e-ISSN 1734-9168
http://www.isez.pan.krakow.pl/en/folia-biologica.html

Folia Biologica (Kraków), vol. 67 (2019), No 3
https://doi.org/10.3409/fb_67-3.13

Cytotoxicity of *Euphorbia peplus* Extract on MCF7 Breast Cancer Cells

Ahmed AL-EMAM, Mubarak AL-SHRAIM, Refaat Ali EID, Abdul-Moneim JAMIL,
Mahmoud Fawzy MOUSTAFA, and Khaled RADAD

Accepted September 25, 2019 *Published online September 27, 2019* *Issue online September 30, 2019*

Original article

AL-EMAM A., AL-SHRAIM M., EID R.A., JAMIL A.-M., MOUSTAFA M. F., RADAD K. 2019. Cytotoxicity of *Euphorbia peplus* extract on MCF7 breast cancer cells. Folia Biologica (Kraków) **67**: 127-139.

In this study, the effect of *Euphorbia peplus* aqueous extract on human breast cancer cell line MCF7 was examined. The short and long term cytotoxicity were evaluated using sulphorhodamine B and clonogenic assays respectively. Scanning and transmission electron microscopy were employed to examine *Euphorbia peplus*-induced ultrastructural changes in MCF7 cells. The sulphorhodamine B assay revealed that *Euphorbia peplus* inhibits the growth of MCF7 with an IC_{50} of 30.32 µg/ml. The clonogenic assay proved that *Euphorbia peplus*' growth inhibitory effect is long lasting. The ultrastructural examination demonstrated that *Euphorbia peplus* extract induces MCF7 cell death. Scanning electron microscopy showed apoptotic blebbing. Transmission electron microscopy displayed cellular shrinkage, the formulation of apoptotic bodies, mitochondrial changes, nuclear shrinkage, chromatin condensation, autophagic vacuoles, and necrotic changes. In summary, *Euphorbia peplus* has displayed growth inhibitory activity against MCF7 cells and induces cell death predominantly via apoptosis and could be exploited as a breast cancer treatment after further evaluation.

Key-words: *Euphorbia peplus*, breast cancer, MCF7 cells, clonogenic survival, ultrastructure.

Ahmed AL-EMAM✉, Department of Pathology, College of Medicine, King Khalid University, Abha, Saudi Arabia; Department of Forensic Medicine and Clinical Toxicology, Faculty of Medicine, Mansoura, University, Mansoura, Egypt.
E-mail: alemam.ahmed@gmail.com
Mubarak AL-SHRAIM, Refaat Ali EID, Abdul-Moneim JAMIL, Khaled RADAD, Department of Pathology, College of Medicine, King Khalid University, Abha, Saudi Arabia.
Mahmoud Fawzy MOUSTAFA, Department of Biology, College of Science, King Khalid University, Abha, Saudi Arabia; Department of Botany, Faculty of Science, South Valley University, Qena, Egypt.

Breast cancer is considered the most commonly diagnosed cancer in women and remains among the leading causes of cancer death worldwide (FERLAY *et al.* 2010; TORRE *et al.* 2015; FERLAY *et al.* 2015). Being characterized by genetic heterogeneity, multi-genotypic breast cancer explains why patients within clinically and morphologically similar classes show a varied response to therapy and the redundancy of the available traditional prognostic factors. Even though some breast cancer groups have been molecularly identified based on molecular gene expression profiles, further distinct molecular classification is still ongoing (PEROU *et al.* 2000; DOUMA *et al.* 2014; EUHUS & DIAZ 2015). So far, four distinct molecular groups have been characterized: luminal epithelial/estrogen receptor (ER) positive, c-erb-B2 (HER2) positive, basal-like, and normal breast-like (PEROU *et al.* 2000). Another study divided the luminal/ER-positive group into three subtypes: luminal A, B, and C (SORLIE *et al.* 2003). The combination of doxorubicin and cyclophosphamide is considered to be one of the most effective chemotherapy regimens for breast cancer (MAMOUNAS *et al.* 2005). However, the use of these conventional chemotherapeutic drugs ignore the molecular classification of the patients and frequently result in serious adverse side effects by producing unnecessary damage to the neighboring normal and healthy cells. These current results shed the

© Institute of Systematics and Evolution of Animals, PAS, Kraków, 2019
Open Access article distributed under the terms of the Creative Commons Attribution License (CC-BY)
http://creativecommons.org/licences/by/4.0
OPEN ACCESS

light on the need to further develop more targeted therapeutic strategies. Indeed, selective estrogen receptor modulators, such as tamoxifen and raloxifen, have been used to treat ER-positive patients, whereas trastuzumab, a HER2-targeted monoclonal antibody, is used on HER2-positive patients (PEER *et al.* 2007). Regrettably, the use of these drugs is associated with high incidence resistance, relapse, and some unwanted side effects (CHANG *et al.* 2011; NORMANNO *et al.* 2005; ADAMO *et al.* 2007). However, it has been shown that medicinal herb extracts may also be used as an adjuvant to inhibit tumor growth and to overcome these side effects (CAI *et al.* 2004). Moreover, combining chemotherapeutic drugs with herbal extracts could lower the chemotherapeutics doses required to treat cancer (NG *et al.* 2014). Herbal extracts result in a variety of effects due to being, anti-allergic, antimicrobial, anti-oxidant and anticancerous due to the presence of various components (BAK *et al.* 2013), and generally exert cytotoxic effects on tumor cells directly, or through the enhancement of the immune response. For this reason, the anti-cancerous activity of numerous herbal extracts is currently being investigated (CHU *et al.* 2009; SHOEMAKER *et al.* 2005).

Euphorbia peplus belongs to the genus *Euphorbia*, which is considered to be the largest among genera of the Euphorbiaceae family or, the spurge family. *E. peplus* is originally native to Europe and North Africa (ZHI-QIN *et al.* 2010). It is well known by the common name of Radium weed in Australia and Petty spurge in the UK. Other common names are milk weed, cancer weed, radium plant, or stinging milk weed. The family in general consists of about 7,500 plant species with a reputation for having several medicinal usages. The majority of its members ooze milky sap (latex) when their stems are cut. The milky sap that is used in traditional medicine for the treatment of nonmelanoma skin cancer and its active compounds has been determined to be diterpene esters (RAMSAY *et al.* 2011). The sap has been used as a treatment for solar keratosis and skin cancers in Australia for decades (ROSEN *et al.* 2012; BRAUN *et al.* 2014). In Europe, it has been used as a laxative and to treat warts, corns, waxy growths, asthma, catarrh, and skin cancers. In Ukraine, it is commonly used to treat gastric, hepatic, and uterine malignancies. Also, in Mauritius, its decoction is used to treat diarrhea and dysentery. Moreover, one of its uses in Saudi Arabia is to lower blood pressure (GREEN & BEARDMORE 1988; TCHINDA 2008). In addition, it has been shown to have antipyretic, analgesic, and antimicrobial effects (ALI *et al.* 2013). Many macrocyclic diterpenes with cytotoxic activity have been isolated from the sap fractionation. For instance, Ingenol 3-mebutate (angelate/PEP005), an ingenane diterpene derived from *E. peplus*, has emerged as a possible anticancer agent (ERSVAER *et al.* 2010). PEP005 has been found to be active against human melanoma xenografts in mice (GILLESPIE *et al.* 2004). Moreover, its effects extend to murine melanoma and lung carcinoma, human prostate, cervical and T-cell leukaemia (OGBOURNE *et al.* 2007). Furthermore, a previous study in murine mice revealed that PEP005 caused mitochondrial swelling and primary necrotic cell death of dysplastic keratinocytes (OGBOURNE *et al.* 2004). A different study showed that PEP005, in contrast to other topical agents for actinic keratosis, appears to target the residual dysplastic keratinocytes using two mechanisms; an immune-mediated response and necrosis (ROSEN *et al.* 2012; COREA *et al.* 2005). Ingenol 3-mebutate, now marketed as Picato gel, has lately been approved by the Food and Drug Administration (FDA), as a treatment of precancerous skin. Therefore, the present study examined the cytotoxic effect of *E. peplus* aqueous extract against MCF7 breast cancer cells by sulphorhodamine B (SRB) and clonogenic survival assays. Furthermore, *E. peplus*-induced ultrastructural changes were examined by both scanning (SEM) and transmission (TEM) electron microscopy.

Materials and Methods

Plant material and extraction

E. peplus stem and leaves from the Asir region, Saudi Arabia (18° 12′ 59″ N, 42° 30′ 19″ E), were collected and authenticated by Professor Mahmoud MOUSTAFA, at the Department of Biology, College of Sciences, King Khalid University, Abha, Saudi Arabia. Ten grams of stem and leaves were washed thoroughly with distilled water and crushed directly using a grinder (Thomas Wiley Laboratory Mill, Model 4) for 15 min. The extract was filtered through 2-layered muslin cloth and the filtrate was diluted with 20 ml of distilled water and left on a rotary shaker at 100 rpm at room temperature for 2 days. The extract was stored in ependorf tubes covered with aluminum foil and was kept at 4°C (ALAMRI & MOUSTAFA 2012).

Cell line and reagents

Human breast adenocarcinoma cell line (MCF7, cat no. 86012803) was purchased from the European Collection of Authenticated Cell Cultures (ECACC; Salisbury, United Kingdom). Dulbecco's modified eagle medium (DMEM), heat inactivated fetal bovine serum (FBS), essential amino acids, L-glutamine and phosphate buffered saline (PBS) without calcium and magnesium, penicil-

lin/streptomycin (PS) solution, trypsin/EDTA, and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (Kuala Lumpur, Malaysia).

Cell culture

MCF7 cells were grown in DMEM supplemented with 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine in 75 cm^2 tissue culture flasks at 37ºC and a 5% CO_2 atmosphere. Cultured cells were trypsinised using 0.25% trypsin-EDTA upon reaching 70-80% confluence (2-3 days) and were used between passages 5-10.

