI, J. & FERREIRA, J. F. S. 2008. Affordable and sensitive determination of artemisinin in *Artemisia annua* L. by gas chromatography with electron-capture detection. *Journal of Chromatography A,* 1190, 302-306.

LIU, W. M., GRAVETT, A. M. & DALGLEISH, A. G. 2011. The antimalarial agent artesunate possesses anticancer properties that can be enhanced by combination strategies. *International Journal of Cancer,* 128, 1471-1480.

LOEWE, S. & MUISCHNEK, H. 1926. Effect of combinations: mathematical basis of problem. *Arch Exp Pathol Pharmakol,* 114, 313-326.

LOPES-LUTZ, D., ALVIANO, D. S., ALVIANO, C. S. & KOLODZIEJCZYK, P. P. 2008. Screening of chemical composition, antimicrobial and antioxidant activities of Artemisia essential oils. *Phytochemistry,* 69, 1732-1738.

MAILLARD, M. P., WOLFENDER, J. L. & HOSTETTMANN, K. 1993. Use of liquid chromatography-thermospray mass spectrometry in phytochemical analysis of crude plant extracts. *Journal of Chromatography A,* 647, 147-154.

MALWADE, C., QU, H., RONG, B.-G. & CHRISTENSEN, L. 2013. Purification of artemisinin from quercetin by anti-solvent crystallization. *Frontiers of Chemical Science and Engineering,* 1-7.

MANNAN, A., AHMED, I., ARSHAD, W., ASIM, M. F., QURESHI, R. A., HUSSAIN, I. & MIRZA, B. 2010a. Survey of artemisinin production by diverse *Artemisia* species in northern Pakistan. *Malaria journal,* 9, 310.

MANNAN, A., LIU, C., ARSENAULT, P. R., TOWLER, M. J., VAIL, D. R., LORENCE, A. & WEATHERS, P. J. 2010b. DMSO triggers the generation of ROS leading to an increase in artemisinin and dihydroartemisinic acid in *Artemisia annua* shoot cultures. *Plant Cell Reports,* 29, 143-152.

MARINOVA, D. & RIBAROVA, F. 2007. HPLC determination of carotenoids in Bulgarian berries. *Journal of Food Composition and Analysis,* 20, 370-374.

MATUSZEWSKI, B. K., CONSTANZER, M. L. & CHAVEZ-ENG, C. M. 2003. Strategies for the Assessment of Matrix Effect in Quantitative Bioanalytical Methods Based on HPLC-MS/MS. *Analytical chemistry,* 75, 3019-3030.

MERCER, A. E., MAGGS, J. L., SUN, X.-M., COHEN, G. M., CHADWICK, J., O'NEILL, P. M. & PARK, B. K. 2007. Evidence for the involvement of carbon-centered radicals in the induction of apoptotic cell death by artemisinin compounds. *Journal of Biological Chemistry,* 282, 9372-9382.

MESHNICK, S. R. 2002. Artemisinin: mechanisms of action, resistance and toxicity. *International Journal for Parasitology,* 32, 1655-1660.

MESHNICK, S. R., TAYLOR, T. & KAMCHONWONGPAISAN, S. 1996. Artemisinin and the antimalarial endoperoxides: from herbal remedy to targeted chemotherapy. *Microbiological reviews,* 60, 301-315.

MIKETOVA, P., SCHRAM, K. H., WHITNEY, J., KEARNS, E. H. & TIMMERMANN, B. N. 1999. Mass spectrometry of 3,5- and 4,5-dicaffeoylquinic acids and selected derivatives. *Journal of Mass Spectrometry,* 34, 1240-1252.

MILLER, J. N. & MILLER, J. C. 2005. *Statistics and chemometrics for analytical chemistry,* Prentice Hall.

MIZUSHINA, Y., TAKEUCHI, T., SUGAWARA, F. & YOSHIDA, H. 2012. Anti-Cancer Targeting Telomerase Inhibitors:-Rubromycin and Oleic Acid. *Mini Reviews in Medicinal Chemistry,* 12, 1135-1143.

MUELLER, M., KARHAGOMBA, I., HIRT, H. & WEMAKOR, E. 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,* 73, 487-493.

MUELLER, M. S., RUNYAMBO, N., WAGNER, I., BORRMANN, S., DIETZ, K. & HEIDE, L. 2004. Randomized controlled trial of a traditional preparation of *Artemisia annua* L.(Annual Wormwood) in the treatment of malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 98, 318-321.

NEAU, S. H., BHANDARKAR, S. V. & HELLMUTH, E. W. 1997. Differential molar heat capacities to test ideal solubility estimations. *Pharmaceutical Research,* 14, 601-605.

NEMEIKAITĖ-ČĖNIENĖ, A., IMBRASAITĖ, A., SERGEDIENĖ, E. & ČĖNAS, N. 2005. Quantitative structure–activity relationships in prooxidant cytotoxicity of polyphenols: role of potential of phenoxyl radical/phenol redox couple. *Archives of biochemistry and biophysics,* 441, 182-190.

NEWMAN, D. J. & CRAGG, G. M. 2007. Natural Products as Sources of New Drugs over the Last 25 Years. *J. Nat. Prod.,* 70, 461-477.

NEWMAN, D. J., CRAGGA, G. M. & SNADERB, K. M. 2000. The influence of natural products upon drug discovery. *Nat. Prod. Rep.,* 17, 215–234.

NOBILI, S., LIPPI, D., WITORT, E., DONNINI, M., BAUSI, L., MINI, E. & CAPACCIOLI, S. 2009. Natural compounds for cancer treatment and prevention. *Pharmacological Research,* 59, 365-378.

NORATTO, G., PORTER, W., BYRNE, D. & CISNEROS-ZEVALLOS, L. 2009. Identifying peach and plum polyphenols with chemopreventive potential against estrogen-independent breast cancer cells. *Journal of agricultural and food chemistry,* 57, 5219-5226.

O'BRIEN, C., HENRICH, P. P., PASSI, N. & FIDOCK, D. A. 2011. Recent clinical and molecular insights into emerging artemisinin resistance in *Plasmodium falciparum. Current opinion in infectious diseases,* 24, 570.

O'NEILL, P. M., BARTON, V. E. & WARD, S. A. 2010a. The molecular mechanism of action of artemisinin—the debate continues. *Molecules,* 15, 1705-1721.

O'NEILL, P. M., BARTON, V. E. & WARD, S. A. 2010b. The Molecular Mechanism of Action of Artemisinin, The Debate Continues. *Molecules,* 15, 1705-1721.

OKSUZ, S., AYYILDIZ, H. & JOHANSSON, C. 1984. 6-Methoxylated and C-glycosyl flavonoids from Centaurea species. *Journal of Natural Products,* 47, 902-903.

OLOFSSON, L., ENGSTRÖM, A., LUNDGREN, A. & BRODELIUS, P. E. 2011. Relative expression of genes of terpene metabolism in different tissues of *Artemisia annua* L. *BMC plant biology,* 11, 45.

PALUSZCZAK, J., KRAJKA-KUŹNIAK, V. & BAER-DUBOWSKA, W. 2010. The effect of dietary polyphenols on the epigenetic regulation of gene expression in MCF7 breast cancer cells. *Toxicology Letters,* 192, 119-125.

PARIJA, S. C. & PRAHARAJ, I. 2011. Drug resistance in malaria. *Indian Journal of Medical Microbiology,* 29, 243-248.

PARK, J. B. 2009. 5-Caffeoylquinic acid and caffeic acid orally administered suppress P-selectin expression on mouse platelets. *The Journal of nutritional biochemistry,* 20, 800-805.

PENG, C. A., FERREIRA, J. F. S. & WOOD, A. J. 2006. Direct analysis of artemisinin from *Artemisia annua* L. using high-performance liquid chromatography with evaporative light scattering detector, and gas chromatography with flame ionization detector. *Journal of chromatography. A,* 1133, 254-258.

PEPLOW, M. 2013. Malaria drug made in yeast causes market ferment. *Nature,* 494, 160-161.

PETERSEN, M. & SIMMONDS, M. S. J. 2003. Rosmarinic acid. *Phytochemistry,* 62, 121-125.

PHYO, A. P., NKHOMA, S., STEPNIEWSKA, K., ASHLEY, E. A., NAIR, S., MCGREADY, R., AL-SAAI, S., DONDORP, A. M., LWIN, K. M. & SINGHASIVANON, P. 2012. Emergence of artemisinin-resistant malaria on the western border of Thailand: a longitudinal study. *The Lancet,* 379, 1960-1966.

QIAN, G. P., YANG, Y. W. & REN, Q. L. 2005. Determination of artemisinin in *Artemisia annua* L. by reversed phase HPLC. *Journal of liquid chromatography & related technologies,* 28, 705-712.

QU, H., CHRISTENSEN, K. B., FRETTÉ, X., TIAN, F., GREVSEN, K., RANTANEN, J. T. & CHRISTENSEN, L. P. Crystallization for the isolation and purification of artemisinin from crude extracts of *Artemisia annua*: Feasibility and challenges. BIWIC 2009 16th International Workshop on Industrial Crystallization, 2009. 37-44.

QU, H., CHRISTENSEN, K. B., FRETTE, X. C., TIAN, F., RANTANEN, J. & CHRISTENSEN, L. P. 2010. A Novel Hybrid Chromatography-Crystallization Process for the Isolation and Purification of a Natural Pharmaceutical Ingredient from a Medicinal Herb. *Organic Process Research & Development,* 14, 585-591.

QUENNOZ, M., BASTIAN, C., SIMONNET, X. & GROGG, A. F. 2010. Quantification of the Total Amount of Artemisinin in Leaf Samples by Thin Layer Chromatography. *CHIMIA International Journal for Chemistry,* 64, 755-757.

RASKIN, I., RIBNICKY, M. D., KOMARNYTSKY, S., IIIC, N., POULEV, A., BORISJUK, N., BRINKER, A., MORENO, D. A., RIPOLL, C. & YAKOBY, N. 2002. Plants

and human health in the twenty first century. *Trend in Biotechnology* 20, 522-531.

RASOANAIVO, P., WRIGHT, C. W., WILLCOX, M. L. & GILBERT, B. 2011. Whole plant extracts versus single compounds for the treatment of malaria: synergy and positive interactions. *Malar J,* 10, S4.

RATH, K., TAXIS, K., WALZ, G., GLEITER, C. H., LI, S. M. & HEIDE, L. 2004. Pharmacokinetic study of artemisinin after oral intake of a traditional preparation of *Artemisia annua* L.(annual wormwood). *The American journal of tropical medicine and hygiene,* 70, 128-132.

REDDY, L., ODHAV, B. & BHOOLA, K. D. 2003 Natural products for cancer prevention a global perspective. *Pharmacology and therapeutics* 99, 1-13.

REUNGPATTHANAPHONG, P. & MANKHETKORN, S. 2002. Modulation of multidrug resistance by artemisinin, artesunate and dihydroartemisinin in K562/adr and GLC4/adr resistant cell lines. *Biological and Pharmaceutical Bulletin,* 25, 1555-1561.

RICHTER, B. E., JONES, B. A., EZZELL, J. L., PORTER, N. L., AVDALOVIC, N. & POHL, C. 1996. Accelerated solvent extraction: a technique for sample preparation. *Analytical Chemistry,* 68, 1033-1039.

RIDDLE, J. M. & ESTES, J. W. 1992. Oral contraceptives in ancient and medieval times. *American Scientist,* 80, 226-233.

RIDLEY, R. G. 2002. Medical need, scientific opportunity and the drive for antimalarial drugs. *Nature,* 415, 686-693.

RIVEIRO, M., DE KIMPE, N., MOGLIONI, A., VAZQUEZ, R., MONCZOR, F., SHAYO, C. & DAVIO, C. 2010. Coumarins: old compounds with novel promising therapeutic perspectives. *Current Medicinal Chemistry,* 17, 1325-1338.

RO, D.-K., PARADISE, E. M., OUELLET, M., FISHER, K. J., NEWMAN, K. L., NDUNGU, J. M., HO, K. A., EACHUS, R. A., HAM, T. S. & KIRBY, J. 2006a. Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature,* 440, 940-943.

RO, D. K., PARADISE, E. M., OUELLET, M., FISHER, K. J., NEWMAN, K. L., NDUNGU, J. M., HO, K. A., EACHUS, R. A., HAM, T. S. & KIRBY, J. 2006b. Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature,* 440, 940-943.

ROBINSON JR, W. E., CORDEIRO, M., ABDEL-MALEK, S., JIA, Q., CHOW, S. A., REINECKE, M. G. & MITCHELL, W. M. 1996. Dicaffeoylquinic acid inhibitors of human immunodeficiency virus integrase: inhibition of the core catalytic domain of human immunodeficiency virus integrase. *Molecular pharmacology,* 50, 846-855.

RODRIGUEZ-AMAYA, D. B. & KIMURA, M. 2004. *HarvestPlus handbook for carotenoid analysis*, International Food Policy Research Institute (IFPRI).

RYDÉN, A.-M. & KAYSER, O. 2007. Chemistry, biosynthesis and biological activity of artemisinin and related natural peroxides. *Bioactive Heterocycles III.* Springer.

SADAVA, D., PHILLIPS, T., LIN, C. & KANE, S. E. 2002. Transferrin overcomes drug resistance to artemisinin in human small-cell lung carcinoma cells. *Cancer Letters,* 179, 151-156.

SANCHEZ, C. P., DAVE, A., STEIN, W. D. & LANZER, M. 2010. Transporters as mediators of drug resistance in *Plasmodium falciparum. International Journal for Parasitology,* 40, 1109-1118.

SARKER, S. D., LATIF, Z. & GRAY, A. I. 2005. Natural product isolation. *Natural Products Isolation.* Springer.

SHILIN, Y., ROBERTS, M. F. & PHILLIPSON, J. D. 1989. Methoxylated flavones and coumarins from *Artemisia annua. Phytochemistry,* 28, 1509-1511.

SHRETTA, R. & YADAV, P. 2012. Stabilizing supply of artemisinin and artemisinin-based combination therapy in an era of wide-spread scale-up. *Malaria journal,* 11, 399.

SIDDIK, Z. H. 2003. Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene,* 22, 7265-7279.

SIDHU, A. B. S., UHLEMANN, A.-C., VALDERRAMOS, S. G., VALDERRAMOS, J.-C., KRISHNA, S. & FIDOCK, D. A. 2006. Decreasing *pfmdr1* copy number in *Plasmodium falciparum* malaria heightens susceptibility to mefloquine, lumefantrine, halofantrine, quinine, and artemisinin. *Journal of Infectious Diseases,* 194, 528-535.

SINGH, N. P. & LAI, H. C. 2004. Artemisinin induces apoptosis in human cancer cells. *Anticancer Research,* 24, 2277-2280.

SIRCHIA, S. M., FERGUSON, A. T., SIRONI, E., SUBRAMANYAN, S., ORLANDI, R., SUKUMAR, S. & SACCHI, N. 2000. Evidence of epigenetic changes affecting the chromatin state of the retinoic acid receptor beta2 promoter in breast cancer cells. *Oncogene,* 19, 1556.

SISA, M., BONNET, S. L., FERREIRA, D. & VAN DER WESTHUIZEN, J. H. 2010. Photochemistry of flavonoids. *Molecules,* 15, 5196-5245.

SONG, R.-Q. & CÖLFEN, H. 2011. Additive controlled crystallization. *CrystEngComm,* 13, 1249-1276.

STRINGHAM, R. W., LYNAM, K. G., MROZINSKI, P., KILBY, G., PELCZER, I. & KRAML, C. 2009a. High performance liquid chromatographic evaluation of

artemisinin, raw material in the synthesis of artesunate and artemether. *Journal of Chromatography A,* 1216, 8918-8925.

STRINGHAM, R. W., LYNAM, K. G., MROZINSKI, P., KILBY, G., PELCZER, I. N. & KRAML, C. 2009b. High performance liquid chromatographic evaluation of artemisinin, raw material in the synthesis of artesunate and artemether. *Journal of Chromatography A,* 1216, 8918-8925.

STRINGHAM, R. W., PENNELL, M., CABRI, W., CARZANA, G., GIORGI, F., LALLI, S., MARAZZI, G. & TORRI, M. 2011. Identification of impurities in artemisinin, their behavior in high performance liquid chromatography and implications for the quality of derived anti-malarial drugs. *Journal of Chromatography A,* 1218, 6838-6842.

SUBERU, J., SONG, L., SLADE, S., SULLIVAN, N., BARKER, G. & LAPKIN, A. A. 2013a. A rapid method for the determination of artemisinin and its biosynthetic precursors in *Artemisia annua* L. crude extracts. *Journal of Pharmaceutical and Biomedical Analysis,* 84, 269-277.

SUBERU, J. O., GORKA, A. P., JACOBS, L., ROEPE, P. D., SULLIVAN, N., BARKER, G. & LAPKIN, A. A. 2013b. Anti-plasmodial polyvalent interactions in *Artemisia annua* L. aqueous extract - possible synergistic and resistance mechanisms. *PLOS ONE (accepted).*

TAKEMURA, T., URUSHISAKI, T., FUKUOKA, M., HOSOKAWA-MUTO, J., HATA, T., OKUDA, Y., HORI, S., TAZAWA, S., ARAKI, Y. & KUWATA, K. 2012. 3, 4-Dicaffeoylquinic Acid, a Major Constituent of Brazilian Propolis, Increases TRAIL Expression and Extends the Lifetimes of Mice Infected with the Influenza A Virus. *Evidence-based Complementary and Alternative Medicine: eCAM,* 2012.

TEGZE, B., SZÁLLÁSI, Z., HALTRICH, I., PÉNZVÁLTÓ, Z., TÓTH, Z., LIKÓ, I. & GYŐRFFY, B. 2012. Parallel Evolution under Chemotherapy Pressure in 29 Breast Cancer Cell Lines Results in Dissimilar Mechanisms of Resistance. *PLoS ONE,* 7, e30804.

TEOH, K. H., POLICHUK, D. R., REED, D. W. & COVELLO, P. S. 2009. Molecular cloning of an aldehyde dehydrogenase implicated in artemisinin biosynthesis in *Artemisia annua* This paper is one of a selection of papers published in a Special Issue from the National Research Council of Canada-Plant Biotechnology Institute. *Botany,* 87, 635-642.

TEPE, B., EMINAGAOGLU, O., AKPULAT, H. A. & AYDIN, E. 2007. Antioxidant potentials and rosmarinic acid levels of the methanolic extracts of *Salvia verticillata* (L.) subsp. *verticillata* and *S. verticillata* (L.) subsp. *amasiaca* (Freyn & Bornm.) Bornm. *Food Chemistry,* 100, 985-989.

TIAN, N., LI, J., LIU, S., HUANG, J., LI, X. & LIU, Z. 2012. Simultaneous isolation of artemisinin and its precursors from *Artemisia annua* L. by preparative RP-HPLC. *Biomedical Chromatography,* 26, 708-713.

TONK, S., BARTARYA, R., KUMARI, K. M., BHATNAGAR, V. & SRIVASTAVA, S. 2006. Effective method for extraction of larvicidal component from leaves of *Azadirachta indica* and *Artemisia annua* Linn. *Journal of Environmental Biology* 27, 103.

TRAGER, W. & JENSON, J. B. 1978. Cultivation of malarial parasites. *Nature,* 273, 621-622.

TUCKER, M. S., MUTKA, T., PATEL, J. & KYLE, D. E. 2012. Phenotypic and genotypic analysis of in vitro-selected artemisinin-resistant progeny of *Plasmodium falciparum. Antimicrobial agents and chemotherapy,* 56, 302-314.

UHLEMANN, A.-C., CAMERON, A., ECKSTEIN-LUDWIG, U., FISCHBARG, J., ISEROVICH, P., ZUNIGA, F. A., EAST, M., LEE, A., BRADY, L. & HAYNES, R. K. 2005. A single amino acid residue can determine the sensitivity of SERCAs to artemisinins. *Nature structural & molecular biology,* 12, 628-629.

VAN DER KOOY, F. & VERPOORTE, R. 2011. The Content of Artemisinin in the *Artemisia annua* Tea Infusion. *Planta Medica-Natural Products and Medicinal Plant Research,* 77, 1754.

VAN EECKHAUT, A., LANCKMANS, K., SARRE, S., SMOLDERS, I. & MICHOTTE, Y. 2009. Validation of bioanalytical LC-MS/MS assays: evaluation of matrix effects. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences,* 877, 2198.

VAN NIEUWERBURGH, F. C. W., VANDE CASTEELE, S. R. F., MAES, L., GOOSSENS, A., INZÈ, D., VAN BOCXLAER, J. & DEFORCE, D. L. D. 2006. Quantitation of artemisinin and its biosynthetic precursors in *Artemisia annua* L. by high performance liquid chromatography-electrospray quadrupole time-of-flight tandem mass spectrometry. *Journal of Chromatography A,* 1118, 180-187.

VERPOORTE, R., CROMMELIN, D., DANHOF, M., GILISSEN, L. J. W. J., SCHUITMAKER, H., VAN DER GREEF, J. & WITKAMP, R. F. 2009. Commentary: "A systems view on the future of medicine: Inspiration from Chinese medicine?". *Journal of Ethnopharmacology,* 121, 479-481.

VICHAI, V. & KIRTIKARA, K. 2006. Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nature protocols,* 1, 1112-1116.

VIVAS, L., RATTRAY, L., STEWART, L., ROBINSON, B., FUGMANN, B., HAYNES, R., PETERS, W. & CROFT, S. 2007. Antimalarial efficacy and drug interactions of the novel semi-synthetic endoperoxide artemisone in vitro and in vivo. *Journal of antimicrobial chemotherapy,* 59, 658-665.

WAGNER, H. 2005. Natural products chemistry and phytomedicine in the 21st century: new developments and challenges. *Pure and applied chemistry,* 77, 1-6.

WAGNER, H. 2011. Synergy research: Approaching a new generation of phytopharmaceuticals. *Fitoterapia,* 82, 34-37.

WAGNER, H. & ULRICH-MERZENICH, G. 2009. Synergy research: Approaching a new generation of phytopharmaceuticals. *Phytomedicine,* 16, 97-110.

WALLAART, T. E., PRAS, N., BEEKMAN AND, A. R. C. & QUAX, W. J. 2000. Seasonal Variation of Artemisinin and its Biosynthetic Precursors in Plants of *Artemisia annua* of Different Geographical Origin: Proof for the Existence of Chemotypes. *Planta Med,* 66, 57,62.

WALLAART, T. E., PRAS, N. & QUAX, W. J. 1999. Isolation and identification of dihydroartemisinic acid hydroperoxide from *Artemisia annua*: a novel biosynthetic precursor of artemisinin. *Journal of Natural Products,* 62, 1160-1162.

WALLE, T. Methoxylated flavones, a superior cancer chemopreventive flavonoid subclass? Seminars in cancer biology, 2007. Elsevier, 354-362.

WALLE, T. 2009. Methylation of dietary flavones increases their metabolic stability and chemopreventive effects. *International journal of molecular sciences,* 10, 5002-5019.

WANG, J., HUANG, L., LI, J., FAN, Q., LONG, Y., LI, Y. & ZHOU, B. 2010a. Artemisinin Directly Targets Malarial Mitochondria through Its Specific Mitochondrial Activation. *PLoS ONE,* 5, e9582.

WANG, L. & WELLER, C. L. 2006. Recent advances in extraction of nutraceuticals from plants. *Trends in Food Science & Technology,* 17, 300-312.

WANG, L.-H., SONG, Y.-T., CHEN, Y. & CHENG, Y.-Y. 2007. Solubility of Artemisinin in Ethanol + Water from (278.2 to 343.2) K. *Journal of Chemical & Engineering Data,* 52, 757-758.

WANG, M., PARK, C. H., WU, Q. & SIMON, J. E. 2005. Analysis of artemisinin in *Artemisia annua* L. by LC-MS with selected ion monitoring. *Journal of Agricultural and Food Chemistry,* 53, 7010-7013.

WANG, S.-J., GAO, Y., CHEN, H., KONG, R., JIANG, H.-C., PAN, S.-H., XUE, D.-B., BAI, X.-W. & SUN, B. 2010b. Dihydroartemisinin inactivates NF-κB and potentiates the anti-tumor effect of gemcitabine on pancreatic cancer both in vitro and in vivo. *Cancer Letters,* 293, 99-108.

WANG, Y. & WEATHERS, P. 2007. Sugars proportionately affect artemisinin production. *Plant Cell Reports,* 26, 1073-1081.

WATSON, A. A., FLEET, G. W. J., ANSO, N., MOLYNEUX, R. J. & NASH, R. J. 2001. Polyhydroxylated alkaloids – natural occurrence and therapeutic application. *Phytochemistry* 56 265-295.

WEATHERS, P., ELKHOLY, S. & WOBBE, K. 2006. Artemisinin: The biosynthetic pathway and its regulation in *Artemisia annua*, a terpenoid-rich species. *In Vitro Cellular & Developmental Biology - Plant*, 42, 309-317.

WEATHERS, P. J., ARSENAULT, P. R., COVELLO, P. S., MCMICKLE, A., TEOH, K. H. & REED, D. W. 2011. Artemisinin production in *Artemisia annua*: studies in planta and results of a novel delivery method for treating malaria and other neglected diseases. *Phytochemistry Reviews*, 10, 173-183.

WEATHERS, P. J. & TOWLER, M. J. 2012. The flavonoids casticin and artemetin are poorly extracted and are unstable in an *Artemisia annua* tea infusion. *Planta medica*, 78, 1024.

WEBSTER, H. K. & LEHNERT, E. K. 1994. Chemistry of artemisinin: an overview. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 88, Supplement 1, 27-29.

WEINA, P. 2008. Artemisinins from Folklore to Modern Medicine-Transforming an Herbal Extract to Life-Saving Drugs. *Parassitologia*, 50, 25.

WEN, X. & WALLE, T. 2006. Methylated flavonoids have greatly
improved intestinal adsorption and metabolic stability. *Drug Metab. Dispos.*, 34, 1786-1792.

WHO 2006. WHO Guideline for the treatment of Malaria. *World Health Organisation Geneva Switzerland*, WHO/HTM/MAL/2006.1108, 1-253.

WHO 2010. World Malaria Report. *World Health Organisation Geneva Switzerland*, 1-137.

WHO 2011. Quality requirements for artemsinin as a starting material in the production of antimalarial active pharmaceutical ingredients (APIs) - Revised Draft for Comment. *World Health Organisation Geneva Switzerland*, Working document QAS/10.349.

WHO 2012. World Malaria Report 2012. *Geneva: World Health Organisation; 2012*
http://www.who.int/malaria/publications/world_malaria_report_2012/report/en/index.html, 1-195.

WILLCOX, M. 2009. *Artemisia* species: from traditional medicines to modern antimalarials-and back again. *The Journal of Alternative and Complementary Medicine*, 15, 101-109.

WILLCOX, M., BODEKER, G., BOURDY, G., DHINGRA, V., FALQUET, J., FERREIRA, J. F. S., GRAZ, B., HIRT, H. M., HSU, E. & DE MAGALHÃES, P. M. 2004. *Artemisia annua* as a traditional herbal antimalarial. *Traditional Medicinal Plants and Malaria*, 43-59.

WILLIAMSON, E. M. 2001. Synergy and other interactions in phytomedicines. *Phytomedicine*, 8, 401-409.

WILLIAMSON, E. M. 2011. *Phytocomplexes versus single-entity drugs : the never-ending dilemma in herbal medicine in Herbal medicines: development and validation of plant-derived medicines for human health (Giacinto Bagetta et al, editor) Pg 401-409* CRC Press ISBN 1439837686.

WITHERS, S. & KEASLING, J. 2007. Biosynthesis and engineering of isoprenoid small molecules. *Applied Microbiology and Biotechnology,* 73, 980-990.

WITKOWSKI, B., LELIÈVRE, J., LÓPEZ BARRAGÁN, M. J., LAURENT, V., SU, X.-Z., BERRY, A. & BENOIT-VICAL, F. 2010. Increased tolerance to artemisinin in *Plasmodium falciparum* is mediated by a quiescence mechanism. *Antimicrobial agents and chemotherapy,* 54, 1872-1877.

WOERDENBAG, H. J., BOS, R., SALOMONS, M. C., HENDRIKS, H., PRAS, N. & MALINGRÉ, T. M. 1993a. Volatile constituents of *Artemisia annua* L.(Asteraceae). *Flavour and fragrance journal,* 8, 131-137.

WOERDENBAG, H. J., MOSKAL, T. A., PRAS, N., MALINGRÉ, T. M., EL-FERALY, F. S., KAMPINGA, H. H. & KONINGS, A. W. T. 1993b. Cytotoxicity of Artemisinin-Related Endoperoxides to Ehrlich Ascites Tumor Cells. *Journal of Natural Products,* 56, 849-856.

WOERDENBAG, H. J., PRAS, N., BOS, R., VISSER, J. F., HENDRIKS, H. & MALINGRÉ, T. M. 1991. Analysis of artemisinin and related sesquiterpenoids from *Artemisia annua* L. by combined gas chromatography/mass spectrometry. *Phytochemical Analysis,* 2, 215-219.

WRIGHT, C. W., LINLEY, P. A., BRUN, R., WITTLIN, S. & HSU, E. 2010. Ancient Chinese methods are remarkably effective for the preparation of artemisinin-rich extracts of Qing Hao with potent antimalarial activity. *Molecules,* 15, 804-812.

WU, G.-D., ZHOU, H.-J. & WU, X.-H. 2004. Apoptosis of human umbilical vein endothelial cells induced by artesunate. *Vascular pharmacology,* 41, 205-212.

XIAO, H., YANG, C. S., LI, S., JIN, H., HO, C. Ä. & PATEL, T. 2009. Monodemethylated polymethoxyflavones from sweet orange (Citrus sinensis) peel inhibit growth of human lung cancer cells by apoptosis. *Molecular Nutrition & Food Research,* 53, 398-406.

XU, J.-G., HU, Q.-P. & LIU, Y. 2012. Antioxidant and DNA-Protective Activities of Chlorogenic Acid Isomers. *Journal of Agricultural and Food Chemistry,* 60, 11625-11630.

YANG, Y., QI, M. & MEI, C. 2004. Endogenous salicylic acid protects rice plants from oxidative damage caused by aging as well as biotic and abiotic stress. *The Plant Journal,* 40, 909-919.

YING-ZI, Y., LITTLE, B. & MESHNICK, S. R. 1994. Alkylation of proteins by artemisinin: Effects of heme, pH, and drug structure. *Biochemical Pharmacology,* 48, 569-573.

