

Beyond Traditional Medicine: Biochemical Insights into the Therapeutic Potential of *Ziziphus spina-christi*

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ABSTRACT

Background *Ziziphus spina-christi* (L.) Desf. is a medicinal plant rich in bioactive phytochemicals that has potential therapeutic applications. **Objective:** The present study was designed to evaluate phytochemical composition and to investigate the antioxidant, antimicrobial, and cytotoxic activities of various solvent fractions of *Ziziphus spina-christi*(L.) Desf. leaves. **Methods:** Leaf extracts were fractionated using hexane, acetone, methanol, and water. Phytochemical screening, total phenolic content, total flavonoid content, antioxidant activity (DPPH assay), antimicrobial activity (MIC determination) and cytotoxic activity against HeLa cells (MTT assay) were evaluated. **Results:** The methanol fraction had the highest phenolic and flavonoid contents, attended by acetone fraction. Significant antioxidant activity was noticed in two fractions, with lower IC₅₀ values compared with the hexane and aqueous fractions. The methanol fraction accompanied the topmost antimicrobial action against all tested microorganisms and the maximal cytotoxic effect against HeLa cells. **Conclusion:** *Ziziphus spina-christi* (L.) Desf. exhibited antioxidant, antimicrobial and cytotoxic activities, particularly the methanol fraction. These findings provide preliminary evidence supporting further investigation of phytochemical composition, biological mechanism, toxicity, and *in vivo* of these fractions.

Keywords: *Ziziphus spina-christi* (L.) Desf.; Phytochemicals; Antioxidant activity; Antimicrobial activity; Anticancer activity.

INTRODUCTION

Medicinal plants continue to be an important source of biologically active compounds with a wide range of potential therapeutic applications. In recent years, interest in identifying of natural products with antioxidant, antimicrobial and anticancer activities has increased, due to the growing concerns about oxidative stress, antimicrobial resistance and cancer^{1,2}.

Oxidative stress, resulting from excessive production of reactive oxygen species (ROS), contributes to the development of various chronic diseases, including cardiovascular disease, diabetes, neurodegenerative diseases and cancer. Therefore, there has been considerable interest in plant-derived antioxidants as potential alternatives to synthetic compounds³.

Ziziphus spina-christi (L.) Desf. is a medicinal plant widely distributed in the Middle East and traditionally used to treat several diseases. Previous phytochemical studies have reported the presence of flavonoids, phenolic compounds, tannins, saponins and other secondary metabolites, which may contribute for its biological activities⁴.

Although several studies have reported the antioxidant and antimicrobial properties of *Ziziphus spina-christi* (L.) Desf., there are limited comprehensive evaluations of different solvent fractions and their potential cytotoxic activity^{5,6}. Understanding the relationship between phytochemical composition and biological activity may help to identify potential bioactive fractions for pharmaceutical applications^{7,8}.

Therefore, this study was conducted to evaluate the phytochemical composition and investigate the

antioxidant, antimicrobial, and cytotoxic activities of various solvent fractions of *Ziziphus spina-christi* (L.) Desf. leaves.

MATERIALS AND METHODS

Plant Collection and Preparation

Fresh leaves of *Ziziphus spina-christi* (L.) Desf. were collected from healthy, mature plants, thoroughly washed with water distilled water, shade-dried at room temperature, and ground into a fine powder. The powdered material was kept in sealed containers until extraction.

Extraction and Fractionation

Approximately 100 g of dried and powdered *Ziziphus spina-christi*(L.) Desf. leaves were subjected to successive maceration with 80% methanol (v/v). For each extraction cycle, 250 mL of 80% methanol was used, corresponding to a solvent-to-drug ratio of 1:2.5 (w/v). The extraction was performed in three successive cycles, with each cycle lasting 36 h at room temperature under shaking. Thus, a total of 750 mL of 80% methanol was used. After each cycle, the extract was separated from the plant residue, and the residue was re-extracted with fresh solvent under the same conditions. The combined extracts were filtered and concentrated under reduced pressure using a rotary evaporator, yielding approximately 25 g of crude methanolic extract (25% w/w).

