Standardized Extract from *Caesalpinia spinosa* is Cytotoxic Over Cancer Stem Cells and Enhance Anticancer Activity of Doxorubicin

Tito A. Sandoval,* Claudia P. Urueña,* Mónica Llano,* Alejandra Gómez-Cadena,*
John F. Hernández,* Luis Gonzalo Sequeda,† Alix E. Loaiza,† Alfonso Barreto,*
Shaoping Li‡ and Susana Fiorentino*

*Department of Microbiology

†Department of Chemistry, Pontificia Universidad Javeriana
Grupo de Investigación Fitoquímica Universidad Javeriana (GIFUJ)
Bogotá, Colombia

‡Institute of Chinese Medical Sciences
University of Macau, Macau, P.R. China

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Abstract: Cancer stem cells (CSC) are the primary cell type responsible for metastasis and relapse. ABC-transporters are integral membrane proteins involved in the translocation of substrates across membranes protecting CSC from chemotherapeutic agents. A plant extract derived from *C. spinosa* (P2Et) previously investigated for its antitumor activity has been shown to reduce lung and spleen metastasis in mice that have been transplanted with breast cancer cells, suggesting that P2Et has a significant activity against cancer stem cells (CSC). P2Et extract was thoroughly characterized by HPLC/MS. The cytotoxicity of P2Et extract was evaluated using a MTT assay in human and murine cell lines with different profiles of resistance, by Pgp overexpression or by enrichment in cancer stem cells. The synergistic effect of P2Et with doxorubicin was evaluated *in vitro* in several cell lines and *in vivo* in mice transplanted with TS/A cells, a highly resistant cell line and enriched in CD44⁺CD24^{low/-} CSC. The chromatographic fingerprint of P2Et extract revealed 13 gal-tannins. We also found that P2Et extract was cytotoxic to cells regardless of their resistant phenotype. Similarly, complementary activities were observed as drug efflux reversion and

Correspondence to: Prof. Susana Fiorentino, Department of Microbiology, Pontificia Universidad Javeriana, Carrera 7 #43-82 Edificio 50 Laboratorio 101, Bogotá, Colombia. Tel/Fax: (+57) 3208320 (ext. 4103). E-mail: susana.fiorentino@javeriana.edu.co

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antioxidant activity. Short-treatment with P2Et extract, revealed a synergistic effect with doxorubicin in resistant cell lines. *In vivo* the P2Et increases mice survival in a TS/A breast cancer model associated with augmentation of calreticulin expression. Our results suggest that P2Et treatment could be used as adjuvant along with conventional chemotherapy to treat tumors with a MDR phenotype or with high frequency of CSC.

Keywords: Gallotannins; *Caesalpinia spinosa*; Multidrug Resistance; Synergy; P-glycoprotein.

Introduction

Over the last century, the goal of conventional medicine has been to identify or create molecules that act on single molecular targets with high efficiencies and few side effects; this concept was postulated by Ehrlich and is referred to as the “magic bullet” (Strebhardt and Ullrich, 2008). However, this reductionist approach is not effective for treating complex diseases where multiple mechanisms are responsible for development, progression and resistance (Efferth and Koch, 2011). Thus, it is not surprising that single molecules fail in controlling cancer late stages (Stensland *et al.*, 2014). Mechanisms underlying multidrug resistance in cancer, such as P-glycoprotein (Pgp) over-expression, are triggered after single drug therapy, increasing the risk of late relapse, and unfortunately, pharmacological Pgp modulation has not been very effective and on the contrary, turned out to be highly toxic (Gottesman, 2002).

Natural products can exert antitumor activity by overcoming drug resistance (Cort and Ozben, 2015). In fact, certain compounds, such as gallic acid derivatives, reverse resistance in cancer cells (Kitagawa *et al.*, 2005) and are thus good candidates for this specific activity.

Phytotherapy is a multi-targeted approach where multiple secondary metabolites exert an antagonistic or a synergistic effect for a final therapeutic response (Rather *et al.*, 2013). Therefore, a complex extract with multiple molecules engaged in complementary activities could become an effective therapy for cancer. Nevertheless, using complex phytotherapeutics require chemical characterization for quality control, safety and efficacy (Li *et al.*, 2011). In addition, assessing the relationship between the chemical components in a complex extract and the antitumor effects may allow us to understand the molecular mechanisms involved, adding strong evidence for using herbal therapy to treat cancer.

Previously, we showed that a gallotannin-rich extract obtained from pods and seeds of *C. spinosa* (P2Et extract), induces a loss of mitochondrial membrane potential ($\Delta\psi_m$), nuclei fragmentation and caspase-dependent apoptosis *in vitro* in several tumor cells (Castaneda *et al.*, 2012). Interestingly, P2Et also reduces lung and spleen metastasis in the 4T1 mouse breast cancer model (Uruena *et al.*, 2013), suggesting that it has some activity against CSC, the primary cell type responsible for tumor metastasis, drug-resistance and relapse (Adorno-Cruz *et al.*, 2015).

In this study, we thoroughly characterized the P2Et extract using HPLC/MS and demonstrated that this group of polyphenols exhibits cytotoxic activity in lines with different profiles of resistance related to MDR pumps or CSC phenotype; also, the synergistic effect of P2Et with doxorubicin *in vitro* and *in vivo* was evaluated.

Materials and Methods

Plant Material

Caesalpinia spinosa (Molina) Kuntze (Divi-divi or tara) pods were collected in Villa de Leyva, Boyacá, Colombia and identified by Luis Carlos Jiménez from the Colombian National Herbarium (voucher specimen number COL 523714, Colombian Environmental Ministry agreement number 0454 related to the use of genetic resources and derived products). The P2Et extract was obtained as previously described (Castaneda *et al.*, 2012).

Reverse-Phase HPLC Fingerprinting

Chromatographic fingerprinting of P2Et was performed in a HPLC Agilent 1200 series instrument with an Agilent 1200 PDA detector ($\lambda = 254$ nm) (Agilent, Wilmington, USA); the compounds were separated using an Agilent Eclipse XD8 C18 column (4.6×250 mm, $5 \mu\text{m}$). Mobile phase gradient of acetonitrile and phosphoric acid 0.1% at flux of 1 mL/min; Data acquisition and analysis were performed using Chemstation v 3.0 Software.

