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In vitro Biological Activity of Germacranolide sesquiterpene lactones

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SUMMARY

Bioactivity-directed isolation has led to the isolation of (-)-*ent*-costunolide (1) as the major active compound from *Hepatostolonophora paucistipula*. This compound (1) was determined by spectroscopic data interpretation. This sesquiterpene lactone (1) inhibited the growth of the dermatophytic fungus *Trichophyton mentagrophytes* ATCC 28185, (4 mm inhibition zone at 15 μg/disc), cytotoxic activity to murine leukaemia cell lines ATCC CCL 46 P 388D1 (IC_{50} 687 ng/ml, at 0.075 μg/disk), BSC monkey kidney cell lines (100% of well at 15 μg/disk) and antiviral activity to *Herpes simplex virus* (0.25 mg/ml, 100% of well at 7.5 μg/disk) and *Polio virus* (0.125 mg/ml, 100% of well at 3.75 μg/disk). These results suggest that (-)-*ent*-costunolide (1) has potential antimicrobial and cytotoxic agents.

Key words: (-)-*ent*-Costunolide (1); *Hepatostolonophora paucistipula; Trichophyton mentagrophytes*; Antifungal activity; Cytotoxic activity; Antiviral activity

INTRODUCTION

Hepatostolonophora paucistipula (Rodw.) J.J. Engel (family Geocalycaceae) is a rich source of sesquiterpenes in the New Zealand liverworts (Asakawa, 1995). There are no literature reports on the biological chemistry of this genus. (-)-*ent*-arbusculin B has isolated from the whole plant of *H. paucistipula,* and its structure has been determined by spectroscopic analysis. Sesquiterpene lactone inhibited the growth of the dermatophytic fungus *Trichophyton mentagrophytes* ATCC 28185, (2 mm inhibition zone at 15 μg/disc) (Kim *et al,* 2005). Increasing attention has been paid to primitive medicinal plants to find new substances with potentially useful biological activities.

Sesquiterpene lactones are natural products isolated from many plant families and most widely distributed within the Compositae. These compounds are known for their various biological activities, including cytotoxicity to tumor cells (Beekman *et al*., 1997). Covalent binding of sesquiterpene lactones to free sulfhydryl groups in proteins and interference with the functions of these macromolecules have been described (Lee *et al*., 1997; Schmidt, 1997). These compounds react with nucleophiles, especially cysteine sulphydryl groups, by a Michael-type addition (Lyss *et al*., 1998). Thus, compounds with

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two α, β-unsaturated carbonyl groups may show considerable cytotoxic properties.

Recently, compounds containinig the R-methylene-γ-lactone functional group, such as sesquiterpene lactones, have attracted much attention during the last 50 years because they display a wide range of biological activities, including antitumor properties (Zhang *et al*, 2005). The isolation of a formal DielsAlder adduct between an unreactive diene such as myrcene and an acid and thermally unstable poor dienophile such as (-)-*ent*-costunolide (**1**) suggests that genepolide is not an isolation artifact but a genuine natural product and that the biogenetic machinery of *A. umbelliformis* contains DielsAlderase enzymatic activity (Appendino *et al,* 2009). This observation is important for the hot debate on the existence of enzymes promoting cycloaddition reactions, since all previously reported DielsAlder adducts involving exomethylene sesquiterpene-γ-lactones involve reactive dienophiles and not a poor partner such as myrcene (Stocking *et al*, 2003). In addition, remarkable differences were observed in the thermal and acid-catalyzed reactivity of the cyclodecadiene moiety of costunolide and genepolide, suggesting that quaternarization at C-11 has a dramatic effect on the reactivity of the medium-sized diene system of these compounds.

In this study, the antiviral, cytotoxic, and antimicrobial activities of (-)-*ent*-costunolide (**1**) from *H. paucistipula* have investigated, and we determined its structure by spectroscopic analysis.