The crude methanolic extract was subsequently subjected to sequential solvent fractionation using hexane, acetone, methanol, and water. The resulting fractions were separately concentrated, yielding approximately 2.0 g of the hexane fraction, 5.5 g of the acetone fraction, 9.0 g of the methanol fraction,

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and 8.5 g of the aqueous fraction. The fractions were stored at 4°C until further phytochemical and biological analyses.

The crude methanolic extract was subjected to sequential solvent fractionation using solvents selected according to their relative polarity. Hexane was used first to recover predominantly non-polar constituents, followed successively by acetone and methanol to obtain fractions containing compounds of intermediate to higher polarity, while water was used as the final solvent to recover the more polar constituents. This sequential fractionation approach was selected to obtain chemically differentiated fractions and to facilitate comparison of their phytochemical composition and biological activities. Similar sequential solvent-fractionation approaches have been reported for *Ziziphus spina-christi* (L.) Desf.

leaves and other plant extracts to separate constituents according to differences in solvent polarity.

Phytochemical Analysis

Total phenolic content (TPC) was determined using the Folin–Ciocalteu colorimetric method. Briefly, appropriately diluted fraction samples were reacted with Folin–Ciocalteu reagent and sodium carbonate, and the reaction mixtures were incubated at room temperature for 30 min. Absorbance was measured spectrophotometrically at 765 nm. Gallic acid was used as the reference standard, and a calibration curve was prepared over a concentration range of 10–100 µg/mL. The TPC was calculated from the linear regression equation of the gallic acid calibration curve ($y = 0.0114x + 0.518$; $R^2 = 0.9932$) and expressed as milligrams of gallic acid equivalents per gram of dry extract (mg GAE/g extract). All determinations were performed in triplicate. Total flavonoid content (TFC) was determined using the aluminium chloride colorimetric method. Appropriately diluted fraction samples were reacted with 10% aluminium chloride and potassium acetate and incubated at room temperature for 30 min. Absorbance was measured at 415 nm. Quercetin was used as the reference standard, and a calibration curve was prepared over a concentration range of 20–100 µg/mL. The TFC was calculated from the linear regression equation of the quercetin calibration curve ($y = 0.015x + 0.005$) and expressed as milligrams of quercetin equivalents per gram of dry extract (mg QE/g extract). All determinations were performed in triplicate, and the results were expressed as mean $\pm$ SD⁹.

Antioxidant Activity

The antioxidant activity of the different fractions was evaluated using the DPPH free-radical scavenging assay. Each fraction was tested at different concentrations and incubated with the DPPH solution. Absorbance was measured spectrophotometrically at 517 nm after the reaction period. Percentage inhibition and IC₅₀ values were determined¹⁰.

Antimicrobial Activity

The antimicrobial activity of the different fractions was evaluated using the broth microdilution method against selected pathogenic microorganisms, including *Staphylococcus aureus*, methicillin opposing *Staphylococcus aureus* (MRSA), *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*. Minimum inhibitory concentrations (MICs) were determined as the lowest concentration of the fractions that inhibited visible microbial growth. MIC determinations were performed in three independent experiments, with each determination conducted in triplicate. The MIC was defined as the lowest concentration of the fraction showing no visible microbial growth. Appropriate growth and sterility controls were included in each assay to verify the validity of the test¹¹.

Cytotoxicity Assay

Standard cell culture conditions were used to maintain human cervical cancer (HeLa) cells. Cytotoxic activity was evaluated using the MTT assay after treatment with the tested fractions at concentrations of 0, 25, 50, 100, 200, 400, 800, and 1000 µg/mL for 24 and 48 h. Cell viability was calculated relative to untreated control cells, and IC₅₀ values were determined from the corresponding dose–response curves. The experiments were performed in three independent replicates¹².

Statistical Analysis

Data are presented as mean $\pm$ from three independent experiments. GraphPad Prism version 10.0 was used for statistical analysis. Differences between groups were assessed using one-way analysis of difference (ANOVA) followed by Tukey's post-hoc test. Statistical significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

Phytochemical Composition and Antioxidant Activity

The results of the phytochemical screening are presented in Table 1 and indicated the presence of various bioactive secondary metabolites including flavonoids, phenolic compounds, tannins, alkaloids, saponins and terpenoids. The methanol and acetone fractions contained the highest levels of phenolic compounds and flavonoids, whereas the aqueous fraction contained lower levels of phytochemicals¹³.