HPLC/MS/UV Analysis

P2Et extract was analyzed using liquid chromatography coupled to mass spectrometry and UV/VIS, an LC system Agilent 1290 series instrument with an Agilent 1290 DAD detector and an Agilent 6410 triple quadrupole detector (Agilent Technologies USA) equipped with an Agilent Zorbax SB-C18 (4.6×50 mm, $1.8 \mu\text{m}$) column. The absorbance was recorded at 254 nm, and the electrospray negative ionization gas temperature was as follows: 325°C gas flow: 10 L/min, capillary voltage 4000 V, nebulizer: 35 psi; mobile phase gradient of acetonitrile and formic acid.

Cell Lines

The phenotyping of all cells was performed by flow cytometry and can be found in Table 1. MES-SA/DX5 (ATCC® CRL-1976™) was used to obtain MES-SA Pgp⁺ and Pgp⁻ via cell sorting using a BD FACS Aria II; K562 Pgp⁺ and Pgp⁻ were obtained as a gift from Professor Karl Tsim (HKSTU, Hong Kong) and the lines were morphologically compared with K562 (ATCC® CCL243™). Pgp expression was confirmed by labelling with Anti-Human P-glycoprotein (BD Pharmingen, 557002). 4T1 was provided by Dr. Alexzander Asea (Texas A&M Health Science Center College of Medicine, Temple, TX) and maintained with 6-tioguanine as method of selection. Additionally, the line was tested in BALB/c mice in order to validate its tumorigenic and metastatic ability. Sub-line 4T1 *side population* was obtained by long-exposure of parental line with doxorubicin and followed cell sorting according to Telford (2013) and was maintained in 6-tioguanine as method of selection. Sub-line 4T1 H17 was obtained from lung metastasis after three *in vivo* passages according to Lelekakis (Lelekakis *et al.*, 1999) and Pulaski and Ostrand-Rosenberg (2001) and cultured in 6-tioguanine to guarantee its identity. TS/A line was

Table 1. Drug-Resistance and Cancer Stem Cell-Related Markers

Cell Line	Species	Sub-line	MDR Proteins				% of Cells			
			Pgp (MFI)	Pgp	BCRP	MRP-1	CD44 ⁺ CD24 ⁻	ALDH ⁺	Sca-1	CD38 ⁻ CD34 ⁺
MES-SA	Human	Pgp ⁺	13299	170	0.90	2.14	1.55	68.9	N/R	N/R
		Pgp ⁻	322	1.0	1.0	1.0	39.0	32.1	N/R	N/R
K562	Human	Pgp ⁺	16480	3175	1.1	1.27	N/R	25.5	N/R	17.8
		Pgp ⁻	286	1.0	1.0	1.0	N/R	22.4	N/R	16.5
4T1	Murine	WT	N/E	1.0	1.0	1.0	0.07	11.4	42.1	N/R
		H17	N/E	0.65	0.44	1.45	5.58	82.4	10.7	N/R
		Side population	N/E	5.86	2.85	0.84	0.30	5.42	14.8	N/R
TS/A	Murine		N/E	0.56	0.84	0.85	87.6	5.14	42	N/R

Note: Human Pgp expression (Medium fluorescence Intensity, MFI) and CSC phenotype (CD44, CD24, ALDH, Sca-1, CD38, and CD34) was evaluated by Flow cytometry. Human BCRP and MRP1 and murine Pgp, BCRP and MRP1 was analyzed by RT-PCR. N/E: not evaluated; N/R: not relevant.

provided by Dr. Clotilde Théry (Curie Institute, Paris, France) and functionally was tested in BALB/c mice in order to validate its tumorigenic and metastatic ability. The B16 cell line was provided by professor Pedro Romero (Lausanne University, Lausanne, Switzerland). This line was tested in C57BL/6 mice in order to validate its tumorigenic and metastatic ability. The cells were tested for mycoplasma using a Myco-Probe Mycoplasma Detection Kit (R&D Systems, Minneapolis, MN) and were maintained with ciprofloxacin (0.5 µg/mL).

In Vitro Cytotoxicity Assays

Cytotoxic effects were evaluated using tumor cells and methylthiazole tetrazolium (MTT) as previously described (Uruena *et al.*, 2013). 3×10^3 cells/well were seeded in 96-wells plates and treated at different concentrations for 48 h; doxorubicin was used as the positive control and ethanol or DMSO, as the negative control.

Real Time and Conventional PCR

Total RNA of cells was extracted using TRIzol LS reagent according to the manufacturer's instructions (Life technologies Corporation, Invitrogen, NY, USA). The quality and quantity of RNA were assessed with a NanoDrop spectrophotometer (NanoDrop Technologies). cDNA was synthesized with SuperScripts III Reverse Transcriptase, following the manufacturer's instructions (Invitrogen). For real-time PCR reaction 30 ng of cDNA, DNA Master Plus SYBRGreenI (Roche Applied Science, IN, USA) and 250 ng of forward and reverse primers in a total volume of 20 µL. Reactions were performed in two independent experiments with duplicates each one using Spectrum 48 Real Time Thermal Cyclers (ESCO). Thermal cycling conditions were: an initial denaturing step at 95°C for 10 min, 40 cycles at 95°C for 10 s, 60°C for 10 s, 72°C for 10 s, followed by a dissociation stage. Amplified product specificity was verified by a post-amplification melt curve analysis. The relative expression level of each transcript was normalized to the endogenous control gene GAPDH. Relative expression was calculated using the comparative method $2^{-\Delta\Delta CT}$.

Flow Cytometry

Phenotype of cancer cells were assessed by flow cytometry in a FACSAria II instrument (BD biosciences). An ALDEFUOR assay kit (StemCell Technologies, Vancouver, BC) was used following manufacturer instructions; anti-mouse-CD44 (BD Biosciences), anti-mouse-CD24 (BD Biosciences), anti-mouse-Sca-1 (Ebiosciences), anti-human-CD38 (BD Biosciences), anti-human-CD34 (BD Biosciences) and anti-human-Pgp (BD Biosciences), results were analysed with FlowJo software (Tree Star Inc). To evaluate cell membrane CRT expression, cells were plated in 6-well plates and treated the following day with P2Et, Dx (positive control), alone or in combination at the IC₅₀ concentrations or ethanol (0,05%) for 24 h. Cells were stained as previously described (Uruena *et al.*, 2015). The

mean fluorescence intensity (MFI) of stained cells was assessed within the live gate with LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Life Technologies, Thermo scientific Eugene US).

