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# CYTOTOXIC POTENTIAL OF *HELLEBORUS PURPURASCENS* L.

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**Abstract:** The genus *Helleborus* (Ranunculaceae), or hellebore, comprises around 20 species of perennial herbs native to Europe and Asia. Our investigations refer to the cytotoxic potential of *Helleborus purpurascens* L., based on the traditional use of this genus on several malignancies. The used alcoholic extract is subject to a national patent application (A/00285/2016). The *in vitro* assays conducted on two cancer cell lines Jurkat (a leukaemic T-cell line) and BT-20 (breast cancer cell line) exposed to alcoholic extract for 24 and 48 hours, respectively, confirmed that this plant is a potential antitumoral agent. The effect is dose-dependent and it is obvious that Jurkat cells are more sensible than BT-20 to exposure. The reference compound, 5-FU (50µg/mL), a widely used anticancer agent, acted slower, the cells viability decreasing from 30-55% after 24h of exposure to 10% after 48h. The studies should be continued in order to investigate the compound(s) in *Helleborus* extract responsible for cytotoxic action.
**Keywords:** HPTLC, MTS, viability, Jurkat, BT-20.

## Introduction

Many species of *Helleborus* are seen today as potential sources for anticancer drugs. Studies involving extracts or chemical compounds gave optimistic results related to cancer inhibition and cytotoxicity (Maior and Dobrota, 2013). Extracts of *H. niger* induced proliferation inhibition of lymphoblastic leukaemia cells (MOLT4; IC50: 171µg/mL), myosarcoma (SK-UT-1b; IC50: 304µg/mL) and melanoma cells (HT-144; IC50: 569µg/mL) due to the induction of apoptosis (Schink et al., 2015). The steroidal fraction obtained from the underground parts of *H. caucasicus* showed cytotoxic activity against human lung cancer (A-549) and colorectal cancer (DLD-1) cell lines (Muzashvili et al., 2006). Hellebrigenin, one of bufadienolides belonging to the family of cardioactive steroids, display *in vitro* toxicity against several cancer cell lines, including HL-60, HCT-8, A549, Hs683, MCF-7, PC-3, KB, HeLa and HepG2 (Moreno et al., 2013; Kamano et al., 1998; Cunha et al., 2010) by inducing DNA damage, mitochondrial collapse, cell cycle arrest and apoptosis (Deng et al., 2014). Also, Lindholm et al., 2002, tested, through a large-scale screening protocol, 100 fractionated plant extracts, seven of them showing interesting cytotoxic properties. The aim of this study is to investigate the cytotoxic potential of *Helleborus purpurascens* alcoholic extract on two cancer cell lines.

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## Material and methods

### Plant material

The plant material (*Helleborus purpurascens* roots) was provided by SC Dacia Plant SRL from their own cultivated resources (Brașov area). For this study, an alcoholic extract was prepared according to patent application no. A/00285/2016.

### Chemicals and reagents

Analytical grade solvents were purchased from Sigma-Aldrich, Germany. Reference compounds for HPTLC analysis were purchased from Sigma, Germany and Phytoplan, Germany. CellTiter 96® Aqueous Non-Radioactive Cell proliferation Assay kit was purchased from Promega, USA. All other materials for maintaining the cell cultures were purchased from Sigma Aldrich, Germany or ATCC, USA and the readings were performed on a LKB Chameleon microplate reader.

### HPTLC analysis

Chromatography was performed on silica gel F254 HPTLC pre-coated plates. Samples were applied on the plates as band of 7mm width using a Camag Linomat V sample applicator at the distance of 14mm from the edge of the plates. The mobile phase was constituted of ethyl acetate-acetic acid-formic acid-water 100:11:11:27 (v/v/v/v) for phenolic compounds and ethyl acetate-methanol-water 20.2/2.75/2 (v/v/v) for ecdysones. After development, plates were dried and derivatised in NP-PEG reagent (for phenolic compounds) or vanillin–sulfuric acid reagent (with heating at 80ºC). The fingerprints were evaluated at 254 and 366nm in fluorescence mode and also in visible mode with a WinCats and VideoScan software. Reference compounds for HPTLC analysis were caffeic acid, chlorogenic acid, rutin, hyperoside, β-ecdysone ($10^{-3}$ M).

