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SELECTIVE CYTOTOXIC AND PRO-APOPTOTIC EFFECTS OF *CECROPIA PACHYSTACHYA* TRÉCUL IN HUMAN LUNG CANCER CELLS

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Abstract

Cecropia pachystachya Trécul, also known as embauba of the family Urticaceae, is traditionally used in Brazilian medicine to manage diabetes, asthma, and cough. Previous studies have highlighted its diverse biological activities, including anti-inflammatory, cardiotonic, leishmanicidal, antidepressant-like, antioxidant, and antinociceptive effects. However, its antineoplastic potential remains unexplored. This study investigated the cytotoxic and apoptotic effects of *C. pachystachya* extract (CPE) on human lung cancer cell lines (H460, A549, and H2023) and non-tumorigenic human foreskin fibroblasts (BJ cells). The extract exhibited concentration-dependent cytotoxicity across all cancer cell lines while sparing non-cancerous BJ cells. Further analyses using acridine orange/ethidium bromide (AO/EB) staining indicated that the observed cytotoxicity was mediated through the induction of apoptosis rather than necrosis. Liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS) analysis identified chlorogenic acid (48%) and orientin (2.9%) as the major constituents of CPE. These findings suggest that *C. pachystachya* harbors bioactive compounds with selective anticancer properties, highlighting its potential as a promising candidate for the development of novel anticancer therapeutics.

Key words: Apoptosis induction; *Cecropia pachystachya*; Lung cancer; Selective cytotoxicity; fibroblast cell

Introduction

Cancer remains one of the leading causes of mortality globally. Data from the National Cancer Institute, Brazil (Primo, 2023), estimate 704,000 new cases in Brazil each year of the triennium 2020-2022, with lung cancer among the five most frequent types, with approximately 30,000 new cases (Anon., 2020). This is an alarming number for public health; therefore, several studies have focused on the discovery of new and efficient methods for successful cancer treatment with minimal side effects (Torres *et al*., 2012). The search for anticancer compounds from plants has yielded promising results (Aragão *et al*., 2012; Fukumasu *et al*., 2006, 2008; Fukumasu *et al*., 2011; Plengsuriyakarn *et al*., 2012). Studies on regional medicinal plants have further strengthened this evidence. Extracts of *Acacia modesta* and *Opuntia monocantha* have demonstrated significant anticancer activity by inducing apoptosis in liver cancer cells (Abid *et al*., 2022), and *Nigella sativa* extracts have exhibited selective pro-apoptotic effects on human cancer cell lines (Maqbool *et al*., 2019). In addition, Maqsood *et al*., (2015) reported that methanolic extracts from regional medicinal plants, including *Withania coagulans* and *Artemesia scoparia,* induced 88–93% cytotoxicity in RD cells, nearly four times more effective than standard chemotherapeutics, highlighting the potential of regional plant extracts to induce apoptosis in cancer cells (Maqsood *et al*., 2015). The antineoplastic properties of plant extracts may be attributed to the presence of flavonoids (do C Maquiaveli *et al*., 2014; Moraes, 2007; Sartori, 2012). Flavonoids have been shown to have antioxidant, anti-inflammatory, antiviral, and antitumor effects, among others (Do C Maquiaveli *et al*., 2014; Gazal *et al*., 2015; Jomova *et al*., 2025; Santos *et al*., 2001).

Cecropia pachystachya Trécul, a member of the familly Urticaceae, is a native Brazilian plant that exhibits anti-inflammatory, renal protective, and cardiotonic effects (Consolini & Migliori, 2005; Consolini *et al*., 2006; do C Maquiaveli *et al*., 2014; Schinella *et al*., 2008), leishmanicidal activity (Cruz *et al*., 2013), antidepressant-like effects (Gazal *et al*., 2014; Ortmann *et al*., 2016), antioxidant properties (Gazal *et al*., 2015; Ortmann *et al*., 2016; Pacheco *et al*., 2014), antinociceptive and cytotoxic activities (Aragão *et al*., 2012), genotoxic effects (Daminani Mendonça *et al*., 2016) and preventive action in bipolar disorder (Gazal *et al*., 2015). This plant, popularly known as *Embauba*, is commonly used by the natives of South America as a natural remedy for diabetes, asthma, and cough (Bigliani *et al*., 2010; Consolini *et al*., 2006; Schinella *et al*., 2008). These therapeutic effects are likely attributable to the presence of polyphenolic compounds, including (+)-catechin, (-)-epicatechin, procyanidin B2, isoorientin, orientin, isovitexin, and chlorogenic acid. (Aragão *et al*., 2010; Costa *et al*., 2011; Cruz *et al*., 2013; Pacheco *et al*., 2014). In addition, a triterpene-enriched

