ANTIPLASMODIAL AND PHYTOCHEMICAL INVESTIGATION OF TRADITIONALLY USED ANTIMALARIAL PLANTS OF THE UNITED STATES

by

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and approved by

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ABSTRACT OF THE DISSERTATION

Antiplasmodial and Phytochemical Investigation of Traditionally

Used Antimalarial Plants of the United States

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Malaria is a devastating parasitic disease that causes over 200 million infections and 600,000 human deaths a year. Increased drug resistance to currently used antimalarial therapies signifies a need to discover and develop novel treatments. Although not a present day issue, malaria was once a major concern in the United States and people often utilized local plant species to treat the disease. Since the eradication of malaria from the U.S. in the 1950's, many herbal remedies have been forgotten and survive only in written records. Such historically reported plants may hold potentially potent and novel antimalarial compounds.

This research project was designed to 1) Identify promising plant species of the United States that have been used to treat malaria; 2) Collect, extract and test the *in vitro* antiplasmodial activity of the selected species; 3) Isolate, identify and characterize the phytochemicals within those plants that are responsible for the observed activity.

This work resulted in the *in vitro* antiplasmodial screening of 243 extracts from 46 plant species representing 28 angiosperm families. Six species were selected for bioassay guided fractionation to isolate the bioactive constituents (of which four are described in detail here). Forty two chemical compounds displaying antiplasmodial activity were isolated in pure form, of which 9 novel compounds were newly described. The IC_{50} values of the compounds ranged from 0.11 μM to over 100 μM. All isolated compounds were also tested for cytotoxicity and several for their *in vitro* ability to inhibit *Leishmania tarentolae* as a proxy for antileishmanial activity.

This work provides pharmacological and phytochemical support for the ethnobotanical use of plant species traditionally used to treat malaria in the United States. It also adds to the number and diversity of plant derived antiplasmodial compounds. The rich ethnobotanical history of the United States has been overshadowed as reliance on modern medicine has become the standard. However, as evidenced by this dissertation, traditional remedies, even those that are no longer used, continue to serve as a potentially important source lead for modern drug discovery.

DEDICATION

I dedicate this work to my wife, Allison.

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CHAPTER 1

Introduction

1.1. Goals of the thesis

The specific goals of this thesis were to: 1) Identify and prioritize plant species from the United States with an ethnobotanical history of malaria treatment, 2) Collect high priority leads and subject the extracts to an *in vitro* antiplasmodial assay to confirm bioactivity, and 3) Apply a bioassay guided fractionation regime to the most promising candidates to determine the chemical constituents responsible for the activity. Further studies regarding the suitability of the compounds as drug candidates were dependent upon their novelty, chemical qualities, and bioactivity. For each species studied and compound isolated compound, these characteristics are discussed.

The outline of this thesis is as follows: A general introduction (Ch. 1) is presented to familiarize the reader with the human impacts and biology of malaria, the role of plants in human medicine, and the potential role of plants in the discovery and development of novel antimalarial medicines. The next chapter (2) is concerned with the selection of plants included in the study and discusses the rationale for choosing and the history of those species. Chapter 2 also discusses the preliminary *in vitro* screening results. The following chapters (3-6) discuss individual species that were shown to be active in the preliminary screens. These species are: *Liriodendron tulipifera* L. (Ch. 3), *Cornus florida* L. (Ch. 4), *Datisca glomerata* (C. Presl.) Baill. (Ch. 5), and *Ptelea glomerata* L (Ch. 6). The compound isolation and activity is discussed as are the results and

conclusions particular to each species. A general conclusion and summary is included (Ch. 7) to discuss the overall impact of the findings as well as to provide suggestions for future research.

1.2. The Global Malaria Burden

Nearly half of the world's population lives in malaria-endemic areas (mostly representing underdeveloped nations) and over 200 million people are infected with the disease annually. More than 600,000 people die each year making malaria one of the most devastating infectious diseases worldwide [1]. Despite vast efforts to eradicate the disease – through vaccine development, avoidance measures and drug therapies – the rate of infection and death caused by malaria has changed little in the past several decades [2]. Furthermore, widespread drug resistant strains of *Plasmodium* spp. (the infectious agent), pesticide resistant *Anopheles* mosquitos (the vector), increasing rates of poverty and population density, climate change and an overall decrease in the availability and efficacy of currently used antimalarial drugs increasingly threaten already faltering malaria eradication paradigm [3].

1.3. The Biology of Malaria

Malaria is an infectious disease caused by several species of the genus *Plasmodium* from the apicomplexa; namely, *P. falciparum, P. malariae, P. ovale,* and *P. vivax. Plasmodium* species are effective pathogens with a complex life cycle in which they alternately reside in humans and *Anopheles* mosquitoes (Figure 1.1). Constant and rapid metamorphosis, intercellular movement and immuno-evasion in red blood cells make it

difficult for the human immune system to combat malaria [4]. If left untreated, several erythrocytic cycles – the hallmark stage of the infection - can quickly lead to high levels of parasitemia, resulting in the typical symptoms of the disease: cycles of fever, chills, nausea, vomiting fatigue, and, more dangerously, anemia, cerebral complications and death [5].

Description of malarial disease symptoms can be traced back to ancient Greek Chinese and Indian texts, however, it wasn't until the 1880s that Charles Louis Alphonse Laveran first observed the parasite in the blood of malaria-infected patients and not until the late 1890's that Ronald Ross associated mosquitos with transmission of the disease. Since then, scientific knowledge of malaria and *Plasmodium* biology has grown exponentially. Ultra structural observations, advances in *in vivo* and *in vitro* culturing techniques, biochemical and molecular characterizations, and genomic work have provided an invaluable cache of tools to investigate malaria. A notable achievement in this field was the development of *in vitro* techniques for culturing *P. falciparum* in human red blood cells, which allowed high throughput screening and the elucidation of many validated and potential drug targets (reviewed by [6]) providing the potential for discovery and development of novel-acting antimalarial therapies.

1.4. The Current Status of Antimalarial Drugs

There are a number of approaches for the prevention and control of malaria. These include vector avoidance (i.e. insecticide treated bed-nets), vector control (residual indoor and outdoor mosquito spraying), vaccine development and chemotherapy.

However, with a successful vaccine still in the works, our current best defense against malaria is chemotherapy, and in populations where prophylactic treatment is simply too expensive, quick acting drugs that clear *Plasmodium* infections are most often used.

Currently used antimalarial drugs fall into several categories: aminoquinolines, arylaminoalcohols, 8-aminoquinolines, artemisinins, antifolates, inhibitors of the respiratory chain and antibiotics [7] (Table 1.1). Quinine, which is derived directly from *Cinchona* spp., was isolated in 1820 and was the only drug in pure form used to treat malaria for more than one hundred years [8]. In the 1940's, chloroquine was introduced as a less expensive and more effective alternative and quickly became the mainstay of antimalarial drug therapy and pioneer of the malaria eradication era in the 1950's. The natural endoperoxide, artemisinin, was discovered from the traditional Chinese medicinal plant *Artemisia annua* in 1972 and influenced the semisynthesis of several analogues, which have become the current first choice in front line antimalarial treatment [9].

Unfortunately, the successes of modern drug development were not long lived. When chloroquine resistant *P. falciparum* strains first began to appear in Africa during the late 1970s [10], our medicinal advantage over malaria began to fade. Since then, antimalarial chemotherapy has been dominated by the cyclical development of new drugs (often as modifications of existing drugs) and the subsequent appearance of drug resistance. This fact is nowhere more disturbing then in reports of resistance to artemisinin in Southeast Asia [11]. The therapeutic 'arms race' between new drug

entities and resistance is a constant concern as the synthetic drug pipeline dries up[7]. Antimalarial drugs are also becoming progressively more costly in a time when increasing world population, climate change, and political distress are exacerbating an overall inability to afford antimalarial treatment [12]. These two problems - resistance and cost - must be addressed for malaria to be successfully controlled.

1.5. The Role of Plants in Antimalarial Drug Discovery

The estimated 300,000 species of higher plants contain a pharmacopeia of complex and unique chemical compounds that are employed *in planta* toward numerous ends from primary metabolism and growth, to antipathogenic activity and reproduction [13]. Humans have taken advantage of this fact by utilizing plants medicinally for millennia [14].

In our current medicinal repertoire, the plant-derived compounds quinine, artemisinin, and their derivatives account for more than half of the WHO's accepted antimalarial remedies (Table 1.1, asterisks) [5]. Quinine, being derived from *Cinchona* spp. and artemisinin, derived from *Artemisia annua*, represent inspiring and triumphant stories of ethnobotanical knowledge influencing the discovery and development of potently bioactive compounds that have been used to save countless lives. However, more than 1,200 plant species are known to be traditionally used for malaria treatment [15]. This ethnobotanical knowledge may similarly serve as a guide in elucidating novel antimalarial compounds of comparable or superior activity to quinine and artemisinin.

In fact, hundreds of antimalarial compounds have already been described from plants and other natural sources, some of which hold clinical appeal (see [16] for a review).

While a select number of these compounds have garnered attention as drug candidates, few have been viewed as integrated botanical therapies. On the one hand, this neglect originates in the highly complex nature of bioactive plant extracts, with may compounds of varied activity. For example, quinine, long thought to be far superior to crude cinchona bark, has been shown to be less effective than a mixture of cinchona alkaloids [17]. On the other hand, plant extracts containing important medicinal compounds are often scientifically dismissed because their activities cannot be traced to a single active chemical. The biological activity of extracts *in situ* may contrast sharply with that measured in bioassays of isolated components [18]. For this reason, drug discovery programs have failed to capture the value of multifunctional, multifaceted agents such as phytomedicines and botanical therapeutics.

Luckily, with a little effort, previously intractable plant extracts can now be subject to isolation and fractionation methods that preserve phytochemical integrity and deliberately seek to quantify incidences of chemical interactions. In this manner, we can begin to understand and exploit the entire range of efficacies of phytochemical mixtures and their key components. This is truly important considering recent calls from the World Health Organization for the discontinuation of all artemisinin monotherapies, while favoring the use of Artemisinin Combination Therapies (ACTs; mixtures of slow acting artemisinins with longer acting quinolone antimalarials in an effort to delay

resistance) [19]. Chemical complexity - found in synthetically, carefully constructed drug therapy programs and naturally in plants - is believed to help delay or avoid the emergence of resistant *Plasmodium* strains.

1.6. Antimalarial Plants of the United States

In malaria endemic regions (i.e. Africa, Southeast Asia, South America) the use of plants to treat malaria is well documented [15], however, until less than a century ago, the distribution of malaria was much greater than it is today and was a major problem throughout the United States (Figure 2.1) [20]. Native Americans and early American settlers had used numerous remedies (most of which were botanical in origin) to treat the disease [21]. With the development of synthetic antimalarials, more effective means of vector control and a general advance in the health care system, malaria was eradicated from most of the continent in the mid-1900's [3]. Consequently, long-used botanical antimalarials were no longer needed and forgotten.

Just as herbal antimalarial remedies of current malaria endemic regions have yielded novel and promising antimalarial remedies, those historically used in the United States have similar potential. This research project was designed to identify and study antimalarial plants of the United States that have not been subject to modern scientific inquiry and hold promise as sources for novel antimalarial agents.

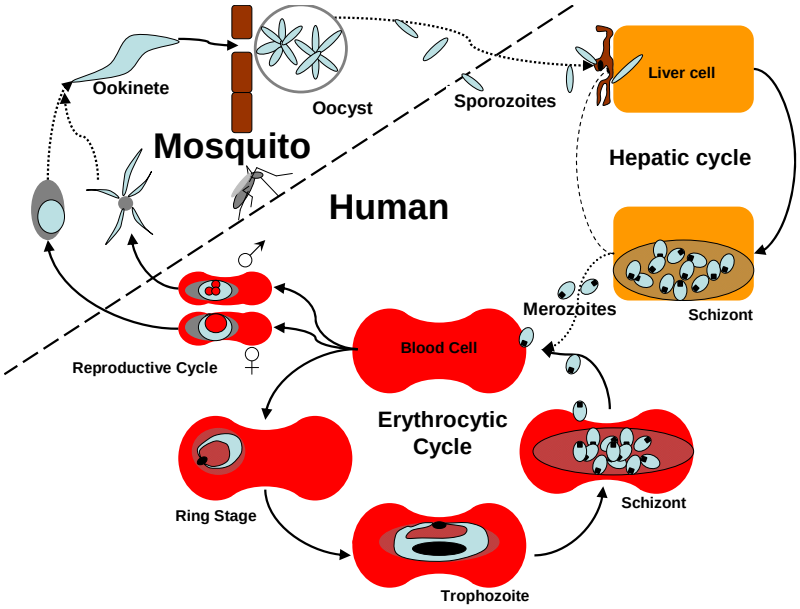
1.7. Tables and Figures

Table 1.1. Currently used antimalarial drugs.

Class	Drug(s)	Target/ Mode of Action
4- Aminoquinolines	**Chloroquine*; Amodiaquine*;**	Complex with heme (toxic by product of hemoglobin digestion) preventing its polymerization to the non-toxic hemazoin.
8- Aminoquinolines	**Primaquine***	Active against sexual stage and (the only drug active against) liver stage.
Arylaminoalcohols	**Quinine*; Mefloquine*; Halofantrine*; Lumefantrine***	Interfere with heme detoxification in digestive vacuole, though mechanisms are not well understood.
Artemisinin and Derivatives	**Artemisinin*; Artemether*; Artesenuate*; Artemotil*; Dihyrdoartemesinin***	Debated, either 1) inhibit an essential plasmodial calcium adenosine triphosphatase, pfATP6, or 2) modify multiple proteins via free radicals generated through intraplasmodial iron sources.
Antifolates	**Sulfadoxine/ Pyrimethamine; Dapsone/ Chloroproguanil**	Dihydropteroate synthase (DHPS) and Dihydrofolate reductase (DHFR), two enzymes involved in the synthesis of folic acid.
Respiratory Chain inhibitors	**Atovaquone/ Proguanil**	Ubiquinone binding site of cytochrome bc_1; blocks electron transport; degradation of mitochondrial membrane potential.
Antibiotics	**Doxycyclin; Clindamycin**	Act on prokaryote-like processes of the mitochondria or the apicoplast

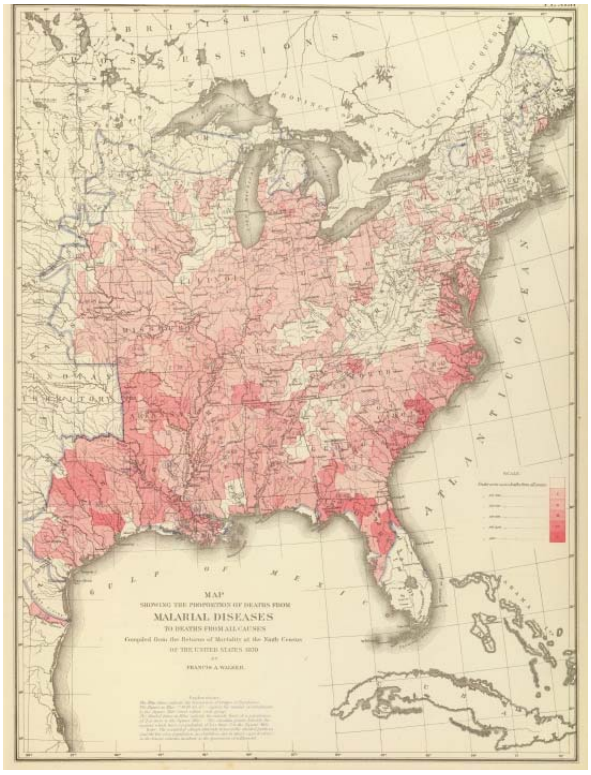
Current antimalarial drugs by class. The target and mode of action are listed where known. Those compounds with an asterisk (*) are directly or indirectly derived from plants.

Figure 1.1. The malaria disease cycle



An infected mosquito injects *Plasmodium* sporozoites during a blood meal, which target and invade hepatocytes. A single sporozoite can produce to 10,000-30,000 merozoites within 7-10 days of infection, which go on to infect erythrocytes. Some merozoites may reinfect hepatocytes causing latent infections. Merozoites enter the circulatory system and invade erythrocytes. Intraerythrocytic *Plasmodium* metabolizes host cell hemoglobin producing a characteristic hemazoin polymer (malaria pigment). Infected erythrocytes (schizonts) rupture simultaneously, releasing secondary merozoites that infect new red blood cells in a cyclical fashion. The coordinated rupturing and release of merozoites is responsible for the recurring bouts of fever. Some parasite enter a sexual cycle forming gametocytes which can be taken up by the mosquito, reproduce and start anew the cycle.

Figure 1.2. Map showing deaths from malaria in the Eastern U.S., 1870.



This map displays the ratio of deaths from malaria to all cause deaths in the eastern United States in 1870. Compiled from the returns of mortality at the ninth census of the United States by Francis A. Walker.Library of Congress.

CHAPTER 2

Species Selection, Collection and Preliminary *in vitro* Screening

2.1. General Overview of Plant Selection

It is not clear if malaria was introduced to the United States by European conquerors or if it was an endemic disease that afflicted the Native Americans [20, 22]. However, it is clear that the disease was a major concern people of the United States from the early 1600s all the way until the mid-1900s. During that period Americans turned to local plant species growing in the United States for relief from malaria when *Cinchona* bark, quinine or other remedies were unavailable. These species have been variously recorded in folk traditions, and eventually into the literature, and many references survive today regarding the most desired, local antimalarial remedies.

With the development of modern healthcare, the improvement of antimalarial drugs and mosquito control, malaria was effectively eradicated from the United States in the 1950s and with it went the need for traditional remedies. Therefore, the selection of plants for investigation in this thesis relied entirely on written records (rather than ethnobotanical field surveys), and much if it dating from before the 1950s. Two sources in particular have contributed immensely to the selection of plants: 1) The plants used by soldiers during the American Civil War to treat malaria and 2) The plants screened by Merck and the New York Botanical Garden during World War II when quinine supplies were limited and novel antimalarials were desperately needed. The rationale

for selecting plants from these sources will be discussed. The preliminary *in vitro* screen will be discussed as will the results of selected plants.

2.2. Plants Used to Treat Malaria during the American Civil War

During the American Civil war, members of the Confederate Army often found themselves in malarious conditions and wartime activity frequently left them without quinine, the preferred treatment of the era [21]. In an effort to mitigate this problem, the Surgeon General's Office of the Confederate Army commissioned future Confederate Surgeon Joseph Jones and surgeon-botanist Francis Peyre Porcher to develop a list of indigenous plant species that could be used as quinine substitutes, which were recorded and disseminated in pamphlets and flyers. The species selected by the surgeons were done in accordance with ethnobotanical practices of the Native Americans and colonists as well as professional experience. Although the records are scattered and incomplete, the most useful plants have been eloquently compiled by Dr. Guy R. Hasegawa in 2007 [21] and again listed here with permission (Table 2.1). A review of the literature has shown that many of these plant species have not been recently investigated for their antimalarial merit using contemporary scientific methods, however, their long history of use (they were generally used long before the war), widespread appeal and ease of collection made them prime targets for this thesis.

2.3. Plants Screened During World War II for Antimalarial Activity

During World War II, the Japanese navy cut off the allied countries access to the Dutch *Cinchona* plantations in Java (the world's main source of quinine) and the German

military took control of quinine stockpiles in Holland. At a time when U.S. troops were battling malaria as much as enemy soldiers, a shortage of antimalarial remedies was disastrous. In response to the shortage, the U.S. Government funded a group of 30 scientists at Merck and the New York Botanical Garden to screen over **600** plants for antimalarial activity in hopes of finding a replacement for quinine [23]. The investigation, involving prophylactic and curative assays of plant extracts in avian malaria (using chicks and ducklings), identified over **88** species with antimalarial activity. Unfortunately, when the war ended and quinine supplies normalized, funding was cut short and the project was largely abandoned.

Before my thesis, more than half of the WWII leads were not subsequently investigated, a neglect that Mrs. Christine Malanga Wilson (pictured in Figure 2.1), who was a major scientific contributor to Mercks efforts during the War, hoped would one day be rectified. At 94 years old, Mrs. Wilson passed on her sole copy of the original work to Dr. Ilya Raskin and me in 2008 with a plea to continue her efforts. She also provided expert inside information to aid the current project.

As international access to plant species was limited by wartime activity, most of the plant species studied by the WWII study were collected from within the United States. They were selected based on literature and anecdotal references to historical antimalarial use. In order to select plants for the current project, a literature review regarding the ethnobotany, phytochemistry, and availability of the plant species was conducted in order to prioritize the active species for further study. **36** of the species,

mostly from the Simaroubaceae and Amaryllidaceae, were deemed unattractive for further studies as they had been already investigated for antimalarial activity or found to be too cytotoxic for human use and were thus discarded from this study. The remaining **52** species were arranged into three tiers and were, with every effort, collected and studied in order of tier (19 of 52 plant species were collected and screened).

The Raskin lab maintains an inclusive collection of plant extracts from Central Asia, which were acquired for an International Cooperative Biodiversity Groups (ICBG)-collaborative, federally funded project. It was found that many of the plants within this collection are close relatives of those identified as active in the WWII studies. As a means to survey the bioactivity of amongst evolutionarily related species, several species from the ICBG collection were selected to extract and bioassay alongside those collected from the WWII list. The species can be compared with those in the WWII studies.

2.4. Plant Collection and Extraction

Plants were obtained by one of several methods: 1) wild collected by the author (R. Graziose) 2) wild collected by colleagues of the author 3) purchased from reputable herbal suppliers who provided authentication or 4) grown from seed or stock in the Rutgers greenhouse. All species studied were validated and authenticated by the author and a voucher specimen from each is stored in the Chrysler Herbarium (CHRB) at Rutgers University. The plants that were wild collected were mostly done within a 5

mile radius of the New Brunswick campus at Rutgers University (Figure 2.2) and all were collected in a sustainable manner and with efforts to avoid contamination.

All collected species were thoroughly dried, either in the air or by lyophilization, immediately after harvesting. Once dry, the plant material (bark, leaves, fruit, roots, etc.) was ground into a fine powder and stored at -4°C until extraction.

For the plants used to treat malaria during the Civil war, the plant material was extracted with a 50% ethanol solution. This preparation method coincides with that described for preparing many drugs during the time period. We therefore chose this method for ethnobotanical and historic accuracy.

For those plants initially screened during World War II, an extraction protocol was adopted that was related to the methods of Spencer et al. [23] in order that our *in vitro* results could be compared to the *in vivo* results of the original work. At the time of the WWII project, it was assumed that novel antimalarial compounds might share similar characteristics to quinine, the only known antimalarial of the day. Quinine is a quinolone alkaloid, and many alkaloids are effectively extracted using an acid-base partitioning method [24]. Therefore, to concentrate plant alkaloids for bioassay, Spencer et al. adopted an acid-base partitioning method.

We now know that many other classes of compounds (i.e. terpenoids, aromatics, polyketides, etc.) have representatives with antiplasmodial activity. Therefore, a slightly modified regime was developed to extract all compound classes, but retain the acid-base

portion of the WWII project, in order to remain consistent with previous methodology while ensuring greater compound coverage.

Extracts preparation is described textually here and graphically in figure 2.3. 10-100 g of freeze-dried and ground plant material is extracted three times with a 1:6 ratio (weight to volume) of hexane under constant agitation for a total of 24 hr. This extract, **Extract 1**, was dried and stored at -20°C until testing. Subsequent extracts were stored similarly. The plant material was air dried of hexane, and extracted three times with a 1:6 ratio (weight to volume) of 80% methanol for a total of 24 hrs. The solution was then filtered and the methanol evaporated. The resulting aqueous solution often contained an insoluble material, which was filtered, collected and dried (**Extract 2**). The aqueous solution was then made alkaline (pH 9-10) and extracted continuously with chloroform until all extractable material had been removed (9-24 hrs). The chloroform fraction was evaporated to dryness and collected (**Extract 3**). The remaining aqueous solution was evaporated to dryness and collected (**Extract 4**).

2.5. *In vitro* antiplasmodial screening methodology

In collaboration with Dr. Peter Smith at the University of Cape Town, South Africa (UCT), plant extracts were tested for their *in vitro* antiplasmodial activity. The Nitro Blue Tetrazolium (NBT) based parasite lactate dehydrogenase (pLDH) assay developed by Makler et al. [25] was used to determine the *in vitro* antiplasmodial activity of the plant extracts with chloroquine (sigma) as a positive control. This assay has been proven effective, accurate, and efficient by over 15 years of successful use.

Plasmodium falciparum was maintained in continuous culture for the duration of the tests as described by Trager and Jensen [26] with a few modifications. The extracts were assayed against the chloroquine sensitive D10 strain.

The concentration of stock plant extracts and drugs for the assay are based on laboratory experience that crude extracts with antiplasmodial activity generally have a 50% inhibitory concentration (IC_{50}) of <20 μg/mL (Table 2.2). Stock solutions of the extracts were prepared and aliquoted for tests doses of 20, 10, 5 and 2 μg/mL concentrations, which were tested against *P. falciparum* cultures for 48 hours, exposing all parasite stages to the extract. A full dose-response was performed for active extracts to determine IC_{50} value, or the concentration that inhibits 50% of *P. falciparum* growth. The concentration of solvent (water, methanol or dimethylsulfoxide) has no measurable effect of parasites (Smith per Corr.). IC_{50}-values were obtained using a non-linear dose-response curve fitting analysis via Graph Pad Prism v.4.0 software.

2.6. Results

The results of preliminary *in vitro* antiplasmodial screening are reported in three separate tables below. Table 2.3 shows the results of the plants used during the American Civil War to treat malaria. Table 2.4 shows the results of the collected species (19) along with those identified as promising from the WWII studies. Table 2.5 shows the results from species collected in Central Asia for the ICBG project and identified as closely related to the WWII plants.

