UNIVERSITY OF MEDICINE AND PHARMACY
"CAROL DAVILA" BUCHAREST
DOCTORAL SCHOOL
PHARMACY FIELD

DOCTORAL THESIS

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PhD student:
IVAN IONUȚ-MĂDĂLIN

2024

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PHYTOCHEMICAL AND BIOLOGICAL RESEARCH ON PLANT EXTRACTS WITH POTENTIAL ANTITUMOR ACTION

DOCTORAL THESIS ABSTRACT

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INTRODUCTION

According to the World Health Organization, through the International Agency for Cancer Research, 19.97 million cases of cancer were registered worldwide (in 185 countries) in 2022, compared to 19.30 million in 2020 [1, 2]. In addition to genetic factors, multiple other risk factors play an important role in the development of carcinogenesis: smoking, alcohol consumption, obesity and sedentary lifestyles, exposure to high doses of radiation, air pollution and diet. Environmental factors play a more important role in the development of cancer than inherited genetic factors [3].

A number of functional mechanisms are altered in carcinogenesis: cell division, apoptosis, cell differentiation, angiogenesis, the body's immune response and the complex process of DNA metabolism [4].

Current treatment methods cause multiple adverse effects, the onset of resistance and toxicity over long periods of time, and can affect the patient's quality of life for a variable duration of time ranging from weeks to years [5]. For these reasons, one direction of research is the development of new molecules, and phytocompounds are inexhaustible sources of well-tolerated therapeutic agents with different mechanisms of action. A large statistical survey carried out for the period 1981-2019 showed that 33.51% of all molecules discovered were derived from natural sources or derived from molecules obtained from natural sources [6]. Among the first approved chemotherapeutic drugs were those whose active substances are alkaloid derivatives, as follows: vinblastine (bronchopulmonary and breast cancer), vincristine (leukemia), vinorelbine (breast cancer) and paclitaxel (breast, ovarian, bronchopulmonary cancer) [7].

Oxidative stress, due to high concentrations of reactive oxygen species, promotes tumor cell hyperproliferation. Polyphenols or flavonoids play an important role in neutralizing free radicals, thus having an anti-proliferative effect. At the same time, polyphenolic compounds have numerous biological actions that can positively influence the management of tumor diseases: anti-inflammatory, antibacterial, hepatoprotective, antiadipogenic and antiviral [8].

Studying the literature data, I focused my attention on some plant raw materials rich in alkaloids with biological properties and polyphenolic compounds, in order to obtain plant

extracts with potential antitumor action. *Berberis vulgaris* L. (barberry) belongs to the plant family Berberidaceae and several polyphenolic compounds and the alkaloid berberine have been identified in the phytochemical composition [9]. *Capsicum annuum* L. (chili pepper) belongs to the Solanaceae plant family and is studied for both its capsaicin and polyphenolic compounds [10]. *Chelidonium majus* L. (greater celandine) belongs to the plant family Papaveraceae and contains numerous biologically active alkaloids (berberine, chelidonine, chelerythrine, sanguinarine) as well as polyphenols [11].

The general scientific research objectives consisted in:

- selection of vegetable raw materials (*Berberidis cortex*, *Capsicii fructus* and *Chelidonii herba*);
- stablishing the methodology for obtaining plant extracts and selecting the optimal solvent for the extraction of the active principles of interest;
- evaluation of the phytochemical profile of dried plant extracts by spectrophotometric assay and qualitative and quantitative high performance chromatographic analysis;
- evaluation of the *in vitro* antioxidant profile of plant extracts by several standardized methods;
- evaluation of *in vitro* cytotoxicity on human tumor cell lines and normal human cells and evaluation of *in vivo* toxicity in an experimental invertebrate model.

This doctoral thesis is composed of two parts, namely: the General Part, as well as Personal Contributions, with the addition of the List of published works, List of abbreviations and symbols, Introduction, Final conclusions and personal contributions, Bibliography and Annexes (Annex no. 1, 2 and 3).

I. GENERAL PART

1. MALIGNANT TUMOR DISEASES

This chapter summarized data on malignant tumor diseases: general aspects, incidence and etiopathogenesis (risk factors, causes and mechanisms of action), treatments and therapeutic perspectives.

2. PLANT PRODUCTS WITH POTENTIAL ANTITUMOR ACTION

In this chapter information has been summarized on: main classes of phytoconstituents, biological actions, and plant extracts of *Berberis vulgaris* L., *Capsicum annuum* L. and *Chelidonium majus* L. (chemical composition, *in vitro* and *in vivo* studies).

II. PERSONAL CONTRIBUTIONS

3. WORKING HYPOTHESIS AND OVERALL OBJECTIVES

Analyzing the literature data from the general part of the thesis and in order to identify other alternative therapies for the treatment of cancerous tumors or adjuvant in chemotherapy, I turned my attention to the following plant products: bark of the barberry (*Berberis vulgaris* L.), chili pepper (*Capsicum annuum* L.) and aerial parts of the greater celandine (*Chelidonium majus* L.).

The proposed general objectives and working hypothesis for the realization of the doctoral studies consisted in:

- selection of plant raw materials;
- identification of the morphological and organoleptic characteristics specific to the vegetable raw materials and qualitative analysis;
- establishing the methodology for obtaining plant extracts and choosing the appropriate solvent for the extraction of the active principles of interest;
- evaluation of the phytochemical profile of dried plant extracts by spectrophotometric assay and qualitative and quantitative chromatographic analysis (UHPLC-HRMS/MS and HPLC-DAD)
- evaluation of the *in vitro* antioxidant profile of plant extracts by standardized methods (DPPH, ABTS and FRAP);
- evaluation of *in vitro* cytotoxicity on human tumor cell lines: Hep G2 (liver), LoVo, HT-29 (colon), MDA-MB-231 (breast), SK-OV-3 (ovary) and PE/CA-PJ49 (tongue);
- evaluation of *in vitro* cytotoxicity on normal human HUVECs;

- evaluation of *in vivo* toxicity on the invertebrate species *Daphnia magna* and *Daphnia pulex*;

- evaluation of *in vivo* toxicity on *Daphnia magna* embryos;

- statistical analysis of the results obtained to establish potential correlations between active principles, antioxidant properties and antiproliferative action;

- publication of scientific articles in international journals with the results of doctoral research.

