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Acute treatment with *Mangifera indica* L. leaf extract attenuates liver inflammation in rats fed a cafeteria diet

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This study investigates the acute anti-inflammatory activity of *Mangifera indica* L. leaf extract and mangiferin in the liver of rats fed a cafeteria diet. This study was a randomized longitudinal experimental study. The animals were divided into three groups – Control: cafeteria diet (CD); Extract: CD + leaf extract (250 mg kg^{-1}); and Mangiferin: CD + mangiferin (40 mg kg^{-1}). Body weight and food intake were measured every week. On day eight, mRNA and protein expression of inflammatory markers were evaluated in the liver. Also, liver weight, SOD activity and malondialdehyde concentration were measured. Treatment for only eight days with mango leaf extract and mangiferin increased SOD activity. Mangiferin intake increased the mRNA expression of PPAR-α and HSP72. The leaf extract treatment enhanced PPAR-α mRNA expression. Mangiferin and leaf extract consumption caused a lower concentration of NFκB (p65) in nuclear extracts, and greater IL-10 mRNA and protein levels. This study highlights the potential of acute treatment with mango leaf extract and mangiferin to prevent liver inflammation caused by fat-rich diets. These results indicate a new use for a product that has low cost, is found in great amounts, and is not routinely used.

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Introduction

The consumption of saturated fatty acids, the main fat present in high-fat diets, is associated with an increase in the inflammatory response in an organism, even when offered in a single meal.[1] Inflammation can also be caused by adipose tissue disturbance as a consequence of excessive fat deposition, macrophage infiltration and increased adipokine/cytokine concentrations, which reach the systemic circulation, contributing to the development of metabolic complications, especially insulin resistance and liver inflammation.[2–4]

The liver is one of the organs most affected by obesity, where metabolically active intra-abdominal fat can induce inflammation due to an increase in macrophage infiltration in the tissue, along with a local production of inflammatory chemokines and cytokines.[5] Also, this tissue is one of the most affected after a single high-fat meal.[1]

In order to reduce inflammation, the organism has an anti-inflammatory mechanism in the form of a heat shock protein (HSP70), which can inactivate NFκB and down-regulate tumour necrosis factor alpha (TNF-α). Studies report that HSP70 plays a cytoprotective role once it negatively regulates TNF-α gene transcription by recruiting heat-shock factor-1 (HSF1).[6–8]

Energetic and lipid homeostasis can be controlled by peroxisome proliferator-activated receptors (PPARs).[9] The PPARs are activated by their natural ligands (saturated and unsaturated fatty acids and their derivatives, prostaglandin and leukotriene) and act on gene expression, promoting fatty acid oxidation, adipocyte differentiation, insulin sensibility, and anti-inflammatory activity.[10,11]

A previous study showed that the pulp, peel, and seed kernels of mango contain high concentrations of antioxidants, which comprise a total of 12 flavonoids and xanthones.[12] Mango stem bark aqueous extract (*Mangifera indica* L.) has received attention from scientists for some time because its anti-inflammatory and antioxidant properties are well estab-

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lished and appear to be specifically related with mangiferin. However, scientific investigations reveal that mangiferin's action does not present the same intensity as extracts, highlighting the importance of synergism among bioactive compounds in a complex matrix with a better effect than isolated compounds.[13,14]

In this context, considering the acute effect of a high-fat diet, the aim of this study was to evaluate the short-term consequences of *Mangifera indica* L. leaf extract and mangiferin in the liver inflammation of rats fed a cafeteria diet.

Experimental

Leaf extract and mangiferin preparation

Leaves of *Mangifera indica* L., Ubá variety, were collected in Viçosa, Minas Gerais, Brazil, and exsiccates deposited in the Herbarium of the Federal University of Viçosa (VIC no 37.611). Leaves were dried at room temperature and ground. To produce a 95% ethanolic extract, the powdered leaves were subjected to exhaustive percolation with 95% ethanol solution and vacuum concentration *via* a rotary evaporator at a maximum temperature of 60 °C (15 °F), followed by lyophilization, yielding about 27% of the end product from dry matter.

