

Effects of Alpha-hederin and Thymoquinone, constituents of *Nigella sativa*, on Human Cancer Cell Lines

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Abstract. The separate effects of alpha-hederin and thymoquinone, the two principal bioactive constituents of *Nigella sativa*, on four human cancer cell lines [A549 (lung carcinoma), HEP-2 (larynx epidermoid carcinoma), HT-29 (colon adenocarcinoma) and MIA PaCa-2 (pancreas carcinoma)] were investigated. Alpha-hederin was also examined as a pro-drug. Each assessment quantified both cytotoxic and apoptotic/necrotic effects. Alpha-hederin and thymoquinone separately induced a dose- and time-dependent effect on the cell lines tested. HEP-2 cells were the most sensitive, exhibiting apoptosis with a higher incidence following thymoquinone treatment. Pre-treatment of cells with alpha-hederin, followed by thymoquinone or cisplatin, did not enhance the cytotoxicity or apoptosis induced by either drug. So, the membrane-perforating properties associated with saponins, here represented by alpha-hederin, enhance neither cytotoxicity nor apoptosis of these cancer cells.

A diet rich in plant foods may protect against cancer, as vegetables, fruits, whole grains, herbs, nuts and seeds with significant terpenoids, sulphur compounds, pigments and other antioxidants are associated with cancer prevention and treatment (1). *Nigella sativa*, or black cumin, is used extensively in Middle and Far Eastern countries as a spice and food preservative (2), with medicinal effects that include, anti-bacterial, anti-fungal, anti-viral, anti-helminthic, anti-inflammatory, immunomodulatory and anti-cancer (3, 4): the latter effects are attributed to alpha-hederin and thymoquinone (2, 4-8).

In vitro treatment with the monodesmosidic, triterpene saponin alpha-hederin (extracted from *Hedera helix*), inhibits proliferation of mouse B16 melanoma cells and non-cancer mouse 3T3 fibroblasts (7) and also induces apoptosis,

intracellular glutathione depletion and reactive oxygen species generation in P388 murine leukemia cells (8). *In vivo*, alpha-hederin, isolated from black seed (34.1 ppm), inhibits growth of implanted LL/2 (Lewis lung carcinoma) cells in BDF1 mouse tumours (6). Thymoquinone, the main constituent of the volatile oil of the *Nigella sativa* seed (736 ppm) (9), induces *in vitro* cytotoxicity and apoptosis in canine osteosarcoma cells, and apoptosis and cell cycle arrest in skin cancer cells, with little effect on non-cancerous cells (2, 4). *In vivo*, thymoquinone inhibits forestomach and fibrosarcoma tumour incidence and multiplicity in mice (10, 11).

However, the toxicity induced by both drugs is highly variable, with IC₅₀ values for alpha-hederin ranging from 5.4-27.1 µM (12) and for thymoquinone from 10-275 µM (4, 5). Accordingly, further investigation of the cytotoxicity of these compounds is warranted, particularly by quantification of apoptosis. In addition, the use of alpha-hederin as a pro-drug should be considered, as membrane-perforating saponins such as alpha-hederin could increase the access of conventional anti-cancer drugs such as cisplatin to cells exhibiting drug resistance, through decreased membrane transportation. The present study investigates the separate effects of alpha-hederin and thymoquinone as lead compounds against four human cancer cell lines in terms of cytotoxicity and apoptosis/necrosis and also assesses the value of alpha-hederin as a pro-drug,

Materials and Methods

Drugs. Alpha-hederin and thymoquinone stock solutions were made up in 100% DMSO and cisplatin in dH₂O. All chemicals were purchased from Sigma-Aldrich Ireland Ltd., unless otherwise stated.

Human cell culture. A549 (lung carcinoma), HEP-2 (larynx carcinoma), HT-29 (colon adenocarcinoma) and MIA PaCa-2 (pancreas carcinoma) were obtained from the National Cell and Tissue Culture Centre, Dublin City University, Dublin, Ireland, and were maintained as previously described (13).

