

ANTIMICROBIAL ASSAY OF A CAPSAICIN - α -CYCLODEXTRIN INCLUSION COMPLEX AGAINST PLANKTONIC AND ADHERENT CELLS

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Abstract

Capsaicin (CAP), (*E*)-*N*-[(4-hydroxy-3-methoxyphenyl)methyl]-8-methylnon-6-enamide, as an active principle and α -cyclodextrin (α -CD) have been used to obtain an inclusion complex with the molar ratio 1:1 that can be subsequently included in semisolid pharmaceutical formulations in order to improve the solubility of this active principle. In the inclusion process no covalent bonds are formed or broken. The results of the antimicrobial activity assay revealed a similar efficiency of the two capsaicin complexes and of non-capsulated CAP against planktonic cells. However, the inclusion complex of capsaicin significantly improves its antibiofilm activity, is thus recommended for the development of preventive or therapeutic strategies for the management of biofilm-associated infections.

Rezumat

Capsaicina (CAP), (*E*)-*N*-[(4-hidroxi-3-metoxifenil)metil]-8-metilnon-6-enamidă, ca principiu activ și α -ciclodextrina (α -CD), au fost utilizate pentru a obține un complex de incluziune cu un raport molar de 1:1 care poate fi ulterior inclus în formulări farmaceutice semisolide pentru a îmbunătăți solubilitatea acestui principiu activ. În urma procesului de incluziune nu s-au format sau nu s-au rupt legături covalente. Activitatea antimicrobiană a acestui complex a fost comparabilă cu cea a unui amestec mecanic și cu a CAP libere față de celulele planctonice, însă net superioară, în ceea ce privește activitatea antibiofilm, demonstrând potențialul acestui complex în elaborarea unor strategii utile pentru prevenția sau terapia infecțiilor asociate biofilmelor microbiene.

Keywords: inclusion complex, capsaicin, α -cyclodextrin, antimicrobial assay

Introduction

A number of new organic compounds have been characterized [22, 24], with the purpose of using them in the biopharmaceutical industry [6, 20], but of all types of biomaterials, cyclodextrins (CDs) have a special place [11]. CDs find a multitude of applications in the pharmaceutical industry, as it has been shown in numerous studies, mainly as good carriers for various types of drugs, for which CDs improve solubility, stability and even their bioavailability [8]. CDs even protect these drugs from the negative effects of various environmental factors (temperature, pH, humidity, UV radiation) [16]. A great advantage of CDs is that they lack side effects, being cyclooligosaccharides resulting from enzymatic degradation of starch, and are also being approved by the Food and Drug Administration (FDA) [26]. Structurally, CDs (α , β , γ) are cyclooligosaccharides

with 6, 7 or 8 glucose residues joined by a 1-4 glycosidic linkage. Carlotti *et al.* [3] noticed that the use of CDs as encapsulating agents for the poorly soluble active principles decreases the minimum active concentration required to achieve a biological effect. Aquil *et al.* state in a recent article that the active principles encapsulated in CDs are released over a significantly longer time period compared with the free, uncapped active principle [1].

Capsaicin (CAP) is part of a group of natural compounds with pharmaceutical properties, of the terpenoids category [10].

CAP acts on pain receptors to trigger the formation of endorphins and enkephalins. The analgesic effect of CAP has been exploited in the pharmaceutical industry by including it at concentrations of 0.025% - 1% in topical preparations having therapeutic indications for pain caused by rheumatoid arthritis,

neuropathic pain caused by certain tissue lesions, cluster type migraines [25]. Reduced doses of CAP have a chemo preventive action and are administered with chemotherapy, potentiate the antineoplastic effects [5]. CAP is a good cardio protector, inhibiting low-density lipoprotein oxidation and thus, lowering the total serum cholesterol levels [15].

Materials and Methods

Synthesis and spectral characterization

CAP and α -CD were purchased from EMD Chemicals Inc Darmstadt, Germany and Merck. HPLC Water-LiChrosolv[®] Merck; Ethanol - HPLC purity - Merck. Initially, a 3.05×10^{-3} M solution was prepared by dissolving CAP in a water-alcohol mixture (v:v = 7:3). We preferred the solubilization of both CAP and CD because it was found that in this way complex formation is facilitated. Complex formation was attempted at different α -CD concentrations ranging from 0 - 2 mM. The two solvents used in the synthesis were removed from the liquid phase by evaporation to dryness.

