Available online on www.ijcpr.com
International Journal of Current Pharmaceutical Review and Research; 9(4); 48-54

ISSN: 0976 822X

Research Article

# Anticancer Potential of (R) -5,7-Dihydroxyflavanone from Leaves of C*hromolaena Leivensis* (Hieron) on Cancer Cells

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*Available Online:25th August, 2018*

**ABSTRACT**

Background: Flavonoids isolated from plants have demonstrated an important role in cancer chemoprevention and chemotherapy. The genus *Cromolaena* has been shown to have active principles against this disease and found in species such as *C. odorata,* and *C. laevigata* in a concentration lower than 100 mg/L, however, flavonoids from *C. leivensis* has not been studied completely as an alternative in cancer treatment. Materials and Methods: The (2R)-5,7-dihydroxy flavanone or (R) Pinocembrin was isolated from leaves of *Chromolaena leivensis* (Hieron) using chromatography methods. Its structure and relative configuration were determined by NMR spectroscopy, gas chromatography coupled to mass spectrometry and X-ray diffraction. We evaluated the (R) Pinocembrin effects on cell proliferation, morphology, DNA damage, and cell cycle progression of cancer cell lines Results: The compound showed a decreasing cell proliferation rate against HT29, PC-3, A549, MDA-MB-231, and SiHa cancer cell lines with an $IC_{50}$ values between 58.9 mg/L, and 30.9 mg/L, causing alterations in the stability of the cytoskeleton and G1-phase cell cycle arrest without affecting significantly the DNA integrity. Conclusion: The (R)-Pinocembrin is a potential molecule to be used in the treatment of cancer with an action on the cytoskeleton. Our study indicate that the medium polarity fraction obtained from *C. leivensis* is a promising fraction which could be used as in the treatment of cancer, especially as a coadjuvant.

**Keywords:** *Chromolaena leivensis* (Hieron), (2R)-5,7-dihydroxy flavanone, Cytotoxicity, Anticancer activity, Cell cycle progression.

## INTRODUCTION

The *Asteraceae* family, with approximately 24,000 species, is considered the largest family of plants with the highest number of species described. *Asteraceae* are usually more abundant in mountainous and arid areas, while more limited in tropical jungles. It contains 1535 genera grouped into 17 tribes, and 3 subfamilies, while the last classification proposed by Funk et al. (2009)[1], based on morphological and molecular characters, recognizes 1600-1700 genera, 43 tribes and twelve subfamilies. The genus *Chromolaena* belongs to the *Eupatorieae* tribe and the *Praxelinae* subtribe and includes about 170 species distributed from the sea level to 3900 meters of altitude from the south of the United States to the south of Argentina, with the highest concentration of species in Brazil. *Chromolaena leivensis* (Hieron) is found growing wild between 1200 and 1800m a.m.s.l and is often referred to as the cancer herb, as it is popularly advocated for the treatment of this disease[2]. Several flavonoids have been previously obtained from the leaves and inflorescences of this plant, mainly flavones, 3,5-dihydroxy-7-methoxyflavone, 3,5,7-trihydroxy-6-methoxyflavone and the flavanone, 3,5-dihydroxy-7-methoxyflavanone. These compounds have an unsubstituted ring B and their cytotoxic activities on some cancer cell lines have already been reported[3].

It is generally accepted that among flavonoids, flavanones have a lowest cytotoxic effect on cancer cells[4], however, this recognized property of flavanones can vary depending on the types of cancers these compounds are acting upon, and the substitution patterns present on rings A and B of the molecule[5]. In this study, another flavonoid from *C. leivensis* (Hieron) has been isolated and identified as (2R)-5,7- dihydroxyflavanone. The antiproliferative activity of the compound has been studied in order to support the commonly claimed antineoplastic properties of the source plant, *C. leivensis* (Hieron).

