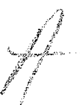

PHYTOCHEMISTRY OF CLUSIACEAE BENZOPHENONES:

NOVEL BIOACTIVE COMPOUNDS FROM *GARCINIA XANTHOCHYMUS*

By

Scott Baggett

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

2005

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Abstract

PHYTOCHEMISTRY OF CLUSIACEAE BENZOPHENONES:

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By

Scott Baggett

Thesis Advisor: Edward J. Kennelly

This project studies the diverse chemistry of the Clusiaceae especially *Garcinia xanthochymus* fruits. In addition, a comprehensive review of benzophenones isolated from angiosperms is also presented. The biosynthesis, sources, isolation, structural determination, and biological activities of benzophenones are explored in detail. Also, the activity-guided isolation and structural elucidation of xanthochymol by 1D and 2D NMR from *Garcinia xanthochymus* fruits is examined.

Fruits of five Clusiaceae species, *Garcinia aristata*, *Garcinia livingstonei*, *Garcinia spicata*, *Rheedia edulis*, and *Rheedia gardneriana*, were studied by LC-MS to prioritize them for further activity-guided isolation. In addition, a dereplication system using the on-line 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay is described.

A methanol extract of *Garcinia xanthochymus* fruits was subjected to activity-guided fractionation yielding two novel benzophenones, guttiferone H and gambogenone. Guttiferone H contains a 7-membered ring attached to the bicyclo[3.3.1]nonane system at positions 7 and 8 and the B/C-ring system in gambogenone is a novel bicyclo[3.3.2]decane system. Both novel compounds displayed cytotoxicity in the SW-480 colon cancer cell line with IC_{50} = 12 and 188 μM, respectively. In addition, both guttiferone H and gambogenone induced apoptosis in SW-480 colon cancer cells. Both

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guttiferone H and gambogenone displayed antioxidant activity in the DPPH assay with IC_{50} = 64 and 38.7 μM, respectively.

Eleven known compounds, aristophenone A, alloathyriol, amentoflavone, 3,8″-biapigenin, cycloxanthochymol, (+/-)-fukugetin, (+/-)-fukugiside, guttiferone E, isoxanthochymol, (+/-)-volkensiflavone, and xanthochymol were also obtained. All eleven known compounds were tested in the SW-480 colon cancer and the DPPH assays. As a group the known benzophenones displayed potent cytotoxicity in the SW-480 colon cancer cell assay (IC_{50} = 7.5 – 33.3 μM) and antioxidant activity in the DPPH assay, IC_{50} = 53 – 73 μM. The biflavonoids were less active in both assays, (SW-480, IC_{50} = 111 – 200 μM; DPPH, IC_{50} = 62 – 400 μM). The xanthone alloathyriol was not active in the DPPH assay and displayed cytotoxicity in the SW-480 assay, IC_{50} = 117 μM.

In summary, six Clusiaceae species were analyzed for their phytochemical constituents and a number of novel and known benzophenones, biflavonoids, and xanthones were identified and isolated from *Garcinia xanthochymus* fruits.

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Chapter 1: Introduction

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1.1. Introduction

Archaeological evidence indicates humans have used plants to treat and prevent diseases for more than 5000 years.[1] Many societies have a rich history of medicinal plant use dating back thousands of years. Today natural products are important sources of new drugs. For example, from 1981-2000, compounds isolated from either plants or microbes have lead to 60% of cancer drugs and 75% of pharmaceuticals for infectious diseases.[2-4]

An increased intake of fruits and vegetables has been associated with a significant reduction in cardiovascular disease (CVD) mortality in the American population in recent years.[5] Increased consumption of fruits and vegetables has also been associated with lower incidences and mortality rates of cancer in several human cohort and case-controlled studies.[6] Studies using human cells and animals also support the antitumorigenic effects of fruits and vegetables.[7] Results from cross-cultural, epidemiological, and experimental studies also suggest the importance of diet in cancer CVD etiology.[8]

1.2. Chemopreventive Effects of Plant Compounds

The protection provided by fruits and vegetables against cancer, CVD, and other diseases has been attributed to the various vitamin and non-vitamin antioxidants produced by plants and consumed by humans. Recent reviews discussing the effects of vitamin and non-vitamin antioxidants on human health have reached mixed conclusions.[9,10] Examples include an inverse correlation between plasma vitamin E levels and deaths from ischemic heart disease reported by Gey *et al.*[11] in cross-cultural studies. Hennekes *et al.*[12] and Omenn[13] found no benefit from long-term supplementation with β-carotene and vitamin A on the incidence of lung cancer and CVD. Critics have

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questioned why only one of the 272 configurational isomers of β-carotene was tested.[14] These complications and contradictions have lead researchers to propose that other compounds, beside vitamins, might be responsible for the disease prevention effects of fruits and vegetables; many researchers have suggested polyphenolic compounds.[7,9,10]

Studies by Wang *et al.*[5] examined the total oxygen radical absorbing capacity (ORAC) of commonly consumed fruits and fruit juices. They showed that the contribution of vitamin C to the ORAC activity of a fruit or fruit juice was less then 15 percent. Their conclusion was "this further suggests that other antioxidants beside vitamins E and C and β-carotene are also responsible for the protection provided by fruits and vegetables against various diseases." They implicated flavonoids, which are common components of the human diet and possess strong antioxidant *in vitro* and *in vivo* activities.

1.3. Flavonoids as Antioxidants and Chemopreventive Agents

Flavonoids, a group of polyphenolic compounds, are found in most plants, with over 4,000 currently isolated. Flavonoids are divided into 12 subclasses[15] and are largely responsible for the oranges, reds, and blue colors of fruits, vegetables, and flowers. As a consequence, flavonoids play a vital role in the pollination and seed distribution of plants by making flowers and fruits attractive to bees and birds.

Humans consume approximately 1 gram of flavonoids per day.[16] After ingestion, flavonoids appear in animal and human cells in pharmacologically significant levels.[16] Reports of flavonoid toxicity are extremely rare because flavonoids do not accumulate *in vivo*. Flavonoids possess many biological activities, including antioxidant and free radical scavenging, antiinflammatory, antiallergic, antihemorrhagic, antiviral, antibacterial,

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antifungal, antitumor, antiplatelet, and antiischemic activities.[17,18] Also, a considerable amount of research has been published detailing the inhibition of numerous enzymes by flavonoids including lipoxygenase, cyclooxygenase, monooxygenase, xanthine oxidase, mitochondrial succinoxidase, NADH-oxidase, phospholipase A_2, and protein kinases.[19,20]

Antioxidants can be divided into two groups: primary and secondary.[21,22] Primary antioxidants reduce the initiation of new radicals by trapping transition metal ions. Secondary antioxidants reduce the chain propagation and amplification of lipid peroxidation. Many compounds act as both primary and secondary antioxidants. For example, vitamin C acts by scavenging oxidizing species and by regenerating oxidized α-tocopherol.

Tea (*Camellia sinensis*), a rich source of antioxidant catechins, has been extensively studied for its anticancer and cardioprotective properties. Tea catechins are a group of six related compounds: catechin, epicatechin, epicatechin gallate, epigallocatechin, and epigallocatechin gallate. The average serving of tea (240 mL) contains 943 mg of catechin flavonoids. Tea catechins act as both primary and secondary antioxidants. The catechins have been shown to regenerate α-tocopherol in human plasma,[23] scavenge free radical species, decrease plasma phosphatidylcholine hydroperoxide levels, and are incorporated into human plasma when consumed.[24] In rats, catechin supplementation increases the activity of the antioxidant enzymes superoxide dismutase and catalase in rat livers.[25] Cao *et al.* showed green and black teas have a 4.5 – 70 fold-higher antioxidant activity than any of the fruits and vegetables studied in the ORAC assay.[5]

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The anticancer properties of flavonoids have been studied extensively. Numerous studies have demonstrated that flavonoids possess antiproliferative effects against human and rodent ovarian, leukemic, intestinal, lung, and breast cancer cell lines.[26] Specifically, the flavonoids genistein, kaempferol, and quercetin display antiproliferation effects against human colon cancer cells.[27,28] Epigallocatechin gallate inhibits cell growth,[29] causes G1 cell cycle arrest, and induces apoptosis in human prostate, lung, colon, gastric, leukemia, and squamous cell carcinomas.[30] In male Swiss albino mice, quercetin and luteolin decreased fibrosarcoma incidence and tumor size following treatment with the carcinogen 20-methylcholanthrene.[31]

A number of epidemiological studies on the role of vitamins in cancer have reached mixed conclusions. Epidemiological evidence indicates that high intake of vitamin C is associated with reduced risk of developing stomach, esophagus, and oral cancers.[32] Some epidemiological studies concluded that increased tea consumption lowers one's cancer risk; however, other investigations proved contradictorily or indicated procarcinogenic effects for tea.[33]

1.4. Benzophenone Bioactivity

The plant family Clusiaceae is known to be a rich source of benzophenones. Benzophenones are a group of *ca.* 146 compounds composed of a 13-member core carbon skeleton. This skeleton is then subject to prenylation and cyclization resulting in a number of bioactive compounds displaying antioxidant, antibacterial, antifungal, antiviral, cytotoxic, molluscicide, and trypanocidal activities. The guttiferones, a subclass of benzophenones, have displayed a number of biological activities including, antibacterial,[34] antioxidant,[35] antiviral,[36,37] and cytotoxicity.[38] The antioxidant and cancer

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preventive effects, as well as other reported biological actives of benzophenones are reviewed in Chapter 2.

1.5. Selection of Plant Material

Tropical fruits with a history of use were targeted because of the decreased likelihood these fruits would contain toxic phytochemicals. Thirty plants were initially screened for their antioxidant activity against the free radical DPPH. Fruits were selected based on ethnobotanical information, previous studies indicating the presence of antioxidants compounds, availability of plant material and Chemical Abstracts, Medline, and Natural Products Alert (NAPRALERT) database searches. The ten most active fruits (Table 1.1) in the DPPH microtiter assay (Section 3.3.4) were selected for further analysis. I selected *Garcinia xanthochymus* for activity-guided isolated based on its activity in the DPPH microtiter assay and cytotoxicity against the SW-480 colon cancer cell line (IC_{50} = 113.3 and 15 μg/mL, respectively), literature searches which established *G. xanthochymus* as a source of unique polyphenolic compounds, and published experimental data showing the likelihood that *G. xanthochymus* could contain novel antioxidant and cytotoxic compounds.

This thesis studies the diverse chemistry of the Clusiaceae. Chapter 2 presents a detailed review of known benzophenones isolated from angiosperms. Chapter 3 discusses the dereplication of five Clusiaceae species for benzophenones and biflavonoids and Chapter 4 details the activity-guided isolation of two novel benzophenones and eleven known compounds from *G. xanthochymus* fruits.

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Table 1.1. DPPH Activities of the Ten Tropical Plants Tested In Dr. Kennelly's Lab

Latin binomial	**Family**	**Common name**	**EtOAc extract IC_{50} (μg/mL)**
Eugenia uniflora	Myrtaceae	Surinam cherry	19.6
Chrysophyllum cainito	Sapotaceae	Star-apple	22.1
Blighia sapida	Sapindaceae	Akee	30.4
Manilkara zapota	Sapotaceae	Sapodilla	33.2
Theobroma grandiflorum	Sterculiaceae	Cupuaçu	47.6
Mammea americana	Clusiaceae	Mammee apple	55.9
Spondias tuberosa	Anacardiaceae	Imbu	84.6
Garcinia xanthochymus	Clusiaceae	Gamboge	113.3
Pouteria campechiana	Sapotaceae	Canistel	142.1
Colocasia esculenta	Convolvulaceae	Taro	169.1
Ascorbic acid			18.3
α-Tocopherol			53.3

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Chapter 2: Benzophenone Review

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2.1. Introduction

To better understand the chemistry of the Clusiaceae a comprehensive review of benzophenones is presented below. Given the structural diversity, bioactivities, and paucity of previous reviews, the benzophenones were subjected to a comprehensive review. The benzophenones are a class of compounds consisting of *ca.* 146 members. Benzophenones have been isolated from the leaves, roots, fruits, bark, and floral resin of plants belonging to at least 13 plant families including: Asteraceae, Clusiaceae, Davalliaceae, Fabaceae, Gentianaceae, Iridaceae, Lauraceae, Magnoliaceae, Moraceae, Rosaceae, and Thymelaeaceae. The majority of benzophenones (96 of 146, 66%) are found in three Clusiaceae genera: *Clusia*, *Garcinia*, and *Hypericum*. Benzophenones have a range of structures but share a common 13-carbon skeleton, Figure 2.1. There is no standard numbering systems for benzophenones. The shikimate-derived A-ring is a benzene ring usually containing 0, 1, or 2 substituents. The acetate-malonate derived B-ring undergoes prenylation and cyclization producing numerous structurally unique compounds containing bi-, tri-, and/or tetracyclic ring systems. Isolated benzophenones exhibit a range of biological activities including antifungal, antioxidant, antimicrobial, antiviral, and are cytotoxic.

Figure 2.1. Basic benzophenone structure

Historically, reviews of benzophenones were included as small sections with the biogenetically related xanthones. Previous reviews include Bennett and Lee[39] who

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discussed the distribution and isolation of 20 benzophenones included in a review focused on xanthones. They also discussed the biosynthetic relationships between xanthones and benzophenones. Kumar *et al.*[40] summarized the chemical composition, medical importance, and biologically active benzophenones, biflavonoids, and xanthones isolated from *Garcinia* species. Two reviews by Waterman and coauthors focused on the chemotaxonomic significance of benzophenones, along with biflavonoids and xanthones, of ten African *Garcinia* species.[41,42] Locksley and Murray[43] discussed the distribution of thirteen benzophenones in higher plants and Sultanbawa[44] included five benzophenones in a review on xanthonoids from tropical plants. Sultanbawa also discussed the biosynthesis of benzophenones and xanthones. Erdelmeier *et al.*[45] discussed 20 polyprenylated benzophenones in a chapter on the various properties of St. John's Wort (*Hypericum perforatum*).

Chapter 2 briefly discusses benzophenone biosynthesis, lists known benzophenones and their sources, and discusses their isolation, structural determination, and biological activities.

2.2. Benzophenone Synthesis

Previous researchers have suggested[44,46] that benzophenones are biosynthesized by condensation of metabolites from the shikimate pathway, forming the A-ring, and the acetate-malonate pathway, creating the B-ring. This produces the basic 13-carbon benzophenone skeleton shown in Figure 2.1. Support for this biosynthetic pathway includes the isolation of benzophenone synthase from *Centaurium erythraea*[47] and research by Atkinson *et al.*[48] who examined benzophenones as intermediates in the synthesis of xanthones. Xanthone biosynthesis is reviewed by Bennett *et al.*[39] Using ^{14}C

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and ^3H labeled precursors, Atkinson *et al.*[48] discovered that the shikimate-derived

Figure 2.2. General pathway for the synthesis of benzophenones

phenylalanine was incorporated into the A-ring and three acetates were condensed to form the B-ring. During these experiments ^{14}C phloroglucinol was also added and was not incorporated into either the A- or B-rings, suggesting the synthesis scheme shown in Figure 2.2. Addition of ^{14}C labeled phloroglucinol is significant because some researchers have suggested that benzophenones are not derived from the basic 13-carbon skeleton shown in Figure 2.1, but from phloroglucinol (1,3,5,-trihydroxybenzene). Examples include the sampsoniones, isolated from *Hypericum sampsonii*, by Hu *et al.* The sampsoniones were initially described as "polyprenylated benzophenones"[49,50] and later called "polyprenylated phloroglucinol"[51-53] derivatives. Hu *et al.* also proposed a

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biosynthetic pathway for the sampsonione type "benzophenones" from the basic 13-carbon benzophenone skeleton shown in Figure 2.1. In their proposed synthesis scheme it is unclear if the basic benzophenone skeleton is derived from the shikimate and the acetate-malonate pathways or from phloroglucinol. Additionally, Fuller *et al.*[54] suggested that the vismiaphenones (**35**, **44**, **45**, **53**) are related to and possible precursors of the guttiferones, a class of polyprenylated benzophenones represented by xanthochymol (**138**).

It remains unclear whether the basic 13-carbon benzophenone is constructed (from the shikimate and acetate pathways) and then prenylated and cyclized or the B-ring is derived from phloroglucinol and later the 7-membered carbon substructure (A-ring) is added. Given the structural diversity of benzophenones, their biosynthesis is suggestive of multiple pathways. Further research is needed in order to fully understand the biosynthesis of benzophenones.

2.3. Isolated Benzophenones

Information on 146 isolated benzophenones is summarized in Table 2.1, sorted by molecular weight from lowest to highest. Each entry contains the common name(s), chemical structure, biological source(s), and reference(s).

For convenience benzophenones are divided into two types: basic benzophenones and polyprenylated benzophenones (PPBs). Basic benzophenones have the 13-membered skeleton, Figure 2.1, with various numbers of -OH, -OMe, prenyl, and geranyl groups attached and are usually uncyclized or have undergone one cylization. Examples include compounds **1-30** and the vismiaphenones (**35**, **44**, **53**). As suggested by Fuller *et al.*[54] vismiaphenones are possible intermediates in the biosynthesis of PPBs, the second type

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of benzophenone. This group has additional prenyl or geranyl groups attached, bi-, tri-, or tetracyclic rings systems, or oxo bridges, peroxide or epoxide groups. Examples are the guttiferones, plukenetiones, and sampsoniones, represented by xanthochymol (**138**), plukenetione A (**76**), and sampsonione A (**126**), respectively. The following discussion highlights features of the basic and polyprenylated benzophenones.

Table 2.1. Chemical Structures, Molecular Weights, and Sources of Isolated Benzophenones

#	Compound	Structure	MW	Species	Ref
		Prenyl = Geranyl =			
1	4-Hydroxy-benzophenone	OH, O	198	*Talauma mexicana*	55
2	2,4,6-Trihyroxy-benzophenone	HO, OH, O, OH	230	*Hypericum sampsonii*	53
3	Cearoin	OH, OMe, O, OH	244	*Dalbergia cearensis*, *Dalbergia coromande-liana*, *Dalbergia odorifera*, *Dalbergia parviflora*, *Dalbergia sissoides*, *Dalbergia volubilis*	56-61
4	Cotoin	HO, OMe, O, OH	244	*Aniba duckei*	62,63

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Table 2.1. Continued

#	Compound	Structure	MW	Species	Ref
5	Isocotoin	MeO, OH, O, OH	244	*Garcinia dulcis, Helichrysum* spp.	64,65
6	2,4,5,4′-Tetrahydroxy-benzophenone	OH, HO, OH, O, OH	246	*Dalbergia melanoxylon*	66
7	2,4,6,3′-Tetrahydroxy-benzopheone	HO, OH, OH, OH, O	246	*Garcinia multiflora, Gentiana lutea*	48,67
8	Iriflophenone	HO, HO, OH, O, OH	246	*Iris florentina, Iris germanica, Iris potaninii, Morus alba*	68-71
9	Hydrocotoin	MeO, OMe, O, OH	258	*Allanblackia floribunda*	43
10	2,6,4′-Trihydroxy-4-methoxybenzo-phenone	HO, HO, OMe, O, OH	260	*Aniba duckei, Anemarrhena asphode-loides*	72,73
11	Melannoin	OH, HO, OMe, O, OH	260	*Dalbergia melanoxylon*	66
12	2,4,6,3′,5′-Pentahydroxy-benzophenone	OH, HO, OH, OH, OH, O	262	*Garcinia pedunculata*	74
13	Macurin	HO, HO, OH, HO, O, OH	262	*Garcinia assugu, Garcinia multiflora*	35,67

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Table 2.1. Continued

#	Compound	Structure	MW	Species	Ref
14	Methlyhydro-cotoin	MeO, OMe, O, OMe	272	*Aniba pseudocoto, Cascara sagrada*	43
15	2,3′-Dihydroxy-4,6-dimethoxy-benzophenone	OH, MeO, OMe, O, OH	274	*Allanblackia floribunda, Garcinia multiflora*	43,67
16	Scleroin	OH, OMe, OMe, O, OH	274	*Machaerium scleroxylon*	75
17	4,6,3′,4′,-Tetrahydroxy-2-methoxy-benzophenone	HO, HO, HO, OH, O, OMe	276	*Garcinia multiflora*	67
18	Annulato-phenone	MeO, OH, OH, OH, OH, O	276	*Hypericum annulatum*	76
19	2′,3′,6-Trihydroxy-2,4-dimethoxy-benzophenone	HO, OMe, HO, OH, O, OMe	290	*Garcinia subelliptica*	77
20	Protocotoin	O, O, MeO, OMe, O, OH	302	*Aniba pseudocoto*	43
21	4′,6-Dihydroxy-2,3′,4-trimethoxy-benzophenone	HO, MeO, MeO, OMe, O, OH	304	*Garcinia subelliptica*	78
22	Methylproto-cotoin	O, O, MeO, OMe, O, OMe	316	*Aniba pseudocoto*	43

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Table 2.1. Continued

#	Compound	Structure	MW	Species	Ref
23	Cudranone	HO OMe HO O OH Prenyl	328	*Cudrania cohochin-chinensis* var. *genontogera*	79
24	2,4,6,3′,4′-Pentamethoxy-benzophenone	OMe MeO OMe OMe OMeO	332	*Garcinia pedunculata*	74
25	2,4,6,3′,5′-Pentamethoxy-benzophenone	MeO MeO OMe MeO O OMe	332	*Garcinia pedunculata*	74
26	4,6,4′-Trihydroxy-2,3′-dimethoxy-3-prenyl-benzophenone	HO HO OH MeO Prenyl O OMe	358	*Garcinia multiflora*	67
27	Clusiaphenone A	O O O OH	362	*Clusia ellipticifolia*, *Clusia sandiensis*	80,81
28	Clusiachromene C	Prenyl O OH O OH	364	*Clusia multiflora*	82
29	Clusiacitran A	O O O OH	364	*Clusia multiflora*	82
30	Clusiacitran B	O O O OH	364	*Clusia multiflora*	82

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Table 2.1. Continued

#	Compound	Structure	MW	Species	Ref
31	Clusiacyclol A		364	*Clusia multiflora*	82
32	Clusiacyclol B		364	*Clusia multiflora*	82
33	Cudraphenone A		364	*Cudrania cochin-chinensis*	83
34	Iso-vismiaphenone B		364	*Clusia ellipticifolia, Vismia decipiens*	80,84
35	Vismiaphenone B		364	*Clusia ellipticifolia, Vismia decipiens*	84
36	Clusiaphenone B		366	*Clusia sandiensis*	81
37	Cudraphenone B		366	*Cudrania cochin-chinensis*	83

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Table 2.1. Continued

#	Compound	Structure	MW	Species	Ref
38	Myrtiaphenone B	O, OMe, Prenyl, O, OH	378	*Garcinia myrtifolia, Garcinia pseudo-guttifera*	85,86
39	4-Geranyloxy-2-hydroxy-6-dimethoxy-benzophenone	MeO, O-Geranyl, O, OH	380	*Helichrysum triplinerve*	64
40	Clusiaphenone C	OH, O, O, O	380	*Clusia ellipticifolia*	80
41	Clusiaphenone D	HO, O, O, O, OH	380	*Clusia ellipticifolia*	80
42	Cudraphenone C	O, OH, HO, O, OH, Prenyl	380	*Cudrania cochin-chinensis*	83
43	Marupone	MeO, OH, Geranyl, O, OH	380	*Moronobea pulchra*	87
44	Vismiaphenone A	Prenyl, HO, OH, Prenyl, O, OMe	380	*Vismia decipiens*	84

