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Anti-inflammatory activity of phenolic extracts from different parts of prickly pear on lipopolysaccharide-stimulated N13 microglial cells

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Abstract

Phytochemicals with health-promoting activities that are components of human diet, have shown to exert a protective effect on the CNS under pathological conditions. In this sense, prickly pears exhibit analgesic and anti-inflammatory properties with neuroprotective effect.

The purpose of this study was to evaluate the potential protective effect of phenolic extracts from different parts of prickly pear on the production of pro-inflammatory mediators by lipopolysaccharide (LPS) -stimulated N13 microglia. Activation of microglia, the hallmark of neuroinflammation, is key to host defence and tissue repair in brain. However, activated microglia secretes cytokines and other factors that are known to contribute to neurodegeneration. To preserve brain integrity, therefore, it is important to keep microglia activation under strict control.

The results show that the extracts studied significantly inhibited the release of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-), interleukin 1-beta (IL-1β), and inducible nitric oxide synthase (iNOS). The present study, however, does not show a clear linear correlation between antioxidant compounds content (total phenolic and flavonoid contents) and anti-inflammatory activity indicates that there must be some additional components within the extracts that play a pivotal role in the anti-inflammatory effect and therefore further characterization is needed. The present study does, however, demonstrate that the phenolic extracts from different parts of prickly pears are potent inhibitors of microglial activation and thus a potential preventive therapeutic agent for neurodegenerative diseases involving neuroinflammation.

Keywords: Prickly pears, phenolic extracts, microglia, LPS, anti-inflammatory activity..

Introduction

Opuntiaficus-indica, (OFI) or prickly pear, is native to the arid and semi-arid regions of Mexico and was introduced into North Africa in the 16th century [1]. Due to its adaptability to difficult growing conditions, the prickly pear is a cactus type that is widely cultivated across the globe and principally exploited for its fruit [2], consisting of a thick peel and an edible juicy pulp with abundant hard seeds. Cactus pear fruit are a rich source of phytochemicals with health-promoting activities [3, 4]. The bioactive composition of Opuntiaficus-indica fruit includes flavonoids such as isorhamnetin, glycosides, quercetin and derivates, as well as two types of betalains, betaxanthins and betacyanins[4, 5]which are also responsible for the fruit's colours.The antioxidant properties of the phenolic compounds in cactus pear plants make them an important product for protecting human health against degenerative diseases such as cancer, diabetes, hypercholesterolemia, arteriosclerosis or cardiovascular and gastric diseases [6-8].

Neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease have been a major focus of neuroscience research for many years, with much effort being devoted to understanding the cellular changes that underlie their pathology. It isnow widely accepted that neuroinflammation plays a major role in neurodegenerative diseases [9]. Microglia,the brain's immune cells, are key mediators in neuro-inflammation and become quickly activated in response to CNS injuries or immunological stimuli. Their activation is similar to that of macrophages, consisting of phagocytosis, antigen presentation, rapid proliferation and cytotoxic secretion. Activated microglia secrete a repertoire of pro-inflammatory and neurotoxic substances such as cytokines, nitric oxide (NO), reactive oxygen species, chemokines, arachidonic acid and its metabolites [10].

Interestingly, it has been reported that extracts from the fruit and stems of Opuntiaficus exhibit analgesic and anti-inflammatory effects [11-13], while flavonoids isolated from *Opuntiaficus-indicavar. saboten* exhibit neuroprotectiveactions [14].

The aim of the present study was, therefore, to determine whether treatment with cactus pear extracts might attenuate the induction of pro-inflammatory mediators in LPS-activated N13 microglia. Thus, the induction of inflammatory mediators, TNF- , IL-1β and iNOSwere evaluated after LPS-activation, analyzing the mRNA of these factors by real-time PCR.

Materials and methods

Chemicals and Solvents

Dimethyl sulfoxide (DMSO), LPS, and 2',7'-dichlorofluorescin diacetate (DFCH-DA) were purchased from Sigma-Aldrich chemie (Steinheim, Germany). RPMI 1640 and PBS were purchased from Oxide (Basingstoke, UK). All other reagents were of analytical grade.

Samples

Prickly pear fruits (O. ficusindica [L.] Mill.), of the red-yellow variety (cladode with spines, ovoid fruit, red-yellow skin, and red-yellow edible portion) were selected for this study.

