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Antibacterial and antifungal activities of different parts of *Tribulus terrestris* L. growing in Iraq

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Abstract: Antimicrobial activity of organic and aqueous extracts from fruits, leaves and roots of *Tribulus terrestris* L., an Iraqi medicinal plant used as urinary anti-infective in folk medicine, was examined against 11 species of pathogenic and non-pathogenic microorganisms: *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Corynebacterium diphtheriae*, *Escherichia coli*, *Proteus vulgaris*, *Serratia marcescens*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Candida albicans* using microdilution method in 96 multiwell microtiter plates. All the extracts from the different parts of the plant showed antimicrobial activity against most tested microorganisms. The most active extract against both Gram-negative and Gram-positive bacteria was ethanol extract from the fruits with a minimal inhibitory concentration (MIC) value of 0.15 mg/ml against *B. subtilis*, *B. cereus*, *P. vulgaris* and *C. diphtheriae*. In addition, the same extract from the same plant part demonstrated the strongest anti-fungal activity against *C. albicans* with an MIC value of 0.15 mg/ml.

Key words: Antimicrobial activity, *Tribulus terrestris*, Urinary tract infections
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INTRODUCTION

Traditional medicine has been practiced for many centuries by a substantial proportion of the population of Iraq. The interest in the study of medicinal plants as a source of pharmacologically active compounds has increased worldwide. It is recognized that in some developing countries, plants are the main medicinal source to treat infectious diseases. Plant extracts represent a continuous effort to find new compounds with the potential to act against multi-resistant bacteria. Approximately 20% of the plants found in the world have been submitted to pharmacological or biological test, and a substantial number of new antibiotics introduced on the market are obtained from natural or semi-synthetic resources (Mothana and Lindequist, 2005).

Tribulus terrestris (Puncture Vine, Caltrop, Yellow Vine and Goathead) is a flowering plant of the Zygophyllaceae family, native to warm temperature and tropical regions of the old world in Southern Europe, Southern Asia, Africa and Northern Australia. It can thrive even in desert climates and poor soil (Abeywickrama and Bean, 1991). In Iraq *T. terrestris* is used in folk medicine as tonic, aphrodisiac, analgesic, astringent, stomachic, anti-hypertensive, diuretic, lithon-triptic and urinary anti-infectives (Majeed and Mahmood, 1988; Saad Aldein, 1986).

Different parts of Turkish and Iranian *T. terrestris* have been reported to have antibacterial activity (Abbasoglu and Tosun, 1994; Kianbakht and Jahaniani, 2003); however, the antimicrobial activity of Iraqi *T. terrestris* has not been studied. In the current study, we evaluated in vitro antimicrobial activity of different parts of *T. terrestris* growing in Iraq using different extracts.

MATERIALS AND METHODS

Plant materials

Fruits, leaves and roots of *T. terrestris* used in

this study were collected at the end of November 2006 from Mosul countryside, Nineveh Province, Iraq, with assistance of local traditional healers, and authenticated by Dr. Abdulazeez Shekho, Department of Biology, University of Mosul, Iraq. Voucher specimen of the plant (No. 62) was dried and deposited at the herbarium of Department of Biology, University of Mosul, Iraq.

Preparation of the extracts

1. Aqueous extracts

The air dried fine powdered plant fruits, leaves and roots (100 g) were infused in distilled water until complete exhaustion. The extract was then filtered using Whatman No. 1 filter paper and the filtrate was evaporated in vacuo and dried using either a rotary evaporator at 60 °C or a freeze drier (Kandil *et al*., 1994). The final dried samples were stored in labeled sterile bottles and kept at −20 °C.

2. Ethanol extracts

Ethanol extracts were accomplished according to established protocols (le Grand *et al*., 1988). Each dried plant sample was ground and extracted in a percolator with 95% ethanol. About 10 ml of ethanol per gram of plant sample was used. The ethanol extract was dried under a reduced pressure at 40 °C. The dried extract was stored in sterile bottles until further use.

3. Chloroform extracts

Powdered samples (100 g) from each plant part were extracted with chloroform using a soxhlet extractor for continuously 10 h or until the used solvent turned pure and colorless (Chhabra *et al*., 1982). The solvent was removed using a rotary vacuum evaporator at 40 °C to give a concentrated extract, which was then frozen and freeze-dried until use.

