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Prenylated Flavonoids: Pharmacology and Biotechnology

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Abstract: Within the flavonoid class of natural products the prenylated sub-class is quite rich in structural variety and pharmacological activity. In the last twenty years a huge number of new structures has been reported, mostly from Leguminosae and Moraceae, with few coming from other genera. The presence, in different forms, of the isoprenoid chain can lead to impressive changes in biological activity, mostly attributed to an increased affinity for biological membranes and to an improved interaction with proteins. Molecules, such as xanthohumol and sophoraflavanone G, while being very structurally simple, show numerous pharmacological applications and are ideal candidates for SAR aimed to the discovery of new drugs.

Only recently the biogenesis of these compounds has been more extensively studied and much attention has been focused on the enzymes involved in the modification and transfer of the prenyl unit.

1. INTRODUCTION

Flavonoids represent an outstanding class of naturally occurring compounds. The antifungal/antimicrobial effect of flavonoids is mainly attributed to the presence of phenolic hydroxyl groups, which have high affinity for proteins and thus may inhibit both microbial enzymes [1] and the NADH dehydrogenase of plant mitochondrial inner membranes [2].

Among this group of phytochemicals, prenylated flavonoids, i.e. featuring C_5 isoprenoid substituents, are attracting more and more attention from the scientific community. These compounds have a relatively narrow distribution in the plant kingdom and are constitutively expressed in plants, as compared with prenylated isoflavonoids, which are produced in response of a damage or an attack (phytoalexins).

The substitution of the flavonoid ring system with prenyl groups increases the lipophilicity and confer to the molecule a strong affinity to biological membranes [3]. As a consequence prenyl flavonoids display a series of interesting biological activities that attracted chemists, biologists, pharmacologists and physicians.

Renewed interest in the isoprenoid chemistry produced in the last years a huge amount of researches on this class of compounds: new structures, varying in the flavonoid skeleton (mainly chalcones, flavanones and flavones), in the position and in the arrangement (cyclic or alicyclic, and at different levels of oxidations) of the isopentenylated substituents, have been reported; promising biological activities shown by some members of the group have been developed into detailed pharmacological studies; investigations, concerning the enzyme involved in the biosynthesis of prenylated flavonoids, have been deepened.

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This paper will deal with an up-to-date review of prenylated flavonoids, with particular emphasis to their biological and pharmacological activities and to their involvement in biotechnological production.

2. PRENYLATED FLAVONOIDS IN THE LITERATURE (SINCE 1995)

The natural distribution and structural variation of prenylated flavonoids have been reviewed by Barron and Ibrahim [4] in 1996. The survey included more than 700 prenylated metabolites, that have been reported up to the end of 1994, and discussed previous aspects of their biological activity. With regard to the biosynthesis, the general statement, that the basic skeleton of the different classes is constructed before any isoprenoid substituents are added [4], seems to have not been further discussed.

Leguminosae and Moraceae were the most studied families in the last years and obviously produced the major number of new metabolites. Umbelliferae, Guttiferae and Euphorbiaceae were the other unusual families represented in the literature.

2.1. Moraceae

The genus *Dorstenia* has been extensively investigated and recently reviewed [5]. The genus contains various species that are used as anti-snakebite, anti-infection and anti-rheumatic remedies in the medicinal plant therapy of many countries in Africa, Central and South America. The members of genus *Dorstenia*, included among the examples of marketed plants from various African countries, as the result of a screening by traditional medicine, appear as a rich source of prenylated flavonoids as well as of their furano-, pyrano- an dihydropyrano derivatives [6].

Two new flavonoids with a modified geranyl substituent, poinsettifolins A (**1a**) and B (**1b**), were among the

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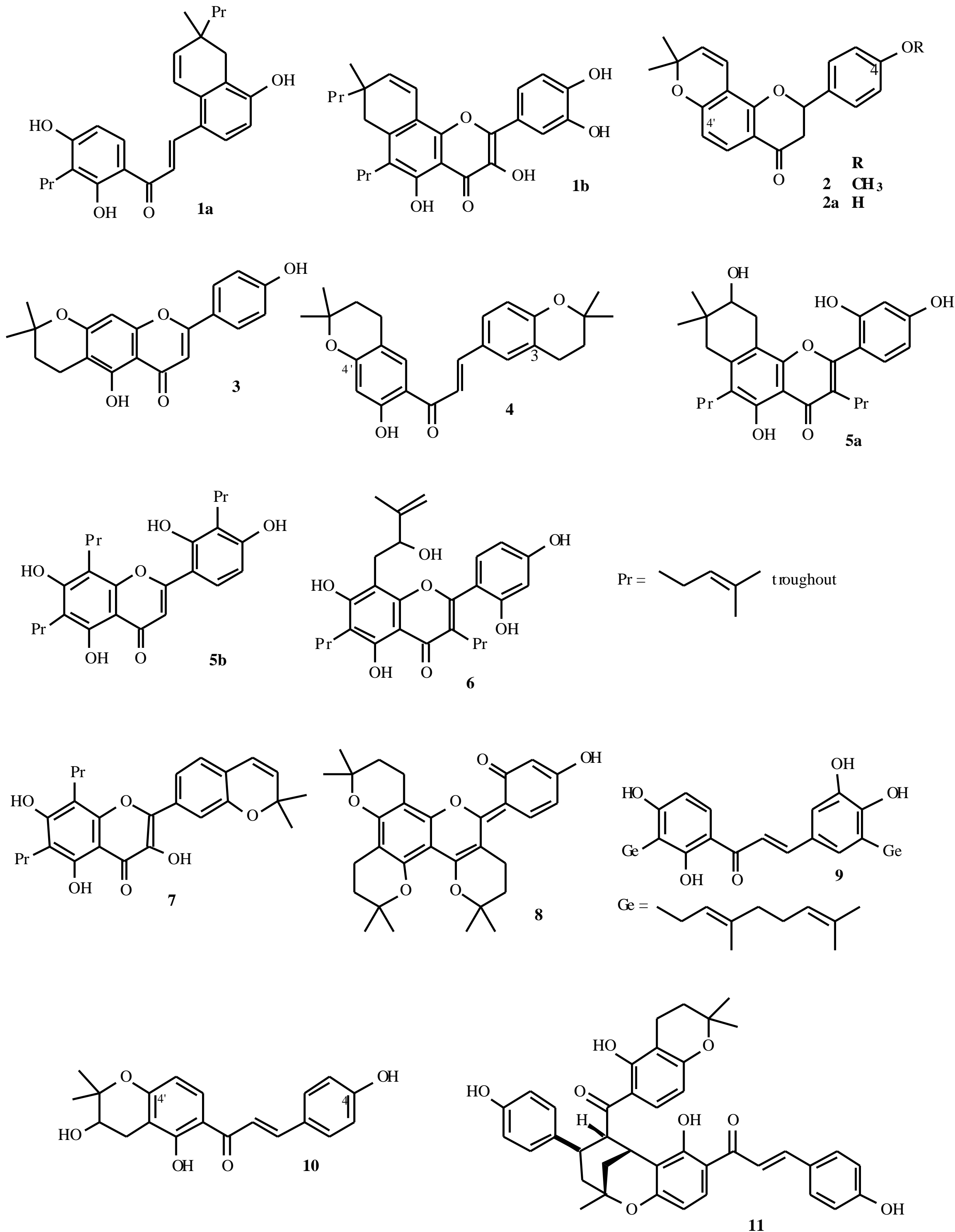
constituents of the extract from the herb *Dorstenia poinsettifolia* Engler [7], whereas 7,8-(2,2-dimethylpyrano)-4'-methoxyflavanone (**2**) was isolated from the twigs of the plant together with 4-hydroxylonchocarpin and its corresponding flavanone (**2a**) [8].

Conversely, two novel products, 6,7-(2,2-dimethylchromano)-5,4'-dihydroxyflavone (**3**) and 3,4-4',5'-bis(2,2-dimetylchromano)-2'-hydroxychalcone (**4**) have been characterized from leaf tissue of *Dorstenia kameruniana* Engler [9].

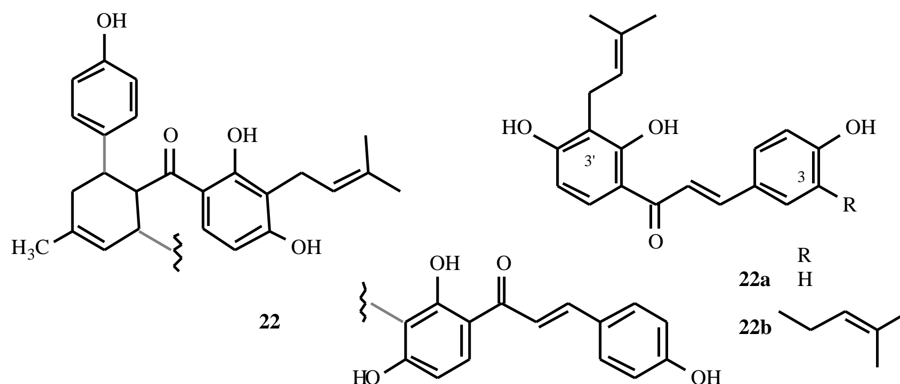
Triprenylated flavonoids have been found only in the root of *Dorstenia psilurus*: dorsilurin A (**5a**) and B (**5b**)[10a], 3,6-diprenyl-8-(2-hydroxy-3-methylbut-3-enyl)-5,7,2',4'-tetrahydroxyflavone (**6**), 6,8-diprenyl-3'-[O]-4'-(2,2-

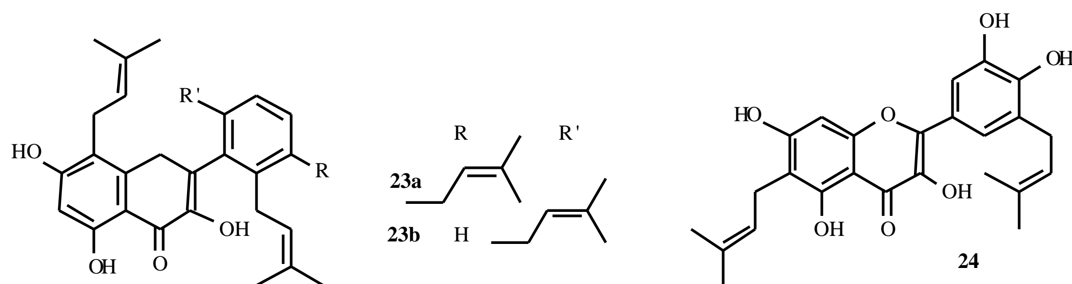


dimethylpyrano)-3,5,7- trihydroxy flavone (**7**) and an unusual B/C rings modified flavonoid with three chromano substituents (**8**) [10b].

5,3'-Di-(3,7-dimethyl-2,6-octadienyl)-3,4.2',4'-tetrahydroxy chalcone (**9**), isolated from the twigs of *Dorstenia prorepens* [11], is the only example of a bis-geranylated chalcone in the literature, while chalcone **10**, that is 3',4'-(3-hydroxy-2,2-dimethyl dihydropyrano)-4,2'-dihydroxychalcone and its related Diels-Alder adduct (**11**) were obtained from *Dorstenia zenkeri* [11].

Investigation of roots [12a] and twigs [12b] of *Dorstenia mannii* gave a series of prenylated or geranylated flavonoids, namely dorsmannin A–E (**12–16**) and F–H (**17–19**), respectively. Dorsmannin F (**17**) and G (**18**) were obtained as





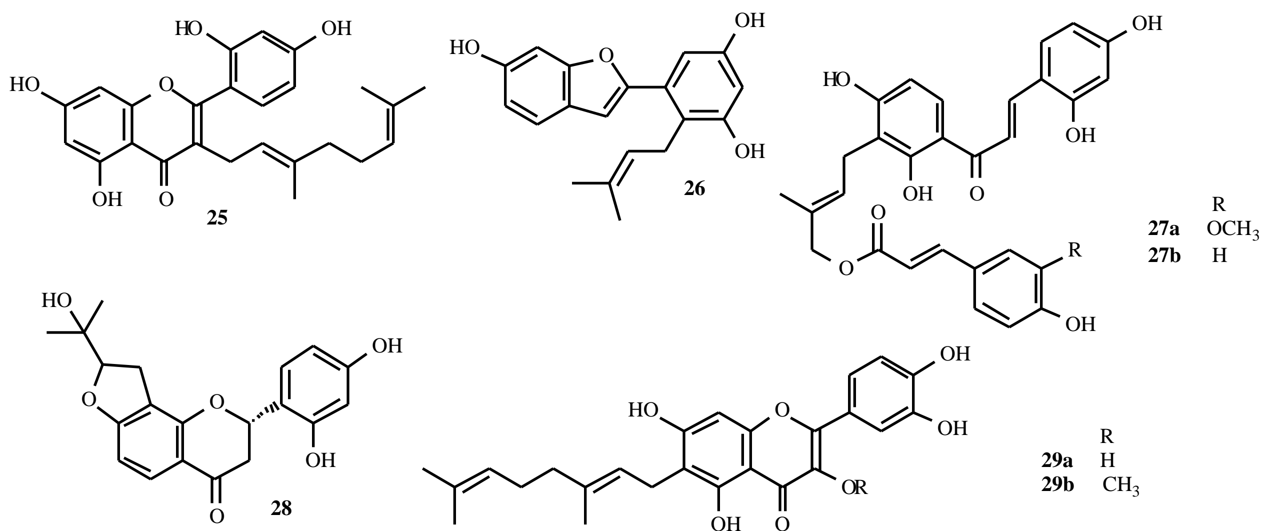
epimeric (at C-2") mixtures. Reinvestigation of a bulk quantity of the aerial parts allowed the isolation of further minor components, which were assigned the structures 6,7-(2,2- dimethylpyrano-8-prenyl-5.3',4'-flavanone (dorsmannin I, **20**), 6,7-(2,2-dimethyl dihydropyrano- 8-prenyl-5.3',4'-flavanone (dorsmannin J, **21**) [12c].

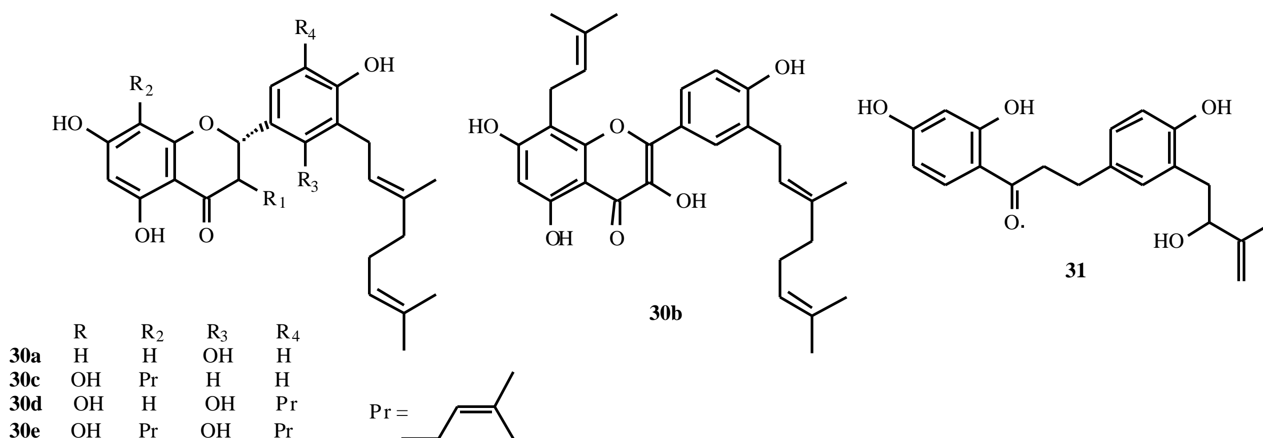
Finally a new Diels-Alder type adduct, dorstenone (**22**) formed by two molecules of isobavachalcone, was isolated from *Dorstenia barteri*, together with isobavachalcone (**22a**), and 4,2',4'-trihydroxy-3,3'-diprenylchalcone (**22b**) [13].