This cactus pear variety is typically cultivated in the area of Bousselam (Setif, Algeria). Fully ripe cactus pears were collected in August from different points of the plant and from plants locatedin various parts of the plot. The samples were selected on the basis of their colour (both pulp and skin) and the shape and presence of cladode spines. The fruits were harvested at a desirable maturity and in good sanitary conditions (pH, 5.95; titratable acidity, 0.09; Brix, 14.22, respectively). They were then carefully washed and peeled manually. The seeds were removed from the pulp and washed with distilled water. Three different parts, corresponding to the edible part of the fruit were studied: seeds, pulp, and the whole fruit (seeds + pulp). They were lyophilized separately (Christ, Alpha 1-4 LD plus, Germany), ground with a crusher (IKA A 11B, Germany), and passed through a 500 μm sieve.

Preparation of extracts

Approximately 0.1 g of the lyophilized samples (seeds, pulp and whole fruit) was extracted with 10 mL of culture medium. The mixture was shaken in a water bath shaker (nüve ST 402, Ankara, Turkey) for 90 min at 37 C. They were then sonicated with an ultrasonic water bath (Ultrasons, SELECTA) at 37 KHz frequency, 50% amplitude for 30 min at 37º C, centrifuged for 15 min at 2250 g (5702 R, Germany) and filtered (Syringe filter: 0.45μm-Millipore). The filtrates were used to determine the total phenolic content (TPC) and flavonoid content, as well as to assay the effect on the production of inflammatory mediators (IL-1β and TNF- , and iNOS) by LPS-activated N13 microglia.

Determination of total phenolic content (TPC) and flavonoid content

TP content was determined using Folin–Ciocalteu reagent according to the method described by [15]. Samples were mixed with 750μL of Folin–Ciocalteu reagent and 600 μL of 7.5% sodium carbonate. Absorbance was measured at 750 nm and TPC was expressed as mg gallic acid equivalents (GAE) per 100 g.

The extracts'flavonoid content was estimated by the method previously described by Quettier-Deleu et al. [16], based on the formation of a flavonoids–aluminium complex. Equal volumes of extract and aluminium chloride solution (2%) were mixed. The absorbance of the reaction mixture was measured at 430 nm after 15 min of incubation. Total flavonoid contents were expressed as mg quercetin equivalents (QE) per 100 g.

N13 Cell Culture and immunostimulation assays

Murine N13 microglia were grown in RPMI 1640 (PAA, Linz, Austria) supplemented with 2 mM glutamine (PAA), 5 % (v/v) foetal bovine serum (PAA), 100 U/mL penicillin and 100 μg/mL streptomycin (PAA) at 37º C and 5 % CO_2.For subculture, cells were removed from the culture flask with a scraper, re-suspended in the culture medium and subcultured in 6-well plates (Nunc, Thermo Fisher Scientif, USA) in culture media at a density of 2.85 x 10^3 cells/well/2 mL. After adhering, cells were treated with the different prickly pear extracts (10 μg/mL on polyphenols) and/or stimulated with LPS (0.01 μg/mL) and finally collected at early (4 hours) and late (6 hours) times after stimulation to extract RNA. Cells treated only with medium but without prickly pears extracts or LPS were used as control.

RNA extraction and reverse transcription

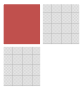
For PCR analysis, total RNA was extracted from the collected cells using the Trisure Isolation Reagent (Roche, Germany) according to the manufacturer's instructions. Briefly, whole cells were collected by adding 0.5 mL/well of Trisure. Reverse transcription (RT) was performed using random hexamers primers, 3 μg of total RNA as a template and the High-Capacity cDNA Archive Kit (Applied Biosystems) following the manufacturer´s recommendations, as previously described [17].

Real-time PCR

After RT, the cDNA was diluted in sterile water and used as the template for the amplification by the polymerase chain reaction. For real time RT-PCR, each specific gene product was amplified employing commercial TaqMan™ probes using the ABI Prism 7000 sequence detector (Applied Biosystems, Madrid, Spain) as previously described [17]. All of them flanked an intronic sequence to ensure the absence of genomic contamination. The cDNA levels were determined using GAPDH as housekeeper. The amplification of the housekeeper was performed in parallel with the gene to be analyzed. Thus, the results were normalized using the GAPDH expression. Threshold cycle (Ct) values were calculated using the software supplied by Applied Biosystems.

