Physico-chemical and Biological Evaluation of Flavonols: Fisetin, Quercetin and Kaempferol Alone and Incorporated in beta Cyclodextrins

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Abstract: Incorporation of fisetin, quercetin and kaempferol in beta cyclodextrins was proved by scanning electron microscopy (SEM), differential scanning calorimetry (DSC) and X-ray powder diffraction (XRPD). Pure compounds and their complexes were tested for antiproliferative and pro-apoptotic activity on the B164A5 murine melanoma cell line and for the antimicrobial properties on the selected strains. The phytocompounds present in a different manner in vitro chemopreventive and weak antimicrobial activity. Incorporation in beta cyclodextrins had a mix effect on the biological activity conducing to decrease, increase or consistent effect compared to pure phytocompound, depending on the screened process and on the chosen combination.

Keywords: Fisetin, quercetin, kaempferol, beta-cyclodextrins, chemopreventive, antimicrobial.

1. INTRODUCTION

Natural compounds and their chemical derivatives have represented among years invaluable sources of both consecrated and new therapeutic agents [1]. Accordingly to a recent report of WHO (World Health Organization) over 80% of world’s populations is using total plant extracts or isolated phytocompounds for their primary health care needs (www.who.int). Flavonoids represent one of the most studied classes of phytocompounds. Studies in the field show their role in the prevention of degenerative diseases including cancers, cardiovascular and neurodegenerative diseases [2]. From the chemical point of view flavonoids comprise a large group of polyphenolic compounds having in common a benzopyrone structure [3]. Flavonols are an important subclass of flavonoids that have a 3-hydroxyflavone backbone [4]. They can be found in an increased number of vegetables and fruits. Statistics have shown that in western population the range value for the intake of flavonols varies between 2-50 mg per day [5, 6]. Fisetin, quercetin and kaempferol are among the prominent representatives of this class.

Fisetin can be found in strawberries, apples, persimmons, mangoes, kiwi, peach, lotus root, onions, cucumbers and other fruits and vegetables [7]. It was reported to prevent Alzheimer’s disease in case of transgenic mice [8]. The flavonol was also depicted for its anti-oxidant, anti-cancer, anti-angiogenic, anti-inflammatory, anti-diabetic neuroprotective and neurotrophic effects [9-11]. It supports ERK-dependent long-term potentiation and enhances memory [12]. Fisetin showed also chemopreventive properties against human colon cancer cells HCT116 [13]. Addition of fisetin to cisplatin proved to be a good therapeutic strategy for the elimination of embryonal carcinoma cells [14].

Quercetin can be found in apples, grapes, red wine, blue and black berries, red cherries, citrus, green leafy vegetables, onions, cocoa, tea [15]. It was described as an antioxidant, antiproliferative and antiangiogenic agent [16, 17]. Quercetin supports cardiovascular health, blocks human platelet aggregation and eicosanoid synthesis [18]. It decrease blood pressure in animal models and humans [19]. It has been depicted as an anti-inflammatory agent by inhibiting STAT-1 and NF-kB activations [20]. Quercetin was found to block the release of substances involved in allergies and to inhibit mast cell secretion [21]. It was also reported as a chemopreventive agent on a wide number of cancers with very good results on prostate and colon cancers [22-24].

Kaempferol can be found in apples, grapes, blackberries, peaches, green tea, tomatoes, potatoes, onions, green leafy vegetables [25]. Epidemiological studies have shown a positive correlation between consumption of nutraceuticals containing kaempferol and a decreased risk of developing several disorders such as cardiovascular diseases and cancer [26]. In vitro and in vivo studies have shown a wide range of biological properties for this flavonol including anti-oxidant, chemo-preventive, cardio-protective, anti-microbial, anti-angiogenic, neuroprotective, anti-diabetic, anti-osteoporotic, anti-allergic properties [26-28]. Kaempferol have proved to have anticancer effects in case of : breast, ovarian, gastric, pancreatic, colorectal, bladder, prostate, lung cancer and leukemia [27, 29].

