

Green extraction of polyphenolic compounds from pistachio hulls: antioxidant potential and identification by HPLC/ESI/MS

Extracción verde de compuestos polifenólicos de ruezno de pistache: potencial antioxidante e identificación mediante HPLC/ESI/MS

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ABSTRACT

Pistachio (*Pistacia vera* L.), a Middle Eastern crop, is a globally popular nut due to its high nutritional value. Pistachio hulls, typically discarded during nut processing, represent approximately 60 % of total production and pose environmental concerns. This study aimed to evaluate the extraction efficiency of phenolic compounds and their antioxidant potential from pistachio hull extracts using green technologies. Three extraction methods were evaluated: ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), and a combined ultrasound-microwave method (UMAE). Extracts were purified via column chromatography to isolate phenolic-rich fractions. The highest extraction efficiency was achieved with MAE, yielding 263.77 ± 0.71 mg of total phenols per g of dry matter (dm), comprising 89.89 ± 2.97 mg EC/g dm of condensed phenols and 173.87 ± 2.39 mg GAE/g dm of hydrolysable phenols. The MAE extract exhibited the highest antioxidant capacity, with values of 520.73 ± 7.91 mg TE/g dm (DPPH) and 747.33 ± 13.48 mg TE/g dm (ABTS). RP-HPLC-ESI-MS analysis identified six bioactive compounds, including geraniin and gallic acid, both recognized for their antioxidant properties. FTIR confirmed the presence of hydroxylated, glycosylated, and esterified phenolics. These findings demonstrate the effectiveness of green technologies in recovering high-value bioactive compounds, supporting the use of pistachio hulls as a sustainable source of natural antioxidants with potential applications in the food, pharmaceutical, and cosmetics industries.

Keywords: Bioactive compounds, DPPH, ABTS, Ultrasound-Microwave-Assisted Extraction.

RESUMEN

El pistache (*Pistacia vera* L.), originario de Medio Oriente, es un fruto seco ampliamente consumido por su alto valor nutricional. Durante su procesamiento, el ruezno de pistache es descartado como subproducto de bajo valor, a pesar de representar aproximadamente el 60 % de la producción

total y generar preocupaciones ambientales. Este estudio evaluó la eficiencia de extracción de compuestos fenólicos y su capacidad antioxidante a partir de ruezno de pistache, utilizando tecnologías verdes. Se aplicaron tres métodos: extracción asistida por ultrasonido (UAE), microondas (MAE) y un método híbrido (UMAE). Los extractos se purificaron mediante cromatografía en columna para obtener fracciones ricas en fenoles. La técnica de MAE mostró la mayor eficiencia, con 263.77 ± 0.71 mg de fenoles totales por g de materia seca, incluyendo 89.89 ± 2.97 mg de fenoles condensados y 173.87 ± 2.39 mg de fenoles hidrolizables. También se registró la mayor capacidad antioxidante con la técnica de MAE: 520.73 ± 7.91 mg TE/g ms (DPPH) y 747.33 ± 13.48 mg TE/g ms (ABTS). El análisis por RP-HPLC-ESI-MS permitió identificar seis compuestos bioactivos, entre ellos la la geranina y el ácido gálico, ambos reconocidos por sus propiedades antioxidantes. Los espectros FTIR confirmaron estructuras hidroxiladas, glicosiladas y esterificadas. Estos resultados respaldan el uso del ruezno de pistache como fuente sostenible de antioxidantes, con potenciales aplicaciones en las industrias alimentaria, farmacéutica y cosmética.

Palabras clave: Compuestos bioactivos, DPPH, ABTS, Extracción asistida por ultrasonido/microondas.

INTRODUCTION

Pistachio (*Pistacia vera* L.) is one of the world's most essential nuts, consumed for over 300,000 years (Mandalari *et al.*, 2021). It is commonly consumed fresh or roasted, as a virgin vegetable oil, spreadable cream, or flour for baking bread, cakes, and cookies (Pakdaman *et al.*, 2021; Mateos *et al.*, 2022). According to the USDA (2025) data, global pistachio production reached 1,178,230 tons, with the United States, Iran, Turkey, and Syria as the leading producers. In Mexico, annual production was estimated at 44.82 tons, with 100 % concentrated in Chihuahua, primarily in the municipalities of Buenaventura, Juárez, Práxedes G. Guerrero, Jiménez, Delicias, and Meoqui (DGSIA, 2024).

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Pistachios are nutritionally valuable, particularly for their high essential fatty acid content and phytochemical compounds (Yuan *et al.*, 2022). Lipids constitute up to 60 % of their chemical composition, primarily unsaturated (~90 %), with oleic and linoleic acids as the dominant free fatty acids (Mateos *et al.*, 2022). These compounds exhibit anti-inflammatory and vasodilatory effects, reducing blood pressure and helping to prevent cardiovascular diseases (Cabo-García *et al.*, 2015). Additionally, pistachios contain 18–23 % protein, 18 % carbohydrates (mainly sucrose), fiber, minerals, and bioactive compounds such as carotenoids, chlorophyll, and phenolics (Martínez-Ruiz, 2019; Mandalari *et al.*, 2021).

Upon harvest, pistachios are divided into an edible fraction (the seed) and a non-edible fraction (the hull), which is typically discarded (Arjeh *et al.*, 2020). The hull constitutes a significant proportion of the fruit, accounting for 45–60 % of its total weight (Hamed *et al.*, 2020; Elhadeb *et al.*, 2021; Cardullo *et al.*, 2021). Studies have shown that after pistachio processing, pistachio hulls retain significant key bioactive compounds (phenolic acids, flavonoids, and tannins) with antioxidant, antiproliferative, antimicrobial, and antimutagenic properties (Abe *et al.*, 2010; Cardullo *et al.*, 2021; Özbek *et al.*, 2020), highlighting their potential as a valuable source of these compounds of interest.

