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Bactericidal activity of *Hemidesmus indicus* R. Br. root extract against clinically isolated uropathogens

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Abstract

Introduction and Aims: The roots of *Hemidesmus indicus* R. Br. (Asclepiadaceae) are rich in tannins, saponin, alkaloids, flavonoids and phenolic compounds etc. It is used as tonic for nutritional disorders, diuretic for urinary problems due to its antimicrobial, antioxidant and anti-inflammatory properties. The present study was carried out to investigate the effect of diverse active constituents present in different solvent fractions of *H. indicus* root i.e. {petroleum benzene (PHI), chloroform (CHI), acetone (AHI), methanol (MHI)} against the clinically isolated uropathogenic bacteria i.e. *Escherichia coli*, *Staphylococcus aureus* and *Enterococcus faecalis*.

Methodology: Clinical isolates were collected from different patients suffering from urinary tract infections. Bioactivities of the different extracts were tested using various antibacterial methods on both solid and liquid media. Different qualitative and quantitative phytochemical tests were carried out using standard method.

Results: Phytochemical analysis of the fractions showed the presence of terpenoid, steroid, alkaloid, tannin, glycoside, coumarin and saponin. Among the 3 test bacteria, *S. aureus* was found to be a MDR strain having resistance to 6 antibiotics (CPM, CFM, CXM, CTX, NX and GEN) and *H. indicus* root extract was more effective against it.

Conclusion: Our results showed that PHI, CHI, AHI and MHI can be used effectively in the treatment of multidrug resistant strains causing urine infection. However, future study can reveal the rationale behind the bactericidal activity and mode of action of the phytoconstituents present in *H. indicus* root extract.

Keywords: *E. coli*, *E. faecalis*, *H. indicus* root, *S. aureus*, urinary tract infections

Introduction

More than 80% of the world's population depend on indigenous medicines for their primary healthcare requirements^[1]. Most of the herbs and spices possess valuable pharmaceuticals that can be used as a substitute for antibiotics against the common bacterial infections^[2]. *Hemidesmus indicus* R. Br. (Asclepiadaceae), commonly called as Indian Sarsaparilla is a climbing twiner plant, found throughout India. Roots are woody, irregularly bent or slightly twisted and have a sweet aroma. It has essential oils, fatty acids, saponins, tannins, resins, flavonoids, sterols and sarsapic acid along with the coumarins like hemidesmin-1, hemidesmin-2, α -amyrin, β -amyrin, lupeol and 2-hydroxy-4-methoxy benzoic acid^[3, 4]. Aborigines use it as a blood purifier, cooling tonic and antidote for snake bite and scorpion sting. It is very useful in dysentery, diarrhoea, impotency, skin disease, menorrhagia, post-partum recovery, stomach ulcer and gastric ailments^[5, 6]. Urinary tract serves to remove waste and excess water from body. It is divided into two parts: upper urinary tract (kidneys and ureters) and lower urinary tract (bladder and urethra). Urinary tract infection (UTI) is defined as an infection in any part of urinary tract due to presence of microbial pathogens. It is usually classified into four categories i.e. bladder infection (cystitis), kidney infection (pyelonephritis), urethra infection (urethritis) and urine infection (Bacteriuria).

Women are more prone to have UTI in comparison to men. UTI is more prevalent in sexually active and reproductive age group women. The length of urethra is about 1.5 and 8 inches in women and men, respectively. Because of their short urethra, the bacterium reaches the bladder easily in women. This infection further increases the risk of pyelonephritis, premature delivery and foetal mortality in pregnant women. UTI is the most common infectious disease after respiratory infections. About 50-60% of women develop one UTI in their lifetime and single infection increases the chances of recurrent infection^[7]. Though the incidence of UTI is

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less in young and adult men but the anatomical and functional abnormality of the urinary tract due to prostate gland enlargement in elderly men increases its probability. It is 25% in >70 years of age and still higher in elderly patients^[8]. UTI is more common in developing and under developed countries in comparison to developed countries^[9].

Escherichia coli is the most common causative agent for UTI and the woman's own faecal flora act as an immediate reservoir for the infecting *E. coli*, indicating a faecal–perineal–urethral route of infection^[10]. The other bacterial species that causes UTI includes both gram negative as well as gram positive bacteria –*Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Klebsiella* species, *Staphylococcus* species, *Proteus mirabilis*, *Enterobacter* species, *Serratia marcescens*, *Citrobacter* species, *Gardnerella vaginalis*, *Mycoplasma* species and *Urea plasma* species^[11, 12]. Some of the opportunists present in the vaginal flora like *Candida* species i.e. *Candida albicans* also cause UTI. After prolonged antibiotic therapy, when the normal microbial flora gets modulated or under immunosuppressive conditions, these opportunistic pathogens invade the bladder and cause the infection^[13]. Acute and recurrent UTI are mostly treated with antibiotics but since the pathogenic bacteria are becoming resistant to antibiotics, emphasis has lain over medicinal plants, which are comparatively safe, cost effective and easy to use. The present study was carried out to investigate the urobactericidal activity of the different solvent extracts of *H. indicus* root.

Methodology

Hemidesmus indicus root extract preparation

The dried roots of *H. indicus* were collected from the herbal store of Bhapur Bazar, Berhampur, Ganjam, Odisha, in the month of September 2017 and authenticated by the botanists of Berhampur University. The powdered roots were subjected to exhaustive soxhlet extraction in different solvents such as petroleum benzene (300 ml), chloroform (300 ml), acetone (300 ml) and methanol (300 ml) for 72 hours at 60-70°C. The filtered extracts were concentrated by a rotary evaporator and then left in the incubator at 37°C for complete solvent evaporation. The crude extracts were stored at 4°C in a desiccator for future use. Petrobenzene and chloroform extracts were mixed with sunflower oil, acetone and methanol extracts were mixed with lukewarm distilled water to prepare the required working concentration of the different extracts depending on the type of study.

Phytochemical analysis

Qualitative test: Qualitative analysis was carried out for the presence of reducing sugar, steroid, tannin, saponin, anthocyanin, coumarin, glycosides and flavonoids using standard protocol^[14].

Quantitative test: Total phenolic compound (TPC):

The total phenolic content of the extract was determined by the folin-ciocalteu (FC) method^[15]. Briefly, 40µl of crude

extract (1mg/ml) was added to 3.16ml of distilled water, mixed thoroughly with 0.2ml of FC reagent for 8min, followed by the addition of 0.6ml of 10% Na₂CO₃. The mixture was allowed to stand for 60min in dark and absorbance was measured at 765nm. The TPC was calculated from the calibration curve, and the results were expressed as mg/g of gallic acid equivalent.

Statistical analysis

Statistical analysis was carried out with MS excel 2010 software and results are expressed as mean ± standard deviation.

