

Full Length Research Paper

In vitro* and *in vivo* assessment of genotoxic activity of *Petiveria alliacea

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***Petiveria alliacea* L. (Phytolaccaceae) is an Amazonian shrub used in traditional medicine for many purposes. This study investigated the genotoxicity of the hydroalcoholic extract of *P. alliacea* (EHPa). In addition, we conducted pharmacognostic characterization and phytochemical investigation. Phytochemical screening and thin layer chromatography (TLC) were used to determine the chemical composition of the extract. Genotoxicity was evaluated using an *in vitro* comet assay in human lymphocytes (50, 100, and 250 mg/ml) and an *in vivo* micronucleus assay in mice orally treated with the extract (50, 100, and 250 mg/kg b.w., p.o.). The phytochemical screening identified the metabolites reducing sugars, alkaloids and saponins. TLC analyses identified organosulfur compounds in EHPa. Comet assay data showed that EHPa induced exacerbated DNA damage at 100 mg/ml treatments (EHPa 100 mg/ml: TL 219.13 ± 48.38 mm, % DNA-t 59.33 ± 18.98%, TM 134.99 ± 61.34, OM 87.48 ± 32.98) as compared with negative control (Roswell Park Memorial Institute (RPMI): TL 37.79 ± 24.79 mm, % DNA-t 9.73 ± 6.56%, TM 4.86 ± 5.79, OM 6.90 ± 5.80; p<0.01). These effects were similar to positive control (hydrogen peroxide). The micronucleus assay, however, showed that EHPa induce slight genotoxicity *in vivo*. However, EHPa induced an exacerbated DNA damage *in vitro*, but this effect was slight in the animal model. These *in vitro* effects may be related to the chemical composition of the extract.**

Key words: *Petiveria alliacea*, genetic toxicity, DNA damage, comet assay, micronucleus assay.

INTRODUCTION

Petiveria alliacea L. (Phytolaccaceae) is an Amazonian indigenous perennial shrub, slender and erect, which reaches about 1 m in height (Rocha et al., 2006).

Popularly, the plant is known as herb to “tame the master”, mucuracaá, guiné, pipi, tipi, anamu, apacin, embayayendo, ouoembo, among others (Camargo, 2007).

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This variety of names is directly related to its large geographical distribution that includes countries in tropical America, Africa, Europe, and the Caribbean, West Indies, and India (Alonso, 2007).

P. alliacea has been used for many purposes in traditional medicine. A decoction of the roots is used in the Amazonian region as an abortifacient, antispasmodic, analgesic, sudorific, and antirheumatic (Corrêa, 1984; Elisabetsky and Castilhos, 1990). An ethnobotanical survey pointed out that the plant has been widely used for the treatment of respiratory diseases, including asthma, bronchitis, chest congestion, and common cold (Vandebroek et al., 2010). Several pharmacological studies have demonstrated the therapeutic potential of *P. alliacea* as antimicrobial, anticancer and immunostimulant agent (Guedes et al., 2009; Mata-Greenwood et al., 2001; Santander et al., 2012). Regarding phytochemistry, *P. alliacea* possesses a broad spectrum of compounds in its constitution, including flavonoids and terpenoids (Delle Monache and Suarez, 1992; Delle Monache et al., 1996; Neves et al., 2011), as well as higher amounts of organosulfur compounds that justifies its pungent smell of garlic (Kubec and Musah, 2001; Kubec et al., 2002, 2010). Among the sulfur-containing compounds present in *P. alliacea*, previous investigations have highlighted the polysulfides and thiosulfonates as responsible for several biological activities shown in the extracts (Benevides et al., 2001; Cifuentes et al., 2009; Kubec and Musah, 2001; Kubec et al., 2002, 2010; Rosado-Aguilar et al., 2010; Uruëña et al., 2008).

The DNA of all living organisms is constantly exposed to injury. Genomic and cellular integrity is maintained by effective maintenance and repair mechanisms. Despite the existence of such pathways, some substances found in medicinal plants and herbal drugs can damage the genetic material and provide genotoxic stress triggering cell aging and carcinogenic processes (Abdelmigid, 2013). International organizations, such as the International Committee on Harmonization (ICH), have recommended the investigation of the potential DNA damaging effects of medicinal plants, through *in vitro* and *in vivo* experimental models (ICH, 2008). In this regard, these *in vitro* and *in vivo* models in the field of genetic toxicology can identify and measure the DNA damage, including frequency of DNA adducts, DNA strand break (single or double), DNA cross-linking, mutations or chromosomal aberrations (Abdelmigid, 2013).

Although *P. alliacea* possesses great pharmacological potential, some studies have pointed out its genotoxic hazards. In this sense, Hoyos et al. (1992) have demonstrated that ethanol extract of *P. alliacea* induces DNA damage evaluated by sister-chromatid exchange assay in *in vitro* and *in vivo* models, and also Soares et al. (2014) reported that different aqueous extracts of the plant caused single- and double-strand breaks in plasmid DNA from an *Escherichia coli* strain. However, both investigations evaluated the genotoxicity of low

concentrations of *P. alliacea* extracts in nonhuman models.

In this spite, this study was conducted to predict the potential genotoxic hazards of high concentrations of the hydroalcoholic extract of *P. alliacea* (EHPa) through *in vitro* and *in vivo* experimental models. In addition, the pharmacognostic parameters of the dry powder of the aerial parts of *P. alliacea* and the phytochemical composition of EHPa was examined.

MATERIALS AND METHODS

Chemicals and reagents

Ethanol, methanol, dimethyl sulfoxide (DMSO), RPMI-1640, Triton X-100, TRIS buffer, sodium hydroxide, sodium chloride (NaCl), ethylenediaminetetraacetic acid (EDTA), trypan blue dye, hydrogen peroxide (H₂O₂), ethidium bromide, low melting-point agarose, and normal melting point agarose (Sigma Chemical Com., St. Louis, MO, USA), fetal bovine serum (Gibco Life Technologies, Lofer, Austria), phytohemagglutinin (Life Technologies, Carlsbad, CA, USA), and cyclophosphamide (Asta Medica, Frankfurt, Germany) were used for the purpose of this study.

Plant

The aerial parts of *P. alliacea* (stem, leaves, and flowers) were collected in April, 2011 at Acará, Pará, Brazil (S 01°29'09.0/W 48°17'94.8). A voucher specimen was identified by the Botanist consultant Mário Augusto G. Jardim and was deposited in the herbarium of the Museu Paraense Emílio Goeldi (MPEG) under registry MG 94354. After collection, the plant material was first dried at room temperature (25°C/24 h) and subsequently, was dried in a forced-air oven to 40°C for 15 h. *P. alliacea* dry aerial parts were then powdered in a knife mill (Willey, Marconi MA 680 model).

