Antiproliferative potential of *Petiveria alliacea* L extract-loaded nanodispersion against cancer cells

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Highlights

- It was obtained and characterized a concentrated extract from the leaves of *Petiveria alliacea* L, which is ready for use in pharmaceutical formulations
- The concentrated extract from the leaves of *Petiveria alliacea* L, and the nanodispersion loaded with the extract did not exhibit a hemolytic effect on red blood cells
- The *Petiveria alliacea* L extract-loaded nanodispersion demonstrates a strong antiproliferative effect and high selectivity against breast, kidney, and liver cancer cells

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**Abstract:** Cancer is characterized by the uncontrolled growth of unhealthy cells that invade tissues and organs, causing thousands of deaths worldwide. In this work, a nanodispersion loaded with soft extract of *Petiveria alliacea* L. was developed for use as an antitumoral. The nanodispersion was prepared using the polymer deposition-solvent displacement method, with Kollicoat MAE 100P as the matrix former polymer. A nanodispersion with a particle size of 147 nm and high homogeneity of size (polydispersity index 0.162) was obtained, along with a ζ-potential of -10.80 mV. The polymer deposition-solvent displacement method allowed for high encapsulation efficiency (86.25%) and high stability on the shelf for a year. The nanodispersion did not show hemolytic effect and inhibited the growth of liver cancer cells (HepG2) with high selectivity (IG\(_{50}\) 18.08 μg/mL, SI 13.82) was obtained, along with a ζ-potential of -10.80 mV. The polymer deposition-solvent displacement method allowed for high encapsulation efficiency (86.25%) and high stability on the shelf for a year. The nanodispersion did not show hemolytic effect and inhibited the growth of liver cancer cells (HepG2) with high selectivity (IG\(_{50}\) 18.08 μg/mL, SI 13.82). The nanodispersion also showed strong antiproliferation activity and high selectivity against breast cancer cells (MDA-MB-231: IG\(_{50}\) 28.22 μg/mL, SI 8.85) and kidney cancer cells (786-0: IG\(_{50}\) 82.38 μg/mL, SI 3.03). Due to the low values of GI\(_{50}\) and selectivity higher than 3, the polymeric nanodispersion loaded with soft extract of *Petiveria alliacea* L. can be a promising product for the treatment of these three types of cancer. However, further studies will need to be conducted to investigate its actual usefulness in anticancer therapy.
Graphical Abstract

PLANT MATERIAL

PERCOLATION

CANCER

ANTIPROLIFERATIVE EFFECT

- Size of 147 nm
- Polydispersity 0.162
- ζ-potential -10.80 mV

NANOPRECIPITATION

Introduction

Cancer is a group of diseases characterized by the uncontrolled growth of unhealthy cells that invade tissues and organs. In 2022, 609 thousand people died from cancer in the United States. Lung cancer caused most deaths (130,180), followed by colorectal cancer (52,580), pancreatic cancer (49,830), and breast cancer (43,780). Other types of cancer caused 32,990 deaths (National Institutes of Health, 2023). In Brazil, according to the National Cancer Institute (INCA), cancer is the second leading cause of mortality (Instituto Nacional de Câncer, 2022a), causing around 232,000 deaths. Each year, approximately 450 thousand cases are diagnosed, and the majority are treated with chemo and radiotherapies (Instituto Nacional de Câncer, 2022b). However, those therapies produce serious adverse effects on patients due to the low selectivity of antineoplastic substances. Thus, there is a need for new, more effective, and selective antineoplastic drugs and more specific treatments that can control the disease with the least amount of patient suffering.

Petiveria alliacea Lineu (Phytolaccaceae) is a medicinal species widespread in South America, Central America, the Caribbean, and the southeastern United States. It is known in Brazil as guiné, erva guiné, mucuracá, tipí, and amansa senhor (Andrade et al., 2012; Duarte & Lopes, 2005; Lima et al., 1991). The traditional communities of those regions have been incorporating the cultivation of this plant in small domestic gardens for its anti-inflammatory properties, especially against rheumatism (Sousa et al., 1990; Low et al., 1999). Hydroalcoholic extracts from the leaves hold tannins, phenols, flavonoids, and coumarins (Taylor, 2002). The roots are rich in sulfur compounds (Taylor, 2002). The leaves contain mineral elements such as selenium, zinc, copper, iron, and magnesium that could contribute to their immunomodulatory effect (Batista et al., 2011). The hydroalcoholic extract from the leaves has analgesic, antipyretic, and anti-inflammatory activity (Germano et al., 1993; Roig, 1974), as well as antimicrobial activity (Benevides et al., 2001), and antifungal activity (Kim et al., 2006). Amino acids, glutamic dipeptides, and cysteine sulfoxide derivatives are other types of compounds present in the roots and stems of this plant (Benevides et al., 2001; Kubec et al., 2002; Kubec & Musah, 2001).

The extracts of Petiveria alliacea L. leaves have shown antitumoral activity (Hernández et al., 2014; Marini et al., 1993). However, regardless of the traditional use of Petiveria alliacea extracts as an antitumor, there is no consensus on which part of the species must be used or the possible mechanisms of the antitumor activity.

