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Cytotoxic Activity by a Pentacyclic Triterpenes-Rich Fraction from *Tabebuia hypoleuca* (C. Wright) Urb. on Cancer Cell Lines

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Abstract

Tabebuia genus has traditionally medicinal properties. Recently, we reported the antitumor activity on some tumor human cells from leaves of *Tabebuia hypoleuca*. The aim of this work was evaluated specificity of cytotoxic activity and apoptosis against cancer cell lines, acute toxicity and genotoxicity on micronucleus from clean ethyl acetate crude, triterpenes fraction isolated of *Tabebuia hypoleuca* leaves. *In vitro* cytotoxic activity was evaluated on human cancer cell lines: HT29 (colon), A549 (lung adenocarcinoma), Hep2 (epidermoid carcinoma), SK-MEL 28 (skin malign melanoma); mice cancer cell: F3II (murine breast carcinoma) and VERO (monkey kidney normal cells) using crystal violet. Annexin V and Propidium Iodide were used to determinate the apoptosis and/or necrosis induction from this active fraction on A549, F3II, HT29 and VERO cells. Oral acute toxicity at 500 mg/Kg of this fraction and genotoxicity (micronucleus) were evaluated. Pentacyclic triterpenes fraction had potent cytotoxic activity with all tumor cells used and some selectivity index respect to VERO cells. Anticancer activity were shown by apoptosis induction in the cell lines, with 94% induction in HT29, 89% in F3II, 74% in A549 and 55% in VERO cells, showing selectivity against normal cells. Active fraction was not toxic in acute toxicity and genotoxicity studies. These studies showed the effectiveness and security from pentacyclic triterpenes fraction from *T. hypoleuca* as anticancer pharmaceutical ingredient.

Keywords: *Tabebuia hypoleuca; In Vitro Cytotoxicity; Apoptosis; Pentacyclic Triterpenes; Acute Toxicity, Genotoxicity*

Abbreviations

PHI: Pharmaceutical Ingredient, Pentacyclic Triterpenes Fraction; DMSO: Dimethyl Sulfoxide; PBS: Phosphate Saline Buffer; OA: Oleanolic Acid; UA: Ursolic Acid; BA: Betulinic Acid; U251: Human Glioma Cell Line; 5-FU: 5-Fluorouracil; Panc-28: Human Pancreatic Cancer Cells; VEGF: Vascular Endothelial Growth Factor; IC_{50}: Inhibitory Concentration 50

Introduction

Cancer, also known as malignant tumor or neoplasm, is a broad term used for a large collection of diseases that can affect any organ or tissue of the body. One of the defining features of cancer is the rapid generation of undifferentiated cells that grow outside their natural boundaries, and which can also invade adjacent or distant tissues or organs of the body (metastasis) [1]. Normally, when cells become old or damaged, they undergo programmed cell death, i.e. apoptosis and new cells replace them to fulfill the need of the body. Whereas in cancer, this orderly process is disrupted and as the cells become old or damaged, instead of dying, they survive. These cells can divide into less specialized cells (tumor) and are able to ignore the signals which stop division or by which apoptosis is started in normal cells [2].

The global cancer burden is estimated to have risen to 18.1 million new cases and 9.6 million deaths in 2018. One in 5 men and one in 6 women worldwide develop cancer during their lifetime and one in 8 men and one in 11 women die from the disease. Worldwide, the total number of people who are alive within 5 years of a cancer diagnosis, called the 5-year prevalence, is estimated to be 43.8 million. Europe accounts for 23.4% of the global cancer cases and 20.3% of the cancer deaths, although it has only 9.0% of the global population. The Americas have 13.3% of the global population and account for 21.0% of incidence and 14.4% of mortality worldwide. Cancers of the lung, female breast, and colorectum are the top three cancer types in terms of incidence and are ranked within the top five in terms of mortality (first, fifth, and second, respectively). Together, these three cancer types are responsible for one third of the cancer incidence and mortality burden worldwide [3,4].

Breast cancer is the most common cancer and also the leading cause of cancer mortality in women worldwide [5]. Breast cancer is characterized by a distinct metastatic pattern involving the regional lymph nodes, bone marrow, lung and liver. Metastasis is the result of several sequential steps and represents a highly organized, non-random and organ-selective process. Although a number of molecules have been implicated in the metastasis of breast cancer, the precise mechanisms determining the directional migration and invasion of tumor cells into specific organs remain to be established [6].

Actually, surgery, chemotherapy, radiation, hormones, and immunotherapy are the main approaches for the cancer treatment, often supplemented by other complementary and alternative therapies, such as herbal medicines. Although chemotherapy is the method most used, for example 5-fluorouracil derivatives, cisplatin, mitomycin, adriamycin, taxol, etc. several problems are associated with its use, including limited efficacy, severe toxicity, and multidrug resistance. After the removal of a malignant tumor by surgical operation, radiation therapy and/or adjuvant therapy with cancer chemotherapy drugs may be curative. However, the removal of certain cancers, for example, breast carcinoma, colon carcinoma and osteogenic sarcoma, may be followed by the rapid growth of distant metastases to lung, liver etc. Therefore, it is necessary to develop new anticancer agents with antitumor and antimetastatic activities but without adverse reactions such as gastrointestinal toxicity, myelotoxicity and immune suppression caused by cancer chemotherapeutic drugs [7,8].

Some medicinal plants and foodstuffs have antitumor and antimetastatic activities, but the basis for this hearsay is unclear. To clarify whether natural products have preventive effects on tumors growth and tumor metastasis, it has been using some biochemical and pharmacological approaches. For example, chalcone derivatives such as xanthoangelol and 4-hydroxyderricin isolated from *Angelica keiskei* roots inhibited the tumor growth and liver metastasis in tumor-bearing mice, through the inhibition of tumor-induced neovascularization and the stimulation of immune functions; the pyroglutamate isolated from the Basidiomycete fungus *Agaricus blazei* as an anti-angiogenic substance, and pyroglutamate inhibited the tumor growth and lung metastasis in tumor-bearing mice [9].

Several potential phytochemicals have been reported for the treatment of esophagus cancer such as icariin, luteolin, curcumin, gallic acid, oridonin, sinomenine, quercetin, apigenin, matrine, berberine, artesunate and have shown promising results by enhancing cancer therapeutics through different mechanisms [10]. These authors reported that the anticancer potential of vegetables and fruits is not just because of few vitamins, minerals, but also because of a group of some other therapeutic agents such as vitamin C, E, isoflavones, beta carotene, selenium, folic acid, dithiolthiones, indole-32-carbinol, isothiocyanate, inositol, phytosterols, lycopene, limonene, protease inhibitors, saponins, and lutein.

Tabebuia impetiginosa, *Tabebuia avellanedae*, *Tabebuia heptaphylla* have quinones, flavonoids and glycosides. The anticancer activity of β-lapachone, a quinone compound isolated from there, may be due to down regulation of COX- 2 (cyclooxygenase) and telomerase activities. It compound induces apoptosis in cancer cells via mitochondrial signaling or by activation of caspase and enzymes. The antimetastatic activity of β- lapachone was shown to be due to decreasing the invasive ability of cancer cells by inducing Egr-1, which is known to suppress metastasis [11].

Tabebuia hypoleuca was reported by our group with anti-inflammatory and anticancer activity [12,13]. The anticancer activity in this plant was associated with a pentacyclic triterpenes rich fraction [14].

