Evaluation of *Polygonum bistorta* for Anticancer Potential Using Selected Cancer Cell Lines

Karuppiah Pillai Manoharan[1], Daiwen Yang [2], Annie Hsu [3] and Benny Tan Kwong Huat[3,*]

[1]*Department of Chemistry, Faculty of Science, National University of Singapore, 3, Science Drive 3, Singapore 117543;* [2]*Department of Biological Sciences, Faculty of Science, National University of Singapore, Science Drive 4, Singapore 117543;* [3]*Department of Pharmacology, Faculty of Medicine, National University of Singapore, Building MD2, 18 Medical Drive, Singapore 119260*

Abstract: The chloroform and hexane fractions and their sub-fractions of *Polygonum bistorta* (Polygonaceae) were evaluated for their cytotoxic activity against P338 (Murine lymphocytic leukaemia), HepG2 (Hepatocellular carcinoma), J82 (Bladder transitional carcinoma), HL60 (Human leukaemia), MCF7 (Human breast cancer) and LL2 (Lewis lung carcinoma) cancer cell lines in culture. Both the chloroform and hexane fractions and a few of their sub-fractions showed moderate to very good activity against P388, HL60 and LL2 cancer cell lines. Both active and non-active fractions were further investigated for their chemical constituents. A total of nine compounds, *viz*. 24(E)-ethylidenecycloartanone **(1)**, 24(E)-ethylidenecycloartan-3α-ol **(2)**, cycloartane-3,24-dione **(3)**, 24-methylenecycloartanone **(4)**, friedelin **(5)**, 3β-friedelinol **(6)**, β-sitosterol **(7)**, γ-sitosterol **(8)** and β-sitosterone **(9)** were isolated. One of the pure compounds, 24(E)-ethylidenecycloartanone **1**, which was obtained in sufficient quantity, was tested for its cytotoxicity against P388, LL2, HL60 and WEHI164 (Murine fibrosarcoma) cancer cell lines but was found to have no activity even at a concentration of 100 μg/mL.

Key Words: *Polygonum bistorta*, cycloartane triterpenoids, β-sitosterol, cytotoxic activity, MTT assay, cancer cell lines.

INTRODUCTION

Drug discovery from medicinal plants has evolved to include numerous fields of inquiry and various methods of analysis. Bio-assay guided fractionation has been employed to isolate and characterise the active compounds, the most promising of which then undergo testing in the *in vivo* hollow fiber assay [1]. Further biological studies were performed to follow-up on compounds of significant interest. Cancer chemoprevention was defined as a strategy of cancer control by the administration of synthetic or natural compounds to reverse or suppress the process of carcinogenesis [2]. Carcinogenesis is a multistage process by which a normal cell is transformed into a cancerous cell. Transformation involves (i) initiation, typically from DNA damaging agents; (ii) promotion, during which cell proliferation is increased; and (iii) progression, involving additional genetic alterations. During the metaphase of cell replication, the daughter chromosomes are split apart and pulled to opposite poles of the cell. The components involved in the pulling are microtubules, which are made up of subunits known as tubulin. Since cancer is caused by uncontrolled division of abnormal cells, it is necessary to dismantle the microtubules so that the cell can proceed with its own division. Chemopreventive agents work by various mechanisms of action, targeting initiation, promotion and/or progression of carcinogenesis. An example is Taxol®, which inhibits depolymerisation of the tubulin subunits and is effective in controlling the spread of malignancy.

Novel approaches to cancer therapy include the prevention of tumour growth by preventing tumour angiogenesis [3,4]. With the availability of different approaches for the discovery of new therapeutics, natural products provide one of the best reservoirs of new structural types that may have novel mechanisms of anticancer action.

Polygonum bistorta, commonly known as Bistort or Snakeroot, belongs to the Polygonaceae family. It is one of the strongest herb astringents and is excellent for the treatment of cholera, diarrhoea and dysentery. When applied to a wound, it can stop bleeding. It has been used in traditional Chinese medicine as a remedy for smallpox, measles, pimples, jaundice, insect stings, snake bites and expelling worms. It is also taken for the treatment of a wide range of complaints including cystitis, irritable bowel syndrome, peptic ulcers and ulcerative colitis. It was reported that the ethanolic extract caused strong anti-inflammatory effect [5]. 5-Glutinen-3-one (alnusenone) and friedelinol were identified as active constituents for such an effect [6]. It was also reported that the aqueous extract strongly inhibits the mutagenicity of Trp-P-1 [7]. We decided to investigate the rhizomes of this plant for possible cytotoxic potential against P338, HepG2, J82, HL60, MCF7 and LL2 cancer cell lines in culture.

