

ANTIPROLIFERATIVE ACTIVITY OF 3,5,7- TRIHYDROXY -6- METHOXY FLAVONE OBTAINED FROM *Chromolaena leivensis* (HIERON) ON CANCER CELL LINES OF BREAST, PROSTATE, LUNG, COLON AND CERVIX.

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Abstract

The flavone 3,5,7-trihydroxy-6-methoxyflavone was isolated from leaf extracts of *Chromolaena leivensis* (Hieron), commonly named plant of cancer. It was identified based on their physicochemical properties and spectroscopic data. This compound presented a methoxylation at C6, this is uncommon in flavonoids and which may confer some specificity of its biological activity. The cytotoxic activity of this flavonoid was determined on PC3 (prostate), MDA- MB-231 (breast), HT29 (colon), SiHa (cervix) and A549 (lung) cancer cells, using MTT assay, to assess if the flavonoid contributes to the anticancer activity previously proposed for *Chromolaena leivensis*. The cytotoxic activity of the 3,5,7-trihydroxy-6- methoxy flavone was similar to that obtained for the flavonoid quercetin but was low compared with the positive control vincristine sulphate. The better value of the inhibitory concentration of fifty percent (IC₅₀) 150 µM, was achieved on SiHa cell line, while the lower activity: 4008 µM, was obtained on HT29 cancer cell line. However, severe morphological changes were detected on cytoskeleton and nucleus of the SiHa cells detected by immunofluorescence microscopy analysis of cells exposed to the half of the IC₅₀ concentration obtained for the flavonoid. Data indicate that the flavonoid contributes to the anticancer activity of the extracts of leaves from *Chromolaena leivensis*, and could broadening the spectrum of flavonoids activity against various types of cancer non hormone-dependent.

Key words: Cancer cells, *Chromolaena leivensis*, antiproliferative activity, 3,5,7-trihydroxy-6-methoxy flavone

Introduction

Chromolaena leivensis is a plant belonging to the *Asteraceae* family that grows abundantly in the Cundiboyacense region of Colombia and has been used in the traditional medicine as an anticancer source [1]. Chemical studies have reported the presence of flavanones and flavones [2], to which it have been studying their cytotoxicity on various cancer cell lines (erythroleukemia (K562) and cells of human melanoma (A375) to determine how each affects the antineoplastic effect of the whole plant. Flavones with methoxyl substituents in C6 and hydroxyl in C7 are rare; usually C7 is the carbon carrying the methoxy group. The presence of the methoxy group in C6 adjacent to C5-OH could generate the special conditions affecting the viability of cancer cells, C6 and C5-OH can facilitate the formation of hydrogen bonds with proteins and nucleic acids to cells which face, also to bind to different sequences of bases in DNA [3].

Aim of the study is to determine the antiproliferative and antioxidant activities of the 3,5,7-trihydroxy-6-methoxy flavone to contribute to the research of biological activity of the *Chromolaena leivensis* on breast, prostate, lung, colon and cervix cancer cell lines.

Methods

Isolation and characterization of the 3,5,7-trihydroxy-6-methoxy flavone

Ethanol extract (5 g) from leaves of *C. leivensis* were chromatographed on silica gel 60 (Merck, 60 kielse gel, 0.063 to 0.2 mm) with mobile phase CH_2Cl_2 and MeOH; in fractions with CH_2Cl_2 :MeOH 9:1, it was isolated a yellow compound which was crystallized from MeOH and identified by their physicochemical and spectroscopic properties. Melting point 233 °C, ^1H NMR [Bruker 400 MHz, DMSO-d_6 , δ ppm]: 3.8s (CH_3O -), 6.6s ($\text{CH}=\text{}$), 7.5m (3 $\text{CH}=\text{}$, J7, 3 Hz), 8.2d (2 $\text{CH}=\text{}$, 7 Hz), 9.7s (OH), 10.8s (OH), 12.5s (OH C5). ^{13}C NMR [Bruker 100 MHz, DMSO-d_6 , δ ppm], 59.9 (OCH_3), 93.8 ($\text{CH}=\text{}$), 103.5 (C=), 127.5 (2 $\text{CH}=\text{}$), 128.4 (2 $\text{CH}=\text{}$), 129.9 ($\text{CH}=\text{}$), 130.9 ($\text{CH}=\text{}$), 136.7, 145.8 (C=), 151.6, 151.7, 157.5, (=C-O), 176.4 (C=O). The data of the NMR spectra and melting point match with reported for 3,5,7-trihydroxy-6-methoxy flavone [4,5].

