EXPERIMENTAL AND THERAPEUTIC MEDICINE 16: 557-566, 2018

In vitro screening of cytotoxic activity of euphol from *Euphorbia tirucalli* on a large panel of human cancer-derived cell lines

VIVIANE ALINE OLIVEIRA SILVA[1], MARCELA NUNES ROSA[1], ALINE TANSINI[1], RENATO J.S. OLIVEIRA[1], OLGA MARTINHO[1-3], JOÃO PAULO LIMA[4], LUIZ F. PIANOWSKI[5] and RUI M. REIS[1-3]

[1]Molecular Oncology Research Center, Barretos Cancer Hospital, Barretos, SP 14784 400, Brazil; [2]Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga 4710-057; [3]ICVS/3B's PT Government Associate Laboratory, Braga/Guimarães 4806-909, Portugal; [4]Medical Oncology Department, AC Camargo Cancer Center, São Paulo, SP 01509-010; [5]Kyolab Laboratório de Pesquisa Farmacêutica Ltda, Valinhos, SP 13273-105, Brazil

Received November 13, 2017; Accepted March 13, 2018

DOI: 10.3892/etm.2018.6244

Abstract. A large number of classic antineoplastic agents are derived from plants. *Euphorbia tirucalli* L. (Euphorbiaceae) is a subtropical and tropical plant, used in Brazilian folk medicine against many diseases, including cancer, yet little is known about its true anticancer properties. The present study evaluated the antitumor effect of the tetracyclic triterpene alcohol, euphol, the main constituent of *E. tirucalli* in a panel of 73 human cancer lines from 15 tumor types. The biological effect of euphol in pancreatic cells was also assessed. The combination index was further used to explore euphol interactions with standard drugs. Euphol showed a cytotoxicity effect against several cancer cell lines (IC_{50} range, 1.41-38.89 μM), particularly in esophageal squamous cell (11.08 μM) and pancreatic carcinoma cells (6.84 μM), followed by prostate, melanoma, and colon cancer. Cytotoxicity effects were seen in all cancer cell lines, with more than half deemed highly sensitive. Euphol inhibited proliferation, motility and colony formation in pancreatic cancer cells. Importantly, euphol exhibited synergistic interactions with gemcitabine and paclitaxel in pancreatic and esophageal cell lines, respectively. To the best of our knowledge, this study constitutes the largest *in vitro* screening of euphol efficacy on cancer cell lines and revealed its *in vitro* anti-cancer properties, particularly in pancreatic and esophageal cell lines, suggesting that euphol, either as a single agent or in combination with conventional chemotherapy, is a potential anti-cancer drug.

Correspondence to: Professor Rui M. Reis, Molecular Oncology Research Center, Barretos Cancer Hospital, 1331 Rua Antenor Duarte Villela, Barretos, SP 14784 400, Brazil
E-mail: ruireis.hcb@gmail.com

Abbreviations: ANOVA, analysis of variance; CI, combination index; DNA, deoxyribonucleic acid; DMEM, dulbecco's modified eagle's medium; DMSO, dimethyl sulfoxide; ETHE, *E. tirucalli* hydroalcoholic extract; FBS, fetal bovine serum; FDA, food and drug administration; g, gram; GI, growth inhibition; HPLC, high performance liquid chromatography analysis; HS, highly sensitive; IC50, half maximal inhibitory concentration; MHz, megahertz; ml, milliliter; MS, moderately sensitive; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; NCI, National Cancer Institute; NMR, nuclear magnetic resonance; P/S, penicillin/streptomycin solution; R, resistant; RPMI-1640, Roswell Park Memorial Institute-1640; SD, standard deviation; STR, short tandem repeat; WHO, World Health Organization; uM, micromolar

Key words: *Euphorbia tirucalli*, anticancer, cytotoxic activity, euphol, pancreatic, esophageal

Introduction

The 20th century saw an extraordinary breakthrough of natural products, especially regarding the application of plants in the field of oncology, enabling the discovery of several substances currently used in cancer therapy (1-3). Plants secondary metabolites and their semi-synthetic derivatives play an important role in current oncology treatment. Of the 250 drugs considered as basic and essential by the World Health Organization (WHO), 11% are derived from medicinal plants (4). Within this list, there are drugs that constitute the backbone of cancer therapy as vinka alcaloids (vinblastine and vincristine), camptothecin derivatives (topotecan and irinotecan), epipodophyllotoxin (etoposide and teniposide), and, more recently, taxanes (docetaxel, paclitaxel and cabazitaxel). Despite these facts, it is estimated that less than 2% of higher plants have been analyzed for their antineoplastic activity, due to the time and resource intensive phenotype-based drug discovery process (1,5).

Brazil has one of largest plant diversity in world, with a myriad of opportunities for phytochemicals production, yet; only approximately 8% it has been studied (6,7). Extracts of species from the genus *Euphorbia* (Euphorbiaceae) are used by traditional healers for the treatment of ulcers, warts and other diseases (7,8). The *Euphorbia* genus is worldwide

spread, used as decorative plant and comprises thousands of different species. Some species of this genus have triggered interest about potential antineoplastic activity, partly based on anedoctic reports stemming from traditional medicine (7-9). Interestingly, a derived from *E. peplus,* the ingenol mebutate (ingenol-3-angelate, PEP005, Picato®; LEO Pharma A/S, Ballerup, Denmark), was recently approved by the FDA for actinic keratosis treatment, a premalignant lesion for sun-related squamous-cell carcinoma (10,11).

Amongst the species under *Euphorbia* genus, *E. tirucalli* has a large use in traditional medicine (7). The main constituent of *E. tirucalli* sap is euphol, a tetracyclic triterpene alcohol, which has shown anti-inflammatory, antiviral, and analgesic properties (12,13). In mice model of acute colitis and arthritic, euphol showed an anti-inflammatory effect (14). Euphol was also reported to exhibit antinociceptive properties in both inflammatory and neuropathic pain of mice and rats models (15). Moreover, euphol showed to inhibit the reverse transcriptase in human immunodeficiency virus type 1.

Recently, euphol was suggested to display an anti-cancer effect. *In vitro* studies in breast and gastric tumor cell reported that euphol decreased cell viability (16,17). In an *in vivo* study of ascitic Ehrlich tumor model, treatment with *E. tirucalli* hydroalcoholic extract (ETHE) leads a higher animal survival (18). These studies have increased the therapeutic interest of *E. tirucalli* compounds, mainly euphol in oncology. On the other hand, some reports suggest that the exposure to *E. tirucalli* crude can be a risk factor for Burkitt's lymphoma, since it act as a genotoxic agent, especially when it contains phorbol ester (7,19). Therefore, further studies are needed to elucidate the potential therapeutic use of euphol.

