Cytotoxic effect of *Semialarium mexicanum* (Miers) Mennega root bark extracts and fractions against breast cancer cells

Juan Maldonado-Cubas¹ · Eduardo San Martín-Martínez¹ · Cinthya Nathaly Quiroz-Reyes¹ · Rocío Guadalupe Casañas-Pimentel²

Received: 22 January 2018 / Revised: 13 June 2018 / Accepted: 17 July 2018 / Published online: 23 July 2018
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Abstract The root bark of *Semialarium mexicanum* (Miers) Mennega (cancerina) is traditionally used in Mexico to treat cancer. However, there are no studies supporting its use. We evaluated whether *S. mexicanum* root bark induces cytotoxicity in breast cancer cells to determine if it has potential applications in the treatment of this disease. Extracts of *S. mexicanum* root bark in petroleum ether, ethanol, and water were obtained by ultrasound-assisted extraction. MTT and WST-1 assays were used to evaluate the cytotoxicity of the extracts toward breast cancer cells (MDA-MB-231 and MCF7), non-tumorigenic breast-derived cells (MCF 10A), and peripheral blood mononuclear cells (PBMCs). For the extract with greatest cytotoxicity, induction of apoptosis and oxidative stress were determined using flow cytometry. The extract was fractionated, and the cytotoxicity of its fractions was evaluated with the four cell types. The fractions were also analyzed by HPLC. Only the petroleum ether extract was cytotoxic for all cell types (MDA-MB-231 > MCF 10A/MCF7 > PBMCs). Cell death occurred by apoptosis, which could be associated with the induction of oxidative stress. Two fractions that were highly cytotoxic for breast cancer cells were obtained from this extract ($IC_{50} \leq 4.15 \mu\text{g/mL}$ for the most active fraction at 72 h). The

MCF 10A cells were less affected, while PBMCs were not affected after 72 h of treatment. Pristimerin was identified in both fractions and may be partially responsible for the cytotoxic effect. These results suggest that *S. mexicanum* root bark has a potential application in breast cancer treatment.

Keywords Cancer · Cytotoxicity · Cancerina · Celestraceae · Pristimerin · Hippocratea

Introduction

Cancer is one of the leading causes of death in the world (Torre et al. 2015). In the last 10 years, the number of cancer cases increased by 33% (Global Burden of Disease Cancer 2017), and it is estimated that the number of cases and deaths will continue to increase (Torre et al. 2015). Breast cancer is the most common type and causes the highest number of deaths among women worldwide (Global Burden of Disease Cancer 2017).

Natural products are important sources of active compounds with medical applications, including the treatment of cancer (Dholwani et al. 2008). More than 60% of the drugs used against cancer were isolated from natural sources (Seelinger et al. 2012), and plants are the most important resources (Alonso-Castro et al. 2011). There is a plethora of active compounds in plants that have yet to be discovered and exploited, which may help to reduce the dramatic cancer statistics expected in the future. A great variety of plant species are traditionally used in Mexico for the treatment of cancer (Alonso-Castro et al. 2011), such as *Semialarium mexicanum* (Miers) Mennega. It is a woody plant that inhabits the tropical deciduous forest of the country (Cáceres-Castillo et al. 2008). It is a member of the

✉ Rocío Guadalupe Casañas-Pimentel
rgcasanaspi@conacyt.mx

¹ Instituto Politécnico Nacional, Centro de Investigación en Ciencia Aplicada y Tecnología Avanzada, Unidad Legaria, Legaria 694, Irrigación. Miguel Hidalgo, 11500 Ciudad de México, México

² CONACYT-Instituto Politécnico Nacional, Centro de Investigación en Ciencia Aplicada y Tecnología Avanzada, Unidad Legaria, Legaria 694, Irrigación. Miguel Hidalgo, 11500 Ciudad de México, México

family Celastraceae and is also known as *Hemiangium excelsum* (Kunth) A.C.Sm., *Hippocratea excelsa* Kunth, *Hippocratea mexicana* Miers, *Hippocratea uniflora* Moc. & Sessé ex DC, and *Prionostemma setuliferum* Miers according to The Plant List (<http://www.theplantlist.org/tpl1.1/record/kew-2480810>). The root bark of this species is popularly known as “cancerina” and has been traditionally used to treat cancer (Alonso-Castro et al. 2011).

Semialarium mexicanum has been poorly studied scientifically to determine if it actually has the anticancer properties attributed by its ethnopharmacological use. There is only one report in which extracts of the whole root of *S. mexicanum* obtained in petroleum ether, ethyl acetate, and methanol were assayed to determine their cytotoxicity in KB (nasopharyngeal carcinoma), UIISO-SQC-1 (squamous cervix carcinoma), and HCT-15 COLADCAR (colon carcinoma) human cell lines. The extract obtained in petroleum ether showed the highest cytotoxic activity in the three cell lines, with IC₅₀ values of 0.76 (HCT-15), 0.004 (KB), and 0.006 (UIISO) µg/mL after 72 h of treatment (Popoca et al. 1998).

Although there are few studies on the cytotoxic activity of *S. mexicanum*, there are several reports about the activity of the quinone methide triterpenes pristimerin and tingenone present in *S. mexicanum*, as well as other members of the Celestraceae family. These two molecules have promising potential as therapeutic and chemopreventive agents against cancer (Gomes et al. 2011). The cytotoxic activity of pristimerin has been evidenced in different cancer-derived cell lines and animal models. It has been shown to inhibit cell proliferation both in vitro and in vivo, as well as angiogenesis and metastatic processes. Pristimerin has effects on the cell cycle, apoptosis, proteasome activity, the generation of reactive oxygen species (ROS), and the NF-κB, AKT/mTOR, and MAPK/ERK pathways (Bashir et al. 2017).

