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Evaluation of the Cytotoxic Effect and Antiproliferative Activity of 3-Carboxylated Coumarins Derivatives in an *In Vitro* Model.

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ABSTRACT

Herein we report an exploratory study based on the cytotoxicity and antiproliferative activity of four previously synthesized coumarins derivatives (compounds **1a-d**). The cytotoxic effect of the compounds was assessed on mononuclear cells, which were obtained from blood samples of healthy donors and measured by XTT method. The antiproliferative activity experiments were developed using HeLa, CaSKi and SiHa cervical cancer cell lines, and was evaluated by the MTT assay. In every single experiment, Cisplatin as internal control was employed. The cytotoxic assessment revealed that the four compounds did not significantly affect the viability on normal cells, whereas the antiproliferative activity on cancer cells was variable, according to the substituent located at position 3 of the coumarin core. It is worth mentioning that compound **1c**, compared with the other products, presented a remarkable effect against CaSKi cell line, likewise **1d** but in HeLa cells. These findings suggest that there is a relationship between biological activity and the alkoxycarbonyl chain since this is the only structural difference among the four tested compounds. The results lead to conclude that butyl group which is the substituent in compound **1d**, was the key element in the antiproliferative effect presented by the molecule against SiHa, CaSKi and HeLa cell lines.

Keywords: Coumarins, Tumor cells, Mononuclear cells, Cis-Platin, Cytotoxicity, MTT.

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INTRODUCTION

Coumarins are natural products abundantly present in a wide variety of medicinal plants. The pharmacological importance of coumarins rises from the diverse biological activities attributed to them, such as anticancer[1,2], anti-inflammatory, anticoagulant among others.[3-4] It has been proposed that the multiple pharmacological function may be associated with determined substituents present in their derivatives.[5] Therefore, it is considered that any modification in a known structure could translate in a new therapeutic property.[6] Cancer development is a series of complex events that include tumor formation, angiogenesis, cell migration, dissemination and growth at a secondary site known as metastasis.[7] In developing countries, Invasive Cervical Cancer (ICC) is considered a serious health problem because of the high incidence rate in women at reproductive age[8]. The main etiological factor associated to onset of ICC is the persistent infection with Human Papillomavirus (HPV) particularly those designed as High-risk genotypes, such as 16 and 18[9]. Despite the continuous improvements in the pharmacological treatment of cervix cancer, the main adverse effect of chemotherapy is the systemic toxicity that compromises the functioning of vital organs, such as bone marrow, oxidative stress in mononuclear cells[10], liver and kidney[11], consequently, the main characteristics, that a new drug, as a promising alternative treatment must include are: to contain a high specificity towards malignant cells and cause the minor side effects in organs and vital systems in sick individuals; however, it is a great challenge, especially in advanced carcinogenic processes

Several studies have reported the biological properties that some natural coumarins exhibited over tumor cells, for instance, the ability to inhibit cell cycle progression, activate apoptosis or reduce telomerase activity. These findings, along with minimal adverse effects observed on normal cells, suggest that coumarins could be employed in the design of molecular targets implicated in cancer onset[12]. In a previous report we presented the synthesis of four coumarins derivatives obtained through the Knoevenagel condensation methodology (Gonzalez, *et al* **2016**). In the current work, we present the *in vitro* cytotoxic evaluation on mononuclear cells and the antiproliferative effect on cervical cancer cell lines (HeLa, CaSKi and SiHa)[13]. Structurally, the four compounds are quite similar as all of them contain the same coumarin core that basically consist of an aromatic ring fused with a cyclic ester. The compounds are different just at three position which is functionalized by an alcoxycarbonyl chain. Thus, the substituents are methyl, (**1a)**, ethyl (**1b)**, isopropyl (**1c)** and *n*-butyl (**1d**). (Figure 1).

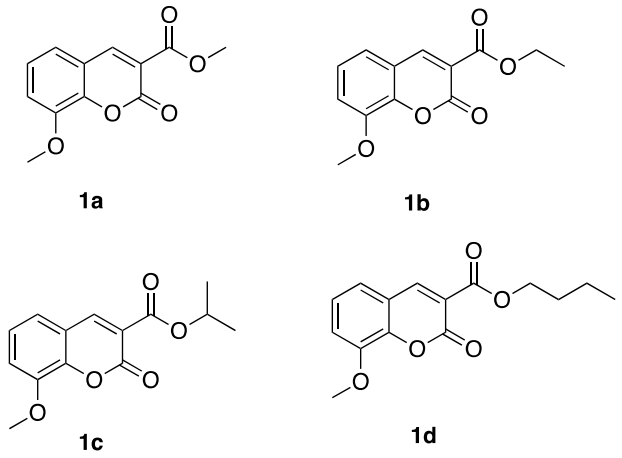


Figure 1: Coumarin derivatives tested in the biological assay

According to recent literature, different substituents in a core structure can cause different biological activity in living organisms[14,15] therefore, there were expected different behaviors of the tested compounds due to the differences in the alkyl chain present in the ester moiety of the alkoxycarbonyl chain located in the coumarin structure. (Figure 2)

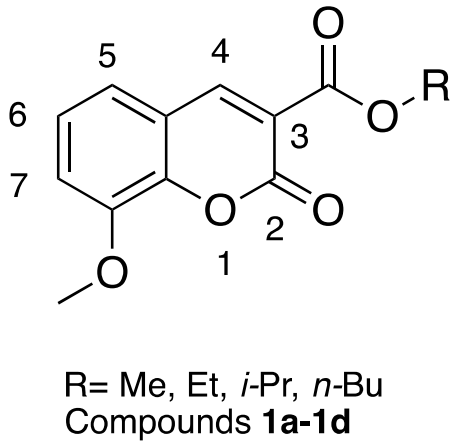
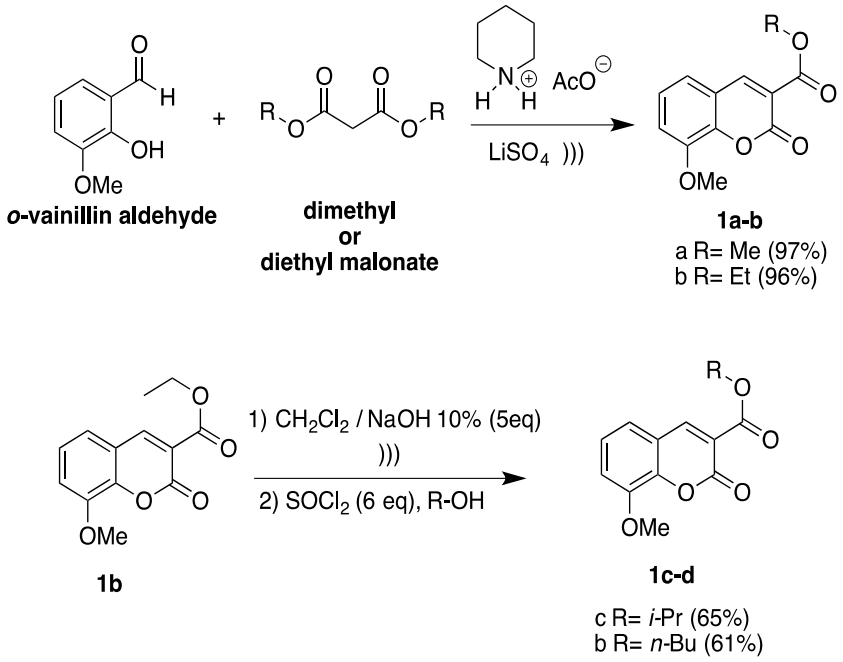


