Phytopharmacology

Inforesights Publishing

Antiproliferative and apoptotic potential of Chinese medicinal plants against MCF-7 (luminal A), HCC1954 (HER2+) and Hs578t breast cancer cells

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Received: 20 January 2012, **Revised**: 28 February 2013, **Accepted**: 1 March 2013

Abstract

Breast cancer is a significant cause of death among women worldwide. Medicinal plant extracts with large amounts of polyphenolic compounds, which are antioxidants, can have an important role in cancer prevention. The purpose of this study is to test *in vitro* the antiproliferative activities of the ethanolic and aqueous extracts of selected Chinese medicinal plants against three human breast cancer cell lines [MCF-7 (luminal A); HCC1954 (HER2+); Hs578t (triple negative)] and one normal cell line [MCF-10A]. The ethanolic extracts of *Polygonum cuspidatum, Rheum officinale, Eucomia ulmoides, Lysinachia christinae, Rhizoma Alpinae, Sarcandra glabre, Scutellaria baicalensis, Smilax glabra* and *Atractyloides macrocephala* showed potent anti-cancer activity with little or no toxicity to normal cells. The apoptosis test was carried out for one of the active plant extract (*S. baicalensis)* and the results were found to be very significant. The plant extracts investigated in this study have significant anticancer activity against the breast cancer cell lines tested. Further investigation is required to isolate and elucidate the structure of the compounds responsible for the observed activity.

Keywords: Rats, amnesia, anxiety, *Piliostigma thoningii*

Introduction

Breast cancer is a heterogeneous and complex disease, being the most common cancer in women worldwide. It is also a major cause of death from cancer among women globally. In 2010, it was estimated that over 1.5 million women worldwide were diagnosed with

Table 1. Molecular classification of breast carcinoma. (EGFR, epidermal growth factor receptor; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; PR, progesterone receptor) (*Cells used in this study).

Classification	Inmunoprofile	Other characteristics	Example cell lines
Luminal A	ER+,PR+/HER2-	Ki67 low, endocrine responsive, often chemotherapy responsive	MCF-7*;T47D; SUM185
Luminal B	ER+,PR+/HER2+	Ki67 high, endocrine responsive, variable to chemotherapy, HER2+ are trastusumab response	BT474, ZR-75
Claudin-low	ER-,PR-, HER2-	Ki67, E-cadherin, claudinin-3, claudinin-4 and claudinin-7 low. Intermediate response to chemotherapy	BT49, Hs578*, SUM1315
Basal	ER-,PR-, HER2-	EGFR+ and/or cytokeratin 5/6+, ki67 high, endocrine nonresponsive, often chemotherapy responsive	MDA-MB-468; SUM190
HER2+	ER+,PR+, HER2+	Ki67 high, trastusumab responsive, chemotherapy responsive	SKBR3; MDA-MB-458; HCC1954*

breast cancer. Based on immunoprofiling, the disease may be classified into at least five subtypes (Holliday, D.L. *et al.* 2011.): luminal A, luminal B, HER2, basal triple negative and claudinlow triple negative (Table 1)

Each subtype has different prognosis and treatment response (Perou, C.M *et al.* 2000). Luminal A and luminal B subtypes are amenable to hormone therapy because ER is a therapeutic target. Likewise the HER2 group is a potential candidate for trasuzumab therapy (Perou, C.M *et al.* 2000). Due to a lack of expression of ERα, PR and HER2, basal and claudin-low groups are difficult to treat, are more aggressive and often have a poor prognosis (Kwan M.L *et al.* 2009). At present, the majority of drugs used in cancer treatment is cytotoxic, have low efficacy with severe side effects and are prone to development of resistance. There is therefore an urgent need for screening programs to discover novel breast cancer therapies by using natural products.

For over 40 years, natural products such as the antracyclines, bleomycin, dactinomycin and mitomycin C have been an important source of cancer chemotherapeutic agents. About two thirds of the drugs used in clinical cancer chemotherapy are derived from natural products (Cragg, G.M. *et al.* 1997; 2002; 2005:2009). The members of four classes of plant-derived compounds are used widely as antitumor agents, namely, the bisindole, alkaloids, the camptothecin, the epipodophyllotoxins and the taxanes [Kinghorn Douglas A *et al.*2009]. The World Health Organization has estimated that the majority of the population in Asia and Africa are still largely depending on complementary and traditional medicinal plant extracts for the treatment of diseases including cancer (WHO Traditional Medicine Strategy 2002-2005). It is also important to mention in this context that phytochemicals, especially phenols and flavonoids which exhibit antioxidant properties have been shown to help reduce the risk of degenerative diseases, such as cancer and cardiovascular diseases (Garcia-Alonso, J. et al. 2006).

For these reasons, the present study aims to investigate the anti-proliferative properties of the water and ethanol extracts of selected Chinese medicinal plants with a view to discover cheaper but effective therapeutic agents. As part of this study, Hs578t monolayer cells (human triple negative breast carcinoma), HCC1954 cells (human HER2+ carcinoma), MCF-7 cells (human breast carcinoma), MCF-10A cells (human non-tumoral breast) (as shown in Figure 1), were selected to test the anti-tumor activities herbal extracts by using the

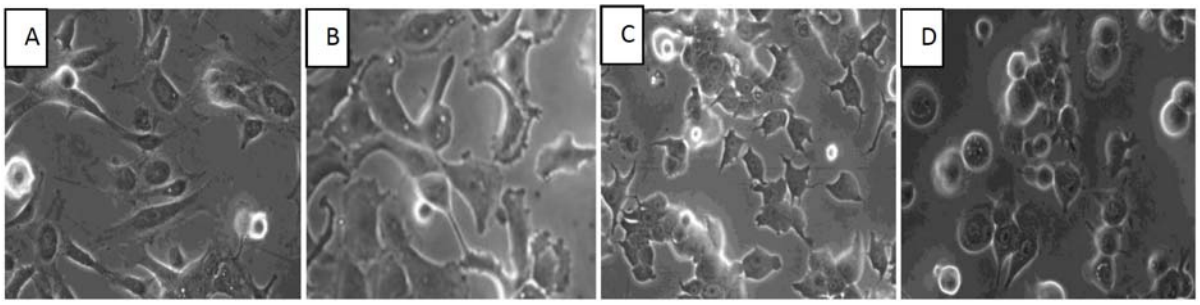


Figure 1. Microscopic images of breast cancer cell lines used in this study with inverted microscope Axiovert 200. A) MCF-10A; B) MCF-7; C) HCC1954; D) Hs578t

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method.

