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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.

Conclusion: C. majus extracts show promising activity, particularly for pancreatic cancer.

Keywords:

Chelidonium majus L.; Papaveraceae; Greater celandine; *In vitro* cytotoxicity; *In vivo* antitumour activity

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

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., 2007). 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 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 randomised 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^{TM} (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 x 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 analysed 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.

Assays

All reagents, including sulforhodamine B, tris(hydroxymethyl)aminomethane (tris) and trichloroacetic acid were purchased from Sigma-Aldrich or Acros Organics. The SRB assay was performed as published before (Capistrano I. et al., 2015) and the NR assay was only used for the cytotoxicity determination on the BEAS-2B cells. This latter experiment was performed at VITO NV in Mol (Belgium). BEAS-2B cells were seeded in 96-well plates (6400 cells/well suspended in 200 µl growth medium). After 48 h of incubation, wells were exposed to various concentrations (1 – 1000 µg/ml) of the extract for 24 h (6 replicate wells per concentration). After incubation, neutral red staining solution (1% dissolved in growth medium) was added to the cells and incubated for 2 h. Cells were subsequently washed with PBS. Neutral red was extracted from the cells by adding 200 µl of 10% acetic acid in ethanol to each well. The optical density was measured at 540 nm using a spectrophotometer. Cell viability inhibition was calculated relative to solvent control.

Experiments carried out on the endometrial cell lines and the mouse fibroblast cells were performed using the *xCELLigence* Real-Time Cell Analysis (RTCA) DP instrument (Roche Diagnostics GmbH, Mannheim, Germany) at the Division of Gynaecologic Oncology, Department of Oncology at KU Leuven. Cell proliferation and cytotoxicity experiments were performed using modified 16-well plates (E-plate, Roche Diagnostics GmbH, Mannheim, Germany) that were placed in the RTCA DP instrument which was kept in a humidified incubator at 37 °C and 5% CO_2/ 95% air. Microelectrodes were attached at the bottom of the wells for impedance-based detection of attachment, spreading and proliferation of the cells. Initially, 100 µl growth medium was added to the wells. The plates were kept at room temperature for 30 min, and then the background impedance for each well was measured. Cells were harvested from exponential phase cultures by trypsinisation with 0.05% Trypsin-EDTA and counted by using a Fuchs Rosenthal Counting Chamber. In every well 100 µl (5000 cells/well for the PC-EM005 and PC-EM002 cell lines, 2000 cells/well for the 3T3 cell line) of the cell suspension was seeded. Water was added to the space surrounding the wells of the E-plate to avoid evaporation from media out of the wells. After leaving the plates at room temperature for 30 min to allow cell attachment, in accordance with the manufacturer's guidelines, they were locked in the RTCA DP device in the incubator. The impedance value of each well was automatically monitored by the *xCELLigence* system and expressed as a cell index value (CI). CI was monitored every 5 or 15 min with the *xCELLigence* system. Twenty-four hours after cell seeding, cells were treated for 48 h with the extract using five different concentrations in triplicate. PBS was added to control wells also in triplicate.

In vitro cytotoxicity

The cytotoxic effect of CM2 was evaluated on various human cancer cell lines *in vitro*, i.e. MDA-MB-231, PANC-1 and HT-29 using the SRB assay. Various concentrations of CM2 (10 – 1000 µg/ml) incubated for 24 and 72 h were tested in three independent experiments. The cytotoxic effect of CM2 was also evaluated on the murine PANC02 cell line using the SRB assay in a concentration range from 1 – 1000 µg/ml (24 h and 72 h incubation time).

The cytotoxic effect of CM2B was tested against the PANC02 cell line *in vitro* in the same concentration range (0.1 – 1000 µg/ml). Three experiments were performed with an incubation time of 24 h and four experiments with an incubation time of 72 h.

The cytotoxic effect of CM2B was evaluated on the BEAS-2B cell line using the NR assay. CM2B was tested at 7 different concentrations (1 - 1000 µg/ml). A 100 mg/ml stock solution of the extract was prepared in 5% DMSO and diluted in media in such a way that the highest end percentage of DMSO did not exceed 0.05%.

The effect of CM2B was also evaluated on two primary endometrial cancer cell lines (PC-EM005 and PC-EM002) and on the mouse fibroblast cells (3T3) using the RTCA system.

Calculation of the IC_{50} values

For the SRB and NR assay, the survival percentages of the cells were calculated from the optical density readings as: (mean OD of treated cells/mean OD of control cells) x 100%. These survival percentages were then fitted in a dose-response curve. The concentration of the crude extract causing 50% of growth inhibition (IC_{50}) was then determined by non-linear regression analysis of the data with the WinNonlin® software (Pharsight, St. Louis, MO, USA)

For the RTCA system, the impedance value of each well was automatically monitored by the *xCELLigence* system in real-time and expressed as CI. A real-time plot of the *xCELLigence* experiment is then graphed by the system and after normalisation of the CI, a dose-response curve was plotted and the IC_{50} value was calculated. The endpoint that was selected to calculate the IC_{50} value was approximately 48 h after addition of the extract.

