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Proline influences the antioxidant capacity of Quelite (*Amaranthus hybridus* L.) and promotes its nutraceutical value

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ABSTRACT

Objective: To identify the proline content and antioxidant capacity in quelites of crop fields and marginal lands, for the delimitation of collection sites of plants with superior nutraceutical quality.

Design/Methodology/Approach: Plants of Mexican quelite (*Amaranthus hybridus* L.) in the fruiting stage were collected in two contrasting site conditions. The variables measured were plant height, number of lateral branches, and (visible) damage caused by insects. Aqueous extracts were obtained to measure the concentration of proline by the Bates free proline extraction method, to estimate antioxidant capacity as a percentage of inhibition of the DPPH radical.

Results: Results express average proline concentrations, 34.34 mg L^{-1} in plants exposed to unfavorable conditions and 32.93 mg L^{-1} in those associated with agricultural crops, this difference was significant ($p \leq 0.05$). Proline content was higher in plants subjected to stress. Regarding the percentage of inhibition of the DPPH radical with Ellagic Acid as a reference, in extracts of plants collected under marginal conditions, the average value was 22% (5.06 mg L^{-1} EAeq), while in extracts of plants collected under favorable conditions it was 15.8% (0.98 mg L^{-1} of EAeq). This is, plant extracts from sites with unfavorable conditions had a greater antioxidant capacity, which is related directly to proline.

Limitations/Implications of the study: The sample size of the quelite collections was small, even for non-parametric statistical tests. It is important to increment sample size to adequately measure the metabolites content of this plant with nutraceutical potential. High contents of proline, a bioactive compound with therapeutic properties place quelites, until now considered as weeds, as a functional food with the potential to improve human health.

Findings/Conclusions: The significant differences in proline concentration between sites, and the relationship of this aminoacid with the antioxidant capacity of quelite, highlight that site conditions increase the nutraceutical potential of these edible wild plants. This fact can lead to public health and food safety criteria for the sustainable use of marginal lands.

Keywords: proline, nutraceuticals, *Amaranthus hybridus*, antioxidant capacity, DPPH, functional foods.

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INTRODUCTION

The Mexican quelite (*Amaranthus hybridus* L.) is an herbaceous plant distributed in Mesoamerica that is consumed for its leaves, tender stems and inflorescences. It has high

biological value, with essential amino acids, iron and calcium, fiber, antioxidant vitamins (A and C), and phenolic compounds with anti-inflammatory, anti-anemic and liver-protective properties (Mateos *et al.*, 2020). Its tolerance to abiotic stress, especially drought, allows quelite to be used as an environmental bioindicator (González *et al.*, 2019). In addition, it reduces concentrations of Cu, Cr and Cd in contaminated soils; and it is outstanding as a useful species in phytoextraction and phytoremediation (Olawepo *et al.*, 2020).

Quelites are consumed fresh or cooked in various traditional dishes (López *et al.*, 2018). Cooking maintains bioactive compounds, such as polysaccharides with high antioxidant activity (Tang *et al.*, 2019). Hydroacetonic, methanolic and aqueous extracts of quelite show antioxidant and antimicrobial capacity due to the presence of terpenoids, saponins and alkaloids (Okoye, 2018; Ndukwe *et al.*, 2020). Some compounds inhibit the xanthine oxidase linked to liver and kidney inflammation (Nana *et al.*, 2012). It also contains squalene, associated with anti-cancer and anti-tumor effects (Sharma & Vijayvergia, 2019). Regarding the amino acid content, the abundance of proline and leucine is highlighted (Aja *et al.*, 2021). Proline accumulates in response to stress, acts as an osmotic protectant, redox regulator, and participant in the synthesis of secondary metabolites. This amino acid is a physiological indicator of plant stress (Hosseinifard *et al.*, 2022). In humans, exogenous proline improves tolerance to oxidative stress in diseases such as cancer and fibrosis (Vettore *et al.*, 2021). In addition, it participates in collagen synthesis (Patriarca *et al.*, 2021) and is essential for the function of the retinal pigment epithelium (Du *et al.*, 2021).

Despite its benefits, quelite consumption has decreased due to dietary changes and perceptions of health risk in contaminated areas (De Loera *et al.*, 2019). In Guanajuato, particularly in Salvatierra, this trend is observed in a productive agricultural context with a persistent tradition of rural farmers (Cárdenas *et al.*, 2019). We proposed the hypothesis that stress increases the proline content and antioxidant potential in this wild plant, toward the sustainable use of this species. The objective of the study was to identify the proline content and antioxidant capacity in quelites of crop fields and marginal lands, for the delimitation of collection sites of plants with superior nutraceutical quality.