YORDI, E. G., PÉREZ, E. M., MATOS, M. J. & VILLARES, E. U. 2012. Antioxidant and Pro-Oxidant Effects of Polyphenolic Compounds and Structure-Activity Relationship Evidence. *in Nutrition, Well-Being and Health, Dr. Jaouad Bouayed (Ed.), ISBN: 978-953-51-0125-3, InTech, DOI: 10.5772/29471. Available from: http://www.intechopen.com/books/nutrition-well-being-and-health/antioxidant-and-prooxidant-effect-of-polyphenol-compounds-and-structure-activity-relationship-eviden.*

YOU, K. M., JONG, H.-G. & KIM, H. P. 1999. Inhibition of cyclooxygenase/lipoxygenase from human platelets by polyhydroxylated/methoxylated flavonoids isolated from medicinal plants. *Archives of pharmacal research,* 22, 18-24.

YOU-YOU, T., MU-YUN, N., YU-RONG, Z., LAN-NA, L., SHU-LIAN, C., MU-QUN, Z., XIU-ZHEN, W., ZHENG, J. & XIAO-TIAN, L. 1982. Studies on the Constituents of *Artemisia annua* Part II*. *Planta Medica,* 44, 143-145.

ZHA, R.-P., XU, W., WANG, W.-Y., DONG, L. & WANG, Y.-P. 2007. Prevention of lipopolysaccharide-induced injury by 3,5-dicaffeoylquinic acid in endothelial cells. *Acta Pharmacol Sin,* 28, 1143-1148.

ZHANG, B., YANG, R. & LIU, C. Z. 2008. Microwave-assisted extraction of chlorogenic acid from flower buds of *Lonicera japonica* Thunb. *Separation and Purification Technology,* 62, 480-483.

ZHU, X. X., YANG, L., LI, Y. J., ZHANG, D., CHEN, Y., KOSTECKÁ, P., KMONÍČKOVÁ, E. & ZÍDEK, Z. 2013. Effects of sesquiterpene, flavonoid and coumarin types of compounds from *Artemisia annua* L. on production of mediators of angiogenesis. *Pharmacological Reports,* 65, 410-420.

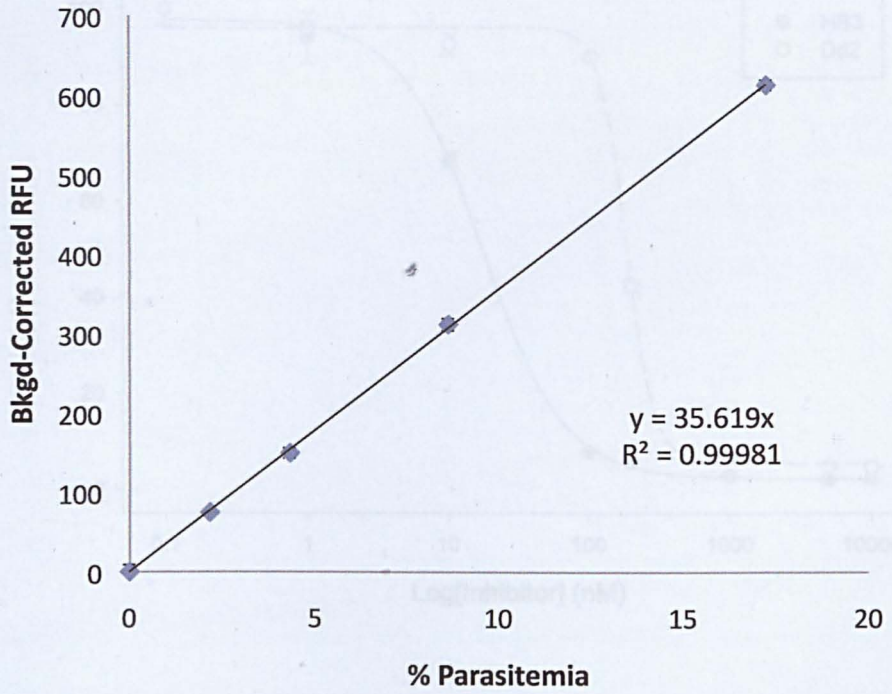
Appendix

Plasmodium Assay

HB3 Calibration Curve

Raw Data			
785.464	795.505	793.950	782.954
486.591	494.702	478.355	493.186
332.790	322.554	330.107	319.136
252.048	247.884	249.553	256.027
180.490	188.080	171.364	168.161

%Parasitemia	RFU	RFU - Bkgd	Error
0	177.024	0.000	9.034
2.1525	251.378	74.354	3.540
4.3050	326.147	149.123	6.374
8.6100	488.209	311.185	7.453
17.2200	789.468	612.445	6.191

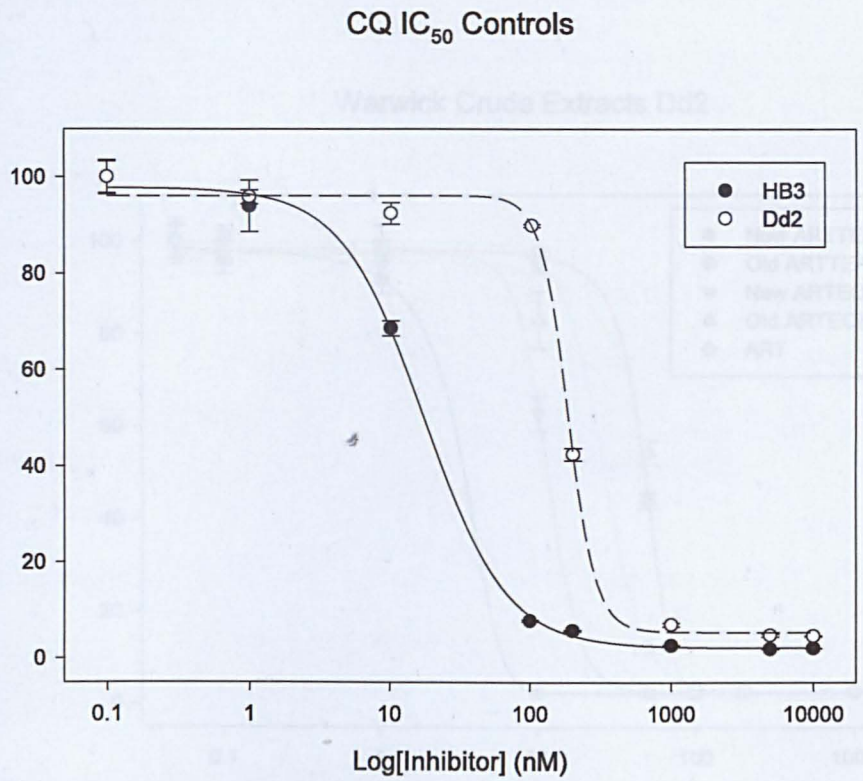


CQ Controls

HB3 Raw Data		
1082.481	1074.566	1020.201
1029.028	1028.297	933.647
721.333	736.068	753.289
116.886	113.634	113.868
92.034	93.767	94.755
61.356	62.511	63.170
54.846	54.356	55.241
56.967	56.062	56.753

Dd2 Raw Data		
1495.855	1401.777	1419.849
1404.221	1368.783	1369.253
1341.646	1296.498	1358.047
1296.096	1292.360	1300.736
619.342	650.905	621.051
128.613	139.502	131.149
98.933	107.309	100.884
102.006	98.980	99.278

CQ % Growth Data				
Conc. (nM)	HB3	Stdev	Dd2	Stdev
0.1	100.000	3.311	100.000	3.556
1	93.936	5.358	95.840	1.448
10	68.534	1.562	92.372	2.270
100	7.777	0.177	89.832	0.299
200	5.699	0.135	42.398	1.264
1000	2.655	0.090	6.975	0.406
5000	1.919	0.043	4.787	0.312
10000	2.093	0.046	4.624	0.119

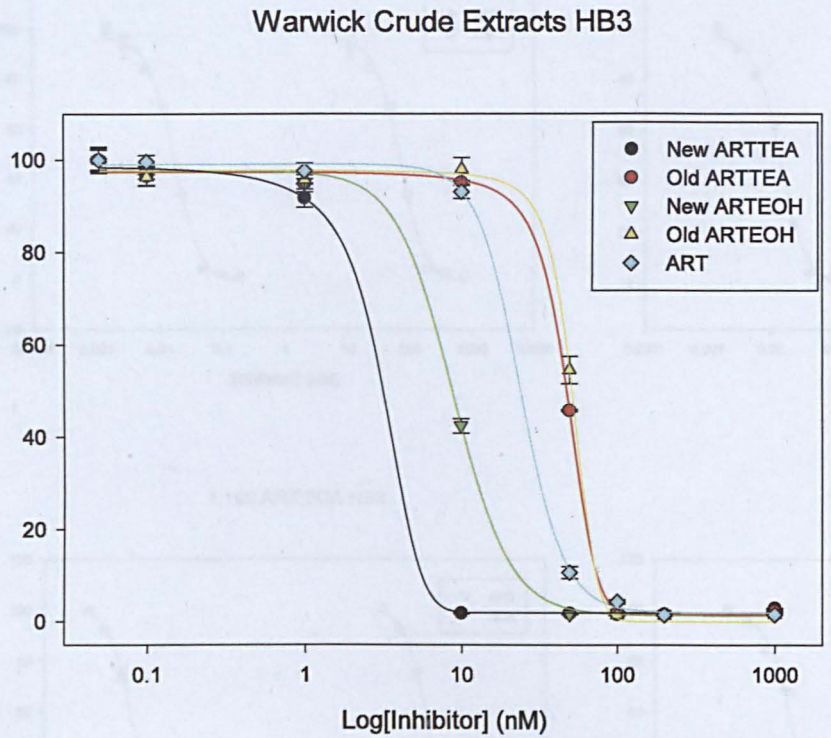


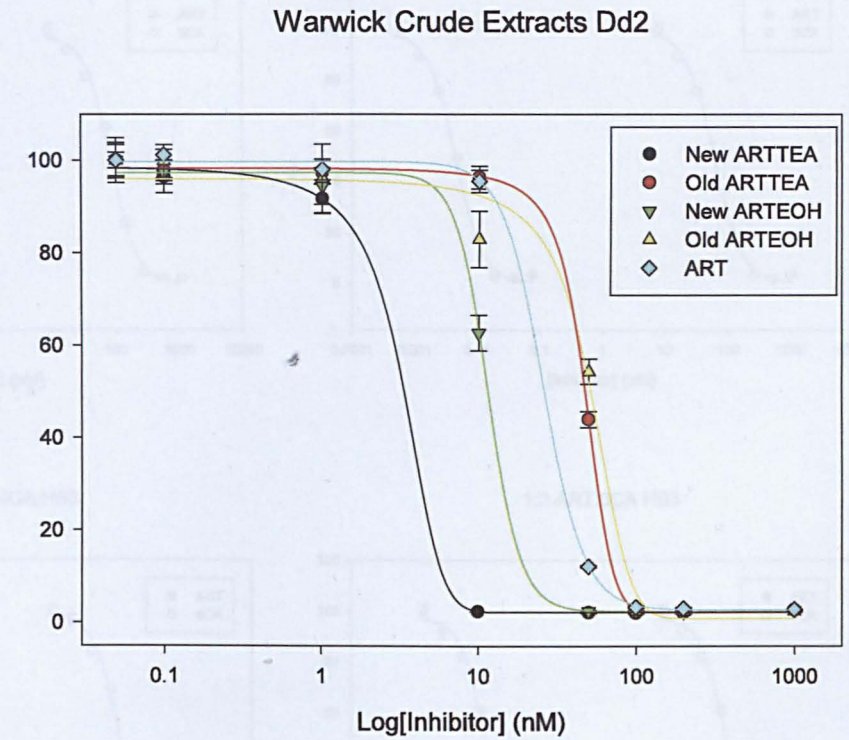
HB3 CQ IC_{50} = 17.2 nM (R^2 = 0.999)

Dd2 CQ IC_{50} = 183.3 nM (R^2 = 0.998)