Materials and methods

Collection of medicinal plants and preparation of their extracts

The dried plant material was purchased from Beijing Tong Ren Tang Chinese Herbal Medicine shop**,** Sydney, Australia. The plant materials were ground to a fine powder using a grinder.

In the preparation of the aqueous extracts, approximately 3 grams of each powdered plant material was autoclaved in 50 ml water at 121°C for 1 hour. The preparations were cooled then centrifuged at 10,447 rpm for 20 min. The supernatant was transferred into a 50 ml volumetric flask and adjusted to volume with sterile water.

In the preparation of the ethanol extracts, approximately 3 grams of powdered plant material was extracted with 95% ethanol on a water bath at 70°C for 6 hours. The samples were centrifuged and the supernatant transferred into a 50 ml volumetric flask and adjusted to volume with 95% ethanol. Both the aqueous and ethanol extracts were stored at -4°C until analysis. All water and ethanol extracts were filtered and dried to remove the solvent prior to analysis. Dried extracts were re-dissolved into the appropriate sterile solvent for the bioassays.

Tumor cell lines

General growth and incubation conditions were the same as those used in earlier experiments (Royo, I. *et al.* 2003; Tormo, J.R et al. 2005) with four cell lines obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Hs578t and HCC1954 monolayer cells were maintained in RPMI medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. The MCF-7 cells were grown in MEM with 10% qualified FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, and 100 µM MEM non-essential amino acids, 0.01 mg/ml bovine insulin and MCF-10A cells, which were cultured routinely in DMEM/F12 medium modified with 10% FBS and 2 mM glutamine. All cell cultures were kept at 37ºC under a humidified atmosphere of 5% CO_2.

Antitumor assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test (Corine, V. *et al.* 1998) was applied to four cell lines for evaluation of cytotoxic activity. The assay is based on the ability of drug-treated cells to reduce the yellow water soluble substrate MTT into a dark blue formazan product that is insoluble in water. NADH is provided directly by the cells, which in turn is required for proper metabolic function. Therefore, the MTT reduction rate is an indicator of the functional integrity of the mitochondria and, hence, of cellular viability. For the *in vitro* cytotoxic activity assay, the number of cells per well were 150,000 for MCF-7, 150,000 for MCF-7, 150,000 for Hs578t, 150,000 for HCC1954 on 96-well plates. Samples were incubated with 60-80% of confluent culture of each cell line for 24 h in an atmosphere of 5% CO2 at 37ºC.

In the first step, each extract was tested at 1mg/ml in triplicate. The active extracts were further tested at different concentrations beginning at 1mg/ml, 6 or 12 point dilutions. After 24 h of incubation with the extracts, the optical density was measured at 570 nm in a Victor2^{TM} Wallac spectrofluorometer.

The inhibition percentage against the tested cell line was determined by the equation

$$Inhibition\ \ percentage = \left(\frac{1 - \left(DOcontrol\ \ negative - DOcontrol\ \ positive \right)}{DOcontrol\ \ negative - DOcontrol\ \ positive} \right) \times 100$$

DOcontrol negative DOcontrol positive

Inhibition percentage DOcontrol negative DOcontrol positive

Medium with 1% DMSO was used as negative control and medium with 2mM of methyl methane sulfonate (MMS) was used as positive control. Internal control curves of actionmycin D (250 μM, 50 μM, 12.5 μM), doxorubicina (250 μM, 50 μM, 12.5 μM), and rotenone (250μM, 125 μM) were used.

Hoechst, YO-PRO1 & PI uptake

The chromatin condensation, membrane permeability and dead cell apoptosis are three parameters involved in the apoptosis pathway. Hoechst 33342, YO-PRO-1 and PI dyes used simultaneously gives information about the apoptosis steps. Green-fluorescent YO-PRO-1 dye can enter apoptotic cells (Idziorek, T. *et al.*1995; Plantin-Carrenard *et al.* 2003) whereas red-fluorescent propidium iodide (PI) cannot. PI can only enter into necrotic cells, when the plasmatic membrane has lost its integrity (Moore, A. *et al.* 1998; Lecoeur, H. 2002). Blue-fluorescent Hoechst 33342 brightly stains the condensed chromatin of apoptotic cells and more dimly stains the normal chromatin of live cells (Ciancio, G. *et al.*1988; Green, D. *et al.*1998; Abraham, V. C. *et al.* 2008)

Cells were washed with serum free medium and incubated with three dyes (1 μl/ml medium) for 30 min at RT. The plates were read in a high content screening BD pathway 855

using YO_PRO-1 excitation/ emition (491 nm / 509 nm); PI (535 nm / 617 nm) and Hoechst 33342 (350 nm /461 nm).

Data presentation and analysis

Data from the MTT screening were analysed using the Genedata Screener program (Genedata AG, Switzerland). This program uses the *Levenberg-Marquardt* algorithm to calculate IC_{50} values. DMSO at the same concentration was used to dissolve the compound and serves as a negative control for all cells assayed, and 2mM MMS functioned as the positive control. In all results the RZ' factor was between 0.7-0.9.