MATERIALS AND METHODS

To determine the variability in proline concentrations, a comparative sampling was implemented, from three sites located within the municipality of Salvatierra, Guanajuato, Mexico; at the geographical coordinates 20° 15ʹ 18” N and 100° 56ʹ 27” W. The criteria for the selection of the sites included two types of soil and climate conditions, an area in association with the crop, with favorable growing conditions due to indirect effects of agronomic management (irrigation, fertilization, pest control), and another in marginal (non-cultivated) lands, with unfavorable conditions for the development of Quelite caused by intense exposure to biotic and abiotic stress factors. The purpose of this strategy was to assess whether environmental stresses, particularly water and biotic stresses, significantly influence endogenous proline content.

The plants were collected during the fruiting stage, in which a large amount of bioactive substances can be found. Development variables, such as plant height and number of lateral branches, as well as the (visible) damage caused by insects were recorded. To measure plant height, a tape measure was used; the number of lateral branches was counted manually, and insect damage was estimated visually. This is, a damaged plant was considered to be one that had leaves severed by the mouthparts of chewing insects. Plants that did not show these characteristics were considered healthy. These procedures were done at each collection site and for each growth condition.

Samples were stripped of leaves, dried in the sun and then ground in a domestic coffee mill. The resulting powder was sifted by a metal kitchen strainer, to ensure the homogeneity of the sample and eliminate coarse particles that interfere with extraction. For the quantification of proline, 24 g of each sample was weighed on an electronic scale (Escali Primo™ model P115M) with an accuracy of 0.1 g, and the compound was extracted with the technique described by Bates in 1973, adapted with the use of a domestic percolator (T-fal® Heliora). The solvent used was purified water in a proportion of 1.8 L per sample; an average volume of 1.75 L of extract per percolation session was obtained.

Before the determination of the proline content, it was necessary to obtain the pre-calibration curve with L-proline at 98.5% purity SAFCTM in progressive dilutions. A Bio-Rad® xMark™ spectrophotometer at a wavelength of 520 nm was used for this purpose. Then the quantification of proline in each of the preparations of the quelite collection was done. The procedure began with the use of a 2-mL Eppendorf-type tube, 600 μL of 3% sulfosalicylic acid was added, stirred to immediately add 400 μL of acid ninhydrin, continued with stirring, and then 400 μL of glacial acetic acid was incorporated. It was heated in a double boiler for an hour and the reaction was stopped with ice for 5 min; 600 μL of toluene were then added and stirred by means of a vortex to separate the phases. The upper or supernatant phase was transferred to a spectrophotometry reading plate with the spectrophotometer previously calibrated at 520 nm. Two measurements were made for both different extracts, each one with three replicates.

To determine the antioxidant capacity of a sample, as percentage of inhibition of the free radical 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$), 150 μL of each extract were placed, then 150 μL of DPPH were added at 150 μM and mixed. Then, absorbance at the 490 nm wavelength was measured at 0, 30, and 60 minutes; three replicates were done per sample. In the end, the 60-minute data were not considered because they did not show changes in antioxidant capacity, compared to the samples measured at 30 minutes. To express the antioxidant capacity, we used the formula:

$$\%\,inhibition = \left(\frac{A_0 - A_s}{A_0}\right) \times 100$$

where, A_0: Absorbance at 0 minutes; A_s: Absorbance at 30 minutes.

The pre-calibration curve with Ellagic acid was obtained to interpolate and express antioxidant activity as Ellagic acid equivalents (EAeq) per gram of sample. The data obtained from proline were analyzed with the statistical program Infostat© version 2020, analysis of variance ($p \leq 0.05$) and post hoc tests were performed with Tukey's multiple test, for comparison of means when required. For the DPPH test, because of the number of data ($n=3$) of each treatment, a nonparametric Mann-Whitney U test was performed to explore significant differences; the error was set at a maximum $\alpha=0.05$.

RESULTS AND DISCUSSION

The morphological comparison between quelite plant collections showed differences in growth and endogenous proline accumulation. The plants obtained within the crop fields (favorable conditions) showed a more robust size, greater plant height, bigger number of leaves and lateral branches (Figure 1A). In contrast, specimens collected in marginal lands showed lower vegetative development, smaller size and number of leaves, as well as the presence of insect damage (Figure 1B).

With the analysis of variance, significant differences ($p\leq0.05$) were found in plant height and percentage of damage by insects; there were non-significant differences in number of lateral branches (Table 1). Tukey means comparison test (Figure 3) confirmed that the greatest damage from partial or total defoliation by insects occurred in plants collected in marginal lands. While those collected in farmlands showed lower incidence of insects. This is probably due to the protective effect of agronomic management provided to the crop of interest in those farmlands.