Nanotechnology allows the development of drug-based preparations that often exhibit more potent activity than the drug in its natural form (i.e., non-nanoparticulate). Nanoformulations usually show pharmacological activities not observed in the counterpart drugs (Rodriguez Amado et al., 2017). Polymeric nanocapsules and nanospheres enhance drug solubility, bioavailability, and stability, especially in vegetal extract-based nanoparticles (Florentino Neto et al., 2021; Rodriguez Amado et al., 2017).
Petiveria alliacea L. has shown pharmacological activities, including hypoglycemicant and cytotoxic in cancer cells. However, there are any preparations of leaves of the species in the literature. No studies have been conducted for using extracts of Petiveria alliacea L as an active ingredient in nanoformulations, which could enhance the stability and bioavailability. Additionally, nanoformulation could add value to the culture of this plant. Thus, we developed a polymeric nanodispersion of the 70% hydroalcoholic extract of the leaves of Petiveria alliacea L. We also evaluated the antiproliferative effect of nanoparticles in cancer cell lines of breast, kidney, prostate, and intestinal cancer.

Material and methods

Plant material

The fresh leaves of the plant were acquired early in the morning in a local market on the outskirts of Campo Grande, MS, Brazil (-20°33'49" S, -54.34'35" W). They were dried in the shade until a constant weight was reached, grounded in a knife mill (~3-5 mm), and used immediately.

Soft extract preparation

First, the fluid extract was prepared by simple percolation (1 kg of drug/1 L of 70% ethanol). The dried and milled leaves were soaked with 15% of the solvent for two hours at room temperature. The maceration time of the drug in the percolator was 24 hours. The percolation rate was 5 mL/minutes. The soft extract was obtained by vacuum concentration of the fluid extract using a rotary evaporator at 45 °C until a drug-solvent ratio of 4:1 (g/mL). The extract was placed in an amber bottle and stored at room temperature, protected from light.

Organoleptic properties

Color, odor, and the appearance of the fluid extract were evaluated according to the Brazilian Pharmacopeia (Agência Nacional de Vigilância Sanitária, 2019).

Phytochemical evaluation

A phytochemical screening was conducted for the qualitative identification of metabolites present in the fluid extract and the soft extract (World Health Organization, 1998).

Physic-chemical properties

Total solids, relative density, total ashes, and the refractive index were evaluated (Agência Nacional de Vigilância Sanitária, 2019; United State Pharmacopeia, 2019). The pH was measured directly using a pH meter (Tecnopon, Brazil), previously calibrated with buffer solutions (pH 4 and 7; Alphatec, Brazil).

Quantitation of phenols

Total phenols (as pyrogallic acid) were determined spectrophotometrically (British Pharmacopeia, 2019). All measurements were performed in triplicate.

Quantitation of flavonoids by HPLC

HPLC analyses were conducted in a Perkin-Elmer chromatograph (USA) equipped with an automatic injector and a UV detector at 360 nm. It was used a Phenomenex C-18 column, 10µm (3.9 x 300 mm) at 40 °C. As mobile phase, methanol-water (1:1) acidified with phosphoric acid to a pH of 2.8 was used. The flow rate was 1 mL/minute. Rutine (Merck, USA) was used as internal standard. For samples preparation 0.1 g of the soft extract was dissolved in 10 mL of the mobile phase. Then, the solution was filtered through a 0.45 Millipore membrane (USA), and 10 uL were injected into the chromatographic system.

ICP-MS

The elemental analysis of Petiveria alliacea L. soft extract was performed by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS). The quantitation was made in SPECTRO ARCOS (Germany) equipment equipped with an axial-view device. All reagents used were from Merck (USA) with spectral quality. For the sample preparation, in a 500 mL volumetric flask, 1.0 g of the fluid extract, 10 mL of distilled water, and 10 mL of concentrated nitric acid were added. The flask was vigorously shaken for 5 minutes and diluted to the mark with distilled water. Aliquots of 3 mL were placed in volumetric flasks of 25 mL, and their volume was completed with deionized water, with conductivity lower than 0.1 µΩ/cm. Calibration curves were constructed to determine each element (Ca, Ba, Ni, Mg, Zn, Cu, Fe, Sn, Si, Pb, Se, Fe, Mn, Bi, Ge, Hg, Al) using appropriated salts. Results were expressed as ppm.

Formulation of the nanodispersion

The nanodispersion was prepared by the polymer deposition/solvent displacement method (Fessi et al., 1989), with modifications. The organic phase was prepared as follows: one gram of polymer (Kollicoat MAE 100P, kindly donated by BASF) and Span 80 (0.5 g) were dissolved in a mixture of 20 mL of 96% ethanol and 10 mL of acetone under magnetic stirring at 500 rpm (Fisaton, SP, Brazil) for 20 minutes. At the same time, an amount of Petiveria alliacea L. soft extract equivalent to 2g of soluble solids was dissolved in 10 mL of 70% ethanol and kept under magnetic stirring (200 rpm). The extract solution was filtered using a Millipore® membrane (45 um) attached to a plastic syringe. A volume of 5 mL of the filtrated extract was slowly dropped into the polymer solution maintaining the agitation (500 rpm) for an additional 10 minutes. The aqueous phase, containing MilliQ water (50 mL) and Tween 80 (0.5 g), was mixed at 400 rpm in a magnetic stirrer. Subsequently, the organic phase was dropped onto the aqueous phase. The stirring was kept for additional 20 minutes, and then
the mixture was homogenized in an ultrasonic apparatus using a potency of 8 Watt, for 5 minutes. Then, the nanodispersion was concentrated in a vacuum rotary evaporator (Buchi, Switzerland) at 45 °C, to remove the ethanol and form the nanospheres in suspension. Volume was adjusted to 50 mL with MilliQ water. The nanodispersion was left at room temperature for 24 hours until its characterization was performed.

**Particle Size and Polydispersity Index**

The particle size and polydispersity index were measured by Photon Correlation Spectroscopy using a Zetasizer (Malvern, UK). The nanoparticles were diluted 1:9 (v/v) in water for injection and filtered through a Millipore® membrane (0.45 mm). The measurement was performed using a laser wavelength of 633 nm, with a scattering angle of 173 ° at 25 °C. Measures were taken in triplicate.