RESULTS AND DISCUSSION

The hexane fraction of *P. bistorta* (obtained from the first batch of plant material, 600g) was screened for cytotoxic activity against P388, HepG2, J82, HL60, MCF7 and LL2

*Address correspondence to this author at the Department of Pharmacology, Faculty of Medicine, National University of Singapore, Building MD2, 18 Medical Drive, Singapore 119260; Tel: +65 6516 3272; Fax: +65 6873 7690; E-mail: phctankh@nus.edu.sg

1573-4064/07 $50.00+.00

cancer cell lines. The IC_{50} values are given in Table **1**. It showed very good activity against P388 cancer cell line with IC_{50} value <10 μg/mL and good activity against HL60 with IC_{50} value of 17.8 μg/mL, but was inactive against all other cell lines screened; the IC_{50} values were 80.6, >100, 72.0 and 62.4 μg/mL against HepG2, J82, MCF7 and LL2 cancer cell lines, respectively. Owing to the promising activity of the hexane extract against P388 and HL60 cancer cell lines, we repeated the experiments with 12 kg of plant material. This time the hexane fraction was chromatographed over a silica gel column using hexane and eluted with solvents of increasing polarity. Five major sub-fractions, (A-E), three minor fractions and one more fraction (A1) of about 90 % purity were obtained. The five major fractions.

(A-E) were screened for activity against the above- mentioned cancer cell lines and the results are also shown in Table **1**.

Fraction A was not active even at a concentration of 100 μg/mL against HepG2, MCF7 and LL2, cancer cell lines. The IC_{50} values with the other three cancer cell lines, P388, J82 and HL60 were 60.8, 80.8 and 72.0 μg/mL, respectively. Fraction B showed weak activity against P388 with IC_{50} value of 40.8 μg/mL and was inactive against all other cancer cell lines tested, with IC_{50} values of 74.3, 64.8, 91.2, 65.6 and > 100 μg/mL against HepG2, J82, HL60, MCF7 and LL2 cancer cell lines, respectively. Fraction C showed very good activity against LL2 with IC_{50} value < 10 μg/mL and good activity against P388 and HL60, with IC_{50} values of 16.2 and 18.6 μg /mL, respectively. But it was inactive against HepG2, J82 and MCF7 cancer cell lines with IC_{50} values of 63.6, 82.1 and 64.8 μg/mL, respectively. Fraction D showed no activity against all the cell lines tested, but the IC_{50} values were 50.2, 52.3 and 40.8 μg/ mL against P388, HepG2 and MCF7 cancer cell lines respectively; the IC_{50} value was >75 μg/mL against both HL60 and LL2, while it was >100 μg/mL against J82 cancer cell line. Fraction E showed good activity against LL2 with IC_{50} value of 19.4 μg/mL and moderate activity against P388, with IC_{50} value 27.6 μg/mL; the IC_{50} values were 70.5, 63.6 and 92.6 μg/mL against HepG2, HL60 and MCF7 cancer cell lines, respectively; while it was >100 μg/mL against J82 cancer cell line.

Further purification of these five major fractions (A-E) led to the isolation of the following compounds (see Fig. **1** for the structures). Fraction A yielded a new cycloartane triterpenoid, 24(E)-ethylidenecycloartanone **1**, whereas fractions B, C and D contained the known compounds, friedelin **5**, 3β-friedelinol **6** and β-sitosterol **7**, respectively. On the other hand, fraction E contained cycloartane-3,24-dione **3**, a rarely encountered compound. Fraction (A1), obtained in about 90 % purity, was not screened for its cytotoxicity and on recrystallisation with acetone yielded the pure compound, 24(E)- ethylidenecycloartanone **1**. The three minor fractions were also not screened for their cytotoxicity due to their small quantities but preparative TLC led to the isolation of one compound from each fraction. These were the known compounds *viz*. β-sitosterol **7** and γ-sitosterol **8** and again the new triterpenoid, 24(E)-ethylidenecycloartanone **1**.

