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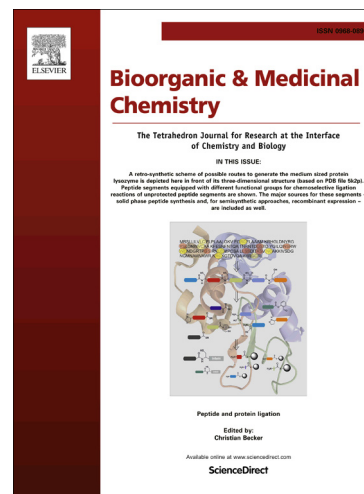
Please cite this article as: Estévez-Sarmiento, F., Said, M., Brouard, I., León, F., García, C., Quintana, J., Estévez, F., 3'-Hydroxy-3,4'-dimethoxyflavone Blocks Tubulin Polymerization and is a Potent Apoptotic Inducer in Human SK-MEL-1 Melanoma Cells, *Bioorganic & Medicinal Chemistry* (2017), doi: https://doi.org/10.1016/j.bmc.2017.09.043

3´-Hydroxy-3,4´-dimethoxyflavone Blocks Tubulin Polymerization and is a Potent Apoptotic Inducer in Human SK-MEL-1 Melanoma Cells

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Abbreviations

MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; PI, propidium iodide.

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Abstract

Flavonoids are naturally occurring polyphenolic compounds and are among the most promising anticancer agents. A series of flavonols and their 3-methyl ether derivatives were synthesized and assessed for cytotoxicity. It was found that 3´-hydroxy-3,4´-dimethoxyflavone (flavonoid **7a**) displayed strong cytotoxicity against human SK-MEL-1 melanoma cells and blocked tubulin polymerization, but had no significant cytotoxic effects against quiescent or proliferating human peripheral blood mononuclear cells. Our analyses showed that flavonoid **7a** induces G_2-M cell cycle arrest and apoptosis in melanoma cells which is associated with cytochrome *c* release and activation of both extrinsic and intrinsic apoptotic pathways of cell death.

1. Introduction

Natural products are important sources of potential new anticancer agents. Approximately 63% of anticancer drugs introduced over the last 25 years are natural products or can be traced back to a natural products source.[1] Flavonoids are secondary metabolites with low molecular weight and phenolic structure, occurring ubiquitously in food plants, and being a common component in the human diet. Many flavonoids display a vast array of biological activities including antitumor properties.[2,3] These properties of flavonoids are mediated by different types of cell cycle arrest and induction of apoptosis. This kind of cell death is characterized by DNA fragmentation, chromatin condensation, and formation of apoptotic bodies. It is mediated by the activation of a class of cysteine proteases, known as caspases, by two main pathways.[4,5] In the intrinsic pathway, diverse pro-apoptotic signals induce mitochondrial cytochrome *c* release to the cytosol, and promote apoptosome assembly and caspase-9 activation. In the extrinsic pathway, apoptosis is mediated by death receptors and involves caspase-8 activation. Both caspase-8 and caspase-9 activate caspase-3, which is responsible for cleaving specific cellular proteins during apoptosis.

Previous research has shown that flavonoids with a hydroxy group at position 3 are potential anticancer agents and that methylation of these compounds increases their antiproliferative activity against human tumor cells.[6-8] Knowledge of the effects of flavonoid substitution profiles on their antitumoral and tubulin polymerization inhibitory activity has witnessed much progress in recent years. Casticin and centaureidin (Fig. 1), are two important members of the family of flavones which show two methoxy groups at the C4´ and C3 positions and one hydroxy at the C3´ position and are promising anticancer agents due to their high cytotoxicity against a variety of cancer cells.
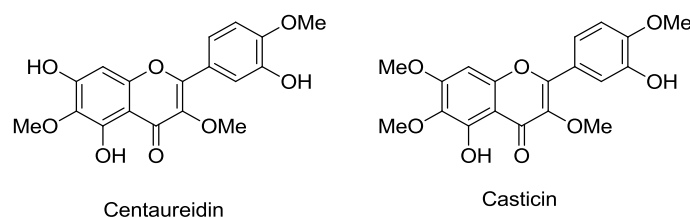
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**Fig. 1.** Chemical structure of anticancer 3-methoxyflavones.

Centaureidin was the first cytotoxic flavone described as a tubulin interactive agent and this was followed by the identification of a flavone which incorporated the same substitution profile as centaureidin and that inhibited the incorporation of tubulin into microtubules.[9,10] Recently, methoxylated flavones have been used as the lead compounds in the design of new analogues as tubulin inhibitors.[11] Analyses of structure and activity showed that i) compounds that contain the previously indicated substitution profile were more active as inhibitors of tubulin polymerization than those that did not, ii) the substitution of a methoxy in C3 for a hydroxy group dramatically decreased the activity of the compound, and iii) substitutions in the A ring do not seem to be relevant at all.[12] Studies in our research group confirmed that the substitution profile (methoxy on C3, C4´ and hydroxy on C3´) leads to compounds with antileukemic activity and significant capacity to inhibit tubulin polymerization.[13]

The potential significance of flavonoids containing this substitution pattern in antimelanoma therapy is largely unexplored to date. The main aim of this work was to investigate the cytotoxic effects of 15 synthetic flavonoids against SK-MEL-1 melanoma cells and elucidate the mechanism of cell death for the most effective compounds. The results show 3´-hydroxy-3,4´-dimethoxyflavone, **7a** to be the most cytotoxic compound against this cell line. We provide new evidence that shows for the first time that this flavonoid induces apoptotic cell death which is mediated by caspase activation and tubulin polymerization inhibition.

2. Results and discussion

2.1. Chemistry

In this manuscript, the design and synthesis of 15 flavonoids was developed with the purpose of determining the minimum structural requirements for a flavone to induce cell death in the human melanoma cell line SK-MEL-1. We focused mainly on the B ring substituents and the C-3 position (C ring). Four compounds were designed to contain the same pair of 3´-hydroxy-4´-methoxy substituents (B ring) and all four possible combinations of methoxy/hydrogen groups at position C-7 (**5a**, **5b**) and hydroxy/methoxy groups at C-3 (**7a**, **7b**). Additionally, the same compounds were prepared with a 4´-hydroxy-3´-methoxy pair of substituents in the B ring (**5c**, **5d** and **7c**, **7d**). Finally, the collection was completed with similar examples containing just one hydroxy group in the B ring (positions 4´ and 2´). Two acetophenones and four aldehydes were used as starting material for this collection of flavones (Scheme 1). The synthetic route did not differ from the traditional one. An aldol condensation reaction in basic media was used to generate eight compounds with a chalcone skeleton (**3a-h**) which were subsequently subjected to the Algar-Flynn-Oyamada cyclization under oxidative conditions to produce eight benzylated flavonols.[14] These compounds were submitted to hydrogenation reaction conditions to obtain the free alcohols (**5a-h**).[15] The methylation reaction employing MeI provided methyl ethers (**6a-g**) which were then deprotected in order to obtain the free alcohols (**7a-g**).[16]
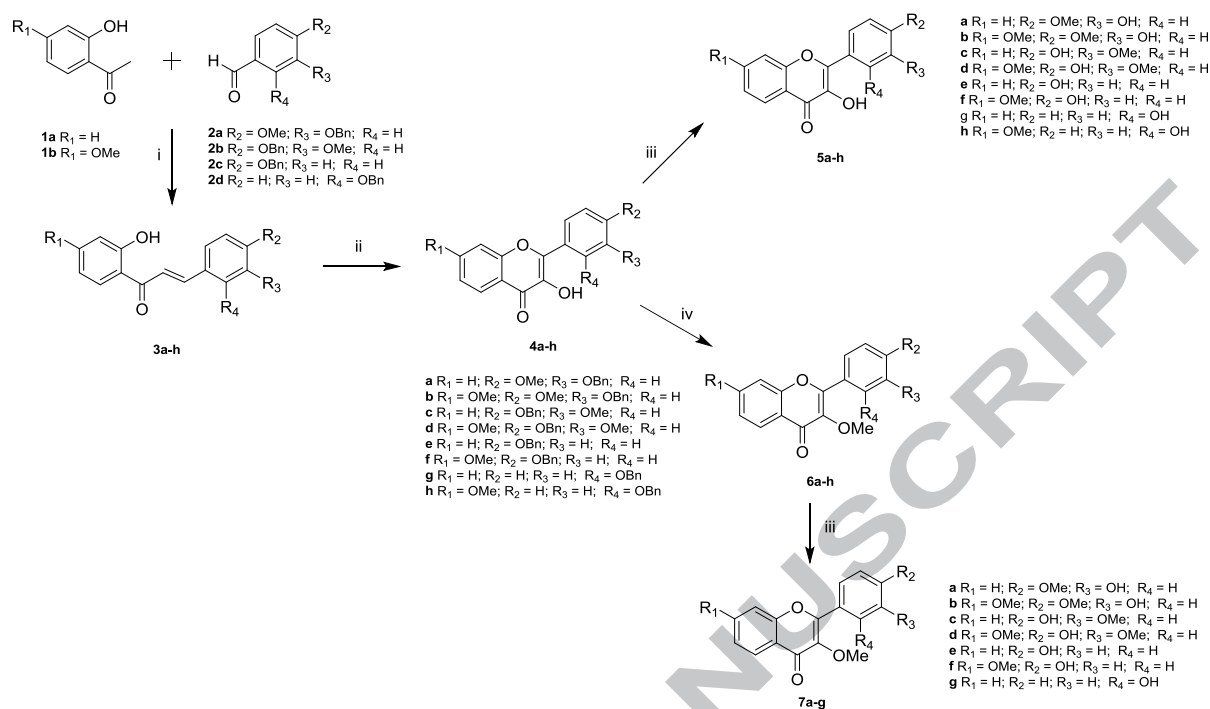
i) NaOHaq (50%), EtOH; ii) KOH/H_2O_2, MeOH; iii) H_2, Pd/C (10%), THF; iv) MeI, K_2CO_3, Acetone.

Scheme 1. Synthesis of the intermediate chalcones and methyl ethers.