R_f = 10.7

Dose Response Curves

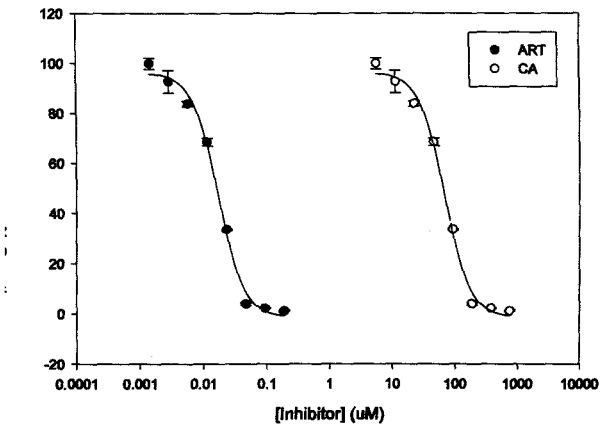




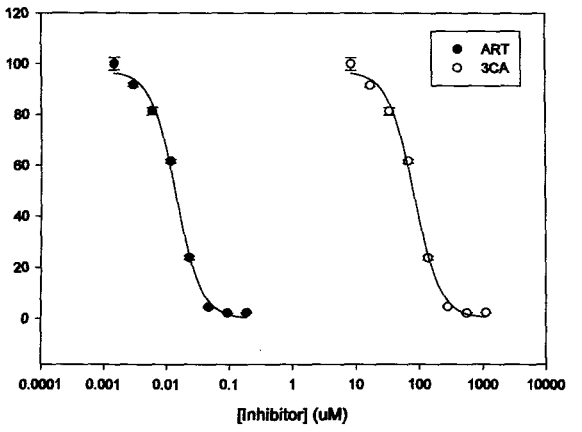
All R^2 values ≥ 0.990

Dose Response Curves

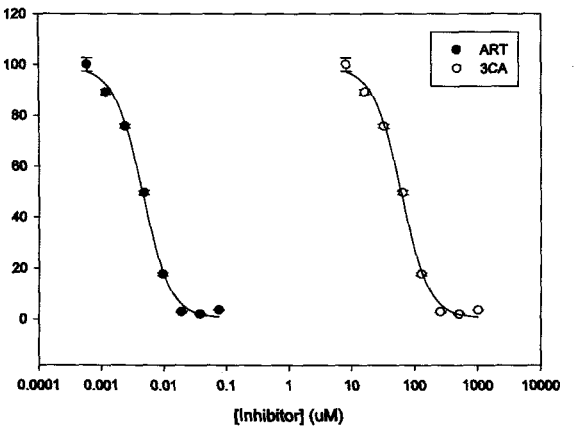
1:3 ART:CA HB3



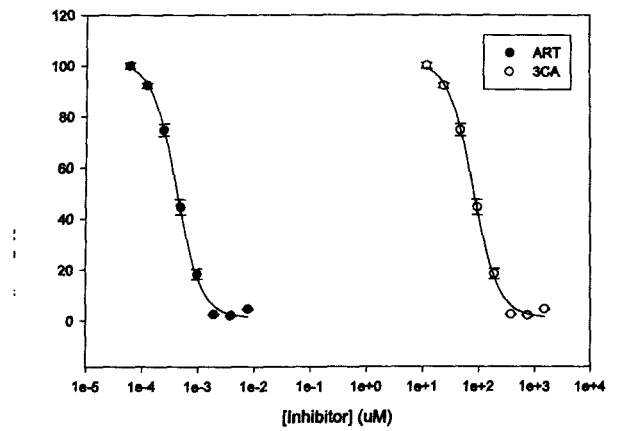
1:3 ART:3CA HB3



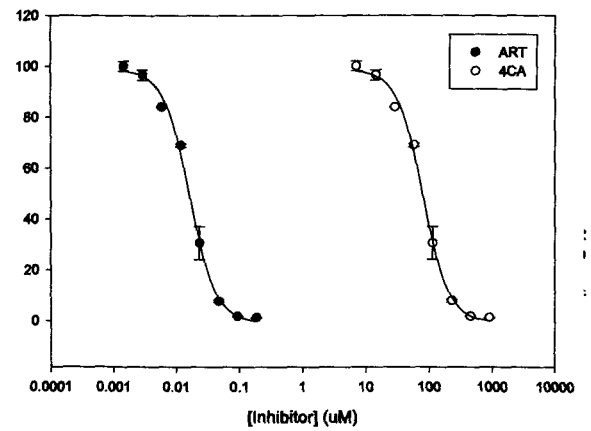
1:10 ART:3CA HB3



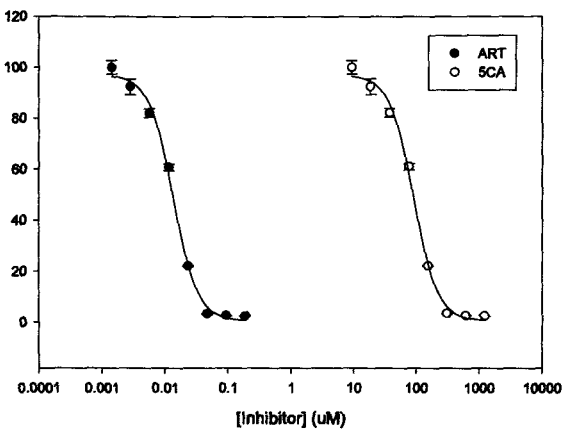
1:100 ART:3CA HB3



1:3 ART:4CA HB3

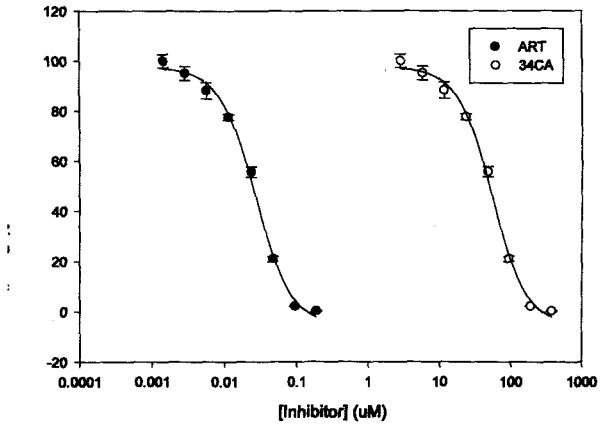


1:3 ART:5CA HB3

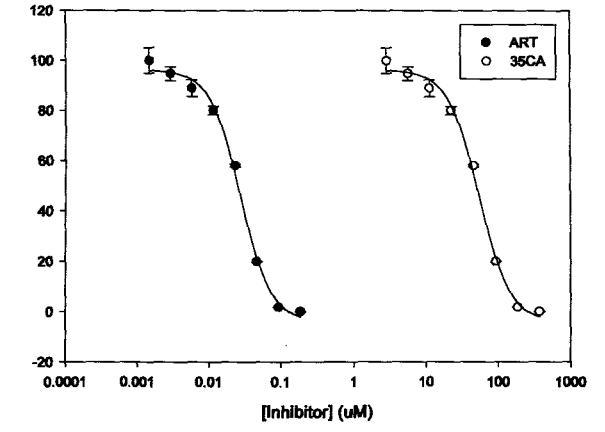


Dose Response Curves

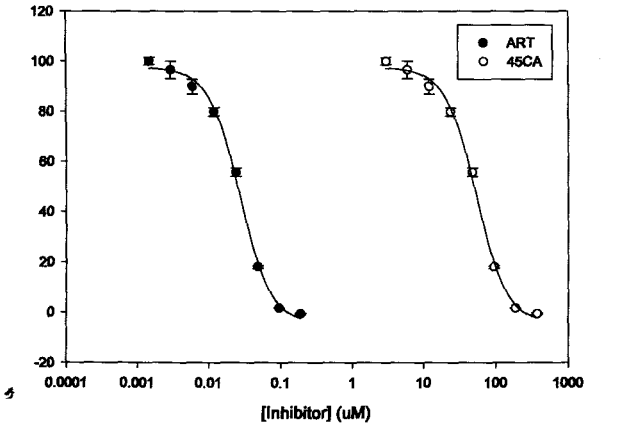
1:3 ART:34CA HB3



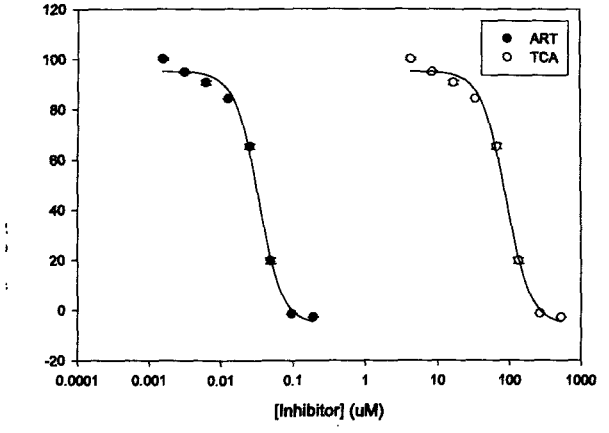
1:3 ART:35CA HB3



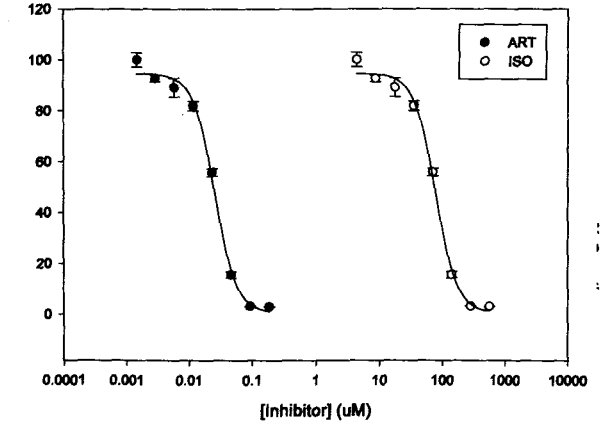
1:3 ART:45CA HB3



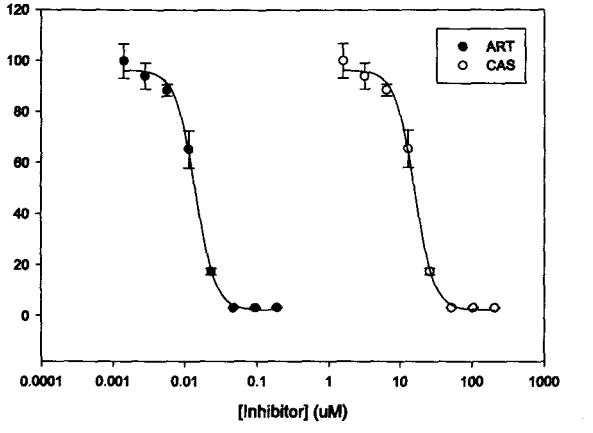
1:3 ART:TCA HB3



1:3 ART:ISO HB3

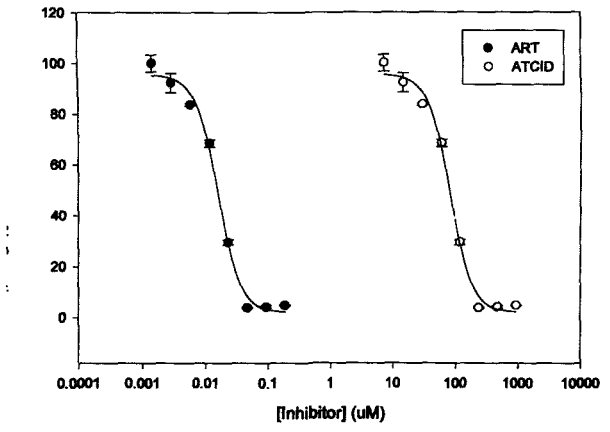


1:3 ART:CAS HB3

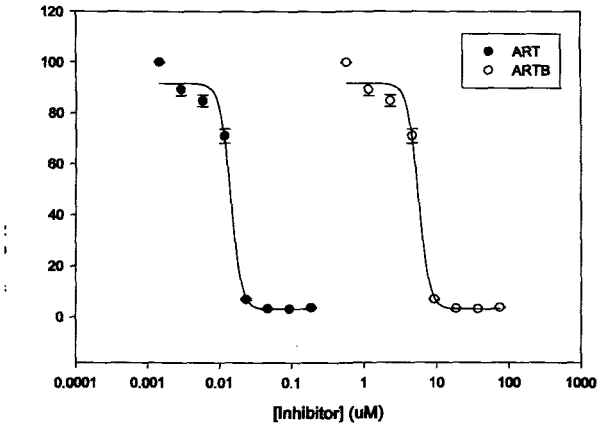


Dose Response Curves

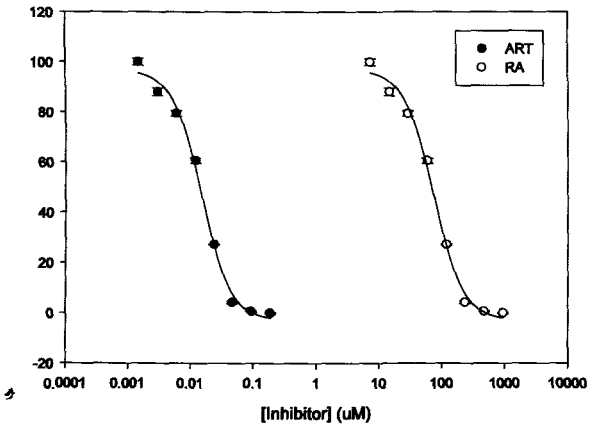
1:3 ART:ATCID HB3



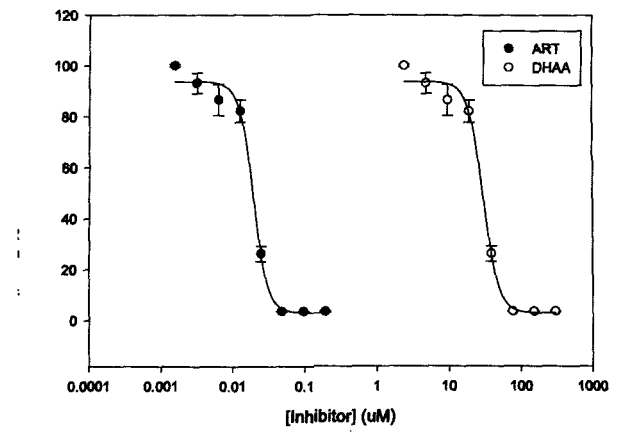
1:3 ART:ARTB HB3



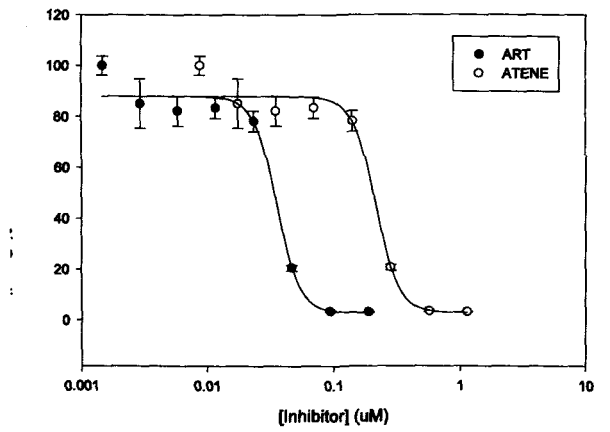
1:3 ART:RA HB3



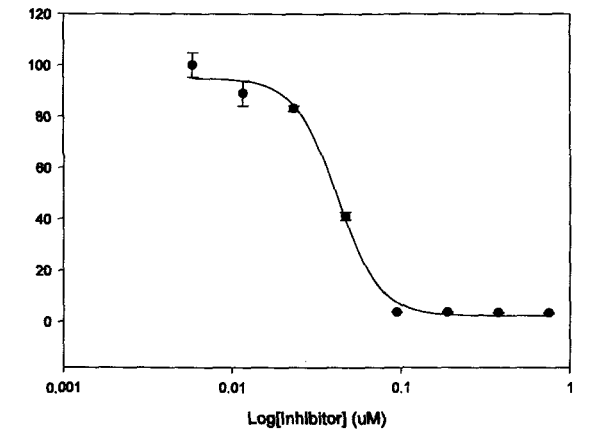
1:3 ART:DHAA HB3



1:3 ART:ATENE HB3

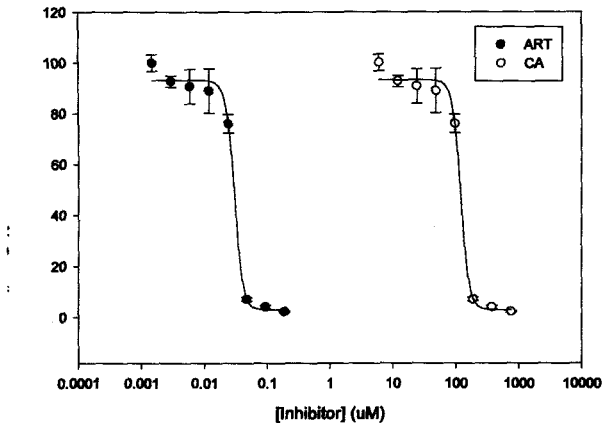


ART HB3

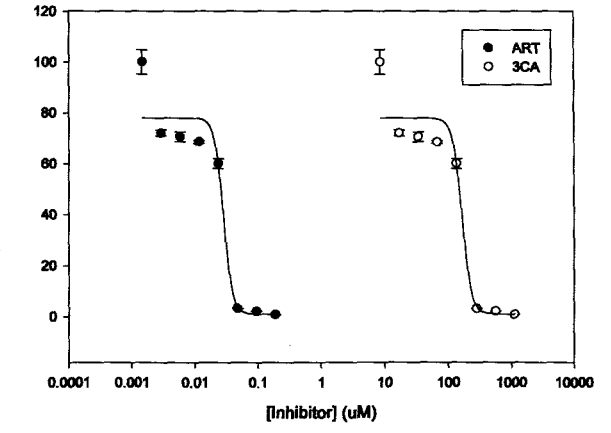


Dose Response Curves

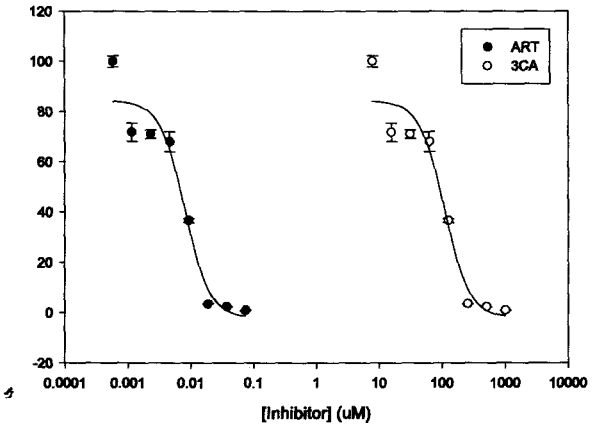
1:3 ART:CA Dd2



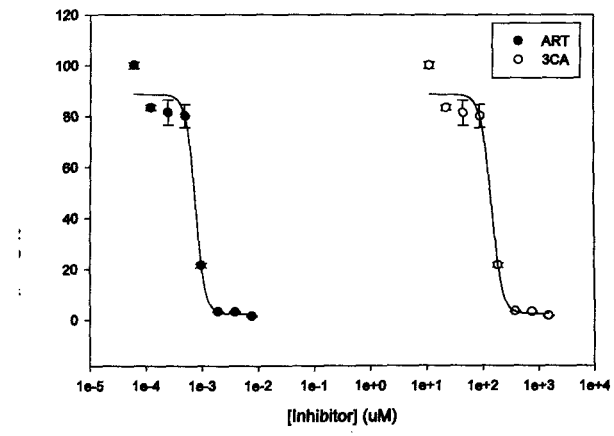
1:3 ART:3CA Dd2



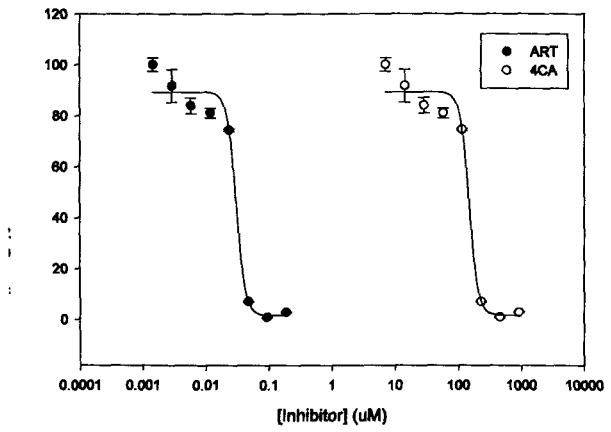
1:10 ART:3CA Dd2



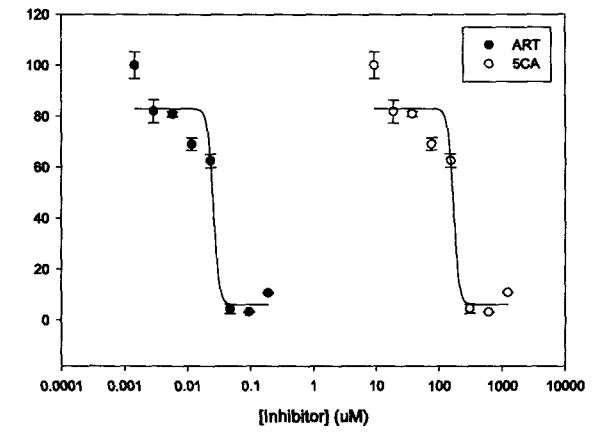
1:100 ART:3CA Dd2



1:3 ART:4CA Dd2

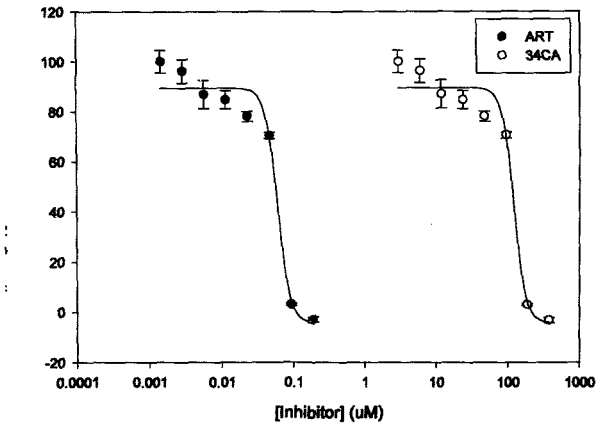


1:3 ART:5CA Dd2

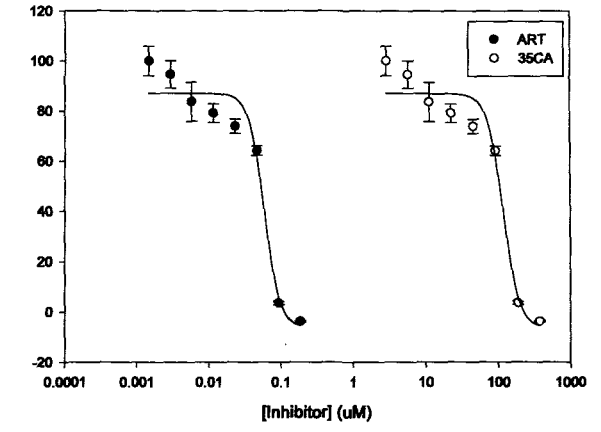


Dose Response Curves

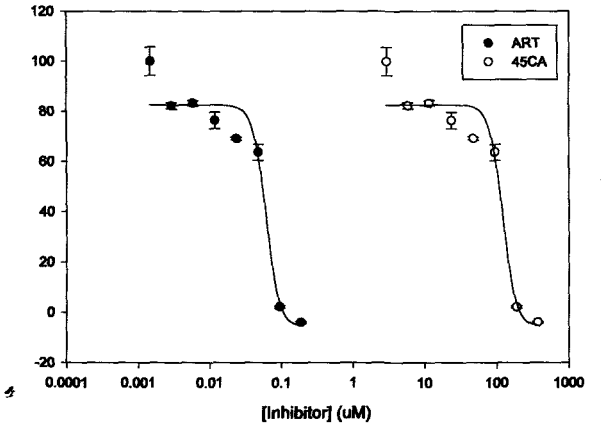
1:3 ART:34CA Dd2



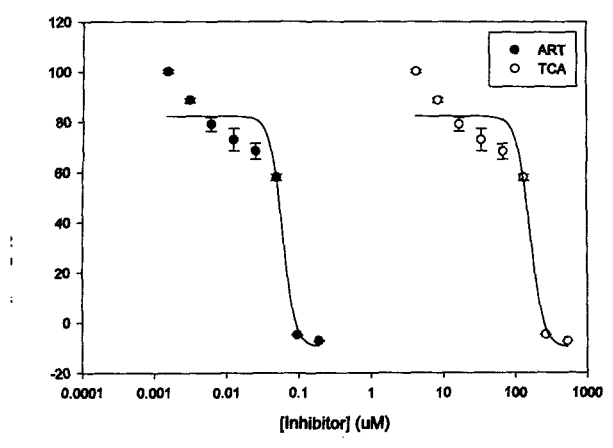
1:3 ART:35CA Dd2



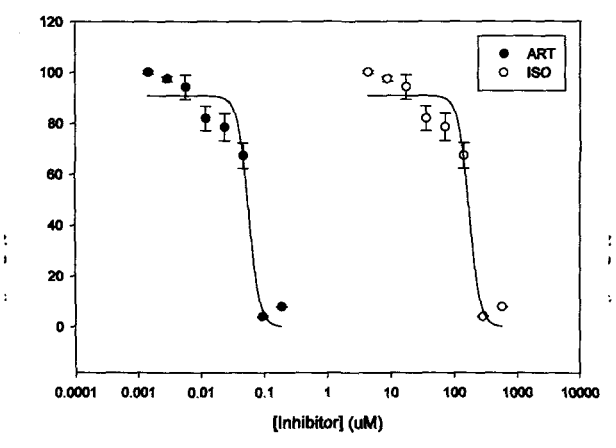
1:3 ART:45CA Dd2



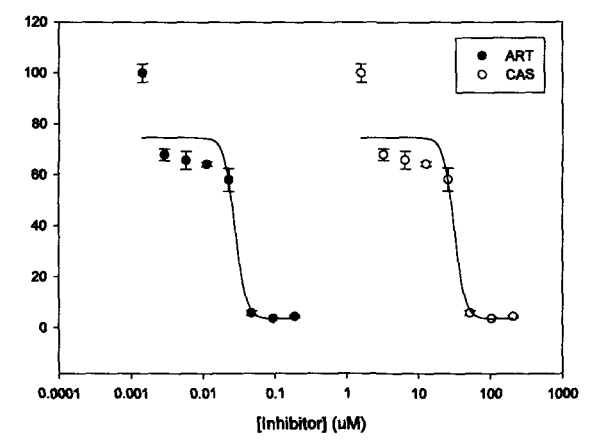
1:3 ART:TCA Dd2



1:3 ART:ISO Dd2

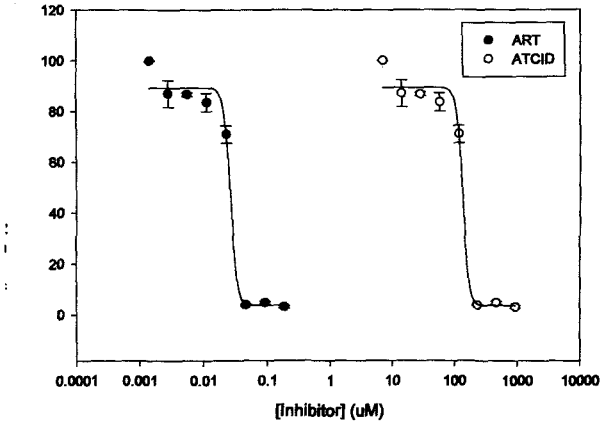


1:3 ART:CAS Dd2

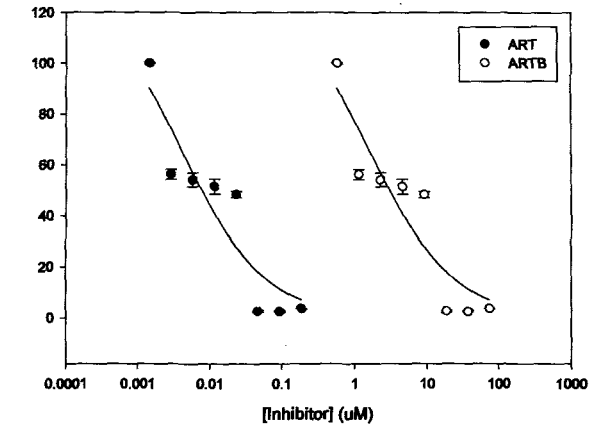


Dose Response Curves

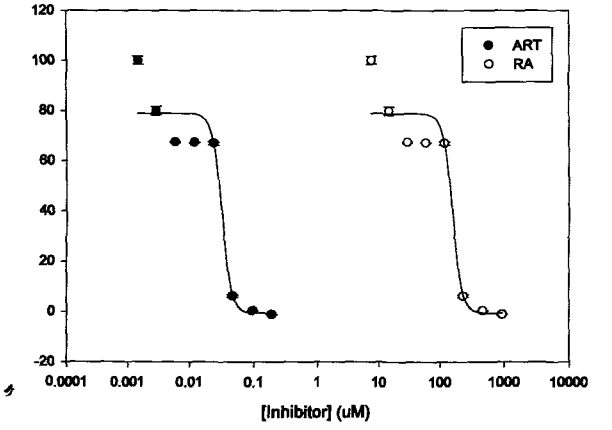
1:3 ART:ATCID Dd2



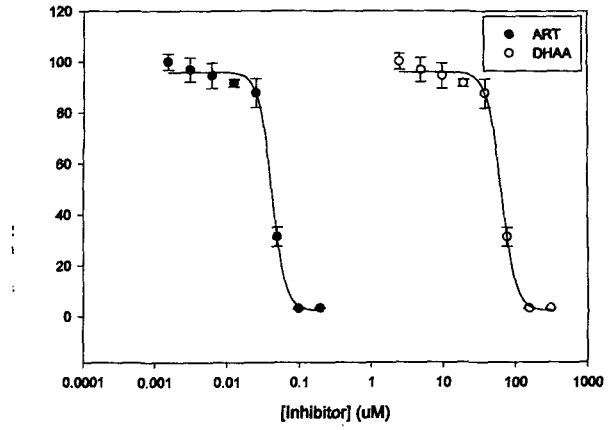
1:3 ART:ARTB Dd2



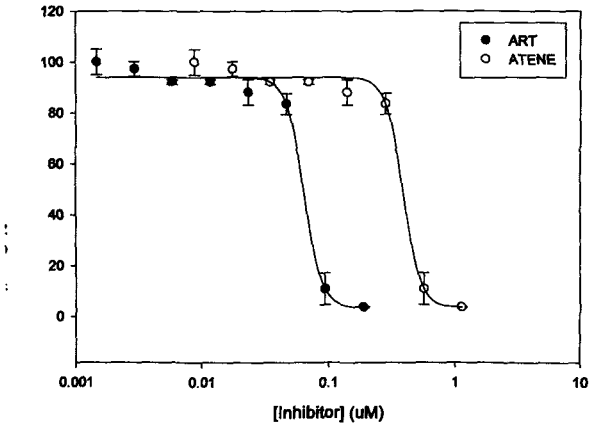
1:3 ART:RA Dd2



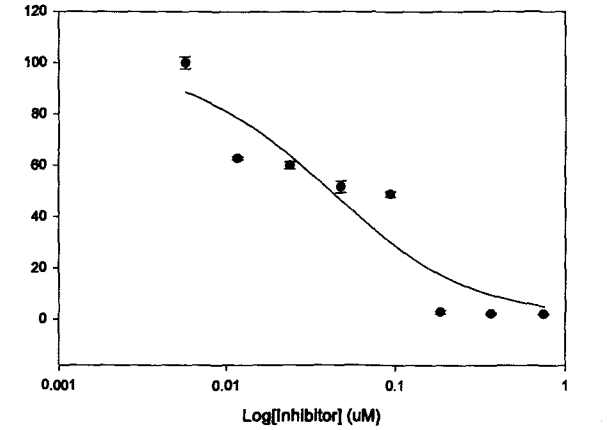
1:3 ART:DHAA Dd2



1:3 ART:ATENE Dd2

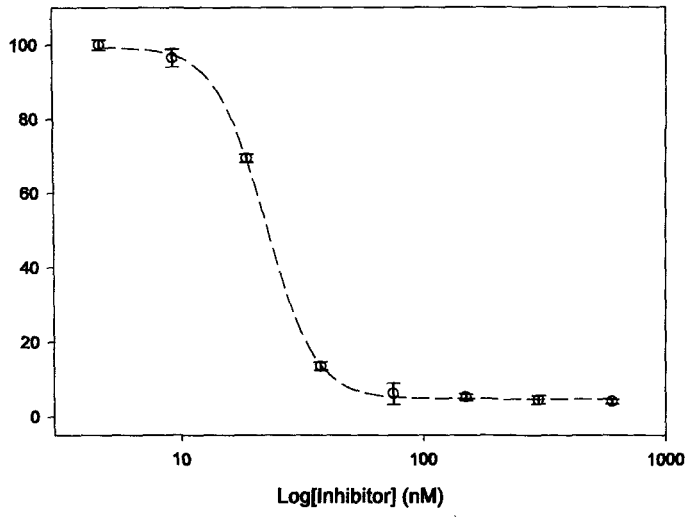


ART Dd2

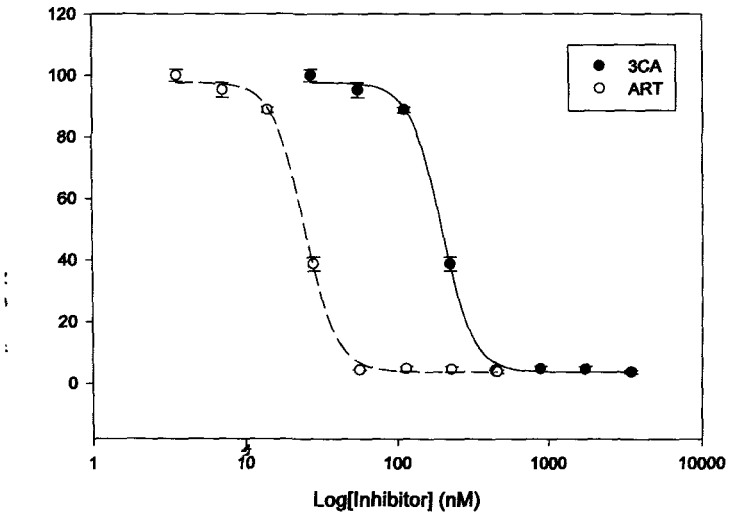


3CA + ART, HB3

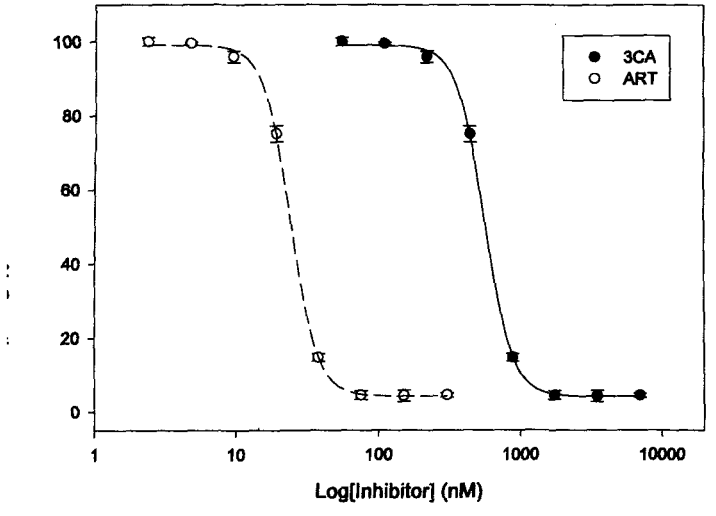
3CA/ART 0:4 HB3



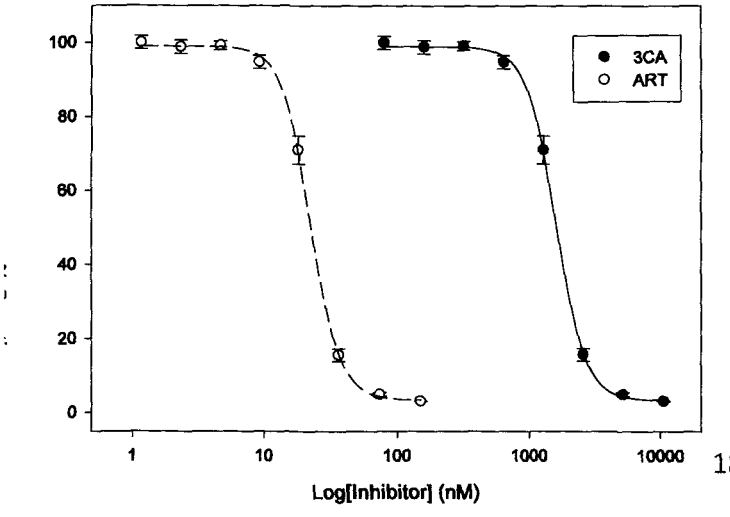
3CA/ART 1:3 HB3



3CA/ART 1:1 HB3

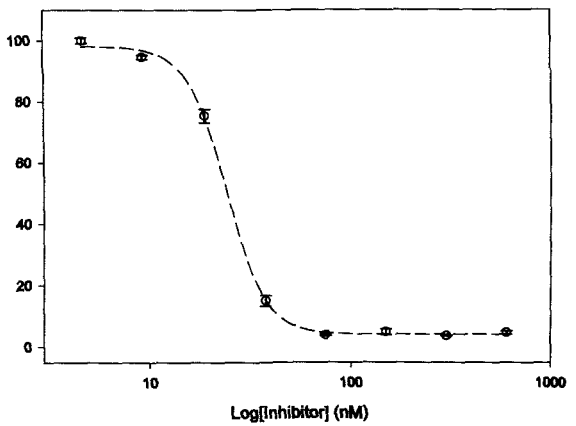


3CA/ART 3:1 HB3

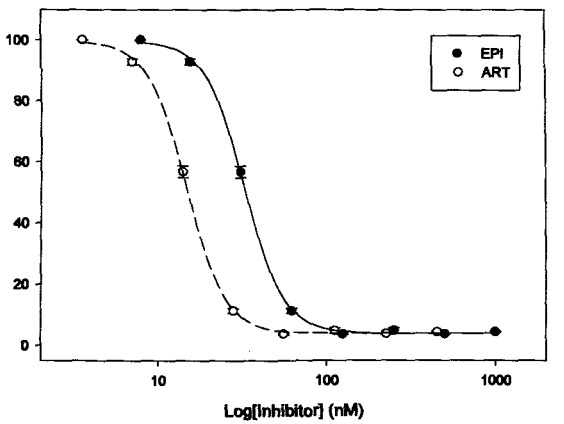


EPI + ART, HB3

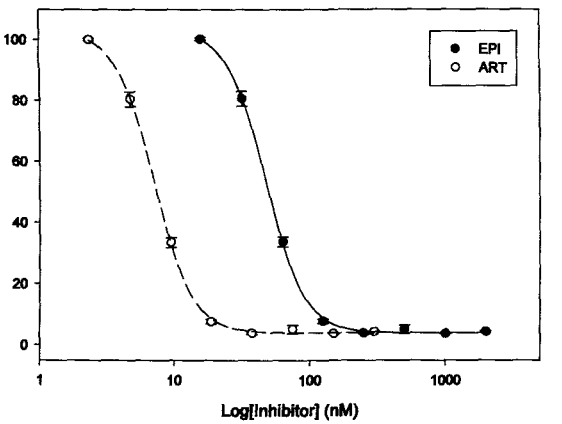
EPI/ART 0:4 HB3



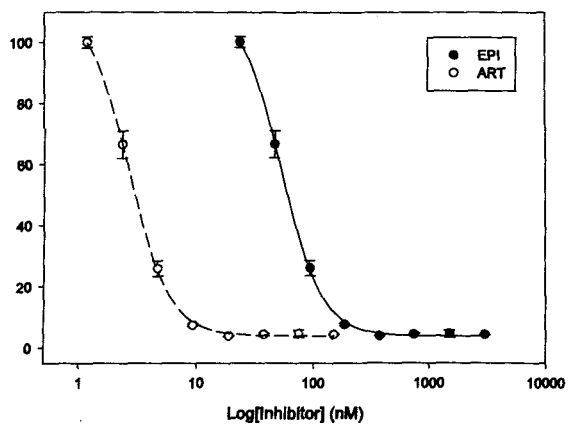
EPI/ART 1:3 HB3



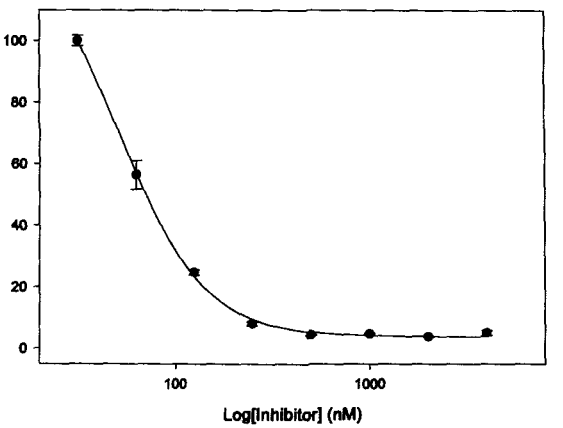
EPI/ART 1:1 HB3



EPI/ART 3:1 HB3

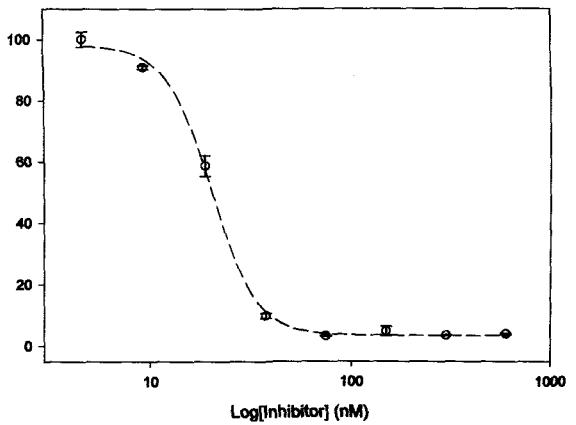


EPI/ART 4:0 HB3

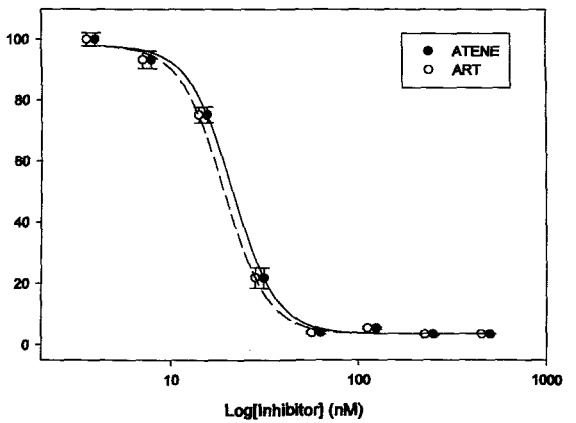


ATENE + ART, HB3

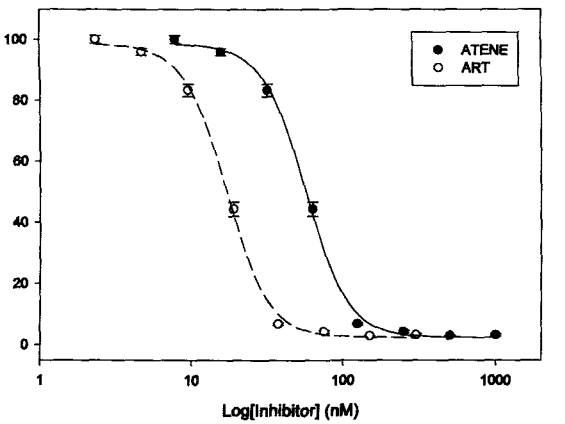
ATENE/ART 0:4 HB3



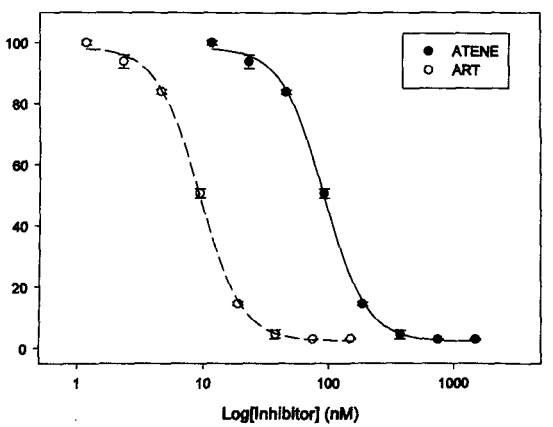
ATENE/ART 1:3 HB3



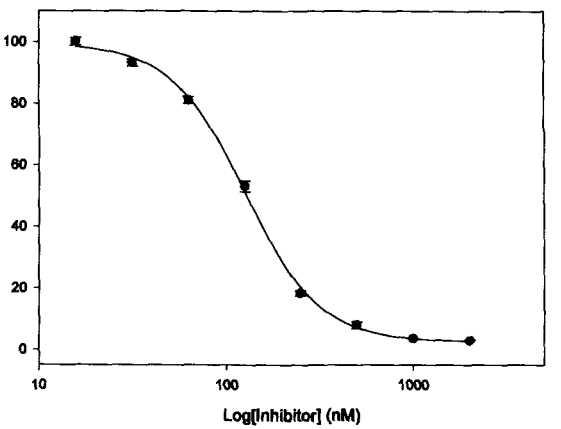
ATENE/ART 1:1 HB3



ATENE/ART 3:1 HB3

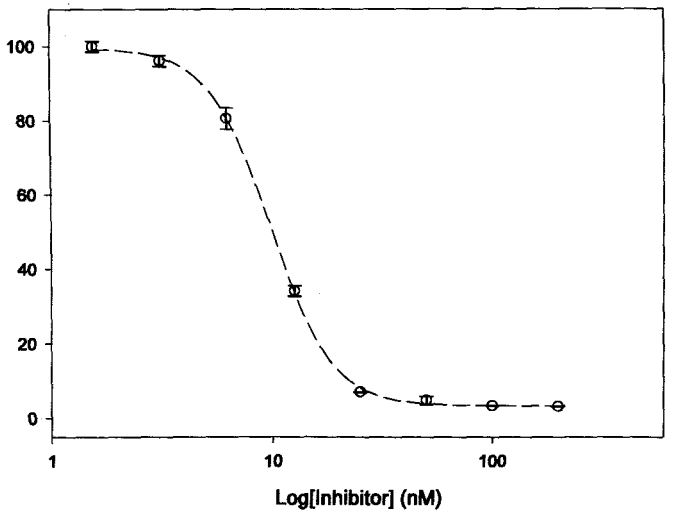


ATENE/ART 4:0 HB3

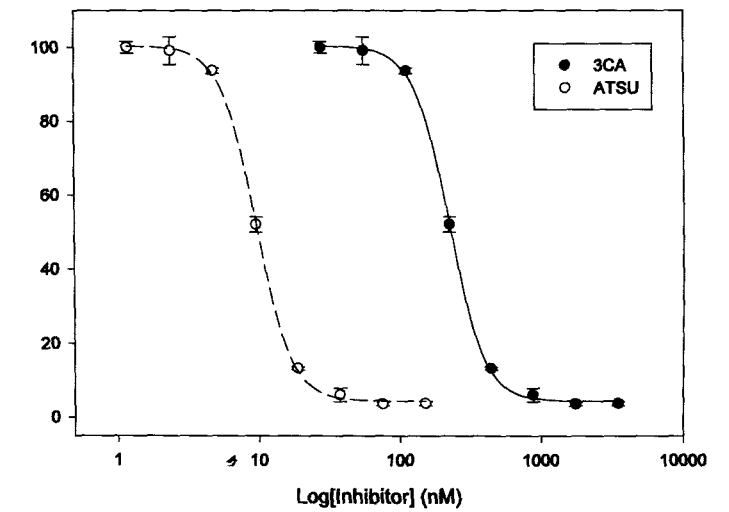


3CA + ATSU, HB3

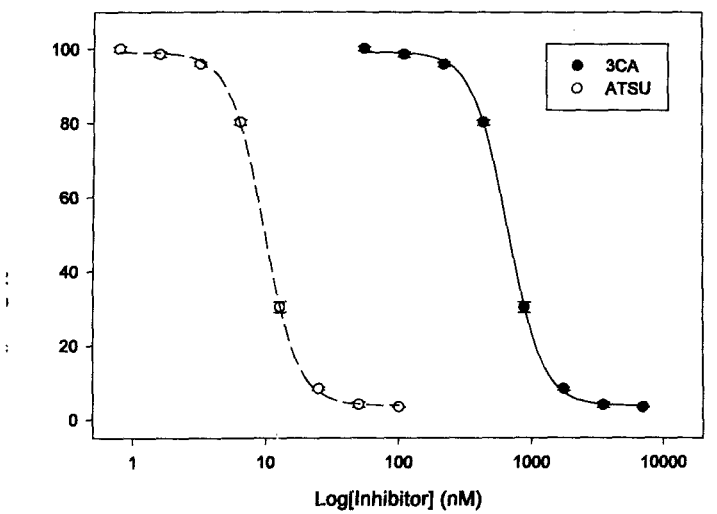
3CA/ATSU 0:4 HB3



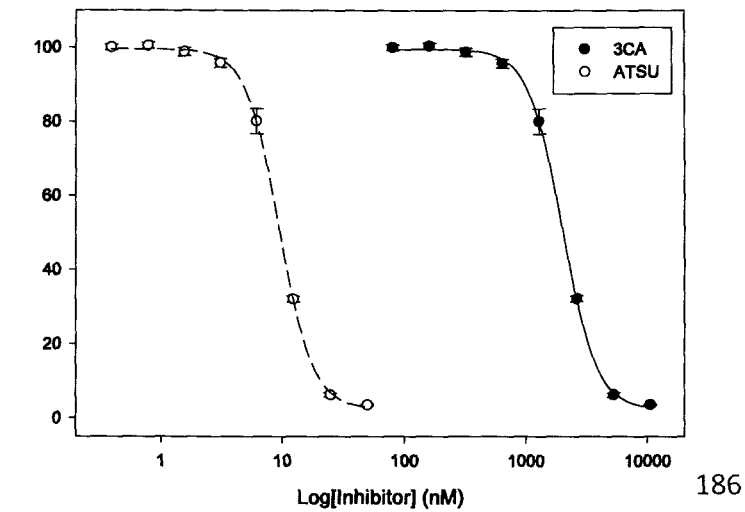
3CA/ATSU 1:3 HB3



3CA/ATSU 1:1 HB3

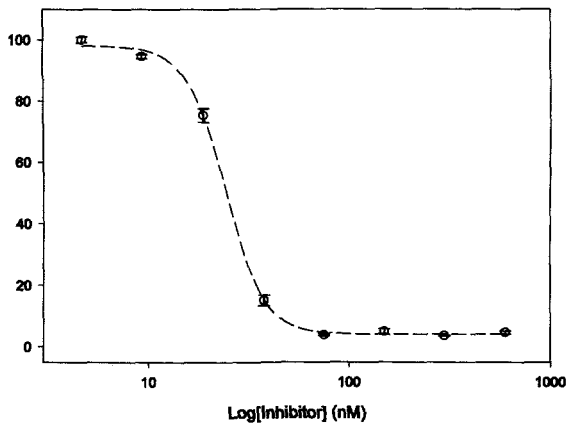


3CA/ATSU 3:1 HB3

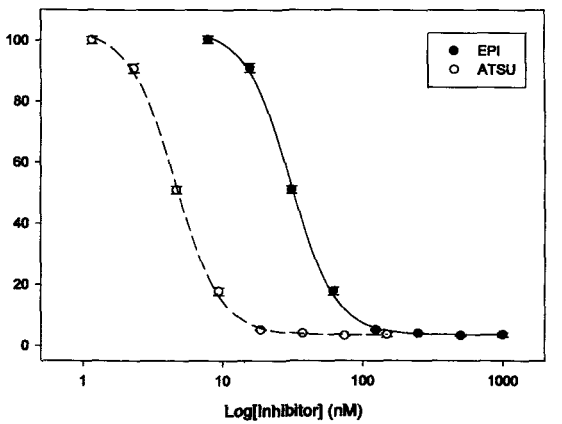


EPI + ATSU, HB3

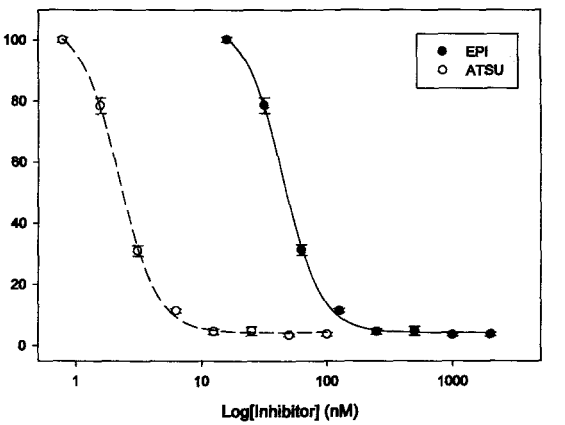
EPI/ART 0:4 HB3



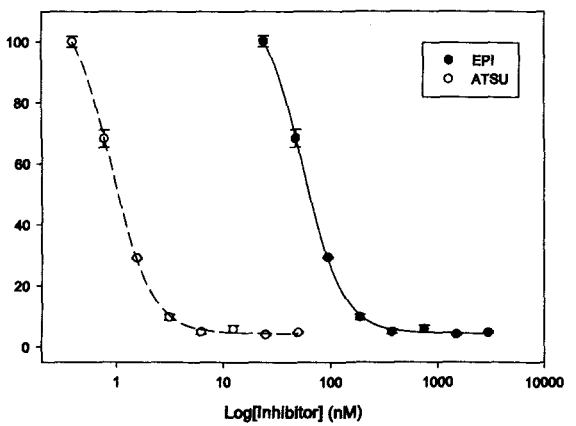
EPI/ATSU 1:3 HB3



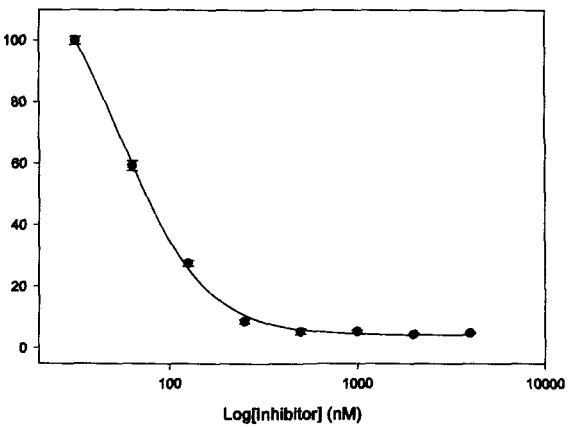
EPI/ATSU 1:1 HB3



EPI/ATSU 3:1 HB3

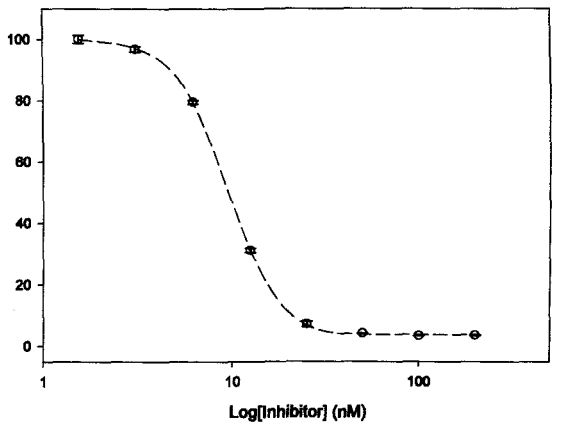


EPI/ATSU 4:0 HB3

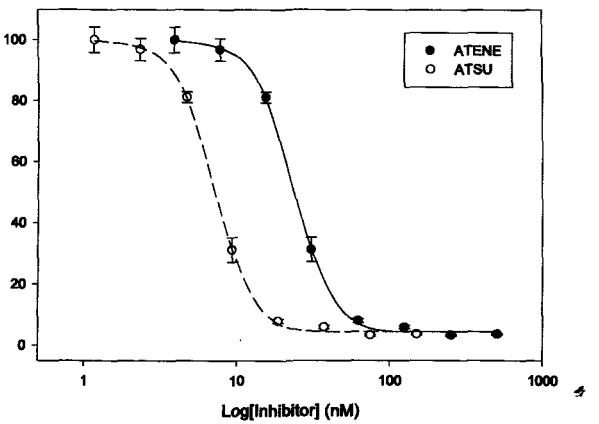


ATENE + ATSU, HB3

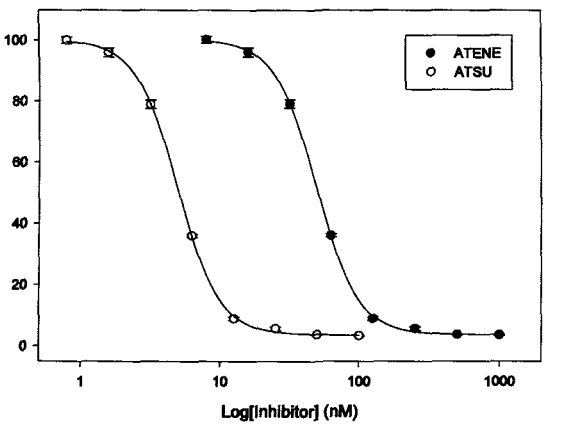
ATENE/ATSU 0:4 HB3



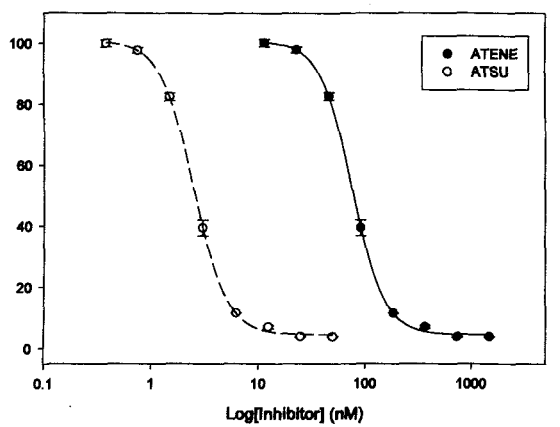
ATENE/ATSU 1:3 HB3



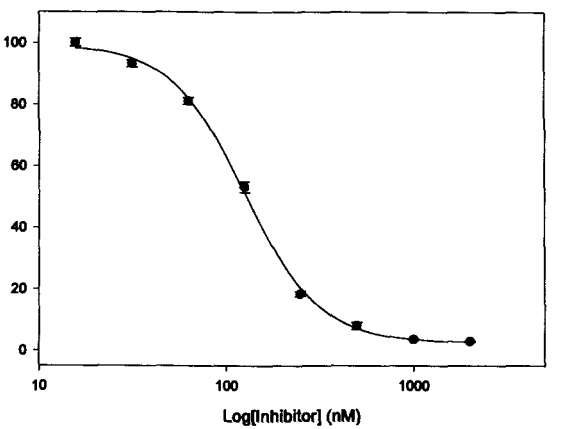
ATENE/ATSU 1:1 HB3



ATENE/ATSU 3:1 HB3

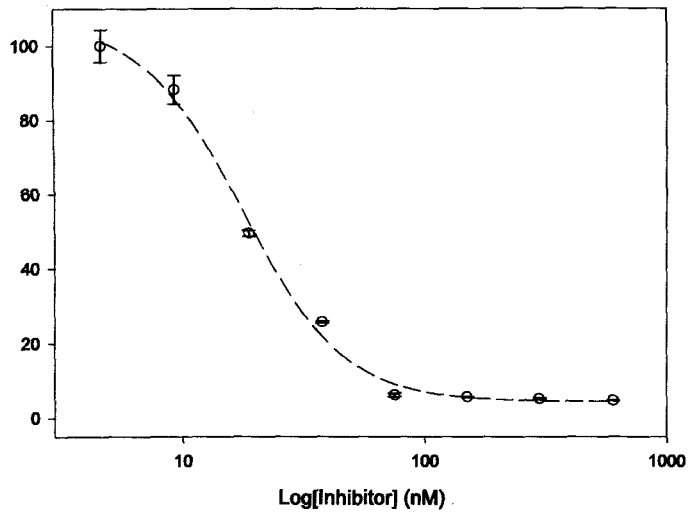


ATENE/ART 4:0 HB3

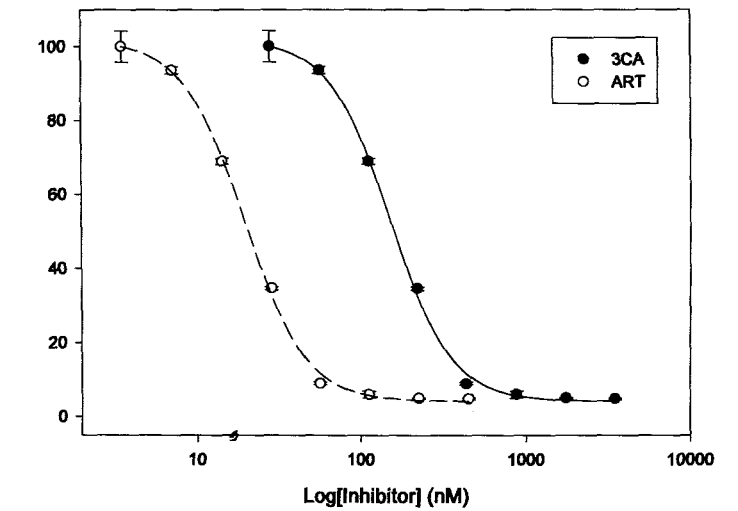


3CA + ART, Dd2

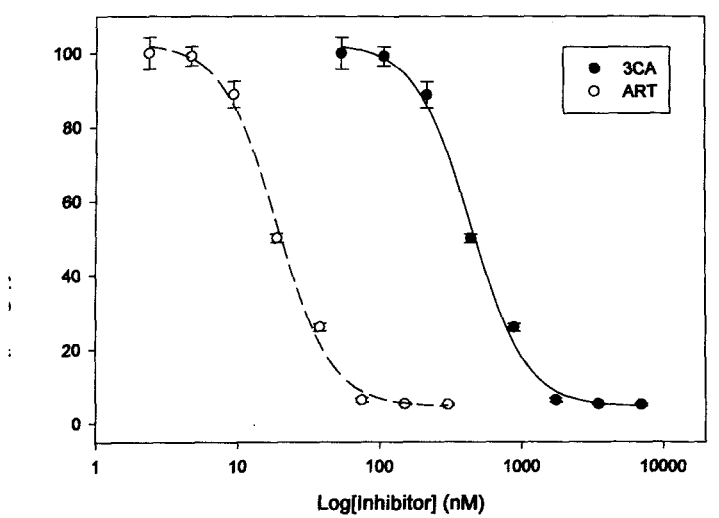
3CA/ART 0:4 Dd2



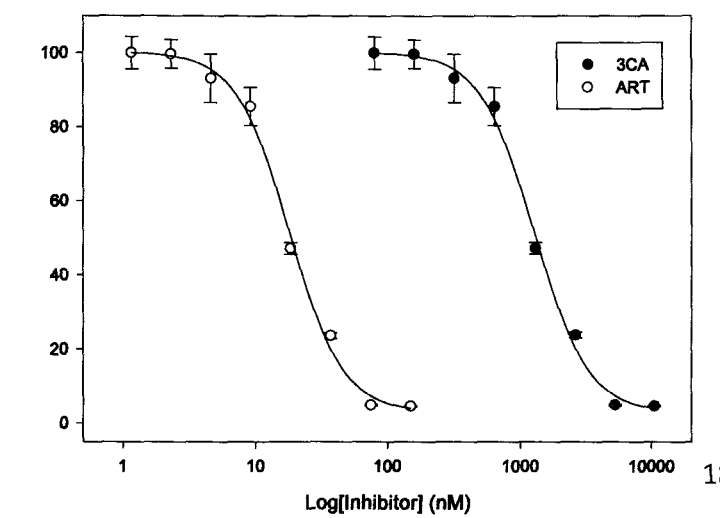
3CA/ART 1:3 Dd2



3CA/ART 1:1 Dd2

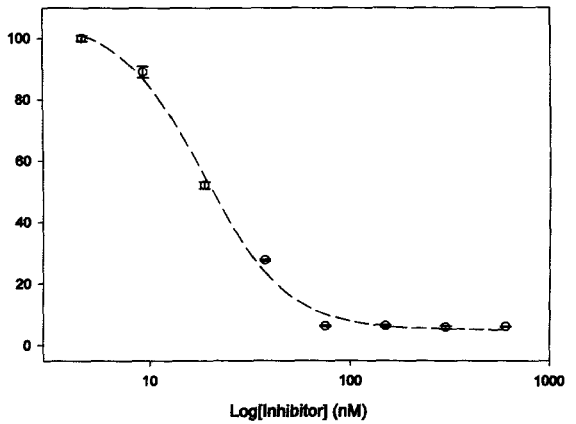


3CA/ART 3:1 Dd2

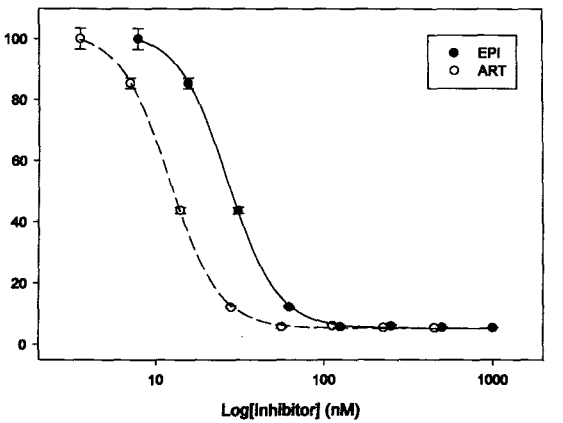


EPI + ART, Dd2

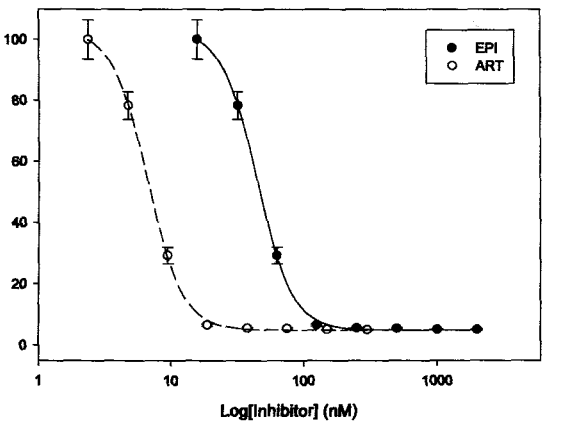
EPI/ART 0:4 Dd2



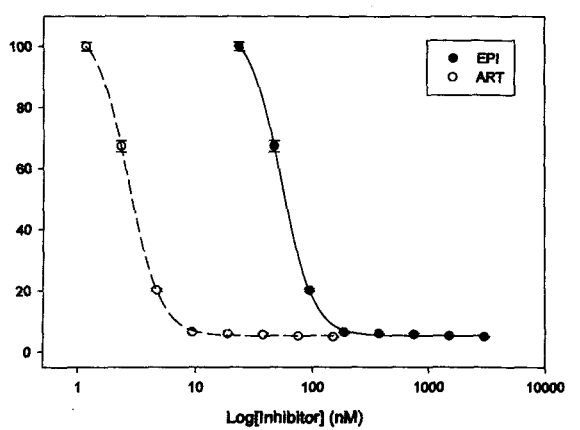
EPI/ART 1:3 Dd2



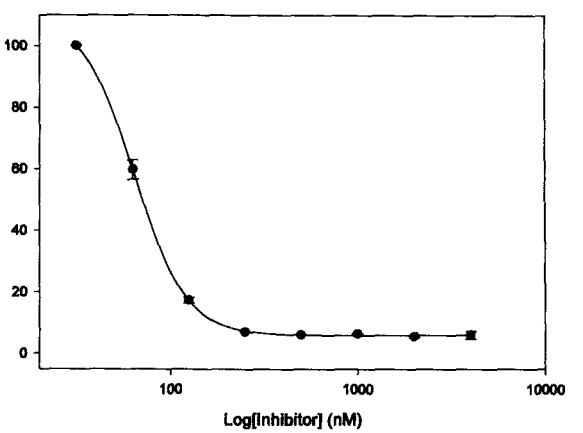
EPI/ART 1:1 Dd2



EPI/ART 3:1 Dd2

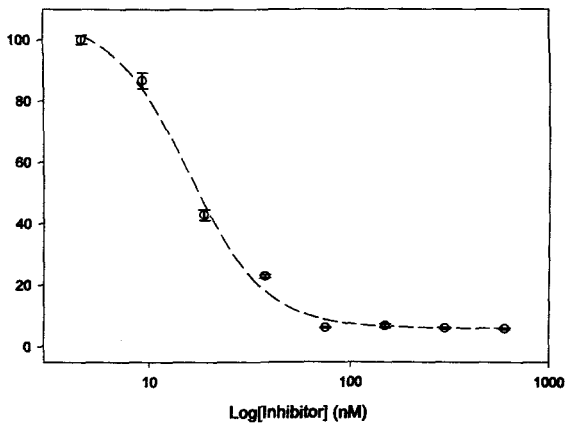


EPI/ART 4:0 Dd2

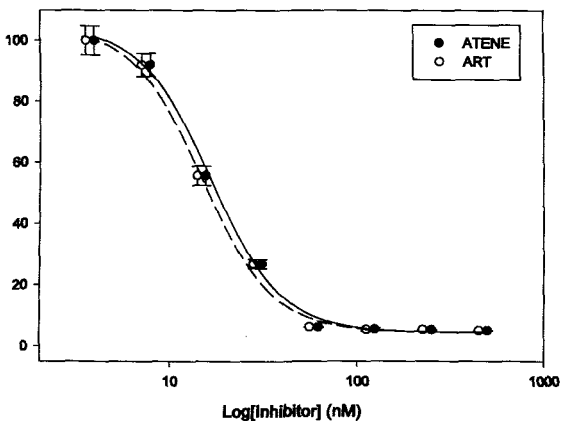


ATENE + ART, Dd2

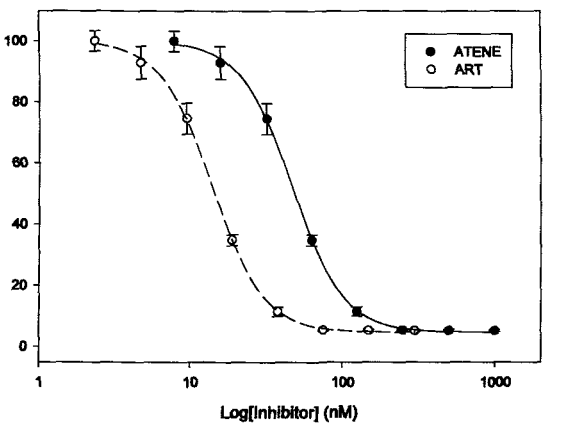
ATENE/ART 0:4 Dd2



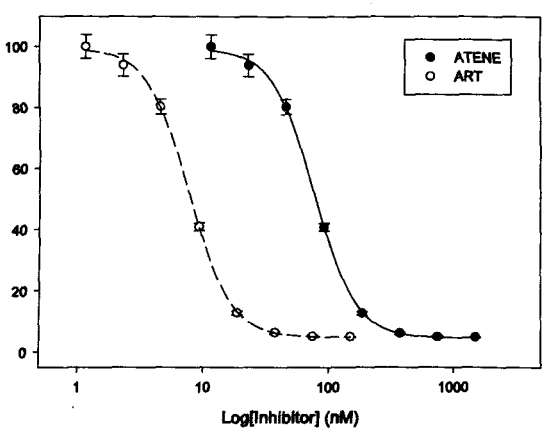
ATENE/ART 1:3 Dd2



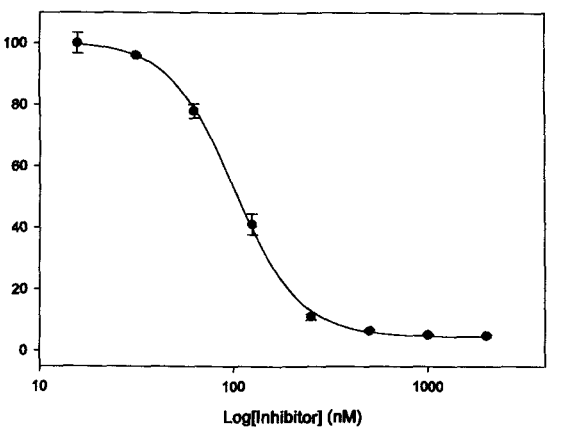
ATENE/ART 1:1 Dd2



ATENE/ART 3:1 Dd2

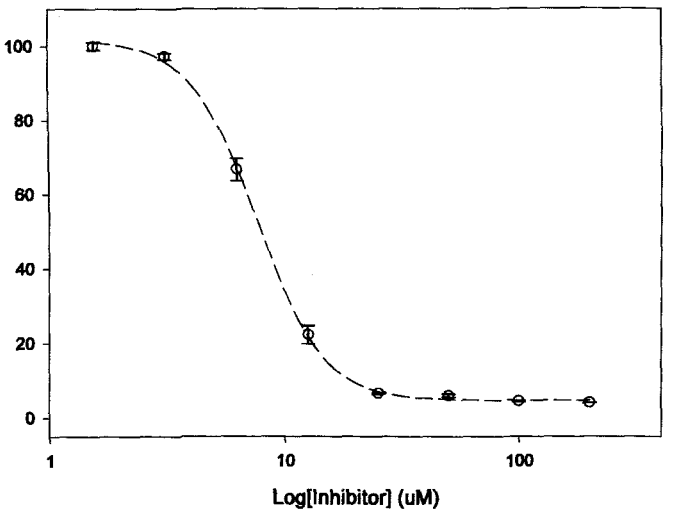


ATENE/ART 4:0 Dd2

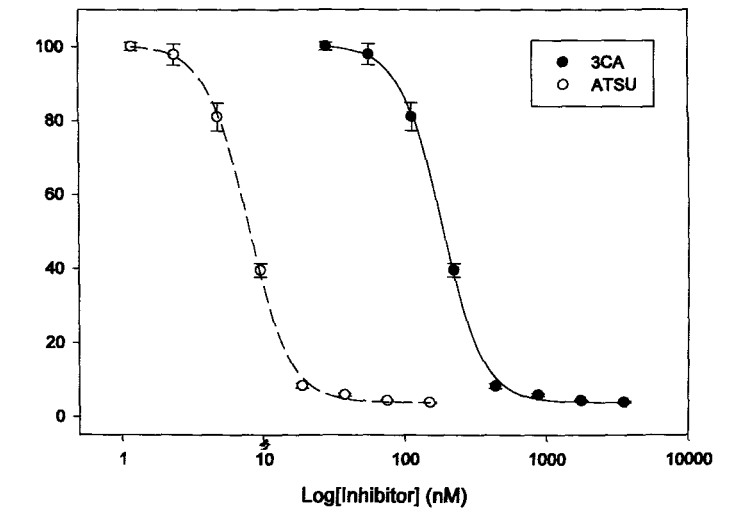


3CA + ATSU, Dd2

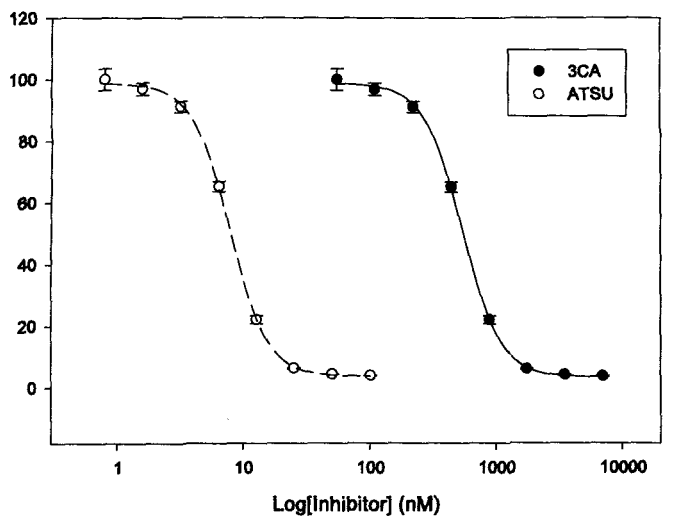
3CA/ATSU 0:4 Dd2



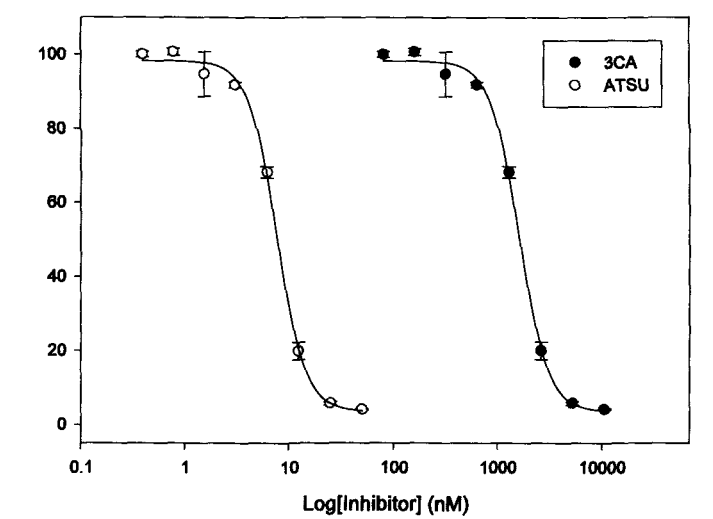
3CA/ATSU 1:3 Dd2



3CA/ATSU 1:1 Dd2

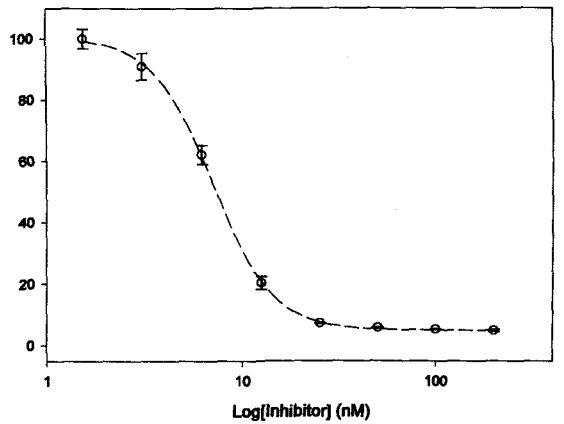


3CA/ATSU 3:1 Dd2

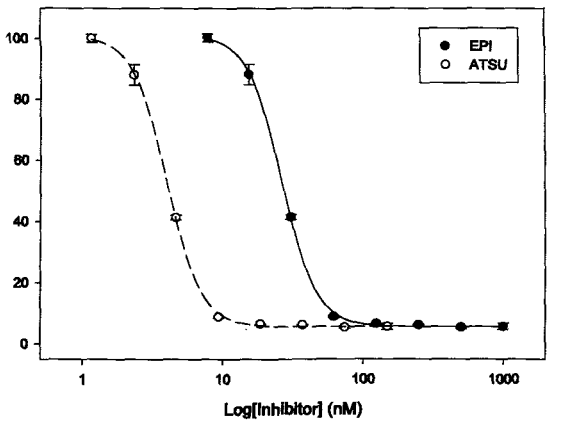


EPI + ATSU, Dd2

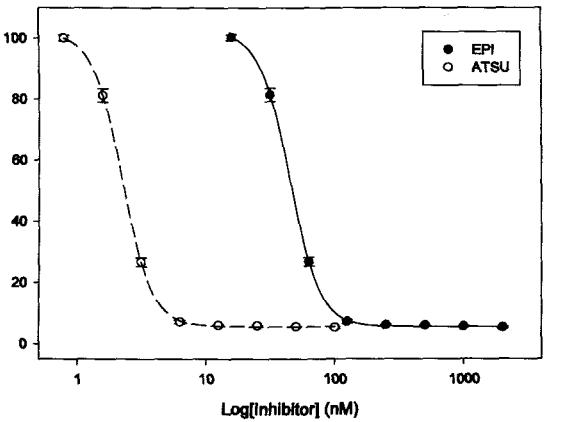
EPI/ATSU 0:4 Dd2



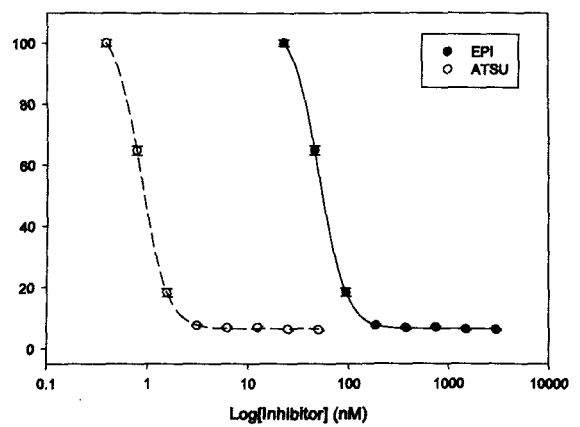
EPI/ATSU 1:3 Dd2



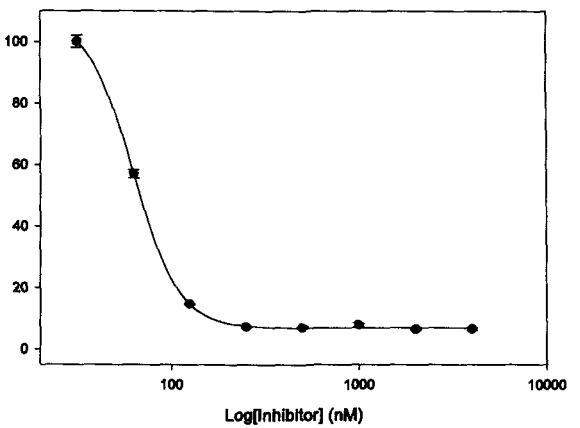
EPI/ATSU 1:1 Dd2



EPI/ATSU 3:1 Dd2

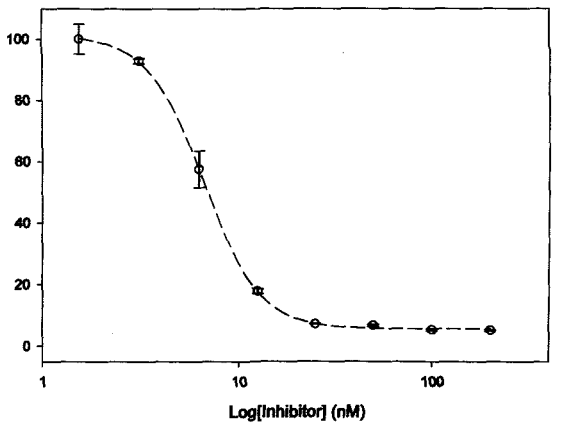


EPI/ATSU 4:0 Dd2

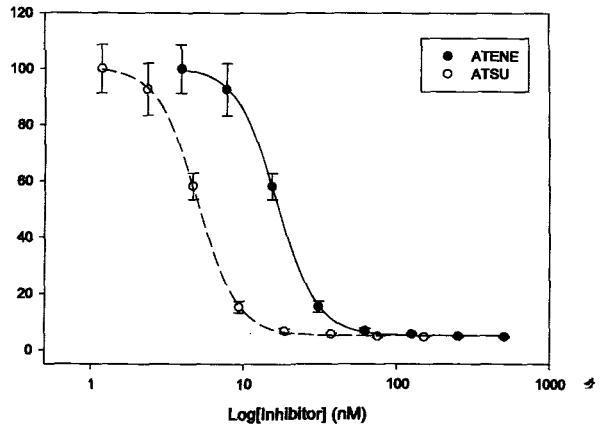


ATENE + ATSU, Dd2

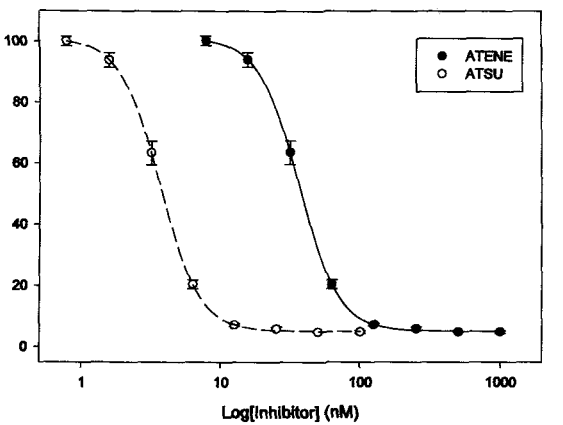
ATENE/ATSU 0:4 Dd2



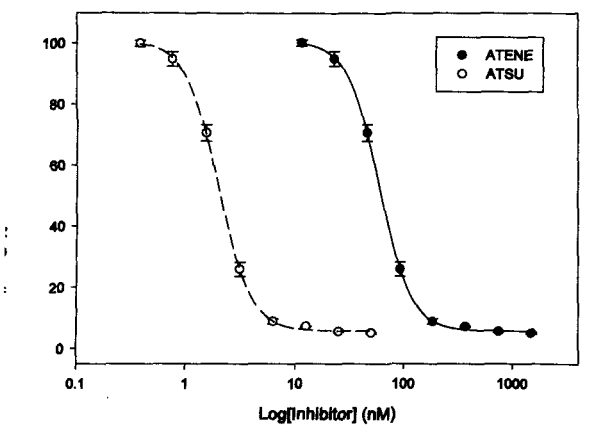
ATENE/ATSU 1:3 Dd2



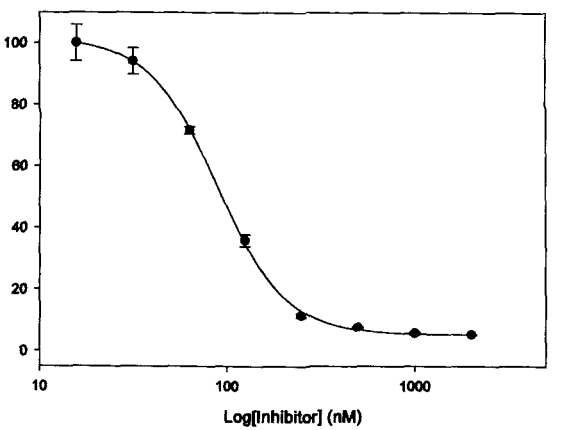
ATENE/ATSU 1:1 Dd2



ATENE/ATSU 3:1 Dd2



ATENE/ATSU 4:0 Dd2



Journal of Pharmaceutical and Biomedical Analysis 84 (2013) 269–277

Contents lists available at SciVerse ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

A rapid method for the determination of artemisinin and its biosynthetic precursors in *Artemisia annua* L. crude extracts

John Suberu[a,c], Lijiang Song[b], Susan Slade[c], Neil Sullivan[d], Guy Barker[c,*], Alexei A. Lapkin[e,**]

[a] School of Engineering, University of Warwick, Coventry CV4 7AL, UK
[b] Department of Chemistry, University of Warwick, Coventry CV4 7AL, UK
[c] School of Life Sciences, University of Warwick, Coventry CV4 7AL, UK
[d] Sensapharm Ltd, Business and Innovation Centre, Sunderland SR5 2TA, UK
[e] Department of Chemical Engineering and Biotechnology, University of Cambridge, CB2 3RA, UK

ARTICLE INFO

Article history:
Received 7 March 2013
Received in revised form 19 June 2013
Accepted 20 June 2013
Available online xxx

Keywords:
Artemisinin
9-Epi-artemisinin
Liquid chromatography
Multiple reaction monitoring (MRM)
Tandem mass spectrometry

ABSTRACT

A rapid high-pressure liquid chromatography (HPLC) tandem mass spectrometry (TQD) method for the determination of artemisinin, 9-epi-artemisinin, artemisitene, dihydroartemisinic acid, artemisinic acid and arteannuin B in *Artemisia annua* extracts is described. Detection and quantification of 9-epi-artemisinin in crude extracts are reported for the first time. In this method all six metabolites are resolved and eluted within 6 min with minimal sample preparation. A recovery of between 96.25% and 103.59% was obtained for all metabolites analysed and the standard curves were linear ($r^2 > 0.99$) over the concentration range of 0.15–10 μg mL^{-1} for artemisinin, 9-epi-artemisinin, artemisitene and arteannuin B, and the range of 3.75–120 μg mL^{-1} for dihydroartemisinic acid and artemisinic acid. All validation indices were satisfactory, showing the method to be robust, quick, sensitive and adequate for a range of applications including high throughput (HTP) analysis.