Statistical analysis

Data were expressed individually, as mean ± SD, or as percentage with respect to control. At least four independent experiments were conducted and analyzed statistically using Student's t test. Different levels of significance (*p< 0.05) are considered statistically significant.



Data were expressed individually, as a percentage with respect to control. For data comparison, LPS-stimulated N13 cells were compared with non-stimulated control cells. Similarly, LPS-stimulated cells treated with the extracts of different parts of prickly pear were compared with LPS-stimulated cells. At least three independent experiments were conducted and analyzed statistically using one-way analysis of variance (ANOVA) followed by a Bonferroni multiple comparisons test. Different levels of significance (*, $p < 0.05$; **, $p < 0.001$) are considered to be statistically significant.

Furthermore, correlation analysis was used to explore the leaner relationship between the anti-inflammatory activities expressed as ARNm (% inhibition of production of TNF- , IL-1β, and iNOS) and total phenolic and flavonoid contents expressed as mg GAE and mg QE/ 100g, respectively).

Results

Total phenolic and flavonoid contents

Based on the absorbance values of extract reacted with the Folin–Ciocalteu reagent, total phenolic contents are given in Figure 1, as gallic acid equivalents by reference to standard curve. Phenolic contents varied depending on "part of the fruit", so seed extract was attributed minor values of total polyphenols (228.58±6,58 mgGAE/100g). Extracts from whole fruit and pulp showed similar contents which were higher than those found in seed extract (553,33±3,72 and 523,05±2,04 mg GAE/100g, respectively). As illustrated in Figure 1, seed extract also showed the lowest value (25,06 ± 1,04 mg QE/100g) in total flavonoid content.

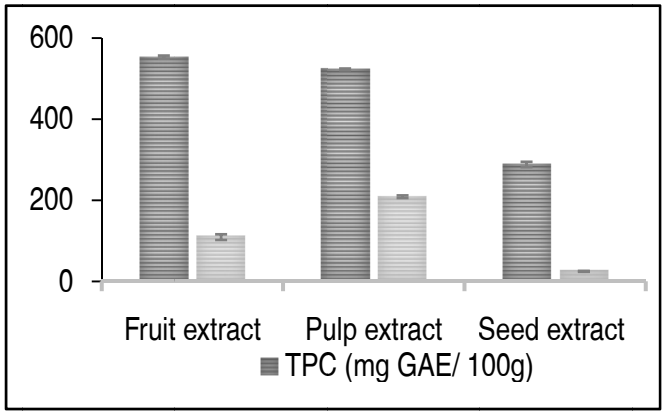
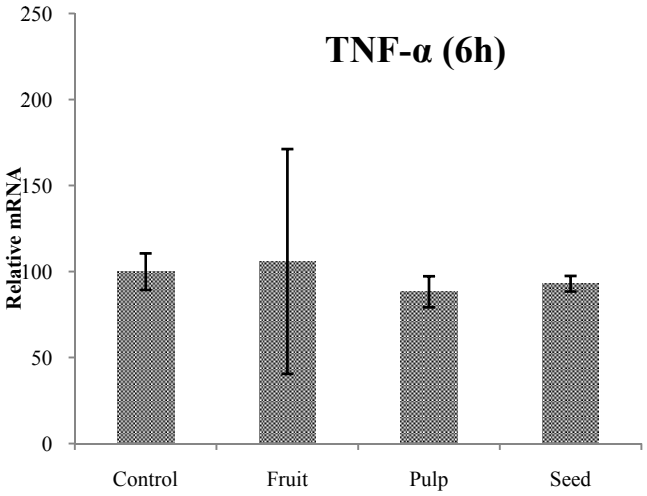
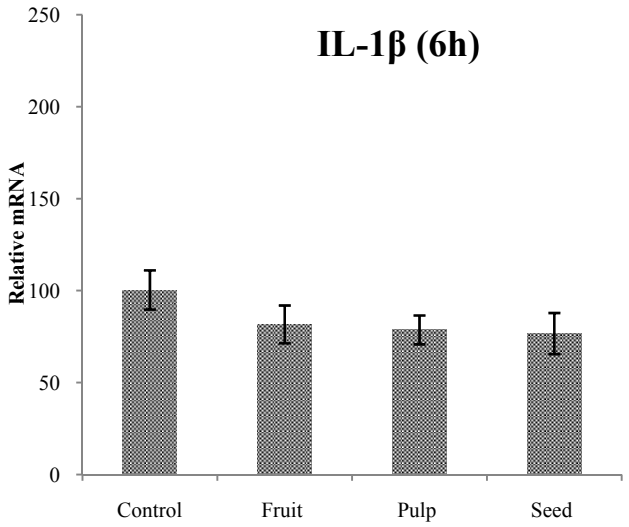


Figure 1. Total phenolic and flavonoid contents in whole fruit, pulp and seedextracts. Total phenolic and flavonoid contents in whole fruit, pulp and seed. Phenolic contents are given as gallic acid equivalents by reference to standard curve. Phenolic contents varied depending on "part of the fruit", with minor values attributed to seed.

