Degree of hydrolization of gallotannins from Sumac (*Rhus coriaria*) on their anti-inflammatory potency *in vitro*

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Presented by

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Abstract. The sumac is a Middle Eastern flavoring spice rich in polyphenols. Polyphenols are compounds present in plants, which according to many investigations have a positive effect on chronic diseases such as cancer. The objective of this study was to hydrolyze a sumac extract in three different levels and evaluate its anti-inflammatory effect in colon cancer cells (HT-29). Completely randomized designs were used for the rhodanine assay for four hours (0-240 min); the proliferation of HT-29 cells with three concentrations of sumac extract (1, 5 and 25 mg / L) and three levels of enzymatic hydrolysis (non-hydrolyzed, partially hydrolyzed and hydrolyzed), and reverse transcriptase polymerase chain reaction using the three levels of hydrolysis with one concentration of sumac extract (5 mg / L). The enzymatic hydrolysis carried out by tannase lasted between 90 and 120 minutes. It degraded the high molecular weight tannins and converted them to gallic acid, from which 34 g/L were obtained along with smaller gallotannins. In the evaluation of cell proliferation, the positive effects of the degree of hydrolysis and the different concentrations used were observed. There was no decrease in the expression of some inflammation markers (NF-κB, TNF-α and IL-1β), while better results were observed with the sample of hydrolyzed particles with the rest of molecular markers (VCAM-1, TRL4, IL -8 and COX-2). It is recommended to continue this study *in vivo* and with other cell lines.
Keywords: Cell proliferation**,** inflammation markers, polyphenols, tannase.

Resumen. El zumaque es una especia aromatizante del Medio Oriente rica en polifenoles. Los polifenoles son compuestos presentes en las plantas. Según muchas investigaciones tienen un efecto positivo en enfermedades crónicas como el cáncer. El objetivo de este estudio fue hidrolizar un extracto de zumaque en tres grados diferentes y evaluar su efecto antiinflamatorio en células de cáncer de colon (HT-29). Se usaron diseños completamente al azar para el ensayo de rodanina durante cuatro horas (0-240 min); la proliferación de células HT-29 con tres concentraciones de extracto de zumaque (1, 5 y 25 mg/L) y tres niveles de hidrólisis enzimática (no hidrolizada, parcialmente hidrolizada e hidrolizada), y reacción en cadena de la transcriptasa-polimerasa inversa usando los tres niveles de hidrólisis antes mencionados y una concentración de extracto de zumaque (5 mg/L). La hidrólisis enzimática realizada por la tanasa duró entre 90 y 120 minutos. Esta degradó los taninos de alto peso molecular y los convirtió en ácido gálico, del cual se obtuvieron 34 g/L y galotaninos de menor tamaño. En la evaluación de la proliferación celular se observaron efectos positivos independientemente del grado de hidrólisis y las diferentes concentraciones utilizadas. No hubo disminución en la expresión de algunos marcadores de inflamación (NF-κB, TNF-α e IL-1β), mientras que se observaron mejores resultados con la muestra parcialmente hidrolizadas con el resto de marcadores moleculares (VCAM-1, TRL4, IL-8 y COX-2). Se recomienda continuar este estudio *in vivo* y con otras líneas celulares.
Palabras claves: Marcadores de inflamación, polifenoles, proliferación celular, tanasa.

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1. INTRODUCTION

Cancer is one of the leading causes of death and effective drug treatment is limited. Since National Cancer Institute began to screen plants for their antitumor activity during 1960s; interest in natural compounds from medicinal plants with anticancer activity has increased (Tohma *et al.* 2019). Colorectal carcinoma is one of the most common cancers and one of the leading causes of cancer-related death in the United States. Pathologic examination of biopsy, polypectomy and resection specimens is crucial to appropriate patient management, prognosis assessment (Fleming *et al.* 2012).

According to Morshedloo *et al.* Sumac (*Rhus coriaria L.*), recognized in Iran and other areas of the Middle East as a very popular flavoring spice, contains a wide range of medicinally active components (Morshedloo *et al.* 2018). The main tannin present in sumac are hydrolysable gallotannins (Zargham and Zargham 2008). Gallotannins, belonging together with ellagitannins to the hydrolysable tannins are of great importance in view of the extent of their use in tannage (el Sisi *et al.* 1971).

Sumac has attracted more attention due to its therapeutic value, because it is rich in phytochemicals, such as, tannins, phenolic acids, flavonoids and organic acids (Athamne *et al.* 2017). The traditional uses of sumac continue to be significant natural therapy for various diseases, this is because it possess antibacterial, antifungal, antioxidant, anti-inflammatory, vasorelaxant, anti-migratory, hypoglycemic, DNA protective, non-mutagenic properties, among others and a wide variety of minerals (Shabbir 2012).

Polyphenols are abundant nutrients in our diet, and evidence for their role in the prevention of degenerative diseases such as cancer and cardiovascular diseases is emerging (Manach *et al.* 2004). According to their structures, tannins are categorized as hydrolysable tannins, condensed tannins or complex tannins (Zhang *et al.* 2009).

Gallotannins are those in which galloyl units or their derivatives are bound to diverse polyol, catechin-, or triterpenoid units. These compounds upon enzymatic hydrolysis yield glucose and gallic acid (Li *et al.* 2005). Gallic acid molecules bind to a core polyol, and the galloyl groups may be further esterified or oxidatively cross-linked to form more complex structures (Zhang *et al.* 2009).

It has been assumed for a long time that tannins are not absorbed due to their high molecular weight and their ability to form insoluble complexes with components of food, such as

proteins (Koleckar 2013). The reduction of the size of the tannin molecule contributes to increase its bioavailability (Ozdal *et al.* 2016).

Tannase (EC. 3.1.1.20) is involved in biodegradation of tannins and has important applications in various industries, because it catalyzes the hydrolysis of ester bonds and depside bonds present in hydrolyzable tannins to form glucose and gallic acid (Chandrasekaran and Beena 2013). As the hydrolysis progresses the quantity of free gallic acid increases as well as the capability of an organism to absorb these compounds (Payán 2018).

In this experiment, a sumac extraction was done, and with different assays its phenolic content was determined. By adding and enzyme, tannase, different degrees of hydrolization were achieved, using the most significant for further analysis. The three levels of hydrolysis used were non-hydrolyzed at 0 minutes, partially-hydrolyzed at 10 minutes and hydrolyzed at 120 minutes. Each of these treatments was diluted to specific concentrations and used for cell viability and RT-PCR. The cell line used for this experiment was colon cancer cells (HT-29). The effect of the Gallotannins over inflammation of the cells were evaluated in order to find out if it could be decreased. The importance of this experiment is that many fruits and vegetables have the natural property of polyphenol content which can help in anti-inflammatory activity to treat chronic diseases, such as cancer. The objectives of the following investigation were:

- Determine the time needed for sumac tannin hydrolysis.
- Evaluate the response of HT29 cell line to sumac gallotannins in different hydrolyzation stages.
- Evaluate the anti-inflammatory effect of sumac gallotannins and its hydrolysates.

2. MATERIALS AND METHODS

Study location.

The study was conducted in the Chemistry of Fruit and Vegetables Laboratory and Cell culture Laboratory of the Department of Nutrition and Food Science. Located in Texas A&M University, College Station, Texas, United States of America.

Extraction.

Ten grams of Sumac powder were weighed in a 50 ml falcon tube. To dissolve tannins in solvent, add 30 ml of acidified methanol with 0.1% formic acid (v/v) and vortex it for 1 min. The sumac powder was ground with the Power Gen 5000 Fisher Scientific homogenizer. The falcon tube containing the sumac and acidified methanol was centrifuged for 10 min at 3,000 rpm at 24 °C. Supernatant was removed from the pellet and it was added to a round flask. Repeat all the steps from adding 30 ml of acidified methanol with 0.1% formic acid (v/v) two more time. The third time this steps were repeated 30 ml of acidified methanol were added and with 10 ml clean particles left from the homogenizer and were added to the tube that was centrifuged and the remaining steps were repeated. After adding all supernatants to the same round flask, glass beads were added and the tannins were separated from the methanol in a Rota-evaporator at 50 °C until the tannins were concentrated enough.

