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Research Paper

A *Petiveria alliacea* standardized fraction induces breast adenocarcinoma cell death by modulating glycolytic metabolism

John Fredy Hernández [a], Claudia Patricia Urueña [a], Maria Claudia Cifuentes [a], Tito Alejandro Sandoval [a], Luis Miguel Pombo [b], Diana Castañeda [a], Alexzander Asea [c], Susana Fiorentino [a,*]

[a] Grupo de Inmunobiología y Biología Celular, Facultad de Ciencias, Pontificia Universidad Javeriana, Carrera 7 N. 43-82 Building 52, Office 608, Bogotá, Colombia
[b] Grupo de Farmacología Vegetal, Fundación Universitaria Juan N. Corpas, Carrera 111N. 159A-61, Bogotá, Colombia
[c] Department of Microbiology, Biochemistry & Immunology, Morehouse School of Medicine, 720 Westview Drive, Atlanta, GA 30310, USA

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ABSTRACT

Ethnopharmacological relevance: Folk medicine uses aqueous and alcoholic extracts from *Petiveria alliacea* (*Phytolaccaceae*) in leukemia and breast cancer treatment in the Caribbean, Central and South America. Herein, we validated the biological activity of a *Petiveria alliacea* fraction using a metastatic breast adenocarcinoma model (4T1).

Materials and methods: Petiveria alliacea fraction biological activity was determined estimating cell proliferation, cell colony growth capacity and apoptosis (caspase-3 activity, DNA fragmentation and mitochondrial membrane potential) in 4T1 cells. *Petiveria alliacea* was used at IC_{50} concentration (29 μg/mL) and 2 dilutions below, doxorubicin at 0.27 μg/mL (positive control) and dibenzyl disulfide at 2.93 μg/mL (IC_{50} fraction marker compound). Proteomic estimations were analyzed by LC–MS–MS. Protein level expression was confirmed by RT-PCR. Glucose and lactate levels were measured by enzymatic assays. LD_{50} was established in BALB/c mice and antitumoral activity evaluated in mice transplanted with GFP-tagged 4T1 cells. Mice were treated with *Petiveria alliacea* fraction via I.P (182 mg/kg corresponding to 1/8 of LD_{50} and 2 dilutions below).

Results: Petiveria alliacea fraction in vitro induces 4T1 cells apoptosis, caspase-3 activation, DNA fragmentation without mitochondria membrane depolarization, and decreases cell colony growth capacity. Also, changes in glycolytic enzymes expression cause a decrease in glucose uptake and lactate production. Fraction also promotes breast primary tumor regression in BALB/c mice transplanted with GFP-tagged 4T1 cells.

Conclusion: A fraction of *Petiveria alliacea* leaves and stems induces in vitro cell death and in vivo tumor regression in a murine breast cancer model. Our results validate in partly, the traditional use of *Petiveria alliacea* in breast cancer treatment, revealing a new way of envisioning *Petiveria alliacea* biological activity. The fraction effect on the glycolytic pathway enzymes contributes to explain the antiproliferative and antitumor activities.

1. Introduction

Petiveria alliacea Linne (*Phytolaccaceae*) is a perennial herb widely used in folk medicine in the Caribbean, Central and South America. Leaf infusions or root powder are known for their anti-spasmodic, anti-rheumatic, anti-inflammatory (Morales et al., 2001), nociception (de Lima et al., 1991), hypoglycemic and abortifacient (Correa and Bernal, 1998) properties. Additionally, aqueous infusions have been used in leukemia and breast cancer treatment (Garcia-Barriga, 1974; Gupta, 1995).

In spite of traditional usage of *Petiveria alliacea* extracts as antitumor agents, the molecular mechanisms by which the plant exerts its activity are not fully understood. Contradicting existing reports, Rossi et al. demonstrated that *Petiveria alliacea* aqueous and alcoholic extracts have cytotoxic activity against leukemia and lymphoma cells lines but not over breast cancer cells (Rossi, 1990; Rossi et al., 1993). Additionally, Ruffa et al. stated that methanolic plant extracts have no cytotoxic activity on cell line HepG2 (human hepatocellular

Abbreviations: 2,4-DNP, 2,4-dinitrophenol; DAPI, 4′,6-diamidino-2-phenylindole; DDS, Dibenzyl disulfide; DTS, Dibenzyl trisulfide; FCS, Fetal calf serum; FBPA, Fructose-bisphosphate aldolase A; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; GFP, Green fluorescence protein; LDH, Lactate dehydrogenase; MMP, Mitochondrial membrane potential ($\Delta\psi_m$); MIF, Macrophage migration inhibitory factor; MTT, Methylthiazol tetrazolium; PBS, Phosphate buffer saline; PK, Pyruvate kinase; TPI1, Triosephosphate isomerase 1.

* Corresponding author. Tel.: +57 1 3208320x4023; fax: +57 1 3208320x4021.
E-mail address: susana.fiorentino@javeriana.edu.co (S. Fiorentino).

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carcinoma) (Ruffa et al., 2002). DTS, a compound present in *Petiveria alliacea*, exhibits antiproliferative and cytotoxic activity against several tumor cell lines such as SH-SY5Y, MCF7, A549, Jurkat and HeLa (An et al., 2006; Williams et al., 2007). Most likely DTS antitumor activity is the result of an irreversible microtubule disassembly on the cytoskeleton (Rosner et al., 2001).

In previous reports, our group has demonstrated cytotoxic activity of a *Petiveria alliacea* fraction derived from leaves and stems over several tumor cell lines: chronic myeloid leukemia (K562, IC_{50} 32 μg/mL), human melanoma (A375, IC_{50} 35 μg/mL), murine melanoma (Mel Ret, IC_{50} 36 μg/mL). Moreover, the fraction has rendered low toxicity to normal fibroblasts and peripheral blood mononuclear cells, even after previous stimulation with phytohemagglutinin (IC_{50} 440 and 151 μg/mL, respectively). A375 cells treated with the fraction underwent cytoskeleton disruption, increased apoptotic cell population, and displayed cell cycle arrest profiles. In addition, changes in cell proteome expression were detected particularly for proteins involved in protein synthesis, cytoskeleton proteins and enzymes regulating metabolic reactions (Urueña et al., 2008).

Metabolic reprogramming provides tumor cells enough energy to survive under variable concentrations of oxygen, glucose and amino acids. Inwards the metabolic reprogramming results in high glycolytic rates, increased glucose uptake and lactate production as well as low oxygen consumption rates. The latter are characteristics describing the "Warburg effect" or aerobic glycolysis and though not present in all tumor types, it develops during active proliferation and/or as a consequence of hypoxia or glucose restraints (Moreno-Sanchez et al., 2007).