Effect of the phenolic extracts of different parts of prickly pear fruit on N13 microglial cells.

To study whether the prickly pear extracts had any effect on microglia cells, cultures treated solely with the extracts were compared with non-treatedN13 cells. The results showed that none of the extracts tested induced the production of pro-inflammatory factors such as TNF- , IL-1β or iNOS at 6 hours after treatment (Figure 2).





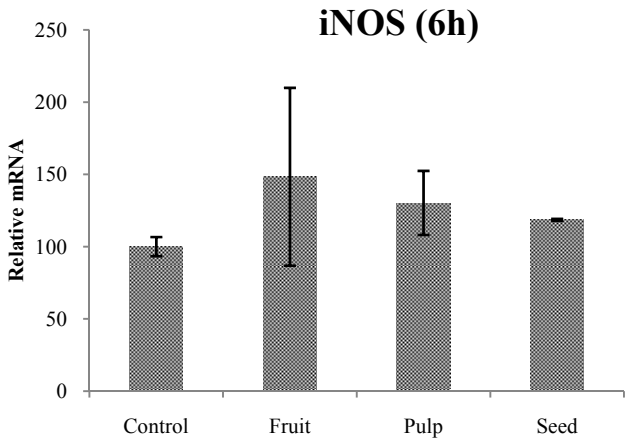


Figure 2. Effect of differentextracts of pricklypear on the expression of TNF- (**A**), IL-1β (**B**) and iNOS (**C**) mRNA in control (non-stimulatedwith LPS) N13 microglia.
No significantdifferencewerefound in the expression of mRNA of any of the proinflammatoryfactorsstudiedbetween control microglia and control microgliatreatedwithpricklypearextracts. All data are presented as the mean±SD of threeindependentexperiments.

Effect of the phenolic extracts of different parts of prickly pear fruit on LPS-stimulated N13 microglial cells

The model of LPS-activated microglia has been widely used as an *in vitro* system for studying the mechanisms thatunderlie neuron damage caused by various mediators released from activated microglia. LPS signals through its Toll-like receptor (TLR-4), leading to a cascade of intracellular events such as the transcription of inflammatory genes [18]. Therefore, to investigate whether prickly pear extracts might attenuate the activation of microglia, we evaluated the transcriptional expression of the pro-inflammatory factors TNF- , IL-1β, and iNOS in LPS-stimulated N13 microglia cells. As expected, LPS stimulation up-regulated the mRNA expression of the pro-inflammatory factors studied. The mRNA of TNF-α and IL-1β reached maximum values at 4 h for TNF- , while iNOS mRNA reached its maximum value at 6 h.
Treatment with extracts from different parts of prickly pear decreased the LPS-induced mRNA expression of pro-inflammatory factors in a time-dependent manner. After four hours of LPS stimulation, only the whole fruit extract significantly attenuated the up-regulation in LPS-induced TNF- mRNA (28.61%, p<0.01). However, after six hours of stimulation, the LPS-induced expression of TNF- mRNA was significantly attenuated by the pulp and seed extracts (43.19%, and 54.49% with respect to LPS-induced cells; p<0.01). These values were statistically different to the value reached with regard to the whole fruit extract (p>0.01) (Figure 3). Interestingly, there was no difference in the TNF-α mRNA values of cells treated with pulp and seed extracts and non-treated control cells.

