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Chemical Composition, Antiradical, Antiproliferative, Proapoptotic and Arrest Cell Cycle Activities of *Bursera linanoe* Extracts

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Abstract. *Bursera linanoe* is a tree widely distributed in the state of Guerrero. The extracts from several species of *Bursera* exhibit a wide range of biological activities, including antiproliferative, apoptotic, and cell-cycle arrest effects, as well as antioxidant properties. This study aimed to determine the phytochemical profile and biological activities of leaf extracts from *Bursera linanoe.* The hexane (EHBl) and dichloromethane (EDBl) extracts were analyzed by Gas Chromatography-Mass Spectrometry analysis and evaluated for their *in vitro* antiradical activity against DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ radicals. Cell cultures of Triple-Negative Breast Cancer (MDA-MB-231) and Human Lung Adenocarcinoma (A549) cells were used to assess antiproliferative activity by the MTT assay. The apoptosis induction and cell-cycle arrest were detected by Annexin/propidium iodide (PI) staining.

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The phytochemical analysis indicated that the major constituents of the EHBl and EDBl extracts are lupeol, τ-sitosterol, and β-amyrin. The EDBl was the most effective at reducing the $ABTS^{\bullet+}$ radical (IC_{50}: 113.29 μg mL^{-1}). In addition, proliferation of MDA-MB-231 and A549 cancer cells was significantly inhibited by EHBl, with IC_{50} values of 78.45 and 266.69 μg mL^{-1}, respectively, and no evidence of toxicity against the non-tumor cell line MCF-10A. Additionally, EHBl extract (200 μg mL^{-1}) increased the percentage of cells in apoptosis and in the G0/G1 phase and arrested the cell cycle in cancer cell lines. In conclusion, this study demonstrates the biological activities of *B. linanoe* extracts, highlighting its pharmacological potential as a source of compounds for cancer treatment.

Resumen. *Bursera linanoe* es un árbol ampliamente distribuido en el estado de Guerrero. Los extractos de varias especies de *Bursera* presentan una amplia gama de actividades biológicas, incluyendo propiedades antiproliferativas, apoptóticas, de detención del ciclo celular y antioxidantes. El objetivo de este estudio fue determinar el perfil fitoquímico y las actividades biológicas de los extractos de hojas de *Bursera linanoe*. Los extractos de hexano (EHBl) y diclorometano (EDBl) se analizaron mediante cromatografía de gases con espectroscopía de masas y se evaluó su actividad antirradical in vitro frente a los radicales $DPPH^{\bullet}$ y $ABTS^{\bullet+}$. El cultivo celular de células de cáncer de mama triple negativo (MDA-MB-231) y de adenocarcinoma de pulmón humano (A549) se utilizó para evaluar la actividad antiproliferativa mediante el ensayo de MTT. La inducción de apoptosis y la detención del ciclo celular se evaluaron mediante tinción con anexina/yoduro de propidio (PI). El análisis fitoquímico indicó que los principales constituyentes de los extractos de EHBl y EDBl son lupeol, τ-sitosterol y β-amirina. El EDBl fue el más eficaz para reducir el radical $ABTS^{\bullet+}$ (CI_{50}: 113.29 μg mL^{-1}). Además, la proliferación de las células cancerosas MDA-MB-231 y A549 se inhibió significativamente con EHBl, con CI_{50} de 78.45 y 266.69 μg mL^{-1}, respectivamente, sin evidencia de toxicidad en las células no tumorales MCF-10A. Además, el extracto de EHBl (200 μg mL^{-1}) aumentó el porcentaje de células en apoptosis y detuvo el ciclo celular en la fase G0/G1 en líneas celulares cancerosas. En conclusión, este estudio demuestra las actividades biológicas de los extractos de *B. linanoe* y evidencia su potencial farmacológico como fuente de compuestos naturales para el tratamiento del cáncer.

Introduction

The World Health Organization (WHO) reports that cancer is a cause of death worldwide, with nearly 10 million deaths in 2020, and has estimated that this number will increase to 12.9 million in 2030 [1,2]. Besides, this disease is considered a public health problem because of the high costs of treatment; the total annual economic cost of cancer in 2010 was estimated at US$ 1.16 trillion [3, 4]. Triple-Negative Breast Cancer (TNBC) and Lung Cancer (LC) have high mortality, presenting 685,000 and 1,80 million deaths in 2020, respectively [2,5]. TNBC is characterized by a lack of estrogen receptors (ER) and affects women worldwide, and is characterized by aggressive behavior, and easily metastasizes to spread to the liver, lung, and central nervous system [5]. The LC is responsible for more deaths than prostate, breast, and colorectal cancer combined. Smoking, air pollution exposure, and occupational exposures were considered the primary risk factors for developing LC. In general, about 13 % of all LC cancers are small cell lung cancer, and 84 % are non-small cell lung cancer. In 2020, the number of deaths for this cancer was 1,796, 144, and the new cases were 2, 206,771. Additionally, LC has a poor prognosis, and studies show that lung cancer causes malignant lesions of bone metastases [6,7].

Conventional treatments for cancer lack specificity due to increased resistance in cancerous cells to antineoplastic drugs. Therefore, alternative therapies are required to identify novel molecules against cancer; in this sense, natural products play a crucial role in the discovery of novel drugs, and the phytochemicals from plant species have been reported to play pleiotropic roles in cancer cells and can also prevent the inflammatory and oxidative stress generated in the process of carcinogenesis. On the other hand, natural compounds enhance the cytotoxic effects of anticancer drugs, promoting synergy in chemoresistant cells [8,9].

