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Natural Inhibitors of Carcinogenesis

Abstract

Previous collaborative work by our group has led to the discovery of several plant isolates and derivatives with activities in *in vivo* models of cancer chemoprevention, including deguelin, resveratrol, bruceantin, brassinin, 4′-bromoflavone, and oxomate. Using a panel of *in vitro* bioassays to monitor chromatographic fractionation, a diverse group of plant secondary metabolites has been identified as potential cancer chemopreventive agents from mainly edible plants. Nearly 50 new compounds have been isolated as bioactive principles in one or more *in vitro* bioassays in work performed over the last five years. Included among these new active compounds are alkaloids, flavonoids, stilbenoids, and withanolides, as well as a novel stilbenolignan and the first representatives of the norwithanolides, which have a 27-carbon atom skeleton. In addition, over 100 active compounds of previously known structure have been obtained. Based on this large pool of potential cancer chemopreventive compounds, structure-activity relationships are discussed in terms of the quinone reductase induction ability of flavonoids and withanolides and the cyclooxygenase-1 and -2 inhibitory activities of flavanones, flavones and stilbenoids. Several of the bioactive compounds were found to be active when evaluated in a mouse mammary organ culture assay, when used as a secondary discriminator in our work. The compounds (2*S*)-abyssinone II, (2*S*)-2′,4′-dihydroxy-2″-(1-hydroxy-1-methylethyl)dihydrofuro[2,3-*h*]-flavanone, 3′-[γ-hydroxymethyl-(*E*)-γ-methylallyl]-2,4,2′,4′-tetrahydroxychalcone 11′-*O*-coumarate, isolicoflavonol, isoliquiritigenin, and ixocarpalactone A are regarded as promising leads as potential cancer chemopreventive agents.

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Introduction

According to the World Health Organization, cancer is the cause of 12% of the world's mortality [1]. This translates to 6.2 million deaths worldwide in the year 2000. In the United States, cancer is the second leading cause of death and is responsible for approximately one in every four deaths. Given our current level of knowledge, it is estimated that at least one-third of all cancers are preventable [2], [3].

Prevention, both primary and secondary, is currently an attractive and promising strategy to reduce the occurrence of this disease [4], [5], [6]. Primary prevention strategies involve removing the causative agent(s) and other life-style modifications that decrease the risk of cancer such as smoking cessation and screening tests to detect precancerous lesions. Unfortunately, not all causative agents are known and other suspected carcinogens are too widespread to feasibly prevent all exposure. Secondary prevention, known as cancer chemoprevention, is the use of non-toxic natural and/or synthetic agents to decrease the risk of malignant tumor development [7], [8].

Cancer chemoprevention is a multidisciplinary field of research that has evolved from numerous scientific observations [9]. For example, epidemiological studies have linked diets high in fresh fruits and vegetables to lower cancer rates. This dietary link is perhaps most strongly supported by studies reporting the cancer risk of migrants from areas of low incidence to high incidence. These studies demonstrated that the incidence of cancer among children of migrants is similar to that of the general population [10]. Another important breakthrough has been the prevention of experimentally induced cancer in laboratory animals. It was subsequently postulated that dietary components, particularly specific nutrients and/or phytochemicals found in fruits and vegetables could be used to prevent cancer in humans [8], [11]. More recently, research in cancer biology has elucidated molecular mechanisms by which cancer chemopreventive agents can act [5], [9], [12]. Much of the theoretical basis for cancer chemoprevention is the understanding that cancer develops over time through a process known as carcinogenesis [13]. This process has been broken down into distinct yet overlapping stages, namely, initiation, promotion, and progression. The evolution of these stages is believed to take 10 to 40 years, during which various genetic mutations must occur [9], [14]. The field of cancer chemoprevention is concerned with reversing, halting, or delaying these stages of carcinogenesis by means of secondary prevention [7], [8], [9].

Cancer chemopreventive agents have been classified according to the stage of carcinogenesis in which they have demonstrated activity and have been broadly termed blocking and suppressing agents [7]. Blocking agents act by preventing the initiation stage through a variety of mechanisms such as directly detoxifying carcinogens, stimulating detoxifying enzymes, and inhibiting carcinogen formation. Suppressing agents act at the promotion and progression stages through mechanisms including: inhibition of arachidonic acid metabolism, induction of cell differentiation, and inhibition of ornithine decarboxylase activity [3], [7], [15]. In the case of hormone-dependent cancers, suppressing agents may act by preventing the hormone from binding to its receptor, as exemplified by the use of the selective estrogen receptor modulators, tamoxifen and raloxifene, for breast cancer prevention [3], [14].

Overview of Cancer Chemoprevention Trials Involving Phytochemicals

Many early cancer chemoprevention studies were focused on nutrients such as vitamin C, calcium, and retinoids [8], [10]. In the last several decades, a great amount of effort has been made to examine non-nutrient phytochemicals found in fruits and vegetables, and a number of promising natural product leads have come from this research effort [14], [16], [17]. For example, green tea extract and pure compounds such as caffeic acid phenethyl ester, capsaicin, curcumin, [6]-gingerol, indole-3-carbinol, lycopene, and perillyl alcohol are undergoing clinical trials for their cancer chemopreventive activities [14], [18], [19]. The United States National Cancer Institute (Center for Cancer Research, Cancer Prevention Studies Branch) is supporting the evaluation of potential cancer chemopreventive agents at different levels of preclinical development and clinical trials [17]. Thus, examples of natural products currently under preclinical or clinical development for cancer chemoprevention include curcumin and lycopene, which are in a phase I study for the prevention of colon cancer, while a soy protein supplement is in a phase II trial for the prevention of prostate cancer in patients with elevated prostate-specific antigens [20]. Moreover, soy isoflavones are also involved in a randomized study in preventing further development of cancer in patients with stage I or stage II prostate cancer [20]. Polyphenon E (green tea extract), in combination with low-dose aspirin, is in a phase II randomized study to prevent cancer in women at high risk for developing breast cancer [21], [22]. Other natural products currently being investigated include *S*-allyl-L-cysteine, epigallocatechin gallate, genistein, folic acid, and quercetin [18], [23].

Collaborative Studies at the University of Illinois at Chicago and Purdue University

The National Cancer Institute has supported a cancer chemoprevention program project entitled "Natural Inhibitors of Carcinogenesis" (1991 – 2004) in our laboratories at the University of Illinois at Chicago and Purdue University. The major aim of this project is the discovery of new cancer chemopreventive agents from plants, particularly those that are edible. The program project itself involves botanical, biological, chemical, biostatistical, and administrative aspects [24], [25], [26]. Plant material select-

ed for investigation in this program project is prioritized based on information in the NAPRALERT database [27]. Edible plants or species with reported biological activity related to cancer chemoprevention, plants with no history of toxicity, and those poorly investigated phytochemically are recorded for preliminary investigation by collecting a small amount of plant material [24], [25], [26].

The panels of *in vitro* bioassays used for the discovery of potential cancer chemopreventive drugs include screening assays that are usually enzyme- or cell-based assays [25], [28]. These assays are adapted to high-throughput measurement techniques performed relatively rapidly in order to uncover the biological properties of a large number of candidate substances [25], [28]. The initial bioassays afford a strategic framework for the evaluation of agents according to defined criteria, and to provide evidence of agent efficacy, and serve to generate valuable dose-response, toxicity, and pharmacokinetic data required prior to phase I clinical safety testing [25], [28], [29].