Figure 2: base structure of coumarin derivatives

MATERIALS AND METHOD

All reagents employed in the synthesis were of analytical grade and used without further purification (Sigma-Aldrich USA). Melting points were measured on a Fischer-Jones apparatus and left uncorrected. The structural characterization was done by High Resolution Mass Spectrometry (HRMS), Infrared Spectroscopy (IR) and ^{1}H and ^{13}C NMR. Mass Spectra were recorded in a Thermo Fischer Scientific LQT-Orbitrap ESI; Infrared spectra (IR) were recorded on a Perkin-Elmer Spectrum One FTMS spectrometer. NMR spectra were recorded on a Varian VX-400 spectrometer with tetramethylsilane (TMS) as an internal standard, and chemical shifts are reported in δ (ppm).

Thin-layer chromatography was carried out with silica gel H (200-300 mesh or 500 mesh). Organic solutions were dried over anhydrous sodium sulfate.



Scheme 1. Synthesis of coumarin derivatives 1a-d

Coumarin derivatives were obtained accordingly to a previously reported method and spectroscopic data of the reaction products correspond to those published in literature.[16] A brief summary of the involved reactions is shown in **Scheme 1.**

Chemistry

The structural characterization of synthesized compounds was done by NMR and Infrared spectroscopy. NMR spectra were recorded on a Varian VX-400 spectrometer with tetramethylsilane (TMS) as an internal standard, and chemical shifts are reported in δ (ppm). Infrared spectrum (IR) was recorded on a Perkin-Elmer Spectrum One FTMS spectrometer. Melting points were measured on a Fisher-Jones apparatus and were not corrected. Thin-layer chromatography (TLC) was performed on silica gel F_{254} plates. Detection was made by immersion in potassium permanganate and UV light irradiation (UV lamp, model UV-IIB). Column chromatography was carried out with Silica gel H (200-300 mesh or 500 mesh). Unless otherwise stated, all reagents were purchased from commercial sources. When necessary, they were purified and dried by standard methods. Organic solutions were dried over anhydrous sodium sulfate.

Characterization of 3-carboxilated coumarins.

Methyl-8-methoxy-2-oxo-2H-chromene-3-carboxylate. 1a. Yellow solid (97% yield), mp 142 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 3.95 (s, 3H-1), 3.97 (s, 3H-11), 7.20 (m, *J*=7.5Hz, 3H-6,7,8), 8.5 (s, 1H-4). ^{13}C NMR (100 MHz, $CDCl_3$) δ = 52.76, 56.15, 115.77, 117.83, 118.23, 120.52, 124.67, 144.67, 146.86, 149.31, 156.04, 163.58

Ethyl-8-methoxy-2-oxo-2*H*-chromene-3-carboxylate. 1b. White solid (96% yield), mp 82 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 1.41 (t, *J*=4Hz, 3H-1), 3.97 (s, 3H-10), 4.41 (q, *J*=4Hz, 2H-2), 7.18 (dd, *J*=1.2Hz, 1H-9), 7.19 (dd, *J*=1.2Hz, 1H-7), 7.26 (dd, *J*=6Hz, 1H-8), 8.5 (s, 1H-5). ^{13}C NMR (100 MHz, $CDCl_3$) δ = 14.42, 56.48, 61.40, 115.68, 118.30, 120.43, 124.62, 144.69, 146.91, 148.73, 156.06, 162.95.

Isopropyl-8-methoxy-2-oxo-2*H*-chromene-3-carboxylate. 1c. Light yellow solid (65% yield), mp 120 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 1.38 (s, 3H-1), 1.40 (s, 3H-2), 3.97 (s, 3H-12), 5.26 (m, *J*=4Hz, 1H-3), 7.25 (m, 3H-8, 9, 10), 8.44 (s, 1H-6). ^{13}C NMR (100 MHz, $CDCl_3$) δ = 21.73, 56.21, 69.56, 115.71, 118.36, 118.75, 120.51, 124.64, 144.69, 146.95, 148.17, 156.13, 162.22.

Butyl-8-methoxy-2-oxo-2*H*-chromene-3-carboxylate. 1d. Yellow solid (61% yield), mp 72 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 0.98 (t, *J*=4 y 8Hz, 3H-1), 1.47 (m, *J*= 8Hz, 2H-2), 1.76 (m, *J*= 4 y 8Hz, 2H-3), 3.97 (s, 3H-13), 4.35 (t, *J*= 4Hz, 2H-4), 7.25 (m, *J*= 4 y 8, 3H-9,10, 11), 8.48 (s, 1H-7). ^{13}C NMR (100 MHz, $CDCl_3$) δ = 13.70, 19.11, 30.56, 56.27, 65.78, 115.73, 118.42, 118.55, 120.55, 124.63, 144.83, 148.65, 156.09, 163.16

NMR spectra (^{1}H and ^{13}C) of coumarins 1a-1d.

