Synthesis of Ursolic Acid Dipeptide Derivates with Potential Biological Activity

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Ursolic acid is a natural compound, a pentacyclic triterpene, found in abundance in many species of native wild flora. The experimental study was intended to extract ursolic acid from Calluna vulgaris, L. Hull, (Ericaceae) and its derivatization to obtain dipeptide compounds with potential biological activity. For this purpose, three dipeptides were used: alanyl-glycine, glycyl-leucine and leucyl-glycine. The biological activity of ursoil-glycyl-leucine has been tested on several cancer cells lines, A2780, A431, HeLa and MCF-7.

Keywords: ursolic acid, dipeptides, biological activity

Ursolic acid, (C_{30}H_{48}O_{3}) (fig. 1) is a hydroxy-pentacyclic triterpene that exhibits good chemo protective activity for the human organism, is widespread in nature and can be isolated from several plant extracts. Ursolic acid was found in many plants, including those that have long been used in traditional medicine, [1], especially the leaves of Calluna vulgaris L. (Hull), (Ericaceae), Bruckenthalia spiculifolia, Salisb., (Ericaceae), Salvia officinalis, (Lamiaceae), Staphylea holocarpa, (Rosaceae), Rosa woodsi, (Rosaceae), Prosopis glandulosa, (Leguminosae), Phoradendron juniperinum, (Loranthaceae), Syzygium clavillorum, (Myrtaceae), Hyptis capitata, (Lamiaceae) and Ternstromia gymnanthera, (Theaceae) and also in the peel of Olearia paniculata, (Asteraceae) and Polylepis australis, (Myrtaceae), Hyptis capitata, (Lamiaceae) and Phoradendron juniperinum, (Loranthaceae), Syzygium clavillorum, (Myrtaceae), Hyptis capitata, (Lamiaceae) and Ternstromia gymnanthera, (Theaceae) and also in the peel of Olearia paniculata, (Asteraceae) and Polylepis australis, (Myrtaceae).

The dried vegetal material was next grinded and the heterogeneous granular mixture was separated into granulometric classes. The extraction evolution was surveyed through refractometry, the influence of various parameters upon the extraction of the ursolic acid from the vegetal mass being analyzed. Within the study, there were used several concentration values of the ethanol-water solution, 30, 50, 60, 70, 80 and 95 %, various granulometric fractions, + 1250, - 1250, + 125, + 90 and - 90 μm and different liquid-solid shares, 10 : 1; 20 : 1; 30 : 1 and 40 : 1, (mL : g).

During the experimental study, both the qualitative and quantitative analysis of the ursolic acid in the extract were performed through reversed phase high performance liquid chromatography, (RP-HPLC). The chromatographic separation was carried out on a column C_{18} (250 × 4.6 mm, 5 μm), the used mobile phase being represented by a mixture of acetonitrile and ultra pure water at a ratio of 90 : 10 (v : v) and a flow rate of 0.8 mL / min. Detection was reached at a wave length of 210 nm.

The dipeptide derivatives were obtained by condensing the ursolic acid with alanyl-glycine, glycyl-leucine and leucyl-glycine as showed in figure 2. The synthesis of the ursolic derivatives occurred over a series of reactions developed on four successive stages, among which the first two, corresponding to the obtaining of the intermediary products, (1a) and (2a), are similar in the research methodology to those corresponding to the obtaining of the ursolicderivatives, (4), (6) and (8).

The first reaction was the treatment of ursolic acid with acetic anhydride resulting in monoacetyl ursolic acid, (1a), to which, during the second stage, thionyl chloride was added and the monoacetyl ursolic acid chloride was obtained, (2a). A quantity of 50 mg ursolic acid was