Cycloartane-3,24-dione **3** was not screened for its cytotoxicity as it was obtained only in small quantity. The known compounds, friedelin **5**, 3β-friedelinol **6**, β-sitosterol **7** and γ-sitosterol **8** were also not tested for their cytotoxicity, since these compounds are very common natural products and there have been several reports citing their cytotoxicity in the literature [8-20]. Of the isolated pure compounds, only the new compound, 24(E)-ethylidenecycloartanone **(1)**, was screened for its cytotoxicity against P388, HL60, LL2 and WEHI164 cancer cell lines. The results are given in Table **2**.

The chloroform fraction of *P. bistorta* (obtained from the first batch of plant materials, 600g) was screened for cytotoxic activity against P388, HepG2, J82, HL60, MCF7 and LL2 cancer cell lines. The IC_{50} values are given in Table **2**. It showed very good activity against both P388 and HL60 can-

Table 1. IC_{50} Values of Hexane Fraction of *P. bistorta* and its Sub-Fractions (A-E) Against Murine and Human Cancer Cell Lines

	IC_{50} values of hexane fraction and its sub-fractions					
Cell lines / Fractions	P388	HepG2	J82	HL60	MCF7	LL2
Hexane fraction	<10	80.6	>100	17.8	72.0	62.4
A	60.8	>100	80.8	72.0	>100	>100
B	40.8	74.3	64.8	91.2	65.6	>100
C	16.2	63.6	82.1	18.6	64.8	<10
D	50.2	52.3	>100	78.8	40.8	75.0
E	27.6	70.5	>100	63.6	92.6	19.4

P338: Murine lymphocytic leukaemia; HL60: Human leukaemia; MCF7: Human breast cancer; LL2: Lewis lung carcinoma; HepG2: Hepatocellular carcinoma; J82: Bladder transitional carcinoma; IC_{50} values are expressed in μg/mL; IC_{50} values less than 30 μg/mL are considered active; NT-Not tested; No. of replicates, n = 8 for each concentration; Hexane fraction was obtained from the first batch of plant materials (600g); Sub-fractions A-E, were obtained from the second batch of plant materials (12kg); 6-Mercaptopurine (6MP) was used as positive control for P388 cancer cell lines (IC_{50} = < 10 μg/mL); Doxorubicin was used as positive control for MCF7 cancer cell lines (IC_{50} = < 10 μg/mL). Positive controls were not maintained throughout experiments. The experiments were conducted over a period of time and the availability of cell lines varied at each time; positive controls were omitted when there was shortage. The 50% inhibitory concentrations (IC_{50}) were measured by MTT assay after 3-days of incubation.