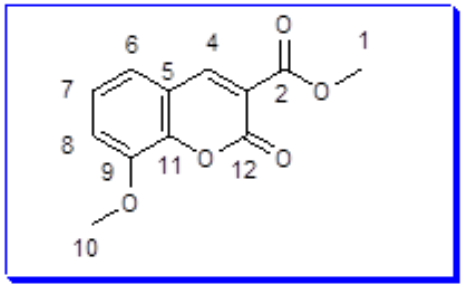
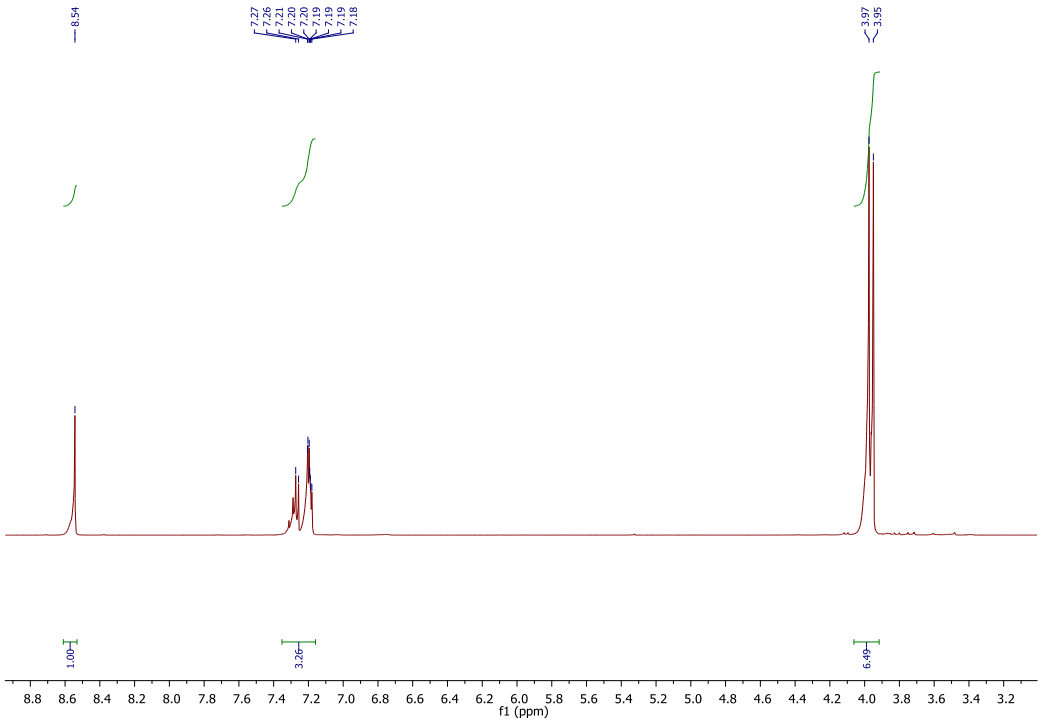
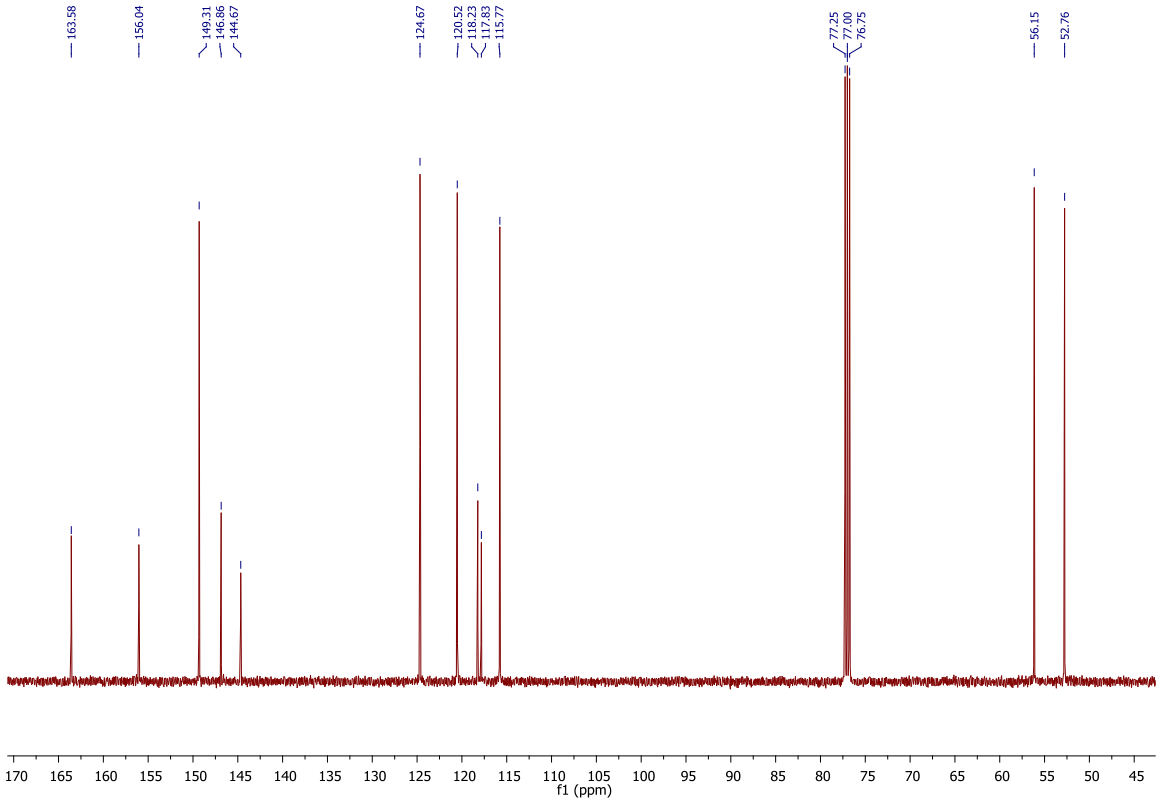


