# ISOLATION, STRUCTURAL ELUCIDATION AND CYTOTOXIC ACTIVITY OF TRICIN, A NATURAL OCCURRING FLAVONE FROM CAUCA´S VALLEY SUGARCANE

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Bachelor Thesis

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## SUMMARY

The Sugarcane can be considered as the most representative crop in the Cauca Valley region southeast of Colombia. The metabolic diversity of this plant makes it suitable as input for several industrial processes, such as ethanol and sugar production. Thus, sugarcane industry is one of the pillars of the local economy.

The study and identification of metabolites present in sugarcane opens a new perspective towards the development of new products with potential pharmaceutical and nutritional value. Through the optimization of the sugarcane exploitation, the Cauca´s Valley economic development would be enhanced.

Previous studies have shown that sugar cane is a potential source of flavonoids, mainly in the C-glycoside form[1-5]. These compounds have special value inside the food and pharmaceutical industries because of their antioxidant properties.
The main objective of this project is to achieve the structural determination of some of these metabolites produced by the Cauca´s Valley sugarcane, by using Nuclear Magnetic Resonance and LC-MS spectrometry.

The study resulted in the full characterization of tricin, a natural occurring flavone which is regarded as a potential therapeutical agent for colorectal cancer treatment.

# 1. GENERAL

## 1.1 Sugarcane industry in Colombia

In Colombia, the Sugarcane fields are concentrated in the southeast of the country, i.e. the geographic area around the Cauca´s River, which comprises 39 municipalities of the provinces of Cauca´s Valley (78%), Cauca (19%), Risaralda (1.6%) and Caldas (1.3%). The total cultivated area is around 200.000 hectares and almost 50% of a 2.3 million tons of sugar produced each year is exported. The region has the necessary microclimatic conditions for a full year cultivation of the crop, unlike other regions where this is limited to 4 to 6 months a year. The cost of sugarcane cultivation in Colombia is less than a half than in other places; which makes the Colombian Sugarcane industry one of the more efficient in the World[6].

## 1.2 The Flavonoids

Flavonoids can be found in almost every superior plant. There are over 8000 known derivatives in vascular plants. Their function is diverse, being antioxidant and antimicrobial agents, photoreceptors, UV protectors, metal chelating agents and insect visual attractors, among others[7].

They were discovered by the Biochemistry Nobel Prize laureate Dr. Albert Szent-Györgi, who first denominated them as "vitamin P". Szent-Györgi discovered that flavonoids favored the function of vitamin C, by enhancing its absorption and protecting it against oxidation. Flavonoids comprise various kinds of natural substances, among which are those who give the yellow, orange, red, violet and blue colors to many flowers, leaves and fruits.

The basic chemical structure of the flavonoids is very characteristic. Figure 1 shows that these molecules have two benzene rings (ring A and ring B), attached through a chain of three carbon atoms. Hence, flavonoids are often denominated as $C_6C_3C_6$ compounds. Flavonoids can contain a central heterocyclic ring (ɤ-pyrones), which is the most common, or an open three carbon chain

(chalcones). The oxygenation pattern can vary and can be in the form of OH, OMe, dioxymethylene and glycosides. Polymerization degree between C-C units intensifies color. Hydroxylation in the aromatic rings is very often in positions 7 and 4´, frequent in positions 5 and 3´, infrequent in 5´ and 8 and very infrequent in 6 and 2´.

Figure 2**a** shows the structure of one of the most common flavonoids found in nature: the Quercetin, a flavonol[8]**.**

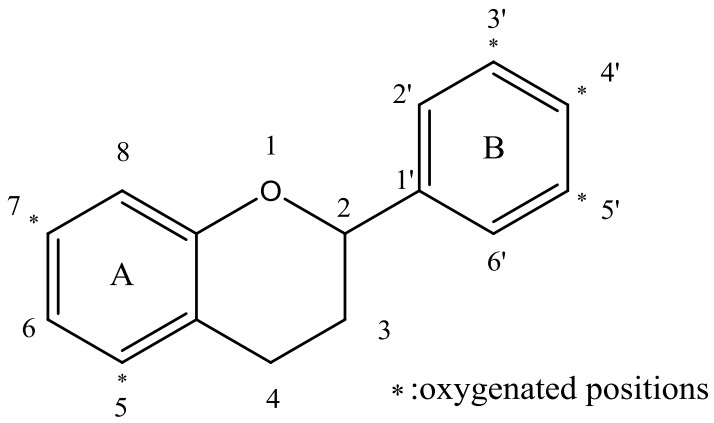



**Figure 1:** The flavonoid nucleus.

As for most of the phenolic type compounds, flavonoids are highly polar. They are located in the cellular cell´s vacuoles, hence they are hydrophilic. Extraction with water or aqueous solvents might be disadvantageous because of co-extraction of other hydro soluble molecules like sugars, peptides or enzymes. Isolation is achieved by using solvent of creasing polarity or directly with alkali.

