International Journal for Development of Science and Technology (IJDST)

Vol. 1, Issue, 8, March 2021 PP.270-280

www.ijdst.com

ISSN: 2456-8503

ISOLATION, IDENTIFICATION OF MICROBIAL ISOLATES FROM URINARY TRACT INFECTION PATIENTS AND EVALUATION OF ANTIMICROBIAL ACTIVITY OF *PUNICA GRANATUM* PEEL EXTRACT

S.SUBIN [1], ANUSHA BHASKAR [2]

1. **Ph.D. Scholar, SRK University, Bhopal, Madhya Pradesh, India.**
2. **Professor, SRK University, Bhopal, Madhya Pradesh, India.**

Abstract

Urinary tract infections (UTI) is a globally important health problem which need attention with respect to treatment. This has become a burden to the health sector because people tend to become resistant to the antibiotics used and constant change in the line of treatment is observed. The present study was aimed to gain knowledge about the type of pathogens responsible for urinary tract infections and evaluation of antimicrobial activity of various plant extracts against the pathogens. 65 samples were collected and processed. Bacterial species isolated from urine samples were *Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Klebsiella pneumoniae, Streptococcus sp.* The antimicrobial activity of methanol, ethanol and aqueous extracts of *Punica granatum* peel extract has been tried against these microorganisms by well diffusion method. The study concluded that the extract was effective against all the organisms irrespective of whether they are gram +ve or –ve. Methanolic extract was

Introduction

Urinary tract infections (UTIs) are health problems that affect a large number of people globally and arise due to the presence of microbial pathogens in the urinary tract. This problem occurs more often in women than men because a women's urethra is shorter. The short urethra makes it easier for bacteria from the anus or genital area to reach the bladder. About 50% women suffer from UTI at least once in their life span and recurrent infections are common [1,2]. While it is more common in women others include patients with catheter or those suffering from complaints of prostatitis. . Transmission occurs in four ways; namely through sexual intercourse, from mother to the foetus via placenta, through poor personal hygiene, and via communal sponge and towel usage [3]. (Onifade *et al*., 2011). Common symptoms include burning micturation and increased frequency of urination with significant pain. In patients with frequent UTI their bacteria may become resistant to antibiotics over time, making careful selection of antibiotic and the full course of treatment essential. In the last three decades, there have been a lot of reports in the scientific literature on the inappropriate use of antimicrobial agents and the spread of bacterial resistance among microorganisms causing urinary tract infections [4].

Bacteria causing UTI express different virulence determinants which include those essential for the initial adhesion and then subsequent colonization of mucosal surfaces [5] . They also invade the host tissues for overcoming the defence mechanisms resulting in persistent and at times chronic infections. The virulent parameters include surface factors (type 1 and P pili, fimbriae and adhesins) as well as secreted factors (polysaccharide coatings, toxins, metabolic enzymes), targeting these factors may be the best approach to treatment[6,7].

Medicinal plant extracts seem to be the most promising alternative to antibiotics and has been explored in the recent times. Available reports illustrate that extracts (aqueous/organic) from different parts of medicinal plants and/or their secondary metabolites have been studied for treatment and/or prevention of UTIs [8-10]. Plants seem to have varied mechanism of action against bacteria, they may reduce bacterial colonization and/or toxin production.

Pomegranate peels (*Punica granatum*) are considered wastes or byproduct obtained through juice processing. It is characterized by significant presence of ellagitannins and polyphenols, gallic acid and ellagic acid as well as flavonoids-associated with biological properties such antimicrobial agents .

Various extracts prepared from pomegranate fruit peels were evaluated for their antimicrobial activity against some food-borne pathogens using several methods (11-14). It was found that 80% methanolic extract of peels was a potent inhibitor for *Listeria monocytogenes, Yersinia enterocolitica, Klebsiella pneumonia, Proteus vulgaris, Bacillus subtilis, Staphyllococcus aureus* and *Escherichia coli* (11-14). Alam Khan and Hanee had shown that ethanolic extract of pomegranate peels has lowest MIC against *E. coli*, *P. aeruginosa* and *S. aureus* compared to MICs of methanolic and hot water extracts (15). The inhibitory zones of all the three extracts were greater than that of the standard antibiotic Tetracycline (18). In contrast Nuamsetti et al. found that the hot water extract of the peels was most potent against *E. coli* compared to 95% ethanol and acetone extracts (16).