Sulphorhodamine B assay

Cultured MCF7 cells were trypsinized, resuspended in fresh medium and counted. Then the cells were plated in 96 well tissue culture plates (5×10^3 cell/well) for 24 hrs. Three plates were used for control for each of the concentrations. After discarding the old medium, serum free medium (100 µl/well) was added to the control plates. Based on a literature search, free medium containing different concentrations of *E. peplus* aqueous extract (0.01, 0.1, 1, 10, 100 µg/ml) were added to the other plates for 48 hrs. After 48 hrs of incubation, the culture medium was removed and cells were fixed with 10% (v/v) trichloroacetic acid (TCA) at 4°C for 1 h. The plates were washed three times with distilled water to remove the TCA and then stained with 70 µl/well of 0.4% sulphorhodamine B (SRB) in 1% acetic acid for 30 min at room temperature. The excess dye was removed by washing 4 times with 1% (v/v) acetic acid and the plates were allowed to dry at room temperature. The protein-bound dye was solubilized in a 10 mM Tris base solution (100 µl/well) and the optical density (OD) of the solution was determined at 570 nm using a microplate reader. The reading was repeated thrice and the mean of three readings was used for analysis. Control values were set at 100% viable and all values were expressed as a percentage of the control. The inhibitory concentration 50% (IC_{50}) of *E. peplus* was calculated using a AAT Bioquest IC_{50} calculator (AAT Bioquest, Inc., Sunnyvale, CA). The values were plotted by using Microsoft Excel (VICHAI & KIRTIKARA 2006).

Clonogenic survival assay

MCF7 cells were grown in two 75 cm^2 cell culture flasks for 24 hrs (until ~70% confluence). After that, the medium was removed. One flask was treated with the IC_{50} of *E. peplus* (30.32 µg/ml) for 48 hrs and the other flask was used as an untreated control. Then, the cells were gently trypsinised and counted using a haemocytometer and serially diluted into 10 ml universals. Cells were seeded out at 100 cell/well into a 6-well plate: three plates for each of the control and treated cells. The 6-well plates were incubated under standard growth conditions for 14 days and stained with crystal violet (0.4% crystal violet, 50% methanol). The number of colonies was counted three times using Image J software (The National Institute of Health, Bethesda, MD) (SILVA *et al.* 2018). Plating efficiencies (PE) were calculated from the ratio between the number of colonies counted and the number of cells seeded. The surviving fraction (SF) was calculated using the formula: SF = PE of treated cells/PE of control × 100 (FRANKEN *et al.* 2006; RAFEHI *et al.* 2011; AL-EMAM *et al.* 2018a).

Scanning electron microscopy SEM

MCF7 cells were grown in complete DMEM, treated with 30.32 µg/ml of *E. peplus* aqueous extract and incubated for 48 hrs in a humidified CO_2 incubator. Non-treated control cells were also cultured in complete DMEM. After 48 hrs, cells were centrifuged and cell pellets were obtained. The cell pellets were immediately fixed in 2.5% (wt/vol) Na cacodylate-buffered glutaraldehyde, pH 7.4 at 4°C for 2 hrs. Then, they were post-fixed in 1% Na cacodylate-buffered osmium tetroxide, pH 7.4 for 1 h. After that, the cells were dehydrated in ascending concentrations of ethanol. Drying was done using the EMITECH-K850 critical-point drying unit. The dried cells were mounted on aluminum stubs with double sided tape and silver glue and then sputter coated with gold by BOC EDWARDS SCANCOAT. Finally, cells were observed using a Jeol SEM (JSM-6390LV, Japan), operated at 2-5 kV at the Electron Microscope Unit, College of Medicine, King Khalid University (EID *et al.* 2012).

Transmission electron microscopy TEM

MCF7 cells were seeded in 6-well culture plates (2×10^5 cell/well). Cultured cells were treated with the IC_{50} of E. *peplus* aqueous extract (30.32 µg/ml) for 48 hrs. Treated cells were then collected in Hank's Balanced Salt Solution (HBSS), centrifuged at 5000 rpm and the resultant cell pellets were fixed in 2.5% (wt/vol) Na cacodylate-buffered glutaraldehyde (pH 7.2) at 4°C for 2 hrs. The cell pellets were then washed in the same buffer and postfixed in 1% osmium tetroxide in Na cacodylate buffer. After ascending series of ethanol dehydration, the pellets were embedded in Spurr's resin. Ultrathin sections stained with uranyl acetate and lead citrate were examined by TEM (Jeol 100CXII, Japan) operated at 80 kV at the Electron Microscope Unit, College of Medicine, King Khalid University (AL-EMAM *et al.* 2018b).

Statistics

All experiments were done in triplicate and the data were presented as mean ± standard deviation (SD). The data were statistically analyzed using one-way ANOVA with Tukey's post hoc tests for the SRB cytotoxicity assay and the Students' *t*-test for the clonogenic survival assay. Analysis was performed using the statistical program IBM SPSS 22. $p \leq 0.05$ was considered as statistically significant.

Results

Effect of *E. peplus* extract on the viability of MCF7 cells

The cytotoxic effect of *E. peplus* aqueous extract on MCF7 cells was examined by the SRB assay, which is one of the short-term cytotoxicity assays. MCF7 cells were treated with different concentrations of *E. peplus* aqueous extract for 48 hrs. The results depicted in Fig. 1A demonstrate that *E. peplus* extract significantly reduced the viability of the cultured MCF7 by 2.79%, 4.65%, 10.30%,

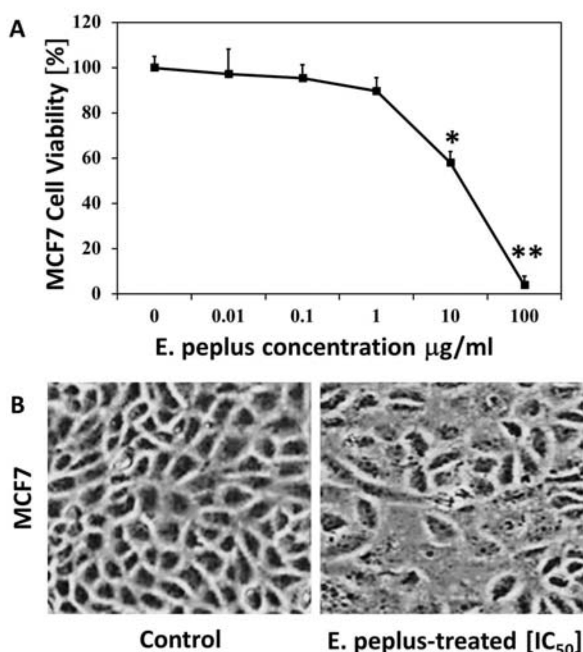

Fig. 1. A, B. Measurement of MCF7 cell viability by SRB. A – MCF7 cells (5000 cells/well) treated with different concentrations of *E. peplus* aqueous extract (0.01, 0.1, 1, 10, 100 µg/ml) for 48 hrs. The percentages of growth inhibition were plotted against the different concentrations and the generated graph was used to calculate the IC_{50} of *E. peplus*. 100% corresponds to the viability of untreated control cells. Error bars represent standard deviation and asterisks represent *p* values (*$p < 0.01$ and **$p < 0.001$). B – Microscopic examination showed that MCF7 treated with the *E. peplus*-calculated IC_{50} (30.32 µg/ml) became rounded and detached compared to the untreated controls.

41.96%, and 96.09% at the concentrations 0.01, 0.1, 1, 10 and 100 µg/ml, respectively. 100% corresponds to the viability of untreated control cells. One-way ANOVA was employed to detect differences between the groups of treated MCF7 cells and the control group. p values <0.05 were considered as statistically significant. Anova showed statistical significance and Tukey's post hoc revealed significant growth inhibition of MCF7 cells treated with 10 and 100 µg/ml concentration of *E. peplus* compared to nontreated control cells with $p = 0.01$ and $p = 0.001$respectively. The IC_{50} was 30.32 µg/ml. Moreover, microscopic examination of the MCF7 cells treated with the IC_{50} for 48 hrs revealed that the majority of the cells shran and became rounded and detached in comparison with untreated control MCF7 cells (Fig. 1B).

Effect of *E. peplus* extract on cell proliferation and colony survival of MCF7 cells

The clonogenic survival assay was employed to assess the long-term cytotoxicity of *E. peplus* aqueous extract on MCF7 cells. MCF7 cells, treated with the IC_{50} of *E. peplus* for 48 hrs, were incubated for 14 days to develop colonies. The number of colonies was counted using Image J. The plating efficiency of MCF7 cells was 80%. The surviving fraction of the colonies treated with the IC_{50} of *E. peplus* had a significantly lower plating effeciency (12±2.7) as compared to the untreated controls (80±2.9) ($p = 0.001$) (Fig. 2).

Ultrastructural changes of *E. peplus*-treated MCF7 cells

Electron microscopy is a multipurpose technique used for the examination of the surface and morphological features in tumor cells. The higher resolution of both SEM and TEM allows documenting the ultrastructural cellular changes during cell death. Moreover, it is listed among the most valid methods that can differentiate between the different modes of cell death alongside various biochemical markers (VENKATESAN *et al.* 2010; MILLER *et al.* 2002; WILLINGHAM 1999). MCF7 cells were treated with the IC_{50} of *E. peplus* for 48 hrs and the cell pellets were processed for both SEM and TEM examination. SEM examination of E. *peplus* treated MCF7 cells revealed characteristic intense apoptotic membrane blebbing as shown in Fig. 3B, whereas the untreated control cells didn't show any surface zeiotic blebbing (Fig. 3A). Apoptotic blebs appear as quasi-spherical, balloon-like protrusions of the cell membrane with the cell membrane still intact.

