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Original Paper

Antitumoral activity of Amazon plant species: extracts of *Apuleia leiocarpa* induce apoptosis and autophagy in lung tumor cell line

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Abstract
Non-small cell lung cancer (NSCLC) is one of the most common malignant tumors. Chemicals and target-directed therapy have been used to treat these tumors, but the development of resistance has hampered patient treatment. Thus, many researchers are seeking new compounds that are capable of reversing resistance. Plants from the Brazilian Amazon, such as *Apuleia leiocarpa*, represent an alternative source of new compounds with the potential to treat lung cancer. Increasing concentrations of *A. leiocarpa* extracts (25, 50 and 100 µg/ml) from stem, sapwood, root, and stem bark were tested against an NSCLC cell line (H460) for 48 h. The dichloromethane-stem (ALE3) and ethanolic-stem bark (ALE5) extracts inhibited cell viability and were further evaluated for apoptosis, loss of mitochondrial membrane potential (MMP), and expression of proteins belonging to the apoptotic and autophagic pathway. The results indicated that ALE3 and ALE5 induced dose-dependent apoptosis and loss of MMP, and while ALE3 induced the expression of apoptotic markers p53 and active caspase 3, ALE5 induced the expression of autophagy markers Beclin-1, ATG12 and LC3II. This study demonstrates for the first time that *Apuleia leiocarpa* possess significant antitumoral potential to fight lung cancer.
Key words: Amazon, apoptosis, *Apuleia leiocarpa*, autophagy, NSCLC.

Resumo
O câncer de pulmão de não-pequenas células (CPNPC) é um dos tumores malignos mais comuns. Produtos químicos e terapia direcionada têm sido usados para tratar esse tumor, mas o desenvolvimento de resistência tem dificultado o tratamento dos pacientes. Assim, muitos pesquisadores buscam novos compostos que sejam capazes de reverter a resistência. Plantas da Amazônia brasileira, como *Apuleia leiocarpa*, representam uma fonte alternativa de novos compostos com potencial para tratar o câncer de pulmão. Concentrações crescentes de extratos de *A. leiocarpa* (25, 50 e 100 µg/ml) de caule, alburno, raiz e casca foram testadas contra a linhagem celular NSCLC (H460) por 48 h. Os extratos diclorometano - caule (ALE3) e etanólico - casca (ALE5) inibiram a viabilidade celular e foram posteriormente avaliados quanto a apoptose, perda de potencial de membrana mitocondrial (MMP) e expressão de proteínas pertencentes à via apoptótica e autofágica. Os resultados mostraram que ALE3 e ALE5 foram capazes de induzir apoptose dependente da dose e perda de potencial de membrana mitocondrial, e enquanto ALE3 induziu a expressão de marcadores apoptóticos como p53 e caspase 3 ativa, ALE5 induziu a expressão de marcadores de autofagia, como Beclin-1, ATG12 e LC3II. Este estudo mostra pela primeira vez que *Apuleia leiocarpa* possui um significativo potencial antitumoral contra o câncer de pulmão.
Palavras-chave: Amazônia, apoptose, *Apuleia leiocarpa*, autofagia, CPNPC.

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Introduction

Lung cancer is a devastating disease characterized by fast-growing undifferentiated cells that spread through metastasis and lead to the patient's death. In Brazil, morbidity due to cancer is surpassed only by cardiovascular diseases (Global Burden of Disease 2019). The WHO estimates that lung cancer was the second most common cancer globally in 2020, with an estimated 2.3 million new cases (11.7% of total cancer cases) and the fifth leader cause of cancer mortality (Sung *et al*. 2021), making this disease a major public health issue. Lung cancer has two major histopathological subtypes: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC); the latter accounts for 80–85% of all lung cancer cases (Zheng 2016). NSCLC treatment involves radiotherapy, the use of platin derivatives in combination with several compounds, and target directed therapy (Ikeda *et al.* 2023). However, treatment effectiveness may be hampered by the phenomenon of tumor resistance to clinically available drugs (Ang *et al.* 2023). The need for new chemical entities as alternatives for treating cancer and other chronic conditions necessitates chemical and biological studies on plants, one of the main sources of natural products (Nasim *et al.* 2022).

Brazil has natural advantages in the bioproducts market. Namely, it has biodiversity associated with social diversity, which is a huge economic wealth (Albuquerque & Hanazaki 2006). There has been considerable speculation regarding the use of biodiversity in the Amazon to generate technology and economic growth, but few efforts have led to concrete results. Many Amazonian species are still under investigation to assess their pharmacological potential. One of these species is *Apuleia leiocarpa.*

Apuleia leiocarpa (Vogel) JF Macbride, popularly known as grápia or garapa, is a tree of the Fabaceae family. It may reach heights of up to 30 meters and is found in the Amazon and in southern Brazil and Uruguay, Argentina, Paraguay, and Bolivia (Carvalho 1994; Marchiori 1997). An important ecological feature of the family is the symbiosis of its roots with bacteria of the genus *Rhizobium* that fix nitrogen from the atmosphere; some species are therefore used for agricultural improvements. Additionally, this tree is used in the production of many types of products, including food, lumber industry, medicines, and pesticides (Abd-Alla, *et al*. 2014).

The stem bark presents antisyphilis properties and is also used to relieve body pain. Garapa is also used in folk medicine as antivenom serum; some authors have also described its analgesic and anti-inflammatory effects (Ruppelt *et al.* 1991). However, its antitumor potential have never been studied. In the present work, we demonstrate for the first time that extracts of *A. leiocarpa* induce apoptosis and autophagy in an NSCLC lung cancer cell line (H460).

Material and Methods

Plant material collection

Several organs (stem, stem bark, sapwood, root) were collected from the *A. leiocarpa* species in September 2010 from Concordia do Pará, state of Pará, Brazil. The plant was identified by Manoel R. Cordeiro from Embrapa Amazônia Oriental, Pará, Brazil, and a voucher specimen (IAN-187048) has been deposited in the herbarium of Embrapa Amazônia Oriental. This investigation was authorized to access the genetic heritage from SISGEN system under the code A678D8C.

Plant material processing

After collection, the plant material was cleaned and air-dried in a chamber at 45 ºC and milled using a knife mill.

Preparation of crude extracts

Apuleia leiocarpa (stem, stem bark, sapwood, and root) dried and ground samples were extracted by maceration. Microextraction was then conducted: 3 g/30 mL of solvent were weighed, and each material was subjected to extraction by maceration. The solvents used were hydrated ethyl alcohol (96º GL), ACS ethyl acetate, and ACS dichloromethane, with two 24-hour extractions. After extraction, the extracts were concentrated in vacuum to evaporate the solvent.

