Uncaria tomentosa (Willd. ex Schult.) DC (Rubiaceae) Sensitizes THP-1 Cells to Radiation-induced Cell Death

Lisa Allen[1,2], Alison Buckner[1,2], Carly A. Buckner[1,2], Pablo Cano[2], Robert M. Lafrenie[1,2,3,4]

[1]Program in Biomolecular Science, Laurentian University, Sudbury, ON P3E 2C6, [2]Health Sciences North, Sudbury, ON P3E 5J1, [3]Division of Medical Science, Northern Ontario School of Medicine, Sudbury, ON P3E 2C6, [4]Health Sciences North Research Institute, Sudbury, ON, P3E 5J1, Canada

ABSTRACT

Background: *Uncaria tomentosa* (Willd. ex Schult.) DC (Rubiaceae), known as Cat's Claw or Uña de gato, is a traditionally used medicinal plant native to Peru. Some studies have shown that *U. tomentosa* can act as an antiapoptotic agent and enhance DNA repair in chemotherapy-treated cells although others have shown that *U. tomentosa* enhanced apoptosis. **Objective:** To determine if treatment with *U. tomentosa* can significantly enhance cell death in THP-1 cells exposed to ionizing radiation. **Materials and Methods:** THP-1 monocyte-like cells were treated with ethanolic extracts of *U. tomentosa* in the presence or absence of bacterial lipopolysaccharide and then exposed to ionizing radiation. Cell proliferation was assessed by MTT and clonogenic assays and the effects on cell cycle measured by flow cytometry and immunoblotting. Changes in cell signaling were determined by immunoblotting and cytokine ELISA and activation of apoptosis measured by caspase activation and DNA fragmentation analysis. **Results:** Treatment of THP-1 cells with *U. tomentosa* had a small effect on cell proliferation. However, when the *U. tomentosa*-pretreated cells were also subjected to 5–9 Gy ionizing radiation, they showed a significant decrease in cell proliferation and increased cellular apoptosis as measured by DNA fragmentation and caspase activation. Treatment with *U. tomentosa* also decreased the expression of Cyclin E and Cyclin B, key regulators of normal cell cycle progression, and decreased the phosphorylation of various stress-activated, cell survival proteins including p38, ERK, and SAP/JNK kinase. **Conclusions:** These results suggest that *U. tomentosa* could be useful in enhancing cell death following anticancer therapies including ionizing radiation.

Key words: Cell signaling, cell survival, cytokine, monocyte, *Uncaria tomentosa*, X-rays

SUMMARY

- Treatment of THP-1 cells with Uncaria tomentosa increases their susceptibility to X-rays. The combination of Uncaria tomentosa and X-ray exposure strongly inhibits cell signaling and promotes apoptosis.
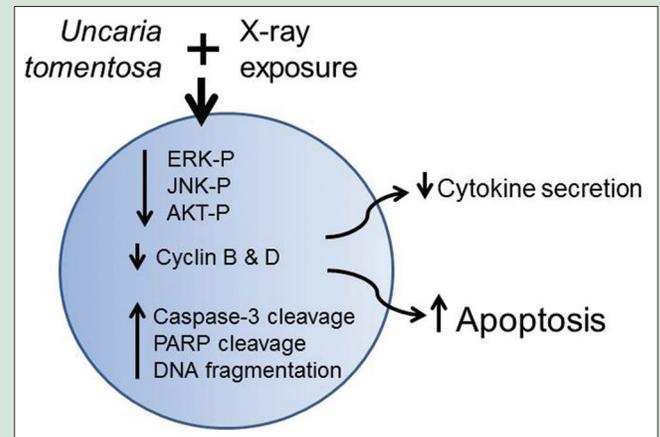
INTRODUCTION

Uncaria tomentosa (Willd. ex Schult.) DC (Rubiaceae) or Uña de Gato is a Peruvian plant that the Ashaninka Indians of South America have used for generations to treat various medical ailments including arthritis, cancer, and premenstrual syndrome.[1,2] The woody vine is prepared and served in a hot water tea-like concoction. The discovery that *U. tomentosa* treatment of monocytes can inhibit the lipopolysaccharide (LPS)-dependent expression of tumor necrosis factor-alpha (TNF-α) highlights its potential as a natural anti-inflammatory agent.[3-9]

We previously showed that treatment of THP-1 monocyte-like cells with *U. tomentosa* decreases LPS-dependent production of TNF-α by more than 50% while augmenting the production of interleukin 1 beta (IL-1β) by more than 25%.[9] Treatment with *U. tomentosa* was shown to inhibit the LPS-dependent activation of all AP-1 subunits and to inhibit p65 and the classical nuclear factor-kappa B (NF-κB) pathway while promoting activation of the p52 nonclassical NF-κB pathway.[10] Inhibition of the p50 subunit of NF-κB, with SN50, partially restored TNF-α secretion in *U. tomentosa*-treated cells but did not affect IL-1β secretion suggesting *U.*

tomentosa is more specific for the classical NF-κB pathway.[10] Inhibition of the classical NF-κB pathway may be important for the prevention and treatment of cancer[11,12] while elevated p52 can enhance cell survival without promoting tumourigenesis.[13-15] Treatment with *U. tomentosa* has been shown to improve outcomes for animals or patients treated with chemotherapeutics or radiation. In some studies, this improvement was associated with a decrease in immune responsiveness to therapy[16-20] while other studies showed the benefit did not involve immune function.[21-23]

Some studies have even shown that *U. tomentosa* can enhance cellular recovery following DNA damage by promoting the repair of both single-strand and double-strand DNA breaks.[24-26]

In the current studies, we report that the treatment of THP-1 cells with *U. tomentosa* sensitized them to ionizing radiation-induced cell death. Treatment of THP-1 cells with *U. tomentosa* alone or in combination with LPS had only modest effects on cell viability. We had previously shown that treatment with LPS-promoted activation of cell signaling pathways associated with cell survival but that inclusion of *U. tomentosa* could inhibit some of these pathways.[9] However, treatment with ionizing radiation following *U. tomentosa* pretreatment inhibited cell signaling, inhibited the expression of cyclin E and cyclin B, prevented accumulation of the cells at any of the cell cycle checkpoints, and increased the frequency of apoptotic cell death.

MATERIALS AND METHODS

Cell culture and treatment

THP-1 cells,[27] obtained from the American Type Culture Collection (ATCC Manassas, VA, USA), were maintained in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone, Login, Utah) and 1% antibiotic/antimycotic solution (Invitrogen, Burlington, ON, Canada) in 5% CO_2 at 37°C. For all experiments, the cells were treated with suspending media or 20–160 µg/ml *U. tomentosa* extract for 24 h. In some experiments, the cells were also co-treated with 2.5 µg/ml bacterial LPS (*Escherichia coli* Serotype 0127, Sigma-Aldrich Chemical, St. Louis, MO, USA) for 24 h. The cells were then treated with 0–15 Gy ionizing radiation using a Gulmay Medical X-ray machine (Scarborough, ON, Canada) and collected for analysis after various incubation times.