Aim of the Study

The aim of this work was evaluated specificity of cytotoxic activity and apoptosis against cancer cell lines, acute toxicity and genotoxicity on micronucleus from clean ethyl acetate crude, triterpenes fraction isolated of *Tabebuia hypoleuca* leaves.

Materials and Methods

Chemical and equipment

Hexane, ethyl acetate, dichloromethane and methanol (P.A. grade, Merck).

Colchicine (Sigma) was used as the DNA damaging agent (positive control) in the micronucleus test. Dulbecco's Modified Eagle Medium Ham's (DMEM), Essential Minimum Medium (MEM), fetal bovine serum (FBS), antibiotic-antimycotic (Penicillin-Streptomycin-Amphotericin B), glutamine and Trypsin-EDTA (0.25%) were supplied from Gibco™.

FITC-Annexin V Apoptosis Detection Kit (Biosciences-BD (USA). Crystal Violet Solution (Biopack). Formaldehyde and acetic acid (Ciccarelli). Doxil (Doxorrubicin HCL, Janssen, USA) was used as positive control in cell viability assay.

Guava Nexin Reagent, Guava Multicaspase kit and Guava Mitosox Red were supplied by Millipore (California, USA).

CLARIOstar® Plus Multi-mode Microplate Reader by BMG LABTECH BMG (Germany) was used to determinate the optical density. Attune NxT Flow Cytometer (Acoustic Focusing Cytometer, A24860, Life Technology) was used to flow cytometry assay. Zeiss Axio Observer Microscope (Zeiss, Oberkochen, Germany) was used to viability assay.

Plant material and extraction

T. hypoleuca were collected at the National Botanical Garden (JBN), in June 2017, Havana Province, Cuba. The identification of the plant was confirmed by the botanist Dr. Eldis R. Becquer and a sample was deposited in the herbarium of the experimental station with the number HFC-88204.

The leaves were separated and dried at room temperature by one week and after at 37ºC. It was milled to 40 mesh, then successively extracted using Soxhlet eqquippment with the following solvents: hexane, ethyl acetate and methanol. Each extract were filtered and concentrated using rotary evaporation at 40ºC, providing the crude hexane (1.15% yield), ethyl acetate (2.59% yield) and methanol (4.09% yields).

Ethyl acetate crude was cleaned using charcoal and Celite by filter, then were obtained a grey powder with 15% of yielding, titled (PHI).

Animals

Male and female Sprague- Dawley rats were supplied by the National Center for Laboratory Animal Production (CENPALAB, Santiago de Las Vegas, Havana, Cuba) for acute toxicology. Balb/c mice were suplied by UNICAMP, Brazil for genotoxicty study. The animals were kept under standard conditions of 23 ± 2°C, 40 - 60% relative humidity, and a 12/12h lightdark cycle, and they were given food and water ad libitum for 7 days. All experimental procedures were performed in accordance with the International Guidelines for the Care and Use of Laboratory Animals and approved by the Animal Ethical Committee of the National Center for Animal and Plant Health (CENSA, Havana, Cuba) and Animal Ethical Committe of CPQBA (University of Campinas, Sao Paolo, Brazil).

In vitro cytotoxicity assay

Cell lines

Human tumor cell lines: HT29 (human colorectal adenocarcinoma), A549 (human lung adenocarcinoma), Hep2 (human epidermoid carcinoma), SK-MEL 28 (human skin malign melanoma) and VERO (monkey kidney normal cells, *Cercopithecus aethiops*) were obtained from National Cancer Institute/USA. F3II (murine breast adenocarcinoma) obtain from Prof. Dr. Daniel Alonso [15], Quilmes University, Argentine [16].

Cell culture

The cells A549 and F3II were maintained in DMEM, supplemented with 1X of glutamine, 10% FBS, 1X antibiotic-antimycotic (Penicillin-Streptomycin-Amphotericin B), at 37°C with 5% CO_2 in an incubator. The other one cell line was maintained in MEM supplemented with 1X of glutamine, 5% FBS, 1X antibiotic-antimycotic (Penicillin-Streptomycin-Amphotericin B).

When the cells were confluent, they were routinely subcultured using 0.25% trypsin-EDTA solution. After trypsin detachment, cells were counted, subcultured at 50000 cells per milliliter on 96-well plates (5000 cell/well) and incubated for 24h at 37°C with 5% CO_2 environment to allow for cell attachment.

Cell viability by crystal violet assay

Cells were culture in two 96 well plates, T0 and T1. They were incubated by 24 h, after this time, plate T0 were determined the viability as is explain below.

Cells in T1 plates (100 µL cells well^{-1}) were exposed to clean ethyl acetate crude (grey powder, PHI) concentrations in DMSO (SIGMA)/ DMEM or MEM (0.25, 2.5, 25, and 250 µg mL^{-1}) and the positive control with doxorubicin (0.0125, 0.25, 2.5 and 25 µgmL^{-1}) at 37ºC, 5% of CO_2 for 48h. Final DMSO concentration did not affect cell viability. All samples were tested in triplicate. Controls with phosphate buffer (PBS, pH 7.0) were carried out and did not effect on cell viability (i.e. 100% survival was registered). Control of 0.01% phenol was examined as positive control of cytotoxicity (i.e. 0% viability).

The viability was evaluated by crystal violet, briefly, medium were aspirate and the plates washed with PBS, added 100 µl of formaldehyde 10% in PBS for 15 minutes; then, the formaldehyde was aspirated and the plates were washed twice with PBS; 50 µl of crystal violet were added for 30 minutes, washed three time with PBS and finally it were added 100 µl of acetic acid 10% in PBS. Sample OD (Optical density) was determinate at 595 nm, 5 minutes after in a spectrophotometer. Viability percentage was determinate considering 100% by the control cells not treatment.

Viability % = 100 x OD_{595s}/OD_{595b}

Where: OD_{595s}: Optical density by the sample

$OD_{450b:}$ Optical density by the control cell

If: T > T1 → cell growing stimulate

T1 > T ≥ T0 → cytostatic activity: % C = 100 x [(T-T0)/(T1-T0)]

T < T0 → cytotoxic activity: % C = 100 x [(T-T0)/T0]

T = OD media from treated cells

T0 = OD by the control cells, in T0 plate.

IC_{50}, media inhibitory concentration values for two different experiments were obtained by nonlinear regression using Graphpad Prism version 5.0 for Windows (Graph-Pad Software, San Diego, California, USA).

Viability % data are expressed as mean ± standard deviation (SD) of three independent experiments with duplicate samples. Statistical analyses were performed with Graphpad Prism version 5.0, Two way ANOVA following.

The morphological changes of cells lines exposed to PHI were observed using a Zeiss Axio Observer Microscope.

Selective index

To determine the specificity of cytotoxic activity against cancer cell lines, it was necessary to obtain information of cytotoxicity against normal cells. Therefore, the sub-fractions were tested for cytotoxicity in the VERO cell line. The selective index (SI) was calculated using the following equation:

$$SI = \frac{IC_{50}\ \text{VERO cells}}{IC_{50}\ \text{cancer cells}}$$

If the valor is > 1, indicate that the samples are more cytotoxic for the tumor cells than for the normal cells, if is < 1, it is the reverse [17].

