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PRODUCTOS NATURALES

Licania arborea fraction bioactive potential assessment in jurkat and cho-k1 cell lines

Valoración del potencial bioactivo de la fracción de Licania arbórea en las líneas celulares Jurkat y Cho-K1

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ABSTRACT

Introduction: Several species of Chrysobalanaceae plants have shown a variety of biological activities, exhibiting a source of interesting compounds from the pharmacological point of view.
Objective: To assess the bioactive potential of *L. arborea* fractions on Jurkat and CHO-K1 cell lines.
Methods: All the experiments were carried out in Jurkat and CHO-K1 (mammalian cell lines). Cytotoxicity of the fractions was assessed via ŧTrypan blue dye and tetrazolium salt (MTT) assays. Genotoxicity was evaluated determining the sister chromatid exchange (SCE). Antiproliferative effect was estimated by clonogenic assay and cell cycle progression. Furthermore,

proliferative kinetics were assessed by SCE of *L. arborea* fractions. **Results:** The IC_{50} of F_8 and F_{10} fractions was approximately 100 µg/mL for Jurkat and CHO-K1 cell lines. Cytotoxicity of the evaluated fractions led to reduced cell viability and cloning capability. Particularly, fraction F_8 showed genotoxic effect reflected in increments of SCEs frequency, mainly on Jurkat cells. **Conclusions:** Jurkat and CHO-K1 cells clearly displayed a dose dependent cytotoxicity and genotoxicity, although the effect was more severe on the lymphoid cell line compared with the noncancerous cells, showing total absence of mitosis in the Jurkat cell line treated with fraction F8 (100µg/mL).

Keywords: Cytotoxicity; antiproliferative effect; genotoxicity; cell lines.

RESUMEN

Introducción: Diversas especies de *Chrysobalanaceae* mostrado una variedad de actividades biológicas que constituyen una fuente de compuestos interesantes desde el punto de vista farmacológico **Objetivo:** Valorar el potencial bioactivo de las fracciones de L.arborea en las líneas celulares Jurkat y CHO-K1. **Métodos:** Todos los experimentos se realizaron en las líneas Jurkat y CHO-K1 (líneas celulares de mamíferos). Se evaluó la citotoxicidad de las fracciones mediante ensayos colorimétricos con sales de tetrazolio y azul de Tripán. La genotoxicidad se determinó con la prueba de intercambio de cromátides hermanas. Se calculó el efecto antiproliferativo por medio del ensayo clonogénico y la progresión del ciclo celular. Asimismo, la valoración de la cinética proliferativa se basó en el intercambio de cromátides hermanas en fracciones de *L. arbórea*. **Resultados:** El valor de la CI_{50} de las fracciones F_8 y F_{10} fue de 100 µg/mL aproximadamente para las líneas celulares Jurkat y CHO-K1. La citotoxicidad de las fracciones evaluadas trajo como resultado la reducción de la viabilidad celular y una menor capacidad de clonación. La fracción F_8 en especial mostró un efecto genotóxico reflejado en los incrementos de la frecuencia de intercambio de las cromátides hermanas, particularmente en las células Jurkat. **Conclusiones:** Las células Jurkat y CHO-K1 muestran con claridad la existencia de citotoxicidad y genotoxicidad dependiente de la dosis, si bien el efecto es más severo en la línea de células linfoides en comparación con las células no cancerosas, lo que demuestra la ausencia total de mitosis en la línea celular Jurkat que recibió tratamiento con la fracción F_8 (100 µg/mL).

Palabras claves: citotoxicidad; efecto antiproliferativo; genotoxicidad; líneas celulares.

