University of Szeged

Faculty of Pharmacy

Department of Pharmacodynamics and Biopharmacy
& Department of Pharmacognosy

Summary of PhD Thesis

Investigation of antitumor properties of semi-synthetic flavonoid derivatives on gynecological cancer cell lines

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Szeged, Hungary

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Doctoral School of Pharmaceutical Sciences
Pharmacodynamics, Biopharmacy, clinical pharmacy PhD programme
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1 INTRODUCTION

Cancer is considered one of the most dangerous diseases that threatens human health and is the second leading cause of death worldwide. Recent statistics reported by the World Health Organization (WHO) indicated that cancer was responsible for an estimated 9.6 million patient death in 2018, and this number will increase to 15 million by 2030. Among different types of cancer, female breast cancer ranked as the first and common type of cancer with 523,000 detected cases, followed by colorectal, lung, and prostate cancer. Moreover, high cancer burden is characteristic of Central and Eastern European countries, including Hungary. While the human cervical cancer is currently one of the crucial global health problems and a leading cause of mortality in women during their reproductive years.

Chemotherapy is one of the best therapeutic strategies to treat cancer disease but adverse effects and acquired drug resistance may lead to failure for standard therapy. There is still an urgent requirement to develop novel effective, reliable and safe anticancer agents for the treatment of a wide range of cancerous disorders. Traditionally attention has been concentrated on the use of natural products and their semi-synthetic derivatives to treat human diseases, including cancer. Plants are substantial sources of natural compounds, which can be used as models for design, and synthesis of innovative drugs. Based on their wide range of bioactivities, low cost, and chemical diversity, they are generally considered an essential factor anticancer drug research. Several plant species and their derivatives exerted strong antiproliferative and proapoptotic effects in different types of cancer cell lines. Additionally, the plants, which contain high amounts of flavonoids in their structure, are accepted in traditional medicine as chemotherapeutic and chemopreventive agents in some countries.

Flavonoids are considered the largest class of polyphenolic secondary metabolites that are widely distributed in nature and have a promising value in cancer research. Flavonoids are classified into different subgroups, which include flavonols, flavones, flavanones, isoflavones, chalcones, and anthocyanidins. During the last few decades, many studies showed that isolated and synthesized flavonoid analogs exerted different pharmacological activities such as antibacterial, antiviral, anti-inflammatory, antioxidant, and antitumor effects against a broad range of human cancer cell lines.

Naringenin, which belongs to the flavanone subclass, is an abundant dietary flavonoid predominantly present in grapes and citrus fruits. It displays several beneficial pharmacological activities on human health, including cardioprotective, anti-inflammatory, antiviral, antioxidant, and anticarcinogenic effects. Recently, several *in vivo* and *in vitro* studies demonstrated that naringenin could effectively inhibit cell proliferation and migration, induce apoptosis, and suppress cell cycle in several types of human cancer cells. However, the anticancer activity of naringenin is not strong enough to apply it clinically. The use of natural naringenin as a cancer chemotherapeutic or chemopreventive agent requires the development of naringenin derivatives that can prompt cytotoxicity at low concentrations. Therefore, based on the chemical structure of naringenin, several research groups have made attempts to design and synthesize new naringenin derivatives to improve their biological properties.

On the other hand, protoflavones represent a rare particular class of natural flavonoids with an unusual non-aromatic B-ring. Such compounds most typically occur in fern species, and their anticancer activity is much more potent than classical flavonoids. Several studies reported the isolation and/or semi-synthetic preparation of new protoflavone derivatives that exert promising bioactivity against different cancer cells *in vitro* and tumor xenografts *in vivo*.

2 SPECIFIC AIMS OF THE STUDY

The present PhD work aimed to study the antitumor potential of a set of new flavonoid derivatives, some (naringenin oxime derivatives) to be prepared and characterized within the scope of this work, and others (protoflavone-chalchone hybrid compounds) prepared and characterized by collaborators. Gynecological (cervical and breast) cancer cell lines were selected to evaluate the compounds' *in vitro* antitumor activity, and we also aimed to evaluate possible mechanism of action for selected compounds. The specific aims were the following.

- Design and synthesis of naringenin oxime and oxime ether derivatives.
- Determination of the *in vitro* antiproliferative activity of the synthesized naringenin oxime derivatives on human gynecological cancer cell lines.
- Investigation of the possible mechanism of action of the most active naringenin derivatives through the antioxidant and proapoptotic properties by determination cell cycle analysis and caspase-3 activity.
- Examination of the antioxidant activity of the synthesized naringenin oxime derivatives based on their efficiency to scavenge diphenyl-2-picrylhydrazyl, oxygen radical absorbance capacity and xanthine oxidase inhibitory assays.
- Evaluation of the antiproliferative activity of protoflavone-chalcone hybrid compounds on gynecological cell lines.
- Evaluation of the cell cycle and caspase-3 activity of gynecological cells upon treatment with protoflavone derivatives. This work was aimed as participation in an international collaboration study on the possible mechanism of action of these compounds.
- Evaluation of the pharmacological benefit of coupling protoflavones and chalcones into hybrid compounds, by performing experimental and virtual combination studies on their relevant fragments.

3 MATERIALS AND METHODS

3.1 Chemicals

3.1.1 Synthesis of naringenin oximes and oxime ethers derivatives

Naringenin oximes and oxime ethers derivatives **(1-8)** were synthesized and characterized at the Department of Pharmacognosy (University of Szeged, Szeged, Hungary). Naringenin was dissolved in 100 mL EtOH, then 3-equiv. of hydroxylamine hydrochloride and 3-equiv. of KOH were added. The reaction mixture was refluxed for 48 h, and then the solvent was evaporated under vacuum. The residue was re-dissolved in water and the aqueous phase extracted with EtOAc. The organic phase was combined, dried over Na_2SO_4 and evaporated. Compounds were purified by flash chromatography on silica with a solvent system of *n*-hexane—EtOAc—formic acid (15:4:0.25, v/v/v).

Naringenin oxime ethers were synthesized by dissolving naringenin in pyridine, then 3-equiv. of the corresponding alkyl or aryloxyhydroxylamine hydrochloride was added and the mixture was refluxed for 48–96 h. completion of the reaction was decided based on continuous TLC monitoring. The solvent was evaporated under vacuum. After that, water was added to the residue and solvent-solvent extraction was performed with EtOAc. Then the combined organic phase was dried over Na_2SO_4, filtered, and evaporated. Each crude mixture was purified with flash chromatography on polyamide with a solvent system of CH_2Cl_2—MeOH (99:1, v/v). Naringenin oximes and oxime ethers were characterized by using NMR and MS.

3.1.2 Protoflavone derivatives obtained from collaborators

Four new hybrid compounds **(10a-d)** were prepared at the Institute of Chemistry (Eötvös Loránd University, Budapest, Hungary) and the Institute of Pharmacognosy, University of Szeged, Szeged, Hungary. Each of these compounds joined two fragments, a protoapigenone 1′-O-propargyl ether **(9)** and a chalchone or ferrocene **(11a-d)** through a triazol function (Figure 1).