Pharmacognostic characterization

The pharmacognostic characterization of *P. alliacea* was conducted from its aerial parts and encompassed the following determinations: granulometric analysis and mean particle diameter, loss on drying (oven/infrared moisture balance), total ash content, foaming index, and content of extractable matter (Allen et al., 2000; Brasil, 2010; World Health Organization 2011).

Extract preparation

The hydroalcoholic extract was chosen because previous studies of our group have demonstrated its potential effects on central nervous system when comparing with traditionally preparations (Andrade et al., 2012; Silva et al., 2015). Crude EHPa was prepared through the maceration method. Thus, plant drug (930 g) was extracted with 70% ethanol (v/v) for 7 days. The hydroalcoholic extract was filtered and evaporated under vacuum (40°C, 167 mbar, and 120 rpm) to provide 92.07 g of EHPa (1 g of extract equivalent to 10.1 g of dried aerial parts).

Preliminary phytochemical screening

The phytochemical screening of EHPa was conducted according to

Table 1. Reactions, reagents, and positive controls used in preliminary phytochemical screening of EHPa according to Barbosa et al. (2001).

Metabolites	Reagent / Reaction	Positive control
Reducing sugars	Formation of a red precipitate with Fehling's solutions	<i>Eupatorium ayapana</i>
Alkaloids	Precipitate formation after addition of Bouchardat's, Dragendorff's, Mayer's or Bertrand's reagents	<i>Psychotria ipecacuanha</i>
Deposides and depsidones	Chromogenic reaction after addition of ferric chloride (1%) to ether extract residue resuspended in methanol	<i>Portulaca pilosa</i>
Sesquiterpene lactones	Chromogenic reaction after addition of ferric chloride (1%) to an acidified solution of extract diluted in methanol plus hydroxylamine hydrochloride (10%) and KOH (10%)	<i>Achryrocline satireioides</i>
Coumarins	Blue fluorescence after addition of NaOH (1 N) drops to ether extract spotted in filter paper	<i>Eupatorium ayapana</i>
Flavonoids	Shinoda reaction	<i>Citrus aurantium</i>
Polysaccharides	Lugol's reagent	<i>Zea mays</i>
Tannins	Precipitate formation after addition of 1% ferric chloride to aqueous extract	<i>Stryphnodendron adstringens</i>

Barbosa et al. (2001) for the identification of the following metabolites: reducing sugars, alkaloids, deposides and depsidones, sesquiterpene lactones, coumarins, flavonoids, polysaccharides, and tannins. The respective reactions and reagents for identification of the metabolites mentioned, as well as vegetable drugs used as positive controls, are shown in Table 1

Thin-layer chromatography of EHPa

Thin-layer chromatography (TLC) was performed on a precoated 20 x 20 cm silica gel 60 F₂₅₄ plate (Merck, Germany) for the identification of sulfur compounds in EHPa as described by Wagner and Bladt (2001). Briefly, aliquots of EHPa dissolved in dichloromethane (10 mg/ml) were spotted on the plate. TLC analysis was developed using toluene-ethyl acetate (100:30, v/v) as mobile phase. As positive controls, the organic phase of aqueous extracts of *Allium sativum* and *Allium cepa* fresh bulbs extracted with dichloromethane were used. After chromatography, the plate was sprayed with 10 ml of vanillin-glacial acid reagent (VGA) and heated at 110°C for 3 min. Then the spots were observed under visible light. The retention factor values (R_f) of each spot were calculated using the equation: R_f = distance the spot moved above the origin/distance the solvent front moved above the origin.

In vitro comet assay

Cell culture and sample preparation for comet assay

Peripheral human blood samples were obtained from healthy volunteers (non-smoking and non-drinking males and females between 20 and 45 years old) after approval by the Research Ethics Committee of UFPA (CAEE 0154.0.073.000-11, Registry 165/11 CEP-ICS/UFPA). The blood samples were obtained as previously described by Albuquerque et al. (2015) for lymphocyte culture. In brief, 0.3 ml of venous blood were added to 4 ml of RPMI-1640 medium containing 20% of Fetal bovine solution (FBS)

and 50 µg/ml phytohemagglutinin (PHA) to stimulate lymphocyte proliferation. The mixture was then incubated under humidified environment at 37°C and 5% CO₂ for 24 h. The lymphocytes cultures (4.5 ml) were then treated with different concentrations of EHPa (50, 100, and 250 mg/ml; 0.4 ml) for 3 h at 37°C. As a negative and positive control, the lymphocytes were treated with RPMI-1640 medium and H₂O₂ at 35%, respectively, for 30 min at 37°C. Afterwards, cell viability was assessed with trypan blue dye exclusion test. Only cell samples whose viability was over 90% were measured by the comet assay (single cell gel electrophoresis).

DNA damage using comet assay

The comet assay was performed as described by Singh et al. (1988) and modified by Anderson et al. (1994). Treated lymphocytes, as already described were mixed with 0.5% low melting-point agarose, and the mixture (100 µl) was pipetted onto pre-coated slides with 1.5% normal melting point agarose. The drop containing the cells was placed under a glass cover slip (24 mm x 24 mm) and left at 4°C for 5 min. The cover slips were gently removed and the slides were treated with a lysis solution (2.5 M NaCl, 100 mM EDTA, 100 mM TRIS, 1% Triton X-100 and 10% DMSO, pH 10.2) for 24 h at 4°C. The slides were immersed in freshly prepared electrophoresis alkaline buffer (2.5 M NaOH, 100 mM EDTA, pH>13) and electrophoresis was performed using an electric field of 23 V/cm for 20 min. After electrophoresis, the slides were neutralized with distilled water for 5 min, fixed with ethanol for 3 min and air-dried. The microscope slides were then stained with ethidium bromide (20 µg/ml), and the cells were visualized and photographed under a fluorescence microscope (ZEISS AxioCam HRC) using a 510 to 560 nm emission/barrier filter at 400x magnification. A total number of 100 cell images (50 cells/slide) were collected randomly for each treatment and analyzed using Comet Score 1.6 (Tritek) software. The parameters of DNA damage analyzed included tail length (TL), percentage of DNA in the tail (% tail DNA), tail moment (TM), and Olive moment (OM). To avoid interference of additional DNA damage this experiment was conducted in a dark room and performed in duplicate.