Of those species used to treat malaria during the civil war, 10 were collected, extracted and subject to antiplasmodial assay. The IC_{50} values of the extracts ranged from 23 to more than 100 μg/mL (Table 2.3). The most active species were *Magnolia tripetela* (23 μg/mL) and *Thuja occidentalis* (28 μg/mL). However, as indicate in table 2.2, these values are rather high to warrant additional follow up studies.

Fifty-two species were identified as promising leads from the WWII studies and they are listed according to priority in table 2.4. Nineteen of those species were collected, extracted and subject to preliminary *in vitro* antiplasmodial assay of which 17 produced one or more active extracts. Of the 116 extracts generated, 41 extracts had tentative IC_{50} values below 20 μg/mL, and of those 29 extracts were below 10 μg/mL. This represents a 'hit rate' of roughly 35%. It was found that, extracts 2 and 3 tended to be active, while extracts 1 and 4 were mostly inactive. This is likely due to the compound classes most probably present in each extract. Extract 1 is a very non-polar fraction (hexane) and likely contains fats, oils, aliphatic compounds and certain volatile components. Extract 4 contains a large amount of carbohydrates, free sugars, and highly glycosylated constituents.

Fourteen species were selected from the ICBG inventory for antiplasmodial assay due to their relation to species identified during the WWII studies as active. From these 14 species, 80 extracts were prepared as described above, of which 31 extracts possessed and activity below 20 μg/mL. This represents a 'hit rate' of 38.8%. Again, it was found that extracts 1 and 4 were mostly inactive, while extracts 2 and 3 were more often active.

2.7. Discussion

It is up for debate whether drug discovery programs that rely on ethnobotanical guidance are more successful than those that simply collect at random [27]. Several observations regarding this issue can be made from the results presented above. From 600 plant species screened by the World War II antimalarial drug discovery program, 88 species were determined to be active. All of the plants selected were done so using ethnobotanical data as a guide. The 'hit rate' of the WWII study was approximately 14.7%. Of those 88, 52 were selected in this study for further investigation, and 19 were actually collected and screened. Of those 19 species, all of which had shown activity *in vitro* in the avian malaria model, 17 species contained one or more active extracts. There were a total of 41 active extracts from 116 total extracts. When comparing these results to ethnobotanically used Civil War antimalarial plants, where none of the 10 species collected generated extracts with an IC_{50} value below 20 μg/mL, one can appreciate the power of utilizing BOTH ethnobotany and preliminary screening data to select lead species.

Of the plants with activity in the preliminary screening just discussed, six were chosen for further study, in particular, bioassay-guided fractionation to determine which plant compound(s) were responsible for the observed antiplasmodial activity. The plants that will be discussed in this thesis are: *Liriodendron tulipifera*, *Cornus florida*, *Datisca glomerata* and *Ptelea trifoliata*. Two additional plants were also subject to bioassay guided fractionation - *Canella winterana* and *Cassia fistula* – and are described in the appendix with links to published papers. The activity of these six species in the World War II *in*

vivo antimalarial screen, their ethnobotanical history, their activity in the preliminary antiplasmodial assay and novelty regarding antimalarial investigation made them ideal candidates for investigation. Each species is discussed individually in the proceeding chapters.

2.8. Tables and Figures

Table 2.1. **Plant species used during the Civil War to treat malaria.**

Acorus calamus L. [Acoraceae]	*Agrimonia eupatoria* L. [Rosaceae]	*Alnus serrulata* (Aiton) Willd. [Betulaceae]
Ambrosia elatior L. [Asteraceae]	*Aristolochia serpentaria* L. [Aristolochiaceae]	*Asclepias syriaca* L. [Apocynaceae]
Lindera benzoin (L.) Blume [Lauraceae]	*Castanea vesca* Gaertn. [Fagaceae]	*Catalpa cordifolia* Moench [Bignoniaceae]
Centaurea benedicta (L.) L. [Asteraceae]	*Chimaphila umbellata* (L.) W.P.C. Barton [Ericaceae]	*Chionanthus virginicus* L. [Oleaceae]
Cornus circinata L'Her. [Cornaceae]	*Cornus florida* L [Cornaceae]	*Cornus sericea* L. [Cornaceae]
Diospyros virginiana L. [Ebenaceae]	*Eupatorium perfoliatum* L [Asteraceae]	*Eupatorium rotundifolium* L. [Asteraceae]
Gelsemium sempervirens (L.) J.St.-Hil. [Gelemiaceae]	*Gentiana catesbaei* Walter [Gentianaceae]	*Gentiana quinqueflora* Hill. [Gentianaceae]
Gossypium herbaceum L. [Malvaceae]	*Ilex opaca* Aiton [Aquifoliaceae]	*Liriodendron tulipifera* L. [Magnoliacaea]
Lycopus europaeus L. [Lamiaceae]	*Magnolia acuminate* (L.) L. [Magnoliacaea]	*Magnolia glauca* (L.) L. [Magnoliaceae]
Magnolia grandiflora L. [Magnoliaceae]	*Magnolia tripetala* (L.) L. [Magnoliaceae]	*Pinckneya pubens* Michx. [Rubiaceae]
Polygonum aviculare L. [Polygonaceae]	*Prinos verticillatus* L. [Aquifoliaceae]	*Quercus falcata* L. [Aquifoliaceae]
Sabbatia angularis Pursh [Lamiaceae]	*Salix alba* L. [Saliaceae]	*Salix nigra* Marshall [Saliaceae]
Swietenia mahogoni Lam. [Meliaceae]	*Thuja occidentalis* L. [Cuppressaceae]	*Zea mays* L. [Poaceae]

Plant species used during the civil war to treat malaria compiled from several sources by Hasegawa (2007) [21]. Species names are uncorrected and are listed as they appear in the reference. Those underlined were collected and assayed in this thesis work

Table 2.2. Thresholds of *in vitro* antiplasmodial activity

IC_{50} (µg/mL)	Level of Activity
<1.0	Potent
1.1-10	Good
11-20	Moderate
20-50	Weak
>50	Inactive

Extracts with IC_{50} values greater than 20µg/mL are not generally followed up on.

Table 2.3 Antiplasmodial activity of antimalarial plants used during the Civil War

Family	Species	Organ	IC_{50} (µg/mL)
Aquifoliaceae	*Ilex verticillata* L.	Leaves	93
	Ilex opaca Aiton	Leaves	66
Cupressaceae	*Thuja occidentalis* L.	Leaves	28
Lauraceae	*Lindera benzoin* (L.) Blume	Leaves	93
Fagaceae	*Castanea dentata* L.	Leaves	59
		Twigs	64
Magnoliaceae	*Magnolia acuminata* L.	Bark	44
	Magnolia glauca L.	Bark	74
	Magnolia grandiflora L.	Bark	78
	Magnolia tripetala L.	Bark	23
Polygonaceae	*Polygonum aviculare* L.	Aerial Parts	>100

Plants were extracted with 50% ethanol.

Figure 2.1 World War II antimalarial plant testing

Mrs. Christine Malanga Wilson, at left, with Dr. Albert Seeler, evaluating the efficacy of plant extracts against avian malaria (1944).

Figure 2.2 Plant collection sites

Location of plant collections in the United States. Wild collected species = 41. Species purchased commercially = 3. Greenhouse grown species = 3.

Figure 2.3. Plant extraction methodology for preliminary studies

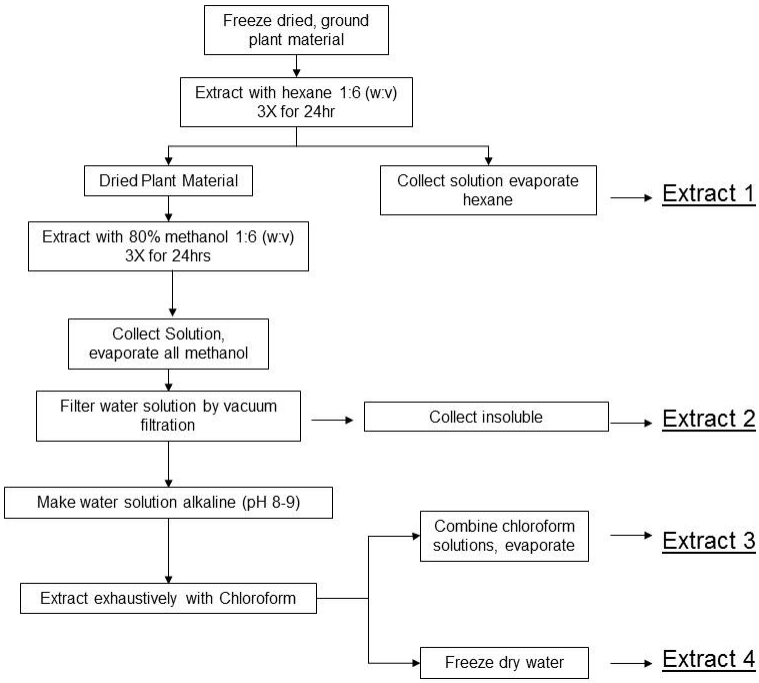


Table 2.4 Antiplasmodial activity of plants investigated during World War II as quinine replacements

Family	Species[a]	Spencer activity[b]	Organ	Extract[c]	% Parasite Growth				IC_{50} (µg/ml)
					20 µg/ml	10 µg/ml	5 µg/ml	2.5 µg/ml	
Priority One Species									
Canellaceae	*Canella winterana* (L.) Gaertn.	*4*	Leaves	1	1.02%	1.50%	37.12%		12.42
				2	0.50%	13.78%	58.03%		15.21
				3	0.40%	1.19%	6.80%		4.99
				4	88.11%	94.09%	81.73%		
			Bark	1	7.96%	10.14%	45.83%		10.09
				2	0.00%	5.56%	36.66%		15.6
				3	0.00%	3.71%	43.92%		8.32
				4	110.25%	107.69%	88.51%		
			Fruit	1	13.76%	10.01%	39.68%		6.43
				2	19.85%	26.27%	65.67%		11.43
				3	11.44%	10.20%	25.02%		2.47
				4	112.65%	96.83%	94.30%		
Cornaceae	*Cornus florida* L.	*4*	Leaves	1	102.82%	100.71%	98.24%		
				2	88.49%	97.94%	110.42%		
				3	100.72%	109.33%	119.75%		
				4	106.77%	111.96%	107.03%		
			Bark	1	81.16%	101.45%	94.93%		
				2	47.68%	91.41%	97.62%		7.52
				3	98.87%	100.48%	85.47%		
				4	99.32%	130.16%	104.05%		
			Fruit	1	115.27%	117.62%	98.46%		
				2	88.04%	84.73%	76.44%		
				3	55.05%	78.56%	93.15%		
				4	111.77%	111.87%	105.80%		
Datiscaceae	*Datisca glomerata* (Presl.) Baill.	*3*	Aerial Parts	1	-	-	-		
				2	61.23%	76.00%	82.30%		
				3	60.65%	81.30%	89.44%		
				4	-	-	-		

Family	Species[a]	Spencer activity[b]	Organ	Extract[c]	% Parasite Growth				IC_{50} (µg/ml)
					20 µg/ml	10 µg/ml	5 µg/ml	2.5 µg/ml	
Priority One Species-*cont.*									
Fabaceae	*Cassia fistula* L.	*2*	Leaves	1	-				
				2	1.11%				9.12
				3	0.00%				7.24
				4	111.71%				
			Bark	1	114.33%				
				2	37.37%				
				3	23.61%				
				4	109.64%				
			Fruit	1	112.99%				
				2	95.01%				
				3	2.35%				6.18
				4	108.49%				
Gentianaceae	*Schultesia lisianthoides* (Griseb.) Benth. & Hook. f. ex Hemsl.	*3*							
Magnoliaceae	*Liriodendron tulipifera* L.	*2*	Leaves	1	77.95%				
				2	0.00%				4.34
				3	0.00%				4.62
				4	121.42%				
Priority two Species									
Asteraceae	*Conyza filaginoides* (DC.) Hieron.	*2*							

Family	Species[a]	Spencer activity[b]	Organ	Extract[c]	% Parasite Growth				IC_{50} (µg/ml)
					20 µg/ml	10 µg/ml	5 µg/ml	2.5 µg/ml	
Priority Two Species-*cont.*									
Apiaceae	*Eryngium foetidum* L.	*3*	Aerial pts	1	24.29%	63.70%	83.10%		
				2	91.93%	103.02%	96.28%		
				3	96.04%	103.78%	105.05%		
				4	122.58%	127.96%	111.79%		
			Roots	1	94.94%	120.03%	131.48%		
				2	110.91%	112.12%	118.78%		
				3	85.22%	97.10%	109.14%		
				4	99.66%	107.75%	98.74%		
Apiaceae	*Eryngium yuccifolium* L.	*2*							
Apocynaceae	*Catharanthus roseus* (L.) G. Don	*2*	Aerial pts	1	x	x	x	x	
				2	x	31.92%	44.59%	79.52%	
				3	x	30.05%	39.55%	56.16%	
				4	x	99.09%	107.35%	102.28%	
Apocynaceae	*Geissospermum vellosii* Allemão	*2*	Bark	1	x	Not tested			
				2	x	80.46%	84.60%	96.10%	
				3	x	27.13%	41.97%	50.09%	
				4	x	Not tested			
Bignoniaceae	*Tabebuia argentea* (Bureau & K. Schum.) Britton	*2*	Bark	1	48.76%	82.67%	98.30%		
				2	23.58%	41.00%	57.17%		
				3	16.88%	40.49%	43.36%		
				4	87.33%	86.41%	84.05%		
			Leaves	1	84.56%	115.47%	103.43%		
				2	19.08	62.19	79.32		
				3	73.59	82.1	86.99		
				4	59.26	68.34	70.86		

Family	Species[a]	Spencer activity[b]	Organ	Extract[c]	% Parasite Growth				IC_{50} (µg/ml)
					20 µg/ml	10 µg/ml	5 µg/ml	2.5 µg/ml	
Priority Two Species-*cont.*									
Menispermaceae	*Cissampelos pareira* L.	*2*	Bark	1	x	19.46%	61.39%	92.87%	
				2	x	21.17%	52.11%	86.43%	
				3	x	35.03%	48.07%	67.19%	
				4	x	92.97%	110.22%	100.08%	
Zygophyllaceae	*Balanites aegyptiacus* var. *tomentosus* (Mildbr. & Schltr.) Sands	*3*							
Priority three species									
Anacardiaceae	*Schinus polygamus* var. *chilensis*	*1*							
Apocynaceae	*Tabernaemontana amygdalifolia* Jacq.	*1*							
Asteraceae	*Achillea millefolium* L.	*1*	Flower	1	1.75	32.59	41.91		
				2	9.65	18.88	38.35		
				3	9.57	25.95	42.51		
				4	71.11	85.22	93		
			Aerial Parts	1	61.97	91.44	98.2		
				2	6.04	18.97	34.34		
				3	14.74	34.41	61.06		
				4	79.08	88.12	88.8		
Asteraceae	*Centaurea calcitrapa* L.	*1*							
Asteraceae	*Eupatorium ligustrinum* DC.	*1*							

Family	Species[a]	Spencer activity[b]	Organ	Extract[c]	% Parasite Growth				IC_{50} (µg/ml)
					20 µg/ml	10 µg/ml	5 µg/ml	2.5 µg/ml	
Priority Three Species-*cont.*									
Asteraceae	*Eupatorium perfoliatum*L.	*1*	leaves	1	89.75	108.19	106.86		
				2	58.44	90.81	103.92		
				3	19.54	58.48	94.67		
				4					
Asteraceae	*Pectis linearis* La Llave	*1*							
Asteraceae	*Spilanthes acmella* (L.) Murray	N/A	Aerial Parts	1	116.9	102.6	_		
				2	0.00	17.3	45.1		10.57
				3	0.76	8.15	33.7		10.3
				4	100.7	101.1	99.1		
Araliaceae	*Hedera helix* L.	*1*							
Betulaceae	*Alnus serrulata* (Aiton) Willd.	N/A	Bark	1	25.5	28.4	51.2		
				2	63.8	89.4	95.8		
				3	7.5	32.0	47.1		
				4					
Boraginaceae	*Ehretia tinifolia* L.	*1*							
Burseraceae	*Bursera simaruba* (L.) Sarg.	*1*							
Cannellaceae	*Capsicodendron dinisii* (Schwacke) Occhioni	*1*							
Caprifoliaceae	*Triosteum perfoliatum* L.	*1*							

Family	Species[a]	Spencer activity[b]	Organ	Extract[c]	% Parasite Growth				IC_{50} (µg/ml)
					20 µg/ml	10 µg/ml	5 µg/ml	2.5 µg/ml	
Priority Three Species-*cont.*									
Celastraceae	*Canotia holacantha* Torr.	*2*							
Cistaceae	*Lechea villosa* Elliott	*1*							
Euphorbiaceae	*Croton californicus* Müll. Arg.	*1*							
Euphorbiaceae	*Croton capitatus Michx.*	*1*							
Fabaceae	*Cercis canadensis* L.	*1*	Leaves	1	98.46	97.81	99.27		
				2	4.73	41.5	65.59		6.19
				3	10.57	4.45	18.66		
				4	96.95	112.6	115.26		
Fabaceae	*Diphysa robinioides*	*1*							
Fabaceae	*Ditremexa leptocarpa (now Senna hirsuta var. leptocarpa)*	*2*							
Fabaceae	*Pericopsis laxiflora (Benth. ex Baker) Meeuwen*	*1*							
Fagaceae	*Quercus alba* L.	N/A	Bark	1	19.79	37.78	49.56		
				2	41.61	85.48	96.2		
				3	5.66	21.32	46.1		
				4	77.83	94	88.51		
Gentianaceae	*Anthocleista frezoulsii* A. Chev.	*1*							

Family	Species[a]	Spencer activity[b]	Organ	Extract[c]	% Parasite Growth				IC_{50} (µg/ml)
					20 µg/ml	10 µg/ml	5 µg/ml	2.5 µg/ml	
Priority Three Species-*cont*									
Gentianaceae	*Centaurium venustum* (A. Gray) B.L. Rob	*1*							
Geraniaceae	*Geranium robertianum* L.	*1*							
Lamiaceae	*Ajuga iva* (L.) Schreb.	*1*							
Lamiaceae	*Callicarpa dichotoma* Raeusch.	N/A	Leaves	1	86.35	87.26	96.15		
				2	26.42	64.24	85.99		
				3	4	14.82	49.73		
				4	118.26	107.8	107.74		
			Bark	1	80.46	90.67	103.61		
				2	45.21	63.12	86.09		
				3	13.13	16.87	88.71		
				4	0	0	5.29		2.23
Lamiaceae	*Marrubium vulgare* L.	*1*							
Lamiaceae	*Ocimum basilicum* L.	*1*							
Picrodendraceae	*Picrodendron baccatum* (L.) Krug & Urb. ex Urb	*2*							
Picrodendraceae	*Picrodendron macrocarpum* (A. Rich.) Britton	*1*							
Rosaceae	*Sorbus scopulina* Greene	*1*							
Rutaceae	*Orixa japonica*	*1*							

Family	Species[a]	Spencer activity[b]	Organ	Extract[c]	% Parasite Growth				IC_{50} (µg/ml)
					20 µg/ml	10 µg/ml	5 µg/ml	2.5 µg/ml	
Priority Three Species-*cont*									
Rutaceae	*Ptelea trifoliata*	*1*	Leaves	1	75.14	81.15	86.82		
				2	1.98	30.7	44.74		
				3	8.1	32.86	42.47		
				4	67.77	78.62	85.23		
Saururaceae	*Anemopsis californica*	*1*							
Saxifragaceae	*Hydrangea arborescens*	*1*	Leaves	1	97.32	97.01	98.21		
				2	65.51	70.52	78.81		
				3	20.03	48.98	70.43		
				4	96.52	100.31	99.67		

Activity of plants collected from those identified as leads from the World War II antimalarial plant screening [23]. Species were arranged into priority groups based upon activity in the WWII screen and literature review of ethnobotanical and phytochemical properties. 19 of 52 species were screened and the results are shown. [a]Plants are identified by currently accepted names. Those in the WWII studies may be identified by synonyms. [b]The activity is presented in the WWII study on a 0-4 scale, with 4 being equal in activity to quinine. [c] The extract number corresponds to that shown in figure

Table 2.5 Antiplasmodial activity of species from ICBG collection (WWII relatives)

Family	Species	Organs	Extract	% Parasite Growth (20µg/mL)
ICBG Plants				
Apiaceae	*Eryngium caucasicum* Trautv.	Roots	1	17.77%
			2	21.78%
			3	NR
			4	NR
		Flowers	1	23.96%
			2	23.16%
			3	16.38%
			4	109.48%
Apiaceae	*Eryngium macrocalyx* Schrenk	Roots	1	28.77%
			2	16.05%
			3	21.46%
			4	104.42%
		Leaves	1	60.96%
			2	18.78%
			3	8.31%
			4	NR
		Flowers	1	26.13%
			2	23.29%
			3	7.97%
			4	23.08%
Asteraceae	*Artemisia absinthium* L.	Roots	1	111.34%
			2	20.81%
			3	69.18%
			4	111.60%
		Aerial Parts	1	76.08%
			2	36.30%
			3	35.89%
			4	98.85%
Asteraceae	*Artemisia annua* L.	Aerial Parts	1	25.25%
			2	16.52%
			3	12.36%
			4	96.10%
Asteraceae	*Artemisia baldshuanica Krasch. & Zapr.*	Aerial Parts	1	78.99%
			2	54.81%
			3	76.90%
			4	90.50%
Asteraceae	*Artemisia dracunculus* L.	Aerial Parts	1	71.23%
			2	42.03%
			3	32.50%
			4	107.13%

Family	Species	Organs	Extract	% Parasite Growth 20mg/ml
ICBG Plants-*cont.*				
Datiscaceae	*Datisca cannabina* L.	Leaves	1	99.98%
			2	39.11%
			3	95.84%
			4	105.74%
		Roots	1	104.68%
			2	64.36%
			3	58.44%
			4	109.57%
Gentianaceae	*Gentiana karelini Griseb.*	Aerial Parts	1	91.64%
			2	48.24%
			3	54.05%
			4	120.12%
Gentianaceae	*Gentiana olivieri* Griseb.	Aerial Parts	1	97.55%
			2	104.70%
			3	35.37%
			4	30.05%
Gentianaceae	*Gentiana turkestanorum* Gand.	Entire	1	105.49%
			2	45.34%
			3	49.24%
			4	106.49%
Gentianaceae	*Swertia lactea* Bunge	Entire	1	112.13%
			2	45.16%
			3	52.39%
			4	97.32%
Lamiaceae	*Ajuga turkestanica* Briq.	Roots	1	77.12%
			2	35.93%
			3	38.10%
			4	102.96%
		Leaves	1	95.85%
			2	23.39%
			3	48.64%
			4	93.63%
Lamiaceae	*Marrubium alternidens Rech. f.*	Aerial Parts	1	71.72%
			2	52.66%
			3	58.62%
			4	97.17%
Rosaceae	*Sorbus tianshanica* (Kom.) McAllister	Aerial Parts	1	70.88%
			2	59.66%
			3	0.00%
			4	91.72%

Antiplasmodial activity of plants collected from Central Asia International Cooperative Biodiversity Groups (ICBG) related to those identified as active in the WWII studies (see text).

CHAPTER 3

Antiplasmodial Activity of Aporphine Alkaloids and Sesquiterpene Lactones from *Liriodendron tulipifera* L.

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3.1. Abstract

Aim of the study: The objective of this study was to isolate and characterize the active constituents of the traditionally used antimalarial plant *Liriodendron tulipifera* by antiplasmodial-assay guided fractionation.

Materials and methods: Bark and leaves were extracted with solvents of increasing polarity. Fractions were generated using flash chromatography, counter current chromatography and preparative HPLC and subjected to in vitro antiplasmodial and cytotoxicity assays. Active fractions were subjected to further fractionation until pure compounds were isolated, for which the IC50 values were calculated.

Results and discussion: Six known aporphine alkaloids, asimilobine (1), norushinsunine (2), norglaucine, (3), liriodenine (4), anonaine (5) and oxoglaucine (6) were found to be responsible for the antiplasmodial activity of the bark. Leaves yielded two known sesquiterpene lactones, peroxyferolide (7) and lipiferolide (8) with antiplasmodial activity. The antiplasmodial activity of (2) (IC_{50} = 29.6 μg/mL), (3) (IC_{50} = 22.0 μg/mL), (6) (IC_{50} = 9.1 μg/mL), (7) (IC_{50} = 6.2 μg/mL) and (8) (IC_{50} = 1.8 μg/mL) are reported for the first time.

Conclusion: This work supports the historical use of *Liriodendron tulipifera* as an antimalarial remedy of the United States and characterizes its antiplasmodial constituents.

3.2. Introduction

Liriodendron tulipifera L. (Magnoliaceae), known as the tulip tree or yellow poplar, is a majestic tree often reaching heights upwards of one hundred feet, that is endemic to the eastern United States [28]. The bark of *L. tulipifera* was used by the Native Americans as a tonic, stimulant and febrifuge, and likely was used to treat the intermittent fevers associated with malaria [29]. It was also adopted by American settlers as a suitable replacement for the imported and often scarce Peruvian bark (*Cinchona* bark) [30]. During the United States Civil War, when the confederate troop's quinine supplies were limited, army surgeons turned to *L. tulipifera* as a substitute [21]. During World War II, a U.S. government-directed program focused on developing quinine replacements confirmed that a crude extract of the tulip tree bark was effective in treating avian malaria [23]. However, while numerous phytochemical constituents, including sesquiterpene lactones [31-36] and aporphine alkaloids [37-42] have been isolated from this species, the antiplasmodial constituents have not been described until now. Although malaria is no longer a pressing issue in the native range of *L. tulipifera*, the antiplasmodial compounds from this species may be of value to future antimalarial drug development as well as to the preservation of traditional knowledge. The goal of this study was to describe the antiplasmodial components of *Liriodendron tulipifera.*

3.3. Materials and Methods

3.3.1. Instrumentation

See appendix A

3.3.2 Plant material

Leaves and bark of *Liriodendron tulipifera* L. (Magnoliaceae) were collected from a single, large tree growing on Rutgers University farm on Ryders Lane in New Brunswick, NJ, which was identified by Lena Struwe, Ph.D. A voucher specimen (RG16) has been deposited in the Chrysler Herbarium (CHRB).