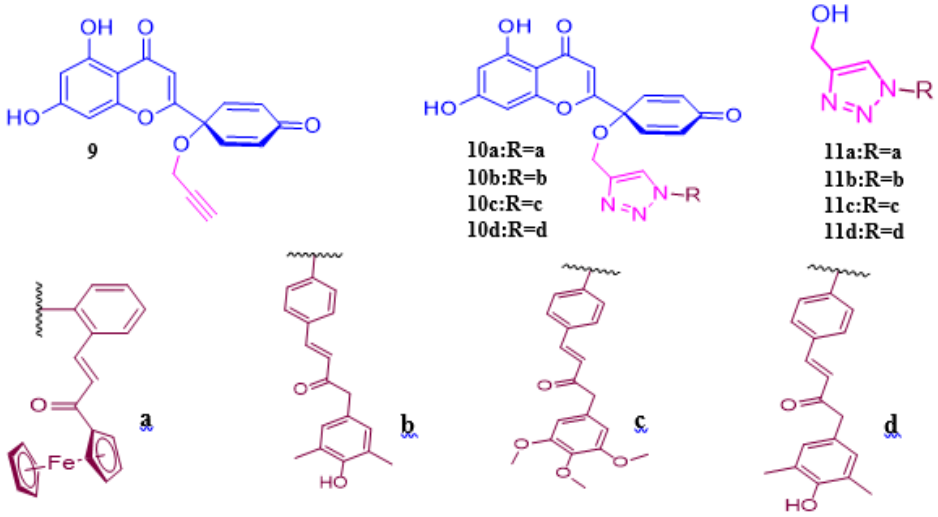


Figure 1. Chemical structure of protoapigenone 1′-O-propargyl ether **(9)** and the protoflavone-chalcone hybrids with triazole linkers **(10a-d).** The hydroxymethyl derivatives **(11a-d)** corresponding to the chalchone fragment were also tested as reference compounds for the hybrids.

3.2 Cell lines and culture conditions

A panel of human gynecological cancer cell lines including: human breast cancer cell lines (MCF-7, MDA-MB-231), cervical carcinoma (HeLa, SiHa), with additional human leukemia cells (HL-60) and non-cancerous mouse embryonic fibroblast cell line (NIH/3T3) were used as *in vitro* design to study the antitumor activity of the tested compounds. All human gynecological and NIH/3T3 cancer cell lines were cultivated in minimal essential medium and enhanced with 10% heat-inactivated fetal bovine serum, 1% antibacterial- antimycotic solution and 1% non-essential amino acids. While the HL-60 leukemia cells cultivated in RPMI 1640 medium, included 10% FBS, 1% antibacterial-antimycotic mixture and 1% L-glutamine. All the cells incubated in a humidified atmosphere at 37°C enclosed with 5% carbon dioxide.

3.3 Treatment with the compounds

The synthesized compounds were solubilized in dimethyl sulfoxide as a 50 and 10 mM standard solution for naringenin oxime and protoflavone derivatives respectively. Two concentrations (25 and 50 µM) were selected for screening the bioactivity of initial naringenin oxime derivatives on breast, cervical and HL-60 cancer cell lines. The values of half-maximal inhibitory concentration were estimated only for those compounds that elicited antiproliferative activity with more than 75% growth inhibition at 50 µM , by repeating adjusted dilutions (1–50 µM) of the compound. In case of protoflavone derivatives, the gynecological cells were exposed to ten different concentrations (0.039, 0.07, 0.1, 0.31, 0.62, 1.25, 2.5, 5, 10 and 20 µM). Cisplatin was used as a positive control (0.1, 0.3, 0.6, 1.25, 2.5, 5, 10, and 20 µM), and untreated cells served as the negative control.

3.4 Antiproliferative activity

The colorimetric MTT assay was employed to estimate the ability of the prepared compounds to suppress the proliferation of treated cancer cells. Cells were seeded and treated in 96-well microplates and incubated for 72 hours. After incubation, 44 µL of MTT solution (5 mg/mL in PBS) was added to each well and incubation continued for another four hours. Subsequently, the supernatant was removed, and the precipitated purple formazan crystals were solubilized by adding 100 µL/well of DMSO and gently shaking them for 1h. Absorbance was measured with ELISA microplate reader at 545 nm. In the case of leukemia cells, the precipitated crystals were dissolved in 10% sodium dodecyl sulfate with acid HCl 0.01 mM and incubated for 24 h, the absorbance read at 545 and 630 nm.

3.5 Cell cycle analysis

The cell cycle distribution was characterized by flow cytometry. The cells were seeded in six-well plates at a density of 4×10^5 cells per well and allowed to grow for 24 h. On the second day, cells were treated with the compounds in two concentrations related to their IC_{50}, with an incubation period of 24 hours. Subsequently, cells were harvested with trypsin, washed with PBS, resuspended, and fixed with 70% cold ethanol. The fixed cells were washed with cold PBS, stained with dye solution containing PI. Incubated in the dark at room temperature for an hour. Finally, detect the DNA contents by flow cytometry, in each experiment, at least 20 000 events per sample were calculated. The distribution of the cells in the different cell cycle phases (subG1, G0/G1, S, and G2/M) was expressed as DNA histograms by using ModFit LT 3.3.11 software.

3.6 Determination of caspase-3 activity

Caspase-3 colorimetric assay kit was used to determine the Caspase-3 in conformity to the manufacturer's instructions. The cells were seeded at a density of 12×10^6 cells in tissue culture flasks and allowed to grow and attach overnight. The cells were treated with the appropriate concentrations of the tested compound and incubated for 24 or 48 h. Cells were harvested, washed with PBS, incubated in lysis buffer on ice for 20 min, and used the supernatant after the cold centrifugation of the lysates. Assays were performed in 96-well plate and incubated for 24 hours. The absorbance was measured at 405 nm with a microplate reader.

3.7 Antioxidant activity

3.7.1 Diphenyl-2-picrylhydrazyl (DPPH) assay

The measurement of the DPPH to the naringenin oxime derivatives was carried out on a 96-well microplate. Series microdilution of samples prepared from the stock solution (10 mM). To each well, added DPPH reagent. The microplate stored in the dark at room temperature for 30 min. Sample absorbance measured at 550 nm.

3.7.2 Oxygen Radical Absorbance Capacity (ORAC) assay

The ORAC assay to the naringenin oxime derivatives were measured in 96 microplate well, mixed a samples standard solution with AAPH and fluorescein solution, and then the fluorescence was calculated through 3 h with 1.5-min cycle intervals by a BMG Labtech FluoStar Optima plate-reader. Trolox used as a standard positive reference.

3.7.3 Xanthine-oxidase inhibitory assay

Xanthine-oxidase inhibitory action of the naringenin oxime derivatives were checked spectrophotometrically at 290 nm on the optima plate-reader. In 96 microplates well mixed 10

μL of samples with 140 μL of the buffer, 100 μL of xanthine and 50 μL of enzyme XO. Allopurinol used as a positive control.

