A petroether extract of the roots of *Onosma paniculatum* induces cell death in a caspase dependent manner

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**A B S T R A C T**

**Aim of the study:** Traditional Chinese medicine (TCM) has become very popular in Western countries during the last years. Zicao, a remedy of TCM, has been traditionally used to treat cancer, and, its main constituents, naphthoquinones, have been reported to possess antitumor activity (Chen et al., 2002; Papageorgiou et al., 1999). Here, we prepared extracts of different polarities of *Onosma paniculatum* Bur. & Franch., a plant which is amongst others used as Zicao, but, much less investigated. The extracts were analyzed concerning their growth inhibitory and apoptosis-inducing activity in various tumor cells.

**Materials and methods:** Cell viability was measured by XTT viability and a growth inhibition assay. Effects on the cell cycle and caspase-3 were determined by flow cytometry.

**Results:** From three different extracts, a petrol ether extract showed significant growth inhibitory effect, cell cycle influence and caspase-3 dependent induction of apoptosis which was time and dose dependent.

**Conclusion:** To further determine the activity and mechanism of action of the petrol ether extract, we would like to isolate and identify the active principle and investigate the effects in more detail.

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1. Introduction

Traditional Chinese medicine (TCM) comprises knowledge of more than 4000 years and has become very popular in Western countries during the last years. TCM has also proven as a rich source of anticancer agents (Efferth et al., 2007a,b, 2008). For example, artesunate, homoharringtonine, arsenic trioxide and cantharidin, which are found in plants traditionally used, have recently been shown to possess anti-inflammatory (Hayashi, 1977; Kundakovic et al., 2006), antitumorigenic (Chen et al., 2002), wound healing, antimicrobial, antithrombotic (Papageorgiou et al., 1999) and antifungal (Sasaki et al., 2002) activities. However, whereas different extracts of *Arnebia* and *Lithospermum* species are already well characterized, *Onosma paniculatum* is hardly investigated towards its anticancer activity.

In TCM, decoctions are recommended for oral use and extracts in vegetable oil are preferred for topical application (Wu, 2005). Therefore, we prepared a decoction of the root and, in addition, extracts composed of non-polar (petrol ether extract) and polar (methanol extract) ingredients. After initial investigations, our interest was especially focused on the non-polar extract. We studied anti-proliferative properties on different cancer cell lines as well as apoptosis-inducing activities.
2. Materials and methods

2.1. Plant material and identification

Dried roots of *Onosma paniculatum* Bur. & Franch. (Boraginaceae) were acquired at the medicinal plant market in Kunming, China, authenticated at the Kunming Institute of Botany in October 2003 and stored at the Joanneum Research Company in Graz. Moreover, the identity of the plant was confirmed using genomic analysis. Therefore, total genomic DNA was extracted from the dried root using a modified CTAB procedure of Doyle and Doyle (1990) with 3% CTAB, 4% genomic analysis. Therefore, total genomic DNA was extracted from the dried root using a modified CTAB procedure of Doyle and Doyle (1990) with 3% CTAB, 4% NaCl and 5% PVP according to the protocol modified by Khan et al. (2007). Two noncoding regions, ITS (ITS1, 5.8S rDNA, ITS2) from nuclear DNA and trnL-F from chloroplast DNA, were chosen for analysis. The markers were amplified from total DNA via the polymerase chain reaction (PCR) using Taq polymerase (Boehringer) and the primer pairs ITSleu1 (5′-GTCCACTGAACCTTATCATTTAG-3′) and ITS4 (5′-TCCGTAGGTGAACCTGCGG-3′) according to White et al. (1990). For amplification of the trnL-F spacer region the forward primer E (5′-GTCCACTGAACCTTATCATTTAG-3′) and reverse primer F (5′-ATT'TGAACTGGTGACACGAG-3′) according to Taberlet et al. (1991) were used.