**ζ-potential**

The ζ-potential and conductivity were measured on a Zetasizer (Malvern, UK). The samples were dissolved in distilled water (1:9). The test was performed at 25 °C using a voltage of 150 V (Rodriguez Amado et al., 2017). The assay was made in triplicate.

**Encapsulation efficiency**

A sample of the nanosuspension (3 mL) was placed in 3 mL ultrafiltration tubes and centrifuged for 15 minutes at 21 krpm using an ultracentrifuge (Optima XPN 80, Brazil). The supernatant was discarded, and the separated nanospheres in the membrane were washed twice with HCl 0.01 mol/L using a vortex stirrer for resuspension and centrifuged each time at 21 krpm for 15 minutes. Afterward, they were dissolved in 1 mL of PBS buffer (pH 7.4) at 45 °C and filtered through a 0.45 um (Millipore, USA) membrane. Then, 10 µL of the filtrate was injected into the chromatographic system. The tests were done in triplicate, using the previously described chromatographic system. The encapsulation efficiency was determined using the following expression:

\[
EE (%) = \left( \frac{(QFN) \times 100}{QFI} \right)
\]

Where QFN is the quantity of flavonoids in the nanospheres, and QFI contained in the extract.

**Effect of pH**

The automatic titrator (MPT-2 Malvern, UK) was coupled to the Zetasizer to evaluate the effect of pH on particle size and ζ-potential. Sodium hydroxide (0.1 mol/L) and hydrochloric acid (0.1 mol/L) were used as titrating solutions. The instrument was calibrated with buffer solutions (pH 4, pH 7, pH 10). The measurements were performed in triplicate at 25 °C.

**Effect of temperature**

The effect of temperature (between 20 and 70 °C) on particle size and ζ-potential was evaluated. The nanosuspension was heated between 20 and 70 °C at intervals of 10 °C. The sample was held at each temperature for 5 minutes before measurement. The particle size, polydispersity index and ζ-potential were measured in triplicate, and the results were expressed as the mean ± standard deviation. The analysis was performed in a Zetasizer (MALVERN, UK). The measurements were performed in triplicate (Rodriguez Amado et al., 2017).

**In-shelf stability**

The in-shelf stability (25-27 °C, relative humidity 50-65%) of the nanodispersion was evaluated over the course of one year. The pH of the nanodispersion was evaluated using a pH meter (Tecnopro, Brazil) previously calibrated with buffer solutions (Alphatech, Brazil). Particle size, polydispersity index, ζ-potential, and encapsulation efficiency were measured on days 0, 15, 30, 45, 60, 180, and 360 after the preparation. The measures were made in triplicate.

**Hemolytic activity**

The hemolytic activity was assessed on the erythrocytes of healthy Wistar male rats 8 weeks old, following the protocol of the Animal Testing Ethics Committee n° 019/2016 of the Federal University of Amazonas, Brazil. For this, 2 mL of blood obtained by tail vein puncture from the animals was placed in heparinized tubes. The blood was centrifuged at 3000 rpm at 5 °C for 15 minutes to obtain the erythrocyte concentrate. After centrifugation, the concentrated was washed with phosphate buffer (PBS; pH 7.4). The procedure was done three times. Finally, the erythrocyte concentrate was suspended in 50 mL of phosphate buffer solution (pH 7.4). Then, 1 mL of erythrocyte suspension was placed in 2 mL test tubes, and at once was added 0.5 mL of the crude extract of Petiveria alliacea L. at concentrations of 0.5, 5, 50, 500 μg/mL. PBS buffer (solvent for erythrocyte dilution) was used as a negative control, and Triton X100 (0.1 and 1 µg/mL) were used as a positive control. The tubes were incubated for one hour at 37 °C. After incubation, the tubes were centrifuged, the supernatant collected, and the absorbance was immediately measured at 540 nm. The hemolytic activity (% of Hemolysis) was calculated using the following formula:

\[
% \text{ of Hemolysis} = \left( \frac{AM - AS}{AC - AS} \right) \times 100
\]

Where: AM is the absorbance of the sample; AS is the absorbance of the solvent, and AC is the absorbance of the positive control. The test was performed in triplicate.

**Evaluation of antiproliferative activity in tumor cells**

Cell lineages used for this assay were: HFF1, normal human foreskin fibroblasts; PC-03, human prostate tumor cells; NIH/3T3, murine fibroblasts; 786-0, human renal adenocarcinoma cells; MCF-7, breast cancer tumor cells;
HepG2, hepatocellular carcinoma cells; and MDA-MB 231, triple-negative breast cancer cells.

The HepG2, HFF-1, and NIH/3T3 cell lines were grown in DMEM medium (Dulbecco’s Modified Eagle Medium, Gibco-Invitrogen, Brazil). The MCF-7, MDA-MB-231, and PC-3 cells were grown in RPMI 1640 medium (Roswell Park Memorial Institute, Gibco-Invitrogen, Brazil) supplemented with 10% inactivated fetal bovine serum (Gibco-Invitrogen, Brazil) and maintained under appropriate conditions (37 °C, 5% of CO₂, and humidity) until the ideal cell density for plating (5x10³/well) was obtained (Skehan et al., 1990). After 24 hours of adaptation, the cells were treated for 48 hours with concentrations of 0.25 to 250 µg/mL of nanodispersion and 0.025 to 25 µg/mL of doxorubicin. For cell fixation, 20% trichloroacetic acid was used, and after rinsing and drying, they were stained with sulforhodamine B at 0.1%. DMSO was used as a negative control, doxorubicin as a positive control, and a blank nanodispersion (nanodispersion prepared without the soft extract) was used as a blank and zero-time reading (before treatment). The optical density was measured at 540 nm in a 96-well microplate reader (Spectramax 190). The growth percentage was calculated using the Monks’ formula (Monks et al., 1991). Origin 6.0 software was used to calculate the GI₅₀. Results were expressed as the mean ± standard deviation of three independent experiments (n = 3).

