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ORIGINAL ARTICLE

Anticancer activities of sesquiterpene lactones from *Cyathocline purpurea* in vitro

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Abstract
Purpose *Cyathocline purpurea* has been traditionally used to treat various diseases including cancers for many years. However, these applications of *C. purpurea* have not been supported by pharmacological investigation. The objective of this study is to investigate the anticancer activities of three main constituents such as santamarine, 9β-acetoxycostunolide and 9β-acetoxyparthenolide isolated from *C. purpurea* in vitro.
Methods Cell viability was determined by trypan blue exclusion and methylene blue assays. Colony formation was assessed by microtitration cloning assay. DNA synthesis was determined by tritiated thymidine incorporation assay. Cell cycle analysis was carried out by flow cytometry. Apoptosis was observed by DAPI staining assay and Caspase 3/7 activities was measured using Caspase-Glo® 3/7 assay kit.
Results Santamarine, 9β-acetoxycostunolide and 9β-acetoxyparthenolide inhibited the growth of L1210 murine leukaemia, CCRF-CEM human leukaemia, KB human nasopharyngeal carcinoma, LS174T human colon adenocarcinoma and MCF 7 human breast adenocarcinoma cells in vitro, with IC_{50} in the range of 0.16–1.3 μg/mL. In L1210 model, santamarine and 9β-acetoxycostunolide inhibited L1210 cell growth, colony formation and [^{3}H]-thymidine incorporation in time- and concentration-dependent manners. Flow cytometry studies showed that santamarine and 9β-acetoxycostunolide blocked L1210 cells in the G_2/M phase of the cell cycle. DAPI staining and caspase activity assays showed santamarine and 9β-acetoxycostunolide induced apoptosis and activated caspase 3 in L1210 cells.
Conclusions These results indicated that santamarine, 9β-acetoxycostunolide and 9β-acetoxyparthenolide exhibit significant anticancer activities in vitro. The inhibitory effects of santamarine and 9β-acetoxycostunolide on L1210 cells are cytotoxic rather than just cytostatic. They block mitosis and reduce uptake of thymidine. The mechanism of the cytotoxicity of santamarine and 9β-acetoxycostunolide to L1210 cells could be related to alkylation of the sulfhydryl enzymes involved in nucleic acids and protein synthesis, as previously found for other sesquiterpenes with the α-methylene-γ-lactone moiety present in santamarine, 9β-acetoxycostunolide and 9β-acetoxyparthenolide. It may also be related to suppression of microtubular proteins. Santamarine and 9β-acetoxycostunolide induced apoptosis of L1210 cells via activation of caspase 3.

Keywords Sesquiterpene lactone · Cell growth · Cytotoxicity · DNA synthesis · Cell cycle · Apoptosis · Caspase

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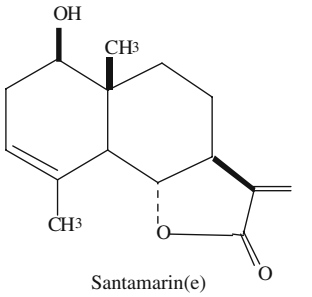
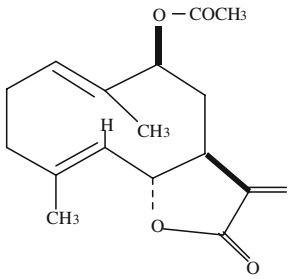
Introduction

Cyathocline purpurea (Compositae), known as "Hong Hao Zhi" in Chinese, is used in traditional Chinese medicine as an herbal remedy for human tuberculosis, malaria, bleeding, rheumatism, swelling and inflammatory diseases [1]. The traditional medicinal practitioners of the Hani ethnic minority in Yunnan, China also commonly use this plant to treat various cancers, according to our visits to the Hani community. Up to now these applications of *C. purpurea* have not been supported by pharmacological investigation. Three sesquiterpene lactones, santamarine, 9β-acetoxycostunolide and 9β-acetoxyparthenolide were isolated from *C. purpurea* by bioactivity-guided fractionation [2]. Their structures are shown in Fig. 1. Sesquiterpene lactones are a class of natural sesquiterpenes which are chemically distinct from other members of the group through the presence of a γ-lactone system, and have a wide range of biological activities including mutagenic, genotoxic, cytotoxic and antitumour actions [3, 4]. Many sesquiterpene lactones have shown significant antineoplastic or cytotoxic effects [5]. Helenalin caused inhibition of growth of Ehrlich ascites cells and produced T/C of 316 for survival of rats with Walker 256 ascites carcinosarcoma [6]. Although various modes of action have been suggested for their biological activities, the mechanism of sesquiterpene lactones as antitumour agents is not established. Previously, it was demonstrated that sesquiterpene lactones or ketones containing an O=C–C=CH_2 moiety had potent antitumour activity [7]. However, some inactive sesquiterpene lactones also have this structural feature. For example, the sesquiterpene arctolide contains an a-methylene-γ-lactone but shows relatively slight cytotoxicity to KB cells [8].

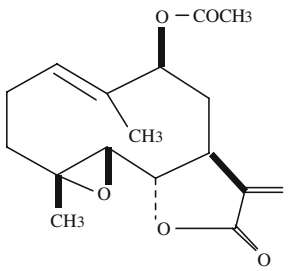
Santamarine and 9β-acetoxycostunolide are sesquiterpene lactones with the α-methylene-γ-lactone moiety in their structure. Previous studies showed that santamarin (e) inhibited the growth of KB human nasopharyngeal cancer cells, P388 murine leukaemia [9], GLC4 human lung carcinoma cells and COLO 320 colorectal cancer cells [10], but no studies on the mode of action have been reported. There have been no reports of cytotoxicity with new compounds of 9β-acetoxycostunolide and 9β-acetoxyparthenolide. We have studied the growth inhibitory activity and possible mechanisms of action of santamarine, 9β-acetoxy-costunolide and 9β-acetoxyparthenolide in vitro against a panel of murine and human tumour cells. Using L1210 cells as a model, inhibition of colony formation, [^{3}H]-thymidine incorporation and cell cycle distribution after exposure to santamarine and 9β-acetoxycostunolide, were investigated. In addition, the mode of cell death induced by santamarine and 9β-acetoxycostunolide was studied.



