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Naturally-occurring Dimers of Flavonoids as Anticarcinogens

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Abstract: Biflavonoids are dimers of flavonoid moieties linked by a C-C or C-O-C bond. Simple, complex, rearranged, natural and ketalized Diels-Alder adducts, benzofuran derivatives, and spirobiflavonoids are some of the structural groups of biflavonoids. These compounds are mainly distributed in the Gymnosperms, Angiosperms (monocots and dicots), ferns (Pteridophyta), and mosses (Bryophyta). Biflavonoids have shown a variety of biological activities, including anticancer, antibacterial, antifungal, antiviral, anti-inflammatory, analgesic, antioxidant, vasorelaxant, anticlotting, among others. This work is focused on probably the most potentially relevant biological activity of biflavonoids, the anticancer activity and the involved mechanisms of action, such as induction of apoptosis [inhibition of cyclic nucleotide phosphodiesterases; effects on NF-κB family of transcription factors; activation of caspase(s); inhibition effects on bcl-2 expression, and upregulation of p53 and caspase-3 gene expression]; inhibition of angiogenesis [anti-proliferative effects; activation of Rho-GTPases and ERK signaling pathways; inhibition of FASN activity]; inhibition of pre-mRNA splicing; inhibition of human DNA topoisomerases I and II-α; anti-inflammatory/ immunoregulatory effects [inhibition of XO; inhibition of proinflammatory enzymes, such as PLA_2 and COX; effects on cytokines mediated COX-2 and iNOS expression]; modulation of immune response; inhibition of protein tyrosine phosphorylation; antioxidant and analgesic activities in relation to the anticarcinogen behavior. For that reason the structures and anticarcinogenic activities of 83 biflavonoids are thoroughly discussed. The results of this work indicate that biflavonoids strongly affect the cancer cells with little effect on normal cell proliferation, suggesting a therapeutic potential against cancer.

Keywords: Anticarcinogens, biflavonoids, structures, mechanisms of action.

INTRODUCTION

Biflavonoids are normally found in Gymnosperms, in a minor extent in Angiosperms (monocots and dicots), and also in ferns (Pteridophyta) and mosses (Bryophyta) [1]. These compounds are homo- and heterodimers of flavonoids linked with a C–C or a C–O–C bond. Biflavonoids are bioactive components found in fruits, vegetables, herbs, roots and leaves, and have been recognized as cancer chemopreventive agents. A variety of biological activities of biflavonoids have been reported, such as anti-inflammatory, antioxidant, antibacterial [2], antiviral [1, 2-8], vasorelaxant [9-12], anticlotting [13, 14], neuroprotective [15, 16], osteoprotective [17], anti-Alzheimer [inhibitors of β-secretase (BACE-1)] [18, 19], hypocholesterolemic [20], and other activities [1]. These compounds, in particular biflavones, have also effects on cancer cells, showing cytotoxic/anticancer activity [21-30], as well as antiviral, antibacterial, antifungal, and antimycobacterial activities [2] similar to flavonoid monomers, as shown in our previous work on the anticarcinogenic and other activities of flavonoids as natural monomers [2, 31]. As the flavonoids, naturally occurring dimers (biflavonoids) have key functions in the regulation of multiple cellular processes, but also regulate apoptosis and neuronal differentiation in pluripotent stem cells [32].

Bioassay-directed fractionation of the ethanolic extract of *Selaginella moellendorffii* has led to the isolation of the biflavone ginkgetin (**1**) (Fig. (**1**)), which showed cytotoxic activity to human ovarian adenocarcinoma (OVCAR)-3 cells, but not to other cells such as HepG2 and HeLa [33]. Nonbioactive fractions yielded four additional biflavones, amentoflavone (**2**), podocarpusflavone A (**3**), kayaflavone (**4**) and I-7,4', II-7,4'-tetra-*O*-methylamentoflavone (**5**) (Fig. (**1**)) [33]. Taiwanhomoflavone-A (**6**) (Fig. (**1**)) showed cytotoxicity against several cancer cell lines [34].

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II-4′-*O*-Methylamentoflavone or podocarpusflavone A (**3**), I-7, II-4′-di-*O*-methylamentoflavone (**7**), and I-7,4′, II-7-tri-*O*-methylamentoflavone (**8**) (Fig. (**1**)) had a moderate cytotoxic activity against human KB (human oral epithelium carcinoma), HeLa (human cervical carcinoma), Hepa (human hepatoma), DLD (colon carcinoma), and A-549 (human lung carcinoma) tumor cell lines [35]. Podocarpusflavone A (**3**) and I-7,II-4'-di-*O*-methylamentoflavone (**7**) (Fig. (**1**)) not only showed significant inhibitions against DLD, KB, MCF-7, but also exhibited activity against HEp-2 tumor cell lines (ED_{50} *ca.* 4.56-16.24 μg/mL), and induced cell apoptosis in MCF-7 *via* mainly sub-G1/S phase arrest. Furthermore, these compounds exhibited moderate Topoisomerase I inhibitory activity [36].

Amentoflavone (**2**) (Fig. (**1**)) is found in a number of plants with medicinal properties, such as *Ginkgo biloba* and *Hypericum perforatum* (St. John's wort). As it is known, biflavones of *H. perforatum* are used in the treatment of inflammation and depression. Recently, amentoflavone and other similar biflavones were quantified in human plasma by HPLC-ESI-MS. The method was effectively applied to pharmacokinetic studies [37].

Pan *et al*. [38] reported the structure-activity relationship (SAR) and binding mechanism of three biflavones, amentoflavone (**2**), II-4′-*O*-methylamentoflavone (**3**) and II-7,4′-di-*O*-methyl-amentoflavone (**9**) (Fig. (**1**)), isolated from *Taxodium mucronatum* as novel natural inhibitors of human cathepsin B with strong inhibitory activities at IC_{50} values of 1.75, 1.68 and 0.55 μM, respectively [38].

Hinokiflavone (**10**) (Fig. (**2**)) was isolated as the cytotoxic principle from the drupes of *Rhus succedanea* L. A comparison of the cytotoxicity of hinokiflavone (**10**) and other related biflavonoids, including amentoflavone (**2**) (Fig. (**1**)), robustaflavone (**11**) (Fig. (**3**)), agathisflavone (**12**) (Fig. (**4**)), rhusflavone (**13**) (Fig. (**4**)), rhusflavanone (**14**) and its hexaacetate (= rhusflavanone I-5,7,4′, II-5,7,4′-hexa-*O*-acetate) (**15**) (Fig. (**4**)), succedaneaflavanone (**16**) and its hexaacetate (= succedaneaflavanone I-5,7,4′, II-5,7,4′-hexa-*O*-acetate) (**17**) (Fig. (**5**)), cupressuflavone (**18**) (Fig. (**6**)), neorhusflavanone (**19**) (Fig. (**6**)), volkensiflavone (**20**) and its hexamethyl ether (= I-5,7,4′, II-5,7,4′-hexa-*O*-methyl- volkensiflavone)

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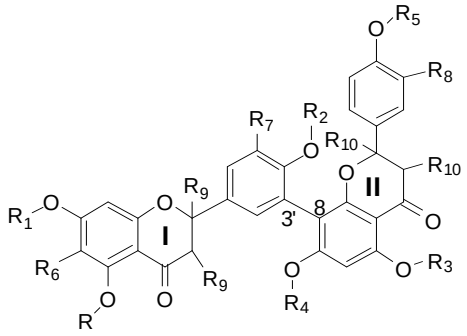
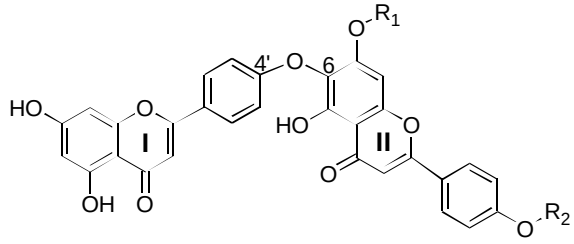


1 Ginkgetin R = R_3 = R_4 = R_5 = R_6 = R_7 = R_8 = H; R_1 = R_2 = Me; R_9 = R_{10} = double bond
2 Amentoflavone R = R_1 = R_2 = R_3 = R_4 = R_5 = R_6 = R_7 = R_8 = H; R_9 = R_{10} = double bond
3 II-4'-*O*-Methylamentoflavone or podocarpusflavone A R = R_1 = R_2 = R_3 = R_4 = R_6 = R_7 = R_8 = H; R_5 = Me; R_9 = R_{10} = double bond
4 Kayaflavone R = R_1 = R_3 = R_6 = R_7 = R_8 = H; R_2 = R_4 = R_5 = Me; R_9 = R_{10} = double bond
5 I-7,4',II-7,4'-Tetra-*O*-methylamentoflavone R = R_3 = R_6 = R_7 = R_8 = H; R_1 = R_2 = R_4 = R_5 = Me; R_9 = R_{10} = double bond
6 Taiwanhomoflavone-A or I-6-methyl-I-7,4'-di-*O*-methylamentoflavone R = R_3 = R_4 = R_5 = R_7 = R_8 = H; R_1 = R_2 = R_6 = Me; R_9 = R_{10} = double bond
7 I-7,II-4'-Di-*O*-methylamentoflavone R = R_2 = R_3 = R_4 = R_6 = R_7 = R_8 = H; R_1 = R_5 = Me; R_9 = R_{10} = double bond
8 I-7,4',II-7-Tri-*O*-methylamentoflavone R = R_3 = R_5 = R_6 = R_7 = R_8 = H; R_1 = R_2 = R_4 = Me; R_9 = R_{10} = double bond
9 II-7,4'-Di-*O*-methylamentoflavone R = R_1 = R_2 = R_3 = R_6 = R_7 = R_8 = H; R_4 = R_5 = Me; R_9 = R_{10} = double bond
31 Isoginkgetin R = R_1 = R_3 = R_4 = R_6 = R_7 = R_8 = H; R_2 = R_5 = Me; R_9 = R_{10} = double bond
52 I-7,4'-Di-*O*-methyl-I-2,3-dihydroamentoflavone R = R_3 = R_4 = R_5 = R_6 = R_7 = R_8 = R_9 = H; R_1 = R_2 = Me; R_{10} = double bond
53 I-7,4', II-7-Tri-*O*-methyl-I-2,3-dihydroamentoflavone R = R_3 = R_5 = R_6 = R_7 = R_8 = R_9 = H; R_1 = R_2 = R_4 = Me; R_{10} = double bond
66 Sciadopitysin R = R_3 = R_6 = R_7 = R_8 = H; R_1 = R_2 = R_5 = Me; R_4 = Glu; R_9 = R_{10} = double bond
71 I-5,7,4',II-5,7,4'-Hexa-*O*-acetylamentoflavone R = R_1 = R_2 = R_3 = R_4 = R_5 = CH_3CO; R_6 = R_7 = R_8 = H; R_9 = R_{10} = double bond
75 I-2,3, II-2,3-Tetrahydroamentoflavone R = R_1 = R_2 = R_3 = R_4 = R_5 = R_6 = R_7 = R_8 = R_9 = R_{10} = H
79 Bilobetin R = R_1 = R_3 = R_4 = R_5 = R_6 = H; R_2 = Me; R_9 = R_{10} = double bond
82 I-2,3-Dihydroamentoflavone R = R_1 = R_2 = R_3 = R_4 = R_5 = R_6 = R_7 = R_8 = R_9 = H; R_{10} = double bond

Fig. (1). Chemical structures of (I-3', II-8) dimers.

(**21**), spicataside (**22**) and its nonaacetate (= spicataside I-5,7,4', II-5,7,4′-nona-*O*-acetate) (**23**), morelloflavone (**24**) and its heptaacetate (= morelloflavone I-5,7,4′, II-5,7,3′,4′-hepta-*O*-acetate) (**25**) and heptamethyl ether (= I-5,7,4′, II-5,7,3′,4′-hepta-*O*-methyl-morelloflavone) (**26**) (Fig. (**7**)), GB_{1a} (**27**), its hexamethyl ether (= I-5,7,4′, II-5,7,4′-hexa-*O*-methyl-GB_{1a}) (**28**) and 7"-*O*-β-glucoside (= GB_{1a} II-7-*O*-β-glucopyranoside) (**29**), and GB_{2a} (**30**) (Fig. (**7**)), indicated that an ether linkage between two moieties of apigenin as seen in hinokiflavone (**10**) (Fig. (**2**)) is structurally required for significant cytotoxicity. Furthermore, a dose-dependent inhibition was observed with rhusflavone (**13**) on the growth of OVCAR-3 cells with 50% inhibition occurring at 1.8 μg/mL [33]. Volkensiflavone hexamethyl ether (**21**) (Fig. (**7**)), and GB_{1a} hexamethyl ether (**28**) (Fig. (**7**)) also demonstrated significant cytotoxicity [21].



