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ANTIBACTERIAL EFFECT OF BOERHAVIA DIFFUSA AND PUNARNAVASAVAM ON URINARY TRACT INFECTION (UTI) CAUSING PATHOGENS

Vineeth T, Deepak M and AB Rema Shree*

Centre for Medicinal Plants Research, Kottakkal Arya Vaidya Sala Kottakkal, Kerala

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***Correspondence for Author**
Dr. A. B. Rema Shree
Centre for Medicinal Plants Research, Kottakkal Arya Vaidya Sala Kottakkal, Kerala

ABSTRACT

The aim of the present study was to determine the antibacterial activity of Boerhavia diffusa leaves and an ayurvedic product Punarnavasavam on five Urinary Tract Infection causing pathogens. Klebsiella species, Pseudomonas species, Enterococcus species, Escherichia coli and Proteus species were isolated from urine samples using standard microbiological procedures. The antimicrobial activity of punarnavasavam which contains Boerhavia diffusa and different extracts of Boerhavia diffusa leaves were investigated on these pathogens using the Disc diffusion method, Well diffusion method and Minimum inhibitory concentration. HPTLC quantification of the Boerhavia diffusa water and ethanolic extracts were also carried out. The ethanolic extract of Boerhavia diffusa and the Polyherbal formulation Punarnavasavam were highly effective against all the bacterial species isolated. Results indicated that Punarnavasavam and ethanolic extract of Boerhavia diffusa leaves contain compounds with therapeutic activity. Antibacterial activity observed higher in Punarnavasavam compared to Boerhavia diffusa leaves.

KEY WORDS: Boerhavia diffusa, Urinary Tract Infection, Punarnavasavam, Antibacterial activity, Minimum inhibitory concentration, HPTLC.

INTRODUCTION

Ayurveda, Siddha, Unani and Folk (tribal) medicines are major systems of indigenous medicines. Among these systems, Ayurveda is most developed and widely practiced in India. Plants, especially used in Ayurveda can provide biologically active molecules and lead structures for the development of modified derivatives with enhanced activity and reduced

toxicity (Joy et al., 1998). Medicines in ayurveda are mainly based on plants, for treating UTI many plants and plant products are used, in which vaccinium macrocapron, vaccinium myrtillus and arctostaphylos uva ursi are most commonly used. Urinary system mainly is composed of organs that regulate the chemical composition and volume of blood. The urinary tract is divided into two major divisions, upper (the kidneys, renal pelvis, and ureters) and lower (urinary bladder and urethra). Upper urinary tract infections are most commonly ascending (that they originate in the urinary bladder and ascend through the ureters to kidneys) (Tortora et al., 2010, Koneman et al., 1997). Pediatric urinary tract infections (UTI) are associated with high morbidity and long term complications. Common uropathogens isolated were Escherichia coli (47.1%), Klebsiella spp. (15.6%), Enterococcus faecalis (8.7%), members of tribe Proteae (5.9%), Pseudomonas aeruginosa (5.9%) and Candida spp. (5.5%) (Neelam et al., 2010). Common treatment for UTI is antibiotic therapy, the over usage and abusage produced resistant bacterial strains.This increasing drug resistance among the bacteria had made therapy of urinary tract infection difficult and lead to greater use of expensive broad spectrum drugs. The resistant problem needs a re-newed effort, resulting in searching antibacterial agents against pathogenic micro organisms resistant to current antibiotics (Soulsby, 2005). Moreover the available synthetic antibiotics are found to have serious side effects like bone marrow depression, anemia and damage to vital organs like liver and kidney. So it is necessary to identify newer antibiotics from herbal sources which are devoid of such serious side effects (WHO, 1993).

Boerhavia diffusa commonly known as punarnava in Sanskrit is an herbaceous plant of the family Nyctaginaceae. The geneus name has been assigned in the honour of Herman Berhaave a famous Dutch physcian of 18th century (Kasushik and Dhiaman, 1999). The whole plant or its specific parts (roots, leaves and stem) are known to have a long history of use by indigenous and tribal people in India. It has many ethno botanical uses and is medicinally used in traditional, Ayurvedic system. Besides, the Boerhavia diffusa plant reported to posses many pharmacological and clinical properties (Awasthi and Verma, 2003). The leaves are used in ophthalmic diseases (Shah and Gopal, 1987), cataract (Balaji Rao et al., 1995), as analgesic, in dropsy, gonorrhea, jaundice, for hastening delivery (Raj and Patel, 1978), in rheumatism (Rao, 1981), hypotension (Jha and Varma, 1996), liver aliments (Gopal and Shah, 1985), for wound healing (Sebastian and Bhandari, 1984) for itch and eczema (Saxena and Vyas, 1981) in dyspepsia and as an antitumor agent (Tripathi et al., 1996). Dried leaves are used in dhoomapana (smoking) in treatment of bronchial asthma.

