Original Article

Investigation of Antimicrobial Effect of *Tribulus terrestris L.* against Some Gram Positive and Negative Bacteria and *Candida spp.*

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Abstract

Background: In this study, antimicrobial effect of total extract of Tribulus terrestris L. and its fraction containing Benzoxazine derivative (Terresoxazine) was studied for the first time in Iran.

Materials and Methods: Total aqueous extract of plant was prepared and in order to separate the components, liquid/liquid extraction method with Petroleum ether was used. Liquid chromatography-mass spectrometry (LC/MS) system proved the existence of Benzoxazine derivative in the water and the third fractions. Antimicrobial effects of all extracts were examined against 10 Gram positive and negative and candida *spp.* by cup plate and disk diffusion methods. Also, the minimum inhibitory concentration (MIC) was determined by micro dilution method.

Results: The total extract showed antibacterial effect on *Escherichia coli*, *Pseudomonas aeruginosa* and *Bacillus subtilis*. The fraction of Benzoxazine had no effect against tested microbes. MIC and minimum bactericidal concentration (MBC) determination showed that *B. subtilis* had the low sensitivity to the total extract. Beside, total extract in water with 1000 mg/ml concentration and total extract in Dimethyl sulfoxide (DMSO) 10% with 750 mg/ml density can be substituted to Penicillin 200 mg/ml to combat the *P. aeruginosa* infections.

Conclusion: Because of antibacterial effects of *Tribulus terrestris L.* against both Gram negative and positive bacteria, and no antibacterial effect of the fraction containing Benzoxazine derivative, it can be concluded that antibacterial effects of the total extract is due to other active ingredients or it is because of the cumulating of different components in total extract. Therefore separation of other components of total extract and determination of their antibacterial effects can be future subjects for researches about this plant.

Keywords: *Tribulus terrestris*, Total extract, Cup plate method, Antimicrobial activity, Benzoxazine

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Introduction

Nowadays, due to increase of bacterial resistance to chemical drugs and their side effects, tend to search on medicinal plants or herbs and identify their active ingredient is rising. These active substances and their associated materials have a biological balance, so don't accumulate in the body and don't cause severe side effects, as a result, have a significant advantage compared with

chemical drugs[1].

Iran is located in a belt of dry weather and has four different climates, this diversity let to various types of plants to grow, *Tribulus terrestris* is a plant from *Zygophylaceae* family with stem lying on the ground and covered with lint, which is abundant in the Kerman province (south east of Iran)[2].

This plant is a strong diuretic has been used to treatment of kidney and bladder stones, increases sexual power, decrease blood pressure, prescribe as an anti-inflammatory, joint pain relief and reduce fever[3].

Recent studies have been demonstrated that various derivatives of Benzoxazine have showed more antibacterial or antifungal effect and even effective against some drug-resistant strains[4,5].

Based on the above information, the antimicrobial effect of different extracts of fruit of *Tribulus terrestris* were studied against some Gram positive and negative bacteria and *Candida spp* for the first time in Iran.

Methods

Plant collection: The fruit of *Tribulus terrestris* was collected from Kerman province (south east of Iran) during spring 2011.

Extraction preparation: 100g fruit *of Tribulus terrestris* L were collected, washed, dried, and crashed. Total aqueous extract of aerial parts of the plant was prepared by boiling method and concentrated by Rotary Evaporator to prepare a volume of 50 ml[6].

By using of water and DMSO 10% separately as second solvents, the followed concentrations (1000-375 mg/mL) of aqueous extract, petroleum ether and Benzoxazine were prepared. Antimicrobial effects of total extract were examined by both cup plate and disk diffusion methods based on Clinical and Laboratory Standards Institute (CLSI) protocol 2010[7].

Benzoxazine fractionation: In this step, 10g from total extract resolved in the 250mL water and poured into a hopper. The aqueous extract was mixed with petroleum ether, and after a few moments the two phases were separated. The result of this separation was aqueous phase, petroleum ether, and the third phase as aqueous phase and organic solvent which had the fraction of Benzoxazine. The extraction with petroleum ether was examined by Liquid chromatography-mass spectrometry (LC/MS) system to track and identify terressoxazine which is a derivate of Benzoxazine[7].

Antimicrobial assay: Both aqueous phase and Benzoxazine fraction were used for antimicrobial tests, separately.

Bacterial strains: *Staphylococcus aureus* PTCC 1112, *Escherichia coli* ATCC 8739, *Staphylococcus epidermidis* ATCC 12228 (PTCC 1114), *Bacillus subtilis* ATCC 6633 (PTCC 1023), *Pseudomonas aeruginosa* ATCC 9027, *Klebsiella pneumonia* ATCC 4352, *Shigella flexneri* PTCC 1234 (NCTC 8516), *Salmonella typhimurium* PTCC 1609, *Candida kruzei* PTCC 5295, *Candida albicans* ATCC 10231.