Cytotoxicity assay. Cellular viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (14) with slight modifications. Briefly, cells in the

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exponential stage of growth, harvested by trypsinisation and centrifugation, were seeded into 96-well plates at 4×10^3 cells/well. After 24 h, alpha-hederin (6-40 μM) and thymoquinone (25-150 μM) were separately added in 8 replicates for 24, 48 and 72 h; cisplatin (5-40 μM) was added in 8 replicates for 24 h. To assess the effect of alpha-hederin as a pro-drug, cells were incubated with a single dose of alpha-hederin for 24 h, gently washed with phosphate-buffered saline (PBS) and further incubated for 24 h with either thymoquinone (5-30 μM) or cisplatin (5-30 μM). Following treatment, the medium was gently aspirated and replaced with 200 ml of fresh medium and 50 μl of MTT (5 mg/ml in PBS), and cells were further incubated for 4 h at 37°C. The solution was then aspirated, and 180 μl of 10% SDS was added to each well, following an overnight incubation to dissolve the formazan crystals. Absorbance was measured in a microplate reader using a filter at 550 nm.

Apoptosis and necrosis quantification. Cells ($0.75 \times 10^5/\text{ml}$) grown on coverslips in 35-mm tissue culture dishes, were separately treated with alpha-hederin, thymoquinone and cisplatin or with alpha-hederin followed by thymoquinone or cisplatin for 24 h. Floating cells, collected by centrifugation, were washed with PBS and included with washed adherent cell populations. Cells were then either: a) fixed in ice-cold methanol: acetic acid (3:1), at 4°C for 5 min, stained for 10 min with Hoechst 33258 (8 $\mu\text{g}/\text{ml}$ in PBS) and analysed for apoptosis by fluorescence microscopy (40x) (15); or b) stained with 0.2% trypan blue and analysed for necrosis by bright field microscopy (40x) (16). At least 400 cells were randomly examined and the percentage of apoptotic or necrotic cells present was determined. The experiments were carried out in duplicate and repeated at least twice.

Statistical analysis. The data are presented as mean \pm S.E.M and were analysed by ANOVA using SPSS followed by Bonferroni *post hoc* analysis for multiple comparisons.

Results

Alpha-hederin inhibits cell proliferation. Alpha-hederin inhibited cellular viability in the four cancer cell lines studied, in a dose- and time-dependent manner as measured by the MTT assay (Table I). The A549 cells were most susceptible with IC_{50} values of $9.6 \mu\text{M} \pm 1.4$ (24 h), $8.2 \mu\text{M} \pm 0.1$ (48 h), $8.3 \mu\text{M} \pm 1.6$ (72 h) and MIA PaCa-2 cells were least susceptible with IC_{50} values of 35.2 ± 6.6 (24 h), $25.5 \mu\text{M} \pm 0.6$ (48 h) and $26 \mu\text{M} \pm 1.2$ (72 h).

Thymoquinone inhibits cell proliferation. Thymoquinone also inhibited cellular viability in the four cell lines in a dose-dependent manner (Table I). HEp-2 cells were most susceptible with IC_{50} values of $34.6 \pm 2.1 \mu\text{M}$ (24 h), $28.5 \pm 2.7 \mu\text{M}$ (48 h) and $22.9 \pm 1.1 \mu\text{M}$ (72 h), and A549 cells were least susceptible with IC_{50} values of $90.7 \text{ mM} \pm 6.6$ (24 h), $72 \mu\text{M} \pm 2.8$ (48 h) and $146 \mu\text{M} \pm 0.4$ (72 h), as assessed by the MTT assay.

Alpha-hederin elicits apoptosis and necrosis. Treatment of cells with alpha-hederin (6-40 μM for 24 h) induced less

than 8% apoptosis in A549, HT-29 and MIA PaCa-2 cells (Figure 1 A, C, D); 16 μM alpha-hederin elicited $13 \pm 0.6\%$ apoptosis and $11.6 \pm 3.4\%$ necrosis in HEp-2 cells (Figures 1B and 2J). Higher concentrations increased necrosis substantially, and more than 90% necrosis was elicited by 30 μM in HT-29 cells, 14 μM in A549 cells and 20 μM in HEp-2 cells, while 40 μM alpha-hederin elicited $22 \pm 4.6\%$ necrosis in MIA PaCa-2 cells.