Tingenone has been shown to have antimitotic effects, it induces apoptosis in vitro (probably because of its interaction with caspases), and it inhibits RNA and protein synthesis (Gomes et al. 2011). Despite the therapeutic potential of these agents, obtaining them involves long and complex laboratory processes that have low yields. In addition, their study has not yet translated into tangible benefits because these active compounds are not in clinical use. However, people continue using the infusions of *S. mexicanum* to complement anticancer therapies, even though there is no scientific evidence to support the use of these infusions with a control of the composition and dosage or knowledge of their adverse effects.

Chemical compounds in the root bark of *S. mexicanum* (Fig. 1) have been isolated and identified using different approaches. Reyes-Chilpa et al. (2003) reported the extraction of metabolites in the root bark of *S. mexicanum*

in petroleum ether by maceration and obtained a yield of 0.73 wt.%. Chemical characterization of the extract indicated the presence of long-chain hydrocarbon compounds and four triterpenoids: β-sitosterol (I), pristimerin (II), canophyllol (III), and friedelin (IV). Other molecules identified include triterpenoids such as canophyllal (V) and canophyllic acid (VI), as well as the quinone methide triterpenes celastrol (VII), tingenone (VIII), and excelsin (IX) (Reyes-Chilpa et al. 2003).

A Soxhlet extraction obtained in a mixture of hexane and diethylether (1:1) allowed for the isolation and identification of *trans*-polyisoprene (X), 21β-hydroxyolean-12-en-3-one (XI), dzununcanone (XII), 21α-hydroxy-3-oxofriedelane (XIII), and xuxarine Eβ (XIV). 68.38 wt.% of the total extract corresponded to *trans*-1,4-polyisoprene (X), a white rubbery material that has been described as the largest component of the nonpolar (Mena-Rejón et al. 2007) and moderately polar (Reyes-Chilpa et al. 2003) extracts of *S. mexicanum* root bark.

The cytotoxic activity of *S. mexicanum* root bark was evaluated to determine if it has potential for application in the treatment of breast cancer. *S. mexicanum* root bark extracts were obtained by ultrasound-assisted extraction. This method significantly reduces the sample processing time and has not been used previously to obtain the active compounds of this plant species. Additionally, a method of fractionation by cryoprecipitation is presented, which makes it possible to obtain active fractions without the excessive use of organic solvents used in fractionation by column chromatography. Two fractions that are highly cytotoxic toward breast cancer cells were obtained, which demonstrates that *S. mexicanum* root bark has potential for the treatment of breast cancer and could be used for the development of anticancer drugs.

Materials and methods

Plant material

Semialarium mexicanum (Miers) Mennega root bark was purchased from the Santo Domingo Herbalist (Mexico City) and classified by the herbarium of the Universidad Autónoma de Chapingo, which retains a voucher specimen (number 33093). The plant material was sanitized, dried, and ground into particles with diameters less than 177 µm.

Obtainment of the extracts

The extraction was performed in an ultrasonic bath (Elma, Germany) at 30 °C and 25 kHz for 30 min using solvents in a sequential order of polarity: petroleum ether, ethanol, and water. First, 75 g of powdered *S. mexicanum* root bark

Fig. 1 Chemical compounds present in non-polar extracts of *S. mexicanum* root bark. β -sitosterol (I), pristimerin (II), canophyllol (III), friedelin (IV), canophyllal (V), canophyllic acid (VI), celastrol (VII), tingenone (VIII), excelsin (IX), *trans*-1,4-polyisoprene (X),

21 β -hydroxyolean-12-en-3-one (XI), dzununcanone (XII), 21 α -hydroxy-3-oxofriedelane (XIII), and xuxarine E β (XIV) (Reyes-Chilpa et al. 2003; Mena-Rejón et al. 2007)

was mixed with 750 mL of solvent and extracted with three washes. The extracts were centrifuged at 522 $\times g$, decanted, filtered (Whatman No. 1), and dried (vacuum oven, Thelco,

Precision Scientific, USA) at 40 °C. All extracts were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich)

and stored at $-20\text{ }^{\circ}\text{C}$ for preservation and sterilization (Cos et al. 2006).

Cell culture and cytotoxicity evaluation of the extracts

Cell lines culture and treatments

Cell lines from mammary adenocarcinoma (MDA-MB-231 and MCF7) and non-tumorigenic cells (MCF 10A) were purchased from the American Type Culture Collection. The MDA-MB-231 and MCF7 cells were grown in high-glucose Dulbecco's Modified Eagle Medium without HEPES (DMEM, Caisson Labs) and supplemented with 10% fetal bovine serum (FBS, ATCC). The MCF 10A cells were cultured in Dulbecco's Modified Eagle Medium-F12 (DMEM-F12, GIBCO) supplemented with 10% FBS, epidermal growth factor (EGF, 20 ng/mL, Sigma-Aldrich), insulin (0.25 U/mL, Pisa Biotech), and hydrocortisone (0.5 $\mu\text{g/mL}$, Sigma-Aldrich).

Cells were seeded in 96-well plates at a confluence of 7000 cells per well for the MDA-MB-231 and MCF7 cell lines and 10,000 cells per well for the MCF 10A cells, with a total volume of 100 μL of the corresponding culture medium. Cultures were incubated at $37\text{ }^{\circ}\text{C}$ in a humidified atmosphere with 5% CO_2 for 24 h to allow the adhesion of the cells to the surface of the plate. The culture medium was then discarded, and 100 μL of medium containing the different treatments were added to each well.

First, the cytotoxicity of the petroleum ether, ethanol, and water extracts was tested on the MDA-MB-231 cells at concentrations of 15, 30, 45, and 60 $\mu\text{g/mL}$. Three controls were used: (a) a negative control of untreated cells; (b) a positive control of cells treated with paclitaxel 0.25 $\mu\text{g/mL}$; and (c) a placebo control of cells treated with 0.2% v/v DMSO. The last control was used to evaluate the effect of the DMSO used to preserve and sterilize the extracts on the viability of the cells. In all cases, the DMSO added together with the extracts achieved a final concentration in the culture medium of less than 0.2% v/v. Once all the treatments were added, cells were incubated for 24 and 72 h at $37\text{ }^{\circ}\text{C}$ in a humidified atmosphere with 5% CO_2 . The cytotoxicity of the extracts was then determined by the MTT assay.