INTRODUCTION

The exploration for antitumor agents in plants started in the early 50´s with the discovery of alkaloids with cytotoxic effects such as Vinblastine and Vincristine obtained from *Catharanthus roseus,* and the isolation of podophyllotoxins present in *Podophyllum peltatum.*

In Venezuela, Brazil, and USA have been found *Licania arborea* and other species from the Chrysobalanaceae family containing great quantities of substances with fungicide, antitumor, antioxidant, antiviral, antibacterial, and anti-inflammatory effects,[2-10] such as terpenes. These compounds have been studied in colon, liver, and melanoma tumor cell lines, and Gram positive bacteria and yeast.[2,4,5,10] Out of the 150 species *Licania* genus,[5] it has also been reported biological activity of *L. licaniaeflora*, *L. heteromorpha*, *L. michauxii* and *L. tomentosa.*[2-6,8-10] Nevertheless, there are not reports on the effect on the cell cycle, cytotoxicity or genotoxicity of compounds present in *L. arborea.*

Therefore, herein we present the results of preliminary studies of the biological activity of dichloromethanolic fractions of *L. arborea* in Jurkat (human acute T cell leukemia) and CHO-K1 (derived from the ovary of Chinese hamster) cell line. Cytotoxicity assays such as Trypan blue dye, Tetrazolium salt (MTT), as genotoxic test SCE were used. The antiproliferative effect by clonogenic assay, cell cycle progression, and proliferative kinetics were tested through sister chromatid exchange (SCE).

METHODS

MATERIALS

Analysis grade solvents were used in the preparation of extracts and fractions. Silica gel F_{254} and silica gel 60 (Merck) as stationary phase and Ethyl acetate-hexane (3:1) and dichloromethane/methanol in different proportions as mobile phase respectively, were used in the fine layer and column chromatography. The extracts were concentrated in rotary evaporator (Heidolph Laborota 4001); and an ultraviolet lamp Mineralight model UVGL-58 multiband UV-254/366 nm, and a 4 % in ethanol solution of phosphomolybdic acid (Merck) with further heating at 110 °C were used as visualizing agents.

SAMPLE COLLECTION

Leaves of *Licania arborea* were collected at the Universidad Nacional de Colombia, in El Volador Campus (Medellín). The taxonomic classification was made in the Herbario MEDEL at the same university. A reference sample was kept in the Laboratorio de Productos Naturales Marinos de la Universidad de Antioquia (Medellín, Colombia) with voucher sample number PNM-29.

PHYTOCHEMICAL MARCH AND EXTRACT PREPARATION

To determine the secondary metabolic types present in the extract of *L. arborea* leaves, a phytochemical march was completed.[11] Leaves from *L. arborea* (w/w 50 g) were dried in stove at 40 ºC and then were ground in a scissor grinder. The sample was successively extracted with methanol and dichloromethane, and dried in a rotary evaporator at 40 °C, with reduced pressure and constant agitation. Each extract was fractioned by fine layer and column chromatography using the conditions previously described.[11] Ten fractions (F1-F10), were obtained and their biological activity was evaluated.

CELL CULTURES

All biological tests were completed on cultures in exponential phase of growth, the Jurkat clone E6-1 cell line (human leucemoid ATCC TIB-152) and CHO-K1-B (ATCC N° CCL-61). The cells were grown in RPMI 1640 (Gibco) culture medium, supplemented with 5 % of fetal bovine serum (Lonza, USA). Cell cultures were incubated at 37 °C in a humidified atmosphere with CO_2 at 5 %.

CYTOTOXIC EFFECT EVALUATION

Jurkat and CHO-K1 cells (6×10^3 and 8×10^3 cells, respectively) were seeded in RPMI-1640 medium supplemented with 5 % fetal calf serum (FCS) in 96-wells culture plates and incubated for 48 hours at 37 °C in humidified atmosphere and CO_2 at 5 %.[12] The viability was assessed by the assays of Trypan blue dye at 0.4 % and MTT.[13,14] The cells in each well were treated during 20 hours with different concentrations (70, 75, 80, 85, 90, 95,100, 105 and 110 µg/mL) of the fractions F_8 and F_{10}, prepared from a stock solution 8 mg/mL in DMSO (the maximum concentration of DMSO —1,2 %— was used as a control). Subsequently, 10 µL of MTT (5 mg/mL, Sigma) was added. The cells were incubated at 37 ºC for 4 hours in the dark and then 100 µL of acid isopropanol (0.1 % fuming HCl, 10 % Triton × 100) was added. Afterwards, the plates were shaken for 4 hours, and absorbance at 570 nm was measured in an spectrophotometer (Multiskan Spectrum, Thermo Scientific). Each experiment was repeated at least two times and

each point was determined in at least six replicates. Viability was calculated using the averages of the experiments through the relationship of the absorbance of the treatments with the corresponding controls. Statistical significance was calculated with Probit test using Statgraphics Centurion XV software.