Bacterial strain, maintenance and storage

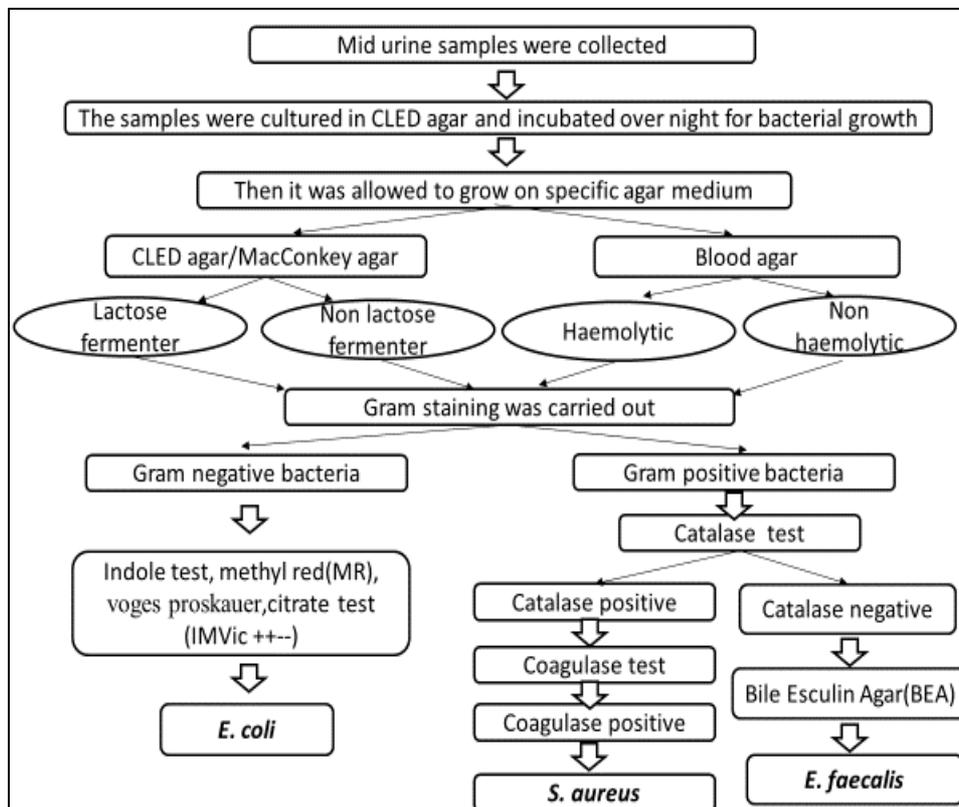
Clinical isolates of *E. coli*, *S. aureus* and *E. faecalis* were procured from MKCG Medical College, Berhampur. For routine use, the cultures were maintained on Mueller Hinton agar (MHA) plates. For long term storage, glycerol stocks were prepared by inoculating a single colony into Nutrient broth (NB) incubated at 37°C for 16 hr. To 0.85 ml of this culture 0.15 ml of 50% sterile glycerol was added, mixed thoroughly and stored at 4°C for 1 hr and then stored at -20°C^[16].

Growth medium and growth condition

The organisms were cultured in MHA (Himedia, Mumbai) plate or slants for routine use. The medium was prepared according to the manufacturer instruction and sterilized by autoclaving at 15lbs for 20 min. An isolated colony was picked carefully and streaked on fresh agar plate to get isolated colonies. The bacteria were grown overnight at 37°C in an incubator.

Urobacteria isolation, identification and biochemical characterization

This was carried out according to standard laboratory procedure followed by the Department of Microbiology, MKCG medical college, Berhampur. Briefly, the mid urine samples of the UTI patients were collected and cultured in cystine lactose electrolyte deficient (CLED) agar then incubated overnight for bacterial growth. Then the bacterial strains were allowed to grow on specific agar medium such as CLED agar, MacConkey agar, Blood agar etc for identification of lactose fermenter, non-lactose fermenter, haemolytic or non-haemolytic bacteria. The bacterial strains were then subjected to gram staining. For confirmation of gram negative bacteria some tests i.e. indole test (I), methyl red (MR), voges proskauer (VP), citrate test (C) were carried out. IMViC (++--) confirmed the presence of *E. coli*. For confirmation of gram positive bacteria catalase test was carried out. Catalase positive reaction followed by positive coagulase test confirmed *S. aureus*. Catalase negative reaction followed by growth in Bile Esculin Agar media confirmed *E. faecalis*. The identification and characterization method is depicted in flowchart 1.



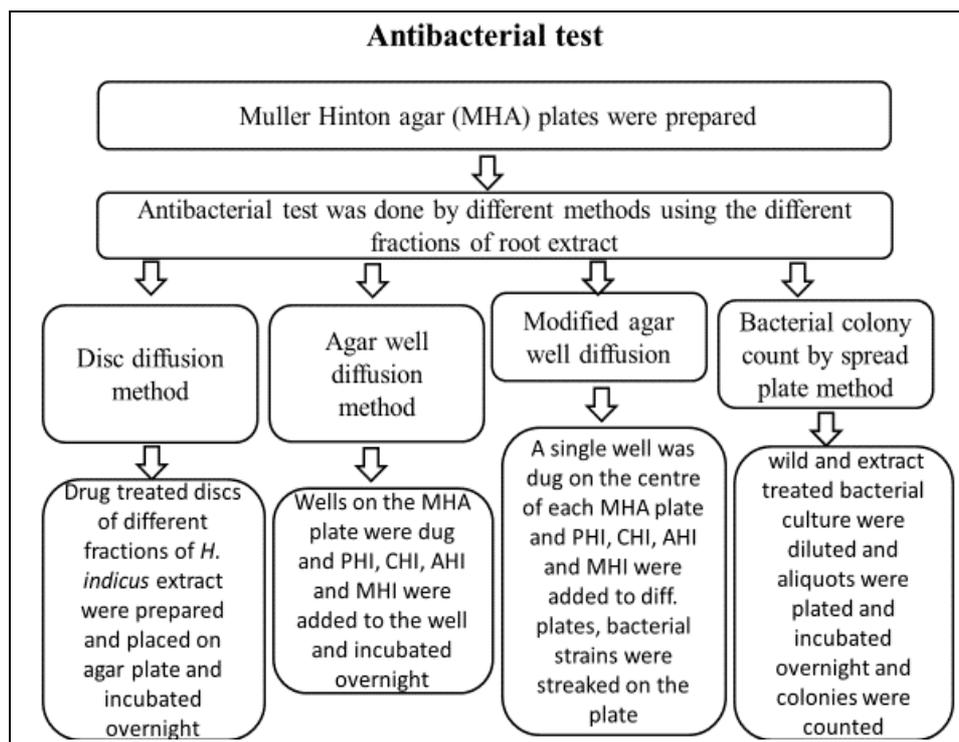
Flow chart 1: Isolation, identification and biochemical characterization of *E. coli*, *S. aureus* and *E. faecalis*

Antibiotic sensitivity test

Standard protocol was followed. Briefly, a single isolated colony was inoculated into 2 ml of sterile saline or peptone water. A sterile cotton swab was dipped into this medium and swabbed on agar plate. Different antibiotic discs (Himedia, Mumbai) were aseptically picked and placed properly on agar plate keeping appreciable gap between the discs. Plates were incubated at 37°C for 18hr. The clear zone formed around the disc was measured, which shows the susceptibility of the organism to the antibiotic at a definite concentration.