Cell death analysis by flow cytometry assay

HT29, A549, VERO and F3II cells lines were seeded at 30000 cells/well in 24 wells microplate and incubated for 24h, followed by treatment with clean ethyl acetate crude (PHI) at various concentrations (2.5, 10 and 25 µg/ml). All samples were tested in triplicate. Controls were carried out: positive cell death control in which 5% H_2O_2 was added to culture medium and basal control in which cells were cultured in pure culture medium. After 48h incubation, medium and floaters from each well were collected and cells were rinsed with PBS and incubated with trypsin/EDTA 0.25% until the cells were detached. All cell suspensions were collected and centrifuged at 800g for 10 minutes. Following centrifugation, the supernatant was removed and pellet was washed with ice-cold PBS and centrifuged at 800g for 10 minutes. The supernatant was discarded.

FITC-Annexin V Apoptosis Detection Kit was used: 100 µL of binding buffer, containing 1 µL of Annexin V-FITC and 0.5 µL of propidium iodide (PI), was added to each sample. It was incubated over 15 minutes in dark, and samples were analyzed in an Attune NxT Flow Cytometer. The % of viable cells, the % of cells in early apoptosis, the % of cells in late apoptosis and the % of cells in necrosis were determined with FlowJo software (TreeStar Inc., Ashland, USA). Total apoptosis (%), as the sum of % of cells in early and late apoptosis, and an apoptotic index were calculated using the following equation:

$$\text{Apoptotic index} = \frac{\%\ \text{total apoptosis for treatment}}{\%\ \text{basal apoptosis}}$$

Statistical analysis

Data are expressed as mean ± standard deviation (SD) of three independent experiments with duplicate samples. Statistical analyses were performed with Infostat 2016 [18], followed by Kruskal-wallis test was used, and p-values less than 0.05 were regarded as significant. It was used Two way ANOVA following by Bonferroni posttests and p-value less than 0.001 were regarded as high significant, for Apoptosis index.

Toxicological study

Acute toxicity

Sprade-Dawley rats were used in this study, females rats weighing 225 ± 25g and males 335 ± 25g. Six animals were employed by group (3 females and 3 males). They were manteined in experimental conditions described by OECD, 2002.

Rats were treated by gavage with clean ethyl acetate crude from leaves, PHI (500 mg/kg) as a treament group and other one as control group (vehicle). Groups were observed during 4h and then daily for 14 days. The following parameters were evaluated: general toxicity signals like body weight loss, locomotion, behavior (agitation, lethargy), respiration, salivation, tearing eyes, cyanosis, and mortality [19,20].

Body weight and relative weight of fundamental organs were determinated to ending the study and macrsocopic anatomopathology to animals.

Genotoxicity (Micronucleous)

Balb C female mice, weithing 22 ± 1g. Mice were keep in conventional experimental conditions. It were used five mice by groups: treatment group with 150 mg/Kg clean ethyl acetate crude, treatment group with 50 mg/Kg of the major compound (PHI) isolated from clean ethyl acetate crude, negative group (vehicle: tween 80 + saline) and positive control with ciclofosfamida 50 mg/Kg bw. It were made three intraperitonial administrations for 3 consecutive days. Animals were sacrifed by eutanasia and samples of bone marrow were taken from the femurs and prepared and stained using established methods (Giemnsa). At least 2000 immature erythrocytes per animal are scored for the incidence of micronucleated immature erythrocytes. It were observed to microspcopy micronucleous (MN) and micronucleated polychromatic erythrocytes (MNPCE) [21].

Statistical analysis

Anova and Newman - Keuls multiple comparison test was use by statitical analysis. P value < 0.05 was considered statistically significant. The statistical tests were carried out using GraphPad Prism, version 6.0 (GraphPad Software, San Diego, CA, USA).

Results and Discussion

Adherent cells detach from cell culture plates during cell death and this characteristic can be used for the indirect quantification of cell death and to determine differences in proliferation upon stimulation with death-inducing agents. One simple method to detect maintained adherence of cells is the staining of attached cells with crystal violet dye, which binds to proteins and DNA. Cells that undergo cell death lose their adherence and are subsequently lost from the population of cells, reducing the amount of crystal violet staining in a culture. Crystal violet staining is a quick and versatile assay for screening cell viability under diverse stimulation conditions. That is suitable for the examination of the impact of chemotherapeutics or other compounds on cell survival and growth inhibition [22,23].

In vitro cytotoxic evaluation using crystal violet assay of clean ethyl acetate crude, PHI on tumor cells and non-tumor cell is described in the table 1. PHI shown a potent activity against all studied cells, according to National Cancer Institute: IC_{50} < 30 µg/mL over tumor cells were positive (IC_{50} ≤ 30 µg mL^{-1}, potent activity) [24]. PHI had the minor IC_{50} valour on lung adenocarcinoma (A549), IC_{50} = 6.83 µm/mL and, following by it strong effect on murine breast carcinoma (F3II) (IC50= 7.0 µg/mL) with a good selectivity index (SI= 1.18)and, a good cytotoxic effect on colon tumor cells (HT29) (IC50= 8.076 µg/mL, SI= 1.03) the best selectivity index (SI = 1.21). In the case of Hep-2 and SK-MEL 28, PHI had a potent cytotoxic activity but not a good selectivity index. This strong *in vitro* antiproliferative activity prompted the study of antitumor activity in murine models.

Sub-fraction	IC_{50} µg/mL (SI)					
	A549 (Lung adenocarcinoma) (SI)	Hep-2(Laryngeal carcinoma)	HT29 (Colon)	F3II (Murine breast carcinoma)	SK-MEL 28 (skin malign melanoma)	VERO (monkey kidney normal cells
PHI	6.83 (1.21)	8.44 (0.98)	8.076 (1.03)	7.0 (1.18)	9.797 (0.84)	8.297
Doxorubicin	0.968 (1.19)	4.060 (0.28)	0.620 (1.86)	0.663 (1.74)	> 250	1.156

Table 1: *Cytotoxic activity against tumor and normal cells (IC_{50}). (SI): Selectivity Index.*

Figure 1 represents viability percentage from PHI and doxorubicin as positive control. PHI shown cytotoxic effect, however doxorubicin had cytostatic effect. The cytotoxic and cytostatic effect were doses- dependent. However, PHI there was high significant by the major concentration (25 µM) respect to positive control with doxorubicin, $p < 0.001$.