Previous studies of *Bursera* genera report the use in traditional medicine for the treatment of pain, inflammation, rheumatism, colds, skin tumors, polyps, and venereal diseases [10-16]. The chemical profile of

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Bursera plants includes flavonoids, triterpenes, sesquiterpenes, diterpenes, short-chain aliphatic alkanes, and predominantly lignans [14,17]. Additionally, extracts and bioactive compounds, from the stems, leaves, and, fruits of *Bursera* species exhibited antiproliferative potential against cancer cells as well as HeLa (Human Cervical Cancer), A549 (Human Lung Adenocarcinoma), C-33 (Human Cervical Cancer), MDA-MB-231 (Triple-Negative Breast Cancer), MCF-7 (Human Breast Adenocarcinoma), PC-3 (Prostate Cancer) and, 9KB (Human Epidermoid Carcinoma of the Nasopharynx) [11,14,17,18]. Likewise, terpenes and lignan compounds can induce mechanisms that activate cell death in cancer cells and inhibit cell proliferation [18]. In the state of Guerrero, Mexico, *Bursera linanoe* (*B. linanoe*) is widely distributed throughout the state; the wood from its trees is used to make artisanal boxes called "cajitas de Olinalá," which are pleasantly fragrant due to the presence of essential oil containing myrcene, limonene, and geraniol [19]. However, wood and fruits are used to obtain essential oils, and the fragrance of the fresh leaves is not like that of the essential oils [20]. Therefore, the objective of this study was to evaluate the phytochemical profile, antiradical and antiproliferative effects of hexane and dichloromethane extracts from *B. linanoe* on MDA-MB-231 and A549 cancer cell lines.

Experimental

Plant material and preparation of extracts

The leaves of *B. linanoe* were collected from trees belonging to the Botany Garden of Universidad Autónoma de Guerrero. The specimen was identified by Angélica Catalán Neria and deposited in the Herbarium of the same University under voucher number 243. The leaves of *B. linanoe* were air-dried in the shade and pulverized. The dried material (100 g) was macerated using hexane (reagent grade, 500 mL) for 24 h, 3 times. The macerated material was filtered, and the organic phase was evaporated under reduced pressure in a rotatory evaporator (digital rotatory evaporator 410, Puebla, Mexico) at 60 °C and 80 rpm to obtain the hexane extract (EHBl). The residues were re-extracted under the same conditions to obtain dichloromethane extract (EDBl). The extracts were stored at 4 °C and protected from light until use.

Preliminary phytochemical analysis by Gas Chromatography-Mass Spectroscopy (GC-MS)

GC-MS analysis of the extracts was performed using an Agilent 6890 series gas chromatograph coupled to an Agilent 5973N mass spectrometer (Agilent Technologies, Inc, USA). Separation was achieved on an HP-5MS capillary nonpolar column (30 m, ID: 0.20 mm, film thickness: 0.25 µm, Agilent 19091S-433). Helio was used as the carrier gas at a constant flow rate of 1.0 mL min^{-1}. The oven temperature was programmed from 50 °C to 230 °C at a rate of 2°C min^{-1}. The injection volume was 1 µL, and samples were dissolved in dichloromethane prior to analysis and were injected in split mode. The instrument was operated in electron impact (EI) mode with an electron energy of 70 eV, and the mass spectral scan range was 20-600 (*m*/*z*).

All results were compared by using the NIST/EPA/NIH Mass Spectral library version 1.7a/ChemStation [21]. Identification was further supported by the corresponding retention times and by comparison with the previously reported phytochemical profile of the genus *Bursera* [14,17,22-25].

Antiradical activity

The ability of extracts to scavenge DPPH$^{\bullet}$ (1,1-diphenyl-2-picrilhydrazyl, D9132, Sigma-Aldrich, St. Luis, USA) radical was determined by the DPPH method [26].

Prior to the antiradical assay, the extracts were dissolved in Dimethyl sulfoxide (DMSO; 1 %) to prepare stock solutions, which were subsequently diluted to the required assay concentration. Briefly, a 0.3 mM DPPH solution in ethanol was prepared. 50 µL of extracts (500, 1000, 2000, and 4000 µg mL^{-1}) were added to 150 µL of the DPPH$^{\bullet}$ solution in each well of a 96-well plate. For the blank, only 50 µL of ethanol was added to the DPPH$^{\bullet}$ solution. The decrease in absorbance was measured at 545 nm after 30 min of incubation in the dark at 37 °C using a Stat Fax microplate reader (Awareness Technology, Palm City, FL, USA). The ABTS$^{\bullet+}$ radical scavenging activity of extracts was determined as follows [27]. The production of the radical ABTS$^{\bullet+}$ was undertaken by preparing a stock solution of ABTS (7 mM, 10102946001 Sigma-Aldrich, St. Luis, USA) in water. This solution, ammonium persulfate (245 mM), was added, and the reaction was allowed to proceed for 16 h in the dark at room temperature to yield a stable blue-green radical solution. 1 mL of the solution was

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diluted in 60 mL of methanol, which was then used as the working solution. 50 µL of extracts (25, 50, 100, 200, 400, and 500 µg mL^{-1}) were added to 150 µL of the $ABTS^{\bullet+}$ working solution in each well of a 96-well plate. For the blank, only 50 µL of methanol was added to the $ABTS^{\bullet+}$ working solution. After 30 min of incubation in the dark, the absorbance was measured at 735 nm using a Multiskan FC microplate reader (Thermo Scientific™). All assays were performed in triplicate. Values were expressed as the sample concentration required to scavenge 50 % of free radicals (IC_{50}).

Antiproliferative activity and morphological changes

The triple-negative human breast cancer cell (MDA-MB-231, ATCC® HTB-26) and the human lung adenocarcinoma cell (A549, ATCC® CCL-185) were cultured in Dulbecco´s Modified Eagle medium (12500-062) from GIBCO, Gaithersburg, MD, USA. The medium was supplemented with 10 % fetal bovine serum (ByProductos, Jalisco, México) and 1 % antibiotic-antimycotic (15240062, GIBCO Gaithersburg, MD), and the cells were maintained in T75 cm^2 flasks at 37 °C in a humidified 5 % CO_2 incubator (Thermo Scientific™ Heracell™ VIOS).