TEM examination of untreated MCF7 cells exhibited spherical morphology with few scattered surface microvilli and normal mitochondrial mor-

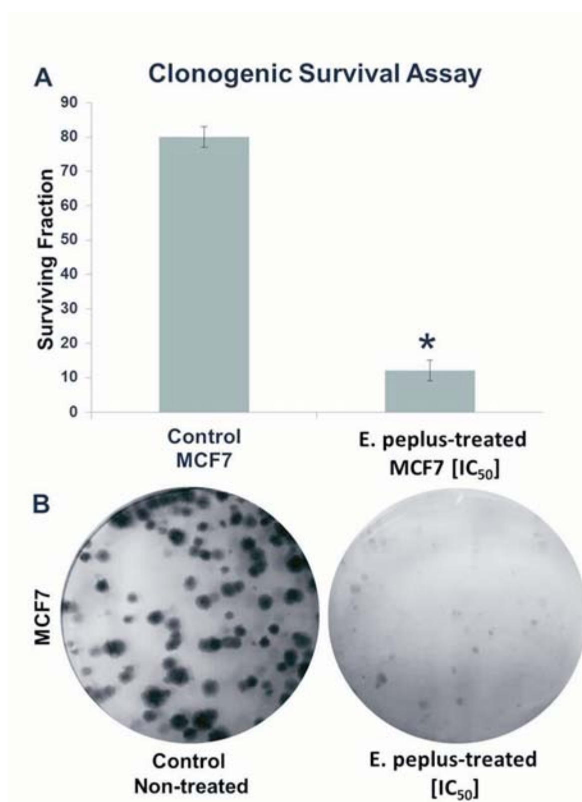

Fig. 2. A, B. Clonogenic survival of *E. peplus*-treated MCF7 cells. MCF7 cells were treated with the IC_{50} of *E. peplus* aqueous extract (30.32 μg/ml) for 48 hrs and incubated for 2 weeks, the colonies were stained with crystal violet, counted with Image J software. A – Bar graph demonstrates the reduced number of colonies formed after treatment compared to non-treated control. Results are expressed as mean ± SD with $*p < 0.001$ indicating significant difference. B – Representative image of clonogenic assay of *E. peplus*-treated MCF7 cells showing a decrease of the number of surviving colonies relative to the untreated control.

phology and distribution. They also had large nuclei with both eu- and heterochromatin (Fig. 4A). On the other hand, *E. peplus*-treated MCF7 cells revealed a loss of surface microvilli (Fig. 4B), mitochondrial distortion in the form of elongation and shrinkage and some electron dense lysosomes (Fig. 4B, C, D, E and Fig. 5B, C, D, E). A majority of treated cells showed the presence of autophagic vacuoles (autophagosomes) in their cytoplasm. Some of these vacuoles were seen containing damaged organelles and mitochondria (Fig. 4 B, C, D, E, F). Nuclear changes in the form of shrinkage, chromatin condensation, and irregular nuclear membrane were constant findings in the *E. peplus*-treated cells (Fig. 4D and E). Moreover, some cells had diffuse areas of cytoplasmic degeneration (Fig. 4B, C and Fig. 5A). Also, apoptotic blebs and apoptotic bodies were frequently captured as shown in Fig. 4B, C and Fig. 5B, C, E, F, G, H. Furthermore, necrotic changes were noticed in many *E. peplus*-treated cells. These changes were in the form of a pyknotic nucleus, cell membrane rupture, the appearance of secondary lysosomes, and autophagic vacuoles indicating cellular digestion of organelles as shown in Fig. 6B, C, D as compared to the untreated control cells in Fig. 6A.

Discussion

The spurge family (Euphorbiaceae) is a large family of plants that have been used over the years for the treatment of various ailments including cancer. The aim of the present study was to examine the cytotoxicity of *E. peplus* aqueous extract against the MCF7 breast cancer cell line. The SRB assay revealed that *E. peplus* aqueous extract is cy-

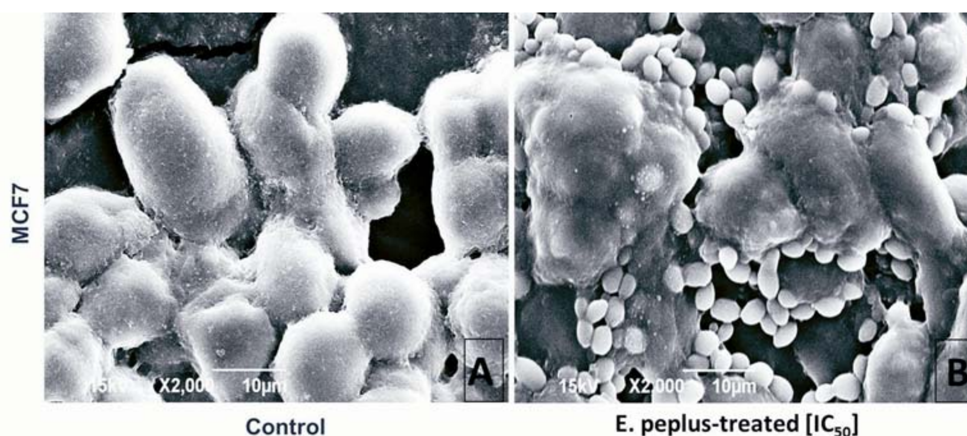

Fig. 3. A, B. Scanning Electron micrograph for non-treated and *E. peplus*-treated MCF7 cells. The MCF7 cells were treated with the IC_{50} of *E. peplus* aqueous extract (30.32 μg/ml) for 48 hrs. The cells were fixed in Na cacodylate-buffered glutaraldehyde, then post-fixed in osmium tetroxide, dehydrated, mounted on aluminum stubs, sputter coated with gold and finally examined under a Jeol SEM. Magnification was x 2000. A – Control non-treated MCF7. B – MCF7 Cells treated with the IC_{50} of *E. peplus* (30.32 μg/ml) showing the formation of characteristic apoptotic blebs. The blebs appear as quasi-spherical, balloon-like cell membrane protrusions. The cell membrane is intact in both the control and treated cells.

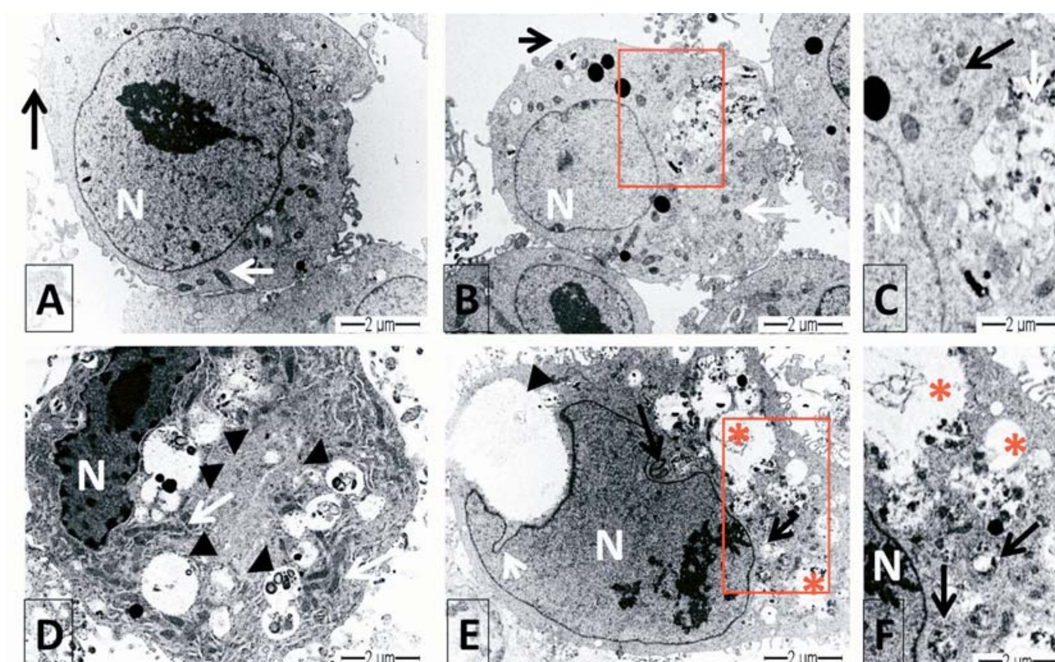

Fig. 4. A-F. TEM micrographs for non-treated and *E. peplus*-treated MCF7 cells. MCF7 cells were treated with the IC_{50} of *E. peplus* aqueous extract (30.32 µg/ml) for 48 hrs. The cells fixed in Na cacodylate-buffered glutaraldehyde, postfixed in osmium tetroxide, dehydrated, embedded in Spurr's resin, stained with uranyl acetate and lead citrate, and finally examined under Jeol 100CXII TEM. Magnification was x 8000. A – a control, non-treated cell showing normal surface microvilli (black arrow), normal mitochondrial morphology and distribution (white arrow) and a normal-shaped nucleus with normal chromatin distribution (N). B – an *E. peplus*-treated cell showing a loss of surface microvilli (black arrow) and few mitochondria (white arrow). C – a magnification of the area delineated by red rectangle in B showing mitochondrial damage (black arrow) and autophagic vacuoles (white arrow). D – an *E. peplus*-treated MCF7 showing a shrunken nucleus with chromatin condensation, autophagic vacuoles containing digested organelles (arrow heads), and elongated mitochondria (white arrows). E – an *E. peplus*-treated cell showing numerous autophagic vacuoles (asterisks), an irregular nuclear membrane (white arrow), distorted mitochondria (black arrow), and the focal area of cytoplasmic lucency (arrow head). The red rectangular area has been magnified in F.

