Development of *Artemisia annua* L. as a crop for production of the antimalarial drug artemisinin

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Quality assessment and sample purification, from raw herb to active pharmaceutical ingredient

Corrinne Burns

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LINK Project 0822: Developing an alternative UK industrial crop *Artemisia annua*, for the extraction of artemisinin to treat multi-drug resistant malaria.

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"120 Years of Phytochemistry"

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List of Acronyms

AAHR	*Artemisia annua* hairy roots
ABA	Abscisic acid
ACT	Artemisinin combination therapy
API	Active pharmaceutical ingredient
ATP	Adenosine triphosphate
atp6	A gene encoding a sarcoplasmic and endoplasmic reticulum calcium ATPase (SERCA)-type protein
bar	Unit of pressure
BDL	Botanical Developments Ltd
CCC	Counter-current chromatography
C_{max}	Maximum serum concentration of a therapeutic drug
COSY	Correlation spectroscopy
CYP	Cytochrome
DAD	Diode-array detection
DCM	Dichloromethane
DDT	Dichlorodiphenyltrichloroethane, a pesticide
DHA	Dihydroartemisinin
DHOase	Dihydroorotase
DMAPP	Dimethylallyl diphosphate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DMU	De Montfort University
DNDi	Drugs for Neglected Diseases Initiative
DW	Dry weight
DXP	Deoxy-xylulose phosphate
EDTA	Ethylenediaminetetraacetic acid
EMR	East Malling Research
ESI	Electrospray Ionisation
FDA	Food and Drug Administration

Fe(II)	Ferrous Iron (also: Fe^{2+})
FIA	Flow Injection Analysis
FPP	Farnesol Diphosphate
FPPS	Farnesol Diphosphate Synthase
GGPP	Geranyl Geranyl Diphosphate
GLY	Glyphosate
GPP	Geranyl Diphosphate
GST	Glandular Secretory Trichome
HDC	Horticultural Development Company
HMG-CoA	β-hydroxy-β-methylglutaryl-CoA
HPLC	High Pressure Liquid Chromatography
IC_{50}	50% inhibitory concentration
IP	International Pharmacopeia
IPP	Isopentenyl Diphosphate
IS	Internal Standard
LCMS	Liquid Chromatography – Mass Spectroscopy
LINK Project 0822	A Defra and HDC-funded project – "Developing an alternative UK industrial crop *Artemisia annua*, for the extraction of artemisinin to treat multi-drug resistant malaria" - under which the work reported in this thesis was completed
LV	Latent Variable
MCF10A	An non-cancerous human breast cell line
MCF7	A human breast cancer cell line
MDA468	A human breast cancer cell line
MEP	2C-methyl-d-erythritol 5-phosphate
MHz	Megahertz
MMV	Malaria Medicines Venture
MS	Murashige and Skoog, a plant nutritional medium
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MVA	Mevalonic Acid
N	Theoretical Plates
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate

NF0613	A Defra-funded pilot project NF0613 – "Field cultivation of *Artemisia annua* and enhanced extraction of artemisinin used in novel antimalarial treatments" – which ran from 2005 until 2006, and preceded LINK 0822
NGO	Non-governmental Organisation
NIAB	National Institute of Agricultural Botany
NMR	Nuclear Magnetic Resonance
NPR	Natural Products Research
ODS	Octadecylsilane
OTC	Over the counter
PC	Principal component
PCA	Principal components analysis
pfatp6	Gene coding for a *Plasmodium falciparum* SERCA
pfcrt	Gene coding for a *Plasmodium falciparum* chloroquine resistance transporter
PfEMP-1	A *Plasmodium falciparum* protein mediating cytoadherence
PfHRP	*Plasmodium falciparum* histidine-rich protein
pfmdr1	Genes coding for *Plasmodium falciparum* multi-drug resistance
PLS-DA	Partial least squares-discriminant analysis
r	Pearson's correlation coefficient
R^2	Linear regression
R*f*	Retention factor
RITAM	Research Initiative on Traditional Antimalarial Methods
RPMI	Roswell Park Memorial Institute, a cell culture medium
SD	Standard Deviation
SERCA	Sarcoplasmic and endoplasmic reticulum calcium ATPase
SIM	Single Ion Monitoring
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
tctp	A gene responsible for the production of translationally-controlled tumour protein
TCTP	Translationally-controlled tumour protein
TIC	Total Ion Current
TLC	Thin Layer Chromatography

UV	Ultra-Violet
v/v	Volume-to-volume
w/w	Weight-to-weight
WHO	World Health Organisation

Abstract

Artemisinin is the parent compound for the latest generation of anti-malarial drugs. In many cases of severe or drug-resistant malaria, the artemisinins are the only effective means of treatment for this common and life-threatening parasitic infection. However, the current supply of artemisinin is unstable, and is predicted to fall far short of demand in the coming years. As artemisinin can currently not be economically synthesised, the only source of the compound is the Chinese herb *Artemisia annua* L.

With the ultimate objective of stabilising the global supply of artemisinin, a Consortium of academics, agronomics and business partners was established. The Consortium, with funding from Defra and the Horticultural Development Company under the name of Project LINK 0822, was to examine the feasibility of establishing an "artemisinin supply chain" – from crop in the field to pure, active pharmaceutical ingredient – based entirely within the UK.

The work presented in this thesis formed part of this Consortium's research – namely, the establishment and validation of rapid, accurate and economical means of quality control of both raw herbal material and purified ingredient. To that end, methods were developed allowing both field-based, triaging quantification and more accurate, high-throughput laboratory-based quantification of sample material. Improved means of artemisinin purification – in particular the removal of the inactive metabolite deoxyartemisinin, which co-elutes and co-crystallises with artemisinin - were also developed.

In addition, the potential pharmacological value of other compounds besides artemisinin was explored – namely, chrysosplenetin. This latter is a methoxylated flavonoid that accumulates with artemisinin in the plant, and has been shown, in this study, to have potential in the treatment of cancer.

Finally, the feasibility of self-medication with *Artemisia annua* L. - in the form of herbal teas and over-the-counter preparations of the plant – was examined, and the amount of artemisinin, deoxyartemisinin and chrysosplenetin in such preparations was examined.

During the four years in which the LINK project ran, the Consortium members worked together to ultimately develop new lines of *Artemisia annua* L, that not only flourished in the UK climate, but also consistently yielded high (> 2.2 % w/DW) levels of artemisinin – almost a twofold increase from that at the beginning of the project. These new lines, together with the improved analytical techniques and means of artemisinin purification, demonstrate clearly that an artemisinin supply chain can indeed be based within the UK, thus contributing to a stable, year-on-year supply of artemisinin.

Prologue – background to this research project

The results presented within this thesis were obtained during four years of collaboration between De Montfort University's Natural Products Research (NPR) laboratory, and a consortium of academic, industrial and business partners. Together, the consortium set the objective of establishing, as a proof of principle, that -

1. The Chinese herb *Artemisia annua* L. – currently the only source of the antimalarial compound artemisinin – could be grown on a large scale in the UK climate;
2. Conventional plant breeding techniques could be used to create new, high artemisinin-yielding varieties of *Artemisia annua* L. that would thrive in the UK;
3. The active pharmaceutical ingredient (API), artemisinin, could be extracted from the crop and purified to International Pharmacopoeia standards (not less than 97 % and not more than the equivalent of 102 % of $C_{15}H_{22}O_5$; melting range**,** 151 – 154 °C) at reasonable cost.

In short, the objective of the consortium was to demonstrate that the UK could host the entire artemisinin supply chain, from field to laboratory, with the end-product of the chain being commercial amounts of pure artemisinin ready for sale to the pharmaceutical industry. The UK was considered an especially suitable host for *Artemisia annua* L. because the plant is a short-day species: that is, a short day length, such as that found in tropical regions, triggers flowering in the plant. As it has been demonstrated that artemisinin levels progressively increase throughout the vegetative period of growth, and then drop sharply upon flowering (Weathers et al., 2006), it was hypothesised that growing the crop under long-day conditions, such as those found in a UK summer, would prolong the vegetative stage, and thus increase the final yields of artemisinin in the crop.

Part of NPR's role in this supply chain was to develop rapid, accurate and inexpensive means of quality control for samples produced throughout the supply chain. The major role of such quality control was to monitor fluctuations in artemisinin levels in growing crops, in order to ascertain optimal crop harvest dates. In addition, it was important to be able to monitor the response of plant artemisinin levels to standard agricultural products; herbicides and growth regulators, for example.

Crop samples came in two forms: fresh leaf sampled at the site of growth (where the analytical objective is rapid, easy-to-use, semi-quantification of artemisinin levels) or dried leaf material assayed in the laboratory (in which more precise quantification is desirable). Dried leaf samples typically represented sampling of an entire crop on a given date or following a given treatment, and such sample sizes were typically greater than 500, necessitating rapid – but still accurate - high-throughput systems.

Additionally, NPR was required to develop means of artemisinin purification from *Artemisia annua* L. dried leaf material. This meant monitoring the presence, in crude and semi-purified extracts, of other compounds, in particular the pharmaceutically inactive deoxyartemisinin – which frequently co-extracts with artemisinin, and has to be removed with minimal artemisinin loss.

Finally, the possibility of adding value to an *Artemisia annua* L. crop, by demonstrating that other potentially useful compounds could be isolated from the leaf, was explored. To that end, the levels of chrysosplenetin, a flavonoid with potential anti-cancer activity, were monitored alongside artemisinin.

This thesis presents the results of investigations into meeting the above objectives, beginning, in Sections 2 – 6, with the analytical procedures which were developed for both field-based and laboratory based quality assessment of samples.

Section 7 describes how the removal of the inactive derivative deoxyartemisinin from artemisinin was achieved.

Section 8 discusses the levels of chrysosplenetin in *Artemisia annua* L, and this compound's potential use in cancer therapy. This section also presents evidence that levels of methoxylated flavonoid aglycones in *Artemisia annua* L. are quantitatively linked to artemisinin levels – providing an additional means of sample quality control.

Section 9 describes a small experiment into the use of exogenous farnesol as a means of increasing artemisinin levels in *Artemisia annua* L. hairy root cultures.

Section 10 discusses the artemisinin, deoxyartemisinin and chrysosplenetin content of herbal teas prepared from *Artemisia annua* L. dried leaf, and the quality of over-the-counter preparations of *Artemisia annua* L.

Finally, the outcomes of the whole LINK project are discussed in Section 11.

But first, a review of the current situation in malaria treatment will be presented, with emphasis on the need for new antimalarial drugs, the efficacy of artemisinins and the mode of action of the drugs, and the economics of the artemisinin supply chain.

1 Introduction

1.1 Natural Products Research - a definition, and a brief history

In colloquial terms, the discipline of natural products research (NPR) – also known as phytochemistry - could be considered to be the point at which herbal medicine meets modern chemistry. The natural products chemist typically takes a plant known, or expected, to have medicinal properties, and systematically attempts to isolate the chemicals within that plant that are responsible for the medicinal properties.

The science has expanded, over time, to take in not just the medicinal plants, but also to consider microbiological sources of medicinal compounds. There are also elements of synthetic chemistry, in which the natural product is modified by the addition or removal of certain functional groups, creating a new semi-synthetic product with improved bioavailability, or reduced side-effect. Sometimes, the active region – or pharmacophore – of a natural product is taken as inspiration for the synthesis, *via* a process called combinatorial chemistry, of vast numbers of related compounds (a combinatorial library), which will then be screened for improved pharmacological action (Ferro and Gray, 2007).

The recent development of artificial neural networks – computer algorithms in which the *in silica* activity screening of natural products can be performed – means that it is now possible to do natural products research without ever handling plant or microbiological material. But, in general, drug discovery within phytochemistry is usually performed as a multi-step process involving first a bioassay on a crude extract, followed by fractionation of the crude extract and further bioassays, and, finally, the isolation and identification – and bioassay – of a single, active compound.

The first reports of the isolation of a pure, crystalline compound with demonstrable pharmaceutical properties – an active pharmaceutical ingredient (API), in modern industrial parlance – came in 1805, when the Austrian apothecary Friedrich Wilhelm Sertüner announced the isolation of what he called the “principium somniferum” from opium – opium being the dried latex of the opium poppy, *Papaver somniferum*. The principium somniferum was, of course, morphine (Sneader, 2005).

Although this isolation of morphine represents the first time an active pharmaceutical ingredient had been isolated from a medicinal plant, scholars do not consider Sertüner himself

the father of natural products research. That honour is, rather, given to two French pharmacists – Pierre-Joseph Pelletier and Joseph-Bienaimé Cavetou – who, in 1820, were responsible for the isolation of quinine from the "Peruvian bark", *Cinchona cordifolia*. It was not so much their successful isolation of the active pharmaceutical ingredient of cinchona bark, but their encouragement of other pharmacists to study single pure compounds rather than crude herbal drugs, that has led scholars to laud the two Frenchmen for setting the course of drug discovery in a new direction (Sneader, 2005).

But it is important to note that this standard *bioassay – fractionation – isolation* process does not take into account the potential synergistic action of multiple active compounds within a crude, or partially purified, extract. Plants had been utilized in this crude manner, for relief from the various human maladies, long before natural products research began in its modern form.

1.1.1 Plants as medicines

In its present form, humanity has been in existence for around sixty thousand years, it appears– sixty thousand years of inventing language, developing agriculture, building cities, and initiating philosophical investigations which would later become religion and science. And all this was achieved alongside sixty thousand years of parasitic infection, tuberculosis, cholera, influenza, a legion of STDs, childbirth, septic wounds and cancer.

And yet, the human race has survived and prospered, and was able to construct something beyond survival – civilization. Certainly the systems of agriculture and animal domestication were major contributors to that, but we must also include the development, by early human societies all over the globe, of medical practice in whatever form it took.
Wherever historical civilizations have developed some form of written record – be that papyrus, codex or cave painting – they have recorded the use of medicinal plants. It is a system that arose over and over again, all over the world, where local diseases were treated with local plants, and represents a human need to understand and classify illness, and our response to it – to make a seemingly random and frightening concept a little less so.

Some of these early records are available to scholars: notably the systematic medicine developed in the great Islamic cities of Cairo and Alexandria, categorised and refined by Ibn Sina and his contemporaries. Less well studied are the codices of pre-Conquest and Conquest-

era Central America: Spanish friars – notably Bernadino de Sahagún – made it their business to record for posterity the details of the daily lives of the Aztec people, in the face of a rapidly collapsing indigenous culture. Sahagún's monumental *General History of the Things of New Spain* (de Sahagún, 1963) stands alongside Martin de la Cruz's *Aztec Herbal of 1552* (de la Cruz, 2000), and Hernandez' later *Rerum* (Varey, 2000), as written testament to a sophisticated plant-based medicine. And, most relevantly to this thesis, we have the ancient system of Chinese medicine, systematically recorded by such ancient physicians as Ge Hong and Li-Shih-Chen. These sources of information – still of great interest to natural product chemists – are discussed in more detail in Section 1.1.5.1.

1.1.1.1 Modern plant-based medicine

It would be incorrect to give the impression, from all this, that the use of crude herbal drugs has been consigned to history. In fact, a large proportion of the modern global population still relies on the use of herbs as a first line of medicine – sometime simply because of tradition, other times because of a lack of access to modern biomedical drugs - or, frequently in the case of indigenous cultures such as the Nahua of Mexico, a mixture of the two (Camey et al., 1996). And the European markets have seen, in recent years, a great upsurge in the use of over-the-counter herbal medicines for self-medication.

Natural products research has a vital role to play in the responsible use of such herbal medicines. In the case of indigenous medical systems, the analysis and identification of active ingredients can help to validate the traditional uses of medicinal plants, the preparation and use of which are often tied closely with ritual and religious cultural practises. Analysis can also help to establish effective means of harvest and preparation of traditional indigenous herbal medicines.

Where Western use of over-the-counter herbal medicines is concerned, a system of quality control is increasingly important as the sale of such products continues to rise – hence the Traditional Medicines Directive which comes into force in 2011, requiring herbal drugs to be subject to the same stringent regulations as any other medication.

So, although natural products research – a science derived from the millennia-old use of medicinal plants – has many roles to play, perhaps the central role is that of drug discovery. In this, it has been remarkably successful.

1.1.1.2 The current picture of plant-derived drugs in conventional medicine

A recent large-scale review by Newman and Cragg (2007), revealed that active pharmaceuticals are still for a large part extracted from natural sources. Newman and Cragg calculated that in the time period ranging from January 1981 until June 2006, of the total of 1010 new chemical entities approved for medicinal use, 275 were either natural products, or derived from natural products. Another 154 were inspired by natural products – that is, although the drugs were produced by total synthesis, the pharmacophore was based upon the skeleton of a natural product. If we just look at the drugs approved for the treatment of cancer, we see that of 100 approved drugs, almost half (43) were either natural products or derived from natural products, and in the case of antiparasitic drugs, of 14 new approved medications, exactly half were natural products or derived from such – including the artemisinins.

1.1.1.3 Example: artemisinin

A striking example of the successful application of a natural product to the treatment of a severe disease lies within the story of artemisinin. Currently existing as a number of semi-synthetic derivatives – artemether, introduced in 1987, artesunate, in the same year, and now as a co-formulated artemisinin-combination therapy (Coartem®; Riamet®) - these drugs represent the latest in a series of drugs designed to destroy the causative agent of malaria: the protozoan *Plasmodium* parasite.

The parent compound of these drugs - artemisinin – is a secondary metabolite of the Chinese herb *Artemisia annua* L. The plant – under the Chinese name of *qing hao*, or *huang hao*, had been known in ancient China as an effective remedy for intermittent fevers, but the active principle was not isolated until the 1970s (Liao, 2009). In less than thirty years following discovery, this molecule has become the single most effective treatment for malaria anywhere in the world.

To appreciate the significance of this discovery, it is necessary to take a closer look at malaria as a disease: its toll on human populations, and the longstanding checkmate between human and parasite.

1.1.2 Malaria: morbidity and mortality

According to the WHO World Malaria Report 2008, there were an estimated 247 million reported cases of malaria in 2006, of which nearly one million were fatal. The deaths, in the main, involved children under 5 years of age. (WHO, 2008c).

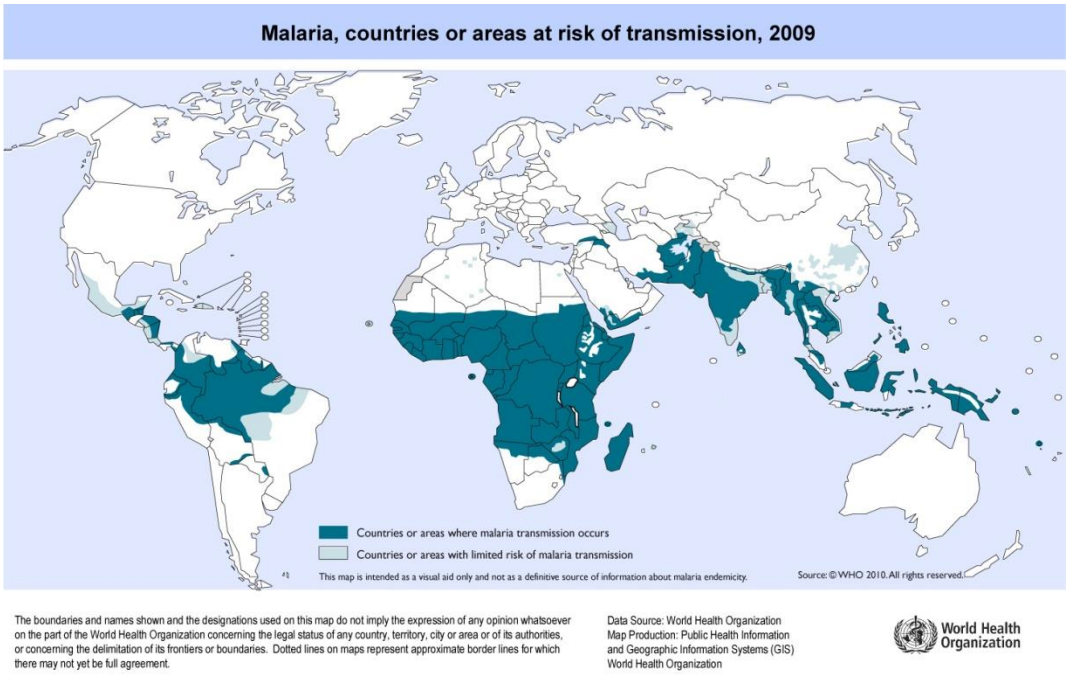


Figure 1. Map showing regions of the world in which the population lives at risk from malarial infection (WHO, International Travel and Health Report 2010). ©WHO 2010

1.1.3 Types of malaria

The numerous references, in the old herbals and early pharmacopoeias, to “intermittent” (as distinguished from “continuous”) fevers, indicate that the medical practitioners of the pre-biomedical era recognised that more than one type of malaria existed. This can be seen in the sub categorization of intermittent fevers – often called the “ague” – into those of a quotidian, tertian or quartan nature. A quotidian fever presented with febrile attacks recurring every 24 hours. Tertian fevers were those in which the febrile attacks recurred every 48 hours, and the

quartan fevers, every 72 hours (Hearsey, 1905). It was these intermittent fevers that were known to recur, sometimes after long intervals, giving rise to the "recurring fever" also frequently described in the old herbals and pharmacopoeias.

It is now known that the difference in frequency of the febrile attacks in tertian and quartan fevers is the result of infection of the red blood cells with different species of *Plasmodium* parasites – an infection with a *Plasmodium* species being the ultimate cause of malaria. The nature of the infection will be discussed in more detail below, using as an example the *Plasmodium falciparum* parasite, which causes the most severe form of malaria. These protozoa, responsible for 80% of all malaria infections and 90% of all deaths from malaria (de Ridder et al., 2008), trigger febrile attacks occurring every 36 to 48 hours: a tertian fever.

Other notable *Plasmodium* species include *P. vivax*, common in the Americas and causing a benign but recurring tertian malaria, with recrudescence occurring even up to 5 years following infection; *P. malariae*, again causing a benign malaria, and *P. ovale* (de Ridder et al., 2008). This latter is rather rare. In recent years, a new form of human malaria has been documented in Malaysian Borneo – *P. knowlesi*. This particular species – now identified as "the fifth human malaria species" – was historically confined to long-tailed and pig-tailed macaques, but has been shown to be naturally transmitted to humans by the *Anopheles* mosquito species *latens* and *cracens*. Although a close relative of *P. vivax*, *P. knowlesi* causes a 24 hour – quotidian – fever, and can cause serious, even fatal, illness (van den Eede et al., 2009).

Finally, it is possible for a patient to be simultaneously infected with more than one species of *Plasmodium*. The WHO noted that in Thailand, an estimated third of patients presenting with acute *P. falciparum* infection were also infected with *P. vivax*. Similarly, 8% of patients presenting with acute *P. vivax* infection were also harbouring parasites of *P. falciparum* (WHO, 2006). The treatment of these mixed infections will be discussed in Section 1.1.6.

1.1.4 Discovery of the cause of malaria

Despite millennia of malarial suffering, the actual cause of the disease remained a mystery until the late 19th Century. Previously, a correlation between the disease and stagnant water,

or foul air arising from such water, had been accepted – such an idea is expressed in the very name of the disease, *mal aire*, and perhaps also in the Aztec "aquatic fever of the night".

As recently as 1834, Mashall Hall, in his "The Principles of Diagnosis", wrote that these intermittent fevers were caused by proximity to stagnant water, marsh gases, and general humidity (Hall, 1834). He was almost correct. Later that century, the causative agent of malaria was finally identified by a French military surgeon, Charles Louis Alphonso Laveran. He performed autopsies in 192 soldiers who were believed to have died from malaria, and found, in the blood of 148 of them, pigmented bodies with moveable filaments or flagella (Haas, 1999). These flagellated bodies were found in the brain, spleen and liver of those soldiers, and led Laveran to hypothesize that these were parasites, and were the causative agent of malaria. Furthermore, he suggested that a species of mosquito could be the vector for transmission of this disease, but this was not demonstrated conclusively until a few years later. In 1897, Laveran's student, Ronald Ross, discovered these same parasites within the stomach wall and salivary glands of the female *Anopheles* mosquito. The life cycle of the newly discovered parasite, from mosquito to human and back again, was elucidated by Ross and two other men, Patrick Manson and Giovanni Grassi. Finally, the cause of so much suffering was understood (Haas, 1999).

Almost a century later, in 1976, the *in vitro* cultivation of *P. falciparum* was achieved, and with this, a new phase in malarial pharmacology was established: the rapid assay of potential new drugs (Wright, 2005).

1.1.5 The Life Cycle of *Plasmodium falciparum*

The causative parasite of *falciparum* malaria, *Plasmodium falciparum*, divides its time between the human body, and the malarial vector – the vector in this case being one of the 30-40 species of *Anopheles* mosquito capable of harbouring the parasite. Across much of Africa, *Anopheles gambiae* s.s is the major vector.

Table 1, overleaf, summarises the stages in the life cycle of *Plasmodium falciparum*.

Table 1. The life cycle of the *Plasmodium falciparum* parasite (Prescott et al., 2002)

Stage of Parasitic Life Cycle	Commentary	Interventions and Medication
The infected female mosquito, in search of a meal, bites the human		• Use of insecticide • Use of mosquito nets • Use of mosquito repellent
Sporozoites enter the human bloodstream from the mosquito's saliva, and immediately head for the liver	These stages are generally asymptomatic. One sporozoite can results in up to 30,000 merozoites.	• Antifolates
Within the heptaocytes, the sporozoites reproduce asexually, in a process called *schizogony*, to form numerous *merozoites*		• Tissue schizontocides • Antifolates
The merozoites escape the hepatocytes and enter the bloodstream, heading for the erythrocytes		• Antifolates
Within the erythrocyte, the merozoite enlarges, to from a *trophozoite* – a stage often described as "ring-form". The trophozoite nucleus then divides repeatedly to form the multi-nucleated *schizont*	This is the erythrocytic stage, and the cyclic activity of this stage (48-72 hours, in *P. falciparum*) accounts for the cyclic nature of the acute symptoms – mainly chills, with shaking, and fever, with sweating. Nacher theorises that "Malaria symptoms offer the vector (the *Anopheles* mosquito) its favorite cues: increased skin temperature, increased lactates, sweating, and CO_2 expiration."	• Antifolates
Each schizont then divides, sharing out its nuclei, and forming, in an echo of the liver stage, mononucleated merozoites		• Blood schizontocides (such as the artemisinin-derived drugs) • Antifolates
The erythrocyte bursts, and the merozoites are released into the blood, heading for new erythrocytes.		• Antifolates
Some merozoites form *gametocytes*, which become full *gametes* upon ingestion by the mosquito		• Use of insecticide • Use of mosquito nets
In the mosquito gut, gametes form diploid zygotes, called *ookinetes*	This process takes 13 days, and is considered a "fragile segment" in the malaria cycle – the cycle is most likely to be broken at this point.	
Ookinetes then form *oocysts*		
Oocysts undergo meiosis, and sporozoites are once again formed. These migrate to the mosquito's salivary gland, and wait for injection, whereupon the cycle begins again.		

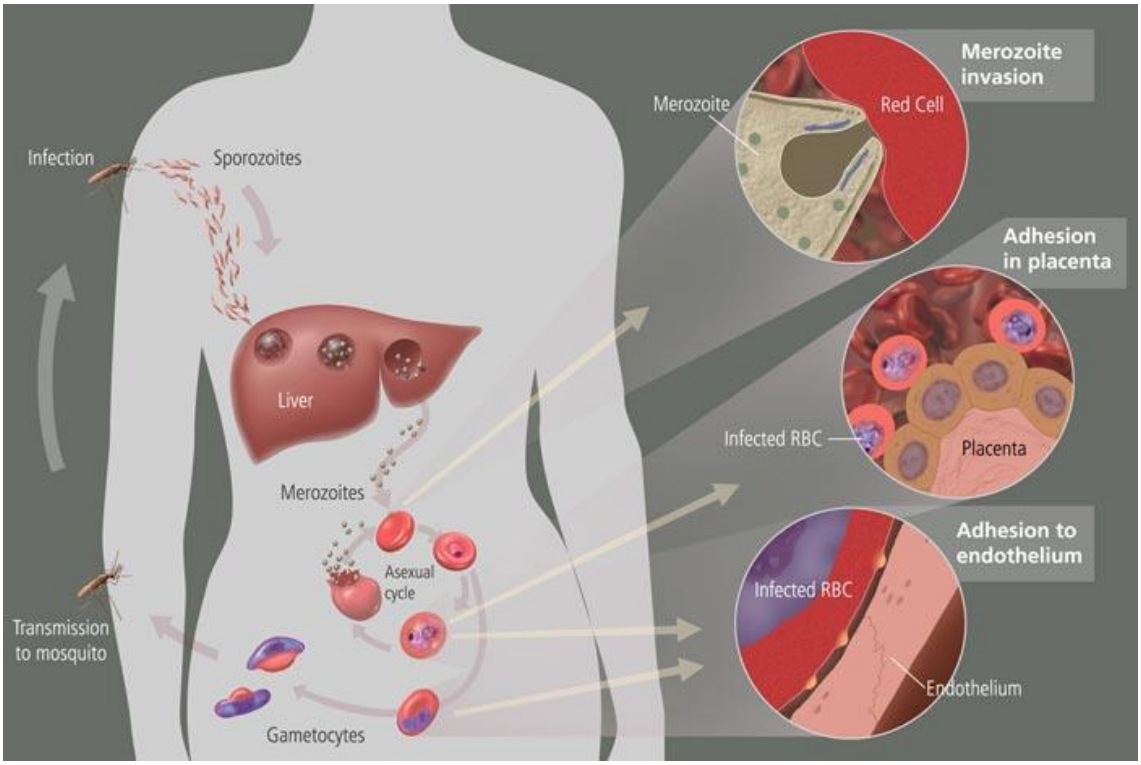


Figure 2. The parasitic life cycle. Image reproduced from the US National Institutes of Health (NIH) at *http://history.nih.gov/exhibits/bowman/SSmalaria.htm*

1.1.6 Symptoms of malaria

The disease, in each of its forms, typically presents initially as a low-grade malaise, accompanied by headache, muscular and joint aches and pains, and abdominal discomfort (WHO, 2006). The similarity of these symptoms to those of many viral infections is notable, and differential diagnosis can be difficult without access to blood tests.

Shortly after the appearance of these vague symptoms of illness, the characteristic features of malarial infection begin to appear. Most striking of these are the periodic fevers and chills, with increased perspiration, vomiting and weight loss adding to the clinical picture.

At this stage, the disease – once it has been confirmed as malaria – is defined as uncomplicated malaria. This indicates that although parasitemia is present, with the associated symptoms of malaria, the disease has not yet caused damage to the internal organs, and is not considered life-threatening. In fact, at this stage, the death rate for *falciparum* infection is around 0.1%, if suitable treatment is provided. If, however, treatment is withheld, or ineffective, the parasite levels in the body increase to the stage where the infection becomes

severe. Patients with severe malaria typically display one or more of the following: coma (i.e. cerebral malaria), metabolic acidosis, severe anaemia, hypoglycaemia, acute renal failure and acute pulmonary oedema. When the disease has progressed to this late stage, fatality rates can be as high as 20% even following administration of suitable medication – and without medication, the disease is invariably fatal (WHO, 2006).

1.1.7 Immunity to malaria

In regions where there is a consistently high level of malarial infection – defined as regions where each individual human is bitten by an infected mosquito more than ten times each year – the human population develops a degree of immunity – partial immunity - to the development of clinical disease. These individuals do develop parasitemia, but the level of parasites in the blood remains fairly low and the disease hence remains mild. This partial immunity is typically seen in sub-Saharan Africa. It must be noted, though, that this immunity is region-specific: if an individual leaves the area and moves to another malarial region, they are once more at risk of developing a serious infection.

Additionally, this immunity takes time to develop in an individual, and until that time, the individual remains at risk of severe disease. It is for this reason that the greatest number of deaths from malaria occur in young children, usually under the age of 5.

By contrast, in areas of low, or fluctuating, malarial infection, partial immunity does not develop in human populations. This is typically the case in most of Asia, and in Central and South America. In these regions – particularly if a malaria epidemic suddenly develops – individuals of all ages can succumb to the severe form of the disease.

Incidentally, molecular biological evidence - the presence of haemoglobin S, giving rise to sickle-cell trait, which gives the carrier some resistance to *P. falciparum* infection, and the absence of the red blood cell Duffy antigen (the lack of which confers resistance to *P. vivax)* - indicates that the *Plasmodium* genus originated and diversified in West African populations, and from there spread to the rest of the world with human migrations (Carter and Mendis, 2002).

1.1.8 Recurrence, Recrudescence and Relapse

Although superficially similar in meaning, it is worth noting that the commonly used terms "recurrence", "relapse", and "recrudescence" do not mean the same thing where malarial infection is concerned. According to the WHO, a recurrence of malaria is defined as the recurrence of asexual parasitemia following treatment. Recurrence may be defined further as a *recrudescence*, a *relapse* (in *P. vivax* and *P. ovale* infections only) or a new infection. Following from this definition, a recrudescence is defined as a recurrence of asexual parasitemia after treatment of the infection, but, crucially, *with the same infection that caused the original illness*. This typically occurs when a course of drugs has not been completed, and a few parasites have survived. A relapse may occur in all types of malarial infection, but is more commonly seen in *vivax* and *ovale* malaria, when a relapse – sometimes several months after the intial infection - occurs when parasites have persisted in the liver, after the infection has been eliminated from the blood (WHO, 2006).

1.1.9 Malaria in the 20th Century

The 20th century saw a rapid decline in malaria levels worldwide, for a number of reasons. Chief amongst these – at least in European nations – was the improved living conditions of much of the population, leaving them less exposed to stagnant water and the attacks of the *Anopheles* mosquito.

In addition to this was the deployment of highly effective insecticides against the *Anopheles* mosquito – notably DDT – and the use of these, in conjunction with effective and affordable quinine-based drugs against the parasite, led to the belief that malaria could actually be eradicated. In fact, by the early 1950s, malaria had actually been eradicated in large areas of previously high malarial infection, including North America and most of Europe (Hay et al., 2004). In Central America, although malaria was not eradicated, the use of DDT ensured a great reduction in malarial infections. However, in South America, no such reduction was possible simply because of the remoteness of most of the malarial regions of the continent.

Similarly, in Africa, the use of such insecticidal measures had little effect because of the sheer numbers of *Anopheles* mosquitoes and the continuous, intense level of malaria transmission. However, some reduction in mortality from malaria was observed in the time period ranging from the early 1950s to the late 1980s – but such a reduction did not last, and the spread of

chloroquine resistant strains of *P. falciparum* meant that malarial mortality was on the rise once again (Carter and Mendis, 2002). In fact, sub-Saharan Africa comprises the only region of the world in which malarial mortality has increased from the beginning to the end of the 20th Century: from 6% of all deaths in 1900, to 9% in 1997 – globally, the figures are 8% of deaths in 1900, and 0.08% in 1997.

It is certain that socio-economic factors in sub-Saharan Africa are a major reason for this rise in malarial mortality – and that, together with the prevalence in Africa of the most dangerous, *falciparum* form of malaria, has made Africa a focus of the latest attempts at the eradication of the disease – for example, the Roll Back Malaria programme, created in 1998 by the WHO, Unicef and the World Bank.

1.1.10 Treatments for malaria

1.1.10.1 China

It is unsurprising that China, with its longstanding medicinal tradition, would have developed a range of medications for the treatment of malarial infection. The Chinese herbal pharmacopoeias described two plant species in particular that may have been efficacious against malaria. The first of these was*changshan*, a Chinese hydrangea first recognised by the West in 1790 for use against "quotidian, tertian and quartan fevers" (Burns, 2008). The second – *qing hao* – is, of course, *Artemisia annua* L.

As was the case elsewhere in the world, the civilisation of China had grown alongside an increasing knowledge of the ways to treat the common and serious illnesses that afflicted its population. First the Han and then the Ming dynasties saw the production of extensive herbals, of which three are particularly revered. The first of these is the *Pen Ts'ao Ching*, or *Classic of Materia Medica* - a product of the latter years of the Han dynasty,a time which spans the years 200 BC to 220 AD - and authored by the Emperor Shen Nung (Wright et al., 2010). A great deal of mythology surrounds Shen Nung: he is regarded as the inventor of agriculture, and it has been claimed that his herbal in fact dates back to three millennia BC, although this is unlikely. What is known is that Shen Nung was the first to introduce the dual concept of warm and cool into Chinese medicine.

The second of the great Chinese herbals is the *Huang Di Neijing*, or *Yellow Emperor's Classic of Internal Medicine*. This document was written around the same time as the *Pen Ts'ao Ching* by an unknown author, and introduces the concept of yin and yang (Entrikin, 1950).

However, neither of these two documents appears to describe a plant bearing any resemblance to *Artemisia annua* L. For that, we have to look to a much older document: *Recipes for 52 Kinds of Disease,* unearthed in a West Han dynasty tomb dating from BC 168. This represents the first known reference to *Artemisia annua* L: identified by the unknown author as *qing hao*, or "blue-green herb". But in this document, no reference to a disease resembling malaria exists in relation to the use of *qing hao*: in fact, the herb was intended to be used in the relief of haemorrhoids

Evidence that *qing hao* was used in ancient China for the treatment of malaria comes from two other, more recent, documents. The first of these is Ge Hong's "Handbook of Prescriptions for Emergency Treatments", dating from some period between 281 and 340 AD. In this, and in Li Shih-Chen's *Pen Ts'ao Kang Mu,* published in 1597, are found references t the use of *qing hao* in the treatment of feverish illnesses (Li et al, 2006). This in itself, of course, is not evidence of antimalarial action: as in the West, Chinese medical practitioners of the pre-biomedical era considered "fever" to be an illness in itself, rather than the symptom of another illness of infection.

1.1.10.1.1 *Qing hao*: the blue-green herb

Botanical descriptions of *qing hao* led Chinese scientists working under the efforts of Program 523 – an initiative sent in motion in 1967 with the objective of searching Chinese traditional medicine for potential sources of antimalarial drugs (Li et al., 2006) – to *Artemisia annua* L; a mountainous annual herb, native to the Chahar and Suiyuan provinces of China, and naturalised in Vietnam. Evidence has been presented by Hsu, though, that in the old Chinese herbals, *qing hao* actually referred to a related species – *Artemisia apiaceae*. *Artemisia annua* L, in the herbals, was indicated by the name *huang hua hao*: "yellow-blossomed herb". It seems that in the oldest herbals – Ge Hong's, for example – *qing hao* actually indicated a herbal preparation rather than a defined species, and both *A. annua* and *A. apiaceae* were acceptable component of the drug *qing hao*. It was not until Li Shih-Chen's time that the two types of *Artemisia* were distinguished by Chinese practitioners – and, surprisingly, it was *A.*

apiaceae, not *A. annua*, that was recommended for Li Shih-Chen for relief from fever (Hsu, 2006).

It is not clear, then, what exactly led Chinese researchers of Program 523 to *A. annua* – but it was serendipitous, as the "correct" herb – *A. apiaceae* – contains comparatively little (0.08% w/DW, compared to up to 1% w/w in *A. annua*) of what was discovered to be the active ingredient - artemisinin.

1.1.10.2 The Americas

Outside of China, other malarial populations had identified medicines based on local resources. Although it is generally accepted that the Americas were free of malaria before the Conquest, there are scholars who doubt this, and claim that that malaria did exist in the Americas before the arrival of the Conquistadors (Bruce-Chwatt, 1965) To support this claim, these scholars point to the numerous references to intermittent fevers and chills in the indigenous languages of Central and South America. Such references are particularly prominent in Nahuatl, the language of the Aztecs - perhaps most strikingly in the case of *atonahuitzli.* This Nahuatl word translates as "the aquatic fever of the night-time" - a disease well known to the Aztecs and characterised by intermittent fevers and chills, frequently complicated by splenomegaly. When splenomegaly is present the diagnosis changes to "*nitic mocomaltia in atonahuitzli*" - literally, "the aquatic fever turns to spleen inside [the patient]". The Aztec Herbal, written in 1552, describes the symptoms of a severe fever as displaying "...various changes...[the face is] at times flushed, at times darkened, again blanched...the body jerks, he turns hither and thither", and that treatment is needed every third or fourth day (de la Cruz, 2000).

Furthermore, the presence of mosquitoes in pre-Columbian Mexico is clear from examples of pre-Columbian pottery depicting the insects, and from the diary of the Spanish conquistador Bernal Diaz. Diaz, in his memoirs, vividly recalls that "there were always many mosquitoes, both the long-legged ones, and the small ones which are called *xexenes* and are worse than the big ones; and together they gave us no sleep." (Diaz, 1963).

A search of the medicinal codices produced by the Spanish in the 15th to 17th centuries, intended to document all the new resources of the conquered American lands, reveals several

plant species identified by indigenous healers as useful in the treatment of the intermittent fevers. The "aquatic fever" described earlier was typically treated with a combination of *Tagetes lucida* and *Artemisia mexicana*. An ethanolic extract of the latter has been investigated by Malagón et al., and was able to clear mice of *Plasmodium yoelii yoelii* infection, a model of human malaria. No artemisinin was detected in the extract, however (Malagón et al., 1997).

Perhaps of most interest are the references to *chillazotl*. This particular plant species – with the Latin designation of *Argemone mexicana*, and colloquially known as the Mexican Prickly Poppy, is currently undergoing trials for use against *falciparum* malaria in parts of Africa, and the Malian government has already approved decoctions of this species as a treatment for uncomplicated *falciparum* malaria (Graz et al., 2010).

1.1.10.3 Europe

It was around 1630 that Europe first became aware of a potential cure for the intermittent (as opposed to continuous) fevers: the "Peruvian bark". This new medicine – known to us now as *Cinchona officinalis* – had been brought to Europe from Peru by Jesuit priests, who had noted how the indigenous people of the province of Quito would drink decoctions of the bark to treat and prevent feverishness and shivering. It should be noted, though, that amongst indigenous Peruvians the medicinal properties of the bark were not well known: only two tribes – the Paltas and the Zaraguros – made use of it, despite the existence in pre-Columbian Peru of a disease called, in Quecha, *chucchu*, or "chill and fever". Numerous local plant species in both South and Central America were identified by the indigenous population as "*chuchupatli*", or "fever-medicine", but curiously, *C. officinalis* was not one of them (Bruce-Chwatt, 1965).

Despite widespread reports amongst colonials in Peru, and then in Spain and Rome, of the bark's efficacy against "the tertian fever", it was some time before the bark became widely adopted as a treatment for malaria. The first record of the bark being prescribed in the UK dates from 1658, when the Cambridge apothecary Professor Robert Brady administered the new medicine during a serious outbreak of the disease (Sneader, 2005).

Efficacious as the Peruvian bark was, physicians soon began to notice that patients varied greatly in their response to the medicine, both in terms of cure rate, and in presence and

severity of unwanted side effects. An additional problem was noted by the 19th century French physiologist François Magendie, who had seen patients die of malignant fevers because they could not ingest sufficient quantities of bark to treat the disease. Such difficulties in standardising this herbal medicine led, in the early 19th century, to attempts by the Portuguese physician Bernardino Gomez to isolate an "active principle" from the Peruvian bark. Gomez succeeded in his efforts: in 1812, using what would now be recognised as a forerunner of the Stas-Otto technique of extraction, he obtained from the bark an amount of silvery-white crystals, which he named cinchonin.

Soon after, in 1820, two French pharmacists – Pierre-Joseph Pelletier and Joseph-Bienaimé Cavetou performed similar extractions of the Peruvian bark, and also of the bark of a related tree: *Cinchona cordifolia*. It was from this latter (colloquially known as the "yellow bark") that the Frenchmen obtained the first pure sample of quinine. Following the announcement of their discovery of quinine in 1820, it was soon discovered that small amounts of this newly isolated compound could arrest the tertian fever, with much higher cure rates than the powdered bark - and moreover, was better tolerated by patients. The efforts of the two men to promote the use of this single compound, rather than the crude bark, has led some to describe their actions as forming "...the prototype for an entire scientific discipline...ethnopharmacology" (Burns, 2008). Quinine became the standard drug for any feverish illness, and quinine derivatives are, to this day, utilised in the treatment of malarial infection (Sneader, 2005).

1.1.11 Current antimalarial drugs

For the conventional treatment of uncomplicated *falciparum* malaria in adults, in which the objective is to reduce parasitemia and fever, the medical practitioner has a choice of three recommended regimens:

1. Atovaquone-proguanil (Malarone®), 4 tablets, each containing 250 mg of atovaquone and 100 mg of proguanil hydrochloride, daily for 3 days (Joint Formulary Committee, 2010);

2. Quinine sulphate, 600 mg every 8 hours, plus a choice of one of the following: Doxycycline, 200 mg orally daily for 7 days; Clindamycin, 450 mg every 8 hours for 7 days, or Sulfadoxine-pyrimethamine (Fansidar®), for use only in children (Joint Formulary Committee, 2010);

3. Artemether-lumefantrine (Riamet®), 4 tablets, each containing artemether at 20 mg and lumefantrine at 120 mg, at 0, 8, 24, 36, 48 and 60 hours (Joint Formulary Committee, 2010);

For non-*falciparum* malaria, a choice of chloroquine or primaquine is available. Mixed malarial infections may be treated "principally for *falciparum* malaria, as quinine and artesunate are effective in all species, whilst resistance to chloroquine by *P. falciparum* is widespread" (Walker et al., 2009).

Table 2 summarises the currently available drugs for the treatment of uncomplicated *falciparum* malaria:

Table 2. Classes of antimalarial drugs (reproduced from "*Human antiparasitic drugs: pharmacology and usage*" by James and Gilles (James and Gilles, 1985)

Drug	***Chemical classification***	***Dynamic classification***
Quinine, mefloquine	4-quinolinemethanols	Rapidly-acting blood schizontocides
Chloroquine, amodiaquine	4-aminoquinolines	
Artemisinin and derivatives	Sesquiterpene lactone	
Sulphadoxine, sulphalene	Sulphonamides	Antifolates (type 1)
Dapsone	Sulphone	
Proguanil, chlorproguanil	Biguanides	Antifolates (type 2)
Pyrimethamine	2:4-diaminopyrimidine	
Primaquine	8-aminoquinoline	Tissue schizontocide

Severe *falciparum* malaria, in which the primary purpose of medical intervention is to save the life of the patient, is treated with either quinine or artesunate, both of which are administered parenterally. Where artesunate is licensed (in South East Asia and sub-Saharan Africa), it is the preferred drug, at a level of 2.4 mg/kg, at 0, 12, 24 hours, and then daily. The authors of a recent British review of current malarial treatment regimens suggest that artesunate will, in all likelihood, become the standard treatment of severe disease in the UK (Walker et al., 2009).

The current unreliability of a source of artesunate, hinted at by the authors of the British report, is the reason for the work undertaken in this thesis. The ultimate source of artesunate, and the other related antimalarials artemether and arteether, are derived from the parent compound artemisinin – a sesquiterpene lactone, found in the leaves of the Chinese herb *Artemisia annua* L.

1.2 Discovery of artemisinin

The active principle of *Artemisia annua* L was identified in 1972, in the research group of You-You Tu - a phytochemist working at the China Academy of Traditional Chinese medicine (Liao, 2009). Tu's isolation of the compound followed a cold diethyl ether extraction of the dried herbal material. Attempts had been made to isolate active compounds by preparing the dried herb according to the Chinese tradition, in which the material is boiled as a tea, but no pharmacological activity in rodent malaria models could be demonstrated in this manner.

Following bioassay guided screening of the diethyl ether extraction, the antimalarial activity of the herb was traced to the presence of colourless, needle-shaped crystals. The structure of this purified crystalline compound – a sesquiterpene lactone with 7 chiral centres and a tetracyclic 1,2,4-trioxane skeleton - was determined in the late 1970s (Liu et al., 1979). Clinical trials followed, and the new drug was given the accolade of "national scientific discovery" by the Chinese Government. In 1986, artemisinin – under the Chinese name of *qing hao su* – was awarded its "new drug certificate"(Liao, 2009), meaning that the drug was now licensed – and the era of artemisinin had begun.

The new compound had a most unusual feature – an endoperoxide bridge which, it would later be discovered, was vital for the pharmacological activity of the molecule.

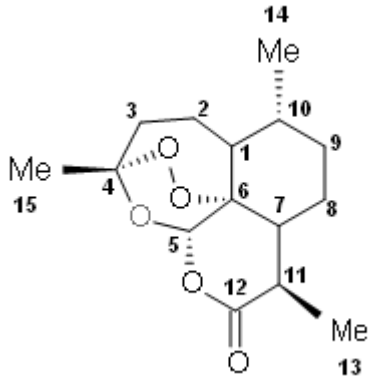


Figure 3. Artemisinin

1.3 Biosynthesis of artemisinin

Although the total synthesis of artemisinin has been achieved in the laboratory, the complex procedures required, and the low yields obtained – 10% in the most recent reported synthesis (Yadav et al., 2003) - mean that for the foreseeable future, *Artemisia annua* itself will remain the only source of the drug. Of particular interest in this regard is the fact that, although 128 stereoisomers of artemisinin are theoretically possible, only one stereoisomer of the antimalarial artemisinin is biosynthesized in *Artemisia annua* L. plants (Julsing et al., 2006).

So, what is known about the biosynthetic pathway to artemisinin?

The biosynthesis of a compound called isopentenyl diphosphate (IPP) is absolutely necessary for the downstream synthesis of the sesquiterpenoids, of which artemisinin is one. Two pathways within the plant cell are capable of synthesising IPP: the mevalonate (MVA) pathway and the deoxyxylulose (1DX) pathway, so named after the crucial intermediates mevalonic acid and deoxyxylulose 5-phosphate, respectively.

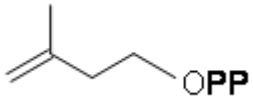


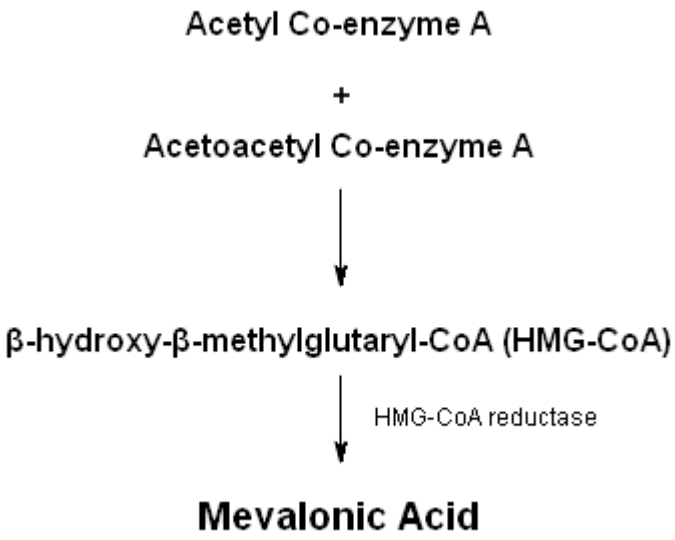
Figure 4. Isopentenyl diphosphate (IPP)

An important difference between the two pathways lies in the fact that the MVA pathway contains all the steps needed to produce artemisinin, whereas the deoxyxylulose pathway does not. This difference will be discussed in more detail in Sections 1.3.1. – 1.3.2., below.

1.3.1 The MVA pathway

Unlike the 1DX pathway, the enzymes of the mevalonate pathway are localised in the cytosol – the aqueous liquid sea in which cell organelles, including the plastids, are embedded. The genes for this pathway are thought to have evolved originally in the Archaebacteria - these prokaryotic cells being the most ancient of the bacterial life forms (Lange et al., 2000).

The activity of the MVA pathway begins when acetyl CoA joins with acetoacetyl CoA, forming the intermediate compound β-hydroxy-β-methylglutaryl-CoA (HMG-CoA). Then, the enzyme HMG-CoA reductase brings about the irreversible two step reduction of HMG-CoA, forming mevalonic acid (MVA) - the first committed step in the production of the terpenoids.



MVA then undergoes a series of phosphorylations, ultimately forming isopentenyl diphosphate (IPP). This latter undergoes geometrical isomerism, resulting in dimethylallyl diphosphate, or DMAPP.

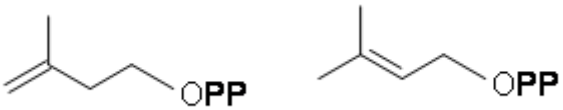


Figure 5. L; IPP. R: DMAPP

The linking of DMAPP with IPP creates a new compound – geranyl diphosphate. The addition of a further IPP unit to geranyl diphosphate then creates farnesyl diphosphate - the starting point for the biosynthesis of the sesquiterpenes, of which artemisinin is one (Dewick, 2001).

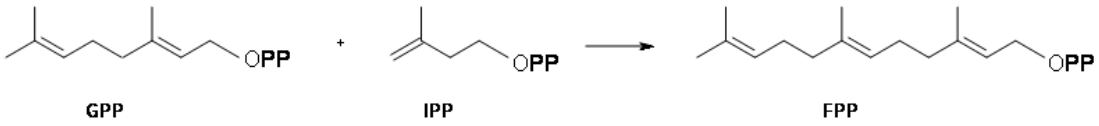


Figure 6. Geranyl diphosphate (GPP) links with isopentenyl diphosphate (IPP) to form farnesyl diphosphate (FPP)

1.3.2 The Deoxyxylulose (1-DX) Pathway

Unlike the MVA pathway, the 1DX pathway – also called the mevalonate-independent pathway - is localised in the plastids. Results of genetic studies provide evidence that this plastidic pathway evolved more recently than the mevalonate pathway, deriving from the Eubacteria – and that the genes for this pathway were actually derived from the cyanobacterial ancestor of plastids (Lange et al., 2000).

The1DX pathway begins with the condensation of D-glyceraldehyde-3-phosphate with activated acetaldehyde – the latter compound itself derived from pyruvate (Dewick, 2001). This condensation forms deoxyxylulose 5-phosphate, and it is from this compound, *via* a series of steps not fully elucidated, that the two important isomers DMAPP and IPP are formed.

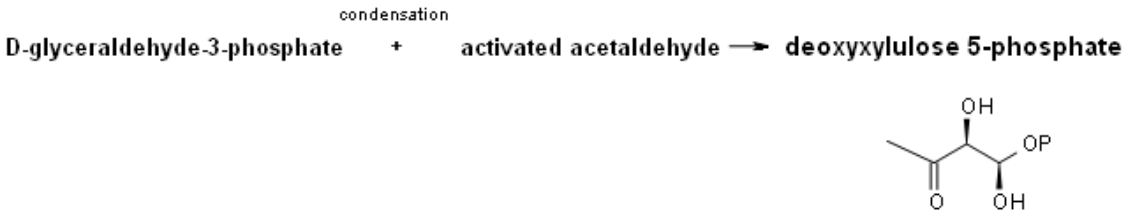


Figure 7. Deoxyxylulose 5-phosphate

Within the deoxyxylulose pathway, still inside the plastid, these two compounds – DMAPP and IPP - can lead to the formation of the chlorophylls, carotenoids, monoterpenes, and abscisic acid (ABA). IPP is, in addition, able to condense with its isomer, DMAPP, to form the 10-carbon compound geranyl diphosphate (GPP), and the 20-carbon geranyl geranyl diphosphate. The

former compound is the starting point for the biosynthesis of the monoterpenes; the latter, for the chlorophylls and carotenoids (Dewick, 2001).

However, the plastidic deoxyxylulose pathway cannot lead to the formation of artemisinin, or any other sesquiterpene for that matter. The deoxyxylulose pathway results in mono- , di- and hemi-terpenes, but sesquiterpene biosynthesis is a function of the MVA pathway (Eisenreich et al, 2001).