Cell viability was evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [28]. A density of 3×10^3 cells was seeded into 96-well plates for 48 h at 37 °C before treatment with the EHBl and EDBl extracts (12.5, 25, 50, 75, 100, and 200 µg mL^{-1}). In addition, the negative control (DMSO 1 %) and the positive control (paclitaxel, 120 nM) were included, MTT (5 mg mL^{-1} in PBS, M6494 Molecular Probes, Carlsbad, USA) was added to wells after 24 h exposure of treatments evaluated, and plates incubated for 3 h afterward the MTT solution was removed, and formazan product was fixed using 10 % of DMSO. Formazan was quantified by measuring absorbance at 545 nm using a 630 nm differential filter in a Stat Fax microplate reader (Awareness Technology, Palm City, FL, USA). The percentage of cell viability was calculated with the equation:

$$\% \text{ viability} = \frac{Abs\ of\ treated\ cells * 100}{Abs\ of\ control\ cells}$$

Where Abs of treated cells is the absorbance of cells with extract added, and Abs control cells is the absorbance of the cells without extract. The inhibitory concentration values (IC_{50}) were determined using linear regression in SigmaPlot.

For the morphological analysis, MDA-MB-231 and A549 cell lines were seeded in 96-well plates at a density of 3×10^3 cells. The cells were treated with EHBl and EDBl extracts (200 µg mL^{-1}) from *B. linanoe* for 24 h. Any morphological changes in the cells were examined and recorded under a light microscope (AE31E, Motic, Hong Kong) and a digital camera (MD500, AmScope, USA).

Annexin V apoptosis assay

MDA-MB-231 and A549 cells (1 x 10^6 cells per well) were cultured and incubated with each extract obtained from *B. linanoe* at 200 µg mL^{-1} for 24 h. After that, 100 µL of Muse ™ Annexin V & Dead Cell reagent (MCH100105, Millipore, Darmstadt, Germany) and 100 µL of cell suspension were added to each tube. Finally, the cells were incubated for 20 min at room temperature. Subsequently, the readings were taken using the Muse® Cell Analyzer (Millipore, Darmstadt, Germany).

Cell cycle assay

MDA-MB-231 and A549 cells were prepared independently with the EHBl and EDBl extracts (200 µg mL^{-1}) for the cell cycle assay, following the manufacturer's instructions. Briefly, after the cell culture and extract incubation, 1×10^6 cells were transferred to each tube, centrifuged at 300 × *g* for 5 min, and washed with 1X PBS. The cell pellet was resuspended, 1 mL of cold 70 % ethanol was added, and the mixture was incubated for 3 h at -20 °C. Subsequently, 200 µL of fixed cells were added to a new tube, centrifuged at 300 × *g* for 5 min, and washed with 1X PBS. 200 µL of Muse™ Cell Cycle reagent (MCH100106, Millipore, Darmstadt, Germany) was added to each tube and incubated for 30 min at room temperature in the dark. After that, the cells were analyzed in the Muse® Cell Analyzer, and the results obtained were graphed.

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Statistical analysis

All experiments were carried out in triplicate and represented as the mean ± SD. GraphPad Prism 7.0 was used for the data analysis. Statistically significant differences between groups were assessed using one-way analysis of variance (ANOVA), followed by the Dunnett test. Values of $P < 0.05$ were considered statistically significant.

Results

Gas Chromatography-Mass Spectroscopy (GC-MS) analysis

The phytochemical components (14) present in the extracts with their name, retention time, molecular formula, molecular weight, and peak area (%) are listed in table 1. The chromatogram of EHBl shows 8 compounds. Among the 8 compounds, the triterpenes such as lupeol 34.32% (Fig. 1**(A)**), τ-Sitosterol 11.36 % (Fig. 1**(C)**), and β-amyrin 10.03 % (Fig. 1**(D)**), and the 1-pyrrolidine butanoic acid 1,2 [1,1-dimethyl ethoxy)-α-nitro-2,6-bis (1,1-dimethyl ethyl)-4 methoxy phenyl ester, [S R* R*)] (13.01 %. Fig. 1**(B)**), were the major phytocompounds. On the other hand, the data indicated that the EDBl contained the triterpenes α-amyrin (13.93 %) and τ-Sitosterol (12.78 %) as major compounds. However, the spathulenol (tricyclic sesquiterpenoid) and the alkane nanocosane were the minor phytocompounds (1.105 and 2.925 %, respectively) presented in the extracts.

Table 1. Phytocomponents identified in the EHBl and EDBl extracts from *Bursera linanoe* by GC-MS.

Extract	Name of compound	RT (min)	Molecular formula	Molecular weight (g/mol)	Peak Area (%)	Ref
EHBl						
1	2-pentadecanone,6,10,14-trimethyl-	17.92	$C_{18}H_{36}O$	268.48	3.586	[14,22]
2	4,8,12,16-tetramethyl-heptadecan-4-olide	22.71	$C_{21}H_{40}O_2$	324.54	1.941	[22]
3	1-pyrrolidine butanoic acid 1,2 [1,1-dimethyl ethoxy)-α-nitro-2,6-bis (1,1-dymethyl ethyl)-4 methoxy phenyl ester, [S R* R*)]	30.00	$C_{28}H_{44}N_2O_7$	520.7	13.01	[22]
4	Nanocosane	30.13	$C_{29}H_{60}$	408.8	2.925	[25]
5	τ-Sitosterol	36.42	$C_{29}H_{50}O$	414.7	11.367	[24]
6	β-amyrin	37.04	$C_{30}H_{50}O$	426.7	10.033	[17]
7	4,4,6a,6b,8a,11,11,14b-octamethyl-1,4,4a,5,6,6a,6b,7,8,8,8a,9,10,11,12,12a,14,14a,14b-octadecahydro-2H-picen-3-one	37.46	$C_{30}H_{48}O$	424.7	9.814	[22]
8	Lupeol	38.30	$C_{30}H_{50}O$	426.72	34.324	[17]

