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Influence of solvent polarity on phenolic and flavonoid content, antioxidant activity, and UPLC-PDA-ESI-MS profiling of wild *Ziziphus spina-christi* leaves

Abstract

Ziziphus spina-christi (*Z. spina*) plant has a long history of traditional use in folk medicine for the treatment of various ailments. In this study, the phenolic and flavonoid components found in wild *Z. spina* leaves from Palestine were separated and identified using Ultra-Performance Liquid Chromatography (UPLC) coupled with Photodiode Array Detection (PDA) and Mass Spectrometry (MS) in both positive and negative electrospray ionization modes (UPLC-PDA-ESI-MS). Additionally, the antioxidant activity (AA), total phenolic content (TPC), and total flavonoids content (TFC) in three different leave extracts were investigated. The TPC was examined using Folin-Ciocalteu method, while the TFC was assessed by the Aluminum chloride method. Four different antioxidant assays were employed, namely, the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging activity, ferric reducing antioxidant power (FRAP), Cupric ion reducing antioxidant capacity (CUPRAC) and free radical scavenging ABTS. All the analyses were conducted using a UV-Visible spectrophotometer. The polarity of the extraction solvent directly affected the TPC, TFC, and AA results. The study revealed that extracting leaves with 80% ethanol yielded superior values for both TPC and AA, whereas absolute ethanol proved to be the optimal for achieving the highest TFC levels. Moreover, the results indicated that TPC ranged from 38.5 to 181.5 mg of gallic acid/g dry weight (DW) while the TFC ranged from 6.3 to 23.1 mg catechin/g DW. AA using FRAP assay was between 9.2 to 28.9 mmol Fe^{+2}/g DW. CUPRAC assay was between 2988 to 7356 µmol Trolox/g DW. DPPH assay ranged from 250.4 to 390.6 µmol Trolox/g DW while ABTS assay was from 29.2 to 56.4 µmol Trolox/g DW. The extracts of wild *Z. spina* are abundant in phenolic compounds, including phenolic acids and flavonoid derivatives, as indicated by the results obtained from UPLC-PDA-ESi-MS analysis.

Keywords: antioxidant activity, *Ziziphus spina-christi,* total flavonoids content, total phenolic content, HPLC-PDA, UPLC-PDA-ESi-MS

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Abbreviations: *Z. spina*, *Ziziphus spina-christi*; UPLC, ultra-performance liquid chromatography; HPLC, high-performance liquid chromatography; PDA, photodiode array detection; MS, mass spectrometry; ESI, electrospray ionization; TFC, total flavonoids content; TPC, total phenolic content; AA, antioxidant activity; DPPH, 1,1-diphenyl-2-picryl-hydrazyl; FRAP, ferric reducing antioxidant power; CUPRAC, cupric ion reducing antioxidant capacity; ABTS, 2,2-azino-di-(3-ethylbenzothiazoline-6-sulphonic acid); DW, dry weight; ACN, acetonitrile; TPTZ, tripyridyltriazine; QDa, Quadrupole mass analyzer; PTFE, polytetrafluoroethylene; UV-Vis, ultraviolet–visible spectroscopy; λ, wavelength; RT, retention time; m/z, mass-to-charge ratio.

Introduction

Medicinal plants have been recognized as an unlimited source that has led to the discovery of numerous successful pharmaceutical drugs.[1,2] Herbal medicine continues to gain attention globally due to its efficacy and affordability. Different parts of the medicinal plant comprise active secondary metabolites and/or precursors that contribute to the development of effective medications.[2]

Although Palestine is geographically small, it is richly endowed with a vast array of diverse natural plants.[3] An important medicinal plant is the *Ziziphus spina-christi* (*Z. spina*), commonly locally known as Sidr, belongs to the Rhamnaceae family. The Ziziphus genus comprises about 200 species indigenous to subtropical and warm-temperate areas including the Middle East.[4,5]

In folk medicine, different parts of *Z. spina*, mainly, its leaves and fruits, have been utilized by decoction with boiling water to address wide range of health issues including skin conditions, digestive problems, hair loss, cough, and rheumatism.[6] These applications reflect both cultural heritage and pharmacological potential of the plant. The plant leaves, bark, fruits, seeds, whole plant, essential oils, and root have recently been extensively reviewed for their antioxidant potential.[6] Moreover, the anti-inflammatory, antimalarial, antidiabetic, antiobesity, anxiolytic, anticancer, and antimicrobial were also reviewed.[6]

Researchers have studied the chemical composition of *Z. spina*, revealing more than 431 compounds.[6] Various parts of the *Z. spina* demonstrated a diverse array of phytochemicals classes such as phenols, flavonoids, alkaloids, saponins, fatty acids and more.[6–8] These findings highlight the plant's abundant biological activity and its potential for use in medicinal applications.