Thymoquinone elicits apoptosis and necrosis. Thymoquinone (25-100 μM for 24 h) induced less than 3% apoptosis in A549, HT-29 and MIA PaCa-2 cells (Figure 1 E, G, H). In the HEp-2 cell line, 25 μM thymoquinone elicited $21.2 \pm 3.3\%$ apoptosis and $4.4 \pm 0.3\%$ necrosis (Figure 1F); and 50 μM elicited $33 \pm 5.6\%$ apoptosis and $16.5 \pm 7.6\%$ necrosis (Figures 1F and 2K). One hundred μM thymoquinone induced more than 90% necrosis in HT-29, MIA PaCa-2 and HEp-2 cells, and $34.5 \pm 1.5\%$ necrosis in A549 cells (Figure 1 E-H).

Alpha-hederin is not a pro-drug of cisplatin or thymoquinone. Alpha-hederin's potential as a pro-drug was evaluated by incubating cells with alpha-hederin prior to incubation with cisplatin or thymoquinone. Cell viability, apoptosis and necrosis were recorded as before. Cisplatin was cytotoxic to all four cell lines after 24 h in a dose-dependent manner, with an IC_{50} value of $18.4 \pm 2.5 \mu\text{M}$ in HEp-2 cells, the most susceptible cell line (Table I). Treatment of cells with 30 μM cisplatin elicited $63 \pm 0.3\%$ apoptosis in HEp-2 cells (Figures 3 and 2L) and $15.4 \pm 0.6\%$ apoptosis in A549 cells. Less than 6% apoptosis was observed in HT-29 and MIA PaCa-2 cells (Figure 3). Alpha-hederin did not enhance the effect of either cisplatin or thymoquinone on any cell line, as assessed by the MTT assay (data not shown) and by apoptosis quantification (Figure 3). Only in HT-29 cells did necrosis increase by 33% as a result of alpha-hederin (15 μM) pre-treatment followed with cisplatin (30 μM) (Figure 3). Similarly, alpha-hederin pre-treatment followed with thymoquinone elicited no significant increase in apoptosis or necrosis (data not shown).

Discussion

Alpha-hederin and thymoquinone, the principal known anti-cancer constituents of *N. sativa*, affect the viability of four human cancer cell lines, A549, HEp-2, HT-29 and MIA PaCa2, in a dose- and time-dependent manner (through both apoptosis and necrosis). This has not been previously shown using these cell lines.

Alpha-hederin (after 24 h) was most cytotoxic and almost four-fold more efficacious against A549 cells than MIA PaCa-2 cells; the reported value of 7.4 $\mu\text{g}/\text{ml}$ (9.9 μM) in A549 cells (17) is consistent with our present data.