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Extract	Name of compound	RT (min)	Molecular formula	Molecular weight (g/mol)	Peak Area (%)	Ref
EDBl						
1	Spathulenol	15.09	$C_{15}H_{24}O$	220.35	1.105	[23]
2	Caryophyllene oxide	15.17	$C_{15}H_{24}O$	220.35	0.883	[17,22]
3	2-pentadecanone,6,10,14-trimethyl-	17.88	$C_{18}H_{36}O$	268.48	7.54	[22]
4	1-pyrrolidine butanoic acid 1,2 [1,1-dimethyl ethoxy)-α-nitro-2,6-bis (1,1-dymethyl ethyl)-4 methoxy phenyl ester, [S R* R*)]	29.73	$C_{28}H_{44}N_2O_7$	520.7	8.479	[22]
5	τ-Sitosterol	29.87	$C_{29}H_{50}O$	414.7	12.78	[24]
6	α-amyrin	36.18	$C_{30}H_{50}O$	426.7	13.936	[17]

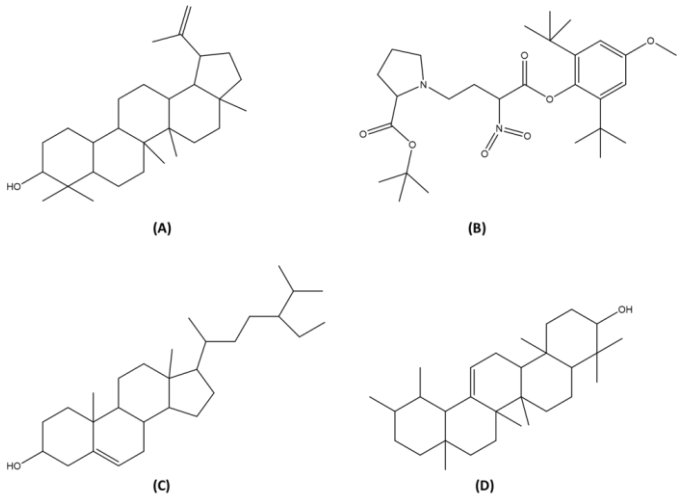


Fig. 1. Structure of main compounds of HBl and EDBl extracts from *Bursera linanoe*. **(A)** lupeol, **(B)** 1-pyrrolidine butanoic acid 1,2 [1,1-dimethyl ethoxy)-α-nitro-2,6-bis (1,1-dimethyl ethyl)-4 methoxy phenyl ester, [S R* R*)]. **(C)** τ-Sitosterol and **(D)** β-amyrin. The chemical structures were drawn using CheBioDraw 13.0 ultra.

Antiradical activity

In our study, the Anti-Radical Scavenging Activity (ARSA) of EHBl and EDBl was assessed using the DPPH and ABTS methods. We observed that the ARSA against $ABTS^{\bullet+}$ radical by the EHBl and EDBl was found to be higher than that of the $DPPH^{\bullet}$ radical. The high IC_{50} of the EHBl and EDBl values (4,180 and 7,800 μg mL^{-1}, respectively) indicates that extracts had lower $DPPH^{\bullet}$ radical scavenging activity. In contrast, EHBl and EDBl showed higher $ABTS^{\bullet+}$ Radical Scavenging Activity, exhibiting IC_{50} values of 119.43 and 113.29 μg mL^{-1}, respectively, against this radical, suggesting that the extracts can act as free-radical $ABTS^{\bullet+}$ inhibitors.

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Antiproliferative activity and morphological changes

The antiproliferative effect of EHBl and EDBl extracts from *B. linanoe* on MDA-MB-231 breast cancer cells was determined using an MTT cell viability assay. The EHBl on MDA-MB-231 cells showed a concentration-dependent effect at 75, 100, and 200 µg mL^{-1} (Fig. 2**(A)**), and cellular viability decreased by 16.57 %, 52.77 %, and 76.08 %, respectively, with an IC_{50} of 78.45 µg mL^{-1}. In contrast, in the EDBl evaluation, we observed a 49.49 % decrease in cell viability at 200 µg mL^{-1} and obtained an IC50 value of 202.33 µg mL^{-1} (Fig. 2**(B)**).

The antiproliferative activity of extracts on the A549 cell line is shown in Fig. 3. The results showed that the EHBl decreased cell viability by 43.14 % at a concentration of 200 µg mL^{-1}, with an IC_{50} value of 266.69 µgmL^{-1} (Fig. 3**(A)**). In addition, the EDBl at 100 µg mL^{-1} decreased cell viability by 25.61 % with an IC_{50} of 1880.60 µg mL^{-1} (Fig. 3**(B)**).

We found that EHBl at 200 µg mL^{-1} significantly reduced cell viability in the MDA-MB-231 and A549 cell lines. Morphological changes associated with the addition of these extracts to MDA-MB-231 and A549 cells are shown in Figures 2**(C)** and 3**(C)**. The cell lines MDA-MB-231 and A549 changed their characteristic epithelial morphology after treatment, adopting an abnormal circular morphology and possible cytoplasmic inclusions, characteristics of apoptotic bodies, like those shown with cells exposed to paclitaxel.

As the EHBl at 200 µg mL^{-1} significantly decreased cell viability in the MDA-MB-231 and A549 cell lines, we decided to evaluate its effect on the non-transformed MCF 10A cell line to determine the cytotoxic effect in normal cells. The results of this test showed that EHBl at 100 and 200 µg mL^{-1} did not significantly decrease the viability of MCF 10A cells (Fig. 4).