2.2. Biological evaluation

2.2.1. A synthetic phenylbenzopyrone derivative, 3´-hydroxy-3,4´-dimethoxyflavone, inhibits the growth and cell viability of human SK-MEL-1 melanoma cells

Previous studies have demonstrated that the methoxyflavones have higher antiproliferative activity than the corresponding hydroxylated derivatives and that the presence of 3´-hydroxy-4´-methoxy groups on the B ring of the flavonoid skeleton enhances the cytotoxicity.[7,12,13,17]

In the present study, the potential cytotoxic properties of a series of 15 flavonoids, including flavonols and 3-methyl ether derivatives with one or two substituents on the B ring (2-phenyl group) and an additional substituent on the A ring were evaluated using the human melanoma cell line SK-MEL-1 (Scheme 1, Table 1). We tested antiproliferative activity with an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl

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tetrazolium bromide] assay. The compounds containing two substituents on the B ring were found to show significant differences in their cytotoxicity between the flavonols and their corresponding 3-methoxy derivatives (**5c** vs **7c** and **5a** vs **7a**). The 3-methylether derivative **7a** is more cytotoxic than the corresponding flavonol **5a**. The IC$_{50}$ values (the concentrations that induce a 50% inhibition of cell growth) for **7a** against SK-MEL-1 cells was 6.9 ± 1.1 μM, while the IC$_{50}$ value for **5a** was 32.6 ± 7.1 μM. The effects of the flavonoid **7a** on SK-MEL-1 cells viability were visualized by phase contrast microscopy. While control cells appeared perfectly round and healthy, there was a clear reduction in the number of cells and they appeared unhealthy after treatment with increasing concentrations of **7a** (Fig. 2A).
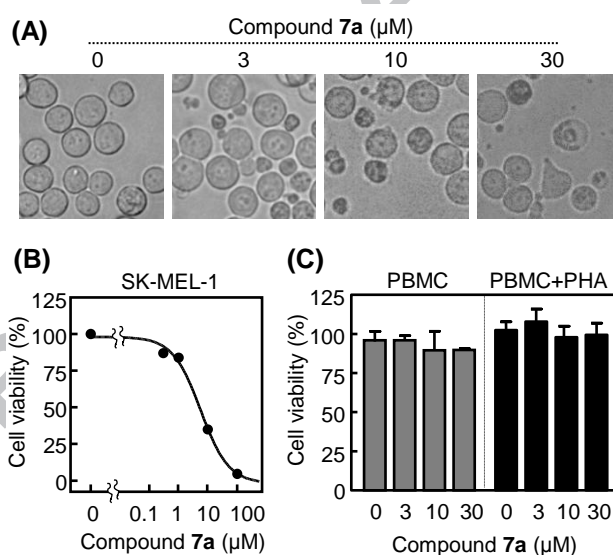

Fig. 2. Effects of **7a** on SK-MEL-1 melanoma cells viability. (A) Cells were incubated with vehicle or the described concentrations of **7a** for 24 h and images were obtained with an inverted phase-contrast microscope. (B) Cells were cultured in the presence of the described concentrations of **7a** for 72 h, and thereafter cell viability was determined by the MTT reduction assay. The results are representative of those obtained in at least three independent experiments. (C) Effect of **7a** on cell viability of peripheral blood mononuclear cells (PBMCs). Quiescent and phytohemagglutinine-activated PBMC cells

from healthy human origin were cultured in the presence of the specified concentrations of **7a** for 24 h. Values represent means ± SE of three independent experiments each performed in triplicate. *$P < 0.05$, significantly different from the untreated control.

Although there were no differences between the flavonols **5c** and **5a**, the position of the hydroxy and methoxy groups on the B ring seems to play a key role in determining the potency of 3-methoxyflavonols on cell viability (**7a** vs **7c**).

For the flavonols containing one substituent at the C7 position on the A ring and one hydroxy group on the B ring (**5f** and **5h**), the position of the hydroxy group on the B ring does not seem to be important, since the 4´-hydroxy derivative was inactive as well as the 2´-hydroxy derivative (**5f** vs **5h**) and methylation of the hydroxy group located at the C3 position did not affect antiproliferative activity either (**5f** vs **7f**). The presence of a methoxy at the C7 position on the A ring apparently is not a major determinant of cytotoxicity (**5g** vs **5h** and **7e** vs **7f**).

In the case of flavonols (**5b** vs **5d**) and their corresponding 3-methylethers (**7b** and **7d**) that contained one substituent in C7 on the A ring and two substituents on the B ring, there were significant differences in cytotoxicity against melanoma cells between the position of the hydroxy and methoxy groups on the B ring (**5d** vs **5b**). In the case of 3-methylether derivatives (**7b** vs **7d**), the substitution pattern of the latter groups on the B ring seems to play a key role in conferring cytotoxicity since the 3´-hydroxy-4´-methoxy derivative (**7b**) exhibited a higher potency than 4´-hydroxy-3´-methoxy derivative **7d**.

In the case of flavonoids containing 3´-hydroxy-4´-methoxy substituents on the B ring, the 3-hydroxy-7-methoxy flavonoid (**5b**) was more potent than the 3,7-dimethoxy

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derivative (**7b**). In all cytotoxicity assays the chemotherapeutic etoposide was used as a positive control (Table 1).

The cytotoxicity experiments revealed that 3´-hydroxy-3,4´-dimethoxyflavone (**7a**) displays strong cytotoxic activity against the human melanoma cell line SK-MEL-1 with an IC_{50} value as low as 6.9 μM (Table 1, Fig. 2B). Moreover, we also assayed the effects of **7a** on the growth of the human lymphocytes and found that it did not significantly affect cell viability of quiescent and proliferating cells (Fig. 2C). Since **7a** was the most cytotoxic compound we decided to investigate whether this flavonoid displays its cytotoxic action on SK-MEL-1 cells through activation of the apoptotic pathway.

Table 1. Effects of flavonoids on the growth of human SK-MEL-1 melanoma cells.

Compound	**5a**	**5b**	**5c**	**5d**	**5e**	**5f**	**5g**	**5h**
IC_{50} (μM)	32.6 ± 7.1	15.5 ± 2.8	22.0 ± 5.8	37.0 ± 5.0	>100	>100	>100	>100
Compound	**7a**	**7b**	**7c**	**7d**	**7e**	**7f**	**7g**	**Etoposide**
IC_{50} (μM)	6.9 ± 1.1	26.2 ± 1.9	79.0 ± 13.1	52.0 ± 8.2	91.0 ± 11.1	>100	>100	8.0 ± 4.1

Cells were cultured for 72 h in presence of the indicated compounds and the IC_{50} values were calculated using a colorimetric MTT assay. The data shown represent the mean ± SEM of 2-3 independent experiments with three determinations in each.

*2.2.2. **7a** induces G_2-M arrest and apoptosis on human SK-MEL-1 melanoma cells*

To determine whether **7a**-induced cytotoxicity involves changes in cell cycle progression, we evaluated the effect of **7a** on cell-cycle phase distribution by flow cytometry. As shown in Table 2 and Fig. 3A, **7a** caused a significant G_2–M arrest at the expense of G_1 phase cell population in SK-MEL-1 cell line at all concentrations analysed (3-30 μM) and an increase in the percentage of sub-G_1 cells (apoptotic cells) as determined by flow cytometry after propidium iodide labeling. Flavonoid **7a** increased apoptosis and this effect is concentration-dependent. The percentage of sub-G_1 increased up to 22 % after 24 h of treatment with 30 μM **7a** (Fig. 3A). The morphological changes characteristic of apoptotic cells (condensed and fragmented

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chromatin) after treatment with **7a** were also visualized by fluorescent microscopy (Fig.

3B). Taken together, these results indicate that **7a** induces cell-cycle arrest in the G_2–M

phase and apoptosis on human melanoma cells.

Table 2. Effect of different durations of treatment with **7a** on cell cycle phase distribution of human SK-MEL-1 melanoma cells.

	% Sub-G_1	% G_1	% S	% G_2-M
Control	4.5 ± 1.6	61.5 ± 1.7	17.7 ± 0.4	16.1 ± 0.2
3 µM **7a**	15.9 ± 1.1*	24.4 ± 0.5*	21.4 ± 1.2*	37.9 ± 2.6*
10 µM **7a**	9.2 ± 1.7*	14.4 ± 1.6*	23.7 ± 0.4	52.2 ± 3.9*
30 µM **7a**	21.9 ± 7.5*	30.7 ± 0.9*	26.8 ± 1.8*	20.2 ± 10.1*

Cells were cultured with increasing concentrations of **7a** for 24 h and the cell cycle phase distribution was determined by flow cytometry. The values are means ± SE of three independent experiments with three determinations in each. Asterisks indicate a significant difference ($P<0.05$) compared with the corresponding controls.
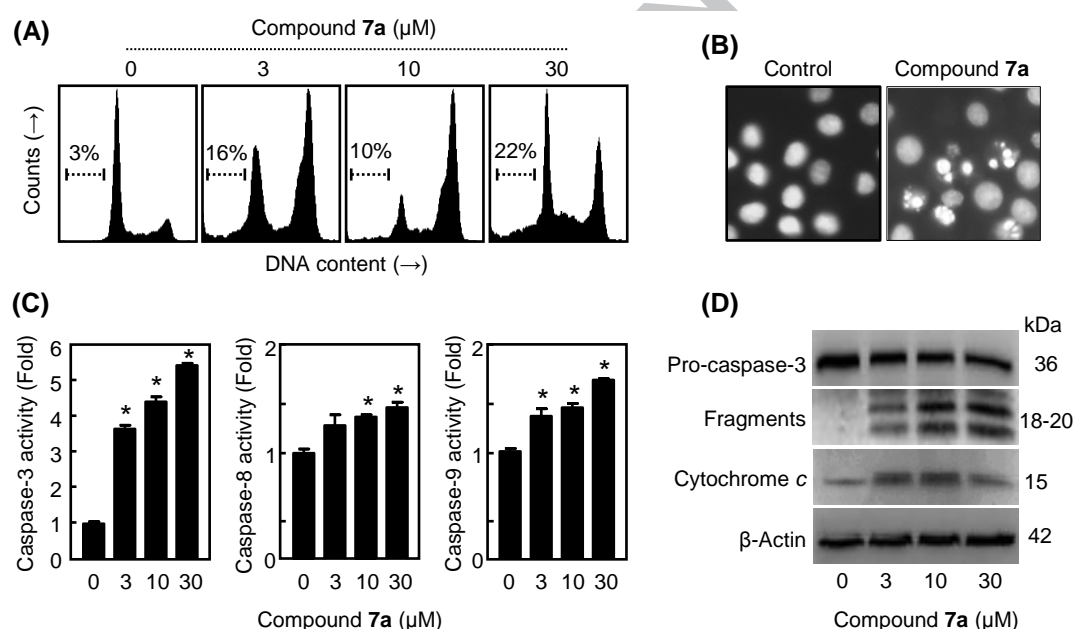

Fig. 3. Effects of **7a** on apoptosis induction in SK-MEL-1 melanoma cells. (A) Cells

were incubated with increasing concentrations of **7a** for 24 h, subjected to flow

cytometry using propidium iodide labeling and the percentage of cells in the sub-G_1

region (apoptotic cells) determined. (B) Photomicrographs of representative fields of

SK-MEL-1 cells stained with bisbenzimide trihydrochloride to evaluate nuclear

chromatin condensation (i.e. apoptosis) after treatment for 24 h with 30 µM of **7a**. (C)

Activation of caspases in response to **7a**. The cells were treated with the described

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concentrations of **7a** and cell lysates were assayed for caspase 3/7, -8 and -9 activities.