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Table 2.1. Continued

#	Compound	Structure	MW	Species	Ref
45	Vismiaphenone C		380	*Garcinia pseudo-guttifera, Garcinia myrtifolia*	85,86
46	Vismiaphenone E		380	*Vismia cayennensis*	54
47	Cudraphenone D		382	*Cudrania cochin-chinensis*	83
48	Vismiaguianone A		382	*Vismia guianesis*	88
49	Vismiaguianone B		382	*Vismia guianesis*	88
50	Vismiaguianone C		382	*Vismia guianesis*	88
51	Garcimangosone D		392	*Garcinia mangostana*	89

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Table 2.1. Continued

#	Compound	Structure	MW	Species	Ref
52	Myrtiaphenone A		394	*Garcinia pseudo-guttifera, Garcinia myrtifolia*	85,86
53	Vismiaphenone F		394	*Vismia cayennensis*	54
54	Pseudo-guttiaphenone A		396	*Garcinia pseudo-guttifera*	86
55	Vismiaphenone D		396	*Vismia cayennensis*	54
56	Annulato-phenonoside		408	*Hypericum annulatum*	76
57	Iriflophenone-2-*O*-β-D-gluco-pyranoside		408	*Coleogyne ramosissima*	90
58	Iriflophenone-4-*O*-β-D-gluco-pyranoside		408	*Davallia solida*	91
59	Vismiaphenone G		412	*Vismia cayennensis*	54

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Table 2.1. Continued

#	Compound	Structure	MW	Species	Ref
60	2,4′,6-Trihydroxy-4-methoxy-benzophenone-2-*O*-glucoside	HO, GlcO, OMe, O, OH	422	*Gnidia involucrata*	92
61	2,3,4′,5,6-Pentahydroxy-benzophenone-4-*C*-glucoside	HO, HO, OH, Glc, OH, O, OH	424	*Gnidia involucrata*	92
62	Hyperico-phenonoside	HO, OH, OGlc, OH, OH, O	424	*Hypericum annulatum*	76
63	Vismiaguianone D	Prenyl, HO, O, O, O, OH	428	*Vismia guianensis*	88
64	Vismiaguianone E	Prenyl, HO, OH, O, O, O	428	*Vismia guianensis*	88
65	Machuone	O, O, Prenyl, Prenyl, O, OH	432	*Clusia sandiensis*	81
66	Grandone	Prenyl, O, OH, Prenyl, Prenyl, O, OH	434	*Clusia grandiflora*	93,94
67	Triptephenoside	MeO, OMe, OGlc, OH, O	436	*Triptero-spermum japonicum*	95

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Table 2.1. Continued

#	Compound	Structure	MW	Species	Ref
68	Acetylannulato-phenonoside		450	*Hypericum annulatum*	96
69	Tovophenone A		464	*Tovomita mangle, Tovomita brevistaminea*	97,98
70	Tovophenone B		464	*Tovomita mangle, Tovomita brevistaminea*	97,98
71	Cassiaphenone, α-2-glucoside		466	*Cassia angustifolia*	99
72	Cassiaphenone, β-2-glucoside		480	*Cassia angustifolia*	99
73	Tovophenone C		480	*Tovomita brevistaminea*	98
74	Garciduol A		486	*Garcinia dulcis*	65,100

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Table 2.1. Continued

#	Compound	Structure	MW	Species	Ref
75	Garciduol C		486	*Garcinia dulcis*	65
76	Plukenetione A		500	*Clusia plukenetii*	101
77	Plukenetione F		500	*Clusia havetioides* var. *stenocarpa*, *Clusia plukenetii*	102, 103
78	Plukenetione G		500	*Clusia havetiodes* var. *stenocarpa*, *Clusia plukenetii*	102, 103
79	Scrobiculatone A		500	*Clusia scrobiculata*	104

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Table 2.1. Continued

#	Compound	Structure	MW	Species	Ref
80	Scrobiculatone B		500	*Clusia scrobiculata*	104
81	7-Epi-clusianone		502	*Rheedia gardneriana*	105-107
82	7-Epi-nemorsone		502	*Clusia insignis*, *Clusia nemorosa*, *Clusia rengger-ioides*	94
83	Clusianone		502	*Clusia spiritu-sanctensis*, *Clusia sandiensis*, *Garcinia assugu*	35,81, 93
84	Garciduol B		502	*Garcinia dulcis*	65,100
85	Hilarianone		502	*Clusia hilariana*	104
86	Kolanone		502	*Garcinia kola*	108

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Table 2.1. Continued

#	Compound	Structure	MW	Species	Ref
87	Lanceolatone	Prenyl; O; O; Prenyl; O; O	502	*Clusia lanceolata*	104
88	Nemorosone	Prenyl; HO; O; R; S; Prenyl; O; O; S; Prenyl	502	*Clusia grandiflora*, *Clusia insignis*, *Clusia nemorosa*, *Clusia rosea*	93,109
89	Nemorosone II	Prenyl; HO; O; S; S; Prenyl; O; O; R; Prenyl	502	*Clusia grandiflora*, *Clusia renggeri-oides*, *Clusia rosea*	94,110
90	Nemorosonol	Prenyl; Prenyl; HO; O; Prenyl; OH; O	502	*Clusia multiflora*, *Clusia nemorosa*	82, 111, 112
91	Plukenetione D	Prenyl; HO; O; R; S; Prenyl; O; O; S; Prenyl	502	*Clusia plukenetii*	103
92	Plukenetione E	Prenyl; O; OH; S; S; Prenyl; O; O; S; Prenyl	502	*Clusia plukenetii*	103

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Table 2.1. Continued

#	Compound	Structure	MW	Species	Ref
93	Propolone A	Prenyl, Prenyl, O, O, O, O	502	Propolis from Cuba	113
94	Weddellianone A	Prenyl, Prenyl, Prenyl, O, O, O, O	502	*Clusia weddelliana*	104
95	28,29-Epoxy-plukenetione A	O, O, Prenyl, O, O, O, Prenyl	516	*Clusia havetioides* var. *stenocarpa*	102
96	Hydroxy-nemorosone	Prenyl, HO, O, HO, Prenyl, O, O, Prenyl	518	*Clusia nemorosa*	93,94
97	Ochrocarpinone B	HO, O, O, Prenyl, O, O, Prenyl	518	*Ochrocarpos punctatus*	114
98	Ochrocarpinone C	OH, O, O, Prenyl, O, O, Prenyl	518	*Ochrocarpos punctatus*	114

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Table 2.1. Continued

#	Compound	Structure	MW	Species	Ref
99	Plukenetione B	R, s, R, s; O; OH; O O; O; Prenyl	518	*Clusia plukenetii*	103
100	Sampsonione B	O; Prenyl; O; O O; OH; Prenyl	518	*Hypericum sampsonii*	50
101	Sampsonione G	R; s; s; O; OH; O O; R; O; Prenyl	518	*Clusia havetioides* var. *stenocarpa*, *Hypericum sampsonii*	49,102
102	Sampsonione L	Prenyl; O; Prenyl; O; O O; OH	518	*Hypericum sampsonii*	51
103	Xerophenone A	Prenyl; O; O; 10; R; 4; Prenyl; OH O; R = OH	518	*Clusia plukenetii*, *Clusia portlandiana*	103, 115
104	Xerophenone B	Prenyl; O; O; 10; R; 4; Prenyl; O OH; R = OH	518	*Clusia portlandiana*	115
105	Sampsonione H	O; O; O O; Geranyl	528	*Hypericum sampsonii*	49

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Table 2.1. Continued

#	Compound	Structure	MW	Species	Ref
106	Nemorosinic acid A	R = Prenyl	530	*Clusia nemorosa*	116
107	Nemorosinic acid B	R = Prenyl	530	*Clusia nemorosa*	116
108	15,16-Dihydro 16-hydroperoxy-isoplukenetione F		534	*Clusia havetioides* var. *stenocarpa*, *Ochrocarpos punctatus*	102, 114
109	Aristophenone A		534	*Garcinia aristata*	117
110	Aristophenone B		534	*Garcinia aristata*	117
111	Ochrocarpinone A		534	*Ochrocarpos punctatus*	114

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Table 2.1. Continued

#	Compound	Structure	MW	Species	Ref
112	Sampsonione E	O O O Geranyl O O	542	*Hypericum sampsonii*	49
113	Plukenetione C	O O O O O O OH	550	*Clusia havetioides* var. *stenocarpa*, *Clusia plukenetii*	102, 103
114	Spiritone	HO Prenyl O Prenyl O O Prenyl	556	*Clusia spiritu-sanctensis*	104
115	Insignone	Prenyl HO O Prenyl O O O O	560	*Clusia insignis*	104
116	Chamone II	O OH R O O Prenyl R = Prenyl	568	*Clusia grandiflora*	110

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Table 2.1. Continued

#	Compound	Structure	MW	Species	Ref
117	Hypersampsone F		568	*Hypericum sampsonii*	53
118	Sampsonione D		568	*Hypericum sampsonii*	49,53
119	Chamone I		570	*Clusia grandiflora*	110
120	Hypersampsone D		570	*Hypericum sampsonii*	53
121	Hypersampsone E		570	*Hypericum sampsonii*	53
122	Weddellianone B		570	*Clusia weddelliana*	104

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Table 2.1. Continued

#	Compound	Structure	MW	Species	Ref
123	33-Hydroperoxy-isoplukenetione C		582	*Clusia havetioides* var. *stenocarpa*	102
124	Sampsonione I		584	*Hypericum sampsonii*	52
125	Sampsonione J		584	*Hypericum sampsonii*	52
126	Sampsonione A		586	*Hypericum sampsonii*	50
127	Sampsonione C		586	*Hypericum sampsonii*	49
128	Sampsonione F		586	*Hypericum sampsonii*	49
129	Sampsonione K		586	*Hypericum sampsonii*	51

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Table 2.1. Continued

#	Compound	Structure	MW	Species	Ref
130	Sampsonione M	Prenyl; OH; Geranyl; O; O; O; O	586	*Hypericum sampsonii*	51
131	(-)-Isoxantho-chymol (Isogarcinol, cambogin)	OH Prenyl; HO; O; O; Prenyl; O; O; Prenyl	602	*Garcinia assugu, Garcinia pedunculata*	38,118
132	(+)-Isoxantho-chymol	OH Prenyl; HO; O; O; Prenyl; O; O; Prenyl	602	*Garcinia indica, Garcinia ovalifolia, Garcinia xantho-chymus, Garcinia xishuan-bannanansis*	36,119 -121
133	Cycloxantho-chymol	OH Prenyl; HO; O; O; Prenyl; O; O; R_1; R_1 =	602	*Garcinia pyrifera, Garcinia subelliptica*	122, 123
134	Garcinol (camboginol)	OH Prenyl; HO; O; O; Prenyl; O; OH; Prenyl	602	*Garcinia cambogia*	124

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Table 2.1. Continued

#	Compound	Structure	MW	Species	Ref
135	Guttiferone A		602	*Calophyllum brasiliense*, *Garcinia intermedia*, *Garcinia livingstonei*, *Garcinia macrophylla*, *Symphonia globulifera*	36, 125-127
136	Guttiferone E		602	*Clusia rosea*, *Garcinia assugu*, *Garcinia huillensis*, *Garcinia ovalifolia*, *Garcinia pedunculata*	35, 36, 118, 128
137	Guttiferone F		602	*Allanblackia stuhlmannii*	129
138	Xanthochymol		602	Clusia rosea, *Garcinia indica*, *Garcinia mannii*, *Garcinia ovalifolia*, *Garcinia staudtii*, *Garcinia xantho-chymus*, *Garcinia xishuan-bannanansis*, *Rheedia madrunno*	36, 119-121, 130-133

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Table 2.1. Continued

#	Compound	Structure	MW	Species	Ref
139	Pedunculol	OH Prenyl, HO, O, O, Prenyl, O, OH, Prenyl	604	*Garcinia pedunculata*	118
140	Garcinol, 13-*O*-methyl ether	Prenyl, OMe, HO, O, O, Prenyl, O, OH, Prenyl	616	*Garcinia assugu*	35
141	Isogarcinol, 13-*O*-methyl ether	OMe Prenyl, HO, O, O, Prenyl, O, O, Prenyl	616	*Garcinia assugu*	35
142	Bronianone	Geranyl, OH, HO, O, Geranyl, O, Prenyl, O, O	670	*Garcinia hombroniana*	46
143	Guttiferone B	OH Prenyl, HO, O, O, Geranyl, Geranyl, O, OH	670	*Symphonia globulifera*	36
144	Guttiferone C	Prenyl, OH Prenyl, Prenyl, HO, O, O, O, OH	670	*Symphonia globulifera*	36

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Table 2.1. Continued

#	Compound	Structure	MW	Species	Ref
145	Guttiferone D	Prenyl, OH Prenyl, Prenyl, HO, O, O, O, OH	670	*Symphonia globulifera*	36
146	Guttiferone G	Prenyl, OH Prenyl, Geranyl, HO, O, O, Prenyl, O, OH	670	*Garcinia macrophylla*	126

Benzophenones and xanthones are known to co-occur in the Clusiaceae,[39] and the garciduols (**74**, **75**, and **84**) are three benzophenone-xanthone dimers.[65,100] The cudraphenones (**33**, **37**, **42**, **37**), a group of benzophenones isolated from the Moraceae family, are prenylated on the A- and B-rings.[83] These are the only four benzophenones that are prenylated on both the A- and B-rings.

Ten benzophenone glycosides, nine *O*-glycosides (**56-58**, **60**, **62**, **67-68**, and **71-72**) and one *C*-glycoside (**61**) have been isolated from five different plant species. These are all basic benzophenones, not prenylated, and the glycoside substituent is attached to either the A- or B-ring.[76,90-92,95,96,99]

The most common polyprenylated benzophenones have a bicyclo[3.3.1]nonane B/C-ring system. Approximately 38 benzophenones with the bicyclo[3.3.1]nonane system have been isolated. A typical bicyclo[3.3.1]nonane benzophenone is xanthochymol (**138**), which is discussed in detail below.

The floral resin of twelve *Clusia* species has yielded at least fourteen (**66**, **79-80**, **82**, **85**, **87-89**, **93-94**, **96**, **114-115**, and **122**) bioactive bicyclo[3.3.1]nonane polyprenylated benzophenones. These compounds have the isoprenyl and benzoyl

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residues attached at different positions to the bicyclo[3.3.1]nonane system.[93,94,104,109,134] In addition, the floral resin of these studied Clusiaceae species, were used by pollinating bees for nest construction.[113]

Four isolated PPBs, with the bicyclo[3.3.1]nonane system, contain peroxide bonds: 15,16-dihydro-16-hydroperoxyisoplukenetione F (**108**), ochrocarpinone A (**111**), plukenetione C (**113**), and 33-hydroperoxyisoplukenetione C (**123**).[102,114]

The tautomeric benzophenone pair xerophenone A (**103**) and B (**104**) present an interesting variation in benzophenone chemistry by having a 7-membered C-ring and an oxo bridge between carbons 4 (C-ring) and 10 (B-ring). These compounds feature an oxatricyclo[4.3.1.1]undecane-7,9-dione system.[115]

A number of PPBs occur as tautomeric pairs, examples include aristophenone A and B (**109** and **110**),[117] plus, plukenetione D (**91**) and E (**92**),[103] which were isolated after acetylation.

Plukenetione A (**76**) was the first PPB isolated with a adamantyl skeleton and an methylpropenyl group.[101] Sampsonione D (**118**) and I (**124**) each have one isopentenyl side chain replaced by an isopropenyl moiety.[49,51,52]

Grossman *et al.* details structural the relationships of the plukenetiones, nemorosone II, and sampsoniones.[135] They showed that plukenetiones B, D, and E are diastereomeric to nemorosone II and sampsonione G.

Nemorosonol (**90**), isolated from *Clusia nemorosa* fruits, has a novel tricyclo[4.3.3.0]decane acetate-derived B-ring.[111] This compound's structure was determined by X-ray crystallography,[112] and is the only isolated benzophenone with a tricyclo[4.3.3.0]decane system.

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There has been considerable confusion in the nomenclature and structural elucidation of garcinol/camboginol (**134**) and isoxanthochymol/isogarcinol (**131**). Their naming history is discussed by Bennett *et al.*[39] and by Fuller *et al.*[129] The synonyms for these compounds are listed in their respective entries in Table 2.1. Details on the structural elucidation of these compounds can be found in 136-145.

2.4. Isolation of Benzophenones

Multiple chromatographies are needed to purify benzophenones, and a variety of normal and reversed-phase solvent systems and solid phases including column chromatography over silica gel, reversed-phase, and Sephadex LH-20, plus preparative TLC and HPLC have been employed. Isolation methods for selected benzophenones are given, and our experiences with the activity-guided isolation of xanthochymol (**138**) from *G. xanthochymus* fruits are discussed in detail.

Typically, the roots, leaves, fruits, wood, or floral resin are extracted with a single solvent, or solvents of, increasing polarity including C_6H_6,[80,111] hexane,[103,117] CH_2Cl_2,[81,114,129] acetone,[81] petroleum ether,[146] MeOH,[81,114,129] and/or EtOH.[51,83] After *in vacuo* concentration, the residue is resuspended in water and sequentially partitioned with solvents of increasing polarity including *n*-hexane, C_6H_6, CH_2Cl_2, EtOAc, and BuOH. After partitioning, extracts are passed over silica gel, either using open columns[103,118] or vacuum-liquid chromatography (VLC), typically with mixtures of hexane–EtOAc or $CHCl_3$–MeOH.[85,86] Separation via Sephadex LH-20 using isocratic systems of MeOH,[65] CH_2Cl_2–MeOH,[36] $CHCl_3$–MeOH,[35,67] or a gradient solvent system of $CH_2Cl_2 \rightarrow CH_2Cl_2$–MeOH,[126] has also been employed. After initial separation benzophenone-enriched fractions are rechromatographed (using preparative TLC, column chromatography, or

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HPLC) over a variety of stationary phases (in order of decreasing times employed) including silica gel, C_{18}, Sephadex LH-20, C_8,[36,88] diol,[36] MCI gel CHP-20P,[90] and Toyopearl HW-40.[90] Usually, multiple chromatographies over the same stationary phase or a combination of stationary phases are used to purify benzophenones. Recrystallization has been used as the final step by a few researchers.[96,117,118] Isolation methods for selected benzophenones are given below.

The anti-HIV guttiferones A-D (**135**, **143-145**) were isolated from *Symphonia globulifera* by extracting with CH_2Cl_2–MeOH and then MeOH. The combined organic layers were partitioned with EtOAc and, after *in vacuo* concentration, were passed over a diol column, eluted with CH_2Cl_2–EtOAc–MeOH. The HIV-active fractions were combined and rechromatographed over a diol column, eluted with CH_2Cl_2. Next, the HIV-active fractions were combined and rechromatographed over a C_{18} column, eluted with 9:1 MeOH–H_2O and 100% MeOH. Final purification was achieved by C_8 HPLC using MeOH–4% H_2O–0.01% TFA.[36]

Sampsoniones A-M (**100-102**, **105**, **112**, **118**, **124-130**) were isolated from whole air-dried *Hypericum sampsonii* which was extracted with 95% EtOH. The EtOH extract was concentrated under reduced pressure and partitioned between CH_2Cl_2 and H_2O. The CH_2Cl_2 phase was chromatographed over silica gel, and eluted with hexane–EtOAc mixtures. The sampsonione enriched fraction(s) were rechromatographed over silica gel, eluted with hexane–$CHCl_3$–acetone mixtures. Individual sampsoniones were isolated by preparative TLC or VLC over C_{18}.[49-52]

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The vismiaphenones (**35**, **44**, **45**, **53**) were isolated using Sephadex LH-20 with 1:1 CH_2Cl_2–MeOH, followed by normal-phase HPLC (17:3 hexane–^{i}PrOH) with a cyano column.[54]

Percolation with hexane[103] or extraction with CH_2Cl_2–MeOH, then MeOH[103] were used in isolating plukenetiones B-G (**77, 78, 91, 92, 99, 113**) and the ochrocarpinones A-C (**97, 98, 111**).[114] After extraction, Henry *et al.* subjected the hexane extract to repeated chromatography over silica gel using Me_2CO–hexanes mixtures to yield plukenetiones B-G (**77, 78, 91, 92, 99, 113**).[103] Chaturvedula *et al.* isolated 15,16-dihydro-16-hydroperoxyisoplukenetione F (**108**) from *Ochrocarpos punctatus* by fractionation over Sephadex LH-20 with *n*-hexane–EtOAc, followed by preparative reversed-phase TLC and HPLC. Chaturvedula *et al.* used a similar procedure to isolate the ochrocarpinones A-C (**97, 98, 111**) from *O. punctatus*.[114]

The benzophenone glycoside iriflophenone-4-*O*-β-D-glucopyranoside (**58**) was isolated from *Davallia solida* by chromatography of the *n*-BuOH layer over Sephadex LH-20, eluted with MeOH.[91] The first fraction was further purified by preparative cellulose TLC and then, using reversed-phase HPLC with MeOH–H_2O to yield **58**.

Porto *et al.* methylated a *Clusia* floral resin extract and then using silica gel and hexane–EtOAc mixtures, followed by hexane–Et_2O, and preparative argentation TLC (5% silver nitrate) with five percent EtOAc in benzene, to isolate seven polyisoprenylated benzophenones.[104] Lokvam *et al.*[110] and de Oliveira *et al.*[94] also methylated a crude extract before isolating chamones I, II (**119**, **116**), and nemorosone II (**89**) from *Clusia* species.

