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HPLC-DAD Profiling of Phenolic Components and Comparative Assessment of Antioxidant Potency in *Opuntia robusta*, *Opuntia dillenii*, and *Opuntia ficus-indica* Cladodes at Diverse Stages of Ripening

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Abstract: Global distribution of prickly pear spans worldwide, with limited cladode exploitation, particularly in Mediterranean regions, excluding American continent nations. Our research reports on three species found in eastern Morocco, including *Opuntia ficus indica* and the two newly introduced species *Opuntia robusta* and *Opuntia dillenii*. The study aims to evaluate the total phenolic content, the amounts of various phenolic compounds classes, and the antioxidant activity across a spectrum of biochemical measurements including the 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant potential (FRAP), 2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and total antioxidant capacity assay (TAC). The results showed that *O. robusta* exhibited the highest total phenolic content (5061.70 mg GAE/100 g) which enhances the significance of this species in terms of nutrition. Differences were also observed in different stage of cladodes maturity, demonstrating that the age factor affects the polyphenol contents. Moreover, *O. robusta* displays the highest amount of total phenolic compounds (11430.35 µg/g) either for flavonoids or phenolic acids. Furthermore, the oldest cladodes show more higher phenolic compounds (22998.62 µg/g) compared to the youngest one's (20438.54 µg/g). Ten phenolic compounds were identified, in which isorhamnetin represent the most abundant flavonoid (6747.85 µg/g), while the chlorogenic acid was determined as the major phenolic acid (2771.69 µg/g) followed by sinapic acid (2047.64 µg/g). The DPPH assay indicates that *O. ficus-indica* possessed the higher hydrophilic antioxidants able to reduction the 2,2-diphenyl-1-picrylhydrazylradical free radical. While *O. robusta* exhibited a greater quantity of antioxidants, which effectively reduced the Fe^{3+} complex of ferric ions $(TPTZ)^{3+}$. In contrast, old cladodes of *O. robusta* and *O. dillenii* demonstrate the higher ability to scavenge the 2,20-azinobis (3-ethylbenzthiazolin-6-sulfonic acid) or $ABTS^{+}$.

Key words: antioxidant capacity, cladode, *O. robusta*, *O. dillenii*, *O. ficus-indica*, polyphenolic compounds

1 Introduction

The challenges posed by climate change, ongoing drought, and the widespread process of global desertification have increasingly drawn the focus of researchers towards plants exhibiting remarkable adaptations to the effects of global warming. These resilient plants offer the potential for the utilization of marginal lands, thereby making substantial contributions to their sustainable development. Among these versatile botanical specimens is prickly pear (*Opuntia* sp.) notable. The global proliferation of the prickly pear is ascribed to its multifaceted attributes[1]. Significantly, its adaptability to diverse soil substrates and its capacity to endure arid environmental conditions have been pivotal in facilitating its extensive dissemination[2]. Furthermore, prickly pear is widely recognized as a versatile plant that has the potential to be used in various sectors such as agrifood, pharmaceuticals, cosmetics and fodder production[3]. Additionally, the nopal

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cactus (*O. ficus-indica*) is recurrently enlisted in combatting soil erosion and desertification, thereby inciting heightened commercial interest in this plant[4].

The utilization of this botanical species exhibits disparity on a global scale, with the fruit emerging as the most commonly used part, compared to cladodes[5,6]. In Mexico, cladodes find ubiquitous application within the alimentary sector, serving as a foundational component for the fabrication of juice, chutney, jam, and fresh consumables[7]. Unfortunately, in the Mediterranean region, prickly pear cladodes are primarily utilized as supplemental animal feed. Despite the recognized health-promoting effects of the bioactive compounds within the cladodes, their use in modern nutrition and medicine remains limited[8].

Cladodes are the flattened, elliptical and narrow segments representing the green stem of the plant[7]. They are a nutritious food choice characterized by their low calorie and phytosterol content, making them a healthy food. Furthermore, these cladodes contain a rich fiber content which is considered as an effective solution for various health conditions including obesity, degenerative disorders, colorectal cancer and coronary heart disease[9]. Moreover, cladodes have demonstrated to exhibited pharmacological properties, such as the ability to reduce cholesterol levels, alleviate inflammation and provide neuroprotective effects. Despite the well-established and acknowledged health-enhancing properties conferred by the bioactive compounds intrinsic to the cladodes, their integration into contemporary nutrition and medical practices remains circumscribed[10].

The evaluation of antioxidant efficacy encompasses a spectrum of tests that leverage diverse mechanisms, including single electron transfer (ET), hydrogen atom transfer (HAT), metal chelation, and reducing power, among other methodologies[11]. Chemical assays, such as ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid)), TAC (total antioxidant capacity assay), FRAP (ferric reducing antioxidant potential), and DPPH (2,2-diphenyl-1-picrylhydrazyl), are commonly employed to quantify antioxidant activity due to their simplicity, ready accessibility, expeditious nature, automation potential, and frequent utilization in the initial screening and assessment of antioxidant compounds. Furthermore, these assays are characterized by their minimal complexity, cost-effectiveness, and independence from advanced laboratory equipment[12,13].

