THYMUS VULGARIS EXTRACT FORMULATED AS CYCLODEXTRIN COMPLEXES: SYNTHESIS, CHARACTERIZATION, ANTIOXIDANT ACTIVITY AND *IN VITRO* CYTOTOXICITY ASSESSMENT

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Abstract

Thymus species are important sources of bioactive compounds but considering their low stability and solubility in aqueous solutions, the pharmaceutical utilization is limited. A method to overcome these drawbacks was proposed in the current study, by encapsulating thyme total hydro-alcoholic extract (TE) in β - and γ - CD (cyclodextrins). It was assessed the antioxidant activity (AOA) in time (DPPH assay) and the *in vitro* cytotoxicity on MCF-7 and MDA-MB-231 breast cancer cells, using the Alamar blue test (cells viability), scratch assay technique (cells migration and proliferation) and Annexin V-FITC assay (cells apoptosis). The TE and its CD complexes showed a significant AOA over a longer time interval indicating a controlled release of the active compounds. *In vitro* tests indicated a significant cytotoxic activity of both CD complexes as compared to TE, even at a low concentration (10 $\mu\text{g/mL}$), the most pronounced activity being recorded for TE γ -CD inclusion complex on MDA-MB-231 cells (32% viable cells at 100 $\mu\text{g/mL}$).

Rezumat

Speciile de *Thymus* sunt surse importante de compuși bioactivi, dar având în vedere stabilitatea și solubilitatea scăzute în soluții apoase, utilizarea farmaceutică a acestora este limitată. În studiul de față, pentru corectarea acestor dezavantaje s-a recurs la încapsularea extractului hidroalcoolic total de cimbru (TE) în β - și γ -CD (ciclodextrine). Ulterior, a fost evaluată activitatea antioxidantă (AOA) în dinamica temporală (prin metoda DPPH) și citotoxicitatea *in vitro* pe celulele de cancer mamar MCF-7 și MDA-MB-231, prin testul Alamar blue (viabilitatea celulară), prin testul *scratch* (migrarea și proliferarea celulară) și testul Annexin V-FITC (apoptoza celulară). TE și complexii acestuia cu CD au prezentat o AOA semnificativă pe un interval de timp mai lung, indicând o eliberare controlată a compușilor activi. Testele *in vitro* au indicat o activitate citotoxică semnificativă a ambilor complecși cu CD comparativ cu TE, chiar și la o concentrație scăzută (10 $\mu\text{g/mL}$), activitatea cea mai pronunțată fiind înregistrată pentru complexul de incluziune TE γ -CD pe celulele MDA-MB-231 (32% celule viabile la 100 $\mu\text{g/mL}$).

Keywords: thyme, cyclodextrin, breast cancer, viability, apoptosis

Introduction

In the last decades, the application of natural and traditional herbal treatments increased remarkably and presents multiple advantages, including improved efficacy in different medical fields. The augmentation of drug resistance determined the researchers to concentrate on finding new sources of effective medicines what led to the development of phytotherapy. Currently, the pharmaceutical industry relies on almost 50% formulations based on plants and herbs, and traditional and complementary and alternative medicine influenced the current approach of many pathologies by highlighting the major role of phytochemicals and nutraceuticals [2, 16]. Extracts obtained from

various aromatic and medicinal plants can serve as new pharmaceutical remedies [12]. Aromatic plants of high therapeutic importance include also *Thymus vulgaris*, one of the ~300 species of the *Thymus* genus. All *Thymus* species are recognized for their antiseptic, antimicrobial and more recently, antitumoral properties, being used as traditional therapy [12]. A compound possesses biological activity if fulfils the following conditions: ability to cross the cell membrane, to keep its integrity during the absorption and distribution processes, and to reach the target site of action regardless of molecular structure. With regard to phytochemicals, they have a low stability and solubility, what leads to a reduced bioavailability, restricting their use in the pharmaceutical field for

prophylactic and curative purposes. A promising alternative to improve their properties is to include them in molecules of natural origin (e.g. cyclodextrins) that are considered safe from a toxicological point of view [13, 15]. Cyclodextrins are part of the class of the most widely used molecules in host-guest chemistry, with application in the food, cosmetic and pharmaceutical fields. Native cyclodextrins, α -, β - and γ -, are obtained by the treatment of starch with amylase isolated from the *Bacillus macerans* strain [14]. The use of cyclodextrins in the pharmaceutical domain is approved by FDA, and are considered exceptional drug carriers, due to their property to encapsulate lipophilic molecules through non-covalent interactions [17, 21].

