

Article

Antitumor Activity of Whole-Plant Extracts from In Vitro Cultured and Wild-Growing *Clinopodium vulgare* Plants on a Panel of Human Tumor Cell Lines

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Abstract: *Clinopodium vulgare* L. is a valuable medicinal plant with various beneficial effects on health. In this study, water extracts from the aerial part of the wild and in vitro cultured *C. vulgare* plants were obtained. The polyphenol, flavonoid content and antioxidant activity of the extracts as well as their antitumor efficiency against a panel of cell lines were analyzed. The ability of *C. vulgare* to inhibit cancer cell migration and induce apoptosis in the tumor cells was examined by wound healing assay and fluorescence microscopic methods. The effect of the extracts on the cell cycle progression of the tumor cells was analyzed by flow cytometry. The presented results show that the antitumor activity of the extracts from in vitro cultured plants was similar to and even exceeded that of the wild plants. The cell viability and migration assays demonstrate the selective anticancer effect of the extract and significant inhibition of cancer cell proliferation and motility. The fluorescence microscopy and cell cycle analyses indicate that the antitumor activity of the in vitro plant extract was related to both antiproliferative and proapoptotic effects. These results show that *C. vulgare* plants obtained by in vitro micropropagation and cultivated ex vitro are promising candidates for anticancer drug therapy.

Keywords: *Clinopodium vulgare*; in vitro cultured plants; antitumor activity; cell viability; apoptosis



Academic Editor: Ipsita Banerjee

Received: 6 November 2024

Revised: 14 January 2025

Accepted: 16 January 2025

Published: 18 January 2025

Citation: Georgieva, A.; Sulikovska, I.; Toshkova-Yotova, T.; Djeliova, V.; Amiri, S.; Tsonevski, N.; Petkova-Kirova, P.; Tasheva, K. Antitumor Activity of Whole-Plant Extracts from In Vitro Cultured and Wild-Growing *Clinopodium vulgare* Plants on a Panel of Human Tumor Cell Lines. *Appl. Sci.* **2025**, *15*, 925. <https://doi.org/10.3390/app15020925>

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1. Introduction

Globally, there has been a significant increase in the number of malignant neoplasms, defined as diseases of modern lifestyle and one of the main causes of premature death. These are serious, socially significant diseases that have a physical, psychological, social and economic impact on patients, their families and caregivers and society as a whole. According to the International Agency for Research on Cancer, both new cancer cases (over 26 million) and cancer deaths (17 million cancer deaths per year) are expected to double by 2030 [1]. In recent years, many new synthetic chemotherapeutic agents have

been discovered and used clinically, but they have failed to meet expectations for effective treatment. Thus, researchers' efforts continue to be strongly focused on the search for new effective and affordable anticancer drugs [2]. The use of natural products to support the therapy of malignant diseases has been applied for decades. Plants, in particular, are a continuous source of biologically active compounds and are used to treat a range of diseases, including cancer [3]. Between 50,000 and 80,000 species are medicinal plants that are applied in medicine and pharmacy worldwide [4]. Over 3000 species are known plants with anticancer properties, and they account for over 60% of the anticancer agents used today [5]. In addition to their therapeutic efficacy, herbal medicines also attract attention due to their accessibility, low cost and little or no side effects.

Due to its geological characteristics and soil, as well as its specific microclimate conditions, Bulgaria has a significant diversity of plant species, including medicinal plants with valuable properties [6]. One such plant is *Clinopodium vulgare* L. (wild basil) of the Lamiaceae family, which in Bulgaria is officially recognized as a herb with medicinal properties under the Medicinal Plants Act, Bulgaria [7], and which in traditional Bulgarian medicine is used to treat warts, skin irritations, wounds, mastitis, diabetes, prostatitis and stomach ulcers [8–10]. In recent years, an increasing number of scientific studies focused on the elucidation of the phytochemical composition and assessment of the biological and pharmacological activities of plant species belonging to the Lamiaceae family, especially the *C. vulgare* species, have been published [11].

Clinopodium vulgare L is rich in flavonoids, monoterpenoids, phenolic acids, diterpenoid quinines, triterpenoid glycosides, triterpenoid saponins and essential oils, including rosmarinic acid, chlorogenic acid, caffeic acid, rutin, kaempferol, quercetin, betulinic and ursolic acids [12,13] known for their anticancer properties [14]. Numerous studies have confirmed the valuable properties of *C. vulgare* plant extracts, including their antioxidant [10,13,15], DNA-protective [16], anti-inflammatory [17–19], anti-bacterial [20–22], antihypertensive [23] and enzyme-inhibiting [10,18] effects. In recent years, its antitumor effect and anticancer activities have also been investigated [8,24–26]. The various methods for the extraction, separation and identification of *C. vulgare* phytoconstituents, as well as the numerous valuable pharmacological activities established in different studies, have been summarized in a recently published review article [11]. Based on analysis of the currently available data, the toxicity assessment of *C. vulgare* has been established as one of the main research priorities for the determination of its safety profile and ensuring the suitability of the plant for therapeutic applications [11].

The phytochemical composition and biological activity of plants of the same species collected from wild populations can vary significantly depending on the conditions of the specific habitat. With the help of in vitro techniques, it is possible to achieve both a constant quality and a change in the content of metabolites in the plant culture [27].

In a previous study [26], we compared the antioxidant and antitumor effects of the extracts of the stems, leaves and flowers of in vitro cultivated *C. vulgare* plants with corresponding extracts of the wild-growing plants. However, due to a probable synergistic action and combined effect of the various components in the different plant parts and the possible improved effectiveness, the aim of the present study was to investigate the antitumor activity of total extracts from the entire aerial part of in vitro cultivated *C. vulgare* plants and compare it with that of the wild species. To extend and strengthen the previous findings indicating the selective anticancer activity of *C. vulgare* extracts and further confirm their lower toxicity on normal cells, the effects of the total plant extracts were assessed using a broader panel of cancer and non-cancerous cell lines. Moreover, the potential of the total plant extract from in vitro cultivated plants to affect cell cycle progression

and trigger apoptosis was analyzed to better elucidate the mechanisms underlying its anticancer effects.

2. Materials and Methods

2.1. Materials

The cell culture medium Dulbecco's modified Eagle's medium (DMEM), acridine orange (AO) and ethidium bromide (EtBr) fluorescent dyes, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent, phosphate-buffered saline (PBS), trypsin-EDTA solution, Murashige and Skoog plant culture medium and the plant growth regulators 6-benzylaminopurine (BAP) and indole-3-butyric acid (IBA) were delivered from Sigma-Aldrich, Schnelldorf, Germany. Fetal bovine serum (Gibco/BRL, Grand Island, NY, USA) was used in the cell culture experiments. 4',6-diamidino-2-phenylindole (DAPI), L-glutamine and the antibiotics penicillin and streptomycin were purchased from AppliChem, Darmstadt, Germany. Cell culture vessels and other disposable plastic consumables (Orange Scientific, Braine-l'Alleud, Belgium) were used. All chemicals for the phytochemical and cell culture analyses were of an analytical grade.

2.2. Plant Specimens and In Vitro Culturing

Clinopodium vulgare plants naturally growing in Bistrica, Bulgaria, were used for seed harvesting and the establishment of in vitro cultures. Murashige and Skoog (MS) medium [28] supplemented with 1 mg/L 6-benzylaminopurine (BAP) and 0.1 mg/L indole-3-butyric acid (IBA) was used for plant propagation. Under these conditions, 100% of the nodal segments developed new shoots, which were then effectively rooted (100%) on a half-strength MS medium devoid of auxins. The plants obtained in vitro were acclimated and grown in the experimental fields located at an altitude of 540 m in Elin Pelin, Bulgaria. Plant cultivation, morphological analysis and measurements were performed using procedures described in previous research [26].

