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in vitro Evaluation of Aqueous and Solvent extract of *Tribulus terrestris* L. leaf against Human bacteria

Kiran.B.[1]*, Lalitha.V.[2],Raveesha.K.A.[3]

[1]PG department of Biosciences,CMR Institute of Management Studies (Autonomous),C.A. #2, 3rd 'C' Cross, 6th 'A' Main, HRBR layout, 2nd Block,Kalyana Nagar Bangalore -560043,Karnataka State,India,

[2]Department of Studies in Botany and Microbiology,Maharanis Science College for Women, Palace Road,Bangalore-560001,Karnataka State,India,

[3]Department of Studies in Botany, Manasagangotri,University of Mysore, Mysore - 570 006,Karnataka State,M,India.

Corres. Author: bkiran2702@gmail.com, kiran_hdtl@rediffmail.com
Phone: +91: 09379267558, 09880698110

Abstract: Antibacterial activity of aqueous (10-50%) and solvent (500, 1000, 1500 and 2000ppm) extract were tested against three Gram positive viz., *Bacillus subtilis*, *Bacillus cereus* and *Staphylococcus aureus* and three Gram negative bacteria viz., *Escherichia coli, Pseudomonas aeruginosa* and *Salmonella typhi in vitro* condition. Among the six pathogens tested, in aqueous extract at 50% concentration, maximum inhibition of 31.0mm was observed in *B. cereus* followed by *E.coli* (29.0mm), *S.aureus* (27.0 mm), *P. aeruginosa* (23.0 mm), *B.subtilis* (19.0mm) and least inhibition was observed in *S.typhi* (18.0 mm). Among the four solvent tested, methanol extract recorded a maximum inhibition of 33.0mm at 2000ppm concentration in *B. cereus*, followed by *E.coli* (31.0 mm) and *S.aureus* (29.0 mm). Moderate activity was observed in petroleum ether extract and chloroform extract. No activity was observed in ethanol extract at different concentration tested. Compared to synthetic antibiotics Gentamicin (25mg), Tetracycline (25mg) and Streptomycin (25mg), both aqueous and solvent extract recorded significant antibacterial activity.
Key words: *Tribulus terrestris,* antibacterial, aqueous extract, solvent extract, antibiotics, medicinal plants.

INTRODUCTION:

Bacterial diseases accounts for high proportion of health problems in the developing countries. To manage the bacterial diseases, many synthetic antibiotics are regularly used. Due to indiscriminate use of synthetic antibiotics, Bacteria have developed resistance to many antibiotics and as a result, immense clinical problem in the treatment of infectious diseases has been created [1]. Resistant pathogens develop and spread and as a result, the effectiveness of the antibiotics is diminished. This type of bacterial resistance to the antimicrobial agents poses a very serious threat to public health, and for all kinds of antibiotics [2]. Researchers are forced to search for new

alternative source to avoid the use of synthetic antibiotics which will support to develop resistance, new pathological races and which is not biodegradable, herbal medicine has been exposed to have genuine utility and about 80% of rural populations depend on it as their primary health care. The world health organization recently compiled and inventory of more than 20,000 species of medicinal plants. There are many reports available where Indian medicinal plants and their products are used to control diverse disease [3]. In India, medicinal plants are widely used by all sections of people either directly as folk remedies or in different indigenous systems of medicine or indirectly in the pharmaceutical preparations of modern medicines. Plant origin herbal medicines are considered as safe alternatives of synthetic drugs. Plants are used in modern medicine where they occupy a very significant place as raw material for important drugs[4]. Plants contain active constituents that are used in the treatment of many human diseases. Plants are rich sources of ecologically developed secondary metabolites, which are potential remedies for different ailments. Extreme interest in plants with antibacterial activity has revived as result of current problems such as resistance associated with the use of antibiotics obtained from microorganisms [5]. Plants used in traditional medicine contain a vast array of substances that can be used to treat chronic and infectious diseases. Plants specifically herbal medicines have received much attention as source of new antibacterial drugs since they are considered as time-tested and comparatively safe both for human use and for environment [6]. Plants are known to produce certain bioactive molecules which react with other organisms in the environment inhibiting bacterial growth. In the present study, aqueous and solvent extracts of leaf of *Tribulus terrestris* L. belongs to family Zygophyllaceae were evaluated for antibacterial activity against five different human pathogens.