To isolate mangiferin, the leaf extract mentioned above was thoroughly degreased with petroleum ether by Soxhlet extraction for 12 hours. After this period, the extract was submitted to successive recrystallization with ethyl acetate, yielding 3.6% of mangiferin from dry matter. The purification method[15] was monitored by thin-layer chromatography and purity was evaluated by HPLC using a mangiferin analytical standard for comparison (Sigma-Aldrich). The methodology used herein was an adaptation of the one described by Bhatt, Sebastian and Joshi,[16] and Muruganandan, Gupta, Kataria, Lal, and Gupta.[17]

Biological assay

This assay was carried out after the institution's Ethics Committee approval (Protocol no. 28/2012) following the National Institutes of Health guide for the care and use of laboratory animals. Twenty-four male weanling Wistar rats, aged twenty-five days, were distributed in collective ventilated cages and kept in an air-conditioned room at 22 ± 2 °C with a 12 hour photoperiod (light/dark). They received the commercial feed Presence Rats and Mouse® (Protein: 23 g per 100 g; Lipids: 4 g per 100 g; Fiber: 5 g per 100 g) for seven days for the adaptation period, after which a cafeteria-diet was introduced, along with the treatments, for an eight day period. The animals were divided into 3 experimental groups: Control (cafeteria diet), Extract (cafeteria diet + leaf extract), and Mangiferin (cafeteria diet + mangiferin), each comprising eight animals (n = 8). Both treatments, mango leaf extract and mangiferin, were administered *via* gavage to the animals at concentrations of 250 mg kg^{-1} and 40 mg kg^{-1}, respectively, using dimethyl sulfoxide (DMSO) as the vehicle. The control group received *via* gavage only the vehicle. This quantity was established according to studies carried out in ref. 18.

The calorie distribution in the cafeteria diet was: 9.8% protein, 31.4% carbohydrate, and 58.8% lipid, and composed of ham pate, potato sticks, bacon, mortadella, corn-starch cookie, chocolate, milk powder, and commercial feed in the proportion 2 : 1 : 1 : 1 : 1 : 1 : 1 : 1. Throughout the entire experiment, the animals were supplied diet and water *ad libitum*.

After 8 days of treatment, the animals were submitted to a 12 hour fasting period and then euthanized. The liver was removed, weighed, and stored at −80 °C for further analysis. The hepatosomatic index was calculated as a relation between liver weight and body weight multiplied by 100.[19]

Parameters evaluated in liver homogenate

Liver homogenates were prepared by suspending 500 mg of tissue in 5 mL of Tris-buffer (0.01 M, pH 7.4) using a homogenizer linked to an ice bath. The homogenates were then centrifuged under refrigeration and the supernatants stored at −80 °C (−176 °F).

The malondialdehyde (MDA) determination was by the thiobarbituric acid reactive substances (TBARS) method.[20] Calculation of final values to minimize the interference of heme and haemoglobin pigments in the measurement of MDA[21] was performed using three wavelengths (510, 560 and 535 nm). The MDA amount was calculated using the molar absorptivity coefficient $E_0 = 1.56 \times 10^5$ M^{-1} cm^{-1}, and results were expressed as nmoles of MDA per mg of protein (MDA/PTN).[21]

The quantification of superoxide dismutase (SOD) was given in relative units, one unit being defined as the SOD enzyme amount that inhibits by 50% the pyrogallol oxidation rate. The calculations were performed using the linear equation obtained from a standard curve, which had five concentrations of SOD (1–5 U). The analysis was performed at 420 nm and the results expressed as U SOD per mg protein.[22] The total protein of the liver homogenate was measured by the Bradford method.[23]