Boerhaavia diffusa leaf extract was reported to posses ant diabetic activity against alloxan induced diabetic rats (Satheesh and Pari, 2004, Chude et al., 2001).The leaves showed presence of proteins, fats, crude fiber, carbohydrates, phosphorus, zinc, manganese, β-carotene, ascorbic acid, oxalic acid, calcium and iron (Theophilus and Arulanatham, 1949, Duhan et al.,1992, Gopalan et al.,1971). Presence of cholinesterase activity was shown in leaves (Gupta and Gupta, 1997). The present study is taken up to investigate antimicrobial activity of Boerhavia diffusa leaves and an herbal formulation Punarnavasavam. Boerhavia diffusa and the Punarnavasavam made out of it, is regularly used for urinary complaints and oedma (sahasrayogam, 2000).

MATERIALS AND METHOD

Culture media- The following media were used, Cysteine Lactose Electrolyte Defficient medium (CLED, HiMedia M1146), Mac Conkey Agar (Merk 105465), 5% sheep Blood agar, Muller Hinton agar (HiMedia, M1657), Citrate agar (HiMedia, M728), Triple Sugar Iron Agar (HiMedia, M021I), Bile Esculin Agar Base (HiMedia, M340), Antibiotics used : Nitrofuratoin (HiMedia SD090), Co-trimoxazole (HiMedia SD010), Cefotaxime (HiMedia,SD040), Gentamicin (HiMedia SD016), Chloramphenicol (HiMedia SD006), For MIC gentamicin (HiMedia, CMS461).

Preparation of plant extracts

The plant materials (leaves) were collected and taxonomically identified from Drug Standardisation Laboratory, Centre for Medicinal Plant Research. The leaves of the plants were thoroughly washed, dried in shade and powered. 200gm of powdered material was successfully extracted using Soxhlet apparatus with 95% ethanol as solvent. After two days of extraction the solvent was evaporated and the residue obtained was used for the studies. The extract was dissolved in Dimethyl sulfoxide (DMSO) and used for antibacterial screening. The water extract of Boerhavia diffusa was prepared by weighing 100g powder and by boil with 300ml of distilled water in a water bath for 24 hours. Then it is filtered and evaporated. The extract obtained was dissolved in sterile distilled water at a concentration of 3g/4ml and used for antibacterial screening.

Isolation of pathogens

Urine samples were collected from patients suffering from UTI from Co-operative Hospital, Thalassery. Mid stream urine was collected in a sterile wide mouth container and transported

using boric acid as preservative. Further evaluation was done in Department of Medical Microbiology, School of Health Sciences, Kannur University, Thalassery campus, palayad.

The calibrated loop method using quarter plates of culture media used for colony counting. In this method urine samples were streaked on a quarter plates of CLED agar using calibrated wire loop (Meta Loop SS2, HiMedia) that holds 0.005 ml. After overnight incubation at 37^0C colony count was done, samples which contain > 10^4 cfu/ml were selected for further study. Based on colony morphology and lactose fermenting ability organisms were differentiated and subjected to biochemical tests. Main tests done Indole test, voges proskauer test, methyl red test, citrate utilization test, Aesculin hydrolysis test, Oxidase test and sugar fermentation ability was detected using Triple Sugar Iron Agar slants. After incubation biochemical characteristics were noted and identification was done using of Bergey's Mannual of Systematic Bacteriology (Kreig and Holt, 1984) The microorganism to be tested is sub cultured on to specific media at 37^0C for 24 hours, a minimum of 4-5 colonies were touched with sterile loop and transferred into peptone water under aseptic conditions and incubated for half an hour. Density of each microbial suspension was adjusted to that of 10^8cfu/ml (Standardized by 0.5 McFarland's standard).