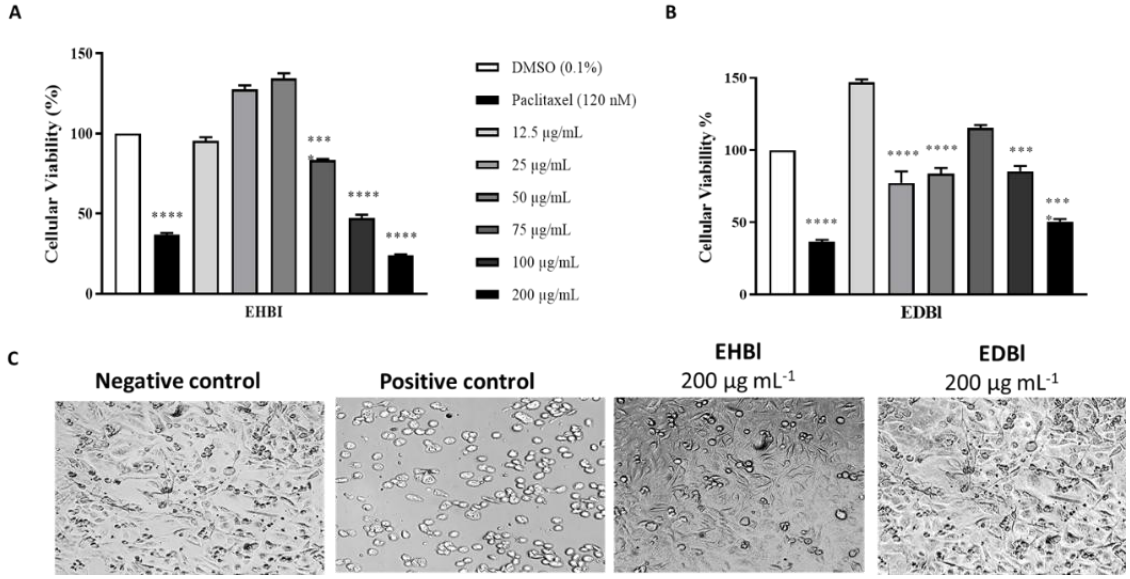


Fig. 2. Determination of the antiproliferative activity of *B. linanoe* extracts in the MDA-MB-231 cells. **(A)** EHBl: Hexanic extract and **(B)** EDBl: Dichloromethane extract. ANOVA test followed by a post-hoc Dunnett analysis. P <0.0001*** and P <0.00001****. **(C)** Morphological changes in MDA-MB-231 cells after treatment with EHBl and EDBl extracts (200 µg mL^{-1}) for 24 h. Negative control cells were treated with 0.1 % DMSO, and positive control cells were treated with Paclitaxel (120 nM). Bar calibration 40µm.

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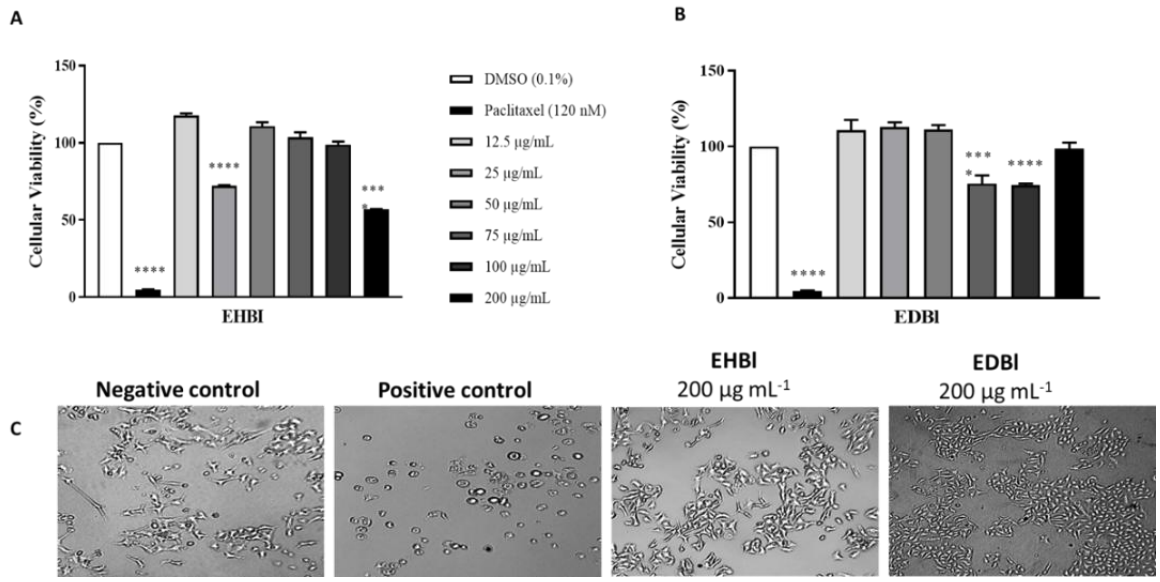


Fig. 3. Determination of the antiproliferative activity of *B. linanoe* extracts in the A549 cells. **(A)** EHBl: Hexanic extract and **(B)** EDBl: Dichloromethane extract. ANOVA test followed by a post-hoc Dunnett analysis. P <0.0001*** and P <0.00001****. **(C)** Morphological changes in A549 cancer cells after treatment with EHBl and EDBl extracts (200 µg mL^{-1}) for 24 h. Negative control cells were treated with 0.1 % DMSO, and positive control cells were treated with Paclitaxel (120 nM). Bar calibration 20µm.