Santamarin(e)



9β-acetoxycostunolide



9β-acetoxyparthenolide

Fig. 1 The structures of santamarine, 9β-acetoxycostunolide and 9β-acetoxyparthenolide

Materials and methods

Chemicals and reagents

Culture media (RPMI 1640 and DMEM) and supplements were purchased from Multicoil Biosciences, Sydney, NSW, Australia. HEPES buffer (*N*-2-hydroxyethyl-1-piperazine-*N*′-ethane sulfonic acid) and phosphate buffered saline (PBS) were obtained from ICN Pharmaceuticals, Costar, Acton MA, USA. 0.2% trypan blue was purchased from Flow Laboratories, North Ryde, NSW, Australia. Sarkosyl [*N*-lauroylsarcosine (*N*-dodecanoyl-*N*-methylglycine], propidium iodide (PI), 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) and ribonuclease (RNAase A-type 1A) were purchased from Sigma, St Louis, MO, USA. [Methyl-^{3}H]-thymidine was obtained from Amersham Pharmacia Biotech (Amersham, Buckinghamshire, UK). Caspase-Glo® 3/7 assay kit was purchased from Promega Corporation, Australia.

Cell lines and cell culture

All of cell lines in this study were purchased from American Type Culture Collection, Rockville MD, USA. L1210 murine leukaemia cells and CCRF-CEM human leukaemia cells were grown in suspension culture at 37°C in RPMI 1640 medium supplemented with 10% non-dialysed fetal calf serum (FCS), 2 mM L-glutamine and 32 μg/ml gentamycin. LS174T human primary colon adenocarcinoma cells, MCF-7 human breast adenocarcinoma cells and KB human nasopharyngeal carcinoma cells were grown as a monolayer in DMEM with 10% FCS.

Cell growth inhibition assay

L1210 cells were set up at 1×10^5 cells/well in Costar 24-well plates. Cells were allowed to grow undisturbed for 24 h before addition of drugs. After 24 h and 48 h incubation with drugs at 37°C, viable cell counts were made by using the trypan blue exclusion method [11] to assess cell viability. CCRF-CEM cells were set up at 2×10^5 cells/well in Costar 24-well plates, and measurements were made as described above for L1210 cells.

KB, MCF-7 and LS174T cells were plated at a density of 5×10^4 cells/well in a 24-well plate and left for 24 h before addition of drugs. After 72 h incubation with drugs at 37°C, the methylene blue assay was performed as described by Finlay et al. [12]. Briefly, culture supernatant was aspirated with vacuum and cells were stained by the addition of 0.2 mL 0.5% methylene blue to each well. After 30 min at room temperature, plates were inverted briefly to allow most of the stain to drain away. Unbound stain was washed off by immersing plates in water. Plates were air-dried and stored until required for further processing. Stained cells were solubilised by the addition of 0.2 mL of 1% sarkosyl into each well, followed by incubation at 37°C for 5 h. 0.1 mL supernatant from each well of a 24-well plate was transferred into each well of a flat-bottom 96-well plate. UV absorbances were read with a microplate reader at a wavelength of 595 nm. Results were expressed as the relative percentage of absorbance detected in treated cells as compared with untreated control cells.

Microtitration cloning assay

L1210 cells were set up at 5×10^4 cells/mL in 25-cm^2 tissue culture flasks (8 mL/flask). Cells were allowed to grow undisturbed for 24 h before addition of drugs. After 2 h, 6 h and 24 h incubation with drugs, L1210 cells were washed once and resuspended in drug-free medium. Cells were counted and the viable cells diluted to the required cell number. Cloning efficiency was determined by plating doubling dilutions of viable cells ranging from 5 to 0.625/well with 24 wells for each dilution. The plates were incubated in humidified 10% CO_2, 5% O_2 and 70% N_2 atmosphere and the wells were inspected for positive colonies after 8 days. Positive colonies were scored if wells contained 50 or more viable cells for 8 days. The cloning efficiency of the cells was calculated from the proportion of negative wells using Poisson statistic and χ^2 minimisation. Cloning results were expressed as colony forming units/ml which was calculated from the percentage cloning efficiency time's viable cell concentration of cultures at the time of cloning. The cloning efficiency of the control culture of L1210 cells was 36.3% in this experiment.

Tritiated thymidine incorporation

L1210 cells were seeded at 5×10^4 cell/mL in 24-well plate (2 mL/well). After 24 h incubation, the cells were treated with the drug (0.1–10 μg/mL) and transferred into 96 well tissue culture plates (200μL/well). The cells were incubated for 2, 6 and 24 h with drugs. During the last 1 h, 1 μCi of [methyl-^{3}H]-thymidine was added into each well. The cells were harvested using an automated multiwell harvester (Skatron, Norway) that aspirated and lysed the cells and transferred the DNA to a glass fibre mat, while allowing unincorporated [^{3}H]-thymidine to be washed away with water. The mat was allowed to dry in an oven at 60–70°C for 1–2 h and put into a plastic bag. Ten millilitre of scintillation cocktail was poured into the plastic bag and then the bag was sealed with heat. The radioactivity was countered on β-plate liquid scintillation counter (LKB Wallac, Norway). Measurement values were determined for each drug concentration performed at least 9 times in three independent experiments and no. of counts of beta emissions per minute plotted as a function of drug concentration.

Cell cycle analysis by flow cytometry

L1210 cells were exposed to the drugs for 2–48 h. Approximately 2×10^5 cells were collected and centrifuged at 800 rpm for 5 min. DNA staining was performed by an addition of 500 μl propidium iodide (200 μg/mL in Triton X-100, 0.2% v/v saline, final concentration: 100 μg/mL) and 50 μL RNAase (final concentration: 40 μg/mL) to 2×10^5 cells. Cellular DNA content was measured by a Facscan flow cytometer. Approximately 10^4 cells were analysed for each DNA content histogram. The samples were excited at 380–410 nm and the resulting fluorescence measured at wavelengths >550 nm. Analysis of the percentage of cells in G_1, S and G_2/M phases of the cell cycle was made by the Cellquist computer program (Dickinson, CA, USA).

