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ASSESSMENT OF ANTIBACTERIAL PROPERTY OF *Tribulus terrestris* (Leaves) FROM TIRUNELVELI DISTRICT, INDIA

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Natural drugs play important and vital role in the modern medicine. They cure ailments which may not be treated by conventional medicine and exhibit many biological activities like antimicrobial. The purpose of our study is to screen phytochemicals and to determine antibacterial activity of hexane, diethyl ether, ethyl acetate and ethanol extracts of *Tribulus terrestris* leaves against five pathogens (*Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae, Salmonella typhimurium* and *Staphylococcus aureus*). The extracts were prepared by cold maceration and the *in vitro* antimicrobial susceptibility testing was done using agar well diffusion method. The inhibition zones were measured for five concentrations (10 mg, 5 mg, 2.5 mg, 1.25 mg and 0.625 mg) of the extract. The phytochemical analysis revealed the presence of phenols, carbohydrates, flavones, saponins, steroids, alkaloids, proteins, quinones and tannins. In antibacterial assay, inhibition zones were detected in all extracts and in all concentrations. Large zones were formed in hexane and diethyl ether extracts. As the concentration of extract increased, the diameter of inhibition zones around the well also increased. Hence our results support the use of all the four extracts of *Tribulus terrestris* as a source of antibiotic substance for the possible treatment of human pathogenic organisms.

Keywords: Plant extract; phytochemical; antimicrobial; agar well diffusion; zone of inhibition.

1. INTRODUCTION

Infectious disease, also known as transmissible disease or communicable disease, is illness resulting from an infection. Infections are caused by infectious agents (pathogens) including: viruses and related agents such as viroids and prions, bacteria, fungi, parasites and arthropods. Infectious diseases are a leading cause of death worldwide, particularly in low income countries, especially in young

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children. Bacterial infection is a proliferation of a harmful strain of bacteria on or inside the body. Bacteria can infect any area of the body. Pneumonia, meningitis, and food poisoning are just a few illnesses that may be caused by harmful bacteria. The symptoms of a bacterial infection depend on the area of the body that is affected. Antibiotics are the most important weapons in fighting bacterial infections and have greatly benefited the health-related quality of human life since their introduction. They exert their therapeutic effect by antagonizing the growth of bacteria with different mechanisms of action including: inhibition of bacteria's cell wall synthesis; inhibition of protein synthesis and as DNA synthesis inhibitors [1]. In the recent years, using antibiotics has led to resistance. Antibiotic-resistant infections are already widespread across the globe [2]. A 2011 national survey of infectious-disease specialists, found that more than 60% of participants had seen a pan-resistant, untreatable bacterial infection within the prior year [3]. Many public health organizations have described the rapid emergence of resistant bacteria as a nightmare scenario that could have catastrophic consequences [4-6].

In this regard, alternative medicine is gaining popularity. Plants especially have been the basis for medical treatments through much of human history, and such traditional medicine is still widely practiced today. Ethnopharmacologists, botanists, microbiologists, and natural-products chemists are searching for phytochemicals which could be developed for treatment of infectious diseases [7]. *Tribulus terrestris* (Family: Zygophyllaceae) is commonly known as puncture vine, land caltrops (English) and Nerinjil (Tamil) [8]. The plant is used in folk medicines as a tonic, aphrodisiac, palliative, astringent, stomachic, antihypertensive, diuretic, lithotriptic, and urinary disinfectant. The dried fruit of the herb is very effective in genitourinary tract disorders and to remove the urinary stones. In Ayurveda used to treat impotence, venereal diseases, and sexual debility. In traditional Chinese medicine, the fruits were used for treatment of eye trouble, edema, abdominal distension, emission, morbid leukorrhea, and sexual dysfunction; in restoring the depressed liver, for treatment of fullness in the chest, mastitis, flatulence, acute conjunctivitis, headache, and vitiligo. In Unani medicine, as diuretic, mild laxative, and general tonic [9]. Experimental study in the plant has proven it to possess diuretic activity, aphrodisiac activity, antiurolithic activity, immunomodulatory activity, antidiabetic activity, absorption enhancer, hypolipidemic activity, activity in cardiac disorders, central nervous system activity, hepatoprotective activity, anti-inflammatory activity, analgesic activity, antispasmodic activity, anticancer activity, antibacterial activity, anhelmintic activity, larvicidal activity and anticariogenic activity [10-12].

