EXTRACTION OF HELLEBRIGENIN 3-ACETATE FROM *HELLEBORI RHIZOMES (HELLEBORUS NIGER* L. SSP. *NIGER)*

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Abstract

There are contradictory reports in literature whether or not *Hellebori rhizomes* contain hellebrigenin 3-acetate, from *Helleborus niger* L. ssp. *niger*. We followed the same method Kupchan *et al.* used for the hellebrigenin 3-acetate extraction from *Bersama abyssinica* Fresen. We are confident that hellebrigenin 3-acetate came from *Helleborus niger* L. ssp. *niger* and not from *Helleborus viridis* L. since we purchased *Helleborus niger* from a nursery and a close taxonomical analysis was performed. Further studies such as Solid State NMR, UV, IR, and MS-MS, acute, sub-acute and chronic study of toxicity will be employed in order to fully characterize the extracted compound.

Rezumat

Datele din literatura de specialitate sunt contradictorii privind conținutul în hellebrigenin 3-acetat în rizomii de *Helleborus niger* L. ssp. *niger*. În cercetările noastre am urmat aceeași tehnică de extracție descrisă de Kupchan și colab. pentru obținerea hellebrigenin 3-acetat din *Bersama abyssinica* Fresen. În studiul nostru putem spune cu certitudine că hellebrigenin 3-acetatul provine din *Helleborus niger* L. ssp. *niger* și nu din *Helleborus viridis* L. Pentru a demonstra acest fapt am procurat planta *Helleborus niger* dintr-o pepinieră și am realizat încadrarea ei taxonomică. Studiile noastre vor continua prin efectuarea analizei RMN în stare solidă, UV, IR și MS-MS, studii de toxicitate acută, subacută și cronică în vederea caracterizării complete a compusului extras.

Keywords: Hellebrigenin 3-acetate, *Helleborus niger* L ssp. *niger*, extraction, elemental analysis.

Introduction

Data from literature are contradictory regarding the presence of hellebrigenin 3-acetate in *Hellebori rhizomes* (*Helleborus niger* L. ssp. *niger*). Wiessner [1] considered that cardiotonic glycosides were not present in *Hellebori rhizomes.* Frohne [2] considered that hellebrigenin 3-acetate came from *Helleborus viridis rhizomes* and not from those of *Helleborus niger*. In 1989 Glombitza *et al*. [3] showed that some fractions of ethanolic extract from *Helleborus niger* presented positive inotropic effect on isolated left and right atria of *Cavia porcellus* (Guineea pig).

Studies on extraction, characterization and testing of cardiotonic/antimitotic compounds were published during 1940-1980 [4-10,15]. Karrer [11] (1943) - "*Über Hellebrin, ein krystallisiertes Glykosid aus Radix Hellebori nigri*", Schmutz [12] (1947) - "*Glykoside und Aglykone, Hellebrin*" opened the road to hellebrigenin study. Schmutz [13] also isolated hellebrigenin 3-acetate and hellebrigenin 3,5-diacetate. Chen and Henderson [14] published comparative studies on cardenolide *vs.* bufadienolide and therefore on hellebrigenin. The structure of most bufadienolides was published during 1967-1995.

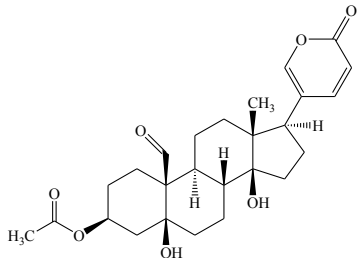

Figure 1
Chemical structure of Hellebrigenin 3-acetate

The present study assessed if *Hellebori rhizomes* contain hellebrigenin 3-acetate. The first step was identifying the sterol structure and the hexa-atomic lactone followed by extraction of what we supposed to be hellebrigenin 3-acetate.

Materials and Methods

Macroscopic characteristics

Helleborus niger L. ssp. *niger* was bought from Sheridan Nurseries, Mississauga, Ontario in order to circumvent any possibility of adulteration with *Helleborus viridis*. Close taxonomical and macroscopic inspections were performed in order to fully certify that the rhizomes took into study only belonged to *Helleborus niger*.

