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Research Article

In Vitro Investigation of Antibacterial Activity of Combined Leaves Extracts of *Alstonia scholaris* and *Fernendoa adenophylla* against Urinary Tract Infection Pathogens

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Abstract

In the present research study, the antibacterial activity of combined leaves extracts of *Alstonia scholaris* and F*ernandoa adenophylla* against urinary tract pathogens was investigated. A total of ten different bacterial strains *(E. coli, Klebsiella spp, Proteus spp, Salmonella spp, Morganella morginii, Pseudomonas aeroginosa, Providencia spp, Staphylococcus aureus* and *Streptococcus pyogene)* were isolated from urine samples. Through disc diffusion method, seven different antibiotics were used against the isolated pathogens to determine their culture sensitivity patterns. Among the seven used antibiotics, Meropenem was most effective and produced the largest ZI 44mm, and was 100% active followed by Azithromycin (90%), Pipracillin (90%), Ciprofloxacin (90%), Amikacin (90%) and Nalidixic acid (80%), while Doxycycline showed average activity and was resistant to five tested bacterial strains. Well diffusion method was used to evaluate the antibacterial activity of leaves extracts of both plants. For this purpose, extracts of both plants were mixed together and five combinations were obtained and were tested against the isolated bacterial strains. Among the five combinations, the combination of (n-hexane extract of *F. adenophylla* + Water extract of *A. scholaris*) showed

broad-spectrum antibacterial activity against all the tested bacterial strains and produced the highest zone of inhibition 18.5mm against *Salmonella spp,* whereas the combination of Methyl alcohol extract of A. scholars mixed with n-hexane extract of *F. adenophylla,* showed no activity. The other three combinations showed average activity against the tested bacterial strains. From the present study it can be concluded that the n-hexane extract obtained from the leaf of *F. adenophylla* mixed with the water extract obtained from the leaf of *A. scholaris* plant, was found to have antibacterial activity against UTI causing bacterial pathogens. So further study in this regard will be definitely helpful in producing plant-based antimicrobial compounds to replace synthetic antibiotics and suppress the emergence of antibiotic resistance strains.

Introduction

Urinary tract represents a system that collect, store and release urine and include kidneys, ureters, bladder and urethra. Urinary tract infections (UTIs) are infections caused by microorganisms anywhere in the urinary tract [1]. UTI is the second most common type of infection in the body. There is an estimated 150 million urinary tract infections per year worldwide [2].

UTI mostly occurs in women than men, except the extremes of age, after 50 years of age, the incidence of UTI is almost as high in men as in women, because of obstruction from prostatic hypertrophy. The reason that women mostly get UTI infection is that women have shorter urethra than that of men, so the pathogen easily get access to the bladder and cause infection [3].

UTI is mostly caused by gram negative bacteria. *Escherichia coli* is the most common cause of UTI, accounts for up to 70% of community acquired and 50% of hospital associated UTIs [4]. Other important uropathogens include *Proteus mirabilis, Proteus vulgaris, Klebsiella spp, Pseudomonas aeruginosa, Acinetobacter, Serrati spp, Provedincia spp, Morganella morganii* [5] *Neisseria spp* and *Salmonella spp* [6]. UTI is also caused by some gram positive bacterial species including *Staphylococcus aureus, Staphylococcus saprophyticus, Streptococcus pyogene, Streptococcus agalactiae, Enterococcus faecalis* and *Bacillus subtalis* [7].

Increasing drug resistance among bacteria that cause UTIs has made therapy of UTI difficult. Bacteria have the genetic ability to transmit and acquire resistance to drugs [8]. The increasing failure of the chemotherapeutic and antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the screening of several medicinal plants for their potential antimicrobial activity [9].

Plants, a key component of traditional medicine, are an important source of valuable medicines. Plants have been used to treat chronic as well as infectious diseases since long time ago [10]. It is estimated that about over 60% of world human population and 80% in the developing countries depends directly on plants for the medicinal purposes [11]. Over 50% of all modern clinical drugs are of natural product origin [12].

