Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

Cytotoxic Activity of *Rauvolfia tetraphylla* L. on Human Cervical Cancer (HeLa) Cells

José Angel Santiago-Cruz, Jesús Arrieta, Jazmín García-Machorro[1], Daniel Arrieta-Baez[2], María Elena Sánchez-Mendoza

Laboratorio De Farmacología De Plantas Medicinales Mexicanas, Escuela Superior De Medicina, Instituto Politécnico Nacional, [1]Laboratorio De Medicina De Conservación, Escuela Superior De Medicina, Instituto Politécnico Nacional, [2]Instituto Politécnico Nacional-CNMN, Ciudad De México, México

Submitted: 13-03-2019 Revised: 22-04-2019 Published: 19-09-2019

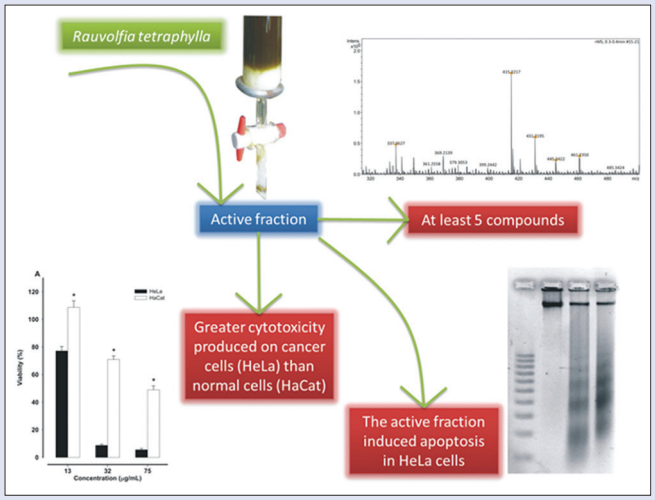
ABSTRACT

Background: Although the plant *Rauvolfia tetraphylla* is used for the treatment of cancer in the traditional medicine of some regions of Mexico, its cytotoxic activity has not been subjected to rigorous investigation. **Objective:** The aim of the present study was to carry out a bioassay-guided fractionation of *Rauvolfia tetraphylla* leaves to evaluate the activity on cervical cancer (HeLa) and normal cells. **Materials and Methods:** Of hexane, dichloromethane and methanol extracts, hexane extract showed the most cytotoxic activity. Its most active fraction was characterized by mass spectrometry and assessed for cytotoxicity (by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assays and trypan blue staining) on cervical cancer (HeLa) cells, normal human (HaCat) cells, and other cancer cell lines: A549, Caco-2, MDA-MB-231, and MCF7. Cisplatin served as the reference drug. Apoptosis was explored as a possible mechanism of action by examining DNA fragmentation and phosphatidylserine translocation. **Results:** The active fraction, composed of at least 5 constituents, was cytotoxic to all cancer cell lines, especially HeLa cells (IC_{30} = 20.10 ± 1.1 µg/mL). To a lesser extent, it was also cytotoxic to normal cells (HaCat; IC_{30} = 40.04 ± 12.78 µg/mL), indicating certain selectivity. Its mechanism of action in HeLa cells involved apoptosis. **Conclusion:** The active fraction of the hexane extract of *R. tetraphylla* was cytotoxic to cancer cells and did not produce excessive damage to normal cells. Apoptosis was apparently part of the mechanism of cytotoxicity.

Key words: Apoptosis, cervical cancer, HeLa, medicinal plants, *Rauvolfia tetraphylla*

SUMMARY

- The active fraction from the hexane extract of *Rauvolfia tetraphylla* has cytotoxic activity against cervical (HeLa), lung (A549), colon (Caco-2), and breast (MDA-MB-231 and MCF7) cancer cell lines
- The active fraction showed much greater cytotoxicity on cervical cancer (HeLa) than normal cells
- A possible mechanism of action is related to apoptosis.



Abbreviations used: DIESI-MS: Direct injection electrospray ionization-mass spectrometry; DMSO: Dimethyl sulfoxide; EtOAc: Ethyl acetate; MS: Mass spectrometry; MTT: 3-(4,5-dimethyl -2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; PBS: Phosphate-buffered saline; *R. tetraphylla*: *Rauvolfia tetraphylla* L.

Correspondence:

Prof. María Elena Sánchez Mendoza, Escuela Superior De Medicina, Instituto Politécnico Nacional, Plan De San Luis Y Díaz Mirón, Colonia Santo Tomás, Delegación Miguel Hidalgo, Ciudad De México 11340, México.
E-mail: mesmendoza@hotmail.com

DOI: 10.4103/pm.pm_106_19

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

Cervical cancer is the second most common malignant tumor found in women. The implementation of early diagnosis programs[1] and new types of anticancer therapy, such as monoclonal antibodies and targeted anticancer drugs,[2] has shown positive results. However, the prognosis for patients diagnosed in the advanced stages of the disease is still poor.[1]

Current chemotherapy treatments can stimulate metastasis to distant organs because of damaging normal tissue along with tumor cells.[3] Therefore, it is necessary to continue the search for new compounds with more selective anticancer activity and fewer side effects. Natural products represent one source of medicinal compounds, whether used in their extracted or chemically modified form. Indeed, over 60% of cancer therapy drugs are either natural products or derived from the same,[4,5] as is the case with vinca alkaloids, taxanes, camptothecins, and epipodophyllotoxins.[6] This evidences the importance of plants as a reservoir of compounds with antineoplastic activity.[7,8]

In traditional medicine, the plant *Rauvolfia tetraphylla* L. (*R. tetraphylla*) is utilized to treat many illnesses, including cardiovascular disease, psychiatric disease, cholera, intestinal disorders, diarrhea, and hypertension.[9] In some regions of Mexico, it is also used to treat cancer. The latter practice was not validated by a study in 2015, in which five alkaloids isolated from the ethanol extract of *R. tetraphylla*