Fluorescence spectra were recorded on Jasco FP-6300 fluorescence spectrofluorometer, while the absorption spectra were recorded on a Jasco V-560 UV-VIS spectrometer.

FT-IR spectra were recorded on an Avatar Nicolet spectrophotometer with KBr in the range 600 cm^{-1} - 3000 cm^{-1} .

α -CD and CAP (reference material), the α -cyclodextrin-capsaicin complex (α -CD-CAP) as well as 1:1 α -CD-CAP mechanical mixture were used.

Antimicrobial activity assays

Microbial strains

The antimicrobial activity was tested on two Gram-positive (*Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC-29212), two Gram-negative (*Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922) bacterial strains and one fungal strain (*Candida albicans* ATCC 10231). The tested samples were represented by 10 mg/mL suspensions in dimethylsulfoxide (DMSO) and encoded as follows: 1) α -CD-CAP mechanical mixture; 2) α -CD-CAP synthesis complex; 3) CAP control; 4) DMSO negative control.

Qualitative screening of the antimicrobial properties

The qualitative screening was performed by an adapted spot diffusion method. Microbial suspensions of 1.5×10^8 colony-forming unit/mL (CFU/mL) or 0.5 McFarland density obtained from 24/48 h bacterial/yeast cultures developed on Nutrient agar/YPD (yeast extract peptone dextrose) solid media, were used in the experiments. Petri dishes with YPD (for yeast strains) or nutrient agar (for bacterial strains) were seeded with cellular inoculums and after seeding, an amount of 10 mL solution of each compound of 10 mg/mL concentration was spotted. The plates were left 10 minutes at

room temperature to ensure the equal diffusion of the active compounds in the culture medium and then incubated at 37°C for 24 - 48 hours. The positive results were read as inhibition zone of microbial colonies growth around the spot.

Quantitative assay of the antimicrobial properties

The binary serial microdilution technique using 96-multiwell microtiter plates was used to determine the minimal inhibitory concentration (MIC) values of the tested compounds against the tested microbial strains. The quantitative assay was performed in YPD broth medium for yeasts and Muller Hinton broth for bacteria, according to Performance Standard for Antimicrobial Susceptibility Testing [7].

The sterile medium was added in sterile 96-multiwell plates and binary dilutions of each tested suspension were performed in a final volume of 100 μ L. After performing the binary dilutions, 10 μ L of microbial suspension adjusted to an optical density of 0.5 McFarland (1.5×10^8 CFU/mL) were added in each well. The MIC values were established by the macroscopic analysis of the wells content and by spectrophotometric measurement of the optical density at 600 nm using a BIOTEK SYNERGY-HTX ELISA multi-mode reader. Each experiment was performed in triplicate and repeated on at least three separate occasions.

The quantitative assessment of the influence of the tested suspensions on the microbial adherence on the inert substratum

The content of the 96-multiwell plastic plates used for the MIC assay were discarded, washed three times with phosphate buffered saline (PBS) and the microbial cells adhered to the plastic walls were fixed with cold methanol for 5 minutes and stained by 1% violet crystal solution for 15 minutes. The coloured biofilm was thereafter re-suspended by 33% acetic acid solution. The optical density of the blue suspension was measured at 490 nm, the obtained values being proportional with the number of the adhered microbial cells.

Cytotoxicity assay

CAP and α -CD-CAP cytotoxicity were tested against MG63 cells (ECACC 86051601) using CellTiter 96[®] Aqueous One Solution Cell Proliferation Assay (Promega G3580) according to the manufacturer indications. Briefly, 10^4 MG63 cells *per* well were plated in a 96-well plate, DMEM:F12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12) supplemented with 10% bovine foetal serum. After recovery, the cells were treated with a binary dilution of α -CD, CAP and α -CD-CAP (between 1 mg/mL and 7.6 μ g/mL) and maintained for 72 h at 37°C, 5% CO₂. The toxicity was evaluated by adding CellTiter 96[®] Aqueous One Solution and maintained the cells for 3 h at 37°C. The absorbance of each well was measured using the plate reader BertholdTech

TriStar2S (Berthold Technologies GmbH & Co. KG, Germany), at 490 nm. The results were presented as % of viability reported to the untreated cells.