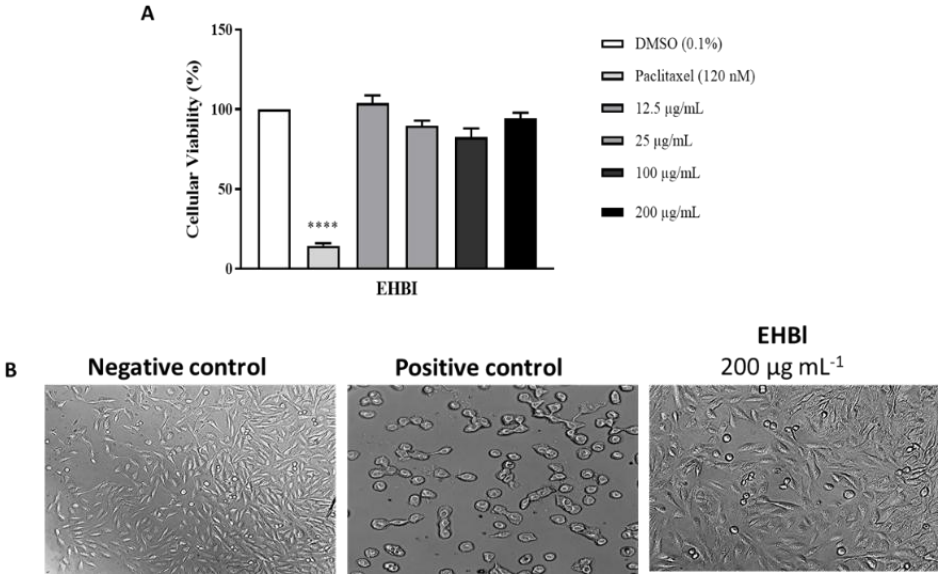


Fig. 4. Determination of the antiproliferative activity of *B. linanoe* extracts in the MCF-10A cells. **(A)** EHBl: Hexanic extract. ANOVA test followed by a post-hoc Dunnett analysis. P <0.0001*** and P <0.00001****. **(B)** Morphological changes in MCF-10A cells after treatment with EHBl extract (200 µg mL^{-1}) for 24 h. Negative control cells were treated with 0.1 % DMSO, and positive control cells were treated with Paclitaxel (120 nM). Bar calibration 40µm.

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Annexin V apoptosis assay

We observed that EHBL and EDBl at 200 µg mL^{-1} significantly decreased cell viability in the MDA-MB-231 and A549 cell lines, so we decided to determine whether the extracts increased the number of apoptotic cells when exposed to this concentration. The Annexin V assay was performed by flow cytometry. In the MDA-MB-231 cells, the result showed that 92.7 % of the negative control cells were live and viable (Fig. 5**(A)**, **(E)**) when the cell population was treated with EHBl decrease 12.41 % of the live cells this effect was accompanied by an increase of 13.9 % of apoptosis in early and late-stage (Fig. 5**(B)**, **(E)**), but the EHBl prominently induced late apoptosis in this line cell. In the A549 cells, the percentage of live cells in the cell population that was treated with EHBl decreased 17.6 % (Fig. 5**(D)**, **(F)**) compared to the negative control (Fig. 5**(C)**, **(F)**), while the percentage of population cells in early apoptosis increased (15 %). There was also an increase in the percentage of cells in late apoptosis (2.4 %) (Fig. 5**(D)**, **(F)**).

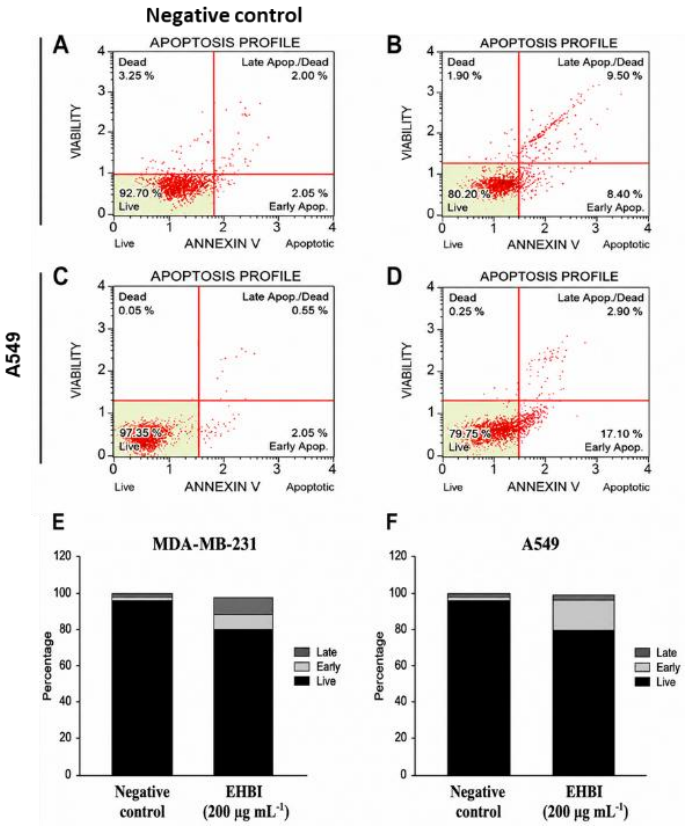


Fig. 5. Flow cytometry of MDA-MB-231 **(A)** and A549 **(C)** cells treated with Annexin V-FITC/PI. The cells were maintained with **EHBl** (200 µg mL^{-1}) for 24 h **(B)** and **(D)**, stained with FITC-annexin V/PI, and analyzed with a Muse® flow cytometer to assess apoptosis/necrosis. Q1: Annexin V negative/PI positive; Q2: Annexin V/PI positive; Q3: Annexin V positive/PI positive; Q4: Annexin V /PI positive negative. **(E)** and **(F)**: live (%), early apoptosis (%), and late apoptosis (%) were calculated compared with the treatment and negative control.

Cell cycle assay

To determine the effect of EHBl treatment on the cell cycle of MDA-MB-231 and A549 cells, DNA content was analyzed by flow cytometry. As shown in Fig. 6, EHBl increased the percentage of MDA-MB-231 and A549 cells in the G0/G1 phase (13 % and 58 %, respectively) and decreased the percentage of both cell lines in the S phase (17.2 % and 34 %, respectively) compared to the negative control cells. It was observed that EHBl increased the percentage of cells A549 at the G2/M phase (3.9 %).