Pgp Drug-Efflux Assay

Pgp drug-efflux activity was measured using perchlorate of tetramethylrhodamine methyl ester (TMRM) based on the report by Minderman *et al.* with small modifications (Minderman *et al.*, 2002). A single cell suspension was obtained via trypsin/EDTA treatment, and 2.5×10^5 cells were incubated with treatments or cyclosporine A (CsA, Sigma-Aldrich, Saint Louis MO) as positive control, for 1 h at 4°C and 40 nM TMRM was then added and incubated for 1 h on ice. Next, the samples were incubated at 37°C for Pgp activation for 3 h. After the incubation, 2 mL of ice-cold PBS was added, and the samples were centrifuged at $200 \times g$ for 5 min at 4°C, washed twice with cold PBS (2% FCS) and analyzed in a BD FACS Aria II cytometer. The analyses were performed with viable cells selected using a LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Life Technologies, Thermo scientific Eugene US).

Synergy Effect for Drug Pre-Treatment

Synergistic effects were determined following the report of Eid *et al.* (2012) with certain modifications. The cells were pre-treated with drugs at concentrations equivalent to the IC_{50} and $0.2 \times IC_{50}$ for 3 h. Next, the media was exchanged for fresh media with doxorubicin (Dx) at serial dilutions and the cells were incubated for 48 h. To determine synergistic effects, viability was plotted against the Dx concentration, and the drug reduction index (DRI) and combination index (CI) were calculated for each combination as follows:

$$DRI = \frac{IC_{50, Dx}}{C_{Dx, 50}} \quad CI = \frac{C_{Dx, 50}}{IC_{50, Dx}} + \frac{C_{P2Et}}{IC_{50, P2Et}}$$

$C_{Dx, 50}$ is the IC_{50} of doxorubicin in combination, C_{P2Et} is the concentration of P2Et used in the combination experiment and $IC_{50, Dx \text{ or } P2Et}$ are the IC_{50} for each alone.

Mammosphere Culture

Mammosphere culture was performed following the procedure of Chen *et al.* (2012) with modifications. In brief, 3×10^3 cells were seeded in a 24-well plate with free-serum media that contains DMEM/F12 1:1 (Gibco, life technologies, NY, USA), N2 supplement (Gibco, life technologies, NY, USA), B27 without vitamin A (Gibco, life technologies, NY, USA), 100 U/mL penicillin and 100 µg/mL streptomycin in ultra-low attachment plates (Corning, NY, USA). Next, the treatment with sub-lethal concentrations of treatments were added every day for 6 days. Finally, on the 7th day of culture, the mammospheres were collected, washed twice with PBS and centrifuged at $100 g \times 5 \text{ min}$ and all the mammospheres with diameter above 50 µm were counted under microscope.

In Vivo Tumor Development Experiments

BALB/c mice were subcutaneously inoculated with 1×10^4 TS/A cells in the mammary fat pad, treatments were administered intraperitoneally (IP), mice were divided in groups as follows: Control (PBS, $n = 6$), doxorubicin group (Dx, 1 mg/Kg IP, once per week, $n = 6$), P2Et group (18.7 mg/Kg, twice per week, $n = 6$) and combined therapy group (Dx, 1 mg/Kg, once per week + P2Et, 18.7 mg/Kg, twice per week, $n = 7$), tumor volume was assessed by measuring with a Vernier caliper and tumor volume was calculated as follows: $V \text{ (mm}^3\text{)} = [(\text{Width})^2 \times \text{Length}]/2$.

Animals

Female BALB/c mice (6 to 12 weeks old) were purchased from Charles Rivers Laboratories International, Inc. (Boston, MA, USA) and housed in an animal research facility following the established protocols of the Ethics Committee of the Science Faculty and National and International Legislation for Live Animal Experimentation (Colombia Republic, Resolution 08430, 1993; National Academy of Sciences, 2010). Mice were housed in polyethylene cages with food and water *ad libitum*, a controlled temperature, and a 12 h light/dark cycle. Before treatment, mice were acclimated for 1 week under standard conditions. This project and the format using animals was approved by the ethics committee of the Science Faculty on May 18, 2012.

Statistical Analysis

All assays were carried out three times, unless otherwise stated. All data are expressed as the mean $\pm$ standard deviation. The IC_{50} values were calculated with a three parameter non-linear regression curve (GraphPad Prism 5.0, GraphPad Software, Inc., CA, USA). A one-way analysis of variance Dunnett's post hoc test was used to analyze the differences within a control group, mammosphere number differences with control were analyzed by Mann-Whitney test. Finally, Kaplan-meier survival analysis were analyzed in (GraphPad Prism 5.0, GraphPad Software, Inc., CA, USA) and differences between survival curves were assessed by comparison log-rank Mantel-Cox test. For all cases a p -value less than 0.05 was considered significant.

Results

Chromatographic Fingerprint for P2Et Extract Showing Galloylquinic Acid Separation

A HPLC/MS/UV analysis for the P2Et extract was performed. TIC, shown in Fig. 1, condenses the data of thirteen primary compounds that were identified by fragmentation analysis and based on the study performed by Clifford (Clifford *et al.*, 2007).

Next, to perform HPLC fingerprint analysis, gallic acid and ethyl gallate were isolated, and their structures were confirmed using ^1H , ^{13}C NMR and mass spectra analyses

Compound ID	Compound Name	t _R (min)	Molecular ion (m/z)	Fragment ions (m/z)	Adducts ions (m/z)
1	n-O-galloylquinic acid	1.406	343	191, 169, 125	
2	gallic acid	2.183	169	125	
3	di-O-galloylquinic acid	4.261	495.1	342.9, 168.9, 130.9	571 [M-2H+Na ⁺]
4	n-galloylquinic acid ethyl ester	5.223	371	343, 320.7, 239, 169.1, 130.7	
5	tri-O-galloylquinic acid	6.296	647	345.1, 174.9, 130.8	668.9[M-2H+Na ⁺]
6	tetra-O-galloylquinic acid	7.073	799	647, 247.1, 174.8, 131.0	668.9[M-2H+Na ⁺]
7	tri-O-galloylquinic acid	7.354	647	494.9, 334.1, 247, 175.1, 131.1	669.1[M-2H+Na ⁺]
8	ethyl gallate	8.101	197	124	
9	*unknown tetra-GCQ	10.099	799.2	523.1, 399, 323, 175.0, 130.9	544.9[523-2H+Na ⁺]
10	tri-O-galloyl quinic acid ethyl ester	13.583	675	475.3, 337.4, 261.1, 174.9, 130.9	697.2[M-2H+Na ⁺]
11	*di-O-galloyl quinic acid ethyl gallate ester	13.901	674.8	474.9, 348.9, 261.0, 197.0, 175.2, 130.9	
12	tri-O-galloyl quinic acid ethyl ester	14.168	675	475.3, 337.4, 261.1, 174.9, 130.9	697.2[M-2H+Na ⁺]
13	*tri-O-galloyl quinic acid ethyl gallate ester	16.306	827	349.0, 197.1	[349-2H+Na ⁺]