Cite this article as: Santiago-Cruz JA, Arrieta J, García-Machorro J, Arrieta-Baez D, Sánchez-Mendoza ME. Cytotoxic activity of *Rauvolfia tetraphylla* L. on human cervical cancer (HeLa) cells. Phcog Mag 2019;15:631-7.

were evaluated against five cancer lines (HL-60, SMMC-7721, A-549, MCF-7, and SW-480) and found to be inactive.[10] Nevertheless, a wide variety of alkaloids has been identified in *R. tetraphylla* through phytochemical studies, and only five were tested in the aforementioned study. Besides, of the numerous alkaloids isolated from this plant, there are a labdane diterpene,[11] tannins, phenolic compounds, flavonoids, and phytosterols.[12] Hence, *R. tetraphylla* may contain cytotoxic compounds that justify the traditional practice of cancer treatment.

The aim of the present study was to conduct a bioassay-guided fractionation of *R. tetraphylla* and evaluate it for cytotoxicity on normal and cancer cells, especially the HeLa cervical cancer cell line. Other cancers cell lines tested were A549, Caco-2, MDA-MB-231, and MCF7. Particular emphasis was given to exploring apoptosis as a possible mechanism of action for reducing cancer cell viability.

MATERIALS AND METHODS

Plant material

The leaves of *R. tetraphylla* (Apocynaceae) were collected in March of 2016 in the city of Tuxpan Iguala de la Independencia, the State of Guerrero, Mexico. A voucher specimen (5155) is available in the UAGC Herbarium of the Universidad Autónoma de Guerrero.

Preparation of the extracts and fractionation

Extraction by maceration was carried out at room temperature (22°C ± 2°C) with *R. tetraphylla* leaves (3 kg), first with hexane (14 L), then dichloromethane (14 L), and finally, methanol (14 L). Extraction was performed three times during 3 days for each solvent, which was successively changed. All solvents were purchased from Arben Chemistry (Mexico State, Mexico) and were of analytical grade. Each of the extracts was filtered and concentrated in a rotary evaporator under reduced pressure and then evaporated to dryness. The yields were 79, 143, and 168 g for the hexane, dichloromethane, and methanol extracts, respectively.

The most active extract (70 g) was subjected to separation on a silica gel (Sigma-Aldrich, Mexico State, Mexico, high-purity grade) column (350 g). Four fractions were obtained from a step gradient with hexane: F1 from hexane (4.2 L), F2 from hexane/ethyl acetate (EtOAc) (9:1, 4.2 L), F3 from hexane/EtOAc (7:3, 4.2 L), and F4 from hexane/EtOAc (1:1, 4.2 L). Each fraction was evaporated to dryness to eliminate the solvent.

Direct injection electrospray ionization–mass spectrometry analysis

For characterization of the most active fraction, 1 mg was added to 1 ml of methanol (Sigma-Aldrich, Mexico State, Mexico, high-performance liquid chromatography grade) and then diluted 1:100 with methanol. This solution was submitted to Direct injection electrospray ionization–mass spectrometry (DIESI-MS) in positive mode using a micrOTOF-QII system (Bruker Daltonics, Billerica, USA). From the sample, 100 µL was loaded into the syringe and injected into the mass spectrometer by means of a 74900-00-05 Cole Palmer syringe pump (Billerica, MA, USA), allowing for a constant volumetric flow rate (8 µL/min). Capillary voltage was set to 4500 V with nitrogen as the drying and nebulizing gas at a flow rate of 4 L/min (0.4 Bar) and a temperature of 180°C. Continuous spectra were collected in a range of 50–1500 m/z, with a 1-min total run time, a 10-s scan time, and a 0.1-s interscan period, producing six spectra per sample. The mass spectrometer was operated at a resolution of 11000 (FWHM) at 622.0290 m/z in positive ion mode at a capillary voltage of 4500 V (positive). The spectrometer was calibrated with an ESI-TOF tuning mix calibrant of the grade for mass spectrometry (Sigma-Aldrich, Mexico State, Mexico). Precursor ion scans (MS/MS) were also conducted, employing positive electrospray ionization (ESI+) and the appropriate mass spectrometry setup.[13] Based on the pattern shown, suitable fragments were examined by a Bruker Compass DataAnalysis 4.0 apparatus (Bruker Daltonics). As a result, a list of possible elemental formulae was provided by Generate Molecular Formula Editor, allowing for a sophisticated comparison between the theoretical and experimental isotope patterns (σ value). This process gave a high degree of confidence in the suggested molecular formula (Bruker Daltonics Technical Note 008, 2004). The widely accepted accuracy threshold was adopted for confirmation of elemental compositions (5 ppm).

Cell cultures

The cancer cell lines included in the study were HeLa (cervical cancer), MCF7 and MDA-MB-231 (breast cancer), A549 (lung cancer), and Caco-2 (colon cancer). All of them were acquired from the American Type Culture Collection (ATCC; Manassas, Virginia, USA). Human keratinocytes (HaCat) served as normal cells and were donated by the Escuela Nacional de Ciencias Biológicas. The cells were grown at 37°C in a humidified atmosphere with 5% CO_2. The medium was DMEM/F12 (Gibco Lab, USA) supplemented with 5% fetal bovine serum (FBS, Atlanta Biological), 2 mM L-glutamine (Sigma-Aldrich, Mexico State, Mexico), 100 U/mL penicillin, and 100 mg/mL streptomycin (Sigma-Aldrich, Mexico State, Mexico). All reactives were suitable for cell culture.