Expression of inflammatory markers

Total RNA extraction was performed using Trizol Reagent (Invitrogen, CA, USA). The concentration and purity were evaluated by a μDrop plate Multiskan™ GO spectrophotometer (Thermo Scientific, DE, USA), and the integrity was confirmed by agarose gel electrophoresis. For cDNA synthesis, an M-MLV Reverse Transcriptase Kit (Invitrogen) was used. Relative quantification of gene expression was performed by RT-qPCR using an ABStep One Real Time PCR System and the reagent 2X SYBR Green Master Mix (Applied Biosystems, CA, USA). The initial parameters used in the run were 15 minutes at 95 °C and then 40 cycles at 95 °C (15 s), 60 °C (30 s), and 72 °C (30 s), followed by melting curve analysis. Sense and antisense primer sequences were ordered (Choma Biotechnologies) to amplify PPAR-α, HSP72, CB1, NFκB, TNF-α and IL10. The relative expression levels of mRNA were normalized to the endogenous control: glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Table 1). All the steps were performed under open conditions with RNase.

Table 1 Sequence of primers used in the RT-PCR analysis

	Oligonucleotide (5′–3′)	
Genes	Forward	Reverse
GAPDH	GGTTGTCTCCTGTCACTTC	CTGTTGCTGTAGCCATATTC
PPAR-α	CCTGCCTTCCCTGTGAACT	ATCTGCTTCAAGTGGGGAGA
HSP72	AGGCCAACAAGATCACCATC	TAGGACTCGAGCGCATTCTT
CB1	TATATTCTCTGGAAGGCTCACAGCC	GAGCATACTGCAGAATGCAAACAC
NFκB	CTTCTGGGCCATATGTGGAGA	TCGCACTTGTAACGGAAACG
TNF- α	ACGGCATGGATCTCAAAGAC	AGATAGCAAATCGGCTGACG
IL-10	ACTACCATAGCCACAACGCA	TTTCTGTTTCCTACGGCGCT

GAPDH: glyceraldehyde 3-phosphate dehydrogenase; PPAR-α: peroxisome proliferator-activated receptor alpha; HSP72: heat shock protein 72; CB1: Cannabinoid Receptor 1; NFκB: factor nuclear kappa B; TNF-α: tumor necrosis factor alpha; IL10: interleukin 10.

Determination of cytokines by flow cytometry

The cytokines were measured in the cytoplasmic extract of liver tissue. We used the NE-PER nuclear and cytoplasmic extraction kit (Pierce Biotechnology/Thermo Fisher Scientific Inc., MA, USA) to separate the nuclear and cytoplasmic fractions of liver homogenates. These fractions were stored at −80 °C until analysis. The concentrations of TNF-α and IL10 were determined by the Cytometric Bead Array method, using commercial kits, according to the manufacturer's protocols (BD Biosciences, CA, USA). The data (ng ml^{-1}) were obtained using the software FCAP Array.

Analysis of p65

The analysis of p65 was performed in nuclear extracts of liver tissues using a specific NFκB (p65) Transcription Factor Assay kit (Cayman Chemical) according to the manufacturer's instructions. The amount of NFκB p65 was evaluated at 450 nm in a Multiskan™ GO spectrophotometer (Thermo Fisher Scientific Inc., MA, USA) and the results obtained according to the absorbance of the positive control.

Statistical analysis

The treatments were conducted in a completely randomized design, with eight repetitions. The results were subjected to analysis of variance at 5% probability. To determine "*F*-value" significance, a *post hoc* Tukey's test was carried out to compare means among the groups. Statistical analyses were performed in the Sigma Plot 11.0 software, version 9.0. Data with a *p*-value ≤ 0.05 were considered statistically significant.

Results

Isolation of mangiferin and structural confirmation

The purification process of the ethanolic extract obtained from *Mangifera indica* leaves provided 21 g of mangiferin, yielding 3.60% from the dry drug and 5.83% from the extract. The compound presented a chromatogram in HPLC with a single peak (RT 8.2 min) and a UV spectrum compatible with the standard (Fig. 1).

Hepatosomatic index, MDA content and SOD activity

The animals presented the same weight gain and liver weight. However, the hepatosomatic index was lowest in the extract group, indicating that mango leaf extract interferes with liver metabolism (Table 2). There was no significant difference in MDA content between the groups. However, the treated groups showed higher SOD activity than the control, with mangiferin intake causing the greatest activity (Table 2).

