Antimalarial Benzophenones and Xanthones from *Garcinia* species

by

James T. Lyles

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Date	Chair of Examining Committee Dr. Edward J. Kennelly, Lehman College
Date	Executive Officer Dr. Laurel Eckhardt
	Dr. Dwight T. Kincaid, Lehman College
	Dr. Michael H. Nee, The New York Botanical Garden
	Dr. John M. Berger, County College of Morris
	Dr. Miguel Cervantes-Cervantes, Rutgers University
	Dr. Dominick V. Basile, Lehman College (Reader)

Supervisory Committee

THE CITY UNIVERSITY OF NEW YORK

Abstract

ANTIMALARIAL BENZOPHENONES AND XANTHONES
FROM *GARCINIA* SPECIES

by

James T. Lyles

Advisor: Dr. Edward J. Kennelly

Garicina, a genus in the Clusiaceae, is a source of antiparasitic and antimalarial phenolic secondary metabolites, including benzophenones and xanthones. The methanolic extracts of *G. livingstonei*, *G. mangostana*, *G. spicata*, and *G. xanthochymus* were tested *in vitro* for antiplasmodial activity against the *P. falciparum* clone D6. The crude methanolic *G. mangostana* extract inhibited the clone by 89%, while the *G. xanthochymus* extract inhibited it by 24%. Neither of the extracts showed any cytotoxicity toward Vero cells. Hexanes, EtOAc, and n-BuOH partitions of a *G. xanthochymus* seed extract were also screened against the *P. falciparum* clone D6, the hexanes partition inhibited the clone by 58%, the EtOAc and n-BuOH partitions showed no inhibitory activity. Additionally, compounds identified from *Garcinia* species were screened in the plasmodial lactase dehydrogenase (pLDH) activity assay. The compounds tested were: aristophenone A (**1**), cycloxanthochymol (**2**), gambogenone (**3**), guttiferone A (**4**), guttiferone E (**5**), guttiferone H (**6**), isoxanthochymol (**7**), xantochymol (**8**), xanthone (**9**), mangiferin (**10**), alloathyriol (**11**), α-mangostin (**12**), β-mangostin (**13**), 3-isomangostin (**14**), 8-desoxygartanin (**15**), 4-methoxyxanthone (**16**), 1,5,6-trihydroxyxanthone (**17**), and 32-hydroxy-ent-guttiferone M (**18**). Only compounds **5**, **6**, **7**, **12**, **13**, and **14** showed antiplasmodial activity. The antiplasmodial activities of

compounds **5**, **6**, and **14** have not been previously reported. This is the first report of **7**, **12**, and **13** having antiplasmodial activity against the chloroquine-sensitive *P. falciparum* clone D6 and chloroquine-resistant clone W2.

For the benzophenones **8** and **2** the absence of a terminal methylene on the C-8 side chain (cf. **5** and **7**) correlates with antiplasmodial activity. Compound **6** with a terminal methylene on C-30 and a cyclohexane from C-7 to C-8 is antiplasmodial. It was concluded that, for benzophenones neither the C-5 nor the C-14 side groups affect antiplasmodial activity. The substitution pattern of both the A and B rings of the xanthones was shown to be important in determining the extent of antiplasmodial activity. An isoprenyl chain was necessary on C-8 for antiplasmodial activity (cf. **12** and **14**). The results indicate that the antiplasmodial activity is determined by factors other than the hydroxylation of C-4 and C-5 alone as the current structure activity studies indicate.

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List of Abbreviations

ACS	American Chemical Association
n-BuOH	n-butanol
CDC	Centers for Disease Control
$CHCl_3$	chloroform
DMSO	dimethyl sulfoxide
ER	endoplamic reticulum
EtOAc	ethyl acetate
EtOH	ethanol
GR	general reagent
HCA	hydroxycitric acid
HPLC	high performance liquid chromatography
HVR	hypervariable region
pLDH	plasmodial lactase dehydrogenase
MeCN	acetonitrile
MeOH	methanol
PDA	photodiode array
PFT	protein farnesyl transferase
PGGT-1	protein geranylgeranyltransferase-I
RP	reversed-phase
rpm	rotations per min

SPE	solid phase extraction
TLC	thin layer chromatography
UV-VIS	ultraviolet-visible spectrum
WHO	World Health Organization

Chapter 1

Introduction

Introduction

Malaria kills between 708,000 to over a million poeple annually. Thirty-five countries account for 98% of the world's malaria deaths (Figure 1-1). Thirty of these countries are in sub-Saharan Africa, corresponding to 89% of the annual death toll. Malaria is the fifth leading cause of death worldwide, but the second leading cause of death from infectious disease in Africa, following HIV/AIDS. There are currently 3.3 billion people, nearly half of the world's population, living in areas that are considered high-risk for the contraction of this often fatal disease. Even the United States is not immune, with 1,500 cases reported annually.[1, 2] The fight against malaria has become a worldwide initiative in recent years. The Roll Back Malaria Partnership aimed to decrease by half the number of deaths inflicted by the disease by 2010. This initiative is implementing four interventions; the distribution of insecticide-treated sleeping nets, the spraying of houses with insecticides, preventative treatment as part of prenatal care, and the use of effective drugs. Overall, The Roll Back Malaria Partnership has spent over $1 billion annually to combat the disease.[3]

Plasmodium species

Malaria is an infectious disease caused by protists in the genus *Plasmodium*. While humans and other vertebrates comprise the primary vector for *Plasmodium*, the transmission and secondary vectors for *Plasmodium* are Culicidae mosquitoes in the Anophaliae subfamily. There are over 3,500 species of mosquitos worldwide. The

Culicidae or mosquito family is divided into two subfamilies: Anophelinae and Culicinae. The Anophelinae is further divided into three genera: *Anopheles*, *Bironella*, and *Chagasia*. While *Anopheles* contains about 430 species, only the females of 30 to 40 species are vectors for malaria that can infect humans.[4] These *Anopheles* species are distributed throughout the world (Figure 1-2), especially in tropical and sub-tropical regions.

The life cycle of an *Anopheles* mosquito begins when a female lays 50 to 200 eggs per oviposition directly on the surface of still or stagnant water. Once the eggs hatch, the mosquito larvae live mainly at the water's surface, breathing through spiracles. Larvae feed on bacteria and microscopic algae. The larvae undergo four instars, or developmental stages, shedding their exoskeletons at each instar. After the fourth instar, a larva undergoes metamorphosis before shedding its exoskeleton and emerging as a pupa. The pupal form is aquatic and does not feed, only coming to the surface to breathe. When a pupa is fully mature, it rises to the water's surface, and the dorsal surface of its cephalothorax splits open, allowing an adult mosquito to emerge. The time spent developing from egg to adult mosquito ranges from 5 to 14 days, depending on environmental conditions. Once adult mosquitoes emerge, the males live an average of 1 week, while females live 1 to 2 weeks in the wild, or over a month when kept in captivity. The adult mosquitoes feed on nectar and other sugar sources. Only adult female mosquitoes take a blood meal, which is necessary for egg development. Female mosquitoes lay eggs 2 to 3 days after a blood meal and can produce eggs during their entire adult lifetime.[5]

When a malaria-infected female *Anopheles* mosquito takes a blood meal it injects *Plasmodium* sporozoites into the human host. The sporozoites are motile for only a few hours and during this time migrate to the liver. Once there, the sporozoite forms a junction with the hepatocyte cell surface and enters through a parasitophorous vacuole.[6, 7] The mechanism used by the parasite to create the vacuole and enter the cell is not completely understood. Once in the cell, sporozoites mature and asexually replicate creating a multinucleated schizont. The hepatic cell later ruptures, releasing merozoites. When the merozoites are freed from the hepatic cell, they move into the host's bloodstream and infect erythrocytes. There the parasites develop into ring-stage trophozoites, which will asexually reproduce to form more merozoites and another multinucleated schizont. Eventually, the infected erythrocyte will rupture, releasing merozoites into the host's blood. The merozoites will then infect more erthyrocytes and the infection process repeats. The presence of the blood stage parasites are clinically diagnosed as malaria.[8]

After a merozoite infects an erythrocyte and develops into a trophozoite, some of the parasites will not continue with asexual reproduction. Rather, some trophozoites will differentiate into sexual erythrocytic stages. The male microgametocytes and the female macrogametocytes remain in the host's blood and are ingested by an *Anopheles* mosquito during another blood meal.

Once the parasite has infected or reinfected a mosquito, it begins the sporogonic cycle, which is the sexual reproductive stage of the *Plasmodium* parasite. The initial steps of this stage occur in a mosquito's gut where a microgamete fuses with a macrogamete to produce a zygote.[9] The zygote develops into an elongated, motile ookinete that invades

the mosquito's midgut wall and continues to mature into an oocyst. The oocyst continues to grow in the midgut wall and eventually ruptures releasing sporozoites. These sporozoites migrate to the mosquito's salivary glands where they will inoculate another person the next time the mosquito takes a blood meal.[8]

Over 200 species of *Plasmodium* have been identified from mammals, birds, and reptiles,[10] but only five species of *Plasmodium* affect humans, namely *P. falciparum*, *P. ovale*, *P. vivax*, *P. malariae*, and *P. knowlesi*.

Plasmodium falciparum occurs worldwide, accounting for 35 to 43% of all infections. It is the dominant species found in Africa and is the likeliest species to result in severe medical complications. This species is responsible for cerebral malaria.[11] *P. vivax* is the most widespread species and can exist in a dormant stage in the liver, which allows *P. vivax* to cause its host a relapse of malaria weeks to even years after initial infection. In order for *P. vivax* to gain entry to the host erythrocyte, the cell membrane must express the Duffy glycoprotein which is a receptor for *P. vivax*. This protein is absent in many human populations, thus preventing the parasite from infecting the host erythrocytes.[12] *P. ovale* was believed to be limited geographically to tropical Africa, New Guinea, Eastern Indonesia, and the Philippines, but infections have been recently reported in a much broader geographic range. Still, *P. ovale* is relatively uncommon even within its complete geographic range.[13] *P. malariae* was first described in 1886 and occurs worldwide. Like *P. vivax,* the parasite can remain dormant in the liver, resulting in a relapse of the disease or a low-level chronic infection. This species is thought to be zoonotic since it is genetically identical to *P. brasilianum* which infects New World

monkeys.[8, 13] Lastly, *P. knowlesi* occurs mainly in Southeast Asia where it is a natural pathogen of macaques, but it can also infect humans.[8]

The Family Clusiaceae

Plants in the family Clusiaceae (formerly the Guttiferae) are distributed nearly worldwide, though most are tropical. The family includes trees up to 40 m tall, shrubs that are often epiphytic, and perennial or more rarely annual herbs. The stems and leaves of the family commonly have a yellow-orange to white latex, and the opposite and thick leaves are usually traversed with latex ducts or dark resin glands.[14, 15]

Ethnobotanically, many Clusiaceae are used for timber or as ornamentals, and the seeds of several species are edible or can be used as a cooking oil.[16] The latex has been used to produce glues, caulks, incense, and a combustible fuel. There are many examples of medicinal compounds from plants in the Clusiaceae, including astringents for diarrhea and dysentery,[17] antidepressants,[18] antimalarials,[19] and antioxidants.[20] In the United States, Clusiaceae plants have provided dietary supplements for weight loss (e.g. *Garcinia cambogia*) and the management of depression (e.g. *Hypericum perforatum*). The Clusiaceae are a well-known source of phenolic secondary metabolites including benzophenones, chromenes, coumarins, flavonoids, triterpenes, and xanthones.[21, 22]

The pantropical genus *Garcinia* consists of over 250 species, which are mainly medium-sized evergreen trees and small shrubs.[23] Their center of diversity is in Africa, Madagascar and Indo-Malasya, and they are generally found in lowland tropical forests.[24] Several species have been reported as medicinal: *G. cambogia, G. conrauana, G. corallina, G. dulcis, G. guineensis, G. hanburyi, G. harmandii, G. lanessanii, G.*

livingstonei, G. mangostana, G. morella, G. olivieri, G. spicata, G. vilersiana, and *G. venulosa*[17]. The best-known species of the genus is *G. mangostana*, which produces the delicious edible fruit, mangosteen.

The genus was revised by Sweeney in 2008 based on floral morphology and molecular phylogenetic studies and was divided into two distinct lineages.[23, 25] Lineage A is distinguished by staminate flowers that lack a well-developed pistillode and have a fleshy disk in the center of the flower. Between the ovary and staminodes are either fleshy antesepalous appendages or a ring. These structures are often described as nectaries and in some species secrete a watery, sweet substance. Lineage B is distinguished from lineage A by its lack of disks, appendages, and rings.

Garcinia cambogia

Garcinia cambogia is a medium to large evergreen tree native to southern India. It has shiny, dark green, glabrous leaves and produces orange-red flowers. The yellow to red fruits are large, globular, and have deep vertical grooves, which divide the fruit into six to eight lobes. The fruits enclose six to eight multi-lobed seeds within their red or white flesh. Majeed, who reviewed the ethnobotanical uses of *G. cambogia* in 1994, noted that the dried rind of the fruit is used as an alternative to tamarind or limes in curries. The rind is also used to polish precious metals and due to its high concentration of acetic acid, to produce rubber latex. The wood is not dense enough for use as commercial timber, but is employed for fence posts and splints. The yellow latex is processed to make a yellow varnish, and the seeds are a source of edible fat. Commercially, the fruit rind of *G. cambogia* is extracted to produce the dietary

supplement for weight loss Citrin.[26] Only two xanthones have been reported in this species: garbogiol and rheediaxanthone A,[27] although several benzophenones have been isolated: camboginol (garcinol), cambogin (isogarcinol), xanthochymol, and isoxanthochymol.[28, 29]

Garcinia livingstonei

Garcinia livingstonei has its center of diversity in southern Africa, where it is known by the common names "muphiphi" and "shanfarood." Inventories of plants used in traditional medicine in this area provide many ethnobotanical uses for the species, including use as a food stuff, a beer additive, a decoction for eye problems, a treatment for toothaches, an aid in childbirth, and as an aphrodisiac.[30-33] Currently, eight xanthones, four biflavones, and one flavonoid have been reported in the fruits, leaves, or rootbark of *G. livingstonei*.[34-37] Various extracts of the fruits and bark have been reported to have cytotoxic activity,[37-39] antifungal activity,[37] as well as anti-HIV properties.[34]

Garcinia spicata

Garcinia spicata (synonym *Rheedia spicata*) has been traditionally used in the dyeing of silks in the Ryukyu Islands.[40] Several flavonoids and biflavonoids, triterpenes, and steroids have been isolated from the bark and leaves of *G. spicata*.[41-45] An extract of the whole plant proved to be an anticholesteremic.[46]

Garcinia xanthochymus

Garcinia xanthochymus is a medium-sized evergreen tree native to India.[47] It has dark, scabrous bark and dark, leathery, large, shiny leaves and produces white flowers. The fruits are the size of a small apple, and, when ripe, are yellow and smooth (Figure 1-3).[17, 47] The fruit contains 3 to 6 seeds, each 2 to 5 cm long. The seeds have a hard, mottled brown seed coat and a cream-white, powdery starch, Figure 1-3. The fruit pulp has been described as both sharp, sour, or sweetish in taste.[17] The pulp is commonly eaten or made into a preserve. It is also used as a tamarind substitute in curries and vinegar. For bilious conditions, the dried fruit is made into a sherbet and administered.[17, 47] Based on Sweeney's revision, *G. xanthochymus* is in lineage A of the genus, having a fleshy disk in the center of the flower and a nectar secreting disk.[25]

Many compounds have been isolated from this species, including the two novel benzophenones, guttiferone H and gambogenone.[48] Additionally, several xanthones have been isolated previously from the bark and stems: 1,5-dihydroxyxanthone,[28] 1,7-dihydroxyanthone (euxanthone),[28] 1,2-dihydroxy-5-methoxyxanthone,[28, 49] 1,5-dihydroxy-2-methoxyxanthone,[28] 1,5-dihydroxy-3-methoxyxanthone,[28] and 1,3,5-trihydroxyanthone.[28, 49]

Benzophenones and Xanthones

Benzophenones are a class of compounds based on a common 13-carbon skeleton (Figure 1-4). They are found in at least 13 plant families including: Asteraceae, Clusiaceae, Davalliaceae, Fabaceae, Gentianaceae, Iridaceae, Lauraceae, Magnoliaceae, Moraceae, Rosaceae, and Thymelaeaceae. Benzophenones have been isolated from

leaves, roots, fruits, bark, and even resins from plants in these families. Of the 146 recently described benzophenones, 66% were found in the Clusiaceae.[48]

The exact nature of benzophenone biosynthesis is still unclear. A mixed biosynthetic pathway with the A-ring derived from the shikimate pathway and the acetate-malonate pathway providing the B-ring is one possibility. This pathway yields the basic 13-carbon skeleton.[50] However, some research indicates that benzophenones are synthesized from phloroglucinol (1,3,5-trihydroxybenzene) rather than a 13-carbon skeleton. A 7-membered carbon ring, the benzophenone A-ring, is added later in the biosynthesis. The phloroglucinol biosynthesis was suggested by Hu *et al.* when studying the sampsoniones, a group of polyprenylated benzophenones.[51] With such varied structural diversity of benzophenones, it is likely that there are multiple biosynthetic pathways.

Xanthones are benzophenone derivatives whose hydroxylation pattern suggests a biosynthesis similar to that of the flavonoids.[52] They have a mixed biosynthetic pathway similar to benzophenones: carbons 1 to 4 are derived through the acetate pathway and designated as ring A; while carbons 5 to 8 arise from the shikimate pathway and form ring B.[28, 52] The basic skeleton and numbering of a xanthone is based on xanthene-9-one (Figure 1-4).[53] Xanthones are classified into five major groups: simple oxygenated xanthones, xanthone glycosides, prenylated and related xanthones, xanthonoligoids, and miscellaneous xanthones.[53] The simple oxygenated xanthones are further subdivided into six groups based on the degree of oxygenation.