ANTIPROLIFERATIVE EFFECT ASSESSMENT BY CLONOGENIC ASSAY

Jurkat and CHO-K1 cells (300 and 200 cells, respectively) were seeded in 6-well plates from a culture treated during 20 hours with concentrations of 100, 50 and 25 µg/mL of the F_8 and F_{10} fractions. Mitomycin C (10 µg/mL, Sigma), 1.2 % DMSO and untreated cultures were used as controls. Absolute cloning efficiency (ACE) was calculated by the relation between the number of observed colonies and the number of plated cells and the relative cloning efficiency (RCE) by the relation of the treatment ACE with the corresponding ACE control.[12] Results were analyzed with simple ANOVA, and the Least Significant Difference (LSD) to compare the means between the treatments.

ANTIPROLIFERATIVE EFFECT DETERMINATION BY CELL CYCLE PROGRESSION

Concentrations of 50 and 25 µg/mL of the F_8 fraction and the corresponding controls in cell cultures treated during 20 hours were assessed. In each culture was added Colcemid (1 %) every two hours during 18 or 14 hours for Jurkat and CHO-K1, respectively.[15] Chromosome slides were obtained and the mitotic index was determined for each treatment. Statistical analysis was made by lineal regression using the method reported by Puck and Steffen.[16] Finally, the differences between slopes of the straight line of each treatment and the controls were compared.

PROLIFERATIVE KINETICS AND SISTER CHROMATID EXCHANGE (SCE)

Concentrations of 100 and 50 µg/mL of the F_8 fraction and the corresponding controls were assessed by SCE in both cell lines. Cells were cultivated in the previously described conditions. Then, 5-bromide-2´-deoxyuridine was added during 48 or 36 hours to Jurkat and CHO-K1 cells, respectively. The cells were treated during 20 hours and one hour before harvesting the antimitotic agent Colcemid (0.1 %, Lab G&M) was added. Later, chromosome slides and differential staining were prepared.[16] Afterwards, around thirty metaphases were classified as follows: half cycle, first cycle, first and a half cycle, second cycle, second and a half

cycle, and third cycle. To calculate the generation time was used the relation between the exposure time to BrdU and the proliferative cell number (PCN) according to reported by Wolff and Perry.[17] SCEs in thirty second cycle mitosis in each treatment and their corresponding controls were also quantified. Statistical analysis was performed with simple ANOVA, and the mean difference among treatments with LSD.

RESULTS

Phytochemical march allowed qualitative determination of the main groups of chemical constituents present in *Licania arborea* leaves. Large amount of amino acids, phenolic compounds, triterpenoids and tannins, moderated presence of flavonoids and leucoantocianidines were detected. However, we found a lack of of nafto- and antroquinones, cardiotonics and alkaloids. The high content of phenolic compounds may be interesting and indicates a high chance of finding antioxidant activity in the polar extracts from *L. arborea* leaves.

Preliminary experiments with MTT with a concentration of 200 µg/mL from eleven fractions of *L. arborea*, indicate that four fractions (F_6, F_8, F_9 and F_{10}) showed viability below 50 percent after the treatment in Jurkat and CHO-K1 cell lines (Table 1). Furthermore, IC_{50} determination via MTT, antiproliferative effect by clonogenic assay were assessed for the F_{10} and F_8 fractions due to the cytotoxic activity and the amount available for further experiments.

Table 1. Viability percentages with MTT in Jurkat and CHO-K1 cells treated with 200 µg/mL of F_1, F_2, F_3, F_4, F_5, F_6, F_7, F_8, F_9, F_{10} and FM_1 Fractions. The results are shown as the mean ± the standard deviation (µg/mL) of at least three replicas and two experiments.