2.3. Preparation of Freeze-Dried Extracts

Plant samples—whole-plant extracts from the aerial part (flower, leaf and stem) of in vitro cultured and wild species were used.

The entire aerial part of *C. vulgare* plants (about 50 g) was air-dried at room temperature and finely powdered using a laboratory grinder (Optimum RK-0150, Warsaw, Al. Witosa 31/22, Poland). The obtained powder was then extracted for 15 min in water heated to 90 °C (5 g/200 mL). After centrifuging the suspensions at 6000× g using a centrifuge MPW-260R (MPW Med. Instruments, Warszawa, Poland), the obtained supernatants were lyophilized for 96 h using a laboratory freeze dryer (Alpha 1–4 LD plus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany).

2.4. Total Polyphenol and Flavonoid Content

The total polyphenol content of the freeze-dried *C. vulgare* extracts was evaluated according to the method described by Singleton and Rossi [29] and is presented as mg gallic acid equivalents (GAE) per 100 g of dry weight (DW) ± SD. The total flavonoid content was measured as described by Chang et al. [30] and is presented as mg rutin equivalents (RE) per 100 g of DW ± SD.

2.5. Antioxidant Activity Assays

Oxygen radical absorbance capacity (ORAC) was determined using a microplate reader, FLUOstar OPTIMA (BMG Labtech, Germany), at 485 nm excitation and 520 nm emission wavelengths, according to a previously published procedure [31]. Hydroxyl

radical averting capacity (HORAC) of the freeze-dried *C. vulgare* extracts was determined following the method of Ou et al. 2002 [32].

2.6. Antitumor Activity

2.6.1. Cell Lines

Cervical carcinoma HeLa (CCL-2), hepatocellular carcinoma Hep-G2 (HB-8065), colorectal adenocarcinoma HT-29 (HTB-38), mammary adenocarcinoma MCF-7 (HTB-22) cell lines and mice fibroblast cells BALB/3T3 (CCL-163) were obtained from the American Type Culture Collection (ATCC). The non-tumorigenic cell line of human keratinocytes HaCaT (CVCL_0038) was obtained from the CLS Cell Lines Service (Eppelheim, Germany). All cell lines were cultivated in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum, the antibiotics penicillin (100 U/mL) and streptomycin (100 µg/mL) and 2 mM glutamine in an incubator with 5% CO₂ at 37 °C.

2.6.2. Cell Viability/Cytotoxicity Assay

The viability of the cancer and non-cancer cells treated with the *C. vulgare* extracts was determined by a standard colorimetric MTT assay. After cell dissociation, using a 0.25% Trypsin-EDTA solution and counting in a hemocytometer, cell suspensions were adjusted to a density of 1×10^5 cells/mL and plated in 96-well plates. The cells were incubated overnight at 37 °C in a humidified atmosphere containing 5% CO₂. The next day, *C. vulgare* extracts were added to the cell cultures at concentrations ranging from 125 µg/mL to 1000 µg/mL. Untreated cells cultivated under the same conditions were used as controls. Each concentration of the extracts was tested in five replicates. After being cultured for 24 h and 48 h in the presence of the extracts, the cells were washed with PBS, and 100 µL of MTT solution in DMEM (5 mg/mL) was added to each well. The cell cultures were then incubated at 37 °C for 3 h, the supernatants were discarded, and 100 µL of lysing solution containing equal volumes of DMSO and ethanol was added to dissolve the obtained purple formazan crystals. The absorbance of the samples was measured using an ELISA plate reader (TECAN, Sunrise™, Grodig/Salzburg, Austria). The viability of the treated cell cultures is presented as a percentage of the respective untreated control.

2.6.3. Cell Migration Assay

A wound-healing scratch assay was performed to examine the effect of the *C. vulgare* extract of in vitro cultured plants on HeLa cancer cell migration. Cancer cells suspended in a complete culture medium at a density of 2×10^5 were seeded in a 24-well plate and cultivated under standard conditions until the formation of a confluent monolayer. A vertical scratch was then made in each plate well using a sterile 200 µL pipet tip. After washing with PBS to remove cell debris, the cells were treated with the extract at a concentration of 300 µg/mL and further cultivated for 72 h. Microphotographs of the treated and control untreated cells were taken at regular time intervals (0 h, 24 h, 48 h and 72 h) to analyze the treatment-induced changes in the ability of the cancer cells to migrate and fill the created monolayer gaps. ImageJ software package, Version 1.42, was used for the measurement of the wound areas at each time point. The wound areas measured at each time point for both control and treated cell cultures are expressed as a percentage of the area in the respective cell culture at the beginning of the experiment.

2.6.4. Fluorescence Microscopy Analysis

Fluorescence microscopy was used to examine the morphological changes induced by the extract of in vitro cultivated *C. vulgare* plants in HeLa carcinoma cells. Cells were grown in 24-well plates on 13 mm cover glasses placed on the bottom of each well, and the extract was added to the cultures for 24 h at a concentration equal to the IC₅₀ value

determined by the MTT test. The cancer cells treated with the extract were subjected to cytomorphological examination using three different fluorescence labeling techniques.

Acridine Orange/Ethidium Bromide Staining

Live/dead double staining with acridine orange (AO) and ethidium bromide (EtBr) was used to determine if the examined extract could cause cytotoxic damage to the cancer cells. Both fluorescent dyes were dissolved in PBS at a concentration of 5 µg/mL and applied to the cell preparations of untreated and *C. vulgare* extract-treated HeLa cells. The standard cytostatic drug doxorubicin was used as a positive control in the study.

DAPI Nuclear Staining

The effects of the *C. vulgare* extracts on the nuclear morphology of the cancer cells were examined after staining the samples with 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI) and compared to those induced by the positive control substance doxorubicin. After being fixed in methanol, the cells were stained with DAPI (1 µg/mL) for 15 min in the dark at room temperature.

Double Staining with Annexin V-FITC/Propidium Iodide (PI)

The ability of the extract of in vitro cultured *C. vulgare* to induce cancer cell apoptosis was analyzed using an Annexin V Apoptosis Detection Kit: sc4252 AK (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) following the manufacturer's protocol. After rinsing with the assay buffer, the cells were incubated for 10 min in the dark with a solution containing Annexin V-FITC (5 µg/mL) and PI (5 µg/mL).

A fluorescence microscope, Leica DM 5000B (Wetzlar, Germany), was used for visualization of the fluorescently stained cell cultures. An excitation filter BP 340–380, a dichromatic mirror 400 and a suppression filter LP 425 were used for DAPI staining. For the AO/EtBr and Annexin V-FITC/PI experiments, the excitation filter BP 450–490, dichromatic mirror 510 and suppression filter LP 515 were applied.

2.6.5. Cell Cycle Analysis

To investigate the effects of the *C. vulgare* extract on the cell cycle progression of HeLa cancer cells, a flow cytometry analysis of propidium iodide-stained cell cultures was performed. For this purpose, semi-confluent cell cultures were treated for 24 h with the extract of in vitro cultured *C. vulgare* plants at concentrations of 600 µg/mL and 800 µg/mL. The culture medium was then aspirated, and the cells were rinsed twice with cold PBS, detached by trypsinization, and centrifuged at $125 \times g$ for 10 min. The obtained cell pellet was washed again with PBS, fixed with 70% ice-cold ethanol, and stored overnight at -20°C . Next, the cells were rinsed with PBS, treated with 20 µg/mL ribonuclease A (Roche Diagnostics GmbH, Mannheim, Germany) for 30 min, stained with 20 µg/mL propidium iodide, and analyzed using a flow cytometer Becton Dickinson (BD Biosciences, San Jose, CA, USA). For each of the control and treated cell samples, 10,000 events were analyzed, and the distribution of the cells in the different phases of the cell cycle was evaluated using FlowJo™ v10.8 software (BD Biosciences, San Jose, CA, USA). The data are expressed as mean $\pm$ SEM of three independent experiments.