It can be seen (Figure 9) that in the mevalonate pathway, IPP remains within the cytosol and continues on down the mevalonate branches, one of which leads to the sesquiterpenes - including artemisinin. But in the deoxyxylulose pathway, some IPP can be transported out of the plastid and into the cytoplasm, to be added to the mevalonate pathway's supply of IPP. This "crosstalk" between the two pathways is a subject of much research interest (Towler and Weathers, 2007). Figure 9 illustrates this proposed process.

1.3.3 The relative contribution of both pathways to artemisinin biosynthesis

It is known that isopentenyl diphosphate (IPP) is a vital intermediate in the biosynthesis of artemisinin. It is also known that IPP may be supplied by both the mevalonate and the DX pathways. Given that the mevalonate pathway carries all the necessary machinery to synthesise artemisinin, what evidence is there for the aforementioned "crosstalk" between the two pathways? Is it certain that at least some of the pool of IPP is contributed from the plastidic 1DX pathway?

Several researchers have looked into this question, making use of labelled intermediates and selective inhibitors for each of the two pathways. The most recent study, published in 2009, found evidence that artemisinin had a mixed biosynthetic origin, but was largely made from (*E*,*E*)-farnesyl diphosphate (FPP) in which the whose central isoprenoid unit came from the 1-DX pathway (Schramek et al, 2009).

The proposed mechanism of mixed biosynthesis is that

1. DMAPP of mevalonate origin is transferred to the plastid,

2. Within the plastid, DMAPP then joins with an IPP unit of 1-DX origin, forming geranyl diphosphate (GPP)
3. This GPP is then exported back to the cytosol and converted into farnesyl diphosphate (FPP), using IPP from the mevalonate pathway.
4. FPP is then further modified to form amorpha-4,11-diene, and, ultimately, artemisinin (Schramek et al, 2009).

1.3.3.1 Farnesyl diphosphate

Farnesyl diphosphate can be converted into amorpha-4,1-diene; the first step along the sesquiterpene branch of the mevalonate pathway, and the road to artemisinin. In fact, it has recently been demonstrated that the overexpression of farnesyl diphosphate synthase in whole plants of *A. annua* results in 34.4% more artemisinin compared to control plants (Han et al., 2006).

Farnesyl diphosphate is cyclised to form amorpha-1-diene: the first committed step of the artemisinin biosynthetic pathway. The enzyme, amorpha-4,11-diene synthase, has been well characterized, following its discovery in *A. annua* in 1999 (Bouwmeester et al., 1999). From farnesyl diphosphate, the enzyme catalyses the production of a number of sesquiterpenes, of which the major product, at 93% in the 1999 study, was amorpha-4,11-diene.

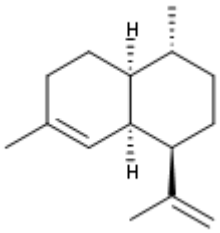


Figure 8. Amorpha-4,11-diene

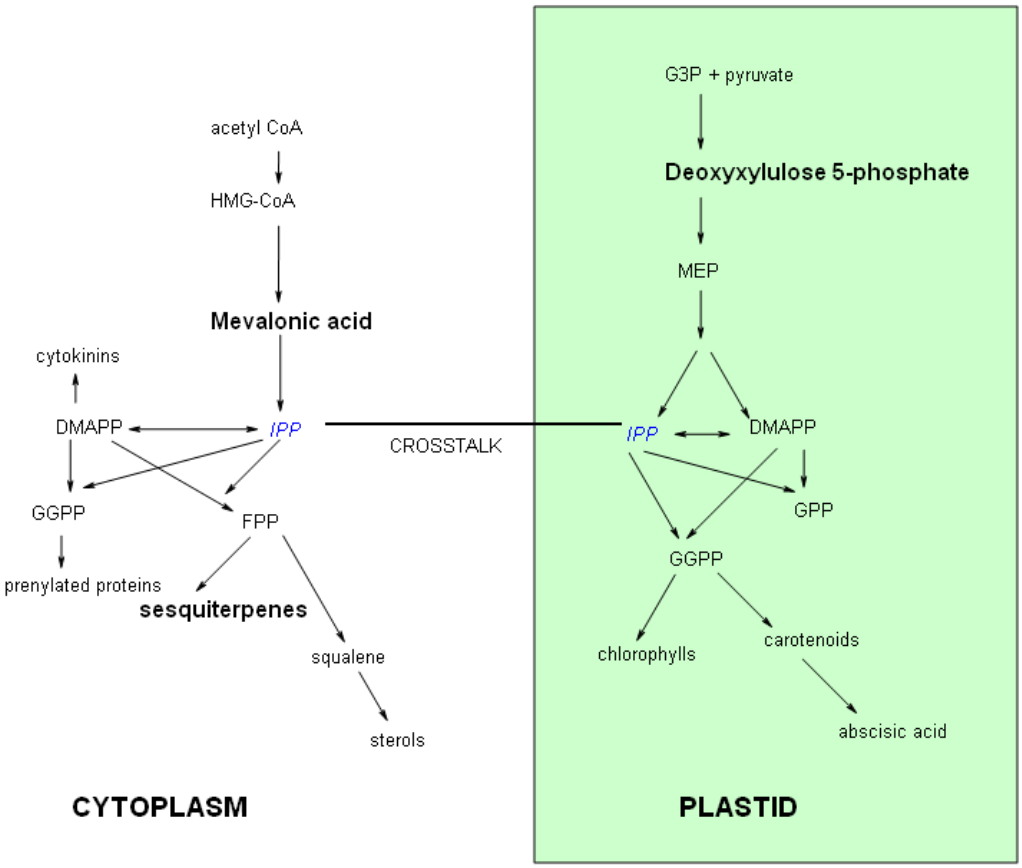


Figure 9. The mevalonate and deoxyxylulose 5-phosphate pathways (reproduced from Towler and Weathers, 2007)

1.3.3.2 From amorpha-4,11-diene to artemisinin

The most recent literature presents evidence that there is a large degree of spontaneous, non-enzymatic conversion in the latter stages of the pathway. But at least one enzyme has been identified in the earlier stages of the artemisinin biosynthetic pathway.

Covello et al. and Teoh et al. (Covello et al., 2007, Teoh et al., 2006) independently presented evidence that a single, CYP-type enzyme, can catalyse multiple conversions in the latter stages of the artemisinin biosynthetic pathway. Following earlier evidence (Bertea et al., 2006) that a cytochrome P450 enzyme from *A. annua* leaf material was involved in the conversion of amorpha-4,11-diene to artemisinic alcohol, both research groups isolated RNA from leaf and flower glandular trichomes, and this isolated RNA eventually led to the discovery of a new

gene, with a coding sequence similar to enzymes of the CYP71D subfamily. The protein product – the enzyme itself - was designated CYP71AV1.

Expression of this enzyme in *Saccharomyces cerevisiae*, followed by substrate feeding, showed that in the presence of NADPH this new enzyme could catalyse the following:

1. Conversion of amorpha-4,11-diene to artemisinic alcohol;
2. Conversion of artemisinic alcohol to artemisinic aldehyde (although this reaction could also be catalysed by a non-membrane-bound enzyme in trichome supernatant);
3. Conversion of artemisinic aldehyde to artemisinic acid.

Artemisinic alcohol, artemisinic aldehyde and artemisinic acid also exist in the reduced forms: dihydroartemisinic alcohol, dihydroartemisinic aldehyde, and dihydroartemisinic acid, respectively.

The final steps from artemisinic acid to artemisinin are still controversial. Is artemisinic acid first converted into dihydroartemisinic acid, which is then converted into artemisinin? Or is artemisinic acid directly converted to artemisinin, as Covello's research suggests? Li (Li et al., 2006) suggests that the 11,13 double bond reduction – ultimately observed in the saturated 11,13 bond in artemisinin – may occur at a late stage, perhaps *via* arteannuin B or artemisitene. But Brown (Brown and Sy, 2007) theorises that high artemisinin levels indicate a good degree of early stage 11,13 reduction, not a late stage reduction. So, there are still a number of questions to be answered.

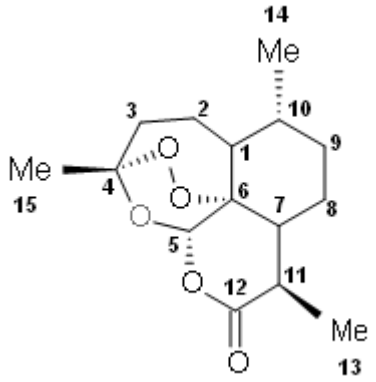


Figure 10. Artemisinin

So that takes care of the alcohols, aldehydes and acids. But extracts of *A. annua* also contain several other compounds, sometimes in respectable amounts – for example, arteannuin B, arteannuins H to M, and dihydroarteannuin B (Brown and Sy, 2007, Brown and Sy, 2004, Li et al., 2006).

Feeding of stable isotope labelled artemisinic acid and dihydroartemisinic acid to whole plants, *via* cut stems and roots respectively (Brown and Sy, 2004; Brown and Sy, 2007), provided evidence that these acidic compounds do not go almost directly to artemisinin, as had been thought in earlier studies. In fact, it was found that arteannuin B – illustrated in Figure 11 - was the major metabolite of artemisinic acid conversion and six other minor metabolites were observed: annulide, isoannulide, deoxyarteannuin B, *seco*-cadinane, artemisinic acid methyl ester, and *epi*-deoxyarteannuin B.

When labelled dihydroartemisinic acid was fed to cut plants, 16 labelled metabolites were observed, of which the major ones were dihydroarteannuin B, dihydro-*epi*-deoxyarteannuin B, and an un-named keto-aldehyde, compound 14. The others were identified as artemisinin, arteannuin M, a tertiary hydroperoxide of dihydroartemisinic acid called compound 15, dihydro-deoxyarteannuin B, a B-epoxy alcohol called compound 17, deoxyartemisinin, arteannuin K, arteannuin L, arteannuin H, arteannuin I, arteannuin J, a-epoxy-dihydroartemisinic acid, and dihydro-*epi*- arteannuin B. The latter was reported as a natural compound for the first time.

A unified form of these arguments implies that

- amorpha-4,11-diene synthase catalyses the conversion of farnesyl diphosphate to amorpha-4,11-diene, and this is the first dedicated step towards artemisinin.
- Following this, a cytochrome P450, designated CYP71AV1, catalyses a series of reactions from amorpha-4,11-diene to artemisinic acid. What is perhaps not yet clear is how the reduced forms of these compounds come about, although in at least one case (conversion of artemisinic alcohol to artemisinic aldehyde), evidence exists for a non-membrane bound enzyme catalysis in trichome supernatant.
- Artemisinic acid can then be converted, perhaps *via* spontaneous autoxidation, into arteannuin B, and dihydroartemisinic acid can be converted, also non-enzymatically, into a lot of compounds, with the major three being dihydroarteannuin B, dihydro-*epi*-deoxyarteannuin B, an un-named keto-aldehyde, and artemisinin

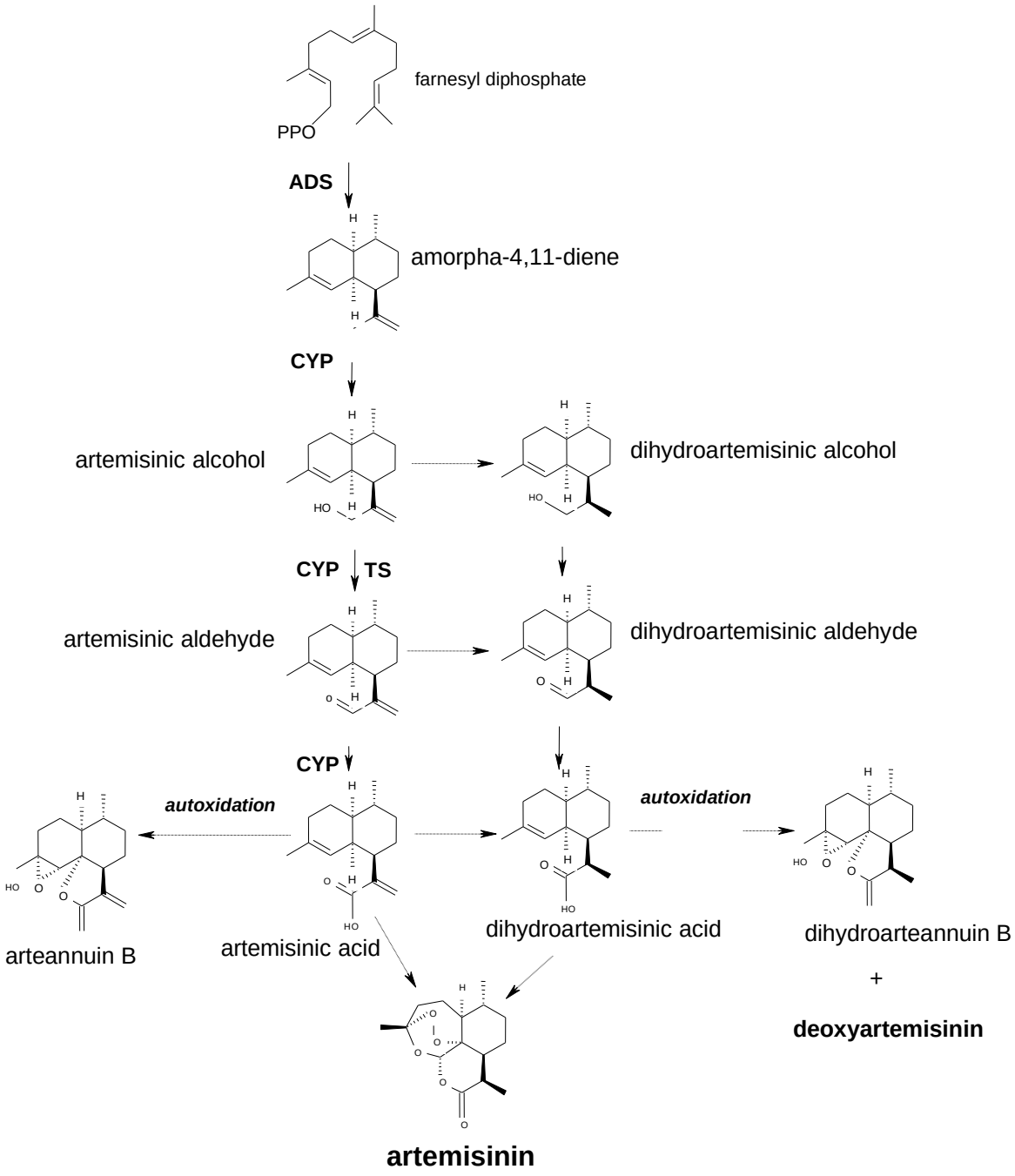


Figure 11. A unified biosynthetic pathway to artemisinin. ADS: amorphadiene synthase; CYP: reactions catalyzed by CYP71AV1; TS: Trichome Supernatant-catalyzed single step reactions (Teoh et al, 2006;Covello et al, 2007).

1.3.4 Trichomes – site of artemisinin biosynthesis

Although the exact site of artemisinin biosynthesis in the plant is unconfirmed, evidence suggests that the latter stages of the pathway, at least, occur within leaf surface structures called glandular trichomes.

Microscopic examination of the leaf epidermis reveals two types of trichome: a distinctive non-glandular T-shaped trichome, looking rather like a windmill, sticking out from the leaf surface – and also observed in other *Artemisia* species -, and an oval-shaped glandular secretory trichome (GST), embedded within the leaf (Fig 7). The latter, with its clear biserate (double-cell) structure, is considered to be the major organ of biosynthesis and storage of artemisinin. The structure of the GSTs has been well studied: they consist of one pair of apical cells, three pairs of subapical, photosynthetic cells, and one pair of basal cells, all on a stalk (Lommen et al., 2006). Surrounding the three pairs of subapical cells and the apical cells is a secretory cavity, where the oily material is stored.

Much evidence suggests that the synthesis of monoterpenes, at least, is dependent upon the activity of photosynthetic plastids such as are found in the subapical cells ((Duke and Paul, 1993). This dependence has of yet not been demonstrated for the sesquiterpenes: indeed, as we have seen, the monoterpenes are produced in a different pathway to the sesquiterpenes. There is enzymatic evidence (the isolation of CYP7AV1 from GST libraries) that the precursors of artemisinin, at least, are made actually within the GSTs (Covello et al., 2009), but we don't know exactly where – although it is known that artemisinin is stored in the subcuticular secretory space, with the monoterpenes (Duke and Paul, 1993).

As an aside, Tellez et al., (Tellez et al., 1999) analysed both normal and glandless (that is, absent in GSTs) *A. annua* leaves for volatile oil content, and found that in the glanded plants, the oil was predominantly monoterpenoid. But in glandless plants, sesquiterpenes - mainly germacrene-D and B-caryophyllene – predominated.

Finally, Duke, in 1994, described how mutant plants, missing glandular trichomes, do not produce artemisinin (Duke, 1994).

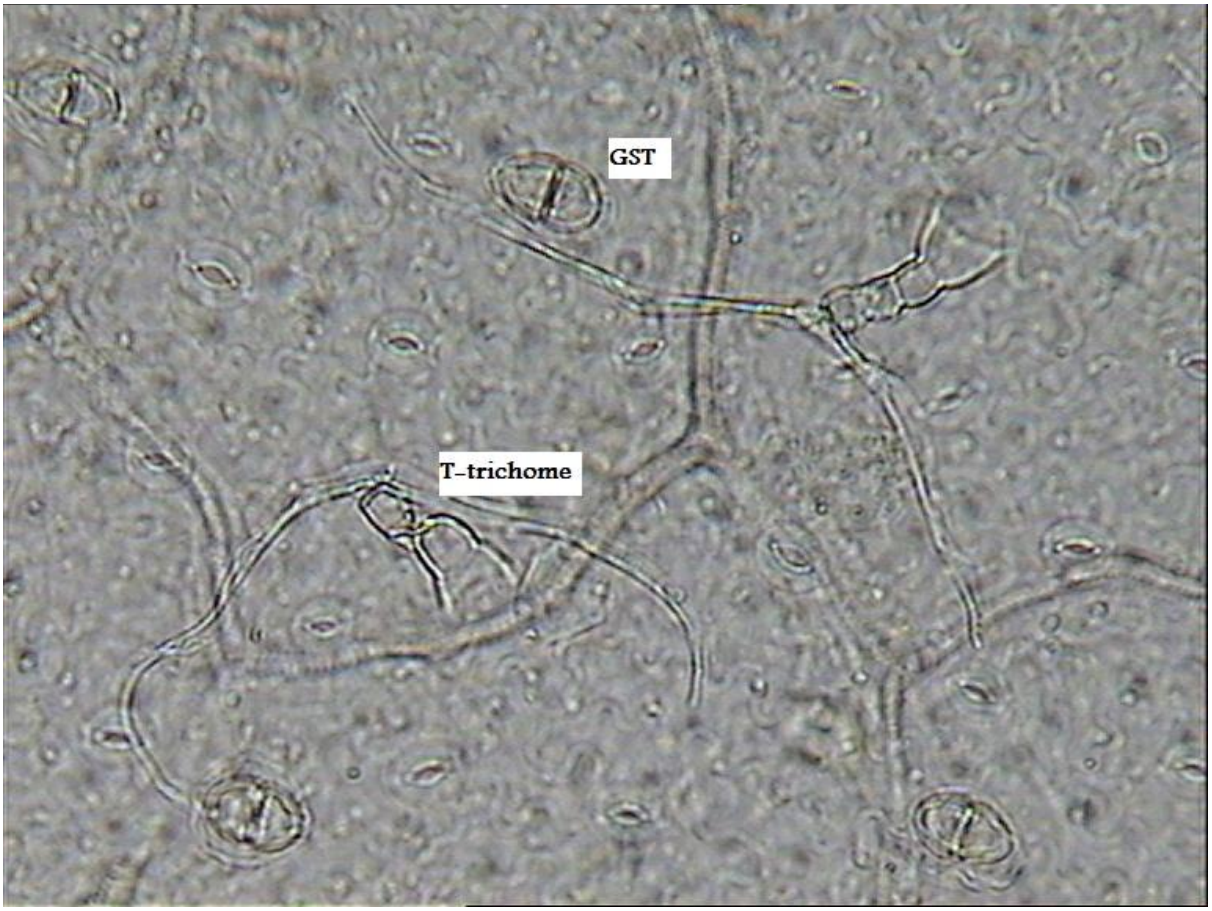


Figure 12. Light microscopy image (x 40) of leaf surface of *A. annua*, showing glandular secretory trichomes (GSTs) and T-trichomes

1.4 Artemisinin-based pharmaceuticals

1.4.1 Use of the drugs

Several licensed forms of artemisinin-derivatives are now widely available in South East Asia and Africa. Artemether-lumefantrine (Coartem®) is the only internationally licensed co-formulated medicine – adopted in the US in 2009 - and also the only form to include artemether. Other licensed artemisinin-based treatment regimes are based around the administration - in separate tablets - of artesunate with amodiaquine (used in African countries); artesunate with mefloquine (used more in Asia), and artesunate with sulfadoxine-pyrimethamine, used in Afghanistan (Greenwood et al., 2005).

It is worth noting that the artemisinins are not suitable for malaria prophylaxis.

1.4.2 Semi-synthetic derivatives of artemisinin, and metabolism thereof

There are five artemisinin-type compounds approved for use in the treatment of malaria: one of these is artemisinin itself. The remaining four are derivatives in which the lactone ring has been modified in some way. The modifications serve to vary the solubility of the compound, and hence affect the means of administration, and are listed below:

a) Dihydroartemisinin (DHA). This compound is actually a metabolite of artemisinin, on which the lactone's oxygen is bonded to a hydrogen atom, forming a lactol (Bhakuni et al., 2002). In addition to being a metabolite the compound can be administered as a drug itself, for oral or suppository use. In fact, it has been shown to be three times more active against *P. falciparum* than artemisinin itself. DHA, additionally, is the first step in the synthesis of the other three derivatives:
b) Arteether, the β-ethyl ether isomer of DHA: an oil-soluble intramuscular form of the drug;
c) Artemether (Figure 13), the β-methyl ether of DHA - another oil-soluble compound for intramuscular or oral use, and the most active of the semi-synthetic derivatives ;
d) Artesunate (Figure 13), which is, more accurately, sodium artesunate: a water-soluble form for oral, intramuscular, intravenous or suppository use.

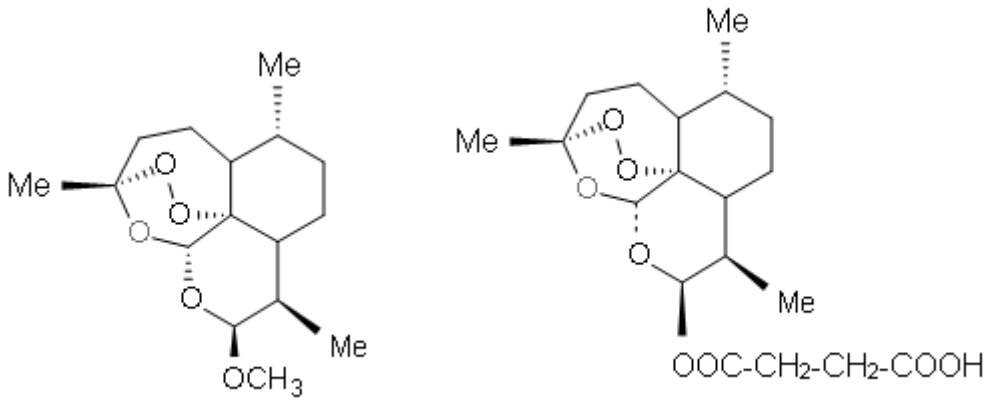


Figure 13. L: Artemether. R: Artesunate

Artemisinin itself is not commonly used in the treatment of malaria; studies show that artemisinin takes longer to achieve parasite clearance, and results in a higher rate of recrudescence, than the semi-synthetic derivatives described above. Alin et al. (1996) found that patients infected with *P. falciparum* who were administered oral artemisinin, at 500 mg on day one, followed by 250 mg twice daily for four days and ending with a final 500 mg dose on day 6, took 31 hours (+- 3.6) to reach full parasite clearance, compared to 26.4 hours (+- 3.6) for those receiving oral artesunate at 100mg on day one, followed by 50 mg twice daily for 5 days. Recrudescence in the artemisinin group was 21.6% (+- 4.6), as compared to 18.9 % (+- 4.0) in the artesunate group (Alin et al., 1996). These differences are small, but clearly significant.

The artemisinin-derived drugs (and artemisinin itself) do act rapidly, reaching maximum plasma concentrations in as little as 1 hour. Alin reported that a C_{max} of 260 ng/ml was obtained 1 hour after dosing with 400 mg of oral artemisinin – a very high figure when it is considered that the IC_{50} for artemisinin against *P. falciparum* in mice is as low as 3.9 ng/ml (Mueller et al., 2004) The drugs are also eliminated rapidly, though, with typical elimination half-lives of 1-2 hours when drugs are given orally – one reason why these drugs are given as combination therapy, together with a longer-lasting drug. Intramuscular forms are available, with typically longer times to elimination, but absorption of drug when delivered in this form has been found to be erratic.

An important difference between artemisinin itself, and the artemisinin-derived drugs, lies in the metabolic products of the compounds. Artemisinin is metabolised in the liver to inactive products - “deoxyartemisinin, deoxydihydroartemisinin, 9,10-dihydrodeoxyartemisinin, and so-called crystal 7) are formed primarily by polymorphic CYP2B6”, according to a 2009 study published in the Lancet (Kerb et al., 2009) - but artesunate and artemether are metabolised first to dihydroartemisinin (DHA), which is more active against *P. falciparum* than the parent compounds. In fact, the WHO states that DHA contributes nearly all the antimalarial activity following artesunate administration (WHO, 2000). DHA is then further metabolised to inactive products.

1.4.2.1 Artemisinin Combination Therapy (ACT)

Typically, these compounds would be administered in combination with a drug with a longer half life: most commonly, mefloquine. The WHO approves the following combinations for uncomplicated *falciparum* malaria:

- artemether + lumefantrine (Coartem®)
- artesunate + amodiaquine
- artesunate + mefloquine
- artesunate + sulfadoxine–pyrimethamine(WHO, 2006).

However, this requires, for outpatients with uncomplicated malaria, the taking of two different tablets (hospital patients suffering severe malaria would receive the intramuscular or suppository forms), and there is a risk of non-compliance: a patient may take only the artemisinin, and not the second drug. There is the additional factor of self-medication: it has been reported that, in areas far from the nearest clinic, individual artemisinin capsules are sold as monotherapy by individuals with little or no medical training: against the advice of the WHO, who disapprove of monotherapy, but state that if it is to be used, then a full 7 day course of treatment is required (WHO, 2006). It is probable that many patients cannot afford this treatment regimen.

To counter this problem, tablets containing both artemisinin and the second drug – the so-called "fixed dose ACTS" – have been introduced. The first WHO-approved fixed-dose ACT was developed by Sanofi-Aventis as combination of two antimalarial drugs in a single tablet. The medicine, Coarsucam®, is a combination of artesunate and amodiaquine, which, like artemisinin, is a blood schizontocide. It was approved for marketing in 2007, and was given WHO prequalification status in 2008.

A fixed dose ACT combining artemisinin and a different longer-acting medicine - naphthoquine – has been trialled in Papua New Guinea for the treatment of uncomplicated malaria. Each tablet contained 125 mg of artemisinin and 50 mg of naphthoquine, and eight tablets were administered as a single dose. This treatment, the authors of the study report, cleared parasitemia in 50% of the patients within 24 hours (Hombhanje et al., 2009).

Finally, in 2009 the US Food and Drug Administration (FDA) approved the use of its Novartis' Coartem® (a fixed-dose combination of artemether and lumefantrine) for the treatment of US-based cases of uncomplicated *falciparum* malaria.

1.4.2.2 Synthetic peroxides

The most well-characterised of the new generation of synthetic peroxide antimalarials are the adamantane-based 1,2,4-trioxanes reported by Tripathi et al., in 2007 – when these were administered to rhesus monkeys infected with *P. knowlesi* (a simian malaria that has recently jumped to humans) total parasite clearance was achieved in a dose regimen of 80 mg/kg for 5 days. However, this is quite a high dose when it is considered that typical artemisinin doses are in the region of 2.4 – 10 mg/kg (Tripathi et al., 2007).

So far, no synthetic peroxide has been licensed for use in the treatment of malaria, although one such compound, designated OZ439, is currently in Phase 1 clinical trials (MMV, 2007). The basic structure of the OZ series of compounds is shown in Figure 14.

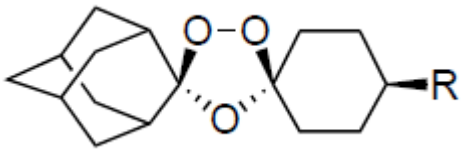


Figure 14. Basic structure of the OZ series of synthetic peroxides (Charman, 2007)

1.4.3 What the drugs do

Artemisinin and its derivatives are known to act on the human blood stages of the *Plasmodium* life cycle: in fact, the drugs are classed as blood schizontocides, putting them in the same class as mefloquine and chloroquine (James and Gilles, 1985).

1.4.3.1 Rapid reduction of parasitemia

The major advantage of the artemisinin-derived drugs – other than the fact that they are able to reduce parasitemia in patients who do not respond to the older antimalarial drugs – is their rapidity of action. The artemisinins are quick to act and very efficaceous against *Plasmodium* parasites, including the youngest (ring form) stages. The drugs also show activity against the precursors of the sexual stages and early stage (I-III) gametocytes (Golenser et al., 2006). Gametocytes, when sequestered in the spleen and bone marrow, pass through 5 morphological stages (Hayward et al., 1999).

The drugs can reduce parasite numbers by a factor of approximately 10,000 in each asexual cycle – a remarkable figure, when it is considered that other antimalarials reduce parasite numbers 100- to 1000-fold per cycle (WHO, 2006) As an aside, parasite numbers in uncomplicated *falciparum* malaria can be over 10,000 per cubic millilitre of blood (Dondorp et al., 2009). It has been reported that the artemisinins can resolve fever and clear parasitemia within 48 hours following the start of treatment – unlike other drugs, which can take several days.

This rapidity of action, though, comes with a complication: rapidity of clearance. Artesunate, a water-soluble derivative, can be eliminated from the body in as little as 10 minutes, although the drug is metabolised to a compound called dihydroartemisinin. However, even this metabolite is eliminated within one hour. So, the artemisinins would typically be administered in combination with a longer-lasting antimalarial. This form of artemisinin combination therapy (ACT) is discussed in more detail in Section 1.4.2.1.

1.4.3.2 Reduction of transmission

Other advantages of the artemisinins compared to other antimalarials have been discussed in the literature, including that of reducing transmission of malarial parasites from patient to

mosquito: the WHO reports that “Artemisinins are the most potent gametocytocidal drugs among those currently being used to treat an asexual blood infection” (WHO, 2006). Such an action would reduce transmission of the disease, because these mature, circulating gametocytes must be ingested by a mosquito before they can become mature gametes. Clearly, destroying a young gametocyte before mosquito ingestion will break this step of the cycle.

However, a note of caution is needed here: although the artemisinins effectively destroy immature gametocytes, as reported by Golenser and described earlier in this section, they cannot destroy those gametocytes that have gone beyond stage III, but have not yet been ingested by a mosquito. So, even with the use of artemisinins, some parasites will survive to reproduce in the mosquito gut, and transmission will continue to some degree. In fact, the only antimalarial that is used specifically for its inhibitory effect on transmission is primaquine: this drug is able to act on mature gametocytes (WHO, 2006).

1.4.3.3 Reduction of cytoadherence

A newly-discovered advantage of artesunate and artemether lies within this drug’s ability to reduce a pathological phenomenon called cytoadherence.

The mortality rate of severe malaria, as discussed in Section 1.1.1., can be as high as 20%, even following hospital admission and appropriate treatment. These deaths typically occur within 24 hours following admission, and are thought to be the result of the binding of infected red blood cells to the microvasculature of the body’s internal organs. As a direct result of this sequestration, blood vessels are blocked and local inflammatory responses can be triggered, with death following from organ failure.

It is known that cytoadherence is mediated by the parasite protein PfEMP-1 – a protein that is expressed to a greater degree in more mature parasites (in the trophozoite stage) compared to younger (early-trophozoite, or ring, stage) parasites. Since artesunate and artemether act on the younger parasites – unlike the older antimalarials, such as lumefantrine, quinine and piperaquine, which act by disrupting the detoxification of haem in mature trophozoites – the drugs prevent the parasites maturing to a stage where cytoadherence is a problem (Hughes et al., 2010).

1.4.4 Mechanism of action

Although the precise mechanism of action of artemisinin against the *Plasmodium* parasite has not been elucidated, the body of evidence is increasing. Current theories postulate both parasite-specific and non-specific mechanisms of action: the former including putative targets in the *Plasmodium* biochemistry, and the latter based upon free radical reactions against a wide range of proteins. The latter will be discussed first.

1.4.4.1 Non-specific reactions

In 2001, Posner wrote that there was now "evidence for a reaction between haem and artemisinin (*qinghaosu*)...for the haem–iron-mediated generation of free radicals from artemisinin...for the alkylation of haem and proteins...for a unified chemical mechanistic paradigm involving carbon-centred radicals; [and] for the putative antimalarial role of a high-valent iron–oxo reactive intermediate" (Posner and Meshnik, 2001). The details of such steps in the mechanism of action of artemisinin will now be discussed in a little more detail.

1.4.4.1.1 The peroxide structure

The necessity of the endoperoxide group for anti-plasmodial action has long been noted: deoxyartemisinin, in which the peroxide bridge has been modified to a single oxygen, in an ether-type structure, displays greatly reduced action against the parasite *in vitro*.

Additionally, artemisinin appears to react, in infected red blood cells, with haem in the Fe(II) form (and also, to a lesser degree, with cytochrome c), in a form of the Fenton reaction – a mechanism that centres upon the reaction of a peroxide moiety with ferrous (II) iron – or ferrous haem, in this case. This hypothesis is further supported by the fact that desferrioxamine, a scavenger of Fe(II), inhibits the antiplasmodial action of peroxides (Krishna et al., 2004).

Further evidence that artemisinin reacts with haem lies with the discovery that artemisinins concentrate in the parasite's food vacuole, where haemoglobin is digested. The parasites consume the globin portion of the haemoglobin molecule so that they can make use of the amino acids therein, but the haem – which is useless and indeed toxic to the parasite – is converted into an inert crystalline polymer, in the Fe(III) form, called haemozoin (the so-called "malaria pigment" so visible under the microscope).

1.4.4.1.2 The Fenton reaction

These two pieces of evidence: the need for a peroxide group, and the apparent link with ferrous haem - led numerous researchers to hypothesise that a Fenton-type reaction was responsible for artemisinin's ability to destroy the parasite.

The presence of Fe(II), whether as part of haem or in the free iron form, is necessary for the Fenton reaction to proceed. In the Fenton reaction, a peroxide group reacts with ferrous iron (such as that found in haem), in the presence of an acid, producing the hydroxyl radical:

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\bullet + OH-$

(Lloyd, 1997)

Current theories postulate the following sequence of events when artemisinin encounters ferrous haem (see also Figure 16):

1. An electron is transferred from the haem Fe(II) ion to the peroxide bridge of artemisinin, forming an artemisinin-haem adduct;

2. The peroxide bond then breaks in one of two homolytic ways, depending on which of the two oxygen atoms the haem has bonded with – designated, for this purpose, O^1 and O^2.

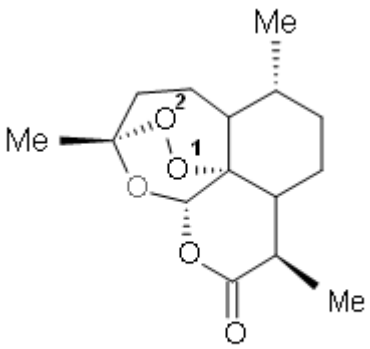


Figure 15. Artemisinin, showing O^1 and O^2

3. If the haem binds with O^1, then a sigmatropic 1,5-H shift takes place, creating a C4 radical intermediate, and in the process, releasing Fe(IV)=O, an iron-oxo species that has been implicated in anti-plasmodial activity (Kapetanaki and Varotsis, 2000). The C4

radical intermediate is quite stable, and has been hypothesised to be the anti-plasmodial species.

4. An intermediate epoxide is formed, with the loss of the unpaired electron (which is accepted by the Fe^{3+} ion).

5. The epoxide structure undergoes rearrangement to 3-hydroxydeoxoartemisinin, the end product of this O^1-based reaction(AG Taranto, 2002)

6. If, however, the haem binds to O^2, then a different series of reactions follows., A **β-scission** of the bond between carbons C3 and C4 takes place, which opens the ring and ultimately creates **arteannuin G** via a C4 radical intermediate (Wright, 2002, Meshnick et al., 1996).

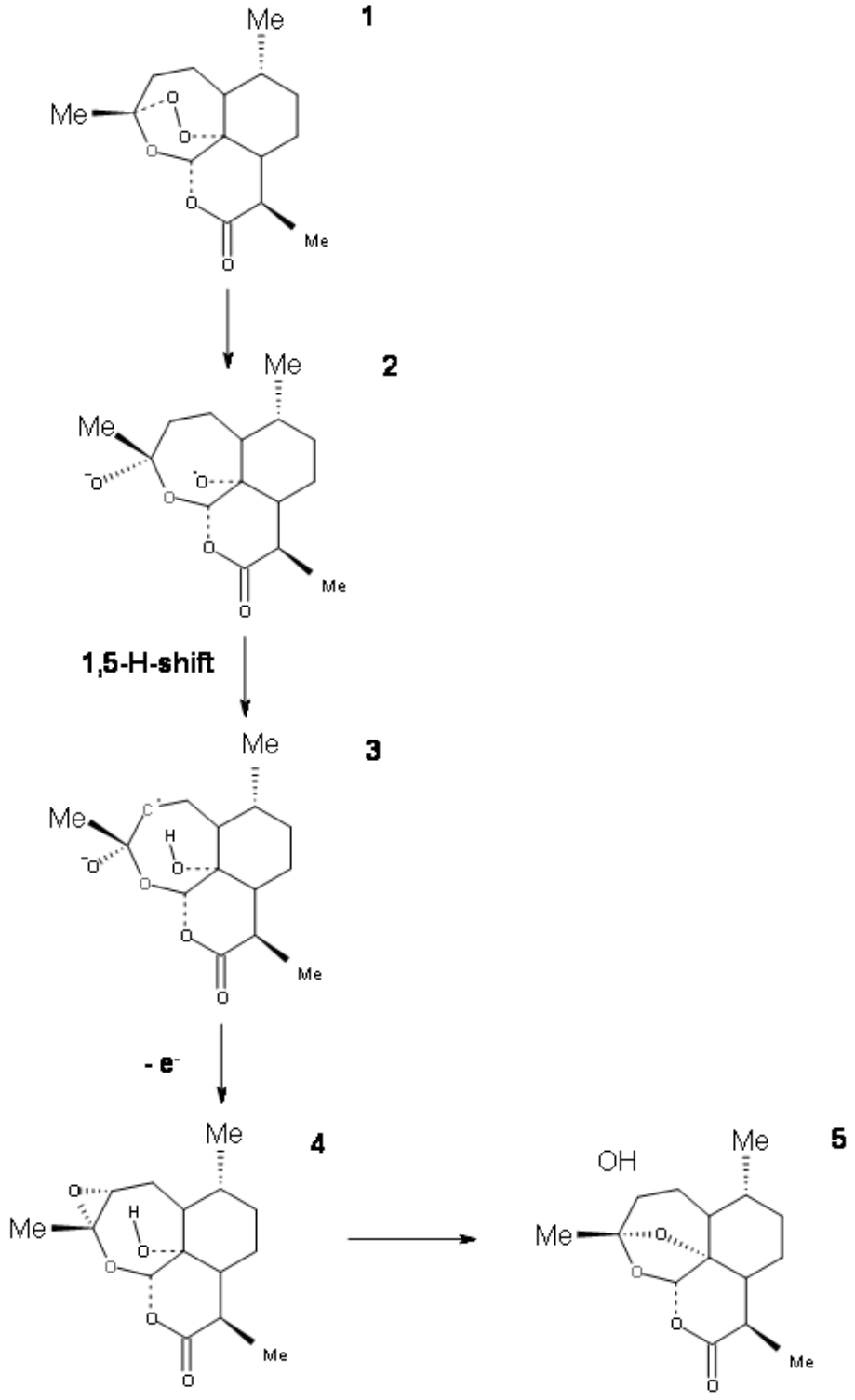


Figure 16. Rearrangement of artemisinin (1) leading to 3-hydroxydeoxoartemisinin (5), following electron transfer to O^1. (Taranto et al., 2002)

1.4.4.1.3 Radical intermediates

Although other reactions, following both O^1 and O^2-haem binding, are possible, evidence supports the prevalence of steps 3 – 4, leading to deoxygenated artemisinins via radical intermediates. It is the intermediate products (the carbon radicals and Fe(IV)=O) that mediate the toxicity within *Plasmodium* parasites (Wright, 2002). As Krishna writes, deoxyartemisinin does not demonstrate antimalarial activiy - and thus antimalarial activity is "claimed to reside in the radicals...by their reacting with 'sensitive biomolecules' in the parasite" (Krishna et al., 2004).

Essentially, it is the formation of an artemisinin-haem adduct, with the binding being between the epoxide bridge and the haem structure, that instigates parasite destruction – brought about by the subsequent generation of intermediate radical species, which mediate parasite death through the alkylation of proteins. "The alkylation of intraparasitic proteins by artemisinin derivatives" has been shown *in vitro*, and is "dependent on the presence of the endoperoxide bridge", according to a 1998 study (Bhisutthibhan et al., 1998).

1.4.4.1.4 Mechanisms of toxicity

The activation of artemisinin, described above, would imply that artemisinin, and its derivatives, are not so much drugs in their original state but prodrugs, requiring activation – in this case, by ferrous haem – to exert their action. Of course, the idea of "prodrugs" is not new: the metabolic activation of methoxylated flavones is theorised to play an important role in cancer prevention.

In this radical-mediated mechanism of action, damage to parasitic proteins by alkylation is the means by which the drug destroys parasites. In fact, the damage is not just directed at proteins - haem itself is vulnerable to alkylation by artemisinin-derived radicals. Haem alkylation, caused by the artemisinin: haem reaction, actually prevents detoxification of haem - normally accomplished by the formation of haemozoin[1] - within the parasite. Haem is both "the trigger and the target of artemisinin", write Selmeczi *et al.* (Selmeczi et al., 2004).

[1] Haemozoin is a polymer composed of haem monomers

Incidentally, this would explain why the artemisinins are more active against younger parasites: artemisinin can react only with haem, not with haemozoin. The bodies of older parasites contain more haemozoin relative to free haem, in their bodies, when compared to younger stages.

Artemisinin does not damage uninfected red blood cells, because the concentrations of free haem in healthy cells do not reach high levels – unlike the situation in the food vacuole of the parasite, wherein haem accumulates before being detoxification.

1.4.4.2 Parasite-specific mechanisms

1.4.4.2.1 SERCAs

Several papers have been published in the last five years highlighting putative parasite-specific targets of artemisinin and its derivatives. One of these targets – an intracellular pump of the sarcoplasmic/endoplasmic reticulum Ca^{2+}ATPase (SERCA) type, encoded for in *P. falciparum* by the gene *pfatp6* – was singled out because it is inhibited by a compound called thapsigargin, structurally very similar to artemisinin. Thapsigargin (Figure 17) is itself a plant-derived sesquiterpene lactone, found in the roots of the Mediterranean species *Thapsia garganica*. Derivatives of thapsigargin are undergoing trials for the treatment of prostate cancer (Denmeade et al., 2003).

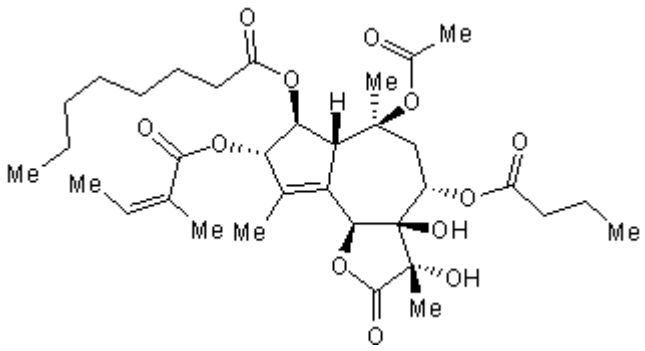


Figure 17. Thapsigargin

SERCAs help to maintain appropriate calcium ion concentrations across organelle membranes, and inhibition of such a protein disrupts cell signalling and protein folding in the parasite. In a 2003 study published in Nature (Eckstein-Ludwig et al., 2003), it was shown that artemisinin and dihydroartemisinin could inhibit the *P. falciparum* SERCA pump, when the protein was

expressed in *Xenopus laevis* oocytes (a standard model system for the analysis of protein function (Heikkila et al., 2007)). Other antimalarials tested had no such inhibitory actions. Moreover, the inhibition of SERCA by artemisinin was specific: even at comparatively high (50µM) levels of artemisinin, no other membrane pumps were inhibited. Finally, it was shown by the Nature study that thapsigargin and artemisinin derivatives were antagonistic to each other, strongly suggesting that both drugs have the same target.

A later study by Uhlemann et al., in 2005, demonstrated that the sensitivity of the plasmodial SERCA to artemisinin could be altered by a single amino acid mutation in the protein. They discovered that an L263E replacement of a malarial by a mammalian residue abolished inhibition by artemisinins (Uhlemann et al., 2005). The L263 residue is found in the region of the protein where thapsigargin is known to bind, further implying that artemisinin binds to the *Plasmodial* SERCA in the same manner.

1.4.4.2.2 The mitochondrial electron transport chain

Another parasite-specific mechanism that has been proposed is that of interference with mitochondrial respiration. It has been demonstrated that artemisinin can inhibit the electron transport chain in mitochondria by inducing a depolarisation of the mitochondrial membrane (Li et al., 2005). Such disruption of membrane potential in mitochondria has also been reported when artemisinin is administered to neuronal cell cultures (Schmuck et al., 2002) – perhaps providing insight into the occasional reports of neurotoxicity in patients following treatment with the artemisinins.

A genetic study demonstrated the involvement of the *nde1* and *ndi1* genes, which code for NADH dehydrogenases in the yeast electron transport chain, in sensitivity to artemisinin. Deletion of these genes induced resistance to artemisinin in yeast, whereas over expression of the same induced increased sensitivity. The *ndi1* gene has an orthologue in *P. falciparum*. The author of the 2005 yeast study proposes the following mechanism:

1. Artemisinin is activated by iron present in the mitochondria;
2. This triggers induction of radical species, as discussed in Section 1.6.4.1.;
3. The radical species trigger depolarisation of the mitochondrial membranes (li et al., 2005).

The mitochondrial disruption hypothesis is supported by a 1999 study by Krungkrai et al., who discovered that artemisinin could inhibit the mitochondrial respiratory chain of both asexual and sexual stages of *P. falciparum* (Krungkrai et al., 1999). As mitochondria are expressed continually, not just in young stages of the parasite, this indicates that artemisinins posses some activity against all *Plasmodial* stages.

1.4.4.2.3 Pyrimidine biosynthesis

Krungkrai and colleagues, in a more recent paper, noted that the *P. falciparum* parasite depends on a *de novo* pyrimidine synthesis pathway: the parasite cannot harvest pyrimidines from the human host. The existence of this pathway obviously suggests a target for antimalarial drugs, and Krungkrai's group discovered differences in the dihydroorotase (DHOase) enzyme in the parasite, and in mammals. This is an enzyme that catalyses ring closure in the pyrimidine biosynthetic pathway. Krungkrai's group reported that specific inhibitors can limit the pyrimidine nucleotide pool in the parasite, without affecting the human host (Krungkrai et al., 2008). However, current drugs targeting the dihydroorotase enzyme – such as Leflunomide, used in rheumatoid arthritis, inhibit mammalian forms of the enzyme, including those present in T-lymphocytes (Ruckemann et al., 1998), and may or may not be suitable.

1.4.4.2.4 Binding to PfHRP II

The histidine-rich proteins of *P. falciparum* - designated PfHRP I, II, III, etc – are capable of binding haem monomers, and play a role in the detoxification of haem in the parasite's food vacuole. Kannan et al. (2002) have shown that the artemisinin-haem adduct binds to PfHRP II, and hence inhibits the detoxification of haem within the parasite(Kannan et al., 2002) This is an example of how haem is, in fact, both a trigger and a target for the mechanism of action of artemisinin.

1.4.5 Resistance to artemisinin

2009 saw the first reports of resistance to artemisinin-based therapy (Dondorp et al., 2009). The reports came from Pailin, near the Thai-Cambodian border, a region previously known as a cradle for *Plasmodium* strains resistant to older drugs, and, crucially, where artemisinin-based

monotherapy has been used for more than 30 years. (Pailin is characterised by low-level seasonal transmission of *falciparum* malaria, indicating that no immunity is likely to be present in the adult population, and hence severe illness can result from infection). According to local news reports, resistance to antimalarials has been a problem in Pailin because the regions mining prospects attract foreign migrants with low immunity, who tended not to seek professional medical help upon infection, but self-medicated (Win, 2010).

It had been predicted that resistance to the artemisinins would eventually emerge– hence the WHO's disapproval of artemisinin monotherapy and restriction of the drugs to the areas of greatest need. In 2005, the Lancet reported, from French Guiana, the first known case of *in vitro* resistance of *P. falciparum* to artemether – in parasites originally obtained from patients and then cultivated in the laboratory. In this case, resistance was defined as greatly increased IC_{50} *in vitro*. However, the resistance was not manifested *in vivo*, for reasons which remain unclear (Jambou, 2005).

1.4.5.1 *In vitro* resistance

In 2006, three years before the emergence of *in vivo* resistant *Plasmodium falciparum*, it was demonstrated that parasites of the rodent malaria model *Plasmodium chabaudi chabaudi* could develop stable – that is, heritable and transmissible – resistance to artemisinin and artesunate under "drug pressure". These rodent parasites – cloned from an artemisinin sensitive strain - were cultivated under pressure from increasing levels of either artemisinin or artesunate, and those parasites that survived the highest doses of drug (up to 300 mg/kg per day) were identified as resistant.

Because the new resistant strain was originally cloned from a drug-sensitive strain, it is clear that, as the authors of the study wrote, the drug-sensitive and drug-resistant parasites were genetically identical except for any mutations involved in resistance (Alfonso et al., 2006). It should then, theoretically, be possible to compare the genetic sequence of the two strains and identify the mutated nucleotide sequences.

Alfonso et al., (2006), searched the nucleotide sequences of the resistant strain of *P. chabaudi chabaudi* for mutations in four genes hypothesized to be potential mediators of artemisinin or artesunate resistance. These were:

a and b) mdr1 and *crt*, orthologues of *pfmdr1* and *pfcrt* - genes for *Plasmodium falciparum* multi-drug resistance and *Plasmodium falciparum* chloroquine resistance transporter respectively, encoding membrane transporter proteins located in the membrane of the parasite's food vacuole. In Cambodia, high copy numbers of *pfmdr1* in *P. falciparum* have been associated with resistance to mefloquine, and have also been implicated in the small decrease in artemisinin sensitivity reported in the Lancet (Price et al., 2004);

c) tctp; a gene responsible for the production of translationally-controlled tumour protein. This protein, with the ability to bind artemisinin, was found to be increased in a resistant strain of *P. yoelii yoelii*. However, the resistance was unstable. It has been demonstrated that artemisinins can bind, *via* alkylation of cysteine residues, to the protein TCTP. The significance of this is not yet known, as levels of TCTP ate highest at the trophozoite stage, and so far, all tests have indicated that artemisinins are most active against the younger, ring-stages (Bhisutthibhan et al., 1998);

d) atp6, a gene encoding a sarcoplasmic and endoplasmic reticulum calcium ATPase (SERCA)-type protein. It has been suggested that this protein is the major target of artemisinin-type drugs. In 2005, a mutated form of this gene in *P. falciparum* isolates obtained from patients in French Guiana (a substitution mutation at S769N) this was shown, *in vitro*, to be an indicator of resistance to artemether, characterised by a greatly increased IC_{50} value for the drug *in vitro* (Jambou, 2005).

However, despite the successful induction of drug resistance that remained stable despite cloning, freeze-thawing, passage in the absence of drug and transmission through mosquitoes (Alfonso et al., 2006), no mutations could be observed in any of the genes examined that would explain the resistant phenotype.

1.4.5.2 Clinical resistance in Pailin

And similarly, a study of the artesunate-resistant *P. falciparum* parasites that developed in Pailin failed to firmly identify a genetic basis for the resistance. The researchers compared the genotype of the resistant parasites – wherein resistance was defined clinically as failure to

clear parasitemia within 3 days following the start of treatment, or recrudescence after 7 days following the end of treatment – with the genotype of sensitive parasites taken from patients in north-west Thailand. The authors did not detect the *PfSERCA* L263E and S769 N polymorphisms, which are proposed to confer artemisinin resistance (Dondorp et al., 2009) – although it was discovered that 80% of parasite isolates from patients in Pailin had the mutant codon Y184F within the *pfmdr1* gene, compared to only 30% of isolates taken from north-western Thailand. But no further research has yet been undertaken regarding this particular mutation.

So, at the moment, it remains unclear precisely how the artemisinin-resistant phenotype develops. It is striking, though, that the two regions where resistant *P. falciparum* parasites have been identified in patients – French Guiana and Pailin – are both regions of "antimalarial drug pressure" (Krishna et al., 2006), where parasites are typically resistant to several antimalarials, and where artemisinin monotherapy has been used.

Krishna et al. propose a hypothetical "cascade" scenario following on from the detection of *in vitro* resistance, beginning with a key mutation in the target enzyme, followed by accumulation of additional mutations, ultimately resulting in High rates of clinical drug failure (Krishna et al., 2006). A similar scenario is proposed by Golenser et al., who suggest that since *pfmdr1* has a generalised importance in antimalarial drug resistance, "induction of resistance to one drug may be followed by resistance to other drugs that are not active by the same mechanism" (Golenser et al., 2006). Such a scenario complicates the search for single mutations mediating artemisinin resistance.

On a related topic, it has been reported that the clinical efficacy of artemether/lumefantrine formulations varies according to the proportion of fat in the patient's diet – a low fat diet, typical in rural parts of South East Asia, can inhibit absorption of lumefantrine (Mukhtar et al., 2007), result in failure of therapy (in effect, an artemether monotherapy was delivered, which is inadvisable in areas prone to artemisinin-type resistance.

1.4.5.3 *In vitro* and *in vivo* resistance - discrepancies

In an inversion of the French Guianan situation, parasite isolates from Pailin showing resistance to artesunate remained susceptible to the drug *in vitro*. (It will be recalled that in French Guiana, *in vitro* resistance was observed whilst *in vivo* resistance was not). The authors

of the Pailin study explain this apparent discrepancy by suggesting that resistance to artesunate developed only in young, ring-stage parasites. (As previously discussed, artemisinin-derived drugs act on the young ring-stage parasites, preventing their maturation into the mature trophozoite stage). As *in vitro* tests typically utilise parasite samples ranging from young ring-stage to mature trophozoite, it is suggested by the authors that only a small percentage of these parasites – the ring-stage – would show resistance to the drug. However, there would seem to be an obvious problem with this explanation. As artesunate acts only on the ring-stage parasites, it is of no consequence that the remainder of the *in vitro* sample consists of mature parasites: these mature stages would not be susceptible to artesunate anyway, and therefore it would be expected that an *in vitro* sample consisting of a proportion of young, artesunate-resistant parasites mixed with a range of older stages, would in fact not be susceptible to the drug.

