Quinovic acid glycosides purified fraction from *Uncaria tomentosa* induces cell death by apoptosis in the T24 human bladder cancer cell line

Fabrícia Dietrich, Samuel Kaiser, Liliana Rockenbach, Fabrício Figueiró, Leticia Scussel Bergamin, Fernanda Monte da Cunha, Fernanda Bueno Morrone, George González Ortega, Ana Maria Oliveira Battastini

**Abstract**

Bladder cancer is the second most prevalent malignancy in the genitourinary tract and remains a therapeutic challenge. In the search for new treatments, researchers have attempted to find compounds with low toxicity. With this goal in mind, *Uncaria tomentosa* is noteworthy because the bark and root of this species are widely used in traditional medicine and in adjuvant therapy for the treatment of numerous diseases. The objective of this study was to investigate the antitumor effect of one purified bioactive fraction of *U. tomentosa* bark on cell proliferation in two human bladder cancer cell lines, T24 and RT4. Quinovic acid glycosides purified fraction (QAPF) of *U. tomentosa* decreased the growth and viability of both T24 and RT4 cell lines. In T24 cells, QAPF induced apoptosis by activating caspase-3 and NF-κB. Further study showed that this fraction does not induce cell cycle arrest and does not alter PTEN and ERK levels. In conclusion, we demonstrated that QAPF of *U. tomentosa* has a potent inhibitory effect on the growth of human bladder cancer cell lines by inducing apoptosis through modulation of NF-κB, and we suggest that QAPF may become a potential therapeutic agent for the prevention and/or treatment of this cancer.

**1. Introduction**

Bladder cancer is the second most common malignancy that affects the genitourinary tract and the seventh most prevalent cancer among men in the world (Ma et al., 2006; Swellam et al., 2003). Some risk factors associated with this cancer include tobacco use, occupational exposure to carcinogens and chronic bladder inflammation, with tobacco being the biggest risk factor for developing this cancer. However, consumption of fruits and vegetables is thought to have a protective effect on bladder carcinogenesis (Jacobs et al., 2010). Among the genetic alterations associated with bladder cancer, it has been shown that mutations resulting in a reduction or deletion in PTEN expression are common (DeGraff et al., 2012; Platt et al., 2009). Furthermore, the dysregulation of the ERK cascade results in several diseases and is thought to be responsible for more than half of all cancers (Wortzel and Seger, 2011). Moreover, overactive signaling of NF-κB has been shown to be involved in development of bladder cancer (Rayet and Gélinas, 1999). Thus, alterations in the protein levels of PTEN, ERK and NF-κB that lead to disruptions...
in the cell signaling pathways associated with bladder cancer are possible targets for the treatment of this disease. Moreover, due to both the ineffectiveness of the various currently available therapeutic strategies used in bladder cancer and the myriad side effects of classical chemotherapeutics drugs, many studies have aimed at discovering more effective drugs demonstrating reduced toxicity (Ma et al., 2006).

**Uncaria tomentosa** (Willd.) DC., usually known as “uña de gato” or “cat’s claw,” belongs to the Rubiaceae family and has been used for centuries by the Asháninka indigenous people (Keplinger et al., 1999). Dietary supplements derived from cat’s claw bark have been used extensively worldwide as a complementary therapy for the treatment of inflammatory diseases and cancer (Craig, 1999; Fennell, 2004; Rosenbaum et al., 2010). Phytochemical studies have shown that *U. tomentosa* bark presents three main fractions of secondary metabolites, polyphenols, alkaloids and triterpene derivatives, which have all been suggested to have antitumor effects (De Martino et al., 2006; Keplinger et al., 1999). Some studies have suggested that *U. tomentosa* can inhibit the proliferation of several cancer cell lines, such as cervical carcinoma, breast cancer, osteosarcoma (De Martino et al., 2006) and leukemia (Cheng et al., 2007), and cell death appears to be mediated through caspase-dependent apoptosis (Cheng et al., 2007; De Martino et al., 2006; Rinner et al., 2009).

