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ORIGINAL PAPER

Variation in bioactive compounds and antiradical activity of *Moringa oleifera* leaves: influence of climatic factors, tree age, and soil parameters

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Abstract *Moringa oleifera* Lam. (Moringa) leaves are a significant source of phytochemicals with different bioactivities carrying human health benefits. This study was undertaken to investigate the effects of the tree age, soil physical and chemical parameters, and climatic factors, on the content of gallic acid, total phenolics, total carotenoids, and ascorbic acid and on the antiradical activity (DPPH and ABTS assays) of ethanolic extracts obtained from Moringa freeze-dried leaves. Multivariate data analysis showed that the bioactive compounds measured as reference and the antiradical activity from Moringa leaves presented correlation with climatic factors (precipitation, humidity, and radiation) and with soil nutrients, principally with the K and P contents. Tree age was positively correlated with the total carotenoids contents, and inversely correlated with the ascorbic acid contents. This information offers an understanding on variations in bioactive compounds and antiradical activity in Moringa leaves influenced by climatic factors, soil, and tree age, which may help in the estimation of the antioxidant potential present in the plants during different harvest times.

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Keywords Climatic factors · Soil parameters · Total carotenoids · Ascorbic acid · Total phenolics · Antiradical activity · *Moringa oleifera* leaves

Abbreviations

TPC	Total phenolic content
COLPOS	Colegio de Postgraduados
DPPH	2,2-diphenyl-1-picrylhydrazyl
ABTS	2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
CEC	Cation exchange capacity
EC	Electrical conductivity
OM	Organic matter
PCA	Principal component analysis
PC	Principal components
TE	Trolox equivalent
GAE	Gallic acid equivalent
db	Dry basis

Introduction

Moringa oleifera Lam. (Moringa) is considered one of the most useful trees in the world, because every part of the Moringa tree can be used for food, medication, or industrial purposes. Leaves can be eaten fresh, cooked, or stored as dried powder for many months without refrigeration, and reportedly without loss of nutritional value [1, 2]. Different activities, such as antispasmodic, anti-inflammatory [3], antiulcer [4], hypocholesterolaemiant [5, 6], antihypertensive [7], hypolipidemic, antiatherosclerotic [8], hypoglycemic [9], antioxidant [10–12], and antidiabetic [13], deriving from different bioactive compounds present in Moringa leaves, have been identified. Moringa leaves are also a significant source of phenolics compounds, β-carotene,

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and ascorbic acid [8, 9, 14–19]. These phytochemicals are important factors to consider Moringa leaves quality, since they are responsible for its antiradical activity and benefits on human health. In particular, some researchers have claimed that Moringa leaves are rich in chlorogenic acid, gallic acid, kaempferol, and quercetin glycosides [1, 2, 12, 20].

Plants contain a wide variety of bioactive compounds, typically these compounds are produced as secondary metabolites, which are a group of compounds other than primary metabolites, believed to help plant to increase their overall ability to survive and overcome local challenges by allowing them to interact with their surroundings [21]. The relative abundance of these phytochemicals determines the contribution of specific plants to prevent deficiencies, improve health, or prevent diseases. However, different studies have shown that the chemical constituents in plants are influenced by the plant species, plant age, harvest seasons, and by other factors that affect tree development, such as soil nutrients, geographical location, and climate conditions [22–27]. Considering the great variations among bioactive compounds and huge number of plant species, it is necessary to develop an integrated approach to screen out these compounds carrying human health benefits.

In this term, Siddhuraju and Becker [28] studied the radical scavenging capacities and antioxidant activities of Moringa freeze-dried leaves extracts from three agroclimatic regions (India, Nicaragua, and Niger). The results shown that the highest polyphenol extraction yield corresponds to a higher antioxidant activity. However, there are not specific data describing the effect of the climatic or other agronomic factors on the antioxidant potential of Moringa leaves. Iqbal and Bhanger [29] reported significant variations in antioxidant activity of methanolic Moringa leaf extracts as function of harvesting seasons and agroclimatic conditions from five locations of Pakistan. In general, an inverse relationship between the environmental temperature and the total phenolic content (TPC) and the antioxidant activity was observed. Nevertheless, the effect of soil properties over these parameters was not investigated, nor was the range of temperature referred to as cold or hot. Jongrungruangchok et al. [18] compared the proximate composition and mineral constituents in Moringa leaves from 11 different agroclimatic regions distributed in Thailand. However, despite having 11 samples from different regions, the authors did not discuss the reasons for the variations in the values of the nutritional profile.

In another study, the major phytochemicals (glucosinolates, phenolics, and flavonoids) and nutrients (total protein, crude fat, fatty acids, and minerals) were estimated for different tissues of Moringa (Root, seed, stem, petiole, and leaf) grown in Ghana, at two distinct developmental stages: vegetative plants and post-flowering/pod production plants [30]. In both developmental stages, the authors report that the leaves had the highest and most complex flavonoid contents. While that phenolics and flavonoids were not detected in roots and seeds. Furthermore, the highest levels of bioactive compounds were detected in the leaves of flowering plants. When these authors compared their results with other reports, they found differences in the bioactive compounds contents, explaining that they might be caused by differences in cultivation and harvesting conditions, but they were not specific about the source of these variations.

Shih et al. [10] described the effect of different parts (leaf, stem, and stalk) and seasons (July 2004 and January 2005) on the chemical compositions and antioxidant activity of Moringa trees grown in Taiwan. Their results showed that the samples collected in January had higher ash content (except the stalk part), calcium and phenolic compounds (except the leaf part), and stronger antioxidant activity than July samples. In both seasons, the leaves expressed the highest antioxidant activity, followed by the petiole and stem. More recently, Ndhlala et al. [31] conducted a study to assess variation in antioxidant, antimicrobial, and phytochemical properties of thirteen Moringa cultivars obtained from different locations: Thailand, Taiwan, South Africa, and United States of America. All accessions were cultivated at the Agricultural Research Council (ARC) experimental farm, Roodeplaat, Pretoria. In general, cultivars from Thailand exhibited the highest amounts of total phenolic compounds and highest values of antioxidant activity. The variations in the obtained data by Ndhlala et al. [31] may suggest differences in the ability of each provenance in establishing themselves in the new environment. However, these authors did not provide information about agroclimatic conditions; therefore, we cannot infer how these factors affected the plants.