## MATERIALS AND METHODS

The plant was collected in flowering time, in the rural area of Bogota-Colombia, close to the town of Chuzaca (4.55; -74.25). A specimen was identified at the National Herbarium of Colombia as *Chromolaena leivensis* (Hieron), King & H. Rob, with registration number Col 535219. The active fraction was obtained by Soxhlet extraction of 660 g of dried leaves, using EtOH (96%) as

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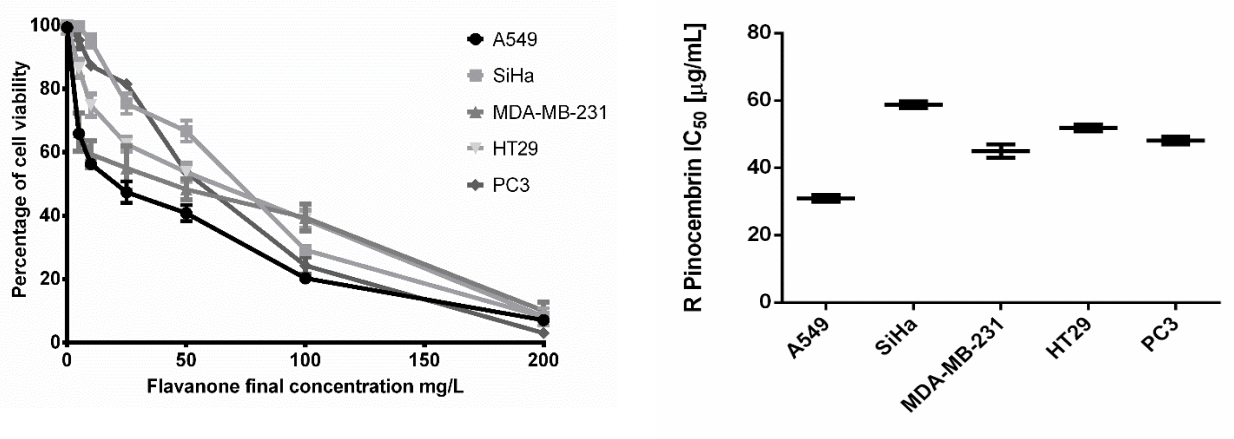


a b

Figure 1: A. Percentage of the relative viability of different cancer cell lines treated with different concentrations of R Pinocembrin. B. The flavanone display cytotoxicity on each cancer cell line studied at different concentrations

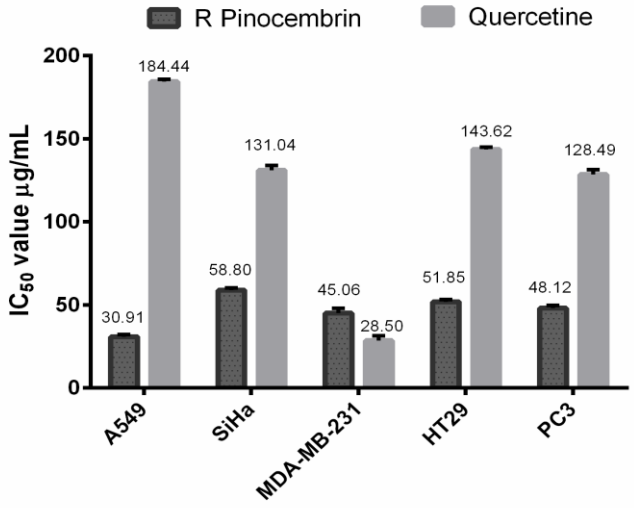


Figure 2: Comparison (2R)-5,7-dihydroxyflavanone cytotoxic activity includes a significant effect on the DNA of A549 cells but not on prostate PC3, breast MDA-MB-231, colon HT29, and cervix SiHa.

the solvent to obtained 27.3 % of yield (180 g ExEtOH). Subsequently, 100 grams of ExEtOH were fractionated in a packed column with three solvents (Petrol, toluene, and AcOEt). Three grams of the AcOEt fraction were fractioned again in a column chromatography (4 x 60 cm) packed with Si-gel (300 g, Kieselgel, Merck 60-200 mesh). The active compound was isolated from the medium polarity fraction and crystallized in n-hexane. The melting point was determined in a Melt-Temp instrument (Electrotermal, Sttafordshire, Great Britain). The Rf on thin-layer chromatography in Si-gel plate was determined using as mobile phase $CHCl_3$: MeOH (9:5). The specific rotation in MeOH $[\alpha]_D^{25} - 70$ was obtained in a polarimeter (Polartronic Schmidt and Haensch, Berlin, Germany). The spectroscopic data were obtained as follows: the UV spectra, using a Cary100, (Agilent, Foster City, CA), mass spectrum using a GC/MS Shimadzu TQ 8045 (Kioto, Japan) with ion trap and IE ionization, at 70 eV, using a capillary column Rtx-5MS (30 m x 0.25 mm x 250 um) (Restek Co USA), $^1$HNMR and $^{13}$CNMR using a Bruker 300MHz were used to obtain the 1D H and $^{13}$C NMR spectra (Bremen, Germany) and XRD of single crystals Data collection, cell refinement, and data reduction: MSC/AFC6S diffractometer control software (Molecular Structure Corp., The Woodlands, TX). The program used to solve structure: SHELXS97; program used to refine structure: SHELXL97; molecular graphics: SHELXTL-PC (G.M Sheldrick, Institute of Inorganic Chemistry Göttingen, Germany).