In vivo evaluation – effect on metastases

An *in vivo* study was performed to evaluate the effect of the *C. majus* defatted extract (CM2B) on a highly metastatic pancreatic murine model. This study was executed at the department of Microbiology and Immunology, Albert Einstein College of Medicine (NY, USA). PANC02 cells were cultured in McCoy's medium supplemented with 10% FBS, glutamine (2 m*M*), nonessential amino acids, sodium pyruvate (1 m*M*), Hepes (10 m*M*), and penicillin/streptomycin (100 U/ml) and were maintained in exponential growth in a humidified 5% CO_2 / 95% air atmosphere at 37 °C. Six-to-eight weeks old female C57BL/6 mice were obtained from Charles River and maintained in the animal husbandry facility of the Albert Einstein College of Medicine according to the Association for Assessment and Accreditation of Laboratory Animal Care guidelines, and according the guidelines of the Albert Einstein Institute for Animal Studies. The mice were housed under a 10/14 h

dark/light cycle at constant temperature and humidity and had access to tap water and food *ad libitum*.

A pilot study was performed to determine the toxicity of *C. majus* on healthy tissues. Non-tumour-bearing C57BL/6 mice (n = 3 per dose) were given different doses of *C. majus* extract (0.4, 1.2 and 2.4 mg/kg BW). These doses were given IP daily for 3 days and the acute toxicity was then evaluated. The mice that received the two lowest doses (0.4 and 1.2 mg/kg BW) did not show any adverse effects. However, two mice died within 3 h after receiving an injection at the highest dose (2.4 mg/kg BW) and the last mouse died immediately after the second injection. Therefore it was preferred to test the cytotoxic effect of CM2B in tumour-bearing mice using the concentration of 1.2 mg CM2B/kg BW.

Cultures of PANC02 cells were harvested using a 0.05% trypsin solution, washed twice in sterile PBS and resuspended in sterile PBS at a concentration of 20 x 10^6 cells per ml. The viable cells were counted by using a haemocytometer and trypan blue. Mice (n = 21) were inoculated with 100 µl of the cell suspension in the mammary fat pad. In this PANC02 metastatic model, the primary tumour extends to the chest cavity lining, which is palpable 5 to 7 days after tumour cell injection, but primary tumours stay relatively small, whereas metastases (visible by eye) predominantly developed in the portal liver, resulting in the production of ascites in the peritoneal cavity within approximately 20 days. In addition, the pancreas is heavily infiltrated with PANC02 tumour cells, but less frequently in the mesenteric lymph nodes, diaphragm, spleen, and kidneys (Quispe-Tintaya, 2013).

Three independent experiments were performed with 2 groups, i.e. a control group, which was given saline (n = 3 mice per experiment) and a treatment group, which was given either CM2B or Ukrain™ or gemcitabine (n = 5 mice per treatment) (Table 1). All mice were injected with PANC02 tumour cells in the mammary fat pad and monitored for the next 3 weeks. Treatment was administered for 19 days, starting 3 days after tumour inoculation. The first day of treatment was assigned “day 1” (Fig. 2). The control mice received 300 µl saline IP daily, while the positive control group was given gemcitabine dissolved in saline 3 times/week. It was administered IP at a dose of 60 mg/kg BW. CM2B was administered IP daily at a dose of 1.2 mg/kg BW and Ukrain™ was given 3 times/week at a dose of 3.6 mg/kg IP. All mice were checked for ascites (swollen abdomen) daily and were sacrificed 23 days

after tumour cell injection. The mice were analysed for ascites, tumour weight and the number of metastases in the peritoneal cavity.

Statistical analysis

Statistical analysis of the *in vivo* results was performed by a non-parametric Mann-Whitney test, using GraphPad Prism 6 (Version 6.01). Values of $p \leq 0.05$ were considered significant.

Results

Cytotoxic effects of CM2 on PANC-1, HT29, MDA-MB-231, and PANC02 tumour cell lines and of CM2B on PANC02 tumour cell line

A survival curve for each cell line and incubation time is shown in **Fout! Verwijzingsbron niet gevonden.**. 3. The calculated IC_{50} values for these cell lines are summarised in Table 2. The *C. majus* crude extract only exerted a moderate effect on the MDA-MB-231 and PANC02 cells with an IC_{50} above 20 µg/ml for both incubation times. A lower IC_{50} value was observed when incubated for 72 h compared to 24 h. A relevant cytotoxic effect of CM2 was observed on PANC-1 and HT-29 at IC_{50} value around 20 µg/ml for both incubation times. The dose-response curve of CM2B against the PANC02 cell line is shown in Fig. 4. The calculated IC_{50} values for this cell line treated with CM2B were 36.0 ± 2.6 for 24 h and 30.1 ± 0.7 for 72 h. The results observed for the defatted *C. majus* extract on the PANC02 cell line did not differ from the results observed for the crude extract. The IC_{50} values for both extracts all ranged around 35 µg/ml and both extracts were considered having a moderate to high cytotoxic effect on the PANC02 cell line.