Disk diffusion method: The blank paper disks were purchased from Padtan teb Co, Iran. The disk diffusion method was done based on CLSI 2010 protocol[7]. In separate plates blank disks were soaked in different concentration of the extracts and kept it at dark dry condition, after drying. The bacterial suspensions were prepared equal to 0.5 McFarland turbidity and cultured on a Muller Hinton agar as a microbial lawn by a sterile swab. A disk of Penicillin (200 mg/ml) was chosen as positive control and the impregnated disks in DMSO 10% and water were used as negative control, simultaneously. All plates were incubated at 37°C for 18-24 hours. Then the diameter zone of

inhibition were measured and reported based on millimeter (mm) unit[7].

Cup plate method: At first by using of a Sterile Pasteur Pipette the wells with a diameter of 5 mm was created. Same as disk diffusion method, the bacterial suspension were prepared equal to 0.5 McFarland turbidity.

All wells were filled by each of prepared aqueous and petroleum ether extract concentrations and Benzoxazine fraction. Penicillin (200 mg/mL) as a positive control and DMSO 10%, and water as negative controls were used simultaneously[7].

Minimum Inhibitory Concentration (MIC) determination: MIC was determined by microdilution method by using 96 wells microplates based on CLSI 2010[7].

The Penicillin powder was used as a positive control and DMSO 10% and water as negative controls.

Validation method: For validation procedure the following steps were performed:

1. Quality control of aqueous extracts and DMSO 10%.
2. Using of negative control in the cup plate and disk diffusion methods.
3. Appling of negative and positive control in the microdilution method.
4. Using of Penicillin 200 mg/ml as a positive control in all antimicrobial tests.
5. All mentioned tests above, were done triplicated and the mean was calculated for statistical analysis.

Statistical analysis: All statistical analysis was done by SPSS software version 16. In order to comparison of the relation between the concentrations of each extract and the diameter zone of inhibition the Kruskal–Wallis nonparametric test were used.

Also, the mean and standard deviation were analysis based on usual descriptive statistic methods and the paired T-test was performed for comparison extracts and antibiotic analysis. Charting was performed by using of Box Plote method.

Results

In this study, the antimicrobial effect of different concentrations (375-1000 mg/mL) of the aqueous and organic extract of *Tribulus terrestris* in two separate solvents, water and DMSO10%, and fraction of Benzocaine were determined against eight Gram positive and negative bacteria and two *Candida spp* by two methods of disk diffusion and cup plate method. Also Penicillin 200 mg/mL (29-45.5mm) was evaluated simultaneously.

No antimicrobial effect was detected by disk diffusion method at three times repeated.

In contrast, total extract that was prepared with water and DMSO 10% as diluents showed the anitibacterial effect only against E. coli, P. aeruginosa and B. subtilis (Tables1-6).

There is a significant difference between the mean of inhibition zone by cup plate method in different concentrations (375 mg/mL (8.3-15.5mm), 750 mg/mL (11.5-19.5mm) and 1000 mg/mL (17.3-22)) of total extract in water solvent in comparison to Penicillin 200 (29.3-47mm) against E. coli, P. aeruginosa and B. subtilis (Tables1-3). Also, there is a significant difference between the mean

Table 1: Mean and SD of effect of total extract of *Tribulus terrestris* in water solvent in concentration of 1000mg/mL comparison to Penicillin 200mg/mL.

Bacteria	Mean of Zone of inhabitation (mm)		p value <0.05
	Extract 1000 mg/mL	Penicillin 200 mg/mL	
	Mean±SD	Mean±SD	
E. coli	22.0±1.0	45.5±1.5	0.001
P. aeruginosa	19.3±2.1	27.7±2.5	0.062
B. subtilis	17.3±0.6	29.0±1.7	0.006

Table 2: Mean and SD of effect of total extract of *Tribulus terrestris* in water solvent in concentration of 750 mg/mL comparison to Penicillin 200 mg/mL.

Bacteria	Mean of Zone of inhabitation (mm)		P value <0.05
	Extract 750 mg/mL	Penicillin 200 mg/mL	
	Mean±SD	Mean±SD	
E. coli	19.5±0.5	45.5±1.5	0.001
P. aeruginosa	16.0±1.0	27.7±2.5	0.013
B. subtilis	11.5±0.9	29.0±1.7	0.002

Table 3: Mean and SD of effect of total extract of *Tribulus terrestris* in water solvent in concentration of 375 mg/mL comparison to Penicillin 200 mg/mL.

Bacteria	Mean of Zone of inhabitation (mm)		p value <0.05
	Extract 375 mg/mL	Penicillin 200 mg/mL	
	Mean±SD	Mean±SD	
E. coli	15.5±0.5	45.5±1.5	0.001
P. aeruginosa	13.7±0.6	27.7±2.5	0.012
B. subtilis	8.3±0.8	29.0±1.7	0.003

of inhibition zone by cup plate method in different concentrations (375 mg/mL (10.8-15.3mm), 750 mg/mL (13-19mm) and 1000 mg/mL (14.7-20.7mm)) of total extract in DMSO 10% solvent in comparison to Penicillin 200 (29.3-47mm) in all 3 mentioned types of bacteria (Tables 4-6).

