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Chromolaena tacotana (Klatt) R. M. King and H. Rob. Source of Flavonoids with Antiproliferative and Antioxidant Activity

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Abstract

Objetive: To determine the antioxidant and antiproferative activity of the flavonoids isolated from the leaves of *Chromolaena tacotana*. **Methods:** Extraction was carried out with dichloromethane (CH_2Cl_2) from the leaves by soxhlet, the flavonoids were isolated by column chromatography (Si gel G 60-200 microns 4 x 60cm) eluted with $CHCl_3$, mixtures of $CHCl_3$: MeOH and MeOH. The molecular structures were elucidated by ^{1}HNMR, ^{13}CNMR mono and 2D and UV spectroscopic methods with displacement reagents. Each flavonoid was evaluated for its antioxidant activity by the methods DPPH$^\bullet$ and ABTS$^{\bullet+}$ and the cytotoxic activity was evaluated by MTT analysis. **Findings:** These compounds were identified as 3,5,4'-trihydroxy-7-methoxyflavone (Ct1), 3,5,8-trihydroxy-7,4'dimethoxyflavone (Ct2), 5,4'-dihydroxy-7-methoxyflavanonol (Ct3) and 5, 7, 3', 4'-tetrahydroxy-3-methoxyflavone (Ct4). The flavonoid that had the best response in antioxidant activity was Ct4 with and IC_{50} of 2.51mg/L of DPPH$^\bullet$ and 2.13 mg/L of ABTS$^{\bullet+}$. Antiproliferative potential showed that MDA-MB-231 breast cancer cells were the most sensitivity cell line to the flavonoids, and Ct2 is the flavonoid with better antineoplastic potential on majority of cells, except for the most resistant SiHa cervix cancer cells. **Application:** The high antioxidant activity of flavonoids isolated from *Chromolaena tacotana* and its antiproliferative activity on different cell lines, make the species a plant with pharmacological potential.

Keywords: Chromolaena tacotana. Flavonoids, Antioxidant Activity, Anticancer potential

1. Introduction

The *Chromolaena tacotana (Asteraceae)* is a shrub that grows between 900-2400 m.a.s.l in the Andean region and can reach 2.5 m in height. The common names in Colombia are "almoraduz", "chicharrón", "chicharrón morado", "chilquilla", "chucha", "mastranto", "salvia morada", "sanalotodo". It is characterized by branches with bark red wine, peciolate leaves, long and dense pilose dorsal or woolly veins, and asymmetrical main veins and sometimes with violet or red wine, secondary veins reticulated or ascending, violet inflorescences with generally sessile chapters. It is commonly used against bacterial infections and against some type of cancer[1]. The genus Chromolaena produces flavonoids and other substances with important biological activities[2–5]. Preliminary studies on this species

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report as the major flavonoid in leaves and inflorescences the 4', 5-dihydroxy-7-methoxy-flavanonol with moderate antibacterial activity[6], and activity against some normal cells[7].

Chromolaena tacotana is a species phylogenetically very close to *Chromolaena leivensis* that produces a variety of flavonoids whose studies have proved useful as agents that may contribute to the treatment of breast, colon, prostate and cervix cancer[8,9]. In the current study it was intended to isolate and identify flavonoids contained in the aerial parts of *C. tacotana* and evaluate their antioxidant activity, they may be similar to those contained in *C. leivensis*[10].

2. Methods

2.1 Isolation and Identification of the Flavonoids

The leaves were extracted in soxhlet with CH_2Cl_2, the extract was flocculated with EtOH: water 1:1 to remove fats and chlorophylls, the aqueous portion was extracted liquid/liquid with CH_2Cl_2 which was concentrated in vacuum to obtain the flavonoid block. By Column chromatography with Si gel (40-60 µm), four flavonoids (Ct1, Ct2, Ct3 and Ct4) were isolated using as eluents $CHCl_3$ and mixtures of $CHCl_3$ and MeOH and purified by crystallization with hexane and MeOH according to the solubility of the flavonoid. Identification was carried by means of U.V spectra with displacement reagents (AcONa, MeONa and H_3BO_3) and mono and two-dimensional ^{1}HNMR and ^{13}CNMR spectra. The evaluation of the antioxidant activity was carried out by means of the techniques DPPH$^\bullet$ and ABTS$^{\bullet+}$[11–13].