Table 2. Pharmacognostic characterization of the dry powder of aerial parts of *P. alliacea*.

Parameter	Mean \pm SD
D_{50} (mm)	0.276 \pm 0.0012
Foaming index	133.95 \pm 12.6219
Loss on drying (%)	
Oven	9.67 \pm 0.2887
Infratest	8.93 \pm 0.1155
Total ash content (%)	9.43 \pm 0.2309
Content of extractable matter (%)	0.75 \pm 2.8868

In vivo micronucleus assays

Experimental animals

All experimental protocols were conducted in accordance with the National Institute of Health (Institute of Laboratory Animal Resources) for animal research and were approved by the Committee for Animal Care and Use of the Universidade Federal do Espírito Santo (UFES, Approval number: 074/2011). Eight-week-old adult male Swiss mice (*Mus musculus*) weighing between 25 and 35 g were obtained from the animal colony at UFES. The animals were housed in polypropylene cages, maintained under *ad libitum* food and water diet, and controlled laboratory conditions (22 to 25°C) with an artificial 12 h light/dark cycle.

Micronucleus assay

The micronucleus assay protocol was modified from Schmid (1975). Briefly, 30 mice were separated into five groups of six mice each. Thus, three groups were administered by oral gavage with EHPa at 50, 100, and 250 mg/kg b.w., and two groups received physiological saline (0.9% NaCl) and cyclophosphamide (50 mg/kg b.w., i.p.). After 24 h the animals were euthanized by cervical dislocation, and their femurs were dissected immediately for bone marrow extraction. The bone marrow samples were homogenized twice with 1 ml of fetal bovine serum (FBS) and centrifuged for 10 min at 1000 rpm. The supernatant was discarded, and the pellet was re-suspended in 0.5 ml of FBS. The cells were dropped on microscope slides and air-dried. Afterward, the slides were fixed with methanol and stained in two different concentrations of Leishman stain to allow visual differentiation between immature polychromatic erythrocytes (PCE) and mature normochromatic erythrocytes (NCE): (a) Leishman at 100% for 3 min., and (b) Leishman-distilled water (1:6, v/v) for 15 min (Krishna and Hayashi, 2000). The microscope slides were air-dried and then analyzed under light microscopy (Olympus, CX41) at a magnification of 1000x. To evaluate the genotoxicity of EHPa, the ratio of micronucleated polychromatic erythrocytes (MNPCE) per 2000 PCE was considered for each treatment and animal. To evaluate the cytotoxic effects of EHPa, 200 erythrocytes (PCE and NCE) were counted for the PCE/(PCE + NCE) ratio following Hayashi et al. (2000).

Data analysis

All results are expressed as mean \pm SD values. For the *in vitro* comet assay, statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey *post hoc* test for

multiple comparisons. The data was analyzed by Graph-Pad Prism software version 5.0 (San Diego, USA). For the *in vivo* micronucleus assay, normality test followed by Kruskal-Wallis test for nonparametric data were performed using Assisat software version 7.6 beta (UFPB, Brazil). In all experiments, differences were considered statistically significant if $p < 0.01$.

RESULTS

Pharmacognostic characterization

Table 2 summarizes the results obtained from pharmacognostic characterization of the dry powder of the aerial parts of *P. alliacea*. As shown, the powder possessed a mean particle diameter (D_{50}) of 0.275 mm. The powder was considered as moderately coarse according to the classification of pharmacopeia. All values for moisture content and total ash content, using the Brazilian Pharmacopoeia (Brazil, 2010) techniques, were within the limits established for plant drugs. The foaming index and the content of extractable matter corresponded to the values for qualitative assays of the plant drug.

Phytochemical screening of EHPa

Preliminary phytochemical screening of the EHPa was regarded as positive for the presence of metabolites, reducing sugars, alkaloids, and saponins.

Thin-layer chromatography of EHPa

TLC of the organic phase of *A. sativum* and *A. cepa* extracts showed the development of organosulfur compounds spots as previously reported by Wagner and Bladt (2001). TLC chromatogram of EHPa revealed the presence of three blue or blue-gray colored majority spots having R_f values of 0.55, 0.87, and 0.95, after spraying with VGA reagent. These spots were similar to the positive controls used and indicate the presence of

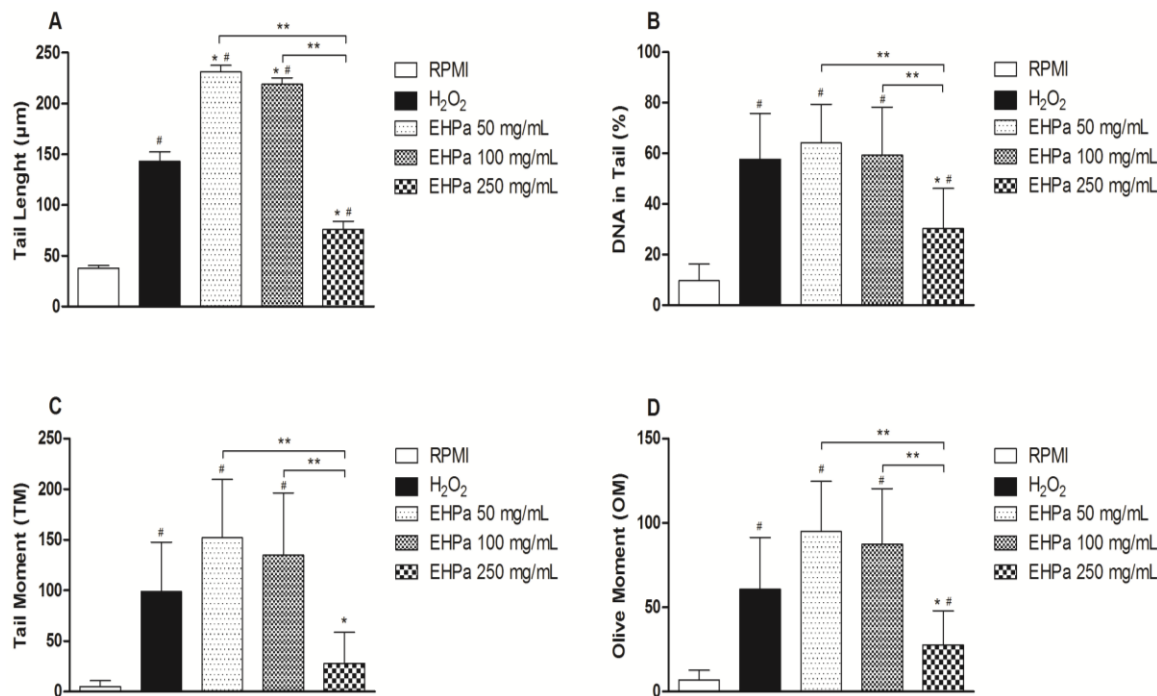


Figure 1. *In vitro* genotoxic effects of *P. alliacea*. The parameters tail length (A), percentage of DNA in the tail (B), tail moment (C) and Olive moment (D) were used as markers for DNA damage in human lymphocytes through comet assay. All values are represented as mean \pm SD. # $p < 0.01$ compared to the negative control (RPMI); * $p < 0.01$ compared to the positive control group (H₂O₂); ** $p < 0.01$ compared to greater EHPa concentration.

sulfur-compounds in EHPa (for example, thiosulfinates).

