

Polyphenols and Antioxidant in Southwest Mexico Agave Leaves

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Currently, the revalorization of the agave leaves produced by the mezcal industry has increased since various studies confirmed the presence of secondary metabolites in this agro-waste. These phytochemicals have shown potential applications in the pharmaceutical and food industries. Therefore, this research aimed to determine and compare the phenolic content and antioxidant activity of different agave leaf species. Leaves from the Agave species *angustifolia*, *americana*, *cupreata*, *karwinskii*, and *potatorum* were macerated in methanol. The extracts were concentrated and characterized by their total phenolic content (TPC: Folin-Ciocalteu) and total flavonoid content (TFC: AlCl₃), antioxidant activity (AA), and phenolic profile. The results evaluated by ANOVA and post hoc Tukey analysis showed that the species and geographical origin significantly influenced the total phenolic content ($p < 0.05$). The extracts of *A. americana* and *A. cupreata* showed high phenolic and flavonoid content within a range of 7.12 ± 1.08 - 3.19 ± 0.13 and 3.01 ± 0.10 - 0.72 ± 0.16 mg GAE/g dry leaf, respectively. Similarly, the AA was influenced by these factors ($p < 0.05$), within ranges from 13.02 ± 0.60 - 4.86 ± 0.95 ; 7.84 ± 1.30 - 3.52 ± 0.97 , 15.72 ± 3.27 - 4.74 ± 0.17 $\mu\text{mol ET/g dry leaf}$ for the ABTS, DPPH, and FRAP assays, respectively. The extracts with the central AA were *A. americana* (Chiapas); *A. angustifolia* (Oaxaca), and *A. cupreata* (Guerrero). In addition, the phenolic profile of these species showed a high content of catechin and (-)-epicatechin, two flavonoids with potent antioxidant and biological activities. The leaves of mezcal agaves are a potential source of diverse secondary metabolites of industrial interest, and they can be exploited for the development of new industrial applications.

Keywords: Agave. Antioxidant Activity. Polyphenols. Byproducts; Extraction.

Agaves are perennial plants that belong to the large family of plants known as *Asparagaceae*. Within the *Agavoideae* subfamily, it is endemic to the American continent and comprises 9 different genera [1]. The Agave genus is the largest, comprising 251 species of plants, which are primarily distributed in Mexico. Therefore, Mexico is the primary country with a diversity of these plants, boasting 177 endemic species distributed throughout the country [1].

The production of alcoholic beverages, such as mezcal, is one of the largest industrial applications of the agave plant. According to the Mexican Mezcal Quality Regulatory Council [2], the production in 2022 totaled 14,165,505 liters, with 81.08% of the production using *Agave angustifolia*,

also known as *maguey espadín*. Additionally, 1.43% and 1.98% came from the species *Agave karwinskii* (*maguey cuishe*) and *Agave potatorum* (*maguey tobalá*), respectively. Other species used in the production of mezcal are the *cupreata* and *americana* Agaves, for which data on their use in mezcal production in 2022 were not reported [2].

The high demand for mezcal has contributed to the growth of agroindustrial waste, which poses an environmental concern [3]. Agave leaves are the primary agricultural waste produced by this industry, as they are discarded without any environmental processing. Approximately 50-60% of the total weight of an agave corresponds to the leaves [4], so its waste translates into economic losses. The production of mezcalero agaves was 393,604.65 tons, generating 196,802.32 tons of leaves [5].

Recently, there has been interest in utilizing agave leaves for industrial purposes, as various studies have reported the presence of secondary metabolites in different species of agaves. These phytochemical compounds have been of interest

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to the pharmaceutical and food industries due to their potential as antioxidants, anti-inflammatory agents, anticancer agents, immunomodulators, and hypolipidemic agents, among others [6-9].

The most studied secondary metabolites from agaves, with their biological potential, are saponins and fructans in commercial agave species used for the production of tequila and mezcal [10,11]. Despite the sufficient research on the characterization of phenolic compounds from Agave (*angustifolia*, *potatorum*, *karwinskii*, *cupreata*, and *americana*) leaves, their antioxidant activity, and phenolic profile need more research about these characteristics. Thus, this research aimed to determine and compare the phenolic content, total flavonoids, as well as the antioxidant activity and phenolic profile of extracts from mezcal agave leaves in the Southwestern region of Mexico, and to provide an overview of the potential uses of these leaves as agrowaste from the mezcal industry.

