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Research Paper

Differential cytotoxic properties of *Helleborus niger* L. on tumour and immunocompetent cells

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ABSTRACT

Ethnopharmacological relevance: In Romanian folk medicine, *Helleborus niger* L. is used for the treatment of rheumatoid arthritis or viral infections and in complementary therapy, especially in anthroposophic medicine (AM), where the plant is administered as an adjuvant to treat malignant diseases. In the present study, we investigated the differential cytotoxic effects of *H. niger* on human tumour and healthy cells of the human immune system *in vitro*.

Material and methods: Protoanemonin and saponins, as significant constituents of *H. niger* extracts, were quantified in five individual batches using validated HPLC methods. Further, the impact of *H. niger* on proliferation capacity (MTT assay) as well as on apoptosis and necrosis induction in a panel of tumour cell lines and human lymphocytes (combined annexin V and propidium iodide staining) was monitored. In addition, NK cell function (degranulation-CD107a assay and IFN-gamma secretion) was also investigated since these immunocompetent cells are important for the control of malignancies within the human body.

Results: Extracts of *H. niger* induced proliferation inhibition not only of lymphoblastic leukaemia cells (MOLT4; IC_{50}: 171 μg/mL) but also of myosarcoma (SK-UT-1b; IC_{50}: 304 μg/mL) and melanoma cells (HT-144; IC_{50}: 569 μg/mL) due to the induction of apoptosis. Purified T cells or NK cells were significantly affected through the presence of high *H. niger* concentrations while bulk lymphocytes were not affected. NK cells' anti-tumour functions expressed by degranulation capacity as well as IFN-γ production were unaffected by the presence of the *H. niger* extract. Since protoanemonin and saponins have been reported in the literature to exert cytotoxic effects, their content was also determined.

Conclusions: H. niger extracts exhibit differential cytotoxicity towards tumour cell lines and healthy human T- and NK-cells.

1. Introduction

Helleborus niger L., commonly known as Christmas rose or black hellebore, belongs to the *Ranunculaceae* plant family. The genus *Helleborus* contains a variety of secondary metabolites including steroidal constituents, *e.g.* β-ecdysone, hellethionins and steroidal saponins (Linde et al., 1971; Milbradt et al., 2003) as well as the ranunculin derivative protoanemonin (Petricić et al., 1971; Bonora et al., 1985). Investigations of the steroidal saponins of *H. viridis* revealed that the leaves contain mainly furostanol glycosides of the diosgenyl type (Braca et al., 2004; Stochmal et al., 2010), whereas the underground parts of *H. caucasicus* bear caucasicosides, which are polyhydroxylated spirostanol and furostanol saponins (Bassarello et al., 2008). The saponin fraction of *H. niger* contains a substantial number of furostanol glycosides, including the bidesmosidic macranthosid I, whose aglycone has a quite similar structure to that of diosgenin (Duckstein and Stintzing, 2014).

In general, saponins possess significant anti-tumour properties, with different types of mechanisms depending on their underlying molecular composition (Man et al., 2010). Steroidal saponins of the diosgenyl type, *e.g.* polyphyllin, dioscin and formosanin C, exhibit

Abbreviations: AM, anthroposophic medicine; HPLC, high performance liquid chromatography; NK cells, natural killer cells; IFN-γ, interferon-gamma; PBMC, peripheral blood mononuclear cells; CD, cluster of differentiation

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very strong anti-tumour properties in their spirostanol form, mainly focussing on leukaemia cell lines, hepatomas and solid tumours (Man et al., 2010). Ranunculin and its enzymatic hydrolysis product protoanemonin are major constituents and are thought to possess systemic cytotoxicity (Turner, 1984).

Standardised *H. niger* extracts are used in complementary medicine, especially in anthroposophic medicine (AM), as subcutaneous injections for supportive treatment of different tumours and haematological malignancies. Additional indications (rheumatoid arthritis or viral infections) are known from Romanian folk medicine (Maior and Dobrota, 2013). *H. niger* preparations possess immunomodulatory properties (Bogdan et al., 1990) and affect the DNA stability of lymphocytes (Büssing and Schweizer, 1998). In addition, cytotoxic and apoptosis-inducing effects on leukaemia cells have been described (Jesse et al., 2009). Still, few publications dealing with the distinct activity of *H. niger* extracts exist. To broaden this knowledge, the present study aimed to investigate the differential cytotoxic properties of *H. niger* on other tumour cells and on healthy T and natural killer cells. Since saponins as well as protoanemonin are characteristic components that may contribute to the biological effects of *Helleborus* extracts, the levels of these substances were quantified by HPLC–DAD.