Solubility may play an important role in the therapeutic efficacy of flavonols. It is well known that in general flavonoids and in this particular discussion fisetin, quercetin and kaempferol have low

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water solubility [3]. This parameter can cause problems in terms of bioavailability of this active agents. Various approaches have been used throughout time in order to modify the dissolution profile of these lipophilic phyto compounds without changing the molecular structure, including: particle size reduction, complexation, use of lipid based system, use of surfactant of delivery in dosage form, incorporation in different types of micro or nano particles, pH modification, solid dispersion, crystal modification [30]. Cyclodextrins (CD’s) have been extensively used in the pharmaceutical field in order to increase the solubility, stability, safety and bioavailability of a wide range of molecules [31, 32]. From the chemical point of view CD’s are a family of cyclic oligosaccharides with the ability to form host-guest inclusion complexes with a wide variety of molecules, having a hydrophilic outer surface and a lipophilic central cavity. β CD’s are naturally occurring CD’s and contain 7 glucopyranose units [31, 33].

The aim of this study is to prove that the three flavonols can be successfully incorporated in β CD’s (beta-cyclodextrin (BCD) and hydroxypropyl-beta-cyclodextrin (HPBCD)) in order to increase their water solubility. A second aim is to evaluate the antimicrobial effect, respectively the anti proliferative and pro apoptotic activity on the B164A5 murine melanoma cell line of pure flavonols and their complexes.

2. MATERIALS AND METHODS

2.1. Reagents

Fisetin, kaempferol, and quercetin were acquired from Sigma Aldrich (purity ≥98%). Beta-cyclodextrin (BCD) and hydroxypropyl-beta-cyclodextrin (HPBCD) were acquired from Cyclolab Hungary. All substances were used as received and stored as indicated by the manufacturer.

2.2. Complexes Preparation

For the preparation of the inclusion complexes the following steps where employed: simple powder mixing, using a mortar and a pestle; kneading with a 50% (w/w) water : ethanol solution until the bulk of solvent evaporated and a paste-type product was formed; the mixture was then dried at room temperature for 24 hours and put in the oven, at 105 °C, for several hours until reaching constant weight. The final product was pulverized and sieved. All the binary products were prepared using a molar ratio of 1:2 active substance : CD (MwFisetin = 286.23 g/mol, MwKaempferol = 286.23 g/mol, MwQuercetin = 302.236 g/mol, MwB2CD = 1134.98 g/mol, MwHPBCD= 1396 g/mol). The molar ratio of 1:2 was chosen in order to achieve a better water solubility for active phytocompounds and their complexes.

2.3. Scanning Electron Microscopy (SEM)

The morphology of the particles was examined by SEM (Hitachi S4700, Hitachi Scientific Ltd., Tokyo, Japan). A sputter coating apparatus (Bio-Rad SC 502, VG Microtech, Uckfield, UK) was applied to induce electric conductivity on the surface of the samples. The air pressure was 1.3-13.0 mPa.

2.4. Differential Scanning Calorimetry (DSC)

The DSC measurements with a Mettler Toledo DSC 821e thermal analysis system with the STAR® thermal analysis program V6.0 (Mettler Inc., Schwerzenbach, Switzerland), approximately 2–5 mg of pure drug or product was examined in the temperature range between 25 °C and 350 °C. The heating rate was 10 °C min⁻¹. Argon was used as carrier gas, at a flow rate of 10 l/h during the DSC investigation.

