

Synthetic and Natural Coumarins as Cytotoxic Agents

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Abstract: Coumarins, an old class of compounds, are naturally occurring benzopyrene derivatives. A lot of coumarins have been identified from natural sources, especially green plants. The pharmacological and biochemical properties and therapeutic applications of simple coumarins depend upon the pattern of substitution. Coumarins have attracted intense interest in recent years because of their diverse pharmacological properties. Among these properties, their cytotoxic effects were most extensively examined. In this review, their broad range of effects on the tumors as shown by various *in vitro* and *in vivo* experiments and clinical studies are discussed. Hence, these cytotoxic coumarins represent an exploitable source of new anticancer agents, which might also help addressing side-toxicity and resistance phenomena. These natural compounds have served as valuable leads for further design and synthesis of more active analogues. In this review, plant derived coumarins and their synthetic analogues were systematically evaluated based on their plant origin, structure-activity relationship and anticancer efficacy. Owing to their diverse effects and inconclusive results from different *in vitro* studies, the mechanism of their action is not yet fully understood and correlation of effects with chemical structures is not conclusive at the moment. It is the objective of this review to summarize experimental data for different coumarins, used as cytotoxic agents, because promising data have been reported for a series of these agents. Yet, the results from different coumarins with various tumor lines are contradictory in part. We therefore conclude that there is still a long way to go until we know which cytotoxic agent will clinically be suitable for what tumor entity for treatment. Their ability to bind metal ions represents an additional means of modulating their pharmacological responses.

Key Words: Synthetic and natural coumarins, cytotoxicity.

INTRODUCTION

Numerous compounds with biological activity have been investigated, however many of them are not suitable for therapeutic use due to their toxic, carcinogenic and mutagenic properties. Nowadays, it is possible to make modifications of active chemical structures, in order to synthesize compounds with improved therapeutic activity and reduced toxicity.

A variety of herbs and herbal extracts contain different phytochemicals with biological activity that can provide therapeutic effects. Several herbs can help to provide some protection against cancer and stimulate the immune system.

Coumarins comprise a group of natural compounds found in a variety of plant sources. The very long association of plant coumarins with various animal species and other organisms throughout evolution may account for the extraordinary range of biochemical and pharmacological activities of these chemicals in mammalian and other biological systems. The coumarins that were studied have diverse biological properties and various effects on the different cellular systems. A lot of biological parameters should be evaluated to increase our understanding of mechanisms by which these coumarins act. Coumarins have important effects in plant biochemistry and physiology, acting as antioxidants, enzyme inhibitors and precursors of toxic substances. In addition, these compounds are involved

in the actions of plant growth hormones and growth regulators, the control of respiration, photosynthesis, as well as defense against infection. The coumarins have long been recognized to possess anti-inflammatory, antioxidant, anti-allergic, hepatoprotective, antithrombotic, antiviral, and anticarcinogenic activities. The hydroxycoumarins are typical phenolic compounds and, therefore, act as potent metal chelators and free radical scavengers. They are powerful chain-breaking antioxidants. The coumarins display a remarkable array of biochemical and pharmacological actions, some of which suggest that certain members of this group of compounds may significantly affect the function of various mammalian cellular systems.

The coumarins are extremely variable in structure, due to the various types of substitutions in their basic structure, which can influence their biological activity. A careful structure-system-activity-relationship study of coumarins with special respect to carcinogenicity, mutagenicity, and cancer-preventing activities should be conducted. Vast majority of coumarins, completely innocuous, may be beneficial in a variety of human disorders, in spite of some ongoing controversy.

The naturally occurring coumarins will be the primary focus of this review, with occasional reference to synthetic compounds. The review is not exhaustive; it is intended to acquaint the reader with this interesting group of natural plant compounds. There has been, in recent years, a major rekindling of interest in pharmacognosy. Coumarins turn out to be present in many natural therapeutically utilized products. They hold a place apart in view of their cytotoxic activity. It was suggested that alterations in the chemical

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structure of coumarins could change their cytotoxic properties.

Actually, *in vitro* cytotoxicity assays with cultured cells are widely used to chemicals including cancer chemotherapeutics, pharmaceuticals, biomaterials, natural toxins, antimicrobial agents and industrial chemicals because they are rapid and economical. These cytotoxicity tests measure the concentration of the substance that damages components, structures or cellular biochemical pathways, and they also allow direct extrapolation of quantitative data to similar *in vitro* situations.

SYNTHETIC AND SEMI-SYNTHETIC SIMPLE COUMARINS

Coumarin (1, 2-benzopyrone) (**1**), the parent molecule of coumarin derivatives, is the simplest compound of a large class of naturally occurring phenolic substances made of fused benzene and -pyrone rings [1]. The investigation of coumarin compounds revealed that a wide spectrum of medicinal plant extracts that were in use as early as 1000 A.D. contains a high content of coumarins. Subsequent analysis of scientific literature revealed numerous reports on the antiproliferative and antitumor activities of a variety of coumarin compounds, e.g., both coumarin itself and 7-hydroxycoumarin (**2**) have been reported to inhibit the proliferation of a number of human malignant cell lines *in vitro* [2-5] and have demonstrated activity against several types of animal tumors [6-10]. These compounds have also been reported in clinical trials to demonstrate activity against prostate cancer, malignant melanoma, and metastatic renal cell carcinoma [11-13].

For coumarins, generally the *in vitro* structure-activity relationship studies have shown that cytotoxicity is found with derivatives containing ortho-dihydroxy substituents (Kolodziej *et al.*, 1997, [14]). Also, the chemical-structure/biological activity study of the coumarins showed that the addition of a catecholic group to the basic structure induces increased cytotoxic activity in tumor cell lines (Kolodziej *et al.*, 1997, [14]). The different cytotoxic values found for the coumarins could be related to presence and the positions of the hydroxyls in their structures.

The cytotoxicity of 22 natural and semi-synthetic simple coumarins was evaluated in GLC4, a human small cell lung carcinoma cell line, and in COLO 320, a human colorectal cancer cell line, using the microculture tetrazolium (MTT) assay [14]. With IC_{50} values $> 100 \mu\text{M}$, following a continuous (96 h) incubation, most coumarins exhibited only low cytotoxicity. Several compounds, however, displayed significant potencies. As far as the structure-cytotoxicity relationship is concerned, it is conspicuous that all the potentially active natural compounds possess at least two phenolic groups in either the 6, 7- or 6, 8-positions. In addition, the 5-formyl-6-hydroxy substituted semi-synthetic analogue was found to be potent, reflecting the importance of at least two polar functions for high cytotoxicity.

Several hydroxylated and/or methoxylated coumarin derivatives were tested for their relative cytotoxicity on four human tumor cell lines (oral squamous cell carcinoma HSC-2, HSC-3, melanoma A-375 and promyelocytic HL-60) and

three normal human cells (gingival fibroblast HGF, periodontal ligament fibroblast HPLF and pulp cell HPC) [15]. Tumor cell-specific cytotoxicity was detected in all 6, 7-dihydroxy-substituted coumarins only. The observations indicate that the tumor-specific cytotoxicity of the naturally occurring coumarin esculetin (6, 7-dihydroxycoumarin) (**3**) can be further enhanced by proper substitutions at 3- and/or 4-position(s) of the molecule. Agarose gel electrophoresis revealed that esculetin and its derivatives with tumor-specific cytotoxicity induce internucleosomal DNA fragmentation in HL-60 cells.

Ratanasavanh D. *et al.* [16] compared the cytotoxic effect of coumarin and its derivatives, 7-hydroxycoumarin, 4-hydroxycoumarin (**4**), o-hydroxyphenyl acetic acid and o-coumaric acid (**5**), on cultured hepatocytes from human, rat, mouse and rabbit liver. At 10^{-5} and $5 \cdot 10^{-5}$ M, coumarin and its derivatives did not give rise to any signs of toxicity on cultured hepatocytes of the four species. At 10^{-4} M, coumarin, but not its derivatives, induced release of lactate dehydrogenase (LDH) into the medium, especially in rat hepatocyte cultures. Intracellular LDH activities were correspondingly reduced. The cytotoxic effect of coumarin in cultured rat hepatocytes was evidenced on morphological examination and from the results of the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium (MTT) reduction test. At higher concentrations ($5 \cdot 10^{-4}$ M), 7-hydroxycoumarin and o-coumaric acid were also found to be cytotoxic in cultured rat hepatocytes. The cytotoxic effect of coumarin ($5 \cdot 10^{-4}$ M) was decreased in the presence of SKF 525-A, a cytochrome P450 inhibitor. Interspecies comparisons showed that rat hepatocytes were the most sensitive to the toxicity of coumarin and its derivatives, whereas human hepatocytes were the most resistant. The results suggest that the cytotoxicity of coumarin is metabolism and species-dependent. Thus, the rat may not be a suitable model for evaluating the pharmacological hazards of coumarin in humans.

Coumarin, 4-hydroxycoumarin and 7-hydroxycoumarin, as well as o-, m-coumaric acid (**6**) and p-coumaric acid (**7**) were tested against P-815 and P-388 tumor cells *in vitro* [17]. In addition, the compounds were investigated in various *in vitro* immunological test systems and genuine coumarin was tested furthermore against the Sarcoma-180 in CD1 mice. *In vivo*, coumarin showed only a moderate antitumor effect against the allogeneic Sarcoma-180 at concentrations of 10 and 40 mg/kg, with inhibition rates of 49 and 60%, respectively. However, both concentrations were markedly toxic. *In vitro* all compounds were more or less cytotoxic against P-815 and P-388 tumor cell lines starting at a concentration of $100 \mu\text{g/mL}$. At subtoxic concentrations (less than or equal to $10 \mu\text{g/mL}$) the samples showed no mitogenic activity against murine spleen lymphocytes and PHA costimulated human peripheral blood lymphocytes. Furthermore, with the coumarin derivatives neither cytotoxic macrophages could be induced against P-815 tumor cells nor an increased release of IL-2 and TNF-alpha could be observed. Only 7-hydroxycoumarin, in concentrations of 2 and $20 \mu\text{g/mL}$, caused a strong increase in phagocytosis of 124 and 84% in both, human peripheral blood granulocytes and murine peritoneal macrophages, respectively.

A selection of natural and synthetic coumarin compounds, including the hydroxylated and nitrated derivatives, were assessed for their cytotoxic potential using the microculture 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay for cellular viability [18]. For the first time this study utilized both human skin malignant melanocytes (SK-MEL-31) and normal human skin fibroblastic cells (HS613.SK), allowing identification of those coumarin derivatives that are selectively toxic. Coumarin was found to exhibit comparatively low toxicity in both cell types, while 7-hydroxycoumarin and coumarin had similar activity in SK-MEL-31 cells. The entire series of hydroxylated coumarins were considerably more toxic in HS613.SK than in SK-MEL-31 cells. Novel synthetic nitrated coumarins, 6-nitro-7-hydroxycoumarin (**8**) and 3, 6, 8-nitro-7-hydroxycoumarin (**9**), were shown to be significantly more toxic to SK-MEL-31 than HS613.SK cells. In the malignant melanocyte skin cell line (SK-MEL-31) the cytotoxic effects of these nitro-derivatives were shown to be dose and time dependent. Therefore, the cytotoxic potential of coumarins appears to be highly dependent on the nature and position of the functional group. In addition, nitration of 7-hydroxycoumarin produced compounds that were cytotoxic to malignant melanocytes, suggesting that these nitro-derivatives may have a chemotherapeutic role in the future.

Finn G.J. *et al.* [19] determined the selective cytotoxicity of eight coumarin compounds to human renal carcinoma cells, relative to non-carcinoma proximal tubular cells. Selectivity cytotoxicity was observed following exposure to 6-nitro-7-hydroxycoumarin and 7, 8-dihydroxycoumarin (**10**). 6-Nitro-7-hydroxycoumarin induced cytotoxicity was irreversible in both cell lines, unlike 7, 8-dihydroxycoumarin, which was reversible in the carcinoma cells only. Mobility shift and BrdU incorporation assays showed that both compounds did not intercalate DNA but had a concentration-dependent inhibitory effect on its synthesis. All coumarins studied were found to be non-mutagenic using the standard Ames test. These results would suggest that 6-nitro-7-hydroxycoumarin and 7, 8-dihydroxycoumarin might have a therapeutic role to play in the treatment of renal cell carcinoma.