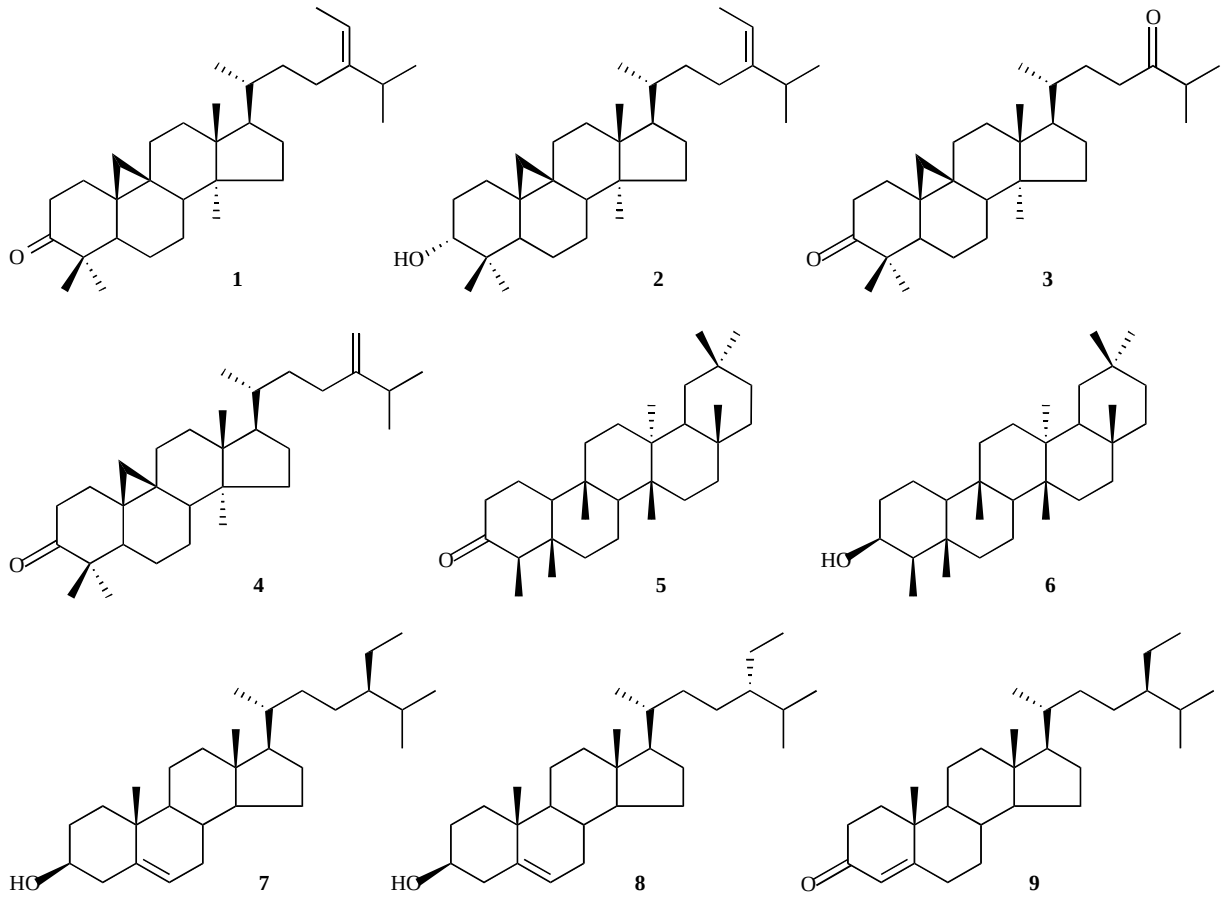

Fig. (1). Structures of isolated pure compounds **1-9** from *Polygonum bistorta*.

cer cell line with IC_{50} value <10 μg/mL, but was inactive against all other cell lines screened; the IC_{50} values were 78.2, >100, 66.6 and 52.3 μg/mL against HepG2, J82, MCF7 and LL2 cancer cell lines, respectively. This chloroform fraction was chromatographed over silica gel using hexane and eluted with solvents of increasing polarity. Further purification of a major sub-fraction by HPLC (hexane/chloroform, 1:9), followed by preparative TLC (chloroform/methanol, 9:1) yielded 24-methylenecycloartanone **4** (7 mg) [21,22].

The chloroform fraction obtained from the second batch of plant material (12 kg) was chromatographed. Three major fractions F, G and H and three minor fractions were obtained. The major fractions were screened for their cytotoxicity against the above- mentioned cancer cell lines. Fraction F showed no activity against P388, HepG2, J82, HL60, MCF7 and LL2 cancer cell lines. The IC_{50} value was found to be >100 μg/mL, against on both MCF7 and HepG2 cancer cell lines; while it was 65.4, 70.4, 76.0 and 90.8 μg/mL against P388, J82, HL60 and LL2 cancer cell lines, respectively. Fraction G showed very good activity against LL2 with IC_{50} value < 10 μg/mL and good activity against HL60 with IC_{50} value of 19.4 μg/mL, but was inactive against all other cancer cell lines; the IC_{50} values were 51.8 and 90.4 μg/mL against P388 and HepG2 cancer cell lines respectively and it was > 100 μg/mL against both MCF7 and J82 cancer cell lines. Fraction H showed borderline activity against MCF7 with IC_{50} value of 35.8 μg/mL, but was inactive against all other cancer cell lines tested; the IC_{50} values were 65.4, 80.2, 92.0, 80.8 and 70.0 μg/mL against P388, HepG2, J82, HL60 and LL2 cancer cell lines, respectively. The minor fractions were not screened for their cytotoxicity as they were obtained in small quantities.

24(E)-ethylidenecycloartanone **1** was again isolated from Fraction F, while 24(E)-ethylidenecycloartan-3α-ol **2** was isolated from Fraction G, which had been shown to have activity against LL2 and HL60 cancer cell lines. However, compound **2** could not be tested for cytotoxicity as it was obtained in a small quantity. A known compound, β-sitosterol 7 was isolated from Fraction H. The known compounds, β-sitosterol **7**, β-sitosterone **9** and 24(E)-ethylidenecycloartanone **1** were again isolated from the three minor fractions, having one compound from each fraction. Compounds **7** and **9** also could not be tested for cytotoxicity due to their poor yield.