1. Introduction

Malaria is a life threatening disease transmitted by mosquitoes with about half of the world's population at the risk of the disease [1]. Although death from malaria has been decreasing globally since 2005 [2], there is the fear of a reversal due to the parasite developing resistance to traditional anti-malaria drugs. The discovery of artemisinin (**1**) three decades ago and the development of the artemisinin-based semi-synthetic drugs used in combination therapies (ACTs) backed by the World Health Organization (WHO) have provided a highly effective treatment against *falciparum*-type malaria in many countries [3–5]. Currently, the worldwide demand for ACT treatments is approximately 100 million doses annually [3–5].

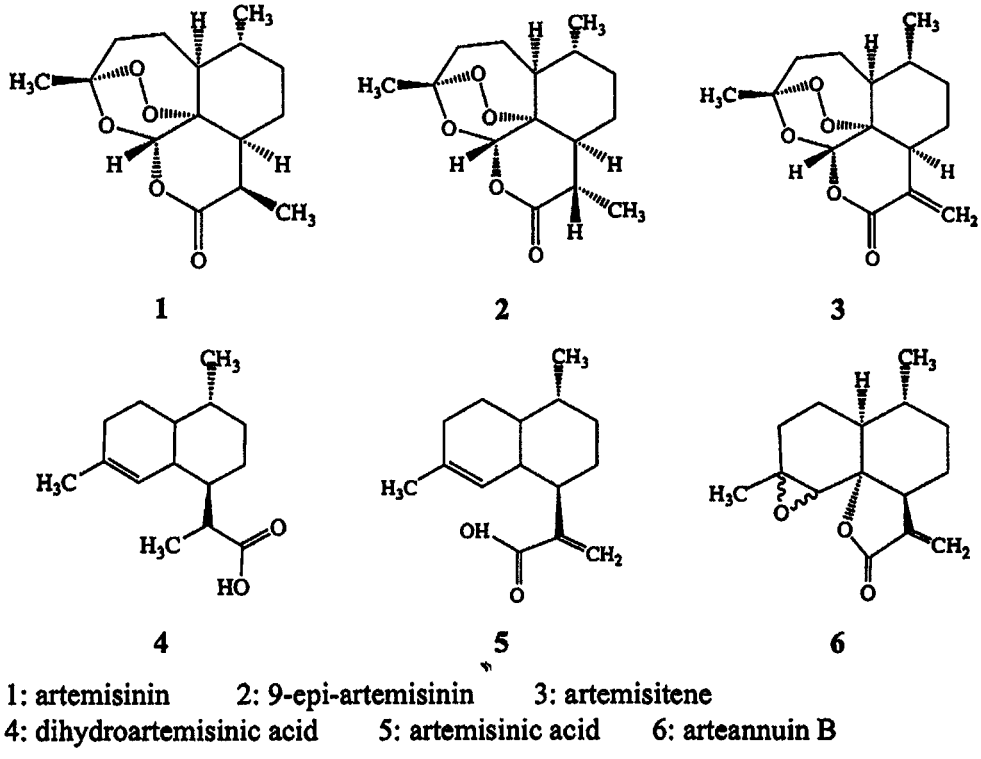
Until recently, the only commercially viable source of (**1**) was *Artemisia* (Asteraceae) plant. *Artemisia annua* L., *Artemisia apiacea* Hance, and *Artemisia lancea* Vanoit are the three species in the genus *Artemisia* that have been reported to contain significant amounts of (**1**) with most of the interest focused on *A. annua* [6,7]. Crystallization from extracts of dried plant biomass is the simplest method of recovery of (**1**) and with improved plant breeding methods reported yields are up to about 1.5% dry weight [8–10]. Commercial interests are also focused on other biosynthetic precursors that are convertible to artemisinin analogues [11].

There are several published methods for the analysis of (**1**) and other related sesquiterpenes including 9-epi-artemisinin (**2**), artemisitene (**3**), dihydroartemisinic acid (**4**), artemisinic acid (**5**), and arteannuin B (**6**). However more rapid, sensitive, accurate and all-in-one methods are still needed for these metabolites (Scheme 1).

Techniques developed and validated for analysis of artemisinin include thin layer chromatography (TLC) [12], high performance liquid chromatography with ultra violet detection (HPLC-UV) [13,14], electrochemical detection (HPLC-ECD) [15], evaporative light scattering detection (HPLC-ELSD) [16], and refractive index (HPLC-RI) [16]. HPLC-UV is the WHO recommended method and the most widely used. However because (**1**) has very weak UV adsorption above 210 nm, the use of UV detection at the end of an HPLC separation requires very careful set-up and calibration, especially for analysis of extracts [15]. An earlier method involved conversion of (**1**) to a UV absorbing compound Q260 to facilitate its detection [17–19]. The disadvantage of this method is that the derivatization procedure from (**1**) to Q260 is time consuming and introduces significant experimental errors [20]. Other methods

* Corresponding author. Tel.: +44 (0)1223 330141; fax: +44 (0)24 765 23568.
** Corresponding author. Fax: +44 (0)1223 334796.
E-mail addresses: guy.barker@warwick.ac.uk (G. Barker), aal35@cam.ac.uk (A.A. Lapkin).

0731-7085/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jpba.2013.06.025



Scheme 1. Chemical structures of metabolites of interest.

include gas chromatography with flame ionization (GC-FID) [21,22], supercritical fluid chromatography with ELSD [23], FID [24] and MS [25] detection. NMR [26] and immunoassay [27] methods have also been reported. An excellent review of these techniques in greater detail was published by Christen and Veuthey [23].

Mass and tandem mass spectrometry based methods have the advantage of high sensitivity and selectivity for metabolites in plant extracts [28]. Several gas chromatography and liquid chromatography methods coupled to mass spectrometry (GC–MS [22], LC–MS [28,29]) and tandem mass spectrometry (GC–MS/MS [30], LC–MS/MS [31]) have been reported for the determination of (**1**) and its derivatives in blood, plasma, serum and plant extracts. The MS/MS method developed by Van Nieuwerburgh et al. [31] for the analysis of (**1**) and its biosynthetic precursors in *A. annua* takes about 20 min to analyse four metabolites.

Here we describe an MS/MS method for the analysis of six analogues of (**1**), including (**2**), in crude plant extract with minimal sample preparation, a simple binary mobile phase solvent system and a short overall analysis time, lending the method to high throughput (HTP) analysis with low consumables costs and a reduced environmental impact.

2. Experimental

2.1. Chemicals

Artemisinin reference standard (98%) was obtained from Sigma–Aldrich (Dorset, UK). Samples of artemisinin were also kindly provided by Neem Biotech (Newport, UK), Chemotechnica (Argentina) and Chengdu (Sichuan Xieli Pharmaceutical Co. Ltd., China). These samples were obtained following purification of extracts from *A. annua* plants cultivated in UK, China and Argentina. Dihydroartemisinic acid (> 99.8%) was gifted by Rodger Stringham (Clinton Foundation, USA). 9-Epi-artemisinin (98%) was sourced from Sensapharm Ltd. (Sunderland, UK), while arteannuin B, artemisitene and artemisinic acid were kindly provided by Walter Reed Army Institute of Research (Washington, USA). LC–MS grade formic acid in water, acetonitrile and HPLC grade acetonitrile were obtained from Fisher Scientific, UK. Purified water (~18 MΩ/cm) was dispensed from a Milli-Q system (Millipore, UK).

2.2. Plant samples

High-yielding *A. annua* biomass samples were obtained from Mediplant (Switzerland), BIONEXX (Madagascar), REAP (Kenya) and ANAMED (Germany). Dried plant leaves were stored at −20 °C. Mutant (glandless) *A. annua* plant samples were kindly provided by Prof. P. Weathers (Worcester Polytechnic Institute, MA, USA). *Hippophae rhamnoides* L. (Elaeagnaceae) (Sea-buckthorn) used as a negative control was obtained from the Centre for Alternative Land Use, CALU (Bangor University, Wales, UK).

2.3. Analytical standards

Standard stock solutions of 1 mg mL^{-1} of artemisinin (**1**), 9-epi-artemisinin (**2**), artemisitene (**3**), dihydroartemisinic acid (**4**), artemisinic acid (**5**) and arteannuin B (**6**) in acetonitrile were prepared. The analytical standard was a mixture of all six standards in a mobile phase spiked with glandless *Artemisia* plant matrix in the concentration range between 0.15 and 10 μg mL^{-1} for (**1**), (**2**), (**3**) and (**6**). For (**4**) and (**5**) the range of 3.75–120 μg mL^{-1} was used. This was to provide a similar matrix for the standards as with the samples minimizing any possible effect due to ion suppression or enhancement. Glandless *A. annua* plant is devoid of artemisinin and related metabolites [32]. β-Artemether was used as internal standard (IS) at 5 μg mL^{-1} to adjust for possible fluctuations in injection volumes. Based on the response from the IS,

Table 1
TQD parameters for MS/MS experiments.

	Cone voltage (V)	Collision voltage (V)	MRM transitions
Artemisinin (**1**)	24	7	283 → 219 + 229 + 247 + 265
9-Epi-artemisinin (**2**)	30	12	283 → 209 + 219 + 247 + 265
Artemisitene (**3**)	30	10	281 → 217 + 227 + 245 + 263
Dihydroartemisinic acid (**4**)	32	12	237 → 190 + 200 + 218
Artemisinic acid (**5**)	32	12	235 → 190 + 200 + 218
Arteannuin B (**6**)	28	9	249 → 189 + 213 + 221 + 231
β-Artemether (IS)	20	5	299 → 221 + 249 + 267

the instrument QuantLynx software automatically adjust for these fluctuations making the method more robust and accurate.