4. OBTAINING AND ASSESSING THE QUALITY OF PLANT EXTRACTS

We have chosen to obtain plant extracts by lyophilization because it ensures a high level of stability and higher concentrations for most of the active compounds, preserving their integrity and therapeutic effect. Low temperatures and pressures are used in lyophilization, so that the solvent present in the plant product sublimates and a dry extract is obtained (*Extracta sicca*) [12]. In order to identify the best solvent for the extraction of various chemical compounds from plant products with different degrees of polarity, several experimental determinations were performed for all plant products. Thus, a 50% ethanolic solution in water was selected as the solvent for extraction, having low toxicity and good dissolution properties of the active principles of interest. The content of active principles (flavones, phenolic acids and polyphenols) was evaluated by spectrophotometric assay to determine the quality of plant products.

Plant extracts were obtained with good yields: 16.35% for *Berberidis extractum* (BVE) 7.65% for *Capsicii extractum* (CAE) and 18.45% for *Chelidonii extractum* (CME) [13, 14]. After quantitative spectrophotometric analysis, the three main classes of active chemical compounds responsible for imprinting the therapeutic effect (PCAs, flavones and total polyphenols) were quantified only in *Chelidonii extractum* (greater celandine extract). In barberry extract, flavones were not found and in chili pepper extract, phenolcarboxylic acids could not be detected by the Arnow technique (the reaction with the Arnow reagent was negative both at high and low sample concentrations) [13, 14]. The total polyphenol content was identified and spectrophotometrically assayed in all plant extracts studied. The highest

concentration of total polyphenols was determined for the extract of barberry (*Berberidis extractum*) (barberry: 17.6780 ± 3.9320 g tannic acid/100 g dry extract) compared to the other extracts (greater celandine: 10.0640 ± 0.2455 g tannic acid/100 g dry extract; chili pepper: 4.7250 ± 1.3619 g tannic acid/100 g dry extract) (Fig. 4.1.). The highest concentrations recorded for flavones were detected in the extract of greater celandine (1.6067 ± 0.0651 g rutozide/100 g dry extract) and then in the extract of chilli (1.1548 ± 0.0442 g rutozide/100 g dry extract), in the extract of barberry they were not detected by spectrophotometric assay techniques (Fig. 4.2.). The detection of phenolcarboxylic acids in *Berberidis extractum* resulted in higher concentrations (barberry: 3.3886 ± 0.3481 g chlorogenic acid/100 g dry extract) compared to the other plant extracts tested (greater celandine: 3.0428 ± 0.2057 g chlorogenic acid/100 g dry extract) (Fig. 4.3.).

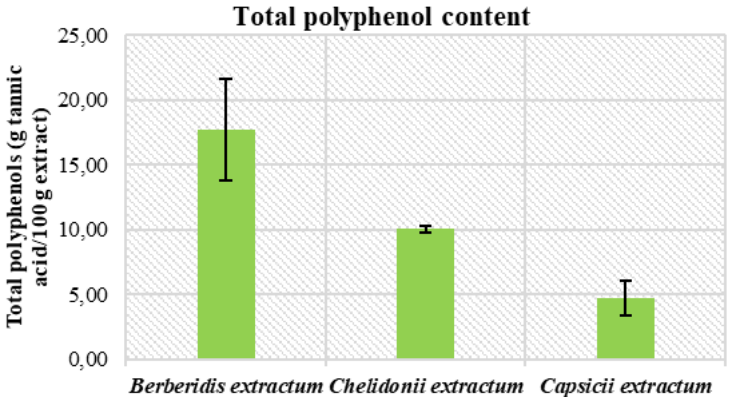

Figure 4.1. Content of total polyphenols in plant extracts

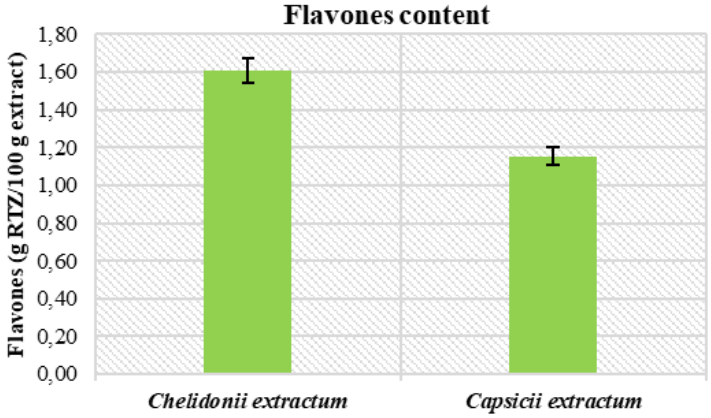

Figure 4.2. Flavones content in plant extracts

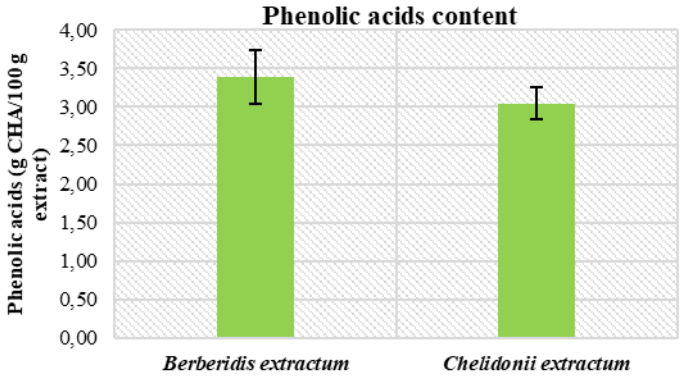

Figure 4.3. Phenolic acids content in plant extracts

5. QUALITATIVE AND QUANTITATIVE CHROMATOGRAPHIC ANALYSIS OF COMPOUNDS IN PLANT EXTRACTS

Taking into account the possible concentration variations of the active principles, we identified and quantified the polyphenolic compounds content of the studied plant extracts by ultra-performance liquid chromatography coupled with high-resolution mass spectrometry (UHPLC-HRMS/MS). The alkaloids were identified either by UHPLC-HRMS/MS or by high performance liquid chromatography coupled to a diode-array (HPLC-DAD). 40 compounds were identified in the case of the hydroethanolic extract of barberry, 70 polyphenolic compounds and 8 capsaicin derivatives in the hydroethanolic extract of chili pepper, and 59 polyphenolic compounds in the hydroethanolic extract of greater celandine [13, 14]. In total, several classes of polyphenolic compounds were identified in plant extracts, totaling 91 compounds.