Breast malignancies are the leading cause of death among women over 40 years old, with an estimated annual diagnosis of over 1 million cases [5, 11]. Moreover, the increased morbidity among patients may be related to the current treatment approach - surgery, both radio- and chemo- therapies [5, 11]. Therefore, finding new therapeutic approaches to reduce morbidity in cancer patients is a challenge for all scientists involved (chemists, biologists, pharmacists, physicians), and is directly related to natural resources, especially medicinal plants.

The main purposes of this study were: to obtain, to characterize, and to assess the *in vitro* activity of hydro-alcoholic total extract from *Thymus vulgaris* (TE), and its inclusion complexes in β - and γ -cyclodextrin (TE/bCD and TE/gCD), on two breast cancer cell lines with different receptor expression - MCF-7 (oestrogen receptor) and MDA-MB-231 (non-oestrogen receptor with epidermal growth factor - EGF and transforming growth factor alpha - TGF alpha expressed).

Materials and Methods

Reagents

β -cyclodextrin and γ -cyclodextrin were purchased from Cyclolab Hungary; 1,1-diphenyl-2-picrylhydrazyl (DPPH) from Sigma Aldrich, Germany; ascorbic acid from Lach-Ner, Czech Republic; Folin-Ciocalteu reagent (Sigma-Aldrich; Merck KGaA), all standard compounds, reagents and solvents were purchased from Sigma-Aldrich, Germany. All substances were used as received.

Extract and inclusion complexes preparation

Thymus vulgaris (thyme) was received as a gift from Favisan Laboratories Lugoj and a voucher specimen (no. CD_004_1) is preserved at the Herbarium of the Faculty of Pharmacy, "Victor Babes" University of Medicine and Pharmacy Timisoara, Romania.

Thyme dried aerial parts (50 g) were extracted by maceration with 70% ethanol (100 mL) for 24 hours. The resulted solution was filtered, subjected to evaporation by means of a rotary evaporator under

vacuum, and the resulting mass was lyophilized and stored at -20°C, until further determinations.

The TE cyclodextrin inclusion complexes were prepared according to a kneading method previously described in the literature [8] with some minor changes: a simple powder mixing of the total lyophilized extract and cyclodextrin in 1:2 molar ratio (the molar ratio was chosen based on the hypothesis that the total extract was a bioactive compound - rutin, $Mm_{Rutin} = 610.52$ g/mol, $Mm_{bCD} = 1134.98$ g/mol and $Mm_{gCD} = 1297.128$ g/mol) using a mortar and a pestle, followed by kneading with an ethanolic solution (50% w/w water: ethanol) until the solvent evaporation and a paste-type mass formation. The final supramolecular system was obtained after drying at room temperature for at least 24 h, followed by drying in the oven at 105°C, for several hours until reaching a constant weight. The physical mixture of the compounds was used for comparative formation studies of inclusion compounds.

Physico-chemical characterization

Total polyphenols, flavonoids, flavonols, and tannins were determined by well-known and frequently used spectrophotometric methods: (1) total phenols (TP) by Folin-Ciocalteu method - extract samples were treated with Folin-Ciocalteu aqueous reagent (1:10 dilution), incubated for 300 seconds at room temperature, followed by addition of sodium carbonate, heated at 50°C and absorbance measurements at 750 nm using an UV-VIS spectrophotometer (Specord 205; Analytik Jena), the results were expressed in mg GAE/g dry material; (2) flavonoids/flavonols (TF/TFv) by Al colorimetric test - extract samples were treated with 2% $AlCl_3$ solution and the absorbance measurements were performed at 417 nm and 445 nm, respectively; the results were expressed as rutin equivalents (RE); (3) total condensed tannin (TT) by vanillin test, extract samples were treated with vanillin solution and the absorbance measurements were registered at 500 nm, the results were expressed as milligrams of (+)-catechin equivalents (mg CE/g extract). All experiments were performed in triplicate.