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The isolation of xanthochymol (**138**), shown in Figure 2.3, illustrates typical methods used to purify benzophenones. Two partitioning methods were developed in the course of our laboratory work with *G. xanthochymus*. The first method dissolved the MeOH extract in 9:1 H_2O–MeOH and this extract was partitioned sequentially with hexane and EtOAc. This was a less-than-optimum system because the benzophenones, biflavonoids, and xanthones were found in both organic phases. An optimized method resuspended the dried MeOH extract in 100% water and partitioned sequentially with

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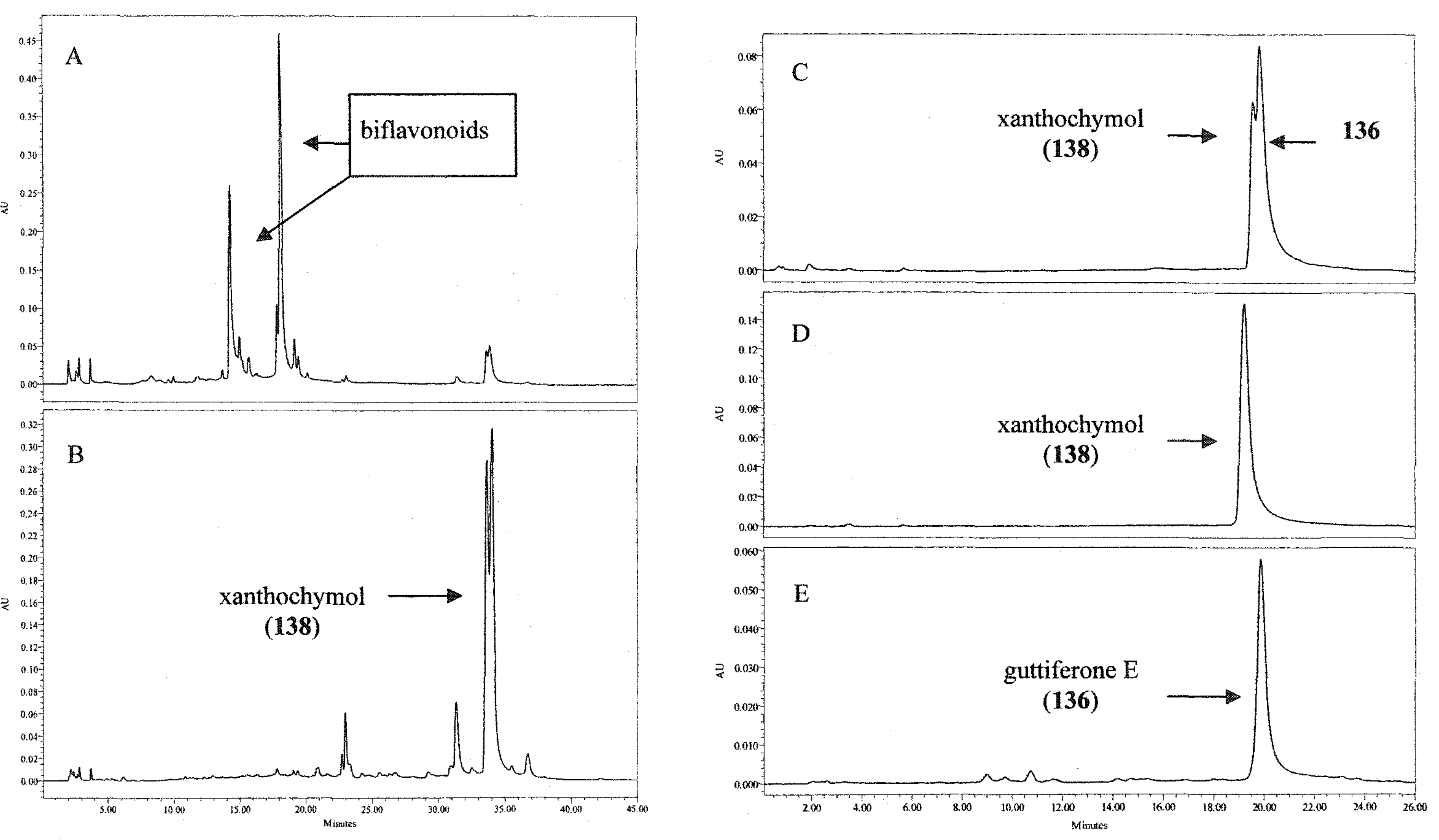

Figure 2.3. Isolation of xanthochymol (**138**). **A**: EtOAc partition, **B**: $CHCl_3$ partition; HPLC system, gradient: 9:1 10 mM ammonium acetate—MeCN to 100% MeCN (see text for details), PDA extracted at 254 nm. **C**. Fraction A, before Ag-TLC **D**. Isolated xanthochymol (**138**) **E**. Isolated guttiferone E (**136**)

$CHCl_3$ and EtOAc concentrating the benzophenone and xanthones in the $CHCl_3$ partition and biflavonoids in the EtOAc layer, Figure 2.3A and 2.3B.

After partitioning, the $CHCl_3$ layer was separated over Sephadex LH-20 and eluted with MeOH. The benzophenone-enriched fraction was repeatedly chromatography over reversed-phase (2:8–0:1 H_2O–MeCN, 5% steps) to yield two novel benzophenones (Chapter 4), the known benzophenone aristophenone A (**109**) and fractions A and B, each a mixture of benzophenone double-bond isomers.[147] Fraction A was a mixture of **136** and **138**, Figure 2.3C, and fraction B was a mixture of **131** and **133**.

Repeated attempts to separate these fractions using normal-phase and reversed-phase preparative TLC; column chromatography over Sephadex LH-20, silica gel, C_{18}, polyamide, or cyano phases; and HPLC over C_{18}, C_8, cyano, phenyl, and silica columns were unsuccessful. Other researchers have encountered difficulties in separating benzophenone double-bond mixtures consisting of **136** and **138** and related compounds.[36,123] After a protracted method development using various types of argentation (silver) chromatography, compounds **136** and **138** were isolated with a quaternary solvent system (40:10:1.25:0.2 hexane–EtOAc–95% EtOH–TFA) over normal-phase TLC impregnated with a 10% solution of $AgNO_3$, Figure 2.3D and 2.3E. This procedure was also used to separate **131** from **133**.[147]

The separation of *G. xanthochymus* was monitored by HPLC (described below) and by TLC. Two C_{18} TLC systems, 1:1 and 15:85 10 mM ammonium acetate–MeCN, were used to combine collected fractions. After development, compounds were visualized with 1% vanillin in acidified EtOH. After heating, benzophenones turned green-yellow.

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These two TLC systems proved very useful in monitoring the separation of both organic phases and for the dereplication of a number of other Clusiaceae species, Chapter 3.

Table 2.2 lists analytical and preparative HPLC methods developed by a number of researchers for the isolation and quantification of benzophenones. Most methods have utilized reversed-phase C_{18} columns with mixtures of MeCN or MeOH and H_2O, with or without an acid modifier. Exceptions include a Nucleodex β-PM,[123] a C_8,[36] and cyano[54] column were used in three different methods.

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Table 2.2. Published HPLC Methods Used for the Isolation and Quantification of Benzophenones

Compound(s) Analyzed	Isolation Methods Column; solvent system	Ref
48-50	MetaChem, Intersil ODS-3 (8 μm, C_{18}, 250 x 20 mm) 10 mL/min; isocratic 4:1 MeOH–H_2O; gradient 70:30 to 90:10 MeOH–H_2O in 30 minutes	88
69	Column not reported; 9:1 MeOH–H_2O	98
93	Waters μBondapack C_{18}, 2 mL/min; 9:1 MeOH–H_2O	113
70, 71	Column not reported; 88:12 MeOH–H_2O	98
97, 98	Shimadzu ODS C_{18} (250 x 10 mm); 70:30 MeCN–H_2O	114
6, 53, 55, 59	Dynamax-cyano (4.1 x 30 cm), 80 mL/min; 17:3 hexane–^{i}PrOH	54
109, 110	Waters μBondapack C_{18}, 2 mL/min; 9:1 MeOH–H_2O	117
108, 111	Shimadzu ODS C_{18} (250 x 10 mm); 75:25 MeCN–H_2O	114
136, 138	Nucleodex β-PM (5 μm, 250 x 10 mm) at 0 °C; 52.5:47.5:0.1% MeCN–H_2O–TFA	123
136[a], **138**[a]	Rainin Dynamax (1.0 x 25 cm); 97:3 MeCN–H_2O	36
117, 120, 121	Cosmosil 75 C_{18} Prep; 9:1 MeOH–H_2O and 1:0 MeOH–H_2O	53
131, 135, 136	Rainin Dynamax (1.0 x 25 cm); 24:1 MeCN–H_2O	36
135, 144, 145	Rainin Dynamax (1.0 x 25 cm); MeOH–4% H_2O–0.01% TFA	36
132, 133, 136, 138	Phenomenex Luna C_{18} (5 μm, 250 x 4.6 mm) 1 mL/min: gradient 9:1 10 mM ammonium acetate–MeCN to 100% MeCN in 45 minutes	147
Compound(s) Analyzed	**Quantification Methods (column; solvent system)**	
66, 88, 96	Waters Novapak C_{18} (4 μm, 3.9 x 150 mm) 1 mL/min; gradient 60:40 to 100:0 MeCN–H_2O in 60 minutes (quantified as Me esters)	94
88	Column not reported; gradient 50:50 to 100:0 MeOH–AcOH 2% in 15 minutes	134
79, 80, 85, 87, 94, 112, 114, 115	Waters Novapak C_{18} (4 μm, 3.9 x 150 mm) 1 mL/min gradient 60:40 to 100:0 MeCN–H_2O in 60 minutes (quantified as Me esters)	104

[a]isolated as a mixture

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During our isolation work with *G. xanthochymus* two HPLC methods were developed. The HPLC methods used a Phenomenex Luna C_{18} (5 μm, 250 x 4.6 mm) column and a solvent system of A = 10 mM ammonium acetate and B = MeCN. In the first method the initial conditions were 9:1 A–B, and a linear gradient was initiated until minute 45. The final solvent mixture was 0:1 A–B. The column was held at 100% B until minute 55, and then the initial conditions (9:1 A–B) were reinitiated at minute 56. The column was equilibrated for 10 minutes before the next injection. In the second system the initial conditions were 1:1 A–B, and a linear gradient was initiated at minute 4 until minute 26. The final solvent mixture was 0:1 A–B. Sample chromatograms are shown in Figure 2.3. Both HPLC systems were used to track compounds isolated from *G. xanthochymus* fruits and to dereplicate the fruits of five Clusiaceae species, Chapter 3.

2.5. Structural Elucidation of Benzophenones

The structures of benzophenones have been established by UV, IR, MS, and, most extensively, by 1D and 2D NMR. The structures of a few benzophenones have been determined by X-ray crystallography including nemorosonol (**88**),[112] epiclusianone (**81**),[107] xanthochymol (**138**),[140] and (-)-isoxanthochymol (**131**).[137,143]

Chemical tests with $FeCl_3$[83] or Gibbs reagent[65] and acetylation[65] or methylation[79] are used to show the phenolic nature of benzophenones. IR has been useful in showing that benzophenones contain hydroxyl groups, both conjugated and nonconjugated ketone groups, and aromatic C=C bonds.

Reported MS losses for benzophenones include an *m/z* at 105 (C_6H_5–CO^+) for a unsubstituted phenyl ketone A-ring,[80,101] *m/z* at 137 ($C_6H_5O_2$–CO^+) for a 3,4-dihydroxybenzophenone moiety, and *m/z* at 68 (C_5H_8) for a prenyl group. Xanthochymol

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(**138**), to our knowledge, is the only benzophenone analyzed for its MS fragmentation behavior.[142] The positive electrospray ionization (ESI) mass spectrum, Figure 2.4, of xanthochymol (**138**) showed a base peak at *m/z* $[M + H]^+ = 603$ and losses at *m/z* 467, loss of the 10-carbon side chain attached at position 8 or the loss of the 3,4-dihydroxybenzophenone moiety. The *m/z* 411 ion likely represents an additional prenyl

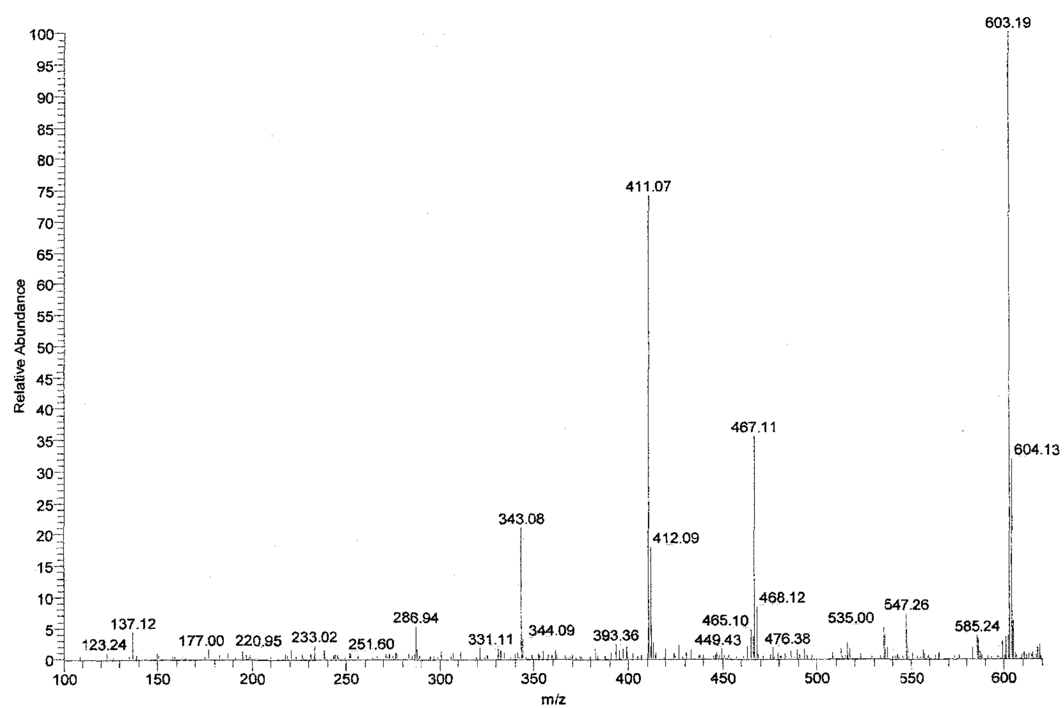

Figure 2.4. Positive ESI mass spectrum of xanthochymol (**138**)

group loss. Additional fragments were observed at *m/z* 343, 286, and 233 likely corresponding to additional losses of 1, 2, or 3 prenyl, or parts of prenyl, groups. The negative ESI mass spectrum (not shown) only displayed a base peak at *m/z* $[M - H]^- =$ 601.

The structures of most benzophenones have been determined by 1D (^1H, ^{13}C, DEPT) and 2D (COSY, HSQC, HMBC, and NOESY) NMR experiments. The majority

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of NMR spectra were recorded in $CDCl_3$ and CD_3OD. Benzene–d_6, pyridine–d_5 or mixtures of benzene–d_6 and $CDCl_3$,[111] have also been used.[36,126] The aforementioned solvents were used to resolve overlapping signals. The addition of deuterated TFA (0.1%) is also used to increase the rate of keto-enol interconversion in benzophenones. We now turn our attention to the structural elucidation of xanthochymol (**138**).

The 500-MHz ^{1}H spectrum of xanthochymol (**138**) is displayed in Figure 2.5. The chemical shifts, coupling data, multiplicity, COSY, and HMBC correlations are shown in Table 2.4. Because of the tautomeric nature of benzophenones, NMR spectra are sometimes acquired after the addition of deuterated TFA (0.1%). We found this to be essential when using $CDCl_3$, but not with CD_3OD. However, carbon spectra recorded with CD_3OD–TFA displayed sharper signals for C-1, 3, 4, 8, and 10.

To aid in the structural elucidation of xanthochymol (**138**), we divided the proton spectrum into three regions. Region one, from δ 0-3.0, contains numerous overlapping signals but yields some valuable information, specifically the number of methyl groups. Due to overlapping signals, the exact number is not conclusive, but 7 or 8 is a good approximation. Furthermore, the methyl signals appear as two groupings, two upfield signals at δ 1.01 and 1.17 and 5-6 signals between δ 1.5-1.8. The two upfield signals are characteristic of geminal-dimethyl protons on an sp^3 carbon.

The number of methyl groups from δ 1.5-1.8, along with data from the next region (δ 4.0-5.2) indicates the number and type (isopent-2-enyl versus isopent-3-enyl) of prenyl groups in xanthochymol (**138**). The ^{1}H spectrum of xanthochymol (**138**) displays signals for a least one olefinic proton at *ca.* δ 5.00 (indicating an isopent-2-enyl group) and two signals for terminal methylene protons, indicating two isopent-3-enyl groups.

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Due to overlapping ^{1}H signals, DEPT-135 and HSQC experiments are needed to confirm the number and type of prenyl groups.

The last region (δ 6.0-8.0) reveals one structural fragment. In polyprenylated benzophenones, the aromatic A-ring typically has either a 3- or 5-spin pattern. Xanthochymol (**138**) clearly displays an aromatic 3-spin system consisting of protons at δ 7.22 d (J = 2.1 Hz), 7.00 dd (2.1, 8.1 Hz), and 6.72 d (8.1 Hz). Even though the ^{1}H spectrum is crowded three important structure features are obtained: i) the type of aromatic A-ring; ii) the approximate number and types of prenyl groups; and iii) the presence of an aliphatic geminal-dimethyl group.

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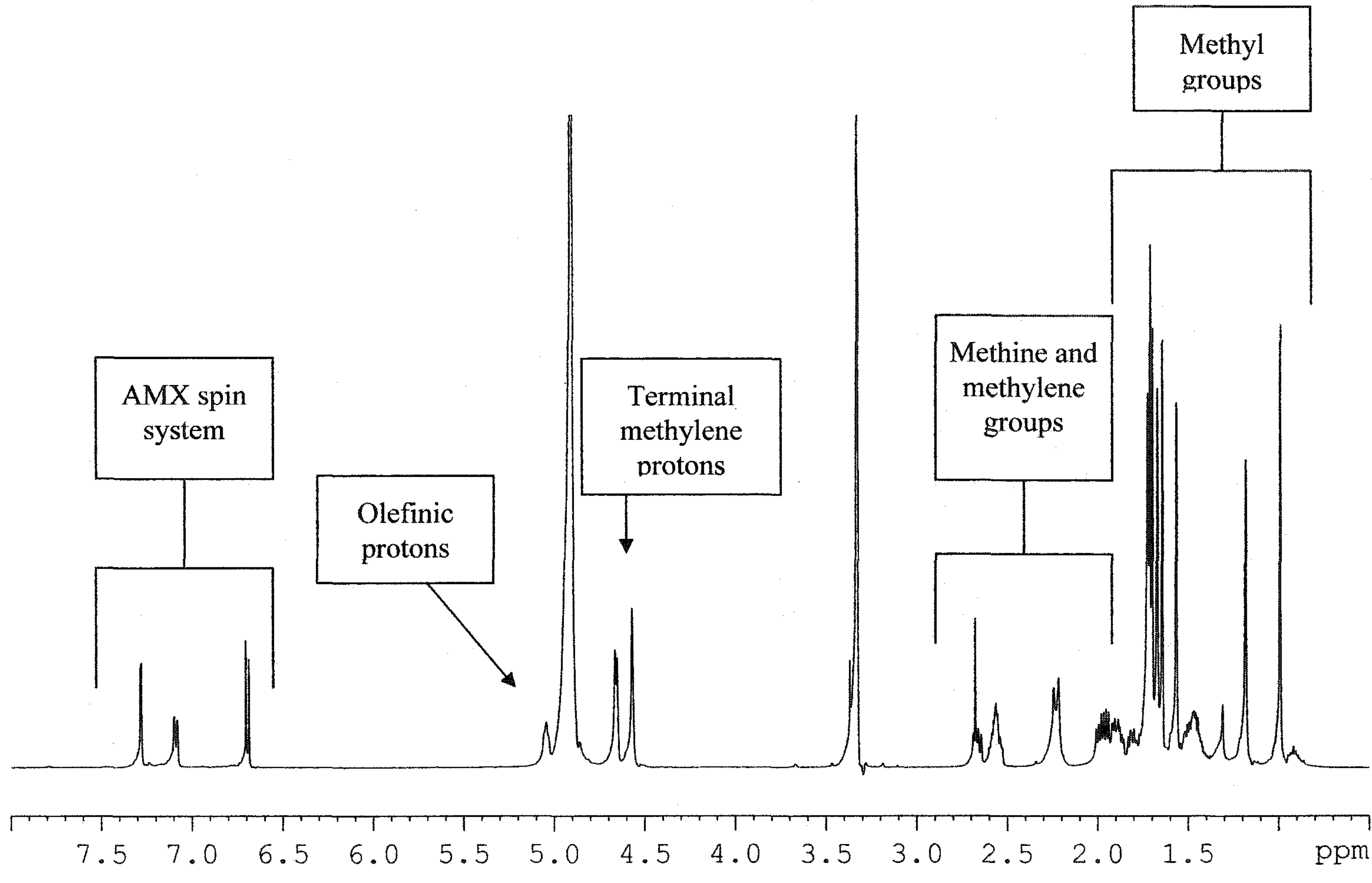

Figure 2.5. ^{1}H Spectrum of xanthochymol (**138**) recorded at 500 MHz in CD_3OD

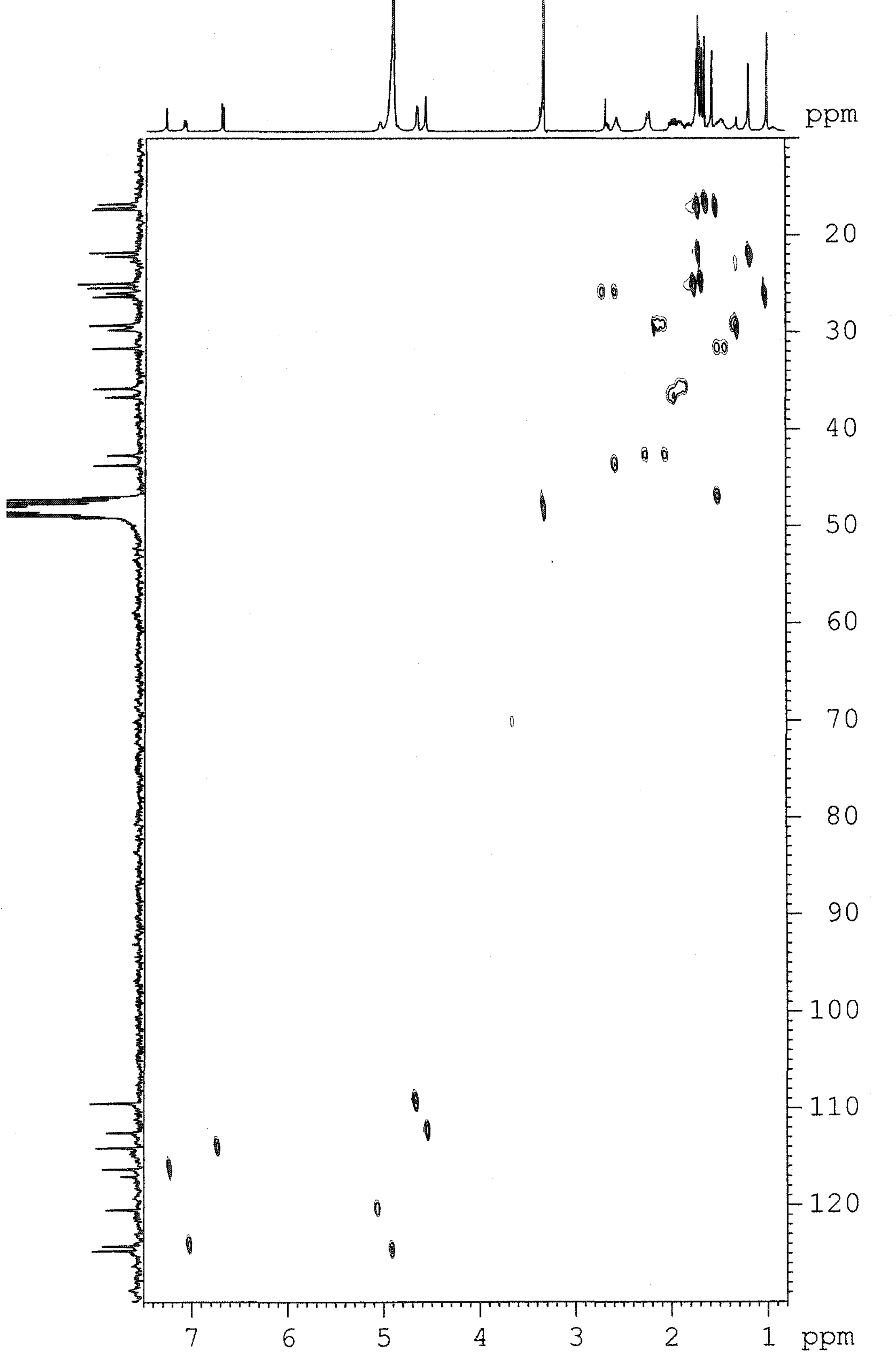

Figure 2.6. HSQC spectrum of xanthochymol (**138**) recorded at 500 MHz in CD_3OD

