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

I. UV and IR spectrometry

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The present paper analyzes the chemical composition of the hellebrigenin 3-acetate methanolic extract obtained from Hellebori Rhizomes. That demonstrated the existence of the 3-acetylated compound. Literature describes its antimitotic action on nasopharynx carcinoma. The bufadienolides use as antineoplastic agents followed their use in therapy as cardiotonic drugs also known for their toxic cardiovascular side effects. Analysis of the methanol extract was subsequently performed by UV and IR spectrometry. Spectral measurements on the methanol extract as compared with data from the literature showed the presence of the hellebrigenin 3-acetate in Hellebori Rhizomes from Helleborus niger L. ssp. niger.

Keywords: bufadienolides, hellebrigenin 3-acetate, Helleborus niger, antimitotics

Bufadienolides are separated from other compounds via extraction, partition and column chromatography which are followed by HPLC and droplet counter-current chromatography (DCCC) [1]. The bufadienolides of Helleborus are all derivatives of hellebrigenin.

Hellebori Rhizomes contains two glucosids: helleborein - a cardiac poison and helleborin - a narcotic poison. Other compounds are: fatty oil and acrid resin among unknown componds. Hellebori Rhizomes contains no tannin. A more active helleborein is present in Helleborus viridis than Helleborus niger. Helleborus viridis yields the largest amount of helleborin (0.04 %). Helleborein was discovered by Husemann and Marmé in 1864. The helleborin was discovered by Bastick in 1853. Those substances were investigated by K. Thaeter in 1898. The isolation of the two substances from the root was effected by means of their opposite behaviour toward water and ether, helleborein being freely soluble in water, but insoluble in ether, while helleborin is insoluble in water and soluble in ether.

Helleborein crystallizes from ethanol as very fine needles: they are not hygroscopic when pure; it has a sweetish taste. Helleborein aqueous solution is precipitated by mercurous nitrate and tannic acid. Helleborein is decomposed into sugar and helleboretin (when boiled with diluted acids). The dark blue flakes of helleboretin are soluble in alcohol with violet colour (Husemann and Marmé) and insoluble in ether and water.

The reaction is:

$$C_{37}H_{56}O_{18}\ (\text{helleborein}) + 5H_2O = C_{19}H_{30}O_5\ (\text{helleboretin}) + 2C_6H_{12}O_6 + 3C_2H_4O_2$$

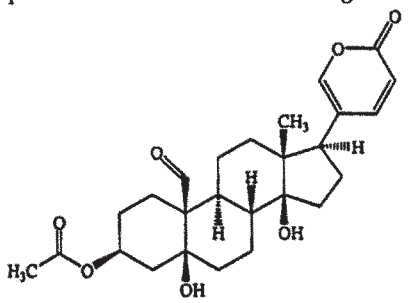
Helleboretin belongs to the fatty series of organic compounds. Helleboretin produces with concentrated nitric acid a characteristic deep-violet colour which, on dilution with water, is persistent for some time. This behaviour is characteristic to helleboretin.

Helleborin has two molecular formulae according to K., Thaeter ($C_{36}H_{42}O_6$) and respectively Husemann and Marmé ($C_6H_{10}O)_n$. Thaeter confirmed all its properties as described by Husemann and Marmé. Helleborin form needles which are odourless but in alcohol solution they are bitter. Helleborin is soluble in chloroform and alcohol but insoluble in cold water. It decomposes into a sugar moiety and helleboresin ($C_{30}H_{38}O_4$) when boiled in diluted acids. In concentrated H_2SO_4 helleborin gives a characteristic violet-red colour; poured into water it precipitates as white flakes.

The literature is lacking in publications on the isolation and testing of antimitotic bufadienolides. Most articles dealing with extraction, characterization and testing of the active substances including some with cardiotonic and/or antimitotic action surfaced during 1940s-1980s [2].

Data from the literature are contradictory with regard to the presence of hellebrigenin-3-acetate in Hellebori Rhizomes from Helleborus niger L. ssp. niger [3, 4]. Compounds found in the Hellebori Rhizomes are: cardiotonic heterosides bufadienolides type (hellebroside and desglucohellebroside); sterolic saponosides (helleborine), ecdisteroides (betaecdisone, macrantoside I), protoanemonine (an irritative lactones that dryes to anemonine, non-irritative); alkaloids (celliamine, sprintiellamine) [5, 6].

There are many authors quoted in the literature who performed studies on the isolation of bufadienolides. The first to perform extraction of hellebrigenin 3-acetate from



Formulae I

Hellebrigenin 3-acetate

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Bersama abyssinica was in [7], with the purpose to demonstrate antimitotic action of bufadienolides, those being known for their cardiotonic action.