Antibacterial activity of *H. indicus* root extract

The effect of the different solvent fractions i.e. petroleum benzene, chloroform, acetone and methanol extract of *H. indicus* root, abbreviated as PHI, CHI, AHI and MHI against the clinically isolated UTI causing bacteria (*E. coli*, *S. aureus*, *E. faecalis*) was determined by disc diffusion, agar well diffusion, modified agar well diffusion method [16]. The cfu/ml was determined in wild and drug treated bacteria by spread plate method [17]. The schematic representation of different tests is depicted in flowchart 2.



Flow chart 2: Antiurobacterial tests of different fractions of *H. indicus* root extract

Disc diffusion method

Discs (5mm diameter) were prepared using whatman filter paper no-1 and sterilized. Different fractions of *H. indicus* root extracts (PHI, CHI, AHI, MHI) of doses 5.0 mg/disc were added on it aseptically and kept for some time for complete drying. MHA plates were prepared and swabbed with sterilized cotton bud with activated culture as mentioned under antibiotic sensitivity test. The drug treated discs were aseptically placed on the swabbed plates. The plates were incubated overnight at 37 °C. The clear zones formed around discs were measured.

Agar well diffusion method

MHA plates with swabbed bacterial culture were prepared as mentioned earlier. Then the plates were kept in the incubator for 15 min. Then wells were dug into it and different fractions of extract (PHI, CHI-12.5mg and AHI, MHI- 25.0mg/well) were loaded into the well. The plates were left at room temperature inside the laminar hood for 1hr for drug diffusion into the media and then incubated overnight at 37 °C. The clear zones formed around the wells were measured.

Modified agar well diffusion method

In order to find sensitivity of different bacteria (*E. coli*, *S. aureus*, *E. coli*) on a single plate this method was used. A MHA plate was prepared. After solidification, a well was dug in the centre. Different organisms were streaked on the plate from periphery towards centre in a zig zag manner. Then a particular dose of the PHI, CHI (12.5mg/well) AHI and MHI (25 mg/well) was loaded into the well. Then the plates were left at room temperature inside the laminar hood for 1 hr for drug diffusion and then incubated overnight at 37°C. The distance between the periphery of the well to the growth start point of the bacterial culture or the diameter of the zone of inhibition was measured.

Cfu/ml determination in control and extract treated bacteria

Bacterial colony count by spread plate method

The stock solutions (PHI, CHI-250mg/ml and AHI, MHI-500mg/ml) were prepared. 3 ml of nutrient broth was taken in different test tube and 300µl of different fractions of root extracts (PHI, CHI, AHI, MHI) were added to each test tube then, 100µl of activated bacterial culture was added to each test tube. A control tube was also prepared by inoculating only bacteria without extract and incubated for 4hrs (log phase study). Aliquots of the culture were taken in eppendorf tube and diluted by adding sterilized distilled water (10 µl of culture + 990 µl of distilled water). MHA plates were prepared and 50 µl of the diluted content of the eppendorf tubes were added to the plate, evenly spread on the plate using a L shaped glass rod. The plates were incubated overnight at 37°C and colonies were counted. For stationary phase study, the same procedure was followed, but the wild type and extract treated bacteria were incubated for 18hrs instead of 4hrs and then the aliquots of the culture were diluted by adding 10µl of culture to 5ml of sterilized distilled water. The same procedure was followed for all the three test organisms.

Results

Hemidesmus indicus root extract preparation

When 50g of powdered shade dried roots were subjected to exhaustive soxhlet extraction in different solvents (petroleum benzene, chloroform, acetone and methanol) followed by extract concentration, sticky masses with mean yield of 3.58%, 1.64%, 3.3% and 10.18%, respectively were obtained, which were used for our following tests. PHI was straw yellow, CHI was dark yellow, AHI and MHI were dark reddish brown in colour.

Phytochemical analysis

Qualitative test: Phytochemical analysis of the fractions showed the presence of terpenoid, steroid, alkaloid, tannin, glycosides, coumarin and saponin, which is summarized in table 1.

Table 1: Phytochemical analysis of *H. indicus* root extract

Phytochemicals	PHI	CHI	AHI	MHI
Reducing sugar	-	-	-	-
Steroid	+	+	+	+
Tannin	+	+	+	+
Saponin	-	-	+	-
Anthocyanin	-	-	-	-
Coumarin	-	+	-	-
Alkaloid	+	+	+	+
Leucoanthocyanin	-	-	-	-
Glycosides	+	+	+	+
Terpenoid	+	+	+	+

+ = Presence and - = absence

Quantitative test

Total phenolic compound:-The TPC of the PHI, CHI, AHI and MHI calculated from the calibration curve ($R^2=0.9982$) were 239 ± 0.0005 , 383 ± 0.0025 , 446 ± 0.0015 and 190 ± 0.0020 mg/g of gallic acid equivalents, respectively.

Antibiotic sensitivity test

E. coli strain used in this study was found to be highly sensitive to antibiotics such as cotrimoxazole (COT) and levofloxacin (LE) and sensitive to many drugs like amikacin (AK), amoxycylav (AMC), cefotaxime (CTX), norfloxacin (NX), gentamycin (GEN) and resistant to linezolid (LZ). Ceflxime (CFM) and cefuroxime (CXM) were the intermediates. (Table 2, Plate 1a).

S. aureus was found to be a multi drug resistant strain (MDR) showing resistant to multiple drugs like cefotaxime (CTX), Ceflxime (CFM), cefuroxime (CXM), gentamycin (GEN), norfloxacin (NX), cefepime (CPM). It was highly sensitive to linezolid (LZ) and sensitive to amikacin (AK), cotrimoxazole (COT). Levofloxacin (LE) and amoxycylav (AMC) were intermediate. (Table 2, Plate 1b).

E. faecalis was highly sensitive to amikacin (AK), levofloxacin (LE), cefuroxime (CXM), amoxycylav (AMC), gentamycin (GEN), linezolid (LZ), cotrimoxazole (COT) and sensitive to norfloxacin (NX), vancomycin (VA). It was resistant to Ceflxime (CFM) (Table 2, Plate 1c).