Materials and Methods

Collection of Plant Material

The origin of the agaves used to obtain extracts is described in Table 1, in which the identification code based on the species and geographical origin of each plant is shown.

Conditioning of Plant Material

Fresh leaves of each plant material were washed and cut into small pieces for subsequent drying at 50°C in a convection oven for 21 h. The dried plant material was milled. The fiber and powder obtained were further reduced in a blender and subsequently sieved using a 40 mesh. Powder was stored at room temperature in plastic bags protected from light until it was used.

Preparation of the Extracts

For the extraction of phenolic compounds, powder of each plant material was defatted with

n-hexane for 24 h in a 1:10 ratio (mass/volume). The defatted material was recovered, dried, and extracted by maceration using methanol in two stages. Maceration was carried out in a 1:5 ratio (m/v) for 24 h. The crude extracts from both extractions were vacuum-filtered, combined, and concentrated using a rotary evaporator. Subsequently, extracts were kept at -18°C and protected from light until use.

Determination of Total Phenolic Content

The determination of total phenols was carried out using the methodology described in Rover & Brown [12] with some modifications, employing the Folin-Ciocalteu reagent. Gallic acid was used at a concentration of 0.5 g/L as a standard solution, with a quantification range of 5-300 mg/L. Aliquots of 20 µL of diluted plant extract were taken in triplicate and reacted with 100 µL of 10% (v/v) Folin's reagent for 5 minutes in the dark. Subsequently, 7.5% (v/v) Na₂CO₃ was added for 90 minutes in the dark at 30°C. The measurements were performed using a UV-VIS spectrophotometer at a wavelength of 765 nm. The results were expressed in milligrams of gallic acid equivalents per gram of dry leaf.

Determination of Total Flavonoid Content

The determination of total flavonoid content was carried out using the procedure as in Dewanto and colleagues [13] with some modifications. Measurements were carried out in duplicate by taking aliquots of diluted plant extract (225 µL) from each plant material, which were reacted with 5% NaNO₂ (70 µL) and 10% AlCl₃·6H₂O (150 µL) for 5 minutes. The reaction was terminated by adding 0.5 mL of 1 M NaOH and incubating for 10 min in the dark. To quantify the total flavonoid content, quercetin was used as a standard (1 mg/ml), and it was quantified in a range of 5-600 µg/ml in a UV-VIS spectrophotometer at 415 nm. The results were expressed as mg quercetin equivalents/g dry leaf.

Table 1. Geographic origin and extracts codification from agave species by region.

Species	Region	Code
<i>Agave angustifolia</i> Haw.	Atetetla, Huitzuco, Guerrero	AAHG
	Lodo Grande, Chilapa de Álvarez, Guerrero	ACCHG1
<i>Agave cupreata</i> Trel & Berg	Los Amates, Chilapa de Álvarez, Guerrero	ACCHG2
	Mazatlán, Chilpancingo, Guerrero.	ACMG
<i>Agave americana</i> 1		AACH1
<i>Agave americana</i> 2	Comitán, Chiapas	AACH2
<i>Agave americana</i> 3		AACH3
<i>Agave karwinskii</i> Zucc.	San Juan del Río	AK
<i>Agave potatorum</i> Zucc.	Tlacoluca, Oaxaca	AP
<i>Agave angustifolia</i> Haw.	San Francisco Sola, Oaxaca	AASO

Evaluation of Antioxidant Activity by the ABTS⁺ Radical Assay

The ABTS⁺ reactive radical was prepared by using the procedure described by Lopez-Romero and colleagues [9]. The ABTS⁺ radical solution was initially adjusted to an absorbance of 0.7 ± 0.02 in a UV-VIS spectrophotometer at 734 nm. Measurements were carried out in triplicate, taking aliquots of diluted plant extract from each sample (20 μ L), which were reacted with adjusted ABTS⁺ (180 μ L) for 5 minutes. Trolox 1M was used as a standard solution with a quantification range of 5-400 μ M. The results were expressed as μ M trolox equivalents/g dry leaf.