The most common type of cancer among women is breast cancer, with a worldwide incidence of 1.38 million new cases per year. Genetic analysis in 295 samples of invasive breast carcinoma stages I and II demonstrated that genes encoding for glycolytic enzymes increase expression in poor prognosis patients with metastatic tumors and recurrence in less than three years (Schramm et al., 2010). In addition, proteomic analysis of breast carcinoma samples in stages I to III showed a significant increased expression in glycolytic enzymes (GAPDH, PK and LDH) as compared to normal breast tissue (Isidoro et al., 2005).

Drugs that exert cytotoxic activity mainly on cells with high proliferation rates, affecting the cell cycle and modifying metabolic pathways might be a suitable therapeutic choice to be used nowadays. Therefore, a fraction containing several metabolites affecting the latter targets would be ideal for tumor therapy. Consequently, herbal drugs are proper candidates, provided that the main biological active components are acknowledged as epigallocatechin gallate and related catechins in green tea (Yang et al., 1998).

Petiveria alliacea infusions are traditionally used in breast cancer treatment. Herein, we evaluated biological activity of a *Petiveria alliacea* fraction derived from leaves and stems. The outcomes of the study were: in vivo tumor regression and in vitro cell death using a 4T1 murine breast adenocarcinoma model.

2. Materials and methods

2.1. Plant extraction and purification

Plant material was collected at Cachipay, Cundinamarca, Colombia on April 2009. The plant material was 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 derived products). *Petiveria alliacea* fraction was obtained using a process derived from a former laboratory methodology developed in our laboratory. The bio-guided methodology allowed us to establish that the biological active extract was derived from ethyl acetate extraction (Cifuentes, 2010; Cifuentes et al., 2009; Urueña et al., 2008). Briefly, dry ground leaves and stems (25 kg) from *Petiveria alliacea* were extracted with 96% ethanol for 15 days (15 ± 5 °C). The extract was filtered and evaporated until half-volume. An equal volume of water was added while heating (65 °C) and placed on ice bath for 12 h. The precipitated material was discarded and supernatant was tested to be chlorophyll-free by TLC at 254 nm. Supernatant (570 mL) was trapped in fumed silica (106 g) and fractionated with ethyl acetate (14.7 L). The latter extract was dried (2.53 g) and trapped in purified sea sand (particle size 0.1–0.3 mm, Merck®, 50 g). Finally, the latter matrix was extracted with methanol:water (7:3) (160 mL) corresponding to *Petiveria alliacea* fraction (2.17 g).

2.2. Chromatographic analysis

Chromatographic analysis was performed using an HP-5MS fused silica capillary column [30 m × 0.25 mm i.d., 0.25 μm film thickness (J&W Scientific, CA, USA)] in an Agilent GC 6850 series II (Agilent, Wilmington, USA) equipped with a mass-selective detector Agilent 5975C VL. To perform the GC–MS analysis, the fraction (10 mg) was acetylated using acetic anhydride (5 mL equivalent to 18 mmol) (Sigma-Aldrich) at 80 °C for 2 h (Kiehlmann and Szczepina, 2011), evaporated to dryness (80 °C) and extracted using ethyl ether (Mallinckrodt). Samples were injected using an Agilent 6850 autoinjector with a split ratio of 10:1. The temperature program began at 45 °C for 5 min, followed by an increase of 5 °C/min until reaching 60 °C. Then an increase of 30 °C/min was held until 190 °C and finally a rise of 40 °C/min till 285 °C was achieved. Injector and transference line temperature were 230 and 325 °C, and carrier gas (helium) was used at constant flow (1.2 mL/min). The MS scan parameters included a mass range of 40–500 m/z and a scan speed of 4.75 scan/seg. Data were analyzed comparing spectra with MSD Chemstation and NIST MS2011 database spectra.

HPLC chromatographic fingerprint analyses were done in a Jasco® PU2089plus with a C18 column (1.6 × 150 mm Waters®) at room temperature and water:acetonitrile –ACN- (t=0–1 90:10; t=25–36 0:100; t=50 90:10 flow 0.4 mL/min) as mobile phase (254 nm). Under the latter conditions retention times of compounds used as standards were established. DDS and DTS standard compounds (Sigma®) were added to *Petiveria alliacea* fraction and the new retention times were estimated. Subsequently, a calibration curve (499, 62.4 and 15.5 ng) was used to estimate DDS and DTS concentrations in *Petiveria alliacea* fraction.

2.3. Cell lines

The breast adenocarcinoma cell line 4T1 (a gift from Prof. Alexzander Asea (Morehouse School of Medicine, Atlanta, GA, USA) was cultured in RPMI-1640 (Eurobio, Tolouse, FR), supplemented with heat-inactivated FCS (10%) (Eurobio Toulouse, FR), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.01 M Hepes buffer, 1 mM sodium pyruvate (Eurobio) and incubated under humidified environment at 37 °C and 5% CO_2 as previously described (Uruena et al., 2013). Tumor cells were proven Mycoplasma-free with a Myco-Probe Mycoplasma Detection Kit (R&D Systems, Minneapolis, MN) and maintained with ciprofloxacin (0.5 μg/mL).

2.3.1. in vitro cytotoxicity assays

Cytotoxic effects were evaluated over tumor cells using trypan blue, methylthiazol tetrazolium (MTT) and neutral red assays (Sigma-Aldrich, Saint Louis MO). 4T1 breast adenocarcinoma cells (5×10^3 cells/well) were seeded in 96-well plates with *Petiveria*

alliacea fraction at different concentrations (125 to 0.95 μg/mL), ethanol (0.02%), doxorubicin (positive control) or test compounds for 48 h. *Petiveria alliacea* fraction vehicle was ethanol, which was used as negative control. After treatment the cells were carefully washed with PBS and MTT (50 μL in PBS) was added to each well and incubated for 4 h at 37 °C. The formazan crystals were dissolved with DMSO. MTT results were seen at 540 nm in a Multiskan MCC/340 (Thermo Labsystems Inc., Waltham, Woburn, MA). For neutral red uptake assay, after treatment the cells were washed with PBS and 100 μL of neutral red working solution in RPMI without phenol red (40 μg/mL) which was added to each well and incubated for 3 h at 37 °C. After incubation, the cells were carefully washed with PBS and 150 μL of destain solution [50% ethanol 96% (Sigma-Aldrich); 49% deionized water, and 1% glacial acetic acid (Mallinckrodt)] was added to each well and the plate was shaken rapidly on an adjustable rocker (Cole Parmer) for 20 min and incubated for additional 5 min. Then optical density was measured at 540 nm in a Multiskan FC (Thermo Scientific). The IC_{50} value was calculated using nonlinear regression analysis (Graph Pad Prism 5 for Windows 2009).