Figure 1. Chromatographic fingerprinting and TIC analysis for P2Et. (A) Identified compounds in HPLC: 1) n-O-galloylquinic acid (t_R : 4.231 min); 2) gallic acid (t_R : 5.724 min); 3) di-O-galloylquinic acid (t_R : 13.619); 4) galloylquinic acid ethyl ester (t_R : 15.002); 5) tri-O-galloylquinic acid (t_R : 18.404 min); 6) tetra-O-galloylquinic acid (t_R : 20.123 min); 7) tri-O-galloylquinic acid (t_R : 20.892 min); and 8) ethyl gallate (t_R : 22.947 min). (B) List of P2Et compounds identified using HPLC/MS: *Compound tentatively assigned, bold numbers indicate the base peak.

(Fig. S1); in this study, both compounds were used as quantitative markers (free gallic acid, 4.88% of total and ethyl gallate, 1.88% of total). Eight of the identified compounds were assigned to HPLC fingerprint as follows (Fig. 1): **1.** n-O-galloylquinic (t_R : 4.231 min); **2.** gallic acid (t_R = 5.64); **3.** di-O-galloylquinic acid (t_R : 13.619); **4.** galloylquinic acid ethyl ester (t_R : 15.002); **5.** tri-O-galloylquinic acid (t_R : 18.404 min); **6.** tetra-O-galloylquinic acid (t_R : 20.123 min); **7.** tri-O-galloylquinic acid (t_R : 20.892 min); **8.** ethyl gallate (t_R = 22.70). IUPAC nomenclature is used for all identified compounds (Table S1).

P2Et and its Isolated Compounds Exhibit Cytotoxic Activity in Resistant and Non-Resistant Cell Lines

P2Et exhibited a significant antioxidant activity (11.76 ± 0.58), similar to ethyl gallate (11.37 ± 0.21) but lower than gallic acid (16.62 ± 4.55). All the results are expressed according to the positive control TROLOX, an analog of vitamin E (Table S2). In addition, P2Et, ethyl gallate and gallic acid exhibited cytotoxic activity on human cells lines with or without a multidrug resistance phenotype: MES-SA/Dx5, K562, 4T1, TS/A cells and two sub-lines developed in our lab: 4T1 H17 cells and 4T1 *side population*. Flow cytometry analysis revealed high expression of Pgp in human cell lines with MDR phenotype; other pumps like BCRP and MRP1 were confirmed at level of mRNA in susceptible and resistant cells with similar expression in all cases. Now, in terms of CSC phenotype, higher rate of ALDH⁺ cells in MES-SA/Dx5 Pgp⁺ compared to Pgp⁻ was observed. In contrast, no changes were observed for K562 between susceptible and resistant variants according to CD38⁻CD34⁺ or ALDH⁺. Finally, in murine cell lines the 4T1 *side population* exhibited the highest expression of MDR pumps and 4T1 WT parental line and *side population* exhibited same rate of cells with CSC phenotype, on the contrary, a higher frequency of CD44⁺CD24^{-/low} and ALDH⁺ cells in the variant 4T1 H17 was observed. Finally, TS/A exhibited a high frequency of CSC CD44⁺CD24^{-/low} cells (Table 1).

Next, the resistance to doxorubicin in cell variants that overexpressed MDR pumps (MES/SA and K562 Pgp⁺) or those which exhibited CSC phenotype (4T1 H17 and TS/A) were confirmed (IC₅₀ above 1.0 μ M, Table 2). Interestingly, P2Et and its compounds were cytotoxic against all cell lines, with only slight changes in the toxicity against cell variants with over-expression of MDR proteins or detoxifying enzyme ALDH. In addition, we

Table 2. Cytotoxic Activity of the P2Et Extract, Gallic Acid and Ethyl Gallate in Resistant and Non-Resistant Cell Lines

Cell Line	Ethyl Gallate	Gallic Acid μ g/ml	P2Et	Doxorubicin μ g/ml (μ M)
MES-SA/Dx5 (Pgp ⁺)	33.69 ± 8.70	50.40 ± 1.00	25.86 ± 7.90	> 5 (>10)
MES-SA/Dx5 (Pgp ⁻)	15.60 ± 4.80	79.14 ± 7.44	64.40 ± 3.60	0.209 ± 0.03 (0.36 ± 0.05)
K562 MDR (Pgp ⁺)	21.17 ± 3.35	68.90 ± 7.90	113.90 ± 11.2	> 5 (>10)
K562 WT (Pgp ⁻)	25.94 ± 3.54	61.39 ± 7.10	88.20 ± 8.1	0.232 ± 0.08 (0.40 ± 0.13)
4T1 <i>side population</i>	13.4 ± 10.0	65.7 ± 10.8	56.10 ± 1.50	3.7 ± 0.06 (6.40 ± 0.10)
4T1 H17	7.97 ± 1.28	112.65 ± 2.35	50.45 ± 4.39	0.753 ± 0.08 (1.30 ± 0.13)
4T1 WT	8.01 ± 3.00	38.57 ± 7.40	25.23 ± 3.10	0.186 ± 0.05 (0.32 ± 0.10)
TS/A	9.43 ± 3.2	40.10 ± 10.9	32.86 ± 0.40	1.108 ± 0.43 (1.910 ± 0.79)

Note: Data are displayed as the mean $\pm$ SD ($n = 3$).

observed that ethyl gallate was the most cytotoxic compound in almost all cases and remarkably P2Et was very effective in MES/SA-Dx5 Pgp⁺ cells.

P2Et and its Compounds Modulates Drug-Efflux Mediated by Pgp

CsA increases TMRM accumulation from 151 ± 15.6 MFI in the control up to 6282 ± 27.6 MFI for CsA treated cells (Fig. S2); in the case of P2Et a dose-dependent effect with marginal but significant increasing in fluorescence up to 225 ± 17.7 MFI equivalent to 5% of the modulation obtained with CsA was observed, similarly to gallic acid that reaches up to 20% inhibition related to CsA. Conversely, ethyl gallate does not exhibit drug-efflux modulation at any concentration (Fig. 2A).