After the extraction part was finished, the extract was fractionated with a C18 column. C18 column was activated by filling it up three times. To clean up the methanol, water was added to the column by filling it up three times. The extract was leveled up to 10 ml using water and it was added to the column three times so the sugars are removed. To collect the tannins, the column was changed to another flask and the column was filled up with methanol three times. The extract was collected in a round flask and five glass beads were added. The methanol was separated from the sumac in a Rota- evaporator a t 50 °C until the tannins are concentrated enough.

Since sumac has a considerable oil content, the last step performed on the extract was defatting. Fifty ml of hexane were added to the extract in a separatory glass funnel. Then, the extract was collected in a round flask. The solvent was evaporated in a Rota- evaporator at 50 °C for about ten minutes. These steps were repeated one more time

.

Polyphenol content.

To measure the concentration of total polyphenols in the samples a follins assay was made. Different dilutions of the four samples were made (100, 200, 400, 800X). Calculations were made for every dilution the µL of each sample were added to the test tubes , and acidified water (0.1% formic acid) was added to complete up to 1 ml. From the 1 ml of each dilution, 100 µL were transferred to three other test tubes, this way each dilution had three repetitions. Then, 1 ml of the follins reagent was added to each test tube. After a three minute wait 1 ml of Na_2CO_3 was added to the sample and waited for 7 minutes. Finally, 5 ml of acidified water were added and waited for 30 minutes. Using the UV/Vis Thermo Scientific Spectrophotometer with 736 nm of wavelength the concentration of each sample was determined.

Gallic acid concentration.

Gallic acid solutions (400, 200, 100, 50, 25, 12, 6) were prepared, diluted with 0.1% formic acid H_2O. Parting form this dilutions the gallic acid standard curve was made. This was used to make a relation between the curve and the samples with the tannase.

For the hydrolysis, 0.3 g of tannase were weighed and added to 5 ml of deionized water. Using the formula for concentration and volume, lower the concentration of the polyphenol content of the extract from the Follins result to 400 ppm in 100ml of acidified water (v/v) with 0.1% formic acid. Water was boiled in a beaker. 10 ml of the sumac solution were added to a test tube. Add 1 ml of the tannase solution to the sumac solution in the beaker. Immediately after the tannase was added 10 ml of the solution were pipetted in a test tube and boiled for five minutes in water to stop the reaction, this was the 0 minutes sample. This same process was repeated with the different samples until 240 minutes after the tannase. To determine the three levels of hydrolysis based on the gallic acid content the Rhodanine Assay was performed on the samples.

This assay was made by adding 0.6 ml of rhodanine (0.667% methanol) and 0.4 ml of sample were added in the test tube (16 × 100 mm). After waiting for 10 minutes add 0.4 ml of 0.5M NaOH. After a 10 minute wait bring it to a final volume of 10 ml using deionized water. Finally, waited for another 10 minutes. Each sample was read in the spectrophotometer (520nm) in quartz cuvettes (10 × 10 × 45mm) using the UV/VIS Thermo Scientific at 520 nm wavelength. After the determination of the three levels of hydrolysis the samples were diluted to 1, 5, and 25 mg/L in 5 mL Eppendorf tubes and frozen for further analysis at -20.

Identification of Gallotannins and Gallic acid in the LC-ESI-MS.

Three mL of each of the degrees of hydrolysis from sumac extracts with tannase samples were collected and filtered through a Whatman filter (0.45µm) PTFE membrane, and characterized and quantified by LC-MS.

Seeding cells.

The following procedure was done in the cell culture room in the biosafety cabinet (Thermo Scientific formal 1400 series) were the surface was previously disinfected with 70% alcohol to avoid any contamination. When the petri dishes containing the colon cancer cell (HT-29) reached an 80% confluence, the cells were detached to be seeded in 96-well plate for cell viability and 12-well plate for PCR. The media, composed of 1% Penicilin/Steptomicin as an antibiotic, 10% FBS (Fetal Bovine Serum) as a source of proteins, and DMEM (Dulbecco´s Modified Eagle Medium), was aspirated and discarded. Then, 5 mL of PBS were added to wash the cells, aspirated and discarded. To detach cells, 2 ml of trypsin were added to the plate, the plate was incubated for 5 minutes, not for a longer time because it could damage the cells. To stop the effect of the trypsin, 8 ml of 10% FBS were added to the plate with the 2 ml of trypsin. The solution was added to a 15 mL falcon tube to centrifuge at 1200 rpm for 2 minutes at 24 °C. The supernatant was aspirated, being careful not to aspirate the pellet. The pellet was suspended 3 ml of media to count the cells.

Using the Invitrogen Countess (automated cell counter) the cells were counted by pipetting 10 µL of cell suspension into a 2 ml Eppendorf tube. Then, 10 µL of the Trypan Blue stain 0.4% were added into the drop of cell suspension inside the Eppendorf and mixed using the pipette by drawing the mixture in and out. The 10 µL of the dyed cell suspension pipetted and putted into one of the sides of the countess chamber slide. Insert the slide in the countess, and use the number of viable cells that the equipment provides.

Make the calculations necessary so the concentration of cells in each well is the same. To get to a specific concentration the following formula [1] for concentration and volume was used:

$$C_1V_1 = C_2V_2 \text{ [1]}$$

Where:
C_1= Concentration of viable cells (countess reading)
V_1= Volume of the suspension
C_2= Concentration desired in each well
V_2= Volume of each well

According to the results of the formula, mix the amount of suspension solution with media to have enough for each analysis.

Cell viability test (MTT).

Cells were seeded in the 96-well plate at a 2×10^4 cells/well (four wells per treatment) with 200 µL of media. The plate was incubated for 24 hours at 37 °C. After the 24 hours the media was aspirated and cells were treated with the different concentrations of sumac extracts what were prepared and stored in the freezer for 48 hours. Following the protocol for MTT of Mosman (1983) and modifications by Wang *et al.* (2010) the MTT solution

was prepared in PBS and 20 µL of the solution were added (2.5 mg/mL) to each well. The plate was re- incubated for 4 hours. After the time passed it was centrifuged for 5 minutes at 1030 x g (Heraeus ™Fresco™21 Microcentrifuge). The supernatant was removed and 200 µL of DMSO were added to each well. The absorbance was measured using the microplate reader (FLUOstar Omega) at 570 nm.

mRNA extraction.

The cells were seeded in a 12 well plate (three wells for each treatment) at a concentration of 1.6×10^5 cells/well and let them incubate at 37 °C until they were confluent enough. During 6 hours they were treated with each of the treatments as mentioned before. After that time the supernatant was discarded and washed with 500 µL of PBS. 350 µL of lysis buffer and 10 µL of β-mercaptoethanol and then stored at -80 °C until the next day. Then, 350 µL of 70% ethanol were added to the lysis in the wells. The 700 µL were placed in a RNeasy Mini spin column placed in a 2 ml collection tubes and centrifuged for 15 seconds at > 8000 xg and the flow through was discarded. 700 µL of Buffer RW1 were added to the RNeasy spin column and centrifuged at ≥ 8000 x g for 15 s and the flow through was discarded. 500 µL of Buffer RPE were added to the RNeasy spin column and centrifuged at ≥ 8000 x g for 15 s and the flow through was discarded. 500 µL of Buffer RPE were added to the RNeasy spin column and centrifuged at ≥ 8000 x g for 15 s and the flow through was discarded. The RNeasy spin column was placed in a new 1.5 ml collection tube. Finally 30 500 µL of Buffer RPE were added to the RNeasy spin column and centrifuged at >8000 x g for 15 s and the flow through was discarded. Finally 30 µL of RNAse free water were added and centrifuged for 1 minute at > 8000 x g.

Quantification of RNA and synthesis of cDNA.

Based on the results of the quantification of the Thermo Scientific Nanodrop 1000, using the concentration formula on all of the treatments they were leveled to 1000ng/µL. Then the 1000ng/µL were divided by the result of the RNA quantification and the calculation indicated the microliters from each of the RNA samples of the different treatments. In the Eppendorf tubes for the synthesis of cDNA, RNAse free water was added up to 12 µL to the RNA µL of the calculation mentioned before. To the 12 µL of the RNA and RNAse free water, 3 µL of reverse transcriptase were added. The tubes were processed in the Thermocycler to obtain the cDNA. Finally, 85 µL of RNAse free water were added to each sample and stored at -80 °C.

mRNA-RT PCR (Reverse transcription polymerase chain reaction).