Analysis of the chromatographic profiles of the extracts by HPTLC

Extract analyzes were submitted to HPTLC, with a hexane and ethyl acetate elution system in a proportion of 40%, to evaluate their chromatographic profiles. Several classes of substances were observed, according to the colors presented on the chromatoplates after derivatization with specific chemical developers for each group of metabolites (Phenolic compounds - NP/PEG and Terpenes - VAS). The VAS-sprayed plates were

heated to a temperature of 100 °C. The bands of those derivatized with NP/PEG were visualized by irradiating ultraviolet light at 254 nm and 366 nm.

Cells and culture conditions

The human non-small cell lung cancer cell line H460 cells was maintained in an RPMI medium (Sigma Chemical Co., St. Louis, MO, USA), supplemented with 10% heat-inactivated fetal calf serum (Gibco, NY, USA), 100 U penicillin, and 100 µg/mL streptomycin in disposable plastic bottles at 37 °C with 5% CO_2.

Chemicals

Propidium iodide, DMSO and MTT were purchased from Sigma-Aldrich.

Viability inhibition assay

The cytotoxicity of *Apuleia leiocarpa* extracts was assessed by the MTT assay. Briefly, 180 µL of cell suspension (10^4 cells per well) was distributed in 96-well plates and pre-incubated for 24 h at 37 °C/5% CO_2 for culture stabilization. The extracts, henceforth referred to as ALE1 (ethanol-stem), ALE2 (ethanol-sapwood), ALE3 (dichloromethane-stem), ALE4 (ethanol-root), and ALE5 (ethanol-stem bark) were dissolved in DMSO and diluted in a medium for use. Cells were treated with either the medium, different concentrations of the extracts (25, 50 and 100 µg/mL), or DMSO (at the same concentration carried by the compounds, control). After 48 h incubation, MTT (5 mg/mL) was added, and the sample was kept for 4 h at 37 °C. The formazan crystals produced by reduction of MTT by viable cells was dissolved in DMSO, and the optical density was measured in an ELISA reader (Spectramax Multi-Mode Microplate Readers) at 570 nm (reference filter 630 nm). Results are expressed as mean ± SD of at least three different experiments performed in triplicate.

DNA fragmentation assay

Apoptosis was assessed by cell cycle analysis using flow cytometry. After 24 h resting, platted cells ($2x10^4$/well) were treated with a medium or different concentrations of ALE3 and ALE5 and incubated for another 48 h. After this time, cells were harvested, resuspended in a hypotonic fluorescent solution (50 µg/mL propidium iodide [PI] and 0.1% Triton X-100 in 0.1% Na Citrate buffer) for 1 h at 4 °C in the dark and the DNA content was measured by flow cytometry (FL-2) (FACSCalibur, Becton Dickinson, San Jose, CA). Data acquisition and analysis were conducted using the Cellquest software version 3.1f. Results are presented as mean ± SD percentage of subdiploid cells of at least two different experiments performed in triplicate.

Loss of $\Delta\psi m$

Mitochondrial membrane potential (MMP) was assessed with the fluorochrome DiOC6(3) (40 nM) as previously described (Amarante-Mendes *et al.* 1998). Cells were plated and treated with ALE3 and ALE5 (25, 50 and 100 µg/mL) and incubated 48 h. Stained cells were analyzed using flow cytometry (FL-1). The results represent the medium ± SD of three experiments.

Immunofluorescence

After 24 h resting, platted cells ($5x10^4$/well) were treated with medium or ALE3 and ALE5 (25, 50 and 100 µg/mL) and incubated for 48 h. After this time, cells were directly fixed in 24 well plates with 4% PFA for 25 minutes, washed twice with PBS, and permeabilized with 0.1% Triton-X in PBS for 15 minutes. The blockage was performed for 1 hour with 1% BSA/PBS in a humidity chamber, and after wash, cells were incubated with primary Ab (1:200 in 1% BSA/PBS) against p53, active caspases-3, Beclin-1, ATG12, and LC3 for 1 hour, followed by a wash and incubation with secondary antibodies conjugated with FITC (green) or Cy5 (red). The images were captured with a Leica DMI 6000B fluorescence microscope.

Image analysis

The images captured via fluorescence microscopy were analyzed using the Cell Profiler software with a custom algorithm (Carpenter *et al*. 2016).

Statistical analysis

All experiments were performed in triplicate and repeated at least two times. Results are expressed as mean ± SD. Statistical comparisons were made using a one-way ANOVA, followed by a Turkey test, with $p < 0.05$ considered significant.

Results and Discussion

Apuleia leiocarpa extracts reduced cell viability in a dose-dependent manner

Due to the impact of the chemotherapy failure in lung cancer treatment, the search for new alternatives to treat this high-incidence and

high-mortality tumor is of the utmost importance. We evaluated five extracts of several parts of *A. leiocarpa* to search for antitumor activity against NSCLC, using an MTT viability inhibition assay. As indicated in Table 1, the dichloromethane extract ALE3, from the stem, had the highest efficiency in reducing cell viability to 75.1±5% (50 μg/mL), and to 66±8% (100 μg/mL). While ALE5 (ethanolic-stem bark) reduced the viability to 71.7±15% at (100 μg/mL). The effect of ALE1, ALE2 and ALE4 in cell viability were not significant. Thus, from this point, the investigation was focused on the extracts with significant antitumoral effect, *i.e.*, ALE3 and ALE5.

Table 1 – Effect of extracts of Apuleia leiocarpa (25–100 μg/mL) on the viability of non-small cell lung cancer cell line H460.

Extract	25 μg/mL	50 μg/mL	100 μg/mL
ALE1	96.3 ± 10	97.5 ± 9	95.2 ± 15
ALE2	105.4 ± 24	99.6 ± 13	88.8 ± 13
ALE3	89.5 ± 12	75.1 ± 5**	66.0 ± 89***
ALE4	99.4 ± 17	91.4 ± 13	83.5 ± 10
ALE5	85.5 ± 10	81.3 ± 16	71.7 ± 15*

Results are expressed as the percentage of control cells. Data represent mean ± SD values of triplicate experiments. Tukey-Kramer Multiple Comparison test; * = p < 0.05; ** = p < 0.01; *** = p < 0.0001

During the experiments designed to evaluate reduction of cell viability, it was observed that the cells had undergone several morphological alterations consistent with apoptosis and autophagy, with the formation of vacuoles in the highest concentrations. Thus, cells were treated at the same concentrations as was done for cell viability, and after 48 h, the cells were photographed in bright field microscopy. As seen in Figure 1, the extract ALE3 induced cell death at 100 μg/mL, as evidenced by a reduction in the number of cells in the field and membrane deformities. On the other hand, the extract ALE5 induced vacuolization of the cells, also at higher concentration (bright spots), indicating the autophagy activation.

