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Effects of the Polyacetylene Capillin on Human Tumour Cell Lines

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Abstract. *We investigated the effects of capillin, a constituent of Artemisia monosperma, on four human tumour cell lines: colon carcinoma HT29, pancreatic carcinoma MIA PaCa-2, epidermoid carcinoma of the larynx HEp-2 and lung carcinoma A549. Cells were treated with capillin to examine both the anti-proliferative and pro-apoptotic effects, as well as the molecular mechanism underlying these effects. Changes in cell proliferation, membrane permeability, macromolecular synthesis, glutathione (GSH), cell cycle and programmed cell death were evaluated. Capillin (1μM-10μM) inhibited cell proliferation and decreased macromolecular synthesis simultaneously, in a dose- and time-dependent manner. Co-incubation with L-buthionine sulfoximine (L-BSO) augmented the efficacy of capillin. Capillin modulated GSH levels, accumulated cells in the $S+G_2$/M-phase of the cell cycle and induced cell death and DNA fragmentation, as indicated by flow cytometry, fluorescence microscopy and DNA fragmentation assay. These findings suggest that capillin has cytotoxic activity and can induce apoptosis in human tumour cell lines.*

Polyacetylenes, identified as a unique class of natural products, possess diverse biological activities including antimicrobial, anti-apoptotic, H^+,K^+-ATPase inhibitory, HIV inhibitory, antimicrobial, antifungal, anti-inflammatory, immuno-suppressive, cytotoxic and antitumour activities (1-8). The genus *Artemisia* (family Compositae) has, in addition to antimicrobial, antifungal, antihelminthic and insecticidal properties, anti-inflammatory, antispasmodic, antihepatotic and antineoplastic effects (5,9). The polyacetylene capillin (1-phenyl-2,4-pentadiyne), a constituent of various *Artemisia* species including *Artemisia monosperma* (Compositae), destroys insect membranes (10), is antifungal and anti-inflammatory (11). Moreover, capillin prevents liver cell apoptosis induced by transforming growth factor β_1 and may be cytostatic through inhibition of macromolecular synthesis (5).

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The above findings led us to investigate whether the polyacetylene could inhibit cell proliferation and induce apoptosis in tumour cell lines. The present report, the first on capillin's efficacy against human tumour cells, describes effects on tumour cell growth, macromolecular synthesis, cell death and the cell cycle using A549, HEp-2, HT29 and MIA PaCa-2 as a model system. Capillin's interactions with glutathione (GSH) and the enhancement of efficacy by the GSH-depleting agent L-buthionine sulfoximine (L-BSO) are also reported. Taxol was used as a reference drug throughout.

Materials and Methods

Drugs and reagents. Capillin, a gift from Glaxo Wellcome Research and Development (Leydon Road, Stevenage, Hertfordshire, UK), was prepared as a stock solution (1.689mg/ml) in 100% acetone. Radioactive-labelled precursors were obtained from Amersham. Unless otherwise stated, all other reagents were from Sigma-Aldrich Ireland Ltd or Gibco (BioScience Ltd, Charlemont Tce, Dun Laoghaire, Co. Dublin, Ireland).

Human tumour cell lines. Colon carcinoma HT29, pancreatic carcinoma MIA PaCa-2, epidermoid carcinoma of the larynx HEp-2 and lung carcinoma A549 were all obtained from the National Cell and Tissue Culture Centre, Dublin City University, Dublin, Ireland. All cells were maintained at 37˚C in 5% CO_2 as subconfluent monolayers in 75-cm^2 culture flasks. A549 were maintained in nutrient mixture F-12 HAM HEPES modification supplemented with 5% foetal bovine serum (FBS), 2% penicillin-streptomycin and 0.5% L-glutamine; HT29 and HEp-2 were grown in minimal essential medium (MEM) supplemented with 10% FBS and 5% FBS, respectively, 2% penicillin-streptomycin and 1% L-glutamine; MIA PaCa-2 were maintained in Dulbeccos MEM supplemented with 10% FBS, 2.5% horse serum, 2% penicillin-streptomycin and 2% L-glutamine.