LC-MS analysis. Quantitative analysis of polyphenolic compounds was conducted on Shimadzu chromatograph: detectors - SPD-10A UV and LC-MS 2010; column - EC 150/2 NUCLEODUR C18 Gravity SB 150 x 2 mm x 5 μ m; chromatographic conditions, mobile phase A - water acidified with HCOOH, mobile phase B - C_2H_5N acidified with HCOOH, gradient program: 0' - 20' 5% B, 20' - 50' 5% -40% B, 5' - 55', 40% - 95% B, 55' - 60' 95% B; f.r. of 0.2 mL/min; temperature 20°C; wavelengths 280 nm and 320 nm. Experiments were conducted in duplicate.

Scanning Electron Microscopy (SEM). To evaluate supramolecular system morphology, the inclusion complexes were examined by scanning electron microscopy (Hitachi S4700, Hitachi Scientific Ltd., Tokyo, Japan) and a sputter coating apparatus was

utilized for the induction of electric conductivity on the surface of the samples.

Differential Scanning Calorimetry (DSC). To verify the complexes behaviour and to confirm the formation of inclusion complexes it was used a thermal analysis system device Mettler Toledo DSC 821e with STARE V6.0 program (Mettler Inc., Schwerzenbach, Switzerland); ~ 2 - 4 mg of tested compounds were examined in the temperature range of 25°C and 350°C with the heating rate 10°C/min and Argon used as carrier gas, at a flow rate of 10 L/h.

FTIR analysis. This test was performed using the FT-IR Jasco 430 spectrophotometer in the frequency range of 4000 cm^{-1} and 400 cm^{-1} . Solid samples subjected to analysis were prepared in potassium bromide pills and IR spectra of supramolecular systems were processed and compared with the spectra of pure β - and γ - cyclodextrins, thyme total extract and their physical mixtures.

Antioxidant activity. Evaluation of the antioxidant activity of these complex systems was performed using separate solutions of TE (1 mg/mL) and TE complexes (4 mg/mL). The antioxidant activity analysis was performed using a T70 UV/VIS Spectrophotometer (PG Instruments Ltd.), and the data obtained were processed using the UV-Win software version V5.2.0.1104. A mixture of 2 mL 96% ethanol (or 2 mL of distilled water), 0.5 mL of sample solution, alcoholic or aqueous, and 0.5 mL of 1 mM DPPH solution was prepared, and the absorbance was recorded at 517 nm for 900s. The solution discolours over time as DPPH reacts with the free radicals or their precursors in the system, and the progress of the reaction is monitored spectrophotometrically. All readings were reported to an alcoholic or aqueous reference solution.

Cell culture

HaCaT - immortalized human keratinocytes (ATCC – American Type Culture Collection) and MDA-MB-231 (ATCC[®] HTB-26[™]) human breast cancer cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium, Sigma Aldrich) supplemented with 10% FCS (foetal bovine serum, Sigma-Aldrich, Germany) and 1% penicillin-streptomycin (Pen/Strep 10,000 U/mL; Gibco). MCF-7 (ATCC[®] HTB-22[™]) human breast cancer cells were cultured in EMEM (Eagle's Minimum Essential Medium, ATCC) supplemented with 10% FCS (foetal bovine serum, Sigma-Aldrich, Germany) and 1% penicillin-streptomycin (Pen/Strep 10,000 U/mL; Gibco). During the experiment, all cell lines were kept in standard conditions: incubation at 37°C in 5% CO₂ atmosphere. The following sample concentrations were tested: 10, 50 and 100 $\mu\text{g/mL}$.

Alamar blue assay. Resazurin sodium salt (Sigma-Aldrich, Germany) was used as bioreagent to determine cell viability, and was therefore dissolved in PBS (Phosphate Buffered Saline, Fisher BioReagents, USA) to obtain a suitable Alamar Blue solution. Cells were

allowed to adhere to 96-well culture plates (1 x 10⁴ cells/well) until the appropriate confluence was reached (24 - 48 h). Then, the cells were stimulated with 200 μL of new medium containing the tested substances (10, 50, and 100 $\mu\text{g/mL}$). Substances were allowed to interact for 24 h with the cells, followed by addition of Alamar Blue solution to each well (20 $\mu\text{L/well}$). After 3 h of incubation at 37°C, absorbance was read at two wavelengths (570 and 600 nm). The spectrometer (TECAN, infinite M 200 PRO, Austria) was used to read the absorbance values. The results were obtained in quadruplicate. Unstimulated cells were used as a reference/control.