## 1.3 Flavonoids in the sugarcane

The flavonoid presence in sugarcane has been reported several decades ago. Studies using capillary electrophoresis, HPLC-UV/PAD[9] and HPLC-UV-MS[10] among others have been performed. It is known that the major flavonoids are flavones, such as luteolin (2**b**), tricin (2**c**), apigenin (2**d**), flavonones like naringenin (2**e**), and its derivatives[11].

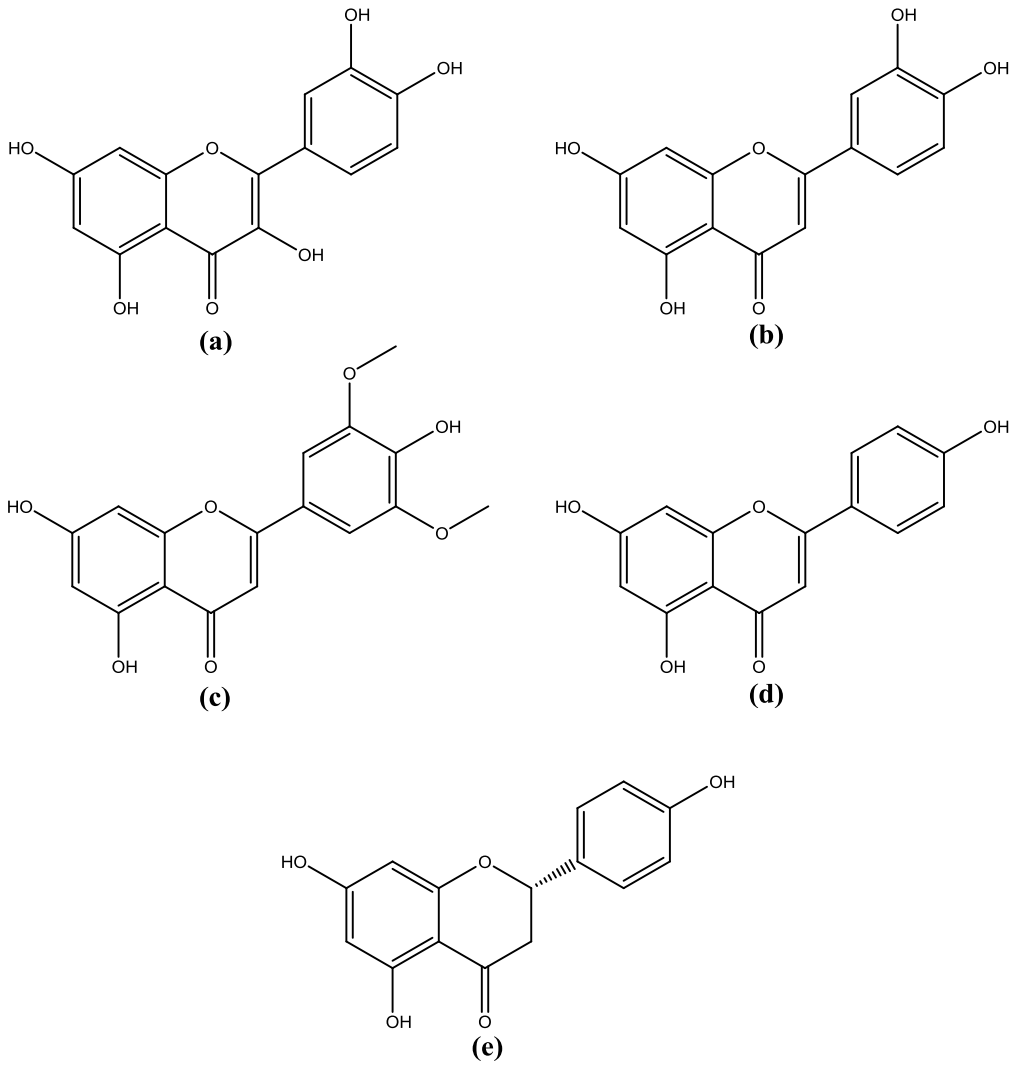



**Figure 2:** **(a)** Quercetin, a flavonol **(b-d)** Flavones commonly found in sugarcane, i.e. luteolin, tricin and apigenin, **(e)** Naringenin, a flavonone.

Diverse species sugar cane are grown around the world, and it is known that there are differences in the flavonoid content between them[12]. Flavonoid expression in sugarcane depends on several factors, such as weather, cultivation conditions, ecologic interactions and variety[13].

## 1.4 Chemopreventive Properties

Several naturally occurring flavonoids show cancer chemopreventive properties in experimental models. Nevertheless, only a few can be regarded as potential candidates for clinical development as cancer chemopreventive agent. For the matter of this study, a special flavone is worth noting: the tricin. This molecule has become focus of investigation, since it was discovered to interfere potently with the growth of human-derived mammary and colonic cancer cells in vitro[14].

