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Chimie şi Inginerie Chimică, Biotehnologii, Industrie Alimentară

Scientific Study & Research
Chemistry & Chemical Engineering, Biotechnology, Food Industry *ISSN 1582-540X*

ORIGINAL RESEARCH PAPER

ANTIPROLIFERATIVE ACTIVITY OF FLAVONOIDS ISOLATED FROM *ECHINOPS GRACILIS* O. HOFFM

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Received: July, 12, 2023
Accepted: September, 21, 2023

Abstract: Apigenin-7-*O*-(4″-feruloyl)-*β*-*D*-glucopyranoside (**1**) were isolated from the methanol extract of aerial part of *Echinops gracilis*, together with apigenin-7-*O*-(4″-*trans*-*p*-hydroxycinnamoyl)-*β*-*D*-glucopyranoside (**2**) and apigenin-7-*O*-*β*-*D*-glucopyranoside (**3**). Compound (**1**) previously displayed antioxidant and antibacterial activity. The present study aims at evaluating the antiproliferative potential of flavonoids, isolated from the aerial part of *E. gracilis* O. Hoffm. The effect of compounds (**1**), (**2**) and (**3**) on the viability of HeLa cells was determined by the method of 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay at different concentrations. The ability of compounds to induce the cell death was evaluated by using acridine orange/ethidium bromide (AO/EtBr) staining. Compound (**1**) induced an effective change in the cell viability of HeLa cells with IC_{50} concentration value of 27.36 $\mu g \cdot mL^{-1}$. Induction of cell death alteration in cell morphology and cancer cell population was observed in cells treated with compound (**1**), which makes it behave as a potent synergistic antiproliferative agent against HeLa cells.

Keywords: *Asteraceae, aerial part, apigenin-7-O-(4''-feruloyl)-β-D-glucopyranoside, cytotoxicity, HeLa cell, cell viability*

INTRODUCTION

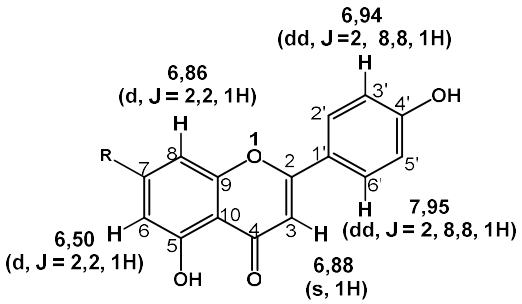
Cervical cancer is the fourth most common cancer in women. In 2018, an estimated 570,000 women were diagnosed with cervical cancer worldwide and about 311,000 women died from this disease [1]. Almost all cervical cancer cases (99 %) are linked to infection with high-risk human papillomaviruses (HPV), an extremely common virus transmitted through sexual contact.

In order to reduce cervical cancer prevalence, many improvements have been made through the cervical cancer elimination initiative. It includes human papillomavirus vaccination of girls, screening and treatment of precancerous lesions, and access to diagnosis and treatment of invasive cancers [2]. However, those measures and improvement are still not enough to eradicate this disease. The remark is done because, each year in the United States, about 13,000 new cases of cervical cancer are diagnosed and about 4,000 women die of this cancer [1].

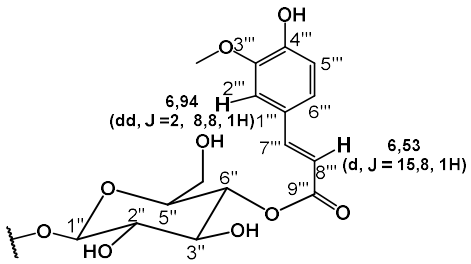
Flavonoids are polyphenolic compounds synthesized in plants as bioactive secondary metabolites [3]. They are potent antioxidants [4] protecting plants from unfavorable environmental conditions [4], therefore they have attracted attention of researchers and have been used in numerous epidemiological and experimental studies to assess their possible beneficial effects in multiple acute and chronic human disorders [5]. The *in vitro* and *in vivo* studies have shown that flavonoids could exert anti-inflammatory, immunomodulatory [6] and strong anticancer activities [7, 8].