2.4. Clonogenic assays

Cell colony growth capacity assays were performed as previously described (Urueña et al., 2008). Briefly, 4T1 breast adenocarcinoma cells (2.5×10^5 cells) were plated (96-well) and treated with *Petiveria alliacea* fraction (29.3, 2.9 and 0.29 μg/mL), vincristine (0.082 μg/mL) or ethanol (negative control 0.02%) and incubated for 12 h under a humidified environment at 37 °C and 5% CO_2. After treatment cells were washed, plated onto 0.5% agar dishes (60 mm, 20,000 cells/dish) and incubated for 14 days (37 °C and 5% CO_2) or plated onto 0.5% agar and treated for 14 days and then stained with violet crystal (0.4% in ethanol). Cell colonies with more than 50 cells were counted. Treatments were performed in triplicate, and results were expressed as mean ± SD.

2.5. Caspase 3 activity assay

Caspase 3 activity was estimated as previously described (Uruena et al., 2013) using the caspase 3 colorimetric assay kit (Sigma-Aldrich), which detects enzyme activity based on the cleavage of Acetyl–Asp–Glu–Val–Asp–p-nitroanilide (Ac-DEVD-pNA). Briefly, cells (2×10^5) were cultured at different concentrations of *Petiveria alliacea* fraction, doxorubicin (positive control) or ethanol (negative control) for 48 h. After treatment cells were ice lysed (10 min) and enzyme activity evaluated on 96-well flat-bottom microplates with 20 μL of supernatant prepared by centrifuging at 10,000*g* for 1 min (100–200 μg of total protein). For caspase 3 positive control 5 μL was used. For the reaction, 70 μL of reaction buffer and 10 μL of caspase 3 colorimetric substrate (Ac-DEVD-pNA) were added to supernatant and control, were incubated for 330 min at 37 °C, and caspase-3 activity was estimated at 405 nm on a spectrophotometer (Multiskan, Labsystems). To some wells caspase 3 inhibitor (Ac-DEVD-CHO) was added. The increase in caspase 3 activity was calculated from a calibration curve prepared with pNA standards, using the following formula: μmol pNA/min/mL = [(μmol pNA × *d*) / (*tv*)], where *d* is dilution factor, *t* is reaction time in minutes and *v* is volume of sample in mL.

2.6. Analysis of DNA fragmentation

Cells (3×10^5) were treated with *Petiveria alliacea* fraction (29.3 μg/mL) or doxorubicin (positive control, 0.94 μM) for 48 h. DAPI (4′,6-diamidino-2-phenylindole, Sigma-Aldrich) stained cells were monitored under a confocal microscope as previously reported (Uruena et al., 2013). Slides were mounted using a prolong anti-fade kit (Molecular Probes) and cells analyzed by a fluorescence confocal microscope (FluoView 1000, Olympus, Japan).

2.7. Evaluation of mitochondrial membrane potential –MMP – ($\Delta\psi_m$)

Cells (3×10^5) were treated with different concentrations of *Petiveria alliacea* fraction (29.3, 14.6 and 7.3 μg/mL) or S2 fraction from *Caesalpinia spinosa* previously obtained (positive control) for 6 and 12 h (Castaneda et al., 2012). MMP was measured using JC-1 (Sigma-Aldrich) dye as previously reported. Red fluorescence was evaluated at 530 nm excitation (Ex)/590 nm emission (Em) and green fluorescence at 485 nm Ex/530 nm Em. Cells were acquired on a FACSAria I (Becton Dickinson, New Jersey, USA) and analyzed with a FlowJo software (Tree Star Inc., Ashland, USA) and the red/green fluorescence ratios were calculated. Duplicate estimates were made and the average was expressed as mean ± SEM in three independent experiments.

2.8. Characterization and identification of proteins

Proteins from 4T1 breast adenocarcinoma cells treated with *Petiveria alliacea* (20 μg/mL) or ethanol (0.02%) as negative control for 24 h were digested, and the extracted peptides injected onto a 1100 Series HPLC-Chip Cube MS interface, and Agilent 6300 Series Ion Trap Chip-LC–MS/MS system (Agilent Technologies). Data are increase or decrease intracellular proteins after treatment with *Petiveria alliacea* fraction as judged by mass spectrometry.

2.8.1. Sample preparation

4T1 breast adenocarcinoma cells treated with *Petiveria alliacea* fraction (20 μg/mL) or ethanol (0.02%) as negative control for 24 h were lysed in lysis buffer, supplemented with phosphatase and proteinase inhibitors. Protein samples were de-salted in 10 K microcon, and diluted with 100 mL of ammonium bicarbonate buffer (100 mM). Cysteine residues were reduced with DTT (10 mM) by incubation at 65 °C for 45 min. After cooling to room temperature, sulfhydryls were alkylated with iodoacetamide (55 mM) for 30 min under dark conditions. The reduced and alkylated sample was diluted (1:1) with water. Trypsin (Promega, Madison, WI) was added at a 1:50 enzyme:substrate ratio, and incubated overnight at 37 °C. Tryptic peptides were completely dried in a SpeedVac and reconstituted with 10 mL of 0.1% TFA.

2.8.2. HPLC-chip/MS analysis

A sample of peptides (1 mL) was injected onto an LC–MS system with an 1100 Series liquid chromatograph, HPLC-Chip Cube MS interface, and 1100 Series LC–MSD Trap XCT Ultra ion trap mass spectrometer (Agilent Technologies). The system is equipped with an HPLC-Chip (Agilent Technologies) incorporated to a 40 nL enrichment column and a Zorbax 300SB-C18 column (43 mm × 75 mm, 5 mm diameter particles). Peptides were loaded onto the enrichment column with 97% solvent A (water with 0.1% formic acid). Then eluted with a gradient from 3% B (acetonitrile with 0.1% formic acid) to 45% B in 25 min, followed by a steep gradient to 90% B in 5 min at a flow rate of 0.3 mL/min. The total runtime, including column reconditioning, was 35 min. The column effluent was directly coupled to an LC–MSD Trap XCT Ultra ion trap mass spectrometer (Agilent Technologies) via a HPLC-Chip Cube nanospray source operated at ~1900 V in ultra-mode. The gain control (ICC) was set to 500,000 with a maximum accumulation time of 150 ms. CID was triggered on the six most abundant, not singly charged peptide ions in the m/z range of 450–1500. Precursors were set in an exclusion list for 1 min after two MS/MS spectra.