Xanthones were historically used as dyes, such as the yellow pigment gentisin from *Gentiana lutea* (Gentianaceae) and the family of rhodamine dyes.[54, 55] Currently,

xanthone dyes are primarily used in analytical work as fluorescent indicators, as many of them are chemiluminescent when oxidized with hydrogen peroxide in an alkaline environment.[56] Xanthones are not limited to use as dyes: rather this group of compounds displays activity as antioxidant,[20] antidepressant,[18] antifungal,[57-59] antimicrobial,[60, 61] anticancer agent,[62] antihypertensive,[63] cardioprotective agent,[64] and in the prevention of vascular thrombosis through antiplatelet activity.[65] Further work has implicated xanthones as strong antiparasitic agents, effective against American trypanosomiasis, leishmaniasis, and malaria.[66-68]

Overall, several hundred xanthones have been found in flowering plants,[54] but they have also been isolated from lichens and fungi.[49, 53, 69] The majority of naturally produced xanthones in vascular plants occur in the Clusiaceae and Gentianaceae,[28, 29] with at least 200 xanthones reported in the Clusiaceae, from 60 well-studied species.[28, 54]

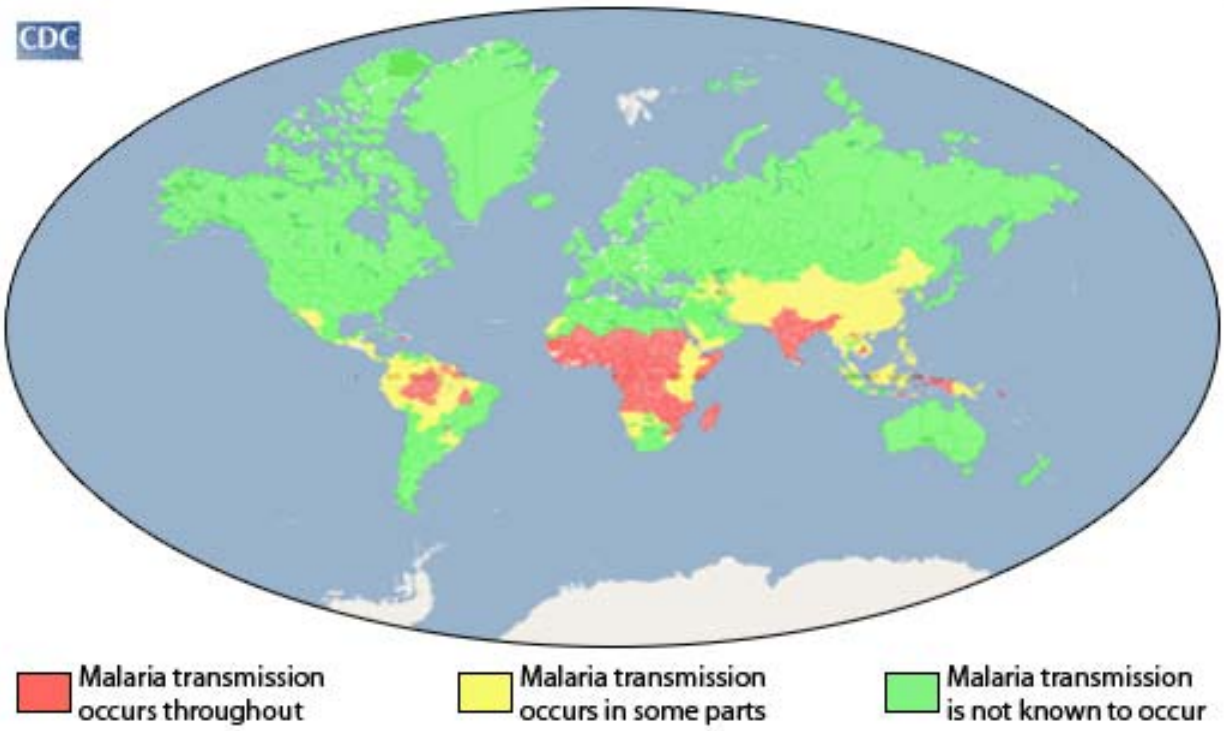


Figure 1-1. Locations of malaria transmission worldwide.[2]

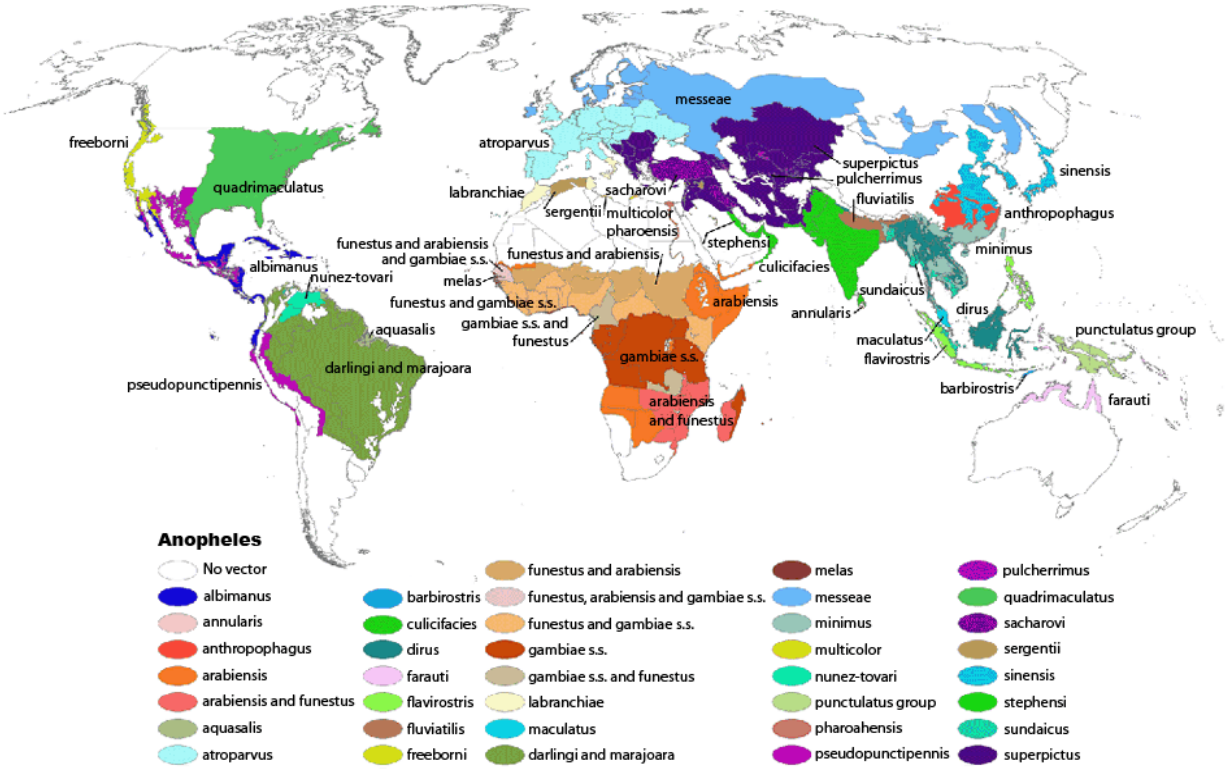


Figure 1-2. Worldwide distribution of *Anopheles* mosquitoes.[70]

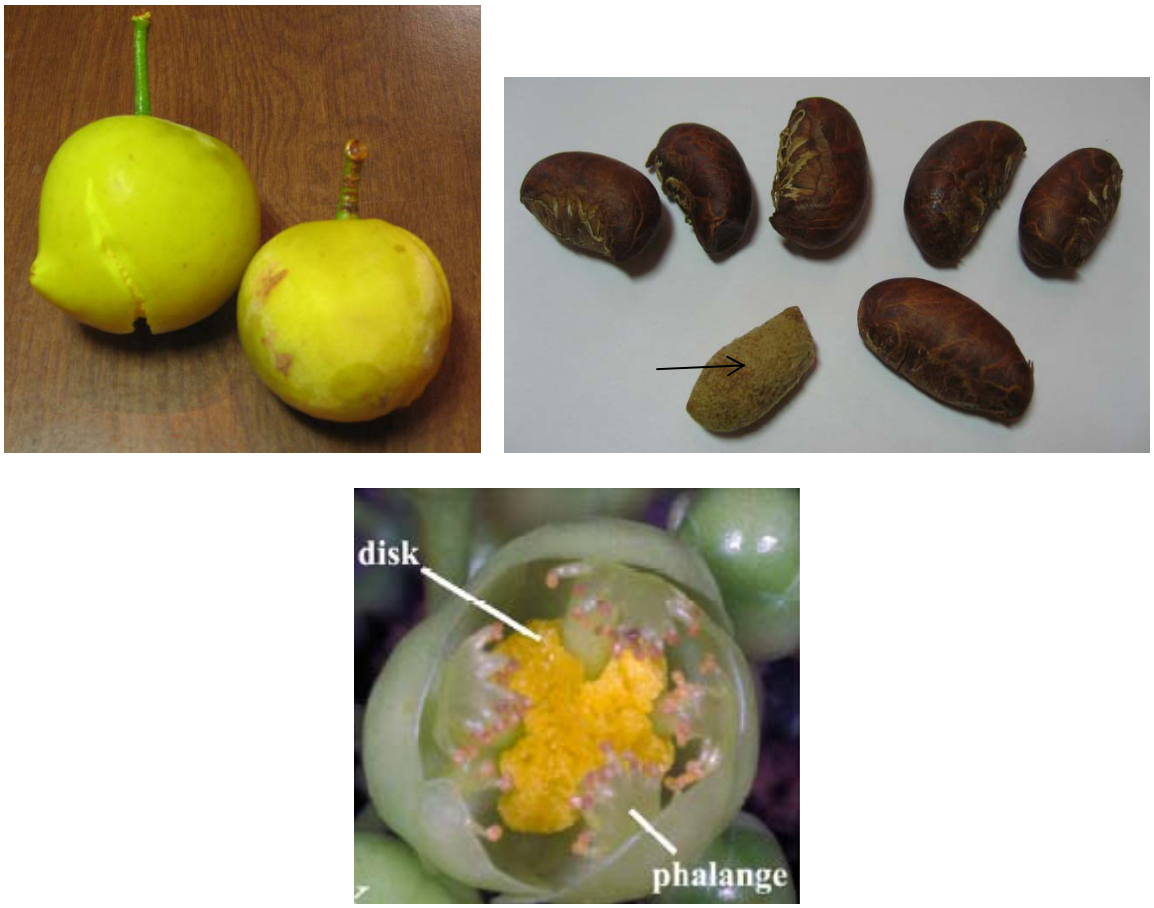


Figure 1-3. *G. xanthochymus* fruits (**top left**), seeds (**top right**), and staminate flower (**bottom**)[25]. The seed coat was removed to reveal the starch on the seed indicated with the arrow.

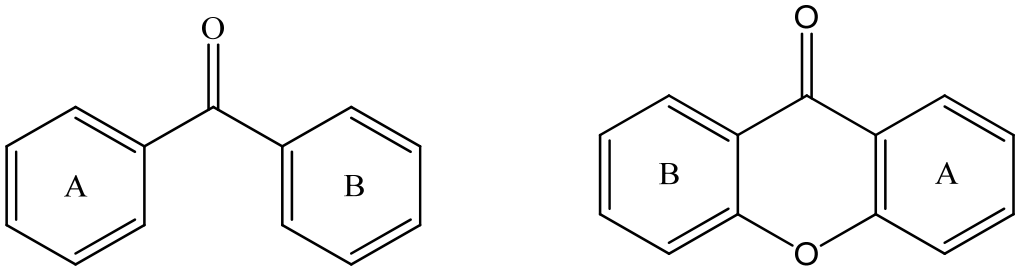


Figure 1-4. Basic skeleton of a benzophenone (**left**) and a xanthone (**right**).

Chapter 2

Phytochemical Analysis

Introduction

In order to identify the phytochemical constituents of *G. cambogia, G. livingstonei*, *G. spicata*, and *G. xanthochymus*, as well as their potential bioactivities, fruit and seed samples were extracted, partitioned, and investigated using classical chromatographic techniques, as well as modern analytical tools. The classical chromatographic techniques of solvent-solvent partitioning, open column chromatography, and TLC were used to separate the compounds found in these plants aiming to simplify the analysis and identify potential antiplasmodial leads. The composition of plant fractions prepared via classical chromatography was analyzed using RP HPLC-PDA and LC-MS.

Previous investigations of *G. xanthochymus* have focused on the fruits, leaves, and bark.[71-73] The benzophenones aristophenone A, cycloxanthochymol, gambogenone, guttiferone E, guttiferone H, isoxanthochymol, and xanthochymol, as well as the xanthone, alloathyriol, were identified from the fruit.[71] Additionally, several xanthones have been isolated previously for the stem bark or leaves: 1,5-dihydroxyxanthone, 1,7-dihydroxyanthone (euxanthone), 1,2-dihydroxy-5-methoxyxanthone, 1,5-dihydroxy-2-methoxyxanthone, 1,5-dihydroxy-3-methoxyxanthone, and 1,3,5-trihydroxyanthone.[74, 75] However, neither Baslas *et al.*[74] nor Venkataran *et al*.[75] investigated the bioactivity of any of the compounds isolated from the plant.

Both the *Garcinia* extracts and purified compounds identified from the extracts were later screened against *P. falciparum* to test for antiplasmodial activity using the BHIA and pLDH activity assays described in Chapter 3.

Results and Discussion

Solvent-Solvent Partitioning

A *G. xanthochymus* seed extract underwent three separate solvent-solvent partitioning schemes (Figure 2-1). In Scheme 1, 6.99 g of the *G. xanthochymus* seed extract was sequentially partitioned with: hexanes, EtOAc, and n-BuOH. The combined yield of the partitions was 5.19 g or 74% of the initial extract. In Scheme 2, 5.92 g of the seed extract was sequentially partitioned with: $CHCl_3$, EtOAc, and n-BuOH. The combined yield of the partitions was 3.95 g or 67% of the initial extract. And in Scheme 3, 8.77 g of the seed extract was sequentially partitioned with: hexanes, $CHCl_3$, and n-BuOH. The combined yield of the partitions was 7.40 g or 84% of the initial extract. The yields for the resulting, dried partitions are shown in Table 2-1. All partition schemes used a modified Kupchan scheme.[76] In order to produce a strong solvent line, the proportion of water was increased in the aqueous phase for each partition. Partition Scheme 1 had the highest yield of the three partition schemes **(**Table 2-1**)**. All nine of the resulting non-aqueous partitions were then evaluated to determine the best scheme for further research.

Table 2-1. Yields of dried partitions from the sequential solvent-solvent partitioning of *G. xanthochymus* seed extract.

Scheme 1 Partitions	**Yield (g)**	**Scheme 2 Partitions**	**Yield (g)**	**Scheme 3 Partitions**	**Yield (g)**
hexanes	2.56	$CHCl_3$	1.73	hexanes	3.00
EtOAc	1.78	EtOAc	1.14	$CHCl_3$	0.25
BuOH	0.83	BuOH	1.08	BuOH	4.15
H_2O	ND	H_2O	ND	H_2O	ND

Chromatograms for each of the nine *G. xanthochymus* partitions from the three solvent-solvent partitioning schemes discussed previously are shown in Figure 2-2.

Figures 2-2a, 2-2b, and 2-2c are the chromatograms for the hexanes, EtOAc, and n-BuOH partitions of Scheme 1, respectively. The chromatogram of the hexanes partition shows most of the compounds eluting off the column at around 25 min, at a high MeCN concentration, and these peaks were determined to be mostly benzophenones by examination of their UV-VIS spectra and later HPLC-spiking experiments. The EtOAc chromatogram has some extremely hydrophilic compounds eluting in the first 5 min of the run with 90% aqueous solvent (Figure 2-2b). The majority of the compounds in the EtOAc partition eluted off the column between 10 and 20 min. Based on the UV-VIS spectra, these are mid-polar biflavonoids and biflavonoid glycosides eluting at nearly 50:50 10 mM aqueous ammonium acetate:MeCN. Due to the presence of the sugar moiety, the biflavonoid glycosides elute slightly earlier, at a higher aqueous concentration, than the less polar biflavonoids. Lastly, the n-BuOH partition has the early eluting hydrophilic compounds that were seen in the EtOAc partition (Figure 2-2c). The presence of these similar hydrophilic compounds leads to the conclusion that they are carried over from the aqueous phase. The chromatogram from the n-BuOH partition also has some peaks eluting around 15 min, which were determined to be biflavonoid glycosides, based on their UV-VIS spectra. Since n-BuOH and EtOAc have such similar polarity indexes, it is not surprising to see similar compounds in these fractions. Additionally, the biflavonoid glycosides spicatiside and fukugiside are present in very high quantities in the seeds and may not have been completely extracted in EtOAc, thus their presence in the n-BuOH phase.

The chromatograms for the $CHCl_3$, EtOAc, and n-BuOH partitions from partition Scheme 2 are shown in Figures 2-2d, 2-2e, and 2-2f, respectively. The compounds in the

$CHCl_3$ partition elute off the column between 18 and 25 min (Figure 2-2d). This partition has more mid-polar compounds than the hexanes partition from Scheme 1 (Figure 2-2a). Many of these mid-polar compounds are also present in the EtOAc partition shown in Figure 2-2e. Also, the early eluting hydrophilic compounds that were found in the EtOAc partition of Scheme 1 are seen in the EtOAc partition of Scheme 2 as well. These hydrophilic compounds also show up in the n-BuOH partition (Figure 2-2f). Compounds from the n-BuOH partition elute at 14, 18, and 20 min. These same compounds are seen in the EtOAc partition and the mid-polar compounds eluting at 18 and 20 min can be found in the $CHCl_3$ partition. As these chromatograms show, partition Scheme 2 does not provide adequate separation of the compounds between the various partitions and therefore is not a suitable partition scheme to use for further research.

