

Biochemical profile, selective cytotoxicity and molecular effects of *Calendula officinalis* extracts on breast cancer cell lines

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Abstract

Calendula officinalis extracts have been known to possess anti-tumor properties, but questions regarding their mechanisms of action still need to be answered. Therefore, the present study aims to investigate the selective cytotoxicity, the biochemical profile and the corresponding molecular effects of two extracts of *C. officinalis*: flowers and leaves, against several breast cancer cell lines *in vitro*. Dry flowers and leaves were subjected to ultrasonication assisted extraction in methanol 70%. The phenolic and volatile profiles of the extracts, determined by HPLC-MS and nontargeted GC-MS, revealed high levels of specific phenolic acids, flavonols and coumarin and several volatile compounds, including mono- and sesquiterpenes, ketones, aldehydes and esters. Both extracts proved to possess selective cytotoxic activities against tumor cells in comparison to healthy endothelial cells, according to the MTT assay. The flower extract was superior in terms of both cytotoxicity and selectivity when compared to the leaf extract, in accordance to their biochemical profiles. The gene expression pattern for 10 genes of interest was evaluated by RT-qPCR. The expression level of several genes involved in apoptosis (*BCL2*, *BAX*, *BBC3*, *ZMAT3*), and cell cycle progression (*NFkB*, *CCND1*, *STAT3*) was modulated by the treatment with both extracts. Therefore, *C. officinalis* extracts proved to be rich in compounds characterized by cancer-related cytotoxicity and are capable of inducing selective cytotoxicity on breast cancer cell lines.

Keywords: apoptosis; breast cancer; *Calendula officinalis*; phenolic constituents; proliferation; selective cytotoxicity; volatile constituents

Introduction

Plants are considered an important resource for medicine in general, and for oncology specifically. They are a prime source of natural compounds, especially secondary metabolites, with wide applications in the

treatment of different affections and diseases. Between 1940 and 2014, 49% of all approved drugs for cancer chemotherapy were derived from natural products (Newman and Cragg, 2016). However, only 10% of all plant species were tested for their pharmacological proprieties (Iqbal *et al.*, 2017), suggesting a great potential for this research area. Besides using plant-derived compounds in current clinical settings as chemotherapeutic drugs, plant extracts, formulations and dietary supplements are also used by cancer patients as complementary and alternative medicine (CAM) products. The overall prevalence of CAM use by cancer patients has increased in the past decades, with 49% of patients using it after the year 2000 (Horneber *et al.*, 2012). Among CAM treatments, herbal medicine is often found to be the most common therapy (Olaku and White, 2011).

Breast cancer has the highest incidence among women worldwide and is the fifth cause of cancer death overall, with a mortality of 0.62 million in 2018 (Bray *et al.*, 2018). Plant-derived drugs, especially taxanes, are often part of the chemotherapy regimens for breast cancer patients. Taxanes improve chemotherapy efficacy, independently of tumor size, grade or nodal status, while regimens based on anthracyclines and taxanes reduce breast cancer mortality by 30% (Peto *et al.*, 2012). Furthermore, plant-derived compounds are widely used by breast cancer patients as CAM products. Even though the effectiveness and safety of CAM products are not always proven (Hu *et al.*, 2005), 40-80% of patients with breast cancer report using CAM for both treating their disease and improving the treatment-related symptoms (Saghatchian *et al.*, 2014). Despite the progress made in breast cancer management in the past decades, therapeutic failures are still common in the current clinical practice. The therapeutic options for advanced stage breast carcinoma and specific subtypes like TNBC (Triple Negative Breast Cancer) are still limited and need to be diversified. In addition, taking into consideration that herbal formulations are increasingly being used by breast cancer patients during and after standard cancer therapies, assessment of both their safety and effectiveness is needed in order to establish their potential in cancer management.

Calendula officinalis L (Asteraceae), known as the pot marigold, is a plant widely used in traditional medicine. Its flowers and leaves are used in the treatment of poorly healing wounds, minor bruises, rashes and burns and also in the discomfort alleviation caused by stomach ulcers or inflammation of the oral and pharyngeal mucosa (Mehta *et al.*, 2012). Several pharmacological activities were proven for *C. officinalis* flower/leaf herbal preparations, including antioxidant (Frankic *et al.*, 2009), immunostimulant (Varlijen, 1989), wound healing activity (Parente *et al.*, 2012), antibacterial (Efstratiou *et al.*, 2012) antifungal (Gazim *et al.*, 2008) and anti-viral activity (Bogdanova *et al.*, 1970).

C. officinalis extracts of flowers, leaves and roots also possess anti-tumor activities, emphasizing their potential in cancer management (Cruceriu *et al.*, 2018). Several studies report cytotoxic and cytostatic effects in different cancer cell lines *in vitro* (Matysik *et al.*, 2005; Jimenez-Medina *et al.*, 2006; dos Santos Junior *et al.*, 2010; Wegiera *et al.*, 2012; Matic *et al.*, 2013; Mouhid *et al.*, 2018), while the flower extracts anti-tumor (Jimenez-Medina *et al.*, 2006), chemoprotective (Barajas-Farias *et al.*, 2006; Ali *et al.*, 2014;) and antimetastatic (Preethi *et al.*, 2010) activities were demonstrated *in vivo*, in animal models. However, several questions regarding the cancer-related cytotoxicity of *C. officinalis* extracts still need to be answered. First of all, the constituents that are responsible for the cytotoxic activity of *C. officinalis*' whole extracts need to be identified. Secondly, the selectivity towards cancer cells compared to healthy cells in regard to the cytotoxic activity needs to be assessed. Thirdly, the molecular mechanisms underlying the cell death and cell cycle arrest effects induced by *C. officinalis* extracts need to be elucidated.

Materials and Methods

Plant material and culture conditions

C. officinalis seeds were collected from Covasna County, Romania, in October 2016. Seeds were germinated and cultivated *ex vitro* in laboratory conditions (temperature: 21 °C; photoperiod: 16 h/ 8 h, watering: three times a week) for 12 weeks. Authentication of the plant species was done at the CL Herbarium, Babes-Bolyai University, Romania (specimen voucher 668431). Plant flowers and leaves were collected separately, dried in a dark room and powdered for further analysis.