Microscopic characteristics

Microscopic examination for the vegetal product was analyzed using Carl Zeiss AXIO IMAGER D1 system and EC - Plan - NEO FLUAR 10x and 40 x lenses.

Hellebori rhizomes dust has been treated with aqueous solution of 80 % chloral hydrate (i.e. clarified) then tinted with hydrochloric aqueous solution of 1 % phloroglucinol.

Sterol and hexa-atomic lactone structure identification

Reagents used

a. For the Lieberman-Burchard reaction, concentrated sulfuric acid, acetic anhydride and chloroform were used.

b. For the Roques reaction, 85 % aqueous solution phosphoric acid, chloroform and acetone were used.

All these reagents were of analytical purity (Merck®).

Working technique

a) The Liebermann-Burchard reaction: a volume of ~ 5 mL chloroform extract solution was evaporated in a capsule on sand bath then the resulting residue reacted with a volume of ~ 2 mL acetic anhydride. The resulting solution was subsequently separated into two tubes: the blank and the sample. A volume of ~2 mL concentrated H_2SO_4 was then added over the sample.

b) The Roques reaction: A volume of ~ 3 mL chloroform extract solution was brought to residue in a water bath, then that was taken up in ~ 2 mL acetone. Then we added 5 drops of H_3PO_4 prior to keeping the sample in a water bath at a temperature of ~35ºC for 15 min.

Extraction of hellebrigenin 3-acetate

We have followed step-by-step the method Kupchan et *al.* developed for hellebrigenin 3-acetate extraction from *Bersama abyssinica* [6]. Rhizomes were graded obtaining an initial amount of 3350 g. This amount was then supplemented with ~3000 g allowing for a sufficient quantity of compound H (Figure 2).

Extraction (Figure 2) was carried out continuously in two halves, with 95% ethanol for a cumulative time of 12 hours. 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 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 and 10 % methanol. 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 (Figure 2).

The extract containing 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 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 and, PbS was removed by filtration. The compound G (2.2 g) was obtained *via* evaporation of the filtrate fraction (Figure 2).

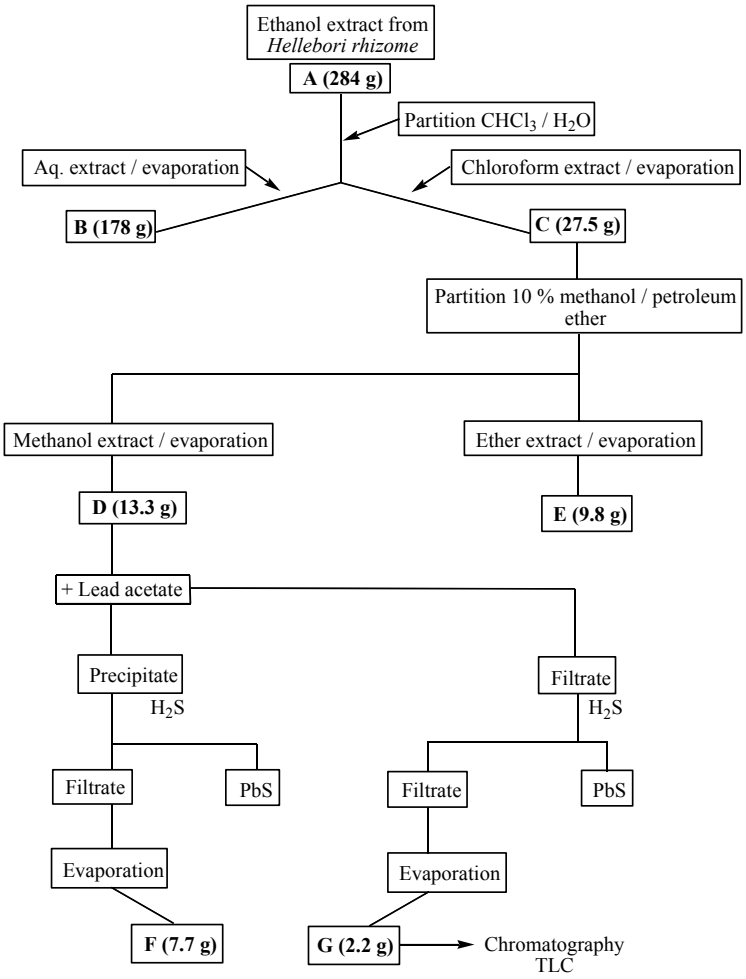

Figure 1 Separation of the ethanolic extract from *Hellebori rhizomes*. The above separation procedure was first used by Kupchan et *al.* [8] for the extraction of hellebrigenin 3-acetate from *Bersama abyssinica.*

Since the amount of the component G was 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 pedo-climatic conditions. Sampling was done under conditions identical to those of the first purchased lot.