According to [7] method, the rhizomes of Bersama abyssinica were extracted continuously with ethanol for 10 h.

Partition of the concentrated ethanolic extract between water and chloroform resulted in concentration of the activity in the chloroform phase. The residue from the chloroform layer was defatted by partitioning between 10 % aqueous methanol and petroleum ether. The material recovered by evaporation of the aqueous methanol layer was dissolved in methanol and treated with a saturated methanolic solution of neutral lead acetate. Removal of the precipitate by centrifugation and of the excess of lead with hydrogen sulfide gave the active extract.

A chloroform solution was collected and examined by Thin Layer Chromatography (TLC) on silicic acid (stationary phase). Re-chromatography on silicic acid lead to the isolation of two crystalline compounds hellebrigenin 3,5-diacetate and hellebrigenin 3-acetate. The last compound, hellebrigenin 3-acetate presented the major cytotoxic activity of the original extract [7].

The researches of Shimada K. et al. (1977) and also Linde H. et al. (1959) were presented in [8]. Shimada presented in various articles bufadienolides isolated from Bufo species (animal sources of bufadienolides, the bufotoxins isolated from the bodies of toads of the genus Bufo). It has been suggested that, by virtue of their potency as digitalis – like inhibitors of Na^+, K^+ - ATP-ase and therefore active monovalent cation transport, bufadienolides and their derivatives may be important in sodium homeostasis in toads that migrate between fresh and salt water environments.

Another authors described a comparative evaluation of the cardiotonic effect of new cardiac glicosides from Far-Eastern Convallaria; cardiac pharmaco-toxicological studies of judaicin, isolated from Artemisia judaica; a new cardiotonic glycoside: proscillaridine A [10].

In a previous article, the authors have described the extraction of hellebrigenin 3-acetate from the rhizomes of the Helleborus niger plant and its elemental analysis. Elemental analysis as well as spectral data showed the identity of the data the authors have obtained with those reported in the literature [11-17].

Experimental part

Materials and methods

Ethanol 95%; chloroform; petroleum ether; methanol; hydrogen sulfide; ceric sulfate (as color reagent); absolute methanol Merck® (UV spectrometry); KBr anhidre Merck® (FT-IR spectrometry);

10% methanol in aqueous solution; saturated solution of lead acetate in methanol; solution of 1, 2 and 5% methanol in chloroform; solution of 7% methanol in chloroform (as mobile phase).

Column chromatography with silicic acid/silicic acid:celite (300 : 50 g) (as stationary phase); column chromatography for HPLC determination was Inertsil ODS-3 C18 (250 x 4.6 mm, i.d., 5μ).

HPLC determination

Verification of purity of the analysis sample, obtained by methanolic extract dilution in *Hellebori Rhizomes*, was realized through HPLC, using a chromatograph CECIL 4300 equipped with UV-Vis detector.

The column was Inertsil ODS-3 C18 (250 x 4.6 mm, i.d., 5μ). Flow rate, detection wavelength and column temperature have been set at 1.0 mL/min, 298 nm and room temperature, respectively. The mobile phase was composed of acetonitrile and 0.3% aqueous acetic acid. The mobile phase composition was maintained constant (isocratic elution mode, 40% : 60 %).

Electronic excitation spectrum (UV-Vis)

From the methanolic extract obtained by the method described previously it was prepared a methanolic solution of 1mg/mL hellebrigenin 3-acetate concentration. This solution was diluted with methanol, obtaining in the end a solution with 10^{-4}mol/L concentration (work solution). The sample was bought in quartz cuvette with optic path of 1 cm. Sample reading was performed against a reference (absolute methanol, Merck®). The sample reading was made at two wavelengths, corresponding to $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions. The spectrometer field reference was between 200-600 nm.

The UV-Vis spectrum of the substance extracted from the plant product has been obtained with UV-Vis spectrophotometers SPECORD® 40.

IR spectra

The sample analysis (working methanolic solution with 1mg/mL concentration) obtained through evaporation of methanolic extract containing hellebrigenin 3-acetate was compressed in pellet KBr and analyzed by vibration-rotation spectrometry (FT-IR) with a Bruker Vertex 70 spectrometer. The analyzed field was between 4000 cm^{-1} and 400 cm^{-1}.

Extraction of hellebrigenin 3-acetate

It has followed step-by-step the method Kupchan et *al.* developed for hellebrigenin 3-acetate extraction from *Bersama abyssinica* [7]. *Hellebori Rhizomes* were graded obtaining an initial amount of 3350 g. This amount was then supplemented with ~3000 g allowing for a sufficient quantity of fraction H.