2.7. Statistics

Analysis of the statistical significance of the data was performed by one-way analysis of variance (ANOVA) and Bonferroni's post hoc test using GraphPad PRISM software, Version 5. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ were considered as the lowest levels of statistical significance. To calculate the IC₅₀ concentrations, nonlinear regression curve fit analysis (GraphPad PRISM) was applied.

3. Results

3.1. Secondary Metabolites Production in Wild and In Vitro Cultured Plants

The total polyphenol and flavonoid content and the antioxidant activity of the water extracts of wild and in vitro cultured plants were determined (Table 1).

Table 1. Total polyphenol and flavonoid content and antioxidant activity of in vitro cultured and wild-growing *C. vulgare* plants.

Sample	Total Polyphenols, mg GAE/100 g DW	Total Flavonoids, mg RE/100 g DW	ORAC, $\mu\text{mol TE/g DW}$	HORAC, $\mu\text{mol GAE/g DW}$
in vitro cultured plants	22402 $\pm$ 812 ^a	3689 $\pm$ 190 ^a	6119 $\pm$ 195 ^a	1538 $\pm$ 89 ^a
wild-growing plants	24957 $\pm$ 1011 ^b	4694 $\pm$ 165 ^b	7115 $\pm$ 665 ^b	2000 $\pm$ 101 ^b

The data are presented as means of 3 samples $\pm$ SD. Different letters indicate significant differences assessed by the Fisher LSD test ($p \leq 0.05$) after performing ANOVA multifactor analysis.

In vitro cultivated plants and wild species showed a high content of total polyphenols and flavonoids, as well as high antioxidant activity as measured by the ORAC and HORAC methods.

3.2. Cell Viability Assay

The extracts obtained from the in vitro cultured and wild *C. vulgare* plants were assessed for antitumor activity on four human cancer cell lines using the MTT cell viability assay. The effects resulting from the treatment with the extracts for 24 and 48 h on the cancer cell viability are presented in Figure 1. As evident from the presented data, the tested extracts induced statistically significant reductions in the cell viability and proliferation of the cancer cell lines compared to the untreated control cells.

The observed cytotoxic effects of the extracts on all cell lines tested were concentration-dependent. The viability of HeLa, HT-29 and MCF-7 cells treated with the highest concentration of the extracts was most strongly decreased and lower than 15% at both time intervals. The extracts obtained from in vitro cultured plants showed stronger antitumor activity compared to the wild plant extracts. A statistically significant difference in the cell viability of the cell lines treated with in vitro cultured and wild plant extracts was observed for the HeLa cell line treated with extracts at a concentration of 500 $\mu\text{g/mL}$ for 24 and 48 h, as well as with a concentration of 250 $\mu\text{g/mL}$ for 48 h and MCF-7 cells treated with 500 $\mu\text{g/mL}$ for 48 h and HepG-2 cells treated for 48 h with concentrations of 250 $\mu\text{g/mL}$, 500 $\mu\text{g/mL}$, and 1000 $\mu\text{g/mL}$. Moreover, the antitumor effects of the in vitro plant extracts increased with the increase in exposure time, while, at some concentrations, the viability of the HeLa and MCF-7 cell lines treated with the extracts of wild plants for 48 h was even slightly increased compared to the 24th h. The strongest time-dependent increase in cytotoxicity was observed for the HepG-2 cells treated with extracts from in vitro cultured plants. The viability of hepatocellular carcinoma cells treated with the highest concentration of this extract was 50% and 14.9% at the 24th and 48th h, respectively. Compared to control untreated cells, both tested extracts induced a statistically significant reduction in the cell viability of HT-29 colorectal carcinoma cells at all concentrations tested, while in HepG2, HeLa and MCF-7 cell lines, significant cytotoxicity of both extracts was established at concentrations higher than 125 $\mu\text{g/mL}$ (HepG2), 250 $\mu\text{g/mL}$ (HeLa) and 250 (24 h) and 500 (48 h) $\mu\text{g/mL}$ (MCF-7).

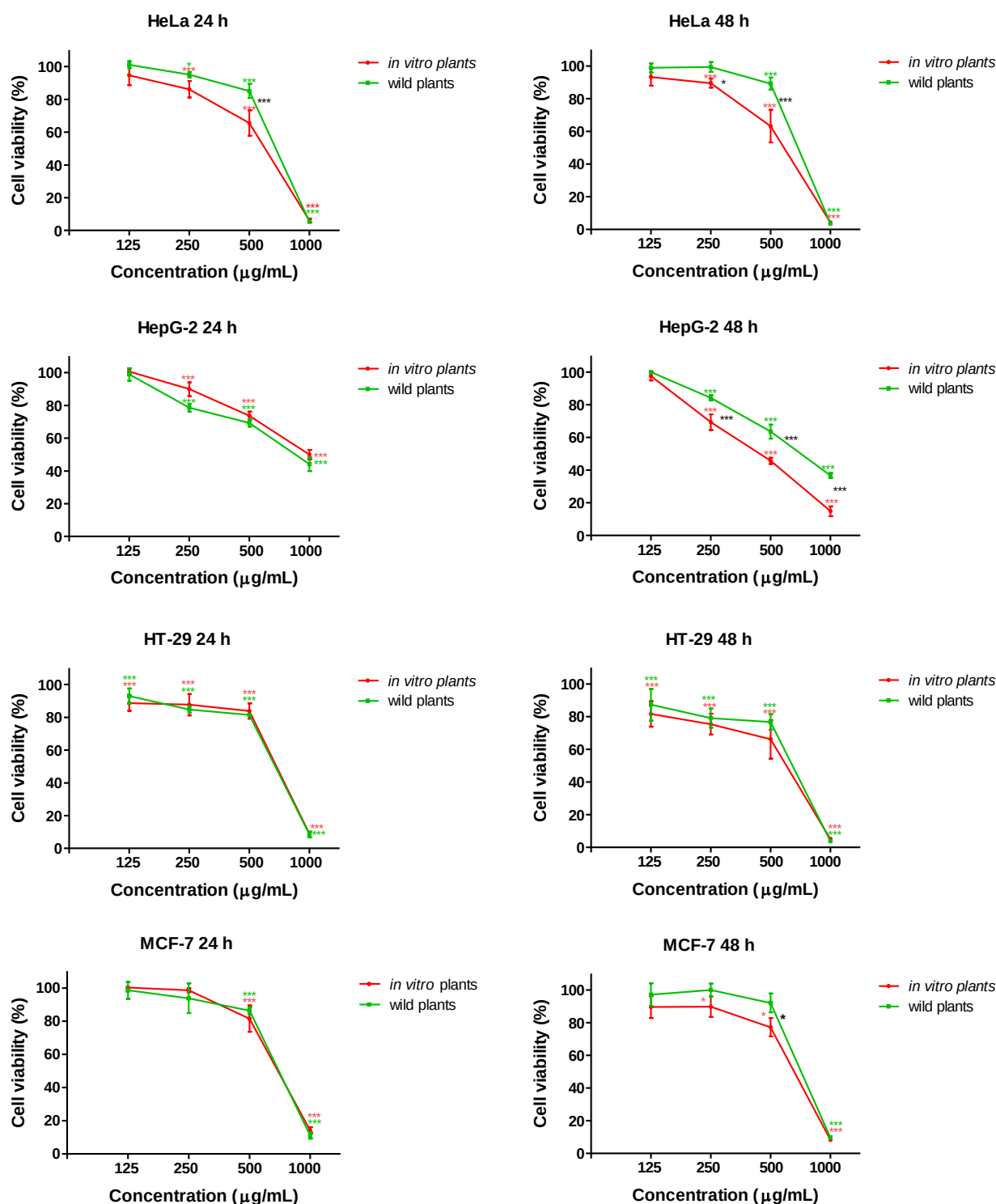


Figure 1. Effects of *C. vulgare* extracts from in vitro cultured and wild-growing plants on the cell viability of human tumor cells HeLa, HepG2, HT-29 and MCF-7 tumor cell lines assessed by the MTT test after 24 and 48 h of exposure. Values are presented as means $\pm$ SD; * $p < 0.05$ and *** $p < 0.001$; green and red asterisks indicate significance of the differences between control and extract-treated cells; and black asterisks indicate significance of the differences between cells treated with extracts from wild and in vitro cultured plants.