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assays

The cell lines were seeded in 96-well plates (1×10^4 cells/well) and incubated overnight. Subsequently, they were exposed to different concentrations of the extracts of *R. tetraphylla* (30, 70, and 100 µg/mL), the fractions (100 µg/mL), F4 (13–75 µg/mL), cisplatin (1.5–7.5 µg/mL), or the vehicle control. The extracts and fractions were dissolved in dimethyl sulfoxide (DMSO) and DMEM/F12 culture medium (using 1% v/v as the maximum final concentration of DMSO), while cisplatin was dissolved in deionized water.

Following 72 h of treatment, viability was measured by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) test.[14] After adding 100 µL of MTT (1 mg/mL of phosphate-buffered saline [PBS]) per well, and incubating at 37°C for 2.5 h, the formazan formed were dissolved in 100 mL of DMSO. All reactives were analytic grade, molecular biology grade, or suitable for cell culture (Sigma-Aldrich, Mexico State, Mexico). Absorbance was evaluated at 540 nm on a microplate reader (Fisher Scientific Thermo Multiskan MCC/340). Cell viability was expressed as the percentage of live treated cells versus live control cells. The 30% and 50% inhibition concentration (IC_{30} and IC_{50}) was calculated by linear regression. All the experiments included the corresponding vehicle control.

Trypan blue staining assays

HeLa and HaCat cell lines were seeded in 24-well culture plates at 1×10^4 cells/well and incubated for 24 h. On completion of this time, the control (DMSO only) and three concentrations of F4 (13, 32, or 75 µg/mL) were tested, adding one of these to each well. The plates were left to stand for 72 h at 37°C and 5% CO_2, followed by the removal of the medium containing dead cells. The plate was then washed with ×1 PBS and stained with trypan blue suitable for cell culture (0.4%, Sigma-Aldrich, Mexico State, Mexico). After 5 min, the excess dye was removed.[15] The number of live (unstained) cells was counted in three quadrants per well under a light microscope. The results are expressed as a percentage of the value of the control group (untreated cells), the later considered as 100%.

DNA fragmentation

HeLa cells were seeded at a density of 5 × 10^5 in 35 mm dishes in the presence of 300 µg/mL of the F4 fraction, 50 µg/mL of cisplatin as the positive control, or the vehicle (DMSO only) as the negative control. On completing 24 h of incubation, the cells were centrifuged at 362 × g for 5 min. Cell pellets were suspended in 200 µL of lysis buffer (50 mM Tris-HCl (pH 7.5), 10 mM EDTA, 0.002% sodium azide, 1 mM $CaCl_2$, and 1% Triton X-100) with 2 µL proteinase K (100 µg/mL) and left to stand at 37°C for 2 h. Subsequently, 200 µL of phenol: chloroform: isoamyl alcohol (25:24:1) was added, and the mixture was gently homogenized and centrifuged at 15294 ×g for 10 min. The supernatant was transferred into a new tube, in which 500 µl of ice-cold isopropanol and 100 µl of ice-cold sodium acetate buffer (3 M, pH 5.2) were placed for overnight incubation at −70°C to allow the DNA to precipitate. Samples of the precipitated DNA were centrifuged at 15294 ×g for 10 min at 4°C, then washed with 70% cold ethanol, and dissolved in 30 µL 10 mM Tris-HCl (pH 7.4). DNA was incubated with RNase solution (20 µg/mL) at 37°C for 3 h, followed by an analysis of its fragmentation by electrophoresis at 100 V for 70 min on 2% agarose gel containing ethidium bromide (0.1 µg/mL). All the reactives were of analytic or molecular biology grade (Sigma-Aldrich, Mexico State, Mexico). A DNA molecular weight marker (Bio-Rad, California, USA) was applied to the gel, and the separated DNA fragments were visualized under ultraviolet light.[16]

Determination of apoptosis

HeLa cells (5 × 10^5 cells/well) were treated with 300 µg/mL of F4 or the negative control (DMSO only) for 24 and 48 h and then harvested, washed with ice-cold ×1 PBS, and stained with Annexin V Alexa Fluor 488/PI (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Apoptotic cell death was evaluated by flow cytometry (BD FACSCalibur, USA). Fluorescence of Annexin V Alexa Fluor 488 and propidium iodide (PI) was monitored at 530 nm and 575 nm, respectively.

Statistical analysis

Statistical significance was examined with one-way analysis of variance, followed by the Dunnett's test or Student's *t*-test, where appropriate. Significant differences were considered at $P \leq 0.05$. All experiments were carried out in triplicate.

RESULTS

Bioassay-guided fractionation and inhibition of HeLa cells

The cytotoxic activity of the hexane, dichloromethane, and methanol extracts of *R. tetraphylla* was determined on the HeLa cell line with the MTT assay [Table 1]. The hexane extract was the most active, reaching a maximal growth inhibition of 39.41% at a concentration of 100 µg/mL. At the same concentration, the dichloromethane and methanol extracts induced only 22.0% and 29.34% inhibition, respectively. Therefore, the hexane extract was separated by column chromatography.

Four fractions were obtained from the hexane extract. F2, F3, and F4 (each at 100 µg/mL) inhibited HeLa cells at 14.12%, 66.61%, and 81.51%, respectively [Figure 1]. The most active fraction, F4, was analyzed by DIESI-MS.

Chemical characterization of F4 by DIESI-MS

The study of F4 by DIESI-MS demonstrated the existence of a main compound at 415.2217 m/z and at least four other compounds at low concentrations [Figure 2]. The main peaks of MS/MS were examined by SmartFormula 3D command with Bruker DataAnalysis. This tool is used to calculate the possible molecular formula for an MS precursor ion by combining the results from SmartFormula operations performed on the precursor ion with those from all of its fragment ions. The proposed formula, afforded by the MS/MS analysis, was processed by the *in silico* fragmenter MetFrag[17] to furnish the structure [Table 2].