In our previous study [9], apigenin-7-*O*-(4″-feruloyl)-*β*-*D*-glucopyranoside (**1**) were isolated from the methanol extract of aerial part of *Echinops gracilis*, together with apigenin-7-*O*-(4″-*trans*-*p*-hydroxycinnamoyl)-*β*-*D*-glucopyranoside (**2**) and apigenin-7-*O*-*β*-*D*-glucopyranoside (**3**) (Figure 1).

In the present work, compound (**1**), (**2**) and (**3**) were evaluated against human cervical cancer (HeLa) cell lines as a contribution to the fight against cervical cancer disease.



1- R=



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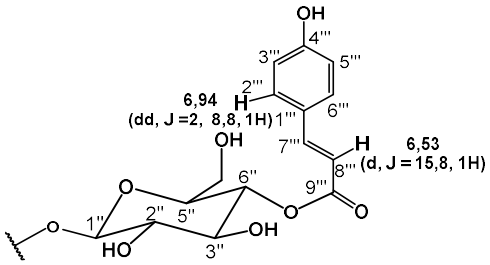

3- R= OH

Figure 1. *Structures of compounds* ***(1)****,* ***(2)*** *and* ***(3)***

MATERIALS AND METHODS

Plant material

The whole plant *Echinops gracilis* was collected from Fongo Tongo, West Cameroon. This species was authenticated by Dr. Tsabang Nole under the reference N°66943/HNC at the National Herbarium in Yaoundé, Cameroon.

Isolation of compounds

Compounds were isolated from the methanol extract of aerial part of *E. gracilis*. The isolation was carried out by the protocol previously reported by Weyepe et *al*. 2021 [9].

Cell line

Human cervical cancer cell lines HeLa were procured from The American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle Medium culture medium with 10 % fetal bovine serum (FBS) at 5 % CO_2 and 37 °C. Cells were passaged using Trypsin EDTA until the culture reaches 70 - 80 % confluence.

Sample preparation

1 mL stock solution of each compound was prepared with DMSO. From the stock, the samples were prepared at different micromolar concentrations (05, 25, 50, 75 and 100) with serum-free medium (SFM) for the test. The concentration of DMSO was aimed not to exceed 0.01 %.

Cell viability assay

HeLa cells were seeded in 96-well plate (5×10^3 cells / well) in medium containing 10 % of FBS and incubated for 24 h under 5 % of CO_2 at 37 °C for attachment. The cells were then washed with 1 × PBS and 100 µL of the prepared samples was added to the wells. 100 µL of SFM was added to the control well and incubated for 24 h. The medium was then removed and washed with PBS. A hundred microliters of 0.5 $mg \cdot mL^{-1}$ MTT solution was added to each well and incubated for 2 - 3 h. After the incubation period, 100 µL of DMSO was added for solubilization of cells and kept in the dark for 1 h. The intensity of the color developed was read at 570 nm using ELISA reader.
The growth inhibition was then calculated as follows in equation (1):

$$\textit{Cell viability (\%)} = \textit{Absorbance of treated cells/Absorbance of control cells} \times 100 \quad (1)$$

Five different observations were carried out, and the IC_{50} values were calculated.

Detection of cell death

The ability of compounds to induce cell death in HeLa cells was determined by AO/EtBr dual staining. The cells were grown on the cover slip in 24-well plate with 1×10^5 cells / well, then the cells were treated with the IC_{50} (80 µM) concentration of compounds for 24 h. After incubation, 5 µL of AO (1 $mg \cdot mL^{-1}$) and 5 µL of EtBr (1 $mg \cdot mL^{-1}$) were added, and the induction of cell death was observed by using a fluorescence microscope.

