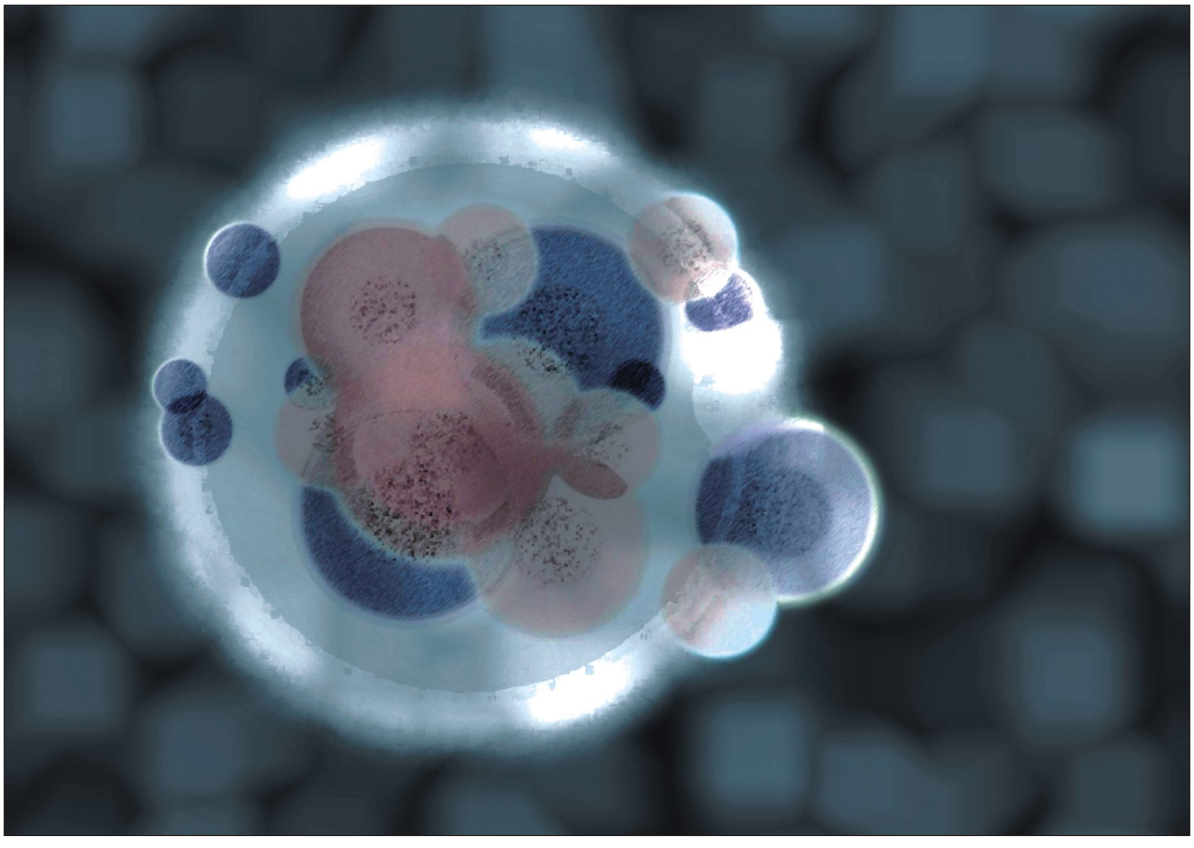

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Research Article
Antimicrobial and Lipid Peroxidation Inhibition Potential of Ziziphus Spina-christi (Sedr), A Jordanian Medicinal Plant