DAPI staining

After treatment of cells with drug for 48 h, the cells were harvested by centrifugation and washed three times with PBS, fixed in a solution of 3.7% formaldehydrate for 10 min, and once in 1 ml of methanol. Fixed cells were stained with 4 μg/ml DAPI for 10 min. The nuclear morphology of cells was observed by fluorescence microscopy.

Measurement of caspase activity

Caspase activity was detected by using Caspase-Glo® 3/7 assay kit (Promega Corporation, Australia). Briefly, L1210 cells (2×10^4cells/well) was seeded in a luminometer plate and incubated for 24 h at 37°C. The time course of caspase activation was evaluated in an initial set of experiments. L1210 cells were treated with 0.1% DMSO (control vehicle) or with santamarine, 9β-acetoxycostunolide for 2, 6, 12, 24 and 48 h. Fifty microlitre of caspase 3/7 reagents were added to each well and incubated for 1 h on rotary shaker at room temperature. Luminescence for each well was recorded.

Results

Effects of santamarine, 9β-acetoxycostunolide and 9β-acetoxyparthenolide on growth of a panel of murine and human tumour cell lines

The growth inhibitory activities of santamarine, 9β-acetoxycostunolide and 9β-acetoxy-parthenolide against a panel of murine and human tumour cell lines were evaluated after 48 h exposure for L1210 and CCRF-CEM cells, and after 72 h exposure for KB, MCF-7 and LS174T cells in vitro. Santamarine, 9β-acetoxycostunolide and 9β-acetoxy-parthenolide inhibited significantly the growth of L1210, CCRF-CEM, KB, MCF-7 and LS174T cells. The inhibitory effects were concentration-dependent. The estimated IC_{50} values are given in Table 1. There were significant differences in the sensitivity of the above five cell lines to the three compounds. KB cells were the most sensitive (IC_{50} 0.16–0.29 μg/mL), followed by CCRF-CEM (IC_{50} 0.41–0.65 μg/mL), and LS174T cells were the least sensitive (IC_{50} 0.92–1.28 μg/mL). The order of sensitivity of the various tumour cells to santamarine was KB > L1210 > MCF-7 > CCRF-CEM > LS174T; the order of sensitivity to 9β-acetoxycostunolide was KB > CCRF-CEM > MCF-7 > L1210 > LS174T and the order of sensitivity to 9β-acetoxyparthenolide was KB > CCRF-CEM ≥ MCF-7 > L1210 > LS174T.

To be considered to have significant cytotoxic activity, a pure compound should have an $IC_{50} \leq 4.0$ μg/mL [13]. On this criterion, santamarine, 9β-acetoxycostunolide and 9β-acetoxyparthenolide are active against the above five cell lines, and thus worthy of further study to determine the characteristics of cell growth inhibition and possible mechanisms of action.

The effects of santamarine and 9β-acetoxycostunolide were further studied to explore their mode of action on L1210 murine leukaemia cells. Although 9β-acetoxyparthenolide exhibited potent growth inhibition of the above cell lines, the amount of 9β-acetoxyparthenolide isolated was too limited to be used for further studies.

The time-dependent effects of santamarine and 9β-acetoxyconstunolide on the growth of L1210 cells

The time course of growth inhibition by santamarine and 9β-acetoxycostunolide was investigated using L1210 murine leukaemia cells. The cells were treated with santamarine and 9β-acetoxycostunolide for 24 and 48 h, and viable cell numbers determined by the trypan blue dye exclusion test. L1210 cell growth was suppressed after 24 h treatment with santamarine and 9β-acetoxyconstunolide, but the inhibitory effect increased after 48 h treatment (Fig. 2a, b). A time- and concentration-dependent effect was evident. At the higher concentrations (3, 10 μg/mL), most cells were dead after 48 h treatment.

Table 1 Inhibitory effects of compounds santamarine, 9β-acetoxycostunolide and 9β-acetoxyparthenolide on the growth of a panel of murine and human tumour cell lines in vitro (48 h drug exposure for L1210 and CCRF-CEM, and 72 h drug exposure for KB, MCF-7 and LS174T cells)

Cell line	Estimated IC_{50} (μg/mL)		
	Santamarine	9β-Acetoxycostunolide	9β-Acetoxyparthenolide
L1210	0.41 ± 0.03	0.89 ± 0.06	0.59 ± 0.05
CCRF-CEM	0.59 ± 0.12	0.41 ± 0.03	0.49 ± 0.07
KB	0.16 ± 0.03	0.25 ± 0.02	0.29 ± 0.01
LS174T	0.92 ± 0.01	1.28 ± 0.05	1.08 ± 0.09
MCF-7	0.53 ± 0.10	0.63 ± 0.0.7	0.50 ± 0.03