Currently, two new resistant species, *O. robusta* and *O. dillenii*, have been successfully introduced in the northeastern region of Morocco. *O. robusta* is a shrub-like to tree-like plant that can grow up to 3 m in height. The stems are cylindrical to oblong, displaying a glaucous blue-green or silvery-blue hue, measuring 1.5 to 2.5 cm in thickness and 14 to 40 cm in length. The fruits are spherical to ellipsoidal, attaining a dark red to purple color when mature, featuring some tubercles with an elongated podarium, measuring 7 to 9 cm in length[14]. Concerning *O. dillenii*, is a typically plants grow to a height ranging from 50 to 200 cm and feature flattened, elliptical, or obovate stem segments. The cladodes, commonly referred to as joints or pads, are distinguished by their abundant spines, which range in color from yellow to brown and measure 1.5 to 6 cm in length. The pear-shaped violet fruits, measuring 4 to 7 cm in length and approximately 3 cm in diameter, are usually devoid of thorns, and their glochids typically shed as the fruit ripens[15]. Both *O. dillenii* and *O. robusta* are among the eight-cochineal scale insect's resistant species that the National Institute of Agronomic Research (INRA) of Morocco has included in the official catalog for use in future plantings and site restoration following devastation by cochineal scale insects. Both *O. robusta* and *O. dillenii* demonstrate high adaptability to agro-ecological diversity of eastern region of Morocco. Consequently, the study's overarching objective is to compare the phenolic profiles and assess the antioxidant activity within the cladodes of *O. robusta* and *O. dillenii*, and subsequently compare these findings with those from the indigenous *O. ficus-indica* cultivated in eastern Morocco.

2 Materiel and Methods

2.1 Plant material

Three distinct populations were collected from the Oujda region in the northeastern part of Morocco (Spring 2023), situated at coordinates 34°41′12.001″N and 1°54′41″W. This geographical area is characterized by a Mediterranean climate typified by cool winters, hot summers, and irregularly distributed precipitation patterns. Meteorological data obtained from the Experimental Station of the Faculty of Sciences in Oujda reveal that July registers as the warmest month, exhibiting an average temperature of 27.24℃, while January is documented as the coldest month, featuring an average temperature of 9.21℃[16]. Healthy cladodes, differentiating between those less than one year old and their older counterparts exceeding two years in age were collected. Afterwards, they were meticulously excised and promptly placed within an isothermal container to ensure their preservation.

2.2 Sample preparation

The cladodes underwent a thorough wash with tap water, and the spines removed using curved forceps. Oven drying was carried out using an oven drier at a constant temperature at 30℃ during 72 hours. The dried samples were subsequently ground in an electric grinder (Type ME 2 B, 800 W; INTERMITTENT 15 S/1 min; made in France). Finally, the resulting powder was stored in an air-tight bottle.

2.3 Extraction and determination of total phenolic content

The extraction of phenolic compounds was conducted following the method outlined by reference[17]. 0.1 g of powder were blended with 5 mL of acetone water 1:1 (v/v), subsequently vortexed during 10 min then was subjected to centrifugation at 5000 rpm for a duration of 15 minutes. This process of extraction was repeated three times to ensure the total extraction of phenolic compounds. Subsequently, the obtained mixture underwent filtration using a microfilter featuring a pore size of 45 μm. To assess the total polyphenol content, 50 μL of the resulting supernatant were utilized. Absorbance measurements were executed employing a Rayleigh UV-1800 spectrophotometer (Beijing Rayleigh Analytical Instrument Corporation, China) at a wavelength of 760 nm. Using the gallic acid as standard, a calibration curve was conducted out and the results were expressed as milligrams of gallic acid equivalent per 100 g of dry matter.

2.4 Antioxidant activity determination

The determination of total antioxidant capacity, employing the [2,2-di (4-*tert*-octylphenyl) -1-picrylhydrazyl] (DPPH) assay, as well as the assessment of ferric reduction antioxidant power and the 2,20-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) test. The results were quantified in terms of milligrams of Trolox Equivalent per gram of dried cladode (mg TE g^{-1}). For optical density measurements, a UV-Vis spectrophotometer, specifically the RAYLEIGH UV1800 model, was utilized.

2.4.1 Total antioxidant capacity assay

Total Antioxidant Capacity (TAC) was determined following the method described by Chaymae Benkirane[18]. The phosphomolybdenum reagent was prepared by combining 4 mM of ammonium molybdate, 28 mM of sodium phosphate, and 0.6 M sulfuric acid in equal proportions. The experimental procedure entailed the mixing of 1.5 mL of this reagent with 50 μL of the extract, followed by thorough vortexing and an incubation period in a water bath set at 95℃ for 90 minutes. Subsequently, the absorbance was promptly recorded at 695 nm, using a blank as a reference for baseline correction.

2.4.2 Radical scavenging activity of 1,1-diphenyl picrylhydrazyl - DPPH assay

The evaluation of DPPH free radical scavenging activity within the samples was carried out spectrophotometrically, following the procedure outlined in reference[19]. This assay relies on the electron transfer ability of antioxidants to counteract the DPPH radical, resulting in a detectable color shift, which is quantified at 517 nm. A reciprocal relationship exists between the absorbance of the reaction mixture and the degree of DPPH free radical scavenging activity. In this study, 50 μL of each sample was introduced into 2 mL of a 0.13 mM methanolic solution of DPPH. The resulting mixture was vigorously vortexed and subsequently incubated in a dark environment at room temperature for a duration of 30 minutes.