Conventional extraction methods, such as maceration and Soxhlet, are time-consuming and require large amounts of solvents, creating a need for more sustainable options. Green technologies have emerged to lower solvent use and increase efficiency (Pereira *et al.*, 2017), offering eco-friendly benefits while boosting yield and quality (Picot-Allain *et al.*, 2021). Ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) are among the most sustainable techniques (Carciochi *et al.*, 2017), providing high efficiency with less solvent and shorter processing times (Nayak *et al.*, 2015). Previous research shows that hydrolyzable phenols (HP) extracted with UAE range from 22.2 to 218 mg gallic acid equivalents per gram of dry matter (mg GAE/g dm) (Garavand *et al.*, 2017; Taghizadeh *et al.*, 2018; Elakremi *et al.*, 2022; Noorolahi *et al.*, 2020), depending on the specific extraction conditions used. In comparison, MAE has produced HP levels between 62.6 and 180 mg GAE/g dm (Tabaraki and Ghadiri, 2016; Garavand *et al.*, 2017). To our knowledge, no studies have yet explored a hybrid ultrasound–microwave-assisted extraction (UMAE) technique for this purpose. Combining microwave and ultrasound irradiation accelerates extraction by simultaneously enhancing heat and mass transfer, resulting in shorter extraction times due to their synergistic effects (Luo *et al.*, 2017). Although there is increasing interest in green extraction techniques, research on the effectiveness of UAE and MAE for recovering phenolic compounds from pistachio hulls remains limited. Additionally, the phytochemical profile and antioxidant capacity of pistachio hulls grown in Chihuahua, Mexico, have not been investigated.

In that matter, this study aimed to assess the effectiveness of green extraction technologies (UAE, MAE, and UMAE) for the extraction of phenolic compounds from *Pistacia vera*

L. hulls cultivated in Chihuahua, Mexico. The findings are expected to contribute to the valorization of pistachio hulls and promote the development of natural antioxidants for use in the food, pharmaceutical, and cosmetic sectors.

MATERIAL AND METHODS

Plant Material

Pistachio hulls (var. Sfax) were collected from a field production site in Meoqui, Chihuahua, Mexico (29°42'00.0"N 104°33'00.0"W) in Delicias, Chihuahua, Mexico. They were dried at 50 °C for 24 h (Shel-Lab 1380FX Forced-Air Oven, Cornelius, OR, USA), pulverized (PULVEX Mini 100, Mexico City, Mexico) to a particle size of 1 mm, and stored in a sealed black polyethylene bottle at room temperature (30 °C) until use.

Chemical Reagents

All chemicals and solvents were of analytical grade. Ethanol and methanol, were obtained from J. T. Baker (New Jersey, US). 2,2-Diphenyl-1-picrylhydrazyl (DPPH[•]), 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS^{•+}), Folin-Ciocalteu, sodium carbonate, HCl-butanol (10 %), TROLOX, gallic acid, iron (II) sulfate heptahydrate (FeSO₄·7H₂O), and ferric reagent (Amberlite XAD-16) were obtained from Sigma-Aldrich (Missouri, US).

Extraction of Phenolic Compounds from Pistachio Hulls

Phenolic compound extractions were conducted using an ATPIO-SM300 ultrasonic microwave reaction system, following the methodology of Hernández-Hernández *et al.* (2020). Pistachio hulls (62.5 g) were placed in the reactor with 1000 mL of distilled water. The extraction process differed depending on the method used: (a) UAE was conducted at 25 kHz for 20 min at room temperature; (b) MAE was performed at 70 °C for 5 min operating at 800 W and 2450 MHz; and (c) UMAE combined both methods sequentially, consisting of 15 min of UAE followed by 5 min of MAE, totaling an extraction time of 20 min. The obtained extracts were then filtered through cotton and filter paper to remove solids, stored in plastic bottles, and kept in the absence of light at -18 °C until use.

Separation and Purification by Column Chromatography

The obtained extracts were purified using Amberlite XAD-16 resin packed in a glass column (3.7 × 50 cm), as reported by Ascacio-Valdés *et al.* (2016). The extract (100 mL) was loaded onto the column, and soluble compounds, including carbohydrates, proteins, and other impurities, were eluted with copious amounts of distilled water (2 L). Ethanol was then used to elute the fraction rich in phenolic compounds. Finally, the samples were concentrated using a rotary evaporator (R-100, BUCHI) at 40°C to remove ethanol and recover the purified sample in a powdered form. The resulting extracts were stored in 1.5 mL vials, protected from light, and refrigerated (4 °C).

ZHydrolyzable Phenols Determination

To determine hydrolyzable phenols (HP), 20 μL of the sample were mixed with 20 μL of Folin-Ciocalteu reagent and incubated for 5 min. Subsequently, 20 μL of sodium carbonate were added, followed by another 5-min incubation. Finally, 125 μL of distilled water were added, and absorbance was measured at 790 nm using a microplate reader (Multiskan GO, Thermo Scientific). The assay was conducted in quadruplicate, and the results were expressed as mg gallic acid equivalents per gram of dry mass (mg GAE/g dm), based on a gallic acid calibration curve ranging from 0 to 0.2 mg/mL (Ramírez-Esparza *et al.*, 2023).

Condensed Phenols Determination

To determine condensed phenols (CP), 250 μL of the sample were placed in test tubes, followed by the addition of 1500 μL of HCl-butanol (1:9, v/v) and 50 μL of ferric reagent. The tubes were then incubated in a water bath inside a pressure cooker for 40 min. After cooling to room temperature, 200 μL were added, and absorbance was measured at 524 nm using a microplate reader (Multiskan GO, Thermo Scientific). The assay was performed in quadruplicate, and results were expressed as mg catechin equivalents per gram of dry mass (mg CE/g dm), based on a catechin calibration curve ranging from 0 to 0.2 mg/mL (Ramírez-Esparza *et al.*, 2023). The total phenolic content was calculated as the sum of HP and CP values and expressed as mg/g dm.

Antioxidant Capacity

DPPH assay

The free radical scavenging capacity was assessed following the method reported by Meléndez *et al.* (2014) using a 60 μM DPPH• (2,2-diphenyl-1-picrylhydrazyl) solution. A mixture of 7 μL of the sample and 193 μL of DPPH solution was prepared. A control (DPPH-ethanol) and a blank (ethanol) were included. The reaction was incubated in the dark for 30 min, and absorbance was recorded at 517 nm using a microplate reader (Multiskan GO, Thermo Scientific). The assay was performed in quadruplicate, and results were expressed as mg Trolox equivalents per g of dry sample (mg TE/g dm) using a Trolox calibration curve (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Sigma-Aldrich®, Mexico) ranging from 0.2 to 1 mg/mL.