As shown in Table 2, the methanol fraction contained the highest total phenolic content (285.4 ± 8.6 mg GAE/g extract) and total flavonoid content (173.2 ± 5.1 mg QE/g extract), followed by the acetone fraction. These fractions also showed stronger antioxidant activity, as indicated by their lower DPPH IC₅₀ values compared with hexane and aqueous fractions.

Figure 1 illustrates the relationship between total phenolic content and DPPH IC₅₀ values across the tested fractions. The four fractions showed negative association, including that fractions with higher phenolic content tended to exhibit lower DPPH IC₅₀ values. ($r = -0.89$, $p < 0.001$). Given the small number of observation ($n=4$), this relationship interpreted descriptively rather than as an inferential statistical association¹⁴.

These results suggest that phenolic and flavonoid compounds are the main compounds responsible for the antioxidant activity of *Ziziphus spina-christi* (L.) Desf.

Phenolics and flavonoids can neutralize reactive oxygen species by hydrogen atom donation and electron transfer mechanisms, thereby reducing oxidative stress and cellular damage. Similar association between polyphenolic content and antioxidant activity have been reported in medicinal plants, where phenolic rich fractions generally show higher radical scavenging activity¹⁵.

Antimicrobial Activity

The antimicrobial activity results summarized in Table 3 revealed that the methanol fraction showed the strongest inhibitory activity against all tested microorganisms. The lowest MIC values were observed against *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA), followed by *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans*. The acetone fraction showed slightly lower but still appreciable antimicrobial activity, whereas the aqueous fraction exhibited the weakest inhibitory effect^{16,17}.

MIC values represent the lowest concentrations showing no visible microbial growth. MIC determinations were performed in three independent experiments, with each determination conducted in triplicate.

Table 1. Phytochemical Screening of Different Fractions of Iraqi *Ziziphus spina-christi* Leaves (L.) Desf

Phytochemical Constituents	Hexane	Acetone	Methanol	Aqueous
Alkaloids	+	++	+++	+
Flavonoids	+	+++	+++	++
Phenolic compounds	+	+++	+++	++
Tannins	-	++	+++	++
Saponins	-	++	+++	++
Terpenoids	+++	++	+	-
Glycosides	-	++	+++	+

Table 2. Total Phenolic Content, Total Flavonoid Content and Antioxidant Activity of Different Fractions

Fraction	Total Phenolic Content (mg GAE/g)	Total Flavonoid Content (mg QE/g)	DPPH IC ₅₀ (μg/mL)
Hexane	92.4 ± 4.3	51.7 ± 2.1	34.6 ± 1.4 ^b
Acetone	241.6 ± 7.2	151.8 ± 4.6	12.4 ± 0.8 ^c
Methanol	285.4 ± 8.6	173.2 ± 5.1	8.7 ± 0.5 ^d
Aqueous	138.2 ± 5.4	82.6 ± 3.5	67.9 ± 2.1 ^a
Trolox	—	—	0.72 ± 0.04

Values are presented as mean ± SD (n = 3). Different superscript letters indicate significant differences among fractions according to one-way ANOVA followed by Tukey's post-hoc test (p < 0.05).

Table 3. Minimum Inhibitory Concentration (MIC) of Different Fractions against Tested Microorganisms (mg/mL)