A derivative of coumarin, 8-nitro-7-hydroxycoumarin (**11**), was synthesised, purified and characterised [20]. The cytostatic and cytotoxic nature of this compound was determined using both human and animal cell lines grown *in vitro* for 96 h in the presence of drug (0-500 μ M, equivalent to 0-104 μ g/mL). 8-Nitro-7-hydroxycoumarin was shown to be cytotoxic to three cell lines, but cytostatic to all cell lines tested. With K562 and HL-60 cells, cell death was found to occur by apoptosis. This cytotoxic effect was found to be irreversible, with cell death continuing to occur following a 96 h recovery period. The cytostatic effects were found to be irreversible in four of the five cell lines tested. 8-Nitro-7-hydroxycoumarin demonstrated its cytostatic effects within 24 or 48 h, while its cytotoxic effects appeared more gradually. The IC_{50} of 8-nitro-7-hydroxycoumarin was 475-880 μ M, depending on the cell line tested. It was shown to exert its cytostatic effect through an alteration of cell cycle. It also inhibited DNA synthesis. The toxicity of 8-nitro-7-hydroxycoumarin does not appear to be mediated through

the multi-drug resistance (MDR) protein since it caused significant cytostatic and cytotoxic effects to CHR5 cells, which have an increased expression of this protein. This compound was shown to be non-mutagenic in a standard Ames test, both with or without a mammalian enzyme activation system. The applications and mode of actions of coumarins are discussed.

Ten 6, 12-dihydro-1-benzopyrano[3, 4-b][1, 4]benzothiazin-6-ones and other related coumarins were compared for their cytotoxic activity and radical intensity [21]. These compounds showed highest cytotoxic activity against human promyelocytic leukemic HL-60 cells. They produced radicals under alkaline conditions, and showed low pi-spin density at S-atom of the molecule, suggesting the delocalization of pi-spin density. These data suggest the possible relation between radical intensity and biological activity. The cytotoxic effects and alkylating activity of a series of 3-[1-(alkylamino)-ethylidene]-chroman-2, 4-dione, 2-methoxy-3-[1-(alkylamino)-ethylidene]-2,3-dihydro-2,4-dioxo-2lambda-(5)-benzo[e][1,2] oxaphosphinane and [2-oxo-4-phenyl(alkyl)-2H-chromen-3-yl]-phosphonic acids dimethyl ester on the leukemia cell lines HL-60 and NALM-6 have been determined [22]. The test compounds are much more toxic to NALM-6 cells than to HL-60 cells. IC_{50} data are up to nine times lower for the NALM-6 than for the HL-60 cell lines. As determined in an *in vitro* Preussmann test phosphonic derivatives [2-oxo-4-phenyl(alkyl)-2H-chromen-3-yl]-phosphonic acids dimethyl ester possess very high alkylating activity, phosphoric derivatives are less active while the derivatives of 3-[1-(alkylamino)-ethylidene]-chroman-2, 4-dione can be included in the group of low activity alkylating agents. Using regression analysis QSAR the authors found a relationship between biological activity and the physicochemical properties of the test compounds. Their cytotoxic effect increases with an increase of the hydrophobic parameters in the region of the substituents at the 2-, 3- and 4-positions of the benzopyrone skeleton of the compounds. Several aromatic seleno lactones have been synthesized and shown to possess significant inhibitory activity against human colon tumor-8r cells in culture at concentrations lower than 1mM [23]. Although all of the compounds tested were found to be active, 5-hydroxy-3-[(phenylseleno)methyl]hydrocoumarin-octanoate and 5-hydroxy-3-[(phenylseleno)methyl]hydrocoumarin-decanoate were found to be the most effective in inhibiting cell growth. In situ formation of the corresponding alpha-methylene lactones is postulated to account for the cytotoxic activity in this class of compounds.

COUMARIN ANTICOAGULANTS AND CYTOTOXICITY

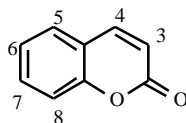
The possibility of a relation between the clotting mechanism and the development of metastases was postulated as early as 1878, when Billroth described cancer cells within a thrombus and interpreted his finding as evidence of the spread of tumor cells by thromboembolism. Only a few published studies of the therapeutic efficacy of anticoagulants against cancer were appropriately designed randomized trials; otherwise, they have been mainly case descriptions, retrospective epidemiologic reviews, and small phase 1 or 2 trials [24]. Moreover, in most trials, the

treatment included a combination of cytotoxic drugs plus an anticoagulant. For this reason, we do not know whether the possible benefit of anticoagulation was due to an effect on the clotting system, a direct cytotoxic activity of the anticoagulant [25], or a change in the pharmacokinetics of the cytotoxic drugs caused by the anticoagulant [26]. Warfarin (**12**) (a coumarin anticoagulant) therapy was reported to improve the survival of patients with small-cell lung cancer, but this finding has never been confirmed [25]. The results, obtained, suggest that there may be some survival benefit to anticoagulation in the treatment of small-cell lung cancer. The effect is probably not large, judging by the difficulty in reaching statistical significance, but there does appear to be a consistent small benefit. The administration of low levels of anticoagulant is, in general, much less toxic and expensive than the addition of another chemotherapy agent, or the use of high-dose chemotherapy with stem cell support. While intriguing, however, the data are not sufficiently convincing to alter current clinical practice. There is renewed interest in determining whether anticoagulation may improve survival in oncology patients. Although there are many hypotheses for *in vitro* antitumor activity of anticoagulants, the practical question is whether anticoagulants affect cancer mortality in a clinical setting.

Coumarin anticoagulants inhibit metastasis in several animal models, but the mechanism of this effect is uncertain. In order to determine the role of cytotoxic and/or cytostatic actions of coumarins on the tumour cells, McCulloch P. *et al.* [27] have studied the effects of warfarin on tumour cell growth in a model in which tumour metastasis is inhibited by this drug. Clonogenic assay, growth curve analysis and thymidine labelling index revealed that warfarin had no effects on Mtn3 mammary carcinoma cell growth *in vitro* at concentrations below 1 mM. The growth rate of subcutaneously implanted Mtn3 tumour deposits in female F344 rats, assessed by weight and by stathmokinetic analysis of the tumour tissue, was identical in warfarin-treated and control animals. Spontaneous metastasis from such tumours to the lungs was, however, significantly reduced in warfarin-treated animals (median 0 pulmonary tumours per animal in warfarin treated, eight tumours per animal in control animals; P less than 0.05, Mann-Whitney). The mean plasma warfarin concentration in warfarin treated rats was 1.63 μ M. These results suggest that warfarin treatment of the host animal can inhibit tumour metastasis without having any direct or indirect effect on the growth rate of the tumour cells.

The coumarin anticoagulants, dicoumarol (Dicoumarol) (**13**) and its synthetic derivative warfarin sodium (Coumadin) (**14**), have been shown to decrease metastases in experimental animals [28]. Warfarin sodium, largely replacing dicoumarol therapeutically as an anticoagulant, has been used for the treatment of a variety of cancers and shown to improve tumor response rates and survival in patients with several types of cancer [29]. However, despite numerous studies, little information has been acquired on the cellular mechanism of action of coumarin compounds in the treatment of malignancies. Possibly for this reason, the coumarin compounds have not received much attention for the treatment of cancer. Earlier studies revealed that coumarin, 7-hydroxycoumarin, and 4-hydroxycoumarin inhibit mitosis in

Allium cepa root tips [30]. Interestingly, 7-hydroxycoumarin disorganized the mitotic spindle microtubules in *A. cepa* cells, leading to the random distribution of the chromosomes at metaphase. This is a form of cytotoxicity common to mitotic spindle poisons that inhibit mitosis by modifying microtubule dynamics. On further investigation of the coumarin literature, it was developed the hypothesis that coumarin compounds might inhibit cell proliferation by interfering with mitotic spindle microtubule function [31]. Dicoumarol, a natural anticoagulant drug chemically designated as 3, 3'-methylenebis[4-hydroxycoumarin], is metabolized from coumarin in the sweet clover (*Melilotus alba* and *Melilotus officinalis*) by molds, such as *Penicillium nigricans* and *Penicillium jensi*. In studies on the anti-proliferative actions of coumarin compounds, Hamta Madari *et al.* [31] discovered that dicoumarol inhibits the first cleavage of *Strongylocentrotus purpuratus* (sea urchin) embryos in a concentration-dependent manner with 50% inhibition occurring at a concentration of 10 μ M. The results suggest that the antiproliferative mechanism of action of dicoumarol and possibly related pharmacophores may be mediated by tubulin binding and the stabilization of spindle microtubule dynamics. Because of its low toxicity and simple chemical structure, there is potential interest to explore combinations of antimitotic coumarins with other chemotherapeutic agents to improve efficacy and lower toxicity. The results suggest that dicoumarol and Taxol have the potential to be used together to improve efficacy and lower toxicity. It is also possible that the coumarin anticoagulants in combination with Taxol may improve cancer survival rates by other mechanisms. Tumor cell invasion is dependent on angiogenesis and requires both cell migration and digestion of basement membrane by proteases. Anticoagulants were thought to reduce the incidence of cancer metastases by inhibiting formation of a fibrin matrix required for the fixation of circulating cancer cells. Because Taxol has been reported to inhibit migration of human ovarian and prostate carcinoma cells and the coumarin anticoagulants inhibit the thrombin-induced release of matrix metalloproteinases that cause the breakdown of extracellular matrix proteins, it is logical to assume that dicoumarol might enhance the antiadhesive activity of taxanes. Coumarin anticoagulants inhibit the pathway involving tissue factor and factor VIIa. Tissue factor VIIa appears to be a major factor in the regulation of angiogenic growth properties of tumor cells, and *in vivo* studies have demonstrated a significant role for warfarin in the regulation of the inhibition of angiogenesis [32]. Taxol also has been shown to display antiangiogenic properties in association with the down-regulation of vascular endothelial growth factor, a pro-angiogenic factor that can act directly on endothelial cells to promote vessel formation. Because these two compounds possess the ability to inhibit angiogenesis by different mechanisms, it is possible that when combined, they can produce a synergistic effect on the inhibition of tumor growth by a mechanism other than stabilization of microtubule dynamic instability. It is however important to stress that although coumarin comprises the substructure of dicoumarol and warfarin, it does not contain any anticoagulant properties. It would be of great interest to further investigate the potential antiangiogenic effect of coumarin anticoagulants and Taxol combinations *in vitro* and *in vivo*. Dicoumarol and coumarins

Table 1. Structures of Coumarins used as Cytotoxic Agents

	Compound	R3	R4	R5	R6	R7	R8
1	Coumarin	H	H	H	H	H	H
2	7-hydroxycoumarin (Umbelliferone)	H	H	H	H	OH	H
3	6,7-dihydroxycoumarin (Esculetin)	H	H	H	OH	OH	H
4	4-hydroxycoumarin	H	OH	H	H	H	H
8	6-nitro-7-hydroxycoumarin	H	H	H	NO ₂	OH	H
9	3,6,8-nitro-7-hydroxycoumarin	NO ₂	H	H	NO ₂	OH	NO ₂
10	7,8-dihydroxycoumarin (Daphnetin)	H	H	H	H	OH	OH
11	8-nitro-7-hydroxycoumarin	H	H	H	H	OH	NO ₂
15	Coumarin-3-carboxylic acid	COOH	H	H	H	H	H
16	6,7-dihydroxy-4,4-dimethylhydrocoumarin	2H	2Me	H	OH	OH	H
17	4-methyl-6,7-dihydroxycoumarin	H	Me	H	OH	OH	H
18	Fraxetin	H	H	H	OMe	OH	OH
20	Esculin	H	H	H	OGl	OH	H
21	Auraptene	H	H	H	H	OGer	H
22	3-methylcoumarin	Me	H	H	H	H	H
23	4-methylcoumarin	H	Me	H	H	H	H
24	3,4-dimethylcoumarin	Me	Me	H	H	H	H
25	4-methyl-7-hydroxycoumarin (Mendiaxon)	H	Me	H	H	OH	H
28	Scopoletin	H	H	H	OMe	OH	H
32	Pervilleanine	Ph	H	H	H	H	H
33	5,7-dihydroxy-6-methyl-4-phenyl-8-(3-phenylpropionyl)coumarin	H	Ph	OH	Me	OH	3-phenylpropionyl
34	5,7-dihydroxy-6-methyl-4-phenyl-8-(3-phenyl-trans-acryloyl)coumarin	H	Ph	OH	Me	OH	3-phenyl-trans-acryloyl
35	5,7-dihydroxy-6-methyl-4-phenyl-8-(2-hydroxy-3-phenylpropionyl)coumarin	H	Ph	OH	Me	OH	2-hydroxy-3-phenylpropionyl
36	5-(4-hydroxyphenethenyl)-4,7-dimethoxycoumarin	H	OMe	4-hydroxy-phenethenyl	H	OMe	H
37	3,8-dimethyl-5-isopropyl-6-methoxycoumarin (mansorin A)	Me	H	Isopropyl	OMe	H	Me
38	3,8-dimethyl-5-isopropyl-6-hydroxycoumarin (mansorin B)	Me	H	Isopropyl	OH	H	Me

could provide a new structural class for synthetic elaboration that could lead to improved antineoplastic drugs. The simple chemical structure of the coumarins allows great potential to clinically explore combinations of coumarin analogs with other microtubule-stabilizing agents in an attempt to improve efficacy. In addition, dicoumarol and possibly other

coumarin compounds that act by stabilizing microtubule dynamics might have entirely new tumor specificity than the currently used antimitotic agents. Their synergistic activity in combination therapy could be the basis for development of rational approaches to new forms of cancer chemotherapy.