2.5. X-ray Powder Diffraction (XRPD)

XRPD spectra were recorded with a BRUKER D8 Advance X-ray diffractometer (Bruker AXS GmbH, Karlsruhe, Germany) system with Cu Kα1 radiation (λ = 1.5406 Å) over the interval 5-30°/2. The measurement conditions were as follows: target, Cu; filter, Ni; voltage, 40 kV; current, 40 mA; time constant, 0.1 s; angular step 0.010°. In the determination of the degree of crystallinity, the total area of the three peaks with largest intensity was examined, after smoothing and background removal

2.6.1. Cell Culture

B16 4A5 murine melanoma cell line (Sigma-Aldrich Company, Ayshire, UK) was cultured in complete growth medium for these cells, containing Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco BRL, Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal calf serum (FCS; PromoCell, Heidelberg, Germany), 1% Penicillin/Streptomycin mixture (Pen/Strep, 10,000 IU/ml, FCS; PromoCell), and 2% HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Gibco BRL). The cells were cultured and expanded in standard conditions, at 37 °C and 5% CO2 atmosphere. Medium replacement was performed every third day and, upon reaching 80% to 90% confluence, the cells were passed using 0.25% Trypsin-EDTA (ethylenediaminetetraacetic acid) solution (Sigma-Aldrich Company) followed by centrifugation (10 minutes, 300 x g) and were replated in T75 culture flasks at a density of 10,000 cells/cm2 to ensure optimal growth and expansion.

2.6.2. MTT Assay

B16 4A5 murine melanoma cell line was seeded onto a 96-well culture plate at a cellular density of 10,000 cells/well and attached to the bottom of the well overnight. After 24 hours, 100 µL of new medium containing Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco BRL, Invitrogen, Carlsbad, CA, USA) and 100 µM of the tested substances (dissolved in dimethyl sulphoxide – DMSO; Sigma-Aldrich Company) were added and incubated for 72 h; the medium was supplemented with 10% fetal calf serum (FCS; PromoCell, Heidelberg, Germany) and 1% Penicillin/Streptomycin mixture (Pen/Strep, 10,000 IU/ml; PromoCell, Heidelberg, Germany). The melanoma cells were then assayed by the addition of 10 µL of 5 mg/ml MTT solution from MTT-based in vitro toxicology assay kit (Tox-1; Sigma-Aldrich Company). The intact mitochondrial reductase converted and precipitated MTT as blue crystals during a 4 h contact period. The precipitated crystals were dissolved in 100 µL of lysis solution provided by the manufacturer (Sigma-Aldrich Company). Finally, the reduced MTT was spectrophotometrically analyzed at 570 nm, using a microplate reader (Bio-Rad, Hercules, CA, USA); wells with untreated cells were considered as reference for viability, while DMSO, which was used to prepare stock solutions of the tested substances, was also added on the cells for evaluation of cellular proliferation. All in vitro experiments were carried out on two microplates in quadruplicates for each substance tested and controls.

2.6.3. Annexin V-PI Assay

For evaluation of fisetin, quercetin, kaempferol, and their CD complexes effects on B164A5 melanoma cells, these cells were seeded on 6-well culture plates at a cellular density of 10,000 cells/cm2. The tested substances were added in culture media at a concentration of 100 µM and left to interact for 72 hours. Annexin V-FITC (Miltenyi Biotec, Gladbach, Germany) was used in cell death flow cytometric studies (apoptosis) combined with propidium iodide staining solution (BD Biosciences, San Jose, CA, USA) following the manufacturer’s protocol. Briefly, 106 cells were washed in 1 × Annexin V Binding Buffer (BD Pharmingen), centrifuged at 300 g for 10 min, resuspended in the same solution and incubated with 10 µL of Annexin V-FITC for 15 min in the dark. After washing the cells with 1 mL specific binding buffer and centrifugation, the cell pellet was resuspended in 500 µL binding buffer, and 1 µg/mL of PI solution was added immediately prior to analysis by flow cytometry. Acquisition of data was performed with
the Cell Quest Pro software (Becton-Dickinson) on a 2 laser-capable flow cytometer (FACSCalibur; BD Biosciences), and data analyses used Flowing Software 2.5.