Preparation and characterization of *Uncaria tomentosa* extracts

U. tomentosa (Willd.) DC (Rubiacae) was obtained as a powdered preparation of the plant's root as identified and provided by Dr. Rosaria Rojas, Lima. Peru. Extracts were prepared through exhaustive percolation with 95% ethanol (100 mg/ml to create the stock concentration) as described.[9] Different preparations of *U. tomentosa* were used and compared by high-performance liquid chromatography (HPLC) to normalize for the quantity of marker components. This resulted in the use of two different final concentrations based on the amount of ground root material used to create the extract. The *U. tomentosa* extract was analyzed using HPLC on a Breeze 2 chromatography system (Waters Inc., Toronto, ON, Canada) fitted with a 4.6 mm × 100 mm Sunfire C18 column 3.5 µm resin [Figure 1]. The solvents used were: (A) 60 volumes 10 mM phosphate buffer, pH 6.6; 20 volumes acetonitrile; and 20 volumes methanol; and (B) 30 volumes 10 mM phosphate buffer, pH 6.6; 25 volumes acetonitrile; and 25 volumes methanol. The solvent gradient was applied at 1 ml/min starting with 100% A and finishing with 100% B over 40 min followed by 10 min with 100% B. The marker components, corresponding to uncarine C, uncarine D, uncarine E, mitraphyline, isomitraphyline, and rhynchophylline, were detected at 245 nm and compared to a group of standards (Planta Analytical) and were very similar to our previous preparations.

Microscopic visualization of THP-1 cells

THP-1 cells were treated with media *U. tomentosa* and/or LPS for 24 h and then exposed to ionizing radiation, as indicated. The cell morphology was examined each day using a Zeiss-Axiophot 100 phase-contrast microscope (Zeiss Canada, Toronto, ON) and images obtained using Northern Eclipse software.

peak	Compound	Retention time	Area	Amount (µg/ml)
1	Uncarine D	9.73	972	390
2	mitraphylline	11.53	1770	459
3	Uncarine C	13.88	1823	270
4	isomitraphylline	14.78	240	32
5	Rhynchophylline	18.70	1138	213
6	Uncarine E	21.01	774	165
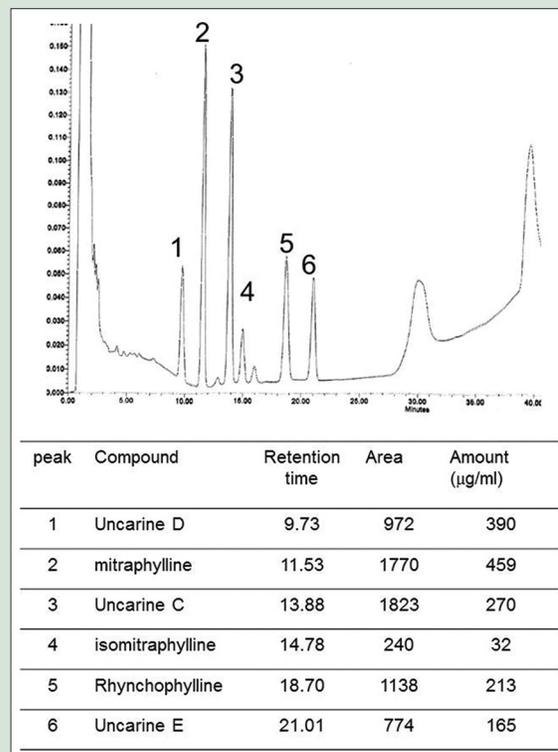
Figure 1: High-performance liquid chromatography analysis of *Uncaria tomentosa* extracts. The root powder was extracted with 95% ethanol and subjected to high-performance liquid chromatography as described. The identification of the indicated peaks is based on the retention times of the indicated standard compounds analyzed under the same conditions

Quantitation of cytokines

THP-1 cells (10^6/ml) were treated with media *U. tomentosa* and/or LPS for 24 h and then exposed to ionizing radiation, as indicated. Following 24 h incubation, the conditioned media were collected and analyzed for the amount of secreted IL-1β and TNF-α using ELISA kits from R and D Systems (Minneapolis, MN, USA).[10] The experiments were performed in triplicate and analyzed using a Students' *t*-test. Similar results were obtained for three independent experiments.

Analysis of cell proliferation using the MTT assay

THP-1 cells (10^3 cells/well) were plated on 96 well plates in quintuplicate and treated with media, *U. tomentosa* and/or LPS for 24 h and then exposed to ionizing radiation, as indicated. On each day of culture, 5 pg of thiazolyl blue tetrazolium bromide (Sigma M2128) was added to each well and incubated at 37°C for 4 h. The media were removed, the MTT crystals dissolved in 200 ml of dimethyl sulfoxide, and the absorbance read at 540 nm each day. Comparisons in the values between treatment conditions were evaluated using a one-way ANOVA. The percent inhibition was reported as the mean ± standard deviation (SD) for three independent experiments.

Clonogenic assays

THP-1 cells (10^6/ml) were treated with media *U. tomentosa* and/or LPS for 24 h and then exposed to ionizing radiation, as indicated. The treated cells (10^5 per sample) were suspended in methylcellulose culture media (20% FBS and 2.8% methylcellulose in Iscove's Modified Dulbecco's Media [Invitrogen]) and 3 ml plated on six well plates. Following 14 days

of incubation at 37°C and 5% CO_2, to allow proliferation of the cells into colonies, a minimum of five microscopic fields/sample were counted. Only those colonies that contained more than fifty cells were counted. The experiment was performed in triplicate, and the results show the mean ± SD for the combination of three independent experiments analyzed using a Students' *t*-test.

Cell cycle analysis by flow cytometry

THP-1 cells (10^6/ml) were treated with media *U. tomentosa* and/or LPS for 24 h, exposed to ionizing radiation, and then incubated for 4–24 h, as indicated. The cells were fixed by suspension in 70% isopropanol at −20°C, and nuclear DNA was stained by incubation in 0.1% propidium iodide in methanol as described.[28] The fluorescent cell population profiles were created on a Beckman-Coulter Epics Elite flow cytometer (Beckman-Coulter, Mississauga, ON, Canada). The percent cells reported in each phase are the mean of three independent experiments.