Microbial cultures

Ten strains of bacteria and one yeast were used as test microorganisms. The bacterial strains included Gram-positive *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus* and *Corynebacterium diphtheriae*; Gram-negative *Escherichia coli*, *Proteus vulgaris*, *Serratia marcescens*, *Salmonella typhimurium*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*; and the yeast *Candida albicans*. All microorganisms were clinical isolates, obtained from the Microbiology Laboratory at Department of Biology, University of Mosul, Iraq, and very carefully identified using standard microbiological methods.

Inoculum preparation

Mueller-Hinton broth and Sabouraud dextrose agar (SDA) were applied for growing and diluting the microorganism suspensions. Bacterial strains were grown to exponential phase in Mueller-Hinton broth at 37 °C for 18 h and adjusted to a final density of 10^8 CFU/ml by diluting fresh cultures and comparing with McFarland density. *C. albicans* was aseptically inoculated on Petri dishes containing autoclaved, cooled and settled SDA medium. The Petri dishes were incubated at 31 °C for 48 h to give white round colonies against a yellowish background. These were aseptically subcultured on SDA slants. The yeast colonies from SDA slants were suspended in sterilized 0.9% sodium chloride solution (normal saline), which was compared with McFarland solution. One ml yeast suspension in normal saline was added to 74 ml of sterile medium, kept at 45 °C, to give concentration of 2×10^7 cells/ml (according to the manufacturer's instructions).

Antimicrobial activity

Extracts were tested against the strains for their inhibitory activity, using a common broth microdilution method in 96 multiwell microtiter plates, in duplicate, as reported by Koneman (1995) and recommended by the National Committee for Clinical Laboratory Standard (NCCLS, 2001).

For susceptibility testing, 50 μl of Mueller-Hinton broth was distributed from the second to the twelfth test tubes. Dry extracts from each plant part were initially dissolved in 100 μl of dimethyl sulfoxide (DMSO) and then in Mueller-Hinton broth, to reach a final concentration of 10 mg/ml; 100 μl of these suspensions were added to the first test well of each microtiter line, and then 50 μl of scalar dilutions were transferred from the second to the ninth well. The 10th well was considered as growth control, since no extracts solutions were added. Then, 50 μl of the bacterial suspensions were added to each well. The final concentrations of the extracts adopted to evaluate the antibacterial activity were 0.01 to 5.00 mg/ml. Plates were incubated for 24 h at 37 °C. Maxipime (Hemofarm) at the concentration range of 0.01~5.00 mg/ml was prepared in Mueller-Hinton

broth and used as standard drug for positive control. As an indicator of bacterial growth, 40 μl *p*-iodonitrotetrazolium (INT) violet dissolved in water was added to the wells and incubated at 37 °C for 30 min (Buwa and van Staden, 2006). The lowest concentration of each extract showing no growth was taken as its minimal inhibitory concentration (MIC) and confirmed by plating 5 μl samples from clear wells on Mueller-Hinton agar medium. The colorless tetrazolium salt acts as an electron acceptor and is reduced to a red-colored formazan product by biologically active organisms (Eloff, 1998). Where bacterial growth was inhibited, the solution in the well remained clear after incubation with INT.

As for *C. albicans* a simple turbidity test was used to determine the MIC values of *T. terrestris* extracts by adding 0.1 ml of each extract (0.01~5.00 mg/ml) from each plant part into tubes containing 9.8 ml sterile Mueller-Hinton broth, and tubes were then inoculated with 0.1 ml of yeast suspension and incubated at 31 °C for 48 h. Amphotericin B (0.01~5.00 mg/ml) was used as positive control. The optical density was determined using a Spectro SC spectrophotometer (LaboMed Inc., USA) at 630 nm. The MIC values were taken as the lowest concentration of the extracts that showed no growth after 48 h of incubation by comparing with the control tube that included 9.8 ml Mueller-Hinton broth and 0.1 ml of yeast suspension in addition to 0.1 ml of each extract in different concentration (un-incubated).