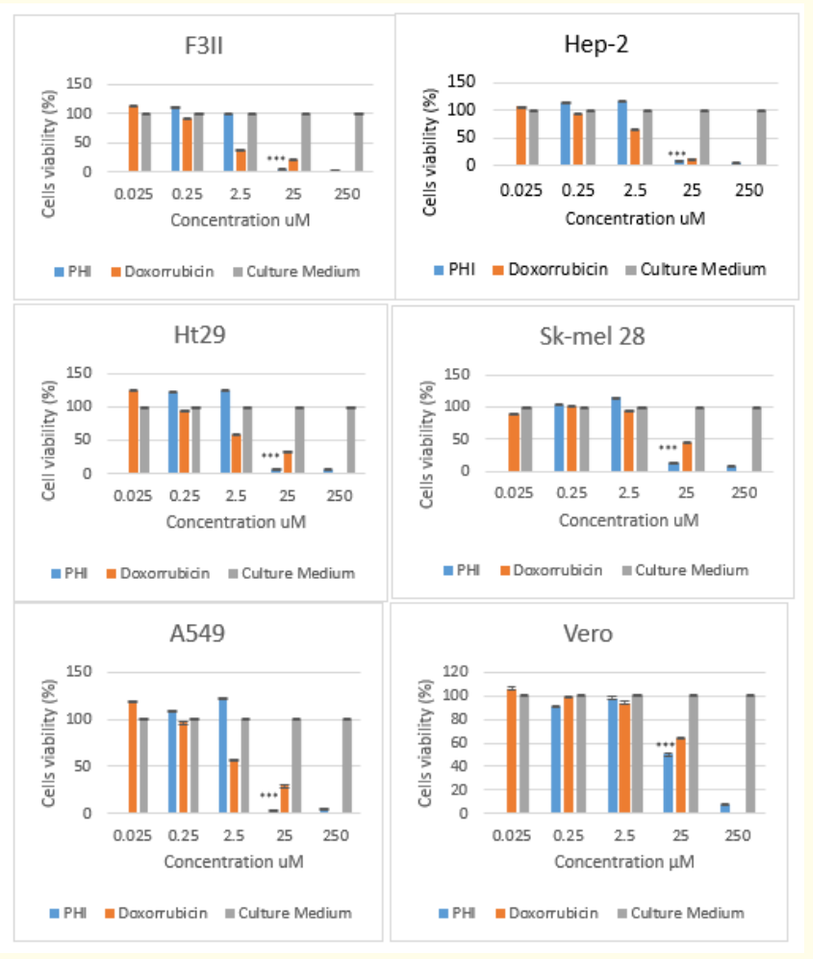

Figure : *Graphs showing viability percentage obtained by adding different concentrations of clean ethyl acetate crude, PHI and Doxorubicin on tumor and non- tumor cells (n = 3 ***p <0.001).*

In figure 2 we shown the morphology of the cells treated with the major doses, 250 µg/ml of PHI, evaluated by microscopy, where are possible to see morphological change in the cells. In figure 2A we shown the morphology of cells treated with the major doses (250 µg/ml of PHI and 25 µg/ml of Doxorubicin) evaluated by microscopy. In positive viability control, cells lines were firmly adhered to the wall. After 48h of the treatment, cells showed completely altered morphology as varying degrees of cell shrinkage, rough edges, rounded cells, smaller size and loss of connectivity, characteristics of cell death mechanism. Positive control of cytotoxicity (Doxorubicin) showed the same morphological alterations as PHI treatment.

Cell death analysis

Flow cytometry (Annexin V/Propidium iodide)

To examine whether the cytotoxic effects of PHI were due to the induction of cells death (apoptosis and/or necrosis), cells were treated with PHI and apoptosis and or necrosis was determined by Annexin V/PI and flow cytometry. The results of the present study demonstrated that PHI induced A549, HT29; F3II and Vero cells apoptosis in a dose-dependent manner. The number of apoptotic cells was determined (Figure 3). The percentage of apoptotic cells increased from 4.30% in the control to 93.77% in the 25 µM PHI-treated group on HT29; 14.63% to 88.83% on F3II cells; 11.39 to 74.34 on A549 and 22.25% to 55.03% on Vero cells. In this result was demonstrated the selectivity of PHI for tumor cells respect to normal cells (Vero).

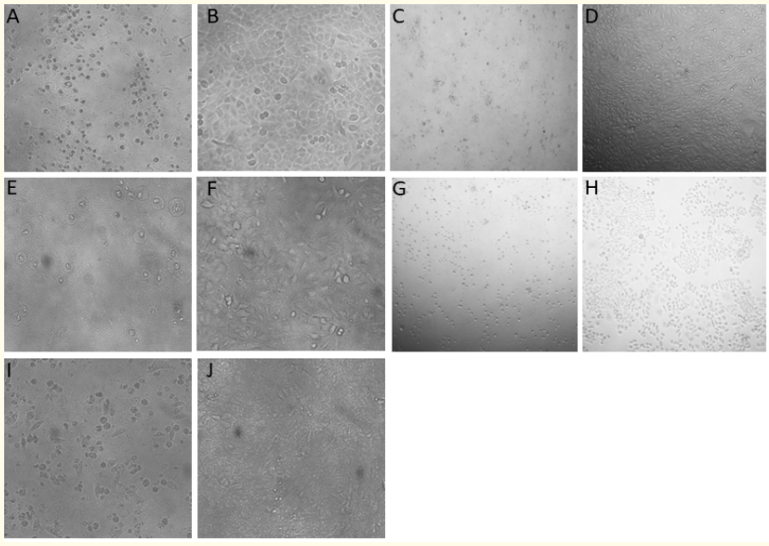

Figure 2: *Morphological changes in cells lines observed under Zeiss Axio Observer Microscope. Effect of PHI at dose of 250 µg/mL on (A) A549, (B) A549, positive viability control; (C) F3II, (D) F3II, positive viability control; (E) Hep2, (F) Hep2, positive viability control; (G) HT29, (H) HT29, positive viability control; (I) Vero, (J) Vero, positive viability control.*

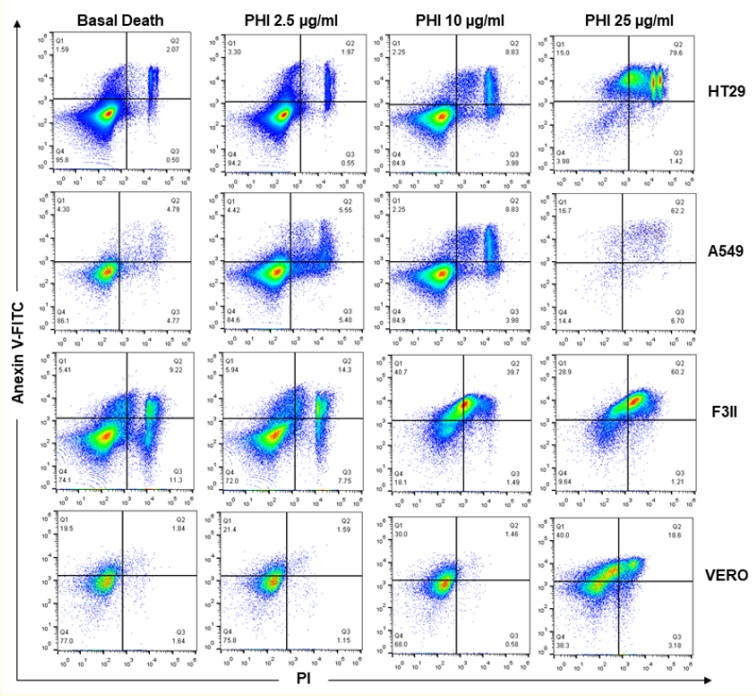

Figure 3: *Representative dot plots showing cell death by apoptosis and/or necrosis using Annexin V-FITC and propidium iodide (PI) stain and evaluated by flow cytometry. The dot plots shows Basal Death and cell death induced by PHI (2.5 µg/ml), PHI (10 µg/ml) and PHI (25 µg/ml) in HT29, A549, F3II and VERO cell lines. In each image Q1 represents early apoptotic cells (Annexin V-FITC +/IP-), Q2 late apoptotic cells (Annexin V-FITC +/IP +), Q3 necrotic cells (Annexin V-FITC-/IP +) and Q4 live cells (Annex V- FITC-/IP-).*

Table 2-5 represent the percentage of viable cells, cells in early apoptosis, cells in late apoptosis, cells in necrosis and total apoptosis. In general, the answer depended to the doses. In all cases, necrosis was low respect to apoptosis. In the case of HT29 cells the necrosis was not different to basal control. This fact would suggest that PHI did not cause cell death by necrosis, which it is considered an undesired mechanism due to cellular material extravasation and inflammation of surrounding tissue [25].