The structure elucidation and/or identification of all these compounds, **1-9**, together with the physical and spectroscopic details have previously been reported [23].

Although 3β-friedelinol **6** was isolated from the active fraction C, our literature search revealed that it was not only inactive against P388 but also A549, MCF7, HT29 and KB cancer cell lines [8]. Friedelin **5** also showed no activity against P388, A549, MCF7, HT29 and KB cancer cell lines

Table 2. IC_{50} Values of Chloroform Fraction of *P. bistorta* and its Sub-Fractions Against Murine and Human Cancer Cell Lines

IC_{50} values of chloroform fraction and its sub-fractions							
Cell lines / Fractions	P388	HepG2	J82	HL60	MCF7	LL2	WEHI164
Chloroform fraction	<10	78.2	>100	<10	66.6	52.3	NT
F	65.4	>100	70.4	76.0	>100	90.8	NT
G	51.8	90.4	>100	19.4	>100	<10	NT
H	65.4	80.2	92.0	80.8	35.8	70.0	NT
*Pure compound	>221	NT	NT	>221	NT	>221	>221

P338: Murine lymphocytic leukaemia; HL60: Human leukaemia; MCF7: Human breast cancer; LL2: Lewis lung carcinoma; HepG2: Hepatocellular carcinoma; J82: Bladder transitional carcinoma; WEHI164: Murine fibrosarcoma; IC_{50} values are expressed in μg/mL; IC_{50} values less than 30 μg/mL are considered active; *Pure compound = 24(E)-ethylidenecycloartanone **(1)**; For the pure compound, 24(E)-ethylidenecycloartanone **(1)**, the IC_{50} values are expressed in μM; NT-Not tested; No. of replicates, n = 8 for each concentration; Chlorofrom fraction was obtained from the first batch of plant materials (600g); Sub-fractions F-H, were obtained from the second batch of plant materials (12kg); 6-Mercaptopurine (6MP) was used as positive control for P388 cancer cell lines (IC_{50} = < 10 μg/mL); Doxorubicin was used as positive control for MCF7 cancer cell lines (IC_{50} = < 10 μg/mL). Positive controls were not maintained throughout experiments. The experiments were conducted over a period of time and the availability of cell lines varied at each time; positive controls were omitted when there was shortage. The 50% inhibitory concentrations (IC_{50}) were measured by MTT assay after 3-days of incubation.

[8]. Friedelin **5**, however was reported to be cytotoxic against PC3 and U251 cancer cell lines [9]. At a concentration of 31μM, the percentage ranges of inhibition caused by friedelin were 61.9, 25.8 and zero on PC3, U251 and K562 cancer cell lines, respectively [9]. Friedelin **5** is reportedly inactive against MCF7 (breast cancer), TK10 (renal) UACC62 (melanoma), NCI-H460 (lung cancer) and SF-268 (CNS) cancer cell lines [10]. β-Sitosterol **7**, the major phytosterol in higher plants, including fruits and vegetables, has shown potential for prevention and therapy of human cancer. β-Sitosterol **7** treatment was reported to inhibit HCT116 human colon cancer cell proliferation in a concentration-dependent manner [11]. Breast cancer cell growth was inhibited by 66% after 3 days and by 80% after 5 days with 16 μM β-sitosterol; supplementation of β-sitosterol **7** for 3 days at 16 μM resulted in an increase in apoptosis in cells; it was reported that β-sitosterol **7**, by a still-unknown mechanism, provides protection from breast cancer by inhibiting growth and stimulating apoptosis [12-14]. This compound effectively inhibits invasion of tumour cells and metastasis [15]. β-Sitosterol **7**-treated premalignant and malignant cells have been reported to accumulate in the G0/G1 and G2/M phases, respectively [16]. β-Sitosterol **7** exhibited a growth suppressing effect against two human cancer cell lines, MCF7 and BT20 [17,18]. It also caused a high degree of growth inhibition on Hep-2 and McCoy cells [19] with cytotoxic activity against human nasopharynx epidermoid cells [20].