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dissolved in dichloromethane and then was stirred up with 0.15 mL acetic anhydride for 6 - 8 h at room temperature, with the obtaining of the monoacetyl ursolic acid, (1a). To this compound, 1 mL of thionyl chloride is added and after 12 h at room temperature, the redundant thionyl chloride was removed through low pressure distillation, at 75 °C, the residue being then recrystallized from hexane under heating, up to a constant melting point. After a drying up session of 2 h at 110 °C, the acetyl ursolic acid chloride was obtained, (2a). By condensing the compound (2a) with alanyl-glycine in the third phase, the intermediary product, 3-acetoxy-ursoloil-alanyl-glycine was obtained (3), then after removing the acetoxy group by treatment with NaOH in the fourth phase, the synthesis of the final product, ursoloil-alanyl-glycine, (4), was achieved. A quantity of 0.89 mmol of alanyl-glycine dissolved in dichloromethane was used in the synthesis process, which was then mixed with 0.1 mL of triethylamine. The resulted solution was poured under stirring over the monoacetyl ursolic acid chloride, (2a), kept over night at room temperature, then diluted with dichloromethane and washed with water and concentrated saline solution. After filtration and drying on anhydrous sodium sulfate (Na2SO4), the residue is then concentrated under vacuum in a rotary evaporator, and the 3-acetoxy-ursoloil-alanyl-glycine, (3) was obtained. The compound (3) is then dissolved within a mixture of tetrahydrofuran (THF) / methanol 50 %, 2 mL, and treated with a mixture of 0.160 g NaOH, (1 mL, 4 M) and 1 mL water under a ratio of NaOH : H2O = 1 : 1. After stirring the solution for 5 h at room temperature, the product was neutralized with 1 mL HCl 1 N up to precipitation then the obtained precipitate was washed with water and dried under vacuum, resulting in a quantity of 38.06 mg of ursoloil-alanyl-glycine, (4), with a synthesis yield of 7.32 %. In a similar manner, 151.52 mg of ursoloil-glycyl-leucine, (6), with a synthesis yield of 35 % and 54.15 mg of ursoloil-leucyl-glycine, (8), with a synthesis yield of 12.51 % were obtained. The compounds (4), (6) and (8) were characterized by two analytical methods, mass spectrometry, (GC-MS), by electron impact ionization, (EI) and infrared spectroscopy, (IR). The results describing the compounds characterization are presented in table 1.

The antiproliferative effects of ursolic acid and its condensed analogue with glycyl-leucine, (6), were measured for the following cell lines: HeLa (cervical adenocarcinoma); MCF-7 (human breast adenocarcinoma); A431 (squamos cell carcinoma) and A2780.
(human ovarian carcinoma). The MTT assay protocol for cell proliferation was used during research, [19, 20].

Measurement of the antiproliferative effects
Within the undertaken experiment, the antiproliferation effects were expressed as percentages. The simultaneous development of several experiments allowed also the calculation of the mean standard deviation, (MSD). For the ursolic acid, the IC50 (μg/mL) values mentioned in previous studies were also pointed out for comparison purposes. As a general rule, the previously mentioned values were given for the same protocol type, MTT, protocol that was also used in the experiments.

Results and discussions
The study for the extraction of ursolic acid from Calluna vulgaris, L. Hull, (Ericaceae), by maceration has been undertaken by following the influence of several parameters upon the process. The following parameters were investigated: solvent concentration, liquid-solid ratio and dimension of the vegetal mass upon the separation process.

The influence of the solvent concentration on the extraction process was carried out by using different concentrations of ethanol-water solutions, 30, 50, 60, 70, 80 and 95%, while maintaining a constant ratio L : S of 20 : 1, (mL : g) and the granulometric fraction - 1250 μm. The variation of the refractive index is showed in figure 3. It was observed that the extraction is carried out with high efficiency when using an ethanol-water solution with a concentration of 95%, in the given experimental conditions, the equilibrium being reached after approximately 4 days.

The influence of liquid-solid ratio on the extraction was studied by using different L : S ratios of 10 : 1; 20 : 1; 30 : 1 and 40 : 1, (mL : g), an ethanol-water solvent concentration of 95 % and the particle size fraction - 1250 μm. Extraction was registered at maximum speed when using the L : S ratio of 10 : 1, (mL : g) and the equilibrium was reached in the given experimental conditions after approximately 4 days.