Only the extract obtained in petroleum ether was assayed for its cytotoxic activity on the MCF7 and MCF 10A cells due to its proven cytotoxicity. The protocol described for the MDA-MB-231 cells was used. The extract concentrations evaluated for these cell lines were 30, 45, 60, and 75 $\mu\text{g/mL}$ in order to increase the cytotoxic effect and to determine the IC_{50} values.

MTT assay

Once the treatments were completed, 0.1 mg of MTT reagent (Sigma-Aldrich) was added to each well, and the plates were incubated for 2 h at $37\text{ }^{\circ}\text{C}$ in humidified atmosphere with 5% CO_2 in darkness. After completing the incubation, the medium was removed, and 100 μL of DMSO were added. The absorbance of the samples was determined at 570 nm in an ELISA reader (Labsystem Multiskan MS, Thermo Scientific, USA). The different treatments were compared against the negative control, and the cell viability percentage was calculated.

PBMCs isolation and treatments

PBMCs were obtained from leukocyte packages of healthy donors (Blood Bank, Central Military Hospital, Mexico City) using the density gradient medium Lymphoprep (Axis-Shield) according to the manufacturer's instructions. The PBMCs were seeded in 96-well plates at a confluence of 50,000 cells per well in a volume of 50 μL of culture medium. The petroleum ether extract was tested for cytotoxicity on these cells by adding 50 μL of X-VIVO medium containing the different treatments to each well to obtain a final volume of 100 μL .

Petroleum ether extract was evaluated on the PBMCs at concentrations of 30, 45, 60, and 75 $\mu\text{g/mL}$. The positive, negative, and placebo controls were used as described for the cell lines. The cytotoxicity of the extract was determined by the WST-1 assay after 24 and 72 h of treatment.

WST-1 assay

After completing the treatments for the PBMCs, 10 μL of WST-1 reagent solution (Clontech) were added to each well. The cells were incubated for 90 min at $37\text{ }^{\circ}\text{C}$ in humidified atmosphere with 5% CO_2 . The plates were then shaken for 5 s, and the absorbance at 450 nm was determined using ELISA equipment. The different treatments were compared to the negative control, and the cell viability percentage was calculated.

Statistical analysis

All cytotoxicity assays were repeated at least 6 times. The results were analyzed through one-way ANOVA followed by Dunnett's multiple comparisons tests using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla California USA).

Analysis of apoptosis by flow cytometry

The induction of apoptosis by the petroleum ether extract was evaluated using the MDA-MB-231, MCF7, and MCF 10A cell lines by flow cytometry (Muse Cell Analyzer, Merck Millipore Corporation) with Annexin V for the detection of apoptotic cells and 7-amino-actinomycin D (7-AAD) to evaluate the loss of cell membrane integrity related to cell death (Schmid et al. 1992) (Muse Annexin V and Dead Cell Assay Kit, Merck Millipore Corporation). To achieve this, 260,000 cells per well were seeded in 6-well plates with 3 mL of the corresponding culture medium. The cells were incubated for 24 h at 37 °C in 5% CO₂ to allow adhesion to the surface of the plate. The culture medium was then removed, and 3 mL of fresh medium was added with 60 µg/mL of the extract. Cells treated with 0.25 µg/mL of paclitaxel and untreated cells were used as controls. Treatments were maintained for 24 and 72 h at 37 °C in 5% CO₂. The culture medium and the trypsinized cells were then collected and centrifuged for 10 min at 140×g, and the cell pellet was resuspended in 200 µL of culture medium. Staining of the cells and flow cytometry assays were carried out according to the manufacturer's protocol.

Oxidative stress evaluation

Oxidative stress induction by the petroleum ether extract was evaluated using the MDA-MB-231, MCF7, and MCF 10A cell lines by flow cytometry (Muse Oxidative Stress kit, Merck Millipore Corporation). The production of intracellular superoxide radicals was determined, the results are expressed in terms of the ROS production, and the ROS(+) and ROS(−) cell percentages are indicated. For this evaluation, 260,000 cells per well were seeded in 6-well plates with 3 mL of the corresponding cell culture medium. Cells were incubated for 24 h at 37 °C in 5% CO₂ to allow adhesion to the surface of the plate. The culture medium was then removed, and 3 mL of fresh medium containing 60 µg/mL of the petroleum ether extract were added. Cells treated with paclitaxel 0.25 µg/mL and untreated cells were used as controls. Treatments were maintained for 24 and 72 h. The culture medium and the trypsinized cells were then collected and centrifuged for 10 min at 140×g, and the cell pellet was resuspended in 40 µL of culture medium. The staining of the cells and the flow cytometry assays were done according to the manufacturer's protocol.

Fractionation by cryoprecipitation

The petroleum ether root bark extract was fractionated by cryoprecipitation (precipitation at low temperature). After

ultrasound-assisted extraction, the petroleum ether extract containing all the metabolites was stored at − 20 °C for 48 h to allow the precipitation of the gum present in it. The extract was then filtered and dried in a vacuum oven. The dried material was resuspended in 40 mL of petroleum ether and centrifuged at 522×g for 15 min. The supernatant was stored at − 20 °C for 48 h and centrifuged again. A final precipitation process was applied to the supernatant at − 20 °C for 24 h. The samples were centrifuged to obtain two fractions: the sediment (a pale yellow powder) and the supernatant. The supernatant was concentrated in a vacuum oven, which produced an oily fraction with an intense red color.