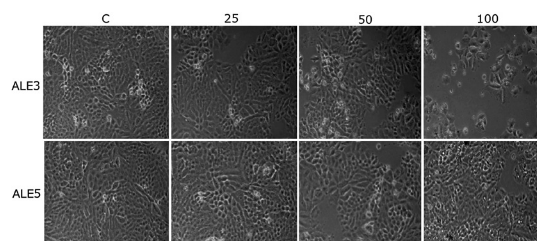

Figure 1 – *Apuleia leiocarpa* extracts induced cell death and vacuolization in lung cancer cell line H460. Cells were treated with 25, 50 and 100 μg/mL of ALE3 or ALE5 for 48 h, and morphological analysis was performed using bright field microscopy.

Previous work has demonstrated that *A. leiocarpa* seed extracts possess antibacterial activity against *Salmonella enteriditis*, *Pseudomonas aeruginosa*, *Klebisiela pneumonia*, among others, in both gran positive and negative species (Carvalho *et al.* 2015). However, the present study is the first to demonstrate that extract from any part of this plant reduced viability of lung cancer cells.

Apuleia leiocarpa extracts induced DNA fragmentation and loss of MMP in a dose-dependent manner

Apoptosis is the main form of cell death in tumor cells treated with chemotherapy drugs (Ricci & Zong 2006). Thus, we tested if the extracts induced DNA fragmentation through the appearance of the sub-G1 cell population, a hallmark of apoptotic cell death, in lung cancer H460 after 48 h. As seen in Figure 2a-b, both extracts induced the appearance of the sub-G1 population at 100 μg/mL (though with different efficiencies). Figure 2c-d include the representative histograms generated by flow cytometry analysis for the population considered for quantification. Analysis of the flow cytometry histograms indicated that there was no significant change in the proportion of G1, S, and G2 phases of the cell cycle, indicating that the extracts did not induce cell arrest.

Apoptosis induced by natural products mostly involves activation of the mitochondrial pathway (Chen *et al.* 2023), which is indicated by the loss of mitochondrial membrane potential. Thus, using flow cytometry, we evaluated the loss of MMP after 48 h of treatment with the extracts ALE3 and ALE5. As seen in Figure 3a-b, both extracts induced loss of MMP in a dose-dependent manner, with ALE3 reducing the potential starting in the lower concentration (25 µg/mL), whereas ALE5 initiated at 50 µg/mL.

After observing that ALE3 and ALE5 treatment caused reduction of cell population in H460 cells, indicating cell death by apoptosis, we tested if the extracts induced DNA fragmentation and loss of MMP, both indications of apoptotic cell death (Bredholt *et al.* 2009). Past work has demonstrated that most natural products and chemotherapeutic drugs used in clinics rely on induction of apoptosis to reduce tumor mass, and they usually reach this effect by inducing the loss of MMP (Wang *et al.* 2023), which leads to the release of cytochrome c and the assembly of the apoptosome, activating caspases and other effector molecules, leading to DNA fragmentation and the generation of sub-G1 population.

Among the effector molecules required for apoptosis execution it is caspase 3. The cells were treated with ALE3 and ALE5, and fluorescence microscopy was performed to

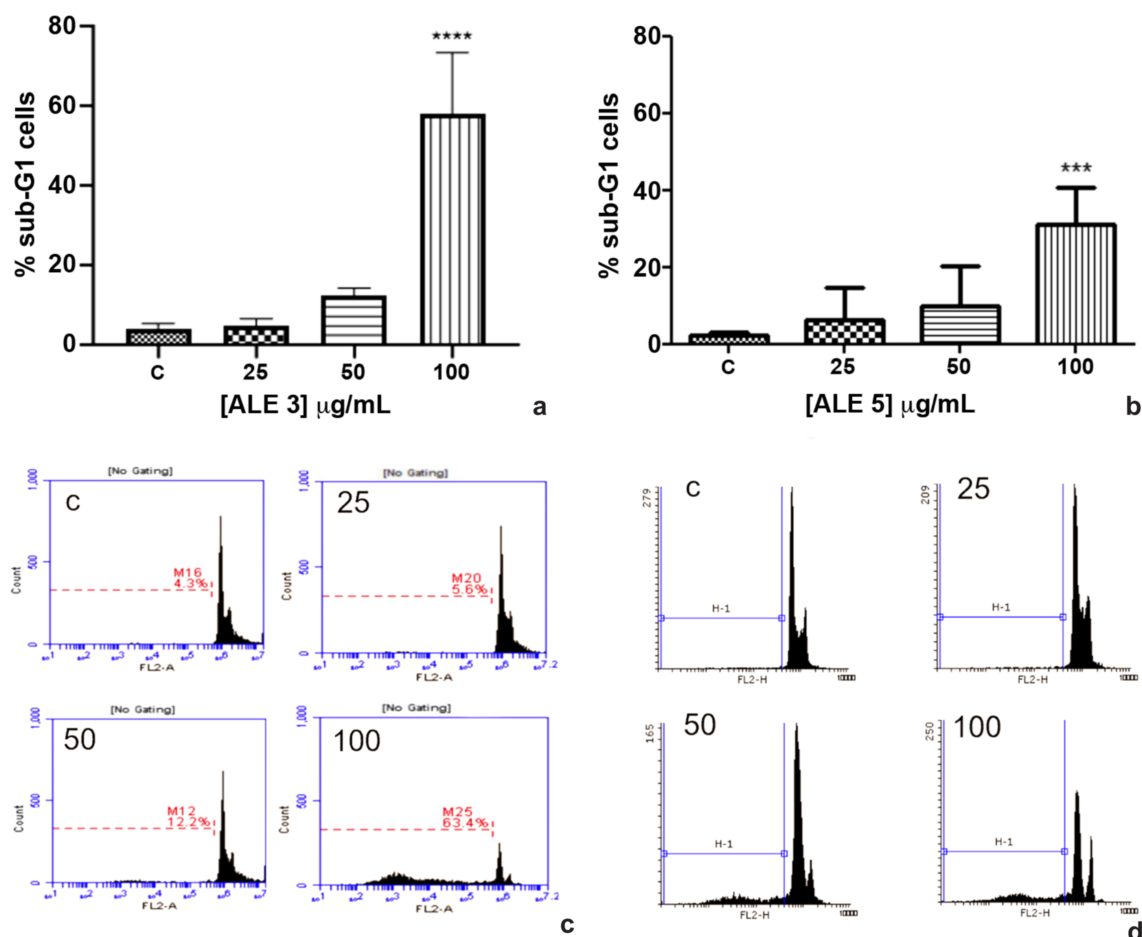

Figure 2 – a-d. *Apuleia leiocarpa* extracts enhanced sub-G1 population of lung cancer cell line H460. Cells were treated with 25, 50 and 100 µg/mL of ALE3 or ALE5 for 48 h. Sub-G1 cell population after treatment was indicative of apoptosis, as measured by flow cytometry – a. cells treated with ALE3; b. cells treated with ALE5; c. representative histograms of the data presented in (a); d. representative histograms of the data presented in (b). The columns represent mean ± SD of three experiments conducted in triplicate. *** = $p < 0.001$; **** = $p < 0.0001$.