2. Materials and methods

2.1. Plant material

The investigated aqueous *Helleborus niger* L. extract is an injectable plant extract which is prepared, provided and also marketed by Helixor Heilmittel GmbH & Co. KG (Rosenfeld, Germany) as a registered product according to §§ 38/39 of the German Drug Law. The extract is manufactured and diluted according to the German Homeopathic Pharmacopoeia (German Homeopathic Pharmacopoeia (GHP), 2011). The raw plant material is collected in summer (rhizome and leaves) or winter (flowers) in wild habitats in a region in Lower Austria. Authentication of plant material is done according to the description of *H. niger* to specific literature (Hager, 2013) and by thin layer chromatography according to internal protocols. Voucher samples were deposited at Helixor Herbarium, Rosenfeld, Germany. Publicly available samples can be found at Herbarium Heidelberg (HEID), voucher no. 405977. The plant material is harvested and transported to Helixor Heilmittel GmbH & Co. KG were it is washed and afterwards frozen. Before further processing the frozen plant material is thawed, crushed and extracted by water at 4 °C for 2 h according to GHP 49. Separate extracts are made from whole plant (rhizome and leaves) and flowers, respectively. Both extracts are mixed 1:1 (Flos rec., 0.5 mL and Plant tota rec., 0.5 mL; 1 mL ampoules) and diluted according to GHP 40b. For our investigations, we used an endotoxin-free aqueous drug extract diluted to D1 and stored until further use. Before marketing release all compulsory controls of the finished product have to meet the current specifications for solutions for injection of the European Pharmacopoeia. All specifications for parenteral medications are fulfilled according to the EP. Good manufacturing practice (GMP) and quality control according to the EP is regularly monitored by local German authorities. No additional substances like preservatives are added, except sodium chloride for ionization.

2.2. Quantification by HPLC–DAD analyses

Protoanemonin and total saponins were quantified in duplicate in five individually prepared *H. niger* batches (A–E) using a validated HPLC–DAD method. For each substance class the HPLC quantification was validated in accordance to ICH guideline Q2 (R1), taking into account specificity, linearity, accuracy and precision as well as the limit of detection (LOD) and quantification (LOQ). For statistical analysis and evaluation, the software MVA® 2.1 (Method Validation in Analytics; Novia GmbH, Frankfurt, Germany) was used. Chromeleon 6.8 and 7.1 software (Thermo Scientific, Dreieich, Germany) was applied for HPLC data processing.

Chemicals used were analytical grade at minimum: HCl (25%, Merck, Darmstadt, Germany), acetonitrile and methanol (HPLC grade; J.T. Baker, Deventer, Netherlands). Purified water was used throughout.

2.2.1. Method I: Quantification of protoanemonin

Protoanemonin is a very instable substance (Bonora et al., 1985) and is not suitable as calibration standard for quantification. Based on the monograph of *Clematis recta* of the French Pharmacopoeia, protoanemonin was quantified on an equivalent basis using the structurally related compound alpha-angelica lactone (PubChem CID:11559) (q-NMR contents of two different batches were 64.1% and 79.0%, PhytoLab, Vestenbergsgreuth, Germany). To account for the different peak responses of protoanemonin and alpha-angelica lactone, we used a constant factor which is based on the comparison of the peak areas at known concentration levels to evaluate the amounts of protoanemonin on the basis of alpha-angelica lactone. To verify the protoanemonin peak in the chromatogram of the sample solution, a synthetic protoanemonin standard with proven identity (1H NMR; Stauris, Jena, Germany) was used.

Chromatographic analyses were carried out on a Summit and an Ultimate 3000 HPLC system (Thermo Scientific, Dreieich, Germany), equipped with an online vacuum degasser, a binary pump, an autosampler, a thermostatic column compartment and a UV-VIS diode array detector. A Reprosil-AQ reversed phase column (5 µm, 250 × 4 mm i.d., Maisch, Ammerbruch, Germany) with a pre-column was used for chromatographic separation at 30 °C. The UV-detection of the reference standard alpha-angelica lactone was performed at 220 nm and protoanemonin at 260 nm.

The mobile phase consisted of water (mobile phase A) and methanol (mobile phase B) with a flow rate of 0.75 mL/min. Starting with 3% B for 1 min, a linear gradient was followed to 8% B at 25 min, keeping 8% B until 30 min, then increasing to 100% B at 31 min, continuing for 4 min, before re-equilibration to starting conditions. The injection volume of each sample was 10 µL.

The sample solutions of *H. niger* extracts were diluted with purified water and filtered through a 0.45 µm GHP acrodisc membrane (PALL Life Sciences, Dreieich Germany) prior to use.

Specificity was proven by comparison of the retention time and UV-spectrum of protoanemonin in the sample solution of *H. niger* extracts with protoanemonin standard solution. Linearity was calculated from 6 calibration solutions of alpha-angelica lactone in acetonitrile in the concentration range from 7.54 to 113.17 µg/mL (corresponds to 0.76 to 11.35 µg/mL protoanemonin), resulting in peak areas from 2.2 to 34.4 mAU*min. The calibration curve ($y = 0.3043x - 0.0165$) showed a correlation coefficient of 0.99998 and a relative standard deviation (RSD) of 0.47%.

The system suitability test with an acceptance criterion of RSD ≤ 1.5% was performed by injecting the alpha-Angelica lactone calibration solution six times.

Repeatability was demonstrated by analyzing 6 independent samples of *H. niger* extracts. An RSD of 1.61% was achieved. Normal distribution (David, 95%) and outliners (Dixon, 95%) were checked.

Stability testing of the alpha-angelica lactone stock solution was proven for 27 days at 2–8 °C with a recovery of > 99%. On the contrary, protoanemonin at the same conditions was only stable for 1 day in the aqueous sample solution with a recovery of > 98% and degraded within 5 days to a recovery of about 90%.