Cell cycle analysis

For cell analysis, 10^5 MG63 cells were seeded in 1 cm² well in the presence of DMEM:F12 containing 10% foetal bovine serum and 10 µg/mL or 50 µg/mL CAP, α -CD, α -CD-CAP 1 (synthesis complex) or α -CD-CAP 2 (mechanical mixture). After 24 h, the cells were harvested, fixed in cold ethanol overnight, and stained with 20 µL FxCycle™ PI/RNase Staining Solution (F10797, Life Technologies). The events were quantified using Beckman Coulter flow cytometer and analysed using FlowJo 7.2.5 software.

Results and Discussion

In the inclusion complex may appear a series of specific interactions, such as hydrogen bonds, van der Waals interactions, or even electrostatic attraction. These interactions can alter the physico-chemical or even physiological properties of guest molecules, potentiating some features such as low solubility of CAP. The results of the antimicrobial activity assay revealed a similar efficiency of the two CAP complexes and of non-capsulated CAP against planktonic cells, the most susceptible microorganism being the *C. albicans* fungal strain, followed by *E. coli* and *S. aureus* strains, with the exception of the better activity of CAP and of the α -CD-CAP mechanical mixture, as compared to the α -CD-CAP mixture obtained by inclusion recorded against the *E. coli* strain in planktonic growth.

Synthesis of α -CD-CAP inclusion complexes

Although CAP can be extremely soluble in alcohols, carbon tetrachloride, ketones or other organic solvent classes, it is practically insoluble in water at room temperature. This inconvenience makes its use in the pharmaceutical field quite limited. There are studies in which solubilization and, implicitly, the increase of CAP bioavailability by different formulations, such as its inclusion in cyclodextrins, have been

attempted and succeeded [4]. Despite the fact that of the three types of CDs, α -CD is suitable for the encapsulation of diverse types of drugs, β -CD is preferred because it has a larger cavity diameter, and it is widely used for encapsulating a large number of guest molecules. In terms of acquisition costs, α -CD is less expensive compared to β -CD and γ -CD. CAP is a relatively small molecule with a mass of 305.71 g/mol, thus making it suitable to form an inclusion complex with α -CD.

There are numerous methods of forming α -CD-CAP inclusion complexes, methods involving neutralizing, coprecipitation, shaking etc. When choosing a method to obtain these inclusion complexes, the physico-chemical properties of the included molecule, CAP, and related costs were taken into account [28]. The inclusion process involves a substitution of water molecules in the hydrophobic cavity with the lipophilic compound molecules. Therefore, in this seemingly simple process, no covalent bonds are formed or broken [17], but other forces such as hydrophobic interactions, sometimes hydrogen bonds are formed, van der Waals forces appear.

Spectral characterization

Using a fluorescence spectrometer and a UV-VIS spectrometer, stoichiometry of the inclusion complex was established. From the point of view of the stoichiometry of the incorporation complex, it has been noted in our previous studies that the ratio of the number of CAP molecules to α -CD is 1:1. This report was also found by other authors who made CAP inclusion complexes with other types of CD [29]. There are also several literature studies in which the ratio of CD:bioactive molecule was 1:2 or 2:1, but these reports are extremely rare [18]. The constant between the non-complexed and the complexed molecules obtained by us in the inclusion complex was 86 M⁻¹, the result is in accord with the literature that states that $K_{1:1}$ is between 50 and 2000 M⁻¹ (Figure 1) [9].

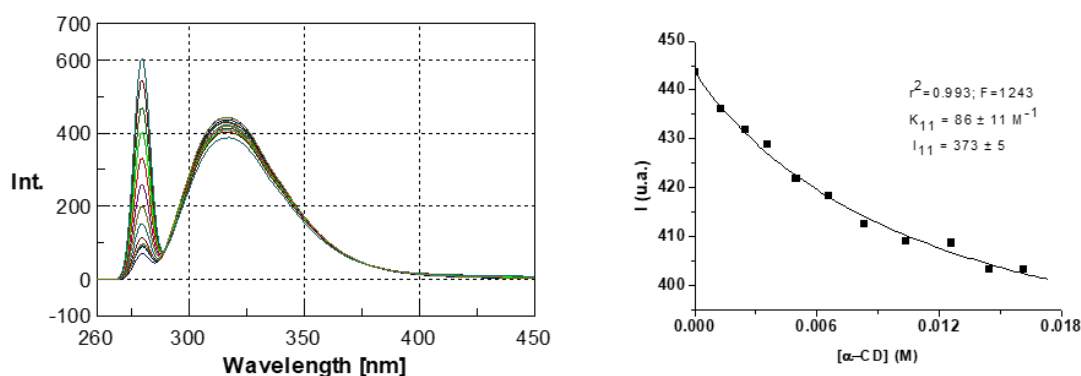


Figure 1.