Some cycloartane-type triterpenoids, mostly in the form of saponins or with several functional groups, have been reported to show cytotoxic activities against several cancer cell lines, *viz*. HepG2, HL60, MCF7, KB Cells, A2780 (Ovarian cancer cells), 26L5 (Murine colon cells), HSC2 (Human oral squamous cells), U251 (CNS), PC3 (Prostate cancer cells), HCT15 (Colon carcinoma cells), K562 (Leukaemia cancer cells) *etc*. [24-33]. However, in contrast, the new compound 24(E)-ethylidenecycloartanone **1**, though it is a cycloartane-type triterpenoid, did not show any activity against a panel of cancer cell lines. It has been noted that this new compound, 24(E)-ethylidenecycloartanone **1**, only has a ketone functional group and one double bond. The lack of functionality could be a probable reason for its inactivity against the cancer cell lines tested.

MATERIALS AND METHODS

Biological Materials and Cells

The sources of most of the chemicals and biochemicals are the same as reported elsewhere [34]. P388 (Murine lymphocytic leukaemia), HL60 (Human leukaemia), MCF7 (Human breast cancer), LL2 (Lewis lung carcinoma), HepG2 (Hepatocellular carcinoma), J82 (Bladder transitional carcinoma) and WEHI164 (Murine fibrosarcoma) cancer cell lines were all obtained from American Type Cell Culture (ATCC) [Manassas, VA, USA].

Instrumental

Melting points were determined on a Buchi Melting point B-540 apparatus. The IR spectra were recorded on a Bio Rad, Class II Laser product. The ^{1}H, ^{13}C NMR and 2D NMR spectra were recorded on Bruker, 300 and/or 500 MHz spectrometers. Standard microprogrammes supplied by Bruker were used to run 1D and 2D NMR spectroscopy. Chemical shifts were reported in parts per million (ppm) with TMS as a reference standard and coupling constants (*J*) expressed in hertz. LREIMS were measured on a Finnigan/MAT MAT 95 XL-T or VG Micromass 7035. HREIMS were measured on Finnigan/MAT MAT 95 XL-T mass spectrometers. HPLC was carried out on a Waters associate, μ-Porasil (300 x 5 mm) column with a Shimadzu RID-10A, refractive index detector. Silica gel 60 (Merck, 0.063- 0.200 m) was used for column chromatography. Lichroprep RP-18 (Merck, 40-63 μm) was used for separation and/or purification. Precoated

silica gel plates (Merck, Kieselgel 60F 254, 0.25 mm or Baker Si250F, 0.25 mm) were used for preparative TLC and/or analytical TLC. Spots were detected using UV light or staining with iodine or by spraying with 50% H_2SO_4, followed by heating at 110˚C for 5 minutes. The following instruments were used at the Traditional Medicine and Natural Products Research Laboratory, Department of Pharmacology: Biological safety cabinet (NUAIRE, Plymouth, USA), ELX 800 Microplate reader (Bio-Tek Instruments Inc., USA), Hemocytometer (Fortuna, Germany), Leitz Fluovert microscope (Ernst Leitz Wetzlar GMBH, Germany), Water incubator (Everbloom Medical & Scientific Pte. Ltd., Singapore), -86˚C Freezer (Forma Scientific), Beckman Avanti J-251 Cenrtifuge (Fullerton, CA, USA), Beckman Optima L-90K and Ultracentrifuge (Fullerton, CA, USA).

Assay of Cell Viability

Cells were cultured in Corning disposable flasks using RPMI-1640 or DEME medium supplemented with 5% fetal bovine serum and streptomycin and incubated at 37˚C in a humidified atmosphere of 5% CO_2. To ensure an exponential growth, cells were resuspended in fresh medium every 36h or 48h. Cell concentration and viability were determined using the trypan blue exclusion test. 20 µL of cell suspension was taken and equal volume of trypan blue solution (0.4%) was added it. The number of live cells was counted using a Hemocytometer under a Leitz light microscope. The cultured cell lines were then diluted to the required concentration with medium. 90 µL of cultured cells were transferred to each well of a 96 well plate and 10 µL extract of various concentrations was added to the cultures and incubated at 37˚C in the humidified atmosphere of 5% CO_2. On day three, 10 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5mg/mL) was added to the culture medium. After a further 4h of incubation, 100 µL solution of 10% sodium dodecyl sulphate (SDS) in 0.01 N HCl was added to each well and the formazan crystals in each well were dissolved. Optical density (absorbance) measurements were made with a microplate reader at 570 nm. Cytotoxicity was calculated by the following formula [35]:

Growth inhibition (GI) = [(OD_{570} of control – OD_{570} of test sample)/ OD_{570} of control] x 100%.