MATERIALS AND METHODS:

Plant Material : Fresh leaves of *T. terrestris* free from diseases were collected from Mysore. The leaves were washed thoroughly 2-3 times with running tap water and once with sterile distilled water, leaf material was then air dried on a sterile blotter under shade and used for extraction.

Extraction

Aqueous extraction: 50 grams of thoroughly washed leaves of *T. terrestris* were macerated with 50 ml of sterile distilled water in a Waring blender (Waring International, New Hartford, CT,USA) for 10min. The macerate was first filtered through double-layered muslin cloth and then centrifuged at 4000 g for 30 minutes. The supernatant was filtered through Whatman No.1 filter paper and sterilized at 120 ^{o}C for 15 minutes. The extract was preserved aseptically in a brown bottle at 5^{o}C until further use [7].

Solvent extraction: Thoroughly washed leaves of *T. terrestris* were dried in shade for five days and then powdered with the help of Waring blender. 25 grams of shade dried powder was filled in the thimble and extracted successively with petroleum ether, chloroform, methanol and ethanol in a Soxhlet extractor for 48 hours. Solvent extracts were concentrated under reduced pressure. After complete evaporation, 1gram of each concentrated solvent extracts were dissolved in 9 ml of methanol and used for antibacterial assay.

Test pathogens: *In vitro* antibacterial activity was examined for aqueous and solvent extracts. Authenticated pure cultures of three Gram negative human bacteria viz., *Escherichia coli, Pseudomonas aeruginosa* and *Salmonella typhi*. Three Gram positive bacteria viz., *Bacillus subtilis*, *Bacillus cereus* and *Staphylococcus aureus* were obtained from Research Center, CMR Institute of Management Studies (Autonomous), Bangalore. All Gram Negative bacteria were grown in Mac Conkey agar medium and Gram Positive bacteria in blood agar medium and maintained at 4^{o}C until further use.

Preparation of Inoculum

Preparation of standard culture inoculums of test organism: All the test Gram Negative bacterial species were inoculated into 2 ml Mac Conkey broth and Gram positive bacteria to nutrient broth and incubated at 37^{o}C for 24 hours till the growth in the broth was equivalent with Mac-Farland standard (0.5%) as recommended by WHO[8].

Antibacterial assay

Aqueous Extract

Agar cup diffusion method: An overnight culture of *E. coli, P. aeruginosa, S. typhi, B. subtilis*, *B.cereus* and *S. aureus* was standardized to contain approximately 107cfu/ml. Gram negative bacteria was inoculated into 20 ml of Mac Conkey broth and Gram positive bacteria was inoculated to Nutrient broth. The culture medium was allowed to set. Thereafter, all the inoculum was swabbed over the surface of the Mac Conkey agar medium for Gram negative bacteria and blood agar medium for Gram positive bacteria plate using sterile cotton swab. Using a sterile cork borer of 5 mm diameter, five wells were made in solidified sterile Mac Conkey agar medium and blood agar

medium plate (one in the centre and four wells at the corner). The agar plugs were removed with a flamed and cooled wire loop. Then 10,20,30,40 and 50µl of aqueous extract of *T. terrestris* leaves were placed in the wells made in inoculated plates. The treatment also includes 50 µl of sterilized distilled water as control. All the plates were incubated for 24hours at 37^{o}C and zone of inhibition if any around the well were measured in millimeter (mm). For each treatment ten replicates were maintained. The same procedure were followed for standard antibiotics Gentamicin (25mg),Tetracycline (25mg) and Streptomycin (25mg) to compare the efficacy of aqueous extract against test organisms[7,9].