Results

The cytotoxic activity on the four breast cell lines in the MTT test was measured 24 h after the treatment with herbal extracts. Two types of extracts were used for this study, ethanol extracts (38 plants) and water extracts (33 plants).

The percentage inhibition of the ethanol extracts at 1mg/ml is presented in Table 2

Table 2. *In vitro* cytotoxicity (% of inhibition at 1 mg/ml) of ethanol extracts of the plants on breast cancer cell lines.

Group	Extract	% Inhibition at 1 mg/ml (Avg# ± Stdv$)				
		Family	Luminal MCF-7	HER2+ HCC1954	TN Hs578t	Non Tumoral MCF10a
A	Akebia quinata (Houtt.)Decne.**	Lardizabalaceae	119.2±20.6	97.3±3.9	99.5±0.3	97.5±5.7
	Artemisia vulgaris L.*	Asteraceae	123.4±16.5	99.6±0.2	99.9±0.3	103.5±8.3
	Ducheshea indica (Andr.) Focke.*	Rosaceae	69.5±14.7	89.9±5.3	86.4±3.9	44.2±13.1
	Ligustrum lucidum Ait.*	Oleaceae	82.3±14.7	92.1±5.5	83.8±7.5	78.8±21.1
	Paeonia suffuticosa Sndr.*	Paeoniaceae	84.9±17.0	72.5±5.4	63.7±7.9	66.3±6.3
	Paris polyphylla (Fr.) Hand.-Mazz. (PPY),*	Liliaceae	123.9±11.5	99.67±0.4	99.9±0.3	107.5±7.9
	Platycodon grandiflorus (Jacq.) A. DC*.	Campanulaceae	122.9±16.0	99.8±0.4	100.2±0.3	107.3±7.7
	Ploygala tenuifolia Willd.*	Polygalaceae	121.9±14.2	99.9±0.2	100.1±0.3	107.2±8.5
	Plantago asiatica L.**	Plantaginaceae	115.8±10.1	97.0±5.8	95.2±8.8	105.3±7.2
	Rabdosia rubescens (Hemls.) Hara *.	Lamiaceae	109.9±19.2	99.3±0.9	98.7±1.3	100.6±8.4
	Salvia miltiorrhiza Bunge.*	Lamiaceae	109.3±10.2	76.6±10.7	95.2±1.7	88.7±11.5
	Solanum lyratum Thunb.**	Solanaceae	119.6±24.8	97.9±2.9	90.5±18.1	96.6±5.9
	Solanum nigrum L.**	Solanaceae	123.8±31.2	99.7±0.4	99.8±0.5	104.3±4.2
	Tussilago farfara L.**	Asteraceae	120.8±21.0	97.7±2.2	93.2±1.7	99.1±9.7
	Uncaria rhyncophylla (Miq.) Jacks *	Rubiaceae	107.2±9.9	92.3±3.9	84.1±9.1	58.7±2.5
	Polygonum aviculare L.*	Polygonaceae	26.33±16.6	79.8±4.6	62.3±6.4	43±11.9
B	Polygonum cuspidatum Houtt.*	Polygonaceae	61.93±9.8	63.6±17.6	40.2±17.7	17.4±5.1
	Rheum officinale L.*	Polygonaceae	84.56±8.6	83.1±5.8	60.1±21.0	17±14.8

	Eucommia ulmoides Oliv.*	Eucommiaceae	119.4±28.6	99.2±0.7	95.3±1.8	34.3±14.5
	Lysinachia christinae Hance.**	Primulaceae	42.8±8.9	97.7±1.0	98±0.9	20.6±11.1
	Alpinae officinarum Hance **	Zingiberaceae	23.75±16.9	92.2±9.1	83.8±8.5	37.2±12.1
	Sarcandra glabre (Thunb.) Nakai*	Chloranthaceae	52.71±16.2	54.9±10.3	73.8±13.5	10.8±11.4
	Scutellaria baicalensis Georgi.*	Lamiaceae	63.65±11.5	74.4±25.6	82.5±15.3	11.1±1.6
	Smilax glabra Roxb*	Smilacaceae	47.77±17.5	55.4±14.7	92.6±7.2	5.9±6.9
	Atractylodes macrocephala Koidz.**	asteraceae	117.8±16.5	71.8±18.9	49.93±10.7	1.6±3.8
	Aster tataricus L.**	Asteraceae	69.22±14.5	59.6±18.0	6.3±8.3	15.9±8.2
	Pinellia ternate (Thunb.) Breit.**	Araceae	65.25±7.1	98.0±0.8	11.6±15.0	30.9±5.3
	Poria cocos (Schw.) Wolf **	Polyporaceae	52.49±10.6	92.5±7.0	4.9±5.5	10.9±13.0
C	Rehmannia glutinosa (Gaertn.) Steud.**	Scrophulariaceae	46.81±19.9	20.9±15.3	6.6±4.6	4.7±11.0
	Scutellaria barbata Don.*	Lamiaceae	115±9.6	81.6±9.9	19.2±13.4	27.2±7.4
	Taxillus chinensis (DC.) Danser**	Fabaceae	37.16±5.6	43.8±7.5	2.5±18.7	3.8±11.5
D	Cynanchum paniculatum (Bge.) Kitag*	Asclepiadaceae	1.555±2.6	61.5±18.5	4.9±1.4	2.3±7.2
E	Lobelia chinensis Lour.**	Campanulaceae	36.09±6.5	5.2±12.9	4.1±5.1	7.7±9.1
	Saposhnikovia divaricata (Turcz.)Schischk **	Apiaceae	35.87±12.5	12.9±10.7	2.9±3.2	10.4±1.8
	Schizandra chinensis (Turcz.) Baill.**	Schisandraceae	51.42±15.2	2.8±16.9	3.2±6.4	11.8±1.4
F	Sanguisorba officinalis L.*	Rosaceae	12.28±2.5	71.6±10.6	58.9±7.8	82.6±6.2
	Sophora japonica (L.) Schott.**	Leguminosae	7.67±1.2	87.4±2.8	64.5±22.1	84.5±6.6
	Spatholobus suberectus Dunn.*	Leguminosae	0	71.3±11.4	57.3±15.6	40.2±7.1

