Cyclodextrin Complexes of Oleanolic and Ursolic Acid
Physico-chemical and biological preliminary evaluation

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Oleanolic and ursolic acids are two isomer triterpenic acids with a large number of various pharmacological activities. Their low water solubilities may explain their low bioavailability. Cyclodextrin complexation was used in order to increase the water solubility of the active substances. The resulted 1:1 complexes were physico-chemically analyzed using thermal analysis, X-ray diffraction and phase solubility studies. Preliminary in vitro investigations were conducted by means of MTT assay on three tumor cell lines: A431, A375 and B16 4A5. The entrapment of triterpenic acids inside the cyclodextrin cavity led to improved solubility as well as higher antiproliferative activity.

Experimental part

**Preparation of complexes**

The two complexes of oleanolic and ursolic acid (Sigma Aldrich, Germany) with OGCD (Cyclolab, Hungary) were prepared in a 1:1 molar ratio using the kneading method. Briefly, the active drug was mixed together with the necessary amount of cyclodextrin in the presence of a hydroalcoholic solution, until a paste-type product was obtained. After 24 h at room temperature, the product was dried in the oven at 105 °C and then pulverized and sieved.

**Phase-solubility studies**

Water solubility studies for both triterpenic acids were conducted by the Higuchi and Connors method [16] using a series of increasing concentrations of oleanolic acid and ursolic acid, respectively, were prepared in the presence of OGCD in a concentration range of 1 to 300 mmol/L. The suspensions were agitated at room temperature for five days and it was assumed that during this period the equilibrium was achieved. After filtration, the resulted solutions were spectrophotometrically analyzed at 210 nm. The following equation was used in order to calculate the stability constants of the two complexes [17]:

$$K = \frac{\tan \alpha}{S_0 (1 - \tan \alpha)}$$

where:

- $S_0$ is the intrinsic water solubility of the active compound
d- $\tan \alpha$ represents the slope of the phase solubility diagram.

**Differential scanning calorimetry (DSC)**

Differential scanning calorimetry was performed using a Mettler Toledo STAR Thermal Analysis System, DSC 821 (Switzerland) and the following parameters: argon as carrier gas with 10 L/h flow rate, 25-300°C temperature range and 5°C/min heating rate. The weight of each sample was between 2 and 5 mg.
X-ray analysis

X-ray analysis was performed by means of a Philips PW 1710 diffractometer equipped with a Cu tube anode, $K\alpha = 1.542\AA$. A 50 kV tube voltage was used as well as 40 mA of tube current in step scan mode (0.035 step size, 1 s / step counting time).

MTT assay

MTT assay was performed for all samples on A431 (skin carcinoma), A375 (human melanoma) and B16 4A5 (murin melanoma) cancer cell lines. A 96-well microplate was used to seed the tumor cells; 200$\mu$L of solution of active compounds in Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco BRL, Invitrogen, Carlsbad, CA, USA) were added, supplemented with 10% fetal calf serum (FCS; PromoCell, Heidelberg, Germany). A mixture of Penicillin and Streptomycin (Pen/Strep, 10,000 IU/mL; PromoCell, Heidelberg, Germany) was added to each sample. After 72 h incubation, 20$\mu$L of 5 mg/mL MTT solution were added to each sample and incubated for 4 h. During this period, the mitochondrial reductase from the intact cells precipitated MTT as its reduced blue form which was later dissolved in DMSO and spectrophotometrically analyzed at 545 nm. Untreated cells were used as control. 10 mM stock solutions of all tested compounds were prepared in DMSO which at the highest riched concentration (0.1%) was proven as a solvent without any significant influence upon cell proliferation.

Paired Student tests or One-way Anova followed by Bonferroni post-tests were used for the statistical analysis of the data; *, ** and *** indicate p<0.05, p<0.01 and p<0.001, respectively, and p<0.05 was considered as level of significance.

Results and discussions

The stability constants of the cyclodextrin complexes are significant values in the evaluation of a product in terms of potential pharmacological use [18]. In the pharmaceutical field a complex between an active drug and a cyclodextrin carrier must exhibit an optimal stability constant which should allow a safe transport within the body fluids as well as a relatively easy release at the target site [19]. Previous reports mentioned an optimal interval of 100-1000 M$^{-1}$ [20] for such pharmaceutical cyclodextrin complexes. In the present study, the stability constants of oleanolic and ursolic acids, respectively, with OGCD are included in table 1, calculated according to the phase solubility diagrams of each acid (fig. 1a, b)).

One can notice that the stability constants have similar values, probably due to the structural similarity of the two compounds, and in the same time are situated within the optimal interval mentioned in the literature [20].

Thermal analysis is an analytical method that quantifies the heat exchange which accompanies a certain process. Differential scanning calorimetry helps assessing if the formation of a true inclusion complex took place, in which case the peaks that characterize the active compound included inside the cyclodextrin disappear or appear shifted.

