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In vitro cytotoxic activity of bark extracts from *Pinus durangensis* Martinez and *Quercus sideroxyla* Bonpl.

Actividad citotóxica in vitro de los extractos de *Pinus durangensis* Martinez y *Quercus sideroxyla* Bonpl.

Marcela Soto-García[1], Martha Rosales-Castro[2], José Rubén García-Sánchez[3], María José Rivas- Arreola*[4]

[1] Universidad Autónoma de Ciudad Juárez, Instituto de Ciencias Biomédicas-DMCU, C.P.32310. Ciudad Juárez, Chih., México.
[2] Instituto Politécnico Nacional, CIIDIR- Unidad Durango, Sigma 119 Fraccionamiento 20 de noviembre, C.P. 34220, Durango, Dgo. México.
[3] Instituto Politécnico Nacional, Sección de Estudios de Posgrado e Investigación. Escuela Superior de Medicina del, Plan de San Luis y Diaz Mirón s/n, Col. Casto de Santo Tomas, Delegación Miguel Hidalgo, C.P. 113340, Ciudad de México, México.
[4] Universidad Iberoamericana Puebla Blvd. del Niño Poblano 2901 Col. Reserva Territorial Atlixcáyolt San Andrés Cholula, Puebla, México C.P. 72820.

ABSTRACT

Nowadays, plants bioactive compounds represent a great potential for discovering novel drugs that could act in cancer treatment. Previous word reports that different pine and oak species possess cytotoxic activities against various cancer cell lines. The present study evaluated the phenolic profile and cytotoxic activities of crude and organic extracts from *P. durangensis* and *Q. sideroxyla* bark. Among ESI-MS identified compounds in extracts from both species, are taxifolin and procyanidin dimers. The cytotoxic activity was performed on MDA-MB-231 (breast cancer), HeLa (cervix cancer), MCF-10A (breast non-tumorous cell), and HSF-1184 (human skin fibroblast cells) by MTT assay. All extracts showed a dose-dependent cytotoxic effect against cancer cell lines and small or no activity against the non-tumorous cells. The results provide important information about the cytotoxic activity of bark extracts from *P. durangensis* and *Q. sideroxyla*. This strong cytotoxic effect represents an opportunity for the valorization of a by-product of the wood industry.

Keywords: Bark extracts, cytotoxicity, phenolic profile.

RESUMEN

En la actualidad, los compuestos bioactivos de las plantas representan un gran potencial para descubrir un fármaco novedoso que pueda actuar en el tratamiento del cáncer. Se ha informado que diferentes especies de pino y roble poseen actividades citotóxicas contra varias líneas celulares cancerosas. En el presente estudio, se evaluó el perfil fenólico y las actividades citotóxicas de extractos crudos y orgánicos de la corteza de *P. durangensis* y *Q. sideroxyla*. Algunos compuestos, como la taxifolina y dimeros de procianidina, fueron identificados en extractos de ambas especies mediante ESI-MS. La actividad citotóxica se realizó en MDA-MB-231 (cáncer de mama), HeLa (cáncer de cuello uterino), MCF-10A (células no tumorales de mama) y HSF-1184 (células de fibroblastos de piel humana) mediante ensayo MTT. Todos los extractos mostraron un efecto citotóxico dependiente de la dosis contra las líneas celulares cancerosas y poca o ninguna actividad contra las no tumorales. Los resultados proporcionan información importante sobre la actividad citotóxica de extractos de corteza de *P. durangensis* y *Q. sideroxyla*. Este fuerte efecto citotóxico representa una oportunidad para la valorización de un subproducto de la industria de la madera.

Palabras clave: Extractos de corteza, citotoxicidad, perfil fenólico

INTRODUCTION

Breast and cervix cancer are the most common cancer-related death cause among females worldwide. Thus, it is crucial to search for newer therapies that can help prevent and treat this illness, besides lessening the side effects of available therapies (Khosropanah *et al.*, 2016; Saenglee *et al.*, 2016). The use of medicinal plants in the treatment of diseases is increasing due to the benefits against chronic diseases like cancer, since plants have bioactive compounds, playing a vital role in the development of pharmaceuticals (Abdulla *et al.*, 2014).