A preliminary evaluation tentatively suggests that tricin possesses a favorable safety profile[15]. This hypothesis is based on three findings. Firstly, tricin failed to produce any sign of general toxicity or specific tissue damage in an acute toxicity study in mice five daily doses of 1,000 mg/kg (by gavage). Second, tricin lacked DNA topoisomerase II-inhibiting properties, unlike quercetin and genistein[16], which is related with low mutagenicity. Thirdly, unlike quercetin, tricin was deficient of genotoxic properties, as reflected by its inability to induce either chromosomal aberrations in Chinese hamster ovary cells, micronuclei in bone marrow erythrocytes in Swiss-Webster mice, or revertant colonies in the Salmonella–E. coli assay.

# 2. OBJECTIVE

## 2.1 General objective

- Determinate the chemical structure of some flavonoids produced by Cauca´s Valley sugarcane.

## 2.2 Specific Objectives

- Purify the main compounds of the obtained extract, using preparative HPLC.
- Identify the chemical structure of the purified fractions based on theirs $^{1}$H-NMR, $^{13}$C-NMR, HSQC, HMBC DEPT and MS spectra.

# 3. EXPERIMENTAL PART

## 3.1 High performance liquid chromatography

### 3.1.1 Chemicals and equipment

The flavonoid extract was purified from sugar cane juice from *Manuelita* plant. The acetonitrile and methanol used was HPLC-grade (VWE International, BDH Prolabo, France). HPLC-grade water was purified using a Millipore Millipak Express 20 system (Millipore Corp., New Bedford, MA, USA). Formic acid 50% solution was purchased from Sigma Aldrich (Switzerland).

The HPLC analyses were carried out on an Agilent Technologies 1100 series system consisting in a Degasser, Bin pump, ALS (1200 series), and DAD detector. The preparative HPLC separations were performed in an Agilent Technologies 1100 series chromatograph connected to an ultraviolet multi wave length detector (MWD). A 150 mm x 4,6 mm (5 µm) Eclipse XDB-C18

and a 150 mm x 4,6 mm, (5 µm) Nucleodur Sphinx columns were used (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Because of the size of the sample, this last column was used in both analytical and preparative scale. The temperature was thermostatically controlled at 30°C in every run.

### 3.1.2 Sample Preparation

The 8 mg flavonoid extract was dissolved in 800µL methanol and 100 µL were taken for analysis. Acetonitrile was used to set the final concentration to 1 mg per ml.

### 3.1.3 HPLC Parameters optimization

Sugarcane is a challenging matrix due to the high content of water-soluble compounds such as sugars. In addition, the flavonoid chromatographic profile on the sample, necessary for preparative HPLC separation, was unknown. For that reason, preliminary HPLC analyses were performed.

The separation was optimized by varying one parameter at a time (flow rate, mobile phase and stationary phase), and analyzing the respective chromatogram.

A 150 mm x 4,6 mm (5 µm) Eclipse XDB-C18 column was preconditioned by injecting 10 µl of blank solution (MeOH / ACN 90:10) with a gradient of water (solvent A) and acetonitrile (solvent B), with the chromatographic program described in table 1. This operation was performed 3 times.

In the first run, 10 µl of flavonoid extract were injected and a UV spectrum was recorded from 200 to 400 nm. This preliminary approach showed one mayor peak and a minimum of eight minor peaks, but poorly separated. No peaks were found after 15 minutes.

| Time (min) | Solvent B (%) |
|---|---|
| 0 | 10 |
| 2 | 10 |
| 3 | 30 |
| 25 | 100 |
| 33 | 80 |
| 37 | 40 |
| 38 | 10 |
| 40 | 10 |

**Table 1:** Starting Chromatographic program

The next improvement was achieved by reducing the analysis time to 30 minutes, injecting 20 µl of the sample, and using the following conditions: 0-5 min, 10% B and 3-30 min, 10-70% B. Nevertheless, this time the peaks appeared all together around 20 min retention time.

The next step was testing a 150 mm x 4,6 mm (5 µm) Nucleodur Sphinx column under the same conditions. Even though the peaks continued to appear together, they had better shape. Because no peaks were found before minute 12, an isocratic mode was used in the further analyses.

An isocratic 70:30% $H_2O$/ACN mode was tested. An injection volume of 20 µl and a flow rate of 1.0 ml/min were set. Here, 4 of the peaks appeared well separated between minutes 6 and 10, while the remaining peaks around minute 12, stilled not well separated. Formic acid was added to a final concentration of 0.1% in the mobile phase, and the flow rate reduced to 0.7 ml/min. In acidic conditions, the separation profile was improved.