Preliminary screening is performed on an ethyl acetate-soluble partition extract using a battery of short-term *in vitro* bioassays [25]. Bioactive extracts are further evaluated in a mouse mammary organ culture model as a secondary discriminator [30], [31]. The battery of short-term *in vitro* assays was developed to monitor tumorigenesis at different stages. Antimutagenicity activity, antioxidant activity, and induction of NADPH: quinone reductase activity are monitored to evaluate inhibition of carcinogenesis at the initiation stage [32], [33], [34]. Monitoring the inhibition of carcinogenesis at the promotion stage is performed by evaluation of the inhibition of phorbol ester-induced ornithine decarboxylase activity, the inhibition of cyclooxygenases-1 and -2 activities, the inhibition of phorbol dibutyrate receptor binding and the inhibition of transformation of JB6 mouse epidermal cells [35], [36], [37], [38]. Induction of HL-60 human promyelocytic leukemia cell differentiation, and inhibition of aromatase, antiestrogenic, estrogenic, and estrone sulfatase activities are all used to monitor inhibition of carcinogenesis at the progression stage [39], [40], [41], [42].

The plant extracts showing potency and/or selectivity in preliminary biological screening procedures are selected for bioassay-guided fractionation to isolate the active principle or principles. Methanolic crude extracts are partitioned using solvents of varying

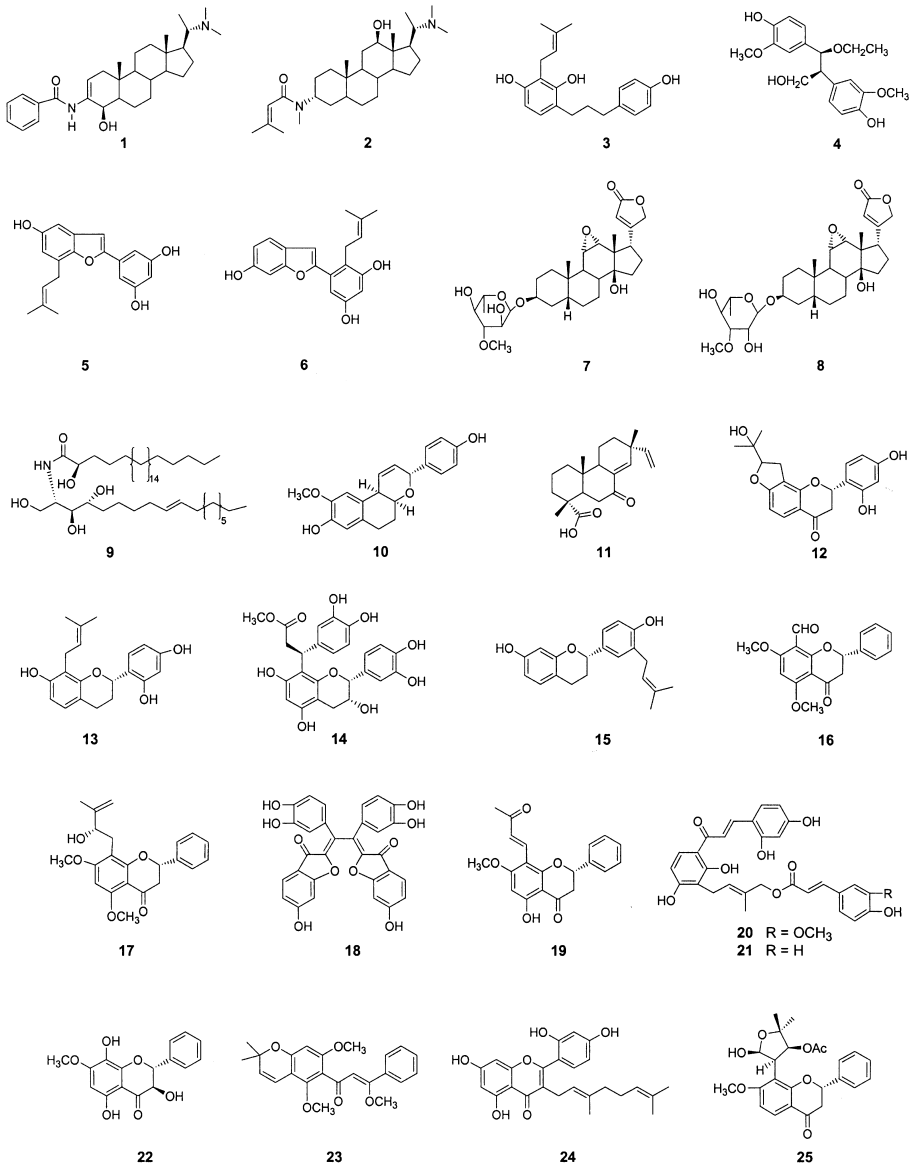


Fig. **1**

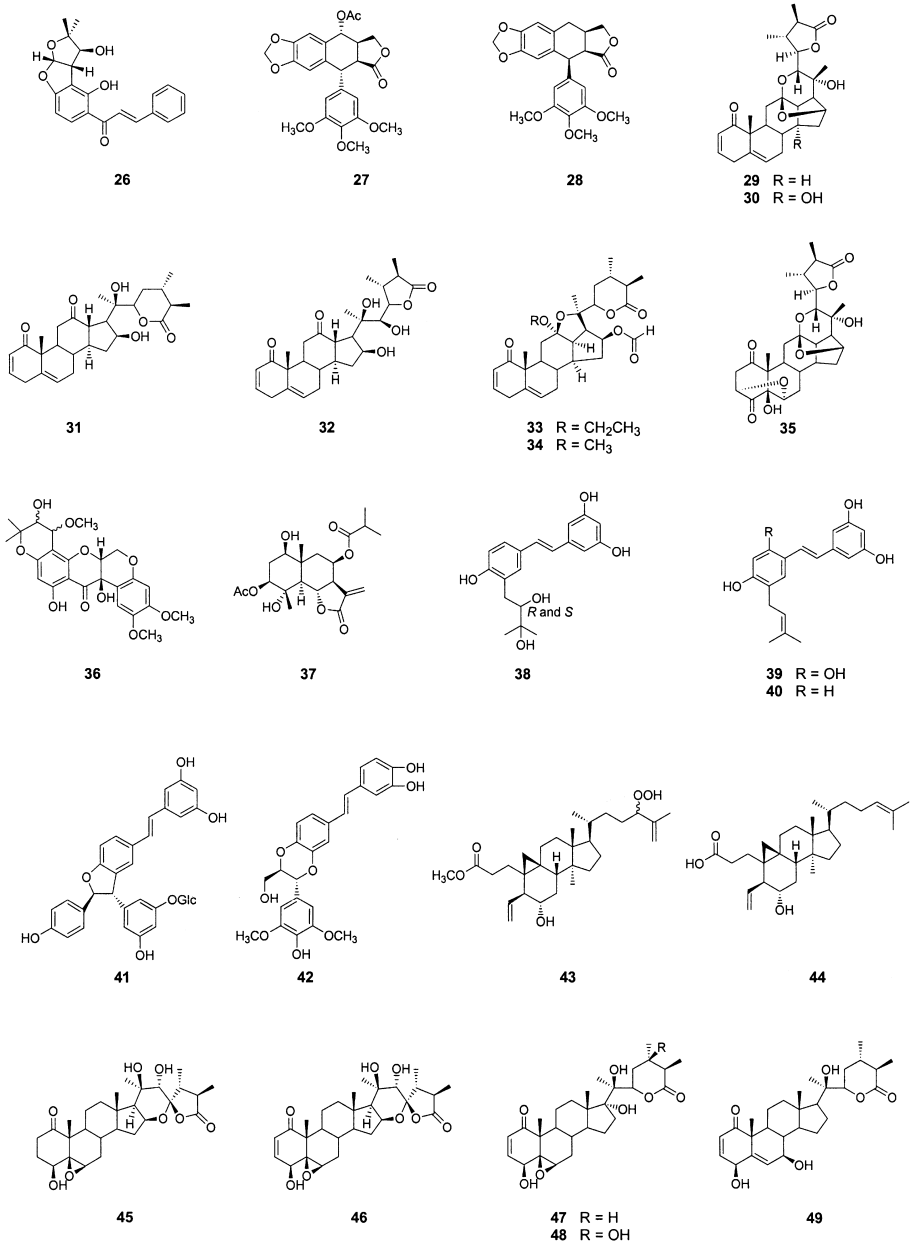


Fig. **1** cont.

polarities and then chromatographed by either gravity, flash, or low-pressure columns over silica, alumina, ion-exchange resins, polyamide, reversed-phase silica gel, size-exclusion gels, or other solid-phase supporting material [26], [43]. Analytical thin-layer and high-pressure liquid chromatography (HPLC) techniques are used to help determine optimal solvent systems for the maximal separation of active components of fractions [44]. Other separation techniques, such as droplet countercurrent chromatography (DCCC), high-speed countercurrent chromatography (HSCCC), and semi-preparative HPLC are used occasionally for complex mixtures of active constituents [26], [44], [45].