**Selectivity Index (SI)**

The selectivity index (SI) was calculated by the ratio of the GI₅₀ of the nanodispersion on the non-tumoral cell line (NIH/3T3) and the GI₅₀ of the nanodispersion on tumor cell lines (Indrayanto et al., 2021). The calculation of the selectivity index was done according to the expression:

$$SI = \frac{GI_{50}^{NIH/3T3}}{GI_{50}^{non-potastic cells}}$$

(3)

**Results**

Qualitative assays suggest that extracts contain a high concentration (+++) of phenols and flavonoids, as well as moderate amounts of triterpenes, steroids, quinones, and alkaloids (++). Saponins were also detected, but in low quantities (+). The soft extract exhibited a higher concentration of metabolites in all cases, as indicated by its more intense color and/or precipitation weather compared with the more diluted fluid extract. The exception was saponin, which was present in both extracts but formed a poor amount of foam, indicating a low concentration.

The soft extract of *Petiveria alliacea* L exhibited a high concentration of phenolic compounds (9.81 mg/mL) and flavonoids (6.30 mg/mL) (Table 2).

### Table 1. Phytochemical screening of fluid and soft extracts of Petiveria alliacea L.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Assay</th>
<th>Positive evidence</th>
<th>FE</th>
<th>SE</th>
</tr>
</thead>
</table>
| Flavonoids           | H₂SO₄ conc.            | Intense Yellow    | (+)| (+++)
|                      | Shinoda                | Intense red       | (+)| (+++)
| Phenols & Tannins    | FeCl₃                  | Dark green        | (++)| (+++)
| Saponins             | Foam formation         | Persistent foam   | (+)| (+) |
| Quinones             | Borntrager             | Pink color        | (+)| (+) |
| Triterpenes & steroids | Liebermann-Burchard   | Dark green        | (+)| (+) |
| Alkaloids            | Dragendorff            | Red precipitate   | (+)| (+) |

**Legend:** (+) indicates a low quantity of metabolites, (++ moderate amount; and (+++) high amount of metabolite.

### Table 2. Physicochemical properties of the soft extract of Petiveria alliacea L.

<table>
<thead>
<tr>
<th>Properties</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aroma</td>
<td>Garlic</td>
</tr>
<tr>
<td>Color</td>
<td>Deep brown</td>
</tr>
<tr>
<td>Appearance</td>
<td>Viscous liquid</td>
</tr>
<tr>
<td>Relative density (25 °C)</td>
<td>1.244 ± 0.005</td>
</tr>
<tr>
<td>Refraction index (25 °C)</td>
<td>1.392 ± 0.037</td>
</tr>
<tr>
<td>pH</td>
<td>5.51 ± 0.5</td>
</tr>
<tr>
<td>Total ash (%)</td>
<td>0.20 ± 0.08</td>
</tr>
<tr>
<td>Total solids (g/100 mL)</td>
<td>58.44 ± 2.42</td>
</tr>
<tr>
<td>Flavonoids (g/100 mL, as rutine)</td>
<td>6.30 ± 1.25</td>
</tr>
<tr>
<td>Phenolics (g/100 mL, as pyrogalic acid)</td>
<td>9.81 ± 0.88</td>
</tr>
</tbody>
</table>
The soft extract of *Petiveria alliacea* L. showed a rich mineral composition, including iron, that was the major element (365.85 ppm), calcium (335.27 ppm), magnesium (325.50 ppm), copper (150.55 ppm), zinc (135.17 ppm), selenium (75.24 ppm), bismuth (35.45 ppm), manganese (26.25 ppm), germanium (15.95 ppm), nickel (6.25 ppm), silica (3.25 ppm) and aluminum (3.05 ppm).

After 24 hours of preparation, the nanodispersion had a light brown color, and was translucent, was slightly opaque. It also had a weak garlic aroma, which is characteristic of the plant.

<table>
<thead>
<tr>
<th>Properties</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle size (nm)</td>
<td>147.80 ± 0.55</td>
</tr>
<tr>
<td>Polydispersity index</td>
<td>0.162 ± 0.013</td>
</tr>
<tr>
<td>ζ-potential (mV)</td>
<td>-10.80 ± 0.40</td>
</tr>
<tr>
<td>pH (25 °C)</td>
<td>4.55 ± 0.28</td>
</tr>
<tr>
<td>Encapsulation efficiency (%)</td>
<td>86.25 ± 2.57</td>
</tr>
</tbody>
</table>

The pH of nanodispersion was 4.55, with a particle size of 147.80 nm, the polydispersity index was 0.162, and the ζ-potential was -10.80 mV (Table 3). The preparation process showed a high encapsulation efficiency of 86.25%. The narrow particle size distribution and a uniform ζ-potential of the *Petiveria alliacea* nanodispersion after 24 h of preparation were observed (Figure 1).