The chromatograms for the hexanes, $CHCl_3$, and n-BuOH partitions from partitioning Scheme 3 are shown in Figures 2-2g, 2-2h, and 2-2i respectively. Most of the compounds from the hexanes partition elute between 22 and 26 min in a poorly resolved series of overlapping peaks. The $CHCl_3$ partition chromatogram is very similar to that of the hexanes partition. Since these two solvents have very similar polarity indexes it is unsurprising that their chromatograms appear so similar. The better resolved peaks seen in the $CHCl_3$ partition are likely due to a lower concentration of these compounds, since most of them would have been previously extracted in the hexanes partition. The n-BuOH partition of Scheme 3 shows the same hydrophilic compounds eluting in the first 5 min, as seen in the n-BuOH partitions of Scheme 1 and 2 (Figures 2-2c and 2-2f, respectively). Peaks corresponding to the mid-polar compounds that elute between 12 and 20 min. are very similar in appearance to those seen in the EtOAc partitions of Scheme 1

and 2 (Figures 2-2b and 2-2e). Due to the similarity of the hexanes and $CHCl_3$ partitions and the broad range of compounds found in the n-BuOH partition, partitioning Scheme 3 was not used for further research.

Based on the HPLC-PDA analysis of the three solvent-solvent partitioning schemes tested with the *G. xanthochymus* seed extract. Partitioning Scheme 1 was determined to provide the best separation of compounds between the three partitions with the least amount of overlap from one partition to the other. Thus, the solvent-solvent partitioning Scheme 1 of hexanes, EtOAc, and n-BuOH was used for further phytochemical analysis.

Chromatography

Once a satisfactory solvent-solvent partitioning scheme was determined, the dried hexanes residue (30 g) was suspended in MeOH using sonication. The material was then chromatographed in a 105 x 2.5 cm open column over Sephadex LH-20 (200 g, dry weight) and eluted with MeOH (Figure 2-4). The resulting 18 fractions, A-R, were analyzed by RP TLC (Figure 2-3a). The plate was examined under UV light and then developed with a vanillin spray reagent. Based on the RP TLC analysis fractions A-C were combined into 2-45-3 since the only spot was seen at the baseline, D-E were combined into 2-45-4 since a faint secondary spot with a low R_f was seen under UV light. Fraction F was not combined since it showed a yellow-orange band that was not seen elsewhere and it became 2-45-5. Fractions G-H were combined into 2-45-6 due to the yellow band at $R_f = 0.3$. Fractions I-N were combined into 2-45-7 as they all contained a pink-red spot at $R_f = 0.3$, rather than the yellow band seen in 2-45-6. Fraction O became 2-45-8, as it was the only fraction with two distinct pink bands. The remaining fractions

P-R were pooled into 2-45-9 and showed no spots under UV or with vanillin. The individual fractions yielded 7 pooled fractions, 2-45-3 to 2-45-9, and dried *in vacuo* at 35-40°C. The pooled fractions were further analyzed by RP HPLC-PDA (Figure 2-5). The yields of each recombined fraction are shown in Table 2-2. The total yield of all the fractions was 27.4 g, which is 90.4% of the starting material.

The dried EtOAc residue (41 g) was redissolved in MeOH and also chromatographed over Sephadex LH-20 (200 g) eluted with MeOH. The resulting 66 fractions, A-NNN, were analyzed by RP TLC and developed with a vanillin spray reagent (Figure 2-3b). The RP TLC analysis allowed the fractions to be recombined into 11 fractions. Fractions A-J were combined into 2-30-1 based on the absence of any bands in the TLC analysis, fractions K-L were combined into 2-30-2 due to the presence of a band at $R_f = 0.7$ and an absence of a large band near the origin; since fractions M-O had both bands they were combined into 2-30-3. Fractions P-T were combined into 2-30-4 due to the presence of a yellow band with $R_f = 0.7$, rather than the gray band seen in 2-30-2. Fractions U-V were combined into 2-30-5 since the band at $R_f = 0.7$ was a yellow-red. Fractions W-Y were combined into 2-30-6 due to the presence of a yellow band near the solvent front and a broad red-orange band at $R_f = 0.56$. Fractions Z-GGG were very similar, but appeared to have an additional band under UV and were therefore combined to 2-30-7. Fractions HHH-MMM lacked the red-orange band at $R_f = 0.56$ and were combined into 2-30-8. Fractions NNN-OOO were very similar to 2-30-8, however the intensity and shade of the yellow band differed and thus they were combined into 2-30-9. Fractions PPP-WWW were combined into 2-30-10 due to the presence of a rust to brown spot at red-orange band at $R_f = 0.60$. The remaining fractions XXX-NNN were combined

into 2-30-11. The resulting 11 pooled fractions, 2-30-1 to 2-30-11, were dried *in vacuo* at 35-40°C and further analyzed by RP HPLC-PDA (Figure 2-6). The yields of the recombined fractions are shown in Table 2-3. The total yield for all of the fractions was 38.77 g or 94.01% of the starting material. Dried, combined fractions 2-30-1 to 2-30-11 and 2-45-3 to 2-45-9 were submitted to antiplasmodial testing to ascertain the potential antimalarial activity of the extract.

Table 2-2. Yields of recombined fractions from the hexanes partition of *G. xanthochymus* seeds.

Fraction	Yield (g)	Fraction	Yield (g)
2-45-3	0.0238	2-45-7	23.25
2-45-4	0.0783	2-45-8	0.4551
2-45-5	0.7169	2-45-9	0.1053
2-45-6	2.8015		

Table 2-3. Yields of recombined fractions from the EtOAc partition of *G. xanthochymus* seeds.

Fraction	Yield (g)	Fraction	Yield (g)
2-30-1	0.1576	2-30-7	18.5883
2-30-2	0.2013	2-30-8	0.5996
2-30-3	1.3761	2-30-9	0.4105
2-30-4	7.1166	2-30-10	1.6594
2-30-5	2.1872	2-30-11	0.2238
2-30-6	6.2521		

Purified standards were used to verify the identity of isolated compounds via HPLC spiking experiments. Figure 2-7 shows the identification of fukugiside using a spiking experiment from a fraction of the EtOAc partition *G. xanthochymus* seeds. Figures 2-7a, 2-7b, and 2-7c show the chromatograms of fukugiside, the *G. xanthochymus* fraction 2-15-8, and the fraction spiked with fukugiside, respectively. Fukugiside's peak area in each chromatogram is shown in Table 2-4. The increase in

peak area of fukugiside spiked when the sample is spiked with the standard is readily apparent. Additionally, the identity of the fukugiside was confirmed with LC-MS, Figure 2-7 shows the positive ESI mass spectrum with *m/z* 719.0 for fukugiside and a second *m/z* of 1436.7. Fukugiside has a molecular mass of 718.6 corresponding to the *m/z* 719.0 peak. The dimer of fulugiside has a mass of 1437.2, twice the mass of fukugiside with a loss of one hydrogen.

Table 2-4. Peak area for fukugiside spiking experiment.

Sample	Retention Time (min)	Peak Area
fukugiside standard	15.23	9,820,570
fraction 2-15-8	15.22	19,648,062
2-15-8 + fukugiside	15.17	28,528,671

Several compounds were previously isolated from a methanolic extract of *G. xanthochymus* fruit pulp. The extract was partitioned with $CHCl_3$ and EtOAc. The $CHCl_3$ partition underwent open column chromatography with Sephadex LH-20 and then open column chromatography with RP C_{18} before being subjected to preparative RP HPLC to purify alloathyriol. Additional, Sephadex LH-20 and RP open column chromatography of the $CHCl_3$ partition yielded aristophenone A, cycloxanthochymol, gambogenone A, guttiferone E, guttiferone H, isoxanthochymol, and xanthochymol. In order to separate the double bond isomer mixture of cycloxanthochymol and isoxanthochymol, and the mixture of guttiferone E and xanthochymol, argentation TLC was necessary. The biflavonoids and their glycosides amentoflavone, fukugetin, fukugiside, and volkensiflavone were also isolated from the EtOAc partition. The EtOAc partition

repeatedly underwent open column chromatography with Sephadex LH-20 and preparative RP HPLC to purify these compounds.[71]

Guttiferone A was isolated from a MeOH extract of the fruit pulp of *G. livingstonei*.[77] The extract was partitioned with H_2O, EtOAc, and n-BuOH. The EtOAc partition was repeatedly chromatographed over Sephadex LH-20 via open column and preparative RP HPLC was used to purify guttiferone A.

The benzophenone 32-hydroxy-*ent*-guttiferone M was isolated from the methanolic seed extract of *G. edulis*.[78] The extract was partitioned with $CHCl_3$, EtOAc, and n-BuOH. The EtOAc was chromatographed over a RP C_{18} open column and then subjected to preparative RP HPLC.

Mangiferin was isolated from the leaves of *Phaleria nisidai*.[79] The leaves were exhaustively extracted with MeOH under reflux. The extract was dried *in vacuo* and then dissolved in H_2O and $CHCl_3$; mangiferin precipitated out of the solution. The precipitate was filtered then washed with $CHCl_3$ and MeOH.

The purity of purchased standards was verified via RP HPLC-PDA. Chromatograms for the xanthone and xanthone glycoside standards used for this research, including 3-isomangostin, α-mangistin, β-mangostin, 4-methoxy-9H-xanthone, 1,5,6-trihydroxyxanthone, and xanthene-9-one are shown in Figure 2-8. Chromatograms used to evaluate the purity of the benzophenones and biflavonoids are shown in Figure 2-9 and 2-10 respectively. The same HPLC-PDA method and gradient system was used for determining the purity of these standards as for the *G. xanthochymus* fractions.

Mass Spectral Analysis

Mass Spectra were obtained by LC-MS using a Waters LCT Premiere XE Time of Flight (ToF) mass spectrometer (Waters MS Technologies, Manchester, UK). Ionization was achieved using a multi-mode EScI source in electrospray (ESI) mode at the following conditions: +ESI capillary 3000 V, cone: 20 V, aperture 1: 0 V, ion guide 1: 0 V, multichannel plate (MCP): 2600 V. Nitrogen was used for both cone and desolvation gases, with a cone gas flow of 20 L/h, and desolvation gas flow of 600 L/h at 400°C. The source temperature was 120°C. Leucine-enkephaline was used as a reference mass and infused by a secondary reference probe. The reference mass was scanned once every five scans for each positive data collection. Positive ESI data was collected using a scan time of 0.5 s, with an interscan time of 0.02 s. MS data was collected and analyzed in centroid mode using the program MassLynx V4.1 Scn 727.

Materials and Methods

Plant Material

Dried, powdered *G. cambogia* fruit extract was obtained from Nature Sunshine Products (Spanish Fork, UT). The *G. cambogia* fruits were extracted in H_2O, and a 50% hydroxycitric acid (HCA) solution was also used in the extraction process. *G. livingstonei* fruits were collected at The Kampong of the National Tropical Botanical Garden (Coconut Grove, FL) and shipped in dry ice to New York City via overnight carrier. *G. spicata* fruits (Lot # 1320) were collected from the Brevard County Rare Fruit and Vegetable Council (FL) and shipped to New York City via overnight carrier. *G.*

xanthochymus fruits were collected at The Fruit and Spice Park (Homestead, FL) and shipped to New York City in dry ice via overnight carrier.

All fresh plant material was weighed, catalogued, and then stored at -20 °C until analyzed. Voucher specimens of *G. livingstonei*, *G. spicata*, and *G. xanthochymus* were previously deposited at the William and Lynda Steere Herbarium of The New York Botanical Garden (Bronx, NY).

Chemicals and Supplies

n-BuOH**,** H_2SO_4, and HPLC grade MeCN, were purchased from Mallinckrodt Baker (Philipsburg, NJ). EtOAc, formic acid, hexanes, HPLC grade MeCN, and MeOH were purchased from EMD Chemicals Inc. (Gibbstown, NJ). Ammonium acetate and vanillin were purchased from Sigma-Aldrich (St. Louis, MO) and EtOH was from Pharmco Products Inc. (Brookfield, CT). Several standards were purchased; 8-desoxygartanin, α-mangostin, β-mangostin, and 3-isomangostin from Chromadex (Irvine, CA), 4-methoxyxanthone from Sigma-Aldrich (St. Louis, MO), and 1,5,6-trihydroxanthone from Quality Phytochemicals LLC (NJ).

Column chromatography was accomplished with Sephadex LH-20 (25-200 μm) purchased from Sigma-Aldrich (St. Louis, MO). Separations were monitored using thin-layer chromatography (TLC) silica gel 60 reversed-phase C_{18} F_{254} plates obtained from EMD Chemicals Inc. (Gibbstown, NJ). HPLC samples were prepared with 15 mm Phenomenex 0.45 μm regenerated cellulose syringe filters (Torrance, CA).

Extraction and Partitioning of Plant Material

In order to partition the *G. cambogia* extract, various solvents were tested to redissolve it, including: H_2O, MeOH:H_2O, EtOH:H_2O, hexanes, varying concentrations of formic acid, and MeOH or EtOH mixtures with aqueous formic acid. The dissolutions with these solvents was either incomplete, with significant particulate matter remaining in solution, or resulted in the production of heat and gas. The powdered extract contained gum acacia as an excipient and was spiked with HCA to at least 50%. Most likely the addition of these compounds prevented the extract from completely redissolving without the acidification of the solvent and resulting hydrolysis. Due to the difficulties in redissolving the *G. cambogia* extract, this fruit was not investigated further.

G. livingstonei and *G. spicata* fruits were washed to remove debris and then deseeded. The fruit pulp and skin were homogenized with MeOH and then extracted for one hour at room temperature. The resulting crude MeOH extracts were filtered and concentrated *in vacuo* at 35-40°C.

Initial extraction of *G. xanthochymus* fruits was performed on deseeded, lyophilized fruit pulp and skin. The fruit was homogenized in a blender with 8:2 MeOH:H_2O and extracted overnight twice at room temperature. The resulting crude MeOH extracts were combined, filtered, and concentrated *in vacuo* at 35-40 °C. A large scale extraction of *G. xanthochymus* seeds previously separated from the fruit pulp was performed by Naturex (South Hackensack, NJ). The seeds were pulverized then homogenized with 80% aqueous EtOH. This crude EtOH extract was then dried to 66.36% solids *in vacuo* at temperatures not exceeding 40 °C.

A sample of the resulting semi-dry seed extract was redissolved into 8:2 MeOH:H_2O, filtered, and then partitioned using three separate solvent-solvent partitioning schemes (Figure 2-1). The resuspended residue was sequentially partitioned with hexanes, EtOAc, and BuOH, or $CHCl_3$, EtOAc, and BuOH, or hexanes, $CHCl_3$, and BuOH. The resulting partitions were dried *in vacuo* at 35-40 °C and evaluated via HPLC-PDA (Figure 2-2). Based on the HPLC-PDA analysis, a modified Kupchan scheme using hexanes, EtOAc, and BuOH was chosen to partition the remaining crude EtOH seed extract.[76] The extract was partitioned as described above, and the resulting partitions were dried *in vacuo* at 35-40°C to yield three dried residues for further study. The dried hexanes, EtOAc, and BuOH residues were submitted to antiplasmodial testing.

Column and TLC Chromatography

Open column chromatography was done over the size-exclusion matrix, Sephadex LH-20 and eluted with MeOH. The resulting fractions were dried *in vacuo* at 35-40°C. TLC analysis was performed using RP TLC plates with either 1:3 10 mM ammonium acetate:MeCN or 1:1 10 mM ammonium acetate:MeCN as the mobile phase. The plates were examined under UV light and then developed with a vanillin spray reagent composed of 1% vanillin in 10% ethanolic sulfuric acid.

HPLC Methods

HPLC analyses of samples was performed on a Waters 2695 Separations Module equipped with a Waters 996 or 2996 photodiode array detector and monitored using Waters Empower 2 software (build 2154) (Milford, MA). The analysis was performed

with either a Phenomenex (Torrance, CA), Nucleosil RP C_{18} column (250 x 4.6 mm, 5 μM) or a Phenomenex Synergi Hydro RP C_{18} (250 x 4.6 mm, 4 μM) column. The HPLC samples were prepared in HPLC grade MeOH and passed through a syringe filter prior to HPLC analysis.

Several HPLC solvent systems were evaluated to optimize the chromatographic separation. First, isocratic solvent systems were used: H_2O:MeOH, H_2O:MeCN, MeOH or MeCN mixtures with aqueous formic acid, and MeCN mixtures with aqueous 10 mM ammonium acetate. A gradient solvent system was tested. The gradient is based on a previously published method using 10 mM ammonium acetate (solvent A) and MeCN (solvent B).[80] The gradient profile was as follows: 0-10 min, 65-55% A; 10-20 min, 55% A; 20-30 min, 55-45% A; 30-35 min, 45% A; 35-40 min, 45-35% A; 60-70 min 35-65% A; concluding with a 10 min column wash and equilibration at 65% A. While this method provided adequate separation of the peaks, a simplified version was evaluated. The gradient profile of the simplified method used 10 mM ammonium acetate (solvent A) and MeCN (solvent B) and was as follows: 0-4, 90% A, 4-34 min, 90-0% A; 34-44 min, 0% A; concluding with 10 min to reestablish initial conditions and equilibrate the column to 90% A. For samples with few polar compounds, an abbreviated method was developed. This method used 10 mM ammonium acetate (solvent A) and MeCN (solvent B) and was as follows: 0-30 min, 90-0% A; 30-36 min, 0-90% A; the last 6 min reestablished initial conditions and equilibrated the column.

The slow return to initial conditions was only necessary when using the Synergi Hydro column in order to prevent the HPLC system from going over pressure. Additional HPLC experiments indicated that additional run times were necessary when using the

Synergi Hydro column, which has a 4 μm pore size; this resulted in an increased resolution.

