Endogenous increase of proline in leaves of quelite (*Amaranthus hybridus* L.) through sun drying

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ABSTRACT

Objective: To evaluate the amount of proline in leaflets of quelite (*Amaranthus hybridus* L.) dried under sunlight.

Design/methodology/approach: Quelite plants were collected in Salvatierra, Guanajuato, Mexico, defoliated, and the leaves were exposed to two drying conditions: one group directly under the sun and another in the shade, for five days. The samples were ground, and 24 g of each were used to prepare aqueous extracts using a homemade percolator (T-fal® Heliora model). Proline content was determined in each extract.

Results: Statistically significant differences were found through ANOVA and Tukey's test, showing that sun drying was a better treatment compared to shade drying.

Limitations of the study/implications: The quelite plants used were exclusively collected from uncultivated sites; it is recommended to conduct comparisons in other growth environments.

Findings/conclusions: Sun drying is an effective technique to achieve an endogenous increase of proline in quelite plants, enhancing their quality for use as a nutritional or nutraceutical product, either in flour or extract form.

Keywords: Proline, *Amaranthus hybridus*, extracts, secondary metabolites, antioxidants.

Citation: Ortega-García, N., Pérez-Olvera, Ma. A., Raya-Pérez, J.C., & Aguirre-Mancilla, C.L. (2025). Endogenous increase of proline in leaves of quelite (*Amaranthus hybridus* l.) through sun drying. *Agro Productividad*. https://doi.org/10.32854/hgkhcm76

Academic Editor: Jorge Cadena Iñiguez
Associate Editor: Dra. Lucero del Mar Ruiz Posadas
Guest Editor: Daniel Alejandro Cadena Zamudio

Received: February 15, 2025.
Accepted: May 14, 2025.
Published on-line: July XX, 2025.

Agro Productividad, 18(6). June. 2025. pp: 239-248.

INTRODUCTION

Plants contain phytochemicals, mainly categorized as secondary metabolites, including terpenes, cyanogenic compounds, glucosinolates, alkaloids, phenolic acids, and glycosides, among others. Most of these compounds possess antioxidant capacity, which has led to their inclusion in daily diets for health care purposes (Gasaly *et al.*, 2020; Santiago *et al.*, 2019; Slabbert & Krüger, 2014; Chagas *et al.*, 2022; Muscolo *et al.*, 2024; Buchanan *et al.*, 2015).

In Mexican cuisine and traditional medicine, the use and utilization of plant species are considered essential for dishes that hold great recognition in local gastronomy, as well as being highly valued by traditional medicine to produce remedies addressing health problems in rural populations (Muscolo *et al.*, 2024; Hernández *et al.*, 2022; Mateos *et al.*, 2020; Santiago *et al.*, 2019).

Quelites are plants that encompass more than 500 species, widely distributed in Mexico. Many of these species are considered undesirable in both agricultural and urban contexts, being categorized as weeds (Bang *et al.*, 2021; Mateos *et al.*, 2020; Santiago *et al.*, 2019; Linares & Bye, 2015; Rzedowski & Calderón, 2004).

In the municipality of Salvatierra, Guanajuato, Mexico, *Amaranthus hybridus* L. is known as quelite (Rzedowski & Calderón, 2004). This plant is considered highly nutritious, with its leaves being a good source of essential amino acids, carbohydrates, and mineral elements (Nwaogu *et al.*, 2006). Its importance for health is increasingly recognized as an alternative for treating diseases affecting the population, thanks to the abundance of secondary metabolites it contains (Bang *et al.*, 2021; Santiago *et al.*, 2019).

This plant serves both as food and medicine for humans and livestock and is often exposed to various types of stress, which promotes the production of phenolic compounds, polysaccharides, and both essential and non-essential amino acids, including proline. This underlines its importance as a nutraceutical food source (González *et al.*, 2019; Santiago *et al.*, 2019; Kumar & Goel, 2019; Zou *et al.*, 2019; Bielach *et al.*, 2017; Slabbert & Krüger, 2014).

