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In vitro and *in vivo* investigations on the antitumour activity of *Chelidonium majus*

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ABSTRACT

Background: *Chelidonium majus* L. (Papaveraceae) (greater celandine) is a medicinal herb that is widely spread in Europe. Antitumoural activity has been reported for *C. majus* extracts.
Hypothesis/Purpose: To investigate the antitumour activity of a *C. majus* extract *in vitro* and *in vivo*.
Study Design: Cytotoxic effects of *C. majus* extracts were evaluated on human cancer cell lines, *i.e.* PANC-1 (pancreas cancer), HT-29 (colon cancer), MDA-MB-231 (breast cancer), PC-EM005 and PC-EM002 (primary endometrium cancer cells), and PANC02 (murine pancreatic adenocarcinoma cells). A preliminary *in vivo* study was performed to evaluate the effect of a defatted *C. majus* extract and Ukrain™ in a highly metastatic murine pancreatic model.
Methods: *Chelidonium majus* L. herb containing 1.26% (dry weight) of total alkaloids expressed as chelidonine was used to prepare an 80% ethanolic extract (CM2). This crude extract was then defatted with *n*-hexane, resulting in a defatted *C. majus* extract (CM2B). Cytotoxic effects of the two extracts (CM2 and CM2B) were evaluated on human and murine cell lines *in vitro*. CM2B and Ukrain™ were evaluated in a highly metastatic murine pancreatic model.
Results: Four main benzylisoquinoline alkaloids were identified in CM2B, *i.e.* chelidonine, sanguinarine, chelerythrine and protopine, using HPLC-UV. CM2 showed a high cytotoxic activity against PANC-1 (IC_{50}, 20.7 μg/ml) and HT-29 (IC_{50}, 20.6 μg/ml), and a moderate cytotoxic activity against MDA-MB-231 (IC_{50}, 73.9 μg/ml). CM2 as well as CM2B showed a moderate to high cytotoxic activity against the PANC02 cell line (IC_{50}, 34.4 and 36.0 μg/ml). Low to almost no cytotoxic effect was observed on primary endometrium cancer cells PC-EM005, PC-EM002 and on normal fibroblast cells 3T3, when treated with CM2B. Significantly less metastases were counted in mice treated with 1.2 mg/kg CM2B, but not with 3.6 mg/kg Ukrain™, compared to the control group. The extract, however, did not affect the weight of the primary tumours.

Introduction

Chelidonium majus L. (Papaveraceae), commonly known as greater celandine, is an herb that is widely spread in Europe and is used in folk medicine against disorders of liver and bile and for treatment of warts. The major secondary metabolites are benzylisoquinoline alkaloids, including benzophenanthridines (*e.g.* chelerythrine, chelidonine, sanguinarine, isochelidonine), protoberberines (*e.g.* berberine, coptisine, stylopine) and protopines (*e.g.* protopine). Sanguinarine and chelerythrine are the most prominent alkaloids obtained from roots while coptisine, chelidonine and berberine are usually obtained from the aerial parts. *Chelidonium* alkaloids have been thoroughly studied and their potential application as anticancer agents has already been reported (Barnes et al., 2007; Colombo and Bosisio, 1996; Kemeny-Beke et al., 2006). The antiproliferative effects of *C. majus* were evaluated *in vitro* on rapidly multiplying human keratinocyte (HaCaT) cell lines resulting in an IC_{50} value of 1.9 μg/ml for the dry extract containing 0.68% alkaloids expressed as

Abbreviations: ATCC, American type culture collection; BEGM, Bronchial epithelial cell growth medium; BW, Body weight; CI, Cell index value; CM2, C. majus crude extract; CM2B, C. majus defatted crude extract; DMSO, Dimethyl sulfoxide; EDTA, Ethylene Diamine Tetra-Acetic Acid; EMA, European Medicines Agency; FBS, Fetal bovine serum; HS, 0.05 M n-Heptanesulfonic acid aqueous solution; NR, Neutral red; PBS, Phosphate Buffer Saline; RCT, Randomised clinical trial; RTCA, Real-Time Cell Analysis; SPE, Solid Phase Extraction; SRB, Sulforhodamine B.

