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Anti-inflammatory effect of chaya extracts fractions

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Patients with Type 2 diabetes often experience chronic inflammation due to the overexpression of NF-kB-regulated pro-inflammatory proteins, such as TNF-alpha, IL-6 and IL-1. Chaya (*Cnidoscolus aconitifolius*) extracts have been reported to decrease this expression, but fractions of these extracts have not been studied. The present work focused on determining the anti-inflammatory effect of fractions of aqueous extracts of chaya. The phenolic content fractions of the sample were determined by solid-phase extraction and traced with HPLC-MS-MS, and the activation of the NF-kB factor and the concentration of the pro-inflammatory proteins TNF-alpha and IL-6 were evaluated using streptozotocin-induced diabetes rats. Two of the fractions showed the best effect, in fact, better than the crude fraction, which contains the highest amount of compounds. The presence of ferulic acid in the determined chemical profile suggests that it may be involved in the observed anti-inflammatory effect, while narigenin and apigenin, which are only present in the crude fraction, may be inhibiting the effect, which could explain why this fraction showed a better performance.

Key words: Chaya, fractions, NF-kB, anti-inflammatory effect, ferulic acid.

INTRODUCTION

Diabetes is a global health problem and, in many countries, it is among the three leading causes of death. It is a multifactorial disease, caused by a complex interplay of environmental, genetic, lifestyle, physical activity, and stress factors (Azevedo and Alla, 2008; Chatterjee et al., 2017). Complications, which include amputations, blindness, neuropathy and kidney damage, usually have their beginnings in inflammatory processes that are associated with the activation of genetic factors, mainly NF-kB (Coto et al., 2018). The molecular mechanisms that are associated with the function of pancreatic beta cells include this NF-kB factor that associated with the expression of pro-inflammatory regulates the expression of a large number of genes proteins (Gupta et al., 2010; Thoma and Lightfoot, 2018).

The NF-kB factor is normally present in an inactive form in the cytoplasm of the cell, bound to a dimer of two subunits, p65 and p50. NF-kB can be activated in two ways: through the presence of reactive oxygen species and through pro-inflammatory proteins such as IL-6, IL-1, and TNF-alpha. When these proteins bind to their protein receptors on the cell membrane they activate the IKK-b complex which in turn acts to separate the p65 and p50 dimer and phosphorylate the factor. The already phosphorylated factor, now called p-NF-KB, migrates to the cell nucleus where it binds to specific regions to

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promote the expression of genes encoding various proteins, including IL-6, IL-1 and TNF -alpha. This leads the patient to chronic inflammation that leads to the aforementioned diabetic complications (Cnop et al., 2005; Thoma et al., 2018).

The presence of various phenolic compounds in vegetables such as fruits, stems, flowers, and roots has demonstrated anti-inflammatory effects when used to treat various diseases. As such, the NF-kB signaling pathway is a potential new target for intervention in the treatment and prevention of degenerative diseases including diabetes, cancer, and cardiovascular disease, as about 750 inhibitors of NF-kB activation have been identified (Zárate and Marrugo, 2017). In recent years, compounds such as phenols showed a physiological protective effect against chronic degenerative diseases by interacting with enzymes, transcription factors and scavenging free radicals. At least in Mexico, one of the most widely used plants with these effects is chaya -*Cnidoscolus aconitifolius*- (Castro et al., 2014; Guzmán et al., 2021). Chaya is a shrub native to the Mayan region of Mexico and Central America. Since ancient times it has been used as food in many different dishes such as salads, omelets, “empanadas”, soups and water. However, it is also recognized for a series of beneficial health properties, including those of diabetes treatment, antimicrobial properties, and as a good antioxidant (Panghal et al., 2021)

Us-Medina et al. (2020) have reported that the anti-inflammatory and antioxidant effect of chaya is mainly due to the presence of phenols, flavonoids, flavanones and hydroflavonols. In their study they report that the aqueous extract showed the highest content of phenols (70.61 ± 0.07 g/100 g of extract) and the ethanolic extract registered the highest content of flavonoids (47.76 ± 4.84 g/100 g of extract), flavanones and dihydroflavonols (70.10 ± 7.29 g/100 g of extract). The acetone extract registered the greatest inhibition of the DPPH radical (49.85 ± 5.30 %) while the ethanolic extract showed the greatest inhibition of the ABTS radical (41.01 ± 3.81 %). The ethanolic and aqueous extracts inhibited ACE. The ethanolic extract had the highest anti-inflammatory activity by reducing TNF-α gene expression by 39.78% and IL-6 by 97.81% and its production by 46% and 48.38% respectively.