Herein, we aimed to study the antitumor effect of euphol on a large panel of human cancer cell lines from a high variety of tumor types, in order to provide insight into the tailoring designing of euphol-based therapies for cancer patients.

Materials and methods

Cell lines and cell culture. Seventy-three immortalized human cancer cell lines from 15 solid tumor models were analysed (Table I). The U87MG was purchased from American Type Culture Collection (ATCC HTB-14; Manassas, VA, USA). Authentication of all cell lines was carried out by the Center for Molecular Diagnostics of Barretos Cancer Hospital (São Paulo, Brazil) as previously reported (20). Shorthly, short tandem repeat (STR) DNA typing was performed according to the International Reference Standard for Authentication of Human Cell Lines previously described (21). The identity of all cell lines was confirmed by genotyping, with the exception of U373, which was shown to be a sub-clone of U251 cell line. All the cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM 1X, high glucose; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) or RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin solution (P/S; Gibco; Thermo Fisher Scientific, Inc.), at 37˚C and 5% CO_2.

Preparation and compound dilution. The sap from *E. tirucalli* L. (accepted name record 82539-Sp. Pl. 452 1753.) was initially extracted with hexane and the resulting precipitate was extracted with n-butanol. The most lipophilic compounds present in the butanol fraction were purified by means of high performance liquid chromatography analysis (HPLC). Further purification of the compounds was carried out using a Sephadex G75 column in a mixture of hexane-ethyl acetate. Recrystallization of the acetate fraction from butanol gave 3.5 g crystals, comprising euphol acetate and filtrate (1.5 g). The chemical structure of the euphol, representeted in Fig. 1, was determined by elemental analyses of 1H NMR and 13C NMR spectral data, and by comparison with their respective authentic compounds using Chemdraw software version 7.0 (22,23) (PubChem CID: 441678). The 1H NMR (500 MHz) and 13C NMR (126 MHz) spectra were recorded on a Bruker 500 MHz instrument. The MS spectra were recorded on a Perkin Elmer instrument, model API 150 and run in ES-MS positive mode: MH+ 427 m/e, MH+ _H2O 409 m/e. The NMR parameters were 13C NMR (CDCl3): 15.7 (C29), 15.8 (C18), 17.9 (C26), 19.1 (C21), 19.2 (C6), 20.4 (C19), 21.7 (C11), 24.9 (C30), 25.0 (C23), 25.9 (C27), 28.0 (C2, C7), 28.2 (C28), 28.3 (C16), 30.0 (C15), 31.1 (C12), 35.4 (C1), 35.7 (C22), 36.1 (C20), 37.5 (C10), 39.2 (C4), 44.3 (C13), 49.9 (C17), 50.2 (C14), 51.2 (C5), 79.2 (C3), 125.4 (C24), 131.1 (C25), 133.8 (C9), 134.3 (C8) and 1H NMR (CDCl3): 0.75 (3H, s, H-18), 0.80 (3H, s, H-29), 0.85 (3H, d, H-21), 0.87 (3H, s, H-30), 0.95 (3H, s, H-19), 1.00 (3H, s, H-28), 1.50 (3H, s, H-26), 1.68 (3H, s, H-27), 3.23 (1H, dd, H-3), 5.09 (1H, bt, H-24). The tetracyclic triterpene euphol used in this study showed >95% purity.

The extract fraction was initially dissolved in dimethyl sulfoxide (DMSO) at a concentration of 50 mg/ml and stored at -20˚C. The intermediate dilutions of the experimental compound were prepared to obtain a concentration of 1% DMSO.

Cell viability assay. The cytotoxicity effect of euphol was assessed by Cell Titer 96 Aqueous cell proliferation assay (MTS assay; Promega Corporation, Madison, WI, USA), following the manufacturer's instructions as previous described (20,24). For experiments, the cells (third and fifth passage) were plated into 96-well plates (until a maximum $5x10^3$ cells/well). The plate was incubated overnight for optimal attachment of adherent lines, and then placed under low serum-starved conditions for 24 h (DMEM supplemented with 0.5% of FBS). Subsequently, the cells were treated with increasing concentrations of the test compound diluted in DMEM (0.5% FBS) and incubated for 72 h. The control groups received the same amount of vehicle (1% DMSO, final concentration). Growth of tumoral cells was quantified by ability of living cells to reduce the yellow dye MTS to a blue formazan product. Two hours before the end of incubation, 10 μl of MTS were added to each well, and the plate further incubated for 2 h at 37˚C. Absorbance was measured in automatic microplate reader Varioskan (Thermo Fisher Scientific, Inc.) at 490 nm. The response to euphol treatment was assessed by standardizing treated groups to the untreated control, and were expressed as a percentage relatively to control cells, in DMSO alone (considered as 100% viability) ± SD.

IC_{50} determination. The results of absorbance values of treated cells were converted to percentage of cell viability in cells in

Table I. Euphol IC_{50} values and percentual of GI at a fixed euphol concentration (15 μM).

Cell line	Mean IC_{50}	SD	GI (%) at 15 μM[a]	GIS	SD	Tumor type
T47D	38.89	9.27	31.8	R	7.0	Breast
MDA-MB-231	9.08	0.87	56.7	MS	14.0	
MDA-MB-468	30.89	6.22	27.5	R	10.3	
BT20	8.96	2.92	66.7	HS	2.9	
HS587T	18.15	8.91	61.4	HS	25.5	
MCF-7	18.76	3.43	52.6	MS	14.1	
MCF7/AZ	33.42	5.01	22.8	R	11.6	
JHU-O22	26.35	7.31	5.6	R	5.1	Head and neck
HN13	8.89	6.53	69.2	HS	6.0	
SCC25	6.65	3.54	74.2	HS	3.6	
SCC4	19.82	1.95	31.5	R	9.9	
SCC14	15.81	2.63	53.3	MS	6.5	
FADU	20.17	2.68	46.7	MS	9.2	
SW480	5.79	0.05	80.7	HS	4.1	Colon
SW620	10.02	4.54	68.8	HS	9.2	
CO115	9.58	2.35	74.1	HS	8.3	
HCT15	5.47	0.81	92.3	HS	1.6	
HT29	6.52	1.37	78.1	HS	4.4	
SK-CO-10	17.53	7.13	58.0	MS	11.9	
DLD1	2.56	1.18	80.0	HS	3.9	
LOVO	11.49	2.39	63.2	HS	5.5	
DIFI	11.38	2.86	67.9	HS	4.3	
Caco2	35.19	5.11	25.1	R	10.0	
U87-MG	26.41	3.19	6.7	R	12.7	Glioma
U373	30.48	3.51	10.0	R	12.1	
U251	29.01	7.82	23.3	R	9.5	
GAMG	8.73	1.87	90.1	HS	0.5	
SW1088	27.12	2.55	7.2	R	7.2	
SW1783	19.62	1.42	44.2	MS	9.6	
RES186	16.70	3.72	41.6	MS	14.8	
RES259	10.34	4.08	70.6	HS	8.6	
KNS42	19.94	0.27	23.3	R	6.2	
UW479	15.26	4.83	53.4	MS	15.3	
SF188	5.98	2.42	74.4	HS	4.3	
PC-3	11.95	4.47	66.7	HS	9.6	Prostate
LNCaP	1.41	0.45	67.7	HS	4.6	
T24	30.72	0.30	9.2	R	3.1	Blader
5637	4.83	1.61	88.3	HS	2.4	
HT1376	25.25	0.41	9.9	R	8.5	
MCR	7.40	2.77	59.1	MS	7.0	
DAOY	5.72	1.37	79.3	HS	1.5	
ONS76	21.72	2.07	10.9	R	16.1	Meduloblastoma
JEG3	16.65	0.86	61.5	HS	7.6	Choriocarcinome
A431	17.79	3.41	40.2	MS	14.8	Epidermoid
H292	13.25	2.16	52.6	MS	5.7	Lung
SKMES1	25.62	0.79	24.9	R	7.4	
A549	11.01	3.11	60.0	HS	10.4	
SK-LU-1	22.83	2.06	4.2	R	8.7	
SIHA	24.74	3.65	6.7	R	5.9	Cervical
CASKI	24.74	2.67	19.3	R	13.1	
C33A	21.32	4.21	52.6	MS	1.7	
HELA	17.55	3.41	44.6	MS	18.0	