The fluorescence spectrum of CAP in the presence of different concentrations of α -CD

The UV-VIS spectrum of the inclusion complex (Figure 2) was slightly modified compared to the CAP UV-VIS spectrum, the maximum absorption in UV being recorded at $\lambda = 279$ nm. The interactions between CAP and α -CD cause changes in absorbance maximum. Other authors have found small or large changes in the UV-VIS spectrum of complexes, depending on the strength of the bonds formed between cyclodextrins and guest molecules [12].

FT-IR characterization of CD-CAP inclusion complexes
The presence in the CAP structure of the secondary amine group results in the occurrence of a stretching vibration in the FT-IR spectrum in the region 3400 - 3300 cm^{-1} [27], as revealed by Figure 3

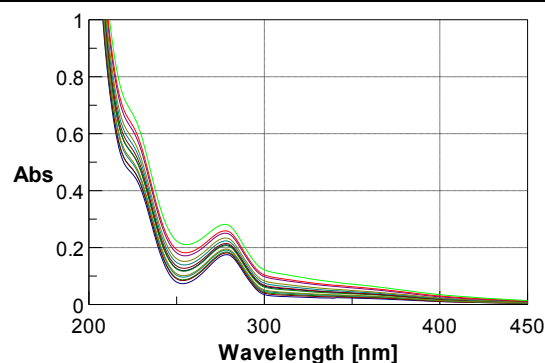


Figure 2.
The UV-VIS spectra of the CD-CAP inclusion complexes

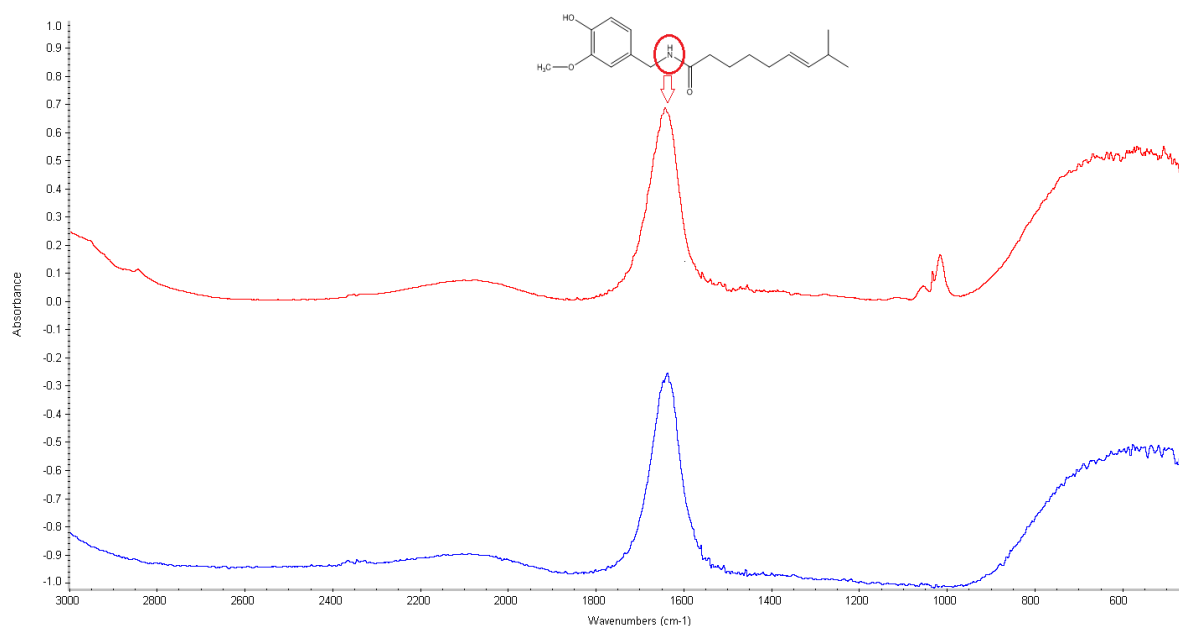


Figure 3.
FT-IR spectra: CAP (red) and the inclusion complex (blue)

At 1636 cm^{-1} it could be observed in the FT-IR spectra a band due to the carbonyl group vibration of the capsaicin structure. This band is present at the same wavelength in both pure capsaicin spectrum (Figure 3 red) and in the spectrum of the inclusion complex (Figure 3 blue). The wavelength at which the stretching vibration of the carbonyl group of the CAP structure remains virtually unchanged, due to the lack of coordination links in the complex.