3.8 Combination study of relevant fragments vs hybrids 10b-d

A virtual combination study was performed, in which the cell viability data obtained from the treatment with the hybrid compounds was further analyzed in comparison with corresponding data of relevant fragments. The hybrid compounds **10b-d** were considered as 1:1 ratio mixtures of compound **9** and reference fragments **11b-d**, respectively, and evaluated by Chou-Talalay method using the CompuSyn software. This method is a well-established mathematical model for the calculation of drug-drug interactions. A classical experimental combination study was also performed as a control, for which the cell viability data were obtained from equimolar mixtures of the tested fragments and results were analyzed in comparison with the corresponding single treatment controls.

3.9 Other bioassays done in collaboration

Cell death analysis by AV/PI labelling, and studies on the effect of compounds **10a-d** on the intracellular ROS/RNS levels, and on the mitochondrial membrane depolarization were performed in cooperation with the group of Dr. Milica Pešić, Institute for Biological Research, Department of Neurobiology, University of Belgrade, Belgrade, Serbia.

DNA damage response studies, evaluating the compounds' effect on the ATR-Chk1 signaling pathway were performed in cooperation with the group of Prof. Hui-Chun Wang, Graduate Institute of Natural products, Kaohsiung Medical University, Kaohsiung, Taiwan.

4 RESULTS

4.1 Naringenin oxime derivatives

Naringenin oxime and oxime ethers were prepared. Two geometric isomers of naringenine oxime (**2** and **3**) were obtained from naringenin (**NG**; **1**) by reacting it with hydroxylamine hydrochloride in ethanol in presence of potassium hydroxide, compound **2** was identified as the *E* isomer, while compound **3** as the minor *Z* isomer that was prepared by us for the first time. Five oxime ether derivatives (**4**–**8**) were also prepared by a similar one-step synthesis from the reaction of **(1)** with ethoxy-, methoxy-, allyloxy-, t-butoxy-, or benzyloxyamine hydrochloride, respectively, in the presence of pyridine. In these reactions, the exclusive formation of the *E* isomers was observed, and they are shown in Figure 2.

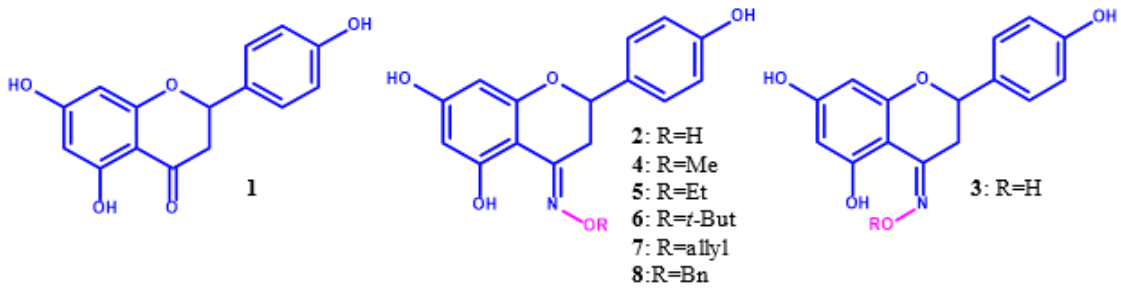


Figure 2. Chemical structures of naringenin (**1**), and the prepared naringenin oximes (**2** and **3**) and oxime ether derivatives (**4-8**).

4.2 Pharmacological activities of naringenin oxime derivatives

4.2.1 Antiproliferative activity of naringenin oxime derivatives

After the initial bioactivity screening by using MTT assay, we found that among all the applied compounds, the *t*-butyl substituted naringenin oxime ether **(6)** exerted substantial and dose-dependent antiproliferative activity against all tested cancer cell lines. Based on our results, we show for the first time that the *E*-oxime ethers are more potent antiproliferative agents as compared with naringenin and naringenin oximes, mainly if the ether contains a bulky alkyl group as in the t-butyl derivative **6.**

Table 1. Antiproliferative activities of naringenin **(1)** and its oxime derivatives **(2-8)** against human gynecological cancer and leukemia cell lines. Cisplatin was used as a positive control; SEM: standard error of the mean; n = 5.

Compound	Conc. (μM)	Growth inhibition (%) ± SEM [Calculated IC_{50} value (μM)]				
		SiHa	HeLa	MDA-MB-231	MCF-7	HL-60
(1)	25	<20	<20	<20	<20	<20
	50	<20	23.9±2.09	<20	<20	<20
(2)	25	<20	<20	<20	<20	<20
	50	<20	28.75±2.44	<20	21.83±3.92	43.40±2.81
(3)	25	<20	<20	<20	<20	<20
	50	<20	<20	<20	<20	<20
(4)	25	<20	<20	<20	<20	37.67±1.29
	50	<20	31.36±2.97	24.35±1.88	48.44±3.27	57.89±1.13
(5)	25	<20	<20	<20	<20	<20
	50	<20	29.36±1.42	<20	44.06±2.18	44.89±0.48
(6)	25	<20	52.37±2.32	27.19±1.78	61.41±1.93	37.31±3.65
	50	88.54±1.51 [35.41]	92.22±1.03 [23.49]	90.33±0.58 [29.74]	87.00±0.61 [19.46]	88.07±0.10 [31.76]
(7)	25	<20	<20	<20	<20	<20
	50	<20	25.04±2.4	<20	33.75±2.45	<20
(8)	25	<20	22.63±0.63	<20	24.29±1.86	<20
	50	<20	37.67±2.01	24.87±3.47	64.47±2.12	<20
Cisplatin	25	86.40±1.02	98.71±0.21	41.37±1.05	90.81±0.22	64.03±0.43
	50	96.72±0.36 [13.63]	99.09±0.24 [11.79]	84.43±0.4 [25.82]	98.49±0.11 [5.15]	84.88±0.41 [5.75]

4.2.2 Effect of naringenin oxime derivatives on the cell cycle distribution

Our results of cell cycle analysis revealed that compound **6** induces disturbance in the cell cycle distribution followed by cell death due to the apoptotic process in HeLa and SiHa cancer cell lines, which was demonstrated by a significant increase in the percentage of hypodiploid (subG1) phase (Figure 3A).

4.2.3 Effect of naringenin oxime derivatives on caspase-3 activity

To verify the proapoptotic activity of the *t*-butyl derivative **(6),** the activity of caspase-3 was additionally determined in HeLa cells after treatment with two concentrations, representing the IC_{50} and its half value, after an incubation time of 24 h. The obtained results indicate that both used concentrations of the tested compound induced a considerable increase in caspase-3 activity in a concentration-dependent manner (Figure 3B).