Table 1: Cytotoxic activity of the extracts from *Rauvolfia tetraphylla* leaves on cervical cancer cells

Treatment	Concentration (µg/mL)	Cell viability (%)±SEM
Control	-	100±3.5
Hexane extract	30	90.74±5.2
	70	83.19±4.9
	100	60.59±2.6*
Dichloromethane extract	30	104.54±5.9
	70	95.65±5.2
	100	78.0±3.6*
Methanol extract	30	94.32±4.4
	70	88.7±3.4
	100	70.66±4.3*

Data are expressed as the mean±SEM of three independent experiments performed in triplicate. *P<0.05 versus the control group. SEM: Standard error of mean

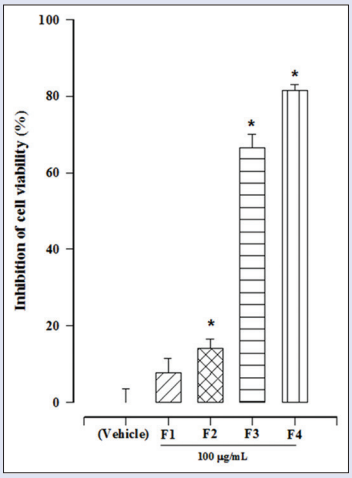


Figure 1: The cytotoxic activity on HeLa cells produced by the fractions obtained from the hexane extract. Data are expressed as the mean ± standard error of the mean of three independent experiments performed in triplicate and were analyzed by analysis of variance followed by the Dunnett's test. *P < 0.05 versus the control group

Cytotoxic activity of F4 on HaCat and HeLa cells

The cytotoxic effect of F4 was assessed on normal human (HaCat) cells and cancerous (HeLa) cells, inhibiting both cell lines in a concentration-dependent manner. The maximum concentration tested (75 µg/mL) caused 48.42% inhibition of HaCat cells (IC_{30} = 40.04 ± 12.78 µg/mL) and 73.43% inhibition of HeLa cells (IC_{30} = 20.10 ± 1.1 µg/mL). The difference between these values was significant (P < 0.001), indicating selectivity for the HeLa cells [Figure 3a]. The maximum concentration of cisplatin (7.5 µg/mL), the reference drug, produced a 79.73% inhibition of HeLa cells and 84.43% inhibition of HaCat cells, representing a nonsignificant difference. However, a significant difference was indeed found at lower concentrations [Figure 3b]. The IC_{50} of cisplatin for HeLa cells was 3.18 ± 1.16 and for HaCat cells 2.43 ± 0.72 µg/mL.

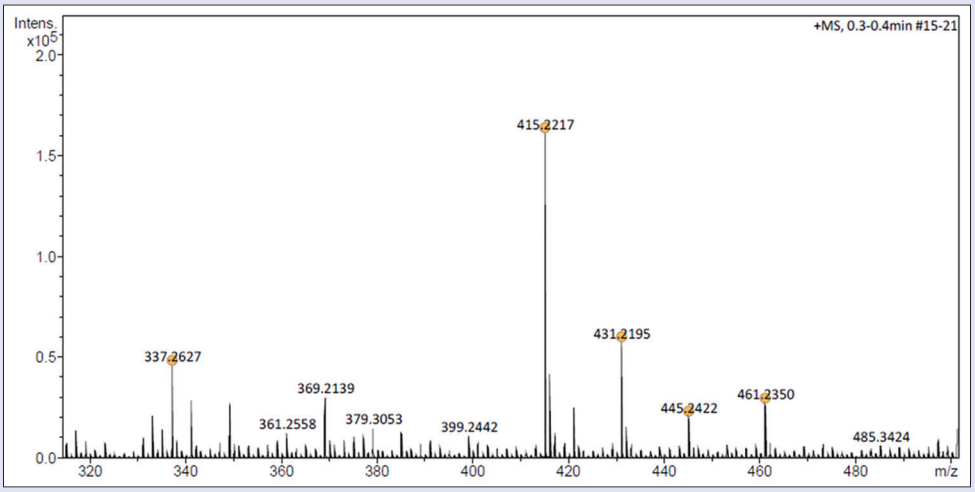


Figure 2: MS-ESI spectrum (positive ion mode) of fraction F4 from the hexane extract of *Rauvolfia tetraphylla*

Table 2: The main chemical constituents of the F4 fraction from the hexane extract of *Rauvolfia tetraphylla*, identified by direct injection electrospray ionization mass spectrometry

Compound	MW fraction F4	MW observed	Error (ppm)	mSigma	% RA	Formula
1,2-Ethanediyl dioctanoate	337.2349	337.2627	5.4	6.1	0.68	$C_{18}H_{34}O_4Na$
Methyl 10,11-dimethoxy-19-methyl-18-oxayohimban-16-carboxylate	415.2227	415.2217	−2.5	16.8	2.14	$C_{23}H_{30}N_2O_5$
$C_{23}H_{30}N_2O_6$	431.2176	431.2195	−4.2	15.2	0.96	$C_{23}H_{30}N_2O_6$
$C_{24}H_{32}N_2O_6$	445.2333	445.2426	−20.8	12.2	0.50	$C_{24}H_{32}N_2O_6$
$C_{24}H_{32}N_2O_7$	461.2282	461.2353	−15.3	15.7	0.63	$C_{24}H_{32}N_2O_7$

MW fraction F4: Molecular weight of the fraction F4; MW observed: Molecular weight observed; Error (ppm): Absolute value of the deviation between measured mass and theoretical mass of the selected peak in ppm; mSigma: Combined value for the standard deviation of the masses and intensities for all peaks, given in milliSigma; % RA: Percentage relative area

The cytotoxic effect of F4

The trypan blue test showed that the exposure of HeLa and HaCat cells to F4 for 72 h led to an increasing number of dead cells in a concentration-dependent manner [Figure 4a]. At a concentration of 13, 32, and 75 μg/mL, this fraction inhibited HeLa cells by 22.86%, 91.36%, and 94.50% and HaCat cells by 0%, 29.06%, and 51.11%, respectively. The HeLa and HaCat cells were viewed on an optical microscope before and after treatments, finding greater cellular damage with higher concentrations of F4. At the maximum concentration tested (75 μg/mL), some HaCat cells maintained their membrane integrity, but only the remains of HeLa cells were observed [Figure 4b].