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chelidonine. Sanguinarine, chelerythrine and chelidonine gave IC_{50} values of 0.2, 3.2 and 3.3 μM, respectively, whereas berberine showed only low potency with an IC_{50} of 30 μM. The lactate dehydrogenase assay showed a cytostatic activity rather than cytotoxic activity (Vavreckova, 1996a, 1996b). Most *in vitro* studies suggested that sanguinarine, chelidonine, chelerythrine and berberine are responsible for the antitumoural effect of the *C. majus* extract. The strongest antitumour agent was found to be sanguinarine, which intercalates strongly with DNA. Chelidonine, chelerythrine and berberine are also active but are less potent than sanguinarine (EMA, 2011).

Ukrain™, a purported anticancer drug, has been described as a semi-synthetic *Chelidonium majus* alkaloid derivative, consisting of three chelidonine molecules combined to thiophosphoric acid (thiotepa). It is claimed that Ukrain™ is effective against a range of cancers and the drug has been licensed only in a few states of the former Soviet Union. Numerous preclinical investigations and randomized clinical trials (RCT) suggest that Ukrain™ is pharmacologically active and clinically effective as an anticancer drug, but many of the Ukrain™ products show several limitations and there are doubts about the validity of the publications. Moreover, Panzer et al. found the mechanisms of action of Ukrain™ to be similar to the *C. majus* alkaloids it is prepared from. Chemical analyses of Ukrain™ were inconsistent with the proposed trimeric structure, and it was demonstrated that at least some commercial preparations of Ukrain™ consisted of a mixture of *C. majus* alkaloids (including chelidonine) (Panzer et al., 2000a, 2000b; Ernst and Schmidt, 2005). In the present work the antitumour activity of a *Chelidonium majus* extract and Ukrain™ was investigated *in vitro* and *in vivo*.

Materials and methods

Plant material and preparation of the extract

Analytical grade ethanol, *n*-hexane and hydrochloric acid (25%) were purchased from Acros Organics (Geel, Belgium). Ammonium formate was purchased from Acros Organics and *n*-heptanesulfonic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol was HPLC grade and purchased from Fisher Scientific (Leicestershire, UK). RiOS water was prepared by reverse osmosis and water for HPLC was dispensed by a *Milli-Q* system from Millipore (Bedford, MA, USA) and passed through a 0.22 μm membrane filter. The reference materials chelidonine (99.2%), sanguinarine (98%), chelerythrine (97%), protopine (100%) and gemcitabine (100%) were purchased from Sigma-Aldrich. Berberine (97.5%) was purchased from Alfa Aesar (Karlsruhe, Germany). Ukrain™ (batch number A012212111) was purchased from Nowicky Pharma (Vienna, Austria).

Uncut dried *Chelidonium majus* herb was identified and provided by Dr. Olaf Kelber from Steigerwald Arneimittelwerk GmbH; a voucher specimen was kept in the laboratory. The herb was ground and passed through a sieve of 1 mm. According to the method described in the European Pharmacopoeia (European Pharmacopoeia, 2012) the plant material contained 1.26% total alkaloids (dry weight) expressed as chelidonine (minimum requirement 0.6%). An amount of 930 g was extracted exhaustively and consecutively with 33.9 L of 80% ethanol by percolation and maceration at room temperature. The ethanol was removed under reduced pressure at 40°C and the aqueous extract was lyophilised, yielding 192 g crude extract (CM2). Approximately 30 g of crude extract was dissolved in 400 ml of water and extracted three times with 400 ml of *n*-hexane. This aqueous phase (CM2B) was then lyophilised and yielded 21.8 g. This defatted extract contained 2.87% total alkaloids expressed as chelidonine. The *C. majus* crude extract (CM2) and the defatted extract (CM2B) were used to evaluate the antitumoural activity of the extract *in vitro*.