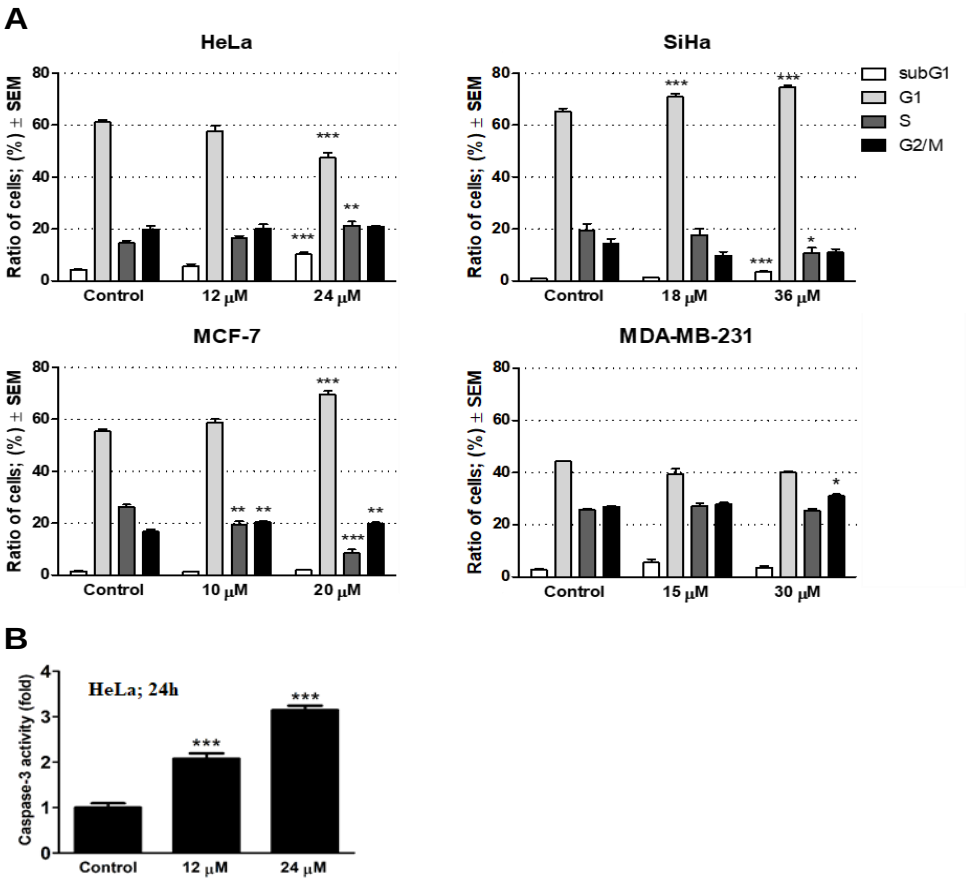


Figure 3. (A) Cell cycle distributions of human gynecological cancer cells after treatment with naringenin t-butyl oxime **(6). (B)** Effect of naringenin t-butyl oxime **(6)** on the caspase-3 activity of HeLa cells. Results from 5 replicates are represented as means ± SEM. *, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

4.2.4 Antioxidant activity of naringenin oxime derivatives

The antioxidant results are shown in Table 2. Except for compound **4**, all synthesized compounds exerted a weak antioxidant activity in all measurement methods when compared with the reference standards rutin or allopurinol. Surprisingly, the oxime methyl ether derivative **4** exhibited the most considerable antioxidant activity in both the ORAC and DPPH assays, and it was also the only one that was more potent in the ORAC assay as compared with the positive control rutin.

4.3 Pharmacological activities of protoflavone derivatives

4.3.1 Antiproliferative activity of protoflavone derivatives

All hybrid compounds displayed more potent cytotoxic activity than the positive control cisplatin (Table 3). The results demonstrated that hybrid compounds (**10a-d**) showed generally more pronounced cytotoxicity compared to their fragments (**9** and **11b-d**). Mainly, among these compounds, hybrids **10b** and **10c** exhibited an excellent antiproliferative activity on the tested cancer cell lines. In general, the breast MDA-MB-231 and the cervical SiHa cells exhibited exceptional susceptibility to the hybrid compounds. The most potent cytotoxic compounds **10b** and **10c** were also tested on non-cancerous mouse embryonic fibroblast NIH/3T3, and their IC_{50} values were 0.99 and 0.89 μM respectively, demonstrating a 3-4 times tumor selectivity as compared to their effect on MDA-MB-231 cells.

Table 2. Antioxidant activity of naringenin **(1)**, and its oxime derivatives (**2–8)** on ORAC, DPPH, and xanthine oxidase assays. TE: Trolox equivalent; n.d.: not determined.

Compound	Antioxidant activity ± SD		
	ORAC (μmolTE/μmol	DPPH EC_{50} (μM)	XO inh (%)
1	11.18±0.46	- [a]	12.31±4.60[b]
2	8.88±0.23	243.45±4.88	7.35±1.32
3	6.95±0.12	1776.00±123.71	2.13±0.78
4	16.63±1.68	212.20±32.59	4.00±1.81
5	5.54±0.41	1437.50±36.06	8.13±2.02
6	3.89±0.87	-	6.95±2.31
7	6.03±2.79	1164.00±226.27	12.84±3.01
8	1.38±0.41	-	9.06±0.79
Allopurinol	n.d.	n.d.	98.23±3.29
Rutin	12.35±0.38	39.88±1.34	n.d.

[a] Compounds eliciting DPPH scavenging activity less than 50% at the highest applied concentration were considered inactive, and the numerical results are not presented; [b] inhibition % at 330 μM

Table 3. Antiproliferative activity of hybrid compounds **(10a-d)** and their corresponding fragments **(9** and **11a-d)** on human gynecological cancer cell lines. C.I.: 95% confidence interval, from two biological replicates.

	IC_{50} [95% C.I.] (μM)			
Compound	MCF-7	MDA-MB-231	HeLa	SiHa
9	1.742 [1.554 – 1.953]	2.525 [2.341 – 2.724]	1.659 [1.391 – 1.977]	2.342 [1.836 – 2.988]
10a	0.4712 [0.4511 – 0.4922]	0.3710 [0.3568 – 0.3858]	3.244 [2.820 – 3.732]	0.4659 [0.4334 – 0.5008]
10b	0.2522 [0.2271 – 0.2801]	0.2913 [0.2725 – 0.3113]	1.104 [0.9689 – 1.257]	0.1772 [0.1627 – 0.1929]
10c	0.2963 [0.2721 – 0.3227]	0.2223 [0.2075 – 0.2382]	0.7083 [0.6332 – 0.7924]	0.1533 [0.1327 – 0.1770]
10d	0.5125 [0.4805 – 0.5466]	0.3241 [0.3023 – 0.3474]	1.013 [0.9537 – 1.075]	0.1965 [0.1762 – 0.2192]
11a[a]	>20	>20	>20	>20
11b	15.07 [13.66 – 16.64]	11.11 [10.53 – 11.71]	23.60 [b] [21.22 – 26.24]	14.63 [13.55 – 15.80]
11c	2.510 [2.235 – 2.818]	4.399 [3.981 – 4.861]	10.32 [9.644 – 11.04]	3.020 [2.755 – 3.312]
11d	11.00 [10.20 – 11.85]	4.921 [4.521 – 5.356]	11.71 [10.93 – 12.53]	8.527 [8.139 – 8.932]
Cisplatin	5.347 [4.965 to 5.758]	26.15 [b] [24.18 to 28.27]	11.86 [10.63 to 13.22]	12.21 [10.90 to 13.69]

[(a)] **11a** exerted less than 10% inhibition on each cell line at the highest tested concentration, therefore, it considered as inactive. [b] Experimental data are available up to 20 μM.