2.8.3. Data analysis

CID data was analyzed using Thermo Scientific Protein Center Software, a web-based data interpretation tool. Proteins identified showed a protein probability value below 80%.

2.9. Real-time PCR expression profiling

4T1 breast adenocarcinoma cells were treated with *Petiveria alliacea* fraction (20 μg/mL) for 4, 6, 12, 16 and 24 h and total RNA was extracted using TRIzol reagent according to manufacturer's instructions (Life technologies Corporation, Invitrogen, NY, USA). The quality and quantity of RNA were assessed with a NanoDrop spectrophotometer (NanoDrop Technologies). cDNA was synthesized with SuperScript® III Reverse Transcriptase, following manufacturer's instructions (Invitrogen). For quantitative real-time PCR (qPCR) reaction 100 ng of cDNA, DNA Master Plus SYBR Green I (Roche Applied Science, IN, USA) and 500 ng of forward and reverse primers (Table 1) in a total volume of 20 uL. Reactions were performed in triplicate using Lyght Cycler 1.5 (Roche). Thermal cycling conditions were: an initial denaturing step at 95 °C for 10 min, 35 cycles at 95 °C for 10 s, 62 °C for 5 s, 72 °C for 5 s, followed by a dissociation stage (95 °C 10 s, 70 °C for 15 s, 99 °C for 5 s). Amplified products specificity was verified by a post-amplification melt curve analysis. The mean cycle threshold (CT) value was calculated for each independent experiment. The expression level of each transcript was normalized to the endogenous control gene relative GAPDH. Relative expression was calculated using the comparative method $2^{-\Delta\Delta CT}$=[(CT gene of interest − CT internal group) Sample A – (CT gene of interest − CT internal group) Sample B)]. Sample A corresponds to treated 4T1 cells and sample B corresponds to non-treated 4T1 cells.

2.10. Glucose and lactate measurement

4T1 breast adenocarcinoma cells (5×10^3) were cultured in low glucose DMEM media (Eurobio), supplemented with heat inactivated FCS (10%) (Eurobio), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.01 M Hepes (Eurobio) and incubated under humidified environment at 37 °C and 5% CO_2. Cells were treated with *Petiveria alliacea* fraction, ethanol (negative control) or 2,4-dinitrophenol (2,4-DNP) 500 μM (oxidative phosphorylation uncoupler) for 48 h. Glucose and lactate were estimated in the supernatant by enzymatic assays, using Accu-Check® Performa and BM Lactate Cobas® test strips (Roche Diagnostics GmbH), detection limit 0.6 and 0.8 mmol/L, respectively. Values are the mean ± SD of three replicates.

2.11. Acute toxicity study

Female BALB/c mice 6 to 12 weeks old, purchased from Charles Rivers Laboratories International Inc (Wilmington, MA) were housed in our animal research facility following established protocols by the Ethics Committee (founded in 2002) of the Science Faculty in August 15, 2007 meeting (Act number 8) and National and International legislation for live animals experimentation (Colombia Republic, Law 84, 1987, Resolution 08430, 1993; National Academy of Sciences, 2010; Canadian Council of Animal Care, 1996). Mice were housed in polyethylene cages with food and water *ad libitum*, controlled temperature with a 12 h light/dark cycle. Before treatments the mice were accustomed for 1 week at standard conditions. Animals were gathered in groups of 5 and inoculated with 5, 50, 500 and 2000 mg/kg of *Petiveria alliacea* fraction injected via intraperitoneal (I.P). After 72 h, deceased animals were counted and LD_{50} was calculated with Probit version 14 (Minitab Inc.).

2.12. GFP-tagged 4T1 breast adenocarcinoma tumor model

Female BALB/c mice (6–8 weeks old) were housed at research facility of Texas A&M Health Science Center College of Medicine (Temple, Tx, USA) following established research protocols. Mice were housed in polyethylene cages with food and water *ad libitum*, controlled temperature (20 ± 2 °C) with a 12 h light/dark cycle. Before treatments the mice were accustomed for 1 week at standard conditions. Animals were randomly assigned in control or treatment groups of 5 individuals. GFP-tagged 4T1 breast adenocarcinoma cells (1×10^4) were injected into the mammary fat pad on day 0. Since day 7 the mice were treated with *Petiveria alliacea* fraction (45.5, 91 and 182 mg/kg) or ethanol (0.02% in PBS) via I.P once a week for 5 weeks. After treatment, mice were lightly anesthetized and the intensity of GFP was measured using an in vivo animal imaging system (Maestro®, Cambridge, MA).

2.13. Statistics

Results are expressed as mean ± SD. One-way ANOVA was used for caspase 3 activity estimations and unpaired *t*-test for the remaining analyses. Statistical analyses were done with Graph Pad Prism 5 with significance at $p < 0.05$.

3. Results

3.1. Ethyl acetate Petiveria alliacea fraction obtained from leaves and stems contains flavonoids and sulfide derivatives

Petiveria alliacea fraction was acetylated and analyzed by GC–MS. Data were analyzed comparing spectra with MSD Chemstation and NIST MS2011 database and the identified compounds were benzaldehyde, leridol, petiveral, myricetin, petiveral 4-ethyl, pinitol, DDS and DTS (Table 2 and Fig. 1A). All of the mention compounds were reported previously in *Petiveria alliacea* (Adesogan, 1974; Ayedoun et al., 1998; De Sousa et al., 1990; Delle Monache and Cuca, 1992, 1996). Triacetin derivative (retention time 16 min) is a sub-product of acetylation.

Petiveria alliacea fraction was standardized using a quantitate active marker compound according to European Medicines Agency (EMEA) Guidelines for Herbal Substances and IUPAC protocol (EMEA, 2006; Mosihuzzaman and Choudhary, 2008). DDS is a compound previously reported in *Petiveria alliacea* having cytotoxic activity (Cifuentes et al., 2009); therefore it was used as marker. The fraction content of DDS was 2.6 mg/g fraction using HPLC spike out methodology (Fig. 1B).

Table 1
Real time primer sequences.

