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Research Article

ANTIMICROBIAL EFFECT OF HYDRO-ALCOHOLIC EXTRACTS OF *TRIBULUS TERRESTRIS, PHYLLANTHUS AMARUS* AND *HEMIDESMUS INDICUS* AGAINST COMMON BACTERIAL URINARY PATHOGENS- AN IN VITRO STUDY

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ABSTRACT

Context: Urinary tract infection (UTI) is one of the most common infectious diseases in the community. Moreover, with rapidly emerging resistance to antibiotics it is becoming increasingly difficult to treat such infections. Herbal extracts against a wider range of bacterial strains can be used as an efficient herbal drug for UTI.

Aims: The primary objective of this study was to detect the *in vitro* activity of selected Hydro-alcoholic (40:60) extracts of three plants viz. *Tribulus terrestris* (Drug-1) (fruit extracts), *Phyllanthus amarus* (Drug-2) (whole plant extracts) and *Hemidesmus indicus* (Drug-3) (Root extracts) individually and in combination against the isolated bacterial pathogens showing antimicrobial resistance

Materials and Methods: This study was conducted for a period of 3 months in the Department of Microbiology. Patients with history of recurrent UTI were included in the study population. Fifty isolates (n=50) showing multidrug resistance isolated from clinical samples are taken for the study

Results: The extracts of *Tribulus terrestris* (Group A), combinations of *Tribulus terrestris* with *Hemidesmus indicus* (Group F) and of *Tribulus terrestris, Phyllanthus amarus* and *Hemidesmus indicus* (Group G) inhibit both Gram positive and Gram negative bacteria, while *Hemidesmus indicus* (Groups C) inhibit only Gram positive bacteria. The Gram positive bacteria are more susceptible to these extracts than the Gram negative bacteria. All the herbal extracts tested showed more than 70 % sensitivity to the study group.

Conclusions: The *hydro-alcoholic* extracts of *Tribulus terrestris*, *Phyllanthus amarus* and *Hemidesmus indicus* can be used effectively in the treatment of multi-drug resistant strains causing urinary tract infection. An effective drug could be developed from combination of these herbal extracts which would be natural, more economic and free of any side- effects.

KEYWORDS: Herbal extracts, UTI, Tribulus terrestris, Phyllanthus amarus and Hemidesmus indicus.

INTRODUCTION

Urinary tract infection (UTI) is one of the most common infectious diseases in community which are caused more commonly by uro-pathogenic *Escherichia coli* (UPEC),^[1] *Staphylococcus saprophyticus*,^[2] *Klebsiella* species, *Staphylococcus epidermidis*, *Enterococcus* species, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and other coliforms.

Females are more vulnerable to get infected due to anatomical reasons, i.e. shorter urethra and its proximity to anus. The chance of urinary tract infection among school going children is 1-3% and this rises with the onset of sexual activity being more common in adolescents and young women^[3]. Chances of recurrence after the first attack of UTI are 25% within 6 months. Currently the most effective treatment for these infections is antibiotic therapy.

Long-term or frequent use of antibiotics may be harmful and moreover, with rapidly emerging

resistance to antibiotics it is becoming increasingly difficult to treat the infection. In this context, 3 plants viz. Tribulus terrestris L., Phyllanthus amarus Sch. and Hemidesmus indicus L.Br. widely used in the treatment of diseases of uro-genital system and reported to be safe^[4-6]. They have been taken up to evaluate antimicrobial activity against the commonly isolated bacteria in recurrent urinary tract infection. According to Ayurveda our ancient health care system, Gokshura (Tribulus terrestris),^[7] Bhumyamalaki (Phyllanthus *amarus*) ^[8] are the plants with diuretic activity used against UTI and Sariva (Hemidesmus indicus)^[9] is indicated for nephropathy for centuries. The natural products from herbal plants act on the body system slowly and very often they do not directly kill the disease-causing organisms but they increase immunity by creating an environment inside the body so that the organism cannot survive, therefore they have to be taken for longer periods. Generally herbal preparations

have to be taken in larger quantity compared to antibiotics.