Cytotoxicity assays of the fractions

Both the oil (supernatant) and powder (sediment) fractions were tested for cytotoxicity using the protocol described above using concentrations of 2.5, 5, 10, 15, 20, and 25 µg/mL.

Selectivity index determination

To determine whether the *S. mexicanum* root bark petroleum ether extract and its fractions are selective for the breast cancer cells, the selectivity index (SI) was calculated using IC₅₀ of the evaluated treatment in normal cells, which was divided by IC₅₀ of the evaluated treatment in cancerous cells to obtain SI. IC₅₀ is the concentration of the extract or fraction needed to reduce the cell viability to 50% with respect to untreated cells (Badisa et al. 2009).

Chemical characterization of the cryoprecipitated gum by FT-IR spectroscopy

The gum removed from the petroleum ether extract was characterized by FT-IR spectroscopy (Agilent Cary 630 FT-IR Spectrometer) in the range of 650–4000 cm^{−1}. The spectrum was analyzed using Essential FT-IR spectroscopy software (Operant LLC, USA).

HPLC analysis

The oily and the powdered fractions were evaluated by UV–Vis spectroscopy to identify the absorbance signals to be used in the chromatographic studies. The samples were then prepared for HPLC analysis as follows. First, 50 mg of the oily fraction was diluted in 1 mL of a mixture of MeOH:H₂O (95:5 v/v) and sonicated (45 kHz) for 10 min. The sample was then centrifuged at 8452×g for 15 min at room temperature. The supernatant was diluted in 1.5 mL of a solution of MeOH/(H₂O:H₃PO₄ (0.5%)) (70:30 v/v). Furthermore, 2.5 mg of the powdered fraction was

dissolved in 1 mL of a solution of MeOH/(H₂O:H₃PO₄ (0.5%)) (80:20 v/v). Both fractions were filtered through a 0.45- μ m PVDF membrane before chromatographic analyses.

Chemical fingerprint of the fractions, identification and quantification of pristimerin (HPLC–DAD)

HPLC analyses were carried out using an Agilent 1200 series HPLC–DAD (Agilent Technologies, Waldbronn, Germany) equipped with a Zorbax Eclipse Plus C18 column (Agilent Technologies, Waldbronn, Germany) (4.6 $\times$ 100 mm, 3.5 μ m). The mobile phase consisted of a mixture of H₂O:H₃PO₄ (99.5:0.5) as eluent A and MeOH as eluent B. The system was run with a gradient program according to the method proposed by Araujo Leon et al. (2015) with minor modifications: 55–70% B, 0–10 min; 70–85% B, 10–15 min; 85–90% B, 15–23 min and 90–100% B, 23–33 min, followed by isocratic elution with 100% B for 3 min. The column temperature was set at 30 °C, the flow rate was set at 0.7 mL/min, and the injection volume was 5 μ L. Chromatograms were acquired at 290 and 420 nm. Pristimerin was identified at 420 nm using a commercial standard (530070, Merck Millipore). A standard calibration curve (0.05–1.0 mg/mL) was prepared and used for quantitative analysis.

Results and discussion

Petroleum ether extract of *S. mexicanum* root bark is cytotoxic for MDA-MB-231, MCF7, and MCF 10A cells

In the MDA-MB-231 cells (Fig. 2a), the *S. mexicanum* root bark petroleum ether extract induced cell proliferation after 24 h of treatment with 15 and 30 μ g/mL. Statistically insignificant changes were observed for 45 μ g/mL with respect to the control of untreated cells, while increasing of the extract concentration to 60 μ g/mL significantly reduced the cell viability to 63%. For 72 h of treatment, the proliferative effect of low concentrations (15 and 30 μ g/mL) observed at 24 h decreased, while the cytotoxic activity of higher concentrations was increased, achieving 26% cell viability at 60 μ g/mL.

All the evaluated concentrations of the ethanolic extract showed time- and concentration-dependent proliferative effects for both 24 and 72 h of treatment. The water extract did not induce statistically significant changes in cell viability at 24 h for all the evaluated concentrations. At 72 h, values greater than or equal to 30 μ g/mL induced higher viability values with respect to the control of untreated cells, but the viability was similar to those obtained for the

control with 0.2% DMSO, suggesting that this effect could be exerted by DMSO (Wen et al. 2015; Singh et al. 2017). Paclitaxel at 0.25 μ g/mL reduced the cell viability to 82% at 24 h of exposure and 34% at 72 h.

The traditional use of *S. mexicanum* suggests drinking an aqueous infusion of the root bark to obtain therapeutic effects (Reyes-Chilpa et al. 2003). However, in our research, only the petroleum ether extract induced cytotoxicity, whereas the aqueous extract was not cytotoxic for the MDA-MB-231 cells. This observation agrees with the report by Popoca et al. (1998), in which the petroleum ether extract of the whole root of *S. mexicanum* showed greater cytotoxic activity toward cancerous cells compared to the extracts obtained in solvents with greater polarity (Popoca et al. 1998).

Because only the petroleum ether extract showed cytotoxicity toward MDA-MB-231 cells, the studies of the other cell types were done using this extract. Concentrations of 30, 45, 60, and 75 μ g/mL were evaluated to calculate the IC₅₀ values. In the MCF7 cells (Fig. 2b), a proliferative effect was observed at 24 h for the lowest concentration, whereas for 45 and 60 μ g/mL, no statistically significant changes were observed with respect to untreated cells. At 75 μ g/mL, the cell viability decreased to 87%. After 72 h of treatment, the proliferative effect observed for 30 μ g/mL at 24 h was enhanced, reaching a viability of 150%. A concentration-dependent cytotoxic effect was observed for higher values, with a viability of 60% observed for 75 μ g/mL. Cells treated with DMSO showed no statistically significant changes in cell viability for the two evaluated times, while paclitaxel reduced cell viability to 78% at 24 h and 49% after 72 h.