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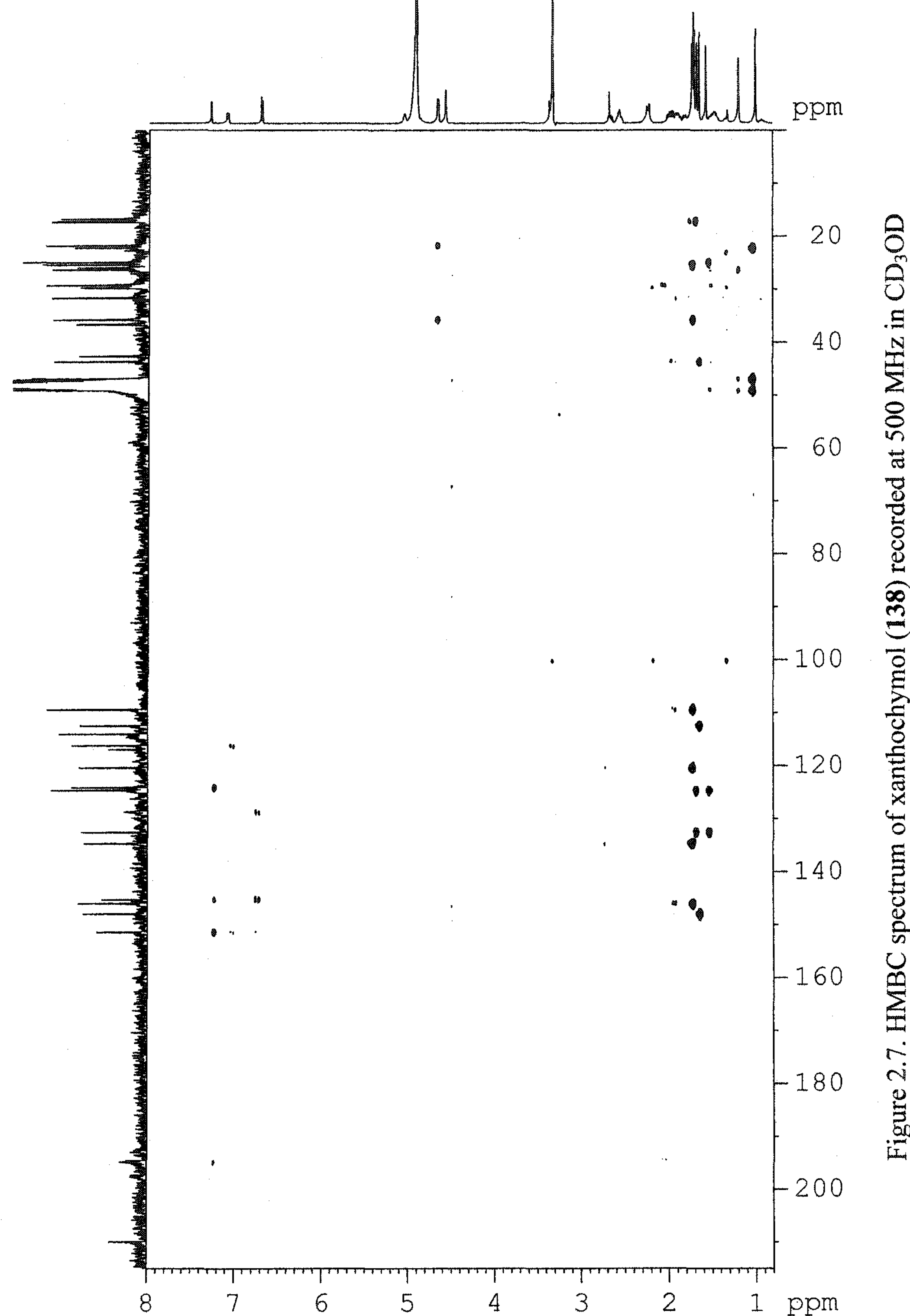

Figure 2.7. HMBC spectrum of xanthochymol (**138**) recorded at 500 MHz in CD_3OD

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The ^{13}C, DEPT-135, and DEPT-90 experiments showed a nonconjugated ketone at δ 209.8, flanked by two quaternary bridgehead carbons (δ 59.5 and 68.9), an enolized 1,3-diketone [δ 117.9 and 194.5 (2x)], a methylene carbon at δ 42.7, and a methine carbon at δ 47.0 which, together with a quaternary carbon at δ 50.4, established the bicyclo[3.3.1]nonane system in xanthochymol (**138**). The DEPT-135 showed five sp^2 methine and six aliphatic methylene carbons. The number of methine carbons, five sp^2 and two aliphatic, was confirmed by a DEPT-90 experiment. The carbon and DEPT experiments further indirectly confirmed the AMX 3-spin system with ^{13}C quaternary aromatic resonances at δ 128.7, 145.3, and 151.4, and the number of prenyl groups as four (two isopent-2-enyl and two isopent-3-enyl groups).

The HSQC spectrum, displayed in Figure 2.6, unequivocally established the number and type of protonated carbons in xanthochymol (**138**). Even though the DEPT-135 experiment displays the number of CH_3, CH_2, and CH carbons, the HSQC spectrum resolves the following: i) an olefinic CH proton is under the H_2O signal, thus **138** contains two isopent-2-enyl groups; ii) xanthochymol (**138**) has two terminal methylene groups, and therefore two isopent-3-enyl groups; and iii) the exact number of CH_3, CH_2, and CH carbons. Therefore, the HSQC spectrum establishes that **138** contains eight methyl, eight methylene, and seven methine carbons. Resonances for C-H pairs are listed in Table 2.3.

The COSY spectrum connects 3 multi-spin networks in xanthochymol (**138**). Analysis of the COSY spectrum shows one olefinic proton coupled to a methylene group. The second olefinic proton is coupled to another methylene group, and, indirectly, to an additional methylene and methine group. Also, a CH_2-CH-CH_2-CH_2 coupled spin system,

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possibly connecting two prenyl groups is observed. The three observed proton spin systems are: i) δ 5.05 (18), 2.73 (17A), and 2.55 (17B); ii) δ 4.92 (25), 2.28 (24A), 2.08 (24B), 1.52 (6), 2.25 (7A), and 2.05 (7B); and iii) δ 2.0 (29AB), 2.6 (30), 1.5 (34A), 1.3 (34B), and 1.9 (35 AB).

Table 2.3. Protonated Carbons Obtained from the HSQC Spectrum

CH_3		CH_2		CH	
$^{\delta}H$	$^{\delta}C$	$^{\delta}H$	$^{\delta}C$	$^{\delta}H$	$^{\delta}C$
1.01	26.4	1.9	35.8	1.52	47.0
1.17	22.2	2.0	36.7	2.6	43.7
1.52	17.2	1.3, 1.5	32.1	4.90	124.1
1.63	16.8	2.05, 2.25	42.7	5.05	120.4
1.67	25.0	2.08, 2.28	29.8	6.72	114.0
1.71	17.3	4.52	112.5	7.00	124.2
1.71	21.8	4.65	109.4	7.22	116.2
1.75	25.4	2.55, 2.73	26.0		

At this point, using the ^{1}H, ^{13}C, DEPT, COSY, and HSQC spectra, we have tentatively identified five structural fragments shown in Figure 2.8. We now use the

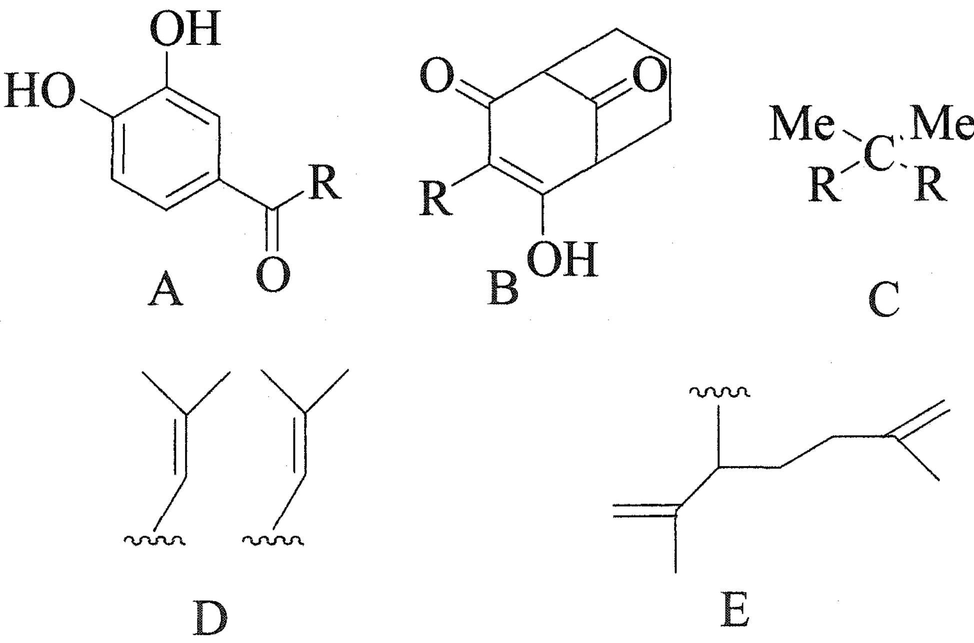

Figure 2.8. Structural fragments deduced by ^{1}H, ^{13}C, DEPT, COSY, and HSQC spectra

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HMBC spectrum, Figure 2.7, to confirm these structural fragments and to assemble the complete structure of xanthochymol (**138**), Figure 2.9.

The HMBC spectrum, Figure 2.7, confirms the following substructures: i) HMBC correlations from H-12 (δ 7.22) to C-13 (δ 145.3) and C-14 (δ 151.4) and from H-15 (δ 6.72) to C-13 (δ 145.3) and C-14 (δ 151.4) confirms the 3,4-dihydroxybenzophenone moiety (Figure 2.8A), and an HMBC cross peak from H-12 (δ 7.22) to C-10 (δ 194.8) connects the AMX system to the carbonyl carbon at C-10 (δ 194.8); ii) HMBC cross peaks from H-7B (δ 2.05) to C-1 (δ 194.5) and from H-6 (δ 1.52) to C-7 (δ 42.7) and C-5 (δ 50.4) confirmed the bicyclo[3.3.1]nonane, Figure 2.8B,; iii) the geminal-dimethyl protons which displayed HMBC cross peaks to each other, Figure 2.8C, iv) HMBC cross peaks from the methyl groups to the olefinic carbons and correlations from the CH_2 groups to their respective olefinic carbons confirmed the two isopent-2-enyl groups, Figure 2.8D; v) HMBC cross peaks from H-29 (δ 2.0) to C-30 (δ 43.7) and C-31 (δ 147.9), from H-35 (δ 1.9) to C-34 (δ 32.1), C-30 (δ 43.7), and C-37 (δ 109.4), and from H-33 (δ 1.63) to C-30 (δ 43.7) confirmed the two isopent-3-enyl groups, Figure 2.8E, are connected at C-30.

Finally, the HMBC spectrum is used to connect the structural fragments to the bicyclo[3.3.1]nonane[1] system and complete the structure of xanthochymol, Figure 2.9. Observed HMBC cross peaks from H-17B (δ 2.55) to C-3 (δ 194.5) and from H-7B (δ 2.05) to C-24 (δ 29.8) placed the isopent-2-enyl groups at C-4 (δ 68.9) and at C-6 (δ 47.0). HMBC cross peaks from Me-22 and Me-23 to C-4, 5, and 6 (δ 68.9, 50.4, 47.0, respectively) placed the geminal-dimethyl group at C-5. An HMBC correlation was

[1] For clarity the stereochemistry of the bicyclo[3.3.1]nonane is not shown, as not to confuse it with the bolded COSY cross peaks.

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observed from H-29 to C-1, thus the two isopent-3-enyl groups, Figure 2.8E, were placed at C-8. Therefore, using 1D and 2D NMR experiments we assigned the structure in Figure 2.9 to xanthochymol.

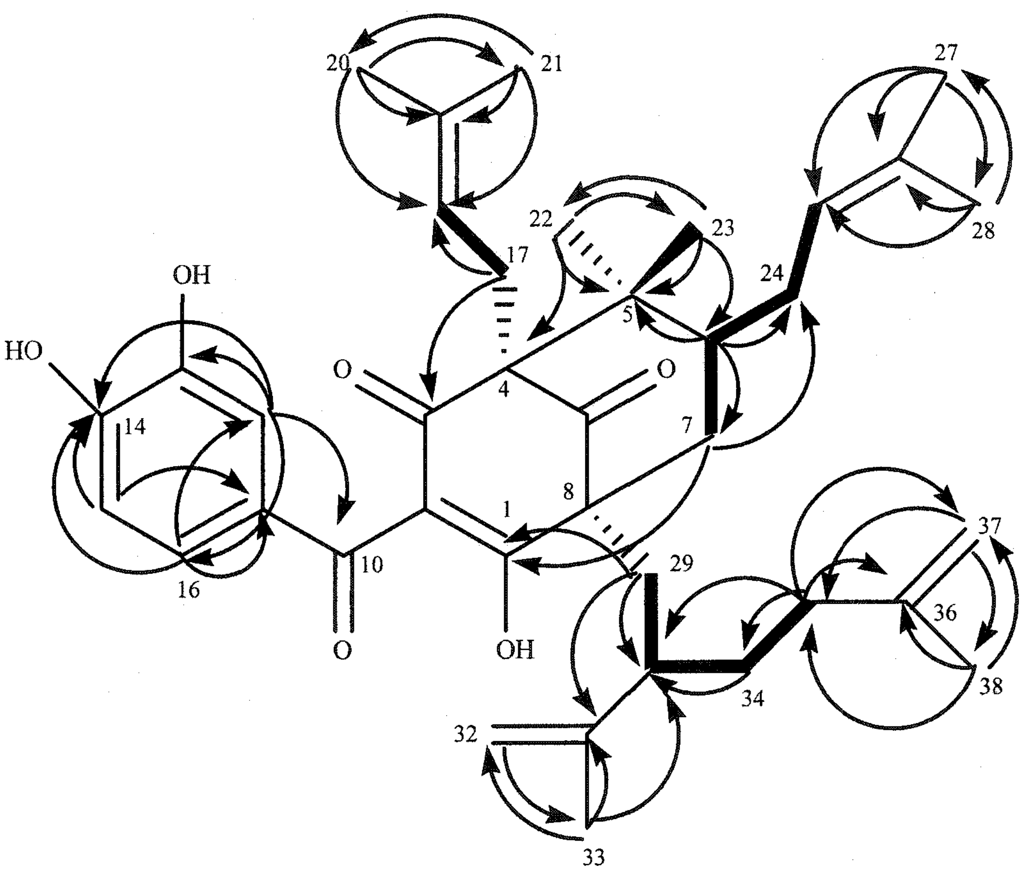

Figure 2.9. Arrows denote key HMBC cross peaks and bold lines indicated COSY correlations for xanthochymol

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Table 2.4. ^{1}H, ^{13}C, DEPT, COSY, and HMBC NMR Data for Xanthochymol (138) in CD_3OD

	H[a] (δ, mult. J= Hz)	C[b] (δ)	DEPT[b]	COSY[a]	HMBC[a]
1		194.5	C		
2		117.9	C		
3		194.5	C		
4		68.9	C		
5		50.4	C		
6	1.52, m	47.0	CH	7, 24	5, 7, 23, 24
7	2.05, 2.25, m (12.8)	42.7	CH_2	6	1, 24
8		59.5	C		
9		209.8	C		
10		194.8	C		
11		128.7	C		
12	7.22, d, (2.1)	116.2	CH	16	10, 13, 14, 16
13		145.3	C		
14		151.4	C		
15	6.72, d, (8.1)	114.0	CH	16	11, 13, 14
16	7.00, dd, 2.1, (8.1)	124.2	CH	12, 15	12, 14
17	2.55, 2.73, m	26.0	CH_2	18	3, 18, 19
18	5.05, t, (5.5)	120.4	CH	17	
19		134.7	C		
20	1.75	25.4	CH_3		18, 19, 21
21	1.71	17.3	CH_3		18, 19, 20
22	1.17	22.2	CH_3		5, 6, 23
23	1.01	26.4	CH_3		4, 5, 6, 22
24	2.08, 2.28	29.8	CH_2	6, 25	
25	4.92,	124.7	CH	24	
26		132.6	C		
27	1.67	25.0	CH_3		25, 26, 28
28	1.52	17.2	CH_3		25, 26, 27
29	2.0, m	36.7	CH_2	30	1, 30, 31
30	2.6, m	43.7	CH	29, 34	
31		147.9	C		
32	4.52, brs	112.5	CH_2		32
33	1.63	16.8	CH_3		30, 31, 32
34	1.3, 1.5, m	32.1	CH_2	30, 35	30
35	1.9, m	35.8	CH_2	34	30, 34, 36, 37
36		145.9	C		
37	4.65, brs	109.4	CH_2		35, 38
38	1.71	21.8	CH_3		35, 36, 37

[a]Recorded at 500 MHz; [b]Recorded at 300 MHz

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2.6. Biological Activities of Benzophenones

Isolated benzophenones display a number of biological activities, Table 2.5. Reported biological activities are grouped into eight categories: antioxidant, antibacterial, antifungal, antiviral, cytotoxic, molluscicide, trypanocidal, and additional. Of the 146 isolated benzophenones only 49, (33%) have been evaluated for their biological activity. This section discusses the various biological activities of isolated benzophenones.

Table 2.5. Reported Biological Activities for Benzophenones

Antioxidant			
Cmpd	**Assay**	**Result**	**Ref**
7	DPPH (1,1-diphenyl-2-picrylhydrazyl)	$IC_{50} = 66.3\ \mu M$	67
13		$IC_{50} = 5.3\ \mu M$	
17		$IC_{50} = 7.8\ \mu M$	
83		$IC_{50} = 100\ \mu M$	35
88		$IC_{50} = 44.1\ \mu M$	134
131		$IC_{50} = 13.3\ \mu M$	35
134		$IC_{50} = 10.2\ \mu M$	35, 148, 149
139		$IC_{50} = 100$	35
140		$IC_{50} = 100$	
131	Peroxide determination	Weak activity	150
134		Weak activity	
134	Hypoxanthine/xanthine oxidase system Fenton reaction system H_2O_2/NaOH/DMSO system	Suppressed the superoxide, hydroxyl, and methyl radicals	148, 151
Antibacterial			
Cmpd	**Assay/organism**	**Result**	**Ref**
86 (1% solution)	*Bacillus subtilis*	14.2 ± 0.2 mm[b]	108
	Pseudomonas aeruginosa	15.0 ± 0.2 mm[b]	
	Staphylococcus aureus	14.4 ± 0.1 mm[b]	
	Streptococcus pneumoniae	14.0 ± 0.2 mm[b]	
	Candida albicans	14.1 ± 0.1 mm[b]	
89	*Paenibacillus larvae*	19.5 mm[b]	110
	Paenibacillus alvei	2.3 mm[c]	
119	*Paenibacillus larvae*	19.7 mm[b]	110
	Paenibacillus alvei	10.0 mm[c]	
93	*Streptomyces chartrensis*, *S. violochromogenes*	50[e]	113

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Antibacterial			
Cmpd	**Assay**	**Result**	**Ref**
131	Methicillin-resistant *Staphylococcus aureus* (MRSA)	12 – 25 μg/mL	122
132		25 μg/mL[a]	
133		25 μg/mL[a]	
134		6.25 – 25 μg/mL	
138		3.1 – 12.5 μg/mL	
134	*Helicobacter pylori*	Complete inhibition at 31.5 μg/mL at 6h and 3.9 μg/mL at 12h	35, 152
138	*Staphylococcus aureus*	Active	121
	Escherichia coli	Inactive	
	Streptococcus faecalis	0.78 μg/mL[d]	34
	Klebsiella pneumonia	1.56 μg/mL[d]	
Antifungal			
Cmpd	**Assay/organism**	**Result**	**Ref**
81	*Cladosporium sphaerospermum*	Inactive	153
86	A rage of organisms	Activite	108
93	*Candida albicans*, *Candida tropicalis*, *Pseudomonas aeuruginosa*	16.2[e]	113
Antiviral			
Cmpd	**Assay**	**Result**	**Ref**
13	Epstein-Bar virus early antigen	52.7 ± 2.3 @ 16 nmol	35
83		52.7 ± 2.3 @ 16 nmol	
131		56.0 ± 1.9 @ 16 nmol	
134		47.0 ± 2.5 @ 16 nmol	
140		54.9 ± 2.6 @ 16 nmol	
141		60.1 ± 2.1 @ 16 nmol	
46	Anti-HIV	Inactive	54
53		Inactive	
55		$EC_{50} \approx 11$ μg/mL	
59		Inactive	
132		Inactive	36
135		$EC_{50} = 1 - 10$ μg/mL	
136		$EC_{50} = 1 - 10$ μg/mL	
137		$EC_{50} = 23$ μg/mL	
141		$EC_{50} = 1 - 10$ μg/mL	
142		$EC_{50} = 1 - 10$ μg/mL	
143		$EC_{50} = 1 - 10$ μg/mL	
117	Anti-HBeAg secretion on MS-G2 hepatoma cell line	10 μg/mL	53
120		10 μg/mL	
121		10 μg/mL	

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Cytotoxic			
Cmpd	**Assay**	**Result**	**Ref**
33	HSC-2 human oral squamous cell	CC_{50} = 0.17 mM	83
	HGF normal human gingival fibroblasts	CC_{50} = 0.43 mM	
37	HSC-2 human oral squamous cell	CC_{50} = 0.036 mM	
	HGF normal human gingival fibroblasts	CC_{50} = 0.090 mM	
42	HSC-2 human oral squamous cell	CC_{50} = 0.092 mM	
	HGF normal human gingival fibroblasts	CC_{50} = 0.19 mM	
47	HSC-2 human oral squamous cell	CC_{50} = 0.052 mM	
	HGF normal human gingival fibroblasts	CC_{50} = 0.19 mM	
48	KB cell line	$EC_{50} > 20$ μg/mL	88
49		$EC_{50} > 20$ μg/mL	
50		$EC_{50} > 20$ μg/mL	
63		$EC_{50} = 2.4 \pm 0.9$ μg/mL	
64		$EC_{50} = 3.3 \pm 1.5$ μg/mL	
69		EC_{50} = 10.0 μg/mL (inactive)	98
70		EC_{50} = 9.0 μg/mL (inactive)	
73		EC_{50} = 8.2 μg/mL (inactive)	
131/133[a]		IC_{50} = 5.8 μM	123
136/138[a]		IC_{50} = 10 μM	
97	A2780 human ovarian cells	$IC_{50} = 7.4 \pm 0.2$ μg/mL	114
98		$IC_{50} = 8.2 \pm 0.2$ μg/mL	
108		$IC_{50} = 8.4 \pm 0.6$ μg/mL	
111		$IC_{50} = 6.9 \pm 0.3$ μg/mL	
135		IC_{50} = 6.8 μg/mL	126
144		IC_{50} = 8.0 μg/mL	
124	P388 cell line	ED_{50} = 6.9 μg/mL	52
125		$ED_{50} > 30$ μg/mL (inactive)	
88	HeLa human cervix carcinoma	IC_{50} = 3.3 μM	134
	Hep-2 human larynx carcinoma	IC_{50} = 3.1 μM	
	PC-3 prostate cancer	IC_{50} = 7.2 μM	
	U251- central nervous system	IC_{50} = 3.9 μM	
134	HL-60 leukemia cells	IC_{50} = 9.42 μM	154
		IC_{50} = 5 – 20 μM	38
	K562 leukemia cells	IC_{50} = 5 – 20 μM	
	NB4 leukemia cells	IC_{50} = 5 – 20 μM	
	U937 leukemia cells	IC_{50} = 5 – 20 μM	