Inflammatory markers

There was a greater PPAR-α mRNA expression in the groups treated with *M. indica* leaf extract (2.04 ± 0.48) and mangiferin (4.58 ± 0.33) when compared to the control (1.00 ± 0.00) (Fig. 2A). Animals that received mangiferin had significantly higher HSP72 mRNA expression (1.22 ± 0.08). The leaf extract administration did not interfere with the chaperone mRNA expression when compared to the control group (Fig. 2B).

Different from expected, mangiferin treatment enhanced CB1 mRNA expression (2.21 ± 0.51) (Fig. 2C), however, with no effect on food consumption, since the groups did not present any difference in dietary intake (data not shown). There was no significant difference in NFκB mRNA expression between the studied groups; however, treatment with leaf extract and mangiferin caused a lower p65 concentration in the nuclear extract (0.88 ± 0.04 and 0.85 ± 0.04 Relative Units, respectively) compared to the control group (1.06 ± 0.08 Relative Units). This result indicates that there was lower concentration of active NFκB in the treated animals (Fig. 3A and B). The leaf extract (254.32 ± 6.58) and mangiferin (244.59 ± 81.98 pg mL^{-1}) did not significantly interfere with TNF-α expression and concentration in comparison to the control group (284.47 ± 95.59 pg mL^{-1}) (Fig. 3C and D).

All treated groups showed lower IL-10 mRNA expression compared to the control; however, the IL-10 protein concentration was significantly higher when the animals received the extract (431.90 ± 40.97 pg mL^{-1}) and mangiferin (161.24 ± 27.53 pg mL^{-1}) (Fig. 3E and F).

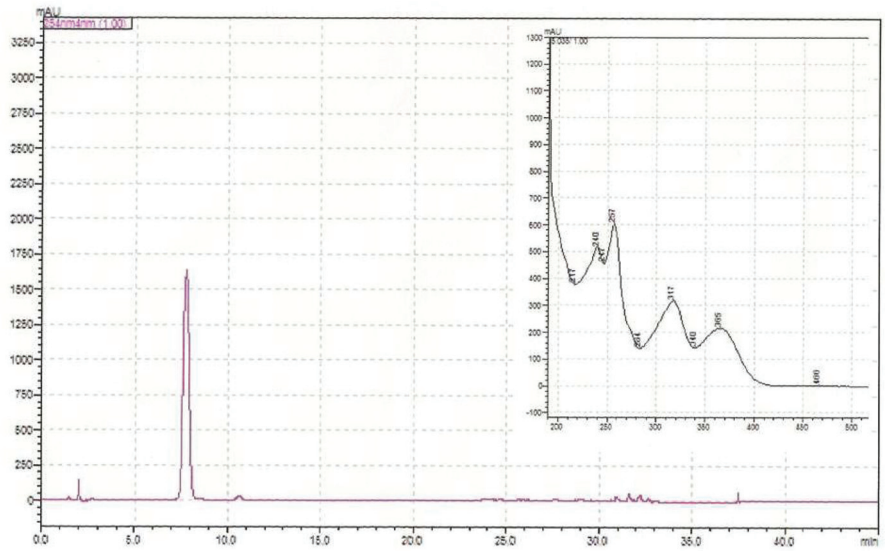

Fig. 1 Chromatogram and UV spectrum of mangiferin purified from the leaves of *M. indica*. Conditions: Diode array detector; C18 column; mobile phase: solvent A, 0.3% acetic acid P.A., and solvent B, HPLC grade acetonitrile; gradient: 10% B for 5 min, 20% B for 15, 100% B for 15 min, 10% B for 10 min; flow rate: 1 ml min^{-1}, injected volume: 50 μl.