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Abstract

Background and Objective: Anti-microbial agents, specially anti-biotics are becoming less potent against microbial infections due to the development of resistance. The need to find new agents never been more pressing. Natural products such as plant extracts are emerging as possible replacement for the outdated anti-biotics. The overall aims of this study were to evaluate the anti-microbial activities of crude methanolic and ethanolic leaf extracts of Ziziphus spina-christi (sedr), collected from Jordan Valley, against Gram-negative bacteria represented by *E. coli* and *P. aeruginosa*, Gram-positive bacteria represented by *S. aureus* and yeast represented by *C. albicans* and to assess the lipid anti-oxidant potential of the plant leaf extracts in linoleic acid model system. **Materials and Methods:** Methanolic and ethanolic leaf extracts of Z. spina-christi were prepared and tested for their anti-oxidant activities against lipid peroxidation using ammonium thiocyanate method. Agar well diffusion and microtiter plate-based anti-bacterial assay incorporating resazurin as an indicator of cell growth were performed to determine the anti-microbial activities. **Results:** Plant leaf extracts exhibited anti-bacterial activity against both Gram-positive and Gram-negative bacteria. Gram-positive bacteria were found to be more susceptible to both extracts. However, no anti-fungal activity against *C. albicans* was detected for both extracts even at the highest working concentration of 70 mg mL^{-1}. Both extracts inhibited the peroxidation of linoleic acid significantly. **Conclusion:** Ziziphus leaf extracts showed *in vitro* anti-microbial and anti-oxidant activities. Further purification of extracts and identification of the active component is necessary to enhance greater biological activities.

Key words: *Ziziphus spina-christi*, linoleic acid model system, lipid anti-oxidant, anti-bacterial, anti-fungal, methanolic and ethanolic leaf extracts

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Anti-biotics, since their discovery in the 1940s have been used to treat infectious diseases. For the last 70 years, these drugs have greatly reduced illness and death from infectious diseases. Unfortunately, bacteria and other infectious organisms have developed resistance to these drugs over time making them less effective. The lack of discoveries of new anti-biotics put patients at risk and even death. The urge to find alternatives to anti-biotics prompted research to explore natural products as anti-microbial compounds. Plant extracts have been tested as anti-microbials against number infectious bacteria[1].

During the past two decades, a growing number of studies have investigated the diverse health benefits and protective effects of bioactive compounds present in various plants. Plants are rich in a wide variety of secondary metabolites such as tannins, terpenoids, alkaloids, flavonoids, glycosides, etc., which have been found *in vitro* to have anti-microbial and anti-oxidant properties[2-4]. Plant natural products which have anti-fungal, anti-bacterial and anti-protozoal activities were investigated in order to eliminate the use of synthetic anti-biotics which cause the resistance of micro-organisms and can exhibit side effects to human health[5,6].

In addition to the infectious agents that cause health problems, oxidative stress can affect human health. Free radicals and lipid peroxidation are associated with aging, membrane damage, heart disease, stroke, emphysema and cancer in living organisms[7,8]. Different plant extracts have shown ability to prevent lipid oxidation in different lipid environments such as human low-density lipoprotein (LDL) and liposome (1)[9,10] and scavenging activity against various artificially generated free radicals[11,12]. Although there are some synthetic anti-oxidant compounds such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) which are commercially used in food processing, it has been reported that these synthetic anti-oxidants are not preferred due to toxicological concerns. For that there have been increasing interests in identifying plant extracts to minimize or retard the oxidation process in food products[13,14].

Different studies have shown that the medicinal properties and phytoconstituents of the plants are influenced by different factors, such as: The plant species, plant age, harvest seasons, soil nutrients, geographical location, climate conditions, genetic variation, plant parts used, collection period, drying and storage conditions, extraction processes and analysis[15-17]. Considering the huge number of plant species and the great variations among bioactive compounds, it is necessary to identify plant extracts and to develop an integrated approach to screen out their active compounds carrying human health benefits.

Ziziphus spina-christi plants are deciduous shrub belongs to the family Rhamnaceae and grows throughout the Middle Eastern region including Jordan. It is commonly known as "Sedr" also known as 'Nabak'[18]. Since ancient times, it has been among the key plants of the Jordanian traditional medicine. Species of the genus *Ziziphus* have also a long history of use in traditional eastern and western herbalism.

Based on the previous knowledge, there are no studies on the potential anti-microbial and lipid anti-oxidant activities of crude extracts of a wild plant of *Z. spina-christi* grown in Jordan. Therefore, the present study was planned to investigate the anti-microbial activities of crude methanolic and ethanolic leaf extracts of *Z. spina-christi* plant collected from Jordan Valley, against Gram-positive bacteria, Gram-negative bacteria and yeast and to assess the lipid peroxidation inhibition properties of the plant leaf extracts using linoleic acid as a model system.