Figure 3



Graph 1



Graph 2

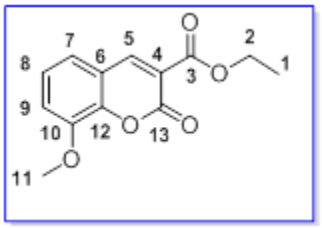
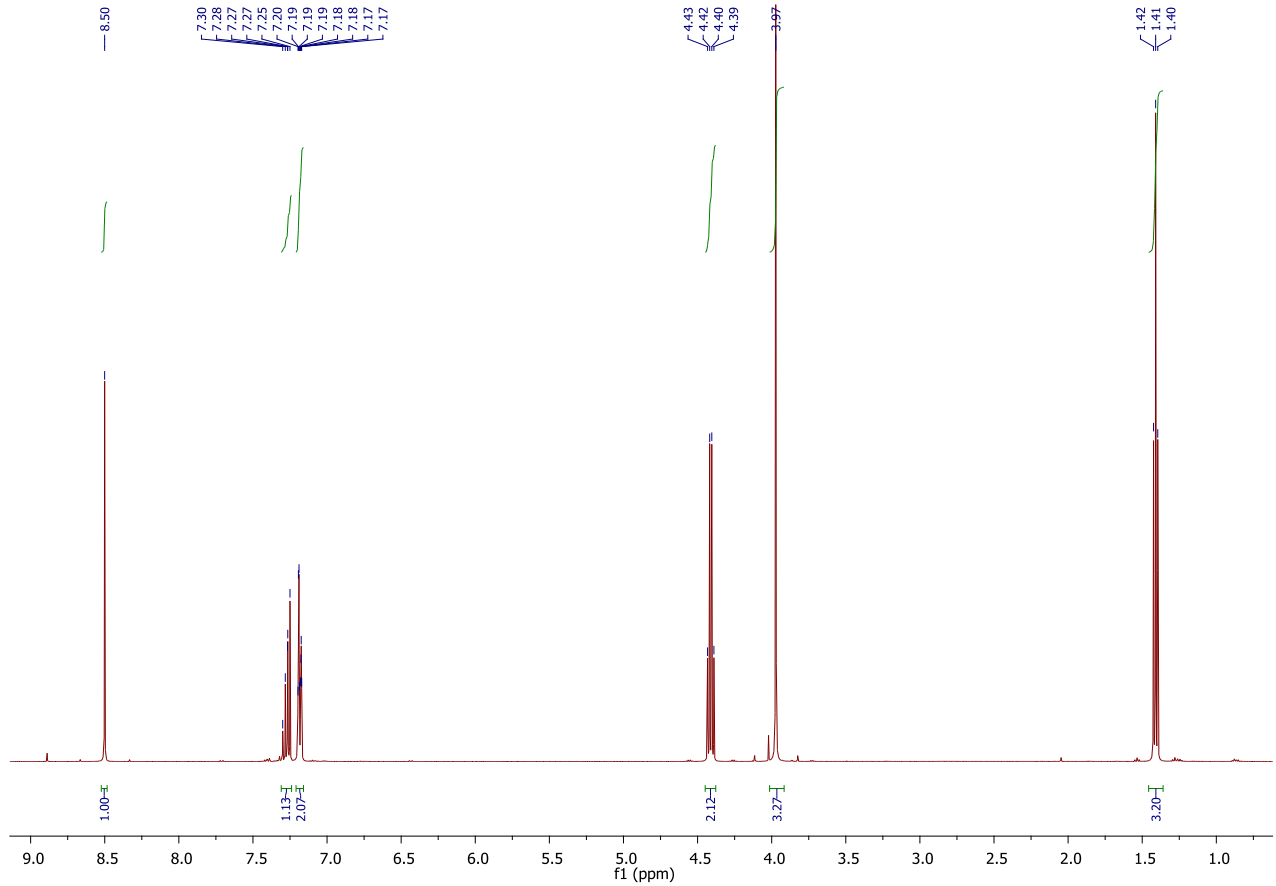
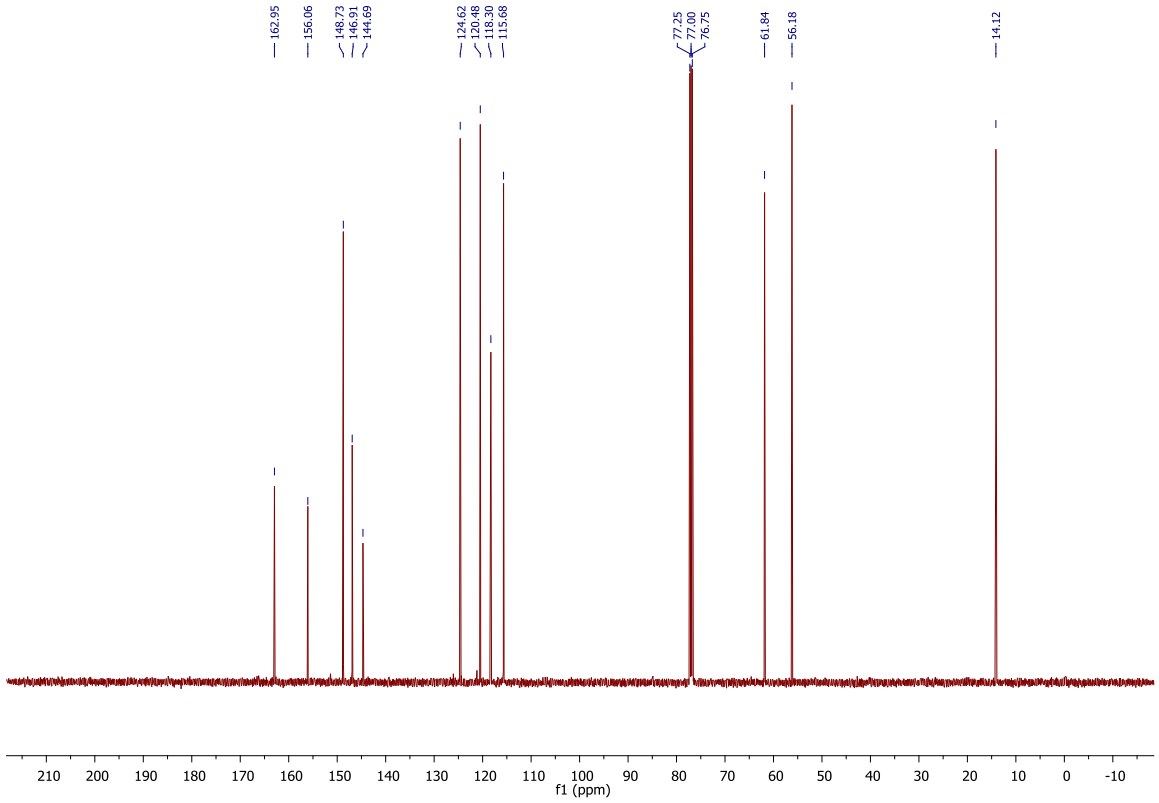


Figure 4



Graph 3



Graph 4

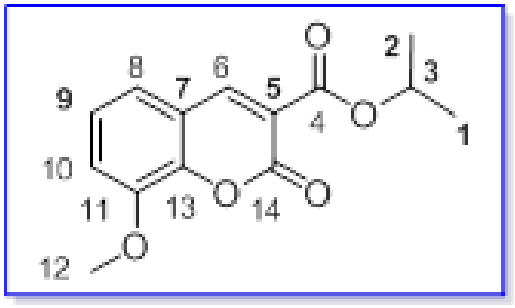
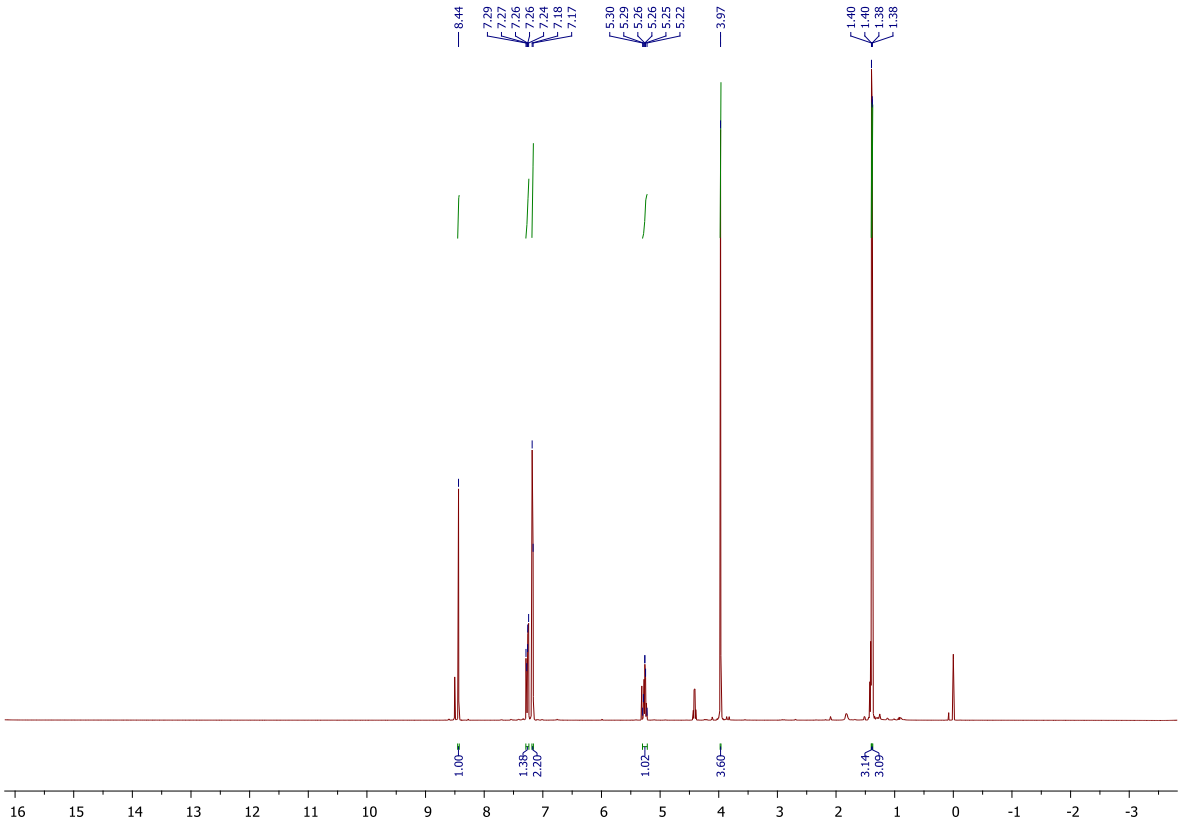