Because *U. tomentosa* is used in traditional medicine as an anticancer agent (Heitzman et al., 2005; Keplinger et al., 1999), the objective of this study was to evaluate the effect of a crude extract and one purified fraction of *U. tomentosa* on the growth of bladder cancer cell lines.

### 2. Materials and methods

#### 2.1. Obtaining the crude extract and purified fraction

*Stem bark of Uncaria tomentosa*, supplied by Laboratorios Induquimica S.A., (Lima, Peru) and collected in Ucayali (Peru), were botanically certified by Peruvian biologist José Ricardo Campos de La Cruz (Universidad Nacional Mayor de San Marcos, Lima, Peru). The crude extract was prepared by 4 days-maceration with ethanol:water solution (40%, v:v) in a plant:solvent ratio of 1:10 (w:v). To prepare the triterpene fraction, the crude extract was pre-purified with cross-linked polyvinylpyrrolidone (PVPP, BASF, Germany) using a dried residue extract:PVPP ratio of 1:10 (w:w). The pre-purified extract was passed through an ion-exchange column filled with a strong anionic resin (Dowex Marathon, Sigma Aldrich, USA), as follows: 300 mL of pre-purified extract was poured onto the column, followed by 100 mL of ethanol:water solution (40%, v:v) at a flow rate of 5.0 mL/min. The eluate obtained (Anionic resin eluate – ARE) was concentrated and freeze-dried and then underwent solid-phase fractionating in a column filled with a poly styrene resin (Diaion HP-20, Supelco, USA), as follows: 300 mg sample of ARE was dissolved in 200 mL of water, followed by the addition of methanol:water solutions in decreasing polarity at flow rate of 2.5 mL/min. The methanol:water fraction (90%, v:v) and methanol fraction were pooled (quinovic acid glycosides purified fraction, QAPF).

#### 2.2. Characterization of freeze-dried samples

**2.2.1. HPLC-PDA analysis**

Previously validated and reverse HPLC-PDA methods were used to determine the oxindole alkaloid content and quinovic acid glycosides content (Kaiser et al., 2013a; Pavet et al., 2012), using mitraphyline (Phytolab, Germany) and α-hederin (ExtraSynthèse, France) as external standards, respectively. Total oxindole alkaloid content was calculated from the sum of the individual alkaloid concentrations of speciophylline, uncarnine F, mitraphyline, isomitrphylmine, pteropodine and isopteropodine (Kaiser et al., 2013a). Total quinovic acid glycosides content was calculated from the sum of the individual concentrations of seven major peaks (Peak 1–7). The results were expressed as gE (w/w, freeze-dried sample) and represent the mean of three determinations.

**2.2.2. UPLC/Q-TOF-MS analysis**

The analysis of the QAPF was performed in a Waters Acquity UPLC system (Waters, USA) coupled with a Waters Q-TOF Premier mass spectrometer (Waters, USA) in accordance with conditions employed by Pavet et al. (2012).

#### 2.3. Cell culture

The human bladder cancer cell lines T24 and RT4 were obtained from the ATCC and maintained in RPMI and DMEM culture medium, respectively. Both mediums contained 0.5 U/mL penicillin/streptomycin and were supplemented with 10% FBS. Cells were cultured in 5% CO2, 95% air atmosphere at 37°C.

#### 2.4. Cell treatment

*U. tomentosa* crude extract and purified fraction were dissolved in DMSO at a maximum final concentration of 0.25% in the culture medium. After reaching semi-confluence, T24 and RT4 cell lines were exposed to the following formulations: *U. tomentosa* crude extract (5, 10, 25, 50, 100 and 150 μg/mL) for 24 or 48 h and QAPF (5, 10, 25, 50, 100 and 150 μg/mL) for 24, 48 or 72 h. Control cultures were prepared with the addition of culture medium or DMSO (vehicle control).

#### 2.5. Cell counting

Cells were treated with *U. tomentosa* crude extract or QAPF as described in Section 2.4. At the end of treatment, cells were washed, and 200 μL of 0.05% trypsin/EDTA solution was added to detach the cells, which were counted in a hemocytometer. The results were expressed as the percentage of cells in relation to DMSO control.