Evaluation of Antioxidant Activity by the DPPH⁺ Radical Assay

The DPPH reactive radical was prepared using the procedure described by Lopez-Romero and colleagues [9]. The DPPH radical solution was initially adjusted to an absorbance of 0.7 ± 0.02 at 515 nm in a spectrophotometer. Measurements were carried out in triplicate by taking aliquots of diluted plant extract from each sample (20 μ L), which were reacted with adjusted DPPH⁺ (180 μ L) for 30

minutes. 1M Trolox was used as a standard solution with a quantification range of 5-400 μ M. The results were expressed as μ M trolox equivalents/g dry leaf.

Iron Reducing Antioxidant Power (FRAP) Assay

The FRAP reagent was prepared according to the procedure described in Zhang and colleagues [14]. Aliquots of diluted plant extract (20 μ L) were taken from each plant material, and the FRAP solution (150 μ L) was reacted for 30 minutes in the dark. The measurements were carried out in triplicate, and 1M Trolox was used as a standard solution with a quantification range of 5-400 μ M. Measurements were performed using a UV-VIS spectrophotometer at a wavelength of 593 nm. The results were expressed as μ M trolox equivalents/g dry leaf.

HPLC-DAD-UV Phenolic Profile Auantification

Thirteen standards of different compounds were used (Sigma-Aldrich®, purities 90- 99.8%), among which there were 4 phenols (gallic acid, 3,4-dihydroxy-benzoic acid, vinylic acid, and ferulic acid) and 9 flavonoids. (myricetin, rutin, quercetin, pueranin, kaempferol, rosmarinic acid,

(+)-catechin, (-)-epicatechin, and phlorizin). The chromatograms of the standards and samples were obtained at wavelengths of 254, 280, 340, and 350 nm. Spectral data for all peaks were accumulated in the range of 100–500 nm. Identification and quantification were carried out based on the retention time and UV spectra of each standard at its maximum wavelength. The quantitative results of each metabolite were subject to the corresponding family of phenolic compounds and flavonoids. They were expressed as the content of flavones, dihydroxychalcones, hydroxybenzoic acids, flavanols, and flavonols per gram of dry leaf.

Statistical Analysis

The dependent variables — total phenolic content, total flavonoids, antioxidant activity, and metabolite content — quantified by HPLC-DAD — were analyzed using one-way analysis of variance. The independent variables in this analysis were geographic region and species. To determine the differences between the means of each variable, Tukey's multiple range test was

used with a significance level of 0.05 (95%). Additionally, the correlation between phenolic and flavonoid content and antioxidant activity was determined using the Pearson correlation coefficient (r^2). Statistical analyses were performed in STATGRAPHICS Centurion XVI software.

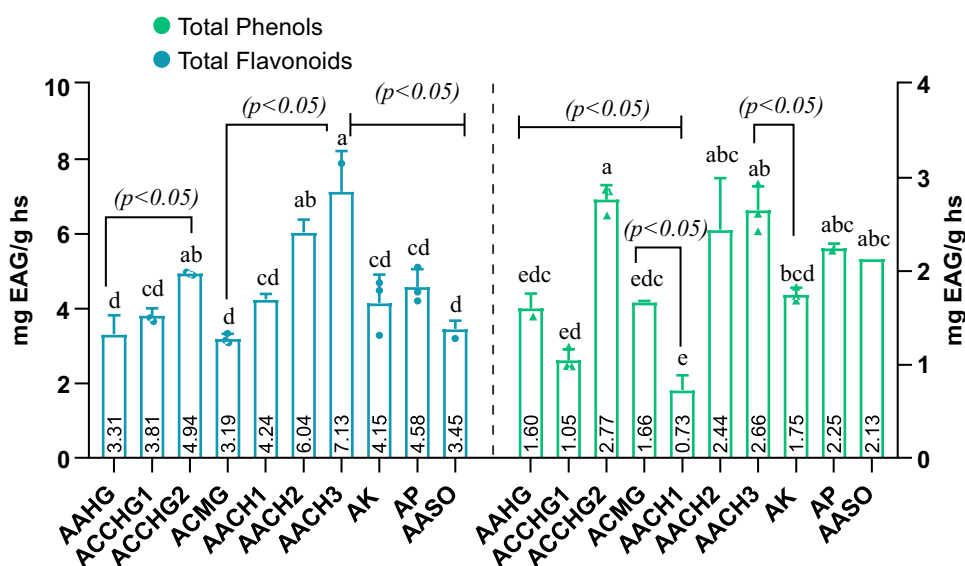
Results and Discussion

Total Flavonoid Phenol Content

According to Figure 1, the analysis of variance (ANOVA) for phenolic and total flavonoid content revealed a significant statistical difference by species ($p < 0.05$).

