

# Antiamoebic Activity of *Petiveria alliacea* Leaves and Their Main Component, Isoarborinol

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*Petiveria alliacea* L. (Phytolacaceae) is a medicinal plant with a broad range of traditional therapeutic properties, including the treatment of dysentery and intestinal infections caused by protozoan parasites. However, its effects against *Entamoeba histolytica* have not been reported yet. We investigated the antiamoebic activity present in the leaves of *P. alliacea*. Antiamoebic activity was evaluated in methanolic and aqueous extracts, as well as in the hexanic, methanolic, and EtOAc fractions. The *P. alliacea* methanolic extract showed a better antiamoebic activity than the aqueous extract with an  $IC_{50} = 0.51$  mg/ml. Likewise, the hexanic fraction was the most effective fraction, showing a dose-dependent activity against *E. histolytica*, with an  $IC_{50} = 0.68$  mg/ml. Hexanic subfraction 12-19 showed the highest antiamoebic activity at 0.8 mg/ml, producing 74.3% growth inhibition without any toxicity in mammal cells. A major component in subfraction 12-19 was identified as isoarborinol, which produced 51.4% *E. histolytica* growth inhibition at 0.05 mg/ml without affecting mammal cells. The *P. alliacea* leaf extract has antiamoebic activity that can be attributed to a major metabolite known as isoarborinol.

**Keywords:** *Petiveria alliacea*, isoarborinol, antiamoebic activity, cytotoxicity

## Introduction

Medicinal plants are considered as an important source of chemotherapeutic agents and useful structures for the development of more effective drugs. Among a variety of plants that have been studied, *Petiveria alliacea* (Phytolacaceae) is extremely interesting because of its multiple biological activities. *P. alliacea* is a shrub widely distributed throughout America and some regions of Africa. Notably, it grows in several parts of Mexico where it is known by many popular names, such as *hierba del zorrillo*, *hoja de zorrillo*, *epazote de zorrillo*, *zorrillo*, *payché*, *hierba de las gallinitas*, or *sanituwan* [1]. Previous phytochemical characterization of *P. alliacea* revealed the presence of triterpenoids, saponins, polyphenols, coumarins, benzaldehyde, benzoic acid, flavonoids, fredelinol, pinitol, and allantoin [2–5].

*P. alliacea* has a broad range of therapeutic properties. It is used as an antileukemic, antispasmodic, antirheumatic (topical use), anticancer, immunostimulant, anti-inflammatory, antinociceptive, and antimicrobial agent. Ethnobotanical data indicate that *P. alliacea* leaves are used in Guatemala for the treatment of dysentery and protozoal infections [6, 7]. In Mexico, it is also used to treat diseases as diverse as the cold, rabies, and dysentery, as well as stomach ache [1, 8]. The antiprotozoal properties of *P. alliacea* have been proved in vitro by Berger *et al.* [6] and Cáceres *et al.* [7] who reported its activity against *Trypanosoma cruzi* epimastigotes and trypomastigotes. Additionally, Echevarría and Torres [9] reported the inhibitory effect of *P. alliacea* on the growth of *Giardia lamblia* in vitro. However, the activity of *P. alliacea* against *Entamoeba histolytica*, the protozoan parasite that is responsible for human amoebiasis, has not previously been reported.

## Materials and Methods

### Collection and Identification of *P. alliiacea*

*Petiveria alliiacea* was collected in Catemaco, Veracruz, Mexico in April and May 2015. Taxonomic identification was confirmed in the herbarium of the Universidad Autonoma Metropolitana Xochimilco, Mexico by M. Sc. Aurora Chimal Hernández, and an herbarium specimen was deposited in the National Herbarium of Mexico (MEXU) (voucher number 1414464).

### Obtaining Extracts and Fractionation

*P. alliiacea* leaves were dried at room temperature. To obtain the aqueous extract, 20 g of leaves was macerated with 300 ml of distilled water and concentrated by lyophilization. For methanolic extract preparation, 1.8 kg of leaves was macerated with 12 L of methanol and concentrated with a vacuum rotary evaporator. Then, fractions of methanol extract were obtained by dissolving 133 g of the extract with 50 ml of MeOH and absorbed in silica gel (0.015–0.04 mm; Macherey-Nagel) to be subsequently fractionated with 250 ml of *n*-hexane, EtOAc, and methanol. The hexanic fraction (6.8 g) was selected and subfractionated through a silica gel column using 400 ml mixtures of *n*-hexane:EtOAc (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 30:70, 20:80) and EtOAc:methanol

(100:0, 80:20, 60:40, 0:100) of increasing polarity, as the mobile phase. Subfractions of 100 ml were collected and analyzed for chromatographic profiling (Fig. 1).