An isocratic flow consisting of 60:40% $H_2O$/ACN was also tested, but the results were not better than the 70:30% system. Thus, the optimal separation conditions were set to an isocratic 70:30% $H_20$/ACN, 0.1% formic acid as mobile phase using a 5 µm Nucleodur Sphinx column, flow rate of 0.5 ml/min and 100 µl of injection volume. This system allowed a good separation of 7 out 9 peaks in the sample (Fig.3)

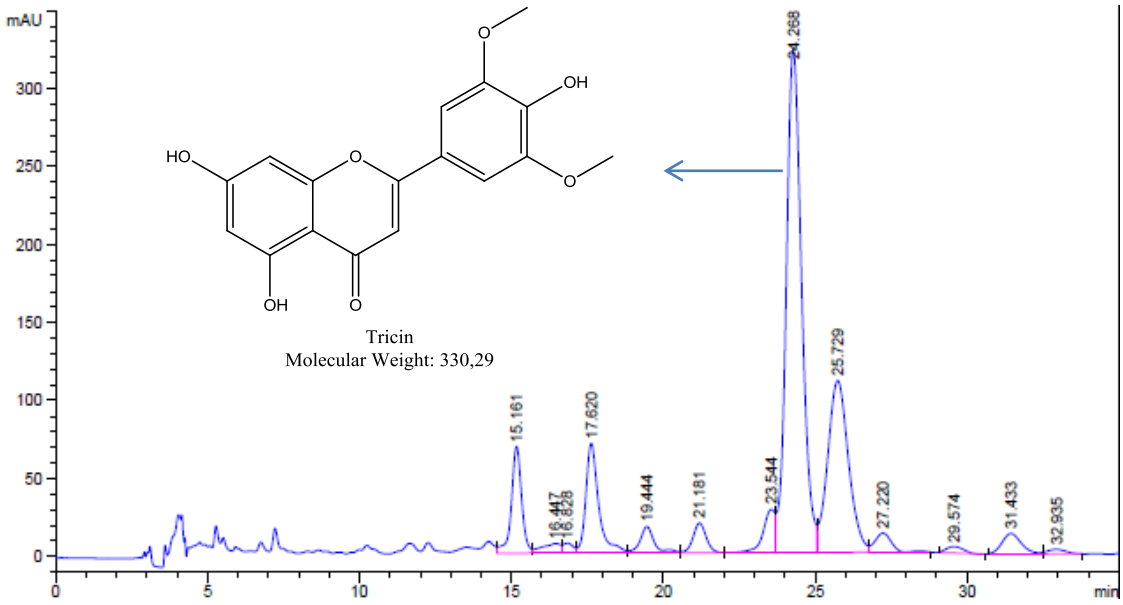



**Figure 3:** Chromatogram showing the optimal separation method for the flavonoids extract. The retention time of tricin is 24.268 min, under these conditions.

### 3.1.4 Preparative HPLC

The optimal chromatographic conditions described above, were scaled up to preparative HPLC, and 9 different fractions corresponding to the 9 chromatographic signals, were collected. Due to the multi wave length detection method of the instrument (instead of DAD) the wave length detection were set to 254 and 330 nm. The solvent was dried at room temperature and the compounds weighted (Table 2)

| Fraction | Dry Weigh (mg) |
|---|---|
| 1 | 0 |
| 2 | 0,4 |
| 3 | 0,4 |
| 4 | 0,3 |
| 5 | 0,2 |
| 6 | 0,8 |
| 7 | 0,6 |
| 8 | 0,4 |
| 9 | 0,5 |

**Table 2:** Weigh of the collected compounds

## 3.2 NMR SPECTROSCOPY

### 3.2.1 Chemicals and equipment

All analyses were performed either in a Bruker NMR 400 MHZ UltraShield or in a DMX 600 Oxford NMR equipped with Cryoprobe. This feature increases the sensitivity of the probe coil and reduces the level of thermal noise generated by electronic circuits, which makes the signal to noise ratio increase by about 4X vs. a room temperature probe[17]. The methanol-$d_4$ and the DMSO-$d_4$ 99.8% were purchased from Deutero GmbH, via Sigma Aldrich.
Due to the low yield on every fraction (table 2), the samples were measured on the 600 MHz NMR in the organic chemistry department, for better resolution. One and two dimensional experiments of fractions 5, 6 and 8 were performed, since structural determination in other fractions could not be achieved because the sample yield was too low for NMR analyses

### 3.3.2 Sample Preparation

The purified samples were dissolved in either methanol-$d_4$ or DMSO-$d_4$ 99.8%. A shaker and an ultrasonic bath were used for this propose, for 5 minutes and 40 seconds, respectively.

## 3.3 LC-MS

### 3.3.1 Chemicals and equipment

The mobile phase for the LC-MS experiment was prepared under the same conditions and with the same chemicals as described in section 3.1.1. The chromatographic conditions were set as described in section 3.1.4. Analyses were performed using an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany) equipped with an 1100 series LC/MSD Trap operated under the

following conditions: positive mode; capillary voltage, 3500 V; drying gas temperature, 350°C; drying gas flow, 8 l/min; nebulizer pressure, 30 psi.

### 3.3.2 Sample Preparation

A stock of the original flavonoid extract was diluted to a final concentration of 7µg/ml, using mobile phase. LC-MS spectra was recorded both in positive and negative mode.