MATERIALS AND METHODS

General experimental procedures

All solvents were distilled before use. Solvents from chromatography fractions were removed by rotary evaporation at temperature up to 40 °C. Initial fractionation of crude plant extract using reverse phase column chromatography was performed with octadecyl-functionalized silica gel (C-18 Aldrich) as the adsorbent. Further column fractionation was performed using Davisil silica 60Å (35-70 μm silica gel, Allth) as adsorbent. TLC was carried out using Merck DC-plastikfolien Kieselgel 60 F_{254} and visualized first with a UV lamp, then dipped in a vanillin solution (1% vanillin, 1% H_2SO_4 in EtOH) followed by heating. Microanalyses were performed by Marianne Dick and Bob McAllister (Campbel Microanalytical Laboratory, Chemistry Department, University of Otago). MS, UV, and IR spetra were recorded on Krato MS-80, Shimadzu UV 240, and Perkin-Elmer 1600 FT-IR instruments respectively. NMR spectra, of $CDCl_3$ solutions at 25 °C, were recorded at 300 MHz for ^{1}H-NMR and 75 MHz for ^{13}C-NMR on a Varian VXR-300 spectrometer. Chemical shifts are given in parts per million on the δ scale referenced to the solvent peak $CHCl_3$ at 7.25 ppm and $CDCl_3$ at 77.08 ppm and are referenced to TMS at 0.00 ppm.

Plant material

H. paucistipula was collected from Port Adventure, Stewart Island, in January 1994. This was identified by D. Glenny, Landcare Research, and a voucher specimen, OTA 046764, has been kept in the Otago University herbarium.

Isolation of (-)-*ent*-costunolide (1)

Air-dried *H. paucistipula* (76.3 g) was grounded and macrated in redistilled ethanol (1,000 ml) in a Waring Blender, then filtered. The residual marc was re-extracted in the same way with more ethanol (3 × 300 ml). The combined filtrates were evaporated under reduced pressure to give a crude extract (1.585 g). A sub-sample (0.836 g) was fractionated over a C_{18}-bonded silica column (10 g), developed with H_2O, 3 : 1, 1 : 1, and 1 : 3 mixtures of H_2O : CH_3CN, then 1 : 1 - CH_3CN : $CHCl_3$, then $CHCl_3$, then Hexane, followed by extra $CHCl_3$ and CH_3CN (2 × 17 ml fractions for each solvent mixture). Most of the antimicrobial activity was found in fractions eluted with 1 : 3 - H_2O : CH_3CN (34 ml). This material (96 mg) was fractionated on a silica gel column (1.0 g), developed with 100% hexane (4 ml), 2% (3 × 4 ml), 5% (6 × 4 ml), 10% (5 × 4 ml),