### Subfraction Analysis

Subfractions were analyzed by thin-layer chromatography using silica gel plates, and mixtures of *n*-hexane/toluene, *n*-hexane/EtOAc, and chloroform/EtOAc/formic acid as the mobile phase. The spots were detected using various techniques and reagents; those with similar profile were mixed and their yield was calculated. An abundant white precipitate observed in the subfraction 12-19, was purified through the recrystallization method, by dissolving it in a methanol/chloroform mixture. The melting point of the compound was determined and it was analyzed by nuclear magnetic resonance of hydrogen and carbon ( $^1\text{H-NMR}$ ,  $^{13}\text{C-NMR}$ ) in a spectrometer (Bruker, Advance DPX400 MHz) at 300 MHz. The spectrum was compared with previously reported data to identify the molecule.

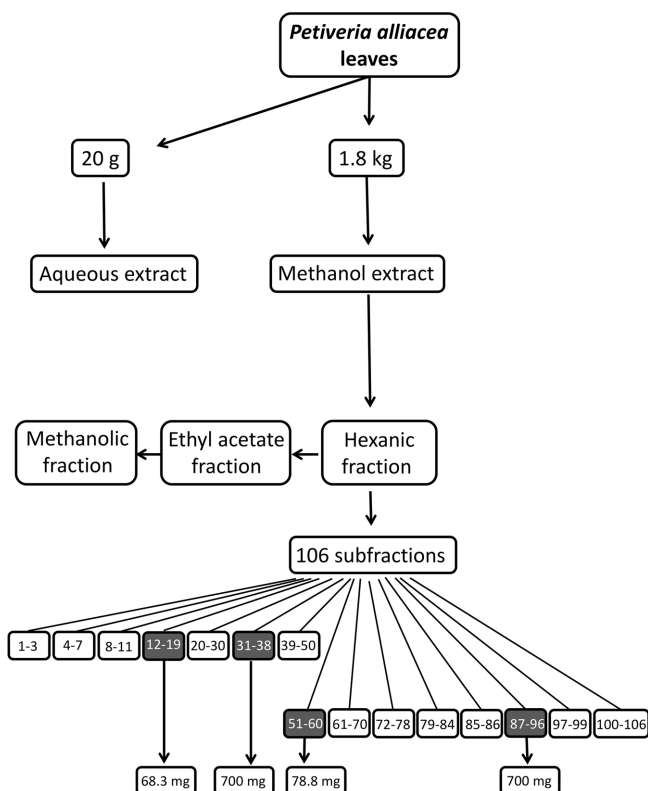
Isobarborinol: colorless crystal with mp 296–298°C.  $^1\text{H NMR}$  (300 MHz,  $\text{CDCl}_3$ )  $\delta$  5.23 (1H, d,  $J$ = 6.2 Hz, H-11), 3.22 (1H, dd,  $J$ =10.2, 3.8 Hz, H-3 $\beta$ ), 1.03 (3H, s, H-25), 0.98 (3H, s, H-23), 0.89 (3H, d,  $J$ = 6.5 Hz, H-29), 0.83 (3H, d,  $J$ = 6.8 Hz, H-30), 0.82 (3H, s, H-24), 0.81 (3H, s, H-26), 0.77 (3H, s, H-27), and 0.76 (3H, s, H-28).  $^{13}\text{C NMR}$  (71.4 MHz,  $\text{CDCl}_3$ )  $\delta$  39.1(C-1), 27.8(C-2), 78.9(C-3), 39.6(C-4), 52.3(C-5), 21.4(C-6), 26.7(C-7), 42.8(C-8), 148.8(C-9), 35.9(C-10), 114.3(C-11), 36.0(C-12), 36.8(C-13), 38.2(C-14), 29.7(C-15), 36.0(C-16), 42.8(C-17), 52.1(C-18), 20.2(C-19), 28.2(C-20), 59.6(C-21), 30.8(C-22), 28.2(C-23), 15.6(C-24), 22.1(C-25), 17.0(C-26), 15.3(C-27), 14.0(C-28), 22.1(C-29), and 23.0(C-30).

### Cell Cultures

*E. histolytica* HM1-IMSS trophozoites were axenically grown at 37°C in TYI-S-33 medium, supplemented with 20% bovine serum [10]. Cells were harvested in the log phase of growth for all experiments. Human epithelial colorectal adenocarcinoma cells (Caco-2, HTB-37; ATCC, USA) were grown in advanced minimum essential medium (MEM; Gibco, USA) supplemented with 5% fetal bovine serum, 200 mM glutamine (Gibco), 0.0125% penicillin, and 0.02% streptomycin. Cultures were maintained at 37°C with a humidified atmosphere of 5%  $\text{CO}_2$ .