In the treatment group with PHI to major doses (25 ug/ml) on A549 cells, early apoptosis cells were not significant respect to basal group and late apoptosis cells were significant respect to basal ((60.83 ± 10.66, p < 0, 05). However, apoptosis cells percentage increase to 74.34 ± 12.62% though it was not significant (Table 2).

Sample	Doses (ug/mL)	Living cells Q4	Early apoptosis Q1	Late apoptosis Q2	Necrosis Q3	% cell apoptosis
Basal		83.8 ± 0.93 A	4.58 ± 0.93 AB	6.81 ± 1.11 A	4,8 ± 0.77 A	11.39 ± 2.02 A
PHI	2.5	80.8 ± 2.84 A	5.9 ± 1.08 AB	6.15 ± 0.99 A	6,16 ± 1.07 A	12.05 ± 1.88 A
	10	82.6 ± 1,67 A	2.35 ± 0.42 A	9.64 ± 0.42AB	5,44 ± 1.06 A	11.98 ± 0.79 A
	25	15.8 ± 6.8 A	13.47 ± 2.78 B	60.87 ± 10.66 B	9,8 ± 5.93 A	74.34 ± 12.62 A

Table 2: *Percentage of viable cells, cells in early apoptosis, cells in late apoptosis, cells in necrosis and percentage of total cell apoptosis for PHI on A549. Values are shown as mean ± standard deviation and different letters indicate statistically significant differences (p < 0.05).*

Table 3 represent the result on HT29 tumor cells. In this case, living cells decrease with the doses with statistical significant at 25 ug/ml respect to basal group. Early apoptosis cells increase with PHI doses, with the major valor to PHI, 25 ug/ml. It were shown statistical significant respect to basal at 10 ug/ml (3.56 ± 0.18, PHI to 1.70 ± 0.12, basal) and 13.43 ± 1.11, PHI to 1.70 ± 0.12, basal at 25 ug/ml. It was shown statistical significant respect to basal (13.43 ± 1.11, PHI to 1.70 ± 0.12, basal). Late apoptosis increase with the doses in PHI treatment group. Cell apoptosis percentage was 93.77 ± 2.02 in the group treated with PHI, 25 ug/ml, with statistical significant to basal group. There were not necrosis cells.

Sample	Doses (ug/mL)	Living cells Q4	Early apoptosis Q1	Late apoptosis Q2	Necrosis Q3	% cell apoptosis
Basal		93.9 ± 1.30 A	1.70 ± 0.12 A	2.59 ± 0.52 AB	1.69 ± 0.90 A	4.30 ± 0.55 A
PHI	2.5	94.4 ± 0.14 A	3.03 ± 0.35 AB	1.86 ± 0.16 A	0.7 ± 0.18 A	4.89 ± 0.31 A
	10	91.7 ± 1.28 AB	3.56 ± 0.18 BC	3.29 ± 0.69 AB	1.41 ± 0.63 A	6.85 ± 0.81 AB
	25	4.57 ± 1.92B	13.43 ± 1.11 C	81.4 ± 2.11 B	1.66 ± 0.22 A	93.77 ± 2.09 B

Table 3: *Percentage of viable cells, cells in early apoptosis, cells in late apoptosis, cells in necrosis and percentage of total cell apoptosis for PHI on HT29. Values are shown as mean ± standard deviation and different letters indicate statistically significant differences (p < 0.05).*

Respect to F3II cells, living cells decrease in doses- dependence. It was statistical significant respect to basal group (with 10 and 25 ug/ml of PHI). Late apoptosis was significant for the major doses (25 ug/ml of PHI). Necrose decrease with the doses in treatment group (Table 4).

Table 5 show the results of PHI treatment groups respect to basal group on normal cells (VERO). Early apoptosis increase with the doses in treatment group, statistical significant by PHI, 25 ug/ml. Later apoptosis were not significant and there were not necrosis. Cell apoptosis percentage was significant by PHI, 25 ug/ml respect to basal, but this effect was less than the observed with tumor cells. These results show the selectivity from PHI against normal cells.

Sample	Doses (ug/mL)	Living cells Q4	Early apoptosis Q1	Late apoptosis Q2	Necrosis Q3	% cell apoptosis
Basal		73.1 ± 2.57 A	4.18 ± 0.43 A	10.45 ± 0.91A	12.23 ± 1.25 A	14.63 ± 1.34 A
PHI	2.5	69.3 ± 2.11 A	4.81 ± 0.27 AB	15.93 ± 0.79 AB	9.95 ± 1.14 AB	20.74 ± 1.04 AB
	10	17.9 ± 2.75 AB	19.57 ± 4.57 B	59.1 ± 5.11 BC	3.43 ± 1.88 BC	78.67 ± 1.76 BC
	25	9.5 ± 0.65 B	20.77 ± 1.05 B	68.07 ± 1.64 C	1.68 ± 0.1 C	88.83 ± 0.68 C

Table 4: *Percentage of viable cells, cells in early apoptosis, cells in late apoptosis, cells in necrosis and percentage of total cell apoptosis for PHI on F3II. Values are shown as mean ± standard deviation and different letters indicate statistically significant differences ($p < 0.05$).*

Sample	Doses (ug/mL)	Living cells Q4	Early apoptosis Q1	Late apoptosis Q2	Necrosis Q3	% cell apoptosis
Basal		76.3 ± 1.42 A	20.47 ± 1.44 A	1.78 ± 0.06 A	1.40 ± 0.23 A	22.25 ± 1.38 A
PHI	2.5	75.07 ± 3.06 A	22.47 ± 2.96 A	1.47 ± 0.19 A	0.99 ± 0.11 A	23.94 ± 3.1 A
	10	66.7 ± 3.82 AB	30.97 ± 3.46 AB	1.63 ± 0.17 A	0.66 ± 0.21 A	32.59 ± 3.60 AB
	25	42.2 ± 3.50 B	39.4 ± 0.45 B	15.67 ± 2.74 A	2.78 ± 0.44 A	55.03 ± 3.12 B

Table 5: *Percentage of viable cells, cells in early apoptosis, cells in late apoptosis, cells in necrosis and percentage of total cell apoptosis for PHI on VERO. Values are shown as mean ± standard deviation and different letters indicate statistically significant differences ($p < 0.05$).*

Figure 4 show apoptosis index, with the major activity against HT29 cells with high significant by 25 ug/ml (p less than 0.001). In the case of F3II tumor cells was observed a major activity by 10 ug/ml thought it was not significant.