3.3.3 Extraction and Isolation

3.3.3.1 Isolation of actives from bark.

The dried and powdered bark of *L. tulipifera* (300 g) was defatted with hexane (3 L) at room temperature for 24 h, dried, and extracted with 95% ethanol (3 L, 2x) at room temperature for 48 h. The ethanolic extract was separated by filtration and concentrated under vacuum to yield 20 g of crude extract that showed an antiplasmodial activity of 10.9 μg/mL. This crude extract was acidified with 3% HCl solution and extracted with chloroform (200 mL, 3x) to remove phenolic compounds, and the remaining aqueous extract was basified with 5 N NH_4OH solution and extracted with chloroform (200 mL, 3x), which was concentrated under vacuum to yield 0.23 g of extract. This basic extract was purified by reverse phase HPLC (Waters RP-8 300 x 19.0mm, 7 μm, 30-95% methanol in water which contains 0.01% TFA, over 60 min, flow rate 10 mL/min; UV

detector, 254 nm) to collect four fractions, Fr-I (3 mg), Fr-II (22 mg), Fr-III (42 mg) and Fr-IV (28 mg) respectively. Fr-II was further purified by reverse phase HPLC (Phenomenex Synergy Hydro-RP 80A 250 x 21.20 mm, 4 μm, 30-95% methanol in water with 0.01% TFA, over 60 min, flow rate 10 mL/min; UV detector, 254 nm) to give 3 sub fractions, Fr-II-1 (2 mg), Fr-II-2 (4 mg) and Fr-II-3 (5 mg) respectively. Fr-II-2 & 3 were further purified by reverse phase HPLC (Phenomenex Synergy Hydro-RP 80 Å 250 x 4.60 mm, 4 μm, 30-95% methanol in water with 0.01% TFA, over 60 min, flow rate 1 mL/min; UV detector, 254 nm) to give compound **1** (2 mg) from Fr-II-2, compounds **2** (2 mg) and **3** (1 mg) from Fr-II-3 respectively. Similarly, Fr-III gave 3 sub fractions, Fr-III-1 (3mg), Fr-III-2 (2mg) and Fr-III-3 (8mg) and Fr-IV gave 4 sub fractions, Fr-IV-1 (5mg), Fr-IV-2 (3 mg), Fr-IV-3 (2 mg) and Fr-IV-4 (2 mg) respectively. Compound **4** (4 mg) was isolated from Fr-III-3 and compounds **5** (2 mg) and **6** (1.5 mg) were isolated from Fr-IV-1 & 2 respectively.

3.3.3.2 Isolation of actives from leaves:

The dried powdered leaves (100 g) were defatted with hexane (1 L) at room temperature for 24 h, dried then extracted with 80% methanol (1L, 2x) at room temperature for 24 h. The combined aqueous methanolic extract was concentrated under vacuum to a volume of 300 mL, which was extracted with 300 mL chloroform three times and dried to yield 2.9 g of crude chloroform extract. This extract, which showed an antiplasmodial activity of 2.0 μg/mL, was separated by flash chromatography (80 g, 230-400 mesh, 60Å Merck, column dimensions 25 x 3cm) and eluted with $CHCl_3$:CH_3OH

[100:0 (250 mL); 99.5:0.5 (375 mL); 99:1 (1 L); 98:2 (375 mL); 97:3 (375 mL); 95:5 (250 mL); 50:50 (250 ml)]; 125 mL fractions were collected. Fractions 16-19, which were the most active (IC_{50} = 4.3 μg/mL), were combined (70 mg) and further separated by Counter Current Chromatography with a 2:1:2:1 HEMWat (Hexane: Ethyl Acetate: Methanol: Water) solvent system using the Elution-Extrusion method with the lower phase as the stationary phase following previously published protocol [43]. Eight fractions were collected according to peaks observed at 254 nm. Fractions I and II (mins 27-31 and 32-40) were further purified by silica gel column chromatography to yield compound **7** (6 mg) and compound **8** (10 mg) respectively.

3.3.4 In vitro assays

3.3.4.1 In vitro *Antiplasmodial assay*

See appendix A

3.3.4.2 In vitro *cytotoxicity assay*

See appendix A

3.4. Results and Discussion

This research is the first systematic isolation and characterization of the antiplasmodial compounds from the tulip tree (*Liriodendron tulipifera*). Six known aporphine alkaloids and two known sesquiterpene lactones (Figure 3.1), which have been previously reported from *L. tulipifera*, were shown to be the antiplasmodial constituents of the bark and leaves, respectively. Asimilobine (**1**) [40, 44], norushinsunine (**2**) [38], norglaucine (**3**) [45], liriodenine (**4**) [46, 47] anonaine (**5**) [44]

oxoglaucine (**6**) [40, 48] were isolated from the bark of *L. tulipifera,* which were confirmed by comparison of spectral data with the literature values. Two sesquiterpene lactones, peroxyferolide (**7**) and lipiferolide (**8**) were isolated from the leaves of *L. tulipifera* and the structures were confirmed by comparison of spectral data previously published data [33-36]. ESI-MS and ^{1}H NMR spectral data for the compounds **1**-**8** can be found below.

All the isolated compounds were evaluated for antiplasmodial activity using both CQ-sensitive (D10) and CQ-resistant (Dd2) plasmodium strains and cytotoxicity using the Chinese Hamster Ovarian (CHO) cell line (Table 3.1).

Several sesquiterpene lactones with antimalarial activity have been previously isolated from plants [49]. However, with the exception of artemisinin and related compounds from *Artemisia annua*, none has proven therapeutically valuable. The antimalarial activity of artemisinin is thought to depend on the endoperoxide bridge [50]. The hydroperoxide moiety of peroxyferolide (**7**) is chemically related to the endoperoxide moiety of, artemisinin; a functionality group which is lacking in lipiferolide (**8**). The antiplasmodial activity of peroxyferolide (**7**) (IC_{50}=6.3 μg/mL) is not significantly different than that of lipiferolide (**8**) (IC_{50}=1.8 μg/mL), suggesting that the hydroperoxide moiety does not contribute to the observed activity. Our results confirm the documented toxicity of α-methylene lactones [51] and suggest that the therapeutic value of both isolated sesquiterpene lactones may be counterbalanced by their relatively high cytotoxicity (Table 3.1).

The aporphine alkaloids isolated by antiplasmodial activity-guided fractionation of *L. tulipifera* bark display *in vitro* antiplasmodial activates with IC_{50} values ranging between 1.2 and 29.6 μg/mL (Table 3.1). The antiplasmodial activity of compounds **1**, **4** and **5** showed considerable antiplasmodial activity against both the CQ sensitive D10 strain (IC_{50} values of 1.2, 4.1 and 1.2 μg/mL, respectively) and the CQ resistant Dd2 strain (IC_{50} values of 5.8, 7.9 and 5.2 μg/mL, respectively). These antiplasmodial values are consistent with previous reports for these compounds [52-54]. The low resistance index (RI) (4.8, 1.9 and 4.2 for **1, 4** and **5,** respectively) suggests a mechanism of resistance different from chloroquine. The favorable selectivity index (SI) for **1** and **5** suggests that these compounds show selective toxicity against *P. falciparum* when compared to mammalian cell lines. Compound **6** showed moderate antiplasmodial activity with an IC_{50} value of 9.1 μg/mL with no observable cytotoxicity. Compounds **2** and **3** show much lower antiplasmodial activity with IC_{50} values of 29.6 and 22.0 μg/mL respectively and no observable cytotoxicity.

Our results suggest that the antiplasmodial activity of crude extracts of *Liriodendron tulipifera* bark (IC_{50} = 10.9 μg/mL) and leaves (IC_{50} = 2.0 μg/mL) can be partially explained by the isolated aporphine alkaloids and sesquiterpene lactones, respectively. However, the crude extract of the leaves showed a comparable antiplasmodial activity to the pure isolated compounds (**7**) or (**8**) (6.2 and 1.8 μg/mL, respectively), suggesting that these compounds may act in combination with other constituents found in the leaves (possibly aporphine alkaloids, which are known to occur throughout the plant).

3.5. Conclusions

The investigation of *L. tulipifera* bark and leaves, a traditional antimalarial remedy used in the United States, yielded six aporphine alkaloids and two sesquiterpene lactones with moderate *in vitro* antiplasmodial activity. The antiplasmodial activity of both isolated sesquiterpene lactones (**7, 8**), along with three of the aporphine alkaloids (**2**, **3** and **6**) are reported here for the first time. While our results show high cytotoxicity of the isolated sesquiterpene lactones, long standing traditional use of *L. tulipifera* extracts may argue against toxicity in humans. It is also possible that the overall antimalarial efficacy and toxicology of the tulip tree extracts are derived from complex interactions of its antimalarial components, rather than from simple additive action. Species endemic to the United States, a region no longer troubled by malaria, are still important in the discovery of novel antimalarial botanical compounds. This work may serve as an example for future studies focused on American antimalarial plant species.

3.6. Chemical Data

Asimilobine (**1**): $C_{17}H_{17}N0_2$. ESI-MS, *m*/*z*: 268.0 $[M+H]^+$. ^1H NMR (500 MHz, $CDOD_3$) δ_H 8.37 (1 H, d, *J* = 7.6 Hz, C-11), 7.33 (3 H, m, C-8,9,10), 6.75 (1 H, s, C-3), 4.29 (1 H, m, C-6α), 3.71 (1 H, dd, *J* = 12.3, 6.2 Hz, C-5), 3.60 (3 H, s, OCH_3), 3.21 (2 H, m, C-5/C-7), 3.08 (1 H, dd, *J* = 13.5 Hz, 4.4, C-4), 2.97 (2 H, m,C-4/C-7).

Norushinsunine (**2**):$C_{17}H_{15}N0_3$. ESI-MS, *m*/*z*: 281.9 $[M+H]^+$. ^1H NMR (400 MHz, $CDOD_3$) δ_H 8.22 (1 H, d, *J* 7.9, C-11), 7.47 (2 H, m, C-8/C-9), 7.38 (1 H, dd, *J* = 10.5 Hz, 4.4, C-10), 6.73

(1 H, s, C-3), 6.17 (1 H, d, *J* = 1.1 Hz, O-CH_2-O), 6.04 (1 H, d, *J* = 1.1 Hz, O-CH_2-O), 4.78 (1 H, d, *J* = 3.4 Hz, C-7), 4.58 (1 H, s, C-6), 3.68 (1 H, dd, *J* = 12.5 Hz, 6.6, C-5), 3.52 (1 H, m, C-5), 3.26 (1 H, m, C-4) 2.99 (1 H, dd, *J* = 17.7 Hz, 5.1, C-4), 1.99 (1 H, d, *J* =1.6, N-H).

Norglaucine (**3**):$C_{20}H_{23}N0_4$. ESI-MS, *m*/*z*: 342.0 $[M+H]^+$. ^1H NMR (500 MHz, $CDOD_3$) δ_H 8.06 (1 H, s, C-11), 6.94 (1 H, s, C-3), 6.86 (1 H, s, C-8), 4.30 (1 H, dd, *J* = 13.8 Hz, 4.4, C-6α), 3.90 (3 H, s, -OCH_3), 3.89 (3 H, s, -OCH_3), 3.86 (3 H, s, -OCH_3), 3.73 (1 H, dd, *J* =12.7 Hz, 5.4, C-5), 3.68 (3 H, s, -OCH_3), 3.39 (1 H, m, C-5), 3.35 (1 H, m, C-7), 3.05 (1 H, m, C-7), 2.89 (1 H, t, *J* = 13.8 Hz, C-4).

Liriodenine (**4**):$C_{20}H_{23}N0_4$. ESI-MS, *m*/*z*: 275.9 $[M+H]^+$. ^1H NMR (500 MHz, $CDCl_3$) δ_H 9.06 (1 H, d, *J* = 5.6 Hz, C-5), 8.67 (1 H, d, *J* = 8.2Hz, C-11), 8.57 (1 H, d, *J*= 6.8 Hz, C-8), 7.97 (1 H, d, *J*= 5.7 Hz, C-4), 7.81 (1 H, t, *J* = 8.0 Hz, C-10), 7.62 (1 H, t, *J*=7.5 Hz, C-9), 7.05 (1 H, s, C-3), 6.46 (2 H, s, -O-CH_3-O-).

Anonaine (**5**):$C_{17}H_{15}N0_2$. ESI-MS, *m*/*z*: 265.9 $[M+H]^+$. ^1H NMR (400 MHz, $CDOD_3$) δ_H 8.13 (1 H, d, *J* = 7.8 Hz, C-11), 7.33 (3 H, m, C-8/ C-9/C-10), 6.74 (1 H, s, C-3), 6.17 (1 H, s, -O-CH_3-O-), 6.02 (1 H, s, O-CH_3-O-), 4.46 (1 H, dd, *J* =13.6 Hz, 3.7, C-6α), 3.74 (1 H, dd, *J* = 12.4 Hz, 6.3, C-5), 3.40 (2 H, m, C-7/ C-5), 3.17 (1 H, dd, *J* =14.2 Hz, 4.8, C-4), 3.01 (2 H, m, C4/C-7).

Oxoglaucine (**6**):$C_{20}H_{17}N0_5$. ESI-MS, *m*/*z*: 351.9 $[M+H]^+$. ^1H NMR (400 MHz, $CDOD_3$) δ_H 8.73 (2 H, s, C-8/ C-11), 8.22 (1 H, s, C-5), 7.82 (1 H, s, C-4), 7.56 (1 H, s, C-3), 4.05 (12 H, m, 4CH_3).

Peroxyferolide (**7**):$C_{17}H_{22}0_7$. ESI-MS, *m*/*z*: 337.0 $[M+H]^+$. ^1H NMR (500 MHz, $CDCl_3$), δ_H 6.31 (1 H, d, *J* = 3.5, C-13), 5.94 (1 H, m, C-8), 5.54 (1 H, d, *J* =3.0 Hz, C-13), 5.50 (1 H, d, *J* = 2.2 Hz, C-14), 5.21 (1 H, s, C-14), 4.38 (1 H, dd, *J* =11.1 Hz, 4.5, C-1), 4.18 (1 H, t, *J* = 9.6 Hz, C-6), 3.70 (1 H, s, C-7), 2.88 (1 H, d, *J* = 9.4 Hz, C-9), 2.75 (1 H, d, *J* = 13.8 Hz, C-5), 2.62 (1 H, s, C-9), 2.06 (3 H, s, Ac), 1.54 (3 H, s, C-15)

Lipiferolide (**8**):$C_{17}H_{22}0_5$. ESI-MS, *m*/*z*: 304.9 $[M+H]^+$. ^1H NMR (500 MHz, $CDCl_3$) δ_H 6.38 (1 H, d, *J* =3.0 Hz, C-13), 5.71 (1 H, d, *J* =3.1 Hz, C-13), 5.69 (1 H, d, *J* =4.5 Hz, C-8), 5.29 (1 H, dd, *J* =11.2 Hz, 3.3, C-1), 4.40 (1 H, t, *J*= 8.4 Hz, C-6), 3.15 (1 H, m, C-7), 2.82 (1 H, d, *J* = 8.7Hz, C-5), 2.76 (1 H, dd, *J* = 14.4 Hz, 4.7, C-9), 2.36 (1 H, dd, *J* =14.3 Hz, 1.9, C-9), 2.06 (3 H, s, Ac), 1.76 (3 H, s, C-15), 1.37 (3 H, s, C-14).

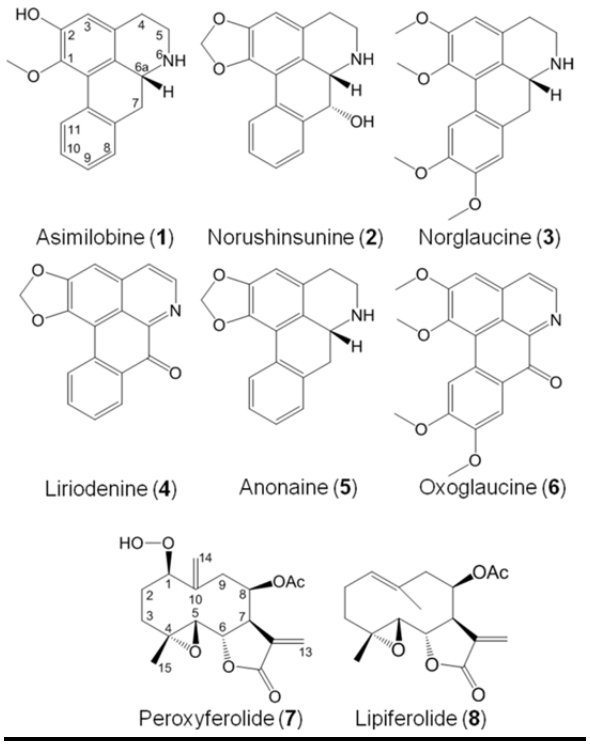
3.7. Tables and Figures

Table 3.1 *In vitro* biological activity of compounds isolated from *Liriodendron tulipifera*

	***Plasmodium falciparum* D10 (IC_{50})**		***Plasmodium falciparum* Dd2: IC_{50}**		**CHO Cell line(IC_{50})**		**SI**	**RI**
	µg/mL	**µM**	**µg/mL**	**µM**	**µg/mL**	**µM**		
1	1.2 ± 0.1	4.5 ± 0.3	5.8 ± 1.3	21.7 ± 6.3	> 100	>300	> 83	4.8
2	29.6 ± 3.9	105.2 ± 13.9	30.9 ± 2.3	109.8 ± 8.1	> 100	>300	> 3	1.0
3	22.0 ± 2.8	64.4 ± 8.2	32.2 ± 1.3	94.3 ± 3.8	> 100	>300	> 5	1.5
4	4.1 ± 1.0	14.9 ± 3.6	7.9 ± 1.1	28.7 ± 4.0	8.1 ± 0.1	29.4 ± .4	2.0	1.9
5	1.2 ± 0.1	4.5 ± 0.3	5.2 ± 0.3	19.6 ± 1.1	>100	>300	> 83	4.2
6	9.1 ± 0.1	25.9 ± 0.2	20.8 ± 2.9	59.2 ± 8.3	> 100	>300	> 11	2.3
7	6.2 ± 0.1	18.3 ± 0.3	4.3 ± 1.2	12.7 ± 3.5	3.2 ± 0.6	9.5 ± 1.8	0.5	0.7
8	1.8 ± 0.7	5.9 ± 2.3	2.3 ± 0.1	7.5 ± 0.3	1.4 ± 0.2	4.6 ± 0.7	0.8	1.3
CQ	.01 ± .01	.03 ± .01	.07 ± .01	.23 ± .01	-	-	-	7.9
Em	-				0.2 ± 0.1	0.4 ± 0.2	-	-

Selectivity index (SI) = IC_{50} CHO / IC_{50} D10
Resistance index (RI) = IC_{50} Dd2 / IC_{50} D10
CQ=Chloroquine
Em= Emetine

Figure 3.1 Antiplasmodial compounds from leaves and bark of *Liriodendron tulipifera* L.



CHAPTER 4

Antiparasitic compounds from *Cornus florida* L. with Activities Against *Plasmodium falciparum* and *Leishmania tarentolae*.

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4.1. Abstract

Aim of the study: The objective of this study was to identify the antiplasmodial constituents from the bark of *Cornus florida* L., a plant traditionally used in the united states for the treatment of malaria.

Methods and Materials: Dried and powdered bark was extracted with 95% ethanol. The resultant extract was subjected to *in vitro* antiplasmodial-guided fractionation against *Plasmodium falciparum* (D10 strain). Antiplasmodial IC_{50} values were calculated for pure compounds. Compounds were also assayed against *Leishmania tarentolae*, and rat skeletal myoblast L6 cells to assess antileishmanial activity and cytotoxicity, respectively.

Results: Antiplasmodial-guided fractionation afforded 8 compounds: betulinic acid (**1**), ursolic acid (**2**), β-sitosterol (**3**), ergosta-4,6,8,22-tetraene-3-one (**4**), 3β-O-acetyl betulinic acid (**5**), 3-epideoxyflindissol (**6**), 3β-O-*cis*-coumaroyl betulinic acid (**7**), 3β-O-*trans*-coumaroyl betulinic acid (**8**), of which, (**6**) is for the first time here isolated from a natural product and (**4**), (**7**) and (**8**) are reported for the first time from this genus. *In vitro* IC_{50} values against *P. falciparum* for (**4**) (61.0 μM) (**6**) (128.0 μM), (**7**) (10.4 μM), (**8**) (15.3

μM) are reported for the first time. Antileishmanial IC_{50} values are reported here for the first time for (**4**) (11.5 μM), (**6**) (1.8 μM), (**7**) (8.3 μM) and (**8**) (2.2 μM).Cytotoxicity against L6 cells is reported for all compounds.

Conclusions: The compounds isolated in this study, while displaying moderate *in vitro* antiplasmodial activity, do not fully support the historical importance of *C. florida* as an antimalarial remedy in North America. The traditional remedy may exert its well documented effects by mechanisms unrelated to direct antiplasmodial action. While not traditionally used to treat Leishmania, this work shows that several constituents of *C. florida* possess promising *in vitro* antileishmanial activity.

4.2. Introduction

Cornus L., commonly known as dogwoods, consists of about 58 species in 10 subgenera belonging to the Cornaceae Bercht. & J. Presl family and can be found across the northern hemisphere [55]. The fruit of several species (including *C. mas* and *C. suecica* in Europe/ Central Asia and *C. officinalis* and *C. kousa* in Eastern Asia) are eaten raw, fermented into alcoholic beverages and variously integrated into the diet [56, 57]. In China *C. officinalis* is an important medicinal plant used for numerous purposes including as an antibacterial, diuretic, hypotensive, urinary antiseptic, antipyretic and to suppress bodily fluids [58]. Several species of dogwood have gained horticultural recognition including *C. canadensis* (flowering ground cover), *C. alba* (border plant) and *C. florida, C. kousa* (year round landscape specimens)[59]. A variety of phytochemicals representing several chemical classes have been described from the genus that include:

iridoids, terpenoids, steroids and flavonoids among others. The distribution of chemicals, most notably iridoids [60-62], have been used as chemotaxonomic markers.

Cornus florida L. is native to eastern North America and is deeply ingrained in the ethnobotany of the region [28]. The Native Americans valued the root bark in treating the periodic fevers we now associate with malaria and an infusion of the flowers was used to 'sweat off fevers' [63]. The early American settlers were quick to realize the dogwood bark's potential in treating malaria, often citing it as of equal or greater value than the bark of *Cinchona*, the source of quinine [29, 56, 64]. Through the American Civil War, confederate surgeons prized the native remedy as a treatment for their troops [21]. During World War II, when the quinine supplies of the United States were cut off by enemy forces, the potential of *C. florida* as a quinine replacement was again considered when it was found to be active in treating avian malaria [23]. However, despite the long history of it use as an antimalarial, no formal investigation of *C. florida* as an antiplasmodial has been undertaken until now. The present work describes the antiplasmodial-assay guided fractionation, isolation, and identification of 8 compounds with antiplasmodial activities that likely contribute to the antimalarial qualities of this plant. In addition, the fractions and compounds were tested against *Leishmania* parasites in our aim to find novel antileishmanial compounds. Leishmaniasis is a neglected tropical disease for which there is an urgent need to find new leishmanicidal agents.

4.3. Methods and Materials

4.3.1. Instrumentation

See appendix A

4.3.2. Plant Material

Bark was collected from *Cornus florida* in September of 2009 at the north end of the field on the tree line in West Hills Park, Huntington Station, NY (40°48'36.38"N, 73°26'14.90"W). The plant material was collected by Rocky Graziose and species identity was confirmed by Dr. Lena Struwe; a voucher (RG #45) is stored at the Chrysler Herbarium (CHRB).

4.3.3. Extraction and Isolation

The dried and powdered bark (1.25 Kg) was extracted with 95% ethanol to yield 80 g of dried extract (Cf, 66,3% inhibition of *P. falciparum* at 20 μg/mL). A portion of this extract (40 g) was suspended in 2 L of distilled water for 2 hours. The insoluble portion was filtered and suspended in 2 L of water and again filtered. The aqueous solutions were combined and lyophilized (CfAS, 20 g, 23.5% inhibition) and the insoluble portion dissolved in ethanol, filtered and evaporated to dryness (CfAI, 18 g, 69.4% inhibition). A portion of the aqueous insoluble extract (CfAI, 10 g) was fractionated on silica gel (500 g) by flash chromatography using a stepwise gradient of methanol in chloroform ($CHCl_3$, 0.5, 1, 2, 5, 10, 20% MeOH). Fractions were obtained based on TLC analysis and assayed for activity. From fraction 7 (CfAI7), white crystals spontaneously formed, which were recrystallized from methanol to give 1,250 mg of betulinic acid (**1**). From fraction 8 (CfAI8) a mixture of two compounds crystallized (1050 mg), a portion of which were separated by RP-HPLC (C8, 19 x 300mm, acetonitrile/ 0.5% acetic acid in water, 85:15, 12 mL/min) to yield an additional amount of (**1**) (t_r = 13 min) along with ursolic acid (**2**) (t_r = 15 min). Upon bioassay, the activity was found in the mother liquor portions of fraction

7 and 8 (eluted with 1% MeOH in $CHCl_3$) (71 and 75% inhibition at 20 μg/mL, respectively), while the crystalized compounds (**1**) and (**2**), exhibited no antiplasmodial activity.