Scratch assay. The cellular ability to migrate and proliferate was assessed by means of a wound healing - type technique - scratch assay. Briefly, 2 x 10⁵ cells/well were seeded in 12-well plates, and when a 90% confluence was obtained, a scratch was drawn in the middle of the well with a 10 μL sterile tip. To observe the effect of the TE, TE/ β CD, and TE/ γ CD (10 $\mu\text{g/mL}$) in terms of proliferation and migration, the difference between the initial and after 24 h wound width, was studied and pictures at 10 $\times$ magnification have been made with an Olympus IX73 inverted microscope equipped with DP74 camera (Olympus, Tokyo, Japan).

Annexin V/PI apoptosis assay. MCF-7 and MDA-MB-231 human breast cancer cells were seeded into 6-well plates (3 x 10⁵ cells/well) in order to study cell apoptosis. For this purpose, flow cytometry analysis were performed using an Annexin V-FITC kit (eBioscience, Viena, Austria). Selected concentrations (10, 50 and 100 $\mu\text{g/mL}$) of tested compounds were added. After 24 h of treatment, cells were washed with 500 μL PBS and centrifugated for 5 min at 1500 rpm. The cells were resuspended in 200 μL , and a mixture of 195 μL Annexin V binding buffer and 5 μL of FITC-conjugated Annexin V was added to cell suspension. The samples were incubated for 15 min at room temperature in the dark. Finally, after two more consecutive wash steps with 200 μL binding buffer, 10 μL of propidium iodide solution (20 $\mu\text{g/mL}$) were added. The samples were analysed by flow cytometry (FACS Calibur; Becton Dickson, Franklin Lakes, NJ, USA). Cells that were not exposed to the tested substances were used as control.

Statistical analyses

The analysis program and software utilized in the current study were GraphPad Prism 7 and Flowing Software Version 2.5.1. Results were interpreted using one-way ANOVA, followed by Tukey's post-tests when appropriate, to establish the statistical difference between experimental and blank lots; *, **, *** and **** indicate $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively. The obtained results are shown as mean value of three experiments $\pm$ standard deviation.

Results and Discussion

The chemical composition of plant extracts varies significantly depending on several parameters, such as: climatic influences, growing and harvesting processes, genetic variability, and the type of processing and extraction methods chosen – solvent, extraction period, temperature, etc. [6, 9, 20].

The extraction yield percentages of the total extracts obtained from aerial parts of thyme by using 70% ethanol (maceration) are presented in Table I, together with the total content of different classes of biologically active compounds. The highest quantity of phenols (TPC), flavonoids (TFC), flavonols (TFvC) and tannins (TT) were detected in the TE.

Table I

Total phenolic, flavonoid, flavonols and tannins content found in the extracts of *Thymus vulgaris* (n = 3, mean ± SD)

Extract	Extraction yields (%)	TPC (mg GAE/g dm)	TFC (mg RE/g dm)	TFvC (mg RE/g dm)	TT (mg CE/g d.m.)
TE	22.7	37.2 ± 1.6	43.8 ± 0.6	3.6 ± 1.1	16.8 ± 0.6

The individual quantitative analysis of bioactive phytochemicals like polyphenols, revealed the amounts of each type of molecule found in the total hydro alcoholic extract of thyme, rosmarinic acid, rutin and kaempferol being the major compounds.

As can be observed in Table II, caffeic acid and quercetin are found in sufficient quantities. The identity of the compounds was certified by HPLC-MS by comparison with analytical standard of each compound.

Table II

Major phenolic compounds (% of total) identified in *Thymus vulgaris* total hydro alcoholic extract by HPLC-MS

Sample	Compound name	Retention time	Concentration (µg/g)
TE	Gallic acid	4.876	1.492
	Protocatechuic acid	11.181	0.626
	Caffeic acid	21.180	52.081
	Epicatechin	22.125	26.618
	p-Coumaric acid	24.573	0.246
	Ferulic acid	23.455	5.417
	Rutin	25.122	1285.389
	Rosmarinic acid	27.418	1490.881
	Resveratrol	29.745	8.637
	Quercetin	31.839	75.329
	Kaempferol	35.451	1083.883