The diluted primers were prepared by adding 10µL of the forward primer, and 10 µL of the reverse primer with 180 µL of RNase free water. Seven primers were chosen: NF-κB, TNF-α, IL-1β, VCAM-1, TRL4, IL-8, COX-2. The mater mix was prepared with 10µL of the iTaq Universal Probes supermix, 2µL of the diluted primer and 8 µL of the cDNA sample.

6 µL of master mix were added to each well plus 4 µL of the diluted RT Rxn. Samples were prepared in the plate and ran in the Bio-Rad Thermal cycler.

Experimental design.

A Completely Randomized Design was used for the cell viability test with a factorial arrangement of 3 × 3 with three different degrees of hydrolysis (non, partially and hydrolyzed) and three concentrations (1, 5, 25 mg/L) of the sumac extract (Table 1), quantifying the absorbance and the number of cycles, respectively. The samples were compared to a negative and a positive control. For the RT-PCR a Completely Randomized Design was used for the cell viability test with a factorial arrange of 3 × 1 with three different degrees of hydrolysis (non, partially and hydrolyzed) and one concentration (5 mg/L) of the sumac extract (Table 2).

Table 1. Experimental design for enzymatic activity.

Degrees of Hydrolysis	**Extracts (mg/L)**		
	1	**5**	**25**
Non-hydrolyzed	TRT 1	TRT 2	TRT 3
Partially-hydrolyzed	TRT 4	TRT 5	TRT 6
Hydrolyzed	TRT 7	TRT 8	TRT 9
[1]Negative control		TRT 10	
[2]Positive control		TRT 11	

†TRT: treatment. [1]Cells with no sumac extract. [2]Cells with no sumac extract and DMSO.

Table 2. Experimental design for RT-PCR.

Degrees of hydrolysis	**Concentration (mg/L)**
	5
Non-hydrolyzed	TRT 1
Partially-hydrolyzed	TRT 2
Hydrolyzed	TRT 3
[1]Control	TRT 4

†TRT: treatment. [1]Cells with no sumac extract.

3. RESULTS AND DISCUSSION

Enzymatic activity.

Tannase hydrolyses esters and depside bonds of hydrolysable tannins and it releases glucose, gallic acid, and galloyl esters (Jana A *et al.* 2014). Tannase catalyses the hydrolysis of gallic acid esters and hydrolysable tannins. This enzyme is produced by plants and microorganisms and it is industrially used as catalysts in the manufacture of gallic acid. Also, it is potentially used in beverage and food processing (Belmares 2004).

The process of hydrolysis occurs when a glucose molecule bind to a certain number of galloyl molecules, breaks this bonds due to the presence of tannase (Figure 1). Tannase (tannin acyl hydrolase, EC 3.1.1.20) catalyzes the hydrolysis of ester bonds from gallotannins, which are also called complex or hydrolysable tannins, producing gallic acid and glucose (Chandrasekaran and Beena 2013).

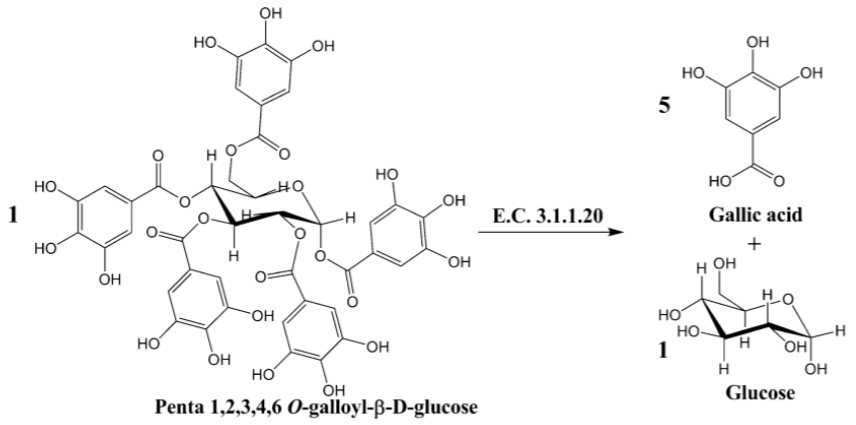

Figure 1. Hydrolysis of 1,2,3,4,6-pentagalloylglucose catalyzed by tannase (Aguilar and Gutiérrez-Sánchez 2001)

Since the times needed for each level of hydrolysis were unknown, tannase was added to a sample of the extracts starting from 0 to 240 minutes as the curve of hydrolysis shows (Figure 2). Parting from absorbance obtained from the rhodanine assay the concentration of the extract was calculated by taking in consideration the slope of the standard curve and the dilution of the extract, with this information each of the levels were established. There was an initial concentration of 34.79 g/L after 90 minutes of the reaction constant results

were monitored. The 120 minutes was picked because it was at this point when it was indicted that the reaction stopped at 90 minutes and the difference between the rests of the concentrations obtained was not significant ($P < 0.05$). A concentration of 15.70 g/L represented half of the hydrolyzed level, which occurred at 10 minutes.

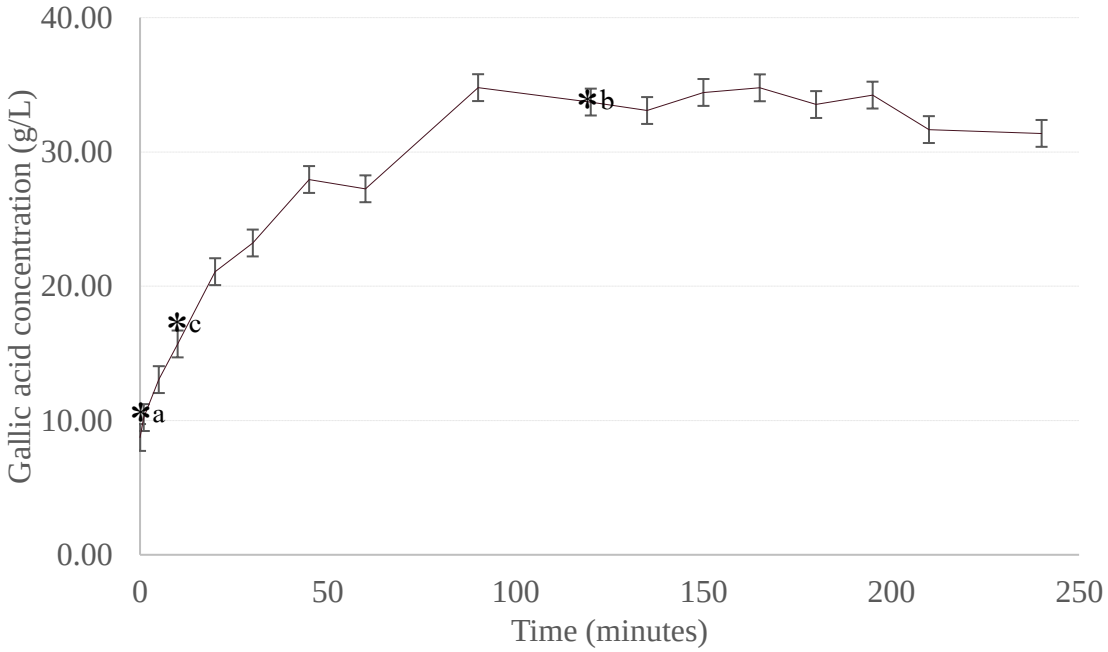

Figure 2. Concentration of Gallic acid in sumac extracts and during its tannase hydrolysis. [a]Initial Gallic acid concentration. [b]Tannase hydrolysis of sumac gallotannins. [c]Partial hydrolysis.

LC-MS.

Using this procedure and examining ions from lower to higher m/z values, the following compounds were identified: the gallate anion at m/z 169, and a set of ions from m/z 331 to m/z 787 (Regazzoni *et al.* 2013).