2.2 Antioxidant Activity

Two methods were used to determine the antioxidant activity of flavonoids, DPPH$^\bullet$ (1,1-diphenyl-2-picrylhydrazyl) and ABTS$^{\bullet+}$ (2,2'-azino-bis- (3ethylbenzthiazoline)-6 Sulfonic acid).

Solutions of 50, 25, 12.5 and 6.25 mg/L of each flavonoid were prepared in MeOH and 0.2 mL of each dilution were mixed with 1.0 mL of solution of DPPH$^\bullet$ or ABTS$^{\bullet+}$ depending on the method to be used. The absorbance of the mixtures was read at 517nm or 734nm respectively, after ten minutes in a spectrophotometer Thermo Scientific, model 4001/4[14–17].

2.3 Cytotoxic Activity

Tumor derived cells were maintained as follows: colon adenocarcinoma cell line HT29 in Dulbecco's modified Eagle's medium with high glucose (Lonza), prostate cancer cell line PC-3 (in Eagle's medium (Lonza), adenocarcinomic human alveolar basal epithelial cell line A549, breast adenocarcinoma cell line MDA MB-231 and cervical squamous carcinoma cell line SiHa 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 experiment, by DAPI staining (Invitrogen) and immunofluorescence microscopy (MOTIC).

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 flavonoids 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 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 and then optical density was measured at 570 nm using a microplate reader (BioRad, Hercules, CA, USA)[18]. 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 in mg/L required to decrease 50% of cell viability (IC_{50}).

3. Results and Discussion

3.1 Compounds Isolated from the Leaves of *C. tacotana*

Four flavonoids were isolated from extracts from the leaves of *C. tacotana* and named Ct1 to Ct4.

Ct1: Yellow solid, eluted in the fractions with $CHCl_3$: MeOH 9.5: 0.5, crystallized from $CHCl_3$, Mp 214-216°C, Soluble in Acetone.

UV in MeOH, λ_m nm (A),: 266 (0.589); 360 (0.551); plus AcONa: 263 (0.694); 376 (0.534) confirms absence of OH in C_7. Plus MeONa 272 (0.668); 427 (0.653) confirms OH at C3. Plus $AlCl_3$ 271 (0.747) and 420 (0.737) OH free in C_5 and C_3.

^{1}H-NMR 300Mz, $(CD_3)_2CO$, δ ppm, m, (J): 3.94, s, CH_3O; 6.33, s; 6,71, s; 7.04, d (9 Hz); 8.19, d (9Hz); 12.16, s, HO-C_5.

^{13}CJMOD NMR, 75Mz, δ ppm (phase): 55.53 (-), 91.84 (-), 97.45 (-), 104.37 (+), 115.96 (-), 122.37 (+), 129.64 (-), 135.98 (+), 146.42 (+), 156.81 (+), 159.35 (+), 160.70 (+), 165,63 (+), 175,77 (+). The data for ^{1}HNMR, ^{13}C NMR and UV match with published data[19–21], for compound 3,5,4'-trihydroxy-7-methoxyflavone.

Ct2: Yellow solid, eluted in $CHCl_3$: MeOH 9: 1, crystallized from $CHCl_3$: hexane, Mp 270 °C Soluble in Acetone.

UV in MeOH, λ_m nm (A): in MeOH: 271 (1.248); 370 (0.426); plus AcONa 270 (1264); 372 (0.408) confirms absence of OH in C_7; plus MeONa 273 (1295); 435 (0.539) there is no OH in C_3 and $C_{4'}$ simultaneously; plus $AlCl_3$ 272 (1.043); 420 (0.578); plus $AlCl_3$ / HCl 272 (1027); 420 (0.559) confirms OH in C_5 and C_3[22].

^{1}H-NMR 300Mz, $(CD_3)_2CO$, δ ppm, m (J): 3.82, s, CH_3O; 4.01, s, CH_3O; 6.87, s; 7.04, d (9); 8,20, d (9); 12.03,s, HO-C_5.