15% (4 × 4 ml), 20% (10 × 4 ml), 30% (3 × 4 ml), 40% (6 × 4 ml), 50% (5 × 4 ml), 75% (4 × 4 ml) mixture of ethyl acetate : hexane, then 100% ethyl actate, then 100% EtOH (5 × 4 ml). Fractions eluted with 5% ethyl acetate : hexane (2 × 4 ml) and 10% ethyl acetate : hexane (5 × 4 ml) were combined and yielded white crystals (44.0 mg, P 388 IC_{50} 302 ng/ml). This material (44.0 mg) was fractionated on a silica gel column (500.0 mg), developed with 100% hexane (4 × 2 ml), 5% (4 × 2 ml), 10% (5 × 2 ml), 15% (5 × 2 ml), 20% (5 × 2 ml), 25% (6 × 2 ml), 30% (6 × 2 ml), mixture of ethyl acetate : hexane. Fractions eluted with 5% ethyl acetate : hexane (4 × 2 ml) and 10% ethyl acetate : hexane (2 × 2 ml) were combined and yielded white crystals (37.1 mg, P 388 IC_{50} 945 ng/ml). The third P 388-active fraction from this column was subjected to preparative Si gel TLC (1 : 9 ethyl acetate : hexane) to give (-)-*ent*-costunolide (**1**, 14.4 mg, P 388 IC_{50} 687 ng/ml): courless gum; $[\alpha]^{22}$-127^0, $[\alpha]^{25}_{577\ nm}$-175^0, $[\alpha]^{25}_{546\ nm}$-233^0, $[\alpha]^{25}_{435\ nm}$-621^0, $[\alpha]^{25}_{405\ nm}$-748^0, (c, 0.075, $CHCl_3$); lit. $[\alpha]_D$-35^0, ($CHCl_3$); silica TLC R_F 0.20 (1 : 9 - ethyl acetate : hexane); $UV_{\lambda max}$ (MeOH) nm (log ε) 224 (4.00); $IR_{\nu max}$ (film), 2921, 2856, 1763, 1665, 1442, 1382, 1289, 1246, 1137, 968, 755 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ 6.27 (1H, d, J=3.6 Hz, 13'-H), 5.53 (1H, d=3.3 Hz, 13-H), 4.86 (1H, brdd, J=6.6, 10.8 Hz, 1-H), 4.75 (1H, brd, J=10.2 Hz, 5-H), 4.58 (1H, dd, J=8.7, 9.6 Hz, 6-H), 2.58 (1H, m, 7-H), 2.46 (1H, brdd, J=3.9, 13.5 Hz, 3'-H), 2.0 - 2.4 (6H, m, 2'-H, 3-H, 8-H, 8'-H, 9-H, 9'-H), 1.71 (3H, s, CH_3), 1.67 (1H, dd, J=2.7, 5.1 Hz, 2-H), 1.43 (3H, s, CH_3); ^{13}C-NMR ($CDCl_3$): δ 170.52 (C-12), 141.52 (C-11), 140.15 (C-4), 137.00 (C-10), 127.33 (C-5), 127.12 (C-1), 119.70 (C-13), 81.97 (C-6), 50.49 (C-7), 41.07 (C-3), 39.54 (C-9), 28.13 (C-2), 26.27(C-8), 17.42 (C-15), 16.19 (C-14); EI-MS (70 eV): 232.1464 (26%, M^+, $C_{15}H_{22}O_2$ requires 232.1463), 217 (25%, M^+-CH_5), 149 (36%), 136 (15%), 121 (100%); A dose of 15 μg/disk and 7.5 μg/disk showed 100% inhibition of *Herpes simplex* and *Polio*. A dose of 3.75 μg/disk showed 75% inhibition of *Herpes simplex* and *Polio*. 15 μg/disk gave a 4 mm zone of inhibition of *Bacillus subtilis*.

Antiviral activity

The compound was applied (30 μL of a 0.5 mg/ml solution) to a small filter-paper disc, dried, and assayed for antiviral activity using Schroeder *et al.* methods (Asakawa *et al*., 1987). The results were observed as either cell death (cytotoxicity), inhibition of virus replication, no effect (i.e., all of the cells show viral infection), or a combination of all three. The results were noted as the approximate size of the circular zone, radiating from the extract sample, from 1+ to 4+ representing 25% through to whole well sized zones. The notation used is inhibition/

Table 1. ^{1}H-NMR spectral data of compounds **1, 2**, and **3** (300 MHz, $CDCl_3$) (Chen, *et al*., 1989)

H	**1**			**2**			**3**		
1	4.86	br dd	1H	4.84	br dd	1H	5.16	t,	1H
2	1.67	dd	1H	1.67	qd	1H	1.76-2.1	m	5H
2	2.0-2.4	m	6H	2.0-2.4	m	6H	-	-	-
3	2.0-2.4	m	6H	2.0-2.4	m	6H	1.76-2.1	m	5H
3	2.46	br dd	1H	2.44	dd	1H	2.14	m	1H
5	4.75	br d	1H	4.73	br d	1H	5.05	dd	1H
6	4.58	dd	1H	4.57	t	1H	4.57	dd	1H
7	2.58	m	1H	2.56	t	1H	2.48	ddq	1H
8	2.0-2.4	m	1H	2.0-2.4	m	6H	1.50	dddd	1H
8	2.0-2.4	m	6H	-	-	6H	2.27	dddd	-
9/9	2.0-2.4	m	6H	2.0-2.4	m	1H	1.76-2.1	m	5H
13	5.53	d	1H	5.51	d	1H	5.41	d	1H
13	6.27	d	1H	6.26	d	1H	6.14	d	1H
14	1.43	s	3H	1.40	s	3H	1.68	d	3H
15	1.71	d	3H	1.70	s	3H	1.81	d	3H