Although *Z. spina* may share many secondary metabolites across different geographical regions, variations in local environmental conditions can influence the uniqueness of its phytochemical profile and, consequently, its biological activity. In particular, wild *Z. spina* growing in Palestine is highly valued in traditional medicine; however,

its phytochemical composition and antioxidant potential have never been examined. Therefore, this endeavor aims to characterize rich phytochemicals present in the leaves using UPLC-PDA-ESI-MS and to measure their antioxidant activity, total phenolic content, and total flavonoid content following extraction with three different solvent systems: water, ethanol, and 80% ethanol.

Materials and methods

Plant materials

Leaves of the Palestinian wild *Z. spina* plant was collected from the West Bank, Palestine and was subsequently authenticated by Professor Khalid Sawalha, director of Biodiversity Research Laboratory, Al-Quds University.

Chemicals and reagents

All chemicals used in this study were purchased from Sigma-Aldrich (Germany) and were of analytical grade. These included Folin–Ciocalteu reagent, sodium carbonate, gallic acid, copper chloride, sodium hydroxide, neocuproine, ferric chloride, ammonium acetate, ferrous sulfate, hydrochloric acid (37%), potassium persulfate, sodium acetate, sodium nitrite, aluminum chloride, methanol, ethanol (99.9%), Trolox, DPPH, TPTZ, and ABTS.

HPLC-grade acetonitrile was obtained from Sigma. LC–MS grade ultrapure water (Milli-Q Millipore), acetonitrile (J.T. Baker), and formic acid (Merck) were also used.

FRAP reagent was prepared as described by Benzie and Strain by mixing 2.5 mL of 10 mM TPTZ solution in 40 mM HCl, 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$, and 25 mL of 0.3 M acetate buffer at pH 3.6. The 0.3 M acetate buffer was prepared by dissolving 16.8 g of acetic acid and 0.8 g of sodium hydroxide in distilled water and adjusting the final volume to 1000 mL.[9]

Extraction of plant material

Fresh leaves of *Z. spina* were carefully washed with distilled water to remove adhering dirt and impurities. The cleaned leaves were then air-dried at room temperature in a shaded area until fully dehydrated. After drying, the plant material was pulverized into a fine powder using a household grinder.

For extraction, 5 g of the powdered leaves were treated separately with 50 mL of each solvent-water, 80% ethanol, and absolute ethanol. The extraction was performed in a water bath maintained at 37°C for three hours with sonication to enhance solvent penetration. Afterward, the mixtures were filtered, and the liquid portions were concentrated under reduced pressure using a rotary evaporator. The concentrated crude extracts were then kept refrigerated until further experimental analysis.

HPLC-PDA and UPLC-PDA-ESI-MS systems

The chemical analysis was conducted using a Waters Alliance HPLC system (e2695 separations module) coupled with a 2998 photodiode array (PDA) detector. Additionally, an Acquity UPLC H-Class system (Waters, USA) was employed, interfaced with both a PDA detector and a quadrupole mass analyzer (QDa), with data acquisition also managed using Empower 3.

Determination of antioxidant activity

FRAP assay

The FRAP assay was carried out using freshly prepared reagent (3.0 mL) that was pre-warmed to 37°C. Each reaction mixture was prepared by combining the reagent with 40 µL of plant extract and then incubating the mixture at 37°C. The absorbance was measured at 593 nm against a blank containing distilled water, which was incubated under the same conditions. Unlike the standard FRAP protocol of 4 minutes, the incubation was extended up to 1 hour to ensure complete reaction. A calibration curve was generated using aqueous solutions of Fe^{2+} at concentrations ranging from 2 to 5 mM, and the antioxidant capacity of the extracts was expressed as mmol Fe^{2+} per gram of sample.

Cupric reducing antioxidant power (CUPRAC assay)

This method was assessed following the method described by Apak et al.[10] For each assay, 100 µL of plant extract was mixed with 1 mL of 10 mM copper chloride, 1 mL of 7.5 mM neocuproine in 99.9% ethanol, 1 mL of 1 M ammonium acetate buffer (pH 7.0), and 1 mL of distilled water, giving a total reaction volume of 4.1 mL. The mixture was allowed to react for 30 minutes, after which the absorbance was recorded at 450 nm using a reagent blank for reference. A calibration curve was prepared using Trolox standards, and the antioxidant capacity of the extracts was reported as µmol Trolox per gram of sample.

Free radical scavenging activity using DPPH (DPPH assay)

The antioxidant activity of the extracts was evaluated using the DPPH radical, based on the method of Brand-Williams et al.[11] In this assay, 100 µL of each plant extract was mixed with 3.9 mL of 0.0634 mM DPPH solution prepared in 95% methanol. The mixture was briefly vortexed for 5-10 seconds, and the reduction in absorbance was tracked at 515 nm over a 30-minute period until the readings stabilized. A 95% methanol solution served as the blank. Trolox was used to construct a standard calibration curve, and the antioxidant capacity of the samples was expressed in µmol Trolox per gram of extract.