Cell lines and culture conditions: Tumor-derived cells originated in multiple tissue sites were maintained as follows: colon adenocarcinoma cell line HT29 (ATCC ® HTB-38™), in Dulbecco's modified Eagle's medium with high glucose (Lonza), prostate cancer cell line PC-3 (ATCC® CRL-1435™) in Eagle's medium (Lonza), adenocarcinomic human alveolar basal epithelial cell line A549 (ATCC® CCL-185™), breast adenocarcinoma cell line MDA-MB-231 (ATCC® HTB-26™) and cervical squamous carcinoma cell line SiHa (ATCC® HTB-35™) were grown in RPMI 1640 medium (Lonza), all medium supplemented with 10% (v/v) Fetal Bovine Serum (Biowest), 2 mM L-glutamine (Lonza), 5,000 UI/ml penicillin and 5 mg/ml streptomycin (Lonza). Cells were screened for mycoplasma contamination before each

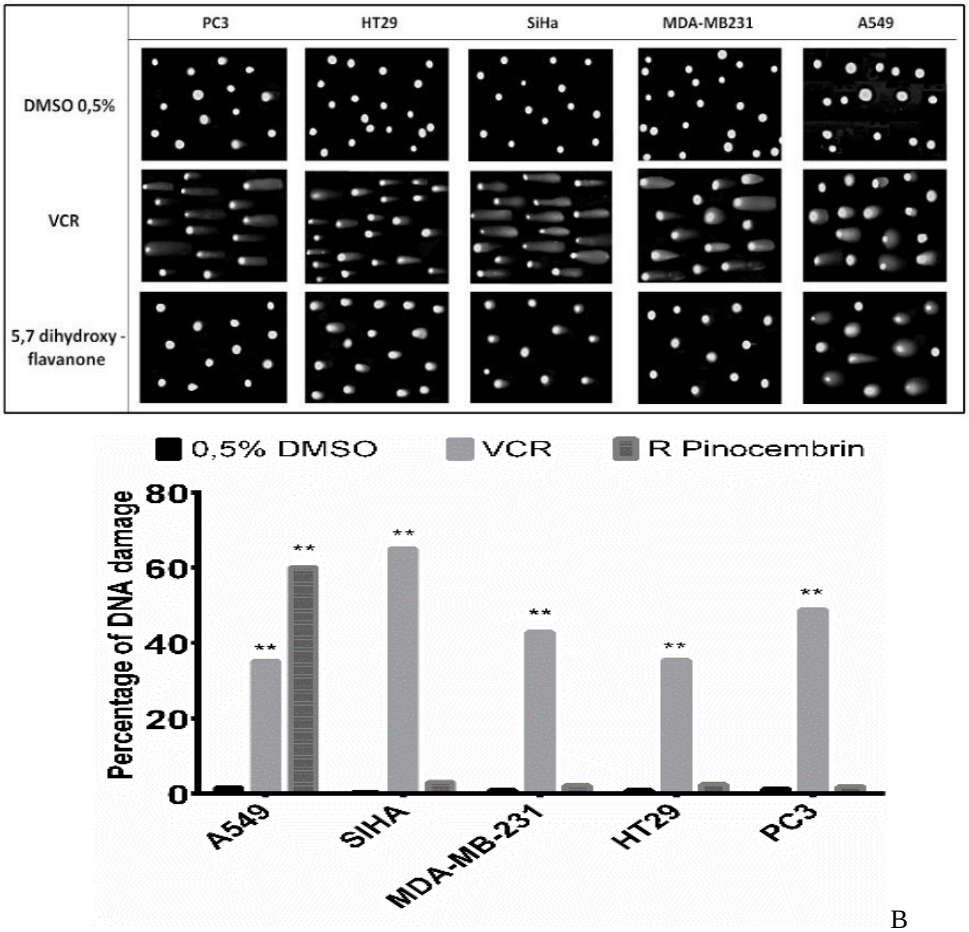


Figure 3: Genotoxic activity of 5,7- dihydroxy- on SiHa, HT29, A549, MDA-MB-231, and PC3 cancer cell lines after 24 hours of incubation. **A**. Comet assay on cells **B**. Mean of the percentage of DNA damage compared with positive (VCR) and negative (DMSO 0.5%) controls.