Different flavonoids were identified in all plant extracts: 6-methoxyluteolin, apigenin-7-O-glycosylglucoside, apigenin (apigenin-7-O-glucuronide and apigenin-8-C-glucoside/isovitexin were identified only in the BVE extract), galangin, kaempferol, kaempferol (or luteolin)-O-glucoside/isomers, kaempferol-3-O-rutinoside (except CME), lehmanin, naringenin, quercitin, quercetin-3-O-glucuronide, rutin (quercetin 3-rutinoside). Of the isoflavones, genistein and glycythein were found in all plant extracts, and genistin, daidzein, biochanin A, sisotrin (biochanin A 7-O-β-D-glucoside), irylon, baptigenin and pratensein in the hydroethanolic extracts of greater celandine and chili pepper. Concerning phenolic acids and

dicarboxylic acids, chili pepper extract was richer in these components compared to the other two plant extracts, gallic acid, chlorogenic/neoclorogenic acid, ferulic acid, *p*-coumaric acid, azelaic acid, rosmarinic acid, abscisic acid and hydroxyferulic acid were found in all the plant extracts studied. Of the diterpenes, carnosol and rosmanol methyl ether were identified in the three extracts, carnosic acid in CME and CAE, lignan in all extracts, cyanidin-3-O-glucoside and cyanidin-3-sambubiozide in CME and CAE.

Regarding the quantitative analysis, in the case of the hydroethanolic extract of greater celandine, CME, among the flavonic compounds, hyperoside (482.93 µg/g), kaempferol (247.87 µg/g), chrysin (194.24 µg/g), rutin (188.52 µg/g), naringenin (146.88 µg/g) and quercitin (113, 82 µg/g) were found in concentrations higher than 100 µg/g, of the isoflavones - only daidzein (194.55 µg/g), and of the phenolic and dicarboxylic acids, gallic acid (1027.84 µg/g), chlorogenic acid (400.40 µg/g), *p*-coumaric acid (290.22 µg/g) and ferulic acid (161.31 µg/g). Concerning the BVE extract, naringenin (90.41 µg/g) and gallic acid (540.00 µg/g) were determined in the highest concentrations. For chili pepper hydroethanolic extract, CAE, kaempferol (377.26 µg/g), quercitin (312.02 µg/g), hesperetin (292.81 µg/g), rutin (240.5 µg/g), hyperoside (212.78 µg/g), naringenin (152.96 µg/g), galangin (102.1 µg/g) and apigenin (101, 31 µg/g) were the flavonic compounds in concentrations higher than 100 µg/g, among the isoflavones, glycytidine (148.91 µg/g), and among the phenolic acids, chlorogenic acid (207.71 µg/g) and *p*-coumaric acid (117.58 µg/g) were present in the highest concentrations. The flavonic compounds quantitatively amounted to 1580.64 µg/g in CME, 440.26 µg/g in BVE and 1863.78 µg/g in CAE, and among all the compounds in the three hydroethanolic extracts, the hyperoside identified in CME was determined in the highest concentration (482.93 µg/g). The highest concentration of isoflavones was calculated for CME (329.16 µg/g), and for phenolic acids the most concentrated extract was CME (1905.71 µg/g), with gallic acid being predominant (1027.84 µg/g).

6. EVALUATION OF THE *IN VITRO* ANTIOXIDANT ACTION OF PLANT EXTRACTS

In order to obtain an antioxidant profile as complex as possible of the studied extracts, which showed a varied phytochemical composition (polyphenols, flavones, phenolic acids and

secondary metabolites), we used three standardized spectrophotometric methods of determination: DPPH, ABTS and FRAP [15]. Thus, the lower the concentration values of an extract, the more pronounced its antioxidant effect, acting as a potent free radical scavenger.

Of the extracts studied, barberry showed the lowest IC_{50} and EC_{50} values in all methods for determining antioxidant activity ($IC_{50,\ DPPH}$ = 0.2610 mg/mL; $IC_{50,\ ABTS}$ = 0.0442 mg/mL; $EC_{50,\ FRAP}$ = 0.1398 mg/mL) (Fig. 6.1.). Barberry extract therefore had the most potent antioxidant action, with an IC_{50} value very close to that of the reference standard, ascorbic acid ($IC_{50,\ acid\ ascorbic}$ = 0.0165 mg/mL) [14]. *Capsicii extractum* showed the weakest antioxidant activity, following the application of antioxidant methods, which resulted in the highest IC_{50} values ($IC_{50,\ DPPH}$ = 1.6699 mg/mL; $IC_{50,\ ABTS}$ = 0.2006 mg/mL; $EC_{50,\ FRAP}$ = 0.5613 mg/mL) (Fig. 6.1.) [13]. For *Chelidonii extractum*, compared to the other extracts analyzed, intermediate values for antioxidant capacity were obtained ($IC_{50,\ DPPH}$ = 0.7643 mg/mL; $IC_{50,\ ABTS}$ = 0.1790 mg/mL; $EC_{50,\ FRAP}$ = 0.2814 mg/mL). In conclusion, the values recorded for all three extracts analyzed reveal a very good antioxidant potency that correlates with the content of active principles present in each plant extract and that may explain their potential association in various pharmaceutical preparations for the prevention or annihilation of oxidative stress in the body.