^{13}C NMR: 75 MHz $(CD_3)_2CO$, δ ppm: 55.93 CH_3O, 59.71 CH_3O, 90.92, 104.32, 115.44, 122.44, 129.64, 132.00, 135.82, 146.42, 146.46, 152.28, 159.33, 159.41, and 175.89.

In the ^{1}HNMR spectrum, two doublet signals at 7.04ppm and 8.20ppm are observed for two hydrogens each, with a coupling constant[3] J_{HH} of 9,00 Hz common for an AX coupling system in an aromatic ring *para* di substituted, corresponding to the B ring of the flavonoid.

The HMBC spectrum show correlations to $^{3}J_{HC}$ between the hydrogens of methoxyl group at 4,01ppm and the carbon of 159.41ppm, also correlated with the hydrogens of 7.04ppm (H-3' and 5') and 8.20 ppm (H-2' and 6'). In addition, the correlation to $^{3}J_{HC}$ between the hydrogens in the 6' and 2' carbons with the alpha carbon to the oxygen having a signal of 146.46 is verified. The 7.04ppm hydrogens show correlation with the carbon of 122.6ppm (C-1') signal, giving us the full displacements of the B ring. Since the hydrogen that is found in 6.87ppm presents correlations to $^{2}J_{HC}$ and $^{3}J_{HC}$ with the carbons of 104.32, 132.00, 152.28 and 159.33 ppm and at $^{4}J_{HC}$ with 175.89ppm, therefore it is confirmed the position of the methoxyls groups in the $C_{4'}$ and in the C_7. Analysis of spectroscopic data allows the identification of flavonoid as the 3,5,8-trihydroxy-7,4'-dimethoxy flavone named also THDMF or Herbacetin 7,4'-dimethyl ether (Figure 1, 2).

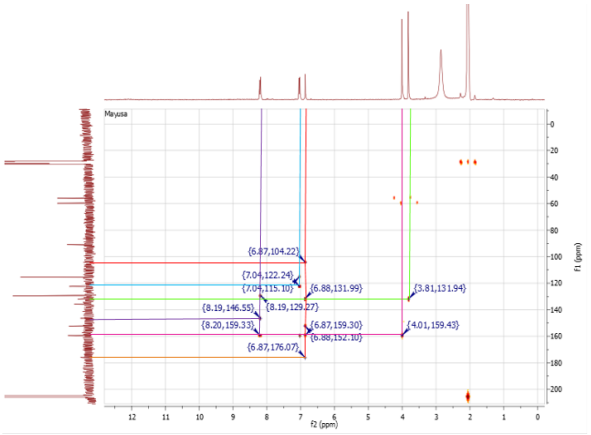

Figure 1. Two-dimensional nuclear magnetic resonance spectrum HMBC of compound Ct2.

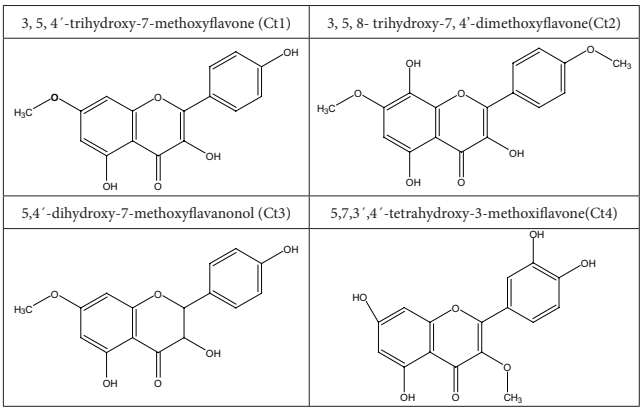

Figure 2. Compounds isolated from the leaves of C. *tacotana*.

Ct3: White solid, eluated in $CHCl_3$: MeOH 9:1, crystallized from $CHCl_3$: Mp 176°C, soluble in acetone.

UV in MeOH, λ_m nm (A), :290 (0.635); 330 (0.502); plus AcONa: 290 (0.642); 330 (0.520), confirm OH not free in C_7. Plus MeONa: 289 (0.614); 360 (0.261) indicates OH at C_4; plus $AlCl_3$: 315 (0.760); 388 (0.186); plus $AlCl_3$ / HCl: 314 (0.726); 388 (0.172) indicates OH at C_5.