Experiments were carried out in triplicate

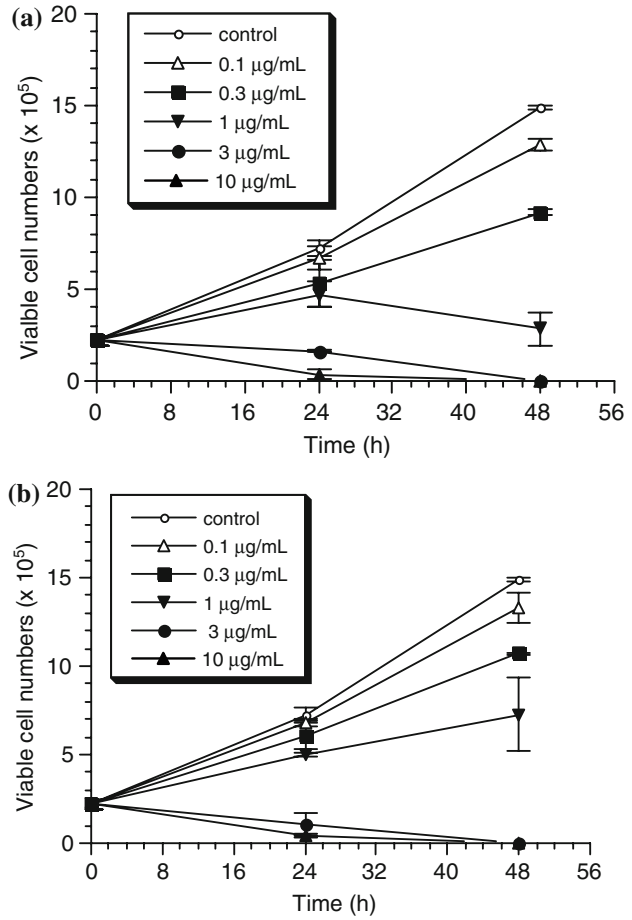


Fig. 2 Time- and concentration-dependent effects of santamarine (**a**) and 9β-acetoxycostunolide (**b**) on L1210 cell growth. Cells were treated with santamarine or 9β-acetoxycostunolide for 24 and 48 h and then cell numbers were determined by the trypan blue exclusion test. Results shown are mean ± SD (*bars*) of triplicate experiments

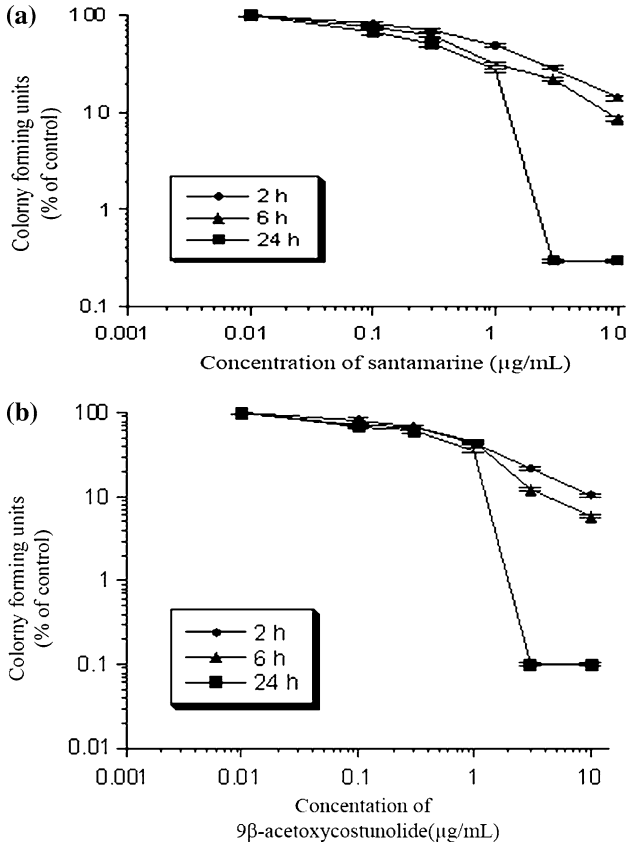


Fig. 3 Effects of santamarine (**a**) and 9β-acetoxycostunolide (**b**) on colony formation of L1210 cells, after exposure for 2, 6 and 24 h, as determined by the microtitration cloning assay. Results are mean ± SD (*bars*) from triplicate experiments

Effects of santamarine and 9β-acetoxycostunolide on colony formation of L1210 cells

To determine if santamarine and 9β-acetoxycostunolide are cytotoxic rather than only cytostatic, colony formation assays were performed. The effects on clonogenicity of L1210 cells of exposure to santamarine and 9β-acetoxycostunolide for 2, 6 and 24 h were studied. The results are shown in Fig. 3a, b. Santamarine and 9β-acetoxycostunolide inhibited colony formation in a concentration- and time-dependent manner. At 2 h exposure to santamarine or 9β-acetoxycostunolide, colony formation units decreased gradually with increasing concentration of santamarine or 9β-acetoxycostunolide. After 24 h exposure, the cytotoxicity was increased significantly.

Effects of santamarine and 9β-acetoxycostunolide on [^{3}H]-thymidine incorporation in L1210 cells

The effects of santamarine and 9β-acetoxycostunolide treatment on DNA synthesis in L1210 cells were measured by the incorporation of [^{3}H]-thymidine. Both santamarine and 9β-acetoxycostunolide significantly inhibited the incorporation of [^{3}H]-thymidine into DNA of L1210 cells in a concentration- and time-dependent manner (Fig. 4a, b). After 2 h incubation with santamarine or 9β-acetoxycostunolide, [^{3}H]-thymidine uptake into L1210 cells decreased and the inhibitory effects increased with extending of exposure time. The maximum observed inhibition was reached at 24 h exposure.

Effects of santamarine and 9β-acetoxycostunolide over time on the cell cycle distribution of L1210 cells

The effects of santamarine and 9β-acetoxycostunolide treatment on the cell cycle were studied by DNA flow