All PCR amplifications were carried out in a MWG thermocycler (Primus). For ITS the following program was chosen: (1) 94 °C for 2 min; 30 s, 40 cycles at 94 °C for 30 s, 54 °C for 30 s, 72 °C for 1 min; 30 s and (2) a terminal extension phase at 72 °C for 10 min. For the trnL-F spacer each cycle consisted of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min 30 s, the other steps remained unchanged. The PCR products were purified with QuantumPrep Gel Slice Kit (Bio-Rad, USA). For sequencing an ABI 377 automated sequencer was used. PCR products were purified and sequenced directly using the same primers as for amplification. Sequencing was performed on an ABI 377 automated sequencer as described elsewhere (Brauecher et al., 2004).

2.2. Extraction procedure and sample preparation

For extract preparation, 71 g powdered root was successively extracted with 500 ml petrol ether and 500 ml methanol by Soxhlet extraction. The extracts were evaporated to dryness under reduced pressure. For decoction, 20 g crude crushed plant material was cooked two times with 250 ml water for 30 min each. Afterwards, the decoction was filtered and lyophilized. For cell assays, the decoction was filtered and lyophilized. For cell assays, the decoction was filtered and lyophilized.

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2.3. Cell culture

Leukemia cells CCRF-CEM and THP-1 and breast cancer cells MDA-MB-231 were cultured in RPMI 1640 medium (Sigma, MO, USA), 2 mM glutamine (Sigma, MO, USA), 10% heat-inactivated fetal bovine serum (FBS, PAA laboratories, Austria) and 1% Pen/Strep. (PAA laboratories, Austria). Glioblastoma cells U251, colon cancer cells HCT 116 and cervix carcinoma cells Hela are cultured in Dulbecco's modified Eagle medium (DMEM, Sigma, MO, USA), 2 mM glutamine, 10% FBS and 1% Pen/Strep. Colon adenocarcinoma cells HT29 are cultured in Ham's F12 medium (Invitrogen, UK), 2 mM glutamine, 10% FBS and 2% Pen/Strep. Kidney carcinoma cells 769-P are cultured in RPMI, 10% FBS, 2 mM l-glutamine, 1% Pen/Strep. and 1 mM sodiumpyruvate. Melanoma cells lines from primary (WM35, SBc-L2) and metastatic (WM9, WM164) lesions are grown in RPMI 1640 medium supplemented with 2% FBS, 2 mM l-glutamine and 2% Pen/Strep. All cells are kept in a 5% CO₂ atmosphere at 37 °C. At 90% confluence cells were passaged.

2.4. XTT viability assay

Cell proliferation kit II (XTT) (Cat. No. 11 465 015 001) was obtained from Roche Diagnostics (Mannheim, Germany). Aliquots (100 μl) of 5 × 10⁴ cells/ml of MDA-MB-231, U251 and HCT 116 cells were seeded in 96-well plates (flat bottom) and grown for 18 h in a 37 °C, 5% CO₂ atmosphere before extracts were added (10 μg/ml). Control cells were treated with 0.5% DMSO. In case of CCRF-CEM cells, aliquots (100 μl) of 1 × 10⁶ cells/ml were seeded in 96-well plates (flat bottom) and extracts were added immediately. All cells were incubated with the extracts for 72 h before XTT solution was added. XTT solution consisted of a XTT labeling reagent and an electron-coupling reagent. XTT is a yellow tetrazolium salt (sodium 3-[(1-phenyliminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro)benzene sulfonic acid hydrate) and cleaved by metabolic active cells into an orange formazan dye. This color changes only occur in viable cells and can be directly quantified using a scanning multiwell spectrophotometer (Scudiero et al., 1988). Numbers of viable cells were determined with the following formula and expressed as percentage of control: (absorbance of treated cells/absorbance of untreated cells) × 100 (Konkimalla et al., 2008).

2.5. Growth inhibition (GI) assay

Growth inhibition assay was performed as described elsewhere (Efferth et al., 2002). In brief, aliquots (2 ml) of 5 × 10⁵ cells/ml of MDA-MB-231, U251 and HCT 116 cells were seeded in 6-well plates and grown for 18 h in a 37 °C, 5% CO₂ atmosphere before extracts were added. In case of CCRF-CEM cells, aliquots (1 ml) of 5 × 10⁵ cells/ml were seeded in 24-well plates and extract was added immediately. After 7 days cells were counted using a Casby Cell Counter (Innovatis, Reutlingen, Germany). Results are presented as viable cells in percentage of control (100%).