Cell lines and culture conditions

Four human cell lines were used in this study: MCF7 (luminal breast cancer cells), MDA-MB-231, Hs578T (triple negative breast cancer cells) and HUVEC (human umbilical vein endothelial cells); cell lines were obtained from the European Collection of Authenticated Cell Cultures. MCF7 cells were cultured in standard conditions in MEM, MDA-MB-231 in RPMI-1640, Hs578T in DMEM and HUVEC in ECM growth media, supplemented with 10% fetal bovine serum (FBS), 1% glutamine and 1% penicillin-streptomycin. Additionally, the MCF7 media was supplemented with 1% non-essential amino-acids (NEAA), while 0.01 mg/ml insulin was added to the Hs578T media. All cell lines were maintained at 37 °C in a humidified atmosphere containing 5% CO₂. All cell culture reagents were obtained from Sigma-Aldrich (München, Germany).

Preparation of plant extracts

Dried powdered plant material (5 g) was subjected to extraction in methanol 70% at a liquid-to-solid ratio of 10:1 ml/g, by an ultrasound-assisted extraction (UAE) procedure. Briefly, plant material in extraction solvent was sonicated at a fixed ultrasonic power of 750 W, at 40 °C, in three successive 10 min cycles (Sonics Vibra-cell VC750). The obtained suspension was left for an additional 24 h period, in the dark, at room temperature for maceration. This homogenate was then centrifuged at 2000 rpm for 5 min and the supernatant was collected and filtered through a 0.45 µm Millipore. The obtained crude methanolic extracts were concentrated to dryness under reduced pressure, at 40 °C, using a vacuum rotary evaporator (Heidolph Laborota 4000 Efficient). Half of the dry residue obtained for each sample (flowers/ leaves) was redissolved in methanol 70% for future biochemical analysis, while the other half was homogenized in dimethyl sulfoxide (DMSO) for *in vitro* experiments, both at a concentration of 100 mg/ml (stock solution). All extracts were filtered once again through a 0.22 µm Millipore, before testing.

HPLC-PDA/-ESI+-MS identification and quantification of phenolic compounds

HPLC analysis of the samples was performed on an Agilent 1200 system equipped with a binary pump delivery system LC-20 AT (Prominence), a degasser DGU-20 A3 (Prominence), a diode array SPD-M20 UV-VIS detector (DAD). Separation of the compounds was achieved on an Eclipse XDB C18 column (4 µm, 4.6 x 150 mm). The mobile phases consisted of solvent A - bidistilled water and 0.1% acetic acid/acetonitrile (99/1) v/v, while solvent B was acetonitrile and acetic acid 0.1%. The gradient elution system was programmed as following: 0-2 min, isocratic with 5% (v/v) eluent B; 2-18 min, linear gradient from 5% to 40% (v/v) eluent B; 18-20 min, linear gradient from 40% to 90% (v/v) eluent B; 20-24 min, isocratic on 90% (v/v) eluent B; 24-25 min, linear gradient from 90% to 5% (v/v) eluent B; 25-30 min, isocratic on 5% (v/v) eluent B. Flow rate was set to 0.5 ml/min and column temperature was maintained at 25 °C. The chromatograms were monitored at 340 nm. Identification of the compounds and peak assignments were done using their retention time, UV-VIS and mass spectra, and also comparing with commercial standards (chlorogenic acid, caffeic acid, quercetin-rutinoside, quercetin-glucoside, ellagic acid, myricetin) and previously published literature. For the mass spectrometric measurements, a single quadrupole 6110 mass spectrometer (Agilent Technologies) equipped

with an ESI probe was used. Measurements were performed in the positive mode with an ion spray voltage of 3000V, and a capillary temperature of 350 °C. Data were collected in full scan mode within the range 280 to 1000 m/z. For quantification of phenolic acids and flavonoids, standard curves of chlorogenic acid, gallic acid and rutin were used, respectively.

Non-targeted ITEX/GC-MS headspace analysis of volatile compounds

The volatile compounds from the headspace of the methanolic extracts of the samples were determined using the in-tube extraction technique (ITEX) coupled with gas-chromatography-mass spectrometry. A volume of each extract was put in a headspace vial and incubated at 60 °C for 20 min. After the incubation, the extraction of the volatile compounds from the headspace phase was performed using a Combi PAL AOC-5000 autosampler (CTC Analytics) with a headspace syringe ITEX-II equipped with a microtrap (ITEX-2TrapTXTA, Tenax TA 80/100 mesh). The volatiles were then directly thermally desorbed into the chromatograph injector. The analysis was carried out on a GCMS QP-2010 (Shimadzu Scientific Instruments) model gas chromatograph - mass spectrometer. The volatile compounds were separated on a Zebtron ZB-5 ms capillary column of 30 m × 0.25 mm i.d and 0.25 µm film thickness. The initial column temperature was set at 30 °C (hold for 2 min), increased to 140 °C with a rate of 5 °C/min and then increased to 270 °C and hold for 5 °C. The carrier gas was helium with a flow rate of 1 mL/min and a split ratio of 1:20. The injector, ion-source and interface temperatures were set at 250 °C. The MS detection used for the qualitative analysis was performed on a quadrupole mass spectrometer operating in the electron ionization mode (EI) at 70 eV. The volatile compounds were recorded in total ion chromatogram (TIC) in full scan (40-450 m/z).

The volatile compounds were tentatively identified by first comparing the mass spectrometric information of each chromatographic peak to NIST27 and NIST147 mass spectra libraries (considering a minimum similarity of 85%) and then whenever possible by comparison with retention indices drawn from www.pherobase.com or www.flavornet.org (for columns with a similar stationary phase to the ZB-5ms column). This technique offers a qualitative assessment of volatile compounds, so the relative percentage of each compound was estimated as a fraction of its integrated ion area from total ion chromatograms (TIC) area (100%).