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Molluscicide			
Cmpd	**Assay/organism**	**Result**	**Ref**
81	*Biomphalaria glabrata*	Inactive	153
Trypanocidal			
Cmpd	**Assay/organism**	**Result**	**Ref**
81	*Trypanosoma cruzi*	LC_{50} = 518 μM (active *in vitro*, not *in vivo* in mice)	153
135	Epimastigotes	100 μM	125
	Trypomastigotes	83 μM	
Additional			
Cmpd	**Assay**	**Result**	**Ref**
7	Brine shrimp lethality	LD_{50} > 100 μM	67
13		LD_{50} = 43.1 μM	
17		LD_{50} > 100 μM	
81		LC_{50} = 49.7 μM	153
10	Testosterone 5α-reductase	IC_{50} > 1.00 mM	73
49	DNA strand-scission activity	43 ± 12% nicked at 2.5 μg/mL	88
131/133[a]	Microtubule disassembly inhibition	Inactive	123
136		IC_{50} = 1.5 μM	
138		IC_{50} = 2 μM	
131	Western blot analysis	Apoptosis activation of caspase-3	38
134			
138			
138	Cardiovascular effects in cats	No CNS effect at 1/5 LD50 (LD_{50} = 1000 mg/kg i.p.)	34
134	Male F344 rats using a aberrant crypt foci (ACF) bioassay w/ azoxymethane (AOM)	Significantly inhibited AOM induced ACF formation	155
	Apoptosis induction in HL-60 cells	EC_{50} = 8.4 μM	149
	Griess reaction	Reduced NO production 49, 87, and 92% at 2.5, 5, and 10 μM, respectively	
	Ulceration induction in rats by indomethacin	Significantly prevented adverse affects	151

[a]tested as a mixture; [b]zone of inhibition; [c]total inhibition, [d]minimum inhibitory concentration, [e]concentration required to eliminate 99% of organism

A number of benzophenones have been assayed for their cytotoxicity towards ovarian,[114,126] leukemia,[38,52] and CNS[134] cancer cell lines. In addition, the guttiferones

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(**132** and **135-142**) have displayed potent cytotoxicity toward leukemia[38] and ovarian cancer cell lines.[35,126]

Garcinol (**134**), also a guttiferone, has been evaluated for a number of biological activities. Garcinol was found to neutralize the superoxide anion, methyl, and hydroxyl radicals in a variety of antioxidant assays[151] and displayed anti-glycation activity in a fructose-BSA assay.[148] Sang *et al.*[149] studied the reaction mechanism between the stable free radical DPPH and **134**. They also isolated the DPPH/garcinol oxidation products[149] and tested **134** in a NO generation, apoptosis, and H_2O_2 antioxidant assays. Recently **134** was found to inhibit histone acetyltransferase *in vitro.*[156]

Two animal studies have been conducted on garcinol (**134**). The first study used F344 male rats and administered **134** at 0.01% or 0.05%. Garcinol provided significant *in vivo* protection against the development of aberrant crypt foci (ACF).[155] Another study, using male Wistar rats, evaluated a *G. cambogia* extract against indomethacin induced gastric ulcers.[157] They concluded that *G. cambogia* extract prevented gastric ulcer formation and maintained the rats at a near normal state. Even though a purified benzophenone was not tested, compounds **131** and **134** have been isolated from *G. cambogia* fruits.[124] (A *G. cambogia* extract has been evaluated as a weight loss aide.[158,159] Hydroxycitric acid is believed to be the active compound in *G. cambogia.* This compound is not a benzophenone.) Other reported activities for garcinol (**134**) include a bactericidal effect on *Helicobacter pylori,*[152] strong cytotoxicity,[38,154] and additional antibacterial activities.[122]

The guttiferones (**132** and **135-142**) displayed partial cytoprotection toward HIV-1 infection in human lymphoblastoid CEM-SS cells; however, no decrease in viral

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replication was observed.[36,129] The vismiaphenones were also assayed for their anti-HIV activity in the NCI primary HIV screen; only **55** was active.[54]

Guttiferone A (**135**) and 7-epiclusianone (**81**) were assayed against *Trypanosoma cruzi*, the etiologic agent of Chagas' disease, and found active.[125,153]

Xanthochymol (**138**) and guttiferone E (**136**) displayed outstanding activity in an *in vitro* microtubule disassembly assay.[123]

Kolanone (**86**), nemorosone II (**89**), propolone A (**93**), chamone I (**119**), and xanthochymol (**138**)[34,108,110,113] displayed significant antimicrobial and antifungal activity against a variety of pathogenic yeasts and bacteria. Compounds **131-134** and **138** displayed antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) at 3.1 – 12.5 μg/mL, nearly equal to the antibiotic vancomycin.[122]

2.7. Conclusion

Benzophenones are an interesting class of compounds displaying much structural diversity and numerous bioactivities. By far, the richest sources of benzophenones have been Clusiaceae species. However, of the approximately 37 genera and 1610 Clusiaceae species,[160] only a few species have been analyzed exhaustively. It is likely that a number of novel bioactive benzophenones are still undiscovered.

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Chapter 3: Dereplication of Five Clusiaceae Species and the LC-DPPH Assay

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3.1. Introduction

Typical activity-directed isolation can take months to identify bioactive constituents from a given plant.[161] One method to reduce time and concentrate efforts on isolating novel compounds uses activity-guided LC-MS database dereplication.[162] This chapter discusses the dereplication of five Clusiaceae species and an alternative to the traditional dereplication system, the on-line LC-DPPH assay.

3.1.1. Dereplication Systems

Dereplication is the rapid identification of known compounds from a semi-purified mixture and activity determination of unknown compounds to prioritize samples for in-depth structural and bioactivity studies. Dereplication methods have been described using electrospray LC/PDA/MS in conjunction with chemical databases (*e.g.* Natural Products Alert and Chemical Abstracts) which enhances the efficiency when searching for novel biologically active constituents.[161] I established two dereplication systems in Dr. Kennelly's laboratory. The first consists of a Waters 2695 HPLC and a 2487 dual wavelength detector, a ThermoFinnigan LCQ MS, and Waters automatic fraction collector II. Experimental data and literature information from publications, Dictionary of Natural Products, and NAPERLET was entered into a Microsoft Access database for further analysis. A detailed description of the LC-APCI dereplication system is presented in Section 3.5.3.

A modification to the traditional dereplication system, the on-line LC-DPPH assay, the second system I established, was used to quickly identify DPPH-active peaks in crude extracts. DPPH-active peaks are detected as negative peaks, using a single

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wavelength detector. The on-line LC-DPPH assay rapidly, in less than 30 minutes, separates complex mixtures and locates DPPH active peaks.

The DPPH method (Figure 3.1) is a rapid and inexpensive method to measure antioxidant capacity. Because of its odd electron DPPH is purple at 517 nm. When an antioxidant compound donates a hydrogen to DPPH a change in color (to yellow) is observed. This reaction has been widely used to predict a compound's antioxidant activity.[163]

Ph Ph N N• O_2N NO_2 NO_2 + Antioxidant Compound Hydrogen Donor —Reduction→ Ph Ph N NH O_2N NO_2 NO_2 + Stable Antioxidant Compound

Purple Yellow

Figure 3.1. Reaction of DPPH with an antioxidant compound

The on-line LC-DPPH method is described below and is illustrated in Figure 3.2. A sample on-line LC-DPPH run, using the EtOAc partition of *Mammea americana*, is shown in Figure 3.3.

3.1.2. Plants Selected for Dereplication

Clusiaceae species are rich sources of benzophenones (see Chapter 2) and biflavonoids.[164,165] Thus, to isolate additional DPPH-active compounds, fruits of six Clusiaceae species, *Garcinia aristata* (Griseb.) Borhidi, *G. livingstonei* T. Anders, *G.*

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spicata Hook. f., *Rheedia edulis* Planch. & Triana., *R. gardneriana* (Planch. & Triana) D. C. Zappi, and *Mammea americana* were selected for dereplication.

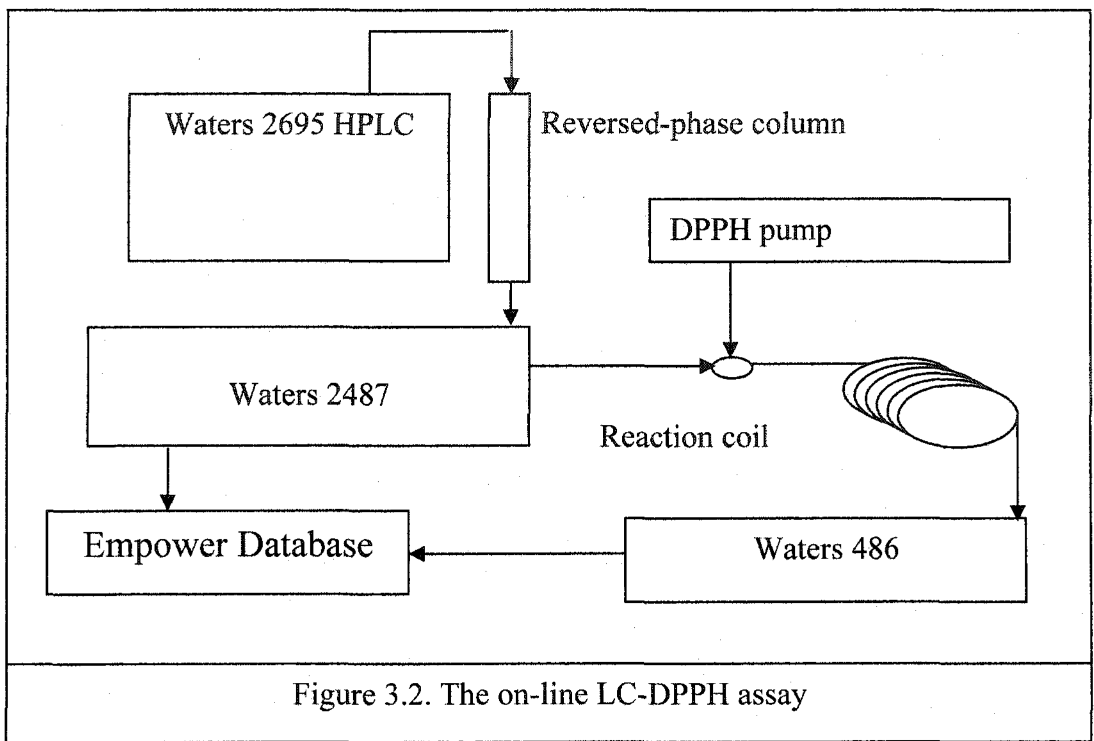

Figure 3.2. The on-line LC-DPPH assay

The fruit of each species was extracted with methanol and subjected to solvent-solvent partitioning, yielding three fractions: chloroform ($CHCl_3$), ethyl acetate (EtOAc) and aqueous. The $CHCl_3$ and EtOAc fractions were tested for their antioxidant activity in the DPPH assay. The $CHCl_3$ and EtOAc fractions displayed activity in the DPPH assay (Table 3.1) ranging from $IC_{50} = 46 - 97$ μg/mL; therefore, the chloroform and EtOAc fractions were dereplicated using LC-APCI MS to prioritize them for further isolation studies.

LC-MS results were compared with *G. xanthochymus* Hook.f., (common name "gamboge"), which displayed excellent activity in the DPPH assay and was subjected to activity-guided isolation. Two novel benzophenones (**1-2**), five known benzophenones (**3**,

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5-8), and five known biflavonoids (**11-12, 14, 20-21**) were isolated, (see Chapter 4).[147,166] This section details the phytochemical screening and dereplication of the five selected Clusiaceae species. Previous isolated compounds from studied fruits are listed in Table 3.2 and their structures are shown in Figure 3.4. Collection ID numbers and yields are listed in Table 3.3.

G. aristata (common name Cuban mangosteen) is endemic to Cuba and resembles a holly, with dark green leaves with hard spiny tips. Trees bear small yellow fruits having slightly sweet white pulp with 1-2 large seeds. There are two reports on the phytochemistry of *G. aristata*, a alcoholic bark extract displayed activity in the DPPH assay[167] (IC_{50} = 41 μg/mL), and Rubio-Cuesta *et al.* isolated aristophenones A and B (**3, 4**) from the fruits.[117]

G. livingstonei (common name "mupimbi") is a small tree, growing in Africa, producing small orange-colored fruits having a thick skin with a small amount of juicy, acidic, edible pulp surrounding a large seed. Among the Venda, a remote tribe of Southern Africa, Arnold *et al.* reported the roots and leaves were used to treat impotency (in combination with other plants), toothache, watering eyes, and as a aphrodisiac.[168] Johns *et al.* reported *G. livingstonei* was used as a wild food plant by the Batemi of Ngorongoro district in Tanzania.[169] A MeOH and dichloromethane extract of "mupimbi" displayed antifungal activity, significant brine shrimp toxicity, and cytotoxicity toward the Co-115 and SW-480 cancer cell lines.[170] Previous phytochemical studies have isolated the benzophenone guttiferone A (**9**) which displayed anti-HIV activity.[90] In addition, four biflavonoids (**11, 14, 21-22**)[171] and a number of prenylated xanthones displaying activity against human colon cancer cell lines have been isolated.[172]

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G. spicata (common name, "fukugi") is an evergreen tree commonly found in Southeast Asia especially in the Ryukyu Islands of Japan. The bark has been used as a raw material in yellow dyes. Six biflavonoids, (+/-)-fukugetin (**11**), (+/-)-3‴-*O*-methyl-fukugetin (**13**), (+/-)-volkensiflavone (**14**), GB-1 (**16**), GB-1a (**17**), GB-2a (**18**), and two biflavonoid glycosides, fukugiside (**12**) and spicataside (**15**) have been isolated from the leaves or the bark of G. *spicata*.[173-175] No benzophenones have been isolated, and no biological activates have been reported for *G. spicata*.

R. edulis is widely distributed in the humid tropical forests of Central America, from southern Mexico to Panama, up to 1,200 meters. "Mameyito" is a tall tree (up to 30 m) with opposite, thick, leathery leaves. The fruits are edible,[176] oval shaped, and orange-yellow in color, 2-3 cm in diameter. The wood is rose-yellow, has copious gummy yellow latex, and is valued for construction because it is nearly immune to insects.[177] The wood is also used for tool handles and fence posts. *R. edulis* has many common names including: "mameyito" (Bralize), "arrayan" and "palo de frutilla" (Guatemala), "waiki plum" (Belize), "chaparrón" (El Salvador), "jorco" (Costa Rica), and "sastra" (Panama).[177] No phytochemical studies detailing the chemistry of *R. edulis* fruits have been published.

R. gardneriana is a small tree, 5-8 m tall, with opposite leaves and small (1-2 cm in diameter) yellow fruits. *R. gardneriana* is widely distributed in Brazil where it is commonly known as "bacupari." "Bacupari" has a history of use in folk medicine to treat infections, inflammations, and pain.[178] Five biflavonoids (**11-12, 14, 18-19**) have been isolated from the leaves of "bacupari," including the first isolation of GB-2a-II-4'-OMe (**19**). Compound **19** showed analgesic activity in the formalin-induced pain mouse

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model.[179,180] The pericarp yielded the novel benzophenone 7-epiclusianone (**10**), which displayed antibacterial activity, brine shrimp lethality, and activity against *Trypanosoma cruzi*, the cause of Chagas' disease.[153] Also, from the wood and roots of *R. gardneriana*, a number xanthones have been isolated.[181,182,183]

Table 3.1. DPPH Values for the Tested Clusiaceae Extracts

Species	**DPPH IC_{50} ($\mu g/mL$)**	
	$CHCL_3$	EtOAc
R. gardneriana	46.3	49.5
G. livingstonei	97.0	97.0
G. spicata	81.7	85.0
G. aristata	38.2	46.4
R. edulis	53.0	57.8
G. xanthochymus	23.0	55.0

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Table 3.2. Benzophenones and Biflavonoids Previously Isolated from the Five Studied Clusiaceae Species

#	Compound Name[a]	Species	Ref
1	Gambogenone	*G. xanthochymus*	147
2	Guttiferone H	*G. xanthochymus*	147
3	Aristophenone A	*G. aristata*	144
4	Aristophenone B	*G. aristata*	144
5	Xanthochymol	*G. xanthochymus*	147
6	Guttiferone E	*G. xanthochymus*	147
7	Cycloxanthochymol	*G. xanthochymus*	147
8	Isoxanthochymol	*G. xanthochymus*	147
9	Guttiferone A	*G. livingstonei*	90
10	7-Epiclusianone	*R. gardneriana*	37,105,106,153
11	Fukugetin	*G. spicata, R. gardneriana, G. livingstonei, G. xanthochymus*	173,174,180,184, 185
12	Fukugiside	*R. gardneriana*; *G. spicata, G. xanthochymus*	175,180,185
13	Fukugetin, 3‴-O-methyl-	*G. spicata*	174
14	Volkensiflavone	*R. gardneriana, G. spicata, G. livingstonei, G. xanthochymus*	174,180,184,185
15	Spicataside	*G. spicata*	174
16	GB-1	*G. spicata*	173
17	GB-1A	*G. spicata*	173,174
18	GB-2A	*G. spicata, R. gardneriana*	173,174,180,185
19	GB-2A-II-4′-OMe	*R. gardneriana*	179
20	3,8'-Biapigenin	*G. xanthochymus*	147
21	Amentoflavone	*G. livingstonei, G. xanthochymus*	171,180
22	Podocarpusflavone A	*G. livingstonei*	171

[a]Structures are shown in Figure 3.4

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3.2. Results and Discussion

Chromatograms of the $CHCl_3$ and EtOAc partition for each species are presented in Figures 3.5 and 3.6, respectively. Using compounds isolated from *G. xanthochymus* fruits as standards and LC-MS experiential data, *G. spicata* fruits contain the benzophenones xanthochymol (**5**) and guttiferone E (**6**). The benzophenones isolated from *G. xanthochymus* fruits were not detected, by LC-MS, in the other four species, *G. aristata*, *G. livingstonei*, *R. edulis*, and *R. gardneriana*. These results are presented in Table 3.3.

Table 3.3. Yields of the Five Studies Clusiaceae Species

ID #	Species	Fruit Pulp (g)	MeOH (g)	$CHCl_3$ (g)	EtOAc (g)	Water (g)
1-259	*G. livingstonei*	175	ND	1.1106	0.1261	2.649
1-304	*R. aristata*	206	0.5053	2.5598	0.1233	ND
1-235	*G. spicata*	312	0.3882	2.6747	0.7648	ND
1-316	*R. edulis*	136	0.2058	0.4902	0.1621	5.8692
1-234	*G. gardneriana*	115	0.1624	0.3535	0.5019	ND
ND = not determined						

The $CHCl_3$ partition of *G. livingstonei* likely contains 4-5 additional benzophenones peaks at *m/z* 602. The major peak, Figure 3.5B, is likely guttiferone A (**9**), previously isolated by Gustafson *et al.*[36] from *G. livingstonei*. The additional *m/z* 602 peaks are likely guttiferone class benzophenone derivatives.

The $CHCl_3$ partition of *G. aristata* contains three groups of peaks. The first group consists of two peaks *m/z* 650 and 520. These molecular weight do not match any known benzophenones, thus, these compounds are possibly new benzophenones or other known or novel compounds. The second group also has two peaks, each with a *m/z* 602. These

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peaks have similar retention times as those in *G. livingstonei*. It is likely these peaks contain guttiferone A (**9**) and/or additional guttiferone class benzophenones. The third group of peaks has an *m/z* 670. There are five previously isolated benzophenones from Clusiaceae species having a molecular weight of 670, Table 2.1, compounds **142-146**. It is likely that this peak is bronianone (**142**), guttiferone B-D (**143-145**), or G (**146**).

The $CHCl_3$ partition of *R. edulis* and *G. livingstonei* are alike in many aspects. The retention time of the major *m/z* 602 peak, in *R. edulis*, is similar to that in *G. livingstonei*. It is likely that this major compound is guttiferone A (**9**). The additional *m/z* 602 peaks are likely other guttiferone-class benzophenones.

The $CHCl_3$ partition of the last species, *R. gardneriana,* contains the least number of peaks. This species has one major peak with *m/z* 602. The retention time of this peak is similar to the major benzophenone peak in *G. livingstonei*. It is likely that this compound is guttiferone A (**9**).

The EtOAc partition of *G. spicata* fruits contains the biflavonoids fukugetin (**11**), volkensiflavone (**14**), and amentoflavone (**21**). None of the biflavonoids (**11, 12, 14, 20,** and **21**) isolated from *G. xanthochymus* fruits were detected in the other four species by LC-MS.

The EtOAc partition of *G. aristata* and *R. gardneriana* showed these species contain a few compounds. It is likely that these two species do not contain any of the biflavonoids isolated from *G. xanthochymus* fruits. The only peak in these two EtOAc partitions have a *m/z* 602 and a similar retention time, in the $CHCl_3$ partition, to the benzophenone guttiferone A (**9**). These peaks are likely guttiferone A (**9**) that was not extracted into the $CHCl_3$ partition.

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I developed a new dereplication system (Figure 3.2) and during method development five different aqueous mobile phases were used to optimize peak shape including: water, 10 mM ammonium acetate, 10 mM formic acid pH = 2.0, 25 mM formic acid pH = 2.0, and 0.1% TFA. The best peak shape for extracts was obtained with TFA > 25 mM formic acid > 10 mM formic acid ≅ 10 mM ammonium acetate > pure water.

HPLC separations with quercetin and kaempferol at 1 mg/mL (using 0.1% TFA produced no DPPH quenching. A similar result was also noticed for plant extracts. I developed two hypotheses to explain these effects: i) TFA drives the pH well below the pK_a of a antioxidant compound, thus suppressing a compound's ability to transfer a proton to DPPH; or ii) TFA, a proton donor, dissociates easily in solution producing numerous free protons; therefore, DPPH is quenched by protons from TFA not an antioxidant compound.

In addition, adding various mobile phase modifiers caused changes in the noise level of the DPPH activity channel. The noise level, going from most to least noise was TFA < 25 mM formic acid < 10 mM formic acid ≅ 10 mM ammonium acetate < pure water. It was concluded that TFA is incompatible with the on-line LC-DPPH system for two reasons, i) using TFA results in a loss of DPPH activity; and ii) TFA produces chromatograms with a low S/N value. Therefore, the best buffer for the on-line LC-DPPH system was 10 mM ammonium acetate.

3.3. Experimental

Fruits from *R. gardneriana*, *G. livingstonei*, and *G. spicata* were collected in July 2002 and *G. aristata* and *R. edulis* were collected in May 2003. All studied species were

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collected from the Fruit and Spice Park in Homestead, Florida. Fresh frozen fruits were shipped to New York City by overnight courier, and stored at –20 °C until extracted. A voucher specimen of each fruit was prepared and will be deposited at the herbarium of The New York Botanical Garden (Bronx, NY).

3.3.1. Fruit Extraction

Fruits (*ca.* 150 g) were homogenized in a blender and extracted with MeOH three times at room temperature. The extracts were filtered and the MeOH was removed *in vacuo*. The remaining residue was resuspended in 9:1 MeOH–H_2O and partitioned sequentially with $CHCl_3$ and EtOAc.

3.3.2. Chemical and Supplies

GR grade MeOH, $CHCl_3$, and EtOAc were purchased from VWR (West Chester, PA). Water for HPLC analysis was drawn from a Milli-Q system and HPLC grade acetonitrile was purchased from VWR (West Chester, PA). A Phenomenex (Torrance, CA) Luna C_{18} 250 x 4.6 mm, 5 μm column was used for HPLC analysis.