Cytotoxicity assay of the plant extract

Cytotoxicity effects of compounds was investigated on HeLa cell line ATCC using resazurin-based assay as previously described by Mosmann [10]. The cells were cultured in Dulbecco's Minimal Essential Medium (DMEM) (Sigma-Aldrich-Germany), supplemented with 5 % Fetal Bovine Serum (Sigma-Aldrich-Germany) and 1 % gentamicin (Sigma-Aldrich-Germany). Monolayer culture reaching a confluence between 80 - 90 % was detached using trypsin solution (1x) (Sigma-Aldrich-Germany) and calibrated with a cell counter (Newbeaur). Calibrated cell suspension was seeded into 96-well tissue culture microtiter plates at a density of 1×10^4 cells / well and incubated overnight at 37 °C in a 5 % CO_2 incubator for cell adhesion. Following incubation, 100 µL of medium was removed from cells and replaced by 100 µL of the fresh one followed by the addition of gradual concentration of extracts at 100, 20, 4, 0.80 and 0.16 $\mu g \cdot mL^{-1}$. Positive control (Podophyllotoxin 10 $\mu mol \cdot L^{-1}$ - Sigma-Aldrich-Germany) and negative control (0.5 % DMSO) were added. Then, plates were incubated for 48 h in the same culture conditions as above described. At the end of the incubation period, cell proliferation and viability were quantified by addition of 10 µL of 0.015 $mg \cdot mL^{-1}$ solution of resazurin in PBS and incubate at 37 °C for 4 h. Fluorescence was measured at excitation of 530 nm and emission of 590 nm using Infinite M200 (TECAM, Swiss) microtiter plate reader. The results were expressed as a percentage of viability of the control cells and IC_{50} values were calculated based on the equation (2):

Inhibition (%) = (1-Absorbance of treated cells/Absorbance of untreated cells) x100 (2)

Statistical analysis

The data was subjected to statistical analysis using one-way Analysis of Variance, followed by Student-Newman-Keuls test to access the significance between groups at a level of $P < 0.05$ using SPSS 17.0 version.

RESULTS AND DISCUSSION

Antiproliferative activity

Results on cytotoxicity of compounds on HeLa cervical cancer cell lines are shown below (Figures 2, 3, 4 and 5). Two of the three tested compounds on the HeLa cells showed cytotoxic activity.

Compound (**1**) showed the highest cytotoxic activity against HeLa cancer cell line. Compound (**3**) exhibited no cytotoxic activity. The IC_{50} values are considered NOT bioactive as they are NOT > 4 $\mu g \cdot mL^{-1}$ as the acceptable threshold for compounds treated between 48 and 72 h, decided by the NCI plant screening program [11].

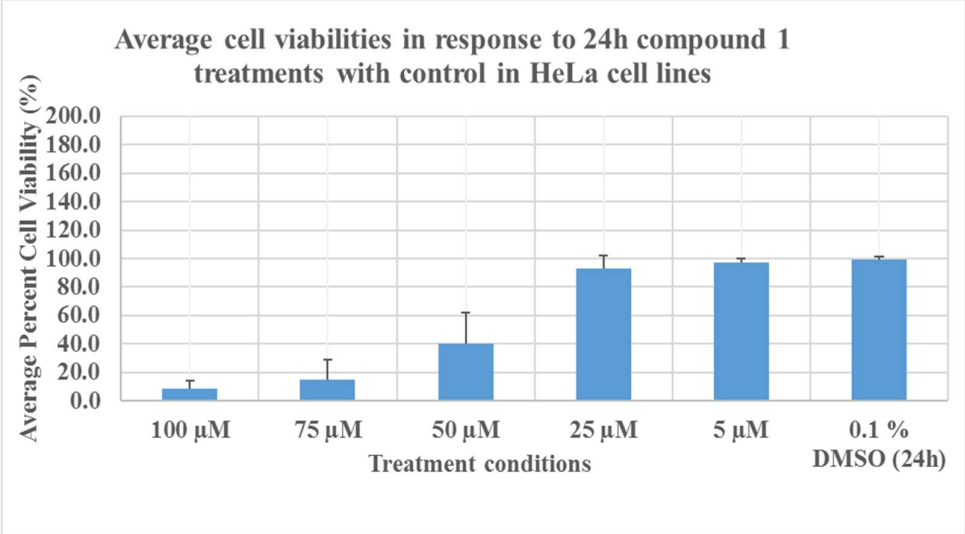

Figure 2. *Average cell viabilities in response to 24 h compound (**1**) treatments with control in HeLa cell lines*