This work aimed to identify the phenolic fractions of chaya aqueous extracts and verify how they affect the expression of the NF-kB factor and the expression of proinflammatory proteins. In addition, it sought to explore the relationship between the phenols present in the different fractions and the observed effect.

MATERIALS AND METHODS

Preparation of plant materials

Fresh leaves of chaya grown in a domestic garden in the city of Durango, Mexico (24°01′30″N 104°40′03″O), were collected in spring 2019. A botanical sample was deposited in the herbarium of CIIDIR-IPN-Durango, Mexico where it was identified as *Cnidoscolus aconitifolius* (Mill). Voucher No. 53,591 was assigned. The plant is listed in http://www.theplantlist.org/tpl1.1/search?q=Cnidoscolus+aconitifolius as Cnidoscolus *aconitifolius* (Mill.) I.M.Johnst. The material was dried by natural convection until it lost 90% of its original weight. Dried leaves (2.5 g) were crushed and boiled for 5 minutes in 100 ml of water. The extract was filtered through a 0.45 µm pore nitrocellulose filter, denoting this as crude fraction (F0). Subsequent fractions were obtained by solid phase extraction as described by Angel (2005). Thermo Scientific Hypersep C8 cartridges were used.

Fractionation procedures

Five ml of F0 were acidified with 0.1 M HCl until pH 3.5 and were eluted through a first C8 cartridge, thus obtaining the F1 fraction (unbound aqueous fraction). Then, the cartridge was washed with pH 7.0 water and the eluent was passed through a second clean cartridge. This second cartridge was then washed with methanol and the eluent was collected and labeled as phenolic acid fraction. Finally, the first cartridge was washed with ethyl acetate obtaining the flavonoids. Therefore, we obtained three fractions wich were classified as unbound aqueous fraction (F1), phenolic acid fraction (F2), and flavonoid fraction (F3). All fractions were evaporated at 45 °C and vacuum to dryness and then reconstituted in drinking water.

Due to the importance that phenols present, all the fractions were subjected to phenol quantification by the Folin Ciocalteu method and according to what was described by Singleton and Rossi (1965), later the spectrophotometric readings were made at 760 nm and the results were reported expressed as equivalent-micrograms of Gallic acid (GA) per gram of dry leaf (µg / eq GA / g dry leaf).

Phytochemical and chromatographic analyses

The chromatographic analysis of the extract was performed using an Agilent 1200 instrument equipped with a binary pump, autosampler, and a Agilent 6410 QQQ-MS/MS tandem mass detector with ESI as the ionization source, as described by Xia et al. (2019) with modifications. The capillary voltage was set to 3000 V. The drying gas was maintained at 200 °C with a flow of 13 L/min and a pressure of 45 psi. The fragmenter was maintained at 135 V and masses ranging from 50 to 380 in positive polarity were analyzed with a scan time of 150 ms. The mobile phase consisted of a solution of formic acid in aqueous solution at 0.1% and ACN in a proportion of 65/35% v/v in an isocratic regime at a flow rate of 0.05 ml/min. Injection volume 12 ul. A zero volume connection at 45°C was used. The results were expressed as percentage of ion abundance. QQQ-MS/MS analysis identifies ions based on mass/charge (m/z) ratio, resulting in ion count detection. The number of ions of each compound is expressed as a percentage of the total ions counted. The compounds were identified based on the m/z relationship reported in the Mass Bank and Merk index databases.

Anti-diabetic studies

Long Evans rats weighing 150 ± 20 g were divided into 6 groups (n=4) as follows: 1) healthy control without treatment (HC); 2) untreated diabetic control (DC); 3) diabetic group treated with F0 (F0); 4) diabetic group treated with F1(F1); 5) diabetic group treated with F2 (F2) and 6) diabetic group treated with F3 (F3). Diabetes was induced by intraperitoneal injection of 60 mg/Kg of streptozotocin (0.1 M citrate buffer solution, pH 4.5). The rats had access to water ad libitum and were fed 30 g of Nestlé-Purina Petcare Company pellets daily. The extracts were administered in

Table 1. Phenol content in the various fractions.