2.7.1. In Vitro Antibacterial Activity

The selected compounds were screened for their antimicrobial activity against 11 bacterial strains: Bacillus cereus (ATCC 14579), Staphylococcus aureus (ATCC 25923), Streptococcus pyogenes (ATCC 19615), Streptococcus pneumoniae (ATCC 49619) Enterococcus faecalis (ATCC 29212), Escherichia coli (ATCC 25922), Yersinia enterocolitica (ATCC 23715), Klebsiella pneumoniae (ATCC 700603), Proteus mirabilis (ATCC 25933), Pseudomonas aeruginosa (ATCC 27853), Candida albicans (ATCC 10231) by the agar disk-diffusion method.

2.7.2. Disk Diffusion Method

The antimicrobial activity of the selected compounds was evaluated according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS, 1997) using agar disk diffusion method. Performance Standards for Antimicrobial Disk Susceptibility Tests, Approved standard M2-A6. NCCLS recommends a bacterial suspension with a density equal to a 0.5 McFarland (which gives a final bacterial concentration of 1-2x10⁸ CFU/ml). The Mueller-Hinton agar plates were inoculated with bacterial suspension using a sterile cotton swab. The same method was used also for Candida albicans but in this case the tested suspension was of 2.2 McFarland and the medium was Sabouraud. Solutions of the tested compounds were prepared in DMSO at a concentration of 10 mM. Within 15 min after the plates were inoculated, sterile Whatman no.1 filter paper disks (6 mm in diameter) impregnated with the solution in DMSO of the test compounds were distributed evenly on the surface, with at least 30 mm (center to center) between them. For negative control we used paper disks impregnated with BCD and HPBCD. Plates inoculated with the bacterial suspensions were incubated at 37°C for 24h. The inhibition zone diameters were measured in millimeters, with a ruler. For all bacterial strains we performed duplicate disk-diffusion tests and average values are reported.

2.8. Statistics

The Prism software package (Graph Pad Prism 4.03 for Windows) was used for data presentation. The experiment was repeated three times and results are presented as mean ± SD. One or two way ANOVA were applied to evaluate statistical significance (∗, p < 0.05; ∗∗, p < 0.01; and ∗∗∗, p < 0.001).

3. RESULTS

SEM pictures are depicted in Fig. 1; one can notice the crystalline structure of all three natural compounds certified by the presence of defined crystal particles of various shapes and sizes. Also, beta-CD reveals crystalline character as proven by the irregular shaped particles in its composition; HPBCD is of amorphous nature, displaying round, partially broken particles with

a clear tendency to aggregate. The beta-CD complexes revealed crystalline structures while the HPBCD based complexes show an amorphous composition of aggregated particles.

The DSC profiles of the natural flavonols, CD and 1:2 complexes are presented in Fig. 2. The two CD’s show an endothermic large peak below 100°C, specific for the loss of crystallization water molecules; above 100°C the two CD’s display a flat profile within the analysis temperature range thus certifying that the melting processes develop above 300°C. Fisetin (Fig. 2A) shows an endothermic sharp peak at 348°C consistent with its melting point; its complexes’ DSC profiles display flat curves which certify the

**Fig. (2).** Differential scanning calorimetry (DSC) for: A) 1. Fisetin and it’s CD complexes; B) Kaempferol and it’s CD complexes; C) Quercetin and it’s CD complexes; D) Raw CD’s.

![Differential scanning calorimetry (DSC) profiles](image1)

**Fig. (3).** X-ray diffractograms for for: A) 1. Fisetin and it’s CD complexes; B) Kaempferol and it’s CD complexes; C) Quercetin and it’s CD complexes; D) Raw CD’s.
absence of thermal processes. For kaempferol (Fig. 2B) the melting process is revealed by the endothermic sharp peak at 286°C which disappears in the DSC profiles of its CD complexes. Fig. 2C displays the DSC curves for quercetin and its 1:2 complexes; one can notice the sharp endothermic peak at 321°C which characterizes the melting point of the pure compound; the DSC profiles of its complexes reveal the absence of thermal processes.