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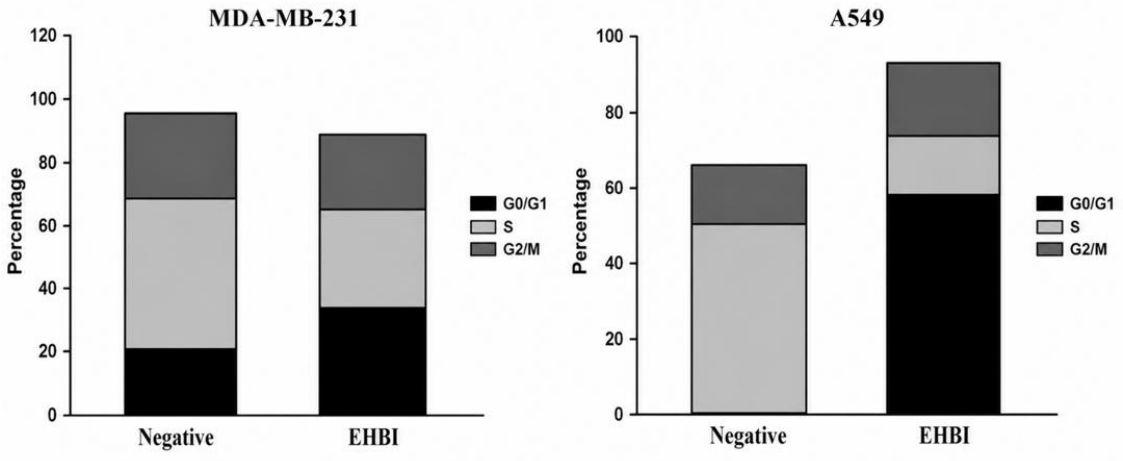


Fig. 6. Determination of the cell cycle phases in the cell lines. **(A)** MDA-MB-231and **(B)** A549 cells treated at 200 μg mL^{-1} of **EHBl**.

Discussion

Despite the advances in research against cancer, the World Health Organization predicts that the incidence of this disease will increase from 19.3 million in 2020 to 24.6 million by the 2030 year [1]. So, the interest in the search, discovery, and use of new compounds with anticancer activity [29]. Recent research highlights the importance of natural compounds as chemotherapeutic or chemopreventive agents against cancer. In this sense, a wide number of natural compounds have been investigated to improve the effectiveness of conventional therapy against cancer [28]. Additionally, it is also known that these biocompounds exhibit other health benefits because they are widely used as an antioxidant; this activity is crucial because a primary mechanism of many drugs against cancer cells is the formation of Reactive Oxygen Species (ROS) and Free Radicals, and the studies of antioxidant compounds suggest that they may reduce these compounds in carcinogenic processes [30].

The plants of the genus *Bursera* are a source of natural compounds with important pharmacological activities [10-13,15,16, 31]. In this study, GC-MS analysis of the EHBl and EDBl extracts detected 14 compounds. The major compounds identified were lupeol, τ-sitosterol, and β-amyrin (Table 1, Fig. 1). Although, the chemical profile of the leaves analyzed in this study differs from that reported for this species in the literature, where linalyl acetate (57.6 %), germacrene D (39.3 %), and β-caryophyllene (1.8 %) were the main leaves components of *B. linanoe* [20]. The probable reason for the discrepancy between our results and the published data is that plant phytochemicals may vary with biotic and abiotic factors and extraction methods [32].

However, several compounds present in the extracts have been reported in other *Bursera* species; for example, spathulenol and caryophyllene oxide (sesquiterpene) have been identified in the leaves and resin of *Bursera simaruba*, *Bursera glabrifolia,* and *Bursera microphylla* [10,33,34]. The compounds identified may contribute to the observed biological activity. Among them, lupeol has been reported to exhibit a wide range of pharmacological activities, including anticancer activity in several experimental models. Specifically, lupeol has shown antiproliferative and pro-apoptotic effects against Human Ovarian cancer cells, hepatocellular carcinoma cells, lung cancer cells, colorectal cancer cells, bladder cancer cells, and osteosarcoma cells. In addition, lupeol possesses analgesic and anti-inflammatory activities, which have been demonstrated in both *in vitro* and *in vivo* studies [35-38]. The α- and β-amyrins have been reported to exhibit a broad spectrum of biological activities. These triterpenes have demonstrated antitumor effects against Hep-G2 hepatocellular carcinoma cells, as well as neuroprotective and anticonvulsant activities in experimental models following oral and intraperitoneal administration during acute and sub-chronic treatments. Additionally, α- and β-amyrin have

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shown anxiolytic and antidepressant effects in murine models, anti-inflammatory and analgesic activity in several animal models, antibacterial activity against different bacterial strains, including *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, and antifungal activity against *Candida albicans*, *Aspergillus flavus*, Microsporum *canis*, *Fusarium solani*, and *Candida glabrata* [39-41]. β-sitosterol, a phytosterol commonly found in plants, has been reported to exhibit a wide range of biological activities. Anticancer effects have been demonstrated in several cancer cell lines, including Hep-G2 hepatocellular carcinoma cells, where β-sitosterol inhibits cell proliferation and promotes apoptosis. In addition, this compound has shown anxiolytic and sedative effects in murine models, antibacterial activity against *Escherichia coli* and *Staphylococcus aureus in vitro,* and antioxidant and antidiabetic properties in experimental animal models [42-44]. Although spathulenol (tricyclic sesquiterpenoid) and n-nanocosane have been reported to possess antimycobacterial activity against drug-resistant clinical isolates of *Mycobacterium tuberculosis*, exhibiting both growth-inhibition and bactericidal effects. In addition, spathulenol has demonstrated anti-nociceptive and anti-inflammatory activities in experimental animal models, including cold-induced nociception and carrageenan-induced paw edema assays [45,46]. This study also evaluated the antiradical activity of EHBl and EDBl extracts because the ability to scavenge free radicals in the living organism is crucial for the treatment of diseases such as cancer, hypertension, and other oxidative stress-related illnesses [47]. $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays were utilized for the measurement of antiradical activity of leaf extracts from *B. linanoe*. The results of the ABTS assay showed that EHBl and EDBl extracts had higher antiradical activity on this radical (IC_{50} values of 119.43 and 113.29 $\mu g mL^{-1}$, respectively) when compared to the $DPPH^{\bullet}$ radical.

