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REZUMAT

BIOLOGICAL AND PHYSICO-CHEMICAL COMPLEX SCREENING OF SEVERAL DERIVATIVES WITH SESQUITERPENIC STRUCTURE

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T i m i ș o a r a
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INTRODUCTION

Despite medical and technological advances, cancer is still one of the leading causes of death worldwide. A main problem associated with the current treatments implemented in the anticancer therapeutic schemes (chemotherapy, radiotherapy or surgical procedures) is represented by the severity of the adverse effects. These can affect both the patients' treatment compliance and their quality of life. As a result, one of the current research directions in the pharmaceutical area is focused on natural substances that have low toxicity levels and high therapeutic efficacy.

Sesquiterpene derivatives present great importance, being currently studied worldwide by researchers in the medico-pharmaceutical field. The basic structure of sesquiterpenoid lactones can be considered to be that of artemisinin, a key compound isolated from *Artemisia annua L.*, a plant used since ancient times for its therapeutic properties in the treatment of malaria. *Artemisia annua L.* is native to Asia and Eastern Europe, but it also currently grows in different European and American countries. Since the end of the 20th century, researchers have shown that artemisinin and its semisynthetic derivatives (artemether, artesunate and dihydroartemisinin) exhibit antiproliferative, proapoptotic and anti-tumor properties. Their biological activity has been correlated with the presence of an endoperoxide bridge within the molecular architecture of the compounds belonging to this class. Their activity is based on their action on neoplastic cells with high proliferation rates and high concentration of iron (II) ions. Since 1990, researchers around the world have studied and presented the cytotoxic effects of sesquiterpene compounds on a significant number of cancer cell lines.

Artemisinin and its derivatives have shown, together with the desired efficacy on different cancer cell lines, reduced levels of toxicity and good tolerability during *in vivo* administration, studies being realized on human subjects and animal models. Unfortunately, their low solubility in aqueous environment raises a number of issues regarding their bioavailability and implicitly pharmacodynamics.

Thus, the present thesis is focused on the study of artemisinin (ART) and its main semisynthetic derivatives (artemether - ARTM, artesunate - ATS and dihydroartemisinin - DHA), which retain the structural elements of the "parent" compound, but have different physico-chemical and biologial profiles. Alongside the pure compounds, the study also presents the development of new formulations that improve the benefits of the compounds, while reducing their disadvantages.

PURPOSE OF RESEARCH

The main scientific objectives established for this PhD thesis are based on the information previously presented and can be resumed as follows:

• Therapeutic target-oriented screening for biologically active compounds with sesquiterpene structure;

• Complete physico-chemical characterization of the selected bioactive molecules, including thermal stability determination, FTIR analysis, kinetic triplet and decomposition mechanism determination through correlation with each compound's chemical structure;
• *In vitro* evaluation of the biological activity by estimating the cytotoxic effect on healthy and pathological human cell lines;
• Evaluation of antioxidant activity and *in vivo* determination of the irritant potential of sesquiterpene compounds;
• Obtaining new formulations in order to improve the solubility of the active substances by forming supramolecular adducts, such as guest-host inclusion complexes and nanoliposomes;
• Physico-chemical characterization of the prepared formulations;
• *In vitro* evaluation of the biological activity for the new formulations.

CONTRIBUTIONS TO THE PHYSICAL-CHEMICAL AND BIOLOGICAL PROFILES OF SESQUITERPEN-LACTONE DERIVATIVES

For each of the four active substances with a sesquiterpenic structure (ART, ARTM, ATS and DHA), a complete physico-chemical profile was obtained. These can provide additional information to the previously reported literature data. Thus, each substance was investigated using thermal analysis (TG/DTG/HF) at five different heating rates β = 5, 7, 10, 12 and 15 °C·min^{-1} and FTIR spectroscopy, an objective evaluation of the mechanism of thermal decomposition being also revealed. The current kinetics concepts concerning determinations in heterogeneous environment were followed, as recommended by the ICTAC international committee through the ICTAC 2000 protocol. Three isoconversional methods were employed (a Friedman differential method, as well as two integral methods, Flynn-Wall-Ozawa and Kissinger-Akahira-Sunose), alongside the non-parametric kinetics method (NPK).