Results are expressed as factorial increases in caspase activity relative to the control.

Values represent the means ± SEs of three independent experiments each performed in

triplicate. $^{*}P$< 0.05, significantly different from untreated control. (D) Effects of **7a** on

processing of pro-caspase-3 and mitochondrial cytochrome c release. Cells were

incubated in the presence of the described concentrations of **7a**, and whole cell lysates

or cytosolic fractions (in the case of cytochrome c) were probed with antibodies raised

against the indicated proteins by immunoblotting. β-Actin was used as a loading

control.

*2.2.3. **7a** induces Cell Death by a Caspase-Dependent Pathway in SK-MEL-1 cells*

To analyze whether the effect of **7a** is mediated by caspases, we examined whether this

flavonoid induces the enzymatic activities of caspase-3-like proteases (caspase-3/7) and

of caspase-8 and -9. To this end, cells were treated with increasing concentrations of **7a**

and cell lysates were assayed for the cleavage of the tetrapeptides DEVD-*p*NA, IETD-

*p*NA and LEHD-*p*NA as specific substrates for caspase-3/7, caspase-8 and caspase-9,

respectively. All these caspase activities were significantly detectable after 24 h of

treatment with **7a** (Fig. 3C). Dose-response experiments show that a low concentration

(3 μM) of **7a** was sufficient to induce caspase-3/7 activation, while caspase-8 and -9

caused lower levels of activation.

To determine whether **7a**-triggered apoptosis involves the processing of caspase-3, cells

were treated with increasing concentrations of the flavonoid for 24 h and this caspase

was analysed by Western blot. As shown in Figure 3D, **7a** stimulated the cleavage of

the main executioner caspase. It was also studied whether this apoptotic effect of **7a**

involves the release of mitochondrial cytochrome c. The results showed a significant

increase in the amount of this proapoptotic protein in the cytosol after treatment with **7a**

in a concentration-dependent manner (Fig. 3D). Taken together these data show that both the intrinsic and the extrinsic pathways play an important role in cell death induced by **7a** in SK-MEL-1 melanoma cells.

*2.2.4. Effect of **7a** on Tubulin Polymerization*

As shown before, concentrations of flavonoid **7a** that inhibited SK-MEL-1 proliferation caused an increase in the number of cells in the G_2–M phase at 24 h, taking into account that these cells have long doubling times of approximately 72 h. Several flavonoids have been reported to exert their antiproliferative activity by targeting microtubules through tubulin binding.[12] One of the structural requirements usually found in flavones for tubulin interaction is the presence of a methoxy group at C-3. Since **7a** contains a methoxy group at C-3, G_2–M arrest induced by this flavonoid might be explained by the inhibition of microtubule formation or by changes in the expression and/or activity of G_2–M cell-cycle regulators. The flavonoid quercetin has been shown to inhibit cancer cell proliferation by depleting cellular microtubules and inhibiting microtubule polymerization through tubulin binding.[18] To confirm this hypothesis we used purified tubulin (containing no microtubule-associated proteins) in our assay and found that **7a** led to a concentration-dependent inhibition of tubulin assembly. Colchicine and taxol were used as positive controls of inhibition and promotion of tubulin polymerization, respectively (Fig. 4). Further studies are needed to determine the effect of this compound on these regulators such as the cyclin-dependent kinase-1, cyclin-dependent kinase inhibitor p21[Cip1], B-type cyclin isoforms, and cdc25C.
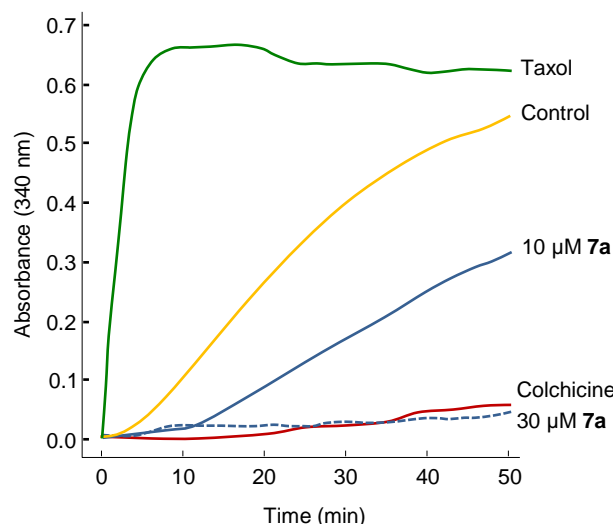
Fig. 4. Flavonoid **7a** blocks the tubulin polymerization *in vitro*. Purified tubulin protein in a reaction buffer was incubated at 37 ºC in the absence (control) or in the presence of taxol (10 μM), colchicine (5 μM) or the indicated concentrations of **7a** and the absorbance at 340 nm was measured using a microplate reader.

3. Conclusions

We have synthesized 15 flavonoids, including eight flavonols and seven 3-methyl ethers, with one or two substituents on the B ring and an additional substituent on the A ring, and evaluated their effects on cell viability of the malignant cell line SK-MEL-1. The B ring substituents included the pairs of 3´-hydroxy-4´-methoxy or 4´-hydroxy-3´-methoxy or just one hydroxy group. The 3-methoxyflavone **7a**, containing a 3´-hydroxy-4´-methoxy pair of substituents in the B ring, was the most cytotoxic compound against melanoma cells but had low cytotoxicity against human lymphocytes. The fact that human melanoma SK-MEL-1 cells were sensitive to **7a** is extremely interesting given that melanoma is the most aggressive and lethal skin cancer and frequently resists chemotherapy.

Relatively few studies have analysed the potential of flavonoids in antimelanoma therapy. Tamarixetin (3,5,7,3´-tetrahydroxy-4´-methoxyflavone) and dihydromyricetin are two known flavonoids that have been reported to display antiproliferative capacity against melanoma cells.[19,20] Tamarixetin exhibits cytotoxic properties on SK-MEL-1 cells but the pathways involved in cell death remain to be explored,[19] and comparative studies indicate that its cytotoxic capacity is three times lower than **7a** (IC_{50} = 23.1 ± 2.1 μM vs. 6.9 ± 1.1 μM). Dihydromyricetin suppress cell proliferation of SK-MEL-28 melanoma cells by inducing cell cycle arrest at the G_1 phase and apoptosis, although this occurs at higher concentrations than were used for flavonoid **7a**.

In summary, the present study on the human melanoma cells SK-MEL-1 demonstrates that the flavonoid **7a** arrested the cell cycle at the G_2-M phase, induced apoptotic cell death and blocked tubulin polymerization. Apoptosis was confirmed by i) fluorescent microscopy, ii) the appearance of a subdiploid DNA content and iii) caspase-3 activation. The antiproliferative effect of **7a** is associated with cytochrome *c* release and with the activation of the initiator caspases involved in the extrinsic and the intrinsic pathways of cell death.
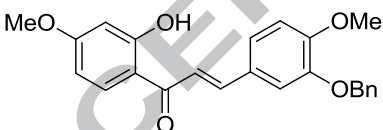
4. Experimental

4.1. General information

Compounds used as starting materials and reagents were obtained from Aldrich Chemical Co., or other chemical companies and utilized without further purification. Melting points are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded at 400 and 500 MHz for ^{1}H and 100 and 125 MHz for ^{13}C, respectively. Chemical shifts (δ) are reported in parts per million relative to the residual solvent signals, and coupling constants (*J*) are reported in hertz. High resolution ESI mass spectra were obtained

from a Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer, an RF-only hexapole ion guide, and an external electrospray ion source. Flash column chromatography was carried out using silica gel 60 (230−400 mesh), and analytical thin layer chromatography (TLC) was performed using silica gel aluminum sheets. All commercially available chemicals were used without further purification.

4.1.2. General procedure for the synthesis of chalcones (3a-3h)

A mixture of the acetophenone (5-10 mmol, 1 equiv) and the corresponding aldehyde (1 equiv) in EtOH (20-40 mL) was stirred at room temperature and a 50% aqueous solution of NaOH (5-8 mL) was added. The reaction mixture was stirred at room temperature until aldehyde consumption. After that, HCl (10%) was added until neutrality. Precipitated chalcones were generally filtered and crystallized from MeOH, although in some cases the product was purified using column chromatography. Chalcones **3a**, **3c**, **3e** and **3f** have been previously described.[21-25]

4.1.2.1. 3-Benzyloxy-2´-hydroxy-4,4´-dimethoxychalcone (3b)

Orange solid, mp 145-146 ºC (65%). IR (KBr, cm^{-1}) v_{max}: 2956, 1716, 1683, 1635, 1607, 1558, 1540, 1508, 1456, 1436, 1374, 1260, 1236, 1156, 1130, 1020, 846. ^{1}H NMR (500 MHz, CDCl$_3$) δ = 13.44 (s, 1H, OH); 7.69 (d, 1H, J = 15.6 Hz, H-β); 7.67 (d, 1H, J = 8.3 Hz, H-6´); 7.40 (d, 2H, J = 7.5 Hz, 2xH-Ph); 7.31 (tt, 2H, J = 7.2, 1.5 Hz, 2xH-Ph); 7.26-7.22 (m, 2H, H-Ph and H-α); 7.15 (dd, 1H, J = 8.3, 1.9 Hz, H-6); 7.10 (d, 1H, J = 2.0 Hz, H-2); 6.83 (d, 1H, J = 8.3 Hz, H-5); 6.41-6.37 (m, 2H, H-5´ and H-3´); 5.12 (s, 2H, CH$_2$-Ph); 3.85 (s, 3H, CH$_3$O-C-4); 3.77 (s, 3H, CH$_3$O-C-4´). ^{13}C NMR (125 MHz, CDCl$_3$) δ = 191.7 (C=O), 166.6 (C-2´), 166.0 (C-4´), 152.3 (C-4), 148.4 (C-3), 144.4 (C-β), 136.8 (C-Ph), 131.1 (C-6´), 128.7 (C-Ph), 128.1 (C-Ph), 127.7 (C-1), 127.4 (C-Ph), 123.6 (C-6), 118.1

(C-α), 114.1 (C-1´), 113.4 (C-2), 111.6 (C-5), 107.6 (C-5´), 101.0 (C-3´), 71.3 ($\underline{C}H_2$-Ph), 56.0 ($\underline{C}H_3O$-C-4), 55.6 ($\underline{C}H_3O$-C-4´). HRMS (ESI-FT-ICR) *m/z*: 413.1371 [M+Na]$^+$; calcd. for $C_{24}H_{22}O_5Na$: 413.1365.