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fraction of *C. pachystachya* has shown selective cytotoxicity against prostate cancer PC3 cells, mainly through senescence induction (Rosa *et al*., 2020). In this study, we aimed to investigate the cytotoxic effects of *C. pachystachya* extract (CPE) on human lung cancer and non-cancerous fibroblast cell lines.

Materials and Methods

Plant material and preparation of *Cecropia pachystachya* extract: The leaves of *C. pachystachya* were collected from the University of São Paulo, *Campus* of Ribeirão Preto, São Paulo, Brazil (-21.167499, -47.861759). The plant identified by Claudia do Carmo Maquiaveli was deposited in the ESA Herbarium at the Luiz de Queiroz College of Agriculture of the University (ESALQ), Piracicaba, São Paulo, Brazil, under voucher no. (132691) as a voucher specimen.

This study employed the aqueous portion obtained from the acetone extract of *C. pachystachya*. Because acetone extraction is known to yield high levels of phenolic compounds (Rockenbach *et al*., 2008; Yilmaz & Toledo, 2006). The leaves were briefly dried at 25°C for three days, powdered, and then macerated. Aliquots of 50 g of dried material were resolubilized and maintained in H_2O-C_3H_6O (3:1) (Synth, Brazil) for 48 h. This mixture was subsequently filtered, and acetone was removed by distillation under reduced pressure (TE-211, Rotary Evaporator, Tecnal, Brazil). The resulting product was subjected to another fractionation using n-butanol (Synth, Brazil) to obtain two fractions: butanolic and aqueous (CPE) fractions. Both fractions were lyophilized (L-202, Lyophilizer – Liotop, Brazil) and resuspended in water for further use.

Cell culture conditions: Three human lung cancer cell lines (A549, H2023, and H460) were kindly donated by Dr. Lucy M. Anderson, with technical assistance from Janet Fields at the Comparative Carcinogenesis Laboratory, National Cancer Institute (Fredericks, US). The cells were maintained in RPMI-1640 medium, which was supplemented with 10% fetal bovine serum (FBS), 2% GlutaMAXTM-I, and 1% antibiotic solution comprising 10,000 Units/ml Penicillin and 10,000 µg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). They were incubated at 37 °C in a humidified environment with 5% CO_2. Non-tumorigenic human foreskin fibroblast cells (BJ) were kindly provided by Dr. Fabiana F. Bressan from the Laboratory of Molecular Morphophysiology and Development (FZEA-USP) and used as a control.

Cytotoxicity assay: To evaluate the cytotoxicity of the CPE, 2×10^3 cells were seeded per well in a 96-well plate and cultured under standard conditions. After 24 h, the medium was removed, and 8 different concentrations of CPE were added [1.6–200.0 µg/ml, six wells per concentration]. The cells were then incubated for an additional 48 h, and the assay was performed in 3 independent experiments, each conducted in triplicate.

Cytotoxicity was assessed using a colorimetric assay with MTT reagent (Sigma-Aldrich, St. Louis, MO, USA). MTT was diluted in DMEM (Invitrogen, Carlsbad, CA, USA) at a concentration of 5 mg/ml, and 10 µL of this solution was added to each well, followed by incubation for 2 h. To dissolve the formazan crystals, the medium was removed by inversion, and 0.04N HCl in 100µl of isopropyl alcohol (Synth, Diadema, SP, Brazil) was added. Absorbance data were acquired using a FLUOstar OPTIMA microplate reader (BMG Labtech, Germany).

Cell death analysis: To determine the mode of action of CPE, treated and non-treated cells were exposed to a solution of 100µg/ml ethidium bromide (EB) (Sigma-Aldrich; St. Louis, MO, USA) and 100µg/ml acridine orange (AO) (Sigma-Aldrich; St Louis, MO, USA), as previously described (Ribble *et al*., 2005). This double-staining technique enabled the simultaneous identification of apoptosis and necrosis based on the properties of nucleic acid-binding dyes.