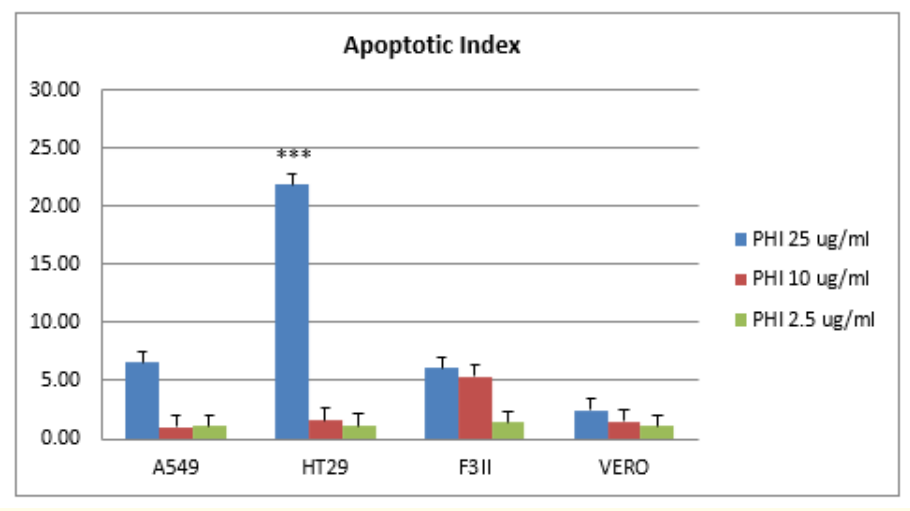

Figure 4: *Apoptosis index of PHI against cell lines: lung A549, colorectal HT29, mice breath F3II, normal VERO.*

In our preview report, we found the presence of three pentacyclic triterpenes in PHI: oleanolic acid, ursolic acid and betulinic acid and it apoptosis effect on U-251 (glioma tumor cells) [14].

In the present work, we separate the active fraction or PHI from ethyl acetate crude extract and we evaluate its cytotoxicity and apoptosis activities against colon, mammalian, lung, laryngeal, skin malign tumor cells and against normal cells (Vero). Their apoptosis action mechanism as anticancer effect was promissory and PHI shown selectivity compared with normal cells.

Pharmacological studies revealed that OA has several biological activities, which determine its therapeutic potential. The triterpenoid has been reported to show an anti-inflammatory, antitumor, hepatoprotective, antidiabetic or antibacterial properties ant it compounds has low toxicity [26]. This author mention at oleanolic acid as a multi-targeted agent and with low toxicity against normal cells that makes it a good candidate for further use as a starting point for a new drug design. It was reported that OA as cytotoxic activity against a number of tumor cell lines including lung, ovarian, brain, colon, breast cancer cells as well as melanoma cells [27-29]. Sasikumar., *et al*. [30] reported the antiproliferative efficacy of oleanolic acid isolated from *Vitis vinifera* against human colon adenocarcinoma HCT-116 cells was assessed using cell viability assay. The minimum inhibitory concentration (IC_{50}) was determined and found to be 40 µg/mL at 48h incubation.

A few different mechanisms of oleanolic acid antitumor activity were described. One of them is cytotoxicity related to the induction of apoptosis. This physiological mechanism protects from uncontrolled process of transformed cells proliferation. Apoptotic death induction became a new target for innovative drug discovery [31]. Induction of tumor cells apoptosis was recognized as one of the most effective methods in anticancer treatment [32]. A detailed study into the cytotoxic/proapoptotic activity of oleanolic acid [33-38] yielded information about its intracellular molecular targets.

The combination of OA and 5-FU was able to increase the growth inhibition of Panc-28 cells and the induction of apoptosis via lysosomal-mediated leakage of cathepsin D. Some apoptotic proteins such as caspase-3, NF-κB, and Bcl-2 play important roles in the apoptotic pathway induced by the combination regimen [36].

Ursolic acid inhibits cell growth via causing apoptosis in U251 cells by a UA triggered TGF-b1/miR-21/PDCD4 pathway [39]. Numerous studies suggest that UA regulates mitochondrial function through the activation of multiple pathways. UA regulates the expression of associated metabolic enzymes, decreases tumor proliferation, promotes ROS production and accumulation in mitochondria under stress conditions, destabilizes mitochondrial membrane potential, activates the p53 pathway and promotes apoptosis in various types of cancer cells. Mitochondria are not only the major center of cell respiration and oxidative phosphorylation, but also the control center of apoptosis. Apoptosis is regulated by two major mechanisms [40]. The apoptosis mechanism which showed UA to have the ability to prevent Nuclear Factor B (NF-B) pathway by p65 phosphorylation suppression, resulting in mandatory decrease in various downstream oncogenes such as B-cell lymphoma-extra-large (Bcl-XL) and B-cell lymphoma-2 (Bcl2) [41].

OA and UA induce of apoptosis in human hepatocellular carcinoma HuH7 cells, inhibit the growth of HuH7 cells with IC_{50} values of 100 and 75 µM, respectively. Cell cycle analysis using flow cytometry indicated that the fraction of HuH7 cells in sub-G1 phase progressively increased with increasing concentrations of OA or UA from 20 to 80 µM. Treatment with OA and UA for 8h induced a dramatic loss of the mitochondria membrane potential and interfered with the ratio of expression levels of pro- and antiapoptotic Bcl-2 family members in HuH7 cells [33]. Apoptotic effects of oleanolic acid (OA) and ursolic acid (UA) on human liver cancer HepG2, Hep3B, Huh7 and HA22T cell lines were reported and concentration-dependently diminished Na+-K+-ATPase activity and VEGF level [34].

Betulinic acid (BA) has apoptosis effect. For example, it were demonstrated induction of cell cycle arrest and apoptosis by betulinic acid-rich fraction from *Dillenia suffruticosa* root in MCF-7cells involvedp53/p21 and mitochondrial signaling pathway [42,43].

Acute toxicity

In this study any toxic effect was not shown in both sex. There were satisfactory increasing in the body weight and body gain from the rats in both group, treatment and control groups. Any macroscopic pathologic sign were observed. The relative weigth of the fundamental organs (liver, heart, spleen, kidneys and ovaries) were not significant diference respect to control group (Figure 5). These results are important to proceed at *in vivo* evaluation on experimental animals.