The particle size of the *Petiveria alliacea* L. nanodispersion remained constant at around 160 nm at a pH below 4. Between pH 4 and 6, there was a significant increase in particle size (up to 550 nm) and polydispersity (which reached a value of 0.910 at pH 6), showing a fully polydisperse system (Figure 2). Between pH 6 and 8, the particle size decreased to remain constant at around 40 nm. From pH 6 onwards, the nanodispersion was fully polydisperse, with polydispersity values close to 1.

Changes in pH from 2 to 5 produced an increase (i.e., a decrease in magnitude) in the ζ-potential from -17 mV at pH 2 to 1.5 mV at pH 5. From pH 5 onward, the ζ-potential decreased to zero and remained constant up to pH 9.

The particle size remained practically constant between 20 and 40 °C (Figure 3A). At temperatures above 40 °C, the size increased to 450 nm at 60 °C. Similarly, temperatures between 20 and 50 °C produced an increase (in moduli) in the ζ-potential from -12 mV to zero. Beyond 50 °C, the nanoparticles lost their electric charge (Figure 3B).

**Figure 1.** Particle size and ζ-potential of *Petiveria alliacea* L. nanodispersion, 24 hours after the preparation.

**Figure 2.** Effect of pH on particle size and ζ-potential of *Petiveria alliacea* L. nanodispersion, 24 hours after preparation. (Polydispersity index in parentheses).
Along a year, the pH remained practically unchanged (Table 4). The particle size decreased slightly from 147.75 nm (t = 0) to 141.30 nm (t = 360), and the polydispersity index diminished from 0.162 (t = 0) to 0.93 (t = 360), indicating a more homogeneous particle size distribution over time. The ζ-potential and encapsulation efficiency remained practically constant throughout the year. Figure 4 shows the distribution of particle size (Figure 4A) and ζ-potential (Figure 4B) of nanodispersion one year after preparation.

Neither the extract nor the nanodispersion showed any hemolytic effect on erythrocytes (Figure 5). However, the positive control (Triton, at a concentration of 1 µg/mL) caused 97% hemolysis in erythrocytes.

### Inhibition of tumor cell proliferation

The nanodispersion was highly effective on the liver cancer cell HepG2 (G1₀ 18.08 µg/mL) and on the triple-negative breast cancer strains MDA-MB-231 (G1₀ 28.22 µg/mL) and MCF-7 (G1₀ 65.01 µg/mL) (Table 5).

The nanodispersion showed good selectivity indexes (SI) over the studied strains. The highest selectivity was on liver carcinoma cells (HepG2) with 13.82, followed by breast cancer cells (MDA-MB 231 and MCF-7) with 8.85 and 3.84, respectively. On human renal adenocarcinoma cells (786-0) was 3.03, and on prostatic tumor cells (PC-03), was 2.51.

### Table 4. Stability study in-shelf of the nanodispersion of *Petiveria alliacea* L.

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>pH</th>
<th>Size (nm)</th>
<th>Polydispersity index</th>
<th>ζ-potential (mV)</th>
<th>E. Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.55 ± 0.10</td>
<td>147.75 ± 0.55</td>
<td>0.162 ± 0.013</td>
<td>-10.80 ± 0.40</td>
<td>86.67 ± 0.80</td>
</tr>
<tr>
<td>15</td>
<td>4.50 ± 0.20</td>
<td>146.25 ± 1.33</td>
<td>0.171 ± 0.085</td>
<td>-10.25 ± 0.81</td>
<td>86.28 ± 0.31</td>
</tr>
<tr>
<td>30</td>
<td>4.52 ± 0.30</td>
<td>146.00 ± 0.95</td>
<td>0.172 ± 0.092</td>
<td>-10.65 ± 0.95</td>
<td>86.80 ± 0.31</td>
</tr>
<tr>
<td>45</td>
<td>4.60 ± 0.30</td>
<td>143.01 ± 1.81</td>
<td>0.101 ± 0.010</td>
<td>-10.92 ± 0.21</td>
<td>86.21 ± 0.54</td>
</tr>
<tr>
<td>60</td>
<td>4.55 ± 0.50</td>
<td>143.27 ± 1.75</td>
<td>0.102 ± 0.015</td>
<td>-10.98 ± 0.33</td>
<td>86.95 ± 0.75</td>
</tr>
<tr>
<td>180</td>
<td>4.55 ± 0.30</td>
<td>141.80 ± 1.12</td>
<td>0.099 ± 0.024</td>
<td>-11.02 ± 0.15</td>
<td>86.05 ± 0.47</td>
</tr>
<tr>
<td>360</td>
<td>4.55 ± 0.20</td>
<td>141.30 ± 1.55</td>
<td>0.093 ± 0.005</td>
<td>-11.28 ± 0.25</td>
<td>86.37 ± 1.02</td>
</tr>
</tbody>
</table>

**Figure 3.** Effect of temperature on particle size and ζ-potential of *Petiveria alliacea* L. nanodispersion 24 hours after preparation.

**Figure 4.** Particle size distribution and ζ-potential of the *Petiveria alliacea* L. nanodispersion after one year of preparation.
The leaves were chosen as raw material because they showed antitumor activity in vitro (Marini et al., 1993). In addition, dried and ground leaves (125 μm) are used in a tablet formulation approved by the Cuban Health Authority (CECMED) as an immunostimulant in cancer patients. Thus, antitumor preparations made with the extracts from leaves could have a double action, which would be highly beneficial for patients. However, powdered leaves can only be used in solid pharmaceutical forms, limiting the possibility of use in other pharmaceutical forms.