* Extracts studied in both water and ethanol.
** Extracts without any activity in water extracts
# Average of triplicate measurements
$ Standard deviation

Group A extracts of 16 plants were active on all cell lines. Group B extracts of nine plant samples showed cyotoxicity effect on tumoral cell lines but not on the non-tumoral cell line MCF-10A. Group C extracts of six samples exhibited cytotoxicity effect on MCF-7 and HCC1954 but not on HS578t and MCF-10A. *Cynanchum paniculatum* (group D) extract showed activity only on HCC1954. Group E extracts showed low inhibition activity but showed specific cytotoxicity effect on MCF-7. Finally, the group (F) extracts exhibited cytotoxic effect on HCC1954, Hs578t and MCF-10A.

The percentage inhibition of the water extracts of the 33 plant species at 1mg/ml concentration is given in Table 3. Group 1 extracts of six plants were active on all the cell lines. Two plant extracts of group 2 exhibited cytotoxic effect on MCF-7, HCC1954 and Hs578t but not on MCF-10A. Six plant extracts of group 3 showed cytotoxicity effect only

on MCF-7 and HCC1954. Three plant extracts belonging to group 4, exerted activity on HCC1954. However, group 5 not only showed activity on HCC1954 but also on the Hs578t cell line. Nine plant extracts of group 6 exhibited activity only on MCF-7 and this activity was high in *Prunella vulgaris* among the group 6 plant extracts. Group 8 plant extracts showed activity on MCF-7 and Hs578t.

Looking at the effect of extraction process on the results, it is observed that the two types of extracts exhibited different activities. Six plants were not active in either the ethanol or water extracts. Twenty one plants of both the ethanol and water extracts showed activity but with different potencies. The ethanol extracts of seventeen plants showed activity but not their water extracts while the other plants exhibited activity in the water extract but not in the

Table 3. *In vitro* cytotoxicity (% of inhibition at 1 mg/ml) of aqueous extracts of the plants on breast cancer cell lines.

Group	Extract	% Inhibition at 1 mg/ml (Avg# ± Stdv$)				
		Family	Luminal	HER2+	TN	Non Tumoral
			MCF-7	HCC1954	Hs578t	MCF10a
1	*Artemisia vulgaris* L.*	Asteraceae	82.6±12.2	92.7±0.4	99.2±0.2	94.4±12.7
	Paris polyphylla (Fr.) Hand.-Mazz. (PPY)*	Liliaceae	123.5±21.2	95.4±5.4	96.7±2.9	106.9±3.0
	Ploygala tenuifolia Willd.*	Polygalaceae	123.9±18.9	100±0.2	100.1±0.2	108.6±3.9
	Sanguisorba officinalis L.*	Rosaceae	63.3±16.5	77.9±5.1	64.3±6.5	87.3±2.4
	Spatholobus suberectus Dunn.*	Leguminosae	46.6±11.5	69±9.1	67.7±8.5	88.3±3.8
	Rabdosia rubescens (Hemls.) Hara *	Lamiaceae	115.8±16.9	63.7±26.5	76.1±15.9	50.6±3.6
2	*Scutellaria baicalensis* Georgi.*	Lamiaceae	95.1±21.5	91.3±7.4	99.2±0.1	29.4±2.8
	Platycodon grandiflorus (Jacq.) A. DC *	Campanulaceae	51.9±15.1	46.3±11.3	42.9±16.5	10.6±1.6
3	*Acanthopanax sessiliflorus* (Rupr. & Maxim.)	Araliaceae	74.1±14.8	53.9±7.8	1.9±1.6	26.4±3.4
	Hedyotis diffusa Willd.**	Rubiaceae	68.4±16.8	97.3±2	2.9±1.5	13.7±9.3
	Leonurus japonicus Houtt**.	Lamiaceae	70.2±16.6	83±22.9	6.5±2.3	29.6±7.1
	Eucommia ulmoides Oliv.*	Eucommiaceae	67.5±16.6	75.3±12.2	0.9±0.4	2.5±2.0
	Pogostemon cablin (Blanco) Benth **	Lamiaceae	43.7±14.4	72.2±1.9	18.6±1.2	21.9±8.6
	Smilax glabra Roxb. *	Smilacaceae	48.9±10.6	43±3.8	17.1±3.1	9.6±5.7
4	*Cynanchum paniculatum* (Bge.) Kitag*	Asclepiadaceae	16.7±7.9	47.4±6.3	16.8±1.9	8.1±2.9
	Actinidia arguta (Sieb.et Zucc.)Flarich.ex Miq.	Actinidiaceae	15.2±2.1	70±19.5	3.9±2.3	2.2±2.6
5	*Rheum officinale* L	Polygonaceae	24.1±14.8	85.3±12.6	16.7±5.7	0.6±4.6
	Andrographis paniculata (Burm.f.) Wall. ex Nees**	Acanthaceae	16.9±5.9	90.9±10.2	50.9±18.4	0.7±14.3
	Salvia miltiorrhiza Bunge.*	Lamiaceae	22.3±11.5	73.5±20.2	79.2±15.5	12.3±2.7
6	*Acanthopanax senticosus* (Rupr. et Maxim) Harms **	Araliaceae	69.4±10.6	20.5±12.2	5.7±7.2	24.7±4.9
	Cyperus rotundus L.**	Cyperaceae	52.3±8.8	19.2±5.6	32.2±7.9	22.6±13.2
	Paeonia suffuticosa Sndr.*	Paeoniaceae	54.1±11.5	25.3±6.7	16.7±3.6	7.4±12.4
	Pleione bulbocadioides (Franch.) Rolfe **	Orchidaceae	40.1±11.6	10.7±6.7	16.2±7.6	6.8±6.3
	Polygonum cuspidatum Houtt.*	Polygonaceae	43.9±28.6	14.3±1.5	21.9±3.7	7.2±4.8
	Prunella vulgaris L**	Lamiaceae	110.1±11.6	1.47±6.9	18.2±7.9	13±9.0
7	*Scutellaria barbata* D.Don*	Lamiaceae	51.6±19.8	14.3±1.2	0.3±1.1	27.3±12.7
	Tussilage fárfara L	Asteraceae	58.9±10.6	0.89±5.8	14.2±4.6	2.8±4.5