1.4.6 WHO advice for the prevention of artemisinin resistance

In the absence of understanding of how resistance to the artemisinin-derived drugs develops, it is crucial that the advice of the WHO regarding administration of these drugs – that they should always be used in combination with an effective partner drug, which has a different mechanism of action - is followed wherever possible.

The use of artemisinin monotherapy is almost certain to hasten the spread of resistance – indeed, one study has shown that where artesunate monotherapy was used to treat non-immune individuals, the IC_{90} value of the drug against recrudescent parasites was 5 times greater than in the initial infection (Jambou, 2005): strongly implying that the parasites that survived the initial infection were in fact resistant to artesunate. The concomitant use of an additional antimalarial would have been likely to destroy these survivors – as the WHOs treatment guidelines explain: “The use of drugs in combination, specifically with artemisinin derivatives, will remove the survival advantage conferred on parasites resistant to a particular drug by the use of that drug as monotherapy... The use of an artemisinin derivative with an effective partner drug will delay the selection and spread of drug resistance” (WHO, 2006).

Of particular concern is the availability, without prescription, of a range of products claiming to contain artemisinin – typically, capsules containing powdered *Artemisia annua* L*,* or pure

artemisinin itself. A number of US and UK-based websites sell such products to the general public, and the implications of this will be further discussed in Section 10 of this thesis.

1.5 The economics of artemisinin

In 2006, the total global production of artemisinin from *Artemisia annua* L reached 150 tons, but by 2009 annual production had fallen to 40 tons. However, the number of artemisinin-based treatments needed per annum has increased in that time, from 100 million in 2006 to 120 million in 2009. Estimates from NGOs suggest that by 2014, 250 million treatments will be needed. Although after 2014, the estimated requirements for artemisinin are expected to reduce – mainly because of expanded use of mosquito nets and other physical interventions, such as spraying of insecticide – the estimated demand for artemisinin is expected to remain above 200 million treatments, and there is no guarantee that sufficient crops of *Artemisia annua* L will be produced to meet this demand (Grewal, 2009).

The understanding that developing countries are entitled to access the medicines that they need to survive malaria is not a recent one: as far back as 1926, Professor Emile Perrot of the Faculty of Pharmacy in Paris wrote of the necessity to "look for the solution, if it exists, to the problem of supplying the poor nations with a medicine to fight malaria" (Burns, 2008). But the fact remains that today, in parts of Africa in particular, many populations either cannot obtain sufficient quantities of artemisinin-based therapies, or cannot afford the drugs – of the "250 million individuals are infected annually.only 3% have access to ACTs" (Talisuna et al., 2009). Mutabingwa, in a similar vein, explains that high costs and an imbalance between supply and demand are major problems in accessing ACTs (Mutabingwa, 2005).

Solutions proposed to this imbalance between supply and demand range from the small-scale - self-medication with locally grown *Artemisia annua* L. teas, for example - to the global: c.f. the Roll Back Malaria programme, which considers that the "scale-up in cultivation of the plant [*Artemisia annua* L.] and capacity to process it are crucial to worldwide malaria control efforts." (Traore, 2005).

The cultivation of the crop in malarial endemic regions has several advantages, not least of which is as a source of employment. Additionally, it is generally the case that transport costs

are reduced if herbal raw material can be grown and processed near the site of intended use. However, two problems in the cultivation of *A. annua* in Africa, in particular, remain:

1. At the moment, few facilities exist in malaria-endemic regions for the processing (i.e. extraction and purification) of artemisinin from *A. annua* L. So shipping costs – to Europe or China, where production of the pharmaceutical dose-form takes place – are high;
2. Tests – (see section 5.3.1.) - have shown that herbal material grown in Tanzania and Ghana contains relatively high levels of deoxyartemisinin, when compared to European-grown herbal material.

So until processing facilities in malaria-endemic improve – and this improvement is another objective of the Roll Back Malaria programme – the establishment of a UK-based artemisinin supply chain, from field to active pharmaceutical ingredient, would have a significant role to play in the stabilisation of the global artemisinin supply.

1.6 Final words

This thesis presents evidence that

1. Artemisinin levels in *Artemisia annua* L. can be rapidly and accurately assessed both in-field and in the laboratory, using high-pressure liquid chromatography-mass-spectrometry (HPLC-MS) in the laboratory and densitometric thin-layer chromatography in the field (Sections 2, 3 and 6);
2. Deoxyartemisinin can be separated from artemisinin on thin-layer chromatography, and on a gram-scale using a simple and inexpensive flash-chromatography technique (Sections 5 and 7);
3. Breeding for increased artemisinin content results in concomitant increases in chrysosplenetin content, as demonstrated by HPLC-diode array detection (DAD) potentially increasing the value of the crop (Section 8);
4. High-pressure liquid chromatography coupled to diode-array detection (DAD), a UV-based detection method, can be used indirectly to quantify artemisinin levels in herbal material (Section 8). This is based upon the direct correlation between

flavonoid content and artemisinin content, and the fact that flavonoids are strong UV-light absorbers.

5. Teas prepared from *Artemisia annua* leaf, and most over the counter preparations of *Artemisia annua*, are very low in artemisinin content. Additionally, the presence of iron salts in water used to prepare teas does not have a detrimental effect on artemisinin content of the tea (Section 10).

Section 9 briefly discusses an attempt to increase artemisinin yields in hairy root cultures of *A. annua* by applying exogenous farnesol. The thesis concludes, in Section 11, with a discussion of the outcomes of the whole LINK project.

Prologue to Sections 1 - 6: Development of analytical methods

Between 2005 and 2006, a Defra-funded pilot project – "Field cultivation of *Artemisia annua* and enhanced extraction of artemisinin used in novel antimalarial treatments" - ran. This project examined the feasibility of establishing, in the UK, an artemisinin supply chain, from crop in the field to active pharmaceutical ingredient (or, more memorably, from farmer to Pharma).

One of the outcomes of project NF0613 was the identification of the need for an improved analytical system – one that could handle the large sample numbers (in the hundreds or thousands) that are generated by these kinds of breeding projects. The requirements for the improved system were:

1. Greater accuracy and precision (i.e. less variation between replicated analyses, and a more reliable value for artemisinin content);
2. Faster - the current HPLC-DAD based method having a 25 minute run-time, as well as requiring much in the way of sample clean-u*p.* A TLC method was in place, but was semi-quantitative at best;
3. More economical; HPLC solvents being expensive to purchase and dispose of.

Additionally, the requirement for a portable, field-based, means of artemisinin quantification was identified. Such a method would not need the high levels of accuracy of the so-called high-throughput laboratory-based analytical system described in points 1-3, above. It would merely need to be able to give a comparative indication, to a plant grower, of artemisinin level. A device like this would be useful not only in large-scale commercial crops, allowing the user to make on-site, rapid decisions about harvesting times, but also for the small grower in the malarial-endemic regions, who frequently do not have access to laboratory equipment.

Section 2, as well as Sections 3, 4, 6 and, indirectly, 8, discuss new means of reliably quantifying artemisinin levels within leaf material, crude extracts, and purified material.

2 Modifications to HPLC-DAD technique for the separation and quantification of artemisinin

Synopsis

Throughout the one-year pilot project (NF0613), the quantification of artemisinin in dried leaf material had utilised the International Pharmacopoeia's HPLC-DAD-based method (which was intended for the analysis of pharmaceutical-grade, crystalline material). Artemisinin's lack of a strong chromophore, together with the presence in crude leaf extracts of many other UV-absorbing compounds, meant that before this method could be used, time-consuming clean-up had to be performed for each sample analysed. Given that the new four-year LINK project was expected to generate samples in the thousands, a complex clean-up stage for each sample would be unfeasible. As part of this LINK project, the author was asked to investigate whether modifications to the HPLC-DAD method could be made that would improve artemisinin separation to the extent that such clean-up was unnecessary.

Several modifications to mobile and stationary phases were made, and column quality (as quantified by theoretical plates) was monitored. It was found that by keeping theoretical plates above a critical level, reasonable precision and accuracy for artemisinin level could be obtained when analysing crude leaf extracts. However, it was concluded that HPLC-DAD, whilst suitable for the quantification of partially-purified and crystalline artemisinin, was unsuitable for the assay of crude leaf material, and that other avenues (notably HPLC-MS) should be explored.

2.1 Introduction

Sample analysis during Project NF0613 had relied on a high pressure liquid chromatographic (HPLC) separation system, coupled to a diode array detector (DAD) detection system, which is based on the absorption of ultra-violet (UV) light by parts of a molecule of interest. The particular method was adapted from that given in the International Pharmacopoeia (IP) for the

quality assessment and quantification of artemisinin in pure, or almost pure, crystalline form (WHO, 2003). Although the technique had been validated for such a purpose, the UV-based quantification of artemisinin is by no means an ideal technique. Artemisinin, containing no real chromophore, does not strongly absorb UV light. In addition, the wavelength of UV that the molecule does absorb - 214 nm – is very close to the UV maxima of acetonitrile, the primary constituent of the mobile phase recommended by the IP.

The net result of this poor UV absorbance, in a region where solvent absorbance is also observed, is that artemisinin can only be reliably quantified by UV-based methods when high (>1 mg/ml) concentrations of highly purified (> 90% w/w) artemisinin are assayed. HPLC-UV shows its limitations when artemisinin must be quantified in a less clean matrix – a crude plant extract, for example. To fulfil the requirements of this LINK project, thousands of *Artemisia annua* L plants needed to be assayed for artemisinin level. The unfeasibility of purifying thousands of crude extracts to >90% purity before analysis became quickly apparent.

As a large number of samples would be generated on a regular basis, it was necessary to develop a rapid, reliable, and economical procedure that would quickly provide useful data from dried herb. Discussions with consortium members raised a number of potential methods (densitometric thin layer chromatography [TLC], high performance liquid chromatography-diode array detection [HPLC-DAD], HPLC-mass spectrometry [HPLC-MS] , immunological techniques), many of which had already been successfully trialled in previous work by consortium members. Other researchers have also made use of an artemisinin derivatisation procedure, which uses “0.2% (by weight) NaOH solution and then 0.08 M acetic acid” to produce a compound which absorbs UV at 260 nm (Qian et al., 2005). However, in the interests of simplicity – and in order to reduce preparation time - for this large-scale analysis of samples, a modified HPLC-DAD assay, with simplified extraction of dried herb, was developed and tested.

The next section will describe attempts that were made to improve the DAD system. Initially, the robustness of the IP method for the analysis of crude extracts was examined. Following this, modifications were made in an attempt to improve robustness.

2.2 Materials and Methods

2.2.1 HPLC-DAD: initial parameters

An Agilent 1100 Series HPLC separation system with diode array detection was utilised for the separation and quantification of artemisinin. An isocratic mobile phase of 60% acetonitrile and 40% water (both HPLC grade, purchased from Fisher) was run at a flow rate of 1ml/minute through a Phenomenex RP-C18 octadecylsilane (ODS) column of dimensions 250 mm x 4.6 mm, and a particle size of 5 µm. The column oven was set to 30°C, and columns were protected with the Phenomenex Security Guard cartridge system. Artemisinin was quantified at 214nm.

These initial HPLC-DAD parameters are based on those of the International Pharmacopeia.

2.2.1.1 Generation of calibration curve

The response factor of artemisinin was calculated, and a calibration curve of artemisinin across a range of concentrations (0.078 – 5 mg/ml) was generated.

2.2.1.2 Extraction of dried Artemisia annua leaf

Dried leaf material was powdered in a pestle and mortar, and mixed with solvent in a 1/10 ratio. Mixtures were macerated under dark conditions over 24 hours, at 27°C, with gentle shaking.

2.2.1.3 Sample Analysis

The crude extract (10µl) was injected onto the column for artemisinin quantification. Samples were bracketed (every 10 samples) with reference samples of artemisinin at 2.5 mg/ml. Theoretical plates were counted before and after each batch analysis.

2.3 Results

2.3.1 Testing of IP method

2.3.1.1 Retention time of artemisinin

Using this system, artemisinin had a retention time of 9.8 minutes.

2.3.1.2 Response factor of artemisinin by DAD

The units of UV absorbance produced by a single µg of artemisinin were calculated for the HPLC method described above (of course, if the flow rate or solvent composition of a method is changed, then the response factor must be re-calculated. It is a function of solute and solvent). It was calculated that 1µg of artemisinin gave rise to a mean value of 52 (with a standard deviation of 0.6) units of UV absorbance.

2.3.1.3 Calibration curve by DAD

The DAD response to artemisinin was linear in the range used for the calibration curve (0.078 – 5 mg/ml), with a R^2 value of 0.999 and an equation of $y = 557.64x - 3.653$ (Figure 18). However, asymmetry of peaks was detected at artemisinin levels of 2.5 mg/ml and above (Table 3).

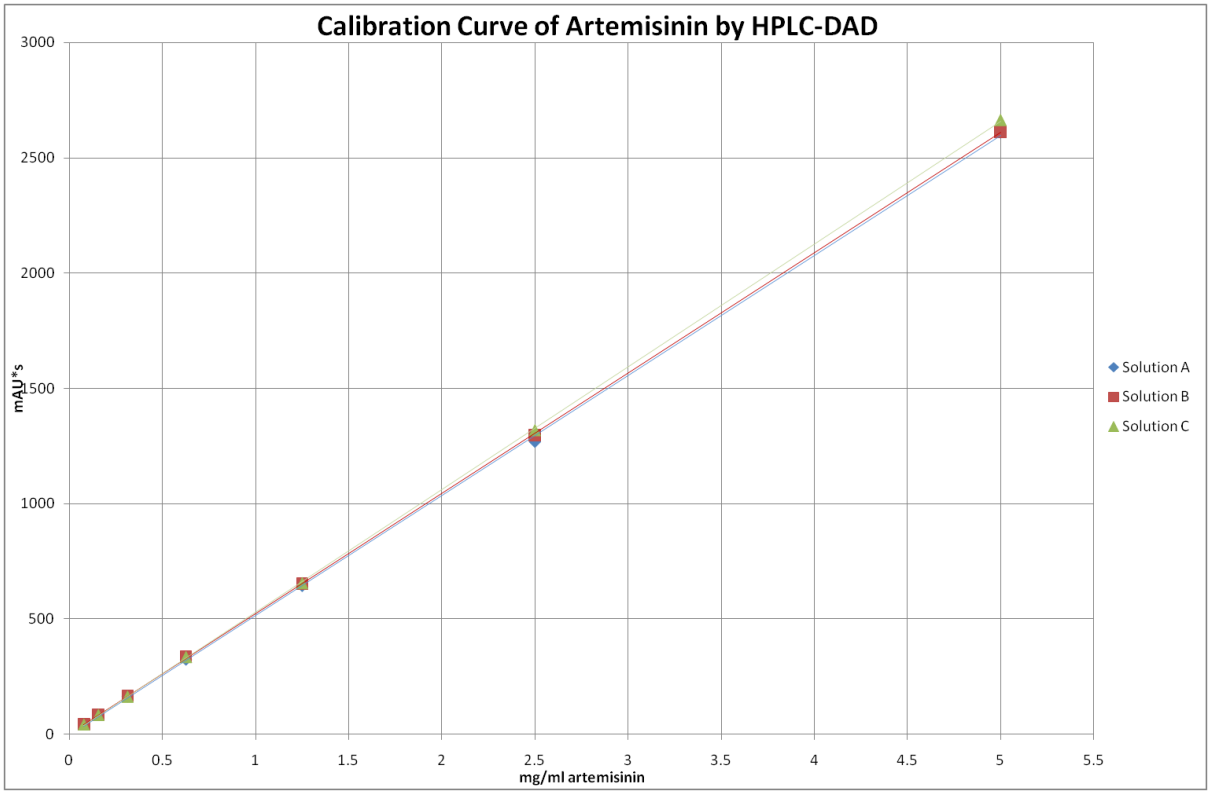


Figure 18. Calibration curve of artemisinin by HPLC-DAD

Table 3. Artemisinin peak symmetry, width and *N*, by HPLC-DAD at 214 nm, across a range of sample concentrations. † Samples were analysed in the order: least concentrated →most concentrated, over a 24 hour period. Therefore the drop in *N* may reflect column conditions deteriorating slightly throughout the sequence, rather than being a function of sample concentration. An asterisk (*) indicates peak tailing.

Conc. artemisinin (mg/ml)	Symmetry	Peak Width	Plate Count†
0.078	0.87	0.1633	66315
0.15	0.86	0.1633	65823
0.31	0.85	0.1650	64075
0.625	0.82	0.1650	63991
1.25	0.77	0.1667	62946
2.5	0.69*	0.1689	60652
5.0	0.56*	0.1689	56224

2.3.1.4 Theoretical plates

One parameter found to be vital in the reliable use of DAD to quantify artemisinin was to keep track of the column's performance, by calculating theoretical plates (*N*). *N* is a numerical measure of how good a column is at resolving closely eluting peaks (i.e. compounds), as calculated by a simple formula:

$N = ([\text{retention time of compound/peak width}]^2) \times 16$

Variation in reported artemisinin levels in crude extracts, even across replicated samples, dogged much of the initial DAD-based work described in this section. This variation could be traced to deterioration in *N*: when this value dropped below 30,000, the reported results for artemisinin level in a sample could not be relied upon to be accurate. Table 4 illustrates this. Crude extracts of *Artemisia annua* were assayed for artemisinin content, in triplicate, on three occasions over a three-week period. Samples labelled as beginning with F were Frontier samples, those beginning with N were NIAB samples, and GBR was a sample of *A. annua* obtained from a local herbalist, and included here for comparative purposes. The table shows reported artemisinin content in each sample, on the three occasions of analysis. It also shows the column plate count (*N*) as calculated at the beginning and end of each batch analysis.

Table 4. Variation in reported artemisinin content of 10 samples of *Artemisia annua* L. leaf, related to *N*

Sample	Week 1	Week 2	Week 3
	Artemisinin % w/w (SD)	Artemisinin % w/w (SD)	Artemisinin % w/w (SD)
FA	0.8 *(0.03)*	0.72 *(0.01)*	0.77 *(0.06)*
FB	2.1 *(0.08)*	1.98 *(0.11)*	2.06 *(0.03)*
FC	1.74 *(0.1)*	1.63 *(0.12)*	1.52 *(0.07)*
FD	0.75 *(0.02)*	0.75 *(0.02)*	0.81 *(0.07)*
GBR	0.2 *(0.004)*	0	0
N65	1.00 *(0.03)*	0.89 *(0.02)*	0.91 *(0.03)*
N200	1.65 *(0.1)*	2.27 *(0.6)*	1.9 *(0.25)*
N409	1.14 *(0.06)*	1.57 *(0.13)*	1.06 *(0.05)*
N812	1.54 *(0.07)*	1.93 *(0.44)*	1.50 *(0.07)*
N950	1.70 *(0.08)*	2.35 *(0.26)*	1.86 *(0.28)*
N at start of analysis	30027	22779	43233
N at end of analysis	29198	20006	41453
N loss	829	2773	1780

The greatest standard deviation in reported artemisinin content is observed in Week 2 – when the plate count was at its lowest. It will be noticed that the reported artemisinin levels in Week 2 differ (typically being reported as higher) from the levels in Weeks 1 and 3. After Week 2, a new guard column was connected to the HPLC column, thus increasing the plate count on Week 3.

If such a DAD-based system were to be used to quantify artemisinin in crude extracts, a report of column performance – based on plate count for artemisinin - calculated before and after each batch analysis would be advisable.

2.3.2 Modifications to IP method

2.3.2.1 Modified Parameters

Modifications were made to mobile phase composition, stationary phase, column oven temperature, flow rate, and gradient of flow rate were made. HPLC-grade methanol was

purchased from Fisher, as was formic acid. Hichrom RP C8 and RP C6 (150 x 4.6 mm, 5 µm) columns were a gift from Nicola Wilsher of the DMU Cancer Drug Discovery Group.

2.3.2.2 Further attempts at utilization of DAD

The temperature of the column oven was varied in steps from 5 °C to 50 °C, but at no point was an improvement in artemisinin separation seen.

2.3.2.3 Solvent extraction of dried leaf material

A consideration of the solvents used by other research groups for the extraction of artemisinin found that the most widely used were toluene, *n*-hexane, *n*-hexane and acetonitrile, chloroform, or petroleum ether. Several solvents were tested for artemisinin extraction efficiency, by simple maceration. A “compromise” solvent was reached, for use in high-throughput screening: acetonitrile. Over a 24-hour maceration, acetonitrile was not quite as efficient as a 1:1 mixture of methanol dichloromethane (DCM), but was as efficient as ethyl acetate and more efficient than hexane. It was cleaner than a methanol: DCM mixture and much easier to use,, as the marc could simply be HPLC-ed without further ado. This method is similar to that of Elferaly et al. (Elferaly et al., 1990), who extract the herb into hexane and then partition into acetonitrile to obtain clean artemisinin.

Table 5. Literature survey of *A. annua* extraction solvents (Flockhart et al., 2005); (Hao et al., 2002); (Hsu, 2006, Kumar et al., 2004, Quispe-Condori et al., 2005, Van Nieuwerburgh et al., 2006, Vandenberghe et al., 1995, Bilia et al., 2006)

Author	Extraction	Clean-Up
Bilia et al. (2006)	Maceration of dried aerial parts with hexane or dichloromethane for 72 hours (room temperature). Dried under vacuum. Re-suspended in acetonitrile.	Sonicated for 20 minutes and filtered through PTFE (0.45µm).
Flockhart et al. (2005)	Toluene or methanol: chloroform (1:1) maceration for 24 hours.	SPE from 100% heptane to 15% ethyl acetate in heptane. Dry under nitrogen and re-suspend in acetonitrile or mobile phase.
Hao et al. (2002)	Microwave-assisted with pet ether (30-60°C, 60-90 °C) cyclohexane, hexane, trichloromethane, ethanol, No. 6 solvent oil, 120 solvent oil.	
Hsu (2006)	Soak entire plant in water, then wring out the plant and ingest the juice. Soak entire plant in urine.	
Kumar et al. (2004)	Hexane extraction of dried powdered material.	
Quispe-Condori et al. (2005)	Supercritical carbon dioxide extraction of leaves. Hexane Soxhlet extraction.	
Van Nieuwenbergh et al. (2006)	Immersion of fresh leaf in chloroform for 1 minute.	
Vandenberghe et al. (1995)	Toluene extraction of dry herb with sonication (x 2).	Silica gel SPE preconditioned with toluene, then washed with pet ether: ethanol (discarded). Eluted with MeCN. Eluate evaporated under nitrogen and re-suspended in 1ml ethanol: water (1:1).

Hexane was commonly employed by other researchers as an extraction solvent, but a simple comparison (Table 6) of hexane with acetonitrile for the extraction of artemisinin from dried leaves showed that hexane was the poorer:

Table 6. Comparison of hexane and acetonitrile as *A. annua* extraction solvents

Sample	Artemisinin % w/w (hexane extract)	Artemisinin % w/w (acetonitrile extract)
NIAB 25	0.4	0.8
NIAB 30	0.3	0.7
NIAB 35	0.2	0.5
NIAB 110	0.2	0.5

Other combinations were tried (hexane: butylmethylether, 50/50; hexane: ethyl acetate, 85/15; 100% methanol, 100% DCM), but consistently, the cleanest extracts, combined with the most efficient artemisinin extraction, were obtained with acetonitrile.

2.3.2.4 Sample mass

Sample mass as a factor influencing variability was also considered, as was the variation between powdered *vs*. non-powdered samples. It was found that sample mass used - between 100 mg and 1400 mg – were unimportant, as long as lower samples sizes were powdered. Slight variability was observed when 100 mg samples were not powdered, but this variability disappeared upon powdering with a pestle and mortar.

2.3.2.5 Gradient of mobile phase

As artemisinin separation by the isocratic IP method was reasonable, as long as other factors (namely *N*) were considered, it was decided to keep the gradient separation quite gentle and fairly close to the conditions of the isocratic method, whilst trying to eliminate the characteristic problems of isocratic separations: a lot of peaks in the first few minutes, a "dead zone" in the middle of the run where little elutes, and a few wide peaks towards the end (Figure 20).

Incidentally, extreme gradients (from 10% water to 90%, for example) were not at all ideal for these crude extracts and were eliminated as possibilities early on.

After much trial and error, the following method was found to produce the best separation of all compounds, when used with a GraceSmart narrow-bore column of 150 x 2.1 mm, 3µm particle size:

Time	Solvent A %	Solvent B%	Flow (ml/minute)
0.00	70	30	0.5
10.00	0	100	0.75
15.00	50	50	0.75
20.00	70	30	0.5
22.00	70	30	0.5

Where solvent A was comprised of 33% acetonitrile and 66% water, and Solvent B was made up of 66 % acetonitrile and 33% water. But this separation still had two drawbacks:

a) although the overall separation was better, artemisinin, being a small peak in a crude extract, was still not reliably separated;
b) baseline drift with increasing acetonitrile was a persistent problem (Figures 21 and 22).

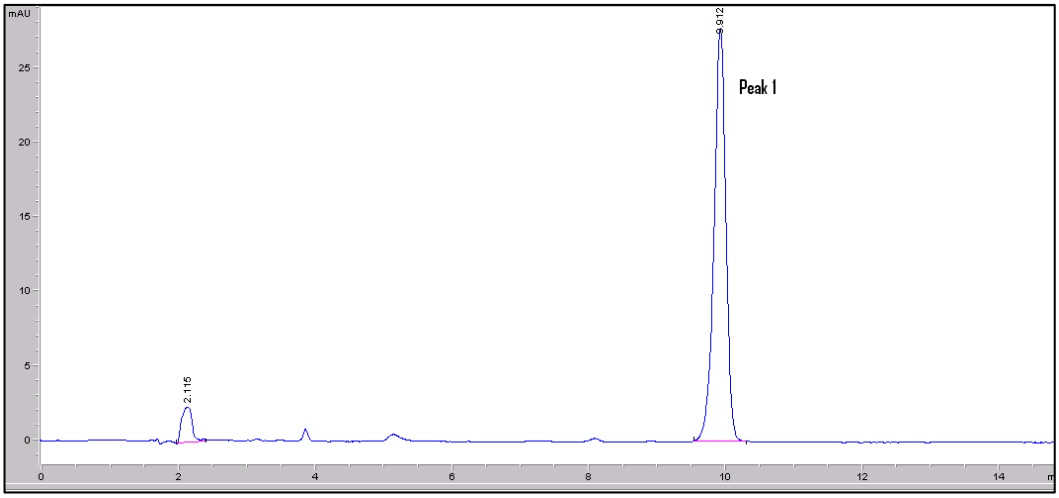


Figure 19. HPLC separation of artemisinin reference using the IP method, and read at 214 nm. Artemisinin elutes at 9.8 m.

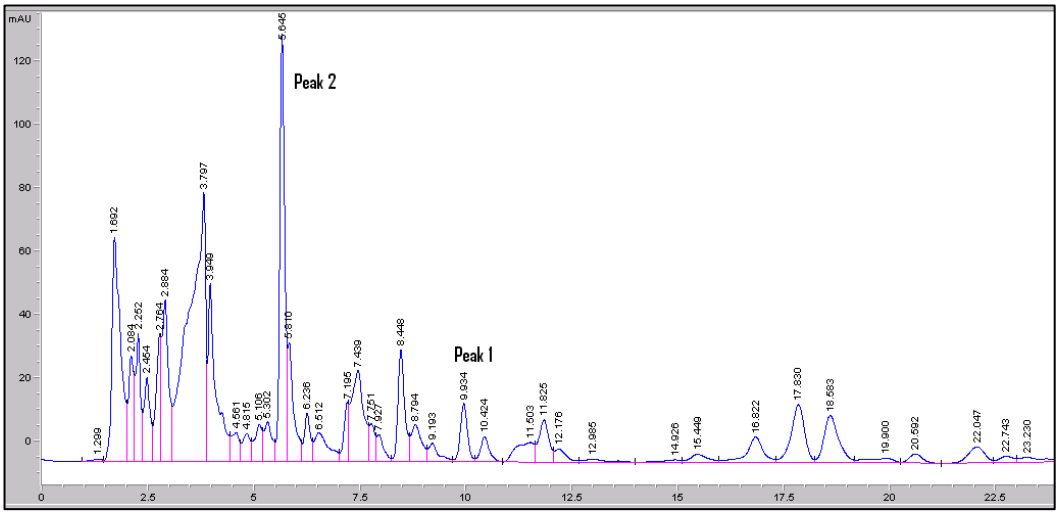


Figure 20. HPLC separation of a crude extract of *Artemisia annua*, using the IP method, and read at 214 nm. Artemisinin (Peak 1) elutes at 9.9m, and chrysosplenetin (Peak 2) at 5.6 m

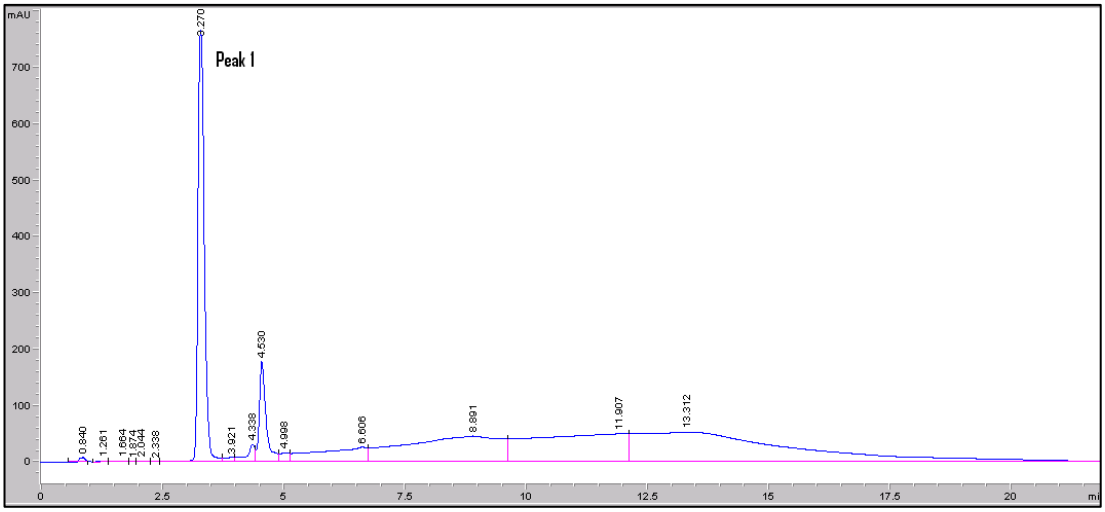


Figure 21. HPLC separation of artemisinin using the newly developed gradient narrow-column separation method, read at 214 nm. Artemisinin elutes at 3.2 min. An impurity is visible at 4.5 min.

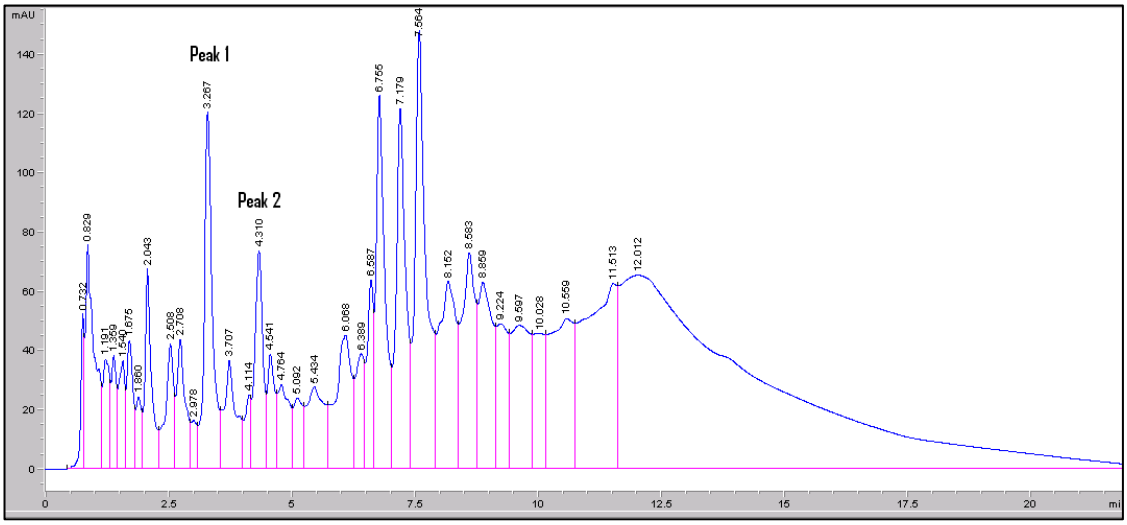


Figure 22. HPLC separation of a crude extract of *Artemisia annua* using the newly developed gradient narrow-column separation method, read at 214 nm. Artemisinin (Peak 1) elutes at 3.2 min. Chrysosplenetin elutes at 4.2 min.

Baseline drift, when the chromatogram was viewed at this low UV region, rendered the gradient method unsuitable for the analysis of artemisinin. However, it was noted that the flavonoid constituents – identified by reference chrysosplenetin, and by UV-profile of unknown flavonoid-type compounds, separated well by this new method. The method was, therefore, taken as a starting point for a flavonoid separation system, discussed more fully in Section 8.

2.4 Discussion

The use of HPLC-DAD for the quantification of artemisinin is by no means ideal: artemisinin has no strong chromophore. Where it does absorb UV - at 214 nm - the absorbance is uncomfortably close to the absorbance of acetonitrile (and indeed all other reverse-phase HPLC solvents), a major component of reverse-phase HPLC mobile phases. It is this low absorbance that has so far prevented the improvement of peak resolution and shortening of the analytical time *via* a solvent gradient - as acetonitrile levels increase throughout a gradient run, the baseline drifts so far to high absorption as to render the quantification of artemisinin unreliable. Subtracting a blank run improved the chromatogram, but not to the extent desired. Despite the efforts made, it was concluded that HPLC-DAD would, no matter how well optimized, never be a suitable means by which to quantify artemisinin in crude extracts of *Artemisia annua* L – a conclusion similarly reached by the Malaria Medicines Venture (MMV), who in a recent report state that after experimentation, they cannot recommend the use of HPLC-UV for the quantification of artemisinin in extracts, but only for determination of the purity of isolated artemisinin (Lapkin et al., 2009).

As artemisinin is easily detected by ESI (electrospray ionization) mass spectroscopy (MS), and a machine of this type had been made available in the department, it was decided that future attempts at the high-throughput quantification of artemisinin would be based on LCMS.

3 HPLC-MS (ESI) separation and quantification of artemisinin

Synopsis

HPLC-MS, using an Agilent 1100 system with electrospray ionisation, was used to develop a high-throughput analytical method for the quantification of artemisinin in dried *Artemisia annua* leaf. The method was externally validated, and was capable of accurately assaying more than 100 samples per day.

3.1 Introduction

Having established that reliable quantification of artemisinin in crude extracts of *Artemisia annua* was not feasible using HPLC-DAD, attention was turned to the use of HPLC-mass spectrometry (HPLC-MS). This had been used by several groups as a means of quantifying artemisinin metabolites in human plasma (Naik et al., 2005), in particular artesunate and dihydroartemisinin, and occasionally for the quantification of artemisinin in crude plant extracts, but at the time that this project began (2006), no validated methods had been published for high-throughput MS-based assay of crude extracts.

At the time of writing, though (March 2010), several recent papers are available detailing such methods, including those by Han et al. (Han et al., 2008) and Lapkin et al. (Lapkin et al., 2009).

As for HPLC-DAD, the International Pharmacopeia method was taken as a starting point, and modifications were made from that point.

A further advantage of HPLC-MS is was expected to be that the method can, unlike HPLC-DAD, discriminate between artemisinin and the pharmaceutically inactive (but co-eluting) deoxyartemisinin.

3.2 Materials and Methods

3.2.1 Extraction and analytical procedure - LCMS

Extraction. Dried *Artemisia annua* leaf material was powdered using a pestle and mortar, and macerated in acetonitrile for 24 hours under dark conditions, with gentle shaking, at a 1/10 weight-to-volume ratio.

Sample Preparation. Analytical samples were prepared by the dilution of 25 µl of original extract in 875 µl of acetonitrile, with addition of 100 µl of santonin stock solution at 0.1 mg/ml – i.e., 10 µg/ml as a final concentration.

3.2.2 HPLC-MS parameters

Artemisinin was quantified using an Agilent Technologies 1100 Series high-performance liquid chromatography system, with a GraceSmart RP18-HPLC column (150 mm x 2.1 mm; pore size of 3 mm), coupled to an Agilent Technologies G1946 single quadruple mass spectrometer with electrospray ionization (ESI), used in positive ion mode, with single ion monitoring (SIM). HPLC separation was achieved with an isocratic mobile phase of 60% acetonitrile and 40% HPLC-grade water, with 0.1% v/v formic acid, at a flow rate of 0.2 cm^3, with a run time of 8 minutes. The column oven was set at 25°C.

Following flow injection analyses (FIA), the ESI spray chamber and mass spectrometer parameters were set as follows: nitrogen flow at 8 L^3/minute; drying gas temperature 350°C; capillary voltage 2800 V in positive mode, 3500 V in negative mode; nebulizer pressure 40 psig; and a fragmentor voltage of 70 V. The mass spectrometer was calibrated and, if necessary, tuned daily, using Agilent's Electrospray Tuning Mix, to ensure peak performance. Generation of calibration curve. Santonin was used as an internal standard, with ions monitored at 173.3 and 247.3. The monitored artemisinin ions were 283.3 and 209.3.

3.2.3 Generation of calibration curve

A calibration curve was generated, in which artemisinin concentration ranged from 2 to 16 µg/ml, and in which santonin, as an internal standard, was kept constant at 10 µg/ml. All calibration levels were prepared in triplicate and analysed three times per sample. The ratio of artemisinin to santonin was calculated for each analysis; linear regression of the resulting

scatter plot was used to establish the calibration curve. The upper limit of quantification was given as the highest level of the calibration curve: beyond this, saturation of response rendered quantification less reliable. The lower detection limit was taken as five times the standard deviation of the measured response to the lowest calibration level, and was calculated to be 0.8 ng.

3.3 Results

3.3.1 Choice of ions for SIM-based quantification

Following assay of artemisinin and santonin references under single ion monitoring (SIM) mode, quantifier and qualifier ions of 265.2 and 283.2, respectively, were selected for the single ion monitoring (SIM) of artemisinin. For santonin, quantifier and qualifier ions were 247.3 and 174.3, respectively.

3.3.2 New column and reduction in mobile phase

The IP HPLC-DAD method utilised a column of dimensions 250 mm x 4.6 mm (particle size: 5 µm), with a flow rate of 1ml/minute. This resulted in a column pressure of ¬80 bar. As this flow rate would have been a little high for electrospray ionisation, a column of smaller dimensions was chosen. The mobile phase composition – 60% acetonitrile, 40% water, with 0.1% v/v formic acid – was kept the same, but using a GraceSmart RP18 narrow-bore column, of length 150 mm and internal diameter of 2.1 mm (particle size: 3µm), allowed reduction of flow rate to 0.2 ml/minute whilst retaining a column pressure of 80 bar.

On this new system, run times were shortened from 25 minutes to 10 minutes, with artemisinin eluting at 4.8 minutes.

3.3.3 Generation of calibration curve

The calibration curve (Figure 23) was linear in the artemisinin range 2.5 – 20 µg/ml, with a slope of $y = 0.057x + 0.024$, and a linear regression of 0.993.

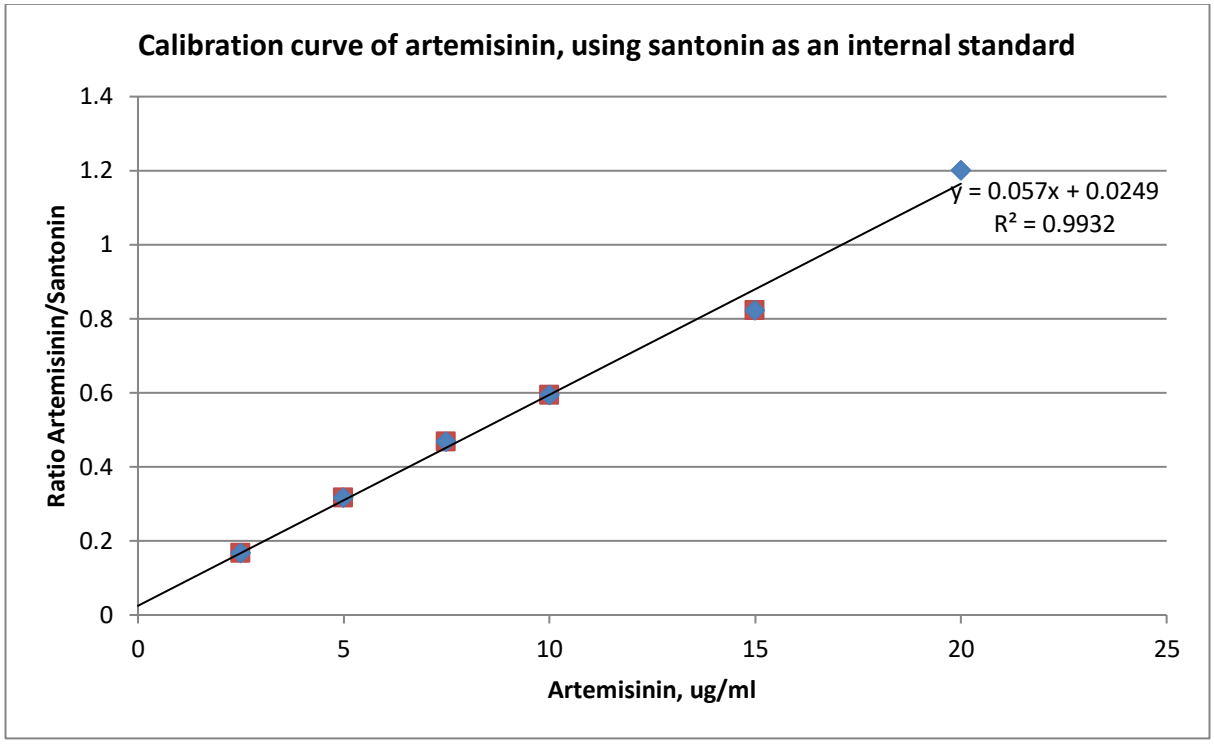


Figure 23. LCMS calibration curve of artemisinin, using santonin as internal standard

The vertical axis shows the ratio of artemisinin to santonin in each reference sample (calculated as the peak area for artemisinin divided by the peak area for santonin). The horizontal axis shows the artemisinin concentration, in µg/ml, of each reference sample.

3.3.4 Precision and accuracy

It was noted that, when using ESI without formic acid in the mobile phase, that the line of least squares did not quite intercept at zero. This phenomenon was observed each time the calibration curve was prepared. Naturally, this meant that at artemisinin levels below a certain threshold, quantification became unreliable – in fact, error became larger, the smaller the artemisinin concentration. Table 7 illustrates this.

Table 7. Actual vs. reported artemisinin content of samples assayed by LCMS

Artemisinin Response/Santonin Response	Reported artemisinin content of sample (µg/ml)	Actual artemisinin content of sample (µg/ml)	Actual artemisinin content of sample, as a percentage of the reported value
0.2	2.8	1.77	63.9
0.3	4.7	3.69	78.7
0.4	6.6	5.6	84.8
0.5	8.5	7.5	88.2
0.6	10.4	9.41	90.4
0.7	12.3	11.53	92.0
0.8	14.2	13.23	93.0
0.9	16.1	15.14	93.8
1	18.0	17.05	94.5

When the artemisinin content of the sample reaches 18 µg/ml, actual artemisinin content is almost 95% of the reported value – an acceptable level of error. But at the lowest artemisinin levels (0.3 µg/ml, and lower), the actual artemisinin content if the sample is significantly lower than the reported value. For this reason, it is recommended that if the reported artemisinin value falls in the range below 0.4 µg/ml, the sample should be re-analysed at a lower dilution factor, to bring it within the reliable limits of quantification. This problem was not observed following addition of formic acid to the mobile phase.

3.3.5 Precision

Samples of crude *Artemisia annua* were prepared in triplicate, and analysed by the new method. Each preparation was injected three times onto the column. Table 8 illustrates the results.

Table 8. Repeatability of sample injections

Sample	Injection 1	Injection 2	Injection 3	Mean	SD
1186 A	1.8	1.8	1.8	*1.8*	*0.03*
1186 B	1.7	1.7	1.8	*1.7*	*0.07*
1186 C	1.7	1.9	1.9	*1.8*	*0.09*
1243 A	2.0	1.9	2.1	*2.0*	*0.1*
1243 B	1.9	1.9	1.9	*1.9*	*0.03*
1243 C	2.1	2.0	2.0	*2.0*	*0.09*

3.3.6 External validation of LCMS technique

Several samples of dried leaf material, which had previously been assayed for artemisinin content using the newly developed LCMS technique, were sent to Sensapharm Ltd, a UK company focused on the development, supply and support of malaria associated drug reference standards and drug detection systems. Samples were randomised and sent to Sensapharm with no information as to the expected artemisinin content.

The artemisinin levels reported by Sensapharm are shown in Table 9, and compared with the levels reported at DMU.

Table 9. Comparison of DMU and Sensapharm results for identical samples

Sample	**Artemisinin % w/w (DMU Analysis)**	**Artemisinin % w/w (Sensapharm Analysis)**
African 1	0.41	0.45
African 2	0.50	0.39
African 3	0.43	0.34
African A	0.53	0.69
African B	0.50	0.59
African C	0.52	0.53
876	1.20	1.21
869	1.10	1.12
A. annua shoot culture	Trace	0.03
981	2.27	2.04
984	2.55	2.28
991	2.46	2.39
1013	2.46	2.52
1029	2.55	2.43
1053	2.41	2.31
1091	2.36	2.40
1097	2.55	2.47
1102	2.46	2.22
Mean	**1.60**	**1.55**
P value	**0.097**	

Using a paired t-test, no significant difference between the two sets of data was observed.

3.4 Discussion

Using high-pressure liquid chromatography coupled to an electrospray ionisation mass spectrometer, a high-throughput means of artemisinin quantification in plant material was developed. The use of a narrow-bore column greatly reduced solvent usage and the cots that are inherent with such usage. In addition, the method was the rapid, requiring less than 10 minutes per run, and selective, in that deoxyartemisinin - which co-elutes with artemisinin and can, by UV, lead to overestimation of artemisinin content – can be screened out with the use of selective ion monitoring.

Unlike HPLC-DAD, it was noticed that at lower sample concentrations, percentage error became unacceptably large – a phenomenon caused by the fact that the line of least squares did not intercept the y axis at zero. The addition of formic acid to the mobile phase improved accuracy of sample reporting in this respect.

Using this method, over 100 samples could be assayed per day assuming the machine was in use for 20 hours per day. It is feasible that the use of smaller columns could further reduce sample run time, and thus increase sample throughput.

4 PCA and PLS-DA analysis of *artemisia annua* L. leaf samples

Synopsis

NMR-based Principal Components Analysis (PCA) and Partial Least Squares-Discriminant Analysis (PLS-DA) was investigated for potential use in the quality control of dried *Artemisia annua* L. leaf samples.

Using raw NMR, poor samples (i.e. those containing no artemisinin, or very little) could immediately be discerned from those containing high levels of artemisinin. However, the discernment of fine grades of artemisinin content was not possible by simply observing raw NMR spectra.

PCA was able to cluster samples by age and post-harvest treatment, but not by artemisinin content. PLS-DA was able to classify most samples into expected groupings based on sample pre-treatment and harvest dates, but validation of this method is needed.

4.1 Introduction

Multivariate analysis was performed on 46 samples of *Artemisia annua* grown within the consortium. This was done using principal components analysis (PCA) and partial least squares discriminant analysis (PLS-DA), based on data obtained by nuclear magnetic resonance (NMR) of crude herbal extracts. The objective behind this analysis was to ascertain whether NMR-based multivariate analysis could allow the rapid identification of high-artemisinin material at all stages of the harvest season, with a minimum of sample preparation. This process could also, in theory, identify the genetic and geographical origins and growth conditions of an unknown sample.

4.1.1 Principal components analysis (PCA)

PCA is a statistical technique used when a large amount of data is to be handled – in this case, every signal generated in more than 50 proton NMR spectra. The PCA process correlates

variables (i.e. NMR signals) in a similar way to a simple ***x* vs. *y*** scatterplot. However, because of the very large number of variables (NMR signals) generated by this method, a 2-D scatterplot, which can only correlate 2 variables, is insufficient. So, using a mathematical concept called hyperspace, in which data can be plotted in more than three spatial dimensions, a multidimensional scatterplot is generated. Statistical software will then identify a regression line – in the same way as in a 2-D scatterplot – that displays the variation between the samples along this line. The line that lies across the greatest degree of variation in the scatterplot is designated as the first principal component, or PC1 – sometimes also called a factor (Alt, 1990).

After the first PC has been created, carrying information as to most of the sample variation, the software will then identify further regression lines that travel through the remaining variation. The generation of these PCs is done via the rotation of graphical axes. PC2 will always be orthogonal to PC1, to ensure that the greatest degree of variation is covered by the PCs.

Typically, 3 or 4 PCs are sufficient to explain all of the variation within the sample data, with PC1 explaining most of the variation, and PCs 2, 3 and 4, progressively less[2] (Jansen et al., 2010).

PCs may be considered as combined variables – they show how individual signals from the molecules in the samples vary in combination with all the other signals[3]. Principal components express in simplified form the relationship between all of the variables in a sample – i.e., the whole metabolome.

[2] Geometrically, PCA involves the rotation of graphical axes on a multi-dimensional graph. Axis one – or PC 1 – is the axis along the length of which most variation in samples can be observed. PC2 is the axis along which the second greatest degree of variation can be observed, and so on.

[3] Each variable (i.e. NMR signal, representing part of a molecule in the extract) will correlate to each principal component either negatively, positively, or not at all.

The principal components (that is, combined variables) may, in the case of plant extracts, relate to terpenoids, or sugars, or lipids, to name a few examples. The advantage of generating principal components is that the multidimensional scatterplots that are initially needed to display all the variables generated by NMR – and which are imposible to visualise – can be greatly simplified, by combining all the variables into a much smaller number of PCs . This means that the more familiar 2-D scatterplots can be used - PC1 will be displayed on the x-axis, and PC2 or PC3 on the y-axis. In this way, it is possible

a) to determine whether the PCs are linked
b) to cluster related samples

Each sample analysed by NMR will correlate to the principal components differently, because each sample varies to a greater or lesser degree in levels of artemisinin, or chrysosplenetin, or sucrose, for example. So, by looking at how two or more different samples relate to the same principal components, similar and differing samples can be easily clustered together.

4.1.1.1 Scores plots and loadings plots

PCA typically generates scores plots and loadings plots. A scores plot shows the co-ordinates of each sample on the axis system of the PCs, and looks like any scatter plot, in that related samples will cluster together. A loadings plot gives information as to which original variables – in NMR, which ppm values – are responsible for the differences between samples (Jansen et al., 2010).

PCA is an unsupervised technique – the samples are analysed “blind”, and the statistical software is given no information as to the nature or expected clustering of the samples. On a scores plot, observed clusters merely show that samples are related in some way – no information on the nature of the relationships between the samples, or the chemical content of the samples, is obtained. Loadings plots, however, do give some chemical information, and can help to identify chemical similarity between clustered samples (Schripsema, 2010).

4.1.2 Partial least squares discriminant analysis (PLS-DA)

PLS-DA is a supervised technique that has similarities to PCA – the major similarly being that PLS-DA also involves rotating axes to give the greatest spread along an axis. But the two techniques differ in a major way, and the clue to the difference is in the designation "supervised".

Whereas in PCA, the axes are rotated so that the greatest sample spread is observed, and the software is not given any information as to the expected results, in PLS-DA, the axes are rotated to give the optimal discrimination between sample groups (Colquhoun, 2007). The algorithm will be told which samples belong to which group, and will be validated according to how well it clusters samples in this group. Another way of saying this is that PCA clusters samples by dependent variables (those variables that depend on what treatment they received – for example, glyphosate-treated plants should contain more artemisinin than those that were not treated. Artemisinin level is a dependent variable). On the other hand, PLS-DA attempts to cluster samples by independent variables – those variables, such as harvesting early or late, that are created by the experimenter and are not a result of the experiment itself (Colquhoun, 2007).

PLS-DA, rather than placing PCs on the axes, creates latent variables (LVs) which are for most purposes similar to PCs (Ciosek et al., 2005).

4.1.3 This study

It was hypothesised that using PCA, samples of *Artemisia annua* L. with a high concentration of artemisinin would cluster; as would samples containing low levels of artemisinin. It was further hypothesised that, ultimately, NMR-based principal components analysis – in particular, PLS-DA - would allow the quality assessment of "blind" samples of *Artemisia annua* L

4.2 Materials and Methods

4.2.1 Sample preparation

Dried, powdered leaf (100 mg) was macerated in 1 ml 70% deuterated methanol for one hour. Samples were then centrifuged at 1200 rpm for 5 minutes, and the supernatant thus obtained was used for NMR analysis.

4.2.2 NMR analysis

Samples were analysed by as per Belton et al., 1998. 500 MHz ^{1}H NMR spectra were recorded at 27°C using a Bruker DRX-500 spectrometer. For quantification, the 1.44 ppm signal (corresponding to H-15 of the methyl group) was used.

4.2.3 Principal components analysis

PCA was carried out as per Belton et al., 1998, in that preliminary data processing was carried out using FELIX software, version 2.30 (Molecular Simulations), on a Silicon Graphics Indigo workstation. Bucketing into bins of 0.04 ppm was carried out using Bruker Biospin's AMIX software, and the spectra that resulted were transferred to a PC for data analysis (Colquhoun, 2007).