Table 2. ^{13}C-NMR spectral data of compounds **1, 2**, and **3** ($CDCl_3$) (Chen, *et al*., 1989)

C	1	2	3
1	127.13	127.01	124,87
2	28.13	28.18	26,71
3	41.07	41.01	38.87
4	140.15	140.04	138.13
5	127.33	127.23	124.45
6	81.97	81.95	80.66
7	50.49	50.50	44.98
8	26.27	26.33	25.41
9	39.54	39.56	25.08
10	137.00	136.93	136.73
11	141.52	141.11	140.1
12	170.52	170.37	170.31
13	119.70	119.65	118.39
14	16.19	16.28	21.91
15	17.42	17.51	17.23

As indicated in Table III, (-)-*ent*-costunolide (**1**) inhibited the growth of the dermatophytic fungus *Trichophyton mentagrophytes* ATCC 28185, (4 mm inhibition zone at 15 μg/disc). The activities are expressed by the diameter of the developed inhibition zones and compared with those of the widely antibious chloramphenicol, gentamycin and nystatin (Table III) (Baek *et al*., 2003). This compound (**1**) shows the strong antiviral activity against *Herpes simplex* Type I *virus* ATCC VR 733 (0.25 mg/ml, 100% of well at 7.5 μg/disk) and *Polio* Type I *virus* (Pfizer vaccine strain, 0.125 mg/ml, 100% of well at 3.75 μg/disk). This compound was more antimicrobial than (-)-*ent*-arbusculin B (**4**) (Kim *et al*., 2005).

antiviral activity. The type of antiviral effect, indicated by a number after the size of the zone, was also considered important and may give some indication as to the mode of cytotoxic action.

Antibacterial and antiyeast activities

Activity against the following bacterial strains and yeast was tested: multiresistant *Bacillus subtilis* (ATCC 19659) *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Cladosporium resinae* (ATCC 52833) and *Candida albicans* (ATCC 14053). The compound was dissolved and diluted in an appropriate solvent (usually ethanol : water) to a concentration of 0.5 mg/ml. Test plates are prepared from Mueller Hinton agar containing extract to give a final concentration of 100 μg extract/ml agar. Activity of the growing cultures of the test strains were diluted in saline to deliver 10^4 colony forming units onto the test, control (solvent), and blank (agar only) plates with a multipoint inoculator. Inoculated plates were incubated overnight at 37 °C. Growth on the blank and control plates were checked and, if satisfactory, growth on the test plates were was scored for each test strain. Solutions of compound for assay were dried onto 6 mm filter paper disks, placed onto seeded agar Petri dishes and then incubated. Activity was observed as a zone of inhibition around the disk, with its width recorded from the edge of the disk in mm. HM and SM refer to the observed margin surrounding the zone of inhibition. (H = hazy, S = sharp).

Antifungal activity

Fungal spore suspensions of *Trichophyton mentagraphytes* (ATCC 28185) were applied to dextrose agar plates. Aliquots of the extract solutions were applied to filter paper discs, at 30 μg sample/disc, and dried at 37 °C for two hours. These discs were applied to the agar plates, two per plate, and incubated at 28 °C.