	Uncaria rhyncophylla (Miq.) Jacks *	Rubiaceae	54.7±8.8	19.3±1.8	0.3±4.5	18.4±13.7
	Ducheshea indica (Andr.) Focke.*	Rosaceae	36.5±5.9	23.4±3.5	56.4±13.9	3.9±8.7
	Mahonia fortunei (Lindl.)Fedde**	Berberidaceae	21.7±9.9	7.4±2.5	36.4±9.4	6.7±6.8
8	*Ligustrum lucidum* Ait.*	Oleaceae	67.9±19.9	26.2±2.1	73.3±12.3	29.4±8
	Polygonum aviculare L*.	Polygonaceae	45.1±9.9	14±6.6	62.7±11.2	14±7.2
	Sarcandra glabra (Thunb) Nakai *	Chloranthaceae	88.2±21.2	11.7±3.0	64.4±5.3	28.1±4.7

*Extracts studied in both water and ethanol. ** Extracts without activity in ethanol extracts

Table 4. IC_{50} values of ethanol extracts on four breast cancer cell lines.

Extracts	IC_{50} mg/ml (Ethanol extracts)			
	MCF 7	HCC1954	Hs578 t	MCF-10
Platycodon grandiflorus	0.82±0.2	0.45±0.1	0.7±0.2	0.42 ±0.2
Paeouis suffuticosa	0.43±0.1	0.32±0.1	0.48±0.1	0.59±0.1
Paris polyohylla	0.0003	0.0004	0.0004	0.0004
Rabdosia rubescens	0.83±0.2	0.86±0.2	0.83±0.3	0.062±0.03
Polygonum aviculare	0.17±0.05	0.13±0.06	0.12±0.08	0.12±0.05
Polygonum cuspidatum	0.17±0.1	0.13±0.1	0.42±0.1	>1
Rheum officinale	0.95±0.3	0.78±0.25	0.27±0.08	>1
Eucomia ulmoides	0.44±0.1	0.29±0.1	0.29±0.1	>1
Lysinachia christinae	>1	0.04±0.01	0.27±0.05	>1
Rhizoma Alpinae	0.64	0.05	0.2	>1
Sarcandra glabre	>1	0.11±0.1	0.47±0.1	>1
Scutellaria baicalensis	0.48±0.1	0.23±0.1	0.25±0.1	>1
Smilax glabra	0.64	0.85	0.94	>1
Atractyloides macrocephala	0.51±0.1	0.33±0.12	0.63±0.09	>1

Table 5. Comparison of IC50 values of ethanol (E) and water (W) extracts.

Extracts	Extract	IC_{50} mg/ml		
		HCC1954	Hs578 t	MCF-10
E	*Atractyloides macrocephala*	0.51±0.1	0.33±0.12	0.63±0.09
W	*Atractyloides macrocephala*	>1	>1	>1
E	*Lysinachia christinae*	>1	0.04±0.01	0.27±0.05
W	*Lysinachia christinae*	>1	>1	>1
E	*Polygonum aviculare*	0.55±0.2	0.08±0.01	0.04±0.01
W	*Polygonum aviculare*	>1	>1	0.87
E	*Rheum officinale*	0.95±0.3	0.78±0.25	0.27±0.08
W	*Rheum officinale*	>1	>1	>1
E	*Scutelleria baicalensis*	0.48±0.11	0.06±0.01	0.40±0.1
W	*Scutelleria baicalensis*	0.64±0.12	0.13±0.02	0.44±0.1

ethanol extract (Tables 2 and 3). In general, the ethanol extracts have higher activity than the water extracts, probably due to more organic compounds present in the ethanol extracts.

This study also examined the dose-response curves of the extracts. Of the 38 ethanol extracts, only fourteen of them had good IC_{50} values (Table 4) and the rest of the plant extracts exhibited very low activity or that activity is lost at the second dilution point. None of

Table 6A. List of medicinal herbs identified in this study whose ethanol extracts have high activity against indicated breast cancer cell lines and minimum toxicity to non-tumoral cells.

Group	Plant name	Activity of ethanol extracts against cell lines
A	*Polygonum aviculare* L	HCC1954, Hs578t
	Ducheshea indica (Andr.) Focke	MCF-7, HCC1954, Hs578t
B	*Polygonum cuspidatum* Houtt	MCF-7, HCC1954
	Rheum officinale L *	MCF-7, HCC1954, Hs578t
	Eucommia ulmoides Oliv *	MCF-7, HCC1954, Hs578t
	Lysinachia christinae Hance	HCC1954, Hs578t
	Alpinae officinarum Hance	HCC1954, Hs578t
	Scutellaria baicalensis Georgi *	MCF-7, HCC1954, Hs578t
	Atractylodes macrocephala Koidz	MCF-7
	Sarcandra glabra (Thunb) Nakai *	Hs578t
C	*Scutellaria barbata* Don	MCF-7, HCC1954
	Aster tataricus L	MCF-7
	Pinellia ternate (Thunb.) Breit	MCF-7, HCC1954
F	*Spatholobus suberectus* Dunn	HCC1954