In this assay, live cells exhibited normal nuclear morphology, organized cytoplasm, and green fluorescence; early apoptotic cells displayed condensed or fragmented chromatin with bright green nuclear staining; and late apoptotic cells showed both chromatin condensation and fragmentation accompanied by red nuclear staining. In contrast, necrotic cells resemble live cells in structure but exhibit red-stained nuclei (Ribble *et al*., 2005). To perform this test, only the cell line that presented the highest CPE cytotoxicity was analyzed. The analysis was performed after 48 h of treatment by adding the EB/AO dye mixture. Cells were immediately analyzed by fluorescence microscopy using an inverted microscope (Nikon Eclipse TS100, Nikon, Japan) at 200× magnification. Images were captured using Nikon DS-Ri1 (Nikon, Tokyo, Japan). Approximately 100 cells per well were counted using the ImageJ software (National Institutes of Health, Bethesda, MD, USA). Six wells were analyzed in each experimental group.

LC-MS analysis of CPE: Liquid Chromatography-Mass Spectrometry (LC-MS) was conducted following the standardized protocol outlined by Cruz *et al*., (2013). The active components in CPE were analyzed using reverse-phase liquid chromatography on an Acquity UPLC I-class system (Waters Corporation, USA) in conjunction with an Esquire 3000 Plus mass spectrometer (Bruker Daltonics, USA). The separation process employed an RP-amide column (Ascentis-Supelco, Sigma–Aldrich; 25 cm × 4.6 mm; 5M).

The samples were prepared at a concentration of 5 mg/mL. Before injection, all samples were filtered through 0.22 µm membrane filters to remove particulate matter and ensure reproducibility. The extract was prepared in 0.1% formic acid, and 10 µL aliquots were injected into the liquid chromatography (LC) system at a flow rate of 1 mL/min. The LC gradient was applied, ranging from 10% to 35% acetonitrile (CH_3CN) in an aqueous solution containing 0.1% formic acid over a period of 30 minutes. Detection was carried out at a wavelength of 280 nm using diode array detection (DAD). The mass spectrometry (MS) analysis was conducted under the following conditions: capillary voltage of 4000 V, nebulizer pressure of 27 psi, dry gas flow of 7.0 L/min, dry temperature of 320°C, and mass flow of 100 L/min. The active constituents were identified by comparing the retention time (RT), mass spectra, and UV–Vis spectra with those of authentic standards.

Statistical analysis

Statistical analyses were conducted using one-way ANOVA, followed by Dunnett's post hoc test. A p-value of less than 0.05 was considered to be statistically significant. All analyses were performed using Prism 5.0 software (GraphPad Software, USA).

Results

***Cecropia pachystachya* extract is cytotoxic for human cancer cells:** CPE induced a concentration-dependent cytotoxic effect in all lung cancer cell lines tested (A549, H460, and H2023), with the H460 cell line being the most sensitive (Fig. 1 and Table 1), exhibiting an IC_{50} of 38.1±3.3 µg/ml. In contrast, CPE had no effect on the non-neoplastic cell line (BJ) used as a control (Fig. 1), even at the highest concentration tested (Table 1).

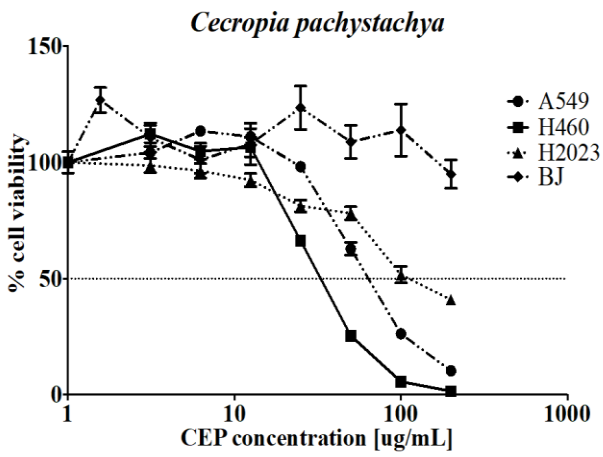

Fig. 1. Cytotoxic effects of *Cecropia pachystachya* extract (CPE) on human lung cancer and non-neoplastic cell lines.

