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Research Article

IN VITRO ANTIMICROBIAL ACTIVITY OF *TRIBULUS TERRESTRIS* L.

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ABSTRACT

Free and bound flavonoids of different parts of *Tribulus terrestris* (Zygophyllaceae) L. have been studied for their antimicrobial activities using disc diffusion assay, against two Gram negative bacteria (*Escherichia coli* MTCC 46 and *Proteus mirabilis* MTCC 1425), one Gram positive bacteria (*Staphylococcus aureus* MTCC 87) and two fungi (*A. flavus* MTCC 277 and *A. niger* MTCC 282). Minimum inhibitory concentration (MIC) of the extract was evaluated by microbroth dilution method, while minimum bactericidal/ fungicidal concentration determined by subculturing the relevant samples. The plant exhibited good activity against tested bacteria while it showed no activity against tested fungi. Free flavonoids were found to be more potent than bound flavonoids. Among 8 extracts tested 7 were found to be active, while 1 extract was found to be inactive at tested concentration. Total activity (TA) was calculated for the extracts, to relate MIC of the extracts with its amount isolated from 1 g dried plant part. Results of the present study indicate that *T. terrestris* can be exploited for future antibacterial drug.

Keywords: Flavonoids, *Tribulus terrestris*, Antimicrobial activity, Disc diffusion assay, Total activity.

INTRODUCTION

Medicinal plants are an indispensable part of the traditional medicine practiced all over the world because of low cost, easy access and ancestral experience [1]. During the past decade, traditional system of medicine has become increasingly important as they are considered to be safe and have long lasting effect. In many developing countries a large part of population relies on herbal medicines. Phytomedicines have maintained popularity for historical and cultural reasons and have attracted attention as an alternative therapy [2, 3].

Irrespective of the decline in the use of plants as herbal medicines after advent of antibiotics in 1900, the importance of botanicals in the evolution of medicines remains unchallenged. Revival of interest in plant derived drugs is mainly due to the current widespread belief that 'Green Medicine' is safe, cheap and more dependable than costly synthetic drugs with adverse side effects, thus making natural therapeutics as an attractive option of synthetic pharmaceuticals [4]. Many researchers have focused the investigation of natural products and plant extracts as source of new bioactive molecules [5, 6].

In recent years, pharmaceutical industries have spent a lot of time and money in developing natural products, extracted from medicinal plants to produce more cost effective remedies that are affordable to a common man. WHO has acknowledged increasing awareness of herbals and recently defined traditional medicine (including herbal drugs and medicinal plants) as comprising therapeutic practices that have been in existence almost for several thousands of years before the development and spread of modern medicine and are still in use[7].

Flavonoids are common and widespread secondary metabolites which have a wide range of biological and physiological activities. They are a group of polyphenolic compounds possessing low molecular weight. They are ubiquitous in photosynthesising cells and are commonly found in fruits, vegetables, nuts, seeds, stems, flowers, tea, wine, propolis and honey. The objective of this study was to evaluate the antimicrobial potential of free and bound flavonoids of *T. terrestris* (root, stem, leaves and fruits).

MATERIALS AND METHODS

Collection and identification of plant material

Tribulus terrestris L. was collected from different localities of Jaipur in the month of June, 2008 and was identified at Department of Botany, UOR. A voucher specimen (RUBL- 20390) was also submitted to the Herbarium of Botany Department, UOR.

Extraction of flavonoids

Flavonoids were extracted from different parts of the plant (root, stem, leaves and fruits) following the well established method of Subramanian and Nagarajan [8]. Hundred grams of finely powdered plant parts were Soxhlet extracted with hot 80% methanol (500 ml) on a water bath for 24 h and filtered. Filtrate was re-extracted successively with petroleum ether, ethyl ether and ethyl acetate. Each step was carried out three times to ensure complete extraction. Petroleum ether fraction was discarded due to being rich in fatty substances and ethyl ether fractions (free flavonoids) were collected. Ethyl acetate fractions were analyzed for bound flavonoids. Each fraction was hydrolyzed in 7% H_2SO_4 for 2 h. Resulting mixture was filtered and filtrate was again extracted with ethyl acetate. The ethyl acetate extract was washed with distilled water till neutrality and collected. The ethyl ether (free flavonoids) and ethyl acetate fractions (bound flavonoids) were dried in *vaccuo*, weighed and stored in glass vials at 4^0C till used.

Test microorganisms

Bacterial strains of *E. coli* (MTCC 46), *S. aureus* (MTCC 87) and *P. mirabilis* (MTCC 1425) were procured from IMTECH, Chandigarh, India. Bacterial strains were grown and maintained on 'Muller-Hinton Agar medium' (Beef extract 2.0 g; Peptone 17.5 g; Starch 1.5 g; Agar 17.0 g; in 1000 ml of distilled water; Final pH 7.4 ± 0.2) at 37±2^0 C while fungal strains were kept on 'Sabouraud Dextrose Agar' medium (Peptone 10 g; Dextrose 20 g; Agar 20 g in 1000 ml of distilled water; pH adjusted to 6.8 - 7.0) at 27±2^0 C.