In vitro evaluation of plants for antimicrobial property is the first step towards achieving the goal for developing eco-friendly management of infectious diseases of humans by new bio-molecules of plant origin. These three plants were screened individually and in combination *in vitro* for antibacterial activity against four human pathogenic bacteria known to be the common cause of UTI in humans.

OBJECTIVES

- 1. Isolate the common bacterial uro-pathogens causing UTI and to see their antibiotic susceptibility pattern.
- 2. Detect the *in vitro* activity of selected plant extracts individually and in combination against the isolated bacterial pathogens.
- 3. Compare the antimicrobial activity of the herbal extracts with the standard urinary antibiotic.

MATERIALS & METHODS

Study Design & Setting - A Prospective study was conducted in the Dept. of Microbiology of MKCG Medical College, Berhampur, Odisha for a period of 3 months from July to September 2011 under Short Term Studentship (STS) Program granted by ICMR, New Delhi [Reference ID: 2011-00868]. The study included patients with history of recurrent UTI (more than one time in 6 months) attending both OPD and IPD of Department of Obstetrics and Gynaecology and Department of Medicine. A total of fifty (n=50) isolated bacteria having antibiotic resistance were taken for the study group.

Exclusion Criteria

Patients with diagnosis of HIV infection, nosocomial infection or neutropenic fever due to anticancer drug therapy were excluded. Clinically diagnosed case of malaria and tuberculosis were also excluded from our study.

IEC Approval

The study protocol was approved by Institutional Ethical Committee (IEC) of the MKCG Medical College, Berhampur, Odisha.

Trial drugs

Hydro-alcoholic (40:60) extracts of three plants viz. *Tribulus terrestris* L. (fruit extracts) (Fig. 1), *Phyllanthus amarus* Sch. (Whole plant extracts) (Fig. 2) and *Hemidesmus indicus* L.Br. (root extracts) (Fig. 3) were used for the study. These were procured in sterilized packets (Fig. 4) from an ISO 9001:2000 laboratory with standardized parameters.

Study procedures

Urine samples were collected aseptically for culture before commencement of antibiotic therapy. The clean catch of midstream urine sample was collected in a sterile container. Collected specimen of urine was transported to the laboratory within 2 hours without delay and processed. Microscopical examination of wet film of un-centrifuged urine was done to determine for the presence of polymorphs (pus cells) .The samples were subjected to Semi-quantitative culture on Mac Conkey and CLED agar. The bacteria were identified based on growth character and biochemical tests.

Antibiotic sensitivity tests were done by Kirby –Baeur's disk diffusion method on Muellor-Hinton agar. Antibiotic disks used in Gram positive bacteria are Amikacin (AK), Amoxicillin/Clavulanic acid (AMC), Cotrimoxazole (COT), Cefoperazone+Sulbactam (CFS), Norfloxacin (NX), Gentamicin (G).

In Gram negative bacteria, Amikacin (AK), Amoxicillin/ Clavulanic acid (AMC), Cotrimoxazole (COT), Norfloxacin (NX), Gentamicin (G), Nitrofurantoin (NF) were used. Out of these bacterial isolates with antimicrobial resistance, 50 isolates (20 isolates of *Escherichia coli*, 10 isolates of *Klebsiella pneumoniae*, 10 isolates of *Staphylococcus aureus* and 10 isolates of *Enterococcus faecalis*) were selected for testing the sensitivity to the herbal extracts.

Standardization of the MIC for herbal extracts

ATCC strains of *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 700603), *Staphylococcus aureus* (ATCC 252923) and *Enterococcus faecalis* (ATCC 29212) were used to determine the minimum inhibitory concentration (MIC) of the herbal extracts and their combinations for these bacteria and determine the MIC of Norfloxacin which is considered as the standard drug for comparison with MIC of the herbal extracts.

Agar Dilution Method

Using Agar dilution method^[10] plates of the following combination of plant extracts were prepared in MHA media at increasing concentrations of 10mg/ml, 25mg /mL, 37.5mg /mL, 50mg /mL, 62.5mg /mL, 75mg /Ml, 82.5mg/ml and tested for antimicrobial activity.