### Antiamoebic Activity

*E. histolytica* trophozoites ( $1.5 \times 10^5$ ) were treated with increasing amounts of *P. alliiacea* leaf extracts, fractions, subfractions, or the purified compound. Extracts were dissolved in culture medium, whereas fractions and subfractions were dissolved in polyvinylpyrrolidone (PVP), and the purified compound was first dissolved in chloroform and polyethylene glycol E-4000 (PEG), and this was followed by evaporation in a speed vacuum and dissolution on TYI-S-33 culture medium. Cultures were incubated at 37°C for 48 h. The trophozoite number and viability were determined [11]. A positive control treated with 0.04  $\mu\text{g/ml}$  of metronidazole, a negative control without treatment, and a



**Fig. 1.** Schematic diagram of *P. alliiacea* fractionation.

Gray boxes denote subfractions containing the highest concentrations of metabolites; numbers below indicate the amount of these subfractions.

vehicle control treated with DMSO, PVP, or PEG were included in each experiment. Experiments were performed twice in triplicates. Data were expressed as the mean  $\pm$  standard deviation (SD).

### Cytotoxicity Assays

**Activity of NADPH-dependent cellular oxidoreductase enzyme.** Caco-2 cells were cultured in a 96-well microplate ( $2.0 \times 10^4$  cells/well) in the presence of different amounts of hexanic subfractions (0.1 to 0.8 mg/ml) or the purified compound (0.05 to 0.3 mg/ml), for 48 h. Then, cells were incubated with 1 mM MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) at 37°C for 4 h. The medium was removed and the formazan dye crystals were solubilized in 100  $\mu$ l of DMSO for 5 min. Cell viability was determined from absorbance at 570 nm wavelength as previously described [11]. Experiments were performed twice in triplicates and results were expressed as the mean  $\pm$  SD.

**Lactate dehydrogenase release.** Caco-2 cells were cultured in the presence of the hexanic subfractions or the pure compound, as described above. Supernatants were collected, centrifuged at 500  $\times$ g for 5 min, and transferred to a microtiter plate (50  $\mu$ l/well) to determine lactate dehydrogenase (LDH) release using the CytoTox 96 Non-radioactive Cytotoxicity Assay (Promega) following manufacturer recommendations. Experiments were performed twice in triplicates and results were expressed as the mean  $\pm$  SD.

### Acute Oral Toxicity

Acute oral toxicity was evaluated in CD1 male mice, weighing 25–30 g (5 mice per group), according to the guidelines for testing chemicals number 423, Organization for Economic Cooperation and Development (OECD), and the Mexican official standard for the production, use and care of laboratory animals (NOM-062-ZOO-1999). The protocol was approved by the Institutional Ethics Committee (January 1<sup>st</sup> 2016). Animals were fasted for 12 h and they received 2 g/kg hexanic fraction through an oral cannula. The control group only received saline solution. Then, they were observed for 1 min to evaluate possible behavioral effects. Toxic effects such as loss of movement, tremors, convulsions, ataxia, respiratory arrest, and death were evaluated at 30 min, every 2 h for 24 h, and every day for 14 days. Additionally, animals were weighed daily in order to observe weight loss.

### Statistical Analyses

All the data were processed using the Graph Pad Prism 6 software. Statistical analyses were performed using one-way ANOVA and Dunnett's and Bonferroni tests. Statistical significance was set at  $p \leq 0.05$ .

## Results

### The *P. alliaceae* Methanolic Extract Has Antiamoebic Effect

To initiate the evaluation of *P. alliaceae* on *E. histolytica* trophozoite cultures, we first compared the effects of aqueous and methanol extracts. The aqueous extract only

had effect at the highest amount tested (1.8 mg/ml), producing 49.7% growth inhibition, in reference to the control group treated with culture medium. By contrast, the methanol extract showed a dose-dependent antiamoebic activity; surprisingly, the effect decreased when the extract concentration was increased. The methanol extract showed an  $IC_{50} = 0.51$  mg/ml (Fig. 2A).