Gene	Forward primer (5′-3′)	Reverse primer (3′-5′)	Size (pb)
GAPDH	TTCGACAGTCAGCCGCATCTTC	GCCCAATACGACCAAATCCGTT	105
FBPA	AGGCTCTTTCCCATCACTCTTCT	AGTTGTTTATTGGCAGTGGGCGG	98
TPI1	TGCCTGCATAGCATGGACTAGGTT	TACAGCGTGGATTAGGCCACAAGA	109
PK isozymes R/L	TCGAGAACCATGAAGGCGTGAAGA	TCTCTGCTGGGATCTCAATGCCAA	102

Table 2
Secondary metabolites detected in *Petiveria alliacea* fraction by de-replication using GC–MS.

Identified secondary metabolites	Molecular weight	Retention time (min)
Benzaldehyde	106	5960
Leridol	314	24,163
Petiveral	314	24,811
Petiveral-4-ethyl	342	27,058
Pinitol	194	31,205
Dibenzyl disulfide	246	31,643
Dibenzyl trisulfide	278	36,334

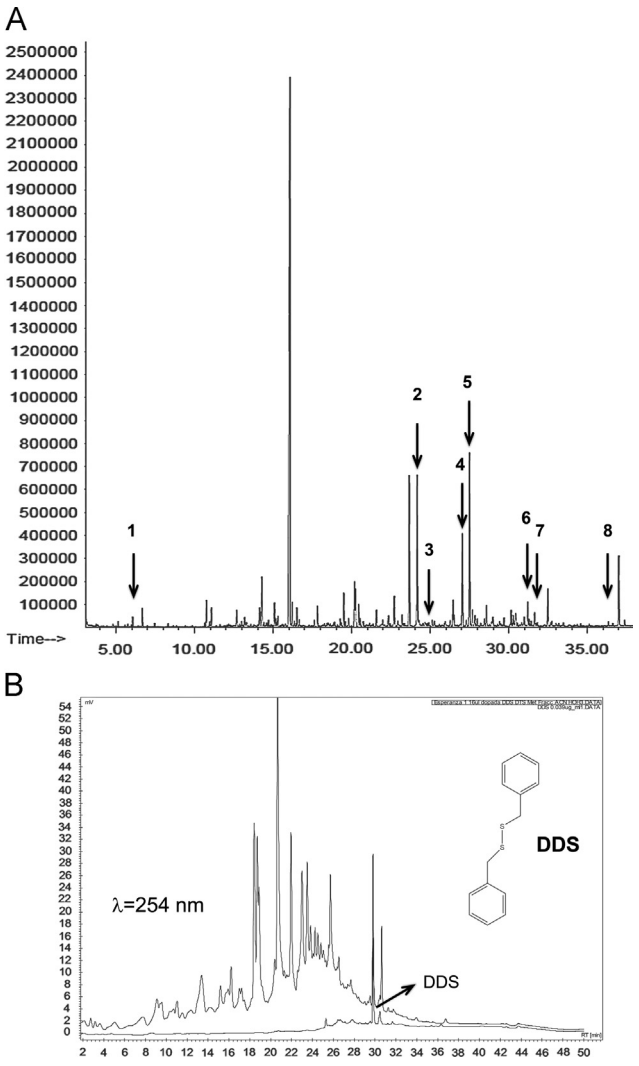

Fig. 1. (A) *Petiveria alliacea* fraction total ion chromatogram by GC–MS (HP-5MS fused silica capillary column, Agilent GC 6850 II, mass detector Agilent 5975C VL). Black arrows show detected compounds: (1) benzaldehyde, (2) leridol, (3) petiveral, (4) petiveral-4-ethyl, (5) benzylmethyldisulfide, (6) pinitol, (7) DDS, (8) DTS. Data were compared with MSD Chemstation and NIST MS2011 database spectra. (B) Fraction chromatographic fingerprint overlapped dibenzyldisulfide (DDS) standard chromatogram (C18 column – 1.6 × 150 mm Waters®-, Jasco® PU2089plus HPLC at 254 nm, water:acetonitrile (t=0–1 90:10; t=25–36 0:100; t=50 90:10), flow 0.4 mL/min).

3.2. *Petiveria alliacea fraction exerts cytotoxic activity against 4T1 cells inducing apoptosis independently of mitochondrial membrane depolarization ($\Delta\psi_m$)*

Petiveria alliacea fraction cytotoxic activity against breast murine adenocarcinoma 4T1 was determined through MTT and neutral red assays. After 48 h of treatment, a significant decrease in cell proliferation was observed in both assays (Table 3). The decrease was dose dependent at concentrations ranging from 125 to 7.8 μg/mL (MTT IC_{50} 29.3 μg/mL; neutral red IC_{50}19.9 μg/mL). Estimated DDS IC_{50} was 2.93 μg/mL (MTT) and 2.12 μg/mL (neutral red). Positive control was doxorubicin (MTT IC_{50} 0.27 μg/mL; neutral red IC_{50} 0.25 μg/mL) and ethanol at 0.02% (fraction diluent) as negative control.

Table 3
IC_{50} estimated for *Petiveria alliacea* fraction, dibenzyl disulfide (DDS) and doxorubicin by MTT assay and neutral red assay in 4T1 cells after 48 h treatment.

Fraction/compound	IC_{50} calculated by MTT assay (μg/mL)	IC_{50} calculated by neutral red assay (μg/mL)
Petiveria alliacea fraction	29.3	19.9
DDS	2.93	2.12
Doxorubicin	0.27	0.25

The cell colony growth capacity was decreased with 29.3 (IC_{50}) and 2.93 μg/mL of *Petiveria alliacea* fraction after treatment for 12 h (Fig. 2A) and 14 days (Fig. 2B). To assess the mechanism by which *Petiveria alliacea* fraction induces cell death, caspase 3 activation, DNA fragmentation and MMP were evaluated. 4T1 cells treated with *Petiveria alliacea* fraction (29.3 and 14.6 μg/mL) showed a significant increase in caspase 3 activity similar to doxorubicin and the internal positive control (Fig. 3A). In contrast, no effect on MMP was determined after 6 or 12 h of treatment at 29.3, 14.6 and 7.3 μg/mL (Fig. 3B).

Nuclear apoptotic bodies were evidenced by DAPI stain, showing condensed chromatin of 4T1 cells DNA after treatment with *Petiveria alliacea* fraction (29.3 μg/mL) or doxorrubicin (0.23 μg/mL) (Fig. 3C). As a whole, results revealed that *Petiveria alliacea* fraction induces apoptosis in 4T1 cells by a mitochondria independent pathway. The latter was evidenced since changes in $\Delta\psi_m$ were not detected.