Protein isolation and immunoblot analysis

THP-1 cells (10^6/ml) were treated with media *U. tomentosa* and/or LPS for 24 h, exposed to ionizing radiation, and incubated for 24 h, as indicated. Cells were collected by centrifugation and lysed by incubation in RIPA buffer (1% Triton X-100, 0.5% sodium dodecyl sulfate [SDS], 0.5% sodium deoxycholate, 150 mM NaCl, 50 mM Tris–HCl, and pH 7.6), supplemented with 5 mM NaF, 2 mM sodium orthovanadate (Sigma–Aldrich), and protease inhibitors (Roche, Laval, QB).[29] The proteins (30 mg/lane) were subjected to electrophoresis on 10% polyacrylamide gels containing SDS and transferred to nitrocellulose membranes: four identical blots were created from each group of samples and probed independently. The membranes were blocked by incubation in 5% bovine serum albumin (BSA) in Tris-buffered saline, pH 7.5, and 0.1% Tween-20 (TBST) and then incubated with antibodies against phospho-ERK1/2 (titer 1:1000, Cell Signaling Lake Placid, NY, USA), phospho-AKT (titer1:1000, Cell Signaling), phospho-SAP/JNK (titer 1:1000, Cell Signaling), P38 (titer1:1000, Cell Signaling), Cyclin B (titer
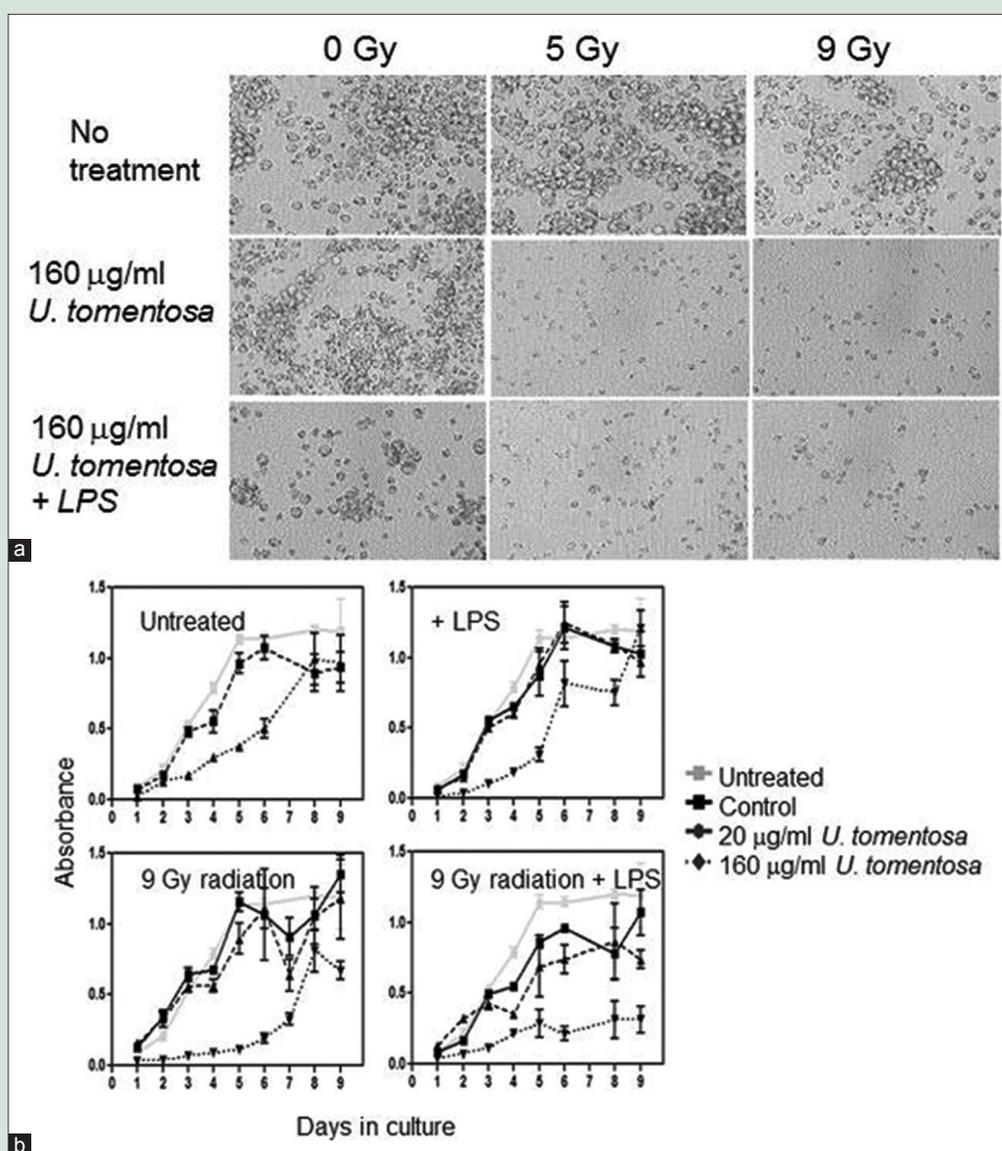
Figure 2: Treatment with *Uncaria tomentosa* and ionizing radiation inhibits cell proliferation. (a) Microscopy of THP-1 cell suspensions treated with *Uncaria tomentosa*, lipopolysaccharide, and/or ionizing radiation for 48 h. (b) The relative number of THP-1 cells (mean ± standard deviation, *n* = 3) was determined using an MTT assay. Each panel shows cells treated with *Uncaria tomentosa* (the gray line shows the number of untreated THP-1 cells). The different panels show cells that were also untreated or treated with lipopolysaccharide, 9 Gy radiation, or 9 Gy radiation and lipopolysaccharide

1:250, Santa Cruz, Biotechnology, Santa Cruz, CA), Cyclin E (titer 1:500, Santa Cruz Biotechnology), poly ADP-ribose polymerase (PARP) (titer 1:250, Santa Cruz Biotechnology), or caspase-3 (titer 1:1000, Cell Signaling) in 1% BSA and TBST. The blots were washed and then incubated in appropriate secondary antibody - horseradish peroxidase (HRP) conjugates in 1% BSA and TBST. HRP was detected by incubation in ECL reagent (Pierce Chemical Co. Rockford, IL, USA) and exposed to ECL X-ray film (Amersham, Baie-d'Urfe, QC). Densitometry of the signal intensity was determined for each blot, normalized to the intensity of β-tubulin staining in a duplicate blot, and the mean relative intensity (to the control set at 1.0) reported for three independent blots.

DNA laddering assay

THP-1 cells (3×10^5/ml) were treated with media, *U. tomentosa* and/or LPS for 24 h, exposed to ionizing radiation, and incubated for 6 h. The cells were collected by centrifugation at 400 ×*g* for 10 min and the pellet lysed by incubation in TE/Triton buffer (0.2% Triton X-100, 10 mM Tris, pH 8.0, and 1 mM ethylenediaminetetraacetic acid) on ice for 10 min.[30] The lysate was subjected to centrifugation at 13,000 ×*g* for 15 min at 4°C. The low molecular weight DNA-containing supernatant was transferred to a fresh microcentrifuge tube treated sequentially with 15 μg/ml RNase A (Sigma-Aldrich) for 1 h at 37°C and 0.025% SDS and 0.10 μg/ml proteinase K for 1 h at 50°C. The DNA was precipitated using cold isopropanol and the pellet resuspended in gel loading buffer (0.01% bromophenol blue and 20% sucrose in TBE) and subjected to electrophoresis on a 2% agarose-TBE gel containing 0.001% ethidium bromide.

Nuclear morphology

THP-1 cells (10^6/ml) were treated with media *U. tomentosa* and/or LPS for 24 h and then exposed to ionizing radiation, as indicated. The cells were collected at 24, 48, and 72 h postirradiation, stained for 10 min in 2.5 μg/ml Hoechst stain, fixed in 3% paraformaldehyde in PBS, pH 7.4, and examined using a Zeiss–Axiophot 100 fluorescence microscope and Northern Eclipse software.

RESULTS

Effect of *Uncaria tomentosa* and ionizing radiation on cell growth and morphology

The morphology of THP-1 cell cultures treated with *U. tomentosa* and LPS for 24 h and then exposed to ionizing radiation and cultured for 48 h is shown in Figure 2a. Exposure to 9 Gy ionizing radiation had very little effect on the morphology of THP-1 cells. The cells were normal in appearance and were able to divide and replicate even after being exposed to doses of ionizing radiation up to 15 Gy (not shown). THP-1 cells treated with 160 mg/ml *U. tomentosa* were smaller and fewer in number but were able to survive and replicate. Treatment of THP-1 cells with both LPS and *U. tomentosa* resulted in fewer cells but those that survived appeared normal and were able to replicate. We have previously shown that LPS treatment alters THP-1 cell signaling,[10] and it has been shown that LPS can change susceptibility to cell death in response to treatment with other agents. Cells treated with *U. tomentosa*, in the presence or absence of LPS, and then exposed to 5 or 9 Gy ionizing radiation, demonstrated significant cell fragmentation, and loss of viability.

The survival of THP-1 cells after exposure to ionizing radiation and treatment with *U. tomentosa* and/or LPS was determined using MTT assays. Relative cell number was determined each day and growth curves plotted [Figure 2b]. Untreated THP-1 cells showed an increase in cell number for the first 5 days when it reached a plateau. Treatment of

cells with 20 μg/ml *U. tomentosa* had very little effect on cell growth, while treatment with 160 μg/ml *U. tomentosa* delayed cell growth by 58% ± 11% on day 6 (based on three independent experiments), although by day 8 treated cell number reached >80% of control. Treatment with both LPS and 160 μg/ml *U. tomentosa* significantly reduced THP-1 growth although the extent was variable between experimental replicates (not shown). Treatment of THP-1 cells with LPS and 9 Gy ionizing radiation also reduced THP-1 cell growth by 16% ± 9% by day 6. The addition of 20 μg/ml *U. tomentosa* to the radiation-treated cells strongly inhibited cell growth by 36% ± 14% while inclusion of 160 μg/ml *U. tomentosa* almost completely inhibited growth.