Free radical scavenging activity using ABTS (ABTS assay)

The ABTS assay was performed using a modified method based on Pellegrini et al.[12] An ABTS stock solution (7 mM) was prepared by reacting 7 mM ABTS with 2.45 mM potassium persulfate to generate the radical cation. This stock solution was then diluted with 99.9% ethanol to produce a working solution with an absorbance of 0.70 ± 0.02 at 734 nm. For the assay, 200 µL of each extract was mixed with 1800 µL of the $ABTS^{+\cdot}$ solution, and the absorbance was measured at 734 nm after 10 minutes of incubation at 30°C. The antioxidant activity of the extracts was expressed as Trolox Equivalent Antioxidant Capacity (TEAC) in µmol Trolox per gram of sample.

Total phenolic content (Folin-Ciocalteau assay)

The total phenolic content of the extracts was determined using the Folin–Ciocalteu method as described by Singleton and Rossi.[13] In brief, 40 µL of *Z. spina* extract or gallic acid standard was combined with 1.8 mL of Folin-Ciocalteu reagent, which had been diluted tenfold with distilled water. The mixture was left at room temperature for 5 minutes, followed by the addition of 1.2 mL of 7.5% (w/v) sodium bicarbonate solution. After incubating at room temperature for 60 minutes, the absorbance was recorded at 765 nm. A calibration curve was prepared using gallic acid solutions ranging from 10 to 500 mg/L, and the phenolic content of the samples was expressed as mg gallic acid equivalents (GAE) per gram of extract.

Total flavonoid content

The total flavonoid content of the extracts was measured using a colorimetric method adapted from Kim et al.[14] In this procedure, 1 mL

of the extract was combined with 4 mL of distilled water in a test tube. Next, 0.3 mL of 5% sodium nitrite solution was added, followed by 0.3 mL of 10% aluminum chloride solution. The mixture was allowed to react at room temperature for 5 minutes, after which 2 mL of 1 M sodium hydroxide was added. The total volume was then brought to 10 mL with distilled water and mixed thoroughly using a test tube shaker. The absorbance of the resulting pink solution was measured at 510 nm. A standard curve was prepared with catechin solutions ranging from 50 to 100 mg/L, and the flavonoid content of the samples was expressed as mg catechin equivalents (CEQ) per gram of extract.

Chromatographic conditions

The crude water, 80% ethanol, and absolute ethanol extracts were analyzed by HPLC using a Waters XBridge ODS column (4.6 × 150 mm, 5 µm). The mobile phase comprised 0.5% acetic acid in water (A) and acetonitrile (B) and was run in a linear gradient. The program began at 100% A, decreasing to 70% over 40 minutes, then to 40% over 20 minutes, followed by a drop to 10% over 2 minutes, which was held for 6 minutes before returning to the initial conditions over 2 minutes. The system was equilibrated for 5 minutes with 100% A before each injection. Samples were filtered through a 0.45 µm PTFE membrane. Detection was performed with a PDA detector scanning 210–500 nm. The flow rate was 1 mL/min, injection volume 20 µL, and the column temperature was maintained at 25°C.

For UPLC-PDA-ESI-MS analysis, formic acid replaced acetic acid in the mobile phase, maintaining a flow rate of 0.5 mL/min. Separation was carried out on an Acquity UPLC BEH C18 column (50 × 2.1 mm, 1.7 µm) with a BEH C18 guard column (2.1 × 5 mm, 1.7 µm). Column and sample temperatures were set at 20°C. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). A linear gradient started at 98% A, decreased to 70% over 3 minutes, then to 30% over 40 minutes, and returned to the starting conditions in 1 minute. A 5-minute delay was applied before injection to allow column equilibration. PDA detection ranged from 210–500 nm, and mass spectrometry was performed in both positive and negative ESI modes over a mass range of 200–1200 Da.

Statistical analysis

Three separate *Z. spina* samples were analyzed in triplicate, with results reported as mean ± standard deviation. Statistical analysis was conducted using SAS 8.02 (SAS Institute, USA). The impact of extraction solvent on TPC, TFC, and antioxidant activity was assessed using one-way ANOVA (GLM), and multiple comparisons were adjusted with the Bonferroni method to maintain a 5% overall error rate. Pearson correlation coefficients were calculated to examine relationships between the measured parameters, and the NOMISS option was applied to ensure consistent data for subsequent regression analyses.