Subsequently, the extract was evaluated with MCF 10A, a non-tumorigenic cell line derived from mammary tissue (Fig. 2c). A cytotoxic effect was not observed in this cell line after 24 h of treatment with the concentrations evaluated, but after 72 h of exposure, a concentration-dependent cytotoxic effect was induced with 45, 60, and 75 μ g/mL. The controls did not show statistically significant changes at 24 h, while at 72 h, a decrease in cell viability was observed for both DMSO (81%) and paclitaxel (75%).

In summary, the petroleum ether extract of *S. mexicanum* root bark induced a proliferative effect for both breast cancer-derived cell lines in concentrations less than or equal to 30 μ g/mL, showing no statistically significant changes in the non-tumorigenic cell line (MCF 10A). Higher concentrations induced cytotoxicity for all three cell lines. This behavior could suggest that the extract contains components that promote the proliferation of the tumor cells and a cytotoxic effect. In that case, it would be desirable to remove the components that exert the proliferative effect to promote the cytotoxicity. IC₅₀ values for the three cell lines after 24 h of treatment were higher than

Fig. 2 Cytotoxicity assessment of *S. mexicanum* root bark extracts in **a** MDA-MB-231, **b** MCF7, **c** MCF 10A, and **d** PBCM after 24 and 72 h of treatment. For each group, $n = 6$, the + symbol indicates a

statistically significant difference from the control with untreated cells ($P < 0.05$), and the * symbol indicates a statistically significant difference from the control of untreated cells ($P < 0.01$)

the evaluated concentrations used, whereas IC_{50} values of 55.5 (MDA-MB-231), 66.8 (MCF 10A), and 75.8 (MCF7) $\mu\text{g/mL}$ were obtained after 72 h of treatment

Finally, the extract was evaluated using PBMCs (Fig. 2d) to explore its toxicity on the immune system. No statistically significant changes were observed after 24 or 72 h of treatment with 30, 45, and 60 $\mu\text{g/mL}$. Viabilities of 86% at 24 h and 79% at 72 h were obtained for the assays with 75 $\mu\text{g/mL}$. Interestingly, the extract did not induce a proliferative effect on non-tumorigenic cells (MCF 10A and PBMCs), and it exerted a much lower cytotoxic effect on PBMCs when compared to the evaluated cell lines. The viability observed for PBMCs treated with 60 $\mu\text{g/mL}$ of the extract after 72 h was 91%, a much higher value than those obtained for the two breast cancer-derived cell lines (26% for MDA-MB-231 and 68% for MCF7).

A proliferative effect was observed for DMSO on the PBMCs at 24 and 72 h of treatment. However, paclitaxel

did not show statistically significant changes at 24 h, whereas it exerted a proliferative effect after 72 h. Other studies have reported that the proliferative effect of paclitaxel on PBMCs corresponds to an immunomodulatory effect (Kaneno et al. 2011). This phenomenon gives advantages to paclitaxel over other antineoplastic agents by promoting the activity of the immune system on tumor cells. However, this effect has also been associated with hypersensitivity reactions experienced by patients treated with this drug (Fitzpatrick and Wheeler 2003).

Petroleum ether extract of *S. mexicanum* root bark induces cell death via apoptosis

Flow cytometry assays were performed to evaluate the induction of apoptosis by the petroleum ether extract (60 $\mu\text{g/mL}$) in MDA-MB-231, MCF7, and MCF 10A cells. The following were used as controls: untreated cells

(negative control) and cells treated with 0.25 $\mu\text{g/mL}$ of paclitaxel (positive control) (Wang et al. 2000). For the interpretation of the apoptosis results, four cell populations were generated as a function of the signal obtained with Annexin V and 7-AAD: (1) live cells (Annexin V-/7-AAD-); (2) cells in early apoptosis (Annexin V+/7-AAD-); (3) cells in late apoptosis/dead (Annexin V+/7-AAD+); and (4) dead cells (Annexin V-/7-AAD+).

After 24 h of incubation, all three cell lines without treatment (negative control, see Fig. 3a, d, and g) showed values of live cells higher than 90% of the population. Minor changes were observed at 72 h of incubation (Fig. 4a, d, and g). Paclitaxel induced cell death by apoptosis in the breast cancer-derived cells after 24 h of treatment (Fig. 3b, e), reducing the percentage of live cells to

72.73 and 50.52% for MDA-MB-231 and MCF7, respectively. MCF 10A cells showed no major changes (Fig. 3h) when compared with the negative control, indicating a minor induction of cell death in this cell line.

After 72 h of treatment, paclitaxel significantly reduced the percentage of live cells in all three cell lines (Fig. 4b, e, and h) when compared to the negative control in the same period of incubation (Fig. 4a, d, and g). Paclitaxel induced significant increases in the percentages of cells in early and late apoptosis for all the experiments. The MDA-MB-231 and MCF7 cell lines (Fig. 4b, e) showed mostly events of late apoptosis or dead (41.04 and 35.39%, respectively), while the MCF 10A cells (Fig. 4h) presented a higher number of events in early apoptosis (53.52%).

Fig. 3 Dot plots of apoptosis assays after 24 h of treatment. Negative control, positive control, and petroleum ether extract of *S. mexicana* root bark were evaluated in MDA-MB-231, MCF7, and MCF 10A cells

Fig. 4 Dot plots of apoptosis assays after 72 h of treatment. Negative control, positive control, and petroleum ether extract of *S. mexicanum* root bark were evaluated in MDA-MB-231, MCF7, and MCF 10A cells

Treatment with the petroleum ether extract for 24 h increased the number of events in early and late apoptosis/dead (14.29, 5.31%, and 23.06, 7.40%) in breast cancer-derived cell lines MDA-MB-231 and MCF7 (Fig. 3c, f) compared to the negative control (Fig. 3a, d). These values are lower than those obtained with the positive control (Fig. 3b, e). However, MCF 10A cells treated with the extract (Fig. 3i) showed no significant changes in comparison to the negative control for the same incubation time.