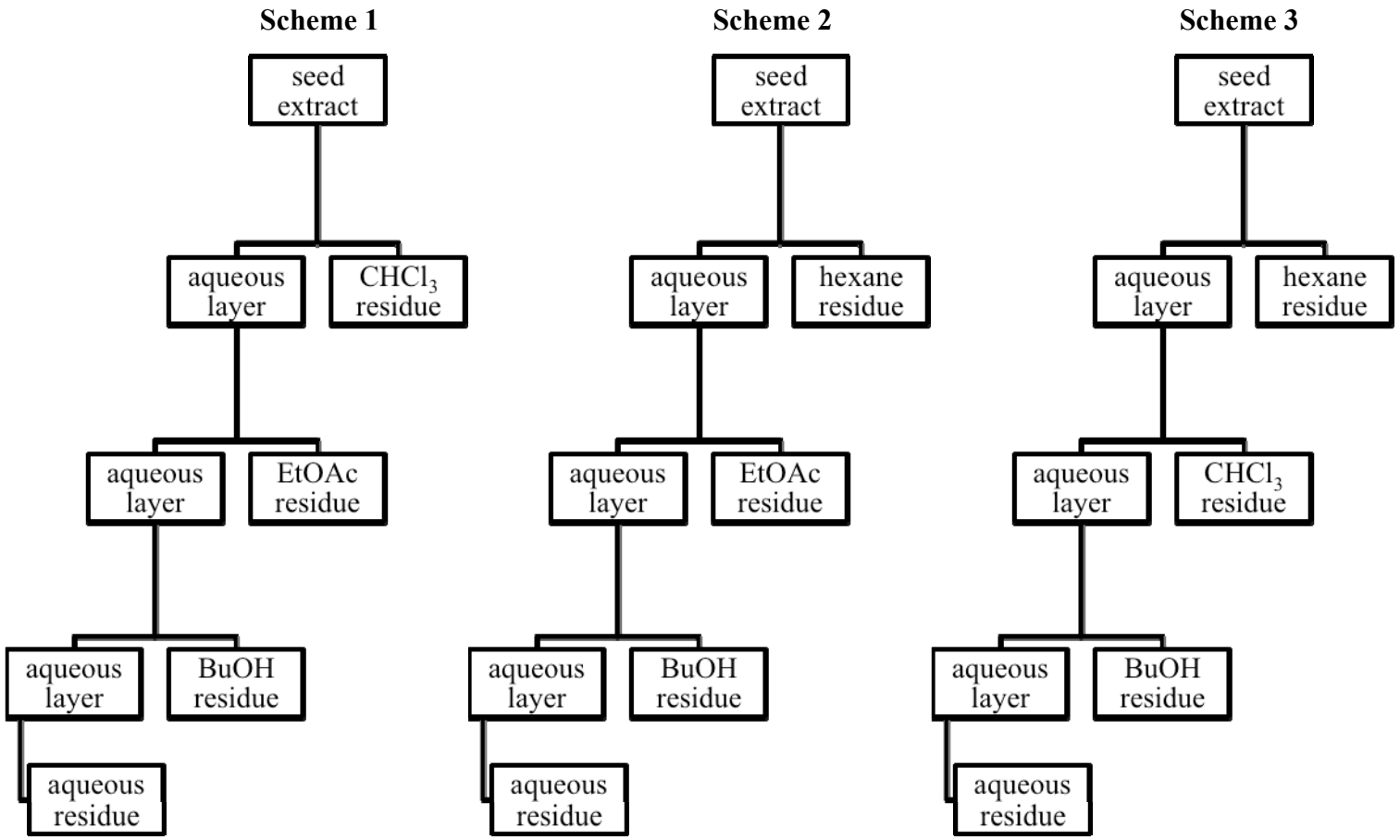


Figure 2-1. Solvent-solvent partition schemes used with *G. xanthochymus* seed extract.

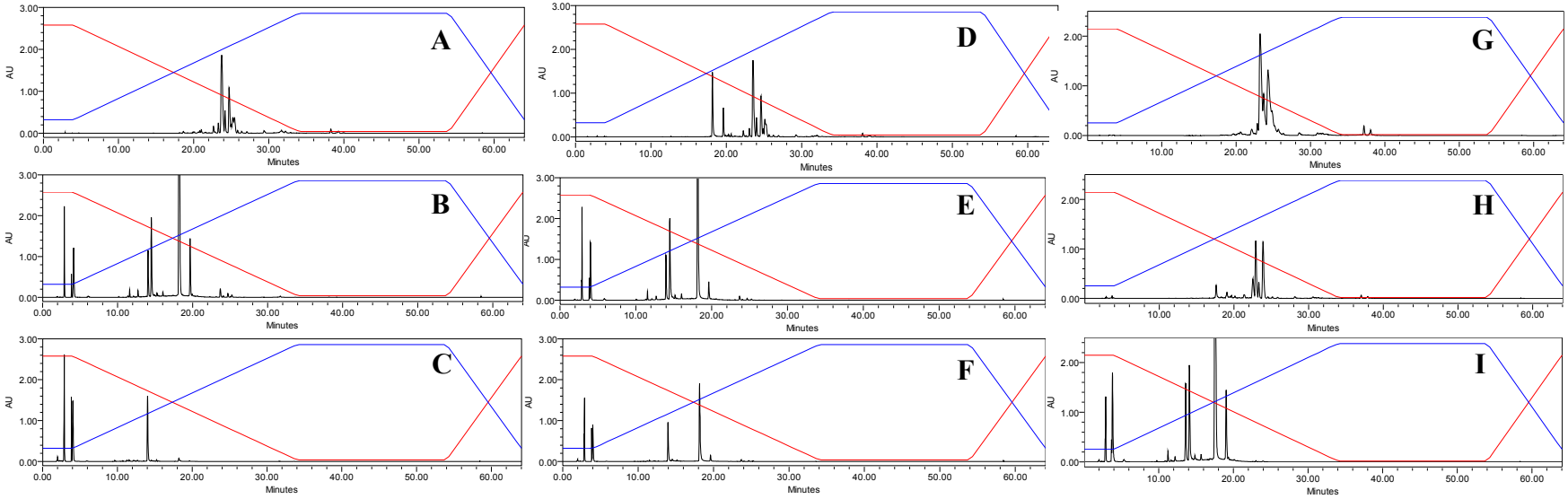


Figure 2-2. HPLC-PDA chromatograms for solvent-solvent partitioning Scheme 1: hexanes (**a**), EtOAc (**b**), and n-BuOH (**c**); Scheme 2: $CHCl_3$ (**d**), EtOAc (**e**), and n-BuOH (**f**); Scheme 3: hexanes (**g**), $CHCl_3$ (**h**), and n-BuOH (**i**). All chromatograms are shown at 320 nm, from a Synergy column with a solvent system of 10mM ammonium acetate (A) and MeCN (B). Initial conditions were set at 90A:10B.

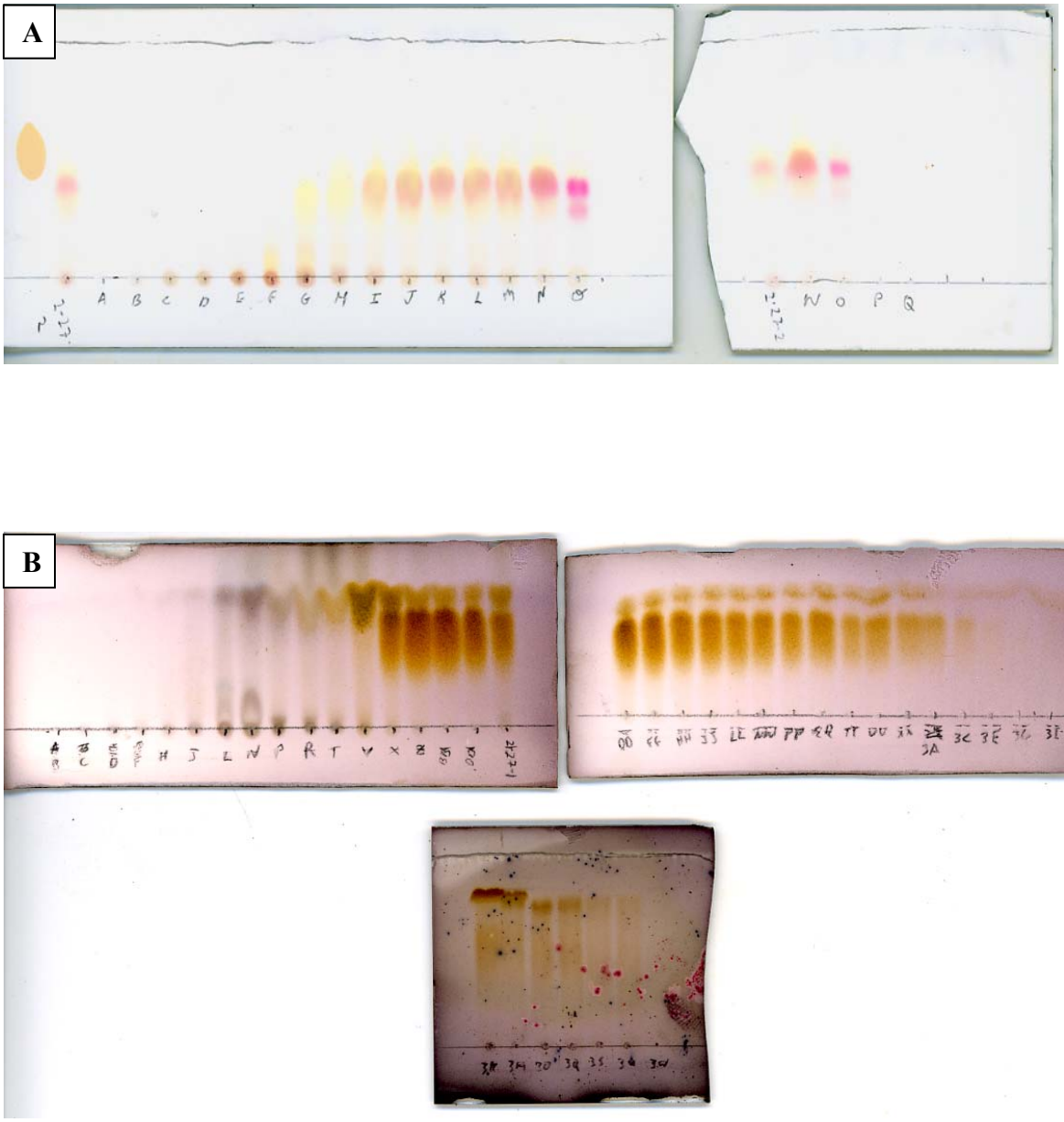


Figure 2-3. RP TLC plates of the fractions from the hexanes (A) and EtOAc (B) partitions of *G. xanthochymus* seeds. The lane labeled 2-27-1 is the hexanes partition and the lane labeled 2-27-2 is the EtOAc partition. All the plates have been developed with a vanillin spray reagent.

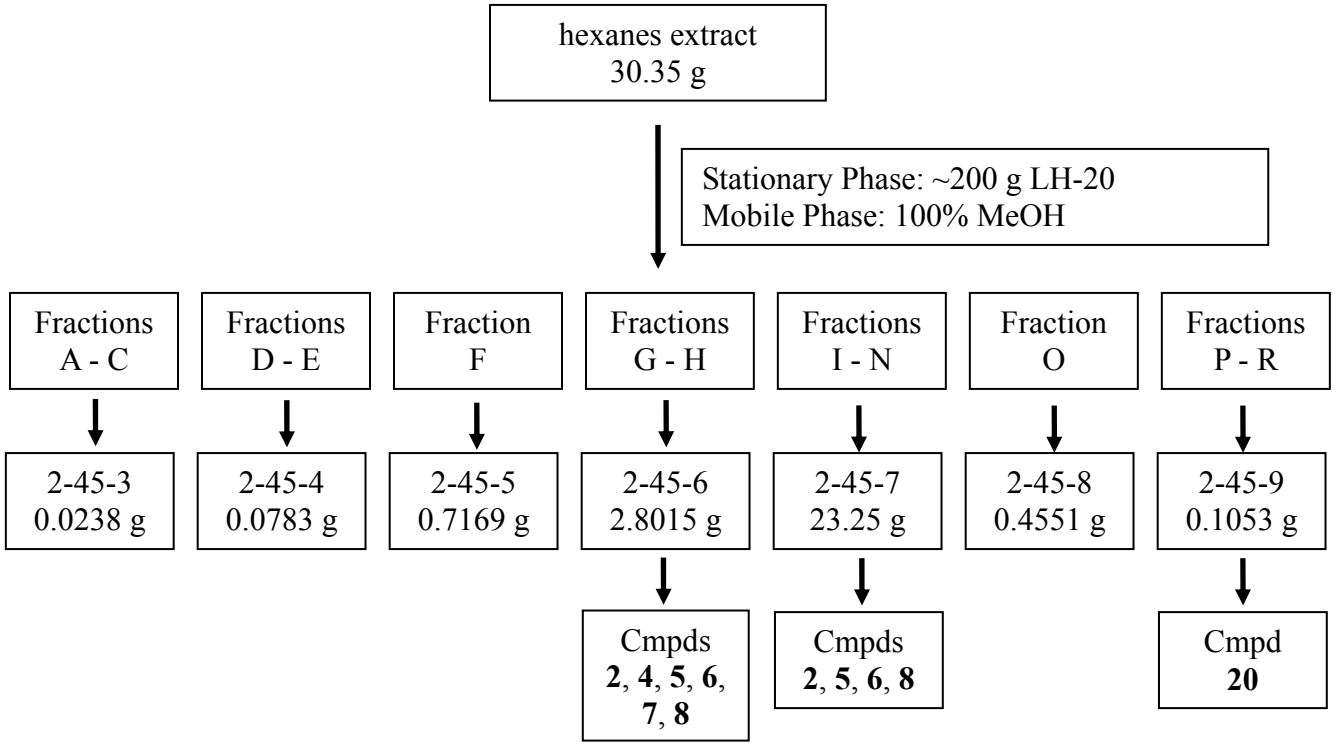


Figure 2-4. Fractions, yields, and resulting compounds from the hexanes partition of the *G. xanthochymus* seeds.

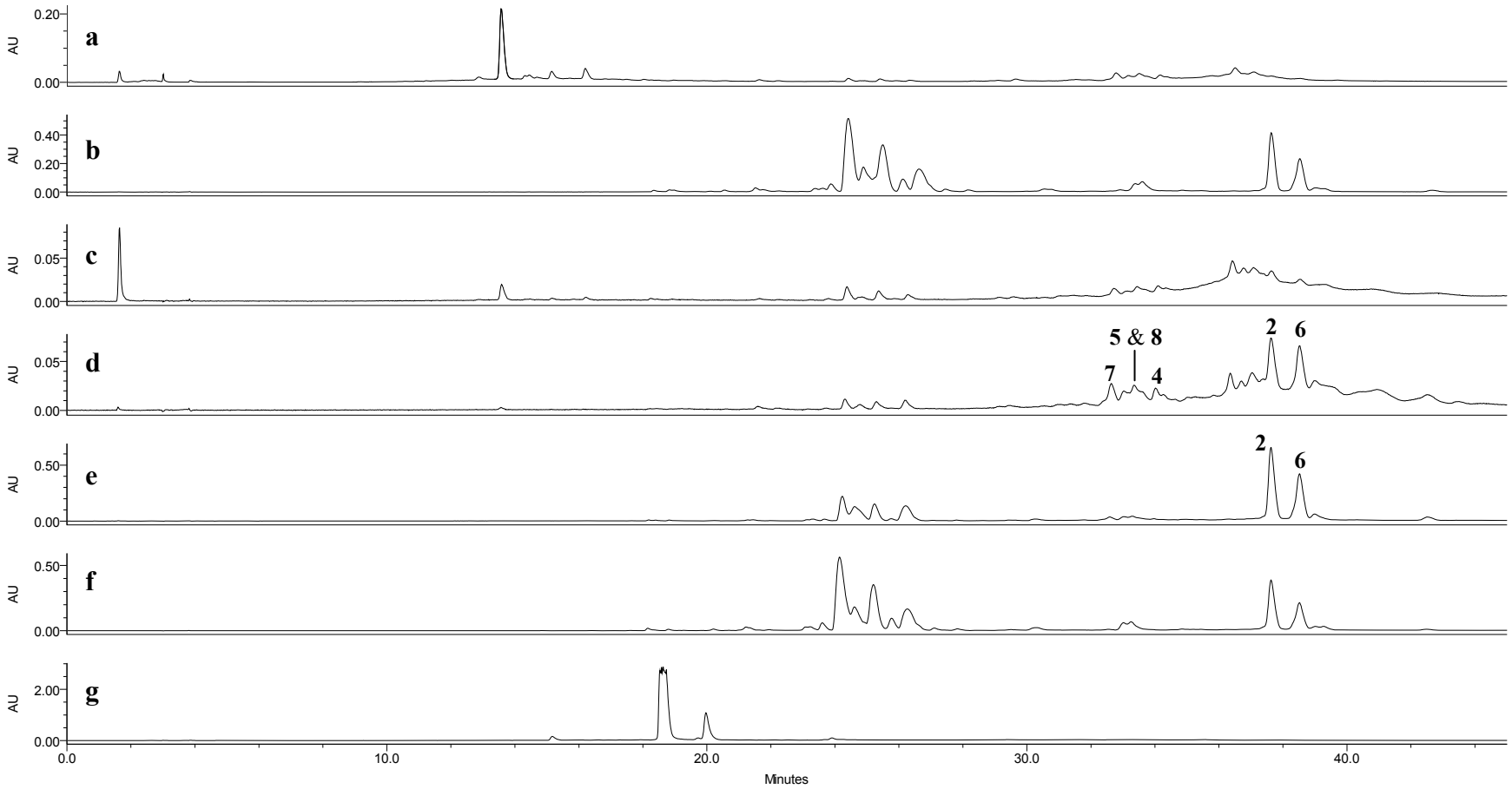


Figure 2-5. RP HPLC-PDA chromatograms of pooled fractions 2-45-3 (**a**), 2-45-4 (**b**), 2-45-5 (**c**), 2-45-6 (**d**), 2-45-7 (**e**), 2-45-8 (**f**), and 2-45-9 (**g**). All chromatograms extracted at 254 nm.