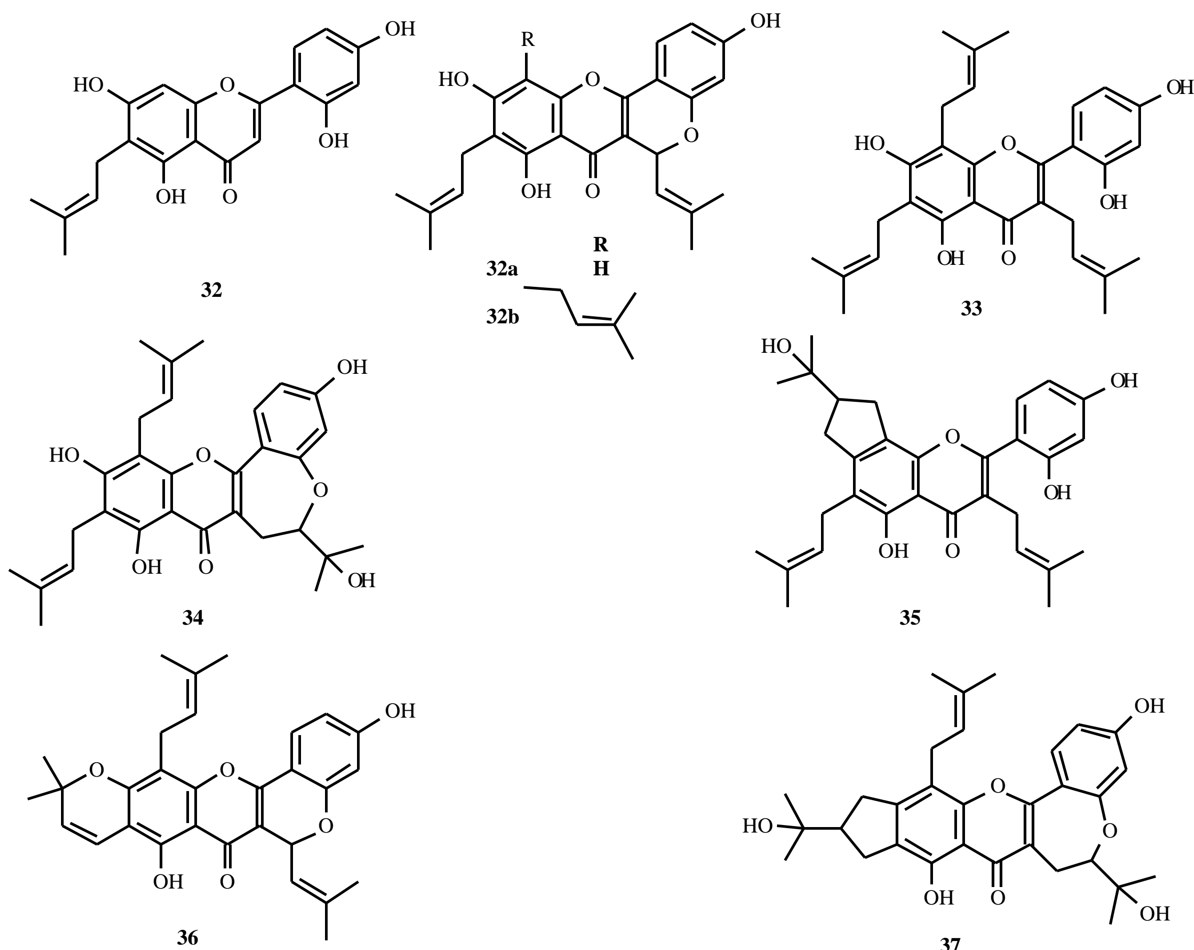
The structure of broussoflavonol E (**23a**), from Formosan *Broussonetia papyrifera* has been revised to be 8,5',6'-triprenyl-3,5,7,3',4'-pentahydroxyflavone (**23b**), also renamed broussoflavonol G [14].

Papyriflavonol A, was isolated from the root bark of the same plant and assigned the structure 5,7,3',4'-tetrahydroxy-6,5'-di-(- -dimethyl allyl)-flavonol (**24**) [15].

Bioassay guided fractionation of an ethyl acetate-soluble extract from the whole plant of *Broussonetia papyrifera*, using an *in vitro* aromatase inhibition assay, led to the isolation of five new active compounds: 5,7,2',4'-tetrahydroxy-3-geranylflavone (**25**), demethylmoracin (**26**), isogemichalcone C (**27a**), 3'-[-hydroxymethyl-(*E*)- -dimethylallyl]-2,4,2',4'-tetrahydroxychalcone 11'-*O*-coumarate (**27b**), and (2*S*)-2',4'-dihydroxy-2"-(1-hydroxy-1-methylethyl)-dihydrofurano-[2,3-*h*]-flavanone (**28**), together with ten known compounds, which also proved to be active [16]. Additionally, five new prenylated flavonoids, including 5,7,3',4'-tetrahydroxy-6-geranylflavonol (**29a**), 5,7,2',4'-







tetrahydroxy-3-methoxy-6-geranylflavone (**29b**), a flavan and two diphenylpropane derivatives, and 21 known compounds were isolated.

Five new isoprenylated flavonoids, sangennols A (**30a**), B (**30b**), C (**30c**), D (**30d**) and E (**30e**) were isolated from root bark of the Chinese *Morus cathayana* [17].

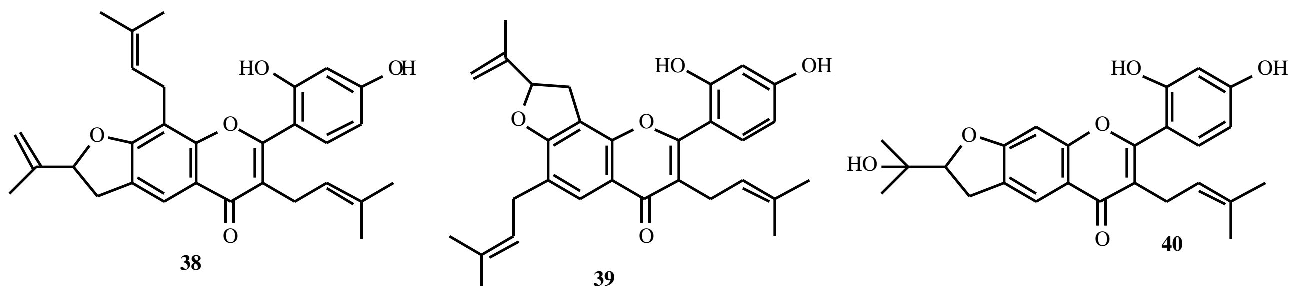
The prenylated flavonoids, including the new natural product, 2',4,4',2''-tetrahydroxy-3'-(3''-methylbut-3''-enyl)chalcone (**31**), isolated from an ethanol extract of the leaves of *Maclura tinctoria* (L.) Gaud. were evaluated against the AIDS-related opportunistic fungal pathogens, *Candida albicans* and *Cryptococcus noformans*: only isobavachalcone (**22a**) resulted active against both yeasts [18].

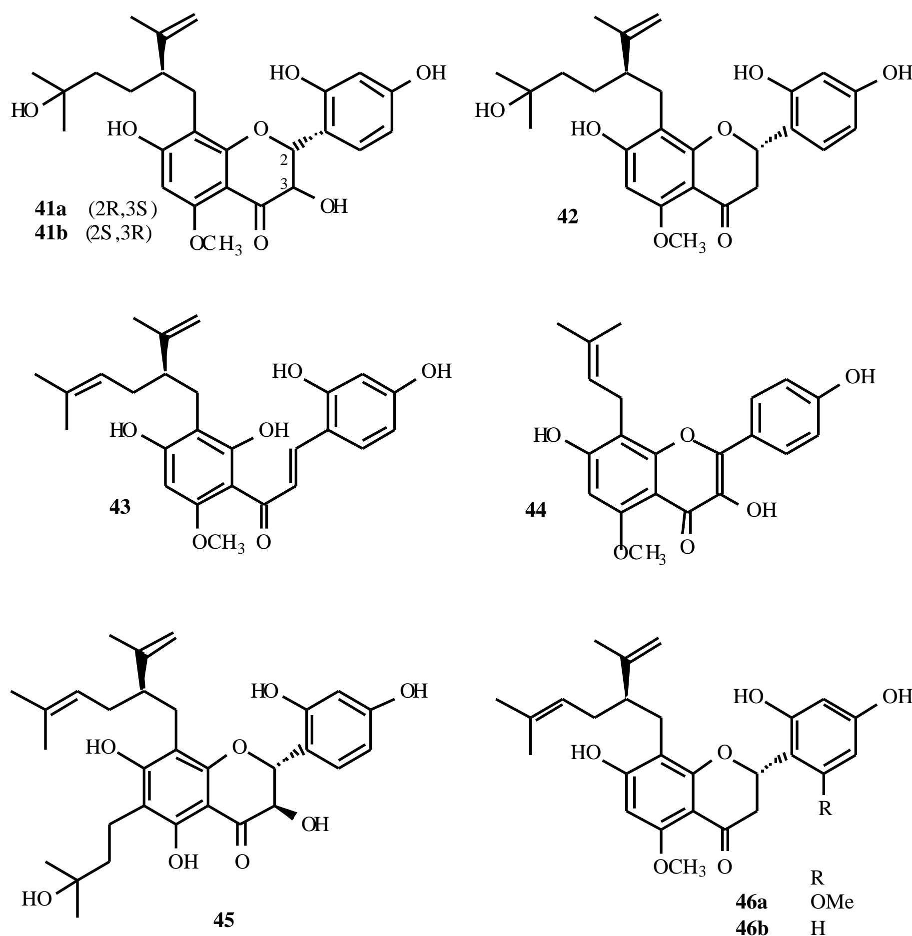
The wood and bark of *Artocarpus* genus, indigenous of the tropical and sub-tropical regions and including more than 50 arboreal species, are very rich in prenylated flavonoids and their derivatives [4]. Kijjoa has reported the isolation of six new flavones from the wood of *Artocarpus elasticus* [19]. These compounds appear to be related to artocarpesin (**32**) and cyclocommunin (**32a**) and have been named artelastin (**32b**), artelasticin (**33**), artelastocarpin (**34**), artelastofuran (**35**), artelastochromene (**36**), and carpelastofuran (**37**). Compounds **32**-**37** were tested for cytotoxicity *in vitro* against three human cell lines [19c]. All the compounds were active, artelastin (**32b**) exhibiting the most potent activity.

Other new prenylated flavones, namely artoindonesianins G, H and I (**38**-**40**) have been reported from *Artocarpus lanceifolius* [20].

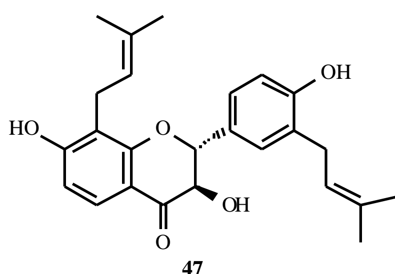
2.2. Leguminosae

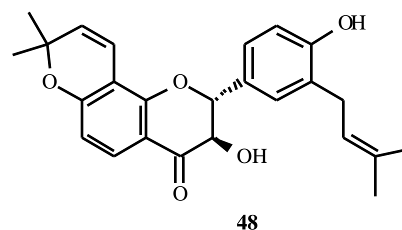
A survey of the chemical constituents of *Sophora* species revealed that prenylated flavonoids were extremely abundant





in their roots. The reported compounds present different carbon skeletons (flavanone, isoflavanone, etc), oxygenation



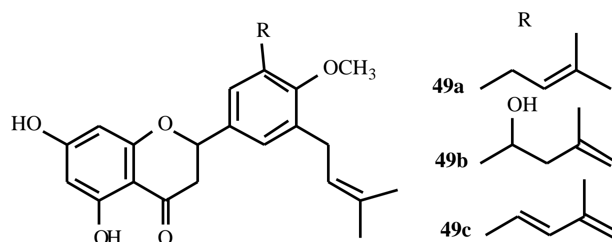


pattern of the B-ring and variations of C5 and/or C10 substituent [21].

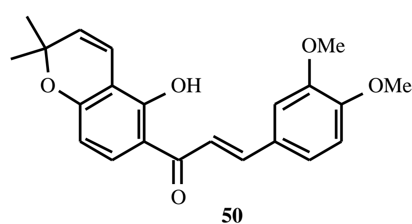
The antiviral activities against HSV-1 and HSV-2 of the phytochemical constituents of *Sophora flavescens*: kushenol H (**41a**), kushenol K (**41b**), kurarinol (**42**), kuraridine (**43**), and sophoflavescenol (**44**), have been reported [22a]. A dihydroflavonol, kosanol A (**45**) [22b] and two further lavandulynated flavanones, (2S)-2'-methoxy kurarinone (**46a**) and (-)-kurarinone (**46b**) [22c] were isolated from the roots of the same plant.

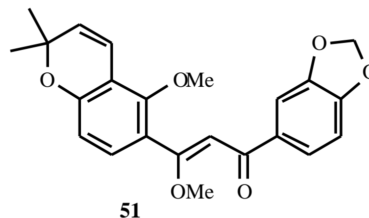
Kanzonol Y (**47**) and Z (**48**), a dihydrochalcone and a flavanonol, respectively, were isolated from cultivated liquorice (*Glycyrrhiza glabra*) [23].

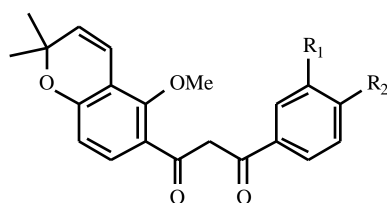
The stem bark of *Erythrina burttii* yielded three new flavanones, which were assigned the structures of 5,7-dhydroxy-4'-methoxy-3',5'-di-(3-methylbut-2-enyl)-flavanone (**49a**), 5,7-dihydroxy-4'-methoxy-3'-(2-hydroxy-3-methylbut-3-enyl)-5'-(3-methylbut-2-enyl)-flavanone (**49b**), and 5,7-dihydroxy-4'-methoxy-3'-(3-methylbutadienyl)-5'-(3-methyl-but-2-enyl)-flavanone (**49c**, burttinone dehydrate) [24].

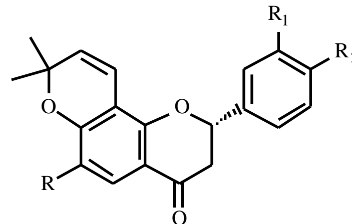


Chalcones (**50-52b**) and flavanones (**53a-53c**) featuring a chromene group in the A ring were obtained from the roots of *Lonchocarpus subglaucecscens* together with a series of furano derivatives and several already known flavonoids [25].

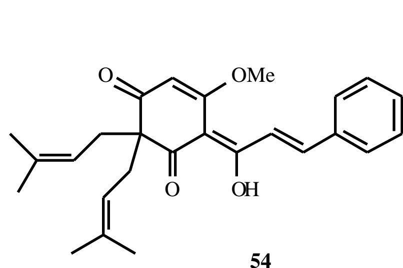


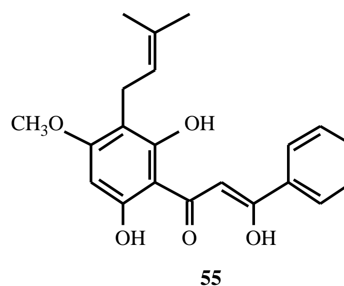




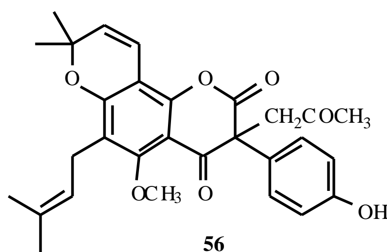


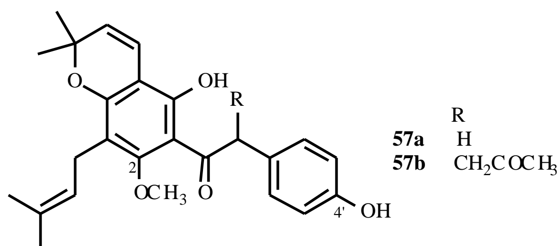
Looking at the genus *Tephrosia*, a 3',3'-di-(– dimethylallyl)-2'-4'-di-oxo-enol-chalcone (tunicatachalcone, **54**) was reported from the roots of *Tephrosia tunicata* together with six known C-alkylated flavonoids [26], while the roots and aerial parts of *Tephrosia major* Micheli afforded the new 2',6'-dihydroxy-4'-methoxy-3'-prenyl- – hydroxychalcone (**55**) [27].