ABTS Assay

The ABTS (2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assay was conducted following the method of López-Cárdenas *et al.* (2023). The ABTS^{•+} radical cation was generated by mixing an aqueous ABTS^{•+} solution with potassium persulfate and incubating the mixture in the dark at room temperature (27 °C) for 12 h before use. The reaction was conducted by adding 190 μL of ABTS solution to 10 μL of the sample, followed by 1 min of incubation. Absorbance was measured at 734 nm using a microplate reader (Multiskan GO, Thermo Scientific). The assay was performed in quadruplicate, and results were expressed as mg Trolox equivalents

per g of dry sample (mg TE/g dm), using a Trolox calibration curve (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) ranging from 0.2 to 1 mg/mL.

IC₅₀ Analysis

To determine IC₅₀, dilutions ranging from 50 to 400 mg/L were prepared from the stock solution (1000 mg/L). The percentage of inhibition was calculated using the following equation:

$$\text{Inhibition (\%)} = 100 - \left(\left(\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100 \right) \quad \text{Eq. (1)}$$

where A_{control} represents the absorbance of the control, and A_{sample} corresponds to the absorbance of each sample. The IC₅₀ values were determined by plotting extract concentration against the % inhibition of DPPH• and ABTS^{•+} radicals. A logarithmic regression model was fitted to the inhibition data, and the IC₅₀ value was obtained by setting Y (% inhibition) = 50 and solving for X (extract concentration, mg/L) (Pérez-Jiménez *et al.*, 2008).

Identification of Phenolic Compounds by RP-HPLC–ESI-MS

The extract was purified using column chromatography with Amberlite XAD-16 (Sigma-Aldrich®, St. Louis, MO, USA), following the methodology described by Ascacio-Valdés *et al.* (2011). The solvent was then evaporated until a powdered extract was obtained, which was subsequently analyzed to determine the TPC profile using RP-HPLC–ESI-MS. Reversed-phase high-performance liquid chromatography (RP-HPLC) was conducted according to the procedure outlined by Cerda-Cejudo *et al.* (2024). The analysis was performed on a Varian HPLC system, which included an autosampler (Varian ProStar 410, Palo Alto, CA, USA), a ternary pump (Varian ProStar 230I, Palo Alto, CA, USA), and a photodiode array detector (Varian ProStar 330, Palo Alto, CA, USA). Pistachio hull extracts and solvent ethanol-water (50:50, v/v) were injected at a 1:20 (v/v) ratio. Chromatographic separation was performed on a Denali C18 column (150 $\times$ 2.1 mm, 3 μm ; Palo Alto, CA, USA) at 30 °C. Methanol served as the mobile phase, with acetonitrile (B) and 3 % acetic acid solution (C) as eluents. The gradient elution started at 3% B and 97 % C, progressively shifting to 9 % B (0–5 min), 16 % B (5–15 min), and 33 % B (15–30 min). From 30 to 35 min, 90 % B was maintained before returning to the initial composition (3 % B, 97 % C) for 35–42 min. The flow rate was set at 1.2 mL/min with an injection volume of 10 μL . For mass spectrometry, the HPLC system was coupled to a Varian 500-MS IT mass spectrometer (Palo Alto, CA, USA) equipped with an electrospray ionization (ESI) source operating in negative ion mode $[\text{M}-\text{H}]^{-1}$. Data acquisition and processing were carried out using MS Workstation software (V 6.9) in full-scan mode, covering a mass-to-charge ratio (m/z) range of 50–2000.

Fourier Transform Infrared Spectroscopy (FTIR) Analysis

The purified polyphenol extract that demonstrated the highest values for TPC and antioxidant capacity, was analyzed using an Agilent Cary 630 FTIR spectrometer equipped with a zinc selenide (ZnSe) ATR crystal, following the protocol described by Bautista-Hernández *et al.* (2021). Spectral data were collected using MicroLab PC software over a range of 4000 to 650 cm^{-1} , with 32 scan cycles at a resolution of 2 cm^{-1} . Functional group identification and spectral analysis were performed using MicroLab Expert software, while OriginPro 8 was used to generate the graphs.

Data Analysis

A Completely Randomized Design (CRD) was employed to assess the effect of the extraction method (MAE, UAE, and UMAE) on total phenol content and antioxidant capacity. Four repetitions were carried out per treatment. A one-way analysis of variance (ANOVA) was conducted using the SAS 9.0 statistical software. Differences between treatments were analyzed using Tukey's multiple range test, with a significance level of $\alpha = 0.05$.

RESULTS AND DISCUSSION

Phenolic Content Assays

Hydrolyzable Phenolic Content (HPC)

The impact of different extraction methods on the hydrolyzable phenolic (HP) content in pistachio hull extracts was assessed. The results are shown in Figure 1.

The HP content varied significantly across the extraction methods, ranging from 100 to 176 mg GAE/g dm. The highest concentration was obtained with MAE, reaching 173.87 ± 2.39 mg GAE/g dm, which was 1.38 and 1.87 times higher than the values registered for UAE and UMAE, respectively. The enhanced efficiency of MAE can be attributed to the dielectric heating mechanism induced by microwave irradiation, which causes the rapid oscillation of solvent dipoles. This results in localized heating that increases cell wall permeability and facilitates the release of intracellular phenolic compounds (Tsevdou *et al.*, 2024). Unlike UAE, which primarily relies on mechanical disruption through cavitation, MAE provides uniform and intense energy transfer, making it particularly effective for extracting more thermally stable polyphenolic compounds (Ballistreri *et al.*, 2024). It is essential to note that the optimal extraction strategy varies depending on the phenolic subclass. While MAE proved superior for hydrolyzable phenolics, other methods, such as UAE, may be more suitable for preserving thermolabile anthocyanins. This distinction is critical in the isolation, characterization, and targeted application of polyphenolic compounds, as multiple factors, including compound structure, degree of polymerization, solvent polarity, temperature, and sample particle size, influence extraction efficiency (Gil-Martín *et al.*, 2022).