^{1}H-NMR 300Mz, $(CD_3)_2CO$, δ ppm, m, (J): 3.87 CH_3O, 4.70, d (12); 4.82, s OH; 5.13, d (12); 6.06, s; 6.10, s, 6.92, d (9); 7.44, d (9); 11.7, s OH at C5.

^{13}C JMOD NMR, 75Mz, δ ppm (phase): 55.48 (-) CH_3O; 72.30 (-) CHOH; 83.55 (-); 93.81 (-); 94.86 (-); 101.23 (+); 115.0 (-); 128.13 (+); 129.44 (-); 157.96 (+); 163.11 (+); 163.80 (+); 168.35 (+); 197.84(+). Analysis of spectroscopical data of ^{1}H NMR^{13} CNMR, UV displacement reagents and the comparison of these with previous investigations[23], the compound coincide with that 5, 4'-dihydroxy-7-methoxy-flavanonol.

Ct4: Yellow Solid, eluted in $CHCl_3$: MeOH 1:1, crystallized from $CHCl_3$: Mp 268°C Soluble in dimethyl sulfoxide.

UV in MeOH, λ_m nm (A): 259 (0.884); 356 (0.509); plus AcONa 273 (0.866); 388 (0.520) OH free in C_7; plus MeONa 271 (0.684); 400 (0.269) indicates OH in $C_{4'}$; plus $AlCl_3$ 273 (0.827); 426 (0.680); $AlCl_3$ / HCl 269 (0.879); 426 (0.689) indicates OH at C_5; H_3BO_3: 262 (0.929); 386 (0.631) indicates two OH ortho on ring B.

^{1}HNMR, 300 MHz, $DMSOd_6$, δ ppm (J): 3.83, s, CH_3O; 6.31, s; 6.67; 6.88, d (9); 7.57, dd (9); 7.70, d (9): 12.46, s OH at C_5.

^{13}CJMOD NMR, 75Mz, $DMSOd_6$, δ ppm (phase): 56.44 (-) CH_3O; 92.30 (-); 97.88 (-); 104.39 (+); 115.60 (-); 116.01 (-); 120.56 (-); 122.30 (+); 136.45 (+); 145.50 (+); 147.68 (+); 148.24 (+); 156.49 (+); 160.74 (+); 165.32 (+); 176.34 (+).The analysis of spectroscopical data of ^{1}H NMR, ^{13}C NMR, UV displacement reagents and data comparison with those already published[24] allow us to propose as structure for Ct4 the 5, 7, 3 ‘, 4’-tetrahydroxy-3-methoxyflavone (3-methoxyquercetin).

3.2 Antioxidant Activity by the Methods DPPH• and ABTS•+

With the results obtained for antioxidant activity of each of the flavonoids (Ct1, Ct2, Ct3 and Ct4) the activity of

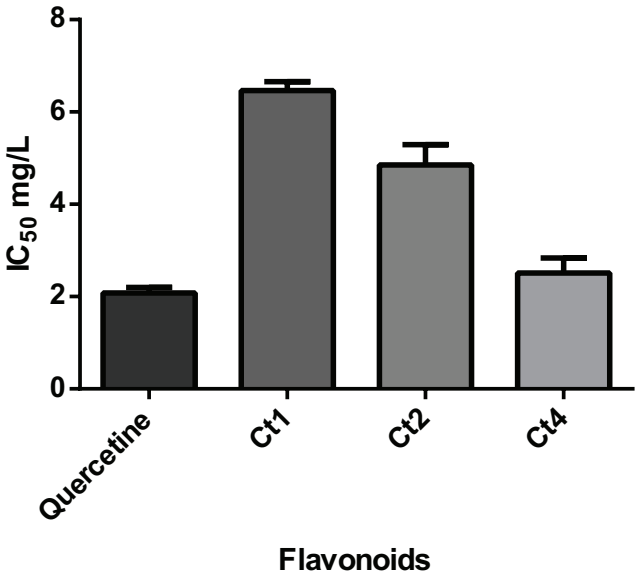

Figure 3. IC_{50} of flavonoids and quercetine, determinated by DPPH• method.