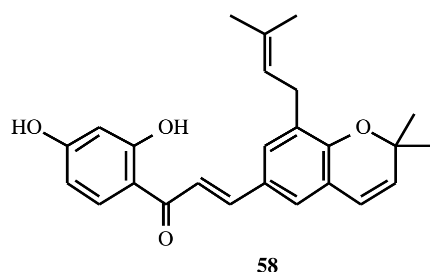


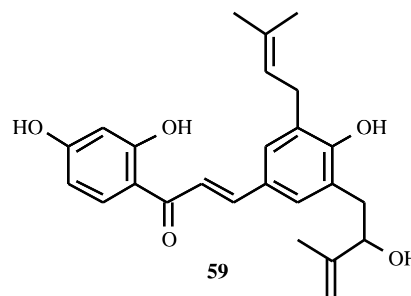


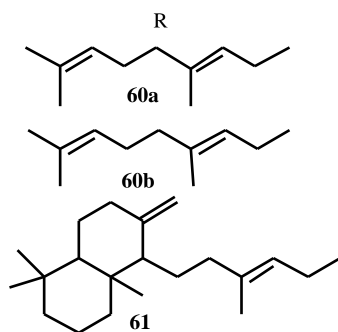
Dichloromethane and petrol extracts from the roots of *Deguelia hatschbachii* A.M.G. Azevedo furnished bioactive (brine shrimp lethality essay) 3-(4'-hydroxyphenyl)-5-methoxy-6-(, -dimethylallyl)-2",2"-dimethyl chromene (5",6",8,7)-3-propyl-2-one)-4H-1-benzo-2,3-dihydropyran-2,4-dione (**56**), 6',4'-dihydroxy-3-(, -dimethylallyl)- 2",2"-dimethyl chromene (5",6",5,4)-2-methoxydeoxybenzoin

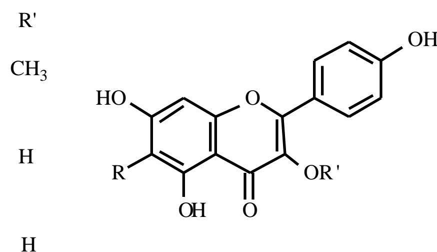










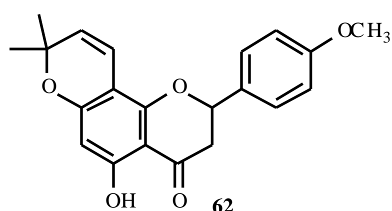


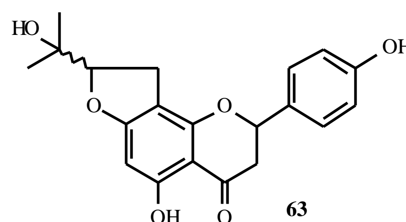
(**57a**), and 6',4'-dihydroxy-3-(, -dimethylallyl)-2",2"-dimethyl chromene (5",6",5,4)-2-methoxy-8-(propyl-2-one) deoxybenzoin (**57b**) [28].

Finally, two new prenylated chalcones, named anthyllisone (**58**) and anthyllin (**59**) were isolated from the aerial parts of *Anthyllis hermanniae* [29].

macarangin (**60b**) and dentilucaflavonol (**61**) possessing a diterpene substituent have been isolated from the leaves of *Macaranga denticulata* [30]. Among the compounds tested for radical scavenging properties, only macarangin showed a pronounced antioxidant activity (IC_{50} 0.032 mM).

Two prenylated flavonoids, 5-hydroxy-4'-methoxy-2",2"-dimethylpyrano (7,8:6",5") flavanone (**62**) and 5,4'-

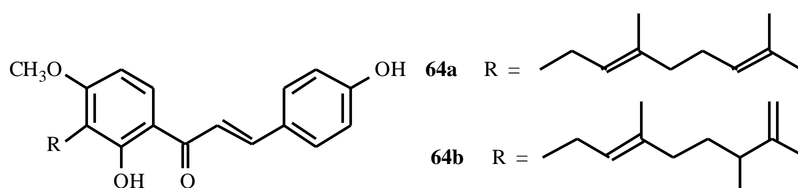


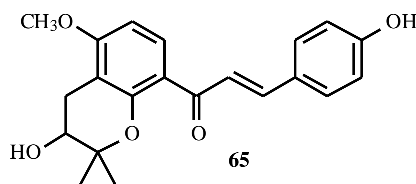


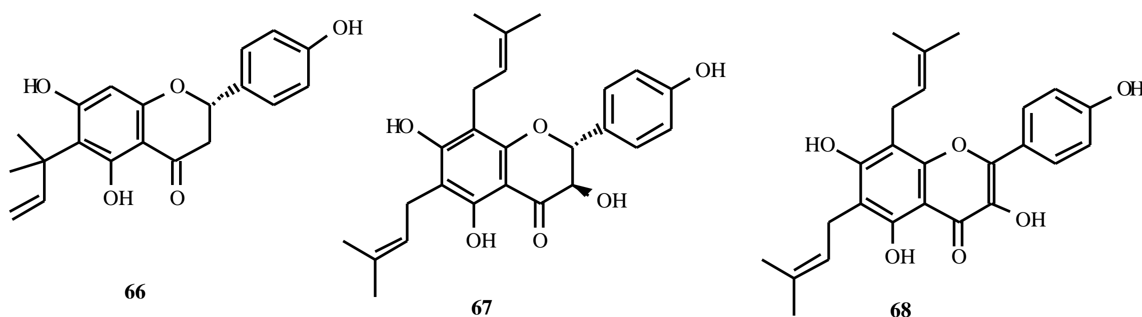
2.3. Euphorbiaceae, Umbelliferae, and Guttiferae

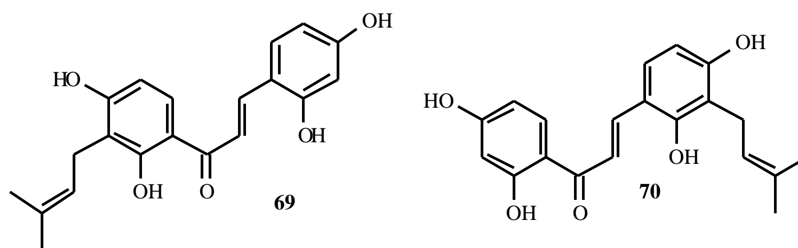
The genus *Macaranga* (Euphorbiaceae) has recently received attention for the interesting biological activity of some constituents. An O-methylated analog (**60a**) of

dihydroxy-[2"-(1-hydroxy-1-methylethyl)dihydrofurano-(7,8:6",5")flavanone (**63**) were isolated from the EtOAc-soluble extracts of the leaves of *Macaranga conifera*, using an *in vitro* activity-guided fractionation procedure based on the inhibition of cyclooxigenase-2 [31].

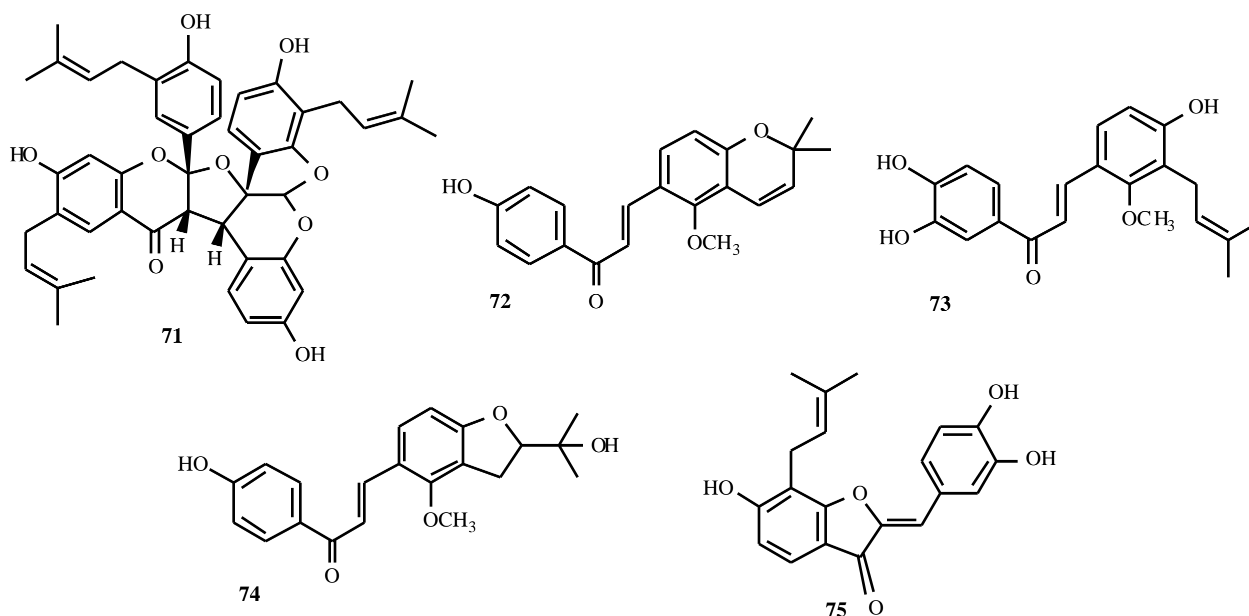








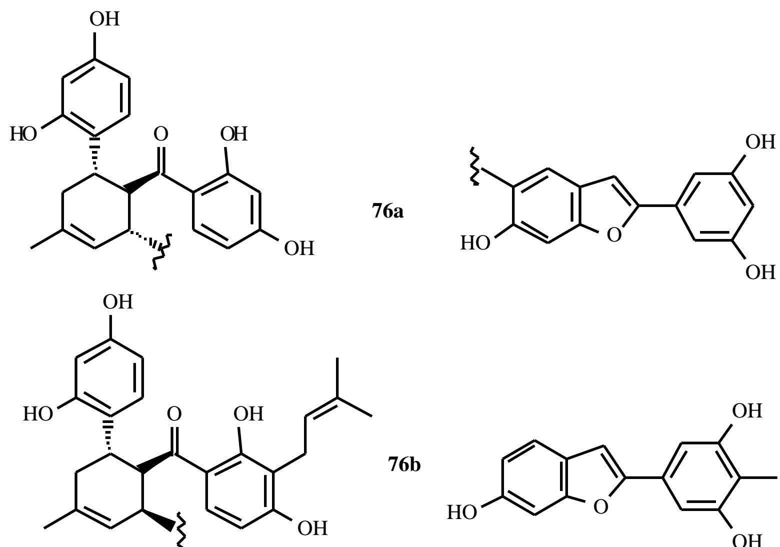
The family Umbelliferae is represented only by *Angelica keiskei*, whose roots yielded three new xanthoangelols F (**64a**) , G (**64b**) and H (**65**) together with isobavachalcone (**22a**) [32].

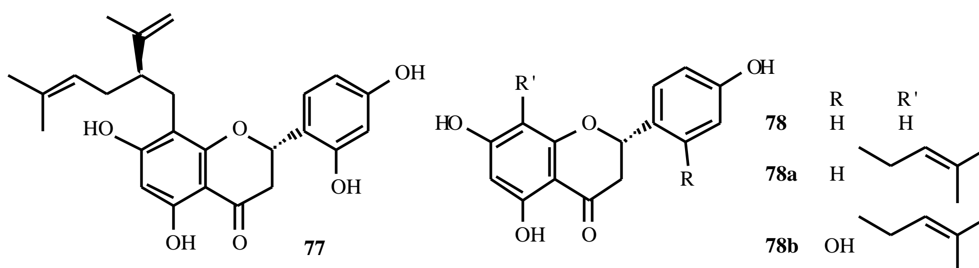


6-(1,1-Dimethylallyl)naringenin (**66**) isolated from *Monotes engleri* [33], 6,8-diprenylaromadendrin (**67**) and 6,8-diprenyl kaempferol (**68**), isolated from *Monotes africanus* [34], are among the few examples of prenylated flavonoids from Guttiferae.

2.4. Hairy Root, Callus and Suspended Cell Cultures

Surprisingly, the review of Barron and Ibrahim (1996) [4] contained only few reports on the production of prenylated flavonoids in callus tissue and cell suspension cultures: 6- and 8-prenyl derivatives of 7,4'-di hydroxyflavone [35] and 5'-prenyllicodione [36] have been obtained in the cell culture of *Glycyrrhiza* species. On the other hand, the 8-lavandulyl derivatives of 5,7,2',4'-tetrahydroxyflavanone (sophora-

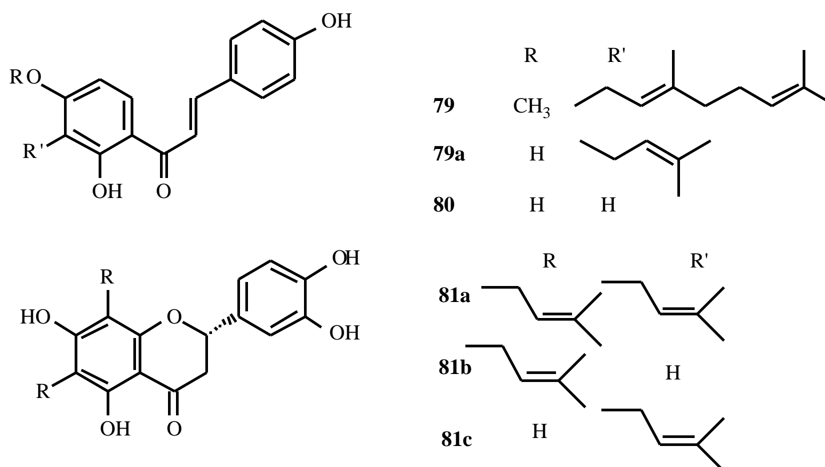




flavanone G) and 7,2',4'-trihydroxyflavanone (lehmannin) have been reported to accumulate in *Sophora flavescens* cells (*vide infra*) [37]. Finally, a leguminous tissue culture, *Epimedium diphyllum*, furnished several 7-O-mono- and diglycosides of 8-prenyl kaempferol (*vide infra*) [38]. To date, the literature on the *in vitro* production of prenylated flavonoids is still limited. Plant tissue cultures of *Maclura pomifera* show a flavonoid accumulation both qualitatively and quantitatively different from that of the parent plant. Prenylated flavones and flavanones are preferentially produced by the suspended cells, but the prenyl substituent was located exclusively on ring A. 2,2',4,4'-tetrahydroxy-3'-(3-methylbut-2-enyl)-chalcone (**69**), isolated from callus and cell culture, is the first example of *in vitro* formation of 6'-deoxychalcones [39].

Table 1. Biological Activities of Some Prenylflavonoids

Biological and/or pharmacological activity	Active molecules and source	Lit.
Antibacterial activity [against Gram-(+) pathogenic bacteria] Antiulcer	Xanthoangelol (**79**) and 4-hydroxy derricin (**79a**) from *Angelica keinskei* Koidrumi	[43]
Antioxidant activity	Macarangin (**60b**) from *M. denticulata* 6,8-diprenyleriodictiol (**81a**), dorsmanin C (**14**) and F (**17**)	[30] [11]
Aromatase inhibition	Synthetic analogues of metabolites from *Broussonetia papyrifera*	[44]
Citotoxicity (against three human tumour cell lines)	Artelastin and similar compounds (**31-37**) from Artocarpus	[19c]
Cyclooxygenase-1 (COX-1) and COX-2 inhibition	Prenyflavonoids (**62-63**) from leaves of *Macaranga conifera*	[31]
DNA strand scission activity	Known prenylated flavonoids from stem bark of *Artocarpus kemando*. Xanthoangelol (**79**)	[45] [43]
Induction activity of DNA damage (rec-assay)	Isoliquiritigenin (2',4,4'-trihydroxychalcone, **80**), 6- (**81b**) and 8-prenyl eriodictiol (**81c**) from liquorice.	[23b]
HIV-inhibition	5,7-Dihydroxy-6,8-diprenylflavonoids (**67-68**) from *Monotes africanus*	[34]
Herpes simplex type 1 (HSV-1) inhibition	Leachianone G (**78b**) from root bark of *Morus alba*	[46]
Tyrosinase inhibition	Kuraridin (**43**) and kurarinon (**42b**) from root bark of *Morus alba*	[47]
Electronic transfer inhibition in mitochondrial inner membrane	Synthetic prenylated flavonoids	[3]



2',4,4'-Trihydroxy-3-(3-methylbut-2-enyl)-chalcone (licoagrochalcone A, **70**) was isolated successively from the hairy root cultures of *Glycyrrhiza glabra* [40a] together with (6aR,11aR)-3-hydroxy-9-methoxy-4-prenyl-pterocarpan(ligrocarpin).

