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LC-ESI-UHR-QqTOF-MS/MS profiling and anti-inflammatory potential of the cultivated *Opuntia ficus-indica* (L.) Mill. and the wild *Opuntia stricta* (Haw.) Haw. fruits from the Algerian region

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ABSTRACT

Opuntia plants are abundant but still underexplored edible resources of the Algerian region. This work chemically characterizes extracts of different parts of the fruit of the commercial *Opuntia ficus-indica* (L.) Mill. and the wild *Opuntia stricta* (Haw.) Haw. growing in Bejaia, and evaluates their anti-inflammatory potential through different cell and cell-free bioassays. The LC-ESI-UHR-QqTOF-MS/MS analysis enabled the identification of 18 compounds, with azelaic acid and 1-*O*-vanilloyl-*β*-D-glucose reported here for the first time. Aqueous extracts of seeds were the most effective in scavenging superoxide anion radical (IC_{50} = 111.08 μg/mL) and presented the best anti-inflammatory potential in LPS-stimulated macrophages (IC_{50} = 206.30 μg/mL). The pulp of *O. stricta* suggested potential for addressing post-inflammatory hyperpigmentation, with piscidic and eucomic acids predicted with the strongest binding affinity towards tyrosinase, exhibiting higher scoring values than the reference inhibitor kojic acid. This pioneer study brings valuable perspectives for the pharmacological, nutritional and economic valorization of the wild *O. stricta* for functional foods.

1. Introduction

The Mediterranean region contains a wide variety of plants endowed with diverse ecologic, nutritional, and medicinal properties, worth of being explored for the development of functional foods, pharmacological and non-pharmacological therapies. However, despite the vast diversity of vegetable sources, many plants remain slightly known, needing to be further explored for their biochemical composition and biological targets (Thompson, 2020).

Cacti are a group of plants originating from the New World. The genus *Opuntia*, commonly known as prickly pears cactus, stand out for being the most widespread, with almost 300 species reported so far, and for producing nutritious fruits and cladodes that can be consumed as vegetables (Giraldo-Silva, Ferreira, Rosa, & Dias, 2023). *Opuntia* sp. have also been used in traditional medicine to alleviate a variety of health conditions, such as burns, edema, wounds, diarrhea, indigestion, hemorrhoids, and so forth (Boudjouan et al., 2021). These potential therapeutic properties can be attributed to the richness of *Opuntia* sp. in many primary and secondary metabolites, with nutritional and biological properties of interest to humans' health, such as vitamins, proteins, polysaccharides, polyunsaturated fatty acids, carotenoids, betalains (Blando, Albano, Jiménez-Martínez, & Cardador-Martínez, 2022; García

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et al., 2020), and phenols, these last playing a central role in the inflammatory process, as recently reviewed by us (Zeghbib, Boudjouan, Vasconcelos, & Lopes, 2022).

Inflammation is a physiologic protective process initiated by the body in response to infection or tissue injury, intended to eliminate the triggering factors and initiate tissue regeneration. The inflammatory response is highly complex, involving a wide range of soluble mediators, matrix molecules and enzymes, and the coordination of different immune cells, acting together to establish tissue repair and replace homeostasis. Upon failure of the immune system or a continuous exposure to the triggering factor, inflammation may become chronic or long lasting, resulting in tissues damage and often leading to the emergence of serious diseases and pathological disorders, such as autoimmunity, diabetes, neurodegenerative disorders, and cancer (Arulselvan et al., 2016; Yahfoufi, Alsadi, Jambi, & Matar, 2018). Nitric oxide ($^{\bullet}NO$) is a pleiotropic compound, product of the nitric oxide synthase (NOS) enzymes. $^{\bullet}NO$ plays crucial roles in many physiological processes, being able to act as a neurotransmitter, a tissue relaxing factor, in blood flow control, stimulating the immune system, and in the regulation of pro-inflammatory cytokines secretion. Whereas, an uncontrolled production of $^{\bullet}NO$ may be implicated in the exacerbation of inflammation and consequent tissues damage resulting, among others, from the continuous production of reactive oxygen species (ROS) such as superoxide anion radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), and reactive nitrogen species (RNS) like peroxynitrite ($ONOO^-$). These reactive species contribute to the disruption of homeostasis, what may result in cells and molecules dysfunction, favoring the appearance of various oxidative stress related-diseases (Mangge, Becker, Fuchs, & Gostner, 2014; Sharma, Al-Omran, & Parvathy, 2007).

The treatment of inflammation relies on the use of anti-inflammatory drugs, known for being associated with undesirable side effects leading to gastric, renal, hepatic, and cardiovascular problems. Thus, the screening for natural substances able to effectively suppress inflammation is an added value, regarding an alternative or complementary approach to the drugs already available as pharmacological treatment (Maroon, Bost, & Maroon, 2010; Zeghbib, Boudjouan, Vasconcelos, & Lopes, 2022).

Some species from the *Opuntia* genera have already been explored for their anti-inflammatory potential (Zeghbib, Boudjouan, Vasconcelos, & Lopes, 2022), nevertheless, none of the studies has focused on the different parts of the fruits, namely pulp and seeds of the same species. Otherwise, to the best of our knowledge, there is no previous study comparing the anti-inflammatory potential of the wild *Opuntia stricta* (Haw.) Haw. and the cultivated *Opuntia ficus-indica* (L.) Mill. growing in Algeria. In this regard, this work explores the qualitative and quantitative phenolic profile of different parts (seeds, pulp, and entire fruit) of cultivated and wild *Opuntia* sp. from the Bejaia region (Algeria), and hypothesizes their contribution to overcome inflammatory conditions, through the exploitation of their anti-inflammatory potential in different cell and cell-free targets, accompanied by a docking study involving the main phenolic compounds identified.

2. Materials and methods

2.1. Standards and reagents

β-Nicotinamide adenine dinucleotide disodium salt reduced form, nitro blue tetrazolium chloride, phenazine methosulfate, sodium formate buffer, tyrosinase from mushroom, L-DOPA-(phenyl-d3), hyaluronidase from Bovine Testes, hyaluronic acid (HA) sodium salt from *Streptococcus equi,* 4-(dimethylamino)benzaldehyde, lipopolysaccharide (LPS) from *Escherichia coli* O127:B8, sulfanilamide, *N*-(1-naphthyl) ethylenediamine dihydrochloride (NEDD), malic acid, citric acid, azelaic acid, vanillic acid, 2,5-dihydroxybenzoic acid (gentisic acid), 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, ferulic acid, *p*-coumaric acid, and kojic acid were acquired from Sigma Aldrich (Darmstadt, Germany). Phosphate buffer (KH_2PO_4) and sodium tetraborate decahydrate were from Fluka (Buchs, Switzerland). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Duchefa Biochemie (Haarlem, The Netherlands). Dulbecco's modified Eagle medium (DMEM) was from Gibco (New York, USA) and the inactivated fetal bovine serum (FBS) and penicillin-streptomycin were from Biochrom (Holliston, MA, USA).

2.2. Sampling

The present study was conducted on fruits of two *Opuntia* species (cultivated and wild) from the Algeria region. The cultivated species, *O. ficus-indica*, is the only one with commercialized fruits that are consumed by the population. The wild species, *O. stricta*, is highly abundant in the Algerian region, but still not commercially explored. An average of 40 fruits from each species were harvested in Bejaia (Algeria), during their respective period of maturity (August for *O. ficus-indica*, and October for *O. stricta*). Unharmed fruits with the same ripening were washed with tap water to remove impurities and glochids present on their surface, and then manually peeled. After blending with a domestic mixer (Moulinex, France), 50% of the amount was passed through a strainer, to separate seeds from pulp, and obtain the two different parts of the fruit, while the other part (edible set, composed by seeds and pulp) was homogenized using an ultra-turrax (IKA T18 Basic ULTRA-TURRAX, Berlin, Germany) for 5 min, to produce a mixture of pulp and seeds, hereinafter called "fruit", which corresponded to the edible parts. The pulp and fruit samples were frozen and lyophilized (Christ, alpha 1–4 LD plus, Germany). Seeds were then washed with tap water, frozen, lyophilized, and crushed (Retsch RM200, Germany). The dehydrated samples were stored at −20 °C until further analysis. The final samples resulting from the different *Opuntia* consisted of: *O. ficus-indica* fruit, *O. ficus-indica* pulp, *O. ficus-indica* seeds, *O. stricta* fruit, *O. stricta* pulp, and *O. stricta* seeds.

2.3. Preparation of the extracts

Two different extracts (ethanolic and aqueous) were prepared for chemical and biological analysis. For the ethanolic extract, 2 g of each lyophilized *Opuntia* sample was suspended in 50 mL of ethanol (99%), and then sonicated (Elma Schmidbauer GmbH, Germany) for 10 min, at room temperature. The supernatant was collected and filtered, while the remaining pellet was resuspended in the same volume of ethanol and sonicated again under the same conditions. The procedure was repeated 4 times. The recovered supernatants were combined, dried using a rotary evaporator (BÜCHI, Switzerland), and stored at −20 °C until further chemical and biological analysis. The aqueous extract followed the optimized conditions of the decoction procedure, recently reported by us (Zeghbib, Boudjouan, & Bachir-bey, 2022). Briefly, the decoction was performed on a stirrer (VELP SCIENTIFICA, Italy), by considering a sample/solvent ratio of 1 g/100 mL, for 30 min, at 90 °C. The resulting extracts were cooled down to room temperature, and then frozen, lyophilized (Telstar, Spain), and stored at −20 °C until further chemical and biological analysis.