Groups

Gr. A: Extract of Drug-1

Gr. B: Extract of Drug-2

Gr. C: Extract of Drug-3

Gr. D: Extracts of Drug-1 & Drug-2 mixed in equal proportion

Gr. E: Extracts of Drug-2 & Drug-3 mixed in equal proportion

Gr. F: Extracts of Drug-1 & Drug-3 mixed in equal proportion

Gr. G: Extracts of Drug-1, Drug-2 & Drug-3 mixed in equal proportion

Screening for the antimicrobial potential of the plant extracts and phyto-chemicals, MHA plates were

prepared having different concentration of the herbal extracts.

The stocked bacterial isolates (*Staphylococcus aureus* -10, *Enterococcus faecalis* -10, *Escherichia coli* - 20 and *Klebsiella pneumoniae* -10) were grown to exponential phase in Mueller-Hinton broth at 37°C for 18 hours and adjusted to a final density of 10⁴ CFU /mL by diluting fresh cultures and comparing with McFarland density. Using a standardized loop the bacteria were spot inoculated on the plates. Appropriate controls of ATCC strains were tested with each batch (Fig. 5). The plates were incubated at 37°C overnight. The following day the plates were examined to note the sensitivity patterns of the different strains.

The MIC of the isolated strains was compared with the ATCC strains and MIC of the Norfloxacin which is considered as standard.

RESULTS

In all the urine samples processed for bacterial culture, isolation and identification, the maximum number of cases were due to *Escherichia coli*, followed by *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Enterococcus faecalis*. A total of fifty (n=50) bacterial isolates were taken for study which includes 10 isolates of *Staphylococcus aureus*, 10 isolates of *Enterococcus faecalis*, 20 isolates of *Escherichia coli* and 10 isolates of *Klebsiella pneumoniae*. The bacterial isolates that were taken up for study are given in Table 1.

Table 1: Bacterial Isolates From Urine Samples (n=50)

Bacteria	Number of samples
Staphylococcus aureus	10
Enterococcus faecalis	10
Escherichia coli	20
Klebsiella pneumoniae	10

The antibiotic susceptibility of Gram positive bacteria i.e. *Staphylococcus aureus* and *Enterococcus faecalis* is shown in Table 2. All the isolates of *Staphylococcus aureus* were sensitive to Amikacin (AK) and 80% are sensitive to Gentamicin (G).

Moreover, 80% of the *Staphylococcus aureus* were resistant to both Amoxicillin/Clavulanic acid (AMC) and Norfloxacin (NX).

All the isolates of *Enterococcus faecalis* were resistant to Gentamicin (G) and Norfloxacin (NX). They also showed resistance to most other antibiotics.

Gram positive bacteria	Antibiotics											
	AMC		G		AK		NX		CFS		СОТ	
	S*	R*	S I	RR	S	R	S	R	S	R	S	R
Staphylococcus aureus (n*=10)	2	8	8	2	10	0	2	8	3	7	5	5
Enterococcus faecalis (n=10)	3	7	0	10	2	8	0	10	1	9	2	8

Table 2: Antibiotic Susceptibility for Gram Positive Bacterial Isolates

(*S=sensitive, R=resistant, n=number)

(AMC-Amoxicillin/Clavulanic acid, G-Gentamicin, AK-Amikacin, NX-Norfloxacin, CFS-Cefoperazone+Sulbactam, COT-Cotrimoxazole)

The antibiotic susceptibility of Gram negative bacteria i.e. *Escherichia coli* and *Klebsiella pneumoniae* is given in Table 3. *Escherichia coli* showed 100% sensitivity to Nitrofurantoin (NF). 95% are resistant to Amoxicillin/Clavulanic acid (AMC), 90% to Cotrimoxazole (COT) and 85% to Norfloxacin (NX).

The isolated *Klebsiella pneumoniae* were sensitive to Amikacin (AK). 80% isolates showed resistance to both Amoxicillin/Clavulanic acid (AMC) and Norfloxacin (NX).