Tukey's multiple range test at $\alpha = 95\%$ indicated that the extracts with the highest total phenolic content in decreasing order were AACH3; $7.13 \pm 1.09 > \text{AACH2}$; $6.04 \pm 0.34 > \text{ACCHG2}$; 4.94 ± 0.05 mg EAG/g dry leaf, respectively. The extracts coded as AP, AACH1, AK, and ACCHG1 showed intermediate concentrations of phenols in a range of 4.58–3.81 mg EAG/g of dry leaf, while the extracts AASO, AAHG, and ACMG showed the lowest phenolic content in a range of 3.45

Figure 1. Total Content of phenols and flavonoids in agave leaf extracts.



Different letters indicate significant statistical differences.

-3.19 mg EAG/g dry leaf.

In Figure 1, the extracts with the highest flavonoid content in decreasing order were ACCHG2 (2.77 ± 0.45) > AACH3 (2.66 ± 0.48) > AACH2 (2.44 ± 0.07) mg EQ/g dry leaf, respectively. The extracts with an intermediate content of these metabolites in decreasing order were AP>AASO>AK, while those with low flavonoid content corresponded to the extracts ACMG>AAHG>ACCHG1>AACH1.

The concentration of total phenolics was influenced by the geographic region ($p < 0.05$); however, this did not occur for the content of total flavonoids. The phenolic and flavonoid content of the AP extract (4.58 ± 0.47 mg EAG/g hs; 2.25 ± 0.05 EQ/g hs) was higher than that reported by Delia and colleagues [15], whose values were 1.73 ± 9.36 mg EAG/g hs; 0.35 ± 6.41 mg EQ/g hs, in the ethanolic extract of *A. potatorum* leaf. On the other hand, Lopez-Romero and colleagues [9] and Ahumada-Santos [16] reported a phenolic content of 21.7 ± 0.08 and 2.06 ± 0.25 mg EAG/g hs in methanolic and ethanolic extract of *A. angustifolia* leaves, respectively. The differences in the AAHG, AAOS, and AP extracts compared to those described by these authors can be attributed to the region of origin of the plant material, the type of solvent used, the extraction method employed, the age of the plant material, and the part of the leaves [15,17,18].

According to the ANOVA results, extracts from Comitán, Chiapas; Chilapa, Guerrero (Predio Los Amates), and San Juan del Río, Oaxaca regions showed an average total phenolic content of 5.80, 4.94, and 4.36 mg EAG/g dry leaf, respectively.

Extracts of *A. americana* from Comitán, Chiapas showed the highest content of total phenols compared to the other sampled regions. In [19], a total phenolic and flavonoid content of 14.70 ± 0.31 mg EAG/g fresh weight and 5.15 ± 0.18 mg rutin equivalents/g fresh weight is reported, which were higher than those found in AACH1, AACH2, and AACH3 of *A. americana* extracts. However, the results and standard quantification for total flavonoids described in Maazoun and colleagues

[19] differed from those in this study, which also contributes to the discrepancy in the results reported in the literature. Maazoun and colleagues [19] and Lopes-Romero and colleagues [20] reported quantifications with different standards and total flavonoids content, such as rutin and hesperidin, which implies the presence of these metabolites in *A. angustifolia* and *A. americana*.