From the same source, *inter alia*, an unusual biflavonoid (licoagrodin, **71**) was obtained along with three prenylated retrochalcones (licoagrochalcones B, C, D; **72-74**), and a prenylated aurone (licoagroaurone, **75**) [40b].

The Diels-Alder type adducts, isolated from the acetone extract of the callus from *Morus nigra*, were totally different from those obtained from the root bark of the plant, not only for the cyclohexene ring substitution, but also for the stereochemistry of the alicyclic ring (*cis-trans in vitro* instead that *trans-trans in vivo*) [41]. Albafuran C (**76a**, from *Morus nigra* root bark) and calcomoracin (**76b**, from *Morus nigra* callus) are two typical examples.

The addition of cork tissue increased the production of sophoraflavanone G (**77**) in cell suspension cultures of *Sophora flavescens* three- to five-fold higher with respect to the cells cultured alone [42a].

The lavandulyl group is not directly transferred to the flavanone skeleton, but it was shown to be formed by two dimethylallylations between which the 2'-hydroxylation occurred: the biosynthetic pathway goes thus from naringenin (**78**) to **77**, *via* 8-prenylnaringenin (sophoraflavanone B, **78a**) and leachianone G (**78b**) [42b].

The two precursors, never detected either in cultured cells or in the original plant, were isolated from the cork tissue where most of sophoraflavanone G also accumulates, together with 13 minor flavonoids, including two new prenylated isoflavonoids as well as a benzofuran [42c].

3. PRENYLATED FLAVONOIDS IN PHARMACOLOGY

Biological and pharmacological activities have been studied sporadically and without going into great detail. For instance, in spite of the anti-inflammatory, analgesic, antioxidant, cytotoxic, anti-snakebite, antirheumatic, and antileishmanial activities reported for extracts of *Dorstenia*, the pharmacological data on this genus are scanty [5]. Some of the biological/pharmacological activities of prenyl flavonoids reported in the literature are summarized in Table **1**.

The following section will be dedicated to the crucial problem of multidrug resistance. In the other sections the studies concerning two lead molecules, xanthohumol and sophoraflavanone G, and other related compounds will be summarized. Finally some structure-activity relationships, reported from various sources, will be again discussed.

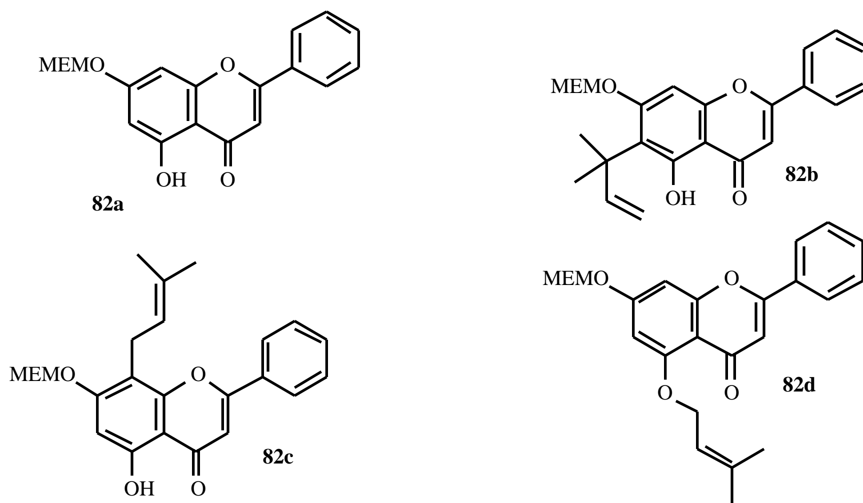
3.1. Multidrug Resistance

The development of multidrug resistance (MDR) by tumor cells is a major impediment to the success of cancer therapy. The over-expression of the P-glycoprotein (Pgp) confers MDR to cancer cells [48]. Tumor cells expressing Pgp are resistant to a number of major cytotoxic agents, including anthracyclines, *Vinca* alkaloids, taxanes, and *epi*podophylotoxins. It is possible to overcome the anticancer drug-escape mechanism of MDR-cells *in vitro* by increasing the anticancer drug in the culture medium. However, this cannot be done *in vivo*, since in clinical practice treatments on cancer patients are already performed with nearly maximal dosage anti-cancer drugs close [49].

Several studies have allowed the identification of a variety of agents which can decrease the anticancer drug resistance of MDR-tumor cells *in vitro*; such chemosensitizers belong to different structural classes, though a high hydrophobicity and an ability to diffuse through the cell membrane seem to constitute common requirements. They include peptides, calcium channel blockers, cardiovascular drugs, immunosuppressive, and anti-fungal agents [50]. Their use has been limited by the fact that most of them are also transported by Pgp [51].

Hydrophobic steroids, such as progesterone and antiprogestin RU 486, are not transported and behave as efficient modulators of cellular MDR inhibiting anticancer drug efflux [52].

Flavonoids constitute also promising potential modulators of multidrug resistance.

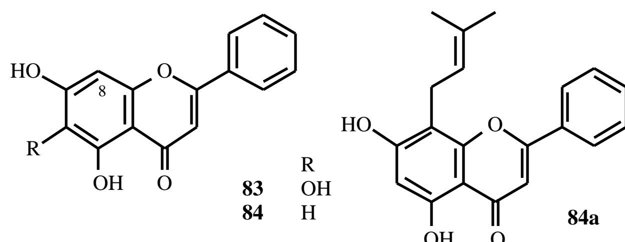


An *in vitro* rational screening of the flavonoid family was based on a four step procedure: i) direct binding to purified recombinant cytosolic NBD (nucleotide binding domain) and/or full length transporter; ii) inhibition of ATP hydrolysis and energy-dependent drug interaction with transporter-enriched membranes; iii) inhibition of cell transporter activity, monitored by flow cytometry; (iv) chemosentitization of cell growth [53].

Chalcones, flavones and flavonols have been shown to bind more strongly to Pgp cytosolic site than flavanones, isoflavones and glycosyl derivatives. The hydroxylation at position 5, in addition to the presence of a ketone at position 4, is essential for the ability of these modulators to mimic the adenine moiety of ATP [54].

The activity as potential P-glycoprotein inhibitors of 7-O-protected prenylated flavones (**82b-d**) was monitored by affinity of direct binding and compared to that of corresponding dimethylallyl derivatives of naturally occurring 1-hydroxy-3,7,8-trimethoxyxanthone (decussatin). Both classes of compounds exhibited the same structure-activity relationship: prenylation at either position 6 (**82b**) or 8 (**82c**) produced a 80-fold increase in affinity as compared to the simple flavone (**82a**), whereas prenylation of the hydroxyl group at position 5 (**82d**) produced a very limited effect [55].

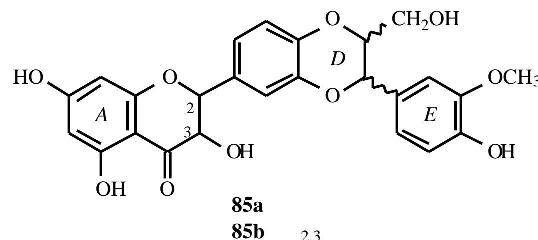
Using a new *in vitro* rational-screening assay, based on measurements of binding affinity toward the C-terminal nucleotide–binding domain (NBD2) of PgP [56] structure-activity relationships of flavonoids as potential MDR modulators were studied. The binding affinity evaluation showed that the activity of several 4-O-alkyl substituted (B ring) chalcones was directly correlated to the chain length when it contained up to eight carbon atoms. C-alkylated chalcones were considered more advantageous than their O-alkylated analogs, because the free hydroxyl groups can play a role in the interaction. Therefore, C-prenylated derivatives were synthesized and again the presence of the prenyl group markedly enhanced the binding affinity. The 3-prenyl derivative binds to NBD2 with 20 folder affinity as compared to the unprenylated 2'-hydroxychalcone [57]. Because of the possible cyclization of chalcones to give the less active flavanones, flavonol with different hydrophobic substituents on B ring were synthesized using 3,5,7-trihydroxyflavone (galangin, **83**) as reference compound. The most striking increase in binding affinity was observed with a 4'-O-*n*-octyl group, which produced a 93-fold higher affinity, as compared with galangin [58].



To investigate structural requirements of the flavonoid A ring toward Pgp modulation a series of C- or O-substituted hydrophobic derivatives of the 5,7-dihydroxyflavone chrisyn (**84**) were synthesized. Increasing lipophilicity at either positions 6, 8, or 7 increased the affinity of *in vitro* binding to a purified cytosolic domain of Pgp, but only benzyl and , -dimethylallyl C-substitution produced a high maximal quenching of the protein intrinsic fluorescence. Inhibition of membrane Pgp within leukemic cells, characterized by intracellular daunomycin accumulation, was specifically produced by isoprenylated derivatives, with 8-(, -dimethylallyl)chrysin (**84a**) being even more efficient than the commonly used cyclosporin A [59].

Notably, the modulating effects of C-prenylated chrysin derivatives were produced by concentrations which were not toxic for the cells, suggesting that these compounds should be investigated *in vivo* as potential Pgp modulator in tumor cells.

The activities of silybin (**85a**), a flavonolignan isolated from fruits of *Silybum marianum* [60] and some of its prenyl and geranyl derivatives were also compared. The absence of 2-3 double bond as well the relative polarities of rings D and E theoretically made silybin a poor candidate for a good interaction with NBD2. However, silybin displayed a surprising good affinity. Oxidation of sylibin to its corresponding flavonol derivative dehydrosylibin (**85b**) increased the activity by a factor 3. As expected, prenylation led to an increase in affinity (six- to nine-fold depending on the position of the substituent), whereas geranylation produced a further two-fold increase [61].



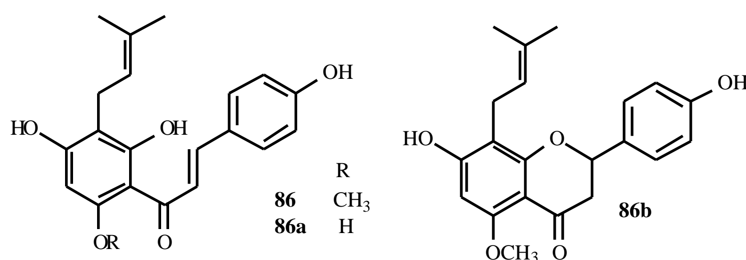
3.2. Xantohumol and 8-Prenylnaringenin

Xantohumol, 2',4,4'-trihydroxy-6'-methoxy-3'-(, -dimethylallyl)-chalcone (**86**), is one of the most important prenylated flavonoid for its large spectrum of biological activities. This chalcone is the main component (80-90% of the total flavonoids) in hops (*Humulus lupulus*) plants, where it is flanked by its flavanone isomer (**86b**), 11 structurally similar chalcones and 10 flavanone isomers of these latter [62].

The amount of xantohumol is largely reduced in the brewing process by losses due to incomplete extraction, adsorption to insoluble malt proteins and to yeast cells during the fermentation. Moreover, xantohumol and the other chalcones are partially converted into their isomeric flavanones, i.e. prenylnaringenin derivatives (like **78a**) [63]. Prenylchalcones and prenylflavanones from hops and beer have been examined for their activities through several studies, which are summarized below.

3.2.1. Estrogenic Activity

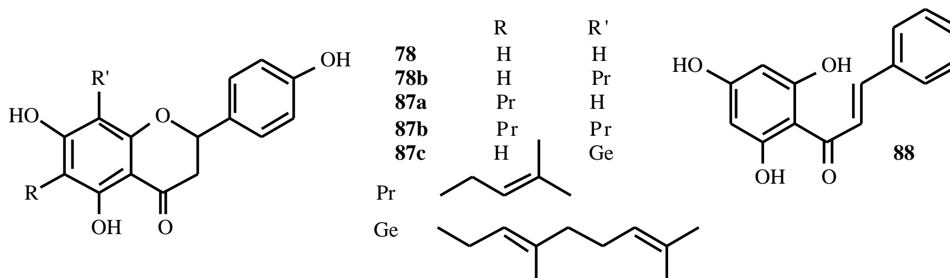
A sensitive *in vitro* bioassay, revealing the ability of hop components to stimulate alkaline phosphatase activity in a human endometrial adenocarcinoma cell line, was used to determine their estrogenic activity [64]. All samples based on hop acids or hop acid-derived compounds were devoid of



any activity. In contrast, polyphenolic extracts stimulated estrogen responsive alkaline phosphatase activity, which, however, could neither be attributed to xanthohumol (**86**), nor to desmethyxanthohumol (**86a**).

The study of some herbal dietary preparations, claiming "breast enhancement" for women, confirmed the strong activity of 8-prenylnaringenin (**78a**) as phytoestrogen, whereas the potency of structurally related flavonoids, 6-prenylnaringenin (**87a**), 6,8-diprenylnaringenin (**87b**) and 8 geranylnaringenin (**87c**) was in comparison less than 1% [65]. 8-Prenylnaringenin (**78a**) alone competed strongly with was consistent with the content of 8-prenylnaringenin [66]. The extract also inhibited the reductive activity of 17 - hydroxysteroid oxidoreductase to a greater extent than a mixture of hop phytoestrogen at the same concentrations. However, the supplement was only weakly active in mouse uterotropic assays at doses of **78a** up to 250 times higher than that recommended.

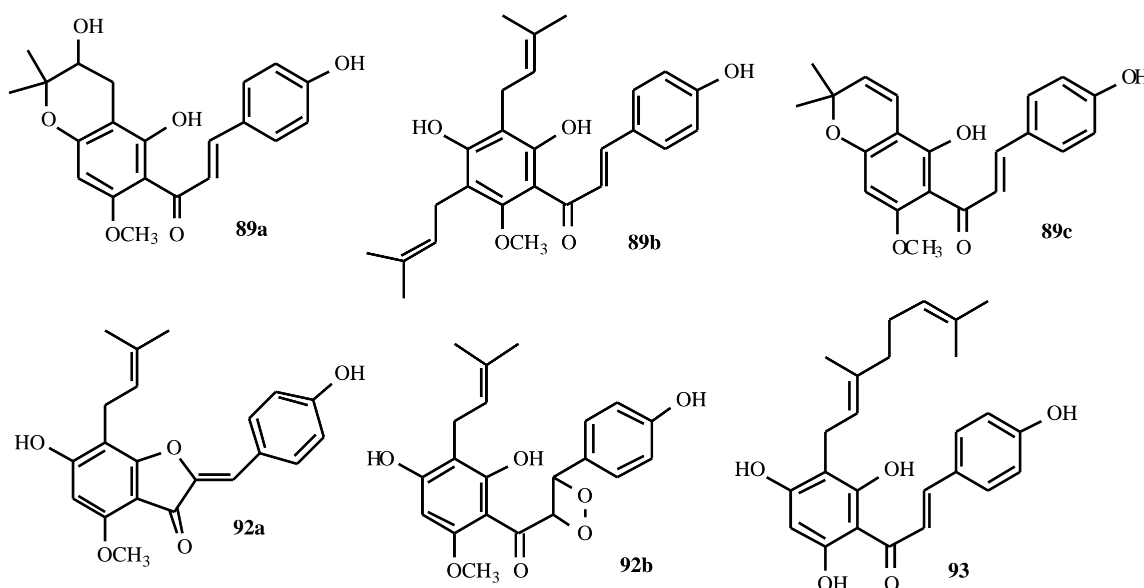
A medicament from the female hop cones for the treatment of physiological disorders, related to perimonopause or menopause but also suitable for dietary food supplements and cosmetic compositions, has been



17 -estradiol for binding to both the and -estrogen receptors, but did not show – as well as the other components, included xanthohumol – progestogenic or androgenic bioactivity.