Table 1. Half-maximal inhibitory concentration (IC_{50}) values of *Cecropia pachystachya* extract in human lung cancer and non-neoplastic cell lines.

Cell lines	*Cecropia pachystachya* (IC_{50} µg/ml)
H460	38.1 ± 3.3
A549	81.9 ± 22.1
H2023	131.5 ± 15.1
BJ Fibroblast	> 200.0

Three human lung cancer cell lines (A549, H460, and H2023) and a non-tumorigenic fibroblast cell line (BJ) were treated with increasing concentrations of CPE. A concentration-dependent cytotoxic effect was observed in all cancer cell lines, whereas the non-neoplastic BJ cells remained unaffected at all tested concentrations.

***Cecropia pachystachya* extract induces apoptosis instead of necrosis in cancer cells:** To determine the mode of action of CPE responsible for its cytotoxic effect in lung cancer cells, the H460 cell line was selected because of its low IC_{50} [38.1 µg/mL] among the tested cell lines. A concentration-dependent increase in apoptosis was observed in H460 lung cancer cells, as evidenced by a significant increase in the number of early and late apoptotic cells (Fig. 2B and C) following exposure to CPE concentrations ranging from 25 to 200 µg/ml (p<0.0001; Fig. 2D).

The control group consisted of live cells exhibiting intact membranes and normal nuclei (Fig. 2A). In contrast, CPE-treated cells showed signs of both early and late apoptosis at 25 and 50µg/ml (Fig. 2B and C). Early apoptotic cells displayed condensed chromatin and bright green nuclear staining, whereas late apoptotic cells showed condensed and fragmented chromatin with red-stained nuclei. Both concentrations resulted in a significant apoptotic index, and no necrotic cells were observed.

These findings indicate that apoptosis induction by CPE is the primary mechanism underlying its cytotoxic effects on lung cancer cells.

Phytochemical constituents of *Cecropia pachystachya* extracts: LC-ESI-MS analysis in the positive ion mode revealed that CPE contained 48% chlorogenic acid and 2.9% orientin. Chlorogenic acid and orientin exhibited retention times (RT) of 11.316 and 15.042 min, respectively. The $[M+H]^+$ ion peaks were observed at m/z 355 and 449 for chlorogenic acid and orientin, respectively. Additionally, chlorogenic acid (CGA) displayed a characteristic fragment ion at 162.9 m/z (Fig. 3). The RT and UV spectra of the authentic standards for chlorogenic acid and orientin were identical to those obtained from the samples.

Discussion

In this study, we demonstrated that the aqueous extract of *C. pachystachya*, which is rich in chlorogenic acid and orientin, selectively induced apoptosis in human lung cancer cell lines while exhibiting no cytotoxic effects on non-neoplastic fibroblast cells. These findings highlight the potential of *C. pachystachya* as a promising source of bioactive compounds with selective anticancer activities. Consistent with our findings, recent work also demonstrated that *C. pachystachya* extract exerts antitumoral activity in glioblastoma, reducing tumor growth *In vivo* and modulating oxidative stress and purinergic signaling (Bona *et al*., 2024).

The biological effects of *C. pachystachya* have been extensively associated with its anti-inflammatory properties, which were initially demonstrated in a mouse model of carrageenan-induced paw edema. (Schinella *et al*., 2008). The authors identified compounds capable of inducing apoptosis in polymorphonuclear cells, contributing to reduced edema. Similarly, Aragão *et al*., (2012) reported significant anti-inflammatory effects of the methanolic extract of *C. pachystachya* (CPM) in a croton oil-induced ear edema model (Aragão *et al*., 2012). Subsequent studies have confirmed the anti-inflammatory efficacy of CPM in various *In vivo* models, which is sometimes comparable to that of standard drugs, such as indomethacin and dexamethasone (Pacheco *et al*., 2014). Another study demonstrated that administration of *C. pachystachya* extract alleviated kidney damage in rats subjected to nephrectomy by diminishing inflammation and lowering renal arginase activity. This protective effect was probably mediated by decreased expression of *TGF-β*, phosphorylated JNK, and arginase-related genes (Maquiaveli *et al*., 2014).