In vitro comet assay

The comet assay was performed for *in vitro* evaluation of some potential DNA damage of EHPa on human lymphocytes. According to the parameters for DNA damage analyzed (Figure 1), EHPa exhibited a dual effect on DNA. Treatment with EHPa at the concentrations of 50 and 100 mg/ml induced significant DNA damage when compared to the negative control (EHPa 50 mg/ml: TL 231.25 ± 48.56 mm, % tail DNA 64.12 ± 15.26 , TM 152.09 ± 57.63 , OM 94.93 ± 29.72 ; EHPa 100 mg/ml: TL 219.13 ± 48.38 mm, % tail DNA 59.33 ± 18.98 , TM 134.99 ± 61.34 , OM 87.48 ± 32.98 ; negative control: TL 37.79 ± 24.79 mm, % tail DNA 9.73 ± 6.56 , TM 4.86 ± 5.79 , OM 6.90 ± 5.80 ; $p < 0.0001$). These values were statistically higher than DNA damage caused by H₂O₂ for TL, TM, and OM parameters (H₂O₂: TL 143.23 ± 89.51 mm, TM 96.54 ± 51.35 , OM 60.67 ± 30.67 ; $p < 0.0001$). On the other hand, the highest concentration evaluated of EHPa (250 mg/mL) exhibited minor effects on DNA for TL, % tail DNA, and OM parameters when comparing to negative control (EHPa 250 mg/mL: TL 76.24 ± 56.77 mm, $p = 0.0017$; % tail DNA 24.01 ± 19.26 , $p = 0.0001$; OM 23.03 ± 20.67 , $p = 0.0021$).

In vivo micronucleus assay

The micronucleus assay was carried out in rodents for assessment of genotoxic and cytotoxic activities of EHPa. Table 3 summarizes the micronucleated polychromatic erythrocytes (MNPCE) frequencies and PCE/(PCE + NCE) ratio - ratio between immature polychromatic erythrocytes (PCE) and immature polychromatic erythrocytes (PCE) plus mature normochromatic erythrocytes (NCE); ratio in bone marrow of mice treated with different doses of EHPa. As for the genotoxic parameter, our results demonstrated that EHPa at doses of 50 and 250 mg/ml induced changes in MNPCE frequency compared to negative control ($p < 0.01$); however, these values were at least threefold lower than the positive control ($p < 0.01$). With regards to the cytotoxicity parameter, EHPa did not decrease significantly the PCE/(PCE + NCE) ratio when comparing mice treated with the negative control for all tested doses ($p > 0.01$). As observed with cyclophosphamide treatment, the PCE/(PCE + NCE) ratio was statistically lower than negative control and EHPa 50 mg/ml ($p < 0.01$), but not significant, compared to the EHPa 100 mg/ml and EHPa 250 mg/ml treatment groups.

DISCUSSION

Petiveria alliacea is an important Amazonian herb with

Table 3. Micronucleated polychromatic erythrocytes (MNPCE) frequencies and polychromatic/normochromatic erythrocytes ratio (PCE/[PCE + NCE]) in mouse bone marrow cells treated with oral doses of EHPa and controls.

Treatment (mg/kg b.w.)	MN/2000 PCE	PCE/(PCE + NCE)
EHPa 50	10.00 ± 2.04 ^b	0.49 ± 0.04 ^b
EHPa 100	8.75 ± 2.60 ^{ab}	0.45 ± 0.04 ^{ab}
EHPa 250	9.58 ± 2.10 ^b	0.43 ± 0.14 ^{ab}
Positive control	32.98 ± 4.13 ^c	0.41 ± 0.05 ^a
Negative control	5.50 ± 1.97 ^a	0.48 ± 0.03 ^b

Values are expressed as mean ± SD. Same letters in the same column – $p > 0.01$; different letters in the same column – $p < 0.01$. Negative control: saline; positive control: cyclophosphamide.

several pharmacological properties already reported. Recent investigations of our group have demonstrated that ethanolic preparations of *P. alliacea* possess anxiolytic, antidepressant and mnemonic effects upon central nervous system (Andrade et al., 2012; Silva et al., 2015). Although previous studies have demonstrated that *P. alliacea* induces genotoxic hazards in low concentrations, there is a lack of information about the effects of high concentrations of ethanolic preparations in both *in vitro* and *in vivo* models.

As alluded to earlier, we performed some pharmacognostic tests aiming to determine the quality control parameters of the powder obtained from *P. alliacea*. With regards to pharmacognostic characterization, powdered dry aerial parts of *P. alliacea* showed quality control parameters which were within the limits set forth in Brazilian Pharmacopoeia. These results are also similar to those observed by Audi et al. (2001) for loss on drying and content of extractable matter tests.

Phytochemical screening encompasses a set of qualitative tests for the identification of metabolites present in medicinal plants preparations. In this study EHPa showed positive results for the presence of reducing sugars, alkaloids, and saponins. Carillo et al. (1997) also identified the presence of alkaloids and saponins in ethanol extract of *P. alliacea*, differently however, we did not identify tannins, flavonoids, and sesquiterpene lactones. On the other hand, Villar et al. (1997) did not identify saponins and alkaloids in *P. alliacea* extracts.