Selective cytotoxicity assay

The cytotoxic activity of both *C. officinalis* extracts was evaluated on all four cell lines by the MTT assay (Sigma-Aldrich München, Germany), according to the manufacturer protocol. Briefly, cells were seeded in 96-well plates at a density of 2×10^4 cells/well. After 24 h, the media was replaced and the extracts were added in nine successive concentrations, varying from 50-1000 µg/ml, in six technical replicates. After a 48h-period of incubation with the extracts, the supernatant was removed, 100 µl of the MTT solution (1 mg/ml) was added onto the cells and left for incubation for 1 h. Subsequently, the MTT solution was removed, 150 µl DMSO was added on cells to solubilize the formed formazan and the absorbance was measured at a wavelength of 570 nm, using a microplate spectrophotometer (Synergy HTX, BioTek, Winooski, VT, USA). All experiments were performed in triplicate.

Cell viability was calculated as the fraction of viable cells compared with the untreated control, based on the absorbance values. The half maximal inhibitory concentration (IC₅₀) values for each extract on each cell line were calculated in GraphPad Prism Software Version 5. The selectivity coefficient in the cytotoxic action for each extract was calculated as the ratio between the IC₅₀ value on the healthy cell line (HUVEC) and the IC₅₀ value obtained for each cancer cell line. HUVEC cells were chosen as a tumor unrelated control, because they are one of the first cell types that come in contact with the anti-tumor compounds administrated *in vivo*, by forming the inner layer of the vasculature.

Separate experiments were conducted in the same conditions to evaluate the cytotoxicity of the DMSO-vehicle on all four cell lines. The final DMSO concentration did not exceed 1% on the extract-treated cells and no DMSO-related cytotoxic effects were observed up to this concentration (data not shown).

Expression analysis of apoptosis- and cell cycle-related genes

The molecular effects of both extracts on MCF7 breast cancer cell line was evaluated by RT-qPCR. Ten well-known genes involved in apoptosis and cell cycle progression were selected for gene expression analysis: BCL2, BAX, BBC3, TP53, CASP3, CASP7, CCND1, NFkB, STAT3 and ZMAT3. Cells were grown in 12-well plates at an initial density of 2×10^5 cells/well, for 24 h. Each extract was added at the IC₅₀ concentration in technical duplicates, and the cells were further incubated with the treatments for 48 h. Cells were lysed with TriReagent solution (Sigma-Aldrich München, Germany) and total RNA was extracted using the phenol-chloroform method. Total RNA quality and quantity were determined using the Bioanalyzer 2100 (Agilent Technologies). For each sample, 500 ng of RNA were used for the cDNA synthesis step (High-Capacity cDNA Reverse Transcription Kit, Applied BioSystems). The PCR reaction mixture (10 μ L) contained 2 μ L LightCycler TaqMan Master (Roche), the specific primer pair at a concentration of 0.5 μ M each, the UPL probe (Roche) at a concentration of 0.2 μ M and 2.5 μ L diluted cDNA (1:10). The primers and the UPL probes used for amplification are presented in Table 1. The RT-qPCR reaction was carried out on a LightCycler 480 (Roche) device. The following cycling conditions were used: denaturation at 95°C for 10 min., amplification including 40 cycles of 10 seconds at 95 °C, 20 seconds at 55 °C and 1 second at 72 °C, final cooling at 40°C for 30 seconds. Each amplification was carried out in technical duplicates. The gene expression (fold change) was calculated with the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001), the housekeeping gene used for normalization being RN18S1.

Table 1. The primers sequences and the UPL probes used for RT-qPCR

| Gene | Primers (Sequence 5'-3') | UPL probe |
|-------|--|-----------|
| BCL2 | F: ttgacagaggatcatgctgtactt R: atctttatttcatgaggcactt | #6 |
| BAX | F: caagaccagggtggttgg R: cactccgccacaagat | #55 |
| BBC3 | F: gacctcaacgcacagtacga R: gagattgtacaggaccctcca | #68 |
| TP53 | F: ctttgaggcggtgtttgtg R: ccctttcttgcggagattc | #6 |
| CASP3 | F: tggaaattgatgcgtgatgtt R: tccaaaaattattccttcttcacc | #68 |
| CASP7 | F: cttggaagagcaagggacag R: ttatgggccaggcttacatc | #2 |
| CCND1 | F: gctgtgcatctacaccgaca R: ttgagcttgttaccaggag | #17 |
| NFkB | F: cagtgaagaccacctctcagg R: tcactgtctcggagctcgt | #16 |
| STAT3 | F: cctctgccggagaacagt R: cattggaagctgtcactgtag | #1 |
| ZMAT3 | F: ccaggaagaagggaatgag R: gcaagagctgcaacataacaat | #2 |

Results

Preparation of plant extracts

Leaves and flowers of *C. officinalis* represented the plant material subjected for extraction. The ultrasound-assisted extraction (UAE) technique was chosen as the extraction method, due to its proved effectiveness in bioactive compounds extraction from plants, with better extraction yields in comparison to classical methods (Chemat *et al.*, 2017). The solvent used for extraction was methanol 70%, an organic, polar solvent capable of extracting a wide variety of phytoconstituents with high efficiency (Sulaiman *et al.*, 2011). Furthermore, methanol proved to extract natural compounds with better yields in comparison to other solvents, such as ethanol, isopropanol (Butnariu and Coradini, 2012), distilled water, acetone, chloroform, ethyl acetate or hexane (Dhawan and Gupta, 2017). The total extraction yields for both extracts are presented in Table 2.

Table 2. Total extraction yields for both *C. officinalis* extracts produced by UAE technique

| Extract | Input - Plant material DW (g) | Output - Extracted powder DW (g) | Extraction yield (%) |
|-------------------------------|-------------------------------|----------------------------------|----------------------|
| <i>C. officinalis</i> flowers | 5 g | 1.205 g | 24.1% |
| <i>C. officinalis</i> leaves | 5 g | 1.600 g | 32% |

Phenolic profile of the C. officinalis extracts

Phenolic compounds in both *C. officinalis* extracts were identified by HPLC-PDA/ESI+-MS and further quantified using standard curves. Fourteen different phenolic compounds were identified in the *C. officinalis* flower extract, while only twelve were found in the leaf extract (Figure 1; Table 3). Both extracts were characterized by the presence of flavonols (68.5% in the flower extract; 59.8% in the leaf extract), quercetin and isorhamnetin derivatives being identified in both extracts. Phenolic acids, namely hydroxybenzoic and hydroxycinnamic acids (25.1% in the flower extract; 35.6% in the leaf extract) and coumarins (6.4% in the flower extract; 4.6% in the leaf extract) were also present in the analyzed extracts (Table 3).