The therapeutic potential of medicinal plants generally associates to the antioxidant activity of phytochemicals, mainly phenols and flavonoids, closely linked to their ability to suppress cancer cells' growth through reduced oxidative stress (Marvibaigi *et al.*, 2016). However, the cellular mechanisms by which the phenols elicit these anticancer effects are multifaceted (Sorice *et al.*, 2016).

Q. sideroxyla and *P. durangensis* are forest timber species in Mexico. Their primary non-timber uses are for edible harvesting seeds and even as medicine in the treatment of disorders like ulcers and inflammatory problems (Bermejo and Pontones 1999; Luna *et al.*, 2003). Previous studies reported antibacterial effects, high phenolic content, and antioxidant properties in *P. durangensis* extracts (Rosales-Castro and González-Laredo, 2003; Rosales-Castro *et al.*, 2006). Extracts from *Q. sideroxyla* have shown hypoglycemic and genotoxic effects, amelioration of oxidative stress evaluated in a murine model, high antioxidant capacity, anti-inflammatory effects, and anticarcinogenic activity in rat colon (Moreno-Jimenez *et al.*, 2015; Soto-García *et al.*, 2016). The main bioactive compound of these species identified is flavan-3-ols in *Q. sideroxyla* (Rosales *et al.*, 2012), and flavonoids like taxifolin

*Autor para correspondencia: María José Rivas Arreola
Correo electrónico: mariajose.rivas@iberopuebla.mx
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and quercetin in *P. durangensis* (Soto-García and Rosales-Castro, 2016).

Many investigations reported catechin, taxifolin, and quercetin as compounds with cytotoxic activities (Moreira *et al.*, 2013; Evacuasiany *et al.*, 2014; Moreno-Jimenez *et al.*, 2015; Alzaharna *et al*., 2017; Rameshthangam and Chitra, 2017). Thus, this research aimed to determine the cytotoxic activities of polyphenolic extracts from *P. durangensis* and *Q. sideroxyla* bark against MDA MB-231, MCF 10A HeLa, and HSF-1184 cell lines.

METHODS

Plant material

The bark collection was in January 2014 in Pueblo Nuevo, Durango, México. María Del Socorro Gonzalez Elizondo (taxonomist) identified the samples, and botanical specimens deposited at the Herbarium in CIIDIR-IPN, Durango, with vouchers number 42842, 428443, 42844 for *Q. sideroxyla* and 93, 97, 99, 100, 101 for *P. durangensis*. The bark preparation consisted in a mixture to make a unique sample, drying at room temperature, milled (mesh 40), and finally stored at 4 °C in paper bags until further use.

Extracts preparation and purification

The bark powder (10 g) was twice soaked with 50% ethanol (ethanol/water 50:50 v/v) (2 x 300 mL) with stirring at room temperature for 24 h, then filtered through a Whatman filter paper no. 1. The extracts were combined, filtered, and then vacuum evaporated at 40 °C until ethanol was removed. A portion of the remaining aqueous extract was dried and identified as crude extract (CE); the other portion went through a liquid partition process with ethyl acetate (3 x 100 mL). The organic phase was vacuum evaporated at 40 °C and identified as the organic extract (OE).

ESI-MS analysis

To obtain the *P. durangensis* and *Q. sideroxyla* OE spectra, we used a micrOTOF-QII mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization source (ESI). The parameters were set as capillary 2700 V, nebulizer pressure 1.2 bar, dry gas flow 11 L/min, and dry gas temperature 200 °C and the sample ran in the negative ion mode. The scan range was from 50 to 3000 m/z.

Cytotoxic evaluation

Cell lines and culture conditions

Human breast cancer (MDA-MB-231), human cervical cancer (HeLa), and non-cancer (MCF-10A and H-1184) cell lines were from the American Tissue Culture Collection (ATCC, USA). The cultures of HeLa, MCF-7, MDA-MB-231, and H-1184 cells were in DMEM high glucose (Gibco Lab, Grand Island, NY) in 10% (MDA-MB-231 was 5%) bovine fetal serum and antibiotic 1X (streptomycin/penicillin 10000 U/mL, Gibco LabGrand Island, NY). The culture of MCF-10A was in DMEM/F12 medium with 10% bovine fetal serum, incubated at 37 °C in a humid atmosphere of 5% CO_2.