Cytotoxic activity

This is a measure of the ability of a sample to inhibit the multiplication of murine leukaemia cells. The sample was dissolved in a suitable solvent, usually ethanol, at 0.5 mg/ml, and 30 μL of this solution was placed in the first well of a multi-well plate. Seven two-fold dilutions were made across the plate. After the addition of the cell solution, the concentration range in the test wells were between 25,000 to 195 ng/ml. After three day incubation, the plates were read using an ELISA palte reader at 540 nm wavelength. Automated reading of the plates was possible with the addition of a MTT tetrazolium salt (yellow color). Healthy cells reduce this salt to MTT formazan (purple color). The concentration of the sample required to inhibit cell growth to 50% of a solvent control was determined using the absorbance obtained upon staining with MTT tetrazolium. Mytomycin C (concentration 0.075

Table 3. Biological activities of (-)-*ent*-costunolide (**1**) from *H. paucistipula*

Assay	Tested material				
	11 **11111** 1	Chloramphenicol	Nystatin	Gentamycin	Mitomycin C
Cytotoxicity					
BSC-1 cells[a]	++++				
P388, IC_{50}	687[b]				34.6[c]
Antiviral activity[d d'])					
Herpes simplex virus	++++(+++)				
Polio virus	++++(+++)				
Antimicrobial activity[e]				0	
B. subtilis	-	SM 13		0	0
E. coli	-	0		0	SM 9
P. aeruginosa	-	0		HM 10	SM 11
C. albicans	-	0		HM 10	0
C. resinae	-	0		SM 6	0
T. mentagrophytes	HM4	0			0

[a]% of well showing cytotoxic effects. @ 0.5 mg/ml, 15 μg/disk; ++++: 100% activity. Hazy margin, SM; Sharp margin, numbers refer to zone of inhibition (mm). [b]Toxicity of sample to murine leukaemia cell lines (ATCC CCL 46 P388D1) in ng/ml at 0.075 μg/disk. P388; Concentration of the sample required to inhibit cell growth to 50% of a solvent control. [c]Toxicity of sample to murine leukaemia cells (ATCC CCL 46 P388D1) in ng/ml at 7.5 μg/disk. [d]Cytotoxicity in antiviral assays. @ 0.25 mg/ml, 7.5 μg/disk; Zone of cytotoxic activity: ++++: 100% activity. [d']Cytotoxicity in antiviral assays. @ 0.125 mg/ml, 3.75 μg/disk; Zone of cytotoxic activity: +++: 75% activity and +++: 75% activity. [e]Width of zone of inhibition in mm; 15 μg/disk; -: not detected, 0: not determined. Chloramphenicol; 30 mcg/disk, Gentamycin; 30 mcg/disk, Nystatin; 100 unit/disk. HM; Hazy margin, SM; Sharp margin, numbers refer to zone of inhibition (mm)

Cytotoxic activity of (-)-*ent*-costunolide (1)

Normal-phase flash column chromatography concentrated the cytotoxic activity in fraction 3 then eluted with 5% and 10% ethyl acetate - hexane. Chromatography on silica gel (0.5 g) with an ethyl acetate - cyclohexane gradient gave four fractions. The second silica gel column chromatography of subfraction 2 gave most of the mass in the less polar fraction, eluted with 5% and 10% ethyl acetate - hexane. Among them, subfraction 2, that eluted with 5% and 10% ethyl acetate - hexane, is the most cytotoxic activity to murine leukaemia cells ATCC CCL 46 P388D1 (37.1 mg, P388 IC_{50} 945 ng/ml at 7.5 μg/disc). A comparison of IC_{50} (ng/ml) values of these subfractions in cancer cells showed susceptibility to these subfractions decreased in the following order; subfraction 2 > 4 > 1 = .3 (Baek *et al*., 2003; Kim *et al*., 2009).

The result shows the potent cytotoxic activity of (-)-*ent*-costunolide (**1**) from *H. paucistipula* against P388 murine leukaemia cell lines (P 388 IC_{50} 687 ng/ml) and BSC monkey kidney cell lines (100% of well at 15 μg/disk). The cytotoxic activity of this sesquiterpene lactone was in a dose-dependent inhibition of cell proliferation. This compound showed a dose-dependent increase of cell antiproliferation after treatment with of (-)-*ent*-costunolide (**1**) (Baek *et al*., 2000; Shin *et al*., 2001). This compound was more cytotoxic than (-)-*ent*-arbusculin B (**4**) (Kim *et al*., 2005). This compound- mediated cytotoxicity was rapidly increased in the MTT method when its concentrations or absorbances were raised from 2.591 to 3.494. However, the other absorbances were most strong cytotoxic. An IC_{50} of 0.57 μg/ml against KB carcinoma cells has been reported for (+)-costunolide (**2**) (Fig. 2) (Kupchan *et al*., 1971).