Hence the present study was aimed to screen the phytochemicals and to evaluate the antibacterial efficacy of the leaves of *Tribulus terrestris* extracted in hexane, diethyl ether, ethyl acetate and ethanol solvents *in vitro* by agar well diffusion technique.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Hexane, diethyl ether, ethyl acetate, ethanol, picric acid, Fehling solution I and II, 10% ammonia, 2% copper sulphate, 95% ethanol, ferric chloride, dilute ammonia, concentrated sulphuric acid, benzene, sodium hydroxide, Mueller-Hinton agar, dimethyl sulfoxide (DMSO) and streptomycin. All the chemicals and reagents used were of analytical grade.

2.2 Plant Collection and Extraction

Tribulus terrestris leaves were collected in Tirunelveli District in the month of January. The plant was authenticated by Dr. Vinod, Assistant Professor in the Department of Botany, St. Xavier's College (Autonomous), Palayamkottai. The leaves were shade-dried, powdered, extracted by cold maceration in hexane, diethyl ether, ethyl acetate and ethanol [13]. The dried extracts were used for further study.

2.3 Phytochemical Analysis

Extracts of *Tribulus terrestris* leaves were subjected to preliminary phytochemical analysis using standard methods to detect the presence of alkaloids, carbohydrates, saponins, proteins, phenols, flavones, steroids, tannins and quinines [14].

2.4 Evaluation of Antibacterial Activity

3.8 g of Mueller-Hinton agar was dissolved in 100 ml distilled water, autoclaved at 121˚C for 20 minutes at 15 lbs pressure and used as media. The bacteriostatic property of the compounds was tested by agar well-diffusion method [15]. The plates were swabbed with cultures of bacteria: *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhimurium* and *Staphylococcus aureus*. The extracts were dissolved in sterile 1% DMSO. Streptomycin (1 mg) used as positive drug control was also dissolved in 1% DMSO. The extracts were serially diluted to obtain concentrations of 10 mg, 5 mg, 2.5 mg, 1.25 mg and 0.625 mg per 50 µl and poured into the pre-formed agar wells. After 24 hrs incubation, the diameter of zone (including the diameter disc) was

measured and recorded in mm as recommended by the National Committee for Clinical laboratory Standards [16].

3. RESULTS AND DISCUSSION

The global rise in antibiotic resistance threatens to undo decades of progress in treating infectious diseases caused by bacterial pathogens [17]. Mortality is higher for children with drug-resistant infections [18]. The misuse of antibiotics has lessened the efficacy of many commonly used antibiotics. The use of plant extracts or pure natural compounds in combination with conventional antibiotics may hold greater promise for rapidly providing affordable treatment options [19]. Hence the present study was aimed to find an alternative agent from medicinal plant that could help in developing an antibiotic to destroy the ever-rising resistant forms of bacteria. We selected *Tribulus terrestris* because of its wide use in all traditional systems of medicine followed in India and around the world [20-24].

Cold maceration extraction technique was preferred since previous studies have had reported significant better antimicrobial activity of extracts obtained through cold maceration than extracts obtained through Soxhlet method. Cold maceration method is also relatively simple and does not involve complex instruments and yet yields better extract [25] and it could be used for the extraction of thermolabile components [26].

Four solvents of different polarity indices were used to extract the constituents. The polarity of the solvent used for extraction play vital roles in both the efficiency and efficacy of pharmacological activities [27]. The solvents were selected based on their increasing order of polarity indices which is a relative measure of the degree of interaction of the solvent with various polar test solutes. Among the four, hexane has the lowest solvent polarity index (0.1) followed by diethyl ether (2.8), ethyl acetate (4.4) and ethanol (5.2) in an increasing order. This would enable comparative study of the impact of solvents on the extraction yield, content of the bioactive components and effectiveness in controlling bacterial growth [28].

Preliminary qualitative phytochemical analysis was carried out to identify the secondary metabolites present in the various extracts [29]. Any pharmacological activity seems to be related to the phytochemicals detected in the extracts. This encourages the identification and isolation of active substances which could be used as lead(s) molecules in the development of new antimicrobial drugs [30].

In our study, the hexane extract of *Tribulus terrestris* leaves contains carbohydrates, flavones, steroids, proteins and tannins. The diethyl ether extract contains carbohydrates, saponins, steroids, proteins and quinones. Ethyl acetate extract contains phenols, carbohydrates, saponins, steroids, proteins, quinones and tannins. Ethanol extract has flavones and alkaloids (Table 1).

To investigate extracts as potential antimicrobial agents, *in vitro* antimicrobial susceptibility testing by agar well diffusion method was done. The well diffusion method is reported to be better than the disc diffusion method for measuring zones of inhibition [31]. Many earlier studies have reported the activity of various parts of *Tribulus terrestris* against a different set of bacteria and different extracts in varying concentrations [32-36].