Assessment of cellular viability. Cellular viability was assessed using the 3-(4,5-dimethyl thiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay (12). Briefly, cells seeded into 96-well microtitre plates were treated with capillin (1μM, 4μM, 8μM, 10μM) for 24, 48 and 72h in this and all subsequent assays. To demonstrate the effect of L-BSO, cells were exposed to L-BSO (8μM) for 24h prior to stimulation with capillin. Following incubation, the medium was aspirated and replaced with 200 ml cell culture medium and 50 ml MTT (5mg/ml). After

Table I. *IC_{50} value of capillin on human tumour cell lines.*

Cell line	A549			HEp-2			HT29			MIA PaCa		
Time	24h	48h	72h	24h	48h	72h	24h	48h	72h	24h	48h	72h
IC^b_{50}(μM)	6.1±1.2	4.5±0.98	1.6±0.4	2.8±0.3	0.8±0.1	0.6±0.1	6.0±1.5	0.8±0.2	6.0±0.8	3.4±0.91	0.8±0.1	1.4±0.9

Cells were grown for 24h, 48h and 72h in the presence of capillin, prior to MTT[a] assay. [a]MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide. [b]IC_{50} (μM) is the amount of drug required to inhibit cell growth by 50% when compared to capillin-free control. Data are mean±SE of four replicates of three independent experiments.

further culture for 4h at 37˚C, the formazan crystals formed were dissolved by the addition of 200μl DMSO and 25μl Sorensen glycine buffer. The formazan generated was quantified from the absorbance at 550nm (13).

Macromolecular synthesis. DNA, RNA and protein synthesis was measured by the incorporation of [*methyl*-^{3}H]-thymidine (1μCi/ml), [5,6-^{3}H]-uridine (1μCi/ml) and L- [4,5-^{3}H]-leucine (1μCi/ml). Following incubation with capillin, log-phase cells were washed and pulsed with either tritiated thymidine, uridine or leucine for 4h at 37˚C. Cells, washed twice with PBS and treated with 5% trichloracetic acid (TCA) for 5min, were washed with PBS and solubilised with 0.3N sodium hydroxide (NaOH). 6N HCl was added to samples to neutralise pH.

Quantification of intracellular glutathione (GSH). Cells (1 x 10^6), harvested from the experimental growth media, were washed twice in PBS (pH 7.2) and lysed in 0.75ml distilled water. One aliquot served for protein estimation by the Bradford method (1976) (14) and the remainder for GSH analysis. After protein precipitation by 12% sulfosalicyclic acid followed by centrifugation at 12,000 x g for 1min, total glutathione in the supernatant was assayed by the method of Griffith (15). Absorbance was monitored at 412nm on a Beckman UV-VIS-NIR spectrophotometer. The glutathione content was determined by comparing the rate observed to a standard curve generated with known amounts of glutathione.

Quantification of cell membrane damage using 2-deoxy-D-glucose. Cells (0.5 x 10^6cells/dish), grown to subconfluency in 60-mm Petri dishes, were washed in PBS, incubated with tritiated 2-deoxy-D-glucose (1mCi/ml) for 2h at 37˚C, washed with PBS and treated with capillin in the presence of appropriate amounts of D-glucose. Liquid scintillation counting took place, 1, 5, 10, 20 and 30 min after addition of capillin, and three 50μl aliquots from each mixture were taken. After dissolving the cells in 1ml 1M NaOH at room temperature for 30min, cell fractions were transferred to new vials.

Cell cycle analysis. Cells in exponential phase of growth were harvested, fixed overnight in 70% ethanol at 4˚C and incubated with 100μl RNase (1mg/ml) and 100μl propidium iodide (PI; 400μg/ml) for 30min at 37˚C. Cells were analysed on a FACStar^{Plus} flow cytometer with laser excitation at 488nm using a 639nm band pass filter to collect the red PI fluorescence. The percentage of cells in the G_0 (resting phase) /G_1 (early growth phase), S (DNA synthesis) and G_2/M (late growth phase/mitosis) cell cycle phases, respectively, were calculated using the Cell Quest software for Macintosh (Becton Dickinson). Cells less intensively stained than G_0/G_1 cells *i.e.* sub-G_0/G_1, were considered apoptotic (16).

Quantification of programmed cell death using fluorescence microscopy and agarose gel electrophoresis. Cells (0.5x10^6) in exponential phase of growth were seeded into 100-mm Petri dishes and incubated with capillin for 24, 48 and 72h. Harvested cells were labelled with 100μg/ml acridine orange and 100μg/ml ethidium bromide prior to examination under 40x dry objectives by epillumination and filter combination. Live cells were identified by the exclusion of ethidium bromide and the presence of a bright intact nucleus. Apoptotic cells were identified by nuclear chromatin condensation, nuclear fragmentation and the presence of apoptotic bodies stained by acridine orange or ethidium bromide. Uniform labelling with ethidium bromide identified necrotic cells. DNA fragmentation assay was carried out by the method of Mc Gahon (17).