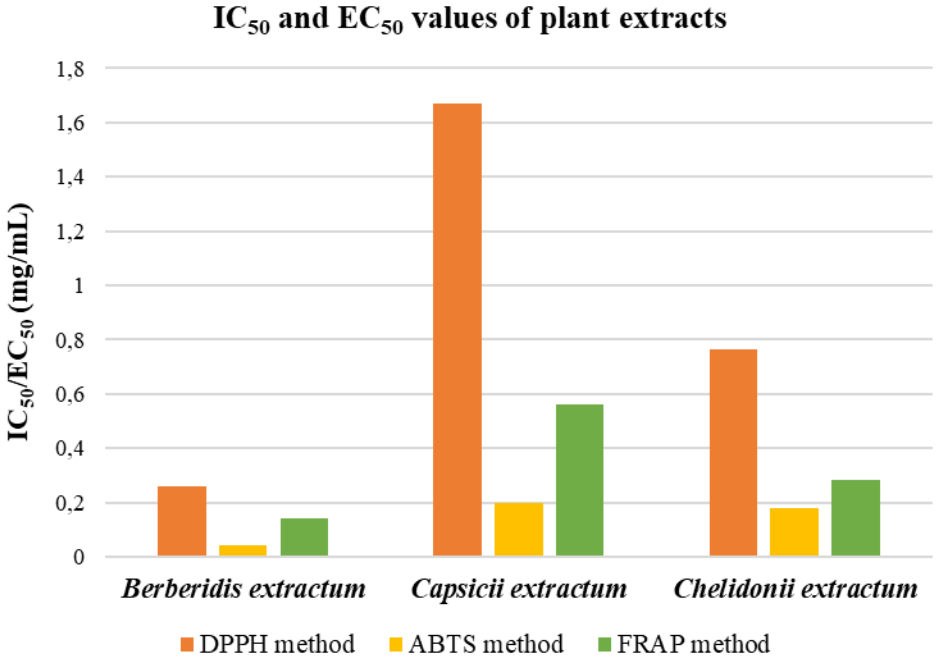

Figure 6.1. IC_{50} and EC_{50} values of plant extracts by the three methods

7. EVALUATION OF *IN VITRO* CYTOTOXICITY OF PLANT EXTRACTS ON HUMAN TUMOR CELL LINES AND NORMAL HUMAN CELLS

According to the World Health Organization, cancers of the liver, colon, breast, ovary and oral cavity had a high prevalence [1]. Cytotoxicity studies are preliminary for the identification of possible new therapies, and the MTS technique, an improved MTT method, is considered to be one of the most sensitive quantitative methods [16]. Determination of *in vitro* cytotoxicity was performed on six standardized tumor lines derived from colon tumors (LoVo, HT-29), breast tumor (MDA-MB-231), ovarian tumor (SK-OV-3), liver tumor (Hep G2), tongue tumor (PE/CA-PJ49) and, as control, on normal human umbilical cord endothelial cells (HUVEC). Their antiproliferative effect was compared with that of the reference cytostatics cisplatin (Cis-Pt), 5-fluorouracil (5-FU) and doxorubicin (DOX).

A concentration- and time-dependent antiproliferative effect was observed on tumor cell lines. The most marked antiproliferative action was observed on the breast adenocarcinoma-derived tumor cell line MDA-MB-231, which was below 80% at 48h, for all plant extracts and at all concentrations, except CME and CAE at 6.25 µg/mL. The hydroethanolic extract BVE induced, at 400 µg/mL and at 48h, a decrease in cell viabilities to 2.99% ± 0.44%, thus also the lowest percentage of cell viabilities in these experiments. Referring to the IC_{50} values, the berberine standard (BS) showed the strongest inhibitory effect on cell proliferation, IC_{50} = 20.22 µg/mL ± 1.28 µg/mL (48h), in contrast the IC_{50} value of the hydroethanolic extract BVE was 117.30 µg/mL ± 2.65 µg/mL. In the case of CME and standardized 2% hydroethanolic extract of greater celandine (CME2), at 24h and 48h, at concentrations of 400 µg/mL and 200 µg/mL, CME extract showed lower percentages of cell viabilities compared to CME2. Comparing the IC_{50} values of the two hydroethanolic extracts, in the first 24h, CME showed a stronger inhibitory effect (CME – 249.56 µg/mL ± 8.34 µg/mL; CME2 – 346.59 µg/mL ± 7.05 µg/mL), but at 48h the two values were close, with the standardized extract showing a slightly lower value (CME – 186.26 µg/mL ± 2.87 µg/mL; CME2 – 179.21 µg/mL ± 5.26 µg/mL). It can be concluded that in the case of the two plant products greater celandine (*Chelidonium majus* L.) and barberry (*Berberis vulgaris* L.), at the

highest concentrations tested, the lowest percentages of cell viability were obtained in the cells treated with total hydroethanol extracts and that the whole phytocomplex contributes to the antitumor action (Fig. 7.1.).

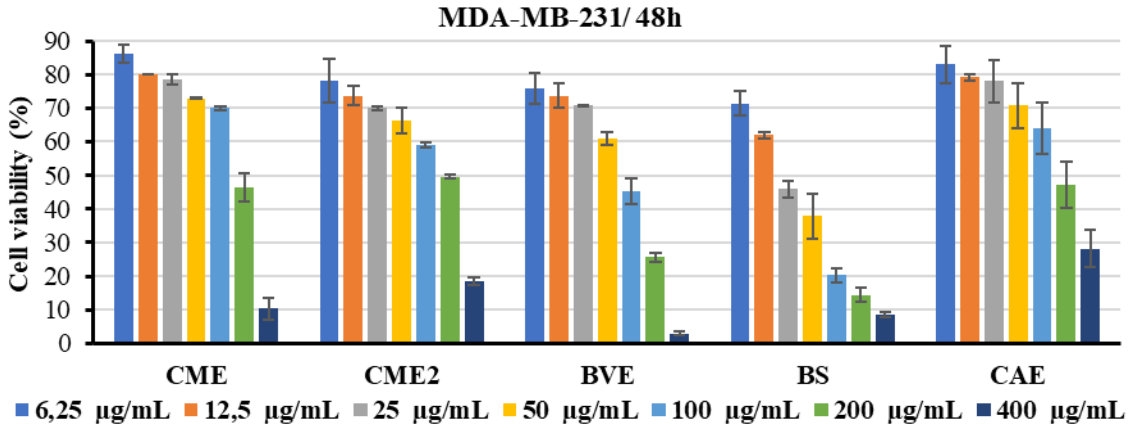

Figure 7.1. Cell viability (%) at 48h of MDA-MB-231 tumor cells treated with plant extracts

The second lowest percentage of cell viability was observed in the colon adenocarcinoma-derived cell line LoVo of BVE-treated cells at 48h and 400 µg/mL, i.e. 3.09% ± 2.66%. The lowest percentage of cell viability of the CME hydroethanolic extract was also obtained in this cell line – 3.63% ± 0.76% at 48h and 400 µg/mL. Also in this tumor line, total hydroethanolic extracts induced the highest cell apoptosis, emphasizing the role of the whole phytocomplex. With regard to IC_{50}, close values were obtained for all plant extracts, but the lowest was obtained for BS (136.78 µg/mL ± 1.97 µg/mL) (Fig. 7.2.).

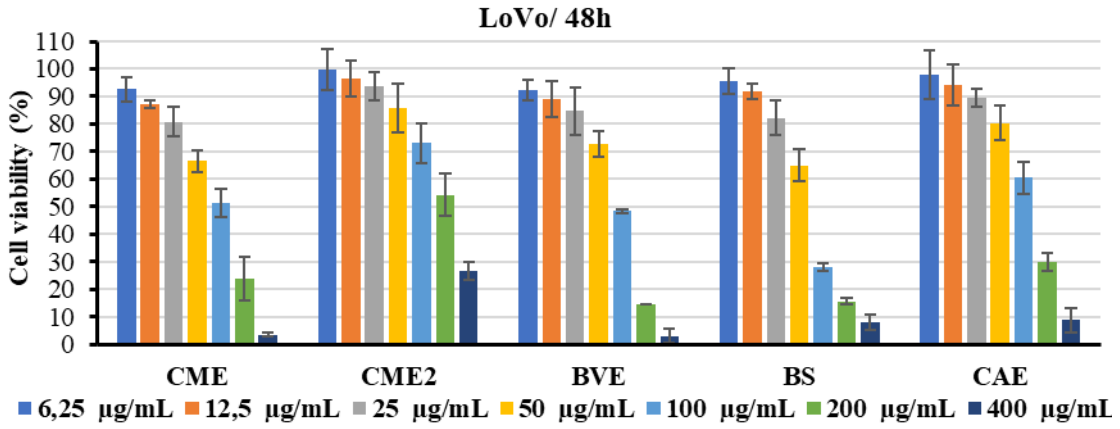

Figure 7.2. Cell viability (%) at 48h of LoVo tumor cells treated with plant extracts