Fraction	Total phenols (µg/eq AG/g dry leaf)
Crude fraction (F0)	21.46 ± 0.803
Unbound aqueous fraction (F1)	13.31 ± 0.008
Phenolic acid fraction (F2)	3.87 ± 0.005
Flavonoid fraction (F3)	5.2 ± 0.006

Source: Authors.

aqueous form as freely available drinking water. The CS and CD groups had access to pure water. For one month, extracts and water were renewed daily during the trial. Every week during the trial, blood samples were taken from the caudal vein and analyzed for glucose content using a One Touch Select plus glucometer. Throughout the experiment, the ethical guidelines for experimentation in laboratory animals established by Mexican NOM-062-ZOO-1999 "Technical specifications for the production, care and use of laboratory animals" were met in accordance with international guidelines, such as the US guidelines set forth by the NIH. The fulfillment of the ethical aspects was certified by MVZ Gerardo del Campo G. (P.C. 975133-R. SAGARPA 10-0006).

Anti-inflammatory studies

To determine the anti-inflammatory effect of the extracts, the activation of the gene expression factor NF-kB and the expression of pro-inflammatory proteins were determined. Once the study time was over, the pancreatic tissue was removed from the rats by abdominal incision and homogenized with 2 mL of lysis buffer. Proteins were separated by centrifugation and 70% ethanol. They were then separated by electrophoresis on 10% polyacrylamide gels under denaturing conditions (SDS-PAGE) to undergo Western blot. Two antibodies (Santa Cruz Biotechnology Inc.) were used: NF-kB 65 for detection of non-activated NF-kB factor and p-NF-kBp65 for detection of activated factor. Bound antibodies were visualized with a chemiluminescence kit (Clarity Western ECL, BIO-RAD) and the emitted light was captured by ChemiDoc XRS equipment with Image Lab software (BIO-RAD). The densities of the bands were used as a basis for determining the presence of one form or another and were expressed as "relative density". A higher density is synonymous with a higher concentration of either form. To compare the concentrations of each form in each treatment, a t-Student test was applied.

To determine the expression of pro-inflammatory proteins, 2 ml fasting blood samples were taken at the beginning and end of the trial and plasma concentrations of pro-inflammatory cytokines TNF-α and IL-6 were quantified by ELISA (kit Peprotech Inc, . USES). Student's t-test for dependent samples was applied to assess the effect.

RESULTS

Phytochemical and chromatographic analyses

The results indicate that the fractionation procedure is correct and reproducible. The separation of different amounts of phenolic compounds was achieved.

Table 1 shows the content of phenolic compounds in the analysis of 5 batches of extracts. As expected, the F0 fraction is the one with the highest content since it is the complete extract, followed by the F1 fraction. Despite the fact that phenolic compounds are not very soluble in water due to their content of -OH radicals, the results could be explained because in this fraction compounds that are more soluble in water were detected, such as coumaric acid and especially gallic acid (Table 2).

Chromatographic analysis resulted in the identification of various compounds, not just phenols. The compound with the highest abundance was protocatechuic acid present in fractions F0 and F1 (Table 2) with a concentration of 13%. Other compounds such as fatty acids were also found, although in low concentrations. Two forms of kaempferol were also detected, one of them (rutinuside) only present in the crude extract

Antiinflamatory and anti-diabetic studies

After induction of diabetes, the groups showed a high increase in glucose (Figure 1). The untreated group (DC) maintained their glicemya at 403±12 while the healthy group (HC) maintained their glicemya at 97±5. Treatment in the other groups lowered the glucose level by approximately the same proportion. Extract F2 and F3 seem to have the best performance lowering glucose from 348 to 186 and 338 to 160 mg/dl respectively.

Regarding the activation of the transcription factor NF-kB, Figure 2 shows the results of the Western blot expressed as relative density, inactive form (NF-kB p65) and activated form (p-NF-kB p65). As can be seen, the HC group shows a small amount of both forms without a significant difference between them ($p>0.05$). A similar situation occurs in the DC group, although, as expected, the concentrations of both forms are higher, especially the activated form, which has the highest concentration in relation to the other groups.

On the other hand, the group treated with the F3 extract showed greater inactivation of the NF-kB factor, presenting a relative density of 70 as opposed to its active form, which only presents 1. A similar situation occurs with F2 and with F0. The F2 and F3 groups seem to have the best effect to prevent the activation of the transcriptional factor without significant differences between them ($p>0.05$).

The results regarding activation are consistent with the decrease in the concentration of pro-inflammatory proteins. Figure 3 shows the percentage in which the

Table 2. Approximate chemical profile of the fractions (percent ionic abundance).