Statistical analysis. Data interdependency required split-plot ANOVA (18) using Genstat 5 for Windows 3.2, 2nd edition (Numerical Algorithms Group (NAG), Oxford, UK). Appropriate means were analysed by the two-tailed *t*-test (19).

Results

Effects of capillin on cell proliferation. Capillin (1μM-10μM) inhibited cell viability in the four human tumour cell lines in a dose- and time-dependent manner as determined by the MTT assay. Capillin was most efficacious against HEp-2 cells with IC_{50} values of 2.8μM±0.3 (24h), 0.8μM±0.1 (48h) and 0.6μM±0.1 (72h) (Table I).

Effects of capillin on macromolecular synthesis. Addition of capillin to cultured cells inhibited DNA, RNA and protein synthesis in a dose- and time-dependent manner. Of all the cell lines tested, capillin at 1, 4, 8 and 10μM maximally decreased [*methyl*-^{3}H]-thymidine incorporation by 96%, 99%, 99% and 99%, respectively, and inhibited [^{3}H]-leucine by 4, 20, 4 and 28%, respectively, and [5,6-^{3}H]-uridine uptake by 9%, 32%, 70% and 76%, respectively, in HT29 at 72h (Figure 1).

Increased cytoxicity elicited by L-BSO. GSH depletion by L-BSO dose-dependently enhanced capillin's cytotoxicity. The

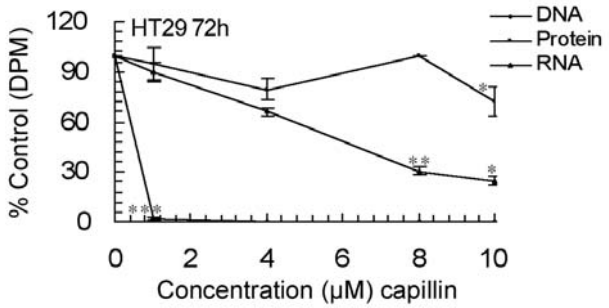


Figure 1. *Effect of capillin on macromolecular synthesis. The incorporation of [methyl-^{3}H]-thymidine, [5,6-^{3}H]-uridine and L-[4,5-^{3}H]-leucine in HT29 cells after 72-h incubation with capillin. Data points for percent control of the DPM of radioactive precursors are the mean±S.E.M. of three triplicates and are representative of three experiments. * p<0.05, ** p<0.01, *** p<0.001; significant difference from control.*

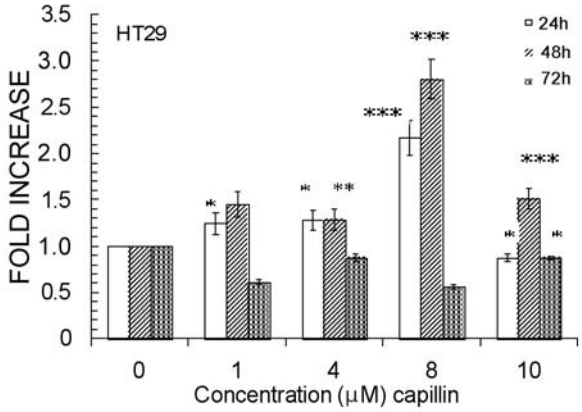


Figure 2. *Effect of increasing concentrations (μM) of capillin on glutathione levels in HT29 cells after exposure to capillin for 24, 48 and 72h. Results are expressed as percentage increase in glutathione levels compared to the untreated cells. Cells were seeded on day 1, exposed to capillin on day 3 and on days 4, 5 and 6, glutathione levels were calculated by the method of Griffith (15). Each point represents the mean of two duplicate experiments±S.E.M. 0μM denotes the control. * p<0.05, ** p<0.01, *** p<0.001; significant difference from control.*

sensitivity enhancement (ratio of IC_{50} without L-BSO to IC_{50} with L-BSO) produced by 8μM L-BSO after 24h was 2.2-fold in A549 and 2.6-fold in HT29 cells (Table II).