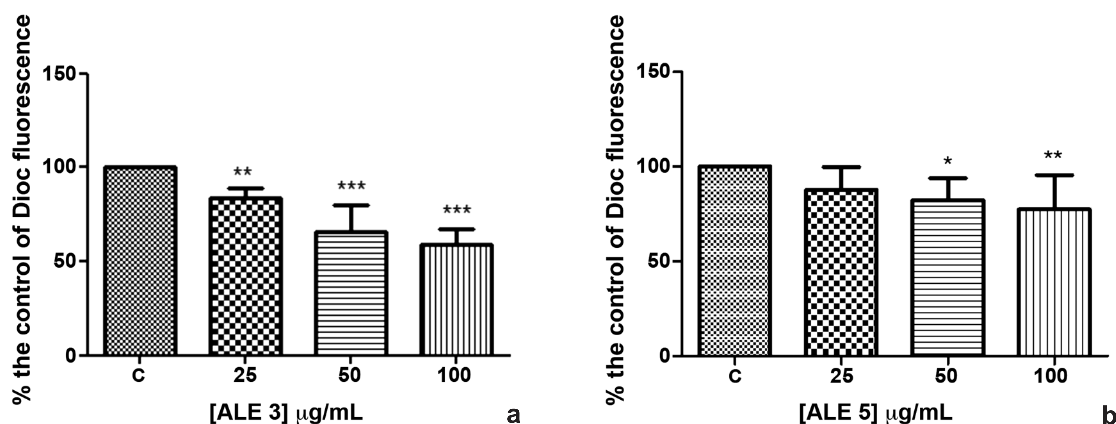

Figure 3 – a-b. Extracts of *Apuleia leiocarpa* reduced mitochondrial membrane potential in H460. Cells were treated with 25, 50 and 100 μg/mL of ALE3 or ALE5 for 48 h, and the loss of mitochondria membrane potential was measured – a. cells treated with ALE3; b. cells treated with ALE5. The columns represent mean ± SD of three experiments conducted in triplicate. *** = $p < 0.001$.

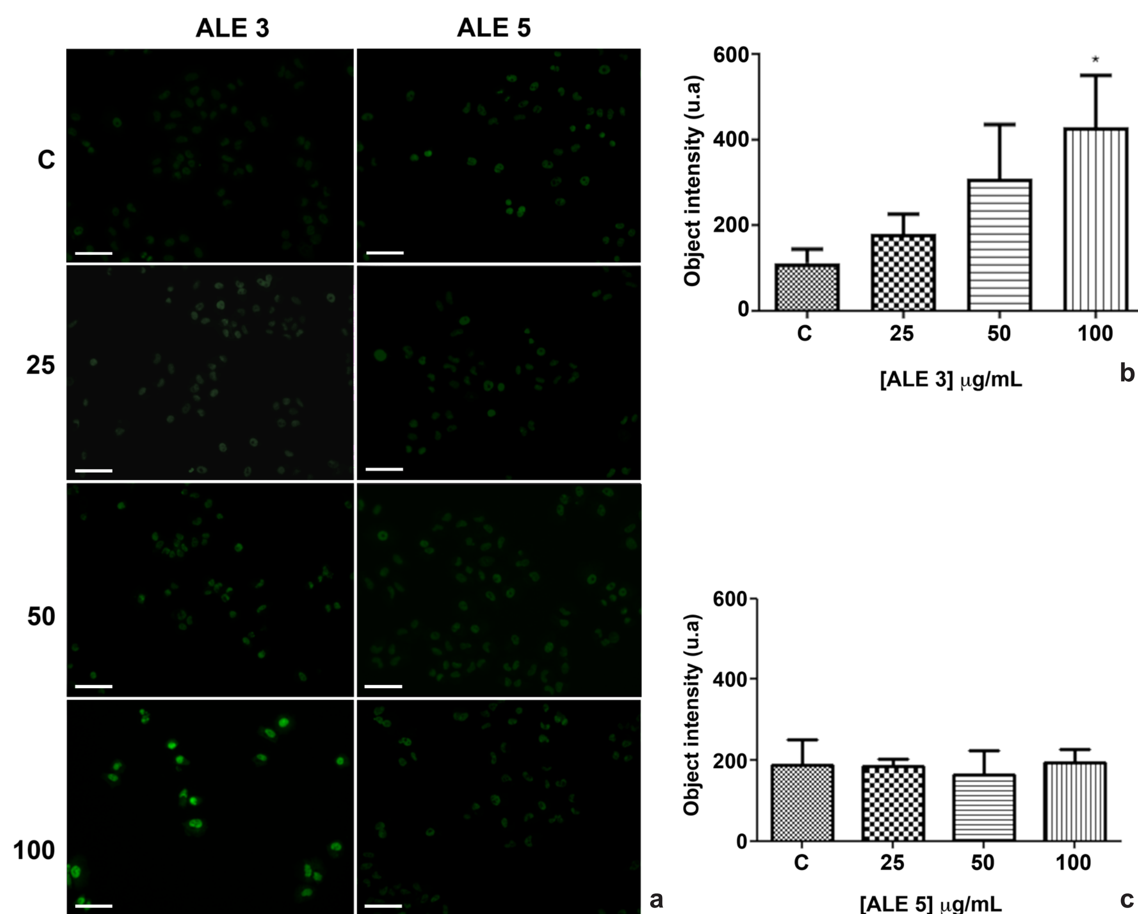

Figure 4 – a-c. *Apuleia leiocarpa* dichloromethane stem (ALE3) extract induced dose-dependent expression of active caspase 3 – a. H460 cells treated with 25, 50 and 100 μg/mL of extracts ALE3 or ALE5. Fluorescence microscopy was performed; b-c. fluorescence quantification related to (a), conducted using the Cell Profiler software. Scale bar = 100 μm (40x magnification). The columns represent one experiment conducted in triplicate. * = $p < 0.05$.

evaluate the presence of activated caspase 3, the protein to which both extrinsic and extrinsic pathways converge (Asadi *et al.* 2022). As seen in Figure 4a-c, only ALE3 induced significant activation of caspase 3, indicating that ALE3 is the extract responsible for inducing apoptosis in H460 cells (Fig. 4a). Along with activation of caspases, another important apoptosis marker is the expression of the tumor suppression protein p53. As can be seen in Figure 5a-c, ALE3 induced the expression of p53 at 100 µg/mL, indicating that more than one apoptotic pathway is activated by the extracts, since the mitochondrial pathway may or may not involve p53 activation (Yu & Zhang 2008). The activations of caspase 3 and p53 were not significant in cells treated with ALE5 (Fig. 5c).