The mother liquor portions of CfAI7 (2.9 g) and CfAI8 (1.9 g) were combined (CfAI78) and a portion (1.5 g) was separated by countercurrent chromatography (elution-extrusion) using hexane/ 2-propanol/ acetonitrile (10:3:7) in the ascending mode (flow rate = 5 mL/min, rpm = 700, elution of upper phase for 1,350mL, extrusion of lower phase for 1,200mL, detection at 254 nm). Using UV and TLC data, 7 fractions were produced and submitted for bioassay. From CfAI78_2 (CCC 75-118 min, 144 mg), β-sitosterol (**3**) (91 mg) was purified by flash chromatography (hexane/ EtOAc, 9:1). From CfAI78_3 (CCC 119-158 min, 54 mg), ergosta-4,6,8,22-tetraene-3-one (**4**) (6 mg, t_r = 28 min) was purified by RP-HPLC (C8, acetonitrile/ 0.5% acetic acid in water, isocratic 95:5, 10 ml/min). Fraction CfAI78_4 (CCC 159- 294 mins, 346 mg) yielded 2 compounds after separation by RP-HPLC (C8, acetonitrile/ 0.5% acetic acid in water, isocratic 90:10, 15 mL/min): 3*β*-*O*-acetyl betulinic acid (**5**) (9mg, t_r = 11mins), and 3- epideoxyflindissol (**6**) (8mg, t_r = 15 min). Fraction CfAI78_6 (CCC 355-406 min, 129 mg) was separated by RP-HPLC (Prodigy 5μm 10 x 250 mm, acetonitrile/ methanol/ 0.5% acetic acid in water, isocratic 45:45:10, 5 mL/min) to yield 3*β*-*O*-*cis*-coumaroyl betulinic acid (**7**) (4 mg, *tr* =19 min) and 3*β*-*O*-*trans*-coumaroyl betulinic acid (**8**) (12 mg, t_r = 20.5 min).

4.3.4. In vitro *assays*

4.3.4.1 In vitro *antiplasmodial assay*

See appendix A

4.3.4.2 In vitro *antileishmanial assay*

See appendix A

4.3.4.3 Cytotoxicity Assay

See appendix A

4.4. Results and Discussion

The antiplasmodial, antileishmanial and cytotoxic activity for all compounds is reported in table 4.1. The IC_{50} value, or the concentration of each compound required to inhibit 50% of *in vitro* growth, are reported in μM for pure compounds. The structures of compounds **1**-**8** can be found in figure 1. LC-MS, physical and NMR data for the isolated compounds are presented and compared to previous reports.

Betulinic acid is a well-known constituent of the bark of certain tree species and may occur as a large percentage of the dry weight, especially in birch trees [65]. Biochemical analysis of the bark of *Cornus florida* suggested that betulinic acid (**1**), along with another common pentacyclic triterpene, ursolic acid (**2**), comprise 2% or more of the dry weight. Neither compound showed considerable *in vitro* antiplasmodial activity (IC_{50}> 200 μM for both compounds). On the contrary, in previous studies it was found that the *in vitro* IC_{50} value of betulinic acid against the W2 strain of *P. falciparum* was 9.89 μM [66] and 43.0 μM and 56.8μM against K1 and T9-96 strains respectively [67]. However, betulinic acid was not active *in vivo* in a murine model against *P. berghei* [67].

3β-O-acetyl betulinic acid (**5**), a common derivative of betulinic acid also isolated from the bark, but in much lower concentrations, was inactive against *P. falciparum*. However, this compound was also previously shown to have antiplasmodial activity with an IC_{50} value of 5.99 μM [66]. de Sá et al. also demonstrated that 3β-O-acetyl betulinic acid was able to reduce the parasitemia of *P. berghei* infected mice by 70% on the seventh day after infection when administered intraperitoneally, but not when administered orally.

While compounds (**2**) and (**5**) were inactive against *P. falciparum,* both showed significant activity against *L. tarentolae,* with IC_{50} values of 9.9 μM and 0.9 μM, respectively. In previous reports, 3β-O-acetyl betulinic acid (**5**) showed an IC_{50} value of 44.9 μM against *L. amazonensis* but was inactive (>200 μM) against *L. braziliensis* [68]. Wert et al. showed a series of synthetic betulin derivatives and their activity against promastigotes and amastigotes of *Leishmania infantum* and *L. donovani*, but none of the compounds exhibited appreciable activity at 50 μM [69].

In previous, studies it was shown that ursolic acid (**2**) had moderate activity against *L. amazonensis* but was inactive against *L. infantum* [70]. Also, Torres-Santos et al. showed that ursolic acid isolated from *Pourouma guianensis* Aublet (Moraceae) had a mild *in vitro* activity (IC_{50} = 59 μM) against intracellular amastigotes of *Leishmania amazonensis*[71].

Our results, which show β-sitosterol (**3**) inactive against both *P. falciparum* and *L. tarentolae*, are in accord with previous reports [72].

Ergosta-4,6,8,22-tetraene-3-one (**4**) is unlikely to be a native compound from *C. florida* bark as ergosterols, the class of compound to which (**4**) belongs, are not known to be synthesized by plants [73]. The collected bark was free from any visible mold and was dried shortly after collection to prevent microbial contamination. However, it is probable that fungi - which possess ergosterol biosynthetic pathways -associated with *C. florida* are the source of this compound (**4**). In preliminary studies (not shown here), bark samples were collected from six different individuals of *C. florida* in New York and New Jersey. Extracts of the bark from all individuals showed comparable *in vitro* antiplasmodial activity, which led us to believe environmental or geographic factors do not dramatically influence the antiplasmodial activity of this species. However, it is not known whether or not fungal infections alter the chemistry of *C. florida*.

The fact that a significant amount of (**4**) could be isolated from bark presents an interesting possibility that fungal compounds contribute to antiparasitic activities of plants. While the compound showed weak activity against *P. falciparum* (IC_{50} = 128 μM) it was quite active against *L. tarentolae* (IC_{50}= 1.8). This is the first report of either activity for this compound.

3- epideoxyflindissol (**6**) is a tirucallane triterpenoid reported here for the first time isolated from a natural product. Deoxyflindissone (21, 23-epoxytirucalla-7,24-diene-3-one) and 3β- acetoxy-epideoxyflidissol (3β-acetoxy-21, 23-epoxytirucalla-7,24-diene) were reported from *C. captiata* where 3-epideoxyflindissol was generated on $NaBH_4$ reduction of deoxyflindissone [74]. More recently, twelve new tirucallane triterpenoids named cornusalterin A-L, were isolated from *C. walterii* [75]. While the antiplasmodial

activity of (**6**) was negligible (IC_{50}= 128.0 μM), it possessed very strong antileishmanial activity (IC_{50}= 1.8 μM), which was comparable to the positive control pentamidine (IC_{50}= 1.6 μM).

Two phenolic esters of betulinic acid, 3*β*-*O*-*cis*-coumaroyl betulinic acid (**7**) and 3*β*-*O*-*trans*-coumaroyl betulinic (**8**), have been isolated by us for the first time from any *Cornus* spp.. Compounds (**7**) and (**8**) show moderate antiplasmodial activity (IC_{50} values of 8.3 μM and 2.2 μM, respectively) and antileishmanial activity (IC_{50} values of 10.4 μM and 15.3 μM, respectively). The considerably increased activity of these compounds over compound (**1**), suggests the addition of a coumaroyl group at the 3 position increases antimicrobial activity. This substitution introduces a large phenolic moiety that alters the structure of the compound as well its physico-chemical properties. It is interesting to note that the stereochemistry of the double bond- either *cis* or *trans*– does not significantly affect the activity.

This observation provides support for the notion that phenolic moieties greatly increase the antiplasmodial activity of pentacyclic triterpenes, which may be effectual regardless of position. For example, it was shown that 3β-O-*trans*-caffeic acid isolated from *Diospyros quaesita* showed considerable *in vitro* antiplasmodial activity against both a chloroquine sensitive (D_6) and resistant (W_2) strain of *P. falciparum* (1.40 and 0.98 μM, respectively), but when the phenolic moiety was chemically cleaved, the activity was reduced 8 fold [76]. In a separate study the *in vitro* antiplasmodial activity of messagenic acids A (2.5 μM) and B (6.3 μM), in which the 27^{th} position of betulinic acid is substituted with *cis*-coumaric acid and *trans*-coumaric acid, respectively, showed greatly increased

antiplasmodial activity over betulinic acid (inactive at 20μg/mL) [77]. The authors also showed that both ursolic acid and oleanolic acid, which were inactive at 20 μg/mL, also have significant activity (each 4.8 μM) when the 27th position is substituted with *trans*-coumaric acid.

Compounds (**5**), (**6**) and (**8**) displayed a similar or better antileishmanicidal activity (IC_{50} = 0.9, 1.8 and 2.2 μM, respectively) compared to the control, pentamidine (IC_{50} =1.6 μM). Plant derived natural products represent an important source for antileishmanial compound discovery, and this report adds to a small but growing list of phytochemicals with activity comparable or better than currently used drugs [78].

4.5. Conclusions

The present study describes the antiplasmodial constituents of *Cornus florida,* a species which held high acclaim as an antimalarial remedy of the eastern United states. It was found that the activity of this species can largely be attributed to phenolic esters of betulinic acid. However, previous studies have suggested that betulinic acid and ursolic acid have antiplasmodial properties as well and it is possible that, because of very high content, these triterpenes add to the antiparasitic activity of *Cornus florida* extracts *in vivo*.

The historical antimalarial value of *C. florida* could not be fully explained compounds isolated in this work. It is very probable that, as firsthand accounts show [79], extracts of *C. florida* bark may affect antimalarial activity not solely by killing the

malaria parasite, but by alternative means (i.e. fever reduction, immune support, etc.) that may warrant further investigation.

We also show here that, while not traditionally used to treat Leishmania, *C. florida* is the source of several highly potent antileishmanial compounds. Further investigation regarding the *in vivo* antileishmanial activity of both the crude extract and pure compounds is warranted as we have shown several compounds with equal or better activity than the pentamidine control.

4.6. Chemical Data

Betulinic Acid (**1**): white crystals

APPI-MS *m/z* 455.4 $[M - H]^-$

^{1}H NMR (pyridine d-5, 500 MHz) δ 4.97 (1H, s, H-29a), 4.79 (1H, s, H-29b), 3.56 (1H, dt, *J*=12.0, 4.5 Hz, H-19), 3.48 (1H, t, *J*=8.0 Hz, H-3), 2.76 (1H, td, *J*=12.0, 3.3 Hz, H-13), 2.65 (1H, dd, *J*=9.6, 3.2 Hz, H-16), 1.82 (3H, s, H-30), 1.25 (3H, s, H-23), 1.10 (3H, s, H-27), 1.09 (3H, s, H-26), 1.03 (3H, s, H-24), 0.85 (3H, s, H-25)

^{13}C NMR (pyridine d-5,126 MHz) δ 177.65, 150.13, 108.77, 76.93, 55.45, 54.74, 49.78, 48.58, 48.49, 46.59, 41.67, 39.94, 38.35, 38.10, 37.43, 36.41, 36.34, 33.65, 31.70, 30.03, 29.10, 27.49, 27.14, 24.93, 20.03, 18.30, 17.61, 15.25, 15.16, 13.73.

Compared with [80, 81].

Ursolic Acid (**2**): white powder

APPI-MS *m/z* 455.4 $[M - H]^-$

^{1}H NMR (pyridine d-5, 500 MHz) δ 5.51 (1H, t, *J*=3.3 Hz, H-19), 3.47 (1H, dd, *J*=10.3, 5.8 Hz, H-3), 2.66 (1H, d, *J*=11.4 Hz, H-18), 2.35 (1H, td, *J*=13.7, 4.9 Hz, H-15), 1.26 (3H, s, H-23), 1.25 (3H, s, H-27), 1.07 (3H, s, H-26), 1.04 (3H, s, H-24), 1.02 (3H, d, *J*=6.3 Hz, H-29), 0.97 (3H, d, *J*=6.0 Hz, H-30), 0.91 (3H, s, H-25)

^{13}C NMR (pyridine d-5,126 MHz) δ 180.24, 139.62, 125.98, 78.46, 56.17, 53.90, 48.40, 42.85, 40.32, 39.84, 39.76, 39.73, 39.43, 37.80, 37.64, 33.93, 31.43, 29.16, 29.04, 28.48, 26.51, 25.27, 24.26, 23.98, 21.76, 19.14, 17.87, 17.81, 16.92, 16.03.

Compared with [82] ESIMS

β-*Sitosterol* (**3**): white powder

EIMS *m*/*z* (%) 414 (100) $[M]^{+\bullet}$, 396 (61), 329 (63), 303 (57)

^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.35 (1H, d, *J*=5.2 Hz, H-6), 3.52 (1H, tt, *J*=11.8, 4.5 Hz, H-3), 1.01 (3H, s, H-19), 0.92 (3H, d, *J*=6.6 Hz, H-21), 0.87 – 0.80 (9H, 3 Me), 0.68 (3H, s, H-18)

^{13}C NMR ($CDCl_3$, 126 MHz) δ 140.75, 121.72, 71.81, 56.78, 56.07, 50.15, 45.85, 42.34, 42.30, 39.79, 37.27, 36.52, 36.16, 33.96, 31.93, 31.92, 31.66, 29.17, 28.26, 26.10, 24.32, 23.08, 21.10, 19.83, 19.41, 19.05, 18.79, 11.99, 11.87.

Compared with [83]

Ergosta-4,6,8,22-tetraene-3-one (**4**): slightly yellow oil

EIMS *m*/*z* (%) 392 (20) $[M]^{+\bullet}$, 268 (100), 253 (22), 214 (19)

^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.61 (1H, d, *J*=9.5 Hz, H-7), 6.03 (1H, d, *J*=9.5 Hz, H-6), 5.74 (1H, s, H-4), 5.26 (1H, dd, *J*=15.2, 7.3 Hz, H-23), 5.21 (1H, dd, *J*=15.3, 8.0 Hz, H-24), 1.06

(3H, d, *J*=6.7 Hz, H-21), 1.00 (3H, s, H-19), 0.96 (3H, s, H-18), 0.93 (3H, d, *J*=6.8 Hz, H-28), 0.85 (3H, d, *J*=6.8 Hz, H-26), 0.83 (3H, d, *J*=6.8 Hz, H-27)

^{13}C NMR ($CDCl_3$, 101 MHz) δ 199.47, 164.34, 156.06, 135.00, 133.99, 132.53, 124.64, 124.46, 123.01, 55.71, 44.34, 44.00, 42.89, 39.30, 36.77, 35.61, 34.15, 34.15, 33.10, 29.74, 27.73, 25.38, 21.24, 19.99, 19.67, 18.99, 17.65, 16.66.

Compared with [84]

3β-O-Acetyl Betulinic Acid (**5**): white powder

APPI-MS *m*/*z* 497.3 $[M - H]^-$

^{1}H NMR ($CDCl_3$, 500 MHz) δ 4.74 (1H, s, H-29a), 4.61 (1H, s, H-29b), 4.47 (1H, dd, *J*=10.6, 5.7 Hz, H-3), 3.00 (1H, td, *J*=10.6, 4.5 Hz, H-19), 2.04 (3H, s, -$OCOCH_3$), 1.69 (3H, s, H-30), 0.97, 0.94, 0.85, 0.84, 0.83 (each 3H, s, 5 x CH_3)

^{13}C NMR ($CDCl_3$, 126 MHz) δ 182.56, 171.03, 150.37, 109.75, 80.95, 55.43, 55.36, 50.42, 49.29, 46.94, 42.44, 40.71, 38.46, 38.41, 37.81, 37.14, 37.06, 34.26, 32.20, 30.57, 29.70, 27.96, 25.46, 23.71, 21.33, 20.87, 19.36, 18.18, 16.48, 16.19, 16.04, 14.66.

Compared to [85]

3-epideoxyflindissol (**6**): colorless amorphous solid

APPI-MS *m*/*z* 441.5 $[M + H]^+$

^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.27 (1H, s, H-7), 5.18 (1H, d, *J*=8.6 Hz, H-24), 4.53 (1H, dt, *J*=14.4, 9.1 Hz, H-23), 3.90 (1H, t, *J*=7.8 Hz, H-21a), 3.42 (1H, t, *J*=8.8 Hz, H-21b), 3.24 (1H,

d, *J*=10.2 Hz, H-3), 1.72 (3H, s, H-26 or H-27), 1.68 (3H, s. H-26 or H-27), 0.97 (6H, s, H-28 and H-30), 0.86 (3H, s, H-29), 0.81 (3H, s, H-18), 0.75 (3H, s, H-19)

^{13}C NMR ($CDCl_3$, 126 MHz) δ 145.41, 135.76, 126.26, 118.16, 79.21, 75.26, 71.44, 51.13, 50.70, 50.67, 48.79, 43.72, 43.54, 40.22, 38.97, 37.15, 35.04, 34.25, 31.65, 27.74, 27.69, 27.60, 27.12, 25.85, 23.96, 22.57, 18.13, 17.51, 14.71, 13.07.

Compared to [74, 86]

3β-O-cis-coumaroyl betulinic acid (**7**): pale yellowish powder

APPI-MS *m/z* 601.6 $[M - H]^-$

^{1}H NMR (pyridine d-5, 500 MHz) δ 8.13 (2H, d, *J*=8.2 Hz, H-5′ and H-9′), 7.22 (Obscured by solvent shift, H-6′ and H-8′), 7.01 (1H, d, *J*=12.9 Hz, H-3′), 6.06 (1H, d, *J*=12.9 Hz, H-2′), 4.94 (1H, s, H-29a), 4.83 (1H, dd, *J*=11.8, 4.5 Hz, H-3), 4.79 (1H, s, H-29b), 3.62 (1H, s, H-19, 1.82 (3H, s, H-30), 1.09 (3H, s, H-27), 1.05 (3H, s, H-26), 0.95 (3H, s, H-23), 0.89 (3H, s, H-24), 0.78 (3H, s, H-25)

^{13}C NMR (pyridine d-5, 126 MHz) δ 171.88, 166.31, 158.40, 150.38, 142.89, 132.48, 132.35, 127.73, 117.99, 115.81, 114.89, 109.74, 80.90, 56.21, 55.53, 50.46, 49.28, 46.90, 42.46, 40.73, 38.46, 38.35, 37.86, 37.15, 37.02, 34.28, 31.90, 30.54, 29.71, 28.00, 25.49, 23.67, 20.90, 19.35, 18.21, 16.50, 16.21, 16.05, 14.12.

Compared to [87]

3β-O-trans-coumaroyl betulinic acid (**8**): pale yellowish powder

APPI-MS *m/z* 601.6 $[M - H]^-$

^{1}H NMR (pyridine d-5, 400 MHz) δ 8.06 (1H, d, *J*=15.9 Hz, H-3′), 7.68 (2H, d, *J*=8.7 Hz, H-5′ and H-9′), 7.21 (2H, d, *J*=8.8 Hz, H-6′ and H-9′), 6.73 (1H, d, *J*=15.9 Hz, H-2′), 4.97 (1H, s, H-29a), 4.91 (1H, dd, *J*=11.6, 4.7 Hz, H-3), 4.80 (1H, s, H-29b), 3.57 (1H, td, *J*=11.2, 5.2 Hz, H-19), 1.82 (3H, s, H-30), 1.12 (3H, s, H-27), 1.06 (3H, s, H-26), 0.98 (6H, s, H-23 and H-24), 0.82 (3H, s, H-25)

^{13}C NMR (pyridine d-5, 101 MHz) δ 179.57, 167.76, 161.96, 151.84, 145.45, 131.19 (2C, H-5′ and H-9′), 126.69, 117.36 (2C, H-6′ and H-8′), 116.32, 110.42, 81.01, 57.15, 56.17, 51.19, 50.23, 48.28, 43.34, 41.55, 39.08, 39.03, 38.75, 38.13, 37.80, 35.11, 33.41, 31.71, 30.75, 28.62, 26.51, 24.79, 21.66, 19.94, 18.95, 17.42, 16.86, 16.78, 15.37.

Compared to [87]

4.7. Tables and Figures

Figure 4.1 Antiplasmodial compounds from leaves and bark of *Cornus florida* L.

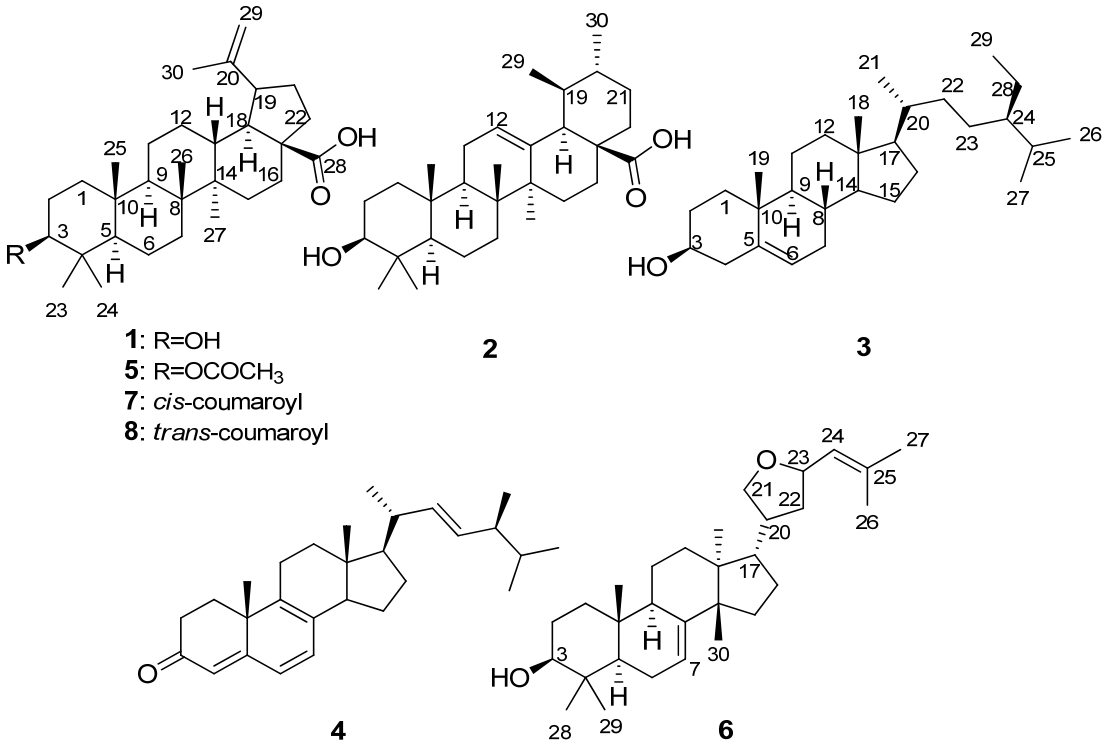


Table 4.1 *In vitro* biological activity of compounds isolated from *Cornus florida*

Sample	IC50 (μM) *L. tarentolae*	*P. falciparum*	L6 cells
Betulinic Acid (**1**)	>40	>200	10.6 ± 2.6
Ursolic Acid (**2**)	9.9 ± 3.2	>200	12.7 ± 0.7
β-Sitosterol (**3**)	>40	>200	6.2 ± 1.0
Ergosta-4,6,8,22-tetraene-3-one (**4**)	11.5 ± 0.4	61.0 ± 6.3	27.0 ± 5.4
3*β*-*O*-Acetyl Betulinic Acid (**5**)	0.9 ± 0.3	>200	5.2 ± 0.1
3- epideoxyflindissol (**6**)	1.8 ± 1.1	128.0 ± 8.1	14.7 ± 1.1
3*β*-*O*-*cis*-coumaroyl betulinic acid (**7**)	8.3 ± 4.0	10.4 ± 1.2	5.6 ± 1.4
3*β*-*O*-*trans*-coumaroyl betulinic acid (**8**)	2.2 ± 0.1	15.3 ± 0.9	9.3 ± 0.7
Pentamidine[a]	1.6 ± 0.1		
Chloroquine[a]		0.02 ± 0.001	
Emetine[a]			0.04 ± 0.001

In vitro antileishmanial (*L. tarentolae)*, antiplasmodial (*P. falciparum,* D10) and cytotoxic (L6 cells) activity of isolated compounds. *Each* sample was tested in triplicate. Data is expressed in μM with standard deviation. [a] Positive control

CHAPTER 5

Cucurbitacin Glycosides from *Datisca glomerata* (C. Presl.) Baill. with Antiplasmodial and Cytotoxic Activities.

Submitted to Phytochemistry July 2012

5.1. Abstract

Aim of the study: The objective of this study was to identify the antiplasmodial constituents from the bark of *Datisca glomerata* (C. Presl.) Baill., a plant traditionally used in the United States for the treatment of malaria.

Methods and Materials: Dried and powdered plant material was extracted subjected to *in vitro* antiplasmodial-guided fractionation against *Plasmodium falciparum* (D10 strain). Antiplasmodial IC_{50} values were calculated for pure compounds. Compounds were also assayed against *Leishmania tarentolae* to determine antileishmanial activity as well as rat skeletal myoblast L6 cells and Chinese ovarian hamster cells (CHO) to assess cytotoxicity.

Results: Seven new cucurbitacin glycosides, datiscosides I-O, along with the two known compounds, datiscoside and datiscoside B, were isolated from the aerial parts of *D. glomerata*. Their structures and relative stereochemistry were determined on the basis of mass spectrometry, 1D and 2D NMR. The antiplasmodial activity of the compounds was moderate and ranged from 7.7 μM to 33.3 μM. None of the compounds showed appreciable antileishmanial activity. The compounds displayed cytotoxicity against L6 but not CHO mammalian cells.

Conclusions: This work supports the traditional use of *Datisca glomerata* as an antimalarial remedy by describing several antiplasmodial of the plant extract. Due to the selective action of these compounds, further studies may be warranted to investigate the mode of action and medicinal applicability of the datiscosides.