HPLC chromatograms were recorded for CM2B on an Agilent 1260 series with degasser, quaternary pump, automatic injection sampler, thermostatic column compartment and a diode array detector (Agilent Technologies), as well as reference standards, *i.e.* chelidonine, sanguinarine, chelerythrine, protopine and berberine. A HPLC method according to Sarközi et al. (2006), with minor modifications, was used. Solid phase extraction was performed before injection. CM2B (98.0 mg) was dissolved in 3 ml 0.5% HCl in methanol. About 1.25 ml from this solution was diluted with 3.75 ml of 0.05 *M* *n*-heptanesulfonic acid aqueous solution (HS) and was homogenised by sonication. The supernatant was loaded onto an octadecyl SPE column (Chromabond, Macherey-Nagel), previously activated with 5 ml of 5% HS (0.05 *M*) in methanol and 5 ml of 100% HS (0.05 *M*). The SPE column was then washed with 5 ml of a 70% HS (0.05 *M*) solution in methanol to remove the matrix. The compounds were then eluted with 2.5 ml of 5% HS (0.05 *M*) in methanol. The reference standards were dissolved in a concentration ranging from 0.08 – 0.23 mg/ml in methanol. Twenty microliter was injected and separation was performed on the Phenomenex Luna C_{18} (250 × 4.6 mm, 5 μm) (Phenomenex, Torrance, CA, USA) coupled with a precolumn. Column temperature was set at 30°C and the flow rate was 1 ml/min. The mobile phases were (A) 30 m*M* ammonium formate (pH 2.80) and (B) methanol. The gradient was 0 min, 5% B; 5 min, 5% B; 55 min, 100% B; 60 min, 100% B. The chromatograms were recorded at 280 nm and retention time and UV spectrum of the reference standards were compared with the compounds found in CM2B. Fig. 1 shows the chromatogram of the defatted *C. majus* extract and the determined alkaloids. The UV spectrum of the major peak was analyzed at the beginning, the apex and the end of the peak and showed that it was a mixture of different compounds; but according to Sarközi et al. (2006) it should mainly consist of coptisine.

Cell lines

All cell culture reagents and media were purchased from Life Technologies (Ghent, Belgium). All cell lines were maintained at 37°C and 5% CO_2/ 95% air in a humidified incubator. Malignant human cell lines of various origins (breast, pancreas and colon) were used for the *in vitro* cytotoxicity assessments. For breast cancer the cell line MDA-MB-231 was used. These cells form loosely cohesive grape-like or stellate structures consistent with the more invasive phenotype, therefore these cells are considered as invasive *in vitro* (Holliday and Speirs, 2011). Other human cancer cell line included the pancreatic epithelioid carcinoma (PANC-1) cells and the colorectal adenocarcinoma cells (HT-29). MDA-MB-231 cells were cultured in RPMI 1640 medium enriched with 10% fetal bovine serum (FBS), 1% L-glutamine, 1% sodium pyruvate and 1% penicillin/streptomycin. PANC-1 and HT-29 cells were cultured in DMEM also supplemented with 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin. Two normal cell lines were also used to investigate the cytotoxicity of the crude extract. Human bronchial epithelial cells (BEAS-2B) were cultured in bronchial epithelial cell growth medium (BEGM) and the mouse fibroblast cells (3T3) were cultured in DMEM with all the supplements mentioned above. All previous mentioned cell lines were obtained from the American type culture collection (ATCC).

Primary endometrial cancer cell cultures (PC-EM005 and PC-EM002) established from patients undergoing surgery at the Division of Gynaecologic Oncology, University Hospital Gasthuisberg, Leuven (Belgium) (approved by the ethical committee, study number: S52732) were cultured in RPMI 1640 medium with all the supplements described above.

The murine pancreatic adenocarcinoma cells (PANC02) were a kind gift from Dr. C. Gravekamp (Albert Einstein College of Medicine, New York, USA) and were cultured in RPMI 1640 medium supplemented with all the supplements described above.