### *P. alliaceae* Fractions Exhibit Differential Activity against *E. histolytica*

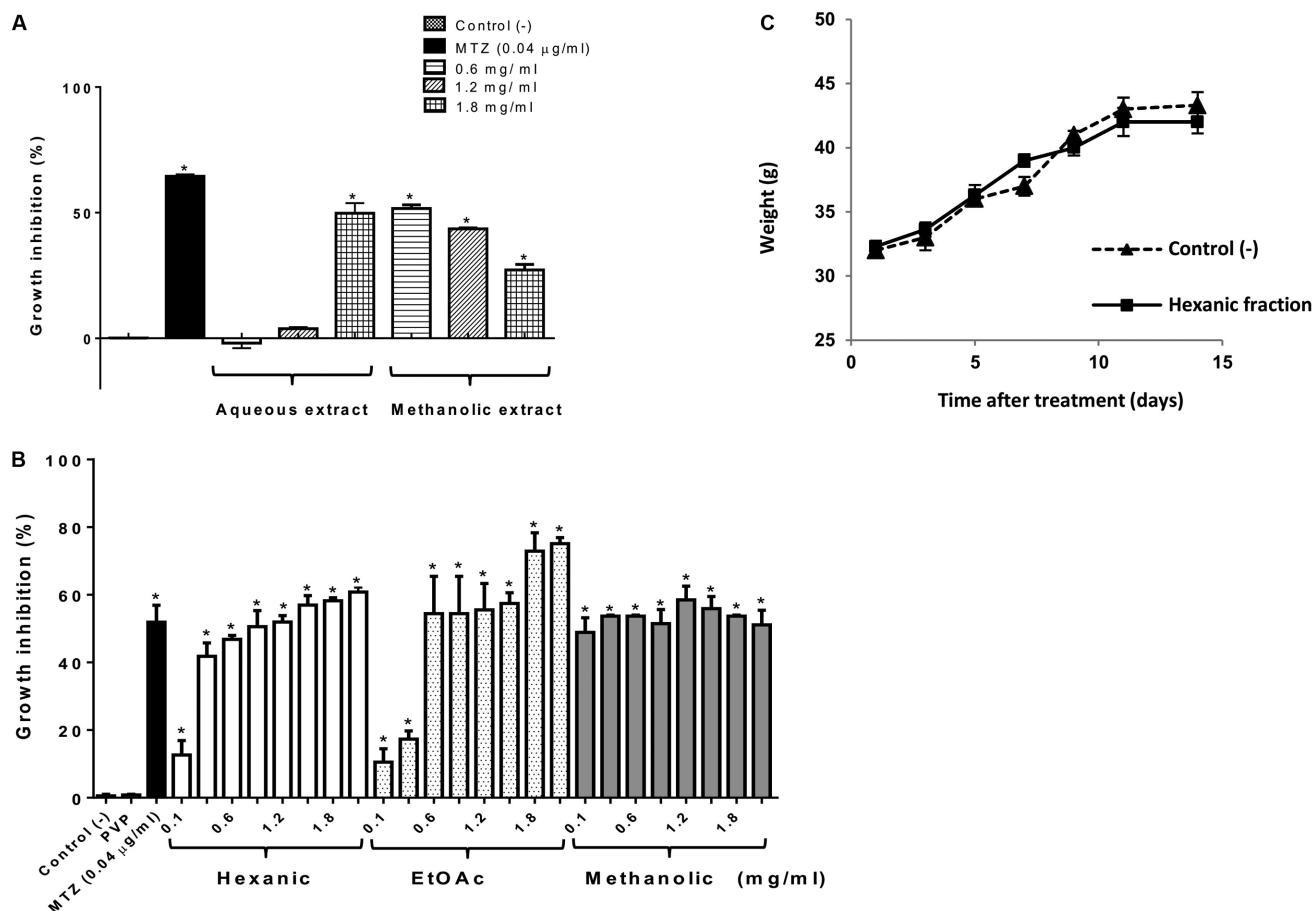
To better characterize the methanol extract, we fractioned it to identify the most active fraction. Results showed that *P. alliaceae* fractions have different effects on *E. histolytica* growth. The hexanic fraction had a dose-dependent effect, producing 60.7% growth inhibition at the major concentration of 2.1 mg/ml; this fraction had an  $IC_{50}$  of 0.68 mg/ml. The EtOAc fraction also had a dose-dependent effect, producing 75% trophozoite growth inhibition at the concentration of 2.1 mg/ml; this fraction had an  $IC_{50}$  of 1.28 mg/ml. In contrast, the methanolic fraction did not show dose-dependent activity, since all concentrations produced about 50% growth inhibition (Fig. 2B). Even though the three fractions had antiamoebic effects, we decided to focus our study on the hexanic fraction that had the lowest  $IC_{50}$  and produced a clear dose-dependent response.

### The *P. alliaceae* Hexanic Fraction Is Not Toxic to Mice

In order to know the potential systemic toxicity of the hexanic fraction, we performed an acute systemic toxicity test in CD1 mice, using a single dose of 2 g/kg by oral route and assessing the general toxic effect within a short observation period. After administration, animals showed no sign or symptom of systemic toxicity, mortality, or macroscopic damage throughout the 14 days of the daily observation period (data not shown). Additionally, no significant difference in weight gain was observed, in comparison with control group animals (Fig. 2C). Thus, the lethal dose 50% ( $LD_{50}$ ) was considered higher than 2 g/kg, which demonstrated the safety of the hexanic fraction under these experimental conditions.

