Revista Brasileira de Farmacognosia 27 (2017) 306–314

Brazilian Journal of Pharmacognosy
REVISTA BRASILEIRA DE FARMACOGNOSIA
www.elsevier.com/locate/bjp

Original article

A cytotoxic *Petiveria alliacea* dry extract induces ATP depletion and decreases β-F1-ATPase expression in breast cancer cells and promotes survival in tumor-bearing mice

John F. Hernández[a,b], Claudia P. Urueña[a], Tito A. Sandoval[a], Maria C. Cifuentes[a], Laura Formentini[b], Jose M. Cuezva[b], Susana Fiorentino[a,*]

[a] *Grupo de Inmunobiología y Biología Celular, Facultad de Ciencias, Pontificia Universidad Javeriana, Bogotá, Colombia*
[b] *Departamento de Biología Molecular, Centro de Biología Molecular Severo Ochoa, Consejo Superior de Investigaciones Científicas-Universidad Autónoma de Madrid, Madrid, Spain*

ARTICLE INFO

Article history:
Received 17 May 2016
Accepted 20 September 2016
Available online 9 February 2017

Keywords:
Petiveria alliacea
Breast cancer
β-F1-ATPase
Respiration
ATP depletion

ABSTRACT

Metabolic plasticity in cancer cells assures cell survival and cell proliferation under variable levels of oxygen and nutrients. Therefore, new anticancer treatments endeavor to target such plasticity by modifying main metabolic pathways as glycolysis or oxidative phosphorylation. In American traditional medicine *Petiveria alliacea* L., Phytolaccacea, leaf extracts have been used for leukemia and breast cancer treatments. Herein, we study cytotoxicity and antitumoral effects of P. *alliacea* extract in tumor/non-tumorigenic cell lines and murine breast cancer model. Breast cancer cells treated with *P. alliacea* dry extract showed reduction in β-F1-ATPase expression, glycolytic flux triggering diminished intracellular ATP levels, mitochondrial basal respiration and oxygen consumption. Consequently, a decline in cell proliferation was observed in conventional and three-dimension spheres breast cancer cells culture. Additionally, *in vivo* treatment of BALB/c mice transplanted with the murine breast cancer TS/A tumor showed that *P. alliacea* extract *via i.p.* decreases the primary tumor growth and increases survival in the TS/A model.

Introduction

Cancer cells may assure survival and proliferation under shifting levels of oxygen and nutrients through metabolic plasticity between glycolysis and oxidative phosphorylation (OxPhos) metabolic pathways. Integrating these pathways in glucose oxidation provides important substrates as ATP, NADH and biosynthetic precursors for the cell housekeeping processes (Formentini et al., 2010). Mitochondrial activity and specifically OxPhos play a relevant role in facilitating the execution of cell death (Cuezva et al., 2009). In the mitochondrial inner membrane is found the ATPase or complex V, a multi-enzymatic complex with two domains: a hydrophobic intramembrane domain F0 and a hydrophilic domain F1 facing to the matrix leaflet. F1 domain has five sub-units $\alpha 3\beta 3\gamma 1\delta 1\varepsilon 1$ and three catalytic sites (subunit β and α/β interface). The electrochemical gradient generated by the mitochondria REDOX reactions, makes possible the proton influx into the matrix through F0 domain providing the necessary energy for ADP phosphorylation (Stock et al., 1999; Gledhill et al., 2007). It is well known that cancer cells can have structural and functional mitochondrial alterations. For instance, it has been shown in human carcinomas that selective repression of β-F1-ATPase is inversely correlated with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels causing decrease in mitochondrial activity and increase in glycolytic flux (Cuezva et al., 2002). Moreover, human breast cancer cells overexpressed lactate dehydrogenase isoform A (LDH-A) leading to an increase in lactate secretion (Koukourakis et al., 2008). Hence high lactate levels are associated to taxol resistance and cell proliferation augmentation under a hypoxic microenvironment (Fantin et al., 2006; Zhou et al., 2010).

Recently, dichloroacetate (a pyruvate dehydrogenase kinase inhibitor) has been proposed as a new anticancer drug specially for glycolytic tumors posing limited side effects (Papandreou et al., 2011) and broadening the spectra for new OxPhos regulators. In this regard, plants could be a source of compounds able to target cancer metabolic pathways.

In fact, *Petiveria alliacea* L., Phytolaccaceae, infusions from leaf and root have been reported to have anti-spasmodic, anti-rheumatic and anti-inflammatory properties (Morales et al., 2001) and particularly used in leukemia and breast cancer treatments

* Corresponding author.
E-mail: susana.fiorentino@javeriana.edu.co (S. Fiorentino).

http://dx.doi.org/10.1016/j.bjp.2016.09.008

(Garcia-Barriga, 1974; Gupta, 1995). It has been shown that *P. alliacea* extracts are cytotoxic on leukemia, lymphoma and melanoma cell lines (Rossi, 1990; Rossi et al., 1993; Urueña et al., 2008), however it poses low toxicity on human fibroblasts and peripheral blood mononuclear cells (Urueña et al., 2008). Recently, we have proposed that antitumor activity of a dry extract from *P. alliacea* can be partly explained by the glycolytic flux shifting of cancer cells, as shown on 4T1 breast cancer model (Hernandez et al., 2014).

Herein, we demonstrated that a dry extract from *P. alliacea* causes changes in mitochondrial activity characterized by a decrease in β-F1-ATPase expression and ATP depletion leading to a decrease in breast cancer cell proliferation *in vitro* and *in vivo*.

Materials and methods

Plant material and extraction procedure

Petiveria alliacea L., Phytolaccaceae, leaves and stems (local name "anamu") were collected in Cachipay, Cundinamarca, Colombia on April 2009 and identified by Carlos Parra from the Colombian National Herbarium; voucher number COL 569765 (Colombian Environmental Ministry agreement number 1927 related to the use of genetic resources and derivatives products). *P. alliacea* extraction procedure and chemical characterization were previously described (Urueña et al., 2008). Briefly, dry ground leaves and stems were extracted with 96% ethanol (15 ± 5 °C), filtered and concentrated under reduced pressure. Ethanolic extract was trapped on fumed silica, fractionated with ethyl acetate and extracted with methanol:water yielding a dry extract (DER genuine: 10000–11000:1). The compounds identified in dry extract from *P. alliacea* were: benzaldehyde, leridol, petiveral, myricetin, petiveral 4-ethyl, pinitol, dibenzyl disulfide and dibenzyl trisulphide. HPLC chromatographic fingerprint was acquired in a Jasco®PU2089plus equipped with a UV detector (254 nm) using a C18 column and water/acetonitrile gradient as mobile phase (Hernandez et al., 2014). To meet EMA guidelines, the active marker selected was dibenzyl disulfide, a reported cytotoxic compound (Cifuentes et al., 2009) found at a concentration of 2.6 mg per g of extract.

Cell lines

4T1, TS/A, 3T3 and HS578T cell lines were cultured in DMEM and HCT116 cell line in McCoy's 5A medium, both supplemented with fetal calf serum (FCS) heat-inactivated (10%), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), HEPES buffer (0.01 M) and sodium pyruvate (1 mM) (Eurobio Toulouse, FR). MCF12F cell line was cultured in DMEM/F-12 medium supplemented with fetal horse serum (5%), epidermal growth factor (20 ng/ml), human insulin (10 μg/ml), hydrocortisone (500 ng/ml), cholera toxin (100 mg/ml), penicillin (100 U/ml) and streptomycin (100 μg/ml). Cell lines were incubated under humidified environment at 37 °C and 5% CO_2.

In vitro *cytotoxicity assays*

Cytotoxic effects were evaluated using methylthiazol tetrazolium (MTT, Sigma-Aldrich, Saint Louis, MO) and trypan blue dye assays. Cells (5×10^3 cells/well) were seeded in 96-wells plates with different concentrations of dry extract from *P. alliacea* (250–0.95 μg/ml) or ethanol (0.02%) as negative control for 48 h. Proliferation was estimated by MTT assay according to procedure previously described (Urueña et al., 2008). The IC_{50} value was estimated with nonlinear regression analysis (Graph Pad Prism 5 for Windows).

