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Original Article

A Comparative Study on the Effects of *Ziziphus Spina-christi* Alcoholic Extracts on Growth and Structural Integrity of Bacterial Pathogens

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Abstract

In different folk medicine *Ziziphus spina-christi* is used for different purposes such as pneumonia, dysentery, scorpion stings, cough, constipation, intestinal worms and fever. The aim of this study was evaluation and comparing the antibacterial activity of methanolic and ethanolic extracts of *Ziziphus spina –christi* as well as subsequent structural changes in affected bacteria. For this purpose, ethanolic and methanolic extracts were prepared by 80% alcoholic solution. Antibacterial activity of these extracts was assessed using standard disc diffusion method against pathogenic bacteria. Sterile filter paper discs (6mm) were saturated by four different concentrations of each extract. The prepared discs were placed on lawn cultures of test bacteria and incubated at 37 ^{0}C for 24 h. After incubation the inhibition zone diameter around each disc was measured in millimeter. The induced changes in shape of affected bacteria were discovered using scanning electron microscopy (SEM). As a result of this study maximum inhibition zone diameter in case of methanolic extract were 18 and 14 against *Staphylococcus aureus*, *Bacillus cereu*, and in case of ethanolic extract was 15 mm for *S. aureus* and *Proteus mirabilis*. The methanolic extract of this plant was more effective against *S. aureus* and *B. cereus* than the ethanolic extract even at high concentration. While the ethanolic extract was more active on *Proteus mirabilis*. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) indexes of both extracts were equal (MIC= MBC=8 $^{mg}/_{ml}$) for *S. aureus*. The SEM analysis revealed cell deformation and irregular shape in both *S. aureus* and *B. cereus*. These results suggest significant antibacterial activity of this plant especially against *S. aureus*, which its resistant strains are currently a

great hazard in infection treatment. So, this plant should be considered as a potential source for finding new antibacterial agents.

*Key word***:** antibacterial activity**,** ethanolic extract, medicinal plant, methanolic extract, MIC, MBC, *Ziziphus spina-christi*.

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1. Introduction

Ziziphus species *(Rhamnaceae)* are widely distributed from Africa to India and are one of the most important trees that grow in the dry parts of tropical Asia, Africa and also South of Iran [1]. This genus approximately consists of 100 species locally known as sidr in Iran [2]. *Z. spina-christi* has very nutritious fruits that fresh ones are usually eaten. The fruits are applied on cuts and ulcers [3, 4]. Pneumonia, dysentery, scorpion stings, cough, constipation, intestinal worms, and fever are some of indications for application of this plant [4, 5]. It has showed that leaves extracts of this plant has antimicrobial, antinociceptive, antidiabetic, and antihyperglycaemic effects [6]. The extract of *Z. spina-christi* was shown to contain beutic acid and ceanothic acid, cyclopeptides, as well as saponin glycoside and flavonoids, lipids, protein, free sugar and mucilage that some of them have antibacterial effects [3, 5].

Nowdays, traditional medicine is most popular in developing countries because several pharmaceutical agents that are presently used in modern medicine have been derived from products initially used in traditional medicine [7]. These drugs entirely consist of single plant extract or its fractions or mixtures of fractions/ extracts from different plant species, which have been carefully formulated based on their safety and efficacy [8]. *Ziziphus spina-christi* is one of the most widespread native plant in Khouzestan, southwest of Iran that grows in mountainous regions of this province and provides a vast and cheaply available source for finding new antibacterial agents [5]. The aim of the present study was evaluation and comparing antibacterial activity of methanolic and ethanolic

extracts of *Ziziphus spina–Christi* leaves and investigation of the structural changes that made following challenging pathogenic bacteria with these extracts.

2. Materials and Methods

2.1. Plant Collection and Identification

The plants were collected from Ahvaz, Iran and were identified based on herbarium present in the department of biology.

2.2. Plant Extracts Preparation

The leaves of this plant were dried at room temperature for 10 days and then powdered using electric blender. Then ethanolic and methanolic extracts were separately prepared using 1 g of each plant powder and 10 mL of 80% ethanol or methanol (ethanol or methanol-distilled water, 8:2 v/v). After 1 min of sever mixing, the samples were centrifuged (3500 rpm, 20 min) and finally, the supernatant was harvested. This process was repeated three times for full extraction and the solvents were then evaporated at room temperature [9, 10].