2.4. Sample extraction and preparation

Samples were extracted using published methods [16,33] with a slight modification. Briefly, 10 mL of n-hexane containing 5% (v/v) ethyl acetate was used to extract 1 g of biomass in a sonication bath (PUL 125, Kerry Ultrasonics, UK) operated at 50 Hz and kept cold with ice for 30 min. The extracts were striped of solvent *in vacuo* and the residue re-suspended in 2 mL acetonitrile. This was then filtered through a 0.2 μm syringe filter (Fisher, UK) to remove waxes and other un-dissolved components. An aliquot of the filtrate was dissolved in the mobile phase and internal standard added for LC–MS/MS analysis. Glanded *A. annua* (sample), glandless *A. annua* (matrix), and *H. rhamnoides* (Sea-buckthorn) used as negative control were all extracted using the above procedure.

In several earlier studies [34–37] solvent extraction was combined with sonication for the extraction of artemisinin and related compounds. Sonication provides mechanical disruption of the contents of the trichomes thereby aiding in extraction. Extraction at cold temperature (<30 °C) minimizes the amount of chlorophyll and other interfering components extracted. Briars and Paniwnyk [37] also observed an increase in the amount of artemisinin in plant extracted using ultrasound at 25 °C compared to extraction at higher temperatures without ultra-sonication and conventional steeping at the same temperature. The use of hexane modified by 5% (v/v) ethyl acetate increases the solubility of artemisinin in the mixture by over 3500% compared with hexane alone [9]. Initially we employed an additional sample purification step using SPE columns, however this led to significant losses for some of the metabolites. Therefore a limited extract purification procedure with acetonitrile was used in all consecutive experiments.

Treated plant extract for high resolution ESI-MS analysis (QToF) was prepared by a 20 min contact of the plant extract (above) with activated carbon (AC) and celite at a ratio of 1 g each of AC and celite to 100 mL of extract. This was filtered *in vacuo* with a 0.2 μm Millipore® filter paper.

2.5. Liquid chromatography method

An Acquity liquid chromatograph coupled to a tandem quadrupole detector (TQD) (Waters Corp., Milford, MA, USA) was used in the analysis. The liquid chromatography system consisted of a binary pump, a cooling auto-sampler set at 10 °C with an injection loop of 10 μL. The column heater was set at 30 °C and a Genesis® Lightn C18 column (100 mm × 2.1 mm; 4 μm) (Grace, IL, USA) protected by an Acquity column in-line filter unit was used for the separation of the metabolites. The mobile phase consisted of A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile. Chromatographic separation was achieved using a linear gradient: 0–7.0 min, 25–98% B; 7–9.5 min, 98% B; 9.5–10 min, 98–25% B; 10–15 min, 25% B; at a flow rate of 0.4 mL min^{-1}. Weak wash solvent was 10% acetonitrile. The strong and needle wash solvents were a mixture of acetonitrile, propan-2-ol, methanol and water (30:30:30:10, v/v/v/v).

2.6. Multiple reaction monitoring (MRM) method

The MS/MS system was operated with an ESI interface in positive ionization mode (ESI^+) and acquisition was performed in MRM mode. The cone and de-solvation gas flow rates were set at 45 L h^{-1} and 800 L h^{-1}, respectively while the capillary voltage, the source and de-solvation temperatures were similar for all analytes at 28 kV, 150 °C and 350 °C respectively. MS parameters were automatically defined using Waters IntelliStart® software for the tuning and calibration of the TQD and subsequently manually optimized as shown in Table 1. Quantification was determined using multiple reaction-monitoring (MRM) modes for the above transitions. The dwell time was automatically set at 0.161 s. Data were acquired by MassLynx V4.1 software and processed for quantification with QuanLynx V4.1 (Waters Corp., Milford, MA, USA).

2.7. Dionex RS 3000 method

A Dionex RS 3000 instrument coupled to a Bruker MaXis high-resolution Q-ToF mass spectrometer was used to confirm results obtained from the TQD instrument and obtain accurate mass measurements. The mobile phase consisted of A: water with 0.1% formic acid and B: methanol with 0.1% formic acid. The flow rate was 0.2 mL min^{-1} and a run time of 29 min. The gradient was as follows: 0–10 min, isocratic 55% B; 10–15.4 min, 55–100% B; 15.4–20.4 min, isocratic 100% B; 20.4–23.4 min, 100–55% B, then isocratic for 5 min before next run. Separation was achieved on a Zorbax RPC18 2.1 mm × 100 mm, particle size 2.2 μm with 2 μL injection volume.

2.8. Q-ToF high resolution – MS

The Bruker MaXis UHR-Q-ToF mass spectrometer was operated under the following conditions: ionization mode: ESI (+); MS scan range: 50–2500 *m/z*; end plate offset: −500 V; capillary: −3000 V. Nebulizer gas (N_2): 0.4 bar; dry gas (N_2): 4 L min^{-1}; dry temperature: 180 °C. Ion transfer conditions: funnel RF: 200 Vpp; multiple RF: 200 Vpp; quadruple low mass: 55 *m/z*; collision energy: 5.0 eV; collision RF: 600 Vpp; ion cooler RF: 50–250 Vpp ramping. Transfer time: 121 μs; Pre-Pulse Storage time: 1 μs. Calibration was achieved before each run through a loop injector of 20 μL of sodium formate (10 mM).

3. Results and discussion

3.1. Chromatography

Gradient and isocratic LC methods were tested to optimize the conditions for resolution of all the metabolites and the internal standard. A buffered system similar to those employed by Van Nieuwerburgh et al. [31] was also tested. The best resolution was obtained with the method described in Section 2.5. All metabolites and internal standard were successfully resolved in the first 6 min

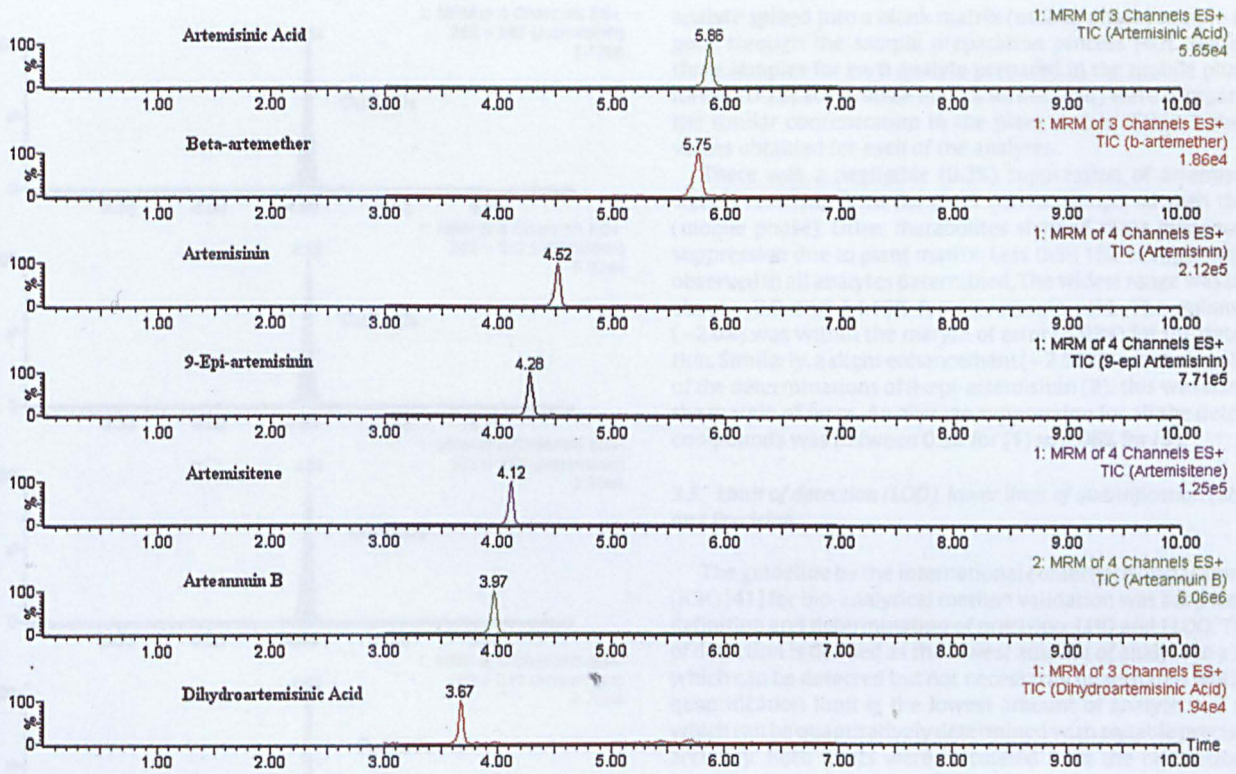


Fig. 1. Total ion current chromatography (TIC) with retention times and chemical structures for all metabolites of interest and the internal standard (β-artemether). The chromatograms were acquired by multiple reaction monitoring (MRM) in positive electro-spray mode using analytical standards at a concentration of 5 μg mL^{-1} for all analytes and internal standard except for artemisinic acid and dihydroartemisinic acid which were determined at 60 μg mL^{-1}.

of run time. Fig. 1 shows the total ion current chromatogram (TIC) for all the analysed compounds.

3.2. Recovery

The recovery was assessed using ten equal samples of 1 g of dried *A. annua* (Madagascar variety). Six of these extracts were not spiked while four were spiked with a mixture of each analyte to give the final concentration in the prepared extract of 2.5 μg mL^{-1} for artemisinin, 5.0 μg mL^{-1} for 9-epi-artemisinin, artemisitene and arteannuin B, 30 μg mL^{-1} for dihydroartemisinic acid and artemisinic acid. Table 2 shows a recovery of between 103.59 and 96.25% was obtained for the analytes investigated.

3.3. Specificity

Fig. 2 shows the possible MRM transitions for artemisinin. Three or four transitions were monitored for the MS/MS experiment to identify and quantify each metabolite. The sum of combined transitions gave the total ion current (MRM) data while the signal with the highest *m/z* value was used for the quantification of each analyte.

MS/MS based assay are inherently specific. However to investigate the specificity of the method further, extracts of glandless *A. annua* and *H. rhamnoides* (Sea-buckthorn) were analysed. In these negative control extracts we found no components of interest in the chromatograms from the MS/MS experiments. The result for

Table 2
Recovery of artemisinin and analogues from *A annua*.