Figure 5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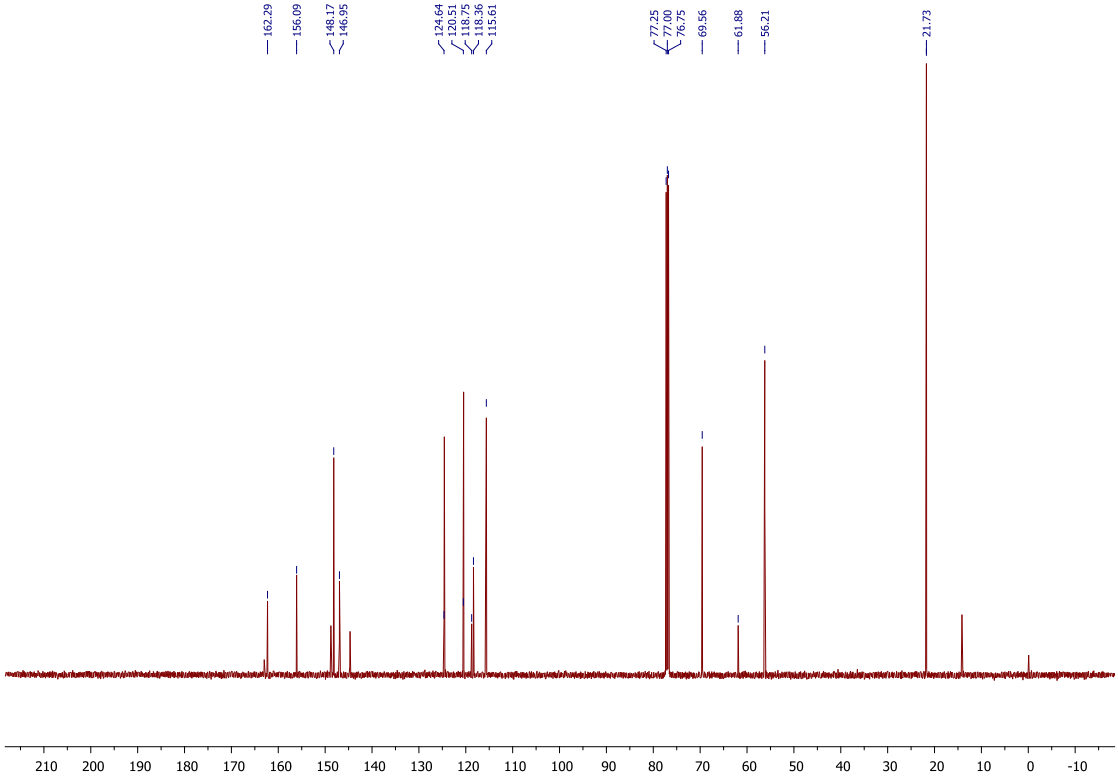
Graph 5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Graph 6

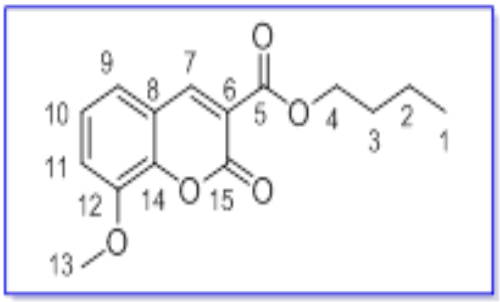
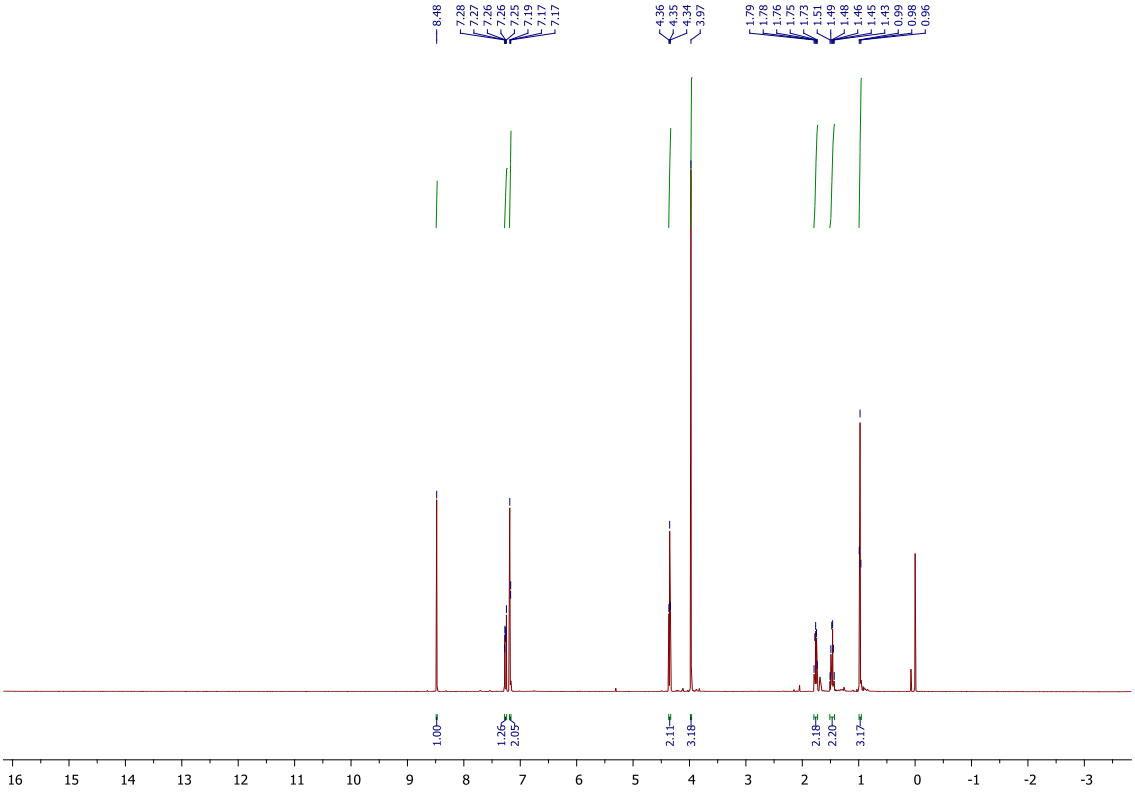
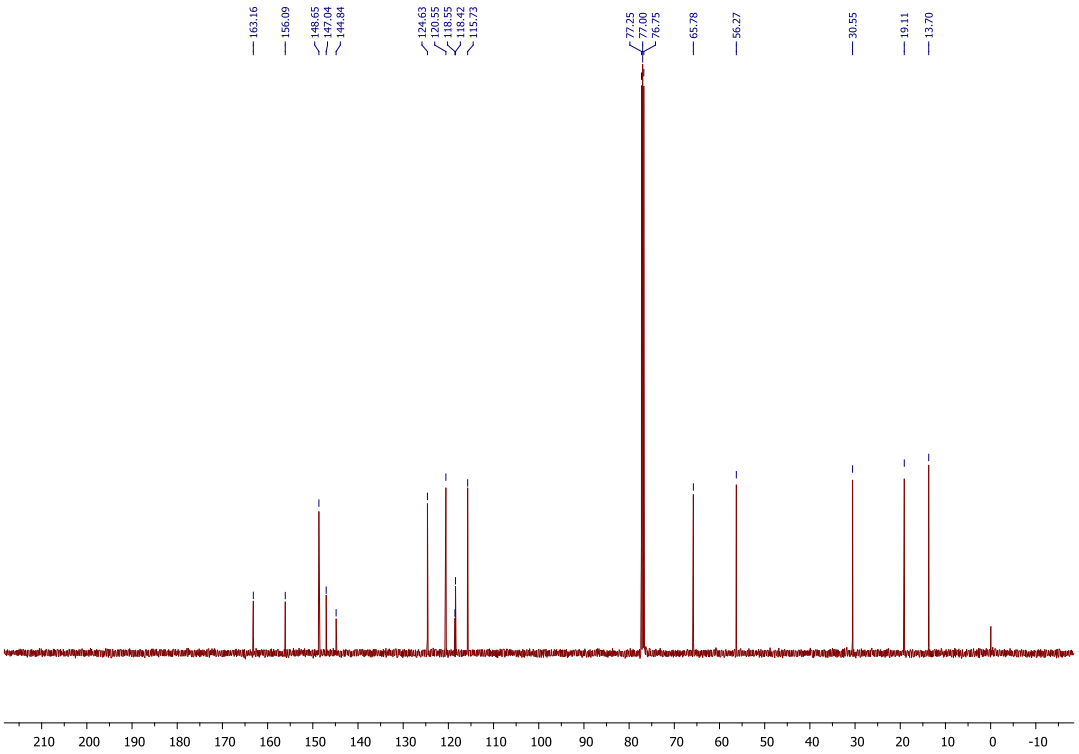