2.3. Bacterial Strains

The test microorganisms used in this study were as follow: Gram-positive bacteria: *Bacillus cereus, Staphylococcus aureus,* and Gram-negative bacteria, *Salmonella* Typhi, *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Proteus mirabilis.* All of these isolates were from clinically isolated origin.

2.4. Determination of Antibacterial Activity

Antibacterial activity of the ethanolic and methanolic extracts was assessed using Kirby-Bauer method [11]. Stock cultures of test bacteria were grown in nutrient broth medium (Merck, Germany) at 37 ^{0}C for 22 h. A lawn culture of understudy bacteria was prepared on Muller-Hinton agar (MHA, Merck) using sterile cotton swab from broth cultures with 0.5 McFarland turbidity [12]. Four concentrations of each extract (100, 200, 400 and 600 $^{mg}/_{mL}$) were prepared and sterile filter paper discs (6 mm diameter) were saturated through adding 40 µL of each concentration. So, final concentration of effective substances in each disc was 4, 8, 16 and 24 mg, respectively [13]. These discs were placed on lawn cultures and plates were left at

room temperature for about 1 h to allow diffusion of extract into culture medium and then were incubated at 37 °C for 24 h. Diameter of inhibition zone around each disc was measured based on millimeter after 24h incubation. Routinely used antibiotic discs including Nafcillin, Carbenicillin, Novobiocin, Doxycyline, and Colistin were also tested parallel to prepared extracts. In order to determine the possible inhibitory effect of solvents, i.e, ethanol and methanol, on test bacteria, discs containing 80% ethanol and methanol were also included [12].

2.5. Minimum Inhibitory Concentration (MIC)

In order to determine the minimum inhibitory concentration (MIC), a twofold serial dilutions (0.5, 1.0, 2.0, 4.0, 8.0, 16.0 and 32 $^{mg}/_{ml}$) of each extract was prepared. These dilutions were prepared in tubes containing 1 mL Muller Hinton broth and 30 µL of bacterial suspension equal to 0.5 McFarland. Then tubes were incubated at 37 °C for 24 h. The MIC of the extract was determined for the most sensitive bacterial species. The minimum concentration of crude extract in broth medium that had inhibited the growth of the test microorganism was considered as MIC [14].

2.6. Minimum Bactericidal Concentration (MBC)

To determine the minimum bactericidal concentration (MBC), a loopful of broth from those tubes without visible growth in the MIC assay was cultured on freshly prepared sterile Muller-Hinton agar and incubated (37 °C, 18-24 h). After incubation, the highest dilution (least concentration) that was able to prevent colony formation on agar was considered as MBC [13].

2.7. SEM Analysis

For finding the possible structural changes induced consequence of bacterial exposure to extracts, a sample from those species that had been inhibited by extract were prepared for scanning electron microscopy (SEM). These samples were carbon coated and studied by SEM at central laboratory of Shahid Chamran University.

3. Results and Discussion

The results of antibacterial activity are presented in Table 1. Methanolic and ethanolic extracts yielded from leaves of *Z. spina-christi*

Table 1. The inhibitory effect of different concentrations of ethanolic and methanolic extracts prepared from *Z.spina-christi* on tested bacteria.

		Concentration of extract ($^{mg}/_{disc}$)							
		Ethanolic				Methanolic			
Bacterial Spp.		4*	8	16	24	4	8	16	24
Gram +	*S. aureus*	8	12	14	15	10	12	14	18
	B. cereus	9	10	12	12	10	12	11	14
Gram -	*S.* Typhi	R	R	R	R	R	R	R	R
	E. coli	R	R	R	R	R	R	R	R
	P. aeruginosa	R	R	R	R	R	R	8	R
	K .pneumoniae	R	R	R	R	R	R	R	R
	P. mirabilis	R	11	13	15	7	7	9	12

R: Resistant, * Inhibition Zone (mm), diameter of disc (6mm).

Table 2. Antibacterial activity of clinically used antibiotics.