MATERIALS AND METHODS

Sample collection: Leaves of *Z. spina-christi* plants used in this study were collected from various regions of Jordan valley during the Spring of 2015. The collected plant material was rinsed with sterile distilled water and dried at room temperature for several days.

Preparation of extracts: The dried leaves of *Z. spina-christi* plants were ground and extracted in methanol and ethanol at 20% (w/v) concentration. The mixtures were mixed on rotary shaker for 2 h and then for 15 min in ultrasonic bath. The mixtures were filtered through Whatman no. 4 and then membrane filter (0.45 μm). The crude concentrated extracts were transferred to brown colored sample vial and stored in a refrigerator until used[19].

Determination of anti-microbial activity

Test organisms: Crude ethanolic and methanolic leaf extracts of *Z. spina-christi* plants were screened for their anti-microbial activities against four opportunistic pathogens: One Gram- positive bacteria, *Staphylococcus aureus* (ATCC 25923), two Gram-negative bacteria, *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) and one yeast, *Candida albicans* (ATCC 7596).

Preparation of bacterial inoculum: Direct colony suspension method was used in preparing the inoculums. Few morphologically similar colonies from fresh Muller Hinton Agar plates were transferred into 5 mL of normal saline in a capped test tube and vortexed. The suspension formed was adjusted to give a turbidity equivalent to that of a 0.5 McFarl and standard ($BaSO_4$ prepared spectrophotometrically) to give an approximate 1.5×10^8 CFU mL^{-1}. Mixed suspension then diluted in Muller Hinton Broth to give a concentration of 1×10^6 CFU mL^{-1}.

Preparation of fungal inoculum: One fungal colony was freshly subcultured on sterile Sabouraud Dextrose Agar and incubated at 30°C for 2-5 days. The resultant cells were washed into sterile normal saline and the turbidity adjusted to a 0.5 McFarland standard equivalent. Mixed suspension then diluted to give a concentration of 1×10^6 CFU mL^{-1}.

Agar well diffusion bioassay: An aliquot of 1.0 mL each from both the prepared bacterial and fungal inoculum were uniformly seeded on the solidified agar surface (15 mL, 4 cm thickness) in Petri dishes. Wells of 6 mm in diameter and about 4 cm apart were punctured in the culture media using cork borers, concentrations of 70 mg mL^{-1} of each of methanolic and ethanolic plant leaf extracts were suspended in dimethyl sulfoxide (DMSO): sterile distilled water (1:1 v/v) solvent. Bacterial and fungal cultures were incubated at 37 and 30°C, respectively for 24 h. After incubation, bioactivity was determined by measuring the diameter of inhibition zone (DIZ) in millimeter. All sample were tested three times to assess the reproducibility of the results. Controls containing sterile DMSO: sterile distilled water without plant extracts were also employed[4,20,21].

Determination of minimum inhibitory concentrations (MICs): The MICs of each plant leaf extract at which the growth of the bacteria/fungi became invisible or undetectable were determined as described previously[4,20,21]. The assay was performed using resazurin microtiter plate-based anti-bacterial assay. Resazurin (BDH Laboratory Supplies) was obtained as a tablet and prepared by dissolving a 270 mg tablet in 40 mL of sterile distilled water (according to the manufacturer's specifications). Briefly, 16 h cultures were prepared and then diluted with a sterile normal saline solution [0.85% (w/v) NaCl] with reference to the 0.5 McFarland standards to obtain an inoculums' size of approximately 10^6 colony forming unit mL^{-1}. Final concentrations ranging from 0-70 mg mL^{-1} of each extract were used to determine MIC. Fifty microliters of resazurin and 50 µL of the bacterial suspension per mL of nutrient broth were inoculated into tubes, homogenized and incubated at 37°C. The MIC value was determined as the lowest concentration of the extract in the broth medium that inhibited the visible growth of the test micro-organism.