Composition	F0 %	F1 %	F2 %	F3%
2',3',6-Trimethoxyflavone	2			1
p-Coumaroyltyramine	2	1	1	
Ferulic acid	2		1	2
Clorogenic acid	1			1
Naringenin	2			
Protocatechuic acid	13	13	1	
Apigenin	1			
Caffeic acid	2	3		
Miristic acid	2		1	
Palmitic acid	1		1	
Riboflavin	2	1		1
Niacin	5	4		
Lauric acid	2	2		
Miristic acid	1		2	
Linoleic acid	2			1
Retinol	1	1		1
Catechin	2	1		2
Kaempferol-3-O-rhamnoside	2	1	1	
Kaempferol-3-O-rutinuside	2			
Total of compounds	19	**9**	**7**	**7**

Source: Authors

concentration of said proteins decreases from the initial time of the experiment to the end of the treatment. For TNF-α, (Figure 2), a decrease of 35% in F2, 30% and 40% for F1 was quantified. There were no significant differences between the three groups (ANOVA, $p>0.05$). A similar situation is taken into account for the quantification of IL-6 (Figure 4) where the decrease was 49 and 51% for the groups treated with F2 and F3, respectively, and there were no differences between F1, F2 and F3.

DISCUSSION

As part of the chromatographic analysis and the fractionation process of the extracts, the fractions F2 and F3 were classified as phenolic acids and flavonoids respectively because the process for obtaining fractions is based on a polarity screening of the various compounds. In addition, the use of cartridges packed with C8 matrices does not ensure separation 100% of each type of compound. Therefore some compounds in F2 are not phenolic acids and that other compounds present in F3 are not flavonoids, however they may have a similar affinity for both phases (mobile and stationary), that is, the solvents and the cartridge packing. In any case, the detection of these compounds is interesting because they increase the information that can explain the sought effect.

The low expression of pro-inflammatory proteins in the healthy control is consistent with that detected in Western blot as indicated. On the contrary, for diabetic control, the activation detected in Western blot is consistent with the increase in both pro-inflammatory proteins.

One fact stands out: the crude extract F0, which contains the largest amount of compounds (Table 2), does not show a great inhibition in the activation of the NF-kB factor or in the expression of pro-inflammatory proteins. However, F2 and F3, which registered 8 to 9 compounds (Table 2) have low phenol content (Table 1), and present the best performance. Thus, on the one hand, the presence of ferulic acid present in the three extracts and, on the other hand, the exclusive presence of Naringenin and Apigenin in F0.

Zheng et al. (2019); Zhou et al. (2020) and Manoharan et al. (2014) agree in pointing out the modulating effect of NF-kB when administering ferulic acid in animal models. In all three studies, a significant decrease in IL-6, TNF-α and IL-1β was reported. Likewise, they report a modulation of the NF-kB factor. Additionally, Zheng et al. (2019) report an increase in the concentration of IL-10, which is a protein that reduces inflammation by preventing immune cells from producing cytokines. Liu et al. (2021) have reinforced this theory by administering ferulic acid to bovine epithelial cells and by inducing apoptosis and cell injury. They observed that the administration of ferulic acid blocks oxidative stress, restores the redox balance, inhibits mitochondrial