Extraction was carried out continuously in two halves, with 95% ethanol for a cumulative time of 12 h. The ethanol extract was concentrated under low pressure to give a semi-solid consistency compound (A = 284 g). The extract obtained was then partitioned with a mixture of chloroform: water (5:1.5 L). After vigorous stirring the mixture was allowed to stand for 8 h.

Evaporation of the two solutions resulted in two solution (B = 178 g) and solution (C = 27.5 g). The chloroform extract of the solution was partitioned with 600 mL of a mixture petroleum ether: 300 mL 10% methanol aqueous solution. The solutions obtained were then evaporated under low pressure. The compound (D = 13.3 g) was separated from the methanol extract. The compound (E = 9.8 g) was separated from the petroleum ether extract.

The extract containing the compound D and a solution of 10% methanol were treated with a saturated solution of lead acetate in methanol. The resulting precipitate was isolated by centrifugation, washed and suspended in methanol and then, treated with H_2S. Lead sulphide (PbS) was separated by filtration. The compound (F = 7.7 g) was obtained through the evaporation of the filtrate fraction. The supernatant from the precipitation of lead acetate was treated with H_2S; PbS was removed by filtration. The compound (G = 2.2 g) was obtained via evaporation of the filtrate fraction.

Since the amount of the component was G small we had to repeat the separation scheme yielding to an amount of compound (G = 4 g) from a new batch of *Helleborus niger* purchased from the same source and that came from the same batch cultivation being grown in identical

pedoclimatic conditions. Sampling was done under conditions identical to those of the first purchased lot.

Separation was done by column chromatography with silicic acid as stationary phase [7, 18]. The column was eluted with chloroform (~18L) and a solution of 1% methanol in chloroform until the eluent was colourless. The procedure was then repeated with a solution of 2% methanol in chloroform (~ 18 L). Fractions obtained were examined by thin layer chromatography (TLC) using as solvent a solution of 7% methanol in chloroform and as developant ceric sulfate. Under mild heating green spots were observed. All fractions with an R_f less than that of the green spot were eluted with 1% methanol in chloroform then concentrated under low pressure. These operations were repeated until the fraction H was obtained (H = 8.3 g).

Large value R_f spots (green spots) were eluted with 2% methanol in chloroform and concentrated under low pressure leading to the fraction (J = 1.08 g).

Low value R_f spots (green spots) were eluted with 2% methanol in chloroform and concentrated under low pressure leading to the fraction (K = 1.12 g).

The solvent was further exchanged with a solution of 5% methanol in chloroform leading to the fraction (M = 1.10 g).

The fraction (J = 1.08 g) was then dissolved in chloroform and applied to a chromatography column having as stationary phase silicic acid:celite (300: 50 h). The eluent used was chloroform (~ 4.5 L). Evaporation of the solvent gave a residue of (58 mg). The solvent was then replaced by a solution of 1% methanol in chloroform. 15 mL fractions were collected and then we performed thin layer chromatography on silica gel G using as solvent a solution of 7% methanol in chloroform. The developant was ceric sulfate.

The crystallization of R fraction from a mixture of methanol:ether resulted in two components: *a)* a white precipitate removed by decantation and *b)* colourless prisms by re-crystallization from methanol. Prisms were identified as hellebrigenin 3,5-diacetate (2.05 mg). The fraction (K = 1.12 g) was dissolved in chloroform and then injected in a column chromatography with silicic acid as a stationary phase. That was eluted with chloroform (~5L) and the solvent was then evaporated, yielding to fraction (U = 46 mg). The solvent was then replaced by a solution of 1% methanol in chloroform. Fractions of ~ 15 mL each were collected and thin layer chromatography on silica gel G was then performed using as solvent a 7% methanol solution. The developant was ceric sulfate.

The crystallization of the ω fraction from methanol:ether resulted in two components: *a)* a white precipitate removed by decantation and *b)* colourless prisms by recrystallization from methanol. Colourless prisms were identified as hellebrigenin 3-acetate (82 mg).

Results and discussions

HPLC determination

The HPLC chromatogram analysis it is observed the presence of a single signal (start time: 18:13,8). Values obtained demonstrated the existence of a single compound in the analyzed sample (purity > 99%) and this is hellebrigenin 3-acetate. It is excluded the presence of other compounds that might interfere UV and IR spectra with analyzed sample (fig. 1).

UV-Vis electronic excitation spectra

In order to certify that the sample contained hellebrigenin 3-acetate, it involved a qualitative analysis (i.e. UV) for getting the figer-print described by Kupchan et al. [7].