Results and discussion

TPC assay by Folin-Ciocalteu reagent

Table 1 summarizes the total phenolic content (TPC) of leaf extracts obtained with three different solvents. The results clearly show that the type of solvent has a major influence on TPC. Statistically significant differences ($p < 0.05$) between the extracts are indicated by different letters (a, b, c). The highest TPC was found in the 80% ethanol extract, at 181.5 ± 1.9 mg/g. In contrast, extracts with 100% ethanol and water yielded much lower TPCs of 56.9 ± 0.9 mg/g and 38.5 ± 1.5 mg/g, respectively, representing only 30% and 21% of the phenolics extracted with 80% ethanol. These findings highlight that phenolic compounds are far more soluble in 80% ethanol, emphasizing that solvent selection is critical for maximizing the recovery of these bioactive compounds.

Table 1 Phenolic and antioxidant activity of *Z. spina* leaves extracted with different solvents

Extract	TPC (mg/g)	TFC (mg/g)	FRAP (mmol/g)	CUPRAC (µmol/g)	DPPH (µmol/g)	ABTS (µmol/g)
Water	38.5^{c} ± 1.5	6.3^{c} ± 0.9	9.2^{c} ± 0.4	2988^{c} ± 51	250.4^{c} ± 2.5	29.2^{c} ± 1.4
80% Ethanol	181.5^{a} ± 1.9	12.4^{b} ± 0.2	28.9^{a} ± 1.4	7356^{a} ± 49	390.6^{a} ± 0.7	56.4^{a} ± 0.9
100% Ethanol	56.9^{b} ± 0.9	23.1^{a} ± 0.9	14.5^{b} ± 0.9	4820^{b} ± 52	310.9^{b} ± 1.1	37.4^{b} ± 0.6

Results are expressed as an average of three samples of *Z. spina*. Different small letters (a, b, c) within column indicate significant difference ($p < 0.05$, $n = 3$). TPC as mg Gallic acid/g DW, TFC as mg catechin/g DW, FRAP as mmol Fe^{+2}/g DW, CUPRAC as µmol Trolox/g DW, DPPH as µmol Trolox/g DW, and ABTS as µmol Trolox/g DW

TFC assay

Table 1 presents the results of the ferric chloride colorimetric assay used to measure the flavonoid content of *Z. spina* leaves. Similar to the TPC analysis, the TFC was subjected to the same statistical analyses, with significant differences ($p < 0.05$) denoted by small letters (a, b, and c) to indicate variations among the plant leaves extracted with the three solvents. The analysis revealed noteworthy distinctions in the flavonoid content based on the choice of extraction solvent. The highest TFC was recorded when the plant material was extracted with 100% ethanol (23.1 ± 0.9 mg/g), which was twice as high as that obtained with 80% ethanol (12.4 ± 0.2 mg/g). Moreover, the TFC extracted with 80% ethanol was also significantly double that of the TFC obtained with water (6.3 ± 0.9 mg/g). A comparative analysis of the trends in solvent effects on TFC and TPC revealed a disparity between the two. The highest TPC was achieved with 80% ethanol, whereas TFC was greater when using 100% ethanol. This difference reflects the influence of solvent polarity and the chemical nature of flavonoids, which favor less polar solvents like absolute ethanol. Overall, solvents with intermediate polarity, such as 80% or 100% ethanol, appear most effective for extracting both phenolic and flavonoid compounds, as these molecules contain a mix of polar and nonpolar functional groups.

Antioxidant activity

The determination of AA holds significant importance in the field of nutrition due to the valuable insights it offers from fruits, vegetables, and medicinal plants.[15] The AA parameter accounts for the redox properties (hydrogen donors), oxygen radical scavenger presence, such as phenolic compounds including flavonoids. In assessing the AA of plant extracts, two typical assay categories are employed. The first type assesses the ability of plant extracts to act as reducing agents, converting oxidants or metal ions such as ferric and cupric ions into their reduced forms. Common assays in this group include FRAP, which evaluates the reduction of ferric to ferrous ions, and CUPRAC,

which measures the conversion of cupric to cuprous ions. The second type evaluates the capacity of extracts to neutralize free radicals. DPPH and ABTS assays, which utilize stable free radicals, are typical examples. These methods are popular because they are quick, simple, reproducible, and produce results that are directly proportional to the antioxidant content of the sample.

Reducing potential of plant extracts

FRAP assay (Ferric reducing-antioxidant power)

This method evaluates the ability of antioxidants to reduce ferric ions. It relies on the conversion of the yellow ferric–TPTZ (2,3,5-triphenyl-1,3,4-triazine-2-azoniacyclopentadienyl chloride) complex into its blue ferrous form by electron-donating compounds, such as phenolics, under acidic conditions. The reaction is tracked by measuring the change in absorbance at 593 nm. The assay was performed according to the procedure of Benzie and Strain. FRAP values are calculated by comparing the absorbance changes of the samples with those of standard Fe^{2+} solutions at known concentrations.[15]

The antioxidant activity of *Z. spina* leaf extracts prepared with three different solvents is summarized in Table 1, expressed as mmol Fe^{2+} per gram of dry plant material. Statistical analysis indicated significant differences in FRAP values depending on the extraction solvent, with different letters (a, b, c) denoting significance at $p < 0.05$. Table 1 highlights the variation in antioxidant activity among the extracts.