3.3. *Petiveria alliacea fraction alters key glycolytic enzymes in 4T1 cells metabolism*

Proteins from 4T1 cells treated with *Petiveria alliacea* (20 μg/mL) were digested, peptides analyzed by LC–MS–MS and compared with the control proteomic profile (ethanol 0.02%). The comparative analysis made with Protein Center Software allows establishment of increase or decrease in protein profile expression (Table 4). Within increased proteins, 78% were metabolic related, mainly glycolytic enzymes (e.g. PK isozymes R/L, FBPA isoform 2, TPI1 and GAPDH), while 22% were non-metabolic related, e.g. proteins associated with microtubules, mRNA processing and ribosomal constituents. PK expression was confirmed by RT-PCR showing a 15 fold increase after *Petiveria alliacea* treatment. Corroboration by RT-PCR was not achieved for the following enzymes: FBPA isoform 2, TPI1 and GAPDH (Fig. 4).

In proteins whose expression was decreased after treatment, we found glycolytic enzymes (e.g. LDH-A and phosphoglycerate kinase 1), cytoskeleton constituents (Tubulin alpha 1B) and mobility and invasiveness related proteins (Profilin 1, cyclophilin A, transgelin 2 and MIF).

3.4. *Petiveria alliacea fraction induces decrease in glucose uptake and lactate production in 4T1 cells*

A quantitative approach of cancer cells' metabolic behavior can be achieved by measuring glucose uptake and lactate production. We found 55% decrease in glucose uptake and 38% decrease in lactate production after *Petiveria alliacea* treatment (3 μg/mL) as compared with control (Fig. 5). A well-known mitochondrial uncoupler agent is 2,4-DNP which increases glucose uptake, boosting glycolytic flux and consequently enhancing lactate

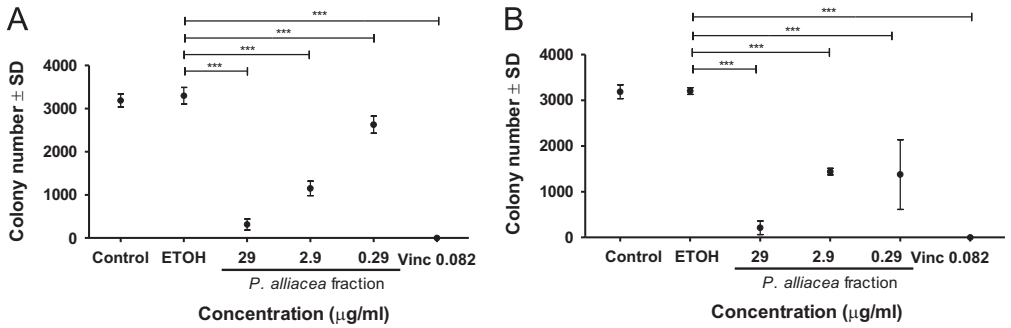

Fig. 2. *Petiveria alliacea* fraction significantly decreases growth of 4T1 colonies. 4T1 cells (2×10^5) were treated with ethanol (0.02%), *Petiveria alliacea* fraction or vincristine for (A) 12 h and (B) 14 days. Data represents the mean of the number of colonies (± SD) from three independent experiments. (***$p < 0.005$).

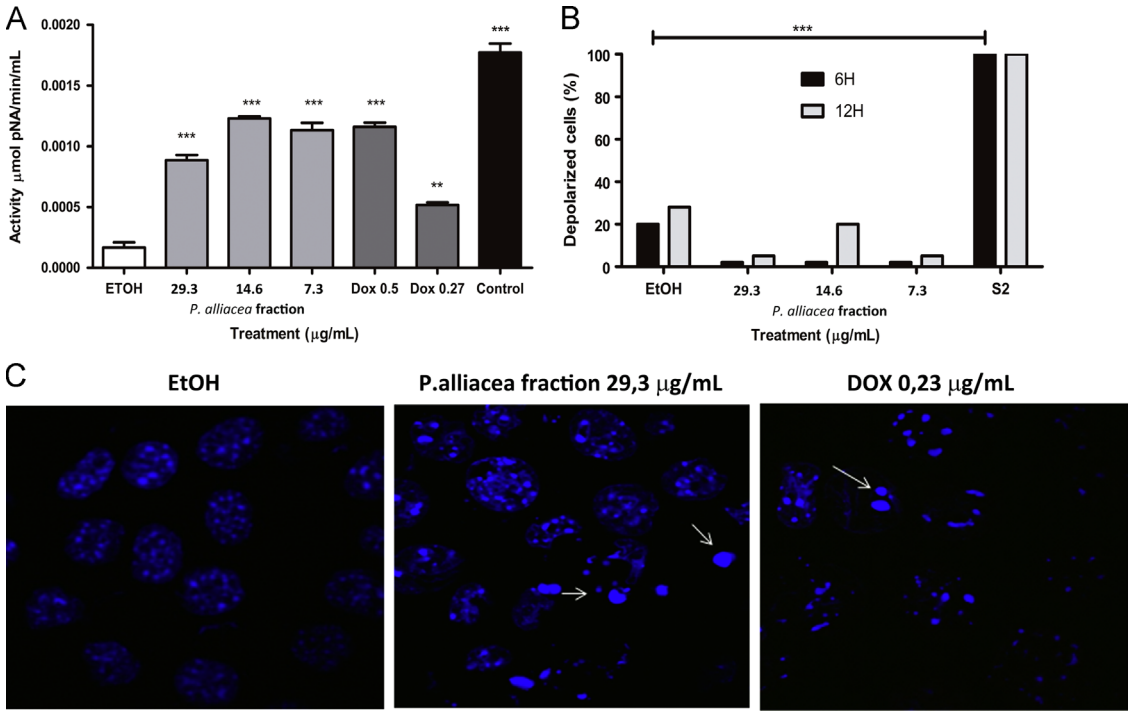

Fig. 3. *Petiveria alliacea* fraction induces apoptosis in 4T1 cells by a mitochondria independent pathway. (A) 4T1 cells (2×10^5) treated with *Petiveria alliacea* fraction, doxorubicin or ethanol (0.02%) for 48 h were ice lysed and caspase 3 activity measured. (B) 3×10^5 cells treated with *Petiveria alliacea* or S2 fraction (6 and 12 h). MMP was measured using JC-1. (C) 3×10^5 cells treated with *Petiveria alliacea* fraction or doxorubicin (48 h) were stained with DAPI and analyzed by confocal microscopy. Arrows show DNA fragmentation.

production. On the contrary, *Petiveria alliacea* fraction decreases glycolytic flux associated with decrease in cell proliferation.