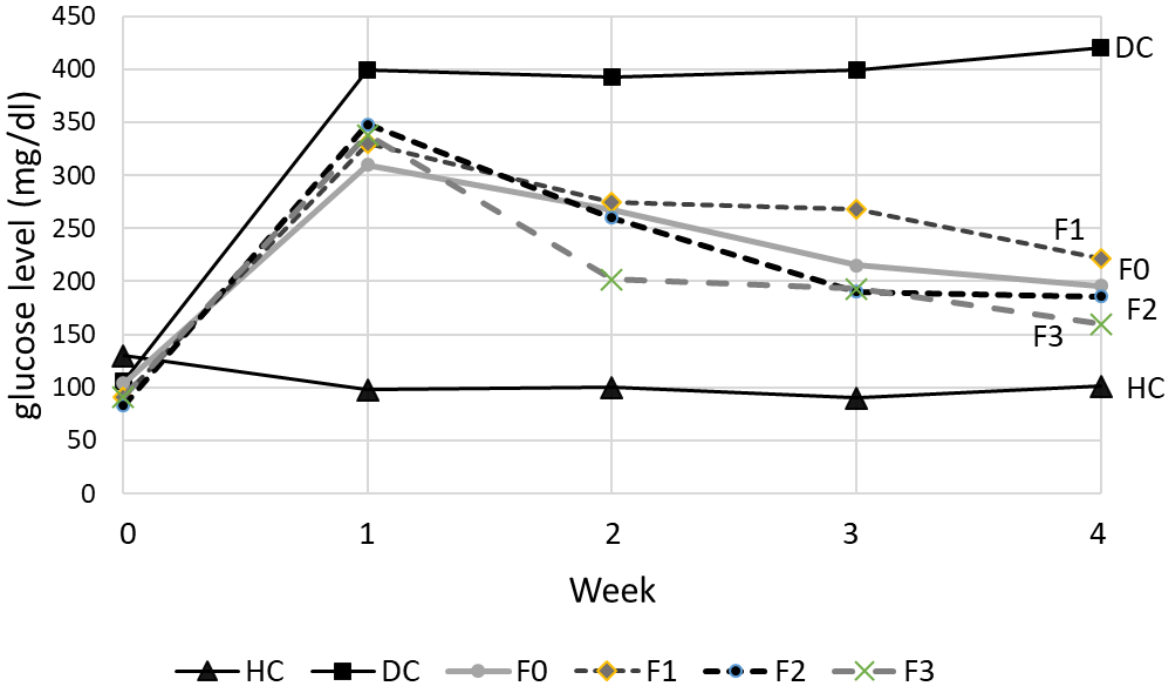

Figure 1. Blood glucose, time zero corresponds to the measurement before the induction of diabetes. The following times (1, 2, 3...) correspond to the weeks of treatment.
Source: Authors.

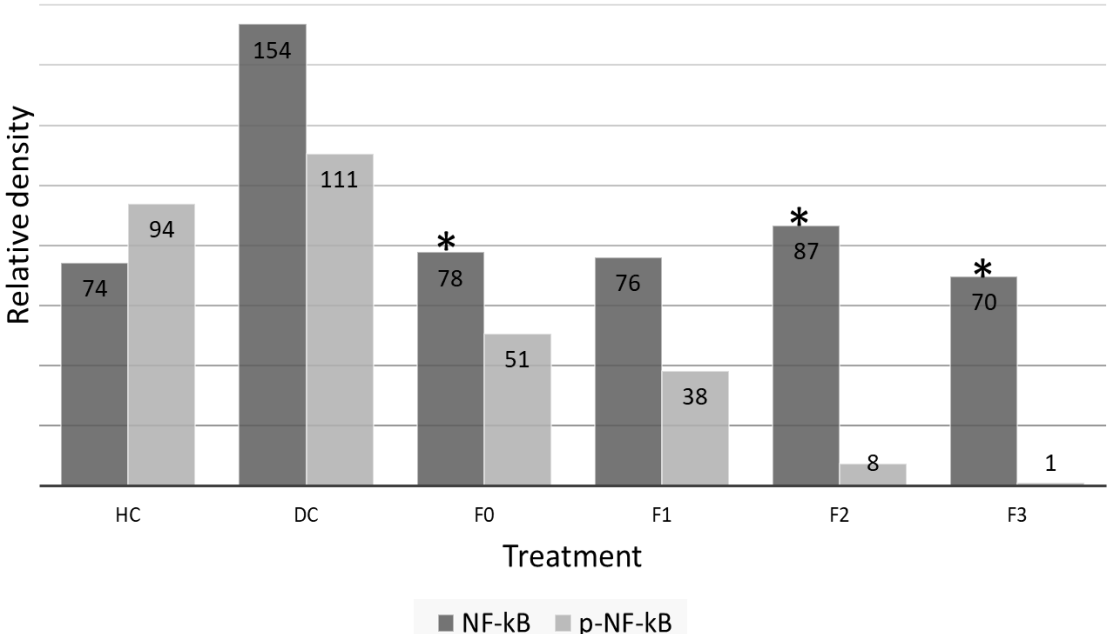

Figure 2. Western blot results identifying inactive (NFkB p65) and active (p-NFkB p65) NF-kB for each group (n=4).
Source: Authors.

dysfunction and, above all, efficiently inhibits NF-kB activation. Therefore, the presence of ferulic acid has an anti-inflammatory effect, but, could another phenol inhibit this effect?