P2Et Drug-Efflux Activity is Governed by Hydrophobic Compounds

Sub-fractions of P2Et with different polarities from F1-24 (highest polarity) to F88-89 (lowest polarity) was obtained (Fig. S3). When drug-efflux modulation of this sub-fractions was evaluated, it was observed that the fractions with higher hydrophobicity have more

	F1-24	F55-59	F63-67	F88-89
Hydrophobicity	Low	Medium/low	Medium/high	high
ABTS (μmol TROLOX/mg)	7.02 ± 0.14	4.10 ± 0.32	4.45 ± 0.32	3.03 ± 0.43
Cytotoxicity (IC ₅₀ , μg/mL)	61.5 ± 5.4	63.34 ± 6.5	57.01 ± 4.3	34.28 ± 5.6

Data are displayed as the mean $\pm$ SD (n=3)

(C)

Figure 2. P2Et modulates the drug efflux mediated by Pgp in MES-SA/Dx5 Pgp⁺ cell lines. (A) Representative drug efflux experiment using flow cytometry. Percentage of accumulated TMRM with respect to CsA after treatments at different concentrations (IC₅₀ or 0.2 $\times$ IC₅₀) of P2Et, gallic acid and ethyl gallate. (B) Percentage of accumulated TMRM with respect to CsA after treatments at different concentrations (IC₅₀ or 0.2 $\times$ IC₅₀) of sub-fractions of P2Et. (C) anti-oxidant and cytotoxic activity of sub-fractions of P2Et. Data are displayed as the mean $\pm$ SD (n = 3).

activity on Pgp (Fig. 2B) and higher cytotoxicity (Fig. 2C). In addition, it was found that the fractions with lower hydrophobicity exhibited higher anti-oxidant activity but lower cytotoxicity (Fig. 2C); interestingly none of the sub-fractions showed the same anti-oxidant potential or cytotoxicity found with the whole P2Et, suggesting a synergistic effect.

Pre-Treatment with P2Et Enhances the Cytotoxic Effect of Doxorubicin

Short-term treatment with P2Et improves the effect of doxorubicin; synergy results can be found in Table 3 and corresponding dose-response graphics are presented in Fig. S4. The drug reduction index (DRI) and combination index (CI) were used to infer synergy, additivity or antagonism. For comparison, synergy results of gallic acid and ethyl gallate were summarized in Table S3.

We observed synergy in MES-SA/Dx5 Pgp⁺ cells but not for Pgp[−] cells; in the case of K562 we could not estimate the synergistic effect in the Pgp⁺ cells due to very high resistance to doxorubicin, however in K562 Pgp[−] cells we observed an antagonistic effect. On the other hand, we observed moderate synergism in 4T1 WT and 4T1 H17. In the case of TS/A we observed an additive effect. In addition, synergistic effect with the combination of gallic acid and ethyl gallate with doxorubicin (Table S3), was observed for K562 Pgp[−] cells only. These results showed that P2Et could act in synergy with chemotherapeutics and improve the efficiency of the therapy.

P2Et Inhibits Mammosphere Formation Ability of CSC

To evaluate whether P2Et inhibits mammosphere formation we exposed 4T1, 4T1 H17 and TS/A to varying sub-lethal concentrations of P2Et and doxorubicin. As shown in Fig. 3, P2Et inhibited the formation of mammosphere *in vitro* in a dose-dependent manner in all cell

Table 3. Synergy of P2Et with Doxorubicin

Cell Line		DRI		CI	Interpretation
		P2Et IC ₅₀ +Dx	P2Et 0.2 × IC ₅₀ +Dx		
MES-SA Dx5	Pgp ⁺	6.86	1.87	0.64	+++
	Pgp [−]	0.83	1.02	0.96	±
K562	Pgp ⁺	N/C	N/C	N/C	N/C
	Pgp [−]	1.28	0.35	3.09	---
4T1	WT	2.22	1.98	0.65	+++
	H17	1.86	1.93	0.72	++
TS/A		0.99	1.03	1.10	±

Note: Drug reduction index (DRI) and combination index (CI) after sensitization with P2Et at concentrations equivalent to IC₅₀ and 0.2 × IC₅₀ for 3 h and doxorubicin for 48 h in different cell lines. CI < 0.1 very strong synergism (+++), 0.1–0.3 strong synergism (+++), 0.3–0.7 synergism (+++), 0.7–0.85 moderate synergism (++), 0.85–0.9 slight synergism (+), 0.9–1.10 nearly additivity (±), 1.10–1.20 slight antagonism (−), 1.20–1.45 moderate antagonism (−), 1.45–3.3 antagonism (−−), 3.3–10 strong antagonism (−−−), and > 10 very strong antagonism (−−−−), N/C: not calculated.

Figure 3. Inhibitory effect of P2Et inhibits Mammosphere growth under serum free and low-adherence culture conditions. *Upper left*: Representative mammosphere growth. 4T1, 4T1 H17 and TS/A, sphere formation efficiency (SFE) normalized to 3×10^3 cells after 7 days of culture. Data are displayed as the mean $\pm$ SD ($n = 3$); $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

lines. In detail, for 4T1 WT we observed that the number of mammospheres declined up to 50% of control ($p > 0.001$) for cells treated with P2Et, while a reduction of 85% was observed with doxorubicin ($p > 0.001$) at the higher concentration. Likewise, in 4T1 H17 cells we observed a reduction of 80% ($p > 0.001$) in the number of mammospheres treated with P2Et and 77% for doxorubicin, both at higher concentration. Finally, we observed a decrease in the number of mammospheres of TS/A treated with P2Et of 41% ($p > 0.001$) and 99% with doxorubicin. Therefore, these results demonstrate that P2Et impacts CSC mammosphere formation at non-toxic concentrations, similar to that of control doxorubicin.

P2Et Exhibited Synergy with Doxorubicin In Vivo and Enhance Survival of Treated Mice

We confirmed that cytotoxicity of P2Et in TS/A cells is mediated by induction of apoptosis after 24 and 48 h (Fig. S4). Afterwards, we transplanted 1×10^4 TS/A cells in the

mammary fat pad of BALB/c mice and then treated them with P2Et twice a week. We observed that P2Et treatment reduces primary tumor volume ($p < 0.001$), with significant differences since the 47th day compared to control (Fig. 4A). Similarly, there was a significant diminution in primary tumor weight ($p < 0.05$) (Fig. 4B) compared to control.