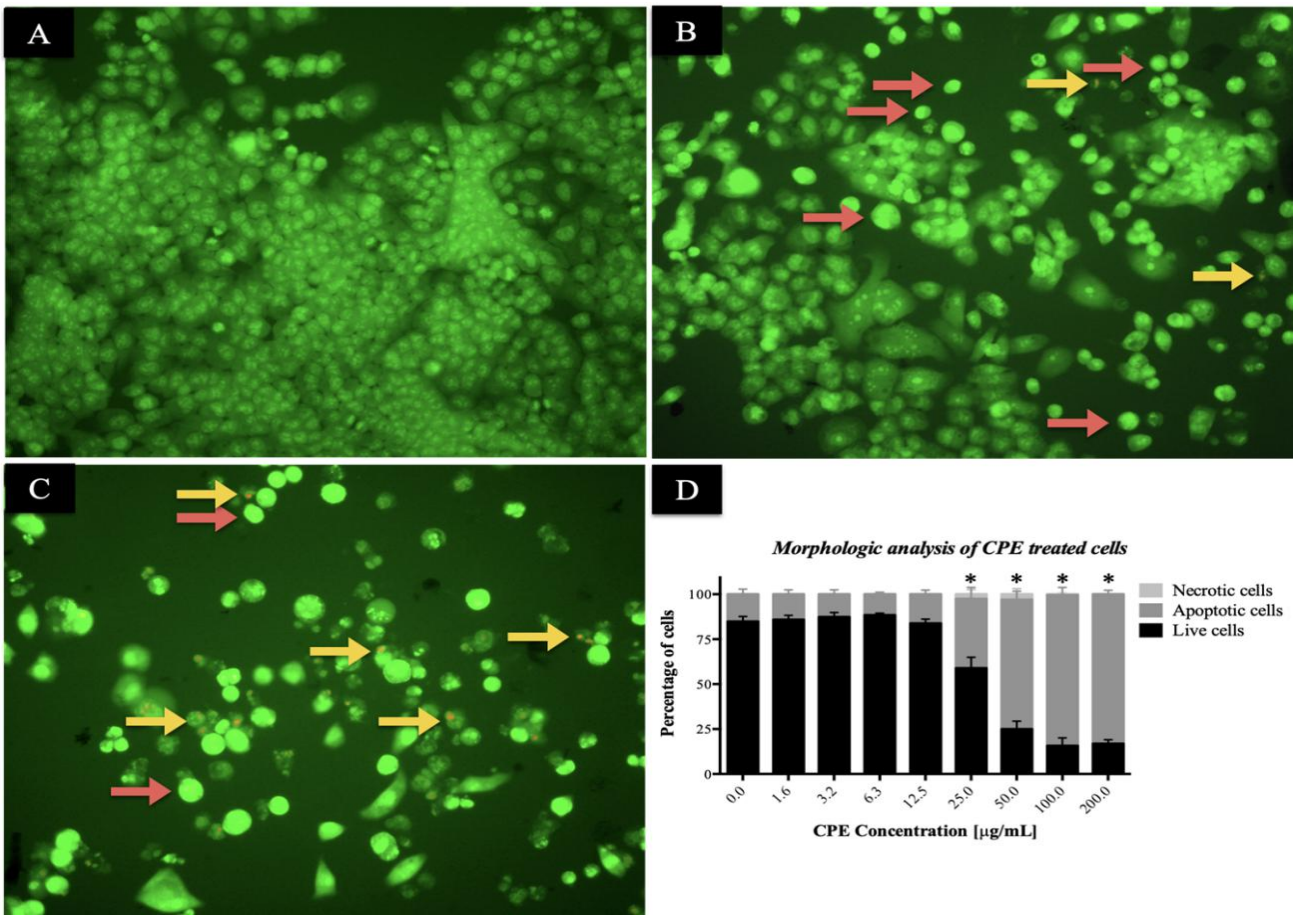

Fig. 2. Induction of apoptosis by *Cecropia pachystachya* extract in H460 lung cancer cells.
(A) Untreated control cells displayed intact membranes and normal nuclei; **(B)** Cells treated with 25 µg/ml CPE showed early apoptotic features, such as condensed chromatin and bright green nuclei (yellow arrows); **(C)** Cells treated with 50 µg/mL CPE exhibited characteristics of late apoptosis, including fragmented chromatin and red-stained nuclei (red arrows); **(D)** Quantification of apoptotic cells revealed a statistically significant increase in early and late apoptosis following CPE treatment (***p<0.001). Magnification 10×. Scale bar = 100 µm