Cytotoxic assay

The cytotoxic assay was measured using MTT (3-(4,5-dimethylth-iazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Each cell line was plated in 96-well plates at a density of 1×10^4 cells/well and incubated for 24 h (37 °C, 5% CO_2 air humidified) to allow them to adhere to the plate. Different concentrations of the CE and OE from *P. durangensis* and *Q. sideroxyla* were prepared (from the stock solution) and added to the culture medium to treat the cells, and cells incubated for another 72 h under the same conditions, using cells without treatment as a negative control. At the end of the experimental period, 100 µL of MTT dissolved in medium (5 mg/mL) were added to each well and incubated for another 2.5 h. Then, the medium was removed, the formazan crystals dissolved in 150 µL DMSO, and the absorbance measured at 550 nm in a spectrophotometer (Sinergy Biotek Instruments, Winooski, VT). In order to determine viability and the concentration leading to 50% inhibition of the viability, the determination of IC_{50} was by regression analysis using the following equation:

% Viability = Abs treated cells / Abs control cells × 100

Statistical analysis

The results reported are as the mean and the standard deviation (SD) of at least two independent experiments. The data analysis was by One-way analysis of variance (ANOVA), followed by post-hoc Turkey test, using a significance level of $\alpha \leq 0.5$ to determine statistical significance. The statistical analysis software used for these analyses was Statistica 7.

RESULTS AND DISCUSSION

The characterization of organic extracts (OE) from *P. durangensis* and *Q. sideroxyla* was by the analysis of the major peaks identified based on elution order, and that of all compounds was by interpreting their mass spectra obtained by the HPLC-ESI-MS, considering previously reported data. Figure 1 (A and B) shows the total ion chromatogram (TIC) of extracts from species studied. Several investigations reported cytotoxic effects of some Pine and oaks species on different cell lines that were related to compounds with the biological activities (Şöhretoğlu *et al.*, 2012; Moradi *et al*., 2016; Li *et al*., 2016; Yang *et al*., 2016; Amessis-Ouchemoukh *et al*., 2017; Sarmeili *et al*., 2017).

Table 1 shows the main constituents of pine bark, where the compounds of the peaks 11, 12, 15, 16, 25, 36, 40, 41, and 58 coincide with compounds recently reported by Rosales (2017) in *P. durangensis* bark, demonstrating the presence of flavonoids like procyanidins and the abundance of taxifolin. Other TIC peak constituents were compared with the relevant literature in order to obtain a tentative identification (most studies on compounds of *Pinus* and *Quercus* bark) finding: kaempferol and eriodictyol ($[M-H]^-$ 285 and 287 respectively; De la Luz Cádiz-Gurrea *et al.*, 2014), taxifolin-o-hexoside ($[M-H]^-$ 465; Cretu *et al.*, 2013), β-hydroxypropiovanillone glucoside ($[M-H]^-$ 357; Karonen et al., 2004), HHDP-galoyl-glucose ($[M-H]^-$ 357; Fernández *et al.*, 2009),

