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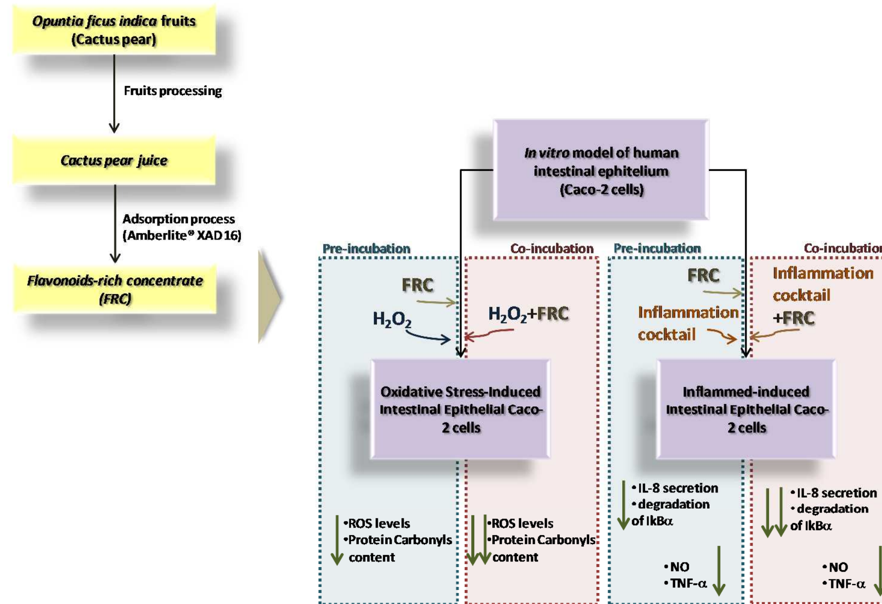


Flavonoids-rich concentrate (FRC) recovered from cactus pear juice is able to modulate intestinal oxidative stress biomarkers and inflammatory mediators suggesting that could be an interesting natural ingredient to attenuate and prevent intestinal chronic inflammation.

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1 Antioxidant and anti-inflammatory activity of a flavonoid-rich concentrate recovered from
2 *Opuntia ficus-indica* juice

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1 **Abstract**

2 In this work, *Opuntia ficus indica* juice was explored as potential source of natural antioxidant and
3 anti-inflammatory ingredients towards intestinal inflammation. An adsorption separation process
4 was used to produce a natural flavonoid-rich concentrate (FRC) from *Opuntia ficus-indica* juice.
5 The FRC effect (co- or pre-incubation) on induced-oxidative stress and induced-inflammation was
6 evaluated in human Caco-2 cells. The main constituents identified and presented in the extract are
7 flavonoids (namely isorhamnetins and its derivatives such as isorhamnetin 3-O-rhamnose-
8 rutinoside and isorhamnetin 3-O-rutinoside) and phenolic acids (such as ferulic, piscidic and
9 eucomic acid). Our results showed that co-incubation of FRC with stress-inductor attenuates
10 radicals production in a more significantly manner than pre-incubation. These results suggest that
11 FRC compounds which cannot pass the cell membrane freely (isorhamnetins derivatives) had
12 ability to inhibit the formation of $H_2O_2$-induced radicals in the surrounding environment of
13 intestinal epithelial cells. The capacity of FRC (co-incubation) in suppressing (at extracellular
14 level) free radicals chain initiation or propagation reaction was probably related with a more
15 pronounced reduction in protein oxidation. A similar response was observed in inflammatory state,
16 where a markedly decrease in IL-8 secretion and blocked degradation of IκBa was achieved for
17 FRC co-incubation. Simultaneously, treatment with FRC significantly reduces NO and TNF-
18 $\alpha$ expression and modulate apparent permeability in Caco-2 cells. In these cases, no significant
19 differences were found between pre- and co-incubation treatments suggesting that bioavailable
20 phenolics, such as ferulic, eucomic and piscidic acid and isorhamnetin acts at intracellular
21 environment.

22 Keywords: intestinal inflammation, oxidative stress, *Opuntia ficus-indica*, flavonoids,
23 isorhamnetins

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1 ## 1 - Introduction

2 Currently, established therapies for inflammatory bowel diseases (IBD) are characterized by a
3 limited therapeutical efficacy and occurrence of adverse side effects, leading patients to
4 increasingly look for complementary and alternative medicines for symptom relief and improved
5 quality of life[1]. Recently, a wide range of phytochemicals have demonstrated their role in the
6 modulation of inflammatory responses[2]. Despite significant advances in the understanding of
7 polyphenols biology, they are still mistakenly regarded as simply antioxidants. However, recent
8 evidences suggest that they play a significant role in decreasing oxidative/inflammatory stress and
9 increasing protective signaling[3].
10 *Opuntia ficus-indica*, also known as cactus pear, is the most common member of *Cactaceae* family
11 and belongs to *Opuntia spp*. Since ancient times, *Opuntia ficus-indica* have been used in traditional
12 medicine but recently their popularity increased in developed countries and is now recognized as
13 a rich source of phytochemicals with health-promoting activities[4]. Bioactive composition of
14 *Opuntia ficus-indica* fruit includes flavonoids such as isorhamnetin glycosides, quercetin and
15 derivates, and also two types of betalains, which are also responsible for the fruit colours:
16 betaxanthins and betacyanins[4]. Recently, a diet rich in betalains and flavonoids, naturally present
17 in *Opuntia ficus-indica* fruits, have been associated with reduced risk of oxidative stress-related
18 diseases, such cancer, cardiovascular and neurodegenerative diseases[5].
19 *Opuntia ficus-indica* is very abundant in Portugal, especially in South of Portugal, at Alentejo, but
20 its culture is practically not explored in contrast with other countries such as Italy and Mexico that
21 use cactus pears as food (in form of beverages, jellies and others), animal forage, natural dyes
22 among others[6].

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1 In a previous work from our lab, juices derived from *Opuntia ficus-indica* fruits collected in
2 different locations on Portugal were evaluated and compared in terms of nutritional content,
3 phytochemical composition and *in vitro* antioxidant activity. This study demonstrated that
4 flavonoids of *Opuntia ficus-indica* juices are resistant to pH variations and enzymatic reactions
5 occurring during gastrointestinal digestion[7] being bioavailable at intestinal level.
6 In an effort to increase the exploitation and consumption of traditional Portuguese *Opuntia ficus-*
7 *indica* fruits, in this work we evaluate the potential of developing from cactus pear's fruit juices,
8 new anti-inflammatory bioingredients. A flavonoid-rich concentrate (FRC) was prepared from an
9 *Opuntia ficus-indica* juice, using an adsorption separation process. FRC was further characterized
10 in terms of phenolic content and antioxidant capacity and its modulatory effects on the oxidative
11 stress biomarkers and inflammatory mediators were studied using Caco-2 cells as model of human
12 intestinal epithelium.
13
14 **2- Results and Discussion**

15 **2.1 – FRC composition and antioxidant activity**

16 Former work involving phytochemical characterization of several *Opuntia ficus indica* juices
17 obtained from wild fruits collected in different sites of Portugal, showed that juice prepared with
18 wild fruits from Beja (N37º56.559, W007º35.246) was a rich source of flavonoids[8]. Aiming at
19 developing promising anti-inflammatory bioingredients from *Opuntia ficus indica* fruits, a
20 flavonoid-rich concentrate (FRC) was prepared from Beja cactus pears juice using a static
21 adsorption process with a macroporous resin Amberlite® XAD16. XAD16 resin demonstrated to
22 be highly efficient in eliminating constituents such as carbohydrates, organic acids and minerals
23 and concentrating in the selected target compounds (flavonoids). FRC was analysed for their