2.6. HPLC analysis of the PE extract

For HPLC analysis, a Merck-Hitachi HPLC system (Darmstadt, Germany) was used: pump: L7100, autosampler: L-2700, photodiode array detector: L7455. The detector was also used for identification of the compound class due to their UV spectrum. A RP18 column (250 mm × 4 mm, 5 μm) was used and the mobile phase consisted of A: acetonitrile and B: water with the following program: 0–35 min; 65–74% B. Flow rate was 1 ml/min.

2.7. Cell morphology

Tumor cells were plated in 12-well plates at a density of 5 × 10⁵ cells/well and grown for 24 h to attach on the plates completely. Afterwards, different concentrations of the petrol ether extract (0, 2.5, 5, 7.5 and 10 μg/ml) were added and cells were grown at 37 °C in a humidified 5% CO₂ atmosphere for different periods of time (24, 48 and 72 h). For cell morphology experiments, the culture plates were examined and photographed by phase microscopy (Olympus IX51, Japan).

2.8. Sub-G1 peak and cell cycle

After incubation with 10 μg/ml petrol ether extract for 24, 48 and 72 h, cells were harvested by trypsinization (except for the suspension cell line THP-1), 5 × 10⁵ cells were fixed with 70% ice cold ethanol for 10 min at 4 °C. After washing, the cell pellet was suspended in PI-staining buffer (50 μg/ml PI, RNase A, Beckman Coulter, USA) and incubated for 15 min at 37 °C. Cells were analyzed by flow cytometry (FACS Calibur, BD, USA).
2.9. Caspase-3 by flow cytometry

After incubation with 10 μg/ml extract for 24, 48 and 72 h, cells were harvested by trypsinization (except for the suspension cell line THP-1) and 2 × 10⁶ cells/ml were fixed and permeabilized with Cytofix/Cytoperm™ solution (BD Biosciences, USA). The pellet was resuspended in Perm/Wash™ buffer and stained with FITC-conjugated monoclonal active caspase-3 antibody (BD Biosciences, USA). Finally, cells were resuspended in 1 ml PBS and analyzed with a FACS Calibur® instrument equipped with a 488 nm argon ion laser and a 635 nm red diode laser (Becton Dickinson, Franklin Lakes, NJ, USA). Untreated cells were used as negative control.

3. Results

3.1. Identification of plant material

The plant material was authenticated at the Kunming Institute of Botany as well as identified using genomic analysis. The ITS2 region of nuclear DNA and trnL-F region of plastid DNA can be potentially used as a standard DNA barcode to identify medicinal plants and their closely related species (Chen et al., 2010). To estimate the reliability of species identification using a DNA barcoding technique, the Basic Local Alignment Search Tool (BLAST) can be used that finds regions of local similarity between sequences. The program compares nucleotide sequences to sequence databases and calculates the statistical significance of matches. The obtained sequences were queried with the NCBI nucleotide database by a BLASTN 2.2.22 search (Zhang et al., 2000), which indicated a significant similarity to *Onosma paniculatum* for both ITS (98% max identity to acc. no. EF199859) and trnL-F spacer (98% max identity to acc. no. EF199851; for the trnL-F 5'-intron 99% max identity to acc. no. EF199875). For *Arnebia euchroma*, a plant also used as Zicao, the similarity values revealed were 84% for ITS and 96% for the trnL-F spacer, respectively.

3.2. XTT assay

In a pharmacological screening, Soxhlet extracts were tested for their growth inhibitory activity on human CCRF-CEM leukemia, human MDA-MB-231 breast, U251 glioblastoma and HCT 116 colon cancer cells (Fig. 1) at a concentration of 10 μg/ml. We investigated the activity after 72 h as well as after 7 days. Cells treated with the petrol ether (PE) extract were strongly inhibited in their growth...
and viability after 72 h as well as after 7 days. After 7 days, hardly any living cells were detected. On the contrary, the methanolic extract of *Onosma paniculatum* limited the growth of the cancer cells neither after 72 h nor after a week of treatment. Furthermore, we also investigated the effect of a traditionally prepared decoction on CCRF-CEM cells since they were the most sensitive cells. However, we could not detect any activity. Therefore, we chose the PE extract for further investigations, analyzed the activity on other cancer cell lines and examined the mechanisms behind this effect.