Cytotoxic effects of CM2B on BEAS-2B, PC-EM005 and PC-EM002 tumour cell lines, and 3T3 cells

CM2B was also evaluated on other cell lines *in vitro* using various methods. Fig. 5 shows the percentage of cell viability inhibition on BEAS-2B cells relative to the solvent control. The IC_{50} as determined from the fitted curve was 64.38 µg/ml, thus CM2B only exerted moderate activity against BEAS-2B cells.

CM2B was evaluated against the two primary endometrial cancer cell lines (PC-EM005 and PC-EM002) and the mouse fibroblast cells (3T3) using the RTCA system. Dose-response curves were plotted and IC_{50} values calculated (Table 3). High IC_{50} values were observed for PC-EM005 and 3T3 cells, thus having no relevant cytotoxic effect on these cell lines. For the PC-EM002 cell line a low cytotoxic effect was observed with an IC_{50} of 0.1 mg/ml. Fig. 6 shows a real-time plot of the cytotoxic effect of CM2B on the PC-EM002 cell line. While no cytotoxic effect of CM2B was found on the PC-EM002 cells at a concentration of 100 µg/ml, a decline in number of cells was observed starting from a concentration of 250 µg/ml. Approximately 15 h after adding treatment, a slight increase in number of cells at a concentration of 250 µg/ml was found, but after some hours a proliferation inhibition was observed. When treated with 500 µg/ml, again a decline in cell number was observed 3 h after the treatment was added, and inhibition of proliferation was observed 24 h later. When cells were treated with 1 mg/ml, a slow decrease in cell number was observed during the entire incubation period.

In vivo evaluation – cytotoxic effect of CM2B on metastases in PANC02 model

Firstly, each mouse was scored based on the degree of ascites (bloody, translucent or no ascites). The gemcitabine-treated mice did not show any ascites. All mice of the saline control groups developed bloody ascites. One mouse of the *C. majus* treated group did not show any ascites, while the four others showed translucent ascites. For the Ukrain™ treated group, 3 mice had bloody ascites and 2 mice had no ascites.

Secondly, all mice were analysed for tumour weight and number of metastases (Fig. 7). Treatment with the *C. majus* extract resulted in significantly less metastases compared to the saline control group. However, the extract did not affect the weight of the primary tumours. No significant effect of Ukrain™ on the number of metastases and tumour weight was observed compared to the control group. In the positive control group that was treated with gemcitabine, significantly less metastases were counted in comparison with the saline control group, but again no significant decrease in tumour weight was observed.

Although the positive control confirmed that the used model was suitable to evaluate the cytotoxic effect of the CM2B on the number of metastases, this was merely a pilot study with promising preliminary results. Therefore further investigation with for example a bigger sample size is needed. The PANC02 model is an extremely aggressive model, and in the future less tumour cells should be injected in order to create a longer treatment time period to fine tune the cytotoxic effects on metastases.

Discussion

In this study the cytotoxic effects of two *Chelidonium majus* L. extracts (CM2 and CM2B) were evaluated on various tumour cell lines *in vitro* and in pancreatic cancer *in vivo*. It was demonstrated that CM2 showed a high cytotoxic activity against PANC-1 and HT-29, and a moderate cytotoxic activity against MDA-MB-231, with IC_{50} values between 20 and 100 µg/ml. CM2 as well as CM2B showed a moderate to high cytotoxic activity against the PANC02 cell line. Low to almost no cytotoxic effect was observed on PC-EM005, PC-EM002 and 3T3 cell lines, when treated with CM2B, with IC_{50} values above 100 µg/ml. In a pilot study in mice with pancreatic cancer (PANC02 model), a significant reduction of the number of metastases was observed compared to the control group, when treated with 1.2 mg CM2B/kg BW. The extract, however, did not affect the weight of the primary tumours. This is most likely due to the poor vascularization of the primary tumours in this model (Quispe-Tintaya et al, 2013). The drug Ukrain™, which was demonstrated to be a preparation consisting of a mixture of *C. majus* alkaloids has been tested in several human clinical trials, and results were considered to be quite promising. Increased survival of patients with colorectal cancer, regression of tumours in patients with bladder cancer, improved survival of patients with pancreatic cancer, and remarkable symptomatic improvement in patients with breast cancer was found (Ernst and Schmidt, 2005). While most primary tumours can be removed by surgery, radiation and/or chemotherapy, for metastases there is still no cure. The significant reduction in the number of metastases with the CM2B extract of *Chelidonium majus* L. in mice with pancreatic cancer is therefore particularly promising. Ninety-five percent of all cancer patients diagnosed with pancreatic cancer will die within 6 months of the diagnosis. Combining CM2B with gemcitabine, which is the most common standard therapy in patients with pancreatic cancer, may be envisaged but is not supported by appropriate experimental data yet.