The major constituents in the flower extract were identified as chlorogenic acid (peak 3; [M+H]⁺, m/z=355) among the phenolic acids, and quercetin-3-O-glucosyl-rhamnosyl-glucoside (peak 5; [M+H]⁺, m/z=773), isorhamnetin-3-O-galactoside (peak 7; [M+H]⁺, m/z=478), isorhamnetin-3-O-glucosyl-rhamnoside (peak 10; [M+H]⁺, m/z=624) and isorhamnetin-7-O-rhamnoside (peak 12; [M+H]⁺, m/z=479) from the flavonol fraction. All these compounds were found in concentrations higher than 1 mg/100 mg extract (DW) in the flowers methanolic extract. The major phenolic constituents of the leaf extracts were the dihydroxybenzoic acid (peak 1; [M+H]⁺, m/z=156), isoquercetin (peak 8; [M+H]⁺, m/z=465) and isorhamnetin-3-O-glucosyl-rhamnoside (peak 10; [M+H]⁺, m/z=624), all being found in concentrations higher than 0.5 mg/100 mg extract (DW).

Comparing the phenolic composition of the two *C. officinalis* extracts, several relevant differences must be highlighted as follow: the hydroxybenzoic acids are the only class of compounds that were found in higher concentrations in the leaf extract, with 57% more than in the flower extract. On the other hand, the hydroxycinnamic acids had more than twice the concentrations in the flower extract, with a difference of 139% in comparison to the leaf extract. The flower extract proved to be far superior in the flavonols quantities as well, having almost three times higher concentrations when compared to the leaf extract (difference of 188%). The differences found in the flavonols content are mainly due to the isorhamnetin derivatives quantities, the quercetin derivatives being found in approximately similar concentrations in both extracts. Furthermore, isorhamnetin and isorhamnetin-3-O-glucoside, two of the flavonol constituents, were only detected and quantified in the flower extract of *C. officinalis*. Regarding coumarins, scopoletin-7-O-glucoside had a more

than three times higher concentration in the flower extract in comparison with the leaf extract (difference of 244%).

Table 3. Identification and quantification of phenolic compounds in the extracts of *C. officinalis* leaves and flowers. R_t – retention time; [M+H]⁺ – molecular ion; UV λ_{max} – wavelengths of maximum absorption in the visible region

| Class of compounds | Peak No. | R _t (min) | [M+H] ⁺ (m/z) | UV λ _{max} (nm) | Compound | Quantification (µg/mL extract) | |
|-----------------------------|-----------------------|----------------------|--------------------------|--------------------------|--|--------------------------------|------------------------------|
| | | | | | | <i>C. officinalis</i> flowers | <i>C. officinalis</i> leaves |
| Hydroxybenzoic acids (BA) | 1 | 3.05 | 156, 139 | 240 | Dihydroxybenzoic acid | 366.89 | 576.00 |
| | Total BA ¹ | | | | | | 366.89 |
| Hydroxycinnamic acids (HBA) | 2 | 9.87 | 355 | 320 | 3-Caffeoylquinic acid (Neochlorogenic acid) | 458.61 | 321.11 |
| | 3 | 11.85 | 355 | 320 | 5-Caffeoylquinic acid (Chlorogenic acid) | 1261.26 | 386.57 |
| | 11 | 17.00 | 517 | 330 | 3,5 Dicafeoylquinic acid (Isochlorogenic acid A) | 842.42 | 365.29 |
| Total HBA ² | | | | | | 2562.29 | 1072.97 |
| Flavonols (FL) | 4 | 13.93 | 757, 303 | 360, 260 | Q-3-O-rhamnosyl-rhamnosyl-glucoside | 401.66 | 103.05 |
| | 5 | 14.68 | 773, 303 | 355, 255 | Q-3-O-glucosyl-rhamnosyl-glucoside | 1165.46 | 423.18 |
| | 6 | 15.34 | 611, 303 | 360, 250 | Q-3-O-rutinoside (Rutin) | 374.52 | 402.98 |
| | 7 | 15.77 | 478, 317 | 350, 250 | I-3-O-galactoside | 1273.79 | 106.05 |
| | 8 | 16.00 | 465, 303 | 361, 251 | Q-3-O-glucoside (Isoquercetin) | 243.65 | 591.78 |
| | 9 | 16.15 | 478, 317 | 350, 250 | I-3-O-glucoside | 634.70 | nd |
| | 10 | 16.53 | 624, 317 | 350, 260 | I-3-O-glucosyl-rhamnoside | 1866.82 | 767.72 |
| | 12 | 17.31 | 479, 317 | 362, 352 | I-7-O-rhamnoside | 1727.26 | 375.56 |
| | 14 | 22.92 | 317 | | Isorhamnetin | 300.53 | nd |
| Total FL ³ | | | | | | 8728.98 | 2985.54 |
| Coumarins (CM) | 13 | 18.07 | 355, 193 | 358, 261 | S-7-O-glucoside | 740.59 | 215.22 |
| TOTAL CM ³ | | | | | | 740.59 | 215.22 |

¹ expressed as µg gallic acid /mL; ² expressed as µg chlorogenic acid /mL; ³ expressed as µg rutin /mL; Q-Quercetin; I-Isorhamnetin; S-Scopoletin; nd-not detected