After pure active isolates have been evaluated in all of the available *in vitro* assays, selected species are evaluated in the *ex vivo* mouse mammary organ culture model as a secondary discriminator [30], [31]. Highly promising compounds may be selected for testing in full-term, animal tumorigenesis models, such as the two-stage mouse skin model using 7,12-dimethylbenz[*a*]anthracene (DMBA) as initiator and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) as promoter, and the rat and mouse mammary carcinogenesis models with DMBA or 1-methyl-1-nitrosourea (MNU) as the carcinogens [24], [25], [37].

Our goal has been to examine plants, primarily edible species, for cancer chemopreventive compounds based on specific mechanisms of action [24]. Wattenberg remarked in his 1985 paper how impressive it was that so many small-molecule compound structural classes have cancer chemopreventive activities [7]. We have certainly found this to be true, and elaborate on additional examples of potential cancer chemopreventive agents from plants in the remainder of this review.

Potential Cancer Chemopreventive Agents from Plants

Over a recent period of approximately five years, a total of 166 active compounds were isolated and biologically evaluated in our laboratories from 32 plant species (Tables **1** – **3**; Figs. **1** – **3**).

Table **1** New bioactive natural products obtained by activity-guided fractionation[a]

Compound code	*Species code*[b]	*Bioassay system*[c]	*Ref.*	*Compound code*	*Species code*[b]	*Bioassay system*[c]	*Ref.*
Alkaloids				**Lignans**			
1	12	ES	[46]	**27**	9	JB6	[60]
2	12	ES	[46]	**28**	17	JB6; ODC	[52]
Benzenoids				**Norwithanolides**			
3	4	AO	[47]	**29**	8	QR	[44]
4	7	QR	[48]	**30**	8	QR; JB6	[44]
Benzofurans				**31**	8	QR; JB6	[44]
5	3	COX-1; COX-2	[49]	**32**	8	QR	[44]
6	4	AR	[47]	**33**	8	QR	[44]
Cardiac glycosides				**34**	8	QR	[44]
7	5	Col2; IA	[42]	**35**	8	QR; JB6	[61]
8	5	Col2; IA	[42]	**Rotenoid**			
Ceramide				**36**	16	QR	[57]
9	13	JB6	[50]	**Sesquiterpene lactone**			
Diarylheptanoid				**37**	18	HL-60	[62]
10	11	QR	[51]	**Stilbenoids**			
Diterpenoid				**38**	3	COX-2	[49]
11	17	JB6; ODC	[52]	**39**	3	COX-1	[49]
Flavonoids				**40**	3	COX-2	[49]
12	4	AR	[47]	**41**	19	COX-1; COX-2	[63]
13	4	ERα; ERβCOX-1	[53]	**Stilbenolignan**			
14	1	AX	[54]	**42**	2	COX-1	[64]
15	6	MCF-7	[53]	**Triterpenes**			
16	14	QR	[55]	**43**	1	COX-1; COX-2	[54]
17	14	QR	[55]	**44**	1	COX-1; COX-2	[54]
18	6	AO	[56]	**Withanolides**			
19	16	QR	[57]	**45**	13	QR	[65]
20	4	AR	[47]	**46**	13	QR	[50]
21	4	AR	[47]	**47**	13	QR; MMOC	[50]
22	10	QR	[58]	**48**	13	QR; MMOC	[50]
23	14	QR	[55]	**49**	13	QR	[65]
24	4	AR	[53]				
25	15	QR	[59]				
26	15	QR	[59]				

[a] The structures of compounds **1** – **49** are shown in Fig. **1**.

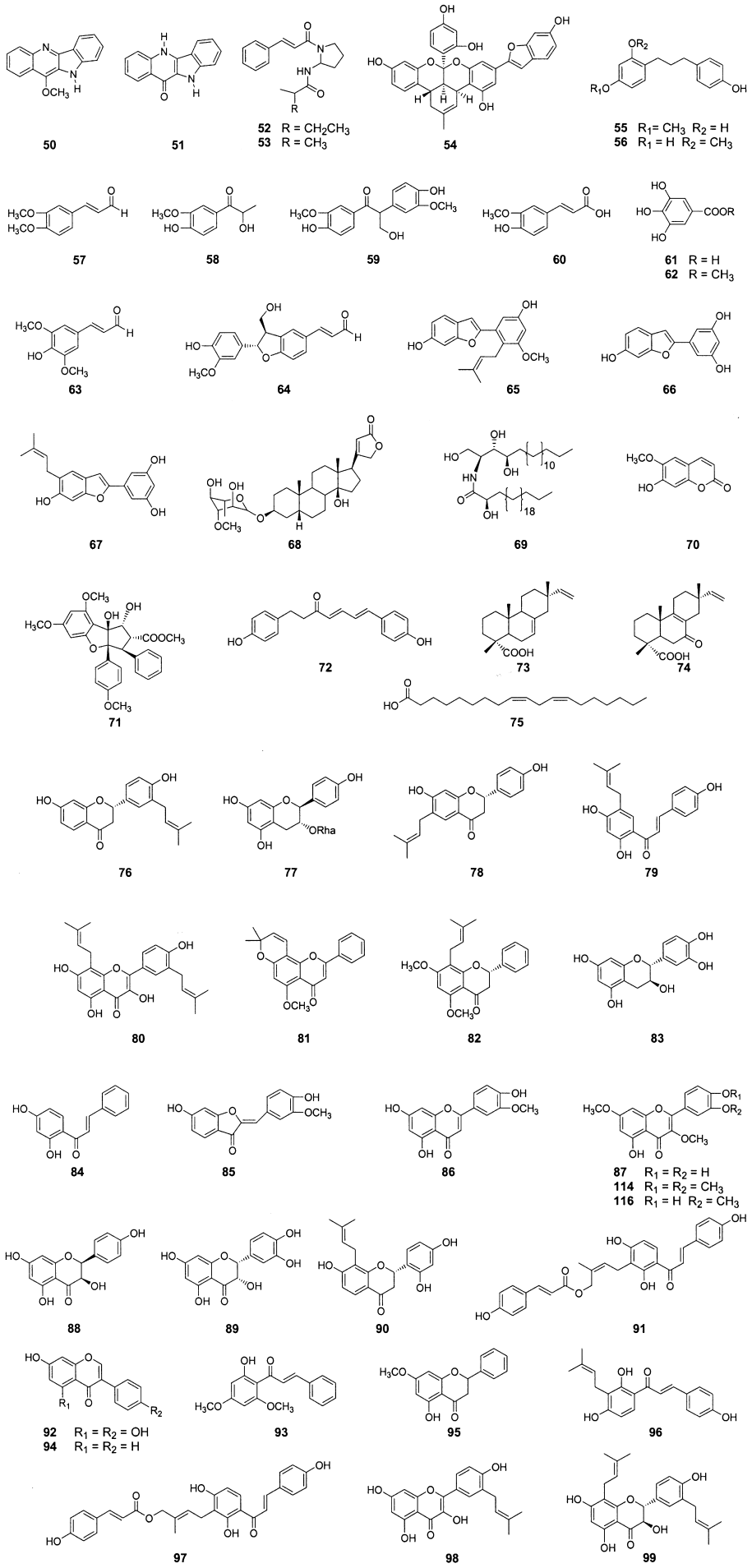
[b] Species code: 1 (*Antirhea acutata*); 2 (*Aiphanes aculeata*); 3 (*Artocarpus dadah*); 4 (*Broussonetia papyrifera*); 5 (*Cerbera manghas*); 6 (*Cotinus coggygria*); 7 (*Couepia ulei*); 8 (*Deprea subtriflora*); 9 (*Hernandia ovigera*); 10 (*Muntingia calabura*); 11 (*Musa x paradisiaca* cultivar); 12 (*Pachysandra procumbens*); 13 (*Physalis philadelphica*); 14 (*Pongamia pinnata*); 15 (*Tephrosia purpurea*); 16 (*Tephrosia toxicaria*); 17 (*Thuja occidentalis*); 18 (*Tithonia diversifolia*); 19 (*Vitis vinifera*, cell culture).

