Biological Screening of Select Puerto Rican Plants for Cytotoxic and Antitumor Activities

Karla Claudio-Campos, BS*; Janibeth Hernández-Rivera, BS*; Jaymie Rivera-Gutierrez, BS†; Isamar Ortiz-Rivera, BS†; Augusto Carvajal-Vélez, MSc*; Marianela Pérez-Torres, PhD‡; Mayra Pagán-Ortiz, PhD†; Claudia A. Ospina-Millán, PhD†

Objective: The objective of this study was to evaluate the cytotoxic and anticancer activities of extracts from 7 species of endemic and native plants from Puerto Rico.

Methods: The plant species selected for this study were *Canella winterana*, *Croton discolor*, *Goetzea elegans*, *Guaiacum officinale*, *Pimenta racemosa*, *Simarouba tulae*, and *Thouinia striata*. The dried plant material was extracted with a 1:1 mixture of CH_2Cl_2-MeOH. The resulting crude extract was suspended in water and extracted with solvents of different polarities. The extracts were evaluated for their cytotoxic effects against *Artemia salina* and 3 breast cancer cell lines.

Results: About 50% of the extracts evaluated against *Artemia salina* exhibited LC50 values of less than or equal to 200 µg/mL. The strongest activity was detected in the chloroform and ethyl acetate extracts of *Guaiacum officinale*, with lethality values below 10 µg/mL. The extracts were further evaluated for their bioactivity as possible inhibitors of several breast cancer cell lines, with the extracts from *Simarouba tulae* and *Croton discolor* showing the highest percentages of growth inhibition. The dose-effect data analysis for the crude extracts of the different plants also confirms the high cytotoxicities of *Guaiacum officinale*, *Simarouba tulae*, and *Croton discolor*.

Conclusion: Based on our results, we concluded that the *Simarouba*, *Croton*, and *Guaiacum* plant extracts show cytotoxic and anticancer activities that merit closer investigation in order to identify the chemical compounds responsible for these bioactivities. [*P R Health Sci J 2015;34:25-30*]

Key words: Endemic and native plants, Puerto Rico, Brine shrimp lethality test, Cytotoxic activity, Cancer inhibition

Cancer is the second leading cause of death in the USA, after cardiovascular disease. Worldwide, breast cancer has the highest incidence and mortality among women. Specifically in Puerto Rico, during the period of 1999 to 2003, an average of 72 women per 100,000 were diagnosed with breast cancer each year (1). These statistics have propelled research directed at its prevention and treatment as well as toward the discovery of new therapeutic agents.

Medicinal plants continue to be an important source of compounds that could be used as drug leads for cancer treatment. It has been estimated that over 60% of all commercial anticancer agents originate from natural sources (2).

At least 2 prior studies describing the cytotoxic activities of several Puerto Rican plant extracts have been documented. The first was reported by Guerrero and Robledo in 1993 (3). In this work, 6 crude extracts (having LC_{50} values ≤ 200 µg/mL against *Artemia salina*) of the Euphorbiaceae, Solanaceae, Myrsinaceae, Polygonaceae, and Polygalaceae families were evaluated. These results suggest that the tested plant extracts possess potentially bioactive compounds. However, no isolation work was conducted during this investigation. Subsequently, Chavez et al. used the Brine Shrimp Lethality Test (BSLT) to assay the dichloromethane portion of the ethanol extracts (at concentrations of 1000 µg/mL) of various Puerto Rican plant species (4). Extracts displaying LC_{50}s that were less than or equal to 1000 µg/mL were further tested against Hela and CHO cell lines. These tests demonstrated that the extracts from Annona glabra, Simarouba tulae, Tithonia diversifolia, Dendropanax arboreous, Piper jacquemontanium, Annona montana, and Polygala hecatantha were active in both assays.

This report highlights the biological importance of Puerto Rican plant extracts as potential sources of secondary metabolites with antitumor activities. Notwithstanding, further work isolating and characterizing the metabolites responsible for these biological activities is required; equally important is the study of additional plant species belonging to these families.