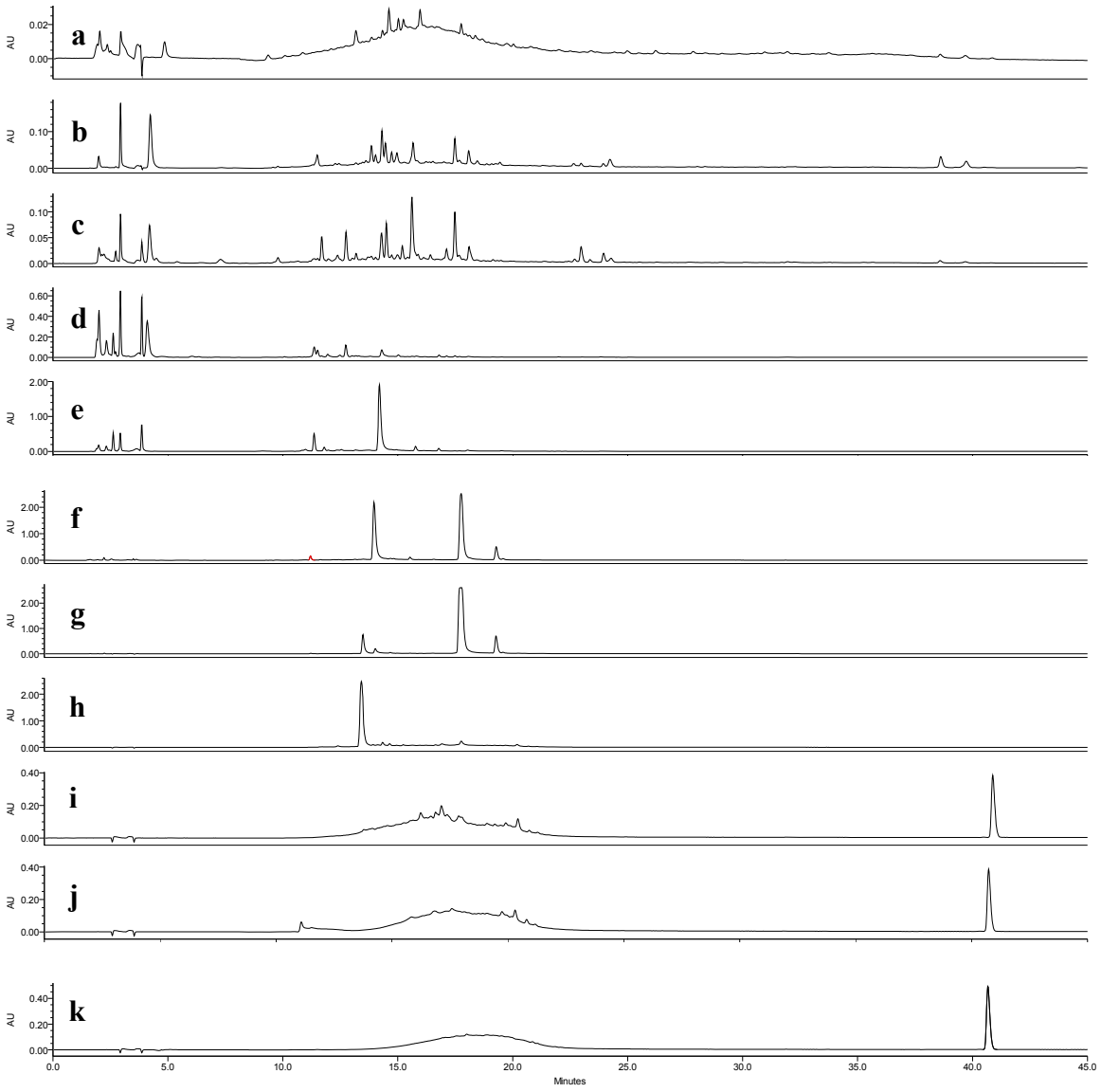


Figure 2-6. RP HPLC-PDA chromatograms of pooled fractions 2-30-1 (**a**), 2-30-2 (**b**), 2-30-3 (**c**), 2-30-4 (**d**), 2-30-5 (**e**), 2-30-6 (**f**), 2-30-7 (**g**), 2-30-8 (**h**), 2-30-9 (**i**), 2-30-10 (**j**), and 2-30-11 (**k**). All chromatograms extracted at 254 nm.

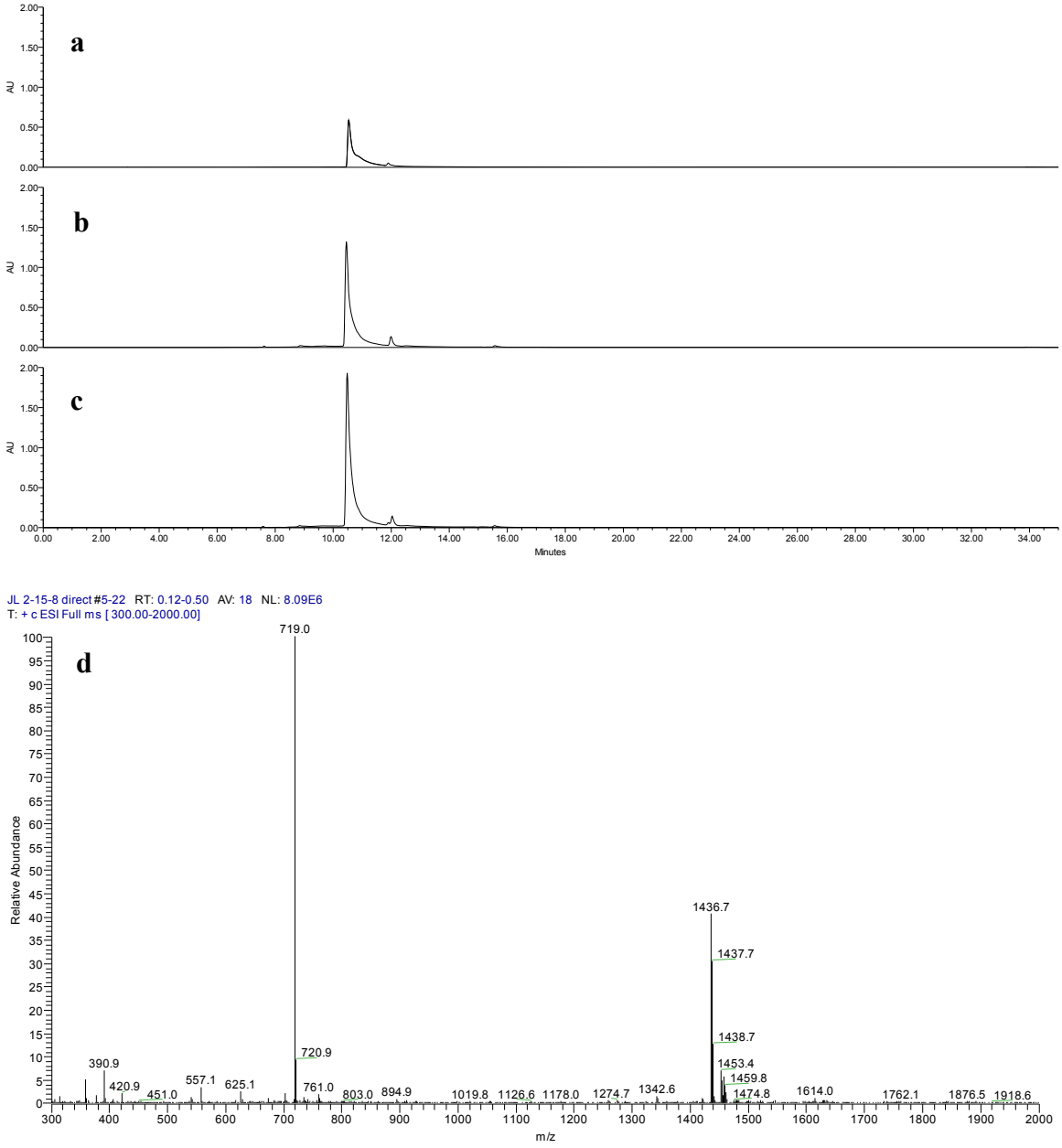


Figure 2-7. Chromatograms, extracted at 254 nm, of fukugiside (**a**), fraction 2-15-8 (**b**), and fraction 2-15-8 spiked with fukugiside (**c**), having retention times of 15.230, 15.218, and 15.170 min. respectively. The positive MS ESI of fraction 2-15-8 (**d**) showing *m/z* 719.0 confirming the molecular mass of fukugiside.

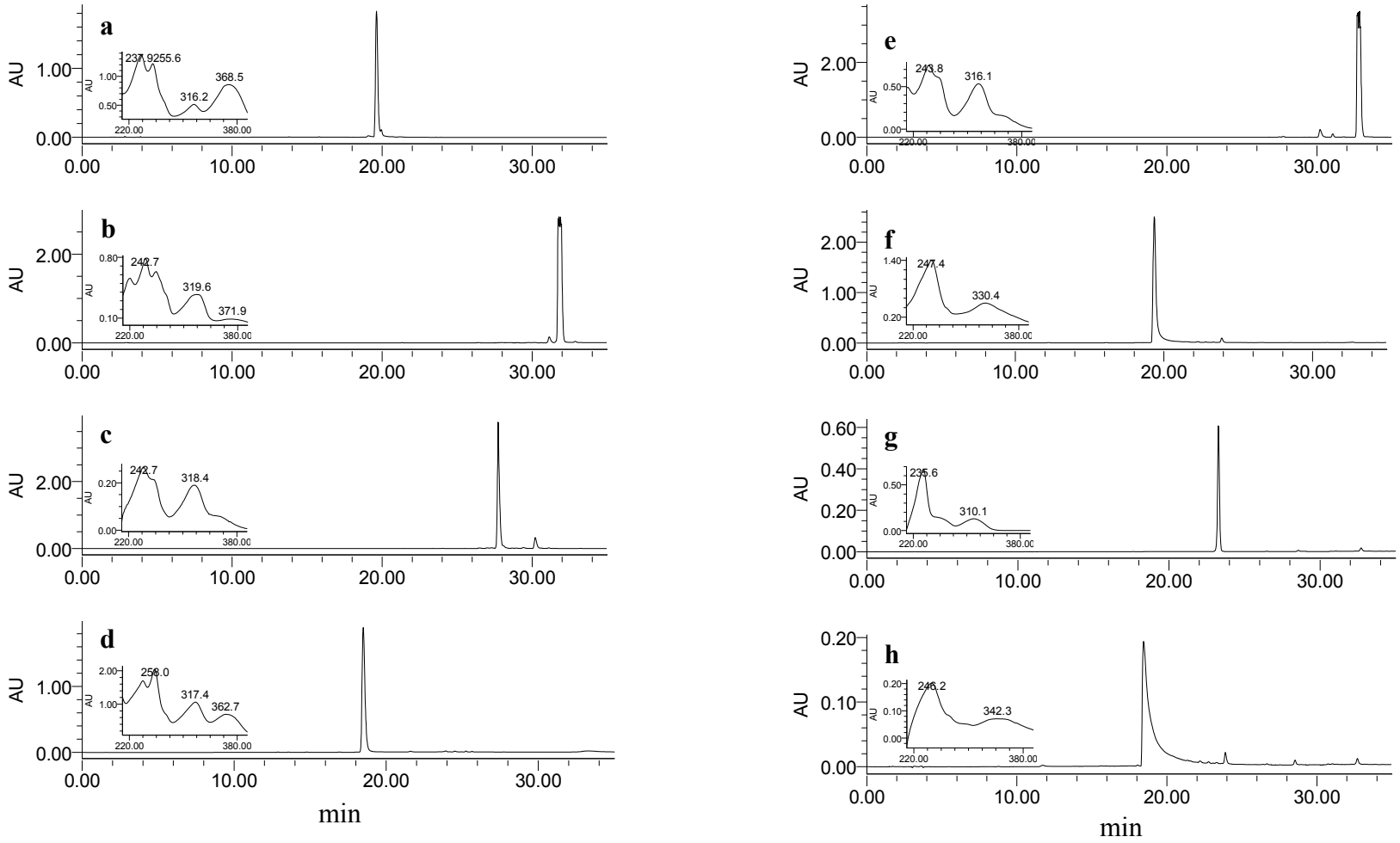


Figure 2-8. RP HPLC-PDA chromatograms and UV spectra of xanthones and xanthone glycosides used in this study: (**a**) alloathyriol, (**b**) 8-desoxygartanin (**c**) 3-isomangostin, (**d**) mangiferin, (**e**) α-mangostin , (**f**) β-mangostin, (**g**) 4-methoxy-9H-xanthone, and (**h**) 1,5,6-trihydroxyxanthone. All chromatograms extracted at 254 nm.

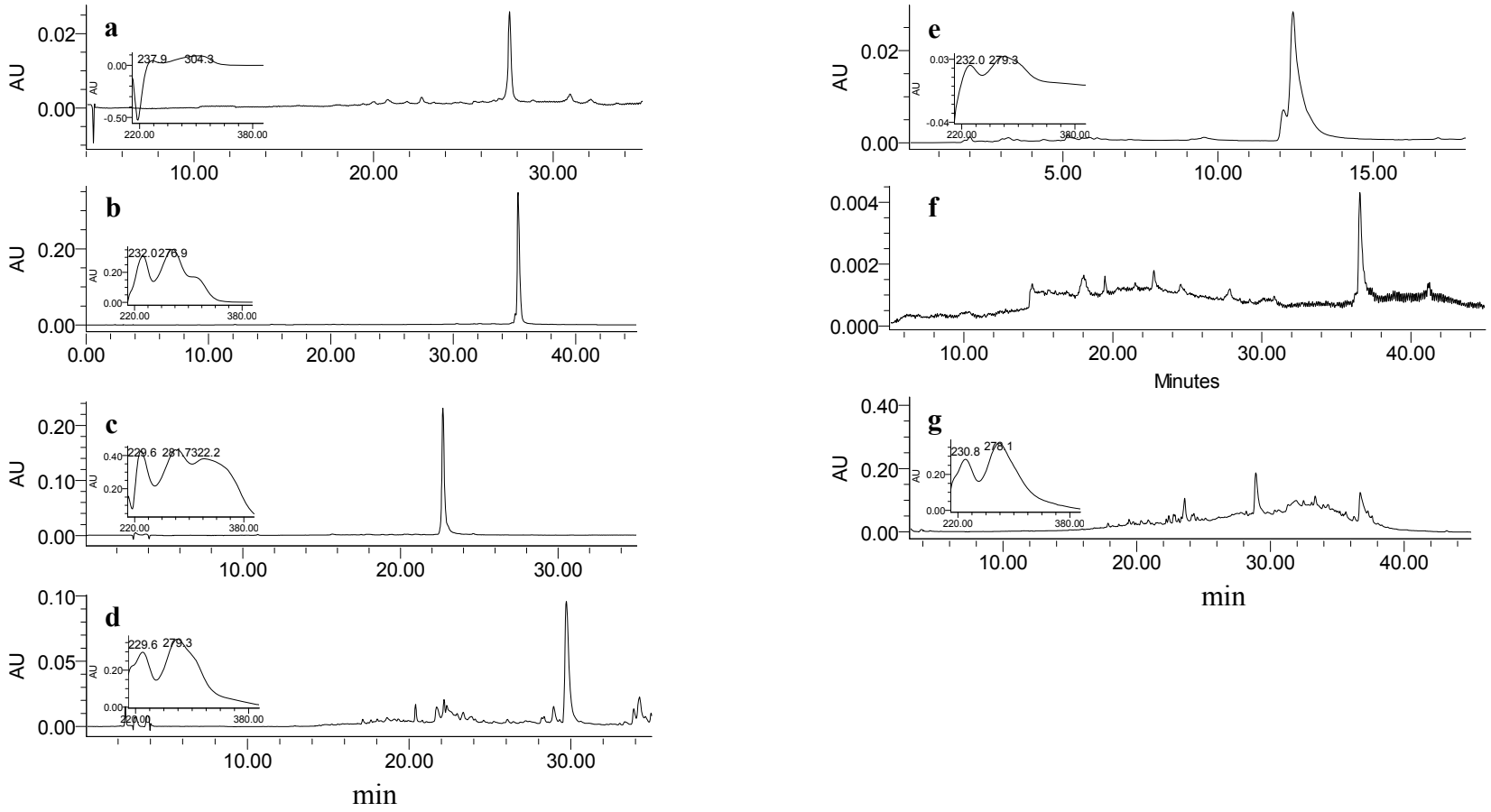


Figure 2-9. RP HPLC-PDA chromatograms and UV spectra of benzophenones used in this study, (**a**) aristophenone A, (**b**) cycloxanthochymol (**c**) gambogenone, (**d**) guttiferone A, (**e**) guttiferone E, (**f**) guttiferone H, and (**g**) isoxanthochymol. All chromatograms extracted at 254 nm.