Proline (Pro) is an amino acid classified as a primary metabolite and has the ability to alleviate the effects of oxidative stress. It is involved in the production of secondary metabolites and acts as an inducer for enzymatic and protein synthesis that counteracts the excess of reactive oxygen species (ROS). Moreover, proline remains the main indicator of the level of stress experienced by an organism (Perassolo *et al.*, 2013; Slabbert & Krüger, 2014; Hosseinifard *et al.*, 2022).

Recent studies have documented that the exogenous application of substances helps to complement the endogenous production of proline. This has allowed more detailed research on the use of proline in efficiently eliminating excess ROS at the cellular level (Pisoschi *et al.*, 2020; Ejiofor *et al.*, 2022; Chaudhary *et al.*, 2023; Muscolo *et al.*, 2024).

Other substances supplied exogenously that have recently gained increased use are secondary metabolites, especially those that prevent cellular damage caused by oxidative stress. These compounds are considered safer and less toxic for humans, as they are naturally found in a wide variety of plants (Gonçalves & De Souza, 2022; Lourenço *et al.*, 2019). Some experts argue that when a product becomes commonly used by people, sooner or later there will be a need to meet demand, generating the necessity to implement biotechnological processes or to seek strategies that enhance its productivity and quality (Narayani & Srivastava, 2017).

Due to the high adaptability and phytochemical content of the quelite plant, some of its chemical components can be utilized as inputs within the food processing and preservation sectors, the pharmaceutical industry, and the manufacture of beauty products, without neglecting the fresh consumption currently practiced by the population (Chaudhary *et al.*,

2023; Gielecińska *et al.*, 2023). The present study aimed to evaluate the amount of proline in leaflets of quelite (*Amaranthus hybridus* L.) dried under sunlight as a strategy to increase endogenous proline in the plant, thereby leveraging the benefits this amino acid offers by enhancing its nutritional and nutraceutical quality. This, in turn, brings benefits to society and positions the plant as a valuable input for the food industry and processing.

MATERIALS AND METHODS

Quelite plants were collected from lands adjacent to cultivated fields in the municipality of Salvatierra, Guanajuato, Mexico, specifically from the ejido San Nicolás de los Agustinos, at coordinates 20.252158° N latitude and −100.961503° W longitude. The plants were uprooted during the fruiting stage from three randomly selected sites and immediately taken to the laboratory for manual defoliation. Subsequently, the leaves were spread out on paper. Half of the sample was left in the shade, while the other half was exposed to sunlight for 5 days, uncovered during the nights (Figure 1).

The sample preparation was carried out using the dried leaflets, which were finely ground using a domestic coffee grinder. The ground material was then sieved to standardize particle size. Twenty-four grams of each sample were weighed using an Escali Primo electronic balance, model P115M, with a capacity of 0 to 5000 g.

The method used for proline determination was that of Bates (1973). A crude extract of each sample was prepared using a homemade percolator, a ®T-fal Heliora model. A total of 1.8 L of water was used to obtain 1.75 L of extract. For the extraction process, the quelite flour sample was placed on filter paper inside the percolator compartment. Then, 1.8 L of purified water was added, the device was turned on, and left running until the water was completely evaporated from the reservoir. The device was turned off once dripping into the collection container ceased completely.

Figure 1. Different types of drying of quelite leaflets (Left: leaflets exposed to shade drying; Right: leaflets dried directly under the sun).

To determine the amount of proline (Pro), a calibration curve was obtained using L-proline at 98.5% purity (SAFC TM) with a Bio-Rad xMark™ spectrophotometer at a wavelength of 520 nm. Subsequently, the Pro content of each quelite extract sample was quantified. For this, 600 μL of the crude extract was placed into a 2 mL Eppendorf tube, followed by the addition of 600 μL of sulfosalicylic acid. The mixture was shaken, then 400 μL of acid ninhydrin was added. After further mixing, 400 μL of glacial acetic acid was incorporated. The mixture was heated in a water bath for one hour, and the reaction was stopped by cooling the tubes on ice for 5 minutes. Then, 600 μL of toluene was added, and the mixture was vortexed. The supernatant was taken for analysis in the previously calibrated plate spectrophotometer at 520 nm. Two measurements were made for each extract, each with three repetitions (Figure 2).