Capillin increases glutathione (GSH) levels in tumour cells. Capillin increased glutathione (GSH) levels in all cells lines in a dose-and time-dependent manner. Maximum induction of GSH occurred in HT29 cells following treatment with capillin. Capillin (1,4 and 8μM) elicited a 1.2, 1.3 and 2.2-fold increase in GSH at 24h and a 1.4, 1.3, 2.8-fold increase in GSH at 48h, respectively, in HT29 cells. No further increase in GSH was seen at 72h (Figure 2).

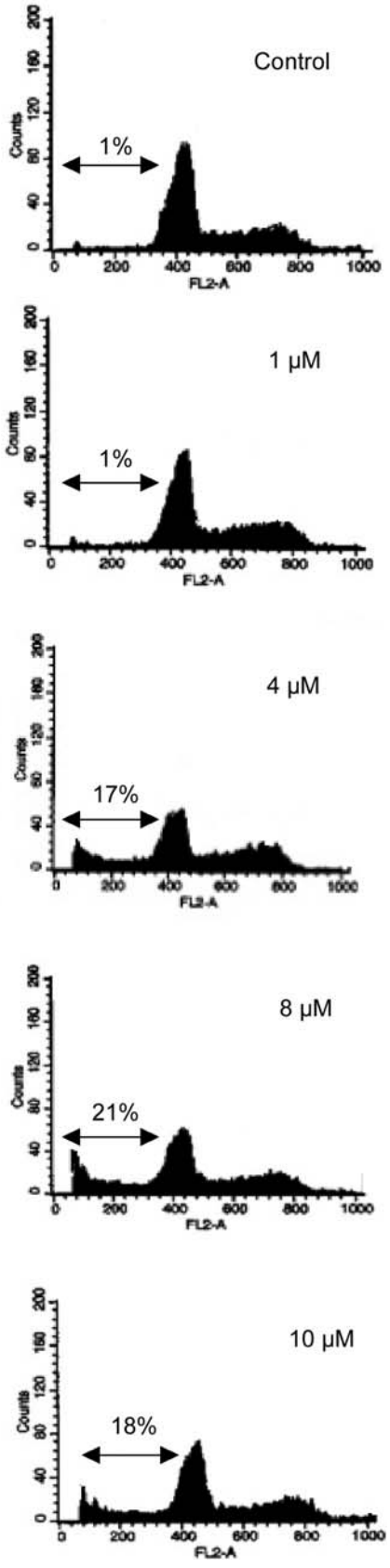
Capillin induces cell death in tumour cells. Some 25% of all treated cells displayed cell shrinkage and loss of cell-to-cell contact after treatment with 1-8μM capillin. By fluorescence microscopy, capillin (1-10μM) induced less than 25% apoptosis in all cell lines at 24, 48 and 72h (data not shown). Apoptotic cell death induced by capillin was additionally confirmed by flow cytometry and agarose gel electrophoresis. Few apoptotic cells occurred in the control (<4%) and capillin elicited a dose - and time-dependent increase in apoptotic cell death. Specifically, capillin (1, 4, 8 and 10 μM) induced 1%, 17%, 21% and 18% apoptosis in HT29 cells at 72h, respectively (Figure 3). Broadly similar increases were observed in MIA PaCa cells also. DNA laddering further confirmed this effect as capillin (4μM and 10μM) induced DNA fragmentation in HT29 cells at 72h (Figure 4).

*Capillin causes accumulation of cells in S+G_2/M-*phase *of the cell cycle.* By flow cytometry, capillin (1, 4, 8, 10 μM) caused a dose - and time-dependent decrease in the percentages of cells in the G_0/G_1-phase (resting) with a concomitant increase in the percentages of cells in the S-phase (DNA synthesis) and G_2/M-phase (mitotic/dividing). In HT29 cells, G_0/G_1-phase decreased from 87% (control) to 72% (1μM) and 51% (4, 8, 10 μM capillin); S-phase increased from 4% (control) to 12,

Table II. *Effects of L-buthionine sulfoximine (L-BSO) on capillin cytotoxicity in human tumour cell lines.*

	A549		HEp-2		HT29		MIA PaCa	
Treatment	IC_{50}	DMF[a]	IC_{50}	DMF	IC_{50}	DMF	IC_{50}	DMF
Control	6.1±1.2	N/Ab	2.8±0.3	N/A	6±1.5	N/A	3.4±0.91	N/A
8μM L-BSO	2.8±0.5	2.2±0.15	3.0±0.5	N/A	2.3±0.8	2.6±0.42	3.5±0.82	N/A

[a] DMF or dose-modifying factor is calculated by dividing the IC_{50} capillin of control cells by that of L-BSO-pretreated cells. All capillin concentrations were in μM. Cells were treated with 8μM L-BSO for 24h prior to treatment with capillin for 24h. Cell viability was assessed by the MTT assay. [b]N/A-not applicable.