*Department of Biology, †Department of Chemistry, University of Puerto Rico at Cayey, Puerto Rico; ‡Department of Pharmaceutical Sciences, School of Pharmacy, University of Puerto Rico Medical Sciences Campus, San Juan, Puerto Rico

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Address correspondence to: Claudia A. Ospina-Millán, PhD, Department of Chemistry, University of Puerto Rico at Cayey, Cayey, PR 00736. Email: claudia.ospina1@upr.edu

In the present study, 7 species of native and endemic plants from Puerto Rico were chemically studied, namely, *Canella winterana*, *Pimenta racemosa*, *Guaiacum officinale*, *Croton discolor*, *Goetzea elegans*, *Thouinia striata*, and *Simarouba tulae*.

Canella winterana is a tree found in Florida and throughout the Caribbean. Important constituents of this plant are monoterpenes such as canellal (1) (5), eugenol (2) (6), eucalyptol (3) (7), and the drimane sesquiterpenoid 4, which is isolated mainly from the stem bark (Figure 1) (8). The latter compounds exhibited phototoxic activity in a Lemna minor bioassay (7). Recently, 5 drimane-type sesquiterpenoids with antimalarial and anticancer activities were isolated from hexane extracts made from the leaves of *Canella winterana* (9).

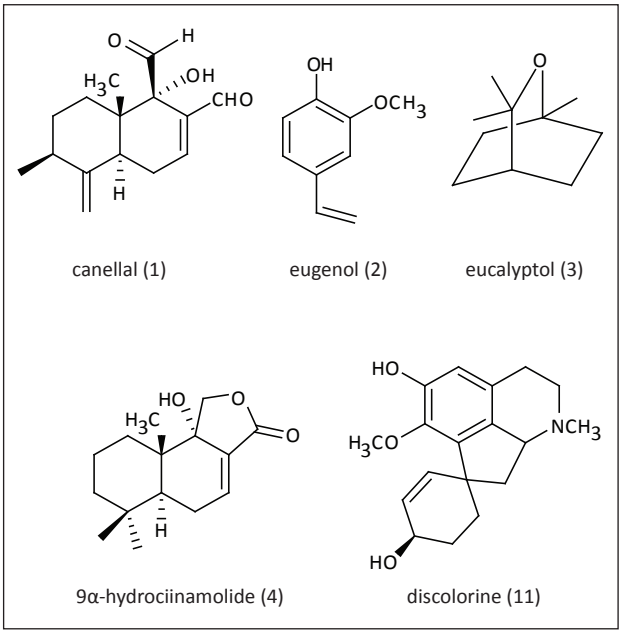

Figure 1. Some compounds isolated from Canella winterana and Croton discolor.

Pimenta racemosa is a native tree found in many Caribbean islands and is used in folk medicine for the treatment of toothache and rheumatism, among other ailments (10). The oil extracted from *Pimenta racemosa* has been reported to have, variously, anti-inflammatory, antibacterial, contraceptive, antiviral, antimicrobial, and antiradical properties, among others (11,12,13,14,15).

Guaiacum officinale is a tree common to the Antilles and other tropical zones of the Americas. The fruit and leaves contain triterpene saponins such as officigenin (5) (16), and the resin is known to contain lignans such as α-guaiaconic acid (6) (Figure 2) (17). Recently, 4 spirocyclic lignans, ramonanins A-D (7-10) (Figure 2), were isolated from *G. officinale* and *G. sanctum*. The ramonanins exhibit cytotoxic activity against human breast cancer cell line MDA-MB- 231, having an IC_{50} value of 18μM and inducing cell death via apoptotic mechanisms.

Croton discolor is a shrub found in the floral regions of Puerto Rico and the Virgin Islands. A proaporphine alkaloid, discolorine (11) (Figure 1), was isolated from this plant and later found in *Croton plumieri* (18,19). No stereochemistry was reported at the spiro carbon in references 18 and 19.

Simarouba tulae is an endemic tree from Puerto Rico belonging to the Simaroubaceae family. In recent years, its potential therapeutic uses have increased because of the (reported) bioactivities of the quassinoids, the principal chemical constituents within the Simaroubaceae family (20). In 1997, Chavez reported the cytotoxic activities of *Simarouba tulae* against Hela and CHO cells, but none of the plant's constituents were reported (4). *Thouinia striata* (Sapindaceae) and *Goetzea elegans* (Solanaceae) are 2 plant species endemic to Puerto Rico. To the best of our knowledge, no studies of the biological properties of extracts obtained from these plant species (or of their secondary metabolite compositions) are available.