These findings are consistent with those reported by Taghizadeh *et al.* (2018), who evaluated pistachio hulls of the Sarakhs variety extracted via maceration (48 h, 25 °C) using different solvents. They reported HP contents of 148.78 mg

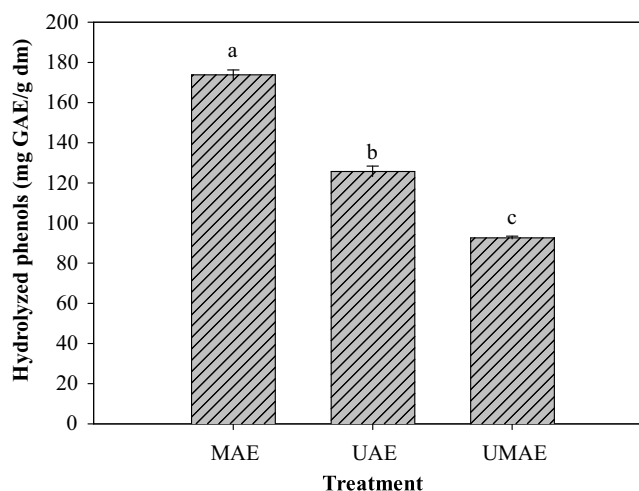


Figure 1. Effect of microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), and hybrid extraction (UMAE) on the hydrolyzable phenolic (HP) content in pistachio hull extracts. Bars represent the mean $\pm$ standard error of four replicates. Different letters indicate statistically significant differences (Tukey, $\alpha < 0.05$).

Figura 1. Efecto de las tecnologías de extracción asistida por microondas (MAE), ultrasonido (UAE) e híbrida (UMAE) sobre el contenido de compuestos fenólicos hidrolizables (HP) en los extractos de ruezno de pistache. Las barras representan el promedio $\pm$ error estándar de cuatro repeticiones. Letras distintas sobre las barras indican diferencias estadísticamente significativas entre tratamientos, de acuerdo con la prueba de Tukey ($\alpha < 0.05$).

GAE/g dm with ultrapure water and 169.53 mg GAE/g dm with ethanol. Similarly, Garavand *et al.* (2017) obtained HP levels of 179 mg GAE/g dm (aqueous) and 120 mg GAE/g dm (ethanolic) from Kalleguchi variety pistachio hull extracts using maceration. However, when applying UAE and MAE with water as the solvent, their results improved significantly, reaching values above 200 (UAE) and 180 (MAE) mg GAE/g dm. Conversely, the highest HP concentration was achieved employing UAE. In line with these extraction values, Elakremi *et al.* (2022) reported an HP content of 218 mg GAE/g using a 50:50 ethanol–water mixture with UAE performed at 50 °C for 1 h. On the other hand, the values obtained in our study were higher than those reported by Tabaraki and Ghadiri (2016), Noorolahi *et al.* (2020) and Elhadeif *et al.* (2021). Tabaraki and Ghadiri (2016), after optimizing their method, reported HP contents of 62.6 mg GAE/g dm (MAE) and 58.1 mg GAE/g dm (UAE) using water as the extraction solvent. Although their extraction values are lower than the obtained in this study, the higher HP content was registered through MAE, as observed in this research. Noorolahi *et al.* (2020) reported an HP content of 27.96 mg GAE/g dm in aqueous extracts from an Ahmad Aghaei variety, obtained via cold maceration (8 h, 25 °C). Elhadeif *et al.* (2021), who also worked with the Sax variety (the same used in the present study), obtained an HP content of 132 mg GAE/g through cold maceration (12 h, 25 °C) with water as the solvent. However, their highest value was reported for the Sidi Bouzid variety, at 197 mg GAE/g.

Differences in results, beyond those attributable to extraction methods and solvent choice, may also be influenced by agronomic factors, including cultivar type, geographic origin, and harvest season (Kaseke *et al.*, 2020).

Condensed Phenolic Content (CPC)

The condensed phenolic (CP) content in hull extracts obtained through different extraction methods is presented in Figure 2. A significant effect of the extraction system was observed, with CPC values ranging from 80 to 100 mg EC/g dry matter (dm). The highest concentration was registered using ultrasound-assisted extraction (UAE) (97.76 ± 1.20 mg EC/g dm), which was significantly higher than the values obtained with microwave-assisted extraction (MAE) and the combined ultrasound-microwave-assisted extraction (UMAE) (Tukey, $\alpha = 0.05$). On average, the CP content extracted via UAE was 1.08 mg EC/g dm higher than that obtained with MAE and UMAE.

The UAE's superior performance can be attributed to the cavitation effect produced by ultrasonic waves. This phenomenon induces the formation and collapse of microbubbles in the solvent, resulting in the mechanical disruption of plant cell walls and enhanced mass transfer, thereby promoting the release of intracellular phenolic compounds (Herrera-Pool *et al.*, 2021). Furthermore, using room temperature during UAE likely contributed to the preservation of heat-sensitive condensed phenolics. Elhadef *et al.* (2021) reported similar results, with a CP content of 79.06 ± 2.46 mg CE/100 g in pistachio hull extracts obtained through cold maceration with conventional water.

Total Phenolic Content (TPC)

The TPC in pistachio hull extracts obtained using the three evaluated extraction methods (MAE, UAE, and UMAE) was determined by summing the CP and HP values. The results are presented in Figure 3.

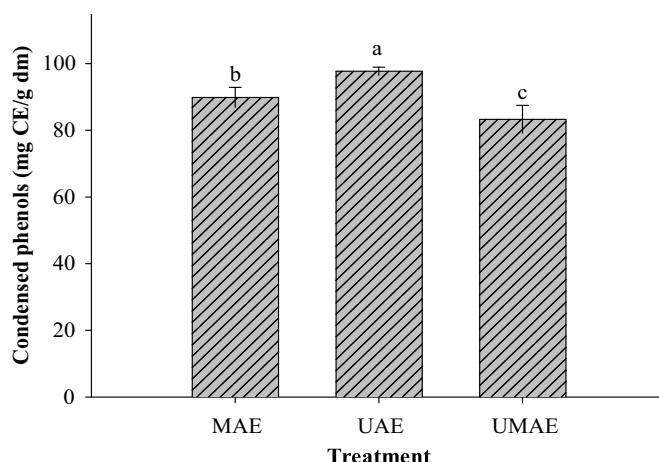


Figure 2. Effect of microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), and hybrid extraction (UMAE) on the condensed phenolic (CP) content in pistachio hull extracts. Bars represent the mean $\pm$ standard error of four replicates. Different letters indicate statistically significant differences (Tukey, $\alpha < 0.05$).