Cell Lines and Culture Conditions

Tumor derived cells that originated in multiple tissue sites were maintained as follow: HT29 (colorectal adenocarcinoma), PC3 (prostate adenocarcinoma) and A549 (Lung: NSCLC alveolar basal epithelial- squamous) in Dulbecco's

modified Eagle's medium with high glucose (Lonza), supplemented with 10 % (v/v) Fetal Bovine Serum (Biowest), 2 mM L-glutamine, 5,000 UI/ml penicillin and 5 mg/ml streptomycin. MDA MB-231 (breast adenocarcinoma) and SiHa (cervical squamous cell carcinoma) were grown in RPMI 1640 medium (Lonza) supplemented with 10% serum (Biowest) and 2 mM L-glutamine, 5,000 UI/ml penicillin and 5 mg/ml streptomycin. Eight thousand cells were seeded in 96 well plates and grown in a 5 % CO_2 atmosphere at 37 °C for 24 h before treatment. Cells were screened for mycoplasma contamination before each experiment, by means of DAPI staining (Invitrogen) and fluorescence microscopy camera (Motic CamPro 282A).

Cell Viability Assay

Inhibition of cancer cell viability by 3,5,7-trihydroxy-6-methoxyflavone was performed by MTT assay [6]. Cells were treated with the flavone 3,5,7-trihydroxy-6-methoxyflavone, dissolved in Dimethyl sulfoxide (DMSO) (Sigma- Aldrich) at concentrations of 5, 10, 25, 50 and 100 $\mu\text{g}/\text{mL}$. The maximum concentration of DMSO was 0.5 % at each treatment. After 48 hours of incubation, 3-(4, 5-methyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma-Aldrich) was added at 500 $\mu\text{g}/\text{mL}$ / well and then incubated for 4 hours. Formazan products were solubilized with 100 μl DMSO (Sigma-Aldrich). Assays were quantified by reading optical density at a wavelength of 570 nm using a microplate reader (BioRad Model 680). Quercetin (Cayman) was used as positive control at IC_{50} determinate by MTT assay under same conditions described above.

The cell viability was expressed as percentage of cells non-inhibited by the compound at different concentrations. The statistical analyses were performed on IBM SPSS 20 software. It was determined the assumptions of the parametric analysis looking for a normal Gauss distribution by Shapiro-Wilk and Kolmogorov-Smirnoff test, with homogeneity of variances ($p > 0.05$). The IC_{50} value was defined as the concentration of the compound which caused a 50 % decrease of the cell viability. The IC_{50} values were derived from a lineal regression after transformation of the concentration to logarithm scale. The values of IC_{50} were submitted to analysis of variance (ANOVA), with post-hoc HSD-Tukey, Scheffé and versus IC_{50} determinate for the positive control quercetin by Dunnett's test. All the experiments were performed in triplicate. Significant differences between cell responses were indicated as $p < 0.05$

Immunofluorescence microscopy analysis

Untreated and treated cells with 3,5,7-trihydroxy-6-methoxyflavone, quercetin and vincristine sulphate for 24 h were fixed in absolute methanol for 10 min, then in acetone for 20 sec at -20°C . Microtubule cell integrity was evaluated by using of the monoclonal mouse anti- α -tubulin clone DM1A (Sigma-Aldrich). After washing with PBS, cells were incubated for 1 h at 37°C with (1:2000) of the primary monoclonal antibody and after blocking with 2 % (w/v) BSA/PBS, cells were incubated with 2 $\mu\text{g}/\text{mL}$ Alexa Fluor 488 goat anti-mouse secondary antibody (Molecular Probes) in 2 % (w/v) BSA/PBS. DNA was stained with DAPI (Invitrogen). Slides were mounted with an antifade solution (Vectashield; Vector Laboratories). Immunofluorescent images were captured with the MoticCamPro 282A and the image analysis was performed with the Motic Image plus 2.0 software.

Results

Chromolaena leivensis contains the flavonoid 3,5,7-trihydroxy-6-methoxyflavone and was identified by its spectroscopic data, it has a methoxy substituent in the C6 which could be related with the inhibition of the cell viability found in this study (Figure 1).

The IC_{50} value was defined as the extract concentration which caused a 50 % decrease of the cell viability. IC_{50} values (Figure 2) were derived from a lineal regression from respective curves of percentage of cell viability (Figure 1).

The cytotoxic activity was expressed as the negative logarithm to base 10 of the IC_{50} (pIC_{50}) and it was compared with the activity of the control quercetin (Figure 3).

The IC_{50} values obtained for the flavonoid 3,5,7-trihydroxy-6-methoxyflavone were significantly different ($p < 0.05$) to the values obtained for the vinca alkaloid vincristine sulphate in the same conditions (Table 1)

Additionally, morphological changes were observed on SiHa cells exposed to $75\ \mu\text{M}$ of 3,5,7-trihydroxy-6-methoxyflavone (Figure 4D). The size of the nucleus and the whole cell increase in presence of the flavone, compared with the cells untreated (Figure 4A), and this nucleus increasing was similar to that observed in the cells exposed to vincristine and quercetin (Figure 4B and C). Another effect was observed in the microtubules integrity indicating losing of proliferation capacity of the cells.