4.3 Results and Discussion

Table 10 shows the samples that were assayed for this part of the research:

Table 10. Samples assayed by multivariate analysis. Samples identified only by numbers indicate NIAB (Cambridge-grown) material. All samples, except 49-50, were of *Artemisia annua* L. GLY: indicates samples were sprayed with Glyphosate before harvest

Sample No.	Details (Sample ID)	Artemisinin (% w/w)	Cross	Harvest Date	Treatment
1	1	0.8	1015	31/07/2008	
2	846	1	1015	22/09/2008	
3	876	1.2	1015	22/09/2008	GLY
4	8	0.7	1001-1	31/07/2008	
5	853	Unknown	1001-1	22/09/2008	
6	878	1.1	1001-1	22/09/2008	GLY
7	20	0.7	1001-3	31/07/2008	
8	865	0.9	1001-3	22/09/2008	
9	879	1	1001-3	22/09/2008	GLY

10	6	0.6	1012-10	31/07/2008	
11	851	0.7	1012-10	22/09/2008	
12	875	0.8	1012-10	22/09/2008	GLY
13	3	1	1038-1	31/07/2008	
14	848	0.9	1038-1	22/09/2008	
15	874	1.3	1038-1	22/09/2008	GLY
16	18	0.8	1045-3	31/07/2008	
17	863	0.9	1045-3	22/09/2008	
18	886	1.2	1045-3	22/09/2008	GLY
19	15	1	1046-7	31/07/2008	
20	860	1.1	1046-7	22/09/2008	
21	873	1.3	1046-7	22/09/2008	GLY
22	7	1.2	1062-1	31/07/2008	
23	852	1.4	1062-1	22/09/2008	
24	871	1.7	1062-1	22/09/2008	GLY
25	12	1	1062-4	31/07/2008	
26	857	1.2	1062-4	22/09/2008	
27	872	1.5	1062-4	22/09/2008	GLY
28	14	1.4	1053-1	31/07/2008	
29	859	0.8	1053-1	22/09/2008	
30	881	1.3	1053-1	22/09/2008	GLY
31	21	0.9	1053-2	31/07/2008	
32	866	0.9	1053-2	22/09/2008	
33	883	1.3	1053-2	22/09/2008	GLY
34	9	0.8	1053-4	31/09/2008	
35	854	1.2	1053-4	22/09/2008	
36	869	1.1	1053-4	22/09/2008	GLY
37	989 (2007)	*1.1*	1015	27/09/2007	
38	1159 (2007)	*1.1*	1015	05/10/2007	
39	145 (2007)	*0.6*	1043-1	01/08/2007	
40	557(2007)	*0.7*	1043-1	30/08/2007	
41	AA15593		Brown herb	060207-01	
42	AA 16195		Green herb	060207-3	
43	Moroccan	*0.5*		160708-01	
44	Moroccan	*0.5*		160809-02	
45	Spent herb a	None		060606-01	
46	Spent herb b	None		060424-01	
47	Shoot	Not known		Shoot culture	
48	Shoot	Not known		Shoot culture	
49	*T. lucida*	N/A			
50	*A. absinthium*	N/A			
51	Artemisinin 2mg				

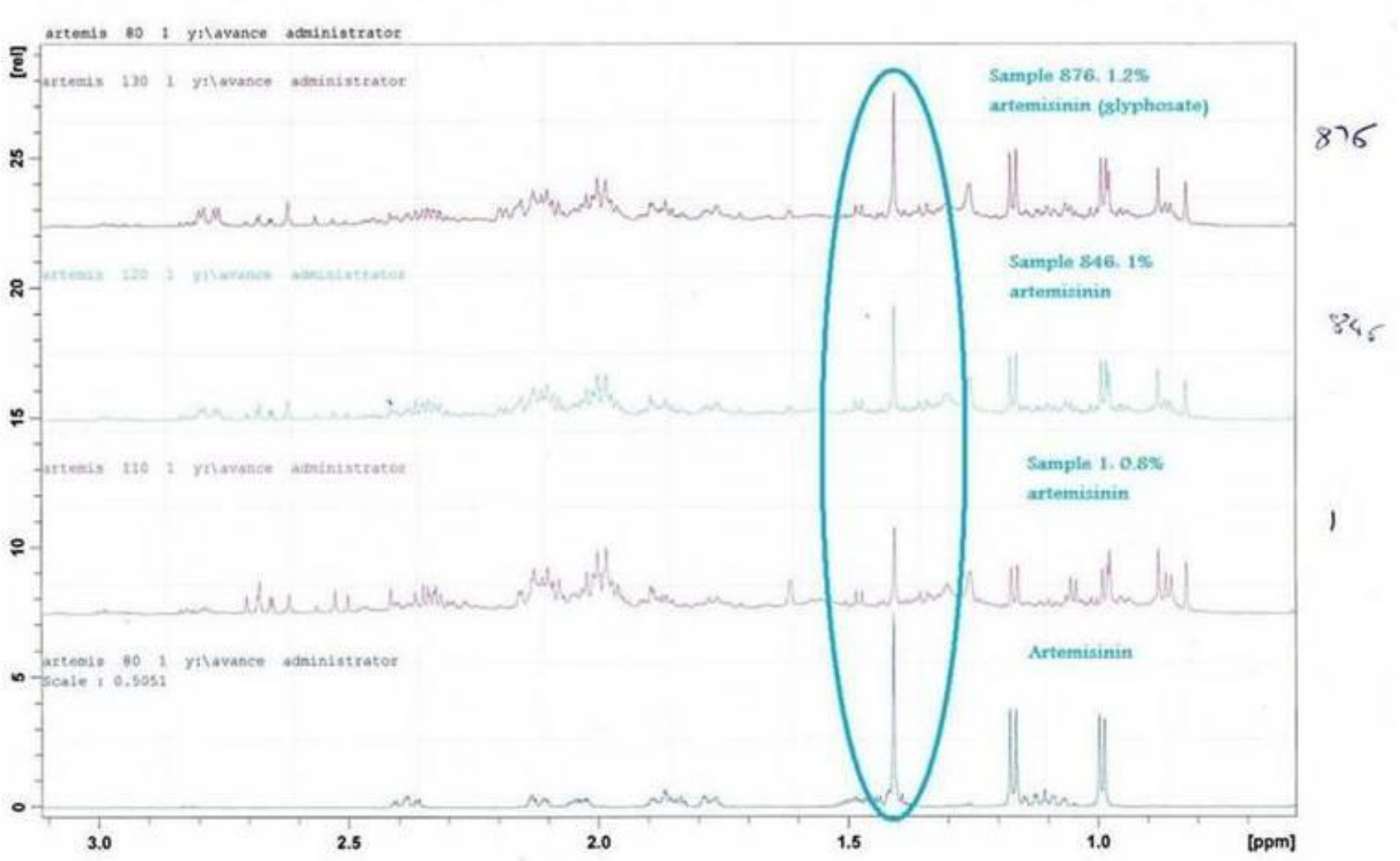


Figure 24. NMR spectra of extracts of 2 *A. annua* samples of differing artemisinin content, against artemisinin reference of 1mg/ml. Note signal at 1.44 ppm

Section 4: NMR-based PCA and PLS-DA of crude *Artemisia annua* extracts

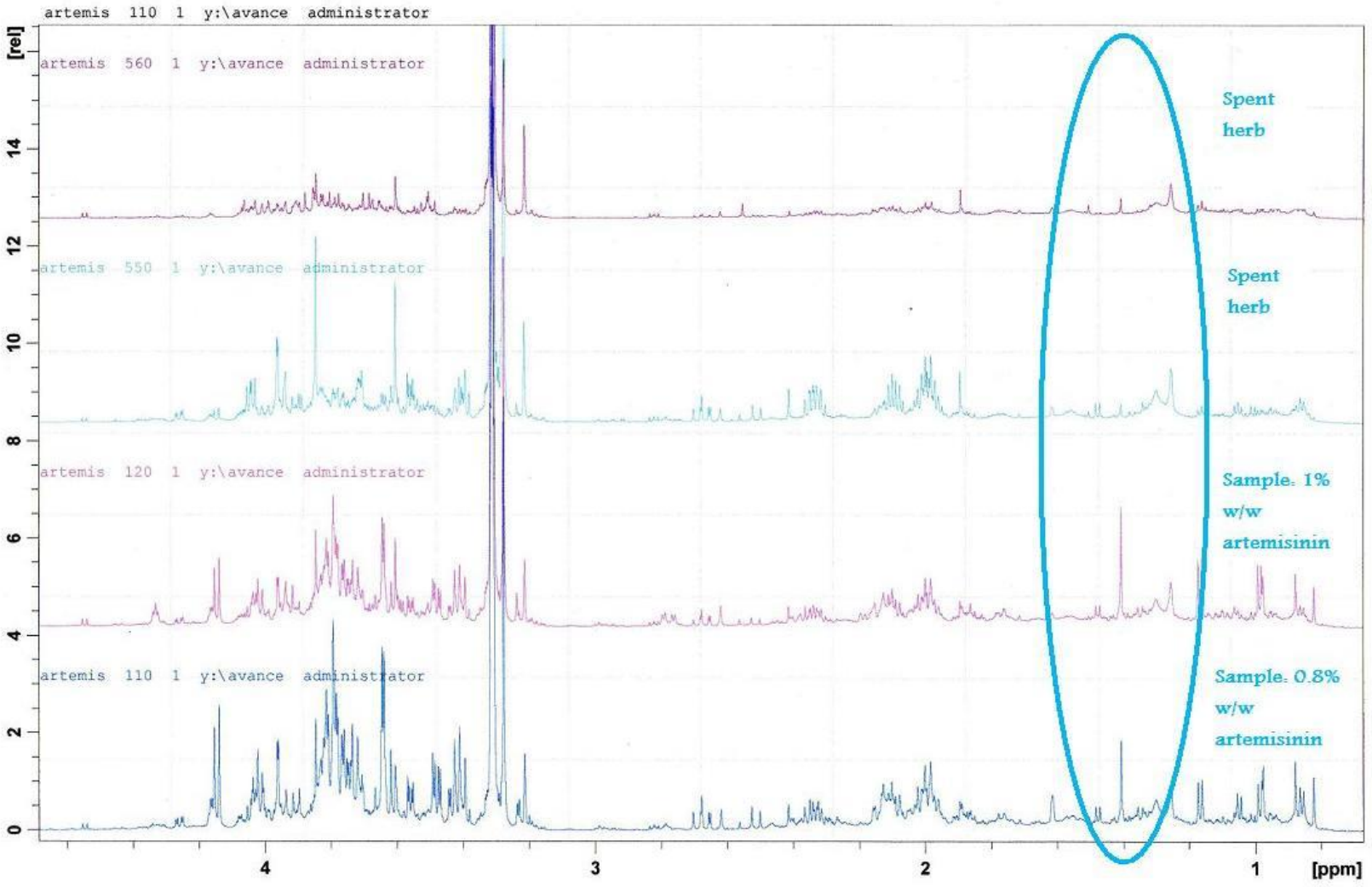


Figure 25. NMR spectra of two samples of spent herb compared to two samples containing artemisinin

Section 4: NMR-based PCA and PLS-DA of crude *Artemisia annua* extracts

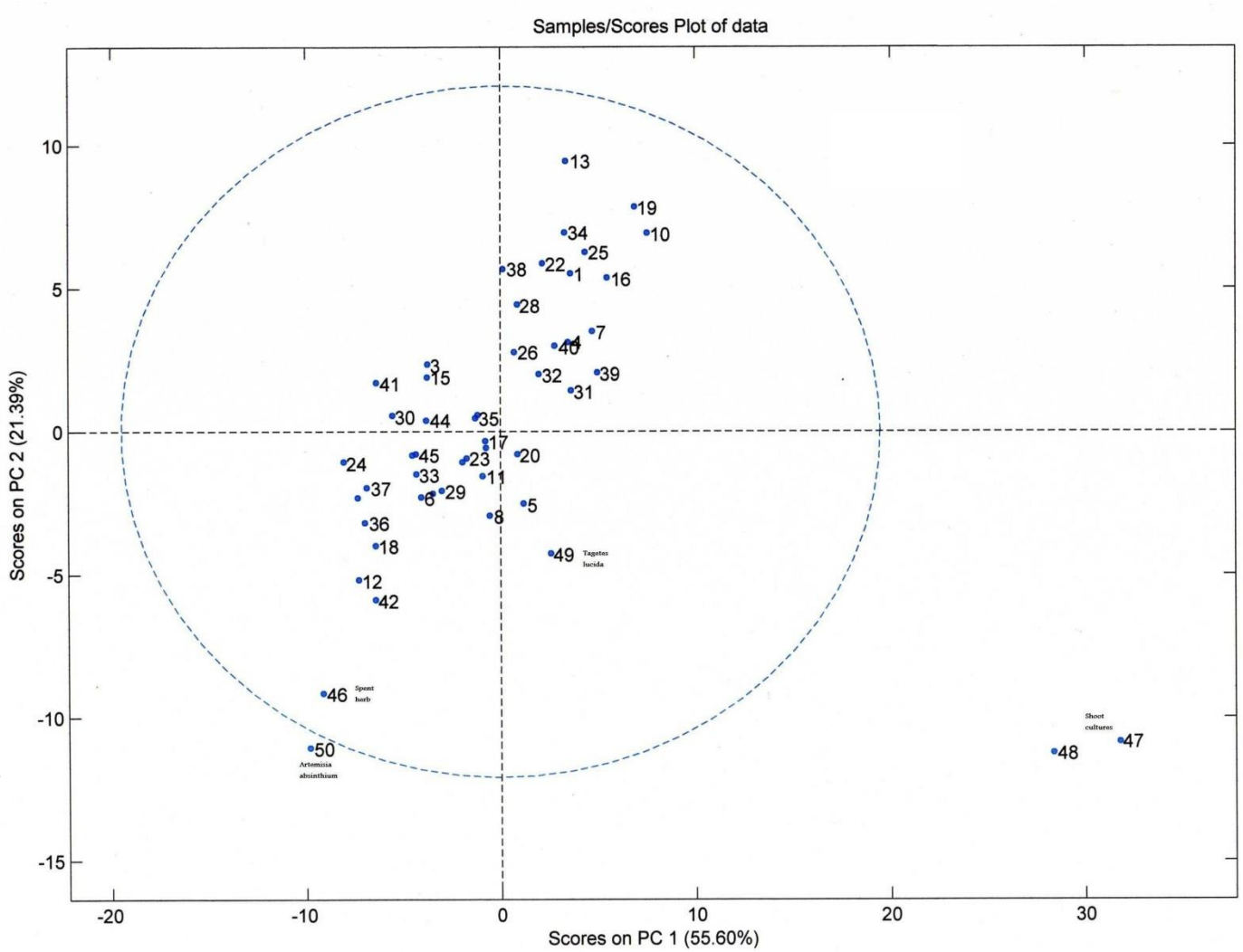


Figure 26. Scores plot 1. Scores plot of samples on PC1 and PC2

Section 4: NMR-based PCA and PLS-DA of crude *Artemisia annua* extracts

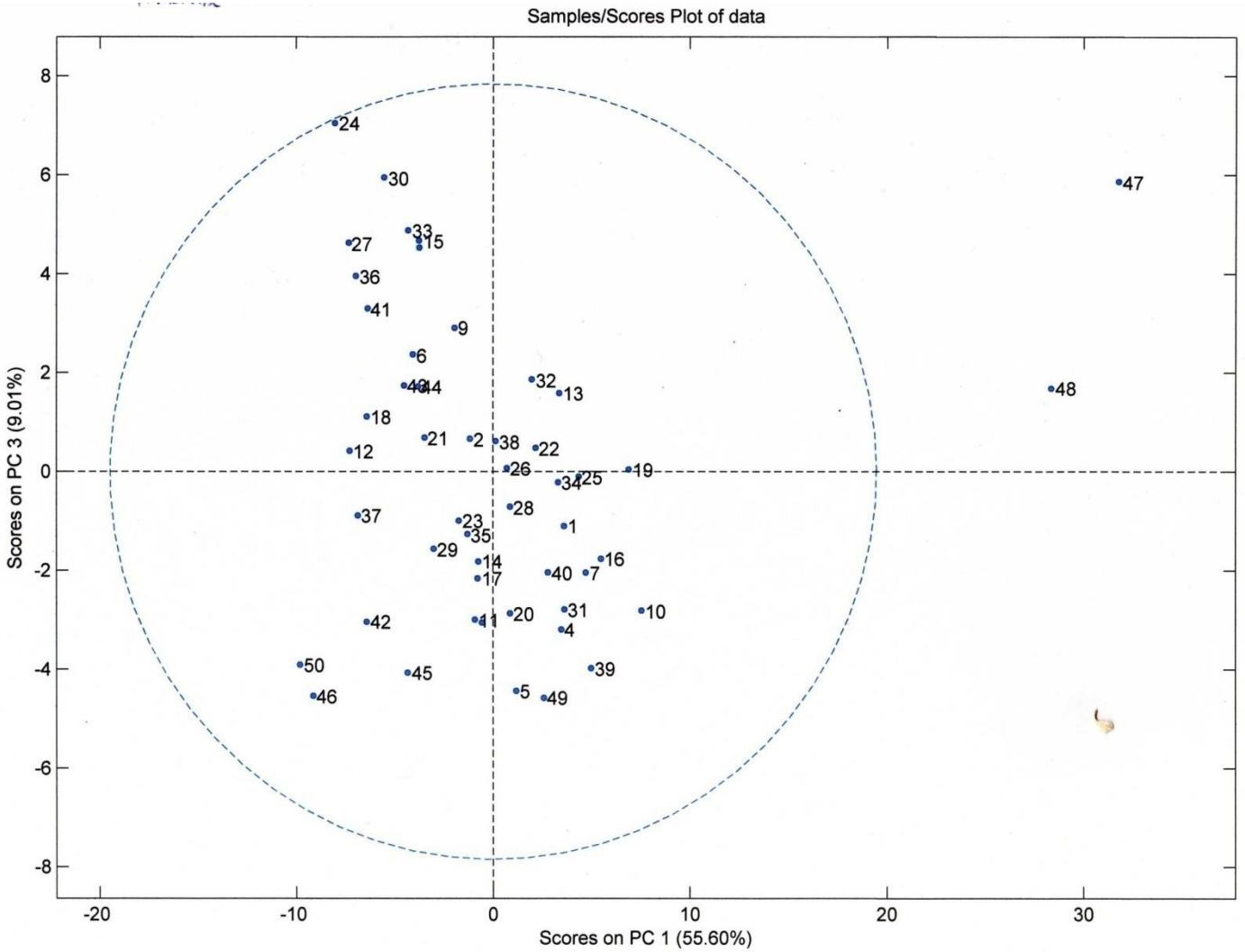


Figure 27. Scores plot 2. Scores plot of samples on PC1 and PC3

Section 4: NMR-based PCA and PLS-DA of crude *Artemisia annua* extracts

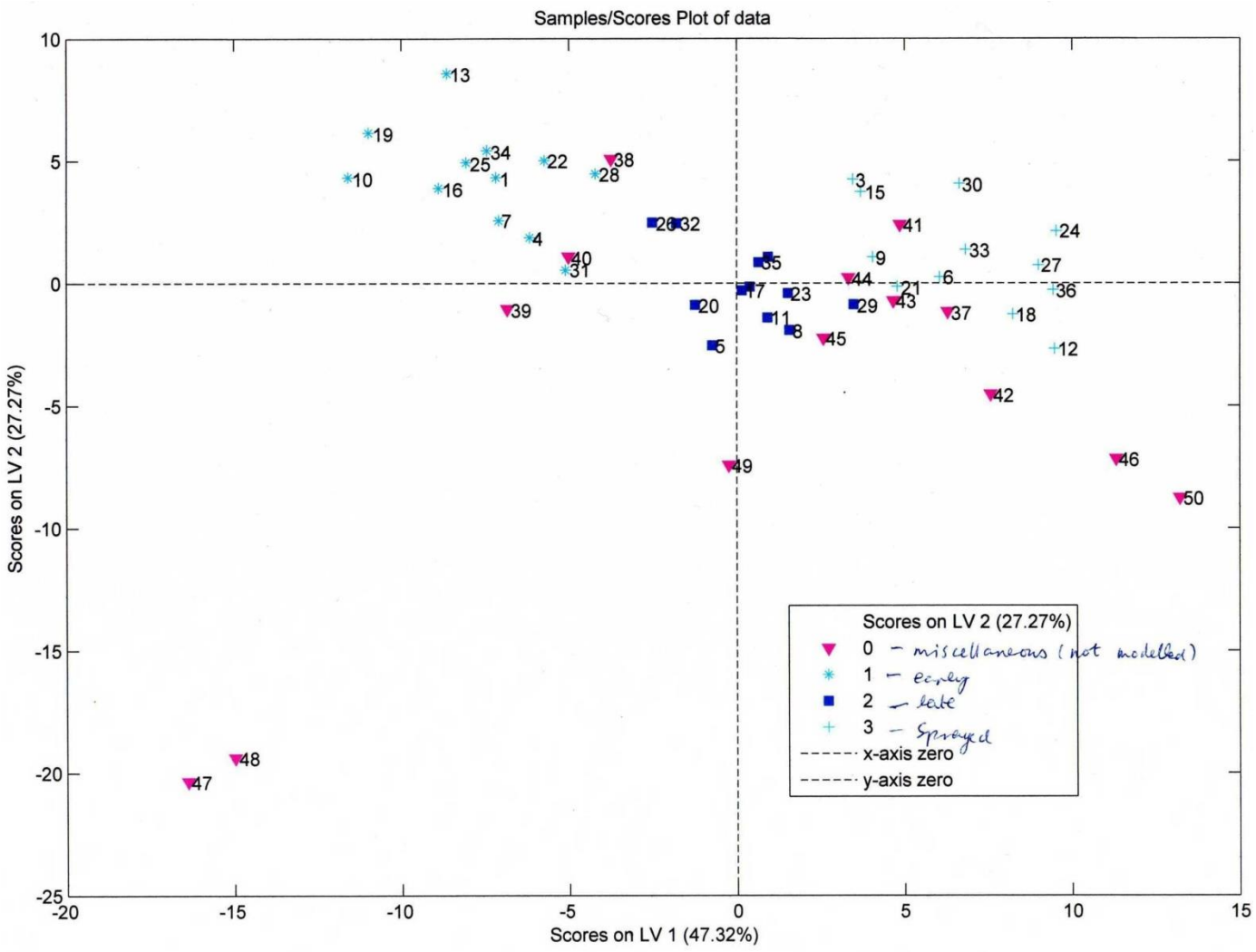


Figure 28. Partial least squares-discriminant analysis scores plot of samples on latent variable (LV) 1 and LV2

This was a short experiment, designed to test whether, in principle, NMR-based multivariate analysis would be of use in the quality control of *Artemisia annua* field samples.

Initially, it was observed that simply comparing the relative heights of the artemisinin signals in the raw NMR spectra, with no data manipulation, could clearly distinguish material of different quality. Figure 24 shows raw NMR data for pure artemisinin, and then three samples of differing artemisinin content, ranging from 0.8% w/w to 1.2% w/w. Differences in the height of the 1.4 ppm signal, which arises from the H-15 methyl group, are apparent, and the height correlates positively to increasing artemisinin level. Raw NMR data could also immediately spot a poor sample, as Figure 25, showing spent herb (that which has been treated to remove artemisinin) shows. It is clear that in the upper two spectra of this image – representing spent herb – the artemisinin signal at 1.4 ppm is absent. However, it is unclear that this method would be able to discriminate fine grades of artemisinin content – whether a sample containing artemisinin at 1.4 % w/w could be distinguished from another sample at 1.6 % w/w, for example.

In addition, if using raw NMR spectra to quantify artemisinin in sample, it is important to ensure that the signal chosen for quantification are indeed exclusive to artemisinin, and not shared by artemisinin and deoxyartemisinin. Castilho et al., (Castilho et al., 2008) for example, used the 1.44 and 5.86 ppm signals to quantify artemisinin– signals which arise from the H-15 methyl and H-5 proton respectively, and, as Section 7.3 discusses, do differ slightly from the deoxyartemisinin signals.

4.3.1 Principal components analysis (PCA)

Initially, samples were assayed using principal components analysis (PCA), an unsupervised clustering technique in which multiple variables are combined into a smaller number of principal components – in this case, three.

PCA involves the rotation of graphical axes on a multi-dimensional graph. Axis one – or PC 1 – is the axis along the length of which most variation in samples can be observed. PC2 is the axis along which the second greatest degree of variation can be observed, and so on.

PCA typically generates scores plots and loadings plots. A scores plot shows the co-ordinates of each sample on the axis system of the PCs, and looks like any scatter plot, in that related samples will cluster together. A loadings plot gives information as to which original variables – in NMR, which ppm values – are responsible for the differences between samples.

In this short experiment, only scores plots were generated. But clustering of samples can be observed. If Scores Plot 1 (Figure 26) is considered, one thing becomes immediately apparent: that a positive correlation exists between PC1 and PC2. Samples with a relatively high figure for PC1 also have a fairly high figure for PC2. However, this tells us nothing about the quality of the samples in terms of artemisinin content: although most of the samples fall into an oblong cluster (from the lower left to the upper right of the plot, the samples within that cluster appear randomly distributed in artemisinin content. Clearly, something else is being correlated here – perhaps related to the age of the material, as the upper right quadrant contains mainly early-harvested material.

Scores plot 2 (Figure 27) is more interesting. PC1 is still on the x-axis, but the y-axis is now PC3. This time, an inverse correlation is observed: as a sample's correlation to PC1 decreases, its PC3 value increases.

On first glance, it appeared that PC3 had pulled out high-artemisinin material: most of the samples in the upper left quadrant are high (>1.3 % w/w) in artemisinin, and most in the lower right quadrant are relatively low (<0.9 % w/w) – in fact, these lower-right quadrant samples are mostly early-harvested material.

But upon closer inspection, it was noticed that, in fact, almost all the samples in the upper left quadrant are actually those samples that were Glyphosate-treated. In addition, the lower left and right quadrants – apparently containing samples of lower artemisinin yield – do contain a number of high-yielding samples, but those samples were not Glyphosate-treated. So it seems that PC3 relates, somehow, to this post-harvest treatment, rather than directly to artemisinin content.

So a foolproof way of using PCA to pull out high-yielders has not been achieved in this limited study – perhaps because the extraction solvent used (70% deuterated methanol) is not ideal

for the extraction of artemisinin, and is likely to extract more polar compounds such as glycosides. But what both scores plots do show is that the wild cards were easily identified: samples 46 and 50 on both plots were "spent herb" – wherein the plant material had been pre-treated to remove the artemisinin – and a sample of *A. absinthium*, respectively. Both these samples lie far outside the clusters, using all three PCs. But is should also be noted that a sample of *Tagetes lucida*, a Mexican marigold with some chemical similarity to *A. annua*, and indicated on the plots as sample 49, did not fall outside the general cluster, whereas two samples of *A. annua* shoot cultures – samples 47 and 48 – did. If loading plots had been generated, the reasons for these anomalies may have become apparent. In a metabolomic study of the quality of *Artemisia annua* plant extracts, Van der Kooy et al. (2008) found, using a loadings plot, that the signals 0.99, 1.21 and 1.44 (representing the three methyl-hydrogens of artemisinin: see Section 5.1.2.3) provided most of the effect of PC1 in their analysis (van der Kooy et al., 2008), but, because of the lack of affinity of artemisinin for the extraction solvent used, this is unlikely to be the case in this study.

4.3.2 Partial least squares discriminant analysis (PLS-DA)

On the PLS-DA plot (Figure 28), an example of supervised clustering can be seen. The software was told of three major groupings (early harvest, late harvests, and late glyphosate-treated harvests). Additionally, a number of samples were left "unmodelled" – that is, the software was not told to which groups these samples belonged. This latter is to act as a means of validation – as PLS-DA does have a tendency to result in false-positive groupings if unvalidated.

The x-axis shows LV1, and the y-axis, LV2. The three sample groupings – early harvests, late harvests and late glyphosate-treated harvests – are distinctly clustered. But the unmodelled samples do not appear to have strong relationships to the clusters into which they have been arranged. Samples 38, 39 and 40 have all fallen into the "early-harvest" group, even though these were all harvested late in the season. It should be noted, though, that these latter three were samples from season 2007, not 2008, unlike the other modelled samples in the cluster. Additionally, samples 45 and 46 were duplicate samples, containing almost no artemisinin, and yet despite being duplicates, are separated by this algorithm.

However, it is notable that samples that are clearly not clustered – 47, 48, 49 and 50 – were very different from all other samples in nature. Samples 47 and 48 represent shoot cultures of

Artemisia annua, which contained very little artemisinin. Samples 49 and 50 were samples of *Tagetes lucida* and *Artemisia absinthium* respectively.

So, although some discrimination was possible using PLS-DA, it is clear than more validation is needed before this technique could be used to confidently assay *Artemisia annua* field samples.

4.3.3 Summary of multivariate analysis

Multivariate analysis looks at the full range of components in a given sample, and compares this to the full range of components in any number of other samples. There are reasons why this would be useful, and they are mainly to do with quality control and sample identification. Samples of *Artemisia annua* with similar biological properties – all harvested in Cambridge in July 2008, for example, or all high in artemisinin – should have a fairly similar metabolic profile, and therefore outliers will be immediately apparent.

But, importantly, it was found that this technique is not faster than LCMS-based quantisation. In fact, each sample needed 10 minutes per NMR scan, which is the same length of time that the LCMS needs per sample - and NMR is much less sensitive than LCMS, so more compound is required to give a signal. This, coupled with the complex interpretation, means that NMR-based multivariate analysis is unlikely to offer any great advantage for the simple quantification of artemisinin.

Where the method does come into its own, though, is in the classification of samples, and the identification of compounds that correlate either positively or negatively to artemisinin – information that may be useful for biosynthetic studies. The technique has also found application in the prediction of anti-plasmodial activity of *Artemisia annua* extracts, as a 2004 paper by Bailey et al. demonstrated. In Bailey's study, samples extracted in 1,1,1,2-tetrafluoroethane could be clustered, using PCA, according to their IC_{50} values for *P. falciparum* strain 3D7 (Bailey et al., 2004). Such studies demonstrate that NMR-based multivariate analysis is an area with great potential in the quality assessment of herbal material, although as it stands the technique is not yet suited to the purposes of this project.

4.4 Acknowledgements

The author would like to thank Ian Colquhoun and his team, at the Institute of Food Research at Norwich Research Park, for assistance with this research.

5 Separation of Artemisinin and Deoxyartemisinin by TLC

Synopsis

Deoxyartemisinin is a pharmaceutically inactive artemisinin degradation product. The compound occurs naturally in the plant, and can also be formed during storage of the leaf and extraction of *Artemisia annua* L. Deoxyartemisinin typically co-elutes with artemisinin in chromatographic systems, and, in TLC, can result in overestimation of artemisinin content. To date, no means of separating the two compounds by TLC have been reported.

This section reports on a new TLC system which can separate the two compounds, thus increasing the accuracy of TLC-based artemisinin quantification.

5.1 Introduction

Although the artemisinin molecule is a fairly complex structure, its anti-plasmodial activity resides within a single moiety: the endoperoxide bridge. A change to this bridge – as occurs in the closely related compound deoxyartemisinin – renders the molecule useless for the treatment of malaria.

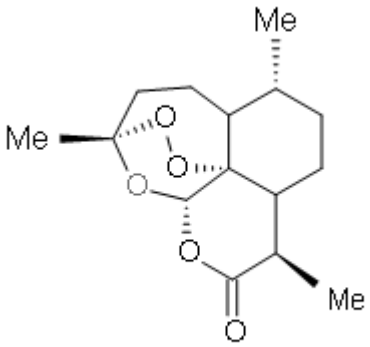


Figure 29. Artemisinin

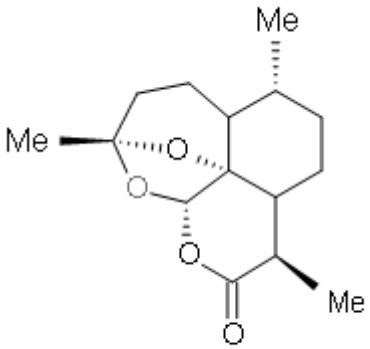


Figure 30. Deoxyartemisinin

Deoxyartemisinin is missing one of the oxygens from the endoperoxide bridge, resulting in an ether moiety (and a reduced melting range, from 152 – 156 °C for artemisinin to 110 °C for the deoxyartemisinin). Incidentally, deoxyartemisinin is not to be confused with the semi-synthetic deoxoartemisinin (McChesney, 1990) – the names may be similar, but deoxoartemisinin has lost the oxygen from the lower ring, not the endoperoxide, and so retains its anti-plasmodial action[4]. The generation of deoxyartemisinin within the leaf is controversial. It is known that incubation of artemisinin with microbial cultures can result in the metabolism of artemisinin to deoxyartemisinin (Lee et al., 1989). So it could be that the deoxy-form occurs as a result of microbial colonisation of the plant in the field. It may also be triggered by the presence of iron within the plant - incubation of artemisinin with ferrous iron ions has been demonstrated to result in a degradation to deoxyartemisinin (Creek et al., 2005).

Investigations of UK-grown *A. annua* by the author have shown that deoxyartemisinin is present in dried plant material at levels averaging 5% of the artemisinin level. African grown material, again investigated by the author, typically shows levels of about 8% of the artemisinin level – even when the material is of the same genetic stock as UK-grown material. It has been hypothesised that the iron-rich soils of Africa are the cause of this relative increase in deoxyartemisinin.

[4] Similarly, deoxoartemisinin is not to be confused with 3-hydroxydeoxoartemisinin. The latter has lost an oxygen from the endoperoxide bridge, and hence has no anti-plasmodial activity – see Section 10.5.1.2.1

But whatever the cause, its presence is a problem. In many chromatographic systems, deoxyartemisinin co-elutes with artemisinin, as reported by Christen and Veuthey (Christen and Veuthey, 2001): “deoxyartemisinin and [artemisinin] are not separated at all and therefore [artemisinin] is systematically overestimated” in HPLC-DAD and TLC. This does, inevitably, result in an over-estimation of artemisinin content of the sample in question.

Although the use of single ion monitoring (SIM) in LCMS does get around this problem, this is of little help to the many laboratories and field scientists who have no access to such an expensive piece of kit. Ideally, a simple and inexpensive means of separating the two compounds is needed. The obvious solution is thin layer chromatography.

5.2 Materials and Methods

5.2.1 LCMS for quantification of artemisinin and deoxyartemisinin

5.2.1.1 Reference standards

Reference artemisinin was provided by AECS, and found to have a melting range of 154 – 156 °C, and a purity of > 98%. Identity of artemisinin was confirmed by electrospray mass spectrometry and 1H nuclear magnetic resonance on a Bruker 400-mHz Ultrashield machine.

5.2.1.2 HPLC-MS Conditions

Artemisinin was identified using an Agilent Technologies 1100 Series high-performance liquid chromatography system, with a GraceSmart RP18-HPLC column (150 mm x 2.1 mm; pore size of 3 mm), coupled to an Agilent Technologies G1946 single quadruple mass spectrometer with electrospray ionization (EI), used in positive ion mode, with single ion monitoring (SIM). HPLC separation was achieved with an isocratic mobile phase of 60% acetonitrile and 40% HPLC-grade water at a flow rate of 0.2ml/minute, with a run time of 8 minutes. The column oven was set at 25°C.

The EI spray chamber and mass spectrometer parameters were as follows: nitrogen flow at 8 L^3/minute; drying gas temperature 350°C; capillary voltage 2800 V in positive mode, 3500 V in negative mode; nebulizer pressure 40 psig; and a fragmentor voltage of 70 V. The mass

spectrometer was calibrated and, if necessary, tuned daily, using Agilent's Electrospray Tuning Mix, to ensure peak performance.

5.2.1.2.1 Generation of artemisinin calibration curve

Santonin was used as an internal standard, with ions monitored at 173.3 and 247.3. The monitored artemisinin ions were 283.3 and 209.3.

A calibration curve was generated, in which artemisinin concentration ranged from 2 to 16 µg/ml, and in which santonin, as an internal standard, was kept constant at 10 µg/ml. All calibration levels were prepared in triplicate and analysed three times per sample. The ratio of artemisinin to santonin was calculated for each analysis; linear regression of the resulting scatter plot was used to establish the calibration curve. The upper limit of quantification was given as the highest level of the calibration curve: beyond this, saturation of response rendered quantification less reliable. The lower detection limit was taken as five times the standard deviation of the measured response to the lowest calibration level, and was calculated to be 0.8 ng.

5.2.1.2.2 Generation of deoxyartemisinin calibration curve

Santonin was used as an internal standard, with ions monitored at 173.3 and 247.3. The monitored deoxyartemisinin ions were 203. 2 and 267.2.

A calibration curve was generated, in which deoxyartemisinin concentration ranged from 2.5 to 15 µg/ml, and in which santonin, as an internal standard, was kept constant at 10 µg/ml. All calibration levels were prepared in triplicate and analysed three times per sample. The ratio of deoxyartemisinin to santonin was calculated for each analysis; linear regression of the resulting scatter plot was used to establish the calibration curve. The upper limit of quantification was given as the highest level of the calibration curve: beyond this, saturation of response rendered quantification less reliable. The lower detection limit was taken as five times the standard deviation of the measured response to the lowest calibration level, and was calculated to be 0.8 ng.

5.2.2 TLC separation of artemisinin and deoxyartemisinin

Artemisinin was purified from a crude extract, using counter current chromatography, by Les Brown of AECS QuickPrep. Deoxyartemisinin was a gift from Neil Sullivan at Sensapharm Ltd.

Silica plates (G260, 0.2 mm with fluorescent indicator) were purchased from Sigma. Solvents were purchased from De Montfort University Chemical Stores and purified by distillation. Spots were visualised with a vanillin-based spray reagent, prepared by dissolving 2g vanillin in 100 ml of ethanol, and acidifying with 2 ml of concentrated sulphuric acid. Plates were dipped in the solution for a few seconds, and then heated in an oven at 105°C for 2 minutes. Alternately, plates can be sprayed with the visualization reagent, but coverage can be less uniform than when the dipping procedure is used.

Hexane was taken as the base for the mobile phase, and a range of solvents – beginning with the very polar (isopropanol) and including ethyl acetate, dichloromethane, dioxane, butyl methyl ether and toluene – were added, either alone or in pairs, and in a range of volume/volume ratios, to build up a large number of 2- or 3-component mobile phases. Each of these phases was tested for its ability to separate a mixture of reference artemisinin and deoxyartemisinin.

5.3 Results

5.3.1 Deoxyartemisinin in UK and African-grown material

Table 11. Deoxyartemisinin content of UK-grown Consortium material (1435, 1437, 1440, 1nd 1442), Moroccan-grown UK material (Moroccan A and Moroccan 1), Ghanaian-grown Ghanaian material (Ghana 1 and 2), and Tanzanian-grown Anamed material (Anamed 1 and Anamed 2)

Sample	Artemisinin % w/w	Deoxyartemisinin % w/w	Deoxyartemisinin as a percentage of artemisinin
1435 (2007)	1.4	0.08	5.7
1437(2007)	1.6	0.08	5.0
1440(2007)	1.4	0.09	6.4
1442(2007)	1.4	0.09	6.4
Moroccan A	0.4	0.03	7.5
Moroccan I	0.5	0.04	8.0
Ghana 1	0.5	0.07	14
Ghana 2	0.5	0.09	18
Anamed 1	0.8	0.07	8.8
Anamed 2	0.8	0.06	7.5

Table 11 shows that African-grown *Artemisia annua* L. contains a higher proportion of deoxyartemisinin when compared to UK-grown material. This is illustrated by the fact that UK-grown samples 1435, 1437, 1440 and 1442, and Moroccan samples A and 1, are actually the same variety of *Artemisia annua*.

5.3.2 Separation of deoxyartemisinin from artemisinin by TLC

After a great deal of trial and error, it was found that the solvent system consisting of hexane: butyl methyl ether: ethyl acetate, at the ratio 80: 15: 5, produced the optimal separation of the two compounds. Deoxyartemisinin elutes with a retention factor (R*f*) of 0.41, and artemisinin moves slightly slower, with an R*f* of 0.33. The two compounds respond slightly differently to the visualization reagent: deoxyartemisinin derivatises to a light green, whilst artemisinin is dark green or even blue, depending on the freshness of the visualization reagent (Figure 31).

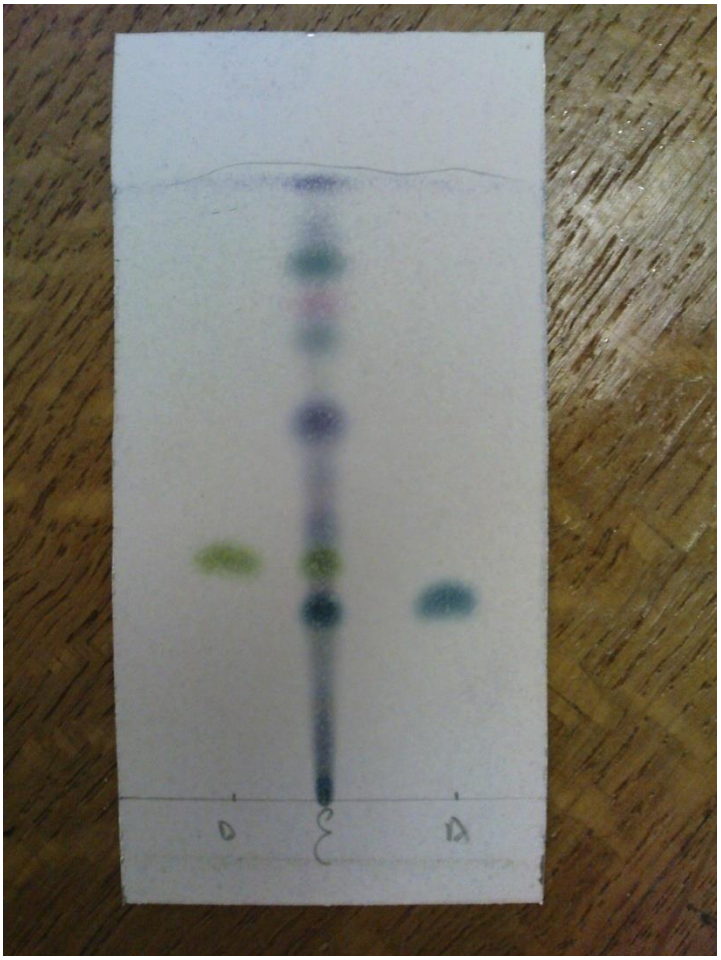


Figure 31. TLC separation of deoxyartemisinin from artemisinin. L-R: deoxyartemisinin, extract from a Tanzanian-grown variety of *A. annua*, and artemisinin.

5.4 Discussion

Despite the close similarity in structure – with deoxyartemisinin differing from artemisinin only by the lack of one oxygen atom – it has been possible to clearly separate the two spots by thin layer chromatography, using standard silica plates and commonplace laboratory solvents. Such a technique should prevent the overestimation of artemisinin content during TLC-based sample analysis – in particular, when extracts of African grown *Artemisia annua* L (in which deoxyartemisinin levels can reach 18% w/w) are analysed.

6 Artemisinin quantification by TLC densitometry

Synopsis

There is a need for a simple, rapid means of *Artemisia annua* quality control that can be used at the site of crop growth, with a minimum of user training and outlay. This section describes the validation of TLC densitometry for such a purpose. Unlike other TLC-densitometric methods in the literature, the method reported here made use of a simple desktop scanner and inexpensive (costing less than £200) Quantiscan® densitometric scanning software.

The TLC-densitometric technique was found, when used carefully, to be comparable in accuracy to LCMS – but the potential for error is greater compared to LCMS. Nonetheless, when used correctly, TLC densitometry with Quantiscan® software is a realistic means of *Artemisia annua* quality control for those without access to the laboratory.

6.1 Introduction

The need for a field-based means of artemisinin quantification, relatively inexpensive and simple to use whilst retaining high levels of reproducibility and accuracy, is apparent from the fact that *Artemisia annua* L. is often cultivated on a small scale in remote areas that are far from the nearest laboratory. Besides this, even those growers with access to a QA laboratory may wish to perform frequent assays throughout a growth season, in order to determine the ideal time for harvest, without sending numerous samples through to a perhaps expensive external assessment.

A portable lateral flow device for field-based artemisinin quantification has been developed by a UK company, and is soon to be brought to market. But such a system is not cheap, and there is room for other, perhaps less complex, means of quantification. One such technique is thin layer chromatography (TLC) coupled with a densitometric scanner – or TLC-densitometry, for short.

Thin-layer chromatography, as applied to artemisinin quantification, is suitable for the semi-quantitative analysis of samples of *Artemisia annua*, but has its limitations. Primary amongst these is that although the human eye alone, looking at a developed and visualised TLC plate, can discriminate samples containing high levels of artemisinin from those containing low levels, the eye cannot easily discern between samples displaying small differences. In quantitative terms, the eye cannot discern accurately between samples containing 1.2 % w/w artemisinin, for example, as compared to 1.4% w/w.

However, the use of densitometric scanning software can overcome such difficulties of human subjectivity. In these types of device, a developed and visualised TLC plate is scanned by a plate reader, and areas of densest staining – indicating the presence of extracted compounds - are converted into numerical values, which will naturally be of greater of lesser magnitude depending on the intensity of the spot. In this way, a numerical value is produced for each stained spot on the plate, and that value, in theory, correlates positively to the quantity of compound present.

Densitometric scanners (usually with ultra-violet imaging) are commercially available – they are used routinely in the reading of electrophoresis gels. TLC densitometry also finds a place in the quantification of natural products (Bodoki et al., 2005) and pharmaceuticals – in 2007, Tayade and Nagarsenker validated the technique for the quantification of artemether in pharmaceutical formulations (Tayade and Nagarsenker, 2007). But the devices are typically bulky (and hence not portable), and also expensive – the Camag TLC Scanner III, used by Tayade and Nagasenker, retails at over €2000, even second-hand.

For this project, Quantiscan® densitometric software was purchased from Biosoft® - this is a quantitative scanning program that currently retails at less than £200, and runs using any desktop scanner. The software enables the scanner, with a standard PC, to be used for quantitative densitometric analysis of developed TLC plates.

To monitor the precision and reproducibility of this new technique, 218 samples were analysed both by TLC densitometry, and by HPLC-MS.

6.2 Materials and Methods

6.2.1 Extraction and analytical procedure – LCMS

Extraction: Dried *Artemisia annua* leaf material was powdered using a pestle and mortar, and macerated in acetonitrile under dark conditions, with gentle shaking, for 24 hours at a 1/10 weight-to-volume ratio.

Sample Preparation: Analytical samples were prepared by the dilution of 25 µl of original extract in 875 µl of acetonitrile, with addition of 100 µl of santonin stock solution at 0.1 mg/ml.

LCMS Parameters: After this time, artemisinin in samples was quantified using an Agilent Technologies 1100 Series high-performance liquid chromatography system, with a GraceSmart RP18-HPLC column (150 mm x 2.1 mm; particle size of 3 µm), coupled to an Agilent Technologies G1946 single quadruple mass spectrometer with electrospray ionization (ESI), used in positive ion mode, with single ion monitoring (SIM). HPLC separation was achieved with an isocratic mobile phase of 60% acetonitrile and 40% HPLC-grade water at a flow rate of 0.2 cm^3, with a run time of 8 minutes. The column oven was set at 25°C.

The EI spray chamber and mass spectrometer parameters were as follows: nitrogen flow at 8 L^3/minute; drying gas temperature 350°C; capillary voltage 2800 V in positive mode, 3500 V in negative mode; nebulizer pressure 40 psig; and a fragmentor voltage of 70 V. The mass spectrometer was calibrated and, if necessary, tuned daily, using Agilent's Electrospray Tuning Mix, to ensure peak performance.

Generation of calibration curve. Santonin was used as an internal standard, with ions monitored at 173.3 and 247.3. The monitored artemisinin ions were 283.3 and 209.3. A calibration curve was generated, in which artemisinin concentration ranged from 2 to 16 µg/ml, and in which santonin, as an internal standard, was kept constant at 10 µg/ml. All calibration levels were prepared in triplicate and analysed three times per sample. The ratio of artemisinin to santonin was calculated for each analysis; linear regression of the resulting scatter plot was used to establish the calibration curve. The upper limit of quantification was given as the highest level of the calibration curve: beyond this, saturation of response rendered quantification less reliable. The lower detection limit was taken as five

times the standard deviation of the measured response to the lowest calibration level, and was calculated to be 0.8 ng.

6.2.2 Extraction and analytical procedures: TLC-densitometry

Quantiscan® Scanning Software. The *Quantiscan® for Windows* software was purchased from Biosoft**,** PO Box 1013, Great Shelford, Cambridge, CB22 5WQ, UK.

Extraction and sample preparation. Dried *Artemisia annua* leaf material was powdered using a pestle and mortar, and macerated in acetonitrile under dark conditions, with gentle shaking, for 24 hours at a 1/40 weight-to-volume ratio. After this time, 5µl of extract were loaded as a single spot onto the baseline of the plate. Analytical samples were bracketed with reference samples at 0.2 and 1 mg/ml.

Generation of calibration curve. Reference samples of artemisinin in acetonitrile - ranging from 0.05 mg/ml to 1 mg/ml - were prepared in quadruplicate Using these, four TLC plates were prepared, on which 5 µl of each calibration level was applied as a single spot. 1.5 cm were allowed between spots. Plates were developed as described below.

TLC development and visualisation conditions. Silica gel plates (aluminium backed; 60A, 0.2 mm thickness with F254 fluorescent indicator) were purchased from Fisher. Plates were kept under desiccation until use. Plates were developed using a mobile phase prepared from diethyl ether: hexane (7:3). After development, plates were dried naturally for 5 minutes, and then dipped for 5 seconds in a 2 % v/v solution of vanillin in acidified ethanol (prepared by adding 2g vanillin to 98 ml of ethanol, and then adding – over ice – 2 ml of concentrated sulphuric acid). Plates were heated in a fan oven set to 105°C for precisely 120 seconds. Plates were removed and allowed to stand for 15 minutes before reading.

TLC Scanning Conditions. Biosoft's Quantiscan® software package (illustrated in Figure 34)was used with a Mustek desktop scanner to calculate artemisinin in analytical samples, based on the calibration curve.

6.3 Results

6.3.1 Calibration curve of artemisinin by TLC densitometry

The calibration curve for artemisinin was linear (*y = 2552x 161.6*, R^2: 0.990) in the concentration range 0.05 – 0.4 mg/ml, where 5 µl of reference solution are spotted onto the plate (Figures 32 and 33).

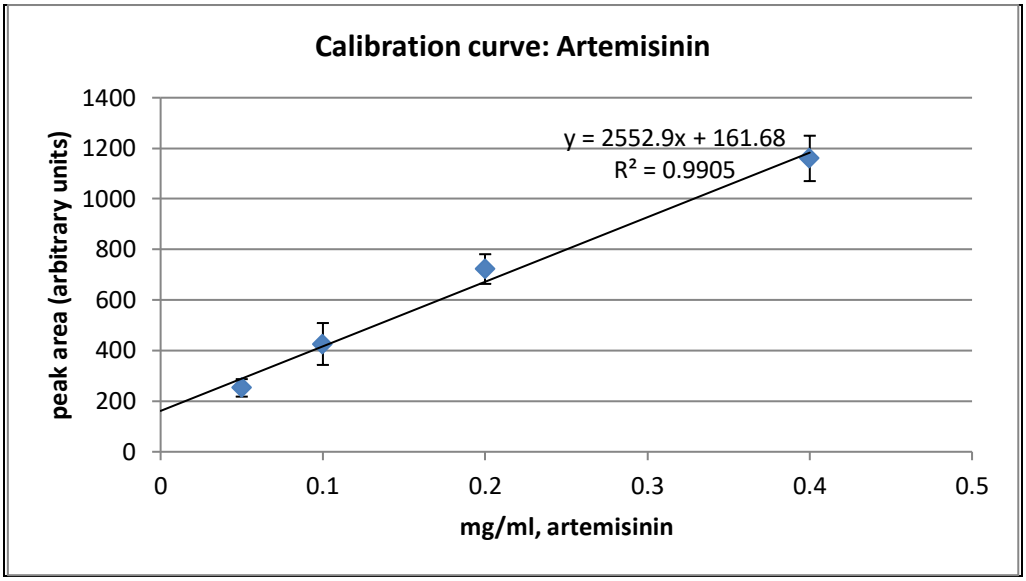


Figure 32. Calibration curve of artemisinin by TLC-densitometry

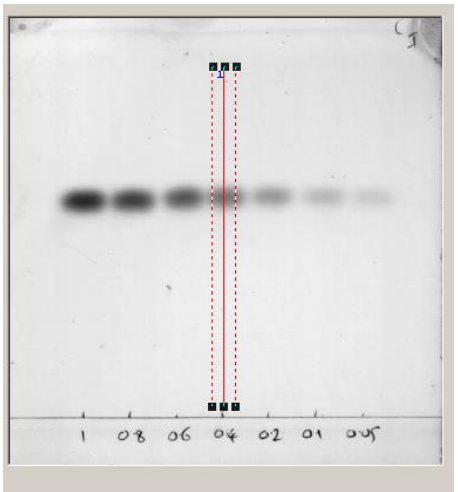


Figure 33. Calibration of artemisinin by TLC densitometry. Samples range from (L-R) 1 - 0.05 mg/ml artemisinin, where 5 µl were spotted onto plate. Plate scanned and imaged using Biosoft® software.

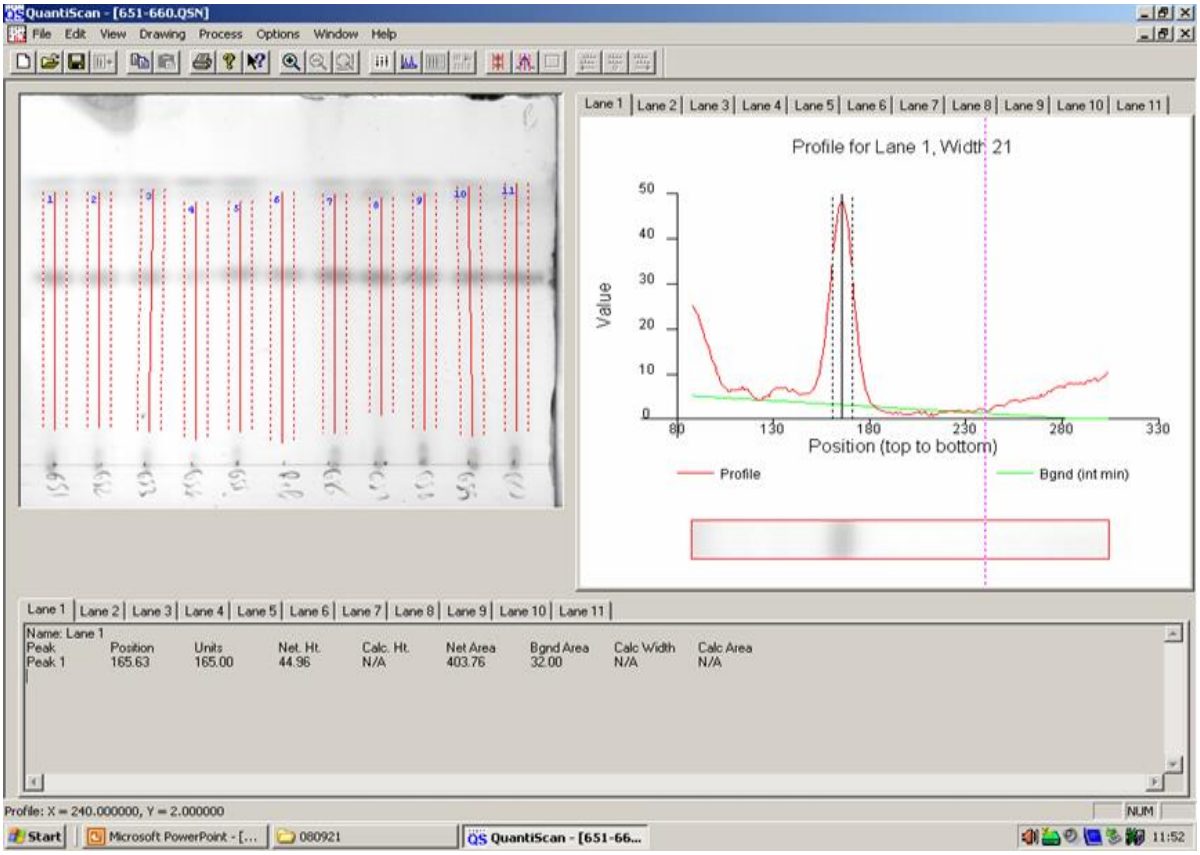


Figure 34. Example of data obtained from Biosoft program. Top left image: scanned TLC plate showing extracts of *A. annua*, with artemisinin spot approximately two-thirds of the way up the plate. Top right image: a profile showing spot density (y-axis). The lower table gives the numerical data for spot density.

6.3.2 Sample analysis

Table 12 shows the data (artemisinin as % w/w) for 218 samples, as assayed by TLC-densitometry and LCMS.