10 Hinokiflavone R_1 = R_2 = H
64 Isocryptomerin R_1 = Me; R_2 = H
65 Cryptomerin B R_1 = R_2 = Me

Fig. (2). Chemical structures of (I-4', II-6)-biflavones.

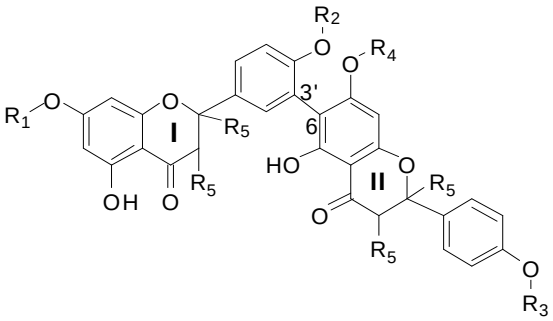
Recently, the biflavones ginkgetin (**1**), isoginkgetin (**31**) (Fig. (**1**)) and I-4′-*O*-methylrobustaflavone (**32**) (Fig. (**3**)), isolated from the herb *Selaginella moellendorffii* Hieron., were reported to have selective cytotoxicity against three human cancer cell lines [39].

Amentoflavone (**2**) (Fig. (**1**)) and II-7-*O*-methylagathisflavone (**33**) (Fig. (**4**)) showed concentration-dependent growth inhibitory activities on Ehrlich carcinoma cells in 45-h culture, tested by the tetrazolium method, with IC_{50} = 26 ± 1 μM for (**2**), and 24 ± 1 μM for (**33**) [40].

The methanolic crude extracts of leaves and stem bark of *Campylospermum flavum* (Ochnaceae) and their constituents displayed a significant cytotoxicity towards *Artemia salina* larvae. Some components of the extract were the biflavone II-4'-*O*-methylagathisflavone (**34**) (Fig. (**4**)) as well as a chalcone dimer, an alkaloid and other ten compounds, including three flavonoids, two biflavonoids, two alkaloids, two nitrile glucosides, and sitosterol 3-*O*-β-*D*-glucopyranoside. The structures of these compounds and their relative configurations were established by 1D and 2D NMR experiments [41].

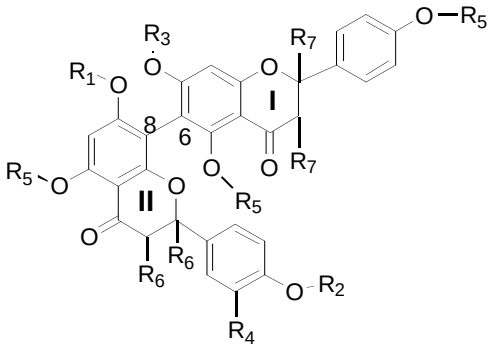
I-7,II-7-Di-*O*-methylagathisflavone (DMGF) (**35**) (Fig. (**4**)), a biflavonoid isolated from the needles of *Taxus* × *media* cv. Hicksii, has shown to induce apoptotic cell death in HT-29 cells, triggered both apoptotic and autophagic death in A549 cells and induced autophagic cell death in HepG2 cells [42].

The biflavanone lateriflavanone (**36**) (Fig. (**4**)) and other compounds were isolated from the stem bark of *Garcinia lateriflora*, collected in Indonesia. The structure of (**36**) was determined by spectroscopic data by analysis of COSY and NOESY NMR and ECD spectra. The biflavonoids exhibited proteasome-inhibitory activity, and the (I-3, II-8)-flavanone-flavone



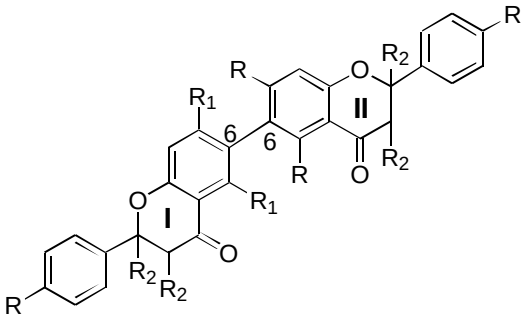
11 Robustaflavone $R_1 = R_2 = R_3 = R_4 = H$; R_5 = double bond
32 I-4'-*O*-Methylrobustaflavone $R_1 = R_3 = R_4 = H$; $R_2 = Me$; R_5 = double bond
50 I-4', II-4'-Di-*O*-methylrobustaflavone $R_1 = R_4 = H$; $R_2 = R_3 = Me$; R_5 = double bond
51 I-7,4', II-4'-Tri-*O*-methylrobustaflavone $R_4 = H$; $R_1 = R_2 = R_3 = Me$; R_5 = double bond
55 I-7,4', II-7-Tri-*O*-methyl-I-2,3, II-2,3-tetrahydrorobustaflavone $R_3 = R_5 = H$; $R_1 = R_2 = R_4 = Me$

Fig. (3). Chemical structures of (I-3', II-6) dimers.



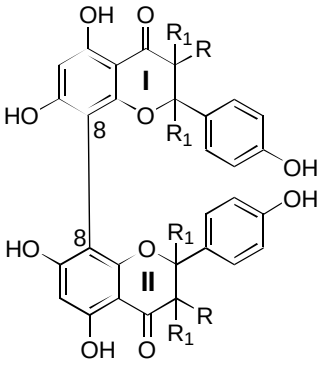
12 Agathisflavone $R_1 = R_2 = R_3 = R_4 = R_5 = H$; $R_6 = R_7$ = double bond
13 Rhusflavone $R_1 = R_2 = R_3 = R_4 = R_5 = R_7 = H$; R_6 = double bond
14 Rhusflavanone $R_1 = R_2 = R_3 = R_4 = R_5 = R_6 = R_7 = H$
15 Rhusflavanone I-5,7,4', II-5,7,4'-hexa-*O*-acetate $R_1 = R_2 = R_3 = R_5 = Ac$; $R_6 = R_7 = H$
33 II-7-*O*-Methylagathisflavone $R_1 = Me$; $R_2 = R_3 = R_8 = R_9 = H$; $R_6 = R_7$ = double bond
34 II-4'-*O*-Methylagathisflavone $R_1 = R_3 = R_4 = R_5 = H$; $R_2 = Me$; $R_6 = R_7$ = double bond
35 I-7,II-7-Di-*O*-methylagathisflavone $R_1 = R_3 = Me$; $R_2 = R_4 = R_5 = H$; $R_6 = R_7$ = double bond
36 Lateriflavanone $R_1 = R_2 = R_3 = R_5 = R_6 = R_7 = H$; $R_4 = OH$

Fig. (4). Chemical structures of (I-6, II-8) dimers.



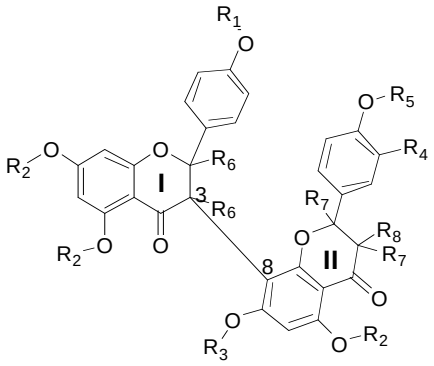
16 Succedaneaflavanone $R = R_1 = OH$; $R_2 = H$
17 Succedaneaflavanone I-5,7,4', II-5,7,4'-hexa-*O*-acetate $R = R_1 = OAc$; $R_2 = H$
77 (I-6, II-6)-Biflavone $R = R_1 = H$; R_2 = double bond
78 I-5,7-Dihydroxy-(I-6, II-6)-biflavone $R = H$; $R_1 = OH$; R_2 = double bond

Fig. (5). Chemical structures of (I-6, II-6) dimers.



18 Cupressuflavone R = H; R_1 = double bond
19 Neorhusflavanone or mesuaferrone A R = R_1 = H
80 I-3,II-3-dihydroxycupressuflavone R = OH; R_1 = double bond

Fig. (6). Chemical structure of (I-8, II-8) dimers.



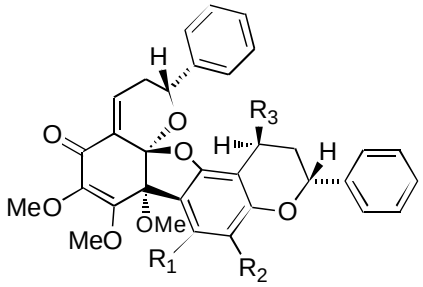
20 (+)-Volkensiflavone [2*R*,3*S*] R_1 = R_2 = R_3 = R_4 = R_5 = R_6 = R_8 = H; R_7 = double bond
21 I-5,7,4', II-5,7,4'-Hexa-*O*-methylvolkensiflavone R_1 = R_2 = R_3 = R_5 = Me; R_4 = R_6 = R_8 = H; R_7 = double bond
22 Spicataside (= volkensiflavone-II-7-*O*-*D*-glucoside) R_1 = R_2 = R_4 = R_5 = R_6 = R_8 = H; R_3 = Glu; R_7 = double bond
23 Spicataside nona-*O*-acetate R_1 = R_2 = R_5 = Ac; R_3 = 2,3,4,6-Tetra-*O*-acetylGlu; R_4 = R_6 = R_8 = H; R_7 = double bond
24 (+)-Morellolavone (= fukugetin) [2*R*,3*S*] R_1 = R_2 = R_3 = R_5 = R_6 = R_8 = H; R_4 = OH; R_7 = double bond
25 Morellolavone I-5,7,4', II-5,7,3',4'-hepta-*O*-acetate R_1 = R_2 = R_3 = R_5 = Ac; R_4 = OAc; R_6 = R_8 = H; R_7 = double bond
26 I-5,7,4', II-5,7,3',4'-Hepta-*O*-methylmorellolavone R_1 = R_2 = R_3 = R_5 = CH_3; R_4 = OCH_3; R_6 = R_8 = H; R_7 = double bond
27 GB_{1a} R_1 = R_2 = R_3 = R_4 = R_5 = R_6 = R_7 = R_8 = H
28 I-5,7,4', II-5,7,4'-Hexa-*O*-methyl-GB_{1a} R_1 = R_2 = R_3 = R_5 = CH_3; R_4 = R_6 = R_7 = R_8 = H
29 GB_{1a}-II-7-*O*-β-*D*-glucopyranoside R_1 = R_3 = R_4 = R_5 = R_6 = R_7 = R_8 = H; R_3 = Glu
30 GB_{2a} R_1 = R_2 = R_3 = R_5 = R_6 = R_7 = R_8 = H; R_4 = OH
49 Pancibiflavonol R_1 = R_2 = R_3 = R_5 = R_6 = H; R_7 = double bond; R_4 = R_8 = OH
67 GB_1 R_1 = R_2 = R_3 = R_4 = R_5 = R_6 = R_7 = H; R_8 = OH
68 GB_2 R_1 = R_2 = R_3 = R_5 = R_6 = R_7 = H; R_4 = R_8 = OH
69 Kolaflavanone R_1 = R_2 = R_3 = R_6 = R_7 = H; R_4 = R_8 = OH; R_5 = CH_3
83 II-4'-*O*-Methyl-GB_{2a} R_1 = R_2 = R_3 = R_6 = R_7 = R_8 = H; R_5 = CH_3; R_4 = OH

Fig. (7). Chemical structures of (I-3, II-8) dimers.

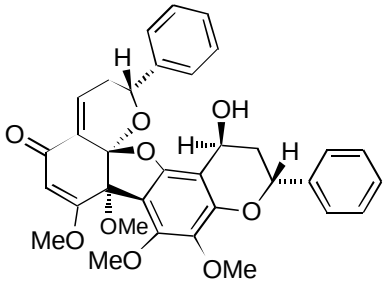
dimer, called morelloflavone (**24**) (Fig. (**7**)), was found to have the highest potency (IC_{50} = 1.3 μM) [43].