Natural bioactive compounds present increased sensitivity to degradation and inactivation, facts that led to a rapid development of protection techniques with the mandatory requirement to secure the bioactive compounds from chemical degradation (oxidation, hydrolysis) without altering their properties [10, 21]. Inclusion in cyclodextrins leads to encapsulated compounds protected from the action of external factors that can be released gradually into the biological aqueous environment due to the association-dissociation equilibrium that occurs [8]. In the case of TE, after the complexing process, high recovery rates were obtained: from 72.3% TE/βCD complex and to 84.6% TE/γCD complex. The βCD complex was obtained with a slightly lower yield, probably due to a higher solubility of this complex in the ethanol-water solution used for complexation. FT-IR spectroscopic analysis confirms the interaction between the extract and the cyclodextrins and implicitly the formation of their complexes. The FT-IR spectra of the complexes were compared to those of pure substances (extract and cyclodextrins). The absorption bands characteristic for extract and cyclodextrins were also found in the spectra of the complexes confirming that the extract was encapsulated. In Figures 1 and 2 are presented the

βCD and TE/βCD complexes, where the characteristic bands of the phenolic hydroxyl groups are less intense for the complex, at 3421 cm⁻¹ and 1600 cm⁻¹, and the characteristic βCD band, ν_{COC} at 1027 cm⁻¹, is also found in complex as equally intense at 1026 cm⁻¹. The same characteristic signals can be noticed within the complex with γCD. The DSC technique can highlight the thermal degradation and thermal-oxidative degradation processes of materials of inorganic and organic nature and the DSC curve is specific to each material; therefore, this approach can be considered as a thermal spectroscopy method by which a material can be identified [19]. The DSC profiles of extract, pure cyclodextrins and their inclusion complexes were analysed. βCD and γCD present a large endothermic peak before 95°C and 80°C, denoting the gradual loss of H₂O while TE/CD complexes displays a very large endothermic peak with the onset around 85 - 90°C (TE/βCD) and 65 - 70°C (TE/γCD). The formation of supramolecular systems is showed by the modification of the water dissociation energy (WDE) from 1290 mJ (βCD) to 892.26 mJ (TE/βCD) and from 435.92 mJ (γCD) to 182.85 mJ (TE/γCD), respectively (see Figures 1 and 2). βCD, TE/βCD, γCD and TE/γCD were investigated by SEM to establish

the morphology of the compounds. The compounds were analysed at 100 μm scale with 800 magnification and the micrographs present the crystalline nature of the CDs with variable dimensions and irregularities in orientation and structure with the aggregation capacity (Figures 1 and 2). The physical mixture of

extract and cyclodextrin (data not included) presents a different shape and structure. Both inclusion complexes exhibited an amorphous form and the presence of small particles with various shaped and cluster formation with different sizes.

Figure 1.

DSC analysis of βCD (up) and TE/ βCD (down) on the temperature range 25 - 350°C; FTIR spectra of βCD (up) and TE/ βCD (down) in the range of 4000 - 400 cm^{-1} ; TEM images of the compounds βCD (up) and TE/ βCD (down) - scale bars are 100 μm

Figure 2.

DSC analysis of γCD (up) and TE/ γCD (down) on the temperature range 25 - 350°C; FTIR spectra of γCD (up) and TE/ γCD (down) in the range of 4000 - 400 cm^{-1} ; TEM images of the compounds γCD (up) and TE/ γCD (down) - scale bars are 100 μm

The antioxidant activity of plant extracts and/or bioactive compounds can be assessed by spectrophotometric methods using the DPPH radical capture method. One aspect which must be considered in these studies is the competitiveness of the reactant at the biocompatible/cyclodextrin-binding/dissociation balance. An important indicator that cumulates these aspects is the reaction rate of DPPH in the presence of samples to be analysed, suggesting the reactivity of the compounds with antioxidant activity, *i.e.* the possibility of their controlled release from the complexes with cyclo-

dextrins. Although the final antioxidant activity is significant, an insignificant reaction rate of DPPH over the final time interval indicates that there is no antioxidant effect (high reactivity and/or no controlled release for the antioxidant compound). The antioxidant activity was calculated as a difference from 100 percent of the absorbance at time *t* relative to initial absorbance (the ratio between “*t*” time absorbance and initial absorbance at 517 nm), expressed as a percentage, as previously published in our studies [18]. To evaluate the behaviour of bioactive compounds

studied in the presence of reactive species in the human body (e.g. reactive oxygen species, ROS, free radicals, etc.), DPPH reaction rates were determined on the pseudo linear portions of the concentration-time variation curves. The antioxidant activity (Figure 3) for

TE and the corresponding β CD and γ CD complexes (at comparable extract concentrations) is ~ 71%, ~ 81% and ~ 56% respectively. However, reaction rates of DPPH at representative time intervals (10 - 60 s, 60 - 180 s and 180 - 900 s) are relatively close.