2.4.3 Ferric reducing/antioxidant power (FRAP) assay

The Ferric Reduction Antioxidant Power (FRAP) assay is grounded in the measurement of an antioxidant's capacity to reduce ferric ions (Fe^{3+}) to form an intensely blue ferrous complex (Fe^{2+}), as delineated in reference[18]. Each sample, consisting of 50 μL, was combined with 0.65 mL of 0.2 M phosphate buffer (pH 6.6) and 0.65 mL of a 1% ferricyanide solution. After 20 min of incubation at 50℃ in a water bath, the reaction was halted by introducing 0.65 mL of 10% trichloroacetic acid. Subsequently, 0.65 mL of the extract was combined with 0.65 mL of water and 0.25 mL of 0.1% solution of $FeCl_3$. Spectrophotometrically, the reducing power of the examined antioxidants can be evaluated, Increased absorption at 700 nm indicates strong antioxidant activity.

2.4.4 ABTS [2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid)] free radical scavenging activity assay

The assessment was conducted to determine the capacity of antioxidants to counteract the stable radical cation, known as 2,20-azinobis (3-ethylbenzthiazolin-6-sulfonic acid) ($ABTS^{\cdot+}$). This radical cation manifests as a blue-green chromophore, exhibiting its highest absorption peak at 734 nm. The presence of antioxidants induces a reduction in the intensity of this chromophore.

The $ABTS^{\cdot+}$ radical was generated by mixing 2.45 mM potassium persulfate with 7 mM of ABTS [2,2'-Azinobis (3-Ethylbenzothiazoline-6-Sulphonic Acid)]. This mixture was left to incubate at room temperature in the absence of light for a period of 16 hours. Subsequently, the working solution, consisting of 50 μL of the extract combined with 2 mL of the $ABTS^{\cdot+}$ solution, was incubated for 30 minutes at room temperature in a light-shielded environment. Finally, the absorbance was measured against a blank at 734 nm using a spectrophotometer.

2.5 HPLC-DAD of phenolic compounds

Chromatographic analyses were conducted utilizing an Agilent 1260 Infinity II high-performance liquid chromatography system equipped with a diode array detector. The chromatographic separation was achieved by employing an Eclipse XDB-C18 column (3.5 μm particle size, 150×4.6 mm internal diameter, USA). The elution solvent consisted of a mixture of water (A) and acetonitrile (B), with the addition of formic acid (1%, v/v) in water. The chromatographic separation was carried out using a gradient elution process. The solvent composition commenced at 2% B and was held constant from 0 to 6 minutes. Subsequently, it was increased to 12.5% B between 23 and 33 minutes, further elevated to 30% B from 33 to 38 minutes, and then to 45% B from 38 to 42 minutes. This was followed by a stepwise increase to 75% B from 42 to 47 minutes and maintaining 100% B from 47 to 49 minutes. Subsequently,

the solvent composition reverted to 2% B from 49 to 50 minutes and remained at 2% B from 50 to 51 minutes. The flow rate was set at 0.6 mL/min, and a 10 μL injection volume was used. The separation of extracts was monitored at multiple wavelengths, specifically 254, 280, 300, and 340 nm. In addition, the UV/visible spectra of each compound were recorded over a range spanning from 190 to 800 nm. For the determination of the compound present in the sample, we compared the retention time of the observed peaks and the UV spectra with the standards (sigma-Aldrich Saint-Louis, United States).

Quantification was performed using an external standard curve of Isorhamnetin (20–200 μg/mL), Chlorogenic acid (20–200 μg/mL), sinapic acid (20–200 μg/mL), Gallic acid (6.25-100 μg/mL), Kaempferol (6.25-100 μg/mL), Ferulic acid (8– 60 μg/mL) and hydroxybenzoic acid (8– 60 μg/mL) were quantified using their commercial standards. The HPLC profiles were visualized and analyzed by the Agilent Openlab CDS software. Results are expressed as mg of standards per 100 g of dry matter.

2.6 Statistical analysis

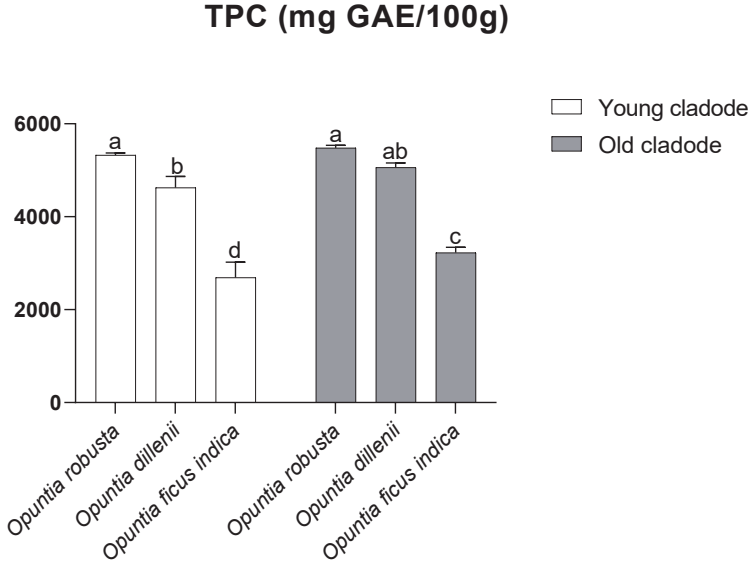
In the present investigation, all analyses of the prickly pear cladode were conducted in triplicate to ensure robustness and reliability of the findings. A one-way analysis of variance (ANOVA) was carried out to ascertain variations among the means of different samples. Statistical significance was considered established when the p-value was less than 0.05, indicating a statistically significant difference. Subsequently, the Tukey post hoc test was employed to perform multiple comparisons of means, enabling a more detailed assessment of the differences among groups. The results are reported as the mean ± standard deviation, and statistical analysis was executed using IBM SPSS Statistics 25 software. Moreover, correlations among the data obtained were computed using Pearson's correlation coefficient (r), providing insights into the relationships between variables under consideration.