Table 12. Comparison of data obtained on 218 samples of dried *A. annua* by TLC-densitometry and LCMS. All data represents artemisinin % w/w

Sample	TLC	MS	Sample	TLC	MS	Sample	TLC	MS	Sample	TLC	MS	Sample	TLC	MS
1	1.0	0.9	51	0.2	0.7	101	0.7	0.8	151	0.9	0.7	201	1.1	0.7
2	0.5	0.4	52	0.3	0.8	102	0.7	0.7	152	0.9	0.8	202	1.1	0.9
3	0.8	0.7	53	0.4	0.9	103	0.8	0.9	153	0.8	0.6	203	1.2	0.8
4	0.7	0.7	54	0.3	0.6	104	0.7	0.9	154	0.8	0.7	204	1.2	1.1
5	0.8	0.8	55	0.4	0.7	105	0.8	1.4	155	0.9	0.9	205	1.3	1.3
6	0.8	0.8	56	0.4	0.9	106	0.6	0.7	156	1.0	1.1	206	1.1	0.9
7	1.1	1.0	57	0.4	0.8	107	0.8	0.9	157	0.8	0.8	207	0.8	0.8
8	0.4	0.3	58	0.5	1.0	108	0.8	0.9	158	1.3	0.9	208	0.8	0.9
9	0.7	0.5	59	0.3	0.8	109	0.8	0.7	159	1.5	1.0	209	1.3	0.7
10	0.8	0.8	60	0.5	1.1	110	1.0	0.9	160	1.1	0.7	210	1.4	0.8

11	0.9	0.6	61	0.6	0.7	111	0.7	0.7	161	1.1	0.9	211	1.1	1.1
12	0.5	0.4	62	0.8	1.0	112	0.7	0.8	162	1.2	0.8	212	0.7	0.7
13	1.2	0.9	63	0.8	0.8	113	0.7	0.8	163	1.2	1.1	213	0.7	0.7
14	1.1	0.9	64	0.7	0.8	114	0.9	1.0	164	1.3	1.3	214	0.9	0.8
15	1.2	1.2	65	0.7	1.0	115	0.7	0.7	165	1.1	0.9	215	1.1	0.9
16	0.6	0.6	66	0.5	0.8	116	0.6	0.6	166	0.8	0.8	216	1.3	1.1
17	0.8	0.9	67	0.6	0.8	117	1.1	1.2	167	0.8	0.9	217	1.1	0.8
18	1.0	1.1	68	0.4	0.6	118	1.1	0.9	168	1.3	0.7	218	1.5	1.0
19	0.8	0.7	69	0.5	0.9	119	0.8	0.9	169	1.4	0.8			
20	0.7	0.6	70	0.9	0.9	120	0.7	0.8	170	1.1	1.1			
21	1.0	0.8	71	0.8	0.8	121	0.6	0.7	171	0.7	0.7			
22	1.0	0.8	72	1.0	1.2	122	0.4	0.4	172	0.7	0.7			
23	1.0	1.1	73	0.8	0.9	123	0.7	0.6	173	0.9	0.8			
24	0.8	0.8	74	0.5	0.6	124	1.0	0.9	174	1.1	0.9			
25	1.0	0.9	75	0.5	0.5	125	1.1	1.1	175	1.3	1.1			
26	0.9	0.6	76	0.8	0.8	126	0.9	0.9	176	1.1	0.8			
27	0.7	0.7	77	0.7	0.7	127	0.8	0.9	177	1.5	1.0			
28	1.4	1.2	78	0.9	0.8	128	0.9	1.0	178	1.0	0.9			
29	1.3	1.4	79	0.7	0.7	129	1.0	0.9	179	0.8	0.6			
30	0.7	1.1	80	1.0	0.9	130	0.6	0.5	180	1.3	1.1			
31	0.7	0.7	81	0.9	0.8	131	0.8	0.9	181	1.0	0.7			
32	0.5	0.5	82	1.0	1.1	132	0.8	0.8	182	1.1	0.7			
33	0.7	0.7	83	0.7	0.6	133	0.7	0.6	183	1.0	0.8			
34	0.7	0.7	84	0.9	0.9	134	1.1	1.1	184	1.0	1.0			
35	0.7	0.7	85	0.8	0.8	135	0.9	0.9	185	0.9	1.0			
36	0.8	0.8	86	0.7	0.7	136	1.1	1.0	186	0.8	0.8			
37	0.7	0.8	87	0.8	0.7	137	1.0	0.9	187	1.0	1.2			
38	0.5	0.6	88	0.7	0.7	138	0.8	0.6	188	1.2	1.1			
39	0.7	0.7	89	0.8	0.8	139	1.3	1.1	189	0.9	0.7			
40	0.8	1.1	90	0.7	0.7	140	1.0	0.7	190	0.8	0.7			
41	0.9	0.8	91	0.8	0.9	141	1.1	0.7	191	0.9	0.8			
42	0.7	0.7	92	0.7	0.8	142	1.0	0.8	192	0.9	0.7			
43	0.7	0.7	93	0.8	1.0	143	1.0	1.0	193	0.9	0.8			
44	0.9	0.9	94	0.6	0.7	144	0.9	1.0	194	0.8	0.6			
45	0.7	0.6	95	0.9	1.1	145	0.8	0.8	195	0.8	0.7			
46	0.8	0.9	96	0.7	0.9	146	1.0	1.2	196	0.9	0.9			
47	0.8	0.8	97	0.5	0.7	147	1.2	1.1	197	1.0	1.1			
48	0.9	1.0	98	0.7	1.0	148	0.9	0.7	198	0.8	0.8			
49	0.8	0.8	99	0.5	0.8	149	0.8	0.7	199	1.3	0.9			
50	0.7	1.0	100	0.9	0.9	150	0.9	0.8	200	1.5	1.0			

An examination of the degree of difference in reported artemisinin content between LCMS and TLC-densitometric assays showed that in 26 cases, reported artemisinin content by TLC was underreported by between 10 and 19% compared to LCMS data. In 45 cases, reported artemisinin content by TLC was either the same as by LCMS, or underreported by up to 9%. In 42 samples, reported artemisinin content by TLC was between 1 and 10% higher than the

LCMS result, and in 37 samples, reported artemisinin content by TLC was between 11 and 20% higher than the LCMS result (Figure 35).

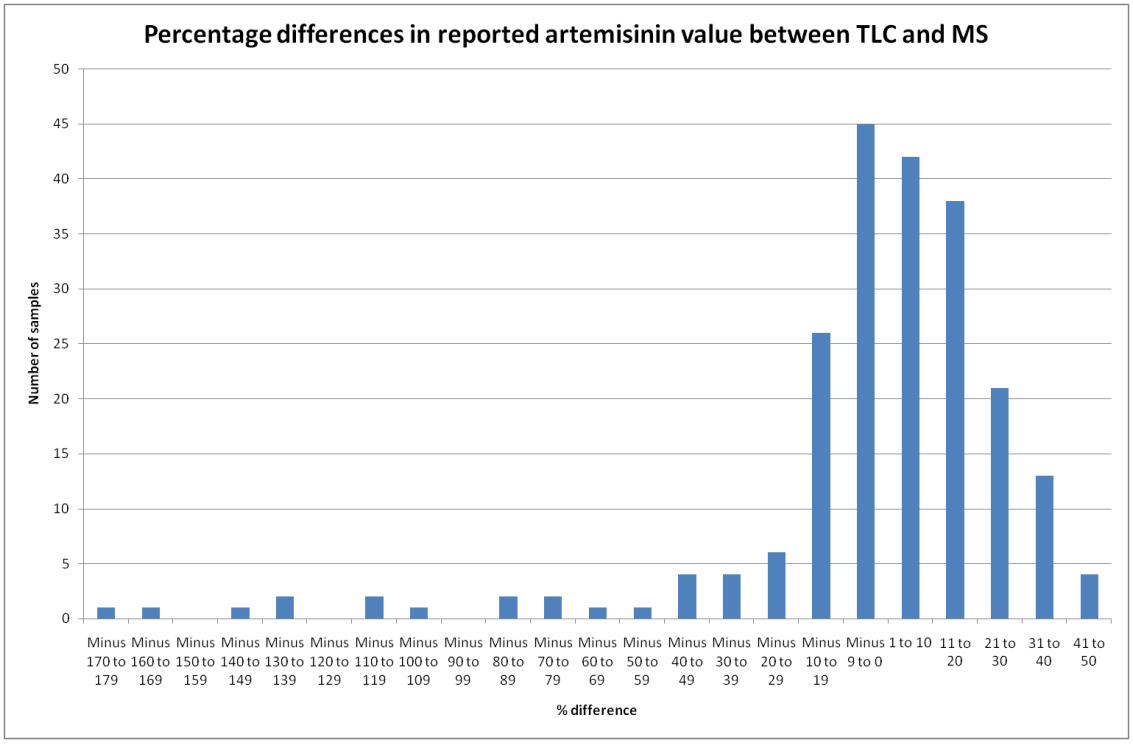


Figure 35. Differences in reported artemisinin content of 217 samples, expressed as (difference between TLC and MS value/TLC value) x 100.

However, a comparison of the mean values obtained by TLC-densitometry and LCMS – 0.86 and 0.84 respectively – shows that the two assay methods produce comparable results. Although the nature of the samples analysed – which were expected to range from low (0.6 % w/w) to high (1.2 % w/w) would naturally produce some variance from the mean, statistical analysis does show that the TLC-densitometry results show more variance than the MS results. In particular, the distribution of the differences between the two test methods shows that TLC can show large underestimations of the data (if we assume that the MS method was the "gold standard", and therefore more likely to be correct). But there was a strong positive correlation coefficient (0.7) between the data obtained from the two methods.

Using a paired t-test, a P-value of 0.054 was observed, indicating no significant difference between LCMS and TLC-densitometric data.

6.3.3 "Leaf-Spot" technique for sample application

A highly experimental, and as yet unvalidated, procedure for very quick quality control of fresh *Artemisia annua* was developed. In this technique, fresh leaves taken from the plant are pressed directly onto the TLC plate, with no extraction step. To ensure that the same area of leaf is pressed onto the TLC plate for each sample, a filter paper with identically-sized holes (cut using a hole-punch) is placed over the plate, and the leaf is pressed over the hole, as illustrated below. The TLC plate is then run and visualised as usual (Figures 36 – 41):

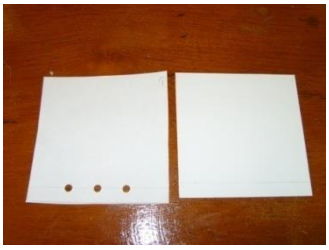


Figure 36. Left: filter paper with holes in base. Right: TLC plate.

Figure 39. Pressing on leaf to deposit trichome content onto TLC plate.

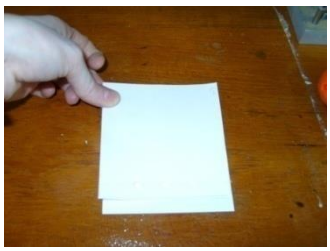


Figure 37. Placing filter paper over TLC plate.

Figure 40. Running plate as usual.

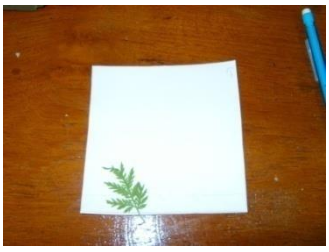


Figure 38. Placing fresh leaf over hole in filter paper.

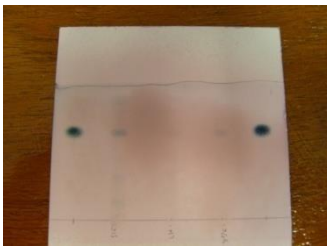


Figure 41. Developed plate. L-R: Artemisinin, 1015, 1012-12, 1046, Artemisinin.

Clearly, this method has limitations: it is only useful if all samples are taken from the sample region of the plant (e.g. the tips) at the same time, to ensure comparability. But the developed

TLC plate, below, indicates that the technique can discriminate between high- and low-quality plants. On this plate, the second spot from the left shows artemisinin from a high-quality plant 1015 (1.2% w/w DW); the third spot from the left is from a poor quality plant, 1012-12 (0.6% w/w DW), and the fourth from the left is from a plant of intermediate quality, 1046 (1.0 % w/w DW). Differences in intensity of artemisinin spot can be discerned.

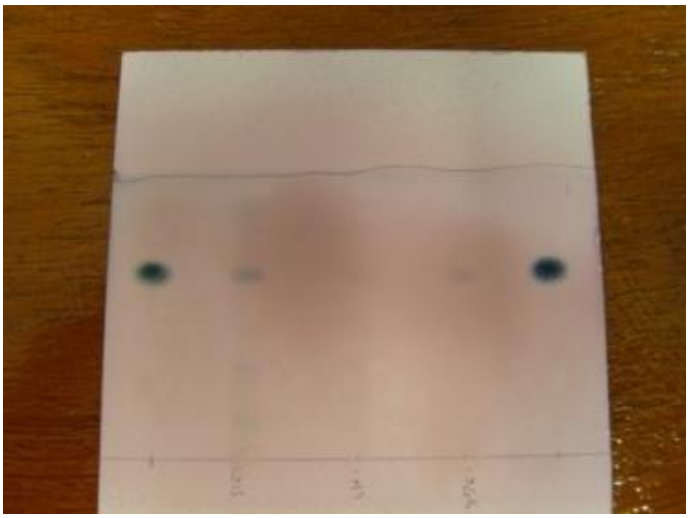


Figure 42. TLC plate comparing three varieties of *Artemisia annua* L. assayed using the LeafSpot technique

6.4 Discussion

This TLC-densitometric method has three major advantages for field laboratories; the two major advantages being the price of the software, and the fact that is can be used on any PC. It currently costs less than £200 – far cheaper than purchasing specialised densitometric apparatus. The third advantage is the rapidity and simplicity of analysis: no specialised training is required, and data can be obtained within one hour.

It must be borne in mind, though, that there is potential for error when using this method, if certain procedures are not followed correctly – particularly in relation to the visualisation ste*p.* It is essential that calibration curves are used, and that control samples, of a known artemisinin concentration, are included on every plate, as occasionally environmental variables (particularly, variations in oven temperature and humidity) can alter the degree of visualisation, resulting in falsely high or low values being given for artemisinin content. The presence of control samples are essential in correcting for these variables.

Additionally, samples must be prepared so that expected artemisinin level falls within the range of accurate quantification. This method has a detection limit of 0.05mg/ml artemisinin, and at levels above 0.6mg/ml, quantification becomes inaccurate due to saturation effects.

The solvent-free “leaf-spot” method, perhaps, has potential in the in-field rapid analysis of fresh material, in combination with a validated densitometric scanning programme, but requires further investigation.

Despite these limitations, though, the Quantiscan software, used appropriately, could provide a reliable and economical means of rapid plant quality control for the small-scale grower.

7 Deoxyartemisinin and artemisinin separation on a preparatory scale

Synopsis

Deoxyartemisinin is a pharmacologically inactive degradation product of artemisinin. As the two compounds are very similar in structure, differing only in the former's lack of an oxygen molecule in the 7-membered ring, they tend to co-elute in chromatographic systems, and can also co-crystallise. This can result in the overestimation of artemisinin content in samples, and in the incorporation of inactive deoxyartemisinin in pharmaceutical preparations.

This section describes a means of separating and crystallising the two compounds, using flash column chromatography. From 4g of crude *Artemisia annua* extract, 350 mg artemisinin and 35 mg deoxyartemisinin were separated and purified. Purity of separated compounds was confirmed by melting point analysis and ^{1}H NMR, and differences in the NMR signals of artemisinin and deoxyartemisinin were noted.

7.1 Introduction

Section 5 presented a method for the thin-layer chromatography (TLC)-based separation of artemisinin from its inactive cousin, deoxyartemisinin. Such a technique is of use to the general laboratory, where more selective techniques such as LCMS are unavailable, as it prevents the overestimation of artemisinin content in sample analysis. But it does not solve an equally pertinent problem: having ascertained that the sample – be it a crude extract, or crystalline matter – does contain a degree of deoxyartemisinin, how can the unwanted deoxyartemisinin be removed on a useful scale? Can the TLC method be adapted for column chromatography, allowing the removal of deoxyartemisinin from gram quantities of impure samples? This chapter will attempt to answer that question.

7.2 Materials and Methods

7.2.1 Column chromatography conditions

Silica (70 – 200 mesh, 60 angstroms) was purchased from Sigma-Aldrich, as was Chromatography Grade silica (35 – 70 mesh, 60 angstroms). Solvents were purchased from De Montfort University Chemical Stores and purified by distillation.

Hexane extract of Tanzanian-growth *Artemisia annua* – which, by TLC, had appeared to be comparatively rich in deoxyartemisinin – was a gift from Ian Flockhart and Colin Hill of Botanical Developments Ltd.

Columns were prepared using 50 g of silica in a column of height 35 cm and internal diameter of 3.5 cm. Silica was mixed with initial mobile phase as a slurry and poured into the column.

Column 1 was prepared using silica with a particle size of 70 – 200 µm. 4g crude *A. annua* extract was dried onto 5g silica and placed on the top of the column. A gradient mobile phase was used, consisting of the following solvent mixtures:

Solvent Mixture	**Volume**
Hexane 100%	100 ml
5% ethyl acetate in hexane	100 ml
10% ethyl acetate in hexane	100 ml
20% ethyl acetate in hexane	100 ml
30% ethyl acetate in hexane	100 ml
40% ethyl acetate in hexane	100 ml
50 % ethyl acetate in hexane	200 ml

Fractions of 20 ml were collected. Following TLC of the fractions, those fractions containing artemisinin and deoxyartemisinin were pooled and concentrated.
Crystalline matter from the pooled fractions was washed with hexane, re-dissolved in 50% ethyl acetate in hexane, and dried onto 1g of 35 – 70 µm particle size silica.

Column 2 was prepared using silica with a particle size of 35 – 70 µm. The column was otherwise prepared as above, with an isocratic mobile phase of hexane: butyl methyl ether: ethyl acetate (80: 15: 5). The artemisinin/deoxyartemisinin mixed crystals – dried onto silica – were placed at the top of the column. A mobile phase reservoir was placed over the column, filled with solvent (Figure 43), and the sample was eluted from the column using flash chromatography. Fractions of 10 ml were collected, and analysed by TLC.

Fractions containing artemisinin were pooled and crystallised, as were those containing deoxyartemisinin.

Figure 43. Flash column, used to separate deoxyartemisinin from artemisinin

7.2.2 LCMS conditions

LCMS techniques for the quantification of artemisinin and deoxyartemisinin are described fully in Section 5.2.1.2.

7.3 Results

Using column 1, with a gradient of ethyl acetate in hexane, a crude extract of *A. annua* was fractionated. Using this system, it was possible to separate artemisinin and deoxyartemisinin from the other sample components, but not from each other. Pooled and concentrated

fractions containing both these compounds were allowed to stand overnight, and from this liquor, 450 mg of opaque, off-white needles (Figure 44), with a melting range of 125 - 141°C, were obtained. TLC analysis showed that the needles were a mixture of artemisinin and deoxyartemisinin, with a small level of other contaminants.

Figure 44. Impure crystals containing both deoxyartemisinin and artemisinin.

These impure crystals were fractioned by flash chromatography on column 2, and TLC of the resulting fractions showed that deoxyartemisinin had been largely separated from artemisinin:

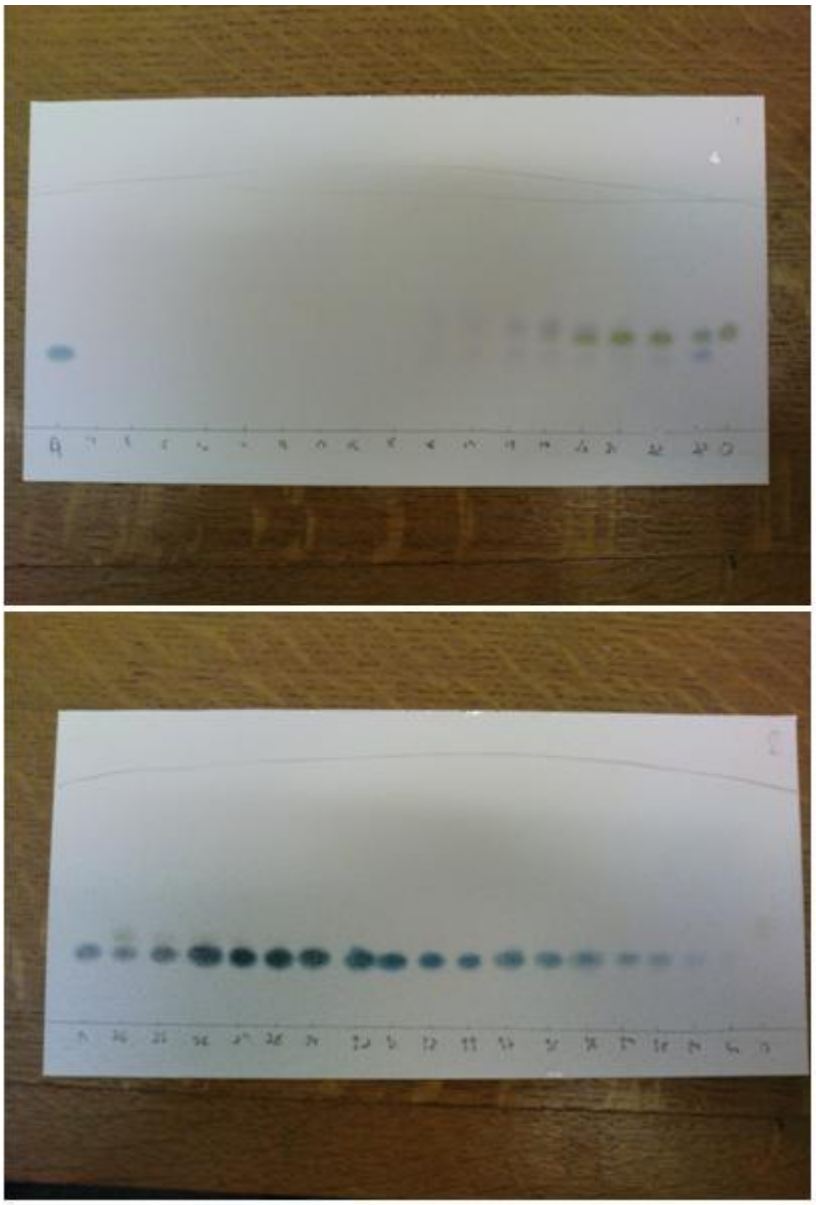


Figure 45. TLC of fractions obtained by flash chromatography of impure crystals of artemisinin and deoxyartemisinin.

Figure 45 shows the TLC plate. Fractions 19 – 23 contained deoxyartemisinin, and a small amount of another compound, and fractions 24 – 39 contained artemisinin. It can be seen that there is a small degree of overlap – fractions 23 and 24 contain both compounds, so baseline separation was not obtained. Fractions 19 – 22 were pooled and concentrated on a rotavapor at 60°C, as were fractions 25 – 39.

Upon concentration to ~ 3 mls, crystals – in the form of opaque white needles - readily formed from fractions 25 – 39. Crystals formed whilst solvent was still warm, and continued forming

as the solvent cooled. After 1 hour, no further crystal development was seen and crystals were dried and weighed. 355 mg of crystals were obtained, with a melting range of 152 – 156°C.

Fractions 19 – 22, containing deoxyartemisinin and another impurity, were reduced in volume on a rotavapor at 60°C. When the volume was ~1 ml, the liquor was allowed to stand. Within 30 minutes, clear white needles had begun to form in the liquor, and within 1 hour no further crystallization was seen. Crystals were dried and weighed. 35 mg of crystals were obtained, with a melting range of 108 - 110°C – identical to that given in the literature for deoxyartemisinin (Bhattacharya et al, 2007). By TLC, a retention factor comparable to a deoxyartemisinin reference was observed. A very small trace of artemisinin could be seen.

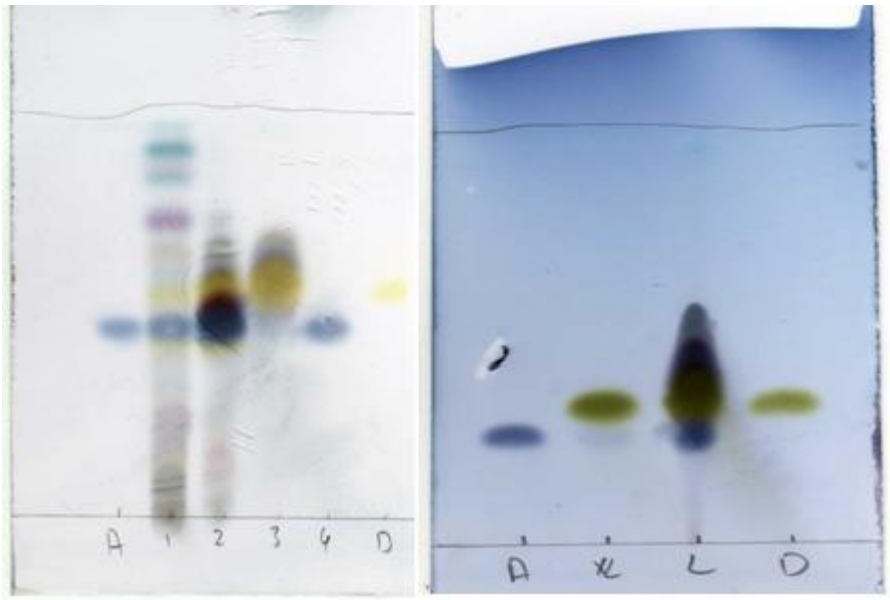


Figure 46. Left image, L-R. A: artemisinin reference, 1: crude extract prior to chromatography, 2: pooled artemisinin and deoxyartemisinin after column 1; 3: pooled deoxyartemisinin fractions after column 2 (but before sample concentration and crystallization), 4: pooled artemisinin fractions after column 2 (but before sample concentration and crystallization), D: deoxyartemisinin reference. Right image. A: artemisinin reference, XL: deoxyartemisinin after crystallization; L: mother liquor of deoxyartemisinin, D: deoxyartemisinin reference.

7.3.1 ^{1}H NMR

^{1}H NMR analysis of these crystals, purported to be deoxyartemisinin, showed signals at ppm values of 1.63 (m, 1H, H-3), 5.70 (s,1H, H-5), 3.19 (m, 1H, H-11), 1.20 (d, 3H, Me-13, *J* = 7.2), 0.94 (d, 3H, Me-14, *J* = 5.6) and 1.53 (s, 3H, Me-15). 2-D ^{1}H NMR showed clear interaction between the H11 and H13 protons.

TLC analyses confirmed that the crystals with a melting range on 152 - 156°C were a single compound, with a TLC retention factor comparable to that of an artemisinin reference. ^{1}H NMR analysis of these crystals, purported to be artemisinin, showed signals (Figure 49) at ppm values of 2.44 (m, 1H, H-3), 5.87 (s, 1H, H-5), 3.39 (m, 1H, H-11), 1.21 (d, 3H, Me-13, J = 7.2), 1.00 (d, 3H, Me-14, J = 5.8), and 1.44 (s, 3H, Me-15). These signals match those given in the literature for artemisinin (Blasko and Cordell, 1998). Interaction between the H11 and H13 protons could clearly be seen by 2-D ^{1}H NMR, as could interaction between H3 and H15. Small but distinct differences in the ppm values could be observed between artemisinin and deoxyartemisinin (Figure 50), particularly in H-3, H-5, H-11 and H-15.

Table 13. Comparison of ^{1}H NMR shifts, in $CDCL_3$, from artemisinin and deoxyartemisinin

Proton	Artemisinin (ppm)	Deoxyartemisinin (ppm)
H-3	2.44 (1H, ddd, J = 14.5, 13.3, 3.6)	1.80 (1H, m)
H-5	5.87 (1H, s)	5.70 (1H, s)
H-11	3.39 (qd, J = 1.4.5.6, 7.2)	3.19 (1H, ddd, J = 4.6, 7.2, 11.8)
H-13 (Methyl)	1.21 (3H, d, J = 7.2)	1.20 (3H,d, J = 7.2)
H-14 (Methyl)	1.00 (3H, d, J = 5.8)	0.94 (3,d, 5.6)
H-15 (Methyl)	1.44 (3H, s)	1.53 (3H,s)

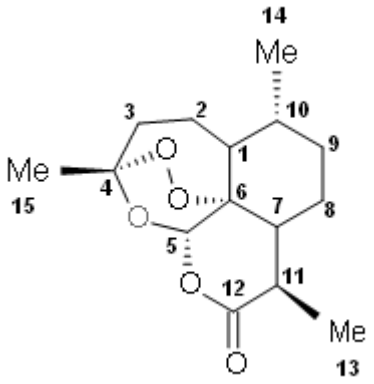


Figure 47. The numbering of the artemisinin molecule

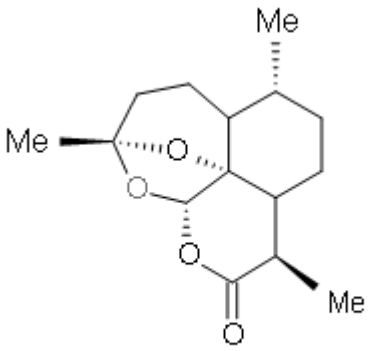


Figure 48. Deoxyartemisinin

Section 8: Prep-scale separation of artemisinin and deoxyartemisinin

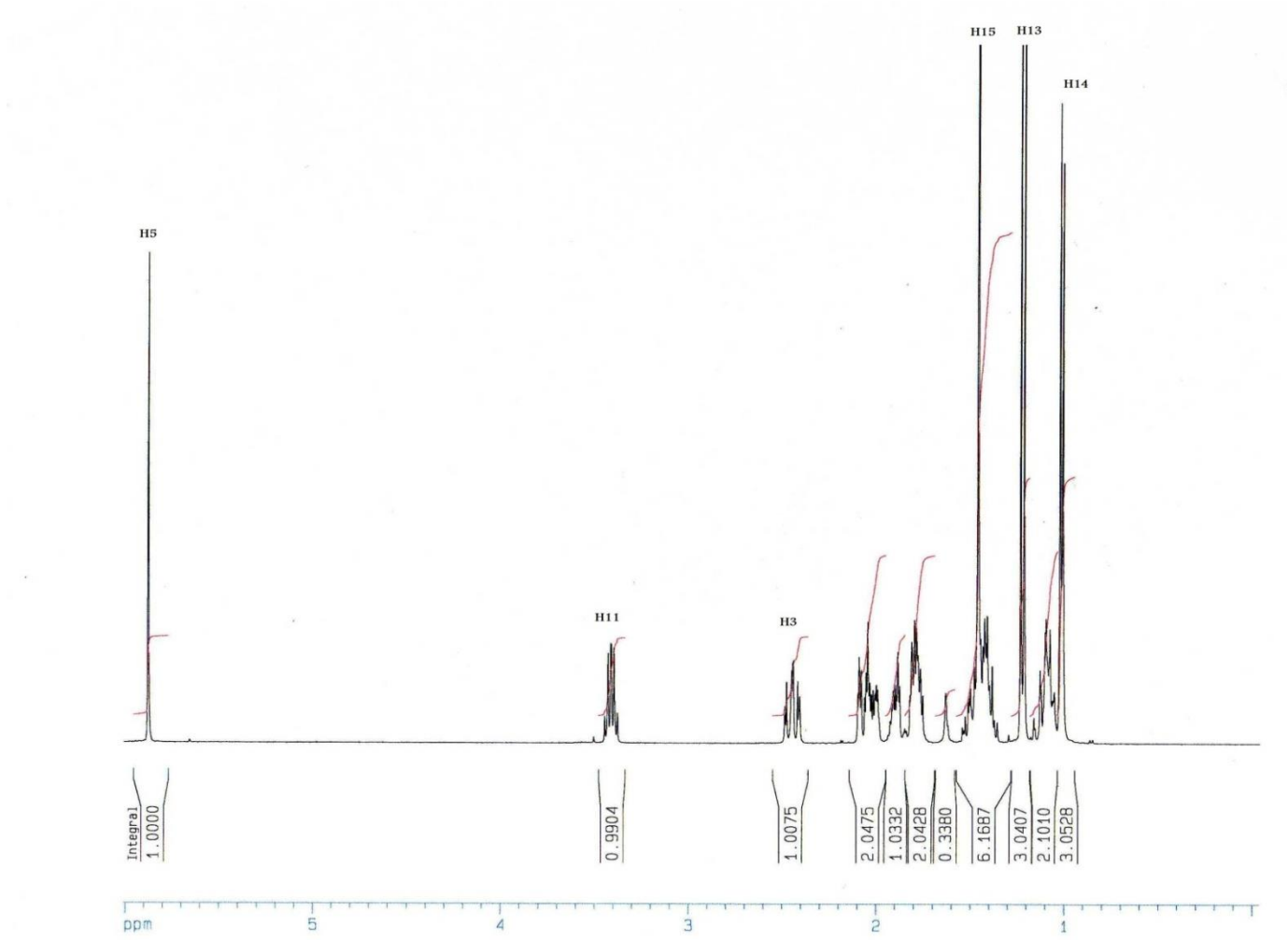


Figure 49. ^{1}H NMR of artemisinin

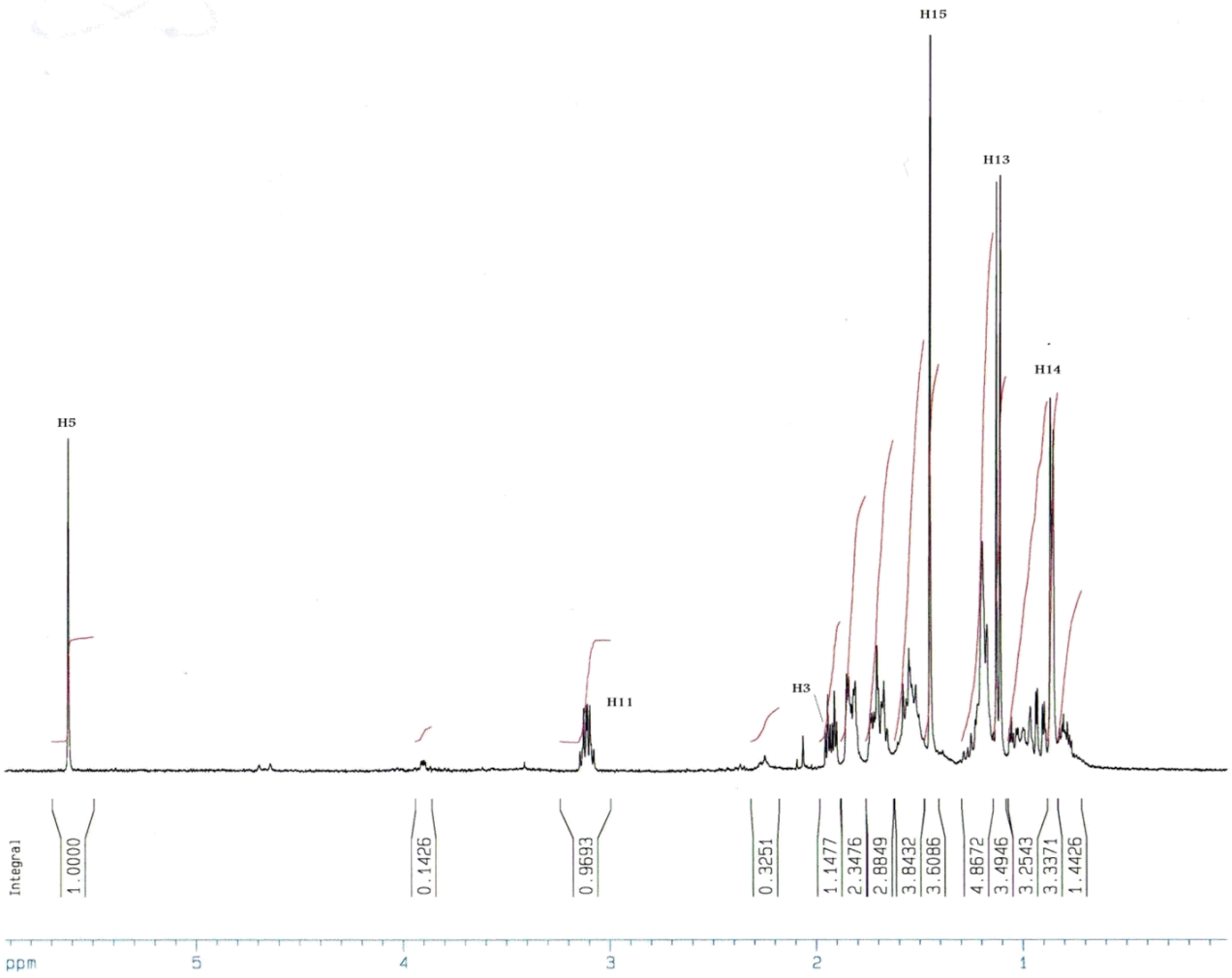


Figure 50. ^{1}H NMR of deoxyartemisinin

7.3.2 Purity by LCMS

LCMS quantification of artemisinin and deoxyartemisinin showed that artemisinin had been purified to 95% w/w, and deoxyartemisinin, to 91% w/w.

7.4 Discussion

Using simple laboratory equipment, it has been demonstrated that it is possible to separate artemisinin from the inactive contaminant deoxyartemisinin. A two step column chromatography set-up is needed: step 1, to isolate the two compounds from the other constituents of a crude extract, and step 2, to pull the two compounds away from each other. For this last step, two modifications to the standard column chromatography set-up were required:

1. The use of silica with a particle size of 35 – 70 µm (as distinguished from the more usual 70 - 200 µm size);
2. The use of flash chromatography.

Use of silica with a finer particle size - and hence a larger surface area - allows for a greater degree of interaction between solute and stationary phase. Figure 51 graphically illustrates the difference in quality between the standard and chromatography grade silica.
It may be hypothesised that the use of flash chromatography - in which the addition of pressure to the column results in a much more rapid elution of compounds than by gravity alone – prevents the dispersion effects that can cause closely eluting compounds to overlap.

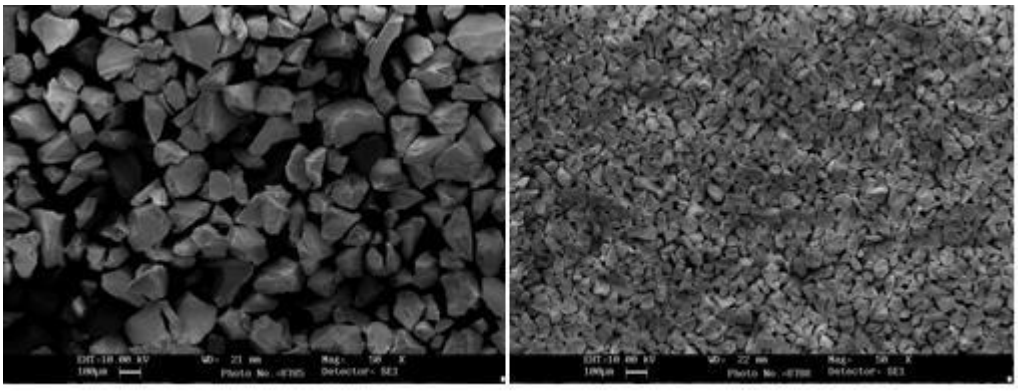


Figure 51. Comparison of particle size and distribution of standard (left) and chromatography grade (right) silicas. Pictures taken at 50 X magnification with a scanning electron microscope.

Although absolute baseline separation was not achieved, it was demonstrated clearly that a high yield of deoxyartemisinin-free artemisinin can be produced using simple, inexpensive methods. Whether this technique can be scaled to gram and kilogram levels has yet to be discovered.

8 Flavonoids as Markers of Quality

Synopsis

During an experiment to quantify levels of methoxylated flavonoids in *Artemisia annua* leaf, a correlation between flavonoid level and artemisinin levels was observed. This correlation was further investigated. A strong positive (>0.6) correlation between artemisinin and chrysosplenetin level was observed in several samples. However, in all samples assayed, a very strong positive correlation (>0.8) was observed between artemisinin and another flavonoid - hypothesised to be either eupatin or chrysosplenol-D.

As the methoxylated flavonoids strongly absorb UV – unlike artemisinin - this correlation may be used as an indirect means of artemisinin quantification by those with access to HPLC-UV, but without access to HPLC-MS.

This section also demonstrates that breeding new varieties of *Artemisia annua* for increased artemisinin content also results in plants with increased flavonoid content, potentially adding value to the crop, because of demonstrated anti-cancer activity of chrysosplenetin in *in vitro* cell viability (MTT) tests.

8.1 Introduction

The aerial parts of *Artemisia annua* contain, in addition to the terpenoid compounds of which artemisinin is one, a range of flavonoid aglycones .Typically, four different compounds are observed in the species, each of which is a methyl ether of quercetagetin.

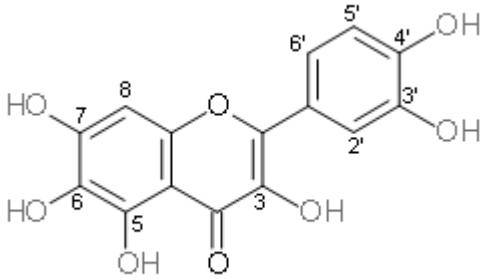


Figure 52 Quercetagetin

Although flavonoids are commonly thought to exist within plants as glycosides (that is, as conjugated to a sugar molecule), within the hydrophobic enclave of the trichome these structures exist without the sugar component. Indeed, exudate (or surface) flavonoids, as these trichome-occurring compounds are known, are typically highly methoxylated structures that do not lend themselves to glycosidation (Taleb-Kontini et al., 2007). A high degree of methoxylation is observed amongst the flavonoids of *A. annua*, as figure 53 shows:

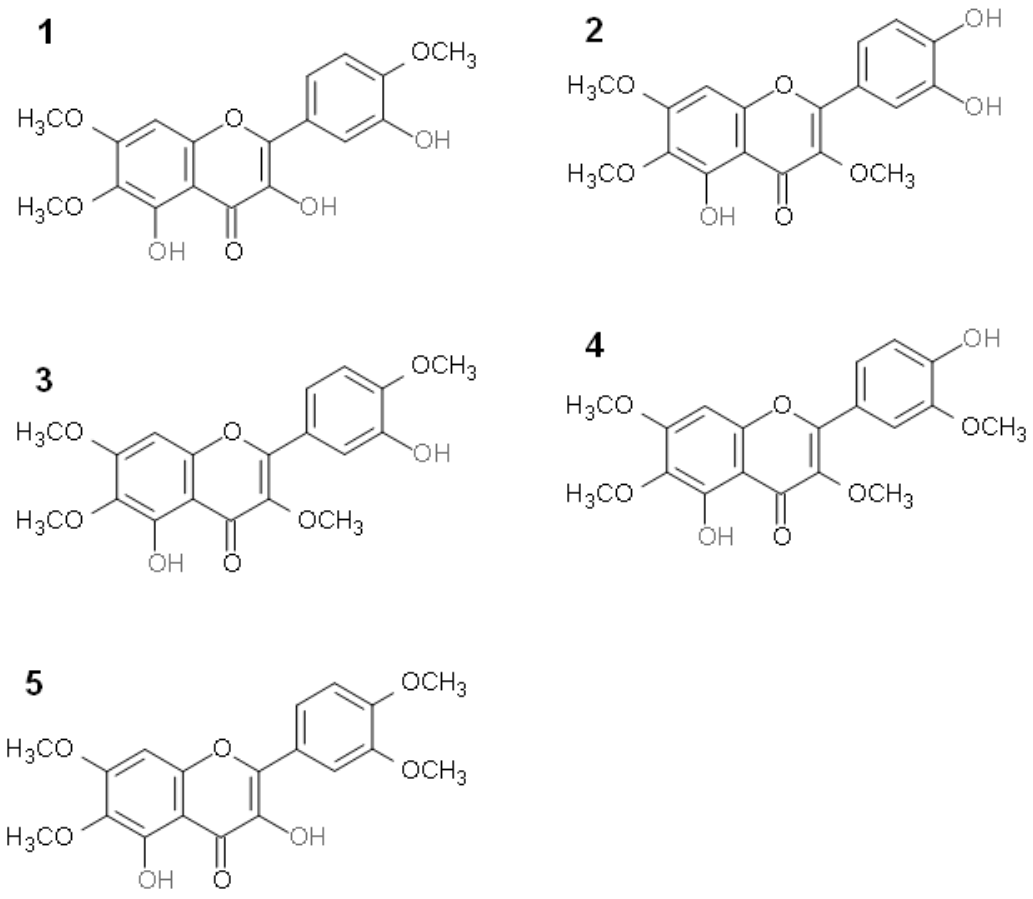


Figure 53. Methoxylated flavonoids of *Artemisia* spp. 1: Eupatin. 2: Chrysosplenol-D. 3: Casticin. 4: Chrysosplenetin. 5: Artemetin

The presence of trichome-stored flavonoids is widespread amongst *Achillea* species (Valent-Vetschera et al., 1994), and has been reported in many other plant subfamilies, including *Artemisia* (Baraldi et al., 2008). It has been proposed that the biosynthesis of flavonoids – highly UV-absorbent compounds that they are – is upregulated by increased intensity of UV-B radiation, and that the compounds act to form a sunscreen (Jaakola et al., 2004). As with many

other secondary metabolites, it has also been speculated that the exudate flavonoids are a form of defence against insecticidal or microbial attack (Tomas-Barberan et al, 1988).

Whatever the function of flavonoids *in vivo*, the flavonoid profile of *Artemisia annua* is of interest. The anti-cancer potential of casticin has been explored by several research groups who have noted its ability to halt cancerous cell growth at the G2/M stage, whilst having no effect on the growth of normal cells (Haidara et al, 2006, Kobayakawa et al, 2004).

It has also been demonstrated that the methoxylated flavonoids casticin and artemetin were able to potentiate the antiparasitic action of artemisinin against *Plasmodium falciparum* cultures (Elford et al., 1987). Therefore, the usefulness of these compounds, and their potential to add value to an *Artemisia annua* crop, renders their quantification in the plant an important piece of research.

It was during such quantification of *A. annua* flavonoids that the author noticed a strong positive correlation between artemisinin levels, and the levels of two flavonoids, in extracts of the plant. Since a major difficulty in the quantification of artemisinin in *A. annua* herbal material is artemisinin's lack of a chromophore (not a problem experienced by the flavonoid researcher), it was hypothesised that this artemisinin: flavonoid correlation, if it could be demonstrated to be valid, would represent a simple way to use UV-based detection to quantify artemisinin in herbal material, suitable for those laboratories that do not have access to the expensive detectors (such as mass spectrometers) that are needed to reliably quantify artemisinin.

How likely is a quantitative link between these two very different classes of compound? A shared biosynthetic pathway, at least at the latter stages of synthesis, is unlikely. But, as Wollenweber has observed, there is a correlation in most cases between levels of flavonoid aglycones and other lipophilic plant products, and the presence of secretory structures such as trichomes (Wollenweber et al., 1981). From this, we can speculate that a quantitative link may well be present simply because plants possessing more trichomes will (in theory) – possess more of the compounds stored or synthesized in trichomes – more of all the compounds. Indirectly, this brings us to a related question: does the level of artemisinin within a plant correlate to the trichome density? It would be logical, given that artemisinin is sequestered

within the glandular secretory trichomes (GSTs) of the leaf, to suppose that it does: particularly since the enzyme CYP7AV1, capable of catalysing multiple reactions in the biosynthetic pathway to artemisinin, has been localised to the GSTs of *A. annua* (Teoh et al., 2006).

Few reports exist of the methoxyflavonoid content of *Artemisia annua*. Bilia (Bilia et al., 2006a) reported a total methoxylated flavonoid content of 2% w/w (dry leaf), with an artemisinin level of 0.4% w/w. Bilia also reports that of the total methoxylated flavonoid content, extracted using hexane, eupatin represented 19.1%, an inseparable mixture of casticin and chrysosplenetin made up 34.3%, and artemetin a further 9.4%.

It is probable that flavonoid chemovars exist: material of differing geographical origin, or differing germplasm, may well produce qualitative and quantitative differences in flavonoid profile. It may also be that even with genetically identical plants, the profile changes with age and exposure to biotic and abiotic insults. Ideally, these putative changes in profile would be shown to be quantitatively linked to changes in artemisinin level.

So, the questions to be addressed are:

1. Is there a clear quantitative link between flavonoid level and artemisinin level?
2. If so, is this link stable even in the presence of environmental changes (the application of herbicides, for example, or differences in geographical location)?

8.2 Materials and Methods

8.2.1 Reagents

Chrysosplenetin was supplied by Colin Hill of Botanical Developments Ltd as an artemisinin/chrysosplenetin mixture. Chrysosplenetin was separated by column chromatography (diethyl ether: hexane at 7:3 over a silica phase) and identity was confirmed by LCMS and ^{1}H NMR. Artemisinin was purified by Les Brown of AECS QuickPrep Ltd, using counter current chromatography, from an extract of *A. annua* supplied by Ian Flockhart of Botanical Developments Ltd; again, identity was confirmed by LCMS and ^{1}H NMR. Santonin was purchased from Sigma-Aldrich. Electrospray tuning mix was purchased from Agilent Technologies Ltd. HPLC solvents (acetonitrile, HPLC gradient grade and water, mass

spectrometry grade) were purchased from Fisher, as was silica (70 – 200 mesh) for column chromatography. Solvents for column chromatography (diethyl ether and hexane) and solvents for sample extraction (dichloromethane, diethyl ether and hexane) were purchased from De Montfort University Chemical Stores.

8.2.2 Choice of extraction solvent

Four solvents – acetonitrile, dichloromethane, hexane, and methanol – were compared for flavonoid aglycone extraction efficiency. Dried and powdered leaf matter was macerated under dark conditions, with gentle shaking, for 24 hours at a 1/10 weight-to-volume ratio. Following this, the samples were filtered using ashless filter paper to remove leaf material, and liquid was dried under nitrogen. Dried extracts were re-dissolved in HPLC mobile phase (66% acetonitrile, 34% methanol) and assayed by HPLC-DAD, with detection at 350 nm.

8.2.3 Extraction of plant material

Following the comparative solvent assay described in Section 8.2.2.., acetonitrile was chosen as the optimal solvent for the extraction of flavonoids from dried herbal material. The herbal matter was powdered using a pestle and mortar, and macerated under dark conditions, with gentle shaking, for 24 hours at a 1/10 weight-to-volume ratio. After this time, samples were filtered using ashless filter paper, and analysed by HPLC-DAD and HPLC-MS.

8.2.4 HPLC-DAD for the quantification of flavonoids

8.2.4.1 Reference standard

Chrysosplenetin, obtained as an impure by-product of artemisinin purification from herbal material, was provided by Colin Hill of Botanical Developments Ltd. The compound was purified over a column consisting of 20 g silica (standard grade, 70 – 200 mesh), with a mobile phase of diethyl ether: hexane (7:3). Chrysosplenetin was re-crystallised to a constant melting range of 177 – 178 °C. Structure was confirmed by ^{1}H nuclear magnetic resonance on a Bruker Ultrashield 400 MHz machine.

8.2.4.2 HPLC Conditions

Separation of flavonoids was achieved using an Agilent 1100 Series HPLC system coupled to a diode array detector. A Grace Alltech column (150 x 2.1 mm, pore size 3 µm) was used with a gradient system utilising two solvent mixtures. Solvent A was prepared using HPLC mass spectrometry grade water with 0.1% orthophosphoric acid. Solvent B was a mixture of acetonitrile and methanol (both HPLC gradient grade) at a ratio of 66: 34.

Separation required the following gradient (designated "Methoxyflavones"), at a constant flow rate of 0.3 ml/minute:

Time (m)	**Solvent A %**	**Solvent B %**
0	70	30
15	50	50
20	50	50
25	40	60
30	30	70
40	20	80
45	70	30
48	70	30
Post run	10 minutes	

Absorption of ultraviolet light by flavonoids was recorded at 350 nm.

8.2.4.2.1 Generation of calibration curve

A series of samples were prepared in which chrysosplenetin level ranged from 5 mg/ml to 0.125 mg/ml. Samples were prepared in triplicate, and each sample was run three times for reproducibility assessment. The resulting curve was linear with a regression index of 0.9987.

8.2.5 HPLC-MS for the identification of flavonoids

A crude, resinous toluene extract of a Madagascan-grown *Artemisia annua* variety (a gift from Dr Colin Hill at Botanical Developments Ltd) was weighed out to 100 mg and mixed with methanol and water (in a 1:1 ratio). This was followed by partial purification, performed by

shaking the solution into dichloromethane. The dichloromethane fraction was dried with anhydrous sodium sulfate, evaporated under nitrogen, and re-dissolved in 250 µl acetonitrile.

25 µl of this preparation were injected onto a Grace Alltech RP18 (octadecylsilane) column, with an internal diameter of 2.1 mm and a length of 150 mm. The column packing had a particle size of 3µm. The mobile phase used was acetonitrile: water (6:4), at 0.2 ml/minute. A multimode spray chamber was used in electrospray ionisation mode, with the following mass spectrometer parameters:
nitrogen flow at 8 L^3/minute; drying gas temperature 350°C; capillary voltage 2800 V in positive mode, 3500 V in negative mode; nebulizer pressure 40 psig, and a fragmentor voltage of 70 V. This fragment voltage was gradually ramped in increments of 25, and mass fragments of the individual peaks observed at each increased voltage.

Detection was in total ion chromatogram mode, with a mass range of 10 to 500 Daltons.

8.2.6 HPLC-MS for the quantification of artemisinin

8.2.6.1 Reference standards

Reference artemisinin was provided by AECS, and found to have a melting range of 154 – 156 °C, and a purity of > 98%. Identity of artemisinin was confirmed by electrospray mass spectrometry and ^{1}H nuclear magnetic resonance on a Bruker 400-MHz Ultrashield machine.

8.2.6.2 HPLC-MS Conditions

Artemisinin was quantified using an Agilent Technologies 1100 Series high-performance liquid chromatography system, with a GraceSmart RP18-HPLC column (150 mm x 2.1 mm; pore size of 3 mm), coupled to an Agilent Technologies G1946 single quadruple mass spectrometer with electrospray ionization (EI), used in positive ion mode, with single ion monitoring (SIM). HPLC separation was achieved with an isocratic mobile phase of 60% acetonitrile and 40% HPLC-grade water at a flow rate of 0.2ml/minute, with a run time of 8 minutes. The column oven was set at 25°C.

The EI spray chamber and mass spectrometer parameters were as follows: nitrogen flow at 8 L^3/minute; drying gas temperature 350°C; capillary voltage 2800 V in positive mode, 3500 V in

negative mode; nebulizer pressure 40 psig; and a fragmentor voltage of 70 V. The mass spectrometer was calibrated and, if necessary, tuned daily, using Agilent's Electrospray Tuning Mix, to ensure peak performance.

Generation of calibration curve. Santonin was used as an internal standard, with ions monitored at 173.3 and 247.3. The monitored artemisinin ions were 283.3 and 209.3.

8.2.6.2.1 Generation of calibration curve

A calibration curve was generated, in which artemisinin concentration ranged from 2 to 16 µg/ml, and in which santonin, as an internal standard, was kept constant at 10 µg/ml. All calibration levels were prepared in triplicate and analysed three times per sample. The ratio of artemisinin to santonin was calculated for each analysis; linear regression of the resulting scatter plot was used to establish the calibration curve. The upper limit of quantification was given as the highest level of the calibration curve: beyond this, saturation of response rendered quantification less reliable. The lower detection limit was taken as five times the standard deviation of the measured response to the lowest calibration level, and was calculated to be 0.8 ng.