		Antibiotic discs				
Bacterial Spp.		NF	CB	NB	DX	CL
Gram +	*S. aureus*	R	13*	30	15	R
	B. cereus	R	7	18	18	R
Gram -	*S.* Typhi	R	23	30	26	R
	E. coli	R	R	17	11	R
	P. aeruginosa	R	R	12	R	15
	K. pneumoniae	R	R	15	R	11
	P. mirabilis	R	15	17	R	R

NF: Nafcillin (1 mcg), CB: Carbenicillin (100 mcg), NB: Novobiocin (30 mcg), DX: Doxycyline (30 mcg), CL: Colistin (10 mcg). * Inhibition Zone (mm).

showed significant antibacterial activity against pathogenic gram positive (*B. cereus* and *S. aureus*) and gram negative (*P. mirabilis*) bacteria. On the other hand, these extracts had no activity against other tested bacteria namely *S.* Typhi, *E. coli*, *P. aeruginosa*, and *K. pneumoniae* even at highest concentration. The maximum inhibition zone diameter was related to *S. aureus* (18 and 15 mm for methanolic and ethanolic extract, respectively) at highest concentration of effective dose (24 mg). The least effect was related to *P. mirabilis* (inhibition zone diameter 7-12 mm) in case of methanolic extract. The methanolic extract was more efficient against *S. aureus* and *B. cereus* but the ethanolic extract had higher inhibitory effect on *P. mirabilis.* The results of antibacterial activity of standard antibiotics are presented in Table 2. Interestingly, the results showed that all of the tested bacteria were resistant to Nafcillin. Highest concentration of methanolic extract from *Z. spina-christi* showed higher antibacterial

Table 3. MIC and MBC indexes for *Z.spina-christi* hydroalcoholic extracts against *S. aureus*.

	Ethanolic extract ($^{mg}/_{mL}$)		**Methanolic extract ($^{mg}/_{mL}$)**	
Bacterial Spp.	MIC	MBC	MIC	MBC
S. aureus	8	8	8	8

MIC: Minimum Inhibitory Concentration, MBC: Minimum Bactericidal Concentration.

activity against *S. aureus* comparing to the activity of standard antibiotics except for Novobiocin. Discs containing 80% ethanol and methanol didn't have any inhibitory effect on the under study bacteria. MIC and MBC values of ethanolic and methanolic extracts for *S. aureus* are shown in Table 3. As we can find, MIC and MBC values of both extracts were equal (MIC= MBC=8 $^{mg}/_{ml}$).

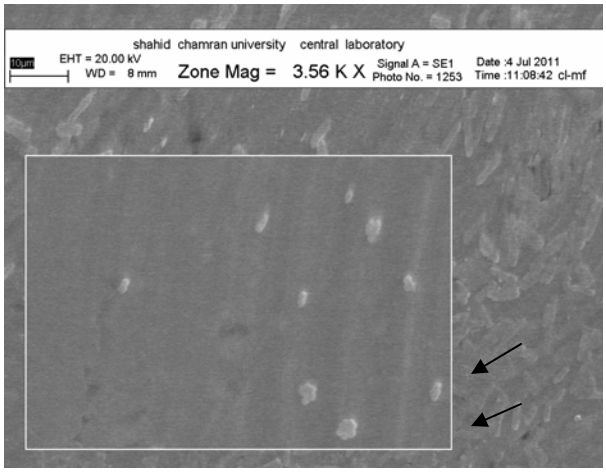

Figure 1. The structural changes of *S. aureus* following exposure to hydroalcoholic extract of *Z.spina-christi.* The arrows show deformed bacterial cells.

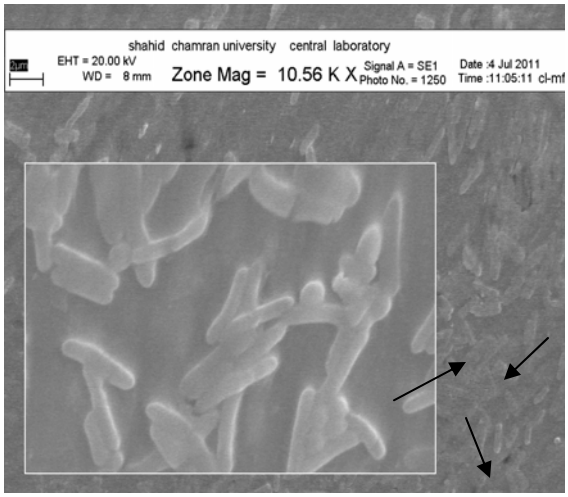

Figure 2. The structural changes of *B. cereus* following exposure to hydroalcoholic extract of *Z.spina-christi.* The shortened and irregular cells are noticeable.