Antibacterial Screening

The antibacterial activity of the leaf extract and Punarnavasavam were screened by Disc diffusion method, Well diffusion method and Minimum Inhibitory Concentration.

Agar disc diffusion method

Standardized bacterial cultures were inoculated in to the surface of the Muller Hinton agar plates using a sterile swab to ensure that the growth is uniform and confluent. Sterile Whatman NO:1 filter paper discs of 6 mm were placed equidistantly on the surface of plates. Each paper discs were impregnated with different concentration of the plant extract and formulation. Cotrimoxazole (25µg) and gentamicin (10µg) discs were used as positive control and 10% dimethyl sulfoxide as negative control. The plates were incubated at 37^0C for 24 hours. After incubation the plates were examined for zone of growth inhibition which is expressed in millimeter (NCCLS, 1997). (Table 2&5).

Well diffusion method

The sterile plates containing Muller Hinton agar medium were spread with fresh standardized bacterial culture by using sterile cotton swab. Wells were made from agar plates using a sterile cork borer. The wells loaded with different concentration of extract and formulation,

the plates were incubated 37^0C for 24hrs. After incubation the plates were observed for the presence of clear zone around the well. Gentamicin and Dimethyl sulfoxide were used as positive and negative control respectively (NCCLS, 1999). (Table 1&4).

Minimum Inhibitory Concentration (MIC)

The MIC of Boerhavia diffusa ethanolic extract, punarnavasavam and standard antibiotics were determined by broth dilution (Broth macro dilution) method. In this test a series of culture tubes(13×100 mm) were prepared using Muller Hinton Broth, Test organisms were grown in Muller Hinton Broth to give a final density of 5×10^5 CFU/mL and these were confirmed by viable counts. First row the ethanolic extract and formulation were serially diluted (Doubling dilution) to give a concentration of 10 to 100 mg/mL. In second row standard antibiotic solution (Gentamicin) was serially diluted (0.25-30µg/mL). The tubes were then inoculated with the 1 mL of Standardized bacterial culture. Fill the control tubes with 1 ml of sterile broth without antimicrobial agent (Sterile control), prepare a growth control with 1 ml of standardized bacterial suspension and 1 ml of sterile broth, incubated all the tubes for overnight at 37^0C. After the incubation the tubes examined for turbidity (Mbosso et al., 2010, Wiegand et al., 2008).

For studying the effect of extract on bacterial growth and growth kinetics turbidity method was used. The method is based upon comparing the turbidity of the liquids with the help of a spectrophotometer. The greater the turbidity, the lesser is the inhibition of microorganisms. About 5ml of Nutrient broth was transferred aseptically into 24 tubes taken in triplicate. Then the organisms were inoculated into the sterile nutrient broth and compared the turbidity. Four different concentrations of Boerhavia diffusa ethanol extract and Punarnavasavam (20µl, 30µl, 40µl and 50µl) were pipette out into the test tubes with bacterial strains and one tube was kept as control for each without adding the plant extract. Tubes were incubated at 37^0C for 24 hours and after which they were shaken properly so as to ensure mixing. A digital spectrophotometer is used for measuring the turbidity and absorbance's were taken at 600 nm. (Table 3&6), (Figure 1&2).

Chromatographic Quantification Of Antimicrobial Triterpene

Antimicrobial triterpene β-sitosterol presence was detected using high performance thin layer chromatography and quantified using camag HPTLC system.

Preparation of sample and standard solutions

Extract solutions were prepared by dissolving 0.5 g of ethanolic extract and water extract in ethanol and water and make up in 10ml in a standard flask.1 mg of standard β-sitosterol was diluted to 10 ml of methanol in a standard flask to obtain the working standard solution.

Chromatographic conditions

A Camag (Muttenz Switzerland) HPTLC system made up of a Automatic TLC Sampler 4 (ATS 4), Automatic Developing Chamber (ADC 2), TLC Scanner 3, TLC Visualizer and winCATS integration software were used. The Samples and were spotted in the form of bands of width 5 mm using Camag ATS 4 on pre-coated silica gel aluminum plate 60F-254 (20 cm× 10 cm with 250 µm thicknesses, Merck, Darmstadt. Germany). Spotted plates were transferred in to the Automatic Developing Chamber with twin trough chamber. The suitable solvent system [toluene: ethyl acetate (8: 2)], was filled in to the ADC 2. Plates after development taken out from ADC 2 was dried and uniformly sprayed with the reagent Anisaldehyde-sulphuric acid. The sprayed plate was heated in hot air oven at 110^0C for 10 minutes to obtain colored bands with out charring. Quantitative evaluation of HPTLC plate is performed densitometrically, in absorption mode, at wave length 550 nm. Quantification of β-sitosterol in sample was carried out by comparing the peak areas of both sample and standard.