5.2. Introduction

Datisca glomerata (Datiscaceae), commonly known as durango root, is a functionally androdioecious [88] perennial herb that habits riparian environments in California, Baja California and other parts of the southwestern United States [89]. *D. glomerata* was used by the Native Americans as an external wash for sores, as a sore throat remedy, and occasionally to treat fevers [63]. *D. cannibina,* the only other species of the family Datiscaceae, is found in central Asia and northern India [89] and has been traditionally considered as a substitute to quinine [90]. During World War II, it was shown that a crude extract of *D. glomerata* had potent *in vivo* antimalarial properties in white leghorn chicks infected with *Plasmodium gallinaceum* [23].

Being a member of the cucurbitales, *D. glomerata* is a prolific producer of cucurbitacins, and previous reports have described unique cucurbitacins glycosides from *D. glomerata* aptly named the datiscosides [91, 92]. The first cucurbitacin reported from *D. glomerata*, named datiscoside, was isolated as an antileukemic principle in 1972 [91] and its molecular structure and absolute configuration was confirmed by X-ray crystallography shortly after [93]. Datiscoside is the 16-*O*- (2'-*O*-acetyl-6'-deoxy-α-L-*gluco*-hex-3'-ulopyranoside) of Cucurbitacin D. The unique structure of datiscoside

prompted further investigation into the chemistry of *D. glomerata*, which yielded an additional 7 cucurbitacin glycosides, named datiscoside B-H, along with datiscoside and the aglycones cucurbitacin D and F as well as cucurbitacin B [92].

Cucurbitacins are known to be cytotoxic [94], and there have been reports of livestock poisoning following consumption of *D. glomerata* [95]. However, the medicinal merit of cucurbitacins is well substantiated by a history of traditional use of species of Cucurbitaceae and numerous research reports of their bioactivity. Interestingly, few reports have been published assessing the antimalarial potential of cucurbitacins, despite the widespread traditional use of species of the cucurbitales to treat malaria [96].

This work describes the antiplasmodial assay guided fractionation of the aerial parts of *D. glomerata*. This traditionally used plant yielded 7 novel cucurbitacin glycosides, named datiscosides I-O along with two known compounds, datiscoside and datiscoside B. The isolation and structure elucidation as well as the antiplasmodial, antileishmanial and cytotoxic activity of these compounds is discussed.

5.3. Methods and Materials

5.3.1. Instrumentation

See Appendix A

5.3.2. Plant material

Datisca glomerata was kindly collected and verified by Jim French, PhD (retired professor, Rutgers University) in July 2011, at Santa Lucia Memorial Park, Los Padres National Forest, Monterey County, CA. The plant material was authenticated by Rocky

Graziose and Lena Struwe, PhD (Rutgers University/Chrysler herbarium). A voucher specimen is stored at the Chrysler herbarium, CHRB, (JF-MP1).

5.3.3. Extraction and Isolation

Dried and powdered aerial parts (230 g) of *D. glomerata* were defatted by extracting with hexane (2 L) twice for a total of 24 hrs. The plant material was then extracted with 2 L of methanol, twice, for a total of 48 hrs. The methanolic extract was concentrated to 150 mL to which 1350 mL distilled water was added and partitioned with hexane (1 L), which was discarded. The aqueous portion was removed of methanol, concentrated to 500 mL and partitioned first with ethyl acetate, then with 2-butanol (2 x 500 mL, each). Both the ethyl acetate and 2-butanol fractions showed similar TLC and comparable (albeit, moderate) inhibition of *P. falciparum:* 24 and 20 % inhibition, respectively at 10 μg/mL.

The ethyl acetate (23 g) and 2-butanol (19 g) fractions were combined, half of which (21 g) was chromatographed over silica gel column (500 g) with a stepwise elution gradient (1 L each 0, 2, 4, 6, 8, 10, 20% MeOH in $CHCl_3$). Subfractions (250 mL) were collected and pooled by TLC similarity ($UV_{254, 366}$, p-anisaldehyde/H_2SO_4) to generate 4 main fractions A-D. Fraction B (subfractions 15-19, 5.4 g) showed a moderately increased activity (35% inhibition at 10 μg/mL) while fraction C (subfractions 20-30, 4.7 g) was about the same as the starting material (23% inhibition at 10 μg/mL)

Fraction B was subject to an additional separation by flash chromatography using dichloromethane-ethyl acetate-methanol (1L each 60:40:0, 60:40:2, 60:40:5, 60:40:10,

60:40:20, 250 mL subfractions) to generate 6 fractions BA-BF. Fractions BC (subfractions 11-12, 1.8 g), BD (subfractions 13-14, 1.3 g) and BE (subfractions 15-18, 0.6 g) showed increased activity over the starting material and inhibited *P. falciparum* by 46, 47, and 53 % at 10 μg/mL, respectively. The three fractions BC, BD and BE were separately purified by centrifugal partition chromatography using hexane: ethyl acetate-methanol-water (1:1:1:1) in the ascending mode (10 mL/min, elute 1.2 L upper phase, extrude 1.2 L lower phase). BC furnished two compounds: **2** (140 mg), which eluted from 121-158 minutes and was crystallized as colorless needles from neat ethanol and **9** (510 mg) which eluted from 159-171 minutes and was crystallized as colorless needles from diethyl ether. BD also afforded two compounds: **8** (50 mg), which eluted from 132-135 minutes, and was further purified by flash chromatography (2% MeOH in dichloromethane) and **1** (142 mg) which eluted from 179-210 minutes and was crystallized as colorless needles from neat ethanol. BE afforded a single compound **5** (84 mg) which eluted from 189-220 minutes, and was crystallized as colorless needles from diethyl ether-hexane.

Fraction C was subject to separation by centrifugal partition chromatography using hexane-ethyl acetate-methanol-water (2:3:2:3) in the ascending mode (10 mL/min, elute 1.2 L upper phase, extrude 1.2 L lower phase). Three separate runs were made with 1.5 g per run, which generated 5 fractions, CA- CE. Fraction CD (108-180 minutes, 700 mg) was subjected to HPLC (250 x 25 mm, C18 column, Acetonitrile: water, 1:1, 10ml/min) to yield 3 compounds: **3** (50 mg, *R*t =11.1 min), **7** (55 mg, *R*t= 12 min), **6** (116 mg, *R*t = 15.3 min). CE (181-210 min, 200 mg) yielded compound **4** (41 mg) after additional HPLC purification (parameters the same as above, *R*t= 12.5 min).

5.3.4. In vitro *assays*

5.3.4.1 In vitro *antiplasmodial assay*

See appendix A

5.3.4.2 In vitro *antileishmanial assay*

See appendix A

5.3.4.3 In vitro *cytotoxicity assays*

5.3.4.3.1 In vitro *cytotoxicity assay using rat skeletal myoblast L6 cells*

See appendix A

5.3.4.3.2 In vitro *cytotoxicity assay using Chinese ovarian hamster CHO cells*

See appendix A

5.4 Results and Discussion

5.4.1. Compound Characterization

The defatted and dried aerial parts from *D. glomerata* were extracted with methanol and the resulting extract was dried, suspended in water and successively partitioned with hexane, ethyl acetate and 2-butanol. The ethyl acetate and 2-butanol fractions were combined and subjected to bioassay guided fractionation against the chloroquine sensitive strain *Plasmodium falciparum* (D10), which led to the isolation of nine cucurbitacin glycosides (**1-9**).

Datiscoside (**1**) was isolated as colorless needles from neat ethanol. High resolution ESI-TOF-MS showed an $[M-H]^-$ ion at *m*/*z* 701.3461 corresponding to a

molecular formula $C_{38}H_{54}O_{12}$ (calculated 701.3543). The ^{13}C and ^{1}H NMR of **1** (Table 1 and 2) as well as the TLC (*R*f = 0.68, chloroform - methanol, 17:3) corresponded well with the published data for the known compound cucurbitacin D 16-*O*-(2'-*O*-acetyl-6'-deoxy-α-L-*gluco*-hex-3'-ulopyranoside) (datiscoside) [91-93] (Figure 1).

Datiscoside I (**2**) possessed a molecular formula $C_{38}H_{54}O_{11}$ based on its HR-ESI-TOFMS signal at *m*/*z* 685.3530 $[M-H]^-$ in the negative ion mode. The molecular weight of **2** showed 16 mass units less than that of **1**, suggesting one less oxygen atom. From the ^{1}H NMR, it was evident that **1** and **2** shared the same aglycone, cucurbitacin D [92]. However, the spectra suggested that compounds **1** and **2** possessed a different sugar moiety (Table 2). The downfield shifts of H-1' (δ_H 5.29) and H-2' (δ_H 5.18) in compound **2** suggested that C-2' was linked to an acetoxy group, similar to that of compound **1**. This was corroborated by the HMBC correlation of H-2' to the acetoxy carbonyl carbon at δ_C 169.4. The coupling constant between H-1' and H-2' (4 Hz), suggested an equatorial (H-1')-axial (H-2') relationship [97], similar to that of datiscoside (**1**) [92]. The C-3' carbonyl group found in **1** was also present in **2**, where it resonated at δ_C 200.2, and was correlated to both H-1' and H-2' in the HMBC. The C-4' resonance (δ_C 48.5), was shifted far upfield in **2** compared to that of **1** (δ_C 78.0), which, along with a negative DEPT peak confirmed that C-4' was missing the hydroxyl group of datiscoside (**1**). H-4' eq and H-4' ax appeared as a broad singlet (δ_H 2.40) and doublet (δ_H 2.39, *J* = 3.4 Hz), respectively. H-5' (δ_H 3.82, dqd, *J* = 11.5, 6.1, 3.2 Hz) resonated slightly further downfield than the H-5' of datiscoside (**1**) (δ_H 3.47, dq *J* = 9.6, 6.0 Hz). The additional splitting of H-5' (*J* = 3.2 Hz) further confirmed the presence of H-4 eq and supported the configuration of the C-5'

methyl group as equatorial. Therefore, it was confirmed that compound **2** is the 16-*O*-(2'-*O*-acetyl-4',6'-dideoxy-*α*-L-*gluco*-hex-3'-ulopyranoside) of cucurbitacin D and was given the name datiscoside I.

Datiscoside B (**3**) gave a low resolution mass signal at *m*/*z* 703 $[M-H]^-$. ^1H , ^{13}C NMR, and TLC (*R*f = 0.51) of **3** corresponded well with the published literature of the known compound datiscoside B [92].

Datiscoside J (**4**) was assigned a molecular formula $C_{38}H_{58}O_{11}$ based on the HR-ESI-TOFMS $[M-H]^-$ ion at *m*/*z* 689.3824 in the negative ion mode. The ^{13}C NMR showed two less resonances in the SP^2 region and two additional resonances at δ_C 80.8 and 67.1 when compared to **2**. Along with the appearance of two proton resonances at δ_H 2.99 and 4.05, this data suggested that the carbonyl groups at C-3 and C-3', respectively, of **2** had been reduced in **4.** Investigation of the ^1H and ^{13}C NMR spectra revealed that the aglycone of **4** was cucurbitacin F, as in **3**, accounting for one reduced ketone at C-3 compared to **1** and **2**. The other was found to be at the C-3' position where the ketone of **2** was reduced to an axial hydroxyl group. H-3' of **4** resonated at δ_H 4.05 as a broad singlet and was coupled to H-2', H-4' ax and H-4'eq as revealed by the COSY spectrum and showed NOE with those resonances as well, confirming the axial position of the hydroxyl group. A noteworthy observation from the ^1H NMR was that the H-24 resonance of **4** (δ_H 7.21) was shifted downfield compared to all other isolated molecules (δ_H 7.01-7.05), which may be attributable to through space interactions with the sugar

moiety, 2'-*O*-acetyl-4',6'-dideoxy-α-L-allopyranoside. Compound **4** was confirmed as cucurbitacin F 16-*O*-(2'-*O*-acetyl-4',6'-dideoxy-α-L-allopyranoside).

Datiscoside K (**5**) possessed a molecular formula of $C_{38}H_{56}O_{11}$ based on the $[M-H]^-$ ion at *m*/*z* 687.3658 from its HR-ESI-TOFMS. ^{1}H NMR indicated that **5** possessed the same aglycone moiety, cucurbitacin F**,** as in **3** and **4** (Figure 1). The ^{1}H and ^{13}C NMR resonances associated with the sugar moiety matched quite closely to that of **2** (Tables 1 and 2). Therefore the structure of **5** was determined to be the 16-*O*-(2'-*O*-acetyl-4',6'-dideoxy-α-L-*gluco*-hex-3'-ulopyranoside) of cucurbitacin F.

Comparison of the ^{1}H and ^{13}C NMR of compounds **6-9** revealed that they shared the same sugar moiety, as the chemical shifts of H-1' through H-6' and C-1' through C-6' matched with almost no variation (Tables 2 and 3). Analysis of the NMR spectra revealed the sugar moiety to be 2',3'-di-*O*-acetyl-4'-deoxy-α-L-allopyranoside, which had been previously reported as attached to cucurbitacin D in datiscoside **F** [92]. The C-2' and C-3' positions were both found to possess acetoxy groups as confirmed through the HMBC correlations of H-2' and H-3', respectively. The α linkage of the anomeric carbon, as is common to all reported datiscoside with the exception of datiscoside G, was confirmed by NOE and further supported by the ^{13}C NMR shift, which is slightly upfield from β-linked anomeric carbons [97]. The relative configuration of the acetoxy groups as C-2' equatorial and C-3' axial was confirmed by the indicative coupling constant between the H-1' and H-2' (*J* = 3.4-4.3 Hz), and H-2' and H-3' (*J* = 3.2-3.9 Hz). The NOESY

correlation between H-1' and H-2', H-2' and H-3', and H-3' to both H-4'ax and H-4' eq further confirmed the configuration.

Datiscoside L (**6**) isolated by reverse phase HPLC, possessed a molecular formula of $C_{40}H_{60}O_{12}$ based on its HRESIMS signal of *m/z* 731.4103 $[M-H]^-$. Analysis of the ^1H and ^{13}C NMR of **6** indicated that the aglycone was cucurbitacin **F**. Based on the assignment of the sugar moiety described above, the structure of **6** was determined to be the 16-*O*-(2',3'-di-*O*-acetyl-4'-deoxy-*α*-L-allopyranoside) of cucurbitacin F.

Datiscoside M (**7**) isolated by reverse phase HPLC, was found to have the identical molecular formula as compound **6** of $C_{40}H_{60}O_{12}$ based on its HRESIMS signal of *m/z* 731.4103 $[M-H]^-$. The compounds separated nicely by TLC (*R*f of **6** = 0.44, and **7** = 0.48) and HPLC (*R*t of **6** = 19.4 min, and **7** =18.4 min, see experimental) and upon spraying with p-anisaldehyde/H_2SO_4, the compounds showed a marked color difference (**6** - purple, **7** - blue). As previously stated, the ^1H and ^{13}C NMR clearly showed that the compounds shared the sugar 2',3'-di-*O*-acetyl-4'-deoxy-*α*-L-allopyranoside. The ^1H NMR spectra of **6** and **7** were largely similar save for the resonances of the A-ring protons. Both H-2 (δ_H 3.92) and H-3 (δ_H 3.47) in **7** were shifted downfield compared to those of **6** (δ_H 3.58 and 2.98, respectively). These shifts could be explained by assigning an equatorial position to H-3 (with concomitant equatorial deshielding) and an axial position to the C-3 hydroxyl group in **7,** which would exert an anti deshielding effect on H-2 [98]. The coupling constant between H-2 and H-3 in **6** (9.1 Hz) suggested diaxial coupling while that of **7** (3.5 Hz) suggested equatorial-axial coupling [97]. The ^{13}C NMR

showed an upfield shift in A ring carbons C-1, C-2 and C-3 in **7** as compared to **6** which can be attributed to the stronger deshielding effect of the equatorial –OH in **6** [99]. The ^{1}H NMR resonances of the C-4 methyl groups were also affected by the relative configuration of the C-3 hydroxyl group. In **6,** the axial methyl group (C-29) on C-4 resonates at δ_H 21.6 where in **7** the shift is δ_H 25.4, which is consistent with a gauche and anti-relationship to the C-3 hydroxyl group, respectively [100]. In compound **6** NOE was observed only between H-3 and the equatorial methyl group H-28, consistent with an axial position for H-3. However, in compound **7** NOE was observed between H-3 and both methyl groups H-28 and H-29, indicating an equatorial position of H-3 (Figure 2). From this data, the aglycone was confirmed to be cucurbitacin O, which was originally assigned as 2α, 3α hydroxyl [101], but, and as this work confirms, was later determined to be 2β, 3β hydroxyl [91]. Datiscoside M, was assigned as cucurbitacin O 16-*O*-(2',3'-Di-*O*-acetyl-4'-deoxy-α-L-allopyranoside).

Datiscoside N (**8**) and Datiscoside O (**9**) were found to have the same molecular formula of $C_{40}H_{60}O_{11}$ based on the $[M-H]^-$ HRESIMS ions of *m*/*z* 715.3965 and 715.3994 respectively (calculated 715.4063). As mentioned above, the sugar moiety of both compounds was identical to that of **6** and **7** (2',3'-di-*O*-acetyl-4'-deoxy-α-L-allopyranoside). Investigation of the ^{13}C NMR revealed the C-2 resonance was shifted far upfield (δ_C 30.6 and 29, for **8** and **9**, respectively) as compared to the previous set of isomers **6** and **7** (δ_C 71.2 and 68.2, respectively) and gave a negative DEPT peak. This data suggested that the C-2 hydroxyl group of **6** and **7** was not present in **8** and **9**. Like the previous pair of isomers, **8** and **9** were chromatographically separable with an *R*f

difference of 0.05 and an *R*t difference of 1.4 min. Also similar to the previous pair, **8** showed a purple color on spraying with p-anisaldehyde/ H_2SO_4, while **9** appeared blue. In **8,** the axial methyl group (C-29) on C-4 resonated at δ_C 20.3, where in compound **9** it was shifted downfield at δ_C 25.5, which is consistent with a gauche and anti-relationship to the C-3 hydroxyl group, respectively [100]. H-3 of compound **8** showed NOE only with H-1 ax and H-28 while compound **9** showed NOE with H-2 ax, H-2 eq, H-28 and H-29 (Figure 2). Interpreting this data in comparison to that of isomers **6** and **7**, it was concluded that datiscoside N (**8**) is 2-deoxycucurbitacin F 16-*O*-(2',3'-di-*O*-acetyl-4'-deoxy-*α*-L-allopyranoside) while datiscoside O (**9**) is 2-deoxycucurbitacin O 16-*O*-(2',3'-di-*O*-acetyl-4'-deoxy-*α*-L-allopyranoside).

5.4.2. General Observations About the Datiscosides

The B, C, and D rings of all datiscosides isolated to date are identical and structural observations based on the NMR spectra are discussed here. The assignment of the methyl groups (C-18, 19, 21, 26, 27, 28 and 29) were in accordance with previously published cucurbitacin structures [102] and were confirmed here based on HMQC and HMBC spectra. The definitive assignment of C-26 vs. C-27 along with their respective protons was not attempted. The carbon and proton shifts of the gem-dimethyl groups, C-28 and C-29 in the A ring, were distinguished on the basis of axial and equatorial shielding effects, hydroxyl γ effects (^{13}C NMR) and NOE correlations.

The stereochemistry of the D ring for all compounds was determined based on comparison with published ^{1}H and ^{13}C NMR data [102] and by analysis of NOESY spectra. Cross peaks in the NOESY between H-17 and H-30, as well as between H-16

and H-18 confirmed the diaxial orientation of the methyl groups as well as the β-orientation of the side chain and the α-orientation of the C-16 sugar moiety.

The NOESY correlation between H-8, H-18, and H-19 confirms that these groups all reside on the same side of the molecule, designated here as alpha in agreement with all previously reported cucurbitacins [94].

The configuration of the C-20 stereocenter was confirmed by NOE of H-23 with H-21, and is in agreement with previous reports [92].

5.4.3. Biological Activity

All isolated compounds were evaluated for their *in vitro* antiplasmodial activity using the chloroquine sensitive D10 strain of *Plasmodium falciparum*. The compounds were also tested for their ability to inhibit *Leishmania tarentolae in vitro*. The compounds were assessed for cytotoxicity against two cell lines: rat skeletal muscle (L6) and Chinese ovarian hamster (CHO). Activities are listed in Table 3.

The antiplasmodial activity of the isolated compounds showed moderate *in vitro* activity and ranged from 7.7 μM to 3.3μM. The most active compound was datiscoside K (**5**) (7.7 μM) while the least active was datiscoside O (**9**) (33.3 μM). Some structure-activity comments can be made based on the structural similarities and differences of the isolated compounds.

The oxidation state of C-3 does not dramatically affect the activity of the compounds, as evidenced by the comparable activities of **1** (13.5 μM), where C-3 has a ketone moiety, and **3** (14.5 μM) where C-3 is hydroxylated. Similarly, the oxidation state

of C-2 does not noticeably affect the activity, as evidenced by comparing compounds **6** vs. **8**, and **7** vs. **9**, in which the former compounds in the pairs are hydroxylated at C-2 where the latter are not.

The nature of the C-3' position moderately effects the antiplasmodial activity where hydroxyl> acetoxy> ketone, as in compounds **5**, **6**, and **4**, IC_{50} values of 7.7, 12.7, and 16.3 μM, respectively.

The most notable trend follows the stereochemistry of the C-3 hydroxyl which appears to play a significant role in the antiplasmodial activity. This can be readily observed when comparing the activity of **6** (IC_{50} = 12.7 μM) vs. **7** (IC_{50} = 33.8 μM) as well as **8** (IC_{50} = 8.8 μM) vs. **9** (IC_{50} = 27.8 μM), where the former compounds possess an α equatorial hydroxyl group while the latter share a β axial hydroxyl group at C-3.

None of the compounds tested exhibit activity below 20μg/mL against *Leishmania tarentolae in vitro*. There have been no reports of this plant being used to treat this illness, so our findings are unsurprising.

The compounds display cytotoxicity against rat skeletal muscle cells (L6) but do not show cytotoxicity toward Chinese ovarian hamster cells (CHO). Datiscoside (**1**), has been previously shown to possess *in vitro* cytotoxicity toward human carcinoma cells of the nasopharynx (KB), (ED_{50}= 0.16 μg/mL) as well as *in vivo* against P-388 leukemia and WM-256 intramuscular carcinosarcoma [91].

Our results suggest that datiscosides I-O may exhibit selective toxicity, as evidenced by their activity against *Plasmodium falciparum* but not *Leishmania tarentolae*, as

well as their cytotoxicity against rat skeletal muscle cells (L6), but not Chinese ovarian hamster cells (CHO).

5.5. Conclusions

In this work, 9 cucurbitacin glycosides were isolated from the aerial parts of *D. glomerata* following bioassay guided fraction, 7 of which (datiscosides I-O) are here reported for the first time. All compounds displayed moderate *in vitro* antiplasmodial activity, with interesting structure activity relationship related to the stereochemistry of the C-3 hydroxyl group. None of the compounds exhibited appreciable activity against *Leishmania tarentolae*, suggesting that toxicity amongst protozoa may be selective. Similarly, we show that datiscosides I-O have cytotoxicity against rat skeletal muscle cells (L6) but not against Chinese ovarian hamster cells. This work supports the traditional use of *Datisca glomerata* as an antimalarial remedy by describing several antiplasmodial of the plant extract. Due to the selective action of these compounds, further studies may be warranted to investigate the mode of action and medicinal applicability of the datiscosides.