Apoptosis of HeLa cells

To evaluate whether the mechanism of cytotoxic action of F4 is related to the apoptosis of HeLa cells, two apoptotic hallmarks were analyzed: DNA fragmentation and phosphatidylserine (PS) translocation. Similar DNA fragmentation profiles were found for HeLa cells incubated with F4 (300 μg/mL) or the positive control, cisplatin (50 μg/mL). Thus, F4 seems to have triggered apoptotic cell death. For untreated cells (DMSO only), the presence of a single band evidenced the lack of apoptosis [Figure 5a].

To further explore the possibility of F4-induced apoptosis, HeLa cells were exposed to F4 or left untreated. According to the subsequent examination of apoptosis by flow cytometry, a higher percentage of cells was found in the Q1, Q2, and Q4 quadrants after 24 and 48 h of F4 treatment (at 300 μg/mL). These three quadrants correspond to cells having undergone necrosis, late apoptosis, and early apoptosis, respectively [Figure 5b]. The highest percentage of stained cells was detected in quadrant 4 (early apoptosis), having increased (versus untreated cells) 38.1% by 24 h and 43.3% by 48 h. Contrarily, only 2.3% of untreated cells showed early apoptosis.

Cytotoxicity of F4 on various human cancer cells

The activity of F4 on human cancer cells other than HeLa was also evaluated with the MTT assay, finding concentration-dependent cytotoxicity in all cases [Table 3]. The greatest cytotoxic effect was produced on cervical cancer cells (HeLa), followed by lung cancer cells (A549), colon cancer cells (Caco-2), and breast cancer cells (MDA-MB-231 and MCF7).

DISCUSSION

Bearing in mind the urgency of developing novel drugs that can provide better alternatives for cancer therapy,[18] a study was herein carried out on *R. tetraphylla*, a plant used in traditional medicine in some regions of the State of Guerrero, Mexico, for the treatment of cancer. Even though five alkaloids isolated from *R. tetraphylla* were previously reported to exhibit no relevant cytotoxicity against five human cancer cell lines, all having an IC_{50} >40 μM,[10] there are numerous alkaloids in this plant. Since other compounds in the plant may be capable of causing cytotoxic activity, plant leaves were examined through a bioassay-guided fractionation and tested on cervical cancer cells and four additional cancer cell lines.

With the MTT assay, the hexane extract proved to be the most cytotoxic (at 100 μg/mL). Although the American National Cancer Institute regards an extract as a good candidate for future bioassay-guided analysis only if it has a significant cytotoxic effect with an IC_{50} ≤30 μg/mL,[19] we considered the possible existence of substances in the hexane extract that interferes with the desired

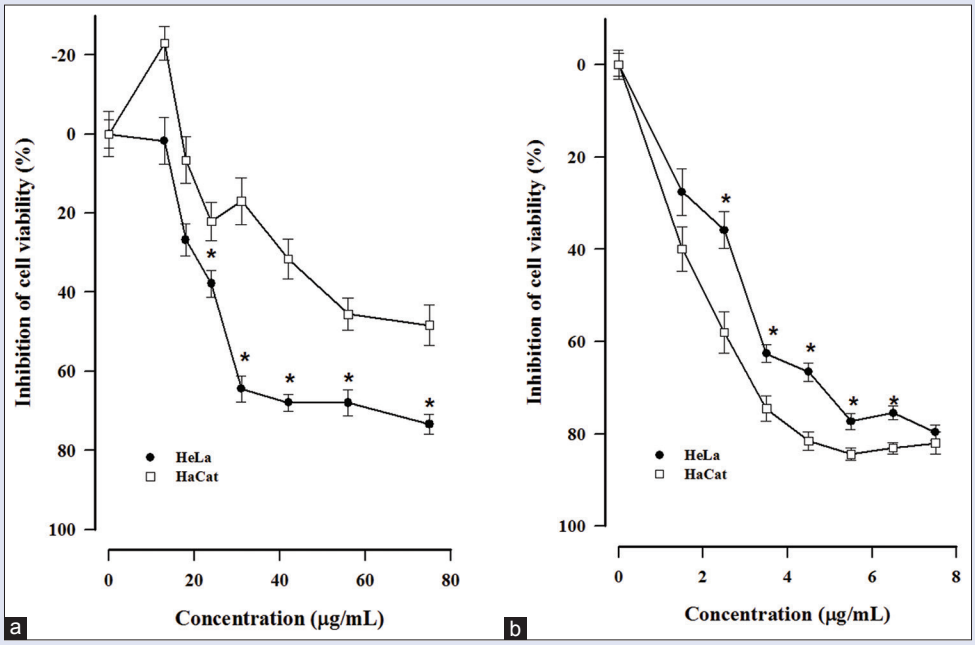


Figure 3: The cytotoxic effect on HeLa and HaCat cells caused by (a) F4 and (b) cisplatin. The data are expressed as the mean ± standard error of the mean of three independent experiments performed in triplicate. The results for each concentration were analyzed by the Student's *t*-test. *$P < 0.05$ comparing HeLa and HaCat cells

Table 3: The half maximal inhibitory concentration of the active fraction (F4) on five cancer cell lines