14, 19, 20%, respectively, and G_2/M-phase increased from 7% (control) to 11, 16, 15 and 14%, respectively, after treatment with 1, 4, 8 and 10 μM capillin at 24h. Capillin (1, 4, 8, 10 μM) further decreased G_0/G_1-phase from 67% (control) to 44, 35, 27 and 32%, respectively; S-phase increased from 15% (control) to 20, 25, 29 and 25%, respectively, and G_2/M-phase increased from 15% (control) to 22%, 26%, 36% and 36%, respectively, at 48h (Figure 5). Cell cycle perturbations also occurred in A549, MIA PaCa and HEp-2 cell lines.

Cell membrane toxicity as indicated by 2-deoxy-D-glucose. Capillin (1-10μM) did not augment cell membrane permeability in HT29, MIA PaCa-2 and A549 cells.

Discussion

Capillin (1-10μM) was cytotoxic to HEp-2, HT29, MIA PaCa-2 and A549 cells in a dose- and time-dependent manner, with greatest effects on the A549 cell line. Such variable effects are not uncommon. For example, HEp-2 cells are more resistant to the stimulatory or inhibitory effects of cisplatin, etoposide and quercetin whereas A549 cells are more susceptible (20). Capillin also inhibited DNA synthesis with inhibition of protein or RNA synthesis occurring as a secondary effect. These results are in agreement with previous studies, suggesting that capillin (a constituent of the herbal medicine Inchinko-to and *Artemisia capillaris*) may act through inhibition of macromolecular synthesis (5). Decreased cell viability was not accompanied by severe membrane damage.

Capillin increased GSH levels in all cell types studied. This could be due to defensive synthesis of GSH or possibly altered feedback-control of synthesis. Alternatively, the high levels may reflect an inhibition of sulphur-containing gamma-glutamyl peptidase, the main enzyme that degrades GSH. Increased GSH levels have been associated with other therapeutic drugs, including alkylating agents and platinum-containing drugs (21).

Depletion of GSH *via* inhibition of GSH synthesis with L-buthionine sulfoximine (L-BSO), an irreversible inhibitor of γ-glutamylcysteinyl synthetase, increases tumour susceptibility to some chemotherapeutic drugs *i.e.* melphalan (16). Co-incubation of capillin with L-BSO counteracts GSH synthesis thus enhancing efficacy in A549 and HT29 cell lines. Increased cytotoxicity induced by L-BSO in conjunction with

Figure 3. *Effect of capillin on apoptosis of HT29 cells by flow cytometric DNA analysis. HT29 cells treated with capillin (1μM-10μM) for 72h were fixed with 70% EtOH and their DNA was labelled using the fluorochrome propidium iodide. Results represent 10,000 events (cells) from a representative experiment. Control, 1μM capillin, 4μM capillin, 8μM capillin, 10μM capillin.*

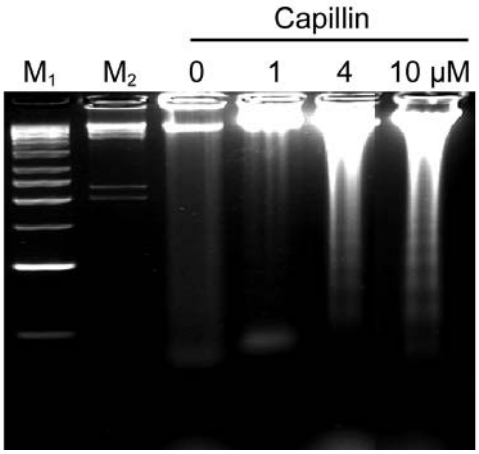


Figure 4. *Effect of capillin on DNA fragmentation in HT29 cells. Following treatment of HT29 with increasing concentrations of capillin for 72h, extracted DNA was separated on a 1.5% agarose gel containing ethidium bromide and visualised under UV light. M_1-Hind lamdba digest marker, M2-1Kb marker.*