Determination of anti-oxidant activity in linoleic acid system: The anti-oxidant activity of *Z. spina christi* plant leaf extracts against lipid peroxidation was measured through ammonium thiocyanate assay. The reaction solution, containing 0.2 mL of 5 mg mL^{-1} leaf extract, 0.2 mL of linoleic acid emulsion (25 mg mL^{-1} in 99% ethanol) and 0.4 mL of 50 mM phosphate buffer (pH 7.4) was incubated in the dark at 40°C. A 0.1 mL aliquot of the reaction solution was then added to 6 mL of 70% (v/v) ethanol and 0.1 mL of 30% (w/v) ammonium thiocyanate. After 3 min the addition of 0.1 mL of 20 mM ferrous chloride in 3.5% (v/v) hydrochloric acid to the reaction mixture, the absorbance of the resulting red color was measured at 500 nm. Aliquots were assayed every 24 h until the day after the absorbance of the control solution (without leaf extract) reached maximum value. Trolox was used as positive control. All determinations were performed in triplicate (n = 3)[22].

Statistical analysis: The one-way analysis of variance (ANOVA) and Dunnett t-tests at the significance level $p<0.05$ were conducted using SPSS statistical software (version 10). Data were reported as Mean±Standard deviations. All experiments were performed in three different sets each in triplicates (n = 3).

RESULTS

Antimicrobial activity: The results of anti-microbial activities of the methanolic and ethanolic leaf extracts of *Z. spina christi* against four pathogens were shown in Table 1 and 2. Using

Table 1: Anti-microbial activity of *Z. spina christi* leaf extracts

	Diameter of zone of inhibition (mm±SD)	
Test organism	Methanolic extract	Ethanolic extract
E. coli	12±1.0	7±0.5
P. aeruginosa	13±1.0	9±1.0
S. aureus	24±1.7	20±1.5
C. albicans	-	-

Table 2: Minimum inhibitory concentrations (MIC) of *Z. spina christi* leaf extracts

	Minimum inhibitory concentrations (mg mL^{-1})	
Test organism	Methanolic extract	Ethanolic extract
E. coli	8.7	17.5
P. aeruginosa	8.7	17.5
S. aureus	<2.2	<2.2
C. albicans	-	-

agar well diffusion method, methanolic leaf extracts gave the highest zone of inhibition compared with ethanolic leaf extracts against *S. aureus* (24 and 20 mm), respectively and against *P. aeruginosa* (13 and 9 mm), respectively, while *E. coli* was the least inhibited by the extracts with a zones of inhibition of 12 and 7 mm for methanolic and ethanolic extracts, respectively (Table 1). Regarding the minimum inhibitory concentration (MIC), methanolic and ethanolic leaf extracts showed that *S. aureus* exhibited growth inhibition at a concentration of <2.2 mg mL^{-1} for both extracts, followed by *E. coli* and *P. aeruginosa* growth inhibition at a concentration of 8.7 and 17.5 mg mL^{-1} for methanolic and ethanolic extracts, respectively (Table 2). On the two methods, no anti-fungal activity against *C. albicans* was detected for both extracts at any concentration used.

Total anti-oxidant activity in linoleic acid system: Figure 1 showed the results of antioxidant activity of methanolic and ethanolic leaf extracts of *Z. spina-christi* against lipid peroxidation using ammonium thiocyanate method. The amount of peroxides formed in emulsion during incubation was determined spectrophotometrically by measuring the absorbance at 500 nm. High absorbance was an indication of high concentration of formed peroxides, while low absorbance indicated high anti-oxidant activity. Both ethanolic and methanolic leaf extracts showed the same anti-oxidant potential till the 3rd day. After that, ethanolic leaf extracts showed a significantly ($p<0.05$) higher anti-oxidant potential than the methanolic leaf extracts as indicated by a higher absorbance in the case of methanolic leaf extracts. Interestingly, both leaf extracts of *Z. spina-christi* showed a significantly ($p<0.05$) higher anti-oxidant potential than Trolox, which was used as a positive control.