Western blots

Cells were suspended in lysis buffer containing 25 mM HEPES, 2.5 mM EDTA, 0.1% Triton X-100, 1 mM PMSF and 5 mg/ml leupeptin. Extracts were centrifuged at 11,000 × *g* for 15 min at 4 °C. Supernatants protein concentration was determined with Bradford protein assay. Cellular proteins (7–20 μg) were fractionated by SDS/PAGE (12%) and transferred onto PVDF membranes. Primary monoclonal antibodies were: anti-β-F1-ATPase (1:50,000) (Cuezva et al., 2002), anti-Hsp60 (1:10,000) and anti-NADH9 (complex I 39 kDa) (1:1000) (Acebo et al., 2009), anti-Complex III subunit Core 2 (1:1000) from Abcam; anti-SDH (succinate dehydrogenase) (1:500) from Life Technologies; anti-β-actin (1:20,000) and anti-tubulin (1:5000) from Sigma-Aldrich. Peroxidase-conjugated anti-mouse or anti-rabbit IgG (Nordic Immunology, 1:3000) were used as secondary antibodies. Blots revealed with luminol electrochemiluminescence (ECL) reagent (Amersham Pharmacia Biotech, Little Chalfont, UK).

Oxygen consumption estimation

Cellular oxygen consumption rates were determined in an XF24 Extracellular Flux Analyzer (Seahorse Bioscience). Cells (4×10^4) were seeded in XF24-well cell culture microplates (Seahorse Bioscience), treated with dry extract from *P. alliacea* or ethanol, incubated at 37 °C and 5% CO_2 for 6 or 24 h. After treatment the following substances were consecutively injected to achieve the indicated final concentration: oligomycin (OLIGO, 6 μM), dinitrophenol (DNP) 0.5 mM, rotenone 1 μM and antimycin 1 μM.

Glycolysis flux estimation

Cells (1.5×10^5) were seeded and allowed to grow until 60% of confluence. To determine glycolytic rates, cells were treated for 3, 6, 24 or 48 h with dry extract from *P. alliacea* or ethanol with or without OLIGO 6 μM. After treatment medium was replaced by fresh one (FCS 0.5%) and cells were allowed to rest during 2 h. Sample medium (100 μl) was precipitated with perchloric acid (6%), neutralized (KOH 20%), and glycine–hydrazine–EDTA buffer (1 M: 0.4 M: 1.3 mM) containing LDH (Roche Diagnostics GmbH) and β-NADH hydrate (Sigma-Aldrich) was added (Govindarajan et al., 2007). Lactate levels were estimated at 340 nm in a Shimadzu spectrophotometer. Protein content was estimated by Bradford protein assay (Sigma-Aldrich).

Intracellular ATP determination

Intracellular ATP was measured using ATP Bioluminescence Assay kit HS II (Roche Diagnostics). Briefly, 1.5×10^5 cells were plated in 6-well plates, treated with dry extract from *P. alliacea*, DOG/metformin HCl (Sigma-Aldrich) or ethanol during 6 h, harvested, counted, and lysed with lysis buffer (50 μl) at 20 °C for 5 min. A dilution (1:100) from sample or standard (50 μl) was transferred to a 96-well plate, and luciferase reagent (50 μl) was added. The emitted light was measured immediately and integrated using a Plate Chameleon V Model 425-156 (Hidex) for 10 s. Blank value (no ATP) was subtracted from each sample's raw data and ATP concentrations were calculated from the standard curve and expressed as μmol per 1×10^5 cells.

Spheres number and area estimation

Single cells (3×10^3 cells/ml) were plated in a Corning® Costar® ultra-low attachment culture plate in serum-free DMEM (glucose 1 g/l)/F12 (1:1) supplemented with B27 lacking vitamin A (1:50; Gibco), N2 (1:100; Gibco), penicillin (100 U/ml) and

streptomycin (100 μg/ml). Cells were daily treated during 6 days with ethanol (negative control), dry extract from *P. alliacea* (11 μg/ml), deoxyglucose (DOG, 0.24 mM) and doxorubicin (DOX, 0.08 μM). After 7 days, spheres were counted by two independent observers using an optical microscope Olympus (10×). Cell culture medium was recovered, centrifuged (100 × *g*) during 3 min and spheres pellet suspended in phosphate buffer saline (PBS) and placed on microscope slide. Spheres' area was measured using Axiovision® software (Carl Zeiss).

Animals

Female BALB/c mice, 6–12 weeks old were purchased from Charles Rivers Laboratories International, Inc. (Boston, MA), and housed in our animal research facility following the established protocols of the Ethics Committee of the Science Faculty and National and International Legislation for Live Animal Experimentation (Colombia Republic, Resolution 8430/1993; National Academy of Sciences, 2010). Mice were housed in polyethylene cages with food and water *ad libitum*, controlled temperature, and a 12-h light/dark cycle. Before treatments, the mice were acclimated for one week under standard conditions. This project was approved by the Ethics Committee of the Science Faculty on 29/04/2009.

Tumor model

TS/A cells (1×10^4) suspended in 100 μl of PBS were injected into the right mammary fat pad (subcutaneously [SC]) on day 0 and then randomly assigned to PBS control group ($n = 9$), DOX group (3 mg/kg Al Pharma®, $n = 9$) or *P. alliacea* extract group (250 mg/kg, $n = 8$). After 5 days of inoculation, treatments were injected intraperitoneally (*i.p.*) once a week for DOX and twice a week for *P. alliacea* extract until 56 days post-inoculation. Tumors were measured with Vernier calipers three times a week, and tumor volume was calculated using the following formula: tumor volume (mm^3) = [(width)^2 × length]/2 Gallotannin-rich *Caesalpinia spinosa*

