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ANTI-COLON CANCER POTENTIAL OF TWO FLAVONE ISOMERS ISOLATED FROM *Chromolaena tacotana* (Klatt) R.M. King & H. Rob.

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Abstract

The anticancer potential of the *Chromolaena tacotana* has been studied in recent years due to its high content of antioxidant and cytotoxic flavonoids against some cancer cells types. This study aimed to analyze the cytotoxicity, genotoxic potential, and apoptosis-inducing properties of two flavone isomers isolated from leaves of *Ch. tacotana:* 3,5,3'-trihydroxy-7,4'-dimethoxyflavone (F1) and 3,5,8-trihydroxy-7,4'dimethoxyflavone (F2) and additionally, their effect against *Campylobacter jejuni* growing, a microorganism that promotes colorectal cancer through production of toxic substances. Cytotoxic effects and selectivity for cancer cells were determinate using colorectal cell lines RKO and HT29 and the normal cells FHs 74 Int - CCL-241 by MTT assay. Genotoxic effects were analyzed by comet assay, apoptosis induction of cancer cells by flow cytometry, and the flavone's activity against *Campylobacter jejuni* growing was evaluated through microdilution in broth. The two flavones F1 and F2 showed a greater cytotoxic effect than quercetin on colon cancer lines, but, the activity of F2 was higher than F1. Concerning genotoxic activity, compound F1 showed greater damage to cell DNA than F2. As quercetin, at IC_{50} values, both flavones are apoptosis-inducing compounds in colon cancer cells. Also, it was observed the inhibition of *Campylobacter jejuni* growing specially induced by F2. This study shows the anti-colon cancer potential of two flavones isolated from *Ch. tacotana* and how the position hydroxyl groups significantly affect the activities evaluated.

Keywords:*Chromolaena tacotana*, flavones, cytotoxicity, genotoxicity, apoptosis, *Campylobacter jejuni*.

Introduction

According to the World Health Organization (WHO), colorectal cancer (CRC) is the third type within the highest number of deaths with a total of 774,000 deaths worldwide; being the third most common in men and the second most common in women, additionally, several side-effects have been reported to be associated with chemotherapeutic agents [1]. Between risk factors, enteric pathogen and pathobionts infections including *Campylobacter jejuni* who have been associated with CRC development through the action of cytolethal distending toxin [2]. The use of medicinal plants has provided a starting point for the development of new drugs that contribute to the prevention and treatment of cancer with less toxic effects on normal cells [3], which is one of the main drawbacks of the current chemotherapy. Between natural compounds, with beneficial effects on human health, flavonoids are well known [4]. These polyphenolic compounds have been related with cancer prevention, antiestrogenic, antiproliferative activity, induction of cell cycle blocking of cancer cells at a given stage and apoptosis, prevention of oxidation, induction of detoxification enzymes, regulation of the immune system, and changes in the cell signaling [4], and other activities that have been influenced or depend on the compound structure [5]. Additionally, flavonoids provide various biochemical functions in seed maturation, protection from different biotic and abiotic stresses, and act as detoxifying and defensive agents. In this respect, researchers have evaluated the relationship between flavonoids structure and antibacterial activity [6], which could depend on the substitutions on the aromatic rings [5]. Common flavonoids as quercetin and galangin have shown inhibition of *Campylobacter jejuni* [7].

The genus *Chromolaena* is considered a great source of flavonoids with antioxidant and cytotoxic activity [8], however, several of the flavonoids isolated from the leaves of the plant species *Chromolaena* have not yet been evaluated on their anti-cancer potential on CRC cell lines as RKO, only have been reported preliminary data against HT-29 cell line, among them, the compound F2 has shown a potent cytotoxic activity for that colon cancer line [8]. RKO and HT-29 are colorectal cell lines that differ specially in the expression of the tumor suppressor protein p53, being the protein status wild type in RKO and mutated in HT-29 [9].

The purpose of this study was to analyze and interpret the relationship between the structure-activity of two flavone positional isomers, isolated from aerial parts of *Ch. tacotana* by comparing the cytotoxicity, genotoxicity, apoptosis-induction potential, and additionally, the inhibition of *Campylobacter jejuni* growing, a pathogen related to the development of colon cancer.