A selective stimulatory effect of dicoumarol on 2, 7-diaminomitosen-DNA adduct formation in EMT6 cells treated with mitomycin C was investigated [33]. Dicoumarol had no effect on alkylation by mitomycin C in cell-free systems, nor did it have significant effects on adduct formation or cell survival for cells treated with 2, 7-diaminomitosen. It is proposed that in the cell dicoumarol stimulates a reductase enzyme located at subcellular sites where the activated mitomycin C species has no direct access to DNA and therefore is diverted into the non-cytotoxic pathway, which leads to the formation of 2, 7-diaminomitosen and its adducts.

COUMARIN ANTIOXIDANTS AND CYTOTOXICITY

Plant phenolics are widely consumed and have received considerable attention as anticarcinogens. Tumor-modulating effects of coumarin antioxidants also have been studied using carcinogens. Protective mechanisms include inhibition of prooxidant carcinogenesis by peroxisome proliferators, antipromotion effects, and induction of detoxifying enzymes. The effects of antioxidants in animal studies are complex, however, and also include tumor promotion, carcinogenic, and co-carcinogenic activities. The most attractive candidate anticarcinogens, however, are those that suppress the rate at which initiated cells progress through the promotion-progression-metastasis pathway without appreciable toxicity, since their application does not require knowledge of the initiating carcinogen and, by definition, will not have tumor promotional properties.

The ability to control the amount and the rate of production of hydroxyl radicals may prove useful for examining the cytotoxic effects of hydroxyl radicals generated in biological systems. The kinetics of the production of hydroxyl radicals during the autoxidation of ferrous ion complexes at pH 7.4 was investigated using the fluorescent probe coumarin-3-carboxylic acid (**15**) [34]. The data presented indicate the usefulness of autoxidation of ferrous ion complexes for generation of hydroxyl radicals in chemical systems.

Nishiyama T. *et al.* [35] compared the antioxidative activities of seven hydrocoumarins with those of alpha-tocopherol for the oxidation of tetralin and linoleic acid in a homogeneous solution. Hydrocoumarins exhibited a higher induction period than that of alpha-Toc in both systems. However, the rate of oxygen absorption during the induction period for alpha-Toc was slower than that of the hydrocoumarins in both systems. In addition, 6, 7-dihydroxy-4, 4-dimethylhydrocoumarin (**16**) showed less cytotoxicity toward human fibroblasts than did 2, 6-di-*t*-butyl-4-methylphenol.

Protective effects of coumarins against cytotoxicity induced by linoleic acid hydroperoxide were examined in cultured human umbilical vein endothelial cells [36]. When the cells were incubated in medium supplemented with linoleic acid hydroperoxide and coumarins, esculetin (6, 7-dihydroxycoumarin) and 4-methylesculetin (**17**) protected cells from injury by linoleic acid hydroperoxide. Fraxetin (**18**) and caffeic acid (**19**) showed a weak protection. Esculin (**20**) as well as esculetin and 4-methylesculetin were effective for protecting cells against linoleic acid hydroperoxide-induced cytotoxicity in the case of pretreatment for 24 h,

however fraxetin and caffeic acid showed no protection. Since esculetin was detected after 24 h treatment with esculin, a sugar moiety in the esculin molecule appears to be hydrolyzed during pretreatment. Coumarins such as umbelliferone (7-hydroxycoumarin) containing only one hydroxyl group showed no protective effect in pretreatment or concurrent treatment. Esculetin and 4-methylesculetin provided synergistic protection against cytotoxicity induced by linoleic acid hydroperoxide with alpha-tocopherol. Furthermore, the radical-scavenging ability of coumarins was examined in electron spin resonance spectrometry. Esculetin, 4-methylesculetin, fraxetin, and caffeic acid showed the quenching effect on the 1, 1-diphenyl-2-picrylhydrazyl radical. These results indicate that the presence of an ortho catechol moiety in the coumarin molecules plays an important role in the protective activities against linoleic acid hydroperoxide-induced cytotoxicity.

It is well known that dietary factors play an important role in enhancement of health status and physical strength in humans. Recently, it has been shown that certain foods have a host defense function related to the immune system and anti-oxidation and anti-tumor activity. The immune system plays an important role in physical and chemical carcinogenesis and in tumor-bearing hosts. The role of host immune function has become increasingly important in our understanding of the mechanisms that are involved in the body's ability to prevent cancer. Although the inter-relationship between diet, immune function and carcinogenesis is not clear, there is increasing evidence that dietary alteration of the host's immune functions is a key component of chemoprevention. Macrophages, lymphocytes (T and B cells), dendritic and Langerhans' cells and natural killer (NK) cells are important cells for the immune system. Macrophages play a major role in inflammation, repair, humoral and cellular immunity and metabolic and neoplastic disease processes. Cytokines, being messenger molecules of the immune system, modulate natural immunity. It is known that several cancer chemopreventive agents can modulate immune function.

An antioxidant auraptene (7-geranyloxy coumarin) (**21**) isolated from the peel of citrus fruit (*Citrus natsudaoidai* Hayata) has been reported to have chemopreventive effects on chemically induced carcinogenesis. Dietary administration of auraptene significantly increased the activities of detoxification (phase II) enzymes, such as quinone reductase and glutathione *S*-transferase, in the liver and colon of rats. In addition, expression of cell proliferation biomarkers, such as ornithine decarboxylase activity and polyamine biosynthesis, in the colonic mucosal epithelium was significantly inhibited by dietary feeding of auraptene. These biological functions of auraptene may contribute to its anti-tumorigenic effect. However, a modulatory effect of auraptene on immune function has not been investigated. The results in the study [37] clearly indicate that oral administration of auraptene effectively enhances both macrophage and lymphocyte functions in mice. The study suggested involvement of the immune response in chemically induced carcinogenesis. Auraptene is a naturally occurring coumarin-related compound. Coumarin derivatives have been reported to have enhancing effects on lymphocyte mitogen responsiveness. Therefore, the results in the study suggest that the

mitogenic activity of auraptene might be due to the coumarin structure, and 200 mg/kg/day might be an appropriate oral dose of auraptene to enhance lymphocyte responsiveness. The results described in [37] may support the hypothesis that auraptene directly activates macrophage activities, whereas it only primes lymphocytes to display a greater immune response following interaction of splenic lymphocytes with another stimulus. The findings suggest that auraptene may exert a part of its cancer chemopreventive activity through enhancement of immune function.

The ingestion of citrus fruit has been reported to be beneficial for the reduction of certain types of human cancer. Several classes of citrus phytochemicals, including monoterpenes, limonoids and flavonoids, have been recognized as effective chemopreventive agents in rodent carcinogenesis models. Auraptene (7-geranyloxycoumarin), a coumarin derivative have been isolated, from citrus fruit (e.g. grapefruit) and have been demonstrated its anti-tumor promoting effect in mouse skin and anti-carcinogenesis activities in rat tongue, esophagus and colon [38]. Murakami A. *et al.* [38] reported that Auraptene suppresses superoxide anion (O_2^-) generation from inflammatory leukocytes in *in vitro* experiments. In the study, they investigated the anti-inflammatory activities of Auraptene using a 12-*O*-tetradecanoylphorbol-13-acetate-treated mouse skin model, and compared them with those of Umbelliferone (7-hydroxycoumarin), a structural analog of Auraptene that is virtually inactive toward O_2^- generation inhibition. Double pre-treatments of mouse skin with Auraptene, but not Umbelliferone, markedly suppressed edema formation, hydrogen peroxide production, leukocyte infiltration, and the rate of proliferating cell nuclear antigen-stained cells. These inhibitory effects by Auraptene are attributable to its selective blockade of the activation stage, as revealed by single pre-treatment experiments. Umbelliferone did not show any inhibitory effect. This contrasting activity profile between Auraptene and Umbelliferone was rationalized to be a result of their distinct differences in cellular uptake efficiencies, i.e. the geranyloxyl group in Auraptene was found to play an essential role in incorporation. Thus, the findings indicate that Auraptene is an effective agent to attenuate the biochemical responsiveness of inflammatory leukocytes, which may be essential for a greater understanding of the action mechanism that underlies its inhibition of inflammation-associated carcinogenesis.

Coumarin, in combination with cimetidine, has been subjected to separate clinical trials for the treatment of advanced renal cell carcinoma, malignant melanoma, and non-small cell lung cancer [39]. While objective tumor regressions were observed only in renal carcinoma, no symptomatic or organ dysfunction toxicity was observed in any of the trials. The purpose of this *in vitro* study was to determine the concentrations of coumarin and 7-hydroxycoumarin that would be toxic to human peripheral blood mononuclear cells (PB-MNC) and human and murine bone marrow (GM) progenitor stem cells. Coumarin was nontoxic for PB-MNC in concentrations up to 100 $\mu\text{g}/\text{mL}$. Concentrations of coumarin or 7-hydroxycoumarin greater than or equal to 200 $\mu\text{g}/\text{mL}$ produced significant suppression of human marrow GM stem cell activity. Coumarin greater than or equal to 25 $\mu\text{g}/\text{mL}$ produced suppression of murine

marrow GM stem cell activity. Differences in human and murine marrow sensitivity probably reflect interspecies differences in metabolism of coumarin. Correlations between toxic concentrations *in vitro* and maximally achievable serum concentration *in vivo* in humans await the results of further clinical trials. An immunostimulant therapy with coumarin and cimetidine was evaluated in 17 patients with advanced malignant melanoma [40, 41]. Induction therapy with coumarin 100 mg daily was given for 8 weeks, after which cimetidine 1000 mg daily was added. No patients had been previously treated with cytotoxic drugs. All patients had good performance status. Sixteen patients experienced progressive disease, and only one patient showed no change lasting for 30 weeks. It is concluded that treatment with this schedule of coumarin and cimetidine is without effect in advanced malignant melanoma.