2.4. Phenolic compounds profiling by LC-ESI- UHR-QqTOF-MS/MS

The aqueous and ethanolic extracts of the different samples of the cultivated (*O. ficus-indica*) and wild (*O. stricta*) cactus were analyzed by Liquid Chromatography – Electrospray Ionization – Ultrahigh-Resolution – Quadrupole Time of Flight – Mass Spectrometry (LC-ESI-UHR-QqTOF-MS/MS), following a methodology proposed before (Oliveira, Barros, Silva Ferreira, & Silva, 2015). Before injection, aqueous extracts were resuspended in ultra-pure water and ethanolic extracts in absolute ethanol, to a final concentration of 25 mg (dry extract)/mL, and filtered through a 0.45 μm nitrocellulose membrane. The separation was performed in a Bruker Elute series liquid

chromatograph, equipped with an UHR-QqTOF mass spectrometer, with 50,000 full-sensitivity resolution (FSR) (Impact II, Bruker Daltonics, Bremen, Germany) and a BRHSC18022100 intensity Solo 2 C18 column (100 × 2.1 mm, 2.2 μm, Bruker). The injection volume was 5 μL and the parameters for MS analysis were defined using negative ionization mode, with spectra acquired from *m/z* 20 to 1000 in an Auto MS scan mode.

The elemental composition for the compounds was confirmed in accordance with the accurate mass and isotope rate calculations designated mSigma (Bruker Daltonics). Phenolic compounds were identified according to their accurate mass $[M-H]^-$ **(Table S1)**. Malic acid, citric acid, azelaic acid, vanillic acid, 2,5-dihydroxybenzoic acid (gentisic acid), 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, ferulic acid, and *p*-coumaric acid were used as external standards. 1-*O*-vanilloyl-β-D-glucose, piscidic acid, eucomic acid, and catechol were quantified using the calibration curve of vanillic acid; gentisic acid 2-*O*-β-glucoside was quantified using the calibration curve of gentisic acid; *O*-hydroxybenzoic acid (salicylic acid) was quantified using the calibration curve of 4-hydroxybenzoic acid; sinapic acid acetate and (+)-pinoresinol were quantified using the calibration curve of ferulic acid and hesperetin was quantified using the calibration curve of naringenin. The results were expressed in μg of the respective phenol *per* gram of dry extract and *per* gram of dry sample, and the calibration curves are present in the supplementary material **(Table S2)**.

2.5. Biological activities

2.5.1. Superoxide anion radical scavenging activity

The antioxidant potential of the aqueous and ethanolic extracts of the different parts of *O. ficus-indica* and *O. stricta* was accessed by evaluating their ability to scavenge the superoxide anion radical ($O_2^{\bullet-}$), as previously described (Lopes, Barbosa, Andrade, & Valentão, 2019). Gallic acid was selected as positive control. The scavenging activity of the extracts was monitored using a Synergy HT Multi-detection Microplate Reader, operated by GEN5™ (Biotek, Bad Friedrichshall, Germany) and the results were expressed in percentage of $O_2^{\bullet-}$ scavenging face to control, as mean ± standard deviation (SD) of at least three independent assays performed in duplicate.

2.5.2. Hyaluronidase inhibition

The capacity of the extracts to inhibit hyaluronidase was evaluated according to the protocol proposed by Ferreres et al. (2012). The absorbance of the reaction product was measured at 560 nm against a blank sample, the results being expressed as the percentage of hyaluronidase activity face to the untreated control. Disodium cromoglicate (DSCG) was used as positive control.

2.5.3. Tyrosinase inhibition

The capacity of the extracts to inhibit tyrosinase was evaluated according to the adapted protocol of Favas, Morone, Martins, Vasconcelos, and Lopes (2021). Briefly, 20 μL of extracts (final concentration 1000 μg/mL) were mixed with 70 μL of tyrosinase (50 U/mL) and 40 μL of phosphate buffer (50 mM, pH 6.5). After an incubation of 5 min at room temperature, 70 μL of L-DOPA (2.5 mM) were added and the absorbance was immediately read at 475 nm (T0) and then after 15 min (T15). The product of reaction was calculated considering the differences in absorbances recorded between T0 and T15. The results were expressed as the percentage of tyrosinase activity compared to the untreated control. Kojic acid was used as a positive control.

2.5.4. Anti-inflammatory potential on RAW 264.7 cells

2.5.4.1. Cell culture and treatments. The murine macrophage cell line RAW 264.7 was cultured in DMEM (GlutaMax™-I), supplemented with 10% (*v/v*) of inactivated FBS and 1% (*v/v*) of PenStrep (Penicillin 100 U/L, Streptomycin 100 μg/mL) at 37 °C, in a humidified atmosphere containing 5% of CO_2. Cell passages were done by scraping at about 80% confluence, and the culture medium was renewed every two days.

Plates were seeded at a density of 3.5×10^4 cells/well in 96-well plates and incubated at 37 °C for 24 h, under a humidified atmosphere of 5% CO_2. *Opuntia* lyophilized extracts were resuspended in water or DMSO, for aqueous and ethanolic extracts, respectively, and sterilized by filtration through a 0.22 μm pore membrane. The final concentration of DMSO in contact with the cells did not surpass 0.5%, in order to not affect cells viability.

2.5.4.2. Cytotoxicity to RAW 264.7 cells. The cytotoxicity of the extracts on RAW 264.7 cells was evaluated using the MTT assay, following the methodology described before (Barbosa et al., 2017). After measuring the $^{\bullet}NO$ in the cells culture supernatant, the adherent cells were incubated during 45 min with MTT (0.5 mg/mL); the resulting formazan salts were dissolved with DMSO and the absorbance was determined at 550 nm, in a Synergy HT Multi-Detection microplate reader operated by GEN5 software. Cytotoxicity was expressed as the percentage of cells viability face to the control. At least 5 independent assays were carried out in duplicate.

2.5.4.3. $^{\bullet}NO$ production by RAW 264.7 cells. The anti-inflammatory potential of *Opuntia* sp. extracts was evaluated through their ability to reduce the $^{\bullet}NO$ produced by LPS-stimulated RAW 264.7 macrophages, following a procedure proposed before (Barbosa et al., 2017). Briefly, Raw 264.7 cells were pre-exposed for 2 h with serial dilutions of extracts (or vehicle), and then stimulated with bacterial LPS (1 μg/mL) and further incubated for 22 h at 37 °C. The $^{\bullet}NO$ production was also evaluated in the absence of LPS, to infer the possible effect of the extracts alone in basal $^{\bullet}NO$ levels. After 22 h of incubation, the amount of $^{\bullet}NO$ released in the culture medium was measured through the Griess reaction, by mixing 75 μL of cells supernatant with 75 μL of Griess reagent [sulfanilamide 1% and NEDD 0.1% prepared in 2% H_3PO_4 solution (*v/v*)] and incubating in the dark for 10 min. The absorbance was determined at 562 nm, and the results were expressed as the percentage of $^{\bullet}NO$ produced by extracts-exposed cells against the untreated control. Dexamethasone (50 μM) was used as positive control. At least 5 independent assays were performed in duplicate. Non LPS-stimulated cells were considered representative of normal basal metabolism.

2.6. Docking studies

In silico evaluation of putative tyrosinase inhibition by *Opuntia* sp. phenolic compounds was evaluated by the use of protein-ligand docking, using a structural model of the tyrosinase enzyme present in *Agaricus bisporus* mushroom, a selection of the phenolic compounds identified in *Opuntia* sp. extracts and tyrosinase reference binders.

2.6.1. Model preparation

Model for the mushroom tyrosinase was built from the crystal structure of *Agaricus bisporus* mushroom tyrosinase, deposited in the Protein Data Bank (RCSB PDB) (https://www.rcsb.org/) with the accession code 2Y9X, with a resolution of 2.78 Å. Water molecules and additional ligands were removed when preparing the structure for protein-ligand docking. The co-crystallized ligand tropolone (code OTR) was saved for subsequent use to optimize the docking protocol. This was done through redocking and comparison of the predicted poses with the experimental ones by root mean square deviation (RMSD) until an optimal superposition of the experimentally determined and docking predicted poses was achieved. Tropolone is a cyclic ketone that is cyclohepta-2,4,6-trien-1-one substituted by a hydroxyl group at position 2. This compound is a toxin produced by the phytopathogen *Burkholderia plantarii*, with known roles as a bacterial metabolite, a toxin and a fungicide.

Tyrosinase structure preparation and addition of the hydrogen atoms was done using the GOLD software suite (Jones, Willett, Glen, Leach, & Taylor, 1997), following the recommended protocol, as in previous studies involving other biological systems (Fernandes, Borges, Gomes, Sousa, & Simões, 2023; Lapaillerie et al., 2022; Leite et al., 2023; Mauro et al., 2023; Teixeira et al., 2023).

2.6.2. Ligand preparation

Structures of the phenolic compounds and reference molecule (Kojic acid) were obtained from the ChEMBL database (Gaulton et al., 2012), converted into 3D sdf format and optimized with Datawarrior (Sander, Freyss, von Korff, & Rufener, 2015), with protonation estimated at pH 7 with open babel (O'Boyle et al., 2011). This strategy for ligand preparation has been widely employed (Martins, Melo, & Sousa, 2021; Vieira, Cerqueira, Simões, & Sousa, 2023).

2.6.3. Docking

Docking was accomplished with GOLD software using the GOLD score scoring function (Verdonk, Cole, Hartshorn, Murray, & Taylor, 2003). The co-crystallized ligand tropolone was used as reference to evaluate the accuracy of the docking protocol in pose prediction and to optimize it. Each ligand conformation was randomized, and the ligands were redocked against tyrosinase model. In order to ensure an accurate reproduction to the pose of the reference ligands, different settings were considered. The optimized parameters included the position of the center and radius of the docking region (centered on the initial tropolone binding region, plus a 10 Å radius), and the number of independent genetic algorithm (GA) runs (1000 runs).