### Cell lines and culture conditions

The cytotoxic effect on Jurkat T cells (a leukaemic T-cell line, ATCC, USA and BT-20 (breast cancer cell line, ATCC, USA) was analyzed by MTS assay, reflecting cell viability, as described in the manufacturer kit (Promega, USA). BT-20-cells were grown at 37°C with 5% $CO_2$ in EMEM media (ATCC, USA) with 10% fetal bovine serum (ATCC, USA), 1% penicillin/streptomycin/neomycin (Sigma, Germany). Jurkat T cells were grown at 37°C with 5% $CO_2$ in RPMI 1648 media (Sigma, Germany) with 10% fetal bovine serum (Sigma, Germany), 1% penicillin/streptomycin/neomycin (Sigma, Germany). Each type of cell line was trypsinized off the culture flask, split, and replated into 96-well plates and grown until confluent. Jurkat T cells ($2.5 \times 10^3$ per well) and BT-20 cells ($7.5 \times 10^3$ per well) were then incubated with extract of several dilutions and 5-fluorouracil (5-FU, 50μg/mL) as reference compound in 96-well plates (all samples were tested in duplicate). After incubation for 24h and 48h, 50μl of the MTS solution was added. The optical density (OD) values of the solutions were measured at 492nm using a plate reader. All data are expressed as the mean + SD. Statistical analysis was done using student's t-test.

## Results and discussion

*Helleborus* species have long been used in traditional medicine to treat various conditions. Several classes of compounds were identified and studied, including: cardiac glycosides (hellebrin, degluco-hellebrin), steroidal saponins, ecdysteroids and γ-lactones (protoanemonin) but few researches regarding polyphenolic compounds were carried out.

Vochita et al. (2011) showed that total and fractionated polyphenolic compounds extracted from roots and rhizomes of *H. purpurascens* exert a very strong cytostatic effect on HeLa cancerous cells with values between 59.46 - 90%.

The fingerprint of the constituents present in our sample was recorded using Camag TLC visualizer and WinCats Software. The chromatogram (Fig. 1) showed characteristic spots for reference polyphenolic compounds. Chlorogenic and caffeic acid appear as intense fluorescent blue spots (Rf-value 0.52 and 0.95, respectively), quercetin glycosides give yellow spots - rutin at Rf~0.45 and hyperoside at Rf~0.67. None of these reference compounds are present in H extract which exhibits only few blue spots (Rf~0.28, 0.63, 0.88, 0.99), probably polyphenolcarboxylic acids.

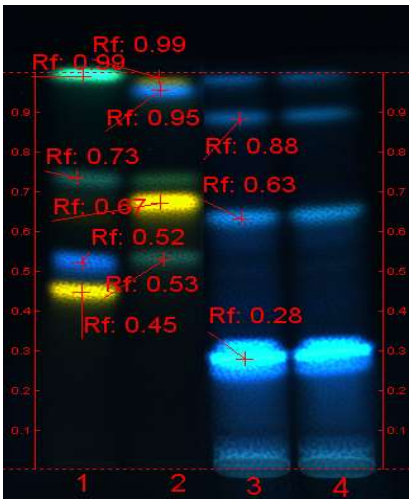


Figure 1. HPTLC fingerprint of H extract, after derivatization with NP-PEG, 366nm.
Tracks 1 and 2 – reference compounds; tracks 3 and 4 – H extract

In the second solvent system, in the extract was identified β-ecdysone (Rf~0.41), a compound widely found in *Helleborus* genus, along with cardiac glycosides.

Exposure of Jurkat cells to *H. purpurascens* alcoholic extract for 24 and 48 hours conducted to a strong decrease of cell viability for all concentrations tested (Fig. 2). The reference compound, 5-FU acts slower, the cells viability decreasing from 30% after 24h of exposure to 10% after 48h. In an experiment conducted on Jurkat cells, it was showed that exposure to *H. niger* extract induces apoptosis via the intrinsic pathway and is independent of Smac overexpression (a mitochondrial protein released into the cytosol when cells undergo apoptosis) (Jesse et al., 2007).

Exposure of BT-20 cells to *H. purpurascens* alcoholic extract for 24 and 48 hours conducted to a decrease of cell viability only for higher concentration of the extract (60-250μg/ mL) (Fig. 3). The reference compound, 5-FU acts slower, the cells viability decreasing from 55% after 24h of exposure to 10% after 48h.