Our study demonstrated the capability of *Tribulus terrestris* to inhibit a broad spectrum of bacterial (Gram negative and Gram positive) growth. The zones of inhibition formed around five different concentrations of *Tribulus terrestris* hexane extract is listed in Table 2. The zone was maximum (34 mm) in *E. faecalis* and *S. typhi* cultures at 10 mg concentration. The smallest zone (18 mm) was observed in *E. coli* culture at the same concentration.

Table 1. Phytochemicals in *Tribulus terrestris* leaves

Phytochemicals	Hexane	Diethyl ether	Ethyl acetate	Ethanol
Phenols	-	-	+	-
Carbohydrates	+	+	+	-
Flavones	+	-	-	+
Saponins	-	+	+	-
Steroids	+	+	+	-
Alkaloids	-	-	-	+
Proteins	+	+	+	-
Quinones	-	+	+	-
Tannins	+	-	+	-

('+' indicates presence; '-' indicates absence)

Table 2. Zones of inhibition formed by *Tribulus terrestris* hexane extract

Bacteria	Zones of inhibition (mm)					
	Standard	**10 mg**	**5 mg**	**2.5 mg**	**1.25 mg**	**0.625 mg**
E. faecalis	18	34	26	22	19	18
E. coli	15	18	15	11	11	9
K. pneumoniae	35	30	25	20	19	16
S. typhimurium	36	34	28	19	19	19
S. aureus	40	30	26	21	20	18

The zones of inhibition formed around five different concentrations of *Tribulus terrestris* diethyl ether extract is given in Table 3. The zone was maximum (40 mm) in *K. pneumoniae* and *S. typhi* (39 mm) cultures at 10 mg concentration. The smallest zone (24 mm) was observed in *E. faecalis* culture at the same concentration.

The zones of inhibition formed around five different concentrations of *Tribulus terrestris* ethyl acetate extract is tabulated in Table 4. The zone was maximum (27 mm) in *S. aureus* and *S. typhi* (20 mm) cultures at 10 mg concentration. The smallest zone (8 mm) was observed in *E. coli* culture at the same concentration.

The zones of inhibition formed around five different concentrations of *Tribulus terrestris* ethanol extract is shown in Table V. The zone was maximum (28 mm) in *S. aureus* and *S. typhi* (26 mm) cultures at 10 mg concentration. The smallest zone (14 mm) was observed in *E. coli* culture at the same concentration.

Among the solvents tested, diethyl ether extract was the best. Hexane extract also showed significant activity similar to diethyl ether but little lesser than it. Ethanol extract exhibited moderate activity. The bacterial activity was low with ethyl acetate extract. This may be attributed to the presence of lipophilic compounds especially steroids in the extracts. The diethyl extract showed maximum inhibition zone (40 mm) against *K. pneumoniae* followed by *S. typhi* (39 mm) and *S. aureus* (37 mm). Hexane extract showed 34 mm zones against *S. typhi* and *E. faecalis* followed by *K. pneumoniae* and *S. aureus* creating 30 mm zones. Medium-diameter zones (28 mm and 27 mm) was observed in *S. aureus* around ethanol and ethyl acetate extracts. 26 mm zone was formed around ethanol extract against *S. typhi*. *E. coli* was susceptible in all the extracts however registering small zones of inhibition: 25 mm in diethyl ether extract followed by 18 mm in hexane, 14 mm in ethanol and 8 in ethyl acetate extract. Among the tested bacterial species, *S. aureus* was the most susceptible organism whose zones of inhibition in all the extracts were greater than 25 mm. Next to this *S. typhi* and *K. pneumoniae* recorded zones of inhibition greater than 25 mm in diethyl ether and hexane extracts. *E. faecalis* had zone of inhibition greater than 25 mm only around hexane extract. *E. coli* was the least susceptible bacteria with all the tested extracts. Among the concentrations tested, 10 mg was

Table 3. Zones of inhibition formed by *Tribulus terrestris* diethyl ether extract

Bacteria	Zones of inhibition (mm)					
	Standard	**10 mg**	**5 mg**	**2.5 mg**	**1.25 mg**	**0.625 mg**
E. faecalis	20	24	20	15	11	9
E. coli	22	25	18	15	14	8
K. pneumoniae	45	40	34	25	20	20
S. typhimurium	36	39	30	30	21	18
S. aureus	40	37	32	16	9	6

Table 4. Zones of inhibition formed by *Tribulus terrestris* ethyl acetate extract