Table 2: Antibiotic sensitivity test of test strains

Antibiotics	Organisms		
	<i>E. coli</i>	<i>S. aureus</i>	<i>E. faecalis</i>
AK	++	++	+++
LE	+++	+	+++
CFM	+	R	R
CXM	+	R	+++
AMC	++	+	+++
CTX	++	R	–
GEN	++	R	+++
COT	+++	++	+++
LZ	R	+++	+++
CPM	–	R	–
NX	++	R	++
VA	–	–	++

R= Resistant, += Less sensitive, ++= Moderately sensitive, +++= Highly sensitive

Antiurobacterial activity

Disc diffusion method

In disc diffusion method, the AHI and MHI were found to be more effective against clinically isolated *E. coli*, *S. aureus* and

E. faecalis than PHI and CHI. *S. aureus* was found to be comparatively more sensitive than *E. coli* and *E. faecalis* (Table 3).

Table 3: Effect of *H. indicus* root extract against different bacterial strains

Concentration of different fractions in mg/disc	ZOI of <i>E. coli</i> in cm	ZOI of <i>S. aureus</i> in cm	ZOI of <i>E. faecalis</i> in cm
PHI (5.0)	0.6	1.2	0.6
CHI (5.0)	0.8	1.0	0.9
AHI (5.0)	2.0	2.2	2.0
MHI (5.0)	1.8	2.0	1.4

(ZOI- zone of inhibition). The results represent the mean value of triplicate set of experiments

Agar well diffusion method

The *S. aureus* was found to be more sensitive to the different

fractions of *H. indicus* root extracts in comparison to *E. coli* and *E. faecalis*. (Table 4, Plate 2a, 2b and 2c).

Table 4: Growth inhibitory effect of *H. indicus* against different bacterial strains

Concentration of different fractions in mg/ml	ZOI of <i>E. coli</i> in cm	ZOI of <i>S. aureus</i> in cm	ZOI of <i>E. faecalis</i> in cm
PHI (12.5)	1.1	1.4	1.1
CHI (12.5)	1.3	1.4	1.4
AHI (25.0)	2.5	2.6	2.6
MHI(25.0)	2.1	2.2	2.1

(ZOI- zone of inhibition). The results represent the mean value of triplicate set of experiments

Modified agar well diffusion method

MHI was found to be more effective against all the 3 test organisms in comparison to other fractions and *E. faecalis*

was comparatively more sensitive to all the solvent fractions of *H. indicus* root in comparison to *E. coli* and *S. aureus*. This method was useful to observe the effect of the extract against different types of bacteria on a single plate (Table 5, Plate 3a, 3b, 3c and 3d).

Table 5: Activity of *H. indicus* root extract against different bacterial strains

Conc. of different fractions in mg/ml	ZOI of <i>E. coli</i> in cm	ZOI of <i>S. aureus</i> in cm	ZOI of <i>E. faecalis</i> in cm
PHI (12.5)	0.5	0.5	0.7
CHI (12.5)	0.7	0.8	0.7
AHI (25.0)	0.5	0.5	0.6
MHI(25.0)	1.0	1.0	1.3

(ZOI- zone of inhibition). The results represent the mean value of triplicate set of experiments

Cfu/ml determination in control and extract treated bacteria by spread plate method:

Cfu/ml determination in control and extract treated bacteria at log phase:

At log phase the cfu/ml was determined as 1.088×10^7 for the wild *E. coli* and 4.8×10^5 , 3.6×10^5 , 8.2×10^5 , 1.92×10^6 for PHI, CHI, AHI and MHI treated *E. coli*, respectively. For wild *S. aureus*, cfu/ml was found to be 6.56×10^6 and 3.2×10^5 ,

6.0×10^5 , 2.4×10^5 , 1.8×10^5 for PHI, CHI, AHI and MHI treated *S. aureus*. For wild *E. faecalis* cfu/ml was found to be 1.50×10^6 and 9.0×10^5 , 8.8×10^5 , 3.2×10^5 , 6.6×10^5 for PHI, CHI, AHI and MHI treated *E. faecalis*, respectively (plate 4, 5 and 6, graph 1A, 1B and 1C).

Cfu/ml determination in control and extract treated bacteria at stationary phase:-

At stationary phase the cfu/ml was determined as 8.0×10^8 for the wild *E. coli* and 5.2×10^8 , 1.6×10^8 , 2.56×10^8 , 6.4×10^8 for PHI, CHI, AHI and MHI treated *E. coli*, respectively. For

wild *S. aureus*, cfu/ml was found to be 4.8×10^8 and 3.2×10^8 , 3.6×10^8 , 2.4×10^8 , 4.0×10^8 for PHI, CHI, AHI and MHI treated *S. aureus*. For wild *E. faecalis* cfu/ml was found to be

1.0×10^9 and 7.5×10^8 , 8.3×10^8 , 3.4×10^6 , 4.7×10^6 for PHI, CHI, AHI and MHI treated *E. faecalis*, respectively (graph 2A, 2B and 2C).

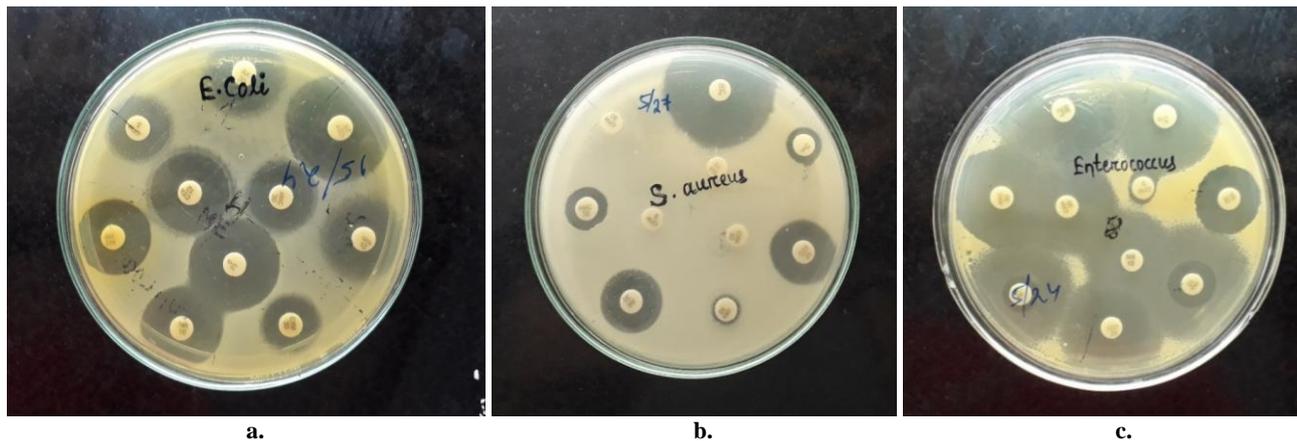


Plate 1: Antibiotic sensitivity test (a) *E. coli* (b) *S. aureus* (c) *E. faecalis*