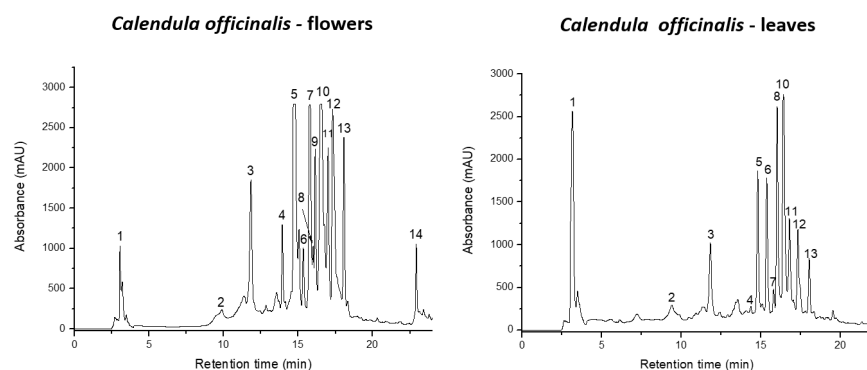


Figure 1. HPLC-PDA chromatograms of phenolic compounds from the extracts of *C. officinalis* flowers and leaves, recorded at 340 nm

Volatile profile of the C. officinalis extracts

The volatile compounds in the methanolic extracts of *C. officinalis* flowers and leaves were identified by ITEX/GC-MS and the relative percentage for each compound was estimated as a fraction of its integrated ion area from total ion chromatograms (TIC) area (100%). A number of 24 volatile constituents were identified in *C. officinalis* flower extract, while the leaf extract contained only 16 volatile compounds (Table 4). The volatile compounds identified in the extracts belong to several important secondary metabolites classes: monoterpenes (p-cymene, limonene, cosmene and alloocimene), sesquiterpenes (cubebene, copaene, muurolene and cadinene), ketones (acetophenone, propiophenone and 2-chloroacetophenone), or volatile aldehydes (benzaldehyde, benzeneacetaldehyde, octanal, decanal and nonanal).

Comparing the volatile profile of the two analyzed extracts, several important differences are observed. First of all, the relative abundance of sesquiterpenes in the flower extract (38.07%) is much higher than in the leaf extract (4.65%). These substantial differences are due both to the different muurolene content and to the fact that other sesquiterpenes, such as cubebene, copaene and cadinene are only present in the flower extract. On the other hand, the monoterpenes were found in larger percentage in the leaf extract (4.58%) compared to the flower extract (1.47%). Nevertheless, the overall relative abundance of the terpenes is significantly higher in the flower extracts. Furthermore, compounds belonging to the volatile aldehydes class (octanal, decanal, nonanal) were found only in the flower extract.

Table 4. Identification and relative quantification of volatile compounds in the extracts of *C. officinalis* flowers and leaves. R_t – retention time; Relative abundance - relative percentage (%) of total peaks area

| Class of compounds | Peak. No. | R _t (min) | Compound | Relative abundance (%) | |
|-------------------------|-----------|----------------------|--|-------------------------------|------------------------------|
| | | | | <i>C. officinalis</i> flowers | <i>C. officinalis</i> leaves |
| (Mono-) Terpenes (MT) | 8 | 14.30 | p-Cymene | 0.35 | 0.96 |
| | 9 | 14.45 | Limonene | 0.32 | 0.38 |
| | 15 | 17.80 | Cosmene | nd | 0.64 |
| | 16 | 18.20 | Alloocimene | 0.8 | 2.6 |
| Total MT | | | | 1.47 | 4.58 |
| (Sesqui-) Terpenes (ST) | 23 | 24.60 | Alpha-Cubebene | 0.59 | nd |
| | 24 | 25.60 | Copaene | 1.01 | nd |
| | 25 | 29.50 | Gamma-Muurolene | 3.15 | 0.73 |
| | 26 | 30.40 | Alpha-Muurolene | 8.81 | 3.92 |
| | 27 | 31.10 | Delta-Cadinene | 24.51 | nd |
| Total ST | | | | 38.07 | 4.65 |
| Aldehydes | 5 | 12.15 | Benzaldehyde | 3.37 | 1.6 |
| | 10 | 15.00 | Benzeneacetaldehyde | 0.62 | 0.26 |
| | 7 | 13.60 | Octanal | 0.28 | nd |
| | 14 | 17.10 | Nonanal | 0.42 | nd |
| | 20 | 20.30 | Decanal | 0.22 | nd |
| Total | | | | 4.91 | 1.86 |
| Ketones | 11 | 15.70 | Acetophenone | 8.89 | 7.08 |
| | 18 | 19.00 | Propiophenone | nd | 0.2 |
| | 21 | 22.70 | 2-Chloroacetophenone | 0.87 | nd |
| Total KT | | | | 9.76 | 7.28 |
| Esters | 2 | 5.90 | Methyl isovalerate | 0.33 | nd |
| | 3 | 7.05 | Butyl acetate | 30.45 | 69.91 |
| | 4 | 10.80 | Methyl hexanoate | 0.1 | nd |
| | 13 | 16.70 | Methyl benzoate | 4.64 | 3.6 |
| | 19 | 19.90 | Methyl Salicylate | 0.31 | nd |
| | 22 | 23.00 | Benzeneacetic acid, .alpha.-oxo-, methyl ester | 1.12 | nd |
| Total | | | | 36.95 | 73.51 |
| Others | 1 | 4.80 | Isobutylaldehyde dimethyl acetal | 0.37 | 0.67 |
| | 6 | 12.70 | 1,1-Dimethoxyhexane | 7.24 | 6.08 |
| | 12 | 16.50 | 2-Methyl-1-phenylpropene | nd | 0.56 |
| | 17 | 18.70 | Benzoic Acid | 1.22 | 0.83 |
| Total | | | | 8.83 | 8.14 |

*nd – not detected

Selective cytotoxicity assay

The two extracts from *C. officinalis* leaves and flowers were evaluated for their cytotoxic activity against MCF7, MDA-MB-231 and Hs578T breast cancer cell lines in 9 successive concentrations (50, 100, 150, 200, 250, 350, 500, 750, 1000 $\mu\text{g/ml}$), at 48 h after administration. Both extracts presented dose-dependent cytotoxicity on all three breast cancer cell lines (Figure 2). The half maximal inhibitory concentrations (IC₅₀ values) for each extract on each cell line are presented in Table 5. Administration of extracts at IC₅₀ values caused substantial morphological changes in the cells, besides reducing the number of viable cells (Figure 3). By comparing the IC₅₀ values obtained for the breast cancer cells and the healthy HUVEC cell line, both extracts proved to possess selective cytotoxicity (Table 5).