Spiked analyte quantities (μg mL^{-1})	Artemisinin* (1)	9-Epi-artemisinin* (2)	Artemisitene (3)	Dihydroartemisinic acid* (4)	Artemisinic acid (5)	Arteannuin B (6)
Mean quantity in un-spiked sample[a]	7.49	0.13	0.05	15.15	0.00	0.39
Spiked quantity[b]	2.50	5.00	5.00	30.00	30.00	2.5
Total quantity in spiked sample[c]	9.99	5.13	5.05	45.15	30.00	2.89
Recovered quantities (μg mL^{-1})	Artemisinin* (1)	9-Epi-artemisin* (2)	Artemisitene (3)	Dihydroartemisinic acid* (4)	Artemisinic acid (5)	Arteannuin B (6)
Spiked sample 1[d]	10.24 (101.56%)	5.08 (98.95%)	5.08 (98.94%)	52.14 (103.89%)	28.65 (92.78%)	3.06 (106.44%)
Spikes sample 2[d]	10.60 (114.72%)	5.24 (102.14%)	4.97 (96.78%)	47.84 (90.74%)	30.62 (99.17%)	2.82 (96.93%)
Spiked sample 3[d]	10.38 (106.53%)	4.92 (95.98%)	5.38 (104.72%)	51.87 (103.04%)	28.30 (91.65%)	3.09 (107.96%)
Spiked sample 4[d]	9.97 (91.53%)	4.98 (97.08%)	5.46 (106.36%)	47.53 (89.80%)	31.31 (101.40%)	2.96 (102.71%)
Mean spiked sample (μg mL^{-1})	10.30 (103.59%)	5.05 (98.53%)	5.22 (101.70%)	49.85 (96.87%)	29.72 (96.25%)	3.05 (103.51%)
Standard deviation (μg mL^{-1})	0.26 (8.44%)	0.14 (2.70%)	0.23 (4.57%)	2.50 (7.64%)	1.47 (4.77%)	0.15 (4.74%)

Ten equal samples of 1 g dried Madagascan *A. annua* leaves were extracted and prepared for analysis. [a]Six of these extracts were un-spiked while 4 were spiked at indicated levels.[b] The total quantity[c] of analyte in samples is calculated as the sum of the mean quantities in six un-spiked sample and the spiked quantity. Analyte levels in individual spiked samples were determined[d] and absolute and percentage (in bracket) recoveries presented.

* Quantitative values corrected for percentage purity.

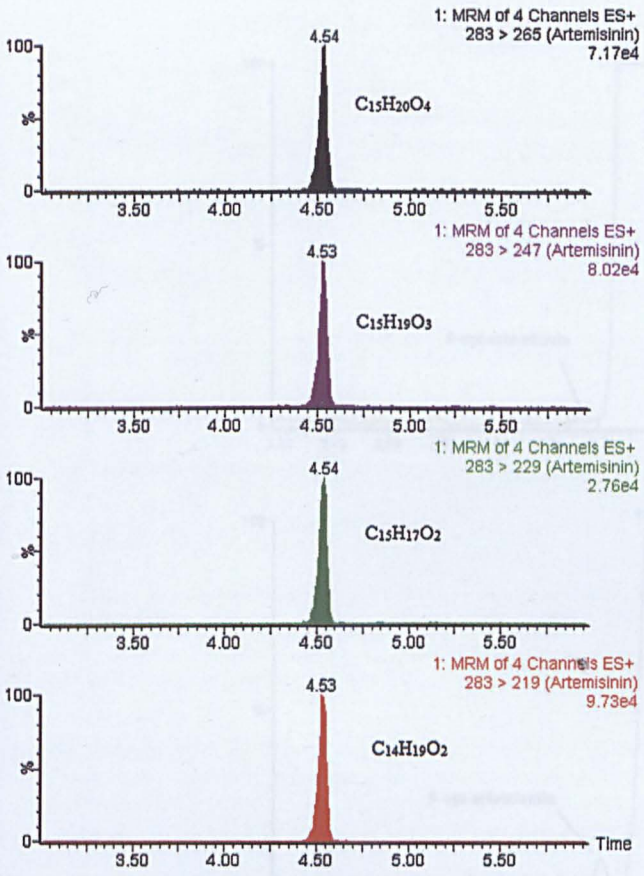


Fig. 2. Multiple reaction monitoring (MRM) chromatogram of standard artemisinin (5 μg mL^{-1}) showing the four transitions chosen for the experiment with their chemical formulas and signal intensities.

the presence of trace level of artemisinin in the glandless biomass was not conclusive and this is being investigated further.

3.4. Ion suppression (matrix effect)

Quantitative analysis of plant and biological samples with positive electro-spray ionization coupled to tandem mass spectrometry is compounded by the presence of matrix components, which can interfere with the analysis hence resulting in ion suppression or enhancement effects. The common methods for the assessment of ion suppression are the post-column infusion method [38] and the post-extraction spike method [39]. The spike method was used in our determination and this was assessed by comparing the response of the analyte in plant matrix to the response of the analyte spiked into a blank matrix (mobile phase) sample that has gone through the sample preparation process [40]. Signals from three samples for each analyte prepared in the mobile phase (25% formic (0.1%) acetonitrile in 0.1% formic acid) were compared with the similar concentration in the plant matrix. Table 3 shows the values obtained for each of the analytes.

There was a negligible (0.3%) suppression of artemisinin (**1**) signals resulting from the plant matrix compared with the blank (mobile phase). Other metabolites showed some evidence of ion suppression due to plant matrix. Less than 15% of suppression was observed in all analytes determined. The widest range was between about −2.0 and 14.13% for artemisinic acid. The enhancement (−2.0%) was within the margin of error (5.02%) for the determination. Similarly, a slight enhancement (−2.58%) was observed for one of the determinations of 9-epi-artemisinin (**2**); this was also within the margin of error. An average suppression for all the determined compounds was between 0.3% for (**1**) to 8.06% for (**5**).

3.5. Limit of detection (LOD), lower limit of quantification (LLOQ), and Precision

The guideline by the international conference on harmonization (ICH) [41] for bio-analytical method validation was adopted for the definition and determination of precision, LOD and LLOQ. The limit of detection is defined as the lowest amount of analyte in a sample, which can be detected but not necessarily quantitated while lower quantification limit is the lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy. Both limits were calculated from the calibration curve following Miller and Miller [42]. Injection precision (repeatability) was calculated with at least six determination of each analyte in a single day. The coefficient of variation (CV) for these determinations is below 10% for all metabolites investigated (Table 4).

Within-day precision was determined for six concentration levels covering the analyte calibration range and making a total of 12–17 analyses on a single day. Between-day precision was calculated for the same calibration range on three different days spread over a month, resulting in a total of 32–40 determinations. The range for the accuracy for both within and between day precision determinations was from 81.42 to 118.81% while the coefficient of variance in both was less than 8.5% (Table 4).

3.6. Regression indices and dynamic range

Good linearity ($r^2 > 0.99$) of the calibration curves for all the analytes of interest in both the mobile phase and the mobile phase spiked with the matrix is indicative of the robustness of the method. The regression and sensitivity indices in Table 4 are for the standards prepared in the mobile phase spiked with extracts of the glandless plant. The method is highly sensitivity for most of the analytes. LLOQ for arteannuin B is about 3.5 pg mL^{-1}. However for

Table 3
Ion suppression due to *A. annua* plant matrix.

	(1)(μg mL^{-1})	(2) (μg mL^{-1})	(3) (μg mL^{-1})	(4) (μg mL^{-1})	(5) (μg mL^{-1})	(6) (μg mL^{-1})
Mean quantity spiked into plant matrix[a]	2.50	5.05	5.08	32.72	30.88	2.49
Quantities in blank matrix[b]						
Sample 1	2.49 (−0.22%)	4.92 (−2.58%)	5.49 (7.95%)	32.86 (0.42%)	35.24 (14.13%)	2.61 (5.03%)
Sample 2	2.52 (0.85%)	5.58 (9.62%)	5.70 (12.14%)	36.17 (9.54%)	34.57 (11.94%)	2.49 (0.0%)
Sample 3	2.51 (0.28%)	5.54 (8.67%)	5.46 (7.37%)	34.20 (4.32%)	30.30 (−1.89%)	2.55 (2.48%)
Mean	2.51 (0.30%)	5.35 (5.30%)	5.58 (6.83%)	34.41 (4.76%)	33.37 (8.06%)	2.55 (2.48%)
Standard error (SE)	0.04 (0.24%)	0.21 (3.95%)	0.08 (1.50%)	0.96 (2.64%)	1.54 (5.02%)	0.04 (1.47%)

[a] Mean of three determinations of spiked standards at 2.5 μg mL^{-1} for artemisinin (**1**) and arteannuin B (**6**), 5 μg mL^{-1} for 9-epi-artemisinin (**2**) and artemisitene (**3**). Dihydroartemisinic acid (**4**) and artemisinic acid (**5**) were spiked at 30 μg mL^{-1} each.

[b] Three determination of blank matrix (mobile phase of 0.1% formic:0.1% formic in acetonitrile, 75:25) spiked with standards at an equivalent level to spiked plant matrix. Percentage suppression or enhancement is shown in brackets.

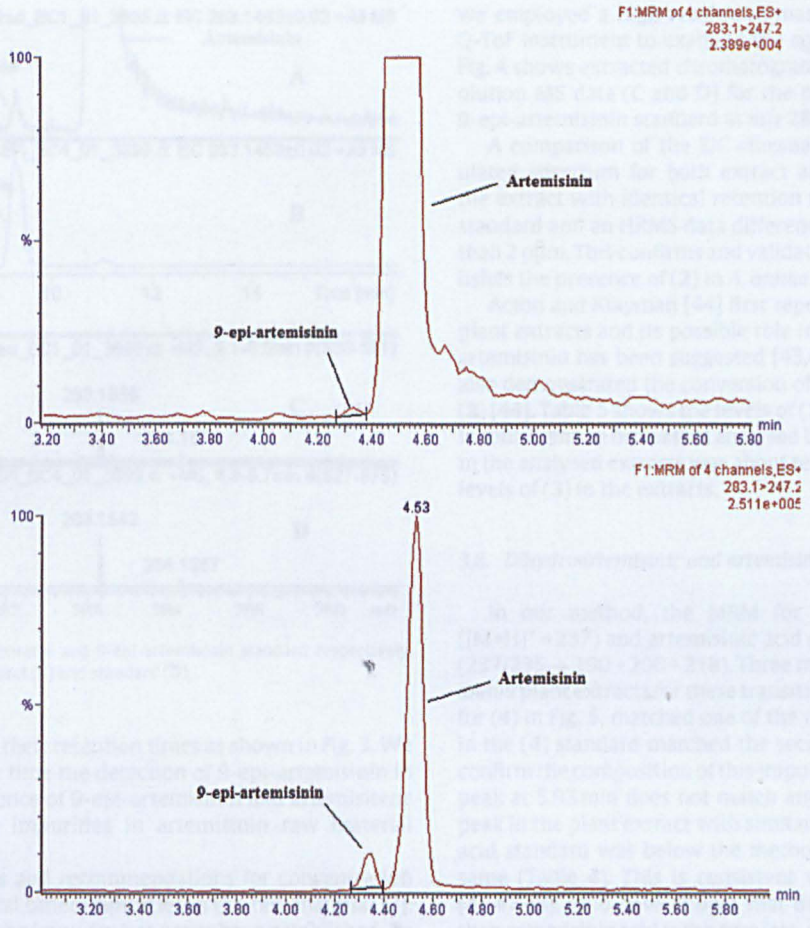


Fig. 3. Top – MRM chromatogram for 9-epi-artemisinin (**2**) in *A. annua* extract also showing artemisinin (**1**) content. Bottom – chromatogram of the same extract spiked at 5 $\mu g\,mL^{-1}$ of (**2**).

dihydroartemisinic acid and artemisinic acid, the LLOQ is much higher at 0.6 and 1.0 $\mu g\,mL^{-1}$ respectively. The dynamic ranges for these compounds reflect the same pattern with a lower range (0.15–10 $\mu g\,mL^{-1}$) for all analytes except dihydroartemisinic acid and artemisinic acid with a range of 3.75–120 $\mu g\,mL^{-1}$.

3.7. Artemisinin, 9-epi-artemisinin and artemisitene

Transitions used for the MS/MS analysis of artemisinin and 9-epi-artemisinin were 283 → 219 + 229 + 247 + 265 and 283 → 209 + 219 + 247 + 265 respectively. The two analytes were

Table 4
LOD, LLOQ, injection precision, within-day and between-day precisions.

	(1)	(2)	(3)	(4)	(5)	(6)
LOD ($\mu g\,mL^{-1}$)[a]	1.3×10^{-4}	1.0×10^{-3}	2.8×10^{-4}	2.0×10^{-1}	3.3×10^{-1}	1.2×10^{-6}
LLOQ ($\mu g\,mL^{-1}$)[a]	4.1×10^{-4}	3.0×10^{-3}	8.4×10^{-4}	6.0×10^{-1}	9.9×10^{-1}	3.5×10^{-6}
Regression equation[a]	$y = 857.32x + 163.1$	$y = 344x - 35.84$	$y = 388.54x - 21.01$	$y = 13.98x + 54.16$	$y = 21.64x + 71.47$	$y = 96645x + 5947.7$
R^2 value	0.99396	0.99623	0.99562	0.99408	0.99803	0.99547
Injection precision[b]						
Mean ($\mu g\,mL^{-1}$)	5.67 ($n = 10$)	0.13 ($n = 8$)	0.24 ($n = 7$)	19.54 ($n = 6$)	n/a	0.32 ($n = 6$)
CV (%)	4.48	4.54	6.24	6.20	n/a	9.89
Within and between-day precision[c]						
Within day range (%)	93.22–114.20 ($n = 12$)	83.09–106.61 ($n = 16$)	86.04–116.35 ($n = 17$)	87.26–111.75 ($n = 14$)	92.73–118.81 ($n = 13$)	82.32–112.69 ($n = 15$)
CV (%)	7.26	5.92	7.60	7.96	7.10	7.70
Between day range (%)	82.78–117.88 ($n = 36$)	83.09–109.07 ($n = 37$)	81.65–116.35 ($n = 40$)	81.42–114.33 ($n = 38$)	92.32–118.81 ($n = 32$)	82.32–119.98 ($n = 37$)
CV (%)	7.86	6.47	7.37	8.30	6.26	8.26

n/a – undetermined values below method's LLOQ.

[a] Calculation based on 12 point calibration graph and the following formulas LOD = $Y_B + 3S_B$ and LLQD = 3LOD, where Y_B is the signal equal to the blank signal (the y intercept) and S_B is standard deviation of the blank (the random error in the y-direction) [41].

[b] Injection precision was assessed by n determination at 100% concentration.

[c] Within and between-day precisions were determined over 6 concentration levels covering the calibration range for both precisions.

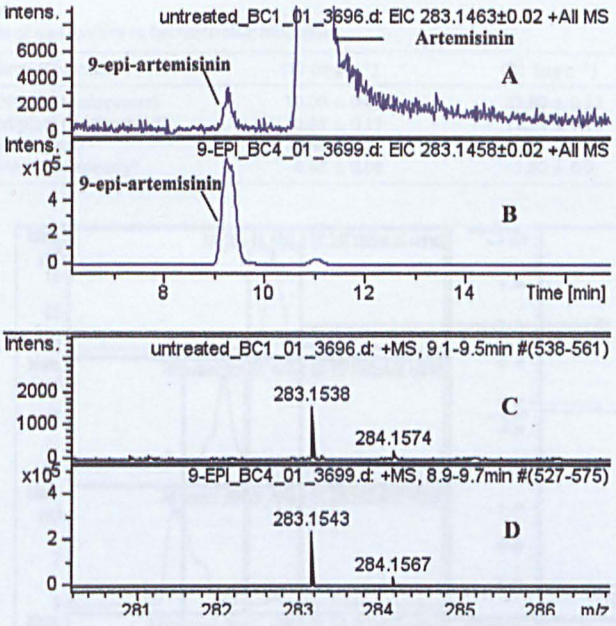


Fig. 4. (A) and (B) EIC for extracts and 9-epi-artemisinin standard respectively. Below are HRMS data for extract (C) and standard (D).

differentiated based on their retention times as shown in Fig. 3. We report here for the first time the detection of 9-epi-artemisinin in plant extract. The presence of 9-epi-artemisinin and artemisitene has been reported as impurities in artemisinin raw material [2,13,43].

WHO has guidelines and recommendations for concentration limits for this isomer and other impurities in the raw material [2]. However the source of the impurity has never been established. To verify the detection of the isomer in plant extract by our method we employed a high-resolution mass accuracy approach using a Q-ToF instrument to examine the compound in the plant extract. Fig. 4 shows extracted chromatograms, EIC (A and B) and high resolution MS data (C and D) for the crude (untreated) extract and 9-epi-artemisinin standard at *m/z* 283.

A comparison of the EIC chromatograms and the HRMS simulated spectrum for both extract and standard show a peak in the extract with identical retention time as the 9-epi-artemisinin standard and an HRMS data difference between both peaks of less than 2 ppm. This confirms and validates the MRM results and establishes the presence of (**2**) in *A. annua* raw extracts.

Acton and Klayman [44] first reported the isolation of (**3**) from plant extracts and its possible role in the biosynthetic pathway of artemisinin has been suggested [45,46]. Acton and Klayman have also demonstrated the conversion of (**1**) into iso-artemisitene and (**2**) [44]. Table 5 shows the levels of (**1**) and the related metabolites in four *A. annua* biomasses analysed by our method. The level of (**2**) in the analysed extracts was about tenfold lower than the detected levels of (**3**) in the extracts.

3.8. Dihydroartemisinic and artemisinic acids

In our method, the MRM for dihydroartemisinic acid (**4**) ($[M+H]^+ = 237$) and artemisinic acid (**5**) ($[M+H]^+ = 235$) are similar (237/235 → 190 + 200 + 218). Three main peaks were observed in *A. annua* plant extracts for these transitions (Fig. 5). The standard peak for (**4**) in Fig. 5, matched one of the main peaks while an impurity in the (**4**) standard matched the second peak. We were unable to confirm the composition of this impurity. Standard artemisinic acid peak at 5.93 min does not match any of the major peaks. A small peak in the plant extract with similar retention time to artemisinic acid standard was below the method's quantitation limit for the same (Table 4). This is consistent with work by Brown and Sy [45,47,48], who have shown that dihydroartemisinic acid rather than artemisinic acid is the true late-stage precursor to artemisinin in some *A. annua* chemotypes.

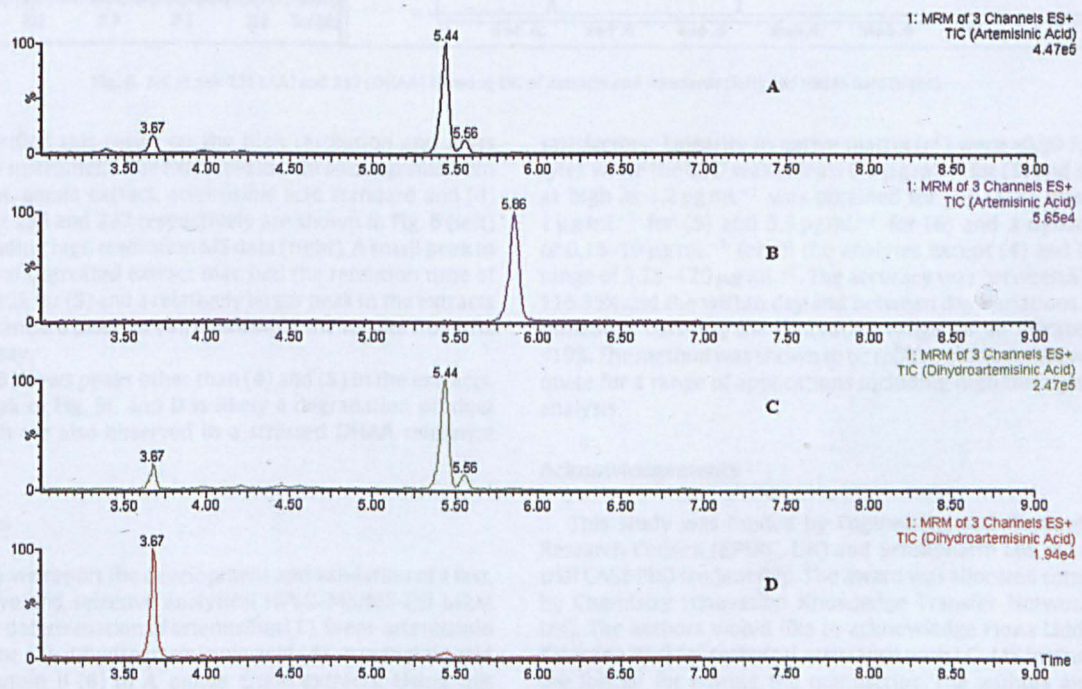


Fig. 5. (A) MRM TIC chromatogram for *A. annua* plant extract monitored for artemisinic acid (235 → 190 + 200 + 218). (B) MRM chromatogram for artemisinic acid standard at 40 μg mL^{-1}. (C) MRM of plant extract monitored for dihydroartemisinic acid (237 → 190 + 200 + 218). (D) MRM of dihydroartemisinic acid standard at 10 μg mL^{-1}.

Table 5
Levels of metabolites in four *Artemisia* biomasses.

Source of biomass	(1) (mg g^{-1})	(2) (μg g^{-1})	(3) (μg g^{-1})	(4) (mg g^{-1})	(6) (μg g^{-1})
BIONEXX (Madagascar)	10.00 ± 0.03	23.80 ± 0.12	290.0 ± 1.0	58.15 ± 1.52	81.10 ± 1.05
Mediplant (Switzerland)	10.63 ± 0.11	12.70 ± 0.07	389.0 ± 4.0	67.38 ± 1.22	168.60 ± 2.11
REAP (Kenya)	10.66 ± 0.01	12.40 ± 0.06	110.0 ± 1.0	68.33 ± 2.64	20.70 ± 0.07
ANAMED (Germany)	6.45 ± 0.08	1.60 ± 0.0	64.0 ± 4.0	37.71 ± 1.01	2.30 ± 0.0

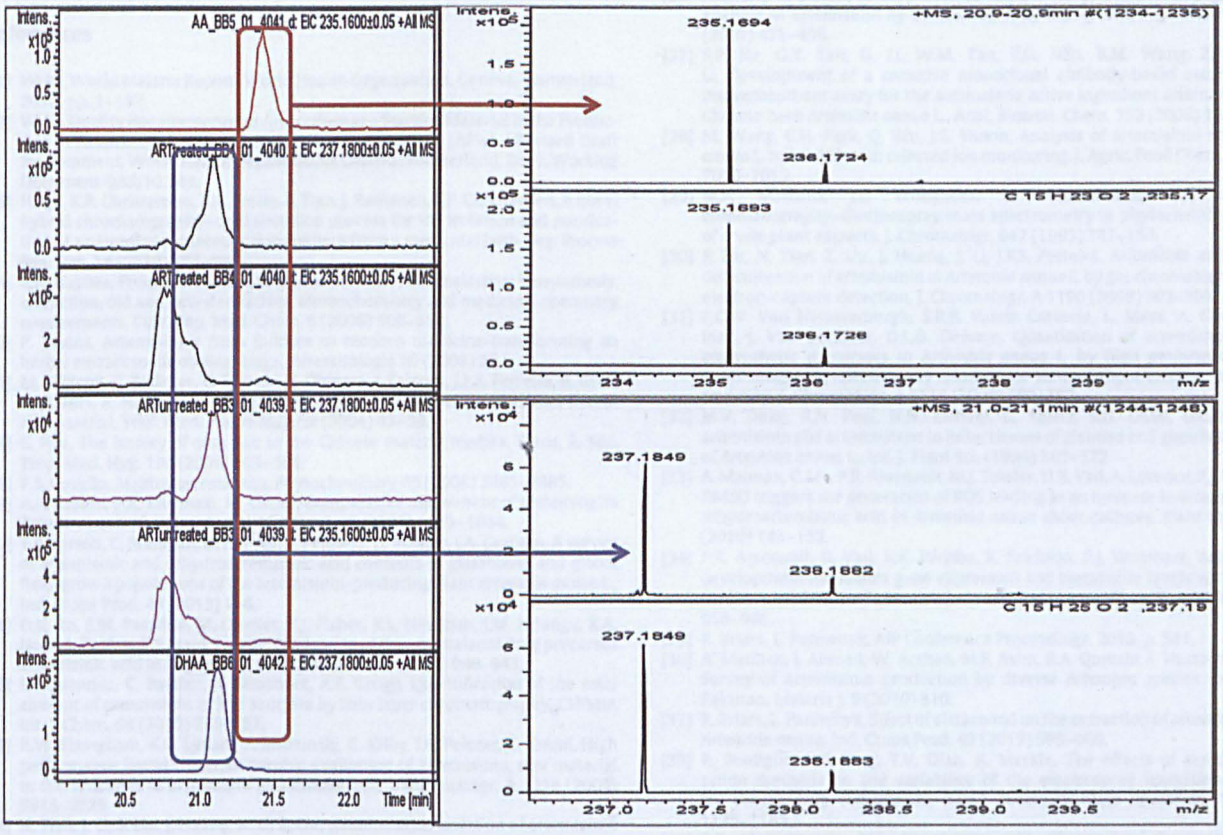


Fig. 6. EIC at *m/z* 235 (AA) and 237 (DHAA) showing EIC of extracts and standards (left) and HRMS data (right).

We also verified this result on the high resolution and mass accuracy Q-ToF instrument. The extracted ion chromatograms from the Q-ToF for *A. annua* extract, artemisinic acid standard and (**4**) standard at *m/z* 235 and 237 respectively are shown in Fig. 6 (left) with corresponding high resolution MS data (right). A small peak in both treated and untreated extract matched the retention time of the standard peak for (**5**) and a relatively larger peak in the extracts matched the standard peak for (**4**), confirming the results from the MRM based assay.

Figs. 5 and 6 shows peaks other than (**4**) and (**5**) in the extracts. The largest peak in Fig. 5C and D is likely a degradation product of DHAA which we also observed in a stressed DHAA reference standard.

4. Conclusions

In this study we report the development and validation of a fast, simple, sensitive and selective analytical HPLC–MS/MS-ESI MRM method for the determination of artemisinin (**1**), 9-epi-artemisinin (**2**), artemisitene (**3**), dihydroartemisinic acid (**4**), artemisinic acid (**5**) and arteannuin B (**6**) in *A. annua* crude extracts. Using this method we report for the first time the presence of 9-epi-artemisinin in *A. annua* extracts. Validation indices evaluated were satisfactory. Linearity in native matrix (r^2) were >0.99 for all analytes while the LOD was at least 0.3 μg mL^{-1} for (**5**) and sensitivity as high as 1.2 pg mL^{-1} was obtained for (**6**). LLOQ was between 1 μg mL^{-1} for (**5**) and 3.5 pg mL^{-1} for (**6**) and a dynamic range of 0.15–10 μg mL^{-1} for all the analytes except (**4**) and (**5**) with a range of 3.75–120 μg mL^{-1}. The accuracy was between 82.32% and 116.35% and the within day and between day variations for determinations covering the calibration range for all metabolites was <19%. The method was shown to be robust, quick, sensitive and adequate for a range of applications including high throughput (HTP) analysis.

Acknowledgements

This study was funded by Engineering and Physical Sciences Research Council (EPSRC, UK) and Sensapharm Ltd. *via* an Industrial CASE PhD studentship. The award was allocated competitively by Chemistry Innovation Knowledge Transfer Network (CIKTN, UK). The authors would like to acknowledge Fiona Liddicoat and Krizstina Radi for technical assistance with LC–MS instrument and the former for editing the manuscript. The authors also greatly appreciate selfless assistance of many companies and individuals in providing artemisinin and biomass samples. In particular, William

Ellis, Charles Giblane, Christian Desmarchelier, Malcolm Cutler, Xavier Simonnet, Roger Sharland, Pamela Weathers and Keith Lindsey.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jpba.2013.06.025.