Isolated compounds, such as betulinic acid, need higher concentrations (502 µg/mL, *i.e.*, 1.1 mM), to achieve similar DNA fragmentation after treatment of the same cell line. Previous work using the same cell line (H460) has demonstrated that cisplatin was able to induce 50% of apoptosis (cells were treated with 10 µg/mL for 48 h), while carboplatin, another platin derivative used to treat lung cancer, can achieve 40% of DNA fragmentation (but cells must be treated with 200 µg/mL, *i.e.*, 538 µM, again for 48 h; Fernandes *et al.* 2019). This finding reinforces the strong antitumoral activity of the dichloromethane

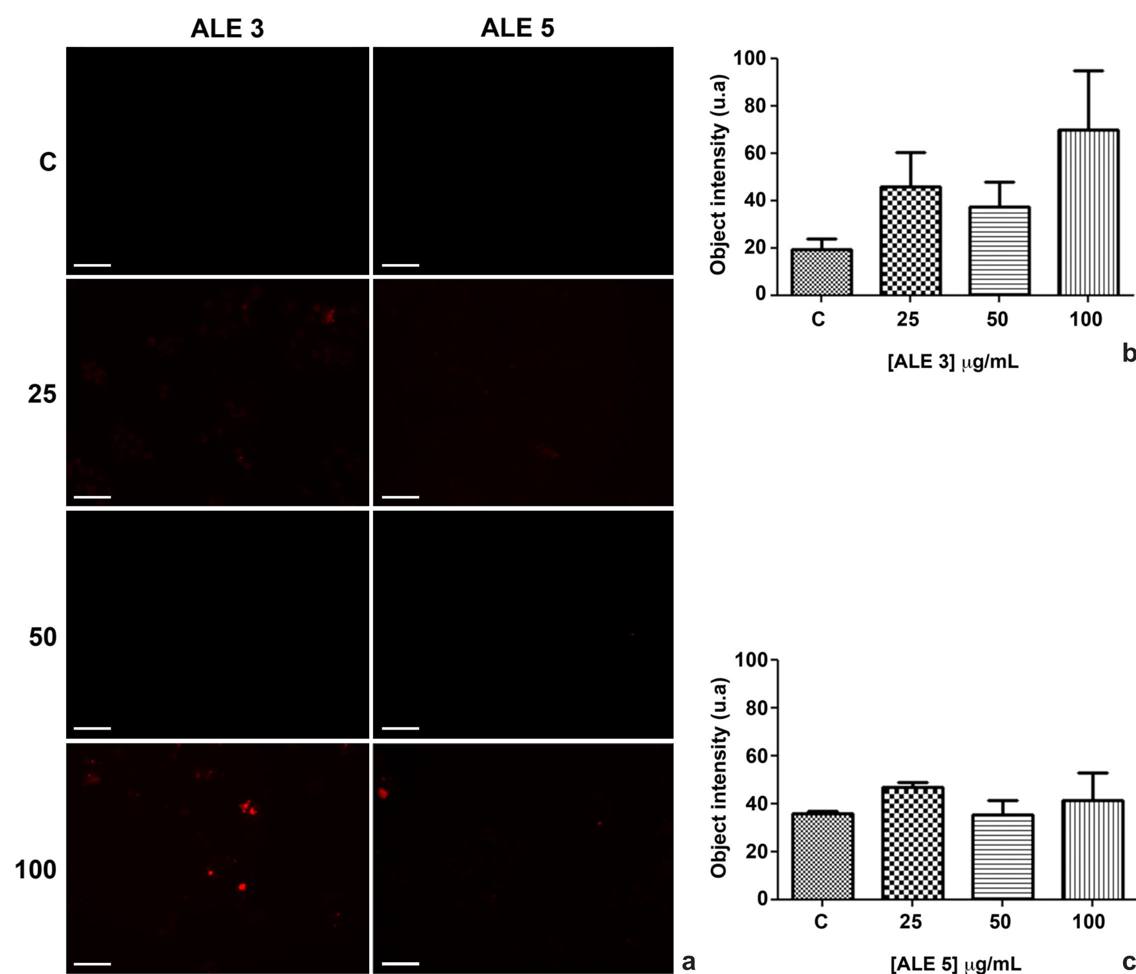

Figure 5 – a-c. *Apuleia leiocarpa* dichloromethane stem (ALE3) extract induced dose-dependent expression of tumor suppressor gene p53 – a. H460 cells treated with 25, 50 and 100 µg/mL of extracts ALE3 or ALE5. Fluorescence microscopy was performed; b-c. fluorescence quantification was conducted using the Cell Profiler software. Scale bar = 100 µm (40x magnification). The columns represent one experiment conducted in triplicate. * = $p < 0.05$.

extract of *A. leiocarpa,* leading to its cell-death-inducing rate in vitro of 50% at 100 µg/mL, close to the concentrations of at least one standard compound already used in clinics (Fernandes *et al.* 2019).

In addition to reducing mitochondrial membrane potential and inducing DNA fragmentation, ALE3 more strongly induced the activation of caspase 3 and enhanced p53 expression than ALE5. Betulinic acid also induces apoptosis involving these pathways. P53's role in tumor suppression is already well established (Green & Kroemer 2009), and its enhanced expression is related to tumor cell death, which has also been observed for other natural compounds (Qin *et al.* 2018).

Altogether, these results distinguish *A. leiocarpa* stem dichloromethane extract as a potent inducer of lung cancer cell death and this species as a potential source of proapoptotic compounds.

Apuleia leiocarpa induced the expression of autophagy markers in H460

Morphological analysis of the cells treated with ALE5 revealed the presence of vacuoles indicative of autophagy. Thus, we treated H460 cells for 48 h with 25, 50 and 100 µg/mL of ALE3 and ALE5. Fluorescence microscopy was performed to evaluate the presence of Beclin-1 and the autophagy-related gene 12 (ATG12), proteins involved in autophagosome development and maturation (Debnath *et al.* 2023; Mizushima 2007). As seen in Figure 6a, cells treated with ALE3 exhibited reduced cell quantity, as evidenced by bright field microscopy for the concentrations 50 and 100 µg/mL (Fig. 6a, BF), but did not express Beclin1, as evidenced by the complete blackened field in the red fluorescence channel (Fig. 6a, Beclin). Even though Atg12 presents basal expression in control cells, its expression did not increase with ALE3 treatment (Fig. 6a, Atg12). On the other hand, cells treated with ALE5 significantly induced the expression of both Beclin-1 and ATG12 (Fig. 6b). The fluorescence experiments for both ALE3 and ALE5 were performed simultaneously.

Another important molecule involved in the final step of the autophagy activation pathway is the expression of the microtubule-associated protein light chain 3 (LC3II) (Debnath *et al.* 2023). As indicated in Figure 7a-c, ALE5, but not ALE3, induced the expression of LC3II. Altogether, these results suggest that the three main markers of the three main steps of autophagy are activated in response to ALE5 treatment.