[c] Key to bioassay systems in which activity was shown: AO (antioxidant assay); AR (aromatase assay); AX (cytotochrome c antioxidant assay); Col2 (antiproliferative human colon cancer assay); COX-1 (cyclooxygenase-1 inhibition assay); COX-2 (cyclooxygenase-2 inhibition assay); ERα/β (estrogen receptor-binding α/β assay); ES (estrone sulfatase assay); HL-60 (differentiation of HL-60 cells); IA (Ishikawa anti-E_2 bioassay); JB6 (soft agar transformation assay with JB6 cells); MCF-7 (antiproliferative human breast cancer cells); ODC (inhibition of TPA-induced ornithine decarboxylase activity with cultured mouse epidermal 308 cells); QR (quinone reductase induction assay in cultured Hepa 1c1c7 mouse hepatoma cells).

The active metabolites were obtained using activity-guided fractionation with a pre-selected *in vitro* assay to monitor their purification process. These active compounds were found to represent 29 major secondary metabolite compound classes including alkaloids (of the β-carboline alkaloid, indoloquinoline alkaloids, and steroidal types), amides, benzenoids, benzofurans, cardiac glycosides, ceramides, a coumarin, diarylheptanoids, diterpenoids, fatty acids, flavonoids (of the aurone, bisaurone, chalcone, flavan, flavanone, flavone, flavonol, flavonone, and isoflavone types), glycerol esters, a β-ionone derivative, an iridoid, lignans, a monoterpenoid, a naphthopyran, norwithanolides, phenylphenalones, a porphyrin derivative, a rocaglamide derivative, rotenoids, sesquiterpene lactones, sesquiterpenoids, simaroubolides, a stilbenolignan, stilbenoids, triterpenoids, and withanolides. Active compounds based on three different types of novel carbon skeletons were obtained during this work, which included seven norwithanolides (**29** – **35**) possessing a new C_{27} skeleton (as opposed to the 28 carbons of the more widespread withanolides) [44], [61], a novel stilbenolignan (**42**) containing a stilbene-phenylpropane unit with a dioxane moiety [64], and two triterpenes (**43** and **44**) based on a 29-*nor*-3,4-*seco*-cycloartane skeleton [54]. Forty-nine new compounds from 19 species (Fig. **1**) were found among the compound classes mentioned above and were classified into 16 major structural classes (Table **1**).

A large number of known bioactive compounds were isolated from the 32 species (Fig. **2**), and can be grouped into 23 major structural classes (Table **2**). Many of these known isolates were

Fig. **2**



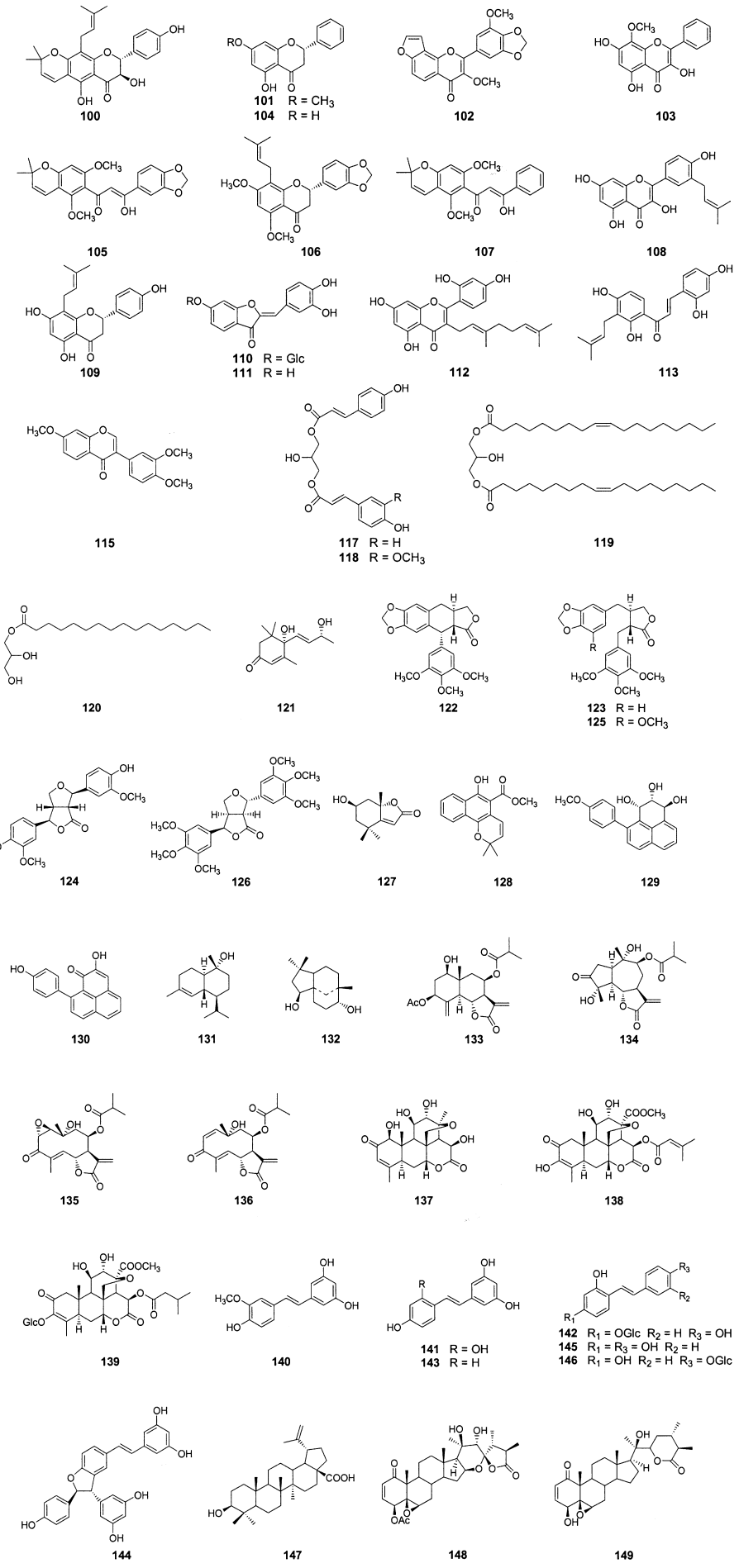


Fig. **2** cont.