Thin layer chromatography was also performed in order to identify the presence of organosulfur compounds in EHPa. TLC chromatograms demonstrated the presence of organosulfur compounds similar to Kubec and Musah (2001) with a spot of $R_f = 0.55$. Other sulfur compound spots identified in the chromatogram may belong to the class of thiosulfinates and other polysulfides, when compared to positive controls *A. cepa* and *A. sativum* spots (Wagner and Bladt, 2001). In this regard, several studies have reported the identification of organosulfur compounds in EHPa, including polysulfides (for example, dibenzyl trisulfide) and thiosulfinates (Benevides et al.,

2001; Hernández et al., 2014; Kubec et al., 2002, 2010; Rosado-Aguilar et al., 2010; Urueña et al., 2008; Cifuentes et al., 2009).

Other phytochemical studies have revealed the presence of different compounds in *P. alliacea*, such as the flavonoids engeletin, astilbin, myricetin, leridal, leridol, and petiveral (Delle Monache and Suarez, 1992; Delle Monache et al., 1996; Hernández et al., 2014); terpenoids; and benzenoids (Neves et al., 2011). The non-identification of some metabolites previously reported in *P. alliacea* does not characterize their absence in EHPa because several factors, such as environmental temperature, time of collection, height, UV radiation, storage, drying, and extraction, influence the constancy of metabolite content in plants (Gobbo-Neto and Lopes, 2007; Sahoo et al., 2010).

In addition to pharmacognostic and phytochemical investigations, the evaluation of toxicity of medicinal plants and their derivatives is an important step in predicting potential hazards to human health (Abdelmigid, 2013; Netto et al., 2006). In the acute toxicity assay (data not shown), we did not observe any death in animals treated with EHPa even at the maximum dose administered (1000 mg/kg b.w.). Hernández et al. (2014) evaluating the acute toxicity of a *P. alliacea* fraction (doses ranged between 5 to 2000 mg/kg b.w., i.p.), calculated an LD₅₀ of 1545 mg/kg b.w.

The possible genotoxic effects of *P. alliacea* was evaluated through the *in vitro* comet assay in the present study; through the *in vitro* comet assay it was observed that EHPa significantly induced DNA damage of human lymphocytes. However, the EHPa induced high DNA damage in low concentrations (50 to 100 mg/ml), while the greatest concentration (250 mg/ml) induced a small DNA damage. This different effect could be due to the synergistic and antagonistic relationship between some substances in the extract, like thiosulfinates (Xiao and Parkin, 2002), that can lead to an oxidative stress imbalance and can also activate DNA repair pathways, reducing the genotoxic effects (Cooke et al., 2003). In this context, Hageman et al. (1997) reported that the water extract of raw garlic and two organosulfur

compounds, diallyl sulfide and S-allylcysteine, were able to inhibit the DNA adduct formation induced by benzo[a]pyrene by antioxidant mechanisms, such as increase of the glutathione S-transferase activity and reduction of ROS. In addition, other studies reported that another antioxidant compound, such as phenolic compounds, mainly caffeoylquinic acid derivatives, from root extracts of *Rhaponticum carthamoides* may stimulate in CHO cells to repair oxidative DNA damage and protect DNA by increased antioxidant gene and protein expression levels (SOD2 and CAT) regulating intracellular antioxidant capacity (Skala et al., 2016).

In this regards, reactive oxygen species (ROS) and free radicals generation may play a pivotal role in DNA damage (Cadet et al., 1999; Del-Rio et al., 2005; Subhashinee et al., 2005). Previous studies have demonstrated that *P. alliacea* exerted both pro-oxidant and antioxidant effects on *in vitro* and *in vivo* models (Desmarchelier et al., 1997; Andrade et al., 2012). Desmarchelier et al. (1997) observed that preparations obtained from aerial parts of *P. alliacea* (1 mg/ml) presented slight anti-oxidant activity with lowest TRAP values (aqueous and dichloromethane extracts: <100 μ M Trolox; methanol extract: 275 μ M Trolox). On the other hand, our group also reported that animals orally treated with 900 mg/kg of *P. alliacea* L. hydroalcoholic extract exhibited pro-oxidant status (Andrade et al., 2012). In this study, it was shown that the extract was able to increase Trolox equivalent antioxidant capacity (TEAC), nitric oxide (NO) and malondialdehyde (MDA) levels, as well as increase methemoglobin formation in the human plasma. These data suggest that *P. alliacea* extract has pro-oxidant action and can lead to cell death (cytotoxicity) *in vivo*, because the increase of MDA is used as a marker of lipid peroxidation and cell damage caused by the action of reactive species in the body, as well as oxidative processes in the membrane (Janero, 1990; Del-Rio et al., 2005). In addition, *in vitro* and *in vivo* studies have reported that the MDA is able to interact with nucleic acid bases, as deoxyguanosine and deoxyadenosine, to form several different adducts, which may be mutagenic and these were quantified in several human tissues (Marnett, 1999a; Marnett, 1999b; Lykkesfeldt, 2007). Thus, high levels of reactive species and MDA formation induced by *P. alliacea* may be one of the mechanisms that lead to the high EHPa-induced DNA damage *in vitro* observed in this study.

In order to confirm the results from *in vitro* comet assay the *in vivo* micronucleus assay was performed using mouse bone marrow. In this study, only the treatment with EHPa at the concentrations of 50 and 250 mg/kg b.w. induced small changes in the frequency of MNPCE compared to negative control. In this regard, the micronucleus assay has been employed for genotoxicity and mutagenicity detection of substances that can cause cytogenetic damage, thus the increased MNPCE frequency can be related to cancer, though the MN can

be a target of carcinogenesis (Bonassi et al., 2006). On the other hand, the comet assay was used to detect genotoxic effect of compound by measuring its DNA damage at single cell (Hartmann et al., 2003). Therefore, the present data showed that *P. alliacea* induces a slight mutagenic effect *in vivo*, but a high genotoxic effect that was detected by Comet assay. Thus, the data suggest that *P. alliacea* is associated with high genotoxicity, but a low risk for carcinogenesis, as described by Lee et al., (2015). These different effects detected by *in vitro* (comet assay) and *in vivo* (micronucleus assay) assays, also was reported by other studies, as Schauss et al. (2015) that showed that the Dahurian larch tree derived lavitol led to DNA damage in marrow cells, liver and blood, but did not increase the number of binucleate cells containing micronuclei. These effects can be explained by the concentrations/doses used in both assays. As well established, herbal derivatives, including plant extracts, possess poor lipid solubility or improper molecular size, which results in poor absorption and consequently poor bioavailability (Kesarwani and Gupta, 2013). In this regard, the activity of some enzymes on *P. alliacea* compounds could decrease their bioavailability on tissues, as well as the time of administration of the plant extract, and as mentioned before, the possible synergistic and antagonistic interactions between the compounds presents in the EHPa and their effects on oxidative balance. These findings may suggest that the oral administration route can allow some substances present in the plant extract to suffer from drug biotransforming reactions involving hepatic enzymes, which can facilitate their excretion and consequently the decrease of plasmatic concentrations. In spite of this, our results suggest that at least a concentration of 50 mg in plasma level could trigger DNA damage, as observed through comet assay.