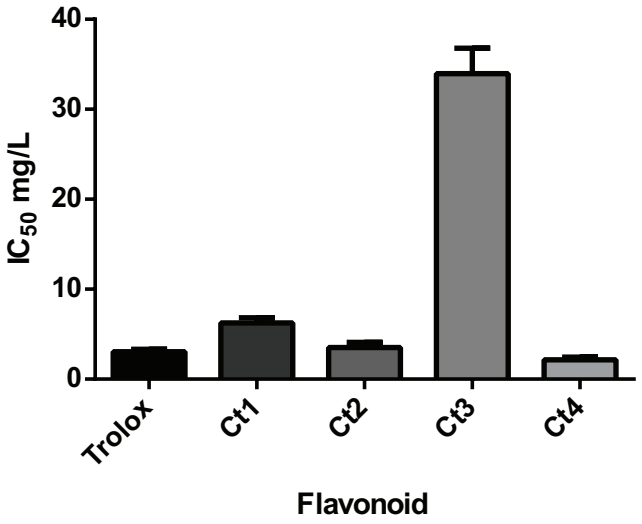

Figure 4. IC_{50} of flavonoids and trolox, determinated by ABTS•+ method.

Ct3 was discarded since it did not show inhibition greater than 50% of the DPPH• and

ABTS•+. The flavonoid that has the best response in antioxidant activity is Ct4 since it is the most polar and has the lowest IC_{50} value, that is, the lowest concentration of this is required in comparison with the other flavonoids to achieve 50% of the inhibition of DPPH radical ● and ABTS•+ (Figure 3, 4).

No significant differences in IC_{50} were observed between the two methods; However, it is observed that the IC_{50} was always lower in ABTS•+ than in DPPH• because of the poor selectivity of ABTS•+ against DPPH• since the latter does not react with flavonoids lacking OH groups in ring B whereas the latter ABTS•+ reacts with any hydroxylated aromatic

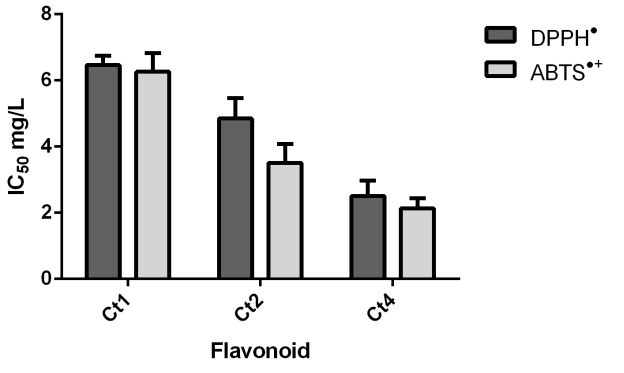

Figure 5. Comparison of IC_{50} of flavonoids with the two DPPH• and ABTS•+ methods.

compounds[25]. The results obtained from antioxidant activity with the methods DPPH• and ABTS•+ are consistent with[26,27] who evidenced the close relationship between flavonoid polarity and antioxidant activity. More polar flavonoids have the greatest potential for capturing free radicals. The Post Hoc tests: HSD Tukey and Scheffé showed that the flavonoid that has the best response in antioxidant activity is Ct4, even better than trolox (Figure 5).

3.3 Cytotoxic Activity

The viability of lung A549 colon HT29 prostate PC3 cervix SiHa breast MDAMB231 cancer cells and MRC5 healthy fibroblast was analyzed 48 hours after treatment with the flavonoids by MTT assay. Data obtained showed an inhibition of the percentage of cell viability and the inhibitory concentration at which we observe 50% in cell viability (IC_{50}) calculated from the non-linear curve regression (Figure 6). Data showed that activity of flavonoids is different for each cell line, however the most sensitivity cell line to them is the breast cancer cell MDA-MB-231, and the most resistant cell line is the cervix cancer cell SiHa (Figure 6). The flavonoids Ct1 named also rhamnocitrin and Ct3 had not reports about its antiproliferative activity on cancer cells, however, between the flavonoids Ct1 is the less cytotoxic, and the most cytotoxic is Ct3, even for healthy MRC5 fibroblast (IC_{50}=14.65 mg/L), in this order we did an analysis of selectivity index to exclude flavonoids with less specificity for tumor cells.