Table 2 Body weight, liver weight, hepatosomatic index, superoxide dismutase activity and malondialdehyde of rats fed a cafeteria diet and submitted to different treatments for 8 days

	Groups		
Variables	Control	Extract	Mangiferin
Body weight (g)	49.25 ± 4.51[a]	40.90 ± 5.26[a]	45.75 ± 5.13[a]
Liver weight (g)	4.84 ± 0.76[a]	3.96 ± 0.58[a]	4.58 ± 0.55[a]
Hepatosomatic index	4.79 ± 0.42[a]	3.94 ± 0.42[b]	4.62 ± 0.38[a]
SOD (U mg^{-1} protein)	34.28 ± 12.77[c]	84.02 ± 3.62[b]	101.48 ± 12.77[a]
MDA (nmol mg^{-1} protein)	1.16 ± 0.09[a]	1.01 ± 0.17[a]	1.02 ± 0.16[a]

Mean ± SD followed by the same letters do not present a significant difference by the Tukey test ($p < 0.05$). SOD: superoxide dismutase; MDA: malondialdehyde.

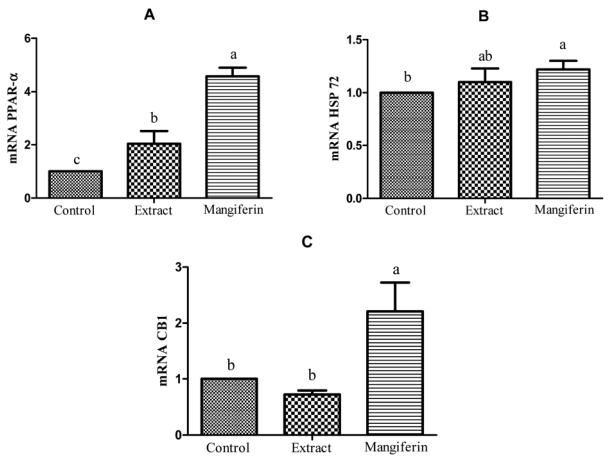

Fig. 2 mRNA expression in the liver of rats subjected to different treatments for 8 days. A: PPAR-α receptor. B: HSP72. C: CB1 endocannabinoid receptor. Control: Cafeteria diet (CD) + DMSO; Extract: CD + mango leaf extract; Mangiferin: CD + mangiferin. Mean ± SD followed by the same letters do not present a significant difference by the Tukey test ($p < 0.05$).

Discussion

Although the animals presented the same weight gain and liver weight, the hepatosomatic index was the lowest in the extract group. Li *et al.*[24] demonstrated that high fat diet-fed rats present higher hepatosomatic index, resulting in greater lipid accumulation in the hepatic tissue, with possible alterations such as steatosis and fibrosis. Thus, reduction of the hepatosomatic index in animals treated with high-fat diets is an indication of lower lipid accumulation, a positive factor for animal health.

In a previous study that evaluated the effect of mango pulp (Ubá variety) intake at two concentrations, it was shown that ingestion of a diet with 3% mango pulp produced lower hepatosomatic index compared to a diet with 10% mango pulp.[25] This indicates that the amount of bioactive compounds in the diet may be critical for liver response. This was also observed in the present study, since mango leaf extract reduced the hepatosomatic index, while the mangiferin group did not exhibit the same reductions. In addition, another factor to consider is the synergistic effect that the leaf extract can present, since there are other bioactive compounds besides mangiferin, which may be acting together and causing a better outcome.

MDA is produced from PUFAs in two different reactions: as an enzymatic eicosanoid formation side product and as an