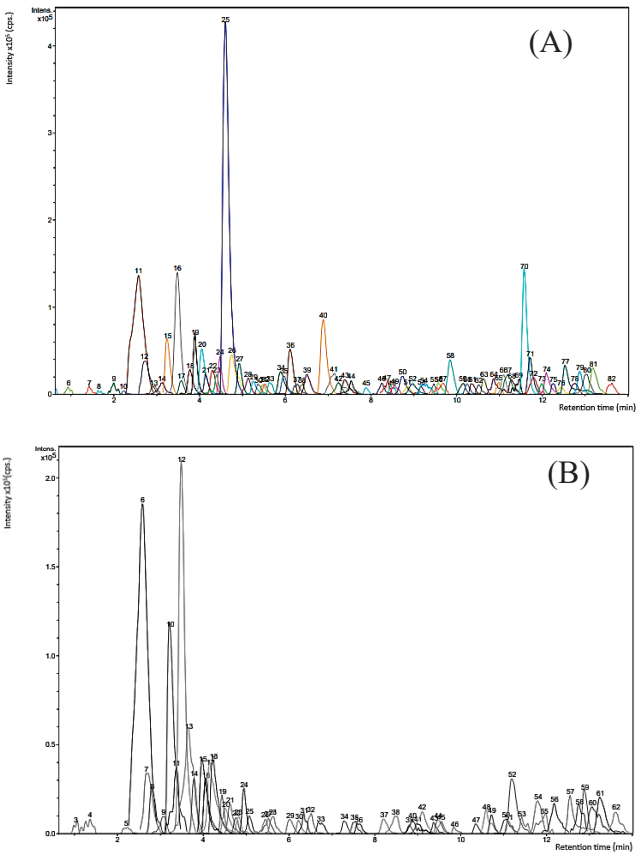

Fig. 1. Total ion chromatogram. (A) OE from *P. durangensis*. 11= Procyanidin dimer, 16= (+)-Catechin/(-)-Epicatechin, 25= Dihydroquercetin (Taxifolin), 40= Myricetin, 70= Kaempferol; (B) OE from *Q. sideroxyla*. 6= Procyanidin dimer, 10= Procyanidin dimer B1, 12= (+)-Catechin/(-)-Epicatechin; Procyanidin dimer, 13= (+)-Catechin/(-)-Epicatechin.
Fig. 1. Cromatografía de iones totales. (A) OE de *P. durangensis*. 11= Dímero de procianidina, 16= (+)-Catequina/(-)-Epicatequina, 25= Dihidroquercetina (Taxifolina), 40= Miricetina, 70= Kaempferol; (B) OE de *Q. sideroxyla*. 6= Dímero procianidina, 10= Dímero procianidina B1, 12= (+)-Catequina/(-)-Epicatequina; dinero Procianidina, 13= (+)-Catequina/(-)-Epicatequina.

trigalloyl glucose ([M-H]-635; Mucilli *et al.*, 2017), (Epi)catechin-3-O-glucoside-gallate ([M-H]- 603; Jiménez-Sánchez *et al.*, 2015), t-caftaric acid ([M-H]- 311; Pardo-García *et al.*, 2014).

The polyphenolic profiles of *Q. sideroxyla* shown in Table 2 includes procyanidin dimer of catechin/epicatechin compounds (peaks 6, 10, 12, and 13) which is, by far, the dominant polyphenols in bark extract from this species, also reported in previous studies (Rosales *et al*., 2012). Other compounds present in the extract also compared with previously reported literature, resulted in the tentative identification of constituents such as Quercetin 3-O-glucoside and t-caftaric acid ([M-H] - 463 and 311 respectively; Pardo-García *et al.*, 2014), Ellagic acid-rhamnoside ([M-H]-447; Santos *et al.*, 2013) gallic acid hexoside ([M-H]- 331; Lorenz *et al.*, 2016), taxifolin ([M-H]- 303; Rosales *et al*., 2017). Other unknown compounds reported in barks detected in both extracts are also included ([M-H] - 293; Mämmelä *et al*., 2000).

In previous studies, we have identified some of the major phenolic compounds of crude *P. durangensis* and *Q. sideroxyla* bark extracts by HPLC-DAD (Soto-Garcia and Rosales-Castro, 2016); these results, together with those obtained in this research, confirm the reproducibility of their bioactive compounds in bark extracts, like catechin in both species.

Table 1. Compounds identified by ESI-MS in the *P. durangensis* bark organic extract.
Tabla 1. Identificación de compuestos por ESI-MS en extractos orgánicos de corteza de *P. durangensis*.