Table 6B. List of medicinal herbs identified in this study whose water extracts have high activity against indicated breast cancer cell lines and minimum toxicity to non-tumoral cells

Group	Plant name	Activity of aqueous extracts against cell lines
2	*Scutellaria baicalensis* Georgi *	MCF-7, HCC1954, Hs578t
3	*Acanthopanax sessiliflorus* (Rupr. & Maxim.)	MCF-7
	Hedyotis diffusa Willd	MCF-7, HCC1954
	Leonurus japonicus Houtt	MCF-7, HCC1954
	Eucommia ulmoides Oliv *	MCF-7, HCC1954
	Pogostemon cablin (Blanco) Benth	HCC1954
4	*Rheum officinale* L *	HCC1954
	Actinidia arguta (Sieb.et Zucc.)Flarich.ex Miq.	HCC1954
5	*Andrographis paniculata* (Burm.f.) Wall. ex Nees	HCC1954
	Salvia miltiorrhiza Bunge.	HCC1954, Hs578t
6	*Acanthopanax senticosus* (Rupr. et Maxim) Harms	MCF-7
	Prunella vulgaris L	MCF-7
8	*Ligustrum lucidum* Ait	MCF-7, Hs578t
	Sarcandra glabra (Thunb) Nakai *	MCF-7, Hs578t

the plant extracts from group B inhibited MCF-10 cells (non-tumoral cells) and this group of herbs is the most interesting for further study involving isolation and structure elucidation for potential anti-cancer compounds. Extracts belonging to group A showed similar activities against tumoral and non-tumoral cell lines and may be interesting for further investigation. A comparison of the IC_{50} values of the active ethanolic extracts and corresponding aqueous extracts is presented in Table 5. The IC_{50} values of both the water and ethanol extracts of *S. baicalensis* are similar in all breast cancer cell lines (Figure 2). The promising anti-cancer activity exhibited by the extract of *S. baicalensis* and the fact that this extract was inactive on normal cell line (MCF10) indicates that it is worthwhile to pursue further studies on this extract (Table 5). The apoptotic studies started with the extract of *S. baicalensis*, due to its good dose-response curves, and the fact that the activity not depend on the extract methods.

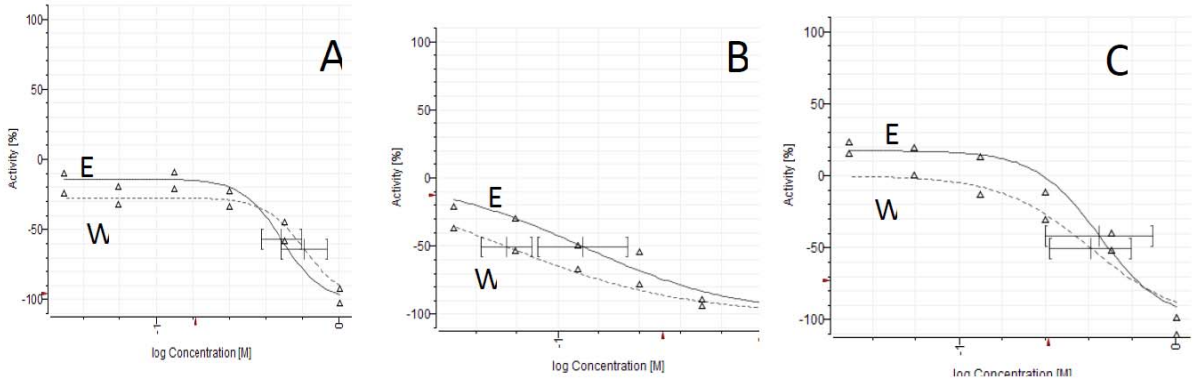
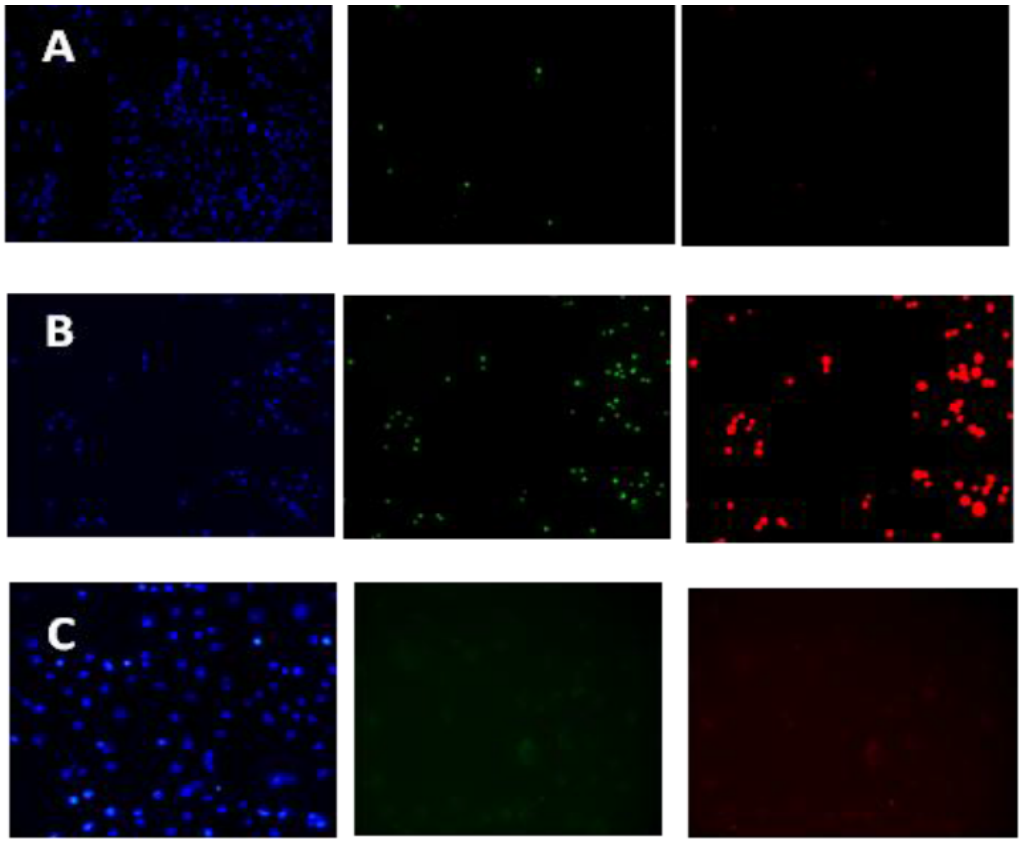