In tongue tumor-derived cells PE/CA-PJ49, the berberine standard BS resulted in the highest inhibition of cell proliferation, viability of 3.95% ± 0.66% at the highest concentration and at 48h. The hydroethanolic extract BVE (4.70% ± 0.51%) showed a value close to the standard at the same concentration and time interval. In contrast, the hydroethanolic extracts CME and CME2 behaved differently in the sense that, at the highest concentration, CME had a more pronounced inhibitory effect on cell proliferation, decreasing cell viabilities to 15.81% ± 6.02% compared to 48.21% ± 1.62% for CME2. At the other concentrations of these extracts of greater celandine (*Chelidonium majus* L.), cell viabilities were closer in value. Comparing the IC_{50} values of the plant extracts, the 48h values for BVE and BS were close, 150.42 µg/mL ± 1.99 µg/mL for BVE and 101.74 µg/mL ± 1.01 µg/mL for BS, and among the hydroethanolic extracts CME, CME2 and CAE, the lowest value was CME, 198.67 µg/mL ± 5.02 µg/mL (Fig. 7.3.).

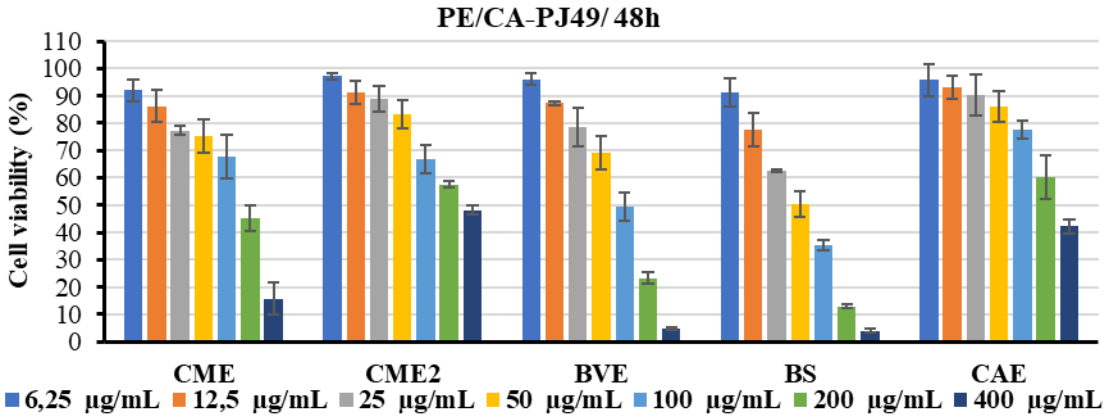

Figure 7.3. Cell viability (%) at 48h of PE/CA-PJ49 tumor cells treated with plant extracts

In the SK-OV-3 cell line, the lowest cell viabilities and IC_{50} values were obtained in cells treated with standard berberine BS 10.05% ± 0.80% at 48h and 400 µg/mL and 141.75 µg/mL ± 1.43 µg/mL, lower than those of the BVE hydroethanolic extract. In the case of the extracts of greater celandine, the hydroethanolic extract CME showed lower values of cell viabilities (32.72% ± 6.98%) than the standardized 2% extract CME2 (51.73% ± 0.27%), and IC_{50} could be calculated only for the total extract (282.29 µg/mL ± 7.31 µg/mL). The CAE extract (20.32% ± 1.92%) inhibited cell proliferation at a higher percentage compared to the two extracts of greater celandine and showed a pronounced inhibitory effect at 48h, within the first 24h all cell viabilities of cells treated with this extract showed viabilities higher than 95% (Fig. 7.4.).

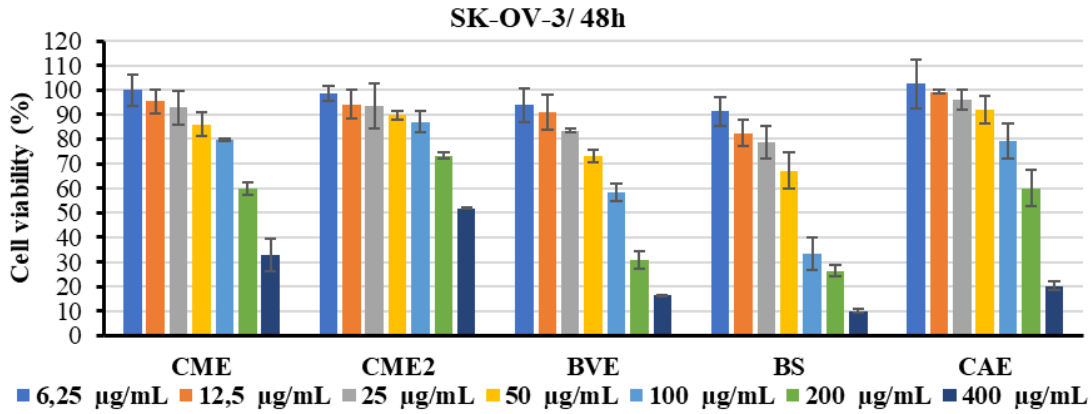

Figure 7.4. Cell viability (%) at 48h of SK-OV-3 tumor cells treated with plant extracts

The inhibitory effect of plant extracts was not so pronounced in the Hep G2 liver tumor-derived cell line, with cell viabilities in CME2 and CAE at both time points being greater than 80%. The lowest percentage was identified in cells treated with BS at 48h and 400 µg/mL, 21.82% ± 6.02%. The weakest inhibitory effect of plant extracts was on the tumor cell line HT-29 (Fig. 7.5. and Fig. 7.6.).