3.3.3. LC-APCI MS

Samples were dissolved in MeOH, filtered and chromatographed over a C_{18} column using a Waters (Milford, MA) 2695 separation system equipped with a Waters 2487 dual wavelength detector operating at 254 nm. For all LC-MS experiments, the flow rate was 1 mL/min, and the column was at room temperature. The following solvent system was used: 10 mM ammonium acetate (A) and acetonitrile (B); the initial conditions were 90% A, 10% B. The initial condition was held for 4 minutes, then a linear gradient was initiated until minute 34; the final solvent mixture was 0% A, 100% B. The column was held at 100% B until minute 44 and then returned to initial conditions

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(90% A, 10% B) at minute 45. The column was reequilibrated for 10 minutes before the next injection. The total acquisition time was 44 minutes. Mass spectroscopic analysis used a ThermoFinnigan LCQ (San Jose, CA) mass spectrometer equipped with an APCI source operating in the positive mode scanning from 100-1500 amu. The capillary temperature was 150 °C and the APCI vaporizer temperature was 450 °C. Nitrogen was used as the sheath gas and helium as the auxiliary gas at flow rates of 80 and 30 units, respectively. The capillary voltage was 10 V, the tube lens offset was 0 V, and the discharge current was 5 μA. Both instruments were controlled by ThermoFinnigan's Xcalibur software version 1.3.

3.3.4. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Microtiter Assay

The DPPH assay was performed on samples as previously described.[186] Reaction mixtures containing plant extracts at four concentrations (12.5 μg/mL, 25 μg/mL, 50 μg/mL, and 100 μg/mL) and 150 μL of 400 μM DPPH are placed in 96-well microtiter plates and incubated at 37 °C for 30 minutes. Ascorbic acid and α-tocopherol were used as positive controls and as activity guides. After incubation, absorbance was read at 515 nm using a $Versa_{max}$ tunable plate reader. Antioxidant activity was determined as percent inhibition by comparison with ascorbic acid and α-tocopherol controls and calculated by % In =[(C-S)/S] x 100, where C is the absorbance by the control and S is sample absorbance. Obtained IC_{50} values (μg/mL) signify the concentration of a sample necessary to scavenge 50% of the DPPH free radical. A sample was considered to have high antioxidant activity if the $IC_{50} \leq 50$ μg/mL, moderate activity if the IC_{50} is ≥ 50 μg/mL, low activity if the IC_{50} value ≤ 150 μg/mL, and no activity if the $IC_{50} \geq 200$ μg/mL.

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3.3.5. The On-Line LC-DPPH Assay

A gradient from 90% water/buffer to 100% MeCN in 30 minutes at a flow of 1 mL/min was run using a Phenomenex Nucleosil C_{18} 100Å, 250 x 46 mm, 5 μm column. A methanolic solution of DPPH (39 mg/L) was added by a post column split supplied by a Perkin-Elmer 250 binary pump. The flow rate of the Perkin-Elmer pump was set to match the Waters 2695 separation unit. Eluding compounds and the free radical DPPH react together in a 50′ x 0.001″ loop of stainless steel tubing. The reaction time was 0.7 minutes. The reaction eluant was passed through a Waters 486 single-wavelength detector set at 517 nm. Active compounds produce a negative peak corresponding to DPPH quenching. This system was adapted from Koleva *et al.*[187] and Snyder *et al.*[188] A standard method for conducting, processing, and reporting the DPPH activity data was also established.

3.4. Conclusion

Of the five species dereplicated, *R. edulis* is the mostly likely to contain novel bioactive compounds. This species, to my knowledge, has not undergone a detailed phytochemical analysis. Even though the $CHCl_3$ partition has few compounds [likely containing only guttiferone A (**9**)], the EtOAc extract has a number of peaks not matching any of the known benzophenones and biflavonoids isolated from *G. xanthochymus* fruits.

By LC-MS analysis, *G. livingstonei* has the most number of peaks; however, this species has been subjected to a number of phytochemicals studies in the past.[36,171,172,189-191] Further analysis of the 4-5 additional peaks with *m/z* 602 and further examination of the EtOAc extract is suggested.

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Also, by LC-MS, *G. aristata* has a number of peaks molecular weights with known and unknown. Even though two benzophenones, aristophenone A and B (**109** and **110**),[117] have been previously isolated, *G. aristata* likely contains different benzophenones and biflavonoids than *G. xanthochymus*. *R. gardneriana* should not be analyzed further because this species contains only guttiferone A (**9**). Also, no further analysis is recommend for *G. spicata* fruits. This species is very similar to *G. xanthochymus* in both its chemistry and bioactivity.

In conclusion, due to the large number of unknown peaks detected by LC-MS dereplication, *R. edulis*, *G. livingstonei*, and *G. aristata* fruits are excellent candidates for in-depth phytochemical analysis. Due to a paucity of peaks detected by LC-MS dereplication and their similarity to *G. xanthochymus*, no further analysis on *R. gardneriana* and *G. spicata* is recommended.

Furthermore, an effort should be made to expand this dereplication study to include all 37 Clusiaceae genera (only two genera were represented in this study).

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Table 3.4. Benzophenones and Biflavonoids Identified in Studied Fruits by LC-MS

	MW	Compound name	*G. xanthochymus*	*G. aristata*	*G. livingstonei*	*G. spicata*	*R. edulis*	*R. gardneriana*
		Benzophenones						
1	602	Guttiferone H	X	—	—	—	—	—
2	452	Gambogenone A	X	—	—	—	—	—
3	534	Aristophenone A	X	—	—	—	—	—
5	602	Xanthochymol	X	—	—	—	—	—
6	602	Guttiferone E	X	—	—	X	—	—
7	602	Cycloxanthochymol	X	—	—	X	—	—
8	602	Isoxanthochymol	X	—	—	—	—	—
		Biflavonoids						
11	556	Fukugetin	X	—	—	X	—	—
12	718	Fukugeside	X	—	—	—	—	—
14	540	Volkensiflavone	X	—	—	X	—	—
20	538	3,8'-Biapigenin	X	—	—	—	—	—
21	540	Amentoflavone	X	—	—	X	—	—

X = detected; — = not detected

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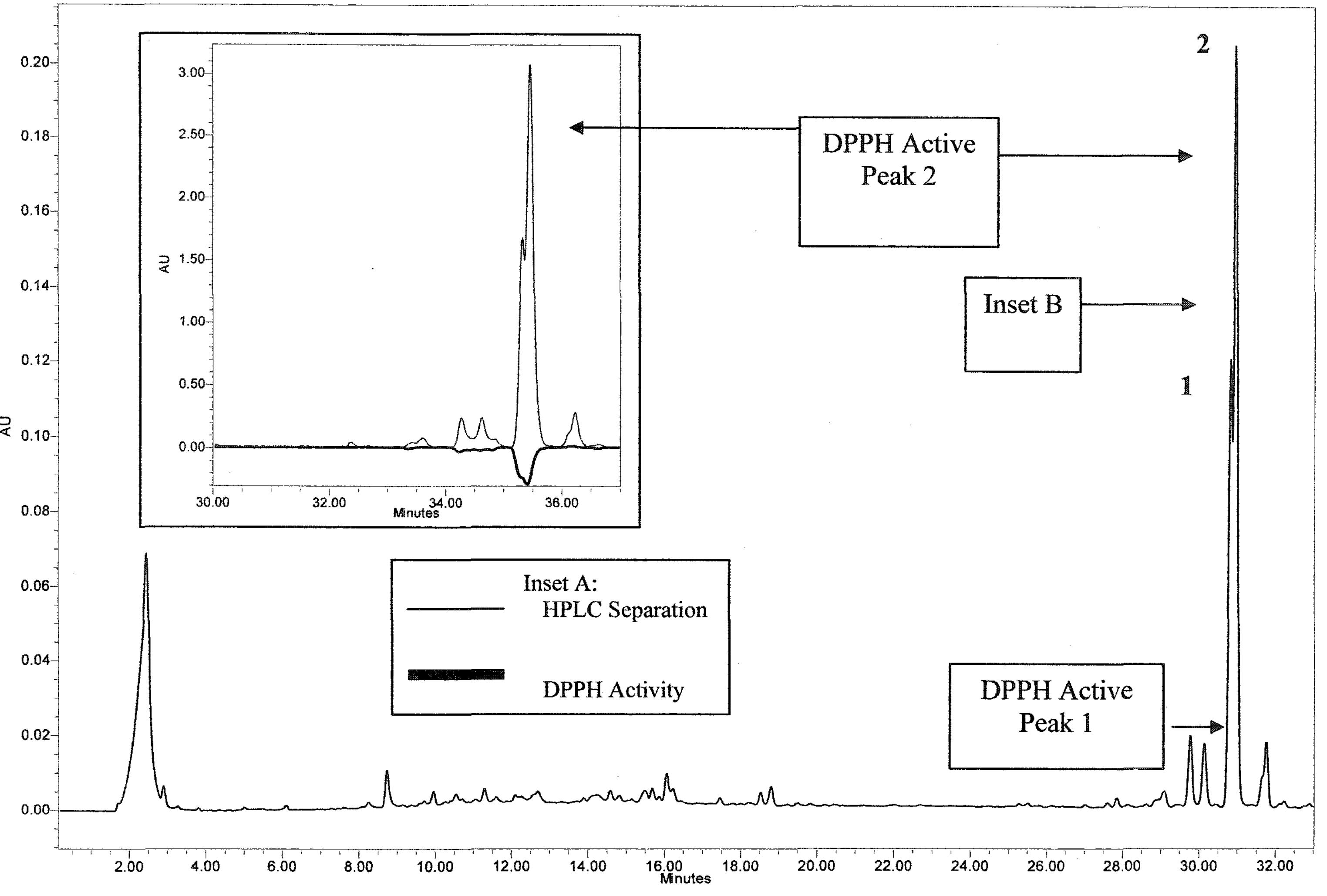

Figure 3.3. HPLC analysis of the *M. americana* EtOAc partition, linear gradient from 10% MeCN to 100% MeCN in 30 minutes. Inset: On-Line DPPH analysis of the *M. americana* EtOAc partition; HPLC analysis, same as above

1 2 3 4

5 R_1=
6 R_1=
7 R_1=
8 R_1=
9
10

11 R_1=H R_2=OH
12 R_1=Glc R_2=OH
13 R_1=H R_2=OMe
14 R_1=R_2=H
15 R_1=Glc R_2=H

16 R_1=OH R_2=H
17 R_1=R_2=H
18 R_1=H R_2=OH
19 R_1=H R_2=OMe

20

21 R_1=H
22 R_2=OMe

Figure 3.4. Structures of compounds previously isolated from studied Clusiaceae fruits

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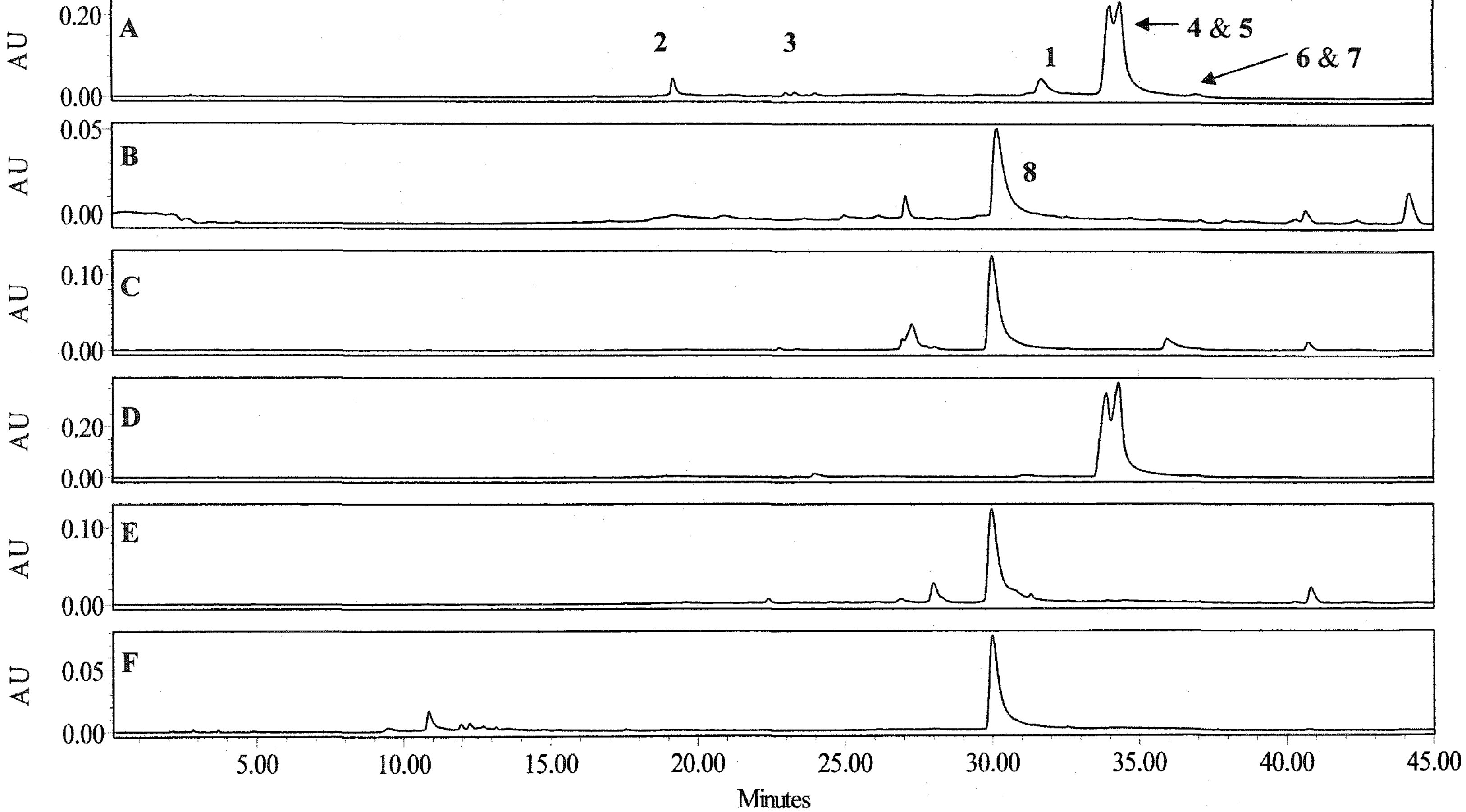

Figure 3.5. HPLC chromatograms of $CHCl_3$ partitions of studied Clusiaceae species; **A**: *G. xanthochymus*, (**1**) = guttiferone H, (**2**) = gambogenone, (**3**) = aristophenone A, (**4**) = xanthochymol, (**5**) = guttiferone E, (**6** & **7**) = cycloxanthochymol/isoxanthochymol; **B**: *G. livingstonei*, guttiferone A = (**8**); **C**: *G. aristata*; **D**: *G. spicata*; **E**: *R. edulis*; **F**: *R. gardneriana*. HPLC system was, gradient 9:1 10 mM ammonium acetate–MeCN to 100% MeCN in 45 minutes, 1 mL/min, UV detection at 254 nm

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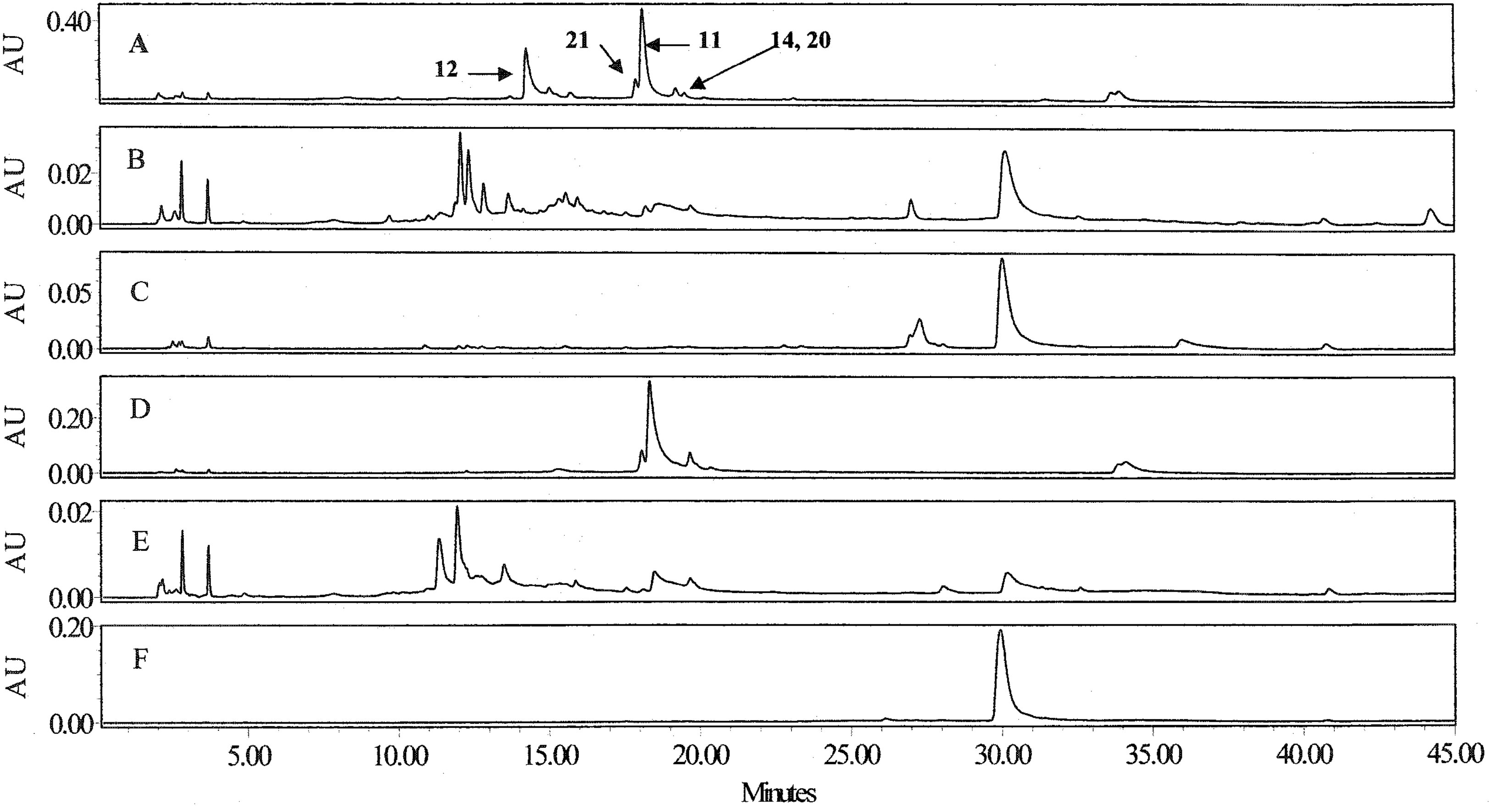

Figure 3.6. HPLC chromatograms of EtOAc partitions of studied Clusiaceae species; A: *G. xanthochymus*, (**11**) = fukugetin, (**12**) = fukugiside, (**14**) = 3,8″-biapigenin, (**20**) = volkensiflavone, (**21**) = amentoflavone; B: *G. livingstonei;* C: *G. aristata*; D: *G. spicata*; E: *R. edulis*; F: *R. gardneriana*. HPLC system, see Figure 3.4

Chapter 4: Novel Bioactive Benzophenones from *Garcinia xanthochymus* Fruits

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4.1. Introduction

Garcinia xanthochymus Hook.f. (Clusiaceae), commonly known as gamboge, is a tree endemic to India growing 8-10 m in height. The trees have dark green leaves, a gummy yellow sap, and bear yellow fruits 6-7 cm in diameter with juicy, acidic, yellow pulp containing two seeds. The acidic fruits are used in jams, preserves, and in vinegar.[192] Gamboge is used in watercolors and as a yellow fabric dye.[193] Medicinally, gamboge fruits are used in traditional medicine for treating diarrhea and dysentery.[194] Previous phytochemical studies of the leaves, seeds, fruits, and heartwood of *G. xanthochymus* have isolated two benzophenones,[142,146,195] xanthochymol (**4**) and isoxanthochymol (**7**), ten biflavonoids,[146,174,195,196] including fukugetin (**11**), fukugiside (**12**), and volkensiflavone (**13**), the flavonoid vitexin,[196] and a number of triterpenes,[197] xanthones,[39,198,199] and lipids.[200] Biological activities, including analgesic,[180] antibacterial,[34] antioxidant,[201] antiviral,[36,37,202] and cytotoxic[38] have been reported for previously isolated benzophenones and biflavonoids. Structures of isolated compounds are shown in Figure 4.1.

As part of a program to isolate novel antioxidant and cytotoxic compounds from plants,[203-205] the chloroform and EtOAc partitions from *G. xanthochymus* fruits were analyzed for their cytotoxic and DPPH activities. Both partitions displayed activity in the DPPH assay (IC_{50} = 32 and 105 μg/mL, respectively), and cytotoxicity against the SW-480 colon cancer cell line (IC_{50} = 15 and 50 μg/mL, respectively) and were selected for further analysis. In this chapter the activity-guided isolation of guttiferone H (**1**) and gambogenone (**2**), two novel benzophenones, and eleven known compounds: aristophenone A (**3**), xanthochymol (**4**), guttiferone E (**5**), cycloxanthochymol (**6**),

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isoxanthochymol (**7**), alloathyriol (**8**), amentoflavone (**9**), 3,8″-biapigenin (**10**), (+/-)-fukugetin (**11**), (+/-)-fukugiside (**12**), and (+/-)-volkensiflavone (**13**) are described. This is the first reported isolation of compounds **3**, **5**, **6**, and **8-10** from *G. xanthochymus* fruits. The antioxidant activity and cytotoxicity of **1-13** are also reported.

4.2. Results and Discussion

The methanol extract of *G. xanthochymus* fruits was sequentially partitioned with chloroform and EtOAc. Chromatography of the chloroform partition over Sephadex LH-20, followed by repeated gradient reversed-phase column chromatography using mixtures of MeOH or MeCN in H_2O led to the isolation of two novel benzophenones **1** and **2**.

Guttiferone H (**1**) was isolated as a yellow oil, and the molecular formula was established as $C_{38}H_{50}O_6$ (corresponding to 14 units of unsaturation) from HRESIMS, ^{13}C and DEPT NMR spectral data, Table 4.1. The UV spectrum of **1** showed maxima at λ_{max} (log ε) 229 (1.87) and 278 nm (2.40), similar to previously isolated polyisoprenylated benzophenones.[36,129] COSY and HMBC experiments (Figure 4.2) established the presence of the following: i) two pairs of geminal-dimethyl groups: the protons at δ 1.20 (Me-22) and 0.97 (Me-23) comprising one group and those at δ 1.21 (Me-37) and 0.95 (Me-38) the other; ii) an isopropenyl moiety composed of alkenic methylene protons at δ 4.81 (35A) and 4.67 (35B) and the methyl group at δ 1.68 (Me-36); iii) two isopent-2-enyl groups: the alkenic proton at δ 5.0 (H-18), methylene protons at δ 2.58 (17A) and 2.52 (17B), and two methyls at δ 1.68 (Me-20) and 1.67 (Me-21) comprising one group and the alkenic proton at δ 5.0 (H-25), methylene protons at δ 2.45 (24A) and 2.28 (24B), and two methyls at δ 1.67 (Me-27) and 1.62 (Me-28) the other; iv) the distinctive, aromatic, 3-spin system consisting of proton signals 12 (δ 7.36 d), 15 (δ 6.70 d), and 16

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(δ 7.21 dd) and carbons 10-16; and v) an enolized β-diketone group (carbons 1 and 3) at δ 190.6 and 190.9.

Strong HMBC cross peaks from the protons of geminal Me-22 and Me-23 to the carbons at δ 67.1, 48.0, and 47.6 established the latter as C-4, C-5, and C-6 while HMBC cross peaks from the geminal Me-37 and Me-38 to the carbons at δ 40.2, 40.9, and 49.4 identified them as C-29, C-30, and C-31. HMBC and COSY connectivities were then sought that would extend the ring system beyond C-6.