The objective of this study was to explore the efficacy of using aqueous pomegranate peel extract to reduce pathogenicity of *E. coli* responsible for UTI and attempt to find a safety method to solve the problem of multi-drug resistance pathogen.

Materials and Methods

Study population

The study population was drawn from patients attending noorul islam institute of medical Sciences, Triuvananathapuram. Patients on antibiotic therapy were excluded from the study.

Isolation and identification of UTI isolates

Urine samples from 65 UTI patients were collected from pathological laboratory. For the isolation of UTI causing strains, loop full of urine sample was streaked on to Nutrient agar and Mac Conkey agar plate and incubated at 37 C for 24hrs. Next day individual colonies were selected and identified on the basis of morphological, cultural and biochemical characteristics.

Identification of organism

To check morphological characteristics, Gram-staining, capsule staining and motility test were performed. To check the growth pattern, different media including Nutrient

agar, MacConkey agar, Eosine Methylene Blue agar, Mannitol Salt agar, Citrimide agar, Bi.G.G.Y agar (Bismuth
Glycine Glucose Yeast agar) and Blood agar base supplemented with 5% sheep blood were used. For biochemical characteristics, sugar fermentation (lactose, glucose, mannitol, maltose, sucrose and xylose), TSI, IMViC (indole, MR, VP, citrate) oxidase, catalase and nitrate tests were performed.

Maintenance of clinical isolates

Stock cultures were maintained in vials on nutrient agar and stored at 4 C for further study.

Pomegranate peel extract

Fresh pomegranates were collected and peeled. The peels were washed well in running water and shade dried for a few days. The dried peels were powdered using a blender and the moisture content was determined using a moisture analyzer balance. Plant powder (35 g) was filled in the thimble and extracted successively with 95 % ethanol in a soxhlet extraction unit for 48 hours. The modified form of extraction process was carried out as per the methods reported earlier by Lin *et al.,* (1999). The plant extracts were filtered and then concentrated using rotary evaporator at 40 ºC and each extract were transferred to glass vials and kept at 4º C before use.

Determination of the antimicrobial activity of medicinal plants

Assay of antimicrobial activity of medicinal plant extract was done by agar well diffusion method and MIC.

Well Diffusion Method

The Mueller Hinton agar plates were prepared and test microbial strains were swabbed on the MHA plates using sterile cotton swabs. Five wells were made with cork borer. Different concentrations (1000µg/ml - 5000µg/ml) of peel extracts (methanol, acetone and ethanol) was poured in the wells. Then the plates were incubated at 37 °C for 24 48 hours. After incubation period, zone of inhibition were measured and recorded. Control plates were prepared without plant extract using only different solvents. The tests were performed in duplicates for each microorganism evaluated and the final results were presented as the arithmetic average. The inhibition zones were measured in millimetres. The results obtained from leaf extract, root extract and seed extracts were compared to know the

effectiveness of leaves, roots or seeds against pathogens.

Minimal inhibitory concentration (MIC)

The MIC estimated and serial dilutions of broth and various concentrations of herbal extracts were made to 3.0 ml in test tube. Then cultures were added. The test tubes were incubated at 37 C for each type of microbial culture. The lowest concentration of the crude drug that inhibited the growth of microorganisms completely was considered as MIC.

Results and Discussion

UTIs are a cause of global health concern as they significantly affect public health. It affects the quality of life and is also implicated in higher mortality rates. Women seem to be more prone to the infection than men. In this study, 49 patients out of 65 were showed to be urine culture positive. There were 35 females and 14 males in patients with urine positive culture. On the Nutrient agar plate and Mc conkey agar plate the colonies were isolated and identified. Age group of 21- 30 were showed maximum infection. Female patients were more when compared with male patients (Table I). Percentage of *E.coli* present was maximum in the urine samples. Microorganisms isolated from urine samples were *E.coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus* sp. The pathogens identified subcultured on nutrient agar slants for further use.

Antimicrobial activity of different extracts of *Punica granatum* peel against pathogens

In our study, we have achieved isolation, identification of pathogens from urine samples with help of normal media, differential media, and selective media and biochemical tests. Evaluation of antimicrobial activity also performed with different plant extracts such as methanol and ethanol and aqueous extracts of *Punica granatum* peel.