The result of the analysis of proline content showed that the highest content of the amino acid was obtained from plants collected under unfavorable conditions (34.34 ± 0.82 mg L^{-1}), compared to those developed in association with a crop of interest (32.93 ± 0.87

Figure 1. Comparison between Quelite plants (*Amaranthus hybridus*) collected in the Ejido San Nicolás de los Agustinos, Salvatierra (Guanajuato) Mexico, in 2022. A: plant developed in farmlands. B: plant developed in marginal lands.

Table 1. Comparison of number of lateral branches in Quelite plants (*Amaranthus hybridus*) collected in sites with different conditions (favorable or unfavorable) in Salvatierra (Guanajuato) Mexico.

Treatment	Number of lateral branches
Plants collected in marginal lands	16.57±1.2 a
Plants collected in farmlands	18.37±1.7 a

Source: data analysis in Infostat© 2020 version. Data are means ± standard deviation.

Figure 2. Morphological characteristics and evidence of damage caused by insects in Quelite plants (*Amaranthus hybridus*) collected in Ejido San Nicolás de los Agustinos, Salvatierra (Guanajuato) Mexico, in 2022. Plant developed in marginal lands, with evidence of insect attack (left). Plant developed under favorable conditions (in farmlands, right).

mg L^{-1}) (Table 1). The difference was significant ($p \leq 0.05$; Figure 4), which validates the initial hypothesis of the study, that stress increases the antioxidant response in Quelite plants. Also, it agrees with what was reported in other physiological models of response to oxidative stress in plants (Xie *et al*., 2019).

When comparing the results obtained with values reported by Aja *et al*. (2021) in an investigation in Africa, where the Quelite plant reached proline contents of up to 0.0051 g per gram of dry matter (DM), the samples of our research showed lower values (0.00205 g of proline per g DM). However, these figures are relevant, because they were obtained through extraction techniques that can be repeated, by people in the community, without the need for specialized instruments.

The percentage of inhibition of DPPH and the interpolation of the equivalence with Ellagic acid concentrations, calculated with the pre-calibration curve, were notable for their high values (Table 2). As well as, because the ellagic acid used as a reference antioxidant has

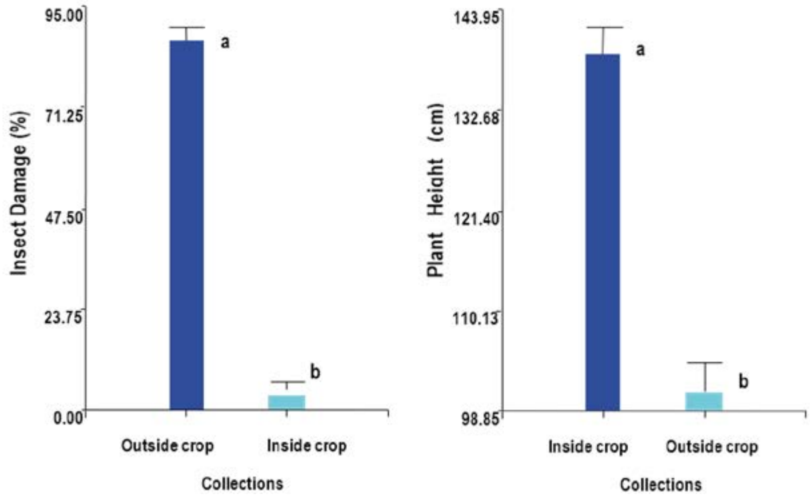

Figure 3. A: Insect damage (%) and B: plant height of Quelite plants (*Amaranthus hybridus*) obtained in favorable and marginal conditions, in Ejido San Nicolás de los Agustinos, Salvatierra (Guanajuato) Mexico, in 2022.

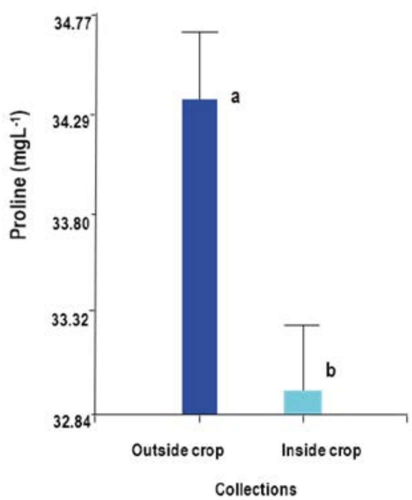

Figure 4. Proline content in Quelite plants (*Amaranthus hybridus*) collected under favorable and marginal conditions in Ejido San Nicolás de los Agustinos, Salvatierra (Guanajuato) Mexico, in 2022.