4.3.2 Effect of protoflavone derivatives on the cell cycle distribution

Flow cytometry was applied to investigate the cytotoxic mechanism of selected compounds **10b** and **10c** and to check their ability to induce alteration in the cell cycle progression and apoptosis induction on SiHa and MDA-MB-231 cell lines. It was found that both cancer cell lines exhibited an increase in the proportion of apoptotic (subG1) cells after treatment with compound **10b** or **10c** in a dose-dependent manner (Figure 4). Based on the portion of subG1 cells, compound **10c** displayed more potent effects in both tested cells.

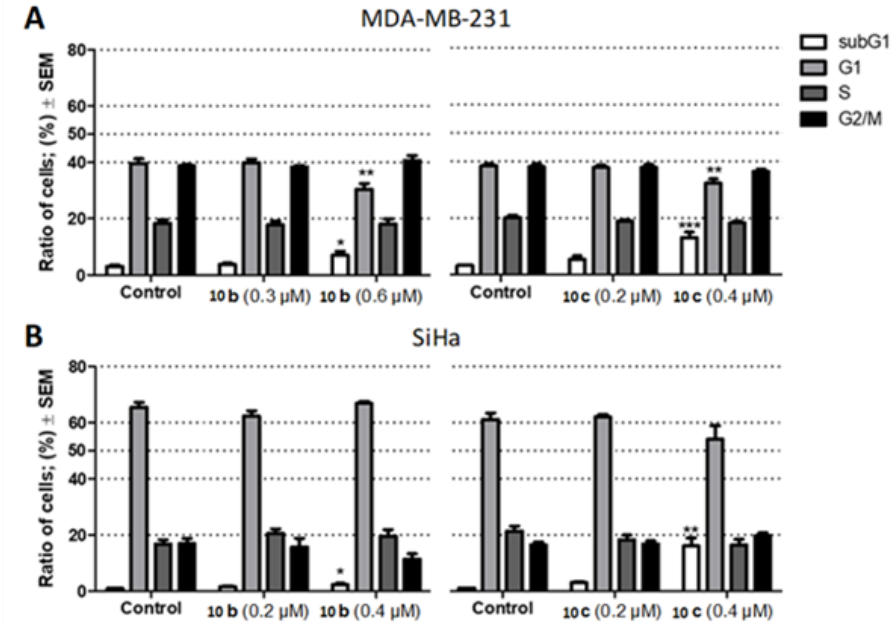


Figure 4. Effect of compounds **(10b and 10c)** on the cell cycle distribution of MDA-MB-231 **(A)** and SiHa **(B)** cells. Cells were treated for 24h; *, ** and ***: $p < 0.05$, $p < 0.01$ and $p < 0.001$.

4.3.3 Caspase-3 activity affected by protoflavone derivatives

The caspase-3 activity was analyzed in MDA-MB-231 cells after exposure to compound **10c** to investigate the apoptotic mechanism. Compound **10c** caused a significant (2-fold) increase in caspase-3 activity at the higher used concentration, after exposure with IC_{50} and double corresponding concentration and incubated for 24 h. Moreover, when the concentration was increased to 0.4 and 1.0 µM and the incubation period to 48 h, the compound produced a significant elevation in the caspase-3 activity in both tested concentrations (4- and 7-fold, respectively) (Figure 5). These results confirm that compound **10c** induced apoptosis in the TNBC cells through activation caspase-3 in a time- and concentration-dependent manner.

4.3.4 Combination study of protoflavone derivatives

The protoflavone hybrid derivatives showed the strongest synergism effects in the virtual combination studies in the MDA-MB-231 and SiHa cells. This study also showed that the hybrids protoflavone derivatives were much more potent than what would be expected by the cytotoxicity of their corresponding building blocks, and this manifested in strong synergism in every case. These results strongly suggest that the hybridization of these fragments offers a significant pharmacological benefit, and that a virtual combination study by Chou-Talalay method can be used to evaluate this (Figure 6).

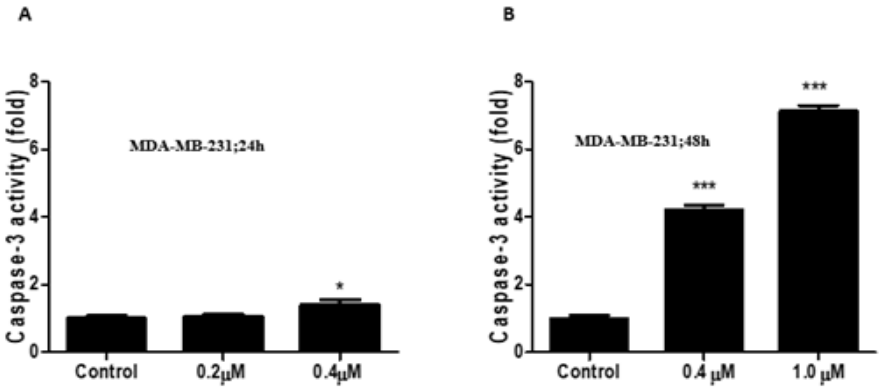
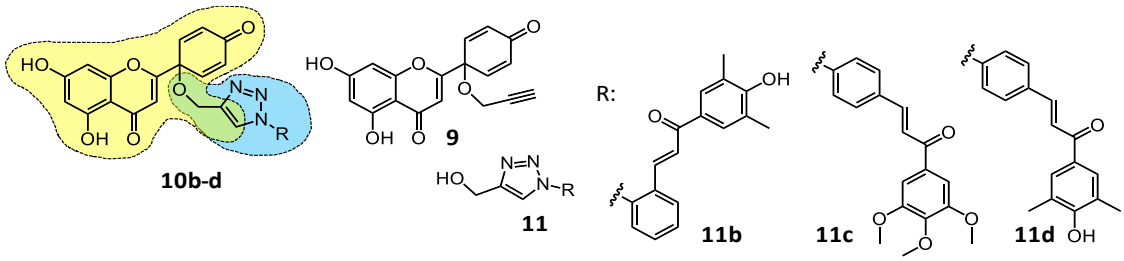


Figure 5. Effect of compound **(10c)** on the caspase-3 activity of MDA-MB-231 cells. **(A)** Cells treated for 24h **(B)** Cells treated for 48h. *, and ***: $p < 0.05$, and $p < 0.001$, respectively.