# Peak	RT (min)	Mass [m/z]-	Suggested compound
11	2.6	577.1320	Procyanidin dimer
12	2.7	289.0699	(+)-Catechin/(-)-Epicatechin
15	3.3	577.1321	Procyanidin dimer
15	3.3	865.1916	Procyanidin trimer
16	3.5	289.0706	(+)-Catechin/(-)-Epicatechin
16	3.5	579.1490	Catechin dimer
19	3.9	465.1041	Taxifolin-O-hexoside
25	4.6	303.0506	Dihydroquercetin (Taxifolin)
34	5.9	633.1210	HHDP-galoyl-glucose
36	6.1	317.0616	Myricetin
36	6.1	635.1369	Trigalloyl glucose
39	6.5	287.0539	Eriodictyol
40	6.9	317.0639	Myricetin
40	6.9	635.1369	Trigalloyl glucose
41	7.1	331.0448	Pinomyricetin (myricetin-6Me)
50	8.7	357.1330	b-hydroxypropiovanillone glucoside
58	9.9	301.0679	Quercetin
58	9.9	603.1440	(Epi)catechin-3-O-glucoside-gallate
70	11.6	285.0759	Kaempferol
77	12.5	311.1854	t-caftaric acid
79	12.9	293.1740	Unknown

The *P. durangensis* and *Q sideroxyla* CE and OE bark cytotoxic effects were also evaluated on a cell line from human breast carcinoma, estrogen receptor-negative (ER-) MDA-MB-231, and cervix carcinoma cell line HeLa. Non-tumorous MCF-10A and HSF-1184 cells, used as controls, allowed comparing the effects produced by the extracts in healthy and tumor cells.

Results showed that crude extract and organic extract of both species have cytotoxic activity on cancer cell lines growth. A concentration-dependent manner (Fig. 2 A, B, C), becomes statistically different ($p \leq 0.05$) between treated and untreated cells (MDA-MB-231 and HeLa cell lines), nevertheless they were not cytotoxic to the non-tumorous HSF-1184 cell line.

The crude extracts showed a slight reduction in cell viability on normal breast MCF-10A cells, 20 % approximately (Fig. 2 D), even at the higher applied doses. These results

Table 2. Compounds identified by ESI-MS in the *Q. sideroxyla* bark organic extract.
Tabla 2. Identificación de compuestos por ESI-MS en extractos orgánicos de corteza de *Q. sideroxyla*.

# Peak	RT (min)	Mass [m/z]-	Suggested compound
6	2.6	577.1319	Procyanidin dimer
7	2.7	289.0702	(+)-Catechin/(-)-Epicatechin
10	3.2	577.1323	Procyanidin dimer B1
12	3.5	289.0704;579.1489	(+)-Catechin/ (-)-Epicatechin; Procyanidin dimer
13	3.7	289.0702	(+)-Catechin/(-)-Epicatechin
14	3.8	577.1307	Procyanidin dimer
15	4.0	463.0873	Quercetin 3-O-glucoside
18	4.2	447.0920	Ellagic acid-rhamnoside
21	4.6	303.0498	Dihydroquercetin
37	8.2	331.2490	Gallic acid hexoside
57	12.5	311.1848	t-caftaric acid
59	12.9	293.1736	Unknow

indicate that the *P. durangensis* and *Q. sideroxyla* extracts preferentially reduced the growth of the tumoral cell while not affecting the healthy cell line. The role of phenolic compounds in the extracts is essential, since it has been observed that polyphenols such as catechins induce apoptotic cell death in MDA MB-231 cell lines but not in normal cells (MCF 10-A) (Farhan *et. al.*, 2016). The CE of both species presents catechin in their compounds, explaining the selective effect on MCF-10A.

Several investigations reported that the effects obtained on cell proliferation relate to dose and cell type, implying a different sensitivity by different cell lines (Gezer *et al.*, 2015; Anlar *et al.*, 2016). It has also been reported that the selective activity of cytotoxic compounds against healthy (MCF- 10A) and tumor cells is due to a broad spectrum of mechanisms responsible for the resistance. These include differences in the subcellular localization of Bik and differences in mitochondrial-mediated apoptosis that explain the sensibility exerted by the bark extracts from *P. durangensis* and *Q. sideroxyla* in the cell lines (Studzinska-Sroka *et al*., 2016).