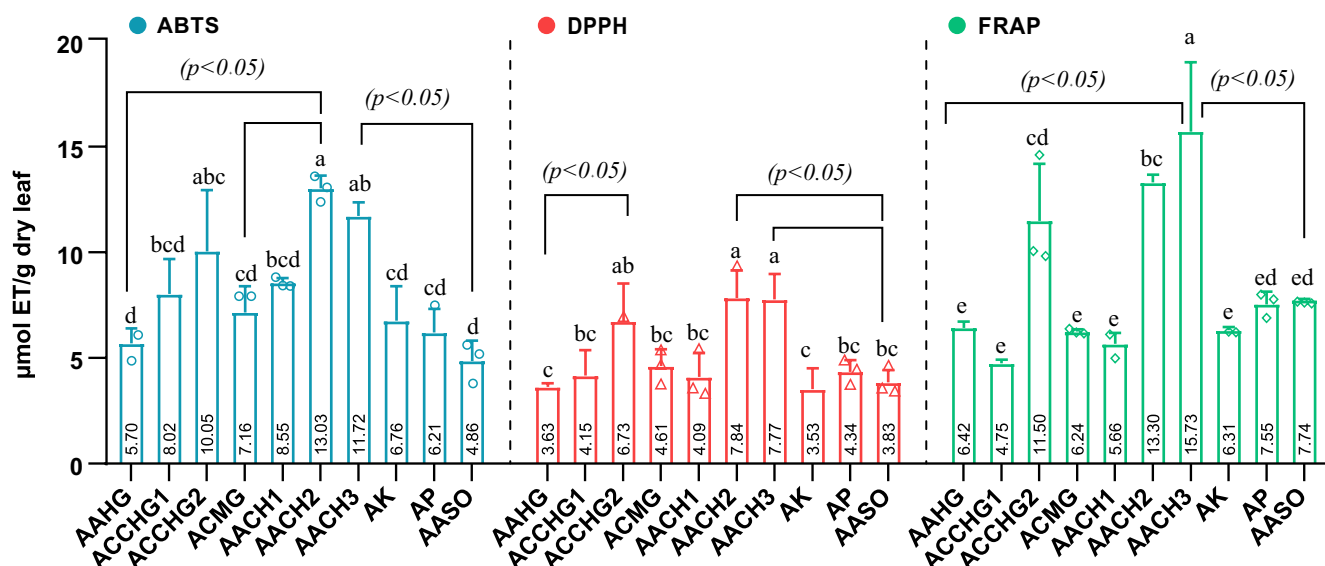
Evaluation of Antioxidant Activity

According to Figure 2 and Table 2, the species significantly influenced ($p < 0.05$) the antioxidant activity (AA) determined in the ABTS, DPPH, and FRAP assays. The same thing happened with the geographic region factor. From the results illustrated in Figure 2, we observe that the species that resulted in the highest AA in the three analyses were the extracts AACH3 (11.72 ± 0.64 /ABTS; 7.77 ± 1.20 /DPPH; 15.73 ± 3.28 /FRAP) > AACH2 (13.03 ± 0.61 /ABTS; 7.84 ± 1.30 /DPPH; 15.30 ± 0.38 /FRAP) > ACCHG2 (10.05 ± 2.89 /ABTS; 6.73 ± 1.80 ; 11.50 ± 2.69 /FRAP) μ mol trolox equivalents/g dry leaf, respectively.

With respect to the region, the ANOVA indicated that the extracts of sampled species from Comitán, Chiapas, and Chilapa, Guerrero (Los Amates), showed better antioxidant activity than those from other regions (data not shown).

It is worth noting that the species exhibiting the most significant antioxidant activity corresponded to those with the highest phenolic and total flavonoid content, suggesting a correlation between phenolic and flavonoid concentration and antioxidant activity. According to the results in Table 2, the total phenolic content exhibited a strong and significant correlation ($p < 0.05$) with the antioxidant activity evaluated by the ABTS, DPPH, and FRAP assays. A strong and significant correlation between phenolic content and antioxidant activity indicates that AA is mainly attributed to phenolic compounds [21], which was confirmed in this study. Furthermore, the reducing and free radical scavenging nature of the phenols present in the plant extracts was also observed in

Figure 2. Antioxidant activity in agave extracts from the southwestern area of Mexico by the ABTS, DPPH, and FRAP assays



Different letters indicate significant statistical differences.

Table 2. Correlation between total phenolic and flavonoid content and antioxidant activity.

Variable	r ²	Variable	r ²
CFT vs. ABTS*	0.732	FT vs. ABTS	0.342
CFT vs. DPPH*	0.757	FT vs. DPPH	0.591
CFT vs. FRAP*	0.836	FT vs. FRAP*	0.775

the three AA assays evaluated; these results are similar to those reported by Rover & Brown [22].