5.6. Chemical Data

*Datiscoside I (**2**)*

Cucurbitacin D 2'-*O*-acetyl-4',6'-dideoxy-*α*-L-*gluco*-hex-3'-ulopyranoside; colorless needles from EtOH; $[\alpha]_D^{20}$ +13.0 (*c* 0.19, MeOH); UV (MeOH) λ_{max} 223 nm; IR *V*max (film) cm^{-1}: 3470, 2970, 1737, 1676, 1625, 1367, 1238, 1055, 983; ^1H NMR data see table

1; ^{13}C see table 2; HR-ESI-TOF-MS (negative ion) *m/z* 685.3530 $[M-H]^-$ (calcd. for $C_{38}H_{54}O_{11}$ 685.3593); *R*f - 0.79 (yellow) ; *R*t - 21.6 min ; 0.120% yield (of dry weight)

*Datiscoside J (**4**)*

Cucurbitacin F 2'-*O*-acetyl-4',6'-dideoxy-α-L-allopyranoside; amorphous powder; $[\alpha]_D^{20}$ +1.5 (*c* 0.13, MeOH);); UV (MeOH) λ_{max} 224 nm; IR *V*max (film) cm^{-1}: 3431, 2970, 1737, 1687, 1625, 1371, 1238, 1055; ^1H NMR data see table 1; ^{13}C see table 2; HR-ESI-TOF-MS (negative ion) *m/z* 689.3824 $[M-H]^-$ (calcd. for $C_{38}H_{58}O_{11}$ 689.3906); *R*f - 0.55 (purple) ; *R*t - 18.5 min; 0.043 % yield (of dry weight)

*Datiscoside K (**5**)*

Cucurbitacin F 2'-*O*-acetyl-4',6'-dideoxy-α-L-*gluco*-hex-3'-ulopyranoside; colorless needles from diethyl ether-hexane; $[\alpha]_D^{20}$ -1.4 (*c* 0.4, MeOH); UV (MeOH) λ_{max} 224 nm; IR *V*max (film) cm^{-1}: 3431, 2970, 1737, 1676, 1625, 1367, 1215, 1107, 1055; ^1H NMR data see table 1; ^{13}C see table 2; HR-ESI-TOF-MS (negative ion) *m/z* 687.3658 $[M-H]^-$ (calcd. for $C_{38}H_{56}O_{11}$ 687.3750); *R*f - 0.56 (purple); *R*t - 19.6 min; 0.073% yield (of dry weight)

*Datiscoside L (**6**)*

Cucurbitacin F 2',3'-Di-*O*-acetyl-4'-deoxy-α-L-allopyranoside; amorphous powder; $[\alpha]_D^{20}$+ 10.6 (*c* 0.3, MeOH);); UV (MeOH) λ_{max} 223 nm; IR *V*max (film) cm^{-1}: 3469, 2970, 1737, 1382, 1215, 1111; ^1H NMR data see table 1; ^{13}C see table 2; HR-ESI-TOF-MS (negative ion) *m/z* 731.4103 $[M-H]^-$ (calcd. for $C_{40}H_{60}O_{12}$ 731.4052); *R*f - 0.44 (purple) ; *R*t - 19.4 min; 0.101 % yield (of dry weight)

*Datiscoside M (**7**)*

Cucurbitacin O 2',3'-Di-*O*-acetyl-4'-deoxy-α-L-allopyranoside amorphous powder; $[\alpha]_D^{20}$ + 3.6 (*c* 0.16, MeOH); UV (MeOH) λ_{max} 223 nm; IR *V*max (film) cm^{-1}: 3431, 2970, 1737, 1687, 1626, 1369, 1232, 1055, 1012; ^{1}H NMR data see table 1; ^{13}C see table 2; HR-ESI-TOF-MS (negative ion) *m*/*z* 731.3927 $[M-H]^-$ (calcd. for $C_{40}H_{60}O_{12}$ 731.4052); *R*f - 0.48 (blue) ; *R*t - 18.4 min; 0.047% yield (of dry weight)

*Datiscoside N (**8**)*

2-deoxycucurbitacin F 2',3'-Di-*O*-acetyl-4'-deoxy-α-L-allopyranoside; amorphous powder; ; $[\alpha]_D^{20}$ +5.8 (*c* 0.3, MeOH); UV (MeOH) λ_{max} 224 nm; IR *V*max (film) cm^{-1}: 3431, 2970, 1737, 1680, 1626, 1369, 1232, 1055, 1022; ^{1}H NMR data see table 1; ^{13}C see table 2; HR-ESI-TOF-MS (negative ion) *m*/*z* 715.3965 $[M-H]^-$ (calcd. for $C_{40}H_{60}O_{11}$ 715.4063); *R*f - 0.70 (purple); *R*t - 22.3 min ; 0.043% yield (of dry weight)

*Datiscoside O (**9**)*

2-deoxycucurbitacin O 2',3'-Di-*O*-acetyl-4'-deoxy-α-L-allopyranoside.; colorless needles from diethyl ether; $[\alpha]_D^{20}$ +25.2 (*c* 0.3, MeOH); UV (MeOH) λ_{max} 229 nm; IR *V*max (film) cm^{-1}: 3431, 2970, 1737, 1680, 1626, 1369, 1217, 1060, 1022; ^{1}H NMR data see table 1; ^{13}C see table 2; HR-ESI-TOF-MS (negative ion) *m*/*z* 715.3994 $[M-H]^-$ (calcd. for $C_{40}H_{60}O_{11}$ 715.4063); *R*f - 0.75 (blue); *R*t - 20.9 min; 0.435% yield (of dry weight)

5.7. Tables and Figures

Table 5.1. 1HNMR resonances of compounds isolated from *D. glomerata* (aglycone protons)

Proton #	1	2	3	4	5	6	7	8	9
1α	2.32ddd (12.6, 5.9, 3.4)	2.33ddd (12.6, 6.3, 3.6)	1.90dd (12.6, 6.7)	1.93m	1.93m	1.92m	1.65m	1.68dd (15.2, 2.5)	1.52m
1β	1.24q (12.7)	1.23m	1.08m	1.09m	1.09m	1.12m	1.48m	1.03d (9.1)	1.48tt (8.5, 3.5)
2α	4.45s	4.44m	3.60ddd (11.0, 9.0, 3.0)	3.61td (10.5, 4.5)	3.60ddd (11.7, 9.0, 4.0)	3.58ddd (11.5, 9.1, 3.7)	3.92ddd (11.0, 3.5, 3.0)	1.80dd (12.8, 3.2)	1.78td (12.0, 4.0)
2β	~	~	~	~	~	~	~	1.62m	1.71q (3.4)
3α	~	~	~	~	~	~	3.47d (3.5)	~	3.50m
3β	~	~	2.99d (9.1)	2.99d (9.13)	3.00d (9.2)	2.98d (9.1)	~	3.23dt (11.2, 3.5)	~
6	5.79dt (4.3, 2.0)	5.79d (5.7)	5.72d (5.6)	5.71d (4.98)	5.73d (5.8)	5.68d (5.7)	5.66d (5.8)	5.65d (5.1)	5.62d (5.8)
7α	2.45ddt (19.4, 8.0, 2.7)	2.45dd (19.9, 8.7)	2.44dd (19.3, 7.2)	2.44dd (20.5, 8.3)	2.41ddd (18.0, 7.8, 3.0)	2.43dd (19.1, 7.5)	2.46ddt (19.7, 6.6, 3.0)	2.41dd (19.4, 7.7)	2.45ddt (19.1, 6.6, 3.0)
7β	1.96dd (19.5, 5.3)	1.98dd (20.8, 6.9)	1.96m	1.86d (6.3)	1.93d (12.43)	1.90m	1.86dd (19.1, 5.8)	1.86dd (19.4, 5.7)	1.85dd (19.1, 6.0)
8	2.01d (8.0)	2.02d (8.2)	2.03d (8.2)	1.96d (8.0)	1.97d (7.9)	1.94d (8.2)	1.96d (7.9)	1.91d (7.9)	1.93d (7.9)
10	2.73dd (12.8, 3)	2.76d (12.0)	2.35d (13.1)	2.36d (12.8)	2.38d (12.8)	2.36d (13.0)	2.35d (12.4)	2.20d (12.2)	2.32d (9.1)
12α	3.24d (14.6)	3.25d (14.7)	3.17d (14.5)	3.22d (14.5)	3.19d (14.6)	3.19d (14.5)	3.18d (14.5)	3.19d (14.9)	3.19d (14.4)
12β	2.75d (14.6)	2.75d (14.8)	2.67d (14.6)	2.70d (14.5)	2.68d (14.6)	2.63d (14.5)	2.62d (14.5)	2.62d (14.3)	2.63d (14.5)
15α	1.64dd (13.8, 7.9)	1.59m	1.61dd (13.3, 7.3)	1.90m	1.58m	1.60dd (13.1, 7.0)	1.59dd (13.1, 7.0	1.57	1.58m
15β	1.55d (13.5)	1.59m	1.53d (13.5)	1.63dd (13.6, 7.8)	1.56	1.42	1.54d (13.0)	1.54d (9.0)	1.54d (10.1)
16	4.43t (7.5)	4.41t (5.79)	4.40t (6.9)	4.41t (6.5)	4.39td (6.45, 2.3)	4.36t (6.6)	4.36t (6.7)	4.35t (5.8)	4.35t (6.6)
17	2.70d (6.7)	2.77d (8.4)	2.67d (6.6)	2.67d (6.2)	2.75d (6.4)	2.66d (6.4)	2.66d (5.5)	2.67d (5.9)	2.67d (6.1)
18	1.02	1.03	1.00	1.01	1.01	0.98	0.97	0.97	0.98
19	1.10	1.10	1.12	1.12	1.12	1.10	1.14	1.08	1.14
21	1.36	1.34	1.34	1.39	1.34	1.42	1.42	1.42	1.41
23	6.48d (15.4)	6.52d (15.3)	6.46d (15.4)	6.59d (15.3)	6.52d (15.3)	6.77d (15.2)	6.76d (15.1)	6.76d (15.5)	6.76d (15.1)
24	7.04d (15.4)	7.02d (15.3)	7.03d (15.4)	7.21d (15.3)	7.01d (15.4)	7.05d (15.1)	7.05d (15.2)	7.04d (15.3)	7.05d (15.2)
26	1.44[a]	1.44[a]	1.44[a]	1.39[a]	1.44[a]	1.41[a]	1.4[a]	1.41[a]	1.42[a]
27	1.43[a]	1.41[a]	1.43[a]	1.38[a]	1.41[a]	1.33[a]	1.33[a]	1.34[a]	1.34[a]
28	1.34	1.35	1.19	1.17	1.20	1.16	1.19	1.13	1.14
29	1.27	1.26	0.95	0.94	0.95	0.93	1.00	0.92	1.02
30	1.35	1.34	1.26	1.31	1.27	1.32	1.32	1.34	1.34

All spectra were taken in $CDCl_3$ at 500Mhz. Shifts are recorded relative to TMS. The assignment of signals with the same letter superscript in ay column may be interchanged.

Table 5.2 1HNMR resonances of compounds isolated from *D. glomerata* (glycoside protons)

Proton #	1	2	3	4	5	6	7	8	9
1'	5.27d (4.9)	5.29d (4.5)	5.26d (4.9)	4.93d (3.5)	5.29d (4.5)	4.70d (4.3)	4.70d (4.1)	4.70d (3.4)	4.70d (4.2)
2'	5.25dd (4.8, 1.4)	5.18d (4.4)	5.24d (4.6)	4.65t (3.5)	5.18d (4.4)	4.79t (3.9)	4.79t (3.9(	4.79t (3.2)	4.79t (3.9)
3'	~	~	~	4.05 brs	~	5.33q (3.5)	5.33q (3.8)	5.34br s	5.34td (3.5, 3.0)
4'	3.87ddd (9.5, 5.6, 1.4)	2.40s, 2.39d (3.4)	3.86dd (8.8, 3.5)	1.82dt (14.3, 3.5), 1.55td (12.8, 3.0)	2.40m, [2H]	1.66m [2H]	1.66m [2H]	1.66m [2H]	1.66m [2H]
5'	3.47dq (9.6, 6.0)	3.82dqd (11.5, 6.1, 3.2)	3.46dq (9.5, 8.0)	3.84dq (11.9, 6.5)	3.83dqd (12.0, 6.1, 2.9)	4.01dqd (11.9, 6.2, 3.0)	4.01 dqd (11.9, 6.1, 2.9)	4.01br q (6.0)	4.01 dqd (11.5, 6.3, 3.5)
6'	1.46d (6.1)	1.32d (6.5)	1.46d (6.1)	1.23d (6.2)	1.33	1.22d (6.2)	1.21d (6.3)	1.22d (6.0)	1.22d (6.3)
2'-OCOCH3	2.11	2.08	2.12	2.09	2.09	2.09[b]	2.08[b]	2.1[b]	2.09[b]
3'-OCOCH3	~	~	~	~	~	2.02[b]	2.02[b]	2.03[b]	2.03[b]

All spectra were taken in $CDCl_3$ at 500Mhz. Shifts are recorded relative to TMS. The assignment of signals with the same letter superscript in ay column may be interchanged.

Table 5.3. ^{13}CNMR resonances of compounds isolated from *D. glomerata*

Carbon #	1	2	3	4	5	6	7	8	9
1	35.9	35.9	33.2	33.2	33.2	33.3	29.2	25.0	21.2
2	71.6	71.7	70.6	71.0	71.0	71.2	68.2	30.6	29.0
3	212.7	212.8	80.8	80.8	80.8	80.8	78.4	76.5	75.9
4	50.4	50.4	41.9	41.9	41.9	40.8	40.9	42.2	41.6
5	140.9	140.8	141.1	141.3	141.1	141.3	138.4	142.4	140.3
6	120.0	120.3	118.6	117.9	118.9	118.8	120.4	118.9	119.7
7	23.9	23.8	23.8	24.0	23.7	24.0	24.1	24.1	24.3
8	42.2	42.2	42.4	42.3	42.4	42.5	42.6	42.5	42.7
9	48.0	48.0	48.0	47.8	48.0	48.2	48.3	48.1	48.2
10	33.9	33.9	34.0	34.0	34.0	34.1	33.8	35.6	35.5
11	211.6	211.7	212.3	212.3	212.4	212.7	212.7	213.0	213.0
12	48.7	48.7	48.7	48.8	48.8	48.7	48.7	48.7	48.7
13	50.2	50.2	50.5	50.9	50.4	50.7	50.7	50.8	50.8
14	48.3	48.3	48.3	48.1	48.2	48.1	42.6	48.6	48.6
15	38.8	38.1	38.8	39.3	38.2	41.9	41.6	40.8	40.9
16	76.3	75.4	76.4	77.8	75.5	78.6	78.6	78.7	78.7
17	55.6	56.0	55.6	55.9	56.0	56.1	56.1	56.0	56.0
18	19.9	20.0	19.9	20.0	20.0	19.8	19.8	19.8	19.8
19	20.1	20.1	20.4	20.3	20.3	20.3	20.2	20.0	20.0
20	77.7	77.9	78.0	77.8	78.0	78.1	78.1	78.1	78.1
21	24.4	24.6	24.8	23.9	24.5	24.2	24.2	24.1	24.4
22	200.7	202.4	200.6	200.1	200.4	201.2	201.2	201.2	201.2
23	119.0	119.3	119.1	118.8	119.3	118.9	118.9	118.0	118.9
24	156.3	156.5	156.2	158.3	156.3	154.7	154.7	154.5	154.5
25	70.6	70.3	72.7	70.2	70.4	71.0	71.2	71.2	71.2
26	29.3[a]	29.2[a]	29.3[a]	28.9	29.2[a]	30.1[a]	30.3[a]	30.1[a]	30.1[a]
27	28.4[a]	28.4[a]	28.4[a]	28.0	28.3[a]	29.0[a]	29.0[a]	29.0[a]	28.6[a]
28	29.5	29.4	24.3	24.8	24.8	25.8	26.6	24.3	27.3
29	21.5	21.3	21.8	21.8	21.7	21.6	25.4	20.3	25.5
30	18.4	18.4	18.2	18.5	18.5	17.7	17.6	17.8	17.7
1'	96.9	96.8	96.9	96.1	96.8	95.9	95.9	95.9	95.9
2'	74.5	75.5	74.5	69.9	75.6	68.8	68.8	68.8	68.8
3'	200.5	200.2	201.8	67.1	200.3	66.1	66.4	66.3	66.3
4'	78.0	48.5	77.7	40.3	48.5	36.1	36.1	36.1	37.0
5'	72.7	67.6	71.0	60.5	67.5	60.9	60.9	60.9	60.9
6'	18.3	21.1	18.5	20.1	21.1	20.0	20.0	20.3	20.2
2'-O<u>C</u>OCH3	169.1	169.4	169.3	169.9	169.5	170.6[b]	170.5[b]	170.4[b]	170.5[b]
3'-O<u>C</u>OCH3	~	~	~		~	169.8[b]	169.8[b]	169.8[b]	169.8[b]
2'-OCO<u>C</u>H3	20.3	20.4	20.3	20.9	20.4	21.2[c]	21.2[c]	21.2[c]	20.7[c]
3'-OCO<u>C</u>H3	~	~	~		~	20.7[c]	21.7[c]	20.7[c]	20.6[c]

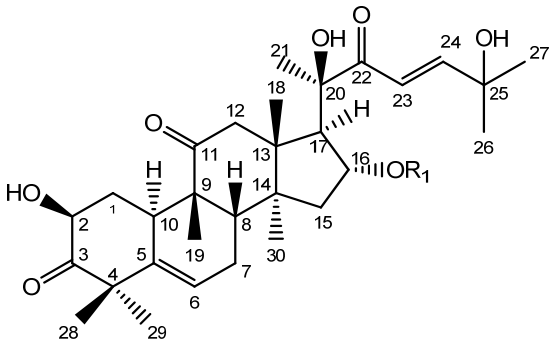
All spectra were taken in $CDCl_3$ at 125Mhz. Shifts are recorded relative to TMS. The assignment of signals with the same letter superscript in ay column may be interchanged.

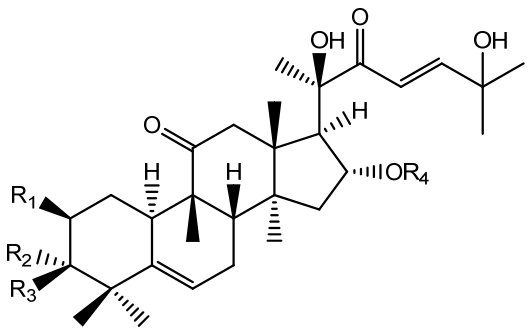
Table 5.4. Biological activity of compounds isolated from *Datisca glomerata*

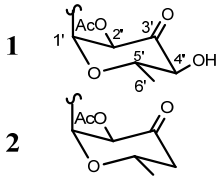
	Antiplasmodial		Cytotoxicity (CHO)		Cytotoxicity (L6)		Antileishmanial	
	IC_{50} (μg/mL)	IC_{50} (μM)	IC_{50} (μg/mL)	IC_{50} (μM)	IC_{50} (μg/mL)	IC_{50} (μM)	IC_{50} (μg/mL)	IC_{50} (μM)
1	9.7 ± 1.3	13.5 ± 1.9	>100	>100	0.24 ± 0.1	0.34 ± 0.1	>20	>20
2	11.0 ± 1.7	16.0 ± 2.5	>100	>100	0.51 ± 0.4	0.74 ± 0.6	>20	>20
3	10.2 ± 2.3	14.5 ± 3.3	>100	>100	0.35 ± 0.3	0.50 ± 0.4	>20	>20
4	11.3 ± 3.8	16.3 ± 5.5	>100	>100	0.12 ± 0.1	0.51 ± 0.1	>20	>20
5	5.3 ± 2.4	7.7 ± 3.5	71.8 ± 2.6	104.4 ± 3.7	0.076 ± 0.1	0.11 ± 0.1	>20	>20
6	9.3 ± 1.8	12.7 ± 2.5	>100	>100	2.51 ± 1.0	3.42 ± 1.4	>20	>20
7	24.4 ± 4.0	33.3 ± 5.5	>100	>100	>20	>20	>20	>20
8	6.3 ± 0.7	8.8 ± 0.9	61.4 ± 3.9	85.8 ± 5.5	8.1 ± 6.0	11.3 ± 8.1	>20	>20
9	19.9 ± 3.1	27.8 ± 4.3	>100	>100	>20	> 20	>20	>20
Chloroquine	0.013 ± 0.001	0.024 ± 0.001	>100	>100	-	-	-	-
Pentamidine	-	-	-	-	-	-	0.30 ± 0.03	0.51 ± 0.005
Emetine	-	-	0.054 ±0.002	0.11 ± 0.004	0.018 ± 0.0034	0.037 ± 0.006	-	-

Results are expressed as IC50 values (μM) ± standard deviations. All experiments were performed in triplicate. Chloroquine was used as positive control for antiplasmodial activity, pentamidine for antileishmanial activity and emetine for cytotoxicity.

Figure 5.1 Antiplasmodial compounds from leaves and bark of *Datisca glomerata*







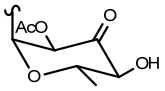
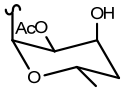
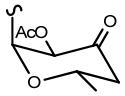
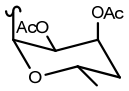
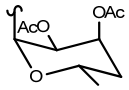
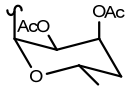
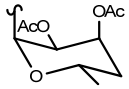
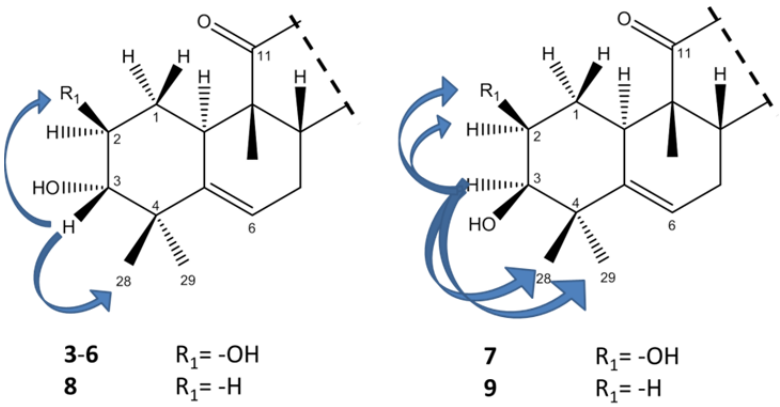
	R_1	R_2	R_3	R_4
3	-OH	-OH	-H	
4	-OH	-OH	-H	
5	-OH	-OH	-H	
6	-OH	-OH	-H	
7	-OH	-H	-OH	
8	-H	-OH	-H	
9	-H	-H	-OH	

Figure 5.2. NOE correlation for H-3 of datiscosides



β hydroxyl at C-3 is in the equatorial position (compounds 3-6 and 8). *α* hydroxyl at C-3 is in the axial position (compounds 7 and 9).

CHAPTER 6

Antiplasmodial Activity of Furoquinoline Alkaloids from *Ptelea trifoliata* L.

6.1. Abstract

Aim of the study: The objective of this study was to identify the antiplasmodial constituents from the bark of *Ptelea trifoliate* L. a plant traditionally used in the United States for the treatment of malaria.

Methods and Materials: Dried and powdered leaves were extracted and subjected to *in vitro* antiplasmodial-guided fractionation against *Plasmodium falciparum* (D10 strain). Antiplasmodial IC_{50} values were calculated for pure compounds. Compounds were also assayed against Chinese ovarian hamster cells (CHO) to assess cytotoxicity.

Results: 7 known furoquinoline alkaloids: neoacutifolin (**1**), hydroxylunine (**2**), skimmianine (**3**), isoptelefolonium (**4**), ptelefolonium (**5**), ptelefolone (**6**) and ptelecultinium (**7**) were isolated. The structures of the isolated compounds were confirmed by the LC-ESI-MS and NMR spectral data and also complied with published reports. Compound **1** is reported here for the first time from this species. Compounds **1**-**7** exhibited potent *in vitro* inhibitory activity against *Plasmodium falciparum* chloroquine-sensitive strain D10 with IC_{50} values ranging from 0.04 and 47.60 μg/mL. The *in vitro* antiplasmodial activity of these compounds [except for (**3**)] is reported for the first time.

Conclusion: The isolation of potent antiplasmodial alkaloids from *P. trifoliata* supports ethnomedical evidence of its use as an antimalarial plant. Three quartenary

alkaloids - isoptelefolonium (**4**), ptelefolonium (**5**), and ptelecultinium (**7**) – show potent *n vitro* antiplasmodial activity and possess structures possibly amenable to medicinal chemistry. Further investigation is warranted to determine the suitability of these structures as drugs.

6.2. Introduction

Ptelea trifoliata L. (Rutaceae), or the common hop tree, is a shrub or small tree that is widely distributed in deciduous forest regions of eastern North America [103]. During the early days of medical and homeopathic practice in the United States, *P. trifoliata* won considerable interest as a native medicinal species [104]. The leaves were used as a vermifuge and vulnerary [29]. As the common name suggests, the fruits were once substituted for the true hops (*Humulus* spp.). The bark was useful as a tonic and to treat various types of ailments including fever [104]. The root bark was substituted for quinine in treating remittent and intermittent fevers associated with malaria [105]; an application confirmed during the World War II antimalarial plant screening project, which showed an extract from the leaves has activity against avian malaria [23]. Phytochemical studies of *P. trifoliata* have resulted in the isolation of numerous compounds including furoquinoline, dihydrofuroquinoline and quinolone alkaloids [106-118]. However, the medicinal components of the plant, in particular those responsible for the antimalarial activity, have not yet been described. The goal of this work is to describe the antiplasmodial components of *Ptelea trifoliata* L.

6.3. Methods and Materials

6.3.1. Instrumentation

See appendix A

6.3.2. Plant Material

Leaves of *Ptelea trifoliata* (Rutaceae) were collected on September 22nd, 2010 in Butler County, Ohio by Dr. Micheal A. Vincent from Michigan State, USA. A voucher specimen (15236) has been deposited in Miami University Herbarium (MU).

6.3.3. Extraction and Isolation

The dried and powdered leaves of *Ptelea trifoliata* (200gm) were defatted with hexane (2L) at room temperature and evaporated under vacuum to yield 3.5 g of crude hexane extract (Pt-1). The remaining air dried material was extracted with methanol (2L x 2) at room temperature for 2 days and the methanolic extract was evaporated under vacuum to yield 48 g of the crude methanolic extract (Pt-2). The crude methanolic extract was dissolved in 3% HCl solution (150 mL) and extracted with chloroform (150 mL x 2) which was concentrated under vacuum to yield 10.5 g of crude acidified chloroform extract (Pt-3). The aqueous portion was basified by using 5N NH_4OH solution (pH ~8-9) and extracted with chloroform (200 mL x 2) to yield 0.45 g of crude basified chloroform extract (Pt-4). This was purified by Fast Centrifugal Partition Chromatography using Armen instrument with a 4:1.5:2 (Chloroform: Methanol: 0.2M HCl) solvent system using the Elution-Extrusion method with the upper phase as the stationary phase following previously published protocol [119]. Four fractions were collected according to peaks observed at 254 nm. Fractions Pt-4-2 and Pt-4-3 (mins 23-60

and 62-100) were collected and concentrated by vacuum to yield 85mg and 24 mg respectively.

The fraction Pt-4-2 was further purified by reverse phase HPLC (Phenomenex Curosil 250 x 21.20mm, 4μm, 20 – 95% acetonitrile in water which contains 0.1% TFA over 60min, flow rate 10mL/min; UV detector 254nm) to give 8 subfractions, Pt-4-2-1 (4 mg), Pt-4-2-2 (3 mg), Pt-4-2-3 (5 mg), Pt-4-2-4 (7 mg), Pt-4-2-5 (5 mg), Pt-4-2-6 (9 mg), Pt-4-2-7 (22 mg) and Pt-4-2-8 (10 mg). Pt-4-2-1 was further purified by reverse phase HPLC (Phenomenex Prodigy ODS (3) 250 x 10.0mm, 5 μm, 20 – 95% MeOH in water which contains 0.1% TFA over 80min, flow rate 5mL/min; UV detector 254nm) to give compound **2** (1.4 mg). Compounds **1** (1.0 mg), **3** (2.2 mg), **4** (4.3 mg), **5** (8.0 mg) and **6** (3.1 mg) were isolated from Pt-4-2-2, Pt-4-2-4, Pt-4-2-6, Pt-4-2-7 and Pt-4-2-8 respectively. Similarly, Pt-4-3 gave 6 subfractions, Pt-4-3-1 (2 mg), Pt-4-3-2 (5.6 mg), Pt-4-3-3 (3.4 mg), Pt-4-3-4 (2 mg), Pt-4-3-5 (3 mg) and Pt-4-3-6 (1.1 mg). Compound **7** (1.3 mg) was isolated from Pt-4-3-3. All the isolated compounds were confirmed by ESI-MS and ^{1}H NMR and ^{13}C NMR spectral data and also comply with literature report [108, 112, 114, 116-118, 120].