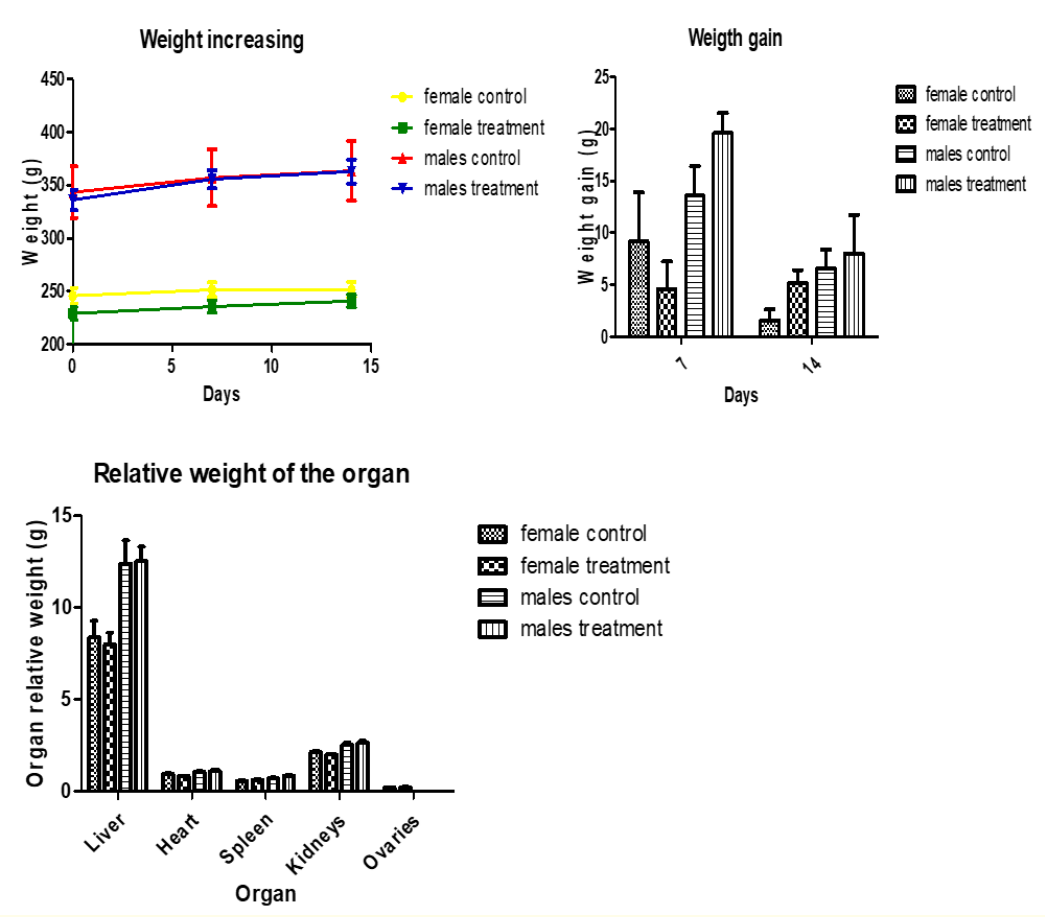

Figure 5: *Body weight, weight gain and relative weigth of the organs in acute toxicity study. Values are expressed by median ± SEM (n = 3).*

Cornejo., *et al*. [44] reported that the mixture UA/OA is indeed safe when administered subcutaneously as a single dose of 300 mg/kg or in repeated doses of 13 mg/kg during 28 days. During the subacute administration, there was no death of animals and no changes were observed in the growth or weight of the different organs when compared to the control groups. Studies of blood chemistry and blood count showed normal levels in all parameters evaluated. The histopathology of major organs showed no changes or abnormalities.

Genotoxicity (Micronucleous)

No difference were found with the treatment of clean ethyl acetate crude and with the major compounds isolated from this crude respect to control group on micronucleated polychromatic erythrocytes (MNPCE) and normochromatic erythrocytes (NCE) (Figure 6). For also, ethyl acetate-clean crude neither the compound isolated from it had not genotoxicolgical effect.

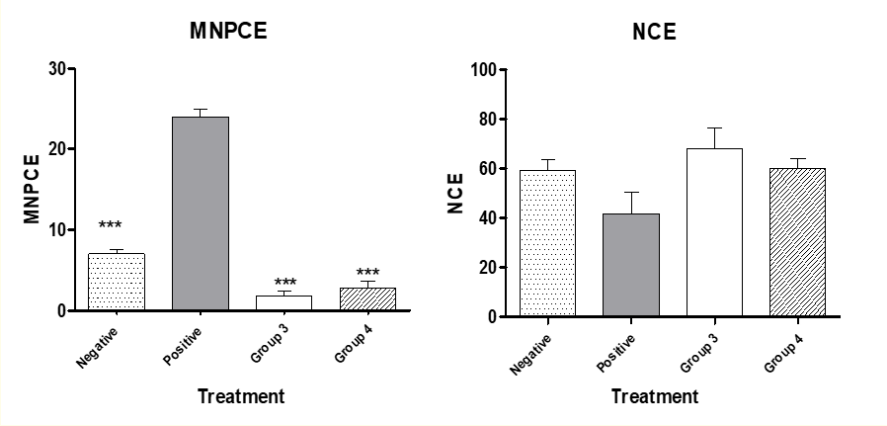

Figure 6: *Number of micronucleated polychromatic erythrocytes (MNPCE) observed in the bone marrow cells of female Balb/C mice treated with negative control (vehicle), positive control (cyclophosphamide, 50 mg/Kg), a clean ethyl acetate crude from Tabebuia hypoleuca (150 mg/Kg, group 3) and the major compound in this crude, PHI (50 mg/Kg, group 4) (i.p.) ($p < 0.05$, $n = 5$).*

These finding are request in the preclinical evaluation of new drugs and to follow the evaluation *in vivo* with experimental animals and to development a new drug to the human use.

Aparecida., *et al*. [45] reported not genotoxicity from UA and OA. They found no significant difference in the frequencies of MNPCEs between animals treated with UA and OA alone or in combination and the negative or solvent control, demonstrating the absence of genotoxic effects of these agent doses used. This study was carried out in peripheral blood and bone marrowof Balb/c mice micronucleous test, using in single doses of 80 mg/Kg b.w, p.o of UA, OA or a mixture of both tritepernes. UA and OA reduced DXR clastogenicity in mouse peripheral blood and bone marrow cells under the experimental conditions used in the present study. These results support the indication of UA and OA as promising candidates in the prevention of various types of cancer and other diseases. Further investigations exploiting anticlastogenesis mechanisms of these triterpenoid compounds should be conducted before their use as chemopreventive agents in humans.

Conclusion

Therefore, this study showed that the active fraction with different pentacyclic triterpenes in *T. hypoleuca* have promising anticancer activity against colon, lung, mama, laryngeal and skin tumor cells with selectivity against normal cell and security by it not toxic or genotoxic effect. We recommend further the study of this fraction by the treatment of cancer and formulations development with this active ingredient.

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Conflict of Interest

Any conflict of interest.

Bibliography

1. American Cancer Society. "Cancer Facts and Figures 2019". Atlanta: American Cancer Society (2019).
2. Kumar V., *et al*. "Robbins Basic Pathology". 9th edition. Philadelphia, PA: Saunders. Neoplasm (2013): 161-214.
3. IARC, WHO International Agency for Research on Cancer and World Health Organization. "Latest global cancer data: Cancer burden rises to 18.1 million new cases and 9.6 million cancer deaths in 2018" (2018): 263.
4. Siegel RL., *et al*. "Cancer statistics, 2019". *CA: A Cancer Journal for Clinicians* 69.1 (2019): 7-34.
5. Shah R., *et al*. "Pathogenesis, prevention, diagnosis and treatment of breast cancer". *World Journal of Clinical Oncology* 5.3 (2014): 283-298.
6. Müller A., *et al*. "Involvement of chemokine receptors in breast cancer metastasis". *Nature* 410 (2001): 50-56.
7. Kimura Y. "New Anticancer Agents: *In Vitro* and *In Vivo* Evaluation of the Antitumor and Antimetastatic Actions of Various Compounds Isolated from Medicinal Plants". *Review in vivo* 19 (2005): 37-60.
8. De Sousa L., *et al*. "Medicinal Plants and Other Living Organisms with Antitumor Potential against Lung Cancer". *Evidence-Based Complementary and Alternative Medicine* (2014): 15.
9. Kimura K. "Antitumor and antimetastatic actions of various natural products". *Studies in Natural Products Chemistry* 34 (2008): 35-76.
10. Abbasi BA., *et al*. "Potential phytochemicals in the prevention and treatment of esophagus cancer: A green therapeutic approach". *Pharmacological Reports* 71 (2019): 644-652.
11. Cassileth BR., *et al*. "Herb-drug Interactions in Oncology". Second Edition. Printed in China by the Peoples´s Medical Publishing House (2010).
12. Regalado AI., *et al*. "Actividad anti-inflamatoria de los extractos metanólicos de hojas y de tallos de *Tabebuia hypoleuca* (C. Wright) Urb". *Journal of Pharmacy Pharmacognosy Research* 3 (2015): 109-117.
13. Sánchez Perera LM., *et al*. "*In vitro* and *In vivo* antiproliferative activity of extracts and fractions of leaves and stem from *Tabebuia hypoleuca* (C. Wright) Urb". *Drug Discovery* 13 (2019): 10- 21.
14. Perera LM., *et al*. "Induction of cell cycle arrest and apoptosis by a pentacyclic triterpenes -rich fraction from *Tabebuia hypoleuca* (C. Wright) Urb. leaves in u-251 glioblastoma cells". *Bio Nature* 39.2 (2019): 70-87.
15. Alonso D., *et al*. "Characterization of F311, a Sarcomatoid Mammary Carcinoma Cell Line Originated From a Clonal Subpopulation of a Mouse Adenocarcinoma". *Journal of Surgical Oncology* 62 (1996): 288-297.