totoxic to breast cancer MCF7 cells as the viability of the *E. peplus*-treated MCF7 cells significantly declined in a concentration-dependent manner. After 48 hrs of treatment the IC_{50} of *E. peplus* was calculated to be 30.32 µg/ml. In consistency with this finding, the active principle of *E. peplus* (PEP005) has been found to be cytotoxic against different cancer cell lines including skin, lung, prostate, cervical and T-cell leukaemia (ERSVAER *et al.* 2010; GILLESPIE *et al.* 2004; OGBOURNE *et al.* 2007). Moreover, different herbal extracts from the genus *Euphorbia* were shown to possess cytotoxic activity against the breast cancer cell line MCF7; for example *E. hirta* (KWAN *et al.* 2016), *E. triaculeata* (AL-FAIFI *et al.* 2017), *E. macrostegia* (BANIADAM *et al.* 2014), *E. fischeriana* (KUANG *et al.* 2016), *E. humifusa* (SHIN *et al.* 2016), and *E. supina* (KO *et al.* 2015). The reduction of MCF7 cells' viability detected by SRB in response to *E. peplus* extract treatment could be attributable to the inhibition of cell proliferation, increased cell death, or both. Although SRB is one of the assays that measure the potency of a cytotoxic agent, it is considered to be a short-term cytotoxicity assay. In addition, it might underestimate the cytotoxicity of a drug or chemical agent when compared with long-term assays for cell growth or cloning efficiency. Moreover, such assays do not take into consideration the reversible damage and/or regrowth of cancer cells that are resistant to treatment (PEREZ *et al.* 1993). Therefore, in order to determine whether the cytotoxic effect of *E. peplus* against MCF7 is long lasting, we selected the clonogenic survival assay which is widely used for testing and predicting the long-term cytotoxicity of anticancer agents (YALKINOGLU *et al.* 1990), as it evaluates the sum of all modes of cell death, and also accounts for delayed growth arrest (MIRZAYANS *et al.* 2007; STRONCEK *et al.* 2007). Indeed our results revealed that the treatment of MCF7 cells with IC_{50} of *E. peplus* for 48 hrs significantly reduced the number of surviving colonies compared to the untreated control cells (Fig. 2). In agreement with this finding, previous studies have revealed that phytochemicals including apigenin, luteolin, and flavone are potent in cancer

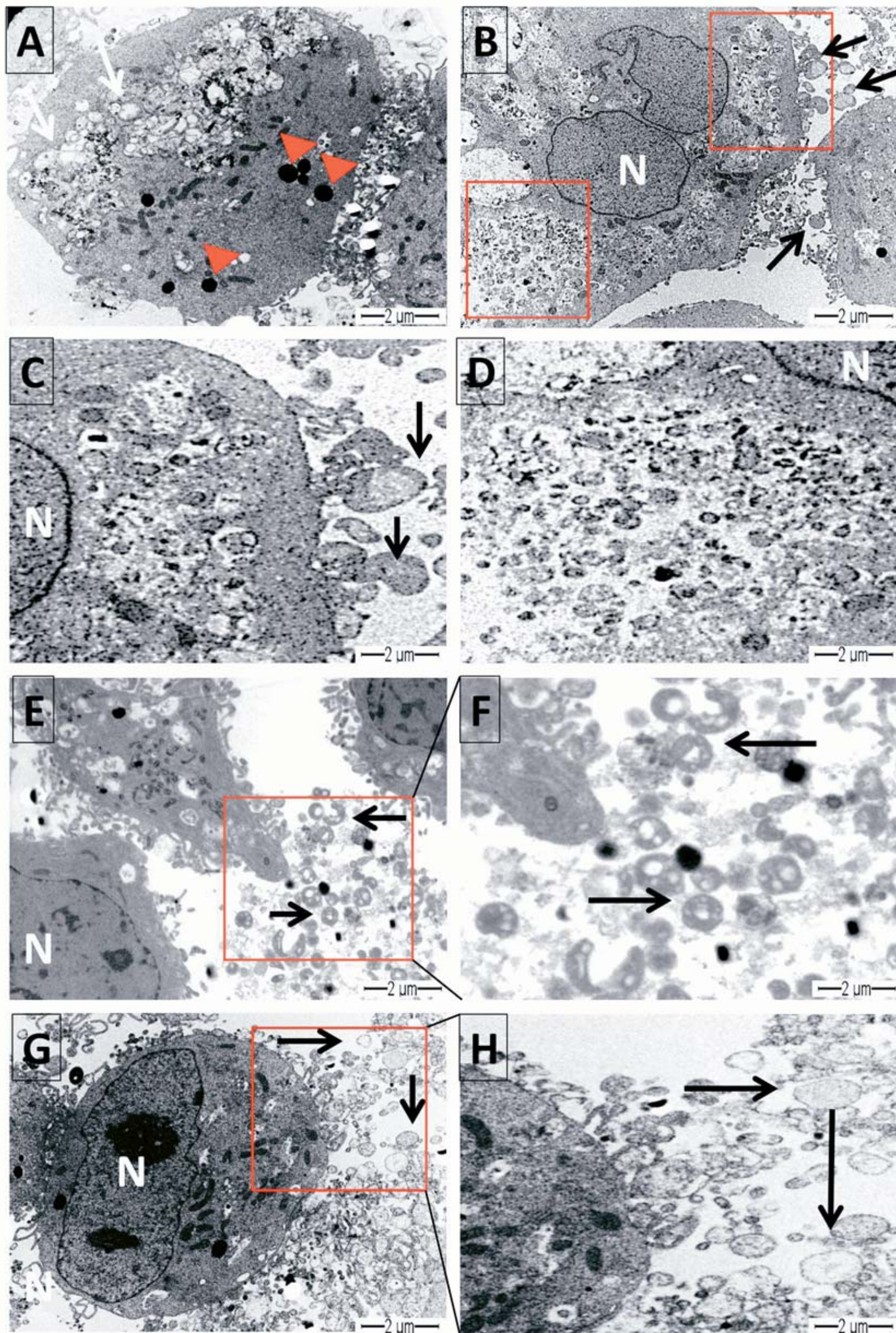

Fig. 5. A-G. TEM micrographs for *E. peplus*-treated MCF7 cells. MCF7 cells were treated with 30.32 µg/ml of *E. peplus* extract for 48 hrs, then fixed in Na cacodylate-buffered glutaraldehyde, postfixed in osmium tetroxide, dehydrated, embedded in Spurr's resin, stained with uranyl acetate and lead citrate, and finally examined under Jeol 100CXII TEM. Magnification was x 8000. A – the treated cell shows a large vacuolated area of cytoplasmic degeneration (white arrows) and electron dense lysosomes (red arrow heads). B – the treated cell shows surface apoptotic blebs (black arrows), autophagic vacuoles in the area delineated by a red rectangle in the upper right corner of the image, which has been magnified in C, and shrunken and damaged mitochondria in the area delineated by a red rectangle in the lower left corner of the image, which has been magnified in D. E & G – apoptotic cells with areas of apoptotic bodies delineated by red boxes which have been magnified in F and H, respectively.

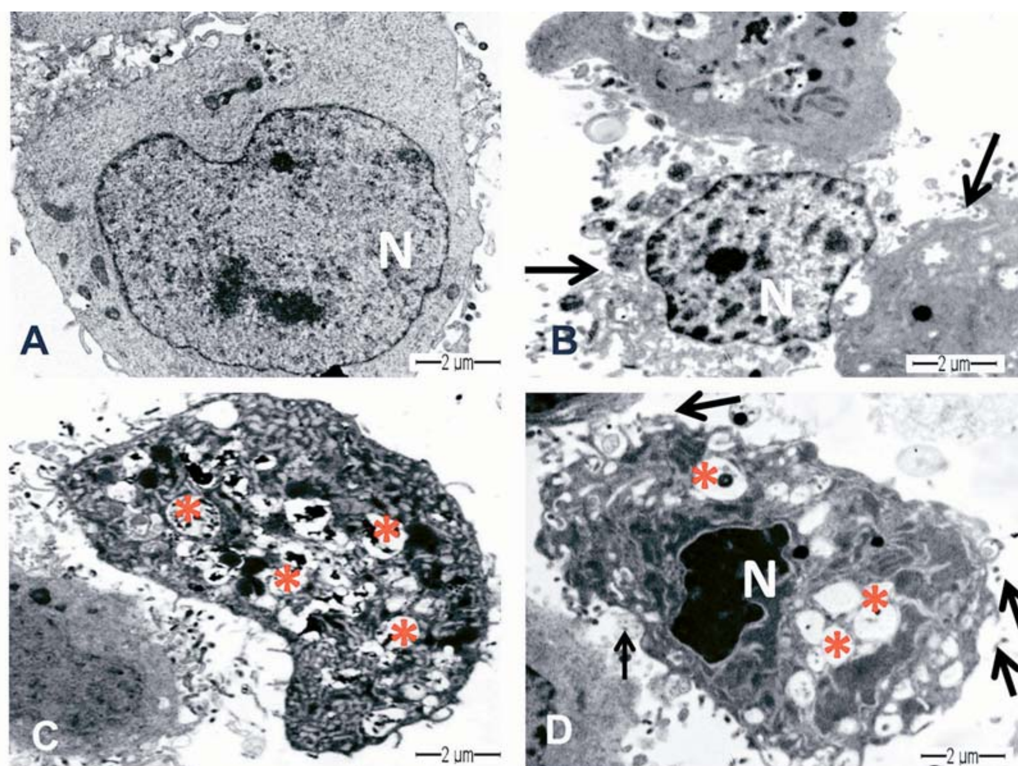

Fig. 6. A-D. TEM micrographs for non-treated control and *E. peplus*-treated MCF7 cells. MCF7 cells were treated with the IC_{50} of *E. peplus* aqueous extract (30.32 µg/ml), and then fixed in Na cacodylate-buffered glutaraldehyde, postfixed in osmium tetroxide, dehydrated, embedded in Spurr's resin, stained with uranyl acetate and lead citrate, and finally examined under Jeol 100CXII TEM. Magnification was x 8000. A – control non-treated cells. B-D – *E. peplus*-treated MCF7 showing necrotic changes: cell membrane rupture indicated by black arrows (B & D), an abundance of autophagic vacuoles indicated by asterisks (C & D), and a pyknotic nucleus (D).

chemoprevention. Their long-term effects on the growth of breast cancer cell lines were tested by clonogenic survival assay and they reduced colony numbers 2-3 fold (LIN *et al.* 2015).