In conclusion, CM2 showed a high cytotoxic activity *in vitro* against various human cancer cell lines. CM2 as well as CM2B showed a moderate to high cytotoxic activity against the PANC02 cell line. In a pilot study with the PANC02 mouse model, *C. majus* (1.2 mg CM2B/kg BW) showed a significant reduction of the number of metastases compared to the control group. The extract, however, did not affect the weight of the primary tumours.

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Conflicts of interest

The authors wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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433

Table 1

Group treatment assignment

Group	# mice	Treatment and dose
Negative control	9	300 µl saline IP, daily
Positive control	5	60 mg/kg BW gemcitabine IP, 3x/week
***Chelidonium majus* extract**	5	1.2 mg/kg BW IP, daily
Ukrain™	5	3.6 mg/kg BW IP, 3x/week

Table 2

IC_{50} of the *C. majus* crude extract on the tested cell lines

		IC_{50} ± SEM (µg/ml)			
		MDA-MB-231	**PANC-1**	**HT-29**	**PANC02**
***C. majus* (CM2)**	24 h	73.9*	20.7 ± 1.0	20.6 ± 2.5	34.4 ± 2.8
	72 h	57.8 ± 5.1	19.8 ± 2.7	19.4 ± 1.6	33.4 ± 1.4

*No standard error is shown since the best-fit value for the slope was difficult to determine, due to the low amount of data point in that area

Table 3

IC_{50} of the defatted *C. majus* extract on the tested cell lines

		IC_{50} ± SEM (µg/ml)				
		PANC02		**PC-EM005**	**PC-EM002**	**3T3**
***defatted C. majus* (CM2B)**	24 h	36.0 ± 2.6	48 h	1400 (R^2: 0.98)	100 (R^2: 0.98)	1600 (R^2: 0.99)
	72 h	30.1 ± 0.7				

Figure legends

Fig. 1. HPLC-UV chromatogram of the defatted *C. majus* extract (CM2B) and the determined alkaloids i.e. protopine, chelidonine, sanguinarine and chelerythrine. The major (unlabeled) peak was not pure, but consisted mainly of coptisine.

Fig. 2. Time line of the *in vivo* metastasis study. Inoculation of tumour cells, start of treatment and euthanisation are indicated by black arrows. The coloured lines above the time line indicated the days certain treatments were given. Ukrain™ and gemcitabine (positive control) were given 3 times/week, while the *C. majus* extract (CM2B) group and the saline group (negative control) were treated daily.

Fig. 3. Dose-response curves of the different cancer cell lines treated with *C. majus* crude extract (CM2). Four cancer cell lines were treated with various doses of CM2 for 24 and 72 h. Three independent experiments were used to fit the curves and to calculate the IC_{50} values.

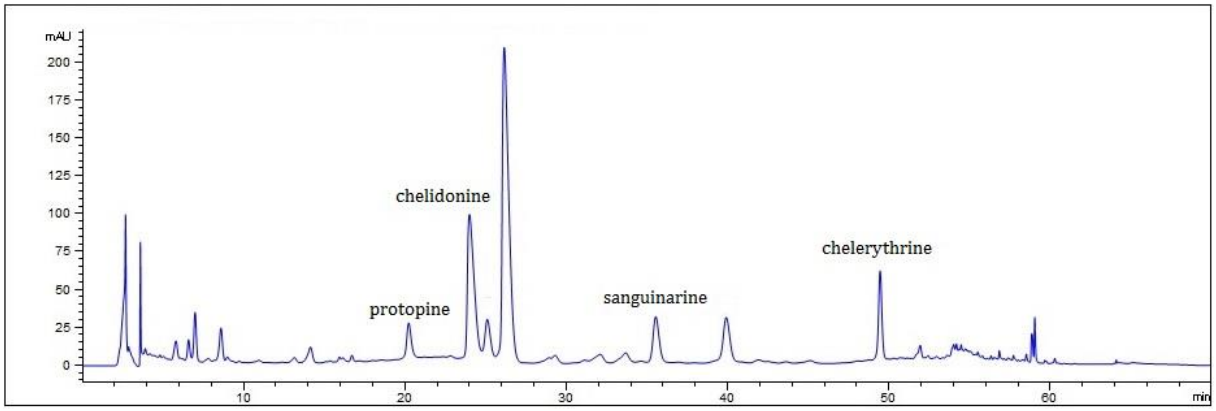
Fig. 4. Dose-response curves of the PANC02 cell lines treated with the defatted *C. majus* extract (CM2B). The PANC02 cells were treated with various doses of CM2B for 24 and 72 h. At least three independent experiments were used to fit the curves and to calculate the IC_{50} values.