The optimized protocol was applied to dock all the phenolic compounds into the tyrosinase model. The most stable predicted conformations were recorded and analyzed. The value of the GOLD score for each molecule was determined and compared with the binding scores of the reference molecules (tropolone and kojic acid). Scoring values determined with GOLD score are non-dimensional, with higher values indicating stronger association.

2.7. Statistical analysis

The statistical analysis and the graphics for the *invitro* assays were performed using GraphPad Prism 8 software. One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison *post-hoc* test, was carried out on data obtained from each sample. A level of statistical significance at $p \leq 0.05$ was used. The amounts of individual organic acids and phenolic compounds quantified by LC-ESI-UHR-QqTOF-MS/MS were analyzed using the *t*-student test, taking a significance level of $p \leq 0.05$.

3. Results and discussion

3.1. Phenolic profile of Opuntia sp. extracts

The phenolic compounds present in *O. ficus-indica* and *O. stricta* extracts were analyzed by LC-ESI- UHR-QqTOF-MS/MS, and their qualitative profiles are displayed in the supplementary **Fig. S1 – S6**. For the first time, azelaic acid and 1-*O*-vanilloyl-*β*-D-glucose were identified in *O. ficus-indica* and *O. stricta* extracts. Their identification was based on detailed fragmentation pathways. Azelaic acid, with a deprotonated molecular ion at *m/z* 187.0927, undergoes primary fragmentation involving the loss of water (*m/z* 169.0822) and cleavage of the carboxylic acid group (*m/z* 143.0666). Secondary and tertiary fragmentations lead to smaller fragments at *m/z* 125.0558, 97.0650, 73.0288, 55.0176, and 45.0332. For 1-*O*-vanilloyl-*β*-D-glucose, the deprotonated molecular ion is at *m/z* 329.1080, with primary fragmentation including the loss of the glucose moiety (*m/z* 167.0350) and formation of deprotonated glucose (*m/z* 179.0555). Secondary fragmentation results in the loss of a methoxy group (*m/z* 152.0101) and CO_2 (*m/z* 123.0443), while tertiary fragmentation produces simpler fragments at *m/z* 108.0211 and 93.0178. The profile of organic acids and phenolic compounds varied between samples, depending on the nature of the extract (aqueous or ethanolic), the part of the plant, and the *Opuntia* species investigated (**Tables 1, 2,** and **3**). Regarding organic acids, for the same part of the fruit, a greater abundance was found in the aqueous extracts when compared to the ethanolic ones, which is expected, since these compounds are more hydrosoluble. Citric acid was the most abundant in aqueous extracts, being mainly found in the pulp part, with nearly 131.057 and 21.478 mg/g for the *O. stricta* pulp and *O. ficus-indica* pulp, respectively (**Table 2**). Regarding ethanolic extracts, apart from the *O. stricta* seeds, which showed a high content of malic acid (2167.58 μg/g) (**Table 1**), the two other parts of wild cactus were richer in citric acid, with 123.505 and 94.637 mg/g for the pulp and fruit, respectively (**Tables 2** and **3**). In contrast, for the cultivated cactus, *O. ficus-indica* seeds had a higher content of citric acid (261.30 μg/g), while malic acid was mainly found in fruit and pulp, with 1855.75 and 865.75 μg/g, respectively. In general, there was a different abundance of organic acids in the samples investigated herein, depending on each *Opuntia* sp. part but also depending on the species of *Opuntia* studied, larger quantities of organic acids being particularly noted in the wild *O. stricta*.

Organic acids are present in all living organisms, constituting important intermediates in the central metabolism of cells, and being involved in various plants' fundamental pathways. Therefore, as the metabolic pathways are carried out differently in each plant compartment, and depending on their stage of development, there will be a different accumulation of these compounds in different plants tissues (Batista-Silva et al., 2018). According to the literature, some organic acids were already reported in *Opuntia* species, like malic and citric acids, which were detected in Mata's et al. (2016) study, that included different *Opuntia* sp. juices; Melgar et al. (2017) found that citric acid ranged from 0.80 to 49.30 mg/100 g fresh weight (FW) in pulps of different *Opuntia* species, while malic acid was found in traces. To the best of our knowledge, there are no records on the presence of azelaic acid in *Opuntia* sp., this compound being reported for the first time herein.

Regarding the hydroxybenzoic acids, three compounds stood out in all the extracts analyzed: eucomic acid, piscidic acid, and 1-*O*-vanilloyl-*β*-D-glucose. Starting with eucomic acid, it was mostly present in the cultivated varieties, namely in aqueous extract of *O. ficus-indica* fruit (47.72 mg/g) and in the ethanolic extracts of the pulp (2.393 mg/g). Piscidic acid, on the other hand, was the major component of the aqueous extract of the *O. ficus-indica*, with 6559.81 and 2302.95 μg/g, for the pulp and seeds, respectively. Furthermore, piscidic acid was prevalent in the ethanol extracts of both fruits, with concentrations of 2804.43 and 331.27 μg/g, for *O. ficus-indica* and *O. stricta* fruits, respectively, and in *O. stricta* pulp with a concentration of 720.23 μg/g. Additionally, 1-*O*-vanilloyl-*β*-D-glucose was predominant in ethanolic extracts of both seeds, with concentrations exceeding 80 μg/g (**Tables 1, 2,** and **3**). In a prior study, Mata et al. (2016) successfully identified eucomic and piscidic acids in *O. ficus-indica* pulps. Subsequently, García-Cayuela, Gómez-Maqueo, Guajardo-Flores, Welti-Chanes, and Cano (2019) observed that among various *O. ficus indica* varieties, piscidic acid emerged as the most prevalent phenolic acid in pulps. Their findings revealed concentrations ranging from 8.05 to 21.33 mg/g DW, surpassing the values obtained in the present study, where the maximum concentration of this compound was found in the aqueous extract (5.18 mg/g DW) (**Table 2**). On the other hand, as far as we know, it is the first time that 1-*O*-vanilloyl-*β*-D-glucose is reported in *Opuntia* species.

Concerning the hydroxycinnamic acids group, the aqueous and ethanolic extracts of both *Opuntia* seeds were richer when compared to the extracts of the other parts of the plant, with particularly high amount of *p*-coumaric and ferulic acids (88.00 and 59.72 μg/g, respectively, for the aqueous extract of the seeds of *O. stricta*) (**Table 1**); it can be noticed that only the aqueous extract of the pulp of *O. ficus-indica* presented a similar content of sinapic acid acetate with 50.58 μg/g (**Table 2**). For the

Table 1
Quantification of some compounds identified in the seeds of *Opuntia stricta* and *Opuntia ficus-indica*, by LC-ESI-UHR-QqTOF-MS/MS[1].

Proposed compound	Aqueous extract				Ethanol extract			
	Opuntia stricta		*Opuntia ficus-indica*		*Opuntia stricta*		*Opuntia ficus-indica*	
	μg/g of dry extract	μg/g of dry sample (DW)	μg/g of dry extract	μg/g of dry sample (DW)	μg/g of dry extract	μg/g of dry sample (DW)	μg/g of dry extract	μg/g of dry sample (DW)
Organic acids								
Malic acid	5816.11 ± 21.36 [a]	267.54 ± 0.98 [a]	2973.95 ± 95.62 [b]	191.82 ± 6.17 [b]	2167.58 ± 55.81 [a]	170.15 ± 4.38 [a]	71.27 ± 11.42 [b]	4.20 ± 0.67 [b]
Citric acid	66,314.49 ± 1280.87 [a]	3050.47 ± 59.92 [a]	11,315.63 ± 692.87 [b]	729.86 ± 44.69 [b]	210.46 ± 0.01 [b]	16.52 ± 0.0 [b]	261.30 ± 0.01 [a]	15.42 ± 0.0 [a]
Azelaic acid	39.47 ± 0.0 [a]	1.82 ± 0.0 [a]	28.02 ± 1.40 [b]	1.81 ± 0.09 [b]	10.89 ± 0.03 [a]	0.86 ± 0.0 [a]	12.16 ± 2.10 [a]	0.73 ± 0.12 [a]
Hydroxybenzoic acids								
o-Hydroxybenzoic acid	0[b]	0[b]	1.58 ± 0.01 [a]	0.10 ± 0.0 [a]	7.03 ± 0.00 [a]	0.55 ± 0.0 [a]	0[b]	0[b]
Gentisic acid 2-*O*-β-glucoside	55.23 ± 0.69 [a]	2.54 ± 0.03 [a]	24.42 ± 0.43 [b]	1.58 ± 0.03 [b]	32.42 ± 2.06 [a]	2.54 ± 0.16 [a]	0[b]	0[b]
1-*O*-Vanilloyl-β-D-glucose	132.88 ± 4.72 [b]	6.11 ± 0.22 [b]	354.80 ± 5.70 [a]	22.88 ± 0.37 [a]	81.21 ± 3.20 [a]	6.38 ± 0.25 [a]	101.56 ± 36.16 [a]	5.99 ± 2.13 [a]
Vanillic acid	38.61 ± 1.77 [a]	1.78 ± 0.08 [a]	40.34 ± 1.61 [a]	2.60 ± 0.10 [a]	0	0	0	0
2,5-Di-hydroxybenzoic acid	30.99 ± 0.41 [a]	1.43 ± 0.02 [a]	25.71 ± 1.01 [b]	1.66 ± 0.06 [b]	10.01 ± 0.45 [a]	0.79 ± 0.04 [a]	7.17 ± 0.45 [b]	0.42 ± 0.03 [b]
Catechol	43.83 ± 0.50 [a]	2.02 ± 0.02 [a]	2.57 ± 0.21 [b]	0.17 ± 0.01 [b]	0	0	0	0
4-Hydroxybenzoic acid	38.17 ± 0.47 [a]	1.76 ± 0.02 [a]	18.51 ± 0.04 [b]	1.19 ± 0.0 [b]	18.22 ± 3.81 [a]	1.43 ± 0.30 [a]	15.51 ± 0.81 [a]	0.92 ± 0.05 [a]
4-Hydroxybenzaldehyde	10.20 ± 0.09 [a]	0.47 ± 0.0 [a]	4.05 ± 0.08 [b]	0.26 ± 0.01 [b]	3.57 ± 0.03 [a]	0.28 ± 0.0 [a]	3.37 ± 0.20 [a]	0.20 ± 0.01 [a]
Hydroxycinnamic acids								
p-Coumaric acid	88.00 ± 2.23 [a]	4.05 ± 0.10 [a]	26.29 ± 1.29 [b]	1.70 ± 0.08 [b]	36.36 ± 1.76 [a]	2.85 ± 0.14 [a]	22.99 ± 0.50 [b]	1.36 ± 0.03 [b]
Ferulic acid	59.72 ± 0.05 [a]	2.75 ± 0.0 [a]	48.03 ± 2.15 [b]	3.10 ± 0.14 [b]	54.82 ± 4.79 [a]	4.30 ± 0.38 [a]	68.70 ± 2.601 [a]	4.05 ± 0.15 [a]
Sinapic acid acetate	0[b]	0[b]	2.78 ± 0.79 [a]	0.82 ± 0.05 [a]	0[b]	0[b]	2.88 ± 0.16 [a]	0.17 ± 0.01 [a]
Flavone								
Hesperetin	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Others								
(+)-Pinoresinol	32.08 ± 0.0 [a]	1.48 ± 0.0 [a]	15.94 ± 0.94 [b]	1.03 ± 0.06 [b]	16.08 ± 0.01 [a]	1.26 ± 0.0 [a]	7.17 ± 0.90 [b]	0.42 ± 0.05 [b]
Piscidic acid	305.93 ± 3.58 [b]	14.07 ± 0.16 [b]	2302.95 ± 72.02 [a]	148.54 ± 4.65 [a]	0	0	0	0
Eucomic acid	61.65 ± 0.71 [b]	2.84 ± 0.03 [b]	916.57 ± 47.53 [a]	59.12 ± 3.07 [a]	54.49 ± 0.26 [b]	4.28 ± 0.02 [b]	75.52 ± 5.95 [a]	4.46 ± 0.3 [a]