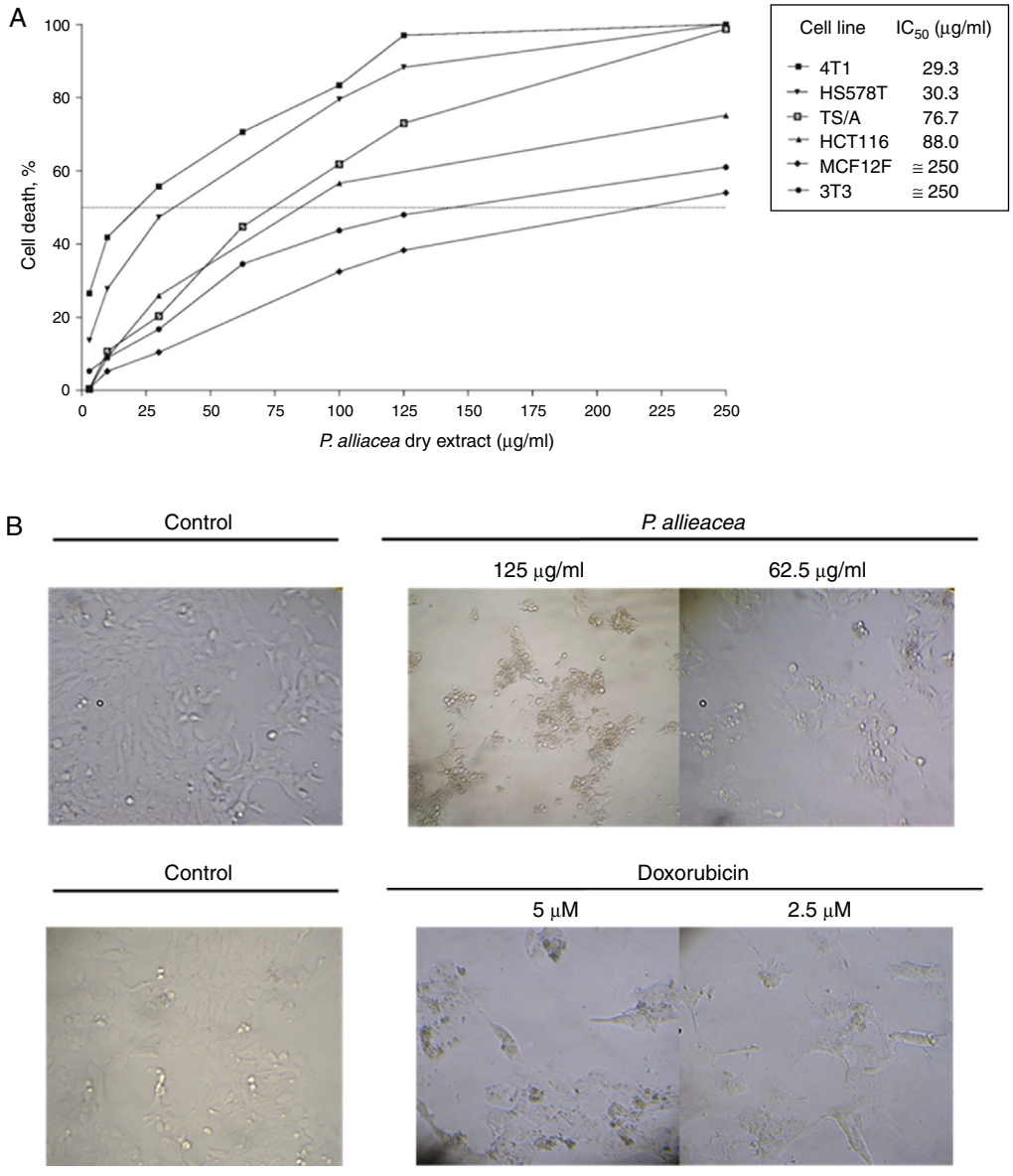

Fig. 1. *Petiveria alliacea* dry extract show cytotoxic activity on breast cancer cell lines. (A) Breast cancer cells (4T1, TS/A HS578T), colon carcinoma (HCT116) and non-tumorigenic (MCF12F and 3T3) cell lines were treated with a *P. alliacea* dry extract during 48 h. Cell viability was determined by MTT assay. Data represent the mean of three independent experiments. (B) Representative images from 4T1 cells treated with DMSO or ethanol (control), doxorubicin (5 and 2.5 μM) and *P. alliacea* dry extract (125 and 62.5 μg/ml) during 48 h under conditions indicated above. Morphological changes were analyzed under invert microscope. Results represent three independent performed experiments.

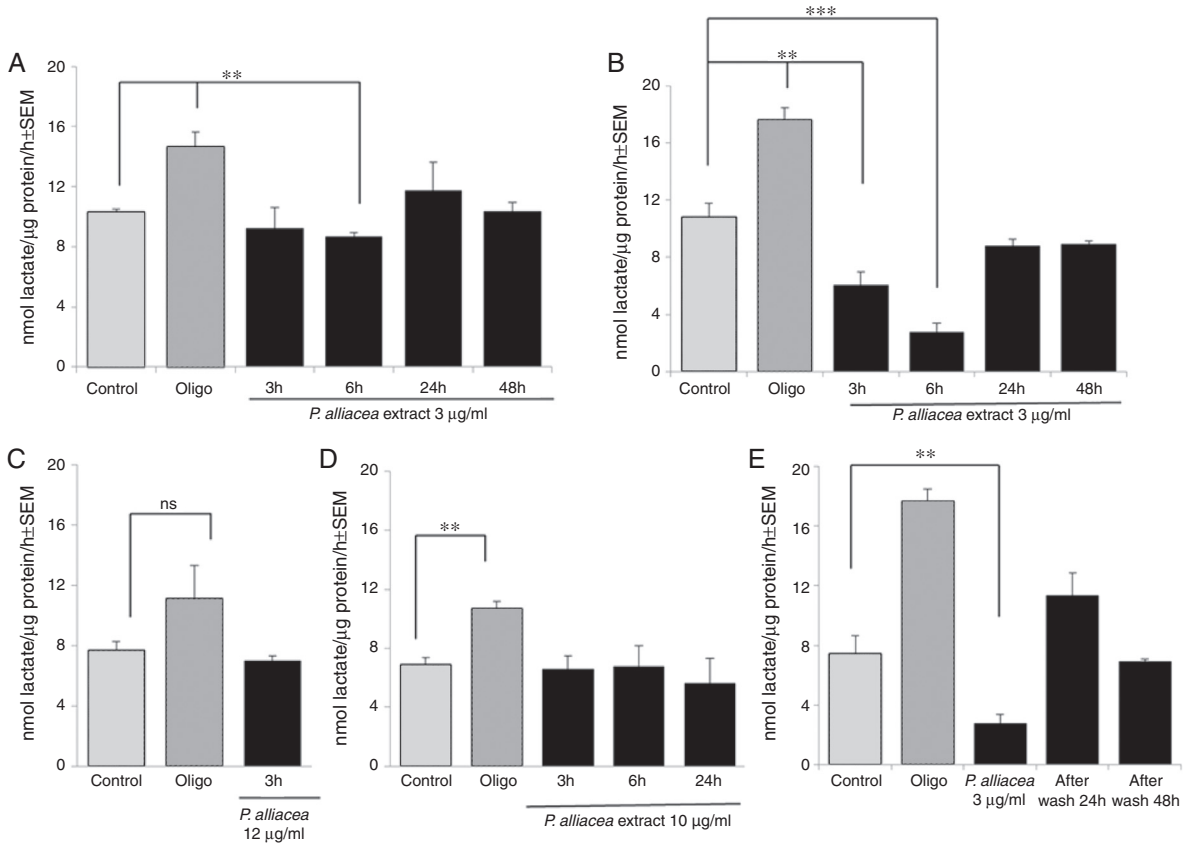

Fig. 2. *Petiveria alliacea* extract decreases glycolytic flux in breast cancer cell lines. (A) 4T1, (B) HS578T, (C) MCF12F, (D) HCT116, (E) HS578T. Cell lines were treated with a *P. alliacea* dry extract ($IC_{50/10}$) during 3, 6, 24 or 48 h. After treatment lactate concentration was evaluated by enzymatic assay. Data represent the mean ± S.E.M. of at least three independent experiments $^{*}p < 0.05$, $^{**}p < 0.001$, $^{***}p < 0.0001$ compared to control using Student's *t* test.

fraction decreases the primary tumor and factors associated with poor prognosis in a murine breast cancer model (Urueña et al., 2013). A study of survival defining the endpoint of each individual according to the criteria of toxicity and animal welfare was conducted. The animals were euthanized in a CO_2 chamber when achieve one or more endpoint criteria.

Statistical analysis

Results are expressed as mean ± S.D. For oxygen consumption estimations and mammosphere analyses two-way ANOVA was used and unpaired *t* test for remaining analyses. Survival curves obtained by the Kaplan–Meier method were statistically analyzed using the Log-rank test. Statistical analyses were done using Graph Pad Prism 5 with a $p < 0.05$ significance.

Results

Petiveria alliacea *dry extract is more cytotoxic to breast cancer cell lines while sparing to fibroblasts and epithelial breast cells*

Dry extract from *P. alliacea* is cytotoxic to breast and colon tumor cell lines in a dose-dependent manner while sparing to fibroblasts (3T3) and non-tumorigenic epithelial breast cell line (MCF12F) (Fig. 1A). The corresponding IC_{50} is 30 µg/ml for human HS578T and murine 4T1 breast cancer cells, 77 µg/ml for murine TS/A breast cancer cell line and 88 µg/ml for colon tumor cell line HCT116 (Fig. 1A). Cytotoxicity observed in breast cancer cell lines was associated to morphological changes like increase of cellular volume, the appearance of refringent vesicles and detachment from the culture surface (Fig. 1B).