The data obtained were analyzed using the statistical software Infostat version 2020. Initially, a normality check was performed using the Q-Q plot test. Once normality was confirmed, an ANOVA was conducted followed by Tukey's multiple comparison test with a significance level of $p \leq 0.05$.

RESULTS AND DISCUSSION

Absorbance data were converted to mg/L using the equation obtained from the previous calibration curve (Table 1). Here, MS1 and MS2 correspond to the sun-drying and shade-drying methods, respectively.

The proline content values of the quelite extracts were subjected to statistical tests (Infostat version 2020) to verify normality using the Q-Q plot test (Figure 3), with this information confirmed, an ANOVA was performed (Table 2) followed by Tukey's multiple comparison test with $p \leq 0.05$ (Figure 4). Statistically significant differences were found between the sun-dried quelite sample (MS1) and the shade-dried sample (MS2).

The sun-drying process of the quelite leaflets (MS1) is statistically considered the best treatment in terms of increasing Pro content, compared to shade drying. This is supported by various research studies involving this plant. Slabbert & Krüger (2014) clearly show

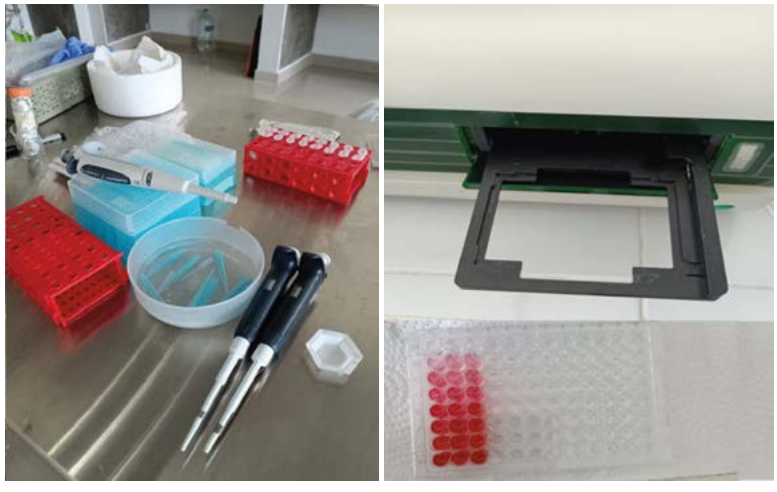


Figure 2. Equipment and materials used for the quantification of proline in quelite extracts.

Table 1. Absorbance data and proline content in mg/L of quelite extracts.

Treatment	Absorbance	Proline (mg/L)
MS1	3.548	33.792
MS1	3.643	34.758
MS1	3.714	35.479
MS1	3.617	34.493
MS1	3.610	34.422
MS1	3.480	33.101
mean	3.602	34.34083
MS2	3.324	31.516
MS2	3.377	32.054
MS2	3.328	31.556
MS2	3.270	30.967
MS2	3.397	32.258
MS2	3.303	31.302
mean	3.3331	31.608

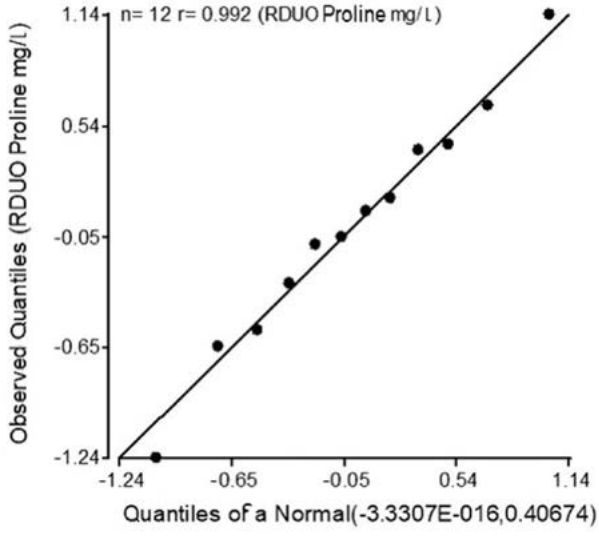
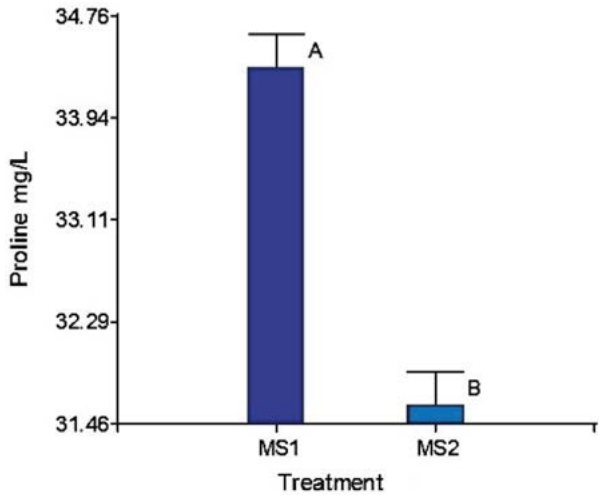