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1 phytochemical content (Table 1 and Figure 1), in particular polyphenols and betaxanthins and
2 antioxidant activity (Table 2). Among all the phenolics recovered, flavonols (with an absorption
3 maximum at ±360 nm) and hydroxycinnamic acids predominates (Table 1). From the main
4 compounds identified the most relevant ones are isorhamnetin and its conjugates, namely,
5 isorhamnetin-3-O-lyxose-rhamnose-glucoside, isorhamnetin-3-O-lyxose-glucoside,
6 isorhamnetin-3-O-glucoside and isorhamnetin-3-O-rutinoside and hydroxycinnamic acids,
7 eucomic acid, piscidic acid and ferulic acid (Figure 1 and Table 1). The phenolic content of FRC
8 was 8-fold higher than non-processed juice (16.5 mgGAE.$g^{-1}$ dry juice) revealing that adsorptive
9 process using XAD16 resin was efficient for polyphenolic concentration. Additionally, FRC
10 presented a total betaxanthins concentration of 0.30mg/g *dw* extract. The chromatographic profile
11 (Figure 1, absorption wavelength of 420 nm) revealed the presence of betaxanthins (small peaks
12 between 50 and 60 min). However, due to lack of standard compound s were impossible to
13 determine which compound s are the identified peaks.
14
15 FRC exhibited stronger activity in scavenging peroxyl radicals than in inhibiting the formation of
16 hydroxyl radicals since FRC's ORAC value was 3.5-fold higher than HORAC (Table 2). In our
17 previous work (data not shown), ferulic acid showed to positively correlate with ORAC value
18 revealing to be a significant contributor of antioxidant capacity. Moreover, isorhamnetin and its
19 glycosides derivates are also recognized as ROS scavengers[9].
20 Comparing to Vitamin C (3220 ± 312 μmolTEAC.$g^{-1}$)[10], FRC possess lower ORAC value.
21 However it is known that ascorbic acid can also act as pro-oxidant and have low stability during
22 gastrointestinal digestion[11, 12]. Indeed, contrarily to Vitamin C, in our previous studies we observed

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1 that antioxidant activity of the *Opuntia ficus-indica* juice, expressed as ORAC value, increases
2 during the gastrointestinal digestion[7].
3
4 **2.2 – Cellular antioxidant activity**
5 The evaluation of FRC activity against free-radical induced cellular oxidative stress was conducted
6 with Caco-2 cells (intestinal epithelial cells model). Caco-2 cells were chosen since they are able
7 to differentiate and express the main characteristics of enterocytes such as digestive enzymatic
8 activities, transporters and receptors expression[13, 14]. As a result, transport and potential
9 metabolism of polyphenols were taking into account during the assays.
10 To investigate the intracellular antioxidant capacity of FRC, three different biomarkers were
11 studied: 1) ROS generation 2) impact on glutathione homeostasis and 3) carbonyl proteins
12 formation. This potential protective effect of FRC was evaluated through two types of treatment,
13 a pre-incubation condition that might reflect a preventive action and a co-incubation treatment
14 which may represent a possible therapeutic administration.
15 In all measurements, the Caco-2 cells were challenged with $H_2O_2$ – radicals inducer and pre- or
16 co-incubated with FRC (50 $mgGAE.L^{-1}$) (Table 2 and Figure 2). The concentration chosen was
17 considered physiological[15] and showed to be non-cytotoxic for Caco-2 cells (data not shown).
18 FRC showed to slightly reduce the generation of $H_2O_2$-induced radicals. However, in co-
19 incubation state the reduction was more pronounced ($p<0.001$) (Table 2).
20 This fact suggests that some compounds couldn't permeate through Caco-2 membranes (or bind
21 to it) but has ability to scavenge or reduce the formation of $H_2O_2$ – induced radicals. Conjugated
22 flavonols, which are known by their low bioavailability, might contribute to the higher antioxidant
23 activity of FRC in co-incubation treatment[16].

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1
2 To better understand the different contributions of the polyphenolic compounds in pre- or co-
3 incubation assays, cellular uptake and transepithelial transport studies were conducted in Caco-2
4 cells for 4hours (Table 3) since it is known that the average intestinal transit time is around 4h ±
5 1.4h[17]. It was observed that hydroxycinnamic acids, namely eucomic and piscidic acid were
6 efficiently uptaked by Caco-2 cells being also identified on the basolateral side during
7 transepithelial transport assay. In the case of flavonols, only isorhamnetin had shown ability to
8 cross Caco-2 cells membrane once it was found inside the cells on cellular uptake assay (Table 3).
9 Nevertheless, even after 4h of incubation isorhamnetin was undetected on basolateral side of Caco-
10 2 cell models (Table 3). On other hand, isorhamnetin conjugates shown to be non-bioavailable.
11
12 Depletion in GSH and consequently decrease of GSH/GSSG is one of the consequences of
13 oxidative stress generated by $H_2O_2$ in Caco-2 cells where the ratio between GSH and GSSG, in
14 Caco-2 incubated with stress inducer ($H_2O_2$), significantly reduced during the 1 h (Figure 2A,
15 p<0.001) of incubation. However, the addition of 50 mgGAE.$L^{-1}$ of FRC did not significantly
16 suppressed the $H_2O_2$-induced depletion of GSH as no significant differences were found in Caco-
17 2 cells treated with FRC compared with control (Figure 2A). The GSH/GSSG remained almost
18 unchanged between pre- and co-incubation experiments.
19 The capacity of FRC to prevent $H_2O_2$–induced protein oxidation in Caco-2 cells was also evaluated
20 (Figure 2B). $H_2O_2$ leads to a 2-fold increase in carbonyl proteins content when compared to
21 negative control whereas the pre-incubation with FRC decreases these oxidation products
22 ($p<0.001$). As observed for $H_2O_2$-induced radicals, when cells are co-incubated with FRC the
23 decrease in carbonyl proteins content is even more pronounced (Figure 2B, $p<0.001$), which again



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1 suggests that the compounds, in particular conjugated forms of flavonols present in FRC
2 (isorhamnetins glucosides) with lower bioavailability, may help to prevent protein oxidation[18].

3 As far as we know, this is the first time that the effects of cactus pear's polyphenols on the
4 modulation of protein oxidation were evaluated in a cell model of intestinal epithelium. The results
5 attained suggest a beneficial role of FRC compounds on the modulation of this oxidative stress
6 biomarker. Our results showed that cactus pear compounds present in FRC may be able to
7 counteract protein oxidation of human intestinal epithelia but no impact was observed in the
8 maintenance of glutathione homeostasis.

9

10 **2.3 – Anti-inflammatory activity**

11 Caco-2 cells, stimulated during 48 h with a specific pro-inflammatory cocktail composed by 50
12 ng.mL$^{-1}$ TNF-α, 25 ng.mL$^{-1}$ IL-1β and 10 µg.mL$^{-1}$ LPS, were applied as *in vitro* model of the
13 inflamed intestinal mucosa. The impact of FRC on inflammation response was determined by
14 quantification of different inflammatory mediators such as IL-8, TNF-α and NO secretion, NF-kB
15 activation and quantification of anti-inflammatory chemokine IL-10. The intestinal barrier
16 dysfunction (which is associated to chronic intestinal inflammation) was also assessed through the
17 determination of apparent permeability of fluorescein ($P_{app}$) across inflamed Caco-2 monolayer
18 (Table 4).

19 The $P_{app}$ was measured in apical to basolateral direction (Table 4) and compared with $P_{app}$
20 determined for non-stimulated intestinal monolayer. The permeability across Caco-2 increased
21 with induced intestinal inflammation but pre-incubation during 4 h or co-incubation with 50
22 mgGAE.L$^{-1}$ of FRC, could maintain the $P_{app}$ (from apical to basolateral side) close to the basal
23 levels (non-stimulated intestinal cells, negative control).

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The results obtained in this work for $P_{app}$ suggests that pre- or co-treatment with FRC could protect Caco-2 cells from barrier dysfunction induced by pro-inflammatory stimuli (Table 4), normally associated to inflammatory pathological symptoms.

These observations shows that polyphenols presented in FRC may reduce inflammatory damages produced at Caco-2 barrier level and are in accordance with the results observed by Tesoriere and co-workers[19] for the effect of an *Opuntia ficus-indica* betaxanthin, indicaxanthin, on the IL-1β induced decrease in tight junctions permeability in Caco-2 cells. Some authors report a potential role of polyphenols from other natural sources on Tight Junctions (TJs) function[20]. However, these effects in case of inflammation are not well explored. For example, incubation of Caco-2 cells with 100 µM of quercetin increase claudin-4 expression, an important TJ protein, in normal cells but this effect was not assessed in inflamed Caco-2 cells[20,,21].