Cell line	Type of combo	Hybrid	Fragments combined	CI value at ED_{50}	CI value at ED_{75}	CI value at ED_{90}	Dm	m	r	CI_{avg}
MCF-7	Virtual	**10b**	**9 + 11b**	0.22	0.19	0.17	0.31	2.48	0.993	0.18
		10c	**9 + 11c**	0.34	0.30	0.27	0.33	2.91	0.997	0.30
		10d	**9 + 11d**	0.39	0.36	0.34	0.53	2.38	0.998	0.35
	Exp.	**10b**	**9 + 11b**	0.70	0.71	0.73	2.18	2.51	0.989	0.72
		10c	**9 + 11c**	1.03	1.06	1.10	1.69	2.47	0.980	1.07
		10d	**9 + 11d**	0.98	0.93	0.87	2.38	2.55	0.997	0.91
MDA-MB-231	Virtual	**10b**	**9 + 11b**	0.18	0.15	0.13	0.25	3.36	0.982	0.14
		10c	**9 + 11c**	0.21	0.17	0.13	0.21	4.37	0.986	0.16
		10d	**9 + 11d**	0.23	0.20	0.17	0.27	3.01	0.982	0.19
	Exp.	**10b**	**9 + 11b**	0.75	0.96	1.22	1.28	2.01	0.972	1.05
		10c	**9 + 11c**	0.86	0.85	0.86	1.67	2.30	0.961	0.85
		10d	**9 + 11d**	0.77	0.79	0.80	1.53	1.86	0.972	0.79
HeLa	Virtual	**10b**	**9 + 11b**	0.48	0.51	0.56	0.74	1.73	0.994	0.53
		10c	**9 + 11c**	0.56	0.47	0.39	0.78	3.31	0.976	0.45
		10d	**9 + 11d**	0.72	0.58	0.48	1.03	3.37	0.997	0.56
	Exp.	**10b**	**9 + 11b**	1.05	1.14	1.32	1.95	1.97	0.962	1.22
		10c	**9 + 11c**	0.77	0.85	0.97	2.06	1.85	0.912	0.90
		10d	**9 + 11d**	0.89	0.97	1.09	2.32	2.31	0.952	1.01
SiHa	Virtual	**10b**	**9 + 11b**	0.10	0.07	0.05	0.25	4.72	0.984	0.06
		10c	**9 + 11c**	0.10	0.08	0.07	0.20	3.52	0.992	0.08
		10d	**9 + 11d**	0.11	0.10	0.09	0.24	2.47	0.994	0.10
	Exp.	**10b**	**9 + 11b**	0.95	1.13	1.40	1.43	1.79	0.964	1.24
		10c	**9 + 11c**	1.09	1.16	1.23	1.60	2.07	0.931	1.18
		10d	**9 + 11d**	0.78	0.94	1.16	1.76	2.90	0.843	1.02

Figure 6. Virtual and experimental comparative analysis of the hybrid compounds **10b-d** with that of their fragments. Combination study shown at 50%, 75% and 90% of inhibition. CI: combination index; $0 < CI < 1$, $CI = 1$ and $CI > 1$ represent synergism, additivity and antagonism, respectively. Dm, m, and r represent antilog of the x-intercept (activity), slope (shape of the dose-effect curve), and linear correlation coefficient (conformity of the data) of the median effect plot, respectively. $CI_{avg} = (CI_{50} + 2 \times CI_{75} + 3 \times CI_{90})/6$. The lowest CI_{avg} value demonstrates the highest added benefit of hybridization in terms of in vitro cytotoxic activity.

5 DISCUSSION

5.1 Naringenin oxime derivatives

5.1.1 Preparation of naringenin oximes and oxime ethers

Several studies reported that oximes or oxime ethers might have stronger antitumor activity than their corresponding oxo-compounds. Therefore, we decided to synthesize such derivatives of naringenin, to possibly improve its anticancer activity. Two naringenin oxime isomers and five different oxime ether derivatives were successfully obtained. In addition, among all the synthesized compounds the *Z*-isomer, ethyl, tert-butyl and allyl ether derivatives were synthesized as new compounds.

5.1.2 Antiproliferative effects of naringenin oximes and oxime ethers

We proved for the first time that elongation of the C-4 *E*-oxime ethers of naringenin, and mainly if the ether is a t-butyl group, naringenin leads to a significant increase in the cytotoxic properties of the compounds. Our results are consistent with previous studies of other authors who explained that modification of the flavanone carbonyl group at C-4 position with oximes led to stronger anticancer activity against different types of cancer cell lines.

5.1.2 Cell cycle analysis

Gynecological cancer cell lines that were treated with prepared compounds showed a considerable increase in the number of hypodiploid cells (subG1 population) in HeLa and SiHa cells after a 24h incubation period, which indicates that the cytotoxic activity of compound **6** in HeLa and SiHa cells occurs via apoptosis. In addition, it also brought about a disruption in the cell cycle in a concentration dependent manner. Our data suggest that the effect of the naringenin oxime ether (**6**) on the cell cycle progression depended on the cancer cell type.

5.1.3 Caspase-3 activity

The antiproliferative and pro-apoptotic activity of the *t*-butyl derivative (**6**) was confirmed by testing the caspase-3 activities in HeLa cells after a 24 h incubation. The present study suggested that compound **6** induced apoptosis in a concentration-dependent manner, which was established by the significant increase in caspase-3 activity besides the considerable rise in the sub-G1 proportion. Our results confirmed that the *t*-butyl ether group on naringenin 4-*E*-oxime significantly enhanced programmed cell death in HeLa cancer cells and thus could be considered as a promising agent against cervical cancer. Further, there is no previous scientific report on the effect of naringenin oxime derivatives on the caspase activity of cancer cells.

5.1.4 Antioxidant activity

Our results revealed that higher DPPH radical scavenging activity than that of naringenin can be observed only in the *E*-oxime and its methyl ether. Concerning the DPPH scavenging activity here, our study was consistent and confirmed earlier reports on the higher antioxidant capacity of naringenin *E*-oxime as compared with its parent compound.

5.2 Protoflavone derivatives

5.2.1 Antiproliferative effects

In the present study, our data suggest that the antiproliferative activity of the new hybrid derivatives were dependent on the position and nature of the fragment on the protoflavone-chalcone hybrids with triazole linker, and showed that the presence of the methoxy group on the hybrid compound structure was beneficial for a potent cytotoxic effect.

5.2.2 Cell cycle analysis

The two protoflavone hybrid compounds **10b** and **10c** exhibited potent cytotoxic effect in cervical SiHa and TNBC MDA-MB-231 cells, and both tested compounds increased the cell population at the subG1 phase. In the case of MDA-MB-231, the effect was associated with a reduction in the percentage of G1 phase cells in a concentration-dependent manner after 24h, which indicates a hallmark of enhanced apoptosis with noticeable effects detected after exposure to compound **10c**. Our study showed that the presence of methoxy groups on the chalcone fragment led to a more potent apoptotic effects as compared with the case when methyl groups were present at the same positions.

5.2.3 Caspase-3 and apoptotic activity

Our results demonstrated that the synthetic hybrid protoflavone **10c** compound was prominent in the cytotoxic and pro-apoptotic effects through triggering caspase-3. We found that **10c** was potent in inducing caspase-3-mediated apoptosis in the TNBCs in a concentration and time-dependent manner. Our data suggest that cytotoxicity of the protoflavone derivatives was due to apoptotic cell death.