Bacteria	Zones of inhibition (mm)					
	Standard	**10 mg**	**5 mg**	**2.5 mg**	**1.25 mg**	**0.625 mg**
E. faecalis	36	17	14	7	6	5
E. coli	15	8	7	6	6	6
K. pneumoniae	32	11	10	7	6	6
S. typhimurium	38	20	17	8	7	6
S. aureus	35	27	17	8	6	6

Table 5. Zones of inhibition formed by *Tribulus terrestris* ethanol extract

Bacteria	Zones of inhibition (mm)					
	Standard	10 mg	5 mg	2.5 mg	1.25 mg	0.625 mg
E. faecalis	33	16	8	7	6	6
E. coli	18	14	8	7	6	6
K. pneumoniae	32	23	17	16	10	6
S. typhimurium	33	26	10	8	7	7
S. aureus	30	28	15	13	7	6

effective forming greater zones and the activity decreased with decrease in concentration up to 0.625 mg, the least concentration in which the extract was tested. Even at 0.625 mg concentration, greater than 15 mm zones were observed around hexane extracts in *E. faecalis* (18 mm), *K. pneumoniae* (16 mm), *S. typhi* (19 mm) and *S. aureus* (18 mm) cultures. Greater than 15 mm zones were also seen around diethyl ether extracts in *K. pneumoniae* (20 mm) and *S. typhi* (18 mm) cultures.

One study has previously reported the antimicrobial activities of the ethanol extract of *Tribulus terrestris* fruit and its mixture with *C. bursa-pastoris* and *G. glabra* against six pathogens that could be effectively used for the treatment of oral infections, although the exact components have not been elucidated [37]. Interestingly, in another work with agar diffusion assay, the methanolic extracts of *T. terrestris* fruits, stems plus leaves and roots have showed considerable activity against four bacteria tested, yet again without revealing the phytochemicals concerned [33]. However, one research has unravelled saponin as the main constituent from the methanol extract of aerial parts of *T. terrestris* when evaluated against clinical isolates of *E. coli* from urinary tract infections [38]. Another group, on screening the antimicrobial activity of the methanolic and aqueous extracts of *Tribulus terrestris* L. fruits have identified alkaloids, tannins, glycosides, flavonoids, saponins, and phenols [39]. There is also an earlier report on methanol extract of *T. terrestris* whole plant with secondary metabolites such as steroids, terpenoids, carbohydrates and glycosides determined for its antimicrobial activity [40]. Nevertheless, our phytochemical analysis on leaf extract reveals the presence of phenols, flavones, saponins, steroids, alkaloids, quinones and tannins. And the inhibitory effect can be rightly attributed to the synergistic effect of the phytochemicals in the extract that has prevented the growth of bacteria [7]. Hence our study affirms that *Tribulus terrestris* leaf extract has prevented the growth of bacteria following one or more of these mechanisms: inhibiting bacterial cell wall synthesis, depolarizing the cell membrane, inhibiting bacterial protein synthesis, inhibiting bacterial nucleic acid synthesis, and/or inhibiting bacterial metabolic pathways. These are the usual pathways taken by an antimicrobial to eliminate bacteria, be it, either Gram-negative or Gram-positive. Further molecular studies should be carried out to elucidate the exact mechanism.

4. CONCLUSION

To conclude, in the present investigation *Tribulus terrestis* leaves is proven scientifically to contain potential antimicrobial components that may be of great use for the development of antibiotics against various infectious diseases. The hexane, diethyl ether, ethyl acetate and ethanol extracts of *Tribulus terrestris* leaves possess significant inhibitory effect against tested pathogens: *Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae, Salmonella typhi* and *Staphylococcus aureus*. Although four different solvents: hexane, diethyl ether, ethyl acetate and ethanol were tested for extraction of these antimicrobial compounds, well-diffusion assay showed that there is little difference in the antimicrobial activities between the solvents used for extraction in this study. However, among the solvents used, diethyl ether extract of *Tribulus terrestris* was potent against all the tested bacterial species and *Staphylococcus aureus* exhibited the highest sensitivity to all the extracts. The results of the study support the folklore claim as an antibacterial and can pave way for the development of new antimicrobial drugs from the plant. Therefore, further studies should focus on the isolation and identification of compounds that may be contributing to inhibition of opportunistic pathogenic bacteria, Additionally, *Tribulus terrestris* leaf with other solvents should also be evaluated.

NOTE

The study highlights the efficacy of "Traditional medicine" which is an ancient tradition, used in some parts of India. This ancient concept should be carefully evaluated in the light of modern medical science and can be utilized partially if found suitable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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