Bufadienolides UV spectra are characterized by a single peak at λ = 298 nm (ε = 5000) and a 220 nm to 200 nm band of similar intensity. At the same time, the UV spectrum indicates a λ_{max} (in methanol) of 298 nm (ε = 5650) in value of 3-acetate hellebrigenin, which is specific to the α-pyrone ring of a bufadienolide.

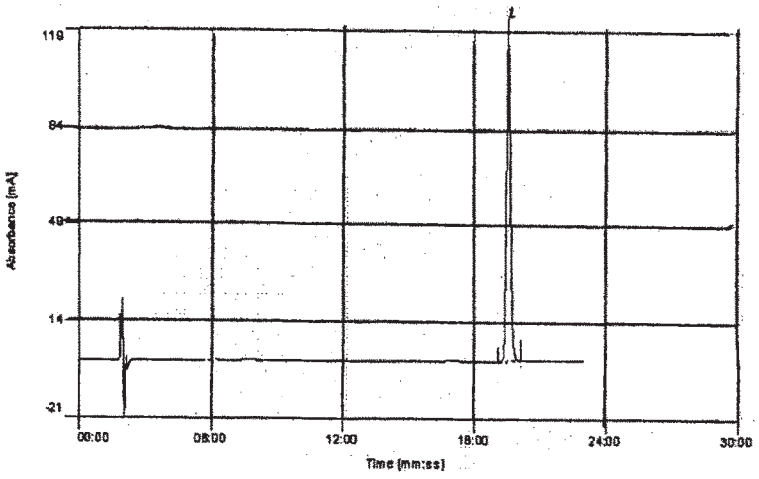
A 200 nm to 220 nm band and a single peak corresponding to λ = 298 can be observed in the spectrum (fig. 2).

Spectral data of hellebrigenin 3-acetate (the extracted substance, formule 1) correspond to spectral data reported in [17] (fig. 3), namely a λ_{max} value (in methanol) at 298 nm (ε = 5650), specific to the α-pyrone ring of a bufadienolide as well as a band between λ= 220 nm and λ = 200 nm of the same intensity.

FT-IR spectra

Because in the literature is not presented a hellebrigenin 3-acetate spectrum but only values of the wave numbers [7, 19] of some of the functional groups, we considered important a full description.

The IR spectrum (fig. 4) confirms the presence of hellebrigenin 3-acetate in the sample analyzed. At high frequencies of the bands can be observed the valence



No.	Peak Name	Ret. Time [mm:ss]	Start Time [mm:ss]	End Time [mm:ss]	Area [mAs]	Height [mA]	Quantity
001	***	01:07,4	18:13,8	19:21,2	1491,4	118,4	N/A

Fig. 1. HPLC chromatogram of the analyzed sample containing hellebrigenin 3-acetate

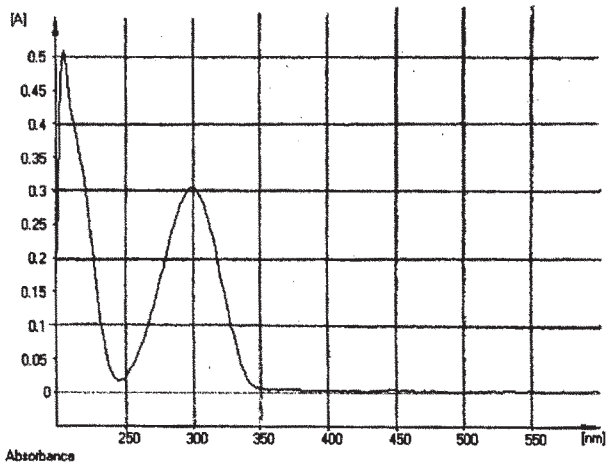


Fig. 2. Electronic excitation spectrum (UV-Vis) of the analyzed sample (methanolic solution 10^{-5} M) containing hellebrigenin 3-acetate (λ = 205.3 nm; A = 0.5103; λ = 297.7 nm; A = 0.3089))

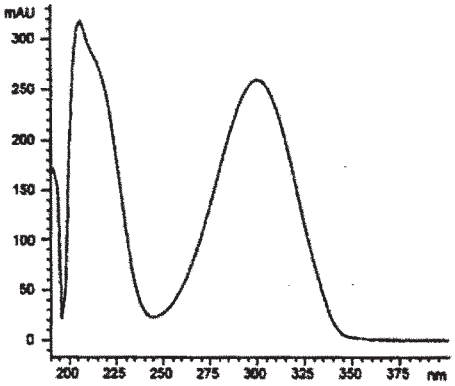


Fig. 3. Bufadienolides UV spectrum [17]