Both extracts exhibited the strongest cytotoxic activity and thus had the highest selectivity coefficient against the luminal breast cancer cell line MCF7. TNBC cell lines were less sensitive to the extracts, but the IC₅₀ values were still lower than the one determined for HUVECs, in the majority of cases. The extract obtained from *C. officinalis* flowers possessed stronger cytotoxic activity than the one produced from leaves, on all three breast cancer cell lines. Furthermore, it proved to be selective against all cancer cell lines tested, while the leaf extract had no selectivity in the antitumor action in the case of Hs578T cell line.

Table 5. The IC₅₀ values and the selectivity coefficients in the cytotoxic activity for *C. officinalis* flower and leaf extracts against MCF7, MDA-MB-231, Hs578T and HUVEC cell lines, at 48 h after administration

| Cell line | Extracts | | | |
|------------|---------------------------------------|-------------------------|---------------------------------------|-------------------------|
| | <i>C. officinalis</i> - flowers | | <i>C. officinalis</i> - leaves | |
| | IC ₅₀ ($\mu\text{g/ml}$) | Selectivity coefficient | IC ₅₀ ($\mu\text{g/ml}$) | Selectivity coefficient |
| MCF7 | 213.4** \pm 13.7 | 3.1 | 252.4** \pm 72.9 | 2.5 |
| MDA-MB-231 | 386.9** \pm 61.8 | 1.68 | 519.7* \pm 20.3 | 1.21 |
| Hs578T | 520.5** \pm 38.3 | 1.25 | 749.4** \pm 99.3 | 0.84 |
| HUVEC | 651.4** \pm 8.8 | - | 631.0** \pm 33.0 | - |

The IC₅₀ values are represented as mean \pm standard deviation for three biological replicates. The statistical significance was assessed by t-test, by comparing the absorbance of the control cells with the absorbance of the cells treated with the closest concentration to the IC₅₀ value in three independent biological replicates ($p^* > 0.05$; $p^{**} > 0.01$).

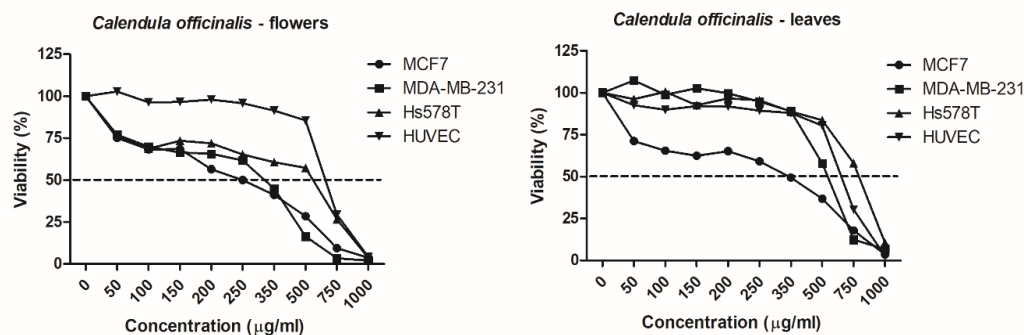


Figure 2. Cytotoxic activity of *C. officinalis* flower and leaf extracts against MCF7, MDA-MB-231, Hs578T and HUVEC cell lines, at 48h after administration

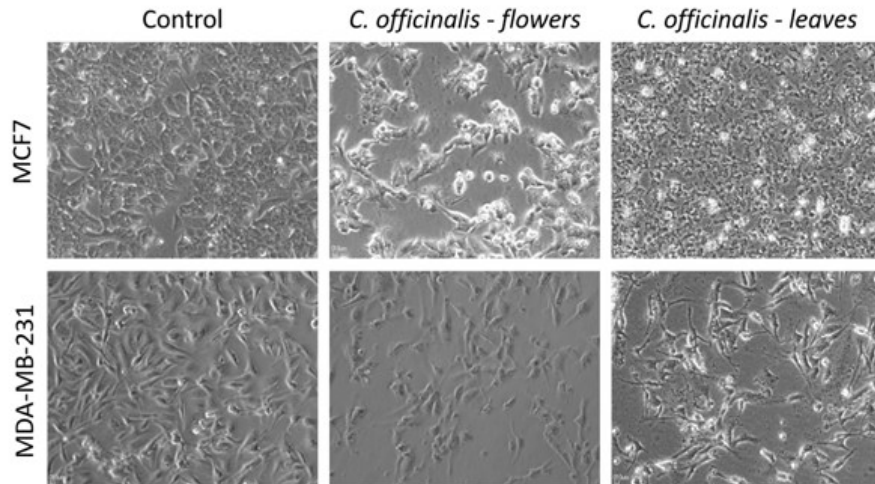


Figure 3. Morphological aspects of MCF7 and MDA-MB-231 cells treated with extracts of *C. officinalis* flowers and leaves, at a concentration equal to IC50 values, at 48h after administration

Expression analysis of apoptosis- and cell cycle-related genes

The relative expression of 7 apoptosis-related genes (*BCL2*, *BAX*, *BBC3*, *TP53*, *CASP3*, *CASP7* and *ZMAT3*) and 3 genes involved in cell cycle progression and proliferation (*CCND1*, *NFkB* and *STAT3*) was evaluated in MCF7 cell line. MCF7 cell line was selected for the expression analysis due to the good results obtained in the selective cytotoxicity assay. The mRNA levels for all genes in response to both *C. officinalis* extracts at IC50 concentration were evaluated by RT-qPCR. The expression level of *BCL2*, *CCND1*, *NFkB*, *STAT3* was significantly down-regulated, while *BAX*, *BBC3* and *ZMAT3* genes were overexpressed in MCF7 cells treated with both extracts. The expression levels of *TP53*, *CASP3* and *CASP7* were unaffected by the administration of any of the extracts. The expression levels (fold change) of each affected gene, for both extracts, are presented in Figure 4.