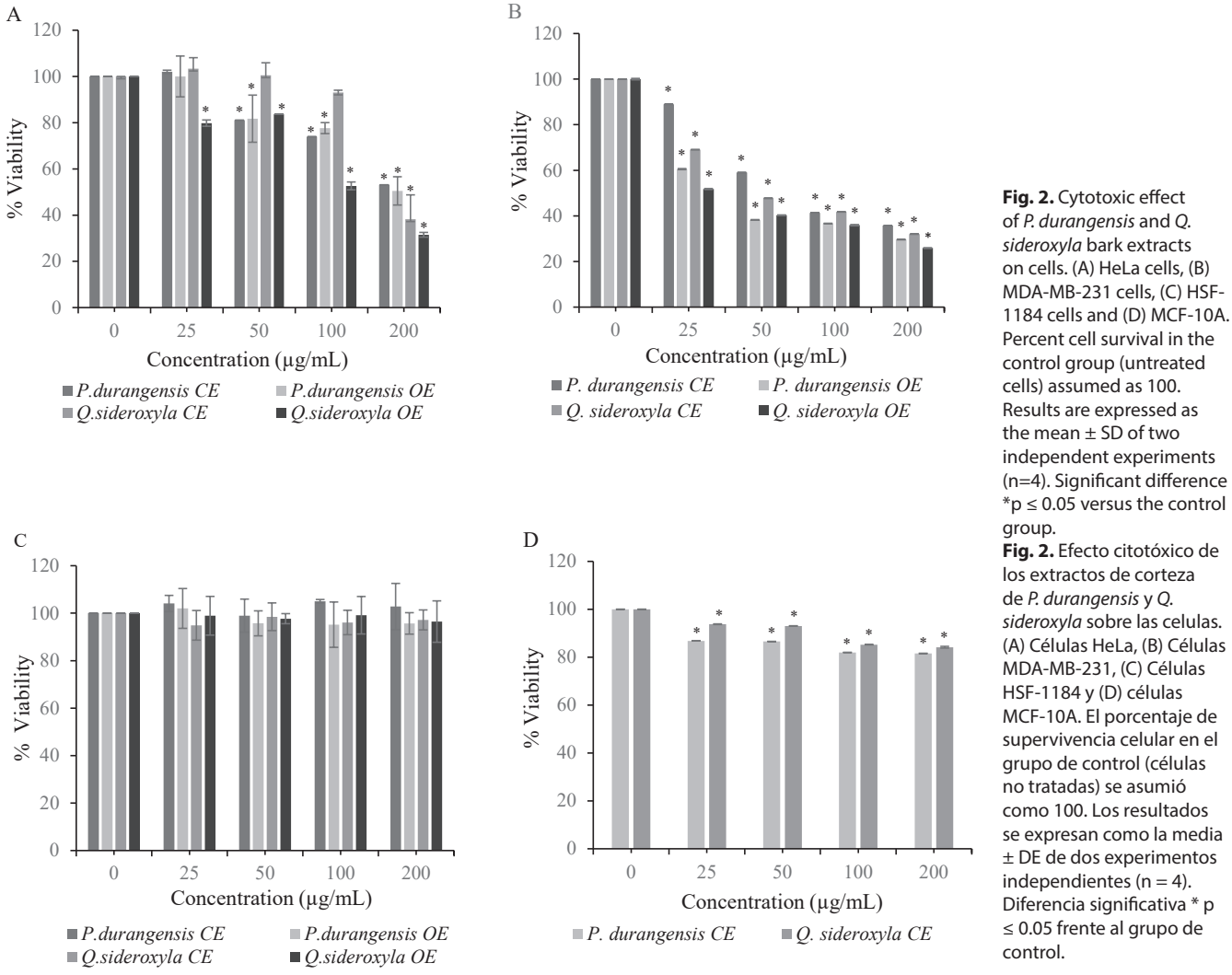

Fig. 2. Cytotoxic effect of *P. durangensis* and *Q. sideroxyla* bark extracts on cells. (A) HeLa cells, (B) MDA-MB-231 cells, (C) HSF-1184 cells and (D) MCF-10A. Percent cell survival in the control group (untreated cells) assumed as 100. Results are expressed as the mean ± SD of two independent experiments (n=4). Significant difference *p ≤ 0.05 versus the control group.
Fig. 2. Efecto citotóxico de los extractos de corteza de *P. durangensis* y *Q. sideroxyla* sobre las celulas. (A) Células HeLa, (B) Células MDA-MB-231, (C) Células HSF-1184 y (D) células MCF-10A. El porcentaje de supervivencia celular en el grupo de control (células no tratadas) se asumió como 100. Los resultados se expresan como la media ± DE de dos experimentos independientes (n = 4). Diferencia significativa * p ≤ 0.05 frente al grupo de control.

IC_{50} determination provides further clarification of both species' crude and organic extracts behavior in different cell lines (Table 3). It shows that organic extracts of both species have a major cytotoxic effect on MDA-MB-231 and HeLa cells, maybe due to the concentration of metabolites exerted by solvent. Previous results report that compounds such as taxifolin and procyanidins, the most abundant present in each of the extracts, play a fundamental role in their cytotoxic activity, inducing apoptosis on upregulating the expression of the proteins in HeLa and MDA-MB-231 cells (Amalinei *et al.*, 2014).

Table 3. IC_{50} for *P. durangensis* and *Q. sideroxyla* bark extracts on cancer cell lines and non-tumorigenic cells.
Tabla 3. IC_{50} de los extractos de *P. durangensis* y *Q. sideroxyla* sobre la viabilidad de líneas celulares cancerosas y no tumorales.

	Cell lines			
Extract	**IC_{50} µg/mL**			
	HeLa	**MDA-MB-231**	**HSF-1184**	**MCF-10A**
P. durangensis CE	210.30±6.75[a]	77.34±4.06[a]	ND	ND
P. durangensis OE	201.50±8.29[a]	37.49±0.95[c]	ND	___
Q. sideroxyla CE	184.03±7.83[b]	46.83±0.73[b]	ND	ND
Q. sideroxyla OE	103.64±3.87[c]	32.82±5.54[c]	ND	___

ND: not detected within the investigated concentration range. Means ± SD in each column followed by different letters are statistically different by Tukey test p≤0.05.