The X-ray diffractograms are showed in Fig. 3; one can notice that all three natural compounds display sharp signals, characteristic for crystalline materials; also, beta-CD has a crystalline structure as proven by the numerous distinct peaks in its diffractogram. HPBCD is an amorphous substance, characterized by almost flat diffractograms. The complexes containing beta-CD as host molecule display distinct sharp peaks, similar to the sum of individual components; however, the appearance of several new peaks as well as the shifting of others can be clearly noticed. The complexes of all three natural compounds with HPBCD are characterized by flat X-ray profiles similar to the corresponding CD.

Additionally the study analyzed the antimicrobial potential of the three flavonols: fisetin, quercetin and kaempferol alone and encapsulated in beta-cyclodextrin (BCD) and hydroxypropyl-beta-cyclodextrin (HPBCD). The strains include: gram negative bacteria (Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Yersinia enterocolitica, Pseudomonas aeruginosa), gram positive bacteria (Bacillus cereus), gram positive cocci (Streptococcus pyogenes, Streptococcus pneumoniae, Staphylococcus aureus, Enterococcus faecalis) and fungus (Candida albicans). Gentamicin 10 µg was used as positive control for bacillus and staphylococcus, gentamicin 120 µg was used as positive control for streptococci and enterococci and fluconazol 25 µg was used as positive control for fungus. For controls diameters were between 16-19 mm. Results are presented in Table 1. A 6 mm diameter show no antibacterial activity. The selected compounds present a weak antibacterial activity against gram positive cocci. The gram negative bacteria and fungus are resistant at this agents. Slight inhibition could be detected for the gram positive bacteria Bacillus cereus but only in case of kaempferol and it’s CD’s complexes. For all three active agents, complexation with the three beta CD’s increased the antibacterial activity different depending on the bacterial strain. For fisetin in case of S. pneumoniae the best activity was recorded after incorporation in BCD, for S. pyogenes after incorporation in HPBCD, for S. aureus both complexes presented a better antibacterial activity while in case of E. faecalis both complexes decreased the antibacterial activity compared to pure fisetin. For kaempferol in case of S. pneumoniae the best activity was recorded after incorporation in HPBCD, for S. pyogenes after incorporation
in HPBCD for *S. aureus* after incorporation in BCD and HPBCD. In case of *E. faecalis* the complexes had the same antibacterial activity compared to pure kaempferol. From the tested flavonols only kaempferol and its CD’s complexes presented antibacterial activity against the gram positive bacteria *Bacillus cereus*, and in this case the best activity was detected after incorporation in HPBCD. For quercetin in case of *S. pneumoniae* and *S. aureus* the complexes had the same antibacterial activity compared to pure compound. For *S. pyogenes* the best activity was recorded after incorporation in BCD while for *E. faecalis* complexation in all three cases decreased the antibacterial activity compared to pure quercetin. Results are presented in Table 1.

Morphological aspect of reference/control B164A5 cells shows polygonal shape, clustering tendency and intense direct contact between cells. The cells are grouping and make variable size placards. When treated with fisetin, quercetin, kaempferol and their CD’s complexes, the cells lose their polygonal shape and become more fusiform, stretching for contact with the neighboring cells. The grouping tendency is reduced, and we can see single cells very often on the microscopic field, more or less, depending on the tested substance. Results are presented in Fig. 4.

The antiproliferative activity of the tested flavonols and their CD’s complexes on B164A5 murine melanoma cell line is rather poor. Among the three tested compounds after 72 h of incubation

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![Fig. 4](image-url)

FIG. 5. Viability (%) of B164A5 mouse melanoma cells after 72 h of incubation with 100 μM of Fisetin (F), Kaempferol (K) and Quercetin (Q) and their cyclodextrin complexes.

and at a concentration of 100 μM, quercetin showed the strongest antiproliferative activity, the mean value for the viability of cells being 51.2 ±14.14, followed by fisetin with 71.5 ±10.60 viable cells and kaempferol with 88.6±16.26 viable cells. Encapsulation of this active agents in beta cyclodextrins did not have a significant activity in terms of increasing the antiproliferative activity. For fisetin the viability was sensitive reduced for both CD complexes. In case of kaempferol the viabiltiy was sensitive reduced for the HPBCD complex, while for the BCD complex the viability was slightly increased. Quercetin was the flavonol whose complexes acted really different, namely the BCD complex decreased the viability and the HPBCD complex significant increased the viability of B164A5 murine melanoma cells. Results can be observed in Fig. 5.