The FRAP values of *Z. spina* extracts increased with solvent polarity, following the order 80% ethanol, 100% ethanol, water. Extracts obtained with 80% ethanol exhibited FRAP values roughly two to three times higher than those from 100% ethanol and water, respectively. This pattern mirrors the trend observed for total phenolic content (TPC) but differs from that of total flavonoid content (TFC), suggesting that phenolics are the main contributors to antioxidant activity. Pearson correlation analysis confirmed a strong and significant relationship between FRAP and TPC, while no significant correlation was observed with TFC (Table 2). Overall, as with TPC and TFC, ethanol-based extractions-whether 80% or 100%-produced higher antioxidant activity compared to water for *Z. spina* leaves.

Table 2 Pearson correlation coefficients among quality indices (TPC, TFC, FRAP, CUPRAC, and DPPH)

	TPC (mg/g)	TFC (mg/g)	FRAP (mmol/g)	CUPRAC (µmol/g)	DPPH (µmol/g)
TPC (mg/g)					
TFC (mg/g)	-0.021				
FRAP (mmol/g)	0.977*	0.129			
CUPRAC (µmol/g)	0.996*	0.0927	0.998*		
DPPH (µmol/g)	0.990*	0.0994	0.989*	0.998*	
ABTS (µmol/g)	0.999*	0.12	0.997*	0.997*	0.996*

*Indicates significance for $p < 0.001$, $n = 9$

Cupric reducing antioxidant power (CUPRAC)

Although the FRAP assay is widely used to assess antioxidant activity, the CUPRAC assay is a more recent method.[10] It utilizes the copper(II)–neocuproine [Cu(II)–Nc] complex as a chromogenic oxidizing agent, measuring the ability of reducing compounds to convert cupric ions to cuprous ions. Table 1 presents the CUPRAC antioxidant activity of *Z. spina* leaf extracts prepared with three different solvents, expressed in µmol Trolox/g. Statistical analysis revealed significant differences in antioxidant activity depending on the extraction solvent, with different letters (a, b, c) indicating significance at $p < 0.05$. The results show that CUPRAC activity follows the order: 80% ethanol > 100% ethanol > water. This pattern is consistent with the trends observed for FRAP activity and total phenolic content (TPC) but differs from total flavonoid content (TFC), suggesting that phenolics are the primary contributors to antioxidant activity. Pearson correlation analysis confirmed a strong correlation between CUPRAC values and TPC, while no significant relationship was found with TFC (Table 2).

Free radical scavenging ability of plant extracts

Free radical scavenging activity using DPPH

DPPH, a stable free radical compound, has found extensive use in assessing the free radical scavenging abilities of various samples.[16] This stable free radical exhibits a characteristic absorption at 517 nm, serving as a marker for studying the radical-scavenging effects of extracts. When antioxidants donate protons to DPPH, the absorption at 517 nm decreases. Antioxidants, upon interaction with DPPH, either donate an electron or a hydrogen atom, thereby neutralizing its free radical character.[17] This interaction causes the color of the solution to change from purple to yellow, accompanied by a decrease in absorbance at the wavelength of 517 nm. The DPPH assay is based on the reaction of the stable free radical 2,2-diphenyl-1-picrylhydrazyl with hydrogen-donating compounds, such as phenolics. The extent of DPPH bleaching increases proportionally with the concentration of the extract.

The DPPH antioxidant activity of *Z. spina* leaf extracts prepared with three different solvents is presented in Table 1, expressed as µmol Trolox/g. Statistical analysis showed significant differences between the extracts, with different letters (a, b, c) indicating significance at $p < 0.05$. The results revealed that antioxidant activity followed the order: 80% ethanol, 100% ethanol, water, consistent with the trends observed in TPC, FRAP, and CUPRAC assays. Correlation analysis confirmed a significant relationship between DPPH activity and TPC, while no significant correlation was found with TFC (Table 2).

Free radical scavenging activity using ABTS

An altered method using ABTS (2,2-azino-di-(3-ethylbenzothialozine-sulphonic acid)) as described by Pellegrini et al was employed in this study.[12] The $ABTS^{+\bullet}$ stock solution (7 mM) was prepared by reacting 7 mM ABTS with 2.45 mM of potassium persulphate as the oxidant agent. The working $ABTS^{+\bullet}$ solution was obtained by diluting the stock solution in ethanol to achieve an absorption of 0.70 ± 0.02 at $\lambda = 734$ nm. A 100 µl sample extract was mixed with 900 µl of $ABTS^{+\bullet}$ solution, and the absorbance readings at 734 nm were taken exactly 10 minutes after initial mixing at 30 °C. The ABTS assay measures the relative antioxidant ability of extracts to scavenge the radical-cation $ABTS^{+\bullet}$ produced by the oxidation of 2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonate. The ABTS antioxidant activity of *Z. spina* leaves extracts using three different