The chromatograms allowed to detect a set of peaks at different retention times (Rt), sharp and well-resolved from mono to tetra-galloyl derivatives while others (from penta to decagalloyl) are broad and poorly resolved, However, the presence of several isomers of gallotannins of low molecular weight and of flavonoids was confirmed by extracting the single ion current for each molecular ion observed in the FI–ESI–HR-MS/MS2 experiments (Regazzoni *et al.* 2013). Clustered gallotannins that could not be identified individually showed up in the first chromatogram, but as the hydrolysis progressed it was reduced.

Several compounds of the sumac extracts were specifically identified and characterized by ESI (electrospray ionization) mass spectroscopy in negative ion mode. For example, free gallic acid was identified based on its molecular weight, fragmentation pattern, and spectral patterns, and compared to an authentic standard. An ion of m/z 169 was most abundant and

MS2 showed a fragmentation pattern with a predominant ion at 125 m/z. Di-galloyl glucose showed a predominant ion with m/z 483, this due to the loss of one molecule of glucose (180g/ml) and a molecule of water (18g/mol) (Talcott and Talcott 2009). The catalytic action of the tannase was successful, degrading some of the gallotannins, both the smaller and larger ones (Payán 2018).

Twelve compounds were identified in the 0 minutes or the non-hydrolyzed sample (Figure 3 A), these ones being; 5 digalloyl glucose, 6 trigalloyl glucose, and 1 tetragalloyl glucose. New compounds were identified in 10 minutes of the partially-hydrolyzed sample , among these: 6 digalloyl glucose, 4 trigalloyl glucose, and 1 tetragalloyl glucose were identified (Figure 3 B). For the 120 minutes or hydrolyzed sample the following fifteen compounds were identified: 5 monogalloylg lycose, 7 digalloyl glucose, 2 trigalloyl glucose, and 1 tetragalloyl glucose (Figure 3C). As it was observed, the elution times started at 5 and finished at 28 minutes. The higher peak, representing Gallic acid, had an elution time of ten minutes. The retention time of MGG (monogalloyl glucose) was lower than gallic acid. This is due to the addition of glucose that gives more polarity, so the elution time is faster within the column (Negrete 2015).

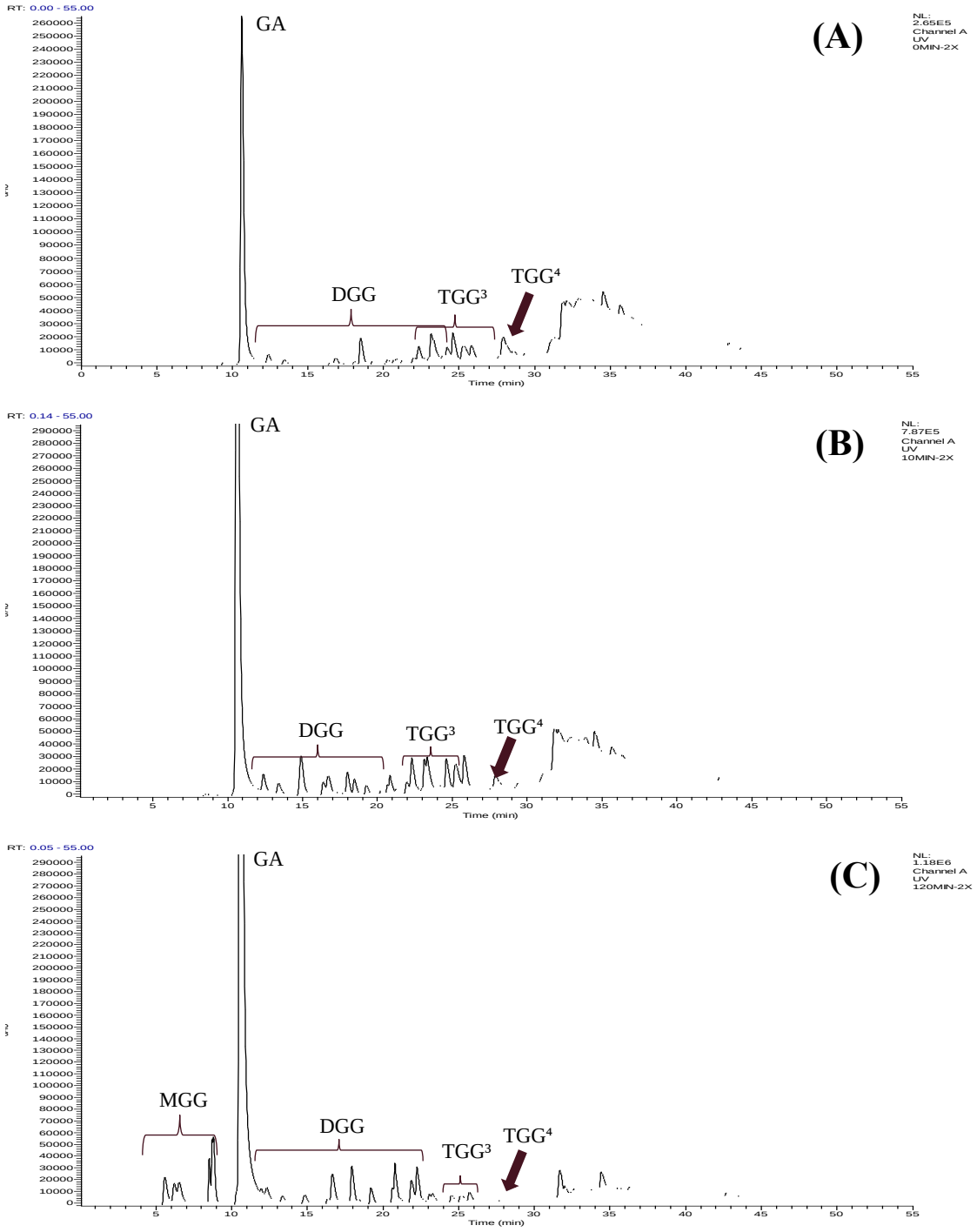

Figure 3. Chromatograms of Sumac polyphenol compounds after 0 (A), 10 (B) and 120 (C) minutes of tannase hydrolisis.
^{GA}Gallic acid; ^{MGG}Monogalloyl glucose; ^{DGG}Digalloyl glucose; $^{TGG^3}$Trigalloyl glucose; $^{TGG^4}$Tetragalloyl glucose.

Cell viability (HT29).

Polyphenol are bioactive compounds found in plants, and one of the most well-known polyphenol is Gallic acid that has importance due to its cytotoxicity with tumor cells (Vermar *et al.* 2013). Activation of signaling pathways and various kinases may mediate differentiation and apoptosis in colon cancer cells (HT29). The presence of a regulatory mechanism controlling differentiation and growth arrest in HT29 cells might have an important clinical implication in treatment in various cancers (Cohen *et al*. 1999). Gallic acid (GA) is a bioactive compound displaying diverse biological properties, including carcinogenic inhibiting activities (Lopez *et al.* 2014). Gallotannis are galloyl unit derivatives bounded to diverse polyol-, yielding gallic acid upon hydrolysis (Swanson 2003). Due to their higher molecular weight and high degree of hydroxylation of aromatic rings, tannins show high antioxidant potential (Koleckar *et al.* 2013). The negative control was media with no sumac extract and the other control was media and DMSO with no sumac extract (Figure 2). The DMSO (Dimethyl sulfoxide) is a colorless liquid found immediate application as a polar, aprotic solvent miscible with water and able to dissolve an enormous catalog of polar and nonpolar small molecules (Capriotti and Capriotti 2012).

According to other studies, sumac was used in vascular smooth muscle cells and no difference between the tannin-treated and untreated groups was found in the number of cells (Zargham and Zargham 2008). According to other studies the use of different extracts rich in polyphenols have a positive effect in the cancer colon cells while cell viability remained constant in healthy colon cells (Chávez 2015). Typical examples in order of increased complexity are resveratrol, an antioxidant in grapes and wine, the large group of flavonoids, also known as proanthocyanidins or condensed tannins or gallo- and ellagitannins, known as hydrolysable tannins (Bors and Michel 2006). Recent studies have shown that many dietary polyphenolic constituents derived from plants are more effective antioxidants *in vitro* than other nutrients, and thus might contribute significantly to the protective effects *in vivo* (Rice-Evans *et al.* 1997). Previous studies have suggested that methanolic extracts of *Rhus coriaria L*. fruits may be a source of natural antioxidants, and as it is well known tannin and its derivatives are strong antioxidants (Zargham and Zargham 2008). According to other investigations, the potent antioxidant action of sumac extract is due to the presence of high amounts of organic acids and/or tannin content (Özcan 2003).

