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Botanical Therapeutics (part II): Antimicrobial and In Vitro Anticancer Activity against MCF7 Human Breast Cancer Cells of Chamomile, Parsley and Celery Alcoholic Extracts

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Abstract: Background: This study was designed as a continuation of a complex investigation about the phytochemical composition and biological activity of chamomile, parsley and celery extracts against A375 human melanoma and dendritic cells.

Objective: The main aim was the evaluation of the antimicrobial potential of selected extracts as well as the in vitro anticancer activity against MCF7 human breast cancer cells.

Methods: In order to complete the picture regarding the phytochemical composition molecular fingerprint was sketched out by the help of FTIR spectroscopy The activity of two enzymes (acetylcholinesterase and butyrylcholinesterase) after incubation with the three extracts was spectrophotometrically assessed. The antimicrobial potential was evaluated by disk diffusion method. The in vitro anti-cancer potential against MCF7 human breast cancer cells was appraised by MTT, LDH, wound healing, cell cycle, DAPI, Annexin-V-PI assays.

Results: Results showed variations between the investigated extracts in terms of inhibitory activity against enzymes such as acetyl- and butyrylcholinesterase. Chamomile and parsley extracts were active only against tested Gram-positive cocci, while all tested extracts displayed antifungal effects. Among the screened samples at the highest tested concentration, namely 60 µg/mL parsley was the most active extract in terms of reducing the viability of MCF7 - human breast

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# First and second author have equal contribution
The Plant Kingdom has been a very important source of medicinal products for millennia, delivering a significant number of drugs developed from vegetal sources used in the medicine of the 21 century [1-3]. Identifying, testing and implementing in the current therapeutic protocols new molecules is a very complex, time-consuming and also very expensive process. Studies have shown that the frame period taken from the moment of the discovery of a new active compound to its reaching the clinic has an average course of twelve years while the amount of money allocated for this ‘road trip’ is in the order of millions of dollars [4]. In many situations, the attention was directed towards the study of plant extracts as it is very well known that in some cases the activity of the phytopharmaceutical is superior to the one of pure phytochemicals due to the synergism of the molecular structures. Traditionally *Matricaria chamomilla* L has been used for its anti-inflammatory, antioxidant, antibacterial, astringent, mild sedative properties for the treatment of gastrointestinal disturbances, different bacterial infections of the skin, oral cavity, respiratory tract, wounds, skin irritations, bruises, eczema, burns, haemorrhoids, rheumatic pain, disorders of the eyes, nasal inflammation, anxiety, insomnia [5-7]. Scientific studies have assigned for the different types of extracts or volatile oil: anti-inflammatory, anticancer, anti-viral, cardioprotective, gastro-protective properties. Also, the beneficial effects in case of haemorrhoids, eczema, osteoporosis, sleep disorders, anxiety, diabetes, vaginitis, have been reported [5-7].

In a previous study our research group have described the antioxidant and biological activity of extracts obtained from this three functional foods on *L.* ligulate flowers extracts obtained by employing different methods, namely: Soxhlet continuous extraction, ultrasound-assisted, microwave-assisted, and subcritical water extraction. The strongest antioxidant activity has been observed for subcritical water extraction. This type of extract was ‘the winner’ also in case of antimicrobial activity, with remarkable effect against *Escherichia coli* and *Aspergillus niger*. Subcritical water extract was the most effective also in case of cytotoxic effect for cell lines derived from human cervix carcinoma Hep2c (HeLa), human rhabdomyosarcoma cells (RD) and cell line derived from murine fibroblast (L2OB) [8].

Parsley, known under the scientific name of *Petroselinum crispum* (Mill.) Fuss presents powerful nutraceutical properties being used both in the culinary as well as in the medical field for its carminative, stomachic, antioxidant, spasmylytic, hepatoprotective, diuretic, antiseptic with tropism for the urinary tract, anti-inflammatory, antibacterial and antifungal properties. The most studied part of the plant because of the complex chemical composition are the seeds [9, 10].

Last but not least celery also known under the scientific name of *Apium graveolens* L. as well as the previous species is considered a nutraceutical plant, the most studied part of the plant because of the complex chemical composition being the seeds and the leaves [11]. In a comprehensive review Kooti *et al.* have described various biological activities like cardioprotective effect, anti-inflammatory, anticoagulant, gastroprotective, diuretic, anti-cancer, antimicrobial potential [12]. Celery was assigned with powerful antioxidant characteristics [13]. Powanda *et al.*, published about the antiarthritic, antiulcer, and antimicrobial potential of alcoholic extract of the seeds [14].

The common element of these three extracts is that they represent the main botanical sources of bulk apigenin [15].

In a previous study our research group have analyzed the activity of extracts obtained from this three functional foods on A375 human melanoma and dendritic cells. Results have shown positive effects in term of *in vitro* anti-melanoma effects, of course with variations of the intensity and mechanism of action [16].

This study was designed as a continuation of the above mentioned research. The main aim was the evaluation of the antimicrobial potential of selected extracts as well as the *in vitro* anticancer activity against MCF7 human breast cancer cells.

In order to complete the picture regarding the phytochemical composition molecular fingerprint was sketched out by the help of FTIR spectroscopy. Moreover, the study aims to analyze the variations between the investigated extracts in terms of inhibitory activity against enzymes such as acetyl- and butyrylcholinesterase, recently discussed in the literature to be involved in tumor development.

