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Phytochemical Screening and Antimicrobial Activity of EthOH/Water *Ziziphus jujuba* Seeds Extracts

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The *Ziziphus* species (Rhamnaceae family) are considered to be multipurpose plants and have been used as foods, folklore medicines, the environmental protection plants. This study was conducted to isolated and evaluate the antimicrobial activity of ethanol extract of *Ziziphus jujuba* seeds against sixe bacterial strains by determining minimum inhibitory concentration (MIC) and analyzed their content by using chromatographic techniques to identify the principal bioactive phytochemicals. GC/MS analysis of ethanol extract of *Ziziphus jujuba* seed revealed the existence of 20component, main components were 13-Heptadecyn-1-ol (12.95%), 7-Ethyl-4-decen-6-one (9.73%), Lineoleoyl chloride (8.54%), Linoleic acid (6.37%), 2,5-Octadecadiynoic acid, methyl ester (5.57%) and Palatinol A (4.81%). The results indicated that the ethanolic extract of *Ziziphus jujuba* seed contains a many bioactive components that could have advantage offer a platform of using *Ziziphus jujuba* seed as herbal alternative for the current synthetic antimicrobial agents.

Key words: Phytochemical Screening, Antibacterial activity, *Ziziphus jujuba*, Medicinal plants.

In the last decade, phytochemicals have attracted a growing attention as alternative traditional medicine[1,2]. Traditional medicine is the oldest method of curing diseases and infections and various plants have been used in different parts of the world to treat human diseases and infections[3-5]. A knowledge of the chemical constituents of plants is desirable, not only for the discovery of therapeutic agents, but also because such information may be of value in disclosing new sources of such economic materials as tannins, oils, gums, precursors for the synthesis complex chemical substances, etc. In addition the knowledge of the chemical constituents' plants would further be valuable in discovering the actual value of folkloric remedies[6].

The *Ziziphus* species (Rhamnaceae family) are considered to be multipurpose plants and have been used as foods, folklore medicines, the environmental protection plants, etc.[7]. *Ziziphus jujuba* Mill. mainly distribute d in the tropical and subtropical regions of Asia and have been employed as essential oriental medicine for thousands of years. Different parts of the plant could be used as remedies in insomnia, fever, diarrhoea, wounds and ulcer, in which the fruits

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were claimed to be beneficial to purify the blood and aid digestion[8,9]. Also it has been widely distributed in northern China. Its fruits and seeds are usually applied in traditional medicine (TCM) for the treatment of various diseases, such as anorexia, lassitude, insomnia, anxiety, etc. [10], and many studies about their chemical constituents[11-13] as well as pharmacology activities[14,15] have been reported.

This study aimed to evaluate the anti-microbial activity of *Z. jujuba* extracts and identify the active compounds of *Z. jujuba* seeds extract. To our knowledge this is first report on the study of anti-microbial components extracted from *Z. jujuba* seeds against the clinical Pathogens action. The assessment might provide a basis for searching the potent active compounds for the antimicrobial related search and improve the therapeutic application of *Ziziphus* species.

MATERIALS AND METHODS

Materials

Fresh *Ziziphus jujuba* seeds were obtained from the local market, Riyadh, Saudi Arabia. The seeds were cleaned, then ground to a fine powder using an electric grinder to pass a 0.4 mm screen. All chemical reagents used in this study were of analytical grade.Deionized water was produced with a Milli-Q system from Millipore (Saint-Quentin-en-Yvelines, France). All solutions were stored at 4°C in the dark.

Methods

Preparation of extracts

About 10 g of *Ziziphus jujuba* seeds powder were soaked in 100 ml solvent with agitation at 40°C overnight, as previously reported[16]. The extract was filtered and dried over anhydrous sodium sulfate and finally evaporated under steam of nitrogen using sample concentrator model Techne DB.3 (Techne, UK). Several solvents (Ethanoland Ethanol /water (1:1)) were tested. Ethanol was tested in our study as it is less toxic than methanol, and so it could be an interesting solvent for the extraction of polar volatile compounds from *Ziziphus jujuba* seeds. The yield of the aqueous-ethanol extract was 1.8 g, the extract was stored at -20°C until further use. Aliquot of the extract was resolved in Dimethyl sulfoxide (DMSO) to a final concentration of 1.0 mg/mL.

