Evaluation of Antioxidant Activity and Phenolic Composition of *Opuntia ficus-indica* Cladodes Collected from Moroccan Settat Region

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ABSTRACT
*Opuntia ficus-indica* cladode growing in Settat region of Morocco was freshly consumed and traditionally used in folk medicine for health benefits by the local people. The antioxidant activity and the chemical compounds of it were studied using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and spectrophotometric techniques. The antioxidant activity screened using three complimentary tests; namely, ABTS, DPPH and FRAP activities. In addition, total phenolic, flavonoid and tannins contents of the extracts were also determined as gallic acid, rutin and tannic acid equivalents, respectively. In the extracts, total phenolic content ranged between 73.1±2.1 and 111.2±5.8 mg/g gallic acid equivalents while flavonoid content between 22±2.0 and 27.0±4.0 mg/g rutin equivalents and tannin content between 5.93±0.07 and 6.17±0.06 mg/g tannic acid equivalents. As a conclusion, the results highlighted that cactus cladode is a source of antioxidant phenolic compounds. Thus, the extracts of cladodes will be probably used for the development of safe food products and/or additives.

Keywords: antioxidant activity, cactus extracts, LC-MS/MS, *opuntia ficus-indica*, phenolic compounds

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INTRODUCTION

Naturally occurring antioxidants can be used in functional food products to prevent oxidation [1]. Additionally, many studies have reported that steady consumption of antioxidants is beneficial to human body [2, 3]. In recent years, scientists focused on cladode and seeds of *Opuntia ficus-indica* which contains major phytochemicals in search of new natural antioxidants. Cactus (*Opuntia* spp.) belonging to Cactaceae family is native to Mexico. However, this plant is widely distributed in arid and semi-arid regions of Africa, Central America, Mediterranean region and South Africa. *Opuntia* genus has more than 200 species [4, 5]. Moreover, its important economic value, fast grows, less water requirement, and adaptation to nutrient-deficient soils made the cactus pear a prominently cultivated crop all over the world [6].

Studies have shown that *Opuntia* was rich in natural antioxidant compounds such as carbohydrates, flavonoids, mineral amino acids (arginine), phenolic acids (caffeic and gallic), polyphenols, quercetin, tannins, tocopherol, and sulfur amino acids (cysteine, methionine, and taurine) [7-15]. Flavonoids of cactus cladodes possessed many notable biological activities; such as inhibition of lipid peroxidation, oxidation of low density lipoproteins [16, 17], anti-inflammatory, anti-carcinogenic, anti-aggregatory, antioxidant, hypoglycemic and antiviral activities [8, 18, 19].

Since, antioxidants and radical scavengers especially polyphenolic compounds are considered as important nutraceuticals due to their promising activity against several diseases [9, 20, 21]. Regarding the chemical composition, there has been an increasing interest to explore the nutritional and pharmacological properties of the cactus cladodes. It should be taken into consideration that same species growing in various regions can have different phytochemical composition. Therefore, in the present study, it is aimed to study the phenolic composition along with the antioxidant activity of Cactus cladodes from Settat region of Morocco. The goal of this study was to investigate the total phenolic, flavonoid and tannin compounds using LC-MS/MS to identify and quantify phenolics of cactus cladodes. Furthermore, the antioxidant activity of the extracts was determined spectrophotometrically using free radical scavenging activity (DPPH assay), reducing antioxidant power (FRAP assay), and cation radical scavenging activity (ABTS assay).

**Materials and methods**

**Chemicals**

The chemical reagents namely Folin–Ciocalteu’s and 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis-3 [ethylbenzothiazoline-6-sulfonic acid] (ABTS), kaempferol (≥97%), 4-hydroxybenzoic acid (≥99%), chrysin (97%), vanillin (99%), rutin (94%), hesperetin (95%), fisetin (≥98%), rosmarinic acid (96%), quercetin (95%), formic acid (≤ 100%), gallic acid (≥99%), hyperoside (≥97%), (L)-malic acid (95-100%), naringenin (95%), quinic acid (98 %), p-coumaric acid (98%), protocatechuic acid (97%), myricetin (≥96%), salicylic acid (≥99%), tannic acid
(puris), coumarin (≥99%), tr-aconitic acid (98%), chlorogenic acid (95%), caffeic acid (98%), α-tocopherol (≥ 95.5%) and sodium carbonate (Na₂CO₃) were obtained from Sigma-Aldrich Corp. (Germany). Rhamnetin (≥99%), luteolin (≥97%), hesperidin (≥97%), Apigenin (≥99%), however, were purchased from R-Biopharm. All used solvents were of analytical grade.