**2. MATERIALS AND METHOD**

**2.1. Extracts preparation, characterization and antimicrobial evaluation**

Extracts were prepared as previously described [16]. The three investigated samples (Matricaria chamomilla L, *Petroselinum crispum* var. radicosum Miller, *Apium graveolens* var. radicosum L.) were acquired from specialized providers. All samples were first tested for the certification of their identity. Briefly, the dried Romanian Chamomillae flos sample (voucher specimen Mc10/2016) was extracted with 60% methanol (thermostatic water bath) in a drug solvent ratio of 2.5 to 100.
(g/mL). Seeds of parsley (voucher specimen Pc01/2016) and celery (voucher specimen Ag05/2016) were powdered and then extracted in a similar manner with the chamomile sample. The final solution was measured in a volumetric flask and a Buchi rotary evaporator was used to obtain the dry extracts. The obtained dried extracts were weighted and stored at 4°C for further testing.

The three extracts (in solvent-free form) were characterized by means of Fourier-transform infrared spectroscopy. The spectra were recorded using a Shimadzu Prestige-21 spectrometer at a resolution of 4 cm⁻¹, in the range 400–4000 cm⁻¹. KBr pellets were prepared for each sample and were further subjected to the infrared analysis. The selected samples were screened for their antimicrobial activity against nine bacterial strains.

Disk diffusion method. The antimicrobial activity of the selected compounds was evaluated, according to other studies, by the disk diffusion method [17]. A Petri dish containing the Mueller-Hinton medium (Sanimed, Bucharest, Romania) was inoculated with 0.1mL of a physiological saline solution containing 108 CFU/mL of the microorganism under study (CFU-colony forming units). Ten microliters from each sample (10 mg/mL in DMSO) was added to a 6 mm diameter sterile blank filter disk, placed on top of the culture media. Plates inoculated with the bacterial suspensions were incubated at 37°C for 24h. The inhibition zone diameters were measured in millimetres, with a ruler. For all bacterial strains (Table 1), we performed duplicate disk-diffusion tests and was taken the average reading. For the positive control we used gentamycin or fluconazole disk (BioMaxima, Lublin, Poland). As a negative control, a disk impregnated with DMSO was used.

Dilution method. The MIC (minimum inhibitory concentration) values were evaluated by the binary microdilution method, in the range of 1.56–50 𝜇g/mL [18]. From stock solutions in DMSO of the tested extracts, serial dilutions of the compounds were prepared and brought, to a final volume of 200 𝜇L with Mueller Hinton broth (Sanimed, Bucharest, Romania). In all tubes 50 𝜇L of bacterial suspensions were added, with a density equal to a 0.5 McFarland. All these tubes were incubated at 37°C for 24 h. The MIC was recorded as the lowest concentration of the compound which inhibited the visible growth of the tested bacteria. For a negative control 50 𝜇L of DMSO was introduced in a tube with 50 𝜇L of bacterial suspension and 100 𝜇L of Mueller Hinton broth.

All the test tubes with no visible growth were inoculated on Columbia agar supplemented with 5% blood in order to determine the MBC (the minimum concentration which killed 99.9% of the bacteria).

Table 1. Reference strains.

<table>
<thead>
<tr>
<th>Bacterial Species</th>
<th>ATCC</th>
<th>Producer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmonella enterica serotype typhimurium</td>
<td>14028</td>
<td>ThermoScientific</td>
</tr>
<tr>
<td>Shigella flexneri serotype 2b</td>
<td>12022</td>
<td>ThermoScientific</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>51299</td>
<td>ThermoScientific</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>25922</td>
<td>ThermoScientific</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>700603</td>
<td>ThermoScientific</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>27853</td>
<td>ThermoScientific</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>25923</td>
<td>ThermoScientific</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>10231</td>
<td>ThermoScientific</td>
</tr>
<tr>
<td>Candida parapsilosis</td>
<td>22019</td>
<td>ThermoScientific</td>
</tr>
</tbody>
</table>

2.2. Inhibitory enzymatic activities

Acetylcholinesterase inhibition. Acetylcholinesterase from Electrophorus electrics (0.2 U/mL in phosphate buffer), acetylthiocholine iodide (0.2 M) and various concentrations of chamomile, parsley and celery extracts diluted in DMSO were employed for the analysis. The ability of the investigated extracts to inhibit acetylcholinesterase was assessed by Ellman’s method by measuring the absorbance variations at 412 for 5 minutes [19, 20]. The activity value was calculated as a percentage of the difference between the absorbance values (enzyme solution with inhibitor at after 5 minutes and the absorbance of the same solution at the initial time).

Butyrylcholinesterase inhibition. For the inhibitory potential against butyrylcholinesterase we used acetylthiocholine iodide (0.2 M) as substrate, but the rest of the assay was conducted similarly to the method described above for acetylcholinesterase evaluation. The measuring time was also 5 minutes and the temperature for determinations was 25°C. Galantamine was used as a positive inhibitor. The values recorded in the test represent the mean and standard deviation of triplicate measurements.

2.3. Cell culture
The MCF7 breast cancer cell line (ECACC; Sigma-Aldrich origin Japan stored in UK) was cultured into Roswell Park Memorial Institute (RPMI) 1640 Medium (Sigma-Aldrich, Germany) supplemented with 10% fetal calf serum (FCS; PromoCell, Heidelberg, Germany) and 1% penicillin/streptomycin mixture (Pen/Strep, 10,000 IU/mL; PromoCell, Heidelberg, Germany). When cells reached confluence stage they were passaged by the help of trypsin EDTA.