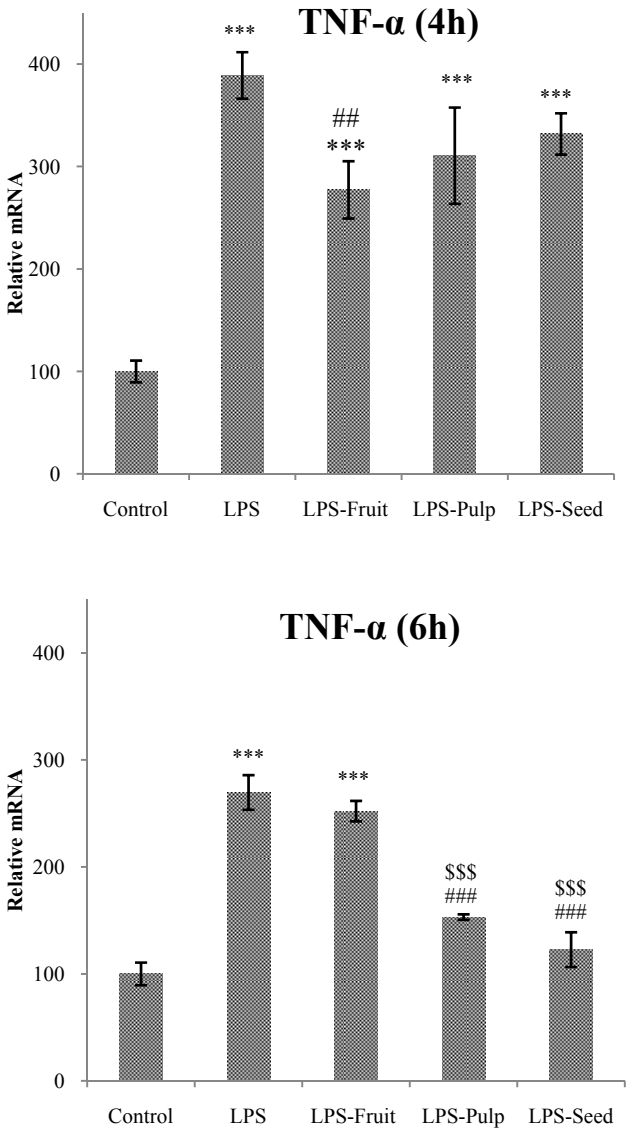


Figure 3. Protective effect of differentextracts of pricklypear on the expression of TNF- mRNA in N13 microgliastimulatedwith LPS.
All data are presented as the mean±SD of at least threeindependentexperiments. Bonferronianalysiswasemployed to compared the differencesbetween the experimental groups: ***p<0.001, **p<0.01 comparedwith control non-stimulatedcells. ###p<0.01, ##p<0.01 comparedwithcellsstimulatedwith LPS. $$$ p<0.001 compared groups stimulatedwith LPS and treatedwithpulp and seedpricklypearextractswith the group treatedwithwhole-fruit extract.

Similar results were found in the expression of IL-1β mRNA. After four hours of LPS stimulation, IL-1β mRNA expression decreased to 38.62% when compared to LPS-stimulated cells ($p<0.05$), but no significant effects were found in the cells treated with phenolic extracts of pulp and seed (8.73%, and 8.29%, respectively). However, after six hours of stimulation, IL-1β mRNA production decreased significantly after treatment with seed and pulp extracts (53.84% and 57.68% ($p<0.01$), respectively, compared to LPS-stimulated cells and reaching values similar to non-stimulated control cells (Figure 4). However, no significant effect was found in the LPS-stimulated cells treated with whole fruit extract.