Reduction in the glycolytic flux was observed after treating 4T1 and HS578T cell lines (3 and 6 h) with *P. alliacea* extract, at subcytotoxic concentrations – IC_{50}/10th – (Fig. 2, panel A and B). The extract effect is early and transient disappearing after 24 h, regardless whether the extract remains or not in the cell culture (Fig. 2, panel E). No effect is observed on MCF12F or HCT116 cell lines (Fig. 2, panel C and D, respectively) after 24 h treatment, neither in the expression of glycolytic enzymes as GAPDH, pyruvate kinase (PK) and LDH (data not shown). The latter suggests that the extract compounds may bind any glycolytic enzyme in a reversible way promoting the transient decrease in the glycolytic flux.

Petiveria alliacea *dry extract treatment causes decrease in mitochondrial respiration, ATP synthase expression and intracellular ATP concentration on breast cancer cell lines*

Mitochondrial OxPhos protein expression of NADH9 (complex I), succinate dehydrogenase (complex II 30 kDa iron–sulfur subunit), cytochrome b-c1 subunit 2 (complex III Core II subunit) and β-F1-ATPase (complex V) were determined to assess *P. alliacea* extract activity. We found that the expression of β-F1-ATPase protein was affected by the treatment in both breast cancer cell lines (Fig. 3).

The oxygen consumption rate (OCR) in 4T1 cells was measured before and after adding pharmacological agents to respiring cells. The calculated basal respiration for the control cells was 57.5 ± 10.8 pmol/min/µg protein while in the treated cells was 39.5 ± 1.9 pmol/min/µg protein, validating that β-F1-ATPase is less functional in the treated cells. The addition of oligomycin, an ATP-linked respiration inhibitor causes a steep decrease in respiration in the control cells while no change was observed in treated cells.

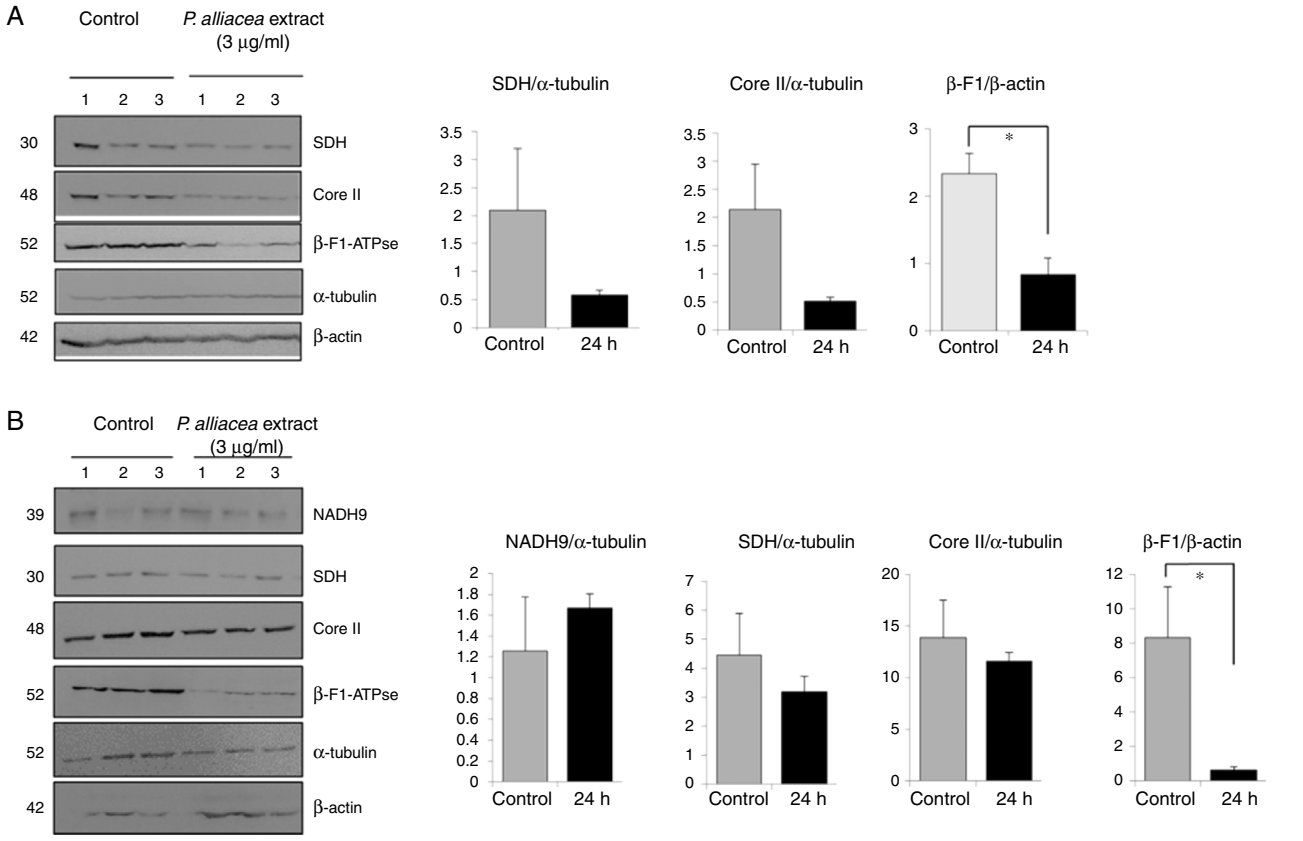

Fig. 3. *Petiveria alliacea* extract decreases β-F1-ATPase expression in breast cancer cell lines. (A) 4T1 and (B) HS578T cell lines treated with a *P. alliacea* dry extract for 24 h. NADH9, SDH, and CORE II protein expression were also determined showing no effect after treatment. Representative Western blots and their corresponding histograms are shown of three independent preparations (lanes 1–3). The left side of blot shows protein molecular mass in (kDa). Black bars represent normalized bands with α-tubulin or β-actin in arbitrary units. Results are the mean ± S.E.M. of three independent experiments. $^{*}p < 0.05$ compared to control using Student's *t* test.