The estrogenic activity of the hop-based dietary supplement was confirmed in a Er reporter gene assay and recently patented [67a]. Analogously, a patent reports the preparation of hop extracts to be used for the prophylaxis and therapy of conditions that are caused by estrogen deficiency or by a disregulation of the sex hormone [67b].

The latest developments on the potential protective properties of phytoestrogens like 8-prenylnaringenin (**78a**),



resveratrol and various isoflavonoids against hormone-dependent breast and prostate cancers and cardiovascular diseases, have been recently reviewed [68].

3.2.2. Apoptosis

Xantohumol (**86**) and humulone (a hop bitter acid) are the active ingredients that inhibited bone resorption in the pit formation assay cc [69]. Both humulone and xanthohumol induced apoptosis to premyocytic leukaemia cell line HL-60. In particular, xanthohumol at low concentration (1–10 µg/mL) killed the cells by induced apoptosis, whereas at the high concentration (100 µg/mL) prevented DNA fragmentation [70].

The chalcone was shown to inhibit chymotrypsin (serine protease) and might thus inhibit cysteine protease such as interleukin-1 . The influence of xantohumol on apopoptosis was compared with that of other bioactive flavonoids by the same author (*vide infra*) [71].

3.2.3. Promoter for Nerve Growth Factor

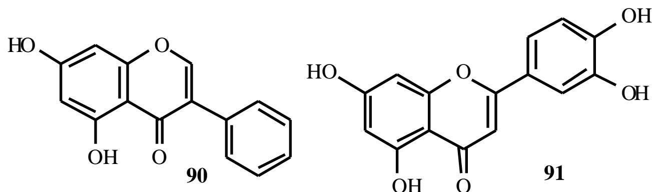
Hop and *Angelica keiskei* extracts and xanthohumol (**86**) have been patented as promoters for nerve growth factor [72]. These drugs could stimulate the production of Nerve Growth Factor (NGV) inside the body (*in vivo*). Foods, drink and feeds, provided with the above active ingredients are useful in maintaining the homeostasis *in vivo* and effective in improving the learning and memory abilities of individuals.

3.2.4. Induction of Quinone Reductase

Prenylchalcones and prenylflavanones were found to induce quinone reductase (QR) in the mouse hepatoma Hepa 1c1c7 cell line. In contrast the non prenylated chalconaringenin (2',4,4',6'-tetrahydroxychalcone, **88**) and naringenin (**78**), were ineffective. The hop chalcones, xanthohumol **86**) and xanthohumol B (**89a**), also induced QR in the Ah-receptor defective mutant cell line, Hepa 1c1c7 bprc1 [73].

3.2.5. Acyltransferase Inhibition

A methanol extract of hops of *Humulus lupulus* showed inhibitory activity against rat liver diacylglycerol acyltransferase (DGAT). Biologically-guided fractonation gave xanthohumol (**86**) and xanthohumol B (**89a**), which showed preferential inhibition of triacetlglycerol formation in intact Raji cells, indicating that they inhibit DGAT activity preferentially in living cells [74].



3.2.6. Antioxidant

Prenylchalcones and prenylflavanones from hops were examined for their ability to inhibit *in vitro* oxidation of human low-density lipoprotein (LDL) [75a]. At 5 and 25 mM, all of the prenylchalcones tested inhibited the oxidation of LDL induced by 2 mM copper sulfate. The prenylflavanones showed less antioxidant activity than the prenylchalcones, both at 5 and 25 mM concentrations. At 25 mM the non prenylated chalconaringenin (**88**) and naringenin (**78**) exerted prooxidant effects on LDL oxidation. The antioxidant activity of xanthohumol (**86**) was higher than -tocopherol and genistein (**90**), but lower than quercetin (**91**). When combined, **86** and -tocopherol completely inhibited copper–mediated LDL oxidation. In conclusion, Miranda *et al.* showed that the prenylation antagonizes the prooxidant effects of the chalcone **88** and the flavanone **78** and protect human LDL from oxidation [75a].

The same group compared also the hops prenylated chalcones with non-prenylated flavonoids (**88**, **78**, **90**, **91**) for their ability to inhibit lipid peroxidation in rat liver microsomes [75b]. Chalcones with prenyl and geranyl groups (5 and 25 mM), as compared with **88**, **78** or **90**, were more effective inhibitors of microsomal lipid peroxidation, when induced by Fe^{2+}/ascorbate; prenylated chalcones were, however, less effective when the oxidation was induced by Fe^{3+}-ADP/NADPH or by *t*-butyl hydroperoxide (TBH). The prooxidant activity of non-prenylated flavonoids was confirmed in the iron-dependent systems: naringenin (5 mM) enhanced Fe^{2+}/ascorbate induced oxidation, as calconaringenin did with Fe^{3+}-ADP/NADPH. None of the flavonoids, except quercetin, inhibited NADPH cytochrome P450-reductase, thus excluding its partecipation to the inhibition mechanism [75b]. Chalcones exhibiting antioxidant activity (25 mM) against TBH-induced lipid peroxidation, such as xanthohumol (**86**) and 5'-prenylxanthohumol (**89b**), protected cultured rat hepatocytes from TBH toxicity. Other antioxidants, desmethylxantho humol (**86a**) and calconaringenin (**88**) in the TBH system were not cytoprotective.

Finally, prenyated chalcones and flavanones from *Humulus lupulus* were shown to inhibit peroxynitrite-mediated oxidation of LDL at low micromolecular concentrations. Oxidation of xanthohumol by peroxinitrite gave mainly the aurone **92a** and an endoperoxy derivative (**92b**), flanked by smaller amounts of their nitrated products. Isoxanthohumol (**86b**), the flavanone isomer of xanthohumol (**86**), unexpectedly showed a slight prooxidant effect instead of an inhibitory effect of LDL oxidation. The flavanone, except for the formation of minor nitrated compounds, remained largely unmodified upon treatment with peroxynitrite, suggesting that the , -unsaturated keto functionality of chalcone is most reactive toward superoxide and peroxynitrite anions [76].

3.2.7. Cancer Chemopreventive Anticarcinogen

Xanthohumol (**86**) and five other related substances – 2',4,4',6-tetrahydroxy-3'-prenylchalcone (**86a**), 2',4,4',6-tetrahydroxy-3'-geranylchalcone (**93**), dehydrocyclo xanthohumol (**89c**), xanthohumol B (**89a**), and isoxanthohumol (**86b**) – have been tested for their antiproliferative activity in human breast cancer (MCF-7), colon cancer (HT-29) and ovarian cancer (A-2780) cells *in vitro* [77].

86, **86b** and **89c** caused a dose-dependent (0.1–100 µM) decrease in the growth of all cancer cells. After a 2-day treatment, the IC_{50}, at which the growth of MCF-7 cells was inhibited, were 13.3, 15.3 and 15.7 µM, respectively. After a 4-day treatment, the IC_{50} for the three compounds were 3.47, 4,69 and 6.87 µM, respectively. Cell counting confirmed the growth inhibition of MCF-7 cells treated with xanthohumol (**86**) and his flavanone isomer (**86b**).

HT-29 cells were more resistant than MCF-7 to these flavonoids. In A-2780 cells, xanthohumol was highly antiproliferative with IC50 values of 5.2 and 0.52 µM after 2 and 4 days of exposure, respectively [77]. The prenylchalcone **86** and the prenylflavanones, 8-prenylnaringenin (**78a**) and isoxanthohumol (**86b**), were shown to strongly inhibit the mutagenic activation of the carcinogen 2-amino-3-methylimidazol[4,5-f]quinoline (IQ) by the cDNA-expressed human CYP1A2, as determined by the Ames Salmonella assay. The three prenylflavonoids also markedly inhibited the human CYP1A2-mediated binding of IQ to metabolites that bind to DNA [78]. In accord with anti-initiating potential, xanthohumol (**86**) potently modulates the activity of enzymes involved in carcinogen metabolism and detoxification. Moreover, the chalcone is able to scavenge reactive oxygen species, including hydroxyl and peroxyl radicals, and to inhibit superoxide anion radical and nitric oxide production. As potential antitumor-promoting mechanisms, it demonstrated anti-inflammatory properties by inhibition of cyclooxygenase-1 and cyclooxygenase-2 activity, and it is antiestrogenic without possessing intrinsic estrogenic potential. Antiproliferative mechanisms of the chalcone to prevent carcinogenesis in the progression phase include inhibition of DNA synthesis and induction of cell cycle arrest in S phase, apoptosis and cell differentiation. Notably, xanthohumol prevents carcinogen-induced preneoplastic lesions in mouse mammary gland organ culture at nanomolar concentrations. In conclusion, xanthohumol is proposed as a novel chemopreventive agent worthy of clinical investigations [79]. More recently, the inhibitory effect of extracts from *Humulus lupulus* on both the production of NO and expression of inducible NO synthase (iNOS) in mouse macrophage RAW 264.7 cells has been investigated. Bioactivity-guided fractionation of the EtOAc soluble fraction gave five chalcones together with other non flavonoid compounds. The chalcones, including xanthohumol, significantly inhibited the production of NO by suppressing the expression of iNOS [80].

3.2.8. Inhibition of Human P450 Enzymes

The chemopreventive properties of xanthohumol and other hop flavonoids are due in part to inhibition of P450 enzymes that activate carcinogens. Prelimiary studies have shown these prenylated flavonoids to be inhibitory (at 100 µM) of P450 mediated activation reactions in a variety of *in vitro* system. The *in vitro* effects of these phytochemicals on cDNA-expressed human CYP1A1, CYP1B1, CYP1A2, CYP3A4, and CYP2E1, were thus examined by the use of diagnostic substrates and the carcinogen AFB1. At 10 µM xanthohumol (**86**) almost completely inhibited the 7-ethoxyresorufin O-deethylase (EROD) activity of CYP1A1. At the same concentration other hop flavonoids decreased the EROD activity by 90.8–27%. At 10 µM, xanthohumol completely eliminated EROD activity of CY1B1, where the other flavonoids showed varying degrees of inhibitory activity ranging from 99.3 to 1.8%. In contrast, the most effective inhibitors of CYP1A2 acetanilide 4-hydroxylase activity were two prenylated flavonoids, isoxanthohumol (**86b**) and 8-prenylnaringenin (**78a**), which produced 90% inhibition when added at concentrations of 10 µM. CYP1A2 metabolism of the carcinogen AFB1 was also inhibited by the last two; moreover, **86b** and **78a** decreased covalent binding of radiolabeled AFB1 to microsomal protein in a concomitant manner.

However, the three prenyl flavonoids were poor inhibitors of CYP2E1 and CYP3A4 by their effect on chorzoxazone hydroxylase and nifedipine oxidase activities, respectively. These results suggest tha the hop flavonoids are potent and selective inhibitors for human cytochrome P450 and warrant further *in vivo* investigation [81].

3.3. Sophoraflavanone G and Prenylated Flavanones of Sophora

Sophoraflavanone G (**77**), isolated for the first time from the root of *Sophora moorcroftiana* Benth. ex Baker [82] is the most diffuse in the series of lavandulyl flavanones from *Sophora*. It was also produced, together with the 5-desoxy derivative lehmannin (**77a**) from callus culture of *Sophora flavescens* var. *angustifolia* [83]. The compound was isolated as a cytotoxic constituent by a bioassay-guided procedure from the roots of *Sophora flavescens* Aiton, and exhibited high cytotoxic activities against A549, HeLa, and K562 cell lines, but showed mild activity (ED_{50} value, 5 µg/mL) against L1210 cells [84]. Among the tested cell lines, A549 cells were the most sensitive (ED_{50} value, 0.78 µg/mL) to the flavanone.

Cytotoxic properties *in vitro* toward human tumor cell lines such as A549, SK-OV-3, SK-Mel-2, XF498, and HCT15, were shown also by other analogous flavonoids from the roots of *Sophora flavescens* [85].

The effect of 11 prenylated flavonoid from the same plant on phospholipase C 1 (PlC 1) was also investigated: the flavonoids exhibited relatively strong inhibitory activity with IC_{50} values ranging from 7.5×10^{-6}–35×10^{-6} M with the exception of kushenol H (**41a**, IC_{50} $>5.3.10^{-4}$ M). With regard to the structure-activity relationship, the presence of C3-OH resulted in a diminution of activity and the configuration of C3-OH was likely to be another factor influencing the activity. Notably, hydration of the C4'''-C5''' double bond of the lavandulyl side chain caused complete loss of activity [86].

In 1999 study by Zheng [87], sophoraflavanone G (**77**) and kurarinone (**46b**) from *Sophora flavescens* were attributed strong insecticidal and fungicidal activity, which was shown to decrease by methylation of 5-hydroxyl group.

On the other hand, four flavonoids with C-8 lavandulyl substituent were tested for their effects on human myeloid leukaemia HL-60 cells and human hepatocarcinoma HepG2 cells, in terms of inhibition of proliferation and induction of apoptosis. They showed potent antiproliferative effects with IC_{50} values from 11.3 µm to 18.5 µm in H-60 cells and from 13.3 µm to 36.2 µm in HepG2 cells. Treatment of H-60 cells with the lavandulyl flavonoids induced apoptosis in a dose – dependent manner [88]. Again the hydration of C4'''-C5''' double bond with or without C3 hydroxylation caused a complete loss of cytotoxicity.

To elucidate the pharmacological mechanism underlying the intensive antibacterial activity of sophoraflavanone G (**77**), the compound was isolated from *Sophora esigua* and compared with less active naringenin (**78**, without

lavandulyl and 2'-hydroxyl groups) in a study of the effecton membrane fluidity using model membranes [89]. Highly purified **78** at 0.05–5 μg/mL concentrations, corresponding to the minimal growth inhibition against various bacteria, significantly increased fluorescence polarization of the liposomes prepared from the 1,2-dipalmitoyl-L- -phosphatidyl choline and 1-palmitoyl-2-oleoyl-L- –phosphatidylcholine. Such increases were found in both liposomes measured with two fluorescent probes to indicate an alteration of membrane fluidity in hydrophilic and hydrophobic regions, suggesting that sophoraflavanone G reduces the fluidity of outer and inner layers of membranes. Although naringenin (**78**) also showed the membrane effect, it required concentrations over 2.5-5 μg/mL to induce a significant reduction of membrane fluidity. The flavanone **77** can be thus considered to exert its antibacterial effect by reducing the fluidity of cellular membranes.

The investigation of the biological activity of ten prenylflavanones purified from *Sophora tomentosa* L. and *Sophora moorcroftiana* Benth. ex Baker evidentiated their medicinal significance [90]. The flavanones with a prenyl-, lavandulyl- or geranyl groups on the A ring showed tumor-specific cytotoxic activity, antimicrobial activity and anti-HIV activity. A relationship was also found between radical generation and the O22-scavenging activity of these prenylflavanones.