2.4. Antiproliferative and pro-apoptotic activities

2.4.1. Cell viability evaluation via MTT assay.

In order to test the cell viability of MCF7 cells treated with test extracts, the consecrated MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was employed. A number of 5000 cells/well was seeded in 96-well plates and left overnight in order to attach to the bottom of the plate. Starting from the second day cells were incubated with different concentrations of extracts (10, 30 and 60 µg/mL) for 72 h. After this step cells were incubated for 4 h with a 5 mg/mL MTT solution. Dimethyl sulfoxide (DMSO) was added and the absorbance of the precipitated formazan crystals solution was measured by the help of a microplate reader at 545 nm.

2.4.2. Proliferative and migratory potential by the means of a wound healing technique.

In order to evaluate the inhibitory activity of the chamomile, parsley and celery extracts on migration and proliferation of human breast adenocarcinoma - MCF7 cells, the scratch assay test was performed. This is a facile and practical technique widely used to express cell to cell interactions and consists in drawing a scratch following the diameter of each well, by using a sterile tip. 2x10^4 cells/well in 1500 µL medium were cultured in a 12-well plate and when the suitable confluence was reached (90%) a scratch was made in the middle of each well. After this step, the cells were stimulated with medium containing the test extracts at a concentration of 60µg/mL. For quantification of the antiproliferative and anti-migratory effect, the cell growth was observed by taking pictures initially and 24h respectively, followed by measuring the wound widths. Pictures at a magnification of 10X were taken using an inverted microscope - Olympus IX73, provided with DP74 camera. The scratch area was determined with CellSense Dimension software and the migration percentage was calculated according to the formula described by Felice et al. 2015 [21].

2.4.3. Cytotoxicity evaluation via lactate dehydrogenase (LDH) release.

Pierce LDH Cytotoxicity assay kit was acquired from ThermoScientific (No 88954). The toxicity of the extracts was assessed by LDH assay, sensitive and well-known technique which detects the extracellular LDH released in the medium when the cellular membrane damage occurs [22]. The quantification of LDH leakage can be measured by a coupled enzymatic reaction, based on the ability of LDH to reduce NAD+ to NADH by conversion of lactate to pyruvate and followed by the reduction of the tetrazolium salt to red formazan via diaphorase which uses NADH formed in the first step of the reaction. 5000 cells/well in the 200µL medium were seeded in a 96-well plate and incubated overnight. After that, the medium was removed by gentle aspiration and the cells were stimulated with 100µL media containing 60µg/mL concentration of extracts, for 72h. On the day of the assay, the cytotoxicity reagents were prepared according to the manufacturer’s protocol. 50µL of all samples were transferred to a new 96 well plate and 50µL of the reaction mixture was added to each well and incubated at room temperature for 30 minutes, followed by addition of a stop solution. After this step, the concentration of formazan (which is directly proportional with the amount of LDH leakage) was measured at 490nm and 680nm wavelengths, via spectrophotometry with a microplate reader (xMarkTMMicroplate, Biorad).

2.4.4. Cell cycle analysis.

Flow cytometry analysis was performed in order to study the percentage of cells in different cell cycle phases. MCF7 human breast cancer cells were seeded onto 9 cm² 6-well plates (3x10⁶ cells/well) and were treated with the selected extracts using the concentrations 30 and 60 µM. After 72 h of exposure, cells were collected, fixed with 70% ethanol and stored for 30 min at 4°C followed by centrifugation at 2000 rpm. In order to wash the cells, cold Phosphate Buffer Saline was used. To obtain DNA staining, 50 µL of Propidium Iodide (50µM) (Carl Roth, Karlsruhe, Germany) was added to the cells and the cells were incubated for 10 min in the dark at room temperature. Next, in order to analyze the DNA content, a FACSCalibur flow cytometer (Becton-Dickinson, Franklin Laked, NJ, USA) was used. The percentage of cells in all cell cycle phases was determined using Flowing Software Version 2.5.1

2.4.5. Annexin V-PI Assay.

In order to evaluate the apoptotic potential of selected extracts, Annexin V-PI assay was employed as previously discussed. A number of 5x10^3 cells/well was seeded into 6 well plates (Greiner Bio-one) and left overnight in order to attach to the bottom of the plate. Starting from the second day cells were incubated with the tested extracts at a concentration of 60 µg/mL for 72 h. Annexin V-FITC -PI kit (Invitrogen, ThermoFisher, Vienna, Austria) was employed for the staining following the manufacturer’s protocol. Briefly, 2-5x10^3 cells were washed twice in 1 x Annexin V Binding Buffer and afterwards centrifuged for 5 min at 1500 RPM. Following this step cells were resuspended in the binding buffer and incubated for 15 min in the dark with 5 µL of Annexin V-FITC. After another step of washing and centrifugation the pellet was resuspended in 190 µL binding buffer, and 10 µL of PI solution was added immediately prior to analysis by flow cytometry.
3. RESULTS

3.1. Physico-chemical characterization and antimicrobial activity

The analysis of FTIR spectra (Figure 1) returned information regarding the composition of the selected extracts: chamomile (CA), celery (C) and parsley (P). All the existing absorption bands were allocated to the specific molecular vibration.