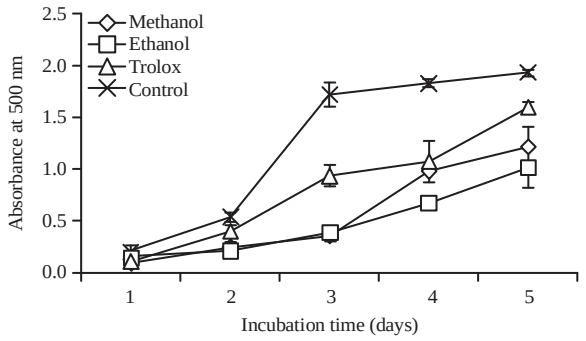

Fig. 1: Anti-oxidant activity *Z. spina-christi* leaf extracts on linoleic acid system

Values are the average of triplicate experiments+SD

DISCUSSION

The utilization of natural anti-biotic and anti-oxidant agents, specially from plant origins, for pharmaceutical purposes had gradually increased in both developing and developed countries, due to their safety and efficacy, in addition to the diminishing efficacy and increasing toxicity of synthetic drugs and increasing occurrence of bacterial resistance against available anti-biotics, it has now become essential to look for newer antibiotics[23,24]. This study explores the anti-microbial and anti-oxidant activities of crude methanolic and ethanolic leaf extracts of *Z. spina-christi* (Sedr), one of the most important traditional medicinal plants in Jordan.

Agar well diffusion and microtiter plate-based anti-bacterial assays were performed to determine the anti-microbial activities of the both leaf extracts of *Z. spina- christi* against opportunistic pathogens (Table 1, 2). In general, the results of this study showed higher anti-bacterial activities of the plant leaf extracts compared with the other studies on the same species in different regions. This might be related to the variation of bioactive compounds profiling and levels in different environments[25]. Methanolic leaf extracts of *Ziziphus* plant were more efficient than ethanolic extract against both Gram-positive and Gram-negative bacteria. Similar results were reported in a study conducted by Ofokansi *et al.*[26], who found that methanolic leaf extracts of *Bryophyllum pinnatum* had a high anti-bacterial activity. This implies that the use of methanol as a solvent release some secondary metabolites which have high activity against some micro-organisms[5,27]. In addition, the results of this study indicated that Gram-positive bacteria were more susceptible to both extracts. These results are not in agreement with that reported by Ohikhena *et al.*[4], that Gram-negative bacteria were more susceptible to the crude extracts of *P. capitata* plant leaves. On the other hand, no anti-fungal activity against *C. albicans* was detected for both extracts even at the highest working concentration of 70 mg mL^{-1}. This result was in supported with those investigations on *Phragmanthera incana* plants by Ogunmefun *et al.*[28], which did not show any anti-fungal activity.

The total antioxidant activity of methanolic and ethanolic leaf extracts of *Z. spina-christi* were determined by using ammonium thiocyanate method. Both extracts can significantly inhibit peroxidation of linoleic acid and reduce formation of hydroperoxide more than Trolox, a widely used commercial anti-oxidant, thus impling that extracts from *Z. spina-christi* are powerful natural anti-oxidants. Several studies have indicated that medicinal plants contain a wide

variety of natural compounds which possess a different biological activities[29-32]. The preliminary phytochemical analysis of *Ziziphus* plant species grown in Jordan reveals the presence of several secondary metabolites such as: Phenols, flavonoids, tannins and alkaloids, which could attribute directly to their anti-oxidant and anti-microbial actions[12,15,33].

By showing a high anti-microbial and lipid anti-oxidant potential of the *Z. spina-christi* plant, that was collected from Jordan Valley, the results of this study may contribute to the production of new and more efficient anti-microbial and anti-oxidant materials and to provide more knowledge about this Jordanian medicinal plant since few studies were done on this plant species and its ecotypes.

CONCLUSION

Crude methanolic and ethanolic leaf extracts of Z. *spina-christi* demonstrated an anti-bacterial activity against Gram-positive and Gram-negative bacteria and showed anti-oxidant potential as indicated by inhibited peroxidation of linoleic acid significantly. Methanolic and ethanolic extracts of *Z. spina-christi* may have a possible role in the treatment of many diseases and the potential use as anti-microbial and anti-oxidant agents. Further purification of the extracts and identification of the active component is necessary to enhance greater biological activities.

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