Autophagy is a complex cellular process that degrades cellular components, damaged organelles, and aggregates of misfolded protein, along with other molecules and subcellular elements. This process involves proteolytic degradation of cytosolic components at the lysosome and promotes cell homeostasis (Mizushima 2007). Autophagy has several main stages, with Beclin1 acting in the initial phase, and ATG12, among other molecules, acting in the subsequent conjugation phase. LC3II acts at the late autophagy stage, with the expansion of phagosome (Glick *et al.* 2010).

While the proapoptotic activity of the *A. leiocarpa* extracts has obvious potential as an alternative treatment of lung cancer, the pro-autophagic activity of the stem bark ethanolic extract (ALE5) represents a significant novel approach to autophagy induction. Autophagy serves to remove damaged proteins, including those involved in neurodegenerative disorders such as Alzheimer disease and Parkinson's disease (Menzies *et al.* 2015). Several studies have found that enhancement of the autophagic flux reduces amyloid plaques responsible for the pathophysiology of both neurodegenerative disorders (Obergasteiger *et al.* 2023; Zhang *et al.* 2021). Autophagy was already observed in H460 lung tumor cell line during treatment with Sufentanil (Jiang *et al*. 2019). The implications of autophagy for cancer mostly depends on the system under investigation, namely the nature and intensity of the autophagic stimulus, since drug-induced autophagy may lead to both tumor cell death (Jiang *et al*. 2019) or tumor survival (Chavez-Dominguez *et al*. 2020). Furthermore, some authors have suggested the combination of autophagy inducers and autophagic inhibitors, as for some systems, the pharmacological inhibition of autophagy may lead to apoptosis (Liu *et al*. 2020).

Analysis of the chromatographic profiles of the extracts by HPTLC

In the analyses, similarities were observed between the ETOH AA and AR extracts (sapwood

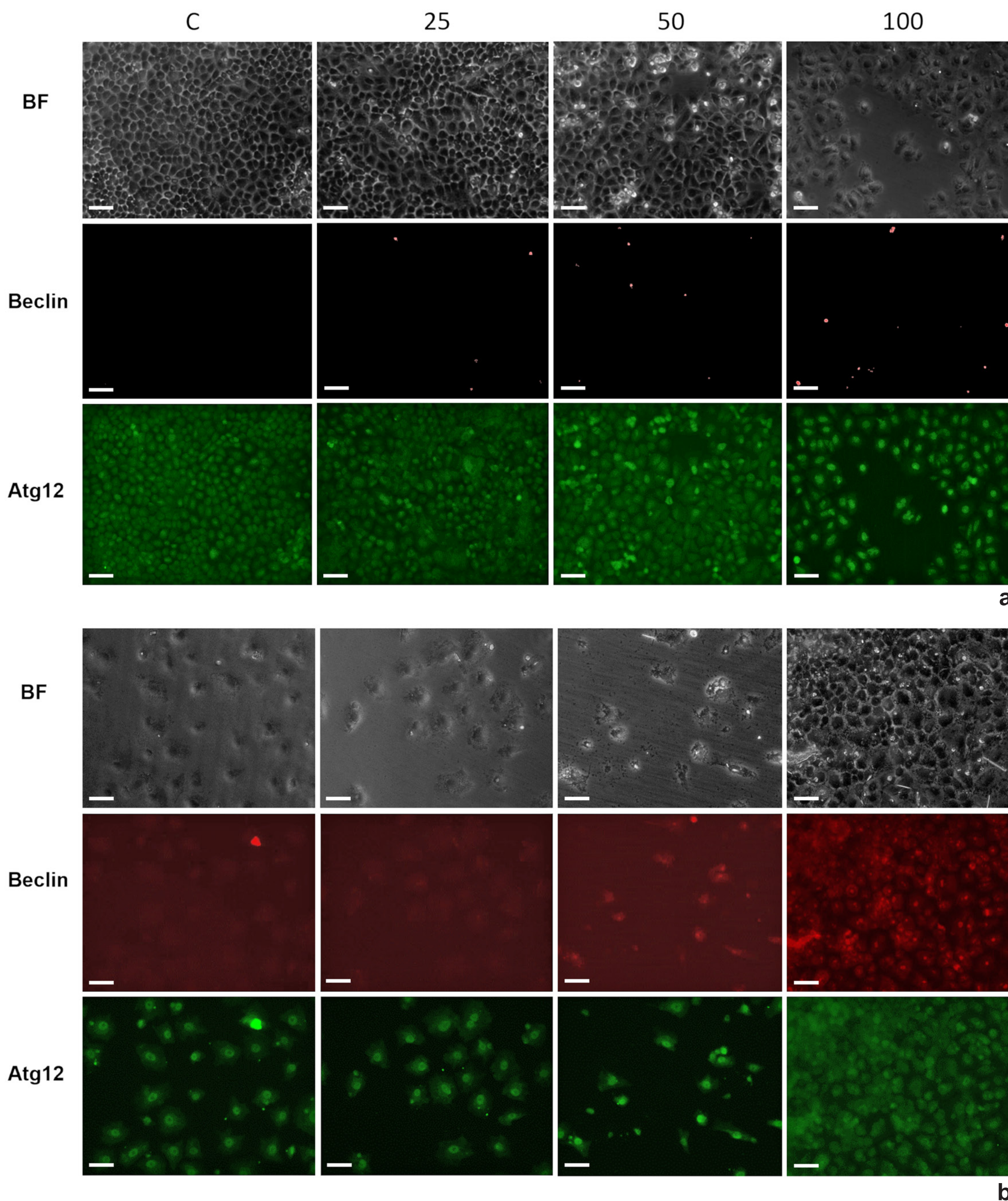

Figure 6 – a-b. *Apuleia leiocarpa* ethanolic stem bark (ALE5) extract induced expression of autophagy markers. H460 cells were treated with 25, 50 and 100 μg/mL of extracts ALE3 or ALE5, and fluorescence microscopy was performed – a. cells treated with ALE3 and marked for Beclin-1 and ATG12; b. cells treated with ALE5 and marked for Beclin-1 and ATG12. BF (Bright field). Scale bar = 100 μm (40x magnification).

and root) and the DCM AC extract (stem), due to the orange and yellow colors that were observed in the same Rf for these extracts, which suggests that there is a predominance of phenolic compounds in these extracts (Fig. 8a - NP/PEG). In the ETOH AC, ACs (stem and bark) and DCM AC (stem) extracts, the several bands were observed, suggesting the presence of several classes of substances, including fatty acids, terpenes, and bands with colored orange for phenolic compounds (Fig. 8b - VAS).