![FTIR spectra of extracts](image)

**Fig. (1).** FTIR spectra extracts: (CA) chamomile alcoholic extract; (P) parsley alcoholic extract and (C) celery alcoholic extract.

It was confirmed by the characteristic vibrations that all the studied extracts contain polyphenols and flavonoids. Correlation of detected bands with other studies presented in the literature which refers to the analyzed extracts is described in the discussion part.

Although establishment of the antibacterial activity of the investigated extracts is difficult to interpret in the absence of standardized values, we considered that the chamomile extract was active only against tested Gram-positive cocci, while the parsley extract inhibited only the S. aureus strain. The celery extract did not show antibacterial activity. All tested preparations showed antifungal activity (Tables 2 and 3).

Table 2. Antibacterial and antifungal activity of selected extracts expressed as inhibition zone (mm).
### Table 3. MIC and MBC values for each extract on bacteria and fungi.

<table>
<thead>
<tr>
<th>Sample</th>
<th>K. pneumoniae</th>
<th>S. flexneri</th>
<th>S. enterica</th>
<th>E. coli</th>
<th>P. aeruginosa</th>
<th>S. aureus</th>
<th>E. faecalis</th>
<th>C. albicans</th>
<th>C. parapsilosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>P</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>10</td>
<td>9</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>C</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

3.2. Enzymatic activity

The activity of each sample on the enzymes included in our assay is presented in Figure 2. One can notice that although the extracts have been used in the same concentration, the inhibitory potential varies from one species to another.

The obtained results indicate that chamomile and celery may represent better agents against the activity of butyrylcholinesterase, whereas, all tested samples act similar against acetylcholinesterase.
3.3. Cell viability inhibition and pro-apoptotic activity

Regarding the cell viability of MCF7 cells, results show (Figure 3) that in the range of tested concentrations the selected extracts present an overall weak cell viability inhibition against MCF7 human breast cancer cell line. Among the screened samples, the inhibitory potential was in the range: parsley > celery > chamomile.

The migratory and proliferative potential of MCF7 - human breast adenocarcinoma cells exposed to the highest concentration (60µg/mL) of chamomile, parsley and celery extracts is significative reduced after 24h post-treatment (Figure 4). Celery extract manifested the most potent anti-migratory and antiproliferative effects, displaying scratch widths of 433µm initially and 392µm after 24h, with a wound closure rate of only 9.30%, while chamomile extract manifested a migratory and proliferative percentage of 14.93%. In addition, the cells treated with chamomile extract presented important morphological alterations. Parsley extract exhibited the lowest inhibitory activity in terms of migratory and proliferative potentials, presenting a scratch closure rate of 39.64% Nevertheless, MCF7 cells exposed to the test extracts reduced statistically significant the scratch closure percentages when compared to control cells (no stimulated cells).

Fig. (2). Inhibitory enzymatic activity of the investigated extracts: (CA) chamomile alcoholic extract; (P) parsley alcoholic extract and (C) celery alcoholic extract. (two tailed paired t-test,**, p = 0,0015, r = 0,9819)
Fig. (3). Cell viability inhibition of MCF7 human breast cancer cell line treated with test extracts (One-way ANOVA with Newman-Keuls posttest; *, **, ***, **** indicates p<0.05, p<0.01, p<0.001 and p<0.0001 respectively, compared to the control group)

Fig. (4). Migratory and proliferative potential of human breast adenocarcinoma - MCF7 cell line, after treatment with test extracts at a concentration of 60µg/mL. Wound closure was imaged by light microscopy initially and 24h, respectively. Scale bars represent 100µm. The bar graphs are expressed as the percentage of wound healing after 24h compared to the initial area.

Exposure of MCF7 - human breast adenocarcinoma cells to chamomile, parsley and celery extracts at a concentration of 60µg/mL caused a significant LDH release only in the case of parsley extract, displaying a cytotoxicity percentage of 10.22% (Figure 5). However, the celery extract manifested a cytotoxicity rate of almost 3%, whereas the chamomile extract showed no cytotoxic effect at all, moreover it displayed a slight proliferative activity. The proliferative effect may be explained by an intracellular alteration of cell function, a process that does not involve LDH release [23].

Table 4. Cell cycle distribution of MCF7 human breast cancer cells after 72 h treatment with selected extracts (mean values of 3 experiments and standard deviation)

<table>
<thead>
<tr>
<th></th>
<th>Viable</th>
<th>Early apoptosis</th>
<th>Late apoptosis</th>
<th>Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>87.90 ± 1.43</td>
<td>1.53 ± 0.38</td>
<td>0.11 ± 0.05</td>
<td>10.46 ± 0.62</td>
</tr>
<tr>
<td>CA</td>
<td>75.26 ± 6.63</td>
<td>5.66 ± 2.74</td>
<td>1.44 ± 1.35</td>
<td>17.65 ± 0.20</td>
</tr>
<tr>
<td>P</td>
<td>80.39 ± 6.58</td>
<td>5.70 ± 2.79</td>
<td>0.93 ± 0.86</td>
<td>12.99 ± 0.13</td>
</tr>
<tr>
<td>C</td>
<td>82.11 ± 7.50</td>
<td>7.53 ± 3.41</td>
<td>1.46 ± 1.26</td>
<td>8.89 ± 0.57</td>
</tr>
</tbody>
</table>
Flow cytometric analysis was conducted in order to study the effect on the distribution of the phases of the cell cycle of MCF7 cells after 72 h of incubation with tested extracts (Table 4 and Figure 6). Results have shown that in the case of CA extract, in both concentrations a slight G0/G1 accumulation can be noticed. Also, P extract elicited the same type of behavior, but in this case, the accumulation of cells in G0/G1 phase was in a dose-dependent manner. Among screened samples, this extract at the highest tested concentration had the most significant effect on the G0/G1 cell cycle arrest. Cells incubated with C extract at the concentration of 30µg/mL led to a G2/M accumulation whereas in case of 60µg/mL a slight G0/G1 accumulation could be detected.