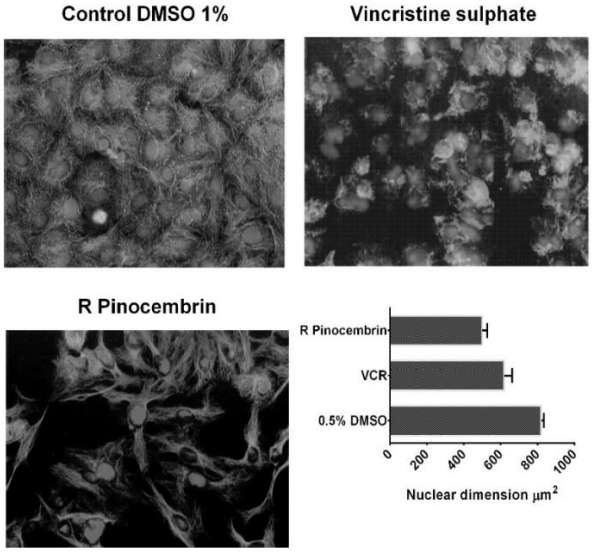


Figure 4: Microtubular and nuclear analysis on A549 lung cancer cell line after 24 hours of incubation with 5,7-dihydroxy-flavanone. Alfa tubulin stained with anti-alpha-tubulin, and DNA stained with DAPI. The nuclear area was analyzed by Motic Image plus 2.0 software.

experiment, by DAPI staining (Invitrogen) and immunofluorescence microscopy (MOTIC).

Cell viability assay: Eight thousand cells were seeded in 96 well plates and grown in 5% $CO_2$ at 37 °C for 24 h. Cells were treated with the flavanone dissolved in dimethyl sulfoxide (DMSO) (Sigma- Aldrich, St Louis MO, USA) at concentrations of 5, 10, 25, 50, 100, 150 and 200 mg/L. The maximum final concentration of DMSO was 1% per