H-6 exhibited both a COSY cross peak to a proton at δ 1.92, suggesting that it was H-7, and HMBC correlations to C-4 and the carbon at δ 60.2. The proton at δ 1.92 also displayed an HMBC connectivity to the carbon at δ 60.2 and, additionally, one to C-6 and the carbon at δ 190.9. These data indicated that C-7 (δ 38.6) is methine in 1, not methylene as in other guttiferone-type benzophenones, and that the carbons at δ 60.2 and 190.9 are C-8 and C-1, respectively. In addition, H-7 showed an HMBC cross peak to the carbon at δ 212.7, which suggested that it is C-9 and that guttiferone H might possess a bicyclo[3.3.1]nonane system similar to those of **4** and other guttiferones.[36,123,129]

The next concern was the location of the isopropenyl and the two isopent-2-enyl side chains. HMBC connectivities from H-17A and H-17B to C-4 and the carbon at δ 190.6 identified the latter as C-3 and placed an isopent-2-enyl group including C-17 at C-4. In addition, H-29A exhibited an HMBC cross peak to the carbon at δ 212.7, lending support to its identity as C-9. Similarly, HMBC correlations between the protons at δ 2.18 (29A) and 1.92 (29B) and the carbons at δ 38.6 and 60.2 confirmed the identities of C-7 and C-8, respectively. Additional cross peaks between these protons and the carbons at δ 190.9 and 212.7 confirmed the assignments of C-1 and C-9, respectively, and required

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that the 3-carbon fragment that includes C-29 (carbons 29, 30, and 31) be positioned at C-8. Moreover, HMBC connectivities between H-6 and H-7 and the carbon at δ 29.7 (C-24) and COSY cross peaks between H-6 and the protons at δ 2.45 (24A) and 2.28 (24B) necessitated that the isopent-2-enyl group that includes C-24 be located at C-6.

At this point only the methylene carbons at δ 28.8 and 32.9 and the isopropenyl group remained unassigned. H-31, at δ 2.34, displayed a multitude of HMBC cross peaks, most notably to the isopropenyl methyl carbon at δ 23.5 (C-36) and alkenic carbons at δ 146.7 (C-34) and 108.3 (C-35). In addition, the protons at δ 1.68 (Me-36) and 4.67 (H-35B) exhibited HMBC connectivities back to the carbon at δ 49.4 (C-31). These correlations all required that the isopropenyl group be placed at C-31. The previously isolated benzophenones sampsonione D and I (Table 2.1, **118** and **124**) are examples of isopentenyl side chains being effectively replaced by an isopropenyl moiety.[49]

In addition, H-31 showed a COSY cross peak to the methylene proton at δ 1.82 (32B) while it, in turn, exhibited HMBC connectivities to both C-31 and the carbon at δ 32.9 (C-33). These correlations suggested that C-32 is attached to both C-31 and C-33. Moreover, the methylene proton at δ 1.76 (33B) displayed HMBC cross peaks to both C-7 and C-8 and the carbon at δ 28.8 (C-32) while its geminal partner (33A) exhibited an HMBC correlation to C-6. These HMBC connectivities together with a COSY correlation between H-32B and H-33A complete the novel 7-membered D-ring by requiring that C-33 be bonded to C-7.

There are no HMBC correlations to C-2 (δ 119.1), however, it is reasonable to depict C-2, together with C-1 and C-3, as an enolized 1,3-diketone comprising part of the B-ring of a bicyclo[3.3.1]nonane system. Note that only one of two possible

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equilibrating, endocyclic, enolized 1,3-diketone structures is depicted in **1**. The nearly identical ^{13}C chemical shifts of carbons 1 and 3 indicate that an equally probable isomer exists in which C-3 is a carbonyl carbon and an endocyclic double bond occurs between carbons 1 and 2.

In addition, a proton spin system was observed from H-31 (δ 2.34) to H-32B (δ 1.82), H-33B (δ 1.76) and to the signals at δ 1.92 (H-7, H-32A, and/or H-33A). This established that C-31, C-32, and C-33 are contiguous. In addition, a coupled spin system was observed consisting of protons H-25 (δ 5.0), H-24AB (δ 2.45 and 2.28), H-6 (δ 1.4), and H-7 (δ 1.92).

Gambogenone (**2**) was isolated as a yellow oil whose molecular formula was established as $C_{27}H_{32}O_6$ (corresponding to 12 units of unsaturation) from HRESIMS, ^{13}C and DEPT NMR spectral data, Table 4.2. The UV spectrum of **2** showed maxima at λ_{max} (log ε) 281 (2.88) and 322 nm (2.58), suggesting that it was also a member of the polyisoprenylated benzophenone family.[36,129] Additional similarities to known benzophenones included: i) two high-field methyl groups at δ 0.83 and 1.08, characteristic of a geminal-dimethyl group (Me-23 and Me-24); ii) a 7.8-Hz triplet at δ 4.99, a methylene group at δ 2.41, and two methyl groups at δ 1.60 and 1.70, indicative of an isopent-2-enyl group (protons 18-22); and iii) a 3,4-dihydroxybenzophenone moiety composed of protons at δ 6.79 d (H-16), 7.25 dd (H-17), and 7.38 d (H-13). COSY and HMBC experiments (Figure 4.2) confirmed the presence of the first and second above-mentioned systems and the 3,4-dihydroxybenzophenone moiety (carbons 11-17). In addition, they revealed that the alkenic methylene protons at δ 4.81 (26A) and 4.64 (26B) and the methyl group at δ 1.64 (Me-27) are part of an isopropenyl group (carbons 25-27).

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Benzophenones, such as xanthochymol (4), typically possess side chains at the 4-, 6-, and 8-positions.[36,123,129,142] Strong HMBC cross peaks from the protons of geminal Me-23 and Me-24 to the carbons at δ 38.2, 40.4, and 48.7 established the latter as C-4, C-5, and C-6, respectively. Surprisingly, C-4 was methine and, therefore, likely lacked a side chain. HMBC connectivities between (i) the methine proton at δ 2.42 (H-4 or H-6) and C-25 and (ii) both H-26B and Me-27 and the methine carbon at δ 48.7 suggested the latter carbon was C-6 and that the isopropenyl group was attached at C-6. In addition, COSY correlations between the proton at δ 2.42 and the diastereotopic methylene protons at δ 2.05 (7A) and 1.67 (7B) not only necessitated that the methylene carbon at δ 26.7 (C-7) be also bonded to the methine carbon at δ 48.7 but also confirmed that this carbon was C-6. This, in turn, confirms that C-4 is methine and lacks its usual side chain.

HMBC cross peaks between H-7A and C-25 confirmed the isopropenyl group was attached to C-6 while those between H-7B to the quaternary carbons at δ 40.4 (C-5) and 54.9 established the latter as C-8. HMBC connectivities (Figure 4.2) between the methylene protons at δ 2.41 of the isopent-2-enyl group (H-18) and both the quaternary carbon at δ 54.9 (C-8) and carbonyl carbon at δ 201.5 (C-1) required that the isopent-2-enyl moiety be placed at C-8.

At this point only carbons at δ 207.6, 173.0, 130.2, and 35.6 remained unassigned. The methylene group (δ_C = 35.6; $\delta_{H(A)}$ = 1.85, $\delta_{H(B)}$ = 1.82) is unusual in that its protons exhibit more combined HMBC correlations than any other protons, namely to C-1, C-4, C-7, C-8, C-10, and C-18. In addition, H-9A and H-9B display weak COSY cross peaks to H-4. Due to the extent of its HMBC connectivities, the 9-methylene group had to be located between C-8 and C-10, creating a novel 7-membered B/C-ring system. Dreiding

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models demonstrate that the COSY cross peaks between H-4 and the methylene protons are due to W-coupling. W-arrangements exist between H-4 and H-9A in one $CH_2C(O)$ bridge conformation and between H-4 and H-9B in the other. While there are no HMBC connectivities to either C-2 (δ 130.2) or C-3 (δ 173.0), it is reasonable to depict them as an enolized 1,3-diketone comprising part of the B-ring of the bicyclo[3.3.2]decane system of **2**.

The NMR data of certain carbons of **2** suggest that the nature of the α,β-unsaturated ketone in its B-ring is substantially different from those of the other isoprenylated benzophenones. Specifically, the chemical shift of C-3 (δ 173.0) is nearly identical to that (δ 173.4) reported for the 3-*O*-methoxyl derivative of xanthochymol (**4**).[123] In addition, the chemical shift of C-11 (δ 191.2) is *ca.* 5 ppm more shielded, while that of C-2 (δ 130.2) is *ca.* 12 ppm more deshielded than those of analogous benzophenones.[36,129] These data suggest that structure **2** is a fairly accurate representation of gambogenone with carbons 1 and 11 being essentially carbonyl carbons with an endocyclic 2,3-double bond largely localized at these two carbons.

In addition, the previously isolated benzophenone **3**, and a xanthone **8** were also obtained. After repeated column and preparative chromatography compounds **4-5** and **6-7** were isolated as isomeric mixtures; their separation was achieved using normal-phase TLC impregnated with Ag^+ ions.

The EtOAc partition was chromatographed over Sephadex LH-20, followed by preparative HPLC affording the biflavonoids **9-13**. The structures (Figure 4.1) of the eleven known compounds (**3-13**) were determined by comparing their spectral data with reported literature values.

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Compounds **1-13** were screened for their cytotoxicity in the SW-480 colon cancer cells and antioxidant activity in the DPPH free radical assay, Table 4.3. The novel benzophenone **1**, as well as its analogs, **3-7**, displayed potent cytotoxicity in the SW-480 colon cancer cells. The effects of **1** on apoptosis and cell cycle distribution in SW-480 colon cancer cells were studied by flow cytometry. Compound **1** exhibited a potent dose-dependent increase in sub-diploid cells at 24 h from 10.2% in untreated controls to 11.8% at 8 μM, 15.6% at 16 μM, and 19.8% at 25 μM. After 48 h of treatment with **1** at 16 μM, 89% of cells were sub-diploid compared to 12% in untreated controls. Additionally, SW-480 cells treated with compound **1** at 16 μM for 3 hours exhibited rapid loss of mitochondrial potential in virtually all cells while controls exhibited active mitochondrial transport indicative of healthy cells. Moreover, SW-480 cells treated with compound **1** at 16 μM for 12 hours also exhibited positive Annexin V staining in 80% of the treated cells but not in controls. Annexin positive cells were virtually negative for propidium iodide staining–an indication of early apoptosis. Previous researchers have reported potent cytotoxicity and apoptosis induction for **4** and related benzophenones in leukemia cell lines.[38]

Garcinol (the enantiomer of **5**) has been shown by other researchers to induce apoptosisin human leukemia cells[38,149] and have chemopreventive activity in a rodent model of colorectal carcinogenesis.[155] In addition, Balasubramanyam *et al.*[156] recently identified garcinol as a potent inhibitor of histone acetyltransferases p300, a key regulatory step in gene expression and cell cycle. Therefore, benzophenones from *G. xanthochymus*, (**1**, **3–7**) may exhibit similar effects.

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Compound **2** was the least cytotoxic (IC_{50} = 188 μM) of the isolated benzophenones, and is structurally different then compounds **1**, **3**–**7**. Compound **2** has a bicyclo[3.3.2]decane system with a isopropenyl group at C-6 and only one prenyl sidechain. It remains undetermined if changes in attached prenyl sidechains or the bicyclo[3.3.2]decane system caused this significant reduction in cytotoxicity.

In addition, the isolated biflavonoids displayed cytotoxicity against SW-480 colon cancer cells, in a range from IC_{50} = 89–185 μM. The biflavonoid glycoside, fukugiside (**12**), displayed weak activity in SW-480 colon cancer assay.

The novel benzophenones **1** and **2** displayed antioxidant activity in the DPPH free radical assay with IC_{50} = 64 and 38.7 μM, respectively. The previously isolated benzophenones, **3-7**, also displayed antioxidant activity in the DPPH assay in a range from IC_{50} = 73 – 125 μM. The biflavonoid fukugetin (**11**) displayed the highest antioxidant activity in the DPPH assay (IC_{50} = 62 μM) and fukugiside (**12**), the glycoside of **11**, was half as active, IC_{50} = 116 μM. The remaining biflavonoids (**9**, **10**, and **13**) displayed different levels of activity (IC_{50} = 184, > 400, and 298 μM, respectively), despite having the same number of phenolic functional groups. To our knowledge these are the first reported DPPH values for the biflavonoids **9-13**.

4.3. Experimental Section

4.3.1. General Experimental Procedures

^{1}H, ^{13}C, DEPT, COSY, HSQC, and HMBC NMR spectra were measured using a JEOL GX 400-MHz, a Bruker DMX 500-MHz, or a Bruker DRX 300-MHz spectrometer. The 2D-TOCSY experiments were recorded at 300 MHz using standard Bruker plus sequences with mixing times of 80, 160, 200, and 300 msec. A Waters 2695

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separation system, equipped with a 996 photodiode array detector, using a Phenomenex (Torrance, CA) Luna C_{18} column (250 x 4.6 mm, 5 μm) or a Nucleosil C_{18} column (250 x 4.6 mm, 5 μm) was used for analytical HPLC. Preparative HPLC separations used a Waters Delta 600 pump, equipped with a Waters 486 single-wavelength detector at 254 nm, and a Phenomenex Nucleosil C_{18} column (250 x 21.1 mm, 10 μm). Molecular weights were determined using a ThermoFinnigan electrospray LCQ mass spectrometer in the positive and negative modes. HRESIMS was performed on a Micromass Q-TOF Ultima mass spectrometer. Optical rotations were measured on a JASCO DIP-140 polarimeter. UV spectra were measured on a Lambda 2 UV/VIS spectrophotometer. Microplates were read using a Molecular Devices $Versa_{Max}$ plate reader at 515 nm. Microplates for MTT assay were read using a Biokinetics plate reader. The MTT assay was purchased from Boeringher-Mannheim (Indianapolis, IN). The SW-480 cell cultures were maintained in Dulbecco's modified Eagle medium (Gibco-BRL, Grand Island, NY). Apoptosis and cell cycle distribution were quantified on a FACScan flow cytometer (Becton-Dickinson, San Jose, CA).

Reversed-phase TLC analysis was performed on RP-18 F_{254} (Merck, Darmstadt, Germany) plates. Normal-phase TLC was performed on silica gel 60 (20 x 20 cm) F_{254}, 250 μm (Merck, Darmstadt, Germany) plates. Compounds were visualized by spraying with a mixture of 1 g vanillin, 10 mL H_2SO_4 (concentrated), and 90 mL ethanol and heated. ACS grade silver nitrate was purchased from Carolina Biological Supply Company (Burlington, NC). Sephadex LH-20 (25–100 μm; Pharmacia Fine Chemicals, Piscataway, NJ), and reversed-phase C_{18} silica gel (40 μm; J. T. Baker, Phillipsburg, NJ) were used for column chromatography. Amentoflavone was purchased from the Indofine

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Chemical Company (Hillsborough, NJ). Propidium iodide, RNAse A, and PBS were purchased from Sigma Chemical Company (St. Louis, MO).

4.3.2. Plant Material

G. xanthochymus fruits were collected from the Fruit and Spice Park (Homestead, FL) in February 2002. Fresh frozen fruits were shipped to New York City by overnight courier and stored at –20 °C until extracted. A voucher specimen of *G. xanthochymus* was prepared and deposited at The William and Lynda Steere Herbarium, New York Botanical Garden (Bronx, NY).

4.3.3. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Microtiter Assay

The DPPH assay was performed on samples as previously described.[186] Reaction mixtures containing plant extracts at four concentrations (12.5 μg/mL, 25 μg/mL, 50 μg/mL, and 100 μg/mL) and 150 μL of 400 µM DPPH are placed in 96-well microtiter plates and incubated at 37 °C for 30 minutes. Ascorbic acid and α-tocopherol are used as positive controls and as activity guides. After incubation, absorbance is read at 515 nm using a $Versa_{max}$ tunable plate reader. Antioxidant activity was determined as percent inhibition by comparison with ascorbic acid and α-tocopherol controls and is calculated by $\% \text{ In} = [(C-S)/S] \times 100$, were C is the absorbance by the control and S is sample absorbance. Obtained IC_{50} values (μg/mL) signify the concentration of a sample necessary to scavenge 50% of the DPPH free radical. A sample was considered to have high antioxidant activity if the $IC_{50} \leq 50$ μg/mL, moderate activity if the IC_{50} is ≥ 50 μg/mL, low activity if the IC_{50} value ≤ 150 μg/mL, and no activity if the $IC_{50} \geq 200$ μg/mL.

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4.3.4. Cell Culture

SW-480 human colon cancer cells were maintained in Dulbecco's modified Eagle medium with 10% fetal bovine serum in a normal atmosphere with 5% CO_2 at 37 °C. Cells were grown to 40% confluence prior to treatment with test compounds. All cultures were passaged weekly, and the media was changed three times a week. No antibiotics were added at any time during the experiment. In all experiments, study compounds were dissolved in DMSO and added to the medium at the start of the incubation.

4.3.5. Microtetrazolium (MTT) Assay

The MTT assay was carried out according to the manufacturer's instructions. Briefly, approximately 30,000 cells were plated in 96-well flat-bottom plates with 100 μL of medium. When cells reached 40% confluence, the medium was changed and cells were exposed to pure compounds or extracts. After 72 h cells were washed 3 times with PBS, and then 100 μL of Dulbecco's modified Eagle medium containing 10 μL of 5 mg/mL MTT solution in PBS was added for 4 h. Finally, 100 μL of MTT solubilization solution was added to each well to dissolve the formazan crystals. Absorbance was read at 570 nm. Octuplet wells were assayed for all tested compounds or extracts. Linear regression analysis was used to determine IC_{50} values.

4.3.6. Flow Cytometry

The flow cytometry procedure is described in detail by Darzynkiewicz *et al.*[206] Briefly, culture media containing the floating cells were harvested. Remaining adherent cells were trypsinized and harvested as well. The cells were then washed twice with PBS and fixed with 70% EtOH chilled at –20 °C and kept in 70% EtOH overnight at –20 °C. Before analysis, cells were washed a third time with PBS, resuspended, and incubated for

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30 minutes in a staining solution containing 0.05 mg/mL propidium iodide and 1 mg/mL RNAse A in PBS. The cells in the suspensions were then analyzed on a flow cytometer. At least 10,000 cells were gated for analysis by flow cytometry. Data was plotted on FL2-A histograms and sub-diploid cells were considered apoptotic. Cell cycle analysis was performed using the FlowJo software version 6.0. Apoptosis is expressed as percentage of sub-diploid cells out of all analyzed cells. Cell cycle distribution is expressed as percentage of analyzed cells in G1, S, or G2/M phase of the cell cycle.

4.3.7. Argentation TLC

Silica gel plates were soaked in a 10% ethanolic solution of $AgNO_3$ for 5 minutes and dried overnight in a 50 °C oven protected from light. The benzophenone mixture was applied at a concentration of 20 mg/mL and developed using a solvent system of 40:10:1.25:0.2 hexane–EtOAc–95% EtOH–TFA. Compounds were scraped from the silica gel plates and eluted with MeOH. To remove the Ag^+ ions the MeOH/compound mix was concentrated *in vacuo* to *ca.* 5 mL and diluted with 95 mL of water. This mixture was partitioned twice with EtOAc. The EtOAc layers were combined and extracted three times with 10% NaCl and three times with H_2O. This procedure was adapted from Lima *et al.*[207] and Momchilova *et al.*[208]

4.3.8. Extraction and Isolation

G. xanthochymus fruit pulp (13 kg) was extracted exhaustively with MeOH and concentrated *in vacuo* to yield 310 g of dark green residue. This residue was resuspended in H_2O and partitioned sequentially with chloroform and EtOAc. The chloroform and EtOAc partitions were concentrated *in vacuo* to give 4.91 g and 4.21 g of residue, respectively.

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The chloroform partition (4.91 g) was chromatographed over Sephadex LH-20 (200 x 2.5 cm), and eluted with MeOH. Seven combined fractions, A–G, were obtained by RP_{18} TLC analysis (1:1 or 15:85 10 mM ammonium acetate–MeCN).

Fraction C (2.5 g) was separated by reversed-phase column chromatography (CC) (200 g; 4:6–0:1 H_2O–MeCN, 5% steps), and collected fractions were combined by RP_{18} TLC analysis (1:1 or 15:85 10 mM ammonium acetate–MeCN) to afford six combined subfractions, A2-F2.

Subfraction C2 (56 mg) was repeatedly chromatographed over Sephadex LH-20 (65 x 1.0 cm), and eluted with MeOH to yield **3** (2.1 mg). Subfraction E2 (130 mg) was rechromatographed over reversed-phase CC (100 g; 3:7–0:1 H_2O–MeCN, 5% steps) and recombined into six subfractions by reversed-phase HPLC to yield subfraction E3 (63 mg), a mixture of **6** and **7**. Similarly, subfraction F2 (1.12 g) was repeatedly chromatographed over reversed-phase CC (100 g; 15:85–0:1 H_2O–MeCN, 5% steps) to yield **2** (13.5 mg) and subfraction F3 (900 mg), a mixture of **4** and **5**. Despite repeated attempts to separate **4** from **5** and **6** from **7** by normal and reversed-phase preparative TLC; Sephadex LH-20, reversed and normal-phase, polyamide, and cyano column chromatography; plus HPLC using C_{18}, C_8, cyano, phenyl, and silica columns, **4** and **5** plus **6** and **7** remained mixtures. Other researchers have encounter difficulties in separating benzophenone double-bond mixtures.[36,122,123] After a protracted method development using various types of argentation chromatography, compounds **4-7** were isolated by argentation TLC. Ag-TLC yielded **6** (2.3 mg) and **7** (2.2 mg) from subfraction E3 and **4** (21.2 mg) and **5** (22.4 mg) from subfraction F3.

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Fraction D (340 mg) was purified over C_{18} CC (100 g; 6:4–0:1 H_2O–MeOH, 10% steps) to yield **1** (38 mg). Fraction G (70 mg) was separated over C_{18} CC (20 g; 8:2–0:1 H_2O–MeOH, 5% steps) and collected fractions were recombined based on RP_{18} TLC (1:1 10 mM ammonium acetate–MeCN) into two subfractions A1 and B1. Subfraction A1 (17 mg) was purified by preparative HPLC (45:55 H_2O–MeOH, 5 mL/min) to yield **8** (3.0 mg).

The EtOAc partition (4.21 g) was chromatographed over Sephadex LH-20 (200 x 2.5 cm) and eluted with MeOH. Fractions were combined using RP_{18} TLC (1:1 10 mM ammonium acetate–MeCN) analysis to give thirteen fractions, A–M. Fraction G (200 mg) was chromatographed over Sephadex LH-20 (200 x 2.5 cm) and eluted with MeOH to yield **12** (42.3 mg). Fraction H (40.4 mg) was separated by preparative HPLC (65:35 H_2O–MeCN, 10 mL/min) to yield **10** (17.5 mg). Fraction J (340 mg) was chromatographed over Sephadex LH-20 (200 x 2.5 cm) and eluted with MeOH yielding **11** (86.3 mg) and subfraction J1 (155 mg); a mixture of **11** and **13**. Subfraction J1 (137 mg) was repeatedly separated by preparative HPLC (65:35 H_2O–MeCN, 10 mL/min) to yield **13** (9.8 mg). Fraction M (50 mg) was chromatographed over of Sephadex LH-20 (30 x 1.5 cm) to yield **9** (23.0 mg).