RESULTS

Three different extracts from fruits, leaves and roots of Iraqi *T. terrestris* were tested at various concentrations (0.01~5.00 mg/ml), and the evaluated MIC values are reported in Table 1. All the plant parts showed antibacterial activity against most tested bacteria. Aqueous extract from *T. terrestris* fruits showed good activity against the tested bacteria and the strongest activity was seen against *C. diphtheriae* (*MIC*=0.62 mg/ml), which was similar to what was achieved by the standard drug Maxipime; meanwhile, *S. typhimurium* was inhibited using the highest extract concentration (*MIC*=5.00 mg/ml). In addition, *S. marcescens* and *P. aeruginosa* resisted all aqueous extracts of various concentrations. Ethanol and chloroform extracts of *T. terrestris* fruits demonstrated very close activities against all reference bacteria. Very strong activity was seen against *S. aureus*, *B. subtilis*, *B. cereus*, *C. diphtheriae*, *E. coli* and *P. vulgaris* using both extracts. The highest antibacterial activity was seen against *B. subtilis*, *B. cereus*, *C. diphtheriae* and *P. vulgaris* in the ethanol extract (*MIC*=0.15 mg/ml), while *B. subtilis*, *B. cereus* and *C. diphtheriae* were the most sensitive bacteria to the chloroform extract (*MIC*=0.31 mg/ml). *S. marcescens*, *S. typhimurium*, *K. pneumoniae* and *P. aeruginosa* were inhibited by high concentrations of ethanol and chloroform extracts (*MIC*=2.50 and 1.25 mg/ml).

Table 1 In vitro antimicrobial activity of different parts of *Tribulus terrestris*

Microorganisms	*MIC* (mg/ml)										
	Fruits			Leaves			Roots			Control	
	A	E	C	A	E	C	A	E	C	M	AB
Gram-positive											
S. aureus	2.50	0.62	0.62	2.50	1.25	2.50	>5.00	0.62	0.31	0.07	NT
B. subtilis	1.25	0.15	0.31	1.25	0.31	0.31	2.50	0.62	0.31	0.01	NT
B. cereus	1.25	0.15	0.31	2.50	0.62	0.62	5.00	1.25	0.62	0.03	NT
C. diphtheriae	0.62	0.15	0.31	1.25	0.62	1.25	2.50	1.25	0.62	0.62	NT
Gram-negative											
P. vulgaris	>5.00	0.15	1.25	2.50	0.31	1.25	>5.00	2.50	1.25	0.15	NT
E. coli	1.25	0.62	0.62	2.50	0.62	1.25	>5.00	2.50	1.25	0.15	NT
S. marcescens	>5.00	2.50	2.50	>5.00	5.00	>5.00	>5.00	>5.00	>5.00	2.50	NT
S. typhimurium	5.00	1.25	2.50	>5.00	1.25	5.00	>5.00	>5.00	2.50	2.50	NT
K. pneumoniae	2.50	1.25	1.25	2.50	0.31	2.50	>5.00	2.50	1.25	1.25	NT
P. aeruginosa	>5.00	1.25	2.50	>5.00	1.25	2.50	5.00	2.50	1.25	2.50	NT
Yeast											
C. albicans	>5.00	0.62	2.50	>5.00	1.25	2.50	>5.00	5.00	2.50	NT	0.15

A: Aqueous extract; E: Ethanol extract; C: Chloroform extract; M: Maxipime; AB: Amphotericin B; NT: Not tested

Aqueous extract from *T. terrestris* leaves revealed close results to the fruit extract, except it was active against *P. vulgaris* with an MIC value of 2.50 mg/ml and resisted by *S. typhimurium*, *S. marcescens* and *P. aeruginosa*. Ethanol and chloroform extracts from leaves also possessed promising results against the tested bacteria with less action compared with fruit ethanol and chloroform extracts. The best MIC values were calculated against *B. subtilis*, *P. vulgaris* and *K. pneumoniae* (*MIC*=0.31 mg/ml) using the ethanol extract and *B. subtilis* (*MIC*=0.31 mg/ml) using chloroform extract. *S. marcescens* resisted the chloroform extract and was inhibited by the highest ethanol extract concentration.

The different extracts from *T. terrestris* roots exhibited from moderate to no activity against tested bacteria. The weakest extract from the roots was the aqueous extract, which was only active against *B. subtilis*, *B. cereus*, *C. diphtheriae* and *P. aeruginosa* using high concentrations (*MIC*=5.00 and 2.50 mg/ml). Chloroform extract showed high antibacterial activity against some of the tested bacteria, with all the calculated MIC values greater than those of ethanol extract from the same plant part. The standard drug Maxipime showed a greater antibacterial activity than that of most used plant extracts, and the strongest action was seen against *B. subtilis* (*MIC*=0.01 mg/ml).

Regarding antifungal activity, all aqueous extracts from different parts of *T. terrestris* were resisted by *C. albicans*. The strongest antifungal activity was observed using the ethanol extract from *T. terrestris* fruits with an MIC value of 0.62 mg/ml. The standard drug Amphotericin B achieved the highest antifungal activity against *C. albicans* (*MIC*=0.15 mg/ml).