6.3.4. In vitro *assays*

6.3.4.1 In vitro *antiplasmodial assay*

See appendix A

6.3.4.2. In vitro *cytotoxicity assay using Chinese ovarian hamster CHO cells*

See appendix A

6.4. Results and Discussion

This research is the first systematic isolation and characterization of the antiplasmodial compounds from the leaves of *Ptelea trifoliata*. The methanolic extract of the leaves of *P. trifoliata* showed significant antiplasmodial activity. (Percentage survival of the chloroquine-sensitive strain of *P. falciparum* [D10] at the concentration of 20μg/mL was 6.12 %). The crude methanolic extract was acidified with 3% HCl solution and extracted with chloroform (Pt-3) which showed antiplasmodial activity with IC_{50} value of 11.87 μg/mL and remaining aqueous portion was basified and extracted with chloroform (Pt-4) and screened for antiplasmodial activity (IC_{50} = 5.78 μg/mL). Pt-4 fraction was further purified by FCPC and 5 subfractions were collected in which 2 fractions Pt-4-2 and Pt-4-3 showed antiplasmodial activity with IC_{50} values of 4.73 and 6.41 μg/mL, respectively. Antiplasmodial activity-guided isolation of these two fractions by reverse phase HPLC produced seven known furoquinoline alkaloids (Figure 6.1), 6 of which have been previously reported from *Ptelea trifoliata*, and 1, neoacutifoline (**1**), which was reported from *Zanthoxylum acutifolium* [120], but is now reported from *P. trifoliata* for the first time. ESI-MS and 1H NMR spectral data for compounds 1-7 is available below.

All the isolated compounds were evaluated for antiplasmodial activity using CQ-sensitive (D10) and cytotoxicity using the Chinese Hamster Ovarian (CHO) cell line (Table 6.1).

The furoquinoline alkaloids isolated by antiplasmodial activity-guided fractionation of *P. trifoliata* leaves displayed *in vitro* antiplasmodial activity with IC_{50} values ranging between 0.04 and 47.60 μg/mL (Table 6.1). Compounds **5** and **7** showed the most potent activity against CQ sensitive D10 strain (IC_{50} values of 0.04 and 0.06 μg/mL, respectively), which is almost equal to standard drug Chloroquine (IC_{50} value of 0.015 μg/mL) and no observable cytotoxicity. Compounds **1** and **4** showed considerable antiplasmodial activity with IC_{50} values of 4.93 μg/mL and 1.85 μg/mL, respectively. Compounds **2, 3** and **6** showed much lower antiplasmodial activity with IC_{50} values of 37.75, 25.31 and 47.60 μg/mL, respectively.

All the isolated compounds share the basic furoquinoline skeleton with small modifications in the substitution pattern. However, a wide range of antiplasmodial activity was observed among the compounds. The main structural difference between the compounds neoacutifolin (**1**) and ptelefolone (**6**) is the presence of a 6-methoxy moiety in (**6**) which may be responsible for the nearly tenfold decrease in antiplasmodial activity [IC_{50} value of (**1**) is 4.93 μg/mL and (**6**) is 47.60 μg/mL]. The compounds **4**, **5** and **7** have quaternary nitrogen atoms in the ring structure and showed most potent antiplasmodial activity, especially compounds **5** and **7** with IC_{50} values of 0.04 and 0.06 μg/mL, respectively. These results confirm the findings of others that indicate the presence of quaternary nitrogen atoms increase antiplasmodial activity [29-30].

6.5. Conclusions

This work confirms the traditional use of *Ptelea trifoliata* as an antimalarial remedy by describing several compounds with potent *in vitro* antiplasmodial activity and no cytotoxicity. Further research may be warranted to ascertain the potential medicinal use of these compounds.

6.6. Chemical Data

Neoacutifolin (**1**)

ESI-MS: m/z = 517 $[M + H]^+$; ^{1}H-NMR (500 MHz, CD_3OD): δ_H 1.87 (3H. s, CH_3), 3.68 (2H, dd, J = 8.2, 15.6 Hz, C$\underline{H}_2$), 4.05 (3H, s, CH_3), 4.10 (1H, dd, J = 8.2, 15.6 Hz, C$\underline{H}_2$), 4.42 (3H, s, CH_3), 5.10 (1H, s, C=C$\underline{H}_2$), 5.25 (1H, s, C=C$\underline{H}_2$), 5.62 (1H, t, J = 8.2 Hz, $CH_2\underline{C}H$-), 7.33 (1H, d, J = 8.8 Hz), 7.46 (1H, m), 7.73 (1H, d, J = 8.8 Hz)

Hydroxylunine (**2**)

ESI-MS: m/z = 517 $[M + H]^+$; ^{1}H-NMR (500 MHz, CD_3OD): δ_H 1.29 (3H. s, CH_3), 1.37 (3H, s, CH_3), 3.76 (2H, ddd, J = 8.2, 15.6, 22.7 Hz, $\underline{C}H_2$CH-), 4.38 (3H, s, CH_3), 4.91 (1H, t, J = 8.2 Hz, $CH_2\underline{C}H$-), 6.20 (2H, s, $\underline{C}H_2$), 7.09 (1H, d, J = 8.8 Hz), 7.71 (1H, d, J = 8.8 Hz)

Skimmianine (**3**)

ESI-MS: m/z = 517 $[M + H]^+$; ^{1}H-NMR (500 MHz, CD_3OD): δ_H 4.00 (3H. s, OCH_3), 4.04 (3H, s, OCH_3), 4.54 (3H, s, OCH_3), 7.41 (1H, d, J = 2.7 Hz), 7.43 (1H, d, J = 9.4 Hz), 7.83 (1H, d, J = 2.8 Hz), 8.10 (1H, d, J = 9.4 Hz)

Isoptelefolonium chloride (**4**)

ESI-MS: m/z = 517 $[M + H]^+$; ^{1}H-NMR (400 MHz, CD_3OD): δ_H 1.90 (3H. s, CH_3), 3.75 (1H, dd, J = 7.0, 15.0 Hz, C$\underline{H}_2$), 3.95 (3H, s, CH_3), 4.06 (3H, s, CH_3), 4.15 (1H, dd, J = 9.3, 15.5 Hz, C$\underline{H}_2$), 4.30 (3H, s, CH_3), 4.46 (3H, s, CH_3), 5.19 (1H, s, C=C$\underline{H}_2$), 5.30 (1H, s, C=C$\underline{H}_2$), 5.79 (1H, t, J = 8.8 Hz, $CH_2\underline{C}$H-), 7.51 (1H, d, J = 9.3 Hz), 8.10 (1H, d, J = 9.3 Hz)

Ptelefolonium chloride (**5**)

ESI-MS: m/z = 517 $[M + H]^+$; ^{1}H-NMR (400 MHz, CD_3OD): δ_H 1.89 (3H. s, CH_3), 3.80 (1H, dd, J = 8.0, 15.8 Hz, C$\underline{H}_2$), 3.93 (3H, s, CH_3), 4.03 (3H, s, CH_3), 4.19 (1H, dd, J = 9.3, 15.8 Hz, C$\underline{H}_2$), 4.30 (3H, s, CH_3), 4.49 (3H, s, CH_3), 5.18 (1H, s, C=C$\underline{H}_2$), 5.30 (1H, s, C=C$\underline{H}_2$), 5.77 (1H, t, J = 8.6 Hz, $CH_2\underline{C}$H-), 7.13 (1H, d, J = 2.7 Hz), 7.27 (1H, d, J = 2.7 Hz)

Ptelefolone (**6**)

ESI-MS: m/z = 517 $[M + H]^+$; ^{1}H-NMR (400 MHz, CD_3OD): δ_H 1.84 (3H. s, CH_3), 3.18 (1H, dd, J = 8.0, 15.8 Hz, C$\underline{H}_2$), 3.57 (1H, dd, J = 9.3, 15.8 Hz, C$\underline{H}_2$), 3.91 (3H, s, CH_3), 3.98 (3H, s, CH_3), 4.15 (3H, s, CH_3), 5.09 (1H, s, C=C$\underline{H}_2$), 5.22 (1H, s, C=C$\underline{H}_2$), 5.68 (1H, t, J = 8.6 Hz, $CH_2\underline{C}$H-), 7.01 (1H, d, J = 2.7 Hz), 7.35 (1H, d, J = 2.7 Hz)

Ptelecultinium chloride (**7**)

ESI-MS: m/z = 517 $[M + H]^+$; ^{1}H-NMR (500 MHz, CD_3OD): δ_H 1.89 (3H. s, CH_3), 3.82 (1H, dd, J = 8.0, 15.7 Hz, C$\underline{H}_2$), 4.06 (3H, s, CH_3), 4.20 (1H, dd, J = 9.8, 15.4 Hz, C$\underline{H}_2$), 4.34 (3H, s, CH_3), 4.49 (3H, s, CH_3), 5.19 (1H, s, C=C$\underline{H}_2$), 5.31 (1H, s, C=C$\underline{H}_2$), 5.81 (1H, t, J = 8.6 Hz, $CH_2\underline{C}$H-), 7.57 (1H, d, J = 8.2 Hz), 7.62 (1H, t, J = 8.1 Hz), 7.91 (1H, d, J = 8.2 Hz)

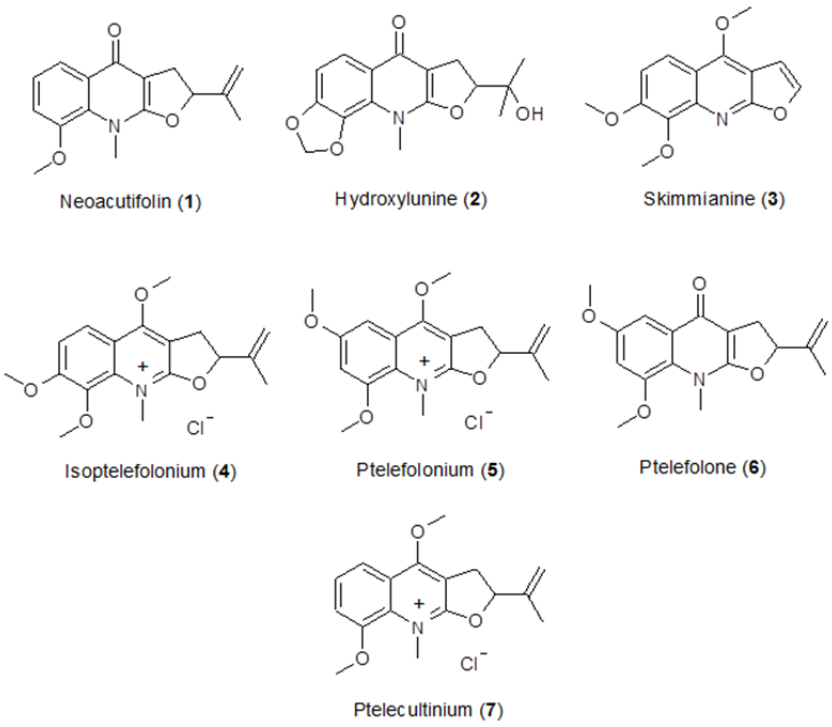
6.7. Tables and Figures

Table. 6.1. *In vitro* biological activity of compounds isolated from *P. trifoliata*

Compounds	*Plasmodium falciparum* D10 (IC_{50})		CHO cell line (IC_{50})	SI
	µg / mL	µM	µg / mL	
1	4.93 ± 1.67	18.17 ± 6.15	NT	-
2	37.75 ± 2.76	124.45 ± 9.09	NT	-
3	25.31 ± 3.49	97.62 ± 13.46	NT	-
4	1.85 ± 0.28	5.24 ± 0.79	NT	-
5	0.04 ± 0.01	0.11 ± 0.03	> 100 ± ND	> 2500
6	47.60 ± 2.86	157.95 ± 9.49	NT	-
7	0.06 ± 0.04	0.18 ± 0.12	> 100 ± ND	> 1667
Chloroquine	0.015 ± 0.003	0.03 ± 0.006	NT	-
Emetine	-	-	0.13 ± 0.02	-

NT = Not Tested; ND = Not Determined
Selectivity Index (SI) = IC_{50} CHO / IC_{50} D10

Figure 6.1. Antiplasmodial compounds from leaves of *P. trifoliata*



CHAPTER 7

General Conclusion

The goal of this thesis was to find novel antimalarial cures from overlooked, but potentially potent Antiparasitic plant species, of the United States. The specific aims were threefold: 1) To identify and prioritize plant species from the United States with an ethnobotanical history of malaria treatment. 2) To collect high priority leads and subject the extracts to an *in vitro* antiplasmodial assay to confirm bioactivity. 3) To apply a bioassay guided fractionation regime to the most promising candidates to determine the chemical constituents are responsible for the activity.

This project resulted in the prioritization of over 100 plant species native to the United States with ethnobotanical history for the treatment of malaria. 46 species representing 28 families were collected, from which 243 extracts were generated and test *in vitro* against *Plasmodium falciparum*. While it is not a present day disease of the country, the historical impact of malaria in the United States has left a legacy of ethnobotanical knowledge that this project has only scratched the surface of.

Antiplasmodial screening of antimalarial plants from the United States has led to several notable observations. For one, this work showed that plant species with a history of ethnobotanical use for malaria are more likely to produce 'hits' in antiplasmodial screens. While this is only an anecdotal observation, and not a statistic that this study sought to obtain, it may warrant additional attention considering the hotly debated

question of whether ethnobotanically based screens are more efficient than random screens. The author surely believes so.

The screening portion of this project also produced valuable information regarding the type of extracts that are generally active in the antiplasmodial screen. Of those species that produced 'hits', extracts 2 and 3 were generally more active than extracts 1 and 4. By following the extraction regime outlined in figure 2.3 one can readily see that extracts 1 and 4 contain the most non-polar and polar plant constituents, respectively, while extracts 2 and 3 contain intermediate polarity and ionizable constituents. According, to Lipinski's rule of 5, the chemical compounds in extract 2 and 3 are more likely to possess 'drug-like' characteristics [121]. Further statistical analysis and literature searches may reveal interesting trends regarding the type of extract most likely to provide active 'hits', which may in turn facilitate high throughput screening in the future.

Six species were selected for bioassay guided fractionation to isolate the bioactive constituents (of which 4 are described in detail within the text and 2 briefly in the appendices). Forty-two chemical compounds displaying antiplasmodial activity were isolated in pure form, of which 9 novel compounds were newly described. The activity of the compounds ranged from 0.11 μM to over 100 μM. All isolated compounds were also tested for cytotoxicity and several for their *in vitro* ability to inhibit *Leishmania tarentolae* as a proxy for antileishmanial activity.

The isolated compounds represent several different classes including sesquiterpenes, triterpenoids, steroids and alkaloids. The bioassay guided approach can be considered 'naïve' for lack of a better word, due to its inherent ability to survey the entire chemical spectrum of a plant extract and follow the activity without major discrimination. This is especially useful in today's marketplace, where drug resistance to compounds of unique skeletons (as compared to similar skeletons) is much less likely to occur. Furthermore, as the diversity of antimalarial compounds grows, so does our ability to investigate unique modes of action, which may in turn facilitate medicinal chemistry and optimization of existing drugs.

If this project was to continue, several different paths could be followed. 1) To conduct additional studies on the isolated compounds. These studies may include animal studies in order to determine the antimalarial activity of the compounds *in vivo*, absorption, distribution, metabolism, extraction and toxicological (AMET) investigation, and mode of action studies. 2) To investigate whether those plant species used traditionally to treat malaria have biological activities beyond antiplasmodial action that may contribute to their effect (i.e. fever reducing or immunomodulatory effects); 3) Investigation of intra and interspecific phytochemistry of the plant species shown active.

The most active compounds isolated in this paper had IC_{50} values in 100 nanomolar range, which is one order of magnitude higher than chloroquine (usually around 10 -20 nanomolar). Hundreds of chemical compounds - either synthesized or isolated from nature - have activities that far exceed those described in this thesis and

some are even more active than chloroquine and the artemisinins [122]. If the main goal of this project was to simply discover and develop the next blockbuster drug, than I would be safe saying it missed the mark. However, the true value of this work rests in its ability to unify ethnobotany, literature review, and modern science to validate traditional knowledge while contributing unique data to the field. When one clear main objective is to help alleviate the world wide malaria burden, no scientific result, charitable donation, or helpful act is too small…