Table I. Continued.

Cell line	Mean IC_{50}	SD	GI (%) at 15 μM[a]	GIS	SD	Tumor type
KYSE30	3.52	1.28	71.7	HS	5.7	Oesophagus
KYSE70	8.77	0.74	78.8	HS	1.8	
KYSE270	10.71	3.95	66.9	HS	12.5	
KYSE410	4.35	2.03	85.5	HS	3.4	
Mia PaCa-2	8.46	0.39	79.9	HS	3.2	Pancreatic
PANC-1	21.47	1.83	49.0	MS	6.6	
PSN-1	3.71	0.17	63.5	HS	4.9	
BXPC-3	5.47	1.64	84.9	HS	2.8	
Capan-1	16.33	2.06	61.0	HS	8.5	
COLO858	14.02	2.94	55.8	MS	7.5	Melanoma
COLO679	8.93	4.45	66.8	HS	9.0	
A375	9.67	1.86	63.6	HS	3.9	
WM1617	16.32	2.95	55.1	MS	11.3	
WM9	9.67	4.11	75.8	HS	6.9	
WM852	7.61	1.08	77.4	HS	4.0	
WM278	27.46	1.48	30.5	R	18.1	
WM35	12.40	3.57	63.9	HS	14.2	
WN793	5.96	0.26	73.3	HS	8.0	
SKMEL-37	10.07	0.06	69.0	HS	9.9	
PA-1	7.97	3.03	68.3	HS	3.4	Ovary
SW626	30.40	5.61	23.9	R	11.5	

[a]GI was defined in comparison with untreated controls. Samples exhibiting more than 60% GI in the presence of 15 μM euphol were classified as HS; MS when located between 40-60%; and R when showing less than 40%. GIS, Growth Inhibition Score; SD, standard deviation; GI, Growth Inhibition; HS, highly sensitive; MS, moderately sensitive; R, resistant.

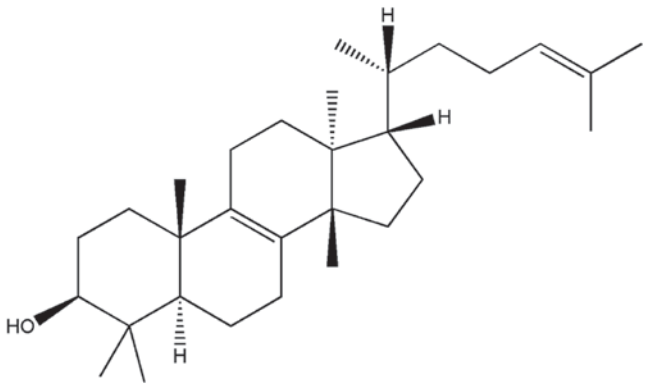

Figure 1. Chemical structure of euphol.

with presence of the vehicle (DMSO), which were used as control, corresponding to 100% survival. The analysis of the non-linear regression curve using GraphPad PRISM version 7 (GraphPad Software, Inc., La Jolla, CA, USA) the was carried out on results of viability, yielding an equation used to calculate the concentration of substance required to produce 50% reduction in cell viability (IC_{50}) as previous determined (25,26).

Drug combination studies. Combination studies were done with fixed concentrations (determinated IC_{50} value) of standard chemotherapeutic paclitaxel (Sigma-T7402; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and gemcitabine hydrochloride (Sigma-G6423; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) (data not shown), exposed simultaneously to increasing concentrations of euphol. The drug interactions were evaluated by the combination index (CI) that was calculated by the Chou-Talalay equation, which takes into account both the potency (Dm or IC_{50}) and the shape of the dose-effect curve (27,28), using CalcuSyn software version 2.0 (Biosoft, Ferguson, MO, USA). In CI analysis, synergy was defined as CI values significantly lower than 1.0; antagonism as CI values significantly higher than 1.0; and additivity as CI values equal to 1.0 at drug IC_{50} value for each cell line.

Proliferation assay. The proliferation effect of euphol was assessed by BrdU assay kit (Roche Applied Science, Mannheim, Germany) following the manufacturer's instructions. In brief, $5x10^3$ cells/well were seeded in a 96-well, flat-bottom plate. The plate was incubated overnight for optimal attachment of adherent lines, and then placed under low serum-starved conditions for 24 h (DMEM-0.5% FBS). Subsequently, the cells were treated with increasing concentrations of the test compound diluted in DMEM (0.5% FBS) and incubated for 72 h. The control groups received the same amount of vehicle (1% DMSO, final concentration). Therefore, the proliferation effect was assessed by BrdU assay kit following the manufacturer's instructions. Experiments were done in triplicate in three independent experiments for each cell line.

The criterion of GI to ascertain the cell line sensibility to euphol was previously described (29). Mean GI values was calculated at fixed dose of 15 μM of euphol (concentration closer to the mean IC_{50} value for all cell lines) and established as 100%-percentage of viable cells at this dose. Samples exhibiting more than 60% GI in the presence of 15 μM euphol were classified as highly sensitive (HS); moderately sensitive (MS) when located between 40-60%; and resistant when showing less than 40%. All the assays were done in triplicate and repeated at least three times for each cell line.