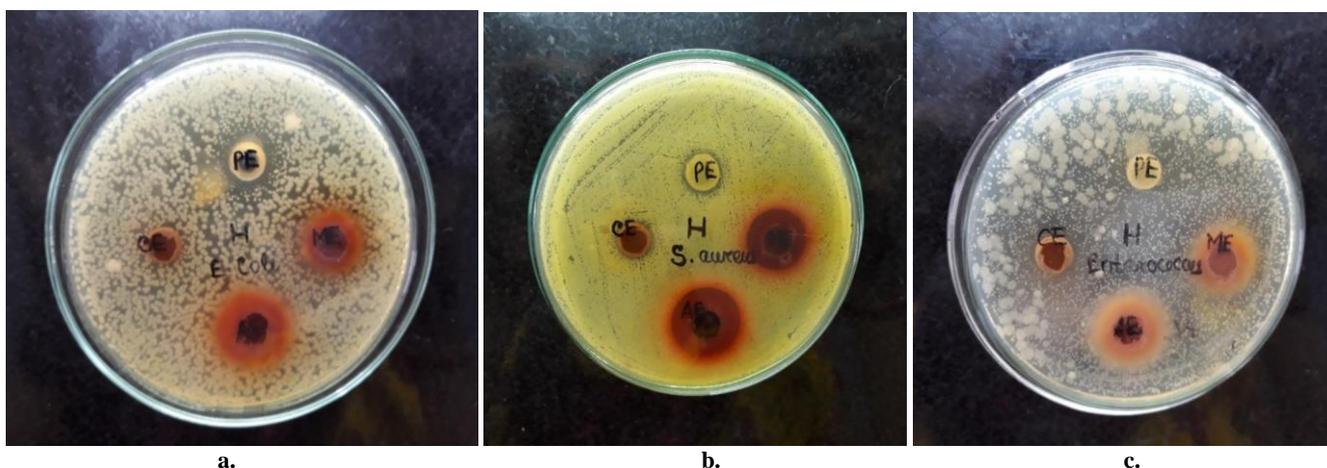


Plate 2: Growth inhibition study of PHI, CHI, AHI and MHI by agar well diffusion method (a) *E. coli* (b) *S. aureus* (c) *E. faecalis*

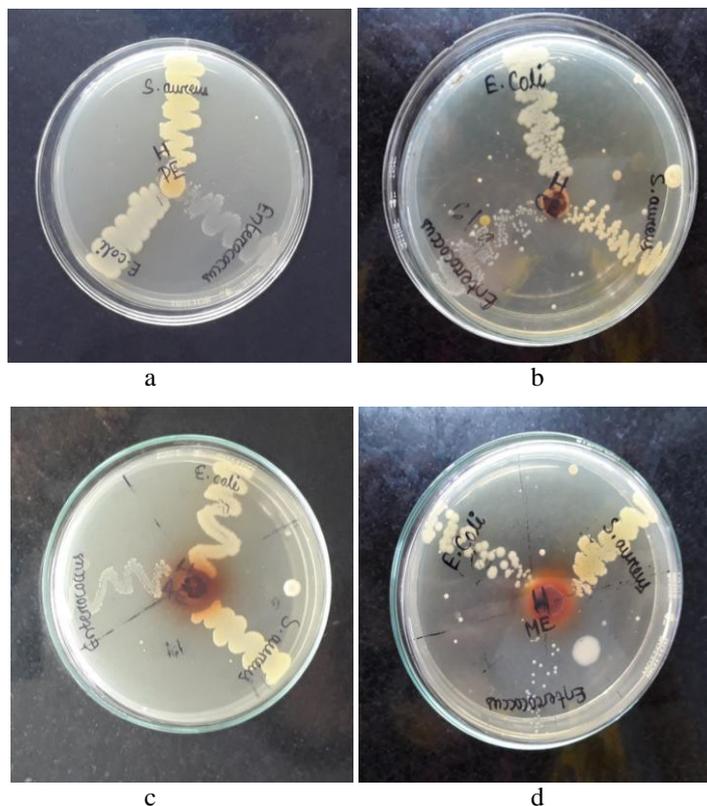


Plate 3: Growth inhibition study by modified agar well diffusion method against *E. coli*, *S. aureus* and *E. faecalis* using different fractions of plant extract. (a) PHI (b) CHI (c) AHI (d) MHI



a.

b.



c.

d.

e.

Plate 4: cfu/ml determination of *E. coli* at log phase (a) Wild (b) PHI (c) CHI (d) AHI (e) MHI treated *E. coli*



a.

b.

c.



d.

e.

Plate 5: cfu/ml determination of *S. aureus* at log phase (a) Wild (b) PHI (c) CHI (d) AHI (e) MHI treated *S. aureus*



a.

b.

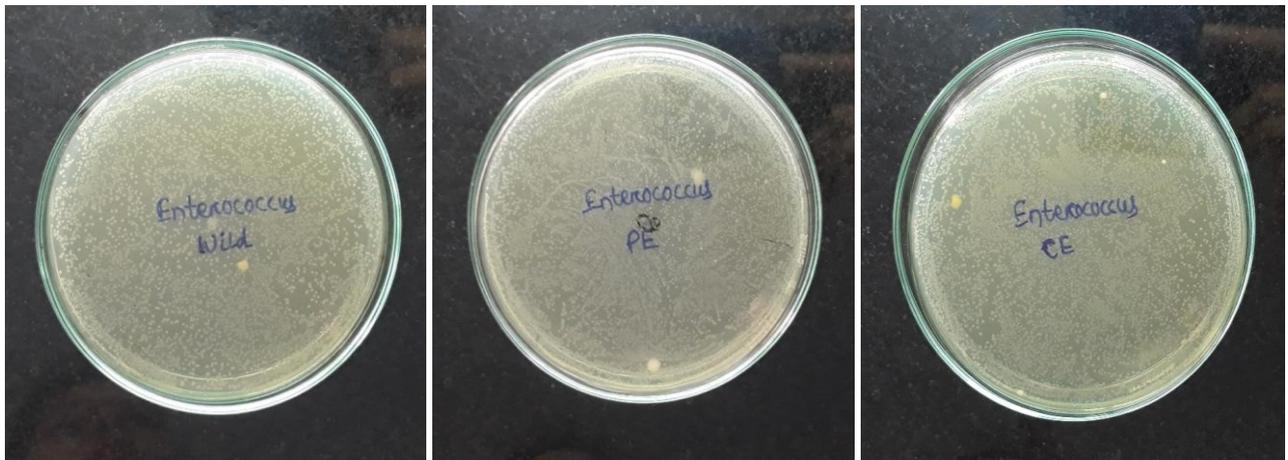
c.



d.

e.