Solvent Extract: One gram of different solvent extract of *T. terrestris* leaves were dissolved in 9 ml of methanol. The sterile Mac Conkey agar medium (for Gram negative bacteria) and Blood agar medium (For Gram positive medium) in petridishes was uniformly smeared with test culture. 5 mm wells were made in each petridish to which 50 µl of 500, 1000, 1500 and 2000ppm of different solvent extracts dissolved in methanol were added. For each treatment ten replicates were maintained. Respective solvents served as control. Standard antibiotics viz., Gentamicin (25mg), Tetracycline (25mg) and Streptomycin (25mg) was used to compare the efficacy of solvent extract against test organisms[7].

Statistical Analysis: The data were subjected to Tukey's HSD analysis. Data on percentages were transformed to arcsine and analysis of variance (Anova) was carried out with transformed values. The means were compared for significance using Tukey's HSD (P=0.05).

Table 1: Antibacterial activity of aqueous extract of *T. terrestris*

Bacteria	Zone of inhibition(mm)							
	Concentration							
	Plant extract					Synthetic antibiotics		
	10 µl	20 µl	30 µl	40 µl	50 µl	Gentamicin (25mg)	Tetracycline (25mg)	Streptomycin (25mg)
B.subtilis	5.0^{a} ±0.1	7.0^{b} ±0.1	11.0^{c} ±0.2	14.0^{d} ±0.1	19.0^{e} ±0.2	32.0^{c} ±0.1	31.0^{b} ±0.1	30.0^{a} ±0.1
E. coli	7.0^{a} ±0.5	13.0^{b} ±0.5	17.0^{c} ±0.5	23.0^{d} ±0.1	29.0^{e} ±0.1	36.0^{c} ±0.2	33.0^{b} ±0.2	31.0^{a} ±0.2
P. aeruginosa	7.0^{a} ±0.1	12.0^{b} ±0.5	14.0^{c} ±0.0	18.0^{d} ±0.1	23.0^{e} ±0.1	32.0^{b} ±0.1	33.0^{c} ±0.1	28.0^{a} ±0.1
B. cereus	8.0^{a} ± 0.2	15.0^{b}± .2	21.0^{c} ±0.1	26.0^{d} ±0.1	31.0^{e} ±0.2	30.0^{a} ±0.3	34.0^{c} ±0.2	32.0^{b} ±0.1
S. typhi	4.0^{a} ±0.1	8.0^{b} ±05	13.0^{c} ±0.1	16.0^{d} ±0.1	18.0^{e} ±0.1	35.0^{c} ±0.2	30.0^{a} ±0.1	33.0^{b} ±0.0
S. aureus	7.0^{a} ±0.4	11.0^{b} ±0.5	15.0^{c} ±0.1	21.0^{d} ±0.1	27.0^{e} ±0.1	30.0^{b} ±0.1	28.0^{a} ±0.1	31.0^{c} ±0.1

- Values are the mean of ten replicates, ±standard error.
- The means followed by the same letter (s) are not significantly different at P 0.05 when subjected to Tukey's HSD.
- Pattern of percentage inhibition increase is not uniform for all the microorganisms