The thermal stability and decomposition processes that occur when the substances undergo controlled heating treatment were evaluated using thermal and kinetic analysis. In accordance with the obtained thermoanalytical curves, ART was found to be stable up to 156-175 °C, ARTM up to 93-112 °C, ATS up to 142-152 °C and DHA up to 132-138 °C. Since the beginning of the decomposition process presents with small variations regarding its starting temperature depending on the chosen heating rate, the temperature up to which the compounds were proven stable is expressed as a range.

Despite the fact that in some cases the TG curve suggests a single mass loss during decomposition, the peaks observed on the DTG and HF curves indicate that the thermo-oxidation of the compounds occurs in several stages. For ART, ARTM and ATS, the endothermic peak visible on the HF curves that marks the melting process appears at temperatures similar to those presented in literature for each individual substance. In the case of DHA, however, the absence of the peak that can be associated with the melting process is most

likely a consequence of the low temperature at which the decomposition begins, probably due to the overlapping of the two types of processes (Fig. 1).

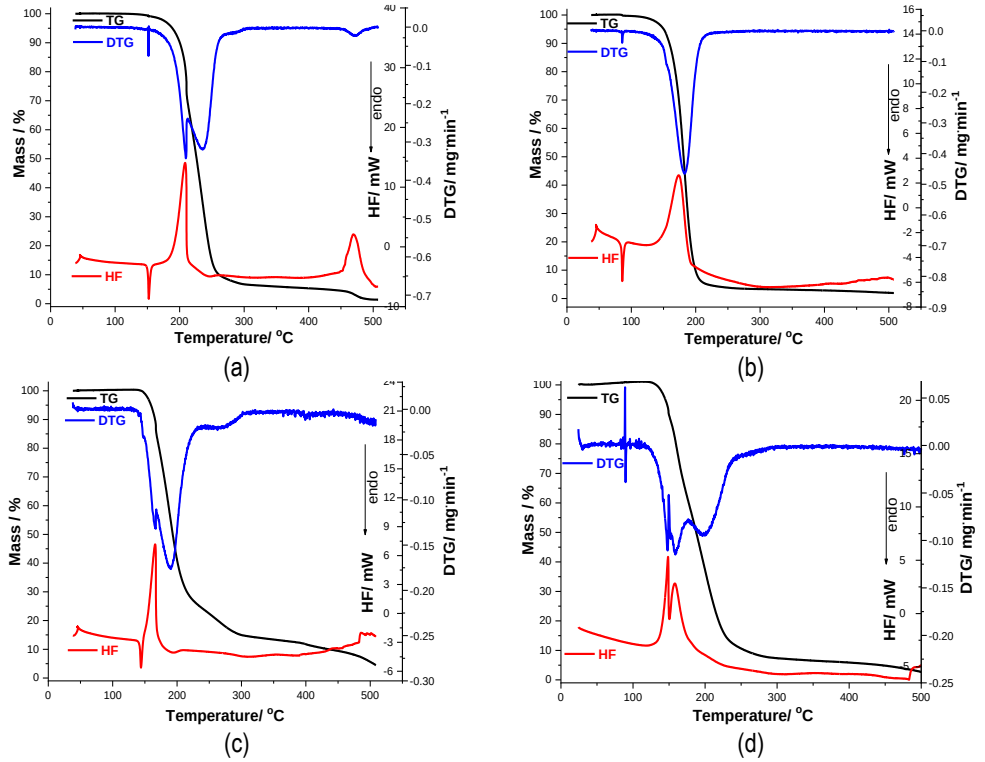


Figure 1. Thermoanalytical data (TG/DTG/HF) determined in synthetic air atmosphere at β = 5 °C·min^{-1} for ART (a), ARTM (b), ATS (c) and DHA (d)

FTIR analysis confirmed the identity and purity of each compound, the data obtained being in accordance with the one found in scientific literature.