Test Microorganisms

Six bacterial strains used in this study, including *Bacillus cereus, Staphylococcus aureus,* as Gram positive bacterium. *Escherichia coli, Pseudomonas aeruginosa, Klebsiella peunomonia* and *Listeria monocytogenes*as Gram negative bacterium. These organisms were obtained from ATCC (American Type and Collection Center). The bacteria rejuvenated in Mueller-Hinton broth MHB (Difco, USA) at 37oC for 18 h and then stocked at 4°C in Mueller-Hinton Agar MHA (Sigma, USA).

Phytochemical Profiling

The phytochemical screening of the sample was carried out as described by[14, 15]. The samples were screened for the following components .

Test for Carbohydrates Molisch's Test

To 2ml of plant extract, 1ml of Molisch's reagent and few drops of concentrated sulphuric acid were added. Formation of purple or reddish ring indicates the presence of carbohydrates.

Test for Tannins

Ferric Chloride Test

To 1ml of plant extract, 2ml of 5% greenish black indicates the presence of tannins.

Test for Saponins

Foam Test

To 1ml of plant extract, 5-10ml of distilled water was added and shaken in a graduated cylinder for 15minutes lengthwise. Formation of 1cm layer of foam indicates the presence of saponins.

Test for Flavonoids

Sulphuric Acid Test

A fraction of the extract was treated with concentrated sulphuric acid and observed for the formation of orange color.

Test for Alkaloids

Mayer's Test

To 2ml of plant extract, 2ml of concentrated hydrochloric acid was added. Then few drops of Mayer's reagent were added. Presence of green color or white precipitate indicates the presence of alkaloids.

Test for Anthocyanin and Betacyanin

Sodium Hydroxide Test

To 2ml of plant extract, 1ml of 2N sodium hydroxide was added and heated for 5minutes at 100°C. Formation of bluish green color indicates

the indicates the presence of betacyanin.

Test for Glycosides

Sulphuric Acid Test

To 2ml of plant extract, 1ml of glacial acetic acid and 5%ferric chloride was added. Then few drops of concentrated sulphuric acid were added.Presence of greenish blue color indicates the presence ofglycosides.Ferric chloride was added. Formation of dark blue orpresence of anthocyanin and formation of yellow color indicates the presence of betacyanin.

Test for Phenols

Ferric Chloride Test

To 1ml of the extract, 2ml of distilled water followed by few drops of 10% ferric chloride was added. Formation of blue or green color indicates presence of phenols.

Antibacterial assay

The method reported by Baqir*et al*.1985[17] has been adopted. The tests were run in triplicate. Petri plates (23x23 mm) were prepared with Trypticase soy agar and an adequate amount of inoculum was flooded onto each plate, excess inoculum was removed and the plates were dried for 30 min at 37°C. Holes (6 mm diameter) were made in the inoculated agar and filled with samples of plant extracts, plates were incubated for 24 hrs at 37°C. Inhibition zones when present were measured in millimeter (Table 2).

Testing for Antimicrobial Activity: Antibacterial activity of the extracts were determined using a modified Kirby-Bauer[18] disc diffusion method. All the bacterial test strains maintained on NA were freshly subcultured for 24-48hrs at 37 °C. Saline suspension of each test strain was prepared and turbidity matched to 0.5 McFarland standard to yield a bacterial suspension of 1.5×10cfu/ml. freshly prepared Mueller Hinton Agar 8MHA plates were seeded with the test inoculums to obtain a lawn culture. Sterile Whatman No. 1 filter paper discs (5mm diameter) impregnated with different concentrations of plant extracts (50 and 100 µg/disc) were placed on the inoculated MHA plates. 5% DMSO served as negative control, Norfloxacin (10µg) and Tetracycline (30µg) were used as standard antibiotics. Post incubation at 37°C for 24-48h, plates were read for zone of inhibition around the disc. Anti-fungal susceptibility testing was carried out as described for antibacterial testing with SDA as the assay medium and Amphotericin B (100U/D) as standard antifungal agent.