The fluid extract has a deep brown color with a garlic aroma characteristic of the plant. Flavonoids, phenols, and tannins; saponins; quinones; triterpenes and steroids; and alkaloids were identified in both the fluid extract and the soft extract. The increase in color intensity and/or quantity precipitated in the correspondent assay with the soft extract, as compared with the fluid extract, indicates a higher concentration of metabolites. That is a consequence of the concentration process from a ratio of 1:1 to 4:1 (gram of dried drug to solvent). The signal observed for saponins in both extracts was weak, indicating a low concentration of these compounds in the extracts.

A qualitative composition similar to the fluid extract obtained in this work has been reported (Akintan & Akinneye, 2020). However, to the best of our knowledge, there are no reports on the qualitative and quantitative composition of the soft extract of Petiveria alliacea L., making this the first report on this preparation. The presence of phenols, tannins, flavonoids, and alkaloids could be responsible for the anti-inflammatory and analgesic activity observed by the population; probably mediated by antioxidant mechanisms. The antitumor activity of the leaves could be attributed to the presence of triterpenes, alkaloids, and flavonoids (Andrade et al., 2012; Olajubutu et al., 2022).

The soft extract of Petiveria alliacea L. leaves contains 9.81 g/100 mL of phenolic compounds and 6.30 g/100 mL of flavonoids. These quantities of phenols and flavonoids are higher when compared to the amounts of these metabolites in other soft extracts reported in the literature. For example, the standardized soft extract of Tamarindus indica L. (Rodriguez et al., 2012) contained 6.19 g/100 mL of phenolics and 0.92 g/100 mL of flavonoids. However, there are few reports on the characterization and standardization of vegetal soft extracts to be used as active ingredients in pharmaceutical preparations. Values reported in Table 2 could be used, preliminarily, as criteria for the quality control of the soft extract of Petiveria alliacea L. leaves.

The mineral elements present in the soft extract were evaluated after total ash determination. Iron was the most abundant mineral, with a concentration of 365.85 ppm. This fact was expected due to the soil composition in the region where the plant material was collected (according to seller information). The soil of the Mato Grosso do Sul region in Brazil is known to be rich in iron and copper (Marini et al., 1993). Calcium ranked second with 335.27 ppm, followed by magnesium at 325.5 ppm. Similarly, the presence of calcium, magnesium, and other elements such as Al, Si, Bi, Mn, Zn, Cu, Ni, Se, and Ge is the result of the composition of the regional soil.

Of particular importance is the presence of nickel at 6.25 ppm, zinc at 135.17 ppm, selenium at 75.24 ppm, and germanium at 15.95 ppm. These microelements participate as enzymatic cofactors of the antioxidant system in the

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Doxorubicin GI&lt;sub&gt;50&lt;/sub&gt; (μg/mL)</th>
<th>Nanodispersion GI&lt;sub&gt;50&lt;/sub&gt; (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH/3T3, normal murine fibroblasts</td>
<td>3.89 ± 0.41</td>
<td>&gt;250</td>
</tr>
<tr>
<td>HFF-1, normal fibroblasts of human foreskin</td>
<td>2.45 ± 0.03</td>
<td>98.79 ± 1.25</td>
</tr>
<tr>
<td>HepG2, liver carcinoma cells</td>
<td>0.25 ± 0.02</td>
<td>18.08 ± 0.36</td>
</tr>
<tr>
<td>MCF-7, breast cancer cells</td>
<td>0.19 ± 0.01</td>
<td>65.01 ± 0.40</td>
</tr>
<tr>
<td>MDA-MB-231, triple negative breast cancer cells</td>
<td>1.51 ± 0.03</td>
<td>28.22 ± 1.02</td>
</tr>
<tr>
<td>PC-3, Prostatic cancer cells</td>
<td>0.28 ± 0.01</td>
<td>97.47 ± 1.13</td>
</tr>
<tr>
<td>786-0, human renal adenocarcinoma cells</td>
<td>0.26 ± 0.02</td>
<td>82.38 ± 2.99</td>
</tr>
</tbody>
</table>

GI<sub>50</sub> (concentration that caused 50% growth inhibition after 48 hours of treatment).

Figure 5. Hemolytic effect of Petiveria alliacea L. soft extract and the nanodispersion loaded with the extract.

Discussion

The leaves were chosen as raw material because they showed antitumor activity in vitro (Marini et al., 1993). In addition, dried and ground leaves (125 μm) are used in a tablet formulation approved by the Cuban Health Authority (CECMED) as an immunostimulant in cancer patients. Thus, antitumor preparations made with the extracts from leaves could have a double action, which would be highly beneficial for patients. However, powdered leaves can only be used in solid pharmaceutical forms, limiting the possibility of use in other pharmaceutical forms.

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human body: their presence could be associated with the immunomodulatory activity of *Petiveria alliacea* L. leaves (Klein & Ladeira, 2004). None of the quantified microelements are present in concentrations that could be considered toxic (Food and Agriculture Organization, 2002).

The soft extract of the leaves of the species *Petiveria alliacea* L., obtained by percolating using a hydroalcoholic solution of 70% followed by vacuum concentration at 45 °C, is suitable for use in all types of pharmaceutical forms.

The physicochemical characterization of the nanodispersion was conducted 24 hours after its preparation to ensure that the color, transparency, and aroma characteristics observed in the newly prepared nanodispersion did not change during the first 24 hours, which is an indicator of stability and the quality of the preparation process.

The nanodispersion exhibited a light brown, translucent color, almost transparent, that was more transparent than the day before. The weak garlic aroma was almost imperceptible, indicating that the extract was entrapped in nanoparticles. The pH of the nanodispersion was 4.55, according to the presence of acidic compounds in the extract that were not entrapped and could be dangling on the particle’s surface. The weak acidity could also be attributed to the weak acid properties of the polymer, which is a mixture of methacrylic acid and ethyl methacrylate in a 1:1 ratio (BASF Pharm, 2019).