Discussion

The effect of 3,5,7-trihydroxy -6-methoxy flavone on cell viability measured at 48 hours was no high but different between the cancer cell lines tested as shown in figure 1. These different effects could be due to the union of this flavonoid in different DNA sequences [3] or the specific phenotypic and genotypic characteristics of cancer cell lines tested. In general, the cytotoxic effect of the flavone against cervical cancer SiHa, breast cancer MDA MB231, colon HT 29, lung A549 and prostate PC3 cancer cell lines was low according with the IC_{50} values obtained (Figure 2 and 3). However significant differences respect to the flavonoid quercetin was obtained on SiHa cervix cancer cells being most active the 3,5,7-trihydroxy -6-methoxy flavone with an IC_{50} of $150\ \mu\text{M}$. Cytotoxic activity of quercetin was significantly higher that the flavone only on colon HT 29 cancer cell line (Figure 3). The other activities obtained on PC3, MDA MB231 and A549 cancer cell lines were similar to quercetin. The IC_{50} values for 3,5,7-trihydroxy- 6- methoxy-flavone, between 150 to $900\ \mu\text{M}$ are low but are in the range of cytotoxic activity of the flavonoid quercetin that is considered as adjuvant treatment to prevent cancer cell proliferation [7]. Other methoxylated on C6 flavonoids such as 5,7-dihydroxy -3,6,8-trimethoxy was tested before against other cancer cell lines [8] showing higher cytotoxicity compared with the 3,5,7-trihydroxy -6-methoxy flavone confirming the low cytotoxic activity of this compound. The lower cytotoxic effect of this flavone was found against colon HT29 and lung A549 cancer cells with an IC_{50} of $4008\ \mu\text{M}$ and IC_{50} $989\ \mu\text{M}$. Interestingly HT29 and A549 are androgen receptor determinate for the positive control quercetin by Dunnett's test. All the experiments were performed in triplicate. Significant differences between cell responses were indicated as $p < 0.05$ expressing cells and these were more resistant to action of the flavonoid, in contrast to androgen independent SiHa, MDA MB231 and PC3 cancer cell lines, indicating that the 3,5,7-trihydroxy -6-methoxy flavone perhaps cannot act as an antagonist for AR as other flavonoids [9], then it cannot limit efficiently the development and progression of hormone-dependent cancer cells. Moreover, the compound 3,5,7-trihydroxy -6-methoxy flavone could be induce apoptosis according with observations on morphology of treated SiHa cells (Figure 4), because the disruption of microtubules affect central functions as cell movement, intracellular trafficking, and mitosis; this is a molecular mechanism of some anticancer drugs [10].

On the other hand, it has been reported before that the inhibition of tumor growth through cell cycle arrest and induction of apoptosis induced by flavonoids is a mechanism related with p53 functions, however, the cells used in this study have different status of this protein [11, 12, 13, 14, 15] in this form the mechanism used for the 3,5,7-trihydroxy -6-methoxy flavone to inhibit cell viability seems to be independent of p53 tumor suppressor functions.

Flavonoids are commonly reported as substances with low cytotoxicity, but its continued use have preventive effect on the appearance of a cancer as referenced in several studies on the anticancer action of these compounds [16].

The different effect of 3,5,7-trihydroxy -6-methoxy flavone on cancer cells is related with the several differences in the oncogenic, phenotypic, metabolic properties of the cells among others.

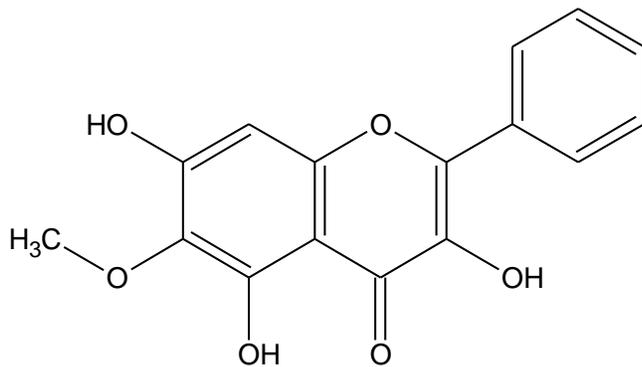
Acknowledgments

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Structure of 3,5,7- trihydroxy-6-methoxy flavone