In SEM analysis of *S. aureus* as it can be found from Figure 1, the bacterial cells were deformed. The round shape of this bacterium changed to oval or irregular shapes. Also, in case of *B. cereus* the rod shaped bacteria have been deformed (Figure 2). The length of bacteria has been shortened and they have gained somehow round, ovoid or irregular shapes. These structural changes can be consequences of cell wall and cell lysis loss of integrity.

Since plants produce a variety of compounds with antimicrobial properties such as antibacterial activity, they are good candidates for exploring new antibacterial drugs [8]. In this study, hydroalcoholic extracts of *Z. spina-christi* showed significant antibacterial activity against

S. *aureus, B. cereus,* and *P. mirabilis.* Nisar *et al* (2010), in Pakistan, had obtained similar results in their experiments on some pathogenic microorganisms such as *Bacillus subtilus, S. aureus,* and some fungi and yeasts such as *Trichophyton longifusus*, *Candida albicans*, *Aspergillus flavus,* and *Microsporum canis* [15]. Mohamed *et al* (2010) reported that methanolic extract of *Z. spina-christi* leaves have significant antibacterial activity against *E. coli, S. aureus,* and *K. pneumoniae* [16]. Abalaka *et al.* (2010) compared the antibacterial effects of ethanolic extracts obtained from leaves of two species of Ziziphus genus in Nigeria and determined that *E. coli* was the most susceptible while *S. aureus* had the least susceptibility [3]. Some differences in these results may be due to ecophysiological and weather conditions, the plant species, time of plant collection, storage conditions, methods of extraction, and different sensitivity of the test strains. The extract of *Z. spina-christi* was shown to contain beutic acid and ceanothic acid, cyclopeptides, as well as saponin glycoside and flavonoids, lipids, protein, free sugar and mucilage [3, 5]. In some studies, it has been reported that some of these detected organic compounds in the extracts including tannins, saponins, polyphenols and alkaloids have antimicrobial activity and could be the main reason for the antibacterial activity of *Z. spina-christi* extract [3, 5, 7, 11, 15, 16, 17, and 18]. Some kinds of flavonoids such as furocoumarins and furanocoumarins are reported in some studies that can inhibit growth of bacteria by disrupting DNA replication. Also, unsaturated fatty acids represent the major component of *Z. spina-christi* and maybe responsible for its antibacterial activity [5, 16]. According to the results, the methanolic extract was more effective against gram positive species than ethanolic extract while the ethanolic extract was more potent on *P. mirabilis*. Both extracts had no antibacterial activity against *S.* Typhi, *E. coli, P. aeruginosa* and *K. pneumoniae* even at the highest concentration. All of these bacteria are gram negative. The cell wall components of gram positive bacteria are different from those of gram negative bacteria and that is due to the presence of outer membrane envelope that surround the cell wall of these types of bacteria. This can acts as a barrier to the entrance of different chemicals including antibacterial

agents [3]. The two extracts have equal MIC and MBC indexes against *S. aureus.* This shows that active constituents present in these extracts are bacteriostatic versus bactericidal. A bactericidal drug kills pathogens at levels only two or four times more than MIC whereas a bacteriostatic drug kills pathogens at much higher concentration [3].

4. Conclusion

Based on the obtained results it can be concluded that hydroalcoholic extracts yielded from *Ziziphus spina-christi* are suitable for fighting bacterial pathogens especially *S. aureus* which its resistance to present antibiotics is an increasing alarm and it might be a life in next future threatening pathogen in both community or hospital acquired infections. Further studies are needed to find the bioactive constituents of this antibacterial plant and to be used in formulation of antibiotics.

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