4.1.2.2. 4-Benzyloxy-2´-hydroxy-3,4´-dimethoxychalcone (3d)
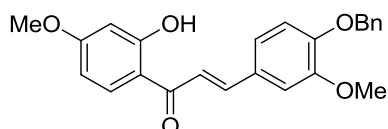
Orange solid, mp 153-155 ºC (70%). IR (KBr, cm^{-1}) v_{max}: 2936, 1633, 1571, 1508, 1454, 1443, 1420, 1370, 1338, 1297, 1259, 1213, 1128, 1019, 959, 846. ^{1}H
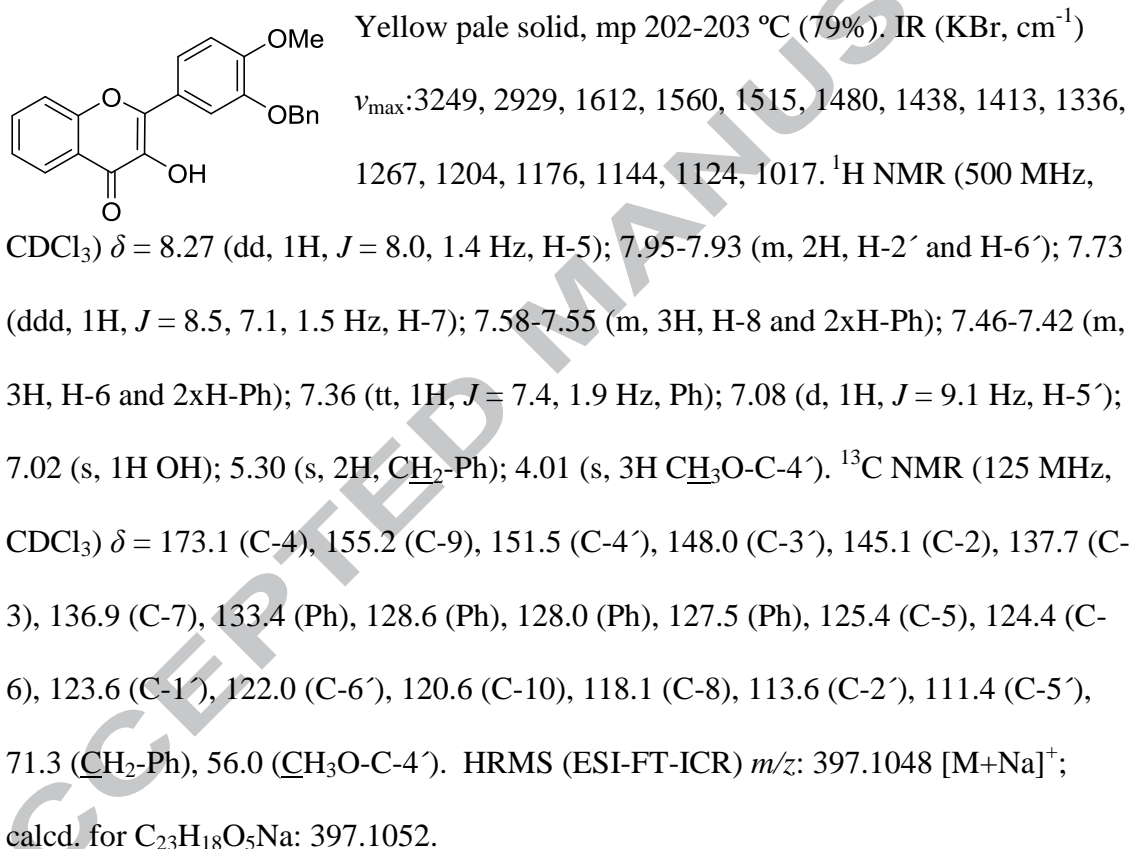
NMR (500 MHz, CDCl$_3$) δ = 13.58 (s, 1H, OH); 7.87 (d, 1H, *J* = 15.3 Hz, H-β); 7.85 (d, 1H, *J* = 8.8 Hz, H-6´); 7.49-7.47 (m, 2H, 2xH-Ph); 7.44-7.42 (m, 3H, H-α and 2xH-Ph); 7.36 (t, 1H, *J* = 7.5 Hz, Ph); 7.22-7.21 (m, 2H, H-2 and H-6); 6.95 (d, 1H, *J* = 8.8 Hz, H-5); 6.53-6.51 (m, 2H, H-5´ and H-3´); 5.24 (s, 2H, C$\underline{H}_2$-Ph); 4.00 (s, 3H, $\underline{C}H_3O$-C-3); 3.88 (s, 3H, $\underline{C}H_3O$-C-4´). ^{13}C NMR (125 MHz, CDCl$_3$) δ = 191.7 (C=O), 166.6 (C-2´), 166.1 (C-3 or C-4´), 150.7 (C-4), 149.8 (C-3 or C-4´), 144.5 (C-β), 136.5 (Ph), 131.1 (C-6´), 128.6 (Ph), 128.1 (C-1), 128.1 (Ph) , 127.2 (Ph), 123.0 (C-2 or C-6), 118.1 (C-α), 114.1 (C-1´), 113.5 (C-5), 110.9 (C-2 or C-6), 107.6 (C-5´), 101.0 (C-3´), 70.9 ($\underline{C}H_2$-Ph), 56.1 ($\underline{C}H_3O$-C-3), 55.6 ($\underline{C}H_3O$-C-4´). HRMS (ESI-FT-ICR) *m/z*: 413.1370 [M+Na]$^+$; calcd. for $C_{24}H_{22}O_5Na$: 413.1365.

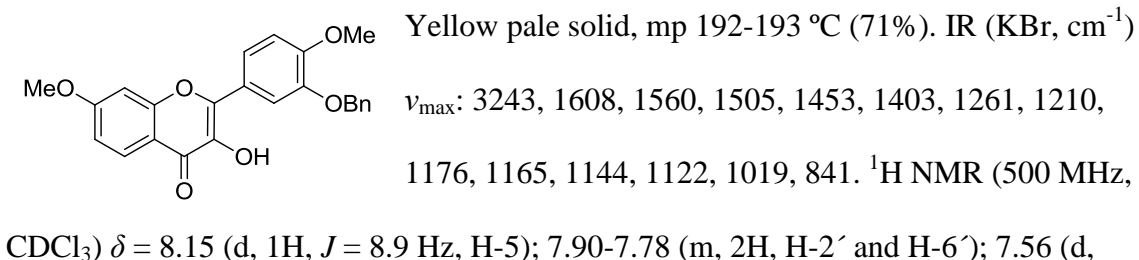### *4.1.3. General procedure for the synthesis of flavonols*

A solution of the corresponding 2-hydroxychalcone (2-4 mmol) in 3.0 M KOH in MeOH (20-30 mL) was cooled at 0 ºC. An aqueous solution of H$_2$O$_2$ (30%) (5-8 mL) was added to the chalcone solution. The resulting mixture was stirred at room temperature, until the starting material was totally consumed, as evidenced by TLC. The reaction mixture was cooled in an ice bath and distilled water (2-4 mL) was added. HCl

(2M) was added until pH 2 and the precipitate was filtered and washed with distilled water and extracted with EtOAc. The organic layer was washed with brine until neutrality and dried with anhydrous $MgSO_4$. The solvent was evaporated in vacuo and the residue was purified using a chromatographic column [SiO_2, petroleum ether:EtOAc (1:0-7:3)]. Flavonols **4c** and **4e** have been previously described.[26,27]

*4.1.3.1. 2-[3-(Benzyloxy)-4-methoxyphenyl]-3-hydroxy-4H-chromen-4-one (**4a**)*

Yellow pale solid, mp 202-203 ºC (79%). IR (KBr, cm^{-1}) v_{max}:3249, 2929, 1612, 1560, 1515, 1480, 1438, 1413, 1336, 1267, 1204, 1176, 1144, 1124, 1017. ^{1}H NMR (500 MHz, CDCl$_3$) δ = 8.27 (dd, 1H, J = 8.0, 1.4 Hz, H-5); 7.95-7.93 (m, 2H, H-2´ and H-6´); 7.73 (ddd, 1H, J = 8.5, 7.1, 1.5 Hz, H-7); 7.58-7.55 (m, 3H, H-8 and 2xH-Ph); 7.46-7.42 (m, 3H, H-6 and 2xH-Ph); 7.36 (tt, 1H, J = 7.4, 1.9 Hz, Ph); 7.08 (d, 1H, J = 9.1 Hz, H-5´); 7.02 (s, 1H OH); 5.30 (s, 2H, C$\underline{H}_2$-Ph); 4.01 (s, 3H C$\underline{H}_3$O-C-4´). ^{13}C NMR (125 MHz, CDCl$_3$) δ = 173.1 (C-4), 155.2 (C-9), 151.5 (C-4´), 148.0 (C-3´), 145.1 (C-2), 137.7 (C-3), 136.9 (C-7), 133.4 (Ph), 128.6 (Ph), 128.0 (Ph), 127.5 (Ph), 125.4 (C-5), 124.4 (C-6), 123.6 (C-1´), 122.0 (C-6´), 120.6 (C-10), 118.1 (C-8), 113.6 (C-2´), 111.4 (C-5´), 71.3 ($\underline{C}H_2$-Ph), 56.0 ($\underline{C}H_3$O-C-4´). HRMS (ESI-FT-ICR) *m/z*: 397.1048 [M+Na]$^+$; calcd. for $C_{23}H_{18}O_5Na$: 397.1052.