8.2.7 MTT Assay for Toxicity of Chrysosplenetin to Breast Cancer Cells

Anti-cancer (MTT) screening assays of chrysosplenetin were carried out by Dr Somchaiya Surichan and Dr Dyan Ankrett, of the DMU Cancer Drug Discovery Group. The protocol is given in Appendix 1.

8.3 Results

8.3.1 Number of flavonoid aglycone compounds present

Four major peaks, with UV spectra characteristic to flavonoids, were observed in all samples (Figure 54).

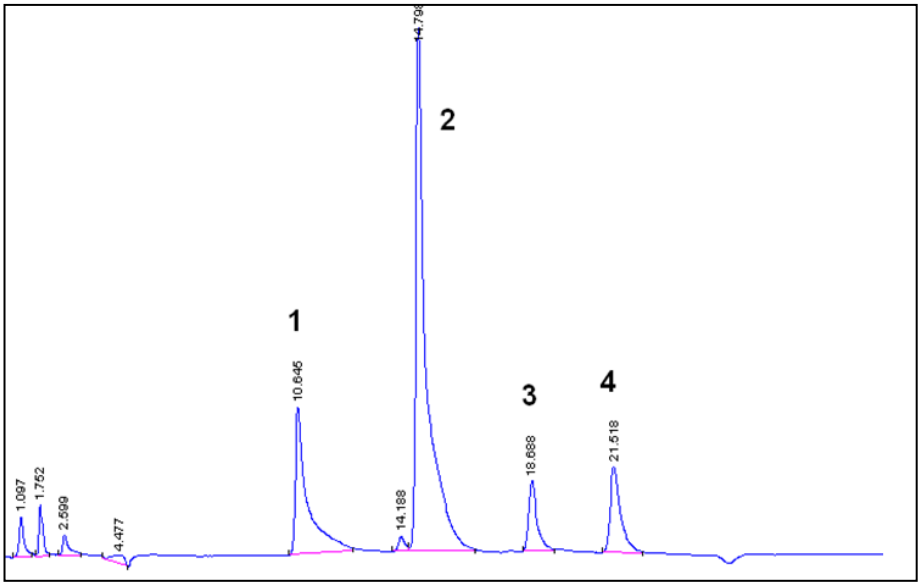


Figure 54. HPLC-DAD chromatogram of extract of *Artemisia annua* L, separated by "Methoxyflavones" method and detected at 350 nm. Peak 1: unknown; hypothesised to be eupatin or chrysosplenol-D. Peak 2: Casticin or chrysosplenetin. Peak 3: Unknown. Peak 4: unknown.

8.3.2 Choice of extraction solvent

Of the four solvents tested, hexane was the least efficient overall in terms of total flavonoids extraction. Notably, hexane failed to extract any eupatin from samples. Acetonitrile and methanol were the most efficient extraction solvents, extracting total flavonoid aglycones of 0.27 and 0.22 % w/w respectively (Table 14). Further tests showed that greater than 90% of the flavonoid content of the leaf material was extracted by this 24-hour maceration with either acetonitrile or methanol. Additionally, acetonitrile-extracted samples contained less green pigment (potentially destructive to HPLC-columns) compared to methanol extracted-samples. As a consequence, acetonitrile was chosen as the extraction solvent, due to its efficiency and similarity to the HPLC mobile phase.

Table 14. Extraction of methoxylated flavonoids from dried *Artemisia annua* leaf matter, expressed as % w/w, using four different solvents. Figures in italics represent standard deviation

Solvent	Peak 1 % w/w	Chrysosplenetin % w/w	Peak 3 % w/w	Peak 4 % w/w	Total Flavonoids % w/w
Acetonitrile	0.05 *(0.02)*	0.17 *(0.007)*	0.02 *(0.001)*	0.03 *(0.0004)*	0.27 *(0.02)*
Methanol 70%	0.04 *(0.005)*	0.14 *(0.0009)*	0.02 *(0.003)*	0.02 *(0.0004)*	0.22 *(0.009)*
Dichloromethane	0.02 *(0.004)*	0.12 *(0.02)*	0.01 *(0.003)*	0.02 *(0.003)*	0.17 *(0.03)*
Hexane	0	0.02 *(0.0013)*	0.003 *(0.0002)*	0.007 *(0.0002)*	0.03 *(0.002)*

It was noted that the hexane extract, in addition to being low in total flavonoid content, did not contain the first of the four peaks (either eupatin or chrysosplenol-D); probably because this compound, eluting before the others, is likely to be the most polar of the four and hence unlikely to be extracted by such a non-polar solvent.

Extraction of the same material in an aqueous methanolic solution, with added β-glucosidase, did not result in any increase in flavonoid content. This adds weight to the assertion by Taleb-Kontini that surface methoxyflavones tend to exist in the free aglycone form.

8.3.3 HPLC-MS for identification of flavonoids

Electrospray ionisation is a soft ionisation technique, and unlike some other ionisation techniques (such as electron impact ionisation), does not generate a large number of fragment ions in the mass spectrum - often, at the default fragmentor voltage of 70 V, only the molecular ion (M + 1) is observed. Such is the case with the methoxylated flavonoids (artemisinin, on the other hand, exhibits significant fragmentation at this voltage).

By using HPLC coupled to both DAD and electrospray ionisation MS, the mass fragments of two early-eluting peaks with flavonoid-type UV spectra could be studied (although four peaks with flavonoid spectra were observed, the two later eluting peaks were not present at sufficiently high levels to allow mass spectral studies). The earliest eluting peak, at 3.3 minutes, had a base peak of 361, and the peak eluting second, at 4.5 minutes, had a base peak of 375. The former figure corresponds to the protonated value expected for either eupatin or chrysosplenol-D, and the latter to that expected for either casticin or chrysosplenetin.

The B-ring of the eupatin molecule differs from the B-ring of chrysosplenol-D, in that the former contains both and O-methyl and a hydroxyl moiety – the ring, if isolated from the rest of the molecule, would have a mass of 123. The B-ring of chrysosplenol-D, on the other hand, contains two hydroxyl moieties, and would have an isolated mass of 109. To identify which of the two compounds was present, the fragmentor voltage of the MS was increased from 70 V to 350 V, and the resulting fragmentation patters of this peak observed. At voltages of 250 and higher, a small fragment of m/z 109.3 appeared in the mass spectrum. The base peak remained the 361, M + 1 fragment. This evidence suggests that the first flavonoid eluting by HPLC is, in fact, chrysosplenol-D (Figure 55).

This increased fragmentation technique would not be applicable to the discernment of casticin from chrysosplenetin (Figure 56), as in this case the B-ring of both molecules contains one hydroxyl and one O-methyl moiety. Other chemical techniques, described below, were used to deduce which compound was present in the extract.

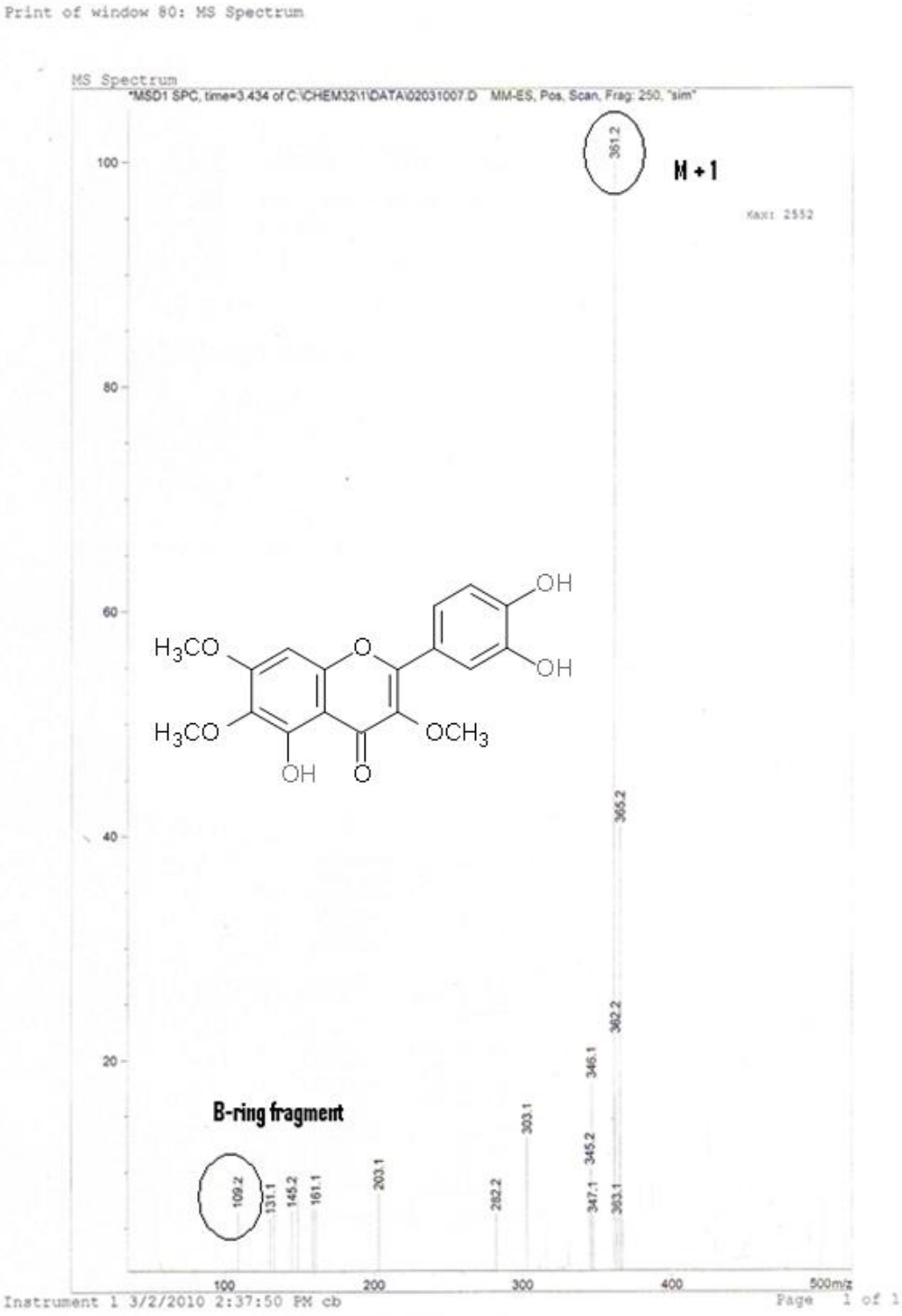


Figure 55. Mass fragments of chrysosplenol-D

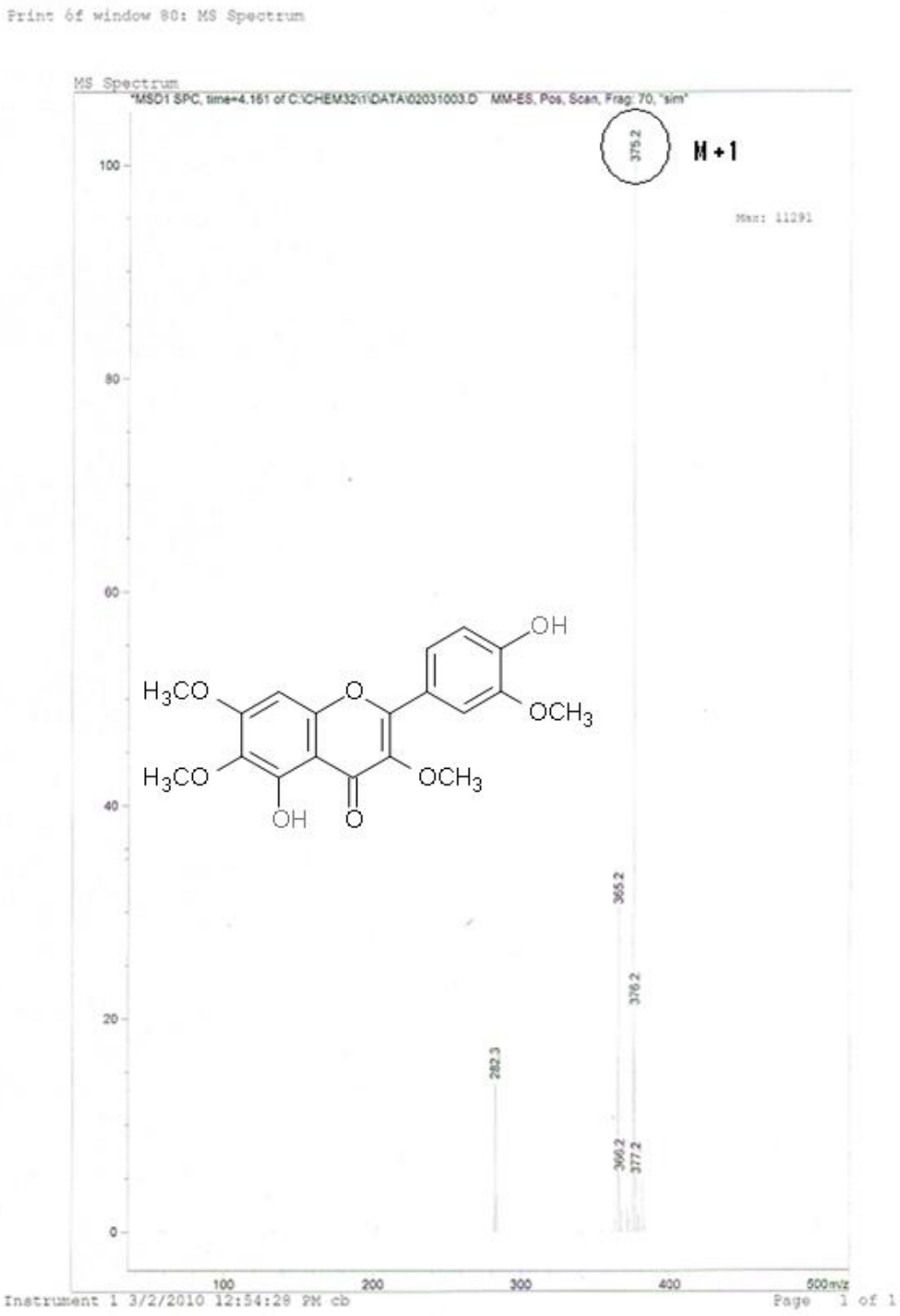


Figure 56. Mass fragments of chrysosplenetin

8.3.4 NMR identification of chrysosplenetin

It is known that both flavonoids - casticin and chrysosplenetin - occur in *A. annua*; Bilia has described the two compounds as inseparable. As both compounds have the same mass (374), and differ only in the position of the B-ring O-methyl group, the two cannot be differentiated by LCMS. The ppm shifts obtained by ^{1}H NMR (Figure 57) of the *Artemisia annua* L. flavonoid were as follows: 3.86 (s, 3'-OCH_3), 3.92 (s, 7-OCH_3), 3.96 (s, 3-OCH_3), 3.98 (s, 6-OCH_3), 6.11 (s, 4'-OH), 6.49 (s, H8), 7.04 (d, *J = 8.4*, H5',H6')), 7.65 (dd, *J* =2.0 [H2',H6'], *J* = 8.4 [H6',H5']), 7.70 (d, *J = 1.97*, H2',H6') and 12.60 (s, 5-OH).

Homonuclear 2-D COSY ^{1}H NMR (Figure 58) confirmed *J*-coupling between the 7.04 ppm (C5') and 7.7 ppm (C2') peaks, but such coupling would be present in both casticin and chrysosplenetin. However, homonuclear 2-D NOESY ^{1}H NMR (Figure 59) demonstrated spatial proximity between H2' and 3'-OCH_3, H8 and 7-OCH_3 and H5' and H6'. The NOESY-interation between H2' and 3'-OCH_3 indicates that these two groups are adjancent in space – providing strong evidence that the compound is in fact chrysosplenetin, not casticin.

Furthermore, the melting range of the material – 177 - 178°C – points toward the material being pure chrysosplenetin. Literature values for the melting range of chrysosplenetin are 177 – 178 °C (Fukui et al., 1969), and that for casticin is given as 188 - 189°C (Azizuddin et al., 2009).

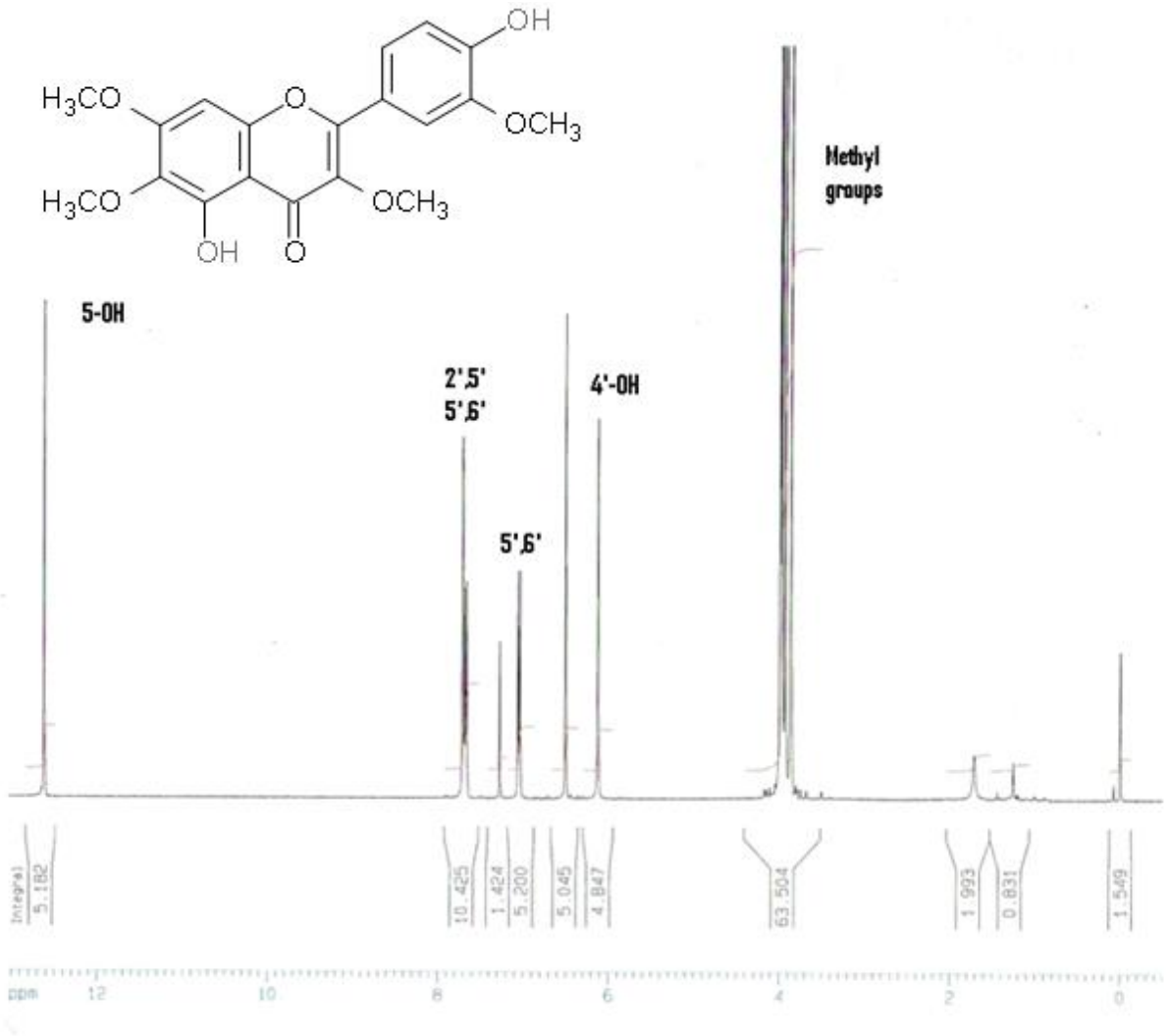


Figure 57. ^{1}H NMR of chrysosplenetin

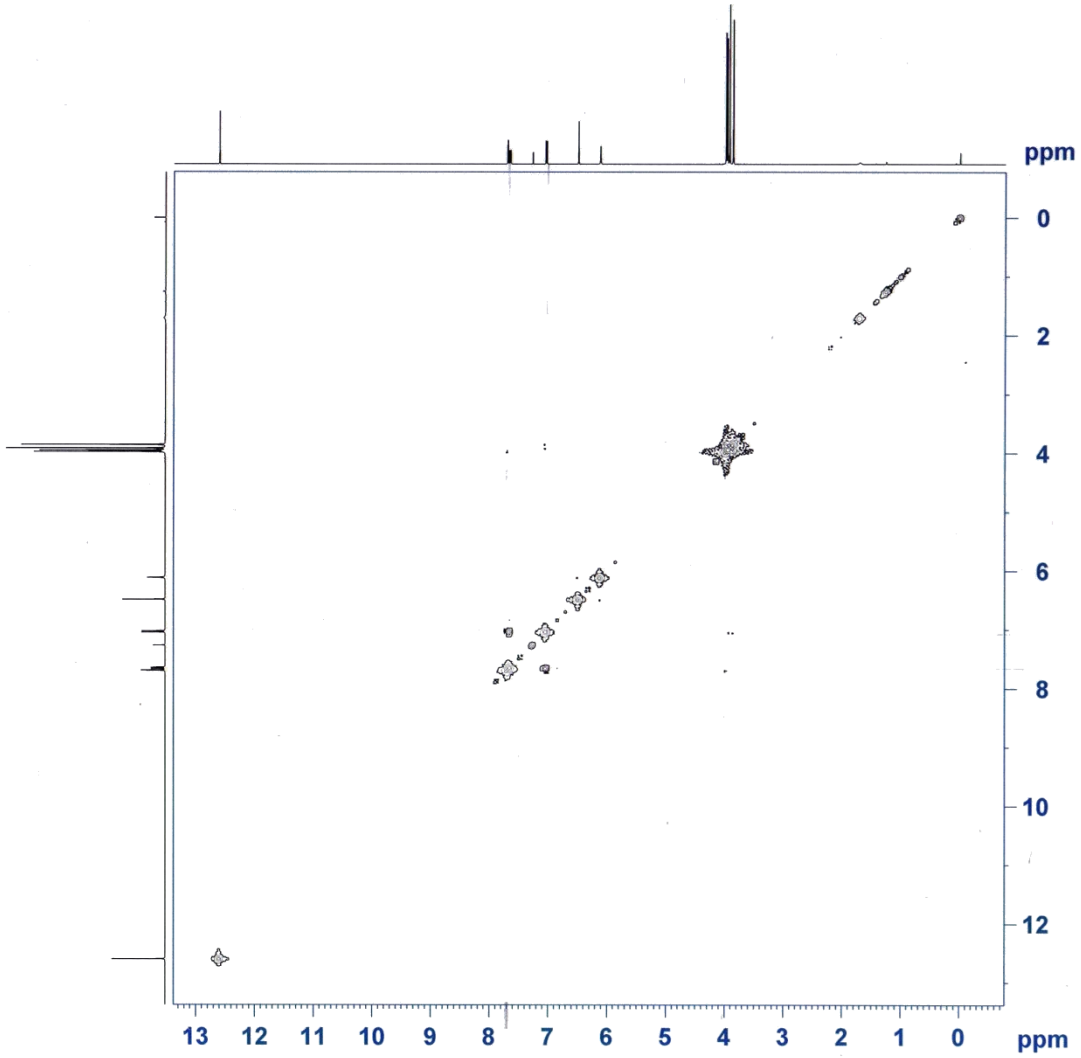


Figure 58. 2-D homonuclear (^{1}H) COSY NMR of chrysosplenetin

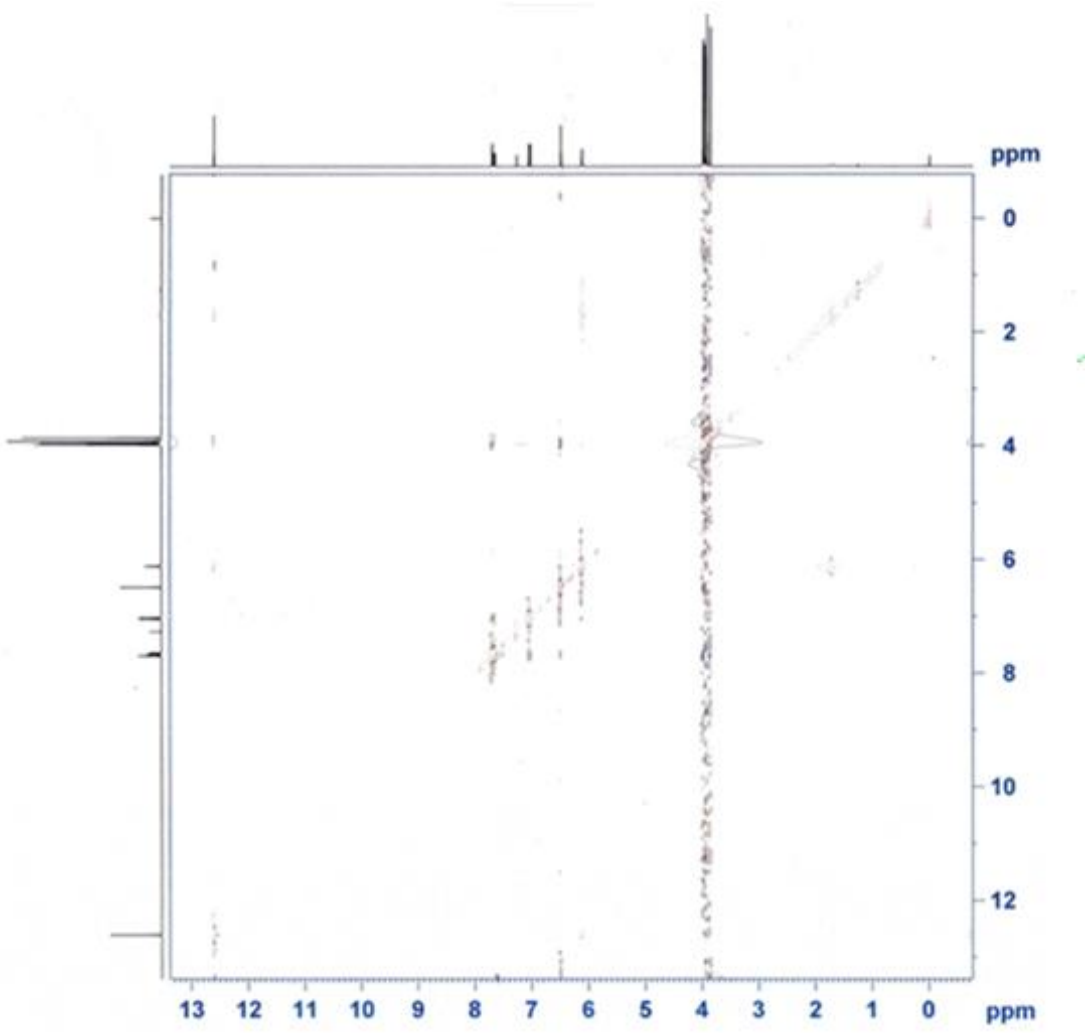


Figure 59. 2-D (^{1}H) NOESY NMR of chrysosplenetin

8.3.4.1 Eupatin or Chrysosplenol-D?

Insufficient sample of chrysosplenol-D was available to perform NMR analysis of this compound, and hence confirm compound identity. However, for the remainder of this section, the compound shall be identified as chrysosplenol-D, based on the preliminary LCMS data.

8.3.5 Artemisinin and flavonoid content in Cambridge, UK, in 2007

Table 15 shows artemisinin, chrysosplenol-D and chrysosplenetin content in *Artemisia annua* plants samples grown in Cambridge, UK, and harvested in 2007:

Table 15. Levels of, and correlation between, artemisinin and two methoxylated flavonoids in three new varieties of *Artemisia annua* across a full harvest season in Cambridge, UK, in 2007. *CH-D*: chrysosplenol-D. *Chryso*: chrysosplenetin

Variety	Harvest Date	ART % w/w	CH-D % w/w	CHRYSO % w/w	Ratio CH-D/Art	Ratio Chryso/Art
1015	25/07/2007	0.7	0.08	0.09	0.11	0.13
	01/08/2007	0.7	0.07	0.09	0.10	0.13
	30/08/2007	1.4	0.11	0.11	0.08	0.08
	06/09/2007	1.2	0.10	0.10	0.08	0.09
	06/09/2007	1	0.1	0.11	0.10	0.11
	13/09/2007	1.1	0.10	0.10	0.09	0.09
	13/09/2007	1.2	0.09	0.09	0.08	0.07
	27/09/2007	1.1	0.10	0.10	0.09	0.09
	27/09/2007	0.9	0.08	0.10	0.09	0.11
	11/10/2007	1.1	0.11	0.11	0.10	0.10
	11/10/2007	1.3	0.12	0.13	0.09	0.10
1046-7	01/08/2007	1	0.07	0.11	0.07	0.11
	01/08/2007	0.9	0.05	0.10	0.06	0.11
	20/08/2007	1.2	0.08	0.13	0.07	0.11
	20/08/2007	1.2	0.09	0.14	0.08	0.12
	30/08/2007	1	0.08	0.12	0.08	0.12
	30/08/2007	1.1	0.09	0.13	0.08	0.12
	06/09/2007	1.2	0.09	0.15	0.08	0.12
	06/09/2007	1.1	0.08	0.12	0.07	0.11
	13/09/2007	1.4	0.12	0.17	0.09	0.12
	13/09/2007	1.3	0.13	0.17	0.10	0.13
	27/09/2007	1.5	0.13	0.18	0.09	0.12
	27/09/2007	1.5	0.11	0.15	0.07	0.10
	11/10/2007	1.2	0.09	0.14	0.08	0.11
	11/10/2007	1.3	0.11	0.16	0.08	0.13
1012-12	30/08/2007	0.4	0.00	0.04	0	0.10
	06/09/2007	0.04	0.00	0.01		0.23
	06/09/2007	0.3	0.01	0.04	0.03	0.14

	13/09/2007	0.4	0.01	0.06	0.03	0.14
	13/09/2007	0.4	0.01	0.05	0.03	0.12
	27/09/2007	0.9	0.03	0.07	0.03	0.08
	27/09/2007	0.5	0.01	0.04	0.02	0.09
	11/10/2007	0.5	0.02	0.05	0.04	0.10
	11/10/2007	0.6	0.01	0.05	0.02	0.08

Correlation Coefficient between Artemisinin and Flavonoid Level	Chrysosplenol-D	Chrysosplenetin
1015	0.9	0.7
1046-7	0.9	0.9
1012-12	0.8	0.9

8.3.5.1 Correlation between artemisinin and flavonoid content in 2007

In all three varieties, a strong positive correlation exists between the level of artemisinin and the level of chrysosplenol-D. In two of the three varieties (1046-7 and 1012-12), a strong correlation exists between the level of artemisinin and the level of chrysosplenetin. These two varieties differ from 1015 in being consortium-bred.

8.3.5.2 Overall flavonoid profile in 2007

In 1015, levels of chrysosplenol-D and chrysosplenetin are nearly equal, with an mean chrysosplenol-D to chrysosplenetin ratio of 0.9 (+-0.08, ranging from 0.80 to 1.0). In 1046-7, chrysosplenol-D levels are a little lower than chrysosplenetin, with a mean ratio of 0.8, (+-0.01, ranging from 0.06 to 0.1). In 1012-12, chrysosplenol-D levels are much lower than chrysosplenetin, with a mean ratio of 0.21 (+- 0.14, ranging from 0 to 0.07).
In two of the three cases (1046-7 and 1012-12) flavonoid levels begin to drop towards the end of the harvest season.

8.3.5.3 The chrysosplenetin: artemisinin ratio

The ratio of chrysosplenetin to artemisinin, when all lines are considered together, ranges from 0.07 to 0.23, with a mean value of 0.11 (+-0.03) –a figure which also represents the most commonly occurring value.

8.3.6 Artemisinin and flavonoid content in Cambridge-grown material in 2008

To verify that this chrysosplenetin to artemisinin ratio of 0.1 is a real and consistent phenomenon, the analysis was expanded in 2008 to include eleven varieties of *A. annua* - both before and after glyphosate treatment. The results are presented in Table 16, after which follows a summary. Table 16shows levels of chrysosplenol-D, chrysosplenetin, and artemisinin in eleven varieties of *A. annua* grown in Cambridge in 2008. Each variety was sampled three times: on 31st July, 22nd September, and 22nd September following previous treatment with Glyphosate (GLY).

Table 16. Artemisinin and flavonoid content of 2008 Cambridge-grown material, some of which (designated GLY) had been exposed to Glyphosate.

Variety	Details	ART % w/w	CH-D % w/w	CHRYSO % w/w	Ratio CH-D /ART	Ratio CHRYSO/ART
1015	31/7/2008	0.8	0.04	0.11	0.05	0.14
	22/9/2008	1	0.04	0.11	0.04	0.11
	22/9/2008 GLY	1.2	0.04	0.11	0.03	0.09
1046-7	31/7/2008	1	0.1	0.15	0.10	0.15
	22/9/2008	1.1	0.1	0.16	0.09	0.15
	22/9/2008 GLY	1.3	0.12	0.19	0.09	0.15
1012-12	31/7/2008	0.6	0.03	0.11	0.05	0.18
	22/9/2008	0.7	0.01	0.09	0.01	0.13
	22/9/2008 GLY	0.8	0.04	0.12	0.05	0.15
1001-3	31/7/2008	0.7	0.01	0.11	0.01	0.16
	22/9/2008	0.9	0.02	0.13	0.02	0.14
	22/9/2008 GLY	1	0.02	0.14	0.02	0.14
1038-1	31/7/2008	1	0.05	0.12	0.05	0.12
	22/9/2008	0.9	0.03	0.1	0.03	0.11
	22/9/2008 GLY	1.3	0.11	0.12	0.08	0.09
1045-3	31/7/2009	0.8	0.07	0.18	0.09	0.23
	22/9/2008	0.9	0.06	0.15	0.07	0.17
	22/9/2008 GLY	1.2	0.08	0.16	0.07	0.13
1062-1	31/7/2008	1.2	0.08	0.16	0.07	0.13
	22/9/2008	1.4	0.1	0.18	0.07	0.13
	22/9/2008 GLY	1.7	0.11	0.22	0.06	0.13
1062-4	31/7/2008	1	0.08	0.11	0.08	0.11
	22/9/2008	1.2	0.15	0.16	0.13	0.13
	22/9/2008 GLY	1.5	0.14	0.15	0.09	0.1
1053-1	31/7/2008	1.4	0.08	0.18	0.06	0.13

	22/9/2008	0.8	0.05	0.14	0.06	0.18
	22/9/2008 GLY	1.3	0.07	0.16	0.05	0.12
1053-2	31/7/2008	0.9	0.05	0.13	0.06	0.14
	22/9/2008	0.9	0.04	0.13	0.04	0.14
	22/9/2008 GLY	1.3	0.06	0.16	0.05	0.12
1053-4	31/7/2008	0.8	0.07	0.08	0.09	0.1
	22/9/2008	1.2	0.08	0.11	0.07	0.09
	22/9/2008 GLY	1.1	0.11	0.11	0.10	0.1

Correlation Coefficient between Artemisinin and Flavonoid Level	Chrysosplenol-D	Chrysosplenetin
Harvest: 31/7/2008	0.8	0.6
Harvest: 22/9/2008	0.8	0.5
Harvest: 22/9/2008, treated with Glyphosate	0.7	0.6

8.3.6.1 Correlation between artemisinin and flavonoid content

The table above shows that, when eleven varieties are considered, a strong positive correlation exists between levels of chrysosplenol-D and levels of artemisinin – although this correlation is somewhat reduced upon application of glyphosate.

The correlation between chrysosplenetin and artemisinin, however, was less pronounced, both with and without glyphosate treatment. This is explained by the fact that the ratios of chrysosplenetin to artemisinin, when all lines and treatments (i.e. with or without glyphosate) are considered together, varies widely from 0.09 to 0.23, although the mean (and median) value is 0.13 – close to that observed in the 2007 material. As methoxylation of the flavonoid skeleton continues, the quantitative link with artemisinin appears to break down.

8.3.6.2 Use of Glyphosate

In four of the eleven varieties assayed, glyphosate treatment did not affect the chrysosplenetin to artemisinin ratio. In three of these cases (1046-7, 1001-3 and 1062-1), a correlated increase in both chrysosplenetin and artemisinin was observed. In the fourth case (1053-4), artemisinin level actually dropped, whilst that of chrysosplenetin increased slightly.

However, in the other seven lines, the ratio of the two compoundswas affected by glyphosate application. In four of these (1012-12, 1045-3, 1053-1 and 1053-2), a small increase in chrysosplenetin was observed, but to a lesser magnitude than the increase in artemisinin. In

two cases (1015, 1038-1), artemisinin increased whilst chrysosplenetin level did not change. In the final line, 1062-4, artemisinin increased whilst chrysosplenetin level dropped.

8.3.6.3 Comparison with 2007 material

Samples of *Artemisia annua* line 1015 showed a different flavonoid profile in 2008 as compared to 2007. In 2008, the levels of chrysosplenol-D were much lower than the levels of chrysosplenetin, whereas in 2007 the levels had been nearly equal. 1012-12 and 1046-7, however, appeared very similar in flavonoid profile in 2008 compared to 2007.

In both 2007and 2008, 1015 produced a maximum chrysosplenetin content of 0.11% w/w. In 2008, 1046-7 contained slightly more chrysosplenetin compared to 2007 (0.19 % w/w and 0.18% w/w respectively). 1012-12, though, showed quite a large increase in chrysosplenetin in 2008 compared to 2007 (0.12% w/w and 0.07 % w/w respectively). This increase was not reflected in the artemisinin levels, which were slightly lower in all three varieties in 2008 compared to 2007.

Such year-to-year variations - small as they are - in the chrysosplenol-D: artemisinin and chrysosplenetin: artemisinin ratios, within the same germplasm, show that environmental conditions are able to override genetic similarity, and reduce the reliability of this indirect means of artemisinin quality assessment.

8.3.6.4 Consortium and non-consortium material

To further explore the effect of environmental variation on the flavonoid profiles of *Artemisia annua*, Consortium-developed material grown in areas other than Cambridge (Surrey, Lincoln and Morocco) were assayed. Table 17 shows the results. Samples 8Z – 8P were grown in Lincoln. 1046-7 and 1001-9 were grown in Surrey. Moroccan 1 and 2 – a Consortium bred line made by crossing 1053 with 1015 - were grown in Morocco.

Table 17. Artemisinin and flavonoid levels in Consortium material grown outside of Cambridge. *Veg*: vegetative plant. *Flower*: flowering plant.

Sample	Details	Art % w/w	CH-D % w/w	Chryso % w/w	Ratio CH-D: Art	Ratio Chryso: Art
8Z	Harvested mid-September 2007	0.3	0.004	0.08	0.01	0.27
8Y		0.3	0.004	0.08	0.01	0.27
8X	Harvested late September 2007	0.6	0.012	0.06	0.02	0.1
8V		0.5	0.006	0.03	0.01	0.06
8P	Harvested late October 2007	0.4	0.003	0.03	0.01	0.075
1046-7 Veg		1.8	0.1	0.14	0.06	0.08
1046-7 Flower		1	0.06	0.08	0.06	0.08
1001-9 Veg		1.8	0.03	0.09	0.02	0.05
1001-9 Flower		1.4	0.1	0.06	0.07	0.04
Moroccan 1		0.5	0.012	0.058	0.02	0.12 (0.06)
Moroccan A		0.4	0.017	0.074	0.04	0.19 (0.008)
Correlation Coefficient Chrysosplenol-D: Artemisinin				0.8		
Correlation Coefficient Chrysosplenetin: Artemisinin				0.6		

8.3.6.5 Correlation between flavonoid and artemisinin level

When all the samples in Table 17 are considered together, a strong positive correlation (0.8) between artemisinin and chrysosplenol-D levels is observed. The correlation between artemisinin and chrysosplenetin is a little lower, standing at 0.6.

8.3.6.6 Chrysosplenetin: artemisinin ratio – non-Cambridge material

The above table (Table 17) shows that across a growth season outside of Cambridge, a wide range of chrysosplenetin: artemisinin ratios are seen, from 0.06 to 0.27. The case of 1046-7 is revealing: when grown in Cambridge, the ratio of chrysosplenetin to artemisinin in this variety ranged from 0.1 to 0.15 in 2007 and 2008 respectively – but in Surrey, in 2008, the ratio dropped to 0.08. The difference can be explained b the fact that in both locations, the chrysosplenetin levels were similar, but artemisinin levels in the Surrey-grown material were higher.

Additionally, the Consortium-developed but Moroccan grown material showed a chrysosplenetin: artemisinin ratio that differed according to drying methods. Both Moroccan samples were of a variety developed by crossing 1053 with 1015, but the sun-dried sample (Moroccan I) showed both a decrease in artemisinin and an increase in chrysosplenetin, compared to the "fast-dried" dried sample (Moroccan A). Both cases, though, showed much less artemisinin compared to Cambridge-grown material, and also much less chrysosplenetin.

Finally, having examined the effects of different location on Consortium-material, the ratio of flavonoid to artemisinin in non-Consortium material was investigated. Table 18 shows the results of four non-Consortium samples.

Table 18. Artemisinin and flavonoid levels in non-Consortium material. CH-D: chrysosplenol-D. Chryso: chrysosplenetin

Sample	ART % w/w	Chrysosplenol-D		Chrysosplenetin		Correlation coefficient, CH-D art	Correlation coefficient, chryso: art
		% w/w	Ratio FLAV: ART	% w/w	Ratio FLAV: ART		
Rutland, UK	0.5 (0.001)	0.01 (0)	*0.02 (0)*	0.059 (0.002)	*0.12 (0.004)*	*0.81*	*0.25*
Anamed (Swiss)	0.8 (0.014)	0.04 (0)	*0.05 (0.0007)*	0.057 (0.0007)	*0.07 (0)*		
Kenyan	0.9 (0.07)	0.06 (0.006)	*0.07*	0.1 (0.06)	*0.11*		
Ghana	0.5 (0.001)	0.037 (0)	*0.07 (0.004)*	0.091 (0.0007)	*0.19 (0.002)*		

The table above shows that in non-Consortium material, chrysosplenetin: artemisinin ratios range from 0.07 to 0.19, with the lowest ratio seen in the Anamed sample, and the highest in the Ghanaian. Striking, though, is the high degree of positive correlation between chrysosplenol-D and artemisinin level – a correlation that is not observed between artemisinin and chrysosplenetin.

8.3.7 Anti-tumour action of chrysosplenetin from *Artemisia annua* L.

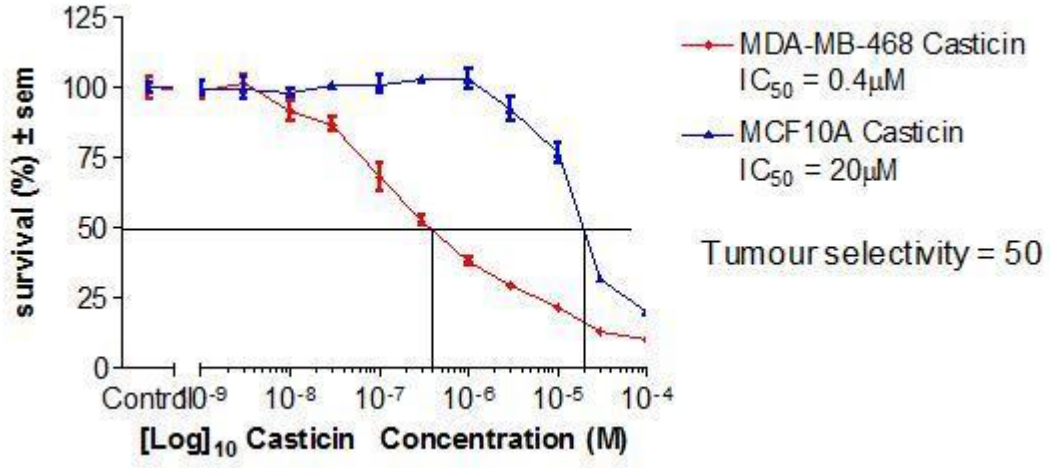


Figure 60. IC50 of chrysosplenetin against human breast cancer and normal human breast cells

Chrysosplenetin – labelled on Figure 60 as "casticin" – showed selective toxicity to breast cancer cells as compared to non-cancerous breast cells. The IC_{50} of chrysosplenetin against cancerous cells (MDA-MB-468, shown in red diamonds) was 0.4 µM, whereas in non-cancerous cells (MCF10A, shown in blue triangles), the IC_{50} was much higher, at 20 µM. As the concentration needed to destroy 50% of normal cells was 50 times higher than that required to destroy 50% of cancer cells, the tumour selectivity of chrysosplenetin is said to be 50.

8.4 Discussion

8.4.1 Why quantify flavonoids of *Artemisia annua*?

This exploration of flavonoid profiles in *Artemisia annua* began in 2007. Previous research had demonstrated the anti-cancer potential of methoxylated flavonoids (Kobayakawa et al., 2004), and it was thought that the presence of chrysosplenetin could add value to *A. annua* as a commercial crop – hence the interest in quantifying this compound in Consortium-grown material. It was during this quantification that the correlation of flavonoid levels with those of artemisinin were noticed.

HPLC-DAD separation of extracts of *A. annua* showed a consistent pattern of four flavonoid-type compounds (Figure 54), of which the peak eluting at 14.7 minutes was demonstrated, by

HPLC-MS and NMR comparison with a reference standard, to be chrysosplenetin. An earlier eluting peak chrysosplenol-D, based on LCMS analysis, but insufficient sample was available to allow for NMR analysis of this compound.

8.4.2 Cambridge-grown material

Initially, chrysosplenol-D and chrysosplenetin levels were quantified in three varieties of *A. annua* across a full harvest season (from late July or early August, to 11th October, 2007). The varieties were 1015 (a variety bred by the Swiss organisation Mediplant), 1046-7 (a high artemisinin variety bred within the consortium) and 1012-12 (a low artemisinin variety bred within the consortium).

Chrysosplenol-D levels ranged from 0 in variety 1012-12, to 0.13% w/w in variety 1046-7. Chrysosplenetin levels ranged from 0.01% w/w, in variety 1012-12, to 0.18% w/w in variety 1046-7.

It was noticed in 2007 that chrysosplenol-D levels correlated positively (0.8 - 0.9) with artemisinin levels, and chrysosplenetin levels correlated positively (0.7 – 0.9) to artemisinin levels. The mean ratio of chrysosplenol-D to artemisinin was 0.07 (+- 0.02)– i.e. for every mg artemisinin, there was about 0.07 mg chrysosplenol-D. The mean ratio of chrysosplenetin to artemisinin was 0.11 (+-0.02). This led to the question of whether chrysosplenetin level could be used as an indirect indicator of artemisinin level: a salient point when it is considered that artemisinin is difficult to quantify by UV-based detection methods, such as HPLC-DAD, and must instead be assayed by such means as LCMS. For those laboratories, particularly in the developing world, that do not have access to these more expensive means of detection, a reliable UV-based method would provide a suitable compromise.

The following year (2008), eleven varieties of *A. annua* were assayed for chrysosplenol-D , chrysosplenetin and artemisinin content. Included in these eleven were the three varieties that had originally been assayed in 2007. Rather than assaying over the entire harvest season – an unfeasible amount of work for eleven varieties – samples were assayed from three environmental conditions:

1. Early in the harvest season (31st July 2008)
2. Late in the harvest season (22nd September 2008
3. Late in the harvest season following exposure to Glyphosate (crops sprayed with Glyphosate on 8th September 2008, and harvested on 22nd September 2008)

Again, a positive correlation between artemisinin and flavonoid content was seen – and the correlation was greatest (0.7 – 0.8) between artemisinin and chrysosplenol-D. The correlation was less strong (0.5 – 0.6) between artemisinin and chrysosplenetin. The mean ratio of chrysosplenol-D to artemisinin was 0.06 (+- 0.02), and the mean ratio of chrysosplenetin to artemisinin was 0.13 (+- 0.03). Both figures are similar to those observed in 2007.

The effect of glyphosate on flavonoid content and hence flavonoid: artemisinin ratios was highly variable between different varieties of *Artemisia annua* – something that would have to be accounted for if flavonoid profiling were to be used to indirectly quantify artemisinin levels.

8.4.3 Other material – Consortium material grown outside of Cambridge, and non-Consortium material

Upon examination of the chrysosplenol-D: artemisinin ratio in both consortium material grown outside of Cambridge, and in non-Consortium material, it became apparent that the chrysosplenetin: artemisinin ratio, whilst averaging at 0.04, shows too much temporal, geographical and genetic variation to provide a consistent, universal means of indirect artemisinin assessment. Certainly, chrysosplenol-D levels could not be used to identify artemisinin levels in an unknown sample.

A similar situation is observed with the chrysosplenetin: artemisinin ratio, which averaged at about 0.12.

However, the results do show that flavonoid: artemisinin ratios are constant within batches (e.g., in material grown in one location during one summer, where material is harvested and dried in the same manner). In this case, the grower would have to determine the ratio for that batch early in the season – which could then be reliably presumed to remain constant. It has also become clear that a strong positive correlation between artemisinin and chrysosplenol-D

exists – this was present in all material assayed, both consortium and non-Consortium, within and outside of Cambridge. Such a link may be due to the fact that both artemisinin and the methoxylated flavonoids are trichome-based – as trichome number increases, the levels of all trichome-based components may be hypothesised to increase, so it may be that by measuring flavonoid level, the user is indirectly performing a trichome count.

As interesting as this information is, the usefulness of flavonoid quantification a as an indirect means of artemisinin quantification is fairly limited because of the extra-batch ratio variation, and it is reasonable to suppose that the TLC densitometry system of artemisinin quantification (described in Section 6) is a more reliable means of quality assessment for those without access to LCMS. However, this experiment does show that breeding new *Artemisia annua* varieties for high artemisinin content also results in plants with a high flavonoid content – potentially increasing the commercial value of the crop, as (discussed below) chrysosplenetin demonstrates potential as an anti-cancer medication.

8.4.4 Anti-cancer potential of chrysosplenetin

The data obtained in this study demonstrates that chrysosplenetin could have potential in the treatment of cancer – the compound was able to selectively inhibit the growth of human breast cancer cells at 0.4 µM, whilst non-cancerous cells remained unaffected up to levels 50 times greater than this. Previous reports exist showing that methoxylated flavonoids can inhibit the growth of cancerous cells – casticin, for example, was shown, at levels of 0.24 µM, to inhibit growth of human squamous carcinoma cells in the G2-M phase. Cleary, this class of compounds deserves further attention as a potential source of anti-cancer drug therapy.

8.5 Acknowledgements

The author would like to thank Somchaiya Surichan and Dyan Ankrett, of the DMU Cancer Drug Discovery Group, for performing the anti-cancer assays of chrysosplenetin.

9 Farnesol as an Inducer of Artemisinin Biosynthesis in *Artemisia annua* Hairy Root Cultures

Synopsis

An attempt was made to increase artemisinin yield in *Artemisia annua* hairy root cultures by feeding with exogenous farnesol. This sesquiterpene has been demonstrated, in cell cultures of *Nicotiana tabacum,* to form part of a signalling pathway that ultimately results in the up-regulation of the mevalonic acid (MVA) pathway. It was hypothesised that uptake of farnesol by *Artemisia annua* hairy roots would result in increased artemisinin level, as a consequence of this up-regulation of the MVA pathway. Farnesol was added, at 12.5 µM to light- and dark-grown cultures. Cultures were harvested after 18 days.

No increase in artemisinin content was observed after this time. However, an increase in another, unidentified compound was observed, with mass fragments – by LCMS (ESI) – of 203.2 and 267.2. These mass fragments match those of deoxyartemisinin, but the retention time of the unknown suggested it was another, as yet unidentified, compound.

9.1 Introduction

Any means by which overall artemisinin yields can be increased are worthy of investigation. This objective has typically been pursued pre-harvest by the spraying of crops with plant hormones, such as the gibberellins (Zhang et al., 2005), or with modification of nutritional regimes (Davies et al., 2009).

Post-harvest, appropriate drying regimes can increase artemisinin yields by optimising the post-harvest conversion of precursors to artemisinin (Ferreira et al., 2010).

For rapid testing of the effects of pre- and post-harvest treatments on artemisinin yield, a model system for the field crop is necessary. Greenhouse grown plants can take several months to reach maturity, and space considerations render use of such plants unwieldy. Axenic shoot cultures of the plant – developed in-house and shown to produce artemisinin at yields of around 10% that of field-grown plants (a figure also reported by other researchers

(van Geldre et al., 1997)) – would be the ideal model system, but they are difficult to maintain and hence the production of the volume of shoot material needed for assay is unlikely.

Hairy root cultures, however, are easy to maintain and fast-growing: large volumes of material can be produced within weeks. The major difficulty in the use of *Artemisia annua* hairy roots (AAHR) as a model system for the whole plant is the low level of artemisinin produced by such cultures. When grown under dark conditions – as is typical for root cultures – artemisinin is typically absent, or present only at trace levels. Reports exist demonstrating that artemisinin content in hairy roots can be increased to 1.8 % w/w (dry weight) when roots are grown under 16 hours of 3000 Lux white light (Liu et al., 2002), and to 3.1% w/w (dry weight) by the utilization of red light (Wang et al., 2001). The former level is comparable to the leaf matter of a high-yielding *Artemisia annua* whole plant, and the latter exceeds any reported value for *A. annua* leaf artemisinin content.

Such reports clearly show that under optimal environmental conditions, biosynthesis of artemisinin can be induced in root cultures of *Artemisia annua* – demonstrating the validity of cultures as a model system for the assay of means of increasing artemisinin production.

In this study, AAHR were cultivated in the presence offarnesol (as a mixture of isomers). The reasoning behind the feeding of farnesol brings us to a brief discussion of the biosynthesis of artemisinin.

9.1.1 Biosynthesis

Farnesyl diphosphate (FPP) – a key intermediate at the point where the mevalonate pathway branches into sterols and sesquiterpenes - can be synthesised, *in vivo*, by two mechanisms - either *via* farnesol phosphokinase-mediated phosphorylation of farnesol, or by farnesyl diphosphate synthase -mediated condensation of IPP with DMAPP or GPP. The former was first demonstrated in 1995, when 100,000 *g* pellets from the micro-alga *Botryococcus braunii* were found to be able to selectively convert farnesol to both the mono- and diphosphorylated forms, in the presence of the cofactor cytidine triphosphate (Inoue et al., 1995), but this mechanism has not yet been demonstrated in *Artemisia annua*.

The latter forms part of the later stages of the mevalonate biosynthetic pathway (MVA pathway) in *Artemisia annua* L, discussed in detail in Section 1.3. In this pathway, FPP – formed from the farnesyl diphosphate synthase-mediated condensation of isopentenyl diphosphate (IPP) with dimethylallyl diphosphate (DMAPP) or geranyl diphosphate (GPP) - is the precursor to both the plant sterols and to the sesquiterpenes; the former, *via* the enzyme squalene synthase, and the latter, *via* amorpha-4,11-diene synthase (Bouwmeester et al., 1999).