Fig. 5. Dose-response curve and calculated IC_{50} for effects on cell viability of BEAS-2B cells treated with defatted *C. majus extract* (CM2B). The BEAS-2B cells were exposed to various concentrations (1 – 1000 µg/ml) of CM2B for 24 h (6 replicate wells per concentration).

Fig. 6. Real-time monitoring of the effect of CM2B on the PC-EM002 cell line. The grey vertical line (at approximately 24 h) indicates the time-point of cell index normalisation and time of treatment. This graph shows the mean cell index measurements per concentration level according to time. The vertical coloured lines show the standard deviation. Effect of the extract on cell proliferation can be observed starting at a concentration of 250 µg/ml.

Fig. 7. Effect of the different treatment on the metastasis expressed as the number of counted metastases ± standard error. The non-parametric Mann-Whitney test was used to determine statistical significance. Values of $p \leq 0.05$ were considered significant.

Fig. 8. Effect of the different treatments on the weight of the primary tumours ± standard error. No significant differences between control and treated groups were observed regarding the weight of the primary tumours when treated with any kind of treatment.


mAU
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150
125
100
75
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25
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chelidonine
protopine
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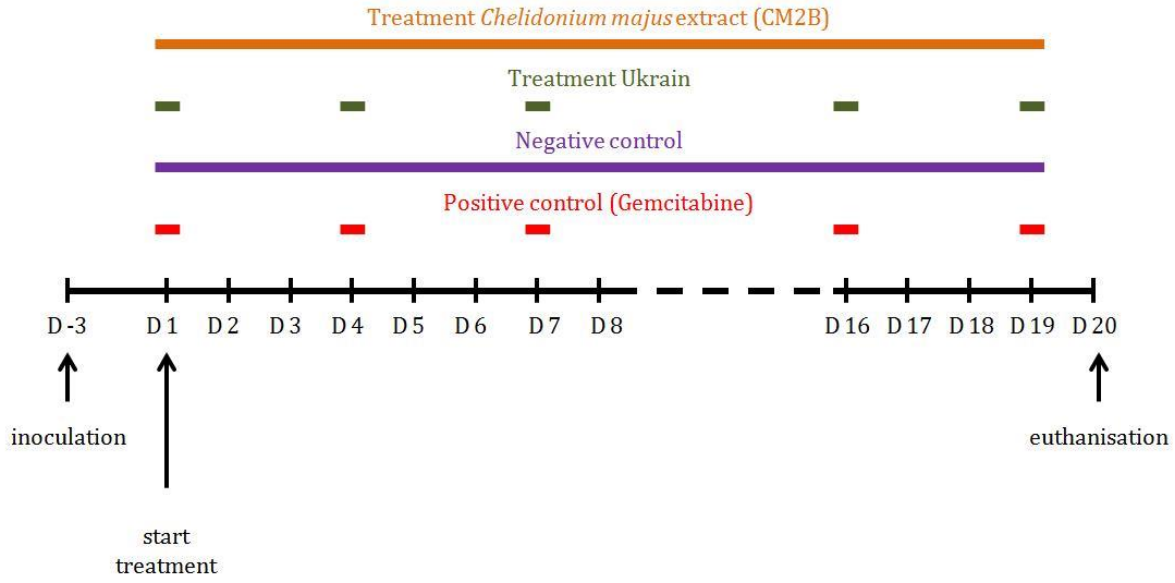
chelerythrine
10
20
30
40
50
60
min