While the content of bioactive compounds in plants varies widely, it can be generalized that Moringa leaves are an important source of them. If we add to this fact that the use of herbal medicines, as a therapeutic alternative of natural origin, has received important consideration worldwide [32], we can state that the Moringa tree represents a very attractive option to be cultivated and widely exploited in tropical and subtropical regions. Given the lack of specific information correlating the agroclimatic conditions with the variations over the bioactive compounds content in Moringa leaves (Table 1), it is necessary to understand how these factors affect their phytochemical profile, providing the knowledge to determine the agronomic conditions that may decrease or increase the performance of these bioactive compounds in Moringa leaves. This study contributes to learn the effects of the tree age, soil physical and chemical parameters, and climatic factors, on the content of total phenolics, total carotenoids, and ascorbic acid and on the antiradical activity of ethanolic extracts obtained from

Table 1 Some works on *Moringa oleifera*, where variations in the bioactive compounds content and other response variables are discussed

Response variables	Evaluated tree parts	Tree origin	Harvest seasons	Developmental stages of tree	Evaluated soil parameters	Evaluated climatic factors	References
Ascorbic acid, total phenolics, total flavonoids, separation of flavonoids by HPLC and antioxidant activity determination by different methods	Leaves	India, Nicaragua and Niger	December 2000	NR	NR	NR	[28]
Total phenolics, total flavonoids, ascorbic acid, reducing power and antioxidant activity determination by different methods	Leaves	Five different provinces in Pakistan	December, March, June, and September (2001 to 2003)	NR	NR	Environmental temperature: coldest and hottest temperatures	[29]
Total phenolics, total flavonoids, total carotenoids, ascorbic acid, tocopherol, antiradical activity and determination of enzymatic antioxidants by different methods	Leaves	India	NR	Mature and tender leaves	NR	NR	[33]
Separation, quantification and identification of glucosinolates, phenolic, flavonoids and various other classes of phytochemicals. Nutrients content: total protein, crude fat, fatty acids and minerals	Root, seed, stem, petiole and leaf	Kumasi, Ghana	Seeds were transplanted in May 2004	Vegetative plants (100 days old) and post-flowering/pod production plants (320 and 380 days old)	NR	Climate is described, but not in relation with phenolics and flavonoids contents	[30]
Proximate composition (moisture, ash, crude fat, crude protein and crude fiber) and mineral constituents (calcium, potassium, iron)	Leaves	11 Different provinces in Thailand	NR	NR	NR	NR	[18]

Table 1 (continued)

Response variables	Evaluated tree parts	Tree origin	Harvest seasons	Developmental stages of tree	Evaluated soil parameters	Evaluated climatic factors	References
Total phenolics, ascorbic acid, proximate composition (crude protein, crude fat, ash and calcium) and antioxidant activity	Leaf, stem and stalk	Taichung, Taiwan	July 2004 and January 2005	NR	NR	NR	[10]
Total phenolics, total flavonoids, antibacterial activity, antifungal activity and antioxidant activity determination by different methods	Leaves	13 Germplasms collected from different locations	NR	NR	NR	All the provenances received the same management practices	[31]
Content of total phenolics, total carotenoids and ascorbic acid; and antiradical activity determination by DPPH and ABTS assays	Leaves	Veracruz, Mexico	June, July and September 2014; January and May 2015	Different samples: 276 to 949 days old	pH, CEC[a], EC[b], K, P, NH_4^+, organic matter, clay, silt, sand	Mean temperature, precipitation, relative humidity, solar radiation, UV radiation	Present work

NR not reported

[a]Cation exchange capacity

[b]Electrical conductivity

Moringa freeze-dried leaves. Gallic acid was also evaluated, because it represents one of the major phenolic components in Moringa leaves.

Materials and methods

Plant material and chemicals

Moringa fresh leaves were collected from El Colegio de Postgraduados (COLPOS), located in the center of the state of Veracruz, at the Mexico's eastern coast (view the geographic coordinates in Table 2) at different harvesting times: June, July, and September 2014; January and May 2015. The leaves were collected from three plots (A, B and C), each one in different initial ages: 608, 458 and 276 days, respectively (June-14). Mature Moringa leaves were hand harvested randomly from middle branches of Moringa tree, as in Sreelatha and Padma [33]. The leaves were washed with distilled water to remove the dirt and the water in excess was removed. After this, the leaves were stored at −32 °C and were processed in less than 48 h. All Moringa plots were subjected to identical cultural practices and environmental conditions to minimize the influence of pre- and post-harvest factors.

2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate, Folin–Ciocalteu reagent, 2,6-dichloroindophenol sodium, metaphosphoric acid, ascorbic acid, trolox, and gallic acid were purchased from Sigma Chemicals, Mexico. Sodium carbonate, sodium bicarbonate, hexane, acetone, methanol, and glacial acetic acid were purchased from JT Baker, Mexico. Magnesium oxide and ethanol were purchased from Golden Bell Reagents, Mexico. Celite was purchased from LABESSA, Mexico. All the solvents used in this study were of analytical grade, except methanol, and glacial acetic acid, which were grade HPLC.

Moringa leaves sample preparation

Solid–liquid extraction is a well-known separation process, which yield can be increased with a previous dehydration stage and a reduction of particle size; moreover in the particular case of bioactive compound extraction from natural sources, the drying process avoids the interference of water in the polyphenols release [34]. For these reasons, the Moringa leaves were freeze-dried at −45 °C and 4.5 Pa using a laboratory freeze-dryer (LABCONCO, FreeZone Mod. 7740060) by 18 h; this drying method was selected to avoid degradation of bioactive compounds by high temperatures. The dried leaves were ground using a coffee grinder (KRUPS, Mod. GX410011), and subsequently, they were sieved to achieve a standard particle size <0.59 mm. The obtained samples were stored at −32 °C until further analyses.

Ultrasound-assisted extraction

For DPPH, ABTS, Folin–Ciocalteu assays, and quantification of gallic acid, a solid–liquid extraction was carried out. The freeze-dried leaf samples were extracted with 80% (w/w) ethanol in water. The solvent was chosen according to the results obtained by Sultana et al. [35], whom found higher yield, phenolic content, and antiradical activity when aqueous organic solvents (80% methanol, 80% ethanol) were used. Methanol was discarded, because it is a toxic solvent for humans and represents a drastic environmental hazard [36]. Ethanolic extracts from Moringa leaves were obtained using a 0.01 (g sample)/(mL solvent) ratio and 30 min of sonication (ultrasound power: 100 W, frequency: 45 kHz). These operation conditions were established in a previous work carried out in our laboratory, where extraction kinetics at different (g sample)/(mL solvent) ratios were evaluated to determinate the time needed to reach the equilibrium between phases and the (g sample)/(mL solvent) ratio needed to obtain the highest yields of solids, TPC, and antiradical activity (DPPH and ABTS

Table 2 Climatic data and harvests on Moringa production site under investigation

Harvest season		June-14	July-14	September-14	January-15	May-15
Average daily temperature (ºC)		26.71	26.83	26.81	21.52	27.41
Total cumulative precipitation (mm)		41.20	897.00	964.80	13.80	25.80
Average daily relative humidity (% HR)		79.96	87.42	86.54	84.92	80.64
Average daily solar radiation (kWh/m^2 day)		6.07	5.54	5.39	2.70	5.04
Average daily UV radiation (Wh/m^2 day)		3.68	4.54	4.62	0.99	6.64
Production site	Altitude	30 m.a.s.l				
	Latitude	19°11′46.19″N				
	Longitude	96°20′17.50″O				

The variables values showed in the table represent the average daily or accumulated of 30 days before of each harvest

assays). The water in the ultrasonic bath was kept at level with solvent surface in the Erlenmeyer flasks, and it was regulated at 25 °C to avoid a rise in temperature caused by the ultrasonics. After the extraction, the extracts were filtrated with Whatman No. 4. All the extracts were kept at −32 °C until further analyses. All experiments were prepared in duplicate.