References

[1] WHO, World Malaria Report, World Health Organisation, Geneva, Switzerland, 2010, pp. 1–137.
[2] WHO, Quality Requirements for Artemsinin as a Starting Material in the Production of Antimalarial Active Pharmaceutical Ingredients (APIs) – Revised Draft for Comment, World Health Organisation, Geneva, Switzerland, 2011, Working Document QAS/10.349.
[3] H. Qu, K.B. Christensen, X.C. Frette, F. Tian, J. Rantanen, L.P. Christensen, A novel hybrid chromatography–crystallization process for the isolation and purification of a natural pharmaceutical ingredient from a medicinal herb, Org. Process Res. Dev. 14 (2010) 585–591.
[4] R.K. Haynes, From artemisinin to new artemisinin antimalarials: biosynthesis, extraction, old and new derivatives, stereochemistry and medicinal chemistry requirements, Curr. Top. Med. Chem. 6 (2006) 509–537.
[5] P. Weina, Artemisinins from folklore to modern medicine-transforming an herbal extract to life-saving drugs, Parassitologia 50 (2008) 25.
[6] M. Willcox, G. Bodeker, G. Bourdy, V. Dhingra, J. Falquet, J.F.S. Ferreira, B. Graz, H.M. Hirt, E. Hsu, P.M. de Magalhães, *Artemisia annua* as a traditional herbal antimalarial, Trad. Med. Plants Malaria (2004) 43–59.
[7] E. Hsu, The history of qing hao in the Chinese materia medica, Trans. R. Soc. Trop. Med. Hyg. 100 (2006) 505–508.
[8] P.S. Covello, Making artemisinin, Phytochemistry 69 (2008) 2881–2885.
[9] A.A. Lapkin, P.K. Plucinski, M. Cutler, Comparative assessment of technologies for extraction of artemisinin, J. Nat. Prod. 69 (2006) 1653–1664.
[10] T.R. Larson, C. Branigan, D. Harvey, T. Penfield, D. Bowles, I.A. Graham, A survey of artemisinic and dihydroartemisinic acid contents in glasshouse and global field-grown populations of the artemisinin-producing plant *Artemisia annua* L., Ind. Crops Prod. 45 (2013) 1–6.
[11] D.K. Ro, E.M. Paradise, M. Ouellet, K.J. Fisher, K.L. Newman, J.M. Ndungu, K.A. Ho, R.A. Eachus, T.S. Ham, J. Kirby, Production of the antimalarial drug precursor artemisinic acid in engineered yeast, Nature 440 (2006) 940–943.
[12] M. Quennoz, C. Bastian, X. Simonnet, A.F. Grogg, Quantification of the total amount of artemisinin in leaf samples by thin layer chromatography, CHIMIA Int. J. Chem. 64 (2010) 755–757.
[13] R.W. Stringham, K.G. Lynam, P. Mrozinski, G. Kilby, I.n. Pelczer, C. Kraml, High performance liquid chromatographic evaluation of artemisinin, raw material in the synthesis of artesunate and artemether, J. Chromatogr. A 1216 (2009) 8918–8925.
[14] N. Tian, J. Li, S. Liu, J. Huang, X. Li, Z. Liu, Simultaneous isolation of artemisinin and its precursors from *Artemisia annua* L. by preparative RP-HPLC, Biomed. Chromatogr. 26 (2012) 708–713.
[15] K.L. Chan, K.H. Yuen, S. Jinadasa, K.K. Peh, W.T. Toh, A high-performance liquid chromatography analysis of plasma artemisinin using a glassy carbon electrode for reductive electrochemical detection, Planta Med.: Nat. Prod. Med. Plant Res. 63 (1997) 66–69.
[16] A.A. Lapkin, A. Walker, N. Sullivan, B. Khambay, B. Mlambo, S. Chemat, Development of HPLC analytical protocols for quantification of artemisinin in biomass and extracts, J. Pharm. Biomed. Anal. 49 (2009) 908–915.
[17] T.E. Wallaart, N. Pras, A.R.C. Beekman, W.J. Quax, Seasonal variation of artemisinin and its biosynthetic precursors in plants of *Artemisia annua* of different geographical origin: proof for the existence of chemotypes, Planta Med. 66 (2000) 57–62.
[18] G.P. Qian, Y.W. Yang, Q.L. Ren, Determination of artemisinin in *Artemisia annua* L. by reversed phase HPLC, J. Liq. Chromatogr. Relat. Technol. 28 (2005) 705–712.
[19] Y. Wang, P. Weathers, Sugars proportionately affect artemisinin production, Plant Cell Rep. 26 (2007) 1073–1081.
[20] Y.Q. Cheng, H.L. Chen, L.Y. Fan, X.G. Chen, Z.D. Hu, On-line conversion and determination of artemisinin and its kinetic parameters using orthogonal design by coupling of flow injection with capillary electrophoresis, Anal. Chim. Acta 525 (2004) 239–245.
[21] C.A. Peng, J.F.S. Ferreira, A.J. Wood, Direct analysis of artemisinin from *Artemisia annua* L. using high-performance liquid chromatography with evaporative light scattering detector, and gas chromatography with flame ionization detector, J. Chromatogr. A 1133 (2006) 254–258.
[22] H.J. Woerdenbag, N. Pras, R. Bos, J.F. Visser, H. Hendriks, T.M. Malingré, Analysis of artemisinin and related sesquiterpenoids from *Artemisia annua* L. by combined gas chromatography/mass spectrometry, Phytochem. Anal. 2 (2007) 215–219.
[23] P. Christen, J. Veuthey, New trends in extraction, identification and quantification of artemisinin and its derivatives, Curr. Med. Chem. 8 (2001) 1827–1839.
[24] M. Kohler, W. Haerdi, P. Christen, J.L. Veuthey, Extraction of artemisinin and artemisinic acid from *Artemisia annua* L. using supercritical carbon dioxide, J. Chromatogr. A 785 (1997) 353–360.
[25] K. Dost, G. Davidson, Analysis of artemisinin by a packed-column supercritical fluid chromatography–atmospheric pressure chemical ionisation mass spectrometry technique, Analyst 128 (2003) 1037–1042.
[26] N.Q. Liu, Y.H. Choi, R. Verpoorte, F. van der Kooy, Comparative quantitative analysis of artemisinin by chromatography and qNMR, Phytochem. Anal. 21 (2010) 451–456.
[27] S.P. He, G.Y. Tan, G. Li, W.M. Tan, T.G. Nan, B.M. Wang, Z.H. Li, Q.X. Li, Development of a sensitive monoclonal antibody-based enzyme-linked immunosorbent assay for the antimalaria active ingredient artemisinin in the Chinese herb *Artemisia annua* L., Anal. Bioanal. Chem. 393 (2009) 1297–1303.
[28] M. Wang, C.H. Park, Q. Wu, J.E. Simon, Analysis of artemisinin in *Artemisia annua* L. by LC–MS with selected ion monitoring, J. Agric. Food Chem. 53 (2005) 7010–7013.
[29] M.P. Maillard, J.L. Wolfender, K. Hostettmann, Use of liquid chromatography–thermospray mass spectrometry in phytochemical analysis of crude plant extracts, J. Chromatogr. 647 (1993) 147–154.
[30] S. Liu, N. Tian, Z. Liu, J. Huang, J. Li, J.F.S. Ferreira, Affordable and sensitive determination of artemisinin in *Artemisia annua* L. by gas chromatography with electron-capture detection, J. Chromatogr. A 1190 (2008) 302–306.
[31] F.C.W. Van Nieuwerburgh, S.R.F. Vande Casteele, L. Maes, A. Goossens, D. Inzè, J. Van Bocxlaer, D.L.D. Deforce, Quantitation of artemisinin and its biosynthetic precursors in *Artemisia annua* L. by high performance liquid chromatography-electrospray quadrupole time-of-flight tandem mass spectrometry, J. Chromatogr. A 1118 (2006) 180–187.
[32] M.V. Duke, R.N. Paul, H.N. Elsohly, G. Sturtz, S.O. Duke, Localization of artemisinin and artemisitene in foliar tissues of glanded and glandless biotypes of *Artemisia annua* L., Int. J. Plant Sci. (1994) 365–372.
[33] A. Mannan, C. Liu, P.R. Arsenault, M.J. Towler, D.R. Vail, A. Lorence, P.J. Weathers, DMSO triggers the generation of ROS leading to an increase in artemisinin and dihydroartemisinic acid in *Artemisia annua* shoot cultures, Plant Cell Rep. 29 (2010) 143–152.
[34] P.R. Arsenault, D. Vail, K.K. Wobbe, K. Erickson, P.J. Weathers, Reproductive development modulates gene expression and metabolite levels with possible feedback inhibition of artemisinin in *Artemisia annua*, Plant Physiol. 154 (2010) 958–968.
[35] R. Briars, L. Paniwnyk, AIP Conference Proceedings, 2012, p. 581.
[36] A. Mannan, I. Ahmed, W. Arshad, M.F. Asim, R.A. Qureshi, I. Hussain, B. Mirza, Survey of artemisinin production by diverse *Artemisia* species in northern Pakistan, Malaria J. 9 (2010) 310.
[37] R. Briars, L. Paniwnyk, Effect of ultrasound on the extraction of artemisinin from *Artemisia annua*, Ind. Crops Prod. 42 (2013) 595–600.
[38] R. Bonfiglio, R.C. King, T.V. Olah, K. Merkle, The effects of sample preparation methods on the variability of the electrospray ionization response for model drug compounds, Rapid Commun. Mass Spectrom. 13 (1999) 1175–1185.
[39] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC–MS/MS, Anal. Chem. 75 (2003) 3019–3030.
[40] A. Van Eeckhaut, K. Lanckmans, S. Sarre, I. Smolders, Y. Michotte, Validation of bioanalytical LC–MS/MS assays: evaluation of matrix effects, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 877 (2009) 2198.
[41] I.C.H.H.T. Guideline, Validation of Analytical Procedures: Text and Methodology Q2 (R1), IFPMA, Geneva, 2005.
[42] J.N. Miller, J.C. Miller, Statistics and Chemometrics for Analytical Chemistry, Prentice Hall, Harlow, Essex, UK, 2005.
[43] R.W. Stringham, M. Pennell, W. Cabri, G. Carzana, F. Giorgi, S. Lalli, G. Marazzi, M. Torri, Identification of impurities in artemisinin, their behavior in high performance liquid chromatography and implications for the quality of derived anti-malarial drugs, J. Chromatogr. A 1218 (2011) 6838–6842.
[44] N. Acton, D.L. Klayman, Conversion of artemisinin (qinghaosu) to iso-artemisitene and to 9-epi-artemisinin1, Planta Med. 53 (1987) 266.
[45] G.D. Brown, The biosynthesis of artemisinin (Qinghaosu) and the phytochemistry of *Artemisia annua* L. (Qinghao), Molecules 15 (2010) 7603–7698.
[46] P.J. Weathers, P.R. Arsenault, P.S. Covello, A. McMickle, K.H. Teoh, D.W. Reed, Artemisinin production in *Artemisia annua*: studies in planta and results of a novel delivery method for treating malaria and other neglected diseases, Phytochem. Rev. 10 (2011) 173–183.
[47] G.D. Brown, L.-K. Sy, Synthesis of labelled dihydroartemisinic acid, Tetrahedron 60 (2004) 1125–1138.
[48] V. Dhingra, M. Lakshmi Narasu, Purification, Characterization of an enzyme involved in biochemical transformation of arteannuin B to artemisinin from *Artemisia annua*, Biochem. Biophys. Res. Commun. 281 (2001) 558–561.

Anti-plasmodial polyvalent interactions in *Artemisia annua* L. aqueous extract – possible synergistic and resistance mechanisms

John O. Suberu,[1] Alexander P. Gorka,[2] Lauren Jacobs,[2] Paul D. Roepe,[2] Neil Sullivan,[3] Guy C. Barker,[1*]· Alexei A. Lapkin[4*]

[1]*School of Life Sciences, University of Warwick, Coventry, CV4 7AL*

[2]*Centre for Infectious Disease, Department of Chemistry, Georgetown University, USA*

[3]*Sensapharm Ltd, Business and Innovation Centre, Sunderland, SR5 2TA, UK*

[4]*Department of Chemical Engineering and Biotechnology, University of Cambridge, CB2 3RA, UK*

*Equal contributions

Abstract

Artemisia annua hot water infusion (tea) has been used in *in vitro* experiments against *P. falciparum* malaria parasites to test potency relative to equivalent pure artemisinin. High performance liquid chromatography (HPLC) and mass spectrometric analyses were employed to determine the metabolite profile of tea including the concentrations of artemisinin (47.5±0.8 mg L^{-1}), dihydroartemisinic acid (70.0±0.3 mg L^{-1}), arteannuin B (1.3±0.0 mg L^{-1}), isovitexin (105.0±7.2 mg L^{-1}) and a range of polyphenolic acids. The tea extract, purified compounds from the extract, and the combination of artemisinin with the purified compounds were tested against chloroquine sensitive and chloroquine resistant strains of *P. falciparum* using the DNA-intercalative SYBR Green I assay. The results of these *in vitro* tests and of isobologram analyses of combination effects showed mild to strong antagonistic interactions between artemisinin and the compounds (9-epi-artemisinin and artemisitene) extracted from *A. annua* with significant (IC_{50} <1 μM) anti-plasmodial activities for the combination range evaluated. Mono-caffeoylquinic acids, tri-caffeoylquinic acid, artemisinic acid and arteannuin B showed additive interaction while rosmarinic acid showed synergistic interaction with artemisinin in the

· Corresponding Author. Email: guy.barker@warwick.ac.uk; Fax: +44 (0)24 765 23568.

chloroquine sensitive strain at a combination ratio of 1:3 (artemisinin to purified compound). In the chloroquine resistant parasite, using the same ratio, these compounds strongly antagonised artemisinin anti-plasmodial activity with the exception of arteannuin B, which was synergistic. This result would suggest a mechanism targeting parasite resistance defenses for arteannuin B's potentiation of artemisinin.

Keywords: malaria, Artemisia annua, artemisinin, synergy, resistance, mechanism

1. Introduction

The use of *Artemisia annua* (*Qing Hao*) in traditional Chinese pharmacopeia includes the treatment of fevers and chills [1,2]. In the 1970s, the active principle in the extract was isolated and identified as artemisinin (**1**), a sesquiterpene lactone. The effectiveness of artemisinin is structurally due to the trioxane pharmacophore and the activation of the compound occurs via the cleavage of the endoperoxide bridge [3]. The mechanism for the activation of artemisinins and their interaction with the parasite are not fully understood. Different but not mutually exclusive mechanistic models have been proposed with evidence for and against each model [4]. A number of studies [5,6] have suggested that artemisinins act by heme dependent activation of the trioxane bridge in the parasites' food vacuole to produce free radicals which then disrupt heme detoxification and therefore lead to parasite toxicity. This hypothesis and other alternative mechanisms for the mode of action of artemisinins have been studied and reviewed by several authors [3,4,7,8,9,10,11,12,13]. Artemisinin and its derivatives have now been established in various combination therapies (ACTs) as effective anti-plasmodial treatments against multidrug-resistant *P. falciparum* infection [14,15].

In some parts of Asia and Africa, a hot water infusion (tea) of the plant is used as a self-medication for malaria. The use of tea in this way has raised concern of the possible development of parasite resistance as a result of un-standardised use of artemisinin in these tea preparations [16]. Consequently, the World Heath Organisation (WHO) in a position statement has called for "extensive fundamental and clinical research" which demonstrates both efficacy and safety for the use of tea

and other non-pharmaceutical forms of *A. annua* extract before recommendation for treating malaria [17].

The recipes in ancient Chinese texts for preparing *Qing Hao* extracts for the treatment of fevers include soaking, followed by wringing or pounding, followed by squeezing the fresh herb [1,2,18]. In their study, Rath *et al* [19] found that adding boiling water to the leaves, stirring briefly and leaving covered for 10 minutes, then filtering and gently squeezing the leaves to release residual water gave the best extraction efficiency (86%) for artemisinin in the preparation, relative to the total amount of the compound in leaves. In the literature, a range of aqueous extraction efficiencies (25-90%) has been reported for artemisinin [19,20,21]. Due to the differences in the content of artemisinin in tea preparation, Van der Kooy and Verpoorte [21] quantified artemisinin in tea prepared by different methods. They observed that the extraction efficiency is temperature-sensitive and that efficiencies of above 90% are attainable.

Figure 1. Structures of some artemisinin related compounds, flavonoids and acids identified in *A. annua* extract.

In some studies evaluating the activity of *A. annua* extracts, the amount of artemisinin in these extracts cannot fully account for its effectiveness against *Plasmodium* parasites *in vitro* and *in vivo* [16,19]. Mouton *et al* however did not find any evidence of improved potency for their extracts relative to the artemisinin content [22]. Apart from artemisinin, there are around 30 other sesquiterpenes and over 36 flavonoids identified in the plant (Figure 1), some of which have shown limited anti-plasmodial properties [23]. Five flavonoids, including casticin (7), have been shown to potentiate the activity of artemisinin [24,25]. Interestingly, the potentiating effect of these flavonoids was not observed with chloroquine (CQ). Billia *et al.* [26] observed that although these flavonoids have no effect on hemin (chloroferriprotoporphyrin IX) themselves, they do catalyse a reaction between artemisinin and hemin.

Weathers and Towler [27] have shown that poly-methoxylated flavonoids like casticin are poorly extracted and unstable in the aqueous tea infusion. This suggests that compounds other than this class of flavonoids are likely to be responsible for the reported improvement in the potency of artemisinin in tea infusion. A recent analysis by Cabonara *et al.* [28] of tea prepared from *A. annua* leaves by infusion in hot water for 1, 24 and 48 hours, identified a series of caffeoyl and feruloyl-quinic acids as main components of the infusion, together with some flavonoids. Chlorogenic or caffeoylquinic acids (CQAs) are esters of caffeic and quinic acids (Figure 1). They possess a broad spectrum of pharmacological properties, including antioxidant, hepato-protectant, antibacterial, anti-histaminic, chemo-preventive and other biological effects [29,30,31,32].

To our knowledge, only the interactions of artemisinin with the poorly extracted poly-methoxylated flavonoids found in *Artemisia* tea have been studied. This study therefore aims at understanding other possible interactions and mechanisms involved in artemisinin activity in the plant extract, and the effects of these interactions on parasite resistance to artemisinin.

2. Materials and Methods

2.1 Chemicals

Reference standards of artemisinin (98%), rosmarinic acid, caffeic acid and casticin were obtained from Sigma-Aldrich (Dorset, UK). Dihydroartemisinic acid (> 96%) was purchased from Apin Chemicals (Oxfordshire, UK). 9-Epi-artemisinin (98%) was sourced from Sensapharm Ltd (Sunderland, UK). Artemisitene, artemisinic acid and arteannuin B were kindly provided by Walter Reed Army Institute of Research (Washington, DC, USA). The chlorogenic acids (>99%) and isovitexin (>99%) were obtained from Biopurify (China). LC-MS grade formic acid in water, acetonitrile and HPLC grade acetonitrile were obtained from Fisher Scientific (UK). Purified water (~18 MΩ cm^{-1}) was dispensed from a Milli-Q system (Millipore, UK).

2.2 Plant materials

High yielding, dried *A. annua* biomass was obtained from BIONEXX Madagascar and stored under dark, cool conditions until use.

2.3 Plant extracts

A. annua tea was prepared according to published methods with slight modification [1,33]. Briefly, 1 L of boiling water was added to 5 g of dried plant material, stirred and stored in the dark for 1 hour. The extract was filtered *in vacuo* and lyophilised after freezing to obtain the dried tea extract which was used in the *Plasmodium* assays and in metabolite profiling.

2.4 Sample preparation – solubility studies

The solubility of artemisinin, artemisitene and 9-epi-artemisinin in aqueous solvent at room temperature (22 °C) was determined by the method employed by Wang *et al.* [34], with modifications. A saturated solution was prepared by dissolving excess amount of the pure (> 99.0%) standard of each material in 1 mL de-ionised water (MS grade, Brucker, UK) and vortexed. This suspension was allowed to settle and the supernatant filtered through a 0.1 µm syringe filter (Fisher Scientific, UK). Appropriate volume of the filtrate was diluted with the mobile phase for mass spectrometry (MS/MS) analysis.

2.5 Mass spectrometry method for artemisinins

The method by Suberu *et al.* [35] was employed. Briefly, the MS/MS system was operated with an ESI interface in positive ionisation mode (ESI+). The cone and desolvation gas flow rates were set at 45 L h^{-1} and 800 L h^{-1}, respectively. The MS parameters were automatically defined using Waters IntelliStart® software for the tuning and calibration of the tandem quardrupole analyser (TQD) and subsequently manually optimized for all analytes. Capillary voltage was set at 2.8 Kilovolts, collision voltage at 7 volts, source temperature was 150 °C and cone voltage was set at 24 volts. A multiple reaction-monitoring (MRM) transition of 283→219+229+247+265, 283→209+219+247+265, 281→217+227+245+263 for artemisinin, 9-epi-artemisinin and artemisitene was employed. Quantification was determined using MRM modes for the above transitions. The dwell time was automatically set at 0.161 seconds. Data were acquired by MassLynx v4.1 software and processed for quantification with QuanLynx v4.1 (Waters Corp., Milford, MA, USA).

The high performance liquid chromatography (HPLC) system coupled to the mass spectrometer consisted of a binary pump, a cooling auto-sampler with an injection loop of 10 µL set at 10 °C. The column heater was set at 30 °C and a Genesis® Lightn C18 column (100 × 2.1 mm; 4 µm) (Grace, IL, USA) protected by an Acquity-LC column in-line filter unit (0.2 µm in-line frit) was used for separation of metabolites. The mobile phase consisted of A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile used in the following gradient: 0–7.00 min, 25-98% B; 7-9.5 min, 98% B; 9.5-10 min, 98-25% B; 10-15 min, 25% B; at a flow rate of 0.4 mL min^{-1}. Weak wash solvent was 10% acetonitrile, strong and needle wash solvent was a mixture of acetonitrile, propan-2-ol, methanol and water (30:30:30:10 v/v/v/v).

2.6 HPLC method for acids and flavonoid

Analysis of acids and flavonoid was performed on an Agilent 1100 series HPLC equipped with a quaternary pump, auto-sampler, photodiode array (PDA) and a degasser. The chromatographic method by Carbonara *et al.* [28] was used in the analysis with slight modifications. Briefly, the solvent system consisted of A (0.1% acetic acid, brought to pH 4 with NaOH) and B (0.1% acetic acid in acetonitrile) using a gradient elusion of 0-60 min: 12-25% B, 60-80 min: 25-60% B, 80-85 min: 60-100% B. The system was equilibrated back to 12% B for 5 minutes before the next run. Analytes were separated and resolved at a flow rate of 1 mL min^{-1} on a Phenomenex Luna C18 column (250 mm x 4.60 mm, 5 μm particle size) attached to a C18 guard column. Detection and quantification was at 310 nm for caffeic acid, chlorogenic acids and isovitexin. Rosmarinic acid was analysed at 330 nm wavelength.

2.7 Plasmodium assay

Determination of 50% growth inhibitory concentration (IC_{50}) values of extracts, compounds and combinations against CQ-sensitive (CQS; HB3) and CQ-resistant (CQR; Dd2) strains of *P. falciparum* was performed at Georgetown University, Washington, DC, USA, using a previously reported protocol [36] with minor modifications. Typically, test samples were dissolved in DMSO to give a stock solution, followed by serial dilution using complete media (RPMI 1640 supplemented with 10% (v/v) type-O^+ human serum, 25 mM HEPES (pH 7.4), 23 mM $NaHCO_3$, 11

mM glucose, 0.75 mM hypoxanthine and 20 μg/L gentamicin) to generate working stocks. 100 μL of these stock solutions were transferred into pre-warmed (37 °C) 96-well plates. 100 μL of asynchronous parasite culture at 2% parasitemia, 4% hematocrit was transferred into each drug (*A. annua* plant extract) pre-loaded well, for a final 1% parasitemia, 2% hematocrit. The final concentration of DMSO was 2.5%. Plates were transferred to a gassed (90% N_2, 5% O_2, 5% CO_2) airtight chamber and incubated at 37 °C for 72 hours. Following this incubation, 50 μL of 10X SYBR Green I dye (diluted with complete media from a 10000X concentrate in DMSO) was added to each well and plates incubated for an additional 1 hour at 37 °C to allow DNA intercalation. Fluorescence was measured at 530 nm (490 excitation) on a Spectra GeminiEM plate reader (Molecular Devices, USA). IC_{50} values were obtained from sigmoidal curves fit of parasite growth vs. drug concentration using SigmaPlot 10.0, and are the average of three replicates. CQ was included as a positive control in the assay.

2.8 Combination analysis

Interactions between compounds were evaluated by isobologram analysis [37,38]. Briefly, a master stock solution is prepared for each compound such that its concentration following four or five twofold dilutions approximates the IC_{50}. These stock solutions were mixed at ratios of 0:4, 1:3, 1:1, 3:1 and 4:0 (v/v) to give working combination stocks. Subsequently, the combination stocks were twofold serially diluted to generate a full dose concentration range for each v/v mixture, which were then analysed under standard growth inhibitory assay conditions (see above) to provide dose response curves and an IC_{50}, for each component of each v/v mixture.

2.9 Data analysis for in vitro combination studies

IC_{50} values for each compound alone and in the combination were used to calculate FICs (fractional inhibition concentrations) as described elsewhere [39,40]. The FICs were summated to obtain the fractional inhibition concentration index (FIC_{index}) for the combination as in the equation below:

$$FIC_{index} = FIC_A + FIC_B$$

where:

$$FIC_A = \frac{IC_{50} \text{ of Drug A in Combination}}{IC_{50} \text{ of Drug A Alone}}$$

$$FIC_B = \frac{IC_{50} \text{ of Drug B in Combination}}{IC_{50} \text{ of Drug B Alone}}$$

The following categorisation was used to determine the type of interactions between compounds evaluated: synergy (FIC_{index} <0.9), additivity (0.9<FIC_{index}<1.5) and antagonism (FIC_{index} >1.5) [39,40].

3. Result and discussion

3.1 Composition of A. annua tea

Table 1 shows the metabolites in the aqueous extract analysed by both MS/MS and HPLC methods and their quantities in milligrams per litre of extract. The compounds analysed were based on the *in extenso* analysis by Carbonara *et al.* [28], who showed them to be among the major metabolites (quantitatively) in *A. annua* tea infusions. Some of these metabolites (like 3-caffeoylquinic acid) also have important dietary profiles [41,42]. In addition, artemisinin-related compounds, which we have previously detected in such extracts, were also analysed. The level of artemisinin reported [2,19,21,28,43] for tea extract is varied and the values obtained in this study (47.5 mg L^{-1}) are within the reported range. These could be due to variation in biomass and the tea preparation method that was employed, but might also be due to differences in the biomass-to-solvent ratio used. Carbonara *et al.* [28] used a solvent to biomass ratio of 26:1 (v/w), while this study, as well as others [19,21], employed the therapeutically recommended ratio (200:1, v/w or 5 g L^{-1}) [44].

Table 1. Metabolites in the aqueous *A. annua* extract analysed by both MS/MS and HPLC methods quantified as milligrams per litre of tea.

Dihydroartemisinic acid (**4**) (70 mg L^{-1}) and arteannuin B (**5**) (1.3 mg L^{-1}) are the only biosynthetic precursors of artemisinin detected in the tea extract using our method

[35]. Therefore artemisinin is the only compound among the metabolites we analysed in the tea with significant (IC_{50} <1 μM) anti-plasmodial activity (Table 2).

3-Caffeoylquinic acid (**11**) was found to be the most abundant (72 mg L^{-1}) of the caffeic derivatives (**11-17**) in the analysed extract, followed by 3,5-di-caffeoylquinic acid (**15**) (57 mg L^{-1}). Caffeic acid (**10**) was the least abundant (0.8 mg L^{-1}) of the evaluated acids. Isovitexin (**8**) was the only flavonoid analysed (105 mg L^{-1}), being relatively abundant in the extract. Some classes of flavonoids have poor aqueous solubility and limited profiles of these compounds in aqueous extract have been reported [27,28]. Lower level of rosmarinic acid (**9**) (1.1 mg L^{-1}) was detected in our samples, compared to the levels found by De Magalhaes *et al* [43]. However, widely different concentrations of the acid were reported in the cultivars and samples they analysed. The acid was not detected in the analysis by Carbonara *et al* [28]. Van der Kooy and Verpoorte [21] have also shown that the method employed in preparing the hot water infusion does affect the amount of artemisinin and therefore other co-metabolites extracted. These differences in profiles and concentration levels of metabolites seem to suggest that composition of prepared tea infusions differ and is significantly influenced by method of preparation and the *Artemisia* cultivar used.

3.2 Anti-plasmodium extracts and bioactive compounds in A. annua

Table 2 shows IC_{50} anti-plasmodial values for pure compounds and extracts of *A. annua* plant. Between three- and seven-fold potentiation of artemisinin activity was observed for *A .annua* aqueous (tea) extract in CQ-sensitive (HB3) and CQ-resistant (Dd2) strains respectively. Only artemisitene (**3**) (IC_{50}, 88.4±9.9/74.1±7.8 nM, HB3/Dd2) and 9-epi-artemisinin (**2**) (IC_{50}, 59.2±1.7/62.2±1.0 nM, HB3/Dd2) showed significant anti-plasmodial activities (IC_{50} <1 μM) among the artemisinin biosynthetic precursors evaluated. 9-Epi-artemisinin and artemisitene respectively showed about one third and one fourth of the activity of artemisinin. Acton *et al.* [45] observed a similarly reduced activity for 9-epi-artemisinin and artemisitene, compared to artemisinin in D6 and W2 strains of *P. falciparum*. Artemisinin has a chiral molecular structure and the bioactivity of the molecule is influenced by its absolute configuration.

Table 2. IC_{50} of extracts and components of *A. annua* in CQ-sensitive (HB3) and resistant (Dd2) strains.

To investigate if solubility of these artemisinin analogues could be partially responsible for the reduced activity, we determined the aqueous solubilities of artemisinin, artemisitene and 9-epi-artemisnin. Table 3 shows the solubility of these compounds at experimental conditions.

Table 3. Solubility of artemisinin, artemisitene and 9-epi-artemisinin in water at 22 °C and atmospheric pressure.

Under these conditions, 9-epi-artemisinin has a higher solubility, about twice that of artemisinin or artemisitene. The lower bioactivity could not be explained based on the solubility data alone, although the experimental data was obtained at 22 °C (Table 3). We do not expect the pattern observed to change significantly at physiological conditions.

Woerdenbag *et al.* [46] observed that the anti-cancer activity of 11-hydroxy-11-epi-artemisinin (C11 in older and C9 in newer references for the structure) was about threefold less than the conformer, which is the same threefold difference we observed in the anti-plasmodial activity for epimerisation at C9 (Table 2). If the threefold activity difference is consistent regardless of the differences in molecular targets and effect, this may suggest a common upstream differentiation point of molecule activation. The lower activity of 9-epi-artemisinin may therefore be due in part to a structural conformation that is relatively more difficult to activate compared to artemisinin.

3.3 Antagonism of artemisinin with biosynthetic precursors

Figure 2 shows the interaction of artemisitene and 9-epi-artemisinin with artemisinin and artesunate (**6**). These biosynthetic precursors of artemisinin have significant (IC_{50} <1 μM) anti-plasmodial activities (Table 2). The interaction of artemisinin with 9-epi-

artemisinin and artemisitene was antagonistic, but the interaction of these compounds with artesunate was additive in both chloroquine sensitive (HB3) and resistant (Dd2) strains.

Figure 2. Isobologram showing the plot of fractional inhibitory concentration (FIC) of 9-epi-artemisinin (EPI) and artemisitene (ATENE) against FIC of artemisinin (ART) and artesunate (ATSU). Panel A – interaction of EPI and ATENE with ART in chloroquine-sensitive (CQS) HB3 strain. Panel B – same as in A but in CQ-resistant (CQR) Dd2 strain. Panel C - interaction of EPI and ATENE with ATSU in HB3. Panel D – same as C but in Dd2 parasite.

The reason for the observed antagonistic interaction with artemisinin at the combinations investigated is unclear. Structurally, artemisinin, 9-epi-artemisinin and artemisitene are differentiated at C9. The difference from artemisinin is epimerisation of the methyl group for 9-epi-artemisinin and a methylene group attached instead for artemisitene (Figure 1). Given the minor structural differences, it is likely that these compounds have identical molecular targets and therefore possibly compete for these when combined. Conversely, due to the relatively large difference in structure and mass of artesunate and 9-epi-artemisinin or artemisitene, these compounds, when combined, may act on the same targets as well as on different molecular targets with the possibility of positive polyvalent interaction. Similarly, Wagner [47,48,49] has reported an *in vitro* synergistic inhibitory effect upon combining ginkgolides A and B from *Ginkgo biloba* extract for PAF-induced thrombocyte-aggregation. The difference between ginkgolide A and B is an oxygen atom (16 Da).

3.4 Analysis of other combinations

Table 4 shows the interaction of co-metabolites in *A. annua* extracts with artemisinin. In the CQ-sensitive (HB3) strain, 3-caffeoylquinic acid (3CA) showed additive interaction at 1:3 (v/v), which became synergistic at higher ratio of the acid to artemisinin (1:10, 1:100 v/v). For casticin, the interaction at 1:3 (artemisinin to casticin, v/v) is antagonistic. Synergistic interaction is however reported [24,25] for combination ratios at the range of 1:10-1000 (artemisinin to casticin, v/v).

Therefore, using the FIC index of casticin (1.9) as a benchmark for potential positive interactions, compounds like isovitexin, caffeic acid and dihydroartemisinic acid that show antagonistic interactions at 1:3 may also, like casticin, interact synergistically at a higher ratio. Rosmarinic acid was synergistic at a 1:3 combination with artemisinin (v/v) and some chlorogenic acids were additive at this combination also. These compounds showing positive interactions with artemisinin may collectively be responsible for the potentiation of artemisinin in the tea extract. However, arteannuin B and artemisinic acid are poorly extracted in the aqueous extract.