In order to examine whether the cytotoxicity of *E. peplus* against MCF7 cells is due to the induction of cell death, we decided to study the ultrastructural changes of MCF7 cells after treatment with the IC_{50} of *E. peplus* for 48 hrs. Interestingly, SEM examination of *E. peplus*-treated MCF7 cells revealed zeiotic membrane blebbing which could be an early event preceding cell death (CHARRAS 2008). Zeiotic blebbing is indeed observed during the execution phase of apoptosis which consequently leads to chromatin condensation, DNA fragmentation, and apoptotic cell death (WYLLIEE *et al.* 1980; EARNSHAW 1995; JACOBSON *et al.* 1997). Moreover previous studies, in agreement with our findings, described the same significant morphological apoptotic characteristics in MCF7 cells in response to cytotoxic herbal treatment. For instance, *Elephantopus scaber* Linn, a herb from the Asteraceae family, showed cytotoxic effects with apoptotic blebbing towards MCF7 cells with an IC_{50} value of 15 µg/ml (HO *et al.* 2011). In addition, the same observation was reported by COLLINS *et al.* (1997). Furthermore, photodynamic therapy induced apoptosis with predominant apoptotic blebs in different cancer cell lines (HeLa, HaCaT, and MCF7) pretreated with photosensitizers (COLLINS *et al.* 1997). However, zeiotic membrane blebbing has been found to be closely associated with cytokinesis (FISHKIND *et al.* 1991), motility (BERGERT *et al.* 2012), cell spreading (NORMAN *et al.* 2010) and mitosis (MOES *et al.* 2011), contractolation (KHAJAH *et al.* 2015), and during early embryogenic migration (TRINKAUS 1973). Also, recent studies revealed that cancer stem cells which undergo apoptosis, create blebbishields from apoptotic bodies, overrule phagocytosis and undergo cellular transformation by blebbishield-mitotic cell, blebbishield-blebbishield, or blebbishield-immune cell fusion and reform tumors in mice. This exciting blebbishield emergency program linked apoptotic cancer stem cells to immune evasion, drug resistance, tumorigenesis, apoptosis evasion, and metastasis (JINESH 2017).

Therefore, in order to verify the lethal effects of *E. peplus* on MCF7 cells, we went further to examine *E. peplus*-treated MCF7 cells with TEM. TEM examination revealed apoptotic changes in the form of mitochondrial elongation, shrinkage and damage, nuclear shrinkage, chromatin condensation, irregular nuclear membrane and characteristic apoptotic blebs and apoptotic bodies as shown in Figs 4 and 5. Moreover, the majority of the *E. peplus*-treated cells displayed an abundance of autophagic vacuoles enclosing damaged organelles and mitochondria (Fig. 4). Furthermore, some cells showed a pyknotic nucleus, cell membrane rupture, the appearance of secondary lysosomes, and autophagic vacuoles (Fig. 6), findings which are indicative of necrosis.

In agreement with our findings, several studies over the years have proved the anticancer activities of different members of the genus *Euphorbia*, predominantly through their pro-apoptotic activities against numerous cancer cell lines (WONGRAKPANICH & CHAROENSUKSAI 2018): for instance, *E. hirta* (KWAN *et al.* 2016), *E. sogdiaa* (AGHAEI *et al.* 2016), *E. fischeriana* (KUANG *et al.* 2016) and *E. humifusa* (SHIN *et al.* 2016) were found to be cytotoxic and induce the apoptotic cell death of breast cancer cell lines. Moreover, ingenol-3-angelate (also known as ingenol mebutate and PEP005), a diterpene isolated from *E. peplus*, has demonstrated anticancer activity against melanoma (OGBOURNE *et al.* 2004) and actinic keratoses (SILLER *et al.* 2009). Its mechanism of action includes primary cell death by necrosis (OGBOURNE *et al.* 2004), initiation of G1 and G2/M cell cycle arrest (COZZI *et al.* 2006), activation of protein kinase C (HAMPSON *et al.* 2005), and neutrophils (HAMPSON *et al.* 2008). It was indeed intriguing as apoptotic changes were the predominant ultrastructural changes in this study as shown in Figs 4 and 5 with a minority of cells showing both autophagic and necrotic changes (Figs 5 and 6). However, it has recently been found that 3-O-angeloyl-20-O-acetyl ingenol, a synthetic derivative of ingenol mebutate known as AAI or PEP008, inhibits cell proliferation, induces G2/M phase arrest, disrupts the mitochondrial membrane potential, and stimulates apoptosis, as well as necrosis in breast cancer and human leukemia cell line via modulation of PKCδ/ERK, JAK/STAT3, and AKT signaling pathways (LIU *et al.* 2016). In addition, GILLESPIE *et al*. (2004) proved that ingenol 3-angelate can trigger necrosis alone or both necrosis and apoptosis in a cell-line-dependent manner, depending on the PKC status of the cells. Also, *E. hirta* aqueous extract has recently been shown to induce both apoptosis and necrosis of breast cancer cell lines (BEHERA *et al.* 2016). Furthermore, there are several examples in literature that documented the occurrence of both autophagic cell death and apoptosis in different cancers. For example, Paratocarpin E, a prenylated chalcone isolated from *E. humifusa* resulted in typical apoptosis of MCF7 cells via activating p38 and JNK and suppressing ERK signaling pathways. Paratocarpin E-treated MCF7 showed plenty of autophagic vacuoles associated with an increased ratio of LC3-II/LC3-I, activation and nuclear translocation of NF-κB, yet a reduced p62 expression. This signposts the strong pro-autophagic effects of paratocarpin E (GAO *et al.* 2016). Noteworthy, characteristic features of apoptosis have been noticed to occur during autophagy in the absence of essential apoptotic players such as caspases or Bax and Bak (GALLUZZI *et al.* 2012). Also, Cepharanthine, a natural alkaloid from *Stephania cepharantha* (GAO *et al.* 2017), and Baicalein, a bioactive compound from *Scutellaria baicalensis* (YAN *et al.* 2018) have been shown to induce apoptotic and autophagic cell death in various cancers in addition to their numerous anticancer properties. For an exhaustive list of natural autophagy inducers, interested readers are referred to a review by LIN *et al.* (2017). In fact, it has been confirmed that different cell death pathways including apoptosis, mitotic catastrophe, autophagy, and necrosis could be evoked by photodynamic therapy (YOO & HA 2012; MROZ *et al.* 2011; CASTANO *et al.* 2005). Moreover, several studies have shown that a shift from apoptosis to necrosis does exist. The switch in the mode of cell death is dependent on a variety of factors, comprising the nature of photosensitizers, cell genotype, and the dose of photodynamic therapy (YOO & HA 2012; MROZ *et al.* 2011; RUIZ-GONZALEZ *et al.* 2013; ACEDO *et al.* 2014). Furthermore, the co-existence of necrosis and apoptosis has been demonstrated in previous studies (LEDDA-COLUMBANO *et al.* 1991; FUKUDA *et al.* 1993). In addition mixed biochemical and morphological characteristics of both necrosis and apoptosis have been revealed in single cell (PENA & PILAR 2000). In fact, the concomitance of apoptotic, autophagic and necrotic changes in our ultrastuctural data could be conceivably explained by the finding that ingenol 3-angelate, an *E. peplus* deriviative, has a dual mechanism inducing both apoptosis and necrosis (GILLESPIE *et al.* 2004). In addition, the abundance of autophagic changes could be viewed as a regulatory switch between the two other *E. peplus*-induced modes of cell death: apoptosis and necrosis. In support of this explanation, the active role of autophagy in MCF7 cell death has recently been demonstrated (GAVLAN *et al.* 2013). For more details on the role of autophagy in breast cancer, interested readers are referred to a review by JAIN *et al*. (2013). However, the more plausible explanation for the variability in terms of cell

death modes observed in our current study and the aforementioned ones could be the plasticity of cell death induction and execution. In other words, cell death could be viewed as a dynamic process with a molecularly overlapping and interactive signaling network rather than a mere static pathway as reviewed by LOOS and ENNGELBRECHT (2009). Indeed, autophagy appears to have a central and upstream role in the cell death process (KARANTZA-WADSWORTH *et al.* 2007; KROEMER *et al.* 2009) and controls the onset of the other cell death modalities (YONEKAWA & THORBURN 2013). For a more detailed discussion about the dynamic regulatory interactions between autophagy and the other cell death machineries, interested readers are referred to these reviews (LIU & LEVINE 2014; GOODALL *et al.* 2016).

In conclusion, the data reported in this study provide sufficient proof that *E. peplus* aqueous extract could inhibit the growth and induce cell death of MCF7 breast cancer cells mainly via apoptosis and that it might be beneficial as an adjuvant therapy alongside conventional breast cancer treatments. The clonogenic survival assay proved that the effect of *E. peplus* on MCF7 cells is long lasting and abrogates the possibility of reversibility of apoptosis. However, it should be emphasized that the apoptotic, autophagic and necrotic changes witnessed by our ultrastructural examination need further investigation to understand the mechanism of cell death induction and execution of *E. peplus* and its derivatives in different cancer cell lines.

Acknowledgement

The authors are thankful to the Deanship of Scientific Research at the King Khalid University for the financial support of project no. KKU-GRP-230-39.

Author Contributions

Research concept and design: A.A., M.A-S., A-M.J., K.R.; Collection and/or assembly of data: A.A., R.A.E., M.F.M.; Data analysis and interpretation: A.A., M.A-S., R.A.E., A-M.J., K.R.; Writing the article: A.A.; Critical revision of the article: A.A., M.A-S., R.A.E., A-M.J., M.F.M. K.R.; Final approval of article: A.A., M.A-S., R.A.E., A-M.J., M.F.M., K.R.