Fraction	Jurkat	CHO-K1
F1	92,19 ± 0,03	88,03 ± 0,03
F2	103,87 ± 0,09	93,64 ± 0,04
F3	101,71 ± 0,21	97,34 ± 0,01
F4	77,05 ± 0,04	66,47 ± 0,02
F5	63,63 ± 0,01	81,69 ± 0,07
F6	41,16 ± 0,07	34,53 ± 0,04
F7	78,67 ± 1,13	62,53 ± 2,08
F8	34,72 ± 0,01	36,74 ± 0,03
F9	28,42 ± 0,07	27,97 ± 0,02
F10	26,73 ± 0,04	56,35 ± 0,01
FM1	83,76 ± 0,02	90,62 ± 0,10

Besides, genotoxic and antiproliferative effects for accumulation function and proliferative kinetic by SCE were only assessed for F_8 fraction. A direct proportional relation between dose and viability was observed in both cell lines and it was evident reduction in the cell viability in both cell lines treated with the two fractions with concentrations of 100 to 120 µg/mL (Figure 1). Data showed absence of mitosis in the Jurkat cell line with 100 µg/mL concentration and all the treatments presented significant differences between them (Table 2).

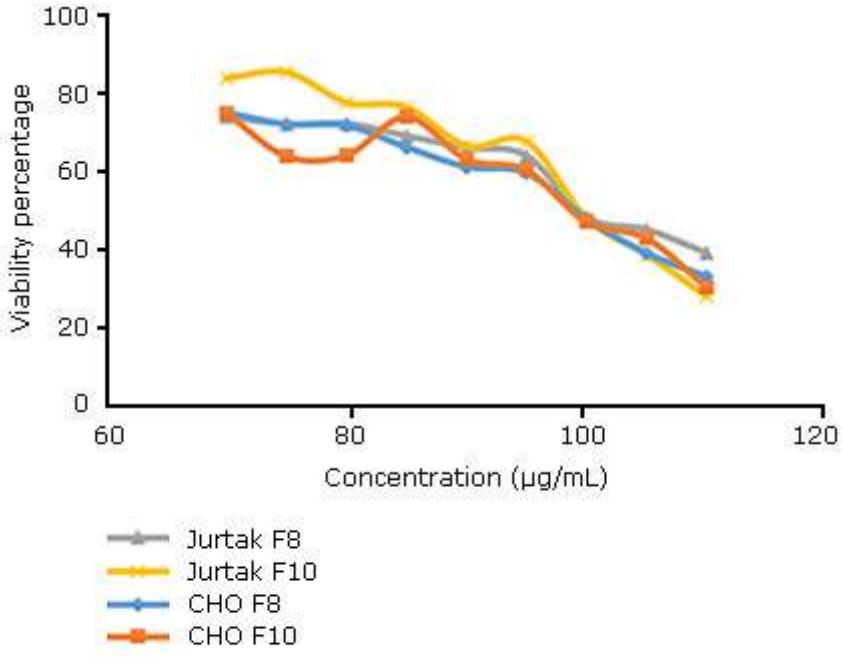

Fig. 1. Viability percentages with MTT in Jurkat and CHO-K1 cells treated with different concentrations (70-110 µg/mL) of F8 and F10 Fractions.

Table 2. Number of cycles and cycle time of Jurkat and CHO cell lines treated with concentrations of 0, 50 and 100 µg/mL of F_8 and DMSO control

Concentration (µg/mL)	Jurkat			CHO		
	Number of cycles	Cycle time	SCE average	Number of cycles	Cycle time	SCE average
0	2.0	24.2	4.8 ± 1.6	2.2	16.4	5.2 ± 0.6
50	2.0	23.1	8.9 ± 2.1	1.8	19.7	6.2 ± 0.5
100	ND*	ND*	ND*	2.1	16.9	6.7 ± 0.6
DMSO (1.2 %)	2.1	22.8	5.1 ± 1.7	1.9	18.8	5.2 ± 0.6

ND*= no determined for mitosis absence.

ACE and RCE were calculated for Jurkat and CHO cell treated with 25, 50 and 100 µg/mL concentrations of F_8 y F_{10} (Table 3) to determine colony generation capability after treatment. Results

show significant differences in the 50 and 100 µg/mL treatments for Jurkat and CHO-K1 cells compared to untreated cells. However, at concentrations of 50 and 100 µg/mL of F_8 fraction showed similar average, while a significant difference in all treatments with F_{10} fraction was observed in Jurkat and CHO-K1 cells. All the tested concentrations in both cell lines showed significant differences compared to untreated cells (Fig. 2).