Next, we evaluated whether the combination of P2Et and doxorubicin could synergistically improve the effect of therapies *in vivo*. Indeed, P2Et or doxorubicin used separately significantly decreases tumor volume compared to control and improved survival, however treatment with the drug combination reduced the tumor volume and

Figure 4. P2Et enhance the survival of treated mice in the TS/A model. (A) Tumor growth is decreased by treatment with P2Et extract. BALB/c mice were subcutaneously inoculated with 1×10^4 TS/A cells. Mice were divided in two groups: control group mice treated with PBS ($n = 10$, black circle) and P2Et group ($n = 9$, black square) treated twice by week (18.7 mg/Kg, I.P). tumor volume was measured three times each week. Results are expressed as the mean value ($\text{mm}^3 \pm \text{ESM}$). (B) mice were euthanized at end-point criteria and primary tumors were surgically removed and weighed. Results are expressed as mean ($\text{g} \pm \text{SD}$). (C) BALB/c mice were subcutaneously inoculated with TS/A cells. Control group (PBS, circle), doxorubicin group (Dx, 1 mg/Kg IP) once each week (triangle), P2Et group (18.7 mg/Kg IP) twice a week and combined therapy group Dx (1 mg/Kg IP) + P2Et (18.7 mg/Kg IP) doxorubicin once and P2Et twice per week. Tumor growth development measure as diameter (mm^3). (D) Kaplan-Meier Survival analysis. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns: non-significant, comparison log-rank Mantel-Cox test. E. TS/A and B16 Surface exposure of CRT determined by flow cytometry, comparisons between negative controls (Dashed line – Black), positive control Dx, P2Et treated, P2Et and Dx treated cells and the isotype control (solid histogram) are shown. MFI is also shown in brackets.

Figure 4. (Continued)

synergistically increased the antitumor effect of each one (Figs. 4C and 4D). Remarkably, the group treated with the combination of P2Et and doxorubicin exhibited an overall survival of 40% with significant differences with the control group ($p < 0.05$). Since no synergistic activity was evidenced *in vitro*, we asked whether synergism could be related to immune system activation *in vivo*, as we have previously shown (Gomez-Cadena *et al.*, 2016; Uruena *et al.*, 2015). In this regard, calreticulin expression at the cell membrane is considered a danger signal, and an “eat me” signal that activates dendritic cells. As expected, P2Et largely induce calreticuline expression, but surprisingly, both on TSA and B16 cells (Fig. 4E), co-treatment with P2Et and Dx at the same time significantly increasing calreticulin membrane expression (Fig. 4E).

Discussion

In the present study we did a comprehensive chemical characterization of the P2Et extract obtained from *Caesalpinia spinosa* in order to understand the molecular mechanisms involved in the anti-metastatic activity. The compounds present in P2Et extract show antioxidant activity, and are cytotoxic to tumor cells lines, whether they have a cancer stem cell phenotype or expression of the drug resistance pump Pgp. A significant synergistic activity with doxorubicin was observed after treating mice that were transplanted with doxorubicin resistant breast cancer cells (TSA). P2Et extract synergistic activity with doxorubicin seems to be related to the induction of calreticulin expression on the plasma membrane of tumor cells.

C. spinosa pods are considered the second most abundant vegetal source of polyphenols, containing 55.1 GAE/100 g, compared to *Galla chinensis*, which contain 61.6 GAE/100 g (Chambi *et al.*, 2013), and seemed to be the most important source until now. In the present study, we identified ten galloylquinic acids, as previously described by Clifford (Clifford *et al.*, 2007) who studied the composition of tara tannin using HPLC-MSⁿ. He identified seven compounds from two primary families (galloylquinic acids and di-galloylquinic acids) and tentatively assigned tri-galloylquinic acids and galloyl-digalloylquinic acids. We also tentatively assigned two possible compounds that were not described previously but their structures need be verified after purification; finally, some galloylquinic common fragments suggest the presence of a tetra-galloylquinic acid. To ensure the safety and efficacy of traditional medicines and natural products is necessary implement strict quality control and regulation (Li *et al.*, 2011). HPLC fingerprinting techniques have been used for the quality control of many components in plant-based medicines and have become mandatory for many traditional medicines (Yang *et al.*, 2011), together with qualitative and quantitative analyses of markers (Li *et al.*, 2011). In this study, we purified gallic acid and ethyl gallate as quantitative markers and other identified compounds as qualitative markers for future quality control of P2Et.

The MDR phenotype it is a barrier for chemotherapy success in cancer (Wind and Holen, 2011), and many efforts in overcoming drug-resistance have attempted to design specific inhibitors of MDR pumps (Cort and Ozben, 2015). However, this approach has not been as successful as expected (Moitra *et al.*, 2011). Multicomponent traditional medicines are ideal to overcome drug resistance, because they affect multiple biological targets simultaneously (Efferth *et al.*, 2007) and in most cases are less toxic than chemotherapeutics (Wargovich *et al.*, 2001). Our results showed that P2Et was effective in almost all cell lines including those cells exhibiting drug resistance mechanisms, and additionally we observed that the most hydrophobic fractions exert the better drug efflux activity, which is correlated with previous reports and our self-results, where gallic acid, one of the most hydrophilic compound present in P2Et does not affect the drug efflux mediated by Pgp (Kitagawa *et al.*, 2005) at low concentrations, but decreases Pgp mRNA expression (Chieli *et al.*, 2010). Additionally, for alkyl gallates, studies have reported that aliphatic chains with more than eight carbons in length can overcome drug efflux mediated by Pgp (Kitagawa *et al.*, 2005); consistent with these results, we observed here that ethyl gallate was not shown to affect drug efflux.

It is possible that some compounds present in P2Et are not targets of the pump, or that a collateral sensitivity phenomenon, principally observed in Pgp⁺ cells, is happening as it has been previously observed (Pluchino *et al.*, 2012). Otherwise, is possible that a particular pattern of Pgp glycosylation in these cells (Greer and Ivey, 2007) together with the changes in cell membrane composition (Clay and Sharom, 2013) could sensitize the cells to different compounds present in P2Et, having antioxidant potential, as previously observed (Chambi *et al.*, 2013). Here, we found a very high anti-oxidant potential for P2Et, which is not due to free gallic acid because of its low concentration in the extract (4.88%). These results suggest that in this extract, hydrophobic compounds should contribute with

high cytotoxicity and drug efflux reversion and hydrophilic compounds contribute with anti-oxidant activity.