## 3.4 Cytotoxicity assay ex-vivo

J774 macrophages were isolated from BALB/c mice, seeded in a 35-mm plate (Tarsons, India) with RPMI-1640 medium (supplemented with 10% heat-inactivated FCS), and kept in a humidified atmosphere of 5% $CO_2$ at 37°C for adherence. After 24 h, the cells were exposed to the flavonoid extract (100, 10 and 1 µg/ml) for 24 h. Afterwards, the cells were washed with PBS and incubated (37ºC) with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) 0.4 mg/ml for 60 min. MTT solution was removed and the cells solubilized in DMSO (100 µl). The extend of reduction of MTT to formazan within cells was quantified by measurement of optical density at 595 nm ($OD_{595}$).The cytotoxic dose 50 ($CD_{50}$) was defined as the concentration of drug, which decreases $OD_{595}$ in 50% in relation to control cultures.

# 4. RESULTS AND DISCUSSION

## 4.1 Spectroscopy data for Flavonoid fraction 6

### 4.1.1 NMR data

**$^1$H-NMR** (MeOH- $d_6$, δ[ppm], J[Hz]): 7.20 (s, 2 H, **H**-a); 6.67 (s, 1 H, **H**-b); 6.49 (d, 1 H, $J_{Hc\text{-}Hd}$ =2.1, **H**-c); 6.21 (d, 1 H, $J_{Hd\text{-}Hc}$ =2.1, **H**-d); 3.96 (s, 6 H, OC**H**$_3$);

**$^{13}$C-NMR** (MeOH- $d_6$, δ[ppm], J[Hz]): 183.86 (s, 1C); 166.30 (s, 1C); 166.09 (s, 1C); 163.25 (s, 1C,); 159.48 (s, 1C); 149,68 (s, 1C); 141.26 (s, 1C); 122.73 (s, 1C); 105.31 (s, 1C); 105.28 (s, 1C,); 104.58 (s, 1C, C-13); 100.22 (s, 1C, C-14); 95.16 (s, 1C); 57.06 (s, 2C)

**Table 3**

$^{1}$H NMR and $^{13}$C NMR spectroscopic data for compound fraction 6 (In methanol- $d_6$; 600 MHz)

| Position | δ$^{13}$C (ppm) | HSQC δ$^{1}$H[ppm], J[Hz]) | COSY | HMBC | DEPT |
|---|---|---|---|---|---|
| 2 | 163.25 | | | H-b(w); H-d(w) | q |
| 3 | 104.58 | 6.67(s) | H-d(w) | | CH |
| 4 | 183.86 | | | H-b(a); H-c(w) | q |
| 5-OH | 166.09 | | | H-a(s); H-b(a) | q |
| 6 | 100.22 | 6.21(d; 2.1) | H- c(a) | H-c(a) | CH |
| 7-OH | 166.30 | | | H-c(w); H-d(w) | q |
| 8 | 95.16 | 6.49(d; 2.1) | | H-d(a) | CH |
| 9 | 159.48 | | | H-c(w) | q |
| 10 | 105.31 | | | | q |
| 1´ | 122.73 | | | H-a(a); H-b(a) | q |
| 2´ | 105.28 | 7.26(s) | H-e(w) | | CH |
| 3´ | 149.68 | | | H-a(s); H-e(s) | q |
| 4´-OH | 141.26 | | | H-a(s) | q |
| 5´ | 149.68 | | | H-a(s); H-e(s) | q |
| 6´ | 105.28 | 7.26(s) | H-e(w) | H-a(s); H-b(s); H-c(a); H-d(a) | CH |
| O-$CH_3$ | 57.00 | 3.96(s) | H-a(w) | | $CH_3$ |

w: Weak, a: Average, s: Strong correlation.