Antimicrobial screening of extracts

Disc diffusion assay (DDA)

Disc diffusion assay (DDA) was performed for antimicrobial screening [9, 10, 11]. MH agar (for bacteria) and SD agar (for fungi) base plates were seeded with the standard size of bacterial and fungal inoculum (1×10^8 CFU/ml for bacteria, 1×10^7 CFU/ml for fungi). Sterile filter paper discs (6 mm in diameter) were impregnated with 100 µl of each of the extract (10 mg/ml concentration) to give a final concentration of 1 mg/disc, left to dry *in vaccuo* to remove residual solvent, which might interfere with the determination. Extract discs were then placed on the seeded agar plates. Each extract was tested in triplicate along with streptomycin (1 mg/disc) and itraconazole (1 mg/disc) as standard for bacteria and fungi, respectively. The plates were kept at 4°C for 1 h for diffusion of extract, thereafter were incubated at 37± 2°C for 24 h; 27 ± 2°C for 48 h for bacteria and fungi, respectively. Zone of inhibition (IZ) or depressed growth of microorganisms was measured and the 'Activity Index' (AI) for each extract was calculated.

Activity Index (AI) = Inhibition zone of the sample / Inhibition zone of the standard

Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) was determined for each plant extract showing antimicrobial activity against test pathogens in disc diffusion assay. Broth microdilution method [12] was followed for determination of MIC values. Plant extracts were re-suspended in acetone (which has no activity against test microorganisms) to make 10 mg/ml final concentration and then was added to broth media of 96-wells of microtiter plates using two fold serial dilution. Thereafter 100 µl inoculum of standard size was added to each well. Bacterial and fungal suspensions were used as negative control, while broth containing standard drugs was used as positive control. The microtiter plates were incubated at 37 ± 2°C for 24 h for bacteria, 27 ± 2°C for fungi. Each extract was assayed in duplicate and each time two sets of microtiter plates were prepared, one was kept for incubation while another set was kept at 4°C for comparing the turbidity in the wells of microtiter plate. The MIC values were taken as the lowest concentration of the extracts in the well of the microtiter plate that showed no turbidity after incubation. The turbidity of the wells in the microtiter plate was interpreted as visible growth of microorganisms.

Minimum bactericidal/fungicidal concentration (MBC/MFC)

Minimum bactericidal/fungicidal concentration (MBC/MFC) was determined by subculturing 50 µl from each well showing no apparent growth. Least concentration of extract showing no visible growth on subculturing was taken as MBC/MFC.

Total Activity (TA)

Total activity for each active extract was calculated by the well established formula [13]. TA value (ml/g) is the volume to which the extract can be diluted retaining the ability to inhibit the growth of microorganisms.

Total Activity (TA) = Amount extracted from 1 g plant material / MIC of the extract

RESULTS AND DISCUSSIONS

Amount of flavonoids extracted from plant parts were calculated and recorded in Table 1. Maximum free and bound flavonoids were observed in fruits and roots, respectively whereas maximum total flavonoid content was observed in roots of the plant.

Antimicrobial activity (assed in terms of IZ, AI, MIC and MBC/MFC) of the flavonoid, tested against selected microorganisms was recorded in Table 2 and 3. In the present study the flavonoid extracts showed only antibacterial activity and were found to be inactive against test fungi. Most susceptible organism in the investigation was *E. coli* against which most of the extracts showed inhibition zone whereas most resistant bacteria was *P. mirabilis*, against which only three extracts showed activity. Best activity against *E. coli* (IZ 12 mm, AI 0.6, MIC 0.156 mg/ml and MBC 0.312 mg/ml), *S. aureus* (IZ 10.5 mm and AI 0.42) with same (0.312 mg/ml) MIC and MBC and *P. Mirabilis* (IZ 16.2 mm, AI 0.67, MIC 0.019 mg/ml and MBC 0.039 mg/ml).

Total activity was also calculated and tabulated in Table 4. Maximum TA values calculated were 18.58, 9.28 and 305.26 ml/g against *E. coli*, *S. aureus* and *P. mirabilis*, respectively.