THP-1 cell growth as determined by clonogenic assay

The ability of single cells to proliferate into colonies was measured using clonogenic, colony-forming assays after treatment with *U. tomentosa*, LPS, and/or ionizing radiation. Treatment of THP-1 cells with *U. tomentosa* decreased colony formation: treatment with 80 μg/ml *U. tomentosa* decreased colony formation by 40% ± 22% (based on three independent experiments), and treatment with 80 μg/ml *U. tomentosa* and LPS decreased colony formation by 46% ± 12% [Figure 3]. Treatment with 5–15 Gy ionizing radiation also decreased colony formation by >35% which was further decreased with the addition of LPS. Treatment with both *U. tomentosa* and ionizing radiation resulted in very large decreases in colony formation, and at higher doses of
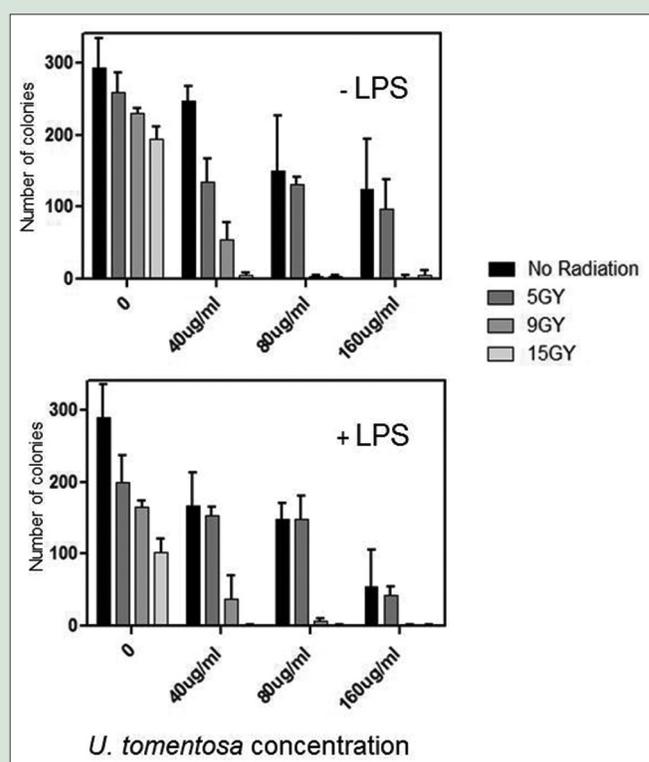
Figure 3: Effect of *Uncaria tomentosa* and ionizing radiation on clonogenic growth. THP-1 cells were treated with *Uncaria tomentosa* extract in the presence of absence or lipopolysaccharide for 24 h and then with ionizing radiation. The treated cells were suspended in methylcellulose growth media and allowed to grow into colonies. The number of colonies (mean ± standard deviation for the combination of three independent experiments) was counted per high power field

U. tomentosa (80 and 160 µg/ml), colony formation was completely inhibited [Figure 3].

Cell cycle analysis of THP-1 cells treated with *Uncaria tomentosa* and ionizing radiation

To determine if the effects on cell growth were mediated through alterations in cell cycle progression, flow cytometry was performed on unsynchronized THP-1 cells stained with propidium iodide. As shown in Figure 4a, there were no significant changes in cell cycle parameters following treatment with *U. tomentosa* for 48 h. Similar results were seen at 6, 12, 24, and 36 h posttreatment in the presence and absence of LPS treatment (not shown). Exposure of THP-1 cells to only 9 Gy ionizing radiation showed a small increase in the number of cells in G2 (but not the expected, strong G2 arrest usually induced by ionizing radiation). The combination of 20 or 100 µg/ml *U. tomentosa* and ionizing radiation increased the sub-G1 population of cells by 2.0 or 3.5 fold ($P < 0.05$), compared to untreated controls (based on average values for three independent experiments). The addition of LPS slightly increased the number of cells in the sub-G1 DNA peak (not shown). In addition, the treated

THP-1 cells showed a small decrease in the proportion of cells in the G2/M phase and S phases.

To identify potential cell cycle targets of *U. tomentosa*, the effects on cyclin expression were examined by immunoblot analysis. Treatment of THP-1 cells with LPS increased cyclin E expression by 1.2 fold (based on densitometry of three independent blots) but had little effect on cyclin B [Figure 4b]. Treatment of THP-1 cells with only *U. tomentosa* caused a small decrease in the amount of cyclin E (0.8 fold) and cyclin B (0.7 fold) expression, and treatment with only 9 Gy ionizing radiation caused an increase in cyclin B (1.8 fold) and a small decrease in cyclin E (0.8 fold). However, in cells exposed to both ionizing radiation and *U. tomentosa* treatment, cyclin B and cyclin E levels were strongly downregulated by 20 µg/ml of *U. tomentosa* and completely blocked by 100 µg/ml of *U. tomentosa*.

Effects of treatment and activation on cell signaling

To identify potential mechanism by which treatment with *U. tomentosa* and ionizing radiation affected cell proliferation, we examined the activation of survival pathway proteins. Treatment of THP-1 cells with LPS increased the activation of the MAP kinase pathways as shown by enhanced phosphorylation of the p38, pERK, and SAP/JNK kinase
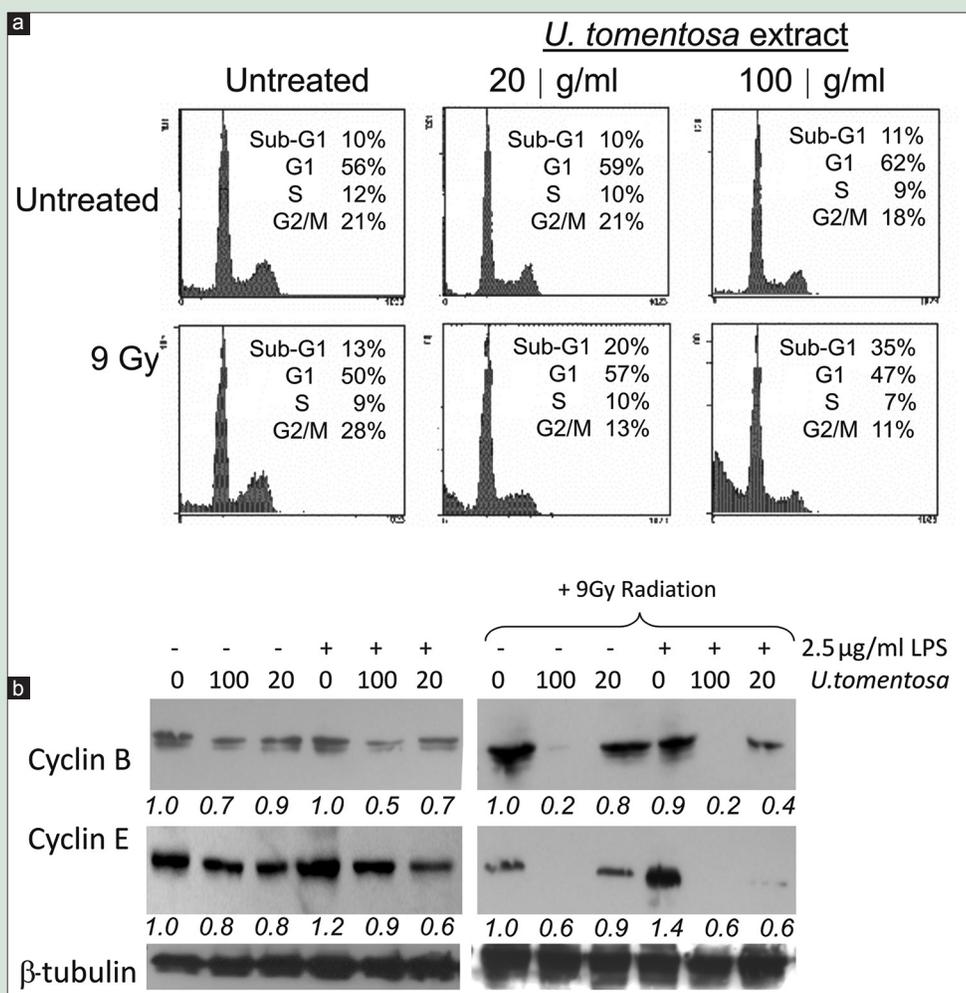
Figure 4: Effect of *Uncaria tomentosa* and ionizing radiation on cell cycle and cyclin expression. (a) THP-1 cells were treated with *Uncaria tomentosa* for 24 h and/or ionizing radiation for a further 24 h and then stained with propidium iodide. The average percent of cells ($n = 3$ experiments) in each of the cell cycle peaks is shown. (b) Immunoblot analysis of cyclin B and cyclin E expression levels for THP-1 cells treated with *Uncaria tomentosa* and/or lipopolysaccharide for 24 h and/or 9 Gy ionizing radiation for a further 24 h. The relative difference in band intensity, normalized for β-tubulin expression, was averaged for three experiments and is shown below each band