Although there are no reports concerning the antiradical activity of extracts of leaves from *B. linanoe*, data from the literature showed that the ethanolic extract of leaves from *B. mycrophyla* exhibits antiradical activity on $DPPH^{\bullet}$ radical with an IC_{50} of 34.7 ± 1.8 $\mu g\ mL^{-1}$ [48]. Additionally, antioxidant activity studies of essential oils and stem extracts from *B. graveolens* reported IC_{50} values of 545.25 $\mu g\ mL^{-1}$ and 22.9 $mg\ mL^{-1}$, respectively, on $DPPH^{\bullet}$ radical [49,50]. On the other hand, different extracts of leaves from *Bursera penicillate* found IC_{50} values in the range 201-142 $\mu g\ mL^{-1}$ for $DPPH^{\bullet}$ and IC_{50} values in the range 39.25-71 $\mu g\ mL^{-1}$ for $ABTS^{\bullet+}$ [51]. In contrast, the hexanic extract of branch bark and aqueous extract of fresh leaves from *B. simaruba* do not exhibit antiradical activity on $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals [52,53]. The difference in antioxidant activity could be due to variability in antiradical activity among species, plant organs, and extraction solvents. Such differences may be associated with variations in phytochemical profile composition, by the abundance of phenolic compounds and terpenoids, environmental conditions, and extraction methodologies [54].

As shown in Figures 2 and 3, EHBl and EDBl extracts exhibited significant inhibitory effects on the proliferation of MDA-MB-231 and A549 cells at concentrations of 100 and 200 $\mu g\ mL^{-1}$. However, the effect of EHBl at 200 $\mu g\ mL^{-1}$ on both cell lines was significant, but not on the proliferation of MCF-10A cells at this concentration. Our results on antiproliferative activity are consistent with the only study reported for this species, in which hexanic and chloroformic extracts of *B. linanoe* bark exhibit IC_{50} values >20 $\mu g\ mL^{-1}$ against MCF-7, HF6, HFS-30, and PC3 cell lines [55].

Other authors reported the antiproliferative activity of *Bursera* species on several cancer cell lines. In this sense, the effects of the hexane fraction of resin extract from *B. mycrophilla* on an acute myeloid cell line (OCI-AML3) at concentrations of 250 and 25 $\mu g\ mL^{-1}$ were able to significantly decrease OCI-AML3 cell number [56] and components of essential oils from *B. glabrifolia* displayed a substantial cytotoxic activity against the cancer cell lines PC-3, OVCAR-3 AND K-562 exhibited $LC_{50} < 100$ $\mu g\ mL^{-1}$ [34]. In the organic extract of leaves from *B. copallifera* against HF6, MCF7, PC-3, Ca Ski and KB cells lines, found IC_{50} values >50 $\mu g\ mL^{-1}$ in the HF6, PC-3 and Ca Ski, and IC_{50} values of 20-34 $\mu g\ mL^{-1}$ on MCF7 and KB [57] and in MDA-MB-231 and MCF7 breast cancer cell line, IC_{50} values were 12.43 and 34.88 $\mu g\ mL^{-1}$ respectively [58]. Additionally, the antiproliferative activity of bark and leaf extracts (petroleum ether) from *B. tomentosa* against CSC-1595 cells (clinically isolated, derived from breast tumor tissue) at a concentration of 300 $\mu g\ mL^{-1}$ showed 7.45 % and 21.83 % viability, respectively [59]. Additionally, the EHBl and EDBl extracts at 200 $\mu g\ mL^{-1}$ prominently induced apoptosis in MDA-MB-231 and A549 cells and also affected their cell cycle by arresting them at the G0/G1 phase.

Reports on the apoptotic effects of *Bursera* species are scarce. However, in an acute myeloid cell line (OCI/AML3), the hexane fraction at 250 and 25 $\mu g\ mL^{-1}$ of *B. microphylla* resin was able to significantly decrease OCI-AML3 cell number caused by apoptosis, as it was correlated with augmented caspase-3

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activation, and this reduced cell number was associated with decreased S phase and blockade of the G2/M phase of the cell cycle [56].

The differences in the chemical profiles of EHBl and EDBl extracts could explain their distinct effects on antiproliferative, apoptotic, and cell-cycle arrest activities. In particular, lupeol, τ-sitosterol, and β-amyrin, major compounds in EHBl and EDBl extracts, exhibited antiproliferative activity and cytotoxic effects against ovarian, melanoma, liver, cervical, lung (non-small cell and A549 cell), and human colon adenocarcinoma (SW620) cancer cells [35,38,57-60]. In addition, the principal mechanisms of these compounds are 1) promoting cell apoptosis principally by activating the mitochondrial-mediated apoptosis pathway, inhibiting the Akt/PKB pathway by MAPK signaling pathway activation, suppressing EGFR/STAT3 activity in human non-small cell lung cancer cells, and inducing mitochondrial hyperfission and 2) inhibiting cancer cell proliferation by inducing cell cycle arrest, regulating the PI3K/Akt and Wnt signaling pathways [35,38,61-64].

Conclusions

In conclusion, the hexane (EHBl) and dichloromethane (EDBl) leaf extracts of *B. linanoe* exhibited antiproliferative activity against MDA-MB-231 and A549 cancer cell lines. Both extracts induced apoptosis and altered cell-cycle progression, suggesting that their cytotoxic effects are associated with the activation of programmed cell death pathways and cell-cycle arrest mechanisms. Preliminary phytochemical profile revealed the presence of triterpenes and sterols, including lupeol, sitosterol, and β-amyrin, compounds previously associated with antiproliferative and proapoptotic activities. Overall, our findings support the potential of *B. linanoe* leaves as a promising source of bioactive compounds with anticancer properties. However, additional studies are required to isolate and identify the active constituents, elucidate their molecular mechanisms of action, and determine their toxicological profile and biological profile (*in vivo* models) before considering therapeutic applications.

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