[1] Values expressed in micrograms/g of dry extract or dry sample represent the mean ± standard deviation. Different superscript letters in the same row denote statistical differences between *Opuntia* sp. samples, for the same extraction solvent, for each compound ($p \leq 0.05$, test of *t*-student). LOQ: Limit of quantification (Table S2).

other analyzed compounds, hesperetin was below the limit of detection, while pinoresinol was found in higher amount in seeds of both *Opuntia*, particularly in the aqueous extract of seeds of the wild species with 32.08 μg/g (**Table 1**). According to the literature, some phenolic acids, namely *p*-hydroxybenzaldehyde, caffeic, ferulic and *p*-coumaric acids, were already identified in ethyl acetate extracts of peels and pulp of *Opuntia megacantha* Salm-Dyck, while *p*-hydroxybenzoic acid was only identified in peel and vanillic acid was only present in pulp (Ndhlala et al., 2007). Chahdoura et al. (2015) reported different ferulic acid derivatives, mainly feruloyl di-hexosides, in the extracts of seeds of some *Opuntia* species, with values ranging from 24.1 to 718.0 μg/g of dry extract, for *Opuntia microdasys* (Lehm.) N.E. Pfeiffer and *Opuntia macrorhiza* Engelm, respectively. For the majority of the derivatives, the values reported by the authors are in the same range of those reported herein for ferulic acid (**Table 1**). Another work from Juhaimi et al. (2020) reported the presence of *p*-coumaric acid in a methanolic extract of *Opuntia ficus-barbarica* A. Berger pulp, its concentration ranging from 2.80 to 13.20 μg/g DW, depending on the month of samples collection. This compound was not detected in the *Opuntia* pulps studied herein, however, it appeared in the seeds of both species analyzed, with concentrations ranging from 1.70 to 4.05 μg/g DW (**Table 1**). Mena et al. (2018) reported the presence of sinapic acid derivatives in pulps of different varieties of *O. ficus indica* with a concentration ranging from 0.06 to 1.70 mg/g DW, which was higher than the values recorded herein for the pulps, were the concentration of sinapic acid acetate ranged between 2.49 and 39.96 μg/g DW (**Table 2**). Furthermore, pinoresinol was only detected in seed oil of *O. stricta* (Koubaa et al., 2017), as well as in ethanol extracts of their cladodes (Zhu & Athmouni, 2022), where its concentration was 0.11 μg/g of dry extract; hence this study confirmed the presence of pinoresinol with higher concentration than that reported in the literature.

According to our recent review (Zeghbib, Boudjouan, Vasconcelos, &

Table 2
Quantification of some compounds identified in the pulps of *Opuntia stricta* and *Opuntia ficus-indica*, by LC-ESI-UHR-QqTOF-MS/MS[1].

Proposed compound	Aqueous extract				Ethanol extract			
	Opuntia stricta		*Opuntia ficus-indica*		*Opuntia stricta*		*Opuntia ficus-indica*	
	µg/g of dry extract	µg/g of dry sample (DW)	µg/g of dry extract	µg/g of dry sample (DW)	µg/g of dry extract	µg/g of dry sample (DW)	µg/g of dry extract	µg/g of dry sample (DW)
Organic acids								
Malic acid	3960.78 ± 77.56 [a]	2994.35 ± 58.64 [a]	3570.57 ± 102.85 [a]	2820.75 ± 81.25 [a]	377.56 ± 25.96 [b]	195.58 ± 13.45 [b]	865.75 ± 56.88 [a]	341.97 ± 22.47 [a]
Citric acid	131,056.95 ± 3153.33 [a]	99,079.05 ± 2383.92 [a]	21,478.16 ± 798.41 [b]	16,967.75 ± 630.74 [b]	123,504.51 ± 9317.41 [a]	63,975.34 ± 4826.42 [a]	0[b]	0[b]
Azelaic acid	11.90 ± 0.25 [b]	8.99 ± 0.19 [b]	20.38 ± 1.91 [a]	16.10 ± 1.51 [a]	5.43 ± 0.35 [a]	2.81 ± 0.18 [a]	7.57 ± 0.50 [a]	2.99 ± 0.20 [a]
Hydroxybenzoic acids								
o-Hydroxybenzoic acid	2.02 ± 0.03 [a]	1.53 ± 0.02 [a]	0.47 ± 0.04 [b]	0.37 ± 0.03 [b]	1.12 ± 0.01 [a]	0.58 ± 0.01 [a]	0[b]	0[b]
Gentisic acid 2-*O*-β-glucoside	11.14 ± 0.50 [b]	8.43 ± 0.38 [b]	15.18 ± 0.37 [a]	11.99 ± 0.30 [a]	14.47 ± 0.70 [a]	7.50 ± 0.36 [a]	5.94 ± 0.60 [b]	2.35 ± 0.24 [b]
1-*O*-Vanilloyl-β-D-glucose	266.56 ± 1.01 [a]	201.52 ± 0.77 [a]	38.71 ± 5.74 [b]	30.58 ± 4.53 [b]	429.89 ± 29.57 [a]	222.68 ± 15.32 [a]	39.20 ± 3.24 [b]	15.48 ± 1.28 [b]
Vanillic acid	27.03 ± 1.12 [a]	20.43 ± 0.85 [a]	6.43 ± 0.18 [b]	5.08 ± 0.14 [b]	0	0	0	0
2,5-Di-hydroxybenzoic acid	0	0	0	0	0	0	0	0
Catechol	14.70 ± 0.59 [a]	11.11 ± 0.45 [a]	0[b]	0[b]	0	0	0	0
4-Hydroxybenzoic acid	0.61 ± 0.03 [a]	0.46 ± 0.02 [a]	0[b]	0[b]	0	0	0	0
4-Hydroxybenzaldehyde	1.10 ± 0.04 [a]	0.83 ± 0.03 [a]	0.80 ± 0.02 [b]	0.63 ± 0.02 [b]	0	0	0	0
Hydroxycinnamic acids								
p-Coumaric acid	<LOQ	<LOQ	0	0	0	0	0	0
Ferulic acid	0.70 ± 0.11 [a]	0.53 ± 0.8 [a]	0[b]	0[b]	0	0	<LOQ	<LOQ
Sinapic acid acetate	6.29 ± 0.64 [b]	4.76 ± 0.48 [b]	50.58 ± 1.79 [a]	39.96 ± 1.41[a]	4.81 ± 0.08 [b]	2.49 ± 0.04 [b]	14.42 ± 2.21 [a]	5.70 ± 0.87 [a]
Flavone								
Hesperetin	0	0	0	0	0	0	0	0
Others								
(+)-Pinoresinol	0	0	0	0	0	0	0	0
Piscidic acid	536.63 ± 8.85 [b]	405.69 ± 6.69 [b]	6559.81 ± 0.54 [a]	5182.25 ± 0.42 [a]	720.23 ± 16.67 [a]	373.08 ± 8.63 [a]	973.66 ± 146.96 [a]	384.60 ± 58.05 [a]
Eucomic acid	52.85 ± 0.22 [b]	39.96 ± 0.17 [b]	3773.86 ± 7.22 [a]	2981.35 ± 5.70 [a]	63.10 ± 3.22 [b]	32.68 ± 1.67 [b]	2393.28 ± 100.48 [a]	945.34 ± 39.69 [a]

[1] Values expressed in micrograms/g of dry extract or dry sample, represent the mean ± standard deviation. Different superscript letters in the same row denote statistical differences between *Opuntia* sp. samples, for the same extraction solvent, for each compound ($p \leq 0.05$, test of *t*-student). LOQ: Limit of quantification (Table S2).