After 72 h of treatment, the extract induced an accumulation of cells in early apoptosis (34.45%) for MDA-MB-231 cells (Fig. 4c), decreasing the percentage of live cells to 50.18%. In MCF7 cells (Fig. 4f), the extract

increased the population of cells in early apoptosis (31.90%) with a percentage of 60.25% for live cells. Finally, for the MCF 10A cells, the treatment with the extract (Fig. 4i) resulted in 73.03% live cells, 23.76% of cells in early apoptosis and 3.09% in late apoptosis/dead. Cancer cells were more sensitive to the induction of apoptosis by the petroleum ether extract. The results suggest that this pathway is the main mechanism of cell death induced by the extract because the percentage of non-apoptotic cell death (Annexin V-/7-AAD+) was negligible for all the experiments.

Petroleum ether extract of *S. mexicanum* root bark induces oxidative stress

The cell death induced by the petroleum ether extract may be associated with the production of ROS if it contains pristimerin (Bashir et al. 2017). For that reason, the induction of oxidative stress was evaluated in the three cell lines (MDA-MB-231, MCF7, and MCF 10A) after 24 and 72 h of incubation. The results are shown in Fig. 5 and 6, where M1 represents the population of ROS(−) cells, and M2 represents the ROS(+) cells.

The basal ROS production for the three cell lines (negative controls) evaluated after 24 h of incubation is shown in Fig. 5a, d, and g. More than 86% of the cells were negative for ROS in these controls. The use of paclitaxel induced a significant increase in the percentage of ROS(+) cells for MDA-MB-231 (21.08%) and MCF 10A (31.92%) cells (Fig. 5b, h), while MCF7 cells (Fig. 5e) showed no

major changes compared to the negative control. ROS generation has been previously described as an important factor in the induction of cell death by paclitaxel (Alexandre et al. 2007; Varbiro et al. 2001). The treatment with the petroleum ether extract induced an increase in the population of ROS(+) cells for the three cell lines (Fig. 5c, f, and i) when compared to the negative controls, with values of 18.33, 26.78, and 22.87% ROS(+) cells for MDA-MB-231, MCF7, and MCF 10A, respectively.

The basal ROS production for the three cell lines (negative controls) evaluated after 72 h of incubation is shown in Fig. 6a, d, and g, where more than 80% of the cells were ROS(−) for the three cell lines. After 72 h of treatment, paclitaxel showed a significant induction of ROS production in the MCF7 and MCF 10A cell lines (Fig. 6e, h) with 48.66 and 44.42% of ROS(+) cells, respectively. The MDA-MB-231 cells (Fig. 6b) showed a lower induction of ROS. The petroleum ether extract increased ROS

Fig. 5 ROS profiles after 24 h of treatment. Negative control, positive control, and petroleum ether extract of *S. mexicanum* root bark were evaluated in MDA-MB-231, MCF7, and MCF 10A cells.

M1 represents the population of ROS(−) cells and M2 represents the population of ROS(+) cells

Fig. 6 ROS profiles after 72 h of treatment. Negative control, positive control, and petroleum ether extract of *S. mexicana* root bark were evaluated in MDA-MB-231, MCF7, and MCF 10A cells.

generation in all three cell lines, with 10.17, 31.16, and 38.42% ROS(+) cells for MDA-MB-231, MCF7, and MCF 10A, respectively (Fig. 6c, f, and i).

It has been proven that a wide range of anticancer agents are active because of their ability to induce the generation of ROS in malignant cells (Yadav et al. 2015). This occurs because the damage produced by oxidative stress leads to the activation of stress-responsive transcription factors that induce apoptosis. This ROS production also affects the mitochondrial activity, inducing a depletion of ATP (Ozben 2007). The petroleum ether extract of *S. mexicana* root bark may induce a significant decrease in the mitochondrial function in the three cell lines evaluated. This is evidenced by the results of the MTT assays, as well as an increased generation of ROS, leading to cell death via apoptosis. All these results suggest that the root bark of *S. mexicana* may have applications in the treatment of breast cancer. Thus, this extract is a candidate for further

M1 represents the population of ROS(–) cells and M2 represents the population of ROS(+) cells

investigations for the possible development of anticancer drugs.

Fractionation of the petroleum ether extract of *S. mexicana* root bark

Fractionation was performed by cryoprecipitation to remove the gum and partially purify the active compounds contained in the petroleum ether extract to improve its cytotoxic activity. This gum was identified by FT-IR spectroscopy as *trans*-1,4-polyisoprene (structure X in Fig. 1), which has previously been identified in *S. mexicana* (Mena-Rejón et al. 2007). The spectrum and the assignment of the signals are shown in Fig. 7.

After removing the gum, two fractions were obtained. The first fraction was obtained as a pale yellow powder, whereas the second fraction showed an intense red color and oily aspect. The method used to obtain these fractions is shown in Fig. 8, including the yields of each step.

Fig. 7 FT-IR spectrum obtained for the gummy material cryoprecipitated from the petroleum ether extract of *S. mexicanum* root bark. The compound was identified as *trans*-1,4-polyisoprene with characteristic peaks in A: asymmetric stretching CH_3 , 2965 cm^{-1} ; B: asymmetric stretching CH_2 , 2913 cm^{-1} ; C: symmetric stretching

CH_2 , 2846 cm^{-1} ; D: stretching vibration of 1,4-unit $\text{CH}=\text{CH}$, 1663 cm^{-1} ; E: deformation CH_2 , 1447 cm^{-1} ; F: Scissoring vibration in *trans*-1,4-unit CH_3 , 1383 cm^{-1} ; G: bending out of plane $=\text{C}-\text{H}$, 876 cm^{-1} ; H: rocking CH_3 , 794 cm^{-1} ; I: rocking CH_2 , 749 cm^{-1} (Chen et al. 2013; Arjunan et al. 2001; dos Santos et al. 2005)