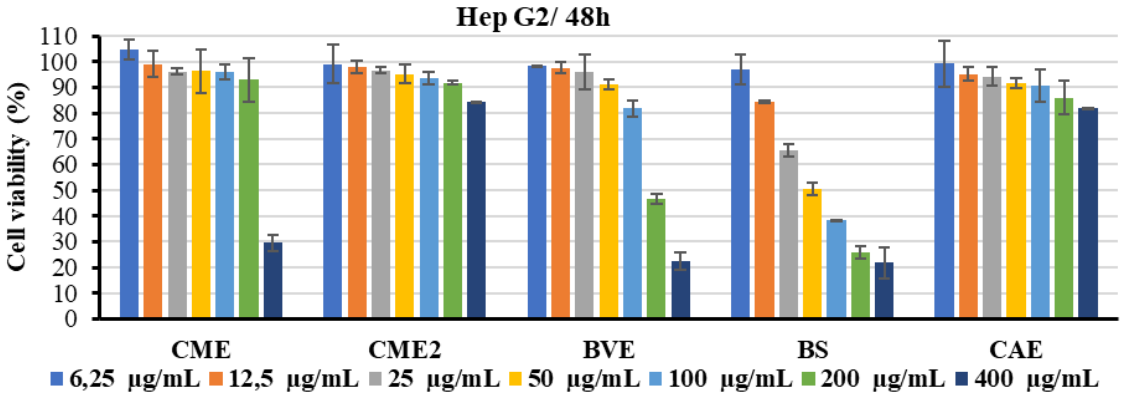

Figure 7.5. Cell viability (%) at 48h of Hep G2 tumor cells treated with the extracts

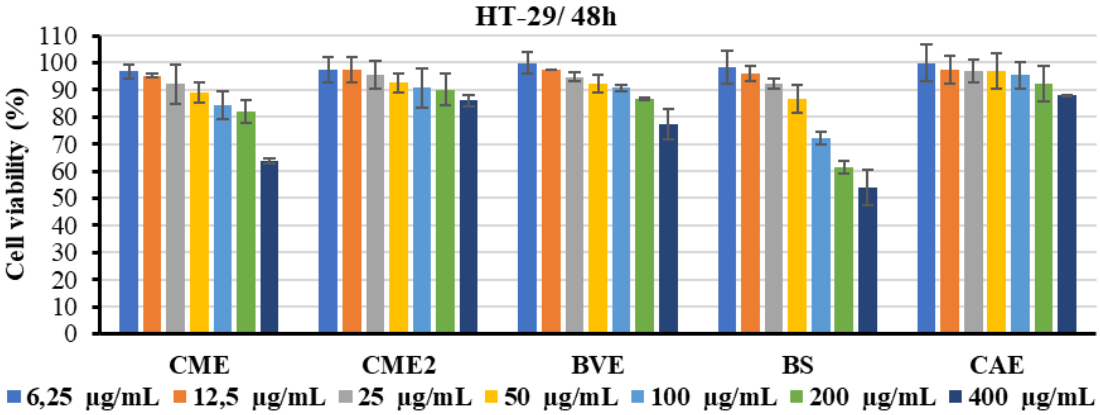

Figure 7.6. Cell viability (%) at 48h of HT-29 tumor cells treated with plant extracts

In human umbilical vein endothelium-derived cells HUVEC, their viability was not influenced by treatment with scaled dilutions of the plant extracts at any concentration or time interval, with a few exceptions, at 48h and 400 µg/mL concentration, BVE (75.48% ± 0.09%), BS (56.10% ± 0.09%) and CAE (52.81% ± 3.59%). Compared to the viabilities of tumor cells treated with these plant extracts, at the same concentration and time interval, their viabilities were lower, except for Hep G2 and HT-29 cells treated with CAE (Fig. 7.7.).

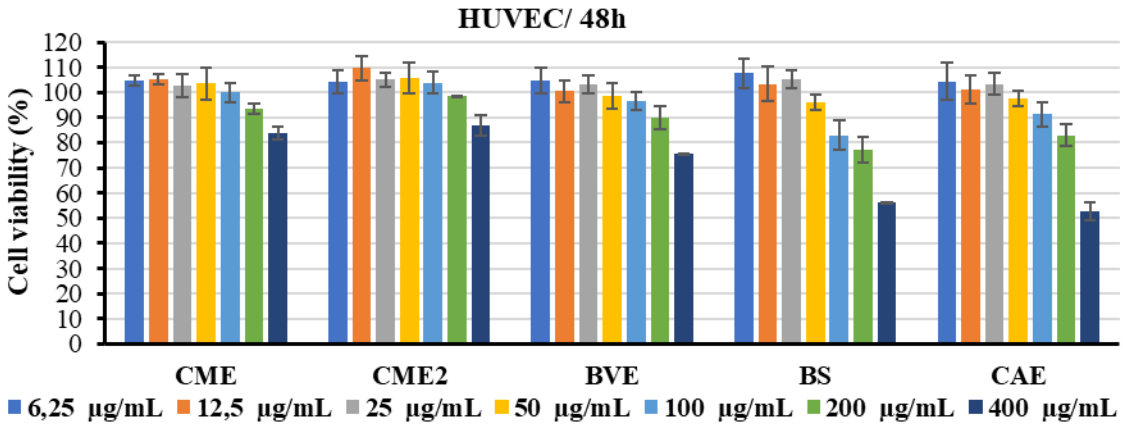

Figure 7.7. Cell viability (%) at 48h of HUVECs treated with plant extracts

Statistical interpretation performed for each plant extract, with regard to phytochemical composition, antioxidant action and antitumor potential, demonstrated a negative correlation between cell viabilities and these variable parameters. Thus, a direct proportionality was established between phytoconstituents, antiradical effect and antitumor properties. In addition, a positive correlation was demonstrated between the antiradical effect and phytochemical composition.

8. EVALUATION OF IN VIVO CYTOTOXICITY OF EXTRACTS IN *DAPHNIA MAGNA* AND *DAPHNIA PULEX* MODELS

Evaluation of *in vivo* cytotoxicity in invertebrates is one of the most accessible and widely used methods, with Daphnid determinations, in particular *Daphnia magna* and *Daphnia pulex*, being the most prominent. Daphnids are used in tests to assess the cytotoxicity of plant extracts, pollutants, drugs or dyes.

The toxicity of plant extracts and standards on the two crustaceans was dependent on exposure time. Of the daphnids studied, the hydroethanolic extract of chili pepper showed slightly higher toxicity to *Daphnia pulex* ($LC_{50, 48h}$ = 148.1 μg/mL) compared to *Daphnia magna* ($LC_{50, 48h}$ = 178.9 μg/mL). Both values were below 200 μg/mL, thus showing a medium toxicity of the extract, and the lethality curves showed a similar profile (Table VIII.1. and Table VIII.2.).