Conflict of Interest

The authors declare no conflict of interest.

References

ACEDO P., STOCKERT J.C., CANETE M., VILLANUEV A. 2014. Two combined photosensitizers: a goal for more effective photodynamic therapy of cancer. Cell Death Dis. **5**: e1122. https://doi.org/10.1038/cddis.2014.77

ADAMO V., IORFIDA M., MONTALTO E., FESTA V., GARIPOLI C., SCIMONE A., ZANGHI M., CARISTI N. 2007. Overview and new strategies in metastatic breast cancer (MBC) for treatment of tamoxifen-resistant patients. Ann. Oncol. **18**: 53-57. https://doi.org/10.1093/annonc/mdm225

AGHAEI M., YAZDINIAPOUR Z., GHANADIAN M., ZOLFAGHARI B., LANZOTTI V., MIRSAFAEE V. 2016. Obtusifoliol related steroids from *Euphorbia sogdiana* with cell growth inhibitory activity and apoptotic effects on breast cancer cells (MCF-7 and MDAMB231). Steroids **115**: 90-97. https://doi.org/10.1016/j.steroids.2016.07.008

ALAMRI S.A., MOUSTAFA M.F. 2012. Antimicrobial properties of 3 medicinal plants from Saudi Arabia against some clinical isolates of bacteria. Saudi Med. J. **33**: 272-277.

AL-EMAM A., ARBON D., JEEVES M., KYSELA B. 2018a. Ku70 N-terminal lysines acetylation/deacetylation is required for radiation-induced DNA-double strand breaks repair. Neoplasma **65**:708-719. https://doi.org/10.4149/neo_2018_171020N673

AL-EMAM A., AL-SHRAIM M., EID R., AL-FAIFI M., AL-SHEHRI M., MOUSTAFA M.F., RADAD K. 2018b. Ultrastructural changes induced by *Solanum incanum* aqueous extract on HCT 116 colon cancer cells. Ultrastruct. Pathol. **42**: 255-261. https://doi.org/10.1080/01913123.2018.1447623

AL-FAIFI Z.I., MASRAHI Y.S., ALY M.S., AL-TURKI T.A., DARDEER T. 2017. Evaluation of Cytotoxic and Genotoxic Effects of *Euphorbia Triaculeata Forssk*. Extract. Asian Pac. J. Cancer Prev. **18**: 771-777. https://doi.org/10.22034/APJCP.2017.18.3.771

ALI A.A., SAYED H.M., IBRAHIM S.R.M., ZAHER A.M. 2013. Chemical constituents, antimicrobial, analgesic, antipyretic and anti-inflammatory activities of *Euphorbia peplus L*. Phytopharmacol. **4**: 69-80.

BAK Y., HAM S., BAATARTSOGT O., JUNG S.H., CHOI K.D., HAN T.Y., HAN I.Y., YOON D.Y. 2013. A1E inhibits proliferation and induces apoptosis in NCI-H460 lung cancer cells via extrinsic and intrinsic pathways. Mol. Biol. Rep. **40**: 4507-4519. https://doi.org/10.1007/s11033-013-2544-0

BANIADAM S., RAHIMINEJAD M.R., GHANNADIAN M., SAEIDI H., AYATOLLAHI A.M., AGHAEI M. 2014. Cycloartane Triterpenoids from *Euphorbia Macrostegia* with their Cytotoxicity against MDA-MB48 and MCF-7 Cancer Cell Lines. Iran. J. Pharm. Res. **13**: 135-141. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3985266/

BEHERA B., DASH J., PRADHAN D., TRIPATHY G., PRADHAN R. 2016. Apoptosis and Necrosis of Human Breast Cancer Cells by an Aqueous Extract of *Euphorbia hirta* leaves. J. Young **8**: 186-193. http://dx.doi.org/10.5530/jyp.2016.3.4

BERGERT M., CHANDRADOSS S.D., DESAI R.A. PALUCH E. 2012. Cell mechanics control rapid transitions between blebs and lamellipodia during migration. Proc. Natl. Acad. Sci. U.S.A. **109**: 14434-14439. https://doi.org/10.1073/pnas.1207968109

BRAUN S.A., HOMEY B., GERBER PA. 2014. Successful treatment of Bowen disease with ingenol mebutate. Hautarzt **65**: 848-50. https://doi.org/10.1007/s00105-014-3509-5

CAI Y., LUO Q., SUN M., CORKE H. 2004. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. Life Sci. **74**: 2157-2184. https://doi.org/10.1016/j.lfs.2003.09.047

CASTANO A.P., DEMIDOVA T.N., HAMBLIN M.R. 2005. Mechanisms in photodynamic therapy: part two-cellular signaling, cell metabolism and modes of cell death. Photodiagnosis Photodyn. Ther. **2**: 1-23. https://doi.org/10.1016/S1572-1000(05)00030-X

CHANG B.Y., KIM S.A., MALLA B., KIM S.Y. 2011. The Effect of Selective Estrogen Receptor Modulators (SERMs) on the Tamoxifen Resistant Breast Cancer Cells. Toxicol. Res. **27**: 85-93. https://dx.doi.org/10.5487%2FTR.2011.27.2.085

CHARRAS G.T. 2008. A short history of blebbing. J. Microsc. **231**: 466-478. https://doi.org/10.1111/j.1365-2818.2008.02059.x

CHU Q., SATOH K., KANAMOTO T., TERAKUBO S., NAKASHIMA H., WANG Q., SAKAGAMI H. 2009. Antitumor potential of three herbal extracts against human oral squamous cell lines. Anticancer Res. **29**: 3211-3219.

COLLINS J.A., SCHANDL C.A., YOUNG K.K., VESELY J., WILLINGHAM M.C. 1997. Major DNA fragmentation is a late event in apoptosis. J. Histochem. Cytochem. **45**: 923-934. https://doi.org/10.1177/002215549704500702

COREA G., FATTORUSSO E., LANZOTTI V., DI MEGLIO P., MAFFIA P., GRASSIA G., LALENTI A., LANARO A. 2005. Discovery and biological evaluation of the novel naturally occurring diterpene pepluanone as anti-inflammatory agent. J. Medicinal Chem. **48**:7055-7062. https://doi.org/10.1021/jm030951y

COZZI S.J., PARSONS P.G., OGBOURNE S.M., PEDLEY J., BOYLE G.M. 2006. Induction of senescence in diterpene ester-treated melanoma cells via protein kinase C-dependent hyperactivation of the mitogen activated protein kinase pathway. Cancer Res. **66**: 10083-10091. https://doi.org/10.1158/0008-5472.CAN-06-0348

DOUMA K.F., MEISER B., KIRK J., MITCHELL G., SAUNDERS C., RAHMAN B., SOUSA M.S., BARLOW-STEWART K., GLEESON M., TUCKER K. 2014. Health professionals' evaluation of delivering treatment-focused genetic testing to women newly diagnosed with breast cancer. Fam. Cancer **14**: 265-272. https://doi.org/10.1007/s10689-014-9770-z

EARNSHAW W.C. 1995. Nuclear changes in apoptosis. Curr. Opin. Cell Biol. **7**: 337-343. https://doi.org/10.1016/0955-0674(95)80088-3

EID R.A., RADAD K., AL-SHRAIM M. 2012. Ultrastructural changes of smooth muscles in varicocele veins. Ultrastruct. Pathol. **36**: 201-206. https://doi.org/10.3109/01913123.2011.637663

ERSVAER E., KITTANG A.O., HAMPSON P., SAND K., GJERTSEN B.T., LORD J.M., BRUSEND O. 2010. The Protein Kinase C Agonist PEP 005 (Ingenol 3-Angelate) in the treatment of human cancer: A balance between Efficacy and Toxicity. Toxins **2**: 174-194.

EUHUS D.M., DIAZ J. 2015. Breast cancer prevention. Breast J. **21**: 76-81. https://doi.org/10.1111/tbj.12352

FERLAY J., SHIN H.R., BRAY F., FORMAN D., MATHERS C., PARKIN D.M. 2010. Estimate of worldwide burden of cancer in 2008. Globacom 2008. Int. J. Cancer **127**: 2893-2917. https://doi.org/10.1002/ijc.25516

FERLAY J., SOERJOMATARAM I., DIKSHIT R., ESER S., MATHERS C., REBELO M., PARKIN D.M., FORMAN D., BRAY F. 2015. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int. J. Cancer **136**: E359-EE86. https://doi.org/10.1002/ijc.29210

FISHKIND D.J., CAO L.G., WANG Y.L. 1991. Microinjection of the catalytic fragment of myosin light chain kinase into dividing cells: effects on mitosis and cytokinesis. J. Cell Biol. **114**: 967-975. https://doi.org/10.1083/jcb.114.5.967

FRANKEN N.A., RODERMOND H.M., STAP J., HAVEMAN J., VAN BREE C. 2006. Clonogenic assay of cells *in vitro*. Nat. Protoc. **1**: 2315-2319. https://doi.org/10.1038/nprot.2006.339

FUKUDA K., KOJIRO M., CHIU J-F. 1993. Demonstration of extensive chromatin cleavage in transplanted Morris hepatoma 7777 tissue: apoptosis or necrosis? Am. J. Pathol. **142**: 935-946. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1886813/

GALLUZZI L., VITALE I., ABRAMS J.M., ALNEMRI E.S., BAEHRECKE E.H., BLAGOSKLONNY M.V., DAWSON T.M., DAWSON V.L., EL-DEIRY W.S., FULDA S., GOTTLIEB E., GREEN D.R., HENGARTNER M.O., KEPP O., KNIGHT R.A., KUMAR S., LIPTON S.A., LU X., MAEO F., MALORNI W., MEHLEN P., NUNEZ G., PETER M.E., PIACENTINI M., RUBINSZTIN D.C., SHOEMAKER Y., SIMON H.U., VANDENABEELE P., WHITE E., YUAN J., ZHIVOTOVSKY B., MELINO G., KROEMER G. 2012. Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. Cell death differ. **19**: 107-120. https://doi.org/10.1038/cdd.2011.96