Fig. 8 Flowchart of the extractive procedures and the fractions generated. The *S. mexicanum* root bark was extracted by ultrasound-assisted extraction using petroleum ether, ethanol, and water. The petroleum ether extract was fractionated by cryoprecipitation, *trans*-1,4-polyisoprene was removed from the extract, and two fractions (powder and oily) containing pristimerin were obtained

Fractions of the *S. mexicanum* root bark petroleum ether extract are highly cytotoxic for breast cancer cells

Both fractions were evaluated for cytotoxicity in the MDA-MB-231 cell line (Fig. 9a) at 2.5, 5, 10, 15, 20, and 25 $\mu\text{g}/\text{mL}$ for 24 and 72 h. The fractionation process markedly increased the cytotoxic effect of fractions when compared to the complete extract, with a decrease in cell viability observed from 2.5 $\mu\text{g}/\text{mL}$. IC_{50} values of 6.83 and 4.80 $\mu\text{g}/\text{mL}$ were obtained for the oily fraction at 24 and 72 h, respectively. The powdered fraction showed a better cytotoxic effect with IC_{50} values of 5.98 and 3.35 $\mu\text{g}/\text{mL}$ at 24 and 72 h, respectively. In both cases, the effect was dependent on the concentration and time.

A concentration- and time-dependent cytotoxic effect was also observed for the MCF7 cell line with the oily fraction (Fig. 9b). At the highest concentration, a viability of 46% was obtained at 24 h of exposure, while 7% was obtained at 72 h. The IC_{50} values were 23.82 and 8.06 $\mu\text{g}/\text{mL}$, respectively. For the powder, the cytotoxic effect was even greater, with a viability of 10% at 24 h for the highest concentration and 0% for treatments $\geq 20\text{ }\mu\text{g}/\text{mL}$ after 72 h. The IC_{50} values were 9.94 and 4.15 $\mu\text{g}/\text{mL}$, respectively.

Fig. 9 Cytotoxicity assessment of the fractions obtained from petroleum ether extract of *S. mexicanum* root bark in **a** MDA-MB-231, **b** MCF7, **c** MCF 10A, and **d** PBCMs after 24 and 72 h of treatment. For each group, $n = 18$, the + symbol indicates a

statistically significant difference from the control with untreated cells ($P < 0.05$), and the * symbol indicates a statistically significant difference from the control with untreated cells ($P < 0.01$)

The non-tumorigenic cells derived from mammary tissue MCF 10A (Fig. 9c) showed a lower sensitivity to the fractions when compared to the tumor cells. At 24 h, the oily fraction induced an effect on cell viability for treatments $\geq 20 \mu\text{g/mL}$ only, decreasing the viability to 67.5% for the highest concentration with an estimated IC_{50} value of $34.6 \mu\text{g/mL}$. The increase in the treatment time (72 h) induced a greater cytotoxic effect, and the cell viability decreased for concentrations $\geq 10 \mu\text{g/mL}$ with an IC_{50} value of $15.55 \mu\text{g/mL}$. The powder showed a greater cytotoxic effect, decreasing cell viability with concentrations $\geq 10 \mu\text{g/mL}$ at 24 h with an IC_{50} value of $21.45 \mu\text{g/mL}$. At 72 h, the cell viability decreased from $5 \mu\text{g/mL}$ with an IC_{50} of $8.97 \mu\text{g/mL}$.

Finally, both fractions were evaluated with PBCMs (Fig. 9d). For the oily fraction, there were no statistically significant changes for the concentrations of 2.5–15 $\mu\text{g/mL}$ at 24 h of treatment, but greater values induced a

concentration-dependent cytotoxic effect, with $\text{IC}_{50} = 24.89 \mu\text{g/mL}$. After 72 h of treatment, a recovery in the cell viability was observed, and none of the evaluated concentrations exerted cytotoxicity for this incubation time. A similar case was observed for the powdered fraction. This fraction showed a greater cytotoxic effect than the oily fraction on PBCMs at 24 h, reaching an IC_{50} of $10.21 \mu\text{g/mL}$. However, at 72 h, the cells recovered their viability, with no cytotoxicity occurring for the evaluated concentrations.

The IC_{50} values obtained for both fractions with cancer-derived cells are important results when taking the recommendations of the US National Cancer Institute (USA NCI) into account. The recommendations suggest that pure compounds with cytotoxic IC_{50} values $\leq 4 \mu\text{g/mL}$ are considered active (Alonso-Castro et al. 2011) and promising for the development of anticancer drugs. This is even

more important when considering that the fractions are not pure substances but mixtures of compounds.

The increase of cytotoxicity when the gum was removed from the extract may have a relationship with previous reports of *trans*-1,4-polyisoprene (the main component of the gutta-percha) as an inducer of cell growth and differentiation (Zhang et al. 2016). Polyisoprenes can participate as initial substrates of the mevalonate pathway and promote cell proliferation, particularly in tumor cells (Mullen et al. 2016).

Fractionations of *S. mexicanum* root bark petroleum ether extract are selective for breast cancer cells

Selectivity indexes were calculated for the whole petroleum ether extract of *S. mexicanum* root bark as well as the powder and oil fractions (Table 1) after 72 h of treatment. IC₅₀ values obtained for the two breast cancer cell lines (MDA-MB-231 and MCF7) were compared to those obtained for two non-tumorigenic cell types (MCF 10A derived from human breast tissue and PBMCs immunological cells). The SI calculation allows for the evaluation of the differential activity of the compounds on tumor cells and normal cells. The greater the SI value is, the more selective the compound is. An SI value less than 2 indicates general toxicity of the analyzed compound (Badisa et al. 2009).