Antiradical activity determination by DPPH assay

The antioxidant activity of Moringa freeze-dried leaf extracts on DPPH radicals was measured in terms of hydrogen-donating or radical scavenging ability according to the method described previously by Siddhuraju and Becker [28]. 0.1 mL of sample extract was added to 3.9 mL of DPPH methanolic solution (0.025 g/L). The sample was incubated for 90 min at 25 °C. The decrease in the absorbance at 517 nm was determined with a UV–visible spectrophotometer (Thermo Scientific, Mod. Genesys 10 S). The reference solution was prepared with 0.1 mL of the extraction solvent and 3.9 mL of DPPH solution. The antiradical activity (*AAR*) in percentage was calculated with Eq. (1):

$$\mathrm{AAR}(\%) = \frac{A_\mathrm{R} - A_\mathrm{S}}{A_\mathrm{R}} 100, \tag{1}$$

where A_s is the absorbance of the samples and A_R is the absorbance of the reference. The results were expressed as trolox equivalents (TE) in grams per 100 g of dry base (g TE/100 g db). The calibration curve was made with a stock solution of trolox (1 mg/mL) with the extraction solvent, which was diluted to give 0.2, 0.1, 0.05, 0.025, and 0.01 mg/mL.

Antiradical activity determination by ABTS assay

The ABTS radical scavenging activity of the Moringa freeze-dried leaf extract was determined according to the previously described procedure by Re et al. [37]. ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at 25 °C for 16 h before use. This solution was diluted with ethanol to generate an absorbance of 0.70 ± 0.05 at 734 nm, and the same solution was used for the assay. The final reaction mixture (1 mL) comprised 980 µL of the ABTS dilute solution and 20 µL of the sample (control or extracts). This reaction mixture was vortexed for 10 s, and after 7 min, the absorbance was recorded at 734 nm using a UV–visible spectrophotometer (Thermo Scientific, Mod. Genesys 10 S) and compared with the control ABTS solution. The *AAR* in percentage was calculated with Eq. (1). The results were expressed as trolox equivalents in grams per 100 g of dry base (g TE/100 g db). The calibration curve was made with a stock solution of trolox (1 mg/mL) with the extraction solvent, which was diluted to give 0.2, 0.1, 0.05, 0.025, and 0.01 mg/mL.

Total phenolics content (TPC) determination

TPC was measured by the Folin–Ciocalteu method reported by Chumark et al. [8]. 100 µL of extract was transferred to a 10 mL volumetric flask and 6 mL of water, and 0.5 mL of Folin–Ciocalteu's phenol reagent were added and mixed. After 5 min, 1.5 mL of sodium carbonate solution (20%) was added and mixed again. The volume was made up to 10 mL with water and mixed thoroughly. The solution was kept for 2 h at 25 °C, and the absorbance was registered at 760 nm. TPC was expressed as gallic acid equivalents in grams per 100 g of dry base (g GAE/100 g db). The calibration curve was made with a stock solution of gallic acid (1 mg/mL) with the extraction solvent, which was diluted to give 0.8, 0.4, 0.2, 0.1, 0.05, and 0.025 mg/mL.

Gallic acid quantification by HPLC

The presence and amount of gallic acid were carried out using an HPLC equip (Varian ProStar, Mod. 240) coupled with a UV–vis detector (Waters, Mod. 2487). The separation was carried out on a C18 reversed phase column (150 mm × 4.6 mm, particle size 5 µm) at 35 °C. The mobile phase consisted of water with 1% acetic acid (solvent A) and methanol (solvent B). The flow rate was kept at 1.3 mL/min using isocratic elution (60% solvent A and 40% solvent B) over 25 min. Samples were filtered through a 0.22 µm membrane filter before injection. The injection volume was 20 µL, and peaks were monitored at 260 nm. Results (mg/g db) were obtained by comparison of peak areas of the samples with that of standard (purity: 98%).

Measurement of total carotenoids

The extraction, separation, and quantification of total carotenoids in the samples were performed according to the AOAC official method 941.15 [38] for dried plants, with slight modifications based on the works of Ranganna [39] and Rodríguez [40]. The techniques are based on the most carotenoids which exhibit absorption in the visible region of the spectrum between 400 and 500 nm and obey the law of Lambert–Beer. 0.5 g db of sample was extracted using 20 mL of acetone/hexane (30:70, v/v). All extracts were sonicated by 5 min with an ultrasound power of 100 W and frequency of 45 kHz (Westprime Systems; Cat. No. B90-055H). Washes were performed in a separatory funnel with water, and supernatant was collected. Supernatants were brought to 20 mL with hexane. Finally, carotenoid

separation was realized by open-column chromatography. The column was prepared according to Ranganna [39]: 3 cm^3 of 1:2 (w/w) magnesium oxide:Celite, 1 cm^3 of 1:4 (w/w) magnesium oxide:Celite and 1 cm^3 of anhydrous sodium sulfate on top. The mixture of carotenoids moves off the column prior to all other pigments and only this portion should be collected, avoiding contamination from the other bands. The absorbance of each main portion collected was measured at 450 nm using a UV–vis spectrophotometer (Thermo Scientific, Mod. Genesys 10 S). Total carotenoids were calculated using Eq. (2), and the results were expressed as milligrams per 100 g of dry base (mg/100 g db):

$$\text{Total carotenoids } (\mu g/gdb) = \frac{A_S V \cdot 1000}{AG}, \quad (2)$$

where A_s is the absorbance of the samples at 450 nm, V is the total volume of extract (mL), A is the absorption coefficient for a mixture of carotenoids (2500), and G is sample weight (g db).

Ascorbic acid content determination

Ascorbic acid determination was carried out following the AOAC official method 967.21 [38] (indophenol titration method). 1 g of samples was homogenized in 35 mL of metaphosphoric acid solution (15 g of metaphosphoric acid and 40 mL of acetic acid brought to 500 mL with distilled water) and was sonicated by 5 min with an ultrasound power of 100 W and 45 kHz (Westprime Systems; Cat. No. B90-055H). 7 mL of each sample were titrated against 2,6-dichlorophenol–indophenol solution in the presence of $NaHCO_3$ (50 mg of 2,6-dichlorophenol–indophenol and 42 mg of $NaHCO_3$ brought to 200 mL with distilled water) to a pink end point. The crude extract samples for analysis were taken according to the AOAC official method cited up. Data were compared with a standard solution of ascorbic acid (1 mg/mL), and the results were expressed as milligrams per 100 g of dry base (mg/100 g db).