Pre- or co-incubation with 50 mgGAE.$L^{-1}$ of FRC with inflammatory stimuli, also significantly decreased the secretion of IL-8 (a chemoattractive chemokine) by inflamed Caco-2 cells (Figure 3A). Nevertheless, when Caco-2 cells are simultaneously exposed to stimuli and FRC (co-incubation assay) the decreased observed was more pronounced and significantly different from pre-incubation assay (Figure 3A, $p<0.001$). In this case, secreted IL-8 presents a 10-fold or 6-fold decrease comparing with the results attained for positive control or pre-incubation assay respectively ($p<0.001$). Several authors mentioned the capacity of polyphenols from different sources to modulate IL-8 secretion[1, 2, 21] and the results presented herein may indicate that polyphenols present in FRC are also able to modulate this important cytokine.

Furthermore, oxidative stress is known as an important component in inflammation due to its capacity to perpetuate and amplify inflammatory cascades[3]. The already discussed capacity of FRC



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1 in decreasing the impact of $H_2O_2$-induced oxidative stress in Caco-2 cells is a possible key behind
2 the modulation of IL-8 secretion.
3 The production of NO, was indirectly measured by the accumulation of nitrite in the culture
4 supernatants, using a colorimetric reaction with the Griess reagent[22]. FRC showed to significantly
5 decrease its production (figure 3B) in both states studied ($p<0.001$). However, no significant
6 differences between pre-incubation and co-incubation assays were observed. This could indicate
7 that uptake polyphenols or those that are able to interact with cellular membrane, such as
8 hydroxycinnamic acids and isorhmanetin (Table 3), may be the main responsible for this activity.
9 Nitric oxide (NO) modulation is one possible mechanism that could clarify the above mentioned
10 barrier protection exerted by FRC. NO has demonstrated to be modulated by polyphenols in
11 different ways: inhibiting iNOS (inducible NO synthase) transcription or decreasing iNOS and
12 NOS activity[23]. Particularly, isorhamnetin, that are more efficiently uptake by cells due to its O-
13 methyl group , has been previously reported as modulator of NO secretion inhibiting iNOS
14 expression through NF- κB activation [24].
15 The impact of FRC on IL-10 secretion, an anti-inflammatory cytokine, by inflamed Caco-2 cells
16 was evaluated in both studied states – pre- and co-incubation (Figure 3C). It could be observed
17 that, in both situations, FRC did not significantly increase IL-10 secretion. Recently, there are
18 evidences that polyphenols can increase IL-10 expression[1] but our data indicate that the ability of
19 FRC phenolic compounds on modulation of inflammatory mediators is not related with the
20 increase on IL-10 secretion.
21 In this work, the hypothesis that FRC compounds could mediate the modulation of the NF-κB
22 activation pathways in the intestine was indirectly evaluated through the quantification of IκBα in
23 inflamed Caco-2 cells (Figure 3D). IκBα is an inhibitor of NF-κB that sequesters it in cytosol,

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1 preventing NF-κB migration to the nucleus where it can bind to DNA and promote pro-
2 inflammatory genes transcription[25, 26]. In presence of pro-inflammatory stimuli, IκBα is
3 phosphorylated and degraded by the proteassome, allowing NF-κB migration to the nucleus[27] .As
4 required, the pro-inflammatory stimuli selected lead to a substantial depletion on IκBα expression
5 (Figure 3D).

6 The pre-treatment with FRC (50 mgGAE.$L^{-1}$) slightly reduce the depletion on IκBα expression
7 while the co-incubation led to a more effective result, carrying to a higher level of IκBα (Figure
8 3D) with a 2- fold increase relatively to the positive control.

9 As the promoters of the IL-8 and NO related genes contain binding sites to NF-κB[23, 25], the obtained
10 results suggest that FRC could control IL-8 and NO secretion through a mechanism depended on
11 NF-κB activation. Some flavonoids, namely quercetin; apigenin, luteolin, among others are
12 reported to inhibit the activation of NF- κB[23, 25]. This suggests that flavonoids (namely the less
13 bioavailable isorhamnetins derivatives) presented on FRC may also take action in this pathway
14 throughout the modulation of oxidative stress.

15

16 The effect of FRC on secretion of TNF-α, another important inflammatory mediator capable to
17 increase barrier dysfunction by induction of TJ disruption[26] was also evaluated (Figure 3E). The
18 pre- and co-incubation with FRC showed to significantly decrease TNF-α secretion ($p$<0.001).
19 Although the applied stimulus was different, the results obtained for barrier dysfunction, where
20 FRC showed the capacity to modulate permeability across inflamed Caco-2 monolayer, could be
21 correlated with results observed for TNF-α secretion (Figure 3E). As pre-and co-incubation
22 treatments results in similar responses in both assays, it is possible that barrier dysfunction was in
23 part mediated by TNF-α secretion, which was mostly modulated by FRC compounds that could



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1 interact with Caco-2 membranes or be bioavailable. This is the case of ferulic, piscidic and
2 eucomic acid and isorhamnetin which are uptake by Caco-2 cells (Table 3).
3

4 **3. Experimental**

5 **3.1 Material**

6 EtOH 96% (AGA, Lisbon, Portugal), distillate water and food grade macroporous resin Amberlite
7 XAD-16 (Sigma-Aldrich, St. Louis, USA) were used for adsorption process. For total polyphenols
8 content, sodium carbonate ($Na_2CO_3$) was purchased from Sigma-Aldrich (St Quentin Fallavier,
9 France), Folin Ciocalteau reagent was acquired from Panreac (Barcelona, Spain) and gallic acid
10 was purchased from Fluka (Germany). Phenolic standards used were from Extrasynthèse (Genay,
11 France). All other reagents and chemicals were obtained from Sigma- Aldrich (St Quentin
12 Fallavier, France). Chemicals used for antioxidant activity assays were: 2′,2′-Azobis (2-
13 amidinopropane) dihydrochloride (AAPH), 6- hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic
14 acid (Trolox), caffeic acid ($C_9H_8O_4$), cobalt floride tetrahydrate ($CoF_2$), hydrogen peroxide ($H_2O_2$)
15 and picolinic acid ($C_6H_5NO_2$) from Sigma-Aldrich (St Quentin Fallavier, France) and $FeCl_3$ from
16 Riedel-de- Haën (Seelze, Germany). Disodium fluorescein (FL) was from TCI Europe (Antwerp,
17 Belgium). Reagents used for phosphate buffer solution (PBS) preparation included sodium
18 chloride (NaCl), potassium chloride (KCl) and monopotassium phosphate ($KH_2PO_4$) from Sigma-
19 Aldrich (St Quentin Fallavier, France) and sodium phosphate dibasic dehydrate ($Na_2HPO_4 \cdot 2H_2O$)
20 from Riedel-de- Haën (Seelze, Germany). All cell culture media and supplements, namely fetal
21 bovine serum (FBS), glutamine, RPMI 1640 and trypsin/EDTA were obtained from Invitrogen
22 (Invitrogen Corporation, Paisley, UK)

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For Elisa and western blots assays: nitrocellulose membrane 0.45µm, fiber pads and filter paper were purchased from BioRad (California, USA), CellTiter 96® AQueous One Solution Cell Proliferation Assay was obtained from Promega (San Luis Obispo, CA, USA), Streptavidin-HRP (Horseradish Peroxydase) was obtained from Millipore (Massachusetts, USA), FemtoMaxTM Chemiluminescent Kit for use with mouse primary antibody, anti-mouse IgG and anti-rabbit IgG antibodies biotin conjugated, Ultra-pure Tween-20, rabbit anti-IL10 antibody, rabbit anti-IL-8 antibody and donkey anti-mouse IgG HRP-labeled antibody were obtained from Rockland (Gilbertsville, PA, USA), mouse monoclonal anti-IκBα (Inhibitor-κB alpha) antibody, mouse monoclonal anti-TNF-α antibody, TNF-α and IL-1β were purchased from Santa Cruz Biotechnology (Heidelberg, Germany).