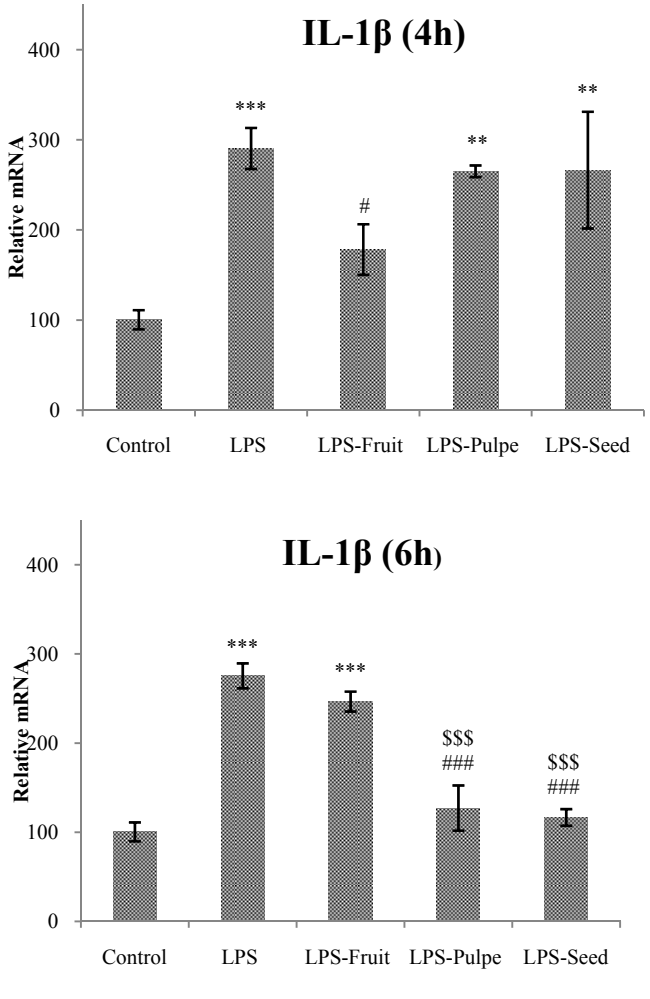


Figure 4. Protective effect of differentextracts of pricklypear on the expression of IL-1β mRNA in N13 microgliastimulatedwith LPS. All data are presented as the mean±SD of at least threeindependentexperiments. Bonferronianalysiswasemployed to compared the differencesbetween the experimental groups: ***$p<0.001$, **$p<0.01$ comparedwith control non-stimulatedcells. ###$p<0.01$, ##$p<0.01$ comparedwithcellsstimulatedwith LPS. $$$$p<0.001$ compared groups stimulatedwith LPS and treatedwithpulp and seedpricklypearextractswith the group treatedwithwhole-fruit extract.

In the case of iNOS mRNA expression, the results showed that all of the extracts studied attenuated the induced expression of iNOS mRNA at 4 and 6 hours post-stimulation. 4 hours after LPS-stimulation the decrease in iNOSmRNA expression varied between 44.47% for phenolic whole fruit extracts and 33.14% and 29.76% for phenolic seed and pulp extracts, respectively, compared with LPS-stimulated cells. After six hours of stimulation, the decrease was at its greatest with a value of 69.32% when comparing pulp-extract-treated cells with LPS-stimulated cells, obtaining values of 38.98%, and 32.67% for whole fruit extracts and seeds extracts ($p<0.001$), respectively, (Figure 5) with no differences when compared with non-stimulated control cells.

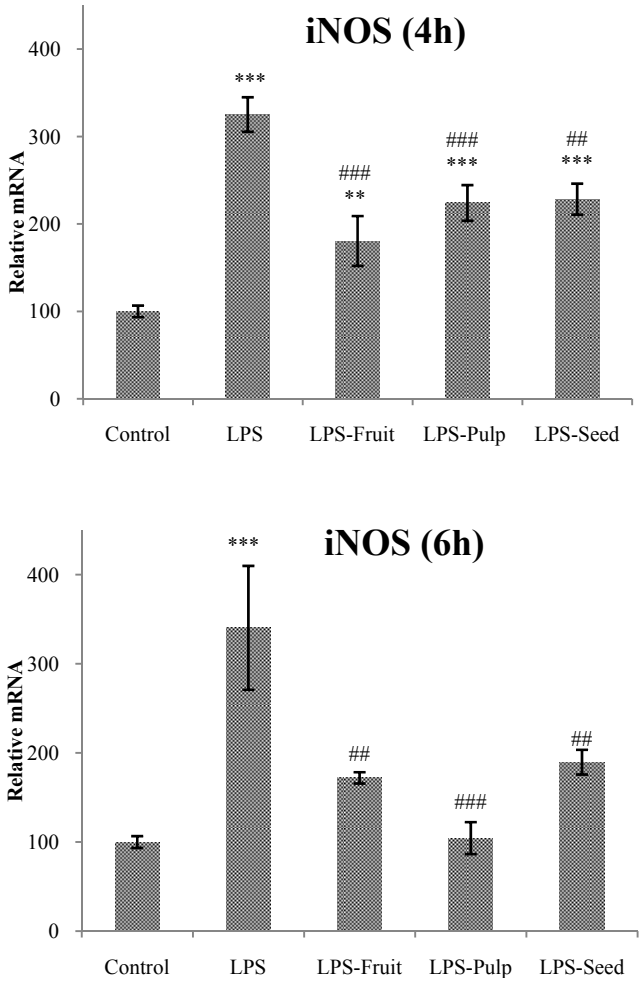


Figure 5. Protective effect of differentextracts of pricklypear on the expression of iNOSmRNA in N13 microgliastimulatedwith LPS. All data are presented as the mean±SD of at least threeindependentexperiments. Bonferronianalysiswasemployed to compared the differencesbetween the experimental groups: ***$p<0.001$, **$p<0.01$ comparedwith control non-stimulatedcells. ###$p<0.01$, ##$p<0.01$ comparedwithcellsstimulatedwith LPS. No