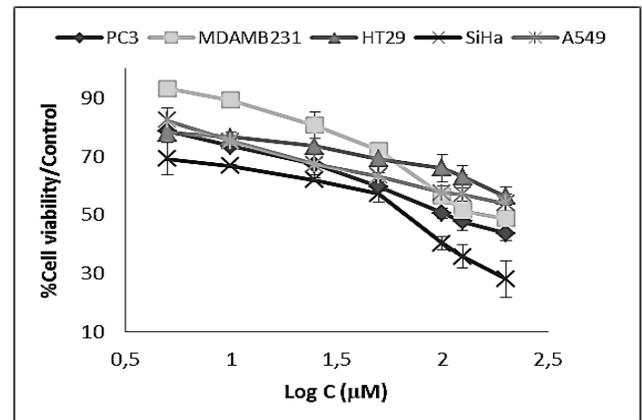


Figure 1. Relative cell viability of various cancer cell lines treated with different concentrations μM , of 3,5,7-trihydroxy-6-methoxyflavone, measured at 48 h, by using of MTT assay.

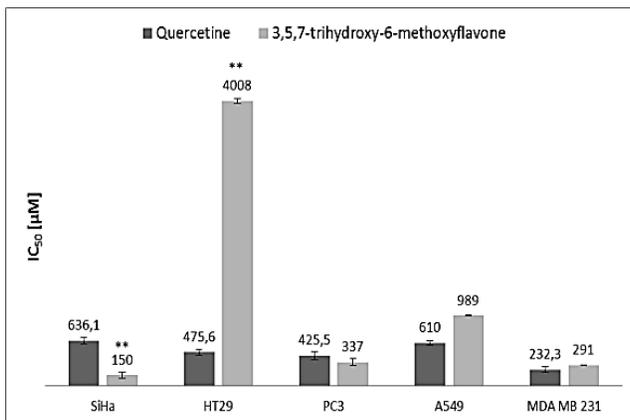


Figure 2. IC_{50} values for the 3,5,7-trihydroxy-6-methoxyflavone in micromolar concentration (μM) calculated by lineal regression equations from the relative cell viability of various cancer cell lines. Values were compared with activity of quercetin (Significant difference $**p < 0.05$)

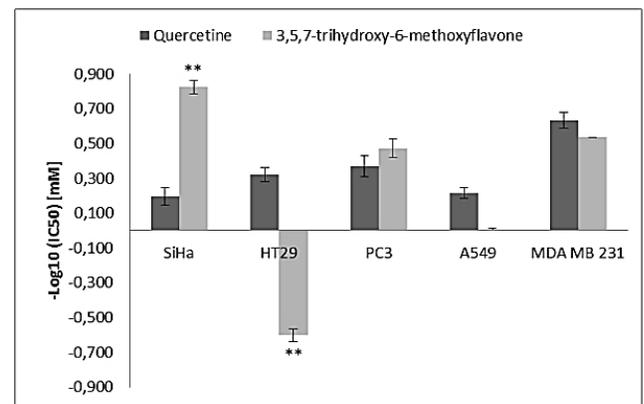


Figure 3. Cytotoxic activity of 3,5,7-trihydroxy-6-methoxyflavone expressed as pD_2 values for the flavone according IC_{50} in millimolar concentration (mM) on various cancer cell lines. (Significant difference $**p < 0.05$)

Cell line	μM VCR	μM Flavone
SiHa	1,040	150**
HT29	0,003	4008**
PC3	0,059	337**
A549	0,042	989**
MDA MB231	0,008	291**

Table 1: Cytotoxic activities of vincristine sulphate and 3,5,7-trihydroxy-6-methoxyflavone. (Significant difference $**p < 0.05$)

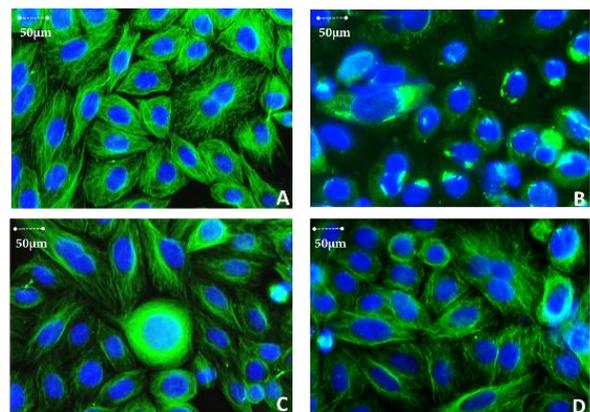


Figure 4. Morphological changes on SiHa cells. A. Untreated cells, B. Cells exposed to $0.5 \mu\text{M}$ of vincristine sulphate C. $300 \mu\text{M}$ of Quercetin D. $75 \mu\text{M}$ of 3,5,7-trihydroxy-6-methoxyflavone. Images were captured with the MoticCamPro 282A and analyzed with the Motic Image plus 2.0 software.