Wound-healing migration assay. Cell migration properties were evaluated by wound-healing assay, as previsously described by our group (26,30). The pancreatic cancer cell lines, Mia-Pa-Ca-2 and Panc-1, were plated in 6-well plates and grown to confluence. A sterile tip was used to create a scratch in monolayer cells. Cells were then incubated with euphol at 8.46 and 21.47 μM. The 'wounded' areas were photographed by phase contrast microscopy (Model IX71; Olympus Corporation, Tokyo, Japan) to evaluate wound closure (0, 24, 48 and 72 h). The migration rate of individual cells was determined by measuring the distances covered from the initial time to the selected time-points (bar of distance tool, DP2-BSW Olympus version 2.2). The percentage of the relative migration distance was calculated as wound area at a given time compared to the initial wound surface. Pictures shown are representative of three independent experiments performed in triplicates.

Colony formation-assay. Inhibition of anchorage-independent was assesed by soft-type-agar assay as reported (30). We placed 1 ml of acellular solution of 0.6% agar (combining equal volumes of 1.2% Noble agar with 2x concentrated DMEM with 20% FBS) into a six-well plate and incubated at 37˚C for 10 min; $2x10^4$ cells of Mia-Pa-Ca-2 and Panc-1 were homogenized in solution containing DMEM supplemented with 0.35% agar (upper layer agar; equal volumes of 0.7% Noble agar and 2X concentrated DMEM with 20% FBS) and seeded onto acellular coating. After solidification 0.5 ml of DMEM medium + 10% FBS was added. The medium was changed every two days, and DMEM medium + 0.5% euphol at 3 and 10 μM was added. The cells were incubated at 37˚C in a humidified atmosphere of 5% CO_2 for 20 days; colonies formed were stained with 0.05% crystal violet for 15 min. Photo-documented colonies were analyzed using the Image J Software. The assay was performed in two biological replicates and the experiments were done in duplicate.

Statistical analysis. The results of *in vitro* experiments are expressed as mean ± standard deviation (SD) of three independent experiments. IC_{50} values were obtained by nonlinear regression. We applied Student's t-test for comparing two different conditions whereas two-way analysis of variance with Tukey's post hoc test was used for assessing differences between more groups. $P<0.05$ was considered to indicate a statistically significant difference. All statistical analyzes were performed using GraphPad PRISM version 7 (GraphPad Software, Inc.).

Results

Euphol promotes cytotoxity in human cancer cell lines. The antitumor effect of euphol *in vitro* was assessed using MTS assay on 73 human cancer lines from 15 solid tumor models (breast, colon, bladder, prostate, lung, pancreas, esophageal, head and neck, cervical, epidermoid carcinoma, meduloblastoma, placental choriocarcinoma, ovarian carcinoma, glioblastoma, and melanoma) (Table I). We generated complete dose-response curves and IC_{50} values for this euphol treatment. Among each tumor type, the distinct cell lines exhibited a heterogeneous profile of response to euphol (Fig. 2A). The mean of IC_{50} values was 15.14 (6.47 μg/ml), but varied significantly between individual cell lines with up to a more than 27-fold difference in the IC_{50} values [IC_{50} range: 1.41-38.89 μM (0.60-16.62 μg/ml)]. Esophageal squamous cell carcinoma and pancreatic carcinomas showed the most sensitive profiles (IC_{50} mean 11.08 and 6.84 μM, respectively, Fig. 2A and Table I), followed by prostate, melanoma and colon cancer cell lines.

To allow a better classification of the cell lines response to euphol, we determined their GI. We found that 50.68% (37/77) were classified as HS, 21.92% (16/73) were MS, and 27.4% (20/77) were resistant (Fig. 2B and Table I). Esophageal (100%), prostate (100%) and pancreatic (80%) cancer models showed a higher percentage of HS cell lines. At variance, glioma (54.5%) followed by breast tumor type (42%) has the most cancer cell lines scored as resistant.

Biological effect of euphol on pancreatic cancer cell lines. We further investigated the biological effect of euphol on pancreatic cancer cell lines, the most sensitive tumoral type in our study. To determine whether the effect of euphol on cancer cells is cytotoxic or cytostatic, its effect was also evaluated on the proliferation of pancreatic cell lines by BrdU incorporation. As shown in Fig. 3A, euphol exhibited dose-dependent effects on proliferation of pancreatic cancer cell lines (Panc-1 and Mia-Pa-Ca-2) but varied significantly between individual cell lines. In both cancer cell lines, low doses of euphol slightly decreased proliferation and the dose of 17.51 μM was able to inhibit almost 50% of the proliferation.

Euphol also exhibited dose-dependent effects on cell viability on pancreatic cancer cell lines (Panc-1 and Mia-Pa-Ca-2). Although euphol decreased the proliferation, the strongest inhibition of proliferation was seen at 35.1 μM concentration of euphol after 72 h, with almost 39.4% for Mia-Pa-Ca-2 cells and 51% for Panc-1 cells (Fig. 3A), whereas the same concentration suppressed cell viability of Mia-Pa-Ca-2 cells by 10.7% and Panc-1 cells by 22.4% (Fig. 3B). Thus, comparing the effects on cell viability in concentrations of the same magnitude, euphol seemed to inhibit growth through a more cytotoxic than cytostatic fashion.

Next, we investigated its biological effect on pancreatic cancer cell lines. In untreated conditions for both cell lines (Mia-Pa-Ca-2 and Panc-1, complete close up of the wound took longer than 72 h, suggesting that these cell lines have an intrinsic low migratory capability. Those two cells lines were treated with euphol at 8.46 and 21.47 μM. In spite of the low migratory feature of the cells, euphol treatment significantly inhibited cell migration in Mia-Pa-Ca-2 at 72 h when compared to control cells (Fig. 3C). We could not evaluate the effect of euphol in Panc-1 cell motility, since this cell present an inherent low motility phenotype hampered an adequate assessment of any inhibition. The findings suggest that euphol