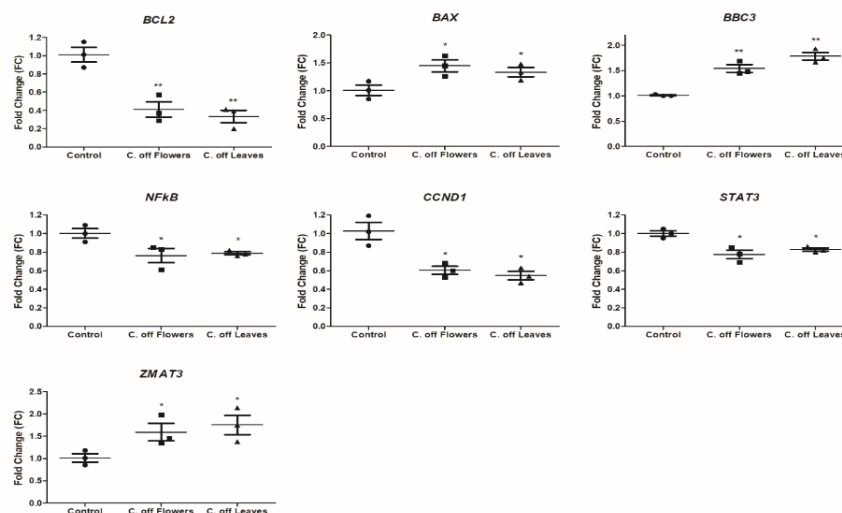


Figure 4. Gene expression of selected apoptosis- and cell cycle-related genes in MCF7 cell line treated with *C. officinalis* flower and leaf extracts at a concentration equal to IC50 values, at 48h after administration. The statistical significance was assessed by t-test (* $p < 0.05$; ** $p < 0.01$)

Discussion

C. officinalis is a well-known plant for its pharmacological activities, including its anti-tumor properties. In this context, this study was focused on the selective cytotoxicity of *C. officinalis* derived extracts against breast cancer cell lines, providing new insights in the biochemical composition of the extracts and the corresponding molecular effects in cancer cells.

The biochemical profile of the extracts

Out of all the phenolic constituents identified in this study, 10 were previously reported in different *C. officinalis* formulations (Mehta *et al.*, 2012; Rigane *et al.*, 2013; Miguel *et al.*, 2016; Olennikov *et al.*, 2017), while four flavonols (dihydroxybenzoic acid, quercetin-3-O-glucosyl-rhamnosyl-glucoside, isorhamnetin-3-O-galactoside and isorhamnetin-7-O-rhamnoside) were identified for the first time in *C. officinalis* extracts. Phenolic compounds are known for their cancer-related cytotoxicity, being capable of inhibiting cell proliferation and inducing apoptosis (Nichenametla *et al.*, 2006; Fantini *et al.*, 2015; Lewandowska *et al.*, 2016). Several phenolic compounds identified in the *C. officinalis* extracts used in this study, were previously reported to possess important cytotoxic activities: chlorogenic acid (Yamagata *et al.*, 2018), quercetin derivatives (Kashyap *et al.*, 2016), isorhamnetin derivatives (Wu *et al.*, 2018) and scopoletin derivatives (Li *et al.*, 2015). Therefore, these constituents contributed substantially to the cytotoxic effects of *C. officinalis* extracts on breast cancer cell lines.

A number of 28 volatile compounds were identified in *C. officinalis* extracts, 24 of them being present in the flower extract and 16 appeared as leaf extract constituents. Our data is consistent with previous reports, which also found cymene, limonene, cubebene, copaene, muurolene, cadinene and nonanal in different *C. officinalis* formulations (Okoh *et al.*, 2007; Gazim *et al.*, 2008; Petrović *et al.*, 2010; Kaškonienė *et al.*, 2011). However, to our knowledge, this is the first-time volatile compounds like octanal and nonanal, cosmene and alloocimene or propiophenone and chloroacetophenone are reported in *C. officinalis* extracts. Volatile compounds, including mono- and sesquiterpenes are recognized for their cytotoxic potential, both as mixtures of compounds and as individual plant constituents (Grey and Hammer, 2015; Dhifi *et al.*, 2016). Individual volatile compounds found in the *C. officinalis* extracts used in this study, such as cadinene (Hui *et al.*, 2015), copaene (Turkez *et al.*, 2014), methyl benzoate and multiple acetophenones (Nakamura *et al.*, 2002) were previously reported to possess important cancer-related cytotoxic activities. Consequently, the volatile profile found in the *C. officinalis* extracts might be responsible, in part, for the cytotoxic effects observed on breast cancer cells.

The cancer-related cytotoxicity of the extracts

The half maximal inhibitory concentration (IC₅₀) values ranged between 213.4 µg/ml for the flower extract, on MCF7 cell line, and 749.4 µg/ml for the leaf extract, on Hs578T cell line. Previous studies, investigating *C. officinalis* extracts obtained through classical methods reported much higher IC₅₀ values on various cancer cell lines (Matic *et al.*, 2013); thus, the UAE technique used in this study is an efficient method for bioactive compounds extraction. However, two other methods, the laser activated extraction (Jimenez-Medina *et al.*, 2006) and the supercritical CO₂ extraction (Mouhid *et al.*, 2018) showed better results in terms of cytotoxicity of *C. officinalis* flower extracts.

The highest cytotoxicity for both extracts was observed against MCF7 cell line. MCF7 is a luminal A breast cancer cell line, a less aggressive molecular subtype, which is also often responsive to chemotherapy. On the other hand, MDA-MB-231 and Hs578T are triple-negative, claudin-low breast cancer cell lines, with a phenotype characterized by intermediate to low chemotherapy responsiveness (Holliday and Speirs, 2011). In

this context, the differences regarding the specific IC₅₀ values between MCF7 cell line and MDA-MB-231/Hs578T were expected.

Both flower and leaf extracts proved to be selective in their anti-tumor action, with the highest selectivity coefficients against MCF7 cell line. Selective cytotoxicity was previously reported for an aqueous extract obtained from *C. officinalis* flowers, by comparing its cytotoxicity on several cancer cell lines and on healthy immunocompetent peripheral blood mononuclear cells (PMBCs) (Matic *et al.*, 2013). However, no selectivity was found for a methanol extract obtained from *C. officinalis* flowers, by comparing its action on breast cancer cells and normal human skin fibroblasts (Matysik *et al.*, 2005). Collectively, these results suggest that *C. officinalis* extracts might be selective against tumor cells, but the results are highly dependent on the extraction procedure, and thus the biochemical composition of the extracts and on the cell types used.