### 3.2 *Opuntia ficus-indica* fruit harvesting

*Opuntia ficus-indica* fruits (Cactus pear) were collected in Beja, Alentejo, Portugal (N37º56.559, W007º35.246), in October of 2010. The identification of the specie was performed according to "The Native Cacti of California" by Lyman Benson (1969). A voucher sample was authenticated and deposited at the herbarium "LISFA" Oeiras, Portugal.

### 3.3 *Opuntia ficus-indica* fruit processing

#### 3.3.1 Opuntia ficus-indica Fruit juice

For juice preparation, spikes were removed with a brush and the fruits were processed using a kitchen robot (UFESA, LC5005, China). Juice was recovered and centrifuged at 8150 *g* for 10 min (Avanti J-26 XPI, Beckman Coulter, USA). The supernatant was collected and preserved under frozen storage (-20 ºC) until the day of the experiments.

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2 **3.3.2 Flavonoid-rich Concentrate (FRC) from *Opuntia ficus-indica* juice**

3 Flavonoid-rich concentrate (FRC) was prepared from *Opuntia ficus-indica* juice through a batch
4 (static) adsorption process using a food grade macroporous resin, Amberlite® XAD-16 as
5 previously reported by Serra et al (2013)[8]. The resulting FRC were kept in a cool, dry and dark
6 environment.

7

8 **3.4 Phytochemical Characterization**

9 **3.4.1 Phenolic Chromatographic Profile**

10 HPLC analysis of phenolic compounds was carried out using a Surveyor apparatus from Thermo
11 Finnigan with a diode array detector (Thermo Finnigan–Surveyor, San Jose, CA, USA) and an
12 electrochemical detector (Dionex, ED40). The data acquisition system was the Chromquest
13 version 4.0 (Thermo Finnigan–Surveyor, San Jose, CA, USA). Identification of compounds was
14 done by comparing retention time, spectra and spiking samples with known amounts of pure
15 standards, whenever available. Identification of compounds was confirmed by LC- MS/MS as
16 previously described[7].

17 Total flavonol content was determined using the 360 nm total peak area above 40 min. Calibration
18 curves with isorhamnetin (0-50 ppm) were performed and final results were expressed in terms of
19 isorhamnetin equivalents per g of extract (dry basis).

20 Total hydroxycinnamic acids content was determined using the 320 nm total peak area between
21 20 and 40 min. Calibration curves with ferulic acid (0-50 ppm) were performed and final results
22 were expressed in terms of ferulic acid equivalents per g of extract (dry basis).

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1 **3.4.2 Total phenolic content by Folin Ciocalteau method**

2 Total concentration of phenolic compounds present in FRC was determined according to the Folin-
3 Ciocalteau colorimetric method as previously described by Serra et al (2008)[28]. Results were
4 expressed as means of triplicates (mg of gallic acid equivalents per g dry extract –mg GAE.$g^{-1}$
5 extract *dw*).

6

7 **3.4.2 Total Betaxanthins content**

8 Total Betaxanthins of FRC were determined by a spectrophotometric method. The absorbance
9 was read at 476 and 538nm and total betaxanthins were calculated using Nilsson equation[29].
10 Results are expressed as mg betaxanthin per g dry extract (mg.$g^{-1}$ extract *dw*)..

11

12 **3.5 Antioxidant Activity**

13 **3.5.1 Oxygen Radical Absorbance Capacity (ORAC)**

14 Peroxyl radical scavenging capacity was determined by the ORAC method. The assay was carried
15 out by following method of Huang et al (2002)[30] modified for the FL800 microplate reader
16 (BioTek Instruments, Winooski, VT, USA)[31]. All data were expressed as micromoles of Trolox
17 equivalent antioxidant capacity per gram of dry extract (µmol TEAC.$g^{-1}$ extract *dw*).

18

19 **3.5.2 Hydroxyl Radical Adverting Capacity (HORAC)**

20 HORAC assay was based on a previously reported method[32] modified for the FL800 microplate
21 fluorescence reader[33]. Data were expressed as micromoles of caffeic acid equivalents antioxidant
22 capacity per gram of extract (dry base) (µmol CAEAC. $g^{-1}$ extract *dw*).

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2 **3.6 Cell-based assays**

3 **3.6.1 Cell culture**

4 Human colon carcinoma Caco-2 cells were purchased from Deutsche Sammlung von
5 Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany), and were routinely grown
6 in a standard medium: RPMI 1640 supplemented with 10 % (v/v) of inactivated FBS (fetal bovine
7 serum), 2 mM of glutamine and 5000 U of penicillin-streptomycin. Stock cells were maintained
8 as monolayers in 175 $cm^2$ culture flasks. Cells were subcultured every week at a split ratio of 1 to
9 20 by treatment with 0.1 % trypsin and 0.02 % EDTA and incubated at 37 ºC in a 5 % $CO_2$
10 humidified atmosphere.

11

12 **3.6.2 Cell stimulation of inflammation**

13 Caco-2 cells were cultivated in a 6-well plate at a seeding density of $2.0x10^5$ cells/well or in 12
14 mm i.d.Transwell® inserts (polycarbonate membrane, 0.4µm pore size, Corning Costar Corp.) at
15 a density of $1.0x10^5$ cells/well. Cells were allowed to grow and differentiate to confluent
16 monolayer for 21 days post seeding by changing the medium three times per week.
17 The state of inflammation was induced on basal side with a pro-inflammatory stimuli composed
18 by LPS, IL-1β and TNF-α (10 $\mu g.mL^{-1}$, 25 $ng.mL^{-1}$ and 50 $ng.mL^{-1}$, respectively) and further
19 exposure for 48 h.

20

21 **3.6.3 Cytotoxicity assay**

22 FRC toxicity in Caco-2 cells was performed using CellTiter ® Reagent accordingly to instructions
23 for a range of 0-50 mg GAE of FRC per mL of cell medium and as briefly described in Serra et al

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1 (2013)[8]. The experiments were performed in triplicate and the results are expressed in percentage
2 to the control with culture medium only.
3

4 **3.6.4 Cellular uptake**

5 The uptake of FRC was performed in Caco-2 cells seeded in 6-well plates at a density of $1.0x10^5$
6 cells/well and cultured in the standard medium during 21 days to obtain fully differentiated cells.
7 After 21 days, cells were washed with PBS and incubated during 4h with FRC. Afterward, FRC
8 was removed, cells were washed with PBS and lysed using cell Lytic™ supplemented with a
9 proteases inhibitor cocktail for 5 min. Lysed cells were removed by scrapping and centrifuged at
10 14000 *g*, for 10 min at 4 ºC (Hettich Zentrifugen MIKRO 220R). Polyphenolic and betaxanthins
11 compounds were analysed as described in 3.4.1 and 3.4.2 section respectively. The experiments
12 were performed in triplicate and the results are expressed in percentage to the control with culture
13 medium only.
14

15 **3.6.5 Transepithelial transport assay**

16 For transepithelial experiments, Caco-2 cells were seeded in 12 mm i.d. Transwell® inserts
17 (polycarbonate membrane, 0.4 µm pore size, Corning Costar Corp.) at a density of $1.0x10^5$
18 cell/well. Cells were allowed to grow and differentiate to confluent monolayer for 21 days post
19 seeding by changing the medium three times *per* week. After 21 days, cells were washed with
20 PBS, and FRC in HBSS was added at the apical side for 4h hours, and basolateral was replaced
21 by HBSS. After that, basolateral medium was removed, centrifuged at 2000 *g* for 10 min at 4 ºC
22 and polyphenolic and betaxanthins compounds were analysed as described in 3.4.1 and 3.4.2
23 section respectively.