solvents was expressed as µmol Trolox/g (Table 1). Statistical analyses revealed significant differences in antioxidant activity among the three extraction solvents, indicated by different small letters (a, b, and c), denoting significance ($p < 0.05$). The results demonstrated that ABTS antioxidant activity of *Z. spina* extracts followed this order: 80% ethanol, 100% ethanol, water, mirroring the trends observed in CUPRAC, FRAP and DPPH antioxidant activities. This trend also aligns with TPC but differs from TFC, suggesting a correlation between ABTS and TPC.

Pearson correlation analyses

A correlation study was performed to explore the relationships between the antioxidant activities (FRAP, CUPRAC, DPPH, and ABTS) and the levels of total phenolics (TPC) and total flavonoids (TFC), as well as the relationship between TPC and TFC (Table 2). The analysis also examined the interrelationships among the four antioxidant assays (Table 2). Pearson correlation results revealed that all measured antioxidant activities were strongly and significantly associated with TPC, whereas no significant relationship was observed with TFC. Additionally, the antioxidant assays themselves showed strong and significant correlations with one another, highlighting their consistent performance in evaluating antioxidant capacity.

HPLC-PDA and UPLC-PDA-ESI-MS analysis of *Z. spina* extracts

Prior to UPLC-PDA-ESI-MS analysis, preliminary HPLC–PDA studies were con-ducted on three extracts (aqueous, methanolic, and 80% ethanolic), to obtain an initial chemical profile. Figure 1 presents the HPLC-PDA chromatogram of the crude aqueous extract of *Z. spina* monitored at 280 nm. Numerous polar phenolic constituents were eluted between 5 and 25 minutes, whereas a limited number of less polar compounds appeared at longer retention times (58-70 minutes). These constituents exhibited maxi-mum UV absorption in the range of 284-305 nm.

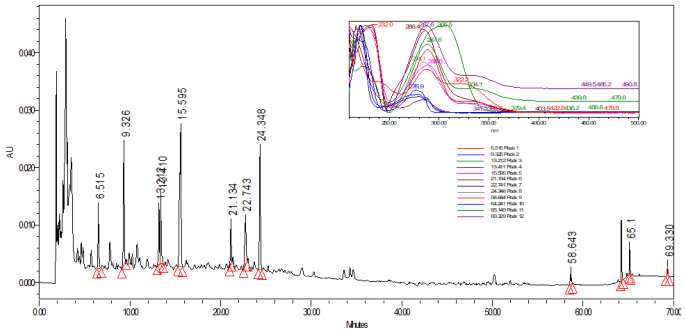

Figure 1 The HPLC-PDA chromatogram of the crude water extract of *Z. spina* at 280 nm, along with their overlaid UV-Vis spectra from 210-500 nm.

As shown in Figure 2, the chromatogram of the crude ethanolic extract recorded at 408 nm revealed a higher abundance of relatively lipophilic compounds compared to the aqueous extract, with elution occurring between 64 and 69 minutes. PDA analysis indicated that these peaks exhibited absorption maxima within the visible region (407-473 nm).

Figure 3 illustrates the HPLC-PDA of the crude 80% ethanolic extract monitored at 408 nm. The overall chromatographic pattern was comparable to that of the pure ethanolic extract, although variations in peak intensities were observed, likely due to differences in solvent composition.

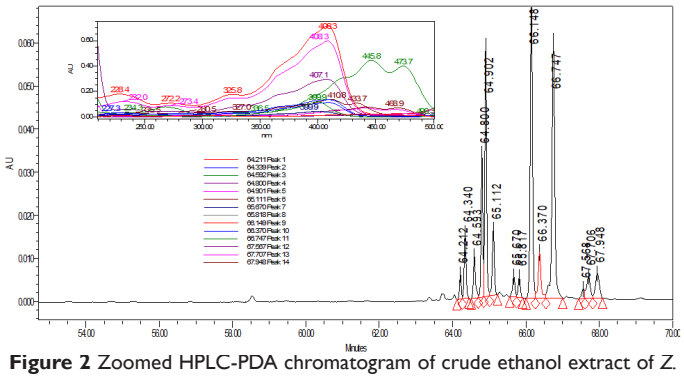

Figure 2 Zoomed HPLC-PDA chromatogram of crude ethanol extract of *Z. spina* at 408 nm, along with their overlaid UV-Vis spectra from 210-500 nm.