Figure 1



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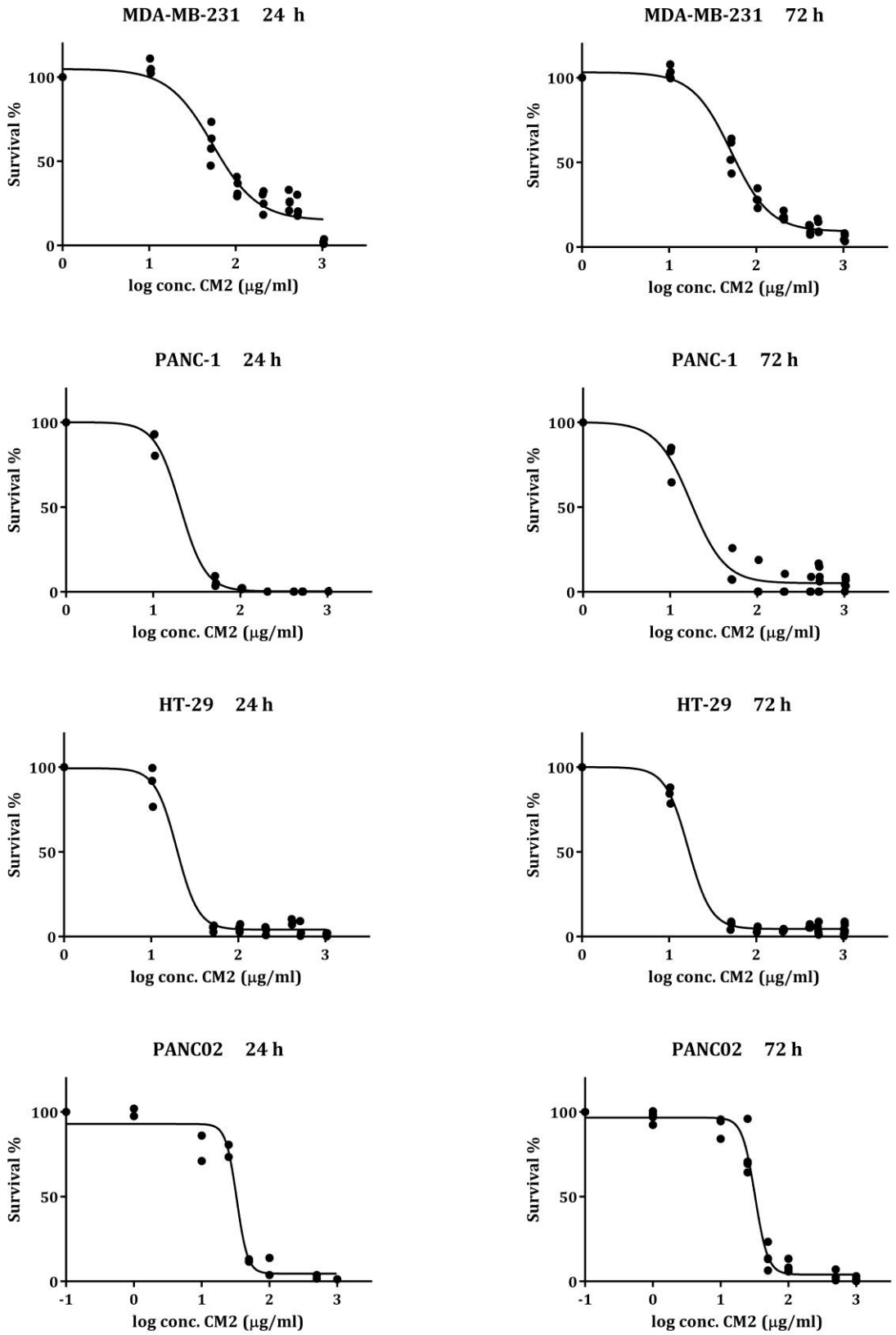

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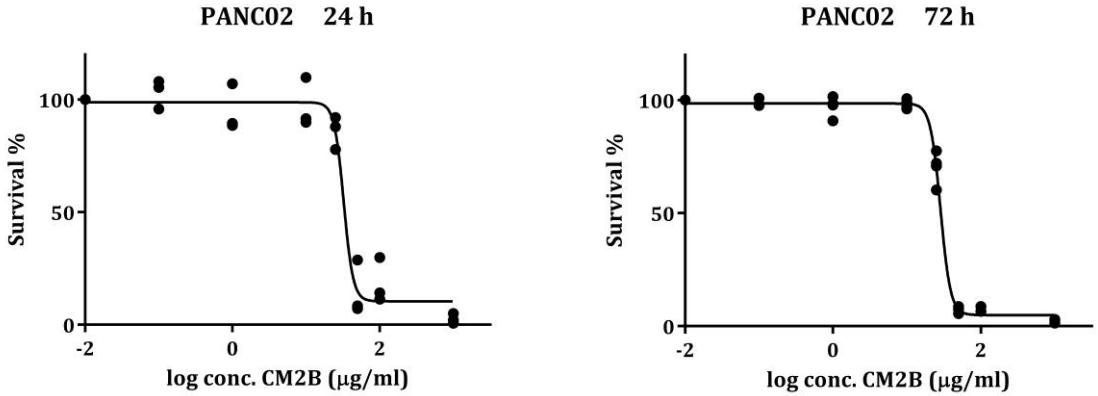
493 Figure 3



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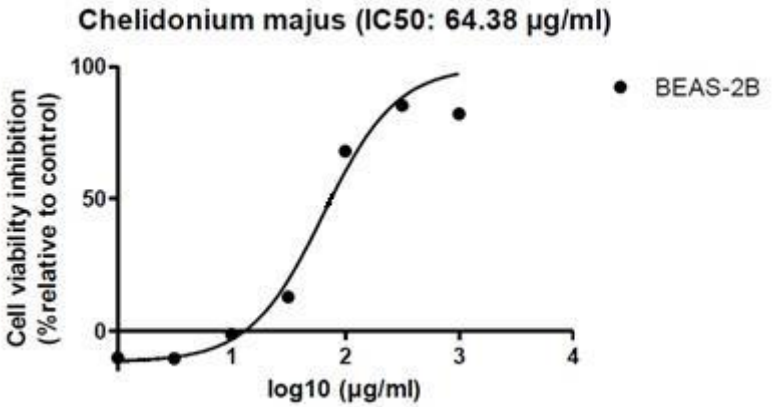