The kinetic analysis revealed that the thermally-induced decomposition is a consequence of two different processes that occur for all the tested compounds, with a difference in the nature of the transformation. In the case of ARTM and ATS, both decomposition processes are represented by chemical degradations, but for ART the main decomposition process is correlated with chemical degradation, while the second one involves both chemical degradation and physical transformation. In the case of DHA, both processes are the consequence of both chemical and physical transformations (Table 1).

Table 1. Data obtained using the NPK method for ART, ARTM, ATS and DHA

Sample	Process	λ /%	A /s^{-1}	E_a /kJ·mol^{-1}	n	m	Šesták-Berggren Eq.	R^2	$\overline{E}_a$ /kJ·mol^{-1}
ART	1	94.7	$3.65 \cdot 10^6$	60.9±2.3	1/3	0	$(1-\alpha)^{1/3}$	0.996	61.3±2.4
	2	5.2	$3.75 \cdot 10^6$	70.0±3.8	1	1	$(1-\alpha)\cdot\alpha$	0.994	
ARTM	1	78.0	$8.22 \cdot 10^9$	84.3±5.4	2/3	0	$(1-\alpha)^{2/3}$	0.9994	92.5±6.18
	2	20.7	$4.28 \cdot 10^{15}$	128.8±0.8	1	0	$(1-\alpha)^1$	0.999	

ATS	1	85.4	$1.36 \cdot 10^9$	77.6±2.9	1/3	0	$(1-\alpha)^{1/3}$	0.996	77.6±3.3
	2	14.6	$1.37 \cdot 10^8$	77.2±0.4	1/3	0	$(1-\alpha)^{1/3}$	0.991	
DHA	1	69.8	$1.96 \cdot 10^6$	54.4±4.7	4/3	1	$(1-\alpha)^{1/3} \cdot \alpha$	0.990	58.0±5.5
	2	30.2	$9.78 \cdot 10^5$	66.2±7.4	4/5	1	$(1-\alpha)^{4/5} \cdot \alpha$	0.993	

The *in vitro* biological activity of ART and ATS was evaluated on two types of cancer, namely melanoma and breast adenocarcinoma. The results obtained were dose and time dependent, the best values being seen after a stimulation period of 72 hours and at the highest concentration tested, namely 25 μM. Under these conditions, ART was found to have a considerable inhibitory effect on the growth of the A375 human melanoma cell line (≈ 65%), affecting to a lesser extent the healthy cell line (HaCaT) (≈ 83%). ATS, however, despite a significant cytotoxic effect on the pathological cell line (≈ 77%), caused a very intense toxic effect on healthy keratinocytes (48%). Regarding the cytotoxic effect of ART on breast cancer, the results showed a slightly more intense effect on the estrogen receptor positive MCF7 cell line (≈ 80%) than on the MDA-MB-231 cell line (estrogen receptor negative, ≈ 89%). The toxic effect on the healthy cells (MCF10A) was relatively low (growth inhibition being ≈ 93%). A more obvious difference between the two tested malignant breast cell lines was observed for ATS, the growth inhibition rate being ≈ 45% for MCF7 and ≈ 75% for MDA-MB-231. However, a rather significant toxic effect was also manifested by ATS on the healthy breast cells, the percentage of cell growth inhibition reaching ≈ 53%.

For ART and ATS, the antioxidant activity was evaluated using the DPPH assay, proving to be around 10% and 13%, respectively, when the results were compared with those determined for ascorbic acid. The irritant potential of ART was determined employing the chorioallantoic membrane test (HET-CAM) using chicken egg embryo. The maximum concentration tested, namely 100 μM, caused a weak irritant effect and a decrease in the viability of the specimen after 24 hours. However, when ART was tested in a a smaller concentration (50 μM), it didn't cause any irritating effect, nor did it affect the angiogenesis process or the viability of the specimen (Fig. 2).