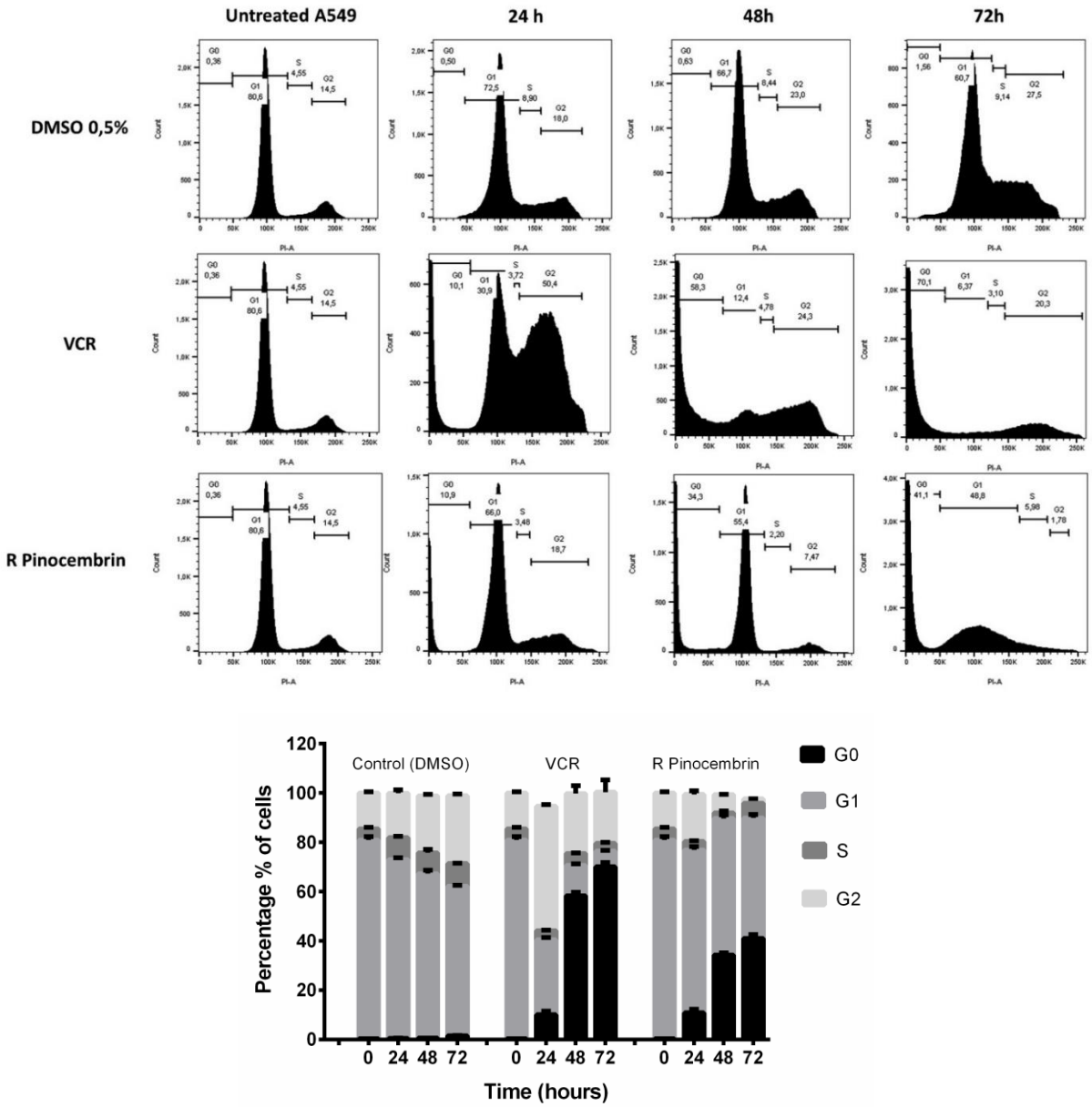


Figure 5: A549 cells distribution in the phases of the cell cycle after treatments with DMSO 0.5% (negative control), VCR (positive control for arresting in G2/M phase), and 5,7-dihydroxy- flavanone (purplin). Cells were incubated by 24, 48 and 72 h and stained with PI. Cells distribution in the Sub-G1 (possible apoptosis), G1, S and G2/M phases of the cell cycle, was analyzed by using the FlowJo_V10 software.

treatment. After 48 hours of incubation, 3-(4,5-methyl-thiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) was added (Sigma- Aldrich, St Louis MO, USA) at 500 mg/L per well and then incubated for 4 hours. Formazan products were solubilized with DMSO[6]. Assays were quantified by reading optical density at a wavelength of 570 nm using a microplate reader (BioRad, Hercules, CA, USA). Statistical analyses were performed using GraphPad Prism 6.0. Data from assays displaying a decrease of cell viability of 50%, were represented as the inhibitory concentration required to decrease 50% of cell viability ($IC_{50}$), and were submitted for analysis of variance (ANOVA), with post-hoc HSD-Tukey and Scheffé tests. All the experiments were performed in triplicate.

Immunofluorescence microscopy analysis: Immunofluorescent assays were performed on A549 cells in order to examine possible microtubule and nuclear damage 24 hours after treatment with the flavanone, according to the methodology suggested previously [7]. One hundred thousand cells per well were seeded on a 24-well plate and grown in 5 % $CO_2$ at 37 °C. Untreated and treated cells with the Pinocembrin or with the positive control vincristine at 0.05 mg/L, were fixed in cold absolute methanol for 10 min, then in acetone for 20 secs at -20 ˚C. Cells were incubated for 1 h at 37 ˚C with 0.2 mg/L of mouse anti-α-tubulin (SIGMA) and then, they were incubated with 2 mg/L Alexa Fluor 488 goat anti-mouse secondary antibody (Molecular Probes, Eugene, Oregon, USA) diluted in 2 % (w/v) BSA/PBS, blocking solution.