Table 3. Relative cloning efficiency (RCE) in Jurkat and CHO cell treated with 25, 50 and 100 µg/mL of F8 and F10, where NC stands for negative control

Treatments		Jurkat		CHO-K1	
Fraction	Concentration (µg/mL)	ACE	RCE	ACE	RCE
NC	0	58.3	-	87.3	-
F8	25	59.6	102.3	94.3	108.0
	50	15.2	26.1	50.5	57.8
	100	9.4	16.2	0.8	1.0
F10	25	36.8	63.2	67.0	76.7
	50	26.3	45.2	62.0	71.0
	100	5.9	10.1	28.0	32.1

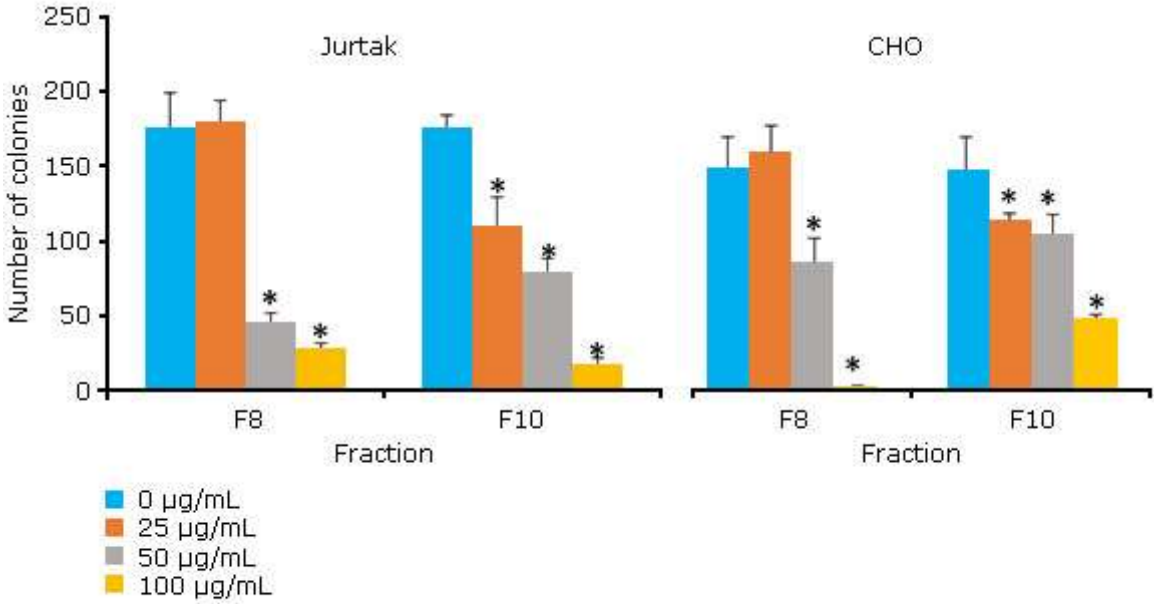

Fig. 2. Number of colonies obtained for the cloning efficiency test for the different treatment (µg/mL) for Jurkat ad CHO-K1 cells. (* indicates significant difference).

Figure 3 shows that both cell lines treated with different concentrations of F_8 behave similarly to the corresponding controls. Slope values close to 0.02 in Jurkat cells and to 0.01 in CHO-K1 cells did not show significant differences. Therefore, delays or reduction of the cell cycle progression with the applied treatments to both cell lines were not evident.