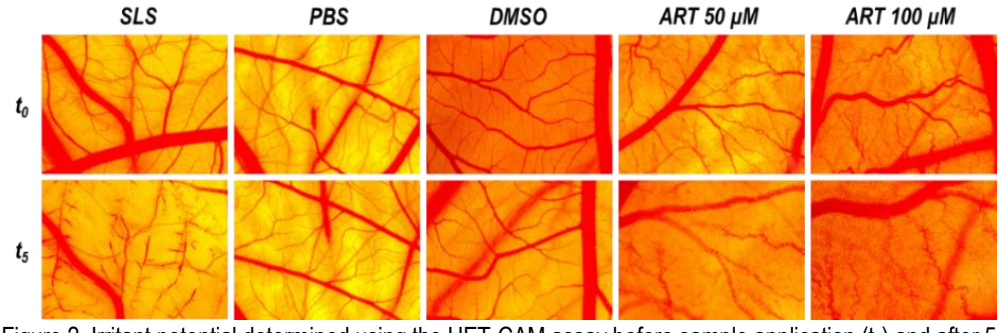


Figure 2. Irritant potential determined using the HET-CAM assay before sample application (t_0) and after 5 min (t_5) of continuous stereomicroscopic evaluation for: controls (SLS- sodium lauryl sulphate, PBS-phosphate buffer solution, DMSO-dimethyl sulfoxide) and ART (50 μM and 100 μM)

CONTRIBUTIONS TO THE PHYSICO-CHEMICAL AND BIOLOGICAL PROFILES OF INCLUSION COMPLEXES AND NANOLIPOSOMES CONTAINING SESQUITERPEN-LACTONE DERIVATIVES

Given the reduced aqueous solubility of sesquiterpene derivatives, their study continued with the aim of obtaining new formulations. These were meant to provide an increased solubility and possibly enhanced cytotoxic effect on malignant cells while reducing toxicity on healthy cells. Thus, two types of formulations were prepared: guest-host inclusion complexes with cyclodextrins and nanoliposomes.

As active substances, ART and ATS were selected for both types of formulations, due to their low solubility and the numerous therapeutic applications indicated in literature. The inclusion complexes were prepared using eight different cyclodextrins, and the two series of obtained structures, namely CPX 1a-8a containing ART and CPX 1b-8b containing ATS, were evaluated using thermal analysis, FTIR spectroscopy and molecular modeling to validate the formation of each guest-host system. FTIR analysis and molecular modeling (Fig. 3) confirmed the formation of all complexes but, for the subsequent biological tests, those with the most favorable physico-chemical characteristics (CPX 6a-8a and CPX 6b-8b) were selected.

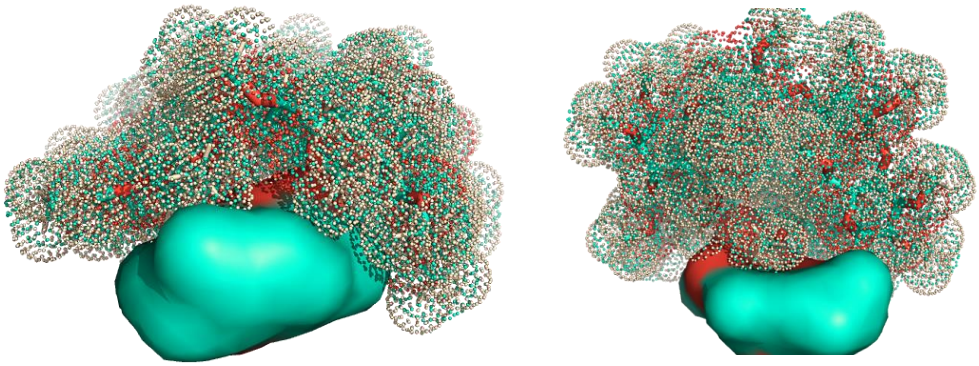


Figure 3. 3D representation of the interactions between ART (solid surface) and heptakis (2,3,6-tri-O-methyl)-β-cyclodextrin (dotted surface) (a), as well as ATS and the same cyclodextrin (b)