Data showed that Ct2 is the flavonoid with better antineoplastic potential, except for the resistant cervix cancer SiHa cells (IC_{50}=58.5 mg/L) (Figure 7). There are

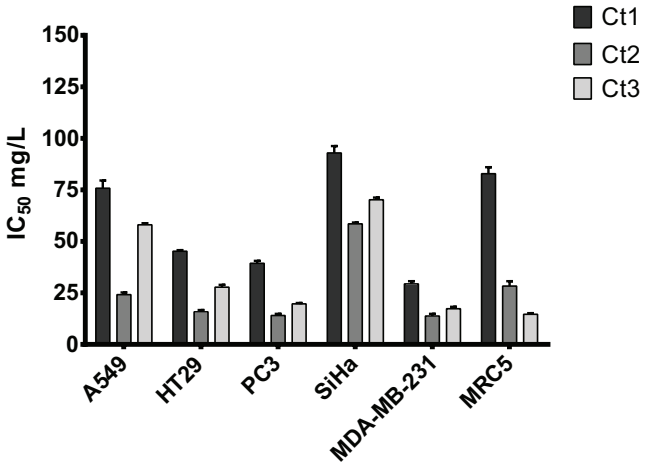

Figure 6. IC_{50} values of flavonoids on cancer cells.

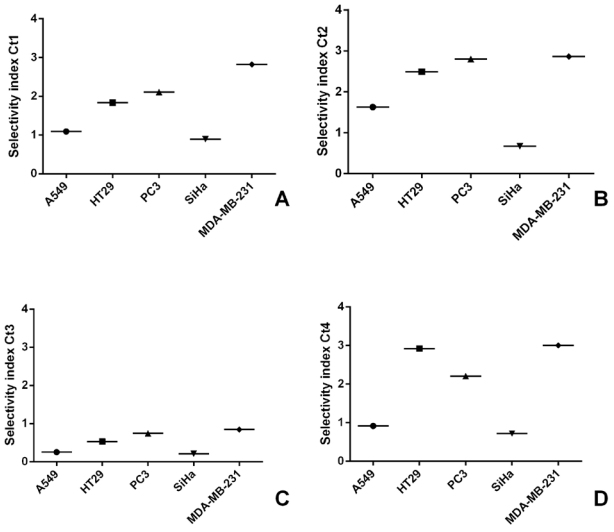

Figure 7. Selectivity index of flavonoids on cancer cells. A. Ct1 B. Ct2 C. Ct3 D. Ct4.

not reports about Ct2 cytotoxic activity, thus is a flavonoid with anticancer potential to be explored. Another promising flavonoid for cancer therapy is the flavonoid Ct4, which had a high activity against the colon HT-29 cell line and prostate PC-3 cell line with IC_{50} of 16.0 and 21.0 mg/L respectively. Recently, the antiproliferative effect of 3-methoxy quercetin was evaluated on the human medulloblastoma (Daoy), human hepatocellular carcinoma (HepG2) and human melanoma (SK-Mel-28), but only effects were found on Daoy cells with IC_{50} of 19.65 mg/L[28,29], corresponding with our findings.

The viability of lung A549 colon HT29 prostate PC3 cervix SiHa breast MDAMB231 cancer cells and

4. Conclusion

The species *Chromolaena tacotana* produces at least three flavonoids:3,5,4'-trihydroxy-7-methoxyflavone,3,5,8-trihydroxy-7,4'dimethoxyflavone and 5,7,3',4'-tetrahydroxy-3-methoxyflavone, with antioxidant activities between 6,46 y 2,51 mg/L de IC_{50} for DPPH• method and between 6,26 and 2,13 mg/L for ABTS•+ method, which are relatively similar to that of the patterns quercetin and trolox respectively. This species is a good source of flavonoids, with antiproliferative activity of cancer cell lines and we found that Ct2 is the flavonoid with better antineoplastic potential to be explored.

5. Acknowledgments

To the University of Applied, sciences and Environmental U.D.C.A, for its financial support, project Chromolaenas Colombianas like source of anticancer substances.

6. Conflict of Interests

The authors declare that this article does not present any conflict of interests

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