proteins and the phosphorylation of AKT [Figure 5]. Treatment with *U. tomentosa* decreased p-Akt, pERK, and SAP/JNK phosphorylation in a dose-dependent manner both in the presence and absence of LPS. Treatment of THP-1 cells with 20 µg/ml and 100 µg/ml of *U. tomentosa* inhibited p38 phosphorylation although 100 µg/ml of *U. tomentosa* was required to inhibit LPS-dependent p38 phosphorylation. Exposure to 9 Gy ionizing radiation resulted in a general inhibition of MAP kinase and AKT phosphorylation. In cells treated with ionizing radiation, *U. tomentosa* was able to further inhibit ERK, p38, and AKT with the same pattern as nonirradiated cells. However, SAP/JNK phosphorylation was activated in cells exposed to ionizing radiation and LPS, both in the presence and absence of *U. tomentosa*.

Cytokine expression as determined by ELISA assay

The activation of cell signaling pathways in response to ionizing radiation was also examined by measuring the effects on cytokine secretion. TNF-α and IL-1β secretion was measured in the conditioned media of THP-1 cells treated with *U. tomentosa,* LPS, and/or ionizing radiation. Consistent with previous results'[10] treatment with LPS increased the secretion of both TNF-α and IL-1β ($P < 0.01$) [Figure 6]. Exposure to 5 or 9 Gy ionizing radiation blocked LPS-dependent TNF-α secretion by 50% (based on three independent experiments). Treatment with 40, 80, or 160 µg/ml *U. tomentosa* almost completely inhibited the LPS-dependent secretion of TNF-α ($P < 0.01$) in the presence or absence of ionizing radiation. In contrast, the secretion of IL-1β was augmented by treatment with *U. tomentosa*. Treatment of cells with both LPS and 80 µg/ml *U. tomentosa* increased IL-1β secretion by 2.1 fold (from three independent experiments). However, exposure to ionizing radiation caused a dose-dependent decrease in IL-1β secretion by THP-1 cells. In cells treated with all doses of *U. tomentosa* and LPS, ionizing radiation significantly blocked IL-1β secretion by 25%–70% ($P < 0.05$).

Apoptosis

To determine if the decrease in cell number following exposure to *U. tomentosa,* LPS, and ionizing radiation was due to an increase in cell death, we examined classical markers of apoptosis including caspase cleavage, PARP cleavage, and DNA fragmentation. Caspase-3 cleavage, which correlates with increased apoptosis, was most significant in cells treated with 100 µg/ml *U. tomentosa*, LPS, and ionizing radiation for 24 h [Figure 7a]. PARP cleavage was present in cells treated with 100 µg/ml *U. tomentosa* or combinations of *U. tomentosa,* LPS, and/or ionizing radiation, *U. tomentosa* and LPS, and *U. tomentosa,* LPS, and ionizing radiation for 24 h.

THP-1 cells treated for 24 h with a combination of 100 µg/ml *U. tomentosa*, LPS, and ionizing radiation showed a much greater amount of DNA laddering than treatment with any of the individual agents [Figure 7b]. This confirmed the flow cytometry results that showed treatment of THP-1 cells with 100 µg/ml *U. tomentosa*, LPS, and ionizing radiation resulted in 3-fold increase in cells in the sub-G1 peak [Figure 4a]. In addition, the nuclear morphology of Hoechst-stained THP-1 cells showed that the nuclei of THP-1 cells treated with only LPS or 9 Gy ionizing radiation appeared intact and that treatment with all of *U. tomentosa*, LPS, and ionizing radiation was required to promote significant nuclear fragmentation typical of apoptosis [Figure 7c].