### *P. alliaceae* Subfractions Exhibit a Greater Antiamoebic Effect than the Hexanic Fraction

To gain insight into the compounds that are responsible for its antiamoebic effect, the hexanic fraction was subfractioned using *n*-hexane, EtOAc, and methanol mixtures. We obtained 106 subfractions, which were clustered according to their thin-layer chromatography profiles (data not shown). Subfractions 12-19, 31-38, 51-60, and 87-96, containing the



**Fig. 2.** Antiamoebic activity and acute toxicity of *P. alliaceae* leaves.

*E. histolytica* growth inhibition by aqueous and methanolic extract (A), and hexanic, EtOAc, and methanolic fractions (B). PVP (polyvinylpyrrolidone), standard medium (-), or metronidazole (MTZ) at 0.04 µg/ml were included as controls. Data represent the mean of two independent experiments performed in triplicates ± SD, \* $p \leq 0.05$  in reference to the negative control. (C) Acute toxicity of the hexanic fraction in CD1 mice. Mice were treated with a single dose of 2 g/kg hexanic fraction on day 1 and the weight was recorded daily (solid line). Saline solution was included as the control (dashed line). Data represent the average weight of five mice ± SD. Statistical significance was set at  $p \leq 0.05$ .

highest metabolite concentration, were chosen to evaluate their activity on *E. histolytica* cultures at two concentrations (0.1 and 0.8 mg/ml). In comparison with control groups, subfraction 12-19 showed the highest effect at 0.8 mg/ml, with 74.3% growth inhibition. The subfraction 31-38 produced 55.3% growth inhibition at 0.1 mg/ml, but its activity decreased to 30% at 0.8 mg/ml. The subfractions 51-60 and 87-96 produced less than 50% growth inhibition at the two concentrations tested (Fig. 3A). Subfraction 12-19, with the highest antiamoebic activity, was selected to evaluate its cytotoxicity on mammal cells.

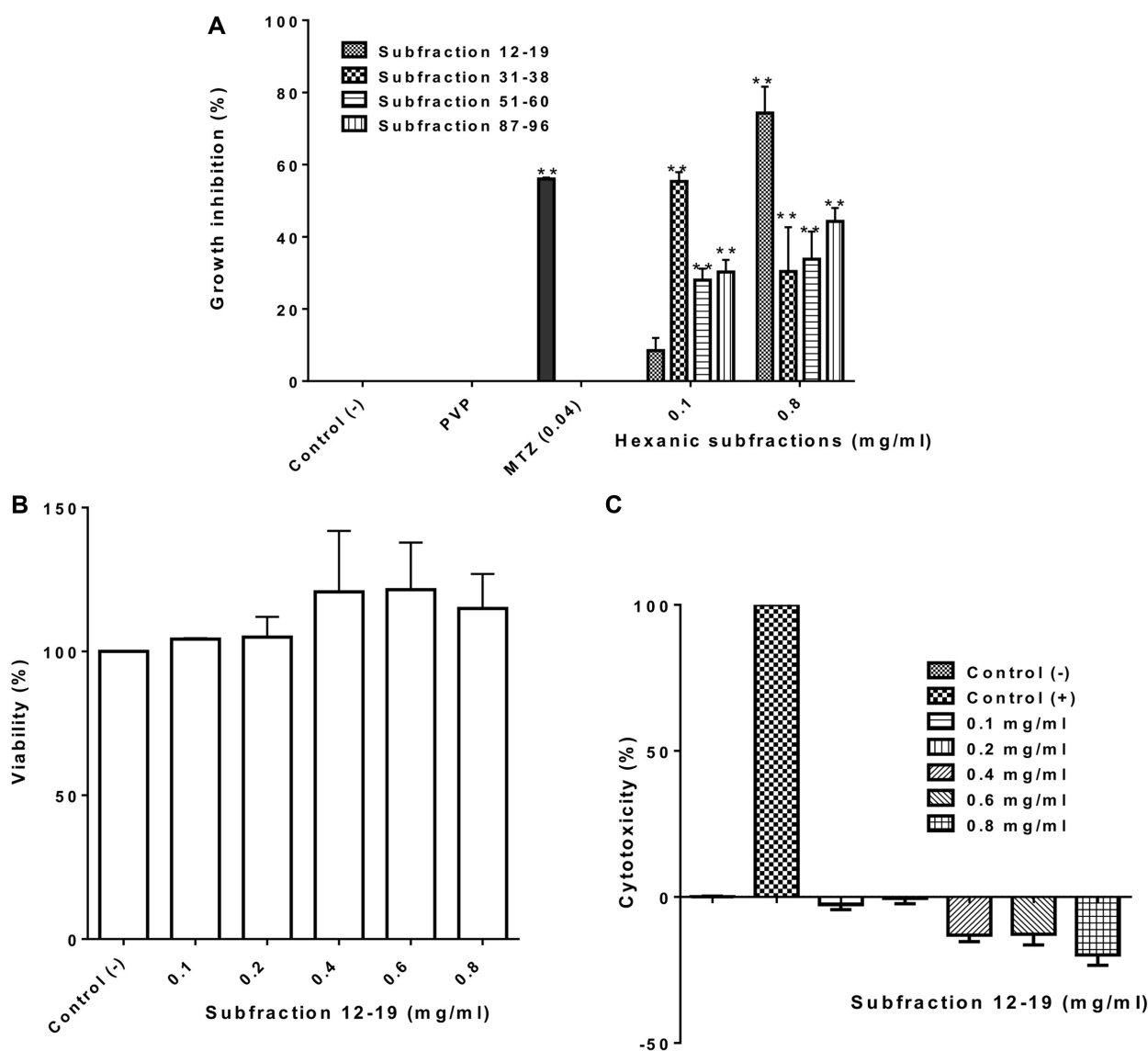
#### *P. alliaceae* Subfraction 12-19 Is Not Cytotoxic toward Mammal Cells