Figure 3.

Representation of antioxidant activity (AOA %) over time for thyme total hydro-alcoholic extract (TE) and its inclusions complexes in β - and γ - cyclodextrins (TE/βCD and TE/γCD)

To evaluate the activity exerted by the thyme total hydro-alcoholic extract and its cyclodextrin inclusion complexes on normal human (HaCaT), and breast cancer (MCF-7 and MDA-MB-231) cells viability, it was selected the Alamar blue test. Viability and proliferation assay with Alamar blue is an indicator based on detection of metabolic activity. Reduction related to growth causes the redox indicator to change from oxidized (blue) form to reduced (red).

Figure 4.

Viability percentage of HaCaT (human keartinocytes) at 24 h post-stimulation with thyme total hydro-alcoholic extract and its inclusions complexes with β - and γ - cyclodextrins (10, 50 and 100 μg/mL)

Stimulation of keratinocytes with TE, TE/βCD and TE/γCD showed a slight increase in cells viability as can be observed in Figure 4, especially for TE/γCD (~ 109 % viable cells at 10 μg/mL, ~ 108% viable cells at 50 μg/mL and ~ 114% viable cells at 100 μg/mL, respectively) as compared to control cells (unstimulated cells). Pure tested cyclodextrins have no noticeable influence on cells viability.

Stimulation of human breast cancer cells (MCF-7 and MDA-MB-231) was performed in the same conditions as applied for normal cells, in order to assess the cytotoxic activity of TE, TE/βCD and TE/γCD (10, 50 and 100 μg/mL) for 24 h. Data revealed that inclusion complexes of thyme total hydro-alcoholic extract exerted a significant decrease in cell viability percentage, cell-type dependent. In the case of MCF-7, TE produced a minor effect on cells viability (~ 95% viable cells at 100 μg/mL), while both inclusion complexes, TE/βCD and TE/γCD induced a reduction of cell viability in a concentration dependent manner. The lowest value of MCF-7 viable cells was obtained for TE/βCD at 100 μg/mL ~ 63%. In the case of MDA-MB-231 cells, it was observed the same activity, but more pronounced even in the case of TE. The following data were obtained: ~ 60% viable cells at 100 μg/mL TE, ~ 49% viable cells at 100 μg/mL TE/βCD and ~ 32% viable cells at 100 μg/mL TE/γCD (see Figure 5).

Figure 5.

Viability percentage of MCF-7 and MDA-MB-231 (human breast cancer cells) at 24 h post-stimulation with thyme total hydro-alcoholic extract and its inclusions complexes with β - and γ - cyclodextrins (10, 50 and 100 $\mu\text{g/mL}$)

Figure 6 reveals the migratory capacity of the normal cells in the presence of TE, TE/ β CD and TE/ γ CD. The concentration applied for this analysis was the lowest, namely 10 $\mu\text{g/mL}$ for better observation of cells behaviour. After TE stimulation, no interference

was observed, the cells grew normally. The same remarks can be made and related to the stimulation with TE/ β CD while in the case of TE/ γ CD a stimulatory effect on the cells was observed (Figure 6).

Figure 6.

The impact of thyme total hydro-alcoholic extract and its inclusions complexes with β - and γ - cyclodextrins (TE, TE/ β CD, and TE/ γ CD 10 $\mu\text{g/mL}$) on the migratory capacity of normal cells (HaCaT)

The *in vitro* scratch assay showed that the breast cancer cells, MCF-7 and MDA-MB-231, migratory capacity was slightly inhibited in a different manner by the tested compounds. After MCF-7 cells stimulation with TE and TE/ γ CD no significant influence was observed; moreover, a stimulatory effect could be noticed, while in the case of TE/ β CD the partial filling with the cells of the trace line can be easily

observed (Figure 7). On the other hand, after stimulation with the samples, MDA-MB-231 cells are clearly influenced in terms of cell migration and proliferation by the TE/ γ CD and for TE and TE/ β CD, there is a slight inhibition of these processes, as it can be seen in the Figure 8, data that are consistent with the results from cells viability assessment.