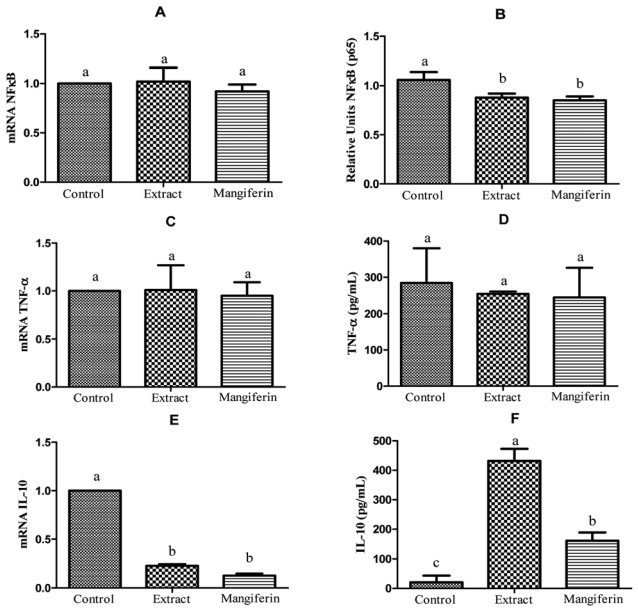

Fig. 3 A, C and E: mRNA expression in the liver of rats subjected to different treatments for 8 days. A: NFκB. C: TNF-α. E: IL10. B, D and F: nuclear (NFκB (p65)) and cytosolic (TNF-α and IL-10) protein concentration in the liver of rats. B: NFκB (p65). D: TNF-α. F: IL-10. Control: Cafeteria diet (CD), Extract: CD + mango leaf extract, Mangiferin: CD + mangiferin. Mean ± SD followed by the same letters do not present a significant difference by the Tukey test ($p < 0.05$).

end product of non-enzymatic, metabolically uncoupled PUFA oxidative degradation.[26] Due to the last reaction cited, there is a quantitative relationship between lipid peroxidation and MDA content,[26] and so MDA is used as a marker of lipid peroxidation. However, there was no difference in MDA content between the groups in the present study. Marineli *et al.*[27] also did not find a reduction in MDA concentration in the liver of animals treated with chia seeds and oil for 6 or 12 weeks, although they verified a reduction in the plasma TBARS concentration and an increase in the antioxidant enzyme activity in hepatic tissue. The authors argued that some tissues have a lower metabolic rate, with a slower reversal process, and therefore there would be no reduction of the lipid peroxidation marker in the liver during this period of treatment. Thus, according to the observations in the previous study, the lack of effect in terms of MDA concentration after treatment could be a result of the short experimental period (only eight days). A lack of difference in MDA content due to the treatment period was also observed by Martins *et al.*[28] in which, with only 14 days of intervention, there was no difference between negative (fed AIN-93 diet) and positive (fed HFD) controls. On the other side, with 28 days of treatment, the negative control showed lower MDA content than the positive control.

Despite the results reported for MDA, as in Marineli *et al.*[27] and Martins *et al.*,[28] greater SOD activity was observed with the treatment. SODs are a group of oxidoreductase enzymes that act as the main cellular defense against superoxide anions, catalysing the dismutation of $O^{2\bullet-}$ into oxygen and H_2O_2, acting in the antioxidant defense of the organism.[29] Thus, the results indicate that the treatment with mango leaf extract and mangiferin can possibly improve the antioxidant defenses, the effect being most pronounced for the isolated compound.

PPARα is a nuclear transcription factor that acts on the expression of β-oxidation-inducing proteins, such as PGC1-α[30] and the hormone-sensitive lipase.[31] Moreover, this nuclear transcription factor seems to be involved in white adipose tissue browning, contributing to the formation of a tissue more susceptible to lipid oxidation for heat production, thus favouring body fat reduction.[30] Still, PPARα activation is involved in the reduction of the inflammatory response, as this nuclear transcription factor inhibits the expression of pro-inflammatory cytokines.[31] In this way, the consumption of the products tested, mango leaf extract and mangiferin, could contribute to body weight and inflammatory response reduction due to greater PPARα expression. The same results were observed in Wistar rats fed with mango leaf tea for 8 weeks. The authors observed that mango leaf tea was able to increase PPAR-α mRNA expression.[32]

The HSP70 chaperone is necessary to degrade p65 and inhibit the NFκB signalling pathway.[8,33] Chung *et al.*[34] found that HSP70 overexpression stops JNK activation and the development of insulin resistance in rats fed a high fat diet, whose body weight was a quarter of the wild lineage. The chaperone HSPs also reduced inflammation and restored glucose homeostasis in a type 2 diabetes (DM2) rat model.[7,35] Accordingly, the induction of heat shock proteins, like HSP70, can reduce inflammation and improve insulin signalling in obesity and DM2.[36] These data are in accordance with the present study, in which the treated groups presented higher HSP72 mRNA expression with less p65 activity (Fig. 2B), suggesting a prominent anti-inflammatory effect correlated with the ingestion of mango leaf extract or mangiferin under the experimental conditions.