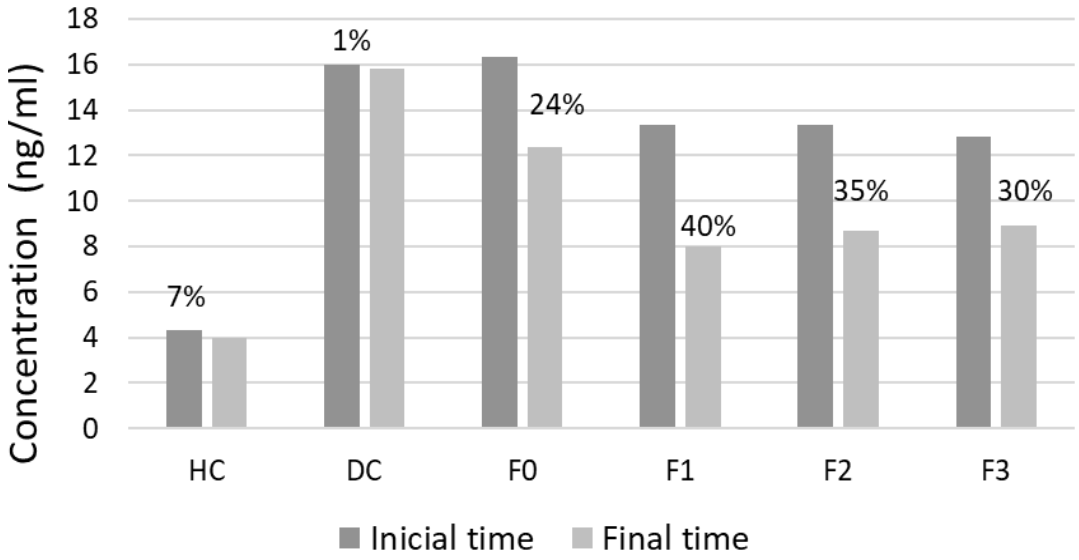

Figure 3. Percentage in the decrease in the presence of TNF-α.
Source: Authors

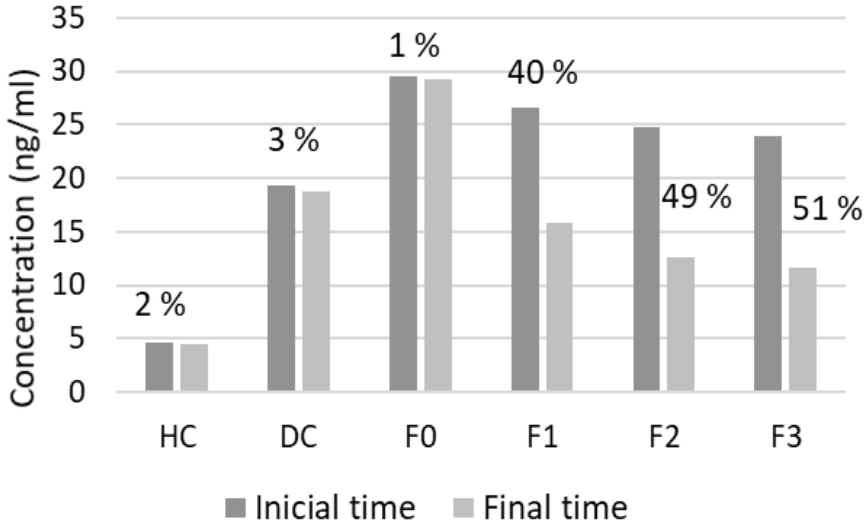

Figure 4. Percentage decrease in the presence of IL-6.
Source: Authors

Yilma et al. (2013) performed a study with infectious processes caused by *Chlamydia trachomatis*, where, the increase in pro-inflammatory proteins such as IL-6, TNF-α and others is reported. Naringenin administration decreased the presence of these proteins, but also decreased the expression of IL-10, so inflammation did not decrease to the extent expected. The presence of Naringenin only in the crude extract (F0) could explain why its anti-inflammatory effect is limited, despite the extract containing more compounds. A similar observation is reported by Palacz et al. (2017) when administering apigenin in cell lines where the expression of IL-10 was restricted. Again, apigenin was only found in in the crude extract in our work.

Conclusion

In conclusion, our results suggest that F2 and F3 fractions have a better anti-inflammatory effect, possibly due to the presence of ferulic acid and other compounds. However, further research is needed to evaluate the presence of compounds such as Naringenin and apigenin that could counteract this effect.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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