The prepared nanoliposomes containing ART or ATS, with or without polyethylene glycol, were subjected to thermal analysis. The results confirmed the formation of the desired systems, aspect revealed by the disappearance of the peaks that characterize the pure compounds from the thermoanalytical curves. Also, the total experimental mass loss was smaller than the hypothetical one calculated for a mixture in which only physical interactions would take place. A significant difference was observed between the PEG-ylated and un-PEG-ylated nanoliposomes, for the first type the decomposition starting temperature being higher and, as such, the formulations presenting improved thermal stability. The

shifts of the characteristic FTIR spectral bands together with the decrease of the peak intensities also proved the formation of the nanoliposomes.

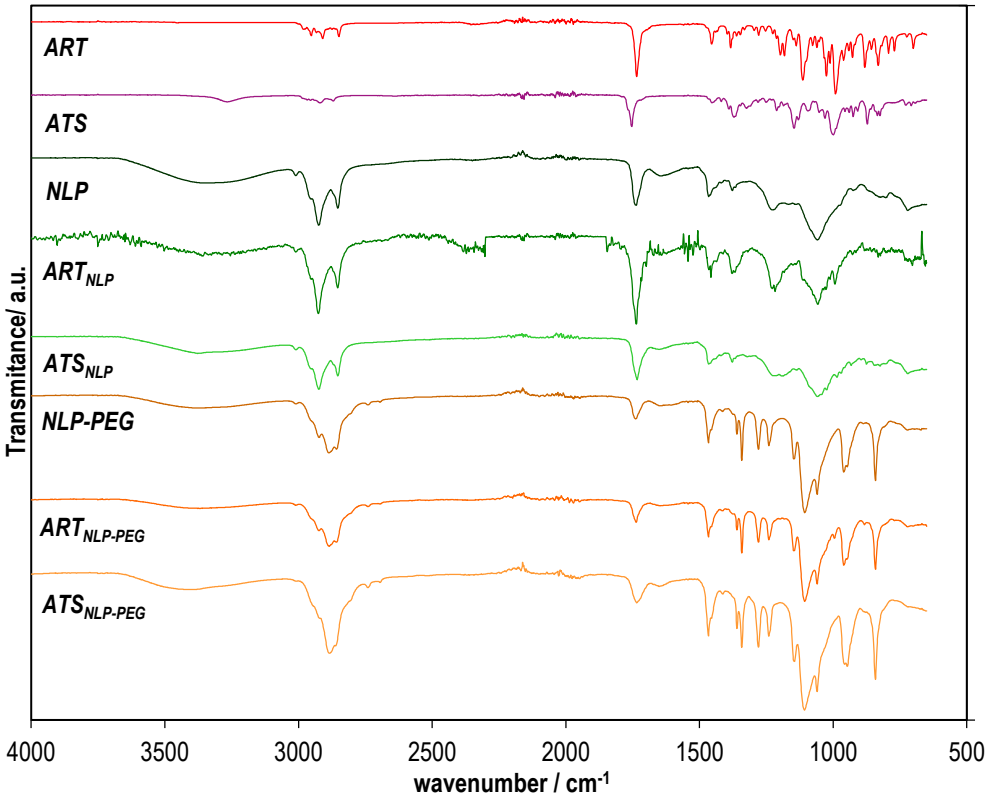


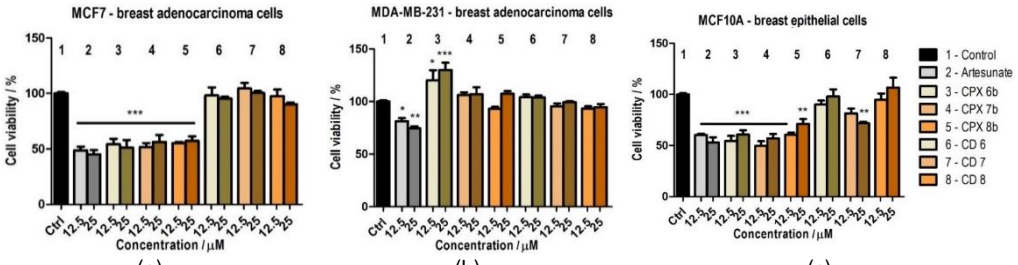
Figure 4. ATR-FTIR spectra obtained for ART, ATS, blank and active substance – loaded NLPs

Zeta potential measurements and particle size determinations showed that immediately after preparation, the nanoliposomes had good stability, each formulation containing populations of homogeneous sizes with particle size values found between 136 nm and 267 nm depending on the composition of the nanoliposome. However, the stability decreased over time and particle size increased, thus determining the need for biological activity evaluation immediately after preparation.

In vitro biological assessments performed for the inclusion complexes containing ART by employing the MTT proliferation method, indicated that out of the three tested complexes, the best potential for cell growth inhibition was presented by CPX 8a on the cell line A375 (≈ 66%), at the highest concentration tested, namely 25µM. Its toxic effect on the HaCaT cell line