Cell line[a]	HeLa	A549	Caco-2	MDA-MB-231	MCF7
IC_{50} (µg/mL)	28.2±1.5	33.8±5.1	40.3±1.7	45.9±2.3	63.3±4.4

[a]HeLa: Cervical cancer cells of a young African-American woman, Henrietta Lacks.; A549: are adenocarcinomic human alveolar basal epithelial cells; Caco 2: line of heterogeneous human epithelial colorectal adenocarcinoma cells; MDA MB 231 is a breast cancer cell line. This cell line was derived at M.D. Anderson in 1976 from a pleural effusion from a 31-year old woman with a history of breast cancer, hence here MDA stands for M.D. Anderson and MB stands for Metastasis Breast cancer and MCF7: Breast cancer cells; is the acronym of Michigan Cancer Foundation-7

activity. In support of this idea, some active compounds isolated from other species of *Rauvolfia* are known to cause cytotoxicity.[20-22] For example, 9-hydroxynoracronycine (an acridone alkaloid) isolated from *Rauvolfia verticillata* decreases human breast cancer cell (MCF-7) proliferation.[20] In addition, reserpine (an indole alkaloid) isolated from *Rauwolfia serpentina*[22] and alstonine (a β-carboline alkaloid) from *Rauwolfia vomitoria*[23] exhibit cytotoxic activity on prostate cancer cell lines (PC3 and LNCaP, respectively).

Through a bioassay-guided fractionation, four fractions were obtained from the hexane extract. The F4 fraction showed the best cytotoxic activity of these four, diminishing cell viability by 81.51%. Moreover, F4 generated a 42.1% greater inhibition of HeLa cells than the whole hexane extract, indicating that chromatography did, in fact, eliminate some compounds interfering with cytotoxicity. Despite not achieving 100% inhibition, the current findings partly support the use of *R. tetraphylla* in traditional medicine.

The separation of the compounds in F4 was not possible because the R_f values of the constituents were similar. The mass spectrometry analysis revealed that F4 contains at least 5 compounds. The probable molecular formula of the main compound is $C_{23}H_{30}N_2O_5$, which apparently corresponds to methyl 10,11-dimethoxy-19-methyl-18-oxayohimban-16-carboxylate (commonly called herbacein). There are no reports, to our knowledge, on the cytotoxicity of this compound.

Notably, F4 exhibited less potent cytotoxic activity on HaCat than HeLa cells, as evidenced by the IC_{30} values. Since selectivity for the target cell reduces the side effects of molecules with antineoplastic activity, it is a key to the success of anticancer treatment. Future research is needed to corroborate the selectivity of F4.

Regarding the cytotoxicity of cisplatin, similar IC_{50} values were found for HaCat and HeLa cells, probably due to the high nucleophilic capacity of the drug. With both cell lines, DNA-cisplatin adducts were formed.[24] As the target is DNA, adverse effects are inevitable because this molecule is the same in healthy and malignant cells.

Since the MTT technique is based on the reduction of MTT to formazan by the action of mitochondrial dehydrogenase enzymes,[25] F4 may only decrease cell proliferation, probably by diminishing the biotransformation of MTT. This question was resolved with trypan blue staining, a dye able to permeate dead cells (because the integrity of the membranes has been lost) but not live cells.[15] The trypan blue assay was performed with three different concentrations of F4 (13, 32, and 75 µg/mL). At these concentrations, the percentage of dead cells ranged from 0, about half and maximum effect, regarding the dose–response curve of F4 on HeLa cells in the MTT assay. The results of trypan blue assay showed that F4 was less cytotoxic for normal cells than cancer cells, which is one of the main requirements of a good cancer drug.[26]

Natural compounds with anticancer activity can give rise to cell death through several mechanisms of action, including the regulation of the cell cycle and the triggering of apoptotic and nonapoptotic pathways (such as autophagy, necrosis, mitotic catastrophe, and senescence). In the case of compounds isolated from plants, the apoptotic pathway has received more attention in research.[27] Programmed death consists of characteristic morphological and biochemical alterations during cell development. Among the morphological changes are decreased cell size, increased cytoplasmic density, the compression of organelles, and the condensation of chromatin. Biochemical changes, on the other hand,

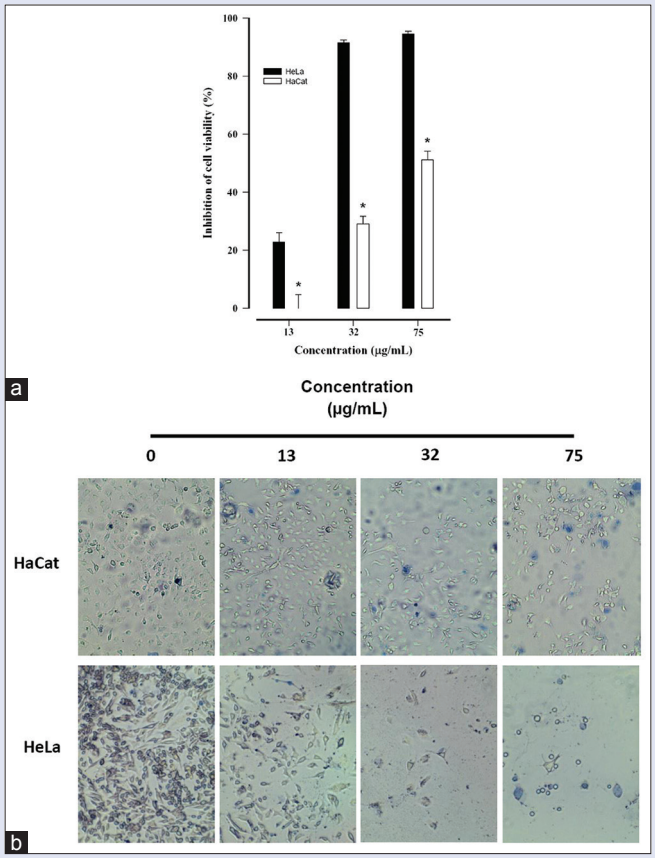


Figure 4: Evaluation by trypan blue staining of the cytotoxic activity of F4 on HeLa and HaCat cells. After treatment with F4 for 72 h, (a) a count was made of viable cells and (b) microphotographs were taken. The data are expressed as the mean ± standard error of the mean of three independent experiments performed in triplicate. The results for each concentration were analyzed by the Student's *t*-test. *$P < 0.05$ comparing HeLa and HaCat cells