The calycopterones, a class of biflavonoids, showed cytotoxicity to human tumor cell lines [44]. Calycopterone (**37**), 4-demethylcalycopterone (**38**), and isocalycopterone (**39**) (Fig. (**8**)) were isolated as cytotoxic constituents from the flowers of *Calycopteris floribunda* Lamk. (Combretaceae), and showed a wide range of activity against a panel of solid tumor cell lines. Among these biflavonoids, calycopterone (**37**) was the main constituent [44].

Neocalycopterone (**40**) and its methyl ether (**41**) (Fig. (**8**)), along with two biflavonoids, calyflorenones A (**42**) and B (**43**) (Fig. (**9**)), were isolated from dried leaves of *C. floribunda*. The relative stereochemistry of calyflorenones A (**42**) and B (**43**) was



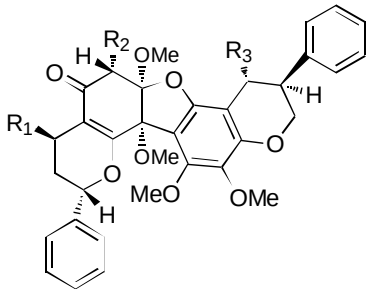
37 Calycopterone $R_1 = OH; R_2 = R_3 = OCH_3$
38 4-Demethylcalycopterone $R_1 = R_3 = OH; R_2 = OCH_3$
39 Isocalycopterone $R_1 = R_3 = OCH_3; R_2 = OH$
40 Neocalycopterone $R_1 = R_2 = OCH_3; R_3 = OH$
41 Neocalycopterone methyl ether $R_1 = R_2 = R_3 = OCH_3$



44 6"-Demethoxyneocalycopterone

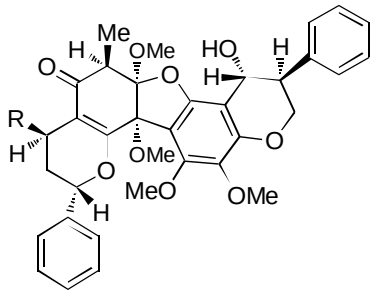
Fig. (8). Chemical structures of calycopterones.

determined by NOE and ROESY NMR experiments, comparative CD and optical rotation evaluations. Cytotoxicity test results of neocalycopterone (**40**) and its methyl ether (**41**) (Fig. (**8**)) were reported [45]. The structures of five biflavonoids, 6"-demethoxyneocalycopterone (**44**) (Fig. (**8**)), calyflorenone C (**45**), calyflorenone D (**46**) (Fig. (**9**)), 6"-*epi*-calyflorenone B (**47**) and 6"-*epi*-calyflorenone C (**48**) (Fig. (**10**)) from the green parts of *C. floribunda* were established by NMR and MS. Their NMR and chiroptical properties (CD, $[\alpha]^{20}_D$) were compared with those of the other biflavonoids from this plant. With regard to one chiral center (C-6"), 6"-*epi*-calyflorenone B (**47**) and 6"-*epi*-calyflorenone C (**48**) (Fig. (**10**)) are the respective epimers of calyflorenones B (**43**) and C (**45**) (Fig. (**9**)) [46].



42 Calyflorenone A $R_1 = R_2 = R_3 = OCH_3$
43 Calyflorenone B $R_1 = R_2 = OCH_3; R_3 = OH$
45 Calyflorenone C $R_1 = R_3 = OH; R_2 = OCH_3$
46 Calyflorenone D $R_1 = R_3 = OH; R_2 = H$

Fig. (9). Chemical structures of calyflorenones.

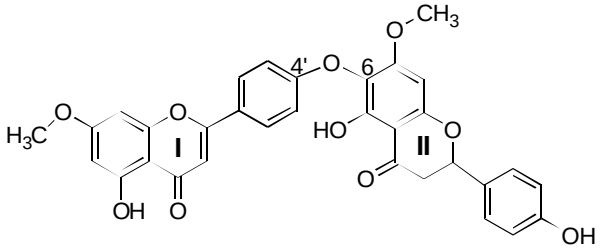


47 6"-*epi*-Calyflorenone B R = OCH_3
48 6"-*epi*-Calyflorenone C R = OH

Fig. (10). Chemical structures of 6-*epi*-calyflorenones.

The biflavonoid pancibiflavonol (**49**) (Fig. (**7**)) was isolated from the ethanolic extract of the stem bark of *Calophyllum panciflorum*, along with other six biflavonoids, and its structure was elucidated by spectroscopic data. All these biflavonoids exhibited significant inhibitory activity against 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced Epstein-Barr virus early antigen activation in Raji cells [47].

Five biflavonoids, I-4′, II-4′-di-*O*-methylrobustaflavone (**50**), I-7,4′, II-4′-tri-*O*-methylrobustaflavone (**51**) (Fig. (**3**)), I-7,4′-di-*O*-methyl-I-2,3-dihydroamentoflavone (**52**), I-7,4', II-7-tri-*O*-methyl-2,3-dihydroamentoflavone (**53**) (Fig. (**1**)), and I-7-*O*-methyl-II-2,3-dihydroisocryptomerin (**54**) (Fig. (**11**)), together with other six compounds have been isolated from the aerial parts of *Selaginella delicatula* [48]. The structures of these new compounds were determined by spectral analysis. Among the isolated compounds, I-4′, II-4′-di-*O*-methylrobustaflavone (**50**) (Fig. (**3**)), I-7,4′-di-*O*-methyl-I-2,3-dihydroamentoflavone (**52**) (Fig. (**1**)), and α-tocopheryl quinone showed cytotoxicities (ED_{50} values < 4 μg/mL) against P-388 and/or HT-29 cell lines *in vitro* [48].



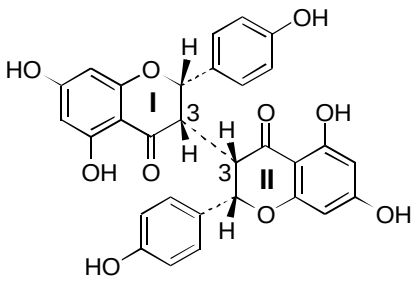
54 I-7-*O*-Methyl-II-2,3-dihydroisocryptomerin

Fig. (11). Chemical structure of a (I-4', *O*, II-6)-flavone-flavanone dimer.

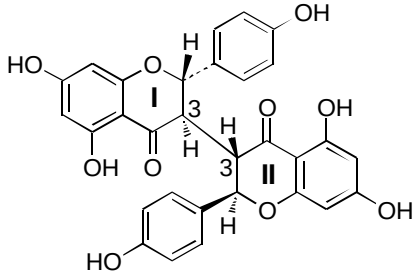
The biflavanone I-7,4', II-7-tri-*O*-methyl-I-2,3, II-2,3-tetrahydrorobustaflavone (**55**) (Fig. (**3**)) was isolated from the whole plant of *Selaginella doederleinii* (Selaginellaceae) together with the biflavonoid, I-7,4′, II-4′-tri-*O*-methylrobustaflavone (**51**) (Fig. (**3**)) as cytotoxic constituents against the three human cancer cell lines, HCT, NCI-H358, and K562 [49].

Neochamaejasmin A (I-2*S*,3*S*, II-2*S*,3*S*) (**56**) (Fig. (**12**)), a biflavanone isolated from the roots of a traditional Chinese medicine, *Stellera chamaejasme*, has been shown to inhibit cellular uptake of [^{3}H]-thymidine (IC_{50} = 12.5 μg/mL) and the subsequent proliferation of human prostate cancer LNCaP cells [50].

A new biflavonoid, 2″-hydroxygenkwanol A (**57**) (Fig. (**13**)), isolated from the aerial parts of *Daphne linearifolia* Hart. showed



56 Neochamaejasmin A [I-2*S*,3*S*, II-2*S*,3*S*]



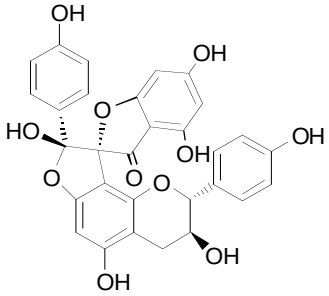
63 Isochamaejasmin [I-2*S*,3*R*, II-2*R*,3*S*]

Fig. (12). Chemical structures of (I-3, II-3)-biflavanones.

to efficiently interact with Hsp90, one of the most promising targets for the modern anti-cancer therapy [51].

MECHANISMS OF ACTION

The mechanisms involved in modulating the effects of mutagens and carcinogens, and the anticancer activity of biflavonoids are shown in the following sections. As it is known, these mechanisms are usually related. Nevertheless, some of them have been grouped for display purposes.



57 2"-Hydroxygenkwanol A

Fig. (13). Chemical structure of 2″-hydroxygenkwanol A.

a. Induction of Apoptosis

(a.1) a.1. Inhibition of Cyclic Nucleotide Phosphodiesterases

Phosphodiesterases (PDEs) are hydrolytic enzymes that have an important role in regulating cAMP cellular levels by cleaving cAMP at the 3'-phosphodiester bond, yielding inactive 5'-adenosine monophosphate [52]. The PDE family is composed of 11 members and over 30 isoforms, distributed in different tissues and expressing different protein levels. The PDE isoforms detected in T-cells were PDE1, PDE2, PDE3, PDE4, PDE5, PDE7, and PDE8 [53], while PDE4 and PDE7 played a role in T-cell activation [52]. Attempts have been made to use selective PDE inhibitors to induce apoptosis in cancer cell lines.

The effect of biflavonoids on PDE isoenzymes has been evaluated [54], showing that almost all tested biflavonoids inhibited PDE activity.

As it is known some biflavonoids and catechins have anti-lymphoproliferative activity [55-57]. There are several types of malignancies related to lymphocytes, such as the lymphoblastic leukemia diseases, which are divided into chronic and acute forms. The leaves of the Cashew plant (*Anacardium occidentale* L.) are used by the folk medicine in South America and West Africa. The crude ethanolic extract of these leaves caused a reduction in blood lymphocytes (lymphopenia) in a 90-day sub-chronic toxicity assay in rats [58]. Fractionation of this extract led to the isolation of the active compound, the biflavone agathisflavone (**12**) (Fig. (**4**)). *In vitro* tests agathisflavone (**12**) induced apoptosis, and showed a strong anti-proliferative effect on lymphocytes.

Previously, de Carvalho *et al.* [59] tested the effect of agathisflavone (**12**) on five cancer cell lines, including HT-29 colon adenocarcinoma, NCI-H460 non-small lungcarcinoma, RXF-393 renal cell carcinoma, MCF-7 breast cancer and OVCAR-3, but all tested cell lines were marginally affected by agathisflavone (**12**), with IC_{50} >40 μg/mL. However, methylated derivatives of agathisflavone exhibited strong cytotoxic effect on these cells [59] and also on the chronic myeloid leukemia cell line K562 [40].

Konan *et al.* [58] reported that agathisflavone (**12**) was cytotoxic against two malignant blood cell lines, Jurkat and HL60. Since agathisflavone (**12**) also induced apoptosis in Jurkat cells, the authors proposed this mechanism for the cytotoxic effect of agathisflavone (**12**). Furthermore, agathisflavone (**12**) gave rise to apoptosis by a likely mechanism of action involving inhibition of cyclic nucleotide PDEs [54].

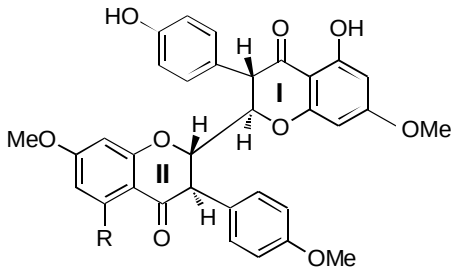
Therefore, the cytotoxic effect of agathisflavone (**12**) on Jurkat T-cells is possibly related to the inhibition of PDEs, such as PDE1 and PDE4. The inhibitory effect of agathisflavone (**12**) on the acute promyeloid leukemia (APL) cell line HL60 was less impressive than in Jurkat cells. The reason for this differential effect may rest in the apparently contradictory roles of PDE in HL60 apoptosis [58].