Some of the phenolic compounds of *A. leiocarpa* were isolated in a mesmerizing phytochemical study of *A. leiocarpa* performed in 1971 by Raimundo Braz and Otto Richard Gotlieb (Braz & Gottlieb 1971). This work identified several constituents as nine flavones and β-sitosterol in the sapwood. The stem bark extract composition was quite different, with pterocarpan and β-sitosterol. Using TLC analysis, they also found flavones. Of interest, D-Pinitol is a known insulin mimicker with potential to be a food supplement because of its ability to lower blood glucose levels with no side effects (Liu & Koyama 2023).

β-sitosterol, found in both stem bark and stem (Braz & Gottlieb 1971), presents antitumoral activity with apoptosis induction in breast cancer (Wang *et al*. 2024), prostate cancer (Macoska 2023), hepatocellular carcinoma (Chen *et al.*

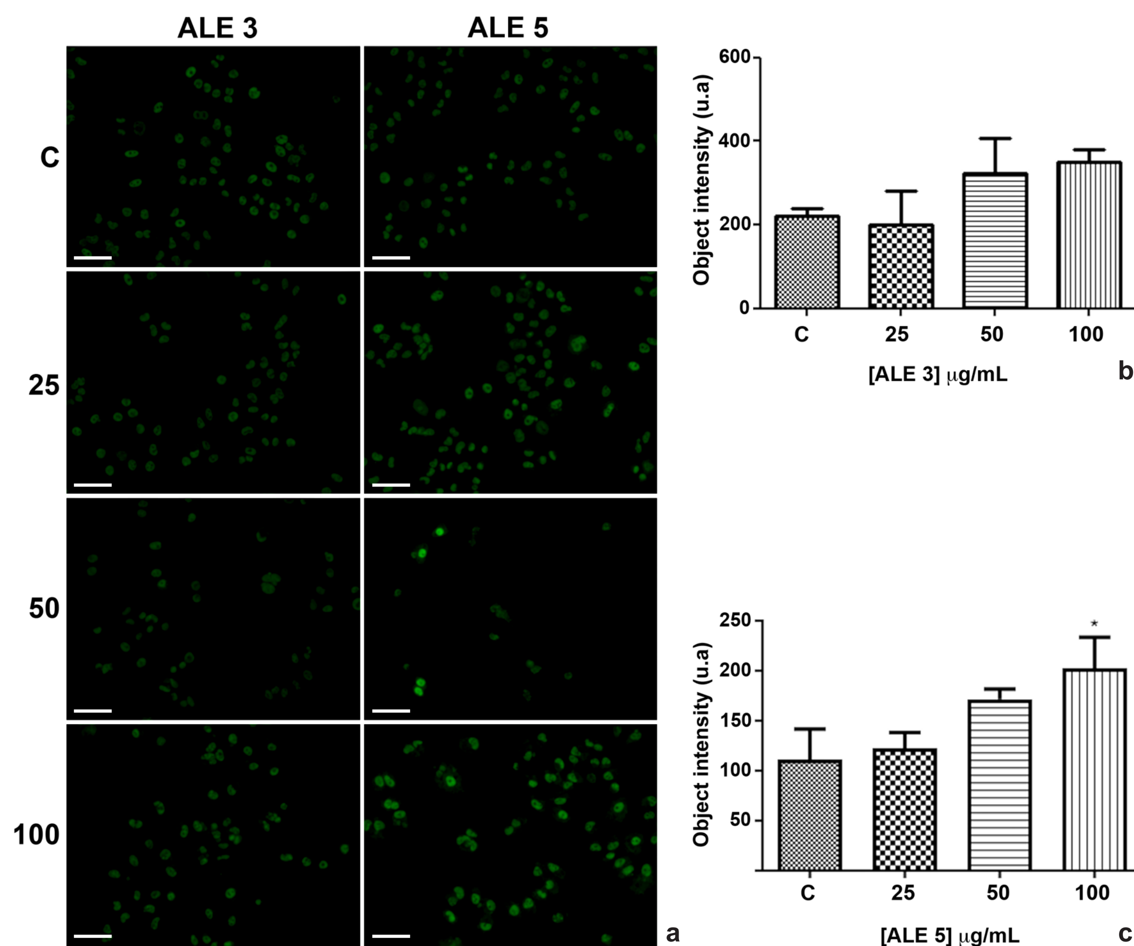

Figure 7 – a-c. *Apuleia leiocarpa* ethanolic stem bark (ALE5) extract induced dose-dependent expression of late autophagy marker LC3II – a. H460 cells were treated with 25, 50 and 100 μg/mL of extracts ALE3 or ALE5, and fluorescence microscopy was performed; b-c. fluorescence quantification using the CellProfiler software. Scale bar = 100 μm (40x magnification). The columns represent one experiment conducted in triplicate. * = $p < 0.05$.

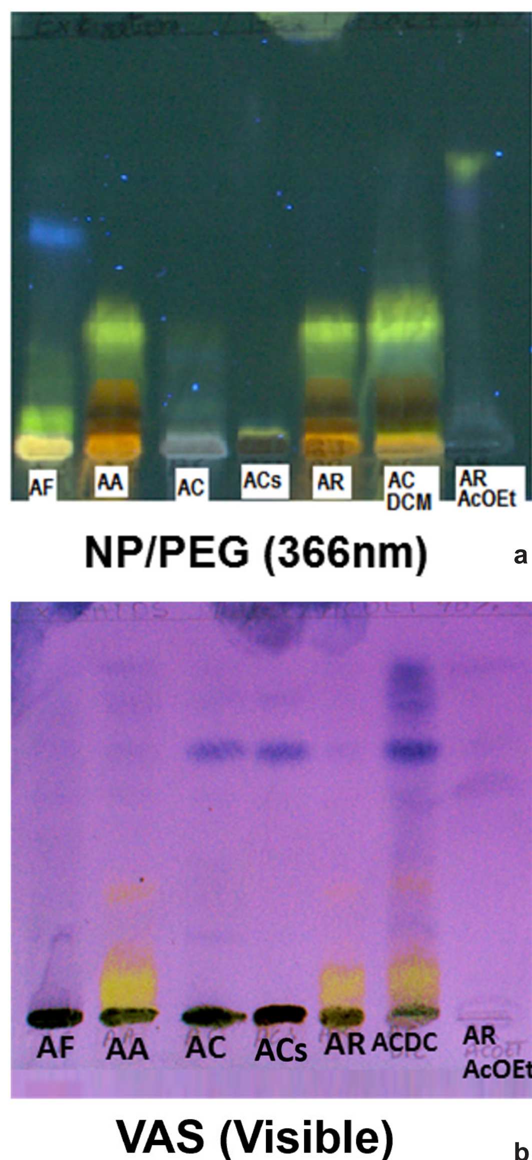

Figure 8 – a-b. Profiles of the *Apuleia leiocarpa* extracts by HPTLC – HPTLC plaque depicted by a) the orange and yellow colors that were observed in the same Rf for these extracts, indicating the predominance of phenolic compounds. b) In the visible spectrum, the presence of several bands was observed, suggesting the presence fatty acids, terpenes, and also bands with colored orange for phenolic compounds.