Lopes, 2022), summarizing the various classes of polyphenols reported so far in *Opuntia* species, it is reported a great variability in both qualitative and quantitative phenolic profile, which depends on several factors, including species-specific factors, environmental interactions, stage of maturity, age, geographical location, vegetative part analyzed, as well as the experimental parameters followed by each author. As far as we are aware, except for the recent study undertaken by our research group (Boudjouan et al., 2022), there is no report about the wild *Opuntia* of the Algerian region. Through our findings, the wild species exhibited higher polyphenol and flavonoid contents than those recorded from the cultivated species. Beyond species-specific factors, certain external conditions can influence plants metabolism, through the upregulation of synthesis and the accumulation of secondary metabolites, which are considered as a mean of defense, and as an adaptive strategy of plants to overcome environmental or pathogenic stressors during their lifetime (Reshi, Ahmad, Lukatkin, & Javed, 2023; Yang et al., 2018).

3.2. Antioxidant potential

The aqueous extracts of *Opuntia* sp. were screened for their ability to scavenge $O_2^{\bullet-}$, at 1000 µg/mL. A strong activity was demonstrated by *O. stricta* seeds, with 95.34% of scavenging, followed by *O. ficus-indica* seeds, and lately by *O. stricta* pulp and fruit, with approximately 70% of $O_2^{\bullet-}$ scavenging, and finally by *O. ficus-indica* fruit (23.12% of scavenging). A range of concentrations of the most promising extracts was thus evaluated, revealing a $O_2^{\bullet-}$ scavenging in a dose-dependent manner (Fig. 1A). The IC_{50} values determined for each extract were estimated at 111.08, 125.09, 559.28 and 669.84 µg/mL for *O. stricta* and *O. ficus-indica* seeds, and *O. stricta* fruit and pulp, respectively. It is worth to highlight the aqueous extracts of seeds of both *Opuntia* sp., which bioactivity was close to that exhibited by the reference compound gallic acid (IC_{50} = 95.60 µg/mL). Concerning the ethanolic extracts, only that of *O. stricta* pulp demonstrated free radical scavenging activity, with nearly 27.24% of $O_2^{\bullet-}$ scavenging for the highest concentration tested.

Superoxide anion radical is involved in the inflammatory response,

Table 3
Quantification of some compounds identified in the fruits of *Opuntia stricta* and *Opuntia ficus-indica*, by LC-ESI-UHR-QqTOF-MS/MS[1].

Proposed compound	Aqueous extract				Ethanol extract			
	Opuntia stricta		*Opuntia ficus-indica*		*Opuntia stricta*		*Opuntia ficus-indica*	
	μg/g of dry extract	μg/g of dry sample (DW)	μg/g of dry extract	μg/g of dry sample (DW)	μg/g of dry extract	μg/g of dry sample (DW)	μg/g of dry extract	μg/g of dry sample (DW)
Organic acids								
Malic acid	4143.98 ± 37.72 ^{b}	1178.96 ± 10.73 ^{b}	25,445.53 ± 2012.37 ^{a}	14,783.85 ± 1169.19 ^{a}	433.27 ± 55.22 ^{b}	147.75 ± 18.83 ^{b}	1855.75 ± 12.73 ^{a}	724.67 ± 4.97 ^{a}
Citric acid	129,129.61 ± 10,153.25 ^{a}	36,737.37 ± 2888.60 ^{a}	4832.61 ± 382.50 ^{b}	2807.75 ± 222.23 ^{b}	94,637.03 ± 1952.49 ^{a}	32,271.23 ± 665.80 ^{a}	0^{b}	0^{b}
Azelaic acid	14.14 ± 0.26 ^{b}	4.02 ± 0.07 ^{b}	88.23 ± 4.22 ^{a}	51.26 ± 2.45 ^{a}	6.81 ± 0.14 ^{a}	2.32 ± 0.05 ^{a}	7.51 ± 0.64 ^{a}	2.93 ± 0.25 ^{a}
Hydroxybenzoic acids								
o-Hydroxybenzoic acid	2.07 ± 0.10 ^{a}	0.59 ± 0.03 ^{a}	0^{b}	0^{b}	1.23 ± 0.11 ^{a}	0.42 ± 0.04 ^{a}	0^{b}	0^{b}
Gentisic acid 2-*O*-β-glucoside	16.79 ± 0.39 ^{b}	4.78 ± 0.11 ^{b}	75.29 ± 1.52 ^{a}	43.74 ± 0.88 ^{a}	12.90 ± 0.69 ^{a}	4.40 ± 0.23 ^{a}	5.00 ± 0.54 ^{b}	1.95 ± 0.21 ^{b}
1-*O*-Vanilloyl-β-D-glucose	237.69 ± 1.15 ^{b}	67.62 ± 0.33 ^{b}	1623.90 ± 198.21 ^{a}	943.49 ± 115.16 ^{a}	199.54 ± 27.75 ^{a}	68.04 ± 9.46 ^{a}	48.76 ± 2.16 ^{b}	19.04 ± 0.84 ^{b}
Vanillic acid	27.68 ± 1.67 ^{a}	7.88 ± 0.47 ^{a}	0^{b}	0^{b}	16.76 ± 2.37 ^{a}	5.71 ± 0.81 ^{a}	0^{b}	0^{b}
2,5-Di-hydroxybenzoic acid	0.94 ± 0.09 ^{a}	0.27 ± 0.03 ^{a}	0^{b}	0^{b}	0	0	0	0
Catechol	17.25 ± 0.35 ^{a}	4.91 ± 0.10 ^{a}	0^{b}	0^{b}	0	0	0	0
4-Hydroxybenzoic acid	9.27 ± 0.21 ^{a}	2.64 ± 0.06 ^{a}	0^{b}	0^{b}	2.68 ± 0.42 ^{a}	0.91 ± 0.14 ^{a}	0^{b}	0^{b}
4-Hydroxybenzaldehyde	3.57 ± 0.04 ^{a}	1.02 ± 0.01 ^{a}	0^{b}	0^{b}	1.21 ± 0.05 ^{a}	0.41 ± 0.02 ^{a}	0^{b}	0^{b}
Hydroxycinnamic acids								
p-Coumaric acid	16.14 ± 0.47 ^{a}	4.59 ± 0.13 ^{a}	0^{b}	0^{b}	2.28 ± 0.19 ^{a}	0.78 ± 0.06 ^{a}	0^{b}	0^{b}
Ferulic acid	2.70 ± 0.17 ^{a}	0.77 ± 0.05 ^{a}	0^{b}	0^{b}	3.49 ± 0.05 ^{b}	1.19 ± 0.02 ^{b}	13.09 ± 0.86 ^{a}	5.11 ± 0.34 ^{a}
Sinapic acid acetate	5.89 ± 0.16 ^{a}	1.68 ± 0.05 ^{a}	0^{b}	0^{b}	3.85 ± 0.07 ^{b}	1.31 ± 0.02 ^{b}	25.12 ± 4.67 ^{a}	9.81 ± 1.82 ^{a}
Flavone								
Hesperetin	<LOQ	<LOQ	0	0	<LOQ	<LOQ	0	0
Others								
(+)-Pinoresinol	13.93 ± 0.66 ^{a}	3.96 ± 0.19 ^{a}	0^{b}	0^{b}	10.12 ± 0.34 ^{a}	3.45 ± 0.11 ^{a}	0^{b}	0^{b}
Piscidic acid	499.39 ± 4.03 ^{a}	142.08 ± 1.15 ^{a}	9896.18 ± 3424.46 ^{a}	5749.68 ± 1989.61 ^{a}	331.27 ± 9.66 ^{b}	112.96 ± 3.29 ^{b}	2804.43 ± 93.24 ^{a}	1095.13 ± 36.41 ^{a}
Eucomic acid	56.12 ± 1.49 ^{b}	15.97 ± 0.42 ^{b}	47,716.52 ± 1840.45 ^{a}	27,723.30 ± 1069.30 ^{a}	53.99 ± 6.07 ^{b}	18.41 ± 2.07 ^{b}	2961.19 ± 67.95 ^{a}	1156.34 ± 26.53 ^{a}

[1] Values expressed in micrograms/g of dry extract or dry sample represent the mean ± standard deviation. Different superscript letters in the same row denote statistical differences between *Opuntia* sp. samples, for the same extraction solvent, for each compound ($p \leq 0.05$, test of *t*-student). LOQ: Limit of quantification (Table S2).

being highly produced by cells involved in the inflammatory process, as well as through the arachidonic acid metabolism, through which, this radical can be highly produced overcoming the capacity of the endogenous antioxidant defenses to neutralize it, at its head the superoxide dismutase (SOD), thus resulting in many damages at molecular and cellular levels (Fazilatun, Nornisah, & Zhari, 2005). It may also enhance pro-inflammatory reactions, and react with $^{\bullet}$NO, forming highly reactive $ONOO^{-}$, endowed by cytotoxic and pro-inflammatory potential, particularly through the deactivation of SOD enzyme, perpetuating the inflammatory response (Salvemini, Ischiropoulos, & Cuzzocrea, 2003).