RESULTS AND DISCUSSION

Results of antimicrobial assay revealed that the ethanolic extract of Boerhavia diffusa and the polyherbal formulation punarnavasavam were highly effective against all the bacterial species isolated from the urine. The highest activity of plant extract and punarnavasavam were exhibited against Pseudomonas species and a lowest zone of diameter was observed against Enterococcus species. But the water extract of Boerhavia diffusa leaves have no antibacterial activity. Results of antibiotic susceptibility showed that nearly all the isolates were resistant against most of the antibiotics tested (Table 7). In the agar disc diffusion method, the maximum zone of growth inhibition was exhibited at a concentration of 50mg of Boerhavia diffusa ethanolic extract (for 2g/3ml). The pseudomonas species shows the maximum zones of inhibition followed by Escherichia coli, Proteus species , Klebsiella species, Enteroccocus species respectively (Table 2). When the results of agar disc diffusion with Boerhavia diffusa ethanolic extract was compared with that of Punarnavasavam (Table 5) showed similar results. Whereas in the agar well diffusion method, the maximum zone of

growth inhibition was obtained at a concentration of 100mg of Boerhavia diffusa ethanolic extract (for 2g/3ml). The Pseudomonas species shows the maximum zones of inhibition followed by Escherichia coli, Klebsiella species, Proteus species and Enterococcus species (Table 1). In the agar well diffusion method, the maximum zone of growth inhibition was exhibited at a concentration of 0.356mg of Punarnavasavam the Pseudomonas species shows the maximum zones of inhibition followed by Escherichia coli, Klebsiella species Enterococcus species and Proteus species (Table 4). The lowest MIC value for Boerhavia diffusa ethanolic extract was recorded for Pseudomonas species and the highest value recorded for Enterococcus species (Table.8) .This will follow the similar pattern of agar diffusion results. By testing different mobile phases for the separation of extract of Boerhavia diffusa and its constituents by HPTLC, the desired resolution of β- sitosterol with symmetrical and reproducible peaks was achieved using toluene: ethyl acetate (8: 2). The R_f value of β- sitosterol was found at 0.39. To ascertain the purity of the peak in test sample, spectrum was compared with that of standard β- sitosterol and found to be super imposable, thus confirming the peak purity. Percentage of β-sitosterol varies from 0.3547-0.3549 percentage in samples.

Urinary tract infection (UTI) is among the most common bacterial infections in women of all ages but the incidence increases with older age (Irene et al., 2010). The presence of bacteria in the urine is called bacteriuria, in significant bacteriuria urine must contain $>10^4$ organisms /ml. it cause frequency, dysuria, suprapubic pain and pyuria, which will later developed in to pyelonephritis, cystitis etc. Escherichia coli are the commonest urinary pathogen (60-90%). UTI caused by Pseudomonas spp, Proteus spp, Klebsiella spp and staphylococcus aureus are associated with hospital acquired infection. Proteus mirabilis, a Gram-negative bacterium, represents a common cause of complicated urinary tract infections in catheterized patients (Greta et al., 2010, Colle et al., 2006, Cheesbrough, 2006). Enterococcus faecalis a ubiquitous lactic acid bacteria commonly associated with the human digestive tract as commensal, accounts for up to 10% of all nosocomial infections of the bloodstream, wounds, urinary tract and heart (Christian et al., 2010). Uropathogenic Escherichia coli (UPEC) afflict nearly 60% of women within their lifetimes (Corinne et al., 2010).