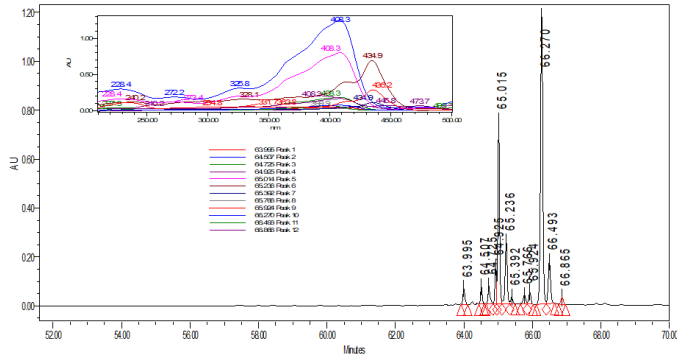

Figure 3 Zoomed HPLC-PDA chromatogram of 80% ethanol extract of *Z. spina* at 408 nm, along with their overlaid UV-Vis spectra from 210-500 nm.

Figure 4 displays the UPLC-PDA chromatographic profile of the 80% ethanolic ex-tract of *Z. spina* recorded at 330 nm, a wavelength selected to enable comprehensive detection of phenolic constituents, including flavonoids and related compounds.

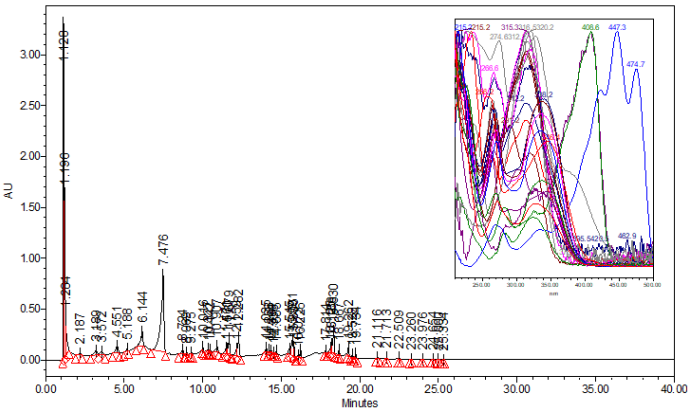

Figure 4 Zoomed UPLC-PDA of *Z. spina* 80% ethanol extract at 330 nm, along with their overlaid UV-Vis spectra from 210-500 nm.

Building on the HPLC-PDA and UPLC-PDA chromatographic profiling described above, Figure 5 presents the expanded UPLC-ESI-MS chromatogram of the *Z. spina* 80% ethanolic extract, acquired in negative ionization mode over a mass range of 200–1200 Da.

Although analyses were conducted in both ESI^+ and ESI^- modes, reliable detection was achieved primarily in the negative ESI mode. The chromatographic and spectroscopic properties of the major metabolites detected are summarized in Table 3. The table includes

retention times (RT), tentative metabolite assignments based on deprotonated molecular ions [M-H]⁻ and comparison with previously reported data.[7,18,19] LC-MS analysis revealed a complex phytochemical profile primarily composed of phenolic acids and flavonoid glycosides (Table 3).

Table 3 Chemical constituents detected in *Z. spina* 80% ethanol extract with corresponding chromatographic and spectral data

Peak	RT (min)	m/z [M–H]⁻	% Area	Tentative identity	Compound class
1	1.128	371	28.31	Hydroxyferuloylquinic acid	Phenolic acid
2	3.189	353	1.41	Chlorogenic acid (caffeoylquinic acid)	Phenolic acid
3	3.572	353	0.99	Chlorogenic acid isomer	Phenolic acid
4	4.551	353, 707	1.89	Chlorogenic acid isomer + dimer	Phenolic acid
5	5.188	707	0.43	Chlorogenic acid isomer dimer	Phenolic acid
6	6.144	707	3.33	Chlorogenic acid isomer dimer	Phenolic acid
7	7.476	353, 707	23.4	Chlorogenic acid isomer + dimer	Phenolic acid
8	10.327	353	0.13	Chlorogenic acid isomer	Phenolic acid
9	10.907	353	1.11	Chlorogenic acid isomer	Phenolic acid
10	14.685	367	0.28	Feruloylquinic acid	Phenolic acid
11	15.701	463	2.79	Quercetin 3-O-glucoside isomer	Flavonoid glycoside
12	15.805	609	0.99	Rutin (quercetin-3-O-rutinoside)	Flavonoid glycoside
13	16.073	463	0.25	Quercetin 3-O-glucoside isomer	Flavonoid glycoside
14	16.225	463	0.88	Quercetin 3-O-glucoside isomer	Flavonoid glycoside
15	18.667	447	0.48	Quercetin 3-O-rhamnoside	Flavonoid glycoside
16	19.262	427, 855	0.81	unidentified + dimer	Flavonoid conjugate