The $O_2^{\bullet-}$ scavenging potential found for aqueous extracts, especially for seeds of both *Opuntia* sp. was lower than those found by Saravanakumar, Ganesh, Peng, Aziz, and Jang (2015), with an IC_{50} of 44.17 μg/mL for a methanolic extract of *O. ficus indica* fruits collected in summer; nevertheless, the authors did not find any efficiency regarding $O_2^{\bullet-}$ scavenging by water extracts of fruits collected in the rainy season. In this sense, it seems plausible that the harvesting season and climatic factors affect the metabolites profile. Thereby, when comparing to the present work, beyond species-specific characteristics, it can be assumed that the differences in plant location and abiotic factors, may significantly influence the plants metabolome and, consequently, the antioxidant potential of their extracts. Otherwise, two other works reported a high $O_2^{\bullet-}$ scavenging potential for *O. ficus indica* cladodes, recording a scavenging of almost 75.70% for a concentration of 1.5 mg/mL (Lee, Kim, Kim, & Jang, 2002), and an estimated IC_{50} of 1.87 mg/mL (Avila-Nava et al., 2014). When comparing these results with those obtained in this study, prickly pears fruit showed a better $O_2^{\bullet-}$ scavenging ability than cladodes. However, due to the lack of reports about the $O_2^{\bullet-}$scavenging ability of *Opuntia* plants, it would be interesting to evaluate this antioxidant potential on other *Opuntia* species and their different vegetative parts, including seeds, fruits, cladodes, and flowers. The statistical analysis between the chemical composition and $O_2^{\bullet-}$

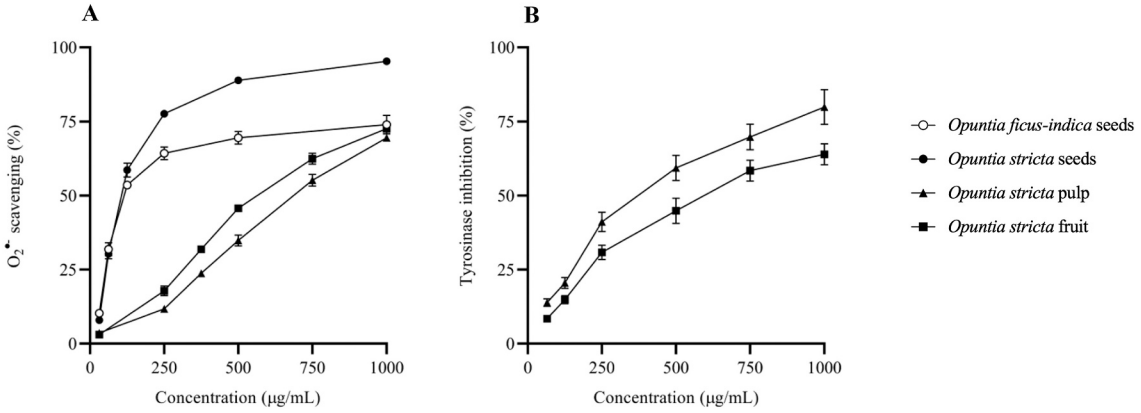

Fig. 1. Superoxide anion radical ($O_2^{\bullet-}$) scavenging activity of *Opuntia stricta* and *Opuntia ficus-indica* aqueous extracts (**A**) and effect of ethanolic extracts of *O. stricta* on tyrosinase inhibition (**B**). Results express the percentage, as Mean ± SD of three independent assays performed in duplicate. Gallic acid was used as positive control for $O_2^{\bullet-}$ scavenging (IC_{50} = 95.60 ± 13.22 µg/mL), and kojic acid was used as positive control for tyrosinase inhibition (IC_{50} = 6.14 ± 0.79 µg/mL).

scavenge (IC_{50}) showed a significant negative correlation with some compounds, particularly vanillic acid (−0.87, $p \leq 0.001$), catechol (−0.78, $p \leq 0.001$), 4-hydroxybenzaldehyde (−0.63, $p \leq 0.001$), 2,5-dihydroxybenzoic (−0.60, $p \leq 0.01$), 4-hydroxybenzoic (−0.54, $p \leq 0.01$), *p*-coumaric acid (−0.54, $p \leq 0.01$), and citric acid (−0.50, $p \leq 0.05$). Some of these compounds have been reported in the literature as good scavengers for $O_2^{\bullet-}$, namely vanillic acid (Liu & Mori, 1993) and 2,5-dihydroxybenzoic acid, which showed the highest $O_2^{\bullet-}$ scavenging potential when compared to other hydroxybenzoic acid derivatives (Velika & Kron, 2012). The authors had also attested that the antioxidant potential depended on the position of the hydroxyl (OH) groups on the benzene skeleton. For the cited molecule, the OH groups are located in the *ortho* and *para* position to the carboxylate group, suggesting that the substitution at these positions favors free radicals neutralization.

3.3. Effect of Opuntia sp. extracts on hyaluronidase

Contrary to the ethanolic extracts, which did not display effect on hyaluronidase, the aqueous extracts of *Opuntia* sp. demonstrated different effects on the enzyme activity: while the *O. stricta* pulp significantly reduced hyaluronidase activity, with approximately 31.56% of inhibition for the highest concentration tested (1 mg/mL), in a similar order of magnitude as that of the positive control DSCG (IC_{50} = 1.105 mg/mL), seeds and fruits of both plants demonstrated enzyme stimulation, leading to an increase in the cleavage of HA into monosaccharides, with an estimated percentage of 36.85%, 44.19%, 47.32%, and 70.22% for *O. stricta* seeds, *O. ficus-indica* and *O. stricta* fruit, and *O. ficus-indica* seeds, respectively **(Fig. S7)**.

Hyaluronidases are endoglycosidases that hydrolyze glycosaminoglycans, including hyaluronan, which is the most abundant constituent of the extracellular matrix, being involved in the induction of immune responses with pro-inflammatory cytokines production (McCook, Dorogi, Vasily, & Cefalo, 2015). Indeed, these enzymes are also related to many biological processes including inflammation, allergy, migration of cancerous cells, and permeability of the vascular system (Kohi et al., 2016). Besides being considered targets for cosmetics development, the hyaluronidase inhibition can also be correlated with the anti-inflammatory potential of extracts or compounds, since their activation promotes the inflammatory process by recruiting different immune cells and releasing of pro-inflammatory cytokines (Gómez-Maqueo, García-Cayuela, Welti-Chanes, & Cano, 2019).

The inhibitory effect recorded for *O. stricta* pulp **(Fig. S7)** was similar to the results obtained by Gómez-Maqueo and co-workers, (2019), who reported an inhibition of 35.80% and 32.40% for the pulp of two varieties of *O. ficus-indica*, the purple-skinned Pelota and the red-skinned Sanguinos, respectively. However, the same authors found a better inhibitory effect for the peels, with 53.60% and 73.40% for Pelota and Sanguinos varieties, respectively. This bioactivity could be attributed to some secondary metabolites, particularly betalains and phenolic compounds, present in each part of cactus fruits, as stipulated by Bhatti and Karim (2021), who reported that some flavonoids, namely flavones, 2-hydroxy-flavone, myricetin, luteolin, quercetin, and apigenin, demonstrated an inhibitory potential on the enzyme. The correlation analysis performed herein showed that some compounds presented a positive correlation with hyaluronidase activity, namely gentisic 2-*O*-β-glucoside acid (0.58, $p \leq 0.01$), 2,5-dihydroxybenzoic acid (0.52, $p \leq 0.01$), azelaic acid (0.51, $p \leq 0.05$), vanillic acid (0.47, $p \leq 0.05$), (+)-pinoresinol (0.45, $p \leq 0.05$), 1-*O*-vanilloyl-β-D-glucose (0.42, $p \leq 0.05$), and malic acid (0.41, $p \leq 0.05$), which can justify, at least in part, the stimulatory effect of some extracts.

Regarding the way that extracts affect the enzyme, and considering the positive correlation values obtained, it can be assumed that the majority of compounds able to increase hyaluronidase activity are present in seeds of both *Opuntia* species, since the fruit parts, constituted by a mixture of seeds and pulp, stimulated the enzyme activity, contrary to the pulp of both prickly pears, which did not show the same effect. According to the literature, no reports have been found regarding the existence of plant extracts with a stimulating effect on hyaluronidase activity. Many beneficial effects associated with the injection of hyaluronidase have been reported, in particular its use in medical applications for over 60 years, which got an approval from the US Food and Drug Administration. It was used as a subcutaneous fluid infusion, acting as an adjuvant for drugs dispersion in subcutaneous tissue (Cavallini, Gazzola, Metalla, & Vaienti, 2013; Jung, 2020). Hyaluronidase has also been used for aesthetic purposes, to dissolve HA fillers in case of patient dissatisfaction, and to treat side effects of inappropriate HA injection, however, some inconveniences such as local pruritus and allergic reactions can occur (Cavallini et al., 2013; Jung, 2020). Therefore, the results obtained herein with aqueous extracts of *Opuntia* sp. seeds may open new research perspectives focusing potential stimulating agents, which could make it possible to reduce the amount of enzyme injected, consequently lowering the risk of allergic complications in patients, particularly in the case of a long-lasting dermal filler resistant to hyaluronidase.

3.4. Effect of Opuntia sp. extracts on tyrosinase

All *Opuntia* sp. extracts were first screened for their ability to inhibit tyrosinase **(Fig. S8)**. At a concentration of 1000 µg/mL, different efficiencies were recorded: the aqueous extracts of *O. stricta* and *O. ficus-indica* seeds, and *O. stricta* pulp and fruit, significantly reduced the enzyme activity, in 27.42, 18.20, 16.27, and 10.94%, respectively;

regarding the ethanolic extracts, *O. ficus-indica* seeds and fruit presented a stimulatory action, increasing the enzymes' activity in almost 18%, while *O. stricta* pulp and fruit showed an inhibitory effect, stronger than that observed for the aqueous extracts, with >83% of inhibition. The most effective samples were tested in a range of concentrations, exhibiting a dose dependent behavior (Fig. 1B), with IC_{50} values of 345.99 ± 30.91 and 576.94 ± 43.00 μg/mL for *O. stricta* pulp and fruit, respectively.