determines a growth inhibition of ≈ 80%. The results are similar to those obtained for pure ART and this, coupled with the improved solubility conferred by complexation makes this compound a suitable choice for further analyses. In the case of the tested breast cell lines, complexation didn't cause a significant change in toxicity towards

healthy cells (growth inhibition > 90%), but the cytotoxic activity on pathological cell lines varied depending on the cyclodextrin used for the complexation. The best results, in which growth inhibition percentage was similar to that obtained for ART, were observed for CPX 8a on MCF7 (80%) and for CPX 6a on MDA-MB-231 (88%).

In the case of the inclusion complexes formed with ATS, the *in vitro* determinations (MTT assay) performed using the samples in the highest tested concentration showed that although the complexation decreased the toxicity of ATS towards healthy keratinocytes, the cytotoxic effect of ATS on the A375 cell line was diminished by the presence of the cyclodextrins. The results obtained for CPX 6b-8b on the two breast adenocarcinoma cell lines showed significant differences. While CPX 6b and 8b caused growth inhibition of the MCF7 cell line (≈ 51% and 57% respectively), the MDA-MB-231 cells were not affected by any of the tested complexes, in all cases the observed effect being cell growth stimulation and not inhibition, as desired. Although complexation decreased somewhat the cytotoxic activity of ATS on the MCF7 cell line, the toxicity reduction on the healthy MCF10A cells was highly relevant, from a growth inhibition of ≈ 53% for pure ATS to ≈ 61% for CPX 6b and ≈ 71% for CPX 8b. These data suggest that, in the case of ATS, complexation not only increased the solubility of the active substance, but also diminished the toxic effect on breast epithelial cells (Fig. 5).



(a) (b) (c)