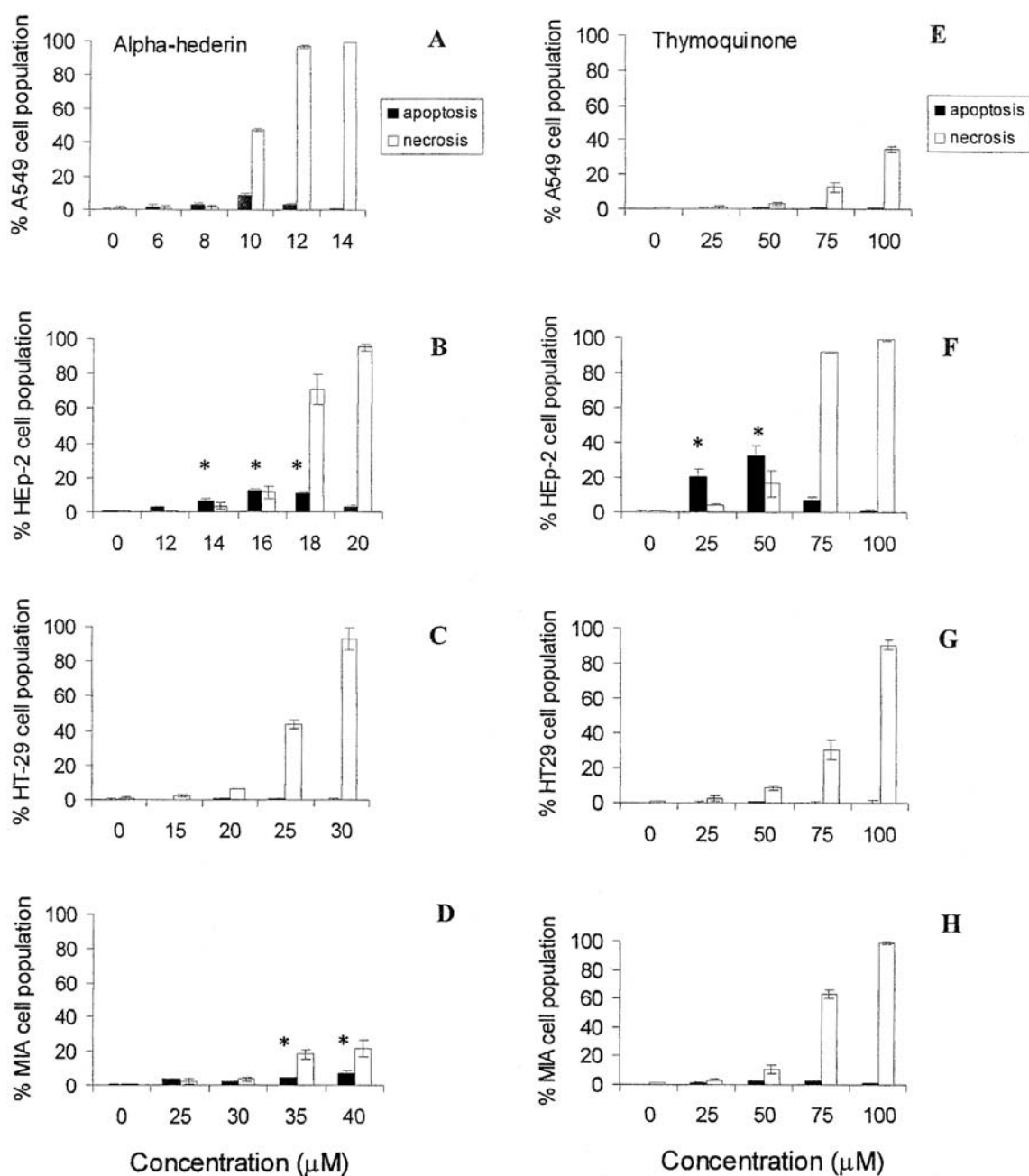


Figure 1. Effect of increasing concentrations (μM) of alpha-hederin (A-D inclusive), and thymoquinone (E-H inclusive) on apoptosis and necrosis of the four cancer cell lines after 24 h. Apoptosis and necrosis were each quantified using Hoechst, and trypan blue staining methods. Values are representative of at least 2 independent experiments ($\pm\text{S.E.M}$). * $p < 0.01$ significant difference from control.

However, the effects on other cell lines clearly differ. Kalopanaxsaponin A, a synonym for alpha-hederin, was cytotoxic and almost four-fold more efficacious after 48 h to HEP-G2 human hepatocellular carcinoma cells at 5.4 μM , than against P388 murine leukaemia cells (12). In contrast, almost equal IC_{50} values were reported for P388 (4.7 $\mu\text{g/ml}$ or 6.3 μM) and HEP-G2 cells (4.8 $\mu\text{g/ml}$ or 6.4 μM) (8).

After 24 h, thymoquinone was most effective at inhibiting HEP-2 cell proliferation, and almost three-fold more effective than against A549 cells. Previously reported IC_{50} values for thymoquinone include: 10 μM and 40 μM against MCF-7 human breast cancer cells and BG1 human ovarian cancer cells respectively after 96 h (4); 35 μM against HCT-116 human colorectal carcinoma cells after 24 h (18); and