Materials and Methods

General experimental procedures

All the solvents and reagents were purchased from Sigma Aldrich. The solid-liquid extractions were performed by crushing the air-dried leaves with a Waring blender. The plant extracts were concentrated in a vacuum using a Yamato rotary evaporator (RE 200). The live shrimp that were used in the BSLT (*Artemia salina*) bioassay were counted by inspection of the well with the aid of a stereo zoom microscope (10X widefield eyepieces, 45-degree inclined body, 7X to 45X magnification range). Brine shrimp eggs were acquired from a local pet shop (San Juan, Puerto Rico), and the yeast was purchased from a local grocery store (Caguas, Puerto Rico).

Plant material

The plant leaves were collected from February through May 2008 from several sites, including a dry forest in Guanica (*C. winterana*, *C. discolor*, *G. officinale*, *P. racemosa*, and *T. striata*), the Botanical Garden of the University of Puerto Rico in Rio Piedras (*G. elegans*), and Patillas, Puerto Rico (*S. tulae*). The plants were identified by one of the authors (Augusto Carvajal, department of biology at UPR-Cayey). Voucher specimens were deposited at the herbarium of the University of Puerto Rico, Rio Piedras (Table 1).

Extract preparation

Plant leaves were dried at 40°C and crushed in a blender with 3 portions of CH_2Cl_2/CH_3OH (1:1, v/v). The solid debris was removed from the combined extracts by vacuum filtration using Celite as a filtering aid. Extracts were concentrated under vacuum, then suspended in water (300–500 mL) and extracted with organic solvents of increasing polarity, namely, hexane, chloroform, and ethyl acetate. The final solvent volume