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1 TEER of all monolayers was monitored before and after experiment to insure their integrity using
2 EVOM$^{TM}$ voltmeter (WPI, Berlin, Germany). Before each experiment, TEER was measured and
3 only monolayers with a TEER value higher than 500 $\Omega.cm^2$ were used. Experiments were done in
4 triplicate
5

6 **3.6.6 Intracellular reactive oxygen species (ROS) reduction**

7 Cellular antioxidant activity of FRC was evaluated monitoring the formation of ROS in Caco-2
8 cells (using the fluorescent probe, DCFH-DA) after treatment with $H_2O_2$ as previously described
9 by Serra et al (2011)[34]. The formation of intracellular ROS was monitored in two different cases:
10 pre- and co-incubation of FRC and stress inducer. Briefly, in pre-incubation treatment,
11 differentiated Caco-2 cells were incubated with FRC diluted in culture medium (50 mgGAE.L$^{-1}$)
12 and DCFH-DA (100 μM). After 1 h, FRC was removed, cells washed with PBS and further
13 incubated with $H_2O_2$ (10 mM) for 1 hour. In co-incubation treatment, cells were incubated during
14 1h with DCFA-DA, washed and FRC and stress inducer added at same time. In both assays,
15 fluorescence (F) was measured for each sample at 0 and 60 min in a fluorescence microplate reader
16 (λex: 485 nm, λem: 530 nm) (BioTek Instruments, Winooski, VT, USA). Experiments were done
17 in triplicate and results are expressed as a percentage of the fluorescence compared to control cells.
18

19 **3.6.7 Determination of protein carbonyls content**

20 Caco-2 cells were seeded in 6-well plates at a density of $1.0x10^5$ cells/well and cultured in the
21 standard medium during 21 days to obtain fully differentiated cells. Cells were washed with
22 phosphate buffer (PBS) and pre- incubated with FRC (50 mg GAE.L$^{-1}$) diluted in the PBS for 1 h.



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1 Negative controls were composed by cells incubated only with PBS. Afterward, FRC were
2 removed and 10 mM of $H_2O_2$ was added to wells (except for control well) for 1 h after which cell
3 Lytic$^{TM}$ supplemented with a proteases inhibitor cocktail was added for 5 min. In the case of co-
4 incubation, the FRC and $H_2O_2$ were simultaneously added to wells for 1 h followed by cell lysis.
5 Lysed cells were removed by scrapping and centrifuged at 14000 *g*, for 10 min at 4 ºC (Hettich
6 Zentrifugen MIKRO 220R). Supernatants were frozen at -80ºC until carbonyl proteins
7 determination. Total protein was determined with Bradford Reagent using Bovine Serum
8 Albumine (BSA) as a reference standard.

9 Carbonylated proteins were determined according to Ramful et al. (2010)[35] with slightly
10 modifications. Briefly, a total of 40 µL of cell lysates (0.6 $mg.mL^{-1}$ total protein) were denatured
11 by 5 µL of 12 % (w/v) sodium dodecyl sulfate (SDS) for 10 min at room temperature. The protein
12 carbonyls groups presented in the samples were derivatized using 80 µL of 5 mM 2,4-
13 dinitrophenylhydrazine (DNPH) in 2M HCl for 20 min at room temperature. 5 µL of samples were
14 diluted and neutralized by adding 1 mL of coating buffer (PBS pH 7.4). For each sample, 100 µL
15 was added to wells of an ELISA plate (NUNC Maxisorp) and incubated during 3 h at 37 ºC. After
16 that, wells were blocked overnight at 4 ºC with 200 µL of Sea Blocking Buffer (Thermo Scientific,
17 Rockford, USA), further probed with a rabbit anti-DNPH antibody (1:5000) for 3 h at room
18 temperature followed by incubation for 1 h at room temperature with HRP conjugated donkey anti-
19 rabbit IgG secondary antibody (1:50000). The tetramethylbenzidine (TMB), HRP substrate was
20 then added and allowed to oxidize for 15 min at room temperature, leading to the formation of a
21 sapphire blue complex. The reaction was stopped by the addition of 2 M HCl and absorbance was
22 measured at 450 nm in a BioTek$^{TM}$ Power Wave XS microplate reader. Experiments were done in
23 triplicate and results are expressed as a percentage of the absorbance compared to control cells.

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1

2 **3.6.8 Glutathione (GSH) and glutathione disulfide (GSSG) quantification**

3 Caco-2 cells were seeded in 6-well plates as mentioned above for determination of protein
4 carbonyls groups. The extracellular media was removed and cells were detached by adding 0.1 %
5 trypsin followed by inactivation with cell medium and centrifugation at 14000 *g*, for 10 min at 4
6 ºC. The cell pellets were ressuspended in PBS and centrifuged at 14000 *g*, for 10 min at 4º C. GSH
7 and GSSG quantification assay was performed as described by Tavares et al (2012)[36].

8

9 **3.6.9 Permeability of fluorescein on the Caco-2 cell monolayer and Transepithelial Electrical**
10 **resistance (TEER) measurement**

11 Caco-2 cells were seeded in 12 mm i.d. Transwell® inserts (polycarbonate membrane, 0.4 µm pore
12 size, Corning Costar Corp.) at a density of $1.0x10^5$ cell/well. Cells were allowed to grow and
13 differentiate to confluent monolayer for 21 days post seeding by changing the medium three times
14 *per* week. Permeability of fluorescein (FL) on the Caco-2 cell monolayer was determined
15 according to Leonard et al (2010)[37] method with slightly modifications. Transport was assessed in
16 apical→basolateral direction. FL (1 $\mu g.mL^{-1}$) was dissolved in transport buffer (RPMI 1640
17 without phenol red supplemented with 2 mM glutamine and 0.5 % (v/v) FBS). Cell monolayers
18 on Transwell fibers were rinsed gently twice and pre-incubated in transport buffer for one hour at
19 37 ºC and 5 % $CO_2$. In case of pre-incubation assay, cells were incubated for 4 h with 50 $mgGAE.L^{-}$
20 $^{1}$ of FRC added to the apical compartment. Wells were further washed with transport buffer, FL
21 was added to the donor compartment and transport buffer was added to the acceptor compartment
22 at the same time of the addition of inflammatory stimuli at the basolateral compartment (50 $ng.mL^{-}$
23 $^{1}$ TNF-α, 25 $ng.mL^{-1}$ IL-1β and 10 $\mu g.mL^{-1}$ LPS). In case of co-incubation assay, FRC and FL



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1 were added simulteously with inflammatory stimuli. At different time points, 50 µL of the samples
2 were taken from the receiver compartment and the volume lost during sampling was replaced with
3 fresh transport buffer. FL amount in the samples was measured using a microplate fluorescence
4 reader (λex: 488 nm, λem: 530 nm). Apparent permeability ($P_{app}$) was calculated using $P_{app}$ =
5 ((dQ/dt) (1/A) (1/$C_0$)) where dQ/dt is the amount of FL transported *per* time, A is the surface area
6 of the monolayer and $C_0$ is FL concentration (µg.mL$^{-1}$) at time 0. A FL calibration curve was used
7 to determine the concentration. Permeability of FL across non-stimulated Caco-2 monolayers was
8 determined as control. TEER of all monolayers was monitored before and after experiment to
9 insure their integrity using EVOM$^{TM}$ voltmeter (WPI, Berlin, Germany). Before each experiment,
10 TEER was measured and only monolayers with a TEER value higher than 500 Ω.cm$^2$ were used.
11 Experiments were done in triplicate and results are expressed as average of obtained values.
12
13 **3.6.10 Quantification of nitrite production, IL-8 and IL-10**
14 Caco-2 cells were seeded in 6 well plates at a density of 2.0x10$^5$ cells/well. Cells were allowed to
15 grow and differentiate to confluent monolayers for 21 days post seeding by changing the medium
16 three times *per* week. Cells were pre- incubated with FRC for 4 h or co-incubated with FRC and
17 inflammatory stimuli (50 ng.mL$^{-1}$ TNF-α, 25 ng.mL$^{-1}$ IL-1β and 10 µg.mL$^{-1}$ LPS) for 48 h.
18 Medium was removed and centrifuged at 2000 *g* for 10 min at 4 ºC. Supernatants were harvested
19 and frozen at -80 ºC until the day of experiments except for nitric oxide (NO) determination that
20 was performed immediately.