The abundance of catechins and the synergy with other metabolites within the phenolic profile of *Q. sideroxyla* could be why it showed the highest cytotoxic activity compared to *P. durangensis*. In addition, there is evidence that flavonoids that possess C2-C3 unsaturated bond and a carbonyl group at position 4 exhibit lower IC_{50}. These two functional groups may increase the compound's activity by affording more stable flavonoid radicals via conjugation and electron delocalization (Sadeghi-Aliabadi *et al.*, 2012).

Several investigations reported cytotoxic effects of some Pinus and oaks species on different cell lines that were related to compounds with the biological activities (Şöhretoğlu *et al.*, 2012; Li *et al.*, 2016; Moradi *et al.*, 2016; Yang *et al.*, 2016; Amessis-Ouchemoukh *et al.*, 2017; Sarmeili *et al.*, 2017).

CONCLUSIONS

Quercus and *Pinus* species studies are intense due to their antioxidant and anti-inflammatory, antimicrobial, and anticancer potential; however, studies on the phenolic profile is scarce, and is associated with the reported effect. This work demonstrated that the phenolic profile and the concentration of specific metabolites such as the procyanidin dimer of catechin/epicatechin compounds was determinant in the cytotoxic activity evaluated in the cell lines used. *Q. sideroxyla* OE showed a potent cytotoxic activity compared to CE and OE from *P. durangensis*. This strong cytotoxic effect represents an opportunity for the valorization of the wood industry by-products, suggesting that OE of *Q. sideroxyla* may represent a good alternative in the search for new agents of natural origin to treat mammary and cervix cancer. Nevertheless, more *in vitro* and *in vivo* studies are necessary in order to understand and establish the mechanism of action.

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ETHICAL DISCLOSURES

The authors declare that this work did not required human or animal experiments, no patient data appear in this article, and declare no conflicts of interest.

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