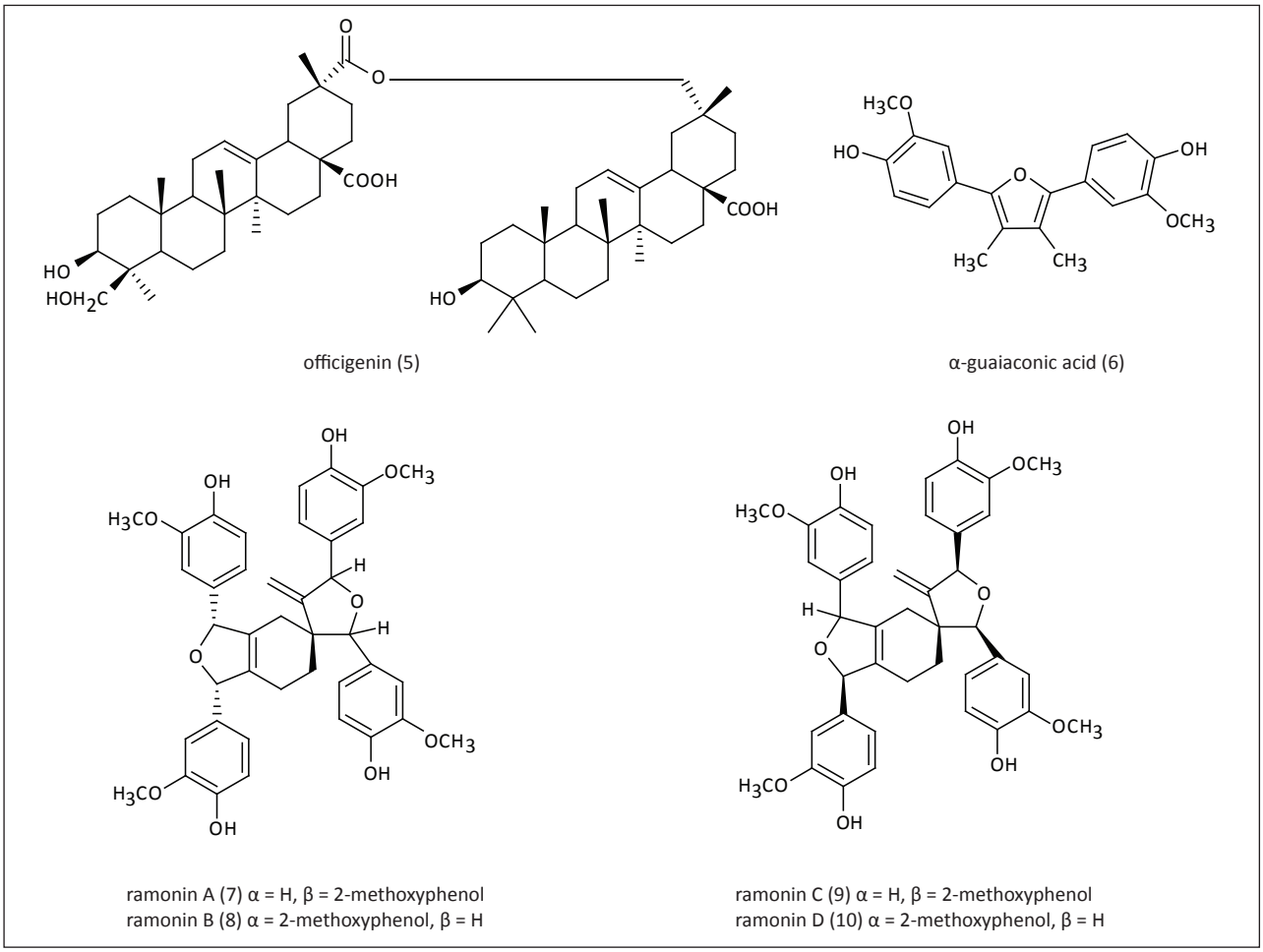

Figure 2. Some compounds isolated from Guaiacum officinale.

Table 1. Puerto Rican plant species and weights of plant extracts

Plant species	Family number	Voucher dry	Leaves weight (g)	Crude extract yield (%)	Hexane extract yield (%)	Chloroform extract yield (%)	Ethyl acetate extract yield (%)
Canella winterana (L.) Gaertn.	Canellaceae	ACCV-2010-02	283.8	22.9	8.8	5.6	0.7
Croton discolor Willd.	Euphorbiaceae	ACCV-2010-01	46.6	18.2	1.6	6.6	0.4
Goetzea elegans Wydler	Solanaceae	ACCV-2010-09	270.1	9.3	2.2	0.4	0.2
Guaiacum officinale L.	Zygophyllaceae	ACCV-2010-03	270.1	16.5	1.9	1.8	0.8
Pimenta racemosa (Mill.) J.W. Moore	Myrtaceae	ACCV-2010-08	279.7	6.1	2.8	0.4	2.3
Simarouba tulae Urb.	Simaroubaceae	ACCV-2010-10	65.7	22.8	3.6	14.0	1.4
Thouinia striata Radlk.	Sapindaceae	ACCV-2010-06	106.4	14.9	3.4	5.7	0.1

was divided into 3 to 4 portions. Each of these extracts was concentrated to dryness by rotoevaporation. The yields for each extract, expressed as the percentage weight of the dried plant material, are shown in Table 1.

Brine shrimp lethality test (BSLT)

Brine shrimp lethality tests were performed to assess the cytotoxicity of the crude plant extracts as well as the hexane, chloroform, and ethyl acetate extracts. The bioassays were performed as reported earlier, but with some minor modifications (21). Brine shrimp eggs were hatched in artificial seawater (0.5 g eggs per liter) in the dark portion of a divided chamber. The artificial seawater was prepared using sea salt (30 g/L) and contained 0.006 g of yeast as a food source. After approximately 48 h, the phototropic nauplii moved through a hole in the division to the portion of the chamber kept under

continuous light. The nauplii were collected with a pipette and concentrated in a beaker. The concentration of the nauplii was adjusted by adding seawater to the beaker until approximately 10 to 15 nauplii were found in 100 µL of solution (measured with an automatic micropipette).