Determination of Minimum Inhibitory Concentrations (MIC)

The anti-microbial activity of the *Z. jujuba* extract, that shows anti-bacterial activity, were determined using micro dilution broth method as described by Brantner and Grein, 1994 [19]. Different antibiotics [Ampicillin, amikacin, gentamicin, kanamycin, and tetracycline (10–32 µg/ml)] were used as reference standards (CLSI, 2011). The *Ziziphus jujuba* extract solution was prepared to obtain final concentrations of 0.25-2.0 mg/ ml for antibacterial testing. One microliter of an overnight culture of each bacterial strain, containing approximately 10^4 CFU, was applied onto a 96-well plate in the presence of MHB. The microtiter plates were incubated at 35°C for 18 h. Observations were performed at least in replicate and results were expressed as the lowest concentration of plant extracts that produced a complete suppression of colony growth, MIC.

RESULTS AND DISCUSSION

With the increase in the incidence of resistance to antibiotics,alternative natural products of plants could be of interest.Some plant extracts and phytochemicals are known to haveantimicrobial properties, which could be of great importancein the therapeutic treatments. In the last years, various studieshave been conducted in different countries, demonstrating theefficacy of this type of treatment[20]. The chemical composition of Ziziphus seeds is given in (Table 1). These seeds are a rich source of fiber and protein. The fiber may be further classed as gum (gel fiber) and neutral detergent fiber. The protein fraction contains the amino acid 4-hydroxyisoleucine, which has been proven to stimulate insulin production.

The preliminary phytochemical screening of seeds extracts of *Z. jujuba* (Table 2) revealed the presence of various chemical compounds such as alkaloids, saponins, flavonoids, phenols, glycosides, Anthocyanin, betacyanin, and steroids,some of which have been previously associated with pharmacology activity[21]. Since there are no reports available exclusively on *Z. jujuba*, the phytochemical content of *Z. jujuba*

in the present study was comparable with the available literature on phytochemical content of related species[22-24] respectively.

Ziziphus jujuba 50% aqueous-ethanol were screened for their antimicrobial activity at two different concentration (50 µl and 100 µl) against *Bacillus cereus, Staphylococcus aureus, Escherechia coli, Pseudomonas aeruginosa, Klebsiella pneumonia* and *Listeria monocytogenes* (Table 3). The results were given in table 2.Aside from concerns with food quality degradation, these microorganisms may be causal agents of intestinal infections in humans. The extract inhibited all the tested bacteria, suggesting a broad antimicrobial activity of *Ziziphus jujuba* extract in a concentration-dependent manner.

The 50% ethanol extract of *Ziziphus jujuba* seeds was evaluated, the results of this work reported that it has different varieties of phytochemicals that could be considered as responsible for the antimicrobial activity. Although they usually occur as complex mixtures, their activity can generally be accounted for in terms of their major components. The antimicrobial activity of the phenolic fractions could be due to the investigated strain sensitivity to tannins, flavonoids and the other phenolic components[25,26]. The tannin components of epicatechin and catechin demonstrated strong antimicrobial activity against bacteria and fungi[27]. Flavonoids are synthesized by plants in response to microbial infection[28] and are effective against a broad range of microorganisms. This study confirms that the seeds of *Ziziphus jujuba* contain high amounts of polyphenol compounds. The phenolic compounds are commonly found in the plant kingdom, and they have been reported to have multiple biological effects including antimicrobial activity.