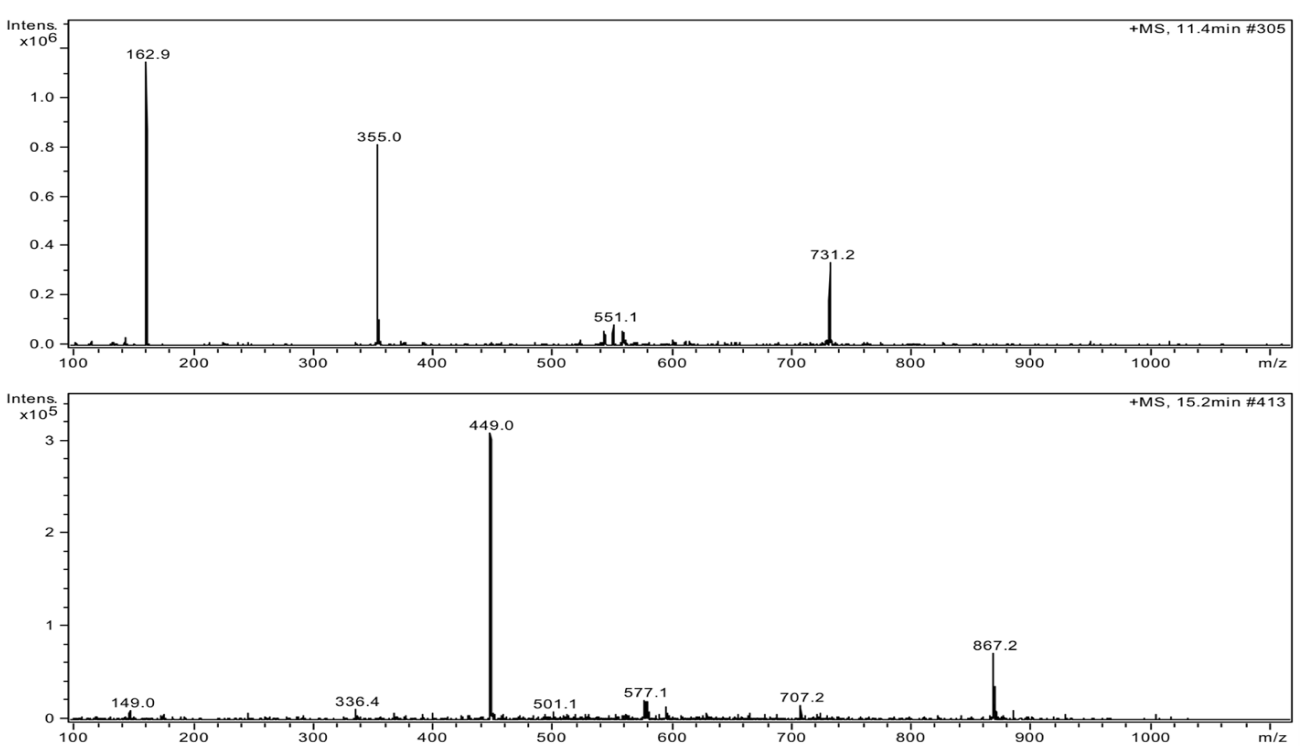

Fig. 3. LC-ESI-MS profile of *Cecropia pachystachya* extract.

Representative chromatographic peaks indicated the presence of chlorogenic acid and orientin, with retention times of 11.316 and 15.042 min, respectively. The [M+H]+ ion for chlorogenic acid appeared at m/z 355, with a characteristic fragment ion at m/z 162.9. Orientin was detected at an m/z of 449. These spectral features matched those of the authenticated standards in the Japan MassBank database (chlorogenic acid: KOX00160; caffeic acid fragment: PR040213).