Aflatoxin B₁ is a potent hepatocarcinogen produced by *Aspergillus flavus*, a mold that frequently contaminates rice and cereal crops in humid areas of the world. In combination with hepatitis B, Aflatoxin B₁ is thought to be largely responsible for the high incidence of hepatocellular carcinoma in southeast China and southern Africa. Like most chemical carcinogens, the mycotoxin requires bioactivation to exert its carcinogenic effects. The ultimate carcinogen of Aflatoxin B₁ is the *exo*-8, 9-epoxide, and once generated by the actions of CYP, it readily forms adducts with DNA. In humans, hepatocellular carcinoma resulting from exposure to Aflatoxin B₁ is associated with mutations in codon 249 of the p53 tumor suppressor gene, whereas in the rat it is associated with mutations in codons 12 and 13 of *ras* oncogenes. Although primates and rats are sensitive to Aflatoxin B₁, the mouse can tolerate high levels of the mycotoxin without showing signs of acute liver damage or of developing liver cancer. Because it is highly improbable that Aflatoxin B₁-producing molds can be eradicated from the environment, chemoprevention is an attractive strategy to protect individuals from the risk of liver cancer caused by exposure to the mycotoxin. Structurally diverse compounds can confer resistance to aflatoxin B₁ hepatocarcinogenesis in the rat. Treatment with either phytochemical coumarin or synthetic antioxidants and other drugs has been found to increase hepatic aldo-keto reductase activity toward Aflatoxin B₁-dialdehyde and glutathione *S*-transferase (GST) activity toward Aflatoxin B₁-8, 9-epoxide in both male and female rats [42]. Under the conditions used, the natural benzopyrone coumarin was a major inducer of the Aflatoxin B₁ aldehyde reductase (AFAR) and the aflatoxin-conjugating class- GST A5 subunit in rat liver, causing elevations of between 25- and 35-fold in hepatic levels of these proteins. Induction was not limited to AFAR and GSTA5: treatment with coumarin caused similar increases in the amount of the class- GST P1 subunit and NAD(P)H:quinone oxidoreductase in rat liver. Immunohistochemistry demonstrated that the overexpression of AFAR, GSTA5, GSTP1, and NAD(P)H:quinone oxidoreductase affected by coumarin is restricted to the centrilobular (periacinar) zone of the lobule, sometimes extending almost as far as the portal tract. This pattern of induction was also observed with ethoxyquin, oltipraz, and trans-stilbene oxide. By contrast, induction of these proteins by *o*-naphthoflavone and diethyl maleate was predominantly periportal. Northern blotting showed that

induction of these phase II drug-metabolizing enzymes by coumarin was accompanied by similar increases in the levels of their mRNAs. To assess the biological significance of enzyme induction by dietary coumarin, two intervention studies were performed in which the ability of the benzopyrone to inhibit either Aflatoxin B₁-initiated preneoplastic nodules (at 13 weeks) or Aflatoxin B₁-initiated liver tumors (at 50 weeks) was investigated. Animals pretreated with coumarin for 2 weeks prior to administration of Aflatoxin B₁, and with continued treatment during exposure to the carcinogen for a further 11 weeks, were protected completely from development of hepatic preneoplastic lesions by 13 weeks. In the longer-term dietary intervention, treatment with coumarin before and during exposure to Aflatoxin B₁ for a total of 24 weeks was found to significantly inhibit the number and size of tumors that subsequently developed by 50 weeks. These data suggest that consumption of a coumarin-containing diet provides substantial protection against the initiation of Aflatoxin B₁ hepatocarcinogenesis in the rat. This report describes the identification of phytochemicals that are effective inducers of Aflatoxin B₁ detoxication enzymes. The study showed that coumarin is highly effective at inducing not only AFAR and GSTA5, but also certain other drug-metabolizing enzymes. On the basis of this information, the hypothesis that enzyme induction by coumarin would confer resistance to Aflatoxin B₁ tumorigenesis was tested in the rat. The results from dietary intervention showed that coumarin consumption does indeed provide protection against initiation of Aflatoxin B₁ hepatocarcinogenesis. The data presented in this report also reveal the ability of different phytochemicals and synthetic drugs to induce different enzymes in the liver in zone- and sex-specific fashions.

The rat hepatic toxicity of coumarin and methyl analogues (3-methylcoumarin (**22**), 4-methylcoumarin (**23**) and 3, 4-dimethylcoumarin (**24**)) has been determined *in vivo* and *in vitro* (freshly-isolated cells) [43]. Coumarin at a dose of approximately 1 mmol/kg produced clear histological evidence of centrilobular necrosis, while the methyl analogues at an equivalent dose were much less toxic. By use of a systematic random sampling protocol and quantitative morphometry it was determined that there was a lobar variation in the extent of hepatic damage but that this exhibited random inter-animal variation. The order of cytotoxicity *in vitro* was identical to that observed *in vivo*. In hepatocytes depleted of glutathione the toxicity of all four compounds was increased. This was particularly marked for the 3-methyl analogues, such that the order of toxicity was different to that observed *in vivo* and in hepatocytes not depleted of glutathione.

METAL COMPLEXES WITH COUMARINS

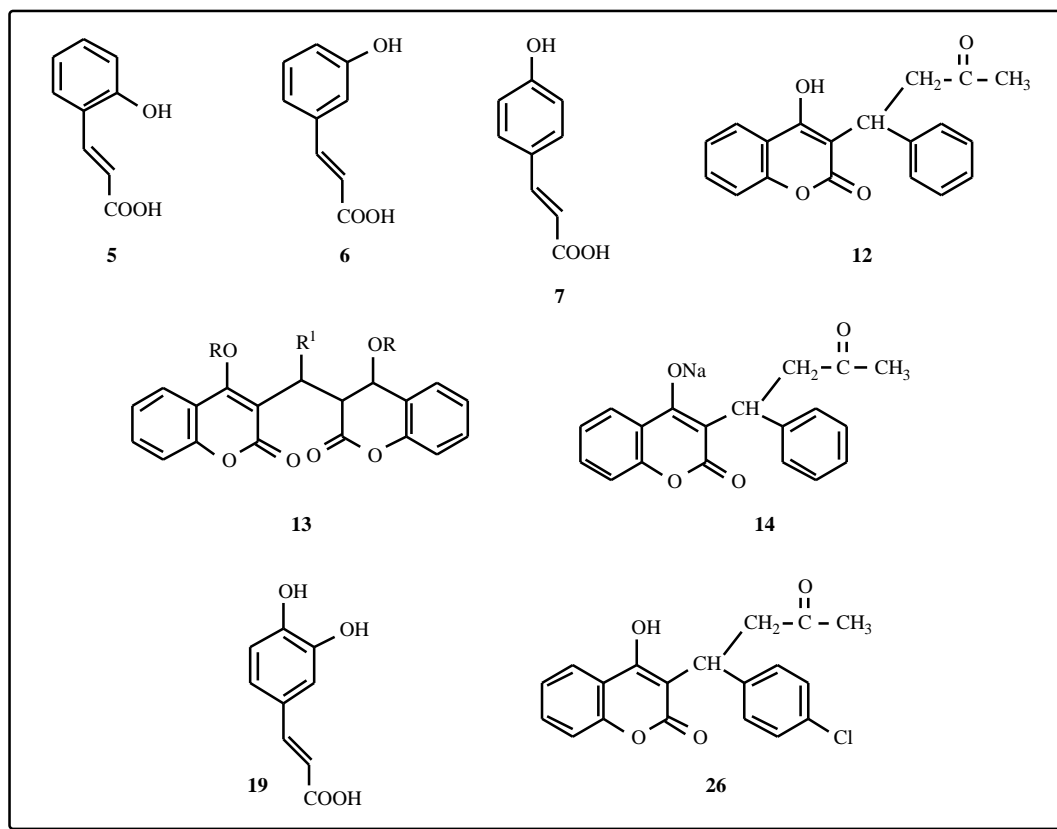
A new, mechanistically based, *in vitro* strategy involving Balb/c 3T3 clone A 31-1-1 mouse embryo fibroblasts, has been proposed for the determination of the carcinogenic potential of inorganic chemicals, in order to establish priority of metal compounds to be tested and, whenever possible, to compare the *in vitro* results with the corresponding *in vivo* data [44]. As a first step in this research, this study reports on the cytotoxic effects of 58 metal compounds in the Balb/3T3 cell line. After harmonisation and standardisation of the

Balb/3T3 protocol, cells were exposed for 72 hours to a fixed dose (100 μ M) of 58 individual compounds. The cytotoxicity induced by some metal compounds was found to be related to their chemical form (for example, Cr(NO₃)₃ and Na₂CrO₄), suggesting that the Balb/3T3 cell line is a valuable cellular model in relation to this aspect of metal speciation. The results of the systematic study on the metal-induced cytotoxic effects in the Balb/3T3 cell line could be arbitrarily classified into three groups according to the degree of cytotoxicity. Group I includes 26 species that induced no observable effect or only a slight cytotoxic effect; Group II includes 13 metal compounds that exhibited an obvious degree of cytotoxicity; and Group III includes 19 metal species that displayed a strong cytotoxic response. Metal compounds of Groups II and III are considered to be of the highest priority for setting of dose-effect relationships for a subsequent *in vitro* study on metal-induced concurrent cytotoxicity and morphological transformation in the Balb/3T3 cell line.

Nowadays, a lot of studies report complexes of coumarin derivatives with metals, which possess biological activity. Rare earth metals, especially lanthanum chloride manifest an antitumor activity. Furthermore, literature data show that the coumarins have also these properties. These previous data from literature are in accordance with our investigations [45-50]. They give our reason to suppose that complexes of coumarins with lanthanides could present interesting metalorganic compounds with antitumor activity. As a result from our earlier work the cytotoxic profile of some complexes of Mendiixon (7-hydroxy-4-methylcoumarin) (**25**), Warfarin (4-hydroxy-3-(3-oxo-1-phenylbutyl)-2H-1-benzopyran-2-one), Coumachlor (4-hydroxy-3-[1-(4-chlorophenyl)-3-oxobutyl]-2H-1-benzopyran-2-one) (**26**) and Niffcoumar (4-hydroxy-3-[1-(4-nitrophenyl)-3-oxobutyl]-2H-1-benzopyran-2-one) (**27**) with lanthanides against P3HR1, K-562 and THP-1 cell lines was proved [45-50]. The complexes of cerium, lanthanum and neodymium with these coumarin ligands induced approximately 30 % reduction of the survival P3HR1 Burkitt lymphoma cells at concentration 100 and 400 μ M. The cerium and lanthanum complexes of Mendiixon and Niffcoumar induce similar low cytotoxic effect on AML derived THP-1 myeloleukemia cells.

Zirconium complexes of Mendiixon, Warfarin, Coumachlor, and Niffcoumar have been synthesized by us [45]. Cytotoxic screening by MTT-assay was carried out. Among these compounds the zirconium complex of mendiixon showed highest cytotoxic activity against human promyelocytic leukemic HL-60 cells. The inorganic salt was found to be active against this cell line.

Cerium complexes of Umbellipherone, Mendiixon, Warfarin, Coumachlor, and Niffcoumar have been synthesized by us [46]. The molecules of the ligands were optimized by means of the semiempirical quantum mechanical method PM3 to the energetically most stable conformers. All the ligands were characterized by molecular and submolecular electronic indices and the putative donor centers are proposed. Cytotoxic screening by MTT assay was carried out. The cerium complexes were found to be more active than the inorganic salts.



Complexes of cerium(III), lanthanum(III) and neodymium(III) with 4-methyl-7-hydroxycoumarin (Mendiixon, Hymecromone) were synthesized by us [47]. The newly synthesized compounds were assayed for acute intraperitoneal and per oral toxicity, influence on blood clotting time and the most active complex was investigated for spasmolytic activity. The complexes of cerium(III) and neodymium(III) showed marginal cytotoxic activity against transformed leukemic cell lines (P3HR1 and THP-1) as compared to the inorganic salts.

With the relatively resistant CML derived erythroleukemic K-562 cell line we obtained very interesting *in vitro* results [48-49]. It is noteworthy that the lanthanum and neodymium complexes with Nifcoumar exert more pronounced cytotoxic effects in comparison to cerium complex. They have a strong cell proliferation inhibiting effects (only about 30% of the cells were survival). This means that the resistant tumor cells may be very good inhibited with lanthanide complexes. This means also that the spectrum of cytotoxicity of these complexes is different from cis-DDP (II) and from Pt (II) complexes. These results are of some interest as a possibility to influence of resistant tumors. The corresponding lanthanide salts are found to be of very low or missing activity. So far we can conclude that the structure metal-ligand determines the antitumor spectrum of the newly complexes. Those *in vitro* effects are not so clearly expressed as it is in the case of cis-DDP (II). Nevertheless their studying is interesting in connection with other cell lines and tumors in order to find out the differences in their spectrum of activity. Following the above

encouraging results we decided to continue our recent investigations regarding antineoplastic/cytotoxic natural and synthetic coumarin derivatives, and especially their cytotoxic lanthanide complexes.