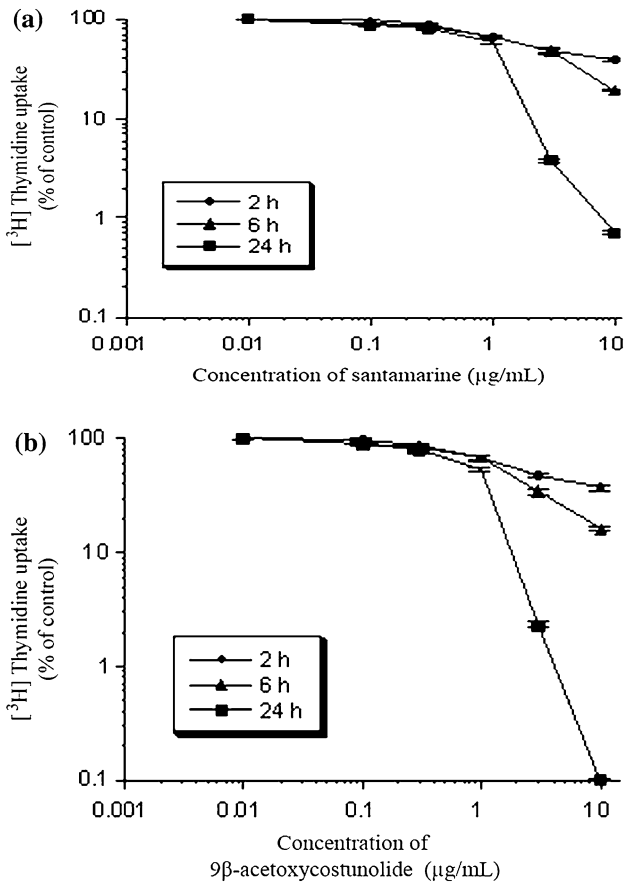


Fig. 4 Effect of santamarine (**a**) and 9β-acetoxycostunolide (**b**) on [^{3}H]-thymidine uptake in L1210 cells after exposure for 2, 6 and 24 h. [^{3}H]-Thymidine was added during the last 1 h of incubation. Results are mean ± SD (*bars*) from triplicate experiments

cytometry. L1210 cells were collected after 2, 6, 24 and 48 h treatment with various concentrations of santamarine or 9β-acetoxycostunolide and analysed by DNA flow cytometry. As shown in Table 2 and 3, santamarine and 9β-acetoxycostunolide blocked L1210 cells in the G_2/M phase of the cell cycle in a concentration- and time-dependent way. After 2 h of santamarine treatment, there was a significant increase of cells in G_2/M phase, with a corresponding decrease in G_1 phase. The number of G_2/M cells reached a maximum at 6 h. These results suggest that cells in the G_2 or M phase were unable to proceed through mitosis to the next G_1 phase, accumulating instead in the G_2/M compartment. The proportion of cells in the S phase was comparatively unchanged by santamarine treatment. After 24 h exposure, there was a significant increase occurred in the proportion of hypodiploid cells (peak prior to G_1 phase) and the appearance of polyploid cells (after G_2/M phase) at the higher concentrations (3, 10 μg/mL of santamarine), while the proportion of cells at G_2/M decreased relatively. By 48 h, most of the cells had reached G_2/M and died. G_1, S, G_2/M phase cells decreased at the higher drug concentrations. The hypodiploid cells are likely dead cells with degraded and/or apoptotic DNA. The polyploid DNA content reflects cells which have not separated at mitosis and hence contain greater DNA content. Figure 5 shows flow cytometry histograms of DNA distribution of untreated control L1210 cells, and of cells exposed to 3 μg/mL of santamarine or 9β-acetoxycostunolide for 2 h. The percentage of cells in G_2/M increased and the percentage of cells in G_0/G_1 phase decreased at 6 h.

Induction of apoptosis

To further examine the mechanism of cell death and to determine whether fragmentation of DNA into nucleosome-sized pieces occurred following santamarine and 9β acetoxycostunolide treatment. DAPI staining was performed. L1210 cells were treated with vehicle or with

Table 2 Effect of santamarine on the cell cycle distribution of L1210 cells among cell cycle phases after 2, 6, 24 and 48 h drug exposure

Incubation time (h)	Concentration (μg/mL)	Percentage of cell cycle distribution (%)				
		HD	G_0/G_1	S	G_2/M	PP
0	0.0	0.7 ± 0.1	39.1 ± 1.3	30.2 ± 1.2	26.0 ± 1.5	4.2 ± 1.1
2	1.0	1.5 ± 0.5	37.2 ± 1.3	27.6 ± 3.4	30.9 ± 1.6	4.3 ± 1.3
	3.0	1.0 ± 0.3	31.0 ± 1.0	28.3 ± 0.6	35.3 ± 2.6	4.9 ± 1.6
	10.0	1.0 ± 0.4	32.2 ± 3.2	27.1 ± 3.7	35.6 ± 2.3	6.5 ± 1. 3
6	1.0	1.1 ± 0.4	39.2 ± 3.1	24.9 ± 4.5	30.3 ± 3.0	4.5 ± 3.1
	3.0	3.1 ± 2.1	23.0 ± 6.2	24.9 ± 5.9	43.7 ± 5.8	5.3 ± 1.3
	10.0	1.4 ± 0.7	22.6 ± 1.3	20.0 ± 5.6	49.9 ± 2.4	5.5 ± 4.7
24	1.0	0.9 ± 0.4	31.2 ± 1.7	27.4 ± 2.1	31.4 ± 2.0	9.3 ± 2.1
	3.0	19.7 ± 7.2	23.1 ± 5.2	20.5 ± 5.6	23.4 ± 6.8	13.5 ± 1.7
	10.0	21.0 ± 3.9	19.3 ± 6.3	25.7 ± 1.4	21.0 ± 8.4	13.0 ± 5.3
48	1.0	2.3 ± 0.1	38.4 ± 1.7	22.9 ± 0.5	25.6 ± 2.4	4.9 ± 1.9
	3.0	31.0 ± 6.3	16.9 ± 4.5	11.8 ± 3.7	21.6 ± 7.3	15.7 ± 0.7
	10.0	44.0 ± 8.6	22.4 ± 4.6	15.3 ± 0.1	16.7 ± 3.8	16.7 ± 3.8

HD hypodiploids, *PP* polyploids
Data are mean ± SD from triplicate experiments

Table 3 Effect of 9β-acetoxycostunolide on the cell cycle distribution of L1210 cells among cell cycle phases after 2, 6, 24 and 48 h drug exposure