Plant Material

The plant materials were purchased from a local market and a voucher specimen (KMano PB 2003) has been deposited in the Department of Biological Sciences, National University of Singapore, Singapore.

Extraction and Isolation

The rhizomes of *P. bistorta* (600g) were grounded into powder and then extracted with chloroform (3L x 4) at room temperature. The residue was dissolved in a water/methanol mixture (95:5) and then extracted successively with hexane and chloroform. After the preliminary screening followed by the isolation of 24-methylenecycloartanone **4**, we carried out further investigation by purchasing 12 kg of plant material and extracted it as previously. Rota-vapour was used to remove solvent from the hexane and chloroform fractions. The hexane extract was chromatographed over silica gel using hexane and eluted in a gradient fashion with increasing polarity. Purification of the eluted fractions afforded friedelin **5** (6 mg)**,** 3β-friedelinol **6** (112 mg), β-sitosterol **7** (1.2 g), γ-sitosterol **8** (4 mg), 24(E)-ethylidenecycloartanone **1** (580 mg) and cycloartane-3,24-dione **3** (ca.1.5 mg). The chloroform extract was chromatographed over Lichroprep RP-18 and eluted in isocratic fashion with methanol. Purification of eluted fractions afforded β-sitosterol **7** (110 mg), β-sitosterone **9** (ca. 0.5 mg), 24(E)-ethylidenecycloartanone **1** (7.0 mg) and 24(E)-ethylidenecycloartan-3α-ol **2** (ca. 1.0 mg). For methanol-water fraction, Rota-vapour was used first to remove as much solvent as possible, followed by the use of Freeze dryer/Lyophiliser to remove the remaining solvent. The residue was chromatographed over Lichroprep RP-18 and eluted in isocratic fashion with methanol. Purification of one of the eluted fractions afforded β-sitosterol **7**.

ACKNOWLEDGEMENT

This research has been supported by a grant from the National University of Singapore. One of the authors, Karuppiah Pillai Manoharan, thanks the National University of Singapore for financial assistance for his Ph.D studies.

REFERENCES

[1] Hollingshead, M.G.; Alley, M.C.; Camalier, R.F.; Abbott, B.J.; Mayo, J.G.; Malspeis, L.; Grever, M.R. *Life Sci*., **1995**, *57*(2), 131.
[2] Sporn, M.B.; Dunlop, N.M.; Newton, D.L.; Smith, J.M. *Fed. Proc*., **1976**, *35*(6), 1332.
[3] Folkman, J. *Nat. Med*., **1995**, *1*, 27.
[4] Ingber, D.; Fujita, T.; Kishimoto, S.; Sudo, K.; Kanamaru, T.; Brem, H.; Folkman, J. *Nature*, **1990**, *8,* 427.
[5] Duwiejua, M.; Zeitlin, I. L.; Gray, A. I.; Waterman, P. G. *Pharm. Pharmacol*., **1994**, 46, 286.
[6] Duwiejua, M.; Zeitlin, I. L.; Gray, A. I.; Waterman, P. G. *Planta Med*., **1999**, *65*, 371.
[7] Miki, N.; A-Fu, W.; Takahiko, S.; Hisamitsu, N.; Hideaki, K. *Nat. Med*., **1995**, *49*, 329.
[8] Guo-Qiang, Z. *Planta Med*., **1994**, *60*, 54.
[9] Reyes-Chilpaa, R.; Estrada-Muniza, E.; Teresa, R. A.; Badia, A.; Andre, A.; Christopher, K. J.; Vazquez-Torresc, M. *Life Sci*., **2004**, *75*, 1635.
[10] Cristina, M.; Fatima, J.; Regina, T.; Marcelo-Curto, M. J.; Helena F. M.; Maria, S. J. N.; Madalena, P.; Fatima, C.; Madalena, M. M. P. *J. Nat. Prod*., **2001**, *64*, 1273.
[11] Choi, Y. H.; Kyu R. K.; Young-Ae, K.; Keun-Ok, J.; Jeong-Ha, K.; Sook-Hee, R.; Kun-Young, P. *Int. J. Oncol*., **2003**, *23*, 1657.
[12] Awad, A. B.; Downie, A. C.; Fink, C. S. *Int. J. Mol. Med*., **2000**, *5*, 541.
[13] Awad, A. B.; Williams, H.; Fink, C. S. *Nutr. Cancer*, **2001**, *40*, 157.
[14] Awad, A. B.; Roy, R.; Fink, C. *Oncol. Rep*., **2003**, *10*, 497.
[15] Ovesna, Z.; Vachalkova, A.; Horvathova, K. *Neoplasma*, **2004**, *51*, 407.
[16] Han, C. H.; Ding, H.; Casto, B.; Stoner, G. D.; D'Ambrosio, S. M. *Nutr. Cancer*, **2005**, *51*, 207.
[17] Madhavi, D. L.; Bomser, J.; Smith, M. A. L.; Singletary, K. *Plant Sci*., **1998**, *131*, 95.
[18] Nguyen, A. T.; Malonne, H.; Duez, P.; Vanhaelen-Fastre, R.; Vanhaelen, M.; Fontaine, J. *Fitoterapia*, **2004**, *75*, 500.
[19] Gomez, M. A.; Garcia, M. D.; Saenz, M. T. *Phytother. Res*., **2001**, *15*, 633.
[20] Bhakuni, D. S.; Mayer, M.; Poyser, K. A.; Poyser, J. P.; Sammes, P. G.; Silva, M. *Quimica*, **1973**, *4*, 166.
[21] Ohtsu, H.; Tanaka, R.; Michida, T.; Shingu, T.; Matsunaga, S. *Phytochemistry*, **1998**, *49*, 1761.
[22] Bohme, F.; Schmidt, J.; Sung, T.V.; Adam, G. *Phytochemistry*, **1997**, *45*, 1041.
[23] Manoharan, K.P.; Tan, B.K.H.; Yang, D.W. *Phytochemistry*, **2005**, *66*, 2304.