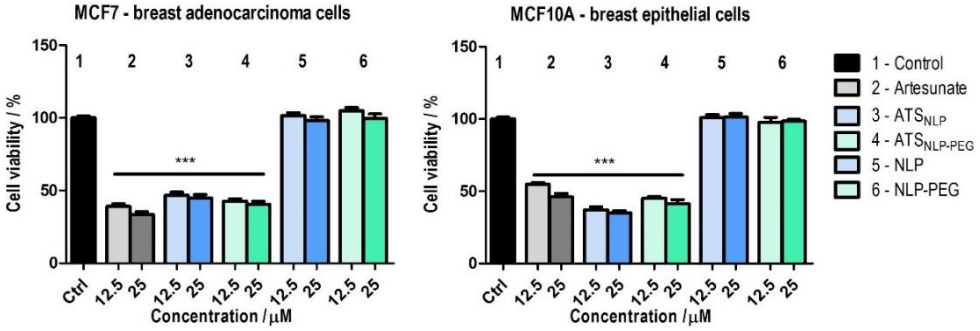
Figure 5. *In vitro* cytotoxicity assessment for ATS, CD6-8 and CPX 6b-8b on MCF7 - human breast adenocarcinoma cell line (estrogen receptor +) (a), MDA-MB-231 - human breast adenocarcinoma cell line (estrogen receptor -) (b) and MCF10A – human breast epithelial cells (c) at 72 h post-stimulation (*p < 0.05; **p < 0.01; ***p < 0.001 vs. control calculated by One-way ANOVA followed by Dunnett's post-test)

In vitro evaluations realized for the prepared nanoliposomes were performed using the Alamar blue method. Skin cell lines (A375 and HaCaT) were used to determine the biological activity of the newly prepared formulations, along with breast cell lines (MCF7 and MCF10A). To certify the lack of toxicity of the blank nanoliposomes, both NLP and NLP-PEG were tested on skin and breast, malignant and healthy cell lines. No significant changes in cell viability were observed. All results obtained when the cells were stimulated with the active substances, in pure form or incorporated in nanoliposomes, were time and dose

dependent, the most relevant results being obtained after a 72-hour stimulation period, at the highest concentration tested, 25 μM.

ART_{NLP} decreased the viability of A375 cells in a more intense manner (≈87%) than that observed for the MCF7 cells (≈ 91%). The addition of PEG to the formulation resulted in an increase in the viability of both malignant cell lines (≈89% and 94% respectively). Unfortunately, both formulations diminished the cytotoxic effect of ART on the tested cell lines. However, both formulations, namely ART_{NLP} and $ART_{NLP-PEG}$, decreased the toxic effect of ART on healthy breast epithelial cells.

Nanoliposomes with ATS caused a decrease in cell viability more intense than the one observed for the corresponding formulations containing ART. Both ATS_{NLP} and $ATS_{NLP-PEG}$ decreased cell viability on the A375 cell line (≈ 59% and 65%, respectively) when compared to ATS (71%), but increased toxicity on HaCaT cells. On the other hand, when used to stimulate breast cell lines, both formulations containing ATS increased the cell viability of the MCF7 cell line, while reducing the cell viability corresponding to the MCF10A cell line (Fig. 6).



(a) (b)

Figure 6. *In vitro* cytotoxicity assessment for ATS, blank NLPs and ATS-loaded NLPs MCF7 - human breast adenocarcinoma cell line (a) and MCF10A – human breast epithelial cells (b) at 72 h post-stimulation (*p < 0.05; **p < 0.01; ***p < 0.001 vs. control calculated by One-way ANOVA followed by Dunnett's post-test)

The evaluation of cell morphology for the cell lines stimulated with ART, ATS, blank nanoliposomes or PEG-ylated nanoliposomes lead to some preliminary conclusions. First, none of the blank nanoliposomes affected the morphology of the tested cells, an expected outcome according to literature data and taking into account the characteristics of the formulation components. Second, ATS, both as a pure compound and incorporated in NLP or NLP-PEG, determined more intense changes in cell morphology compared to ART and its formulations as nanoliposomes. Third, despite the intense cytotoxic effect manifested by ATS and its formulations on malignant cells, they also produced morphological changes in the healthy cells. ART and its formulations, although

less effective on cancer cell lines, determined a lower cytotoxic effect on healthy cells.