Table 5. Viability (mean values) of MCF7 human breast cancer cell line using Annexin V-PI analysis.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
</tr>
</thead>
<tbody>
<tr>
<td>0(CTRL)</td>
<td>61.12 ± 1.38%</td>
<td>21.96 ± 0.25%</td>
<td>16.74 ± 1.66%</td>
</tr>
<tr>
<td>CA 30 µg/mL</td>
<td>69.41 ± 4.02%</td>
<td>14.83 ± 2.08%</td>
<td>15.12 ± 1.42%</td>
</tr>
<tr>
<td>CA 60 µg/mL</td>
<td>69.33 ± 1.00%</td>
<td>13.87 ± 2.63%</td>
<td>16.71 ± 1.63%</td>
</tr>
<tr>
<td>P 30 µg/mL</td>
<td>69.00 ± 0.53%</td>
<td>11.24 ± 0.25%</td>
<td>19.15 ± 0.90%</td>
</tr>
<tr>
<td>P 60 µg/mL</td>
<td>75.82 ± 0.58%</td>
<td>11.66 ± 0.79%</td>
<td>12.21 ± 0.27%</td>
</tr>
<tr>
<td>C 30 µg/mL</td>
<td>57.99 ± 0.41%</td>
<td>21.75 ± 0.11%</td>
<td>19.83 ± 0.49%</td>
</tr>
<tr>
<td>C 60 µg/mL</td>
<td>66.11 ± 2.47%</td>
<td>16.27 ± 1.60%</td>
<td>17.36 ± 0.99%</td>
</tr>
</tbody>
</table>
In order to check the pro-apoptotic potential of selected extracts including phenomena of early apoptosis, late apoptosis and necrosis the Annexin-PI double staining was employed. Among the three extracts, CA caused the highest percentage of necrosis, namely 17.65% ± 0.20. In terms of total apoptotic events C was the most potent extract, inducing a percentage of 8.99% ±4.67 cells. Results are presented in Table 5 and representative dot-plots are displayed in Figure 7.
DISCUSSION

In a previous comprehensive study regarding the phytochemical composition and biological activity of chamomile, parsley and celery extracts against A375 human melanoma and dendritic cells we have shown by the help of RP-UHPLC that the main compounds that were identified in the three extracts belong to the polyphenolic acids and flavone groups. Briefly in case of CA chlorogenic acid, caffeic acid, catechin, apigenin glucoside, rutin, cinaroside, luteolin and apigenin could be detected. On the other hand for P and C extracts chlorogenic acid, caffeic acid, apigenin glucoside, cinaroside, luteolin apigenin and kaempferol could be fingerprinted [16]. Moreover spectrophotometric quantification showed the presence of flavonoids and polyphenols. The radical scavenger capacity of selected extracts was shown by DPPH and ABTS assay. The extracts showed iron chelation potential and lipoxygenase inhibition activity [16].

The quantification of the most important bioactive compounds indicated interesting information related to the metabolism of the investigated samples [24]. There is an important difference between the chamomile inflorescence and the celery and parsley seeds that specifies to the user if one intend to obtain richer extracts in glycosides or in aglycons. The mature flowers contain almost a double amount of apigenin-7-O-glucoside as compared to celery and parsley. Nevertheless, the seeds are richer in aglycons such as apigenin and kaempferol. Lower quantities of polyphenolic acids (caffeic and chlorogenic) were also determined in all samples. In figure 8 are presented the chemical structures for the main compounds that were identified by RP-UHPLC in our previous study [16].

Fig. (7). Representative dot-plots of MCF7 human breast cancer cell line for: (a) Control; (b) incubated with CA 60 µg/mL; (c) incubated with P 60 µg/mL; (d) incubated with C 60 µg/mL.
Fig. (8). Chemical structures of the major polyphenolic compounds identified in chamomile, parsley, and celery methanolic extracts

Characterization of natural extracts through infrared spectroscopy has gained much popularity among other evaluation methods [25-28], although it’s mainly used for isolated compounds than for mixtures. The literature provides many studies that help the interpretation of infrared spectra, but sometimes because of the overlapping bands it is difficult to correlate the vibration frequencies to the compound especially when the analyzed sample is an extract formed from a mixture of compounds and substances. Another fact that worth mention is the fact that Fourier transformed infrared analysis emphasis on the quality of the spectra and it is possible by this method to misidentify the active substances that are present in very low concentrations [27]. For a better characterization and analysis several physico-chemical analysis have to be correlated.