Some of the tumor cell lines exhibited ALDH, which defines a CSC population and plays an important role in drug resistance to cyclophosphamide and anthracyclines by scavenging aldehyde intermediaries, ROS and usually is concomitant with overexpression of multidrug resistance pumps (Cojoc *et al.*, 2015; Mizuno *et al.*, 2015). As expected, gallic acid, which induces ROS in spite of its antioxidant activity (Yen *et al.*, 2002), was not cytotoxic against ALDH⁺ cells, however P2Et does. These facts allow us to hypothesize that P2Et may be cytotoxic by mechanisms other than ROS induction. Finally, it has been reported that CSC with phenotype CD44⁺CD24⁻ exhibited different mechanism of drug-resistance (Charafe-Jauffret *et al.*, 2009). In this regard, P2Et was also cytotoxic against TS/A cells, which also exhibited high resistance to doxorubicin. P2Et impairs sphere formation commonly attributed to highly metastatic CSC (Uruena *et al.*, 2013).

P2Et induces immunogenic cell death in the 4T1 breast cancer model (Uruena *et al.*, 2015) and also B16 melanoma cells (Gomez-Cadena *et al.*, 2016). In this work we observed that, in spite of a non-pharmacological synergy *in vitro* between P2Et and doxorubicin in the TS/A model, we identified a better expression of the danger signal calreticulin on the cell membrane, suggesting that a better immunogenicity of tumor cells might be implicated in the *in vivo* response after combined treatment (Nanni *et al.*, 1983). It will be necessary to evaluate if the immune system is playing a role in tumor control in this model. However, our previous results highly suggest that not only a direct antitumor activity is necessary to diminish tumor progression but also well-chosen drug combinations, as those present in phytomedicines is better than a single drug (Wagner, 2011).

In conclusion, P2Et treatment could be used as adjuvant therapy together with conventional chemotherapy in tumors with a MDR phenotype or with high frequency of CSC.

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Supplementary Materials

Figure S1. ¹H and ¹³C NMR and MS spectra for purified: A–C Gallic acid; D–F Ethyl gallate.

Figure S2. P2Et modulates drug efflux mediated by Pgp in MES-SA/DX5 Pgp⁺ cell lines (A) representative drug efflux experiment using flow cytometry in MES-SA-DX5 Pgp⁺ cells through co-incubating TMRM with concentrations of treatment equivalent to IC₅₀. Filled-grey: vehicle; solid line: cyclosporine A 5 μ M, P2Et 25.8 μ g/ml, gallic acid 50.4 μ g/ml and ethyl gallate 33.69 μ g/ml. (B) representative drug efflux experiment using flow cytometry in MES-SA-DX5 Pgp⁺ cells by co-incubating TMRM with concentrations of treatment equivalent to IC₅₀ of sub-fractions of P2Et.

Figure S3. TLC profile sub-fractions obtained from P2Et fraction. Retention factor (RF) of bands obtained by TLC, flavonoids and triterpenoids evidenced by vanillin reagent and tannins by FeCl₃ reagent. The total phenols in P2Et were estimated using the Folin-Ciocalteu colorimetric method. Results are expressed as grams of gallic acid equivalent (GAE) per 100 g of dry extract, and the phenolic compounds in the P2Et extract were 94.47 $\pm$ 4.23 g GAE/100 g.

Figure S4. Synergy between P2Et and doxorubicin in cell lines with different resistance profiles. Cells were pre-treated for 3 h with a P2Et extract, ethyl gallate and gallic acid (concentrations of $0.2 \times IC_{50}$ and IC_{50}); then, the cell culture media was exchanged for fresh media with doxorubicin. The cells were treated for 48 h at $37^{\circ}C$ and $5\% CO_2$ after an incubation MTT assay was performed. Viability (%) was plotted against the doxorubicin concentration. The drug reduction index (DRI) is shown for each drug combination.

(A)

(B)

Figure S5. Phosphatidil serine externalization in TS/A cells, induced by P2Et fraction treatment. Phosphatidylserine externalization in TS/A cells by flow cytometry after treatment for 24 h and 48 h with P2Et fraction, doxorubicin (DX, positive control), or ethanol (negative control). The top panel is a representative dot plot showing the treatments; bottom is a representative histogram showing percent (%) PS/PI cells after 24 and 48 h of treatment. Results were analyzed with FlowJo software (Tree Star Inc) and the experiments were performed in triplicate.

Table S1. Compound ID, Common Nomenclature Name and IUPAC Name for Identified Compounds for P2Et Fraction

Compound ID	IUPAC Name
1	n-O-galloylquinic acid 1,3,4-trihydroxy-5-(3,4,5-trihydroxybenzoyl)oxycyclohexane-1-carboxylic acid 1,3,5-trihydroxy-1-(3,4,5-trihydroxybenzoyl)oxycyclohexane-1-carboxylic acid 1,3,5-trihydroxy-3-(3,4,5-trihydroxybenzoyl)oxycyclohexane-1-carboxylic acid 1,3,5-trihydroxy-4-(3,4,5-trihydroxybenzoyl)oxycyclohexane-1-carboxylic acid 3,4,5-trihydroxybenzoic acid 1,4-dihydroxy-3,5-bis((3,4,5-trihydroxybenzoyl)oxycyclohexane-1-carboxylic acid 1,5-dihydroxy-3,4-bis((3,4,5-trihydroxybenzoyl)oxycyclohexane-1-carboxylic acid 3,5-dihydroxy-1,4-bis((3,4,5-trihydroxybenzoyl)oxycyclohexane-1-carboxylic acid 4,5-dihydroxy-1,3-bis((3,4,5-trihydroxybenzoyl)oxycyclohexane-1-carboxylic acid 4-(ethoxycarbonyl)-2,4,6-trihydroxycyclohexyl 3,4,5-trihydroxybenzoate 5-(ethoxycarbonyl)-2,3,5-trihydroxycyclohexyl 3,4,5-trihydroxybenzoate 3-hydroxy-1,4,5-tris((3,4,5-trihydroxybenzoyl)oxycyclohexane-1-carboxylic acid 4-hydroxy-1,3,5-tris((3,4,5-trihydroxybenzoyl)oxycyclohexane-1-carboxylic acid 5-hydroxy-1,3,4-tris((3,4,5-trihydroxybenzoyl)oxycyclohexane-1-carboxylic acid 1,3,4,5-tetrakis((3,4,5-trihydroxybenzoyl)oxycyclohexane-1-carboxylic acid 1-hydroxy-3,4,5-tris((3,4,5-trihydroxybenzoyl)oxycyclohexane-1-carboxylic acid ethyl 3,4,5-trihydroxybenzoate 3-(3,5-dihydroxy-4-((3,4,5-trihydroxybenzoyl)oxy)benzoyl)oxy)-1-hydroxy-4,5-bis((3,4,5-trihydroxybenzoyl)oxy)cyclohexane-1-carboxylic acid 4-(3,5-dihydroxy-4-((3,4,5-trihydroxybenzoyl)oxy)benzoyl)oxy)-1-hydroxy-3,5-bis((3,4,5-trihydroxybenzoyl)oxy)cyclohexane-1-carboxylic acid 5-(3,5-dihydroxy-4-((3,4,5-trihydroxybenzoyl)oxy)benzoyl)oxy)-1-hydroxy-3,4-bis((3,4,5-trihydroxybenzoyl)oxy)cyclohexane-1-carboxylic acid 1-(ethoxycarbonyl)-4-hydroxycyclohexane-1,3,5-triyl tris(3,4,5-trihydroxybenzoate) 4-(ethoxycarbonyl)-6-hydroxycyclohexane-1,2,4-triyl tris(3,4,5-trihydroxybenzoate) 5-(5-(5-(ethoxycarbonyl)-2,3-dihydroxyphenoxy)carbonyl)-2,3-dihydroxyphenoxy)carbonyl)-2,3,5-trihydroxycyclohexyl 3,4,5-trihydroxybenzoate 5-(5-(ethoxycarbonyl)-2,3-dihydroxyphenoxy)carbonyl)-2,3-dihydroxyphenoxy)carbonyl)-2,4,6-trihydroxycyclohexyl 3,4,5-trihydroxybenzoate
2	gallic acid
3	di-O-galloylquinic acid
4	galloylquinic acid ethyl ester
5	tri-O-galloylquinic acid
6	tetra-O-galloylquinic acid
7	tri-O-galloylquinic acid
8	ethyl gallate
9	*di-O-galloyl(digalloyl) quinic acid (unknown)
10	tri-O-galloylquinic ethyl ester
11	*3-galloylquinic ethyl digallate ester