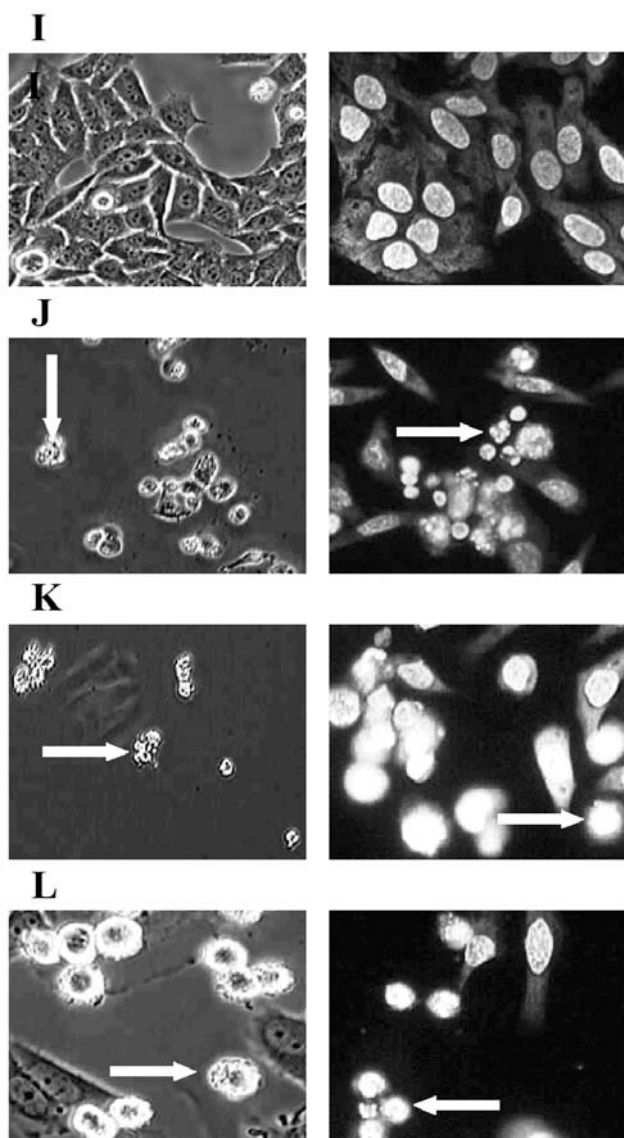


Figure 2. Phase contrast (left) and fluorescent (Hoechst staining) microscopy images of HEp-2 cells (24 h) after the following treatment. I; untreated control cells, J; 16 μ M alpha-hederin, K; 50 μ M thymoquinone, L; 30 μ M cisplatin (apoptotic positive control). White arrows indicate apoptotic cells.

188 μ M and 275 μ M against MES-SA human uterine sarcoma cells and K562 human leukaemia cell lines, respectively, after 72 h (5). Of the treatments tested after 24 h, the most efficacious for alpha-hederin was 9.6 μ M in A549 lung carcinoma cells and 34.6 μ M thymoquinone in HEp-2 larynx carcinoma cells. Comparable *in vitro* IC₅₀ value for other plant constituents against cancer cells are: artemisinin (29.8 μ M) (19); its derivatives artesunate (range 15.4-49.7 μ M) and dihydroartemisinin (range 8.5-32.9 μ M) (20);

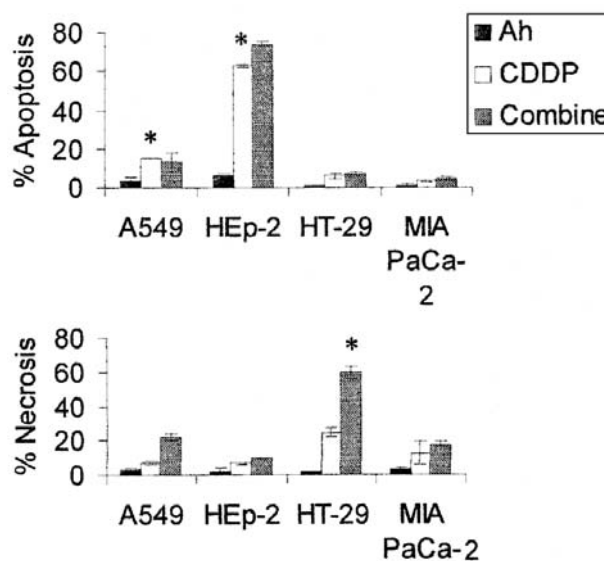


Figure 3. The effects of alpha-hederin only (Ah), 10 μ M in A549 cells, 14 μ M in HEp-2 cells, 15 μ M in HT-29 cells and 30 μ M in MIA PaCa-2 cells); 30 μ M cisplatin only (CDDP), and corresponding alpha-hederin pretreatments followed by 30 μ M cisplatin (combine) on apoptosis and necrosis of the four cell lines. Apoptosis and necrosis were quantified using Hoechst, and trypan blue staining methods as above. Values are representative of at least 2 independent experiments (\pm S.E.M). * $p < 0.01$ significant difference from control /single treatments.

capillin, also from *Artemisia* (range 0.1- 6.1 μ M) (13); and curcumin from turmeric (39.1 μ M) (21).