The whole petroleum ether extract of the *S. mexicanum* root bark was not selective for breast cancer cells when compared to normal breast-derived cells (MCF 10A). The selectivity of this extract when breast cancer cells were compared to the PBMCs was not determined because the IC₅₀ value for the PBCMs was higher than the concentrations evaluated. However, the results show that the cytotoxicity of the whole petroleum ether extract of the *S.*

mexicanum root bark on the breast cancer cells is equal to or greater than that exerted on the PBMCs.

Fractionation of the extract markedly increased the selectivity. Both the powder and oil fractions were highly selective for MDA-MB-231 cells when compared to MCF 10A and PBMCs. The powder fraction was selective for MCF7 cells when compared to MCF 10A cells, but the oily fraction was not selective. However, there is a high selectivity of both fractions between MCF7 and PBMCs. These results suggest that the obtained fractions are potential candidates for the development of drugs to treat breast cancer.

Pristimerin is present in the fractions of the *S. mexicanum* root bark petroleum ether extract

Both fractions were evaluated by HPLC at 290 and 420 nm. The chromatograms are presented in Fig. 10. Pristimerin was identified at 420 nm with a retention time of 24.435 min by comparison to the retention time and maximum UV absorption (420 nm) with a commercial standard. An internal standard was used to corroborate the pristimerin identity. A standard calibration curve with a linearity range of 0.05–1.00 mg/mL ($R^2 = 0.9936$) was prepared and used for quantitative analysis.

Pristimerin was a predominant compound with the highest percentage of peak area in both fractions at 420 nm: 38.36% for the powdered fraction and 63.73% for the oily fraction. For the oily fraction, a yield of 8.90 ± 1.02 mg of pristimerin/kg of dry vegetal material was obtained. For the powdered fraction, a yield of 9.68 ± 1.31 mg of pristimerin/kg of dry vegetal material was determined. The differences in yields between fractions were not significant. The cytotoxic effect of the petroleum ether extract may be explained by its content of

Table 1 Selectivity indexes of petroleum ether extract of *S. mexicanum* root bark and its fractions (powder and oil) for breast cancer cells MDA-MB-231 and MCF7 compared to non-tumorigenic cells MCF 10A and PBMCs after 72 h of treatment. SI = IC₅₀ of the evaluated treatment in normal cells/IC₅₀ of the evaluated treatment in cancerous cells. IC₅₀ is the concentration of the extract or fraction needed to reduce the cell viability to 50% with respect to untreated cells. (Reproduced with permission from Badisa et al. 2009)

	Petroleum ether extract of <i>S. mexicanum</i> root bark	Fractions	
		Powder	Oil
IC ₅₀ MDA-MB-231 (μg/mL)	55.5	3.35	4.80
IC ₅₀ MCF7 (μg/mL)	75.8	4.15	8.06
IC ₅₀ MCF 10A (μg/mL)	66.8	8.97	15.55
IC ₅₀ PBMCs (μg/mL)	> 75	> 25	> 25
Selectivity index (MDA-MB-231 vs. MCF 10A)	1.2	2.7	3.2
Selectivity index (MDA-MB-231 vs. PBMCs)	> 1.35	> 7.5	> 5.2
Selectivity index (MCF7 vs. MCF 10A)	0.9	2.2	1.9
Selectivity index (MCF7 vs. PBMCs)	> 0.99	> 6	> 3

Fig. 10 HPLC-DAD chromatograms of the fractions of petroleum ether extract of *S. mexicanum* root bark. Oily fraction at 290 (a) and 420 nm (b). Powder fraction at 290 (c) and 420 nm (d). Pristimerin commercial standard at 420 nm (e). Pristimerin was identified in the oily and powder fractions at 420 nm with a retention time of 24.435 min. The analysis was carried out using a C18 column with a

mobile phase consisting of a $\text{H}_2\text{O}:\text{H}_3\text{PO}_4$ (99.5:0.5) mixture as eluent A and MeOH as eluent B. 55–70% B, 0–10 min; 70–85% B, 10–15 min; 85–90% B, 15–23 min and 90–100% B, 23–33 min, followed by isocratic elution with 100% B for 3 min. Column temperature 30 °C. Flow rate 0.7 mL/min. Injection volume 5 μL

pristimerin, which induces both the apoptosis and oxidative stress phenomena observed for the extract (Bashir et al. 2017). However, tingenone as well as other compounds with cytotoxic activity could be present in the extract and produce a synergistic cytotoxic effect.

The fractionation of the extract by cryoprecipitation allowed for the elimination of *trans*-1,4-polyisoprene and the generation of two fractions that are highly cytotoxic

and specific for breast cancer cells. The cytotoxic effect of these two fractions could be exerted by pristimerin as well as other components present in the fractions. Future investigations should be developed to identify the other components. This research supports the potential use of *S. mexicanum* root bark for the development of drugs to treat breast cancer.

Conclusions

The petroleum ether extract of *S. mexicanum* root bark induced cell death by apoptosis in MDA-MB-231, MCF7, and MCF 10A cells. This cell death was associated with decreased metabolic activity and the generation of ROS. The extract was fractionated by cryoprecipitation, and two fractions that are highly cytotoxic and specific for breast cancer cells were obtained. Both fractions showed IC₅₀ values that are low enough for them to be considered as potential candidates for the treatment of cancer according to the recommendations of the US NCI. Pristimerin is present in both fractions and may be partially responsible for the cytotoxicity induced by the extract and its fractions. The obtained fractions are promising for the development of anticancer drugs, not only because of the low IC₅₀ values, but also because their obtainment is simple and does not require chromatographic techniques or the use of large quantities of solvents.

Acknowledgements This work was supported by the Instituto Politécnico Nacional and the National Council of Science and Technology, Mexico (CONACYT, Proyecto de Cátedras No. 1728).

Author Contributions Statement ESM, JM-C, CNQ-R and RGC-P conceived, designed and performed the experiments. JM-C, CNQ-R and RGC-P analyzed the data. ESM contributed with reagents, materials and analysis tools. JM-C, RGC-P, CNQ-R and ESM wrote the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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