Cytotoxicity toward Caco-2 cells was evaluated using

increasing concentrations of subfraction 12-19, from 0.1 to 0.8 mg/ml. None of the concentrations tested occasioned significant changes in cell viability compared with the group without treatment (Fig. 3B). Likewise, this subfraction did not produce any cellular cytotoxicity in reference with the group treated with Triton X-100, which caused 100% cellular lysis (Fig. 3C).

#### Identification of the Major Compound of Subfraction 12-19

To identify the main component of subfraction 12-19, we first submitted it to a thin-layer chromatography analysis. The chromatographic profile was visualized with p-anisaldehyde, suggesting that the major component could be a terpenoid (data not shown). The compound was purified by the recrystallization method, obtaining a total

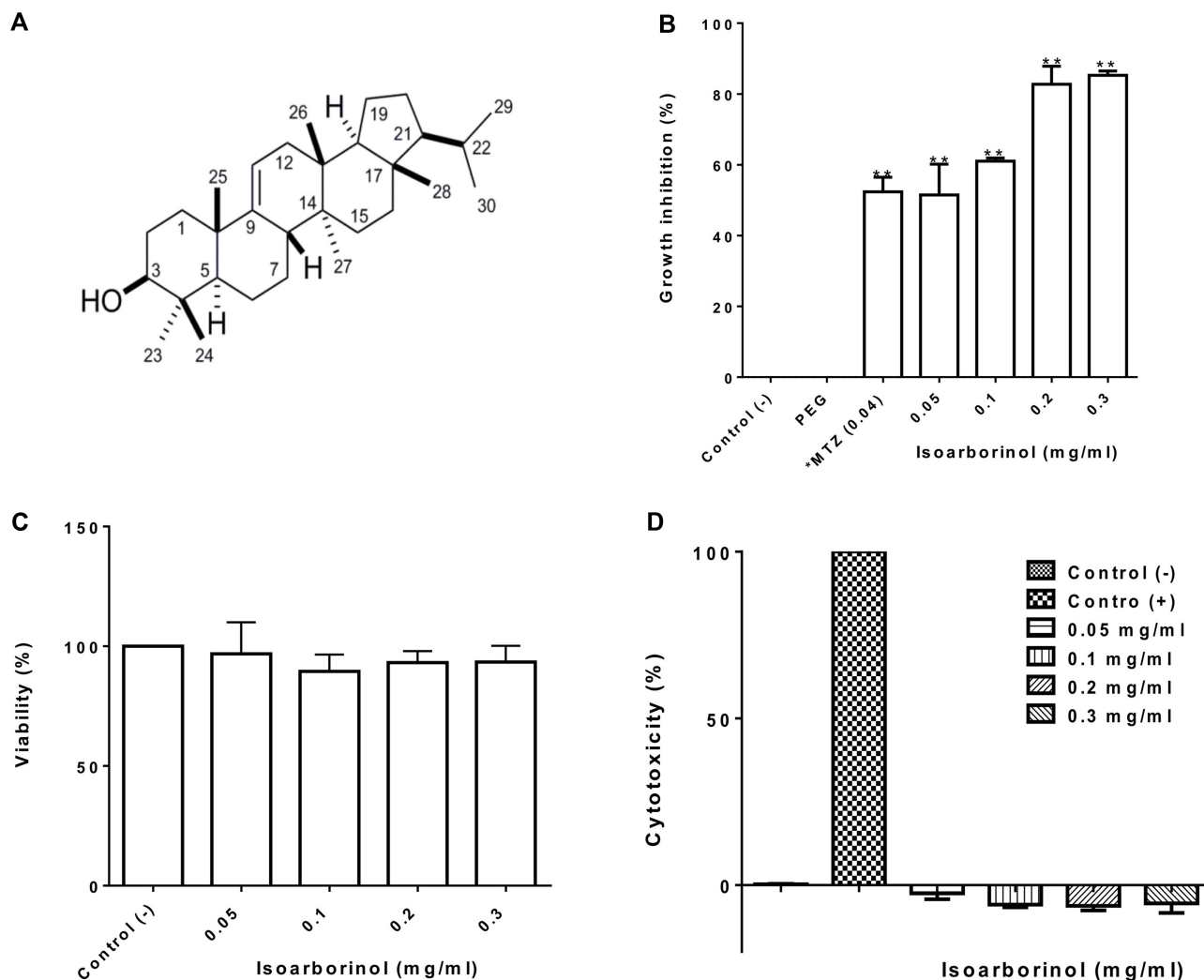


**Fig. 3.** Antiamoebic activity and cytotoxicity of *P. alliacea* subfractions.

(A) Antiamoebic activity of 12-19, 31-38, 51-60, and 87-96 subfractions. PVP (polyvinylpyrrolidone) and metronidazole (MTZ) at 0.04 µg/ml were included as controls. Data represent the average of two independent experiments performed in triplicates ± SD. \*\**p* ≤ 0.05. (B and C) Subfraction 12-19 cytotoxicity was determined in Caco-2 cells. (B) Viability was determined by MTT assay and (C) cytotoxicity was evaluated using the Cyto Tox 96 Non-radioactive Cytotoxicity Kit. MEM (control -), Triton X-100 (control +), and DMSO were included as controls. Experiments were performed twice in triplicates. Data corresponding to the mean value ± SD were expressed in percentage in relation to the number of cells grown in culture medium.

amount of 68.3 mg. The compound melting point was calculated as 296–298°C. The structure of the compound was elucidated by <sup>1</sup>H NMR and <sup>13</sup>C NMR, and it was identified as the pentacyclic triterpenoid isoarborinol by comparison of spectroscopic data with previously reported spectra [12, 13]. Briefly, the <sup>1</sup>H NMR spectrum of isoarborinol revealed the presence of six singlets at low frequency: 0.76,

0.77, 0.81, 0.82, 0.98, and 1.03 δ<sub>H</sub> assigned to methyl groups of C-28, C-27, C-26, C-24, C-23, and C-25 δ<sub>C</sub>, respectively. Furthermore, two doublets at 0.83 and 0.89 δ<sub>H</sub> corresponding to two methyl groups of the isopropyl fragment and olefinic and methine signals at 5.23 δ<sub>H</sub> and 3.22 δ<sub>H</sub> for H-11 and H-3 were easily recognized. Additionally, the concordance of all the signals of <sup>13</sup>C NMR with those described previously