The extracts used for the cell viability test represent three different levels of sumac hydrolysis (non, partially and hydrolyzed). For each level of hydrolysis of the sumac extract three concentrations were tested (1, 5 and 25 mg/L). According to the cell viability test all of these concentrations had a positive effect in HT29 cells compared to the controls. For the first level of hydrolysis (non-hydrolyzed) as the concentration increased no significant effect was observed on the cells, the 1 and 25 mg/L had no statistical difference. For the second level (partially-hydrolyzed) where it can be observed that as the concentration increases, the population decreases; the 25 mg/L samples had the best results. Finally, when the tannase reaction stopped (hydrolyzed) a clear tendency was observed, as the concentration increases the population gradually decreases.

The most significant differences were found in the partially and hydrolyzed sumac samples at a concentration of 25 mg/L GAE. The samples that contained higher levels of Gallic acid showed a decrease in cell population. Gallic acid has a diverse effect on tumor cells, it possess a selective toxicity that compared to the one in normal cells is less (Koleckar *et al.* 2013). When Gallic acid was directly applied to the cell culture medium of HT29 cells a time dependent decrease of detectable compound was observed with about 50% loss already after 1 h of incubation, which exhibited substantial growth inhibitory properties (Kern *et al.* 2001). In larger molecules the scavenging activity increases with the number and position of hydroxyls, and the size of the tannin molecule, the smaller the better (Koleckar *et al.* 2008). The cell viability was an initial assessment in order to decide the sumac extracts concentration for further analysis (PCR). Although the best results were obtained with the 25 mg/L extracts, 5 mg/L of each level of hydrolysis chosen for further analysis because there was still enough cell population to observe an anti-inflammatory effect. At 5 mg/L a consistent pattern for cell proliferation was detected, there was a significant difference between the first level and the other two at this concentration, in the last two the population was lower than the first one (Figure 4).

The various mono to penta- substituted esters are often classified as simple galloylglucoses to allow their discrimination from complex ones, also known as gallotannins (Niemetz and Gross 2004). Phenolic acids with small-molecular weight such as gallic acid and isoflavones are easily absorbed through the tract. On the contrary, large polyphenols such as tannins are poorly absorbed (Martin and Apple 2009). As the tannase hydrolysis progressed, smaller compounds started to appear. Gut microbiota increases bioavalability by transforming the phenols into smaller compounds, but larger compounds in the colon are beneficial, in colon epithelial cells to have an effect against inflammation diseases as colorectal cancer (Ozdal *et al.* 2016). In contrast to the condensed tannins, the ones extracted from Sumac is hydrolysable and therefore may be easier to digest and absorb (Zargham and Zargham 2008). The antioxidant activity of phenolic compounds is due to their ease to give hydrogen atoms and form an aromatic hydroxyl group to a free radical and the possibility to relocate the charges in the double bonds of the aromatic ring (Gallego 2016).

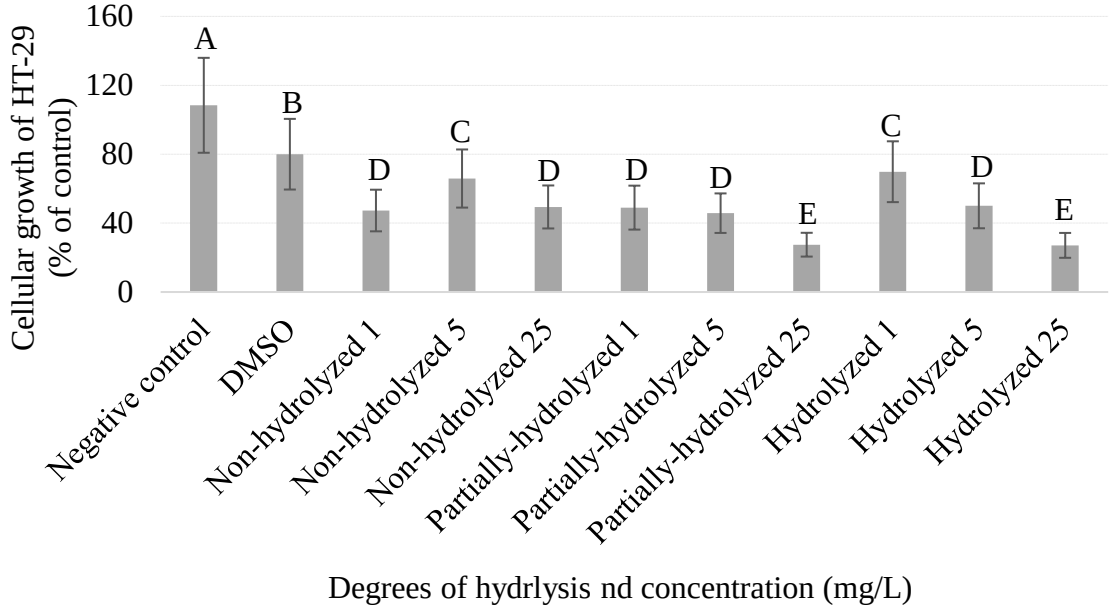

Figure 4. Cell viability test (MTT) in colon cancer cells (HT-29) with different concentrations with 1, 5, and 25 mg/L of the sumac extract.
Different letter indicates statistical difference between treatments ($P < 0.05$).

Reverse transcription polymerase chain reaction.

The data obtained in the extraction of RNA was variable due to the limited quantity of RNA analyzed (101-155 ng/µL). This could have resulted from different factors, among them, a bad extraction, deficient disinfection; water quality pipettes and the pipetting that had an effect in the quantity of the RNA that was collected. Nevertheless, the importance relied in the quality of RNA extracted, which was determined in its absorbance relation A_{260}/A_{280}, which should be between 1.0 and 2.0, indicating purity or lack of polysaccharide (Garrido Gutiérrez n.d). These are just values displayed by the Nanodrop that indicate polysaccharide and polyphenolic contamination, but the quality of RNA was determined by its application (Armijos 2013).

The messenger RNA (mRNA) of high quality was used for the synthesis of complementary DNA (cDNA), which is a double chain DNA, used for Reverse transcription polymerase chain reaction (RT-PCR) (Armijos 2013. Primers increase PCR amplification efficiency as well as isolate the targeted sequence of interest with higher specificity, the binds to the DNA fragments to be amplified (Li and Brownley 2010). Seven primers were used: NF-κB, TNF-α, IL-1β, VCAM-1, TRL4, IL-8, COX-2, to start the PCR reaction.

The nuclear factor (NF-κB) signaling pathways plays a major role in the development, maintenance, and progressions of most chronic diseases, it also controls the expression of genes that are involved in physiological reactions, among them, immune inflammatory responses (Gupta 2010). The expression of the NF-κB in colon cancer cells (HT-29) was not affected ($P = 0.7872$) by the presence of the sumac polyphenols regardless to their level

of hydrolysis (Figure 5). Other studies showed that the protein expression of NF-κB in HT-29 cells decreased when compared with control (Liu *et al.* 2018).