Table **2** Bioactive compounds of known structures obtained by activity-guided fractionation[a]

Compound code	*Species code*[b]	*Bioassay system*[c]	*Ref.*	*Compound code*	*Species code*[b]	*Bioassay system*[c]	*Ref.*
Alkaloids				**97**	4	COX-1	[49]
50	26	QR	[66]	**98**	16	COX-2	[73]
51	26	QR	[66]	**99**	6	COX-1	[47]
Amides				**Flavonoids**			
52	1	QR	[67]	**100**	16	COX-1	[73]
53	1	QR	[67]	**101**	19	QR	[58]
Benzenoids				**102**	23	QR	[55]
54	6	AR	[47]	**103**	19	QR	[58]
55	6	AR	[47]	**104**	6	AR	[47]
56	6	ERα; ERβ	[47]	**105**	23	QR	[55]
57	14	JB6	[68]	**106**	23	QR	[55]
58	26	QR	[66]	**107**	23	QR	[55]
59	10	QR	[48]	**108**	6	AR	[47]
60	5	COX-1	[69]	**109**	16	COX-1	[73]
61	9, 13	AO; AO	[56]; [45]	**110**	9	AO	[56]
62	9	AO	[56]	**111**	9	AO	[56]
63	1	QR	[67]	**112**	6	AR	[47]
Benzofurans				**113**	6	AR	[47]
64	12	QR	[70]	**114**	17	QR	[71]
65	6	ERα; ERβ; MCF-7; COX-1	[47]; [53]	**115**	19	QR	[58]
66	4	COX-1	[49]	**116**	17	QR	[71]
67	6	AR	[47]	**Glycerol esters**			
Cardiac glycoside				**117**	5	COX-1	[69]
68	8	Col2	[42]	**118**	5	COX-1	[69]
Ceramide				**119**	7	COX-2	[74]
69	22	QR	[50]	**120**	5	COX-1; COX-2	[69]
Coumarin				**β-Ionone**			
70	26	QR	[66]	**121**	26	QR	[66]
Cyclopenta[*b*] benzofuran				**Lignans**			
71	1	IA	[67]	**122**	29	ODC	[52]
Diarylheptanoid				**123**	29	ODC	[52]
72	20	QR	[51]	**124**	26	QR	[66]
Diterpenoids				**125**	18	COX-2	[75]
73	29	ODC	[52]	**126**	15	JB6	[60]
74	29	JB6; ODC	[52]	**Monoterpene**			
Fatty acid				**127**	26	QR	[66]
75	5	COX-1; COX-2	[69]	**Naphthopyran**			
Flavonoids				**128**	25	Col2	[76]
76	6	AR	[47]	**Phenylphenalones**			
77	4	COX-1; COX-2	[49]	**129**	20	QR	[51]
78	6	COX-1	[47]	**130**	20	QR	[51]
79	6	ERα; ERβ	[53]	**Sesquiterpenes**			
80	6	MCF-7; COX-1; AO; AR	[47, 53]	**131**	23	QR	[55]
81	23	QR	[55]	**132**	17	COX-2	[71]
82	23	QR	[55]	**133**	30	HL-60	[62]
83	31	COX-1	[63]	**134**	30	HL-60	[62]
84	19	QR	[58]	**135**	30	Col2	[62]
85	12	QR	[70]	**136**	30	Col2	[62]
86	12	QR	[70]	**Simaroubolides**			
87	17	QR	[71]	**137**	7	HL-60	[74]
88	4	COX-1	[49]	**138**	7	HL-60	[74]
89	31	COX-1	[63]	**139**	7	HL-60	[74]
90	6	AR	[47]	**Stilbenoids**			
91	4	COX-1	[49]	**140**	3	COX-1	[64]
92	28	QR	[57]	**141**	4	COX-1	[49]
93	24	QR	[72]	**142**	31	COX-1	[77]
94	19	QR	[58]	**143**	4	COX-1	[49]
95	24	QR	[72]	**144**	31	COX-1; COX-2	[63]
96	6	ERα; ERβ	[47]	**145**	31	COX-1	[77]

Table **2** cont.

Compound code	*Species code*[b]	*Bioassay system*[c]	*Ref.*	*Compound code*	*Species code*[b]	*Bioassay system*[c]	*Ref.*
146	31	COX-1	[77]	**Withanolides**			
Triterpene				**148**	22	QR	[50]
147	32	COX-2	[78]	**149**	11	QR	[61]

[a] The structures of compounds **50** – **149** are shown in Fig. **2**.

[b] Species code: 1 (*Aglaia ponapensis*); 2 (*Antirhea acutata*); 3 (*Aiphanes aculeata*); 4 (*Artocarpus dadah*); 5 (*Asparagus officinalis*); 6 (*Broussonetia papyrifera*); 7 (*Brucea javanica*); 8 (*Cerbera manghas*); 9 (*Cotinus coggygria*); 10 (*Couepia ulei*); 11 (*Deprea subtriflora*); 12 (*Dipteryx odorata*); 13 (*Eugenia sandwicensis*); 14 (*Euphorbia quinquecostata*); 15 (*Hernandia ovigera*); 16 (*Macaranga conifera*); 17 (*Macaranga triloba*); 18 (*Macrococculus pomiferus*); 19 (*Muntingia calabura*); 20 (*Musa x paradisiaca* cultivar); 21 (*Pachysandra procumbens*); 22 (*Physalis philadelphica*); 23 (*Pongamia pinnata*); 24 (*Renealmia nicolaioides*); 25 (*Rubia cordifolia*); 26 (*Sida acuta*); 27 (*Tephrosia purpurea*); 28 (*Tephrosia toxicaria*); 29 (*Thuja occidentalis*); 30 (*Tithonia diversifolia*); 31 (*Vitis vinifera,* cell culture); 32 (*Ziziphus jujuba*)

[c] Key to bioassay systems in which activity was shown: AO (antioxidant assay); AR (aromatase assay); Col2 (antiproliferative human colon cancer assay); COX-1 (cyclooxygenase-1 inhibition assay); COX-2 (cyclooxygenase-2 inhibition assay); ERα/β (estrogen receptor-binding α/β assay); HL-60 (differentiation of HL-60 cells); IA (Ishikawa anti-E_2 bioassay); JB6 (soft agar transformation assay with JB6 cells); MCF-7 (antiproliferative human breast cancer cells); MMOC (mouse mammary organ culture assay); ODC (inhibition of TPA-induced ornithine decarboxylase activity with cultured mouse epidermal 308 cells); QR (quinone reductase induction assay in cultured Hepa 1c1c7 mouse hepatoma cells).

Table **3** Natural product lead compounds active in the mouse mammary culture assay[a]