Regarding the evaluation of the antioxidant activity for the inclusion complexes whose biological activity had been tested on cell lines, the results showed that, while all three complexes containing ART (CPX6a-8a) presented with an antioxidant activity superior to the one observed for the pure compound (Fig. 7), only CPX 6b improved the AOA of pure ATS.

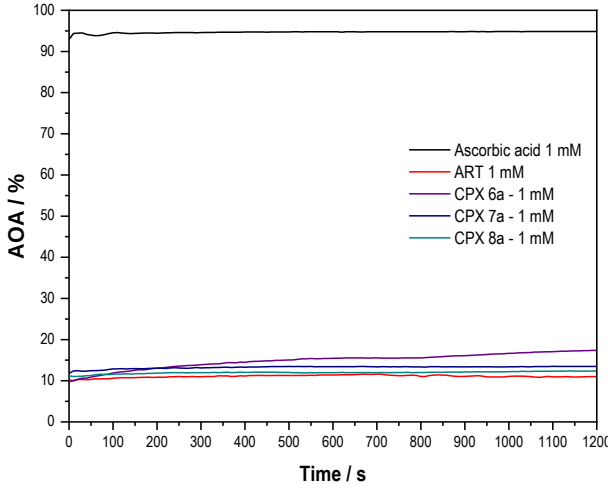


Figure 7. Antioxidant activity evaluation for ART and CPX 6a-8a using the DPPH assay

CONCLUSIONS

Considering the previously mentioned aspects, it can be said that all of the scientific objectives of the present thesis were reached and several research directions were revealed. A further *in vivo* evaluation may be able to give a more comprehensive image of the biological effect determined by the new formulations. Also, an evaluation of the complexation process for artemether and dihydroartemisinin with the discussed cyclodextrins may represent a future research direction.

In conclusion, the present thesis presents a series of comprehensive physico-chemical profiles obtained for artemisinin and its three presented derivatives, artemether, artesunate and dihydroartemisinin. Biological *in vitro* cytotoxicity evaluations were also performed for ART and ATS. Two new types of formulations (guest-host inclusion complexes with cyclodextrins and nanoliposomes – PEG-ylated and un-PEG-ylated) containing artemisinin and artesunate were prepared and evaluated using both experimental directions.

Overall, important physico-chemical data was brought to light in the current study regarding the pure sesquiterpene derivatives and the formation and characterization of the new prepared formulations. ART presented with the highest thermal stability and a moderate dose-dependent cytotoxic effect manifested especially on the A375 and MCF7 cell line, while maintaining a low

toxic effect on the tested healthy cell lines (HaCaT and MCF10A). Out of the two types of supramolecular structures, the inclusion complex CPX 8a containing ART and heptakis(2,3,6-tri-O-methyl)-β-cyclodextrin presented the highest potential as a new improved formulation for ART. The antioxidant activity of ART was enhanced during complexation and the pure compound showed no irritant potential *in vivo* at the concentrations used for the *in vitro* cytotoxicity evaluation.

A relatively high thermal stability was also observed for ATS, but the derivative presented a remarkably significant cytotoxic effect on the tested cell lines even at low concentrations, being highly effective on the MCF7 cell line and moderately cytotoxic on the A375 melanoma cell line. Complexation of ATS with the tested CDs maintained its cytotoxic effect on the MCF7 cell line at approximately the same levels, while significantly reducing its toxic activity on the healthy MCF10A cells. The nanoformulations containing ATS determined an increase in the cytotoxicity levels on the A375 cells, but did not reduce its toxic effect on the healthy keratinocytes. The results indicated that when compared to the “parent” sesquiterpene compound ART, the biological properties of the derivative ATS are potentially superior. The obtained results support further evaluations regarding the supramolecular formulations of ART, ATS and similar sesquiterpene derivatives as potential anticancer agents.

All in all, the present thesis represents an important contribution to the data required in the preformulation stages of drug design, the supramolecular formulations being able to open new pathways in order to develop therapeutic alternatives in the clinical treatment of neoplastic disease.