5.2.4 Combination study

Using the Chou-Talalay method as a mathematical tool to perform a quantitative comparison between the bioactivity of two fragments and their corresponding hybrid is, to the best of our knowledge, a novel approach. We believe that with an appropriate selection of fragments to evaluate, such a virtual combination study provides a reasonable and easy-to-use platform to assess the bioactivity of hybrid compounds in general; therefore, we suggest an extension for the applicability of the Chou-Talalay method to analyze related bioactivity data.

5.2.5 Collaboration findings

The hybrid compounds were further tested within an international collaboration network, and their findings provided independent confirmation of our results, and provided some mechanistical background to the observed potent antitumor activity of the hybrid compounds.

The potential of hybrid compounds **10a-d** to induce cell death in both tested breast cancer cell lines is shown in Figure 7.

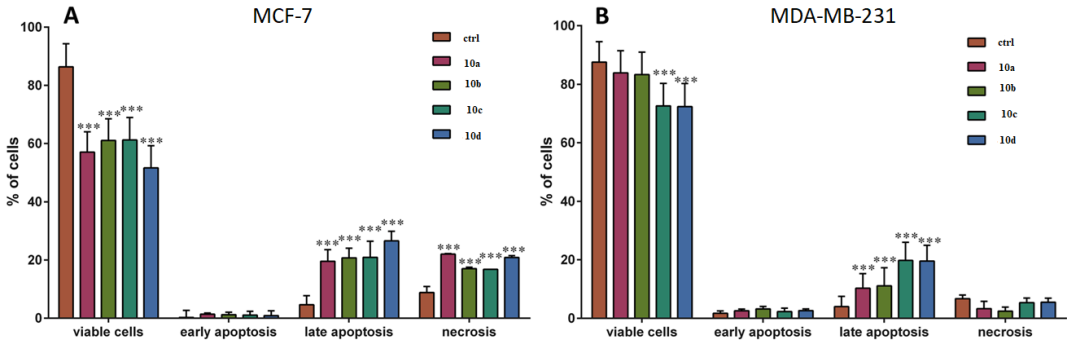


Figure 7. Cell death induction on the **(A)** In MCF-7 and **(B)** MDA-MB-231 cells by the hybrid compounds **(10a-d).** After treatment with 500 nM of each compound for 72 h.

Figure 8A shows that treatment with compounds **10a-d** exhibited a significant suppression of cisplatin-induced Chk1-S345 phosphorylation, and the activity was more potent than that of the positive control. Also, the intracellular ROS and RNS levels in the breast cancer cells were investigated after treatment with protoflavone derivatives (Figure 8B-E). The integrity of mitochondrial membrane potential (MMP) after the treatment of both cancer cell lines with the hybrid compounds **10a-d** was also tested (Figure 9).

Our cooperator's *in vitro* findings further demonstrated that all hybrid compounds induced significant late apoptosis in both cell lines, while a substantial increase in primary necrosis presented only in the MCF-7 cells.

Moreover, it was demonstrated that the hybrid compounds might have a chemo-sensitizing activity when used as an adjuvant with cisplatin chemotherapy. Under these results in the current study, we suggest that DNA damage plays an important role in the cytotoxicity of the hybrid compounds **10a-d**.

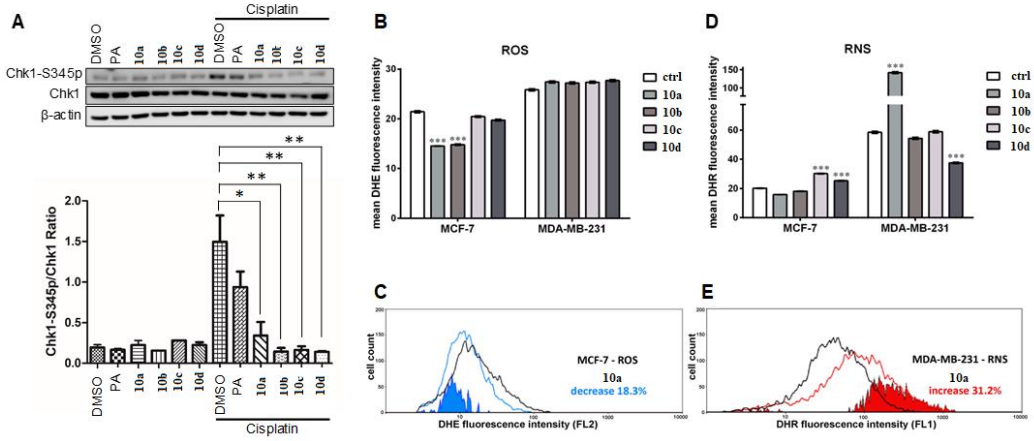


Figure 8. Effects of compounds **(10a-d)** on DNA damage response and redox balance in breast cancer cells. **(A)** Effect on Chk1 phosphorylation in MCF-7 cells, PA (protoapigenone); **(B)** Effect on ROS production; **(C)** Decrease in ROS production by **10a**; **(D)** Effect on RNS production; **(E)** Increase in RNS production by **10a**.

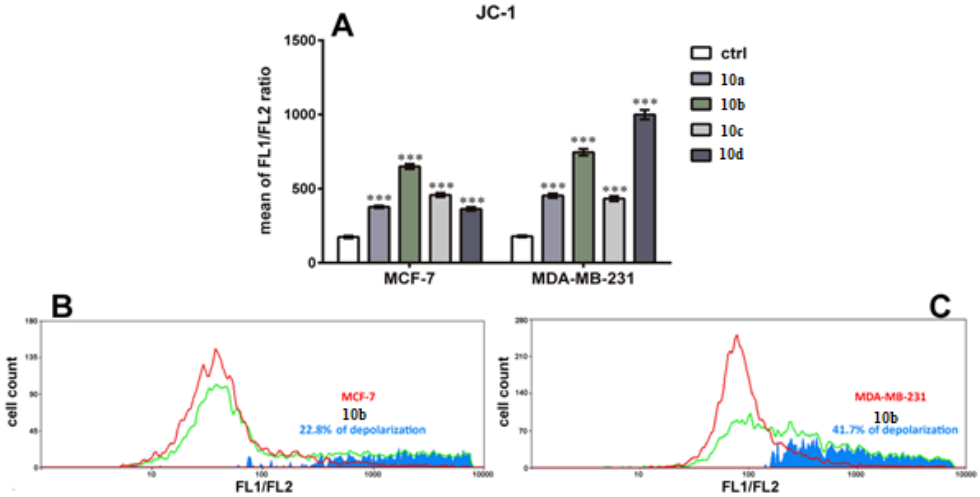


Figure 9. Hybrid-protoflavones induce mitochondrial membrane depolarization. **(A)** Increase in the ratio of green to red fluorescence (FL1/FL2) assessed by JC-1 staining in MCF-7 and MDA-MB-231 cells after 24 h treatments with one μM of compounds **10a-d**; **(B)** Illustration of the effect induced by **10b** in MCF-7 cells; **(C)** Illustration of effects caused by **10b** in MDA-MB-231 cells.