GAO S., LI X., DING X., QI W., ANG Q. 2017. Cepharanthine Induces Autophagy, Apoptosis and Cell Cycle Arrest in Breast Cancer Cells. Cell Physiol. Biochem. **41**: 1633-1648. https://doi.org/10.1159/000471234

GAO S., SUN D., WANG G., ZHANG J., JIANG Y., LI G., ZHANG K., WANG L., HUANG J., CHEN L. 2016. Growth inhibitory effect of paratocarpin E, a prenylated chalcone isolated from *Euphorbia humifusa Wild*, by induction of autophagy and apoptosis in human breast cancer cells. Bioorg. Chem. **69**: 121-128. https://doi.org/10.1016/j.bioorg.2016.10.005

GAVLAN E., SANCHEZ-AGUAYO I., DAZA P., RUANO D. 2013. GSK-3β signaling determines autophagy activation in the breast tumor cell line MCF7 and inclusion formation in the non-tumor cell line MCF10A in response to proteasome inhibition. Cell Death Dis. **4**: e572. https://doi.org/10.1038/cddis.2013.95

GILLESPIE S.K., ZHANG X.D., HERSEY P. 2004. Ingenol 3-angelate induces dual modes of cell death and differentially regulates tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in melanoma cells. Mol. Cancer Therap. **3**: 1651.

GOODALL M.L., FITZWALTER B.E., ZAHEDI S., WU M., RODERIGUEZ D., MULCAHY-LEVY J.M., GREEN D.R., MORGAN M., CRAMER S.D., THORBURN A. 2016. The Autophagy Machinery Controls Cell Death Switching between Apoptosis and Necroptosis. Dev. Cell **37**: 337-349. https://doi.org/10.1016/j.devcel.2016.04.018

GREEN A.C., BEARDMORE G.L. 1988. Home treatment of skin cancer and solar keratosis. Australas. J. Derm. **29**: 127-130. https://doi.org/10.1111/j.1440-0960.1988.tb00383.x

HAMPSON P., CHAHAL H., KHANIM F., HAYDEN R., MULDER A., ASSI L.K., BUNCE C.M., LORD J.M. 2005. PEP005, a selective small-molecule activator of protein kinase C, has potent antileukemic activity mediated via the delta isoform of PKC. Blood **106**: 1362-1368. https://doi.org/10.1182/blood-2004-10-4117

HAMPSON P., KAVANAGH D., SMITH E., WANG K., LORD J.M., ED RAINGER G. 2008. The anti-tumor agent, ingenol-3-angelate (PEP005), promotes the recruitment of cytotoxic neutrophils by activation of vascular endothelial cells in a PKC-delta dependent manner. Cancer Immunol. Immunother. **57**: 1241-1251. https://doi.org/10.1007/s00262-008-0458-9

HO W.Y., YEAP S.K., HO C.L., RAHA A.R., SURAINI A.A., ALITHEEN N.B. 2011. *Elephantopus scaber* induces cytotoxicity in MCF-7 human breast cancer cells via p53-induced apoptosis. J. Medicinal Plants Res. **5**: 5741-5749.

JACOBSON M.D., WEIL M., RAFF M.C. 1997. Programmed cell death in animal development. Cell **88**: 347-354. https://doi.org/10.1016/S0092-8674(00)81873-5

JAIN K., PARANANDI K.S., SRIDHARAN S., BASU A. 2013. Autophagy in breast cancer and its implications for therapy. Am. J. Cancer Res. **3**: 251-65. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3696532/

JINESH G.G. 2017. Exposing the deadly dark side of apoptotic cancer stem cells. Oncoscience **4**: 124-125. https://dx.doi.org/10.18632%2Foncoscience.369

KARANTZA-WADSWORTH V., PATEL S., KRAVCHUK O., CHEN G., MATHEW R., JIN S., WHITE E. 2007. Autophagy mitigates metabolic stress and genome damage in MAMmary tumorigenesis. Genes Dev. **21**: 1621-1635. https://doi.org/10.1101/gad.1565707

KHAJAH M.A., MATHEW P.M., ALAM-ELDIN N.S., LUQMANI Y.A. 2015. Bleb formation is induced by alkaline but not acidic pH in estrogen receptor silenced breast cancer cells. Int. J. Oncol. **46**: 1685-1698. https://doi.org/10.3892/ijo.2015.2884

KO Y.S., LEE W.S., JOO Y.N., CHOI Y.H., KIM G.S., JUNG J.M., RYU C.H., SHIN S.C., KIM H.J. 2015. Polyphenol mixtures of *Euphorbia supina* the inhibit invasion and metastasis of highly metastatic breast cancer MDA-MB- 231 cells. Oncol. Rep. **34**: 3035-3042. https://doi.org/10.3892/or.2015.4304

KROEMER G., GALLUZZI L., VANDENABEELE P., ABRAMS J., ALNEMRI E.S., BAECHRECKE E.H., BLAGOSKLONNY M.V., EL-DEIRY W.S., GOLSTEIN P., GREEN D.R., HENGARTNER M.O., KNIGHT R.A., KUMAR S., LIPTON S.A., MALORNI W., NUNEZ G., PETER M.E. TSCHOPP J., YUAN J., PIACENTINI M., ZHIVOTOVSKY B., MELINO G. 2009. Nomenclature Committee on Cell Death 2009 (2008). Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. Cell Death Differ. **16**: 3-11. https://doi.org/10.1038/cdd.2008.150

KUANG X., LI W., KANNO Y., YAMASHITA N., NEMOTO K., ASADA Y., KOIKE K. 2016. ent-Atisane Diterpenoids from *Euphorbia fischeriana* Inhibit Mammosphere Formation in MCF-7 Cells. J. Nat. Med. **70**: 120-126. https://doi.org/10.1007/s11418-015-0940-6

KWAN Y.P., SAITO T., BRAHIM D., AL-HASSAN F.M.S., EIN OON C., CHEN Y., JOTHY S.L., KANWAR J.R., SASIDHARAN S. 2016. Evaluation of the cytotoxicity, cell-cycle arrest, and apoptotic induction by *Euphorbia hirta* in MCF-7 breast cancer cells. Pharmaceut. Biol. **54**: 1223-1236. https://doi.org/10.3109/13880209.2015.1064451

LEDDA-COLUMBANO G.M., CONI P., CURTO M., GIACOMINI L., FAA G., OLIVERIO S., PIACENTINI M., COLUMBANO A. 1991. Induction of two different modes of cell death, apoptosis and necrosis, in rat liver after a single dose of thioacetamide. Am. J. Path. **139:** 1099-109. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1886348/

LIN C.H., CHANG C.Y., LEE K.R., LIN H.J., CHEN T.H., WAN L. 2015. Flavones inhibit breast cancer proliferation through the Akt/FOXO3a signaling pathway. BMC Cancer **15**: 958. https://doi.org/10.1186/s12885-015-1965-7

LIN S.R., FU Y.S., TSAI M.J., CHENG H., WENG C.F. 2017. Natural Compounds from Herbs that can Potentially Execute as Autophagy Inducers for Cancer Therapy. Int. J. Mol. Sci. **18**: 1412. https://doi.org/10.3390/ijms18071412

LIU M., CHEN F., YU R., ZHANG W., HAN M., LIU F., WU J., ZHAO X., MIAO J. 2016. Synthesis and Cytotoxicity against K562 Cells of 3-O-Angeloyl-20-O-acetyl Ingenol, a Derivative of Ingenol Mebutate. Int. J. Mol. Sci. **17**: 1348. https://doi.org/10.3390/ijms17081348

LIU Y., LEVINE B. 2014. Autosis and autophagic cell death: the dark side of autophagy. Cell Death Differ. **22**: 367-276. https://doi.org/10.1038/cdd.2014.143

LOOS B., ENGELBRECHT A.M. 2009. Cell death: a dynamic response concept. Autophagy **5**: 590-603. https://doi.org/10.4161/auto.5.5.8479

MAMOUNAS E.P., BRYANT J., LEMBERSKY B., FEHERENBACHER L., SEDALCEK S.M., FISHER B., WICCKERHAM D.L., YOTHERS G., SORAN A., WOLMARK N. 2005. Paclitaxel after doxorubicin plus cyclophosphamide as adjuvant chemotherapy for node-positive breast cancer: results from NSABP B-28. J. Clin. Oncol. **23**: 3686-3696. https://doi.org/10.1200/JCO.2005.10.517

MILLER M.L., ANDRINGA A., DIXON K., CARTY M.P. 2002. Insights into UV-induced apoptosis: ultrastructure, trichrome stain and spectral imaging. Micron **33**: 157-166. https://doi.org/10.1016/S0968-4328(01)00014-2

MIRZAYANS R., ANDRAIS B., SCOTT A., TESSIER A., MURRAY D. 2007. A sensitive assay for the evaluation of cytotoxicity and its pharmacologic modulation in human solid tumor-derived cell lines exposed to cancertherapeutic agents. J. Pharm. Pharm. Sci. **10**: 298s-311s.