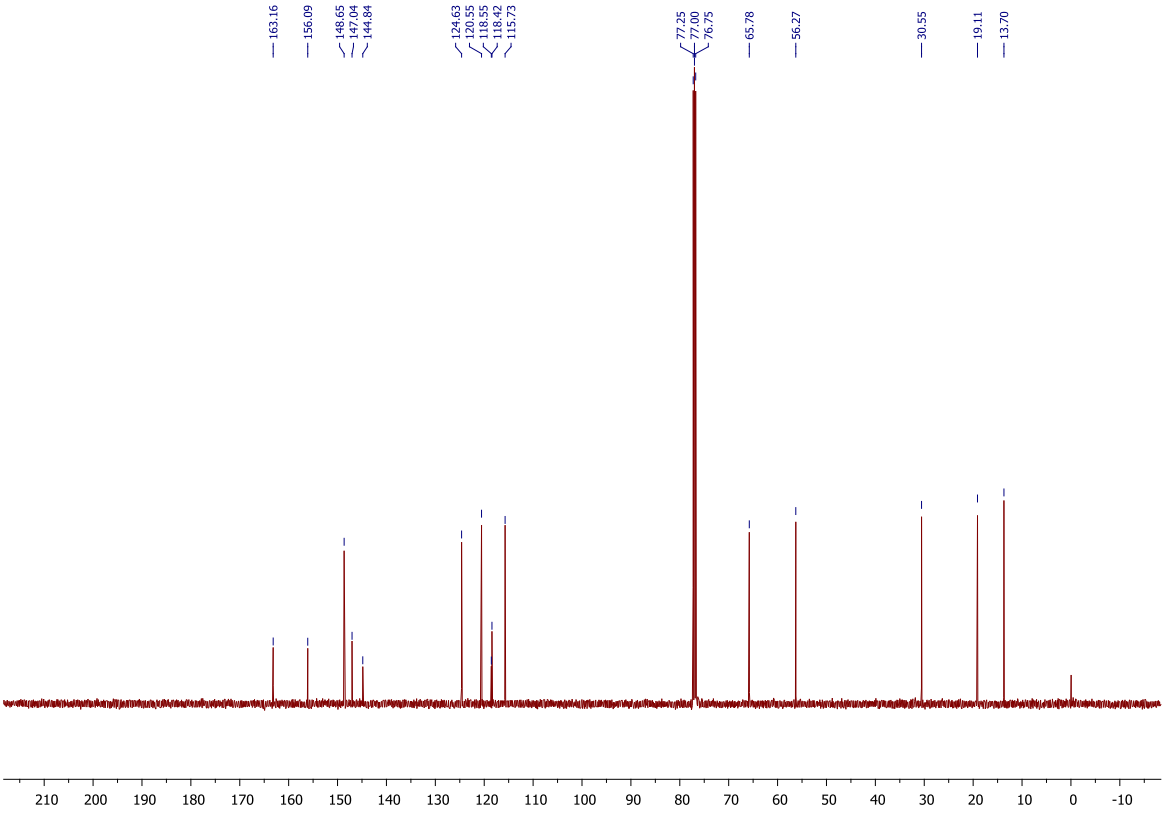
Figure 6



Graph 7



Graph 8



Graph 9

Biological test

Cytotoxicity assay

Primary mononuclear cells were isolated using density gradient separation with the reagent Histopaque-1077 (Sigma-Aldrich, USA), according to the manufacturer′s instructions. All experiments were set up with mononuclear cells extracted from normal individuals (n=5). Cells were cultured with RPMI 1640 medium plus 10% of Fetal Bovine Serum, 10mM Penicillin Streptomycin, and 10mM of L-glutamine (Thermofisher Scientific, USA) in a 37ºC, 5% CO_2 humidified incubator. Cell viability was evaluated by the trypan blue exclusion assay. Briefly, cells were seeded in a 96-well plate at a density of 5 x 10^3cells /well, and the compounds were incorporated into the medium at a concentration of 10, 15 and 20µg/ml. The compounds employed for the treatments were freshly prepared in Dimethyl sulfoxide (DMSO); control cells were only incubated with DMSO. After 48 hrs of treatment, the number of viable cells was assessed with trypan blue at 0.4% by manual counting in an inverted microscopy Axio Vert.A1 (Carl Zeiss, GE) using a hemocytometer (Sigma-Aldrich, USA). The percentage of viable cells in the group of treatments was adjusted to the value obtained in the control group. All assays were performed in triplicate.

Cell proliferation Assay

The cervical cancer cell lines (HeLa, SiHa, and CaSKI) were purchased from the ATCC, and cultured in DMEM/F12 medium (Invitrogen, life technologies) supplemented with 10% of fetal bovine serum (Invitrogen, Life Technologies), penicillin-streptomycin at 37°C in a humidified 5% CO2 atmosphere. Compounds were dissolved in Ethanol (Sigma-Aldrich, Molecular Biology) previous to the treatment.