*4.1.3.2. 2-[3-(Benzyloxy)-4-methoxyphenyl]-3-hydroxy-4H-chromen-4-one (**4b**)*

Yellow pale solid, mp 192-193 ºC (71%). IR (KBr, cm^{-1}) v_{max}: 3243, 1608, 1560, 1505, 1453, 1403, 1261, 1210, 1176, 1165, 1144, 1122, 1019, 841. ^{1}H NMR (500 MHz, CDCl$_3$) δ = 8.15 (d, 1H, J = 8.9 Hz, H-5); 7.90-7.78 (m, 2H, H-2´ and H-6´); 7.56 (d,

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2H, J = 8.4 Hz, 2xH-Ph); 7.45-7.42 (m, 2xH-Ph); 7.39-7.34 (m, 1H, Ph); 7.05 (d, 1H, J = 8.6 Hz, H-5´); 7.01 (dd, 1H, J = 8.9, 2.2 Hz, H-6); 6.92 (d, 1H, J = 2.3 Hz, H-8); 5.29 (s, 2H, C$\underline{H}_2$-Ph); 4.00 (s, 3H, C$\underline{H}_3$O-C-4´); 3.97 (s, 3H, C$\underline{H}_3$O-C-7). ^{13}C NMR (125 MHz, CDCl$_3$) δ = 172.4 (C-4), 164.1 (C-7), 157.1 (C-9), 151.2 (C-4´), 147.9 (C-3´), 144.4 (C-2), 137.3 (C-3), 136.9 (Ph), 128.6 (Ph), 128.0 (Ph), 127.6 (Ph), 126.7 (C-5), 123.7 (C-1´), 121.6 (C-6´), 114.6 (C-6), 114.6 (C-10), 113.7 (C-2´), 111.4 (C-5´), 99.8 (C-8), 71.3 ($\underline{C}$H$_2$-Ph), 56.0 ($\underline{C}$H$_3$O-C-4´), 55.8 ($\underline{C}$H$_3$O-C-7). HRMS (ESI-FT-ICR) m/z: 427.1162 [M+Na]$^+$; calcd. for C$_{24}$H$_{20}$O$_6$Na: 427.1158.

4.1.3.3. 2-[4-(Benzyloxy)-3-methoxyphenyl]-3-hydroxy-7-methoxy-4H-chromen-4-one (4d)
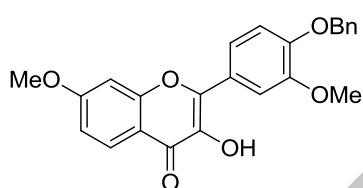
Yellow pale solid, mp 160-162 ºC (55%). IR (KBr, cm^{-1}) ν_{max}: 3422, 2923, 2851, 2009, 1651, 1616, 1506, 1455, 1401, 1262, 1213, 1012. ^{1}H NMR (500 MHz, CDCl$_3$) δ = 8.11 (d, 1H, J = 8.8 Hz, H-5); 7.84 (d, 1H, J = 2.0 Hz, H-2´); 7.77 (dd, 1H, J = 8.6, 2.1 Hz, H-6´); 7.46 (d, 2H, J = 7.2 Hz, 2xH-Ph); 7.38 (t, 2H, J = 7.4 Hz, 2xH-Ph); 7.35–7.29 (m, 1H, Ph); 7.00 (d, 1H, J = 8.6 Hz, H-5´); 6.98 (dd, 1H, J = 8.9, 2.3 Hz, H-6); 6.92 (d, 1H, J = 2.4 Hz, H-8); 5.24 (s, 2H, C$\underline{H}_2$-Ph); 3.99 (s, 3H, C$\underline{H}_3$O-C-3´); 3.92 (s, 3H, C$\underline{H}_3$O-C-7). ^{13}C NMR (125 MHz, CDCl$_3$) δ = 172.6 (C-4), 164.1 (C-7), 157.1 (C-9), 149.6 (C-4´), 149.4 (C-3´), 144.5 (C-2), 137.4 (C-3), 136.6 (Ph), 128.6 (Ph), 128.0 (Ph), 127.2 (Ph), 126.7 (C-5), 124.2 (C-1´), 120.9 (C-6´), 114.7 (C-6), 114.6 (C-10), 113.3 (C-5´), 111.1 (C-1´), 99.8 (C-8), 70.8 ($\underline{C}$H$_2$-Ph), 56.2 ($\underline{C}$H$_3$O-C-3´), 55.8 ($\underline{C}$H$_3$O-C-7). HRMS (ESI-FT-ICR) m/z: 427.1153 [M+Na]$^+$; calcd. for C$_{24}$H$_{20}$O$_6$Na: 427.1158.

### 4.1.3.4. 2-[4-(Benzyloxy)phenyl]-3-hydroxy-7-methoxy-4H-chromen-4-one (4f)
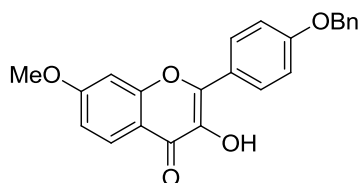
Yellow pale solid, mp 163-164 °C (52%). IR (KBr, cm^{-1}) v_{max}: 3274, 1603, 1560, 1508, 1452, 1423, 1401, 1378, 1315, 1257, 1209, 1179, 1170, 1119, 1027, 1015, 953, 882, 832. ^{1}H NMR (500 MHz, CDCl$_3$) δ = 8.24 (d, 2H, J = 8.9 Hz, H-2´ and H-6´); 8.16 (d, 1H, J = 8.9 Hz, H-5); 7.50 (d, 2H, J = 7.2 Hz, 2xH-Ph); 7.46-7.43 (m, 2H, 2xH-Ph); 7.39 (tt, 1H, J = 6.2, 1.2 Hz, Ph); 7.15 (d, 2H, J = 9.0 Hz, H-3´ and H-5´); 7.02 (dd, 1H, J = 8.9, 2.3 Hz, H-6); 6.97 (d, 1H, J = 2.3 Hz, H-8); 5.19 (s, 2H, C$\underline{H}_2$-Ph); 3.96 (s, 3H, C$\underline{H}_3$O-C-7). ^{13}C NMR (125 MHz, CDCl$_3$) δ = 172.5 (C-4), 164.1 (C-7), 160.0 (C-4´), 157.2 (C-9), 144.6 (C-2), 137.3 (C-3), 136.5 (Ph), 129.2 (C-2´ and C-6´), 128.7 (Ph), 128.1 (Ph), 127.5 (Ph), 126.7 (C-5), 123.9 (C-1´), 114.9 (C-3´ and C-5´), 114.7 (C-6 and C-10), 99.8 (C-8), 70.1 ($\underline{C}$H$_2$-Ph), 55.8 ($\underline{C}$H$_3$O-C-4´). HRMS (ESI-FT-ICR) *m/z*: 397.1059 [M+Na]$^+$; calcd. for C$_{23}$H$_{18}$O$_5$Na: 397.1052.

4.1.3.5. 2-[2-(Benzyloxy)phenyl]-3-hydroxy-4H-chromen-4-one (4g)
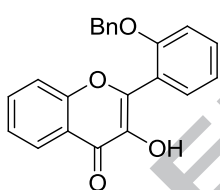
Yellow pale solid, mp 138-139 °C (64%). IR (KBr, cm^{-1}) v_{max}: 3289, 3066, 3029, 2929, 2872, 1949, 1910, 1815, 1633, 1614, 1606, 1567, 1486, 1469, 1417, 1382, 1347, 1287, 1211, 1135, 1102, 1050, 1024, 900, 859. ^{1}H NMR (500 MHz, CDCl$_3$) δ = 8.18 (dd, 1H, J = 8.1, 1.1 Hz, H-5); 7.57-7.54 (m, 2H, H-4´ or H-6´ and H-7); 7.37 (d, 1H, J = 8.4 Hz, H-8); 7.36 (dt, 1H, J = 9.0, 1.5 Hz, H-6´ or H-4´); 7.31 (t, 1H, J = 7.4 Hz, H-6); 7.27 (d, 2H, J = 7.1 Hz, Ph); 7.20-7.13 (m, 3H, 3xH-Ph); 7.03-7.00 (m, 2H, H-3 and H-5´); 6.52 (s, 1H, OH); 5.08 (s, 2H, C$\underline{H}_2$-Ph). ^{13}C NMR (125 MHz, CDCl$_3$) δ = 173.3 (C-4), 156.6 (C-2´), 156.0 (C-9), 145.9 (C-2), 138.9 (C-3), 136.7 (Ph), 133.3 (C-7), 132.0 (C-4´ or C-6´), 131.1 (C-6´ or C-4´), 128.5 (Ph), 127.8 (Ph), 126.9 (Ph), 125.5 (C-5), 124.3 (C-6), 121.4 (C-10), 120.9

(C-5´), 120.2 (C-1´), 118.4 (C-8), 113.4 (C-3´), 70.5 ($\underline{C}H_2$-Ph). HRMS (ESI-FT-ICR)

*m/z*: 367.0944 [M+Na]$^+$; calcd. for $C_{22}H_{16}O_4Na$: 367.0946.

*4.1.3.5. 2-[2-(Benzyloxy)phenyl]-3-hydroxy-7-methoxy-4*H*-chromen-4-one (**4h**)*
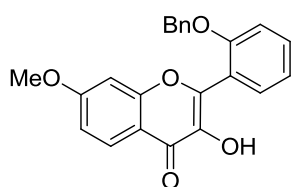
White solid, mp 161-162 ºC (60%). IR (KBr, cm^{-1}) v_{max}: 3275, 3032, 2936, 2841, 1608, 1567, 1505, 1454, 1408, 1318, 1276, 1257, 1230, 1204, 1168, 1130, 1112, 1048, 1026, 995, 954, 885, 835. ^{1}H NMR (500 MHz, CDCl$_3$) δ = 8.20 (d, 1H, *J* = 8.9 Hz, H-5); 7.68 (dd, 1H, *J* = 7.5, 1.6 Hz, H-6´); 7.49 (dt, 1H, *J* = 7.7, 1.7 Hz, H-4´); 7.42 (d, 2H, *J* = 6.9 Hz, 2xH-Ph); 7.35-7.28 (m, 3H, 3xH-Ph); 7.18-7.14 (m, 2H, H-3´ and H-5´); 7.03 (dd, 1H, *J* = 8.9, 2.3 Hz, H-6); 6.87 (d, 1H, *J* = 2.3 Hz, H-8); 6.62 (s, 1H, OH); 5.22 (s, 2H, C$\underline{H}_2$-Ph); 3.90 (s, 3H, C$\underline{H}_3$O-C-7). ^{13}C NMR (125 MHz, CDCl$_3$) δ = 172.7 (C-4), 164.0 (C-7), 157.9 (C-9), 156.5 (C-2´), 145.1 (C-2), 138.5 (C-3), 136.8 (Ph), 131.8 (C-4´), 131.1 (C-6´), 128.4 (Ph), 127.8 (Ph), 126.9 (Ph), 126.8 (C-5), 120.9 (C-3´ or C-5´), 120.3 (C-1´), 115.3 (C-10), 114.7 (C-6), 113.5 (C-5´ or C-3´), 99.8 (C-8), 70.5 ($\underline{C}H_2$-Ph), 55.8 ($\underline{Me}$O-C-7). HRMS (ESI-FT-ICR) *m/z*: 397.1059 [M+Na]$^+$; calcd. for $C_{23}H_{18}O_5Na$: 397.1052.