DISCUSSION

Patients frequently include natural products, such as *U. tomentosa*, in treatment regimens for a variety of medical conditions, including inflammatory diseases and cancer.[1,2] We have previously shown that *U. tomentosa* inhibits the expression of the potent pro-inflammatory cytokine TNF-α (and augments IL-1β) and differentially regulates activation of NF-κB transcription factor subunits suggesting that *U. tomentosa* elicits its cellular response through the NF-κB-mediated pathway.[10] The inhibition of NF-κB has been shown to enhance apoptosis in malignant cells treated with chemotherapy and/or radiation treatment.[11,20,31-34] Further, treatment with *U. tomentosa* was
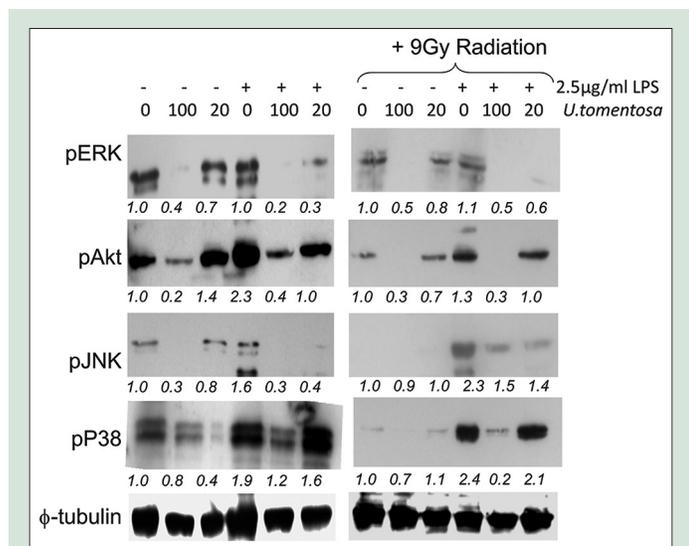
Figure 5: Effect of *Uncaria tomentosa* and ionizing radiation on the activation of stress signaling molecules. Immunoblot analysis of pERK, pAKT, pJNK, and pP38, expression levels for THP-1 cells treated with *Uncaria tomentosa* and/or lipopolysaccharide for 24 h and/or 9 Gy ionizing radiation for a further 24 h. The relative difference in band intensity, normalized for β-tubulin expression, was averaged for three experiments and is shown below each band
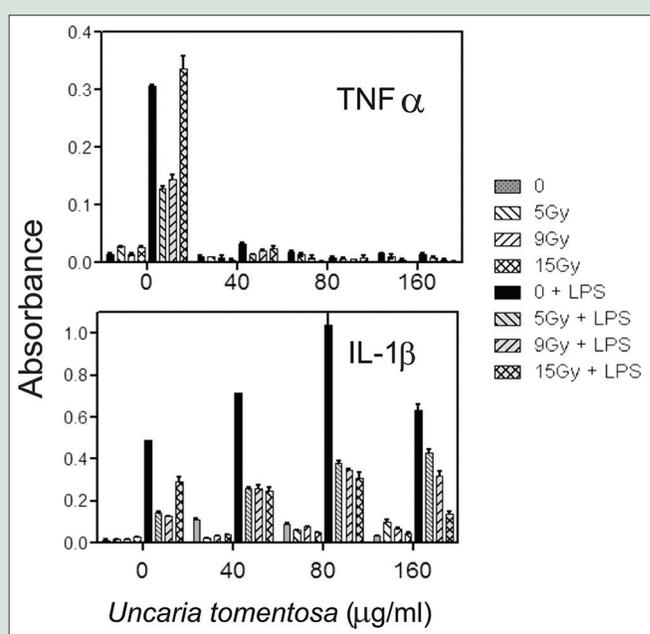
Figure 6: Effect of *Uncaria tomentosa* and ionization radiation on tumor necrosis factor-alpha and interleukin 1 beta secretion. THP-1 cells were treated with *Uncaria tomentosa* and/or lipopolysaccharide for 24 h and/or ionizing radiation and then incubated in serum-free media for a further 24 h. The amount of cytokine (mean + standard deviation, *n* = 3) was measured in the conditioned media using ELISA analysis for tumor necrosis factor-alpha and interleukin 1 beta
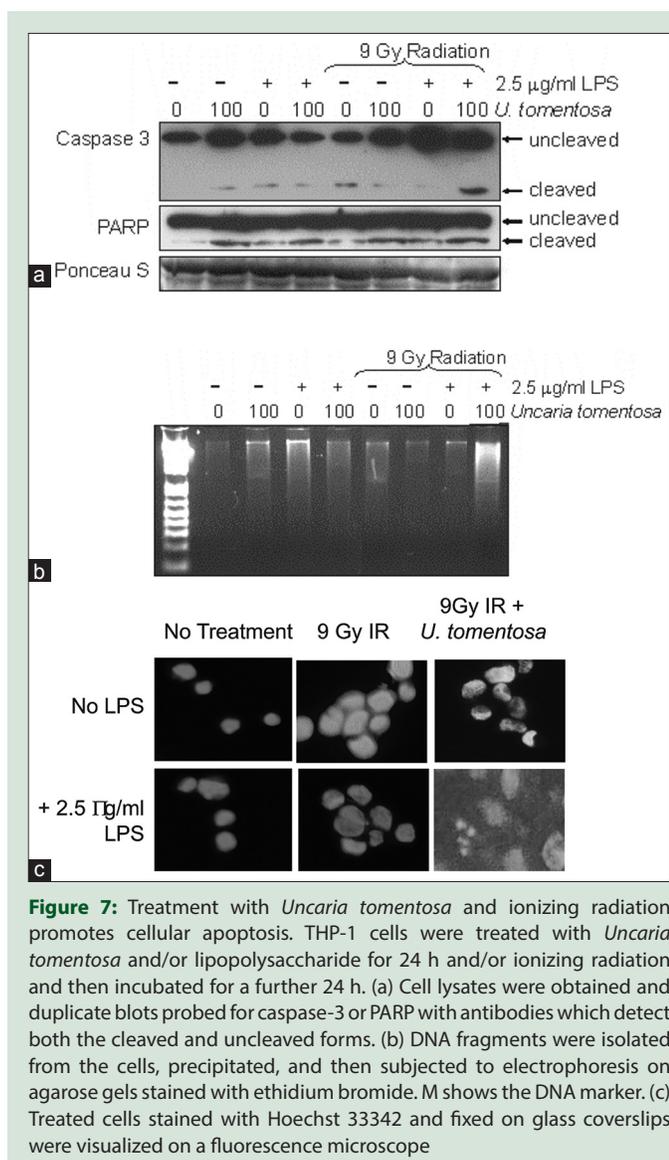
Figure 7: Treatment with *Uncaria tomentosa* and ionizing radiation promotes cellular apoptosis. THP-1 cells were treated with *Uncaria tomentosa* and/or lipopolysaccharide for 24 h and/or ionizing radiation and then incubated for a further 24 h. (a) Cell lysates were obtained and duplicate blots probed for caspase-3 or PARP with antibodies which detect both the cleaved and uncleaved forms. (b) DNA fragments were isolated from the cells, precipitated, and then subjected to electrophoresis on agarose gels stained with ethidium bromide. M shows the DNA marker. (c) Treated cells stained with Hoechst 33342 and fixed on glass coverslips were visualized on a fluorescence microscope

Combining *U. tomentosa* treatment and ionizing radiation had a potent effect on cell signaling pathways which contribute to apoptosis. Treatment of THP-1 cells with both *U. tomentosa* and ionizing radiation had a much stronger effect on blocking activation of ERK, p38, and AKT phosphorylation and on inhibiting cytokine expression. In addition, LPS treatment appeared to increase the sensitivity of THP-1 cells to cell death in response to *U. tomentosa* and/or ionizing radiation. Treatment of THP-1 cells with *U. tomentosa* inhibited the phosphorylation of ERK, p38, and Akt (and decreased cyclin B and E expression), and this was exaggerated by LPS treatment. Although LPS treatment was shown to promote IL-1β and TNF-α secretion, likely through activation of NF-kB,[10] secretion of TNF-α was potently inhibited by *U. tomentosa* consistent with our previous results.[9,10] Previous studies showed ionizing radiation alone can activate TNF-α and IL-1β through activation of NF-κB.[40] However, our studies showed that ionizing radiation only marginally enhanced cytokine expression. The most significant effect of ionizing radiation in this study was its ability to inhibit phosphorylation of ERK, p38, and Akt and decrease secretion of both IL-1β and TNF-α induced by LPS. Therefore, THP-1 cells treated with all of *U. tomentosa*, ionizing radiation, and LPS showed strong inhibition of ERK, p38, and Akt and the highest levels of apoptosis. Previous results have shown that inhibition of NF-kB can inhibit prosurvival and antiapoptotic pathways[15,41-43] and that inhibition of the MAP kinase and AKT survival pathways can result in the enhancement of cell death following exposure to ionizing radiation.[44] Exposure of THP-1 cells to only ionizing radiation inhibited SAP/JNK phosphorylation but treatment with ionizing radiation and *U. tomentosa* preserved LPS-dependent SAP/JNK phosphorylation. This is consistent with previous studies that showed a combination of pERK inhibition and SAP/JNK activation could activate caspase-3 and promote apoptosis.[45-47]

THP-1 cells treated with ionizing radiation do not show the typical arrest of the cell cycle at the G2/M phase[40,48] and show only a small accumulation of cells in G2 6–8 h posttreatment. Pretreatment with *U. tomentosa* completely blocked even this small accumulation in G2 and cells treated with *U. tomentosa* and ionizing radiation showed an increase in the number of cells in the sub-G1 peak which correlates to cellular apoptosis. An examination of cyclin E (which is enhanced in cells in G1[49]) and cyclin B (which is enhanced in G2/M[50]) expression supports the idea that treatment of THP-1 cells with both *U. tomentosa* and ionizing radiation is required to have significant effects on cell cycle regulation and apoptosis. Treatment of THP-1 cells with *U. tomentosa* had a minor effect on cyclin E and cyclin B suggesting normal progression through the cell cycle and cells treated with only ionizing radiation showed a small accumulation of cyclin B which indicates an accumulation at G2. However, exposure to ionizing radiation and *U. tomentosa* completely inhibited the expression of both cyclin E and cyclin B. The lack of cyclin E and B protein expression combined with the increase in the sub-G1 peak suggested that the THP-1 cells did not complete the mitotic cycle and underwent apoptosis.

