Identification and Characterization of the Methanolic Extract of Hellebrigenin 3-acetate from Hellebori Rhizomes

II. Mass spectrometry

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The antimitotic action of hellebrigenin 3-acetate (a bufadienolide compound extracted from Bersama abyssinica Fresen.) on nasopharynx carcinoma was described in the late 60s. Literature data are contradictory with regard to the presence of hellebrigenin 3-acetate in Hellebori Rhizomes. In the current study we present the analysis of the methanolic extract obtained from Hellebori Rhizomes by mass spectrometry (MS), with a view to complete the UV and IR results analysis of the methanolic extract of hellebrigenin 3-acetate from Hellebori Rhizomes, presented in Part I of this study. These determinations enabled to clear up the structure of compound in analyzed sample. Spectral measurements on the methanolic extract as compared with data from the existing literature confirmed the presence of hellebrigenin 3-acetate in Hellebori Rhizomes, from Helleborus niger L. ssp. niger.

Keywords: bufadienolides, hellebrigenin 3-acetate, antimitotic activity, mass spectrometry

The literature is largely lacking in publications on the isolation and testing of bufadienolide antineoplastic drugs a class that includes hellebrigenin 3-acetate [1 - 3]. Most articles on extraction, characterization and testing of active substances including some with cardiac and/or antimitotic action were published between 1940s and 1980s [4-6].

Data from the literature are contradictory with regard to the presence of hellebrigenin-3-acetate in Hellebori Rhizomes [7, 8]. Wiessner [7] considered that cardiotonic glycosides were not present in Hellebori Rhizomes. Frohne [9] considered that hellebrigenin 3-acetate (1) came from Helleborus viridis Rhizomes and not from those of Helleborus niger.

In 1989 year Glombitza et al. [8] showed that some fractions of ethanolic extract from Helleborus niger presented positive inotropic effect on isolated left and right atria of Caviaporcellus (Guinea pig).

The aim of this paper is to establish the existence of hellebrigenin 3-acetate in Hellebori Rhizomes. Elemental analysis as well as spectral data have shown the identity of the data the authors have obtained with those reported in the literature [12]. The compound isolated with methanol and purified, was characterized by elemental analysis, UV, IR spectroscopy and mass spectrometry (MS). Obtained data were in a good agreement with those reported in the literature on hellebrigenin 3-acetate [1,2,9,11,13-16].

Experimental part

Equipment and methods

Mass spectra were obtained with a triple quadrupole mass spectrometer model API3200 (AB Sciex) operated in positive electrospray ionisation (ESI). Hellebrigenin 3-acetate extract diluted at 5 μg / mL in methanol/water (50/50, v/v) was directly infused in the ESI interface at a 10 μL/min flow rate, using the built-in syringe pump. Source parameters were as follows: curtain gas - 15 psi; nebulizer gas - 25 psi; IS voltage - 5500 V. Nitrogen was used as curtain and collision activated dissociation (CAD) gas, while nebulizer and heated gas were supplied with zero grade air. The protonated molecular ion [M + H]+ = 459.1 Da was identified in the full scan mass spectrum obtained by scanning the first quadrupole from m/z 100 to 600 Da.

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The MS/MS spectrum was collected after fragmentation of the protonated molecular ion with collision energy of 55...
Identification of the molecular ion.

The expected protonated molecular ion \([M+H]^+\) 459.1 Da corresponding to hellebrigenin 3-acetate, exact mass 458.2305 (1), is clearly present in the ESI positive ionization full scan LC-MS spectrum of the extracted compound (fig. 1 and scheme 1).

Brown et al. (1972) [13] obtained a cation radical with 458 Da by FAB-TOF-MS.

Identification of fragments

Fragment cations and cation radicals obtained are shown in figure 2 and table 1. Values correlate well with the fragmentation described by Brown et al. [13], taking into account the two different mass-spectrometric techniques used. Electrospray ionisation used in LC-MS involves protonation, while electron-impact or fast-atom-bombardment used in GC-MS involves ion-radicals.

Fragmentation of the molecular ion is influenced by the presence of substituents on the Cyclopentanoperhydro-phenanthrene (Cyclopentane-perhydro-phenanthrene) structure, by the greater lability of the D ring, and last but not least by the MS technique used. Generally for bufadienolides, there are three possible types of molecular ion splitting involving the D ring.

The ring D fragmentation (1), the least stable, with formation of cation fragments corresponding the pyrone ring is presented in scheme 2. In the spectrum of molecular ion fragmentation have been identified two fragments at \(m/z\) 109.2 and 135.1, respectively. These fragments are described in the literature [13].