Figura 2. Efecto de las tecnologías de extracción asistida por microondas (MAE), ultrasonido (UAE) e extracción híbrida (UMAE) sobre el contenido de compuestos fenólicos condensados (CP) en los extractos de ruezno de pistache. Las barras representan el promedio $\pm$ error estándar de cuatro repeticiones. Letras distintas sobre las barras indican diferencias estadísticamente significativas entre tratamientos, de acuerdo con la prueba de Tukey ($\alpha < 0.05$).

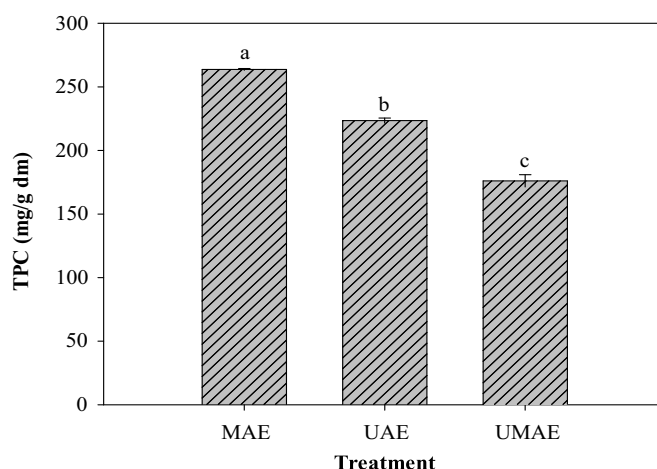


Figure 3. Effect of microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), and hybrid extraction (UMAE) on the total phenolic content (TPC) in pistachio hull extracts. Bars represent the mean $\pm$ standard error of four replicates. Different letters indicate statistically significant differences (Tukey, $\alpha < 0.05$).

Figura 3. Efecto de las tecnologías de extracción asistida por microondas (MAE), ultrasonido (UAE) e híbrida (UMAE) sobre el contenido de compuestos fenólicos totales (TPC) en los extractos de ruezno de pistache. Las barras representan el promedio $\pm$ error estándar de cuatro repeticiones. Letras distintas sobre las barras indican diferencias estadísticamente significativas entre los tratamientos, según la prueba de Tukey ($\alpha < 0.05$).

A significant effect of the extraction method was detected ($p \leq 0.05$), as the highest recovery of total phenolics was observed in the MAE treatment, reaching 263.77 ± 0.71 mg/g dm. This value was 1.17 and 1.49 times higher than those obtained using UAE and the hybrid UMAE method, respectively. This increased recovery may be attributed to the selective heating effect during MAE, where the microwave equipment's energy dissipation enhances the extractability and solubility of bioactive compounds (Wen *et al.*, 2020). Although UMAE theoretically benefits from the synergistic effects of ultrasound-induced cavitation, which enhances solvent penetration, and microwave dielectric heating, which accelerates mass transfer (Carciochi *et al.*, 2017), the UMAE method yielded the lowest TPC contents compared to those obtained with MAE and UAE.

Previous studies have similarly reported that hybrid techniques can improve extraction kinetics and reduce processing times, but may not always outperform optimized single-method protocols in terms of yield. Alaydi *et al.* (2023) reported comparable findings when evaluating the influence of UAE, MAE, and UMAE on phenolic compound recovery from Sitka spruce (*Picea sitchensis*). In their study, UAE achieved the highest total phenolic content (TPC) at 106 ± 2.5 mg GAE/g dm, followed closely by MAE (105 ± 3.2 mg GAE/g dm), whereas UMAE yielded lower levels (93.4 ± 2.5 mg GAE/g dm). Similarly, Dong *et al.* (2021) compared these three extraction techniques in green coffee oil obtained from Arabica coffee beans. Their results indicated that MAE enabled the highest TPC recovery (16.34 ± 1.31 mg GAE/100 g oil), followed by UMAE (14.81 ± 0.13 mg GAE/100 g oil), while UAE produced the lowest yield (14.40 ± 0.65 mg GAE/100 g oil). Conversely,

Zhang *et al.* (2022) found no statistically significant differences among MAE (3.81 mg GAE/g dm), UMAE (3.75 mg GAE/g dm), and UAE (3.76 mg GAE/g dm) regarding TPC extraction. However, they observed a notable decrease in TPC with prolonged UAE application, attributing this reduction to ultrasonic-induced degradation of phenolic compounds.

Therefore, while UMAE represents a promising strategy when the preservation of structurally delicate compounds is prioritized, our findings demonstrate that, under the evaluated conditions, MAE remains the most effective technique for maximizing the extraction of TPC from pistachio hulls. Moreover, its cost-effectiveness makes it a viable preliminary approach for targeted phenolic compound screening, eliminating the need for expensive, high-end equipment (Puertas-Mejía *et al.*, 2013). Consequently, the extracts obtained via MAE were further analyzed for their antioxidant capacity and bioactive compound profile.

Antioxidant Capacity

The pistachio hull extracts obtained by the three methodologies exhibited antioxidant activity. For DPPH assay, values of 520.73 ± 7.91 , 358.47 ± 7.42 and 212.25 ± 6.91 mg TE/g dm were obtained for MAE, UAE and UMAE treatments, respectively. In the case of ABTS assay, similar behavior was obtained, with the highest antioxidant activity for MAE system, with a value of 747.33 ± 13.48 mg TE/g dm, followed by UMAE (699.71 ± 5.09 mg TE/g dm) and UAE (544.79 ± 16.75 mg TE/g dm) treatments. Antioxidant capacity is commonly expressed as IC_{50} , which represents the minimum concentration required to reduce the initial radical concentration by 50 % (Munteanu and Apetrei, 2021). To determine this, the inhibition of DPPH[•] and ABTS^{•+} radicals was assessed at different concentrations of the pistachio hull extracts obtained through the three methodologies, and the results are presented in Figure 4. Lower IC_{50} values indicate a higher antioxidant potential, highlighting the efficacy of the extracted compounds (Djeghim *et al.*, 2024). Table 1 summarizes the IC_{50} values for antioxidant capacity by extraction method.