21 NO production was measured by the accumulation of nitrite in the culture supernatants, using a
22 colorimetric reaction with the Griess reagent[22]. Briefly, 100 µL of sample was mixed with 100 µL
23 of modified Griess reagent and the absorbance was read at 540 nm after 15 min using a microplate

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1 reader. Culture medium was used as blank and medium of cells without inflammatory stimuli was
2 used as negative control.

3 For ELISA IL-8 and IL-10 quantification, 100 μL of supernatants diluted in PBS (5 μg total
4 protein) were added to wells of an ELISA plate (NUNC Maxisorp) for 3 h at 37 ºC, washed 3 times
5 with PBS-T (PBS 0.05 % ultra-pure tween-20) and blocked with 200 μL of PBS with 1% (m/v)
6 BSA overnight at 4 ºC. Wells were washed 3 times with PBS-T and 100 μL of anti IL-8 (1:2500
7 rabbit antibody) or anti IL-10 (1:20000 rabbit antibody) were added to each well for 1 h at room
8 temperature, followed by wash and addition of secondary antibody (1:150000 anti-rabbit IgG
9 Biotin conjugated) for 1 h at room temperature. Wells were washed with PBS-T, 100 μL of
10 Streptavidin-HRP solution (1:1000) were added to each well for 1 h at room temperature and
11 washed again. TMB substrate was then added for 15 min at room temperature. The reaction was
12 stopped by the addition of 2M HCl and absorbance was measured at 450 nm in a BioTek$^{TM}$ Power
13 Wave XS microplate reader. All experiments were done in triplicate and results are expressed as
14 a percentage of the absorbance compared to control cells.
15

16 **3.6.11 Western blot quantification of Inhibitor- κB alpha (IκBα)**

17 Caco-2 cells were seeded in 6 well plates at a density of $2.0x10^5$cells/well, and submitted to the
18 same procedure and treatments as above described in NO, IL-8 and IL-10 quantification. Cells
19 extracts were frozen at -80 ºC until the day of experiment. Proteins (30 μg) were separated by SDS-
20 PAGE in a 12% (w/v) acrylamide gel and transfer onto a nitrocellulose membrane (45 μm) using
21 a Mini Trans-Blot® system from BioRad. After blocking with 1% BSA (w/v) for 1 h in Tris
22 buffered saline solution containing 0.1 % (v/v) of ultra-pure Tween-20 (TBST) with slightly
23 agitation, the membrane was incubated overnight at 4 ºC with primary antibody against IκBα



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1 (1:100 mouse antibody) or with primary antibody against β-actin (1:2000) (loading control). The
2 membrane was washed three times with TBST for 5 min and incubated with FemtoMax™
3 Chemiluminescent Kit for use with mouse primary antibody and revealed according to the
4 manufacture's protocol using ChemiDoc® from BioRad. Relative intensities were calculated using
5 ImageLab® software from BioRad.
6
7 **3.6.12 ELISA quantification of TNF-α**
8 Caco-2 cells were seeded in 6 well plates at a density of $2.0x10^5$ cells/well and submitted to the
9 same procedure and treatments as above described in NO, IL-8 and IL-10 quantification, with the
10 exception that inflammatory stimuli was composed only by 50 $ng.mL^{-1}$ IL-1β. Briefly 100 μL of
11 supernatants diluted in PBS (5 μg of total protein) were added to wells of an ELISA plate (NUNC
12 Maxisorp) for 3 h at 37 ºC, washed 3 times with PBS-T (PBS with 0,05% ultra-pure tween-20)
13 and blocked with 200 μL of PBS with 1% BSA overnight at 4 ºC. Wells were washed 3 times with
14 PBS-T and 100 μL of anti-TNF-α (1:1500 mouse antibody) were added to each well for 1 h at
15 room temperature, followed by wash and addition of secondary antibody (1:100000 anti-mouse
16 IgG Biotin conjugated) for 1 h at room temperature. Wells were washed with PBS-T and 100 μL
17 of Streptavidin-HRP solution were added to each one for 1 h at room temperature. After washing
18 with PBS-T, TMB substrate was added for 15 min at room temperature. The reaction was stopped
19 by the addition of 2M HCl and absorbance was measured at 450nm in a microplate reader.
20 Experiments were done in triplicate and results are expressed as a percentage of the absorbance
21 compared to control cells.
22
23

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1 **3.7 Statistical analysis**

2 All data are expressed as means ± standard deviation (SD) and individual experiments were
3 performed at least in triplicate. The statistical analyses were done using SigmaStat 3.0® software.
4 All values were tested for normal distribution and equal variance. When homogeneous variances
5 were confirmed, data were analysed by One Way Analysis of Variance (ANOVA) coupled with
6 the Tukey's post-hoc analysis to identify means with significant differences. Two sided one p-
7 values of $p<0.001$ and $p<0.05$ were considered significant.

8

9 **4. Conclusion**

10 The results presented herein strongly support scientific knowledge regarding the positive effect of
11 using cactus pear fruit as raw material for the production of functional ingredients for the oxidative
12 stress and inflammation related diseases.

13 To our knowledge, there are very few data concerning the effect of fruit or plant extracts on
14 inflammatory mediators in inflamed Caco-2 cells and no study has been previously conducted
15 regarding the effect of a cactus pear's flavonoid rich concentrate on modulation the studied
16 oxidative stress biomarkers and inflammatory mediators using an *in vitro* cell based model of
17 intestinal epithelium.

18 These results suggest that FRC could be used as a functional food or natural ingredient for
19 nutraceuticals formulation with potential application in prevention of inflammatory disorders such
20 as Inflammatory Bowel Diseases.

21

22



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7

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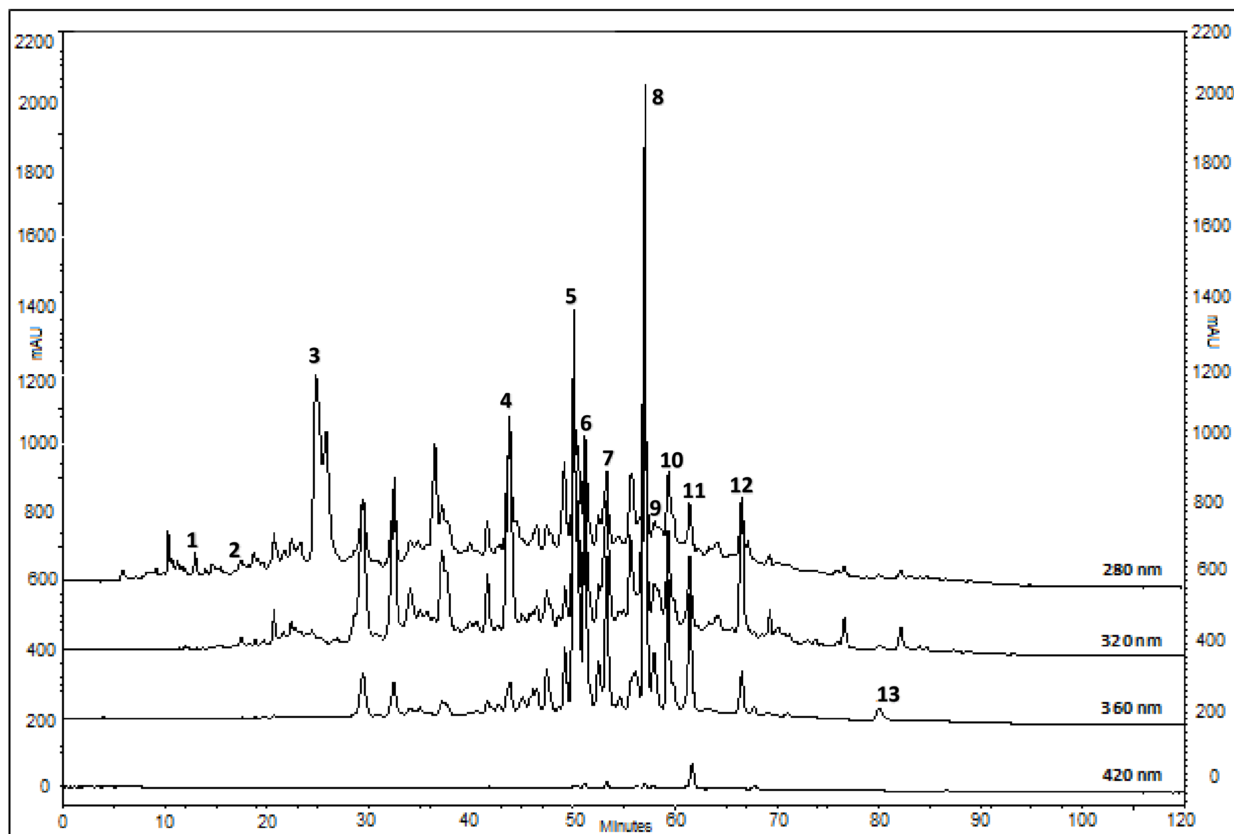


2 Figure 1 – Chromatografic profile of FRC obtained by HPLC-DAD at 280 nm, 320 nm, 360 nm

3 and 420 nm.