#### 2.6. Cell viability assay

After the treatment, the cells were washed and 90 μL of culture medium, and 10 μL of MTT (5 mg/mL) was added to each well. The cells were incubated for 3 h. A total of 100 μL of DMSO was added to the wells, and the optical absorbance was measured at 492 nm using a plate reader (Spectramax M5, Molecular Devices, USA). The results were expressed as the percentage of cell viability in relation to DMSO control.

#### 2.7. Cell cycle analysis

After reaching semi-confluence, T24 cells were treated with 50, 100 or 150 μg/mL of QAPF for 48 h. At the end of the treatment, the medium and the cells were harvested and centrifuged at 400g for 6 min. Cells were resuspended in PBS, and 1 × 106 cells were fixed with 70% ethanol and stored at −20°C. On the day of analysis, PBS containing 50 μg/mL of propidium iodide and 100 μg/mL of RNase was added to the sample for 30 min in the dark. Finally, the cells were analyzed by flow cytometry using a FACS Calibur cytometry system (FACS Calibur, BD Bioscience, USA). The data obtained were analyzed with FLOWJO® (flow cytometry analysis software).

#### 2.8. Annexin V/PI flow cytometric staining technique

Apoptotic or necrotic cells were quantified using an annexin V-FITC-propidium iodide (PI) double staining kit. At the end of the treatment, the medium and the cells were harvested and centrifuged at 400g for 6 min. A total of 1 × 106 cells were fixed and resuspended in a binding buffer containing FITC-conjugated annexin V and PI. The samples were incubated for 15 min in the dark prior to analysis by flow cytometry. The quantification of apoptotic or necrotic cells was assessed by dual-color flow cytometry using a FACS Calibur cytometry system (FACS Calibur, BD Bioscience, USA), and the data were analyzed with FLOWJO® (flow cytometry analysis software). Cells were classified as follows: live (Annexin −/PI −), early apoptotic (Annexin+/PI −), late apoptotic (Annexin+/PI +) or necrotic (Annexin+/PI +).

#### 2.9. Caspase-3 activity

Caspase-3 activity was determined by fluorometric measurement of the kinetics of 7-amido-4-trifluoromethylcoumarin (AFC) release from the fluorogenic substrate Ac-DEVD-AFC from cell lysates. The cells were lysed, and the extracts were clarified by centrifugation at 10,000g for 5 min at 4°C. Supernatants were collected, and protein concentrations were determined (Peterson, 1975). For assays, 30 μg of protein diluted in lysis buffer was mixed with 50 μL of the assay buffer and 10 μL of the substrate solution (0.2 mg/mL). Caspase-3-mediated substrate cleavage was monitored for 40 min (37°C) in a fluorometric reader (excitation 390 nm/emission 520 nm).