NATURAL COUMARINS

Herbs have been used as food and for medicinal purposes for centuries. Research interest has focused on various herbs that possess hypolipidemic, antiplatelet, antitumor, or immune-stimulating properties that may be useful adjuncts in helping reduce the risk of cardiovascular disease and cancer. In different herbs, a wide variety of active phytochemicals, including coumarins, have been identified. Many of the herbs contain potent antioxidant compounds that provide significant protection against chronic diseases. Today we are witnessing a great deal of public interest in the use of herbal remedies. Herbal medicine is based on the premise that plants contain natural substances that can promote health and alleviate illness. In herbal medicine the term herbs is used loosely to refer not only to herbaceous plants but also to bark; roots; leaves; seeds; flowers and fruit of trees, shrubs, and woody vines; and extracts of the same that are valued for their savory, aromatic, or medicinal qualities. The botanical term herb refers to seed-producing plants with nonwoody stems that die down at the end of the growing season. Plants have played a significant role in maintaining human health and improving the quality of human life for thousands of years, and have served humans well as valuable components of seasonings, beverages, cosmetics, dyes, and medicines. Thousands of coumarins have been identified in plants.

Coumarins have extensive biological properties that promote human health and help reduce the risk of disease. Coumarins act as antioxidants, inhibit platelet aggregation, and act as antiinflammatory and antitumor agents.

Kofinas C, *et al.* [51] isolated seven coumarins from the aerial parts of *Tordylium apulum* and established their structures by spectroscopic means. All compounds were tested *in vitro* for their cytotoxicity against two cell line systems. The antiproliferative effects for three of them were studied at the level of the cell cycle in asynchronous cells of the NSCLC-N6 line with a flow cytometry apparatus. Uchiyama T. *et al.* [52] isolated six coumarins from the roots of *Dorstenia brasiliensis*. Their structures were elucidated on the basis of their spectral data. They showed moderate cytotoxicity against leukemia cells (L-1210 and HL-60). Some coumarin-, flavonol- and flavanon-acetic acids were described by Valenti P. *et al.* [53]. The cytotoxicity of the synthesized compounds was determined on human colon carcinoma cell line (LoVo) through evaluation of neutral red uptake, performed by the Riddel method. All tested derivatives were able to induce a statistically significant reduction of lysosomal neutral red uptake at 5.10^{-5} M concentration. Some compounds were more active than the reference compound flavon-8-acetic acid. Nine known compounds, seven kaurenic acid-type diterpenes, a coumarin and a flavone, were isolated, from the aerial parts of *Mikania hirsutissima* DC (Compositae) [54]. The structures of new norhumulenes were determined by spectroscopic means. The cytotoxic activities of isolated compounds against leukemia cells (L 1210) were investigated. They showed relatively strong cytotoxicity. New cytotoxic compounds, scopoletin (**28**) and the novel coumarino-lignan cleomiscosin A were isolated from the wood of *Simaba multiflora* (Simaroubaceae) [55]. Two antileukemic daphnane esters, Pimelea factor P2 and the new compound dircin, were isolated from the twigs and flowers of *Dirca occidentalis* A. Gray (Thymelaeaceae) [56]. Three lignans, (-)-medioresinol, (+)-syringaresinol and (-)-lariciresinol, as well as the coumarin daphnoretin, were found to be additional cytotoxic constituents of this taxon. A limonoid, clausenolide-1-ethyl ether and two coumarins, dentatin and nor-dentatin, were isolated from *Clausena excavata* [57]. Limonoid was obtained from the crude ethanol extract of the rhizomes and the roots but had not previously been isolated from *C. excavata* and exhibited HIV-1 inhibitory activity. Coumarins dentatin and nor-dentatin, with their structures related to an anti-HIV-1 substance, (+)-calanolide A (**29**), were obtained from the crude chloroform extract of the rhizomes. Both induced toxicity to cells used in a syncytium assay for anti-HIV-1 activity. These compounds, did not show any cytotoxic effect against KB and BC-1 cell lines (IC_{50} value > 20 $\mu\text{g/mL}$).

The purpose of the study [58] was to investigate the potential neuroprotective effects of myricetin (flavonoid) and fraxetin (coumarin) on rotenone-induced apoptosis in SH-SY5Y cells, and the possible signal pathway involved in a neuronal cell model of Parkinson's disease. These two compounds were compared to N-acetylcysteine. The viability of cells was assessed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), and cytotoxicity was assayed by lactate dehydrogenase (LDH)

released into the culture medium. Parameters related to apoptosis, such as caspase-3 activity, the cleavage of poly(ADP-ribose) polymerase and the levels of reactive oxygen species were also determined. Rotenone caused a time- and dose-dependent decrease in cell viability and the degree of LDH release was proportionally to the effects on cell viability. Cells were pretreated with fraxetin, myricetin and N-acetylcysteine at different concentrations for 30 min before exposure to rotenone. Cytotoxicity of rotenone (5 μM) for 16 h was significantly diminished as well as the release of LDH into the medium, by the effect of fraxetin, myricetin and N-acetylcysteine, with fraxetin (100 μM) and N-acetylcysteine (100 μM) being more effective than myricetin (50 μM). Rotenone-induced apoptosis in SH-SY5Y cells was detected by an increase in caspase-3 activity and in the cleavage of poly(ADP-ribose) polymerase. After exposing these cells to rotenone, a significant increase in reactive oxygen species preceded apoptotic events. Fraxetin (100 μM) and N-acetylcysteine (100 μM) not only reduced rotenone-induced reactive oxygen species formation, but also attenuated caspase-3 activity and poly(ADP-ribose) polymerase cleavage at 16 h against rotenone-induced apoptosis. The effect of fraxetin in both experiments was similar to that of N-acetylcysteine. These results demonstrated the protective action of fraxetin and suggest that it can reduce apoptosis, possibly by decreasing free radical generation in SH-SY5Y cells. Myricetin at 100 μM was without any preventive effect.

The cytotoxicity and in-vitro antiviral activity of a series of compounds in samples extracted from various parts of the Indian holy tree, Bael (*Aegle marmelos* corr.) were evaluated for their efficacy against human coxsackieviruses B1-B6 [59]. The inhibitory concentrations IC_{50} for leaves (L1 and L2) stem and stem bark (S1, S2, S3 and S4), fruit (F1 and F2micro), root and root bark (R1 and R2) and pure compound, the marmelide were 1000 $\mu\text{g/mL}$ (for L1 and L2), 1000 $\mu\text{g/mL}$ (for S1, S2, S3 and S4), 1000 $\mu\text{g/mL}$ (for F1) and 500 $\mu\text{g/mL}$ (for F2) 250 $\mu\text{g/mL}$ (for R1) and 500 $\mu\text{g/mL}$ (for R2) and 62.5 $\mu\text{g/mL}$ for marmelide respectively by plaque inhibition assay at 96 hrs. On the other hand, the corresponding value for Ribavirin, a standard antiviral drug, was 2000 $\mu\text{g/mL}$ for the same viruses at the same time period. These concentrations did not exhibit any toxicity to Vero cells, the host subtoxic concentrations were 5000 $\mu\text{g/mL}$ for leaf and stem fractions 2000 $\mu\text{g/mL}$ for fruit fractions 500 and 1000 $\mu\text{g/mL}$ for root fractions 250 $\mu\text{g/mL}$ for marmelide and 2000 $\mu\text{g/mL}$ for Ribavirin. The cytotoxic concentrations were 8000 $\mu\text{g/mL}$ for leaf and stem compounds 4000 $\mu\text{g/mL}$ for fruit; 1000 $\mu\text{g/mL}$ and 2000 $\mu\text{g/mL}$ for root 500 $\mu\text{g/mL}$ for marmelide and 4000 $\mu\text{g/mL}$ for ribavirin at 96 hrs. Additionally pretreatment of host cells, virus inactivation, yield reduction and effect of time of addition assays against coxsackievirus B3, suggested that marmelide was most effective as a virucidal agent besides interfering at early events of its replicative cycle like adsorption, penetration, at various steps in single cycle growth curve and effect of time of addition.

Geiparvarin (**30**), containing coumarin moiety, is an antiproliferative compound isolated from the leaves of *Geijera parviflora*, and may represent a new drug which targets tubulin. To better explore the potential use of this

agent, Miglietta A. *et al.* [60] investigated the antimicrotubular and cytotoxic effects of new synthetic aromatic derivatives of geiparvarin. These drugs inhibited polymerization of microtubular protein, particularly when the assembly was induced by paclitaxel. The microtubular network organization of fibroblasts was altered more effectively by some drugs. Normal microtubule architecture completely disappeared when the cells were treated simultaneously with drugs and paclitaxel: microtubules depolymerized or were reorganized into bundles, in a similar but more disarrayed fashion than that observed after treatment with paclitaxel alone. Cytotoxicity studies showed a dose-dependent effect, whereas combined administration of drugs and paclitaxel increased cytotoxicity, more effectively in paclitaxel versus derivatives administration alone. Some simple geiparvarin analogues, in which the coumarin moiety has been replaced with an X-substituted benzene ring, are described [61]. The compounds were tested on LoVo cells (human colon carcinoma cell line) and some of them show a cytotoxicity comparable with that of the prototype. A QSAR analysis was also attempted, but it did not provide satisfactory results, mainly because of the limited range of variation of the biological activity.

Bioassay-guided fractionation of a CH_2Cl_2 -methanol extract of the bark of *Ochrocarpos punctatus* resulted in the isolation of seven new coumarins, ochrocarpins A-G, three new benzophenone derivatives, ochrocarpinones A-C, and five known coumarins, mammea A/AC cyclo F, mammea A/AD cyclo D, mammea A/AB cyclo F, mammea A/AA cyclo F, mammea A/AB cyclo D and 15, 16-dihydro-16-hydroperoxyplukenetione [62]. The structures of the compounds were established on the basis of extensive 1D and 2D NMR spectroscopic data interpretation. All compounds exhibited cytotoxicity against the A2780 ovarian cancer cell line. Two new naturally occurring coumarins, isomesuol and mammearin A, together with nine known *Mammea* coumarins were isolated from the ethylacetate extract of the leaves and twigs of *Mammea harmandii* [63]. The coumarins showed cytotoxicity against a panel of mammalian cancer cell lines. Their structures were determined by spectroscopic methods. The assignments of ^{13}C NMR signals of isomesuol, which was isolated for the first time as a natural product, have been revised.

Five new coumaronochromones, euchretins J-N, along with twelve known compounds, euchretins A, C, D, F, H, I, (+)-matrine, (-)-cytisine, quercetin (**31**), trifolirhizin, retusin 8-methyl ether, and genistein, were isolated from the methanolic extracts of *Euchresta formosana* [64]. The structures of the compounds were established by spectroscopic analyses. The compounds were demonstrated to have cytotoxicity against 59T cell line. Bocca C. *et al.* [65] investigated biological activity of ferulenol, a prenylated 4-hydroxycoumarin from *Ferula communis*. Ferulenol stimulates tubulin polymerization *in vitro*, and inhibits the binding of radiolabeled colchicine to tubulin. It rearranges cellular microtubule network into short fibres, and alters nuclear morphology. Remarkably, ferulenol exerts a dose-dependent cytotoxic activity against various human tumor cell lines. From the root bark of *Milletia pervilleana*, which had shown significant cytotoxic activity, a 3-phenylcoumarin, named pervilleanine (**32**), two new pterocarpan,

pervilline and pervillinine, and one known, emoroidocarpan, were isolated in addition to rotenone and 3 α -hydroxyrotenone [66]. The anticancer activity of two previously isolated isoflavanones, pervilleanone and 3'-O-demethylpervilleanone is reported. Chemical investigation of the ethanol extract of *Morus alba* L. (Moraceae), as guided by free radical scavenging activity, furnished 5, 7-dihydroxycoumarin 7-methyl ether, two prenylflavones, cudraflavone B and cudraflavone C, and oxyresveratrol [67]. Compounds 5, 7-dihydroxycoumarin 7-methyl ether and oxyresveratrol showed superoxide scavenging effects with the IC_{50} values of 19.1 \pm 3.6 and 3.81 \pm 0.5 μM , respectively. Compound oxyresveratrol exhibited a DPPH free radical scavenging effect (IC_{50}) = 23.4 \pm 1.5 μM). Compounds cudraflavone B and oxyresveratrol showed hepatoprotective effects with EC_{50} values of 10.3 \pm 0.42 and 32.3 \pm 2.62 μM , respectively, on tacrine-induced cytotoxicity in human liver-derived Hep G2 cells.