4.1.4. General procedure for the 3-methylation of flavonols

Methyl iodide (1.2 equiv.) and potassium carbonate (2 equiv.) were added to a solution of the flavonols (1 equiv.) in acetone (0.1M) under argon atmosphere and stirred. After standing overnight, the resulting mixture was filtered and the solvent was removed under vacuum to leave a yellow pale solid, which was purified by flash column chromatography or recrystallized from EtOH. 3-Methoxyflavones **6d-f** have been previously described.[28]

### 4.1.4.1. 3´-Benzyloxy-3,4´-dimethoxyflavone (*6a*)
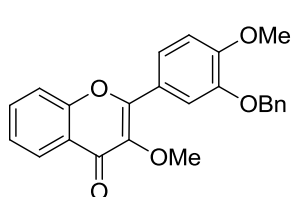
Yellow pale solid, mp 104-106 ºC (65%). IR (KBr, cm^{-1}) ν_{max}: 2931, 2839, 1637, 1615, 1601, 1560, 1511, 1468, 1438, 1378, 1329, 1267, 1243, 1212, 1171, 1134, 1020, 974, 891, 812. ^{1}H NMR (500 MHz, CDCl$_3$) δ = 8.24 (dd, 1H, J = 7.9, 1.7 Hz, H-5); 7.79-7.76 (m, 2H, H-2´ and H-6´); 7.65 (ddd, 1H, J = 8.6, 7.1, 1.7 Hz, H-7); 7.50-7.46 (m, 3H, H-8 and 2xH-Ph); 7.41-7.35 (m, 3H, H-6 and 2xH-Ph); 7.32 (t, 1H. J = 7.4 Hz, Ph); 7.02 (d, 1H, J = 8.4 Hz, H-5´); 5.26 (s, 2H, C$\underline{H}_2$-Ph); 3.98 (s, 3H, C$\underline{H}_3$O-C-4´); 3.73 (s, 3H, C$\underline{H}_3$O-C-3). ^{13}C NMR (125 MHz, CDCl$_3$) δ = 174.9 (C-4), 155.3 (C-2), 155.1 (C-9), 151.8 (C-4´), 147.7 (C-3´), 140.9 (C-3), 136.8 (Ph), 133.2 (C-7), 128.6 (Ph), 128.0 (Ph), 127.2 (Ph), 125.8 (C-5), 124.5 (C-6), 124.1 (C-10), 123.3 (C-1´), 122.5 (C-6´), 117.8 (C-8), 114.3 (C-2´), 111.3 (C-5´), 71.1 ($\underline{C}$H$_2$-Ph), 59.8 ($\underline{C}$H$_3$O-C-3), 56.0 ($\underline{C}$H$_3$O-C-4´). HRMS (ESI-FT-ICR) m/z: 411.1219 [M+Na]$^{+}$; calcd. for C$_{24}$H$_{20}$O$_5$Na: 411.1208.

4.1.4.2. 3´-Benzyloxy-3,7,4´-Trimethoxyflavone (*6b*)
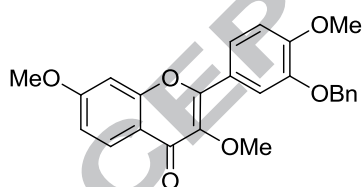
Yellow pale solid, mp 154-155 ºC (68%). IR (KBr, cm^{-1}) ν_{max}: 3014, 2931, 2847, 1611, 1513, 1445, 1382, 1364, 1327, 1266, 1256, 1215, 1170, 1133, 1103, 1022, 953, 836. ^{1}H NMR (500 MHz, CDCl$_3$) δ = 8.06 (d, 1H, J = 8.9 Hz, H-5); 7.68-7.65 (m, 2H, H-2´ and H-6´); 7.41 (d, 2H, J = 7.2 Hz, 2xH-Ph); 7.31 (t, 2H, J = 7.5 Hz, 2xH-Ph); 7.24 (t, 1H, J = 7.3 Hz, Ph); 6.94 (d, 1H, J = 8.5 Hz, H-5´); 6.88 (dd, 1H, J = 8.9, 2.2 Hz, H-6); 6.77 (d, 1H, J = 2.3 Hz, H-8); 5.18 (s, 2H, C$\underline{H}_2$-Ph); 3.90 (s, 3H, C$\underline{H}_3$O-C-4´); 3.84 (s, 3H, C$\underline{H}_3$O-C-7); 3.64 (s, 3H, C$\underline{H}_3$O-C-3). ^{13}C NMR (125 MHz, CDCl$_3$) δ = 174.4 (C-4), 163.9 (C-7), 156.8 (C-9), 154.8 (C-2), 151.7 (C-4´), 147.7 (C-3´), 140.7 (C-3), 136.9 (Ph), 128.6 (Ph), 127.9 (Ph), 127.2 (Ph), 127.1 (C-5), 123.4 (C-1´), 122.3

(C-6´), 118.0 (C-10), 114.2 (C-6 or C-2´), 114.2 (C-2´ or C-6), 111.2 (C-5´), 99.9 (C-8), 71.1 ($\underline{C}H_2$-Ph), 59.9 ($\underline{C}H_3$O-C-4´), 56.0 ($\underline{C}H_3$O-C-7), 55.8 ($\underline{C}H_3$O-C-3). HRMS (ESI-FT-ICR) *m/z*: 419.1483 [M+H]$^+$; calcd. for $C_{25}H_{23}O_6$: 419.1495.

4.1.4.3. 4´-Benzyloxy-3,3´-dimethoxyflavone (6c)
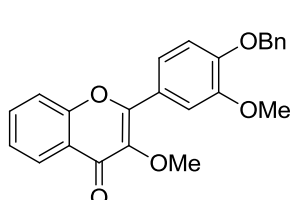
White solid, mp 128-129 ºC (80%). IR (KBr, cm^{-1}) ν_{max}: 3064, 3005, 2931, 2849, 1733, 1638, 1615, 1601, 1561, 1511, 1469, 1438, 1419, 1379, 1330, 1270, 1241, 1212, 1170, 1135, 1012, 972, 915, 889, 879. ^{1}H NMR (500 MHz, CDCl$_3$) δ = 8.30 (dd, 1H, *J* = 7.9,1.5 Hz, H-5); 7.81 (d, 1H, *J* = 1.9 Hz, H-2´); 7.74 (dd, 1H, *J* = 8.5, 1.9 Hz, H-6´); 7.70 (ddd, 1H, *J* = 8.5, 7.2, 1.5 Hz, H-7); 7.56 (d, 1H, *J* = 8.3 Hz, H-8); 7.51 (d, 2H, *J* = 7.2 Hz, 2xH-Ph); 7.45-7.42 (m, 3H, H-6 and 2xH-Ph); 7.36 (t, 1H, *J* = 7.4 Hz, Ph); 7.05 (d, 1H, *J* = 8.6 Hz, H-5´); 5.30 (s, 2H, C$\underline{H}_2$-Ph); 4.02 (s, 3H, C$\underline{H}_3$O-C-3´); 3.93 (s, 3H, C$\underline{H}_3$O-C-3). ^{13}C NMR (125 MHz, CDCl$_3$) δ = 174.9 (C-4), 155.4 (C-2), 155.1 (C-9), 150.4 (C-4´), 149.2 (C-3´), 141.0 (C-3), 136.5 (C-Ph), 133.3 (C-7), 128.7 (C-Ph), 128.1 (C-Ph), 127.2 (C-Ph), 125.8 (C-5), 124.6 (C-6), 124.2 (C-10), 123.7 (C-1´), 122.0 (C-6´), 117.8 (C-8), 113.1 (C-5´), 112.1 (C-2´), 70.8 ($\underline{C}H_2$-Ph), 60.0 ($\underline{C}H_3$O-C-3), 56.2 ($\underline{C}H_3$O-C-3´). HRMS (ESI-FT-ICR) *m/z*: 389.1385 [M+H]$^+$; calcd. for $C_{24}H_{21}O_5$: 389.1389.

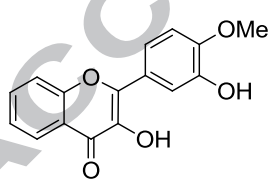### 4.1.4.4. 2´-Benzyloxy-3-methoxyflavone (6g)
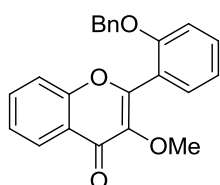
White solid, mp 115 ºC (59 %). IR (KBr, cm^{-1}) ν_{max}: 3064, 3033, 3005, 2936, 1643, 1622, 1610, 1581, 1570, 1489, 1466, 1450, 1385, 1278, 1254, 1230, 1212, 1171, 1148, 1111, 1048, 1008, 955, 900. ^{1}H NMR (500 MHz, CDCl$_3$) δ = 8.34 (dd, 1H, *J* = 8.0, 1.6 Hz, H-5); 7.69 (ddd, 1H, *J* = 8.6, 7.1, 1.6 Hz, H-7); 7.55 (dd, 1H, *J* = 7.4, 1.5 Hz, H-6´); 7.51-7.48 (m, 2H, H-8 and H-

4´); 7.44 (ddd, 1H, *J* = 8.7, 7.9, 0.9, Hz, H-6); 7.38 (d, 2H, *J* = 7.1 Hz, 2xH-Ph); 7.33-

7.27 (m, 3H, 3xH-Ph); 7.15-7.12 (m, 2H, H-3´ and H-5´); 5.21 (s, 2H, C$\underline{H}_2$-Ph); 3.83 (s,

3H, C$\underline{H}_3$O-C-2´). ^{13}C NMR (125 MHz, CDCl$_3$) *δ* = 174.9 (C-4), 156.5 (C-2´), 156.2 (C-

2), 155.7 (C-9), 142.0 (C-3), 136.6 (Ph), 133.2 (C-7), 131.9 (C-4´), 131.0 (C-6´), 128.5

(Ph), 127.8 (Ph), 126.9 (Ph), 125.9 (C-5), 124.6 (C-10), 124.5 (C-6), 120.7 (C-3´ or C-

5´), 120.7 (C-1´), 118.1 (C-8), 113.1 (C-5´ or C-3´), 70.4 (C$\underline{H}_2$-Ph), 60.5 (C$\underline{H}_3$O-C2´).

HRMS (ESI-FT-ICR) *m/z*: 381.1107 [M+Na]$^+$; calcd. for C$_{23}$H$_{18}$O$_4$Na: 381.1103.

### *4.1.5. General procedure for debenzylation*

20 mg of 10% Pd/C was added to a solution of the required compound in THF (0.1M).