The medicinal importance of plants is due to the presence of chemical compounds in them. Some of the important bioactive compounds are Alkaloids, Glycosides, Triterpenoids, Terpenoids, Flavonids, Phenols, Reducing sugars, Saponins and Tannins. Plants containing such type of compounds are used for Ayurvedic, Unani and other treatments in rural areas [13].

Here the plant source is used as *Alstonia scholaris* and *Fernandoa adenophylla*. Both are medicinally important plants. In Thai traditional medicine, the leaves of *F. adenophylla* are used for the treatment of different skin diseases [14]. The fruits are cooked before eating while the flowers are used as such for vegetable and salad purposes [15]. Whereas *Alstoniascholaris* traditionally being used in debility, arthritis, impotence, wounds and earache, asthma, leucorrhoea, dog bite, fever, cancer, tumor, jaundice, hepatitis, malaria, skin diseases, diarrhea, leprosy, mental disorders, cardiopathy, helminthiasis, pruritus, agalactia, hypertension, dental or gum problem, abdominal pain after delivery and swelling. It is also used as aphrodisiac, antidote to poison, abortifacient, astringent, thermogenic, cardio tonic, stomachic and expectorant. Reports are available on its ethno veterinary use such as fever in cattle. Ayurvedic use is found in phosphaturia and as a blood purifier [16].

Materials and Methods

Plant Collection and Identification

The leaves of *Alstonia scholaris* and *Fernandoa adenophylla* were collected from the main campus of University of Peshawar. The leaves were identified in the department of Botany, University of Peshawar while the plants were processed in Microbiology laboratory, Abasyn University Peshawar.

Preparation of Plant Material

The dried leaves of *Alstonia scholaris* and *Fernandoa adenophylla* used as plant sample for extract preparation were first thoroughly washed with water and soaked in detergent to remove the microbial load on the surface of leaves sample. These were then powdered in mortar and pestle and were sieved with fine cloth [17].

Extraction of Plant Material

The fine powder of one hundred gram (100g) of both leaves was soaked in separate flasks containing methanol (ME), ethanol (EE), ethyl acetate (EAE), n-hexane (nHE) and distilled water (DWE) and were vigorously shake until powder was mixed well in solvents [18]. After the completion of soaking period, the supernatant solvent was then collected in a conical flask with the help of coarse cloth as a filter medium. The crude extract collected was then shifted to the rotary evaporator and was processed at 40-50˚C to afford a blackish green mass. The extracts thus obtained were stored in labelled sterile bottles and preserved at 4˚C until further use for the screening of antimicrobial activity. The dried extract was then dissolved (1mg/ml solvent) in Dimethyl Sulfoxide (DMSO) to get semi solid extract [19].

Specimen Collection

Urine samples were collected from patients attending different hospitals and diagnostic centres of Peshawar. Midstream urine samples were collected in sterile containers. The sterile containers containing samples were immediately transported to the Microbiology laboratory, Abasyn University Peshawar for further processing.

Inoculation of Samples

The collected urine samples were inoculated on nutrient agar plates using sterile swabs and were incubated at 37°C for 24 hours.

Isolation and Identification of Bacterial Strains

After 24 hours of incubation, bacterial growth was noticed on the plates. Ten different bacterial strains were isolated and identified i.e *E. coli, Klebsiella spp, Proteus spp, Pseudomonas spp, Salmonella spp, Morganella morgini, Providencia spp, Neisseria gonorrhea, Staphylococcus aureus* and *Streptococcus pyogene.* Isolated bacterial strains were sub-cultured on differential and selective media e.g. Cysteine Lactose Electrolyte-Deficient (CLED) agar, MacConkey agar, Eusine methylene blue (EMB) agar, Blood agar and Salmonella Shigella (SS) agar and were identified according to their specific morphological, staining and biochemical reactions up to genus/species levels wherever applicable [20].