Table 4. Anti-plasmodial interactions of co-metabolites with artemisinin in CQ-sensitive (HB3) and CQ-resistant (Dd2) strains.

Casticin and 3-caffeoylquinic acid (3CA) are polyphenolic compounds that are natural anti-oxidants. Anti-oxidants at cellular redox sites are considered a "double edged sword" able to act either as anti-oxidant or pro-oxidant depending on conditions, such as dosage levels and presence of metal ions [50,51]. This "double edged sword" characteristic of anti-oxidant polyphenols could help explain our observation. At a lower combination with artemisinin, casticin and 3CA were anti-oxidative towards the ROS and carbon-centred radicals formed from artemisinin activation and, as a result, countered artemisinin activity *in vitro*. Conversely, at a higher concentration ratio to artemisinin, casticin and 3CA were pro-oxidative, enhancing the oxidative stress resulting from artemisinin's activation, leading to improvement in artemisinin's potency. A schematic isobologram to describe the interaction between an active pharmaceutical ingredient (API) like artemisinin (A) and synergists like casticin and 3CA (B, non-API) is shown in Figure 3.

Figure 3. A schematic isobologram of the interaction of artemisinin (API) with anti-oxidant synergist (Non-API).

3.5 Possible role of anti-oxidant defence network in resistance

Rosmarinic acid at the combination ratio evaluated had a potentiating effect (FIC_{index} 0.89) on artemisinin in the CQ-sensitive (HB3) strain (Table 4) but this effect was not reproduced in the resistant (Dd2) strain; rather a strong antagonistic effect (FIC_{index}

4.95) was observed. The effect of rosmarinic acid on artemisinin's ability to mitigate the resistance mechanism of the parasite could be partly explained by the finding of Cul *et al.* [52] and others [53] who observed that *in vitro* resistance in *P. falciparum* is associated with increased *pfmdr-1* copy number and anti-oxidant activity. Some experiments with rosmarinic acid have reported strong anti-oxidant activity for the compound that is over three times that of trolox [54,55,56]. In the presence of rosmarinic acid, anti-oxidant activity may further be elevated thereby promoting increased resistance. A similar trend of activity in sensitive and resistant parasite strains in combination with artemisinin was observed for caffeic acid, 4-caffeoyl-quinic acid (**12**) and isovitexin with reported anti-oxidant properties [57,58,59]. This supports the possible role of the anti-oxidant defence network in parasite resistance to artemisinin [60]

3.6 Arteannuin B selectively potentiates the activity of artemisinin against parasite defence system

Arteannuin B at 3:1 (v/v) combination with artemisinin showed additive or no interaction (FIC_{index} 1.25) in the CQ-sensitive strain and a synergistic interaction (FIC_{index} 0.34) in the resistant parasite strain (Table 4). This is about a three-fold improvement in artemisinin's potency against CQ-resistant *P. falciparum*. This is not reproduced in the CQ-sensitive strain. The potentiation of artemisinin by arteannuin B seems to be selectively directed at the parasites' chloroquine resistance mechanism. This combination could therefore help to better understand the mechanism(s) involved in parasite defence network. Reproducing this three-fold improvement in potency with other artemisinin analogues could also help in the development of therapeutics effective against emerging drug-resistant strains.

Arteannuin B is an unusual α-methylene-γ-lactone, transfused via a tertiary hydroxyl group [61]. This structure could account for its easy fragmentation/ionisation observed in mass spectrometry and reported facile rearrangement in acidic conditions [35,62].

4. Conclusions

In this study we examine interactions between artemisinin and co-metabolites found in *A. annua* plant extracts for chloroquine sensitive (CQS; HB3) and resistant (CQR; Dd2) *P. falciparum* malarial parasites. The aqueous extract (tea) showed about three to seven-fold potentiation in the parasite strains. When pure compounds were combined, 9-epi-artemisinin and artemisitene interacted antagonistically with artemisinin at the combinations evaluated. 9-epi-artemisinin and artemisitene were the only artemisinin-related metabolites with significant anti-plasmodial activity (IC_{50} <1 μM) among those evaluated. In CQS parasites, caffeic acids and their chlorogenic acid derivatives showed additive interactions with artemisinin at the combination ratio evaluated. 3-Caffeoylquinic acid's interaction with artemisinin turned synergistic with the increased ratio of the former in the combination. Rosmarinic acid showed synergistic interaction with artemisinin in the drug sensitive strain but the interaction with artemisinin in the drug resistant strain was strongly antagonistic at the same level of combination. This antagonistic interaction in CQR parasites was also observed for caffeic acid and some of its derivatives known to have anti-oxidant properties. The observation supports literature evidence [52,53] for a potential role of anti-oxidants in parasite drug resistance. Therefore the effect of dietary anti-oxidants on artemisinin combination therapies used in the management of drug resistant *P. falciparium* malaria may need to be further investigated.

Arteannuin B was found to selectively potentiate the activity of artemisinin in Dd2 parasites, suggesting some interaction with the CQR mechanism, since the potentiation of artemisinin by arteannuin B was not reproduced in CQS parasites. As a result of this specificity, arteannuin B could potentially be used as a probe to better understand parasite drug resistance mechanisms and the combination might prove useful for treating CQR strains of malaria.

Acknowledgements

This study was funded by Engineering and Physical Sciences Research Council (EPSRC, UK) and SensaPharm Ltd *via* an Industrial CASE PhD studentship. The award was allocated competitively by Chemistry Innovation Knowledge Transfer Network (CIKTN, UK). The authors would like to acknowledge earlier assistance with *in vitro* assay done in Prof. Steve Ward's laboratory (Liverpool School of Tropical Medicine) by Mrs. Jill Davies, which provided a foundation for the current work. The authors also thank Ms. Katy Sherlach (Georgetown University, USA) for technical help and helpful discussions and are grateful to BIONEXX (Madagascar) and Charles Giblane for supply of *A. annua* biomass.

References

1. Hsu E (2006) The history of qing hao in the Chinese materia medica. Transactions of the Royal Society of Tropical Medicine and Hygiene 100: 505-508.

2. Wright CW, Linley PA, Brun R, Wittlin S, Hsu E (2010) Ancient Chinese methods are remarkably effective for the preparation of artemisinin-rich extracts of Qing Hao with potent antimalarial activity. Molecules 15: 804-812.

3. O'Neill PM, Barton VE, Ward SA (2010) The molecular mechanism of action of artemisinin—the debate continues. Molecules 15: 1705-1721.

4. Ding XC, Beck H-P, Raso G (2011) Plasmodium sensitivity to artemisinins: magic bullets hit elusive targets. Trends in parasitology 27: 73-81.

5. Pandey AV, Tekwani BL, Singh RL, Chauhan VS (1999) Artemisinin, an endoperoxide antimalarial, disrupts the hemoglobin catabolism and heme detoxification systems in malarial parasite. Journal of Biological Chemistry 274: 19383-19388.

6. Jefford CW (2001) Why artemisinin and certain synthetic peroxides are potent antimalarials. Implications for the mode of action. Current Medicinal Chemistry 8: 1803-1826.

7. Eckstein-Ludwig U, Webb R, Van Goethem I, East J, Lee A, et al. (2003) Artemisinins target the SERCA of Plasmodium falciparum. Nature 424: 957-961.

8. Wang J, Huang L, Li J, Fan Q, Long Y, et al. (2010) Artemisinin Directly Targets Malarial Mitochondria through Its Specific Mitochondrial Activation. PLoS ONE 5: e9582.

9. Li W, Mo W, Shen D, Sun L, Wang J, et al. (2005) Yeast Model Uncovers Dual Roles of Mitochondria in the Action of Artemisinin. PLoS Genet 1: e36.

10. Haynes RK, Monti D, Taramelli D, Basilico N, Parapini S, et al. (2003) Artemisinin antimalarials do not inhibit hemozoin formation. Antimicrobial agents and chemotherapy 47: 1175-1175.

11. Golenser J, Waknine JH, Krugliak M, Hunt NH, Grau GE (2006) Current perspectives on the mechanism of action of artemisinins. International Journal for Parasitology 36: 1427-1441.

12. Eastman RT, Fidock DA (2009) Artemisinin-based combination therapies: a vital tool in efforts to eliminate malaria. Nature Reviews Microbiology 7: 864-874.

13. Klonis N, Crespo-Ortiz MP, Bottova I, Abu-Bakar N, Kenny S, et al. (2011) Artemisinin activity against Plasmodium falciparum requires hemoglobin

uptake and digestion. Proceedings of the National Academy of Sciences 108: 11405-11410.

14. Haynes RK (2006) From artemisinin to new artemisinin antimalarials: biosynthesis, extraction, old and new derivatives, stereochemistry and medicinal chemistry requirements. Current topics in medicinal chemistry 6: 509-537.

15. Weina P (2008) Artemisinins from Folklore to Modern Medicine-Transforming an Herbal Extract to Life-Saving Drugs. Parassitologia 50: 25.

16. Jansen F (2006) The herbal tea approach for artemisinin as a therapy for malaria? Transactions of the Royal Society of Tropical Medicine and Hygiene 100: 285.

17. WHO Report (2012) WHO position statement on effectiveness of non-pharmaceutical forms of *Artemisia annua* L. against malaria. Available at http://www.who.int/malaria/position_statement_herbal_remedy_artemisia_annua_l.pdf.

18. Hsu E (2006) Reflections on the discovery of the antimalarial qinghao. British journal of clinical pharmacology 61: 666-670.

19. Rath K, Taxis K, Walz G, Gleiter CH, Li SM, et al. (2004) Pharmacokinetic study of artemisinin after oral intake of a traditional preparation of Artemisia annua L.(annual wormwood). The American journal of tropical medicine and hygiene 70: 128-132.

20. De Ridder S, Van der Kooy F, Verpoorte R (2008) Artemisia annua as a self-reliant treatment for malaria in developing countries. Journal of Ethnopharmacology 120: 302-314.

21. Van der Kooy F, Verpoorte R (2011) The Content of Artemisinin in the Artemisia annua Tea Infusion. Planta Medica-Natural Products and Medicinal Plant Research 77: 1754.

22. Mouton J, Jansen O, Frédérich M, van der Kooy F (2013) Is Artemisinin the Only Antiplasmodial Compound in the Artemisia annua Tea Infusion? An in Vitro Study. Planta Medica 79: 468-470.

23. Willcox M, Bodeker G, Bourdy G, Dhingra V, Falquet J, et al. (2004) Artemisia annua as a traditional herbal antimalarial. Traditional Medicinal Plants and Malaria: 43-59.

24. Liu K, Yang S-L, Roberts M, Elford B, Phillipson J (1992) Antimalarial activity of Artemisia annua flavonoids from whole plants and cell cultures. Plant Cell Reports 11: 637-640.

25. Elford BC, Roberts MF, Phillipson JD, Wilson RJM (1987) Potentiation of the antimalarial activity of qinghaosu by methoxylated flavones. Transactions of the Royal Society of Tropical Medicine and Hygiene 81: 434.

26. Bilia AR, Lazari D, Messori L, Taglioli V, Temperini C, et al. (2002) Simple and rapid physico-chemical methods to examine action of antimalarial drugs with hemin: its application to Artemisia annua constituents. Life Sciences 70: 769-778.

27. Weathers PJ, Towler MJ (2012) The flavonoids casticin and artemetin are poorly extracted and are unstable in an Artemisia annua tea infusion. Planta medica 78: 1024.

28. Carbonara T, Pascale R, Argentieri MP, Papadia P, Fanizzi FP, et al. (2012) Phytochemical analysis of a herbal tea from Artemisia annua L. Journal of Pharmaceutical and Biomedical Analysis 62: 79-86.

29. Zhang B, Yang R, Liu CZ (2008) Microwave-assisted extraction of chlorogenic acid from flower buds of *Lonicera japonica* Thunb. Separation and Purification Technology 62: 480-483.

30. Feng R, Lu Y, Bowman LL, Qian Y, Castranova V, et al. (2005) Inhibition of activator protein-1, NF-kappaB, and MAPKs and induction of phase 2 detoxifying enzyme activity by chlorogenic acid. Journal of Biological Chemistry 280: 27888-27895.

31. Miketova P, Schram KH, Whitney J, Kearns EH, Timmermann BN (1999) Mass spectrometry of 3,5- and 4,5-dicaffeoylquinic acids and selected derivatives. Journal of Mass Spectrometry 34: 1240-1252.

32. Belkaid A, Currie J-C, Desgagnés J, Annabi B (2006) The chemopreventive properties of chlorogenic acid reveal a potential new role for the microsomal glucose-6-phosphate translocase in brain tumor progression. Cancer cell international 6: 7.

33. De Donno A, Grassi T, Idolo A, Guido M, Papadia P, et al. (2012) First-time comparison of the in vitro antimalarial activity of Artemisia annua herbal tea and artemisinin. Transactions of the Royal Society of Tropical Medicine and Hygiene 106: 696-700.

34. Wang L-H, Song Y-T, Chen Y, Cheng Y-Y (2007) Solubility of Artemisinin in Ethanol + Water from (278.2 to 343.2) K. Journal of Chemical & Engineering Data 52: 757-758.

35. Suberu J, Song L, Slade S, Sullivan N, Barker G, et al. (2013) A rapid method for the determination of artemisinin and its biosynthetic precursors in Artemisia annua L. crude extracts. Journal of Pharmaceutical and Biomedical Analysis 84: 269-277.

36. Bennett TN, Paguio M, Gligorijevic B, Seudieu C, Kosar AD, et al. (2004) Novel, rapid, and inexpensive cell-based quantification of antimalarial drug efficacy. Antimicrobial agents and chemotherapy 48: 1807-1810.

37. Bray PG, Deed S, Fox E, Kalkanidis M, Mungthin M, et al. (2005) Primaquine synergises the activity of chloroquine against chloroquine-resistant *P. falciparum*. Biochemical Pharmacology 70: 1158-1166.

38. Berenbaum M (1978) A method for testing for synergy with any number of agents. Journal of Infectious Diseases 137: 122-130.

39. Vivas L, Rattray L, Stewart L, Robinson B, Fugmann B, et al. (2007) Antimalarial efficacy and drug interactions of the novel semi-synthetic endoperoxide artemisone in vitro and in vivo. Journal of antimicrobial chemotherapy 59: 658-665.

40. 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: 4097-4102.

41. Friedman M (1997) Chemistry, biochemistry, and dietary role of potato polyphenols. A review. Journal of Agricultural and Food Chemistry 45: 1523-1540.

42. Noratto G, Porter W, Byrne D, Cisneros-Zevallos L (2009) Identifying peach and plum polyphenols with chemopreventive potential against estrogen-independent breast cancer cells. Journal of agricultural and food chemistry 57: 5219-5226.

43. De Magalhaes PM, Dupont I, Hendrickx A, Joly A, Raas T, et al. (2012) Anti-inflammatory effect and modulation of cytochrome P450 activities by Artemisia annua tea infusions in human intestinal Caco-2 cells. Food Chemistry 134: 864-871.

44. Willcox M (2009) Artemisia species: from traditional medicines to modern antimalarials-and back again. The Journal of Alternative and Complementary Medicine 15: 101-109.

45. Acton N, Klayman DL (1987) Conversion of artemisinin (qinghaosu) to iso-artemisitene and to 9-epi-artemisinin1. Planta medica 53: 266.

46. Woerdenbag HJ, Moskal TA, Pras N, Malingré TM, El-Feraly FS, et al. (1993) Cytotoxicity of Artemisinin-Related Endoperoxides to Ehrlich Ascites Tumor Cells. Journal of Natural Products 56: 849-856.

47. Wagner H, Ulrich-Merzenich G (2009) Synergy research: Approaching a new generation of phytopharmaceuticals. Phytomedicine 16: 97-110.

48. Wagner H (2011) Synergy research: Approaching a new generation of phytopharmaceuticals. Fitoterapia 82: 34-37.

49. Wagner H (2005) Natural products chemistry and phytomedicine in the 21st century: new developments and challenges. Pure and applied chemistry 77: 1-6.

50. Yordi EG, Pérez EM, Matos MJ, Villares EU (2012) Antioxidant and Pro-Oxidant Effects of Polyphenolic Compounds and Structure-Activity Relationship Evidence. Nutrition, Well-Being and Health: InTech.

51. Nemeikaitė-Čėnienė A, Imbrasaitė A, Sergedienė E, Čėnas N (2005) Quantitative structure–activity relationships in prooxidant cytotoxicity of polyphenols: role of potential of phenoxyl radical/phenol redox couple. Archives of biochemistry and biophysics 441: 182-190.

52. Cui L, Wang ZL, Miao J, Miao M, Chandra R, et al. (2012) Mechanisms of in vitro resistance to dihydroartemisinin in Plasmodium falciparum. Molecular Microbiology 86: 111-128.

53. Sidhu ABS, Uhlemann A-C, Valderramos SG, Valderramos J-C, Krishna S, et al. (2006) Decreasing pfmdr1 copy number in Plasmodium falciparum malaria heightens susceptibility to mefloquine, lumefantrine, halofantrine, quinine, and artemisinin. Journal of Infectious Diseases 194: 528-535.

54. Erkan N, Ayranci G, Ayranci E (2008) Antioxidant activities of rosemary (Rosmarinus Officinalis L.) extract, blackseed (Nigella sativa L.) essential oil, carnosic acid, rosmarinic acid and sesamol. Food Chemistry 110: 76-82.

55. Petersen M, Simmonds MSJ (2003) Rosmarinic acid. Phytochemistry 62: 121-125.

56. Tepe B, Eminagaoglu O, Akpulat HA, Aydin E (2007) Antioxidant potentials and rosmarinic acid levels of the methanolic extracts of Salvia verticillata (L.) subsp. verticillata and S. verticillata (L.) subsp. amasiaca (Freyn & Bornm.) Bornm. Food Chemistry 100: 985-989.

57. Gülçin İ (2006) Antioxidant activity of caffeic acid (3,4-dihydroxycinnamic acid). Toxicology 217: 213-220.

58. Xu J-G, Hu Q-P, Liu Y (2012) Antioxidant and DNA-Protective Activities of Chlorogenic Acid Isomers. Journal of Agricultural and Food Chemistry 60: 11625-11630.

59. Cao D, Li H, Yi J, Zhang J, Che H, et al. (2011) Antioxidant Properties of the Mung Bean Flavonoids on Alleviating Heat Stress. PLoS ONE 6: e21071.

60. Bozdech Z, Ginsburg H (2004) Antioxidant defense in Plasmodium falciparum–data mining of the transcriptome. Malaria journal 3: 23.

61. Agrawal PK, Vishwakarma RA, Jain DC, Roy R (1991) High field NMR spectroscopic studies of arteannuin B and a reappraisal of the structure of arteannuin C. Phytochemistry 30: 3469-3471.

62. Lansbury PT, Mojica CA (1986) Total synthesis of (±)-arteannuin B. Tetrahedron letters 27: 3967-3970.

Tables:

Table 1: Metabolites in the aqueous *A. annua* extract analysed by both MS/MS and HPLC methods quantified as milligrams per litre of tea.

Compound	Amount (mg L^{-1} of tea)*
Artemisinin	47.5±0.8
Arteannuin B	1.3±0.0
Dihydroartemisinic acid	70.0±0.3
Caffeic acid	0.8±0.00
3,5-Di-caffeoylquinic acid	57.0±1.7
3-Caffeoylquinic acid	72.0±1.6
4-Caffeoylquinic acid	20.4±1.6
4,5-Di-caffeoylquinic acid	31.6±4.0
5-Caffeoylquinic acid	9.0±0.7
Isovitexin	105.0±7.2
Rosmarinic acid	1.1±0.0

*Values are an average of triplicate determinations with ± S.E.M.

Table 2. IC_{50} of extracts and components of *A. annua* in CQ-sensitive (HB3) and resistant (Dd2) strains.

	IC_{50} (nM)[a]	
Compound/extracts	HB3 strain	Dd2 strain
Chloroquine (CQ)	21.8 ± 2.4	202.9 ± 10.7
Artemisinin	22.6 ± 0.7	21.2 ± 2.3
Artesunate	8.8 ± 0.3	5.6 ± 0.6
Artemisitene	88.4 ± 9.9	74.1 ± 7.8
9-epi-artemisinin	59.2 ± 1.7	62.2 ± 1.0
Artemisia aqueous extract (Tea)[b]	7.6 ± 3.4	2.9 ± 0.4
	IC_{50} (μM)[a]	
Artemisinic acid	77.8 ± 1.5	61.6 ± 7.5
Arteannuin B	3.2 ± 0.1	4.8 ± 0.4
Dihydroartemisinic acid	21.1 ± 0.7	17.7 ± 4.2
Caffeic acid	60.4 ± 4.3	47.5 ± 8.8
3-Caffeoylquinic acid	69.4 ± 6.4	61.4 ± 4.3
4-Caffeoylquinic acid	61.4 ± 4.3	53.6 ± 5.0
5-Caffeoylquinic acid	84.8 ± 6.4	85.3 ± 4.2
3,4-Caffeoylquinic acid	36.2 ± 1.0	49.0 ± 6.8
4,5-Caffeoylquinic acid	29.3 ± 2.4	43.2 ± 4.2
3,4,5-Caffeoylquinic acid	181.4 ± 2.1	88.2 ± 6.2
Rosmarinic acid	65.1 ± 5.0	65.0 ± 7.0
Isovitexin	72.5 ± 6.8	48.1 ± 4.5
Casticin	17.9 ± 4.7	12.2 ± 1.8

[a]IC_{50} values are an average of at least three independent measurements each performed in triplicate, and are shown ± S.E.M of the three independent experiments.
[b]IC_{50} of extract determined based on the artemisinin content (i.e. ART IC_{50} of extract) see Table 2.

Table 3: Solubility of artemisinin, artemisitene and 9-epi-artemisinin in water at 22 °C and atmospheric pressure.

Compound	Solubility [mg L^{-1}]* at 22 °C
Artemisinin	74.27±2.10
Artemisitene	74.21±2.99
9-Epi-artemisinin	133.08±5.44

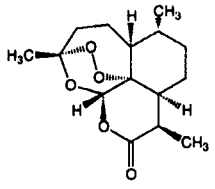
*Values are an average of triplicate determinations with ± S.E.M.

Table 4. Anti-plasmodial interactions of co-metabolites with artemisinin in CQ-sensitive (HB3) and CQ-resistant (Dd2) strains.

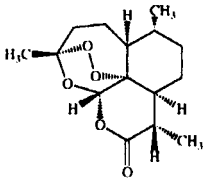
Art = artemisinin, CA = caffeic acid, 3CA = 3-caffeoylquinic acid, 4CA = 4-caffeoylquinic acid, 5CA = 5-caffeoylquinic acid, 3,4 CA = 3,4-di-caffeoylquinic acid, 3,5CA = 3,5-di-caffeoylquinic acid, 4,5CA = 4,5-di-caffeoylquinic acid, TCA = 3,4,5-tri-caffeoylquinic acid, ISO = siovitexin, CAS = casticin, ATCID = artemisinic acid, ARTB = arteannuin B, RA = rosmarinic acid, DHAA = dihydroartemisinic acid, ATENE = artemisitene.

	HB3		Dd2	
Combination	FIC_{index}	Interaction	FIC_{index}	Interaction
1:3 ART:CA	1.570	Antagonistic	4.046	Antagonistic
1:3 ART:3CA	1.172	Additive	2.088	Antagonistic
1:10 ART:3CA	0.685	Synergistic	1.087	Additive
1:100 ART:3CA	0.781	Synergistic	1.177	Additive
1:3 ART:4CA	1.088	Additive	4.266	Antagonistic
1:3 ART:5CA	0.928	Additive	2.460	Antagonistic
1:3 ART:34CA	2.253	Antagonistic	4.862	Antagonistic
1:3 ART:35CA	2.312	Antagonistic	4.749	Antagonistic
1:3 ART:45CA	2.315	Antagonistic	4.844	Antagonistic
1:3 ART:TCA	1.220	Additive	3.041	Antagonistic
1:3 ART:ISO	1.534	Antagonistic	4.829	Antagonistic
1:3 ART:CAS	1.921	Antagonistic	3.034	Antagonistic
1:3 ART:ATCID	1.467	Additive	4.152	Antagonistic
1:3 ART:ARTB	1.250	Additive	0.342	Synergistic
1:3 ART:RA	0.890	Synergistic	4.952	Antagonistic
1:3 ART:DHAA	1.801	Antagonistic	2.861	Antagonistic
1:3 ART:ATENE	3.480	Antagonistic	7.002	Antagonistic
ART	1	-	1	-

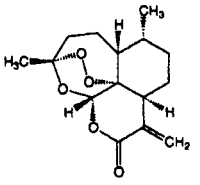
Figures:



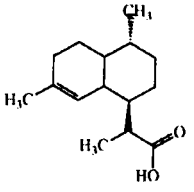
(1) artemisinin



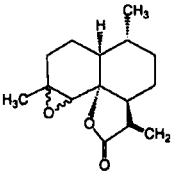
(2) 9-epi-artemisinin



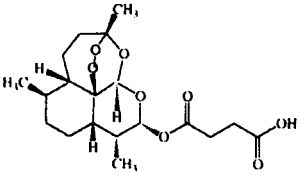
(3) artemisitene



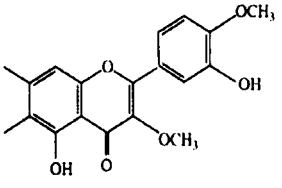
(4) dihydroartemisinic acid



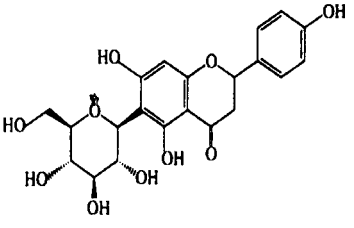
(5) arteannuin B



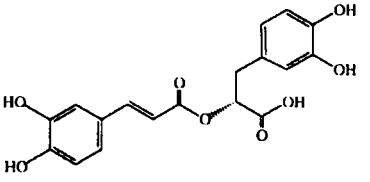
(6) artesunate



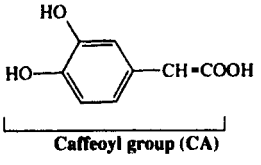
(7) casticin



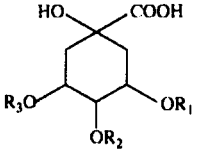
(8) isovitexin



(9) rosmarinic acid



(10) caffeic acid



(11-17) quinic acid

	R_1	R_2	R_3
(11) 3-caffeoylquinic acid	CA	H	H
(12) 4-caffeoylquinic acid	H	CA	H
(13) 5-caffeoylquinic acid	H	H	CA
(14) 3,4-di-caffeoylquinic acid	CA	CA	H
(15) 3,5-di-caffeoylquinic acid	CA	H	CA
(16) 4,5-di-caffeoylquinic acid	H	CA	CA
(17) 3,4,5-tri-caffeoylquinic acid	CA	CA	CA

Figure 1. Structures of some artemisinin related compounds, flavonoids and acids identified in *A. annua* extract.

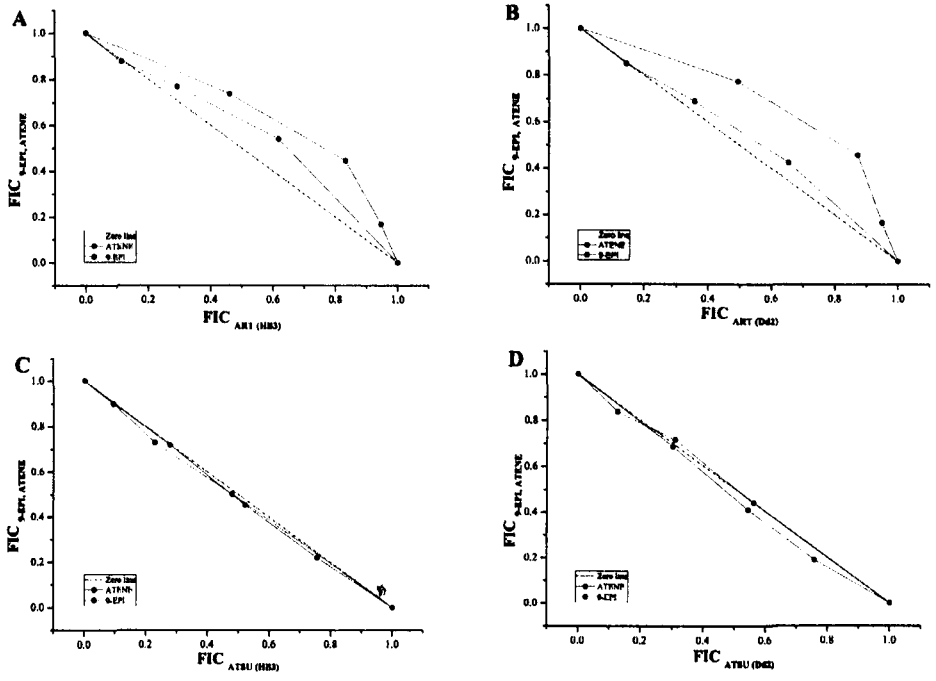


Figure 2. Isobologram showing the plot of fractional inhibitory concentration (FIC) of 9-epi-artemisinin (EPI) and artemisitene (ATENE) against FIC of artemisinin (ART) and artesunate (ATSU). Panel A – interaction of EPI and ATENE with ART in chloroquine-sensitive (CQS) HB3 strain. Panel B – same as in A but in CQ-resistant (CQR) Dd2 strain. Panel C - interaction of EPI and ATENE with ATSU in HB3. Panel D – same as C but in Dd2 parasite

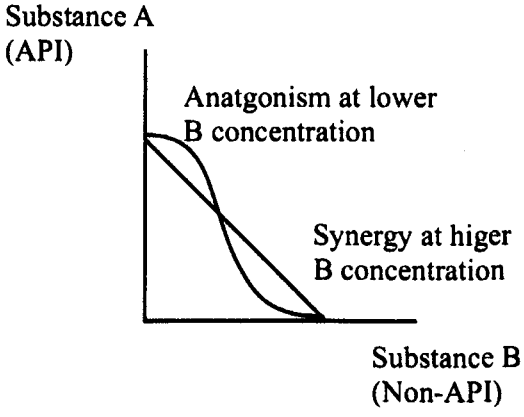


Figure 3. A schematic isobologram of the interaction of artemisinin (API) with anti-oxidant synergist (Non-API