The preparation process allowed obtain nanoparticles (nanospheres dispersed in water with the help of Tween 80) with a size of 147.8 nm and a low polydispersity index of 0.162, as shown in Figure 1A. The lower the polydispersity index, the greater the homogeneity of the particle size. High homogeneity in particle size allows for greater accuracy in dosage, particularly in cases where the dose must be based on surface area, such as in pulmonary applications. The nanoparticles showed a negative ζ-potential (-10.80 mV, Figure 1B), which can be attributed to the anionic polymer used, Kollicoat® MAE 100P, as other nanoparticles produced with this polymer have shown negative ζ-potential (Florentino Neto et al., 2021; Rodriguez Amado et al., 2017).

The preparation process showed a high encapsulation efficiency of 86.25%, which can be considered excellent (Cheong et al., 2008; Organisation for Economic Co-operation and Development, 2009; Florentino Neto et al., 2021; Rodriguez Amado et al., 2017). A similar result in terms of entrapped efficiency using the same polymer and methodology was reported (Florentino Neto et al., 2021).

The pH strongly affected the particle size and ζ-potential of the *Petiveria alliacea* L. nanodispersion. At pH < 4, the particle size kept constant, around 160 nm (Figure 2A). This behavior has been observed in nanoparticles coated with polymer derivatives of methacrylic acid, such as Kollicoat MAE 100P (Florentino Neto et al., 2021; Rodriguez Amado et al., 2017). When the pH is higher than four, the particle size and polydispersity index increase, reaching 550 nm at pH 6, with a polydispersity index close to one. This behavior indicates that the nanoparticles begin swell and permeabilize on forward pH 4, allowing the liquid to penetrate their interior. This process breaks the particles and is more intense as the pH is more basic (pH > 6). In this process, larger particles break off to form smaller particles, resulting in a complete polydisperse system.

The statements in the previous paragraph can be supported by observing the behavior of the ζ-potential (Figure 2B). As the pH increases, the ζ-potential increases (decreases in modular value) until it loses the electric charge at pH 6 (ζ-potential equal to 0). The loss of the electric charges of particles occurs due to a process of solubilization of the active ingredient after the particles break down into smaller particles, increasing the dielectric constant of the medium and causing the electric double layer around the particles to no longer exist (Samimi et al., 2019).

Temperature strongly affects the nanodispersion particle size and ζ-potential (Figure 3). Heating above 40 °C produces a significant increase in size and ζ-potential. At 50 °C, the particle loses its electric charge. The ζ-potential of a nanoparticle is the electric potential at the particle shear plane, the point that separates the nanoparticle from the surrounding medium. It depends on the composition and characteristics of the liquid medium in which the nanoparticles are dispersed (Samimi et al., 2019).

The ζ-potential is a property that contributes to the stabilization of nanoparticulate system, and the higher its absolute value, the more stable the systems (Samimi et al., 2019). Temperatures higher than 40 °C increased the nanoparticle size, causing an increase in liquid permeability inside the particles, increasing the solubility of the extract and its release into the liquid medium. Thus, the concentration of molecules increased in the liquid medium, affecting the surface structure of the particles, viscosity, and ionic strength. In that process, the double electric layer disappears (Samimi et al., 2019), and particles lose their charge at temperatures above 50 °C (Figure 3B).

The nanodispersion kept its properties intact over a period of one year (Table 4). All parameters evaluated in the shelf stability study remained almost constant. The stability of pH suggests that the liquid medium is in equilibrium, keeping the composition intact. This fact suggests that there was no leakage of extract from the core of nanoparticles to the medium, which indicates a consolidated nanoparticle structure. The pH stability between 4.50 and 4.60 warrants the integrity of the nanoparticle, maintaining the encapsulation efficiency almost constant (86.75 ± 0.80%) at time 0 and (86.37 ± 1.02%) after a year. In the same way, decreasing the polydispersity index along a year suggests a more homogeneous particle size over time due to the establishment of a kinetic equilibrium in the nanodispersed system. This fact could be supported by the slight decrease in particle size (from 147.75 nm at time 0 to 141.30 nm after one year). The results agree with those reported for nanoparticles loaded with loratadine and recovered using Kollicoat MAE 100P (Rodriguez Amado et al., 2017). In Figure 4A, particle size distribution and ζ-potential showed a narrow base, indicating homogeneity in both parameters (Figure 4B), which suggests a good stability of particles in the nanodispersion after 12 months of preparation.

The findings suggest that for optimal stability of the *Petiveria alliacea* L. nanodispersion, storage at temperatures below 30 °C and pH 5 is recommended, as these conditions allow for excellent stability for up to one year.

Saponins are substances characterized by hemolytic activity, causing lysis in erythrocytes (Fibach & Rachmilewitz, 2008). Saponins cause hemolysis by membrane permeation, inducing the formation of free radicals within cells (Fibach & Rachmilewitz, 2008; Grisson & Berlin, 2013). The presence of saponins in plant extracts always concerns researchers.
For that reason, the hemolytic effect of both the soft extract and the soft extract-loaded nanodispersion was assessed. Figure 5 shows that, despite containing saponins, neither the soft extract nor the nanodispersion at concentrations up to 500 μg/mL caused hemolysis.