Is well known from the literature that chamomile, parsley and celery have rich content in polyphenols, flavonoids, tannins and glycosides [13, 29-31]. Chemically, this composition could be expressed by aromatic compounds formed from hydroxyl groups, substituted phenyl rings, oxygen and nitrogen-containing heterocycles. Grasel et al. [32] concluded that polyphenolic extracts present a high content of aromatic hydroxyls and this gives them the capacity to compose complexes alongside proteins. According to these existent studies, the absorption bands characteristic for the phenolic compounds present an extended interval on the interval on infrared spectra thus overlapping with other vibration characteristics from others chemical compounds [33].
Matricaria chamomilla L. extract points out five absorption bands on the FTIR spectrum, characteristic for structural and functional groups. The broadband located at 3381 cm\(^{-1}\) denote the –OH stretching vibration from phenolic compounds and thus Al-Maliki [29] concluded that the hydrogen bond belongs to tannins and flavonoids. The band located at 1609 cm\(^{-1}\) resembled the C=O stretching vibration specific in aromatic compounds. The small shoulder at 2931 cm\(^{-1}\) can be attributed to the C-H aromatic stretching vibration and the bands at 1069 cm\(^{-1}\), 1408 cm\(^{-1}\) and 1609 cm\(^{-1}\) are considered symmetric stretching vibration of COO- groups, present in a non-esterified form [30]. The band at 623 cm\(^{-1}\) is characteristic for the vibration of the aromatic group, respectively the bending of C-H bond.

The study realized by K. Roy et al. [34] regarding the leaf extract of Apium graveolens L, revealed on the infrared spectra some important bands along the considerate range 4000-400 cm\(^{-1}\). The vibration recorded at 3396 cm\(^{-1}\) and at 1605 cm\(^{-1}\) can be assigned to the stretching vibration of C=O from tertiary amide and the O-H stretching vibration of the compounds that contain in their structure benzene ring like phenols, confirming in this way the polyphenolic content. The bands below and above 3000 cm\(^{-1}\) suggest the existence of aliphatic and aromatic C-H groups. In the region between 3100-2700 cm\(^{-1}\) usually are bands of C-H stretching vibration, so the bands located at 2934 cm\(^{-1}\) and 2872 cm\(^{-1}\) corresponds the C-H stretching vibration of an aldehyde group. The band present at 1751 cm\(^{-1}\) can be attributed to C=O stretching in aldehyde or ketone. The study conducted by Iswantini [35] allocates the C=C stretching vibration at a wavenumber around 1600 cm\(^{-1}\), the stretching vibration of C-O in the cyclic ether around 1070 cm\(^{-1}\) and around 600 cm\(^{-1}\) the bending vibration of C-H in an aromatic group. The band present at 1425 cm\(^{-1}\) can be attributed to CH2 symmetric bending vibration [36].

The infrared spectra resulted from analyzing the Petroselinum crispum (Mill.) Fuss extract leads to the formation of nine distinct bands on the investigated range. The bands located at 2930 cm\(^{-1}\) and 1609 cm\(^{-1}\) resemble with the C=O stretching vibration from tertiary amides and C-H stretching in aldehydes [34]. The O-H stretching vibration in phenols, alcohols could be retrieved in the absorption band located at 3404 cm\(^{-1}\). The presence of bands between 1600 cm\(^{-1}\) and 1400 cm\(^{-1}\), 830 cm\(^{-1}\) and 670 cm\(^{-1}\) denote the existence of aromatic compounds, such as apigenin and its glycosides according to Pápay [37]. The well-defined band at 1053 cm\(^{-1}\) denote the in-plane -C-H bending vibration and at 1246 cm\(^{-1}\) can be identified the -OH bending vibration [38].

Nevertheless, every absorption band present on the infrared spectra due to the vibration of the functional group has correspondence in characteristic frequencies. Therefore, such spectra can be used for fingerprinting a standardized extract in which the chemical composition is stable and well-known. This method can certify the quality of an extract without the necessity of more quantification and is more economic, but it implies the existence of a certified standard for comparison. Corroborated with the RP-UHPLC analysis these parameters can be considered in the future as standard fingerprints for extracts that contain either apigenin-7-glucoside or apigenin in high amount.

It can be said that the extracts are more active on fungi than on bacteria. A similar approach reported about the antibacterial effect of different type of extracts (using as solvent methanol, ethanol, diethyl ether and hexane), respectively essential oil obtained by hydrodistillation, from the flowers of Egyptian Matricaria chamomilla L. The essential oil led to the smallest values for the minimum inhibitory concentration, and the sensitive strains were the fungus C. albicans and the gram-positive strains B. cereus and S. aureus [39]. It is well known that plant-derived volatile oils are powerful antibacterial and antifungal agents [40]. Both extract and essential oil obtained from Anthemis nobilis L. were described to possess antimicrobial effect against P. gingivalis, a bacteria present in the case of periodontitis [41]. The antibacterial potential against B. subtilis and E. coli of freeze-dried and irradiated parsley leaves and stems were assessed on methanol and water extracts by determining bacterial cell damage and bacterial growth inhibition. Parsley leaf methanol extract was able to induce cell damage against both tested strains [42]. The group of Wahba et al., showed that among other aromatic plants parsley presents antibacterial potential against S. aureus as well as natural microflora, yeast and moulds in Kareish cheese [43]. In a recent study Linde et al., analyzing the antifungal and antibacterial potential of the volatile oil obtained from parsley have concluded that it is a powerful bacteriostatic agent against S. aureus, L. monocytogenes and S. enterica, has powerful bactericidal activity against S. aureus. and also strong fungistatic activity against P. ochrochloron and T. viride [44]. The effect of volatile oil of celery was tested against 21 pathogenic strains. Results have shown bactericidal potential with the gram-positive strains presenting increased sensitivity towards gram-negative strains [45].