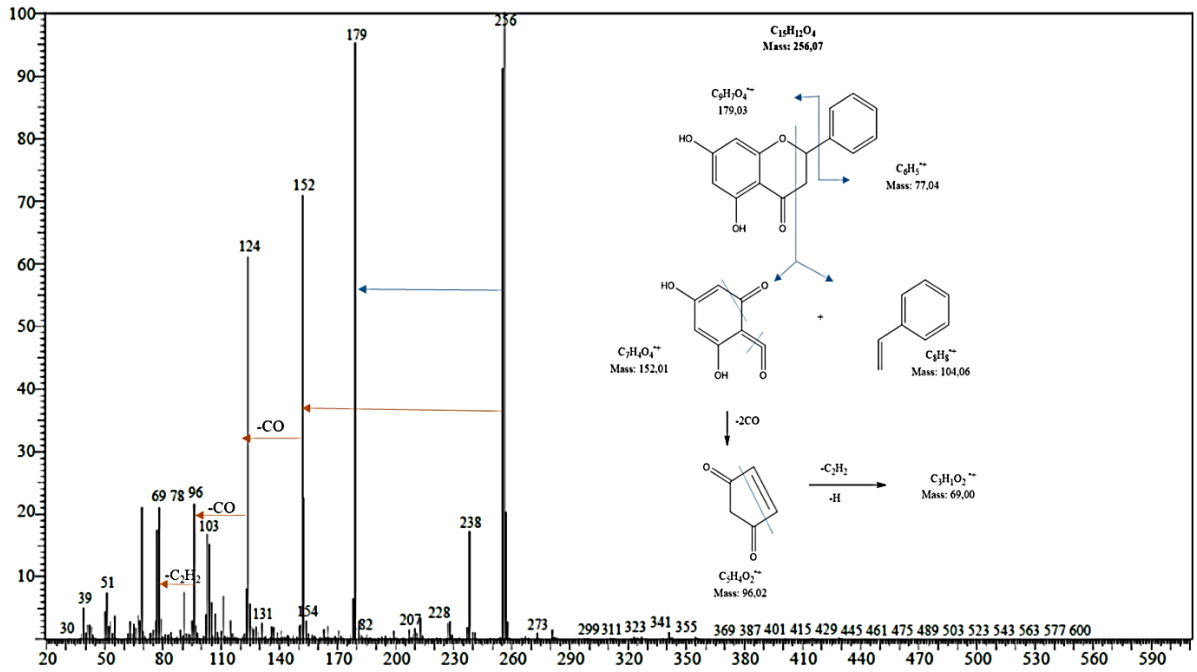


Figure 6: Mass spectrum flavanone and their comparison with reference spectra in the database Nist 11, obtained in the spectrometer GCMS Shimadzu 2000. Main peaks of flavanone 256(100%), 179(96%), 152(72%), 124(64%), 69(24%).

DNA was stained with DAPI 1 mg/L (Invitrogen). Slides were mounted in antifade solution from Vectashield (Vector Laboratories, Burlingame, CA, USA). Immunofluorescent images were monitored with a Motic AE31 microscope, captured with a MoticCamPro 282A and analyzed with the Motic Image plus 2.0 software.

Genotoxicity Assay via Comet Assay, Single Cell Gel Electrophoresis: Single cell gel electrophoresis (SCGE) or comet assay, was used to determine the (2R)-5,7-dihydroxy-flavone genotoxic potential. DNA damage was determined by measuring the displacement between the genetic material of the nucleus (comet head) and resulting tail (tail DNA). At least 50 individual cells were analyzed from each sample. Vincristine sulfate was used as a positive control for genotoxicity [8]. Tests were performed at 24 h after exposure to the flavanone at a concentration of 40 mg/L. The procedure was performed according to the instructions of the OxiSelect™ Comet Assay Kit (Cell Biolabs). Briefly, it was performed using TBE electrophoresis buffer, and Vista Green DNA staining solution was used to visualize the cells on a fluorescence microscope (Motic). Images were captured with the MoticCamPro 282A microscopy and analyzed with Comet Score software (Tritek Corp, Sumerduck, VA USA). Results are expressed as a mean of DNA migration or tail DNA percent.

$$Tail\ DNA\% = 100\ x\ Tail\ DNA\ Intensity/Cell\ DNA\ Intensity.$$

Means of the percentage of tail DNA were submitted for analysis of variance (ANOVA), with post-hoc HSD-Tukey and Scheffé tests for determination of genotoxicity.

Analysis of cell cycle distribution: The analysis of cell cycle distribution was performed on synchronized A549 cells after treatments with either, the flavanone, DMSO 0.5% (negative control) or Vincristine as the positive control for cell cycle arrest at the G2/M phase. The compound was used a concentration corresponding to the $IC_{50}$ value determined previously by MTT assay. Cells were incubated by 24, 48 and 72h at 37 ˚C in 5 % $CO_2$ and were then fixed with cold 70% ethanol dropwise while gently vortexing, and incubated in the dark for 30 min with propidium iodide (PI) staining solution: 3.8 mM sodium citrate, 50 mg/L PI in PBS and RNase A stock solution: 10 mg/L RNase A. DNA content analysis was performed in a BD- FACS Aria™ cytometer (Franklin Lakes, NJ, USA). Cell distribution in the Sub-G1/G0 (possible apoptosis), G1, S and G2/M phases of the cell cycle, was determined by using FlowJo_V10 software (Ashland, OR, USA).