Meteorological data collection

Climatic factors (mean temperature, relative humidity, precipitation, solar radiation, and UV radiation) were recorded from a Davis-Vantage Pro-2 equipment located inside the COLPOS Campus. Average data of 30 days before each harvest were registered, because this period of time considers the complete maduration of the Moringa leaves and the climatic factors may show effect on their development.

Soil analysis

Five samples of soil in each experimental cultivar were collected, following a zigzag pattern. At a distance of 30 cm from the stem of the tree, it was excavated to a depth of 15 cm and samples of 0.5 kg were taken at each sampling point. The corresponding samples were pooled, homogenized, and divided to produce three representative soil samples of 1 kg, designated as A, B, and C. The soil composition analyses were carried out on September 2014, using standardized procedures of the Laboratory of Soil, Plants and Water Analysis; COLPOS, Campus Montecillo, Mexico, Mex. For each soil sample, it was analyzed: pH, cation exchange capacity (CEC), electrical conductivity (EC), the content of organic matter (OM), and the levels of K, P, and NH_4^+. The clay, silt and sand content were also assessed. The evaluated variables and analytical methods are presented in Table 3.

Statistical analysis

The exploratory analysis of the data using principal component analysis (PCA) was performed with the aid of Minitab software version 14 (Minitab Inc., State College, PA,

Table 3 Evaluated variables in three different soil types used in this study and their analytical methods

Evaluated variable	Analytical methods	Measurement
pH	1:2 Soil:water relations	Potentiometer
Cation exchange capacity (CEC)	AS-12 with ammonium acetate	Titration with HCl
Electrical conductivity (EC)	Electrolytic measuring	Conductimeter
K	AS-12 with ammonium acetate	Flame photometer
P	Olsen	Spectrophotometer (882 nm)
NH_4^+	Micro-Kjeldahl	Titration with H_2SO_4
Organic matter (OM)	Walkley and Black	Titration with $FeSO_4$
Clay	Bouyoucos	Hydrometer Bouyoucos
Silt	Bouyoucos	Hydrometer Bouyoucos
Sand	Bouyoucos	Hydrometer Bouyoucos

The analytical methods used by the COLPOS, Campus Montecillo, Mexico, Mex., are established in the NOM-021-SEMARNAT-2000 Mexican Official Standard

USA). PCA was used to establish the simplest mathematical model capable of describing the data set satisfactorily. PCA represents the most appropriate statistical approach when the goal is to establish the relative importance of individual variables in determining the data structure. A total of 12 variables were defined with respect to climatic factors: average temperature, relative humidity, precipitation, solar radiation, and UV radiation; and characteristics of the vegetable matrix: tree age, DPPH, and ABTS radical scavenging, and the TPC, gallic acid, ascorbic acid, and total carotenoids. To evaluate the correlation of the vegetable matrix with the physical and chemical soil parameters, ten more variables were included: pH, CEC, EC, and levels of clay, silt, sand, OM, K, P, and NH_4^+. To remove possible distortions arising from the different magnitudes of the numerical values in the evaluated observations, all variables by subtracting the mean value and dividing by the standard deviation were standardized. Finally, experimental results were given as mean ± standard deviation and statistical analyses were performed by one-way ANOVA followed by Tukey's pairwise test. Differences were considered statistically significant at the value of probability less than 5% ($p < 0.05$).

Results and discussion

Table 2 shows the climatic data registered during the experimental time. The results of the soil analysis carried out on September 2014 are shown in Table 4. The variations in the bioactive compound contents (total phenolics, gallic acid, ascorbic acid, and total carotenoids) and antiradical activity (DPPH and ABTS assays) of Moringa leaf extracts from different soils and harvest seasons are presented in Table 5.

Table 4 Evaluated characteristics on September (2014) of three different soil types used in this study for the Moringa trees plots

Evaluated variables	Soil types		
	A	*B*	*C*
pH	6.49	6.65	6.43
CEC[a] (meq/100 g)	26.52	25.56	31.16
EC[b] (dS/m)	0.12	0.20	0.10
K (meq/100 g)	0.41	0.21	0.21
P (ppm)	16.62	9.02	3.80
NH_4^+ (ppm)	65.80	61.60	47.60
Organic matter (% OM)	5.47	5.47	2.73
Clay (%)	46.72	44.72	48.72
Silt (%)	23.64	22.64	27.64
Sand (%)	29.64	32.64	23.64

[a]Cation exchange capacity

[b]Electrical conductivity

From the PCA, the number of principal components (PC) to retain was determined using the eigenvalue of each PC (Table 6), which shows that the decline is stabilized after the fifth principal component (PC5). However, the eigenvalues of PC4 and PC5 are close to 1; therefore, these were also dismissed. PC1 to PC3 are the most significant in describing the data set; together, they account for 78.5% of the total variance (Table 6). Indeed, most of the information about the system (i.e., 61.9% of total variance) was provided by PC1 (39.7% of the variance) and PC2 (22.2% of the variance); therefore, PC3 was also dismissed hence. Both PC1 and PC2 were considered for building (Fig. 1) to facilitate the interpretation of results. The same principle was applied to retain the number of PC when the soil parameters, and the characteristics of the vegetable matrix were evaluated to investigate possible correlations between these variables on September 2014 (Table 7; Fig. 2).

As shown in Fig. 1b, PC1 has the highest positive correlation with all vegetable matrix variables, except for tree age, whose correlation with the PC1 is almost null. Since they positively correlate, when any of these variables show high values, the other variables also increase their values. As expected, bioactive compound contents (gallic acid, total phenolics, and, to a lesser extent, total carotenoids and ascorbic acid) are closely correlated with the ability to capture ABTS and DPPH radicals. This phenomenon has been widely reported by other authors [28, 41–43], who positively correlated the content of antioxidant bioactive compounds obtained from different vegetable matrices (*Morus nigra, Vaccinium* spp. L., *Rubus* spp. L., *Ribes* spp. L., and *Moringa oleifera* Lam.) with their ability to inhibit radicals. Therefore, our results suggest that both DPPH and ABTS assays are valid to determine or represent the antiradical activity of extracts from Moringa leaves.