DISCUSSION

All the extracts were able to inhibit the growth of one or more of the tested microorganisms. The Gram-positive bacteria *B. subtilis*, *B. cereus* and *C. diphtheriae* were the most sensitive strains to the ethanol extract of *T. terrestris* fruits (*MIC*=0.15 mg/ml), while the strongest activity was demonstrated against the Gram-negative bacterium *P. vulgaris* (*MIC*=0.15 mg/ml) using the same extract.

The activity of the plant against both Gram-positive and Gram-negative bacteria may be indicative to the presence of broad spectrum antibiotic compounds or simply general metabolic toxins in the plant, in addition to the plant (fruits, leaves and root) content of pharmacological active metabolites like furostanol and spirostanol saponins (Kostova and Dinchev, 2005), flavonoid glycosides (Saleh *et al*., 1982), phytosterols and some amides (Xu *et al*., 2000). The plant also contains a mixture of B-carboline alkaloids: harmane, norharmane, tetrahydroharmane, harmine, harmaline, harmol, harmalol, ruin and dihydroruin (Bourke *et al*., 1990).

Saponins have been reported to possess a wide range of biological activities. The toxicity of saponins to insects (insecticidal activity), parasite worms (anthelmintic activity), molluscs (molluscicidal), and fish (piscidal activity), and their antifungal, antiviral, and antibacterial activities are well documented (Lacaille-Dubois and Wagner, 1996; Milgate and Roberts, 1995). The mode of action of antibacterial effects of saponins seems to involve membranolytic properties, rather than simply altering the surface tension of the extracellular medium, thus being influenced by microbial population density (Killeen *et al*., 1998).

Flavonoids are phenolic structures containing one carbonyl group. Since they are known to be synthesized by plants in response to microbial infection (Dixon *et al*., 1983), it should not be surprising that they have been found in vitro to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls (Cowan, 1999). More lipophilic flavonoids may also disrupt microbial membranes (Tsuchiya *et al*., 1996).

Alkaloids isolated from plant are commonly found to have antimicrobial properties (Omulokoli *et al*., 1997). Alkaloids may be useful against HIV infection (Sethi, 1979) as well as intestinal infections associated with AIDS (McDevitt *et al*., 1996). Berberine and harmane are important representatives of the alkaloid group. The mechanism of action of highly aromatic planar quaternary alkaloids such as berberine and harmane is attributed to their ability to intercalate with DNA (Phillipson and O′Neill, 1987).

The best MIC values against most bacteria were

seen in the ethanol extract from the plant fruits followed by leaf extracts and finally root extracts which were resisted by most of the Gram-negative bacteria specially *S. marcescens*.

Candida albicans remains the most common infection-causing fungus; about 45% of clinical fungal infections are caused by *C. albicans* (Gupta *et al*., 2004). The present study shows that ethanol extract from *T. terrestris* fruits had potent anti-*C. albicans* activity with an MIC value of 0.62 mg/ml. The antifungal activity of *T. terrestris* may be attributed to various chemicals detectable in its extracts such as saponins (Zhang *et al*., 2006). The action mechanisms of saponins may lie in damage to the membrane and leakage of cellular materials, ultimately leading to cell death (Mshvildadze *et al*., 2000).

All parts (fruits, stems plus leaves and roots) of Turkish and Iranian *T. terrestris* showed antibacterial activity against *Enterococcus faecalis*, *S. aureus*, *E. coli* and *P. aeruginosa* (Abbasoglu and Tosun, 1994; Kianbakht and Jahaniani, 2003) in contrast to aerial parts of Yemeni *T. terrestris* which had no detectable antibacterial activity against these bacteria (Awadh-Ali *et al*., 2001), and only fruits and leaves of Indian *T. terrestris* were active exclusively against *E. coli* and *S. aureus* (Williamson, 2002). It can be argued that antibacterial activity of Iraqi *T. terrestris* is similar to Turkish and Iranian *T. terrestris*. Different results concerning the antibacterial activity of *T. terrestris* might be due to different geographic sources of the plant used, different types of strains used, and different assay methods.

CONCLUSION

Since Iraqi *T. terrestris* demonstrated activity against the most prevalent Gram-negative bacteria in urinary infections, namely *E. coli*, the use of the plant as a urinary anti-infective is validated, scientifically supported by the results obtained in this work.

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