Incubation time (h)	Concentration (μg/mL)	Percentage of cell cycle distribution (%)				
		HD	G_0/G_1	S	G_2/M	PP
0	0.0	0.7 ± 0.1	39.1 ± 1.3	30.2 ± 1.2	26.0 ± 1.5	4.2 ± 1.1
2	1.0	1.5 ± 0.6	36.9 ± 3.4	30.6 ± 1.8	27.4 ± 0.9	3.6 ± 0.9
	3.0	1.8 ± 0.7	30.6 ± 2.5	27.4 ± 3.8	35.3 ± 4.8	4.9 ± 0.7
	10.0	2.0 ± 0.9	26.7 ± 2.7	27.6 ± 2.5	38.9 ± 2.3	3.9 ± 2.1
6	1.0	1.2 ± 0.8	40.9 ± 1.4	18.8 ± 2.6	35.8 ± 1.5	3.3 ± 1.2
	3.0	2.1 ± 0.5	25.2 ± 4.6	26.1 ± 2.9	45.6 ± 3.2	3.0 ± 0.5
	10.0	2.8 ± 2.4	21.9 ± 5.8	26.3 ± 4.1	48.3 ± 4.1	4.7 ± 1.5
24	1.0	0.6 ± 0.2	33.2 ± 2.3	24.3 ± 4.3	33.9 ± 1.8	11.0 ± 0.3
	3.0	13.6 ± 6.5	16.9 ± 2.7	19.5 ± 9.7	28.0 ± 3.0	22.0 ± 3.6
	10.0	18.1 ± 7.8	19.9 ± 9.8	22.2 ± 1.8	25.2 ± 3.4	15.0 ± 4.3
48	1.0	2.2 ± 0.1	30.2 ± 1.3	24.4 ± 0.2	21.5 ± 0.9	21.1 ± 1.9
	3.0	19.8 ± 4.7	22.7 ± 7.5	13.7 ± 2.4	25.2 ± 0.9	19.1 ± 0.7
	10.0	66.5 ± 0.0	10.3 ± 4.7	9.6 ± 2.3	6.1 ± 0.5	7.6 ± 7.2

HD hypodiploids, *PP* polyploids

Data are mean ± SD from triplicate experiments

3 μg/ml of santamarine or 9β acetoxycostunolide for 48 h. The results are shown in Fig. 6. The cells treated with santamarine or 9β acetoxycostunolide display condensed and fragmented nuclei, typical of apoptosis in L1210 cells.

Activation of caspase activity

To investigate whether caspase 3 is activated in L1210 cells treated with santamarine or 9β acetoxycostunolide, caspase activity was measured using Caspase-Glo® 3/7 assay kit. Figure 7 showed that santamarine or 9β acetoxycostunolide induced a time-dependent increase in caspase 3 activities in L1210 cells. Caspase 3/7 were activated at 6 h after 3 μg/mL of santamarine or 9β acetoxycostunolide treatment. Maximum activity was seen at 24 h and caspase 3/7 activity gradually deceased at 48 h.

Discussion

In this study, the inhibition of cell growth by santamarine, 9β-acetoxycostunolide and 9β-acetoxyparthenolide treatment were examined in five cultured cell lines, i.e. L1210 murine leukaemia, CCRF-CEM human leukaemia, KB human nasopharyngeal cancer, LS174T human colon adenocarcinoma and MCF-7 human breast cancer. Santamarine, 9β-acetoxycostunolide and 9β-acetoxyparthenolide all inhibited significantly the growth of the five cell lines in a concentration-dependent manner. KB cells were to be more sensitive than the other cell lines, suggesting that these compounds may be active more against human nasopharyngeal carcinoma. These results on growth inhibition are in accordance with the postulates and findings dating back to the late 60s that the cytotoxic activity of many sesquiterpene lactones is dependent on the presence of an α-methylene-γ-lactone moiety [14–16].

It was observed that germacranolides were generally more cytotoxic than eudesmanolides to a small lung carcinoma cell line [17], and than guaianolides to KB and HeLa cells [18]. However, for the five cell lines used in this study the IC_{50} values of 9β-acetoxycostunolide and 9β-acetoxyparthenolide, which have a germacranolide, are not lower than those of santamarine, which is a eudesmanolide (Table 1).

Santamarine and 9β-acetoxycostunolide were further studied to examine their mode of action in L1210 cells. First, the effects of santamarine and 9β-acetoxycostunolide on cell growth and colony formation of L1210 cells were studied. Cell growth inhibition test reveals only the changes in viability of cells toward exposure to drugs. Clonogenic assay demonstrates the metabolic or proliferative capacity of cells after removal of drugs, i.e. long-term reproductive capacity. It reflects the cytotoxic activity of drugs to cancer cells. It was found that santamarine and 9β-acetoxycostunolide significantly inhibited the growth and colony formation of L1210 cells in a time- and concentration-dependent manner. This suggests that the growth inhibition is cytotoxic rather than just cytostatic.

Usually cytotoxic agents exert their biological effects by influencing cell metabolism, i.e. inhibiting DNA, RNA or protein synthesis. The results in Fig. 4 showed that santamarine and 9β-acetoxycostunolide decreased [^{3}H]-thymidine incorporation into L1210 cells in a time- and concentration-dependent manner raising the proposal to that santamarine and 9β-acetoxycostunolide could inhibit DNA synthesis. However, flow cytometry (Fig. 5) showed that santamarine and 9β-acetoxycostunolide blocked L1210 cells in the G_2/M phase, suggesting that the inhibition of