### 3.3. HPLC fingerprint of the PE extract

Since the PE extract was active, we proceeded in HPLC investigations of this extract. It appeared that this extract was composed of three main compounds (retention times: 10.8 min, 22.7 min and 24.3 min, respectively) and some other compounds of less content (retention times: 8.5 min, 9.4 min and 19.1 min). According to their UV spectrum and literature data (Hu et al., 2006; Cui et al., 2007), we assume that these compounds belong to naphthoquinone derivatives and that the three main compounds are presumably acetylshikonin, dimethylacrylshikonin and epoxyshikonin (Fig. 2).

### 3.4. Morphological changes

To study morphological changes of different cancer cells during PE extract exposure, Hela, HT-29, THP-1, 769-P, SBc-L2, WM9, WM164 and WM35 cells were treated for 24, 48 and 72 h with different concentrations of PE extract, afterwards, they were gently rinsed with PBS and analyzed under a phase-contrast microscope for size, shape, and integrity of cell membrane, cytoplasm, and nuclei. First morphological changes occurred after 24 h and continued up to 72 h. The effect was time and dose dependent. In case of melanoma cells, non-metastatic cells (SBc-L2) were influenced already after 24 h exposure, whereas in metastatic melanoma cells (WM164) a delay was observed not until 48 h. Although after 72 h, WM9, WM164 and WM35 cells looked quite well in cell morphology, they were much less dense than control cells. The other investigated cells showed both, less density and a significant changed morphology after 72 h (Fig. 3).

### 3.5. Cell cycle

Changes in cell cycle after treatment with the PE extract were studied in Hela, 769-P, HT-29, THP-1 and different melanoma cell lines from different tumor stages (WM35, WM9 and WM164). The percentage of the G2/M-phase cells of 769-P, HT-29, Hela and WM9 increased after 72 h, accompanied by a decrease in the number of G1-phase cells. The G2/M-phase cells of WM35 and WM164 increased slightly. In THP-1 cells, G2/M arrested cells where hardly influence by the treatment (Fig. 4). The representative data from two independent experiments are shown.

### 3.6. Cleaved caspase-3

Active caspase-3, a marker for cells undergoing apoptosis, consists of a heterodimer of 17 and 12 kDa subunits which are derived from the 32 kDa proenzyme. Activation of caspase-3 requires proteolytic processing of its inactive yzomogen into activated p17 and...
p12 fragments. Different tumor cell lines showed high level of cleaved caspase-3 compared to the untreated control cells when they were treated with 10 μg/ml PE extract. The percentage of cleaved caspase-3 was depending on the tumor stages (Fig. 5).

4. Discussion

In traditional Chinese medicine, the remedy Zicao is orally used as decoction as well as vegetable oil extraction for external use (Wu, 2005). Creams and ointments are clinically used for the treatment of carbuncles, furuncles, abscesses, lesions, eczema and burns. In the Chinese Pharmacopoeia, Zicao is officially derived from the roots of *Arnebia euchroma* or *Arnebia guttata* (State Pharmacopoeia Commission of the PRC, 2005). However, especially in the southwest areas of China, also different *Onosma* species are used as Zicao by the native peoples (Hu et al., 2006). For this study, roots of *Onosma paniculatum* were acquired at the medicinal plant market in Kunming and not only authenticated by macroscopic characteristics but also by genomic analysis because of the macroscopic similarities between the different Boraginaceous species. Comparison of the ITS2 region of nuclear DNA and trnL-F region of plastid DNA of the plant material and the NCBI nucleotide database revealed the accordance with *Onosma paniculatum*.