#### 2.10. Western blot assay

After 48 h treatment, T24 cells were lysed and β-mercaptoethanol was added. To evaluate NF-κB protein levels, nuclear and cytosolic fractions were prepared. Cells were homogenized in lysis buffer (10 mM Heps, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.5% NP40), incubated for 15 min on ice and then centrifuged for 5 min at 13,000g at 4°C. The supernatant was collected and used as the soluble cytosolic fraction. The pellet was suspended in nuclear fraction buffer (20 mM
Table 1
Characterization of the crude extract and the quinovic acid glycosides purified fraction (QAPF) obtained from cat’s claw bark.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Content g% ± SD</th>
<th>Key fragment ions (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crude extract</td>
<td>QAPF</td>
</tr>
<tr>
<td>Peak 1</td>
<td>2.12 ± 0.01</td>
<td>7.73 ± 0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>655.4208 [M+Na]⁺ (19.2%) → 633.4451 [M+H]⁺ (15.4%) → 487.3660 [M–DES]⁺ (75.0%) → 469.3746 [M–DES–H₂O]⁺ (100%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>979.5467 [M+Na]⁺ (100%) → 957.5175 [M+H]⁺ (13.5%) → 795.4985 [M–HEX]⁺ (19.2%) → 633.4451 [M–HEX–HEX]⁺ (32.7%) → 487.3767 [M–HEX–HEX–DES]⁺ (38.5%) → 469.3567 [M–HEX–HEX–DES–H₂O]⁺ (63.5%)</td>
</tr>
<tr>
<td>Peak 2</td>
<td>1.24 ± 0.03</td>
<td>3.75 ± 0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>617.3830 [M+K]⁺ (74.3%) → 595.3238 [M+H]⁺ (4.6%) → 487.3564 [M–DES]⁺ (61.1%) → 487.3746 [M–DES–DES]⁺ (25.9%) → 469.3567 [M–DES–DES–H₂O]⁺ (100%)</td>
</tr>
<tr>
<td>Peak 3</td>
<td>0.79 ± 0.01</td>
<td>1.76 ± 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>817.4702 [M+K]⁺ (62.0%) → 795.5203 [M+H]⁺ (4.6%) → 633.4572 [M–DES]⁺ (100%)</td>
</tr>
<tr>
<td>Peak 4</td>
<td>0.49 ± 0.01</td>
<td>1.76 ± 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>671.3830 [M+K]⁺ (74.3%) → 649.3451 [M+H]⁺ (2.7%) → 487.3554 [M–DES]⁺ (100%) → 469.3432 [M–DES–H₂O]⁺ (73.0%)</td>
</tr>
<tr>
<td>Peak 5</td>
<td>0.32 ± 0.01</td>
<td>1.10 ± 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>817.4840 [M+Na]⁺ (83.5%) → 795.4712 [M+H]⁺ (7.6%) → 649.4351 [M–DES]⁺ (5.3%) → 487.3554 [M–DES–HEX]⁺ (59.1%) → 469.3567 [M–DES–HEX–DES]⁺ (100%)</td>
</tr>
<tr>
<td>Peak 6</td>
<td>0.84 ± 0.03</td>
<td>2.42 ± 0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>655.3961 [M+Na]⁺ (10.1%) → 633.4694 [M+H]⁺ (15.2%) → 487.3767 [M–DES]⁺ (34.2%) → 469.3432 [M–DES–H₂O]⁺ (27.7%)</td>
</tr>
<tr>
<td>Peak 7</td>
<td>0.98 ± 0.04</td>
<td>3.28 ± 0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>655.4703 [M+Na]⁺ (3.1%) → 633.4328 [M+H]⁺ (3.0%) → 487.3554 [M–DES]⁺ (100%) → 469.3432 [M–DES–H₂O]⁺ (27.7%)</td>
</tr>
<tr>
<td>Total quinovic acid glycosides</td>
<td>6.78 ± 0.14</td>
<td>21.78 ± 0.05</td>
</tr>
<tr>
<td>Total oxindole alkaloids</td>
<td>1.53 ± 0.19</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND = not detected; HEX = Hexose; DES = 6-desoxy-hexose.

a obtained from HPLC-PDA analysis and expressed as mean ± standard deviation (n = 3).
b fragmentation patterns obtained by UPLC/Q-TOF-MS analysis.
c calculated from the sum of the individual concentrations expressed as α-hederin.
d calculated from the sum of the individual concentrations expressed as mitraphylline.

Fig. 1. UPLC/Q-TOF-MS chromatograms from the QAPF analysis. (A) total ion current profile (TIC) (m/z range 200–1000); and (B) reconstructed ion chromatogram at m/z 487 (quinovic acid aglycone).

Fig. 2. Crude extract of Uncaria tomentosa decreases the cell growth of bladder cancer cells. (A) T24 cells treated with 150 μg/mL of the crude extract for 48 h were observed under phase-contrast microscopy (100 × ). (B) Cells were treated with crude extract or DMSO for 24 or 48 h. After treatment, cells were detached with 0.05% trypsin–EDTA and counted in a hemocytometer. * *p < 0.001.
Hepes, 1.5 mM MgCl₂, 300 mM NaCl, 0.25 mM EDTA, 25% glycerol, 0.5 mM DTT), incubated for 20 min on ice and centrifuged for 20 min at 13,000 g at 4°C. The supernatant was collected and used as the nuclear-protein enriched fraction. Protein concentrations were determined as described (Peterson, 1979). Appropriate amounts of protein (55–75 μg per lane) were resolved on 10% SDS–PAGE gels. After electrophoresis, proteins were transferred to nitrocellulose membranes. Membranes were incubated for 60 min at 4°C in blocking solution and then were incubated overnight at 4°C with the appropriate primary antibody. Primary antibodies against the proteins pERK42/44 (1:1000), ERK42/44 (1:1000), PTEN (1:1000), pNF-κB p65 (1:1000), anti-β-actin (1:1000) and anti-Lamin B1 (1:10,000) were used. Next, membranes were incubated with the corresponding horseradish peroxidase-conjugated secondary antibody at 1:1000 or 1:5000 dilutions. Chemiluminescence was detected using X-ray films. The films were scanned and analyzed using the Optiquant software (Packard Instrument).