Methods

Flavonoids: The two flavonoids were obtained by PRONAUDCA research group who isolated from leaves of *C. tacotana*. The flavonoids were identified as 3,5,3'-trihydroxy-7,4'-dimethoxyflavone (F1) and and 3,5,8-trihydroxy-7,4'dimethoxyflavone (F2). The flavonoids spectroscopy data were already described previously [8, 10]. Quercetin (Sigma-Aldrich) was used as a positive control in the different tests.

Culture conditions and inhibition of colon cancer cells viability: The 3-(4,5-methyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay (MTT) was used to evaluate the effect on cell viability of HT-29 - HTB-38™ and RKO - CRL-2577™ colorectal cancer cells and against FHs74Int normal colon cells all of them maintained according to ATCC recommendations and 10% (v/v) Fetal Bovine Serum (Biowest), 2 mM L-glutamine, 5,000 IU/mL penicillin and 5 mg/mL streptomycin (Lonza). Cells were seeded in a 96-well plate in a density of 70% of confluence and incubated at 37°C and 5% CO_2. Flavones F1 and F2, and quercetin were added in concentrations ranging between 6.25-300 µg/mL followed by 48h incubation. MTT solution (Sigma-Aldrich) was added and incubated for an additional 4h. Formazan crystals were dissolved with 100 µl of DMSO. The results were determined by the optical density determined by the absorbance at 570 nm. Estimation of the half-maximal inhibitory concentration (IC50) was done using non-linear regression from plotting cell survival (%) versus flavonoids concentration [µg/mL] using Graph Pad 8.0 software. All the experiments were performed in triplicate and at least two independent replicates.

The selectivity index (SI), was applied as exclusion criteria for fractions [11, 12] calculated as follows: Selectivity Index (SI)= (normal cell IC50)/ (tumoral cell IC50).

Genotoxic activity: Single cell gel electrophoresis (SCGE) and the comet assay: To determine the genotoxic potential of flavonoids, that is, the DNA damage of cells exposed to them, the displacement between the genetic material of the nucleus (head of the comet) and the resulting tail (tail DNA) from the fluorescence emitted by the DNA of at least 50 individual cells that were subjected to electrophoresis and subsequently processed using the OxiSelect ™ Comet Assay Kit (Cell Biolabs) according to the manufacturer's instructions. Cells were stained with Vista Green DNA from the Kit for visualization by fluorescence microscopy. Images were captured with a MoticCamPro 282A and analyzed with Comet Score software (Tritek Corp, Sumerduck, VA, USA). The results were expressed as a mean of DNA migration or the percentage of tail DNA. $Tail\ DNA\% = 100\ x\ Tail\ DNA\ Intensity\ /\ Cell\ DNA\ Intensity$
The means of the percentage of tail DNA were subjected to analysis of variance (ANOVA), with post-hoc tests of HSD-Tukey and Scheffé for the determination of genotoxicity [13]

Apoptosis induction Annexin-V assay: To determine the viable cell population versus those in a state of early and late apoptosis after exposure to compounds, a flow cytometry analysis was performed using the Muse® Annexin-V & Dead Cell Reagent kit (Luminex Corporation), and the readings were performed on the Guava® Muse® Cell Analyzer (Luminex Corporation) (Luminex Corporation, 2019). The RKO and HT-29 cells were treated and incubated for 24 h with the quercetin control and the two flavonoids at the corresponding IC_{50}. The samples were prepared according to the manufacturer's recommendations, mixing 100 µl of the Muse® Annexin V reagent to each tube containing 100 µl of the 100,000 cells/ml cell suspension and incubated for 20 min at room temperature in the dark. Later the samples were analyzed in the MUSE cytometer.