In a pharmacological screening, a decoction, a polar and a non-polar extract of *Onosma paniculatum* were subjected to viability assays. Only the non-polar PE extract showed strong inhibitory activity towards different cancer cell lines. This also implies that the vegetable oil extractions used for topical application contain compounds able to fight cancer. However, the traditional decoction could not inhibit the growth of cancer cells, which indicates that this form of application is probably not able to fight cancer. Naphthoquinones have been identified as bioactive compounds in Zicao plants (Papageorgiou et al., 1999). Shikonin has been reported to possess an IC50 of 1.1 ± 0.1 μg/ml on MCF-7 breast cancer cells after 72 h, determined by MTT viability assay (Hou et al., 2006), an activity comparable to the PE extract.

An HPLC fingerprint of this extract indicated the presence of three main compounds. According to their UV spectrum and already described constituents of this and related species (Hu et al., 2006; Cui et al., 2007), we assume three different naphthoquinone derivatives (probably acetylshikonin, dimethylacrylshikonin and epoxyshikonin) which has to be isolated and indentified by NMR experiments for future experiments.

In this study, the PE extract was also investigated regarding its effects on cell morphology, cell cycle and caspase-3 activation in various cancer cell lines. In CCRF-CEM, MDA-MB-231, U251, HCT 116, HT29, Hela, 769-P, SBc-L2, WM35, WM9 and WM164 cells, cell viability, growth and morphology were influenced at the latest after 72 h. The used melanoma cell lines (SBc-L2, WM35, WM9 and WM164) were primarily isolated from different stages of melanoma progression. We observed that non-metastatic SBc-L2 cells respond faster to the exposure than metastatic WM164
Fig. 5. The y-axis denotes cell counts and the x-axis represents fluorescence intensity of FITC antibody. The histograms were done by using FCS3-express. Green line represents untreated tumor cells, red line represents tumor cells treated with 10 μg/ml PE extract for 72 h. Compared to the untreated control cells, high level of cleaved caspase-3 was detected in treated cells.

melanoma cells indicating that the intensity of the observed effects is tumor-stage depending. We could show that not only the viability and morphology was stage-dependently influenced, but also the cell cycle, which was measured by flow cytometry analyses. In 769-P, HT-29 and Hela cells the cell cycle was influence as well. An extract from Arnebia euchroma, called naphthoquinone pigment-L III, was also reported to inhibit the proliferation of stomach cancer cell line and esophagus cancer cell line. At the effective concentration of 5 μg/ml this extract inhibited the mitotic index and growth curve of normal human cells without showing any DNA damage indicating acceptable toxic side effects of the active constituents (Lu and Liao, 1990).

In some cell lines, caspase-3 plays a direct role in proteolytic cleavage of cellular proteins responsible for progression to apoptosis. In an attempt to identify the pathway of apoptosis induction in melanoma cells in response to Onosma paniculatum, caspase-3 activation was investigated and we detected a caspase-3 depending apoptosis. It is reported that shikonin induces apoptosis via activation of caspase-3 in leukemia, bladder and cervical cancer cells (Wu, 2005; Yeh et al., 2007; Yoon et al., 1999) and inhibited the
activity of topoisomerase II and NF-κB, which are potential targets for chemotherapy (Fuji et al., 1992; Min et al., 2008). In addition, some anticancer drugs are able to intercalate with DNA in cancer cells and arrest them in the cell cycle (Bachur et al., 1978). In case of shikonin, data from microarray experiments suggests that it affects DNA (Efferth et al., 2007a,b). Our data indicated that Onosma paniculatum interferes with the cell cycle at the G2/M phase and induces a cleavage of pro-caspase-3 in Hela, 769-P, THP-1, SBC-12, WM35, WM9 and WM 164 in a dose and time dependant manner and, therefore, leads to death of cancer cells.

Based on those data, we would like to isolate and identify the active principles of the PE extract and investigate the main compounds regarding their cellular mechanisms. We assume that our PE extract consists of three main and probably some other naphthoquinone derivatives. From other Zicao plants, many different naphthoquinone pigments were identified which are responsible for anticancer activity (Chen et al., 2002; Papageorghiou et al., 1999). We will analyze the naphthoquinones in our extract concerning growth inhibitory activity and targets in the different cancer cells.

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References