Compared to the UAE and UMAE systems, the MAE system achieved a higher inhibition percentage in both assays, with IC_{50} values of 148.94 ± 7.67 mg/L for the DPPH assay and 92.98 ± 5.42 mg/L for the ABTS assay. The lower IC_{50} value observed in the ABTS assay compared to DPPH suggests that the phenolic-rich fraction extracted from pistachio hulls primarily contains hydrophilic antioxidants. Since ABTS^{•+} is reactive toward both hydrophilic and lipophilic compounds, while DPPH[•] favors more lipophilic antioxidants, the differential response reinforces the predominance of water-soluble phenolics in the extract (Munteanu and Apetrei, 2021).

A study by Valero-Mendoza *et al.* (2022), which used water as solvent to extract antioxidants from pomegranate peel, reported DPPH radical inhibition values of 79.18 % at 1000 mg/L and 75.22 % at 500 mg/L. In comparison, the results of the present study exceeded these values. Similarly, Noorolahi *et al.* (2020) reported IC_{50} values of 171.15 mg/L

and 430 mg/L for DPPH[•] and ABTS^{•+} radicals, respectively, in an aqueous extract of pistachio hull. Özbek *et al.* (2020) obtained hydroalcoholic extracts from pistachio hull using conventional extraction and MAE. The authors reported IC_{50} values of 1060 mg/L and 1590 mg/L for the MAE and conventional extracts, respectively. These findings highlight the importance of sample preparation and the significant impact of the extraction method on the recovery of antioxidant compounds. It is important to note that the extracts in this study underwent partial purification using Amberlite XAD-16 column chromatography, resulting in a phenolic-rich fraction with enhanced antioxidant activity. This purification step selectively retained phenolic compounds while removing non-antioxidant constituents such as sugars and organic acids, thereby increasing the concentration of bioactive compounds responsible for the observed antioxidant capacity (da R. Rodrigues *et al.*, 2023).

Polyphenolic Compounds Profile

Table 2 summarizes the phenolic compounds identified in pistachio hull extracts obtained using different extraction techniques. The UAE profile revealed the presence of four distinct phenolic compounds. In contrast, the MAE and UMAE profiles each comprised three compounds. Notably, MAE and UMAE exhibited identical phenolic profiles, whereas UAE recovered a more diverse set of compounds, including an anthocyanin absent in the other treatments.

The variation in phenolic compound profiles observed across the different extraction techniques (UAE, MAE, and UMAE) can be attributed to the distinct physicochemical mechanisms through which each method facilitates the release of bioactive compounds from the plant matrix (Luo *et al.*, 2017; da Rosa *et al.*, 2019). Notably, the phenolic composition correlates well with the IC_{50} values obtained in antioxidant assays. The predominance of compounds such as ellagitannins, gallic acid derivatives, and glycosylated flavonoids, characterized by multiple hydroxyl groups and high aqueous solubility, likely accounts for the higher scavenging activity observed against the ABTS^{•+} radical.

Among the three methods, UAE produced the most diverse phenolic profile, identifying three prominent phenolic families: hydroxybenzoic acids, ellagitannins, and anthocyanins. This broader extractability is likely due to the acoustic cavitation effect. Additionally, the use of room temperature further supports the recovery of highly polar and thermolabile molecules, such as anthocyanins, by minimizing the risk of heat-induced degradation (Hernández-Hernández *et al.*, 2020). Conversely, both MAE and UMAE yielded a more limited phenolic profile. This suggests a selective extraction of more thermally stable or larger molecular weight compounds (such as the ellagic acid-hexoside), likely due to the dielectric heating generated by microwave radiation. This rapid and uniform heating enhances solvent-matrix interactions by breaking hydrogen bonds and increasing the solubility of compounds, particularly for tannins (Fraga-Corral *et al.*, 2020). In this study, the presence of gallic acid 4-O-glucoside