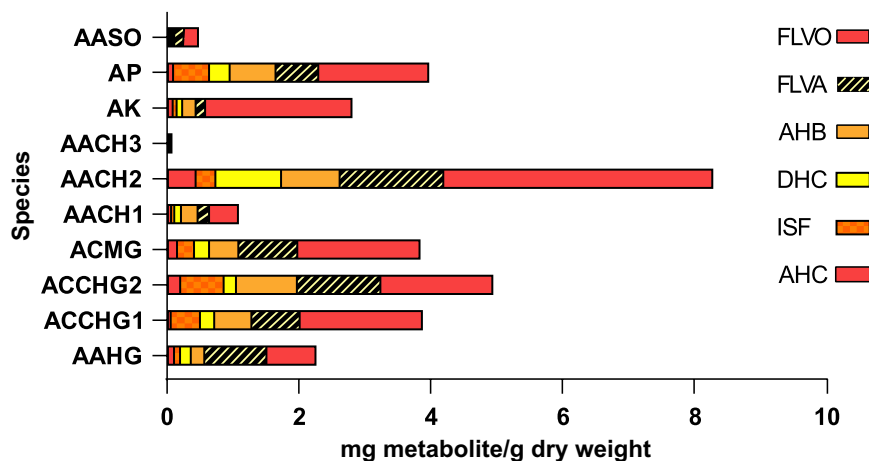
Determination of Phenolic Profile by HPLC-DAD-UV

The phenolic profiles of each plant extract from each agave species differed, as confirmed by the content of total phenolics and flavonoids (Figure 3). Likewise, it can be observed that, in nine plant extracts, the main compounds were flavonols (FLVO), where the concentrations of the routine standards, quercetin, myricetin, and kaempferol, are weighted. The following abundant compounds were flavonols (FLVA), where the concentrations of catechin and (+)-epicatechin are weighted. It is essential to note that the AACH3 extract exhibited a low richness in phenols and flavonoids, despite

its high antioxidant activity. This agrees with the low correlations between the content of flavonoids and antioxidant activity, indicating that even if these metabolites are not present in abundance, they will still have low antioxidant activity.

However, it is essential to note that the extracts with the highest antioxidant activity (AACH2 and ACCHG2) not only exhibited a high abundance of flavonols and flavanols but also contained other families of phenolic compounds. This indicates that, when more varieties of phenols and flavonoids are present in agave, the greater the antioxidant activity they have. Morreeuw and colleagues [23] reported that *A. lechuguilla* contains a rich variety of phenols and flavonoids, using methanol as a solvent. However, the highest abundance of the metabolites quantified by this author was hesperidin, isorhamnetin, and glycosylated forms of kaempferol, myricetin, and quercetin. However, the content of each quantified metabolite varied by region, which is consistent with the results of this research. El-Hawary and colleagues [6] reported a high abundance of flavonoids, phenolic acids, flavonoids, homoisoflavonoids and saponins in *A. angustifolia* var. *marginata* and *A. americana*, and

Figure 3. Phenolic and flavonoid profile obtained by HPLC-DAD-UV from southwestern Mexico agave extracts.



FLVO: Flavonols; FLVA: Flavanols, AHB: Hydroxybenzoic acids; DHC: Dihydrochalcones; ISF: Isoflavones.

the anti-inflammatory and immunomodulatory activities attributed to these metabolites. Although the biological potential of the metabolites identified in the agave species was not evaluated in this work, the predominant metabolites, such as catechin, (-)-epicatechin, quercetin, myricetin, rutin, and kaempferol, are flavonoids related to these biological activities [24,25].

Conclusion

The agaves used in this study, the phenol content depends on the species and the geographical sampling area. The species with the highest phenolic and flavonoid content were *A. americana* (AACH3) from Comitán, Chiapas, and *A. cupreata* (ACCHG2) from Chilapa, Guerrero (Los Amates).

The species with the highest content of phenols and flavonoids exhibited high antioxidant activity, indicating a significant correlation with the antioxidant activity evaluated by the three chemical assays. This result confirmed that these metabolites are mainly responsible for this activity in all the species evaluated.

In the phenolic chemical profile, the samples that presented a variety and abundance of phenolic acids and flavonoids were the species *A. americana* (AACH3) from Comitán, Chiapas, and *A. cupreata*

(ACCHG2) from Chilapa, Guerrero (Los Amates). It confirmed that the greater the variety in phenols and flavonoids, the greater the antioxidant activity.

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