**Plant material**

Nopal cladodes were collected from the experimental station of the regional center-INRA Settat Morocco. Nopal cladodes were washed with distilled water, dried at 50°C in an oven (XU980, France-Etuves, Chelles, France) and mechanically milled (SK 100, Retsch, France). The obtained powder was stored in a closed container at room temperature until further analyses.

**Preparation of Ethanol and acetone extracts from powder cladode**

Ethanol extract was prepared by the method of Radi et al. [22]. A fine dried powder (5 g) was extracted using 100 mL of ethanol /water mixture (70: 30 v/v) for 4 days at 4°C, and then filtered (Whatman No. 4). The solvents were evaporated at 40°C under reduced pressure (Rotary evaporator Buchi R-210). The residue was lyophilized (LABCONCO, Frezone-105°C 4.5 L. Cascade Benchtop Freeze Dry System) and stored at 4°C. The extraction yield was 19.8%.

Acetone extract was obtained by performing the procedure of Zhang et al. [23]. Fine dried powder (5 g) was extracted using 100 mL of acetone /water (70: 30 v/v) mixture with stirring at room temperature for 4 days at 4°C. The extract was filtered (Whatman No.4). The solvents were evaporated at 38°C under reduced pressure (Rotary evaporator Buchi R-210). The residue was lyophilized (LABCONCO, Frezone-105°C 4.5 L Cascade Benchtop Freeze Dry System) and stored at 4°C until further analyses. The extraction yield was 16.6%.

**Determination of total phenolic, flavonoid, and tannins contents**

**Determination of total phenolic content**

Total phenolic content of the extracts was performed by using the Folin-Ciocalteu reagent and spectrophotometer method of Singleton and Rossi [24] that was modified by Tel et al. [25]. An aliquot of the extract was mixed with distilled water (3.5 mL) and Folin-Ciocalteu reagent (0.25 mL). The mixture was shaken and allowed to stand for 6 minutes. After addition 0.5 mL of 7% Na₂CO₃ the final volume was completed to 5 mL. After 30 minutes of incubation, the absorbance was read at 725 nm. Total polyphenols contents of the extracts were expressed as milligrams of gallic acid equivalents per gram of lyophilized fraction. Three parallel measurements were performed for all samples. The results were given as mean value ± standard error meaning (SEM).

**Determination of flavonoid contents**

The total flavonoid content in the extracts was determined spectrophotometrically based on flavonoids-aluminum complexation at 430 nm [25]. Rutin was used standard. The results
of flavonoid content were given in milligrams per gram of rutin equivalent (mg RE/g lyophilized extract). Each couple was analyzed in triplicate.

**Determination of tannins contents**

The total tannin content in the extracts was carried out by modified method of Ganjiwale et al. [26]. The sample (0.1 mL) was mixed with Folin-Denis reagent (0.5 mL), Na₂CO₃ solution (0.5%, w/v, 1 mL) and distilled water (5 mL). The λₘₐₓ was measured at 755 nm within 30 min of reaction. The total tannin results were given as the equivalent to mg of tannic acid by g of extract (mg tannic acid/g lyophilized extract).

**Identification and quantification of phenolic compounds**

**LC-MS/MS Instrumentation and chromatographic conditions**

The Nexera UHPLC system attached to an LC-MS 8040 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan) was used to analyze the phenolic contents of the extracts. The system consists of a degasser (DGU-20A3R), binary pumps (LC-30AD), an auto sampler (SIL-30AC) and a column oven (CTO-10ASvp). The chromatographic separation was achieved using a C18 reversed-phase Inertsil ODS-4 (150 mm×4.6 mm, 3µm) column. The flow rate was adjusted to 0.5 mL/min while the injection volume was 4µL and the temperature was 40°C. The mobile phase A (water, 5 mmol/L ammonium formate, and 0.1% formic acid) and mobile phase B (methanol, 5mmol/L ammonium formate and 0.1% formic acid) were used as gradient elution. The gradient program was as follows: t (min), %B: (0: 40), (20:90), (23.99: 90), (24: 40), (29: 40).