The peak corresponding to \(m/z\) 135.1 also described in literature for hellebrigenin 3-acetate can be noted in the spectrum (fig. 2 and scheme 2) [13]. In this paper, are proposed the other two possibilities for fragmentation. These possibilities were described for other bufadienolides in literature, using the FAB technique. It is the authors’ opinion that the split of the D ring can also be achieved by elimination of the pyrone ring as neutral molecule [13].

In scheme 3, the molecular ion loses one molecule of water successively, the 3-position acetyl group, formyl cation \(^{\text{CHO}}\) \((m/z\) 29), while the cleavage of ring D cation follows fragment at \(m/z\) 211 characteristic of the bufadienolides series.

Another important fragmentation pathway leading to relatively high abundance peaks, characteristic to the bufadienolides series, is loss of water molecules by the molecular ion. Fragmentations have also been highlighted leading to characteristic peaks of \(m/z\) 255.3, and 197.2,

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### Table 1

<table>
<thead>
<tr>
<th>(m/z) Experimental value, ESI-MS-MS at 55 eV (Da)</th>
<th>(m/z) literature (Brown et al.) [10] value (m/z) of EI-TOF-MS, at 70 eV (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>43.1</td>
<td>43</td>
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<tr>
<td>81.2</td>
<td>79</td>
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<tr>
<td>91.2</td>
<td>91</td>
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<tr>
<td>105.3</td>
<td>105</td>
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<tr>
<td>109.0</td>
<td>109.0</td>
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<tr>
<td>119.3</td>
<td>-</td>
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<tr>
<td>135.1</td>
<td>135</td>
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<tr>
<td>145.3</td>
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<td>171.0</td>
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<tr>
<td>289.1</td>
<td>287 (M-171)</td>
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<tr>
<td>-</td>
<td>333 (M-125)</td>
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<td>335.1 (M-124)</td>
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<td>345.4 (M-144)</td>
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<td>363.3 (M-96)</td>
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<td>381.2 (M-78)</td>
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<tr>
<td>441.1 (M-17)</td>
<td>440</td>
</tr>
</tbody>
</table>

* protonated species
Fig. 1. Molecular ion \([M+N]^+\) of hellebrigenin 3-acetate obtained in full scan, positive ESI

Fig. 2. Fragmentation of the molecular ion of hellebrigenin 3-acetate

Scheme 2

Scheme 3
respectively, corresponding to literature data (schemes 4, 5).

In scheme 4 we propose a fragmentation that leads to cation structure that is conserved pyrone ring. Molecular ion at m/z 459.2 can lose a water molecule passing the cation at m/z 441.1, by loosening the ring B. The cleavage is favored by the presence of the formyl group at C10. Further cleavage occurs between positions 5 and 6 of ring B leads to cations fragments at m/z 255.3, respectively m/z 185.3.

Comparing the results with those from literature determination [13], proposed another pathway to split the molecular ion (scheme 5). Lossing one molecule of water and a molecule of acetic acid, the cation may be formed at m/z 255.3.

The spectrum also identified fragments with m/z 197.2 and m/z 195.2 to verify the literature data [13].

Conclusions

Using ESI positive ionization full scan LC-MS technique, was determined the molecular ion of the sample analyzed at 458.2305 Da. The result corresponds to the determinations in the literature, when using FAB-TOF-MS technique for a sample hellebrigenin 3-acetate, extracted from another plant species, *Bersama abyssinica*.

Analyzing MS spectrum of the molecular ion and considering the resulting fragments are cations (and not cation radicals obtained by FAB-TOF-MS technique, Brown et al.), were identified the main pathways of fragmentation.

It is estimated that rings B and D break down the easiest resulting in a first step the two types of cation fragments: fragments containing the pyrone ring and others which do not contain. Comparing the obtained results with literature data, were identified the main characteristic fragments of bufadienolides series.

In conclusion, the sample determined by ESI positive ionization full scan LC-MS contains hellebrigenin 3-acetate. This proves that the species *Helleborus niger* L. ssp. *niger* contains hellebrigenin 3-acetate.

This rule some data from the literature that argues that this compound would result in the contamination of the plant product *Hellebori Rhizomes* from *Helleborus niger* L. ssp. *Niger* with another species, namely *Helleborus viridis*.

Acknowledgements: The authors express their gratitude to Prof. Gabriela Milu, MSc Taxonomy for certifying that the *Helleborus niger* L. ssp. *Niger* subject to our study was indeed *Helleborus niger* L. ssp. *niger* and not *Helleborus viridis*.

References