Table 4 shows the effectiveness of the different extracts of pomegranate on the organisms. Crude methanolic extract seems to be more antibacterial against the organisms studied, while the ethanolic and aqueous extract also exhibited significant activity.

These findings and our result clearly demonstrated and confirmed the effectiveness of pomegranate peel on inhibition of microbial activity. In general, the extent of the inhibitory effects of the pomegranate extracts could be attributed to their phenolic and anthocyanin content. The bioactivity of extracts on the microorganisms tested has high total flavonols, phenolics, anthocyanins and organic acids. Similarly, Shoko *et al*. [17] confirmed that phenolics and gallics were the most effective compounds against tested bacteria. The inhibitory effect of phenolic compounds could be explained by adsorption to cell membranes, interaction with enzymes, substrate and metal ion deprivation [18].

Plants exhibit a number of biological properties and have been investigated for their varied efficacy. Plants being rich in phytoconstituents are chemically complex and often two or more compounds may act synergistically. Moreover, different compounds may have different targets. The synergism and multi-targeting not only increases the efficacy but also minimizes the possibility of pathogens developing resistance [19,20].

Most of the antibiotics usually used for the treatment of UTI are resistant. This necessitates the development of new antimicrobials and therapeutic agents having high effectiveness with no side effects, easy availability and less expensive. The present study helps to understand the presence of bioactive compounds in the plants and application of these plants in the pharmaceutical industry in future.

Table1. Distribution of UTI patients

S. No	Age group (years)	No. of Male	No. of Female
1	1-10	0	0
2	11-20	0	3
3	21-30	5	14
4	31-40	4	10
5	41-50	3	5
6	51-60	2	3

Table 2: Percentage of Gram positive and Gram negative bacteria isolated from UTI patients

Bacterial isolates	**Colony morphology**	**Total organisms**	**Total % of UTI isolates**	**Individual % of G(-) and G(+) UTI isolates**
Gram (-)ve		**31**		
E.Coli	Circular, small, slightly raised, smooth	15	30.61	48.3
K.pneumonia	Circular, mucoid convex, capsulated, small colonies	8	16.3	25.8
P. aeruginosa	Small, rough colonies, flat edges	7	14.28	22.58
Gram (+)ve		**18**		
S. aureus	Circular, convex with pinheaded colonies	9	18.36	50.0
S. epidermidis	Circular, whitish, pinheaded colonies, convex with entire margins	4	8.16	22.2
Streptococcus sp.	Circular with entire margin with depressed centres	5	10.2	27.7

Table 3. Biochemical tests for the identification of microbial isolates

Biochemical test	*E. coli*	*S.aureus*	*S.epidermidis*	*Sreptococcus sp.*	*K.pnuemoniae*	*P.aeruginosa*
Indole	+	-	-	+	-	-
MR	+	+	-	-	-	-
VP	-	+	+	-	+	-
Citrate	-	-	-	-	+	+
Nitrate	+	+	+	-	+	+
Urease	-	-	+	+	+	-
TSI	A(slant, butt) H_2S^-	A(slant, butt) H_2S^-	A butt, alkaline slant H_2S^-	Aslant, H_2S^-	A(slant, butt) H_2S^-	Alkaline slant and butt
Catalase	+	+	+	-	+	+
Oxidase	-	-	-	-	-	+
Glucose	AG	A	A	A	AG	A
Lactose	AG	A	A	A	AG	-
Xylose	A	-	-	-	-	-
Mannitol	AG	A	-	-	AG	-
Maltose	-	A	A	-	-	-
Sucrose	-	A	A	A	AG	-
Motility	+	-	-	-	-	+

(AG – Acid, Gas produced)

Table 4: Antibacterial activity of the Pomegranate Peel Extracts on Bacterial UTI Isolates

Punica granatum Extracts	Diameter of Inhibition Zone (mm)					
	E.Coli	*S.aureus*	*S.epidermidis*	*Sreptococcus sp.*	*K.pnuemoniae*	*P.aeruginosa*
Methanolic extract	19.66±0.57	10.25±0.52	15.2±0.62	16.3±0.52	17.5±0.70	12.6±0.42
Ethanolic extract	18.25±0.65	17.5±0.57	10.52±0.63	9.54±0.60	12.5±0.63	13.0±0.64
Aqueous extract	11.66 ± 0.54	8.21±0.63	7.25±0.52	5.80±0.60	9.67±0.45	5.70±0.52

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