<table>
<thead>
<tr>
<th>Table 1</th>
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<tr>
<td>CHARACTERIZATION OF THE SYNTHESIS COMPOUNDS (4), (6) AND (8)</td>
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<th>Table 2</th>
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<tr>
<td>ANTIPROLIFERATION EFFECT OF URSOLIC ACID, [23-26], AND ITS DERIVATIVE URSOLOGLYCYL-GLYCYLEUCINE,(6)</td>
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<tr>
<th>Cell type</th>
<th>Ursolic acid % antiproliferation effect</th>
<th>Urs-Gly-Leu (6) % antiproliferation effect</th>
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<tr>
<td>IC50 (μM)</td>
<td>10μM</td>
<td>30μM</td>
</tr>
<tr>
<td>MCF-7</td>
<td>26.6 [31]</td>
<td>± 4.74</td>
</tr>
<tr>
<td>MTT</td>
<td>± 0.60</td>
<td>± 1.86</td>
</tr>
<tr>
<td>A431</td>
<td>6.8 [34]</td>
<td>35.11</td>
</tr>
<tr>
<td>5200 [34]</td>
<td>± 0.90</td>
<td>± 0.40</td>
</tr>
<tr>
<td>MTT 48h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HeLa</td>
<td>&gt;10 [25]</td>
<td>48.47</td>
</tr>
<tr>
<td>MTT 96h</td>
<td>± 2.13</td>
<td>± 0.15</td>
</tr>
<tr>
<td>A2780</td>
<td>9.5 [23]</td>
<td>*</td>
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The influence of vegetal mass dimension on extraction was studied by using several granulometric fractions, +1250 - 1250 + 125 + 90 - 90 μm. The ethanol-water solvent concentration was kept at 95 % and the L : S ratio at 20 : 1 (mL : g). Extraction was carried out at maximum speed when using the granulometric fraction - 1250 μm, in the given experimental conditions, the equilibrium being reached after 6 days. The variation of the refractive index is showed in figure 5.

The experimental study led to the identification of optimal parameters of the extraction process, respectively the water-ethanol solution with a concentration of 95 %, ratio L : S of 10 : 1, (mL : g) and granulometric fraction - 1250 μm.

Qualitative analysis of ursolic acid was performed based on the retention time (Rt) of 16.10 min, and the quantitative analysis was carried out through the external standard method, by using the calibration curve. The standard solutions used for calibration, 50, 100, 250, 500 and 1000 μg / mL were prepared from the stock solution of 1000 μg / mL through successive dilution with methanol. The sensitivity of the method was expressed as the limit of detection (LOD) of 1.06 μg / mL and the limit of quantification of 3.21 μg / mL. As a result of the extraction, 22.35 mg of ursolic acid were obtained, in a yield of 2.32 % on the vegetal material processed.

The synthesis of ursolic acid derivatives through “one-pot” procedure involved conducting a series of reactions which were carried out in four successive stages. The first two, respectively obtaining intermediate product (1a) and (2a), (fig.2), are similar in methodology as obtaining of the three ursolic derivatives, (4), (6) and (8). In all syntheses, the same ratios and amount of substances were used. The best synthesis performance was obtained for compound (6), the lowest being obtained for compound (4). No explanation was found for the differences arising between the three values of efficiency in the synthesis of ursolic derivatives.

The measuring of antiproliferative effects was carried out for compound (6), and the analysis led to the conclusion that there are no dramatic differences between the activity of ursolic acid and its substituted derivate, in particular for cell lines, A431 and HeLa. For MCF-7 there is a slight tendency to increase the antiproliferative effect, for lower concentration, 10 μM.

Conclusions
Ursolic acid extraction from Calluna vulgaris, L. Hull, (Ericaceae) was performed through maceration, by following the influence of several parameters upon the process, such as solvent concentration, liquid-solid ratio and dimension of the vegetal mass. Experimentally, it has been stated that after a period of about 6 days, at 22°C, the extraction is completed and variations in the refractive index are insignificant. The highest extraction efficiency was attained in the case of ethanol-water solvent with 95 % concentration, to a ratio L : S of 10 : 1, (mL : g) and the granulometric fraction - 1250μm. A quantity of 22.35 mg ursolic acid was obtained as a result of optimal parameter extraction, in a yield of 2.32 %, amount determined through the liquid chromatography analysis, (HPLC).
Three ursolic derivatives were synthesized and were obtained 38.06 mg ursoloil-alanyl-glycine, (4), with a yield of 7.32 %, 151.52 mg ursoloil-glycyl-leucine, (6), with a yield of 35 %, and 54.15 mg ursoloil-leucyl-glycine (8) with a yield of 12.51 %.

The antiproliferative effects were tested for the compound (6), results showing that there are no major differences between the activity of ursolic acid and its substituted derivatives, in particular cell lines, A431 and HeLa, only in the case of MCF-7 where a vague trend of growth in the antiproliferative character for the lower concentration, 10 μM was observed.

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