Antibiotic Susceptibility Testing

Before the evaluation of antimicrobial activity of plant extracts, all the microorganisms were first tested against commonly used antibiotics. Disc diffusion method was used for measuring the antimicrobial activity and the media used was the Muller Hinton agar. The sensitivity of seven antibiotics was tested against the previously mentioned 10 bacterial strains and the process was repeated thrice. All the plates were incubated at 37°C for 24h [21].

Evaluation of Antimicrobial Activity of Plant Extracts by Mixing the Leaf Extracts of *A. scholaris* with the Leaf Extracts of *F. adenophylla*

Leaves extracts from both plants dissolved in different solvents were mixed together and five combinations of extracts were obtained. These five combinations were shortly named as C1, C2, C3, C4 and C5 for our own ease. These five combinations are as follow;

C1) Ethyl alcohol extract of *F. adenophylla* was mixed with the n-hexane extract of *A. scholaris,*

C2) Ethyl acetate extract of *F. adenophylla* was mixed with Ethyl alcohol extract of *A. scholaris,*

C3) n-hexane extract of *F. adenophylla* was mixed with the Water extract of *A. scholaris,*

C4) Methyl alcohol extract of *A. scholars* was mixed with n-hexane extract of *F. adenophylla,*

C5) Methyl alcohol extract of *F. adenophylla* was mixed with Ethyl acetate extract of *A. scholaris.*

Well diffusion method was followed for the assessment of antimicrobial activities of methanol, ethanol, ethyl acetate, n-hexane and water extracts of *A. scholaris* and *F. adenophylla* [22]. Approximately 1mg of the combined plant extracts was dissolved in 1ml of Dimethyl Sulfoxide (DMSO). Pre autoclaved Muller Hinton agar plates were inoculated with a 10^{-5} dilution of bacterial cultures, using sterile cotton swabs to achieve uniform growth. Sterile cork borer was used to bore wells in the agar, to test the activity of plant

extracts. Sixty (60µl) of each extract i.e., C1, C2, C3, C4, and C5 was introduced through micropipette aseptically into specifically marked wells in the agar plates. All the plates were incubated at 37°C for 24h and the process was repeated three times.

Results and Discussion

Antibiotic Susceptibility Testing

The isolated strains were identified as *E. coli, Staphylococcus aureus, S. pyogenes, Klebsiella pneumonia, Pseudomonas aeroginosa, Proteus spp, Morganella morginii, Salmonella spp, Providencia spp* and *Neisseria gonorrhea.*

These isolated bacterial strains were tested for antibiotic sensitivity patterns against seven commonly used antibiotics. The used antibiotics showed varying results (Table 2) against the tested bacterial strains. Among the seven antibiotics, Meropenem was the most effective and produce the largest ZI 44mm against *Providencia spp* and showed 100% broad-spectrum antibacterial activity against all the tested bacterial strains followed by Azithromycin (90%), Pipracillin (90%), Ciprofloxacin (90%), Amikacin (90%) and Nalidixic acid (80%), while Doxycycline showed average activity and was resistant to five tested bacterial strains. Among the ten tested bacterial strains, *E. coli* was the most resistant specie (71%) followed by *Streptococcus pyogenes* (28%), *Providencia spp* (28%), *Staphylococcus aureus* (15%), *Klebsiella spp* (15%), *Neisseria gonorrhea* (15%) likewise *Morganella morginii, Pseudomonas aeruginos, Salmonella spp* and *Proteus spp* were 100% sensitive to all the seven antibiotics.