Table 1: Flavonoid content of different parts of *T. terrestris*

Part	Flavonoids (mg/g.d.w.)		
	Free	Bound	Total
Root	3.3	5	8.3
Stem	2.82	0.4	3.22
Leaf	2.73	0.13	2.86
Fruit	5.8	1.46	7.2

Table 2: IZ and AI of flavonoids of *T. Terrestris*

Microorganisms		*E. coli*		*S. aureus*		*P. mirabilis*		*A. flavus*		*A.niger*	
Parts	**Extract**	**IZ**	**A1**	**IZ**	**AI**	**IZ**	**AI**	**IZ**	**AI**	**IZ**	**AI**
Root	F	9	0.45	10	0.4	-	-	-	-	-	-
	B	8.2	0.41	-	-	-	-	-	-	-	-
Stem	F	8.4	0.42	-	-	9.4	0.39	-	-	-	-
	B	-	-	-	-	-	-	-	-	-	-
Leaf	F	12	0.6	-	-	10.2	0.42	-	-	-	-
	B	-	-	8.6	0.34	-	-	-	-	-	-
Fruit	F	10	0.5	9	0.36	16.2	0.67	-	-	-	-
	B	-	-	10.5	0.42	-	-	-	-	-	-
Standard		20		25		24		15		10	

IZ=Inhibition zone (mm)
AI= (IZ developed by extract/IZ developed by standard),
F = Free flavonoids; B = Bound flavonoids,
(–) = No activity,
Standards= Streptomycin for bacteria and Itraconazole for fungi.

Table 3: MIC and MBC/MFC of flavonoids of *T. terrestris*

Microorganisms		*E. coli*		*S. aureus*		*P. mirabilis*		*A. flavus*		*A.niger*	
Parts	**Extract**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MFC**	**MIC**	**MFC**
Root	F	0.312	0.625	0.312	0.625	-	-	-	-	-	-
	B	0.625	1.25	-	-	-	-	-	-	-	-
Stem	F	0.625	1.25	-	-	0.625	1.25	-	-	-	-
	B	-	-	-	-	-	-	-	-	-	-
Leaf	F	0.156	0.312	-	-	0.312	0.625	-	-	-	-
	B	-	-	0.312	0.625	-	-	-	-	-	-
Fruit	F	0.312	0.625	0.625	1.25	0.019	0.039	-	-	-	-
	B	0.312	0.625	0.312	0.312	-	-	-	-	-	-

MIC = Minimum inhibitory concentration (mg/ml)
MBC= Minimum bactericidal concentration (mg/ml)
MFC= Minimum fungicidal concentration (mg/ml)
F = Free flavonoids; B = Bound flavonoids,
(–) = No activity.

Table 4: TA of flavonoids of *T. Terrestris*

Parts	Extract	Total Activity (ml/g)				
		E. coli	*S. aureus*	*P. mirabilis*	*A. flavus*	*A. niger*
Root	F	10.57	10.57	-	-	-
	B	8	-	-	-	-
Stem	F	4.51	-	4.51	-	-
	B	-	-	-	-	-
Leaf	F	17.5	-	8.75	-	-
	B	-	0.41	-	-	-
Fruit	F	18.58	9.28	305.26	-	-
	B	-	4.67	-	-	-

F = Free flavonoids; B = Bound flavonoids,
(–) = No activity,
TA (ml/g) =weight of extract (mg/g plant material)/ MIC (mg/ml) of extract

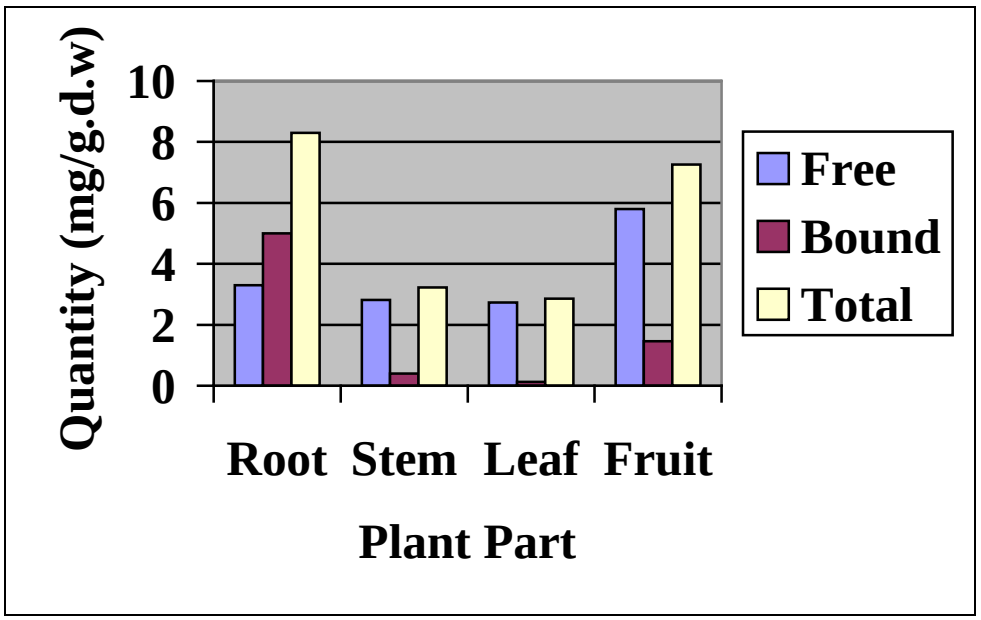

Fig. 1: Flavonoid content of different parts of *T. Terrestris*

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