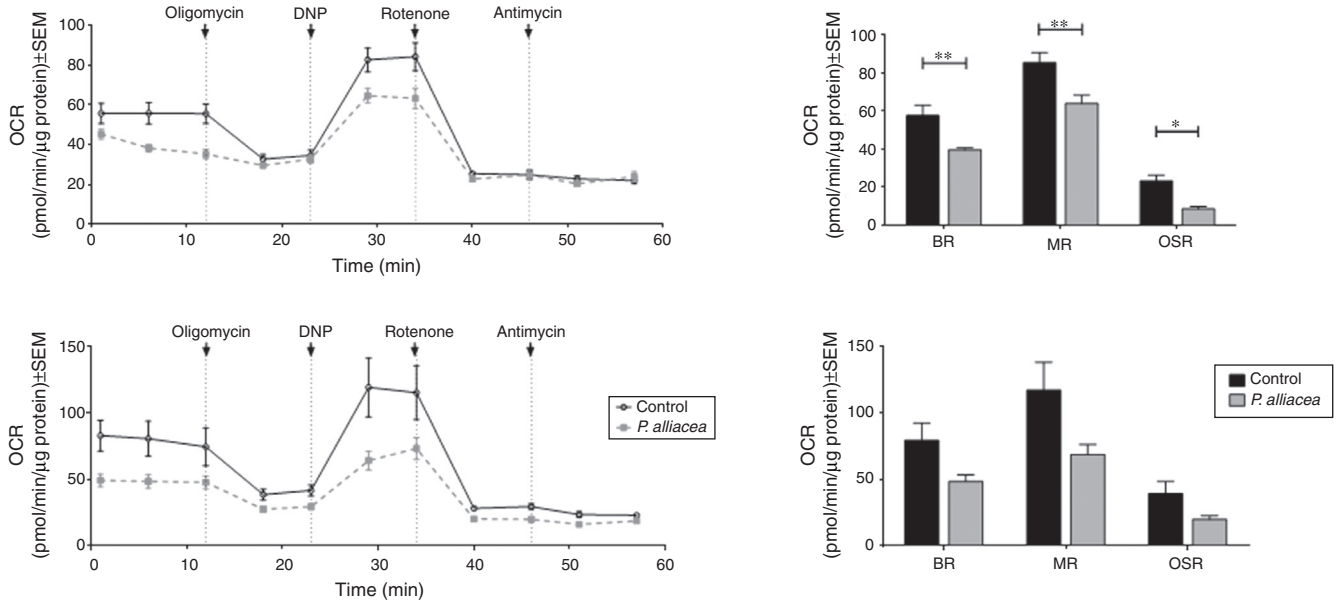

Fig. 4. *Petiveria alliacea* extract treatment reduces basal respiration, maximal respiration and oxygen consumption rate (OCR) associated to ATP production in 4T1 cells. After 6 h (upper panel) and 24 h treatment (lower panel) the OCR was measured following the addition of the indicated agents (left). Histograms show the comparison in basal respiration (BR), maximal respiration (MR) and oligomycin sensitive respiration (OSR) between control and *P. alliacea* extract cells treated. Results are expressed as the mean ± SEM of two independent experiments. $^{*}p < 0.05$, $^{**}p < 0.001$ compared to control using a two-way ANOVA.

However, it must be taken into account that treatment significantly lowers basal respiration explaining the no change in respiration. DNP, is a proton ionophore that induces electron transport chain to function at its maximum rate by collapsing mitochondrial membrane proton gradient. After the addition of DNP, the maximal respiration was measure. Control cells maximal respiration was 85.3 ± 10.7 pmol/min/μg protein, whereas in treated cells was 63.9 ± 8.6 pmol/min/μg protein, suggesting once more that

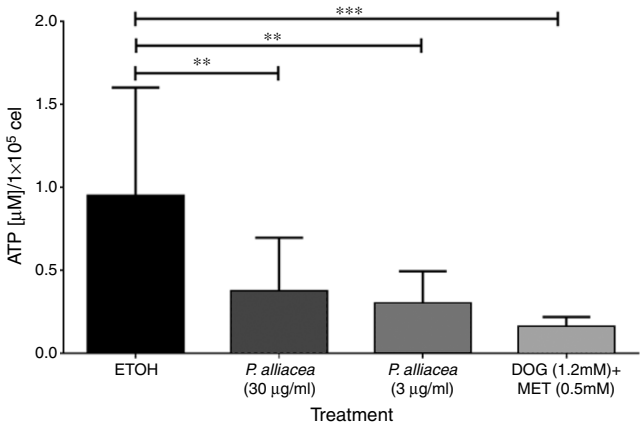

Fig. 5. *Petiveria alliacea* extract treatment decreases intracellular ATP. 4T1 cells were treated with *P. alliacea* dry extract or deoxyglucose plus metformin (DOG + MET) during 6 h. ATP was measured by a bioluminescence assay. ATP concentration is expressed as µmol per 1×10^5 cells. The figure represents one from three independent experiments. Results are expressed as the mean ± S.D. $^{**}p < 0.001$, $^{***}p < 0.0001$ compared to control using Student's *t* test.

expression level or functionality of β-F1-ATPase is altered (Fig. 4, upper panel). Similar results were obtained after 24 h treatment (Fig. 4, lower panel).

Intracellular ATP level in 4T1 cells was measured after using DOG/metformin (1.2 mM/0.5 mM) as a severe ATP synthesis inhibitor, which causes a 5-fold reduction in intracellular ATP. Our treatment showed a 2.5-fold decrease (Fig. 5) in 4T1 cells, confirming that mitochondrial ATP synthesis is decrease by *P. alliacea* extract treatment.

A dry extract from Petiveria alliacea *decreases 4T1 spheres and conventional cell culture proliferation*

An outstanding model for drug screening are the spheres since they are midway between conventional cultures and *in vivo* tumors (Pampaloni et al., 2007). After six days of *P. alliacea* treatment at sub-cytotoxic concentrations, a significant decrease in spheres' number nearly 74% and 75% in area was observed (Fig. 6A and B). A comparable behavior was observed with DOG and DOX treatments. Decrease in viable cells number (60%) was observed in 4T1 conventional cell culture after 6 days treatment with DOG or *P. alliacea* extract at sub-cytotoxic concentrations (Fig. 7).

Petiveria alliacea *dry extract decreases the primary tumor and promotes survival in a TS/A murine breast cancer model*

To assess the antitumor effect of *P. alliacea* extract, a murine model of metastatic breast cancer was used. Previously, an estimated lethal dose 50 (LD_{50}) of 1545 mg/kg for the extract was reported (Hernandez et al., 2014), thus therapeutic dose evaluated was 6-fold lower to assure low toxicity. Female BALB/c mice were inoculated with SC injection of 1×10^4 TS/A cells. After five days, the tumors were palpable, and the mice were treated with *P. alliacea* extract (250 mg/kg, twice a week), vehicle (PBS) or positive control (DOX, 3 mg/kg once a week). Fig. 8A shows that *P. alliacea* extract significantly reduced tumor growth compared with the control, becoming increasingly marked from day 32, at day 42 the tumor achieves a volume of 881 mm^3 in control group compare to 343 mm^3 in *P. alliacea* treatment. Likewise, DOX treatment reduces tumor volume in a significantly manner showing a volume of 87 mm^3 at day 42 with a 67% of animals free of tumor. Due to a

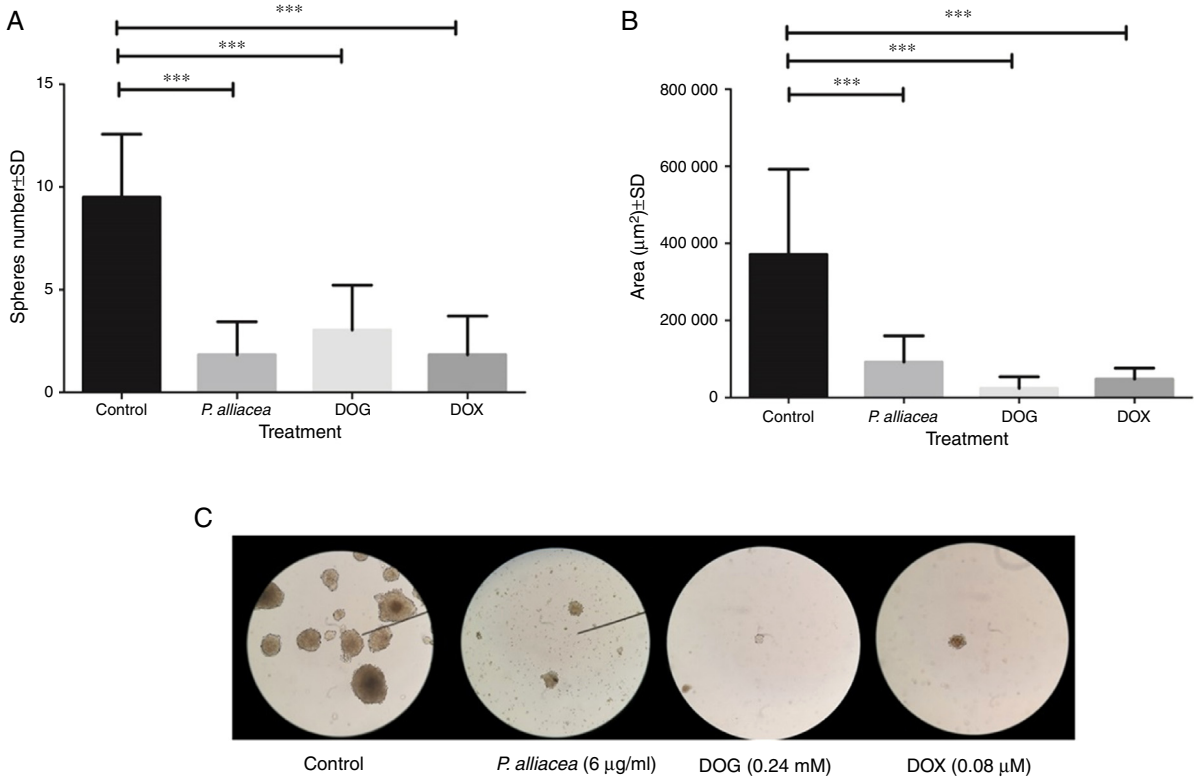

Fig. 6. *Petiveria alliacea* extract treatment decreases the spheres in number and area. 4T1 single cells culture on ultra-low attachment plates were treated with a *P. alliacea* dry extract ($IC_{50/5}$), deoxyglucose (DOG, 0.24 mM)) or doxorubicin (DOX, 0.08 µM) during six days. (A) Spheres were counted by two independent observers on day 7th using an optical microscopy Olympus (10×). (B) Spheres area was determined using an optical microscopy Olympus (10×) and images analyzed with software Axiovision®. (C) Representative images from control and treated 4T1 mammospheres at day 7th (10×). Results are expressed as mean ± S.D. from three independent experiments. $^{***}p < 0.0001$ compared to control using a two-way ANOVA.