Table 3: Antimicrobial Susceptibility for Gram Negative Isolates

Gram negative bacteria	Antibiotics											
	AMC		G		AK NX		СОТ			NF		
	S*	R*	S	R	S	R	S	R	S	R	S	R
Escherichia coli (n*=20)	1	19	11	9	17	3	3	17	2	18	20	0
Klebsiella pneumoniae (n=10)	2	8	5	5	10	0	2	8	5	5	7	3

(*S=sensitive, R=resistant, n=number)

(AMC- Amoxicillin/Clavulanic acid, G-Gentamicin, AK-Amikacin, NX-Norfloxacin, COT- Cotrimoxazole, NF-Nitrofurantoin)

Staphylococcus aureus (ATCC252923) and *Enterococcus faecalis* (ATCC 29212) were tested with the herbal extracts to find out the Minimum Inhibitory Concentration (MIC) of Gram positive bacteria as depicted in Table 4.

Gram positive bacteria	MIC (mg/ml)							
	Gr. A#	Gr. B	Gr. C	Gr. D	Gr. E	Gr. F	Gr. G	
Staphylococcus aureus (ATCC 252923)	50	75	50	62.5	62.5	50	50	
Enterococcus faecalis (ATCC 29212)	62.5	62.5	50	62.5	50	50	62.5	

Table 4: Minimum Inhibitory Concentration (Mic) of the Groups of Herbal Extracts for Gram Positive Bacteria

(# Groups are as mentioned in materials and methods)

Staphylococcus aureus is inhibited by Groups A, C, F and G.

Enterococcus faecalis is inhibited by Groups C, E and F.

The single extracts that inhibit *Staphylococcus aureus* at lowest concentration are *Tribulus terrestris* (Group A) (Fig. 6) and *Hemidesmus indicus* (Group C) (Fig. 7), both at concentration of 50mg/mL. The double drug combination effective at lowest concentration was that of *Tribulus terrestris* and *Hemidesmus indicus* (Group F) at concentration of 50 mg/mL; and the combination of the three extracts i.e. *Tribulus terrestris, Phyllanthus amarus* and *Hemidesmus indicus* (Group G) was also effective at 50 mg/mL. Thus the drug combinations showed no synergistic effect here.

For *Enterococcus faecalis, Hemidesmus indicus* (Group C) was the most effective single drug at 50 mg/mL; the effective double drug combinations were *Phyllanthus amarus* and *Hemidesmus indicus* (Group E) and *Tribulus terrestris* and *Hemidesmus indicus* (Group F) at 50 mg/mL; the triple drug combination of *Tribulus terrestris, Phyllanthus amarus* and *Hemidesmus indicus* indicus became effective at a higher concentration of 62.5 mg/mL.

Table 5 shows the Minimum Inhibitory Concentration (MIC) of the herbal extracts for Gram negative bacteria *Escherichia coli* (ATCC 25922) and *Klebsiella pneumoniae* (ATCC700603). The single drug that inhibited *Escherichia coli* was *Tribulus terrestris* (Group A) at 75 mg/mL; the effective double drug combination was *Tribulus terrestris*, and *Hemidesmus indicus* (Group F) at 75 mg/mL; and the triple drug combination i.e. *Tribulus terrestris*, *Phyllanthus amarus* and *Hemidesmus indicus* (Group G) inhibited at 75 mg/mL. The single drugs *Tribulus terrestris* (Group A) and *Hemidesmus indicus* (Group C) inhibited *Klebsiella pneumoniae* at 75 mg/mL; double drug combinations of *Phyllanthus amarus* with *Hemidesmus indicus* (Group E) (Fig. 8) *and Tribulus terrestris* with *Hemidesmus indicus* (Group F) were effective at 75 mg/mL; and the same for triple drug combination of *Tribulus terrestris* with *Hemidesmus indicus* (Group G).