Medicinal plants represent a rich source of antimicrobial agents; plants are used medicinally in different countries and are source of many potent and powerful drugs (Srivstava et al., 1996). A wide range of medicinal plant parts is used for extract as a raw drug and they

possess varied medicinal properties. The different parts used include root, stem, flower, leaves, fruits, twigs exudates and modified plant organs (Uniyal et al., 2006). Different extracts of Drynaria quercifolia showed good antimicrobial activity against gram positive urinary tract pathogens (Mithrga et al., 2012). Antibacterial activity of Salvia plebia granules against urinary pathogens also reported (Peng et al., 2010). Ajwain oil, Cinnamon oil, Clove oil, Fennel oil and Peppermint oil had activity on selected UTI causing microorganisms (Kumar et al., 2012).

Table .1: Zone of growth inhibition by the ethanolic extract of Boerhavia diffusa Well diffusion method.

ORGANISM	ZONE OF INHIBITION IN (mm)			
	40mg	60mg	80mg	100mg
Proteus spp	10	11	13	16
Klebsiella spp	10	14	15	17
Pseudomonas spp	10	12	15	18
Escherichia coli	10	11	14	17
Enterococcus spp	9	11	13	16

Table .2: Zone of growth inhibition by the ethanolic extract of Boerhavia diffusa Disc diffusion method.

ORGANISM	ZONE OF INHIBITION IN (mm)			
	20mg	30mg	40mg	50mg
Proteus spp	9	10	11	12
Klebsiella spp	8	9	11	12
Pseudomonas spp	7	9	11	13
Escherichia coli	6	7	10	13
Enterococcus spps	6	8	10	11

Table.3: Absorbance obtained with ethanolic extract of Boerhavia diffusa

ORGANISM	ABSORBANCE AT 600 nm						
	0 mg	20 µl (13.3mg)	30 µl (20mg)	40 µl (26.7mg)	50 µl (33.3mg)	60 µl (40mg)	70 µl (46.7mg)
Proteus spp	0.335	0.297	0.222	0.089	0.029	0.022	0
Klebsiella spp	0.347	0.313	0.224	0.188	0.153	0.028	0
Pseudomonas spp	0.33	0.172	0.157	0.082	0.026	0	0
Escherichia coli	0.36	0.345	0.258	0.158	0.03	0.012	0
Enterococcus spp	0.29	0.233	0.166	0.088	0.045	0.026	0

Table .4: Zone of growth inhibition by the Punarnavasavam - well diffusion method.

ORGANISM	ZONE OF INHIBITION IN (mm)			
	125µl (0.223mg)	150 µl (0.267mg)	175 µl (0.312mg)	200 µl (0.356mg)
Proteus spp	10	11	13	14
Klebsiella spp	10	12	13	15
Pseudomonas spp	23	25	26	30
Escherichia coli	23	24	26	28
Enterococcus spp	14	15	17	19

Table .5: Zone of growth inhibition by the Punarnavasavam - Disc diffusion method.

ORGANISM	ZONE OF INHIBITION IN (mm)			
	30µl (0.053mg)	45 µl (0.08mg)	60 µl (0.107mg)	75µl (0.134 mg)
Proteus spp	-	8	9	10
Klebsiella spp	-	8	9	11
Pseudomonas spp	8	10	13	15
Escherichia coli	8	9	11	14
Enterococcus spp	-	-	8	9

Table .6: Absorbance obtained with Punarnavasavam

ORGANISM	ABSORBANCE AT 600 nm						
	0 mg	20 µl (0.036mg)	30 µl (0.053mg)	40 µl (0.071mg)	50 µl (0.089mg)	60 µl (0.11mg)	70 µl (0.12mg)
Proteus spp	0.335	0.125	0.083	0.062	0.038	0.015	0
Klebsiella spp	0.341	0.147	0.136	0.082	0.061	0.028	0
Pseudomonas spp	0.33	0.093	0.075	0.063	0.024	0	0
Escherichia coli	0.36	0.149	0.123	0.081	0.062	0.016	0
Enterococcus spp	0.29	0.151	0.132	0.093	0.071	0.022	0

Table.7: Antibiotic sensitivity test

ORGANISM	ZONE OF INHIBITION IN (mm)				
	Nitrofuratoin (200 µg)	Co-trimoxazole (25 µg)	Cefotaxime (30 µg)	Gentamicin (10 µg)	Chloramphenicol (30 µg)
Proteus spp	16 (I)	30 (s)	15 (I)	18(S)	25(S)
Klebsiella spp	16 (I)	11 (I)	6 (R)	17 (S)	22 (S)
Pseudomonas spp	6 (R)	6 (R)	7 (R)	6 (R)	(R)
Escherichia coli	25 (S)	(R)	(R)	20 (S)	(R)
Enterococcus spp	19 (S)	25 (S)	9 (R)	16 (S)	19 (S)