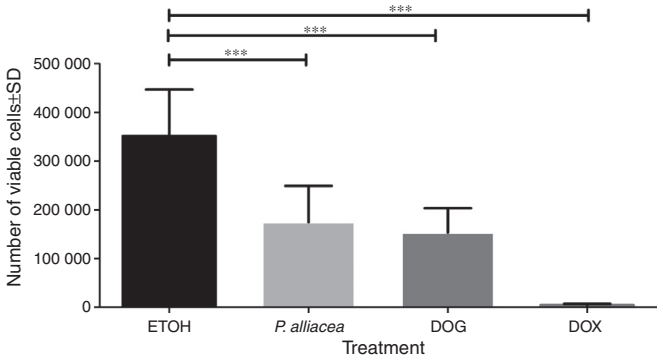

Fig. 7. Continuous treatment with *Petiveria alliacea* dry extract using sub-cytotoxic concentrations decreases 4T1 cell viability. 4T1 cells were treated with *P. alliacea* dry extract ($IC_{50/5}$), deoxyglucose (DOG, 0.24 mM)) or doxorubicin (DOX, 0.08 μM) during six days. At day 7th viable cells were counted using trypan blue dye. Results are expressed as mean ± S.D. from three independent experiments. ***$p < 0.0001$ compared to control using a two-way ANOVA.

marked decrease in the control group population before day 50th (77%), we evaluate the survival of treatments groups following the endpoint criteria of toxicity and animal welfare for each individual. As shown in Fig. 8B *P. alliacea* extract increases survival to 48.5 days compared to 38 days in control group ($p = 0.0055$ Log-rank test) while DOX treatment improves survival to 70 days compared to control ($p = 0.0004$ Log-rank test).

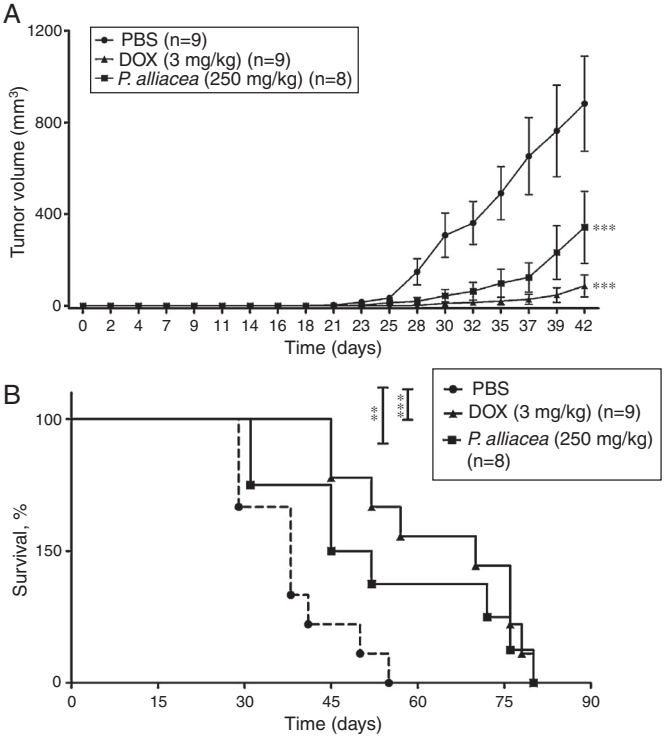

Fig. 8. Tumor growth inhibition and increase in survival in BALB/c mice by *Petiveria alliacea* extract. BALB/c mice implanted SC with 1×10^4 TS/A cells for five days and randomly divided into three groups. Group 1 was treated with PBS (vehicle), group 2 was treated with 3 mg/kg of doxorubicin, and group 3 was treated with 250 mg/kg of *P. alliacea* extract. (A) Mean tumor volume. The graph represents the mean tumor volume (mm^3) ± S.D. from each group with 8–9 animals per group. ***$p < 0.0001$ compared to control using Student's *t* test. (B) Kaplan–Meier survival curves representing % survival of mice bearing subcutaneous TS/A breast tumors. Statistical analysis was done using log rank test. **$p < 0.001$, ***$p < 0.0001$ compared to control.

Discussion

Our group aims to validate *P. alliacea* traditional use for cancer treatment (Chirinos, 1992; Gupta, 1995; Correa and Bernal, 1998). Previously, *P. alliacea* extract has been proven to induce apoptosis and to decrease colony cell growth in 4T1 cells (Urueña et al., 2008). In addition, herein we showed that the dry extract from *P. alliacea* is cytotoxic in a dose-dependent manner to human breast (IC_{50} 30 μg/ml) and colon (IC_{50} 88 μg/ml) cancer cell lines. However, the plant extract is less cytotoxic to murine fibroblasts and non-tumorigenic epithelial breast cell line, when cultured at high xenobiotic concentration (Fig. 1). The cytotoxicity shown in breast but not colon cancer cells is associated with the decrease in glycolytic rate, given that the lactate production is reduced (Fig. 2A). Although, we did not find changes in glycolytic enzymes expression, the fluctuations in rate were acute and reversible.

Suolinna et al. (1975) have demonstrated that flavonoids having hydroxyl groups at 3′, 4′, 7 either 3 or 5 positions, like fisetin, luteolin or quercetin decrease glycolytic rate in Erhlich ascites tumor cells. Such behavior has been explained by the loss of Na^+–K^+-ATPase activity that lowers intracellular ADP and Pi, required for glycolytic rate maintenance (Suolinna et al., 1975). Previously we have shown, that *P. alliacea* extract contains flavonoids as leridol, petiveral and 4-ethyl petiveral which have hydroxyl groups at 5, 7 and 6 positions respectively, although methoxyl substituents may be present at 5 or 7 positions (Urueña et al., 2008). This unique flavonoid combination could be responsible for the lactate secretion decrease in breast cancer cell lines.

Herein, we have demonstrated a decrease in β-F1-ATPase expression and mitochondrial respiration (basal and after OLIGO addition) in human and murine breast cancer cell lines after *P. alliacea* extract treatment (Figs. 3 and 4). Also, changes in maximum respiration can be accounted by the effect of *P. alliacea* extract on respiratory complexes expression (Fig. 3). In mammalian cells β-F1-ATPase expression is primarily regulated by mechanisms controlled at the level of translation (Willers and Cuezva, 2011). Hence, we suggest that *P. alliacea* extract might partially inhibit translation of β-F1-ATPase mRNA.

Moreover, flavonoids like quercetin, kaempferol and morin affect mitochondrial ATPase activity (Zheng and Ramirez, 2000). Specifically, quercetin binds to the hydrophobic pocket between γ- and βTP-subunit by means of van der Waals forces and H-bonds preventing the rotation of F1 catalytic domain (Gledhill et al., 2007). As discussed above, *P. alliacea* extract contains several flavonoids having a planar conformation like quercetin. We hypothesized that *P. alliacea* extract flavonoids behave in a quercetin-like manner such as the one reported by Zheng and Ramirez (2000), by binding to F1 hydrophobic pockets and decreasing the respiration rate.