Figure 7.

The impact of thyme total hydro-alcoholic extract and its inclusions complexes with β - and γ - cyclodextrins (TE, TE/βCD, and TE/γCD 10 $\mu\text{g/mL}$) on the migratory capacity of tumoral cells (MCF-7)

Figure 8.

The impact of thyme total hydro-alcoholic extract and its inclusions complexes with β - and γ - cyclodextrins (TE, TE/βCD, and TE/γCD 10 $\mu\text{g/mL}$) on the migratory capacity of tumoral cells (MDA-MB-231)

In Figure 9 is presented the effect of the samples on MCF-7 and MDA-MB-231 breast cancer cells apoptotic status. At 100 $\mu\text{g/mL}$ it was recorded a significant increase of dead cells, a pronounced pro-apoptotic effect, and the highest percentage of apoptotic cells was determined by inclusion complexes in both cell types: $\sim 31\%$ (TE/βCD) and $\sim 37\%$ (TE/γCD) MCF-7 apoptotic cells, and $\sim 37\%$ (TE/βCD) and $\sim 86\%$

(TE/γCD) MDA-MB-231 apoptotic cells (Figure 9). The cells status was assessed by a FACS technique, represented in four quadrants: Q1 with necrotic cells, Q2 with late apoptotic cells, Q3 with early apoptotic cells, and Q4 with viable cells. In Figure 9 are expressed only early apoptotic cells which are calculated as percentage of apoptotic cells compared to untreated cells.

Figure 9.

Representative dot plots of the apoptotic events induced by thyme total hydro-alcoholic extract and its inclusions complexes with β - and γ - cyclodextrins (TE, TE/ β CD, and TE/ γ CD 10, 50, and 100 μ g/mL) in MCF-7 and MDA-MB-231 breast cancer cells after a 24 h stimulation

Biologically active compounds permanently attract the attention of researchers in the medical/pharmaceutical fields due to the promising activity they exert on the tumour cells. Thyme extract and its individual bioactive constituents represented the subject of various studies conducted in the field. For example, Abaza *et al.* studied the activity of isolated naringenin from *Thymus vulgaris* extract on colorectal and breast cancer cells and showed that the compound induces apoptosis and potentiates the viability of tumoral cells through multifactorial mechanisms (e.g. cell cycle arrest, up-regulation and down-regulation specific genes, inhibition of pro-survival signalling pathways); Arunasree presented the anti-proliferative effect of carvacrol in human breast cancer cells, with an IC_{50} value of 100 μ M [1, 3]. Bozkurt *et al.* stimulated human breast cancer cells, MCF-7 and MDA-MB-231, with *Thymus serpyllum* methanolic extract and, at 72 h post-stimulation the IC_{50} values obtained were 509 μ g/mL and 276 μ g/mL [7]. In another study regarding the evaluation of MCF-7 cells viability in the presence of different extracts from *Lamiaceae* family was observed that *Thymus vulgaris* and *Thymus serpyllum* aqueous extracts performed almost similar activity, the IC_{50} values recorded being 407 and 399 μ g/mL, respectively [4].

Conclusions

In summary, related to the considerable biological effect of phytochemicals on pathologies considered for the time being incurable, the current research was performed in order to evaluate the antioxidant, anti-proliferative and antitumoral effects of TE on human breast cancer cells. To enhance the stability and solubility of TE, the inclusion complexes with β -

and γ - cyclodextrins were prepared successfully and the formation of the supramolecular systems was confirmed by SEM, DSC and FTIR. The compounds shown significant antioxidant activity over different time intervals, which highlights the controlled release of biologically active compounds from extract. TE/ β CD and TE/ γ CD denoted a significant cytotoxic activity on MCF-7 and MDA-MB-231 cells, with the most notable activity of the TE/ γ CD complex on cells that expressed epidermal growth factor (EGF) and transforming growth factor alpha (TGF alpha).

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