## RESULTS

A white compound (120 mg) was isolated from the medium polarity fraction. Its melting point was determined as 192-193ºC and its Rf on thin-layer chromatography in Si-gel plate was 0.40. The crystals revealed a purple stain after exposure to $NH_3$ vapors for 24 hours. The retention time in the ion current chromatogram was 24.57 min.

Spectroscopic data: The isolated compound showed maximum absorption peaks in UV analysis in MeOH $\lambda_m$ nm (A), 289 (0.350). 340 (0.040) and MeOH+ AcONa 295 (0.360), MeOH+ MeONa 343 (0.365). The fragmentation pattern obtained at 70 eV in a GC/MS-TQ corresponded to a molecular ion (m/z) $M^+$ 256, 07 (100), and fragment ions 179(95), 152(72), 124 (64), 69(50). The analysis of $^1$HNMR (Bruker 300MHz) [($DMSOd_6$. ppm. m.J Hz] showed: 2.78 dd 18 and 3 CHa; 3.24 dd 18 and 12 CHb; 5.57 dd 12 and 3 -O-CH; 5.90 s. =CH; 5.93 s =CH; 7.40. m 3(=CH); 7.51. d 2(=CH); 12.11 s HO on C5, and $^{13}$CNMR -JMOD (Bruker. 75 MHz) [$DMSOd_6$. σ ppm]

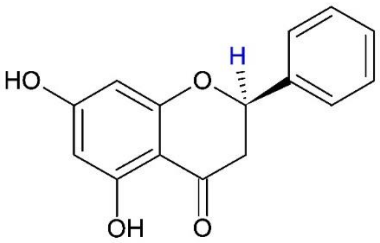


Figure 7: Structure of (2R) -5,7- dihydroxyflavanone from ChemSketch.

showed: 42.51 -$CH_2$-; 78.84 -CH-O; 95.52 =CH-; 96.40 =CH-; 102.25 =C-; 127.06 2(=CH-); 129.04 2(=CH-); 139.08 =C-; 163.17 =C-O; 163.92 =C-O; 167.17 =C-O; 196.40 - C=O.

(2R)-5,7-dihydroxyflavanone effectively inhibited cell viability in cancer cells of the prostate, breast, colon, cervix, and lung: The viability of PC3 prostate, MDA-MB-231 breast, HT29 colon, SiHa cervix and A549 lung cells was analyzed 48 hours after treatment at concentrations between 5 and 200 mg/L of flavanone via MTT assay. Data obtained showed an inhibition of the percentage of cell viability compared to control (Figure 1A). The inhibitory concentration at which we observe 50% in cell viability ($IC_{50}$) calculated from the non-linear curve regression showed that (R) pinocembrin was more cytotoxic against A549 and MDA-MB-231 with $IC_{50}$ values of 30.91 mg/L and 45.06 mg/L respectively (Figure 1B). Further, the $IC_{50}$ values for (R) pinocembrin, were consistently lower than those for quercetin on cells except for breast cancer cell line MDA-MB-231, where the $IC_{50}$ for quercetin was 28.5 mg/L. The most significant difference was observed in A549 lung cells where the $IC_{50}$ value of quercetin was 184.44 mg/L (Figure 2).

According to the results of comet assay, the flavanone had not significant genotoxic effects in the majority of the cells tested only on A549 lung cells with 61 % of DNA damage, comparatively to the effect of the positive control VCR, with a 65 % of DNA damage. (Figure 3).

To complement genotoxicity and cytotoxicity data, it was done a morphological analysis on A549 cells by immunofluorescence microscopy, finding changes in the shape and integrity of the cytoskeleton, increasing of the nucleus area, and inhibition of the cell growth in response to damage occurring after treatment with the flavanone (Figure 4).

(2R)-5,7-dihydroxyflavanone induced cell cycle arrest on A549 cells in G1 phase: Additionally, cell cycle distribution analysis was done on A549 cells by flow cytometry and it resulted in G1 phase arresting during 24 and 48 h of treatment, and consequent cell death at 72 h of treatment (Figure 5).