Effect of the climatic factors and tree age on the bioactive compound contents and antiradical activity of extracts from Moringa leaves

Climatic data (Table 2) show small variations during May 2015 and June 2014, but differed between May 2015 and September 2014, mainly due to precipitation amounts, relative humidity, and UV radiation. January 2015 differed from all the other months by its lowest temperatures, radiation, and precipitation. The clustering of these climatic variations together with the evaluated characteristics in Moringa leaves (Table 5) are shown in Fig. 1a (PCA score plot); hence, the following behavior can be summarized: (1) from June to July 2014, the expression of bioactive compounds and antiradical activity was increased; in addition, the precipitation, relative humidity, and UV radiation were higher, but the mean temperature and solar radiation remained stable. (2) On September 2014, the highest

Table 5 Variations in bioactive compounds and antiradical activity of *Moringa oleifera* leaves grown in three soil types and harvested in different months

Soil types	Harvest Months	Tree age (days)	Antiradical activity (gTE/100 g db)		Total phenolics (gGAE/100 g db)	Gallic acid (mg/g db)	Ascorbic acid (mg/100 g db)	Total carotenoids (mg/100 g db)
			DPPH assay	ABTS assay				
A	June-14	608	4.07 ± 0.06 b	4.13 ± 0.38 cde	3.15 ± 0.10 c	6.33 ± 0.14 efg	38.35 ± 0.53	86.49 ± 0.74 de
	July-14	641	4.11 ± 0.29 b	4.74 ± 0.38 c	2.49 ± 0.03 e	7.53 ± 0.07 def	107.68 ± 0.47	137.30 ± 2.37 a
	September-14	706	6.47 ± 0.03 a	8.54 ± 0.15 a	4.18 ± 0.11 a	12.65 ± 0.17 a	142.89 ± 0.56 b	132.13 ± 1.68 a
	January-15	831	2.51 ± 0.08 c	2.80 ± 0.16 g	2.01 ± 0.03 f	9.10 ± 0.71 bcd	168.17 ± 5.71	93.11 ± 3.56 cd
	May-15	949	3.41 ± 0.07 bc	3.14 ± 0.10 efg	2.87 ± 0.10 d	9.08 ± 0.59 bcd	132.13 ± 0.71 c	65.78 ± 5.23 gh
B	June-14	458	3.40 ± 0.50 bc	3.25 ± 0.35 defg	2.31 ± 0.03 e	5.07 ± 0.10 g	80.76 ± 0.94	70.04 ± 3.18 fg
	July-14	491	3.66 ± 0.69 bc	4.29 ± 0.25 cd	2.58 ± 0.02 e	9.46 ± 0.39 bcd	143.07 ± 0.49 b	106.97 ± 1.59 b
	September-14	556	7.63 ± 0.33 a	5.08 ± 0.06 c	3.67 ± 0.06 b	10.76 ± 0.28 ab	180.47 ± 0.76 a	105.01 ± 3.27 bc
	January-15	681	3.32 ± 0.10 bc	3.23 ± 0.01 defg	2.43 ± 0.001 e	8.28 ± 0.85 cde	98.78 ± 0.87	51.41 ± 5.84 i
	May-15	799	4.52 ± 0.02 b	4.20 ± 0.65 cde	3.74 ± 0.11 b	12.93 ± 1.10 a	180.87 ± 2.68 a	70.18 ± 3.50 fg
C	June-14	276	4.57 ± 0.25 b	4.26 ± 0.32 cd	3.20 ± 0.03 c	5.32 ± 0.28 fg	71.01 ± 0.89	56.80 ± 1.12 hi
	July-14	309	3.50 ± 0.14 bc	2.69 ± 0.12 g	3.08 ± 0.01 cd	5.57 ± 0.15 fg	140.69 ± 0.84 b	77.92 ± 1.24 ef
	September-14	374	4.28 ± 0.85 b	6.69 ± 0.25 b	3.69 ± 0.08 b	10.29 ± 1.27 bc	190.13 ± 1.90 a	66.35 ± 3.30 fgh
	January-15	499	3.34 ± 0.04 bc	2.97 ± 0.10 fg	2.49 ± 0.03 e	6.71 ± 0.42 efg	117.44 0.83	92.57 ± 0.92 d
	May-15	617	4.69 ± 0.10 b	4.04 ± 0.05 cdef	3.32 ± 0.10 c	10.84 ± 0.03 ab	138.62 ± 0.21 bc	69.42 ± 0.88 fg

The values are the means of duplicates and standard deviations. Different letters in the same column indicate statistically significant differences between groups ($p < 0.05$)

TE trolox equivalent, *GAE* gallic acid equivalent, *db* dry basis

Table 6 Correlation matrix of the first ten principal components (PC) of the data set obtained when the climatic factors and the characteristics of the vegetable matrix were evaluated

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10
Eigenvalue	4.7671	2.6585	1.9888	0.9451	0.9154	0.3262	0.1527	0.1174	0.079	0.0485
Proportion	0.397	0.222	0.166	0.079	0.076	0.027	0.013	0.01	0.007	0.004
Cumulative	0.397	0.619	0.785	0.863	0.94	0.967	0.979	0.989	0.996	1

antiradical activity, total phenolics, galic acid, and total carotenoids were obtained, together with the highest precipitation and humidity (except for UV radiation and solar radiation). (3) On January 2015, the minor values of all evaluated variables were presented, except for ascorbic acid and relative humidity. (4) From January to May 2015, an increase of mean temperature, precipitation, UV radiation, and solar radiation was registered, as well as an increased expression of bioactive compounds with antiradical activity. Finally, with the statistical analysis (Tukey's pairwise test) shown in Table 5, it is confirmed that there are significant differences ($p < 0.05$) among the clusters shown in Fig. 1a representing the harvesting periods and the concentration of bioactive substances and their antiradical activity