Figure 3. Normality analysis using the Q-Q plot method. (Source: Own data using Infostat software version 2020).

Table 2. Analysis of variance (Type III SS) of proline values obtained from quelite extracts considering the type of plant drying.

SV	SS	Gl	MS	F	p-value
Model	22.39	1	22.39	50.05	<0.0001
Treatment	22.39	1	22.39	50.05	<0.0001
Error	4.47	10	0.45		
Total	26.87	11			

N=12; R^2=0.83; Adjusted R^2=0.82; CV=2.03; p≤0.05.



Means sharing a common letter are not significantly different ($p \leq 0.05$).

Figura 4. Tukey's multiple comparison test for proline content in mg/L in quelite extract considering the type of plant drying (Source: Own data using Infostat 2020 software).

that when plants are exposed to a period of induced water stress, they produce a higher amount of Pro. Vwioko *et al.* (2018) found that when quelite plants were kept inside two chambers —one with NO_2 and another with SO_2— for one hour daily over three weeks, the amount of proline increased compared to the control, by more than 105% under SO_2 and by 74.8% under NO_2. Asadollahzadeh *et al.* (2023) determined over two consecutive years that in quelite plants subjected to different levels of water stress, the Pro content increased as the degree of stress increased (with a decrease in available water by 25%, 50%, 75%, and 87.5%, respectively).

The results show that quelite plants subjected to stress caused by cutting the plant, defoliation, and direct sun exposure significantly increased the amount of endogenous Pro, opening new possibilities for its use as a nutritional supplement for other organisms. This is supported by Dawood *et al.* (2014), who found that applications of exogenous Pro increase the level of endogenous Pro, enhancing antioxidant activity and alleviating the toxic effects of stress caused by seawater use in faba bean (*Vicia faba*) cultivation. It has also been demonstrated that adding Pro exogenously to the human diet increases stress tolerance and helps maintain redox homeostasis, allowing proper cell function in both cancers and conditions associated with inflammation and fibrosis (Vettore *et al.*, 2021).

Within the results of the present research, Pro reached average values of 34.34 mg/L for the sun-drying process and 31.61 mg/L (in both cases, the values transformed to the Proline content per gram of flour used for the extract, considering the amount of solvent, correspond to 0.002504 g/g of flour) for shade drying. These data are close to those found by Akubugwo *et al.* (2007) in quelites collected from cultivated fields in Nigeria, which were also sun-dried (Table 3).

Perassolo *et al.* (2013) mentioned that Pro promotes the production of secondary metabolites. Valdés *et al.* (2012) conducted experiments with oregano plants (*Lippia graveolens*) grown under four different conditions (commercial nutrient solution, water,

Table 3. Components of the quelite plant reported in research studies. (considering 100 g of dry weight sample).