The sample (0.01 g) to be tested was dissolved in 2 mL DMSO and diluted with seawater (8 mL) to a concentration of 1 mg/mL. Berberine chloride (90% purity; Sigma Aldrich) was used as a positive control. The blank solution (negative control) was prepared by diluting 1 mL of DMSO in 4 mL of seawater. The bioassay was conducted in a 96-microwell plate. Several solutions of each sample, ranging from 2 µg/mL to 500 µg/mL, were prepared in triplicate. About 10 to 15 nauplii were added to each solution containing the samples, negative control, and positive control. The 96-microwell plate was incubated at room temperature for 24 h under constant lighting.

After 24 h, the dead nauplii were counted with the aid of a microscope, and the lethal concentration (LC_{50} value) was calculated by probit analysis using the SPSS program (22). The dose-effect curves and the EC_{50} were calculated using GraphPad Prism software.

In vitro anticancer activity assay

Breast adenocarcinoma cell lines MCF-7, ZR-75-1, and T47D were purchased from the American Type Culture Collection (Manassas, VA). Each cell line was grown in its corresponding culture media (DMEM:F12 for MCF-7 and RPMI-1640 for ZR-75-1 and T47D cells) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 50 U/mL penicillin, and 50 µg/mL streptomycin and 0.3 µg/mL fungizone. All cell lines were maintained at 37°C under a 5% CO2 atmosphere until 90% of confluence. The cells were trypsinized with Trypsin-EDTA, detached from the cell plates, and counted in a hemocytometer. A total of 5 x 103 cells were seeded in 96-well flat-bottom plates and incubated overnight at 37°C under a 5% CO_2 atmosphere.

The samples used for the in vitro anticancer activity were dried under high vacuum to remove the solvents used in the extraction procedure. The cells were treated with 10 µM (or 4 µM) of each extract (in quadruplicate) for 72 h. After treatment, in order to measure the amount of live cells, 20 µL of 5 mg/mL MTT was added to each well and incubated for 4 h at 37°C. Then, the media was aspirated and crystals were dissolved with 100 µL of DMSO. The absorbance was measured at 570 nm and 630 nm using a Benchmark Plus spectrophotometer (Bio-Rad, Hercules CA). Viability was expressed as a percentage of control cells (treated with vehicle). The average and standard deviations were calculated from samples treated in quadruplicate and from control samples. Control percentage was calculated by dividing the absorbance average of each treated sample by the absorbance average of the control, then multiplying by 100. Tamoxifen (99%, Sigma Aldrich) was used as a positive control.

Results and Discussion

The plant species selection for this study was based primarily on the lack of information regarding their chemical composition and the fact that some of the species chosen belong to families known to contain bioactive compounds. In addition, most of the selected species are native, with 2 being endemic, to Puerto Rico.

We used a bioassay-guided fractionation scheme (the brine shrimp lethality test) and tested the extracts for cytotoxic activity. Table 2 shows that 12 extracts spread across 5 plant species exhibited LC50 values below 200 µg/mL. The most active crude extracts were those of S. tulae, G. officinale, and C. discolor, with these having lethality values of 23.7, 26.1, and 111 µg/mL, respectively. These plant species belong to families whose genera have been demonstrated to possess anticancer properties, which properties are attributable to the presence in those genera of quassinoids, lignans, and cembranes, among others compounds. The most active extracts resulting from solvent extraction were those of G. officinale, whose values ranged from 0.6 to 30.8 µg/mL. Generally, the most polar fractions exhibited higher cytotoxicities. The ethyl acetate

Table 2. Cytotoxicities of all the extracts generated from Puerto Rican plant species*

Plant species	Extract	LC_{50} in µg/mL†
Canella winterana (L.) Gaertn.	Crude	≥200
	Hexane	≥200
	Chloroform	≥200
	Ethyl Acetate	≥200
Croton discolor Willd.	Crude	111
	Hexane	165.6
	Chloroform	≥200
	Ethyl Acetate	≥200
Goetzea elegans Wydler	Crude	≥200
	Hexane	141.5
	Chloroform	≥200
	Ethyl Acetate	≥200
Guaiacum officinale L.	Crude	26.1
	Hexane	30.8
	Chloroform	0.7
	Ethyl Acetate	4.5
Pimenta racemosa (Mill.) J.W. Moore	Crude	≥200
	Hexane	63.9
	Chloroform	111.5
	Ethyl Acetate	28.1
Simarouba tulae Urb.	Crude	23.7
	Hexane	≥200
	Chloroform	157.1
	Ethyl Acetate	26.2
Thouinia striata Radlk.	Crude	≥200
	Hexane	≥200
	Chloroform	≥200
	Ethyl Acetate	≥200