[24] Parra-Delgado, H.; Garcia-Pillado, F.; Sordo, M.; Ramirez-Apan, T.; Martinez- Vazquez, M.; Ostrosky-Wegman, P. *Life Sci*., **2005**, *77*, 2855.
[25] Yesilada, E.; Bedir, E.; Calis, I.; Takaishi, Y.; Ohmoto, Y. *J. Ethnopharmacol*., **2005**, *96*, 71.
[26] Watanabe, K.; Mimaki, Y.; Sakagami, H.; Sashida, Y.; *Chem. Pharm. Bull*., **2002**, *50*, 121.
[27] Banskota, A. H.; Tezuka, Y.; Phung, L. K.; Tran, K. Q.; Saiki, I.; Miwa, Y.; Taga, T.; Kadota, S. *Bioorg. Med. Chem. Lett.*, **1998**, *8*, 3519.
[28] Omobuwajo, O. R.; Martin, M. T.; Perromat, G.; Sevenet, T.; Awang, K.; Pais, M. *Phytochemistry*, **1996**, *41*, 1325.
[29] Ozipek, M.; Donmez, A. A.; Calis, I.; Brun, R.; Ruedi, P.; Tasdemir, D. *Phytochemistry*, **2005**, *66*, 1168.
[30] Nishizawa, M.; Emura, M.; Yamada, H.; Shiro, M.; Chairul, Y. H.; Harukuni, T. V. *Tetrahedron Lett*., **1989**, *30*, 5615.
[31] Radwan, M. M.; El-Sebakhy, N. A.; Asaad, A. M.; Toaima, S. M.; Kingston, D. G. I. *Phytochemistry*, **2004**, *65*, 2909.
[32] Mohamad, K.; Martin, M. T.; Leroy, E.; Tempete, C.; Sevenet, T.; Awang, K.; Pais, M. *J. Nat. Prod*., **1997**, *60*, 81.
[33] Tian, Z.; Yang, M.; Huang, F.; Li, K.; Si, J.; Shi, L.; Chen, S.; Xiao, P. *Cancer Lett*., **2005**, *226*, 65.
[34] Nam, K.S.; Su Jo, Y.; Ho Kim, Y.; Hyun, J.W.; Kim, H.W. *Life Sci*., **2001**, *69*, 229.
[35] De-Lean, A.; Munson, P. J.; Rodbard, D. *Am. J. Physiol*., **1978**, *235*, 97.

Received: 29 September, 2006 Revised: 24 December, 2006 Accepted: 08 January, 2007