Figure 2. Comparative dose-response curves between ethanol extracts and water extracts of *S. baicalensis*. A) MCF-7 B) Hcc1954 and C) Hs578t.

The results of Hoeschst, Yo-Pro-1 and PI uptake studies are presented in Figure 3. The results with 1% DMSO showed low intensity of Hoechst, Yo-Pro and PI (Figure 3A). The HCC1954 cells treated with doxorubicin, showed an increase of intensity in Hoechst and the yo-pro and the PI signal appeared. The *S. baicalensis* extract was tested on MCF-10A (Figure 3C), HCC1954 (Figure 3D) and Hs578t (Figure 3E) cell lines. *S. baicalensis* did not



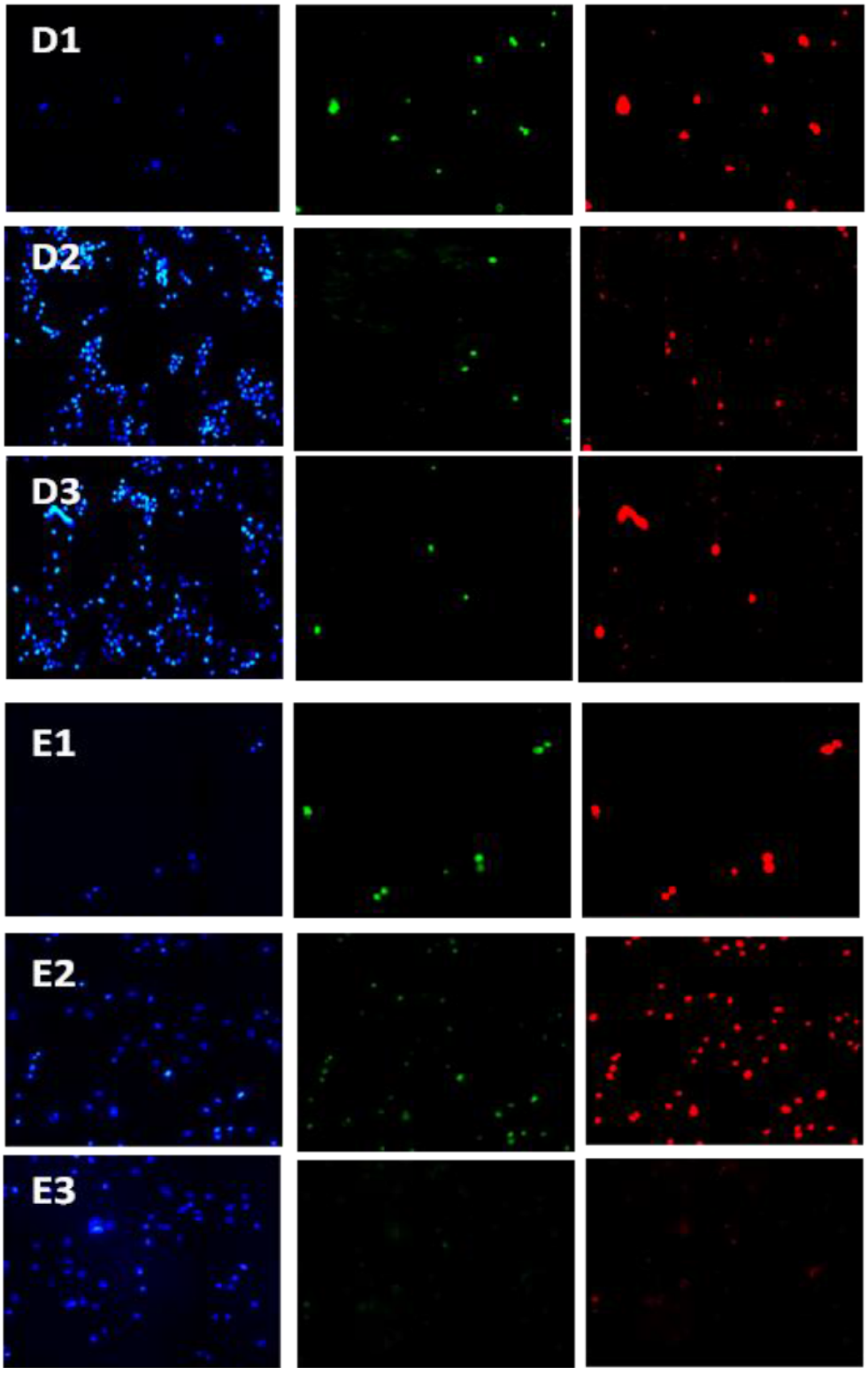


Figure 3. Images of HCS of Hoechst 3342 (blue) Yo-Pro_1 (green) and IP (red). A) DMSO 1%. B) Doxorubicin. C) *Stulleraria baicalensis* at 1mg/ml on MCF-10A. D1) *Stulleraria baicalensis* at 1mg/ml on Hcc1954 D2) *Stulleraria baicalensis* at 0.25mg/ml on Hcc1954 D3) *Stulleraria baicalensis* at 0.031mg/ml on Hcc1954 E1) *Stulleraria baicalensis* at 1mg/ml on Hs578t; E2) *Stulleraria baicalensis* at 0.25mg/ml on Hs578t; E3) *Stulleraria baicalensis* at 0.031mg/ml on Hs578t.