The non-tumor human keratinocytes HaCaT cell line was used as a control cell line to assess the selectivity of the cytotoxicity toward cancer cells (Figure 2). The plant extracts had considerably lower cytotoxic effects on the non-tumor cell line HaCaT compared to all the tumor cell lines, especially at the 24th h and 1000 μ g/mL. In the non-tumor cell line,

statistically significant differences between the effects of the wild and in vitro plant extracts were found only at the 48th h after treatment with the highest concentration. In contrast to tumor cells, normal HaCaT cells appeared to be more sensitive to the effect of the wild plant extract.

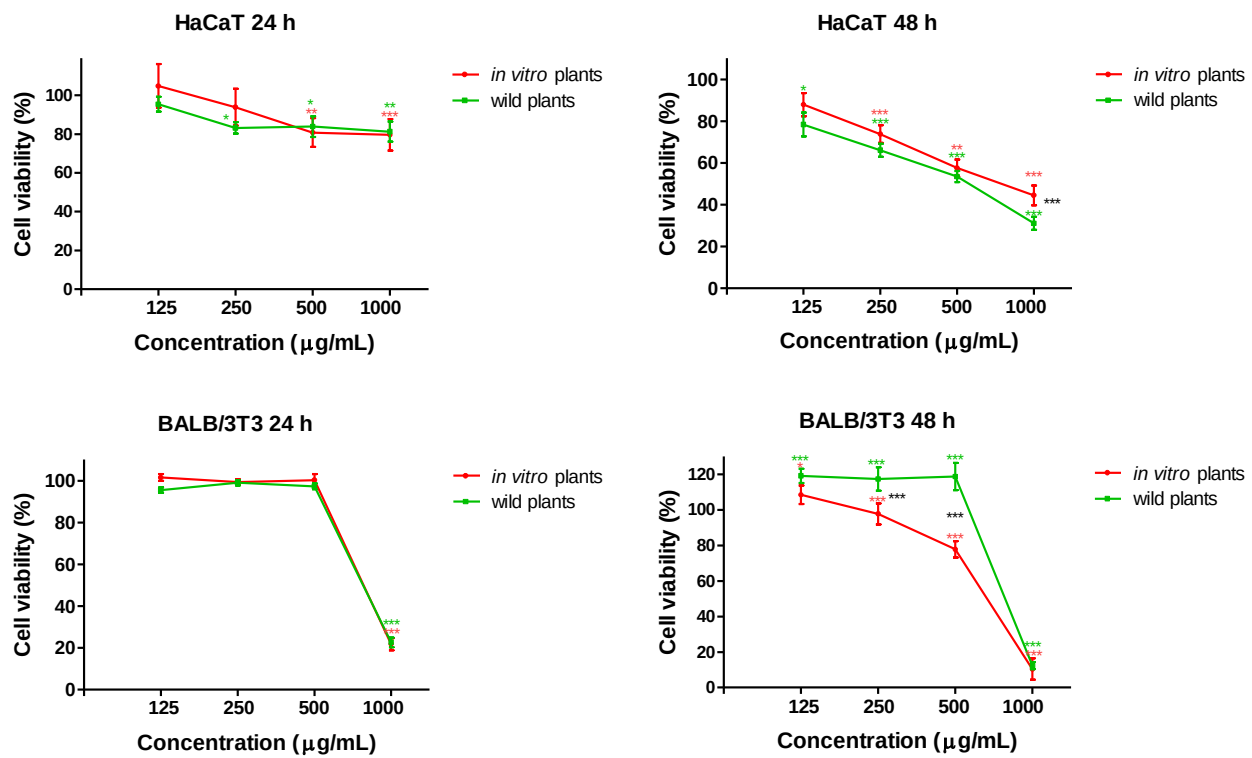


Figure 2. Effects of *C. vulgare* extracts on the cell viability of non-tumor cell lines HaCaT and BALB/3T3 assessed by MTT test after 24 and 48 h of exposure. Values are presented as means $\pm$ SD; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$; green and red asterisks indicate significance of the differences between control and extract-treated cells; and black asterisks indicate significance of the differences between cells treated with extracts from wild and in vitro cultured plants.

The effects of the plant extracts were also assessed on a non-tumor BALB/3T3 cell line, a model system commonly used for in vitro cytotoxicity screening of chemical compounds. Cytotoxic effects at the 24th h were detected only when the cells were treated with the extracts at a concentration of 1000 µg/mL. At the other tested concentrations, significant differences in the cell viability between control and treated cells were not established. After 48 h of treatment, an enhanced effect of the extract from in vitro cultured plants was found. Statistically significant differences between the effects of the extracts obtained from in vitro and wild plants were observed at concentrations of 250 and 500 µg/mL.

The half-maximal inhibitory concentrations (IC_{50}) of the tested *C. vulgare* extracts were determined for each cell line using nonlinear regression curve fit analysis and presented in Table 2.

For each cell line, the wild and in vitro plant extracts showed quite similar IC_{50} values at the 24th h. However, after 48 h of exposure, the IC_{50} values of the extracts of in vitro plants were lower than those of the wild plant extracts for all cell lines tested except for the non-tumor cell line HaCaT. From the tested tumor cell lines, the lowest IC_{50} values were established for HeLa and HepG-2 cells treated with extracts of in vitro plants for 24 and 48 h, respectively.

Table 2. Inhibitory concentrations (IC₅₀) (µg/mL) of extracts from in vitro cultured and wild-growing *C. vulgare*.

Cell Line	In Vitro Plants		Wild Plants	
	24 h	48 h	24 h	48 h
HeLa	568.7	556.6	649.7	656.9
HepG2	977.3	461.1	924.2	740.7
HT-29	665.9	509.2	652.6	611.7
MCF-7	682.7	633.1	691.7	719.3
HaCaT	>1000	744.0	>1000	499.5
BALB/3T3	816.1	716.8	821.3	759.3

3.3. Cell Migration Assay

The migration ability of the cervical carcinoma cells HeLa treated with the extract of in vitro cultured *C. vulgare* plants was evaluated using the wound-healing scratch method. The differences between the wound-healing capacity of the control and extract-treated cell cultures detected at the three time intervals were evaluated and are presented in Figure 3.