**Fig. 4.** Isoarborinol structure and its effect on *E. histolytica* and Caco-2 cells.

(A) Isoarborinol structure. (B) *E. histolytica* growth inhibition by isoarborinol. Trophozoites growing in standard medium, polyethylene glycol (PEG), and metronidazole (MTZ) at 0.04  $\mu\text{g/ml}$  were included as controls. (C and D) Caco-2 cell viability and cytotoxicity in response to isoarborinol. (C) Cell viability was determined by MTT assay and (D) cytotoxicity was evaluated using the CytoTox 96 Non-Radioactive Cytotoxicity Kit. MEM (control -) and Triton X-100 (Control +) were included as controls. Experiments were performed twice in triplicates. Data corresponding to the mean value  $\pm$  SD are expressed in percentage.  $**p \leq 0.05$  in reference to the negative control.

confirmed the isoarborinol structure (Fig. 4A). Copies of the original spectra are obtainable from the corresponding author.

#### Isoarborinol Has Antiamoebic Activity

To confirm that the activity of subfraction 12-19 was due to isoarborinol, *E. histolytica* trophozoites were cultured in the presence of isoarborinol. The cell count showed that isoarborinol has a dose-dependent antiamoebic activity, producing 51.4% *E. histolytica* growth inhibition at 0.05 mg/ml and 85.2% at 0.3 mg/ml, in reference to control groups

(Fig. 4B). Interestingly, isoarborinol did not produce any toxicity toward Caco-2 cells since none of the concentrations used affected cell viability through alteration of mitochondrial enzyme activity (Fig. 4C) nor caused cell lysis (Fig. 4D).

#### Discussion

The effect of *P. alliacea* extracts against the protozoan parasites *T. cruzi* and *G. lamblia* have been previously reported [6, 7, 9], but this is the first study that reveals its activity against *E. histolytica*, the parasite that causes human



amoebiasis.

Although we chose the *P. alliacea* hexanic fraction for further analysis, it is important to mention that the methanol and EtAOc fractions also have antiamoebic activity, which suggests that *P. alliacea* contains metabolites with different polarity that are active against *E. histolytica*. These may include isoarborinol and other triterpenes previously identified in this plant, such as isoarborinol-acetate and isoarborinol-cinnamate.