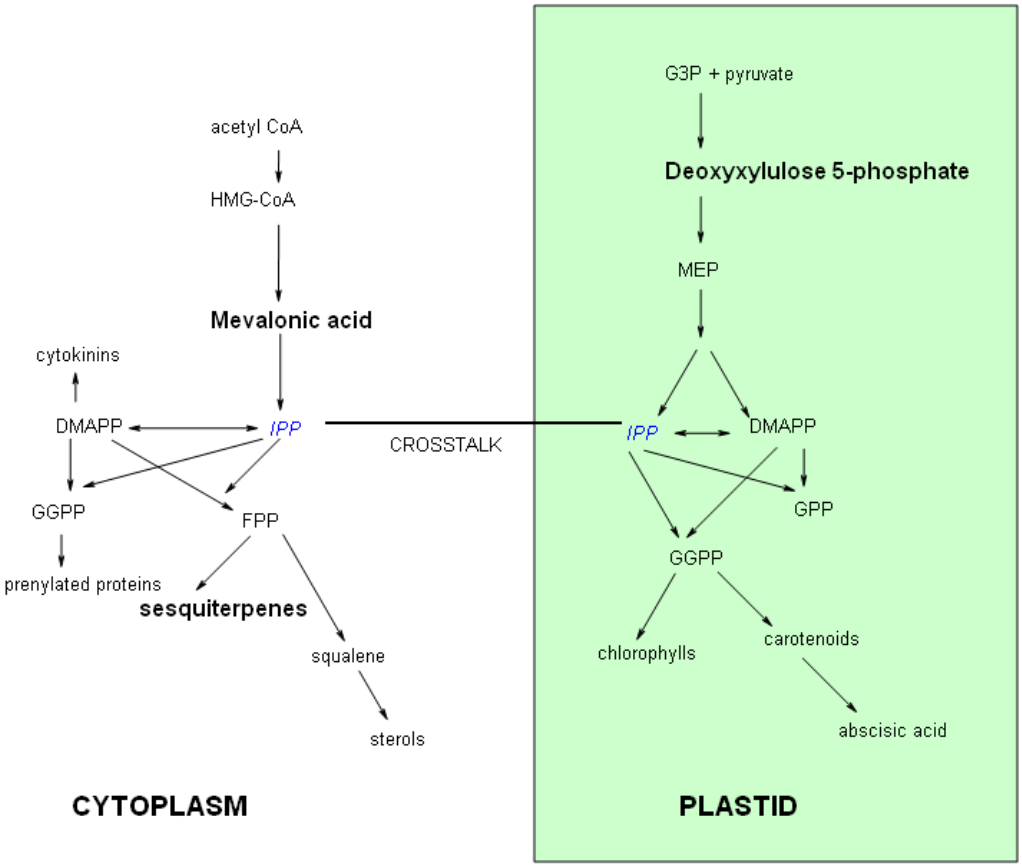


Figure 61. The two pathways involved in artemisinin biosynthesis. From Towler and Weathers (2007)

9.1.2 Farnesol as an inducer of the mevalonate pathway

It had previously been demonstrated that feeding of 1-^{3}H labelled *E-E*-farnesol to cell cultures of *Nicotiana tabacum* resulted in similarly labelled free sterols - the predominant end product of the mevalonate pathway (Hartmann and Bach, 2001). This experiment elegantly demonstrated that exogenous farnesol is capable of being utilised in the biosynthesis of MVA-derived compounds.

The question remains, though, of whether there is a quantitative advantage to feeding of exogenous farnesol – that is, are the levels of compounds downstream of FPP actually increased as a result of such feeding? Farnesol itself is not an elicitor (that is, it is not part of the plant defensive signalling pathway, unlike, for example, salicylic acid, although it is a signalling molecule in bacterial quorum sensing). But Hemmerlin and Bach (Hemmerlin and Bach, 2000), reporting that exogenous farnesol could partially reverse mevinolin[5] -induced inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR)[6] claim that that farnesol may have a regulatory effect on an early step of isoprenoid biosynthesis.

They reached this conclusion from the surprising observation that, contrary to previous reports of a farnesol-mediated degradation of HMGR in mammalian cells (Correll et al., 1994), in *N. tabacum* cell cultures, exogenous farnesol (at levels below 12.5 µM) induced microsomal HMGR activity. Crucially, addition of farnesol at this same concentration, but to an *in vitro* preparation of the enzyme, did not induce enzyme stimulation. This observation prompted the study's authors to claim that the "previously observed activation [of HMGR, by farnesol] was not due to a direct interaction of the compound with the protein", but was in fact part of a cell signalling pathway leading to the upregulation of the enzyme. As such, it is feasible that addition of exogenous farnesol to *Artemisia annua* hairy root cultures can, by up-regulating this rate-controlling enzyme, result in increased levels of artemisinin in the roots.

9.1.3 This study

It was hypothesized that adding exogenous farnesol to the nutrient medium in which AAHR were cultivated, and thereby increasing the pool of farnesol to the cell's machinery, would result in increased biosynthesis of FPP by providing a substrate for farnesol phosphokinase. Furthermore, as Hemmerlin and Bach report, farnesol is capable of up-regulating the activity of the MVA pathway in plant cells. So, it was further hypothesised that via both of these

[5] Mevinolin is a fungal metabolite isolated from *Aspergillus terreus*. It acts to inhibit the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR; also HMC-CoA reductase [see Footnote 4]), the rate-controlling enzyme of the mevalonate biosynthetic pathway. Mevinolin blocks the formation of mevalonic acid from HMG-CoA. Because of this function, mevinolin – under the generic name of Lovastatin - has found applications in the pharmaceutical industry as an inhibitor of cholesterol biosynthesis (HR Valera 2005).

[6] "3-hydroxy-3-methylglutaryl-coenzyme A" and "β-hydroxy-β-methylglutaryl-CoA" are synonyms; both are written in shorthand as HMG-CoA. HMGR – also written as HMG-CoA reductase - refers to the enzyme that catalyses the reduction of this compound.

mechanisms, the artemisinin levels of AAHR would be increased following exposure to exogenous farnesol.

As several reports exist demonstrating that hairy roots of *A. annua* grown under light conditions produce more artemisinin than those grown under dark conditions, it was decided to compare the effect of exogenous farnesol on both light and dark grown roots. Souret (Souret et al, 2003) showed that transcription of the enzyme farnesyl diphosphate synthase (FPPS), which catalyses the formation of FPP *via* the condensation of IPP and DMAPP, was slightly upregulated in light-grown hairy roots compared to dark-grown, and that transcription levels of this enzyme correlated positively with artemisinin level[7].

In this experiment, hairy roots of *Artemisia annua* were cultivated under the following conditions (all at 27°C):

1. Under dark conditions
2. Under dark conditions, in the presence of farnesol at a level hypothesised to induce HMGR
3. Under light conditions
4. Under light conditions, in the presence of farnesol at a level hypothesised to induce HMGR

Under dark conditions, it would be expected that FPPS is transcribed only at low levels, if at all, and so downstream products such as artemisinin would not be predicted to be present at detectable levels unless another enzyme (a putative farnesol phosphokinase for example) was active. Under light conditions, FPPS would be predicted to be upregulated, therefore producing FPP – and, downstream, artemisinin at detectable levels - even in the absence of farnesol phosphokinase-mediated mechanism of FPP formation.

[7] It should be pointed out that this light-induced, FPPS-catalysed formation of FPP is distinct from the farnesol phosphokinase- catalysed mechanism of FPP formation that is hypothesised to be affected by exogenous farnesol. The latter mechanism has not been shown to be affected by light.

In the absence of exogenous farnesol, no farnesol-induced upregulation of HMGR would be predicted. But if exogenous farnesol does, as Hemmerlin and Bach claim, upregulate this enzyme, then an increase in MVA pathway activity and hence artemisinin level may be observed.

It was hypothesized that condition 4 – in which both farnesol-phosphokinase, FPPS and HMGR activity can be speculated to be present - would produce the highest levels of artemisinin in *Artemisia annua* hairy roots.

9.2 Materials and Methods

9.2.1 Production of hairy roots of *A. annua*

Seeds of *A. annua* 1057 – a gift from Steven Bentley at the National Institute of Agricultural Botany - were sterilized by first swirling in 70% ethanol for 5 minutes, and then swirling in a 5% aqueous mixture of hypochlorite for 15 minutes. Seeds were then washed several times with double distilled, sterilized water and axenically placed into small jars partly filled with MS medium, pH adjusted with 1M sodium hydroxide to pH 5.8, and solidified with Phytagel. Jars were capped, and the seedlings left to geminate under a 16 hour photoperiod.
At six weeks, seedlings were inoculated with *Agrobacterium rhizogenes* LB902. *A. rhizogenes* was prepared by plating out cultured bacterium onto yeast mannitol plates supplemented with acetosyringone. The bacterium was cultivated for 48 hours on this medium, after which it was used for inoculation. To inoculate the seedlings, a small wound was made in the stem of each seedling using a sterile scalpel. To this wound was applied a small amount of inoculum.
After 4 weeks, some seedlings showed the appearance of hairy roots at the wound site. These roots were removed and placed onto Murashige and Skoog (MS) basal cell culture media, solidified with Phytagel and supplemented with sucrose at 3% w/v and cefotaxime at 0.1g/L. The roots were cultured in the dark at 27°C and transferred onto new cefotaxime-containing media every 3 days until axenic growth was established; thereafter roots were cultured on the same media without cefotaxime.

9.2.2 Culturing of hairy roots in the presence of exogenous farnesol

To 250 ml Erlenmeyer flaks containing 50 ml of sterilized liquid MS media, supplemented with 3% sucrose, 1 g of *A. annua* hairy roots tips were added. Tips were taken from cultures grown for 18 days on solidified MS and sucrose solidified media, as described above. To half of the liquid cultures farnesol, prepared as a solution in DMSO, was added at a final concentration of 12. 5 µM. To control cultures, equal volumes of DMSO were added.

9.2.3 LCMS for the quantification of artemisinin

LCMS was performed as described in Section 3.2.2. – 3.2.3.

9.2.4 Fluorescence quantification

Fluorescence of root media was quantified with the use of a SpectraMax 96-well plate reader, using SoftMax Pro software. Wells were filled with 200 µl media. Excitation was set at 350 nm, and emission was read at 400 nm.

9.3 Results

Table 19. Root growth and chemical profile of *Artemisia annua* hairy root cultures, with and without exposure to exogenous farnesol

Treatment	Mass of roots (dry weight, g)	Loss on drying (%)	Artemisinin as mg/kg dry weight	Fluorescence of media (Fl. units/ml)	Putative unknown g/kg dry weight
Dark	0.2 (0.07)	89.9 (1.2)	1.0 (1.7)	0.61 (0.10)	3.8 (0.2)
Dark and Farnesol	0.3 (0.07)	90.8 (0.3)	0	0.38 (0.12)	13.1 (8.1)
Light	0.6 (0.07)	93.4 (0.7)	2.0 (0.6)	0.23 (0.12)	1.3 (0.6)
Light and Farnesol	0.5 (0.06)	92.7 (0.6)	1.6 (0.9)	0.24 (0.05)	4.7 (0.3)

9.3.1 Growth of hairy roots

As Table 19 shows, light-grown hairy roots reached a greater final mass, as compared to those kept under dark conditions – at the end of the growth cycle, the mass of light-grown roots ranged from an average of 0.6 g dry weight in control root cultures, to 0.5 g in light-grown roots exposed to exogenous farnesol. The mass of dark-grown root cultures ranged from 0.2 g to 0.3 g dry weight, with the former being control cultures and the latter exposed to exogenous farnesol.

Farnesol could not be demonstrated to have any significant effect, positive or negative, on final root mass, regardless of whether roots were cultivated under light or dark conditions. However, growing roots under light conditions did slightly increase the water content of those roots, as evinced by the increased loss on drying observed in light-grown root cultures.

9.3.2 Chemical content of hairy roots in farnesol-exposed and control samples

9.3.2.1 Artemisinin

Artemisinin content was very low in all cases, ranging from undetectable in dark-grown roots exposed to farnesol, to 2.0 mg/kg DW in light-grown roots without exogenous farnesol. It will be noted that the standard deviation for this mean artemisinin content of dark-grown roots without farnesol is actually higher than the mean itself, because of large difference in individual results within this sample set. No significant difference between the artemisinin content of light-grown roots, whether treated with farnesol or not, could be observed. Deoxyartemisinin was not detected in any cultures.

9.3.2.2 Fluorescent root exudates

It was observed by the author that roots of *Artemisia annua* L. – both *in vitro* and *in vivo* – exude a fluorescent compound or compounds into the aqueous surroundings – soil, in the case of whole plants, or liquid media in the case of hairy roots (Figures 62 – 63). The fluorescence displays a maximum in the region of 350 – 400 nm.

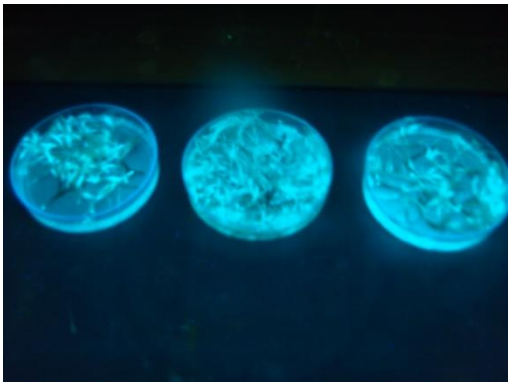


Figure 62. Hairy root cultures of *A. annua*, growing on Phytagel solidified MS media containing 3% w/v sucrose, exhibitng root fluorescen

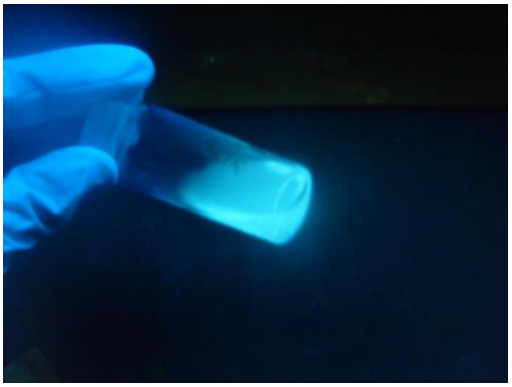


Figure 63. Liquid MS medium in which hairy root cultures of *A. annua* had grown for 21 days, exhibiting fluorescent exudates.

Difficulties in isolation of these highly polar compounds have prevented separation and purification of the compound/s responsible for this rare phenomenon of root fluorescence. To date, all that can safely be claimed as regards the nature of the fluorescent compound/s is that, as a positive response to ninhydrin is observed, a nitrogenous class of compound can be postulated – perhaps an iminosugar, or an amino-acid derivative.

The level of fluorescent root exudates, which were measured in arbitrary units of fluorescence and converted here to units of fluorescence/mg root (DW), appears upon first glance to be highest in those roots that were grown under dark conditions, without farnesol. But it should be borne in mind that the actual root mass was considerably lower in these dark grown roots, artificially inflating the fluorescence/root mass ratio. What this may show, though, is that fluorescent root exudates build up to a final concentration in the earliest days after inoculation, and do not increase further after that point, regardless of whether root mass continues to increase.

9.3.2.3 Unknown compound

Finally, an unknown compound, with a mass spectrum containing two major fragments of mass 203.2 and 267.2, was observed. Although these fragments match those obtained by the electrospray ionisation-induced fragmentation of deoxyartemisinin, the retention time of this unknown compound, at 6.8 minutes, differed from deoxyartemisinin (which under this separation system elutes at 5.2 minutes).

A clear difference between levels of this compound was observed in the sample sets: notably, those roots treated with farnesol showed greater levels of this compound as compared to equivalent roots that were not exposed to farnesol. A strong inverse correlation of -0.8 was observed, in dark-grown roots exposed to farnesol, between artemisinin levels, and levels of this unknown: that is, the higher the artemisinin level, the lower the level of this unknown. The correlation was -0.6 in light-grown roots with no exogenous farnesol. No correlation was observed in dark-grown roots without farnesol.

It should be noted that as no reference compound for this unknown was available, the levels of the compound were calculated using the deoxyartemisinin calibration curve, which may not be appropriate – hence the description of "putative" level. However, the correlation between artemisinin and this unknown should still hold.

9.4 Discussion

It is clear from this short experiment that the application of exogenous farnesol to hairy root cultures of *Artemisia annua* L. did not result in an increase in artemisinin levels: if anything, a slight decrease was observed, although this decrease was not significant. It was also notable that artemisinin level, even in light-grown roots, were very low: certainly not at the levels (up to 3.1 % w/w DW, equivalent 31 g/kg) that have occasionally been reported by other researchers, described more fully in Section 9.1. However, these authors did utilise light either quantified in Lux, or of limited wavelength (i.e. red light). In the study reported here, roots were grown under general plant growth room lighting.

In dark-grown roots with exposure to exogenous farnesol, and in light-grown roots without this exposure, an inverse correlation between artemisinin level, and level of an unknown

compound which may have the same molecular mass as deoxyartemisinin, was observed. But it is difficult to explain why such correlations were not observed in dark-grown roots without exposure to farnesol. It may be that both light and exogenous farnesol, independently, trigger production of this compound, which may be a degradation product of artemisinin, as deoxyartemisinin, and 10-hyrdroxydeoxyartemisinin, are hypothesised to be (Creek et al., 2005).

9.4.1 Why no increase in artemisinin following farnesol exposure?

It may be speculated that addition of exogenous farnesol, in an attempt to increase the pool of farnesyl diphosphate available to the mevalonate pathway, could not increase final artemisinin levels directly, because current research demonstrates that artemisinin has a mixed biosynthetic origin – that is, the component parts of the molecule are derived from both the mevalonate and the deoxyxylulose pathways, as discussed in Section 1.3 and illustrated in Figure 61. The most that this experiment may have done is increased levels of a precursor to artemisinin.

Additionally, it may be that sterols were increased during this experiment, instead of sesquiterpenes. As no sterols were quantified, this is a question that cannot be answered.

It is more likely, perhaps, that – as has been reported in other papers – the presence of farnesol, or a product thereof actually acted to induce HMGR degradation. This is a situation known to occur in yeast and mammalian cells, although, as described in the introduction to this section, in *Nicotiana tabacum* cell cultures, farnesol actually upregulated HMGR. However, as Cronin et al. report (Cronin et al, 2000), HMGR can undergo "regulated degradation...including control by a signal derived from the mevalonate pathway product farnesyl pyrophosphate" – as part of a feedback mechanism. So, excess FPP can actually inhibit the MVA pathway and hence reduce levels of MVA-derived compounds.

9.4.2 Summary

It is clear from this short experiment that much more work is needed to determine the effect of exogenous farnesol on the biosynthesis of artemisinin - and also to determine whether the phosphokinase-mediated conversion of farnesol to farnesyl diphosphate even occurs in

Artemisia annua, or whether – as is perhaps more probable – FPP is only formed via condensation of IPP and DMAPP.

Additionally, greatly increasing the sample size, to reduce statistical variation, and sampling root cultures more frequently throughout the growth cycle, would have improved the clarity of the data in this experiment.

Other research groups have had more success with feeding of salicylic acid to *Artemisia annua* axenic plantlets. This compound has recently been shown to up-regulate expression of the HMGR gene in the 24 hours following administration, and it also triggered a temporary increase in expression of the gene for amorphadiene synthase, the first enzyme of the branch of the MVA pathway leading to artemisinin. However salicylic acid did not affect regulation of farnesyl diphosphate synthase (Pu et al., 2009). Pu et al., and Guo et al. a year later, showed that salicylic acid also increases artemisinin levels by inducing an oxidative burst: a shower of singlet oxygen, which triggers non-enzymatic conversion of dihydroartemisinic acid into artemisinin. But whether the application of such compounds will be feasible on an agricultural scale is still to be decided.

10 *Artemisia annua* as Self-Medication

Synopsis

Dried leaf material of *Artemisia annua*, known to contain artemisinin at 0.6% w/w, was used to prepare herbal teas. The teas were produced either by decoction or by infusion, and the preparations were assayed for artemisinin, deoxyartemisinin and chrysosplenetin content. Teas were also prepared using water containing dissolved ferrous salts, to test the effect of these common water contaminants on artemisinin stability in the traditional preparations. Over-the-counter preparations of *Artemisia annua* (in the form of tinctures, capsules of dried powdered leaf and capsules of active pharmaceutical ingredient) were similarly assayed for relevant phytochemicals.

Artemisinin levels were higher in infusions than decoctions, but the reverse was true for chrysosplenetin. Preparations made using iron-contaminated water contained more artemisinin than those prepared using double-distilled water. Deoxyartemisinin levels did not increase as artemisinin levels decreased, indicating that the latter is not degraded to the former.

Over the counter preparations were generally of poor quality, some containing no detectable artemisinin or chrysosplenetin. The exception was a batch of capsules advertised as containing 100mg pure artemisinin, which contained on average 80 mg of the active pharmaceutical ingredient.

10.1 Introduction

Artemisinin was first isolated and identified in 1972, and the molecule was licensed in China in 1986. In the few years that have followed the drug's discovery, artemisinin has come to form the basis of front-line antimalarial therapy in much of the world, in the form of artemisinin-combination therapy, or ACT (Schlitzer, 2008)

10.1.1 Non-tablet forms of *Artemisia annua* L.

Despite the undisputed efficacy of ACT, the drugs do remain out of the reach of many who need them, whether for economical or geographical reasons (Bate and Hess, 2009). For that reason, there are those who propose that self-medication with preparations of *Artemisia annua* L. is a suitable alternative for ACT, under some circumstances. Amongst those making these case are the Research Initiative into Traditional Antimalarial Medicines (RITAM), who argue that the use of teas made of *Artemisia annua* leaf is sustainable as an emergency treatment for malaria for those living in remote areas (Willcox, 2004). Action for Natural Medicine, or Anamed, a charity based on Germany, provide *Artemisia annua* "starter kits", consisting of seeds and instruction for growth, drying and use of the dried herb as an infusion, although they do recommend that the tea is used in combination with another antimalarial, and that only those resident in malarial-endemic regions make use of the tea – i.e. tourists should not use it as prophylaxis.

Both RITAM and Anamed are concerned with the health of locals living in malaria-endemic regions, and not with those wishing only to travel on leisure or business. However, numerous internet-based herbalists target Western tourists, promoting preparations of *Artemisia annua* as tinctures, capsules containing powdered leaf, and capsules containing purified artemisinin. A few sites carry testimonials from users of these products, like that from one user: "*I am happy to have found a natural substance that can help protect me on my business travels to Asia and Africa; I have never enjoyed using the regular anti-malaria tablets".*

The World Health Organisation's stance on these alternative preparations of *Artemisia annua* L. is one of disapproval. In a statement, the WHO writes that "Since the World Health Organization (WHO) recommended the use of artemisinin-based combination therapies for malaria in 2001, a number of other forms of *Artemisia annua L* for use as anti-malaria 'remedies' have appeared", and that the "WHO does not recommend the oral use of any form of artemisinin other than capsules and tablets". In particular, they note that it is virtually impossible for a tea bag to contain the amount of artemisinin required to effectively treat malaria (WHO, 2008).

10.1.2 This Study

In this study, the chemical profiles of a range of non-tablet forms of *Artemisia annua* L. were assayed. In the first part of the study, teas were prepared from dried leaf of *Artemisia annua* L. Teas were prepared as 1g herb in 40 ml water, and were prepared as either infusions or decoctions. In addition, the effect of the presence of iron salts in the water used to prepare the tea was studied. It is known that the peroxide group in artemisinin reacts with ferrous iron in a Fenton-type reaction, yielding radical intermediates and deoxygenated (and therefore pharmaceutically inactive) end-products, such as deoxyartemisinin and 3-hydroxydeoxoartemisinin. Whilst this reaction is essential to the anti-malarial activity of artemisinin in the infected red blood cell, it would be unfortunate if the reaction took place - and artemisinin was deoxygenated - before the medication has even entered the body.

The levels of ferrous salts added to water were chosen to reflect levels typically found in drinking water. The highest level added (600 µg/L) was chosen as it exceeds palatable level (WHO, 2008).

In the second part of the study, over-the-counter (OTC) preparations of *Artemisia annua* L. were purchased from online retailers. Tinctures, capsules of powdered dried leaf, and capsules of purified artemisinin were assayed.

All samples – teas and OTC preparations – were assayed for artemisinin, deoxyartemisinin, and chrysosplenetin.

10.2 Materials and Methods

10.2.1 Infusions

Extraction: distilled water. 1g of dried *Artemisia annua* was weighed and placed into a 100 ml Erlenmeyer flask. Distilled water was heated on a hot plate to a temperature of 90°C, upon which 40 ml was added to the dried herb. The mixture was stirred with a glass rod and set aside for 10 minutes, with regular stirring. After this time, the mixture was filtered through muslin and the herb squeezed to remove all excess liquid. Filtrate was cooled on ice and extracted in 3 x 40 ml dichloromethane. Pooled dichloromethane fractions were dried on a

rotavapor at 50°C, and assayed by LCMS for artemisinin and deoxyartemisinin content, and HPLC-DAD for chrysosplenetin content. All samples were prepared in quadruplicate.

Extraction: water containing iron salts. High iron: To 1L distilled water was added 600 µg of either ferrous sulfate or ferrous chloride tetrahydrate. *Low iron:* To 1L distilled water was added 150 µg of either ferrous sulfate or ferrous chloride tetrahydrate. Samples using iron-contaminated water were prepared as above.

10.2.2 Decoctions

Extraction: distilled water. 1g of dried *Artemisia annua* was weighed and placed into a 100 ml Erlenmeyer flask. To this was added 40ml of water. The mixture was stirred with a glass rod and brought to the boil on a hotplate. The mixture was kept boiling, with regular stirring, for 10 minutes. After this time, the mixture was filtered through muslin and the herb squeezed to remove all excess liquid. Filtrate was cooled on ice and extracted in 3 x 40 ml dichloromethane. Pooled dichloromethane fractions were dried on a rotavapor at 50°C, and assayed by LCMS for artemisinin and deoxyartemisinin content, and HPLC-DAD for chrysosplenetin content. All samples were prepared in quadruplicate.

Extraction: water containing iron salts. High iron: To 1L distilled water was added 600 µg of either ferrous sulfate or ferrous chloride tetrahydrate. *Low iron:* To 1L distilled water was added 150 µg of either ferrous sulfate or ferrous chloride tetrahydrate. Samples using iron-contaminated water were prepared as above.

10.2.3 Analytical conditions

LCMS conditions for the assay of artemisinin are described in Section 3.2.3. LCMS conditions for the assay of deoxyartemisinin are identical, except that the ions quantified for deoxyartemisinin were 203.2 and 267.2. A separate calibration curve of deoxyartemisinin over santonin was prepared, using reference samples of deoxyartemisinin ranging from 2.5 to 15 µg/ml, and santonin as internal standard at 20 µg/ml. HPLC-DAD conditions for the assay of flavonoids are described in Section 8.4.3.2.

10.3 Results

Table 20, below, shows the amount, in mg, of artemisinin, deoxyartemisinin and chrysosplenetin extracted from 1g of dried *Artemisia annua* by 40 ml water infusion or water decoction.

Table 20. Artemisinin, deoxyartemisinin and chrysosplenetin levels in teas of *Artemisia annua* L. A dichloromethane extract, prepared under the same conditions (i.e. 1g in 40 ml DCM) is shown for comparative purposes

Sample	Artemisinin mg	Deoxyartemisinin mg	Chrysosplenetin mg
Water Decoction	0.5 (0.1)	0.08 (0.02)	0.06 (0.01)
Ferrous Sulphate Decoction Low	1.24 (0.06)	0.29 (0.02)	0.04 (0.005)
Ferrous Chloride Decoction Low	0.82 (0.07)	0.28 (0.02)	0.04 (0.003)
Ferrous Sulphate Decoction High	0.61 (0.16)	0.13 (0.03)	0.06 (0.1)
Ferrous Chloride Decoction High	0.83 (0.13)	0.17 (0.02)	0.07 (0.002)
Water Infusion	0.84 (0.13)	0.14 (0.03)	0.03 (0.001)
Ferrous Sulphate Infusion Low	1.67 (0.09)	0.3 (0.06)	0.03 (0.003)
Ferrous Chloride Infusion Low	1.65 (0.15)	0.33 (0.02)	0.04 (0.002)
Ferrous Sulphate Infusion High	1.03 (0.08)	0.16 (0.03)	0.03 (0.001)
Ferrous Chloride Infusion High	1.29 (0.09)	0.15 (0.05)	0.04 (0.004)
Dichloromethane	5.8 (0.3)	0.7 (0.06)	0.48 (0.07)

Table 21 shows the artemisinin, deoxyartemisinin and chrysosplenetin level, as % w/w, in three over-the-counter capsules prepared from *Artemisia annua* L.

Table 21. Artemisinin, deoxyartemisinin and chrysosplenetin content of capsules of prepared *Artemisia annua* L.

Capsules	Artemisinin % w/w	Deoxyartemisinin % w/w	Chrysosplenetin % w/w
Manufacturer A, Powdered Herb Capsules. 425 mg/capsule.	0.008 (0.002)	0.007 (0.002)	0.06 (0.001)
Manufacturer B, Powdered Herb Capsules 750 mg *Artemisia* leaf/3 capsules.	0	0	0
Manufacturer A, Artemisinin Capsules 100 mg art per capsule	37.8 (1.4)	0.2 (0.002)	0

Table 22 shows the artemisinin, deoxyartemisinin and chrysosplenetin level, as µg/ml, in two over-the-counter tinctures of *Artemisia annua* L.

Table 22. Artemisinin, deoxyartemisinin and chrysosplenetin content in tinctures of *Artemisia annua* L.

Tinctures	Artemisinin µg/ml	Deoxyartemisinin µg/ml	Chrysosplenetin µg/ml
Manufacturer C, 1:5 Tincture, 45% Ethanol	0	7.2 (2.5)	30 (0.07)
Manufacturer D, 1:4 tincture, 55% Ethanol	20.5 (2.4)	0.9 (0.5)	200 (0.5)

10.3.1 *Artemisia annua* tea

10.3.1.1 Extraction efficiency

Solvent extraction of *Artemisia annua* L. by dichloromethane resulted in the highest recovery of artemisinin – 5.8 mg from 1g of dried herb. Compared to this, the lowest extraction

efficiency for artemisinin – 8.6 % - was observed in decoctions prepared using distilled water. The highest extraction efficiency for artemisinin – 28.8 % - was observed in infusions prepared using low levels of ferrous sulfate.

Solvent extraction of *Artemisia annua* L. by dichloromethane resulted in the highest recovery of chrysosplenetin – 0.48 mg from 1g of dried herb. Compared to this, the lowest extraction efficiencies for chrysosplenetin – 6.3 % - were observed in infusions prepared using distilled water, and infusions prepared with low and high levels of ferrous sulfate. The highest extraction efficiency for chrysosplenetin – 14.6 % - was observed in decoctions prepared using high levels of ferrous chloride.

10.3.1.2 Tea prepared from distilled water – infusions vs. decoctions

It is clear from the results that water infusions of *Artemisia annua* L. contain more artemisinin than water decoctions – 0.84 mg on average, as compared to 0.5 mg. Whether this is because of higher extraction efficiency of infusions as compared to decoctions, or increased degradation of artemisinin in decoctions compared to infusions, is unclear. What is clear is that although more deoxyartemisinin is observed in the decoction – which may indicate degradation of artemisinin – in fact, the overall ratios of artemisinin to deoxyartemisinin do not change between infusions and decoctions. Both contained deoxyartemisinin at around 14% of the artemisinin level (for comparison, deoxyartemisinin in the solvent extract was 10% of the artemisinin level).

This evidence suggests that if artemisinin is degrading in the decoctions, it is not degrading to deoxyartemisinin – as if it were, then deoxyartemisinin levels would be expected to rise concurrently with a decrease in artemisinin levels. Higher levels of chrysosplenetin were observed in decoctions compared to infusions.

10.3.1.3 Effect of ferrous sulfate on artemisinin and deoxyartemisinin level

The presence of ferrous sulfate resulted in increased levels of artemisinin and deoxyartemisinin in the extracts, as compared to extracts prepared using distilled water. This was true for both infusions and decoctions, and for both low and high levels of ferrous sulfate.

At low ferrous sulfate levels, infusions contained an average of 1.67 mg artemisinin, compared to 1.24 mg in decoctions. At high levels of ferrous sulfate, infusions contained 1.03 mg, compared to 0.61 mg in decoctions. So it is apparent that a dose-dependent effect of ferrous sulfate on artemisinin content is seen - high levels of ferrous sulfate result in lower artemisinin in extracts compared to lower ferrous sulfate levels. But even extracts containing high ferrous sulfate levels contained more artemisinin than extracts prepared using distilled water alone.

Deoxyartemisinin content did not vary between infusions and decoctions prepared in low levels of ferrous sulfate, being about 0.3 mg in both types of extraction. Deoxyartemisinin content did not significantly vary between infusions and decoctions prepared in high levels of ferrous sulfate, being about 0.16 in the former and 0.13 mg in the latter. This is evidence that if the low artemisinin level in decocted samples containing higher levels of ferrous sulfate is due to degradation of artemisinin, then artemisinin is not degraded to deoxyartemisinin but to some other compound.

In decoctions, a reduced amount of chrysosplenetin was observed in samples prepared using low ferrous sulfate levels compared to those prepared in either water, or water containing high ferrous sulfate levels. The presence of ferrous sulfate, at either level, had no effect on chrysosplenetin level of infusions.

10.3.1.4 Effect of ferrous chloride on artemisinin and deoxyartemisinin level

The presence of ferrous chloride resulted in increased levels of artemisinin and deoxyartemisinin in the extracts, as compared to extracts prepared using distilled water. This was true for both infusions and decoctions, and for both low and high levels of ferrous chloride.

At low levels of ferrous chloride, a large difference between artemisinin content in decoctions as compared to infusions was observed – 0.82 mg in the former, 1.65mg in the latter. Artemisinin content did not change significantly when high levels (as compared to low levels) of ferrous chloride were used in decoctions – an average of 0.83 mg was observed in these samples – but high ferrous chloride levels in infusions resulted in a drop in artemisinin contents compared to low ferrous sulfate infusions (1.29 mg in the former, compared to 1.65 mg in the latter).

Despite the striking difference in artemisinin content in infusions vs. decoctions prepared using low ferrous chloride levels (1.65 mg compared to 0.82 mg), very little difference in deoxyartemisinin content was observed (0.33 mg in infusions, 0.29 mg in decoctions). Again this suggests that if artemisinin is degraded in decoctions, the degradation product is not deoxyartemisinin.

Further evidence for this is presented by the fact that a large difference in artemisinin content was observed between decoctions and infusions prepared using high levels of ferrous chloride (0.83 mg compared to 1.29 mg, respectively), but no significant change in deoxyartemisinin level is observed between these two types of sample. Again, it would be expected that of ferrous salts catalyse the conversion of artemisinin to deoxyartemisinin, that deoxyartemisinin levels would rise as artemisinin levels fall. In fact, when all samples prepared in this tea study are taken into account, a positive correlation of 0.9 is observed between artemisinin and deoxyartemisinin levels – strongly suggesting that one is not converted to the other.

In decoctions, an increased amount of chrysosplenetin was observed in extractions prepared using high levels of ferrous chloride as compared to low (0.07 mg as compared to 0.04 mg, respectively). No difference in chrysosplenetin content between high and low levels of ferrous chloride was observed in infusions.

10.3.2 Summary of tea

In summary, it was found that the presence of iron salts, at either 150µg/L or 600 µg/L, resulted in higher artemisinin levels in the final extract than distilled water alone. This was true for both infusions and decoctions, although in all cases infusions contained higher artemisinin levels as compared to decoctions. At the higher iron salt level, less artemisinin was seen in both infusions and decoctions than at the lower salt level. However, even in these cases artemisinin levels were still higher than that observed in pure distilled water.

Deoxyartemisinin levels were independent of these sampling variations.

Chrysosplenetin levels were higher in decoctions as compared to infusions, where both distilled water and high iron salt level was used. Low iron salt level in decoctions did reduce chrysosplenetin levels as compared to decoctions prepared with water or high salt levels.

10.3.3 Over the counter preparations of *Artemisia annua* L.

10.3.3.1 Capsules

Capsules advertised as containing powdered *Artemisia annua* herb were purchased from two manufacturers. Those purchased from Manufacturer A were sold as containing only *A. annua* leaf material. Those from manufacturer B were advertised as containing *Artemisia annua* leaf as part of a mixture of other herbs, and claimed a total content of *A. annua* leaf per dose (3 capsules) of 750 mg.

Capsules from Manufacturer A did contain discernible levels of artemisinin, but the level was low, at 0.008 % w/w. Deoxyartemisinin was present in almost equal amounts. Chrysosplenetin was present at 0.06 % w/w: unusually, this flavonoid was present at higher levels than artemisinin. All previous leaf material tested throughout this investigation contained flavonoids at levels lower than artemisinin: typically, at only 10% of the artemisinin level.

No artemisinin, deoxyartemisinin or chrysosplenetin could be detected in capsules supplied by Manufacturer B.

Capsules advertised as containing pure artemisinin, isolated from *Artemisia annua* leaf material, were purchased from Manufacturer A. The capsules were sold as containing 100 mg artemisinin per capsule, plus bulking agents.

The content of the capsules were weighed and found to contain, on average, 211 mg of white powder. The powder contained artemisinin at 37 % w/w, which amounts to an artemisinin content of 80 mg per capsule.

10.3.3.2 Tinctures

Tinctures of *Artemisia annua* L. leaf material were purchased from Manufacturers C and D. The tincture purchased from Manufacturer C had a tincture strength of 1: 5 (i.e. 1 weight of herb to

5 volumes of liquid), and 45% alcohol was used as the solvent. That purchased from Manufacturer D had a tincture strength of 1:4, and 55% alcohol was used as the solvent.
No artemisinin was detectable in the tincture purchased from Manufacturer C. 7.2 µg/ml of deoxyartemisinin were detected and 30 µg/ml of chrysosplenetin.
The tincture purchased from manufacturer D contained 20.5 µg/ml artemisinin and 200 µg/ml chrysosplenetin. Very little deoxyartemisinin was found: only 0.9 µg/ml. It will be noted that in both these cases, as in the powdered herb capsules, the chrysosplenetin content was higher than the artemisinin content.

10.4 Discussion

Note: in the following discussion, the phrase "DMU study" is used to demote the work carried out by the author and reported in this Section.

10.4.1 Artemisinin levels in *Artemisia annua* herbal teas

10.4.1.1 The literature

In 2004, Mueller et al. published details of a study in which they found that a tea prepared from *Artemisia annua* L. dried leaf material contained up to 94 mg of artemisinin, when 9g dried leaf was prepared as a 10-minute infusion in 1 litre of water. When 5g herb per litre of water was used, the extract contained 47 mg artemisinin (Mueller et al., 2004).

These levels are high; considerably higher than the results reported in this thesis. The *Artemisia annua* dried leaf used by Mueller was reported as containing artemisinin at 1.4 % w/w, which is higher than the 0.6 % w/w reported from the Consortium-grown herb used in the DMU study. Additionally, in the DMU study, 1g of herb was extracted, rather than the 5g or 9g used by Mueller. But even if 9g of Consortium-grown herb had been extracted, it can be extrapolated that maximum artemisinin levels would have been no higher than *(1.67 mg x 9 =)* 15.03 mg.

Mueller's results were questioned by Jansen, in recent correspondence in *Transactions of the Royal Society of Tropical Medicine and Hygiene.* Jansen repeated Muller's experiment using dried leaf material of similar artemisinin concentration, and found that the concentrations of artemisinin in the teas - 24.2 mg/l - were much lower than the 94 mg/L claimed by Mueller.

Jansen concludes that "...the herbal tea approach to artemisinin as a therapy for malaria is totally misleading and should be forgotten as soon as possible" (Jansen, 2006).

Several responses to Jansen's letter were published in the following months, including one by researchers from the Research Initiative on Traditional Antimalarial Methods (RITAM) *Artemisia annua* Task Force, who argued that Jansen's "statement is totally misleading and should be forgotten as soon as possible" (Willcox, 2007). Their reasons for the support of this use of tea include the hypothesis that artemisinin is only one of a range of antimalarial compounds acting in synergy, and that in reality much higher concentrations of *A. annua* tea would be prepared. Mueller et al. responded that in their trial, in 10 human volunteers, a peak plasma level of 240 ng artemisinin per ml was detected - 26 times higher than the minimum artemisinin concentration of 9 ng/ml required for growth inhibition of *Plasmodium falciparum in vitro* (Heide, 2006). In the light of this, it is difficult to explain the high recrudescence rates observed in Mueller's study.

It is also worth noting that even in the original 2004 paper, Mueller notes that the daily recommended dose of 500 mg of artemisinin is not reached, "the traditional *Artemisia* preparations contained at best 94 mg artemisinin/L... i.e. 19% of the usual clinical dose of pure artemisinin (500 mg/d), and resulted in unacceptable recrudescence rates". For that reason, Mueller did not actually recommend the use of *A. annua* tea as a substitute for ACT (Mueller et al., 2004).

10.4.1.1.1 Traditional preparations?

It is worth noting that almost all studies performed on "traditional" preparations of *Artemisia annua* have assumed that traditional preparations involved dry leaf matter, prepared as either decoction or infusion. But a recent paper by Wright et al. (Wright et al., 2010) took a slightly different approach to "traditional" preparations of *Artemisia annua*. The authors followed the precise instructions given in Ge Hong's 1st century text *Zhou hou bei ji fang*, which required that the fresh herb be soaked in water for up to 12 hours, and then wrung, or squeezed, to extract a concentrated juice. No heat was applied. Using this process, Wright et al. found, in the expressed liquid, an artemisinin content of 72.6 mg/L – which compared favourably with that found in an infusion of dried leaf (14.5 mg/L). However, this product, whilst suppressing growth of *P. falciparum in vitro* (a 1: 222,222 dilution of the juice, containing 0.33 ng/ml

artemisinin, inhibited 50% of growth), was not able to reduce parasitemia in *P. berghei* infected mice. But a more concentrated preparation, made not by wringing but by pounding the fresh herb in a pestle and mortar, was able to reduce parasitemia in mice by up to 96%. This pounded preparation contained artemisinin at 293 mg/L, and a 1: 500,000 dilution, containing 0.59 ng/ml artemisinin, was able to inhibit 50% of parasitic growth *in vitro*. For comparison, Wright et al. found that the IC_{50} of artemisinin control was 3.8 ng/ml – considerably higher than the artemisinin content of the effective doses of either type of juice. This implies synergistic action, in the extracts, of artemisinin and at least one other compound.

However, as interesting as this study on the use of fresh *Artemisia annua* leaf is, those bodies that recommend the use of *Artemisia annua* tea do promote the infusion or decoction of dried leaf matter – perhaps to ensure a year-round supply of the herb.

10.4.1.2 The DMU study

The results presented in this study show that *Artemisia annua* tea preparations – whether infusions or decoctions - could not realistically reach the recommended doses of 250 - 500 mg. The highest artemisinin level observed in the DMU study, in an infusion prepared using water containing low levels of ferrous sulfate, was only 1.67 mg from 1 g of herb, or 0.04 mg/ml artemisinin. At such an extraction efficiency, almost 300 g of herb would be required to obtained 500 mg artemisinin in a preparation of tea, if dried leaf material containing artemisinin at 0.6 % w/w was used.

10.4.1.2.1 Ferrous salts

An unexpected outcome of the DMU study was the observation that the presence of low levels of ferrous salts, especially ferrous sulfate, improved the extraction efficiency for artemisinin. This phenomenon was observed in both infusions and decoctions. Although higher levels of ferrous salts somewhat reduced extraction efficiency as compared to low levels, the artemisinin content of all teas prepared in the presence of ferrous salts was greater than equivalent control (i.e. no iron salts) teas.

No evidence that artemisinin was converted to deoxyartemisinin, in the presence of ferrous salts, was observed. It had previously been suggested that the presence of ferrous ions in an

artemisinin solution was likely to induce the degradation of artemisinin to deoxyartemisinin. This observation was made in a study of the microbial metabolism of artemisinin, in which artemisinin was supplied to cultures of *Rhizopus chinensis* and *Cunninghamella elegans*. Deoxyartemisinin was detected in experimental controls which contained no added micro-organisms. The authors of that study hypothesized that artemisinin degradation to deoxyartemisinin was triggered by ferrous salts in the nutrient medium (J-X Zhan, 2002). From this, the author had speculated that the presence of ferrous ions in water used to prepare *Artemisia annua* tea may also trigger this degradation. But this did not appear to be the case.

Creek et al., in a 2005 study (Creek et al., 2005), found that artemisinin was indeed degraded by the ferrous ions, in an aqueous solution, but not to deoxyartemisinin. In fact, the major product formed during the reaction was 3-hydroxydeoxoartemisinin:

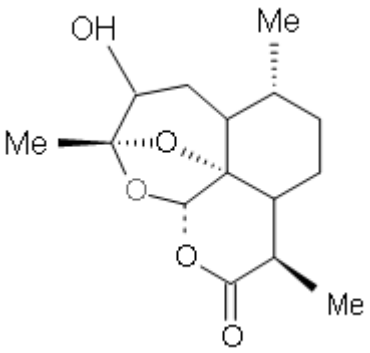


Figure 64. 3-hydroxydeoxoartemisinin

As this compound has the same mass – 282 – as artemisinin, it would have been detected by LCMS at the M + 1 ion: appearing as an additional peak (assuming that the retention time of this compound was different to that of artemisinin). But no such peak was observed in the chromatograms.

It is more likely that the concentrations of ferrous salts used were insufficiently high to induce degradation of artemisinin to the inactive metabolites. In the Creek study, artemisinin was present at 0.03 mM and ferrous sulphate at 3mM – i.e. the ferrous sulfate was in excess, which was not the case in the DMU study, in which ferrous chloride tetrahydrate levels ranged from

0.75 to 3 µM, and ferrous sulfate heptahydrate from 0.5 to 2 µM, but artemisinin reached 237 µM in the highest sample.

10.4.1.2.2 Chrysosplenetin levels in *Artemisia annua* herbal tea

The highest level of chrysosplenetin was seen in a tea prepared by decoction, in the presence of high levels of ferrous chloride. On average, higher levels of this flavonoid were observed in teas prepared by decoction rather than infusion, although some overlap between chrysosplenetin levels in the two types of preparation was observed when low levels of ferrous salts were used. In teas prepared in distilled water, the decoctions extracted twice as much flavonoid as the infusion. However, the levels were low compared to the solvent control.

10.4.2 OTCs

The over the counter preparations of *Artemisia annua* varied widely in quality. Using the capsules of pure artemisinin sold by Manufacturer A, the WHO recommended dose could be reached – but, as this would constitute, monotherapy, would not be recommended. Equally disturbing is the possibility of consumers using these capsules as a form of supplement, or prophylaxis – the use of artemisinin as prophylaxis being not only inefficient but also potentially dangerous for public health, as it increases the likelihood that resistance will develop.

WHO recommended dosages could not be reached with either of the powdered herb capsules tested. However, as those supplied by Manufacturer A did contain low levels of artemisinin, the danger of resistance induction as a consequence of suboptimal dosing remains. A similar concern can be raised by the use of tinctures, in particular that sold by Manufacturer D.

10.4.3 Artemisinin monotherapies

All of the *Artemisia annua* preparations discussed in this section – teas, capsules and tinctures – would be classed as artemisinin monotherapies. Although it has been demonstrated *in vitro* that chrysosplenetin, another constituent of *Artemisia annua* L., can potentiate the anti-plasmodial activity of artemisinin (Elford et al., 1987) until the optimal dosages of chrysosplenetin (and the mechanism of action of this compound) have been elucidated, the presence of this compound in an extract is not sufficient to describe the extract as "combination therapy".

It is also notable that, except for the capsules of pure artemisinin sold by Manufacturer A, none of the preparations discussed would be capable of reaching recommended dosages of artemisinin. Therefore, all of the preparations constitute artemisinin monotherapy, and all but one constitute monotherapy at a suboptimal dose – and, as Bate and Hess write, "exposure to artemisinin monotherapy tablets and substandard drugs has the potential to accelerate parasite resistance to artemisinin" (Bate and Hess, 2009)

The implications of casual use of such preparations vary according to geographical location. In northern Europe, where unregulated use of *Artemisia annua* is likely to be based upon capsules and tinctures, the *Anopheles* mosquito cannot survive, and the ingestion of such products is unlikely to be harmful. The risk is greater when these products are used by visitors to malaria-endemic regions, and the health risks apply to both the visitor, and to the local population.

In malaria-endemic regions, though, the use of *Artemisia annua* tea is more probable than the use of OTCs. As long as licensed artemisinin-combination therapy remains expensive and out of the reach of those who need it, advice such as that given by Anamed – "If you live and work where malaria is a daily hazard, we invite you to grow and use *Artemisia annua anamed* yourself...Harvest the leaves, dry them and store them for use whenever required" - is perhaps understandable. But, apart from the fact that the quality of *Artemisia annua* leaf material varies from year to year depending on environmental factors, there are also pharmacological problems with such usage. As Barnes et al. say, for artemisinin-derived therapies to be effective, "Drug concentrations must exceed minimum parasiticidal concentrations (MPCs) until all parasites are eliminated; this usually takes at least four 48-h asexual life cycles in *P. falciparum"* (Barnes et al., 2008). Whether any form of self-medication will achieve these consistently high drug concentrations has yet to be demonstrated.

11 Discussion: The LINK Project

11.1 *Artemisia annua* L. as a new crop for the UK

11.1.1 Background

The Defra-funded pilot project NF0613 – "Field cultivation of *Artemisia annua* and enhanced extraction of artemisinin used in novel antimalarial treatments" – ran from 2005 until 2006. This £140,046 project, which ran as a collaboration between the National Institute for Agricultural Botany (NIAB), Botanical Developments Ltd (BDL) and East Malling Research (EMR), examined the feasibility of establishing an artemisinin supply chain, from crop in the field to purified active pharmaceutical ingredient (API), based entirely within the UK.

A major component of this pilot project was to "determine the quality and biodiversity of available *Artemisia annua* germplasm" (Defra, 2006). To that end, supplies of *Artemisia annua* germplasm – in the form of seed – were obtained from as many international sources as could be identified, with the double aim of

a) understanding the quality of material currently available, and
b) obtaining genetically diverse material suitable for a large-scale breeding programme.

A total of 32 different lines of *Artemisia annua* were grown in field trials during Project NF0613 (a "line", in this sense, indicating an inbreeding plant variety, in which all the offspring are almost genetically identical). The resulting plant material was scrutinised on two levels: first, for ability to survive and grow well in the UK climate, and second, for artemisinin content – expressed as weight for weight percentage artemisinin in dried plant material. Previous field trials in other countries indicated that artemisinin content in *Artemisia annua* ranged from 0.06 – 1.38 % w/w, as Table 23 illustrates.

Table 23. Artemisinin content in geographically diverse *Artemisia annua* L. (Delabays et al, 2002)

Geographical region of growth	Artemisinin (% w/dry weight)
USA	0.06 – 0.21
Argentina	0.1
Spain	0.24
China	0.79 – 1.07
Vietnam	0.86
Switzerland (Mediplant)	1.38

Analysis of the 32 lines grown in Project NF0613 showed a maximum artemisinin level of 1.2%, in the Swiss Mediplant line. This line was propagated from cuttings and acted as the main control sample in all the analytical work. In addition, three of the 26 lines tested in 2005 had both high artemisinin content and good agronomic characteristics, and these lines were designated 1001, 1019, and 1012.

NF0613 demonstrated that certain *Artemisia annua* lines grew very well under UK weather conditions (summer 2005) and a few of the better adapted lines also had high levels of artemisinin. The fact that some *Artemisia annua* lines could not only grow vigorously here in the UK, but also produce high artemisinin levels, was enough to persuade the members of this new consortium that here was a project that could be taken much further. In fact, estimates based on the artemisinin levels produced in these test crops demonstrated that from one hectare of land, 11kg of artesunate (the drug that is derived from artemisinin) could be produced: enough for 18,000 doses of drug. This compared very favourably to the situation in China, wherein one hectare could, at current productivity, contribute only 3 – 4.5 kg of artesunate (Grewal, 2009).

11.1.2 Next steps – new partners

Project NF0613 - wholly Defra-funded - led to the instigation of project LK0822, which was focused on “Developing an alternative UK industrial crop *Artemisia annua*, for the extraction of artemisinin to treat multi-drug resistant malaria”. This latter, £1,094,395, four-year project

was jointly funded by Defra, the Horticultural Development Council (HDC), and Frontier Agriculture Ltd.

Project LK0822, in line with its greater objectives, included a larger number of partners than the pilot project NF0613. New to the consortium were Humber VHB, a commercial supplier of herbs and salad seedlings, who would evaluate seed production by the newly developed varieties; Frontier Agriculture Ltd, who specialise in helping farmers develop and market their produce, and who would, in this project, concentrate on agronomic techniques for the optimal growth of the crop. De Montfort University's Natural Products Research laboratory, as new partners, would be required to develop improved analytical techniques for the high-throughput analysis of plant samples, as well as devising ways to purify artemisinin from pharmaceutically inactive congeners.

Continuing with the project were the National Institute for Agricultural Botany (NIAB), East Malling Research (EMR) and Botanical Developments Ltd (BDL). NIAB were to take charge of the breeding programme, whilst studies of the plant's nutritional requirements were carried out by EMR. As before, BDL would concentrate on sample analysis and artemisinin purification, in collaboration with DMU.

11.1.3 Next Steps - expanded objectives

Project NF0613 had succeeded in its objective to characterise the available germplasm. Project LK0822, in line with the objective to establish *Artemisia annua* as a viable UK crop, was now to take this further. On the breeding side, researchers at NIAB would instigate a breeding programme, using the best of the germplasm identified in NF0613, to produce a brand new *Artemisia annua* line. This, ideally, would demonstrate vigorous growth combined with a consistently high artemisinin content. It would also produce large amounts of seed, as quantified by Humber VHB. Frontier Agriculture Ltd would take the new varieties and develop optimal sowing and harvesting techniques, and EMR would vary the crop's micronutrient regimes. Such explorations of the agronomic preferences of the new varieties would allow these new plants to reach their full potential in terms of artemisinin yield per hectare.

On the analytical side, Natural Products Research (NPR) at De Montfort University would quantify artemisinin levels in all of the plant material produced by NIAB, Frontier and EMR, and report the results to the relevant department.
NPR would also report the levels of other potentially useful compounds, such as the methoxylated flavonoids, and the levels of unwanted material – deoxyartemisinin, in the main. Such regular feedback would ensure that the breeding programme and agronomic techniques were effective, and heading along the right direction.

NPR would also work on improved means for the isolation of artemisinin from the plant, with a particular focus on the removal of deoxyartemisinin – a pharmaceutically inactive compound that in all previous assays had been found to co-elute and co-crystallise with artemisinin.

11.1.4 Outcomes

The large-scale cultivation of *Artemisia annua* is an essential step in the stabilisation of the global supply of artemisinin. However, the plant is, essentially, a weed – and a weed hailing from the mountainous regions of China, a climate quite different to that of the UK. Although the pilot project had shown that the growth of the plant in the UK was feasible, it was felt that continued improvements in artemisinin yield could be made by a combination of breeding new varieties and optimising agronomic practises.

Over the last four years, the combined efforts of the Consortium partners have produced, year-on-year, new varieties that show consistently increased artemisinin levels as a weight/weight percentage. A maximum level of 1.2 % w/w was measured during the 2005 - 2006 pilot project. By the end of 2009, after the growth and analysis of around 6000 plants, a variety that produced artemisinin at a level of 2.2% w/w - almost a 100% increase – was developed, and the artemisinin content of this new line was externally validated (see Section 3.3.4). Moreover, seed yields from the newly developed varieties – initially as low as 7g/plant – were now as high as 30g/plant. Agronomic techniques had also helped to improve overall yield per hectare, as described in the Consortium's 2009 Annals of Botany publication. Assuming a constant artemisinin level of 2% w/w, one hectare of *Artemisia annua* could yield up to 60 kg of artemisinin (Colin Hill, personal communication). These figures compare well with the 2010 industry average of 0.8 % w/w (dry weight) artemisinin in leaf material of *Artemisia annua*, from which a maximum artemisinin yield per hectare would not exceed 24 kg.