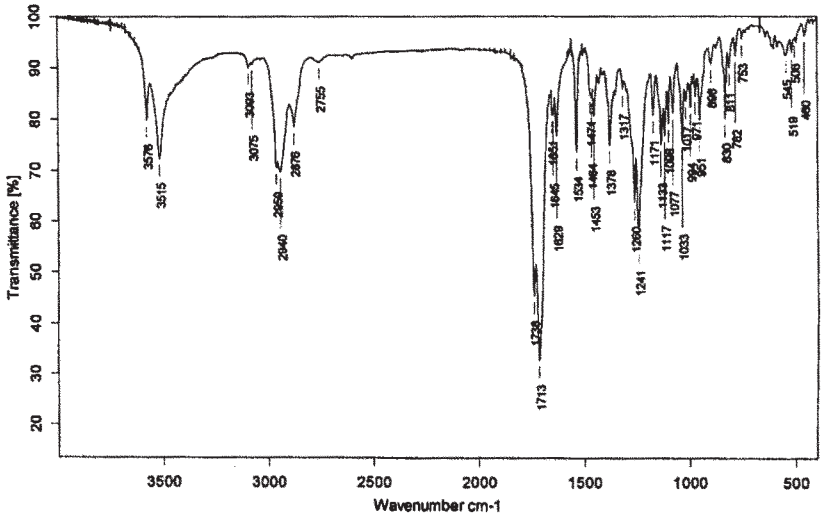


Fig. 4. FT-IR spectra of the analyzed sample containing hellebrigenin 3-acetate, carried out with a Bruker Vertex 70

The IR spectrum (fig. 4) confirms the presence of hellebrigenin 3-acetate in the sample analyzed. At high frequencies of the bands can be observed the valence vibrations of unassociated hydroxyl groups at 3515 cm^{-1} and 3576 cm^{-1} respectively, which proves the reduction of the formation of hydrogen bonds.

In spectrum, methyl groups determine ν_{C-H} symmetric stretching vibrations at ca. 2960 cm^{-1} (overlapping band) and anti symmetric, respectively, at 2959 cm^{-1}.

The methyl groups also determine a δ_{C-H} symmetric, less important, band in the imprint area at 1378 - 1380 cm^{-1} and partially overlapping the δ_{O-H} deformation band (tertiary alcohol). The band at 1453 cm^{-1} can be attributed to the δ_{C-H} antisymmetric vibration of methyl groups.

Methylene groups determine bands corresponding to antisymmetric stretching vibration at 2940 cm^{-1} and symmetric ones at ca. 2876 cm^{-1}, respectively. Values of the wave numbers of the C-H stretching vibrations of methylene groups in untensioned saturated cycles are generally close to those of the methylene groups of alkanes.

In the imprint region, bands due to δ_{C-H} shear vibrations of the methylene groups can be identified at 1464 cm^{-1} and 1455 cm^{-1}, respectively.

The acetyl ester group can be assessed by means of the bands due to the $\nu_{C=O}$ vibration at 1738 cm^{-1} and by the ν_{C-O} valence vibration band at 1133 cm^{-1}, respectively.

The pironic ring with conjugated bonds determines specific bands at 1645 cm^{-1} and 1713 cm^{-1}, respectively. In this region, band $\nu_{C=O}$ stretching vibration of the formyl group at ca. 1730 cm^{-1} is present as well, superimposed on the bands mentioned in the ester group and the pironic ring.

Characteristic bands of C-O bonds in tertiary hydroxyl groups can be observed in the imprint area as well. The values of the wave number of ca. 1033 cm^{-1} suggest their axial arrangement, which confirms the structure of hellebrigenin 3-acetate.

The data are in perfect agreement with those reported in the literature by Kupchan *et al.* for hellebrigenin 3-acetate extracted from *Bersama abyssinica* [7].

Conclusions

Spectral data of hellebrigenin 3-acetate (the extracted compound) correspond to spectral data reported by Ye et al. for UV, and Kamano et al., Kupchan et al. the IR spectrum, respectively.

The above spectral methods of analysis used have shown a good correlation between our data and those published in the literature.

The experimental data have confirmed for the first time the presence of hellebrigenin 3-acetate in *Hellebori Rhizomes* (*Helleborus niger* L. ssp. *niger*); until now this compound was confirmed only in *Hellebori Rhizomes* from *Helleborus viridis*.

These data suggest hellebrigenin 3-acetate extraction method and this compound to be used in therapy for antimitotic action.

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