Cells were seeded into 96-well microplates (Corning) at 4×10^3 cells/well into 0.1 ml of complete medium and then treated for 72 hours. After the treatment, medium was aspirated and cells were washed and fixed with PBS and 10% of formaldehyde, respectively. Fixed cells were incubated with 100µl of 0.1% crystal violet (Sigma–Aldrich, ACS reagent) for 2 hours. Then, cells were washed with distillated water, air-dried and the dye eluted with 100µl acetic acid solution, 10% (Merck-Millipore, ACS reagent). Cell proliferation was assessed by dye absorbance measured at 590 nm on a microplate reader (Epoch, Bioteck). All assays were performed in triplicate. The cytotoxic effect of each treatment was expressed as a percentage of cell proliferation relative to untreated control cells.

Cytotoxic evaluation.

To assess the toxic effect of coumarins in normal cells, mononuclear cells from blood samples of healthy individuals, were isolated by density gradient and cultured in RPMI (Roswell Park Memorial Institute medium) supplemented with 10% fetal bovine serum, penicillin streptomycin 10 □M and 10 □M L-glutamine (Thermo Fisher Scientific, USA) at 37 °C in a humidified incubator at 5 % CO_2. Twenty-four hours previous to the cytotoxic treatments, cells were seeded in a 96 well microplate at a density of 5 x 10^4 cells/well (Corning, USA). Treatments were performed with freshly prepared solutions of the compounds. Solutions were prepared solving them in ethanol at concentrations of 10, 50, 100 and 200 µM. After 48 h of treatment, the number of live cells was determined by trypan blue exclusion at 0.4% counting cells in a hemocytometer using an inverted microscope Axio Vert A1 (Carl Zeiss, GE). Additionally, the cytotoxic effect was evaluated by the Cell Proliferation Assay Kit II according to manufacturer's instructions (Sigma-Aldrich, USA). The percentage of living cells obtained in each group after treatment, was adjusted to the value obtained in the control group, which consisted only of mononuclear cells suspended in ethanol as solvent. All assays were performed by triplicate. Statistical analysis of the different treatments was performed with a two tail ANOVA and Tukey test, p-values smaller than 0.05 was considered significant.

Antiproliferative assay with tumor cells.

To identify the coumarin analogue with the highest activity on malignant cells, the synthetic derivatives **1a-1d** were tested against cervix cancer cell lines (HeLa, CaSKi and SiHa). The viability of cancer cells was investigated using the MTT assay[17,18]. The cancer cell lines were purchased from ATCC and cultured in DMEM/F12 medium (Modified Basal Medium Eagle's Medium) (Invitrogen, Life Technologies) supplemented with 10% of fetal bovine serum (Invitrogen Life Technologies) and penicillin-streptomycin 10 µM in a humidified incubator at 37 °C with 5% CO_2.

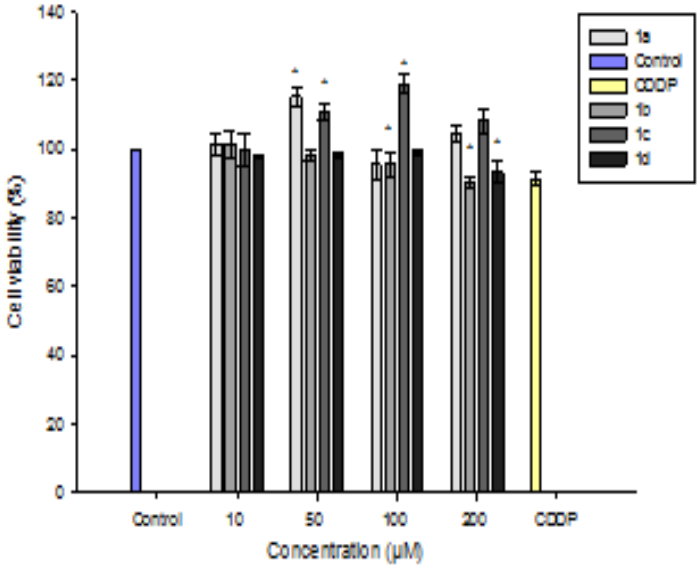
For the cell proliferation assay, cells were seeded in 96-well microplates (Corning) with a density of 54 × 10^3 cells/well in 0.1 ml complete medium. 24 h previous to the treatment, cells were incubated for 72 hours with the different compounds (**1a**, **1b**, **1c** and **1d)** at the before mentioned concentrations (10, 50, 100 and 200 □M). After treatment, the medium was aspirated and cells were washed and fixed with PBS and 10% formaldehyde, respectively. Fixed cells were incubated with 100 µL of 0.1 % crystal violet for two hours (Sigma-Aldrich, USA) then, they were washed with distilled water, air dried, and the dye was eluted with 100 µL of 10 % acetic acid (Merck-Millipore, ACS reagent). Cell proliferation was assessed by dye absorbance measured at 590 nm in a microplate reader (Epoch, Bioteck). All assays were performed by

triplicate. The antiproliferative effect of each treatment is expressed as a mean of the cell proliferation percentage relative to untreated control cells. All assays included cells treated with cisplatin 10 µM as a reference treatment (CCDP)

RESULTS AND DISCUSSION

Cytotoxic effect of coumarin derivatives in normal cells

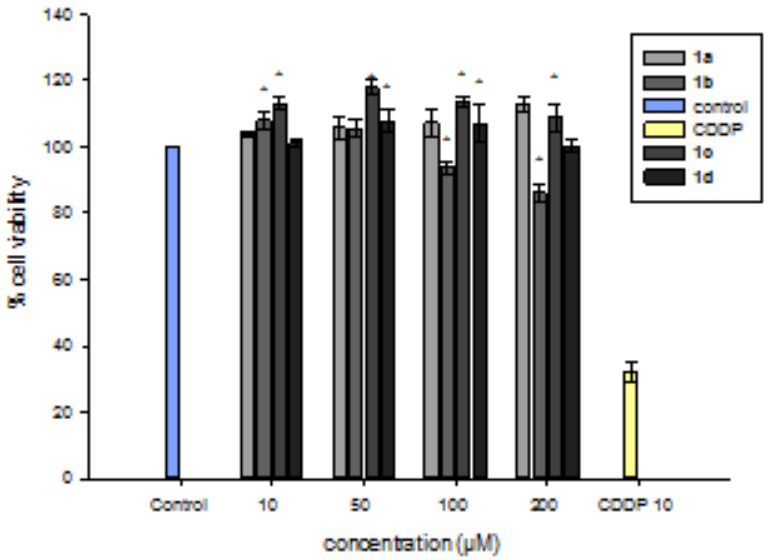
The treatment of the mononuclear cells with the four derivatives showed a similar biologic behavior characterized by a non-toxic effect at the lowest dose (10 □M) and a slight reduction of the viability at the highest concentrations (200 □M) with compounds **1b** and **1d** although it was not significant**.** The non-toxic effect can be seen when comparing the percentage of living mononuclear cells of the control group, with those that were exposed to the derivatives at the concentrations already mentioned before. It is worth to mention that compounds **1a** and **1c** significantly incremented the viability of the normal cells at 50 □M. **See graph 1**



Graph 1. Cytotoxic effect of coumarin derivatives 1a-d on normal cells. Cell viability was determined using the tetrazolium salt XTT. *p≤0.05