Therefore, agathisflavone (**12**) can behave either as a pro- or an anti-apoptotic agent in HL60 cells, and this dichotomy is likely to explain its smaller effect on HL60 when compared to Jurkat cells. Raji B cells from Burkitt lymphoma were not significantly inhibited. Therefore, either PDEs did not play a role in cell cycling control in these cells or Raji's PDEs were not sensitive to agathisflavone (**12**) inhibition. Accordingly, specific inhibitors of PDE4 and/or PDE7 did not elicit any cytotoxic effect in B-cells [60].

In conclusion, agathisflavone (**12**) caused lymphopenia *in vivo* and selectively inhibited growth of leukemia cells by inducing apoptosis in these cells. The cashew ethanolic extract displayed low toxicity in rats, suggesting that its active compound agathisflavone (**12**) might potentially be used in therapy [58].

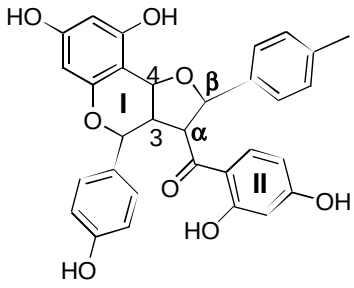
a.2. Effects on NF-κB Family of Transcription Factors

The NF-κB family of transcription factors plays a key role in the regulation of cell growth and survival. In quiescent cells, NF-κB is sequestered in the cytoplasm by the IκB family of inhibitory proteins. Following exposure of cells to a variety of stimuli, including TNF-α, IL-1, and LPS, activation of NF-κB is accomplished by a mechanism involving site-specific phosphorylation, ubiquitination, and proteasomal degradation of IκB. Release of IκB reveals a nuclear localization signal on NF-κB, which allows NF-κB to translocate to the nucleus, where it serves as a transcriptional regulator.



58 Dehydroxyhexaspermone C R = H
59 Hexaspermone C R = OH
Both compounds: [I-2*R*,3*S*, II-2*R*,2*S*]

Fig. (14). Chemical structures of (I-2, II-2)-bi-isoflavanones.



60 Cordigol

Fig. (15). Chemical structures of a tetrahydrofuro[3,2-*c*]benzopyran derivative.

Neochamaejasmin A (**56**) (Fig. (**12**)) was shown to inhibit cellular ^{3}H-thymidine incorporation (IC_{50} 12.5 μg/mL) and subsequent proliferation of human prostate cancer LNCaP cells. Treatment of LNCaP cells with low doses of (**56**) (≤ 6.25 μg/mL) suppressed DNA-binding activities of the transcription factors NF-κB and AP-1 to the promoter of cyclin D and also inhibited expression of the cell cycle regulatory proteins cyclin D, proliferating cell nuclear antigen, and nucleolin, thus arresting cells in G_1 phase of the cell cycle. A lengthy exposure with higher doses of (**56**) (≥ 12.5 μg/mL) revealed the ROS production, dissipation of the mitochondrial membrane potential, upregulation of cyclin-dependent kinase inhibitor p21, and induction of cell apoptosis. An aggregation of Fas-procaspase 8-procaspase 3 and p21-procaspase 3 proteins by coimmunoprecipitation, immunoblotting analysis, and MALDI-MS indicated the involvement of Fas and p21 in (**56**)-mediated cytotoxicity, and pretreatment of cells with antisense FasL oligonucleotides partially abolished apoptosis. Therefore, neochamaejasmin A (**56**) blocked cell cycle progression at the G_1 phase by activating the p21 protein and ultimately promoting the Fas-caspase-8-caspase-3 apoptotic machinery [50].

The ethanolic extract of the bark of *Ochna macrocalyx* showed NF-κB inhibitory activity [61]. The constituents of the bark were the (I-2, II-2)-bi-isoflavanones dehydroxyhexaspermone C (**58**) and hexaspermone C (**59**) (Fig. (**14**)) [62], the tetrahydrofuro[3,2-*c*]benzopyran derivative cordigol (**60**) (Fig. (**15**)), and the biflavonoids calodenin A (**61**) and calodenin B (**62**) (Fig. (**16**)). These compounds were tested for cytotoxic activity on MCF-7 breast cancer cells using the MTT reduction assay method. Calodenin B (**62**) showed better cytotoxic activity (7 ± 0.5 μM) than the other compounds [61].

The root of *Stellera chamaejasme* is a traditional Chinese herb termed Rui Xiang Lang Du and has been used to treat solid tumors, tuberculosis and psoriasis. Tian *et al*. [63] examined four biflavonoids isolated from *S. chamaejasme*, including isochamaejasmin (I-2*S*,3*R*, II-2*R*,3*S*) (**63**) (Fig. (**12**)), two of its stereoisomers and a methyl derivative, in functional assays originally designed to screen ligands for the G protein-coupled formyl peptide receptor-like 1 (FPRL1). The latter is implicated in antibacterial inflammatory responses and malignant glioma metastasis.

Isochamaejasmin (**63**) was found to induce the expression of a NF-κB-directed reporter gene in transfected HeLa cells with an EC_{50} of 3.23 μM, independently of FPRL1 [63]. The isochamaejasmin-stimulated NF-κB reporter activity was accompanied by nuclear translocation of NF-κB proteins and was blocked by a dominant-negative construct of IκBα. Isochamaejasmin (**63**) also induced time-dependent phosphorylation of the mitogen-activated protein kinases extracellular signal-regulated kinase 1/2 and p38, and a novel protein kinase C (PKCδ). Likewise, inhibition of these kinases with the respective pharmacological inhibitors significantly reduced the isochamaejasmin-stimulated NF-κB activation. It is noteworthy that the two stereoisomers and the methyl derivative did not induce detectable activation of NF-κB, and were more cytotoxic than isochamaejasmin, which could partially rescue cycloheximide-induced apoptosis. Inhibition of NF-κB activation reversed the anti-apoptotic effect of isochamaejasmin (**63**). Therefore, a potential mechanism of action by *S. chamaejasme* L. showed that structurally similar compounds of this plant may have different pharmacological properties.

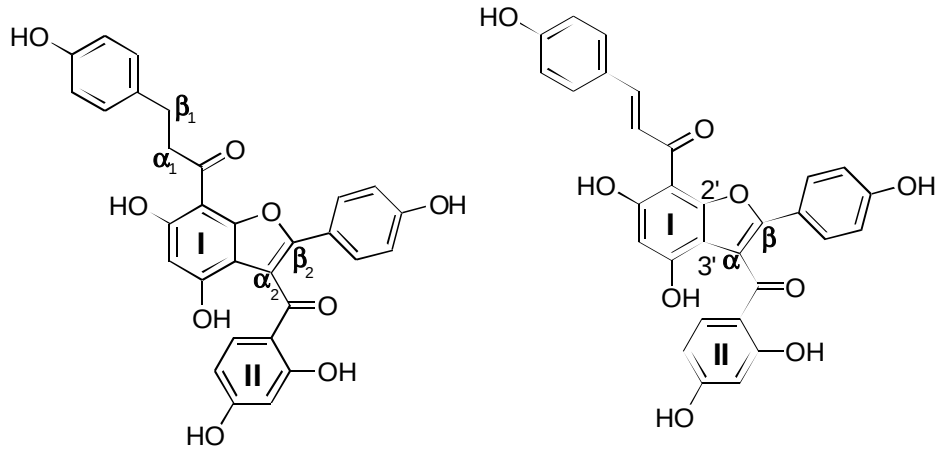
Chalcone compounds are known to behave as potent NF-κB inhibitors and to inhibit nitric oxide (NO) production. Therefore, bichalcone analogs were synthesized and were evaluated for inhibition of NADPH oxidase (NOX)-dependent ROS production and NOS-dependent NO production in LPS-activated microglial cells.

These compounds were also evaluated for *in vitro* anticancer activity using four human cancer cell lines, and for DPPH radical scavenging capacity. These authors investigated the expression of iNOS protein and NF-κB p65 and p65 in the presence of the bichalcone analogs [30].

To summarize the initial SAR observations on the bichalcone analogs, a methoxy group at C-3 in the A-ring resulted in weaker NO production inhibition activity compared with nonsubstitution at this position. Concerning B-ring, those compounds with 2-pyridyl and 3-pyridyl B-rings, respectively, showed improved activity with IC_{50} values of 1.4 and 0.3 μM, respectively. Adding methyl groups at C-3 or C-5 of the thiophene or furan rings decreased the compounds' activity. The introduction of a methoxy group at C-4 of the B-ring resulted in increased inhibition of NO production (IC_{50} = 0.5 μM), while addition of chloro substituents on the B-ring led to decreased inhibition of NO production (IC_{50} = 30.5 and 25.7 μM, respectively).

Two bichalcone analogs significantly blocked the nuclear translocation of NF-κB p65 at 1.0 μM concentration and decreased the iNOS protein expression, suggesting that both compounds may target the NF-κB signaling pathway to block iNOS upregulation, which in turn suppresses the NO production [30].

All compounds were also evaluated for cytotoxicity against four human cancer cell lines, DU145 (prostate cancer), A549 (non small cell lung cancer), KB (nasopharyngeal carcinoma) and HCT-8 (ileocecal). The B-ring pyridyl moiety was important for the significant activity of the bichalcone analogs. The compound bearing a single B-ring pyridyl moiety showed activity, but with higher GI_{50} values (9.77–13.1 μM). Since the response of the most active compound was associated with apoptosis, the effects on the signaling pathway were investigated. All active caspases were detected after 24 h treatment with the bichalcone analog. To verify the involvement of caspases-3, -8, and -9 in the bichalcone-induced apoptosis of HT-29 cells, inhibitors of each caspase were used. The results were consistent with bichalcone-inducing apoptosis through



61 Calodenin A **62** Calodenin B

Fig. (16). Chemical structures of a (I-2′, *O*, II-β; I-3′, II-α)-dihydrochalcone-chalcone dimer and a (I-2′, *O*, II-β; I-3′, II-α)-bichalcone.

the activation of caspases-3, -8, and -9. This compound increased the protein expression of Fas/CD95, FADD, cytosolic cytochrome c, Apaf-1, AIF, Endo G, caspase-3, Bax (Bcl2-associated X protein), and t-Bid, and decreased the protein levels of pro-caspase-8, pro-caspase-9 and Bcl-2. Therefore, this bichalcone induced apoptosis in HT-29 cells *via* both death receptor (Fas/CD95) and mitochondrial-dependent pathways.

In summary, bichalcones with a piperazine Mannich base linkage were prepared and evaluated for inhibition of NO production in microglial cells and for *in vitro* anticancer activity. Two compounds were potent inhibitors of cellular NO production in LPS-activated microglial cells, likely indirectly *via* blockade of NF-κB p65 nuclear translocation. Several bichalcone analogs with a pyridyl moiety also showed significant activity against human tumor cell replication. Exploration of the mechanism of action showed that the most active compound likely acted *via* the Fas/CD95 apoptosis signaling pathway [30].

a.3. Activation of Caspase(s)

Apoptotic cell death by caspase activation was involved in the cytotoxic effects of the biflavone ginkgetin (**1**) (Fig. (**1**)) [64], which were evaluated by the MTT assay in three different human cell lines, e.g., OVCAR-3, HeLa and foreskin fibroblast (FS-5). The EC_{50} values of ginkgetin (**1**) in these cell lines were 3.0, 5.2, and 8.3 μg/mL, respectively. Morphological changes in cells and their nuclei, DNA fragmentation with a characteristic pattern of inter-nucleosomal ladder, and double-stranded DNA breaks were detected following treatment with 3 μg/mL of this biflavone for 24 h. Incubation with 5 μg/mL ginkgetin (**1**) led to increased intracellular levels of hydrogen peroxide as early as 30 min. The cytotoxicity of ginkgetin (**1**) was partially inhibited by pretreating cells with vitamin C, vitamin E or CAT. CAT not only afforded the best protective effect among three antioxidants, but also reduced both the DNA fragmentation and double-stranded DNA breakage induced by ginkgetin (**1**). Moreover, the involvement of caspase(s) in ginkgetin-induced apoptosis was demonstrated by the activation of caspase-3 after drug treatment, and the suppression of cell death by a broad-spectrum caspase inhibitor, z-VAD-fmk. However, the protective effects of z-VAD-fmk and CAT were not additive. Therefore, the apoptosis induced by ginkgetin (**1**), especially at 5 μg/mL, was mediated mainly through the activation of caspase(s) by the hydrogen peroxide generated possibly through autooxidation of this biflavone [64].