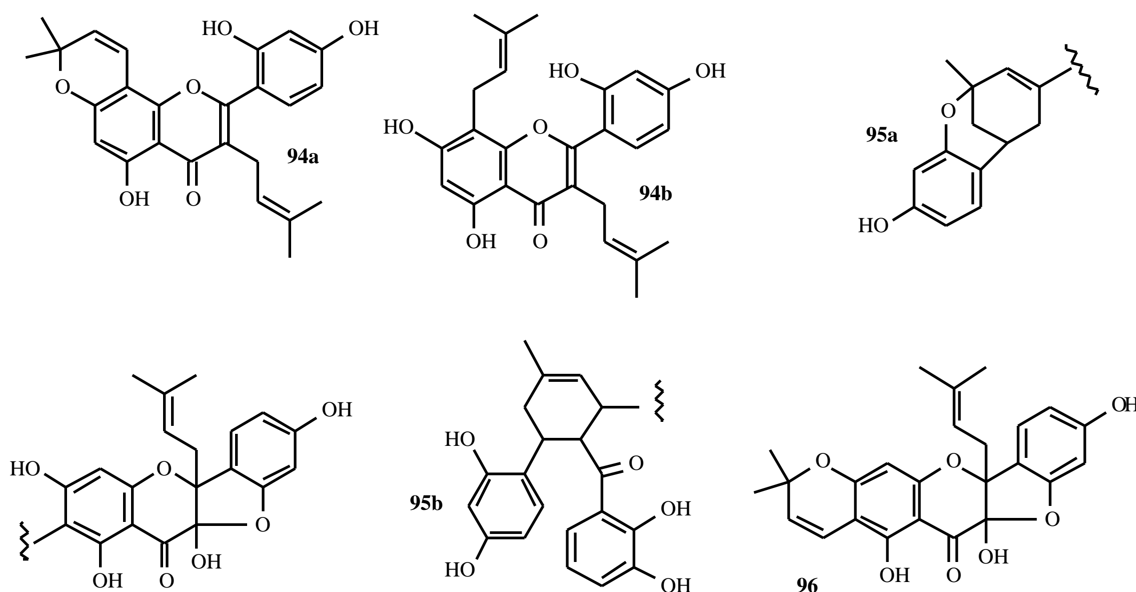
Preliminary screening with fifteen test bacterial strains showed that YS06 was the most active agent among two flavanones (YS01–YS02) and eight prenylflavanones (YS03–YS10) [91] YS06 exhibited highly significant antimicrobial action when tested against 228 bacterial strains comprising two Gram-pos and six Gram-neg genera. Twenty-two of fifty strains of *Staphylococcus aureus* were inhibited at 25 to 50 μg/mL of the agent. YS06 also inhibited strains of *Salmonella*, *Shigella*; a few strains of *Escherichia co*li were also highly sensitive to YS06, while *Klebsiella* species and *Pseudomona aeruginosa* were much less sensitive. In *in vivo* studies, YS06 offered significant protection to Swiss albino mice challenger with 50 min. LD (MLD, virulent bacterium) at concentrations of 160 and 80 μg/mouse [91].

The effect of 19 naturally occurring prenylated flavonoids, isolated from medicinal plants, on cyclooxygenase (COX)-1 and COX-2 and on 5-lipoxygenase (LOX) and 12-LOX was investigated using [14C] arachidonic acid as a substrate [92]. Morusin (**94a**), kuwanon C (**94b**), sanggenon B (**95a**), sanggenon D (**95b**) and kazinol B (**96**) inhibited COX-2 activity (IC_{50} = 73-100 μM), but the potencies were far less than that of NS-398 (IC_{50} = 2.9 μM). In contrast, many prenylated flavonoids inhibited COX-1 activity. Among them, kuraridin (**43**), kuwanon C (**95**) and sophoraflavanone G (**77**), all having a C-8 lavandulyl side chain, showed potent activity (IC_{50} = 0.1 to 1 μM), comparable to that of indomethacin (IC_{50} = 0.7 μM). Most of the tested prenylated flavonoids inhibited 5-LOX activity with IC_{50} values ranging from 0.09 to 100 μM. Moreover, only papyriflavonol (**24**), sophoraflavanone G (**78**) and kuwanon C (**94b**) showed inhibitory activity against 12-LOX at low concentration ranges (IC_{50} = 19-69 M), comparable to that of NDGA (IC_{50} = 2.6 M).

These results once again suggest that the position and the nature of the prenyl substitution greatly influence *in vitro* biological activities of these molecules.

Prenyl flavonoids, such as morusin (**94a**) and kuwanon C (**94b**), were also found to inhibit NO production from lypopolysaccharide (LPS)-induced RAW 264.7 cells at 10μ . Inhibition of nitric oxide production was mediated by suppression of iNOs enzyme induction, but not by direct inhibition of iNOs enzyme activity. While most prenylated flavonoids showed cytotoxicity to RAW cells at 10-100 μM, in the same test biflavonoids were not cytotoxic [93]. Since NO produced by inducible iNOs plays an important role in inflammatory disorders, inhibition of NO production by these flavonoids may contribute, at least in part, to their anti-inflammatory and immunoregulating potential *in vivo*.

The anticariotic activity of the EtOAc soluble extract of *Sophora flavescens* has been investigated [94]. One of the fractions showed growth inhibition activity against *Streptococcus mutans* (MIC, 3.13 μg/mL). The glucosyltransferase activity of the active fraction inhibited the formation of glucan and showed 77% of the antiproliferative



effect at 100 μg/mL. Two flavanones were isolated from the active fraction and assigned the structures (-)-kuraninone (**46b**) and (2S)-2´-methoxy kurarinone (**46a**).

On the other hand, the methanolic extract of *Sophora flavescens*, when administered perorally (p.o.) at a dose of 50 - 200 mg/Kg, significantly inhibited a serotonin (5-HT)-induced itch-related response (scratching) in a dose-dependent manner, without any effect on the locomotory activity [95]. The extract also widely affected the spontaneous scratching of a mouse model of atopic dermatitis. *Sophora flavescens* and its constituents were thus proposed as new antipruritic agents

Prenylated flavonoids containing the resorcinol moiety were isolated as tyrosinase inhibitors from the roots of *Sophora flavescens* by activity-guided fractionation. Among the 12 compounds isolated, kuraridin (**43**) and kurarinone (**46b**) showed stronger inhibitory potencies (IC50 = 1.1, 1.3 μM, respectively) than that of kojic acid (IC50 = 11.3 μM), a well known tyrosinase inhibitor [96]. Substitution of a lavandulyl or hydroxylavandulyl group at the C-8 position and a methoxy or hydroxy group at the C-5 position are essential for the inhibitory effect.

The inhibitory effect on mushroom tyrosinase of ethanol extract and dichloromethane fraction from *Sophora flavescens* was evaluated in parallel by another group [97]. As a result, kuraridin (**43**), kurarinone (**46b**) and sophoraflavanone G (**77**) were isolated from the dichloromethane fraction and shown to possess more potent inhibitory activity (IC_{50} = 0.6, 6.2 and 6.6 μM, respectively) than kojic acid (IC_{50} = 20.5 μM).

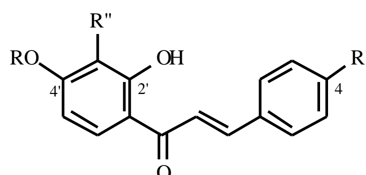
3.4. Structure-Activity Relationship Studies

A first analysis of a structure-activity relationship (SAR) was reported by Barron and Ibrahim [4], who noticed that

Table 2. Binding Affinity for Type II EBS and Ability to Inhibit Cell Proliferation in OVCA 433 cells [100].

Chalcone*	IC_{50} (μM)	Di_{50} (μM)
97a	2.5	0.62
97b	5.0	3.2
97c	1.2	1.2
97d	-	9.0
97e	17.0	-
97f	17.0	10.6
97g	5.0	12.0
97h	4.2	18.0
97i	-	-
97l	6.0	4.2
97m	-	-
97n	-	-

*Chalcones **97b**, **97d**, and **97f** have been previously reported with the numbers **80**, **22a**, and **79a**, respectively.



	R	R'	R"	Other
97a	H	H	H	-
97b	H	OH	H	-
97c	H	H	Pr	-
97d	H	OH	Pr	-
97e	CH_3	H	Pr	-
97f	CH_3	OH	Pr	-
97g	CH_3	OH	Pr	3-OH
97h	CH_3	OH	Pr	2-OH
97i	CH_3	OCH_3	Pr	-
97l	Pr	H	H	-
97m	Pr	OH	H	-
97n	Pr	H	H	, -dihydro

although no particular pattern for SAR could be established, it was generally agreed that at least one phenolic hydroxyl group and a certain degree of lipophilicity were required for the antifungal/antimicrobial activity of flavonoid compounds. The biocidal effect of flavonoids, however, is not specifically dependent on the substitution pattern as much as on the nature of the flavonoid in question; e.g. isoxanthoumol (**85a**) is 60 times less active than the corresponding chalcone (xanthoumol, **85**) [98].

Investigation of 150 natural and synthetic polyphenols, including flavonoids, for cytotoxic activity against normal, tumor and human immunodeficiency virus (HIV)-infected cells revealed higher cytotoxic activity against human oral squamous cell carcinoma HSC-2 and salivary gland tumor HSG cell lines than against normal human gingival fibroblasts HGF. Many of the active compounds had a hydrophilic group (hydroxyl groups) in the vicinity of a hydrophobic group (prenyl, phenyl, methylcyclohexene, etc.), e.g. isoprenoid substituted flavones [99].

Substitution of the hydrophobic group (prenyl or geranyl group) did not significantly change the cytotoxic activity of chalcones, flavanones or other non-flavonoid compounds. However, the prenylation of an isoflavone and a 2-arylbenzouran significantly enhanced the cyotoxic activity. Most of the polyphenols failed to reduce the cytopathic effect of HIV infection in MT-4 cells [99].

The effect of 12 different chalcones (mostly prenylated) on both established and primary ovarian cancer cells expressing type II oestrogen binding site (type II EBS) and the binding affinity of substrates for type II EBS, has been tested in a structure-activity study [100]. At concentrations from 0.1 to 10 μM, chalcones inhibited ovarian cancer proliferation and [3H] oestradiol ([3H]E2) binding to type II EBS. In Table **2**, IC_{50} (concentration corresponding to a 50% inhibition of the growth) and Di_{50} (concentration giving a 50% displacement of [3H]E2 bound to type II EBS) for the tested chalcones (**97a** – **97n**) are reported.

Although no definite structure-activity relationship could be determined, some conclusions on the structural changes that may influence both antiproliferative and binding activity were drawn.

- Isocordoin (**97c**) and 2',4'-dihydroxychalcone (**97a**) are the most active compounds to inhibit cell proliferation.
- The 4'-hydroxyl (A ring) seems to be critical to activity, which is decreased by methylation (**97c** to **97e**) or O-prenylation (**97c** to **97l**).
- A 4-hydroxy substituent (B ring), as in **97b** and **97d** decreases the activity of **97a** and **97c**, respectively, whereas it seems not to influence the activity of derricin (**97e** to **97f**). Methylation of 4-hydroxyl group, however, results in a complete loss of activity for **97f** (to **97i**).
- 3-Hydroxy and 2-hydroxy substituents (B ring) increase (2-OH > 3-OH) the activity of derricin (**97e** to **97g** or **97h**).
- Saturation of the - double bond decreases the activity of the chalcone.
- C-prenylation seems to have a negative effect going from **97b** to **97d**, but the opposite effect is observed going from **97a** to **97c**.

The data of Table **2** suggested that chalcones may regulate cell growth through a binding interaction with type II EBS.

Experiments on primary tumors with isocordoin (**97c**) and cordoin (**97l**), which had been proved to have the highest and the lowest detectable activity in a 0.1 – 10 μM range of concentrations, demonstrated that the antiproliferative effect of chalcones was not limited to established cancer cell line. Infact, both the compounds inhibited colony formation of cells derived from primary ovarian tumours. In four out of five cases the IC_{50} of isocordoin was lower than that of cordoin.

The important role of the chalcone skeleton in preventing DNA fragmentation on H-60 cells was also pointed out, when the relationship between apoptosis –inducing and –preventing activities of xanthohumol and other bioactive flavonoids (chalcone, phloretin, genistein, quercetin, naringenin and baicalein) was studied [101].

On the other hand, the study on the inhibition by the hop chalcones of lipid peroxidation in various *in vitro* systems revealed the importance of one prenyl group in the antioxidant activity, which was, in contrast, decreased by an increase of prenyl substituents [102].

The importance of substitution pattern and stereochemistry was revealed in a study on the fungitoxicity of several prenylated flavanones, by the TLC plate bioassay method [103]: 8- and 3'-prenylated naringenins (**78a** and **98a**, respectively) were shown to be strongly antifungal, whereas 6-prenyl-naringenin (**87a**) was inactive [104]; diprenylated naringenins, i.e. lonchocarpol A (6,8-diprenyl, **87b**) and euchrestaflavone (6,3'-diprenyl, **98b**) had very weak antifungal activity; flavanones with cyclic (furano, dihydrofurano) or acyclic structures like 2-hydroxy-3-methyl-3-butenyl were either inactive or weakly antifungal; lonchocarpol D1 (**99a**) was strongly antifungal, whereas the diastereoisomeric lonchocarpol D2 (**99b**) was not.

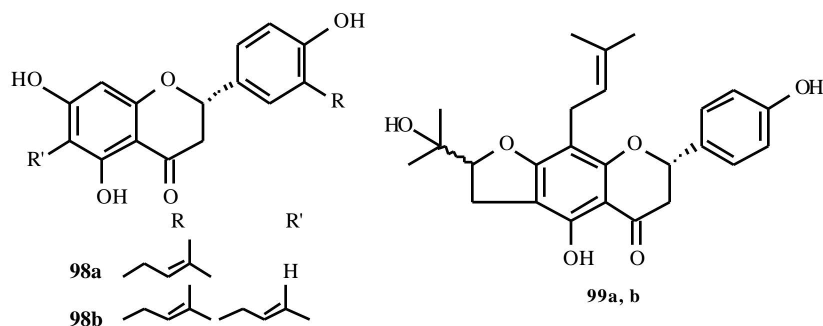
In a XTT-based whole cell screen, only 5,7-dihydroxy-6,8-diprenylflavonoids (**67**, **68**, and **87b**), isolated from *Monotes africanus* [34], exhibited HIV-inhibitory activity.

In order to evaluate a possible SAR between substitution at position 8 of the flavone nucleus, 4 known 8-prenylated flavones isolated from *Epimedium perralchicum* were screened for their anti-tumor activity against sarcoma 180 in mice, as compared with the flavone acetic acid (FAA), carrying an acetic acid group at C-8. The natural compounds had no effect in the tumor model. The COOH group at position 8 of FAA, rather than the prenyl group, seems to be essential for the antitumor/immuno modulatory activity [105].

4. PRENYLATED FLAVONOIDS IN BIOTECHNOLOGY

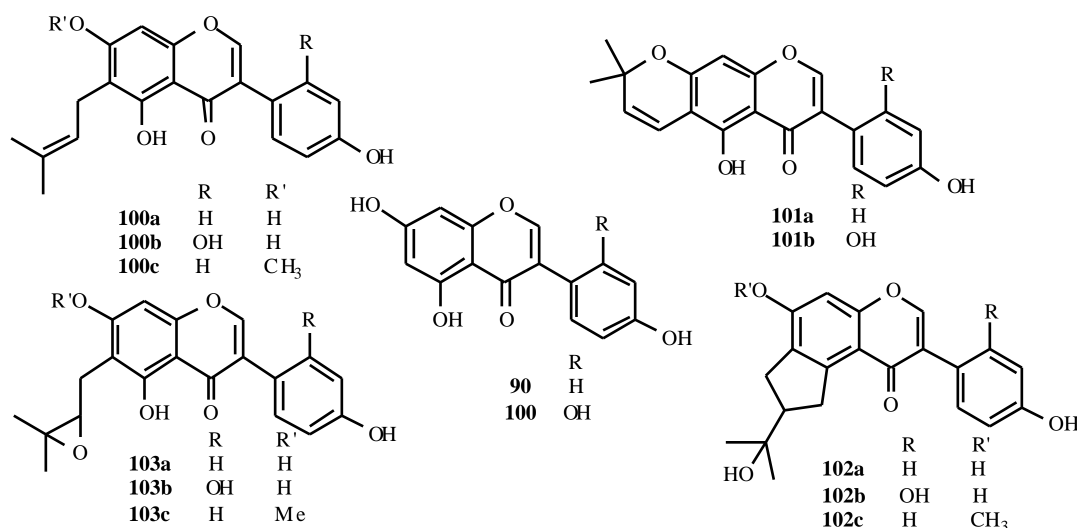
4.1. Biotransformations of the Side Chain

Modifications of the , -dimethylallyl chain have been investigated in biogenetic studies, but few biotransformation



reactions concerning the prenyl group have appeared in the literature so far.