Table 1: *Antibiotic sensitivity pattern of tested bacterial strains*

S.No	Microorganism	Antibiotic discs with ZI (mm) representing sensitivity, while (-) represents resistance						
		1	2	3	4	5	6	7
		AZM	MEM	NA	PIP	CIP	AK	DO
1	*S. aureus*	25mm	21mm	18mm	20mm	21mm	10mm	-
2	*S. pyogenes*	24mm	32mm	-	14mm	26mm	24mm	-
3	*Providencia spp*	14mm	44mm	-	15mm	30mm	22mm	-
4	*M. morginii*	18mm	31mm	14mm	13mm	21mm	26mm	22mm
5	*P. aeroginosa*	22mm	32mm	14mm	14mm	22mm	24mm	18mm
6	*Salmonella spp*	10mm	33mm	16mm	19mm	21mm	15mm	13mm
7	*N. gonorrhea*	19mm	16mm	13mm	25mm	32mm	29mm	-
8	*Proteus spp*	15mm	32mm	24mm	23mm	33mm	20mm	18mm
9	*Klebsiellaspp*	20mm	19mm	25mm	19mm	22mm	28mm	-
10	*E.coli*	-	38mm	29mm	-	-	-	-

Abbreviations: AZM-Azithromycin, MEM-Meropenem, NA-Nalidixic acid, PIP-Pipracillin, CIP-Ciprofloxacin, AK-Amikacin, DO-Doxycycline, mm-millimeter, (-)-No ZI.

Table 2: *Standard zone of inhibition of different antibiotics*

S.No	Antimicrobial Disc	Disc Potency	Resis	Inter	Suscep
1.	Amikacin AN-30	30 µg	≤14	15 - 16	≥17
2.	Azithromycin AZM-15	15 µg	≤13	14 - 17	≥18
3.	Nalidixic Acid NA-30	30 µg	≤13	14 - 18	≥19
4.	Meropenem MEM-10	10 µg	≤13	14 - 15	≥16
5.	Piperacillin PIP-100	100 µg	≤17	18 - 20	≥21
6.	Ciprofloxacin CIP-5	5 µg	≤15	16 - 20	≥21
7.	Doxycycline ee D-30	30 µg	≤12	13 - 15	≥16

MDR strains isolated from urine samples were tested against commercially available antibiotics. *E.coli* and *P.auroginosa* were 71% resistant to the tested antibiotics, *Klebsiella pneumonia, Proteus spp* and *S.aureus* showed 57% resistance, *E.fecalis* and *S.saprophyticus* were 42% resistant while *S.marcescens* was found to be only 14% resistant [23].