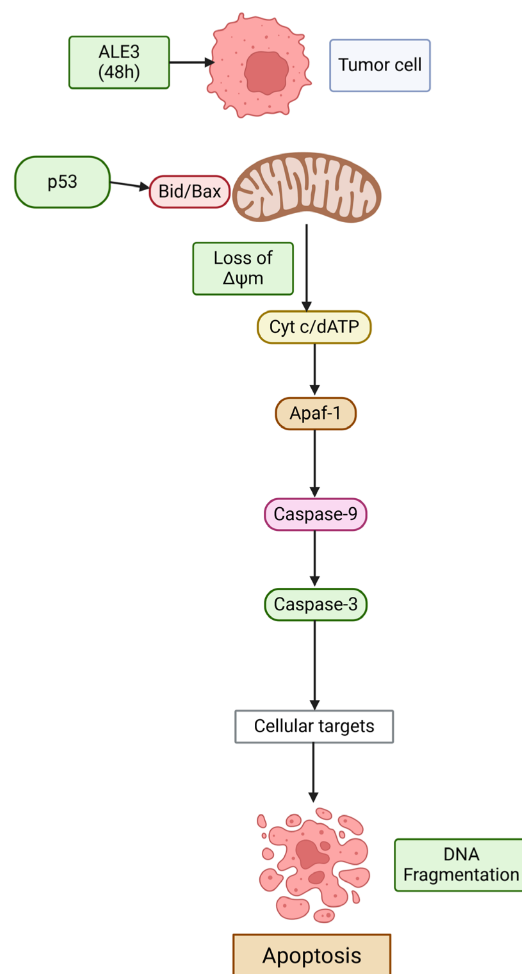

Figure 9 – Proposed apoptotic pathway involved in *Apuleia leiocarpa* dichloromethane stem extract (ALE3) activity in lung cancer. Light green indicates which apoptotic pathway markers were affected by the ALE extract according to the data presented in this study: under ALE3 treatment, H460 cells exhibited enhanced p53 expression that also reduced MMP, a key feature of the intrinsic pathway, contributing directly to the apoptosome formation and DNA fragmentation, both important hallmarks of apoptosis.

2024), among others. β-sitosterol also induced autophagy in hepatocellular carcinoma, leading to cell death (Chen *et al*. 2023). Altogether, these data indicate that *A. leiocarpa* apoptotic and autophagic activities may be due to the presence of these compounds in the extracts.

Altogether, our data indicate that the studied *A. leiocarpa* extracts significatively reduced lung cancer H460 cell viability; induced apoptosis through mitochondrial pathway, culminating in DNA fragmentation (dichloromethane extract from stem - ALE3; Fig. 9); and induced autophagy (ethanolic extract from stem bark - ALE5) with the increased expression of three proteins involved in three major steps of the autophagy

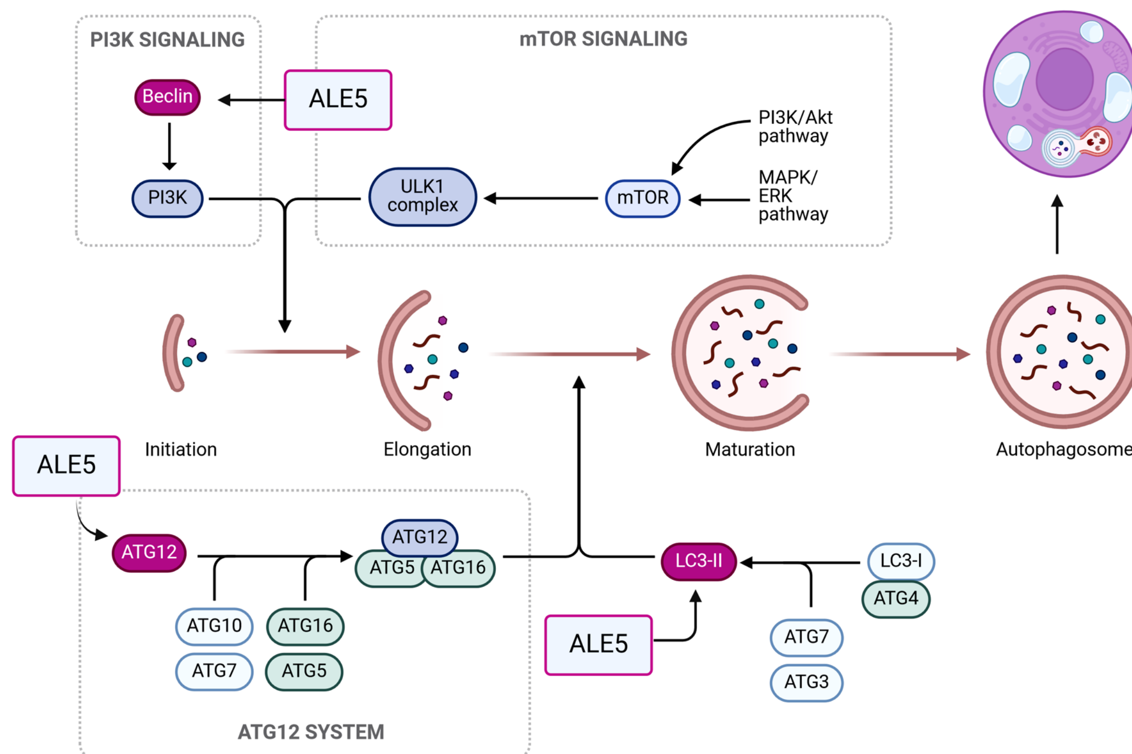

Figure 10 – Autophagic pathways in cancer affected by the extracts of *Apuleia leiocarpa* (ALE5). The development of autophagy as a result of several types of stimuli put the cell on a pathway involving three main phases: initiation, elongation, and maturation of the autophagosome. Beclin is involved in the first phase and works with other proteins to control the membrane nucleation stage. This process leads to the formation of the phagophore, the initial structure that will be further elongated with the participation of ATG12 along with other ATGs. At the end of the elongation phase, the protease ATG4 converts LC3 (microtubule-associated protein 1A/1B-light chain 3) to LC3I, which is then converted by additional ATGs to LC3II, the lipidated form of LC3, which is associated with the autophagosome membrane. Magenta indicates which autophagy pathway markers were affected by the ALE5 extract according to the data presented in this study.

induction: Beclin, ATG12 and LC3II (Fig. 10). These results are related to the compound classes present in the samples, pointing to the antitumoral properties of this species and its autophagy-inducing potential.

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Data availability statement

In accordance with Open Science communication practices, the authors inform that all data are available within the manuscript.

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