3 Results

3.1 Substantial variation of TPC in cladodes

Significant variations in the Total Phenolic Content (TPC) of cladodes were observed among the studied species (Fig. 1). *O. robusta* exhibited the highest TPC, with values of 5484.40 mg GAE/100 g for young cladodes (less than one year) and 5327.30 mg GAE/100 g for old cladodes (more than two years), surpassing the TPC of the other examined species (Supplemental Table 1). *O. dillenii* demonstrated moderate TPC results, with values of 4629.28 mg GAE/100 g for young cladodes and 5061.70 mg GAE/100 g for old cladodes. In contrast, *O. ficus-indica* displayed the lowest TPC values for both young and old cladodes, measuring 2693.93 mg GAE/100 g and 3226.76 mg GAE/100 g, respectively.

Concerning the age of cladodes, except for *O. ficus indica* notable differences were observed between young and old cladodes. Older cladodes exhibited a higher TPC compared to their younger counterparts.



According to the analysis of variance, means followed by the same letters are not significantly different ($p \leq 0.05$)
Error bar: Standard error
Young cladode : less than one years old
Old cladodes : more than two years old

Fig. 1 Total phenolic content of *O. ficus-indica*, *O. robusta* and *O. dillenii* cladodes at different maturity stages.

3.2 Assessment of antioxidant activity in young and mature cladodes of various O. species

Four methods for assessing antioxidant activity were employed to evaluate the antioxidative potential of cladodes at different growth stages within diverse species:

3.2.1 DPPH Radical scavenging activity

The DPPH radical scavenging activities of the species under investigation ranged from 0.41 to 0.87 mg TE per g (Supplemental Table 2). Notably, no significant difference was observed between the young and old cladodes of the various species, except for *O. dillenii*, where the younger cladodes displayed a higher percentage of inhibition compared to the older ones (Fig. 2).

In this specific antioxidant test, *O. ficus-indica* exhibited a superior antioxidant capacity in both young and old cladodes, measuring 0.8 and 0.87 mg TE per g for young and old cladodes, respectively. Conversely, *O. robusta* displayed the lowest free-radical scavenging activity, recording values of 0.54 and 0.41 mg TE per g for young and old cladodes, respectively.

3.2.2 ABTS Free radical scavenging activity assay

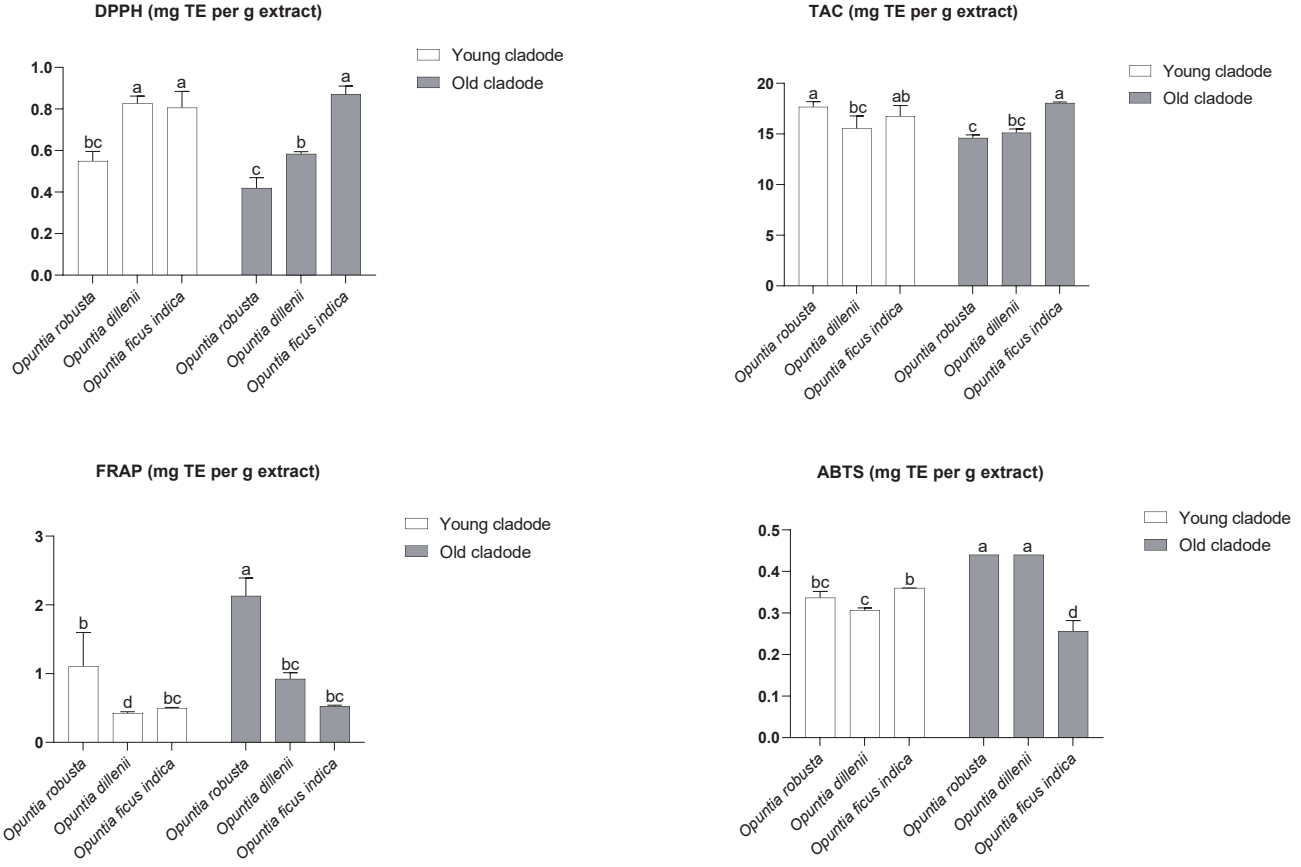
The ABTS values ranged from 0.25 to 0.43 mg Trolox equivalent per gram of the dried cladode (Supplemental Table 2). Notably, significant differences were discerned among cladodes of varying ages within the same species (Fig. 2).