Separation was performed by column chromatography with silicic acid as stationary phase. The column was eluted with chloroform (~18 L) 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 ceric sulfate as developant. 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 and then concentrated under low pressure. These operations were repeated until the fraction H was obtained (H = 8.3 g).

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

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 g). 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 (TLC) on silica gel G using as solvent a solution of 7% methanol in chloroform. The developant was ceric sulfate.

The fraction K (1.12g) was disolved in chloroform, 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 ~ 15mL each were collected and thin layer chromatography (TLC) was then performed.

Thin layer chromatography (TLC) conditions

*Stationary phase: silica gel G, precoated TLC plates Merck (20cm x10cm);
*Mobile phase: solution of 7% methanol in chloroform (MPh 1);
*Reference substances: hellebrigenin 3-acetate (H), strophanthine G (S_G) (alcoholic solutions 1.0 mg/mL);
*Reagents: 3% solution of ceric sulfate in H_2SO_4 3N (reagent 1). The chromatograms were examined in visible spectrum and in UV spectrum at 365nm.

Elemental analysis

Quantitative elemental analysis was performed on a PerkinElmer 2400 Series II CHNS/O Elemental Analyzer (Shelton, CT, USA).

Results and Discussion

Macroscopic characteristics

Hellebori rhizomes presented wrinckles with intricate roots. One can see the libero-wood beams on a circular matrix (in cross-section). *Hellebori rhizomes* are of brownish look at exterior and of a white-yellow colour at interior. *Hellebori rhizomes* are odour less and have a bitter taste (Figure 3 and Figure 4).

Figure 3
Hellebori rhizomes, general view

Figure 4
Hellebori rhizomes, close-up view

Microscopic characteristics

By microscopic examination of *Hellebori rhizomes* one can see now the wooden vessels of large caliber, fragments of cortical parenchyma, and bast fibers (Figure 5 and Figure 6).

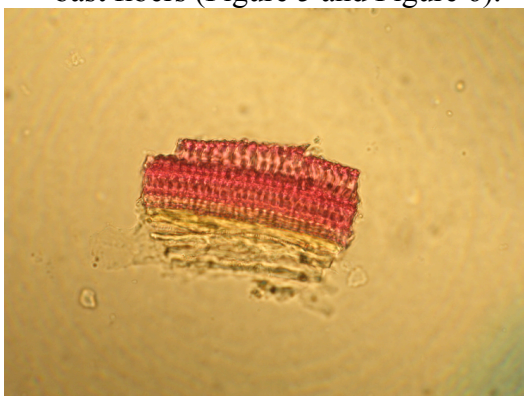

Figure 5
Wooden vessels of large caliber (in red)

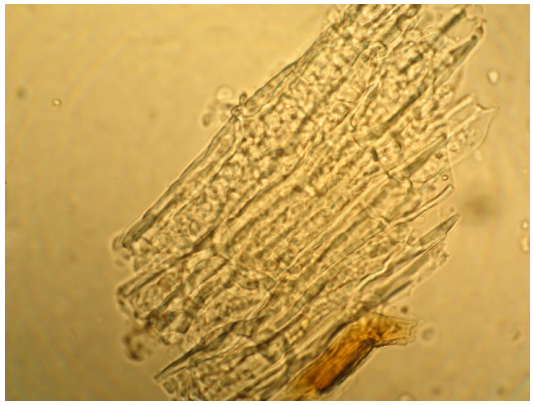

Figure 6
Bast fibers (in orange)