Table S1. (Continued)

Compound ID	IUPAC Name
	4-((4-(5-(ethoxycarbonyl)-2,3-dihydroxyphenoxy)carbonyl)-2,6-dihydroxyphenoxy)carbonyl)-2,4,6-trihydroxycyclohexyl 3,4,5-trihydroxybenzoate
	4-((4-((4-(ethoxycarbonyl)-2,6-dihydroxyphenoxy)carbonyl)-2,6-dihydroxyphenoxy)carbonyl)-2,4,6-trihydroxycyclohexyl 3,4,5-trihydroxybenzoate
	5-(5-(ethoxycarbonyl)-2,3-dihydroxyphenoxy)carbonyl)-3,5-dihydroxycyclohexane-1,2-diyl bis(3,4,5-trihydroxybenzoate)
	Other Isomers
12 tri-O-galloylquinic ethyl ester	5-(ethoxycarbonyl)-5-hydroxycyclohexane-1,2,3-triyl tris(3,4,5-trihydroxybenzoate)
13 *di-galloylquinic ethyl digallate ester	5-((4-((4-(ethoxycarbonyl)-2,6-dihydroxyphenyl)peroxy)carbonyl)-2,6-dihydroxyphenoxy)carbonyl)-2,5-dihydroxycyclohexane-1,3-diyl bis(3,4,5-trihydroxybenzoate)
	5-(5-((4-(ethoxycarbonyl)-2,6-dihydroxyphenyl)peroxy)carbonyl)-2,3-dihydroxyphenoxy)carbonyl)-2,5-dihydroxycyclohexane-1,3-diyl bis(3,4,5-trihydroxybenzoate)
	5-(5-(5-(ethoxycarbonyl)-2,3-dihydroxyphenoxy)carbonyl)-2,3-dihydroxyphenoxy)carbonyl)-2,5-dihydroxycyclohexane-1,3-diyl bis(3,4,5-trihydroxybenzoate)
	5-((4-((4-(ethoxycarbonyl)-2,6-dihydroxyphenyl)peroxy)carbonyl)-2,6-dihydroxyphenoxy)carbonyl)-3,5-dihydroxycyclohexane-1,2-diyl bis(3,4,5-trihydroxybenzoate)
	Other Isomers

*Compound tentatively assigned.

Table S2. Anti-oxidant Activity of the P2Et Extract Evaluated Using ABTS and ORAC Methods

	Anti-oxidant Activity ($\mu\text{mol TROLOX/mg}$)	
	ABTS	ORAC
P2Et extract	11.76 ± 0.58	4.99 ± 0.55
Ethyl gallate	11.37 ± 0.21	4.67 ± 0.82
Gallic acid	16.62 ± 4.55	9.66 ± 0.19

Note: Data are displayed as the mean $\pm$ SD ($n = 3$).

Table S3. Synergy of Gallic Acid and Ethyl Gallate with Doxorubicin

Gallic acid(+Dox)		DRI		CI	Interpretation
		IC ₅₀	$0.2 \times \text{IC}_{50}$		
MES-SA Dx5	P-gp ⁺	0.72	0.89	1.24	—
	P-gp [−]	0.9	1.3	1.06	±
K562	P-gp ⁺	N/C	0.874	1.34	—
	P-gp [−]	0.672	1.72	0.78	++
4T1	WT	1.205	1.182	1.04	±
	H17	0.986	1.23	1.01	±
TS/A		0.988	1.01	1.18	—
Ethyl gallate(+Dox)		IC ₅₀	$0.2 \times \text{IC}_{50}$		
MES-SA Dx5	P-gp ⁺	0.89	0.95	1.64	— — —
	P-gp [−]	1.04	0.8	1.56	— — —
K562	P-gp ⁺	N/C	N/C	N/C	N/C
	P-gp [−]	8.33	1.72	0.32	+++
4T1	WT	N/C	2.933	0.541	++
	H17	1.45	1.20	1.03	±
TS/A		0.986	1.00	1.15	—

Note: Drug reduction index (DRI) and combination index (CI) after sensitization with P2Et at concentrations equivalent to IC₅₀ and $0.2 \times \text{IC}_{50}$ for 3 h and doxorubicin for 48 h in different cell lines. Results are interpreted as Chou (2006). CI < 0.1 means very strong synergism (+++), 0.1–0.3 strong synergism (+++), 0.3–0.7 synergism (+++), 0.7–0.85 moderate synergism (++), 0.85–0.9 slight synergism (+), 0.9–1.10 nearly additivity (±), 1.10–1.20 slight antagonism (−), 1.20–1.45 moderate antagonism (−), 1.45–3.3 antagonism (− −), 3.3–10 strong antagonism (− − −), and >10 very strong antagonism (− − − −), N/C: not calculated.