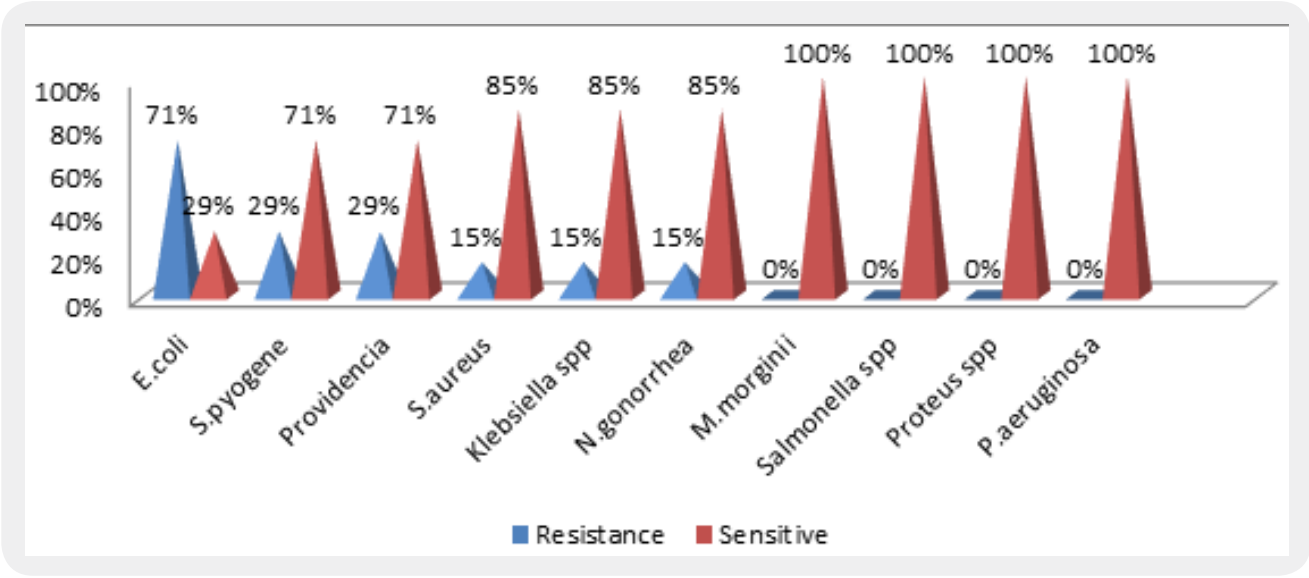

Figure 1: *Percentage antibiotic resistance and sensitivity of the tested bacterial strains*

Evaluation of Antimicrobial Activity of Combined Plant Extracts

Antimicrobial activity of *F. adenophylla* (leaves & seeds) against normal strains of bacteria including *B. subtilis* (ATCC 6633), *S. aureus* (ATCC 25923), *S. epidermidis* (ATCC 12228), *P. aeruginosa* (ATCC 27853) and *E. coli* (ATCC 8739) [24]. Antimicrobial effects of n-hexane fraction of crude methanolic extract of *A. scholaris* were tested on *Shigella dysentery, Enterobacter cloacae, Enterobacteriaceae bacterium* and *Serratiamarcescens* [25]. In the present study, strain specific inhibition zone diameter of leaves extracts of *A. scholaris* combined with the leave extract of *F. adenophylla* dissolved in different organic solvents and distilled water tested against the ten tested bacterial strain are shown in table No.3. Among the five combinations obtained from the combination of both plant leaves extracts, C3 (n-hexane extract of *F. adenophylla* + Water extract of *A. scholaris*) showed broad-spectrum antibacterial activity against all the tested bacterial strains and produced the highest zone of inhibition 18.5mm against *Salmonella spp,* 18mm against *Pseudomonas areoginosa* and *Morganella morginii,* 17.5mm against P*roteus spp,* 16.4mm against *N. gonorrhea,* 16mm against *Providencia spp,* 15.3mm against *S. aureus,* 14.3mm against *S. pyogene* and 13mm against *E. coli.* The other four combinations were found to be sensitive to most of the tested bacterial strains. C1 produced a 14mm zone against *M. morginii* and was sensitive to rest of the tested bacterial strains. C2 produced small zones of 10.3mm against *E. coli* and 11mm against *N. gonorrhea* while found to be sensitive to rest of the tested pathogens. C4 showed no activity and was completely sensitive to all tested species. Whereas C5 produced small zones of 11.33mm against *E. coli* and 10mm against *M. morginii.*

Table 3: *Antibacterial activity of combined plant leaves extracts*

Microorganisms	Plant extracts with ZI (mm) representing sensitivity, while (-) represents resistance				
	C1	C2	C3	C4	C5
Klebsiellaspp	-	-	16.75mm	-	-
Proteus spp	-	-	17.5mm	-	-
Providenciaspp	-	-	16mm	-	-
S.aureus	-	-	15.33mm	-	-
S.pyogenes	-	-	14.33mm	-	-
N. gonorrhea	-	11mm	16.4mm	-	-
E.coli	-	10.3mm	13mm	-	11.33mm
M.morginii	14mm	-	18mm	-	10mm
P.aeroginosa	-	-	18mm	-	-
Salmonella spp	-	-	18.5mm	-	10mm