Parameter evaluated	Akubugwo *et al*. (2007)	Asaolu *et al*. (2012)
Calorific value (kcal $100g^{-1}$)	268.92	
Moisture Content (%)	84.48	
Ash Content (%)	13.80	15.55
Crude Protein (%)	17.92	49.02
Crude Fibre (%)	8.61	8.05
Available Carbohydrate (%)	52.18	3.36
Crude Lipid (%)	4.65	14.02
Na (mg)	7.43	88.00
K (mg)	54.20	168.96
Ca (mg)	44.15	70.40
Mg (mg)	231.22	249.92
Fe (mg)	13.58	39.04
Zn (mg)	3.80	21.68
P (mg)	34.91	32.63
Mn (mg)		10.06
Ni (mg)		12.24
Cu (mg)		0.08
Ascorbic acid (mg)	25.40	
Proline (g of protein*)	3.43	
Alkaloid (mg)	3.54	
Flavonoid (mg)	0.83	
Saponin (mg)	1.68	
Tannins (mg)	0.49	
Phenols (mg)	0.35	

* The values transformed to the proline content per gram of sample used, considering the protein content, are 0.006147 g/g of sample.

moderate stress, and intermediate stress) and exposed to varying concentrations of NaCl, Fe^{2+}, and Cu^{2+}. They concluded that plants subjected to more stressful conditions caused by NaCl and these chemical ions showed greater accumulation of Proline in the roots and increased production of secondary metabolites, which was reflected in an enhanced production of essential oils (thymol and carvacrol). These studies support the idea that plants with higher concentrations of secondary metabolites possess greater antioxidant capacity. In the case of quelite, this is no exception, as several works have evaluated its antioxidant properties using the plant in extractions with different types of solvents, resulting in various types of extracts (ethanolic, ethyl acetate, methanolic, hydroacetonics, hexanoic, dichloromethane, and aqueous) (Ihezie *et al.*, 2023; Bang *et al.*, 2021; Ndukwe *et al.*, 2020; Nana *et al.*, 2012; Maiyo *et al.*, 2009). These studies have focused on analyzing parameters that allow the detection of this vegetable's ability to scavenge the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), ABTS (2,2'-azino-bis(3-

ethylbenzothiazoline-6-sulfonic acid) ammonium salt), total flavonoid content (TFC), or total polyphenol content (TPC) (Vázquez *et al.*, 2022; Bang *et al.*, 2021; Guija *et al.*, 2015), as well as reducing capacity, hydroxyl radical inhibition, and phosphomolybdate scavenging (Ndukwe *et al.*, 2020).

In 2021, Bang *et al.* demonstrated that this plant has an antioxidant capacity to scavenge the free radical DPPH that is equal to or greater than that of amaranth, commonly known as "alegría" (*Amaranthus hypochondriacus*). Additionally, it is known that hydroacetonic, methanolic, and aqueous extracts of quelite, besides their antioxidant power, exhibit high antimicrobial activity attributable to terpenoids, alkaloids, and saponins, effective against certain pathogens that affect humans. They also inhibit enzymes such as xanthine oxidase, whose excessive activity can cause liver and kidney damage (Bang *et al.*, 2021; Nana *et al.*, 2012). Similarly, it has been found that this plant contains a large amount of squalene, a compound with anticancer and antitumor properties, a discovery that positions it as an alternative in traditional medicine and the pharmaceutical industry with new nutraceutical benefits (Tang, 2020). All of the above reveals a broad potential for the quelite plant as a high-quality product and raw material, both for society and industry, since it was confirmed that the internal proline content can be increased by exposing the plant to greater stress while it is still alive. Finally, it is necessary to mention that this research raises questions to be explored in the near future, especially in fields of knowledge that may enhance the metabolic activity of this plant, which has been mistakenly labeled as a "weed."

CONCLUSIONS

Drying quelite plant material directly under the sun is recommended to increase the proline content in flour or extract, which can be used for nutraceutical products. In addition to proline, quelite plant material provides a large amount of antioxidants included in secondary metabolites, proteins, minerals, and ascorbic acid, which can also increase due to the content of this amino acid. The use of this plant, either directly as food or as a raw material for the food industry, presents great nutraceutical potential. It can be used fresh, dehydrated as flour, or as an extract.

ACKNOWLEDGMENTS

We thank SECIHTI for the support provided through the National Scholarship program. We also thank the Roque Technological Institute for the facilities to carry out the laboratory work, as well as Dr. Adriana Basilio for her support in adapting the proline determination technique.

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