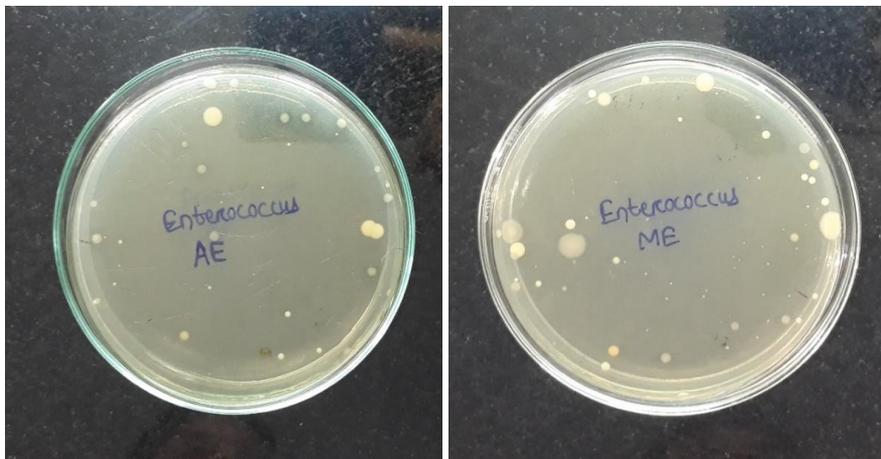
Plate 6: cfu/ml determination of *E. faecalis* at log phase (a) Wild (b) PHI (c) CHI (d) AHI (e) MHI treated *E. faecalis*



a

b

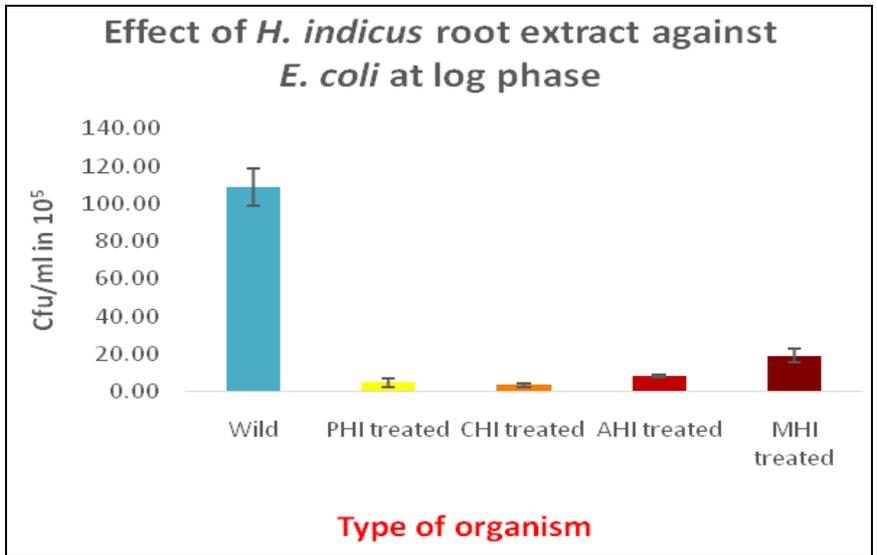
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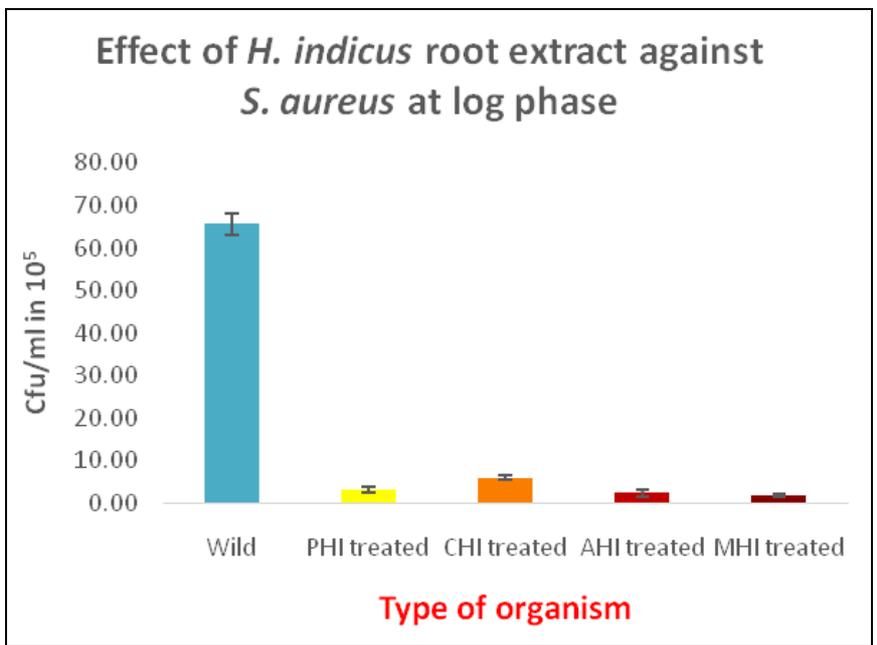
d

e

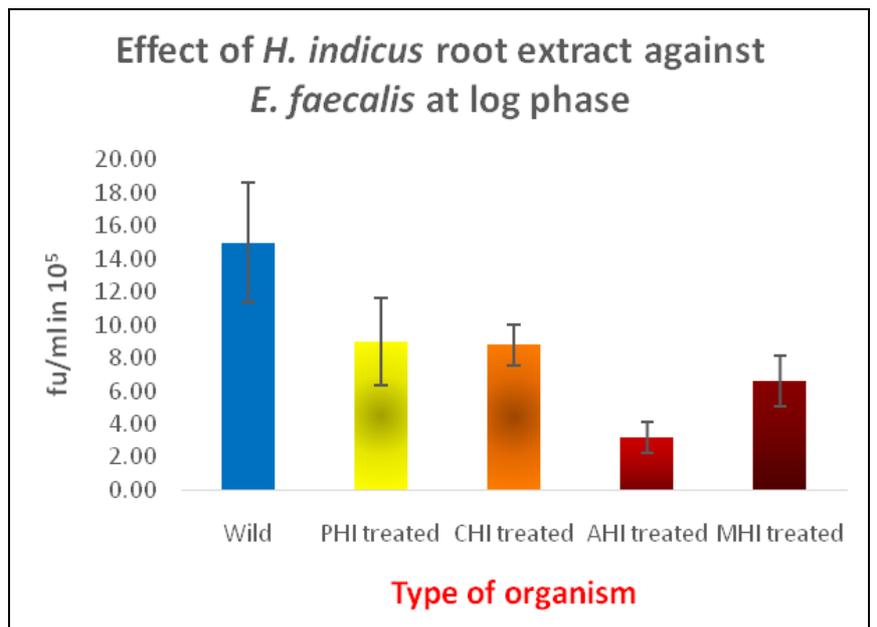
Plate 7: cfu/ml determination of *E. faecalis* at stationary phase (a) wild (b) PHI (c) CHI (d) AHI (e) MHI treated *E. faecalis*