Table 4: Mean and SD of effect of total extract of *Tribulus terrestris* in DMSO 10% solvent in concentration of 1000mg/ml comparison to Penicillin 200 mg/mL.

Bacteria	Mean of Zone of inhabitation (mm)		p value <0.05
	Extract 1000 mg/mL	Penicillin 200 mg/mL	
	Mean±SD	Mean±SD	
E. coli	20.7±1.2	47.0±0.0	0.001
P. aeruginosa	21.3±1.2	27.7±2.5	0.019
B. subtilis	14.7±0.6	29.3±1.2	0.001

Based on the statistical analysis, with increasing in concentrations of the total extract (without any difference between two solvents) the antimicrobial activity increased (Tables1-6). Based on the microdilution method, MIC and MBC for the both solvents were evaluated and the best result was

Table 5: Mean and SD of effect of total extract of *Tribulus terrestris* in DMSO 10% solvent in concentration of 750 mg/mL comparison to Penicillin 200 mg/mL.

Bacteria	Mean of Zone of inhabitation (mm)		p value <0.05
	Extract 1000 mg/mL	Penicillin 200 mg/mL	
	Mean±SD	Mean±SD	
E. coli	19.0±1.0	47.0±0.0	0.001
P. aeruginosa	18.7±1.2	27.7±2.5	0.05
B. subtilis	13.0±1.0	29.3±1.2	0.005

Table 6: Mean and SD of effect of total extract of *Tribulus terrestris* in DMSO 10% solvent in concentration of 375 mg/mL comparison to Penicillin 200 mg/mL.

Bacteria	Mean of Zone of inhabitation (mm)		p value <0.05
	Extract 1000 mg/mL	Penicillin 200 mg/mL	
	Mean±SD	Mean±SD	
E. coli	15.3±0.6	47.0±0.0	<0.001
P. aeruginosa	14.0±1.0	27.7±2.5	0.021
B. subtilis	10.8±1.9	29.3±1.2	0.009

Table 7: MIC and MBC results of total extract of *Tribulus terrestris* in water solvent.

Bacteria	MIC (mg/mL)	MBC (mg/mL)
E. coli	125.0	250.0
P. aeruginosa	125.0	250.0
B. subtilis	250.0	500.0

Table 8: MIC and MBC results of total extract of *Tribulus terrestris* in DMSO 10% solvent.

Bacteria	MIC (mg/mL)	MBC (mg/mL)
E. coli	125.0	250.0
P. aeruginosa	62.5	125.0
B. subtilis	500.0	1000.0

detected against *P. aeruginosa* with MIC 62.5 mg/mL and MBC 125 mg/mL. Also, the difference

between DMSO 10% and water was noticeable because of MIC decreasing from 125 mg/mL when water was used in comparison to 62.5 mg/mL for DMSO 10% (Tables 7-8).

Discussion

As it was mentioned before, the aim of this study was evaluation the antimicrobial effect of different extracts of *Tribulus terrestris* fruits against some Gram positive and Gram negative bacteria and two *Candida* species.

Disk diffusion method was chosen for antimicrobial evaluation due to its easiest and cheapest procedure with less error during the test, but as the results were not satisfying, so cup plate method was substituted[9].

Similarly, in the study of Lin et al. 2011, different agar and broth methods were used for evaluation the antibacterial effect of Chitosan because no any reference for evaluation the extracts and essences. He has identified diffusion of some extracts will be banned in agar based methods so must be evaluate by broth based methods[10].

Terresoxazine is member of the Benzoxazine group with antibacterial, antifungal and insecticide properties[4,5]. In this study, Benzoxazine fraction was extracted by liquid/liquid extraction with petroleum ether method.

So, the other aim of this study was to evaluate the antimicrobial effect of Benzoxazine fraction of *Tribulus terrestris*. LC/MS system proved the existence of Benzoxazine derivative in the third fraction of water.

Based on the results of cup plate and microdilution methods, this fraction has not any antibacterial effect. Also this fraction has not any anticandidal effect too. The results of this study were correlated to the study of Zhang et al. which explained that anticandidal properties of this plant is due to the presence of other compounds (TTS-12and TTS-15) of *Tribulus terrestris* [11].

Kiran et al. 2011 mentioned that to the leaf extract antibacterial effect of *Tribulus terrestris.* They showed that the methanolic extract was more effective compared to the other extracts against *B. cereus, E. coli* and *S. aureus*[12]. However in this study the total extract of the *Tribulus terrestris* fruits was evaluated and showed good antibacterial effect against *P. aeruginosa, E. coli* and *B. subtilis* but no any effect against *S. aureus*. This difference may be related to the different parts of plant which were used for extraction between the recent study and Kiran's study. Also geographical difference may affect on the antibacterial properties of the medicinal plant.

In conclusion, the following suggestions recommend for further studies: (a) Isolation of total extract components and identification its combinations other than Benzoxazine, (b) Investigation of antimicrobial effects of other components present in the total extract, (c) Study of cytotoxicity effects of total extract on cell cultures, and (d) Investigation the antimicrobial effects of total extracts against other fungi and bacteria.

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