The Annexin-PI double staining show that after a period of incubation of 72 h at a concentration of 100 μM all three flavonols conduced to early and late phenomena of apoptosis. The effects where quite close however, kaempferol was the most active in terms of early apoptotic events with a percentage of 26,55±8,48 early apoptotic cells, followed by fisetin with a percentage of 21,42±7,19 early apoptotic cells and quercetin with a percentage of 18,4±4,24 early apoptotic cells. Regarding the late apoptotic events results are quite similar for the three tested compounds, namely : 9,56±2,83 late apoptotic cells after incubation with fisetin, 7,15±3,04 late apoptotic cells after incubation with kaempferol and 5,54 ±4,87 late apoptotic cells after incubation with quercetin. Incorporation in beta cyclodextrins did not increase the number of early or late apoptotic cells for any of the three complexes. Representative plots of Annexin V-PI staining can be observed in Fig. 6 while mean of the three experiments can be observed in Fig. 7.

4. DISCUSSIONS

The physico-chemical analysis use to demonstrate that incorporation took place, are very known “tools” in the pharmaceutical and biomedical field [34-36]. Scanning electron microscopy is a very useful technique to characterize the morphology of solid samples; although it cannot provide strict evidence in terms of complex formation the analysis offers reliable information in the assessment of the final products’ composition [37]. All three natural principles reveal a crystalline structure characterized by the presence of distinct particles, some of parallellepped shape and similar size. Beta-CD is also composed of crystalline particles while HPBCD is an amorphous substances, displaying round particles that form bigger aggregates. The kneaded products reveal a significant change of structure; thus, beta-CD complexes are of crystalline nature, most particular when quercetin is involved. However, the aspect of individual particles is clearly different as compared to the pure compounds. The products obtained using HPBCD as host-molecules reveal an amorphous structure, similar to the pure cyclodextrins, thus leading to the conclusion that a solid state interaction took place. The presence of small particles in the final products together with the aggregation tendency is characteristic for single component amorphous samples [38]; therefore, one can assume that the complexation procedure led to the formation of true inclusion complexes.

Thermal analysis such as differential scanning calorimetry is currently involved in cyclodextrin complexes evaluation due to the valuable information it can provide [38]. In this regard, it has been established that following the inclusion process the normal thermal behavior of the guest-molecule changes significantly as a result to its accommodation inside the cyclodextrin’s cavity [33]. Analyzing the DSC profiles of the pure compounds and cyclodextrins versus the DSC profiles of the newly prepared complexes, one can notice that in all three cases the sharp peak indicating the melting point of the substance alone disappears as a result on 1:2 complexation; thus, the DSC profiles of the 1:2 products are practically identical to the curves recorded for the pure cyclodextrins. The disappearance of the endothermal processes characteristic for the pure compounds in the DSC profiles of 1:2 products is a reliable evidence of the total inclusion of the phytocompound inside the respective cyclodextrin.

In order to complete the evaluation of the 1:2 products the XRD analysis was perfomed; it usually provides significant information in terms of crystalline changes due to intermolecular interactions [39]. In the X-ray diffractograms of pure fisetin, kaempferol and quercetine, respectively, as well as beta-CD, the presence of sharp distinct peaks characterizes the crystalline nature of the compound. HPBCD revealed an amorphous nature as proven by the almost flat X-ray profiles. The 1:2 product of beta-CD with fisetin, kaempferol and quercetin, respectively, show crystalline characteristics; however one can notice the appearance of new peaks as well as the shifting in position of some existing individual peaks, thus proving the formation of a new crystalline phase. The presence of new peaks, of different position and intensity as compared to the individual compounds can be regarded as evidence of the formation of the inclusion complex.