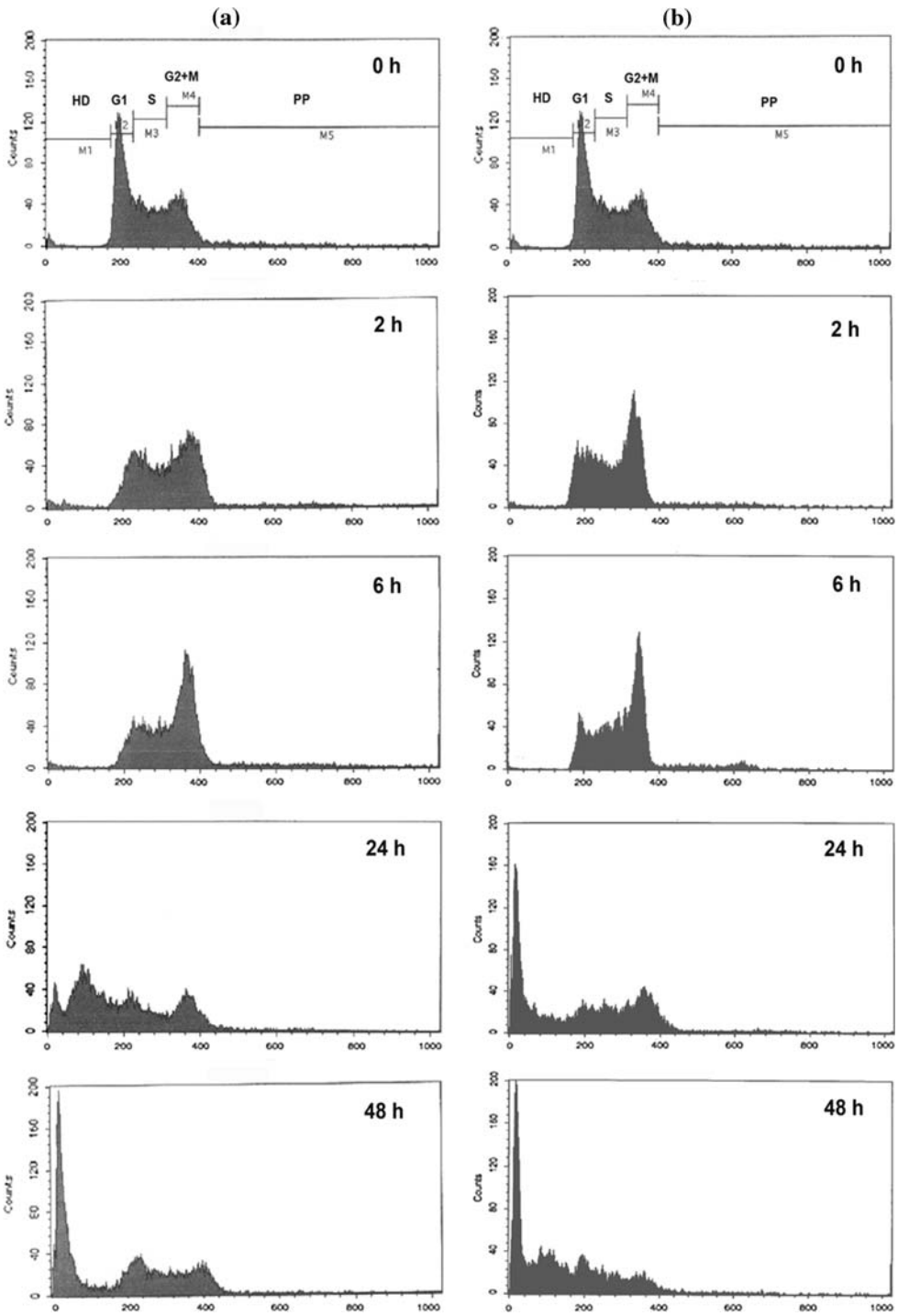


Fig. 5 Flow cytometry histograms of cell cycle distribution of L1210 cells of untreated control and of santamarine (**a**) or 9β-acetoxycostunolide (**b**) treated group at 3 μg/mL. Progressive reduction of cells in G_0/G_1 phase occurred with a concomitant increase of cells arrested in the G_2/M phase and which were unable to proceed through mitosis to next the G_1 phase, instead accumulating in the G_2/M compartment. The proportion of cells in the S phase was comparatively unchanged by santamarine treatment

growth and colony formation by both compounds may be related to effects on cell mitosis. This result is consistent with our recent result [19].

It was shown that (although not exclusively) the O=C–C=CH_2 structure, whether it involves a lactone or ketone, is cytotoxic [8, 20]. Studies cited above hypothesised that the inhibition of tumour growth by highly electrophilic cytotoxic agents may be attributable to selective alkylation of thiol groups in key enzymes which control cell division [21]. In a related study, Lee and coworkers found that the sesquiterpene tenulin, which has a cyclopentenone but not an α-methylene-γ-lactone moiety, also underwent Michael-type addition reaction with the thiol groups of key regulatory enzymes of nucleic acid and chromatin metabolism [22]. In apparently the only previous study involving L1210 cells, various sesquiterpene lactones inhibited thiol-bearing enzymes of nucleic acid metabolism, e.g. α-DNA polymerase, IMP dehydrogenase and ribonucleoside reductase.