Cell death is addressed to necrosis or apoptosis depending upon ATP levels. Strong ATP depletion (>50%) is a commitment to necrosis while higher ATP levels favors apoptosis (Leist et al., 1997). Also, we have established a 2.5-fold decrease in ATP levels associated to a decline in glycolytic flux and OCR in 4T1 cells (Fig. 5). Previously, our group has reported that *P. alliacea* extract induces apoptotic cell death (Hernandez et al., 2014) where ATP depletion could be implied.

Finally, continuous treatment with *P. alliacea* extract (6 days $IC_{50/5}$) decreases spheres' number and area (Fig. 6), parameters associated to self-renewal potential and progenitor cell proliferation (Gong et al., 2013). Recently, DOG a glycolysis inhibitor has been shown to be cytotoxic to human breast cancer stem cells (Ciavardelli et al., 2014); therefore we suggest that changes in glycolytic flux (Fig. 2) and mitochondrial respiration (Fig. 4) induced by *P. alliacea* extract could be accounted for decrease in self-renewal and proliferation of cancer stem cells, known to be highly glycolytic.

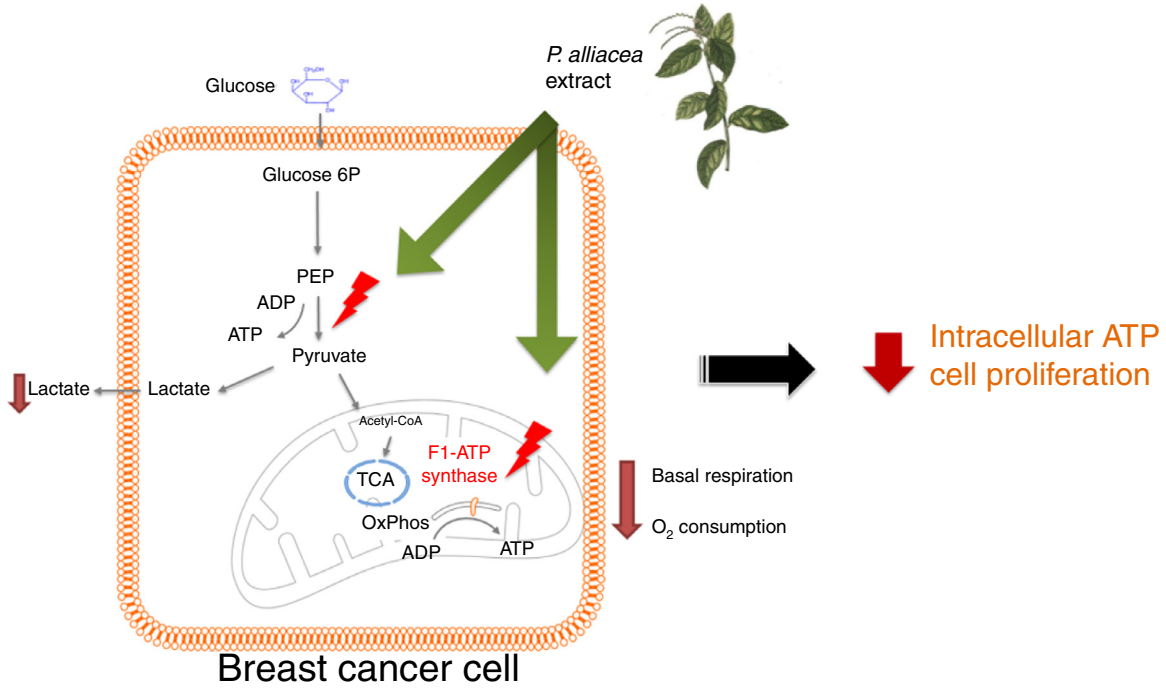

Fig. 9. A model for the proposed metabolic mechanism of action of the cytotoxic extract of *Petiveria alliacea*.

In vivo assay showed that treatment with *P. alliacea* extract twice a week *via i.p.* with a dose equivalent to the $LD_{50/6}$ decreases the primary tumor growth and increases survival in the TS/A murine breast cancer model. TS/A is a highly heterogeneous mammary adenocarcinoma originated spontaneously in a BALB/c mice that develops spontaneous lung metastases and generates 100% of tumor development after inoculation of 10^5 cells *via* SC (Nanni et al., 1983). Our results showed that *P. alliacea* extract increases significantly the median survival of mice with an interesting 50% of population free of tumor until 42 days, suggesting that ATP depletion caused by *P. alliacea* treatment could arrest the tumor growth at the beginning phase, a stage characterized by a highly glycolytic and mitochondrial activity. Overall we hypothesize that continuous treatment of cancer cells with *P. alliacea* extract decrease β-F1-ATPase expression and glycolytic flux triggering diminished ATP levels and finally decreasing cell proliferation. So ATP depletion in breast cancer cell lines could partially explain *P. alliacea* dry extract cytotoxic and antitumoral activity. A proposed model displaying the metabolism mechanism of action of the extract is shown in Fig. 9.

Anticancer drugs that block energy production or mimic low energy condition represent a new class of cancer drug therapy (Jose and Rossignol, 2013). Particularly, drugs affecting mitochondrial complexes (I, II and IV) like VLX600 an indol-1,2,4-triazine have shown antitumor activity in colon tumor xenografts (Zhang et al., 2014). Similarly, a cytotoxic aqueous extract from *Scutellaria barbata* containing flavonoids, terpenes and alkaloids reduces basal respiration and glycolytic flux in breast cancer cell lines (Chen et al., 2012).

Conclusions

Here we have demonstrated that ATP depletion and decrease in mitochondrial expression of β-F1-ATPase could partly explain the *P. alliacea* dry extract cytotoxic activity. Such mechanism seems specific to epithelial breast cancer cell lines having no effect on non-tumorigenic counterparts. Currently, we are studying if ATP depletion is only due to mitochondrial effects or Na^+–K^+-ATPase changes are involved in a mechanism similar to the described by Suolinna for hydroxyl substituted flavonoids.

Author contributions

SF, JMC, LF and JFH participate in the study conception and experiments design. JFH and TAS perform the acquisition of data. JFH, CPU, MCC and TAS participate in drafting of manuscript. SF, MCC and JMC accomplish the critical revision of manuscript. All the authors contribute to analysis and interpretation of data.

Conflicts of interest

The authors declare no conflicts of interest.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors have obtained the written informed consent of the patients or subjects mentioned in the article. The corresponding author is in possession of this document.

Acknowledgments

The authors acknowledge Colombian Environmental Ministry for the usage of genetic resources and derivatives products (agreement number 1927 07/10/2009). We are thankful to Departamento

Administrativo de Ciencia, Tecnología e Innovación "COLCIENCIAS" (120348925341) and PUJ (ID 00004753) for their financial support. Some experiments were supported by Ministerio de Economía y Competitividad (SAF2013-41945-R), Spain. LF was financially supported by Asociación Española Contra el Cáncer (AECC), Spain. PhD students JFH and TAS were financially supported by COLCIENCIAS.

References

Acebo, P., Giner, D., Calvo, P., Blanco-Rivero, A., Ortega, Á.D., Fernández, P.L., Roncador, G., Fernández-Malavé, E., Chamorro, M., Cuezva, J.M., 2009. Cancer abolishes the tissue type-specific differences in the phenotype of energetic metabolism. Transl. Oncol. 2, 138–145.

Chen, V., Staub, R.E., Fong, S., Tagliaferri, M., Cohen, I., Shtivelman, E., 2012. Bezielle selectively targets mitochondria of cancer cells to inhibit glycolysis and OXPHOS. PLoS ONE 7, e30300.