Given the fact that chamomile has been previously studied for various pharmacological properties, we compared the results in our tests to parsley and celery as sources of apigenin derivatives. There are no previous studies on the seeds of parsley and celery concerning their potential against acetyl- and butyrylcholinesterase. In our previous study we have also described the activity of selected extracts against lipoxygenase [16]. The results obtained in the activity against the enzymes, indicates that the chamomile and the celery extract possess a similar inhibitory potential against lipoxygenase, but a stronger activity against acetylcholinesterase is given by chamomile. Nevertheless, parsley has a higher inhibitory activity against lipoxygenase, but this is probably due to the proportion between the active compounds which are similar to celery but vary in the amount. Butyrylcholinesterase is a non-specific cholinesterase that is spread throughout the body. Its main role is to hydrolyze choline esters especially in the liver, pancreas, blood serum and brain [46]. Past research has indicated that both cholinesterases present extensive gene amplification in various malignant tumors (ovarian, brain), but the exact mechanisms remained unknown. Nevertheless, there was a noted an interesting correlation to these enzymes coamplification and transitory altered mode of expression located in the proliferation tissue, thus indicating a higher selectivity for such cell processes [47]. Recently, butyrylcholinesterase has been associated with prognosis for different diseases, some researchers sustain the used of this
enzyme’s activity a biomarker for therapeutic response in certain types of cancer [48]. Moreover in a comprehensive review, the group of Lazarevic-Pasti et al. have discussed about the fact that in different types of tumors an irregular expression of acetylcholinesterase could be detected, suggesting a direct correlation between the expression of this enzyme and tumor development. Thus, an inhibitory activity towards this enzyme can be one of the multiple mechanisms of action of an anti-cancer drug [49]. Supporting this idea Xi et al., have completed the ‘picture’ about the anti-cancer mechanism of this enzyme pointing other important activities such as: regulator of proliferation, apoptosis, cell adhesion and differentiation [50]. As well it is very well known and very well debated in the literature the relation between inflammation induced cancer and cancer induced inflammation [51]. Therefore, inhibitors of lipoxygenase can be considered as possible candidates for cancer therapeutics [52]. Wisastra et al., expressed in a very plastic way the aforementioned mechanisms stating that cancer, inflammation and oxidative lipoxygenase activity are intimately linked [53].

Therefore, the use of this enzyme in the research is of extreme importance in terms of the biological activity of the investigated extracts.

Our results come in hand for future approach in regards to the development of new therapeutic approaches that use natural compounds as the active drug. Moreover, the identification of rich natural sources is of use for both food and pharmaceutical industries. The data presented in this study indicated that the percentage of viable MCF7 cells was most affected after treatment with the highest concentration (60µg/mL) of parsley extract (36.47% viability inhibition), followed by the effect induced by celery extract (21.58%), while the smallest effect was recorded after treatment of cells with chamomile extract (18.95%) (Figure 3). However, the data obtained for chamomile extract was not endorsed by the LDH technique, employed for cytotoxicity testing of the extracts. In this case, a slight proliferative effect of MCF7 cells was observed which may be explained by an intracellular alteration of cells function, that could not be quantified by the LDH assay [23]. In this situation the annexin V-PI technique provided more relevant information regarding the percentage of necrotic cells, showing that chamomile extract induced the higher rate of necrotic events (17.65%), followed by parsley (12.99%) and celery (8.89%) extracts.

Chamomile and celery extracts presented strong anti-migratory and antiproliferative potential 24h post-treatment, MCF7 cells expressing a wound closure rate of 14.93% and 9.30%, respectively. However, the cells stimulated with parsley extract displayed a higher rate of wound closure, 39.64%, compared with chamomile and celery extracts (Figure 4) which may be caused by the short incubation time (24h) performed for the scratch assay technique, compared with the stimulation time used for all other techniques (72h) accomplished in this study.

To evaluate DNA content and its distribution inside the nuclei of MCF7 cells treated with test extracts, DAPI staining was employed as a preliminary assay. The method revealed that chamomile extract induced the most noxious activity on MCF7 cells - numerous nuclei undergoing alterations, while parsley and celery extracts induced only several nuclei with condensed chromatin (data not shown). The cellular death markers screened through DAPI staining were further differentiated and quantified using flow cytometry, thus confirming the highest rate of necrosis recorded for MCF7 cells stimulated with the chamomile extract at concentration of 60 µg/mL. Nevertheless, MCF7 cells stimulated with parsley and celery extracts developed total apoptotic events of 6.63% and 8.99%, respectively. It is important to make a precise differentiation between the types of cellular death (early-apoptosis/late-apoptosis and necrosis) induced by test extracts to gain insight into their mechanism of action which can modulate further the cellular response showing different expression of the cellular markers [54]. Based on these results, the cytotoxic mechanism induced by the chamomile extract on MCF7 cells could be considered necrosis, while the parsley and celery extracts showed several apoptotic events. The distinctive cytotoxic mechanism manifested by chamomile extract versus parsley and celery extracts could be related to the phytochemical composition of extracts; chamomile extract presents a high amount of apigenin-7-O-glucoside, while parsley and celery extracts contain high percentage of apigenin. Our results are in agreement with the ones presented by Smiljkovica et al., who revealed that the effect induced by apigenin-7-O-glucoside on HCT116 cells led to an increased biologically effect when compared with apigenin, showing that HCT116 cells exhibited important necrotic events [55]. These results support our hypothesis that the chamomile extract induce necrosis due to a high amount of apigenin-7-O-glucoside. In the same study apigenin has been associated to induce apoptosis through p53 pathway and to regulate Bax expression in colon cancer HCT116 cells, thus offering an answer to the possible molecular mechanism of how apigenin-high percentage extracts, namely parsley and celery extracts, affect tumourcells.