The highest antioxidant activity was observed in the old cladodes of both *O. robusta* and *O. dillenii*, registering a value of 0.43 mg TE/g. In contrast, the old cladodes of *O. ficus-indica* displayed the lowest antioxidant activity in this assessment.

3.2.3 Ferric reducing/antioxidant power (FRAP) assay

The results for ferric reducing capacities ranged from 0.42 to 2.13 mg TE/g (Supplemental Table 2). Notably, significant differences were observed between the young and old cladodes of the studied species, with the exception of *O. ficus-indica* (Fig. 2).

O. robusta exhibited the highest antioxidant capacity



According to the analysis of variance, means followed by the same letters are not significantly different ($p \leq 0.05$)
Error bar: Standard error
Young cladode : less than one years old
Old cladodes : more than two years old

Fig. 2 Antioxidant assays (DPPH, TAC, FRAP, ABTS) of *O. robusta*, *O. dillenii* and *O. ficus-indica* old and young cladodes.

for both young and old cladodes, with values of 1.1 mg TE/g and 2.13 mg TE/g, respectively. *O. ficus-indica* displayed the second-highest FRAP findings, measuring 0.49 mg TE/g for young cladodes and 0.52 mg TE/g for old cladodes, respectively. In contrast, the lowest ferric reducing capacities were observed in the young cladodes of *O. dillenii*, with a value of 0.42 mg TE/g.

3.2.4 Total antioxidant capacity (TAC) assay

In the TAC assay, the obtained results ranged from 14.6 to 18.05 mg TE per gram of cladode (Supplemental Table 2). Notably, significant differences were observed between the young and old cladodes of *O. robusta* and *O. ficus-indica* (Fig. 2).

O. ficus-indica, in both its young and old cladodes, as well as the young cladodes of *O. robusta*, exhibited the highest Total Antioxidant Activity. In contrast, *O. dillenii* displayed the weakest antioxidant activity among the species under investigation.

3.3 Quantification of phenolic compounds by HPLC-DAD

In the analysis of phenolic compounds, ten distinct compounds were identified, encompassing two flavonoids and eight phenolic acids (Table 1, Supplemental Fig. 1). For both young and old cladodes, HPLC analysis facilitated the identification of Isorhamnetin as the most abundant flavonoid, with levels ranging from 5691.28 to 6747.85 μg/g of dried cladode, while Kaempferol represented the lowest amount, measuring from 35.29 to 331.67 μg/g. In contrast to flavonoids, a range of phenolic acids were identified, including gallic acid, chlorogenic acid, syringic acid, vanillic acid, coumaric acid, sinapic acid, ferulic acid, and hydroxybenzoic acid. Chlorogenic acid was the most abundant phenolic acid, with levels ranging from 1052.38 to 2771.69 μg/g, followed by Sinapic acid, which ranged from 531.49 to 2047.64 μg/g, for both young and old cladodes.

Significant disparities were observed among the examined species, as well as within cladodes of the same species at different stages of maturity (Table 1). Concerning the total phenolic compounds, the older cladodes exhibited higher concentrations at 22998.62 μg/g, while the younger cladodes displayed lower values at 20438.54 μg/g. In comparison to aged cladodes, the youthful cladodes contained a higher content of phenolic acids, measuring 11503.78 μg/g, whereas flavonoids were present in lower concentrations at 8934.75 μg/g. Conversely, the old cladodes were characterized by a higher concentration of flavonoids, totaling 13804.42 μg/g, while phenolic acids exhibited lower concentrations at 9194.19 μg/g.

Among the species under investigation, *O. robusta* displayed the highest concentration of total phenolic compounds, followed by *O. dillenii*, which exhibited moderate levels of both flavonoids and phenolic acids. In contrast, *O. ficus-indica* showcased the lowest content of total phenolic compounds, flavonoids, and phenolic acids.

4 Discussion

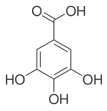
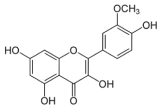
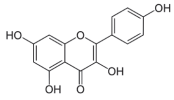
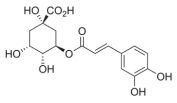
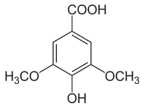
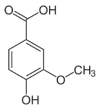
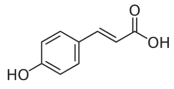
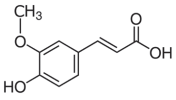
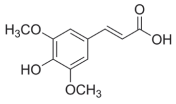
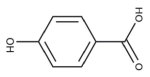
Phenolic compounds represent a category of secondary metabolites ubiquitously encountered within the plant kingdom. The synthesis of phenolic compounds is an adaptive response to both environmental perturbations and physiological stimuli[20]. In beside to their anti-inflammatory and antineoplastic effects, phenols are recognized for their antioxidative properties. The association of "antioxidants" with several fields including the chemical industry, food industry and most notably human health, has heightened the importance of antioxidants[21, 22]. As a result, there has been substantial interest in phenolic compounds due to their potential as antioxidants and their ability to neutralize free radicals. In effect, phenols contribute to the prevention of heart disease, a reduction in the incidence of diabetes, cancers and inflammation. Furthermore research by[23, 24] documented that the phenolic antioxidants safeguard ascorbic acid from oxidative degradation.