The 1:2 products with HPBCD, exhibit an almost flat diffractogram, therefore an amorphous structure; this result can be regarded as a proof of complex formation but also of an amorphisation process that took place due to the kneading procedure.

The selected flavonols have been previously reported for their antimicrobial activity. Fisetin at the cone of 8 μg/mL, was reported to inhibit the oral pathogen S. mutans. However no activity was recorded in case of P. gingivalis or E. coli [40]. It was depicted also as an antiviral agent against Dengue virus type-2 [41]. A recent study showed that fisetin inhibits L. monocytogenes virulence [42]. It was published that fisetin inhibit arginase, a central enzyme in L. amazonensis infection [43]. In vitro antifungal activity against H. courbaril was also described [44]. A range of seven kaempferol rhamnosides were reported for their antimicrobial activity against S. aureus, P. aeruginosa, S. typhi, C. albicans, C. parapsilosis and C. neoformans [45]. Kaempferol was found to inhibit herpes simplex virus, human coronavirus and rotavirus [46]. This flavonol showed synergic activity with clindamycin against Propionibacterium acnes [47]. Previous studies discuss that kaempferol did not inhibit E. coli and S. epidermidis but demonstrate antimicrobial activity against S. aureus [48]. Debating this results, the group of Panizzi et al., showed that kaempferol present no antimicrobial activity against S. aureus and E. coli [49]. The group of Hirai et al., screened the antibacterial activity of quercetin against gram-positive and gram-negative bacteria. The flavonol was found to be selective on
Fig. (6). Representative plots of Annexin V-PI staining. A) 1. Control/reference untreated cells; 2. Cells treated with the highest amount of DMSO used for preparing stock solution of the tested substances; B) 1. Cells treated with BCD; 2. Cells treated with HPBCD; C) 1. Fisetin; 2. BCD complex of Fisetin; 3. HPBCD complex of Fisetin; D) 1. Quercetin; 2. BCD complex of Quercetin; 3. HPBCD complex of Quercetin; E) 1. Kaempferol; 2. BCD complex of Kaempferol; 3. HPBCD complex of Kaempferol.

Fig. (7). Mean of the three experiments of Annexin V-PI staining for A) Fisetin and it’s CD complexes (Two ways Anova-Interaction-p= 0.0404, Row factor-p > 0.9999, Colum factor p < 0.0001); B) Quercetin and it’s CD complexes (Two ways Anova-Interaction-p= 0.0926, Row factor-p > 0.9999, Colum factor p < 0.0001); C) Kaempferol and it’s CD complexes (Two ways Anova-Interaction-p= 0.0232, Row factor-p > 0.9999, Colum factor-p < 0.0001)
S. aureus, Methicillin-resistant Staphylococcus aureus (MRSA) and S. epidermidis [50]. In addition, new quercetin-derived oxidation products presented antibacterial activity against H. pylori and MRSA strains [51]. The phytocompound was evaluated also for its antimicrobial activity against the main pathogens of the main oral infectious diseases and was found to be very efficient against S. mutans, S. sobrinus, A. actinomycetemcomitans [52]. The uptake and recognition by specific cellular receptors and transport systems for different types of cyclodextrins have been previously discussed [53]. The group of Lehner et al., showed that for liphilic compounds complexation with hydroxypropyl-beta-cyclodextrin leads to a stronger antimicrobial activity [54]. Increase antimicrobial activity after incorporation in BCD and HPBCD was reported also for cefdinir [55]. It was published that CD’s used as excipients in tooth pastes increase the availability of the antimicrobial agent triclosan [56]. The beneficial effects of complexation with beta-cyclodextrins were brought to light also for six antimycotic imidazole derivatives [57]. In a study of the group of Athanassiou et al. including several beta-lactam antibiotics it was proved that the activity of the cyclodextrin-included beta-lactam antibiotics was increased, particularly against gram-negative strains [53].