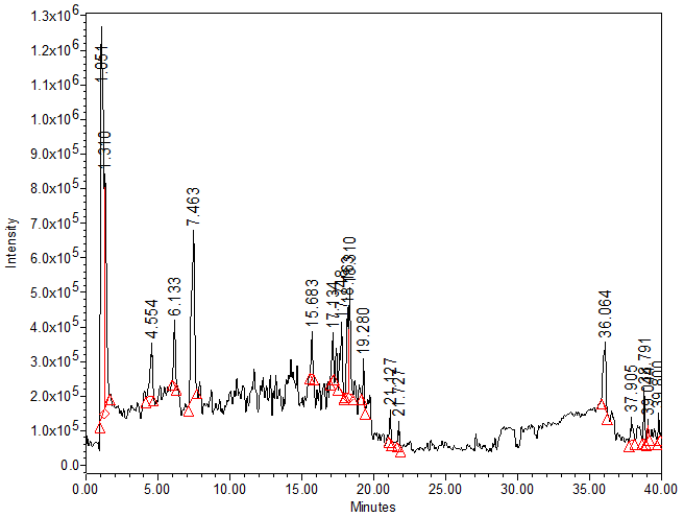

Figure 5 Zoomed UPLC-ESI-MS of *Z. spina* 80% ethanol extract scanned over a mass range of 200-1200Da in the negative mode.

Early-eluting peaks (RT 1.1-7.5 min) were mainly phenolic acids, including hydroxyferuloylquinic acid (m/z 371 Da), the most abundant constituent (28.31% relative area). Multiple peaks corresponding to chlorogenic acid (m/z 353 Da), its positional isomers, and associated dimers ([2M–H]⁻, m/z 707 Da) were also detected. The observed dimers are likely due to the high concentration of these compounds in the extract. Additional phenolic acids, including feruloylquinic acid (m/z 367), were detected at later retention times, further confirming the predominance of hydroxycinnamate derivatives in the polar fraction of the extract.

Later-eluting peaks (RT 15.70–19.26 min) were mainly attributed to flavonoid glycosides. These included several quercetin 3-O-glucoside isomers (m/z 463), rutin (quer-cetin-3-O-rutinoside, m/z 609), and quercetin 3-O-rhamnoside (m/z 447). A minor signal at m/z 427, together with its corresponding dimer at m/z 855, was tentatively assigned to an unidentified flavonoid conjugate.

As a representative example, Figure 6 illustrates the negative-ion ESI mass spectra of a chlorogenic acid isomer and its dimer, eluting at 6.14 and 7.46 min, respectively, thereby supporting their confident detection in the extract.

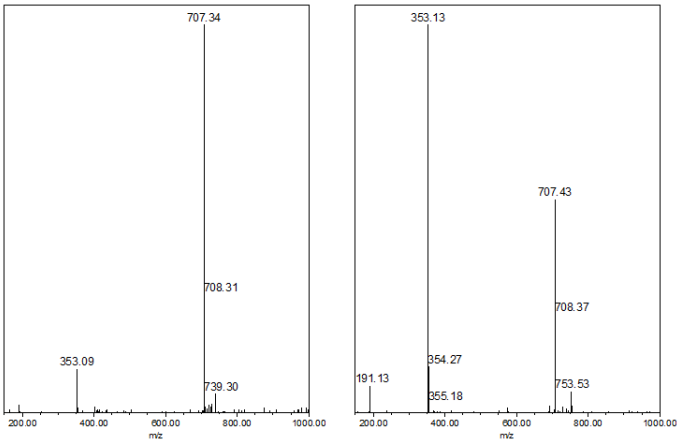

Figure 6 Negative-ion ESI mass spectra of a chlorogenic acid isomer and its dimer identified in *Z. spina* 80% ethanol extract.

Overall, the LC–MS profile demonstrates that *Ziziphus spina-christi* leaf extract is particularly rich in phenolic acids-especially chlorogenic acid derivatives-and quercetin-based flavonoid glycosides. The diversity and abundance of these polyphenolic constituents are consistent with the plant's reported antioxidant and pharmacological properties. The tentative compound assignments and their relative abundances are summarized in Table 3, providing a comprehensive overview of the phytochemical composition of the extract.

Conclusions

This study marks the first exploration of the antioxidant activity of wild *Z. spina* native to Palestine. The findings revealed that the plant extracts exhibited moderate to substantial phenolic content and demonstrated satisfactory DPPH and ABTS radical scavenging activities. Additionally, they displayed notable reducing abilities, as evidenced by the positive results in FRAP and CUPRAC assays. Notably, the extracts yielded the highest concentrations of phenolic and flavonoid compounds when 80% ethanol was used as the extraction solvent. The elevated levels of phenolic compounds and the significant linear correlation observed between the concentration of phenolic compounds and antioxidant activity strongly suggest the substantial contribution of these compounds to the potent antioxidant activity exhibited by the extracts. The UPLC-PDA-ESi-MS analysis of the extracts revealed they contain many active polyphenols particularly flavonoids and anthocyanins.

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None.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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