Anti-Campylobacter jejuni test: The evaluation of the flavone's activity against *Campylobacter jejuni* growing, was done through microdilution in broth. A strain of *Campylobacter jejuni* obtained from molecular laboratory ceparium of the University of Applied and Environmental Sciences was used at a concentration of $1x10^8$ CFU / ml (standard 0.5 of the Mc Farland scale) belonging to the microbiology strain. The bacteria were initially cultivated in Bolton broth and incubated at 42°C in microaerophilic for 48 hours. Subsequently, they were isolated on 5% lamb blood agar and soybean trypticase agar, with incubation at 42 °C for 3 days, with a 10 % CO_2 atmosphere and microaerophilia, for which a generator envelope from this environment was used. Quality control of the bacteria was carried out through Gram stain and biochemical tests such as cytochrome oxidase and catalase were carried out [14, 15]. The compounds including the positive control quercetin were diluted in dimethyl sulfoxide and serial dilutions were done in a range of 2.5 µg/ml to 0.3 µg/ml. Dilutions were mixed with the bacteria (1:1) in a final volume of 100 µl. After 48 hours of incubation, the minimum inhibitory concentrations (MIC) were determined as the lowest concentration of the flavonoid that inhibits *C. jejuni* growth, marked change in turbidity for the control. Results were confirmed by cultivating the samples in Bolton broth medium and Wilkins Chalgren agar under the aforementioned conditions [15].

Results and Discussion

The structural differences of flavones F1 (figure 1A), and F2 (figure 1B) are in the substituents of the benzoyl system (ring A) and the cinnamoyl system (ring B). For F1 the cinnamoyl system is more oxygenated, while for F2 the most oxygenated system is benzoyl, this difference in oxygenation is possibly facilitating the cells viability effects and the interaction between F2 and molecular targets for the induction of apoptosis in both CRC cell lines [8, 10]. In this respect, it has been suggested that the hydroxyl groups in ring A induce and improve the activity of the compound, playing an important role in modulating the activity [5].

The data showed that F1 and F2 suppressed cell viability of HT29 and RKO cells, more than quercetin,

but it is F2, the flavonoid with the highest cytotoxic activity on CRC cells (figure 2A). The selectivity index (SI) is a ratio that allows comparing the cytotoxicity on normal cells and cancer cells, which indicates that the higher the ratio, the compound is more effective and safer during a treatment. In this respect, the F2 selectivity index ratio for RKO cells exceeds the value of 10 (figure 2B), indicating that its activity on these cells is possibly favored by the expression of the wild-type p53 protein. Mutations in p53 or the loss of its functionality occur mainly in the transition from adenoma to cancer, and the frequency of alterations in the gene increases with the corresponding progression of the lesion [16], this could be closely related to the resistance observed in mutated R273H/HT29 cells.

The in vitro genotoxicity results expressed as a percentage of DNA damage exhibited a greater displacement between the genetic material of the nucleus (comet head) and the resulting tail (tail DNA) in the cells after 24 h of exposure to F1 (figure 3). These data indicate that once F1 enters cells, nuclear integrity is affected, a process that accompanies the mechanism of cell death. The flavonoid F2 showed a lesser effect in both CRC cell lines.

Previously was found that quercetin could down-regulate Bcl-2 as well as up-regulate Bax, which may contribute to its apoptosis induction [17]. As the quercetin, the two flavonoids induce apoptosis in CRC cells, however, treatment with F2 at 24 h results in a higher percentage of cells in early and late apoptosis (figure 4). The flow cytometry analysis showed resistance of HT-29 cells to programmed death which could be mediated by the overexpression of the mutated P53 gene, a condition that is not presented by RKO. Other hydroxy methoxylated flavonoids have shown similar effects on colon cancer cells, as the 3', 5-dihydroxy-3,4',6,7-tetramethoxyflavone or casticin that has been evaluated in the HT-29 cells and induced intrinsic apoptosis by downregulation of c-FLIP, Bcl-2, x -IAP, and survivin and cell cycle arrest by altering the Bax / Bcl-2 ratio and activating caspase-3 [18].

The evaluation of the activity of the flavonoids against the growth of *Campylobacter jejuni* was carried out through microdilution in broth. The quercetin positive control showed the highest activity on bacteria but no significant difference was found in the activity of the flavonoid F2 (figure 5), while comparing the results obtained with F1, these were significantly different.