The hexanic fraction of *P. alliacea* leaves had a dose-dependent antiamoebic activity, producing 50% growth inhibition at 0.68 mg/ml. Echevarría and Torres [9] previously reported this dose-response activity of *P. alliacea* methanolic extracts on *G. lamblia*, finding an  $IC_{50}$  of 2.05 mg/ml. In contrast, *P. alliacea* seems to be more effective against trypanosomatids, since Berger *et al.* [6], using hexanic and ethanol extracts of *P. alliacea* found a marked inhibition of *T. cruzi* trypomastigotes at  $IC_{90}$  of 285.6 and 692.2  $\mu\text{g/ml}$ , respectively. Additionally Cáceres *et al.* [7], reported that the dichloromethane extract of *P. alliacea* was active in vitro against epimastigote and trypomastigote stages of *T. cruzi* with  $IC_{90} = 1.0$  mg/ml, which was not toxic for *Artemia salina*. In addition, García *et al.* [14] found that the ethanolic extract from *P. alliacea* leaves was active against the amastigote stage of *Leishmania amazonensis* with an  $IC_{50}$  of 151.5  $\mu\text{g/ml}$ . Similarly, the *P. alliacea* efficiency has been proved against *Plasmodium falciparum*. Ruiz *et al.* [15] reported an antiplasmodial activity with an  $IC_{50} > 10$   $\mu\text{g/ml}$  and they demonstrated that the *P. alliacea* leaf hydroalcoholic extract at the  $IC_{50}$  of 3.8  $\mu\text{g/ml}$  was able to inhibit ferriprotoporphyrin (FP) biomineralization, a *Plasmodium*-specific process of heme detoxification in which FP derived from the digestion of ingested hemoglobin is converted to hemozoin (b-hematin).

The similarity between the  $IC_{50}$  of *P. alliacea* extracts found in *E. histolytica* and *G. lamblia* could be related to similar action mechanisms in these two amitochondriate protozoa. In the case of trypanosomatids and *P. falciparum*, the lower inhibitory concentrations suggest that the mechanism of action could involve mitochondria.

As we previously mentioned, *P. alliacea* contains many secondary metabolites that can produce different biological effects in humans and microorganisms. In the leaves of *P. alliacea*, the presence of steroids, terpenoids (isoarborinol, isoarborinol-acetate, isoarborinol-cinnamate), saponins, polyphenols, and tannins has been reported [16], as well as allantoin, linoleic acid, lignoceryl alcohol, lignoceryc acid, lignoceryl ester,  $\alpha$ -freidelinol, nonadecanoic acid, oleic, palmitic and stearic acids [4], glucosides, and alkaloids

[17]. However, none of the previous studies identified the metabolites responsible for the antiprotozoal activity. Therefore, we decided to investigate which phytochemical molecules are responsible for the antiamoebic activity of *P. alliacea*.

The antiamoebic activity was increased after extract fractioning, as the early fractions (fraction 12-19) showed 74.3% growth inhibition with 0.8 mg/ml, suggesting that active metabolites were concentrated in this fraction. Indeed, we found that it contains isoarborinol ( $C_{30}H_{50}O$ ), a pentacyclic triterpenoid whose molecular weight is 426.7 g/mol. It is a class of 30-carbon isopentenoid (isoprenoid) that can function like sterols, as structural components of membranes [18].

The antiprotozoal activity of other pentacyclic triterpenoids has been previously reported. Maslinic acid, a pentacyclic derivative present in the olive fruit (*Olea europaea*), is capable of blocking the entry of *Toxoplasma gondii* tachyzoites into the cell and can inhibit some of its proteases. It also produces gliding motility and ultrastructure alterations in parasites [19]. Additionally, the antiprotozoal activity of oleanolic acid and its isomer, ursolic acid, has been documented in *P. falciparum* [20–24], *Leishmania donovani*, *L. major*, *L. amazonensis* [25], *L. infantum* [26], and *T. cruzi* [27, 28].

Isoarborinol has been reported in *P. alliacea* extracts, however, its specific antiamoebic activity was not known. Here, we found that isoarborinol has a high effect against *E. histolytica* trophozoites since a concentration of 0.05 mg/ml produced 51.4% growth inhibition. Although this concentration is not comparable to the one of metronidazole ( $IC_{50} = 0.04$   $\mu\text{g/ml}$ ), isoarborinol is not toxic toward human cells at the concentrations effective against amoeba, which could be an advantage over metronidazole toxicity. Therefore, it would be interesting to investigate whether the effects of isoarborinol in amoeba are similar to those produced by other triterpenes in other protozoan parasites. Forthcoming investigations will allow us to obtain information regarding the mechanisms of action of this molecule in *E. histolytica* trophozoites.

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