Several studies show that strong antioxidant substances such as phenols and flavonoids protect cells from the hemolytic effect of saponins. The unlocalized electron cloud of phenolics and flavonoids could neutralize free radicals and reduce the stress caused by saponins (Fibach & Rachmilewitz, 2008; Grisson & Berlin, 2013). The high concentrations of phenolic compounds and flavonoids in the soft extract of *Petiveria alliacea* protected erythrocytes from the potential hemolytic effect of saponins. The same process can occur with nanoparticles loaded with the extract of *Petiveria alliacea*, as the nanodispersion also did not cause hemolysis during this assay (Figure 5).

The nanodispersion loaded with the extract from the leaves of *Petiveria alliacea* showed potent cytotoxicity against the HepG2 liver cancer cells (GI$_{50}$ of 18.08 μg/mL) and high selectivity (SI of 13.82). It also showed potent activity against the triple-negative breast cancer cell MDA-MB-231 (GI$_{50}$ 28.22 μg/mL, SI of 8.5) and the MCF-7 breast cancer cell (GI$_{50}$ 65.01 μg/mL, SI of 3.84). It also exhibited moderate cytotoxic activity against the human renal adenocarcinoma cell (786-0) and the prostate tumor cell line PC-03.

The chemical composition of the extract could be responsible for the antiproliferative effect of *Petiveria alliacea* extract-loaded nanodispersion. Flavonoids produce strong antioxidant and anti-inflammatory effects that can protect cells from the oxidative stress caused by cancer. For example, liver cancer produces inflammation and high oxidative stress (Gomes et al., 2013). Flavonoids reduce lipid peroxidation and reduce liver inflammation (Rodriguez et al., 2012). They can also induce apoptosis in cancer cells (Abotaleb et al., 2018; Ajji et al., 2020). Triterpenes have a strong antioxidant, anti-inflammatory, and immunomodulatory effect and can induce cell apoptosis in breast cancer cells via COX-2/PGE2 (Han et al., 2013). Lastly, the alkaloids present in the extract may contribute to the antiproliferative activity of the studied cancer cells (Iqbal et al., 2017). On the other side, those metabolites are enclosed in particles of nanometric size, which commonly potentiate the effect of the encapsulated actives by different mechanisms (Florentino Neto et al., 2021; Rodriguez Amado et al., 2017).

Some kinds of alkaloids can inhibit cell proliferation by inducing autophagic cell death and causing cell cycle arrest in the G1 or G2/M phase, inhibiting the growth of breast cancer cells (Hu et al., 2022). The phytochemicals of all the metabolites contained in the soft extract of *Petiveria alliacea* L. and in the nanodispersion loaded with this extract can act synergistically to inhibit the proliferation of cancer cells (Ayaz et al., 2022). The activity in these processes can be potentiated by the nanometric size and by a slower release of the active compound (Florentino Neto et al., 2021; Rodriguez Amado et al., 2017).

In general way, the use of IC$_{50}$, EC$_{50}$, and GI$_{50}$ data without the inclusion of SI values of bioactive substances have limited value (Cos et al., 2006). It is accepted that substances with GI$_{50} < 100$ μg/mL are active (promising), while substances with GI$_{50} > 100$ μg/mL can be considered moderately active, and substances with GI$_{50} > 250$ μg/mL are inactive (Cos et al., 2006; Indrayanto et al., 2021).

There is no consensus on the ideal value for SI. Some authors recommend that substances with an SI > 10 can be considered promising for further investigation (Awouafack et al., 2013; Peña-Morán et al., 2016). On the other hand, Weerapreeyakul et al. (2012) suggest that antitumor substances with a SI > 3 can be considered promising and should be further investigated. In the literature, all these authors agree that the higher the SI, the more active the substance evaluated is on cancer cells compared to normal cells. This suggests the absence of toxicity in normal cells and a reduction in side effects (Indrayanto et al., 2021).

The nanodispersion loaded with soft extract from the leaves of *Petiveria alliacea* L. showed SI > 3 in liver carcinoma cells, breast cancer cell and renal adenocarcinoma cells. Considering this fact, this nanodispersion can be considered promising for three types of cancer that cause the most deaths worldwide: liver, breast, and kidney cancer.

**Conclusions**

This paper describes the process of obtaining, characterizing, and evaluating the antiproliferative activity of a polymer nanodispersion loaded with the soft extract of *Petiveria alliacea* L. The particle size obtained was 147 nm with high homogeneity of size (polydispersity index, 0.162) and a ζ-potential of -10.80 mV. The polymer deposition-solvent displacement method allowed for high encapsulation efficiency (86.25%) and high stability in-shelf for a year. The nanodispersion did not show any hemolytic effect and inhibited the growth of liver cancer cells (HepG2) with high selectivity (IG$_{50}$ 18.08 μg/mL, SI 13.82). The nanodispersion also showed strong antiproliferation activity and high selectivity against breast cancer cells (MDA-MB-231: IG$_{50}$ 28.22 μg/mL, SI 8.85) and kidney cancer cells (786-0: IG$_{50}$ 82.38 μg/mL, SI 3.03). Due to the low values of GI$_{50}$ and selectivity higher than three, the polymeric nanodispersion loaded with the soft extract of *Petiveria alliacea* L. can be a promising product for the treatment of these three types of cancer. However, further studies will need to be conducted to investigate its actual usefulness in anticancer therapy.

**Conflict of interests**

The authors declare no competing interest.

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References


Instituto Nacional de Cáncer. (2022a). *Atlas de mortalidade por câncer.* INCA.

Instituto Nacional de Cáncer. (2022b). *Gastos federais atuais e futuros com os cânceres atribuíveis aos fatores de risco relacionados à alimentação, nutrição e atividade física no Brasil.* INCA.


Organisation for Economic Co-operation and Development. (2009). Preliminary review of OECD test guidelines for their applicability to manufactured nanomaterials. OECD.