2.11. Statistical analysis

All experiments were performed at least three times. The results were expressed as the mean ± SD and analyzed by one-way ANOVA, followed by Tukey’s post-hoc test. The comparisons were considered significant in relation to the control when p < 0.05.

3. Results

3.1. Characterization of freeze-dried samples

The total content of quinovic acid glycosides found in the QAPF was four times higher than that present in the crude extract (Table 1). The seven major peaks found in the QAPF could be characterized as quinovic acid derivatives by monitoring the m/z at the 487 ion (quinovic acid aglycone) using UPLC/Q-TOF-MS analysis (Fig. 1). Peaks were characterized as follows: Peak 1 (m/z 655.4208 [M+Na]⁺), Peak 4 (m/z 671.3830 [M+K]⁺), Peak 6 (m/z 655.3961 [M+Na]⁺) and Peak 7 (m/z 655.4703 [M+Na]⁺) as monoglycosylated derivatives; Peak 3 (m/z 817.4702 [M+K]⁺) and Peak 5 (m/z 817.4840 [M+Na]⁺) as diglycosylated derivatives; and Peak 2 (m/z 979.5467 [M+Na]⁺) as a triglycosylated derivative (Table 1). Previously, fourteen quinovic acid glycosides were isolated from cat’s claw bark; three of them were triglycosylated derivatives, nine were diglycosylated derivatives and two were monoglycosylated derivatives (Aquino et al., 1989, 1991, 1997; Cerri et al., 1988; Pavei et al., 2012). Peaks 1, 4, 6 and 7 showed fragmentation patterns comparable to that of compound 4 described previously by Aquino et al. (1997), and the glycosidic chain was composed of one 6-desoxy-hexose (α-L-rhamnopyranosyl at C-3). The fragmentation pattern of Peak 5 was compatible with the nine diglycosylated derivatives (the glycosidic chain composed of hexose and 6-desoxy-hexose) previously isolated from cat’s claw bark, while Peak 3 showed two 6-desoxy-hexoses...
at the glycosidic chain. Conversely, Peak 2 showed a glycosidic chain composed of one 6-desoxy-hexose and two hexoses similar to compound 8 (α-L-rhamnopyranosyl-(3 → 1)-β-D-glucopyranosyl and β-D-glucopyranosyl) linked to C-3 and C-28, respectively) described earlier by Aquino et al. (1997) (Table 1 and Fig. 1). These results are in accordance with those previously described by Pavei et al. (2012).

3.2. The crude extract of U. tomentosa reduces cell number of T24 cells

Because numerous studies have shown that U. tomentosa inhibits the proliferation of certain cancer cells (De Martino et al., 2006; Pilarski et al., 2007), we first investigated the antiproliferative effects of crude extract of U. tomentosa on T24 cells. Cell counting showed a significant decrease in the cell number (47.98% ± 13.83) at the highest concentration tested (150 μg/mL) after 48 h of treatment (Fig. 2A and B).

3.3. The QAPF of U. tomentosa reduces cell number and decreases the cell viability of T24 and RT4

Because the highest concentration of the crude extract resulted in a significant decrease in cell number when compared to the DMSO control, we decided to investigate whether the QAPF of U. tomentosa would be able to inhibit the growth of the T24 cell line more effectively. As shown in Fig. 3A, QAPF significantly inhibited T24 cell growth. We found that 100 and 150 μg/mL concentrations of QAPF significantly reduced the cell number after treatment for 24, 48 and 72 h. Moreover, the T24 cell number was diminished after 72 h of treatment with 50 μg/mL of QAPF (Fig. 3A).