The MIC as low as µg mL^{-1} of a semi-purified fraction against gram negative and positive bacteria is suggestive of good antibacterial potential of the compounds of *Ziziphus jujuba*. Hence *Ziziphus jujuba* may yield potential molecules in the treatment of infections caused by pathogenic bacteria which have developed resistance against the known antibiotics, Singleton, 1999[29].

Results showed that the growth of *B.cereus* and*S. aureus* was inhibited at a MIC value of 72.5 and 41.25µg/ml respectively,followed by *E.*

Table 1. Proximate Composition (%) of *Ziziphus jujuba* Seeds extract

Component	Seeds
Moisture (%)	87.5
Total Soild	12.32
Ash	5.1
Carbohydrate (%)	15.45
Protein	3.31
lipid	1.3
Total Sugar (%)	12.9

Table 2. Phytochemical analysis of *Ziziphus jujuba* seeds extract

Phytochemical	EthOH/Water extract
Alkaloid	+
Tannins	-
Flavonoids	+
Saponins	+
Triterpenes	+
Glycosides	-
Anthocyanin	none
cholesterol	-

Table 3. Mean inhibition zone diameter (mm) of 50% aqueous-ethanol of *Ziziphus jujuba* on tested microorganisms by disc diffusion method with respect to various concentrations in µg/ml

Bacteria	50 % Eth./H_2O Extract		
	50	250	1000
Bacillus cereus	7.0	6.0	13.0
Staphylococcus aureus	-	-	10.0
Escherichia coli	6.0	8.0	17.5
Pseudomonas aeruginosa	-	-	-
Klebsiella pneumonia	-	9.0	17.0
Listeria monocytogenes	-	7.0	16.5

Table 4. Minimal inhibitory concentration (MIC) of 50% ethanolic extract of *Ziziphus jujuba* seeds against different strains. (µg/ml)

Bacteria	MIC
Bacillus subtilis	72.5
Staphylococcus aureus	41.25
Escherichia coli	52.5
Pseudomonas aeruginosa	89.25
Klebsiella pneumonia	42.5
Listeria monocytogenes	51.25

coli, *P. vulgaris* and *K. pneumonia*, while *Ps. aeruginosa* showed highest MIC value of 89.25µg/ml. The poor activity of the 50 % ethanol/water extract against most bacterial strains investigated in this study is in agreement with previous reports[30,31]. This could be due to the insolubility of the active compounds in water or the hot water could have caused denaturation of the active compounds. It is also observed from the results that the ethanol/water extract had wide antibacterial activity (Table 2) against both gram positive and gram negative bacteria *S. aureus* and *S. typhi*, respectively, (Table 4). The activity of the extracts against the Gram negative bacteria is noteworthy as these bacteria are known to exhibit high degree of resistance to conventional antibiotics[32]. The few variations in results between the disc diffusion and MIC results can be due to the different susceptibility of the bacterium to the plant extract, the rate of growth of bacteria, solvents used to extract the plant compounds and the rate of seeds extract diffusion[33].

To the best of our knowledge this isone of the studies evaluated the phytochemical and antimicrobial effects of the 50 % ethanol/water extracts of *Ziziphus jujuba* seeds against various pathogens are reported. Finding of this present study constitute supportive evidence to validate folkloric use of this plant as a remedy for various infections. Further investigations are required to isolate the active constituents responsible for the observed antimicrobial activity.

CONCLUSION

In conclusion, the 50 % ethanolic extract of *Ziziphus jujuba* seeds has antimicrobial activity against various microorganisms. It is expected that using natural products as therapeutic agents will probably not elicit resistance in microorganisms. This can explain the rationale for the use of the plant in treating infections in traditional medicine. The plant could be a veritable and cheaper substitute for conventional drugs since the plant is easily obtainable and the extract can easily be made via a simple process of maceration or infusion. It is essential that research should continue to isolate and purify the active components of thisnatural herb and use in experimental animals.

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