Three new coumarin derivatives, compounds, three new furanocoumarins and a novel dioxocane derivative were isolated from the fern *Cyclosorus interruptus* (Willd.) H. Ito [68]. Based on spectrometric and spectroscopic analysis (FAB or EI mass spectrometry as well as 1D and 2D NMR experiments) their structures were characterised as 5,7-dihydroxy-6-methyl-4-phenyl-8-(3-phenylpropionyl)-1-benzopyran-2-one (**33**), 5, 7-dihydroxy-6-methyl-4-phenyl-8-(3-phenyl-trans-acryloyl)-1-benzopyran-2-one (**34**), 5,7-dihydroxy-8-(2-hydroxy-3-phenylpropionyl)-6-methyl-4-phenyl-1-benzopyran-2-one (**35**), 8-benzyl-5, 8-dihydroxy-6-methyl-4-phenylfuro[2,3-h]-1-benzopyran-2,9-dione, 8-benzyl-5,8 beta, 9 beta-trihydroxy-6-methyl-4-phenyl-8,9-dihydro-furo[2,3-h]-1-benzopyran-2-one, 8-benzyl-5, 8 beta, 9 alpha-trihydroxy-6-methyl-4-phenyl-8,9-dihydro-furo[2,3-h]-1-benzopyran-2-one and 5,11-dihydroxy-6-methyl-4-phenyl-11-(1-phenylmethyl)-7,10-dioxocane[5,6-h]-1-benzopyran-2,12-dione. For these compounds the authors propose the trivial names interruptins A-F. Compounds 5,7-dihydroxy-6-methyl-4-phenyl-8-(3-phenylpropionyl)-1-benzopyran-2-one and 5, 7-dihydroxy-6-methyl-4-phenyl-8-(3-phenyl-trans-acryloyl)-1-benzopyran-2-one, were cytotoxic to a KB cell line.

The CH_2Cl_2 extract of the stem bark of *Kielmeyera albopunctata* was subjected to a bioassay-linked LC-MS dereplication procedure using the KB cell line to afford the new coumarins 4-(1-methylpropyl)-5, 7-dihydroxy-8-(4-hydroxy-3-methylbutyryl)-6-(3-methylbut-2-enyl)chromen-2-one, 9-(1-methylpropyl)-4-hydroxy-5-(4-hydroxy-3-methylbutyryl)-2-(1-hydroxy-1-methylethyl)-2,3-dihydrofuro-[2,3-f]chromen-7-one and 5,7-dihydroxy-8-(4-hydroxy-3-methylbutyryl)-6-(3-methylbut-2-enyl)-4-phenylchromen-2-one [69]. Coumarins 4-(1-methylpropyl)-5, 7-dihydroxy-8-(4-hydroxy-3-methylbutyryl)-6-(3-methylbut-2-enyl)chromen-2-one and 5, 7-dihydroxy-8-(4-hydroxy-3-methylbutyryl)-6-(3-methylbut-2-enyl)-4-phenylchromen-2-one showed moderate cytotoxicity, while 9-(1-methylpropyl)-4-hydroxy-5-(4-hydroxy-3-methylbutyryl)-2-(1-hydroxy-1-methylethyl)-2,3-dihydrofuro[2,3-f]chromen-7-one was inactive at 20 $\mu\text{g}/\text{mL}$.

The ether soluble fraction of the roots of *Ononis vaginalis* Vahl. Symb. afforded three new compounds: 3-

hydroxy-4, 9-dimethoxycoumestan, maginaldehyde [2-(4-hydroxy-2-methoxyphenyl)-5, 6-dimethoxy-3-benzofuran-carboxaldehyde] and 5, 7, 4'-trihydroxy-4-styrylcoumarin [70]. The styrylcoumarin derivative showed significant antiviral activity against Herpes simplex type 1 and weak cytotoxicity. A new coumarin, 5-(4-hydroxyphenethenyl)-4, 7-dimethoxycoumarin (**36**) was isolated from the combined ethylacetate extracts of the root bark, root wood and stem bark of *Monotes engleri*, and found to be cytotoxic against two cell lines in a human tumor panel [71]. Its structure was determined on the basis of spectroscopic methods. Three coumarins and three known mansonones were isolated from the heartwood of *Mansonia gagei* Drumm [72]. The structures of the three coumarins were elucidated as 3, 8-dimethyl-5-isopropyl-6-methoxycoumarin (mansonrin A) (**37**), 3, 8-dimethyl-5-isopropyl-6-hydroxycoumarin (mansonrin B) (**38**) and 2, 3-dihydro-3, 6, 9-trimethyl naphtho[1, 8-bc]pyran-7-oxa-8-one (mansonrin C) by analyses of physical properties and spectroscopic data. The cytotoxicity of the isolated compounds against brine shrimp *Artemia salina* Linn. was also evaluated. Salloum R.M. *et al.* [73] examined the effects of a new antiangiogenic isocoumarin, as a radiation modifier *in vitro* and *in vivo*. The studies demonstrate that this isocoumarin is cytotoxic to human umbilical vein endothelial cells (HUVECs) but not to Lewis lung carcinoma (LLC) cells nor Seg-1, esophageal adenocarcinoma cells, in clonogenic survival assays.

N-Tosyl-L-phenylalanyl chloromethyl ketone, an inhibitor of chymotrypsin-like serine protease (CSP), prevents DNA fragmentation and apoptotic cell death in certain blood cell lines and was reported to reduce hippocampal neuronal damage caused by cerebral ischemia. Movsesyan V.A. *et al.* [74] examined the role of CSP on recovery after lateral fluid percussion-induced traumatic brain injury (TBI) in rats, as well as on cell survival in various *in vitro* models of neuronal cell death. TBI caused significant time-dependent upregulation of CSP activity, but not trypsin-like serine protease activity in injured cortex. Intracerebroventricular administration of N-tosyl-L-phenylalanyl chloromethyl ketone to rats after TBI did not significantly affect deficits of spatial learning but exacerbated motor dysfunction after injury. Moreover, N-tosyl-L-phenylalanyl chloromethyl ketone did not prevent apoptotic neuronal cell death caused by serum/K(+) deprivation or by application of staurosporine or etoposide in cultured rat cerebellar granule cells, rat cortical neurons, or in the human neuroblastoma SH-SY5Y cell line. Instead, at doses from 10 to 100 μM , N-tosyl-L-phenylalanyl chloromethyl ketone was cytotoxic in all cultures tested. Similar results were obtained in cultures treated with another CSP inhibitor, 3, 4-dichloroisocoumarin (**39**). Cell death caused by CSP inhibitors was neither caspase-dependent nor associated with oligonucleosomal DNA fragmentation. Taken together, these data do not support a neuroprotective role for CSP inhibitors. Rather, they suggest that CSPs may serve an endogenous neuroprotective role, possibly by modulating necrotic cell death.

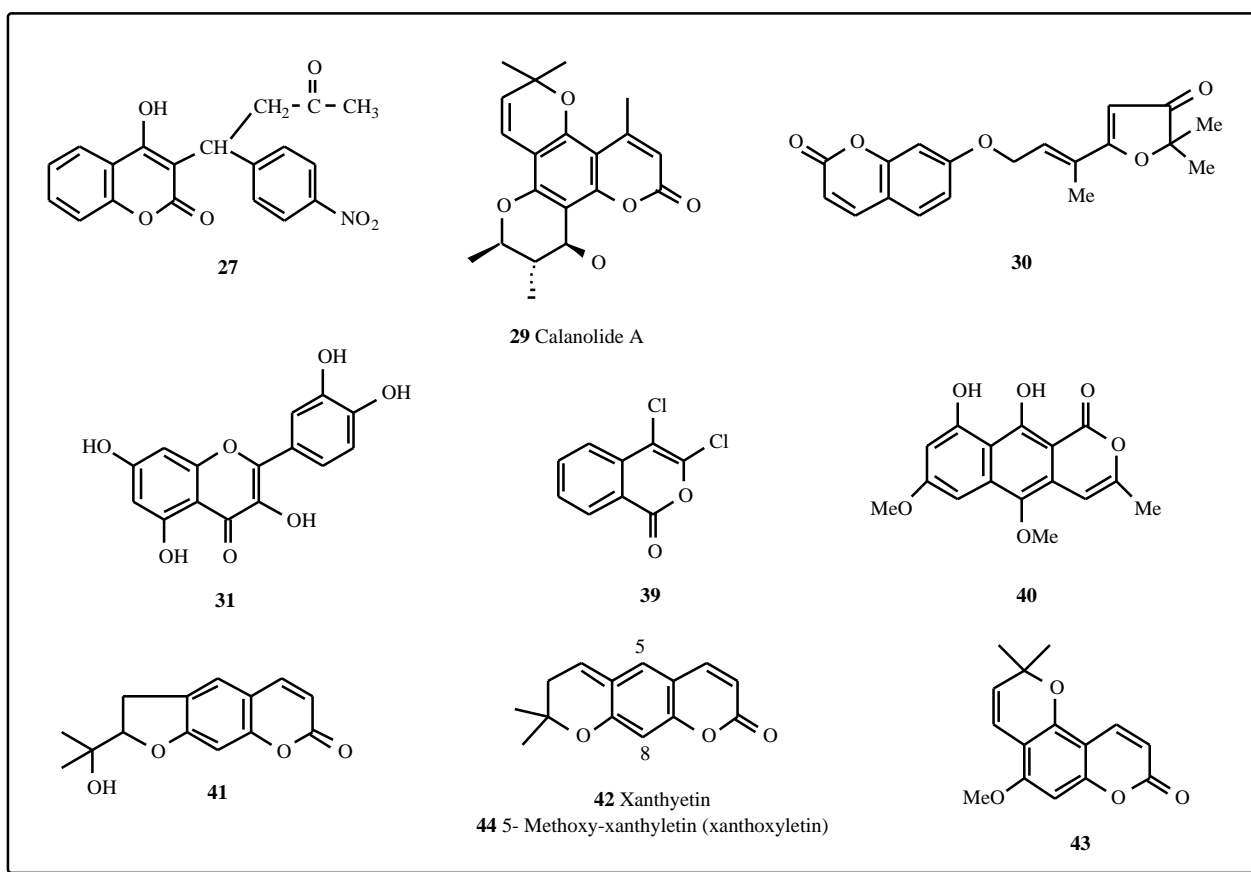
NATURAL ISOCOUMARINS

Devienne K.F. *et al.* [75] investigated structural parameters that could affect the cytotoxicity of isocoumarins similar to 9, 10-dihydroxy-5, 7-dimethoxy-1H-naphtho(2,

3c)pyran-1-one (paepalantine) (**40**). Paepalantine has antimicrobial activity, as well as significant *in vitro* cytotoxic effects in the McCoy cell line. Two other natural and two semi-synthetic isocoumarins with similar structures obtained from the capitula of *Paepalanthus bromelioides* were tested on the same cell line by the neutral red assay. Substitution of the 9 and/or 10-hydroxy group made these compounds less cytotoxic. A new isocoumarin with antimicrobial activity was isolated from *Paepalanthus vellozioides* (a native Brazilian plant) and called paepalantine [76]. This study was carried out to assess the mutagenic activity of this new agent in assays with *Salmonella typhimurium* TA100, TA98, and TA102 and in Chinese hamster ovary (CHO) cell cultures, as well as cytotoxicity to McCoy cells. Paepalantine caused a significant dose-dependent increase in the frequency of revertants in the three strains used in the assay, both with and without S9 mix, in concentrations varying from 2 to 128 $\mu\text{g}/\text{plate}$. The mutagenicity was confirmed in assays with CHO cells treated in the G1, S, and G2 phases of the cell cycle. There was an increase in the chromosomal aberration frequency, mainly in the G2 phase. Furthermore, the mitotic index of the treated cultures (40, 80, and 160 $\mu\text{g}/\text{mL}$) was significantly lower, indicating cytotoxicity. The midpoint cytotoxicity values of McCoy cells by the neutral red (NR) and microculture tetrazolium (MTT) techniques resulted in a NR50 and MTT50 of 30 and 38 micrograms $\mu\text{g}/\text{mL}$, respectively. Alterations to the paepalantine structure are suggested to reduce its mutagenic and cytotoxic activity in investigations for its antineoplastic potential. Dioscorone A and a new isocoumarin derivative were isolated from the rhizome of *Dioscorea futschauensis* R. Kunth [77]. The structures of the compounds were elucidated on the basis of detailed analysis of NMR spectra. Their anti-fungal activity against *Pyricularia oryzae* and cytotoxic activity on K562 and HCT-15 cell lines were evaluated *in vitro*.