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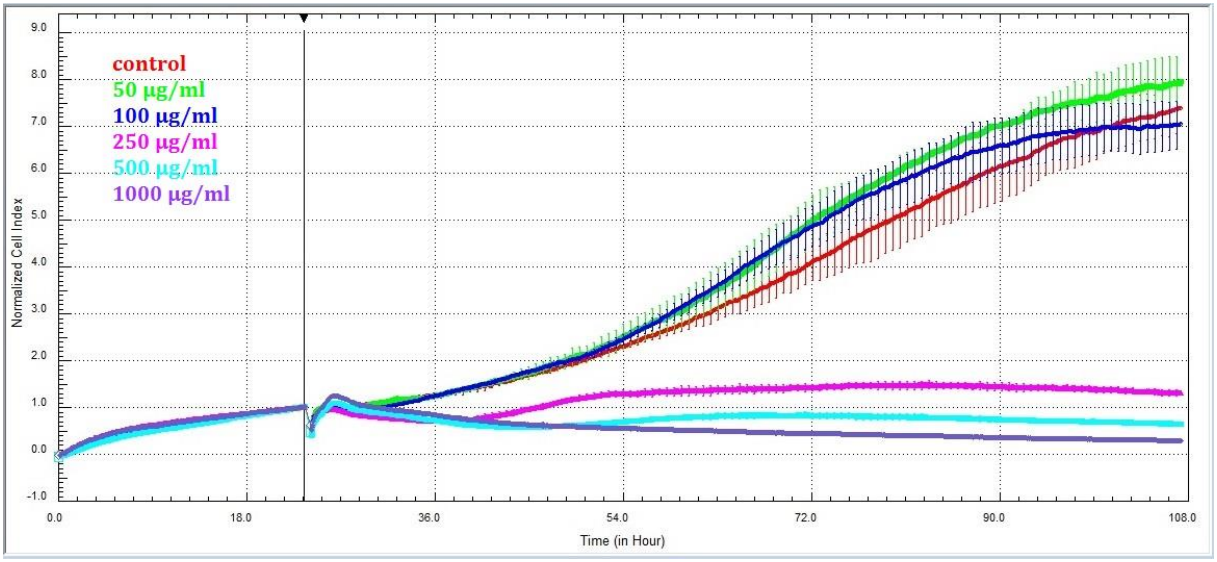

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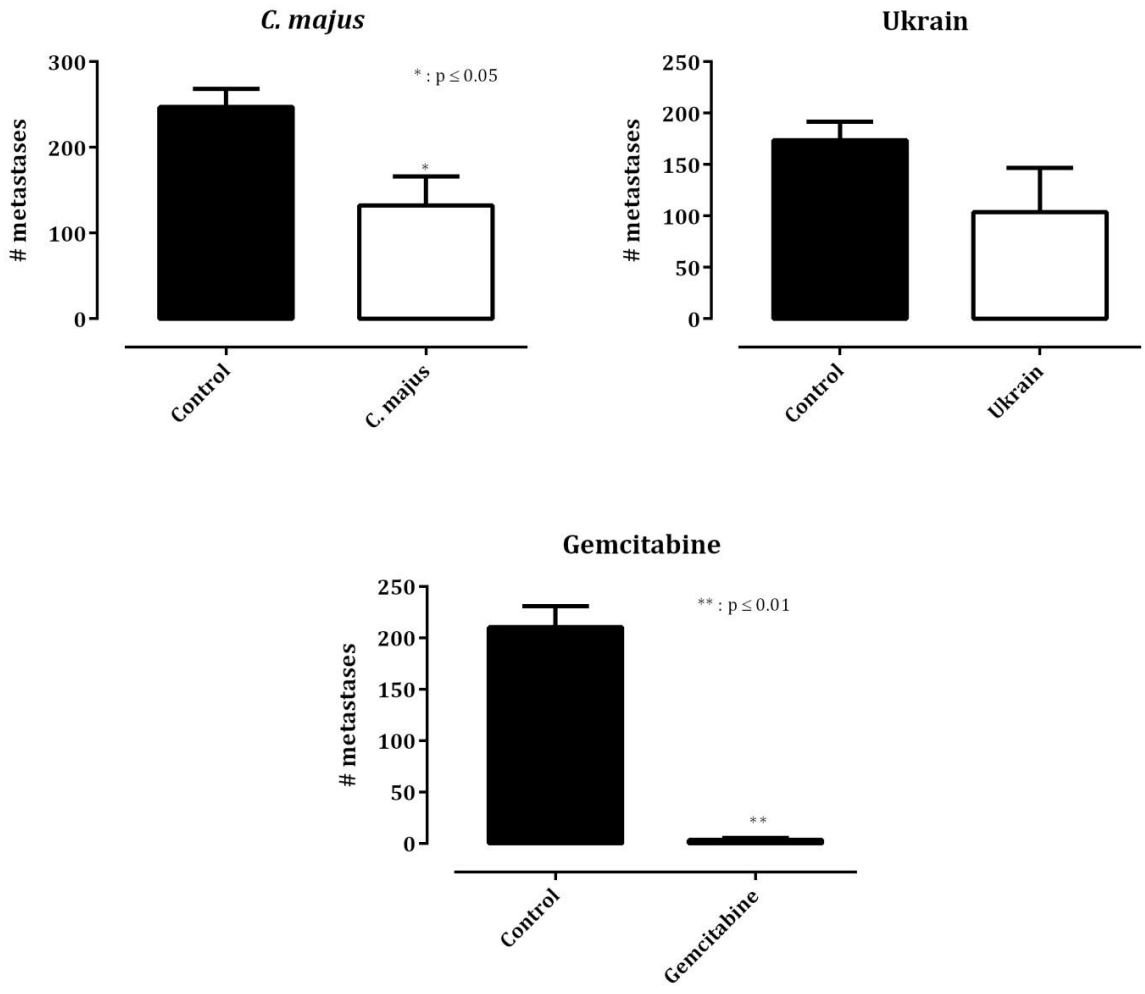
499 Figure 6