**DISCUSSION**

The 5,7-dihydroxyflavanone has been isolated from several plants among them *Alpinia mutica, Litchi chinensis, Lippia graveolens, Lippia origanoides, Dalea elegans, Oxytropis falcate, Glycyrrhiza glabra L., Sparattosperma leucanthum, Cleome droserifolia, Lychnophora markgravii, Helichrysum gymnocomum,* and others (8). We identified the flavonoid 5,7-dihydroxyflavanone from leaves of *Chromolaena leivensis* after being purified by column chromatography and crystallization in n-hexane.

The mass spectrum showed a molecular ion and peak base in m/z 256 (100%) corresponding to a condensed formula $C_{15}H_{12}O_4$. The first fragmentation showed a peak m/z 179 (96). By Retro Diels Alder is obtained the fragment m/z 152 (76), for consecutive loss of two CO molecules is obtained the fragments m/z 124 (64) and m/z 96 (24) that loss the fragment $C_2H_2$ to obtain the peak m/z 69 (24) (Figure 6), that matches that determined by $^{13}$CJMOD.

$^1$HNMR spectrum presents signals at for an AMX coupling system at 2.78 dd 18 and 3 Hz CHa; 3.24 dd 18 and 12Hz CHb; 5.57 dd 12 and 3Hz -O-CH for ring C of a flavanone; signals at 5,90 ppm and 5,93 ppm, corresponding to = CH at C6 and C8 of the A ring and signals 7,51ppm 7,40 that integrates for 5(= CH) corresponding to the protons of B ring, the signal at 12,11 ppm is characteristic of OH at C5 of a flavonoid. The signals at 163,17ppm, 163,92 ppm, and 167,17 ppm in the spectrum $^{13}$CJMOD are quaternary carbons of a benzene ring, which are bonded to oxygen, 3(= C-O) corresponding to the C 5,6,9 of the ring A. The signals at 42,51ppm (-$CH_2$); - 78,84 ppm (CH-O) and 196,40ppm (- C = O) confirm the flavanone (Figures 7).

The results suggest that the compound identified in *Ch. leivensis* is a flavanone 5,7 dihydroxylated without substitutions on the ring B. The relative configuration was assigned based on the configuration of the 3,5 dihydroxy-7-methoxyflavanone that was previously isolated from *Ch. leivensis* and was determined by XRD (4). The flavanone was identified as (2R) -5,7-dihydroxy-2,3-dihydro-2-phenyl-4H-chromen-4-one or commonly named (2R) -5,7-dihydroxyflavanone.

A large variety of pharmacological activities have been attributed to pinocembrin, including its antineoplastic potential[9]. In this respect, several studies have been carried out to evaluate the cytotoxic potential against various cancer cells including, prostate[10], colon[11-12], leukemia[13-14], however, there are significant variances in the cytotoxic activity, suggesting that relative configuration could be responsible for that different effects on cancer cells.

We used the comet assay (single-cell gel electrophoresis) for studying DNA damage on cancer cells exposed to (R) pinocembrin, and results suggest that the compound induces a cytotoxicity that mainly affects the cytoskeleton, and in a smaller proportion to the nucleus as occurred for the majority of cells (Figures 3 and 4), except for A549 cells, the most

susceptible cancer cell line to the flavonoid whose data coincide with those previously obtained for colon cancer that showed cytotoxicity accompanied with condensed and fragmented nuclei[12].

Previously it was shown that pinocembrin inhibited the proliferation of LNCaP cells through arresting cell cycle at S and G2/M phases in prostate cancer cells[10], contrastingly to our data that demonstrated the cell cycle arrest on G1 phase of A549 cells induced by (R)-pinocembrin (Figure 3), it would depend on the manner as the compound modulates cyclins pathways indicating that cell cycle

arrest mechanism could be related with the relative configuration of pinocembrin.

## CONCLUSIONS

The flavanone obtained from *Chromolaena leivensis* (Hieron) was identified as (2R) -5,7-dihydroxy-2,3-dihydro-2-phenyl-4H-chromen-4-one, commonly denominate (R) Pinocembrin, which effectively inhibited the cell viability in cancer cells lines of the prostate PC3, breast MDA-MB-231, colon HT-29, cervix SiHa, and lung A549. The cytotoxic activity of the compound includes a significant effect on the DNA of A549 cells but not on prostate, breast, colon, and cervix, also induced cell cycle arrest on A549 cells in G1 phase. Data indicate that (2R)-5,7-dihydroxy flavanone has an antitumor potential to be explored.

## ACKNOWLEDGMENTS

This work was financially supported by Universidad de Ciencias Aplicadas y Ambientales. U.D.C.A.

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