A

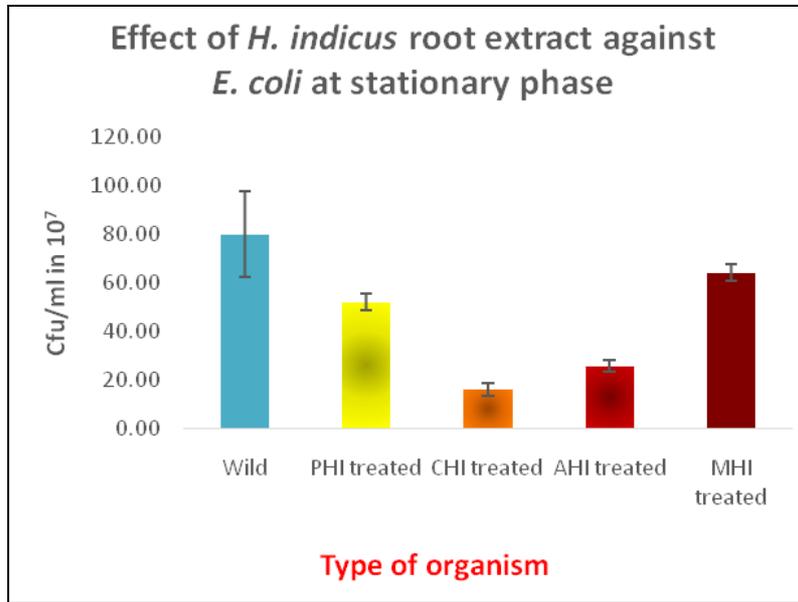


B

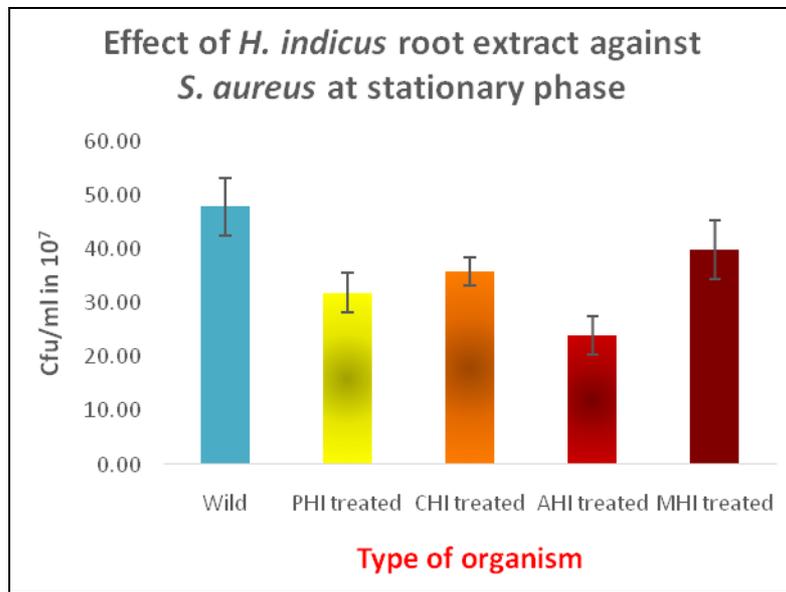


C

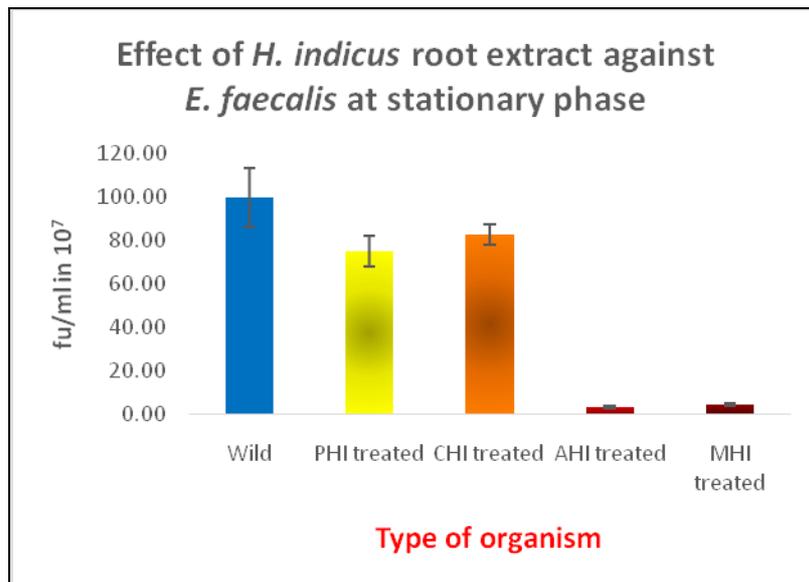
Fig 1: cfu/ml determination at log phase (A) *E. coli* (B) *S. aureus* (C) *E. faecalis*
~ 188 ~



A



B



C

Fig 2: cfu/ml determination at stationary phase (A) *E. coli* (B) *S. aureus* (C) *E. faecalis*

Discussion

Urinary tract infection is the second most common infectious disease in the world and mostly affect women. *E. coli* alone contribute to 80% of cases where as in *S. aureus*, it is 11% and in *E. faecalis*, it is 2-3%. Moreover, with the rapid emergence of multidrug resistant (MDR) pathogenic bacteria, it is high time to look for some alternative medicines. Nature is having a huge variety of plants with medicinal properties, which were often proved to be very effective against the most dreaded diseases. The emerging alternative to antibiotics are the natural plant products with little or no side effects. Plants are a rich source of diverse chemicals, with different solubility and multifunctional utility. There is no single suitable solvent system to isolate all compounds. So, we used various solvent systems with increasing polarity i.e. petrobenezene, chloroform, acetone and methanol. Mean yield and composition of different extracts (PHI, CHI, AHI and MHI) were compared. Maximum phytochemical extraction was observed in MHI followed by PHI and AHI and the yield was minimum in CHI (MHI>PHI>AHI>CHI). Methanol is considered to be an ideal solvent for extraction of phytochemicals. In an antibacterial activity study of petroleum ether, chloroform, methanol and water extract of *Punica granatum* L., methanolic extract was reported to be most effective against all the test microorganisms^[18].

Phytochemical analysis revealed that glycosides, steroids, alkaloids, tannins and terpenoides were present and reducing sugar and anthocyanins were absent in all the extracts. Total phenolic content (TPC) was maximum in AHI and minimum in MHI (AHI>CHI>PHI>MHI). PHI and CHI were found to be hydrophobic and AHI and MHI were hydrophilic in nature. So, for testing the antibacterial activity of different extracts, they should be dissolved in safe vehicles. Therefore, PHI and CHI were dissolved in sunflower oil and AHI and MHI were dissolved in hot water to prepare their working solutions with known concentration.