Abbreviations: mm-millimeter, (-)- No ZI, C1) Ethyl alcohol extract of *F.adenophylla* + n-hexane extract of *A.scholaris,* C2)Ethyl acetate extract of *F.adenophylla* + Ethyl alcohol extract of *A.scholaris,* C3) n-hexane extract of *F.adenophylla* + water extract of *A.scholaris,* C4) Methyl alcohol extract of *A.scholaris* + n-hexane extract of *F.adenophylla,* C5) Methyl alcohol extract of *F.adenophylla* + Ethyl acetate extract of *A.scholaris.*

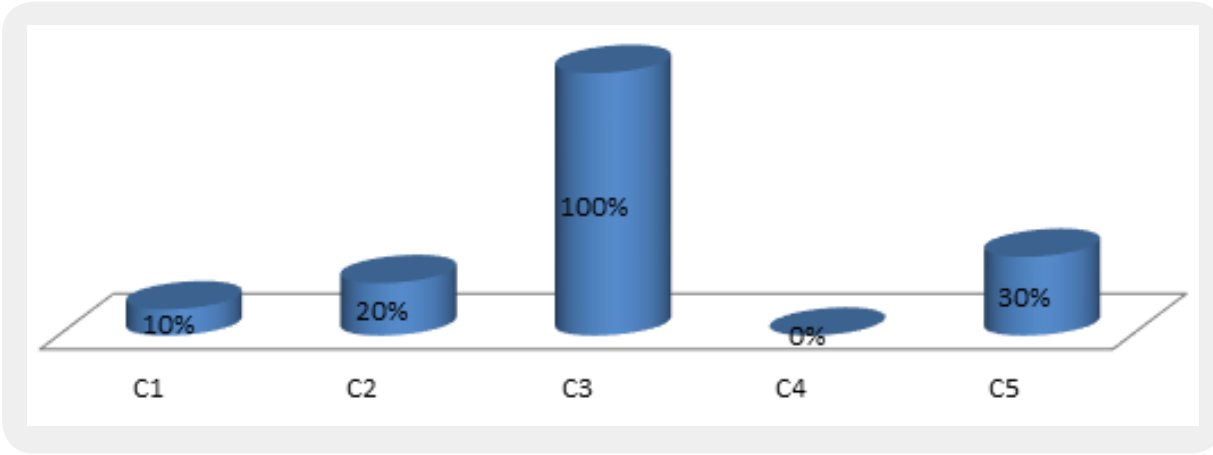

Figure 2: *Percentage antibacterial activity of combined plant extracts*

C1) Ethyl alcohol extract of *F.adenophylla* + n-hexane extract of *A.scholaris.*
C2) Ethyl acetate extract of F.*adenophylla* + Ethyl alcohol extract of *A.scholari.,*
C3) n-hexane extract of *F.adenophylla* + Water extract of *A.scholaris.*
C4) Methyl alcohol extract of *A.scholaris* + n-hexane extract of *F.adenophylla.*
C5) Methyl alcohol extracts of *F.adenophylla* + Ethyl acetate extract of *A.scholaris.*

Conclusion

It was concluded that Meropenem antibiotic was most effective while Doxycycline showed average activity and was resistant to five tested bacterial strains used against bacterial pathogens isolated from urinary tract infection samples. Among the five combinations, the combination of (n-hexane extract of *F. adenophylla* + Water extract of *A. scholaris*) showed broad-spectrum antibacterial activity against all the tested bacterial strains and produced the highest zone of inhibition 18.5mm against *Salmonella spp,* whereas the combination of Methyl alcohol extract of *A. scholars* mixed with n-hexane extract of *F. adenophylla,* showed no activity. From the present study it can be concluded that the n-hexane extract obtained from the leaf of *F. adenophylla* mixed with the water extract obtained from the leaf of *A. scholaris* plant, was found to have antibacterial activity against UTI causing bacterial pathogens. So further study in this regard will be definitely helpful in producing plant-based antimicrobial compounds to replace synthetic antibiotics and suppress the emergence of antibiotic resistance strains.

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