*Generated using the BSLT, †LC50 values greater than 200 µg/ml are not considered cytotoxic. Berberine chloride was used as a positive control, showing an LC50 value of 37 µg/ml.

extract of S. tulae also showed significant activity, with an LC50 of 26.2 μg/mL. Interestingly, while the crude extract of C. discolor displayed cytotoxicity in the BSLT assay, with an LC50 value of 111 μg/mL, none of its extracts showed significant activity. This could be a result of the synergistic effects of the metabolites present in the mixture.

In contrast, the whole extracts of G. elegans and P. racemosa did not show activity against Artemia salina, but some extracts resulting from solvent extraction showed cytotoxic activity. Specifically, the hexane and ethyl acetate extracts of P. racemosa showed LC50 values of 63.9 and 28.1 μg/mL, respectively, and the hexane extract of G. elegans showed an LC50 value of 141.4 μg/mL. This may have been caused by the antagonistic effects of the complex mixture of compounds present in the crude extract. From these preliminary screenings, we have identified several new Puerto Rican plant species with promising cytotoxic activities, which species will now be scrutinized further for the isolation and identification of their active principles.

The dose–effect analysis for the BSLT correlated positively with the cytotoxic activities identified by probit analysis. The most active crude extracts were those of S. tulae, G. officinale, and C. discolor, which had effective concentration values of 26.10, 23.41, and 79.98 μg/mL, respectively. Figure 3 shows the dose-response curves for these plant species. As expected the crude extracts of Canella winterana, Goetzea elegans, Pimenta racemosa, and Thouinia striata did not show significant activities, with EC50 values greater than 200 μg/mL.

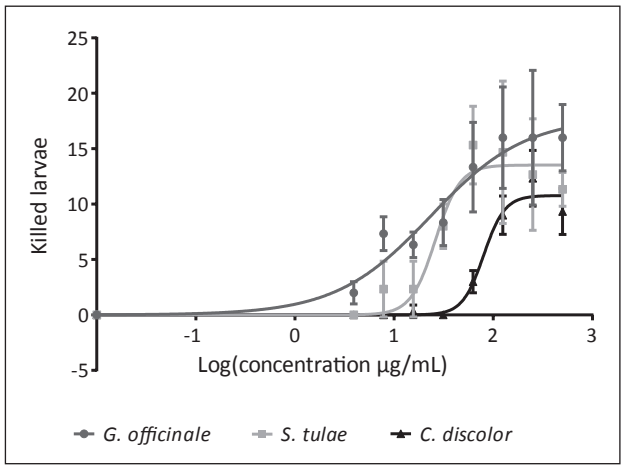

Figure 3. Mortality dose–response curves for the crude extracts of G. officinale, S. tulae, and C. discolor.

We also tested the cytotoxicity of the present plant extracts against 3 breast cancer cell lines (Table 3). A total of 21 extracts were evaluated at a single dose of 100 μg/mL. The strongest cytotoxic activity against the MCF-7 cell line was observed for the crude extracts of C. winterana and T. striata and the chloroform extracts of G. elegans, S. tulae, and T. striata, all of which showed a percentage of growth inhibition equal to or greater than 90%. On the other hand, all the extracts from P. racemosa turned out to be inactive against this cell line. Moreover, C. winterana and T. striata and the crude extracts of G. elegans and P. racemosa exhibited only moderate activity against the ZR-75-1 cell line, showing a percentage of growth inhibition ranging from 80 to 89%. However, even though the extracts of C. discolor and S. tulae were not active against the ZR-75-1 cell line, they showed significant growth inhibition against the MCF-7 cell line. The activities of these 2 species were also tested against the T47D cell line, showing promising results as cell growth inhibitors of this malignant cell line. The crude and chloroform extracts of C. discolor showed inhibitory activities of 84 and 89%, respectively, while all the extracts from S. tulae tested against this cell line demonstrated a level of inhibitory activity greater than or equal to 92%. In general, of the 7 plant species examined, the extracts of C. discolor and S. tulae were the most active, indicating that these plants might contain secondary metabolites with promising activity against breast cancer. These findings, albeit preliminary, points towards the possibility that some or all these plants might yield new leads in terms of cancer treatment.