3.5. Primary tumor reversion in BALB/c mice transplanted with 4T1-GFP cells was accomplished after treatment with Petiveria alliacea fraction

First, acute toxicity via I.P for *Petiveria alliacea* fraction was established in BALB/c mice. Different doses of the fraction were inoculated in 4 groups of mice ($n=5$) and after 72 h of treatment the deceased animals were counted and the data analyzed with Probit test (Minitab software). The LD_{50} calculated was 1545 mg/kg and according to Hodge and Sterner toxicity scale the fraction was classified as slightly toxic (Hodge and Sterner, 1943).

Second, 4T1 cells GFP-tagged were injected into mice mammary fat pad on day 0. *Petiveria alliacea* treatment began on day 7, when the primary tumor was palpable. Mice treatment and GFP intensity were measured weekly. After 3 weeks of treatment at the highest dose (182 mg/kg), 60% of the animals showed primary tumor reversion, while the control had the expected primary tumor growth (Fig. 6). Interestingly, at the medium dose (91 mg/kg) all mice showed primary tumor reversion maintained until day 35, when the mice were sacrificed.

4. Discussion

Petiveria alliacea leaves and roots infusions have been widely used in folk medicine for cancer treatment (Chirinos, 1992; Correa and Bernal, 1998; Gupta, 1995). Currently, their biological activity has been associated with cytoskeleton alterations (Rosner et al., 2001) and cell cycle arrest (Urueña et al., 2008). On the other hand biological activities of DTS, a metabolite present in *Petiveria alliacea*, have been extensively studied. Even DTS has been used as a lead compound in trisulfide derivates synthesis such as fluoropacin (bis(4-fluorobenzyl)trisulfide), a microtubule polymerization inhibitor (Xu et al., 2009).

Previous reports have shown that extracts of *Petiveria alliacea* exhibited cytotoxic activity against cancer cell lines (e.g. melanoma, lymphoma and leukemia) (Cifuentes et al., 2009; Pérez-Leal

et al., 2006; Rossi, 1990; Urueña et al., 2008); however, no effect has been detected on breast tumor cell line proliferation (Pérez-Leal et al., 2006; Rossi, 1990). Herein, we showed decrease in proliferation of a murine breast tumor cell line induced by a *Petiveria alliacea* fraction having an IC_{50} near 30 μg/mL.

Breast carcinoma cell line (4T1) has been widely used as an outstanding model for metastatic tumor studies as it spreads in vivo to liver, lung, bone marrow and brain through the hematogenous route (Heppner et al., 2000). Moreover, *Petiveria alliacea* fraction induces 4T1 cells apoptosis evidenced by caspase-3 activation and DNA fragmentation without mitochondria membrane depolarization (Fig. 3), as shown by Urueña et al. (2008) using a leukemia cell line. Furthermore, *Petiveria alliacea* decreases cell colony growth, a test that evaluates cell proliferation and colony forming capacity, after 12 h or 14 days of treatment at a concentration corresponding to 1/100th of the estimated IC_{50}.

Petiveria alliacea fraction induces changes in cellular expression of several glycolytic enzymes pathways (Table 4). Formerly, Christofk et al. assured that a decrease in tumorigenicity correlates with a change in tumor pyruvate kinase expression into a normal isoform, increasing oxygen consumption rate and decreasing lactate production (Christofk et al., 2008). The present study has proven an increase in normal PK isoform expression and reduction in lactate production confirmed by a decrease in LDH-A expression (Fig. 5).

No antitumor activity was observed in albino mice transplanted with sarcoma 180, sarcoma 37, Ehrlich ascites and breast carcinoma treated with ethanol extracts or decoctions from *Petiveria alliacea* leaves according to Cuban Oncology and Radiology Institute in 1976 (Gupta, 1995). On the contrary, here a *Petiveria alliacea* fraction induces breast primary tumor regression in BALB/c mice transplanted with 4T1-GFP cells at 91 and 182 mg/kg (Fig. 6). This apparent controversy could be explained by differences between extraction procedures as well as the origin of plant material. The bio-guided methodology used allowed cytotoxic metabolites enrichment compared to a crude ethanol extract as previously shown for the ethyl acetate derived fraction (Cifuentes, 2010).

4T1 in vivo tumor growth in BALB/c mice exhibits a high glycolytic phenotype producing vast amounts of lactate between days 8 and 15 (Serganova et al., 2011). Herein, we showed a decrease in primary tumor growth after treatment with *Petiveria alliacea* fraction (91 mg/kg) between days 7 and 14. Hence, change in expression of glycolytic enzymes endorsing decrease in glycolytic flux besides apoptosis induction could be accountable for the primary tumor reversion. Also, *Petiveria alliacea* treatment decreases expression in profilin 1, cyclophilin A and transgelin 2, proteins associated with tumor capacity, to invade and proliferate. Moreover, cytokines associated with tumor progression like MIF (over-expressed in breast cancer tissues (Xu et al., 2008)) decrease in expression after *Petiveria alliacea* treatment.

We hypothesized that glycolytic alterations induced by the ethyl acetate *Petiveria alliacea* fraction in 4T1 cells increase carbon flux towards Krebs cycle, boosting an oxidative metabolism and consequently decreasing glycolytic intermediates. Such scenario will diminish the carbon flux for nucleotides and amino acids synthesis needed for high proliferation rates. Therefore, *Petiveria alliacea* antiproliferative activity might be explained by a drop in carbon precursor's availability.

Unpurified extracts contain a mixture of compounds that might generate either pharmacokinetic or pharmacodynamic synergic

Table 4
Petiveria alliacea treatment affects protein expression. Treated 4T1 cells showed an increase (left column) or a decrease (right column) of intracellular proteins expression as compared to proteomic control profile (ethanol).