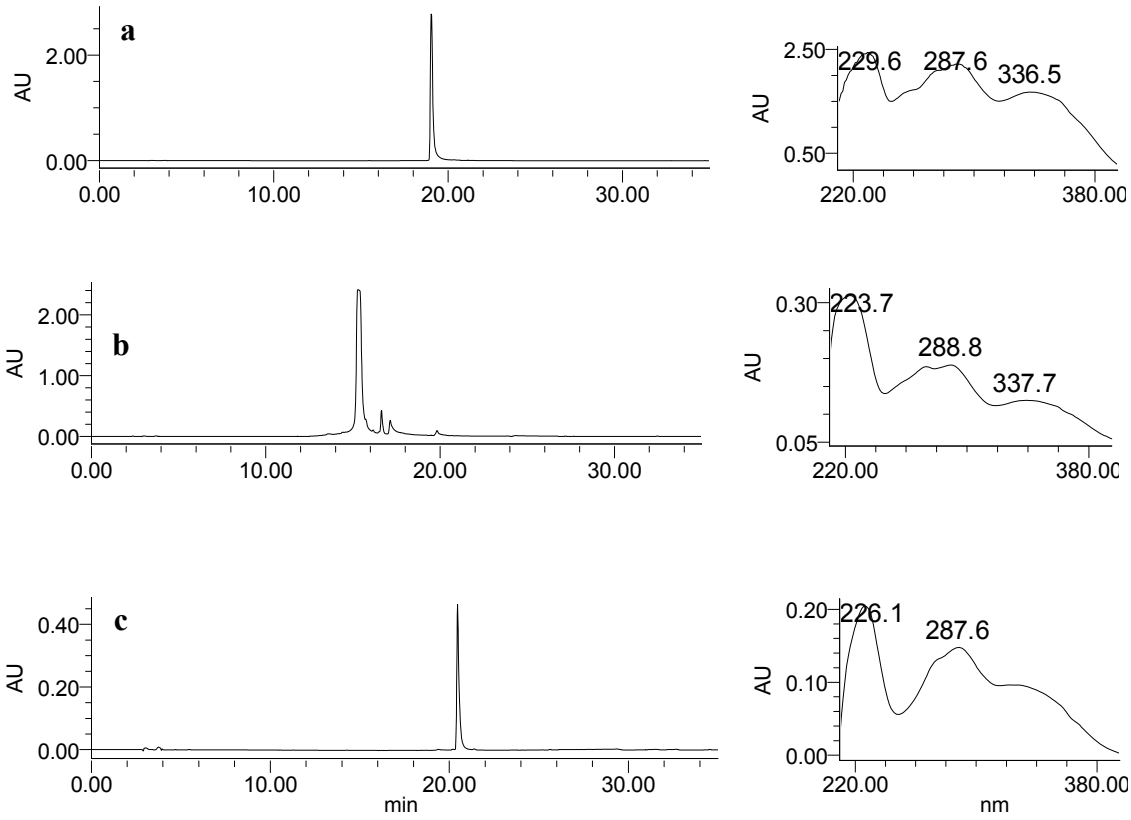


Figure 2-10. RP HPLC-PDA chromatograms and UV spectra of biflavonoids used in this study: (**a**) fukugetin, (**b**) fukugiside, and (**c**) volkensiflavone. All chromatograms extracted at 254 nm.

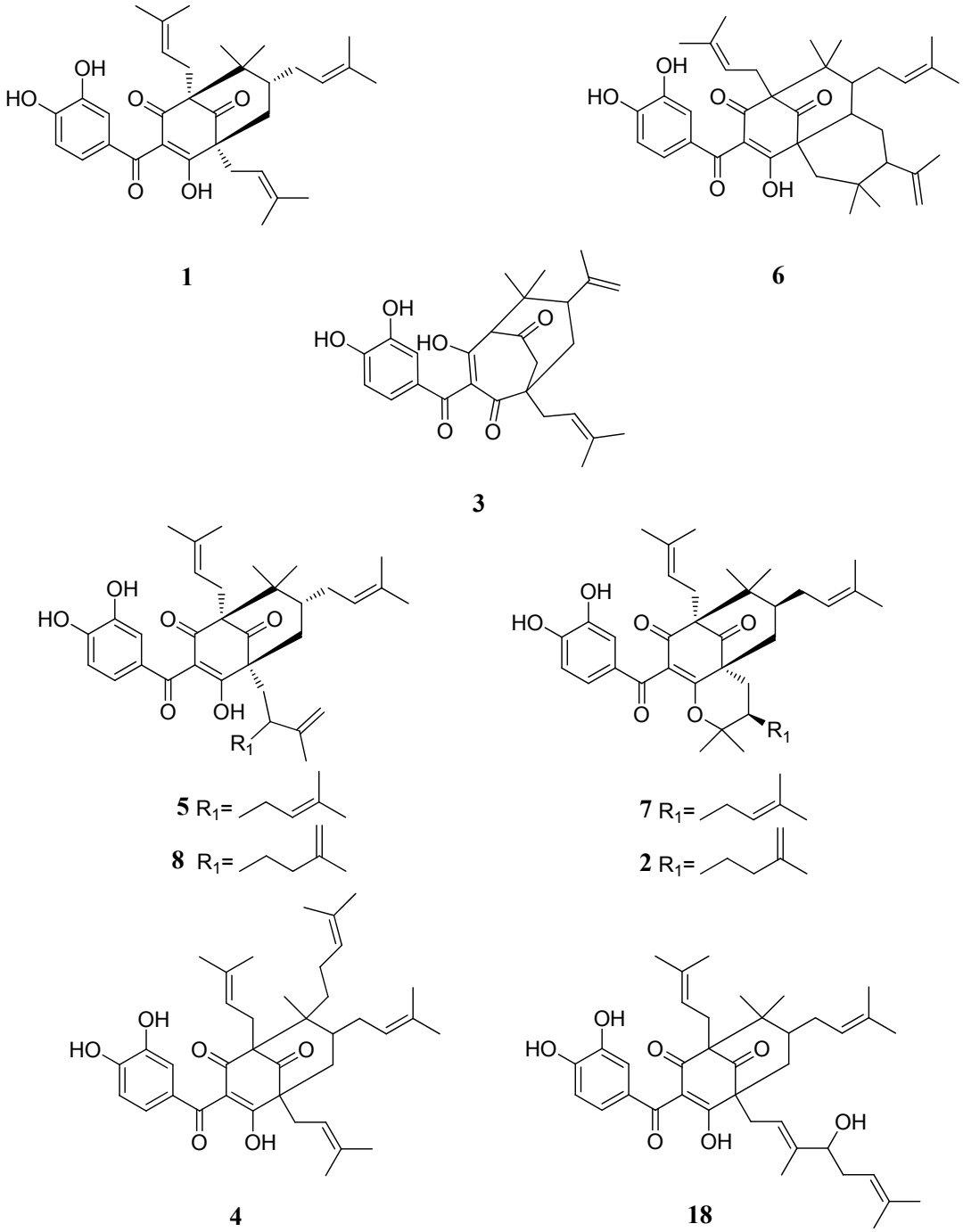
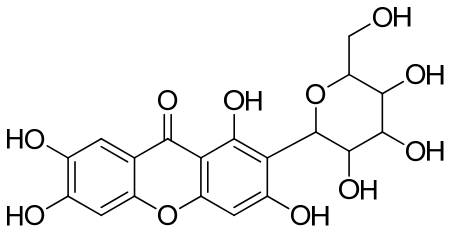


Figure 2-11. Structures of benzophenones used in this study: aristophenone A (**1**), cycloxanthochymol (**2**), gambogenone (**3**), guttiferone A (**4**), guttiferone E (**5**), guttiferone H (**6**), isoxanthochymol (**7**), xantochymol (**8**) and 32-hydroxy-ent-guttiferone M (**18**).



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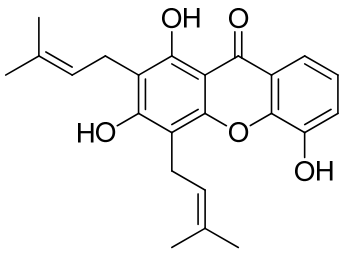

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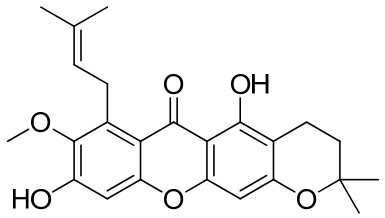

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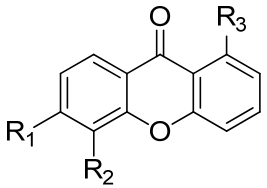
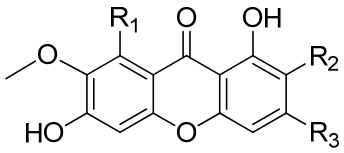


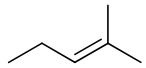
9 $R_1=R_2=R_3=H$

16 $R_1=R_3=H$, $R_2=OCH_3$

17 $R_1=R_2=R_3=OH$



11 $R_1=R_2=H$, $R_3=OH$

12 $R_1=R_2=$  $R_3=OH$

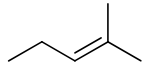
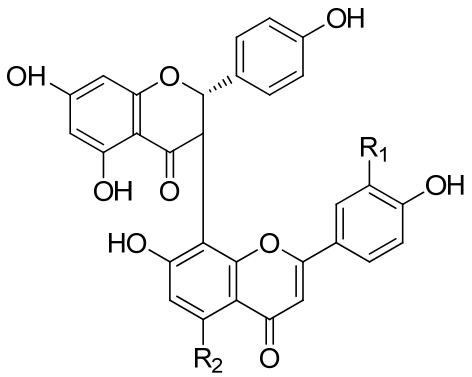
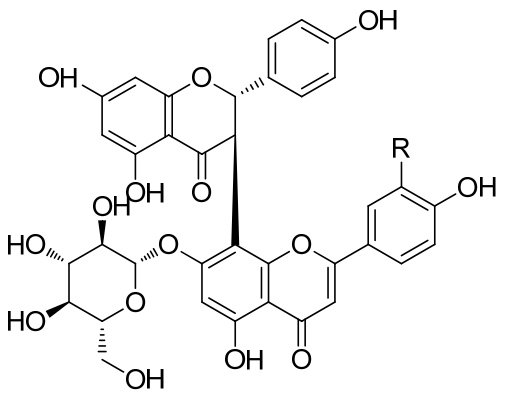
13 $R_1=R_2=$  $R_3=OCH_3$

Figure 2-12. Structures of xanthones used in this study: xanthone (**9**), mangiferin (**10**), alloathyriol (**11**), α-mangostin (**12**), β-mangostin (**13**), 3-isomangostin (**14**), 8-desoxygartanin (**15**), 4-methoxyxanthone (**16**), and 1,5,6-trihydroxyxanthone (**17**).



20 $R_1=R_2=OH$

21 $R_1=R_2=H$



22 R=OH

23 R=H

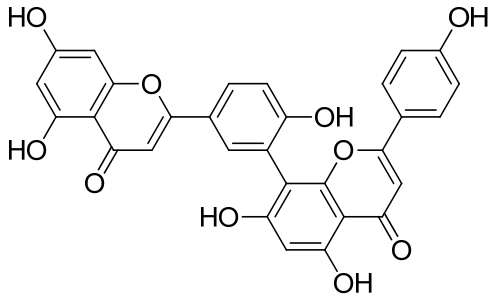


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Figure 2-13. Structures of biflavonoids and biflavonoid glycosides used in this study: amentoflavone (**19**), fukugetin (**20**), volkensiflavone (**21**), fukugiside (**22**), and spicataside (**23**).

Chapter 3

Antiplasmodial Studies

Introduction

The antiplasmodial activities of *G. xanthochymus* extracts and compounds identified from them, as well as related benzophenones and xanthones, were evaluated. The β-haematin inhibitory activity assay was utilized as an antiplasmodial screen for use in this study, but early on was found to be incompatible with the sample matrices. Rather, all samples were evaluated in the plasmodial lactase dehydrogenase (pLDH) activity assay to screen for antiplasmodial activity. The compounds tested were: aristophenone A (**1**), cycloxanthochymol (**2**), gambogenone (**3**), guttiferone A (**4**), guttiferone E (**5**), guttiferone H (**6**), isoxanthochymol (**7**), xantochymol (**8**), xanthone (**9**), mangiferin (**10**), alloathyriol (**11**), α-mangostin (**12**), β-mangostin (**13**), 3-isomangostin (**14**), 8-desoxygartanin (**15**), 4-methoxyxanthone (**16**), 1,5,6-trihydroxyxanthone (**17**), and 32-hydroxy-ent-guttiferone M (**18**).

Xanthone Mechanism of Action

Several studies established xanthones as effective antimalarials and proposed mechanisms of action.[21, 81] The "xanthone hypothesis" was proposed by Winter *et al.* as a mechanism for the antimalarial properties of this class of compounds.[82, 83] The work by Winter and collaborators surmises that a xanthone precursor is transported into the erythrocyte where a prooxidant creates a hydroxyl radical-rich environment suitable for the production of xanthones, which subsequently act as the antimalarial compound. This hydroxyl radical-rich environment is created from the prooxidant interacting with iron and oxygen found in the digestive vacuole that *Plasmodium* creates within the erythrocyte.

Other work has shown that xanthones can function as antimalarial drugs by using a mechanism similar to that exerted by chloroquine and other related quinoline drugs (Figure 3-1).[84] Once inside an erythrocyte, the *Plasmodium* parasite cannot produce enough amino acids to support itself and multiply. It relies on the proteolysis of hemoglobin for the release of amino acids, which are used both to produce more merozoites and as a food source. The hydrolysis of hemoglobin releases the heme moiety that oxidizes to hematin, often referred to as the "malaria pigment." Hematin is toxic to the parasite and damages cellular metabolism: it inhibits certain enzymes, causes peroxidation of the membranes, and produces free radicals in the acidic digestive vacuole. *Plasmodium* converts the toxic hematin to the inert crystal hemozin. However, several antimalarial drugs prevent this mechanism, thus destroying the parasite. Chloroquine and other quinolines stabilize the hematin μ-oxo-dimer thus preventing the crystallization of toxic hematin to hemozin. The death of the parasite results from damage incurred by the free hematin.[84, 85] The heme mechanism for antimalarial drugs has gathered support, due to research implicating this mechanism of action for both quinoline drugs[86] and artemisinin derivative drugs.[87] Spectroscopic,[68] structure-activity studies,[81] receptor binding studies,[88] and electronic profiles[84] have indicated that xanthones indeed bind heme, preventing its aggregation into hematin both *in vitro* and *in vivo*.

Protein Farnesyltransferases

Many pathogenic parasites require intracellular prenylated proteins for survival. Post-translational prenylation reactions are necessary to attach the farnesyl (C_{15}) or geranylgeranyl (C_{20}) groups to the C-terminal cysteine residue on several GTPase

signaling proteins. The reaction is catalyzed by at least three different cytoplasmic protein transferases. The resulting farnesylated and geranylgeranylated protein ends promote membrane association, allowing the proteins to function as signaling molecules involved in DNA replication and cell cycling.[89, 90]

Surveys of the *Plasmodium falciparum* genome reveal the absence of protein geranylgeranyltransferase-I (PGGT-1). Thus many vital signaling proteins that are geranylgeranylated by PGGT-1 in other organisms must be post-translationally modified by protein farnesyl transferase (PFT). Inhibiting both PGGT-1 and PFT is extremely toxic to mammalian cells; but the exclusive inhibition of PFT proves toxic to *P. falciparum* and not to mammalian cells.[91] PFT activity is present in all stages of the *Plasmodium* parasite (i.e. ring, trophozoite, and schizont) and it is therefore an excellent target.[90, 92]

For a more complete understanding of the importance of PFT as an antiplasmodial target, a mammalian model is necessary (Figure 3-2). Ras proteins are small GTPases, that are activated when loaded with GTP. The activated Ras can then bind numerous downstream effector proteins involved in cell growth, proliferation, and differentiation. The most prolific Ras proteins are H-ras, N-ras, and K-ras. Although all the Ras isoforms interact with a common set of activators and effectors, each provides a distinct signaling output. The sequence of all three isoforms is highly homologous, except for the 25 amino acyl residues of the C-terminus that constitute the hypervariable region (HVR).

The initial modification and transport to the plasma membrane is common to all Ras isoforms. Ras proteins are synthesized as cytosolic precursors with a C-terminal CAAX motif that will direct further modifications. The CAAX motif is a tetrapeptide

consisting of a cysteine residue (C), two aliphatic regions (AA), and a variable amino acid residue (X). First, a cytosolic PFT attaches a farnesyl group to the cysteine residue of CAAX. Then the farnesylated CAAX directs the Ras to the cytosolic surface of the ER. Once associated with the ER the endopeptidase Rce1 removes the AAX tripeptide. Lastly, the α-carboxyl group on the carboxy-terminal farnesyl-cysteine is methylated by isoprenylcysteine carboxyl methyltransferase (Icmt).

A second targeting signal, located on the amino-terminal region with respect to the farnesylated-cystine, directs the isoform's transport to the plasma membrane. The H-ras and N-ras take a route to the cell membrane different than the K-ras isoform. The H-ras and N-ras undergo palmitoylation of the HVR, that is, post-translational addition of palmitic acid as a thioester to the thiol group of the cysteine residues. This modification directs the two isoforms to the exocytic pathway via the Golgi apparatus to the plasma membrane. The K-ras lacks the cysteine residues in the HVR, instead displaying a polylysine sequence. It therefore does not undergo palmitoylation and bypasses the Golgi. K-ras is trafficked to the plasma membrane by an undetermined pathway.[92]

Weisner *et al.*[93] showed a series of 13 synthesized benzophenone derivatives with PFT inhibitory activity that were effective against multi-resistant *P. falciparum*. These 13 benzophenone derivatives varied from no effect to 100% inhibition. The most effective compound had an $IC_{50} = 2.7$ μM. Docking studies showed that benzophenone-based PFT inhibitors adopt similar binding conformations and competitively inhibit the farnesyltransferase binding pocket (Figure 3-3).[94] Other benzophenone PFT inhibitors have shown activity against *Plasmodium spp.* both *in vitro* and *in vivo*.[89]

Results and Discussion

The β-hematin inhibitory activity (BHIA) assay was used for initial screens of plant extracts and column fractions. Several problems with the BHIA assay were discovered and it proved to be unsuitable for large-scale antiplasmodial activity screening. First, unreacted hemin was difficult to reliably remove from the 96-well plates. After the initial centrifugation of the plate, the supernatant is removed and the remaining pellet resuspended in DMSO. The unreacted hemin should dissolve into the DMSO, thus allowing its removal with the supernatant after additional centrifugation. Despite various methods for washing the pellet and several centrifugation speeds and durations, the hemin was not quantitatively removed from the pellet. It seemed that the unreacted hemin would stick to the precipitated hematin during the DMSO wash, decreasing its solubility. This introduced a random error to the results. Formation of the pellet also proved unreliable, a problem that was more pronounced when crude plant extracts and partitions were tested rather than column fractions. Despite varying methods of centrifugation and incubation, a film would float on the top of the well. The film appeared to be flocculated hematin that did not go into the pellet. This hematin would be lost with the supernatant or stick to the autopipette tips, again introducing a random error. Lastly, the results provided by the BHIA assay were not consistent with antiplasmodial tests of pLDH activity performed in erythrocytes. For the reasons above, the BHIA assay was considered unsuitable and, instead, the pLDH assay was used to determine antiplasmodial activity in this study.