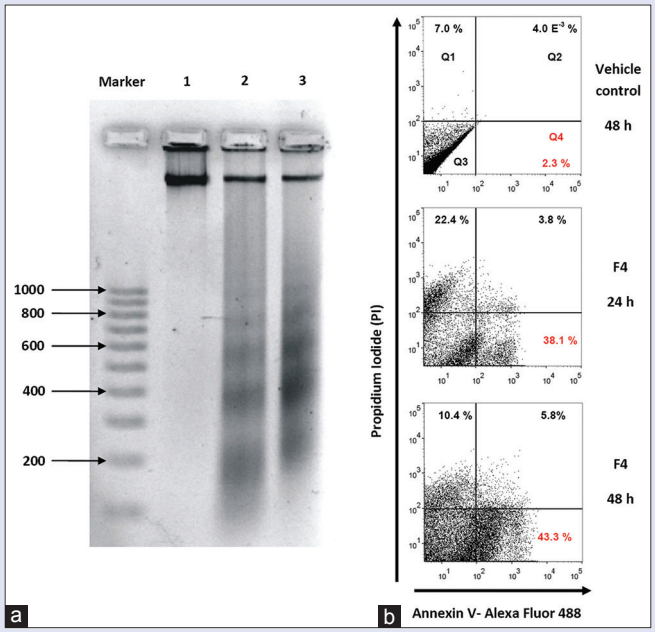


Figure 5: (a) Induction of apoptosis, showing the expected ladder pattern (100–1000 pb) on the left. On the right, DNA fragmentation of HeLa cells produced by: (1) the vehicle control, (2) F4 (obtained from the hexane extract of *Rauvolfia tetraphylla* leaves) and (3) cisplatin. (b) The translocation of phosphatidylserine was measured by flow cytometry. Q1 displays PI (+) annexin V (–), representing necrosis. Q2 portrays PI (+) annexin V (+), representing late apoptosis. Q3 depicts PI (–) annexin V (–), representing healthy cells. Q4 illustrates PI (–) annexin V (+), representing early apoptosis

encompass protein cleavage, protein cross-linking, DNA fragmentation, mitochondrial fragmentation, and phagocytic recognition.[28,29] The two main apoptotic pathways involved, the intrinsic (mitochondrial) and extrinsic (death receptor) pathways, overlap and interact. Possible subpathways are the p53 and lysosomal pathways.[30]

Two apoptosis markers were employed in this study: (a) DNA degradation by a nuclease known as caspase-activated DNase, which generates the 180 bp laddering of DNA and (b) the externalization of a phospholipid, PS, on the outer plasma membrane of cells.[29] Concerning DNA fragmentation, the 180 bp ladder was observed in HeLa cells exposed to 300 µg/mL of F4, indicating the existence of apoptosis-triggering compounds in this fraction. As can be appreciated, a much greater concentration of F4 was necessary to trigger apoptosis than to reach a maximum effect in the viability tests (75 µg/mL). This is probably due to the limited number of cells used in the viability versus apoptosis assay (1×10^4 cells vs. 5×10^5 cells). For the latter assay, a larger quantity of cells was necessary for DNA extraction. Another plausible explanation is that apoptosis was not the only mechanism leading to the death of cancer cells, as described in the case of the extract of *Rauvolfia vomitoria*.[23]

The annexin V/PI staining assay confirmed the triggering of apoptosis by F4 and established a time-dependent effect. Since apoptotic pathways are blocked in cancer cells, their activation is critical for the efficacy of anticancer drugs.[26]

In HeLa cells, F4 generated not only apoptosis but also necrosis. According to recent research, small molecule inhibitors/activators may be able to modulate necrosis.[31] Consequently, the mixture of compounds in F4 might have other molecular targets. Moreover, it is possible that the increased concentration required to carry out this assay produced the observed necrosis, since the degree of stimuli determines whether cells die by apoptosis or necrosis. A cytotoxic anticancer drug can induce apoptosis at low doses and necrosis at higher doses.[28]

The current findings provide evidence of the importance of F4 as a possible chemoprophylactic agent, as suggested by its cytotoxicity on all cancer cell lines herein examined. A correlation has been reported between chemoprevention and the cytotoxicity of plants.[26]

CONCLUSION

R. tetraphylla exhibited cytotoxicity for all five cancer cell lines tested, especially for HeLa cells. The hexane extract was the most active and one of its fractions (F4, composed of at least five compounds) showed even greater cytotoxicity than the extract. The results suggest that cytotoxicity was related to the capacity of F4 to induce apoptosis.

Acknowledgements

We are grateful to PhD Leopoldo Aguilar Faisal and PhD Saúl Rojas Hernández for their support in the present project.