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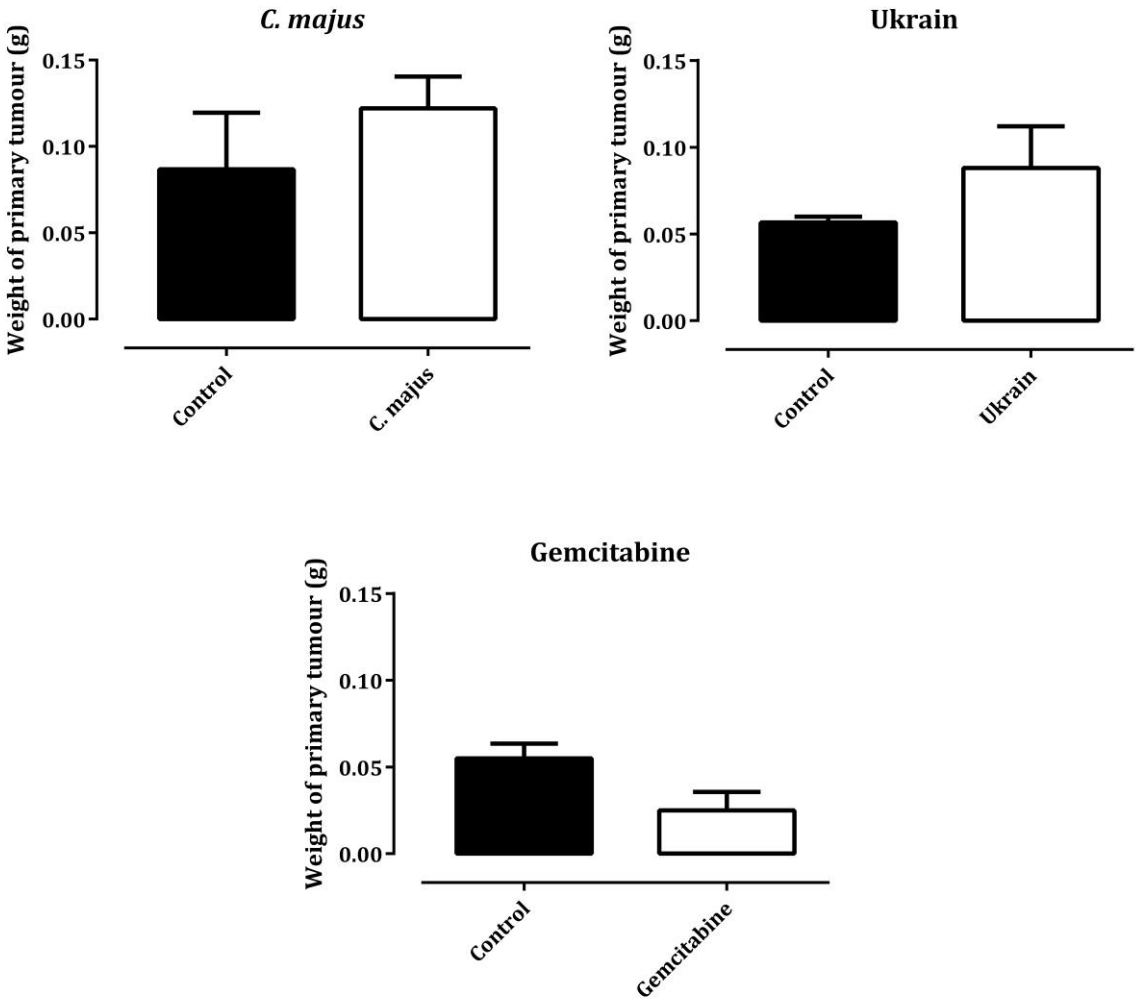
501 Figure 7



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503 Figure 8