NATURAL PYRANOCOUMARINS

A new (+)-norepinephrine derivative, syncarpamide, along with a known coumarin, (+)-S-marmesin (**41**), and one known alkaloid, decarine, have been isolated from the stem of *Zanthoxylum syncarpum* [78]. The structure of syncarpamide was elucidated on the basis of 1D and 2D NMR, MS, IR, optical rotation, and CD analyses. Its absolute stereochemistry was elucidated by synthesis of its enantiomer and subsequent comparison of CD data. Characterizations of compounds (+)-S-marmesin and decarine were based on spectral analysis and comparison with reported data. Compounds syncarpamide and decarine showed antiplasmodial activity, with IC_{50} values of 2.04 and 1.44 μM against *Plasmodium falciparum* D(6) clone and 3.06 and 0.88 μM against *P. falciparum* W(2) clone, respectively. Decarine showed cytotoxicity at 56.42 μM , whereas syncarpamide was not cytotoxic at 10.42 μM . Syncarpamide was tested for hypotensive activity, but no activity was observed. 3, 5-Dimethoxy-4-geranyloxycinnamyl alcohol, 8-methoxy-N-methylflindersine, xanthyletin (**42**) and sesamin have been isolated from petroleum ether extract of the stem bark of *Zanthoxylum rhesta* [79]. The petroleum ether extract and 8-methoxy-N-methylflindersine showed cytotoxicity on brine shrimp nauplii. Four pyranocoumarins: dipetaline, alloxanthoxyletin (**43**), xanthoxyletin (**44**) and



xanthyletin; and two lignans: sesamin and asarinin were isolated from the Northern prickly ash, *Zanthoxylum americanum* [80]. To varying degrees, all inhibited the incorporation of tritiated thymidine into human leukaemia (HL-60) cells. Dipetaline was the most active with an IC_{50} of 0.68 ppm, followed by alloxanthoxyletin (1.31 ppm), sesamin (2.71 ppm), asarinin (4.12 ppm), xanthoxyletin (3.48 ppm) and xanthylletin (3.84 ppm).

Cnidium monnieri is a plant, which grows in China whose seeds are used in classical traditional Chinese medicine for skin problems and as a natural sex booster. *Cnidium* is known in China as She Chuang Zi. *Cnidium* seed contains several compounds including coumarins. Preliminary studies show some of the compounds in *Cnidium* may have anti-histamine, anti-itch, anti-fungal, and anti-bacterial effects, along with having an influence on the pituitary-adrenocortex axis. Additional studies on rodents indicate that *Cnidium* may improve bone strength (coumarins from fruits of *Cnidium* inhibit formation and differentiation of multinucleated osteoclasts of rats). *Cnidium* may also have anti-cancer properties. *Cnidium monnieri* Fructus [*Cnidium monnieri* (L.) Cusson] is used as a tonic agent in traditional Chinese medicine [81]. In a Chinese herb-cytotoxicity screening test, the ethanol extract of *Cnidium monnieri* Fructus exhibited strong effects on human leukemia (HL-60), cervical carcinoma (HeLa) and colorectal carcinoma (CoLo 205) cells. Then, the *Cnidium monnieri* Fructus extract was subjected to silica gel column chromatography and recrystallization to give five coumarins:

osthol (45), imperatorin (46), bergapten (47), isopimpinellin (48), and xanthotoxin (49). Among these compounds, osthol showed the strongest cytotoxic activity on tumor cell lines. The structure-activity relationship established from the results indicated that the prenyl group has an important role in the cytotoxic effects. However, imperatorin showed the highest sensitivity to HL-60 cells and the least cytotoxicity to normal PBMCs. Osthol and imperatorin both caused apoptotic bodies, DNA fragmentation, and enhanced PARP degradation in HL-60 cells by biochemical analysis. These results indicate that osthol and imperatorin can induce apoptosis in HL-60 cells. Therefore, osthol and imperatorin are cytotoxic marker substances in the fruits of *Cnidium monnieri*.

Four flavonoid glycosides, flavaprin, evodioside B, vitexin, and hesperidin, as well as the coumarins bergapten, xanthotoxin, and isopimpinellin, the lignan simplexoside, the steroids beta-sitosterol and daucosterol, the limonoids isolimonexic acid and limonin, and uracil and myo-inositol have been isolated from *Euodia daniellii* [82]. The structures of these compounds were established from spectral data. Among the isolates, bergapten showed cyclooxygenase-2 inhibitory activity with an IC_{50} value of 6.2 $\mu\text{g/mL}$. Flavonoids isolated from this plant exhibited no cytotoxic activity against the human tumor cell lines, A549, SKOV-3, SKMEL-2, XF498, and HCT15.

Four known coumarins, dentatin, nor-dentatin, clausenidin and xanthoxyletin, and six known carbazole derivatives,

3-formylcarbazole, mukonal, 3-methoxycarbonylcarbazole, murrayanine, 2-hydroxy-3-formyl-7-methoxycarbazole and clauszoline J were isolated from *Clausena excavata* [83]. Compounds dentatin and 3-formylcarbazole were first isolated from the crude chloroform extract of the rhizomes. All compounds demonstrated no cytotoxicity against KB and BC-1 cell lines. Five coumarins (seselin (50), 5-methoxy-seselin (51), suberosin (52), xanthyletin and xanthoxyletin) were isolated from the roots of *Plumbago zeylanica* [84]. All coumarins were not previously found in this plant. Cytotoxicity of these compounds to various tumor cells lines was evaluated, and they were significantly suppressed growth of Raji, Calu-1, HeLa, and Wish tumor cell lines. The synthesis of known and new coumarins in the seselin and xanthyletin series is described [85]. The cytotoxic activity of the compounds was carried out *in vitro* on L-1210 cells. The most active compounds in the seselin series and in the xanthyletin series were determined. Structure-activity relationships are discussed. Gunatilaka, A.A. *et al.* [86] isolated twelve coumarins from plants of the Rutaceae collected in Sri Lanka. Coumarins have been subjected to a mechanism-based anticancer bioassay employing DNA repair-deficient and repair-proficient yeasts. Of these, seselin and xanthyletin were found to be active. Seselin also exhibited moderate cytotoxicity. Four coumarin derivatives, theraphins A, B, C, and D, along with three known xanthenes, 2-hydroxyxanthone, 1, 7-dihydroxyxanthone, and 5-hydroxy-1-methoxyxanthone, were isolated from the bark of *Kayea assamica* (Clusiaceae) native to Myanmar [87]. Their structures were determined using spectroscopic and chemical techniques. The absolute configuration of theraphins A was established by the modified Mosher ester method. Theraphins A, B, and C exhibited good cytotoxicity against Col2, KB, and LNCaP human cancer cell lines. Theraphin D showed mild activity only against the KB cell line.

NATURAL FUROCUMARINS

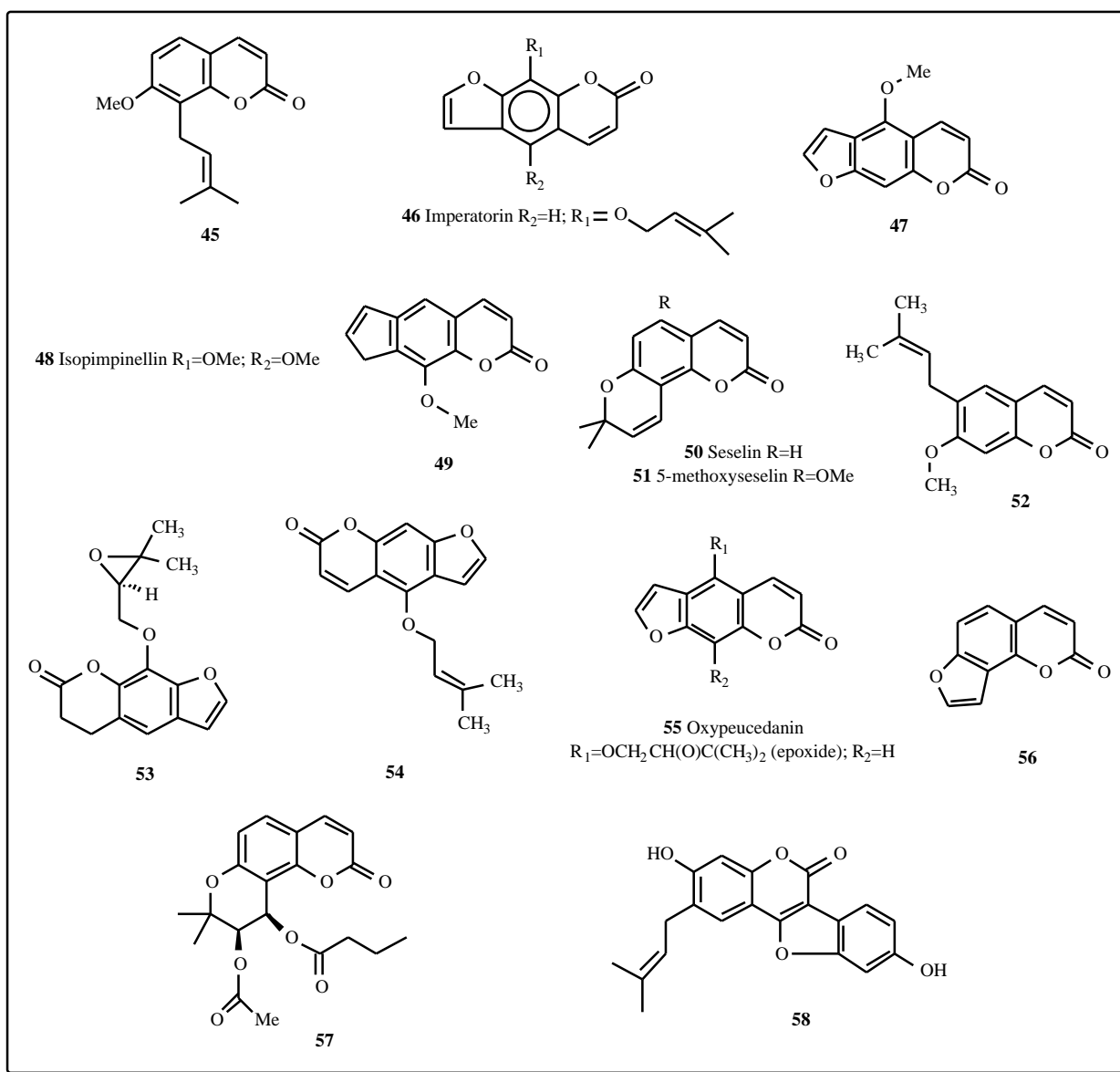
Bark samples from several individuals of the understory tree, *Stauranthus perforatus* (Rutaceae), were collected from lower montane moist forest at c.a 1350 m a.s.l. at Monteverde, Costa Rica [88]. The cytotoxic compounds proved to be the quinoline alkaloids skimmianine and veprisine, and the furocoumarin heraclenin (53). Fractionation of the methanol extract of *Angelica dahurica* Benth et Hook resulted in the isolation of six furocoumarins, imperatorin, isoimperatorin (54), (+/-)-byakangelicol, (+)-oxypeucedanin (55), (+)-byakangelicin and (+)-aviprin [89]. Among these, compounds imperatorin and (+)-byakangelicin exhibited strong hepatoprotective activities, displaying EC₅₀ values of 36.6 +/- 0.98 and 47.9 +/- 4.6 μM, respectively. Compounds (+/-)-byakangelicol and (+)-oxypeucedanin showed moderate activities with EC₅₀ values of 112.7 +/- 5.35 and 286.7 +/- 6.36 μM, respectively. Silybin as a positive control showed the EC₅₀ value with 69.0 +/- 3.4 μM. Comparison of hepatoprotective activities for six furocoumarins suggested that oxy-substitution at the C-9 position increased the hepatoprotective activity.