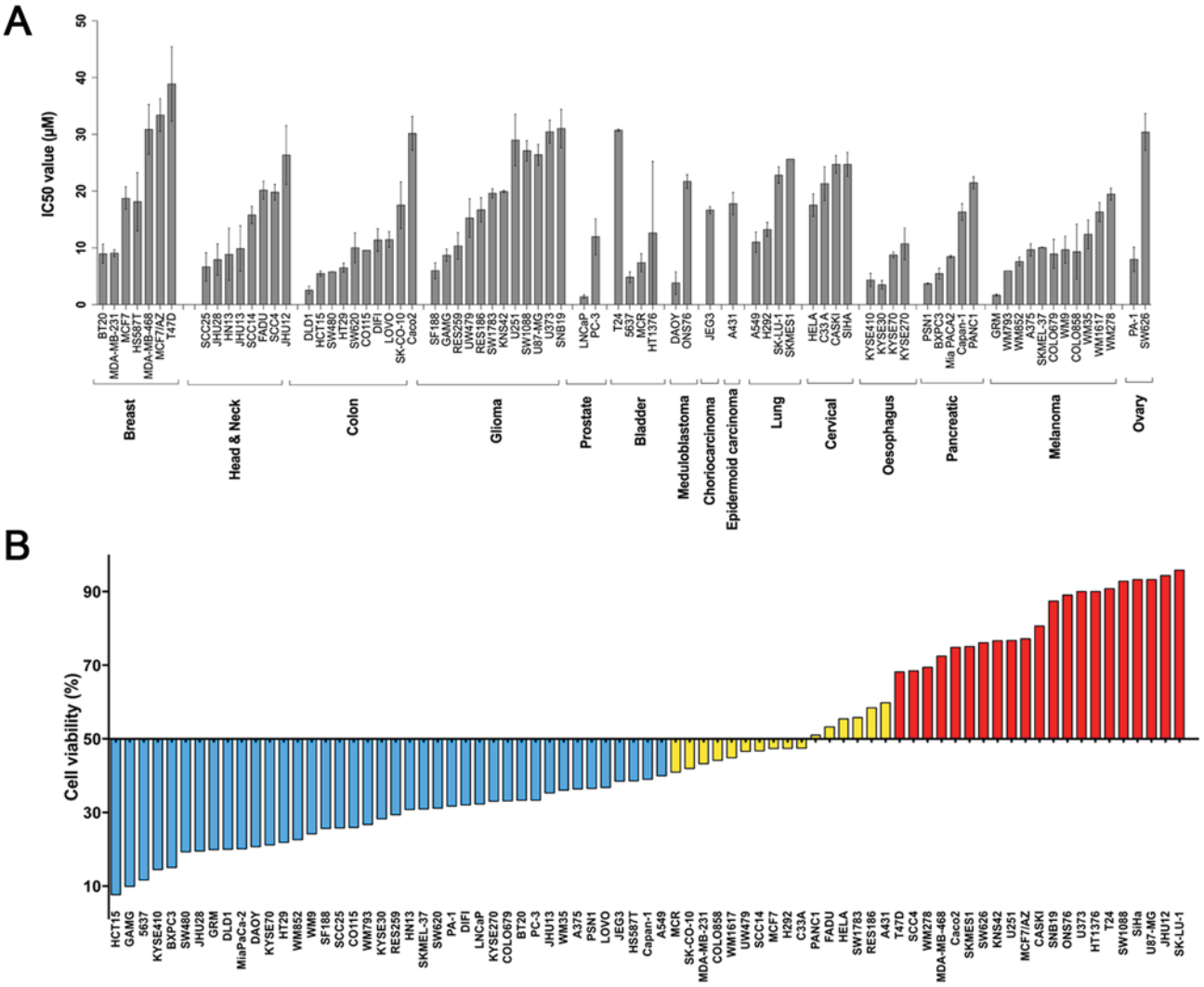

Figure 2. Effect of euphol compound on human cancer cell lines survival. (A) Cellular viability was measured at 72 h by MTS assay. The results were expressed as the mean percentage ± SD of three independent experiments of viable cells relatively to the DMSO alone (considered as 100% viability). The IC_{50} values were calculated by nonlinear regression analysis using GraphPad Prism software. Data represent the mean of at least three independent experiments done in triplicate. (B) Growth inhibition (GI) profile of human cancer-derived cell lines exposed to euphol. Bars represent the cell viability at 15 μM of euphol. Colors represent the GI score classification. Blue (HS, highly sensitive); Yellow (MS, moderate sensitive) and Red (R, resistant).

has a cytotoxic effect but were not able to inhibit the migration of this cell line (Fig. 3D).

In addition to cell proliferation (anchorage-dependent growth) and cell migration, colony formation (anchorage-independent growth) is one of the typical characteristics of the metastatic potential of cancer cells *in vitro* and strongly correlates with tumorigenesis *in vivo* (31). Therefore, we evaluated the effect of euphol in anchorage-independent growth of Panc-1 cancer cells. Panc-1 cells formed colonies on agar after 20 days of incubation, and the presence of euphol at IC_{50} value resulted in a significant suppression of number and size of colonies ($P<0.05$; Fig. 3E). The ability to form colony was reduced by 90% compared to the untreated control. Similarly, we proceeded the same way with Mia-Pa-Ca-2 cell line, however we can not observe the colonies formation even in the control condition suggesting a low tumorigenic potential of this cell line (data not shown).

Euphol potentiates chemotherapeutic-induced decrease in cell viability. We also evaluated the potential combinatorial value of euphol in the context of standard esophageal and pancreatic tumor therapy. We found that euphol and gemcitabine combination treatment showed a synergistic effect (CI<1) in 50% of pancreatic cells lines investigated (mean CI values, range: 0.76-0.8; Table II), being the combination more effective than single agents. Likewise, euphol was able to synergistically sensitize most esophageal cells lines to paclitaxel treatment (mean CI values, range: 0.37-0.55; Table III).

Discussion

In the present study, we investigated the cytotoxity effects of euphol on a large panel of 73 human cancer cell lines, derived from 15 solid tumor models including breast, colon, bladder, prostate, lung, pancreas, esophagus, head and neck, cervical cancer as well as glioma and melanoma. We observed that euphol exhibited dose-dependent cytotoxic effects on all human cancer cell lines tested with the highest effect (reduced viability) for pancreas and esophageal cancer lines, followed by prostate, melanoma and colon cancer cell lines. According to