Methanolic extracts of *G. livingstonei*, *G. mangostana*, *G. spicata*, and *G. xanthochymus* were tested *in vitro* for antiplasmodial activity against the *Plasmodium*

falciparum clone D6, which is chloroquinine-sensitive (Table 3-1**)**. The *G. mangostana* extract inhibited the growth of the *P. falciparum* clone D6 by 89%, while the *G. xanthochymus* extract inhibited it by 24%, as measured by the pLDH activity assay. Neither of the extracts showed any cytotoxicity toward Vero cells (monkey kidney fibroblasts). The hexanes, EtOAc, and n-BuOH partitions of an ethanolic GX seed extract were also screened the *P. falciparum* clones D6 (Table 3-2). The hexanes partition inhibited the *P. falciparum* clone D6 by 58%, the EtOAc and n-BuOH partitions showed no inhibitory activity.

Table 3-1. Antiplasmodial activity and cytotoxicity of *Garcina* species extracts.

Plant Extract	**D6 Clone**	**Cytotoxicity**[a]
	% Inhibition[b]	**% Inhibition**[b]
G. livingstonei	0	NC[c]
G. mangostana	89	NC
G. spicata	0	NC
G. xanthochymus	24	NC

[a] Vero Cells
[b] Samples showing % Inhibition < 50 are considered inactive at 15.9 μg/μΛ
[c] No cytotoxicity

Table 3-2. Antiplasmodial activity of *G. xanthochymus* seed partitions.

***G. xanthochymus* partition**	**D6 Clone**
	% Inhibition[a]
hexanes	58
EtOAc	0
n-BuOH	0

[a] Samples showing % Inhibition < 50 are considered inactive at 15.9 μg/mL

Compounds **1**-**8**, and **18** (Figure 2-11) were tested *in vitro* for antiplasmodial activity against the *Plasmodium falciparum* clones D6 and W2, which are chloroquinine-sensitive and chloroquinine-resistant, respectively (Table 3-3**)**. Benzophenones **5**-**7** showed activity against both clones. The effect of the C-8 side chain on antiplasmodial activity can be discussed for compounds with the 3-(3,4-dihydroxybenzoyl)-4-hydroxy-8,8-dimethyl-1,7-bis(3-methyl-2-buten-1-yl)bicyclo[3.3.1]non-3-ene-2,9-dione benzophenone base skeleton (Figure 3-4) which is numbered according to Baggett *et al*[71] rather than the IUPAC system to simplify discussion. Compound **5** with isopentenyl and dimethylallyl chains present at C-8 is active against both the D6 and W2 clones. Compound **8** with two isopentenyl chains on C-8, i.e. two terminal methylene groups, is not active against either clone. Of note, a 60:40 mixture of compounds **8** and **5** was inactive against both clones.

Further support for the role of a terminal methylene in antiplasmodial activity can be seen with the cyclized isoprenyl chain from C-1 to C-8 of **2** and **7**. The same trend of antiplasmodial activity is observed as **5** and **8**. Compound **2** with an isopentenyl chain on C-30 and a terminal methylene is inactive. Compound **7** with a dimethylallyl chain on C-30 which lacks the terminal methylene is active. Compound **6** has a cyclohexane ring from C-7 to C-8 and a terminal methylene on C-35 on the base skeleton and has activity against both the D6 and W2 clones. This finding indicates that the position of the terminal methylene on the base skeleton, in addition to its presence determines antiplasmodial activity.

The functional group present at C-5 does not affect the antiplasmodial activity. Neither the presence of two methyl groups (cf. **2**) nor the addition of an isopentenyl chain,

as in **4** and **18**, are active against either D6 or W2 clones. Thus, the C-5 site is not likely to be involved in the antiplasmodial activity of benzophenones.

The presence or absence of a hydroxyl group on C-14 does not influence the antiplasmodial activity of the basic skeleton. Compounds **5**, **6**, and **7** have a hydroxyl group at C-14 and show varying antiplasmodial activities. However, compounds **1**, **2**, **4**, **8**, and **18** also have a C-14 hydroxyl and are inactive against *P. falciparum* D6 and W2 clones. Compounds **4** and **18** are structurally similar, with **18** differing only by the absence of a hydroxyl on C-14. Neither **4**, with the hydroxyl group, nor **18**, without the hydroxyl group are active antiplasmodials.

Table 3-3. Antiplasmodial activity and cytotoxicity of benzophenones.

Compound	D6 Clone		W2 Clone		Cytotoxicity[a]
	IC_{50} (ng/mL) Mean ± SD	SI[b]	IC_{50} (ng/mL) Mean ± SD	SI[b]	IC_{50} (ng/mL)
1	NA[c]	–	NA	–	NC[d]
2	NA	–	NA	–	NC
3	NA	–	NA	–	NC
4	NA	–	NA	–	NC
5	4760 ± 0	>1	4500 ± 325	>1.1	NC
6	3200 ± 212	>1.5	3200 ± 0	>1.5	NC
7	4200 ± 891	>1.1	4760 ± 0	>1.0	NC
8	NA	–	NA	–	NC
18	NA	–	NA	–	NC

[a] Vero cells
[b] Selectivity Index (SI) = IC_{50} Vero cells/IC_{50} *P. falciparum*
[c] No antimalarial activity
[d] No cytotoxicity

Xanthones **9** to **17** (Figures 2-12 and 3-4**)** were also tested for antiplasmodial activity against *P. falciparum* clones D6 and W2 (Table 3-4). Compounds **12** and **14** showed activity against both clones. The structural similarity of compound **11** with **14** indicates that the substitution patterns on both the A and B ring influence the antiplasmodial activity of the compound. The lack of an isoprenyl chain at C-8 on **11** when compared of **12** and **14**, both having an isoprenyl chain at C-8 and being active, suggests the importance of this isoprenyl group. While both **12** and **14** showed activities

against the D6 and W2 clones, **14** with the cyclization between C-2 and C-3 had a lower IC_{50} against both clones. The current xanthone hypothesis emphasizes the requirement of hydroxyl groups at C-4 and C-5 for antiplasmodial activity.[95] The spectroscopic study of the binding between 4,5-dihydroxy xanthone and heme has shown the importance of the C-4 and C-5 hydroxyl groups for stabilizing the hematin μ-oxo-dimer.[68] In Figure 3-5, the red bar and circle show the π–π interactions of the xanthone carbonyl-heme iron interaction, the blue bar and circle denote the xanthone C-4 and C-5 hydroxy-heme carboxylate interactions, and the yellow bar and circle denote the heme carboxylate-heme iron interaction. However, the results presented here indicate that other positions, mainly C-8, also affect the activity of xanthones against *P. falciparum*.

Table 3-4. Antiplasmodial activity and cytotoxicity of xanthones.

Compound	D6 Clone		W2 Clone		Cytotoxicity[a]
	IC_{50} (ng/mL) Mean ± SD	SI[b]	IC_{50} (ng/mL) Mean ± SD	SI[b]	IC_{50} (ng/mL)
9	NA[c]	–	NA	–	NC[d]
10	NA	–	NA	–	NC
11	NA	–	NA	–	NC
12	4670 ± 0	–	4180 ± 820	>1.1	NC
13	3150 ± 212	>1.5	2000 ± 283	>2.5	NC
14	3233 ± 773	>1.0	2525 ± 991	>1.6	NC
15	NA	–	NA	–	NC
16	NA	–	NA	–	NC
17	NA	–	NA	–	NC

[a] Vero cells
[b] Selectivity Index (SI) = IC_{50} Vero cells/IC_{50} *P. falciparum*
[c] No antimalarial activity
[d] No cytotoxicity

Materials and Methods

Chemicals and Supplies

Hematin (haematin or ferriprotoporphyrin IX hydroxide), hemin (or haemin) chloride, and chloroquine diphosphate were purchased from Sigma-Aldrich (St. Louis, MO). Acetic acid was purchased from J. T. Baker (Phillipsburg, NJ) and dimethyl sulfoxide from EMD Chemicals (Gibbstown, NJ). Sodium hydroxide was purchased from Fisher Scientific (Pittsburg, PA). The H_2O used for the bioactivity assays was purified using a Milli-Q filtration system (Billerica, MA). The 96-well microtiter plates were

purchased from VWR International (Radnor, PA) and spectrophotometric analysis was performed on a Molecular Devices Versamax microplate reader (SunnyVale, CA).

Assay for *in vitro* antimalarial activity using β-haematin inhibitory activity assay

The BHIA assay was based on the method of Parapini *et al*.[96] For experimental wells, 50 μL of each test compound or plant extract dissolved in DMSO, in doses ranging from 1 to 20 molar equivalents to hemin, was added to duplicate wells. Chloroquinine diphosphate (CQ) was used as a reference compound by serially diluting an aqueous 160 mM CQ solution with H_2O into duplicate wells yielding concentrations from 2 to 40 mM CQ. The positive control and standard curve series was prepared using an 8 mM hematin solution serially diluted with 0.1 M NaOH to yield five sets of duplicate wells with concentrations ranging from 0 to 0.225 mM hematin/well. Blanks were prepared in duplicate with both 50 μL of DMSO and 50 μL of H_2O. After preparation of the plate, 50 μL of DMSO was added to the positive control wells. Final well concentration of 0.4 mM hemin/well in the experimental wells was achieved by adding 50 μL of an 8 mM hemin solution dissolved in DMSO. β-Hematin formation was initiated by adding 8 M acetic acid buffer, pH 5.0, to all wells, bringing total well volume to 200 μL. Plates were incubated for 18 hours at 37°C.

After incubations, plates were centrifuged for 20 min at 2,300 rpm at room tempature. The supernatant containing unreacted hemin was decanted and the remaining hematin pellet was resuspended in 200 μL of DMSO. The plate was again centrifuged for 20 min at 2,300 rpm and the resulting supernatant decanted. The washed hematin pellet was dissolved in 200 μL of 0.1 M NaOH and the absorbance measured at 405 nm. A

standard curve of hematin concentration versus A_{405} was made using the data generated by the positive control.

Assay for *in vitro* antimalarial activity and cytotoxicity using pLDH activity assay

The antimalarial activity was determined by measuring plasmodial lactase dehydrogenase (pLDH) activity as described in the literature.[96] A suspension of red blood cells infected with D6 or W2 strains of *P. falciparum* (200 µL, with 2% parasitemia and 2% hematocrit in RPMI 1640 medium supplemented with 10% human serum and 60 µg/mL Amikacin) was added to duplicate wells of a 96-well plate containing 10 µL of serially diluted test samples (plant extracts, column fractions or pure compounds). The plate was flushed with a gas mixture of 90% N_2, 5% O_2, and 5% CO_2. The plate was subsequently placed in a modular incubation chamber (Billups-Rothenberg, CA) and incubated for 72 h at 37 ºC. Parasitic LDH activity was determined by using Malstat™ reagent (Flow Inc., Portland, OR) according to the procedure of Makler and Hinrichs.[97] Briefly, 20 µL of the incubation mixture was mixed with 100 µL of the Malstat™ reagent and incubated at room temperature for 30 min. Twenty microliters of a 1:1 mixture of nitroblue tetrazolium salt and phenazine ethosulfate (NBT/PES; Sigma, St. Louis, MO) was then added and the plate is further incubated in the dark for 1 h. The reaction was then stopped by the addition of 100 µL of 5% acetic acid solution (v/v). The plate was read at 650 nm. Artemisinin and chloroquine were included in each assay as the drug controls. IC_{50} values were computed from the dose response curves. To determine the selectivity index of antimalarial activity of the samples their *in vitro* cytotoxicity to mammalian cells was also determined. The assay was performed in 96-well tissue

culture-treated plates as described earlier.[98] Vero cells were seeded to the wells of 96-well plate at a density of 25,000 cells/well and incubated for 24 h. Samples at different concentrations were added and plates were again incubated for 48 h. The number of viable cells was determined by Neutral Red assay. IC_{50} values were obtained from dose response curves. Doxorubicin was used as a positive control.

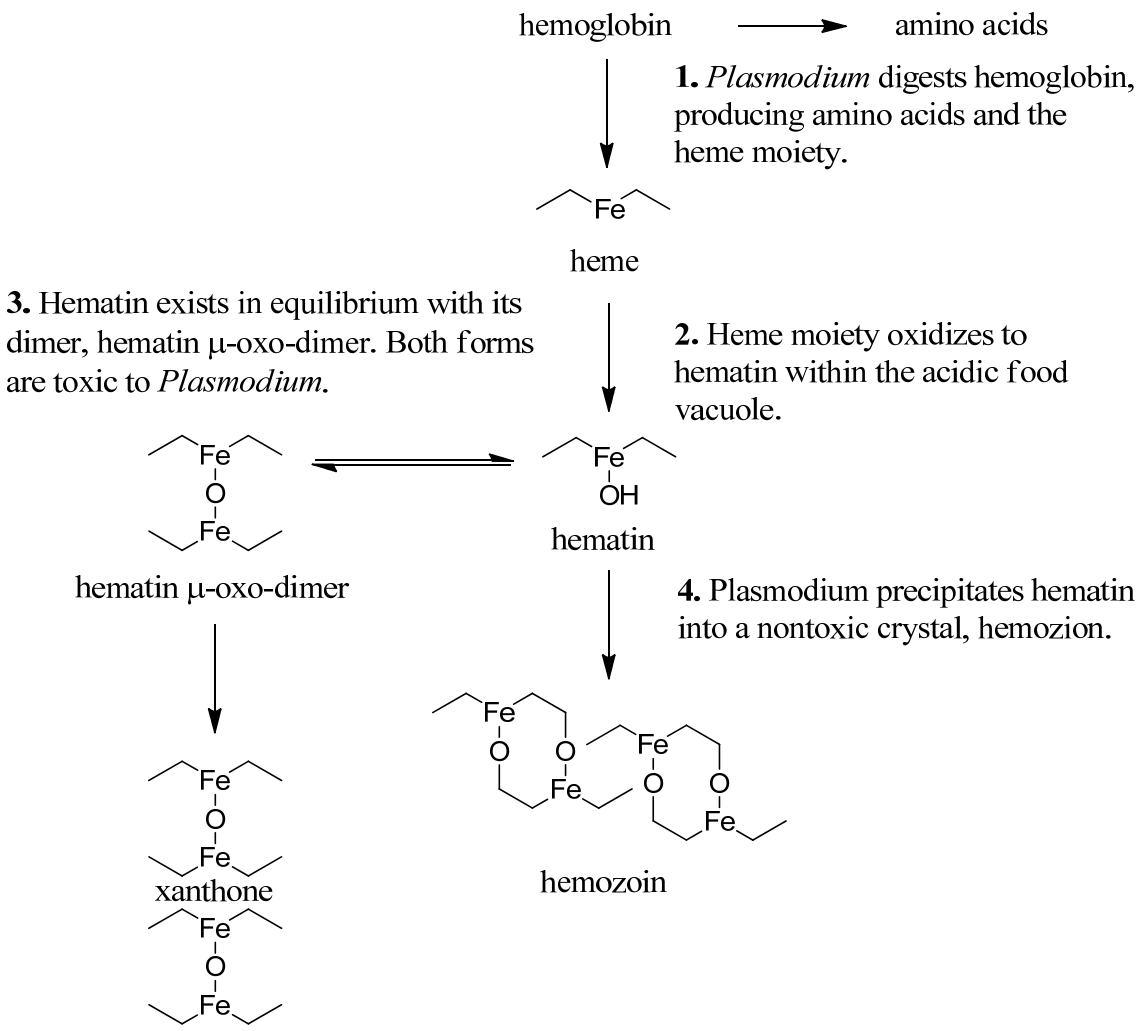


Figure 3-1. Mechanism of hematin stabilization by xanthones.[84]