Although our study was conducted with only 7 species, it is important to note that the results obtained already demonstrate the potential of these plants as natural sources for new anticancer compounds. Specifically, the Guaiacum, Croton, and Simarouba species have shown both significant cytotoxic activity and the ability to inhibit the cellular proliferation of breast cancer cells. Currently, we are pursuing the characterization and the further

Table 3. Percentages of plant extracts-induced growth inhibition of 3 breast cancer cell lines*

Plant species	Extract	% of GI† in breast cancer cell lines		
		MCF-7	ZR-75-1	T47D
Canella winterana (L.) Gaertn.	Crude	93	82	NT
	Hexane	82	86	NT
	Chloroform	81	87	NT
Croton discolor Willd.	Crude	≤80	≤80	84
	Hexane	85	≤80	≤80
	Chloroform	86	≤80	89
	Ethyl Acetate	86	≤80	≤80
Goetzea elegans Wydler	Crude	86	≤80	NT
Guaiacum officinale L.	Crude	81	80	NT
	Chloroform	91	≤80	NT
Pimenta racemosa (Mill.) J.W. Moore	Crude	≤80	84	NT
	Hexane	≤80	≤80	NT
	Chloroform	≤80	≤80	NT
Simarouba tulae Urb.	Crude	82	≤80	94
	Hexane	87	≤80	92
	Chloroform	95	≤80	97
	Ethyl Acetate	78	≤80	92
Thouinia striata Radlk.	Crude	92	84	NT
	Hexane	≤80	80	NT
	Chloroform	90	≤80	NT
	Ethyl Acetate	≤80	≤80	NT

*Generated using the MTT assay, †All extracts were evaluated at a single dose of 100 μg/mL. NT (not tested). Tamoxifen was employed as a positive control, inhibiting more than 91% of cell growth in the cell lines tested.

isolation and analysis of the compounds that have already been isolated from the bioassay-guided fractionation of those plant extracts having the highest inhibitory activity. Thus far, preliminary NMR analyses of the crude extract of Simarouba tulae suggest that structurally complex terpenoids may be the main constituents of this plant.

Resumen

Objetivo: El objetivo de este estudio es evaluar la actividad citotóxica y anticáncer de los extractos de siete especies de plantas endémicas y nativas de Puerto Rico. Metodología: Las especies seleccionadas para este estudio fueron *Canella winterana*, *Croton discolor*, *Goetzea elegans*, *Guaiacum officinale*, *Pimenta racemosa*, *Simarouba tulae* y *Thouinia striata*. El material seco de la planta fue extraído con una mezcla de CH_2Cl_2-MeOH en proporción de 1:1. El extracto crudo obtenido fue suspendido en agua y extraído con solventes de diferentes polaridades. Los efectos citotóxicos de los extractos hacia *Artemia salina* y tres líneas celulares de cáncer de seno fueron evaluados. Resultados: Aproximadamente 50% de los extractos evaluados contra *Artemia salina* exhibieron valores de $LC_{50} \leq$ 200 µg/mL. La actividad más fuerte se encontró en los extractos de cloroformo y acetato de etilo de *Guaiacum officinale* con valores de letalidad bajo 10 µg/mL. En adición, los extractos fueron evaluados para conocer su bioactividad como posibles inhibidores de varias líneas de cáncer de seno, siendo los extractos de *Simarouba tulae* y *Croton discolor* los que mostraron el mayor porciento de inhibición en crecimiento. El análisis de dosis-efecto para el extracto crudo de las plantas confirmó la alta citotoxicidad para *Guaiacum officinale*, *Simarouba tulae* y *Croton discolor*. Conclusión: De acuerdo a nuestros resultados concluimos que los extractos de las plantas *Simarouba*, *Croton* y *Guaiacum* demostraron actividades citotóxicas y anticancerosas que ameritan una investigación detallada para identificar los compuestos químicos responsables de dichas bioactividades.

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