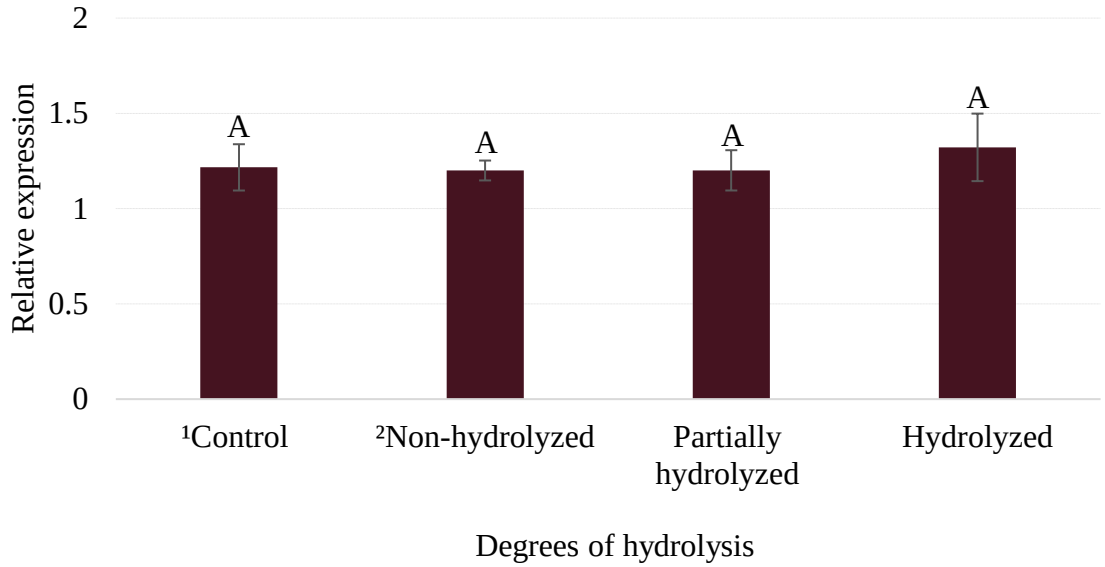

Figure 5. Gene expression (NF- κB) in HT-29 cells with sumac extracts application. Different letter indicates statistical difference between treatments ($P < 0.05$)
[1]Cells with no sumac extracts. [2]Cells with sumac extracts with tannase hydrolysis at 0 minutes (Non-hydrolyzed); 10 minutes (Partially hydrolyzed); and 120 minutes (hydrolyzed).

The next primer evaluated was the Tumor necrosis factor α (TNF α). The inflammatory component of a developing tumor may include a diverse leukocyte population, among them, lymphocytes. These are capable of producing an assorted array of cytokines, cytotoxic mediators including reactive oxygen species, serine and cysteine proteases, and soluble mediators of cell killing (Coussens and Werb 2002). Several pro-inflammatory gene products have been identified that mediate a critical role in suppression of apoptosis, proliferation, angiogenesis, invasion, and metastasis. Among these gene products are TNF and members of its superfamily interleukins, chemokines, cyclooxygenases, among others (Aggarwal *et al.* 2006).

The expression of TNF-α in HT-29 was not affected by the presence of the sumac polyphenols despite the degree of their hydrolysis (Figure 6). According to other studies, the response to TNF-a mRNA expression and protein secretion were sharply increased compared to the control (McCracken 2002). Other studies showed that in comparison with untreated control cells, TNF-α inhibited the cell growth of HT-29 (Kovaříková *et al.* 2000).

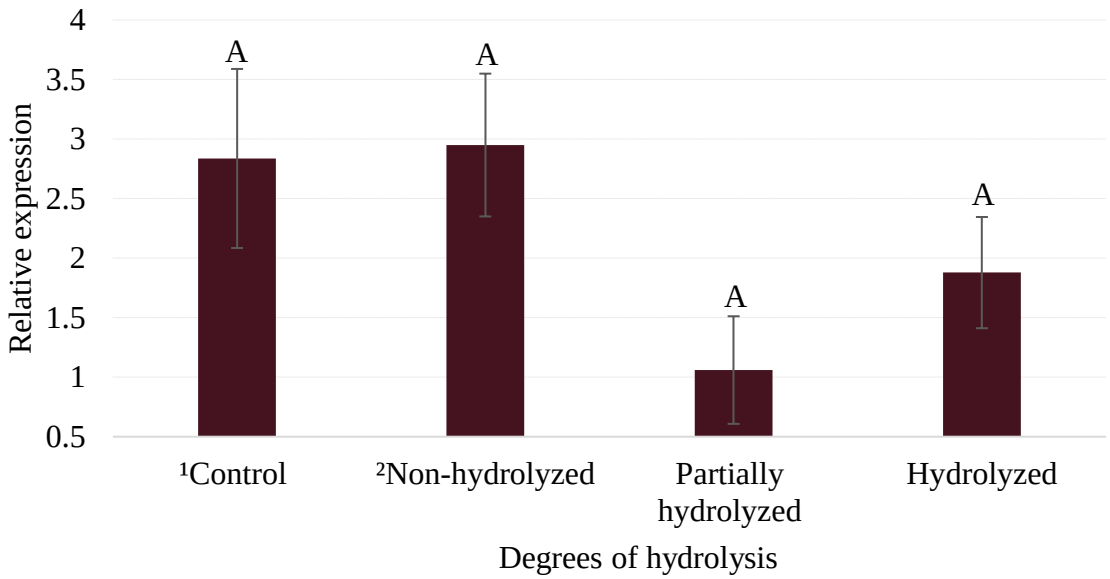

Figure 6. Gene expression (TNF-α) in HT-29 cells with sumac extracts application. Different letter indicates statistical difference between treatments ($P < 0.05$)
[1]Cells with no sumac extracts. [2]Cells with sumac extracts with tannase hydrolysis at 0 minutes (Non-hydrolyzed); 10 (Partially hydrolyzed); and 120 (hydrolyzed).

Two primers belonging to the interleukins (IL´s) were used, and they are secreted proteins that play a role in the communication among leukocytes. Belonging to this family there are the IL-1β and the IL-8, the first one mediates inflammatory diseases by initiating and potentiating immune and inflammatory responses (Akdis *et al.* 2011) and the latter (IL8) major effector functions of IL-8 are activation and recruitment of neutrophils to the site of infection or injury (Akdis *et al.* 2011). The expression of IL-1β in HT-29 was not affected by the presence of the sumac polyphenols despite the degree of their hydrolysis (Figure 7). In contrast IL 8 presented a positive statistical effect in the suppression of the gene, by reducing inflammation. There was no significant difference between treatments, which meant that despite the compounds present in each treatment, they all reduced inflammation. According to other studies the IL-8 concentrations of the gene expression decreased (Boesten *et al.* 2011). Another paper concluded that PGG can inhibit IL-8 gene expression in HT-29 cells (Koleckar *et al.* 2008).