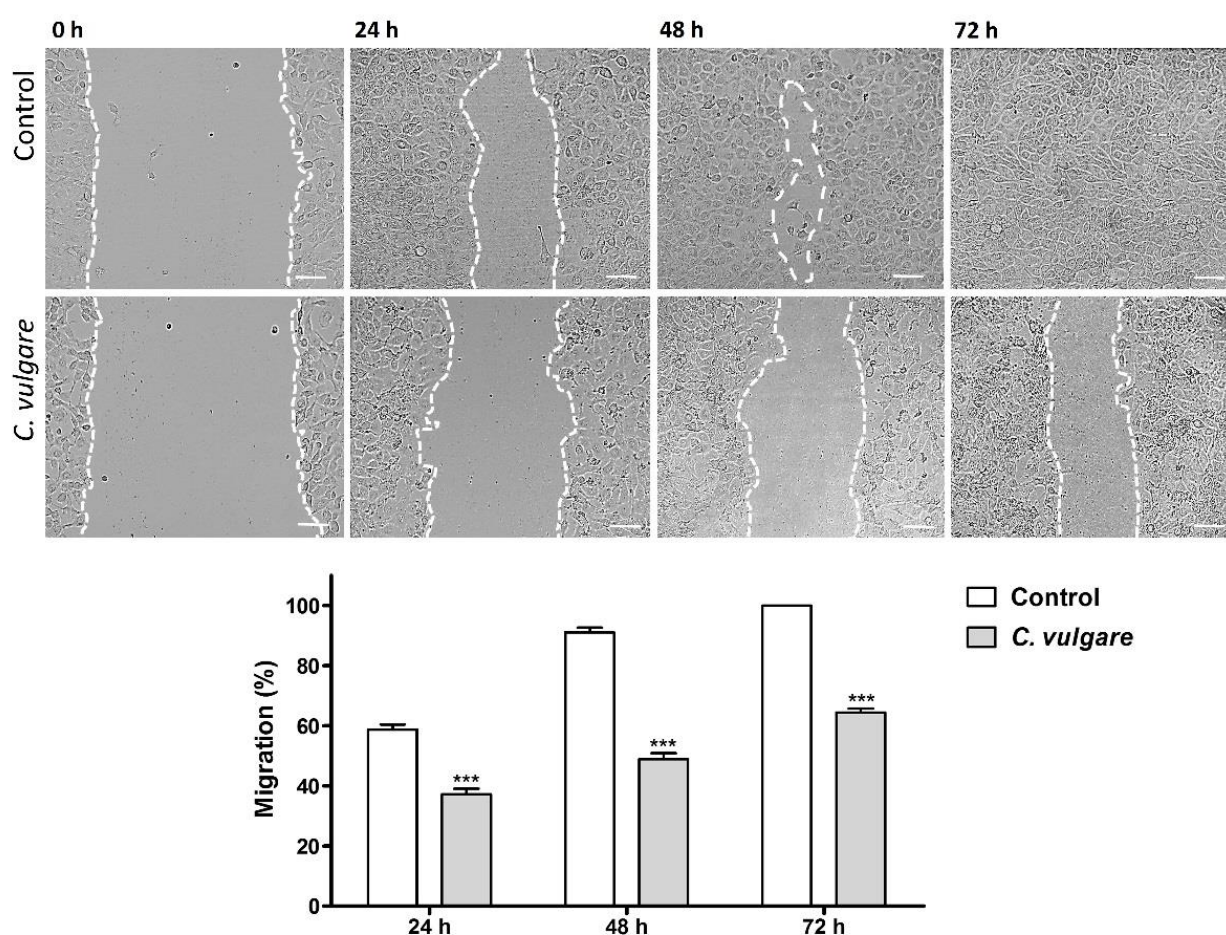


Figure 3. Effect of the extract of in vitro cultured *C. vulgare* plants on the migration ability of HeLa cancer cells. Upper panel: Microphotography images of untreated cell cultures and cell cultures treated with *C. vulgare* extract (300 µg/mL); scale bar = 100 µm. (Lower panel). Graphical representation of the effect of *C. vulgare* extract on the HeLa cancer cells migration. Data are presented as mean ± SD; *** $p < 0.001$ compared to the untreated control.

The presented results indicate that the *C. vulgare* extract induced statistically significant inhibition of the cancer cell migration at the three studied time points. The migration percentages of the untreated cell cultures were $58.74 \pm 1.64\%$, $91.08 \pm 1.59\%$ and 100% for 24 h, 48 h and 72 h. The wound areas in cell cultures treated with the extract were notably wider, and the percentage wound-healing measured at the same time intervals was $37.26 \pm 1.81\%$, $48.97 \pm 1.88\%$ and $64.42 \pm 1.28\%$, respectively.

3.4. Fluorescence Microscopy

To investigate the mechanisms underlying the detected suppressive effects on cancer cell growth, HeLa cells treated for 24 h with the extract of in vitro cultured plants and stained with AO/EtBr were analyzed by fluorescence microscopy. The cellular morphology of the cancer cells treated with *C. vulgare* extract markedly differed compared to the control (Figure 4). Untreated HeLa cells were homogeneously green-stained and displayed normal monolayer growth and typical morphological characteristics (Figure 4a). Fluorescent staining with the DNA-binding dye AO allowed the detection of numerous cells in a phase of mitosis. In contrast, in the cell cultures treated with *C. vulgare* extract, a large number of red-stained dead and late apoptotic cells with condensed chromatin and fragmented nuclei were observed, and the monolayer confluency was greatly reduced (Figure 4b). The observed morphological alterations demonstrate the ability of the tested extract to inhibit proliferation and induce apoptotic cell death.

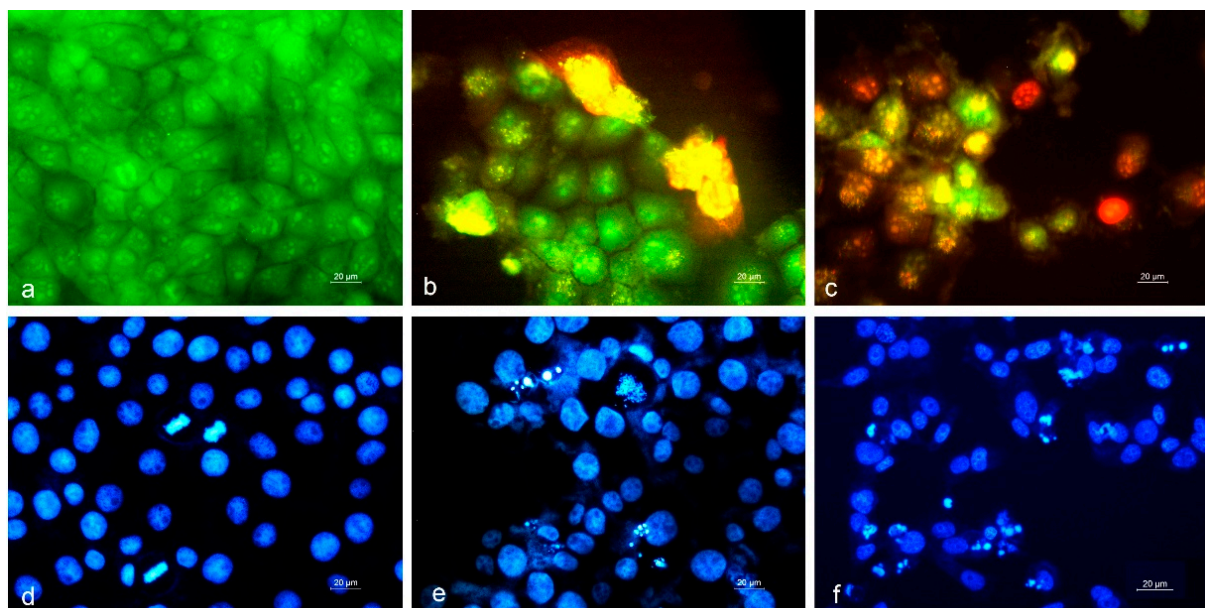


Figure 4. Cytomorphological alterations induced by the extract of in vitro cultured *C. vulgare* plants in HeLa cervical carcinoma cells. (a,d) Negative control (untreated cells); (b,e) cells treated with *C. vulgare* extract (600 µg/mL); (c,f) positive control (cells treated with 2.25 µg/mL doxorubicin); (a–c) AO/EB staining; and (c–f) DAPI staining. Fluorescence microscopy; objective 40×.