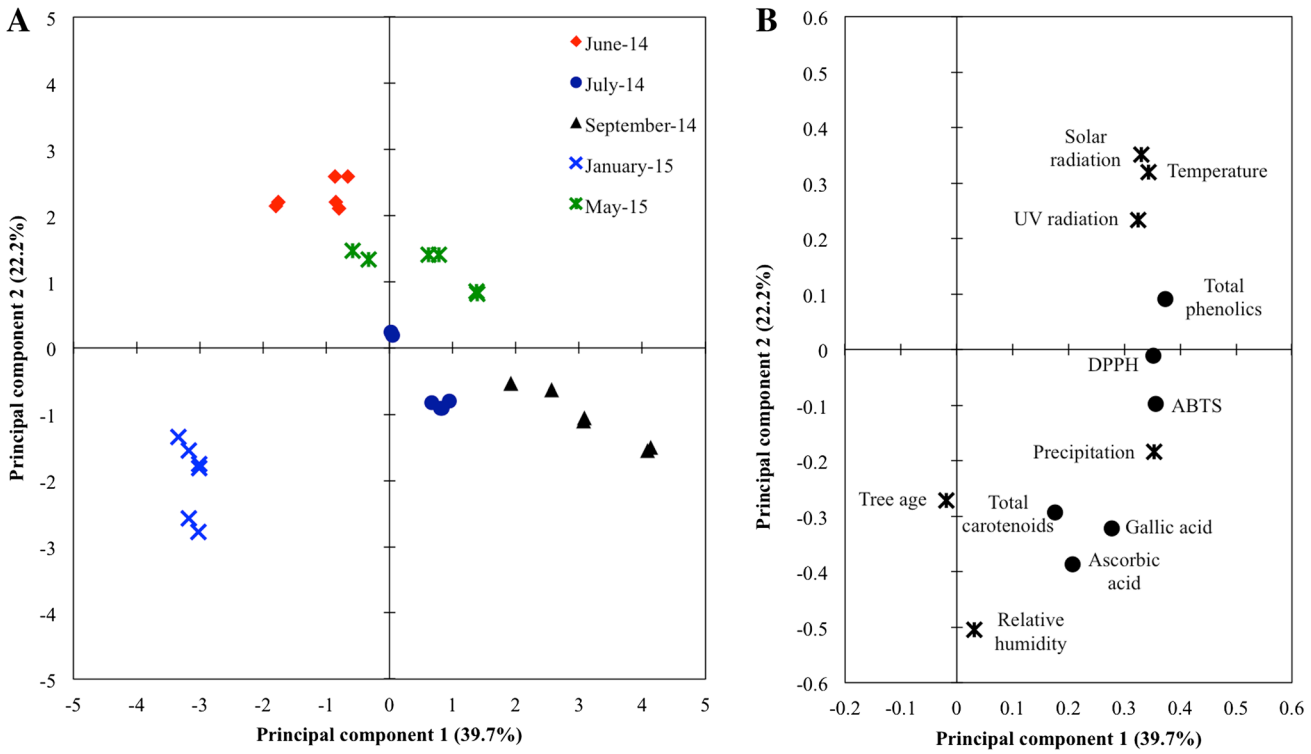

Fig. 1 PCA score plot (**a**) and loading plot (**b**) of the evaluated variables: climatic factors and evaluated characteristics in the Moringa leaves harvested in different months. Evaluated groups: June, July, and September 2014; January and May 2015

in Moringa leaves. Therefore, the evaluated climatic factors and tree age are correlated with the content of bioactive compounds and antiradical activity of extracts obtained from Moringa leaves, which explain these significant differences ($p < 0.05$).

PCA loading plot (Fig. 1b) shows that the climatic factors evaluated (solar radiation, precipitation, UV radiation, and mean temperature) have positive correlation in PC1 with the evaluated variables in the Moringa leaves (total phenolics, gallic acid, ascorbic acid, total carotenoids, and antiradical activity). That is, while solar radiation, mean temperature, UV radiation, and precipitation increased, bioactive compound contents with antiradical activity in the Moringa leaves also increased. These results suggest that higher temperatures and radiation stressed the trees. Exposure of plants to stress results in the generation of reactive oxygen species (ROS). ROS tend to oxidize various cellular molecules, such as proteins, nucleic acids, and lipids, a process that threatens the existence of the cell nucleus. To avoid the accumulation of these ROS and sustain their own survival, plants have developed an intricate antioxidant defense system. This system comprises various enzymatic and non-enzymatic molecules produced to counter the adverse effects of environmental stresses [44]. This expression of antioxidant secondary metabolites allows to explain the results in this investigation, and it is consistent with other results obtained by different authors that positively correlate the bioactive compounds content with environmental stress conditions to which the evaluated plants (*Plantago lanceolata* L., *Nepeta cataria* L. *f. citriodora, Melissa officinalis* L., *Salvia officinalis* L., *Solanum melongena* L., and *Ananas comosus* L. Merr.) were subjected [26, 45–47].

This study confirms that solar and UV radiations are important external factors that affect the production of bioactive compounds with antiradical activity in Moringa trees. The results showed that when the level of radiation increases, the productivity of total phenolics and gallic acid was positively influenced, and in less proportion, the ascorbic acid and total carotenoid contents also were affected (Fig. 1b). At the same time, and as is related to bioactive compounds content (discussed in the previous section), the

Table 7 Correlation matrix of the first five principal components (PC) of the data set obtained when the soil parameters and the characteristics of the vegetable matrix were evaluated on September 2014

	PC1	PC2	PC3	PC4	PC5
Eigenvalue	11.01	5.688	0.238	0.045	0.018
Proportion	0.648	0.335	0.014	0.003	0.001
Cumulative	0.648	0.982	0.996	0.999	1

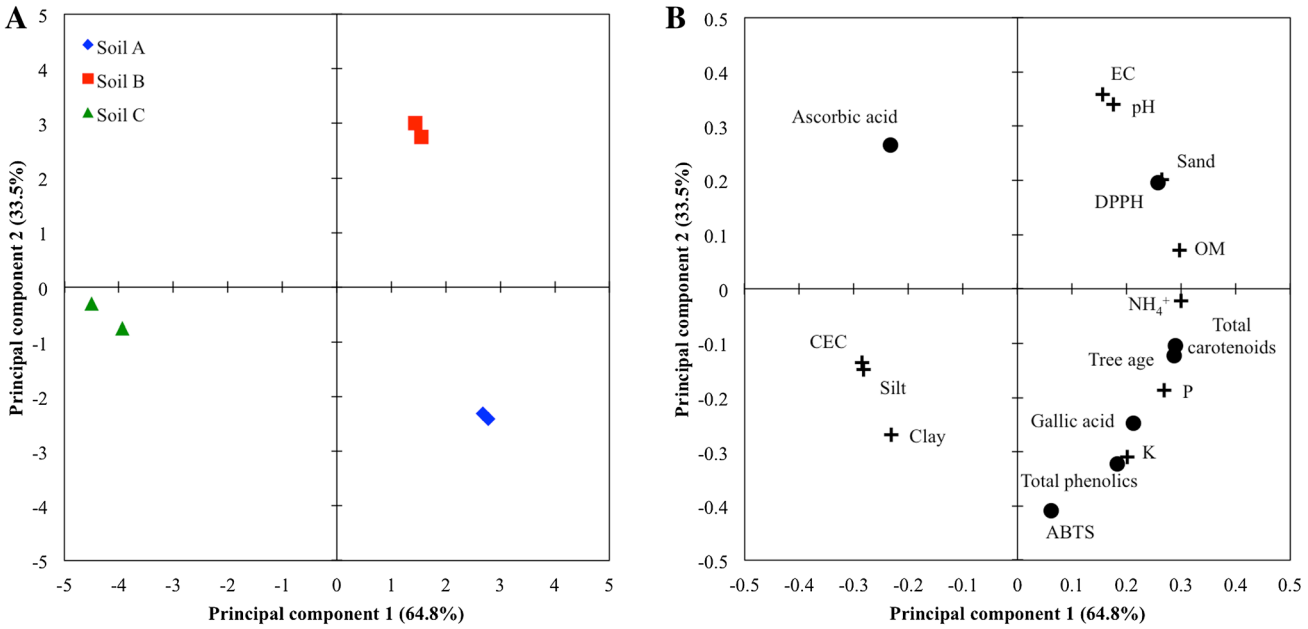

Fig. 2 PCA score plot (**a**) and loading plot (**b**) of the evaluated variables: soil parameters and evaluated characteristics in the Moringa leaves harvested on September 2014. Evaluated groups: three soils designated as A, B, and C each one

antiradical activity also increased (Fig. 1b). In this context, such physiological and biochemical changes and intensive biosynthesis of certain bioactive compounds in plants are an essential part of plant adaptation to environmental stress, such as solar and UV radiation [46].