11.1.5 The future

Given the success of this four-year LINK project, the Consortium is presently applying to Defra for extended funding, with the combined objectives of continuing to raise artemisinin yields by improved breeding and agronomic techniques, and the isolation and testing of other bioactive compounds from *Artemisia annua* L. It is also to be hoped, now that the expertise is in place, that this "artemisinin supply chain" can act as a model for the development of other non-food crops for the UK agricultural market.

12 Summary

12.1 The economics of artemisinin

Artemisia annua L. is, at present and for the foreseeable future, the only source of artemisinin for artemisinin combination therapy (ACT). And, as ACTs are, in several regions of the world, the only effective antimalarial drugs left, a stable supply of this parent compound is essential.

However, a stable supply of artemisinin has not yet been established. 2006 was the last year in which global supply of artemisinin exceeded global use of ACT treatments – since that time, demand for the drug has steadily increased, whilst global supply of artemisinin has decreased (Grewal, 2009) Part of the reason for this decrease in artemisinin production is that, initially, high prices for the compound resulted in global overproduction. This overproduction led to something of a glut in the market, and, naturally, reduced prices followed, leading many small-scale growers of *Artemisia annua* L. to abandon the cro*p.* This "boom and bust" pattern is predicted to continue until the global supply of the compound is stabilised, and it has been estimated that by 2015, a "significant increase in supply [of artemisinin will be] needed to meet steady state demand" (Grewal , 2009).

12.2 A UK-based supply chain – issues of quality control

The objective of the Consortium was to demonstrate that an artemisinin supply chain – from crop in the field to pure artemisinin – could be established by large-scale commercial growers within the UK – helping to ensure a guarantee artemisinin yield and hence stabilising global supply. But for a supply chain to succeed, rapid, reliable and economical quality assessment had to be put in place, for all stages of the chain – from fresh herb in the field, to dried leaf in the laboratory, and for partially-purified and purified artemisinin.

This thesis presents methods developed for:

a) Field-based quantification of artemisinin in dried leaf, based on TLC-densitometry
b) Laboratory-based high-throughput quantification of artemisinin in dried leaf and partially pure and purified extracts, based on LCMS-ESI

The former technique, requiring a minimum of user training and utilising a basic desktop scanner, would be applicable not only to UK-based artemisinin supply chains, but also to small-scale growers in developing countries. Whilst densitometry as a means of quality control is not a new technique, the work presented in this thesis represents the first time that inexpensive Biosoft® software – which costs less than £200 – and a basic desktop scanner have been utilised for this purpose.

The latter, LCMS-based technique, does require training and investment on the part of the end-user. But this thesis demonstrates that by using narrow-bore columns and reducing run times, large savings can be made on solvent purchase and disposal with no reduction in precision and accuracy. Validation of this method by an external laboratory confirms the efficacy of this high-throughput technique.

Furthermore, Sections 5 and 7 demonstrate that, having identified material with a high artemisinin content, the artemisinin can be isolated from the closely-eluting, pharmaceutically inactive deoxyartemisinin, using standard flash chromatography equipment.

To persuade commercial growers to adopt *Artemisia annua* as a crop, added value – as in other compounds, besides artemisinin, that can be isolated and marketed form the crop – is an important issue. As part of the work submitted in this thesis, the levels of chrysosplenetin – a flavonoid found within leaf trichomes of the plant – were quantified, and chrysosplenetin itself was assayed against human breast cancer cells. It was shown that chrysosplenetin, which is found in *Artemisia annua* at levels of up to 0.17% w/w, was able to inhibit grown of human breast cancer cells at an IC50 of 0.4 µM – compared to an IC_{50} in control, non-cancerous cells, of 20 µM.

Additionally, whilst carrying out the quantification of chrysosplenetin, a correlation between artemisinin levels, and level of another flavonoid-type compound – hypothesised to be eupatin – was observed. This correlation – greater than 0.8 - was present in all leaf material analysed, and may well provide an indirect means of artemisinin quantification. Artemisinin has no UV-chromophore and therefore requires more complex and expensive means of analysis – typically MS-based. For those without access to LCMS, flavonoid quantification may provide an alternate means of quality control.

12.3 Self-medication

Aside from quality control techniques, a separate theme explored within this work is that of self-medication with *Artemisia annua* L. Despite the WHOs disapproval of this practise, the promotion of *Artemisia annua* tea is widespread, and it is easily to purchase preparations – tinctures, capsules and so on – of the plant. In Section 10, a sample set of *Artemisia annua* teas and over-the-counter products were assayed for artemisinin, deoxyartemisinin and chrysosplenetin content. Although literature reports exist of artemisinin in herbal teas, to the author's knowledge, this thesis represents the first report of deoxyartemisinin and chrysosplenetin levels in tea – and also the first time that the effect of using iron-contaminated water to prepare tea, had been studied.

Section 10 shows that, as others have found, artemisinin content in tea, whether prepared as an infusion or decoction, is very low – in the region of 0.02 – 0.04 mg/ml, and chrysosplenetin levels are even lower, at a maximum of 0.002 mg/ml. As expected, deoxyartemisinin levels were about 1/10th of artemisinin levels, and, perhaps surprisingly, were not increased in the presence of iron salts – even when salts were present at 600 µg/L – a high level for drinking water. This suggests that using iron-contaminated water at these fairly high levels will not be detrimental to the quality of the tea, but the efficacy of tea, considering the low levels of artemisinin, is questionable.

Section 10 also showed that the quality of OTCs varies widely, from tinctures and capsules of dried leaf that contained no detectable artemisinin or chrysosplenetin, to capsules of pure artemisinin containing 80mg/capsule of the compound. But all of these preparations, even those high in artemisinin, containing monotherapy – a practise strongly disapproved of by the WHO, because of the risk of artemisinin-resistant strains of *P. falciparum* developing, a situation that has begun to become reality in Cambodia.

12.4 Reflections

It is clear that, given time and resources, much more work could be done on the potential of establishing *A. annua* in the UK. In particular the levels of more compounds – precursors such as artemisinic acid, and dihydroartemisinic acid, which can be converted to artemisinin – could be quantified. Lack of sufficient reference material prevented such studies being undertaken

for this research. It would also have been illuminating to further explore the use of exogenous compounds to increase levels of artemisinin within the plant. Although Section 8 describes a small experiment of this nature, a much larger experiments using whole plants and a wider range of exogenous compounds – chitosan, or methyl jasmonate, for example – would provide useful information as whether artemisinin levels in a crop can be economically increased.

The stability of artemisinin in stored, dried *Artemisia annua* leaf is certainly worth exploring – an experiment monitoring levels of artemisinin, deoxyartemisinin and 3-hydroxyartemisinin (the latter two being putative microbial degradation products) would either reassure growers that the dried crop is indeed stable in storage, or would provide motivation to either process the leaf immediately upon harvest, or improve storage conditions. If funding became available, this assay would be a matter of priority.

Finally, the anti-cancer potential of chrysosplenetin could also be explored further – perhaps taking into account potential synergistic actions of chrysosplenetin with artemisinin.

Future research, focussing on one or all of these issues, would be most valuable in confirming the feasibility of *Artemisia annua* L. as a new non-food crop for the UK. In the meantime, it is hoped that the work presented in this thesis goes a small way toward this ultimate goal.

Appendix 1. Anti-Cancer Assay of Chrysosplenetin against Human Breast Cancer Cells

MCF7 cells were grown in RPMI 1640 with phenol red and MDA468 cells were grown in RPMI 1640 without phenol red supplemented with L-glutamine (2mM). MCF10A cells were grown in DMEM: Ham's F-12 supplemented with human insulin (10μg/mL^{-1}), human epidermal growth factor (20ng/mL^{-1}) and hydrocortisone (500ng/mL^{-1}). All media were supplemented with 10% (v/v) heat inactivated foetal calf serum (56°C for 45 minutes to inactivate complement components). Cells were maintained at 37°C, 5% CO_2 in air with 100% humidity. Cells at subconfluence were harvested for experimental use as indicated in section 3.2.4.

Cytotoxicity screen

To harvest adhered cells, medium was aspirated and 1mL of a 1% trypsin-EDTA solution was added to the cells and gently agitated for 30 seconds. The trypsin-EDTA solution was subsequently removed and immediately replaced by a further 1mL and the cells incubated at 37°C for approximately 5 minutes or until cells were visibly non-adherent. The resultant cell suspension was placed in a sterile container with 5mL of fresh medium. To determine the density of the cell suspension, an aliquot (100μL) was added to 100μL of a trypan-blue solution (0.4%) and the number of viable cells determined using a Neubauer haemocytometer (depth 0.1mm, 1/400mm^2). The cell suspension was diluted with medium to give a cell count of 2x10^{3}cells.mL^{-1} and aliquots (100μL) were dispensed into sterile, 96-well microtitre plates. With the exception of MCF7 cells, all cells were incubated for 24 hours prior to the addition of the compounds of interest.

For MCF7 cells, after allowing approximately 4 hours for cells to adhere, 100μL of medium containing 10μM TCDD (from 100mM stock in DMSO, giving a final concentration of 10nM) or medium with 0.2% (v/v) DMSO only as a control was added to each well containing cells to give a final concentration of 10nM TCDD and 0.1% (v/v) DMSO, for 24 hours to induce CYP expression. The medium was then aspirated and 100μL fresh medium added.

Chrysosplenetin added to the cells was ten-fold serially diluted in warm medium (37°C), under subdued light, from an appropriate stock (in DMSO) to give final concentrations of 100, 30, 10,

3, 1, 0.3, 0.1, 0.03, 0.01, 0.003, 0.001 and 0.0003μM (in quadruplicate). The final concentration of DMSO did not exceed 0.1% (v/v). Cells were then allowed to grow in the presence of either chrysosplenetin or 0.1% DMSO (controls) for 96 hours to attain 80-90% confluence in the control wells.

After 96 hours 50μL of 2mg/mL^{-1} MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide in sterile phosphate buffer) was added to each well and incubated at 37°C for 2 hours. All medium was then aspirated and the product (reduction of the yellow tetrazolium salt by mitochondrial dehydrogenase of viable cells, forming an insoluble intracellular purple formazan) was solubilised with 150μL DMSO. Plates were vortexed and the absorbance at 540nm determined using a Molecular Devices SpectraMax M5 plate reader with SoftMax® Pro software, version 4.8.

Relative toxicities of chrysosplenetin within each cell line were evaluated by determining 50% of growth inhibition (IC_{50}). The absorbance at 540nm was plotted using non-linear regression curve fit of the percentage of the control value *versus* the negative logarithm of the molar drug concentration range using GraphPad Prism version 3.00 for Windows.

Appendix 2. Reuters News Article on LINK 0822

REUTERS UK

INTERVIEW-Crop scientists treble yield of anti-malaria drug

Wed Dec 2, 2009 10:14am GMT

* Experiments show artemisinin yield of 2.2 percent

* UK crop scientists say want to talk to drug companies

* Artemisinin combination therapy drugs best malaria weapon

By Kate Kelland

CAMBRIDGE, England, Dec 2 (Reuters) - British crop scientists say they have trebled the yield of a key malaria drug ingredient and want to talk to drug companies about how their work could help in the fight against the killer disease.

Steven Bentley, a researcher at Britain's National Institute of Agricultural Botany (NIAB), says four years of experiments in cross breeding wormwood plants has greatly increased the yield of artemisinin, a natural anti-malaria drug.

"Results from this year's harvested trials indicate that we've increased the concentration of artemisinin to in excess of 2.2 percent -- almost three times the industry average of 0.8 percent," he told Reuters in an interview.

"We've made progress year on year, we know where we're going and we seem to able to notch it up with some consistency."

Artemisinin, derived from the sweet wormwood plant, is the best drug available against malaria, especially when used in artemisinin combination therapy (ACT) medicines made by companies such as Swiss drugmaker Novartis AG (NOVN.VX) and France's Sanofi-Aventis SA (SASY.PA).

But low artemisinin yields in the usual growing areas in Africa and Asia have made prices stubbornly high and contributed to a slow roll-out of the treatments across the world.

Around 40 percent of the world's population is at risk of malaria, a potentially deadly disease transmitted via mosquito bites. It kills more than 1 million worldwide each year and children account for about 90 percent of the deaths in the worst affected areas of sub-Saharan Africa and parts of Asia.

A report last month said millions were dying from malaria because they can't afford life-saving ACT medicines. [ID:nL187891]

Bentley, whose work on artemisinin is funded through a British government-backed research project, said he and his colleagues at the NIAB now want a closer relationship with drug companies who use the ingredient.

"We've got great plants, we can do seed production, and we want to work with them," he said.

ESTIMATED NEED

Experts say around 6,500 hectares of land -- most of it in China, Vietnam, Africa and India -- was devoted to wormwood crops in 2009, producing 30 tonnes of artemisinin a year -- enough for 60 million treatments.

The need in 2010 is estimated at 260 million treatments, requiring 130 tonnes of artemisinin or 28,000 hectares of the cro*p.*

Novartis, which Bentley says is already in contact and has been supportive of the NIAB's work, says it is keen to improve access to ACTs and has the capacity to provide 100 million treatments of its Coartem drug a year if orders come in on time.

Bentley said scientists had in the past been dubious about whether good quality artemisinin could be produced in Britain. But with the results he has achieved so far, and trials of NIAB's seed taking place in countries such as Morocco, Canada and Mozambique among others, he says such doubts should fade.

"Many people were understandably sceptical about the ability of anybody in the UK to make a serious contribution, but now we have been able to prove our credentials," he said.

NIAB's yield results have been independently confirmed and Bentley is sure there is more to come.

"We are confident that in our plant breeding material we have better lines coming along that could yield 2.5 percent."

He says there is no great secret to their success other than rigorous scientific methodology and ensuring each cross breed is made using parents with the best genetic potential.

"You've got to have the right genes, just like with humans," he said. "You've got to have the right potential in the plants and then give them the conditions to express that potential."

Weather conditions in Cambridge in eastern England where Bentley works are typical of England -- not too hot, not too cold and with variable rainfall.

Bentley has brought what he calls his "elite parents" into a normal glass greenhouse for

the winter and will plant out new cross-breeds in June for harvest in September.

Once the plants are established, their needs are simple and variations in rainfall don't appear to affect them too much.

"Basically, it really is a very good weed," he said. "It is very vigorous and we don't have many problems with them at all ... The only thing we really need is a scarecrow, because the rooks sometimes come and dig them u*p.*" (Editing by David Holmes) ((kate.kelland@thomsonreuters.com; +44 (0)7990 567 059))

From http://uk.reuters.com/article/idUKGEE5B01SB. Article accessed on 1st March 2010.

Appendix 3. Davies *et el* (2009). Enhancement of artemisinin concentration and yield in response to optimization of nitrogen and potassium supply to *Artemisia annua. Annals of Botany* **104 (2)** pp 315-323

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ANNALS OF BOTANY Founded 1887

Enhancement of artemisinin concentration and yield in response to optimization of nitrogen and potassium supply to *Artemisia annua*

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• *Background and Aims* The resurgence of malaria, particularly in the developing world, is considerable and exacerbated by the development of single-gene multi-drug resistances to chemicals such as chloroquinone. Drug therapies, as recommended by the World Health Organization, now include the use of antimalarial compounds derived from *Artemisia annua* – in particular, the use of artemisinin-based ingredients. Despite our limited knowledge of its mode of action or biosynthesis there is a need to secure a supply and enhance yields of artemisinin. The present study aims to determine how plant biomass can be enhanced while maximizing artemisinin concentration by understanding the plant's nutritional requirements for nitrogen and potassium.
• *Methods* Experiments were carried out, the first with differing concentrations of nitrogen, at 6, 31, 56, 106, 206 or 306 mg L^{-1} being applied, while the other differing in potassium concentration (51, 153 or 301 mg L^{-1}). Nutrients were supplied in irrigation water to plants in pots and after a growth period biomass production and leaf artemisinin concentration were measured. These data were used to determine optimal nutrient requirements for artemisinin yield.
• *Key Results* Nitrogen nutrition enhanced plant nitrogen concentration and biomass production successively up to 106 mg N L^{-1} for biomass and 206 mg N L^{-1} for leaf nitrogen; further increases in nitrogen had no influence. Artemisinin concentration in dried leaf material, measured by HPLC mass spectroscopy, was maximal at a nitrogen application of 106 mg L^{-1}, but declined at higher concentrations. Increasing potassium application from 51 to 153 mg L^{-1} increased total plant biomass, but not at higher applications. Potassium application enhanced leaf potassium concentration, but there was no effect on leaf artemisinin concentration or leaf artemisinin yield.
• *Conclusions* Artemisinin concentration declined beyond an optimal point with increasing plant nitrogen concentration. Maximization of artemisinin yield (amount per plant) requires optimization of plant biomass via control of nitrogen nutrition.

Key words: *Artemisia*, fertigation, malaria, nitrogen, nutrition, potassium.

INTRODUCTION

Current figures suggest that more than 2 billion people are at risk from malaria with somewhere between 300 and 500 million people affected, of whom 2 million die annually (1 million children) (WHO, 2001; Greenwood *et al.*, 2008). Efforts to reduce the impact of the disease are now hampered by single-gene drug-resistant *Plasmodium* (Kindermans *et al.*, 2007). Therapies now include the use of antimalarial compounds derived from *Artemisia annua* (*qinghao* or sweet wormwood), a member of the Asteraceae (Ferreira, 2007; Greenwood *et al.*, 2008). The medicinal properties of this plant have been part of traditional Chinese medicine for at least 1000 years. The World Health Organization (WHO) now recommends the use of artemisinin-based combination therapies (ACT) in regions where the tropical malarial parasite has developed multi-drug resistance to the more common antimalarial drugs (WHO, 2000; Kindermans *et al.*, 2007). Experience clearly shows that single drug treatment approaches are always likely to induce potential resistance and artemisinin appears to be no exception (see Greenwood *et al.*, 2008).

Originally a native of Asia, *Artemisia* now grows wild throughout Europe, North and South America and Australia. The active principal compound (0·1–0·6 % dry weight, Putalun *et al.*, 2007) in *A. annua*, along with an array of other terpenoids, is 'artemisinin' or *qinghaosu* – ([3*R*-(3α, 5αβ, 6β, 8αβ, 9α, 12β, 12α*R*)]-octahydro-3,6,9-trimethyl-, 3,12-epoxy-12*H*-pyrano[4,3-j]-1,2-benzodioxepin-10(3*H*)-one) – a sesquiterpene lactone (Klayman, 1985). This compound was only isolated, identified and clinically evaluated in the 1970s; its biosynthesis has not yet been completely elucidated, but is suggested to involve the cytosolic mevalonate pathway, and the plastid-located deoxyxylulose (also called the methylerythritol 4-phosphate, or MEP) pathway (Covello *et al.*, 2007; Towler and Weathers, 2007). Artemisinin's mode of action is also a subject of much debate and research, but it

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is suggested to include the involvement of the peroxidic oxygen atom and the generation of reactive oxygen species (ROS; see mechanisms review of Krishna *et al.*, 2004; Denisov *et al.*, 2005). There is, however, clear evidence of the inhibitor effects of artemisinin and its derivatives on the development and viability of the protozoan parasite. The uses of plant-based artemisinin compounds are not, however, an insignificant cost, particularly in the developing world where the need is greatest. These costs are in part linked to limitations in the quality of plant material, the climate and general agronomic management. Despite the low productivity of *A. annua* (1·5–2 tonnes ha^{-1}) and artemisinin concentrations (<1 %), production of between 6 and 14 kg of artemisinin per hectare is possible (Kindermans *et al.*, 2007).

There are a number of reasons why we should endeavour to enhance the concentration and yield of this beneficial compound, along with a number of routes by which enhancement might be achieved. However, the aim here is to examine the agronomic requirements for growing *A. annua* in temperate regions of northern Europe. This work forms part of a larger programme devised to enhance artemisinin concentration and supply by conventional breeding and field management.

Changes in the production of secondary metabolites are frequently linked to a number of aspects of the plant's interaction with its environment. It is therefore no surprise that agronomic factors which influence plant growth and development may also influence the accumulation of some secondary metabolites. The involvement of nitrogen nutrition in influencing plant growth and development and biomass production is universally acknowledged (see review of Fernandes and Rossiello, 1995). Based on this knowledge, it seems entirely appropriate to examine the effects of nitrogen as an interactive factor in driving photosynthesis (via Rubisco content), growth and acting as resource of secondary metabolite production (Wang and Weathers, 2007). It has also be hypothesized that environmental stress, such as nutrient deficiency, via the production of ROS, which are themselves scavenged by dihydroartemisinic acid, produces the stable end product artemisinin (Ferreira, 2007).

Some attention has already been given to the importance of mineral nutrition (nitrogen and potassium) in perturbing artemisinin production (Ferreira, 2007; Ferreira *et al.*, 2007). Given the importance of nitrogen in influencing plant biomass production, and of potassium on growth through its role in the regulation of carbohydrate and protein synthesis, it is critically important that we understand clearly how best to maximize biomass production against leaf artemisinin concentration to achieve optimal yield per plant of the active component. The aims of this experiment are to describe how artemisinin yield can be optimized through nitrogen and potassium nutrition when grown in the UK.

MATERIALS AND METHODS

Plant material and experimental set-up

Seeds of *Artemisia annua* L. were produced by Humber VHB (Chichester, UK) in a glasshouse in winter 2006/07 and pelleted with a specialist vegetable pellet and sown and grown individually in 2·5-cm × 2·5-cm and 3-cm-deep modules by Frontier Agriculture Ltd (lot B/2007). Six hundred individual seedlings of *A. annua* were transferred to East Malling Research, where the plants were subsequently grown. In late June, 400 seedlings were selected and potted into 7·5-L pots using Klassman medium Irish graded peat with no added N, P or K, to which 1·9 g of $CaCO_3$ per litre of peat was added to raise the pH to between 5·8 and 6. Chemical analysis of the compost revealed that concentrations of N, P and K were respectively 30, <0·6 and 5·3 mg L^{-1} of compost. Plants were placed in a naturally lit unheated glasshouse for 3 weeks to establish. They were then sorted according to size and placed outside onto a freely draining gravel bed (11 × 4·8 m) in 15 rows, on 19 July; the pots were staggered along the row, with 20 pots per row. Rain infiltration was excluded from the pots, which were covered with plastic. Plants at the end of each row acted as experimental guards; each experimental block was guarded by a complete row north and south, removing edge effects from the experiment.

Experimental treatments

Nitrogen experiment. Nitrogen was supplied to the plants, via a liquid feed system, at six different levels: 6 (N1), 31 (N2), 56 (N3), 106 (N4), 206 (N5) and 306 mg L^{-1} (N6). Ammonium nitrate was used as the source of nitrogen. All other macro- and micronutrients were kept constant and were supplied, as liquid feed, at the following rates: P at 40 mg L^{-1}, K at 156 mg L^{-1}, Ca at 80 mg L^{-1}, Na at 33 mg L^{-1}, Zn at 0·1 mg L^{-1}, B at 0·3 mg L^{-1}, Cu at 0·1 mg L^{-1}, S at 112 mg L^{-1}, Fe at 2·8 mg L^{-1} and Cl at 3·5 mg L^{-1}. The design was a randomized complete block: eight blocks × six treatments; each plot contained three plants, with a total 144 (8 × 6 × 3) plants. The accuracy of the feed systems used to deliver irrigation and nutrition was checked. Nutrient solutions were prepared within the tank and in manner to ensure no precipitation, evaporation (sealed around the injectors) or algal degradation (blackened tanks). Total irrigation volume, theoretically applied, was calculated based on measured inputs from the feed tank system to the pots, on a daily basis, and summed over the experiment. This calculated estimate agreed closely (within <10 %) with the actual quantity of nutrient solution used, based on measuring the tank contents. Total plant nutrient budgets for N and K calculated after chemical tissue analysis, at the end of the experiment, also agreed closely with calculated total applied N and K.

Potassium experiment. Potassium was supplied to the plants, via a fertigation system, at three different levels: 53 (K1), 155 (K2) and 301 mg L^{-1} (K3). Potassium sulphate was used as the source of potassium. Sulphur was applied at a concentration of between 69 and 170 mg L^{-1}, with K1 plants having the lowest concentration and K3 having the highest. All other macro- and micronutrients were kept constant and were supplied, as liquid feed as described above, with N at 202 mg L^{-1}. The design was a randomized complete block: eight blocks × three treatments; each plot contained three plants, with a total 72 (8 × 3 × 3) plants.

For both the nitrogen and the potassium experiments each pot received the fertigation solution via one 2 L h^{-1} dripper. Fertigation started in late July. The plants were inspected

3–4 times a week and the amount of irrigation the plants were given was adjusted to ensure the plants were receiving adequate amounts of water. Daily irrigation times were recorded throughout the experiment to calculate the total amount of water and nitrogen applied to each plant.

Plant measurements

Main stem heights were measured at 7–12-d intervals from late July to mid-September and finally in early October. To determine plant biomass, artemisinin concentration, artemisinin yield and leaf mineral concentration, plants from both experiments were harvested in October. Leaves and stems from a portion of the main stem of each plant, e.g. one side, were removed from the plant base to the tip. The leaf and stem material was collected from each of the three plants per plot and bulked. The fresh weight of this material was recorded. The material was then dried, in a ventilated oven, at 40–42 °C for at least 48 h, and then passed through a sieve (5-mm mesh) to separate the leaf lamina from pedicles. The dry weights of the two components were recorded. Leaf and stem material was stored, at room temperature, in sealed polythene grip bags to await mineral and artemisinin analysis. The stems and the leaves that remained on the three plants were removed and the fresh weight of the material was recorded. The dry weight of this portion of the plant was estimated using the fresh weight to dry weight ratio obtained from the portion of the plant that was dried. The fresh weight of the main stems, for the three plants, was also bulked and recorded, and these were dried as above. Total fresh weight and estimated dry weight per plant were determined.

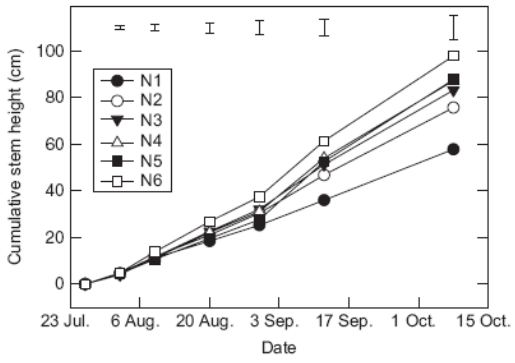

FIG. 1. Changes in stem height of *Artemisia annua* plants grown in pots and fertigated with nutrient solutions containing different concentrations of nitrogen. N1 = 6 mg L^{-1}, N2 = 31 mg L^{-1}, N3 = 56 mg L^{-1}, N4 = 106 mg L^{-1}, N5 = 206 mg L^{-1}, N6 = 306 mg L^{-1}. Bars show l.s.d. at 5 % level.

Plant tissue sampling and analysis of artemisinin by HPLC mass spectroscopy

Reagents and equipment. Santonin was purchased from Sigma (St Louis, MO, USA). Electrospray tuning mix was purchased from Agilent Technologies Ltd (West Lothian, UK). Acetonitrile (HPLC gradient grade) and water (mass spectrometry grade) were purchased from Fisher (Loughborough, UK). Reference artemisinin, in bulk, was provided by AECS Quickprep (Bridgend, UK). The compound was crystallized to a constant melting point of 154 °C and a purity of greater than 99 %. Identity of compounds was confirmed by electrospray mass spectrometry and ^{1}H nuclear magnetic resonance on a Bruker 400-mHz Ultrashield.

Artemisinin (3*a*,5,5*a*,9*b*-tetrahydroxy-3,5*a*,9-trimethylnaphthol[1,2-*b*]furan-2,8(3*H*,4*H*)-dione; $C_{15}H_{22}0_{54}$, mol. wt 282·33) was identified and analysed using an Agilent Technologies 1100 Series high-performance liquid chromatography system, with a GraceSmart RP18-HPLC column (150 mm × 2·1 mm; pore size of 3 μm), coupled to an Agilent Technologies G1946 single quadrupole mass spectrometer with electrospray ionization, used in positive ion mode, with single ion monitoring (SIM). HPLC separation was achieved with an isocratic mobile phase of 60 % acetonitrile and 40 % HPLC-grade water at a flow rate of 0·2 cm^3 min^{-1}. The column oven was set at 25 °C. The spray chamber and mass spectrometer parameters were as follows: nitrogen flow at 8 L m^{-1}; drying gas temperature 350 °C; capillary voltage 2800 V in positive mode, 3500 V in negative mode; nebulizer pressure 40 psig; and fragmentor voltage 70 V. The mass spectrometer was

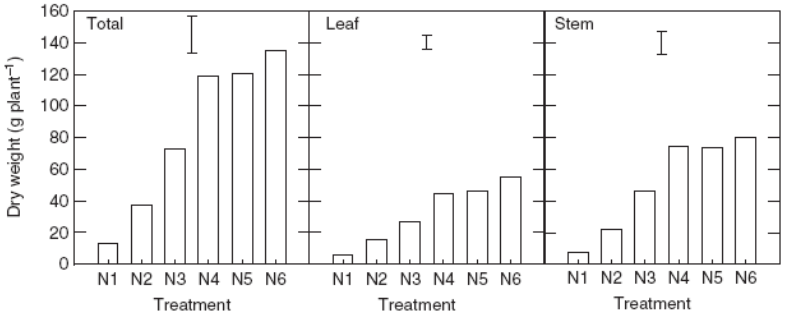

FIG. 2. Total plant dry weight, leaf dry weight and stem dry weight of *Artemisia annua* plants when supplied with differing concentrations of nitrogen. N1 = 6 mg L^{-1}, N2 = 31 mg L^{-1}, N3 = 56 mg L^{-1}, N4 = 106 mg L^{-1}, N5 = 206 mg L^{-1}, N6 = 306 mg L^{-1}. Bars show l.s.d. at 5 % level.

calibrated and, if necessary, tuned daily, using Agilent's Electrospray Tuning Mix, to ensure peak performance.

Generation of calibration curve. Santonin ((3*S*)-3*a*,5,5*a*,9*b*β-tetrahydro-3α,5*a*β,-9-trimethylnaphthol[1,2-*b*]furan-2,8(3*H*,4*H*)-dione; $C_{15}H_{18}0_3$, mol. wt 246·31) was used as an internal standard, with ions monitored at 173·3 and 247·3. The monitored artemisinin ions were 283·3 and 209·3. A calibration curve was generated, in which artemisinin concentration ranged from 2 to 16 μg cm^{-3}, and in which santonin, as an internal standard, was kept constant at 10 μg cm^{-3}. All calibration levels were prepared in triplicate and analysed three times per sample. The ratio of artemisinin to santonin was calculated for each analysis; linear regression of the resulting scatter plot was used to establish the calibration curve. The upper limit of quantification was given as the highest level of the calibration curve: beyond this, saturation of response rendered quantification less reliable. The lower detection limit was taken as five times the standard deviation of the measured response to the lowest calibration level, and was calculated to be 0·8 ng.

Sample analysis. Dried leaf material was ground with a pestle and mortar, and around 100 mg weighed accurately into a 4-cm^3 glass vial (Fisher), to which was added 1 cm^3 of acetonitrile and the vial capped prior to shaking at around 50 r.p.m. for 24 h in the dark. This technique has been found to consistently extract >90% of the artemisinin in a herbal sample. After this maceration 10 μL of liquid was removed and placed in a 2-cm^3 crimp vial with 100 μL of santonin stock solution (0·1 mg cm^{-3} in acetonitrile) and the sample was made up to 1 cm^3 with HPLC-grade acetonitrile. Samples were analysed blind, in a programme that periodically included previously analysed samples to monitor consistency. Precision and accuracy of analyses were measured by the inclusion of samples of known artemisinin concentration.

Statistical analysis

Differences between plants receiving different nitrogen concentrations and different potassium concentrations were determined using analysis of variance. Statistical analyses were performed using Genstat software (v. 9.1, Rothamsted Experimental Station, Rothamsted, UK). Where appropriate, least significant differences (l.s.d.) were calculated and displayed on the figures.

RESULTS

Nitrogen

Differences in stem height of *A. annua* grown under the six different nitrogen treatments were apparent 4 weeks after the start of the treatment applications (Fig. 1). Significant differences in stem heights were apparent during September and at the final harvest in October, with the tallest plants within the highest N concentration (N6) and the shortest at the lowest N concentration (N1). This difference in stem height correlated well with differences in dry matter production in terms of total plant, leaf and stem biomass at harvest (Fig. 2). Increasing the applied N concentration from 6 mg L^{-1} (N1) to 106 mg L^{-1} (N4) increased total plant biomass eight-fold (from 13·4 to 119·2 g), leaf biomass (from 5·6 to 44·7 g) and stem biomass (from 7·8 to 74·5 g). Increasing applied N concentration from 106 mg L^{-1} (N4) to 306 mg L^{-1} (N6) did not significantly increase total plant or stem biomass any further.

Increasing applied nitrogen from a concentration of 6 mg L^{-1} (N1) to 206 mg L^{-1} (N5) increased leaf N concentration from 3 to 6·7% (w/w), while increasing N application above 206 mg L^{-1} had no further effect (Fig. 3). Total amount of leaf N (nitrogen concentration × leaf dry weight) increased with successive increases in N application from 171 mg per plant for the N1 treatment to 3727 mg per plant for N6 treatment. The mean

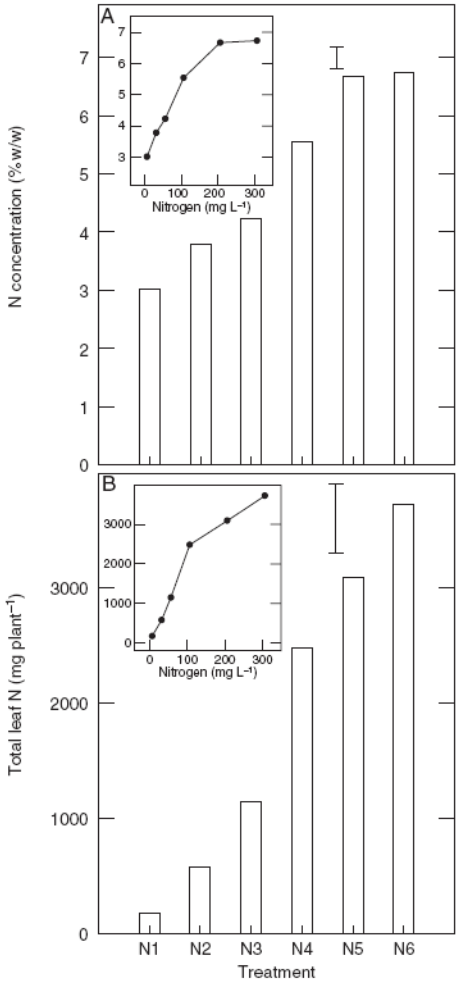

FIG. 3. (A) Nitrogen concentration in leaves and (B) total leaf nitrogen content (nitrogen concentration × leaf dry weight), at harvest, for *Artemisia annua* plants when supplied with differing concentrations of nitrogen. N1 = 6 mg L^{-1}, N2 = 31 mg L^{-1}, N3 = 56 mg L^{-1}, N4 = 106 mg L^{-1}, N5 = 206 mg L^{-1}, N6 = 306 mg L^{-1}. Bars show l.s.d. at 5% level.

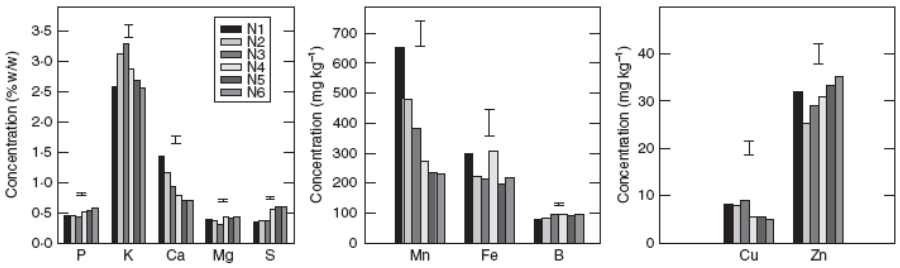

FIG. 4. Phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulphur (S), manganese (Mn), iron (Fe), boron (B), copper (Cu) and zinc (Zn) concentration in leaves at harvest for *Artemisia annua* plants when supplied with differing concentrations of nitrogen. N1 = 6 mg L^{-1}, N2 = 31 mg L^{-1}, N3 = 56 mg L^{-1}, N4 = 106 mg L^{-1}, N5 = 206 mg L^{-1}, N6 = 306 mg L^{-1}. Bars show l.s.d. at 5 % level.

total amount of N applied to each plant was 0·17, 1·05, 2·1, 3·9, 7·6 and 10·7 g for the N1, N2, N3, N4, N5 and N6 treatments, respectively.

Leaf mineral concentrations of calcium (Ca), manganese (Mn) and copper (Cu) all decreased with increasing concentration of applied N (Fig. 4). Leaf K concentration declined with increasing nitrogen application above 56 mg L^{-1}. Phosphorus (P) and sulphur (S) concentrations were higher in leaves of plants receiving N at concentrations above 56 mg L^{-1}. Boron (B) concentrations were higher in leaves of plants receiving N at concentrations above 31 mg L^{-1}.

The concentration of artemisinin in leaves of *A. annua* was at its highest in plants receiving between 6 and 56 mg L^{-1} of nitrogen (N1–N3), where it ranged from 0·86 to 0·93 % (w/w; Fig. 5). As nitrogen concentration increased from 56 to 106 mg L^{-1} the concentration of artemisinin declined to 0·69 % (w/w). A further increase in nitrogen application reduced artemisinin concentration to between 0·53 and 0·56 % (w/w). However, the total yield of artemisinin (artemisinin concentration × leaf biomass) per plant increased, from 0·053 to 0·31 g per plant, as the concentration of nitrogen supplied to the plants increased from 6 to 106 mg L^{-1}. Increasing nitrogen concentrations from 106 to 306 mg L^{-1} did not increase artemisinin yields further.

Potassium

There were no significant K treatment differences in stem height throughout the experiment (Fig. 6). However, increasing K concentration from 53 to 155 mg L^{-1} significantly increased total plant biomass (Fig. 7); this difference was due to an increase in stem weight, there being no significant difference between leaf dry weights. Increasing K concentration from 155 to 303 mg L^{-1} did not increase biomass production further.

Increasing K supply from a concentration of 53 mg L^{-1} (K1) to 303 mg L^{-1} (K3) increased leaf K concentration from 1·8 to 3·4 % (w/w; Fig. 8). The total amount of leaf K (leaf K concentration × leaf dry weight) increased with K application from 706 mg per plant for plants grown with a potassium concentration of 53 mg L^{-1} (K1) to 1524 mg per plant for those supplied with 303 mg L^{-1} potassium (K3). The mean total amount of K supplied to each plant was 2·2, 6·9 and 12·4 g for the K1, K2 and K3 treatments, respectively. Leaf mineral concentrations of N, P, Ca, Mg, Mn and B decreased with increasing K application (Fig. 9). There were no significant differences in leaf mineral concentration for Fe, Cu or Zn.

Concentration of artemisinin in leaves did not significantly differ between potassium treatments (Fig. 10). However, this concentration of artemisinin was lower (0·55 %, w/w) than the maximal apparent for the nitrogen treatments. However, leaf nitrogen concentration measured in the potassium-treated plants was around 6 % (w/w), i.e. the concentration achieved for the N5 and N6 treatments. All K-treated plants were supplied in the N5 range, i.e. at 206 mg L^{-1}, which gave a leaf concentration of 5·5 % N, which supports the expectation of N and K treatment comparability. These nitrogen treatments had very comparable artemisinin concentrations at around 0·55 % (w/w) with respect to the potassium treatments. Total yield of artemisinin (leaf artemisinin concentration × leaf dry weight) was lower for the K1 treatment, but there were no statistically significant differences between treatments (Fig. 10).

DISCUSSION

This experiment shows how nitrogen application enhances plant height and dry matter production in *A. annua*. Nitrogen fertilization appears to increase the allocation of dry matter more to the stem than to the leaf. However, this enhancement in biomass plateaus with the two highest N treatment applications (N5 and N6) and there was no significant increase in stem height or plant biomass above the N4, 106 mg L^{-1}, treatment. Measurement of leaf nitrogen concentration and total leaf nitrogen content (mg per plant) showed, with the exception of the highest N application (N6), that leaf N concentration was directly proportional to the concentration of the applied solution. That is to say, there was no evidence that uptake or demand had been saturated at the higher nitrogen treatments (N4–N6) and that this provided an explanation for a limitation in further growth enhancement at the highest N treatments. Enhanced dry matter production induced by elevated plant nitrogen is extremely well documented and probably driven by increases in photosynthetic efficiency.

An examination of the other key minerals showed that higher nitrogen application was linked to increases in leaf

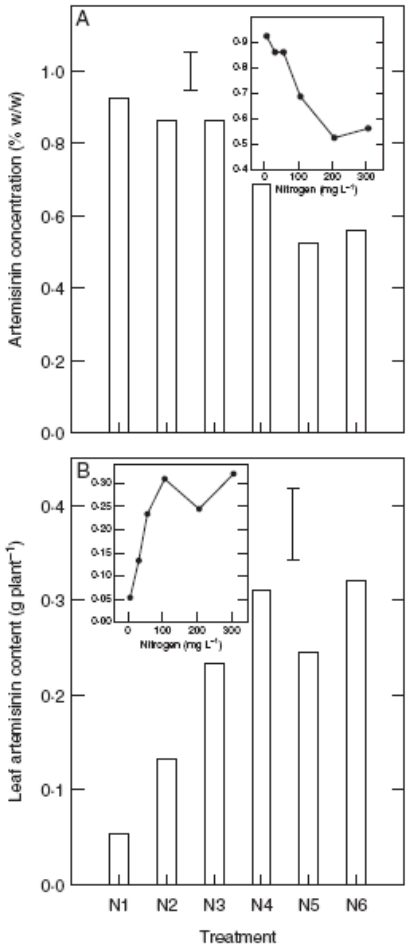

FIG. 5. (A) Artemisinin concentration in leaves and (B) total artemisinin content of leaves (mineral concentration × leaf dry weight) at harvest for *Artemisia annua* plants when supplied with differing concentrations of nitrogen. N1 = 6 mg L^{-1}, N2 = 31 mg L^{-1}, N3 = 56 mg L^{-1}, N4 = 106 mg L^{-1}, N5 = 206 mg L^{-1}, N6 = 306 mg L^{-1}. Bars show l.s.d. at 5 % level.

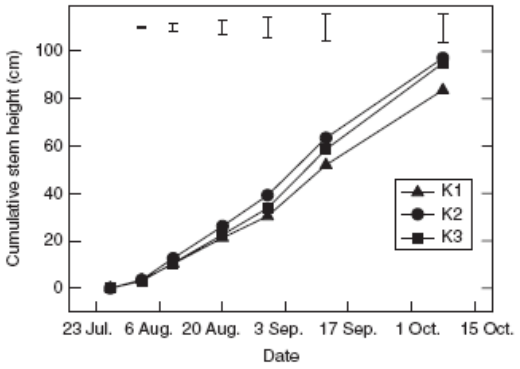

FIG. 6. Changes in stem height of *Artemisia annua* plants grown in pots and fertigated with nutrient solutions containing different concentrations of nitrogen. K1 = 53 mg L^{-1}, K2 = 155 mg L^{-1}, K3 = 303 mg L^{-1}. Bars show l.s.d. at 5 % level.

tissue S concentration and declines in K, Ca and, to a lesser extent, Mn. This may be due to 'dilution effects'; for example, as the growth-limiting element, or nutrient, is applied, the relative rate of dry matter accumulation increases more than the rate of uptake of a particular element causing its concentration to decline (see Jarrell and Beverly, 1981).

Although there was acidification of the compost to pH 4·8, which might be in response to the application of the ammonium nitrate treatment, this effect would have been much the same across all treatments. There did not appear to be any suggestion that either toxicity or deficiency, in regard to S or K, Ca and Mn, respectively, could explain limitations in further nitrogen-induced growth enhancement. We therefore conclude, with respect to growth, that nitrogen application at approximately 100 mg L^{-1}, and a leaf concentration of approximately 5·7 % (w/w), was optimal for *A. annua* growth.

Measurements of leaf artemisinin concentration were highest (around 0·9 %) at the low application rates of nitrogen (N1). As the treatment rate of nitrogen increased, artemisinin concentration declined significantly and was lowest at around 0·5 % for the N5 and N6 treatments. One suggestion is that the increase plant nitrogen status stimulates growth, which dilutes the artemisinin concentration. The interlinking of carbon and nitrogen metabolism beyond the requirements for growth and development are well known (Van Dam *et al.*, 1996; Fritz *et al.*, 2006 and references within). For example, the glucose/fructose ratio may also have a greater stimulatory influence on artemisinin synthesis relative to that of photosynthetically derived sucrose (Wang and Weathers, 2007). Enhanced nitrogen application is well documented in encouraging cell expansion, increases in leaf lamina area and canopy development. Vacuolar sequestration of nitrate appears to be the most likely mechanism driving leaf expansion (Millard, 1988). With monocots (e.g. *Festuca arundinacea*) the increase in leaf elongation rate induced by increased nitrogen supply extends the cell division phase, giving rise to a greater increase in leaf mesophyll cell number compared with adjacent epidermal cells. This was accompanied by a 'compensatory' increase in epidermal cell length (MacAdam *et al.*, 1989). The situation with dicots (e.g. *Ricinus communis*) is, not surprisingly, dependent on the timing of nitrogen enhancement in relation to leaf development stage. Once the full cellular complement of a leaf has been achieved (predetermined) nitrogen status only influences leaf cell size and not cell number (Roggatz *et al.*, 1999).

The influence that nitrogen supply can have on leaf growth provides some insight into a possible mechanism, at least indirectly, by which the observed decline in leaf artemisinin concentration came about. However, it should be noted that a high nitrogen concentration was also apparent in potassium-treated plants, where no increase in leaf biomass occurred. Therefore, simple nitrogen dilution of the leaf artemisinin content may not be the only explanation of the observed

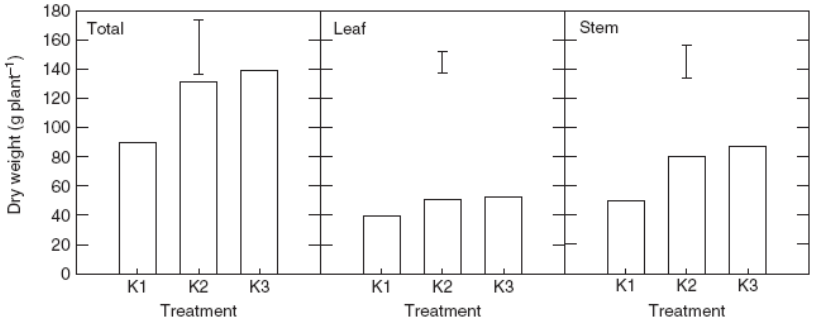

FIG. 7. Total plant dry weight, leaf dry weight and stem dry weight of *Artemisia annua* plants when supplied with differing concentrations of potassium. K1 = 53 mg L^{-1}, K2 = 155 mg L^{-1}, K3 = 303 mg L^{-1}. Bars show l.s.d. at 5 % level.

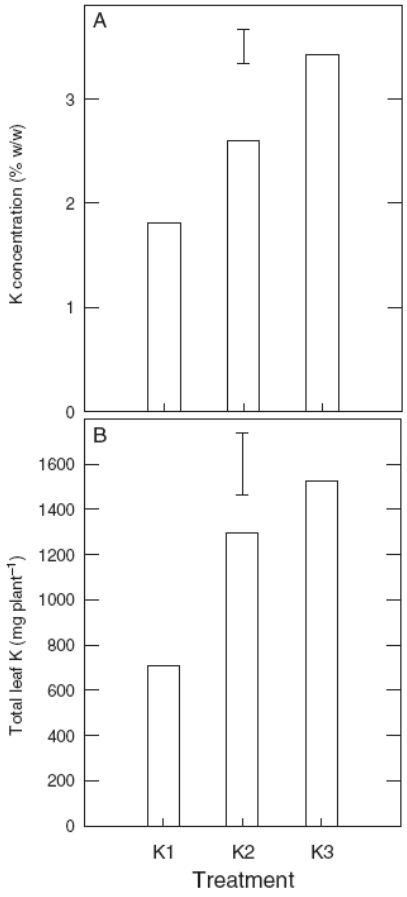

FIG. 8. (A) Potassium concentration in leaves and (B) total leaf potassium content (potassium concentration × leaf dry weight), at harvest, for *Artemisia annua* plants when supplied with differing concentrations of potassium. K1 = 53 mg L^{-1}, K2 = 155 mg L^{-1}, K3 = 303 mg L^{-1}. Bars show l.s.d. at 5 % level.

nitrogen effects. As yet, however, we can only speculate as to how the biosynthesis of sesquiterpenes, such as artemisinin derived from acetyl coenzyme A and glycolytic intermediates, are linked to both nitrogen and carbon supplies.

Artemisinin is found sequestered in the upper sub-cuticular spaces of glandular trichomes, predominantly on leaves and to a lesser extent on flowers (Duke and Paul, 1993; Duke *et al.*, 1994). Glandular trichomes, in general, have been shown to accumulate large quantities of potentially toxic, secondary metabolites (Wagner, 1991; Iijima *et al.*, 2004). Trichomes accumulate and store these compounds, in specific cellular locations, outside metabolically active compartments of the plant; the diverse nature of their chemistry fuels a multi-functional understanding of plant defence options. It is clear that these glands contain cells with metabolically functional chloroplasts containing starch, which in some specific cases are shown to be involved in terpenoid production (Wagner, 1991; Duke and Paul, 1993). This is supported by more recent evidence of terpenoid biosynthetic *in situ* gland enzyme activity, which has developed our understanding of metabolite accumulation in relation to gene transcript levels of various terpene synthases (Iijima *et al.*, 2004).

There are important questions regarding how glandular trichome number might be controlled or what opportunities exist to enhance their number. Trichome morphogenesis is well dissected in *Arabidopsis thaliana* mutants with the role of 40 plus regulatory genes identified (Folkers *et al.*, 1997; Schwab *et al.*, 2000; Ishida *et al.*, 2008) with complementary molecular links to root hair development (see review of Wagner *et al.*, 2004 and references within). Again, it is more recent work with *Arabidopsis* which suggests that epidermal cells produce trichomes in a random fashion (Scheres, 2002). But despite this apparent random origin they subsequently inhibit neighbouring epidermal cells from also producing trichomes. At least for some species (*Betula* spp.) it is suggested that trichome development begins at a very early stage of leaf development; they become apparent at the leaf primordia stage (Valkama *et al.*, 2004). Again in some species (e.g. *Plectranthus*, Laminaceae) this means that final trichome number is fixed early in development (Ascensao and Pais, 1987), while in others cases (e.g. *Mentha* spp.), the ceasing of gland initiation can be directly linked to termination of

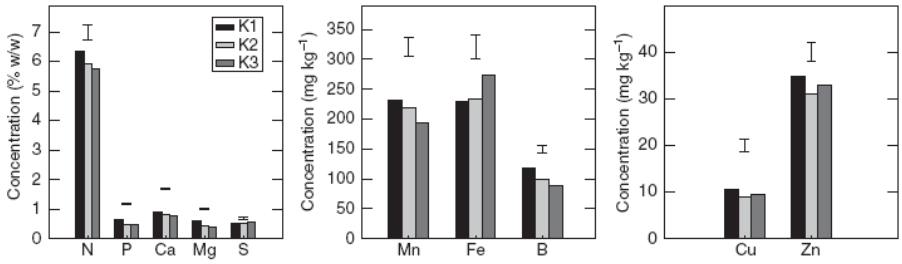

FIG. 9. Phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulphur (S), manganese (Mn), iron (Fe), boron (B), copper (Cu) and zinc (Zn) concentration in leaves at harvest for *Artemisia annua* plants when supplied with differing concentrations of potassium. K1 = 53 mg L^{-1}, K2 = 155 mg L^{-1}, K3 = 303 mg L^{-1}. Bars show l.s.d. at 5 % level.

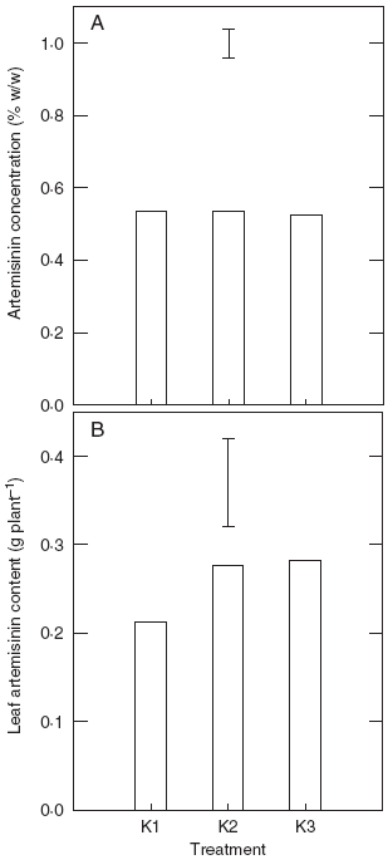

FIG. 10. (A) Artemisinin concentration in leaves and (B) total artemisinin content of leaves (mineral concentration × leaf dry weight) at harvest for *Artemisia annua* plants when supplied with differing concentration of potassium. K1 = 53 mg L^{-1}, K2 = 155 mg L^{-1}, K3 = 303 mg L^{-1}. Bars show l.s.d. at 5 % level.

leaf expansion (Maffei *et al.*, 1989; Turner *et al.*, 2000). In this species there were also clear differences in gland density with leaf node position, early leaves showing much lower densities than those at higher nodes. In *Artemisia* it appears that these glands are present on flowers and leaves at an early stage of development, with leaf primordia being densely covered (Ferreira and Janick, 1996). During later development the early capitulate glands become less obvious as filamentous trichomes develop, with the adaxial leaf surfaces showing rows of trichomes and glands (Duke and Paul, 1993). Conversely, the abaxial surface showed a random pattern of glands. A further complication to understanding the relationship between gland density, development stage and metabolite concentration is the suggestion that artemisinin accumulates predominantly at organ physiological maturity during anthesis (Ferreira and Janick, 1996). How nitrogen nutrition might influence *Artemisia* leaf trichome density is still unclear, but warrants further investigation.

In a practical sense we are particularly interested in optimizing artemisinin yield irrespective of whether this be on a per-plant or per-land area basis. One way to achieve this requires nitrogen optimization based on artemisinin yield per plant, and this can be calculated from the multiplication of leaf artemisinin concentration by leaf dry matter yield. From this the total yield of artemisinin, on a per-plant basis, increased with increasing nitrogen application up to 106 mg L^{-1}. Increasing nitrogen concentration application above 106 mg L^{-1} did not increase artemisinin yield. At this concentration of nitrogen, the plant had a leaf nitrogen concentration of 5·5 % (w/w) at the time of harvest, which was adequate for maximizing artemisinin yields.

Artemisia annua plants treated with different concentrations of potassium showed increased concentrations in their leaves and total amounts of potassium per plant. Optimal *A. annua* growth occurred with the application of potassium at approximately 155 mg L^{-1} (equating to 6·9 g K per plant). Potassium concentrations above this did not increase dry matter biomass, and nor was there any change in leaf artemisinin concentration. However, the total yield of artemisinin, per plant, was slightly higher when potassium application was raised from 53 to 155 mg L^{-1}, although this increase was not statistically significant. A concentration of potassium in leaves of 2·6 % (w/w) at

harvest was adequate for maximizing artemisinin yield per plant.

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