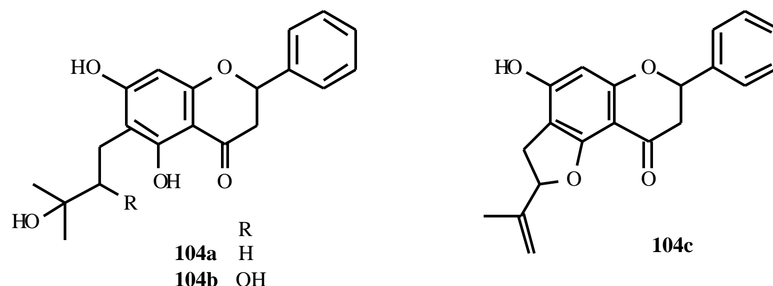
Fungitoxic prenylated isoflavones, wighteone (100a) and luteone (100b), derived from genistein (90) and 2'-hydroxygenistein (100), respectively, were metabolised into the corresponding dihydropyrano (101a-b) and dihydrofurano (102a-b) derivatives, co-occurring in the roots of white lupin, by a cell-free extract of *Botrytis cynerea* [106]. The latter to those dependent on FAD. The enzyme activity was markedly enhanced by pretreating the mycelia with the flavonoid 6-prenylnaringenin (87a) and was dependent on NADPH and O_2 [108]. The resulting epoxide 103c was slowly converted in the corresponding dihydrofuran derivative 102c in a buffer solution at pH 7.5. Preparations of cell-free extract of *Botrytis cynerea* without preincubation of 6-prenyl naringenin in the mycelia, showed, even in the presence of the flavanone a minimum epoxidizing activity,

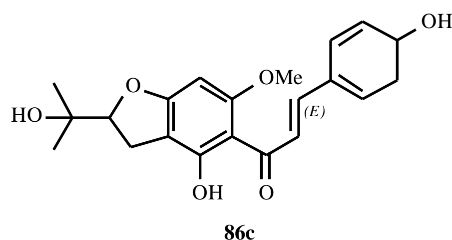


compounds are presumably formed *via* epoxidation of the prenyl double bond, as in 103a-b. An epoxide intermediate has long been postulated also for the reaction of ortho-prenylated phenols to cyclic ether products [107].

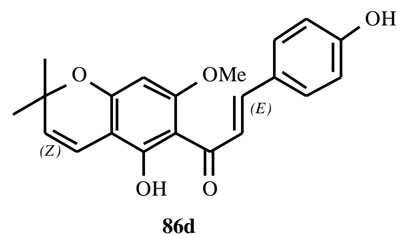
Actually, 7-O-methylluteone (100c) in the cell-free system of *Botrytis cynerea* yielded an epoxy intermediate, the transformation being initially catalysed by a microsomal mono oxygenase; the latter one apparently does not belong to the monooxygenases dependent on cytchrome P450, but revealing that the substrate analog 87a has a role as an enzyme inducer rather than stabilizer [108b].

When incubated in liquid culture of *Aspergillus flavus*, 6-prenylnaringenin (87a) was converted, slowly during isolation by TLC and rapidly by treatment with a base, to derivatives containing 2,3-dihydrodihydroxyprenyl- (104a), prenyl hydrate- (104b) and dihydrofurano-substituents (104c). The last compound was the major product in cultures of *Botrytis cynerea* [109].

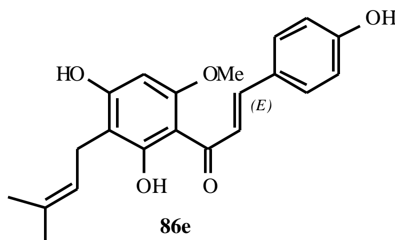




86c

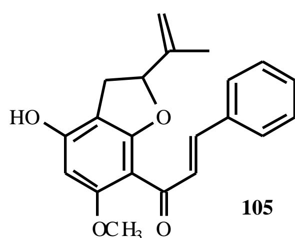


86d

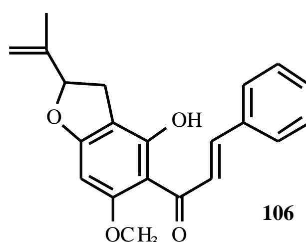


86e

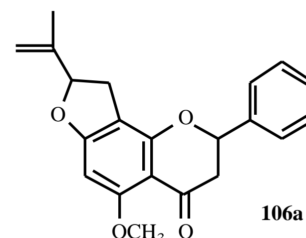
A second biotransformation of the prenyl chain involved the chalcone xanthohumol (86), which was treated with rat liver microsomes in an attempt to generate its mammalian metabolites by microbial models. Three major polar metabolites were produced by liver microsomes from either untreated rats or phenobarbital-pretreated rats and were tentatively identified as derivatives of 86 (86c-e), and a B-



105



106



106a

ring dihydroxylated xanthohumol. Treatment of the chalcone with liver microsomes from isosafrole- and -naphtoflavone-pretreated rats yielded a fourth major nonpolar metabolite, identified as dehydrocycloxanthohumol (89c) [110].

Conversely, the microbial biotransformation of xanthohumol obtained using the culture broth of *Pichia membranifaciens* yielded 2'-(2''-hydroxyisopropyl)-dihydrofurano[5',4':2',3']-4,4'-dihydroxy-6'-methoxychalcone (**105**), 2'-(2''-hydroxy isopropyl)-dihydrofurano [4',5':3',4']-2',4-dihydroxy-6'-methoxychalcone (**106**) and the corresponding flavanone (**106a**) [111].

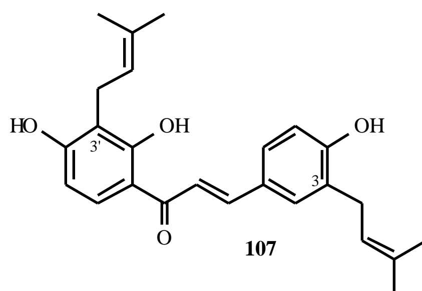
More recently, in *Morus alba* cell culture, the C-3 prenyl substituent of kanzonol C (**107**) was converted into a 3-methylbutadienyl chain (**107a**). Compound **107** is clearly an intemediate for the [4 +2] cycloaddition reaction leading to the chalcone adducts of *Morus alba* [112].

The most important biotransformation for prenyl flavonoids is the introduction of an isopentenyl substituent into the flavonoid skeleton, a reaction catalysed by a dimethylallyl transferase or prenyl transferase.

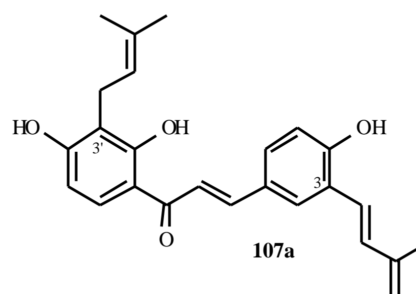
4.2. Prenyltransferase

The term prenyltransferase (PT) defines all those enzymes that catalyze the transfer of prenyl groups to a wide variety of acceptors (isoprenoid groups, aromatic compounds, proteins etc.). PTs are widely distributed in all the living kingdoms and participate in most of the metabolic routes leading to side chains of respiratory coenzymes [113], carotenoids, terpens and polymers such as rubbers [114].

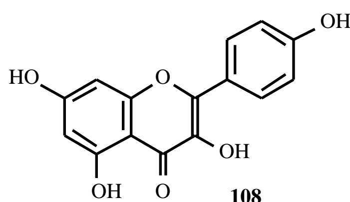
Prenyl transferases have gained more recognition after the renewed interest in isoprenoid chemistry, boosted almost simultaneously from various directions. Among these are: the discovery of a new terpenoid pathway [115,116], the

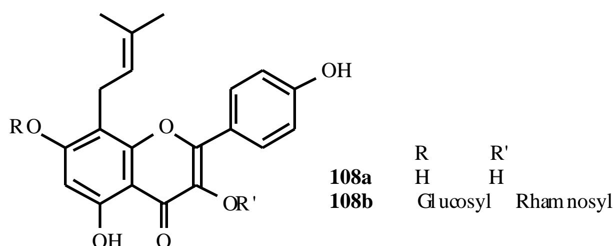


107



107a





discovery of new methods in molecular biology and X-ray chrystallography allowing the study of enzymes like squalene synthase in detail [117]; and the recognition of the importance of prenylation for the regulation and targeting of bioactive compounds in the cell, e.g. the farnesylation of proteins in signal transduction cascades involved in carcinogenesis [118].

PTs are peculiar enzymes because they not only create a new C-C bond, a reaction which only some aldolases and lyases have been previously used for [119], but also introduce a double bond in the framework of the final product, a feature which is often associated with the activation or the enhancement of biological properties [48,50,53,54]. Moreover, the chemical synthesis of prenylated aromatic compounds is quite difficult to achieve in good yield and with the regio or stereo specificity essential for biological activity and usually requires the use of protective groups. Therefore, the possibility of manipulating such an enzymatic catalyst represents an interesting tool for the organic synthesis of biologically active compounds.

4.2.1. Aromatic Prenyl Transferases

Phenol-oligoprenyldiphosphate is the prenyl transferase that allow the prenylation of aromatic compounds, mostly hydroxybenzoic acids and hydroxyphenylketones in a Friedel-Crafts-like reaction [120].

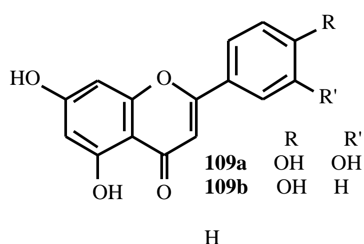
The prenylated aromatic products comprehend the ubiquinones (coenzymes Q), which play a crucial role in redox processes as a part of the respiratory chain [121], the bitter acids of *Humulus lupulus* [122], the precursors of vitamin E (tocopherols), of the Japanese drug and dye shikonin [123], of various alkaloids and of many fungal metabolites.

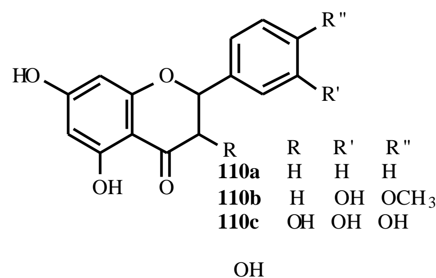
The enzymatic prenylation of a variety of phenolic compounds includes also the 6-prenylation of umbelliferone in *Ruta graveolens* [124] and the C-6 and O-7 prenylation of the same coumarin by two distinct enzymes in *Ammi majus* [125]. However, the studies on conjugated flavonoid compounds seem to be limited to pterocarpans [126] and to isoflavones. For instance, Griseback *et al.* have reported the presence of an isoflavone dimethylallyl transferase in a particulate (4800*g*) fraction of lupine (*Lupinus albus*) hypocotyls [127]. The enzyme catalysed the prenylation of genistein (90) and 2'-hydroxygenistein (90a) with dimethylallyl pyrophosphate to the antifungal wighteone (6-prenylgenistein, 100a) and luteone (2'-hydroxy-6-prenylgenistein, 100b). Successively, microsomial preparations of white lupin radicles and cell suspension cultures were found to catalyse the prenylation at positions 6, 8 and 3' of the two isoflavones 90 and 90a [128]. The enzymatic prenylation required dimethyl allyl pyrophosphate (DMAPP) and 12 mM Mn^{2+} with optimum activity at pH 7.5 in Tris-HCl buffer. Isopentenyl pyrophosphate was a competitive inhibitor of the prenylation reaction. The bulk of enzymatic activity was associated with the membrane fraction and could only be solubilized in the presence of a detergent. The differences observed in PT activity ratios in relation to the source of the enzyme and the type of detergent used suggested that prenylation at positions 6, 8 and 3' of isoflavones is catalysed by a number of distinct enzymes.

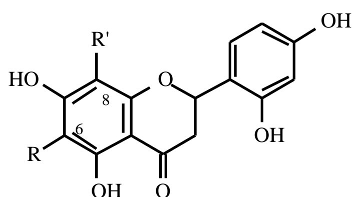
4.2.2. Flavonoid Prenyltransferase

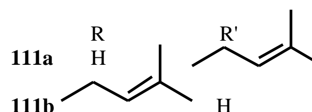
The prenylation of flavonoids was first studied on cell suspension cultures of *Epimedium diphyllum*, which produced a large amount of des-O-methylanhydro icaritin glycosides, such as epidemoside A (108b) [129].

To clarify the order of prenylation and glycosilation, crude cell-free extract of E. diphyllum cell cultures was incubated with 1mM kaempferol (5,7,4'-trihydroxy flavonol, 108), 2mM dimethylallyl diphosphate (DMAPP) and 10 mM $MgCl_2$ for 1 h at 25 °C. The analysis by a reverse-phase HPLC-photodiode array system revealed the presence of a new peak, corresponding (retention time, UV absorption) to des-O-methyl anhydroicaritin (108a). Scaling-up of the reaction gave again the 8-prenylated kaempferol and confirmed the presence of dimethylallyl diphosphate: kaempferol dimethylallyltransferase [130]. The reaction was dependent on the presence of intact enzyme and optimal concentrations of kaempferol (1mM), DMAPP (2-5 mM) and Mg^{2+} (10 mM). The apparent K_m values for DMAPP and kaempferol, 0.58 and 0.13 mM, respectively, were calculated from the Lineweaver-Burk plot with varying concentrations.









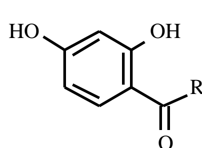
The enzyme activity reached its maximum at pH 7.5 and was nearly consistent up to pH 10 (Tris-HCl buffer), but in strong alkaline conditions 108 and 108a were unstable. Mg^{2+} was the most effective metal ion for the activity. Mn^{2+} (59% of Mg^{2+}) and to a lesser extent Zn^{2+} (35%) were also effective in the reaction. Ca^{2+} (5%) and Cu^{2+} (2%) gave only minor activity. In the fractionation of the crude cell-free extract by ultracentrifugation *ca* 70% of the total prenylation activity was found in the twice washed 156000*g* pellets, indicating that the prenyltransferase was tightly bound to the membrane fraction of the cells. Incubation with isopentenyl diphosphate (IPP) yielded 108a, 30% of that obtained with DMAPP, whereas no prenylayed products were obtained with lavandulyl diphosphate (LPP).

Several flavonoids were examined as putative prenyl acceptors (at 1 mM concentration) with microsomal enzyme preparation. The enzyme was also active with quercetin (5,7,3',4'-tetrahydroxyflavonol, 91), luteolin (5,7,3',4'-tetrahydroxy flavone, 109a), and apigenin (5,7,4'-trihydroxyflavone, 109b), with activities 39, 34 and 60%, respectively, of that obtained with kaempferol. The position of the prenylation (examined by EI-MS spectroscopy) was not determined. Naringenin (5,7,4'-trihydroxyflavanone, 78) and genistein (5,7,4'-trihydroxyisoflavone, 90) as well as kaempferol glycosides were not prenylated.

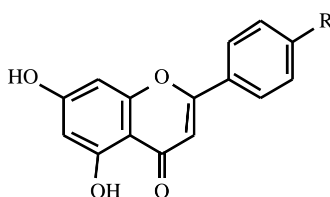
On consideration that neither quercetin nor apigenin prenyl derivatives have been isolated from the intact plant and from cell cultures of *Epimedium diphyllum*, the occurrence of different prenyltransferase in the microsomal preparation appeared unlikely. The prenylation reaction, in conclusion, was shown to precede glycosylation.

4.2.3. Prenyltransferase from Sophora Flavescens Cell Cultures

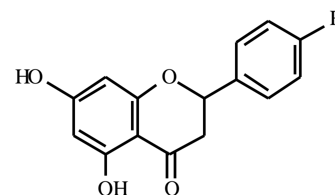
The crude cell free extract of *Sophora flavescens* cultured cells, which had been shown to produce prenylated flavanones such as sophoraflavanone G (77) and lehmanin (77a) [83], was incubated with naringenin (1 mM, 78), DAMPP (2mM), and Mg^{2+} for 2h at 25 °C. HPLC analysis revealed the presence of 8-(3,3-dimethylally)-naringenin (78b). A microsomal preparation obtained by ultracentrifugation, consisting of a twice-washed 100000*g* pellets (85% of the total prenylation activity) incubated with commercial optically inactive naringenin (78), DMAPP and Mg^{2+}, gave optically active (2*S*)-78b. This finding demonstrated the presence in *S. flavescens* cultured cells of a stereospecific naringenin dimethylallyl transferase enzyme [131]. The optimum pH of the enzymatic reaction was 9-10 in Trs-HCl buffer, but experiments were preferentially carried out at pH 9, because in alkaline solution naringenin was liable to be converted into the corresponding chalcone. Mn^{2+} was less effective (32%) than Mg^{2+}, while additon of Ca^{2+} (6%), Co^{2+} (5%), Zn^{2+} (2%) or Cu^{2+} (1%) gave only very low activities. DMAPP was shown, in comparison with IPP and LPP, to be the sole prenyl donor. Saturation for Mg^{2+} was reached at 10 mM, while maximal conversion rates were observed at 10 mM of DMAPP and 1 mM of naringenin, with apparent K_m values 120 and 36 μM, respectively, from the Lineweaver-Burk plot.