As depicted in the results section we can stain that the three flavonols do not have a strong antiproliferative activity against B164A5 murinic melanoma cell line. Among the three compounds quercetin proved to be the best candidate, but the events could be seen at a high dose, namely 100 µM and after a long period of incubation, namely 72 h. On the other hand in terms of apoptosis, using the same conditions the effects where quite close for the three flavonols. The explanation could point towards the different mechanism that this natural compounds have for chemoprevention, targeting the mitochondrial dehydrogenase enzyme for anti-proliferative activity or the mithochondrial regulation or direct signal transduction for triggering apoptosis. The group of Zhang et al. have shown that quercetin induces apoptosis against B16-BL6 cell line by targeting mitochondria, inhibiting expression of PKC-alpha and translocating PKC-delta [58]. In association with tamoxifen, quercetin was described to sensitize human melanoma cells to hyperthermia [59]. A recent study have shown that the mechanism by which quercetin exerts anti-melanoma effect involves the inhibition of STAT3 signaling [60]. This information was completed by the group of Cao et al., who showed that the phytocompound inhibits HGF/c-Met signaling and HGF-stimulated melanoma cell invasion and migration [61]. In vivo, using the B16-BL6 model it was also proved to inhibit melanoma growth and metastatic potential [62]. Fisetin was reported to be an active agent against human melanoma cells by targeting MAPK and NFkB signaling pathways and by promoting the mesenchymal to epithelial transition [63]. The studies regarding the mechanism of action where completed by the group of Syed et al. who have showed that fisetin acts as an anti-melanoma compound through direct binding to p70S6K and mTOR [64]. Kaempferol was described with anti-inflammatory and depigmenting effect [65]. It was shown that it regulates the cyclin-dependent kinases CDK2 and CDK1 in vitro for human melanoma OCM-1 cell line [66]. It was also showed to inhibit melanogenesis in B164A5 melanoma cell line [67]. Cyclodextrin encapsulation did not have any spectacular effect for none of the three phytochemicals concerning both proliferation and apoptosis. Research in the field have shown that for some active compounds this kind of modulations can increase the in vitro chemopreventive activity by higher cellular uptake and transfection efficiency [68, 69]. However the discussion can suffer intense debate because some papaers in the field report increased in vitro chemopreventive activity after incorporation in beta-cyclodextrins, others have noticed no change or even decreased chemopreventive activity after this kind of modulation [70-72].

5. CONCLUSION

The three flavonols: fisetin, quercetin and kaempferol were successfully incorporated in beta-cyclodextrin (BCD) and hydroxylpropyl-beta-cyclodextrin (HPBCD). The phytocompounds present in a different manner in vitro antiproliferative and pro-apoptotic activities on the B164A5 murinic melanoma cell line and weak antimicrobial activity on the selected strains. Incorporation in beta cyclodextrins had a mix effect on the biological activity conuding to decrease, increase or consistent effect compared to pure phytophouding depending on the screened process and on the chosen combination.

CONFLICT OF INTEREST

Authors declare no conflict of interest. The study was financed by the UMFT grant –Parteneriate în cercetarea fundamentală inovativă-PII-C2-PCFI-2015/2016acronim FLAVOFORM.

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DC and CD designed the study and where involved in the article’s drafting. RA, DM and IC performed the experiments. FB and MC analyzed the data. CS and VP reviewed the draft for important intellectual content.

LIST OF ABBREVIATIONS

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<tr>
<th>Abbreviation</th>
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<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
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<td>DSC</td>
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<td>XRPD</td>
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REFERENCES

Fisetin, Quercetin and Kaempferol and their CD’s Complexes