show cytotoxicity on MCF-10 and also did not show apoptosis signals on this cell line. The *S. baicalensis* extract (1mg/ml) showed a very low signal in HCC1954 and Hs578t due to high cel1 death (95%). At lower concentrations, this extract also exhibited significant cell death in HCC1954 cell line (65% at 0.25 mg/ml and 13% at 0.03mg/ml). However, at these concentrations, we could detect cells in a early apoptosis phases, due to an increase in both Hoechst and Yo-Pro intensities were detected. At the same concentrations, some cells were in the necrosis phase, so PI was inside the cells, indicated that the plasmatic membrane had lost its integrity. The activity of *S. baicalensis* extract on Hs578t was similar to HCC1954 at 1mg/ml but 35% of cell death was noticed at the concentration of 0.25 mg/ml on Hs578t and the extract also exerted high levels of apoptosis and necrosis. An increase of Hoechst was observed at low concentration of *S. baicalensi* on Hs578t only.

Discussion

Anti-cancer activity of the aqueous and ethanol extracts of selected medicinal plants has been evaluated using the MTT assay against three cancer cell lines (MCF7, Hcc1954, Hs578t) and one control non-tumoral cell line (MCF-10A). The MTT assay is a commonly used method to study the activity of natural products on cell proliferation, cell viability and cytotoxicity. Sixteen ethanol extracts and nine aqueous extracts showed significant anti-cancer activity against all the cell lines studied, including non-tumoral cell lines (Tables 2 and 3). The dose response (IC_{50}) experiments showed difference in activity at the different concentrations studied (Table 4).

Ethanol extracts of the nine plants - *P. cuspidatum; R. officinale; E. ulmoides; L. christinae; Rhizoma A. officinarum; S. glabre; S. baicalensis; S. glabra and A. macrocphala* and aqueous extracts of two plants - *S. baicalensis* and *Platycodon grandiflorum* showed activity on all breast cancer cell lines studied but did not exert cytotoxic activity against non-tumoral cell lines. The anti-tumoral properties of some of these extracts have been reported in the past (Ajaya Kumar *et al.* 2004; Yanwen *et al.* 2005; Hsu, *et al.* 2008; Cho WCS, 2010).

As can be seen from the results, many of the plants investigated here exhibited activity against several breast cancer cell lines without significant toxicity towards normal cells. The plants identified in this study with high activity against various breast cancer cell lines and with minimum toxicity to normal cells are listed in Table 6. As can be seen from Table 6, ethanol extracts are active against more cancer cell lines than those of water extracts. In addition ethanol extracts have generally exhibited higher activity than water extracts (Table 5). Breast cancer activity results summarized in Table 6 also reveal that the ethanol extracts of "Group B" herbs and the water extracts of "Group 3" herbs are extremely active against several cell lines and have minimum toxicity to non-tumoral cells. These groups of herbs are therefore of great interest for further investigations involving isolation and characterization of novel anticancer therapeutics. This is also expected to lead the way towards a detailed study of structure– activity relationship of isolated anti-cancer agents. Some plant extracts inhibited only one or two cell lines, which indicate their narrow spectrum of activity, and this could be due to a specific profile of compounds responsible for the activity. These extracts could be of interest for future studies.

In general, ethanol extracts presented higher activity than the aqueous extracts (Tables 2, 3 and 5), although some plants presented similar percentage of inhibition in both extra-

cts at 1 mg/ml. In the apoptosis study, the ethanol extract of *S. baicalensis* produced high level of cell death at high concentration, but at the lower concentration the effects leading to cell death, namely, chromatin condensation, membrane permeability and finally loss of membrane integrity was observed. This effect was more evident on Hs578t; at 1mg/ml *S. baicalensis* where a high level of cell death was observed and all the cells still alive showed signs of initial apoptosis as can be seen from the Figure 3 E1. However for the 0.25 mg/ml ethanol extract of *S. baicalensis*, the cell death measured with MTT was not significant at 35%, but as shown Figure 3 E2, there is high levels of Yo-Pro inside the nucleus and even high levels of PI. This indicates that the plasmatic membrane not only had increased permeability, but had also lost structural integrity; two characteristics leading to apoptosis. A similar effect was detected in HCC1954, although the level of apoptosis under the same conditions was lower.

These results show that *S. baicalensis*, has antitumoral effect over these cell lines; so although the IC_{50} over HCC1954 was lower than in Hs578t, the latter still gave high apoptosis level. This result is particularly interesting because the apoptosis signal was not observed over the normal cell line control (MCF10A). Therefore the *S. baicalensis* extract causes cell death in Hs578t and Hcc1954 but not in MCF10A.

In conclusion, this study demonstrates that the plants are an excellent source for the discovery of potent antitumor agents against breast tumor cell lines. Some of the plants studied here have presented high specificity and high selectivity in cytotoxic effect between cancer cell lines and non-cancerous cell lines. This presents the prospect that these plants are extremely likely to contain anti-cancer compounds which could serve as a lead for discovering novel anti-cancer drugs. Further studies are required to isolate the active constituents and to gain a better knowledge of the mechanism of action to understand these interesting natural products as a pathway to future anti-cancer drugs.

Acknowledgements

This work was funded by the University of Western Sydney and the Fundación MEDINA, a public-private partnership of Merck Sharp & Dohme de España S.A./Universidad de Granada/Junta de Andalucía.

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