Table 2: Antibacterial activity of solvent extracts of *T. terrestris*

Bacteria	Zone of inhibition(mm)										
	Concentration										
	Petroleum ether extract				Chloroform extract				Synthetic antibiotics		
	500 ppm	1000ppm	1500ppm	2000ppm	500 ppm	1000 ppm	1500 ppm	2000 ppm	Gentamicin (25mg)	Tetracycline (25mg)	Streptomycin (25mg)
B.subtilis	5.0^{a} ±0.1	10.0^{b} ±0.2	13.0^{c} ±0.2	18.0^{d} ±0.2	3.0^{a} ±0.2	6.0^{b} ±0.1	8.0^{c} ±0.1	11.0^{d} ±0.1	32.0^{c} ±0.1	31.0^{b} ±0.1	30.0^{a} ±0.1
E. coli	7.0^{a} ±0.1	13.0^{b} ±0.1	17.0^{c} ±0.1	25.0^{d} ±0.1	4.0^{a} ±0.1	8.0^{b} ±02	13.0^{c} ±0.2	17.0^{d} ±0.2	36.0^{c} ±0.2	33.0^{b} ±0.2	31.0^{a} ±0.2
P. aeruginosa	5.0^{a} ±0.0	12.0^{b} ±0.1	17.0^{c} ±0.1	20.0^{d} ±0.2	3.0^{a} ±0.1	7.0^{b} ±0.1	11.0^{c} ±0.3	15.0^{d} ±0.1	32.0^{b} ±0.1	33.0^{c} ±0.1	28.0^{a} ±0.1
B. cereus	8.0^{a} ±0.0	15.0^{b} ±0.1	23.0^{c} ±0.2	28.0^{d} ±0.1	5.0^{a} ±0.2	9.0^{b} ±0.1	15.0^{c} ±0.1	20.0^{d} ±0.1	30.0^{a} ±0.3	34.0^{c} ±0.2	32.0^{b} ±0.1
S. typhi	5.0^{a} ±0.3	8.0^{b} ±0.2	12.0^{c} ±0.1	17.0^{d} ±0.1	3.0^{a} ±0.1	5.0^{b} ±0.1	6.0^{c} ±0.2	8.0^{d} ±0.2	35.0^{c} ±0.2	30.0^{a} ±0.1	33.0^{b} ±0.0
S. aureus	4.0^{a} ±02	13.0^{b} ±0.1	20.0^{c} ±0.1	25.0^{d} ±0.1	3.0^{a} ±0.2	7.0^{b} ±0.1	13.0^{c} ±0.1	16.0^{d} ±0.1	30.0^{b} ±0.1	28.0^{a} ±0.1	31.0^{c} ±0.1

- Values are the mean of ten replicates, ±standard error.
- The means followed by the same letter (s) are not significantly different at P 0.05 when subjected to Tukey's HSD.
- Pattern of percentage inhibition increase is not uniform for all the microorganisms

Table 3: Antibacterial activity of solvent extracts of *T. terrestris*

Bacteria	Zone of inhibition(mm)										
	Concentration										
	Methanol extract				Ethanol extract				Synthetic antibiotics		
	500 ppm	1000 ppm	1500 ppm	2000ppm	500 ppm	1000 ppm	1500 ppm	2000 ppm	Gentamicin (25mg)	Tetracycline (25mg)	Streptomycin (25mg)
B.subtilis	8.0^{a} ±0.1	12.0^{b} ±0.2	15.0^{c} ±0.2	21.0^{d} ±0.2	0.0	0.0	0.0	0.0	32.0^{c} ±0.1	31.0^{b} ±0.1	30.0^{a} ±0.1
E. coli	9.0^{a} ±0.2	17.0^{b} ±0.1	25.0^{c} ±0.2	31.0^{d} ±0.1	0.0	0.0	0.0	0.0	36.0^{c} ±0.2	33.0^{b} ±0.2	31.0^{a} ±0.2
P. aeruginosa	7.0^{a} ±0.1	15.0^{b} ±0.1	20.0^{c} ±0.1	23.0^{d} ±0.1	0.0	0.0	0.0	0.0	32.0^{b} ±0.1	33.0^{c} ±0.1	28.0^{a} ±0.1
B. cereus	10.0^{a} ±0.2	18.0^{b} ±0.1	27.0^{c} ±0.1	33.0^{d} ±0.1	0.0	0.0	0.0	0.0	30.0^{a} ±0.3	34.0^{c} ±0.2	32.0^{b} ±0.1
S. typhi	5.0^{a} ±0.1	10.0^{b} ±0.1	14.0^{c} ±0.1	20.0^{d} ±0.1	0.0	0.0	0.0	0.0	35.0^{c} ±0.2	30.0^{a} ±0.1	33.0^{b} ±0.0
S. aureus	6.0^{a} ±0.1	16.0^{b} ±0.1	23.0^{c} ±0.1	29.0^{d} ±0.1	0.0	0.0	0.0	0.0	30.0^{b} ±0.1	28.0^{a} ±0.1	31.0^{c} ±0.1