In conclusion, this study provides clear evidence that positional isomerism especially of hydroxyl groups influences significantly the activities evaluated in order to verify the inhibitory potential of two flavones on colon cancer cells progression.

Acknowledgments

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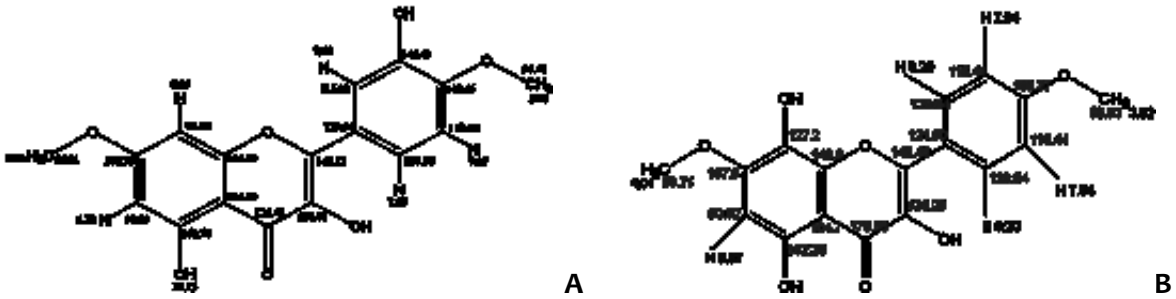

Figure 1. Flavonoids structure. A. 3,5,3'-trihydroxy-7,4'-dimethoxyflavone (F1), and B. 3,5,8-trihydroxy-7,4'dimethoxyflavone (F2).

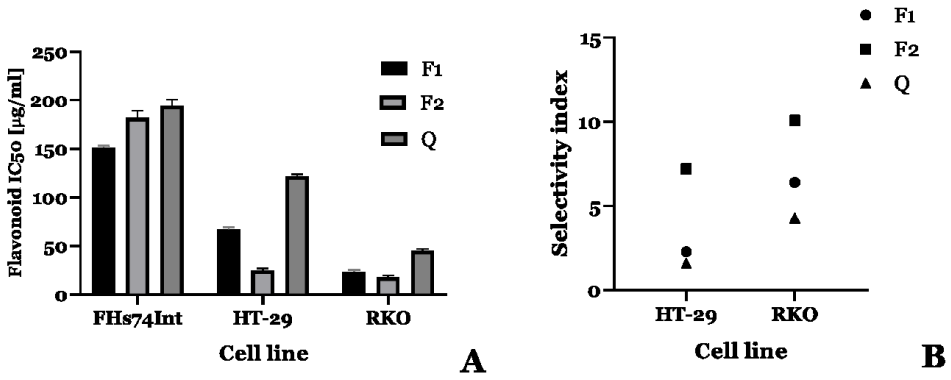

Figure 2. Cytotoxic activity of F1 and F2 on Ht-29 and RKO CRC cells. A. IC50 values including quercetin the positive control. B. Selectivity index by comparing IC50 values with FHS-74-Int normal cells.

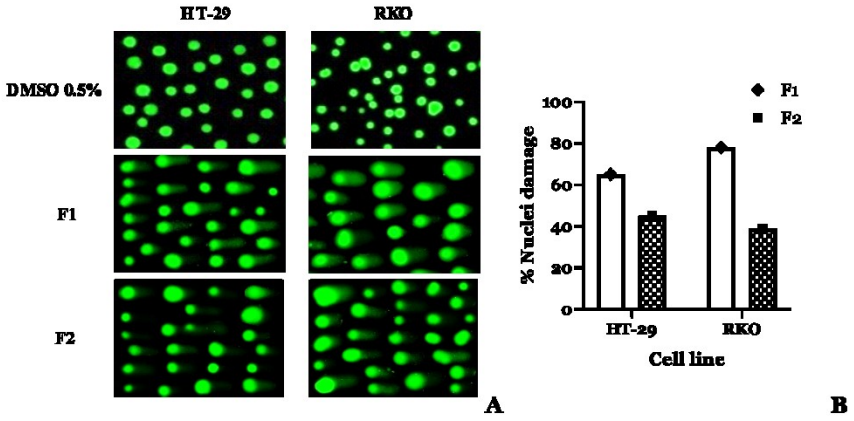

Figure 3. Genotoxic activity of F1 and F2 on Ht-29 and RKO CRC cells. A. Comet assay images captured with a MoticCamPro 282A, DNA was stained with Vista green. B. Selectivity index by comparing IC_{50} values with FHS-74-Int normal cells.