Furthermore, other factors that justify the differences obtained in the *in vivo* versus *in vitro* experiments cannot be excluded. These factors include the DNA repair mechanisms present in biological environment, namely base excision repair, nucleotide excision repair, mismatch repair, direct repair, and recombination repair, which could repair some DNA damage induced by EHPa in animal model (Ramos et al., 2011). Furthermore, it was hypothesized that the DNA damage induced by EHPa can also lead to cell death because it is already well-reported that *P. alliacea* extracts exhibited cytotoxic activity against normal fibroblasts and peripheral blood mononuclear cells (Urueña et al., 2008), and several tumor cell lines, such as melanoma, leukemia, and lymphoma (Pérez-Leal et al., 2006; Rossi, 1990). Regarding cellular toxicity, our data showed that none of the doses led to cytotoxic effects. However, there was a tendency to increase the cytotoxicity with increasing concentration of extract, as seen in Table 3. Urueña et al. (2008) observed that an aerial parts fraction of *P. alliacea* exhibited low toxicity to normal fibroblasts and peripheral

blood mononuclear cells in the presence or absence of phytohemagglutinin stimulation (IC₅₀ of 440, 151, and 121 µg/ml, respectively). Differing from our results, previous studies using the crustaceans *Artemia salina* and *Thamnocephalus platyurus* have demonstrated cytotoxic activity in *P. alliacea* extracts (Desmarchelier et al., 1996; Berger et al., 1998; Mayorga et al., 2010). These differences could be due to the different variables involved in the preparation of *P. alliacea* extracts (that is, plant part, period of collection, nature of solvent, method of extraction, and time of extraction).

In this context, Hernández et al. (2014) also reported other mechanisms by which the *P. alliacea* induced 4T1 cells apoptosis, such as caspase-3 activation and DNA fragmentation without mitochondrial membrane depolarization, this was also shown by Uruña et al. (2008) using a leukemia cell line. Accordingly, Hartmann et al. (2003) and Azqueta and Collins (2013) also reported that the cytotoxicity is associated with increased levels of DNA damage, which leads to the generation of false positive results. In addition, a previous study showed that a *P. alliacea* ethanol extract was able to induce sister-chromatid exchanges in human lymphocytes at the final concentration of 100 µg/ml, as compared with the positive (N-ethyl-N-nitrosourea) and saline control groups (Hoyos et al., 1992).

Therefore, the variation in these factors may yield extracts with different chemical composition which could explain the different pharmacological profiles found in the cytotoxic evaluation. The susceptibility of each cell model employed in these studies may also explain these differences.

Conclusion

Our data suggest that *P. alliacea* induces DNA damage *in vitro*, but this effect was slight in an *in vivo* model. The different effect of the EHPa in *in vitro* model may be related to the chemical composition of the extract, which includes thiosulfinates compounds. Further investigations on the mechanism of the DNA damage induced by *P. alliacea* are required to understand if it could trigger deleterious effects, such as an induction of mutations and carcinogenic processes.

Conflict of interests

The authors have not declared any conflict of interest

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REFERENCES

- Abdelmigid HM (2013). New trends in genotoxicity testing of herbal medicinal plants. In: Gowder S (Ed.). *New Insights into Toxicity and Drug Testing*. InTech. pp. 89-120.
- Albuquerque RV, Malcher NS, Amado LL, Coleman MD, Dos Santos DC, Borges RS, Valente SA, Valente VC, Monteiro MC (2015). In Vitro Protective Effect and Antioxidant Mechanism of Resveratrol Induced by Dapsone Hydroxylamine in Human Cells. *PLoS One* 10(8):e0134768.
- Allen LV, Popovich NC, Ansel HC (2000). Pós e grânulos. In: Allen LV, Popovich NC, Ansel HC (Eds.). *Farmacotécnica, formas farmacêuticas e sistemas de liberação de fármacos*, ninth ed. Porto Alegre: Artmed. pp. 187-228.
- Alonso J (2007). *Tratado de fitofármacos y nutracéuticos*. Rosário: Corpus Editorial y Distribuidora. pp. 828-832.
- Anderson D, Yu T-W, Phillips BJ, Schmezer P (1994). The effect of various antioxidants and other modifying agents on oxygen-radical-generated DNA damage in human lymphocytes in the Comet assay. *Mutat. Res.* 307(1):261-271.
- Andrade TM, Melo AS, Dias RGC, Varela ELP, Oliveira FR, Vieira JLF, Andrade MA, Baetas AC, Monteiro MC, Maia CSF (2012). Potential behavioral and pro-oxidant effects of *Petiveria alliacea* L. extract in adult rats. *J Ethnopharmacol.* 143(3):604-610.
- Audi EA, Vieira de Campos, EJ, Rufino M, Garcia Cortez D, Bersani-Amado CA, Lira Soares LA, Ros Petrovick P, Palazzo de Mello JC (2001). *Petiveria alliacea* L.: Plant drug quality control, hydroalcoholic extract standardization and pharmacological assay of lyophilized extract. *Lat Am. J. Pharm.* 20(3):225-232.
- Azqueta A, Collins AR (2013). The essential comet assay: a comprehensive guide to measuring DNA damage and repair. *Arch Toxicol.* 87(6):949-968.
- Barbosa WLR, Quignard E, Tavares ICC, Pinto LN, Oliveira FQ, Oliveira RM (2001). Manual para análise fitoquímica e cromatográfica de extratos vegetais. *Rev. Cient. UFGA* 4:1-19.
- Benevides PJC, Young MCM, Giesbrecht AM, Roque NF, Bolzani VS (2001). Antifungal polysulphides from *Petiveria alliacea* L. *Phytochemistry* 57(5):743-747.
- Berger I, Barrientos AC, Cáceres A, Hernández M, Rastrelli L, Passreiter CM, Kubelka W (1998). Plants used in Guatemala for the treatment of protozoal infections II. Activity of extracts and fractions of five Guatemalan plants against *Trypanosoma cruzi*. *J. Ethnopharmacol.* 62(2):107-115.
- Bonassi S, Znaor A, Ceppi M, Lando C, Chang WP, Holland N, Kirsch-Volders M, Zeiger E, Ban S, Barale R, Bigatti MP, Bolognesi C, Cebulska-Wasilewska A, Fabianova E, Fucic A, Hagmar L, Joksic G, Martelli A, Migliore L, Mirkova E, Scarfi MR, Zijno A, Norppa H, Fenech M (2006). An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans. *Carcinogenesis* 28:625-631.
- Brasil (2010). *Farmacopeia Brasileira*, fifth ed. Brasília: Agência Nacional de Vigilância Sanitária.
- Cadet JD, Douki T, Gasparutto T, Pouget D, Ravanat JP, Sauvaigo JL (1999). Hydroxyl radicals and DNA base damage. *Mutat. Res.* 424(1-2):9-21.
- Camargo MTLA (2007). Amansa-senhor: a arma dos negros contra seus senhores. *Rev. Pós-Ciências Soc.* 4(8):31-42.
- Carillo CM, Chinchilla EA, González LA, Toledo RA, Zambrana HG (1997). Prevención del cólera aviar, con extractos acuosos de plantas, en pollos de engorde. *Agron. Mesoam.* 8(2):152-158.
- Cifuentes MC, Castañeda DM, Uruña CP, Fiorentino S (2009). A fraction from *Petiveria alliacea* induces apoptosis via a mitochondria-dependent pathway and regulates HSP70 expression. *Univ. Sci.* 14(2):125-134.
- Cooke MS, Evans MD, Dizdaroglu M, Lunec J (2003). Oxidative DNA damage: mechanisms, mutation, and disease. *FASEB J.* 17(10):1195-1214.