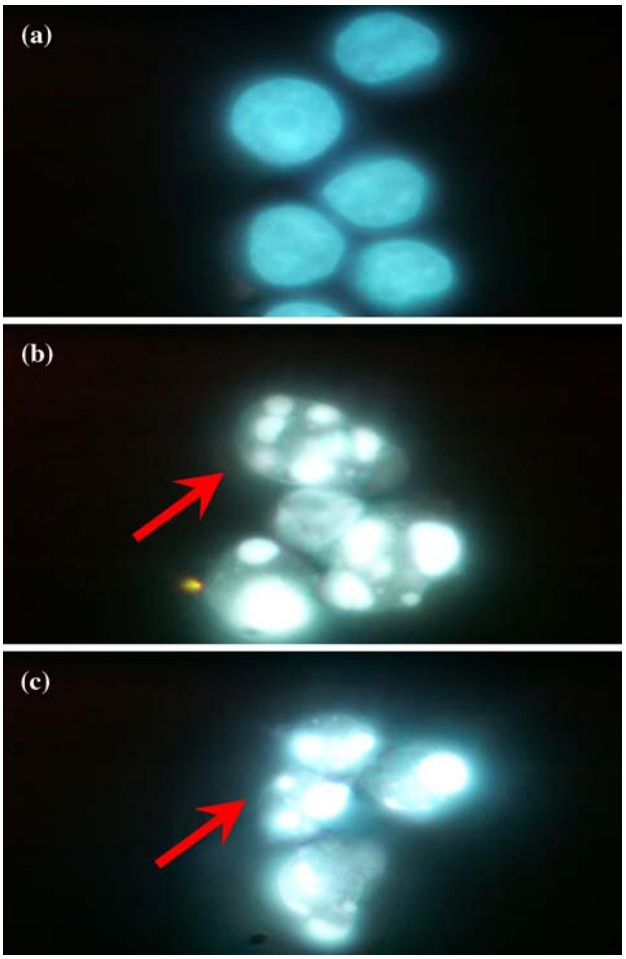


Fig. 6 Induction of apoptosis by santamarine and 9β-acetoxycostunolide in L1210 cells. The cells were treated with vehicle (**a**) or with 3 μg/mL of santamarine (**b**) or 9β-acetoxycostunolide (**c**) for 48 h. Cells were harvested and washed with ice-cold PBS, followed by fixation in 3.7% formaldehyde and methanol. Fixed cells were incubated with 4 μg/ml of DAPI. Nuclear morphology was examined by using a fluorescent microscope. *Arrow* indicates apoptotic cells with condensed and fragmented nuclei

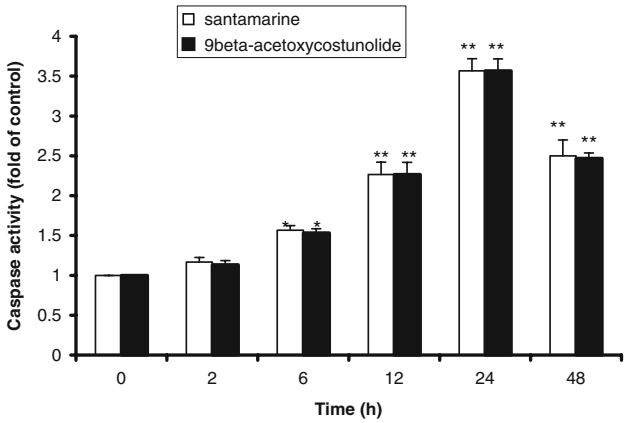


Fig. 7 Activation of casapase 3 in santamarine or 9β-acetoxycostunolide-treated L1210 cells. L1210 cells were treated with or without santamarine or 9β-acetoxycostunolide (3 μg/mL) for 2, 6, 12, 24 and 48 h. Enzymatic activity of caspase 3 were determined by adding 50 μl of caspase reagents. The activity of caspase 3 is expressed as the value relative to untreated cells. The results shown were mean ± SD (*bars*) of triplicate experiments. * $P < 0.05$; ** $P < 0.01$

The existence of more than one mode of action was considered [23].

Figure 4a, b show that santamarine or 9β-acetoxycostunolide at concentrations of more than 1 μg/mL, inhibited thymidine uptake but the maximum effect was not observed until after 6 h. This may suggest that the reduction in DNA synthesis is an indirect process. On the other hand, inhibition of mitosis was more rapid (Fig. 4) build-up of G_2/M phase occurred as early as after 2 h exposure. It is known that proteins involved in microtubule assembly contain thiol (sulfhydryl) groups. One might perhaps that santamarine and 9β-acetoxycostunolide alkylate exposed thiol groups of microtubular proteins of the mitotic apparatus, preventing microtubule assembly or disassembly, leading to blockage of mitosis. The observed reduction in [^{3}H]-thymidine uptake may reflect partly the resultant decrease in surviving cells. Further study is needed to confirm or disprove the above possibility.

There are two known mechanisms of cell death: necrosis and apoptosis. Necrosis is usually considered to result from physical injury. Apoptosis is a deliberate and genetically controlled cellular response to developmental and environmental stimuli, it is characterized by cell shrink and chromatin condenses, followed by fragmentation of nuclear components [24]. Induction of apoptosis in cancer cells or malignant tissues could be an efficient strategy for cancer chemotherapy. In this study, apoptosis was detected within 48 h of drug treatment by DAPI staining assay at cytotoxic concentrations. Santamarine and 9β-acetoxycostunolide induced extensive nuclear condensation and DNA fragmentation. The results of studies by flow cytometry show that there was much hypodiploid debris, perhaps from apoptotic cells. These investigations indicate that the mechanism of santamarine and 9β-acetoxycostunolide might be related to induction of apoptosis.

Caspase 3 is the most prevalent caspase within cells and it is responsible for the majority of apoptotic effects [25]. The activation of caspase 3 induced PARP cleavage, chromosomal DNA break and finally the occurrence of apoptosis [26]. In an attempt to identify the pathway of apoptosis in L1210 cells in response to santamarine or 9β-acetoxycostunolide, caspase 3/7 activation was investigated. The results from Fig. 7 show that santamarine or 9β-acetoxycostunolide could induce caspase 3 activation. The result suggests that caspase 3 might have a critical role in the apoptosis induced by santamarine or 9β-acetoxycostunolide. As shown in this study, santamarine or 9β-acetoxycostunolide exerts apoptotic effects by acting on caspase 3.

Santamarine or 9β-acetoxycostunolide initiates activation of caspases and in turn the caspase activation leads to apoptotic cell death.

In summary, santamarine, 9β-acetoxycostunolide and 9β-acetoxyparthenolide inhibited the growth of L1210, CCRF-CEM, KB, LS174T and MCF-7 cells. In L1210 cells, santamarine and 9β-acetoxycostunolide were cytotoxic. Santamarine and 9β-acetoxycostunolide inhibited mitosis and reduced thymidine uptake in L1210 cells. The mechanism of the cytotoxicity of santamarine and 9β-acetoxycostunolide might be related to suppression of microtubular protein formation and activation of caspase 3, induction of cell cycle blockage and apoptosis. It has been mentioned in the introduction section that *C. purpurea* has been traditionally used to treat various diseases related to inflammation including cancers for many years without any reports of toxicity to humans [1], suggesting that it is not harmful to humans. This investigation provides pharmacological support to its use in cancers. However, further investigation on the exact mechanisms of the anticancer effects of the three components and their in vivo therapeutic efficacy and toxicity are warranted.

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