Table 2. Percentage of inhibition of the DPPH radical (antioxidant capacity) in plant extracts of Quelite (*Amaranthus hybridus*) in two contrasting collection sites in Salvatierra (Guanajuato) Mexico, in 2022.

Extract	Replicate	Inhibition of the DPPH radical (%)
Marginal lands	1	31.60
	2	18.10
	3	16.80
Average		22.00
Farmlands	1	20.90
	2	15.70
	3	10.90
Average		15.80

shown more promise than ascorbic acid, α-tocopherol, BTH (butyl-hydroxytoluene) and BHA (butyl-hydroxyanisole) in their antioxidant capacity, which coincides with Kilic *et al.* (2014). Arró *et al.* (2021) also proved this effect by testing extracts of the shiny oysterwood (or crabwood, *Gymnanthes lucida* Sw.) with the DPPH method; Those authors found that ellagic acid had an average inhibition capacity of 11.75 ± 0.28 μg mL^{-1} and a reducing power of 107% compared to ascorbic acid.

Our data in this research showed a greater antioxidant capacity in the extract of plants collected in sites with greater abiotic deficiencies for the growth of quelite. In plants collected outside farmlands, the average inhibition value was 22%, equivalent to 5.06 mg L^{-1} of ellagic acid. On the other hand, the extract of the plants collected in the sites with better conditions for growth (in farmlands) showed 15.8% inhibition of DPPH, equivalent to 0.98 mg L^{-1} of ellagic acid. These indicate that plant extracts from sites with unfavorable conditions have a greater antioxidant capacity inhibiting the DPPH radical.

According to the results of the non-parametric Mann-Whitney U test (Table 3), although the percentage of inhibition of the DPPH radical was higher in extracts obtained from marginal lands, this test did not show significant differences ($U=2$, $p=0.40$). However, a slight trend towards greater antioxidant capacity was observed, suggesting a possible effect that should be confirmed with a larger sample size.

These results appear to confirm what was expressed at the beginning of this study, showing that the greater the stress condition, the greater the amount of proline and the greater the antioxidant capacity. This shows that the collection sites with the most unfavorable conditions are places with wide potential for the benefit of society. Although they do not have improved conditions from agronomic management, quelites collected there provide food, are innocuous, and cure ailments in the human population. These are valid foundations that can increase interest in the revaluation of many wild plants that are commonly considered weeds.

Our most important result is that proline content in quelite plant is significantly influenced by the ecological conditions of the growing site. Plants collected in marginal environments, exposed to higher levels of biotic and abiotic stress, accumulated higher levels of proline compared to those developed in association with crops, which agrees with Vargas *et al.* (2021). Statistical analyses supported the validity of using proline as a biomarker of nutraceutical quality in this edible species.

From a functional perspective, these findings reinforce the potential of quelites as a nutraceutical food, especially for sectors of the Mexican population that face economic and

Table 3. Results of the nonparametric Mann-Whitney *U* Test for percent of inhibition of the DPPH radical in Quelite (*Amaranthus hybridus*) plant extracts (Salvatierra, Guanajuato, México) in 2022.

U-Statistic	Value
U1	2
U2	7
The smallest *U* value	2
Critical *U* value at $\alpha=0.05$	2

nutritional constraints. The ability of proline to act as an antioxidant, cell stabilizer, and redox stress modulator places quelite plant (*Amaranthus hybridus*) as a valuable component in diets aimed at preventing chronic non-communicable diseases, including cardiovascular and inflammatory conditions, and certain types of cancer (due to squalene contents yet to be explored). This opens a window of opportunity to design evidence-based interventions that promote its consumption. As well as to articulate food education strategies with a view to sustainable agroecological management.

CONCLUSIONS

The Mexican quelite has an interesting nutritional profile for people to be consumed as a functional food. Such a profile highlights proline as an indicator and bioactive agent of nutraceutical interest. Adverse environmental growing conditions can favor the biosynthesis of beneficial compounds for human health in this plant. It appears as necessary to give a strategic value to systematic collection of quelite in wild environments.

Despite it is considered as weed, quelite represents a valuable and abundant resource, not only an accessible source of essential nutrients, but also a bio-cultural plant resource that can be integrated into public policies aimed at combating malnutrition, increasing food sovereignty and at the revaluation of local agricultural biodiversity. It is considered as a priority to establish sustainable collection protocols; to standardize processing methods that guarantee the nutraceutical quality of the plant, and to promote awareness campaigns that identify quelite as an accessible, culturally rooted functional food with scientific validation.

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