Financial support and sponsorship

This study was supported by grants 20195147 and 20195148 from the SIP-IPN.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Wang SJ, Zheng CJ, Peng C, Zhang H, Jiang YP, Han T, *et al*. Plants and cervical cancer: An overview. Expert Opin Investig Drugs 2013;22:1133-56.
2. Jaudan A, Sharma S, Malek SN, Dixit A. Induction of apoptosis by pinostrobin in human cervical cancer cells: Possible mechanism of action. PLoS One 2018;13:e0191523.
3. Zhao B, Hu M. Gallic acid reduces cell viability, proliferation, invasion and angiogenesis in human cervical cancer cells. Oncol Lett 2013;6:1749-55.
4. Tan G, Gyllenhaal C, Soejarto DD. Biodiversity as a source of anticancer drugs. Curr Drug Targets 2006;7:265-77.
5. Gordaliza M. Natural products as leads to anticancer drugs. Clin Transl Oncol 2007;9:767-76.
6. Kinghorn AD, Chin YW, Swanson SM. Discovery of natural product anticancer agents from biodiverse organisms. Curr Opin Drug Discov Devel 2009;12:189-96.
7. Mahata S, Maru S, Shukla S, Pandey A, Mugesh G, Das BC, *et al*. Anticancer property of *Bryophyllum pinnata* (Lam.) Oken. Leaf on human cervical cancer cells. BMC Complement Altern Med 2012;12:15.
8. Choudhari AS, Suryavanshi SA, Kaul-Ghanekar R. The aqueous extract of *Ficus religiosa* induces cell cycle arrest in human cervical cancer cell lines SiHa (HPV-16 positive) and apoptosis in HeLa (HPV-18 positive). PLoS One 2013;8:e70127.
9. Gupta S, Khanna VK, Maurya A, Bawankule DU, Shukla RK, Pal A, *et al*. Bioactivity guided isolation of antipsychotic constituents from the leaves of *Rauwolfia tetraphylla* L. Fitoterapia 2012;83:1092-9.
10. Gao Y, Zhou DS, Hai P, Li Y, Wang F. Hybrid monoterpenoid indole alkaloids obtained as artifacts from *Rauvolfia tetraphylla*. Nat Prod Bioprospect 2015;5:247-53.
11. Brahmachari G, Mandal LC, Gorai D, Mondal A, Sarkar S, Majhi S. A new labdane diterpene from *Rauvolfia tetraphylla* Linn. (Apocynaceae). J Chem Res 2011;35:678-80.
12. Rohela GK, Bylla P, Korra R, Reuben C. Phytochemical screening and antimicrobial activity of leaf, stem, root and their callus extracts in *Rauwolfia tetraphylla*. Int J Agric Biol 2016;18:521-8.
13. Dams R, Murphy CM, Lambert WE, Huestis MA. Urine drug testing for opioids, cocaine, and metabolites by direct injection liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom 2003;17:1665-70.
14. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55-63.
15. Strober W. Trypan blue exclusion test of cell viability. Curr Protoc Immunol 2001;Appendix 3:Appendix 3B. doi: 10.1002/0471142735.ima03bs21.
16. Xu Z, Chen X, Fu S, Bao J, Dang Y, Huang M, *et al*. Dehydrocorydaline inhibits breast cancer cells proliferation by inducing apoptosis in MCF-7 cells. Am J Chin Med 2012;40:177-85.
17. Ruttkies C, Schymanski EL, Wolf S, Hollender J, Neumann S. MetFrag relaunched: Incorporating strategies beyond in silico fragmentation. J Cheminform 2016;8:3.
18. Roy M, Mukherjee A, Sarkar R, Mukherjee S, Biswas J. In search of natural remediation for cervical cancer. Anticancer Agents Med Chem 2015;15:57-65.
19. Alonso-Castro AJ, Villarreal ML, Salazar-Olivo LA, Gomez-Sanchez M, Dominguez F, Garcia-Carranca A. Mexican medicinal plants used for cancer treatment: Pharmacological, phytochemical and ethnobotanical studies. J Ethnopharmacol 2011;133:945-72.
20. Hong B, Gao J, Wu J, Zhao C. Chemical constituents from *Rauvolfia verticillata* and bioactivities research. Chem Nat Compd 2012;48:276-80.
21. Yu J, Ma Y, Drisko J, Chen Q. Antitumor activities of *Rauwolfia vomitoria* extract and potentiation of carboplatin effects against ovarian cancer. Curr Ther Res Clin Exp 2013;75:8-14.
22. Ramamoorthy MD, Kumar A, Ayyavu M, Dhiraviam KN. Reserpine induces apoptosis and cell cycle arrest in hormone independent prostate cancer cells through mitochondrial membrane potential failure. Anticancer Agents Med Chem 2018;18:1313-22.
23. Bemis DL, Capodice JL, Gorroochurn P, Katz AE, Buttyan R. Anti-prostate cancer activity of a beta-carboline alkaloid enriched extract from *Rauwolfia vomitoria*. Int J Oncol 2006;29:1065-73.
24. Alderden RA, Hall MD, Hambley TW. The discovery and development of cisplatin. J Chem Educ 2006;83:728-34.
25. Berridge MV, Tan AS. Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): Subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. Arch Biochem Biophys 1993;303:474-82.
26. Taraphdar AK, Roy M, Bhattacharya RK. Natural products as inducers of apoptosis: Implication for cancer therapy and prevention. Curr Sci 2001;80:1387-96.
27. Gali-Muhtasib H, Hmadi R, Kareh M, Tohme R, Darwiche N. Cell death mechanisms of plant-derived anticancer drugs: Beyond apoptosis. Apoptosis 2015;20:1531-62.
28. Elmore S. Apoptosis: A review of programmed cell death. Toxicol Pathol 2007;35:495-516.
29. Henry CM, Hollville E, Martin SJ. Measuring apoptosis by microscopy and flow cytometry. Methods 2013;61:90-7.
30. Moffitt KL, Martin SL, Walker B. From sentencing to execution – The processes of apoptosis. J Pharm Pharmacol 2010;62:547-62.
31. Aki T, Funakoshi T, Uemura K. Regulated necrosis and its implications in toxicology. Toxicology 2015;333:118-26.