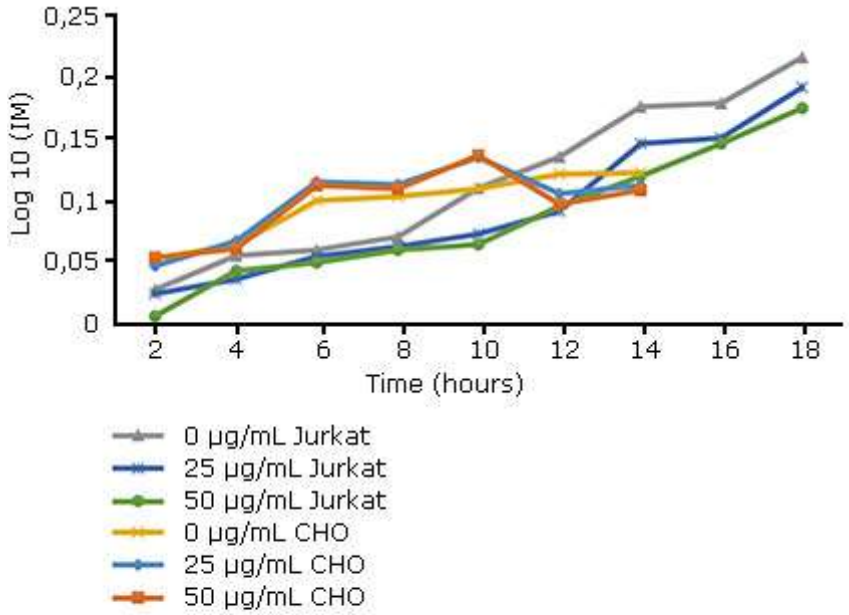

Fig. 3. Mitotic cell accumulation curves vs time, in Jurkat and CHO-K1 cell lines treated with 0, 25 and 50 µg/mL of F_8.

SCE results in number and time cycle in both cell lines do not reveal changes in cells treated compared to the control. CHO-K1 cell line analyzed with conventional ANOVA and LSD multiple range analysis did not show significant difference with 50 and 100 µg/mL of the F_8 fraction. However, there was increment in the SCEs frequency compared with the control. This genotoxic effect was more noticeable with 50 µg/mL concentration in Jurkat cells compared with CHO-K1 cells (Table 2).

DISCUSSION

Results showed a significant cytotoxic effect of the F_8 and F_{10} fractions in Jurkat and CHO-K1 cell lines, being the greater effect observed in the Jurkat cell line. Nevertheless, an approximated IC_{50} of 100 µg/mL of both fractions showed inhibition on the two cell lines. The F_8 and F_{10} fractions reduced the cloning capability in both cell lines, in a dose depending fashion and additionally 100 µg/mL of the F_8 fraction caused total growth inhibition of CHO-K1 cells. These results are similar to others reports on the chloromethanolic fraction (betulinic, pomolic or oleanolic acids and triterpenoids) from leaves of *L. tomentosa* which shows inhibition to the growth in a dose-dependent manner (1, 10, 25, 100 mg/mL), and induced apoptosis in K562, an erythroleukemia cell line.[8] A possible explanation could be the assessment time of the assay used, for instance, short term assays, such as MTT and Trypan blue, assess direct cytotoxic action to mitochondrial and membrane level, respectively, while long term tests, such as

cloning efficiency, SCE, and accumulation function, assess repairing or cell death.

In contrast, antiproliferative potential determined by cell cycle progression analysis did not cause change in the cell cycle time in both cell lines. Therefore, these data are consistent with the SCE proliferative kinetics, which, did not show changes on Jurkat and CHO-K1 cells generation times. However, a greater number of SCE in Jurkat lymphoid line was found, while SCE number was lower for CHO-K1 line, particularly, with 100 µg/mL concentration, mitotic index was 0 % for Jurkat cell line. Besides, SCE results showed dose dependent differential effect between cell lines. These data are similar to other reports, in which it is revealed that fractions and extracts of leaves and fruits of Chrysobalanaceae family plants cause genotoxic effect in the topological conformation of plasmids and in bacteria transformation efficiency with previously treated plasmids.[18]

In conclusion, according to the dichloromethanolic fractions reports of *L. tomentosa*, these results suggest the importance of purification and determination of substances present in F_8 and F_{10} fractions such as phenolic compounds, triterpenoids and tannins, flavonoids and leucoantocianidines that might cause high genotoxicity levels. Further studies are also required to verify bioactivity in other tumor cell lines, as well as to assesss other activities such as, antifungal, antibacterial, and antiviral, among others.

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