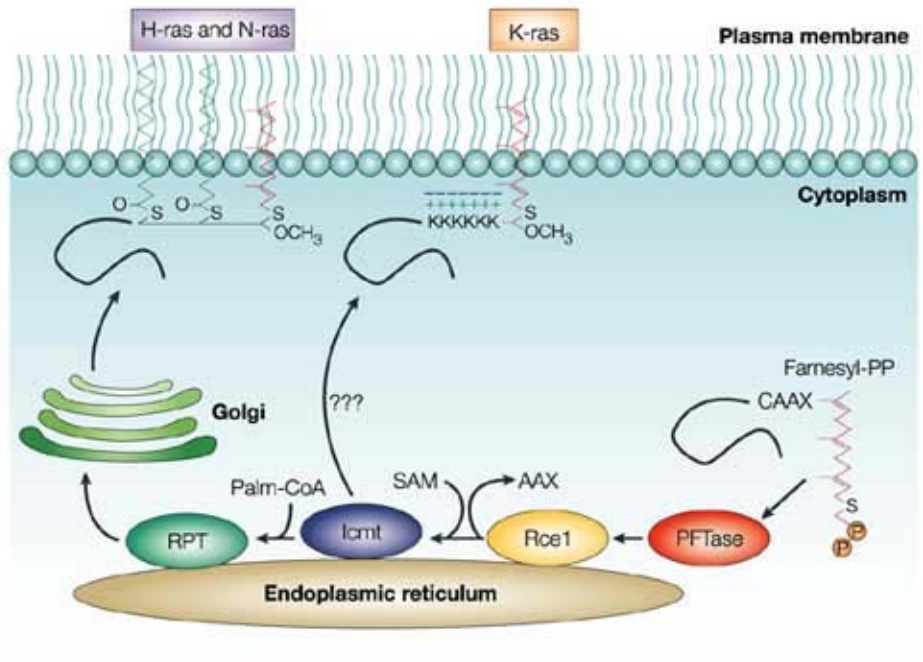
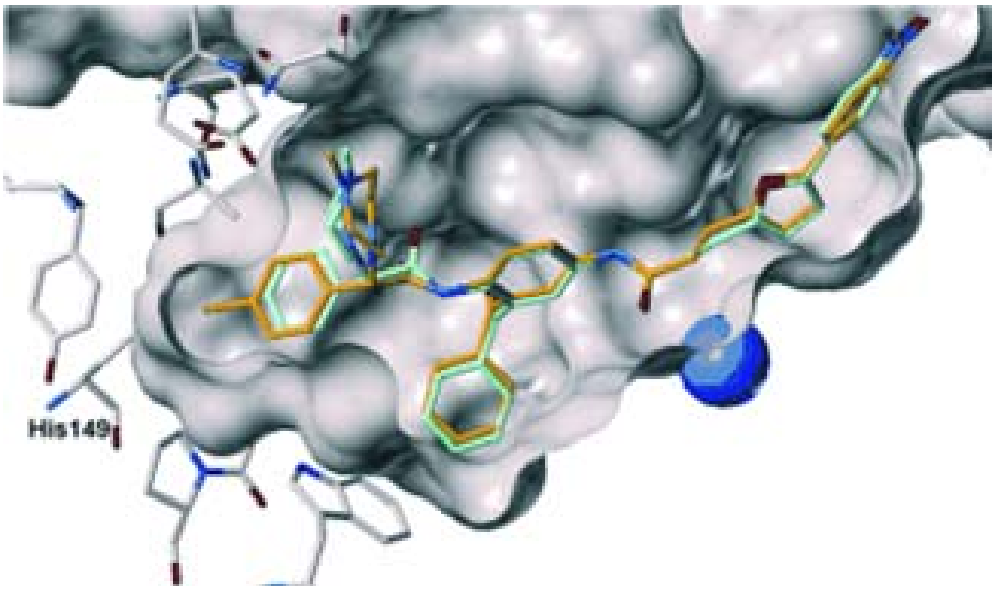
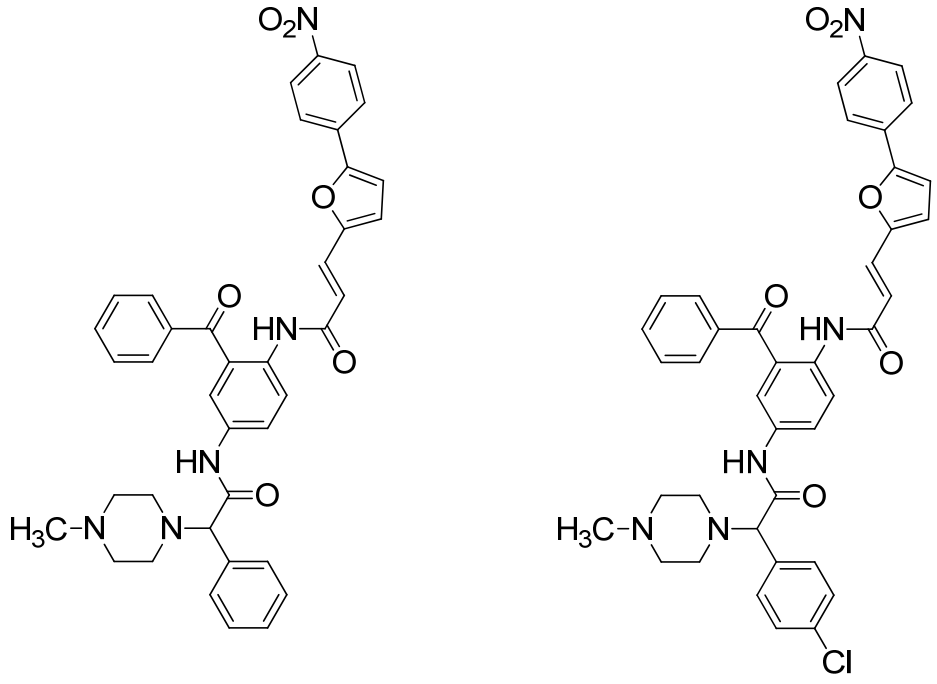


Figure 3-2. The pathway for Ras protein trafficking to the plasma membrane via PFTase.[92]





green yellow

Figure 3-3. Molecular docking experiment between two benzophenone based PFT inhibitors (green and yellow) and the binding pocket of farnesyltransferase.[94]

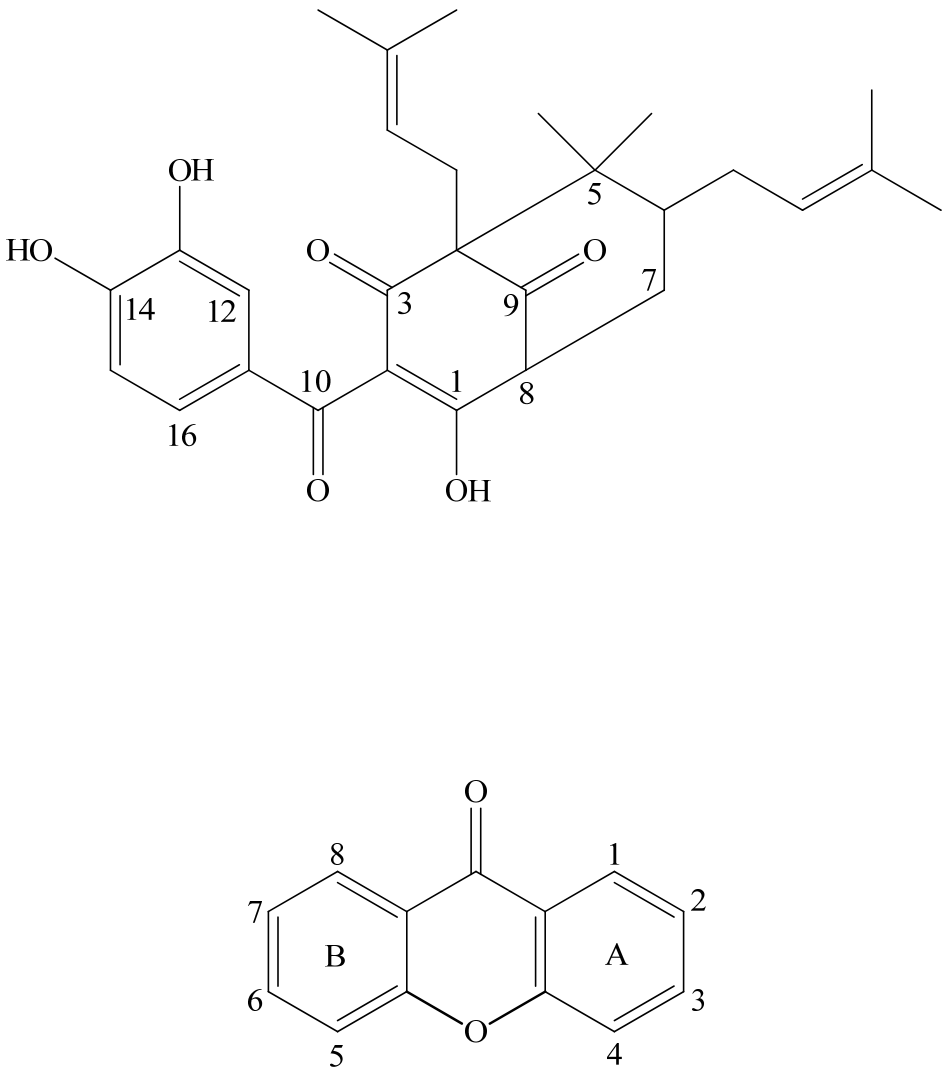
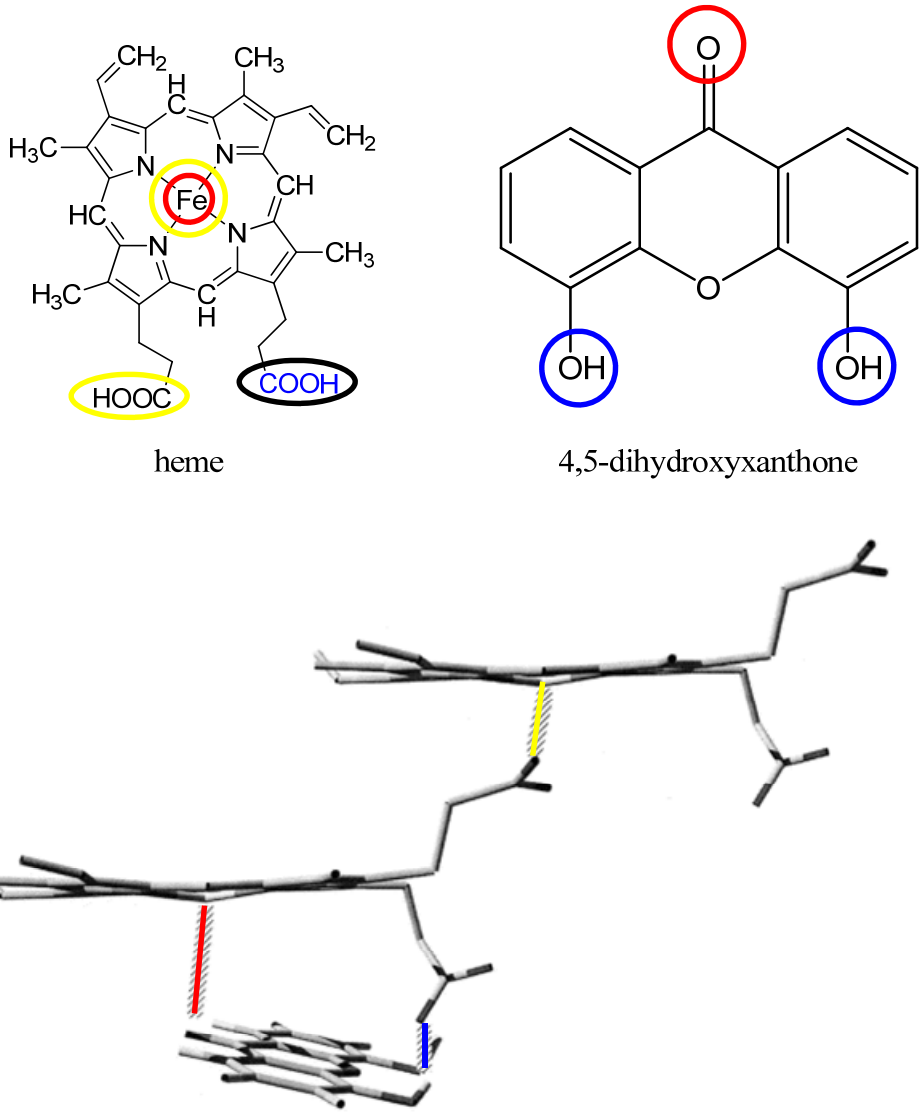


Figure 3-4. The 3-(3,4-dihydroxybenzoyl)-4-hydroxy-8,8-dimethyl-1,7-bis(3-methyl-2-buten-1-yl)bicyclo[3.3.1]non-3-ene-2,9-dione benzophenone skeleton (**top**) and the basic xanthone skeleton (**bottom**).



heme 4,5-dihydroxyxanthone

Figure 3-5. Binding interactions between heme and 4,5-dihydroxyxanthone. The red bar and circle show the π-π interactions of the xanthone carbonyl-heme iron interaction, the blue bar and circle denote the xanthone C-4 and C-5 hydroxy-heme carboxylate interactions, and the yellow bar and circle denote the heme carboxylate-heme iron interaction.[68]

Chapter 4

Conclusions

Natural products are a rich source of bioactive compounds and have played an important role in the development of new drugs and therapies. Between 1981 and 2002, 75% of new approved drugs for infectious diseases were of natural origin. Many therapies for other diseases were also based on natural products, amounting to 26% of the new anti-inflammatory drugs and 62% of cancer chemotherapies. During this 21-year period 67% of all new drugs approved were based or developed on a natural product. These 1,031 compounds were either isolated from natural sources, a modification of a natural product, or synthetic modifications based on natural compounds.[99, 100]

Chemoprevention of disease has been demonstrated against cancer, coronary heart disease, and other diseases with an increased consumption of fruits and vegetables. In their epidemiological study, Hertog *et al.* demonstrated that a diet rich in plant-based products, including fruits and vegetables, correlated with a reduced risk of coronary heart disease.[101, 102] Other epidemiological studies suggest that the "Mediterranean diet," rich in plants and high in fiber with low fat intake, lowers the incidence of several chronic diseases.[103, 104] Several reviews have discussed the effects of vitamin and non-vitamin antioxidants on human health, concluding that they can aid in the prevention of atherosclerosis and prevent oxidative damage to LDL lipoproteins.[105] Additionally, the research on resveratrol as the chemopreventative agent responsible for the "French Paradox" indicates that the consumption of bioactive natural products from food can lower the occurrence of chronic diseases.[106, 107]

Foods are ideal for chemoprevention of disease as they are, indispensable, readily available, and relatively inexpensive. Malaria is a disease of the developing world. Thirty out of the thirty-five nations with the highest malarial mortality are found in sub-Saharan

Africa.[108] The World Bank estimates the GDP of all of sub-Saharan Africa at 946,000 million (US dollars). For comparison, the GDP of the United States is 14,119,000 million and all of Europe has a GDP of 12,443,594 million.[109] Chemoprevention of disease in low-income areas and with restricted access to commercial medicines can be effected through food consumption, making the nutritional chemoprevention of malaria so potentially beneficial in those areas most impacted by the disease.

This work focused on compounds found in *Garcinia xanthochymus* and *G. mangostana,* both of which are often consumed in various forms throughout their distribution ranges and are exported in a variety of products, they make ideal chemopreventives for malaria. Three benzophenones, guttiferone E (**5**), guttiferone H (**6**), or isoxanthochymol (**7**), from *G. xanthochymus* were identified as antiplasmodial. The antiplasmodial activity of compounds **5** and **6** has not been previously reported. Antiplasmodial activity against the drug sensitive *Plasmodium falciparum* isolate NF54 was previously reported for **7**.[110] This is the first report of Compound **7** having antiplasmodial activity against the chloroquine-sensitive *P. falciparum* clone D6 and chloroquine-resistant clone W2.

Additionally, since a small library of structurally similar benzophenones were screened for antiplasmodial activity, the effect of side-chains on the 3-(3,4-dihydroxybenzoyl)-4-hydroxy-8,8-dimethyl-1,7-bis(3-methyl-2-buten-1-yl)bicyclo[3.3.1]non-3-ene-2,9-dione benzophenone skeleton can be discussed. For the benzophenones **8** and **2** the absence of a terminal methylene on the C-8 side chain (cf. **5** and **7**) correlates with antiplasmodial activity. Compound **6** with a terminal methylene on

C-30 and a cyclohexane from C-7 to C-8 is antiplasmodial. It was concluded that, for benzophenones, neither the C-5 nor the C-14 side groups affect antiplasmodial activity.

The three xanthones, α-mangostin (**12**), β-mangostin (**13**), and 3-isomangostin (**14**), were identified as antiplasmodial. The antiplasmodial activity of compound **12** was reported for various nonresistant and drug-resistant clones of *P. falciparum*.[111-115] The antiplasmodial activity of compound **13** was previously reported against the drug-resistant *P. falciparum* clone K_1.[113, 116] Neither compound **12,** nor **13** has previously been screened against the *P. falciparum* clones D6 and W2. Compound **14** has not been previously screened for antiplasmodial activity.

For the xanthones, the substitution pattern of both the A and B was shown to be important in determining the extent of antiplasmodial activity. An isoprenyl chain was necessary on C-8 for antiplasmodial activity (cf. **12** and **14**). The results indicate that the antiplasmodial activity is determined by factors other than the hydroxylation of C-4 and C-5 alone as the current structure activity studies indicate.[68] Other work on synthetic analogues of compound **12** suggest that a C-3 hydroxyl (cf. **12**) or C-2 prenyl side-chain (cf. **12** and **13**) enhance the antiplasmodial activity of the basic xanthone skeleton.[114] This suggests a more complex mechanism of action than that proposed by Kelly *et al.*[68]

While none of the benzophenones found in *G. xanthochymus,* **5**, **6**, or **7**, nor the xanthones found in *G. mangostana,* **12**, **13**, and **14**, were strong antiplasmodial compounds, they still can be used in chemotherapy there is evidence for their bioavailability. After the consumption of a xanthone-rich *G. mangostana* rind product an increase in the blood serum levels of **12** was identified via LC-MS.[117] The serum levels of **12** peaked 1-2 hours after consumption, but still remained elevated 6 hours after

consumption of the product. The antioxidant capacity of the blood serum, as measured by the ORAC assay, was also significantly increased over the 6 hours after consumption of the *G. mangostana* product. A recent patent outlines the production of a fermented *G. mangostana* product that increases the bioavailability of xanthones.[117] The formation of oligosaccharides during the fermentation process is suspected of enhancing the absorption of xanthones in the intestinal tract. The benzophenone 4,4'-dihydroxybenzophenone-2,4-dinitrophenylhydrazone was shown to be orally bioavailability in rats and monkeys as part of preclinical cancer trials.[118] The common sunscreen component, benzophenone-3, is applied topically and its metabolite can be detected in urine samples.[119] Based on these previous studies, the benzophenones **5**, **6**, and **7**, as well as, xanthones **12**, **13**, and **14** are likely bioavailable chemotherapies. Further research is necessary to determine the absorption rate of the compounds and their metabolites.

Since all species of *Plasmodium* have a quiescent developmental stage in the liver, the hepatic sporozoite is an ideal target for prophylactic treatment. A diet rich in the phytochemicals studied in this work eaten during or prior to this stage of the infection may reduce the duration and severity of the disease. Even if the treatment does not entirely prevent the infection, a reduction in the parasite load in the liver has been shown to reduce the severity of the overall infection, in turn leading to a lower mortality and a faster recovery.[120, 121]

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