Manukyan [46] reported that the impact of UV radiation on biosynthesis of some pharmaceutical compounds in medicinal plants could be described as positive, negative, or neutral depending on evaluated bioactive compound in the studied plant. For example, he mentions that intensive doses of UV-B radiation (2.5 kJ/m^2 day) are favorable for the synthesis of essential oils in the studied herbs, while UV-B radiation at low doses (1 kJ/m^2 day) favored increasing polyphenols content in the case of three evaluated species: lemon catmint (*Nepeta cataria* L. *f. citriodora*), lemon balm (*Melissa officinalis* L.), and sage (*Salvia officinalis* L.). However, it has been reported that specific flavonoid compounds, such as the kaempferol derivatives, [48], whose presence in the Moringa leaves has been corroborated [2], may also function as protective agents of plants against the intense radiation. This explains the increasing of total phenolics in Moringa leaves with the radiation, in the present research.

Ascorbic acid and phenolic compounds are considered the more important bioactive compounds in Moringa leaves and, therefore, are mainly responsible for their antioxidant capacity, as shown in Fig. 1b. The results of this research suggest that ascorbic acid fraction depends positively of the climatic factors evaluated. Crespo et al. [25] found similar results with the citric acid content of strawberries being positively influenced by the radiation and the number of sunshine hours the day before the harvest.

Carotenoid composition in green leaves is quite constant, while in mature fruits, noticeable qualitative and quantitative variability is observed [49]. However, differences in the carotenoid content from leafy vegetables are reported by Azevedo-Meleiro and Rodriguez-Amaya [50], who report that the carotenoid content in the endive (*Cichorium endivia*) and New Zealand spinach (*Tetragonia expansa*) was significantly higher in the summer than in the winter, reflecting seasonal effects. In the plants, the carotenoids play three essential protective roles in the photosynthetic apparatus: First protective role of carotenoids is their ability to quench triplet molecules and return to basal status. Second function is to extinguish the excitation energy of oxygen in simple excited state (highly destructive), returning to its normal triplet status. Third protective function is the extinction of reaction centers of photosystems when they are overexcited by intense radiation. In this case, the carotenoids protect the leaves of excessive radiation due to their ability to extinguish or dissipate the excitation energy from other molecules as heat to avoid damage to the plant cell [51, 52]. This explains the obtained results in this work: when excessive solar and UV radiations occurred during the hottest summer, the synthesis of carotenoids in the Moringa leaves was increased (Fig. 1b) to provide protection against ROS generated by the stress caused by the

climatic condition. This way the leaves can continue with the photosynthetic work.

PCA loading plot (Fig. 1b) suggests that the mean temperature and the precipitation are also positively correlated with the bioactive compounds content and the antiradical activity in Moringa leaves. Expression of secondary metabolites increases when the values of these variables are increased, indicating a possible state of stress in the plant. However, the correlation "relative humidity" vs "bioactive compounds content" and "antiradical activity" was practically null (Fig. 1b). These results suggest that the stress in Moringa trees due to the variation in mean temperatures and precipitation is more related to the nutritional transport and their physiological characteristics. When the mean temperature increases, the absorption of K is modified and virtually remains immobile, thus the functional capacity of the plant decreases. By the other side, when the precipitation and/or the relative humidity are high, the plant reduces the water evaporation through the leaves, then the roots absorb less water through the ground and this action significantly reduces the nutrients absorption that travels with the water [53]. Therefore, the expression of bioactive compounds would be favored by the synergism between the mean temperature and the precipitation.

The correlation of tree age with the bioactive compounds contents with antiradical activity of the Moringa leaf extracts was almost null (Fig. 1b). The literature [33] indicates that the antioxidant contents and the antiradical activity vary with the stage of maturity in Moringa leaves, since the activities of all of the bioactive compounds studied showed higher values at the mature stage than at the tender stage. Therefore, it was decided in the present study to work with Moringa leaves in mature stage, but the harvest was carried out on different tree ages. Changes in the bioactive compounds content with respect to the Moringa tree age have not been reported before; therefore, the results of this work suggest that the expression of secondary metabolites with antiradical activity could be independent of the tree age when the Moringa leaves are harvested at mature stage, then the expression of bioactive compounds from Moringa leaves depends principally on the climatic factors.

Effect of the soil parameters and tree age on the bioactive compound contents and antiradical activity of extracts from Moringa leaves

As discussed, the climatic factors have a significant effect on the bioactive compounds contents with antiradical activity in the evaluated Moringa plots; however, variations in the soil parameters also may impact on vegetable bioactive compounds content [54]. The understanding of the relationship between the bioactive compounds contents with antiradical activity and the soil composition can be critical for increasing crop productivity of Moringa trees with adequate content of phytochemicals to ensure the bioactivities on human health through consumption of their products; therefore, in this study, we evaluated three Moringa plots of different ages in three soils, referred to hereinafter as *A, B*, and *C*; the study was carried out on September 2014 when the expression of bioactive compounds with antiradical activity was highest (Table 5). As the Moringa samples were equally grown in this stage, differences due to climatic factors are discarded; it was assumed that the variable values showed changes ascribed exclusively to soil parameters and tree age.

PCA analysis of the soil samples (A, B and C) and Moringa samples obtained on September 2014 is shown in Fig. 2. PCA score plot (Fig. 2a) together with Tables 4 and 5 indicates the following relations: (1) the highest values of OM, NH_4^+, P, K, tree age, total phenolics, gallic acid, total carotenoids, and antiradical activity (ABTS assay) were registered on the Soil A, while lower values of ascorbic acid, cation exchange capacity (CEC), electrical conductivity (EC), and pH were registered in this soil. (2) Soil B presented the highest values of EC, pH, sand, antiradical activity (DPPH assay), and OM, as well as minor values of CEC, silts, clay, and the mean values of the rest of evaluated variables. (3) Soil C has the minor values of tree age, total phenolics, antiradical activity (DPPH assay), OM, NH_4^+, P, K, pH, and EC, but has the highest values of silt, clay, CEC, and ascorbic acid. In Table 5, it is confirmed that there are significant differences ($p < 0.05$) among the clusters shown in Fig. 2a with respect to the evaluated characteristics in the vegetable matrix harvested on September 2014.