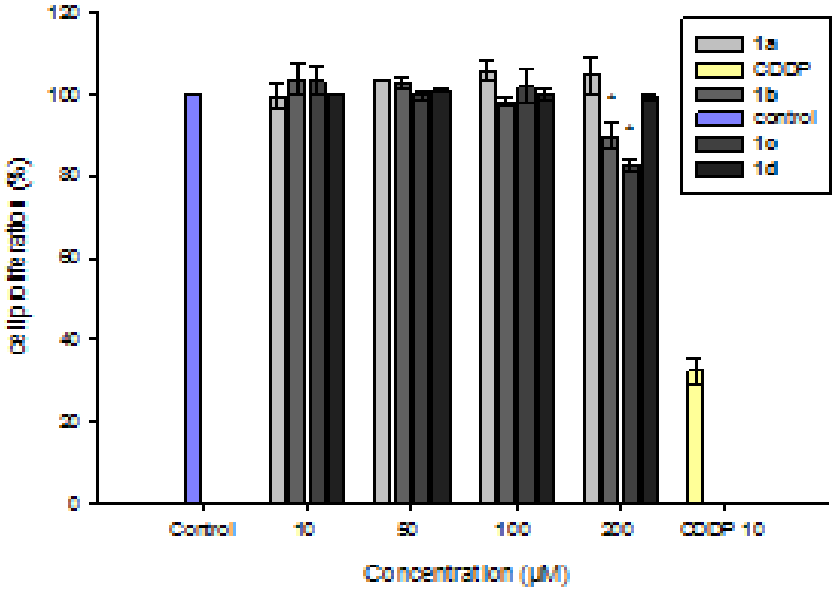
Antiproliferative activity of coumarin derivatives in cervical cancer cell lines.

The inhibitory behavior of the tested compounds over the different cervical cancer cell lines was marked by a diverse pattern of responses. In SiHa cell line, compounds **1b** and **1c** induced a slight, but significant increment in cell proliferation at low concentrations (10 and 50 µM) which turned into meaningful reductions in cell proliferation at higher concentrations (100 and 200 µM) in case of **1b**. **Graph 2.**



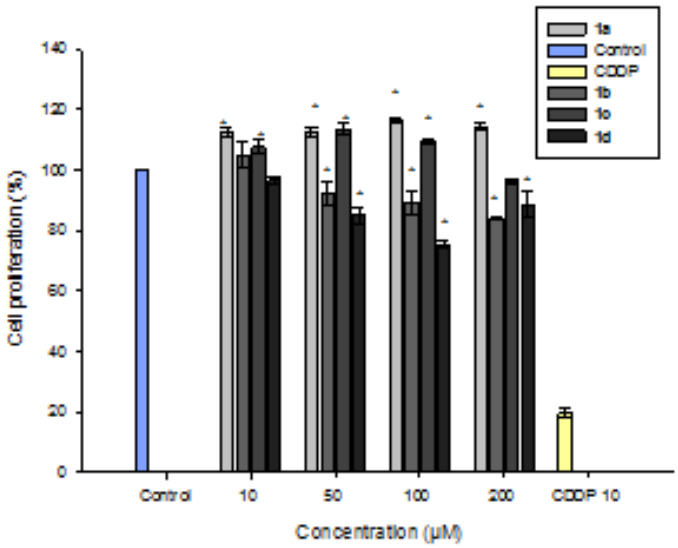
Graph 2. Inhibitory activity of coumarins compounds on SiHa cell line was determined utilizing MTT. *p≤0.05

In CaSKi cells, a cell line with a similar histologic origin (Epidermoid cervical carcinoma cells), compounds **1b** and **1c** showed a moderate antiproliferative effect at a concentration of 200 µM. **1c** had the highest inhibitory effect of the test (20%). In the case of compounds **1a** and **1d**, none of them showed a significant cell growth inhibition. See graph 3.



Graph 3. Antiproliferative activity of the coumarin derivatives on CaSki cells utilizing MTT. *p≤0.05

When the HeLa cell line was treated, two of the derivatives showed an increasing antiproliferative activity from 50 to 200 µM (compounds **1b** and **1d**). Derivative **1d** showed the highest antiproliferative effect among the molecules tested in the study with a 28% reduction at a 100 µM concentration. However, this reduction was not maintained at 200 µM. **Graph**



Graph 4. Antiproliferative activity of the coumarin derivatives on HeLa cells utilizing MTT. *p≤0.05

A brief summary of the *in vitro* antiproliferative activity of coumarins derivatives (**1b-1d**) on before mentioned tumor cells is presented in **table 1**.

Table 1. *in vitro* antiproliferative activity data for coumarins derivatives 1b-1d

Coumarin Derivative	Concentration(µM)	Antiproliferative effect(%)	Cell line
1b	10		**SiHa**
	50		
	100	10	
	200	20	
1b	10		**CaSKi**
	50		
	100		
	200	15	
1c	10		
	50		
	100		
	200	20	
1b	10		**HeLa**
	50	12	
	100	13	
	200	20	

1d	10	
	50	17
	100	28
	200	15

Values are expressed as mean in comparison to the control cells (Percentages)

It was not observed antiproliferative effect.

This study describes the relationship found between alkoxycarbonyle chain in coumarin derivatives, and their biological activity against cancer cervix cell lines. In the cytotoxic assay with mononuclear cells, it could be seen that the coumarin derivatives are inoffensive at all tested concentrations and this is a convenient condition as the mononuclear cells are essential in human immunological system[19]. The results of our compounds, in the antiproliferative experiments, allowed to prove that the relationship already mentioned exist. Starting the analysis of compounds performance, it can be noticed that derivative 1a did not showed any affect at any dose in none cancer cell line, nevertheless the rest of derivatives though at moderate extension elicited variable effects. Such is the case of derivative 1b which at concentration of 200 µM, significantly reduced the growth in SiHa cell line. At this concentration 1c developed a similar inhibitory effect in CaSki cells. In a concentration depending manner up to 100 µM, 1d showed an antiproliferative activity against HeLa cells. This behavior was not seen at the highest concentration of 200 µM and the mechanism remain in study. We have the hypothesis that this phenomenon is due to complex formation between molecules during entry through cell membrane. On the other hand, it can be noticed that in all experiment, cisplatin at a concentration of 10 µM, remained as the most potent inhibitor as he reduced cellular proliferation at a higher extension. However, these molecules offer an initial approach to the treatment of cancer, mainly in the early stages of the disease at the same time they provide an overview to design the synthesis of more potent derivatives.

CONCLUSION

We have explored the influence of four previously synthesized coumarins derivatives, on mononuclear cells and cancer cervix cell lines. These derivatives proved to be safe in normal cells and have moderate activity against growth in HeLA, CaSKi and SiHa cells (1b, 1c and 1d) The cytotoxic effect of the four coumarins on mononuclear cells, were assessed by XTT assay. The antiproliferative effect was measured by MTT assay. The synthesis of the tested compounds is depicted in scheme 1 and it is worth mentioning that were obtained in a previous work from accessible and economical starting materials. In HeLa cells compound 1d showed the best antiproliferative effect. In case of CaSKi cells, only compound 1c showed effect. Similarly, 1b in

SiHa cell line, was the unique product that caused effect. With this information we can generalize that butyl group was the key element in the improvement of the antiproliferative effect presented by molecule 1d on the tumor cell lines[20]

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