In this study, clinically isolated test organisms (*E. coli*, *S. aureus* and *E. faecalis*) were used to investigate their degree of sensitivity towards the phytochemicals derived from *H. indicus* root. Before undertaking the antibacterial activity of *H. indicus* root extract, it is essential to know the biochemical characters as well as their antibiotic sensitivity profile of the test organisms. Therefore, *E. coli*, *S. aureus* and *E. faecalis* were tested for their antibiotic sensitivity. It was found that *E. coli* (Plate 1a) was resistant to linezolid (LZ) whereas *S. aureus* (Plate 1b) was found to be resistant to cefotaxime (CTX), ceftaxime (CFM), cefuroxime (CXM), gentamycin (GEN), norfloxacin (NX), cefepime (CPM) and *E. faecalis* was found to be resistant to ceftaxime (CFM) (Plate 1c). *E. faecalis* was the most sensitive test bacteria followed by *E. coli* and *S. aureus* was a multi drug resistant (MDR) strain. In vancomycin resistant *Enterococcus*, several genes work together to create the resistant phenotype^[19]. So, probably in our test MDR *S. aureus* strain, a complex biochemical and genetic system might be operating that could be responsible for its MDR attribute.

In disc diffusion method and agar well diffusion (Table 1 and 2, Plate 2), *S. aureus*, which was a MDR strain was found to be more sensitive in comparison to *E. coli* and *E. faecalis* and both AHI and MHI, which had hydrophilic phytochemicals had maximum activity against the test strains. There may be variation in results on different plates. So modified agar well diffusion method was used to test the sensitivity of the organisms on a single plate. In modified agar well diffusion assay (Plate 3), *E. faecalis* was found to be more sensitive in

comparison to *S. aureus* and *E. coli* and all the strains were inhibited considerably by AHI and MHI. This could be due to the better diffusion of hydrophilic compounds into the agar media and possibly the hydrophobic nature of PHI and CHI compounds inhibit their diffusion into the media, which could be the reason for their least inhibitory activity on agar diffusion method.

Antibacterial activity study of any phytochemical depends on its solubility and diffusibility in solid agar media. So, we choose the liquid broth media for antibacterial activity study in which the phytochemicals are evenly mixed with media for maximum exposure to bacteria. At Log or exponential growth phase, bacteria divide at a maximum rate and at stationary phase, they were at maximum adaptable condition. Therefore, we used 4h (log phase) and 18h (stationary phase) culture to study the activity of different phytoconstituents present in PHI, CHI, AHI and MHI. Though all the extracts were found to have significant bactericidal activity against *E. coli* (Plate 4) and *S. aureus* (Plate 5), PHI and CHI inhibited growth of *E. coli*, which is the major causative agent of UTI significantly. All the fractions repressed growth of *S. aureus* and *E. faecalis* considerably at log phase, but AHI and MHI had profound effect against *E. faecalis* at log phase (Plate 6 and figure 1). At stationary phase, again all the extracts showed bactericidal and bacteriostatic effect against all the test strains, but AHI and MHI had maximum growth inhibitory activity against *E. faecalis* (Plate 7 and figure 2). Significant growth inhibitory effects were observed at log phase, since the bacteria are more vulnerable to any toxic chemical in their dividing state whereas in stationary phase, their rate of division is decreased and they had started adjusting to the toxic chemicals or any harsh environment probably by altering their natural metabolic pathways, which helps them to survive but their pathogenic ability and virulence capacity is probably lost, which could be explored by further study.

The indiscriminate use of antibiotics has led to the development of multidrug-resistant pathogens. Antibiotic resistance is considered as the major problem in recent era^[20, 21]. So, novel antibacterial substances, especially from natural sources should be explored. Unlike synthetic drugs, antimicrobials of plant origin are diverse in nature, not associated with any major side effects and have a great therapeutic potential to heal many infectious diseases^[22, 23]. They have many advantages i.e. cheap, renewable with fewer side effects, better patient tolerance and acceptance^[24]. Plant antimicrobials were reported to act synergistically with other drugs^[25]. Few plant extracts and phytochemicals exhibited synergistic interaction with antibiotics against Gram-positive bacteria^[26, 27]. So, combination therapy of antibiotic with plant extracts against resistant bacteria is the new choice for the treatment of dreaded infectious diseases^[28]. Some novel antibacterial actions of phytochemicals were reported earlier, which include inhibition of MDR-efflux pump^[29] and β -lactamase inhibiting activity^[30], antibiotic resistance modulation properties^[31] and R-plasmid elimination^[32]. Polyphenols (epicatechin gallate and catechin gallate) have been reported to reverse beta-lactam resistance in Methicillin resistant *S. aureus* (MRSA)^[33]. Diterpenes, triterpenes, alkyl gallates, flavones and pyridines have also been reported to have resistance modulating abilities on various antibiotics against resistant strains of *S. aureus*^[34, 35]. Presence of significant amount of polyphenolic compounds in PHI, CHI, AHI and MHI would be the possible reason for their antibacterial activity especially against the MDR *S.*

aureus test strain used in our study.

Tannins and saponins isolated from plant had excellent antibacterial activity against pathogenic bacterial strains [36]. The roots of *Boerhavia diffusa* (punarnava) with β -sitosterol was effective against UTI causing bacteria like *Proteus*, *Klebsiella*, *Pseudomonas*, *E. coli* and *Enterococcus* when compared to antibiotic gentamycin [37]. The presence of tannins and saponins along with both α and β -sitosterol in *H. indicus* root could be reason for its antiurobacterial activity.

Das *et al.* (2003) studied the antierobacterial activity of methanolic extract of *H. indicus* roots against *E. coli*, *Salmonella typhimurium*, *Shigella flexneri* [5]. Urobactericidal activity of methanolic extract of *H. indicus* root was reported earlier against *E. coli* and *K. pneumoniae* [38]. Kotnis *et al.* (2004) reported its potency for the treatment of kidney disorders [39]. Zulfiker *et al.* (2011) had reported the phenol content varied from 679.102 to 964.230 mg/GA/g of the dry weight in the methanol extract of different plants including *H. indicus* root [40]. In our study it was observed that the total phenol content (TPC) was found to be in the following descending order (AHI>CHI>PHI>MHI) and ranged between 190 to 446 mg/GA/g of the dry weight of extract. Presence of different phytoconstituents such as steroids, tannins, alkaloids, coumarins, saponins, glycosides and terpenoids etc. were confirmed in PHI, CHI, AHI and MHI, which might be acting synergistically to inhibit the different urobacteria. There were many reports that active phytochemicals were effective against MDR strains in comparison to sensitive strains. In this present study, *S. aureus*, which was a MDR strain found to be significantly inhibited by PHI, CHI, AHI and MHI as evident from the results of disc diffusion, agar well, modified agar well and cfu/ml determination study at log and stationary phase. However, further study is needed to understand the biochemical and molecular mechanism of action of these phytoconstituents against these urobacteria and also to identify their molecular targets that contribute significantly to uropathogenesis.

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Conflict of interest statement

This is our collective research work and there is no conflict of interest with any person or organization.

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