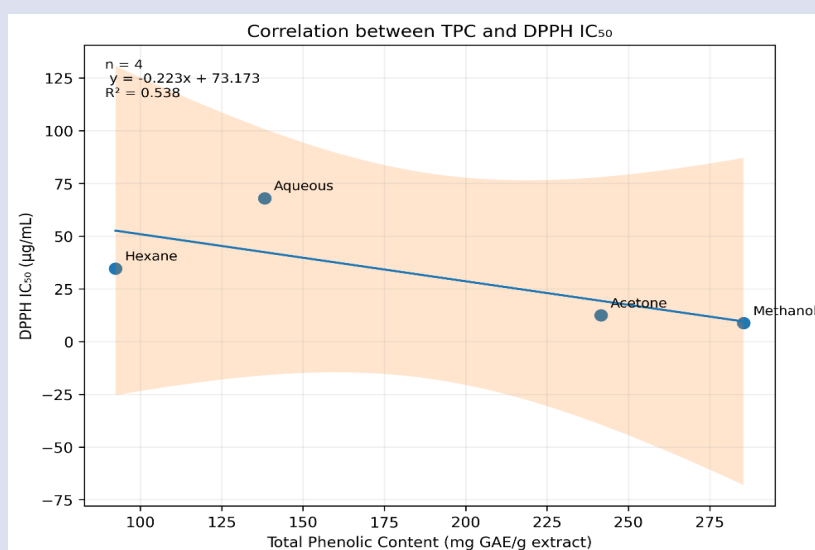
Microorganism	Hexane	Acetone	Methanol	Aqueous
<i>Staphylococcus aureus</i>	1.56	0.78	0.39	3.12
MRSA	3.12	0.78	0.39	6.25
<i>Escherichia coli</i>	1.56	0.78	0.78	3.12
<i>Pseudomonas aeruginosa</i>	3.12	1.56	0.78	6.25
<i>Candida albicans</i>	1.56	0.78	0.78	3.12

Table 4. Cytotoxic Activity of Different Fractions Against HeLa Cells

Fraction	Cell Viability (%) at 24 h	Cell Viability (%) at 48 h	IC ₅₀ at 48 h (μg/mL)
Hexane	68.4 ± 3.2	45.3 ± 2.4	124.8 ± 5.3
Acetone	52.6 ± 2.8	23.5 ± 1.8	78.5 ± 3.1
Methanol	45.2 ± 2.4	13.5 ± 1.2	61.3 ± 2.8
Aqueous	81.4 ± 3.9	63.7 ± 3.1	246.2 ± 8.4

Table 5. Correlation Analysis Between Phytochemical Contents and Biological Activities.

Variables	Correlation Coefficient (r)
Total Phenolic Content vs DPPH IC ₅₀	-0.89
Total Flavonoid Content vs DPPH IC ₅₀	-0.86
Total Phenolic Content vs HeLa Cell Viability	-0.82
Total Flavonoid Content vs MIC Values	-0.79


Figure 1. Correlation between total phenolic content and DPPH IC₅₀

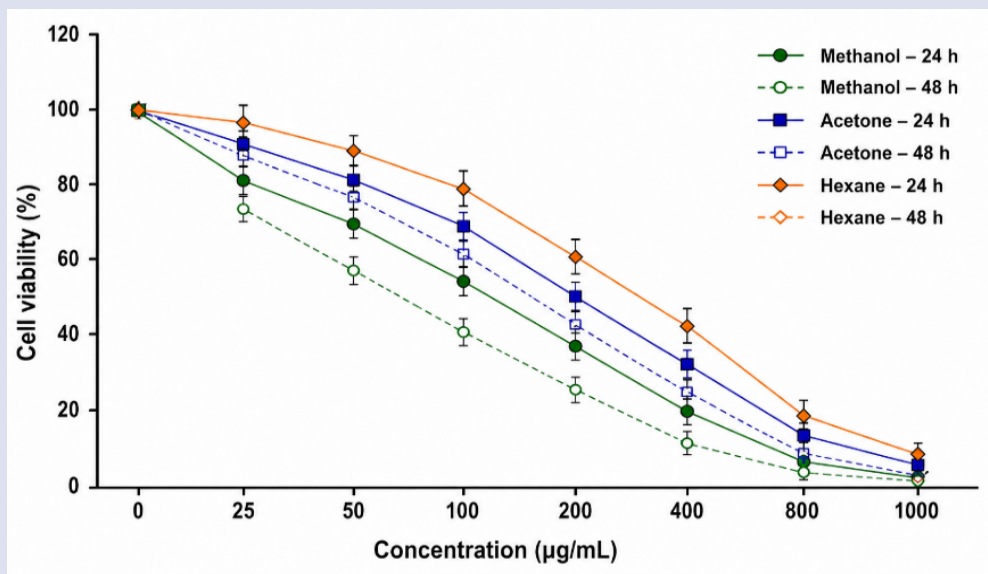


Figure 2. Dose-response curves of selected fractions against HeLa cells. Data are presented as mean $\pm$ SD ($n = 3$)