MOES M.J., BIJVELT J.J., BOONSTRA J. 2011. Attachment of HeLa cells during early G1 phase. Histochem. Cell Biol. **136**: 399-411. https://dx.doi.org/10.1007%2Fs00418-011-0852-9

MROZ P., YAROSLAVSKY A., KHARKWAL G.B., HAMBLIN M.R. 2011. Cell Death Pathways in Photodynamic Therapy of Cancer. Cancers **3**: 2516-2539. https://doi.org/10.3390/cancers3022516

NG P.L., RAJAB N.F., THEN S.M., MOHD YUSOF Y.A., WAN NGAH W.Z., PIN K.Y., LOOI M.L. 2014. Piper betle leaf extract enhances the cytotoxicity effect of 5-fluorouracil in inhibiting the growth of HT29 and HCT116 colon cancer cells. J. Zhejiang Univ. Sci. **15**: 692-700. https://doi.org/10.1631/jzus.B1300303

NORMAN L.L., BRUGUES J., SENGUPTA K., SENS P., ARAND ESPINOZA H. 2010. Cell blebbing and membrane area homeostasis in spreading and retracting cells. Biophys. J. **99**: 1726-1733. https://dx.doi.org/10.1016%2Fj.bpj.2010.07.031

NORMANNO N., DI MAIO M., DE MAIO E., DE LUCA A., DE MATTEIS A., GIORDANO A., PERRONE F. 2005. Mechanisms of endocrine resistance and novel therapeutic strategies in breast cancer. Endocr. Relat. Cancer **12**: 721-747. https://doi.org/10.1677/erc.1.00857

OGBOURNE S.M., HAMPSON P., LORD J.M., PARSON P., DE WITTE P.A., SUHRBIER A. 2007. Proceedings of the First International Conference on PEP005. Anticancer Drugs **18**: 357-362.

OGBOURNE S.M., SUHRBIER A., JONES B. 2004. Antitumour activity of 3-Ingenyl angelate: plasma memebrane and mitochondrial disruption and necrotic cell death. Cancer Res. **68**: 2833-2839. https://doi.org/10.1158/0008-5472.CAN-03-2837

PEER D., KARP J.M., HONG S., FAROKHZAD O.C., MARGALET R., LANGER R. 2007. Nanocarriers as an emerging platform for cancer therapy. Nat. Nanotechnol. **2**: 751-760. https://doi.org/10.1038/nnano.2007.387

PENA C., PILAR G. 2000. Early morphological alterations in trophically deprived neuronal death in vitro occur without alterations in Ca2. J. Comp. Neurol. **424**: 377-396. https://doi.org/10.1002/1096-9861(20000828)424:3%3C377::AID-CNE1%3E3.0.CO;2-F

PEREZ R.P., GODWIN A.K., HANDEL L.M., HAMILTON T.C. 1993. A comparison of clonogenic, microtetrazolium and sulforhodamine B assays for determination of cisplatin cyto-

toxicity in human ovarian carcinoma cell lines. Eur. J. Cancer **29A**: 395-399.
https://doi.org/10.1016/0959-8049(93)90394-U

PEROU C.M., SORLIE T., EISEN M.B., VAN DE RIJN M., JEFFREY S.S., REES C.A., POLLACK J.R., ROSS D.T., JOHNSEN H., AKSLEN L.A., FLUGE O., PERGAMENSCHIKOV A., WILLIAMS C., ZHU S.X., LONNING P.E., BORRESEN-DALE A.L., BROWN P.O., BOTSTEIN D. 2000. Molecular portraits of human breast tumours. Nature **406**: 747-752. https://doi.org/10.1038/35021093

RAFEHI H., ORLOWSKI C., GEORGIADIS G.T., VERVERIS K., EL-OSTA A., KARAGIANNIS T.C. 2011. Clonogenic assay: adherent cells. J. Vis. Exp. **49**: 2573.
https://doi.org/10.3791/2573

RAMSAY J.R., SUHRBIER A., AYLWAROL J.H., OGBOURNE S., COZZI S.J., POULSEN M.G., BAUMANN K.C., WELBURN P., REDLICH G.L., PARSONS P.G. 2011. The sap of *Euphorbia peplus* is effective against human non melanoma skin cancers. Brit. J. Derm. **164**: 633-636.
https://doi.org/10.1111/j.1365-2133.2010.10184.x

ROSEN R.H., GUPTA A.K., TYRING S.K. 2012. Dual mechanism of action of Ingenol mebutate gel for topical treatment of actinic keratosis: Rapid lesion necrosis followed by lesion-specific immune response. J. Am. Acad. Derm. **66**: 486-493. https://doi.org/10.1016/j.jaad.2010.12.038

RUIZ-GONZALEZ R., ACEDO P., SANCHEZ-GARCIA D., NONELL S., CANETE M., STOCKERT J.C., VILLANUEVA A. 2013. Efficient induction of apoptosis in HeLa cells by a novel cationic porphycene photosensitizer. Eur. J. Med. Chem. **63**: 401-414. https://doi.org/10.1016/j.ejmech.2013.02.028

SHIN S.Y., KIM C.G., JUNG Y.J., JUNG Y., JUNG H., IM J., LIM Y., LEE Y.H. 2016. *Euphorbia humifusa Wild* exerts inhibition of breast cancer cell invasion and metastasis through inhibition of TNF alpha- induced MMP-9 expression. BMC Complement. Altern. Med. **16**: 413.
https://dx.doi.org/10.1186%2Fs12906-016-1404-6

SHOEMAKER M., HAMILTON B., DAIRKEE S.H., COHEN I., CAMPBELL M.J. 2005. *In vitro* anticancer activity of twelve Chinese medicinal herbs. Phytother. Res. **19**: 649-651. https://doi.org/10.1002/ptr.1702

SILLER G., GEBAUER K., KATSAMAS J., OGBOURNE S.M. 2009. PEP005 (ingenol mebutate) gel, a novel agent for the treatment of actinic keratosis: results of a randomized, double-blind, vehicle-controlled, multicentre, phase IIa study. Australas. J. Dermatol. **50**: 16-22.
https://doi.org/10.1111/j.1440-0960.2008.00497.x

SILVA V.A., ROSA M.N., TANSINI A., OLIVEIRA R.J., MARTINHO O., LIMA J., PIANOWSKI L.F., REIS R.M. 2018. *In vitro* screening of cytotoxic activity of euphol from *Euphorbia tirucalli* on a large panel of human cancer-derived cell lines. Exp. Ther. Med. **16**: 557-566.
https://doi.org/10.3892/etm.2018.6244

SORLIE T., TIBSHIRANI R., PARKER J., HASTIE T., MARRON J.S., NOBEL A., DENG S., JOHNSEN H., PESICH R., GEISLER S., DEMETER J., PEROU C.M., LONNING P.E., BROWN P.O., BORRESEN-DALE A.L., BOTSTEIN D. 2003. Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc. Natl. Acad. Sci. U.S.A. **100**: 8418-8423.
https://doi.org/10.1073/pnas.0932692100

STRONCEK D.F., JIN P., WANG E., JETT B. 2007. Potency analysis of cellular therapies: the emerging role of molecular assays. J. Transl. Med. **5**: 24-29.
https://dx.doi.org/10.1186%2F1479-5876-5-24

TCHINDA A.T. 2008. *Euphorbia peplus* L. Record from PROTA4U. PROTA (Plant Resources of Tropical Africa), Wageningen, Netherlands.
http://www.prota4u.org/search.asp

TORRE L.A., BRAY F., SIEGEL R.L., FERLAY J., LORTET-TIEULENT J., JEMAL A. 2015, Global cancer statistics, 2012. CA Cancer J Clin. **65**: 87-108.
https://doi.org/10.3322/caac.21262

TRINKAUS J.P. 1973. Surface activity and locomotion of Fundulus deep cells during blastula and gastrula stages. Dev. Biol. **30**: 69-103.
https://doi.org/10.1016/0012-1606(73)90049-3

VENKATESAN P., DAS S., KRISHNAN M.M.R., CHAKRABORTY C., CHAUDHURY K., MANDAL M. 2010. Effect of AEE788 and/or Celecoxib on colon cancer cell morphology using advanced microscopic techniques. Micron **41**: 247-256.
https://doi.org/10.1016/j.micron.2009.10.008

VICHAI V., KIRTIKARA K. 2006. Sulforhodamine B colorimetric assay for cytotoxicity screening. Nat. Protoc. **1**: 1112-1116. https://doi.org/10.1038/nprot.2006.179

WILLINGHAM M.C. 1999. Cytochemical methods for the detection of apoptosis. J. Histochem. Cytochem. **47**: 101-1109.
https://doi.org/10.1177/002215549904700901

WONGRAKPANICH A., CHAROENSUKSAI P. 2018. Induction of apoptosis in cancer by plants in the genus *Euphorbia*. Thai Bull. Pharm. Sci. **13**: 1-11.

WYLLIEE A.H., KERR J.F., CURRIE A.R. 1980. Cell death: the significance of apoptosis. Int. Rev. Cytol. **68**: 251-306.
https://doi.org/10.1016/S0074-7696(08)62312-8

YALKINOGLU A.O., SCHLEHOFER J.R., ZUR HAUSEN H. 1990. Inhibition of N-methyl-N'-nitro-N-nitrosoguanidine-induced methotrexate and adriamycin resistance in CHO cells by adeno-associated virus type 2. Int. J. Cancer **45**: 1195-1203.
https://doi.org/10.1002/ijc.2910450634

YAN W., MA X., ZHAO X., ZHANG S. 2018. Baicalein induces apoptosis and autophagy of breast cancer cells via inhibiting PI3K/AKT pathway *in vivo* and *vitro.* Drug Des. Devel. Ther. **12**: 3961-3972.
https://doi.org/10.2147/DDDT.S181939

YONEKAWA T., THORBURN A. 2013. Autophagy and cell death. Essays Biochem. **55**: 105-117.
https://doi.org/10.1042/bse0550105

YOO J.O., HA K.S. 2012. New insights into the mechanisms for photodynamic therapy-induced cancer cell death. Int. Rev. Cell Mol. Biol. **295**: 139-174.
https://doi.org/10.1016/B978-0-12-394306-4.00010-1

ZHOEMAKER-QIN S., SHU-ZHEN M., YING-TONG D., XIAO-JIANG H. 2010. A new jatrophane diterpenoid from *Euphorbia peplus*. Chin. J. Nat. Med. **8**: 81-83.
https://doi.org/10.1016/S1875-5364(10)60009-X