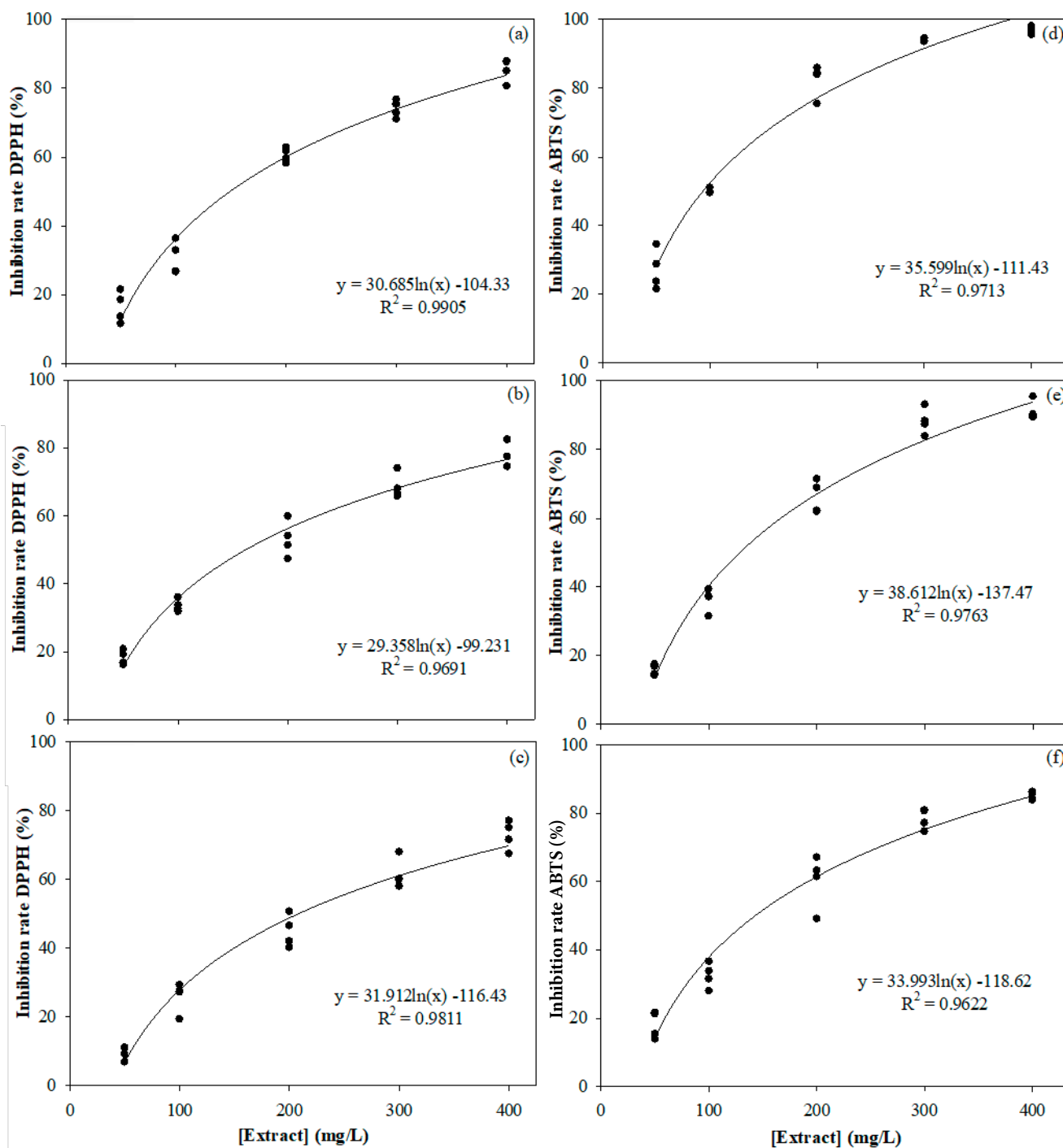


Figure 4. Effect of pistachio hull extract concentration obtained by MAE (a, d), UAE (b, e) and UMAE (c, f) on DPPH and ABTS radical inhibition

Figura 4. Efecto de la concentración del extracto de ruezno de pistache obtenido mediante EAM (a, d), EAU (b, e) y EAUM (c, f) sobre la inhibición del radical DPPH y ABTS.

Table 1. IC₅₀ values for antioxidant capacity by extraction method.

Tabla 1. Valores de IC₅₀ para capacidad antioxidante por método de extracción.

Treatment	DPPH IC ₅₀ (mg/L)	ABTS IC ₅₀ (mg/L)
MAE	148.94 ± 7.67a	92.98 ± 5.42a
UAE	162.01 ± 10.0ab	128.76 ± 8.67b
UMAE	209.01 ± 21.87c	142.94 ± 15.92b

was detected only in extracts obtained through MAE and UMAE, which involve thermal input, suggesting that this compound was naturally present in the plant tissue but was only released or became detectable due to the combined effect of heat and microwave radiation.

In contrast, free gallic acid was exclusively identified in the UAE extract, which was carried out at room temperature. This difference may be attributed to the mechanical action of acoustic cavitation generated during UAE, which can partially break glycosidic linkages, releasing gallic acid from its conjugated glucoside form (Mehta *et al.*, 2022). A similar phenomenon may apply to di-galloyl glucose; applying temperature in MAE and UMAE could lead to partial thermal degradation or structural rearrangement of gallic acid moieties in the presence of glucose, potentially promoting the formation of galloylated derivatives. Supporting this hypothesis, Erşan *et al.* (2017) extracted phenolic constituents from pistachio hull using subcritical water extraction and stated that temperature had a significant effect on gallotannin yields.

However, the absence of anthocyanins and other heat-sensitive compounds in these extracts likely reflects thermal degradation during microwave exposure. Interestingly, the UMAE method did not enhance compound diversity relative to its individual components. Instead, its profile mirrored that of MAE, indicating that the microwave phase may have dominated the extraction outcome, possibly offsetting the mechanical effects introduced by ultrasonication. This outcome may be related to the timing and sequence of the hybrid process or to the thermal conditions established during MAE, which may have degraded or masked phenolics initially mobilized during the ultrasound phase.

These findings highlight the critical influence of extraction techniques and parameters (temperature, time, and energy input) on the chemical profile of phenolic extracts. Results also demonstrate the UAE's ability to preserve heat-sensitive compounds, underscoring its suitability for comprehensive phenolic profiling. On the other hand, MAE and UMAE appear better suited for the targeted recovery of stable phenolic families, such as ellagitannins.

Phenolic compounds are valuable across multiple industries, particularly in the pharmaceutical, cosmetic, and food sectors, where they are commonly used for their antioxidant properties. Their effectiveness lies in their ability to neutralize free radicals by donating hydrogen atoms or electrons, interrupting oxidative chain reactions. This antioxidant activity has been associated with the prevention and management of various chronic diseases, including cancer, hypertension, and diabetes, making phenolic compounds promising candidates for therapeutic and preventive formulations (Ordoñez-Cano *et al.*, 2025).

Several phenolic compounds identified in the pistachio hull extracts exhibit a broad spectrum of bioactivities, highlighting their potential for diverse industrial applications. Gallic acid is well-documented for its antioxidant, anti-inflammatory, antimicrobial, anticancer, and neuroprotective properties. It is utilized in pharmaceuticals (e.g., analgesics, astringents), food preservation (e.g., trimethoprim), cosmetics (anti-aging formulations), and dietary supplements (Choubey *et al.*, 2018). Geraniin, a potent ellagitannin, demonstrates multiple biological effects, including antimicrobial, antiviral, anticancer, hepatoprotective, neuroprotective, and metabolic regulatory functions, making it valuable in pharmaceutical and nutraceutical applications (Perera *et al.*, 2015; Cheng *et al.*, 2017). Cyanidin 3-O-sambubioside exhibits antioxidant, cardioprotective, neuroprotective, and antimicrobial properties, with applications in food, cosmetics, and smart packaging (Galvano *et al.*, 2004; Lazalde-Cruz *et al.*, 2021; Schmutz *et al.*, 2023; Patra *et al.*, 2025). Ellagic acid-hexoside exhibits anticancer, anti-inflammatory, and antidepressant effects and has applications in functional foods,

Table 2. Identification of phenolic compounds by extraction method.

Tabla 2. Identificación de compuestos fenólicos por método de extracción.