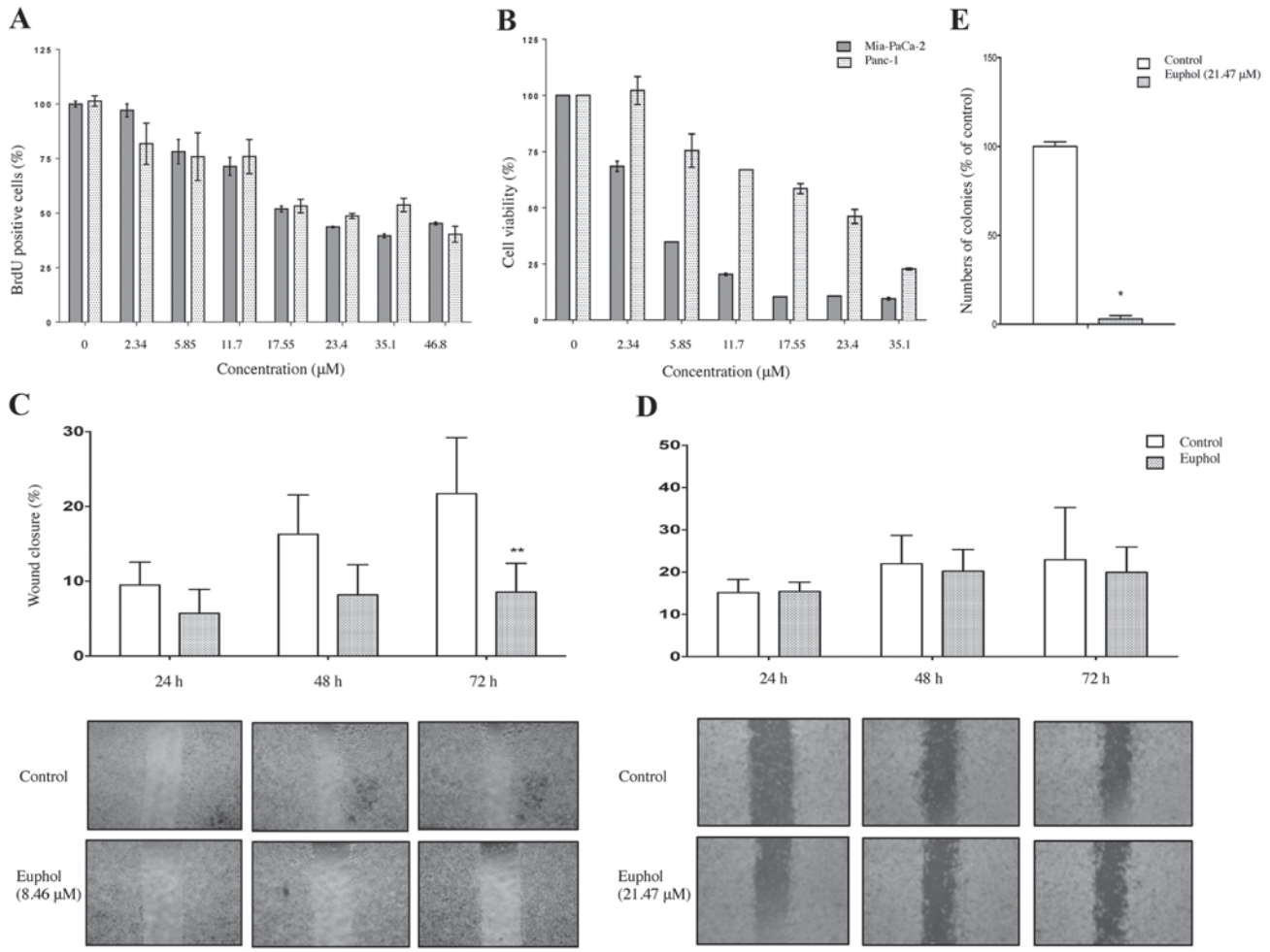

Figure 3. Effect of euphol on pancreatic cancer cell. (A) Cell proliferation was measured with BrdU assay and (B) cell viability was measured with MTS assay on Mia-Pa-Ca-2 and Panc-1 cell lines after 72 h of euphol treatment. The proliferation of the untreated cells and the viability of the untreated cells were regarded as 100%. Results shown are the means ± SD of three independent experiments. Cell motility of (C) Mia-Pa-Ca-2 and (D) Panc-1 was evaluated with wound healing assay. In this assay, a standardized scratch (wound) was applied to cell monolayers and digital images, of the same areas, were taken at several time points (0, 24, 48 and 72 h) after euphol treatment. The distance in pixels was measured in each time point and the percentage of wound closure was calculated according to the distance on initial time point (0 h). Figures are representative of three independent experiments. **Differences with P<0.005 in the Student's t test were considered statistically significant. (E) Effect of euphol on colony formation assay in pancreatic cancer cells. Panc-1 cells were seeded and grown in soft agar medium containing euphol. The number of colonies in each well was determined 20 days after initial euphol treatment. The figures are representative of two experiments performed in duplicate. *Differences with P<0.05 in the Student's t test were considered statistically significant.

the American National Cancer Institute criteria, euphol would be a promising compound for further analysis since its IC_{50} values were lower than 30 μg/ml (http://www.cancer.gov) (32). The cytotoxic and/or growth-inhibition effects of euphol were identified at low IC_{50} values, being lower than 30 μg/ml for 71 out 73 cancer cell lines tested. Our results are in agreement with earlier *in vitro* reports that suggested *E. tirucalli* crude extracts or euphol may have antitumoral effect. The relative toxicity of the *E. tirucalli* crude extracts on Mia-Pa-Ca-2 was also evaluated by Munro *et al,* which demonstrated that methanol extracts exerted a significant decrease in cell viability at 25 μg/ml (33). Also, Choene *et al,* investigated *E. tirucali* crude extracts that contains different types of secondary metabolites mainly terpenes and flavonoids, and reported its effect on breast cancer (MCF-7 and MDA-MB-231) cell cycle arrest (34). Lin *et al*, reported the effect of euphol in gastric cancer cell lines (CSN, CS12, AGS and MKN45) with an IC_{50} values of 49.6, 12.8, 14.7 and 14.4 (μg/ml), respectively (17). The authors also reported that euphol induced apoptosis by upregulation of ERK signaling (17). Another study analyzing the T47D breast cancer cells showed that euphol has an anti-proliferative activity, with IC_{50} values of 260 μM (16). The results suggest a cytostatic effect for euphol that induced GI by cell cycle arrest at the G0/G1 phase. In our study we observed a lower IC_{50} value (38.89 μM) in the T47D cells, yet, also above the limite considered effective by NCI. It is worthy of note that although the extract of *E. tirucalli* containing 64% euphol (7), in our study breast cancer cell lines were the less responsive to euphol in which the HPLC purity revealed above 95%. This finding seems to be in accordance with the potential of the compound found in phytochemical evaluation, which indicated that it is a tetracyclic triterpene alcohol (7,35).

To gain more insight into the role played by euphol in tumorigenesis, we investigated the biological effect of euphol on pancreatic cancer cell lines. Euphol inhibited cell proliferation (anchorage-dependent growth) as well as colony formation (anchorage-independent growth) of pancreatic cancer cells. We also showed that euphol inhibits cell migration of Mia-Pa-Ca-2

Table II. Drug combination studies in pancreatic cancer cell lines.

Cell line	Euphol Mean IC_{50} (μM)	SD	GEM Mean IC_{50} (μM)	SD	CI GEM+Euphol[a]
Mia-PaCa-2	8.46	0.39	1.65	0.51	5.16
PANC-1	21.47	1.83	10.37	2.62	1.5
PSN-1	3.71	0.17	0.51	0.02	0.8
BXPC-3	5.47	1.64	0.61	0.05	0.76

[a]CI was analyzed using CalcuSyn Software version 2.0. The CI value significantly lower than 1.0, indicates drug synergism; CI value significantly higher than 1.0, drug antagonism; and CI value equal to 1.0, additive effect. CI, combination index; SD, standard deviation; GEM, Gemcitabine.

Table III. Drug combination studies in Oesophagus cancer cell lines.