As described in the comprehensive review of Srivastava et al., an increasing number of papers about the anti-cancer activity of chamomile extract involve studies towards the flavone apigenin, one of the most bioactive constituents. In vitro and in vivo studies have shown beneficial effects by eliciting growth inhibitory potential in case of skin, prostate, ovarian and breast cancer models [5]. The same author has evaluated the antiproliferative and pro-apoptotic potential of aqueous and methanolic extracts against different human cancer cell lines and underlined the positive effect on cancerous cells associated with minimal growth inhibitory effect for normal cells [56]. In a complex approach about the in vitro cytotoxic (PC-3- human prostate, A-549 - human lung carcinoma and MCF7 - human breast cancer cells) and antibacterial potential (Propionibacterium acnes) of ten volatile oils, chamomile oil was also included. The essential oil at a concentration of 0.200% (v/v) exhibited a strong cytotoxic effect against PC-3 - human prostate cancer cells. Also the viability of MCF7 - human breast cancer cells was reduced to 6.93% [57]. Kandelous et al., have shown that extracts obtained from the aerial part of 'Roman chamomile', known under the scientific name of Chamaemelum nobile (L.) all in the range of concentrations of (0.001 - 0.25 mg/mL) elicit antiproliferative effect against MCF7 cells [58]. Also when parsley and celery are mentioned, an increased number of studies related to the chemopreventive potential for in vitro and in vivo models of breast cancer of apigenin [59, 60]. In a similar approach Farshori
et al., have screened the anticancer activity of alcoholic extracts and oil of parsley seeds against MCF7 - human breast cancer cells. The study concluded that both the alcoholic extract as well as the oil have significantly reduced cell viability in a dose-dependent manner. Moreover, doses over 50 μg/mL for the extract and 100 μg/mL for the oil were found to be cytotoxic for this breast cancer cell line [61]. In a recent study Tang et al., have analyzed the effect of five types of extracts obtained from leaf and stem of (Mill.) Fuss towards normal 3T3-L1 fibroblasts and MCF-7, MDA-MB-231 (breast) and HT-29 (colorectal) cancer cell lines. Results have shown a weak cytotoxic activity, dichloromethane being the best type of extract, conducting at the highest tested concentration, namely 500 μg/mL to a percentage of inhibition of 48.4% ± 1.8% for MCF7 cells, 25.5% ± 3.0% for MDA-MB-231 cells and 49.9% ± 1.0% for HT-29 cells. Furthermore, at the concentration of 300 μg/mL, the extract inhibited H2O2-induced MCF7 cell migration thus assigning the extract with protective effects against metastasis [62]. Schröder et al., discussed the dose-dependent effect of root extract for the proliferation of MCF7 cells and concluded that in the range of concentrations of [0.01 μg/mL - 100 μg/mL] the extract does not elicit a cytotoxic effect, however at 500 μg/mL the extract was cytotoxic for more than 70% of cells [63]. Regarding the celery extract, literature is poor in information about the effect against breast cancer cells, however antiproliferative and proapoptotic activity against other cancer cell lines was previously reported [64, 65].

CONCLUSION

The screened samples contain phytochemicals belonging in majority to the class of flavonoids and polyphenols. In terms of activity on enzymes an inhibition of acetylcholinesterase and butyrylcholinesterase could be detected. Chamomile and parsley extracts were active only against tested Gram-positive cocci, while all tested extracts displayed antifungal effects. At the highest tested concentration, namely 60 μg/mL parsley was the most active extract in terms of reducing the viability of MCF7 human breast adenocarcinoma cell line and inducing the release of lactate dehydrogenase. In the set experimental conditions chamomile and celery extracts present relevant and moderate antiproliferative and cytotoxic potential. On the other hand celery extract manifested the most potent anti-migratory effect and was the most active extract in terms of total apoptotic events (both early and late). This preliminary study conducted for the assessment of the various biological effects of screened samples show that further steps need to be undertaken for a clear understanding of the complex mechanisms of this bioactive phytocomplexes.

LIST OF ABBREVIATIONS

MCF7 = Human breast cancer cell
FTIR = Fourier-transform infrared spectroscopy
CLSI = Clinical Laboratory Standards Institute
LDH = Lactate dehydrogenase
RP-UHPLC = Reversed Phase-Ultra-High Performance Liquid Chromatography
DPPH = 2,2-diphenyl-1-picrylhydrazyl
ABTS = 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
GRAS = generally recognized as safe
DMSO = Dimethyl sulfoxide
MIC = minimum inhibitory concentration
ECACC = the European Collection of Authenticated Cell Cultures
MTT = (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS


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