- Corrêa MP (1984). Dicionário das plantas úteis do Brasil e das exóticas cultivadas. Rio de Janeiro: Ministério da Agricultura, Instituto Brasileiro de Desenvolvimento Florestal-Imprensa Nacional.
- Delle Monache F, Cuca Suarez LE (1992). 6-C-formyl and 6-C-hydroxymethyl flavanones from *Petiveria alliacea*. *Phytochemistry* 31(7):2481-2482.
- Delle Monache F, Menichini F, Cuca Suarez LE (1996). *Petiveria alliacea*. Part 2. Further flavonoids and triterpenes. *Gazz Chim Ital* 126:275-278.
- Del-Rio D, Stewart AJ, Pellegrini N (2005). A review of recent studies on malondialdehyde as toxic molecule and biological markers of oxidative stress. *Nutr. Metab. Cardiovasc. Dis.* 15(4):316-328.
- Desmarchelier C, Mongelli E, Coussio J, Ciccía G (1996). Studies on the cytotoxicity, antimicrobial and DNA-binding activities of plants used by the Esé'ejás. *J. Ethnopharmacol.* 50(2):91-96.
- Elisabetsky E, Castilhos ZC (1990). Plants used as analgesics by amazonian caboclos as a basis for selecting plants for investigation. *Int. J. Crude Drug Res.* 28(4):309-320.
- Gobbo-Neto L, Lopes NP (2007). Plantas medicinais: fatores de influência no conteúdo de metabólitos secundários. *Quím. Nova* 30(2):374-381.
- Guedes RCM, Nogueira NGP, Fusco-Almeida AM, Souza CRF, Oliveira WP (2009). Atividade antimicrobiana de extratos brutos de *Petiveria alliacea* L. *Lat. Am. J. Pharm.* 28(4):520-524.
- Hageman GJ, van Herwijnen MH, Schilderman PA, Rhijsburger EH, Moonen EJ, Kleinjans JC (1997). Reducing effects of garlic constituents on DNA adduct formation in human lymphocytes *in vitro*. *Nutr. Cancer* 27(2):177-85.
- Hartmann A, Agurell E, Beevers C, Brendler-Schwaab S, Burlinson B, Clay P, Collins A, Smith A, Speit G, Thybaud V, Tice RR (2003). Recommendations for conducting the *in vivo* alkaline Comet assay. *Mutagenesis* 18(1):45-51.
- Hayashi M, Macgregor JT, Gatehouse DG, Adler ID, Blakey DH, Dertinger SD, Krishna G, Morita T, Russo A, Sutou S (2000). *In vivo* rodent erythrocyte micronucleus assay. II. Some aspects of protocol design including repeated treatments, integration with toxicity testing and automated scoring. *Environ. Mol. Mutagen.* 35(3):234-252.
- Hernández JF, Urueña CP, Cifuentes MC, Sandoval TA, Pombo LM, Castañeda D, Asea A, Fiorentino S (2014). A *Petiveria alliacea* standardized fraction induces breast adenocarcinoma cell death by modulating glycolytic metabolism. *J. Ethnopharmacol.* 153(3):641-649.
- Hoyos LS, Au WW, Heo MY, Morris DL, Legator MS (1992). Evaluation of the genotoxic effects of a folk medicine, *Petiveria alliacea* (Anamu). *Mutat. Res.* 280(1):29-34.
- Institute of Laboratory Animal Resources (US). Committee on Care, Use of Laboratory Animals, & National Institutes of Health (US). Division of Research Resources. (1985). Guide for the care and use of laboratory animals. National Academies.
- International Conference on Harmonization (ICH) (2008). Guidance on genotoxicity testing and data interpretation for pharmaceuticals intended for human use S2 (R1). Available at: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Safety/S2_R1/Step4/S2R1_Step4.pdf.
- Janero DR (1990). Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. *Free Radic. Biol. Med.* 9(6):515-540.
- Kesarwani K, Gupta R (2013). Bioavailability enhancers of herbal origin: An overview. *Asian Pac. J. Trop. Biomed.* 3(4):253-266.
- Krishna G, Hayashi M (2000). *In vivo* rodent micronucleus assay: protocol, conduct and data interpretation. *Mutat. Res.* 455(1-2):155-166.
- Kubec R, Cody RB, Dane AJ, Musah RA, Schraml J, Attekkatte A, Block E (2010). Applications of Direct Analysis in Real Time-Mass Spectrometry (DART-MS) in *Allium* chemistry. (Z)-Butanethial S-oxide and 1-butenyl thiosulfonates and their S-(E)-1-butenylcysteine S-oxide precursor from *Allium siculum*. *J. Agric. Food Chem.* 58(2):1121-1128.
- Kubec R, Kim S, Musah RA (2002). S-Substituted cysteine derivatives and thiosulfonate formation in *Petiveria alliacea*—part II. *Phytochemistry* 61(6):675-680.
- Kubec R, Musah RA (2001). Cysteine sulfoxide derivatives in *Petiveria alliacea* L. *Phytochemistry* 58(6):981-985.
- Lee M-Y, Seo C-S, Kim J-Y, Shin H-K (2015). Genotoxicity evaluation of Oryeong-san water extract using *in vitro* and *in vivo* tests. *BMC Complement. Altern. Med.* 15:273.
- Lykkesfeldt J (2007). Malondialdehyde as biomarker of oxidative damage to lipids caused by smoking. *Clin. Chim. Acta* 380(1-2):50-58.
- Marnett LJ (1999a). Chemistry and biology of DNA damage by malondialdehyde. *IARC Sci. Publication* 150:17-27.