16. Rojas L. "Evaluación de la actividad citotóxica de los extractos y fracciones de pulpa y semillas de Annona muricata sobre líneas celulares tumorales y normales". [Thesis de pregrado]. Bogotá: Universidad Distrital Francisco José de Caldas (2015): 76.

17. Di Rienzo JA., *et al*. "InfoStat version". Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina (2016).

18. Lapa AJ., *et al*. "Métodos de avaliação da atividade farmacológica de plantas medicinais". Porto Alegre: Metrópole (2003).

19. Litchfield JT and Wilcoxson F. "A simplified method of evaluating dose effect experiments". *Journal of Pharmacology and Experimental Therapeutics* 194.996 (1949): 99-113.

20. Oecd 474. "Oecd Guideline For The Testing Of Chemicals". Mammalian Erythrocyte Micronucleus Test (1997).

21. Geserick P., *et al*. "Cellular IAPs inhibit a cryptic CD95-induced cell death by limiting RIP1 kinase recruitment". *Journal of Cell Biology* 187 (2009): 1037-1054.

22. Feoktistova Maria., *et al*. "Crystal Violet Assay for Determining Viability of Cultured Cells". *Cold Spring Harbor Protocols* (2016).

23. Oskoueian E., *et al*. "Antioxidant, anti-inflammatory and anticancer activities of methanolic extracts from *Jatropha curcas* Linn". *Journal of Medicinal Plants Research* 5 (2011): 49-57.

24. Kumar V., *et al*. "Respuestas celulares ante el estrés y las agresiones por tóxicos: adaptación, lesión y muerte in Robbins y Cotran". Patología estructural y funcional - Octava Edición (2010): 3-42.

25. Paszel-Jaworska A., *et al*. "Molecular Mechanisms of Biological Activity of Oleanolic Acid - A Source of Inspiration for A New Drugs Design". *Mini-Reviews in Organic Chemistry* 11 (2014): 330-342.

26. Jung HJ., *et al*. "Structure activity relationship of oleanane disaccharides isolated from Akebia quinata versus cytotoxicity against cancer cells and NO inhibition". *Biological and Pharmaceutical Bulletin* 27.5 (2004): 744-747.

27. Setzer WN and Setzer MC. "Plant-derived triterpenoids as potential antineoplastic agents". *Mini-Reviews in Medicinal Chemistry* 3.6 (2003): 540-556.

28. Hsu HF., *et al*. "Antioxidant activity, and DNA information of *Glossogyne tenuifolia*". *Journal of Agricultural and Food Chemistry* 53 (2005): 6117-6125.

29. Sasikumar K., *et al*. "Oleanolic acid from black raisins, Vitis vinifera with antioxidant and antiproliferative potentials on HCT 116 colon cancer cell line". *Brazilian Journal of Pharmaceutical Sciences* 56 (2020): e17158.

30. Senderowicz AM. "Targeting cell cycle and apoptosis for the treatment of human malignancies". *Current Opinion in Cell Biology* 16.6 (2004): 670-678.

31. Huang T., *et al*. "Induction of apoptosis by a combination of paclitaxel and carboplatin in the presence of hyperthermia". *Asian Pacific Journal of Cancer Prevention* 13.1 (2012): 81-85.

32. Shyu MH., *et al*. "Oleanolic acid and ursolic acid induce apoptosis in HuH7 human hepatocellular carcinoma cells through a mitochondrial-dependent pathway and downregulation of XIAP". *Journal of Agricultural and Food Chemistry* 58.10 (2010): 6110-6118.

33. Yan SL., *et al*. "Oleanolic acid and ursolic acid induce apoptosis in four human liver cancer cell lines". *Toxicology in Vitro* 24.3 (2010): 842-848.

34. Wei J., *et al*. "Oleanolic acid arrests cell cycle and induces apoptosis via ROS-mediated mitochondrial depolarization and lysosomal membrane permeabilization in human pancreatic cancer cells". *Journal of Applied Toxicology* 33.8 (2013): 756-765.

35. Wei J., *et al*. "Oleanolic acid potentiates the antitumor activity of 5- fluorouracil in pancreatic cancer cells". *Oncology Reports* 28.4 (2012): 1339-1345.

36. Lúcio KA., *et al*. "Oleanolic acid initiates apoptosis in non-small cell lung cancer cell lines and reduces metastasis of a B16F10 melanoma model *In vivo*". *PLoS One* 6.12 (2011): e28596.

37. Feng L., *et al*. "Oleanolic acid from Prunella vulgaris L. induces SPC-A-1 cell line apoptosis via regulation of Bax; Bad and Bcl-2 expression". *Asian Pacific Journal of Cancer Prevention* 12.2 (2011): 403-408.

38. Wang J., *et al*. "Ursolic Acid Inhibits Proliferation and Induces Apoptosis in Human Glioblastoma Cell Lines U251 by Suppressing TGF-b1/miR-21/PDCD4 Pathway". *Basic and Clinical Pharmacology and Toxicology* 111 (2012): 106-112.

39. Feng XM and Lan XS. "Anticancer effect of ursolic acid via mitochondria-dependent pathways (Review)". *Oncology Letters* 17 (2019): 4761-4767.

40. Mlala S., *et al*. "Ursolic Acid and Its Derivatives as Bioactive Agents". *Molecules* 24 (2019): 2751.

41. Foo JB., *et al*. "Induction of cell cycle arrest and apoptosis by betulinic acid-rich fraction from Dillenia suffruticosa root in MCF-7cells involved p53/p21 and mitochondrial signalling pathway". *Journal of Ethnopharmacology* 166 (2015): 270-278.

42. Hordyjewska A., *et al*. "Betulin and betulinic acid: triterpenoids derivatives with a powerful biological potential". *Phytochemistry Reviews* 18 (2019): 929-951.

43. Cornejo J., *et al*. "Acute and subacute toxicity (28 days) of a mixture of ursolic acid and oleanolic acid obtained from Bouvardia ternifolia in mice". *The Boletin Latinoamericano Caribe Plant Med Aromat* 11.1 (2012): 91-102.

44. Aparecida F., *et al*. "Antimutagenicity of ursolic acid and oleanolic acid against doxorubicin-induced clastogenesis in Balb/c mice". *Life Sciences* 79 (2006): 1268-1273.

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