For a more detailed study of the nuclear morphology of tumor cells treated with the extract from in vitro cultured *C. vulgare*, fluorescence microscopic analysis was performed after staining with the DNA-binding dye DAPI. The nuclei of untreated cells were oval-shaped, with smooth edges and homogenous blue staining (Figure 4d). After treatment with the *C. vulgare* extract, cells with shrunk nuclei, condensed chromatin, and intense fluorescent staining (early apoptotic cells), as well as nuclear fragmentation and the formation of apoptotic bodies (late apoptosis), occurred (Figure 4e).

The cytomorphological alterations induced by the tested extract were similar to the changes observed in HeLa cells treated with the standard cytostatic drug doxorubicin (Figure 4c,f). Doxorubicin was also applied at a concentration equal to the IC₅₀ value determined by the MTT assay.

To confirm the ability of the studied extract to induce apoptotic cell death, annexin V/propidium iodide fluorescent staining was applied (Figure 5). In control untreated tumor cells, no fluorescence was observed (Figure 5a), as the cell membrane was impermeable to propidium iodide and FITC-conjugated annexin V. After treatment with the extract from in vitro cultured *C. vulgare*, the tumor cell cultures showed the presence of early apoptotic cells with green, fluorescent staining (Figure 5b) due to the translocation of phosphatidylserine from the inner to the outer surface of the cell membrane, which makes possible the binding of fluorescently labeled annexin V.

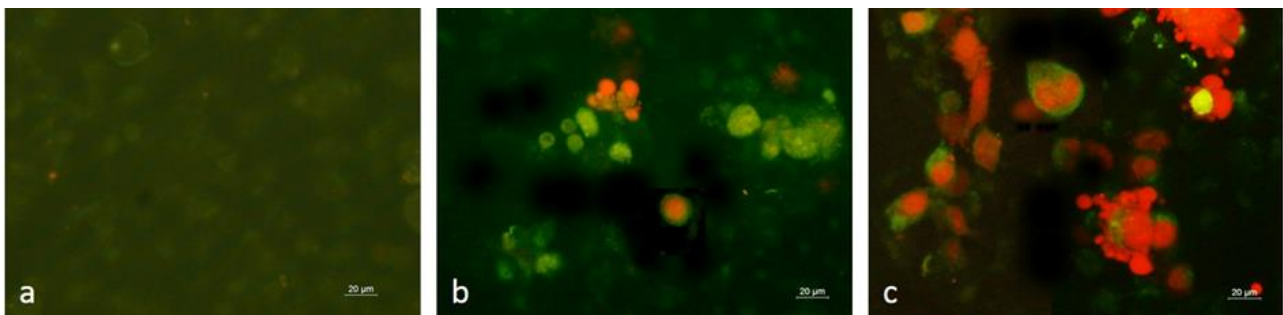


Figure 5. Fluorescence microscopy of the apoptotic alterations in HeLa cervical carcinoma cells induced by the extract of *C. vulgare* in vitro cultured plants. (a) Control untreated cells; (b) cells treated with *C. vulgare* extract 600 µg/mL; and (c) cells treated with *C. vulgare* extract and 800 µg/mL Annexin V/propidium iodide staining. Fluorescence microscopy; objective 40×.

Late apoptotic cells were also identified, with green-labeled membranes and red-stained nuclei (Figure 5c), as in the late stages of apoptosis, the loss of membrane integrity allows propidium iodide to enter the cell and bind to nuclear DNA. The results of the AV/PI fluorescence microscopic analysis of HeLa cervical carcinoma cells are consistent with the results obtained by the other cytomorphological methods used and are proof of the ability of the studied extract of in vitro cultured *C. vulgare* plants to induce tumor cell death via apoptosis.

3.5. Flow Cytometric Analysis

To study the effect of the extract on the cell cycle, flow cytometric analysis to evaluate the distribution of the cells in different phases of the cell cycle was performed. HeLa cancer cells were treated for 48 h with an extract of in vitro cultured *C. vulgare* plants at concentrations of 600 µg/mL and 800 µg/mL and were analyzed using a Becton Dickinson flow cytometer according to a standard protocol. The obtained results show that treatment with *C. vulgare* extract induces a redistribution of the cancer cells in the different phases of the cell cycle (Figure 6).

As can be seen from the data presented, treatment with *C. vulgare* extract resulted in a significant decrease in the percentage of cells in the G1 phase, while a statistically significant increase in the number of cells in the S and G2/M phases of the cell cycle was found. Furthermore, treatment with the extract at a concentration of 800 µg/mL showed a statistically significant increase in the percentage of cells with a lower DNA content (<2 N).

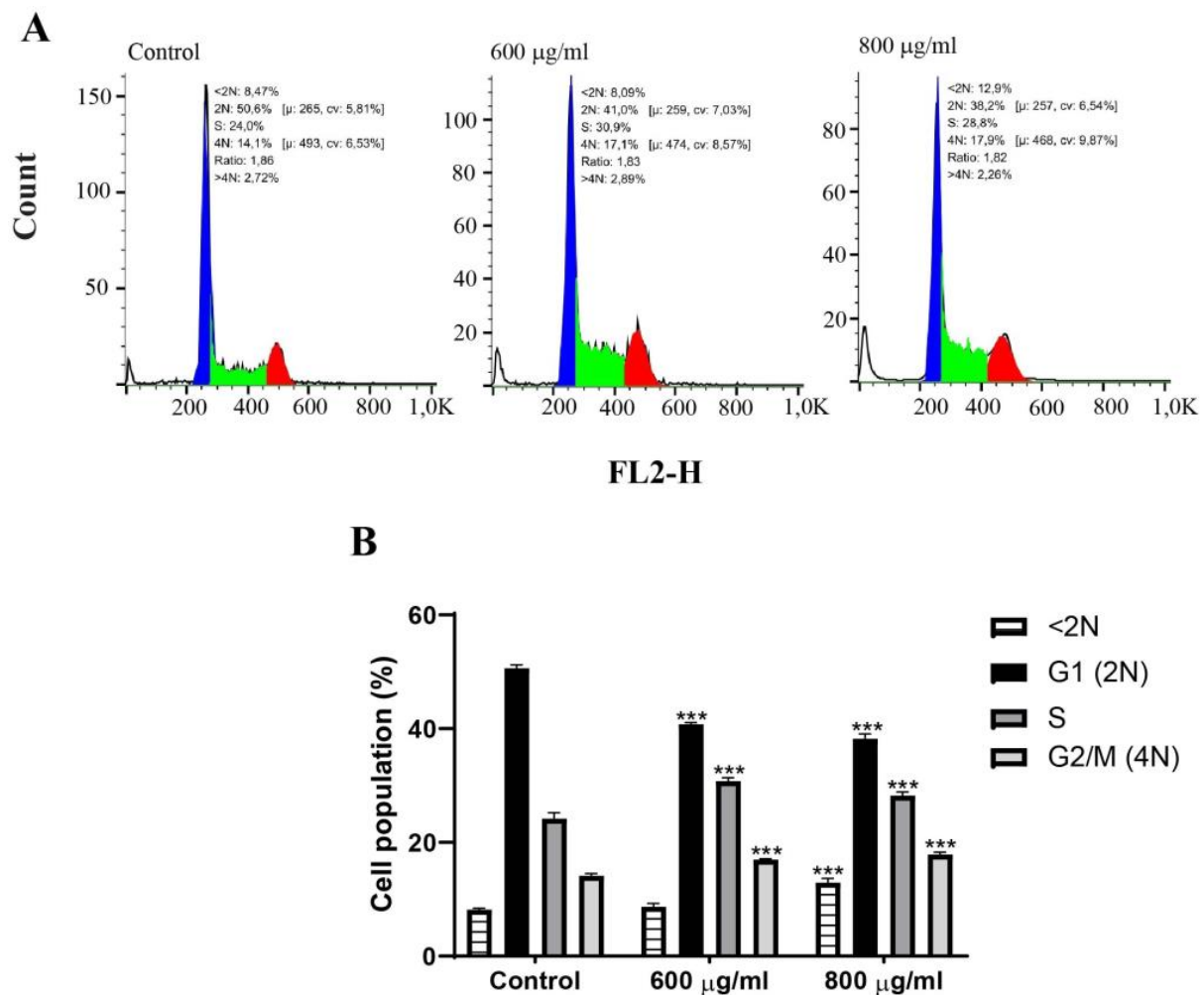


Figure 6. Effects of a total aqueous extract of in vitro cultured *C. vulgare* plants on the cell cycle of HeLa cervical carcinoma cells. **(A)** Histograms of control untreated cells and cells treated with the extract at concentrations of 600 µg/mL and 800 µg/mL; **(B)** graphic representation of the distribution of cells in the different phases of the cell cycle. Data are presented as mean $\pm$ SD from three independent experiments. Stars indicate a difference with the respective phase in control cells at $p < 0.001$.