Based on the acquired results, *O. robusta* cladodes exhibit the highest concentration of total phenolic compounds. In contrast, cladodes of *O. ficus-indica* demonstrate the lowest total phenolic content. The noteworthy elevation of polyphenolic constituents in *O. robusta* underscores the nutritional significance attributed to this particular species. The production of phenolic compounds, considered secondary metabolites, is contingent upon a complex interplay of biotic and abiotic factors[25]. Notably, the specimens under investigation were all harvested from identical environmental conditions, thereby substantiating that the observed distinctions among these species primarily stem from their inherent biochemical characteristics. With the exception of *O. robusta*, a conspicuous delineation becomes apparent when comparing the oldest and youngest cladodes. This observation underscores the significant influence of age on polyphenolic content, which demonstrates a progressive increase as mature cladodes. The influence of cladode maturity on phenolic content was also noted by Marely G Figueroa-Pérez[26], who reported that the age of the cladodes significantly affects phenolic content.

Conversely, *O. albicarpa*, *O. megacantha*, *O. streptacantha*, and *O. hyptiacantha* showcased notably elevated total phenolic compound (TPC) levels, measuring at 694.08, 694.08, 966.81, and 582.2 mg/100 g, respectively. In an additional study by[27], a substantial reduction in total phenolic content was documented for the Spanish *O. ficus-indica*, reporting values of 1890 and 1480 mg/100 g dry weight for young and mature cladodes, respectively. The observed variations in TPC levels are likely attributed to the interplay of genotypic differences and the complex web of biotic and abiotic factors.

The inherent reactivity of an antioxidant towards free radicals is intricately molded by its chemical architecture, thereby exerting a profound influence on its antioxidant

Table 1 Identification and quantification of phenolic compounds of *O. robusta*, *O. dillenii* and *O. ficus-indica*.

				Young Cladode (μg/g)			Old Cladode (μg/g)		
N	RT (min)	Compound name	Structural formula	*O. ficus indica*	*O. robusta*	*O. dillenii*	*O. ficus indica*	*O. robusta*	*O. dillenii*
1	8.595	Gallic acid		223.285 4.46%	87.35 0.76%	179.93 4.49%	273.53 5.11%	314.51 3.03%	203.66 2.78%
2	11.344	Isorhamnetin		972.58 19.43%	5691.28 49.79%	1572.28 39.26%	2299.14 42.99%	6747.85 65.22%	4051.35 55.46%
3	12.519	Kaempferol		331.67 6.62%	35.29 0.3%	331.63 8.28%	328.52 6.14%	174.99 1.69%	202.54 2.77%
4	15.898	Chlorogenic acid		1753.83 35.04%	2771.69 24.24%	1052.38 26.28%	1133.63 21.19%	1309.71 12.65%	1235.05 16.9%
5	18.501	Syringic acid		44.13 0.88%	34.09 0.29%	38.15 0.95%	18.28 0.34%	17.60 0.17%	18.85 0.25%
6	21.491	Vanillic acid		289.47 5.7%	446.39 3.9%	129.40 3.2%	193.30 3.61%	344.02 3.32%	287.31 3.9%
7	22.781	Coumaric acid		30.44 0.6%	32.57 0.28%	4.16 0.1%	21.59 0.4%	4.04 0.03%	3.66 0.05%
8	22.925	Ferulic acid		24.94 0.49%	131.63 1.15%	–	82.87 1.54%	140.89 1.36%	143.13 1.95%
9	24.429	Sinapic acid		1209.99 24.18%	2047.64 17.91%	531.49 13.27%	877.74 16.41%	1065.26 10.29%	985.33 13.48%
10	30.476	Hydroxybenzoic acid		123.47 2.46%	152.37 1.33%	164.86 4.11%	119.40 2.23%	226.86 2.19%	173.86 2.38%
		Total flavonoids		1304.26	5726.57	1903.9155	2627.676	6922.852	4253.8991
		Total			8934.75857			13804.42634	
		Total phenolic acids		3699.5912	5703.7781	2100.4120	2720.3823	3422.9265	3050.8907
		Total			11503.78143			9194.19969	
		Total compounds		5003.85	11430.35	4004.3274	5348.0581	10345.778	7304.7898
		Total			20438.54			22998.626	

efficacy[13]. Consequently, the evaluation of antioxidant activity should not be contingent upon a singular testing method. Instead, a comprehensive approach necessitates the execution of multiple antioxidant assays to meticulously gauge the antioxidative potential inherent to the specimen under investigation. In this study, four distinct spectrophotometric assays were deployed, recognized for their precision owing to their reliance on discerning variations in absorbance values[13]. The divergent outcomes discerned among these diverse antioxidant tests may be ascribed to

the appropriateness of each specific antioxidant assay employed.

The DPPH assay unveiled that *O. ficus-indica* displayed the most pronounced percentage of antioxidants proficient in quenching the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. Conversely, *O. robusta* exhibited the least robust antioxidant capacity in mitigating this particular category of free radicals.