Sterol and hexa-atomic lactone structure identification

The results of the identification of the sterol structure *via* Liebermann-Burchard reaction (a) and that of the hexa-atomic lactone *via* Roques reaction (b) are presented below:

a) The Liebermann-Burchard reaction was positive after ~ 8 min. In the presence of concentrated H_2SO_4 and acetic anhydride, bufadienolide compounds react *via* dehydration, dehydrogenation and isomerization to form a blue-greenish coloured dimer. This colour was an indicative of the sterol structure.

b) The Roques reaction: In the presence of H_3PO_4, bufadienolide compounds that present a hexa-atomic lactone in their structure form a bright-red coloured compound. This colour was an indicative of the hexa-atomic lactone.

Extraction of hellebrigenin 3-acetate

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

Thin layer chromatography (TLC)

The fractions P, Q, R extracted with methanol were collected and labeled as presented in table I.

Table I

Methanolic fraction extract, quantity and spot obtained

Fraction	Symbol	Quantity	Spot
1-124	P	127 mg	No spot
125-147	Q	217 mg	Brown
148-164	R	561 mg	Green and large R_f

P, Q, R are radom symbols from the obtained fraction

The chromatograms have presented UV fluorescence, examination at 365 nm. Using as developer reagent a 3 % solution of ceric sulfate in 3 N sulfuric acid we obtained large green spots for fraction R, corresponding to hellebrigenin 3-acetate (R_f 0.85) and brown spots for fraction Q,

corresponding to strophanthine G (R_f 0.15), and no spot for fraction P (Figure 7 and Figure 8).

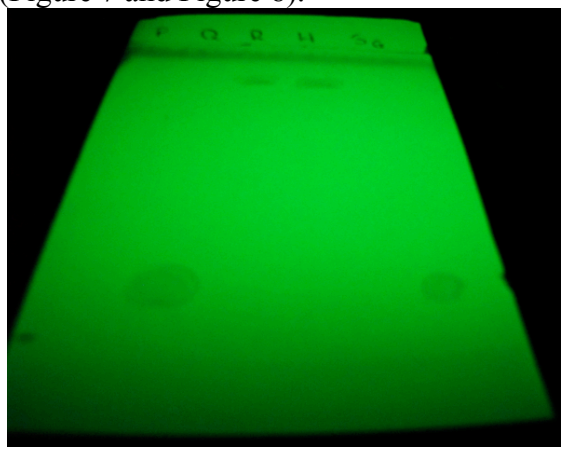

Figure 7
TLC separation of P, Q, R fractions (UV fluorescence, examination at 365 nm; MPh, 1, without reagent 1); P-fraction 1-124, Q-fraction 125-147, R-fraction 148-164, H-Hellebrigenin 3-acetate, S_G-Strophanthine G

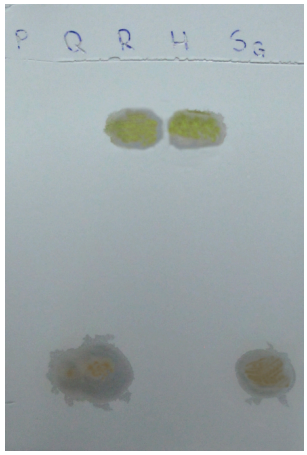

Figure 8
TLC separation of P, Q, R fractions (MPh, 1, reagent 1); P-fraction 1-124, Q-fraction 125-147, R-fraction 148-164, H-Hellebrigenin 3-acetate, S_G-Strophanthine G

The fractions V, W extracted using ether as a solvent were collected and labeled as presented in table II.

Table II
Ether fraction extract, quantity and spot obtained

Fraction	Symbol	Quantity	Spot
1-145	V	124mg	White
146-152	W	703mg	Green and small R_f

V, W are random symbols from the obtained fraction

The chromatograms have presented UV fluorescence, examination at 365 nm. Using as developer reagent a 3 % solution of ceric sulfate in 3 N sulfuric acid we obtained green spots for fraction W, corresponding hellebrigenin 3-acetate (R_f 0.35) and white spots for fraction V, corresponding strophanthine G (R_f 0.65).

Elemental analysis

Elemental analysis was performed on colourless prisms. The resulting data are listed in the table below (Table III). The results were within ±0.4% of the theoretical values, confirming the compound's purity. The values corroborate with results from literature [8].