Comparing the two *C. officinalis* preparations used in this study, the flower extract showed better results in terms of the anti-tumor properties on all three breast cancer cell lines. These results are consistent with the only other study that compared the cytotoxicity of the leaves and the flower extracts obtained from *C. officinalis* on cancer cells, which also found lower cytotoxicity for the leaf extracts, with IC₅₀ values in the same range as the ones reported here (Wegiera *et al.*, 2012). These differences regarding the exerted cytotoxicity might be explained, at least in part, by the distinct phenolic and volatile profiles of the two extracts. The flower extract was superior in regard to both diversity and concentration of phenolic constituents, compounds known for their anti-tumor activity (Nichenamela *et al.*, 2006; Fantini *et al.*, 2015; Lewandowska *et al.*, 2016). It contained two additional flavonols, isorhamnetin and isorhamnetin-3-O-glucoside, had twice as much hydroxycinnamic acids and three times higher concentrations of flavonols and coumarins. Furthermore, several volatile compounds that were previously reported as anti-cancer agents, such as delta-cadinene (Hui *et al.*, 2015) and copaene (Turkez *et al.*, 2014), were identified in the flower extract but not in the leaf extract.

Molecular effects triggered by the extracts in breast cancer cells

C. officinalis extracts exert their cytotoxic activity on cancer cells by induction of apoptosis (Jimenez-Medina *et al.*, 2006; Wegiera *et al.*, 2012; Mouhid *et al.*, 2018) and cell cycle arrest in the G₀/G₁ phase (Jimenez-Medina *et al.*, 2006). It has been demonstrated that both caspase 3 and caspase 7 are activated (cleaved) at the protein level in response to *C. officinalis* extracts administration, and thus apoptosis is induced in a caspase3/7-dependent manner (Jimenez-Medina *et al.*, 2006; Mouhid *et al.*, 2018). On the other hand, the cell cycle arrest effect of *C. officinalis* extracts on cancer cells is induced by the down-regulation of cyclin D1, D3, A, E and several cyclin-dependent kinases (CDKs) (Jimenez-Medina *et al.*, 2006). Besides these molecular effects, there is no other data in the literature regarding the mechanistic underlying *C. officinalis* cytotoxic actions.

BCL2, *BAX* and *BBC3* genes encode proteins members of the BCL2 family, which form homo- and heterodimers and function as apoptosis regulators by controlling the mitochondrial membrane permeability, cytochrome c release from the mitochondria and thus caspase activation. Our data have revealed that *BCL2*, one of the most important antiapoptotic genes, was down-regulated in breast cancer cells treated with *C. officinalis* extracts. On the other hand, *BAX* and *BBC3*, two proapoptotic genes, were up-regulated in response to *C. officinalis* extracts. Therefore, cytochrome c release from the mitochondria could be stimulated by the both tested extracts, and thus apoptosis is most probably implemented through caspase activation, as was shown by previous reports (Jimenez-Medina *et al.*, 2006; Mouhid *et al.*, 2018). The expression of *ZMAT3*, a gene involved in both tumor cell growth and apoptosis (Bersani *et al.*, 2014), was also investigated in relation with *C. officinalis* extracts. Our results confirm the induction of this gene in response to both *C. officinalis* extracts, emphasizing the role of these bioactive formulations in the modulation of tumor cell growth inhibition and induction of apoptosis.

Cell proliferation is controlled by multiple signaling networks, several regulatory proteins as cyclins and transcription factors like NFκB or STAT3 being crucial for cell cycle progression. *CCND1* encodes cyclin D1, the regulatory component of the cyclin D-CDK4/6 complex, which induces the G1/S transition during the cell cycle. Cyclin D1 was found down-regulated at the protein level in response to *C. officinalis* extracts (Jimenez-Medina *et al.*, 2006), while this report shows that its expression is down-regulated starting at the mRNA level. NFκB is a transcription factor that is activated by a wide variety of internal and external stimuli, its activation being associated with cell proliferation. STAT3 is another transcription activator that mediates cellular responses to cytokines and various growth factors, being involved in the G1/S transition of the cell cycle. Its activation leads to the expression of key genes for proliferation, like *CCND1*. Our data confirmed the down regulation of *CCND1* in relation with *C. officinalis* extracts, but also demonstrated the down-regulation of both *NFκB* and *STAT3* transcription factors. Taken together, these data highlight important molecular changes that contribute to the inhibition of cell cycle progression induced by *C. officinalis* extracts.

No significant deregulation in the expression levels was observed at the mRNA level for caspases 3 and 7 or for the *TP53* gene, in response to the administration of the *C. officinalis* extracts. *TP53* encodes a tumor suppressor protein which is activated by multiple cellular stresses and induces cell cycle arrest, apoptosis, senescence and DNA repair. Even though it is not up-regulated at the mRNA level in response to *C. officinalis* extracts, its activation state might be affected by the treatment. Further investigations are needed in this regard. Caspase 3 and 7, effector proteins in the apoptotic process, are activated at the protein level by both intrinsic and extrinsic mechanisms to induce the degradation of cellular components. In *C. officinalis* treated cells, they are activated at the protein level and thus apoptosis is induced (Mouhid *et al.*, 2018), but no deregulation is observed at the mRNA level according to our results.

Conclusions

Collectively, our results bring new useful information regarding the biochemical profile and the molecular effects on breast cancer cells of *C. officinalis* extracts. Several compounds belonging to the phenolic and volatile classes of compounds were identified for the first time in *C. officinalis* formulations. Both leaf and flower extracts proved to possess selective cytotoxic activity against breast cancer cell lines, the extract obtained from flowers being superior regarding the exerted cytotoxicity. Both extracts were able to modulate the expression of several proliferation- and apoptosis-related genes, proving, at the molecular level, the cellular effects previously reported for *C. officinalis* preparations.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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