The ionization of the mass spectrometer was ESI (ElectroSpray Ionization) source running in positive and negative ionization modes. MRM mode (multiple reaction monitoring) was used to process and to quantify the data. At least two or three transitions for each compound were obtained for analysis. The first transition was for quantitative purposes while the second and/or the third ones were for confirmation.

**Optimization of LC-MS/MS method**

After different combinations of trials, the gradient elution was used as water, 5 mmol/L ammonium formate and 0.1% formic acid (solvent A) and methanol,5 mmol/L ammonium formate and 0.1% formic acid (solvent B) in order to have rich ionization and molecule separation. ESI mode was chosen since the phenolic compounds were comparatively polar and small. In addition, liquid chromatography tandem mass spectrometry was applied through its stability of ion fragmentation [27, 28].

The working parameters of ESI were optimized as follows: interface temperature, 350°C; desolvation line (DL) temperature, 250°C; heat block temperature, 400°C; nebulizer gas flow (Nitrogen), 3L/min and drying gas flow (Nitrogen), 15 L/min.
In this study, twenty-four phenolic compounds (flavonoids, flavonoid glycosides, phenolic acids, phenolic aldehyde, coumarin) and three non-phenolic organic acids were qualified and quantified in cactus extracts. Table 1 shows the equations of rectilinear regression and the linearity studies of the standard compounds [28]. Correlation coefficients were all higher than 0.99 indicate good linearity. The limit of detection (LOD) and limit of quantitation (LOQ) of the reported analytical procedure are also given in Table 1. As can be seen from Table 1, LOD ranged between 0.05 and 25.8 µg/L and LOQ ranged between 0.17 and 85.9 µg/L for the studied compounds. Furthermore, the recovery of 27 target compounds ranged from 96.9 to 106.2%.

### Table 1. Analytical parameters of the UPLC-MS/MS method

| Analyte                  | LOD (µg/L)\(^a\) | LOQ (µg/L)\(^b\) | Recovery (%) | RSD\(\%\) | U\(^c\) |
|--------------------------|-------------------|------------------|--------------|-----------|--------|
| Quinic acid              | 22.3              | 74.5             | 103.3        | 0.0388    | 4.8    |
| Malic acid               | 19.2              | 64.1             | 101.4        | 0.1214    | 5.3    |
| tere-Aconitic acid       | 15.6              | 51.9             | 102.8        | 0.3908    | 4.9    |
| Gallic acid              | 4.8               | 15.9             | 102.3        | 0.4734    | 5.1    |
| Chlorogenic acid         | 7.3               | 24.3             | 99.7         | 0.1882    | 4.9    |
| Protocatechuic acid      | 25.8              | 85.9             | 100.2        | 0.5958    | 5.1    |
| Tannic acid              | 10.2              | 34.2             | 97.8         | 0.9075    | 5.1    |
| tere- caffeic acid       | 4.4               | 14.7             | 98.6         | 1.0080    | 5.2    |
| Vanillin                 | 10.1              | 33.7             | 99.2         | 0.4094    | 4.9    |
| p-Coumaric acid          | 15.2              | 50.8             | 98.4         | 1.1358    | 5.1    |
| Rosmarinic acid          | 10.4              | 34.8             | 101.7        | 0.5220    | 4.9    |
| Rutin                    | 17.0              | 56.6             | 102.2        | 0.8146    | 5.0    |
| Hesperidin               | 21.6              | 71.9             | 100.2        | 0.1363    | 4.9    |
| Hyperoside               | 12.4              | 41.4             | 98.5         | 0.2135    | 4.9    |
| 4-OH Benzoic acid        | 3.0               | 10.0             | 106.2        | 1.4013    | 5.2    |
| Salicylic acid           | 4.0               | 13.3             | 106.2        | 0.6619    | 5.0    |
| Myricetin                | 9.9               | 32.9             | 106.0        | 2.8247    | 5.9    |
| Fisetin                  | 10.7              | 35.6             | 96.9         | 2.4262    | 5.5    |
| Coumarin                 | 9.1               | 30.4             | 104.4        | 0.4203    | 4.9    |
| Quercetin                | 2.0               | 6.8              | 98.9         | 4.3149    | 7.1    |
| Naringenin               | 2.6               | 8.8              | 97.0         | 2.0200    | 5.5    |
| Hesperetin               | 3.3               | 11.0             | 102.4        | 1.0164    | 5.3    |
| Luteolin                 | 5.8               | 19.4             | 105.4        | 3.9487    | 6.9    |
| Kaempferol               | 2.0               | 6.6              | 99.1         | 0.5885    | 5.2    |
| Apigenin                 | 0.1               | 0.3              | 98.9         | 0.6782    | 5.3    |
| Rhamnetin                | 0.2               | 0.7              | 100.8        | 2.5678    | 6.1    |
| Chrysin                  | 0.05              | 0.17             | 102.2        | 1.5530    | 5.3    |