Table III

Results of elemental analysis for hellebrigenin 3-acetate

Compound	*mp*	$[\alpha]_D$ $CHCl_3$	$C_{calc.}$	$C_{det.}$	$H_{calc.}$	$H_{det.}$
Hellebrigenin 3-acetate	231.6°C	+30°	69.21	69.03	7.74	7.80

Conclusions

Physical parameters, elemental analysis and identification reactions (Liebermann-Burchard and Roques) confirmed that the extracted compound had a molecular weight, a melting point and a composition identical to that of hellebrigenin 3-acetate extracted from the *Bersama abyssinica* bark.

The experimental data have confirmed the presence of hellebrigenin 3-acetate in *Hellebori Rhizomes* (*Helleborus niger* L. ssp. *niger*).

Future research will employ Solid State-NMR, UV, IR, and MS-MS in order to fully characterize the extracted compound.

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References

1. Wiessner,W. Kating, H. Untersuchungen über die Hellebrinfuhrung der unterirdischen Organe von Helleborus Arten, *Planta Med* (1971) 20, 433.
2. Frohne, D. Pfander, H. J., *A Colour Atlas of Poisonous Plants. A Handbook for Pharmacists, Doctors, Toxicologist and Biologists*, Wolfe Publishing, London (1984).
3. Glombitza, K.-W., Kucera-Waldmann, C., Fricke, U. , Do Roots of *Helleborus niger* Contain Cardioactive Substances?, *Planta Medica* (1989) 55, 107-108.
4. Shimada, K., Umezawa, E., Nambara, T., Kupchan, S. M., Structures of novel bufadienolides in the eggs of a toad, *Bufa marinus, Chem. Pharm. Bull.* (1979) 27 (12), 3111-3114.
5. Lichstein, D., Kachalsky, S., Deutch, J., Identification of ouabain-like compound in toad skin and plasma as a bufadienolide derivative, Life Sci (1986), 38:1261-1270
6. Kupchan, S.M., Hemingway,R. J., Hemingway J.C., Tumor Inhibitors. XLIV[1a]. The Isolation and Characterization of Hellebrigenin 3-Acetate and Hellebrigenin 3,5-Diacetate, Bufadienolide Tumor Inhibitors from *Bersama abyssinica*[1b] *J. Org. Chem.* (1969) 34 (12) 3894-3898.
7. Kupchan, S.M., Hemingway,R. J., Smith, R. H., Tumor Inhibitors. XLIV[1a]. Crotepoxide, a Novel Cyclohexane Diepoxide Tumor Inhibitor from *Croton macrostachys*[1b], *J. Org. Chem.* (1969) 34 (12) 3898.
8. Kupchan, S.M., Hemingway,R. J., Doskotch,. R. W., Tumor Inhibitors. IV. Apocannoside and Cymarin, the Cytotoxic principles of *Apocynum cannabinum* L., *J. Med. Chem.* (1964) 7, 803-804.
9. Kelly, R. B., Daniels, E.G., Spaulding, L.B., Cytotoxicity of cardiac principles, *J. Med. Chem.* (1965) 8, 547-548.

10. Kupchan, S.M., Morotoff, M., Sandhu, R.S. and Hokin, L.E., Tumor Inhibitors. XXIII.[1] The Cytotoxic Principles of *Marah oreganus H.*[2], *J. Med. Chem.* (1967) 10, 1025.
11. Karrer, W., "Über Hellebrin, ein krystallisiertes Glykosid aus Radix Hellebori nigri", *Helv. Chim. Acta* (1943) 26 (5-7) 1353-1367.
12. Von Schmutz, J., - Glykoside und Aglykone, Hellebrin, *J. Pharm. Acta Helv.* (1947) 22 373-22 380.
13. Von Schmutz, J., - Die Konstitution des Hellebrigenins. Glykoside und Aglykone, *Helv. Chim. Acta* (1949) 32, 1442-1452.
14. Chen, K. K., Henderson, F. G., Comparative activity of bufadienolides, *J. Med. Pharm. Chem.* (1961) 3: 111

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