In the case of the hydroethanolic extract of barberry and the alkaloid berberine, *Daphnia magna* crustaceans showed higher sensitivity at both exposure ranges ($LC_{50, 24h, BVE, D. magna}$ = 30.5 μg/mL, $LC_{50, 24h, BVE, D. pulex}$ = 143.0 μg/mL, $LC_{50, 48h, BVE, D. magna}$ = 6.7 μg/mL, $LC_{50, 48h, BVE, D. pulex}$ = 37.4 μg/mL, $LC_{50, 24h, BS, D. magna}$ = 8,7 μg/mL, $LC_{50, 24h, BS, D. pulex}$ = ND μg/mL, $LC_{50, 48h, BS, D. magna}$ = 5,3 μg/mL, $LC_{50, 48h, BS, D. pulex}$ = 6,6 μg/mL). BS showed a more pronounced toxic effect compared to the hydroethanolic extract on the two crustaceans, but at 48h on *Daphnia magna* they showed close LC_{50} values, the effect being correlated with the exposure time in both cases (Table VIII.1. and Table VIII.2.).

Regarding CME and standardized CME2 hydroethanolic extract, crustaceans were more sensitive to the action of CME. Of the two invertebrate LC_{50}s were lower on *Daphnia pulex* at both time intervals ($LC_{50, 24h, CME, D. magna}$ = 982.0 μg/mL, $LC_{50, 24h, CME, D. pulex}$ = 680.6 μg/mL, $LC_{50, 48h, CME, D. magna}$ = 295.2 μg/mL, $LC_{50, 48h, CME, D. pulex}$ = 383.4 μg/mL). Comparing all plant extracts and standards, the lowest LC_{50} values were calculated for BVE and BS at 48h on *Daphnia magna* ($LC_{50, 48h, BVE, D. magna}$ = 6.7 μg/mL, $LC_{50, 48h, BS, D. magna}$ = 5.3 μg/mL). CAE and CME showed medium toxicity, with a more enhanced effect on Daphnia pulex (Table VIII.1. and Table VIII.2.).

Regarding the development of *Daphnia magna* embryos treated with the plant extracts and standards, the greatest morphological changes were observed in those treated with BVE, an overall inhibition of more than 80%. Compound eye formation was the main teratogenic effect observed when treated with CAE and CME. If in the case of CAE and CME, the teratogenic effect of the extracts could be explained due to the presence of alkaloids, effects comparable to the standards, in the case of BVE, this extract had a much more pronounced toxic effect compared to BS, thus indicating that the teratogenicity is due to the whole phytocomplex.

Table VIII.1. Values of lethal concentrations 50 (LC_{50}) and 95% confidence intervals (95%CI) of plant extracts and standards on *Daphnia magna* crustaceans

Extract	LC_{50}, 24h	95%CI, 24h	LC_{50}, 48h	95%CI, 24h
BVE	30.5 µg/mL	22.6–41.1 µg/mL	6.7 µg/mL	3.7–11.9 µg/mL
BS	8.7 µg/mL	6.5–11.8 µg/mL	~5.3 µg/mL	ND**
CAE	311.0 µg/mL	133.2–726.2 µg/mL	178.9 µg/mL	150.5–212.8 µg/mL
Capsaicin	ND*	ND*	ND*	ND*
CME	~ 982.0 µg/mL	ND**	295.2 µg/mL	216.8–402.0 µg/mL
CME2	ND*	ND*	ND*	ND*

ND = not determined, * = lethality < 10%, ** = wide range

Table VIII.2. Values of lethal concentrations 50 (LC_{50}) and 95% confidence intervals (95%CI) of plant extracts and standards on *Daphnia pulex* crustaceans

Extract	LC_{50}, 24h	95%CI, 24h	LC_{50}, 48h	95%CI, 24h
BVE	~143.0 µg/mL	ND**	37.4 µg/mL	28.8–48.7 µg/mL
BS	ND*	ND*	6.6 µg/mL	4.6–9.5 µg/mL
CAE	261.7 µg/mL	204.7–334.6 µg/mL	148.1 µg/mL	125.4–175.0 µg/mL
Capsaicin	ND*	ND*	ND*	ND*
CME	680.6 µg/mL	592.2–782.0 µg/mL	383.4 µg/mL	351.3–418.5 µg/mL
CME2	ND***	~ 1162 µg/mL	ND***	ND**

ND = not determined, * = lethality < 10%, ** = wide range, *** = lethality < 40%

FINAL CONCLUSIONS AND PERSONAL CONTRIBUTIONS

Conclusions

A significant number of new molecules derived from natural sources or their derivatives have been discovered and introduced into therapy, making phytocompounds an inexhaustible source of therapeutic solutions. Cancer is an intense preoccupation of many researchers, both in terms of the mechanisms of action involved and from the perspective of treatments, which are often quite difficult to tolerate, with multiple side effects, resistance and marked toxicity with long-term administration. In order to identify plant extracts with therapeutic potential, the proposed scientific objectives, which consisted in the selection of plant raw materials (*Berberidis cortex*, *Capsicii fructus* and *Chelidonii herba*), determination of the methodology for obtaining plant extracts and selection of the optimal solvent for the extraction of the active principles of interest, determination of the phytochemical profile of dried plant extracts by

spectrophotometric assay and qualitative and quantitative high-performance chromatographic analysis, determination of the *in vitro* antioxidant profile of plant extracts by several standardized methods, determination of *in vitro* cytotoxicity on six human tumour cell lines and on normal human cells, and determination of *in vivo* toxicity on invertebrate species *Daphnia magna* and *Daphnia pulex*, I consider them fulfilled.

Personal contributions

In chapter 4, we presented the extraction of the extracts by lyophilization using a 50% aqueous ethanolic ethanolic solution as solvent for extraction. The extraction yields were good (16.35% for *Berberidis extractum*, 7.65% for *Capsicii extractum* and 18.45% for *Chelidonii extractum*) and the qualitative analysis of the main classes of active principles supported the choice of solvent. The highest concentrations of phenolic acids (3.3886 ± 0.3481 g chlorogenic acid/100 g extract) and total polyphenols (17.6780 ± 3.9320 g tannic acid/100 g extract) were determined in the barberry extract, and the greater celandine extract was the richest in flavones (1.6067 ± 0.0651 g rutozide/100 g extract).

In chapter 5, a total of 91 polyphenolic compounds and 9 alkaloids were identified in plant extracts by UHPLC-HRMS/MS and HPLC-DAD. In *Berberidis extractum* 39 polyphenolic compounds and berberine, in *Capsicii extractum* 70 polyphenolic compounds and 8 capsaicin derivatives, and in *Chelidonii extractum* 59 polyphenolic compounds were determined. Among the compounds identified were gallic acid, apigenin, quercitin, kaempferol known in the literature for their antitumor properties. Quantitative analysis revealed a concentration of polyphenols of 1091.68 µg/g (barberry), 2459.39 µg/g (chili pepper) and 3815.50 µg/g (greater celandine).