\(^a\)LOD(µg/L): Limit of detection
\(^b\)LOQ (µg/L): Limit of quantification
\(^c\)U (%): Percent relative uncertainty at 95% confidence level (k=2).
Estimation of uncertainty and Identification of uncertainty sources

Evaluation and quantification of the uncertainty sources of the applied method were achieved by using equation (1) according to the procedure of Ertaş et al. [28].

\[ u(C) = \sqrt{u^2(cal) + u^2(pur) + u^2(Css) + u^2(w) + u^2(rep) + u^2(rec)} \]  

(1)

where \( rec \) is the recovery, \( Css \) is the stock solutions, \( rep \) is the repeatability, \( cal \) is the calibration curves, \( w \) is the sample weights and \( pur \) is the purity of reference standards.

The main sources of uncertainty were the calibration curve and the purity of standards. The standard combined uncertainties were multiplied by two in order to express expanded uncertainties within 95% confidence interval \((k:2)\). The results of uncertainties are shown in Table 1.

Antioxidant activity analysis

ABTS assay

The ABTS assays of samples were performed as described elsewhere [29, 30]. The ABTS cation radical scavenging activity of samples was expressed as TEAC values (Trolox equivalent antioxidant capacity) as a function of the concentration of Trolox and antioxidants.

DPPH assay

The DPPH free radical scavenging activity of the extracts was determined spectrophotometrically [29, 30]. However, the DPPH free radical scavenging activity of samples was expressed as TEAC values as a function of the concentration of Trolox and antioxidants.

FRAP assay

The ferric ions (Fe\(^{3+}\)) reducing antioxidant power (FRAP) method was performed as described by Oyaizu [31] with a slight modification, which covers the presence of extracts to reduce the ferricyanide complex to the ferrous form. The results were expressed as ascorbic acid equivalents (mg Asco/g lyophilized extract).

Statistical methods

All analyses were performed in triplicates and the results were statistically analyzed. Results are presented as means ± SEM. The obtained data were analyzed using the Student’s \( t \)-test \((p < 0.05)\).

RESULTS AND DISCUSSION

Total phenolic, flavonoid and tannin content

It is well known that the antioxidant action is related to the amount of phenolic compounds [32]. Polyphenols are a group of secondary metabolites which were considered as
the most bioactive compounds [33]. Therefore, it is important to determine and quantify the phenolic content of plant extracts.

The phenolic, flavonoid and tannin content of ethanol and acetone extracts of cladodes were summarized in Figure 1. In ethanol extract, the total phenolic, flavonoid and tannin contents represent 111.2±5.8 mg gallic acid, 27.0±4.0 mg rutin and 5.93±0.07 mg tannic acid/g of lyophilized extract, respectively. While in acetone extract, 1 mg of lyophilized cladode extract provided 73.1±2.1 μg of gallic acid, 22±2.0 μg of rutin and 6.17±0.06 μg of tannic acid.