Compound type	*Species of origin*	*Bioassay system*[b]	*Reference*
Alkaloids			
Cryptolepinone (**150**)	*Sida acuta*	QR: CD = 0.02 μg/mL MMOC: 83 % at 10 μg/mL	[66]
Flazin (**151**)	*Brucea javanica*	MMOC: 75 % at 4 μg/mL	[74]
Amide			
N-trans-Feruloyltyramine (**152**)	*Sida acuta*	QR: CD = 8.5 μg/mL MMOC: 75 % at 10 μg/mL	[66]
Flavonoids			
Dihydromorin (**153**)	*Artocarpus dadah*	COX-1: IC_{50} = 20.4 μg/mL MMOC: 82 % at 10 μg/mL	[49]
Isoliquiritigenin (**154**)	*Muntingia calabura*	QR: CD = 1.4 μg/mL MMOC: 76 % at 10 μg/mL	[58], [70]
Lonchocarpol A (**155**)	*Macaranga conifera*	COX-2: IC_{50} = 3.9 μg/mL MMOC: 86 % at 10 μg/mL	[73]
Norartocarpetin (**156**)	*Artocarpus dadah*	COX-1: IC_{50} = 4.0 μg/mL MMOC: 85 % at 10 μg/mL	[49]
Steppogenin (**157**)	*Artocarpus dadah*	COX-1: IC_{50} = 1.7 μg/mL MMOC: 67 % at 10 μg/mL	[49]
Tomentosanol D (**158**)	*Macaranga conifera*	COX-2: IC_{50} = 9.8 μg/mL MMOC: 68 % at 10 μg/mL	[73]
Porphyrin derivative			
Chlorophyllide *a* (**159**)	*Physalis philadelphica*	JB6: IC_{50} = 0.30 μg/mL MMOC: 58 % at 10 μg/mL	[50]
Rotenoids			
11-Hydroxytephrosin (**160**)	*Tephrosia toxicaria*	MMOC: 60 % at 10 μg/mL	[57]
α-Toxicarol (**161**)	*Tephrosia toxicaria*	MMOC: 80 % at 10 μg/mL	[57]
Triterpene			
(3*Z*)-Coumaroylarjunolic acid (**162**)	*Eugenia sandwicensis*	MMOC: 79 % at 10 μg/mL	[45]
Withanolides			
18-Hydroxywithanolide D (**163**)	*Physalis philadelphica*	QR: IC_{50} = 0.029 μg/mL JB6: IC_{50} = 0.31 μg/mL MMOC: 63 % at 10 μg/mL	[50]
Ixocarpalactone A (**164**)	*Physalis philadelphica*	QR: IC_{50} = 0.16 μg/mL JB6: IC_{50} = 0.13 μg/mL MMOC: *ca*. 60 % at 10 μg/mL	[50]
Withanone (**165**)	*Physalis philadelphica*	MMOC: 69 % at 10 μg/mL	[50]
Withaphysacarpin (**166**)	*Physalis philadelphica*	QR: IC_{50} = 0.015 μg/mL JB6: IC_{50} = 0.020 μg/mL MMOC: 88 % at 10 μg/mL	[50]

[a] The structures of compounds **150** – **166** are shown in Fig. **3**.

[b] Key to bioassay systems in which activity was shown: COX-1 (cyclooxygenase-1 inhibition assay); COX-2 (cyclooxygenase-2 inhibition assay); JB6 (soft agar transformation assay with JB6 cells); MMOC (mouse mammary organ culture assay); QR (quinone reductase assay with cultured Hepa 1c1c7 mouse hepatoma cells).

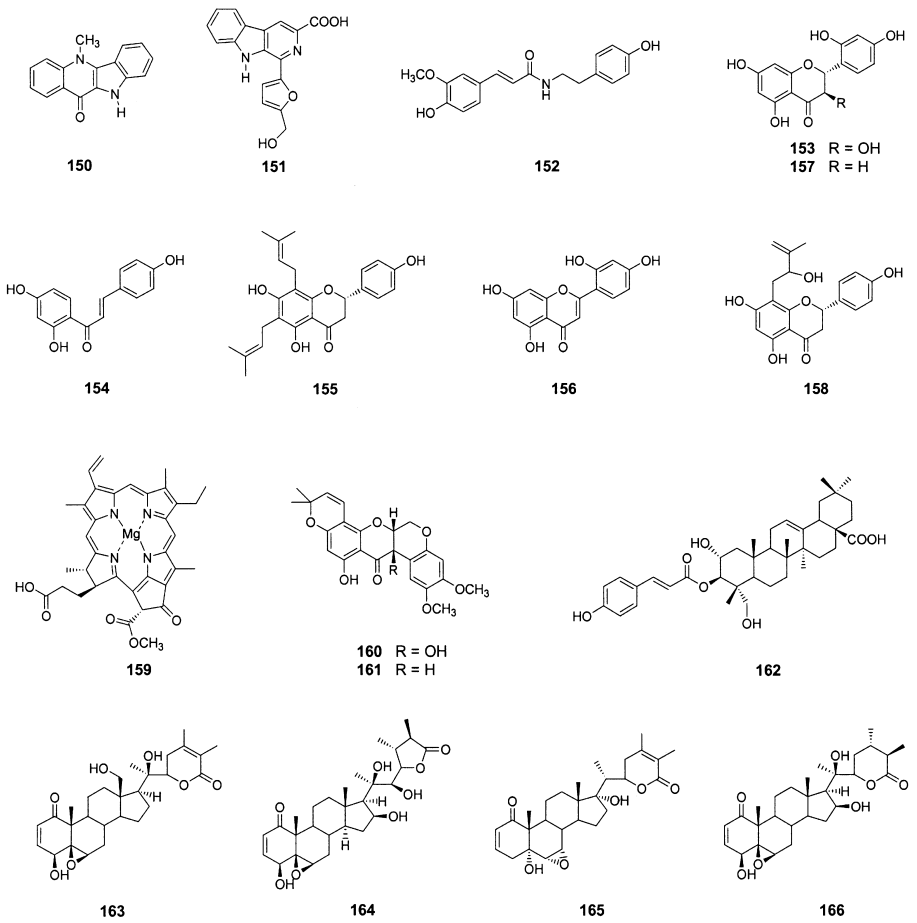


Fig. **3**

accompanied in their plant of origin by inactive substances with new structures.

Natural product lead isolates found active in the mouse mammary organ culture assay (MMOC assay) used as a secondary discriminator assay [30], [31] in our project are presented in Table **3**. These include an indoloquinoline alkaloid (**150**), a β-carboline alkaloid (**151**), an amide (**152**), six flavonoids (**153** – **158**), a porphyrin derivative (**159**), two rotenoids (**160** and **161**), a triterpene (**162**), and four withanolides (**163** – **166**) (Fig. **3**). In the MMOC assay, the inhibition of 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced preneoplastic lesion formation is evaluated, and compounds were generally tested at 10 μg/mL, with, on the basis of historical controls, inhibition of >60% being considered significant. The activity of chemopreventive agents in this *ex vivo* system is known to demonstrate a good correlation with *in vivo* animal models [30], [31].

Structure-Activity Relationships

Since the establishment of our cancer chemoprevention program project in 1991, a battery of cancer chemopreventive *in vitro* bioassays has been used to screen plant extracts, to direct the fractionation and purification of the active fractions, and to evaluate the cancer chemoprevention potential of the pure isolates. Our most recent work has been focused primarily on inducers of quinone reductase (QR) and inhibitors of cyclooxygenases-1 and -2 (COX-1 and COX-2). Using these *in vitro* assays, we have isolated a variety of active compounds from a number of plants collected throughout the world.

QR Induction Activity of Flavonoids

The induction of QR, using the cultured Hepa 1c1c7 (mouse hepatoma) cell line, is a sensitive and rapid method to evaluate the potential of isolates to induce phase II detoxifying enzymes [79]. Phase II enzymes are important in the metabolic detoxification of chemical carcinogens and other harmful oxidants. Furthermore, QR protects the cell from redox cycling of quinones by catalyzing their reduction to hydroquinones. It has also been proposed that QR is involved in maintaining the antioxidant potential of coenzyme Q reductase and vitamin E [79]. Therefore, induction of QR is suggestive of potential cancer prevention at the tumor initiation stage [80]. A great deal of interest has been generated by the role the phase II enzymes play in protecting the cell since their upregulation is considered a major mechanism to prevent the initiation of carcinogenesis [81], [82], [83], [84], [85]. The role of QR and other phase II enzymes in cancer chemoprevention has been reviewed extensively [80], [86], [87]. The majority of the QR active compounds isolated in our work have been either flavonoids or withanolides.