Guttiferone H (1): yellow oil; $[\alpha]_D$ +94° (*c* 0.0061, $CHCl_3$); $[\alpha]_D$ +57° (*c* 0.0061, MeOH); UV (MeOH) λ_{max} (log ε) 278 (2.40) and 229 (1.87) nm; ^1H and ^{13}C NMR spectral data, see Table 4.1: Negative ESIMS *m/z* 601 $[M - H]^-$. Positive HRESIMS *m/z* $[M + H]^+$ 603.3672 (calcd for $C_{38}H_{50}O_6 + H$, 603.3686).

Gambogenone (2): yellow oil; $[\alpha]_D$ –5° (*c* 0.0034, MeOH); UV (MeOH) λ_{max} (log ε) 281 (2.88) 322 (2.58) nm; ^1H and ^{13}C NMR spectral data, see Table 4.2: Negative

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ESIMS *m/z* 451 $[M - H]^-$. Positive HRESIMS *m/z* $[M + H]^+$ 453.2294 (calcd for $C_{27}H_{32}O_6 + H$, 453.2277).

Aristophenone A (3): light yellow oil; Negative ESIMS *m/z* 533 $[M - H]^-$. ^1H and ^{13}C NMR data are consistent with previously published data.[117]

Xanthochymol (4): yellow oil; Negative ESIMS *m/z* 601 $[M - H]^-$. ^1H and ^{13}C NMR data and UV data are consistent with previously published data.[123,142] The identification was further supported by HSQC and HMBC experiments.

Guttiferone E (5): yellow oil; Negative ESIMS *m/z* 601 $[M - H]^-$. $[\alpha]_D$ +106° (*c* 0.0065, $CHCl_3$); $[\alpha]_D$ +120° (*c* 0.0065, MeOH). ^1H and ^{13}C NMR data and UV data are consistent with previously published data.[36]

Cycloxanthochymol (6): yellow oil; Negative ESIMS *m/z* 601 $[M - H]^-$. ^1H and ^{13}C NMR data are consistent with previously published data.[122]

Isoxanthochymol (7): yellow oil; Negative ESIMS *m/z* 601 $[M - H]^-$. ^1H and ^{13}C NMR data are consistent with previously published data.[36]

Alloathyriol (8): Light yellow powder. Negative ESIMS *m/z* 273 $[M - H]^-$. ^1H and NOE NMR data and UV spectrum are consistent with previously published data.[65]

Amentoflavone (9): Light yellow powder; Negative ESIMS *m/z* 537 $[M - H]^-$. ^1H and ^{13}C NMR data are consistent with previously published data.[209-211] The identification was further support by comparison of ^1H data and HPLC analysis (65:35 10 mM ammonium acetate–MeCN) with purchased standard (Indofine Chemical Company, Hillsborough, NJ).

3,8″-Biapigenin (10): Light brown powder; Negative ESIMS *m/z* 537 $[M - H]^-$. ^1H and ^{13}C NMR data and UV spectrum are consistent with previously published data.[212]

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(+/-)-Fukugetin (11): Light yellow powder; Negative ESIMS *m/z* 555 $[M - H]^-$. ^1H and ^{13}C NMR data are consistent with previously published data.[213,214]

(+/-)-Fukugiside (12): Red-brown powder; Negative ESIMS *m/z* 717 $[M - H]^-$. ^1H and ^{13}C NMR data are consistent with previously published data.[175]

(+/-)-Volkensiflavone (13): Light brown powder. Negative ESIMS *m/z* 539 $[M - H]^-$. ^1H and ^{13}C NMR data, and UV spectrum are consistent with previously published data.[209]

4.4. Conclusion

Two novel compounds as well as eleven known compounds were isolated from *G. xanthochymus* fruits. Guttiferone H and gambogenone represent two novel variations in benzophenone chemistry. Isolated benzophenones displayed potent cytotoxicity in the SW-480 cancer cell assay and antioxidant activity in the DPPH assay. The biflavonoids were also active in both *in vitro* assays.

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1

2

3

4 R_1=

5 R_1=

6 R_1=

7 R_1=

8

9

10

11 R_1=OH R_2=H
12 R_1=OH R_2=Glc
13 R_1=R_2=H

Figure 4.1. Compounds isolated from *G. xanthochymus* fruits

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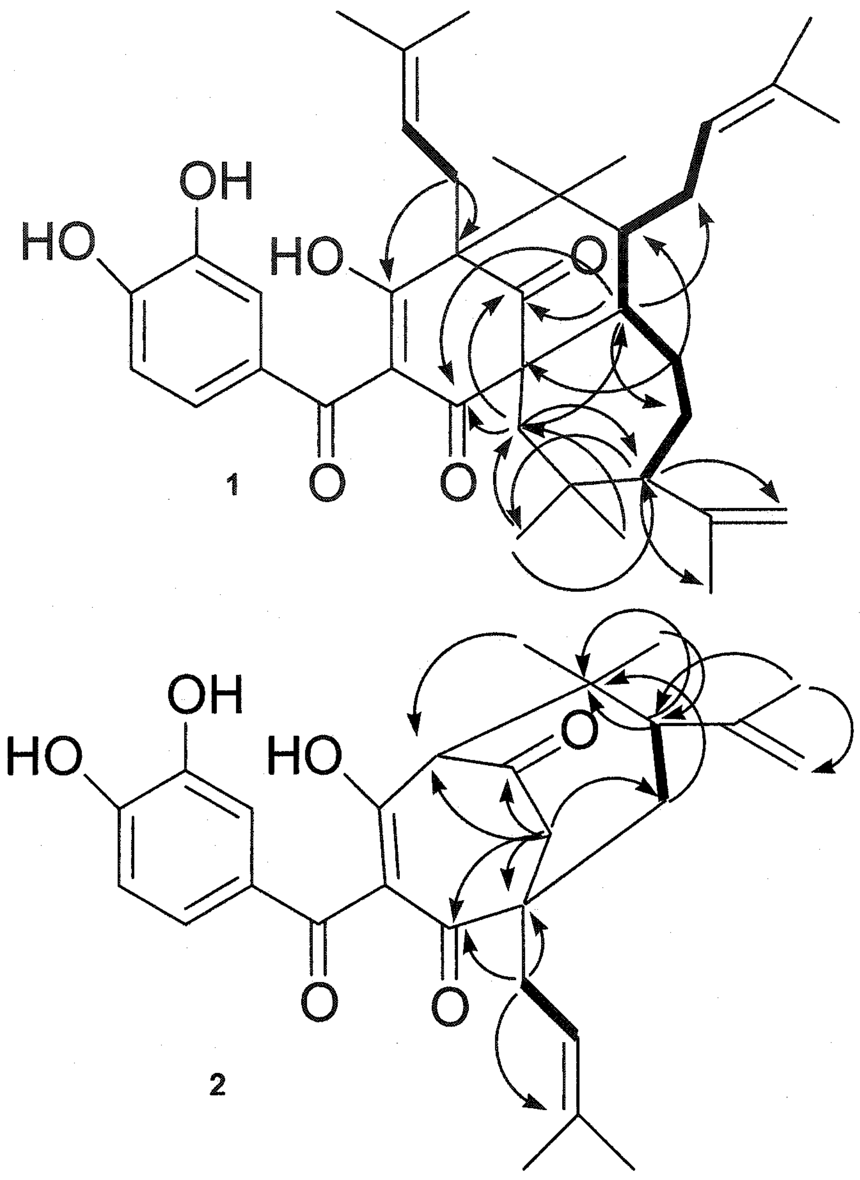

Figure 4.2. Arrows denote key HMBC cross peaks and bold lines indicated COSY correlations for **1** and **2**

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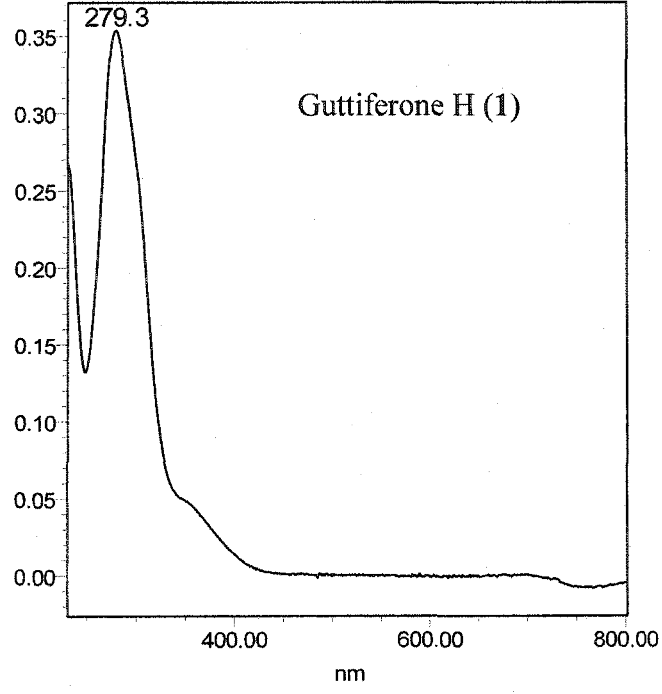

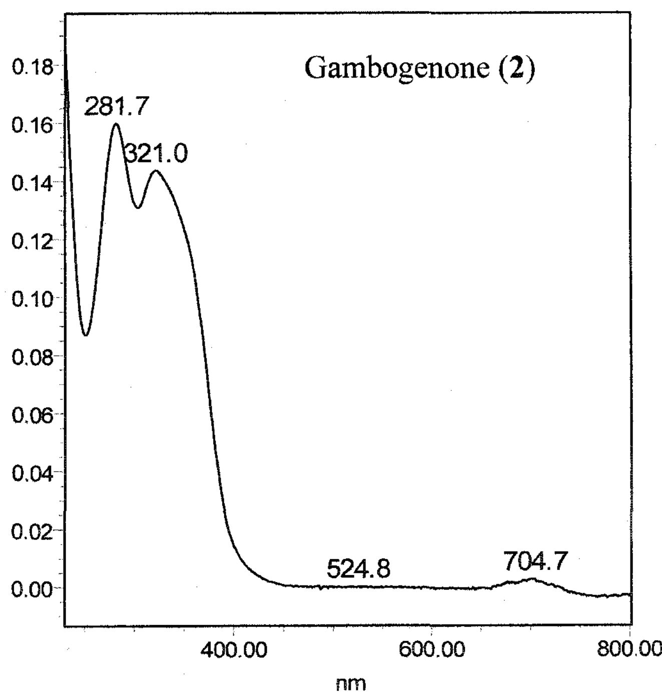

Figure 4.3. The PDA spectrum, recorded from 210-800 nm, of novel benzophenones **1** and **2**

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Table 4.1. NMR Spectral Data for Guttiferone H (1) in CD_3OD

Position	$^{13}C^{a}$ (δ)	$^{1}H^{b}$ (δ, mult., J in Hz)	HMBC connectivities[b,c]
1	190.9		7, 29A, 29B
2	119.1		
3	190.6		17A, 17B
4	67.1		6, 17A, 17B, 22, 23
5	48.0		22, 23
6	47.6	1.40 m	7, 22, 23, 33A
7	38.6	1.92 m	32B, δ 1.92
8	60.2		6, 7, 29A, 29B, 33B
9	212.7		7, 17A, 29A, 29B
10	198.0		12, 16
11	131.6		15
12	115.5	7.36 d (1.8)	16
13	145.1		15
14	150.4		12, 16
15	114.1	6.70 d (8.2)	
16	124.1	7.21 dd (8.2, 1.8)	12
17	25.8	2.58 dd (14, 6.5) 2.52 dd (14, 6)	
18	121.8	5.0, dd (6.5, 6)	17A, 20, 21
19	131.9		20, 21
20	25.4	1.68 s	21
21	17.4	1.67 s	20
22	22.6	1.20 s	23
23	26.6	0.97 s	22
24	29.7	2.45 ddd (14, 6, 5) 2.28 ddd (14, 6, 1)	6, 7
25	126.0	5.0 t (6)	27, 28
26	132.5		27, 28
27	24.5	1.67 s	28
28	17.4	1.62 s	25, 27
29	40.2	2.18 d (14) 1.90 d (14)	37, 38
30	40.9		29B, 31, 32A, 37, 38
31	49.4	2.34 d (3.6)	29A, 32B, 35A(w), 35B, 36, 37, 38
32	28.8	1.93 m 1.82 dd (12, 3.6)	33B, δ 1.92
33	32.9	1.92 m 1.76 dd (14, 8.7)	32B
34	146.7		31, 32A, 36

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Position	$^{13}C^{a}$ (δ)	$^{1}H^{b}$ (δ, mult., J in Hz)	HMBC connectivities[b,c]
35	108.3	4.81 brs 4.67 brs	31, 36
36	23.5	1.68 s	31, 35A, 35B(w)
37	25.1	1.21 s	31, 38
38	23.6	0.95 s	31, 37

[a]Recorded at 75 MHz. [b]Recorded at 500 MHz. [c]Protons correlating with carbon resonance; (w) = weak.

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Table 4.2. NMR Spectral Data for Gambogenone (2) in CD_3OD

Position	$^{13}C^a$ (δ)	$^1H^b$ (δ, mult., J in Hz)	HMBC connectivities[b,c]
1	201.5		9, 18
2	130.2		
3	173.0		
4	38.2	1.62 s	9, 23, 24
5	40.4		4, 6, 7B, 23, 24
6	48.7	2.42 dd (10, 7)	23, 24, 26B, 27
7	26.7	2.05 dd (12, 7) 1.67 dd (12, 10)	9
8	54.9		7B, 9, 18
9	35.6	1.85 d (10.5) 1.82 d (10.5)	
10	207.6		9
11	191.2		13, 17
12	129.9		16
13	115.6	7.38 d (1.7)	17
14	145.6		13, 16
15	151.8		13, 16
16	114.5	6.79 d (8.3)	
17	124.3	7.25 dd (8.3, 1.7)	13
18	34.1	2.41 d (8)	9
19	118.2	4.99 t (8)	21, 22
20	135.9		18, 21, 22
21	16.8	1.60 s	22
22	25.2	1.70 s	21
23	23.4	1.08 s	24
24	24.0	0.83 s	23
25	145.8		6, 7A, 27
26	108.8	4.81 brs 4.64 brs	27
27	23.0	1.64 s	26A

[a]Recorded at 75 MHz. [b]Recorded at 500 MHz. [c]Protons correlating with carbon resonance.

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Table 4.3. DPPH and SW-480 Cytotoxicity IC_{50} Values for Isolated Compounds

Compound	DPPH IC_{50} μM (± SD)	SW-480 IC_{50} μM (95% CI)
1	64 (2.1)	12.4 (10.5-12.0)
2	38.7 (2.3)	188 (172-214)
3	125 (4.1)	33.3 (24-33)
4	53 (1.0)	8.3 (7.0-8.2)
5	68 (0.33)	7.5 (6.1-7.8)
6 & 7[a]	73 (1.5)	16.6 (15-17.3)
8	na[b]	117 (101-123)
9	184 (5.0)	111 (102-122)
10	>> 400	185 (170-203)
11	62 (5.1)	89 (82-98)
12	116 (9.0)	> 200
13	298 (13.8)	185 (170-203)

[a]mixture, [b]na = not active

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Chapter 5: Conclusion

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The activity-guided isolation and identification of two novel benzophenones, guttiferone H and gambogenone, and eleven known compounds from *Garcinia xanthochymus* fruits are described. The identification of the two novel compounds was established by extensive 1D and 2D NMR data analysis.

The first novel compound, guttiferone H, is a variation in the guttiferone class of benzophenones. Guttiferone H is the first guttiferone class derivative containing a 7-membered D-ring attached to the bicyclo[3.3.1]nonane system at positions 7 and 8. Guttiferone H displayed potent cytotoxicity in the *in vitro* SW-480 colon cancer cell assay, $IC_{50} = 12\ \mu M$, and antioxidant activity in the free radical DPPH assay, $IC_{50} = 64\ \mu M$.

The second novel compound, gambogenone, has a novel bicyclo[3.3.2]decane system. This is the first reported benzophenone with a bicyclo[3.3.2]decane B/C-ring system. Gambogenone was less cytotoxic than guttiferone H in the SW-480 colon cancer cell line, $IC_{50} = 188\ \mu M$. Even though gambogenone displayed less cytotoxicity than guttiferone H, gambogenone displayed high antioxidant activity in the free radical DPPH assay, $IC_{50} = 38.7\ \mu M$.

The related benzophenone garcinol (**134**) [Chapter 2], has displayed a number of biological activities including *in vitro* cytotoxicity[38,154] and *in vivo* cancer chemopreventive effects.[155] Garcinol exhibited chemopreventive effects against azoxymethane (AOM) induced colonic aberrant crypt foci (ACF) in F344 rats with minimum side effects.[155] The authors concluded that "garcinol from *G. indica* significantly inhibits AOM-induced ACT formation in male F344 rats without causing

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any adverse effects."[155] Our laboratory is currently exploring additional *in vivo* animal studies on the chemopreventive effects of benzophenones.

Recently garcinol was found to inhibit histone acetyltransferase, repress chromatin transcription, and alter global gene expression.[156] It is likely that the novel and known benzophenones, isolated from *G. xanthochymus* fruits, have a similar mechanism of action. Studies are underway in our laboratory to identify the molecular target(s) of isolated benzophenones.

Eleven known compounds, aristophenone A, alloathyriol, amentoflavone, 3,8″-biapigenin, cycloxanthochymol, (+/-)-fukugetin, (+/-)-fukugiside, guttiferone E, isoxanthochymol, (+/-)-volkensiflavone, and xanthochymol were also isolated. Their structures were determined by comparing their experimental data (molecular weight, ^{1}H and ^{13}C NMR) with literature values. The eleven known compounds were tested in the SW-480 colon cancer and in the DPPH assays. The known benzophenones, **3-7**, displayed potent cytotoxicity in the SW-480 colon cancer assay ranging from $IC_{50} = 7.5 – 33.3\ \mu M$.

Benzophenones are a interesting class of compounds displaying much structural diversity and numerous bioactivities. By far, the richest sources of benzophenones have been Clusiaceae species; however, of the approximately 37 genera and 1610 Clusiaceae species[160] only the genera *Clusia*, *Garcinia*, and *Rheedia* have been analyzed extensively.

Five Clusiaceae species were dereplicated using the compounds obtained from *G. xanthochymus* fruits as standards and literature data. Three species, *G. aristata*, *G. livingstonei*, and *R. edulis*, appear to be good candidates for activity-guided fractionation.

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Two species, *G. spicata* and *R. gardneriana*, are lower priority because they appear to contain only known benzophenones.

New benzophenones are being isolated each year. For example, at the 2004 American Society of Pharmacognosy International Congress on Natural Products four presentations discussed the isolation of eleven new benzophenones from plant[166,215,216] and microbial[217] sources. Clusiaceae species are likely to yield a number of novel bioactive compounds.

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Appendix I: Standard Procedure for On-Line DPPH Assay

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A. Preparation of DPPH Solution

1. Weigh out 39 mg/liter of DPPH (make only the amount of DPPH you will need for the day because it can not be saved).
2. Using volumetric glassware dissolve DPPH in HPLC grade methanol.
3. Mix for 10 minutes.
4. Filter DPPH solution with 0.45 μm nylon filter.
5. Transfer filtered DPPH solution to a amber glass bottle, place in –30 °C freezer for 20 minutes.
6. Remove DPPH from freezer and place on ice.
7. Place DPPH solution and ice bucket in the metal tray above Perkin-Elmer 250 binary pump.
8. Place the inlet line and spare line in the DPPH solution.
9. Turn on the Waters 717 pump controller.
10. Enable reservoir C and set sparging rate to 100 mL/min (see 717 procedure for instructions).
11. Open the valve on the helium tank. **NOTE**: You must close this valve when you are finished.
12. Sparge DPPH solution for 15 minutes with helium.
13. After sparging for 15 minutes set sparging rate to 20 mL/min.

B. Priming the Perkin-Elmer 250 Binary Pump

1. Insert syringe into metal hole and open priming valve by turning one turn to the left.
2. Start pump at 1 mL/min (see Perkin-Elmer 250 binary pump quick reference cards for instruction for running Perkin-Elmer 250 binary pump) NOTE: Once you have started

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the pump and opened the valve fluid will come out of the primping valve. The pump is located on top of the LCQ, be EXTREMELY careful not to drip liquid onto the LCQ.

3. Pull about 20 mL of DPPH solution into the syringe.
4. Close the primping valve and stop the pump.

C. Running a DPPH Method Set, Processing, and Reporting of Data

Note: This report process and prints a sample summary box and chromatogram with the 996 and 486 channels, plus overlaid gradient information. To obtain retention times of peaks, you need to create a new processing method in Empower for each channel of acquired data, then reprocess and print your data.

1. Log into Empower, click on the run samples box and select the system 996_DPPH assay@D119_HPLC2.
2. Prepare DPPH assay for first injection by setting the flow rate of the DPPH solution to match the flow rate of the 2695 system. Allow 10 minutes for system to equilibrate.
3. Start the DPPH pump and setup the 2695 as usual by selecting the correct instrument method in the run samples and pressing setup.
4. Turn on the lamps for the 486 and 996 detectors. Allow 30 minutes for the lamps to warm up before the first injection.
5. In the method set column select, 996 DPPH SOP 30 minutes run. If this selection is not available copy this method set from the project Scottdefault. If you do not know how to copy methods from one project to another press F1 for instructions.
6. Set run time for 32 minutes.

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7. Set delay time for 10 minutes.
8. Under the system tab pull down, select run and report.
9. After samples are entered, press run samples and select printer for reports. (note: please see DPPH positive control section below before starting your sample set)

D. Creating a (New) DPPH Instrument Method

You must create an instrument method which contains the 486 and 996 detectors plus the 2695 separation unit. If you do not you will get an instrument failed message.

Setup of Individual Components

1. Waters 486 detector
   a. Under the General tab| Description| type DPPH Activity.
   b. Under the General tab| Wavelength| change the wavelength to 518 nm.
   c. Leave all other values at their defaults.
   d. Under the events tab: Column| Time| Type 0.00.
   e. Under the events tab: Column| Event| Select: autozero.
   f. Leave all other values at their defaults.
2. Waters 996 Detector
   a. No changes in setup are necessary, suggest scanning from 230-550 nm.
3. Waters 2695 unit.
   a. No changes in setup are necessary.

E. Controls for the DPPH Assay

During the first injection, for reasons that I cannot determine, the baseline for the 486 is always off, therefore a blank must be run before your first sample. You only need to run this blank once to solve this problem, not a blank at the start of each sample set.

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F. Suggested Sample Set

a. Line One: Function: Purge injector: using method set 100% C (create if not in your project or copy from Scottdefaults.) **Note**: do not set any delay time for this method or your first injection will be off.

b. Line Two: Blank control.

c. Line Three: Start of first sample or standard.

G. Shutdown of DPPH System

For the Perkin-Elmer 290 Binary Pump

You must be present to shutdown the Perkin-Elmer 250 binary pump. This pump is NOT controlled by Empower, all control must be done using the firmware.

1. Remove any unused DPPH solution and discard.
2. Fill a flask with 50 mL of HPLC grade methanol.
3. Flush the pump for 30 minutes with HPLC Grade methanol, at flow rate of 0.8 mL/min.

For the Waters 717 Gradient Pump

1. Turn off Waters 717 Gradient Pump.
2. Close valve on the helium tank.

For the 996_DPPH System

You must use the shutdown method appropriated for the column and the system in use. The shutdown method is labeled **shut down analytical bore DPPH**. Add this to the last line of a sample set, as a function to condition columns for 31 minutes or run as a single inject. This method flushes the column with solvent C for 20 minutes and with solvent D for 10 minutes, at a flow rate of 0.8 mL/min. A higher flow rate will cause the system to overpressure.

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Chapter 6: References

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