The DPPH assay represents a swift, extensively recognized, cost-efficient, and widely embraced technique, albeit necessitating the dissolution of the radical in an organic solvent. Consequently, this method finds its primary utility in the evaluation of hydrophilic antioxidants. Within the cohort of investigated species, *O. ficus-indica* showcases the most substantial concentration of hydrophilic antioxidants, with *O. dillenii* and *O. robusta* closely following suit.

The DPPH assay is a frequently used technique for evaluating the antioxidant potential of phenolic compounds[28]. Subsequently, a correlation analysis was executed between the DPPH test and the total phenolic content (Supplemental Table 3). As a result, a remarkably robust positive correlation between the DPPH assay and the total phenolic content across all the various species examined was unveiled (Table S3), with correlation coefficients spanning from 0.89 to 0.99. This strong correlation underscores a direct and pronounced connection between phenols and their capacity to effectively scavenge the DPPH free radical. This finding aligns with the conclusions drawn by a prior study conducted by[29], which also highlighted the substantial scavenging effects of phenolic compounds against the DPPH free radical.

Among the array of tests centered around transitional metals, the ferric reduction of antioxidant power (FRAP) assay stands out as the most widely employed method[30, 31]. This particular assay is extensively utilized on a large scale, furnishing invaluable insights into the antioxidant content within the studied extract. Notably, the redox system involving Fe(III)/Fe(II) in the FRAP assay conforms to the standard reversible redox principles in chemistry[13]. The FRAP assay shares a common trait with the DPPH radical assay, as it reveals the reducing capabilities of hydrophilic antioxidants.

In our study, *O. robusta* showcased the highest concentration of antioxidants capable of effectively reducing the Fe^{3+} complex of ferric ions $(TPTZ)^{3+}$ to the ferrous complex $Fe(TPTZ)^{2+}$. Following closely, *O. dillenii* also displayed substantial reducing power, particularly in the case of older cladodes. In contrast, *O. ficus-indica* exhibited the lowest antioxidant activity among the studied species.

In the context of cladode age, a notable divergence was evident in the cases of *O. robusta* and *O. dillenii*, where the maturation of cladodes exerted a discernible influence on the reducing capacity of the Fe^{3+} complex. In contrast, no discernible variation was observed in the cladodes of *O. ficus-indica* at different stages of maturity, implying that cladode age does not exert an influence on the reducing power of the Fe^{3+} complex.

With the exception of young cladodes of *O. robusta*, a robust positive correlation was identified between the results of the antioxidant assay and the total phenolic content, as outlined in Table S3. The correlation coefficients ranged from 0.81 to 0.99, underscoring a clear and significant association between phenolic compounds and their capacity to reduce the Fe^{3+} complex of ferric ions $(TPTZ)^{3+}$.

The ABTS test stands as one of the pivotal methods for quantifying antioxidants in sampled substances. Its applicability distinguishes it from the two preceding assays (DPPH and FRAP), mainly due to the solubility of the $ABTS^{+}$ cation radical in both aqueous and lipophilic phases. This unique characteristic enables the ABTS assay to evaluate the presence of both hydrophilic and hydrophobic antioxidants within a sample. Concerning the outcomes, it becomes evident that the mature cladodes of *O. robusta* and *O. dillenii* exhibit a superior capacity to scavenge the 2,2-azinobis (3-ethylbenzthiazolin-6-sulfonic acid) or $ABTS^{+}$ radical. In contrast, *O. ficus-indica* displays the lowest antioxidant capacity. In terms of cladode age, the older ones demonstrate heightened antioxidant activity in comparison to their younger counterparts. Conversely, the young cladodes of *O. ficus-indica* register a higher antioxidant capacity than their older counterparts, as also reported in another study (Blando *et al.* 2019). Notably, a strong positive correlation was observed between the total phenolic content and the ABTS test of both *O. robusta* and *O. ficus-indica*, as indicated in Table S3. On the other hand, a moderate positive correlation was identified between the total phenolic content and the ABTS assay of *O. dillenii*.

Regarding the phosphomolybdenum assay (TAC), *O. ficus-indica* demonstrated the highest level of antioxidant activity, while *O. robusta* exhibited moderate results. Conversely, *O. dillenii* recorded the lowest antioxidant capacity, hinting at a potential susceptibility to oxidative stress or an increased risk of oxidative damage. It is essential to note that all the studied species were collected from the same region and subjected to an identical extract preparation protocol, indicating uniform edaphoclimatic conditions. Consequently, the observed disparities among the species can be predominantly attributed to genetic differences. The TAC assay evaluates antioxidant capacity in both water-soluble and fat-soluble antioxidants, accounting for the distinctions between TAC and other examined antioxidant assays such as DPPH, FRAP, and ABTS. In terms of cladode age, *O. robusta* and *O. dillenii* exhibit higher antioxidant results in young cladodes, while *O. ficus-indi-*

ca demonstrates greater values in older cladodes as opposed to younger ones. Regarding the correlation between the studied species and total phenolic content, a strong positive correlation was evident in both young and old cladodes of *O. ficus-indica* (Supplemental Table 3). In contrast, *O. robusta* displayed a notably higher positive correlation only in its older cladodes, while *O. dillenii* showed a moderate positive correlation in both young and old cladodes.

Based on the results obtained, *O. robusta* emerges as the species with the highest concentration of phenolic compounds, closely followed by *O. dillenii*, while *O. ficus-indica* displays the lowest content of these compounds. It's crucial to highlight that all the studied species were procured from the same geographical location, indicating uniform environmental conditions. Consequently, the discerned differences in secondary metabolites cannot be ascribed to external factors such as abiotic or biotic influences but are primarily rooted in the inherent biochemical properties of the species under scrutiny.