### 4.1.1 LC/UV-DAD data

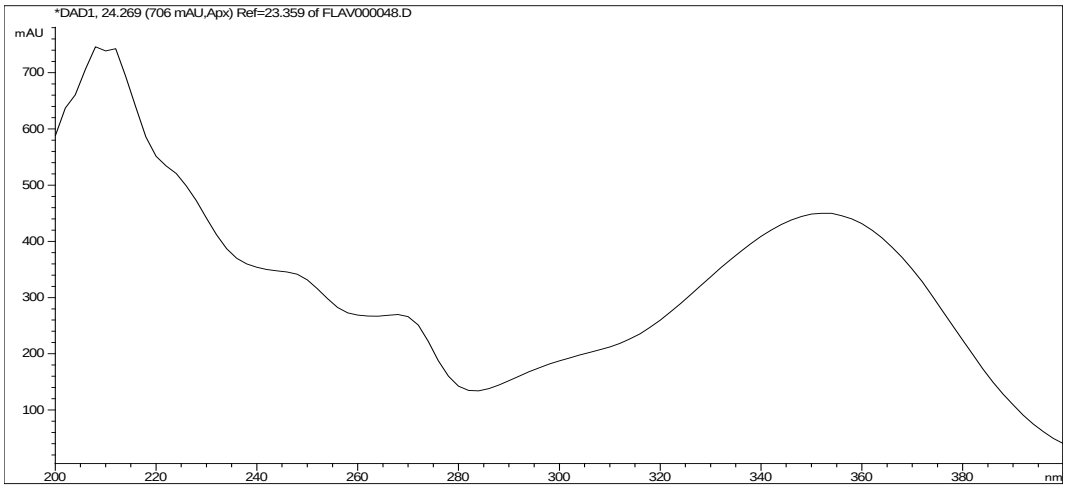



**Figure 4.** UV spectrum recorded during the LC/UV-DAD experiment. It shows bands at $\lambda_{max}$ 270 (band II) and 350 (band I).

### 4.1.2 LC/MS data

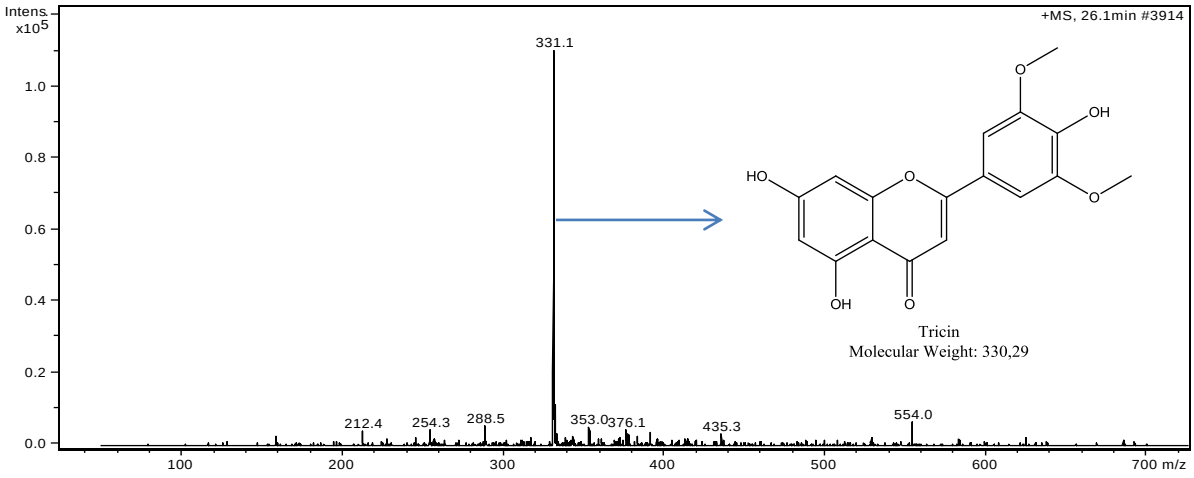



**Figure 5.** LC-MS data for compound fraction 6 (In positive mode). The positive ion mode gave an intense peak at *m/z* 331.1

## 4.2 Cytotoxicity to macrophages

The cytotoxicity of the test samples was determined by the ex-vivo MTT-reduction assay. The results indicated that the extract has cytotoxic activity against J774 cells ($CD_{50}$ = 62,8 μg/mL).
Since tricin was the major compound in the extract (fig.5), there is a high chance that this molecule is the responsible of the biological activity. This might be related with the previous findings about tricin´s chemopreventive properties, in the sense that it is able to interfere with the growth of colon cancer cells (Section 1.4). However, is worth noting that the experiments performed in this study were done in an *in vitro* model for a single cell strain, hence, is hard to make solid hypothesis based on these results. As long as we do not elucidate tricin´s cytotoxic mechanism at molecular level, any affirmations about its potential as an anticancer drug or chemopreventive agent would remain as speculations.

## 4.3 Data analysis and structure proposal

Even though most of the flavonoid NMR spectra reported in the literature are taken in DMSO-$d_6$, due to the relevance of sample recovery for further biological tests, MeOH-$d_6$ was used in this study. In addition, the characteristic residual water peak of DMSO-$d_6$ was occluding the methoxyl signals around 3.9 ppm, making the data analysis difficult.

The NMR data of the Flavonoids fraction Nr. 6 are presented in table 3. Thanks to the reported[7] characteristic shifts on C-4 (173-184 ppm), C-3 (103-112 ppm) and C-2 (160-165 ppm) on the flavonoid basic structure, the compound was set to be either a flavone or a flavonol. Nevertheless, the presence of a carbon signal within the range of C-3 on the flavonoid core at 104.58 ppm ($^{13}$C-NMR), correlated to the proton at 6.67 ppm (HSQC), suggested that C-3 was more likely to be H attached, rather than OH attached. In addition the UV-max absorption at 350 nm for this fraction, obtained in the previous chromatogram, is also characteristic of flavones. Information in the literature also allowed the assignment of signals at 183.86 and 163.25 ppm as C-4 and C-2 in the main structure. With the signal at 104.58 ppm and it´s respective proton at

6.67 (H-b), as starting point and the information provided by the HSQC, position of C-9 was identified as the signal at 159.48 ppm, which is also coherent with a carbon attached to an oxygen heteroatom in the central ring.