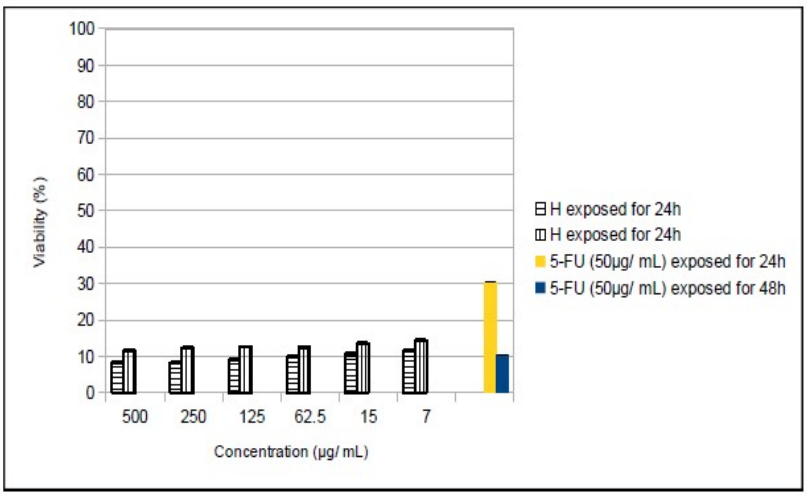


Figure 2. The effect of different concentrations of *H. purpurascens* alcoholic extract on Jurkat cells after exposure times of 24 and 48h. The values are expressed as the means ± S.D. (n = 3). Error bars represent standard error.

It was showed that in other breast cancer cell line, MCF-7, hellethionin C (belonging to a class of peptides found in *Helleborus* genus), at very low concentration (2µg/ml), causes a clear inhibition of proliferation, suggesting that this compound could be responsible for cytotoxic action (Kerek, 2010).

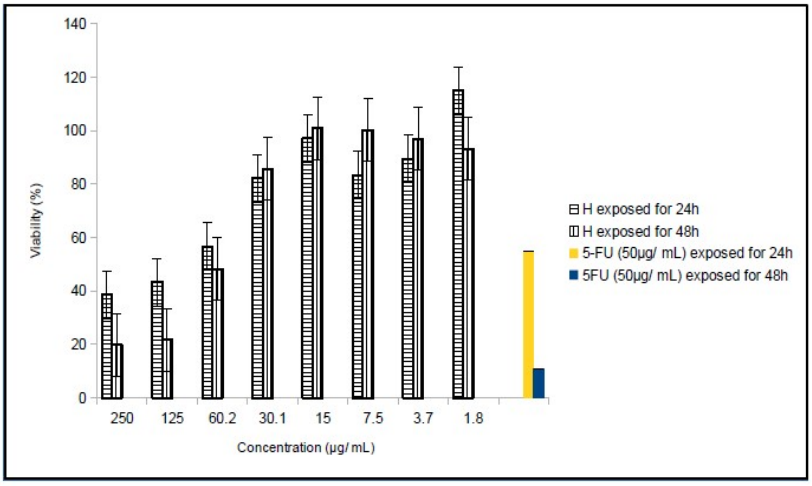


Figure 3. The effect of different concentrations of *H. purpurascens* alcoholic extract on BT-20 cells after exposure times of 24 and 48h. The values are expressed as the means ± S.D. (n = 3). Error bars represent standard error.

Even if *H. purpurascens* is an extremely toxic plant, there are few studies related to its antiproliferative potential. Most of the researches were carried out on HeLa cultures (Vochita et al., 2011; Segneanu et al., 2015) and the authors suggested that responsible compounds for inhibitory action are thionins or phenolic compounds. In this study we investigated the cytotoxic potential of an alcoholic extract on two cancerous cell lines and the results confirmed the efficiency in inhibition of cell proliferation and also the presence in the extract of polyphenolic compounds, ecdysones and cardiac glycosides; but further chemical analysis are required for elucidating the compound(s) that influence(s) this action.

## Conclusions

*In vitro* assays carried out on two cancer cell lines confirmed the cytotoxic potential of the *H. purpurascens* alcoholic extract. The effect is dose-dependent and it is obvious that Jurkat cells are more sensible than BT-20 to exposure. The studies should be continued in order to investigate the compound(s) responsible for this action.

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