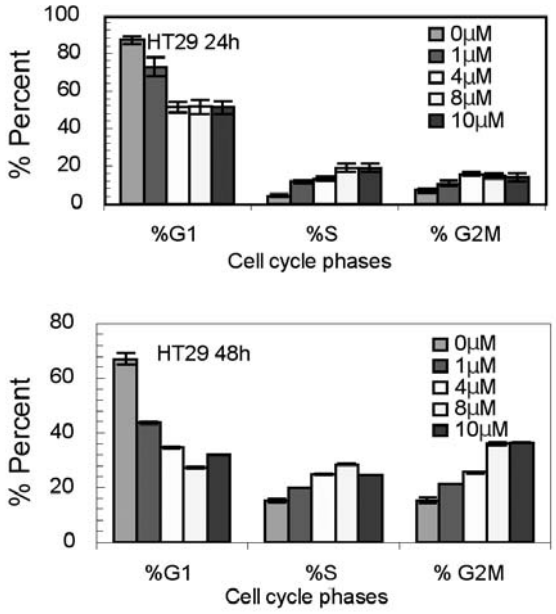


Figure 5. *DNA cell cycle analysis. HT29 cells treated with capillin (1μM-10μM) for 24h and 48h were analysed by flow cytometry. The percentage of cells in the G_0/G_1-, S - and G_2/M-phase was calculated using the Cell Quest software for Macintosh (Becton Dickinson). Results are expressed as mean±S.D. 0μM denotes the control.*

capillin occurs with other chemotherapeutic drugs. L-BSO (10-100μM) increased melphalan cytoxicity in colorectal and ovarian cells (22) and also increased the toxicity of titanocene dichloride in ovarian cells (23). GSH depletion enhances anthracycline toxicity in human ovarian tumour cell lines (24). Thus, the efficacy of capillin might be further improved by limiting glutathione synthesis *via* inhibitors of γ-glutamylcysteine synthetase.

Capillin arrested cells in the S- and G_2/M-phase of the cell cycle, but the former effect was transient in HEp-2, HT29 and MIA PaCa-2 cells. Other antineoplastic agents elicit an S+G_2/M arrest. For example, cisplatin arrests cells in S- and G_2/M-phase of the cell cycle in a concentration - and time-dependent manner. These findings suggest that capillin could induce either an S- or G_2/M-phase arrest depending on the cell type under study.

Morphological examination revealed the presence of apoptotic cell death following treatment with capillin. Cell shrinkage, loss of cell-to-cell contact, membrane blebbing, chromatin condensation and decreased anchorage-independent growth were elicited by increasing capillin concentration and length of exposure. These results were additionally confirmed by fluorescence microscopy and flow cytometry. Loss of substrate adherence is an early event in apoptosis of colonic adenoma cells after treatment with an apoptotic–inducing agent (25). By agarose gel electrophoresis, capillin induced internucleosomal DNA fragments, characteristic of apoptosis in HT29 cells, which were absent in A549, HEp-2 and MIA PaCa-2 cell lines. The absence of DNA laddering may be due to the fact that changes typical of apoptosis are visualised earlier by DNA flow cytometry than those detected by DNA agarose gel electrophoresis (26). Cleavage of DNA into a nucleosomal DNA ladder is not a universal process in programmed cell death as DNA laddering did not occur in epithelial cells that had undergone apoptosis (27). Staurosporine, a protein kinase inhibitor, induces apoptosis in MOLT-4 cells without DNA fragmentation (28).

Capillin can prevent liver cell apoptosis in hepatocytes (5), whereas in our study capillin induced cell cycle arrest, inhibition of macromolecular synthesis and cell death in tumour cells in a dose- and time-dependent manner. Capillin might have a cytoprotective effect in normal cells and induce apoptosis and/or cell cycle arrest in tumour cells. This differential response has been seen with other compounds. For example, epigallocatechin-3-gallate induces apoptosis in cancer cells but not in normal cells (29). The polyacetylenes panaxynol and panaxytriol demonstrate stronger growth inhibitory activity against malignant cells than normal cells with cytotoxicity at high concentrations and cytostatic activity at low concentrations (15). Based on the above findings, we suggest that capillin may contribute to the antineoplastic activity of various *Artemisia* species, but the molecular basis for such effects needs to be further addressed. Although the precise binding sites on the tumour cell and signal transduction still remain unresolved in this study, the anti-proliferative activity provides a basis for further investigation of this polyacetylene.

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