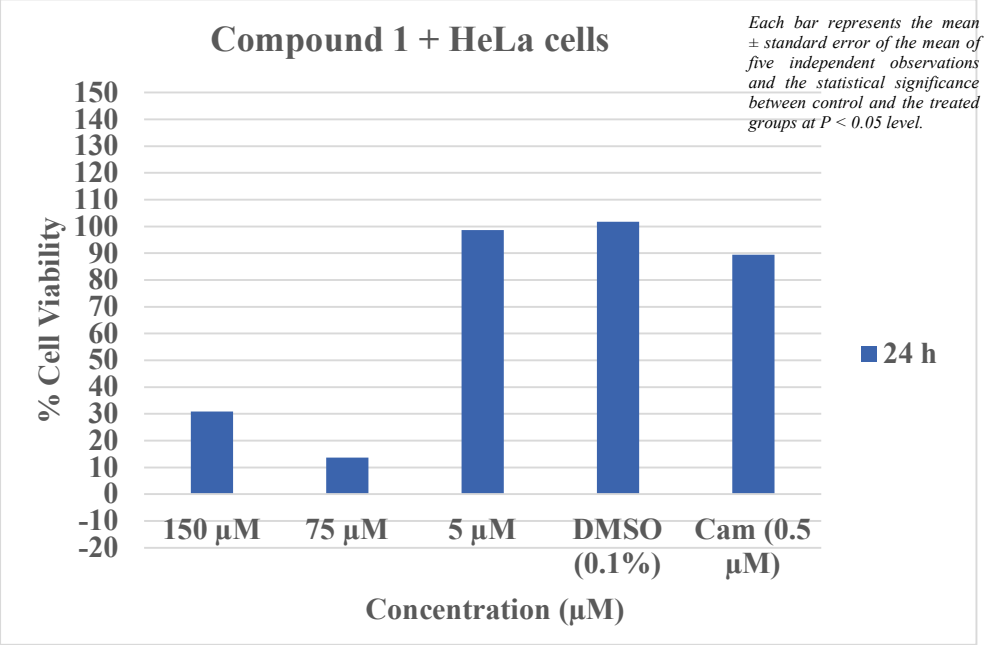

Figure 3. *Effect of compound (**1**) on HeLa cell viability using MTT assay*

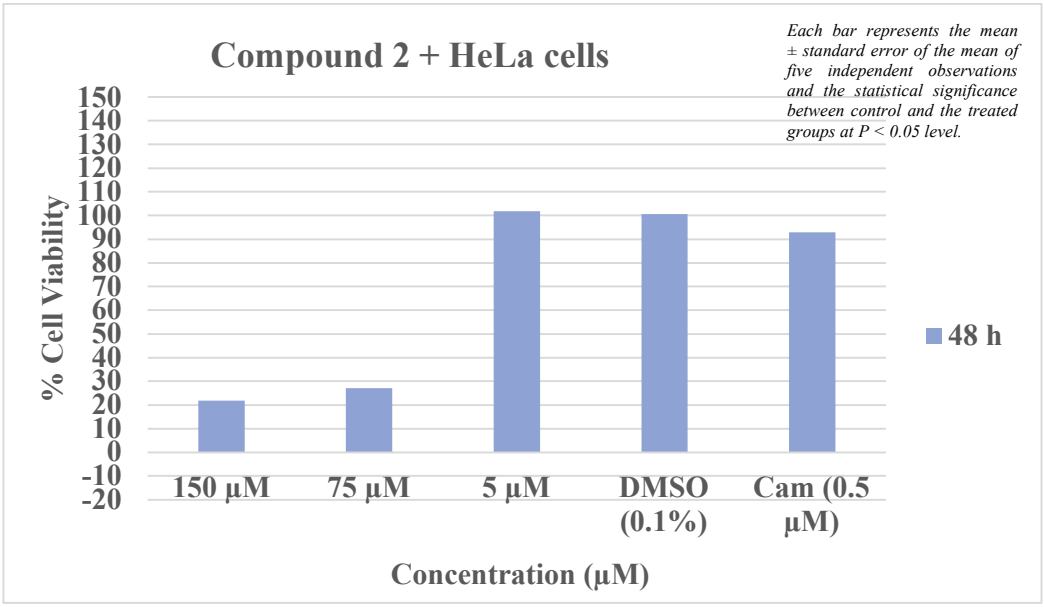

Figure 4. *Effect of compound (**2**) on HeLa cell viability using MTT assay*

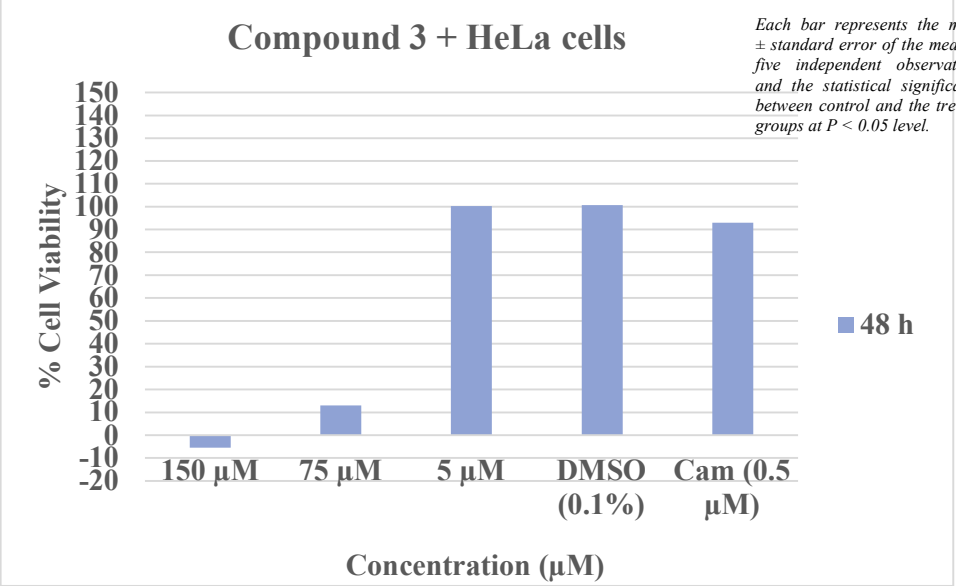

Figure 5. *Effect of compound (**3**) on HeLa cell viability using MTT assay*

Compound (**1**) and (**2**) exhibited a substantial antiproliferative effect on HeLa cells (Table 1). Dose-dependent studies showed IC_{50} of 27.36 µg·mL^{-1} and 40.32 µg·mL^{-1}, respectively.

The main aims of analyzing are to identify bioactive compounds that can be used as lead substances in the preparation of semi synthetic drugs. A large number of novel anticancer drugs have been discovered from natural products in the past and new ones are continually being developed.

Table 1. *Antiproliferative results for studied compounds* [11]

Cytotoxicity of phyto-compounds			
Compounds	**IC_{50} in HeLa cells [µM]**	**IC_{50} in HeLa cells [µg·mL^{-1}]**	**Time of treatment [h]**
(**1**)	45.0	27.36	24
(**2**)	70.0	40.32	48
(**3**)	100.0	43.00	
Camptothecin	25.0	-	

These cytotoxic natural products may be able to play a significant role in treating selected cancers by working in concert with conventional chemotherapeutic drugs, thereby improving their efficacy or reducing their toxicity. Following the fact that cancer drugs are commonly administrated in the form of mixture, Boik et *al.* [12] quantified synergism using nonlinear mixed effect. They concluded that the MixLow method can be used to quantify drug combinations study that includes within-group and between-group replicates, and where responses follow a sigmoidal pattern.

The results of our study show that compound (**1**) have an IC_{50} values within the recommended range for potentially synergistic compounds (i.e. compounds that exert stronger bioactivity when treated together) against Cervical cancer cell. This recommended range is from 1 to 50 [12].

Compound (**1**) exhibited highest cytotoxic effect. Therefore, it could be considered as a promising anticancer agent if the cytotoxicity evaluation on Vero normal cells gives better results with regards to selective index considerations.

CONCLUSION

The present study concludes the antiproliferative efficacy of the plant active ingredient, apigenin-7-*O*-(4″-feruloyl)-*β*-*D*-glucopyranoside (**1**), isolated from the methanol aerial part extract of *E. gracilis*. This compound possesses a leading effect on the growth and survival of the ER-*α* positive HeLa cells and this is reported for the first time.
As the current research has initiated as an idea, the identification of the antiproliferativ effect of apigenin-7-*O*-(4''-feruloyl)-*β*-*D*-glucopyranoside, more *in vitro* and *in vivo* studies are required for the identification of other targets of the compound.
Further investigation of its effect on other cancer signaling pathways may provide effective knowledge on the compound's use in cervical cancer treatment.

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