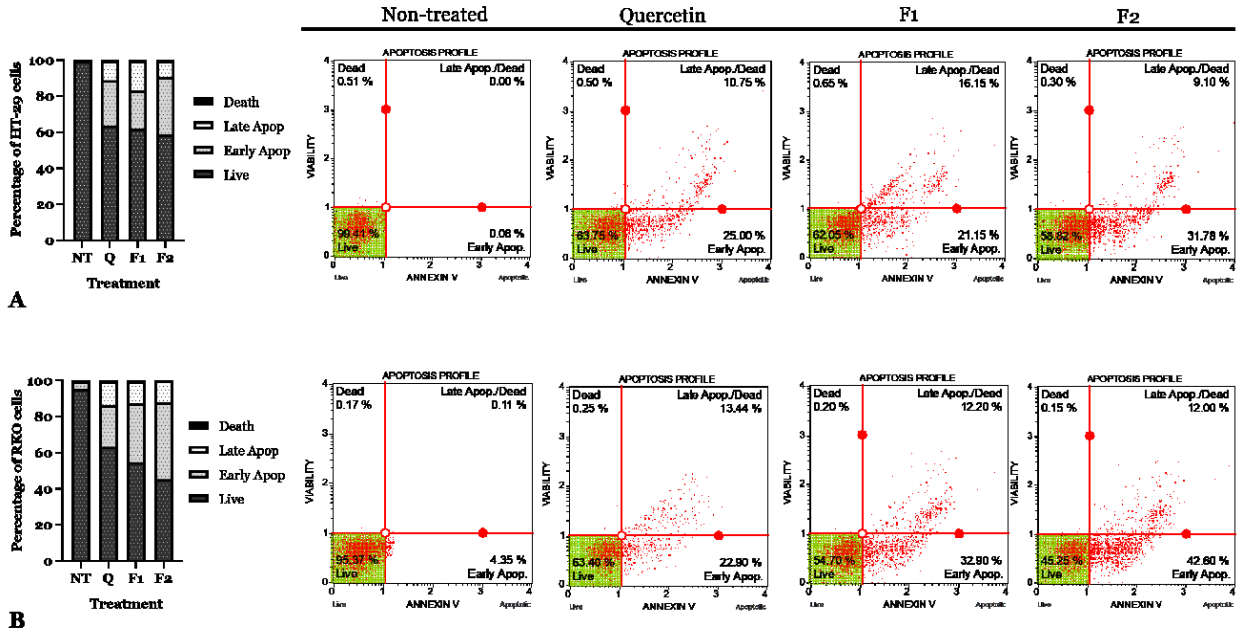

Figure 4. Percentage of CRC cells in early and late apoptosis induced by F1 and F2 compounds (representative plots). A. RKO. B. HT-29.

Flavone Treatment	Turbidity 48 h		
[µg/ ml]	Quercetin	F1	F2
0.00	+++	+++	+++
0.31	---	++	++
0.62	---	+	—
1.25	---	—	—
2.50	---	—	—
MIC value [µg/ ml]	0.3	1.2	0.6

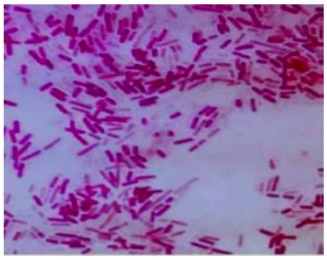

Figure 5. *Campylobacter jejuni* growing inhibition by F1 and F2. Values expressed as minimum inhibitory concentrations (MIC).