Table 5: Minimum Inhibitory Concentration (Mic) of the Groups of Herbal Extracts for Gram Negative Bacteria

Gram negative bacteria	MIC (mg/ml)								
	Gr. A [#] Gr. B Gr. C Gr. D Gr. E Gr. F						Gr. G		
Escherichia coli (ATCC 25922)	75	87.5	87.5	87.5	87.5	75	75		
Klebsiella pneumoniae (ATCC 700603)	75	87.5	75	87.5	75	75	75		

(# Groups are as mentioned in materials and methods)

Escherichia coli is inhibited by Groups A, F and G.

Klebsiella pneumoniae is inhibited by Groups A, C, E, F and G.

Taking the MIC obtained in Table 4 and Table 5 as standard, the fifty number (n=50) of isolates i.e. *Staphylococcus aureus* (n=10), *Enterococcus faecalis* (n=10), *Escherichia coli* (n=20) and *Klebsiella pneumoniae* (n=10) were tested with different groups of herbal extracts for antibacterial sensitivity. Table 6 gives the susceptibility of the bacterial isolates to the herbal extracts.

Table 6: Susceptibility Of The Bacterial Isolates To Herbal Extracts

	Gram positi	ve bacteria		Gram negative bacteria					
Groups	Staphylococci (n=10)	us aureus	Enterococcus faecalis (n=10)		Escherichia (n=20)	coli	Klebsiella pneumoniae (n=10)		
	S*	R*	S	R	S	R	S	R	
Gr. A	9	1	8	2	18	2	8	2	
Gr. B	7	3	6	4	13	7	7	3	
Gr. C	8	2	7	3	17	3	7	3	
Gr. D	8	2	7	3	15	5	7	3	
Gr. E	7	3	7	3	14	6	7	3	
Gr. F	9	1	9	1	18	2	8	2	
Gr. G	9	1	8	2	18	2	7	3	

N.B.: More than 70% isolates are sensitive to all groups of herbal extracts.

It was observed that among single drugs (Groups A, B and C) the drug that most effectively inhibited *Staphylococcus aureus* (90%), *Enterococcus faecalis*(80%), *Escherichia coli* (90%) and *Klebsiella pneumoniae* (80%) was *Tribulus terrestris* (Group A).

Among the double drug combinations, better inhibition is shown by *Tribulus terrestris* and *Hemidesmus indicus* (Group F) for *Enterococcus fecalis* (90%).

The triple drug combination has similar inhibitory effect as Group A on *Staphyloccus aureus*, *Escherichia coli* and *Enterococcus faecalis*

The hydro- alcoholic extract of *Tribulus terrestris* inhibited the growth of five isolates viz. *Staphylococcus aureus* (three), *Escherichia coli* and *Klebsiella pneumoniae* at the dose of 50 mg/mL. Further, it also inhibited the growth of six isolates viz *Staphylococcus aureus* (three), *Escherichia coli* (two) and *Klebsiella pneumoniae* and showed complete inhibition of all the twelve isolates viz. *Staphylococcus aureus* (three), *Enterococcus faecalis* (two), *Escherichia coli* (five) and *Klebsiella pneumonia* (two) at 62.5 mg/mL and 75 mg/mL respectively.

Antimicrobial activity of *Tribulus terrestris, Hemidesmus indicus* and *Phyllanthus amarus* was comparable to standard antibiotic Norfloxacin although at a lesser degree in overall inhibitory activity.

DISCUSSION

In nature, there are a huge variety of herbs, having medicinal properties and they can be used safely, without any side effects.

There is a need to develop new and better therapies for UTIs by understanding diverse pathogenesis of these infections. Keeping in the view of above facts our study was concentrated on the three important medicinal plants which have significant antimicrobial activity against the most prevalent Gram-Positive and Gram-negative bacteria in urinary infections ^[11, 12, 13].

The above study reveals that among the three herbal extracts, all the herbal extracts tested showed more than 70% sensitivity to the study groups. The Gram positive isolates showed greater susceptibility to the herbal extracts compared to the Gram negative isolates. *Tribulus terrestris* (Group A) is the most effective single drug for the multi-drug resistant isolates of *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli* and *Klebsiella pneumoniae*.