Fig. 5. QAPF does not induce cell cycle arrest but does induce apoptotic cell death. T24 cells were treated with DMSO or QAPF for 48 h and cell cycle, cell death and caspase-3 activity assays were performed. (A) Representative images containing the average relative number of cells in subG1, G1, S and G2/M phases of cell cycle after treatments. (B) Representative images containing the average relative number of cells. The gate settings distinguish between viable cells (bottom left), necrotic cells (top left), early apoptotic cells (bottom right) and late apoptotic cells (top right). (C) Quantitative analysis of early apoptosis in cells treated with QAPF. (D) Detection of caspase-3 activity was measured after treatment. *p < 0.05.
As QAPF demonstrated the ability to reduce the cell number of the T24 cell line, we decided to evaluate whether this fraction would have a similar effect in the RT4 cell line. A significant inhibition of growth was observed at concentrations of 50, 100 and 150 μg/mL, indicating that QAPF exerts a similar effect on RT4 cells (Fig. 3B). However, the IC50 values for T24 cells were lower than RT4 cells at all timepoints evaluated (Table 2), demonstrating that cells derived from an invasive bladder tumor with metastatic potential (T24 cells) are more sensitive to treatment with the QAPF.

Subsequently, cell viability was evaluated by MTT assay. The results showed that QAPF significantly decreased cell viability at the higher concentrations, following the same trend observed from the cell counting for both cell lines (Fig. 4A and B). Based on these results, we selected the T24 cell line, the three highest concentrations and the 48 h timepoint for the following experiments.

3.4. QAPF does not induce cell cycle arrest, but induces apoptotic death in T24 cells

Considering that QAPF inhibits cell growth in T24 cells, we decided to assess whether this inhibition could be due to a blockade in cell cycle progression. Flow cytometry analysis showed that QAPF does not alter the distribution of DNA content in 48 h (Fig. 5A), indicating that it did not affect the cell cycle.

Next, we decided to measure the cell death of T24 cells treated with QAPF. As shown in Fig. 5B, the percentage of viable cells was reduced from 86.89% to 76.59%, and consequently, the percentage of early apoptotic cells (Fig. 5C) increased from 3.59% to 12.61% after this treatment.

To evaluate whether caspase-3, a key regulator of the intracellular signaling of apoptosis, was activated in T24 cells treated with QAPF, caspase-3 activity was measured. Fig. 5D shows the parallel profiles of caspase-3 activation and early apoptosis induction after treatment with the same concentrations of QAPF, suggesting that QAPF induces cell death in T24 cells through activation of a caspase-3-dependent apoptotic pathway.

3.5. QAPF does not alter protein levels of ERK and PTEN

To study the involvement of signal transduction pathways after QAPF treatment in bladder cancer cells, phosphorylated and total levels of ERK and total levels of PTEN were analyzed by Western blot. As shown in Fig. 6A and B, respectively, no significant alterations in ERK phosphorylation/activation or PTEN were detected after the treatment with QAPF for 48 h.

3.6. QAPF induces NF-κB activation in T24 cells

The NF-κB pathway plays a crucial role in controlling cellular growth and apoptotic cell death, depending on type cell and activation status (Allen-Hall et al., 2010; Shishodia and Aggarwal, 2004; Stark et al., 2001). Therefore, we evaluated the levels of NF-κB in cytosolic and nuclear protein fractions to verify whether QAPF would affect NF-κB after 48 h of treatment. As shown in Fig. 7A–D, at the highest concentration tested (150 μg/mL), treatment with QAPF decreased the cytoplasmic levels of phosphorylated NF-κB p65 (Fig. 7A and B) and consequently increased the nuclear translocation of phosphorylated NF-κB p65 (Fig. 7C and D), indicating an activation of the NF-κB pathway by treatment with QAPF.