differenceswerefoundbetweenexperimental groups stimulatedwith LPS and treatedwithwhole-fruit, pulp or seedextracts.

Correlation

The present results did not show a clear correlation between the anti-inflammatory activity and the total phenolic and flavonoids contents; r: 0.52 and r: 0.66, respectively, (Figure 6).

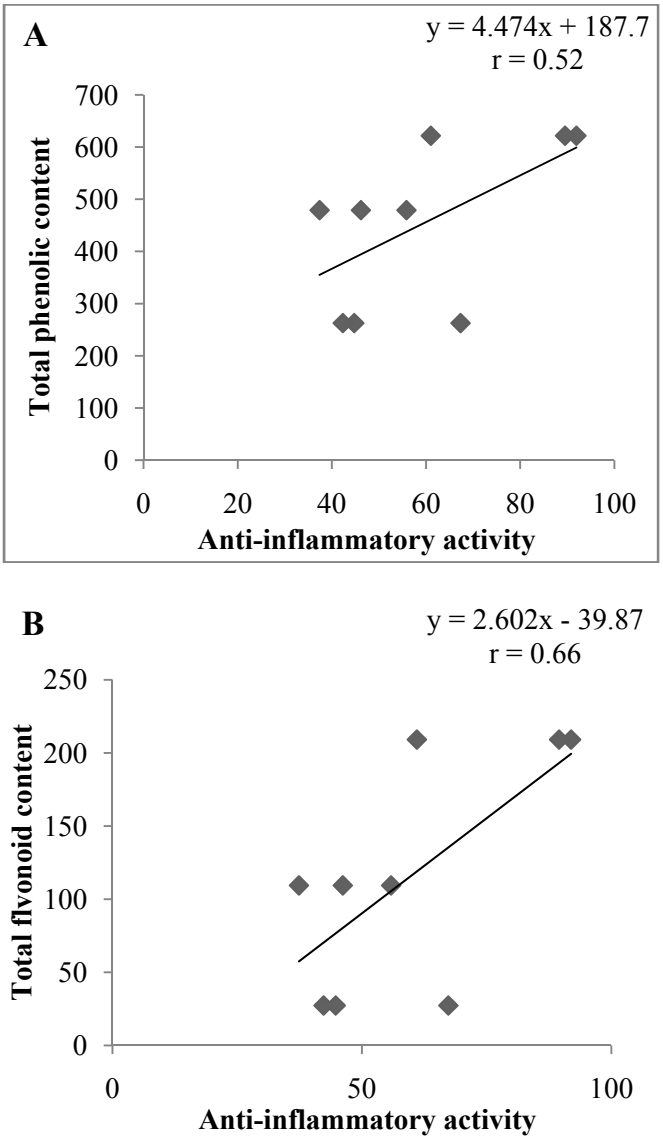


Figure 6. Correlationbetweenantioxidant compounds (total phenolic (A) and flavonoid (B) contents) and anti-inflammatoryactivity of pricklypearextracts.

Discussion

It is widely known that microglial activation is the brain's principal defence against immune challenges, but activated microglia may also contribute to neurodegeneration through the release of pro-inflammatory and/or cytotoxic factors such as IL-1β, TNF- and iNOs[19]. In fact, these processes exacerbate brain injury and cause neuroinflammation, which have been shown to be a risk factor in neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease [20, 21].Hence, it can be assumed that a degree of brain inflammation is required in order to repair damaged tissue, but excessive inflammation causes neuronal cell death. Therefore, the search for molecules that could help to control inflammation in the central nervous system [10, 20, 21]would be of great interest. In this context, and at low physiological concentrations, naturally occurring food chemicals such as phenolic compounds are able to exert neuroprotective actions via their interactionswith critical neuronal/glial intracellular signalling pathways that are pivotal in controlling neuronal resistance to neurotoxins, including oxidants [22]and inflammatory mediators [23]. There is also evidence showing a close link between antioxidant and anti-inflammatory activities [24]. In this sense, natural bioactive compounds rich in phenolics and flavonoids, such as those found in extracts of Chinese medicinal plants [25], have been shown to possess both antioxidant and anti-inflammatory activities. Prickly pear extracts are rich in phenolic compounds and possess beneficial properties such as antioxidant activities [5, 26, 27], therefore, in this study we have evaluated the potential anti-inflammatory property of extracts from different parts of prickly pears. Interestingly, anti-inflammatory activities were found in the different fractions of extracts from the red-yellow variety.