Our results revealed that all tested lung cancer cell lines were sensitive to CPE, with H460 cells displaying at least twice the sensitivity compared to the other lines. This observation suggests variations in the molecular targets or resistance mechanisms across different lung cancer cells. Importantly, CPE exhibited no toxicity toward non-neoplastic fibroblast cells within the tested concentration range (0-200 µg/ml), supporting its potential for the development of selective anticancer agents. Similarly, Ahmad *et al*., (2021) demonstrated that *Terminalia chebula* exerts pronounced cytotoxic effects (~79% cell death) against rhabdomyosarcoma cells, primarily via apoptotic pathways, reinforcing the relevance of phytochemical apoptotic agents in regional medicinal plants (Ahmad *et al*., 2021). These findings are consistent with those reported for *Euphorbia hirta* extracts, which induced apoptosis and selective cytotoxicity against lung cancer NCI-H460 cells without affecting non-cancerous cells (Tran *et al*., 2020).

A previous study by Aragão *et al*., (2012) reported the cytotoxic effects of CPM against human leukemia cells, including drug-resistant HL60 cells. Bcl-2 cell line. However, their evaluation was limited to a single concentration (20 µg/mL) and was based solely on cell viability assays without mechanistic characterization. They suggested that CPM might harbor pro-apoptotic compounds because of the similar inhibition percentages observed across cell lines, despite HL60.Bcl-2's resistance to etoposide (Aragão *et al*., 2012). Our study expands on this by demonstrating that CPE induces apoptosis rather than necrosis, confirming the mechanism of cancer cell death.

CGA, the major constituent of CPE along with orientin, has demonstrated chemopreventive activity in various models of chemical carcinogenesis in rats and mice (Belkaid *et al*., 2006; Matsunaga *et al*., 2002; Shimizu *et al*., 1999). Several studies have revealed CGA's distinctive anticancer mechanisms, including the inhibition of matrix metalloproteinase expression (Jin *et al*., 2005), induction of apoptosis via caspase activation (Yang *et al*., 2012), activation and proliferation of immune cells (Dąbrowska *et al*., 2023), and inhibition of extracellular signal-regulated kinase (ERK) pathways (Yan *et al*., 2015). Moreover, CGA, which is abundant in coffee, has been demonstrated to trigger DNA damage preferentially in lung cancer cells compared to fibroblasts, reflecting its selective toxicity (Burgos-Morón *et al*., 2012). Orientin has been recognized for its radioprotective properties, protecting cellular components from radiation-induced damage (Nayak & Devi, 2005; Uma Devi & Satyamitra, 2004; Vrinda & Uma Devi, 2001). Orientin, found in *Gleditsia triacanthos* leaves exhibited cytotoxic activity against liver, breast, and colon cancer cells by promoting cell cycle arrest and apoptosis (Mohammed *et al*., 2014), which is consistent with our observations in lung cancer cells.

Although CGA and orientin were identified as the major constituents of CPE, but in the present the study these compounds were not isolated individually. Therefore, the specific molecule or combination primarily driving the observed apoptotic effects remains unclear. In addition, no mechanistic assays, such as caspase activity or pathway validation, were performed to define the molecular basis of apoptosis induction. Finally, the results were limited to in vitro models, and *In vivo* validation will be essential to establish translational relevance. Future research should focus on isolating individual bioactive compounds, performing pathway-specific assays, and extending investigations into animal models to confirm the therapeutic potential of CPE.

Conclusions

In conclusion, our findings demonstrate that CPE, which is rich in CGA and orientin, selectively induces apoptosis in human lung cancer cells while sparing non-cancerous fibroblasts. This supports its potential as a source for developing novel and selective anticancer therapeutics.

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Conflict of Interest: The authors declare no conflict of interest.

Author's Contribution: Arina Lázaro Rochetti performed the experiments, analyzed the data, and drafted the manuscript; Pedro Luiz Porfirio Xavier and Pedro Ratto Lisboa Pires assisted with cell culture experiments and cytotoxicity assays; Claudia do Carmo Maquiaveli contributed to plant identification, extract preparation, and phytochemical analysis; Edson Roberto da Silva conducted the LC-ESI-MS analysis and interpretation of phytochemical data; Alia Gul contributed to data interpretation and critical revision of the manuscript; Talal Jamil Qazi assisted with data analysis, manuscript editing, and literature review; Heidge Fukumasu conceptualized the study, supervised the research, secured funding, and critically revised and approved the final manuscript.

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