In chapter 6, the evaluation of the antioxidant properties was performed by three methods (DPPH, ABTS, FRAP). The extract of barberry was distinguished with the most pronounced antioxidant properties: DPPH (IC_{50} = 0.2610 mg/mL), ABTS (IC_{50} = 0.0442 mg/mL) and FRAP (EC_{50} = 0.1398 mg/mL). Extracts of greater celandine ($IC_{50,\ DPPH}$ = 0.7643 mg/mL, $IC_{50,\ ABTS}$ = 0.1790 mg/mL and $EC_{50,\ FRAP}$ = 0.2814 mg/mL) and chili pepper ($IC_{50,\ DPPH}$ = 1.6699 mg/mL, $IC_{50,\ ABTS}$ = 0.2006 mg/mL and $EC_{50,\ FRAP}$ = 0.5613 mg/mL) showed a medium effect.

In chapter 7, the results demonstrated a concentration and exposure time dependent inhibitory effect on cell proliferation. The most pronounced antiproliferative effect was observed on the breast adenocarcinoma-derived tumor cell line MDA-MB-231, these were

below 80% at 48h for all plant extracts. Also, on the same cell line, the hydroethanolic extract of barberry at a concentration of 400 µg/mL and at 48h inhibited cell proliferation most markedly, decreasing the cell viabilities to 2.99% ± 0.44%, the lowest viabilities recorded in these experiments (IC_{50} = 117.30 µg/mL ± 2.65 µg/mL). The second lowest percentage of cell viability, 3.09% ± 2.66% (IC_{50} = 146.32 µg/mL ± 3.45 µg/mL), was obtained with *Berberidis extractum* at 400 µg/mL and 48h on the colon adenocarcinoma-derived cell line LoVo. The extract of greater celandine, at the same concentration and time, showed a close value, 3.63% ± 0.76% (IC_{50} = 149.12 µg/mL ± 6.12 µg/mL), being also the lowest calculated viability for it (*Chelidonii extractum*) among all the lines studied. Also, and in cells derived from tongue tumor PE/CA-PJ49, the hydroethanolic extract of barberry inhibited cell proliferation similarly to the other lines mentioned above, decreasing viability up to 4.70% ± 0.51% (IC_{50} = 150.42 µg/mL ± 1.99 µg/mL) at 400 µg/mL and 48h. The hydroethanolic extract of greater celandine, at the highest concentration, had a pronounced cell proliferation inhibitory effect, decreasing cell viabilities to 15.81% ± 6.02% (IC_{50} = 198.67 µg/mL ± 5.02 µg/mL). In the SK-OV-3 cell line, *Berberidis extractum* (16.29% ± 0.37%) and *Capsicum extractum* (20.32% ± 1.92%) at 48h and 400 µg/mL showed close cell viabilities. For chili pepper extract a marked enhancement of proliferation inhibition was observed by prolongation of the treatment time, in the first 24h cell viabilities were above 95% at all tested concentrations. The hydroethanolic extract of greater celandine had a lower inhibitory effect on cell proliferation (cell viabilities 32.72% ± 6.98%). The inhibitory effect of plant extracts was not so pronounced in the Hep G2 liver tumor derived cell line, the extract of barberry showed, at 48h and 400 µg/mL, a cell viabilities value of 22.62% ± 3.36%, and that of greater celandine of 29.61% ± 3.09%. Plant extracts did not show a good antiproliferative effect on the tumor cell line derived from colon tumor HT-29, the viabilities of the treated cells were above 50% at 24h and 48h at all tested concentrations. In the case of normal cells derived from human umbilical vein endothelium HUVEC, viabilities were generally not influenced by treatment with scaled dilutions of the plant extracts, illustrating their selectivity. The antiproliferative action of the extracts was compared with that of reference drugs (cisplatin, doxorubicin and 5-fluorouracil), but also with berberine sulphate or commercially available standardized 2% extract of greater celandine. The results showed, particularly on the MDA-MB-231 and LoVo cell lines, a more promising action of the hydroethanolic extracts compared to the references, thus indicating a synergistic effect of all phytoconstituents.

Statistical interpretation performed for each plant extract, with respect to phytochemical composition (total polyphenols, flavones and phenolcarboxylic acids), antioxidant action (DPPH, ABTS, FRAP methods) and antitumor action, demonstrated a negative correlation between cell viabilities and these variable parameters. Thus, a direct proportionality was established between phytoconstituents, antiradical effect and antitumor properties. In addition, a positive correlation was demonstrated between the antiradical effect and phytochemical composition.

In chapter 8, the sensitivity of daphnids to the action of plant extracts was concentration and exposure time dependent. The values of lethal concentrations 50 were lower on *Daphnia pulex*, with the exception of the hydroethanolic extract of barberry which showed the most pronounced toxic effect of the extracts studied on both invertebrates ($LC_{50,\ 48h,\ BVE,\ D.\ magna}$ = 6.7 μg/mL, $LC_{50,\ 48h,\ BVE,\ D.\ pulex}$ = 37.4 μg/mL). Toxicity assessment on *Daphnia magna* embryos identified, for all extracts, difficulties in the formation of the compound eye, and an overall inhibition of more than 80% of embryo development was observed for those treated with *Berberidis extractum*. Berberine sulfate did not have this marked retardation effect at the same concentration range, showing a possible synergism of the phytocomplex.

Outlook

In the future I intend to study the antitumor properties of these extracts on other tumor cell lines, evaluation of antiproliferative mechanisms, *in vivo* studies on murine model, association of the studied plant products in order to formulate a phytopreparate, but also to extend the studies on other plant products from the autochthonous flora.

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1. Ivan IM, Popovici V, Chițescu CL, Popescu L, Luță EA, Ilie EI, Brașoveanu LI, Hotnog CM, Olaru OT, Nițulescu GM, Boscencu R, Gîrd CE. Phytochemical profile, antioxidant and cytotoxic potential of *Capsicum annuum* (L.) dry hydro-ethanolic extract. *Pharmaceutics*, 16 (2), 245, 2024. IF_{2023} = 4,9; https://doi.org/10.3390/pharmaceutics16020245 (chapters 4, 5, 6, 7 and 8).

2. Ivan IM, Olaru OT, Popovici V, Chițescu CL, Popescu L, Luță EA, Ilie EI, Brașoveanu LI, Hotnog CM, Nițulescu GM, Boscencu R, Gîrd CE. Antioxidant and cytotoxic properties of *Berberis vulgaris* (L.) stem bark dry extract. *Molecules*, 29, 2053, 2024. IF_{2023} = 4,2; https://doi.org/10.3390/molecules29092053 (chapters 4, 5, 6, 7 and 8).

Participation in projects

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