4. Discussion

Examining toxicity is a crucial step in the safety evaluation of chemical substances and their mixtures, aiming to identify potential adverse effects and determine the appropriate dosage for medicinal applications. Despite the discovered diverse beneficial biological effects of *C. vulgare*, its potential toxicity has not been thoroughly investigated. The main difficulty in the safety evaluation of wild-growing medicinal plants compared to cultivated plants is that their chemical composition and the concentration of the individual components vary widely depending on many different external factors, such as geographical and climate conditions, the presence of stress factors, time of collection, conditions of storage and others. In the case of cultivated plants, the controlled conditions of cultivation allow a higher consistency of the plant's phytochemical composition. In a previous study, the acute and subacute toxicity of an aqueous extract of wild-growing *C. vulgare* plants was assessed through in vivo experiments in mice and rats [9]. The study demonstrated that the oral application of the extract did not induce any toxic side effects in the organs and systems of the experimental animals [9]. In our study, the cytotoxicity of the tested *C. vulgare* extracts was evaluated on BALB/3T3 clone A31 murine embryo fibroblast cells. This cell line was chosen as it is one of the in vitro systems approved by the European Centre of Validation of

Alternative Methods (ECVAM) as a suitable model for safety assessment studies of chemical compounds [33,34]. The results of our study at the 24th h demonstrate a complete absence of cytotoxicity of both tested extracts on the BALB/3T3 cells in the whole concentration range tested, except for the highest concentration (1000 µg/mL). The extension of the time of exposure to the extract of wild-growing plants to 48 h, except at 1000 µg/mL, did not lead to a reduction in cell viability compared to the untreated control cells, and cell viability was even slightly increased. The viability of the cell cultures treated for 48 h with the extract of in vitro cultivated plants, except at 1000 µg/mL, was not decreased at the lowest concentrations of the extract (125 µg/mL and 250 µg/mL (it even slightly increased at 125 µg/mL)) and slightly decreased at the higher concentration (500 µg/mL). The results of our study are in agreement with the in vivo-obtained data indicating low toxicity of the aqueous *C. vulgare* extracts [9].

In addition to the study on BALB/3T3 cells, the cytotoxicity of both extracts was also evaluated using a panel of human tumor and non-tumor cell lines in order to assess their potential anticancer activity. Cervical, hepatocellular, colorectal and mammary carcinoma cell lines were used in the study as in vitro models of some of the most common types of cancer diseases in humans. The results of the analysis show that the tested *C. vulgare* extracts induced a statistically significant and concentration-dependent decrease in the cell viability of all tumor cell lines tested; however, the extent of the observed effects varied depending on the type of cancer cells used and the extract applied. After 24 h of treatment, both extracts induced comparable cytotoxic effects on the tumor cells, except for the HeLa cell line, where the extract of in vitro cultivated plants showed a much better effect than the extract from wild plants, decreasing significantly the cell viability of the cervical carcinoma cells at 500 µg/mL, confirmed also by the lower IC₅₀ for the in vitro plants. The decrease in the tumor cell viability determined at 48 h was more pronounced for the extracts obtained from in vitro cultivated plants for all cell lines used. Moreover, a time-dependent increase in the cytotoxicity was found for all types of cancer cells treated with the in vitro plant extracts, while after 48 h treatment of HeLa and MCF-7 cells with the wild plant extract, a slight decrease in the cytotoxic effect compared to the 24th h was observed. Regarding the effect of the extract of in vitro cultivated plants at the 48th h, the most pronounced was the influence on the HepG-2 cell line, followed by HeLa, which at 500 µg/mL reached a 54% and 37% decrease in cell viability, respectively (the effect at 1000 µg/mL, although strongest, is disregarded as at this concentration a significant decrease in the cell viability of non-tumor cell lines was also observed). Comparing the effects of the extracts on the different types of tumor cells, it was found that MCF-7 mammary carcinoma cells were the least sensitive. The lowest IC₅₀ value corresponding to the highest cytotoxicity at the 24th and 48th h was established for HeLa and HepG-2 cells, respectively. In contrast to the other cancer cell lines, in HT-29 colorectal carcinoma cells, a statistically significant decrease in cell viability compared to controls was found for all concentrations of the tested extracts at both treatment time intervals studied.

To assess the selectivity of the cytotoxic effects induced by the tested *C. vulgare* extracts toward cancer cells, the human non-tumor cell line HaCaT was included as a control in the panel of human cancer cell lines. The normal cells demonstrated lower sensitivity to the cytotoxic effects of the tested plant extracts compared to the tumor cells. Statistically significant differences between the effects of the wild and in vitro plant extracts were found only after 48 h of treatment and at the highest concentration (1000 µg/mL). Interestingly, in contrast to all other cell lines used in the present study, the effect of the wild plant extract on HaCaT cells was more pronounced than that of the extract of in vitro cultured *C. vulgare* plants, pointing to a less detrimental influence on normal, non-tumor cells of the extract of in vitro cultured plants compared to wild plants.

Our results are consistent with previously published data demonstrating the selective anticancer effects of different types of *C. vulgare* extracts against various cancer cell types. Aqueous extracts of *C. vulgare* have been shown to exhibit high cytotoxic activity against HEP-2 human larynx epidermoid carcinoma, A2058 human metastatic melanoma and L5178Y mouse lymphoma cells, while FL human normal amniotic cells and 3T3 murine embryonic fibroblast cells were significantly less affected [24]. Another study examined the antitumor activity of acidified, alkalized and neutral water extracts, as well as lipophilic *C. vulgare* extracts, on HeLa cervical carcinoma, HT-29 colorectal carcinoma and CaOV testicular cystadenocarcinoma [8]. The results of this study demonstrated the highest activity for the acidified and lipophilic extracts. Both extracts showed concentration-dependent cytotoxic effects on cancer cells and a lack of cytotoxicity toward the FL human amniotic cells used in the study as a non-tumor control cell line [8]. The in vitro antitumor activity of methanolic *C. vulgare* extracts against A549 lung carcinoma, HepG-2 hepatocellular carcinoma, CaCo colorectal carcinoma and MCF-7 mammary carcinoma has also been reported [25,35]. Although several plant species belonging to the Lamiaceae family have shown in vivo antitumor activity [36–38], thus far, the *C. vulgare* plant extracts have not been studied in animal cancer models. The results obtained in our study demonstrating selective anticancer effects on a panel of cell lines and the observed inhibition of cancer cell migration could serve as a good basis for conducting future in vivo experiments with laboratory animals to confirm the safety and efficacy of *C. vulgare* extracts and validate their antitumor effects in more complex cancer model systems.