4. Discussion

Several groups have reported that most biological actions of U. tomentosa are related to alkaloid constituents (Pilarski et al., 2007; Rinner et al., 2009). Similarly, recently our group has also demonstrated that the oxindole alkaloid purified fraction significantly inhibited cell viability of the T24 and RT4 human bladder cancer cell lines (Kaiser et al., 2013b). However, other compounds present in the cat’s claw crude extract can have an antiproliferative effect. In the present work, we observe that quinovic acid glycosides purified fraction (QAPF) of U. tomentosa significantly inhibits T24 and RT4 cell growth (Fig. 3A and B). In addition, the results showed that QAPF inhibited the viability of both cell lines (Fig. 4A and B), which is consistent with previous studies that have shown that U. tomentosa inhibits the growth of a variety of tumor cell lines (De Martino et al., 2006; Pilarski et al., 2010). It is important to note that the QAPF used in this study was a fraction rich in quinovic acid glycosides (Table 1).

Some studies indicate that U. tomentosa induces a cell cycle arrest in the G0/G1 or G2/M phases, depending on the extract, fraction or isolated compound used (Cheng et al., 2007; Pilarski et al., 2010; Rinner et al., 2009), but others have demonstrated that U. tomentosa has no effect on cell cycle (Akeson et al., 2003; Pilarski et al., 2010). In agreement with the latter two studies, we demonstrated that QAPF did not induce cell cycle arrest (Fig. 5A). It is important to note that, in most cases, the tumor cell response to a cytostatic drug can be cell cycle arrest, which is one reason why chemotherapy is often ineffective: the cancer cell can continue to proliferate after the end of the treatment. Based on this awareness, current studies have focused on the discovery of drugs that selectively induce apoptosis in proliferating cancer cells (Cheng et al., 2007; Pilarski et al., 2010). In agreement with this idea and with the literature (Cheng et al., 2007; De Martino et al., 2006; Rinner et al., 2009), we showed that QAPF-induced cell death in T24 cells (150 μg/mL) occurred through caspase-3-dependent apoptosis (Fig. 5B–D).

To determine the possible molecular mechanism by which QAPF induces cell death, the effect of this treatment on cell signaling proteins was evaluated. The results demonstrated that PTEN protein levels were not altered by the treatment with different concentrations of QAPF (Fig. 6B), nor was there any effect on the phosphorylation/activation of ERK (Fig. 6A), which is involved in cell proliferation. Because ERK can phosphorylate many proteins...
involved in cell cycle regulation (McCubrey et al., 2007; Tang et al., 2002), this result is in agreement with the lack of effect on the cell cycle after QAPF treatment. Therefore, these data indicate that the PTEN and ERK signaling pathways do not seem to be involved in the apoptosis induced by QAPF.

Previous work has already shown that the hydroethanolic extract of U. tomentosa inhibited the activation of NF-κB, a transcription factor, in LPS-stimulated THP-1 cells (Allen-Hall et al., 2010). Based on this, we decided to investigate whether QAPF of U. tomentosa would also inhibit the nuclear translocation of NF-κB. Our data appear to contradict the study above, as we observed that the QAPF induced the nuclear translocation of NF-κB (Fig. 7C and D). The hypothesis that the nuclear translocation of NF-κB induces apoptosis in bladder cancer cells is intriguing and is in agreement with other studies that have demonstrated that the activation of NF-κB causes cancer cell apoptosis (Ashikawa et al., 2004; Stark et al., 2001). The mechanism by which NF-κB mediates apoptosis is not totally understood, but activation of NF-κB can promote the expression of Fas, c-myc and p53, which may be involved in the induction of apoptosis (Shishodia and Aggarwal, 2004).

In summary, from all of the possible mechanisms investigated, the present study demonstrated that QAPF has great potential to inhibit human bladder cancer cell growth by activating the caspase-3-dependent apoptotic pathway and through a mechanism that involves translocation of NF-κB to the nucleus. Although further investigation is necessary, the results presented here imply that QAPF from U. tomentosa can be used for prevention and/or as an adjuvant therapy for treatment of urinary bladder cancer.

Conflict of Interest

The authors have no conflicts of interest to disclose.

Acknowledgements

This study was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes). We thank L. R. Blazina, M. Pugliese and M.S. Quevedo for their technical assistance.

References