The reaction mixture was stirred at room temperature for 1 h under an atmosphere of 1.5

atm H$_2$(g) after replacement of the air by nitrogen. The Pd/C was filtered through celite

and the solvent was evaporated under reduced pressure. The crude was purified by flash

column chromatography or recrystallized from EtOH. Flavonoids **5b**, **5c**, **5d**, **5e**, **5f**, **5g**,

7c, **7d** and **7f** have been previously described.[26, 29-32]

*4.1.5.1. 3-Hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4*H*-chromen-4-one (5a)*

White solid, mp 201-203 ºC (80%). IR (KBr, cm^{-1}) *ν*$_{max}$: 3296, 3028, 3205, 3028, 1584, 1537, 1523, 1487, 1464, 1417, 1369, 1350, 1329, 1291, 1257, 1207, 1182, 1142, 1121, 1111, 1041, 1026, 883, 811. ^{1}H NMR (500 MHz, DMSO-d$_6$) *δ* = 9.38 (s, 1H, OH-C-3); 9.34 (s, 1H,

OH-C-3´); 8.10 (dd, 1H, *J* = 8.0, 1.5 Hz, H-5); 7.79-7.75 (m, 2H, H-7 and H-2´); 7.72-

7.70 (m, 2H, H-8 and H-6´); 7.44 (dt, 1H, *J* = 7.9, 0.9 Hz, H-6); 7.10 (d, 1H, *J* = 8.7 Hz,

H-5´); 3.85 (s, 3H, C$\underline{H}_3$O-C-4´). ^{13}C NMR (125 MHz, DMSO-d$_6$) *δ* =172.5 (C-4), 154.3

(C-9), 149.2 (C-4´), 146.1 (C-3´), 145.6 (C-2), 138.1 (C-3), 133.4 (C-7), 124.6 (C-5),

124.3 (C-6), 123.7 (C-1´), 121.2 (C-10), 119.7 (C-6´), 118.1 (C-8), 114.7 (C-2´), 111.7

(C-5´), 55.5 ($\underline{C}H_3O$-C4´). HRMS (ESI-FT-ICR) *m/z*: 285.0768 [M+H]$^+$; calcd. for

$C_{16}H_{13}O_5$: 285.0763.

4.1.5.2. *3-Hydroxy-2-(3-hydroxy-4-methoxyphenyl)-7-methoxy-4*H-*chromen-4-one (5b)*
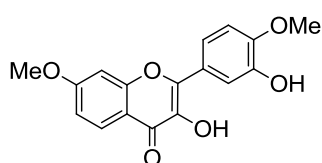
White solid, mp 179-181 ºC (82%). IR (KBr, cm^{-1}) ν_{max}:
3389, 3013, 2936, 2842, 1614, 1563, 1557, 1506, 1455,
1407, 1335, 1261, 1212, 1167, 1138, 1121, 1027, 1010, 960,

836. ^{1}H NMR (500 MHz, DMSO-d$_6$) δ = 9.27 (s, 1H, OH-C-3´); 9.23 (s, 1H, OH-C-3);

7.98 (d, 1H, *J* = 8.9 Hz, H-5); 7.74 (d, 1H, *J* = 2.2 Hz, H-2´); 7.70 (dd, 1H, *J* = 8.6, 2.2

Hz, H-6´); 7.23 (d, 1H, *J* = 2.3 Hz, H-8); 7.09 (d, 1H, *J* = 8.7 Hz, H-5´); 7.03 (dd, 1H, *J*

= 8.85, 2.3 Hz, H-6); 3.91 (s, 3H, C$\underline{H}_3$O-C-7), 3.85 (s, 3H, C$\underline{H}_3$O-C4´). ^{13}C NMR (125

MHz, DMSO-d$_6$) δ = 172.0 (C-4), 163.4 (C-7), 156.2 (C-9), 149.0 (C-4´), 146.1 (C-3´),

144.9 (C-2), 137.8 (C-3), 126.0 (C-5), 123.8 (C-1´), 119.4 (C-6´), 115.0 (C-10), 114.6

(C-2´), 114.4 (C-6), 111.7 (C-5´), 100.1 (C-8), 56.0 ($\underline{C}H_3O$-C-7), 55.6 ($\underline{C}H_3O$-C-4´).

HRMS (ESI-FT-ICR) *m/z*: 337.0680 [M+Na]$^+$; calcd. for $C_{17}H_{14}O_6Na$: 337.0688.
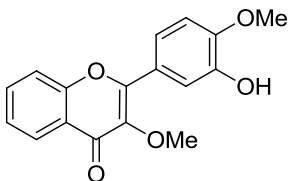
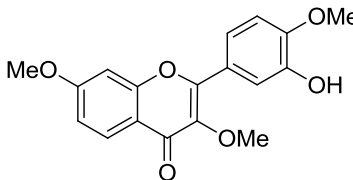### 4.1.5.3. *3-Hydroxy-2-(2-hydroxyphenyl)-7-methoxy-4*H-*chromen-4-one (5h)*
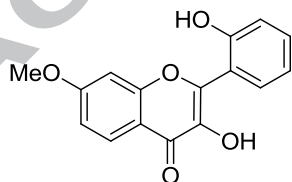
White solid, mp 165-166 ºC (75%). IR (KBr, cm^{-1}) ν_{max}: 3233,
2929, 1618, 1610, 1507, 1450, 1406, 1260, 1206, 1169, 1131,
1111, 1026, 996, 954, 885, 834. ^{1}H NMR (500 MHz, DMSO-

d$_6$) δ = 9.32 (brs, 1H, OH); 8.02 (d, 1H, *J* = 8.9 Hz, H-5); 7.43 (dd, 1H, *J* = 7.6, 1.5 Hz,

H-6´); 7.34 (dt, 1H, *J* = 8.3, 1.6 Hz, H-4´); 7.10 (d, 1H, *J* = 2.2 Hz, H-8); 7.04 (dd, 1H, *J*

= 8.9, 2.2 Hz, H-6); 6.98 (d, 1H, *J* = 7.9 Hz, H-3´); 6.94 (t, 1H, *J* = 7.4 Hz, H-5´); 3.88

(s, 3H, C$\underline{H}_3$O-C-7); 3.35 (brs, 1H, OH). ^{13}C NMR (125 MHz, DMSO-d$_6$) δ = 172.1 (C-

4), 163.3 (C-7), 156.9 (C-9), 155.3 (C-2´), 146.7 (C-2), 138.5 (C-3), 131.2 (C-4´), 130.8 (C-6´), 126.1 (C-5), 118.6 (C-5´), 118.3 (C-1´), 116.3 (C-3´), 115.8 (C-10), 114.3 (C-6), 100.1 (C-8), 55.9 ($\underline{C}H_3O$-C-7). HRMS (ESI-FT-ICR) m/z: 285.0766 [M+H]$^+$; calcd. for $C_{16}H_{13}O_5$: 285.0763. Anal. Calcd for $C_{16}H_{12}O_5$: C 67.60; H 4.25. Found: C 67.58; H 4.50.

*4.1.5.4. 2-(3-Hydroxy-4-methoxyphenyl)-3-methoxy-4*H-*chromen-4-one (7a)*

White solid, mp 138-139 ºC (78 %). IR (KBr, cm^{-1}) v_{max}: 3358, 2933, 2840, 1613, 1557, 1509, 1469, 1438, 1385, 1337, 1275, 1253, 1213, 1172, 1126, 1035, 1023, 1011, 979, 892. ^{1}H NMR (400 MHz, CDCl$_3$) δ = 8.24 (dd, 1H, J = 8.0, 1.8 Hz, H-5); 7.76-7.74 (m, 2H, 2´ and H-6´); 7.65 (ddd, 1H, J = 8.5, 7.2, 1.4 Hz, H-7); 7.51 (d, 1H, J = 8.2 Hz, H-8); 7.37 (t, 1H, J = 7.8 Hz, H-6); 6.96 (d, 1H, J = 9.2 Hz, H-5´); 5.88 (s, 1H, OH); 3.97 (s, 3H, C$\underline{H}_3$O-C-3); 3.89 (s, 3H, C$\underline{H}_3$O-C-4´). ^{13}C NMR (100 MHz, CDCl$_3$) δ = 175.3 (C-4), 155.2 (C-2 or C-9), 155.1 (C-9 or C-2), 148.9 (C-3), 145.6 (C-3´), 141.3 (C-4´), 133.2 (C-7), 125.7 (C-5), 124.5 (*C*-6), 124.2 (C-1´ or C-10), 124.1 (C-10 or C-1´), 121.6 (C-6´), 117.8 (C-8), 114.6 (C-2´), 110.4 (C-5´), 59.8 ($\underline{C}H_3O$-C-4´), 56.0 ($\underline{C}H_3O$-C-3). HRMS (ESI-FT-ICR) m/z: 321.0725 [M+Na]$^+$; calcd. for $C_{17}H_{14}O_5Na$: 321.0739. Anal. Calcd for $C_{17}H_{14}O_5$: C 68.45; H 4.73. Found: C 68.41; H 5.09.

*4.1.5.5. 2-(3-Hydroxy-4-methoxyphenyl)-3,7-dimethoxy-4*H-*chromen-4-one (7b)*

White solid, mp 174-175 ºC (75 %). IR (KBr, cm^{-1}) v_{max}: 3234, 2932, 2840, 1605, 1560, 1508, 1438, 1386, 1367, 1274, 1255, 1213, 1169, 1128, 1103, 1017, 982, 952, 836.

^{1}H NMR (400 MHz, CDCl$_3$) δ = 8.06 (d, 1H, J = 8.8 Hz, H-5); 7.65 (dd, 1H, J = 6.8,

3.5 Hz, H-6´); 7.63 (brs, 1H, H-2´); 6.97 (d, 1H, J = Hz, H-5´); 6.96 (d, 1H, J = Hz, H-6); 6.81 (d, 1H, J = 2.3 Hz, H-8); 5.86 (s, 1H, OH); 3.89 (s, 3H, C$\underline{H}_3$O-C-4´); 3.83 (s, 3H, C$\underline{H}_3$O-C-7); 3.80 (s, 3H, C$\underline{H}_3$O-C-3). ^{13}C NMR (100 MHz, CDCl$_3$) δ = 174.5 (C-4), 163.9 (C-7), 156.8 (C-9), 154.8 (C-2), 148.5 (C-4´), 145.5 (C-3´), 140.9 (C-3), 127.0 (C-5), 124.1 (C-1´), 121.3 (C-6´), 118.0 (C-10), 114.4 (C-2´), 114.2 (C-6), 110.3 (C-5´), 99.9 (C-8), 59.9 ($\underline{C}H_3$O-C-3), 56.0 ($\underline{C}H_3$O-C-4´), 55.7 ($\underline{C}H_3$O-C-7). HRMS (ESI-FT-ICR) m/z: 351.0844 [M]$^+$; calcd. for C$_{18}$H$_{16}$O$_6$: 351.0845. Anal. Calcd for C$_{18}$H$_{16}$O$_6$: C 65.85; H 4.91. Found: C 65.85; H 5.09.