HEp-2 cells treated with alpha-hederin (14 μ M and 16 μ M) for 24 h exhibited significant apoptosis, including shrinkage and rounding, loss of adherence, nuclear condensation, membrane blebbing and formation of apoptotic bodies. However, necrosis rather than apoptosis was the dominant form of cell death for this and the other three cell lines after treatment, displaying cell swelling, nuclear swelling and cell lysis. In contrast, 8 μ g/ml (10.7 μ M) alpha-hederin has induced up to 73% apoptosis in P388 murine leukaemia cells after 24 h (8).

HEp-2 cells treated with thymoquinone (25 μ M and 50 μ M) induced significantly more apoptosis than when treated with alpha-hederin. Only with 75 μ M thymoquinone or higher was necrosis dominant in HEp-2 cells. However, as with alpha-hederin, the other three cell lines displayed necrosis rather than apoptosis as the principal recorded response. Treatment with 100 μ M thymoquinone for 24 h has induced 13% apoptosis and 43% necrosis in the COS31 canine osteosarcoma cisplatin-resistant cell line (4), whereas 100 μ M for 24 h induced >90% apoptosis and no necrosis in HCT-116 colorectal carcinoma cells (18). Clearly, the principal mode of action varies with the cell line investigated.

Table I. IC_{50}^a values of alpha-hederin (Ah) and thymoquinone (Tq) after 24, 48, 72 h and IC_{50} values of cisplatin (CDDP) after 24 h against four human cancer cell lines.

Cell line	A549			HEp-2			HT-29			MIA PaCa-2		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Ah IC_{50} (μ M)	9.6 \pm 1.4	8.2 \pm 0.1	8.3 \pm 1.6	15.9 \pm 1.9	13.1 \pm 1.9	11.3 \pm 0.4	21.6 \pm 1.4	16.7 \pm 2.2	12.9 \pm 3.5	35.2 \pm 6.6	25.5 \pm 0.6	25.5 \pm 1.2
Tq IC_{50} (μ M)	90.7 \pm 6.6	72 \pm 2.8	146 \pm 0.4	34.6 \pm 2.1	28.5 \pm 2.7	22.9 \pm 1.1	51.6 \pm 3.0	53.3 \pm 1.7	51 \pm 0.7	63.4 \pm 1.4	60 \pm 1.0	67.9 \pm 3.6
CDDP IC_{50} (μ M)	38 \pm 1.3			18.4 \pm 2.5			36.3 \pm 2.5			30.1 \pm 6.1		

^a IC_{50} (μ M) is the amount of drug required to inhibit cell growth by 50% compared to untreated control cells as assessed by the MTT assay. Data are the mean \pm S.E.M of eight replicates of two independent experiments.

After treatment with 30 μ M cisplatin, a clinically relevant dose, HEp-2 and A549 cells were most susceptible, whereas HT-29 and MIA PaCa-2 cells were least susceptible in terms of apoptosis. Triterpene saponins were reported as *in vitro* potentiators of cisplatin cytotoxicity in HT-29 cells (22). Thymoquinone potentiates the anti-tumour activity of cisplatin and ifosfamide *in vivo* and protects against the unwanted side-effects of doxorubicin, ifosfamide and cisplatin in rats (9, 23, 24). However, our data show that alpha-hederin failed to enhance apoptosis by either thymoquinone or cisplatin in any cell line, and actually increased the incidence of necrosis in cisplatin-treated HT-29 cells; this is inconsistent with the clinical use of these combinations, as chemotherapeutic drugs should elicit apoptosis rather than necrosis. Such data highlight the value of combining the assessment of viability by the MTT assay with quantification of apoptotic and necrotic cell death in a range of cell lines, when assessing potential lead compounds.

Overall, thymoquinone is more effective *in vitro* than alpha-hederin against HEp-2 laryngeal cancer cells and the higher incidence of apoptosis suggests it is a better potential lead compound against laryngeal cancer.

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