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IF YOU THINK YOU'RE TOO SMALL TO HAVE AN IMPACT,

TRY GOING TO BED WITH A MOSQUITO IN THE ROOM

~~~

Anita Roddick

APPENDIX A

A.1. Instrumentation

A.1.1 Solvents and Reagents

Solvents were of HPLC grade and purchased from VWR, Sigma Aldrich or Fisher scientific. Reagents were purchased from Sigma Aldrich unless otherwise noted.

A.1.2. Counter Current Chromatography

Counter current chromatography was performed on a Bench Scale Fast Centrifugal Partition Chromatography (FCPC) Kromaton® v. 1.0 (1 Liter column). Detection was achieved by single wavelength UV monitoring. A fraction collector was employed to collect eluent by time and fractions were pooled according to the UV chromatogram and TLC monitoring.

A.1.3 Flash chromatography

Flash chromatography was performed using Silica gel (230-400 mesh 60 Å Merck). Positive air flow pressure was used to facilitate elution. Fraction were collected at 1/10 the column volume and pooled according to TLC.

A.1.4. Thin Layer Chromatography (TLC)

TLC was performed on using pre-coated Si25 F254 plates (Fluka Analytical). Development using standard solvent systems was employed. Detection of spots was achieved with UV at 254 and 366nm or by staining with p-anisaldehyde/ H_2SO_4 and heating, vanillin/ H_2SO_4/ Acetic acid and heating, Draggendorf reagent, or idodine.

A.1.5. LC-MS

High Resolution mass spectral data was acquired by Electrospray Ionization Ion-Trap Time-of-Flight mass spectrometry, ESI-IT-TOF-MS, on a Shimadzu LC-MS-IT-TOF (Scientific Instruments, Columbia, MD) instrument equipped with a Prominence HPLC system (SIL-20A HT autosampler, LC-20AD pump system, SDP-M20A diode array detector). Ionization was performed using a conventional ESI source in the negative ionization mode. Shimadzu's LCMS Solution software was used for data analysis. The formula predictor function of LCMS Solution was used in generating the molecular formulae.

Low resolution mass spectral data was collected using a UPLC/MS system including the Dionex® UltiMate 3000 RSLC ultra-high pressure liquid chromatography system, a workstation with Dionex®'s Chromeleon v. 6.8 software package, solvent rack/degasser SRD-3400, pulseless chromatography pump HPG-3400RS, autosampler WPS-3000RS, column compartment TCC-3000RS, and photodiode array detector DAD-3000RS. After the photodiode array detector the eluent flow is guided to a Varian 1200L (Varian Inc., Palo Alto, CA) triple quadrupole mass detector with electrospray ionization interface (ESI), operated in negative ionization mode.

Compounds were separated on a Phenomenex™ C8 reverse phase column, size 150 x 2 mm, particle size 3 μm, pore size 100 Å, from which retention times (*R*t) were obtained. The mobile phase consisted of 2 components: solvent A (0.5% ACS grade acetic acid in double distilled de-ionized water, pH 3-3.5), and Solvent B (100% Acetonitrile). The mobile phase flow was 0.20 ml/min, and a gradient mode was used for

all analyses. The initial conditions of the gradient were 95% A and 5% B; for 30 minutes the proportion reaches 5% A and 95% B which was kept for the next 8 minutes, and during the following 4 minutes the ratio was brought to initial conditions. An 8 minute equilibration interval was included between subsequent injections. The average pump pressure using these parameters was typically around 3300 psi for the initial conditions.

A.1.6. Infrared Spectroscopy(IR)

IR spectra were recorded on a Shimadzu IR Prestige-21 FTIR-8400S spectrophotometer (Kyoto, Japan).

A.1.7. Optical Rotation

Optical rotations were measured on a JASCO P-2000 polarimeter (Tokyo, Japan) with a Spectra Manager software.

A.1.8. Nuclear Magnetic Resonance (NMR)

^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$, Methanol-d, or Pyridine at 500 o and 125 MHz, respectively on a 500 Varian VNMRS 500 MHz or 400 and 100 MHz, respectively on a 400 Varian VNMRS 400 MHz. ^{1}H and ^{13}C chemical shifts are given in ppm relative to TMS, with coupling constants (*J*) reported in Hz. For the HMQC and HMBC experiments the delay was 3.5 and 62.5 ms, respectively. For the NOESY and NOESY1D the mixing time was 500ms. Fourier transformation, spectral processing and all interpretation was achieved using MestReNova's NMR processing software.

A.2. *In vitro* biological assays

All *in vitro* antiplasmodial assays were completed by Dr. Peter J. Smith and colleagues at the University of Cape Town (UCT) South Africa. The author owes a debt of graditude to all UCT colleagues without whom this work would be impossible. *In vitro* cytotoxicity was either 1) completed at UCT (CHO cell line) or 2) At Rutgers University by the author, Dr. Patricio Rojas-Silva or Dr. Slavko Komarnystky. *In vitro* antileishmanial assays were completed at Rutgers University by Dr. Patricio Rojas-Silva.

A.2.1. In vitro *antiplasmodial assay*

All samples were tested in triplicate against chloroquine sensitive or chloroquine resistant (Dd2) strain of *Plasmodium falciparum* (D10). Cultures of asexual erythrocyte stages of *P. falciparum* were maintained continuously *in vitro* using a modified method of Trager and Jensen [26]. Antiplasmodial activity was determined using a modified version of the parasite lactate dehydrogenase assay [25]. Samples were prepared as a 20 mg/ml stock solution in 100% DMSO and stored at -20°C. Dilutions were prepared on the day of the experiment. Chloroquine diphosphate (CQ) was used as the reference drug in all experiments. A full dose-response was performed for pure compounds to determine the concentration inhibiting 50% of parasite growth (IC_{50} value). Samples were tested at a starting concentration of 100 μg/mL, which was then serially diluted 2-fold in complete medium to give 10 concentrations; with the lowest concentration being 0.2 μg/mL. CQ was tested at a starting concentration of 100 ng/mL and similarly diluted 2-fold to give 10 concentrations; the lowest being 0.2 ng/mL. Fractions were tested at 20, 10, 5 and/or 2.5 μg/mL to facilitate bioassay guided fractionation. The IC_{50}-values were

obtained using a non-linear dose-response curve fitting analysis via Graph Pad Prism v.4.0 software.

A.2.2. In vitro cytotoxicity assays

A.2.2.1. Rat skeletal myoblast (L6) cytotoxicity assay

Rat skeletal myoblast L6 cells were used to evaluate the cytotoxicity of isolated compounds. The cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 environment. The assay was conducted in 96-well plates. Each well was filled with 100 μL of culture medium with ca. 1 X 10^4 cells per mL. After 2 hours, the compounds were added to triplicate wells at 10 concentrations (serial dilution from 20 to 0.01 μg/mL). After 72 hours of incubation, the plates were checked under an inverted microscope to assure growth in sterile conditions. Then, 10 μL of a sterile solution (5 mg/mL) of MTT dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were added and incubation continued for another 4 hours. Finally, absorbance was read using a Synergy™ HT multidetection microplate reader (BioTek® Instruments, Inc. Winooski, VT) at 570 nm. Data were analyzed using the software Graph Pad Prism 5.04 (GraphPad Software Inc., La Jolla, CA). The IC_{50} values were calculated from the sigmoidal inhibition curves. Emetine (Sigma) was used as reference drug.

A.2.2.2 Chinese Hamster Ovarian (CHO) cytotoxicity assay

Cytotoxicity was assessed against a Chinese Hamster Ovarian (CHO) cell line using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay[123]. Emetine hydrochloride (Sigma) was used as a positive control. IC_{50} values of

pure compounds were tested in triplicate and were calculated from a 10-point dose response curve (samples were serially diluted 2× from 100 to 0.2 μg/mL, emetine hydrochloride was serial diluted 2× from 100 to 0.001 μg/mL) using a nonlinear dose-response curve fitting analysis via GraphPad Prism v.4.0 software (Graph Pad Software Inc., La Jolla, CA).

A.2.3. In vitro *antileishmanial assay*

The fractions and compounds were tested against promastigotes of *Leishmania tarentolae* strain UC (kindly provided by Dr. Larry Simpson, University of California, Los Angeles). The parasites were cultured in brain heart infusion (BHI) supplemented with hemin (10 μg/mL). A parasite suspension of 100 μL with $1x10^6$ promastigotes were seeded in 96-well plates. Serial dilutions of the compounds were prepared in 100% ethanol covering a range of 12 points from 20 to 0.125 μg/mL and all doses were tested in triplicate. The plates were incubated at 27 ºC in the dark for 48 hours. After this period, the plates were inspected under an inverted microscope to assure sterile conditions. The viability of parasites was evaluated with the dye MTT, following a previously described micromethod [124]. Pentamidine (Sigma) was used as a positive control using a serial dilution of 13 points from 3.75 to 0.125 μg/mL. The dose responses were analyzed with Graph Pad Prism 5.04 (Graph Pad Software Inc., La Jolla, CA).

APPENDIX B

Antiplasmodial and Cytotoxic Activities of Drimane Sesquiterpenes from *Canella winterana*

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Abstract

The hexane extract from the leaves of *Canella winterana* exhibited strong activity against the chloroquine sensitive (CQS) strain of *Plasmodium falciparum* (D10) *in vitro* (IC50 2.53 μg/mL). Bioassay guided fractionation of this extract has led to the isolation of 5 drimane-type sesquiterpenoids: 9-epideoxymuzigadial, 9-deoxymuzigadial, muzigadial, 3-*β*-acetoxypolygodial and the newly isolated hemiacetal, named muzigodiol, with IC50-values of 1.01, 2.19, 0.31, 2.77 and 7.43 μg/mL, respectively. The first four compounds were tested for their cytotoxicity using Chinese Hamster Ovarian (CHO) cells, where they showed IC50-values of 1.82, 33.69, 1.18, and 58.31 μg/mL, respectively. A structure-activity relationship is discussed.

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APPENDIX C

Antiplasmodial Activity of the Ethnobotanical Plant *Cassia fistula*

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Abstract

In our ongoing investigation of new compounds with activity against malaria parasites, we tested the *in vitro* antiplasmodial activity of fractions and purified compounds from *Cassia fistula* L.; a plant traditionally used by native populations of Tanzania, Zimbabwe, Mozambique and Brazil to treat malaria or symptoms associated with this disease. Crude extracts from leaves, bark and fruits were tested for their antiplasmodial activity against the chloroquine-sensitive strain of *Plasmodium falciparum* (D10), where leaf extracts showed the highest activity. The chloroform extract of the leaves was further bioassay-guided fractionated using a combination of centrifugal partition chromatography and flash column chromatography. Three main antiplasmodial principles; phytol (**1**) (IC_{50} 18.9 ± 0.60 μM), lutein (**2**) (IC_{50} 12.5 ± 0.35 μM), and di-lineolylgalactopyranosyl-glycerol (DLGG) (IC_{50} 5.8 ± 0.27 μM) (**3**) were isolated and identified using spectroscopic methods. When the three active principles were tested for their cytotoxicity using a Chinese Hamster Ovarian (CHO) cell line,

compound **3** showed very weak toxicity (IC_{50} 75.9 ± 0.28 μM), while the other two compounds were non toxic even at the highest concentration tested. The study provides evidence to support the use of *Cassia fistula* as an antimalarial remedy and describes the antiplasmodial constituents from the leaves.

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LITERATURE CITED

1. World Health Organization., *World Malaria Report 2011.*

2. World Health Organization., *World Malaria Report 2008.*

3. Hay, S.I., et al., *The global distribution and population at risk of malaria: past, present, and future.* The Lancet Infectious Diseases, 2004. **4**(6): 327-336.

4. Taubes, G., *Vaccines. Malaria parasite outwits the immune system.* Science, 2000. **290**(5491): 435.

5. World Health Organization., *Guidelines for the Treatment of Malaria*, O. World Health, Editor. 2006, World Health Organization: Geneva.

6. Sahu, N.K., S. Sahu, and D.V. Kohli, *Novel molecular targets for antimalarial drug development.* Chemical Biology & Drug Design, 2008. **71**(4):287-297.

7. Schlitzer, M., *Antimalarial drugs - What is in use and what is in the pipeline.* Archiv der Pharmazie, 2008. **341**(3): 149-163.

8. Rocco, F., *The miraculous fever-tree : malaria and the quest for a cure that changed the world / Fiammetta Rocco*. 2003, New York, NY :: HarperCollins.

9. Graziose, R., M.A. Lila, and I. Raskin, *Merging traditional Chinese medicine with modern drug discovery technologies to find novel drugs and functional foods.* Current Drug Discovery Technologies, 2010. **7**(1): 2-12.

10. Campbell, C.C., et al., *Chloroquine-resistant Plasmodium falciparum from East Africa: cultivation and drug sensitivity of the Tanzanian I/CDC strain from an American tourist.* Lancet, 1979. **2**(8153): 1151-4.

11. Noedl, H., D. Socheat, and W. Satimai, *Artemisinin-Resistant Malaria in Asia.* New England Journal of Medicine, 2009. **361**(5): 540-541.

12. Stratton, L., et al., *The persistent problem of malaria: Addressing the fundamental causes of a global killer.* Part Special Issue: Ethics and the ethnography of medical research in Africa, 2008. **67**(5): 854-862.

13. Fabricant, D.S. and N.R. Farnsworth, *The value of plants used in traditional medicine for drug discovery.* Environmental health perspectives, 2001. **109**: 69-75.

14. Balick, M.J. and P.A. Cox, *Plants, People, and Culture: The Science of Ethnobotany*. 2005: W.H. Freeman & Company.

15. Willcox, M.L., G. Burford, and G. Bodeker, *An overview of ethnobotanical studies on plants used for the treatment of malaria*, in *Traditional Medicinal Plants and Malaria*, M.L. Willcox, G. Bodeker, and P. Rasoanaivo, Editors. 2004, CRC Press LLC: Boca Raton. p. 187.

16. Kaur, K., et al., *Antimalarials from nature.* Bioorganic and Medicinal Chemistry, 2009. **17**(9): 3229-56.

17. Honigsbaum, M. and M.L. Willcox, *Cinchona*, in *Traditional Medicinal Plants and Malaria*, M.L. Willcox, G. Bodeker, and P. Rasoanaivo, Editors. 2004, CRC Press LLC: Boca Raton. p. 21.

18. Tyler, V.E., *Phytomedicines: Back to the future.* Journal of natural products, 1999. **62**(11): 1589-1592.

19. World Health Orgaization., *Guidelines for the treatment of malaria, Second edition*. 2010.

20. Boyd, M.F., *An Historical Sketch of the Prevalence of Malaria in North America.* American Journal of Tropical Medicine and Hygiene, 1941. **s1-21**(2): 223-244.

21. Hasegawa, G.R., *Quinine Substitutes in the Confederate Army.* Military medicine, 2007. **172**(6; 6): 650-655.

22. Faust, E.C., *The History of Malaria in the United States.* American Scientist, 1951. **39**(1): 121-130.

23. Spencer, C.F., et al., *Survey of Plants for Antimalarial Activity.* Lloydia, 1947. **10**(3): 145-174.

24. Harwood, L.M. and C.J. Moody, *Experimental Organic Chemistry: Principles and Practice*. 1989: Blackwell Scientific Publications.

25. Makler, M.T. and D.J. Hinrichs, *Measurement of the Lactate Dehydrogenase Activity of Plasmodium falciparum as an Assessment of Parasitemia.* American Journal of Tropical Medicine and Hygiene, 1993. **48**(2): 205-210.

26. Trager, W. and J.B. Jensen, *Human Malaria Parasites in Continuous Culture.* Science, 1976. **193**(4254): 673-675.

27. Gyllenhaal, C., et al., *Ethnobotanical approach versus random approach in the search for new bioactive compounds: support of a hypothesis.* Pharm Biol, 2012. **50**(1): 30-41.

28. Keeler, H.L., *Our native trees and how to identify them; a popular study of their habits and their peculiarities*. 1902, New York: Scribner.

29. Rafinesque, C.S., et al., *Medical flora, or, Manual of the medical botany of the United States of North America* 1828, Philadelphia, Alexander.

30. Thacher, J., *American medical biography; or, Memoirs of eminent physicians who have flourished in America, to which is prefixed a succinct history of medical science in the United States from the first settlement of the country*. A Milford House reprint. 1967, New York: Milford House.

31. Doskotch, R.W. and F.S. el-Feraly, *Antitumor agents. II. Tulipinolide, a new germacranolide sesquiterpene, and constunolide. Two cytotoxic substances from Liriodendron tulipifera L.* Journal of Pharmaceutical Sciences, 1969. **58**(7): 877-80.

32. Doskotch, R.W. and F.S. el-Feraly, *The structure of tulipinolide and epitulipinolide. Cytotoxic sesquiterpenes from Liriodendron tulipifera L.* Journal of Organic Chemistry, 1970. **35**(6): 1928-36.

33. Doskotch, R.W., et al., *Peroxyferolide : a Cytotoxic Gerrnacranolide Hydroperoxide from Liriodendron tulipifera.* Journal of the Chemical Society, Chemical Communications, 1976: 402- 403.

34. Doskotch, R.W., et al., *Isolation and Characterization of Peroxyferolide, a Hydroperoxy Sesquiterpene Lactone from Liriodendron tulipifera.* Journal of Organic Chemistry, 1977. **42**(22): 3614- 3618.

35. Doskotch, R.W., et al., *New sesquiterpene lactones from Liriodendron tulipifera.* Phytochemistry, 1975. **14**(3): 769-773.

36. Muhammad, I. and C.D. Hufford, *Phenylpropanoids, Sesquiterpenes, and Alkaloids from the Seeds of Liriodendron tulipifera.* Journal of Natural Products, 1989. **52**(5): 1177-1179.

37. Buchanan, M.A. and E.E. Dickey, *Liriodenine, A Nitrogen-Containing Pigment of Yellow Poplar Heartwood (Liriodendron tulipifera, L.).* The Journal of Organic Chemistry, 1960. **25**(8): 1389-1391.

38. Chen, C.-L., C. Hou-Min, and E.B. Cowling, *Aporphine alkaloids and lignans in heartwood of Liriodendron tulipifera.* Phytochemistry, 1976. **15**(4): 547-550.

39. Chen-Loung, C. and C. Hou-Min, *Lignans and aporphine alkaloids in bark of Liriodendron tulipifera.* Phytochemistry, 1978. **17**(4): 779-782.

40. Chen-Loung, C., et al., *Aporphine alkaloids and lignans formed in response to injury of sapwood in Liriodendron tulipifera.* Phytochemistry, 1976. **15**(7): 1161-1167.

41. Cohen, J., W. Von Langenthal, and W. Taylor, *Notes-The Alkaloids of Liriodendron tulipifera L. The Structure and Synthesis of the Unnamed Yellow Alkaloid and the Isolation of d-Glaucine.* The Journal of Organic Chemistry, 1961. **26**(10): 4143-4144.

42. Taylor, W.I., *The structure and synthesis of liriodenine, a new type of isoquinoline alkaloid.* Tetrahedron, 1961. **14**(1-2): 42-45.

43. Berthod, A., M. Hassoun, and G. Harris, *Using the Liquid Nature of the Stationary Phase: The Elution-Extrusion Method.* Journal of Liquid Chromatography & Related Technologies, 2005. **28**(12): 1851 - 1866.

44. Leboeuf, M., et al., *Alkaloids of Annonaceae. XXXV. Alkaloids of Desmos Tiebaghiensis.* Journal of Natural Products, 1982. **45**(5): 617-623.

45. Cava, M.P., I. Noguchi, and K.T. Buck, *Novel Pschorr reaction in the papaverine series.* The Journal of Organic Chemistry, 1973. **38**(13): 2394-2397.

46. Costa, E.V.a., et al., *7,7-Dimethylaporphine Alkaloids from the Stem of Guatteriopsis friesiana.* Journal of Natural Products, 2009. **72**(8): 1516-1519.

47. Harrigan, G.G., et al., *Isolation of bioactive and other oxoaporphine alkaloids from two annonaceous plants, Xylopia aethiopica and Miliusa cf. banacea.* Journal of Natural Products, 1994. **57**(1): 68-73.

48. Singh, O.V., et al., *Manganese(III) acetate mediated oxidation of aporphines: a convenient and useful synthesis of oxoaporphines.* Tetrahedron Letters, 2007. **48**(46): 8166-8169.

49. Bero, J., M. Frederich, and J. Quetin-Leclercq, *Antimalarial compounds isolated from plants used in traditional medicine.* Journal of Pharmacy and Pharmacology, 2009. **61**(11): 1401-33.

50. Cui, L. and X.Z. Su, *Discovery, mechanisms of action and combination therapy of artemisinin.* Expert Review of Anti-infective Therapy, 2009. **7**(8): 999-1013.

51. Chagonda, L., et al., *Cytotoxic action of alpha-methylene lactones towards HeLa cells in culture.* Phytotherapy Research, 1989. **3**(5): 196-200.

52. del Rayo Camacho, M., et al., *Oxoaporphine Alkaloids and Quinones from Stephania dinklagei and Evaluation of Their Antiprotozoal Activities.* Planta Medica, 2000. **66**(05): 478-480.

53. Likhitwitayawuid, K., et al., *Cytotoxic and Antimalarial Alkaloids from the Tubers of Stephania pierrei.* Journal of Natural Products, 1993. **56**(9): 1468-1478.

54. Wirasathien, L., et al., *Biological Activities of Alkaloids from Pseuduvaria setosa.* Pharmaceutical Biology, 2006. **44**(4): 274-278.

55. Xiang, J.Q., et al., *Species level phylogeny of the genus Cornus (Cornaceae) based on molecular and morphological evidence - implications for taxonomy and Tertiary intercontinental migration.* Taxon, 2006. **55**(1): 9-30.

56. Millspaugh, C.F., *American Medicinal Plants*. 1887, New York: Boericke and Tafel.

57. Seeram, N.P., et al., *Characterization, quantification, and bioactivities of anthocyanins in Cornus species.* Journal of Agricultural and Food Chemistry, 2002. **50**(9): 2519-2523.

58. Duke, J.A., *Medicinal plants of China*, E.S. Ayensu, Editor. 1985, Reference Publications: Algonac, Mich. :.

59. Dirr, M.A., *Manual of Woody Landscape Plants: Their Identification, Ornamental Characteristics, Culture, Propagation, and Uses*. 5 ed. 1998, Champaign, IL: Stipes Publishing. 1250.

60. Bate-Smith, E.C., et al., *Phytochemical Interrelationships in the Cornaceae.* Biochemical Systematics and Ecology, 1975. **3**(2): 79-89.

61. Eyde, R.H., *Comprehending Cornus: Puzzles and Progress in the Systematics of the Dogwoods.* Botanical Review, 1988. **54**(3): 233-351.

62. Jensen, S.R., A. Kjaer, and B.J. Nielsen, *The genus Comus: Non-flavonoid glucosides as taxonomic markers.* Biochemical Systematics and Ecology, 1975. **3**(2): 75-78.

63. Moerman, D., *Native American Medicinal Plants: An Ethnobotanical Dictionary*. 2009, Portland: Timber Press.

64. Barton, W.P.C., H.C. Carey, and I. Lea, *Vegetable materia medica of the United States, or, Medical botany :containing a botanical, general, and medical history of medicinal plants indigenous to the United States.* Vol. v. 1. 1825, Philadelphia :: H.C. Carey & I. Lea--Chesnut Street.

65. Yogeeswari, P. and D. Sriram, *Betulinic acid and its derivatives: a review on their biological properties.* Current Medicinal Chemistry, 2005. **12**(6): 657-66.

66. de Sá, M., et al., *Antimalarial activity of betulinic acid and derivatives in vitro against Plasmodium falciparum and in vivo in P. berghei-infected mice.* Parasitology Research, 2009. **105**(1): 275-279.

67. Steele, J.C., et al., *In vitro and in vivo evaluation of betulinic acid as an antimalarial.* Phytotherapy research : PTR, 1999. **13**(2): 115-9.

68. Domínguez-Carmona, D.B., et al., *Antiprotozoal activity of Betulinic acid derivatives.* Phytomedicine, 2010. **17**(5): 379-382.

69. Wert, L., et al., *Toxicity of betulin derivatives and in vitro effect on promastigotes and amastigotes of Leishmania infantum and L. donovani.* Journal of Antibiotics, 2011. **64**(7): 475-81.

70. Gnoatto, S.C.B., et al., *Synthesis and preliminary evaluation of new ursolic and oleanolic acids derivatives as antileishmanial agents.* Journal of Enzyme Inhibition and Medicinal Chemistry, 2008. **23**(5): 604-610.

71. Torres-Santos, E.C., et al., *Antileishmanial activity of isolated triterpenoids from Pourouma guianensis.* Phytomedicine, 2004. **11**(2-3): 114-20.

72. Kirmizibekmez, H., et al., *Antiprotozoal activity of Melampyrum arvense and its metabolites.* Phytotherapy Research, 2011. **25**(1): 142-146.

73. Bjurman, J., *Ergosterol as an indicator of mould growth on wood in relation to culture age, humidity stress and nutrient level.* International Biodeterioration & Biodegradation, 1994. **33**(4): 355-368.

74. Bhakuni, R.S., Y. N. Shukla, and R. S. Thakur, *Triterpenoids from Cornus capitata.* Phytochemistry, 1987. **26**(9): 2607-2610.

75. Kim, K.H., et al., *Tirucallane Triterpenoids from Cornus walteri.* Journal of Natural Products, 2011. **74:** 54-59.

76. Ma, C.Y., et al., *Study of antimalarial activity of chemical constituents from Diospyros quaesita.* Chem Biodivers, 2008. **5**(11): 2442-8.

77. Suksamrarn, A., T. Tanachatchairatana, and S. Kanokmedhakul, *Antiplasmodial triterpenes from twigs of Gardenia saxatilis.* Journal of Ethnopharmacology, 2003. **88**(2-3): 275-277.

78. Rocha, L.G., et al., *A review of natural products with antileishmanial activity.* Phytomedicine : international journal of phytotherapy and phytopharmacology, 2005. **12**(6): 514-535.

79. Walker, J.M., et al., *An experimental inquiry into the similarity in virtue between the Cornus florida and sericea : and the Cinchona officinalis of Linnaeus, together with an inquiry into the modus operandi of astringent vegetables in preventing the putrefactive fermentation*. 1803, H. Maxwell ... Philadelphia.

80. Galgon, T., D. Höke, and B. Dräger, *Identification and Quantification of Betulinic Acid.* Phytochemical Analysis, 1999. **10**(4): 187-190.

81. Yili, A., et al., *Betulinic acid and sterols from Astragalus altaicus.* Chemistry of Natural Compounds, 2009. **45**(4): 592-594.

82. Seebacher, W., et al., *Complete assignments of 1H and 13C NMR resonances of oleanolic acid, 18α-oleanolic acid, ursolic acid and their 11-oxo derivatives.* Magnetic Resonance in Chemistry, 2003. **41**(8): 636-638.

83. Jayaprakasha, G.K., et al., *Inhibition of colon cancer cell growth and antioxidant activity of bioactive compounds from Poncirus trifoliata (L.) Raf.* Bioorganic and Medicinal Chemistry, 2007. **15**(14): 4923-4932.

84. Chobot, V., et al., *Ergosta-4,6,8,22-tetraen-3-one from the edible fungus, Pleurotus ostreatus (oyster fungus).* Phytochemistry, 1997. **45**(8): 1669-1671.

85. Ahmad, F.B.H., et al., *Anticancer Activity of 3-O-Acylated Betulinic Acid Derivatives Obtained by Enzymatic Synthesis.* Bioscience, Biotechnology, and Biochemistry, 2010. **74**(5): 1025-1029.

86. Birch, A.J., et al., *513. The structure of flindissol. Some remarks on the elemi acids.* Journal of the Chemical Society (Resumed), 1963: 2762-2772.

87. Chien, N.Q., et al., *New 3-O-Acyl Betulinic Acids from Strychnos vanprukii Craib.* Journal of Natural Products, 2004. **67**(6): 994-998.

88. Listen, A., L.H. Rieseberg, and T.S. Elias, *Functional androdioecy in the flowering plant Datisca glomerata.* Nature, 1990. **343**(6259): 641-642.

89. Davidson, C., *An antomical and morphological study of the Datiscaceae.* Aliso, 1973. **8**: 49-110.

90. Bradley, T., R. Batty, and A.A. Noehden, *The London Medical And Physical Journal, vol.1.* 1799.

91. Kupchan, S.M., et al., *Tumor inhibitors. LXXII. Datiscoside, a novel antileukemic cucurbitacin glycoside from Datisca glomerata.* J. Amer. Chem. Soc., 1972. **94**p. 1353-4.

92. Sasamori, H., et al., *New cytotoxic principles from Datisca glomerata.* Journal of the Chemical Society, Perkin Transactions 1, 1983:1333-1347.

93. Restivo, R.J., R.F. Bryan, and S.M. Kupchan, *Stereochemistry of the cucurbitacins. Crystal and molecular structure and absolute configuration of datiscoside bis-(p-*

iodobenzoate) dihydrate. Journal of the Chemical Society, Perkin Transactions 2, 1973(7): 892-897.

94. Chen, J.C., et al., *Cucurbitacins and cucurbitane glycosides: structures and biological activities.* Natural Product Reports, 2005. **22**(3).

95. Galey, F.D., et al., *A case of Datisca glomerata poisoning in beef cattle.* Veterinary and Human Toxicology, 1990. **32**(6): 575-6.

96. Bourdy, G., et al., *Ethnopharmacology and malaria: New hypothetical leads or old efficient antimalarials?* International Journal for Parasitology, 2008. **38**(1): 33-41.

97. Bubb, W.A., *NMR spectroscopy in the study of carbohydrates: Characterizing the structural complexity.* Concepts in Magnetic Resonance Part A, 2003. **19A**(1): 1-19.

98. Ceccarelli, G., et al., *Additive NMR shielding parameters for some substituted cyclohexanols.* Organic Magnetic Resonance, 1975. **7**(11): 548-553.

99. Ramos, S.S., et al., *13C NMR spectral assignment of five epimeric 3α- versus 3β-functionalized cholestane pairs.* Magnetic Resonance in Chemistry, 2005. **43**(10): 861-863.

100. Crews, P. and E. Kho-Wiseman, *Stereochemical assignments in marine natural products by 13C NMR γ effects.* Tetrahedron Letters, 1978. **19**(28): 2483-2486.

101. Kupchan, S.M., et al., *Tumor inhibitors. LVI. Cucurbitacins O, P, and Q, the cytotoxic principles of Brandegea bigelovii.* The Journal of Organic Chemistry, 1970. **35**(9): 2891-2894.

102. Che, C.-t., et al., *High-Field 1H-nmr Spectral Analysis of Some Cucurbitacins.* Journal of Natural Products, 1985. **48**(3): 429-434.

103. Bailey, V., *Revision of the genus Ptelea (rutaceae).* Brittonia, 1962. **14**(1): 1-45.

104. Bailey, V., *Historical review of ptelea trifoliata in botanical and medical literature1.* Economic Botany, 1958. **14**(3): 180-188.

105. Anonymous, *Journal of Materia Medica Volume 10*. 2010: Nabu Press.

106. Dreyer, D.L., *Coumarins and alkaloids of the genus ptelea.* Phytochemistry, 1969. **8**(6): 1013-1020.

107. Reisch, J., et al., *Inhaltsstoffe der blüten von ptelea trifoliata: Arctigenin-methyläther, (+)-hydroxylunin und ptelefolin.* Tetrahedron Letters, 1969. **10**(43): 3803-3806.

108. Reisch, J., et al., *Über die strukturen weiterer chinolon-(2)-alkaloide aus ptelea trifoliata.* Tetrahedron Letters, 1970. **11**(22): 1945-1948.

109. Reisch, J., et al., *O-methylptelefolonium als Ptelea-[`]leitalkaloid'.* Phytochemistry, 1973. **12**(10): 2552-2553.

110. Szendrei, K., et al., *Coumarins and alkaloids from Ptelea trifoliata ssp. pallida var. confinis.* Lloydia, 1973. **36**(3): 333-7.

111. Mitscher, L.A., et al., *Antimicrobial agents from higher plants. The quaternary alkaloids of Ptelea trifoliata.* Lloydia, 1975. **38**(2): 109-16.

112. Mitscher, L.A., et al., *Antimicrobial agents from higher plants. The antimicrobially inactive components of Ptelea trifoliata L.* Lloydia, 1975. **38**(2): 117-24.

113. Reisch, J., et al., *7,8-methylendioxy-chinolin-derivate als nebenalkaloide der blüten von Ptelea trifoliata.* Phytochemistry, 1975. **14**(12): 2722-2723.

114. Rideau, M., et al., *Alcaloïdes dihydrofuroquinoleiques de quelques rutaceae: Isolement, structure, proprietes biologiques.* Phytochemistry, 1979. **18**(1): 155-159.

115. Grundon, M.F., D.M. Harrison, and S.A. Surgenor, *The synthesis of ptelefoline and ptelefoline methyl ether.* Tetrahedron Letters, 1979. **20**(19): 1713-1714.

116. Neville, C.F., et al., *Quinoline alkaloids. Part 27. Synthesis of the Ptelea alkaloids pteleflorine, neohydroxylunine, O-methylhydroxyluninium salt and hydroxylunine.* Journal of the Chemical Society, Perkin Transactions 1, 1991(2): 259-262.

117. Petit-Paly, G., et al., *New Alkaloids from Ptelea trifoliata.* Planta Medica, 1989. **55**: 209- 210.

118. Neville, C.F., et al., *Quinoline Alkaloids. Part 28. The Biosynthesis of Furoquinolines and Other Hemiterpenoids in Ptelea trifoliata.* ChemInform, 1991. **22**(51).

119. Yang, F., et al., *Application of analytical and preparative high-speed counter-current chromatography for separation of alkaloids from Coptis chinensis Franch.* Journal of Chromatography A, 1998. **829**(1-2): 137-141.

120. Arruda, M.S.P., et al., *Quinolone alkaloids from Zanthoxylum acutifolium.* Phytochemistry, 1992. **31**(10): 3617-3619.

121. Lipinski, C.A., et al., *Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings.* Adv Drug Deliv Rev, 2001. **46**(1-3): 3-26.

122. Gamo, F.-J., et al., *Thousands of chemical starting points for antimalarial lead identification.* Nature, 2010. **465**(7296): 305-310.

123. Mosmann, T., *Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays.* Journal of Immunological Methods, 1983. **65**(1-2): 55-63.

124. Sereno, D. and J.L. Lemesre, *Use of an enzymatic micromethod to quantify amastigote stage of Leishmania amazonensis in vitro.* Parasitology Research, 1997. **83**(4): 401-3.