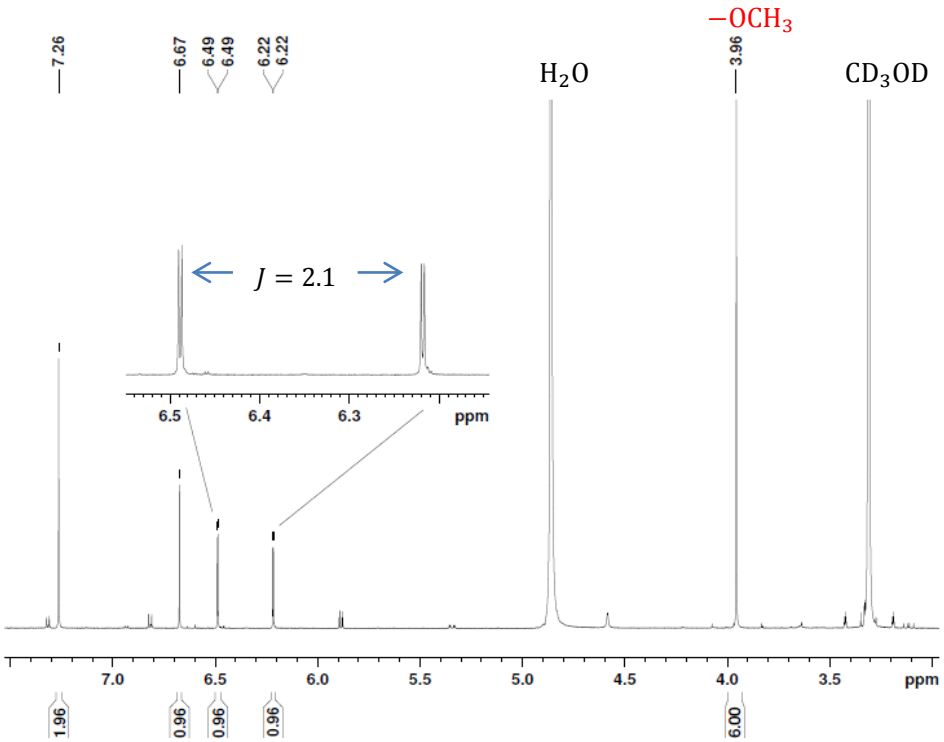



**Figure 6.** [1]H-NMR spectra for compound fraction 6

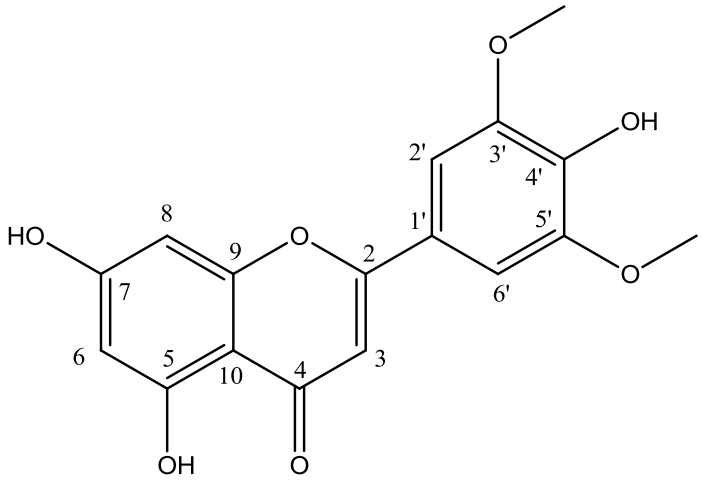
The presence of a singlet representing two protons at 7.26 ppm, indicated two equivalent protons. This signal is more likely to belong to the B ring. This is because the protons in the A ring are locked between ether type oxygen and carbonyl type oxygen, which makes difficult to have equivalent chemical environments. Thus, the doublets at 6.49 and 6.21 ppm must belong to the A ring. Their coupling constant of 2.1 Hz meta position (figure 6). Taking into account that 7-OH and 5-OH is the most common hydroxylation pattern, these protons must be in 8 and 6 position. These proton signals are not visible in the spectra, since they undergo in deuterium exchange with the deuterated methanol. The two methoxyl groups were assigned to the B ring, so the signals of carbons 3´and 5´ and the $OCH_3$ protons are equivalent and because the HMBC showed correlation of these carbons with H-e and H-a, which belonged to the B ring. The remaining

quaternary carbons (C-7, C-5 and C-4´) were assumed to be hydroxylated because this proton signals are not seen in the spectra.

Carbon signals at 131.11, 129.04, 116.32, and 80.49 ppm were labeled as impurities because they showed correlation with protons with incoherent integration profile in the HSQC and HMBC spectra.