4.1.5.6. 2-(4-Hydroxyphenyl)-3-methoxy-4H-chromen-4-one (7e)
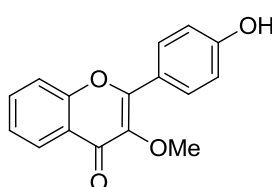
White solid, mp 220 ºC (80 %). IR (KBr, cm^{-1}) ν_{max}: 3217, 2917, 2849, 1603, 1555, 1509, 1469, 1385, 1283, 1242, 1213, 1177, 1149, 1110, 1034, 1007, 953, 898, 811. ^{1}H NMR (500 MHz, DMSO-d$_6$) δ = 10.2 (brs, 1H, OH); 8.07 (dd, 1H, J = 7.9, 1.6 Hz, H-5); 7.98 (d, 2H, J = 8.9 Hz, H-2´ and H-6´); 7.79 (ddd, 1H, J = 8.5, 7.0, 1.6 Hz, H-7); 7.71 (dd, 1H, J = 8.4, 0.6 Hz, H-8); 7.46 (ddd, 1H, J = 8.0, 7.0, 1.0 Hz, H-6); 6.96 (d, 2H. J = 8.9 Hz, H3´ and H-5´); 3.80 (s, 3H, C$\underline{H}_3$O-C-3). ^{13}C NMR (125 MHz, DMSO-d$_6$) δ =173.5 (C-4), 159.9(C-2), 155.2 (C-4´), 154.5 (C-9), 139.7 (C-3), 133.7 (C-7), 130.1 (C-2´ and C-6´), 124.8 (C-5 or C-6), 124.8 (C-6 or C-5), 123.4 (C-10), 120.9 (C-1´), 118.2 (C-8), 115.5 (C-3´ and C-5´), 59.3 ($\underline{C}H_3$O-C-3). HRMS (ESI-FT-ICR) m/z: 269.0818 [M+H]$^+$; calcd. for C$_{16}$H$_{13}$O$_4$: 269.0814. Anal. Calcd for C$_{16}$H$_{12}$O$_4$: C 71.64; H 4.51. Found: C 71.91; H 4.76.

4.1.5.7. 2-(2-Hydroxyphenyl)-3-methoxy-4H-chromen-4-one (7g)
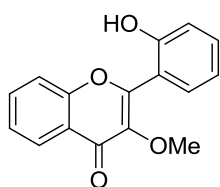
White solid, mp 191-192 ℃ (77 %). IR (KBr, cm^{-1}) v_{max}: 3235, 3007, 2940, 1612, 1560, 1466, 1454, 1388, 1293, 1252, 1212, 1147, 1111, 1037, 1007, 955, 901. ^{1}H NMR (500 MHz, DMSO-d$_6$) δ = 9.96 (s, 1H, OH); 8.13 (dd, 1H, J = 8.1, 1.6 Hz, H-5); 7.80 (ddd, 1H, J = 8.6, 7.1, 1.6 Hz, H-7); 7.63 (d, 1H, J = 8.4 Hz, H-8); 7.50 (ddd, 1H, J = 8.0, 7.3, 0.9 Hz, H-6); 7.41 (dd, 1H, J = 7.6, 1.6 Hz, H-6´); 7.37 (ddd, 1H, J = 8.2, 7.5, 1.6 Hz, H-4´); 7.00 (d, 1H, J = 8.2 Hz, H-3´); 6.94 (dt, 1H, J = 7.5, 0.8 Hz, H-5´); 3.73 (s, 3H, CH$_3$O-C-3). ^{13}C NMR (125 MHz, DMSO-d$_6$) δ = 173.6 (C-4), 156.4 (C-2), 155.3 (C-2´), 155.1 (C-9), 141.1 (C-3), 133.8 (C-7), 131.7 (C-4´), 130.4 (C-6´), 124.9 (C-5), 124.8 (C-6), 123.9 (C-10), 118.7 (C-5´), 118.3 (C-8), 118.0 (C-1´), 116.1 (C-3´), 59.5 (CH$_3$O-C-3). HRMS (ESI-FT-ICR) m/z: 269. [M+H]$^+$; calcd. for C$_{16}$H$_{13}$O$_4$: 269.0814. Anal. Calcd for C$_{16}$H$_{12}$O$_4$: C 71.67; H 4.51. Found: C 71.30; H 4.44.

4.2. Biological assays

4.2.1. Cell culture and cytotoxicity assays

SK-MEL-1 melanoma cells were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and grown in RPMI 1640 containing 2 mM L-glutamine supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 100 units/mL penicillin and 100 µg/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO$_2$. Human peripheral blood mononuclear cells (PBMCs) were isolated from heparin-anticoagulated blood of healthy volunteers by centrifugation with Ficoll-Paque Plus (GE Healthcare Bio-Sciences AB; Uppsala, Sweden). PBMCs were also stimulated with phytohemagglutinine (PHA, 2 µg/mL) for 48 h before experimental treatment.

The cytotoxicity of compounds was evaluated by using a colorimetric 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay. Briefly, 1×10^4 exponentially growing cells were seeded in 96-well microculture plates with various compounds concentrations. After the addition of MTT (0.5 mg/mL), cells were incubated at 37 °C for 4 h. Sodium dodecyl sulfate (10% w/v) in 0.05 M HCl was added to the wells and then incubated at room temperature overnight under dark conditions. The absorbance was measured at 570 nm. Concentrations inducing a 50% inhibition of cell growth (IC_{50}) were determined graphically for each experiment by a nonlinear regression using the curve-fitting routine implemented within the software Prism 4.0 (GraphPad). Values are means ± SE from at least three independent experiments, with each performed in triplicate.

4.2.2. Evaluation and quantification of apoptosis and analysis of the cell cycle

Fluorescent microscopy was used to identify nuclear changes associated with apoptosis as previously described.[33] To study changes in the cell DNA content, flow cytometric analysis of propidium iodide-stained nuclei was carried out using a BD FACSVerse[TM] cytometer (BD Biosciences, San Jose, CA, USA) as described.[8] Briefly, cells were collected and centrifuged at 500 ×g, washed with PBS and resuspended in 50 μL of PBS. Following dropwise addition of 1 mL of ice-cold 75% ethanol, fixed cells were stored at −20 °C for 1 h. Samples were then centrifuged at 500 ×g and washed with PBS and finally resuspended in 1 mL of PBS containing 50 μg/mL propidium iodide and 100 μg/mL RNase A and incubated for 1 h at 37 °C in the dark. The percentage of cells with decreased DNA staining, composed of apoptotic cells resulting from either fragmentation or decreased chromatin, was counted (minimum of 10,000 cells per experimental condition). Cell debris was excluded from analysis by selective gating based on anterior and right angle scattering.

4.2.3. Western blot analysis

Immunoblot analysis of caspase-3 and cytochrome *c* was performed as previously described.[34] Briefly, after treatments, cells were washed twice with PBS and then resuspended in ice-cold buffer [20 mM HEPES (pH 7.5), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 5 μg/mL leupeptin, aprotinin, and pepstatin A] containing 250 mM sucrose. After 15 min on ice, cells were lysed by pushing them several times through a 22-gauge needle, and the lysate was spun down at 1000x*g* for 5 min at 4 °C to eliminate nuclei and unbroken cells. The supernatant fraction was centrifuged at 105000x*g* for 45 min at 4 °C, and the resulting supernatant was used as the soluble cytosolic fraction.

4.2.4. Assay of caspase acivity

After treatments, cells were harvested by centrifugation at 1000x*g* for 5 min at 4 °C and washed with PBS, and the cell pellets were kept on ice. The cells were resuspended in cell lysis buffer (50 mM HEPES, pH 7.4, 1 mM dithiothreitol, 0.1 mM EDTA, 0.1% Chaps) and held on ice for 5 min. After centrifugation for 10 min at 17,000×*g* at 4 °C, the supernatants were analysed for protein concentration by the Bradford dye binding assay and stored at −20 °C until used to study caspase colorimetric enzymatic activity. Equal amounts of protein from different treatments were used, and the assays were set up on ice. The net increase of absorbance at 405 nm after incubation at 37 °C was indicative of enzyme activity. Specific colorimetric substrates for caspase-3, -8 and -9 activities were *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroaniline (DEVD-*p*NA), *N*-acetyl-Ile-Glu-Thr-Asp-*p*-nitroaniline (IETD-*p*NA) and *N*-acetyl-Leu-Glu-His-Asp-*p*-nitroaniline (LEHD-*p*NA), respectively.

4.2.5. In vitro tubulin polymerization assay

In vitro tubulin polymerization assays were performed with reagents as described by the manufacturer (Cytoskeleton Inc., Denver, CO, USA). Briefly, different concentrations of **7a** were incubated with purified bovine tubulin in 80 mM PIPES buffer (pH 7.0) containing 1 mM GTP, 1 mM EGTA, 1 mM $MgCl_2$, and 10% glycerol, and the increase in absorbance was measured at 340 nm in a Beckman Coulter DTX880 microplate reader at 37 ºC and recorded every 30 s for 50 min. Taxol (10 µM) and colchicine (5 µM) were used as positive controls of promotion and inhibition of tubulin polymerization, respectively.

4.2.6. Statistical analysis

Student's t test was used to determine the statistical significance of differences between means of control and treated samples, with p values of <0.05 considered significant.

Acknowledgments

A. Supplementary data

Spectral and analytical data associated with this article.

Conflicts of interest: none.

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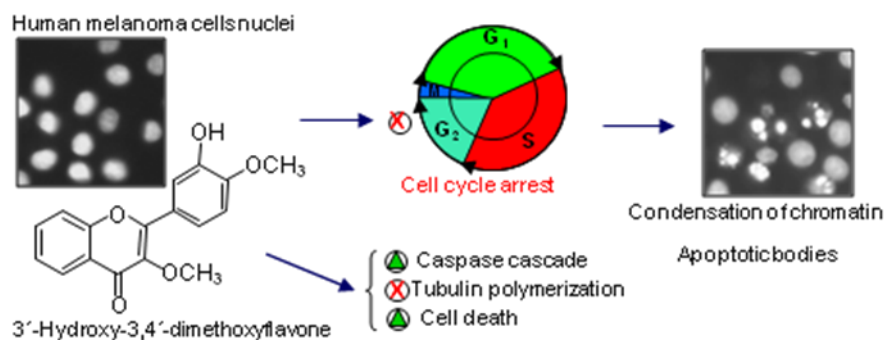
Highlights

3´-Hydroxy-3,4´-dimethoxyflavone is an apoptotic inducer of melanoma cells > It causes arrest in the G_2-M phase of the cell cycle> Cell death was associated with the activation of both initiator caspases > It activates the extrinsic and the intrinsic apoptotic pathways of cell death.