	R
119a	H
119b	Me



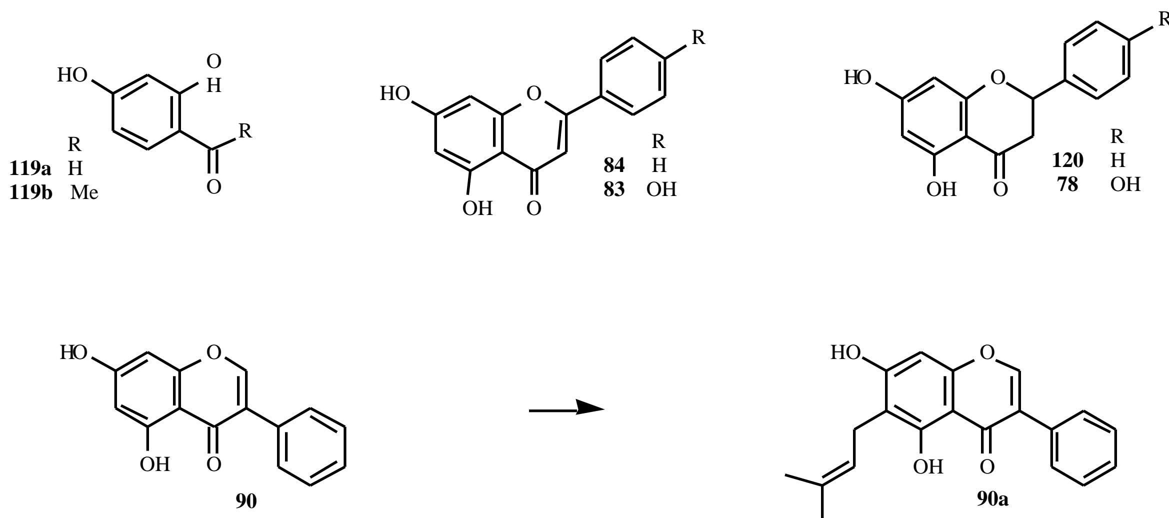
	R
84	H
83	OH



	R
120	H
78	OH

Table 3. Specificity Tests with Chalcones

Precursor	**97a**	**97b**	**112**	**113**	**114**	**115**	**116**	**117**	**118**
Product	**97c**	**97d**	**112a**	**-**	**-**	**-**	**116b**	**117b**	**118b**
Yields (%)									
Whole cells	80	70	60	0	0	0	25	20	40
Microsomes	85	80	75	0	0	0	0	0	0



The prenylation activities of the twice-washed microsomial fraction were less effective, as compared with naringenin, for liquiritigenin (5,7-dihydroxyflavanone, 110a), hesperitin (5,7,3'-hydroxy-4'-methoxyflavanone, 110ab) and taxifolin (3,5,7,3'4'-pentahydroxyflavanone, 110c), although the transfer of the dimethylallyl group to the C-8 position was in any case catalysed.

Notably, when 2'-hydroxynaringenin (78b) was used as a prenyl acceptor, besides the 8-dimethylallylderivative (111a), the 6-dimethylallyl derivative (111b) and sophoraflavanone G (the 8-lavandulyl derivative, 77) were formed. These results suggest that the 2'-hydroxy group plays an important role for the formation of the lavandulyl group.

4.2.4. Prenyltransferase from Morus Nigra Cell Cultures

Cell cultures of *M. nigra* had been shown to convert in very good yields 2´,4´-dihydroxy-chalcone (**97a**) in the anticancer derivative isocordoin (**97c**) [132].

Further studies on the catalytic properties of the PT enzyme system, were performed both with whole cells in feeding experiments and with a preparation obtained by ultra centrifugation of the crude extract and corresponding to the microsomial fraction. In a first set of experiments, nine diversely substituted chalcones were used as substrates. The results, summarized in Table **3**, revealed that prenylation had occurred only with chalcones (**97a, 97b**, **112**) bearing two hydroxyl groups (C-2´, C-4´) on ring A.

Among them, the position of the substituents in ring B appeared to be critical for the prenylation. A hydroxyl group in C-4 (**97c, 112**) did not modify the fate of the reaction as for unsubstituted chalcone **97**. By contrast, the presence of a hydroxyl group in C-2 (**113**) or C-3 (**114**) inhibited the reaction.

Notably, when 4´-hydroxychalcone (**116**) and 2´-hydroxychalcone (**117**) were inoculated in the cells, they did not undergo prenylation, but gave the corresponding dihydroderivatives **116a** and **117a**, with 25 and 20% yields, respectively (Scheme 1); similarly 2´,4´-dimethoxychalcone **118** gave the dihydrochalcone **118a** in fair yield (40%). When microsomes were employed as enzyme source, no product was detected.

To explore whether the whole chalcone structure was necessary to the enzyme recognition, simpler compounds than **97a** with an identical substitution pattern in the ring A, i.e. 2´,4´-dihydroxy-benzaldeyde (**119a**) and 2´,4´-dihydroxy-acetophenone (**119b**), were used as substrates both *in vivo* (feeding) and *in vitro* (microsomes) trials. Since no reaction occurred with either **119a** or **119b**, the importance of the B ring of chalcones for the interaction with the active site of the prenyltransferase with possible stabilization of the enzyme-substrate complex was confirmed.

In order to verify whether the PT from *Morus nigra* cell cultures was able to biotransform flavonoids other than chalcones, the cells were fed with flavanones **78** and **120** and flavones **83-84**. No prenylated compound was isolated in any case, the starting flavonoids being completely recoveredfrom the reaction mixtures. The same results were obtained when the same four compounds were used for reactions with microsomial preparations.

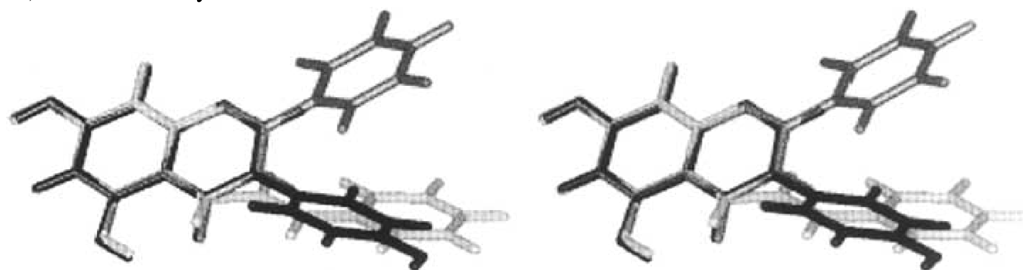


Fig. (1). Stereo view of the superimposition between genistein (**90**, black), 2',4'-dihydroxychalcone (**97a**, light grey) and 5,7-dihydroxyflavone (**84a,** dark grey).

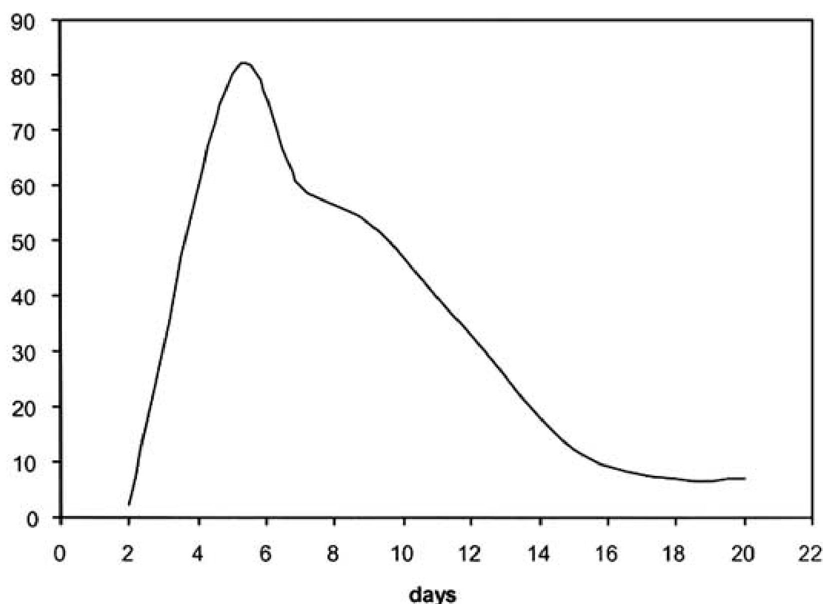


Fig. (2). PT activity versus cell growth age. The relative activities were calculated toward 2',4'-dihydroxychalcone (**97a**), during all the growth curve.

By contrast, genistein (5,7-dihydroxy-isoflavone, **90**) gave the corresponding 6-prenyl derivative **90a** (Scheme 3) both in feeding experiments (30% yield) and with microsomes (40% yield). These results must be emphasized, because they show that the PT enzyme system is able to recognize compounds exogenous for *Morus nigra* both *in vivo* and *in vitro*.

4.2.5. Computational Studies

In order to shed light on the findings described so far, genistein (**90**) and 5,7-dihydroxyflavone (**84**) were chosen as representatives of the class of biologically active 2´,4´-dihydroxy chalcones were investigated by a 3D-comparative study. Alculations and graphic manipulations were performed on Iris 4D/35 and Indigo R4000 Silicon Graphics workstations using the software packages InsightII and Discover by MSI (Molecular Simulations Inc, 9685 Scranton Road, San Diego, CA 92121, USA).

The three lowest energy gas-phase conformers obtained by a conformational analysis performed in a 3.0 kcal/mol energetic window (Search/Compare module) [133] were superimposed, as depicted in Fig. (**1**).

The aromatic ring A of **97a** was fitted atom by atom with the ring A of genistein (**90**), so that the positions 2´ and 4´ of the former were coincident with positions 5 and 7 of the latter, while the remaining moiety was free to be oriented in the 3D-space.

A very good structural similarity was observed between compounds **97a** and **90** (Fig. **1**). Genistein appeared to possess a rigid structure in which ring B was rotated by about 49° with respect to the condensed bicyclic system due to a steric repulsion between the carbonyl oxygen atom and the 3-D related hydrogen (H-2´ or H-6´). Analogously, the extended system of   electrons of 2´,4´-dihydroxy chalcone forced the molecule to adopt a fairly planar conformation in which ring B was rotated of around 46° with respect to ring A as a result of three rotations around the single bonds connecting ring B to ring A. The main rotation of the three was the one involving the bond connecting ring B to the carbonyl group (36°).

4.2.6. Enzyme Localization and Partial Characterization

A suspension culture was grown from calli of *Morus nigra* [41] using a MS62 medium enriched with sucrose

Table 4. Effects of Different Detergents in Solubilizing PT Activity

		Activity	
Solution	**Concentration**	***Pellet***	**Supernatant**
Triton X-100	0.2%	94%	6%
CHAPS	0.2%	95%	5%
Digitonin	0.2%	85%	15%
Octyl- -D-glucopyranoside	0.1%	57%	43%
NaCl	0.5 and 1 M	98%	n. d.

Only the higher activities for each agent used are listed. The percentage of activity was calculated as rate of bioconversion of compound 97a.

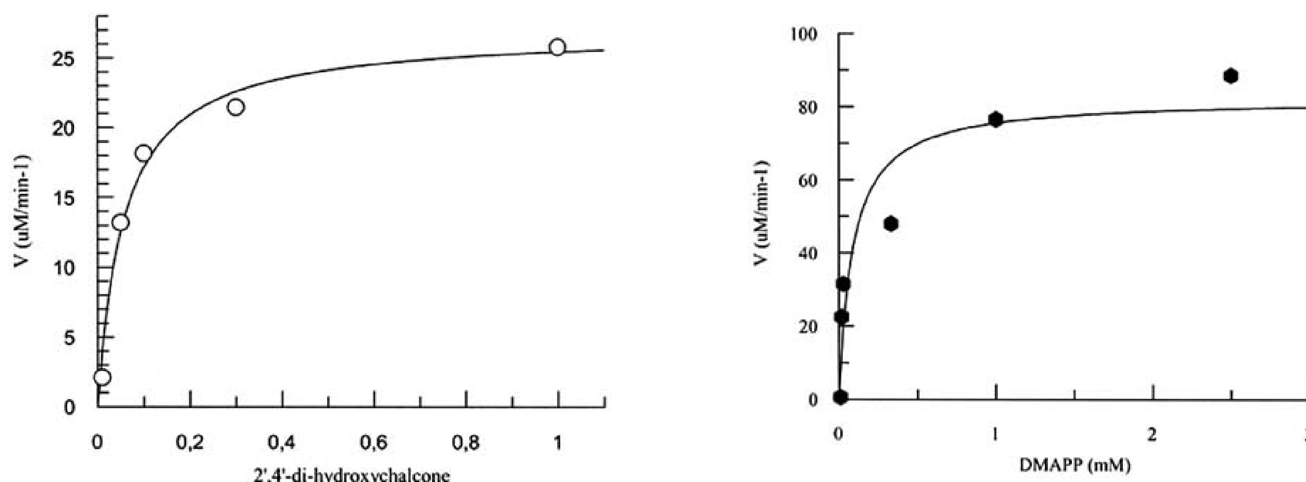


Fig. (3). Dependency of PT activity on the concentration of chalcone (**97a**) (left graph) and DMAPP (right graph). The apparent Kms were calculated by a Lineweaver-Burk plot with different concentrations (0.015 to 2.5 mM) of DMAPP and (0.01 to 1 mM) chalcone (**97a**).

Microsomial fractions were employed for both the experiments.

(3%) and a triade of hormones: 2,4-D (0.2 ppm), NAA (0.1 ppm), and kinetin (0.75 ppm). The medium was selected after trials with different media, as the one showing the maximum enzymatic activity, the latter being expressed in the first 10 days of growth (Fig. **2**).

Since many , -dimethylallyl-transferases have been reported [128,130,131,135] to be tightly associated with the microsomial fraction, a series of experiments were performed with the pellets obtained after ultra centrifugation of crude extract.

Attempts to solubilize the PT with detergents as Triton-X100, CHAPS and with strong ionic solutions (NaCl 1M), resulted in a very low recovery of the enzymatic activity (Table **4**); these findings could be explained either by a strong interaction with cell membranes or by a loss of activity after the detergent treatment; this aspect needs further investigation in view of the enzyme purification.

As for other PTs [128, 130], the reactions performed with the microsomes required the presence of a divalent cation. In our case Mg^{2+} at a concentration of 20 mM gave remarkable results in term of yields of **97c**. In a set of experiments, performed with other cations such as Mn^{2+}, Ca^{2+}, Co^{2+}, Zn^{2+} and Ni^{2+} at the fixed concentration of 20 mM, minor activity values, as compared with Mg^{2+} (100%), were obtained for Mn^{2+}(80%), Co^{2+} (28%), Ca^{2+} (11%), Ni^{2+} (11%), and Zn^{2+}(0%).

In all the successful reactions isocordoin (**97c**) was the only product to be obtained, suggesting that the different cations modify the reaction rate but not the enzyme specificity. The optimum pH was found to be ca. 7.5 in a Tris-HCl buffer, even if at pH 9 activity is still retained. The enzyme activity was well maintained even though the microsomial fractions were kept at –20 °C for several weeks.

The apparent kinetic parameters of the enzyme were obtained via HPLC measurements, with microsomial fractions by calculation of a Lineweaver-Burk plot. As a result, the apparent K_m values of 63 and 142 µ were obtained for , -dimethylallyl-diphosphate and 2',4'-dihydroxychalcone, respectively (Fig. **3**).

4.2.7. Conclusions

Cumulatively, the metabolites pattern produced from both plant and cell cultures of *Morus nigra* [41], the experimental results of the biotransformations and the molecular modeling studies suggest that only one kind of prenyltransferase is present in *Morus nigra* cells and not a series of PT enzymes, as reported elsewhere [128]. For this peculiar enzyme the name of chalcone-prenyltransferase was proposed.

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