- Values are the mean of ten replicates, ±standard error.
- The means followed by the same letter (s) are not significantly different at P 0.05 when subjected to Tukey's HSD.
- Pattern of percentage inhibition increase is not uniform for all the microorganisms

RESULT AND DISCUSSION:

Aqueous extract: Among six bacteria tested, maximum inhibition was recorded in *B.cereus* which showed 8.0mm inhibition at 10µl concentration and 31.0mm at 50 µl concentration. Compared to synthetic antibiotics, Gentamicin recorded 32.0mm, Tetracycline recorded 31.0mm and Streptomycin recorded 30.0mm inhibition at recommended 25 µg concentration. Significant activity was also observed in 20, 30 and 40 µl concentration. *B.cereus* is followed by *E.coli* which recorded a maximum inhibition of 29.0mm at 50 µl concentration. Compared to synthetic antibiotics, Gentamicin recorded 36.0mm, Tetracycline recorded 33.0mm and Streptomycin recorded 31.0mm inhibition. Moderate activity was observed in *S. aureus* and recorded 27.0mm inhibition at 50 µl concentration. Compared to synthetic antibiotics, Gentamicin recorded 31.0mm, Tetracycline recorded 33.0mm and Streptomycin recorded 31.0mm inhibition. Significant activity was observed in *P. aeruginosa* (23.0mm), *S. typhi* (19.0mm) and *B. subtilis* (18.0mm) at 50 µl concentration. The zone of inhibition goes on increasing with increasing in concentration (Table 1).

Solvent extract: Maximum inhibition was observed in methanol extract followed by petroleum ether extract and chloroform extract. No inhibition was observed in ethanol extract tested at 500,1000, 1500 and 2000ppm concentration. In methanol extract, maximum inhibition was observed in *B.cereus* at 2000ppm concentration and recorded 33.0mm inhibition. Compared to synthetic antibiotics Gentamicin recorded 30.0mm, Tetracycline recorded 34.0mm and Streptomycin recorded 32.0mm inhibition. *B. cereus* is followed by *E.coli* (31.0mm), *S. aureus* (29.0mm), *P. aeruginosa* (23.0mm), *B. subtilis* (21.0mm) and *S. typhi* (29.0mm). Significant activity was also observed in 500, 1000 and 1500ppm concentration in all the test bacteria (Table 3). In petroleum ether extract, at 2000ppm concentration, *B.cereus* recorded a maximum inhibition of 28.0mm, followed by *E.coli*(25.0mm), *S. aureus* (25.0mm), *P. aeruginosa* (20.0mm), *B. subtilis* (18.0mm) and *S. typhi* (17.0mm). Compared to synthetic antibiotics, it recorded a significant activity (Table 2). In chloroform extract at 2000ppm concentration, *B.cereus* showed 20.0mm inhibition, *E.coli*(17.0mm), *S. aureus* (16.0mm), *P. aeruginosa* (15.0mm), *B. subtilis* (11.0mm) and *S. typhi* (8.0mm) (Table 2). No significant activity was observed in ethanol extract (Table 3). The zone of inhibition goes on increasing with increase in concentration.

Herbal medicine is the oldest form of healthcare known to mankind. Herbs had been used by all cultures throughout history. It was an integral part of the development of modern civilization. Primitive man observed and appreciated the great diversity of plants available to him. The World Health Organization (WHO) estimates that 4 billion people, 80 percent of the world population, presently use herbal medicine for some aspect of primary health care. Plants have been evaluated *in vitro* for their antibacterial potency against some important human pathogenic bacteria. In the present study *Tribulus terrestris* L which is a potent medicinal plants was evaluated for antibacterial activity both in aqueous and solvent extract. It was observed that in 10 to 50 µl concentration tested, significant activity was observed in all the test bacteria which is nearer and equal to synthetic antibiotics which is not safe for human health and ecosystem. In solvent extract, maximum activity was observed in 2000ppm concentration among all the test bacteria. Further investigation is necessary to isolate the bioactive compound in methanol extract and aqueous which can shoe a better broad spectrum antibacterial activity which will help to avoid the use of synthetic antibiotics.

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