In other studies, several hinokiflavone (**10**)-type biflavonoids, such as isocryptomerin (**64**) and cryptomerin B (**65**) (Fig. (**2**)), exhibited potent cytotoxic effects probably by apoptotic death at low μM concentrations [57]. In contrast, some biflavones such as ginkgetin (**1**) and sciadopitysin (**66**) (Fig. (**1**)) enhanced proliferation of normal human skin fibroblasts and increased collagen production [65].

The ethanolic extract of Cashew leaves, in a sub-acute toxicity, elicited lymphopenia in rats, as mentioned above. The extract was also found to be cytotoxic and to induce apoptosis in Jurkat (acute lymphoblastic leukemia) cells. [^{3}H]-Thymidine incorporation assays and flow cytometry analysis showed that the isolated compound agathisflavone (**12**) (Fig. (**4**)) displayed a high anti-proliferative effect in Jurkat cells with an IC_{50} of 2.4 μg/mL (4.45 μM). The effect of agathisflavone (**12**) on the acute promyelocytic leukemia cell line HL60, Burkitt lymphoma Raji cells and Hep-2 laryngeal carcinoma cells was also tested. The two latter ones were only mildly affected by agathisflavone (**12**). It was also shown that agathisflavone (**12**) induced apoptosis in Jurkat cells, and it was proposed that this may be the mechanism of agathisflavone specific cytotoxicity [58].

a.4. Inhibition Effects on bcl-2 Expression, and Upregulation of p53 and Caspase-3 Gene Expression

Guruvayoorappan and Kuttan [66] reported the regulatory effect of amentoflavone (**2**) (Fig. (**1**)), a biflavone from *Biophytum sensitivum*, on the apoptotic process in B16F-10 melanoma cells, and the production of NO and cytokines in B16F-10 cells, tumor-associated macrophages (TAMs) and peritoneal macrophages. Amentoflavone (**2**) at a concentration of 10 μg/mL could significantly ($p < 0.001$) inhibit the production of NO and proinflammatory cytokines (IL-1β, IL-6, GM-CSF and TNF-α) in B16F-10 cells, TAMs and peritoneal macrophages. Incubation of B16F-10 cells with amentoflavone (**2**) showed the presence of apoptotic bodies and induced DNA fragmentation. Furthermore, amentoflavone (**2**) showed inhibitory effects on bcl-2 expression, and upregulated p53 and caspase-3 gene expression in B16F-10 melanoma cells. These results suggested that amentoflavone (**2**) stimulated apoptosis by regulating bcl-2, caspase-3 and p53 genes in B16F-10 melanoma cells and regulated NO and proinflammatory cytokine production in B16F-10 cells, TAMs and peritoneal macrophages [66].

b. Inhibition of Angiogenesis

The formation of new capillaries from existing blood vessels is critical for tumor growth and metastasis. As example, amentoflavone (**2**) (Fig. (**1**)) at non-toxic concentrations (0.05-0.20

μg/mL) showed a significant inhibition of proliferation, migration and tube formation of endothelial cells, which are key events in angiogenesis [67].

b.1. Anti-proliferative Effects (Inhibition of Tumor Metastasis in vivo)

Guruvayoorappan and Kuttan [68] studied the antimetastatic activity of amentoflavone (**2**) (Fig. (**1**)) using B16F-10 melanoma-induced experimental lung metastasis in C57BL/6 mice. Amentoflavone (**2**) treatment significantly reduced tumor nodule formation accompanied by reduced levels of lung collagen hydroxyproline, hexosamine, and uronic acid. Serum sialic acid and γ-glutamyl transpeptidase levels were also significantly inhibited after treatment. Amentoflavone (**2**) treatment upregulated the lung tissue inhibitor of metalloprotease-1 and tissue inhibitor of metalloprotease-2 expression [68].

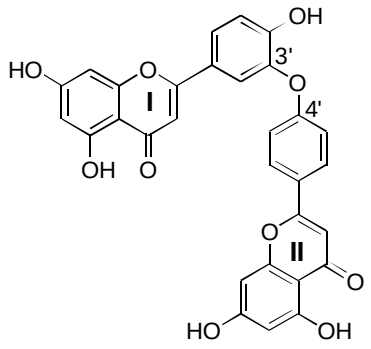
C57BL/6 mice were injected once with B16F-10 melanoma cells *via* tail vein, followed by treatment with amentoflavone (**2**) (50 mg/kg bw) for 10 consecutive days [69]. Twenty-one days after tumor injection, animals were sacrificed, and tumor metastasis was confined to the lungs. Amentoflavone (**2**) treatment significantly lowered the number of lung nodules ($p<0.001$) compared with tumor controls. Amentoflavone (**2**) treatment markedly decreased the mRNA expression of MMP-2, MMP-9, prolyl hydroxylase, lysyl oxidase, VEGF, ERK-1, ERK-2, TNF-α, IL-1β, IL-6, and GM-CSF in lung tissues. However, amentoflavone (**2**) treatment increased mRNA expression of STAT-1 and nm23 in lung tissues. *In vitro* studies also indicated that this treatment inhibited tumor cell invasion and migration. Therefore, treatment with amentoflavone (**2**) reduced experimental tumor metastasis, and such action was associated with attenuation of tumor invasion, proliferation and angiogenesis [69]. Additionally, amentoflavone (**2**) has shown to inhibit intracellular cell signaling ERK pathway leading to the prevention of MMP-1 expression in human skin fibroblasts [70].

Atrazine (ATR) is a widespread agrochemical contaminant frequently detected in water systems and kolaviron is a seed-derived mixture of biflavonoids, e.g., mainly GB_1 (**67**), GB_2 (**68**), and kolaflavanone (**69**) (Fig. (**7**)), which has been reported to modulate the effects of many mutagens and carcinogens. The protective effects of kolaviron on ATR-induced cell death in the human neuroblastoma cell line (SHY-SY5Y) were studied. Kolaviron prevented ATR-induced generation of ROS, cell death and inhibited cell proliferation by reduction of cell proliferation. Furthermore, ATR-induced levels of MDA, CAT, GSH-Px, and GR activities, increased LDH leakage, inhibited cellular LDH activity and depleted GSH levels in SHY-SY5Y cells were blocked by kolaviron. Comparable to the control, kolaviron increased GR, but not GSH-Px activities. ATR mediated nuclear changes associated with apoptosis; including nuclear fragmentation, condensation, DNA laddering, and increased caspase-3 activity were blocked on addition of kolaviron. ATR-induced changes in the expressions of p53, Bax, Bcl-2, p21, and mRNA levels of caspase-3 and caspase-9 were prevented by kolaviron. Based on these results, a model was proposed for the protective effect of kolaviron on ATR-induced cell injury in neuronal cell [71].

b.2. Activation of Rho-GTPases and ERK Signaling Pathways

Morelloflavone (**24**) (Fig. (**7**)) has shown antiangiogenic activity. This biflavonoid could inhibit VEGF-induced cell proliferation, migration, invasion, and capillary-like tube formation of primary cultured human umbilical vascular endothelial cells in a dose-dependent manner. Morelloflavone (**24**) effectively inhibited microvessel sprouting of endothelial cells in the mouse aortic ring assay and the formation of new blood microvessels induced by VEGF in the mouse Matrigel plug assay.

Furthermore, morelloflavone (**24**) inhibited tumor growth and tumor angiogenesis of prostate cancer cells (PC-3) in xenograft mouse tumor model *in vivo*. To understand the underlying inhibitory mechanism of morelloflavone (**24**), Pang *et al.* [72] showed that morelloflavone (**24**) could inhibit the activation of both RhoA and Rac1 GTPases, but have little effect on the activation of Cdc42 GTPase. Additionally, morelloflavone (**24**) inhibited the phosphorylation and activation of Raf/mitogen-activated protein kinase/ERK/ERK pathway without affecting VEGF receptor 2 activity. Therefore, the anticancer action of morelloflavone (**24**) is based on its antiangiogenic action by targeting the activation of Rho-GTPases and ERK signaling pathways [72]. Moreover, ochnaflavone (**70**) (Fig. (**17**)) inhibited the proliferation of HCT-15 cell line of human colon cancer with an IC_{50} value of 4.1 μM [73].



70 Ochnaflavone

Fig. (17). Chemical structure of a (I-3', *O*, II-4')-biflavone.

b.3. Inhibition of FASN Activity

FASN is highly expressed in breast carcinomas to support their continuous growth and proliferation, but has low expression level in normal tissues. Considerable interest has been aroused in the search for novel FASN inhibitors as a therapeutic target for breast cancer. Amentoflavone (**2**) (Fig. (**1**)) was isolated from *Selaginella tamariscina*, a traditional oriental medicine that has been used to treat cancer for many years, and was found to significantly inhibit the FASN enzymatic activity *in vitro* at concentrations above 50 μM [74]. Amentoflavone (**2**) was also found to decrease fatty acid synthesis by the reduction of [^{3}H]-acetyl-CoA incorporation into lipids in FASN-overexpressed SK-BR-3 human breast cancer cells. Furthermore, amentoflavone (**2**), at a concentration higher than 75 μM, increased the cleavage-activity of caspase-3 and poly (ADP-ribose) polymerase (PARP), and administration of pan-caspase inhibitor z-VAD-fmk completely rescued the SK-BR-3 cells from PARP cleavages [74].

Growth inhibition of cancer by amentoflavone (**2**) was dose-dependent, showing a slight reduction at 50 μM and a significant reduction at concentrations of 75 and 100 μM. The growth of FASN-nonexpressed NIH-3T3 normal cells was not decreased by treatment with amentoflavone (**2**) in a dose-time-dependent manner. Therefore, amentoflavone (**2**) induced breast cancer apoptosis by blocking fatty acid synthesis [74].

c. Inhibition of pre-mRNA Splicing

Membrane-permeable compounds that reversibly inhibit a particular step in gene expression are highly useful tools for cell biological and biochemical/structural studies. In comparison with other gene expression steps where multiple small molecule effectors are available, very few compounds have been described to behave as general inhibitors of pre-mRNA splicing. Isoginkgetin (**31**) (Fig. (**1**)) has been identified as a general inhibitor of pre-mRNA splicing using an *in vivo* screening test [75].

O'Brien *et al.* [76] reported construction and validation of a set of mammalian cell lines suitable for the identification of small molecule inhibitors of pre-mRNA splicing. Using these cell lines, the authors identified isoginkgetin (**31**) as a general inhibitor of both major and minor spliceosomes. Isoginkgetin (**31**) inhibited splicing *in vivo* and *in vitro* at similar micromolar concentrations. It appeared to do so by preventing stable recruitment of the U4/U5/U6 three-small nuclear ribonucleoprotein complex, resulting in the accumulation of prespliceosomal A complex. As two recently reported general pre-mRNA splicing inhibitors, isoginkgetin (**31**) has been previously described as an antitumor agent. These results suggest that splicing inhibition is the mechanistic basis of the antitumor activity of isoginkgetin (**31**). Therefore, pre-mRNA splicing inhibitors may represent a novel pathway for the development of new anti-cancer agents [76].

d. Inhibition of Human DNA Topoisomerases I and II-α

Topoisomerase inhibitors are agents with anticancer activity. Amentoflavone (**2**) (Fig. (**1**)) and II-7-*O*-methylagathisflavone (**33**) (Fig. (**4**)) were isolated from the Brazilian plants *Ouratea semiserrata* and *O. hexasperma*, respectively. These biflavonoids and the acetyl derivative of (**2**) (= I-5,7,4', II-5,7,4'-hexa-*O*-acetylamentoflavone) (**71**) (Fig. (**1**)) are inhibitors of human DNA topoisomerases I at 200 μM, as demonstrated by the relaxation assay of supercoiled DNA, and only compound (**33**) at 200 μM also inhibited DNA topoisomerases II-α, as observed by decatenation and relaxation assays. These biflavonoids showed concentration-dependent growth inhibitory activities on Ehrlich carcinoma cells in 45-h culture, assayed by a tetrazolium method, with IC_{50} = 24 ± 1.4 μM for (**33**), 26 ± 1.1 μM for (**2**) and 10 ± 0.7 μM for (**71**). These biflavonoids were assayed against human K562 leukemia cells in 45-h culture, but only (**33**) showed 42% growth inhibitory activity at 90 μM. Consequently, these biflavonoids are targets for DNA topoisomerases and their cytotoxicity is dependent on tumor cell type [40].