μg/ml) was used as a positive control and inhibited the growth of P388 cells by 43 - 75%.

RESULTS AND DISCUSSION

Isolation and identification of (-)-*ent*-costunolide (1)

We have surveyed the New Zealand liverwort for plant products with potential medicinal and agricultural applications. One of the plant extracts that showed activity in antiviral (*Herpes simplex*) and antimicrobial (*Trichophyton mentagrophyte* and *Bacillus subtilis*) screens came from the liverwort of

Jungermannia species. This liverwort is a rich source of diterpenoids belonging to the pimarane, clerodane and kaurane classess (Asakawa, 1995). A sub-sample of the extract was subjected to reverse-phase (C-18) silica gel column chromatography. The column fractions were combined based on visually similar TLC results. These combined fractions were assayed against P388 murine leukaemia cell lines (ATCC CCL 46 P388D1) and the activity was found to be spread over six fractions that were eluted with 1 : 1 H_2O/MeCN, 1 : 3 H_2O/MeCN, MeCN, 1 : 1 MeCN/ $CHCl_3$, Hex/$CHCl_3$ and MeCN (Baek *et al*., 1998). Because of this, the fraction 4 was chromatographed on a silica gel column using a ethyl acetate - hexane gradient. The fraction 4-3 with high activity was shown by TLC and ^{1}H-NMR spectrum to consist of one main UV-active compound. The subfraction was chromatographed on a silica gel column using a ethyl acetate - hexane gradient. The column subfractions were combined based on visually similar TLC results. These combined subfractions were assayed against P 388 murine leukaemia cell lines (ATCC CCL 46 P388D1) and the activity was found to be spread over four subfractions that were eluted with 100% hexane, 5-10% ethyl acetate /hexane, 10-15% ethyl acetate / hexane, and 20-30% ethyl acetate / hexane. High cytotoxic activity from one main UV-active compound was shown by TLC and ^{1}H-NMR spectrum from the fraction 4-3-2. Cytotoxic activity was spread across ethyl acetate - hexane 10 : 90 (R_f 0.20) band containing (-)-*ent*-costunolide (**1**) in thin-layer chromatography. The MS supported a molecular of $C_{15}H_{22}O_2$. The ^{1}H-NMR spectrum of **1** showed the presence of olefinic group with signals at δ5.53 (1H, d, J = 3.3 Hz) and δ6.27 (1H, d, J = 3.6 Hz) and the protons at C-6 in a allylic bond as one-proton double doublet at δ4.75 (brd, J = 10.2 Hz) and δ4.86 (brdd, J=6.6, 10.8 Hz) together with two methyl groups at δ1.43 and 1.71 (Kraut *et al*., 1994). (-)-*ent*-Costunolide (**1**) from *H. paucistipula* has known the major sesquiterpene lactone which had 1H and ^{13}C-NMR data matching those reported for (+)-costunolide (**3**) (EI-Feraly *et al*., 1979; Kim *et al*., 1996). However, the optical rotation ($[\alpha]_D$-127^0) was opposite to the literature values for **3** (+117^0, +129^0) (Kim *et al*., 1996; Park *et al*., 1996). The minor sesquiterpene lactone was identified as (-)-*ent*-arbusculin B (**4**) since it showed the same ^{1}H and ^{13}C-NMR data as those reported for (+)-arbusculin B (**5**) (Greene *et al*., 1974; Kim *et al*., 1996), but had a negative optical rotation. We could only find two previous reports of the isolation of **4**, also known as (-)-γ-cyclocostunolide, from two *Frullania* species of liverwort methods (Asakawa *et al*., 1976; Asakawa *et al*., 1987). It is not surprising that germacranolide (**1)** and eudesmanolide, with the same rare 7α stereochemistry should co-occur, since eudesmanes are thought to be biosynthesised