S= sensitive R= resistant I= intermediate

Table.8: Minimum inhibitory concentration

ORGANISM	MINIMUM INHIBITORY CONCENTRATION(MIC)		
	Boerhavia diffusa ethanolic extract mg/mL	Punarnavasavam mg/mL	Gentamicin µg/mL
Escherichia coli	35	0.09	0.95
Klebsiella spps	35	0.09	0.75
Pseudomonas spps	30	0.07	1.26
Proteus spp	40	0.11	0.85
Enterococcus spp	45	0.12	0.73

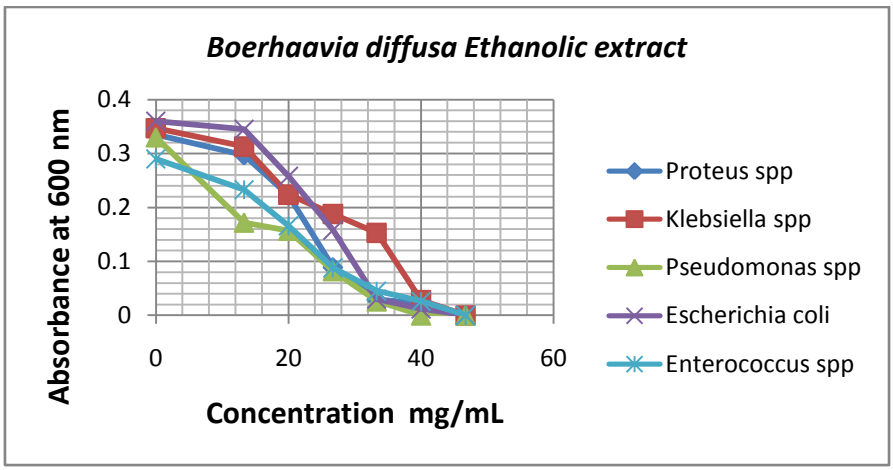

Figure 1. Effect of Boerhavia diffusa ethanolic extract on bacterial growth

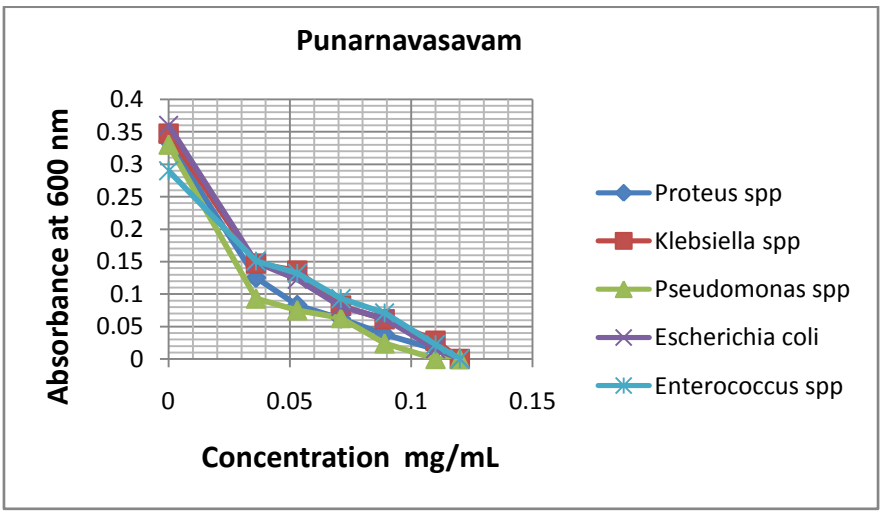

Figure 2. Effect of Punarnavasavam on bacterial growth

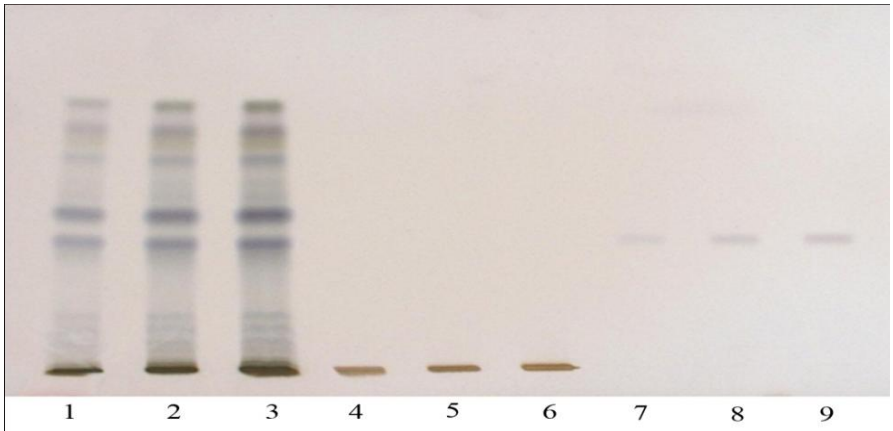

Figure 4: HPTLC PROFILE

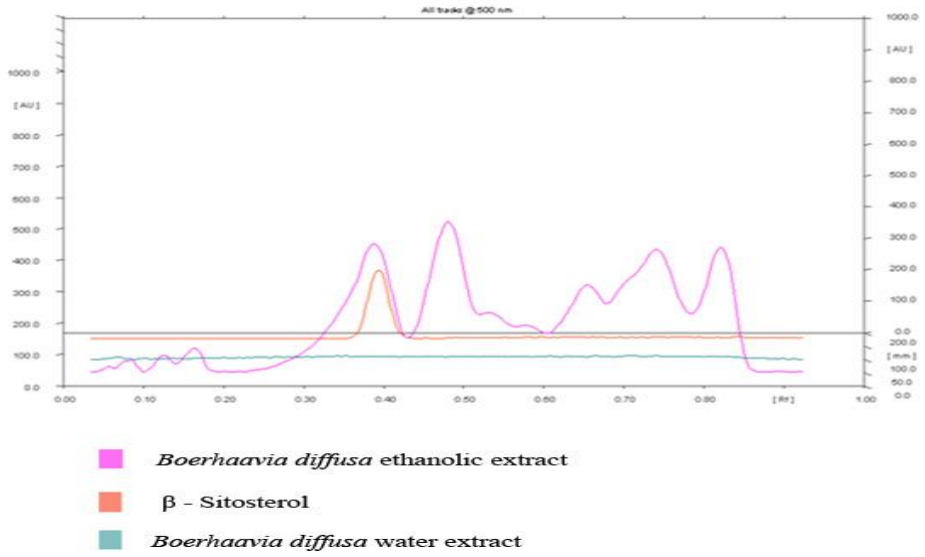

Figure 5: HPTLC chromatogram

CONCLUSION

The wide spectrum of activity of plant extract and Punarnavasavam when compared to the commercial antibiotics is an indication of their antibacterial potential in medicine. There are no reports of severe toxicity in human from consuming Boerhavia diffusa or Punarnavasavam. The observed resistance to most of the antibiotics was not unexpected because these drugs are relatively cheap and therefore commonly available to the population who tend to abuse them heralding the emergence of resistance. The demonstrable antibacterial activity of the plant extract to the pathogens is therefore an interesting and important finding since they are cheap, safe and easily affordable by the population. The results of the present investigation clearly indicating the antimicrobial activity of Boerhavia diffusa ethanolic extract against urinary pathogens. Due to this property of the plant, the

Polyherbal formulation which contains Boerhavia diffusa also showed the antimicrobial activity. The Boerhavia diffusa ethanolic extract and Punarnavasavam showing similar effect on tested urinary pathogens. The aqueous extract of Boerhavia diffusa do not shows any antibacterial activity. So it was concluded that the antimicrobial activity was due to the presence of compounds that dissolve in organic solvent like ethanol. HPTLC profiling of ethanolic extract showed the presence of β- sitosterol. But the β- sitosterol is absent in water extract. Many researchers proved the antibacterial activity of β- sitosterol (Kiprono et al., 2000). So the antibacterial activity of Boerhavia diffusa may be due to the presence of this compound or the synergistic effect of β- sitosterol with other active constituents in the plant. The above studies showed that Boerhavia diffusa may play a beneficial role managing bacterial urinary tract infections.

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