<table>
<thead>
<tr>
<th>Active compound</th>
<th>Stability constant</th>
</tr>
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<tbody>
<tr>
<td>Oleanolic acid</td>
<td>140 M$^{-1}$</td>
</tr>
<tr>
<td>Ursolic acid</td>
<td>145 M$^{-1}$</td>
</tr>
</tbody>
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Fig. 1. Phase solubility diagrams for: a) OA and b) UA in complex with OGCD [21]. The measurements were conducted within the temperature range 25-300 °C, where no peaks were noticed for the active agents (fig. 2), showing that the melting points of the two acids rank outside of the studied temperature interval. The two complexes reveal a flat DSC curve, offering poor information in terms of proving the inclusion process; however, the association OA:OGCD exhibits an endothermic peak around 285 °C, indicating a melting process at a lower temperature than both compounds, OA and OGCD. The decrease of the melting temperature may be an indicator of an interaction at molecular level between the two compounds.

X-ray analysis stands as a useful method in the cyclodextrin complexation field, indicating the decrease of the cristallinity degree or the shift of the main peaks of an active substance following cyclodextrin complexation [22]. Measurements were conducted on both active agents, OA and UA as well as on their 1:1 complexes with OGCD (fig. 3 a, b)).

Both titerpenic acids display sharp, distinctive peaks, characteristic for a crystalline structure. Following cyclodextrin inclusion, in both cases an amorphisation process can be noticed, revealed by the disappearance of the substances' specific peaks. The X-ray diffractogram of the cyclodextrin itself (not shown) exhibit an amorphous structure. The major change of the active agents' diffractograms as a result of cyclodextrin complexation may be interpreted as a proof of the inclusion process; once included inside the cyclodextrin cavity, the active drug can no longer exhibit its specific X-ray profile.

MTT assay

The final goal in preparing cyclodextrin complexes of an active substance is to improve its pharmacokinetic profile, mainly its bioavailability; also, the process aims to increase the biological activity of the active drug. Pentacyclic triterpenes were the subject of several in vitro studies, alone or in combination with hidrophilic cyclodextrins [23-26]. MTT assay was performed on three cancer cell lines, A431, A375 and B16 4A5, which proved sensitive to other pentacyclic triterpenes as well [23-25, 27-29] (fig. 4).
Previous reports [30-33] have revealed the antiproliferative activity of relatively high dosage of oleanolic and ursolic acids on several tumor cell lines (A375, A2058, A2780); ursolic acid has revealed cytotoxic effects on A431 cell line [34]. Also, cyclodextrin complexes were prepared between the triterpenic acids and various hydrophilic cyclodextrins [31, 33], the process leading to an improved biological effect and a higher bioavailability. The choice of the three tumor cell lines in the present experiment was made based on their previously reported sensitivity against oleanolic and ursolic acid [30, 34]; the antiproliferative activity of the two substances alone was used as reference for the cytotoxic effect of the two complexes, respectively. The MTT assay shows a poor biological activity for oleanolic acid on A375 cell line, in agreement with previous studies [30], activity that improves as a result of cyclodextrin complexation; the same improvement can be noticed as well on the A431 cell line. Ursolic acid displays a rather good activity on both tumor cell lines with a decrease of cell viability following cyclodextrin complexation. The best results and therefore the strongest antiproliferative activity were noticed for the

Fig. 2. DSC curves of: 1 – OA, 2 – UA, 3 – OA:OGCD, 4 – UA:OGCD

Fig. 3. X-ray diffractograms of: a) OA and OA:OGCD complex and b) UA and UA:OGCD complex
B16 4A5 tumor cell line, for both substances as such or in complex with OGCD. Also, an increase of the cytotoxic activity was induced by the cyclodextrin complexation of both oleanolic and ursolic acid, respectively, as compared to the pure substances.

In vitro studies represent a preliminary evaluation of the biological activity of a potential pharmacological agent, MTT assay being a useful tool in assessing the benefits that cyclodextrins might have on the antiproliferative activity of both oleanolic and ursolic acids on the two cancer cell lines involved in the study. By means of MTT, we were able to prove that cyclodextrin complexation led in both cases to a decrease of cell viability as compared to the pure substances, therefore a stronger antitumor activity.

Conclusions
The present paper deals with inclusion complexation of two triterpenic acids, oleanolic and ursolic acid and an amphiphilic cyclodextrin. Physico-chemical analysis has proven the formation of 1:1 complexes between the two compounds and the cyclodextrin. In vitro MTT preliminary test was used in order to emphasize the increase of biological activity of both compounds following cyclodextrin complexation. As previously stated by our research group, cyclodextrin complexation can be a useful tool in achieving a better pharmacokinetic profile and a stronger pharmacological effect.

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Fig. 4. MTT assay of OA, UA and their complexes on A431, A375 and B16 4A5 cell lines


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