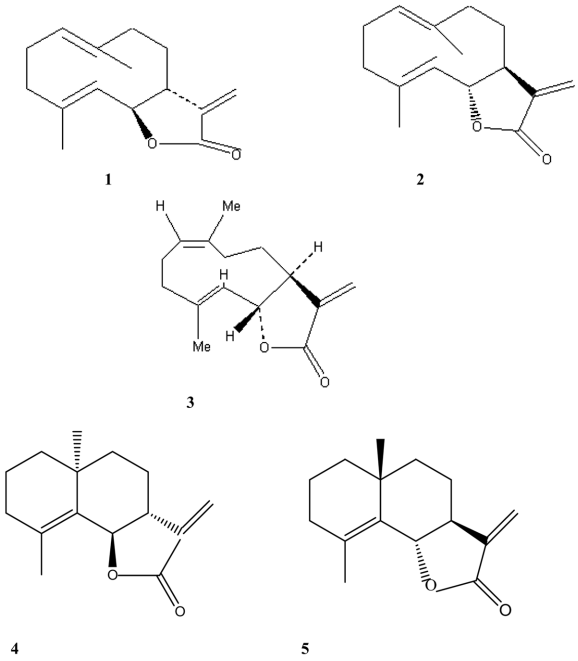

Fig. 1. The molecular structures of (-)-*ent*-costunolide (**1**), (+)-costunolide (**2**), 1(10)-cis-costunolide (**3**), (-)-*ent*-arbusculin B (**4**) and (+)-arbusculin B (**5**). The sesquiterpene lactones (**1**, and **2**) have the same molecular formulas ($C_{15}H_{20}O_2$) and similar spectroscopic data which suggest similar structures. Differences however, exist in mp, optical rotation and NMR data. 1(10)-Cis-costunolide (**3**) has the different spectroscopic data and structure. However, Compound (**3**) has the same molecular formula.

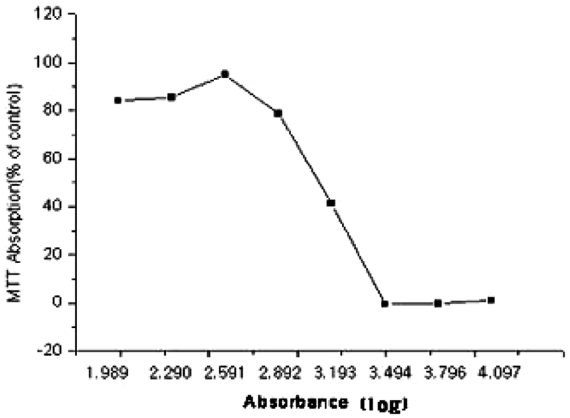

Fig. 2. *In vitro* cytotoxic effect of (-)-*ent*-costunolide (**1**) from *H. paucistipula* by the MTT method. This compound (**1**) was serially diluted in RPMI-1640 with 10% FBS and mixed with equal volume of murine leukaemia cells ATCC CCL 46 P388D1 (7.5 μg/disk). The colometric method was performed as described in materials and methods. Data are mean values of results obtained from three sets of experiments. In conclusion, (-)-*ent*-costunolide (**1**) has isolated from the whole plant of *H. paucistipula,* and we determined its structure by spectroscopic analysis. This sesquiterpene lactone (**1**) inhibited the growth of the dermatophytic fungus *Trichophyton mentagrophytes* ATCC 28185, (4 mm inhibition zone at 15 μg/disc), cytotoxic activity to murine leukaemia cell lines ATCC CCL 46 P 388D1 (IC_{50} 687 ng/Ml, at 0.075 μg/disk), BSC monkey kidney cell lines (100% of well at 15 μg/disk) and antiviral activity to *Herpes simplex virus* (0.25 mg/ml, 100% of well at 7.5 μg/disk) and *Polio virus* (0.125 mg/ml, 100% of well at 3.75 μg/disk).

via germacrane intermediates (Fig. 1) (Cane *et al*., 1999).

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