The biflavonoid II-2,3-dihydroochnaflavone (**72**) (Fig. (**18**)), isolated from the leaves of *Luxemburgia nobilis*, was cytotoxic to murine Ehrlich carcinoma (IC_{50} = 17.2 μM) and human leukemia K562 cells (IC_{50} = 89.0 μM) in a concentration-dependent manner in 45-h cell culture. The acetyl (= I-5,7,4′, II-5,7-penta-*O*-acetyl-II-2,3-dihydroochnaflavone) (**73**) and methyl (= I-7,4′, II-7-tri-*O*-methyl-II-2,3-dihydroochnaflavone) (**74**) (Fig. (**18**)) derivatives of (**72**) were not cytotoxic to these tumor cells at concentrations of 67.0 and 82.0 μM, respectively. The biflavonoids (**72**) and (**73**) inhibited the activity of human DNA topoisomerases I and II-α, as observed in relaxation and decatenation assays. In addition, the authors showed that (**72**) is a DNA interacting agent, thus causing DNA unwinding in a test with topoisomerase I. Also, the spectrophotometric titration of (**72**) with DNA resulted in a pronounced hypochromic effect [77].

e. Anti-inflammatory/Immunoregulatory Effects

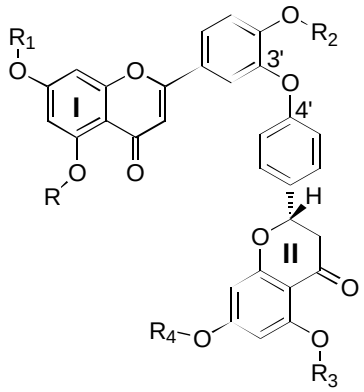
Biflavonoids behave as anticarcinogens by multiple anti-inflammatory mechanisms, as demonstrated *in vivo* [78-90]. The *Garcinia* biflavanones, GB_1 [= I-5,7,4', II-3,5,7,4'-heptahydroxy-(I-3, II-8)-biflavanone] (**67**) and GB_2 (**68**) (Fig. (**7**)), showed *in vivo* anti-inflammatory activity at 50 mg/kg i.p. against CGN-induced edema [81]. GB_1 (**67**) has been recently considered a potential dietary supplement or phytomedicine for the prevention of breast cancer and type 2 diabetes mellitus [91].

When topically applied, the *Ginkgo* biflavonoids, ginkgetin (**1**), amentoflavone (**2**), and sciadopitysin (**66**) (Fig. (**1**)), showed anti-inflammatory activity against croton-oil-induced ear edema. These compounds exhibited higher anti-inflammatory activity when a liposome formulation was used [78].

Morelloflavone (**24**) (Fig. (**7**)) and I-2,3, II-2,3-tetrahydro-amentoflavone (**75**) (Fig. (**1**)) showed *in vivo* anti-inflammatory activity by oral administration [79, 92]. However, in some studies, oral treatment resulted in a much reduced or no activity, suggesting that the oral bioavailability of biflavonoids may be very low [57, 93]. In contrast, i.p. administration resulted in higher anti-inflammatory activity. Topical treatment also yielded positive results [78, 80, 94, 95].

Amentoflavone (**2**) (Fig. (**1**)), a biflavone also isolated from *Selaginella* species, showed potent anti-inflammatory activity *in vivo*; by i.p. route had approximately 1/2-1/5 of the anti-inflammatory activity of indomethacin or prednisolone against several animal models of acute inflammation, including acetic acid-induced writhings in mice. However, amentoflavone (**2**) did not significantly reduce adjuvant-induced arthritis (AIA) in rats [80]. Additionally amentoflavone (**2**) produced significant (p<0.05) dose-dependent inhibition of oedema in the carrageenan-induced inflammation [96].

Morelloflavone (= fukugetin) (**24**) (Fig. (**7**)) and GB_{2a} (**30**) (Fig. (**7**)) prevented the carrageenan-induced paw oedema [97]. The synthetic (I-6, *O*, II-7)-biflavone (**76**) (Fig. (**19**)) showed potential as an anti-inflammatory agent [98]. A synthetic biflavone with a C-C (I-6, II-6)-linkage (BF6-6) (**77**) (Fig. (**5**)), having considerable anti-inflammatory activity, was enhanced by further substitution leading to I-5,7-dihydroxy-(I-6, II-6)-biflavone (G168) (**78**) (Fig. (**5**)) that showed a much stronger activity [99].

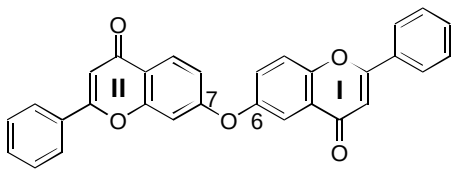


72 II-2,3-Dihydroochnaflavone $R = R_1 = R_2 = R_3 = R_4 = H$

73 I-5,7,4', II-5,7-Penta-*O*-acetyl-II-2,3-dihydroochnaflavone $R = R_1 = R_2 = R_3 = R_4 = Ac$

74 I-7,4', II-7-Tri-*O*-methyl-II-2,3-dihydroochnaflavone $R = R_3 = H$; $R_1 = R_2 = R_4 = Me$

Fig. (18). Chemical structures of (I-3', II-4')-flavone-flavanone dimers.



76 (I-6, *O*, II-7)-Biflavone

Fig. (19). Chemical structure of a (I-6, *O*, II-7)-biflavone.

Otuki *et al*. [100] have recently reported the effect of *Garcinia gardneriana* extracts from leaves, bark and seeds and two isolated compounds in ear oedema and leucocytes migration caused by croton oil. This plant is popularly used in skin disorders. The topical application of the leaf extract was able to reduce (70 ± 3%, and ID_{50} = 0.33 mg/ear) ear oedema, while the seed (51 ± 5%) and the wood (60 ± 12%) extracts were less effective. In a time-course evaluation, the leaf extract (1 mg/ear) was effective when applied 2 h before and until 3 h after the stimulation, presenting a higher effectiveness when applied right after croton oil (83 ± 7% inhibition). In addition, the leaf extract was able to diminish the myeloperoxidase (MPO) activity in 64 ± 13%, which suggested the inhibition of leucocyte infiltration that was confirmed by histological analysis. Also, both biflavonoids isolated from the leaves, morelloflavone (= fukugetin) (**24**) (Fig. (**7**)) and I-3-naringenin-II-8-eriodictyol (= GB_{2a}) (**30**) (Fig. (**7**)), were able to reduce ear oedema, with ID_{50} values of 0.18 (0.10-0.28) and 0.22 (0.15-0.31) mg/ear, respectively, besides the inhibition of MPO activity of 52 ± 6% and 64 ± 5%, respectively. Using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate, the leaf extract, morelloflavone (**24**) (Fig. (**7**)) and GB_{2a} (**30**) (Fig. (**7**)) topically applied to the ear treated with croton oil reduced 52 ± 15%, 63 ± 17% and 83 ± 4%, respectively, the ROS production of the skin. These results revealed the anti-inflammatory effect of *G. gardneriana* leaves for topical use, and both biflavonoids were responsible for this effect [100].

The anti-inflammatory effects of the biflavonoids ginkgetin (**1**) and isoginkgetin (**31**) (Fig. (**1**)) from caper fruits (*Capparis spinosa* L.; Capparidaceae) were evaluated by secreted placental alkaline phosphatase (SEAP) reporter assay, which was designed to measure NF-κB activation. These fruits have been widely used as food and folk medicine in the Mediterranean basin and in central and west Asia. Ginkgetin (**1**) and isoginkgetin (**31**) showed inhibitory effects in initial screen at 20 μM, while the effect of ginkgetin (**1**) was much higher than that of isoginkgetin (**31**). In a dose-response experiment, the IC_{50} value of ginkgetin (**1**) was estimated at 7.5 μM, suggesting it could be a strong NF-κB inhibitor and worthy of study *in vivo* [101].

e.1. Inhibition of Xanthine Oxidase (XO)

The seed of *Semecarpus anacardium* L. is widely used in Indian traditional medicine, Ayurveda and Sidha, for treatment of inflammatory disorders and gout. The activity guided fractionation was conducted using liquid-liquid partition and preparative HPLC. The fractions were evaluated for their XO inhibition, overexpression of which led to inflammation and gout, and antioxidant activity. The ethyl acetate fraction with the highest XO activity yielded tetrahydroamentoflavone (THA) (**75**) (Fig. (**1**)). IC_{50} values of THA for XO inhibition was 92 nM and its value for Ki was 0.982 μM. As a conclusion, THA is a potent XO inhibitor that could be considered as a drug candidate or chemopreventive agent after pharmacological and clinical evaluation. These results support the claim of the traditional medicine with respect to the efficacy of *S. anacardium* seed against inflammation and gout [102].

e.2. Inhibition of Proinflammatory Enzymes, Such as PLA_2 and COX

Research on anti-inflammatory biflavonoids is in the early stages and in continuous development. Based on initial studies, biflavonoids seem to use multiple anti-inflammatory mechanisms. Affect inflammatory cells such as mast cells and lymphocytes. Inhibit proinflammatory enzymes such as PLA_2 [103] and COX [104]. Some biflavonoids have been found to possess inhibitory effects on PLA_2 activity and lymphocytes proliferation, suggesting their anti-inflammatory/immunoregulatory potential.

PLA_2 is a growing family of distinct enzymes that exhibit different substrate specificities, cofactor requirement, subcellular localization, and cellular functions. Thus so far, 10 genes coding for structurally related and enzymatically active $sPLA_2$s have been identified in mammals (groups IB, IIA, IIC, IID, II, IIF, III, V, X, and XII). Since $sPLA_2$ is a pivotal enzyme to generate arachidonic acid that is converted further to proinflammatory eicosanoids, $sPLA_2$ inhibitors may show favorable anti-inflammatory activity. Actually, some of these biflavonoids were found to possess promising anti-inflammatory activity *in vivo*. In this respect, several biflavonoids have been synthesized and their inhibitory activities on $sPLA_2$-IIA were evaluated [105].

Lee *et al.* [106] studied the effects of several biflavonoids on arachidonic acid release from rat peritoneal macrophages, because arachidonic acid released from the activated macrophages is one of the indices of inflammatory conditions. When resident peritoneal macrophages labeled with [^{3}H]-arachidonic acid were activated by phorbol 12-myristate 13-acetate (PMA) or calcium ionophore, A23187, radioactivity released in the medium was increased 4.1 approximately 7.3 fold after 120 min incubation compared to the spontaneous release in the control incubation. In this condition, biflavonoids (10 μM), such as ochnaflavone (**70**) (Fig. (**17**)), ginkgetin (**1**) and isoginkgetin (**31**) (Fig. (**1**)), showed inhibition of arachidonate release from macrophages activated by PMA (32.5 approximately 40.0% inhibition) or A23187 (21.7 approximately 41.7% inhibition). Amentoflavone (**2**) (Fig. (**1**)) showed protection only against PMA-induced arachidonate release, while apigenin, a monomer of these biflavonoids, did not show the significant inhibition up to 10 μM. Staurosporin (1 μM), a PKC inhibitor, showed an inhibitory effect only against PMA-induced arachidonate release (96.8% inhibition). Inhibition of arachidonate release from the activated macrophages may contribute to an anti-inflammatory potential of biflavonoids *in vivo* [106].

Morelloflavone (**24**) (Fig. (**7**)) was reported as an inhibitor of $sPLA_2$ with selectivity for groups II and III enzymes. In addition, it showed anti-inflammatory activity apparently not related to the synthesis of eicosanoids, but likely dependent on other mechanisms, such as ROS scavenging [79].

Several biflavones such as amentoflavone (**2**) (Fig. (**1**)) and ochnaflavone (**70**) (Fig. (**17**)) have been also demonstrated as inhibitors of group II $sPLA_2$ ($sPLA_2$-IIA) [105].

Four classes of C–C biflavones, which have flavone-flavone subunit linkages at A ring–A ring, A ring–B ring, B ring–B ring, and B ring–C ring, were synthesized. The synthetic biflavones exhibited somewhat different inhibitory activities against $sPLA_2$-IIA depending on their chemical structures. Among them, the biflavone having a C–C (I-4', II-4')-linkage showed a potent inhibition comparable with that of the natural biflavonoid, ochnaflavone (**70**), and 7-fold stronger than that of amentoflavone (**2**) [105].