Our results show that cytokines induction, principally TNF-α, preceded the upregulation of iNOS. We have previously reported two stages in the LPS-induced inflammatory response, both *in vivo*[28]and *in vitro*[29, 30]. The peak for TNF- mRNA and IL-1β was 4 h post LPS stimulation and 6 h for iNOS. Cytokines in glial cells are very potent iNOS inductors [31]. It is therefore conceivable that after LPS stimulation, proinflammatory cytokines trigger iNOS induction. Our results also show that all of the prickly pear extracts studied attenuated the induction of TNF-α, Il-1β and iNOS mRNA in N13 microglia after LPS stimulation. NO and TNF- released by activated microglia are considered as markers for active proinflammatory responses. Interestingly, our results are in agreement with previous works reporting that *Opuntiaficusindica var. saboten* inhibited the degradation of I-κB- in LPS-activated microglia, resulting in the inhibition of iNOS expression [32].

However, in addition to iNOS/NO, activated microglia also produce cytokines such as TNF- and IL-1β that may contribute to neuronal damage. In fact, cytokines not only enhance the expression of iNOS but also may contribute to neuronal death via their binding to specific cell surface receptors expressed in neurons that activate pro-apoptotic pathways [33]. The treatment of N13 microglial cells with extracts of different fractions of prickly pears significantly attenuated the production of both TNF- and IL-1β in a dose-dependent manner.

The present results do not indicate a clear correlation between the anti-inflammatory activity and the total phenolic and flavonoids

contents (Figure 6). It must therefore be taken into account that the prickly pear is also rich in other bioactive components [34]. Consequently, not only must the anti-inflammatory effect be due to phenolic and polyphenols compounds, but there must also be some additional components in the extracts that play a pivotal role in the anti-inflammatory effect. So, we have previously described that different varieties of *Opuntiaficus-indica* are rich in betalains that are associated to biological activities such as antioxidant activity, antiviral, anti-inflammatoryandanticarcinogenic effects [5]. As a result, further characterization is needed. In fact, we found that although seed extracts were attributed minor values of both total phenolic and flavonoid content, they also exerted a clear anti-inflammatory effect. The antioxidant and antiradical properties of prickly pear seeds [26, 27]have been described and a clear correlation between total phenolic content and antioxidant activities of similar prickly pear seed extracts [26]have been found. Interestingly, several studies have reported a close link between antioxidant and anti-inflammatory activities [24, 25, 30]. Furthermore, due to its capacity to perpetuate and amplify inflammatory cascades [35], oxidative stress is known to be an important component in inflammation. Thus, combinations of agents that act at sequential stages in the neurodegenerative process may have neuroprotective effects [24]. Therefore, both the antioxidant and antiradical capacity of prickly pears [26, 27], as well as the anti-inflammatory effect described here, make prickly pear fruit a good candidate for the prevention of inflammation-linked neurodegenerative processes.

Conclusion

Overall, our results show that phenolic extracts of different prickly pear fractions exhibit pharmacological activities via an inhibitory effect on the production of LPS-induced inflammatory mediators by activated N13 microglia cells. As well as the known antiradical effects of prickly pear, this property makes it a good candidate for use as a source of potential preventive therapeutic agents that ameliorate the deleterious effects associated with microglial activation in the brain.

Authors´ contributions

ACconceived of the study and participated in its design and coordination, collection of data and drafted the manuscript.
DR and JPparticipated in the design and coordination of the studyand helped to draft the manuscript.
MCcarried out the experiments and participated in the data analysis and the drafted the manuscript
EGcarried out the experiments and participated in the drafted the manuscript
HLhelped to draft the manuscript and reviseditcritically.
All authors have approved the final manuscript.

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Conflict of interest

The authors declare no potential conflicts of interest.

Acknowledgments

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