The combination of *Tribulus terrestris* and *Hemidesmus indicus* (Group F) extracts showed better result against the isolates. Presence of tannins and saponins in higher concentration than the other phytochemicals suggests that these phytochemicals could likely be responsible for the antibacterial activity ^[13, 14]. Our study shows antibacterial activity of *Tribulus terrestris* against all the bacteria of the study group.

This study shows antibacterial activity of *Phyllanthus amarus* against all the bacteria of the study group.

The present study shows antibacterial activity of *Hemidesmus indicus* against all the bacteria of the study group. It corroborates the earlier studies having anti-microbial potentials of this plant ^[14, 16, 17].

The hydro-alcoholic extracts of three Indian medicinal plants *Tribulus terrestris* (fruit extracts), *Phyllanthus amarus* (whole plant extracts) and *Hemidesmus indicus* (root extracts) showed significant anti-bacterial activity against Gram positive (*Staphylococcus aureus* and *Enterococcus faecalis*) and Gram negative bacteria (*Escherichia coli* and *Klebsiella pneumoniae*).

The extracts of *Tribulus terrestris* (Group A) showed maximum inhibitory activity among the three single extracts. Among all the drug combinations, *Tribulus terrestris* and *Hemidesmus indicus* (Group F) was most effective.

Overall, the extracts of Tribulus terrestris (Group A), and combinations of *Tribulus terrestris* with Hemidesmus indicus (Group F) and of Tribulus terrestris, *Phyllanthus amarus* and *Hemidesmus indicus* (Group G) potentially inhibit both the Gram positive and Gram negative bacteria, while *Hemidesmus indicus* (Group C) inhibits Gram positive bacteria. The Gram positive bacteria are more susceptible to these extracts than the Gram negative bacteria. All the herbal extracts tested showed more than 70% sensitivity to the study group. The antibacterial susceptibility testing was done in *in* vitro conditions taking care of all the technical issues. Though we are not able to compare the bacterial isolates having resistance to all types of urinary antibiotics, but somehow the isolates having resistance to three or four urinary antibiotics are tested which shows sensitivity to the herbal extracts. All the aureus. bacterial isolates i.e. Staphylococcus Enterococcus faecalis, Escherichia coli and Klebsiella pneumoniae are multi drug resistant but showed sensitivity to the herbal extracts. So these herbal extracts can be used in the treatment of multi-drug resistant strains causing urinary tract infection.

Long-term studies on *in vitro, in vivo* antibacterial activities of these herbal extracts are necessary to establish their activity in recurrent Urinary tract infection (UTI). An effective drug for recurrent Urinary tract infection (UTI) could be developed from these herbal extracts, which would be natural, easily available, more economic and free of any side- effects

CONCLUSION

In this study the hydro - alcoholic extracts of *Tribulus terrestris* (fruit), *Phyllanthus amarus* (whole plant) and *Hemidesmus indicus* (root) showed an encouraging *in vitro* antibacterial activity against the selected bacterial isolates. This is a short term prospective study. Long term prospective studies

including both *in vitro* and *in vivo* conditions will enable to establish the therapeutic strategy of the herbal extracts for chronic UTI.

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Study Photographs



Fig. 1: Tribulus terrestris L. (fruit extracts)



Fig. 2: Phyllanthus amarus Sch. (Whole plant extracts)



Fig. 3: Hemidesmus indicus L.Br. (root extracts)



Fig. 4: Sterile packets containing drug extracts



Fig. 5:Petri dish- Control plate of MHA



Fig. 6: Petri dish- MHA plate containing *Tribulus terrestris* 50 mg/mL showing inhibition of five out of twelve isolates inoculated



Fig. 7: Petri dish-MHA plate containing *Hemidesmus indicus* 50 mg/mL showing inhibition of six out of twelve isolates inoculated



Fig. 8: Petri dishs-MHA plate containing *Phyllanthus amarus* with *Hemidesmus indicus* 75mg/mL showing inhibition of all twelve isolates inoculated