Cell line	Euphol Mean IC_{50} (μM)	SD	Paclitaxel Mean IC_{50} (μM)	SD	CI PC+Euphol[a]
KYSE30	3.52	1.28	0.015	0.003	0.54
KYSE70	8.77	0.74	0.009	0.001	0.37
KYSE270	10.71	3.95	0.018	0.002	0.55
KYSE410	4.35	2.03	0.023	0.008	2.02

[a]CI was analyzed using CalcuSyn Software version 2.0. The CI value significantly lower than 1.0, indicates drug synergism; CI value significantly higher than 1.0, drug antagonism; and CI value equal to 1.0, additive effect. PC, paclitaxel; CI, combination index; SD, standard deiviation.

cancer cell line. One of the suitable molecular cancer targets is protein kinase (ERK), which is an important factor in the regulation of cell migration of numerous cell types. The ERK pathway inhibitors PD98059 and U0126 inhibit the migration of diverse cell types in response to cell matrix proteins, such as fibronectin, vitronectin and collagen (36). Supporting a possible role of ERK inhibition on migration modulation by euphol, Passos *et al*, showed that, at the intracellular level, euphol reduced TPA-induced extracellular signal-regulated ERK activation in skin inflammation in mice (13). However, these data are in disagreement with Lin *et al*, which showed that euphol selectively induced gastric cancer cells apoptosis by activation of ERK signaling (17). Taken together, these findings provided further support that euphol effect may depend on the cellular context and showed that further investigation regarding euphol in other cancer cell lines and in other experimental model are required.

In addition, we investigated the combination of euphol to chemotherapy in pancreas and esophageal cancer lines and we found that euphol when combined with a gemcitabine and paclitaxel treatment seems to have a synergistic effect (chemo-sensitization) leading to lower doses of therapeutic agents. This synergy (chemo-sensitization) is of major interest since those two standard chemotherapy drugs formed the therapy backbone for those cancers, the level of responses seen in practice is still suboptimal and there is an urgent need for improvement (37,38).

The present study constitutes, to our knowledge, the first largest screening of euphol efficacy on human cancer cell lines. We showed that euphol could be a promising agent on large number of tumor types, in particularly in esophageal and pancreatic cancer. One important limitation of the present study is the lack of normal counterpart cells of the distinct tumor types evaluated. Therefore, additional studies are warranted to address this topic. This study also revealed the inhitbition/reduction of some hallmark events, such as proliferation and migration as part of the mechanism of action of this compound on pancreatic cancer cells. Finally, the euphol also showed synergistic interactions with chemotherapeutic drugs used in clinical practice. Our results provide insights for further studies suggesting euphol as an interesting antineoplastic alone or in combination for cancer treatment.

Acknowledgements

The authors would like to acknowledge the discussions of Amazônia Fitomedicamentos Scientific Committee, in particular Dr. Amilcar Tanuri.

Funding

This study was supported by grants from Amazônia Fitomedicamentos Ltda. (grant no. FITO 05/2012) and Barretos Cancer Hospital, all from Brazil.

Availability of data and material

All data generated or analyzed during this study are included in this published article.

Authors' contributions

VAOS designed the experiments, and participated in data acquisition and interpretation. VAOS and MNR carried out

the studies of cell culture, including cytotoxicity and proliferation assays, wound healing migration assay, colony formation assay, drug combination studies and statistical analysis. AT helped to carry out the cell viability assay. RJSO and OM contributed to the design of some experiments, interpretation of data and were involved in critically revising the manuscript. JPL helped to design the drug combination experiments, and helped to draft and critically revise the manuscript. LFP was responsible for the preparation of extracts and contributed to the discussion of cytotoxicity results. RMR conceived the study, participated in its design and coordination, interpreted the data, drafted the manuscript and was involved in revising it critically for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The drug euphol was provided by Amazônia Fitomedicamentos Ltda. LFP is one of the authors and also one of the inventors of euphol's patent. The Amazônia Fitomedicamentos Ltda. is the sole and exclusive owner of the respective intellectual property rights. This study was supported by grants from Amazônia Fitomedicamentos Ltda as part of the euphol pre-clinical studies and VAOS and MNR received a scholarship from Amazônia Fitomedicamentos Ltda. to conduct of the study.