Through the work of our group and the laboratories of others, it has become evident that flavonoids have numerous biological activities of potential health benefits, including the induction of QR [88]. Efforts have been made to determine structure-activity

relationship of flavonoids based on relative activity among different structural variants. Flavonoids are generally considered to be bifunctional inducers, meaning they induce both phase I and phase II enzymes [89]. However, recent evidence suggests this activity may also be dependent upon structural type and that some flavonoids may be monofunctional, phase II selective, enzyme inducers [90]. In recent years, using the QR bioassay, our laboratory has isolated and tested a total of 60 flavonoids, of which 28 were active [using the cut-off value for concentration to double quinone reductase (CD) as < 10 μg/mL] and 32 were inactive. These 60 flavonoids are predominately flavones, flavanones, chalcones, and isoflavonoids. From this relatively large pool of compounds, a few observations can be concluded based on the activities and structures of these isolates. First of all, all chalcones (**23**, **26**, **84**, **93**, **107**, and **154**) tested were active [55], [58], [59], [72], whereas the 2,3-dihydrochalcones were inactive or marginally active [58]. It has been suggested that the 2,3 double bond in flavonoids is a requirement for QR induction [82]. A caveat to this rule is found in the 7-methoxyflavanones. Of the 21 flavanones tested, 13 were found inactive and nine were active. The active flavanones all contained a methoxy group in the C-7 position although compounds with a second methoxy at either the C-6 or the C-8 position did not maintain this activity. An excellent example of this is the potent activity of pinostrobin, (*S*)-5-hydroxy-7-methoxyflavanone (**101**), which was isolated from the leaves of *Muntingia calabura*, and was found to be highly active in the QR assay (CD <0.56 μM) [58]. Pinostrobin and its enantiomeric isomer were isolated as a racemic mixture (**95**) from the roots of *Renealmia nicolaioides* [72], and a comparable CD value (3.36 μM) was obtained for this racemic mixture [72]. However, the flavanone pinocembin (5,7-dihydroxyflavanone), which possesses a hydroxy group at the C-7 position instead of a methoxy group as in pinostrobin, was found to be inactive (CD >10 μg/mL) [58]. The quite different QR induction activities of 5-hydroxy-7-methoxyflavanone (CD = 0.5 μM, strongly active) and 5,7-dihydroxyflavanone (CD = 110 μM, inactive) were also reported by another group recently [91]. Isoflavonoids are another group of flavonoids that consistently show QR induction activity. All isoflavonoids isolated were active (**90**, **92**, and **113**) with CD values of 22.9, 5.7, and 4.6 μM, respectively. Work by Wang et al. compared the activity of the isoflavonoids genistein and daidzein. The QR activity was lost when the 5-hydroxy group in genistein was removed [83]. In the QR bioassay, no flavonoid glycosides have been found to be active. However, this may be pertinent *in vivo* since the glycosides can be converted to the aglycone and there is evidence to suggest that the glycoside derivatives may have an increased bioavailability [88].

QR Induction Activity of Withanolides

In the course of our cancer chemoprevention research, the chloroform-soluble extracts of two Solanaceous plants, the leaves and stems of *Physalis philadelphica* Lam. [50], [65] and the whole plants of *Deprea subtriflora* (Ruiz & Pavon) D'Arcy [44], [61] were found to be highly active in the QR assay. Bioassay-guided fractionation of these extracts led to the isolation of 12 withanolides [50], [61], [65] and 12 C_{27} norwithanolides [44], [61]. The QR induction abilities of all these isolates and ixocarpalactone B 4-monoacetate (**148**) were evaluated. The test results indicated that withanolides (**46**–**49**, **148**, **149**) and norwithanolides (**29**–**34**), which all possess an α,β-unsaturated ketone structural unit in their A-rings, were significantly active with CD values in the range of 0.03 – 3.5 μM [44], [50], [65]. However, one withanolide, withanone [50], and one norwithanolide, subtrifloralactone L [61], were found to be inactive in the QR assay (CD > 10 μg/mL), although the α,β-unsaturated ketone structure units are present in the molecules of these two compounds. Withanone was the only withanolide tested possessing a 6α,7α-epoxy group, while subtrifloralactone L was the only norwithanolide evaluated with a 2,4-dien-1-one structural functionality in its A-ring. The cell differentiation inducing activity against M1 cells was reported for a number of withanolides isolated from the seeds of *Withania somnifera* (L.) Dun. [92]. The four most active withanolides against M1 cells also contain an α,β-unsaturated ketone structural unit in their A-ring [92]. Hence, a ring A α,β-unsaturated ketone structural unit appears to be necessary for the QR induction activity of both withanolides and norwithanolides, although other functionalities present may mediate this effect [93].

Inhibitory Activity on Cyclooxygenases-1 and -2 by Phenolic Compounds

An increase in prostaglandin (PG) synthesis may influence tumor growth in both humans and experimental animals [94], and numerous studies have demonstrated the effect of PG synthesis on carcinogen metabolism, tumor cell proliferation and metastatic potential [95], [96]. As a result, inhibition of PG synthesis has been investigated as a means of preventing tumor development [96], [97]. PGs produced by cyclooxygenases-1 and -2 (COX-1 and COX-2) are represented by a large series of compounds which enhance mainly cancer development and progression, acting as carcinogens or tumor promoters, with profound effects on carcinogenesis [98]. In the course of our work in this area, several classes of natural products mainly including alkaloids, fatty acids, flavonoids, glycerol lipid esters, lignans, phenylpropanoids, quassinoids, sesquiterpenoids, steroids, stilbenoids and triterpenoids have been isolated and the inhibitory activities against COX-1 and COX-2 of the isolates obtained were evaluated [47], [49], [53], [63], [64], [69], [71], [73], [77], [78]. The biological test results indicated a total of 42 compounds to be active (inhibition % > 50 at 100 μg/mL) against COX-1 and/or COX-2. These isolates include a benzenoid (**60**), three benzofurans (**5**, **65**, **66**), a fatty acid (**75**), 16 flavonoids (**13**, **77**, **78**, **80**, **83**, **88**, **89**, **91**, **97**–**99**, **108**, **153**, **155**, **156**, **158**), four glycerol lipid esters (**117**–**120**), a lignan (**125**), a sesquiterpene (**132**), 11 stilbenoids (**38**–**41**, **140**–**146**), a stilbenolignan (**42**), and three triterpenes (**43**, **44**, **147**). It is evident that most of the active isolates are phenolic compounds, and are mainly flavanones, flavones and stilbenoids.

It appears that the relationships of the cyclooxygenase-inhibitory activities and the structures of flavanones and flavones are very complex. Based on our obtained results, several conclusions can be made, as follows: 1) Flavanones appear to be more active than flavones with a given substitution pattern, as may be seen by comparison of the IC_{50} values of steppogenin (**157**, 5.9 and 46.4 μM for COX-1 and COX-2, respectively) and norartocarpetin

(**156**, 14.0 and >100 μM for COX-1 and COX-2, respectively) [49]; 2) A hydroxy group present at the C-3 position of a flavanone decreases the cyclooxygenase-inhibitory activity (**153**: IC_{50} = 67.1 and >100 μM for COX-1 and COX-2, respectively; **157**: IC_{50} = 5.9 and 46.4 μM for COX-1 and COX-2, respectively) [49]; 3) A few B-ring 2,4-dihydroxy substituted flavanones and flavones (**153**, **156**, **157**) were found to be not only active against *in vitro* COX-1 and/or COX-2 assays but also active in the *in vivo* MMOC model (Table **3**). The acetylated product of chrysin, 5,7-diacetylflavone, has been found to be a selective COX-2 inhibitor (IC_{50} = 2.7 μM for COX-2 and 68 μM for COX-1, respectively), while its analogues, 5,7-dihydroxyflavone and 5-hydroxy-7-methoxyflavone, were inactive for both COX-1 and COX-2 [99]. The activity of 5,7-diacetylflavone, as attributed to the presence of an acetyl group at C-7 position, was analyzed by a three-dimensional modeling method [99].