Recent research also showed that biflavonoids suppressed the expression of proinflammatory molecules. Therefore, biflavonoids have potential as anti-inflammatory drugs, especially for the treatment of chronic inflammatory disorders. Through more intensive studies with modern pharmacological techniques are

required, new types of anti-inflammatory agents based on biflavonoid structures may be successfully developed [57].

Recently, leaf and stem extracts of four species of South African *Podocarpus* species, e.g., *P. elongatus*, *P. falcatus*, *P. henkelii* and *P. arpus latifolius*, were evaluated for their anti-inflammatory, antioxidant and tyrosinase inhibitory activities [107]. DPPH, FRAP and β-carotene-linoleic acid assays were used to determine the antioxidant/radical scavenging activities of these species, which are used traditionally in folk medicine. Anti-inflammatory activity of these species was assayed against two cyclooxygenase enzymes (COX-1 and COX-2). Tyrosinase inhibition activity was analyzed using the modified dopachrome method with *L*-DOPA as the substrate. Phenolics were quantitatively determined using spectrophotometric methods. Stems of *Podocarpus latifolius* exhibited the lowest EC_{50} (0.84 μg/mL) inhibition against DPPH. The percentage antioxidant activity based on the bleaching rate of β-carotene ranged from 96% to 99%. High ferric reducing power was observed in all extracts. For COX-1, the lowest EC_{50} value was exhibited by stem extracts of *P. elongatus* (5.02 μg/mL) and leaf extract of *P. latifolius* showed the lowest EC_{50} against COX-2 (5.13 μg/mL). All extracts inhibited tyrosinase activity in a dose-dependent manner, with stem extract of *P. elongatus* being the most potent with an EC_{50} value of 0.14 mg/mL. The total phenolic content ranged from 2.38 to 6.94 mg of GAE/g dry sample. Therefore, the four species exhibited significant anti-inflammatory, antioxidant and anti-tyrosinase activity [107].

e.3. Effects on Cytokines Mediated COX-2 and iNOS Expression

Some flavonoid derivatives possess anti-inflammatory activity *in vitro* and *in vivo*. Besides their antioxidant properties and effects on the arachidonic acid metabolism, including COX/LOX inhibition, some flavones and flavonols were previously found to show inhibitory activity on NO production by iNOS (NOS type 2) through suppression of iNOS induction. The effects of prenylated flavonoids and biflavonoids on NO production from LPS-induced macrophage cell line (RAW 264.7) were evaluated in order to establish their inhibitory activity on NO production. This action correlated with the *in vivo* anti-inflammatory potential [108]. Among the derivatives tested, prenylated compounds, including morusin, kuwanon C, and sanggenon D, and biflavonoids such as ginkgetin (**1**) and bilobetin (**79**) (Fig. (**1**)) were found to inhibit NO production from LPS-induced RAW 264.7 cells at > 10 μM. Inhibition of NO production was mediated by suppression of iNOS enzyme induction, but not by direct inhibition of iNOS enzyme activity. While most prenylated derivatives showed cytotoxicity to RAW cells at 10-100 μM, all biflavonoids tested were not cytotoxic. Since NO produced by 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* [108].

Banerjee *et al.* [109] have previously demonstrated the potential effects of different flavonoids on cytokines mediated COX-2 and iNOS expression and activities on A549 cell line using quercetin, amentoflavone (**2**) (Fig. (**1**)) and flavanone. These data revealed that quercetin, at a concentration of 50 μM, inhibited PGE_2 biosynthesis by A549 very strongly with little effect on COX-2 mRNA and protein expression. Unlike quercetin, amentoflavone (**2**) inhibited PGE_2 biosynthesis as well as COX-2 mRNA and protein expression strongly. In another series of experiments, quercetin inhibited iNOS protein expression completely without affecting iNOS mRNA expression. Although amentoflavone (**2**) exerted no inhibitory effect on iNOS mRNA expression, weakly inhibited iNOS protein expression. Flavanone had no inhibitory effect on either enzyme at the same concentration. Taken together, these data indicated that amentoflavone (**2**) and quercetin differentially exerted supression of PGE_2 biosynthesis *via* downregulation of COX-2/iNOS expression [109].

Amentoflavone (**2**) (Fig. (**1**)) has been reported to induce apoptosis, and furthermore, this biflavone regulated nitric oxide and proinflammatory cytokine production in B16F-10 cells, TAMs and peritoneal macrophages [66].

Ochnaflavone (**70**) (Fig. (**17**)), a naturally occurring biflavonoid with anti-inflammatory activity [55], was isolated from *Lonicera japonica* and its effects on iNOS gene expression was examined in RAW264.7 cells. An inhibitor of ERK, U0126, significantly downregulated LPS-induced iNOS expression and promoter activity. Transactivation of LPS-stimulated NF-κB was inhibited by U0126. These results suggested that the transcription factor NF-κB is involved in ERK-mediated iNOS regulation and that activation of the Ras/ERK pathway contributed to the induction of iNOS expression in RAW264.7 cells in response to LPS. Ochnaflavone (**70**) treatment inhibited the production of NO in a concentration-dependent manner and also blocked the LPS-induced expression of iNOS. These inhibitory effects were associated with reduced ERK1/2 activity. Ochnaflavone (**70**) inhibited the phosphorylation of c-Jun NH_2-terminal kinase (JNK) and p38 mitogen-activated protein kinase. Suh *et al.* [110] showed that the inhibition of LPS-induced ERK1/2 activation may be a contributing factor to the main mechanisms by which ochnaflavone (**70**) inhibits RAW264.7. To clarify the mechanistic basis for its ability to inhibit iNOS induction, the authors examined the effect of ochnaflavone (**70**) on the transactivation of the iNOS gene by luciferase reporter activity. Ochnaflavone (**70**) potently suppressed reporter gene activity. Furthermore, LPS-induced iNOS expression was abolished by ochnaflavone (**70**) in RAW264.7 cells by blocking the inhibition of transcription factor NF-κB binding activities. These activities were associated with the downregulation of IκB kinase (IKK) activity by ochnaflavone (**70**) (6 μM), thus inhibiting LPS-induced phosphorylation as well as the degradation of IκBα. These findings suggested that the inhibition of LPS-induced NO formation by ochnaflavone (**70**) is due to its inhibition of NF-κB, thus being the likely mechanism for anti-inflammatory effects [110].

To find anti-inflammatory agents, the effects of six synthetic C-C biflavonoids linked with different C-C bonds between flavone monomers, e.g., I-4', II-4"; I-4', II-3"; I-4', II-6; I-3', II-6; I-6, II-6; and I-4', II-3, were examined on PGE_2 and NO production from LPS-treated macrophages, RAW 264.7 [111]. Among the compounds tested, the (I-3', II-6)-, (I-6, II-6)-, and (I-4', II-3)-linked biflavonoids showed a considerable inhibition of COX-2-mediated PGE_2 production at concentrations up to 50 μM, while the (I-4', II-6)-linked derivative exerted cytotoxic effects on RAW cells. Especially, the (I-6, II-6)-linked biflavonoid possessed the most potent inhibitory activity of PGE_2 production with an IC_{50} of 3.7 μM, compared with an IC_{50} of 8.2-20.7 μM of natural ginkgetin (**1**) (Fig. (**1**)). Western blot and reverse transcriptase-polymerase chain reaction analyses have shown that the inhibition of PGE_2 production by these synthetic derivatives was mediated at least in part by COX-2 inhibition, but not by COX-2 downregulation. These synthetic biflavonoids did not considerably inhibit iNOS-mediated NO production at concentrations up to 50 μM. When i.p. administered, the (I-6, II-6)-linked biflavonoid showed a significant anti-inflammatory activity (22.2% inhibition) against rat carrageenan-induced paw oedema at 5 mg/kg. Therefore, the (I-6, II-6)-linked biflavonoid may be used as a synthetic lead for developing new anti-inflammatory agents [111].

f. Modulation of Immune Response

The modulation of the immune response is highly relevant in tumor cell destruction. The research of Guruvayoorappan and Kuttan [112] focused on the effect of amentoflavone (**2**) (Fig. (**1**)), from *Biophytum sensitivum*, on cell-mediated immune responses in normal and tumor-bearing control animals. The treatment with amentoflavone (**2**) significantly enhanced the activity of natural killer cells in normal (42.8% cell lysis) and tumor-bearing animals

(48.2% cell lysis) on the fifth day, which was much earlier compared to tumor-bearing control animals (20.2% cell lysis on day 9). Antibody-dependent cellular cytotoxicity also increased in amentoflavone (**2**)-treated normal (41% cell lysis on day 9) and tumor-bearing animals (43.8% cell lysis on day 9) compared to untreated tumor-bearing control animals (maximum of 15.2% cell lysis on day 13) [112].

Amentoflavone (**2**) administration could significantly enhance the mitogen-induced splenocyte, thymocyte, and bone marrow cell proliferation. Treatment of amentoflavone (**2**) significantly elevated the production of IL-2 and interferon-γ in normal and Ehrlich ascites carcinoma-bearing animals. Moreover, amentoflavone (**2**) treatment significantly reduced the elevated levels of serum sialic acid and serum γ-glutamyl transpeptidase activity in tumor bearing animals [112].

g. Inhibition of Protein Tyrosine Phosphorylation

Protein tyrosine phosphorylation is one of the key mechanisms involved in signal transduction pathways, especially key oncogenic signaling pathways involved in cell proliferation, apoptosis, migration, and invasion. The balanced action of both protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) is responsible for its regulation. Deregulation of either one of these key enzymes leads to abnormal cellular signaling, which is largely associated with human pathologies. In cancer, oncogenic activation of tyrosine kinases is a common feature [113].

Although the role of PTKs in cancer is well established, less is known about the involvement of PTPs in carcinogenesis and tumor progression. PTPs have both positive and negative effects on signaling pathways. Several PTPs have been identified as critical oncoproteins in human malignancies that may be targeted with small chemical inhibitors as a therapeutic strategy [114]. Tumor suppressor PTPs, e.g., the phosphatase and tensin homolog (PTEN), have also been discovered as contributing factors in cancer development [115]. In addition, PTPs have been identified as key negative regulators of cytokines or immune cells. PTPs are considered to be involved in the etiology of diabetes mellitus, neurodegenerative diseases, regulation of allergy and inflammation, and they are even considered to be responsible for the pathogen virulence *in vivo* [116]. Targeting these negative PTPases may improve the efficacy of cytokine therapy and immunotherapy, which currently have modest response rates and limited survival benefit [114, 117].

Several inhibitors targeting PTKs have demonstrated their value in cancer treatment, while PTP inhibitors have only recently been developed [116, 118, 119]. Inhibition of PTP1B has been proposed as a therapy for treatment of type-2 diabetes and obesity, and also plays a significant role in carcinogenesis [116]. Prenylated flavanones and chalcones isolated from *Erythrina mildbraedii* have shown to behave as PTP1B inhibitors [120]. The occurrence of the prenyl group on the B ring of flavonoids is important for suppressing the enzyme PTP1B [121]. Recently, *ca.* 300 natural and synthetic secondary metabolites with inhibitory activity against PTP1B have been reviewed [122]. In particular, PTP inhibitory activity of the biflavonoid amentoflavone has been reported [116, 123]. Therefore, biflavonoids are potentially attractive PTP's targets for developing novel cancer therapeutics.

h. Antioxidant Activity

Another property with potential applicability is the antioxidant activity of biflavonoids. However, the antioxidant potency of biflavonoids appears to be lower than that of monoflavonoids despite the occurrence of almost twice the number of OH phenolic groups [124].