- Marnett LJ (1999b). Lipid peroxidation-DNA damage by malondialdehyde. *Mutat. Res.* 424(1-2):83-95.
- Mata-Greenwood E, Ito A, Westenberg H, Cui B, Mehta RG, Kinghorn AD, Pezzuto JM (2001). Discovery of novel inducers of cellular differentiation using HL-60 promyelocytic cells. *Anticancer Res.* 21(3B):1763-1770.
- Mayorga P, Pérez KR, Cruz SM, Cáceres A (2010). Comparison of bioassays using the anostracan crustaceans *Artemia salina* and *Thamnocephalus platyurus* for plant extract toxicity screening. *Rev. Bras. Farmacogn.* 20(6):897-903.
- Netto EM, Shuqair NSMSAQ, Balbino EE, Carvalho ACB (2006). Comentários sobre o registro de fitoterápicos. *Rev. Fitos* 1:9-17.
- Neves IA, Câmara CAG, Oliveira JCS, Almeida AV (2011). Acaricidal activity and essential oil composition of *Petiveria alliacea* L. from Pernambuco (northeast Brazil). *J. Essent. Oil Res.* 23(1):23-26.
- Pérez-Leal R, García-Mateos M, Martínez-Vásquez M, Soto-Hernández M (2006). Actividad citotóxica y antioxidante de *Petiveria alliacea* L. *Rev. Chapingo Ser. Hortic.* 12:51-56.
- Ramos AA, Lima CF, Pereira-Wilson C (2011). DNA Damage protection and induction of repair by dietary phytochemicals and cancer prevention: What do we know? In: Chen C (Ed.). *Selected Topics in DNA Repair*. InTech. pp. 237-270.
- Rocha LD, Maranhão LT, Preussler KH (2006). Organização estrutural do caule e lâmina foliar de *Petiveria alliacea* L., Phytolaccaceae. *Braz. J. Pharm.* 87(3):98-101.
- Rosado-Aguilar JA, Aguilar-Caballero A, Rodríguez-Vivas RI, Borges-Argaez R, Garcia-Vazquez Z, Mendez-Gonzalez M. (2010). Acaricidal activity of extracts from *Petiveria alliacea* (Phytolaccaceae) against the cattle tick, *Rhipicephalus (Boophilus) microplus* (Acari: ixodidae). *Vet. Parasitol.* 168: 299-303.
- Rossi V (1990). Antiproliferative effects of *Petiveria alliacea* on several tumoral lines. *Pharmacol. Res.* 22:434.
- Sahoo N, Manchikanti P, Dey S (2010). Herbal drugs: standards and regulation. *Fitoterapia* 81(6):462-471.
- Santander SP, Hernández JF, Barreto CC, Masayuki A, Moins-Teisserenc H, Fiorentino S (2012). Immunomodulatory effects of aqueous and organic fractions from *Petiveria alliacea* on human dendritic cells. *Am. J. Chin. Med.* 40(4):833-844.
- Schauss AG, Tselyico SS, Kuznetsova VA, Yegorova I (2015). Toxicological and Genotoxicity Assessment of a Dihydroquercetin-Rich Dahurian Larch Tree (*Larix gmelinii* Rupr) Extract (Lavitol). *Int. J. Toxicol.* 34(2):162-181.
- Schmid W (1975). The micronucleus test. *Mutat. Res.* 31(1):9-15.
- Silva ML, Luz DA, Paixão TP, Silva JPB, Belém-Filho IJA, Fernandes LMP, Gonçalves ACB, Fontes-Júnior EA, Andrade MA, Maia CSF (2015). *Petiveria alliacea* exerts mnemonic and learning effects on rats. *J. Ethnopharmacol.* 169:124-129.
- Singh NP, McCoy MT, Tice RR, Schneider EL (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.* 175(1):184-191.
- Skafa E, Sitarek P, Rózsalski M, Krajewska U, Szemraj J, Wysockińska H, Śliwiński T (2016). Antioxidant and DNA Repair Stimulating Effect of Extracts from Transformed and Normal Roots of *Rhaponticum carthamoides* against Induced Oxidative Stress and DNA Damage in CHO Cells. *Oxid. Med. Cell. Longev.* P 11.
- Soares BO, Oliveira MBN, Mansur E, Dantas FJS, De Mattos JCP, Caldeira-de-Araujo A, Gagliardi R (2014). Effect of extracts from field and *in vitro* plants of *Petiveria alliacea* L. on plasmidial DNA. *J. Med. Plants Res.* 8(35):1101-1109.
- Subhashinee SK, Wijeratne LC, Vikcy S (2005). Hydrogen peroxide induced oxidative stress damage and antioxidant enzyme response in Caco-2 human colon cells. *J. Agric. Food Chem.* 53(22):8768-8774.

- Urueña C, Cifuentes C, Castañeda 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.* 8:60.
- Vandebroek I, Balick MJ, Ososki A, Kronenberg F, Yukes J, Wade C, Jiménez F, Peguero B, Castillo D (2010). The importance of botellas and other plant mixtures in Dominican traditional medicine. *J. Ethnopharmacol.* 128(1):20-41.
- Villar R, Calleja JM, Morales C, Cáceres A (1997). Screening of 17 Guatemalan medicinal plants for platelet antiaggregant activity. *Phytother. Res.* 11(6):441-445.
- Wagner H, Bladt S (2001). *Plant Drug Analysis: A Thin Layer Chromatography Atlas*. 2nd ed. Germany: Springer.
- World Health Organization (WHO) (2011). *Quality control methods for medicinal plant materials*, update ed. Geneva: WHO.
- Xiao H, Parkin KL (2002). Antioxidant functions of selected allium thiosulfinates and S-alk(en)yl-L-cysteine sulfoxides. *J. Agric. Food Chem.* 50(9):2488-2493.