Antimicrobial activity

After the qualitative analysis of the antibacterial activity of the tested compounds, it can be noticed that four tested suspensions induced the occurrence of different growth inhibition zones, depending on the tested strain. The most susceptible proved to be *S. aureus* and *E. coli* strains, and CAP control produced the most evident growth inhibition zones [2, 23].

In the quantitative assay, the MIC values were read by wells observations and confirmed by the decreased value of the optical density measured at 600 nm. In the wells containing high concentrations of compounds the culture growth was no visible, the microbial cells being killed or inhibited by the tested suspensions [19, 21].

The results of the quantitative assay revealed that the most susceptible microorganism was the *C. albicans* fungal strain, despite that fact that no growth inhibition zone was obtained in the qualitative screening. The second most susceptible strains, were, in agreement with the qualitative assays, *E. coli* and *S. aureus* strains. No differences were recorded among three tested suspensions, excepting the better activity of CAP and of the α -CD-CAP 2, as compared to the α -CD-CAP 1, against the *E. coli* strain in planktonic growth state (Table I).

Table I

No.	Microbial strains	Minimal inhibitory concentration values (mg/mL)			
		α -CD-CAP 2	α -CD-CAP 1	CAP	DMSO
		1	1.25	1.25	1.25
2	5	5	5	5	
3	<i>Pseudomonas aeruginosa</i> (ATCC 27853)	2.5	2.5	2.5	2.5
4	<i>Escherichia coli</i> (ATCC 25922)	0.62	1.25	0.62	5
5	<i>Candida albicans</i> (ATCC 9023)	0.62	0.62	0.62	1.25

Concerning the inhibitory activity of the tested suspensions against *S. aureus* biofilm development, the inclusion complex proved to be the most efficient,

inhibiting the biofilm development in the range of 5 - 1.25 mg/mL (Figure 4-A).