Angelicin (56), a naturally occurring furanocoumarin, that showed antifungal activity, was considered as a lead structure for a group of synthetic coumarins [90]. Antifungal

activities of the synthesized coumarins and angelicin derivatives were reported against *Candida albicans*, *Cryptococcus neoformans*, *Saccharomyces cerevisiae* and *Aspergillus niger*. Human cell line cytotoxicity of several coumarins was evaluated against KB cells. Angelicin and several potent antifungals showed to be non-toxic in this assay. The methanolic extract from the roots of *Angelica furcijuga* Kitagawa was found to exhibit protective effects on liver injury induced by D-galactosamine (D-GalN) and lipopolysaccharide (LPS) [91]. From the methanolic extract, seventeen coumarins, two phenylpropanoids, and two polyacetylenes were isolated and examined their *in vitro* and *in vivo* hepatoprotective effects and inhibitory activity of NO production in macrophages. A acylated khellactone, isoepoxypteryxin, showed protective activity against D-GalN-induced cytotoxicity in primary cultured rat hepatocytes. On the other hand, six acylated khellactones (hyuganins A, B, C, and D, anomalin, isopteryxin) and two polyacetylenes [(-)-falcarinol and falcarindiol] strongly inhibited NO production induced by LPS in cultured mouse peritoneal macrophages, and also other acylated khellactones (isoepoxypteryxin, pteryxin, and suksdorfin (57)) and a coumarin glycosides (praeroside II) were found to show the activity. By comparison of the inhibitory activities for acylated khellactones with those for other coumarins, acyl groups were found to be essential to exerting potent activity. Aqueous extracts of *Angelica sinensis* root, a herb commonly used in the treatment of vitiligo in Traditional Chinese Medicine, were tested for their activity on mouse melanocyte proliferation in culture [92]. At concentrations of 0.5-2500 μg/mL, these extracts were not able to stimulate melanocyte cell division. On the contrary, they exerted a general cytotoxicity to the cells at higher concentrations. Cytotoxicity was reduced by prior treatment of the extract with polyvinylpyrrolidone, which was shown by thin layer chromatography to reduce the coumarin content.

A coumestan derivative, psoralidin (58) was found to be a cytotoxic principle of the seeds of *Psoralea corylifolia* L. (Leguminosae) with the IC₅₀ values of 0.3 and 0.4 μg/mL against the HT-29 (colon) and MCF-7 (breast) human cancer cell lines, respectively [93]. A coumarin, angelicin was also isolated as a marginally cytotoxic agent along with an inactive compound, psoralen from the plant. The isolates were not active against the A541 (lung) and HepG2 (liver hepatoma) cancer cell lines. The IC₅₀ values of psoralidin against SNU-1 and SNU-16 carcinoma cell lines were 53 and 203 μg/mL, respectively, indicating cytotoxic activity against stomach carcinoma cell lines [94]. Bioassay-guided fractionation of the H₂O extract of the seeds of *Psoralea corylifolia* furnished one hepatoprotective compound, bakuchiol, together with two moderately active compounds, bakuchicin and psoralen, on tacrine-induced cytotoxicity in human liver-derived Hep G2 cells [95]. The EC₅₀ values of the compounds are 1.0, 47.0, 50.0 μg/mL, respectively. Silymarin as a positive control showed the EC₅₀ value with 5.0 μg/mL.

The synthesis of new tetrahydrobenzo- and benzo-psoralen derivatives carrying at position 5 or 8 of the furocoumarin moiety a methoxy, hydroxy, or dimethylaminopropoxy side chain was reported [96]. The study of their photoantiproliferative activity and ability to induce



erythema on guinea pig skin allows to state that the derivatives carrying the dimethylaminopropoxy side chain exhibit a very interesting photobiological pattern. Indeed, if compared with the lead compounds 5-MOP and 8-MOP, they exert a higher cytotoxic activity devoid of significant skin phototoxicity. Between them, the more interesting appears to be a nonphototoxic compound whose antiproliferative activity on HeLa cells is 2 orders of magnitude higher than that of the reference drug 8-MOP. Photoreaction experiments have revealed that, like classic furocoumarins, AT is the preferred nucleic base pair in its photobinding. Moreover, the extent of covalent photoaddition to DNA correlates well with the photobiological activity. Evaluation of the ability to induce DNA cleavage in the presence of human topoisomerase II has suggested that this enzyme is probably the target accountable for this effect. The synthesis, DNA binding and *in vitro* photoinduced cytotoxic properties of a number of minor groove and sequence-directed psoralen and coumarin conjugates of pyrrole- and imidazole-

containing distamycin analogues are described [97]. Results from an ethidium displacement assay on calf thymus and T4 DNA suggest that like distamycin these agents bind strongly to the minor groove of DNA. The data show that these conjugates exhibit a lower AT preference than distamycin and the decrease is significantly greater for the imidazole-containing compounds. All of the compounds along with 8-methoxypsoralen, were relatively noncytotoxic in the dark with only the imidazole-psoralen compound giving an IC_{50} value below 100 μM . Following UV activation, all compounds showed an increased potency with photoinduced dose modifications in the human chronic myeloid leukemia K562 cells under the UV irradiation conditions employed. The psoralen-pyrrole analogue was over 300 times more active following UV activation than agent 8-methoxypsoralen, 250 times more potent than the corresponding coumarin conjugate, and 15-fold more potent than its imidazole analogue. Data from CD dilution (with DMF) studies show that upon irradiation with light at 366 nm, the

compounds bind irreversibly to DNA. As expected coumarin conjugates did not produce any cross-linked DNA under any conditions. Since the psoralen conjugates are more phototoxic than their coumarin analogues, these results suggest that DNA interstrand cross-link formation may be an important mechanism by which they exert their biological activity in cells.

Guilet D. *et al.* [98] isolated six coumarins from the fruits and the stem bark of *Calophyllum dispar* (Clusiaceae). The structures of these minor components were established by means of spectroscopic analysis, including extensive 2D NMR studies. Some of these coumarins exhibited a significant cytotoxic activity against KB cells. Eight new 4-phenylfuranocoumarins have been isolated from the stem bark and the fruits of *Calophyllum dispar*, together with three known coumarins [99]. The structures of the compounds were established by means of spectroscopic analysis, including extensive 2D NMR studies. Some of these furanocoumarins exhibited significant cytotoxic activity against KB cells. The studies on the stem bark of *Calophyllum mucigerum* (Guttiferae) have yielded a new coumarin mucigerin, a prenylated xanthone cudraxanthone C and the common steroidal triterpenes friedelin and stigmasterol [100]. Structural elucidations of these compounds were achieved using ¹H NMR, ¹³C NMR, DEPT, COSY, HETCOR and HMBC experiments while MS gave the molecular masses. Cytotoxic assays using CEM-SS cell line (T-lymphoblastic leukemia) on the crude extracts of the stem bark indicated some activity. The crude extracts were also found to be moderately toxic against the larvae of *Aedes aegypti*. In a search for anti-tumor-promoting agents, Itoigawa M. *et al.* [101] carried out a primary screening of ten 4-phenylcoumarins isolated from *Calophyllum inophyllum* L. (Guttiferae), by examining their possible inhibitory effects on Epstein-Barr virus early antigen (EBV-EA) activation induced by 12-O-tetradecanoylphorbol-13-acetate in Raji cells. All of the compounds tested in this study showed inhibitory activity against EBV, without showing any cytotoxicity. Calocoumarin-A showed more potent activity than the other compounds tested. Furthermore, calocoumarin-A exhibited a marked inhibitory effect on mouse skin tumor promotion in an *in vivo* two-stage carcinogenesis test. The results of the investigation indicate that some of these 4-phenylcoumarins might be valuable as potential cancer chemopreventive agents (anti-tumor-promoters).

Plants have formed the basis for the treatment of diseases in traditional medicine systems for thousands of years, and continue to play a major role in the primary health care of about 80% of the world's inhabitants. The ability of chemical agents to prevent the development of cancer has provoked much interest as a means of reducing the incidence of neoplastic disease in human populations. In the area of cancer treatment, many claims have been made for the beneficial effects of plants, though many of these claims may be viewed with some skepticism since cancer, as a specific disease entity, is likely to be poorly defined in terms of folklore and traditional medicine. Nevertheless, the discovery and development of efficacious anticancer agents, provided convincing evidence that plants could be a source of novel cancer chemotherapeutic agents. While the natural

product isolated as the active compound might not be suitable for development as an effective drug, it can provide a suitable lead for conversion into a clinically useful agent.

CONCLUSION

Coumarins comprise a vast array of biologically active compounds ubiquitous in plants, many of which have been used in traditional medicine for thousands of years. The coumarins constitute an important class of compounds, with several types of pharmacological agents possessing anti-cancer, anti-HIV, anticoagulant, spasmolytic and antibacterial activity among others. Of the many actions of coumarins, antioxidant and antiproliferative effects stand out. A large number of structurally novel coumarin derivatives have ultimately been reported to show substantial cytotoxic activity *in vitro* and *in vivo*. Moreover, the inhibitory action on inflammatory cells appears to surpass any other clinically available compounds. Given that certain substituents are known to be required or increase their actions, the therapeutic potential of select coumarins is fairly obvious.

ABBREVIATIONS

GLC4	= Human small cell lung carcinoma
COLO 320	= Human colorectal cancer cell line
MTT	= Microculture 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide reduction test
HSC-2, HSC-3	= Oral squamous carcinoma
HL-60	= Human promyelocytic leukemic cells
HGF	= Gingival fibroblast
HPLF	= Periodontal ligament fibroblast
HPC	= Pulp cells
LDH	= Lactate dehydrogenase
SKF 525-A	= Cytochrome P450 inhibitor
P-815, P-388	= Tumor cell lines
TNF	= Tumor necrosis factor
SK-MEL-31	= Human skin malignant melanocytes
HS613.SK	= Normal human skin fibroblastic cells
K-562	= Resistant CML derived erythroleukemic cell line
MDR	= Multi-drug resistance
NALM-6	= Leukemia cell line
Mtln3	= Mammary carcinoma cell
F344	= Female rats
alpha-Toc	= Alpha-tocopherol
NK	= Natural killer
PB-MNC	= Human peripheral blood mononuclear cells
GM	= Human and murine bone marrow progenitor stem cells GST-glutathione S-transferase

AFAR	= Aflatoxin B ₁ aldehyde reductase
P3HR1	= Burkitt lymphoma cells
THP-1	= AML derived myeloleukemia cells
PM3	= Semiempirical quantum mechanical method
cis-DDP (II)	= Cis-dichlordiaminoplatinum
LDL	= Low density lipoproteins
L-1210	= Leukemia cells
LoVo	= Human colon carcinoma cell line
HIV-1	= Human immunodeficiency virus
KB, BC-1	= Cell lines
DPPH	= 1, 1-diphenyl-2-picrylhydrazyl
LC-MS	= Liquid chromatography-mass spectrometry
SH-SY5Y	= Human neuroblastoma cell line
Hep G2	= Human liver-derived cells
HUVECs	= Human umbilical vein endothelial cells
LLC	= Lewis lung carcinoma cells
Seg-1	= Esophageal adenocarcinoma cells
CSP	= Chymotrypsin-like serine protease
TBI	= Traumatic brain injury
McCoy	= Cell line
TA100, TA98,	= Salmonella typhimurium cells and TA102
CHO	= Chinese hamster ovary
NR	= Neutral red
HCT-15	= Cell line
CD	= Cluster determinant
HeLa	= Cervical carcinoma cells
CoLo 205	= Colorectal carcinoma cells
A549, SKOV-3,	= Human tumor cell lines SKMEL-2, XF498, HCT15
Raji, Calu-1,	= Tumor cell lines HeLa, and Wish
Col2, KB, and	= Human cancer cell lines LNCaP
D-GalN	= D-galactosamine
LPS	= Lipopolysaccharide
HT-29	= Colon human cancer cell line
MCF-7	= Breast human cancer cell line
A541	= Lung cancer cell line
SNU-1, SNU-16	= Carcinoma cell lines
CEM-SS	= T-lymphoblastic leukemia cell line
EBV-EA	= Epstein-Barr virus early antigen.

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