Further, the most noticeable effect on RNS levels was observed with **10a** suggesting its significant pro-oxidant activity in MDA-MB-231 cells. On the other hand, **10d** significantly reduced the RNS levels in the TNBC cells. The results showed that the protoflavone-chalcone hybrids can scavenge ROS and induce RNS production in both tested breast cancer cell lines and suggest that the apoptosis effects of protoflavone derivatives were not or not directly dependent on their pro-oxidant activity.

While compounds **10a-c** displayed potent mitochondrial membrane depolarization in MDA-MB-231 and MCF-7 cancer cells, compound **10d** showed pronounced selectivity

towards MDA-MB-231 cells in this regard. These results indicated that the apoptotic effects of protoflavone compounds involve mitochondrial-mediated apoptotic pathways with depolarization of MMP, generation of RNS levels in both breast cancer cell lines.

SUMMARY

The present PhD work aimed to contribute to the knowledge available on the antitumor properties of flavonoids through the *in vitro* evaluation of the bioactivity of some uncommon semi-synthetic derivatives against a panel of breast and cervical cancer cell lines that are well-established models for certain types of gynecological cancer. In summary, our work led to the following results.

- Two naringenin oxime isomers and five oxime ether derivatives were synthesized, purified and characterized. Four of these compounds, such as the minor product naringenin Z-oxime, and naringenin E- ethyl, allyl, and tert-butyl oxime ethers were prepared as new compounds.
- When evaluating the *in vitro* cytotoxicity of the prepared derivatives of naringenin, tert-butyl oxime ether **(6)** showed the most potent effects on different gynecological cancer cells, with significant activity against MCF-7 and HeLa cells.
- The flow cytometric analysis of compound **(6)** on gynecological cancer cells revealed significant accumulation of cells in the hypodiploid (sub-G1) phase in HeLa & SiHa cell lines, indicating the apoptosis induction effect, and induced cycle suppression at G2/M stage in MCF-7 cancer cells. Further, the proapoptotic activity of this compound was confirmed in HeLa cells by detecting the increased activity of caspase-3.
- To our surprise, naringenin methyl oxime ether was more potent in the ORAC assay than its parent compound, while all other analogs were up to an order of magnitude less active. This suggests good peroxyl radical scavenging capacity for this compound.
- There was no apparent correlation between the *in vitro* cytotoxic and antioxidant activities of the tested compounds, suggesting that their anticancer effects are likely not related to their antioxidant properties.
- Four protoflavone hybrid compounds were identified as promising antitumor lead compounds based on their prominent *in vitro* cytotoxic effects and their selectivity on different breast and cervical cancer cells with antiproliferative effects better than cisplatin.
- The most potent compounds have an intense proapoptotic effect on TNBC, as evidenced by flow cytometric investigation and caspase-3 activity. It was shown that compound **10c** induces a considerable expansion in caspase-3 activity in a concentration-time dependent manner with a significant increase in the sub G1 phase.
- A novel approach was used to evaluate the bioactivity of the hybrid compounds in comparison with that of their corresponding fragments. A virtual combination study was performed by using the Chou-Talalay method as a mathematical tool, and results were compared to the corresponding experimental combinations of the cells with non-coupled fragments. This gave valuable extra information as virtual combination index values and confirmed that the studied hybrid compounds are much more potent than what would be expected by a mathematical sum of the bioactivity of their fragments.
- Based on the above, we demonstrated the use of a novel approach to evaluate the bioactivity of hybrid compounds in general, and suggested an extension of the applicability of the Chou-Talalay method, one of the currently available most popular platforms for drug-drug combination studies.

SCIENTIFIC PUBLICATION RELATED TO THE SUBJECTS OF THE THESIS

1. **Latif AD**, Jenei T, Podolski-Renić A, Kuo CY, Vágvölgyi M, Girst G, Zupkó I, Develi S, Ulukaya E, Wang HC, Pešić M, Csámpai A, Hunyadi A: Protoflavone-Chalcone Hybrids Exhibit Enhanced Antitumor Action Through Modulating Redox Balance, Depolarizing Mitochondrial Membrane and Inhibiting ATR-Dependent Signaling. *Antioxidants*. 2020; 09: 519. (IF_{2019}: 5.014; Clinical biochemistry - Q1)

2. **Latif AD**, Gonda T, Vágvölgyi M, Kúsz N, Kulmány Á, Ocsovszki I, Zomborszki ZP, Zupkó I, Hunyadi A: Synthesis and in vitro antitumor activity of naringenin oxime and oxime ether derivatives. *Int J Mol Sci*. 2019; 20: 2184. (IF_{2019}: 4.556; Organic chemistry - Q1)

ADDITIONAL PUBLICATIONS

1. Keglevich A, Dányi L, Rieder A, Horváth D, Szigetvári Á, Dékány M, Szántay C, **Latif AD**, Hunyadi A, Zupkó I, Keglevich P, Hazai L: Synthesis and cytotoxic activity of new vindoline derivatives coupled to natural and synthetic pharmacophores. Molecules. 2020; 25: 1010. (IF_{2019}: 3.267; Pharmaceutical science - Q1)

PRESENTATIONS AND POSTER RELATED TO THE THESIS

1. **Latif AD** ,Vágvölgyi M , Girst G, Jenei T, Zupkó I, Csámpai A, Hunyadi A. In vitro antitumor activity of protoflavone-based hybrid compounds on human gynecological cancer cell lines. Trends in Natural Product Research - PSE Young Scientists' Meeting on Biochemistry, Molecular Aspects and Pharmacology of Bioactive Natural Products, Budapest, 2019, oral presentation.

2. Fási L#, Vágvölgyi M#, **Latif AD#**, Issaadi M, Zoofishan Z, Zupkó I, Spengler G, Martins A, Hunyadi A. Natural product inspired chemical approaches against MDR cancer. First Working-Group Meeting: New Diagnostic and Therapeutic Tools against Multidrug- Resistant Tumours, Turin, Italy, 2019, oral presentation.

3. **Latif AD**, Gonda T, Creeper N, Kulmány Á, Zupkó I, Hunyadi A. Synthesis and evaluation of cytotoxic and antioxidant effects of naringenin oxime relative to naringenin on human cancer cell lines. Forum of Young Herbalists: Scientific conference of the Department of Herbs of the Hungarian Pharmaceutical Society. Szeged, Hungary, 2018, oral presentation.

4. **Latif AD**, Gonda T, Creeper N, Kulmány Á, Zupkó I, Hunyadi A. Synthesis and biological activity of naringenin-oxime derivatives. GA: The 66th Annual Meeting of the Society for Medicinal Plant and Natural Product Research, Shanghai, China, 2018, poster presentation.

5. **Latif AD**, Gonda T, Creeper N, Kulmány Á, Zupkó I, Hunyadi A. Anticancer and antioxidant effects of naringenin and its semi-synthetic oxime ethers. Serbian Biochemical Society Eighth Conference, Novi Sad, Serbia, 2018, poster presentation.