Although tyrosinase activity is not a triggering factor of the inflammatory process, its activity can be affected by a variety of mediators involved in the inflammatory response, resulting in unpleasant variations in pigmentation after an inflammatory process occurring superficially and affecting visible areas of the skin. As so, dyschromatosis after inflammation are a clinically common symptom and, consequently, treatments that modulate specific inflammatory mediators may have important clinical utility in their treatment (Fu et al., 2020).

Many tyrosinase inhibitors have been commercially available, especially in cosmetic industries, as skin lightening agents and also as topical treatment for post-inflammatory hyperpigmentation disorders, like hydroquinone (HQ), arbutin, kojic, azelaic, ascorbic and ellagic acids; however, they present certain drawbacks like irritation, burning, prickling sensation, and may also present potential carcinogenic effects and instability during their storage (Pillaiyar, Manickam, & Namasivayam, 2017; Roggenkamp et al., 2021; Sowash & Alster, 2023). Thus, it is important to find alternative tyrosinase inhibitors, safe and effective, to replace/reduce the actual existing drugs.

Only two studies were found for tyrosinase enzyme inhibition by *Opuntia* species, which demonstrated the scarcity of information found for this enzyme. Souza et al. (2014) reported an inhibition for *O. ficus indica* cladodes (1 mg/mL) of almost 11.39%, while Hwang and Lee (2007) did not find any effect for the fruits of the same species. Thus, the results reported herein contribute to enriching the scientific knowledge on tyrosinase inhibitors from cactus fruit, especially from the wild *Opuntia.* Regarding, the stimulatory action of some plant extracts, Blom van Staden, Oosthuizen, and Lall (2021) reported that some components found in *Aspalathus linearis* (Burm.f.) R. Dahlgren, namely aspalathin and catechin were able to increase tyrosinase activity, explaining this by a probably subversive action of these compounds which reacted with tyrosinase to form other products, rather than improving enzyme activity to increase L-dopa conversion to L-dopaquinone. According to the chromatographic profile, citric acid, which was also found in high amount in *O. stricta* pulp, demonstrated a significant correlation (−0.71, $p \leq 0.001$) with the tyrosinase inhibition, which is *per* with Al-Abbasy, Idrees Ali, Rashan, and Al-Bajari (2021) study, reporting that citric and ascorbic acids demonstrated an effective inhibition of tyrosinase enzyme compared to other tested inhibitors. Overall, *Opuntia* fruits, especially those from the wild species, constitute a valuable source of compounds that present an inhibitory effect on tyrosinase. Thus, according to these results, *O. stricta* appears as a promising plant for potential pharmaceutical and cosmetic applications to prevent hyperpigmentation.

3.4.1. Docking results

To evaluate the putative binding affinity of the different compounds identified in *Opuntia* sp. as tyrosinase inhibitors, protein-ligand docking was used, taking the X-ray crystallographic structure of mushroom tyrosinase as putative target.

Table S4 shows the docking scores obtained with the popular Goldscore scoring function, available in GOLD software (Jones et al., 1997), one of the most widely used docking programs for protein-ligand docking (Sousa et al., 2013). With this scoring function, higher scoring values are indicative of stronger binding.

Tropolone was used in the docking procedure as a control for pose prediction, while kojic acid, a known tyrosinase inhibitor, was used as a positive control. From Table S4 it is evident that several of the compounds evaluated exhibited higher scoring values to tyrosinase than kojic acid (scoring 50.16), suggesting *Opuntia* sp. as a good source of phenolic compounds with tyrosinase inhibition capacity, and with a binding affinity comparable or superior to kojic acid.

Five different hydroxybenzoic acids resulted in higher scoring values than the control molecule, namely piscidic acid (61.54), eucomic acid (61.03), 1-*O*-vanilloyl-*β*-D-glucose (57.44), vanillic acid (52.88), and 2,5-dihydroxybenzoic acid (50.44). Among the other classes of compounds, azelaic acid (scoring 59.84), sinapic acid acetate (scoring 56.06), hesperetin (scoring 58.64) and (+)-pinoresinol (scoring 60.84) also exhibited higher scoring values than kojic acid. From the molecules tested, piscidic and eucomic acids are predicted as the compounds with the strongest binding affinity towards tyrosinase. Overall, the results suggest that several compounds present in *Opuntia* sp. extracts can be putative tyrosinase inhibitors, explaining the inhibition values observed for the extracts. Fig. 2 illustrates the most likely binding modes of piscidic and eucomic acids to tyrosinase, interacting in the binding pocket of this enzyme, close to the dicopper center.

3.5. Anti-inflammatory potential of Opuntia sp. extracts

The anti-inflammatory potential of cactus fruits on the LPS-stimulated RAW 264.7 cells was evaluated by measuring •NO levels released to the culture medium. Due to its high production during the inflammatory process, •NO is considered a marker of inflammation that may contribute to its pathogenesis, leading to serious tissue damages (Sharma et al., 2007). A first screening carried out with all *Opuntia* sp. extracts (1000 μg/mL) alone, revealed no cytotoxicity and no interference with •NO basal levels. When RAW 264.7 cells were stimulated with LPS in the presence of *Opuntia* sp. extracts, a decrease in •NO production, relative to control, was observed. The aqueous extracts of seeds from both species lead to a significant decrease in •NO production, with 74.98 and 60.48% for *O. stricta* and *O. ficus-indica* seeds, respectively, followed by *O. stricta* fruit extract with 33.75%, a low effect for *O. stricta* pulp extract with 13.56%, and no effect for both *O. ficus-indica* extracts of pulp and fruit. For the ethanolic extracts, a lower effect was observed, with a decrease in •NO production of 60.10, 52.48, and 15.92% for *O. stricta* and *O. ficus-indica* seeds, and *O. stricta* fruit, respectively. Serial dilutions of the most effective extracts were evaluated, and a dose-dependent inhibition of •NO production was observed (Fig. 3). Accordingly, IC_{50} values were only reached for the aqueous extracts of seeds, with 750.10 and 447.50 μg/mL for the cultivated and wild seeds, respectively; the fruit of the wild species exhibited a lower inhibitory effect, only reaching IC_{25} (612.71 μg/mL); the IC_{50} for the ethanolic extracts of *O. ficus-indica* and *O. stricta* seeds were, respectively, 856.214 and 777.38 μg/mL (**Table S3**). Furthermore, the statistical analysis confirmed that some compounds were significantly correlated with •NO reduction, namely 4-hydroxybenzaldehyde (−0.89, $p \leq 0.001$), and 4-hydroxybenzoic (−0.95, $p \leq 0.001$), ferulic (−0.90, $p \leq 0.001$), *p*-coumaric (−0.88, $p \leq 0.001$), and 2,5-dihydroxybenzoic acids (−0.86, $p \leq 0.001$); other compounds were also implicated, like (+)-pinoresinol (−0.57, $p \leq 0.01$), as well as catechol (−0.51, $p \leq 0.05$) and vanillic acid (−0.51, $p \leq 0.05$).

As reported in the literature, all the compounds mentioned before and present in the *Opuntia* sp. extracts analyzed, have shown anti-inflammatory capacity, acting by inactivating the NF-κB signaling pathway, and leading to the suppression of pro-inflammatory cytokines expression (Lim et al., 2008; Yun et al., 2008; During, Debouche, Raas, & Larondelle, 2012; Chang et al., 2014; Calixto-Campos et al., 2015; Zhao et al., 2016; Han et al., 2021; Kang et al., 2021; Shi, Chen, Qiang, Su, & Li, 2021; Xu et al., 2021; Y. Liu, Shi, Qiu, & Shi, 2022).

Among other cells, macrophages integrate the first line of defense during tissue damaging and pathogens invasion, playing an essential role in the inflammatory response. They trigger inflammatory reactions through the secretion of pro-inflammatory mediators like •NO, and also modulate the expression of different pro-inflammatory cytokines, such as interleukin-1beta (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) (Cheng et al., 2017; Lopes, Clarinha, &