Extraction Technique	RT (min)	[M-H] ⁻¹ (m/z)	Phenolic compound	Family
UAE	5.87	168.9	Gallic acid	Hydroxybenzoic acids
	19.41	950.5	Geraniin	Ellagitannins
	22.79	614.8	Cyanidin 3-O-sambubioside	Anthocyanins
	25.37	462.9	Ellagic acid-hexoside	Ellagitannins
UMAE	5.69	330.9	Gallic acid 4-O-glucoside	Hydroxybenzoic acids
	16.02	482	Di-galloyl glucose	Gallotannins
	25.91	462.9	Ellagic acid-hexoside	Ellagitannins
MAE	5.42	330.9	Gallic acid 4-O-glucoside	Hydroxybenzoic acids
	17.46	482.8	Di-galloyl glucose	Gallotannins
	25.46	462.8	Ellagic acid-hexoside	Ellagitannins

pharmaceuticals, and bioengineered materials (Evtyugin *et al.*, 2020). Gallic acid 4-O-glucoside and di-galloyl glucose, both derivatives of gallic acid, retain similar bioactivities, notably antioxidant and antimicrobial properties, with relevance in the food and pharmaceutical sectors (Wu *et al.*, 2018; Yao *et al.*, 2021; Zhao *et al.*, 2022; Rippin *et al.*, 2023).

FTIR spectral analysis

The FTIR spectrum of the MAE sample (Figure 5) exhibited characteristic absorption bands consistent with the presence of polyphenolic and bioactive compounds previously identified by HPLC-MS, including geraniin, di-galloyl glucose, ellagic acid derivatives, cyanidin 3-O-sambubioside, and 1-eicosanol. A broad and intense band centered at 3293 cm⁻¹ corresponds to O–H stretching vibrations, typical of phenolic hydroxyl and carboxylic acid groups, which indicates extensive hydrogen bonding (Bautista-Hernández *et al.*, 2021). This observation aligns with the polyhydroxylated nature of ellagitannins and flavonoid glycosides, as detected by chromatography. These hydroxyl groups are relevant to antioxidant activity due to their ability to donate electrons and stabilize free radicals (Moazzen *et al.*, 2022).

The absorption band at 1600.9 cm⁻¹ is attributed to C=C stretching in aromatic and conjugated systems, confirming the abundance of phenolic structures, such as gallic and ellagic acid derivatives. This region also overlaps with signals associated with C=O functional groups, previously reported in pectin and other phenolic matrices (Lucarini *et al.*, 2020). The signal at 1448.1 cm⁻¹, located within the range of carbonyl-related vibrations, has been linked to phenolic constituents and was similarly observed in pistachio hull extracts (Haque-Manirul, 2022). A strong signal at 1198.3 cm⁻¹ corresponds to C–O stretching vibrations (Tucureanu *et al.*, 2016), which may be attributed to aryl ether linkages, glycosidic bonds, and ester groups. This is consistent with the structural features of compounds such as digalloyl glucose, cyanidin 3-O-sambubioside, and geraniin, all of which contain multiple esterified or glycosylated hydroxyl groups.

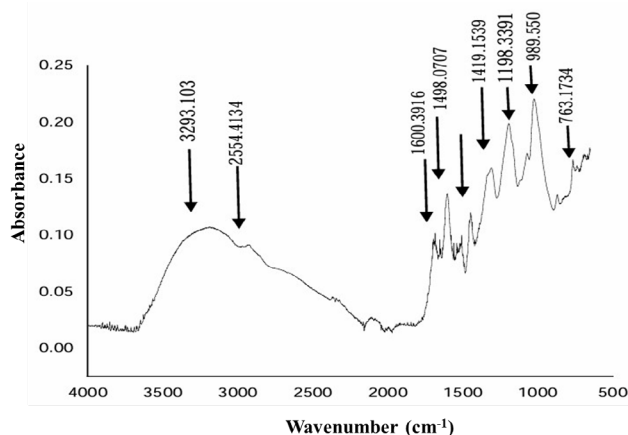


Figure 5. Infrared spectrum of purified polyphenols from pistachio hull obtained by MAE.

Figura 5. Espectro infrarrojo de polifenoles purificados obtenidos a partir del ruezno de pistache mediante EAM.

Additional bands observed in the 1150–1000 cm⁻¹ region are indicative of C–O–H stretching vibrations from alcohols, supporting the presence of phenolic and glycosidic structures, as also noted by Bautista-Hernández *et al.* (2021). A distinct absorption peak at 989.6 cm⁻¹ is attributed to =C–H out-of-plane bending, typical of isolated or conjugated alkene systems (Atyah *et al.*, 2018). This band was absent in simpler structures such as gallic acid and its mono-glucoside, but agrees with the dehydrohexahydroxydiphenoyl (DHHDP) units found in geraniin, offering spectroscopic evidence of non-aromatic conjugation. The signal at 763.2 cm⁻¹, corresponding to aromatic C–H out-of-plane bending (Atyah *et al.*, 2018), further confirms the presence of substituted benzene rings within the extract. The polyphenolic profile obtained in this study is further supported by the findings of Hamidpour *et al.* (2018), whose absorbance peaks did not significantly differ from those reported here despite analyzing a different pistachio by-product.

CONCLUSIONS

Pistachio hulls, a major by-product of the pistachio industry, were confirmed to be a valuable natural source of polyphenolic compounds with significant antioxidant potential. Among the extraction techniques assessed, MAE proved the most effective, yielding the highest phenolic content (263.77 ± 0.71 mg/g dm) and strong antioxidant activity, as indicated by IC₅₀ values in DPPH (148.94 ± 7.67 mg/L) and ABTS (92.98 ± 5.42 mg/L) assays. Furthermore, the extraction method was found to influence both the yield and the qualitative profile of polyphenolic compounds, resulting in the identification of two hydroxybenzoic acids, two ellagitannins, one anthocyanin, and one gallotannin. Although the UAE extract showed a lower overall phenolic content, it revealed a broader spectrum of phenolic compounds, including the presence of geraniin, which was absent in the other extracts. Complementary FTIR analysis confirmed the presence of functional groups corresponding to the phenolics identified by HPLC, thereby supporting their structural characterization. Collectively, the results highlight MAE as an efficient and environmentally sustainable method for the recovery of high-value bioactive compounds from pistachio hulls, with potential applications in the formulation of functional ingredients and nutraceutical products.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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