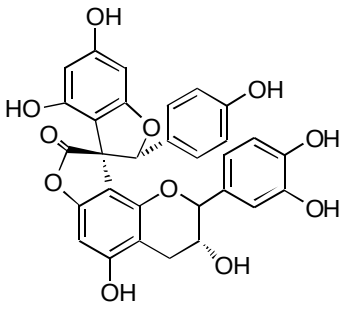
Nevertheless, a recent study showed that kolaviron, a mixture of biflavonoids, *e.g.*, GB_{1a} (**27**) (Fig. (**7**)), GB_1 (**67**), GB_2 (**68**), and kolaflavanone (**69**) (Fig. (**7**)), extracted from *Garcinia kola* seeds, has a protective effect against γ-radiation-induced oxidative stress in the brain of exposed rats [125]. Moreover, the protective effects of kolaviron on brain weight, behavioral performance, and against the neuro-destructive action of methamphetamine on hippocampal neurons of adult rats have been recently reported [126]. Different studies gave further evidence of the antioxidant potential of biflavonoids from the same source [127-133]. Also, morelloflavone (**24**) (Fig. (**7**)) showed strong antioxidant effects in both Fe^{2+}-mediated and non-metal-induced human LDL oxidations, exhibiting higher potency than the well-known antioxidant vitamin E in the same test systems [134]. Recently, morelloflavone (**24**), together with a glycosyl derivative and other natural compounds, were isolated from *Garcinia brasiliensis*, and synthetic penta-*O*-acetyl, penta-*O*-methyl, and penta-*O*-butanoyl derivatives of morelloflavone (**24**) were prepared, all showing leishmanicidal, antiproteolytic, and antioxidant activities in addition to low cytotoxicity. However, the activity was lower than that of butylhydroxytoluene (BHT), probably due to the reduced number of phenolic hydroxyl groups [135].

Bioactive biflavonoids were also recently obtained from other *Garcinia* species [136-140].

The antioxidant potential of the alcoholic extract of *Polyalthia cerasoides*, rich in phenolics, was evaluated using DPPH, hydroxyl radical, and superoxide anion scavenging. The extract showed significant ROS scavenging activity in all *in vitro* antioxidant assays. For *in vivo* genotoxic evaluation, Swiss albino mice were treated with the extract at the concentration of 40 mg/kg bw. Frequency of aberration was compared with control. Both sets did not show genotoxic effect. Furthermore, the extract was subjected to a cytotoxic study using MTT assay, showing moderate cytotoxicity against L929 cell line [141].

Recently, a biflavonoid identified as I-3,II-3-dihydroxy-cupressuflavone (**80**) (Fig. (**6**)), from *Camellia oleifera* Abel. showed stronger scavenging activity of DPPH and ABTS radicals than kaempferol. In addition, MDA decreased, SOD and GSH-Px activity increased significantly in serum ($p < 0.01$) and brain tissue ($p < 0.05$) of mice after intragastric administration of this biflavonoid at 200 mg/kg/day for 30 days [142].

Cancer chemoprevention, the prevention of cancer by ingestion of chemical agents that reduce the risk of carcinogenesis, is one of the potent ways to reduce morbidity and mortality. Wada *et al.* [143] have been searching for cancer chemopreventive agents from the leaves and barks of coniferous trees that were treated as waste in the forestry industry. Spirobiflavonoids, named abiesinols, occurring in the methanolic extract of the bark of *Abies sachalinensis*, were tested for inhibitory effects on the activation of (±)-(*E*)-methyl-2-[(*E*)-hydroxyimino]-5-nitro-6-methoxyhex-3-enamide (NOR 1), a NO donor, as a primary screening test for antitumor initiators. All compounds tested exhibited potent inhibitory effects on NOR 1 activation. Abiesinol A (**81**) (Fig. (**20**)) showed remarkable antitumor-initiating activity in the *in vivo* two-



81 Abiesinol A

Fig. (20). Chemical structure of the spirobiflavonoid abiesinol A.

stage mouse skin carcinogenesis test using peroxynitrite ($ONOO^-$) as the initiator and TPA as the promoter [143].

Recent fractionation of the methanolic extract of *Selaginella sinensis* (Selaginellaceae), which is extensively used in traditional Chinese medicine, led to an ethyl acetate fraction with a potent DPPH radical scavenging activity with the IC_{50} value of 44.9 μM [144]. Antioxidant peaks were screened using DPPH spiking test by HPLC (DPPH-HPLC), and two flavonoids and six biflavonoids were obtained, *e.g.*, quercetin, apigenin, ginkgetin (**1**), amentoflavone (**2**) (Fig. (**1**)), hinokiflavone (**10**) (Fig. (**2**)), robustaflavone (**11**), I-4'-*O*-methyl-robustaflavone (**32**) (Fig. (**3**)), and I-2,3-dihydro-amentoflavone (**82**) (Fig. (**1**)), which were separated by high-speed counter-current chromatography (HSCCC). The structures were identified by ESI-MS and NMR analysis. The antioxidant activity of the eight isolated compounds was assessed by the radical scavenging effect on DPPH radical, quercetin showing the strongest antioxidant activity with IC_{50} of 3.2 μM, while apigenin and compounds (**1**), (**2**) (Fig. (**1**)); (**10**) (Fig. (**2**)); (**11**), (**32**) (Fig. (**3**)); and (**82**) (Fig. (**1**)) showed weak antioxidant activities [144].

Recently, the analysis of the mechanism of intramolecular proton transfer in polar and nonpolar biflavonoid solutions by steady state luminescence and femtosecond spectroscopy has been reported, which may be important in relation to their antioxidant activity [145].

A recent report showed the effect of sciadopitysin (**66**) on osteoblast function by experiments in osteoblastic MC3T3-E1 cells [146]. Sciadopitysin (**66**) caused a significant increase of alkaline phosphatase activity, collagen synthesis, osteocalcin production, mineralization, and glutathione content in the cells (p <0.05). Sciadopitysin (**66**) also decreased the production of tumor necrosis factor-alpha (TNF-α) induced by antimycin A, a mitochondrial electron transport inhibitor. All experiments also demonstrated that sciadopitysin (**66**) may be useful for protecting mitochondria against a burst of oxidative stress. Moreover, sciadopitysin (**66**) increased phosphorylation of cAMP-response element-binding protein (CREB) inhibited by antimycin A. Therefore, sciadopitysin (**66**) may reduce or prevent osteoblasts degeneration *via* its antioxidant properties [146].

The number of known biflavonoids with potent antioxidant activity it is expected to considerably increase in the near future.

i. Analgesic Activity

The analgesic activity can lead to the development of superior anti-inflammatory agents. Some biflavonoids possess peripheral analgesic activity by i.p. injection. Ginkgetin (**1**) and amentoflavone (**2**) (Fig. (**1**)) showed a potent analgesic activity against writhings by i.p. injection, but not by oral administration [80, 84]. Similarly, GB_{1a} (**27**) (Fig. (**7**)) showed antinociceptive activity by i.p. injection on writhing test and formalin test [147]. II-4′-*O*-Methyl-GB_{2a} [naringenin-(I-3, II-8)-II-4'-*O*-methyleriodictyol] (**83**) (Fig. (**7**)) isolated from *Rheedia gardneriana* leaves also showed analgesic activity by i.p. injection [148]. The newly synthesized biflavonoid, I-5,7-dihydroxy-(I-6, II-6)-biflavone (**78**) (Fig. (**5**)), showed analgesic activity on acetic acid-induced writhing in mice [99].

Amentoflavone (**2**) (Fig. (**1**)) was isolated from the bioactive fractions of *Cnestis ferruginea*, used in the Traditional African Medicine for the treatment of various painful and inflammatory conditions. The analgesic activity was evaluated by the acetic acid-induced writhing and hot plate tests in mice, while carrageenan-induced paw oedema test was used for the anti-inflammatory action. Amentoflavone showed to be responsible for the analgesic and anti-inflammatory activity of this shrub [149].

A bimolecular kaempferol structure was obtained from shells of *Camellia oleifera*, and the anti-inflammatory and analgesic effects were evaluated [150]. Showing a dose-dependent anti-inflammatory activity by carrageenin-induced paw oedema in rats and croton oil induced ear inflammation in mice, and analgesic activity by hot plate test and acetic acid induced writhing. The mechanism of anti-inflammation of the biflavonoid was related to both bradykinin and prostaglandins synthesis inhibition. The biflavonoid showed both central and peripheral analgesic effects different from aspirin, inhibition of the synthesis or action of prostaglandins may contribute to the analgesic effect of biflavonoid. The biflavonoid significantly decreased malonaldehyde (MDA) and increased superoxidase dismutase (SOD) and Glutathione peroxidase (GSH-Px) activity in serum (p < 0.01), revealing a strong free radical scavenging activity *in vivo*. Therefore, this biflavonoid can control inflammation and pain by eliminating free radicals so as to inhibit the mediators, decreasing the prostaglandins [150].

CONCLUSIONS

Biflavonois have shown several bioactivities, e.g., anticancer, antibacterial, antifungal, antiviral, anti-inflammatory, analgesic, antioxidant, vasorelaxant, and anticlotting. Biflavonoids as anticarcinogens have called our attention, thus giving rise to the analysis and discussion of the mechanisms of action involved, such as induction of apoptosis [inhibition of cyclic nucleotide phosphodiesterases; effects on NF-κB family of transcription factors; activation of caspase(s); inhibition effects on bcl-2 expression, and upregulation of p53 and caspase-3 gene expression]; inhibition of angiogenesis [anti-proliferative effects; activation of Rho-GTPases and ERK signaling pathways; inhibition of FASN activity]; inhibition of pre-mRNA splicing; inhibition of human DNA topoisomerases I and II-α; anti-inflammatory/ immuno-regulatory effects [inhibition of XO; inhibition of proinflammatory enzymes, such as PLA_2 and COX; effects on cytokines mediated COX-2 and iNOS expression]; modulation of immune response; inhibition of protein tyrosine phosphorylation; antioxidant and analgesic activities in relation to the anticarcinogen behavior.

All these results indicate that biflavonoids strongly affect the cancer cells with little effect on normal cell proliferation, suggesting a therapeutic potential against cancer.

CONFLICT OF INTEREST

There are no potential conflicts of interest.

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ABBREVIATIONS

A-549	=	Human lung carcinoma
Bax	=	Bcl2-associated X protein
bw	=	Body weight
CAT	=	Catalase
COX	=	Cyclooxygenase
EC_{50}	=	Effective concentration to induce 50% death
ED_{50}	=	Effective dose that produces a therapeutic response in 50% of the subjects
ERK	=	Extracellular signal-regulated kinase
ESI-MS	=	Electrospray ionization mass spectrometry
DPPH	=	1,1-diphenyl-2-picrylhydrazyl radical
FASN	=	Fatty acid synthase
FPRL1	=	formyl peptide receptor-like 1
FS-5	=	foreskin fibroblast

GR	=	glutathione reductase
GSH	=	glutathione
GSH-Px	=	glutathione peroxidase
HeLa	=	human cervical carcinoma
HL60	=	acute promyelocytic leukemia cell line
HPLC	=	high-performance liquid chromatography
IC_{50}	=	half maximal inhibitory concentration of a substance
IκB	=	inhibitor *kappa*
B IL	=	interleukin
iNOS	=	inducible nitric oxide synthase
i.p.	=	intraperitoneal
JNK	=	c-Jun NH_2-terminal kinase
Jurkat cells	=	acute lymphoblastic leukemia cells
K562	=	chronic myeloid leukemia cell line
LDH	=	lactate dehydrogenase
LDL	=	low-density lipoprotein
LOX	=	lipoxygenase
LPS	=	lipopolysaccharide
MS	=	mass spectrometry
MDA	=	malondialdehyde
MPO	=	myeloperoxidase
MTT assay	=	3-(4:5-dimethylthiazol-2-yl)-2:5-diphenyltetrazolium bromide assay
NF-κB	=	nuclear factor *kappa* B
NMR	=	nuclear magnetic resonance
NO	=	nitric oxide
NOS	=	NO synthase
NOX	=	NADPH oxidase
OVCAR-3	=	human ovarian adenocarcinoma
PDEs	=	phosphodiesterases
PKC	=	protein kinase C
PLA_2	=	phospholipase A_2
PMA	=	phorbol 12-myristate 13-acetate
Raji cells	=	Raji B cells from Burkitt lymphoma
ROS	=	reactive oxygen species
SAR	=	structure-activity relationship
$sPLA_2$	=	secretory PLA_2
$sPLA_2$-IIA	=	group II $sPLA_2$
TAMs	=	tumor-associated macrophages
TNF-α	=	tumor necrosis factor *alpha*
TPA	=	12-*O*-tetradecanoylphorbol-13-acetate
VEGF	=	vascular endothelial growth factor
XO	=	xanthine oxidase
z-VAD-fmk	=	benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

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