PCA loading plot (Fig. 2b) shows that the tree age and the levels of NH_4^+, OM, and P from the evaluated soils presented a positive correlation (PC1) with the total carotenoids and the antiradical activity of Moringa leaf extracts; and to a lesser extent with the total phenolics and gallic acid in Moringa leaves, whose correlation with the PC1 is near to 0.2, which is similar to the K content. That is, in the A and B soils with the highest content of NH_4^+, OM, and P with respect to soil C (Table 4), principally, the total carotenoids contents and the ability to capture DPPH free radicals tend to rise (Fig. 2b; Table 5). P is part of nucleic acids and is involved in protein synthesis, and is also involved in all metabolic processes of energy transfer. While K is the cofactor or activator of over 50 enzymes that metabolize carbohydrates and proteins also participate in the ionic balance and osmotic regulation [53]. According to the comparison of the results obtained of the soil analysis with the literature [53], the Soil A shows medium levels of K and P, while both Soils B and C show low levels of these nutrients (Table 4). According to this, it is evident that a deficit in P and K has a negative effect on the metabolism of the plant,

and thus, they have an impact on crop yield and bioactive compounds contents [53]. This stress to which the plant is subjected by a deficit in P and K could explain the higher expression of total carotenoids (it is correlated with the P content principally), total phenolics (it is correlated with the K content principally), and gallic acid, as a result of the defense strategy of the Moringa trees (view the correlations in Fig. 2b), at least in the evaluated range. According to Mexican Official Standard [55], the OM and NH_4^+ values from the evaluated soils were found into medium (Soil C) and high (Soil A and B) fertility levels. Some authors report that NH_4^+ and OM contents are positively correlated with crop productivity, because they are involved in all enzymatic reactions and metabolic processes (protein synthesis, respiration, and photosynthesis) [49, 53]. Moreover, according to Fig. 2b, it can be stated that the high levels of OM and NH_4^+ from the evaluated soils are not completely adequate for the synthesis of phenolic compounds, but they are more correlated with the total carotenoids contents. These results agree with those obtained by Chludil et al. [56], who found that in deteriorated soils, the flavonoids content in Quinoa (*Chenopodium album*) was increased; in particular, the compounds derived from quercetin and kaempferol. Other authors [49] reported a similar behavior in lettuce (*Lactuca sativa* L.) samples cultivated in soils with different fertilization treatments; they report that the nitrogen in the soil adversely affects the synthesis of phenolic compounds, furthermore, their results show that the carotenoid contents in lettuce were higher in all the fertilized soils than in the untreated soil; they concluded that the carotenoid biosynthesis depends positively on the nitrogen supply.

With respect to tree age, PCA loading plot (Fig. 2b) shows that it is primarily correlated with the total carotenoids contents in Moringa leaves, and correlated inversely with the ascorbic acid contents. The results show that when the tree age increases, the carotenoid contents increases (view the values on September in Table 5); in this context, Azevedo-Meleiro and Rodriguez-Amaya [50] also reported a heavy reliance on carotenoid levels with the ripeness of the endive (*Cichorium endivia*) and lettuce (*Lactuca sativa*); their results show that the carotenoid concentrations were two-to-four times higher in the mature leaves as compared to young leaves. Maturation or ripening in fruits and vegetables is usually accompanied by enhanced carotenogenesis. This phenomenon has been attributed to an upregulation of carotenoid gene expression (phytoene synthase) with ripening [24, 50] and this could explain our results obtained when the Moringa leaves were harvested as mature leaves but in different tree ages. Opposing findings were showed for the ascorbic acid contents from Moringa leaves. Ascorbic acid content is predominant in the initial tree development, but decreases with the increases of tree age (view the values on September in the Table 5). It is known that L-ascorbic acid content in plants changes with light, hour of the day, age, plant tissue, and cell compartment. Theoretically, all these changes should be explained by the functioning of a complete metabolic network of L-ascorbic acid biosynthesis, catabolism, and recycling [57]. The literature reports that the ascorbic acid is rapidly synthesized during seed germination [58] and cell division [59] and continues to be produced in regions of active growth throughout the life of the plant. However, one might argue that in certain tissues and/or plant organs as the leaves, the metabolic breakdown of ascorbic acid fails to keep pace with biosynthesis, or the key enzymatic activities for such processes are lost or inhibited [58]. For example, studies on the maize root quiescent center, which comprises non-dividing cells, have shown that it contains high levels of ascorbate oxidase and this is correlated with low or undetectable levels of L-ascorbic acid [59]. It is evident that the biosynthetic pathway of L-ascorbic acid in plants is very complex, but the above-mentioned could explain the results obtained in this work.

As shown in Table 4, the pH values ranged between 6.43 and 6.65 for all samples. These pH values are acceptable for proper development of the plants, because in this range, the solubility of most nutrients in the soil is appropriate to facilitate its absorption by the plants [49, 53]. EC expresses the concentration of total soluble salts in the soil (sodium, magnesium, calcium, chloride, sulfate, and bicarbonate), which in high concentrations (>45 mEq/L, i.e.: EC values >4 dS/m) could cause wilt, collapse, and death of plant. Saline conditions are known to suppress plant growth, because sodium and chloride ions reduce the water availability, due to the high osmotic pressure of the external medium, and restrict the availability, mobility, and transport of potassium and calcium ions to the growing parts of plants affecting the quality of both vegetative and reproductive organs and consequently reduce the yield and quality of the crop [49]. Alcántar-González and Trejo-Téllez [53] report that EC values between 0 and 2 dS/m correspond to salt concentrations of between 0 and 20 meq/L; in this range, the effect over crop tolerance is considered negligible. According to our results (Table 4), EC values in the evaluated soils were not higher than 0.2 dS/m; therefore, these soils are considered adequate for the cultivation of this crop. CEC is the ability of a soil to retain and release positive ions, and is correlated with soil clay content, which is consistent with our results (Fig. 2b). CEC values of the evaluated soils range between 25.56 meq/100 g and 31.16 meq/100 g (Table 4), typical values of clay soils. These soils have the characteristic of properly retain the nutrients, which prevents leaching through the water; thus, the availability of soil nutrients is maintained, so that the plant can use them [53]. Given the above, it can be argued

that the pH, EC, the CEC, and the clay content from evaluated soils are found within levels that allow essential nutrients to be absorbed by plant roots to allow their development.

It is clear that more studies under controlled conditions are required to evaluate the effect of soil parameters on the expression of bioactive compounds with antiradical activity from Moringa leaves that would help researchers and agricultural producers understand how synthesis of bioactive secondary metabolites could be enhanced at Moringa crops.

Conclusions

We can conclude that Moringa leaves contain significant amounts of bioactive compounds (total phenolics, gallic acid, total carotenoids, and ascorbic acid) with antiradical activity (DPPH and ABTS assays) especially when plants have grown in soils with low content of P and K and that these compounds are not affected when the pH, CEC, and EC values, clay, sand, and silt contents from soil are within the range to be considered as suitable for this crop. Climatic factors (solar radiation, UV radiation, precipitation, and mean temperature) modulated positively the expression of bioactive compounds with antiradical activity present in the Moringa leaves. The results of this work help to expand the perspective for efficiently Moringa cultivation in tropical and subtropical regions.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Compliance with ethics requirements This article does not contain any studies with animal or human subjects.

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