Intracellular proteins increased in expression	Intracellular proteins decreased in expression
PK isozymes R/L	LDH-A
FBPA	Phosphoglycerate kinase 1
TPI1	Tubulin alpha 1B
GAPDH	Profilin 1
GRP78 (BiP)	Cyclophilin A
Tubulin alpha 8	Transgelin 2
Annexin A2	Macrophage migration inhibitory factor
Heterogeneous nuclear ribonucleo-protein A2/B1 isoform 2	
60S acidic ribosomal protein P2	
Ribosomal protein S5, isoform CRA_a	
60S ribosomal protein L29	

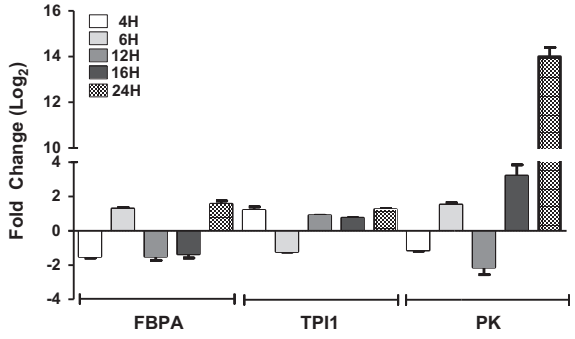

Fig. 4. RT-PCR gene expression in 4T1 cells treated with *Petiveria alliacea* (20 μg/mL). FBPA, TPI1 and PK expression levels were analyzed after 4, 6, 12, 16 and 24 h of treatment. Gene expression level in treated cells was compared with control. Relative gene expression was calculated by the method $2^{-\Delta\Delta CT}$. Data represent the mean of 2 independent experiments.

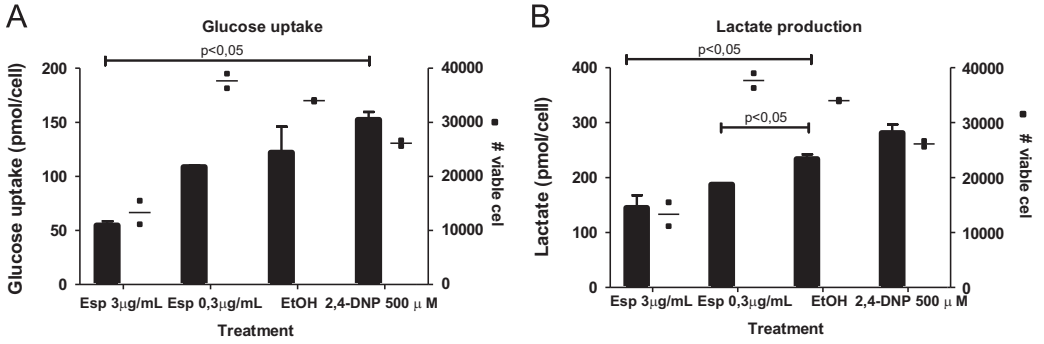

Fig. 5. *Petiveria alliacea* fraction decreases glucose uptake and lactate production in 4T1 cells. The estimation of glucose and lactate concentrations was performed on the cell culture supernatants after 48 h treatment with *Petiveria alliacea* fraction, ethanol or 2,4-DNP. (A) Glucose uptake and (B) lactate production. Data represent the mean ± SD of three replicates for glucose and lactate quantitation and for proliferation assay two replicates.

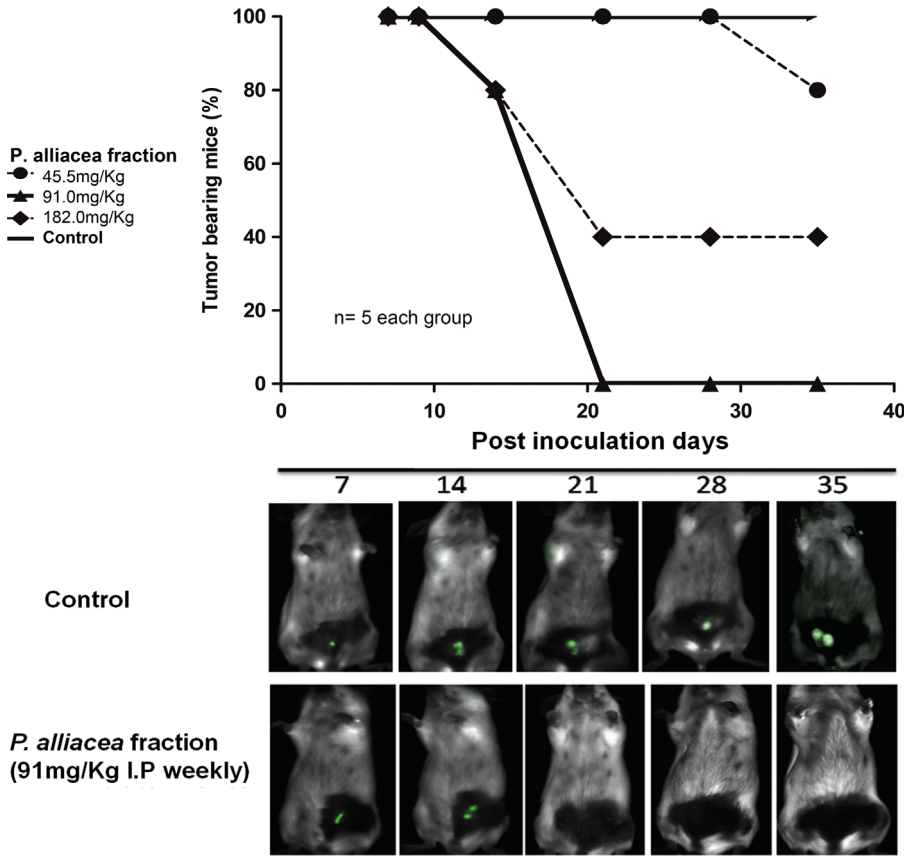

Fig. 6. *Petiveria alliacea* treatment induces primary tumor reversion in BALB/c mice. GFP-tagged 4T1 cells (1×10^4) were injected into mice mammary fat pad on day 0 and treatment began on day 7. Upper panel displays tumor bearing mice percentage during post-inoculation time. Data represent two independent experiments. Lower panel shows progression of fluorescent-tagged tumor growth in control and treated mice. Images were captured weekly with an in vivo imaging system (MaestroTM, Cambridge, MA). Images represent two independent experiments.

effects (Williamson, 2001). Since *Petiveria alliacea* fraction influences glycolytic phenotype, regulates cell cycle and has effect on the cytoskeleton, it is obvious that the potential advantages arise over elimination of tumor cell as compared to a purified molecule acting on a specific target.

Currently, we are evaluating *Petiveria alliacea* activity on oxygen consumption rate, ROS production and fatty acid synthesis to complete the biological scope activity study.

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