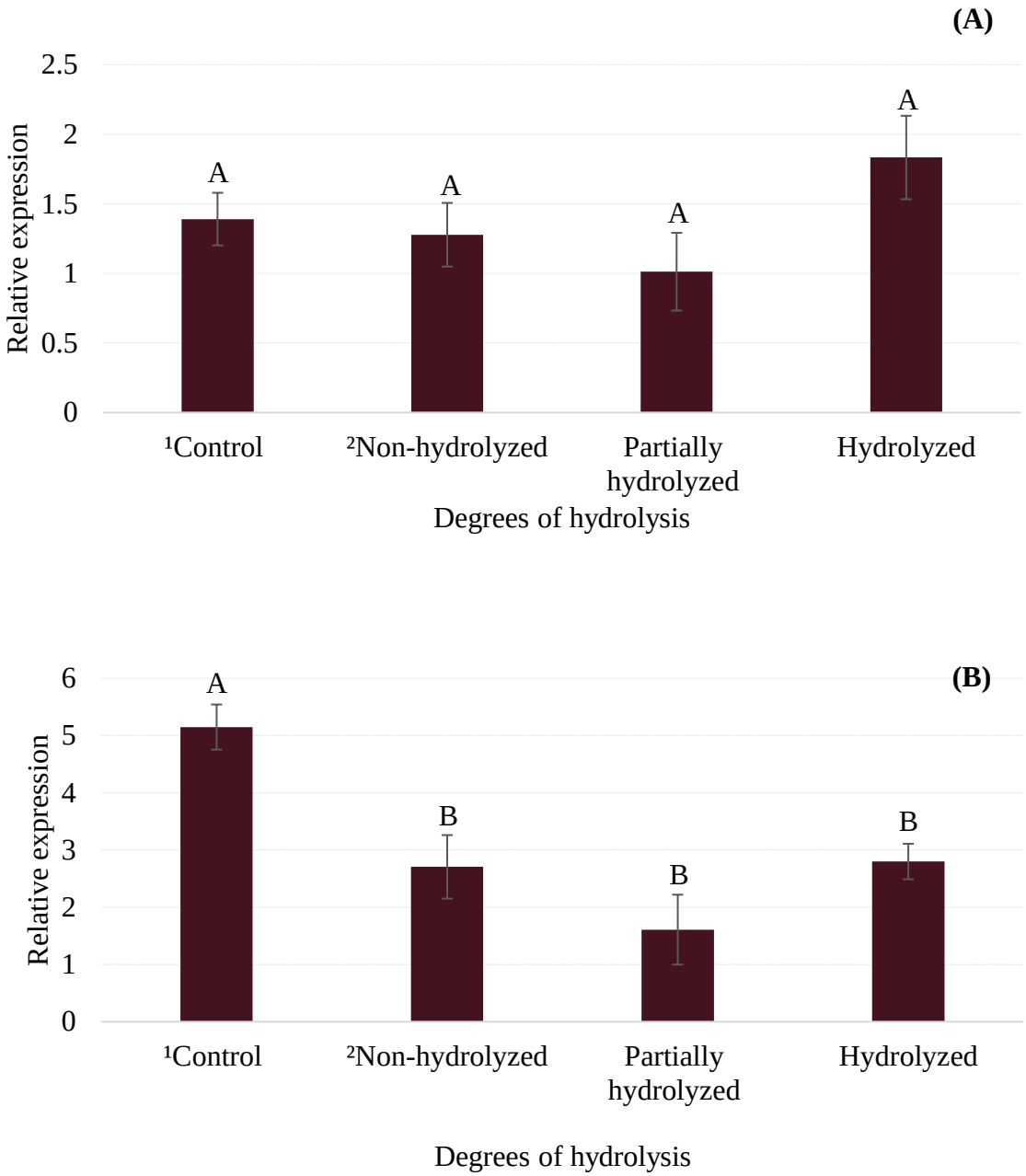

Figure 7. Gene expression (IL-1β)(A) and (IL-8)(B) in HT-29 cells with sumac extracts application.
Different letter indicates statistical difference between treatments ($P < 0.05$)
[1]Cells with no sumac extracts. [2]Cells with sumac extracts with tannase hydrolysis at 0 minutes (Non-hydrolyzed); 10 (Partially hydrolyzed); and 120 (hydrolyzed).

VCAM-1 mediates adhesion of endothelial cells and circulating lymphocytes *in vitro* and *in vivo*, VCAM-1 is expressed on activated endothelia in inflamed tissues, thereby promoting extravasation of lymphocytes into inflamed tissue (Garmy-Susini 2005). The sumac extracts presented a suppression on the gene expression VCAM-1 ($P < 0.05$). There was a positive effect on inflammation on both samples with hydrolyzed sumac gallotannins

(Figure 8). There was no statistical difference between the control and the non-hydrolyzed sample; there was a statistical difference between the partially hydrolyzed and the two previously mentioned. The best results were observed with the partially hydrolyzed sample, effectiveness is due not only to gallic acid presence, but the mixture of smaller compounds as tri- galloyl glucose and di-galloyl glucose were better in reducing VCAM-1 expression. It was observed that there was no statistical difference between the fully-hydrolyzed and the other two treatments with the control.

There are several factors that affect tannase production such as the substrates, and the molecular size, the larger the size the activity is reduced. Gallotannins are considered productive substrates, contrary to nonproductive substrates as gallic acid, also the fungal species use for tannase can have an influence because different species tolerate different induction ratios (Li *et al.* 2006). On the basis of concentrations of phenol groups, penta galloyl glucose was much more active than monogallyolglucose or gallic acid, because the depside bonds between galloyl groups are less stable than the aliphatic ester bonds between galloyl groups and glucose core (Zhang *et al.* 2009).

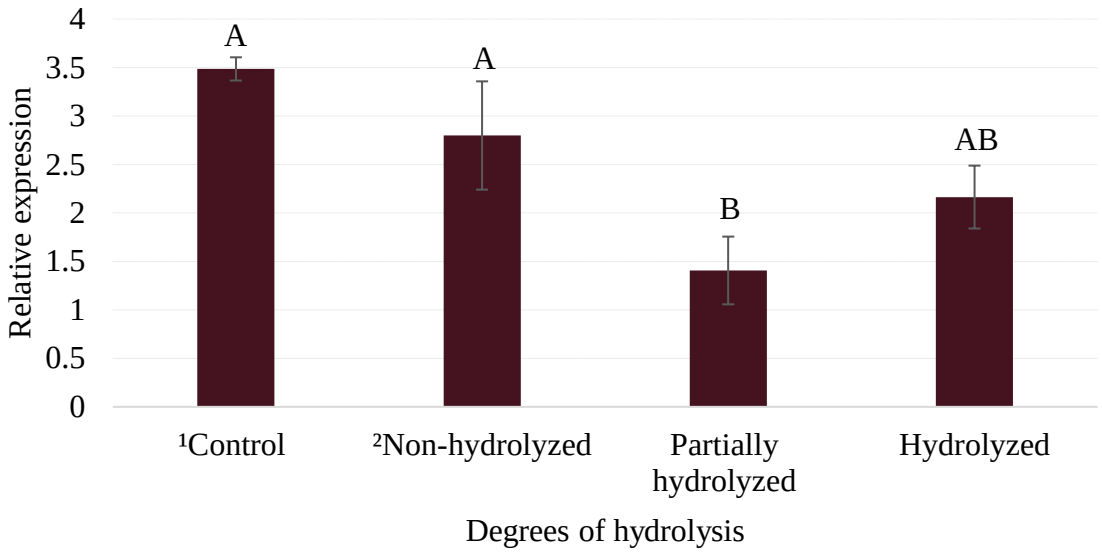

Figure 8. Gene expression (VCAM-1) in HT-29 cells with sumac extracts application. Different letter indicates statistical difference between treatments ($P < 0.05$)
[1]Cells with no sumac extracts. [2]Cells with sumac extracts with tannase hydrolysis at 0 minutes (Non-hydrolyzed); 10 (Partially hydrolyzed); and 120 (hydrolyzed).

Cyclooxygenase-2 (COX-2) is an enzyme that regulates prostaglandin synthesis and it is highly inducible at cites of inflammation and cancer (Turini and DuBois 2002). COX-2 expression in human tumors can be induced by various growth factors, cytokines, oncogenes, and other factors. IL-1β has been reported to upregulate COX-2 expression in human colorectal cancer cells via multiple signaling pathways, treatments in HT-29 cells with IL-1b induced expression of COX-2 mRNA and protein. (Aggarwal *et al.* 2006). The

same results obtained for the VCAM-1 gene were observed with the COX-2 (Figure 9). There was a significant difference in the suppression of the expression, the same as it was observed in VCAM-1, between the control and the partially hydrolyzed sample. This is due as observed on the chromatograms, to the presence of various small compounds as di-galloyl glucose and tri-galloyl glucose rather than just gallic acid. There was no significant differences between the control and the non-hydrolyzed sample.

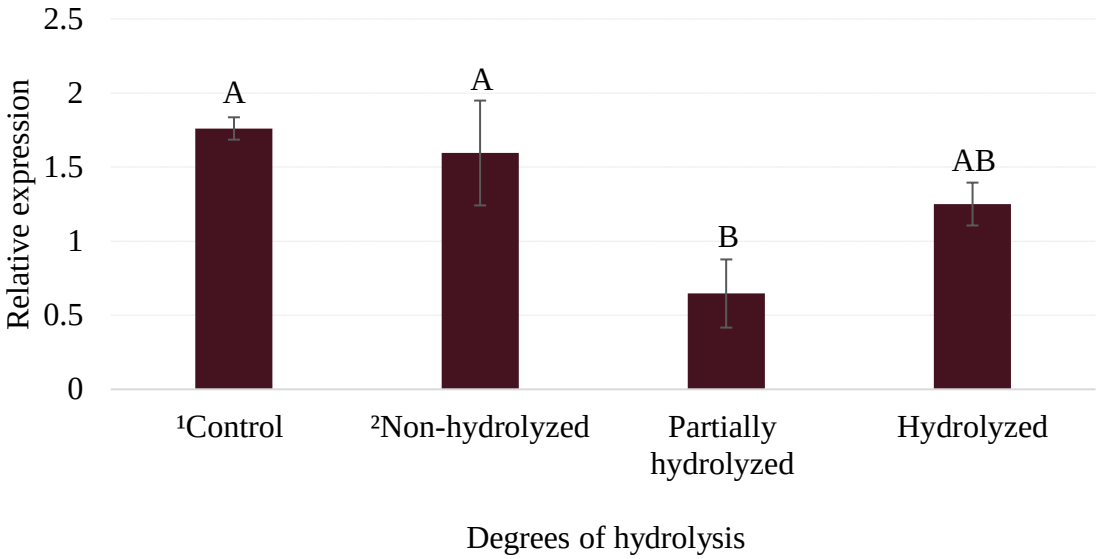

Figure 9. Gene expression (COX-2) in HT-29 cells with sumac extracts application. Different letter indicates statistical difference between treatments ($P < 0.05$)
[1]Cells with no sumac extracts. [2]Cells with sumac extracts with tannase hydrolysis at 0 minutes (Non-hydrolyzed); 10 (Partially hydrolyzed); and 120 (hydrolyzed).