Chirinos, D.N., 1992. El milagro de los vegetales: Petiveria alliacea, 3rd ed. Bienes Lacónica, Caracas, Venezuela.

Ciavardelli, D., Rossi, C., Barcaroli, D., Volpe, S., Consalvo, A., Zucchelli, M., De Cola, A., Scavo, E., Carollo, R., D'Agostino, D., Forli, F., D'Aguanno, S., Todaro, M., Stassi, G., Di Ilio, C., De Laurenzi, V., Urbani, A., 2014. Breast cancer stem cells rely on fermentative glycolysis and are sensitive to 2-deoxyglucose treatment. Cell Death Dis. 5, e1336.

Cifuentes, C., Castañeda, D., Urueña, C., Fiorentino, S., 2009. A fraction from *Petiveria alliacea* induces apoptosis via a mitochondria dependent pathway and regulates Hsp70 expression. Universitas Sci. 14, 125–134.

Correa, J., Bernal, H., 1998. Especies Vegetales Promisorias de Paises del convenio Andrés Bello, Bogotá, Colombia.

Cuezva, J., Krajewska, M., de Heredia, M., Krajewski, S., Santamaria, G., Kim, H., Zapata, J., Marusawa, H., Chamorro, M., Reed, J., 2002. The bioenergetic signature of cancer: a marker of tumor progression. Cancer Res. 62, 6674–6681.

Cuezva, J.M., Ortega, A., Willers, I., Sanchez-Cenizo, L., Aldea, M., Sanchez-Arago, M., 2009. The tumor suppressor function of mitochondria: translation into the clinics. Biochim. Biophys. Acta 1792, 1145–1158.

Fantin, V., St-Pierre, J., Leder, P., 2006. Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance. Cancer Cell 9, 425–434.

Formentini, L., Martínez-Reyes, I., Cuezva, J., 2010. The mitochondrial bioenergetic capacity of carcinomas. IUBMB Life 62, 554–560.

Garcia-Barriga, H., 1974. Flora Medicinal de Colombia. Instituto de Ciencias Naturales. Universidad Nacional, Bogotá, Colombia.

Gledhill, J., Montgomery, M., Leslie, A., Walker, J., 2007. Mechanism of inhibition of bovine F1-ATPase by resveratrol and related polyphenols. PNAS 104, 13632–13637.

Gong, C., Bauvy, C., Tonelli, G., Yue, W., Delomenie, C., Nicolas, V., Zhu, Y., Domergue, V., Marin-Esteban, V., Tharinger, H., Delbos, L., Gary-Gouy, H., Morel, A., Ghavami, S., Song, E., Codogno, P., Mehrpour, M., 2013. Beclin 1 and autophagy are required for the tumorigenicity of breast cancer stem-like/progenitor cells. Oncogene 32, 2261–2272.

Govindarajan, B., Sligh, J., Vincent, B., Li, M., Canter, J., Nickoloff, B., Rodenburg, R., Smeitink, J., Oberley, L., Zhang, Y., Slingerland, J., Arnold, R., Lambeth, J., Cohen, C., Hilenski, L., Griendling, K., Martinez-Diez, M., Cuezva, J., Arbiser, J., 2007. Overexpression of Akt converts radial growth melanoma to vertical growth melanoma. J. Clin. Invest. 117, 719–729.

Gupta, M., 1995. 270 Plantas medicinales Iberoamericanas. Convenio Andrés Bello. Convenio Andres Bello y Subprograma X del CYTED, Bogotá, Colombia.

Hernandez, J., Urueña, C., Cifuentes, C., Sandoval, T., Pombo, L., Castaneda, D., Asea, A., Fiorentino, S., 2014. A *Petiveria alliacea* standardized fraction induces breast adenocarcinoma cell death by modulating glycolytic metabolism. J. Ethnopharmacol. 153, 641–649.

Jose, C., Rossignol, R., 2013. Rationale for mitochondria-targeting strategies in cancer bioenergetic therapies. Int. J. Biochem. Cell Biol. 45, 123–129.

Koukourakis, M., Kontomanolis, E., Giatromanolaki, A., Sivridis, E., Liberis, V., 2008. Serum and tissue LDH levels in patients with breast/gynaecological cancer and benign diseases. Gynecol. Obstet. Invest. 67, 162–168.

Leist, M., Single, B., Castoldi, A.S., Kuhnle, S., Nicotera, P., 1997. Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. J. Exp. Med. 185, 1481–1486.

Morales, C., Gomez-Serranillos, M., Iglesias, I., Villar, A., Caceres, A., 2001. Preliminary screening of five ethnomedicinal plants of Guatemala. Farmaco 56, 523–526.

Nanni, P., de Giovanni, C., Lollini, P., Nicoletti, G., Prodi, G., 1983. TS/A: a new metastasizing cell line from a BALB/c spontaneous mammary adenocarcinoma. Clin. Exp. Metastasis 1, 373–380.

Pampaloni, F., Reynaud, E., Stelzer, E., 2007. The third dimension bridges the gap between cell culture and live tissue. Nat. Rev. Mol. Cell Biol. 8, 839–845.

Papandreou, I., Goliasova, T., Denko, N., 2011. Anticancer drugs that target metabolism: is dichloroacetate the new paradigm? Int. J. Cancer 128, 1001–1008.

Rossi, V., 1990. Antiproliferative effects of *Petiveria alliacea* on several tumoral lines. Pharmacol. Res. 22, 434.

Rossi, V., Marini, S., Jovicevic, L., D'Atri, S., Turri, M., Giardina, B., 1993. Effects of *Petiveria alliacea* L. on cell immunity. Pharmacol. Res. 27 (Supplement 1), 111–112.

Stock, D., Leslie, A., Walker, J., 1999. Molecular architecture of the rotary motor in ATP synthase. Science 286, 1700–1705.

Suolinna, E., Buchsbaum, R., Racker, E., 1975. The effect of flavonoids on aerobic glycolysis and growth of tumor cells. Cancer Res. 35, 1865–1872.

Urueña, C., Cifuentes, C., Castaneda, D., Arango, A., Kaur, P., Asea, A., Fiorentino, S., 2008. *Petiveria alliacea* extracts uses multiple mechanisms to inhibit growth of human and mouse tumoral cells. BMC Complement. Altern. Med. 8, http://dx.doi.org/10.1186/1472-6882-8-60.

Urueña, C., Mancipe, J., Hernandez, J., Castaneda, D., Pombo, L., Gomez, Asea, A., Fiorentino, S., 2013. Gallotannin-rich *Caesalpinia spinosa* fraction decreases the primary tumor and factors associated with poor prognosis in a murine breast cancer model. BMC Complement. Altern. Med. 13, http://dx.doi.org/10.1186/1472-6882-13-74.

Willers, I., Cuezva, J., 2011. Post-transcriptional regulation of the mitochondrial H^+ATP synthase: a key regulator of the metabolic phenotype in cancer. Biochim. Biophys. Acta 1807, 543–551.

Zhang, X., Fryknäs, M., Hernlund, E., Fayad, W., De Milito, A., Olofsson, M., Gogvadze, V., Dang, L., Påhlman, S., Schughart, L., 2014. Induction of mitochondrial dysfunction as a strategy for targeting tumour cells in metabolically compromised microenvironments. Nat. Commun. 5, http://dx.doi.org/10.1038/ncomms4295.

Zheng, J., Ramirez, V., 2000. Inhibition of mitochondrial proton F0F1-ATPase/ATP synthase by polyphenolic phytochemicals. Br. J. Pharmacol. 130, 1115–1123.

Zhou, M., Zhao, Y., Ding, Y., Liu, H., Liu, Z., Fodstad, O., Riker, A., Kamarajugadda, S., Lu, J., Owen, L., 2010. Warburg effect in chemosensitivity: targeting lactate dehydrogenase-A re-sensitizes taxol-resistant cancer cells to taxol. Mol. Cancer 9, http://dx.doi.org/10.1186/1476-4598-9-33.