The extraction efficacy and the chemical complexity of extracts can be influenced by the type of solvent. In fact, extracts obtained by various solvents can behave differently [34]. There are several studies concerning the extraction of cactus cladodes; for example, with acetonitrile, acetone, ethyl acetate, dichloromethane, hexane, methanol and water. The previous studies show that most of the phenolic compounds were in the ethanol extract while tannins were mainly found in acetone extract, which were similar to the results in this study. Alimi et al. [35] reported 57.65 mg of polyphenol per g of methanol extract of roots of *Opuntia ficus-indica* f. *inermis*. According to Medina-Torres et al. [34], the total flavonoid content in Nopal cactus (*O. ficus-indica*) cladodes ranged from 10.80 ± 0.33 to 23.40±1.83 mg g⁻¹. On the contrary, our results indicate different amounts as 5.4 mg flavonoids per gram of dry sample in ethanol extract and 3.7 mg in acetone extract. However, it is difficult to ascertain the amount of polyphenols because there is a diversity of factors involved such as environmental factors, difference of species, the development stage (maturity) of the Nopal at the time of collection and the extraction and analytical methods undergone before proximate analysis [19, 33].
The next step was to identify and quantify the phenolic compounds of ethanol and acetone extracts of *Opuntia ficus-indica*. Determinations of phenolic compounds were performed by the LC-ESI-MS/MS instrument for 24 phenolic compounds (coumarin, flavonoids, flavonoid glycosides, phenolic acids, phenolic aldehyde) and 3 non-phenolic organic acids (*Table 1*).

The results show that cactus extracts presented a chemical profile composed of 18 phenolic compounds, including, rutin, quercetin, caffeic acid, tannic acid, hesperidin, hyperoside, malic acid, *trans*-aconitic acid and quinic acid (*Table 2*).

The HPLC chromatogram of all standards, ethanol and acetone extracts were given in *Figure 2*. To the best of our knowledge, the malic acid and quinic acid were determined as the major phenolic acids. Regarding phenolic acids, the results showed that cactus extract contains a high amount of malic acid which is in agreement with the results of [19, 37]. They also reported that cactus cladodes are featured by high amounts of malic acid vibrating due to a CAM-based diurnal rhythm. Further, Guevara-Figueroa et al. [38] determined gallic acid, *p*-

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**Table 2.** Identification and quantification of phenolic compounds of cactus extracts by UPLC-MS/MS

| Analyte          | Parent ion(m/z) | MS²(CE) | Quantification (µg analyte / g extract) | Analyte ion(m/z) | MS²(CE) | Quantification (µg analyte / g extract) |
|------------------|----------------|---------|----------------------------------------|------------------|---------|----------------------------------------|
| Quinic acid      | 190,95         | 85 (22),93 (22) | 1770.83±84.9 | Ethanol extract | Acetone extract |
| Malic acid       | 133,05         | 115 (14),71 (17) | 44217.0±2343.5 | 4369.69±209.7 |
| *tr*-Aconitic acid | 172,85       | 85 (12),129 (9) | 88.63±4.3 | 77.15±3.7 |
| Gallic acid      | 169,05         | 125 (14),79 (25) | D.      | D. |
| Protocatechuic acid | 152,95   | 109 (16),108 (26) | D.      | D. |
| Tannic acid      | 182,95         | 124 (22),78 (34) | D.      | D. |
| *tr*-Caffeic acid | 178,95         | 135 (15),134 (24),89 (31) | D.      | D. |
| Vanilin          | 151,05         | 136 (17),92 (21) | D.      | D. |
| *p*-Coumaric acid | 162,95        | 119 (15),93 (31) | D.      | D. |
| Rutin            | 609,1          | 300 (37),271 (51),301 (38) | 110.98±5.5 | 110.5±5.5 |
| Hesperidin       | 611,1          | 303 (24),465 (12) | 161.77±7.9 | 174.59±8.5 |
| Hyperoside       | 463,1          | 300 (27),301 (26) | 53.97±2.6 | 62.09±3.1 |
| Quercetin        | 300,9          | 179 (19),151 (21),121 (28) | 6.98±0.5 | 6.81±0.5 |
| Naringenin       | 270,95         | 151 (18),119 (24),107 (26) | D.      | D. |
| Kaempferol       | 284,95         | 217 (29),133 (32),151 (23) | D.      | D. |
| Apigenin         | 268,95         | 151 (25),117 (35) | D.      | D. |
| Rhamnetin        | 314,95         | 165 (23),121 (28),300 (22) | D.      | D. |
| Chrysin          | 253            | 143 (29),119 (32),107 (26) | D.      | D. |

aParent ion (m/z): Molecular ions of the standard compounds (mass to charge ratio)
bMS²(CE): MRM fragments for the related molecular ions (CE refers to related collision energies of the fragment ions)
cValues in µg/g (w/w) of plant extract
dD: peak observed, concentration is lower than the LOQ but higher than the LOD