CONCLUSION

We previously showed that *U. tomentosa* has the potential to be an effective anti-inflammatory agent and can inhibit TNF-α secretion and AP-1 and NF-κB activation in THP-1 cells.[10] We now show that treatment with *U. tomentosa* can promote radiation-induced apoptosis in THP-1 cells. While treatment of THP-1 cells with 20 μg/ml *U. tomentosa* or 9 Gy ionizing radiation did not significantly change cell growth, exposure to both *U. tomentosa* and ionizing radiation significantly decreased cell growth and promoted apoptosis. The observation that even high doses of *U. tomentosa* have relatively low toxicity until combined with ionizing

shown to induce apoptosis in sarcoma, medullary thyroid, and breast cancer cells.[35-39] However, others have reported that *U. tomentosa* can enhance cell survival after radiation-induced DNA damage.[25,26]

The results of the current experiments showed that treatment of THP-1 cells with *U. tomentosa* (and LPS) followed by exposure to ionizing radiation significantly enhanced cellular apoptosis. We discovered that THP-1 cells were extremely resistant to exposure to ionizing radiation or to treatment with *U. tomentosa*. The cells remained viable after exposure to 15 Gy radiation or following treatment with 20 μg/ml *U. tomentosa* (treatment with 160 μg/ml delayed growth for several days). However, when the THP-1 cells were pretreated with *U. tomentosa* and then treated with ionizing radiation, they showed a profound decrease in cell growth and an increase in apoptosis which was further enhanced by the addition of LPS. The increase in apoptosis was measured as cleavage and activation of caspase-3, cleavage of PARP, and DNA laddering (nucleosome laddering was best seen at early time points and DNA appeared as "smears" at later time points, likely due to the ability of THP-1 monocyte-like cells to phagocytose and further degrade any apoptotic body generated during the experiment).

radiation suggest that it may have some promise in future studies as a therapeutic.

Financial support and sponsorship

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Keplinger K, Laus G, Wurm M, Dierich MP, Teppner H. *Uncaria tomentosa* (Willd.) DC. – Ethnomedicinal use and new pharmacological, toxicological and botanical results. J Ethnopharmacol 1999;64:23-34.

2. Heitzman ME, Neto CC, Winiarz E, Vaisberg AJ, Hammond GB. Ethnobotany, phytochemistry and pharmacology of *Uncaria* (Rubiaceae). Phytochemistry 2005;66:5-29.

3. Sandoval-Chacón M, Thompson JH, Zhang XJ, Liu X, Mannick EE, Sadowska-Krowicka H, *et al.* Antiinflammatory actions of cat's claw: The role of NF-kappaB. Aliment Pharmacol Ther 1998;12:1279-89.

4. Sandoval M, Charbonnet RM, Okuhama NN, Roberts J, Krenova Z, Trentacosti AM, *et al.* Cat's claw inhibits TNFalpha production and scavenges free radicals: Role in cytoprotection. Free Radic Biol Med 2000;29:71-8.

5. Sandoval M, Okuhama NN, Zhang XJ, Condezo LA, Lao J, Angeles' FM, *et al.* Anti-inflammatory and antioxidant activities of cat's claw (*Uncaria tomentosa* and *Uncaria guianensis*) are independent of their alkaloid content. Phytomedicine 2002;9:325-37.

6. Gonçalves C, Dinis T, Batista MT. Antioxidant properties of proanthocyanidins of *Uncaria tomentosa* bark decoction: A mechanism for anti-inflammatory activity. Phytochemistry 2005;66:89-98.

7. Reis SR, Valente LM, Sampaio AL, Siani AC, Gandini M, Azeredo EL, *et al.* Immunomodulating and antiviral activities of *Uncaria tomentosa* on human monocytes infected with dengue virus-2. Int Immunopharmacol 2008;8:468-76.

8. Rojas-Duran R, González-Aspajo G, Ruiz-Martel C, Bourdy G, Doroteo-Ortega VH, Alban-Castillo J, *et al.* Anti-inflammatory activity of mitraphylline isolated from *Uncaria tomentosa* bark. J Ethnopharmacol 2012;143:801-4.

9. Allen-Hall L, Cano P, Arnason JT, Rojas R, Lock O, Lafrenie RM. Treatment of THP-1 cells with *Uncaria tomentosa* extracts differentially regulates the expression if IL-1beta and TNF-alpha. J Ethnopharmacol 2007;109:312-7.

10. Allen-Hall L, Arnason JT, Cano P, Lafrenie RM. *Uncaria tomentosa* acts as a potent TNF-alpha inhibitor through NF-kappaB. J Ethnopharmacol 2010;127:685-93.

11. Leger DY, Liagre B, Beneytout JL. Role of MAPKs and NF-kappaB in diosgenin-induced megakaryocytic differentiation and subsequent apoptosis in HEL cells. Int J Oncol 2006;28:201-7.

12. Lwin T, Hazlehurst LA, Li Z, Dessureault S, Sotomayor E, Moscinski LC, *et al.* Bone marrow stromal cells prevent apoptosis of lymphoma cells by upregulation of anti-apoptotic proteins associated with activation of NF-kappaB (RelB/p52) in non-Hodgkin's lymphoma cells. Leukemia 2007;21:1521-31.

13. Wang Y, Cui H, Schroering A, Ding JL, Lane WS, McGill G, *et al.* NF-kappa B2 p100 is a pro-apoptotic protein with anti-oncogenic function. Nat Cell Biol 2002;4:888-93.

14. Wang Z, Zhang B, Yang L, Ding J, Ding HF. Constitutive production of NF-kappaB2 p52 is not tumorigenic but predisposes mice to inflammatory autoimmune disease by repressing Bim expression. J Biol Chem 2008;283:10698-706.

15. Zhang J, Warren MA, Shoemaker SF, Ip MM. NFkappaB1/p50 is not required for tumor necrosis factor-stimulated growth of primary mammary epithelial cells: Implications for NFkappaB2/p52 and RelB. Endocrinology 2007;148:268-78.

16. Sheng Y, Pero RW, Wagner H. Treatment of chemotherapy-induced leukopenia in a rat model with aqueous extract from *Uncaria tomentosa*. Phytomedicine 2000;7:137-43.

17. Groom SN, Johns T, Oldfield PR. The potency of immunomodulatory herbs may be primarily dependent upon macrophage activation. J Med Food 2007;10:73-9.

18. Dreifuss AA, Bastos-Pereira AL, Avila TV, Soley Bda S, Rivero AJ, Aguilar JL, *et al.* Antitumoral and antioxidant effects of a hydroalcoholic extract of cat's claw (*Uncaria tomentosa*) (Willd. Ex Roem. & Schult) in an *in vivo* carcinosarcoma model. J Ethnopharmacol 2010;130:127-33.

19. Santos Araújo Mdo C, Farias IL, Gutierres J, Dalmora SL, Flores N, Farias J, *et al. Uncaria tomentosa*-adjuvant treatment for breast cancer: Clinical trial. Evid Based Complement Alternat Med 2012;2012:676984.