References

1. Lee JA, Uhlik MT, Moxham CM, Tomandl D and Sall DJ: Modern phenotypic drug discovery is a viable, neoclassic pharma strategy. J Med Chem 55: 4527-4538, 2012.
2. Khazir J, Riley DL, Pilcher LA, De-Maayer P and Mir BA: Anticancer agents from diverse natural sources. Nat Prod Commun 9: 1655-1669, 2014.
3. Cragg GM, Newman DJ and Yang SS: Natural product extracts of plant and marine origin having antileukemia potential. The NCI experience. J Nat Prod 69: 488-498, 2006.
4. WHO Model List of Essential Medicines Home Page. accessed Aug 4, 2016.
5. Hopkins AL: Network pharmacology: The next paradigm in drug discovery. Nat Chem Biol 4: 682-690, 2008.
6. Braz-Filho R: Contribuição da fitoquímica para o desenvolvimento de um país emergente. Quim Nova 33: 229-239, 2010.
7. Dutra RC, Campos MM, Santos AR and Calixto JB: Medicinal plants in Brazil: Pharmacological studies, drug discovery, challenges and perspectives. Pharmacol Res 112: 4-29, 2016.
8. Franco-Salla GB, Prates J, Cardin LT, Dos Santos AR, Silva WA Jr, da Cunha BR, Tajara EH, Oliani SM and Rodrigues-Lisoni FC: *Euphorbia tirucalli* modulates gene expression in larynx squamous cell carcinoma. BMC Complement Altern Med 16: 136, 2016.
9. Prakash E and Gupta DK: Cytotoxic activities of extracts of medicinal plants of euphorbiacae family studied on seven human cancer cell lines. Univers J Plant Sci 1: 113-117, 2013.
10. Keating GM: Ingenol mebutate gel 0.015 and 0.05%: In actinic keratosis. Drugs 72: 2397-2405, 2012.
11. Lebwohl M, Swanson N, Anderson LL, Melgaard A, Xu Z and Berman B: Ingenol mebutate gel for actinic keratosis. N Engl J Med 366: 1010-1019, 2012.
12. Dutra RC, Bicca MA, Segat GC, Silva KA, Motta EM, Pianowski LF, Costa R and Calixto JB: The antinociceptive effects of the tetracyclic triterpene euphol in inflammatory and neuropathic pain models: The potential role of PKCepsilon. Neuroscience 303: 126-137, 2015.
13. Passos GF, Medeiros R, Marcon R, Nascimento AF, Calixto JB and Pianowski LF: The role of PKC/ERK1/2 signaling in the anti-inflammatory effect of tetracyclic triterpene euphol on TPA-induced skin inflammation in mice. Eur J Pharmacol 698: 413-420, 2013.
14. Bani S, Kaul A, Khan B, Gupta VK, Satti NK, Suri KA and Qazi GN: Anti-arthritic activity of a biopolymeric fraction from *Euphorbia tirucalli*. J Ethnopharmacol 110: 92-98, 2007.
15. Dutra RC, de Souza PR, Bento AF, Marcon R, Bicca MA, Pianowski LF and Calixto JB: Euphol prevents experimental autoimmune encephalomyelitis in mice: Evidence for the underlying mechanisms. Biochem Pharmacol 83: 531-542, 2012.
16. Wang L, Wang G, Yang D, Guo X, Xu Y, Feng B and Kang J: Euphol arrests breast cancer cells at the G1 phase through the modulation of cyclin D1, p21 and p27 expression. Mol Med Rep 8: 1279-1285, 2013.
17. Lin MW, Lin AS, Wu DC, Wang SS, Chang FR, Wu YC and Huang YB: Euphol from *Euphorbia tirucalli* selectively inhibits human gastric cancer cell growth through the induction of ERK1/2-mediated apoptosis. Food Chem Toxicol 50: 4333-4339, 2012.
18. Santos OJ, Sauaia Filho EN, Nascimento FR, Júnior FC, Fialho EM, Santos RH, Santos RA and Serra IC: Use of raw *Euphorbia tirucalli* extract for inhibition of ascitic Ehrlich tumor. Rev Col Bras Cir 43: 18-21, 2016 (In English, Portuguese).
19. MacNeil A, Sumba OP, Lutzke ML, Moormann A and Rochford R: Activation of the Epstein-Barr virus lytic cycle by the latex of the plant *Euphorbia tirucalli*. Br J Cancer 88: 1566-1569, 2003.
20. Silva-Oliveira RJ, Silva VA, Martinho O, Cruvinel-Carloni A, Melendez ME, Rosa MN, de Paula FE, de Souza Viana L, Carvalho AL and Reis RM: Cytotoxicity of allitinib, an irreversible anti-EGFR agent, in a large panel of human cancer-derived cell lines: KRAS mutation status as a predictive biomarker. Cell Oncol(Dordr) 39: 253-263, 2016.
21. Dirks WG, Faehnrich S, Estella IA and Drexler HG: Short tandem repeat DNA typing provides an international reference standard for authentication of human cell lines. Altex 22: 103-109, 2005.
22. Yasukawa K, Akihisa T, Yoshida ZY and Takido M: Inhibitory effect of euphol, a triterpene alcohol from the roots of *Euphorbia kansui*, on tumour promotion by 12-O-tetradecanoylphorbol-13-acetate in two-stage carcinogenesis in mouse skin. J Pharm Pharmacol 52: 119-124, 2000.
23. Dutra RC, Simao da Silva KA, Bento AF, Marcon R, Paszcuk AF, Meotti FC, Pianowski LF and Calixto JB: Euphol, a tetracyclic triterpene produces antinociceptive effects in inflammatory and neuropathic pain: The involvement of cannabinoid system. Neuropharmacology 63: 593-605, 2012.
24. Teixeira TL, Oliveira Silva VA, da Cunha DB, Polettini FL, Thomaz CD, Pianca AA, Zambom FL, da Silva Leitão Mazzi DP, Reis RM and Mazzi MV: Isolation, characterization and screening of the in vitro cytotoxic activity of a novel L-amino acid oxidase (LAAOcdt) from Crotalus durissus terrificus venom on human cancer cell lines. Toxicon 119: 203-217, 2016.
25. Martinho O, Zucca LE and Reis RM: AXL as a modulator of sunitinib response in glioblastoma cell lines. Exp Cell Res 332: 1-10, 2015.
26. Martinho O, Silva-Oliveira R, Miranda-Goncalves V, Clara C, Almeida JR, Carvalho AL, Barata JT and Reis RM: In vitro and in vivo analysis of RTK inhibitor efficacy and identification of its novel targets in glioblastomas. Transl Oncol 6: 187-196, 2013.
27. Chou TC and Talalay P: Quantitative analysis of dose-effect relationships: The combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 22: 27-55, 1984.
28. Bruzzese F, Di Gennaro E, Avallone A, Pepe S, Arra C, Caraglia M, Tagliaferri P and Budillon A: Synergistic antitumor activity of epidermal growth factor receptor tyrosine kinase inhibitor gefitinib and IFN-alpha in head and neck cancer cells in vitro and in vivo. Clin Cancer Res 12: 617-625, 2006.
29. Konecny GE, Glas R, Dering J, Manivong K, Qi J, Finn RS, Yang GR, Hong KL, Ginther C, Winterhoff B, *et al*: Activity of the multikinase inhibitor dasatinib against ovarian cancer cells. Br J Cancer 101: 1699-1708, 2009.

30. Moniz S, Martinho O, Pinto F, Sousa B, Loureiro C, Oliveira MJ, Moita LF, Honavar M, Pinheiro C, Pires M, *et al*: Loss of WNK2 expression by promoter gene methylation occurs in adult gliomas and triggers Rac1-mediated tumour cell invasiveness. Hum Mol Genet 22: 84-95, 2013.
31. Freedman VH and Shin SI: Cellular tumorigenicity in nude mice: Correlation with cell growth in semi-solid medium. Cell 3: 355-359, 1974.
32. Trendowski M: Recent advances in the development of anti-neoplastic agents derived from natural products. Drugs 75: 1993-2016, 2015.
33. Munro B, Vuong QV, Chalmers AC, Goldsmith CD, Bowyer MC and Scarlett CJ: Phytochemical, antioxidant and anti-cancer properties of *Euphorbia tirucalli* methanolic and aqueous extracts. Antioxidants (Basel) 4: 647-661, 2015.
34. Choene M and Motadi L: Validation of the antiproliferative effects of *Euphorbia tirucalli* extracts in breast cancer cell lines. Mol Biol (Mosk) 50: 115-127, 2016.
35. Silva AC, de Faria DE, Borges NB, de Souza IA, Peters VM and Guerra Mde O: Toxicological screening of *Euphorbia tirucalli* L: Developmental toxicity studies in rats. J Ethnopharmacol 110: 154-159, 2007.
36. Klemke RL, Cai S, Giannini AL, Gallagher PJ, de Lanerolle P and Cheresh DA: Regulation of cell motility by mitogen-activated protein kinase. J Cell Biol 137: 481-492, 1997.
37. Voutsadakis IA: Molecular predictors of gemcitabine response in pancreatic cancer. World J Gastrointest Oncol 3: 153-164, 2011.
38. Wiedmann MW and Mossner J: New and emerging combination therapies for esophageal cancer. Cancer Manag Res 5: 133-146, 2013.