The observed antimicrobial activity may be associated with the relatively high concentrations of flavonoids, tannins, alkaloids, and saponins detected in the methanol and acetone fractions (Table 1). Furthermore, the correlation analysis presented in Table 5 showed a negative association between total flavonoid content and MIC values ($r = -0.79$), indicating that higher flavonoid content was associated with stronger antimicrobial activity. The antimicrobial effects of these phytochemicals may be related to disruption of microbial cell membranes, inhibition of nucleic acid synthesis, interference with protein production, and impairment of essential metabolic pathways. The greater susceptibility observed among Gram-positive microorganisms compared accompanying Gram-negative microorganisms may be related to differences in cell wall structure, as the outer membrane of Gram-negative bacteria provides an additional permeability barrier¹⁸.

Cytotoxic Activity against HeLa Cells

The cytotoxicity results are summarized in Table 4. Among all fractions, the methanol fraction shown the strongest antiproliferative biological activities against HeLa cells, followed for one acetone and hexane fractions. In contrast, the aqueous fraction exhibited relatively weak cytotoxic activity, as reflected by its significantly higher IC₅₀ value ($246.2 \pm 8.4 \mu\text{g/mL}$). The concentration depended - and time-dependent inhibitory effects of the methanol, acetone, and hexane fractions are illustrated in Figure 2. Increasing extract concentrations resulted in progressive reductions in HeLa cell viability after both 24 h and 48 h of exposure. Moreover, cell being was usually lower after 48 h than later 24 h, displaying a pronounced period-contingent cytotoxic reaction. The aqueous fraction was excluded from the graphical presentation because of its comparatively weak cytotoxic activity and markedly higher IC₅₀ value¹⁹. Nevertheless, its complete cytotoxicity data are presented in Table 4 for comparative purposes.

A limitation of the present study is that cytotoxicity was evaluated only against HeLa cells without inclusion of a normal cell line. Therefore, cancer-cell selectivity and a selectivity index could not be determined. Further studies including appropriate normal cells are required to assess the selectivity and safety profile of the tested fractions.

The superior antiproliferative activity observed in the methanol fraction may be associated with its higher concentrations of phenolic compounds and flavonoids (Table 2). Numerous plant-derived

polyphenols have been reported to promote apoptosis, inhibit cell cycle progression, restrain angiogenesis, modulate angiogenesis, and influence oxidative stress pathways in tumor cells. Consequently, the high phytochemical content of the methanol fraction may contribute to its potent cytotoxic activity^{20,21}.

Correlation Analysis and Biological Significance

Descriptive correlation analysis showed negative associations between phytochemical content and biological activity across the four tested fractions ($n = 4$). TPC was negatively associated with DPPH IC₅₀ ($r = -0.89$), while TFC showed a negative association with DPPH IC₅₀ ($r = -0.86$). TPC and TFC were also negatively associated with HeLa cell viability ($r = -0.82$) and MIC values ($r = -0.79$), respectively (Table 5). However, because only four fractions were included, these correlation coefficients should be interpreted cautiously and are considered descriptive rather than inferential^{22,23}.

Correlation coefficients were calculated using the four tested fractions ($n = 4$) and are presented descriptively. Given the small number of observations, statistical significance was not inferred²⁴.

The combined findings from Tables 1–5 and Figures 1–2 indicate that *Ziziphus spina-christi* (L.) Desf. leaves contain bioactive phytochemicals associated with antioxidant, antimicrobial, and cytotoxic activities. The methanol fraction showed the most prominent overall biological activity, supporting further investigation of its phytochemical composition and biological mechanisms²⁵.

CONCLUSION

Ziziphus spina-christi (L.) Desf. leaf fractions demonstrated antioxidant, antimicrobial, and cytotoxic activities under the experimental conditions of the present in vitro study, with the methanol fraction showing the most prominent overall biological activity. These findings provide preliminary evidence of the biological potential of the plant fractions and support further investigation of their phytochemical composition and underlying mechanisms. However, cytotoxicity was evaluated only against HeLa cells; therefore, cancer-cell selectivity and a selectivity index could not be established. Further studies involving appropriate normal cell lines, detailed chemical characterization, mechanistic investigations, toxicity assessment, and in vivo validation are required before any therapeutic applications can be proposed.

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