20. de Oliveira LZ, Farias IL, Rigo ML, Glanzner WG, Gonçalves PB,

Cadoná FC, *et al.* Effect of *Uncaria tomentosa* extract on apoptosis triggered by oxaliplatin exposure on HT29 cells. Evid Based Complement Alternat Med 2014;2014:274786.

21. Akesson CH, Pero RW, Ivars F. C-Med 100, a hot water extract of *Uncaria tomentosa*, prolongs lymphocyte survival *in vivo*. Phytomedicine 2003;10:23-33.

22. Farias IL, Araújo MC, Farias JG, Rossato LV, Elsenbach LI, Dalmora SL, *et al. Uncaria tomentosa* for reducing side effects caused by chemotherapy in CRC patients: Clinical trial. Evid Based Complement Alternat Med 2012;2012:892182.

23. de Paula LC, Fonseca F, Perazzo F, Cruz FM, Cubero D, Trufelli DC, *et al. Uncaria tomentosa* (cat's claw) improves quality of life in patients with advanced solid tumors. J Altern Complement Med 2015;21:22-30.

24. Sheng Y, Akesson C, Holmgren K, Bryngelsson C, Giamapa V, Pero RW. An active ingredient of Cat's claw water extracts identification and efficacy of quinic acid. J Ethnopharmacol 2005;96:577-84.

25. Mammone T, Akesson C, Gan D, Giampapa V, Pero RW. A water soluble extract from *Uncaria tomentosa* (Cat's Claw) is a potent enhancer of DNA repair in primary organ cultures of human skin. Phytother Res 2006;20:178-83.

26. Emanuel P, Scheinfeld N. A review of DNA repair and possible DNA-repair adjuvants and selected natural anti-oxidants. Dermatol Online J 2007;13:10.

27. Tsuchiya S, Yamabe M, Yamaguchi Y, Kobayashi Y, Konno T, Tada K. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). Int J Cancer 1980;26:171-6.

28. Guo B, Lee H. Cloning and characterization of Chinese hamster CDC7 (ChCDC7). Somat Cell Mol Genet 1999;25:159-71.

29. Lam K, Zhang L, Bewick M, Lafrenie RM. HSG cells differentiated by culture on extracellular matrix involves induction of S-adenosylmethione decarboxylase and ornithine decarboxylase. J Cell Physiol 2005;203:353-61.

30. Charbonneau JR, Gauthier ER. Prolongation of murine hybridoma cell survival in stationary batch culture by Bcl-xL expression. Cytotechnology 2000;34:131-9.

31. De Martino L, Martinot JL, Franceschelli S, Leone A, Pizza C, De Feo V. Proapoptotic effect of *Uncaria tomentosa* extracts. J Ethnopharmacol 2006;107:91-4.

32. Singh M, Singh N. Molecular mechanism of curcumin induced cytotoxicity in human cervical carcinoma cells. Mol Cell Biochem 2009;325:107-19.

33. Pilarski R, Zielinski H, Ciesiolka D, Gulewicz K. Antioxidant activity of ethanolic and aqueous extracts of *Uncaria tomentosa* (Willd.) DC. J Ethnopharmacol 2006;104:18-23.

34. Singh S, Shi Q, Bailey ST, Palczewski MJ, Pardee AB, Iglehart JD, *et al.* Nuclear factor-kappaB activation: A molecular therapeutic target for estrogen receptor-negative and epidermal growth factor receptor family receptor-positive human breast cancer. Mol Cancer Ther 2007;6:1973-82.

35. Sheng Y, Pero RW, Amiri A, Bryngelsson C. Induction of apoptosis and inhibition of proliferation in human tumor cells treated with extracts of *Uncaria tomentosa*. Anticancer Res 1998;18:3363-8.

36. Riva L, Coradini D, Di Fronzo G, De Feo V, De Tommasi N, De Simone F, *et al.* The antiproliferative effects of *Uncaria tomentosa* extracts and fractions on the growth of breast cancer cell line. Anticancer Res 2001;21:2457-61.

37. García Giménez D, García Prado E, Sáenz Rodríguez T, Fernández Arche A, De la Puerta R. Cytotoxic effect of the pentacyclic oxindole alkaloid mitraphylline isolated from *Uncaria tomentosa* bark on human Ewing's sarcoma and breast cancer cell lines. Planta Med 2010;76:133-6.

38. Pilarski R, Filip B, Wietrzyk J, Kuras M, Gulewicz K. Anticancer activity of the *Uncaria tomentosa* (Willd.) DC. Preparations with different oxindole alkaloid composition. Phytomedicine 2010;17:1133-9.

39. Rinner B, Li ZX, Haas H, Siegl V, Sturm S, Stuppner H, *et al.* Antiproliferative and pro-apoptotic effects of *Uncaria tomentosa* in human medullary thyroid carcinoma cells. Anticancer Res 2009;29:4519-28.

40. Chendil D, Ranga RS, Meigooni D, Sathishkumar S, Ahmed MM. Curcumin confers radiosensitizing effect in prostate cancer cell line PC-3. Oncogene 2004;23:1599-607.

41. Mittal A, Papa S, Franzoso G, Sen R. NF-kappaB-dependent regulation of the timing of activation-induced cell death of T lymphocytes. J Immunol 2006;176:2183-9.

42. Takada Y, Ichikawa H, Pataer A, Swisher S, Aggarwal BB. Genetic deletion of PKR abrogates TNF-induced activation of IkappaBalpha kinase, JNK, Akt and cell proliferation but potentiates p44/p42 MAPK and p38 MAPK activation. Oncogene 2007;26:1201-12.

43. Zhang SY, Park KW, Oh S, Cho HJ, Cho HJ, Park JS, *et al.* NF-kappaB decoy potentiates the effects of radiation on vascular smooth muscle cells by enhancing apoptosis. Exp Mol Med 2005;37:18-26.

44. Yacoub A, Mitchell C, Lebedeva IV, Sarkar D, Su ZZ, McKinstry R, *et al.* mda-7 (IL-24) inhibits growth and enhances radiosensitivity of glioma cells *in vitro* via JNK signaling. Cancer Biol Ther 2003;2:347-53.

45. Brantley-Finley C, Lyle CS, Du L, Goodwin ME, Hall T, Szwedo D, *et al.* The JNK,

ERK and p53 pathways play distinct roles in apoptosis mediated by the antitumor agents vinblastine, doxorubicin, and etoposide. Biochem Pharmacol 2003;66:459-69.

46. Inanami O, Takahashi K, Kuwabara M. Attenuation of caspase-3-dependent apoptosis by Trolox post-treatment of x-irradiated MOLT-4 cells. Int J Radiat Biol 1999;75:155-63.

47. Pickering BM, de Mel S, Lee M, Howell M, Habens F, Dallman CL, *et al*. Pharmacological inhibitors of NF-kappaB accelerate apoptosis in chronic lymphocytic leukaemia cells. Oncogene 2007;26:1166-77.

48. Mathé G, Reizenstein P, Eriguchi M. Adjuvant treatment of minimal residual tumors. A comparison of chemotherapy and immunotherapy. Bull Soc Sci Med Grand Duche Luxemb 1989;126:75-92.

49. Bresnahan WA, Boldogh I, Ma T, Albrecht T, Thompson EA. Cyclin E/Cdk2 activity is controlled by different mechanisms in the G0 and G1 phases of the cell cycle. Cell Growth Differ 1996;7:1283-90.

50. Mitra J, Enders GH. Cyclin A/Cdk2 complexes regulate activation of Cdk1 and Cdc25 phosphatases in human cells. Oncogene 2004;23:3361-7.