The cancer chemoprevention potential of *trans*-resveratrol (**143**, 3,5,4′-trihydroxy-*trans*-stilbene) in various assays reflective of the three major stages of carcinogenesis has been established in our previous work [37], [100], [101]. The *trans*-stilbenoids possess more potent cyclooxygenase-inhibitory ability compared to *cis*-stilbenoids, as can be concluded from the activity of *trans*-piceid (**142**, 95% inhibition at 70 μg/mL, IC_{50} = 10.6 μM for COX-1) and *cis*-piceid (inactive, 32% inhibition at 70 μg/mL for COX-1), *trans*-resveratrol (**143**, IC_{50} = 14.9 μM for COX-1) and *cis*-resveratrol (IC_{50} = 55.4 μM for COX-1), *trans*-resveratroloside (**146**, IC_{50} = 4.8 μM for COX-1) and *cis*-resveratroloside (IC_{50} = 17.8 μM for COX-1) [77]. The hydroxy group at the C-3′ position greatly decreases the inhibitory activity of stilbenoids, since, for example, *trans*-piceid (5,4′-dihydroxy-3-β-D-glucopyranosyloxystilbene) was found to be active against COX-1 (IC_{50} = 10.6 μM), while *trans*-astringin (3′-hydroxy-*trans*-piceid, 3-β-D-glucopyranosyloxy-5,3′,4′-trihydroxystilbene) was found to be inactive [77]. Similarly, when compared to the promising chemoprevention agent, *trans*-resveratrol, 3′-hydroxy-*trans*-resveratrol (*trans*-piceatannol) was demonstrated to be inactive against both COX-1 and COX-2 [64], [77]. However, inhibitory activity (IC_{50} = 1.5 μM) was observed for *trans*-isorhapontigenin, which possesses a methoxy group at the C-3′ position instead of the hydroxy group in the molecule of *trans*-piceatannol [64]. In addition, a structurally novel stilbene and phenylpropanoid adduct, aiphanol (**42**), which was isolated in our work from the seeds of *Aiphanes aculeata*, exhibited promising inhibitory activity against COX-1 and COX-2, with IC_{50} values of 1.9 and 9.9 μM, respectively [64]. Due to the structural novelty and significant inhibitory activity of aiphanol (**42**), this compound has been recently synthesized by other groups as its racemic mixture [102], [103]. Biological test results indicated that the synthetic (±)-aiphanol was selectively active against COX-1 and COX-2, with IC_{50} values of 7.3 and 0.17 μM, respectively [102]. Accordingly, it would be worth establishing the absolute stereochemistry of aiphanol (**42**), to synthesize the enantiomeric pure form of this stilbenolignan, and to evaluate the cyclooxygenase-inhibitory activity of the racemic isomers.

Inhibitory Activity on Cyclooxygenases-1 and -2 by Fatty Acids

The inhibitory effects against both COX-1 and COX-2 of some naturally occurring and modified fatty acids have been reported recently [104], [105]. In our previous work, a fatty acid mixture of linoleic, oleic and stearic acids isolated from the seeds of *Ziziphus jujuba* was found to be active against both COX-1 and COX-2 [78]. However, the methylation product of the fatty acid mixture was inactive in these assays [78]. Based on our test results for the COX-1 and COX-2 inhibitory activities of pure linoleic, oleic and stearic acids [69], [78] as well as the values reported by other groups [104], [105], it appears that fatty acids with higher unsaturation values possess more potent activity against COX-1 and COX-2 than their saturated analogues. Furthermore, the unsaturated fatty acids are selectively active against COX-2 [69], [78], [104], [105]. For example, the initial petroleum ether extract of the stems of *Macrococculus pomiferus* was found to significantly inhibit COX-2 (the % inhibition was 86 for both extracts at 100 μg/mL) [75]. The major constituents of this extract were also determined to be the common fatty acids, linoleic acid, linolenic acid, oleic acid and stearic acid, based on the NMR and LC-MS data obtained [75]. Some of these *in vitro*-active fatty acids, however, have been found to be totally inactive when tested by our group in a follow-up *ex vivo* MMOC assay [30], [31]. In the course of our search for cancer chemopreventive compounds directed using COX-1 and/or -2 assays, a number of plant petroleum ether-soluble extracts were demonstrated to be active against COX-1 and/or COX-2. However, the major active compounds then obtained by further purification were often fatty acids [69], [78]. Accordingly, to avoid unnecessary time-consuming activity-guided fractionation, it is recommended that fatty acids are removed from plant extracts by defatting with a suitable solvent prior to their evaluation in the COX-1 and COX-2 bioassays. Alternatively, these non-polar extracts may be subjected to LC-MS dereplication [78].

Conclusions

As a result of previous collaborative work in our project designed to identify new natural product cancer chemopreventive agents, several plant-derived isolates or derivatives, such as deguelin, resveratrol, bruceantin, brassinin, 4′-bromoflavone, and oxomate, have demonstrated activity with *in vivo* carcinogenesis inhibition models, and are considered as promising leads for further development [80], [81].

In continuing work to develop cancer chemopreventive agents from plant sources [26], [93], [106], [107], a large number of structurally diverse plant metabolites with significant activity in one or more *in vitro* assays germane to cancer chemoprevention have been isolated in our most recent work (Tables **1** – **3**; Figs. **1** – **3**). Nineteen plant species provided a total of 49 new active natural products evaluated in 14 different *in vitro* assay systems as shown in Table **1**.

All *in vitro*-active compounds isolated from the present work in sufficient quantity (>2 mg) were tested not only in primary *in vitro* bioassays, but also in the mouse mammary organ culture (MMOC) assay, which has been found to be a useful discriminator for metabolites found to be active in these preliminary assays.

The active agents in this *ex vivo* system are known to demonstrate a good correlation with *in vivo* animal models, and therefore, these promising isolates are considered as good leads for further *in vivo* biological studies (Table **3**).

Ixocarpalactone A, a withanolide occurring in relative high natural abundance from *Physalis philadelphica*, has been chosen for further biological studies using advanced biological models. Four aromatase inhibitors, the flavonoids (2*S*)-abyssinone II (**76**), (2*S*)-2′,4′-dihydroxy-2″-(1-hydroxy-1-methylethyl)dihydrofuro[2,3-*h*]flavanone (**12**), 3′-[γ-hydroxymethyl-(*E*)-γ-methylallyl]-2,4,2′,4′-tetrahydroxychalcone 11′-*O*-coumarate (**20**), and isolicoflavonol (**98**) from *Broussonetia papyrifera*, have been selected for further development towards preclinical trials by the Division of Cancer Prevention of the United States National Cancer Institute through the Rapid Access to Preventive Intervention Development (RAPID) program (http://www3.cancer.gov/-prevention/rapid). Of the compounds listed in Table **3**, the quinone reductase inducer and MMOC inhibitor, isoliquiritigenin (**154**), has also demonstrated inhibition of azomethane (AOM)-induced murine colon carcinogenesis and AOM-induced murine colon aberrant crypt focus formation [108]. Moreover, isoliquiritigenin was found to suppress metastasis in a pulmonary metastasis model of mouse renal cell carcinoma [109].

The triterpenoid derivative isolated from *Eugenia sandwicensis*, 3β-*trans*-coumaroyloxy-2α,23-dihydroxyolean-12-en-28-oic acid [(3*Z*)-coumaroylarjunolic acid, **162**], was found to be significantly active in MMOC assay with the inhibition value of 79.2% at 4 μg/mL [45]. However, its *cis*-isomer, 3β-*cis*-coumaroyloxy-2α,23-dihydroxyolean-12-en-28-oic acid [(3*E*)-coumaroylarjunolic acid], was demonstrated to be inactive in the same assay with an inhibition value of only 36.6% even at 10 μg/mL [45].

As demonstrated by the work described in this review, secondary metabolite constituents with broad chemical diversity and interesting biological activity have been discovered from edible and other plants. The results are of interest not only in terms of enriching chemotaxonomic knowledge, but also one or more of the isolates may lead to the development of new cancer chemopreventive agents from natural sources.

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