4 Legend: 1 – Piscidic acid; 2- Betaxanthin; 3- Eucomic acid; 4-Ferulic acid; 5-Isorhamnetin 3-O-

5 rhamnose-rutinoside; 6- Isorhamnetin 3-O-lyxose-rhamnose-glucoside; 7- Isorhamnetin 3-O-

6 lyxose-glucoside; 8- Isorhamnetin 3-O-rutinoside; 9- Isorhamnetin 3-O-glucoside; 10, 11 and 12

7 - Isorhamnetin derivatives; 13- Isorhamnetin.

8

9

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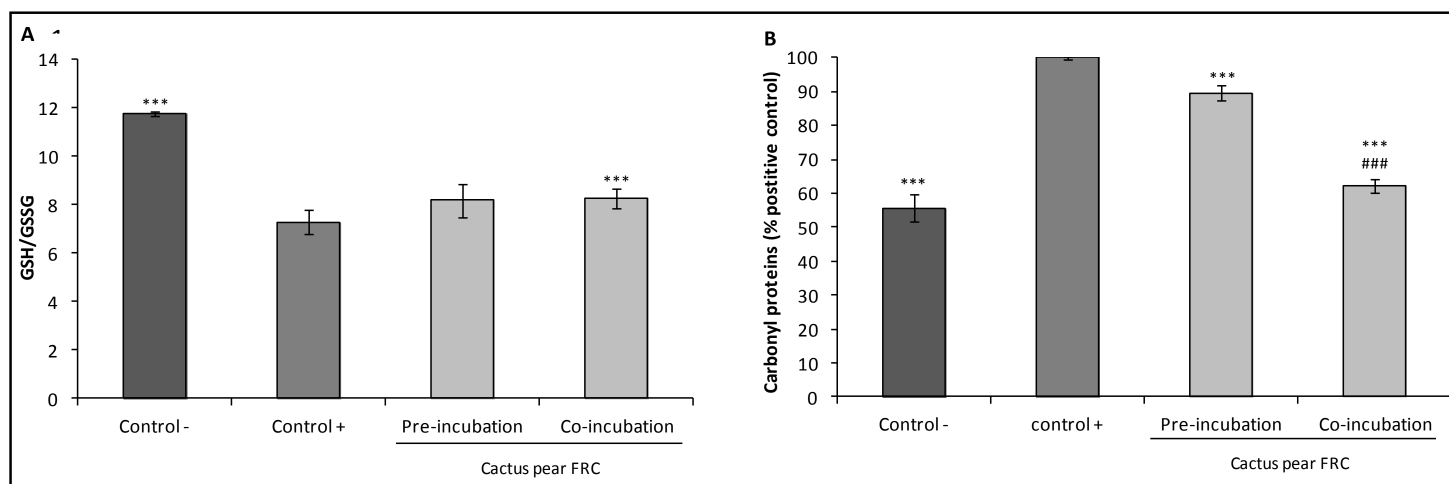


10

11 Figure 2 – Intracellular Antioxidant activity in Caco-2 cells - Comparison between co- and pre-

12 incubation treatments. Effects of cactus pear's FRC (50 mg $GAE.L^{-1}$) on: (**A**) ratio between GSH

13 and its oxidized form GSSG (upon $H_2O_2$ stress induction - 10mM); (**B**) Protein oxidation (upon

14 $H_2O_2$ stress induction - carbonyl proteins formation). Negative control (-) represents normal cells

15 non-challenged with stress inducer ($H_2O_2$) or FRC and positive control (+) represents cells

16 challenged with stress inducer ($H_2O_2$) and not treated with FRC. Statistical differences between

17 Stress (control +) and cells treated with FRC or without any treatment (control -) are denoted as

18 $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Statistical differences between pre- and co-incubation are

19 denoted as $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$. All values are means of three independent

20 experiments ± SD.

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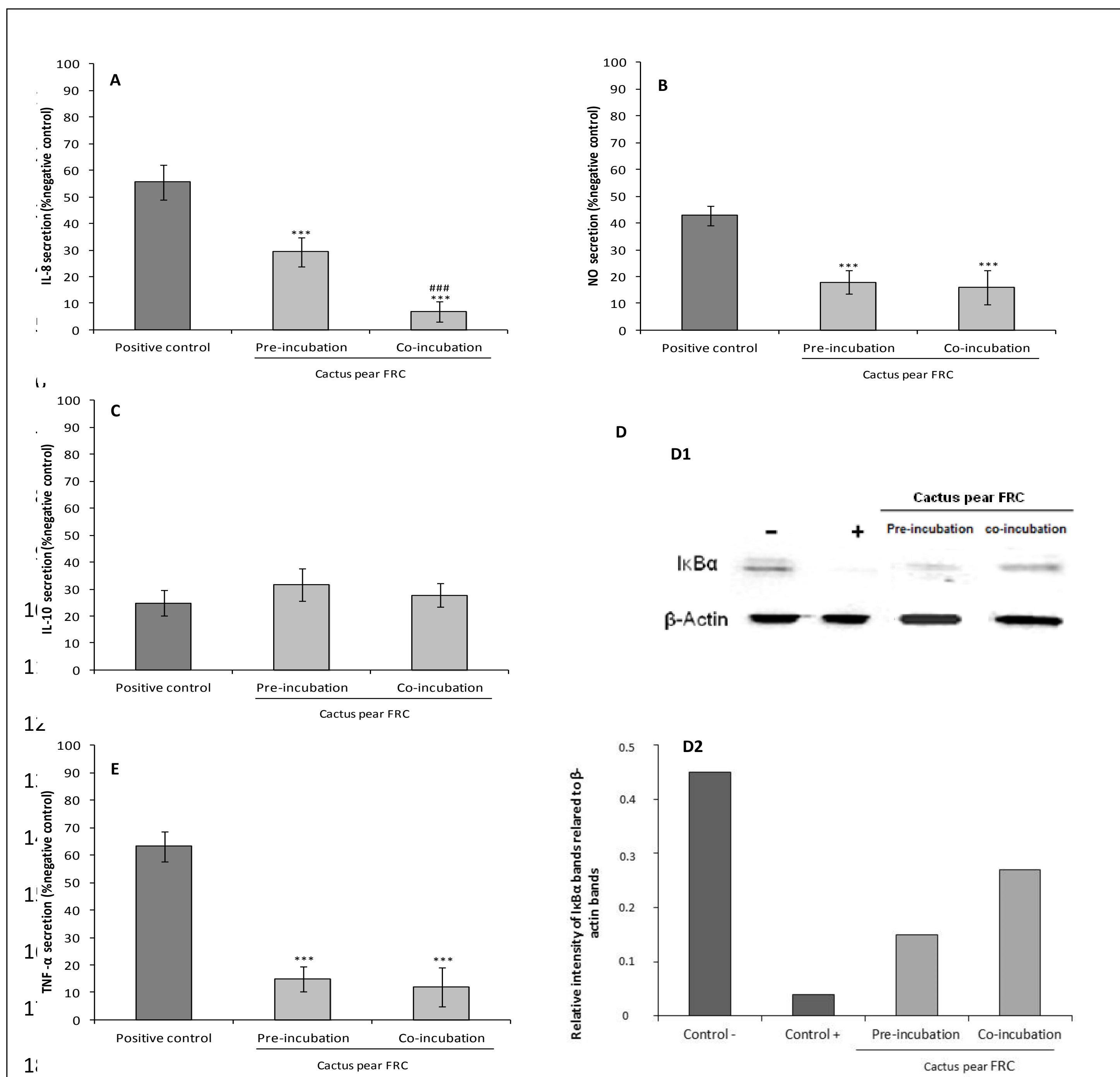