**Identification and quantification of phenolic compounds by LC-MS/MS**

The next step was to identify and quantify the phenolic compounds of ethanol and acetone extracts of *Opuntia ficus-indica*. Determinations of phenolic compounds were performed by the LC-ESI-MS/MS instrument for 24 phenolic compounds (coumarin, flavonoids, flavonoid glycosides, phenolic acids, phenolic aldehyde) and 3 non-phenolic organic acids (*Table 1*).

The results show that cactus extracts presented a chemical profile composed of 18 phenolic compounds, including, rutin, quercetin, caffeic acid, tannic acid, hesperidin, hyperoside, malic acid, *trans*-aconitic acid and quinic acid (*Table 2*).
coumaric acid, protocatechuic as phenolic acids in cactus *Opuntia ficus-indica* cladodes which are comparable to our findings.

As shown in Table 2 the flavonoids contained in cactus cladodes are rutin, quercetin and kaempferol which were in agreement with the results described by Guevara-Figueroa et al. [38] and Stintzing and Carle [19] in *Opuntia ficus-indica* cladodes. *Opuntia ficus-indica* cladodes in Italy and Tunis, were studied by Ginestra et al. [39] and Yeddes et al.[40]. In these studies, cactus cladodes contain quercetin and kaempferol.

The quercetin prevents the generation of the chelates iron, superoxide ion, and suppresses the formation of lipid peroxyl radical [41]. Cho et al. [42] reported that quercetin and its derivatives act significant roles in the antioxidant effects of the cactus. In a parallel investigation, the presence of quercetin in cladodes from *Opuntia ficus-indica* var. *saboten* have been shown to have an antioxidant effect and was reported to be an effective radical scavenger against the neuronal cell damage caused by hydrogen peroxide and xanthin/xanthin oxidase [41].

**Antioxidant assay**

ABTS, DPPH and FRAP assays results are given in Figure 3. The results showed that the ethanol extract have higher antioxidant activity than the acetone extract. These methods presented high positive correlation (p<0.05) of DPPH / ABTS (R= 0.89), ABTS / FRAP (R= 0.90) and DPPH / FRAP (R= 0.79). This means that the used analytical methods presented a very similar response and can be used without distinction to quantify antioxidants activity in this part of cactus. These results are comparable to the earlier report [44].
The correlation between ABTS, DPPH and FRAP methods and the total polyphenol, flavonoid and tannin concentration are presented in Figure 3. A positive correlation \((p<0.05)\) was detected between the mean of antioxidant activity by the ABTS, DPPH and FRAP methods and the total polyphenol concentration. The correlation with the level of flavonoid and tannin cannot explain the antioxidant effect of cactus. Therefore, other phenolic and / or non-phenol compound probably causes this effect. All these results explain the high potential antioxidant of ethanol extract that is rich in polyphenols compared to the acetone extract rich in tannins which showed low antioxidant potential. In fact, it has been shown that polyphenol has a reduced ability to donate hydrogen and could be used for the free radical scavenging activity [20].

CONCLUSION

The results presented in this study are the first information on the phenolic profile and antioxidant activity *Opuntia ficus-indica* cladode growing in Settat, Morocco. *Opuntia ficus-indica* cladodes are a precious source of bioactive substances which can be a part of daily human diet. The tested *Opuntia ficus-indica* cladode extracts were shown to be having high phenolic content and potent antioxidant activities achieved by ABTS, DPPH and FRAP assays. The results clearly demonstrated that ethanol extract was rich in polyphenols while the acetone extract was rich in tannins. It can be concluded that the determined contents are found at different amounts due to preferred extraction solvent. Furthermore, the results presented herein support scientific knowledge regarding phenolic compounds can conduce to the antioxidant activities of *Opuntia ficus-indica* cladode. Genetics and environmental conditions are the anticipated factors which can influence the variation of antioxidant capacity by
affecting the presence of different phenolic compounds. In conclusion, Moroccan Opuntia ficus-indica cladode might be used directly in health promoting diets as a readily attainable source of natural antioxidant agents.

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