For NFkB and TNF-α, the treatment did not cause any difference in mRNA expression; however, it is possible to observe a lower amount of active NFκB in the nucleus, and a tendency of lower protein concentration of TNF-α. In this way, the leaf extract and mangiferin may have exerted their action on post-translational and/or post-transduction events. Furthermore, it is known that NFκB after its synthesis is present in the cytoplasm in an inactive form, and only after IκB phosphorylation can this nuclear transcription factor enter the nucleus and cause pro-inflammatory cytokine expression.[37] In this way, the samples tested in the present study could be acting in this step, inhibiting IκB phosphorylation, and preventing the activation of NFκB. Confirming this hypothesis, studies performed by Márquez *et al.*[38] and Das *et al.*[39] also showed that mangiferin could inhibit the NFκB pathway, *via* IκB phosphorylation. Furthermore, Kim *et al.*[40] observed that administration of mango beverages in rats suppressed NF-κB and p-NF-κB protein expression, protected against docusate-induced colonic inflammation, and significantly attenuated the expression of pro-inflammatory cytokines such as TNF-α, IL-1β, and iNOS at the mRNA and protein levels. In accordance

with these results, Ramírez *et al.*[32] showed that the consumption of mango leaf tea for 8 weeks decreased NF-κB p65 activation and also inhibited NF-κB p65 gene expression in Wistar rats.

IL-10 is a cytokine with an anti-inflammatory activity that can be produced in the liver by hepatocytes, sinusoidal endothelial cells, Kupffer cells, hepatic stellate cells and liver-associated lymphocytes. Its synthesis is stimulated by endogenous and exogenous factors such as stress, exotoxins, TNF-α, catecholamines, and cAMP-elevating drugs. IL-10 activates Jak1 and Tyk2, inhibiting NFκB activity,[41] and as a consequence inhibits the expression of pro-inflammatory cytokine, such as TNF-α.[42] In our study, the higher IL-10 concentration after the leaf extract and mangiferin treatment downregulated the NFκB concentration, demonstrating an anti-inflammatory effect.

The apparent contradiction between IL-10 mRNA expression and protein concentration in the liver tissue of the animals has already been reported in the literature, as a higher mRNA expression is not necessarily translated into greater protein concentration. Shebl *et al.*[43] observed that there is no correlation between mRNA expression and protein content for some cytokines, including IL-10. According to the authors, this difference can be explained by post-transcriptional and post-translational regulation. This difference caused by changes in the post transcriptional level of the cytokines was also described by Iyer and Cheng.[44]

The results demonstrate that both mango leaf extract and mangiferin may affect this anti-inflammatory cytokine, increasing the IL-10 level. Still, it is possible that greater CB1 receptor expression may have influenced the lower IL-10 concentration in the mangiferin group compared to the leaf extract treatment, since this receptor interferes in the expression of the cytokine.[45,46] Despite this, since mangiferin acts in other pathways, the final result was an anti-inflammatory effect after its administration.

Conclusions

Previous studies have indicated the anti-inflammatory and antioxidant effect of mango using semi-purified mangiferin or fruit pulp. In the present research, we demonstrate that the extract obtained from mango leaf also has beneficial effects on health. These results indicate a new use for a product that has low cost, is found in great amounts, and is not routinely used.

Treatment for only eight days with mango leaf extract and mangiferin was able to increase SOD activity, possibly promoting improvement in the antioxidant defenses. Furthermore, there was an anti-inflammatory effect, with an increase in PPAR-α and HSP-72 expression, a reduction in the nuclear levels of NFκB and an increase in IL-10. In this way, the results indicate an anti-inflammatory potential of mango leaf extract, in the liver of rats fed a cafeteria diet.

Finally, we suggest that future research could be conducted using a standard drug as a comparison and studies could be performed using lower doses of mangifera leaf extract, since the dose used (250 mg per kg rat body weight) is too high to be extrapolated to humans.

Conflicts of interest

There are no conflicts to declare.

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