The proposed structure (5,7-dihydroxy-2-(4-hydroxy-3,5-dimethoxyphenyl)-4H-chromen-4-one or *Tricin*) with a molecular mass of 330,29 was consistent with the LC/MS experiment for the Flavonoid fraction 6, which shows a peak at 331.1 m/z in positive mode. This exact same peak was obtained by Brazilian investigators for the same compound[19].

This structure has been previously reported[20]. Despite some minor shift differences due to the solvent used (Acetone-$d_6$) all 14 carbon signals correspond (Table 4). The proton shifts of the compound are also coherent with the reported in the literature for Tricin[21]. Figure 7 shows the proposed structure with the correspondent signal assignment.




5,7-dihydroxy-2-(4-hydroxy-3,5-dimethoxyphenyl)-4*H*-chromen-4-one (Tricin)
Chemical Formula: $C_{17}H_{14}O_7$
Molecular Weight: 330,29

**Figure 7**. Proposed structure and signal assignment for flavonoid fraction 6

| Position | $\delta^{13}C$ (ppm) | Theoretical[20] $\delta^{13}C$ (ppm) | $\delta^{1}H$ (ppm) | Theoretical[21] $\delta^{1}H$ (ppm) |
|---|---|---|---|---|
| 2 | 163.25 | 165.1 | q | q |
| 3 | 104.58 | 104.7 | 6.67 | 6.96 |
| 4 | 183.86 | 183.1 | q | q |
| 5 | 166.09 | 163.4 | OH | OH |
| 6 | 100.22 | 99.7 | 6.21 | 6.21 |
| 7 | 166.30 | 164.9 | OH | OH |
| 8 | 95.16 | 94.9 | 6.49 | 6.56 |
| 9 | 159.48 | 158.8 | q | q |
| 10 | 105.31 | 105.3 | q | q |
| 1´ | 122.73 | 122.4 | q | q |
| 2´ | 105.28 | 105.3 | 7.20 | 7.32 |
| 3´ | 149.68 | 149.1 | q | q |
| 4´ | 141.26 | 141.0 | OH | OH |
| 5´ | 149.68 | 149.1 | q | q |
| 6´ | 105.28 | 105.3 | 7.20 | 7.32 |
| O-$CH_3$ | 57.00 | 57.0 | 3.96 | 3.95 |

**Table 4.** Comparative chart of experimental and theoretical NMR signals of tricin (q= Quaternary carbon)

Structural determination in other fractions could not be achieved because the sample yield was too low for NMR analyses. Fraction 1 and 2 failed to show proton signals after 8 hours scanning in the Bruker NMR 400 MHZ. Fractions 3, 4, 7 and 9 showed weak proton signals, but the low sample concentration made impossible the measuring of a $^{13}C$-NMR spectrum. Fraction 5 showed the same signals but weaker intensities that fraction 6 (tricin), and fraction 8 seems to be has similar signals as fraction 6, in addition with two doublets and one double doublet that could belong to the an aromatic ring. This suggests that this compound could be a derivative of tricin. Unfortunately, the carbon and two dimensional spectra were too weak to identify the nature of the modification or its position. The spectrum for this last two samples was recorded using the DMX 600. One possible explanation is that the molecules contained in the original extract were, either tricin, or some tricin modificated molecules such as glycosides. It is possible that during the sample preparation, transportation and purification, these molecules suffered hydrolysis from

the tricin nucleus. This could explain why the tricin peak was so high, compared to other peaks obtained in the preliminary chromatographic analyses (section 3).

Even though tricin was the only molecule that could be identified in the extract, this finding alone can be regarded as a remarkable result because its potential as a chemopreventive agent. The presence of substances with such properties in the sugarcane juice should encourage the Caucas´s Valley sugar industries to support further research in this field. Even is tricin has been previously reported in sugarcane juice by Brazilian investigators, there is no warranty that the secondary metabolite expression profile should be identical. Thus, it can be expected that the concentration of tricin and other metabolites in Cauca´s Valley sugarcane, are different from Brazil. Further quantifications should be performed. Although all indicates that tricin has a favorable safety profile, it would be necessary to elucidate its molecular modes of action in order to determinate the viability of the development of tricin-based anticancer drugs. The results of this work allow us conclude that there is still a lot to investigate about the flavonoids in the sugar cane juice.

## 5. CONCLUSIONS

- An unexploited flavonoid with great potential for colon cancer treatment, has been detected and isolated and fully characterized from Cauca´s Valley sugar cane.
- There is still a lot to discover about the flavonoids and other secondary metabolites from the sugar cane. Local industries should be encouraged to invest in this field of research.

## 6. ACKNOLEDGEMENTS

This work was supported by the department of Pharmaceutical Chemistry from the University of Wuerzburg. I would like to thank Prof. Dr. Ulrike Holzgrabe and collaborators for supplying the space and materials for the present work, and for their scientific excellence and human quality.

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