The expression of TLR4 was positive, regardless the level of hydrolysis of the treatments, they all reduced inflammation. There is a statistical difference between the control and the partially-hydrolyzed treatment, while the non and hydrolyzed treatment are statistical similar to both the control and the partially hydrolyzed (Figure 10). HT-29 cells were found to have a more complete repertoire of TLR4 signaling molecules (Rich *et al.* 2011). This increase was due expression by the inflammatory infiltrate rather than expression by epithelial cells (Furrie *et al.* 2005). As the size of the molecules were reduced they become bioavailable for their use, also the variety of compounds present in the partially hydrolyzed sample had a better effect in reducing TLR4 expression. The last three primers evaluated showed significant difference only between the control and the partially hydrolyzed samples.

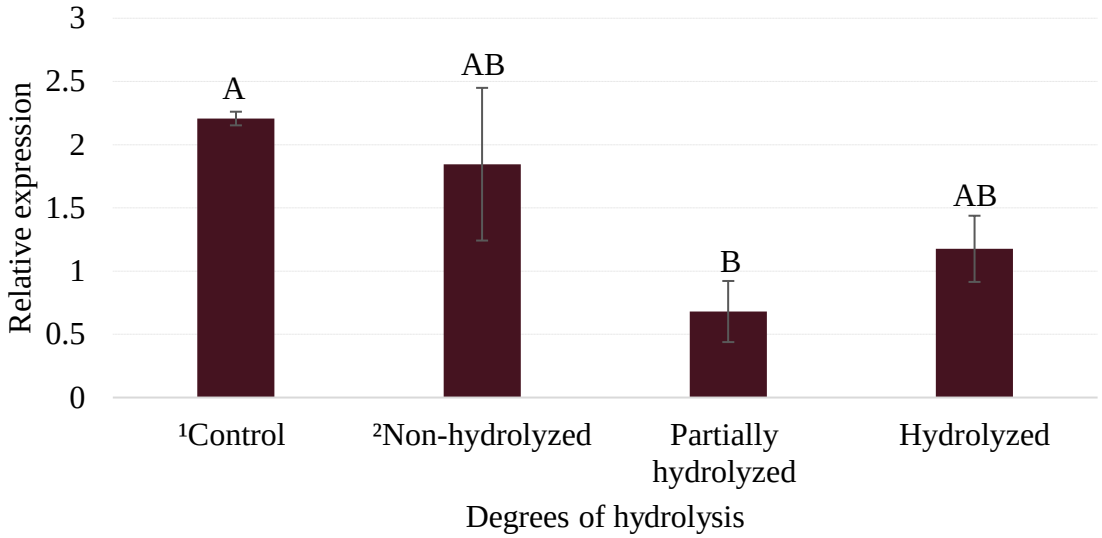

Figure 10. Gene expression (TLR4) in HT-29 cells with sumac extracts application. Different letter indicates statistical difference between treatments ($P < 0.05$)
[1]Cells with no sumac extracts. [2]Cells with sumac extracts with tannase hydrolysis at 0 minutes (Non-hydrolyzed); 10 (Partially hydrolyzed); and 120 (hydrolyzed).

4. CONCLUSIONS

- Addition of the enzyme tannase hydrolyzed the compounds of the sumac extract at ninety minutes, after that, gallic acid levels remained constant.

- Sumac extract used in the cell viability assessment had positive effects in HT-29 cells regardless the stage of hydrolysis and concentrations used.

- There was no significant anti-inflammatory effect for NF-κB, TNF-α, IL-1β genes, while for IL-8 positive effects were observed regardless of the hydrolysis level in sumac extract.

- For VCAM-1, COX-2 and TLR4 partially hydrolyzed extracts had better results in reducing gene expression.

5. RECOMMENDATIONS

- Use different solvents for the extraction of the sumac, such as acetone, to extract other compounds and identify them in the LC-MS.

- Increase the pH of the sumac extracts before adding it to the cells to monitor the influence it has on them.

- Evaluate the different degrees of hydrolysis of the sumac extracts with different cancer cell lines.

- Evaluate the effect of the sumac extracts *in vivo* using rats.

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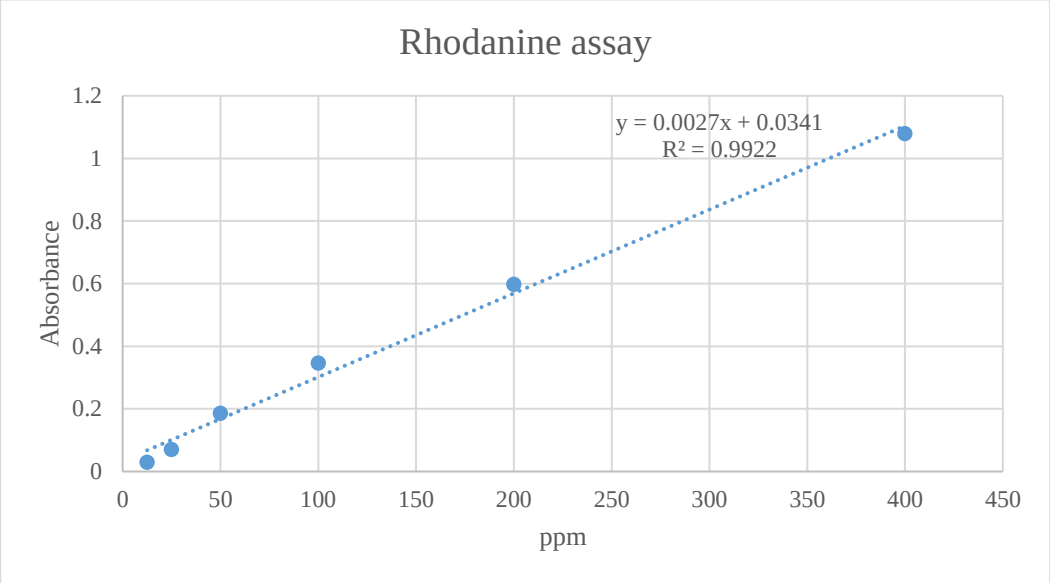
7. APPENDICES

Appendix 1. Enzymatic tannase hydrolysis in sumac extract for 240 minutes

Time (minutes)	Average absorbance	Concentration (apparent)	Concentration (Real) in mg/L	Concentration (Real) in g/L
No tannase	0.237	66.75	6675.31	6.68
0	0.293	87.37	8737.04	8.74
1	0.333	102.19	10218.52	10.22
5	0.409	130.46	13045.68	13.05
10	0.481	157.00	15700.00	15.70
20	0.626	210.83	21082.72	21.08
30	0.684	232.19	23218.52	23.22
45	0.812	279.47	27946.91	27.95
60	0.793	272.56	27255.56	27.26
90	0.996	347.86	34786.42	34.79
120	0.967	337.12	33712.35	33.71
135	0.950	330.83	33082.72	33.08
150	0.987	344.28	34428.40	34.43
165	0.996	347.74	34774.07	34.77
180	0.962	335.27	33527.16	33.53
195	0.981	342.31	34230.86	34.23
210	0.912	316.63	31662.96	31.66
240	0.904	313.79	31379.01	31.38

Appendix 2. Gallic acid standard curve for Rhodanine assay.



Appendix 3. Gallic acid standard curve for LC-MS.