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20 Figure 3 – Modulation of inflammatory mediators by Cactus pear's FRC (50 mgGAE.L$^{-1}$) in Caco-

21 2 cells stimulated by 50 ng.mL$^{-1}$ TNF-α, 25 ng.mL$^{-1}$ IL-1β and 10 µg.mL$^{-1}$ LPS (A,B,C and D) or

22 50 ng.mL$^{-1}$ IL-1β (E) during 48 h – comparison between pre-incubation and co-incubation

23 treatments. (**A**) IL-8 secretion by stimulated Caco-2 cells (determined using ELISA assay)



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1 expressed as percentage of negative control ; (**B**) NO secretion by stimulated Caco-2 cells (using
2 Griess reagent) expressed as percentage of negative control; (**C**) IL-10 secretion by stimulated
3 Caco-2 cells (determined using ELISA assay) expressed as percentage of negative control.(**D**)
4 Modulation of NF-κB activation: (D1) Immunoblot of IκBα and β-actin (30μg protein per well)
5 (D2) Intensity of IκBα band relative to correspondent β-actin band expressed as relative intensity.
6 (**E**) TNF-α secretion by stimulated Caco-2 cells expressed as percentage of negative control.
7 Negative control (-) represents non-stimulated cells while positive control (+) represents cells
8 incubated with pro-inflammatory stimuli. Statistical differences between control + and cells treated
9 with FRC are denoted as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and statistical differences between
10 pre- and co-incubation are denoted as $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$. All values are means of
11 three independent experiments ± SD.
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Table 1 – Total phenolic compounds and Total Betaxanthins from cactus pear FRC. Peak assignment according to profile in Figure 1, retention time (RT), molecular ion ([M-H]$^-$) , MS/MS fragmentation and concentration of compounds identified by LC-MS/MS and quantified by HPLC-DAD

| **Total Phenolic** | | | | | 137.5 ± 6.9 (mg GAE.g$^{-1}$ extract *dw*) |
|---|---|---|---|---|---|
| **Total Flavonols** | | | | | 54.07 ± 2.7 (mg IE.g$^{-1}$ extract *dw*) |
| | **RT** | **[M-H]$^-$ *m/z*** | **MS/MS *m/z*** | **Peak (Figure 1)** | **Concentration (mg.g$^{-1}$ extract *dw*)** |
| **Isorhamnetin 3-O-rhamnose-rutinoside** | 48.8 | 769 | MS$^2$ [769]: 315 | 4 | * |
| **Isorhamnetin 3-O-lyxose-rhamnose-glucoside** | 49 | 755 | MS$^2$ [755]: 315 | 5 | * |
| **Isorhamnetin 3-O-lyx-glu** | 50.7 | 609 | MS$^2$ [609]: 314 | 6 | * |
| **Isorhamnetin 3-O-rutinoside** | 53.8 | 623 | MS$^2$ [623]: 315, 300 | 7 | 2.33 |
| **Isorhamnetin 3-O-glucoside** | 54.6 | 477 | MS$^2$ [477]: 314 | 8 | 0.71 |
| **Isorhamnetin derivative** | 56 | 753 | MS$^2$ [753]: 651, 609, 315 | 9 | n.a |
| **Isorhamnetin derivative** | 56.7 | 593 | MS$^2$ [593]: 314, 315 | 10 | n.a |
| **Isorhamnetin derivative** | 59 | 621 | MS$^2$ [621]: 315, 477, 518 | 11 | n.a |
| **Isorhamnetin** | 75 | 315 | MS$^2$ [315]: 300 | 12 | 1.17 |
| **Total Hydroxycinnamic Acids** | | | | | 40.26 (mg FAE.g$^{-1}$ extract *dw*) |
| | **RT** | **[M-H]$^-$ *m/z*** | **MS/MS *m/z*** | **Peak (Figure 1)** | **Concentration (mg.g$^{-1}$ extract *dw*)** |
| **Eucomic acid** | 24 | 239 | MS$^2$ [ 239]: 179, 149, 107, 133 | 2 | * |
| **Ferulic acid** | 44 | 193 | MS$^2$ [193]: 134, 149 | 3 | 0.265 |
| **Piscidic acid** | 15 | 255 | MS$^2$ [255]: 165, 193 | 13 | * |
| **Total Betaxantins** | | | | | 0.30 (mg $^1$ betaxanthins.*dw*) |

* Compound not quantified due to the unavailability of commercial standards. Calculation of these concentrations by extrapolation using another standard compounds would lead to high errors.

n.a – not applicable. GAE-Gallic Acid Equivalents; IE – Isorhamnetin Equivalents; FAE – Ferulic Acid Equivalents.

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Table 2 – Chemical and Intracellular antioxidant activity of Cactus pear FRC

| | | |
|---|---|---|
| **Chemical Antioxidant Activity** | | |
| **ORAC** | | 1923 ± 141.2 (µmol TEAC.g$^{-1}$ extract *dw*) |
| **HORAC** | | 526 ± 67.7 (µmol CAEAC.g$^{-1}$ extract *dw*) |
| **Intracellular Antioxidant Activity** | | |
| **ROS inhibition (%)*** | Pre-incubation | 6 ± 3.3 |
| | Co-incubation | 13 ± 0.9 |

*Intracellular antioxidant activity in Caco-2 cells. Effects of pre- and co-incubation with FRC (50 mg GAE.mL-1) on ROS inhibition measured by DCFH-DA oxidation upon $H_2O_2$ stress induction (10mM).

Legend: TEAC – Trolox Equivalents Antioxidant Activity; CAEAC – Caffeic Acid Equivalents Antioxidant Activity

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Table 3 – Cellular uptake and transepithelial transport of main phenolic compounds present on FRC in the human intestinal cell line, Caco-2

| Compound | [M-H]- m/z | MS/MS m/z | Cellular uptake | Transepithelial Transport |
|---|---|---|---|---|
| Piscidic acid | 255 | $MS^2$ [255]: 165, 193 | 10.0% | 7.2% |
| Eucomic acid | 239 | $MS^2$ [239]: 179, 149, 107, 133 | 7.1% | 6.4% |
| Ferulic acid | 193 | $MS^2$ [193]: 134, 149 | <LOD | >LOD |
| Isorhamnetin | 315 | $MS^2$ [315]: 300, 151 | 4.1% | n.d |

Legend: (n.d) not detected

LOD detection limit of the method

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Table 4 - Apparent Permeability ($P_{app}$) of fluorescein across Caco-2 monolayer after 4 h of pre-incubation or 48h of co-incubation with Cactus pear FRC (50 mg GAE.$L^{-1}$).

| | | **$P_{app}$ ($x10^{-6}cm.s^{-1}$) Apical → Basolateral** |
|---|---|---|
| **Control -** [1] | | $0.93 \pm 0.05^a$ |
| **Control +** [2] | | $1.14 \pm 0.03$ |
| **Cactus pear FRC** | Pre-incubation | $0.89 \pm 0.01^a$ |
| | Co-incubation | $0.88 \pm 0.01^a$ |

[1] Negative control represents non-stimulated cells.

[2] Positive control represents cells incubated with pro-inflammatory stimuli composed by 50 ng.$mL^{-1}$ TNF-α, 25 ng.$mL^{-1}$ IL-1β and 10 µg.$mL^{-1}$ LPS during 48 h and applied at the basolateral side.

Data with the same superscript letter in the same column are not statistically different.

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