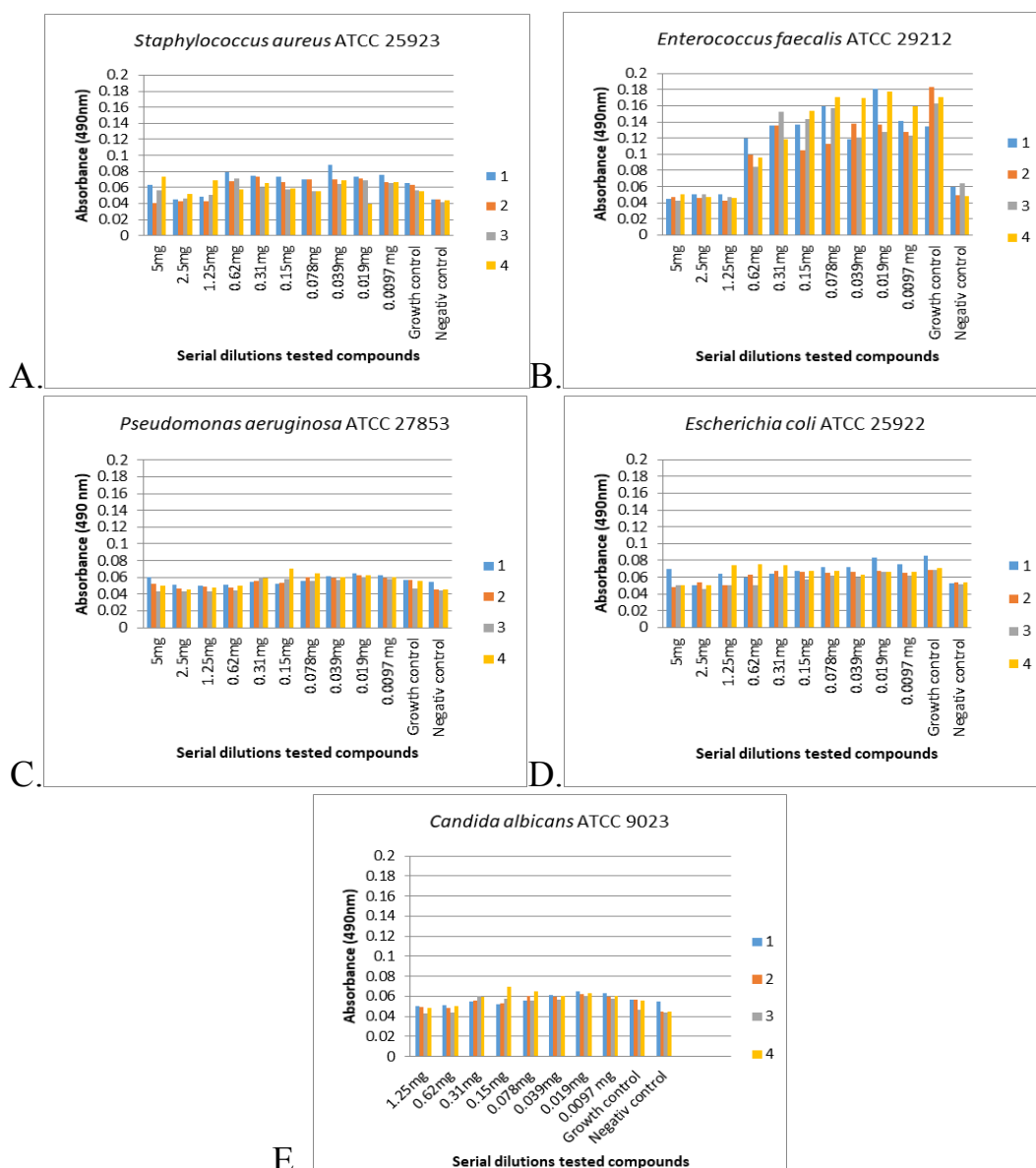


Figure 4.

Viscosity curves and the statistical mean of differences expressed by the regression lines

Concerning the inhibitory activity of the tested suspensions against *E. faecalis* biofilm development, all three suspensions proved to be very efficient, drastically inhibiting the biofilm development in the

range of 5 - 1.25 mg/mL (Figure 4-B). However, at the lower concentrations, the inclusion complex preserved its antibiofilm properties on the entire range of the tested concentrations.

Concerning the efficiency against the biofilm-development activity of the Gram-negative tested strains, no antibiofilm activity was recorded against the *P. aeruginosa* strain (Figure 4-C), while in the case of the *E. coli* strains, the inclusion complex was active in the range 5 - 1.25 mg/mL (Figure 4-D). No antibiofilm effect was obtained for the texted suspensions in case of *C. albicans* fungal strain (Figure 4-E).

Cytotoxicity assay

Cytotoxicity assay has proved that treatment of MG63 cell with α -CD for 72 h enters this substance into the non-toxic category ($IC_{50} = 258.63$). Instead, the toxicity of CAP was increased ($IC_{50} = 88.437$) and α -CD provided only a small protection in the case of α -CD-CAP 1 ($IC_{50} = 76.77$) or α -CD-CAP 2 ($IC_{50} = 78.533$).

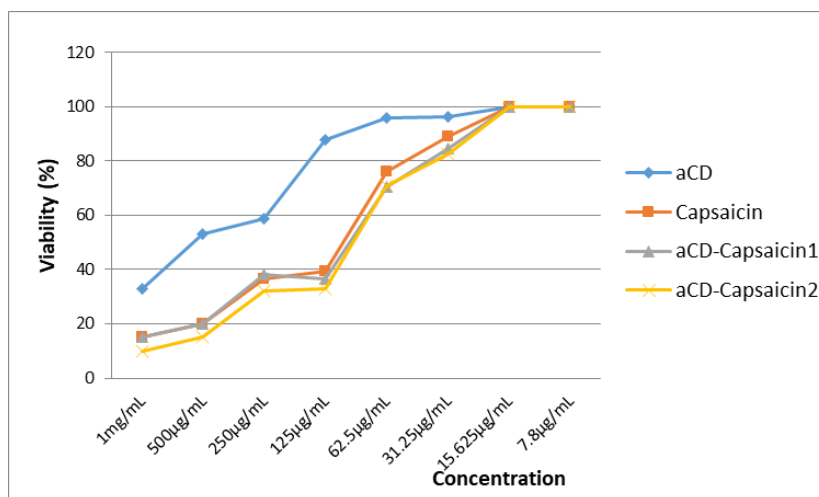


Figure 5.