The HPLC-DAD analysis unveiled the presence of two distinct flavonoids, with isorhamnetin constituting the major component and kaempferol as the minor constituent, a finding consistent with the reports of[32]. Acquiring these substances might serve as a means to boost the profitability of cactus pear production while generating employment opportunities and adding value to rural regions[33]. In the broader literature, other Opuntia species, including *O. megacantha*, *O. ficus-indica*, and *O. streptacantha*, have been documented to contain additional flavonoids such as isorhamnetin and kaempferol[7, 34, 35]. Moreover, Rocchetti *et al.*[36] has reported the presence of quercetin, rutin, and Zeghbib *et al.*[37] identified cyanidin, pelargonidin, catechin, myricetin, and apigenin in these species. These flavonoids, recognized as inherent regulators in plants, offer substantial health benefits, serving as protective agents against conditions such as cancer, cardiovascular diseases, inflammation, allergies, capillary fragility, and the aggregation of human platelets[7].

Isorhamnetin, a distinguished member of the flavonoid family and more precisely classified as a flavonol, constitutes approximately half of the identified phenolic compounds, as indicated in Table 1. The recorded results spanned from 972.58 to 6747.85 μg/g, surpassing the values reported in the literature for other species, which typically fall within the range of 1250 to 4140 μg/g[37]. Consequently, *O. robusta* can be rightfully recognized as particularly rich in isorhamnetin, especially in the older cladodes. Isorhamnetin possesses a wide spectrum of pharmacological effects, encompassing advantages for nerve health, cardiocerebrovascular function, neurodegenerative protection, antitumor properties, and significant impacts on obesity[38–40]. Given its multifaceted potential for clinical prevention and treatment, isorhamnetin emerges as a valuable candidate for further development and practical applications.

Chlorogenic acid stands out as the most abundant phenolic acid identified within the studied species, including *O. robusta*, *O. dillenii* and *O. ficus-indica*. Notably, *O. robusta* exhibited the highest concentration of chlorogenic acid. In contrast to other species in the literature, ferulic acid and caffeic acid have been reported as the predominant phenolic acids[35, 41]. Chlorogenic acid is renowned for its diverse therapeutic functions, contributing to a wide array of health benefits. It exhibits antimutagenic, antioxidant, antiviral, antibacterial, hepatoprotective properties, and also serves as a lipid-lowering, cardioprotective, antipyretic, neuroprotective, immunomodulatory, and anti-hypertensive substance[42, 43]. Consequently, phenolic acids, including chlorogenic acid, have garnered significant attention, particularly in light of the growing emphasis on natural substances and concerns regarding the use of chemicals within the food industry[44].

Sinapic acid is widely recognized for its versatile range of beneficial properties, which encompass antioxidant, antiglycemic, anti-inflammatory, neuroprotective, antimutagenic, antibacterial, and anticancer activities[45–47]. This valuable compound is also among the significant phenolic acids found in the cladodes of the species under investigation, with concentrations ranging from 985.33 to 2047.64 ug/g. Notably, all the studied species exhibited higher levels of sinapic acid in comparison to those reported for *O. ficus-indica*, which typically ranged from 40 to 470 μg/g[48], Furthermore, *O. robusta* demonstrated superior results in sinapic acid content when compared to *O. dillenii* and *O. ficus-indica*, emphasizing its potential richness in sinapic acid and underscoring the significance of this particular species.

5 Conclusion

The current study represents the exploration of phenolic compounds and antioxidant activity within the cladodes of *O. robusta*, *O. dillenii*, and *O. ficus-indica*. The insights garnered regarding phenolic compounds and their associated antioxidant capabilities are of considerable value in identifying species that hold promise for yielding compounds with nutritional and medicinal benefits. *O. robusta*, distinguished by its rich phenolic compound content and robust antioxidant properties, exhibits a noteworthy potential for promoting health.

It is crucial to note that each assay used in this study assesses diverse antioxidant capacities against specific free radical types. This underscores the need to perform multiple assays to precisely measure the antioxidant effectiveness. The disparities noted among the species under examination primarily stem from genetic factors.

In summary, the cladodes of these examined species,

particularly *O. robusta*, emerge as promising candidates for health-promoting foods. Nevertheless, further research is warranted to explore other essential constituents in these species, including fatty acids and minerals, which can contribute to a comprehensive understanding of their nutritional and medicinal potential.

Supplementary Materials

This segment comprises supplementary data and statistical assessments that offer a deeper and more comprehensive insight into the findings presented in the primary section, enhancing overall comprehension.

These materials are available free of charge via the Internet at doi: 10.5650/jos.ess24034

Author Contributions

Conceptualization, A.M., M.A. (Malika Abid) and M.A. (Mohamed Addi); methodology, A.M and Y.R.; software, A.A and A.T.; validation, M.A. (Malika Abid), and M.A. (Mohamed Addi); formal analysis, A.A. and M.B.; investigation, A.M.; resources, M.A. (Malika Abid); data curation, M.B.; writing-original draft preparation, A.M.; writing-review and editing, M.A. (Malika Abid) and M.A. (Mohamed Addi); visualization, A.B. and R.M.; supervision, M.A. (Malika Abid) and M.A. (Mohamed Addi); project administration, M.A. (Mohamed Addi); review and editing: A.E. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors affirm that they do not possess any conflicting interests.

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