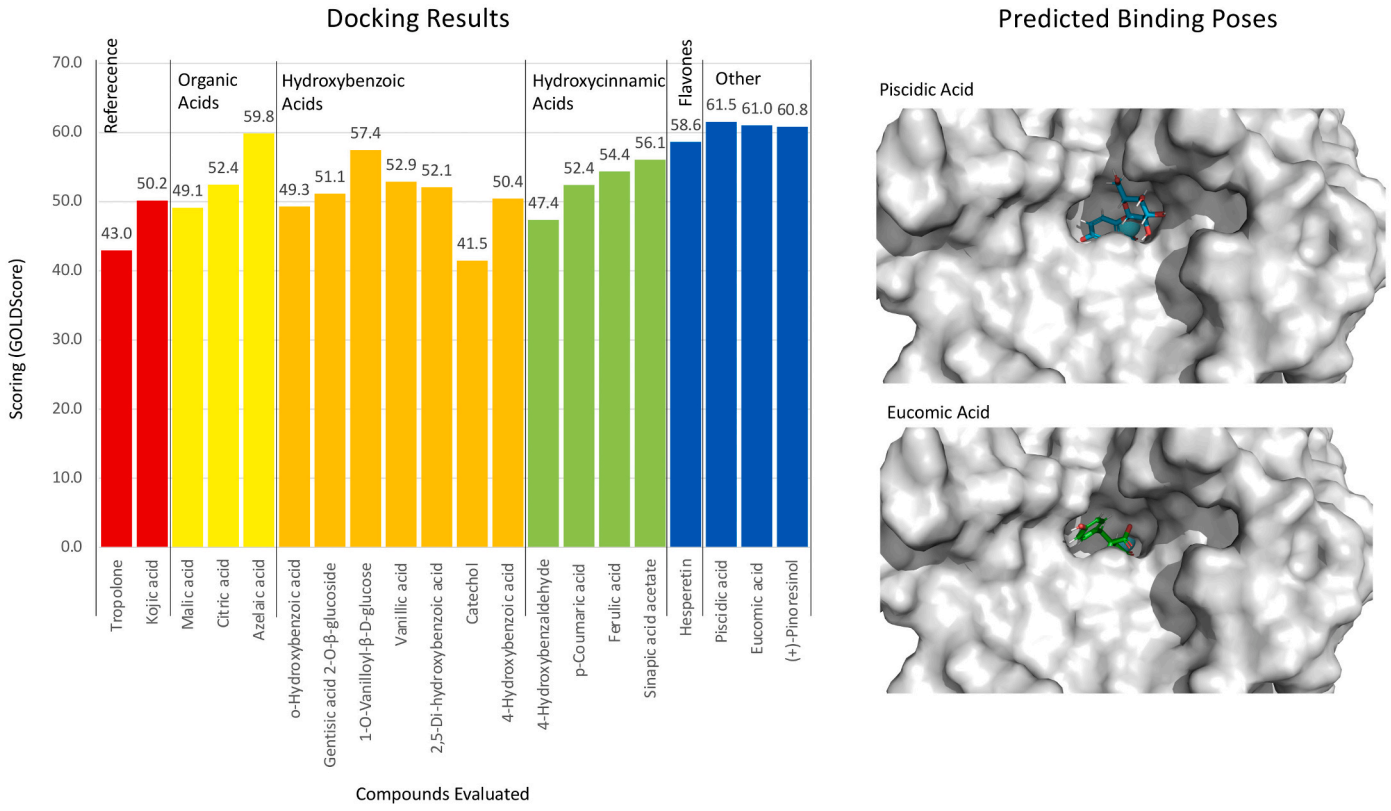

Fig. 2. Target-Ligand docking results of the different compounds evaluated against mushroom tyrosine, with GOLD software and with the GOLDscore scoring function. Higher values indicate stronger binding affinity. Predicted binding poses for strongest binders. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Vasconcelos, 2020). In this study, the anti-inflammatory potential of the different *Opuntia* sp. extracts was evaluated using the mouse macrophage RAW 264.7 cells model, upon LPS stimulation, a well-established *invitro* model for inflammation research studies (Cheng et al., 2017; Lopes et al., 2020). Some studies have reported the anti-inflammatory potential of *Opuntia* plants using RAW 264.7 cells, however, none of them was carried out using the entire fruit or pulp of the studied species. In fact, among the available works, flowers from *O. ficus-indica* exhibited a reduction of $^{\bullet}$NO levels in LPS-stimulated macrophages (Benayad, Martinez-Villaluenga, Frias, Gomez-Cordoves, & Es-Safi, 2014). *Opuntia humifusa* Raf. Leaf also demonstrated ability to modulate the expression of iNOS as well as of inflammatory-related genes like IL-1β and IL-6 (Cho et al., 2006). Furthermore, another study from Yeo, Hwang, and Park (2020) reported the anti-inflammatory potential of *O. humifusa* seeds methanolic extract, reducing $^{\bullet}$NO production by 50% compared to that in the LPS-treated control groups. These authors were also able to isolate and identify isoamericanin A, which demonstrated a lowering effect on $^{\bullet}$NO levels (almost 60%) through the decrease of iNOS expression in RAW 264.7 cells, accompanied by an inhibition of pro-inflammatory cytokines TNF-α and IL-6 secretion. This effect was devoted to the inhibition of IκB phosphorylation and to the decrease in NF-κB translocation into the nucleus (Yeo et al., 2020).

The current investigation showed that, in the LPS-induced RAW 264.7 macrophages, extracts from both *Opuntia* sp. fruits, either cultivated and wild, had low cytotoxicity and displayed anti-inflammatory action, especially the seeds, which could be more valorized and may be considered as a source of compounds with a potential medicinal value for the treatment of chronic inflammatory diseases.

4. Conclusions

The aqueous extracts of seeds from both cultivated and wild *Opuntia* yielded a remarkable result by inducing an increase in hyaluronidase activity, an occurrence not previously documented in the literature. Additionally, these extracts demonstrated an inhibitory effect on tyrosinase, a discovery supported by docking studies and with significant potential, particularly in the realms of aesthetic conditions and the treatment of post-inflammatory hyperpigmentation disorders. Besides the radicals scavenging potential of *Opuntia* sp. aqueous extracts towards $O_2^{\bullet-}$, cactus seeds also promoted a reduction in the production of $^{\bullet}$NO by LPS-stimulated macrophages. The LC-MS/MS analysis of the different samples allowed the establishment of their phenolic profile and, as supported by the statistical correlation, the undervalued wild variety contains important amounts of phenolic compounds that seem to be implicated in the important biological activities analyzed herein. Altogether, the present study emphasizes the economic potential of the wild *O. stricta*, an abundant resource that remains poorly exploited in Algeria, by pointing out their possible valorization as functional food, as well as in the cosmetic and pharmaceutical fields.

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CRediT authorship contribution statement

Walid Zeghbib: Formal analysis, Investigation, Methodology, Writing – original draft. **Fares Boudjouan:** Resources, Supervision, Writing – review & editing. **João Carneiro:** Writing – review & editing. **Ana L.S. Oliveira:** Formal analysis, Methodology, Writing – review & editing. **Sérgio F. Sousa:** Formal analysis, Methodology, Writing –

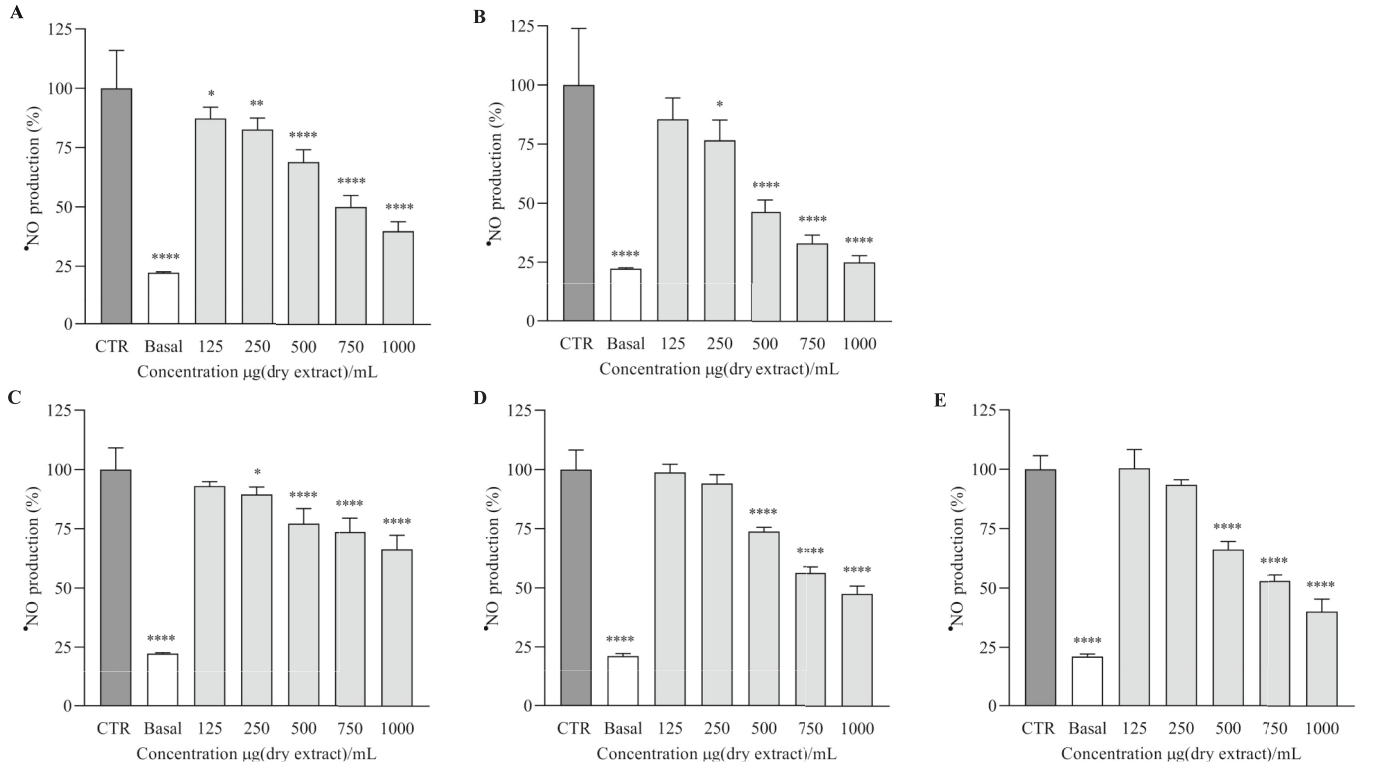

Fig. 3. Effect of *Opuntia* extracts on •NO production in LPS-stimulated RAW 264.7 cells. **A)** Aqueous extract of *Opuntia ficus-indica* seeds; **B)** Aqueous extract of *Opuntia stricta* seeds; **C)** Aqueous extract of the *O. stricta* fruit; **D)** Ethanolic extract of *O. ficus-indica* seeds; **E)** Ethanolic extract of the *O. stricta* seeds. Results are expressed as % of •NO relative to the LPS-stimulated control. "Basal" represents the •NO produced by RAW 264.7 cells without LPS stimulation. Results are expressed as the Mean ± SD of four independent assays, performed in duplicate. * $p \leq 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$ (ANOVA, Dunnett's multiple comparison test).

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.140414.

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