Despite the increasing amount of experimental evidence revealing the potential of *C. vulgare* extracts to decrease tumor cell survival and proliferation, the mechanisms underlying their anticancer effects remain unclear. A recently published study investigated the involvement of the apoptosis pathway as a possible mechanism of the anticancer effects of a methanolic extract of the plant on the A549 human lung carcinoma cell line [25]. The results of the performed analysis showed that the treatment of the tumor cells with *C. vulgare* methanolic extracts did not lead to discernible alteration in the expression of the apoptosis-related genes Bax and Bcl-2. Moreover, the flow cytometric analysis of treated tumor cells stained with Annexin V/propidium iodide indicated that the observed anticancer effects were due to the induction of necrosis rather than apoptosis [25]. In contrast, applying three different fluorescent staining methods in combination with cell cycle analysis, the present study clearly demonstrates the ability of the tested aqueous extract obtained from in vitro cultured *C. vulgare* plants to induce cancer cell apoptosis. The discrepancies between our findings and the lack of detectable apoptotic alterations in the previous study [25] could be explained with the use of different extraction procedures and different in vitro cancer models.

Apoptotic cells are characterized by the occurrence of specific alterations in the cellular and nuclear morphology, allowing their identification by cytomorphological methods [39]. The AO/EB and DAPI staining used in our study showed chromatin condensation, nuclear fragmentation, membrane blabbing and the formation of apoptotic bodies in the HeLa cells treated with the extract from in vitro cultured *C. vulgare* plants, demonstrating the ability of the tested extract to induce apoptosis. The proapoptotic activity of the tested *C. vulgare* extract was also confirmed by fluorescence microscopy after staining with Annexin V/propidium iodide. This method allows the detection of apoptosis-specific alterations in the cell membrane expressed in the translocation of phosphatidylserine from the inner to the outer surface of the plasma membrane. The results of our analysis reveal the presence of early and late apoptotic cells in the HeLa cell cultures treated with the *C. vulgare* extract.

Cell cycle analysis was performed to further elaborate on the molecular mechanisms of the detected cytotoxicity, inhibition of cell migration and proapoptotic activity. The flow

cytometry results additionally suggest the induction of apoptosis by the *C. vulgare* extract as evidenced by the increase in the sub-G1 population. Moreover, statistically significant and concentration-dependent alterations in the distribution of cells in the different phases of the cell cycle were identified in the extract-treated cell cultures compared to the untreated control. The accumulation of cells in the S and G2/M phases and the decrease in the G1 cell population could be explained by the activation of checkpoint and repair mechanisms in response to cellular stress or damage induced by the *C. vulgare* extract. Further detailed molecular studies of the potential of *C. vulgare* extract to induce DNA damage in cancer cells and alter the expression of checkpoints, stress-response and repair genes or proteomics analysis to detect differentially expressed proteins would be necessary to identify the exact pathways and mechanisms involved.

Examining the chemical composition of the extracts of the in vitro cultivated and wild plants shows that the total content of both flavonoids and polyphenols, as well as the antioxidant activity, are higher for the wild plants compared to the in vitro cultivated plants, which cannot explain the higher cytotoxic effect of the in vitro plant extract on the HeLa cell line at the 24th h and the consistently higher cytotoxic effect of the same extract on all cancer cell lines at the 48th h. However, our previous research [26] has shown that it is only the in vitro cultivated plants (leaves and flowers) and not the wild-growing plants that contain catechin. Furthermore, neochlorogenic acid (flowers), quercetin (stems) and apigenin (stems) are significantly higher in the in vitro cultivated plants than in the wild plants [26]. Many studies have shown the protective and anticarcinogenic effects of catechins against a number of cancers, amongst which are gynecological cancers such as ovarian and endometrial cancer, breast cancer, oropharyngeal and laryngeal cancer, digestive tract cancers such as stomach and colorectal cancer, incident glioma, liver and gallbladder cancers, lung cancer and others [40]. For example, epigallocatechin gallate (EGCG), the most abundant catechin in fresh tea leaves [41], has been reported to inhibit tumor invasion and angiogenesis essential for tumor growth and metastasis [42]. Similarly, neochlorogenic acid or extracts rich in it have been reported to possess strong anticancer activity against several types of human cancers, like gastric cancer and human glioma, with some of the underlying mechanisms being the loss of mitochondrial membrane potential, reactive oxygen species generation and the induction of apoptosis partly through DNA damage and the regulation of epigenetic factors in human cancer cells [43,44]. Regarding apigenin and quercetin, their anticancer potential has been confirmed through their ability to modulate various cell signaling pathways related to cancer, including tumor suppressor genes and the PI3K/AKT, NF- κ B, MAPK/ERK and the STAT3 pathways [45,46]. Thus, for example, quercetin has been demonstrated to exert an anti-tumor immune effect in breast cancer by regulating the IL-6/JAK2/STAT3 signaling pathway to deplete regulatory (Treg) cells, which in the tumor microenvironment are reprogrammed to acquire higher immunosuppressive capacity, and a large number of infiltrated Treg cells in the tumor microenvironment is often related to poor prognosis [46]. It could be that one of the above biologically active substances, or rather their combination, is responsible for the better cytotoxic efficiency of the extract of the in vitro cultivated plants compared to the wild-growing plants.

The tendency toward the use of total plant extracts for anticancer activity screening [14,47,48] is justified and useful as the pharmacological (protective) effect of the extract is very often the result of the complex, multi-targeted action of the different active substances and, frequently, of the synergistic relations between them. The specific mechanisms of action of the active substances are variable and dependent on their structure and concentration in the extract as well as on the different physicochemical environmental conditions. Thus, although in a previous study [26] we examined the antioxidant and

antitumor activity of different parts (stems, leaves and flowers) of in vitro cultivated and wild *C. vulgare* plants, in the present research, we turned our attention to evaluating the cytotoxicity and anticancer potential of total extracts, comprising the entire aerial part, of wild and in vitro cultivated *C. vulgare* plants. A comparison between the two studies demonstrates that the extracts from in vitro cultivated plants consistently show stronger anticancer effects compared to the respective extracts from wild-growing plants. Furthermore, leaves and flowers of in vitro cultivated plants show higher cytotoxicity toward cancer cells than total extracts of in vitro cultivated plants, which, in turn, show better effects than stems of in vitro cultivated plants. An important advantage of the total extract from in vitro cultivated plants is that it showed the lowest cytotoxicity toward normal cells compared to all the other tested extracts. Thus, the overall selectivity of its anticancer action increases, making it more suitable for therapeutic applications.

5. Conclusions

The results of our study evaluating the cytotoxic and antitumor activity of water whole-plant extracts of wild and in vitro cultivated *C. vulgare* plants on a panel of four cancer and two control non-cancer cell lines demonstrate consistently better anticancer effects for the in vitro cultured plants. The detected reduction in cancer cell viability and migration was associated with suppression of cell cycle progression and induction of apoptosis. Further research is needed to fully elucidate the anticancer mechanisms and identify the main bioactive phytocomponents involved.

Author Contributions: Conceptualization, K.T. and A.G.; methodology, A.G., I.S., S.A. and N.T.; software, A.G., I.S. and V.D.; validation, A.G., K.T.; formal analysis, T.T.-Y., S.A. and N.T.; investigation, K.T., A.G., I.S., S.A. and N.T.; data curation, A.G. and I.S.; writing—original draft preparation, A.G., K.T. and P.P.-K.; writing—review and editing, A.G., K.T. and P.P.-K.; visualization, A.G., I.S. and V.D.; supervision, K.T.; project administration, K.T.; funding acquisition, K.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Bulgarian National Science Fund, grant number KP-06-N56/16.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data are contained in the manuscript.

Acknowledgments: The authors are grateful to Maria Petrova, Lyudmila Dimitrova and Margarita Dimitrova (Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences) for their invaluable assistance in obtaining the in vitro plants and their cultivation in the field.

Conflicts of Interest: The authors declare no conflicts of interest.

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