The effects of CAP, α -CD, α -CD-CAP 1 and α -CD-CAP 2 on MG63 cells

Cell cycle analysis

The cells have a doubling time between 18 and 24 hours. Thus, as observed in the Figure 6, at 24 h, from passage, many of the MG63 cells are found in

G2/M. The treatment with 50 μ g/mL CAP makes it possible to decrease the percent of cells in G2/M associated with increase of G0/G1 cells percent and the appearance of a small peak sub-G0.

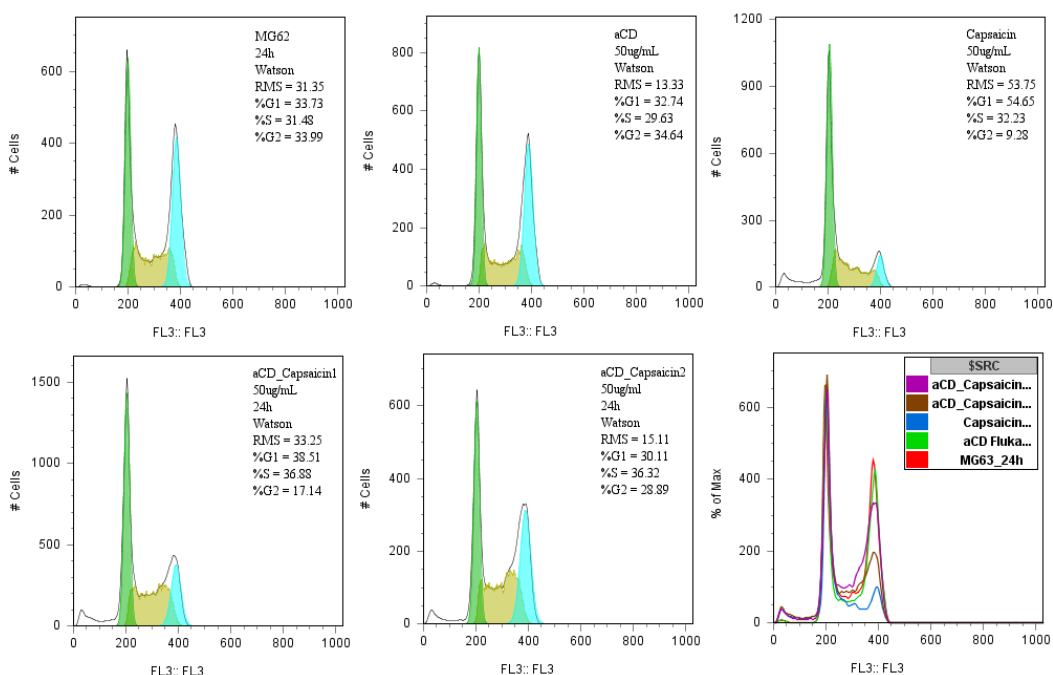


Figure 6.

The effects of CAP, α -CD, α -CD-CAP 1 and α -CD-CAP 2 on MG63 cells cycle

The anti-proliferative effect of CAP *via* inducing cell cycle G0/G1 phase arrest was observed in other studies using colon cancer cells [13]. The proposed mechanism was the stabilization and activation of tumour protein p53, and induction of apoptosis associated with an increase of p21 (cyclin-dependent kinase inhibitor 1), Bax (bcl-2-like protein 4) and cleaved PARP (Poly (ADP-ribose) polymerase) [12]. Another study demonstrated that CAP significantly reduced cell viability associated with apoptosis through mitochondrial membrane permeabilization and caspase activation and cell cycle arrest at G2/M phase [14].

Conclusions

The inclusion complex proved to be the most efficient against the biofilms formed by the two Gram-positive tested strains, i.e. *S. aureus* (5 - 1.25 mg/mL) and *E. faecalis* (the entire range of the tested concentrations, i.e. 5 - 0.0097 mg/mL), as well as on the *E. coli* biofilm (5 - 1.25 mg/mL). The α -CD-CAP 2 mixture proved also a better anti-biofilm activity, as compared with the non-encapsulated CAP, but mainly at the highest tested concentrations. Therefore, we could conclude that the inclusion complex of CAP significantly improves its antibiofilm activity, being thus recommended for the development of preventive or therapeutic strategies for the proper management of biofilm-associated infections.

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