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ANTI-PSEUDOMONAS AERUGINOSA DRUG;

TO EVALUATE BACTERICIDAL ACTIVITY OF TABEBUIA IMPETIGINOSA AGAINST PSEUDOMONAS AERUGINOSA AND ITS SYNERGISTIC EFFECT WITH COMMON ANTI-PSEUDOMONAS AERUGINOSA DRUG.

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ABSTRACT… Objectives: In this research we assess in-vitro susceptibility of *Pseudomonas aeruginosa* (*P. aeruginosa*) using the ethanolic extract of medicinal plant Tabebuia impetiginosa (dried inner bark). To evaluate the synergistic effect of ethanolic extract of Tabebuia impetiginosa combine with ciprofloxacin In-vitro anti-*Pseudomonas aeruginosa* activities of the extracts and ciprofloxacin were confirmed, and synergism was verified for this combine extracts. **Study Design:** Experimental study. **Period:** October 2016 to February 2017. **Place of Experiment:** Rashid Latif Medical College, Lahore. **Method:** Diffusion method tests are mostly qualitative methods that are used to identify the antimicrobial activity, resistance and synergistic effect. The fresh plants inner bark was grinded and soaked in 95% ethanol for extraction. The antibacterial sensitivity of this compound against *P. aeruginosa* was assessed using the diffusion method. About 1000mg of grinded menstruum was added in 800ml of petroleum ether in a conical flask and adjust in rotary shaker at 100 rpm for 12 hours and then the final extract was filtered with 0.45mμ filter membrane and centrifuged at 2000rpm for 15 minutes. The final extract was redissolved in ciprofloxacin solution ($10^{\mu g/ml}$) for bioassay analysis. **Results:** We concluded that the fresh ethanolic extract of Tabebuia impetiginosa with ciprofloxacin has high antibacterial potency against *P. aeruginosa which is prominent then a single*. However this was not pure extract and if it is refined then it might gives significant antibacterial activity at low concentration. There is still need to test Tabebuia impetiginosa extract for antibacterial activity and to check synergistic effect with other drugs in-vivo against *Pseudomonas aeruginosa*. Extract presented the highest synergism rate with antimicrobial drug. **Conclusion:** In-vitro study showed Tabebuia impetiginosa fresh inner bark extract with ciprofloxacin dilution have significant antibacterial activity against *Pseudomonas aeruginosa* with P.value <0.001. Isolates were susceptible to this combine solution with mean zone diameter of 16.15 ± 0.95 mm and no regrowth was noticed. In the present research the synergistic effect of ciprofloxacin antibiotic with Tabebuia impetiginosa ethanolic extract were observe against *Pseudomonas aeruginosa* bacteria.

Key words: Antibacterial, Ethanolic, Pseudomonas Aeruginosa, Tabebuia Impetiginosa.

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INTRODUCTION

Hospital infections are important health problems in all over the world, because of their high morbidity and mortality, and prolonging the time of hospitalization and increasing the cost of treatment. Pseudomonas aeruginosa (P. aeruginosa) is recognised as one of the leading causes of severe hospital-acquired infections.[1] P. aeruginosa exhibits high-level resistance to many antimicrobials, and resistance can develop during therapy. Combination antibiotic treatment is preferred to provide larger spectrum antimicrobial effect and to prevent the rapid emergence of resistance in nosocomial infections caused by P. aeruginosa.[2] Combinations usually comprise an anti-pseudomonal beta-lactam and an aminoglycoside or a fluoroquinolone.[3] P. aeruginosa is a versatile microorganism, ubiquitously distributed in different environments, including terrestrial, aquatic, animal, human, and plant.[4] It is a Gram-negative opportunist pathogen in hospitalized or immune-compromised patients, causing infections, such as pneumonia, burn, wound, urinary tract and gastrointestinal

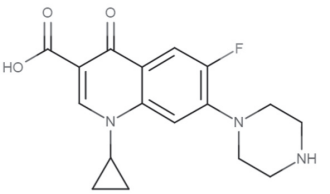
infections, otitis media, and keratitis.[5]

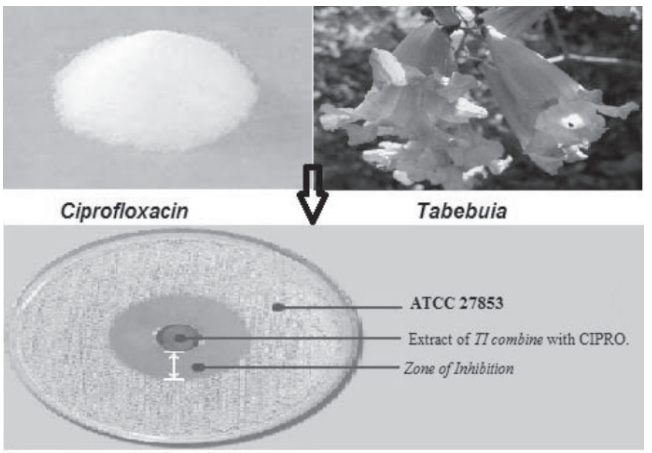
The use of plants for medicinal purposes is as old as the emergence of human species on earth.[6] Historically, first civilizations realized that some plants contained active ingredients in their essences, which empirically revealed their healing power when they were tested in disease.[7]

Medicinal plants have been studied as alternatives to the treatment of different dermatological diseases, especially in those with complex healing process of resolution.[8]

This complexity involves some factors that can affect the tissue repair process, which are systemic and local.[9] Red Lapacho (Tabebuia impetiginosa, syn. Tabebuia avellanedae), a canopy tree indigenous to the Amazonian rainforest and other parts of South America, has been acclaimed to be one of the "miraculous" cures for cancer and tumours.[10] Natural sciences interest in the plant also began in the 1960s when the United States National Cancer Institute (NCI) systematically began researching plant extracts all over the world looking for active compounds against cancer and looked at Tabebuia impetiginosa in considerable detail.[10] Two main bioactive components have been isolated from Tabebuia impetiginosa: lapachol and beta-lapachone. beta-Lapachone is considered to be the main anti-tumour compound, and pro-apoptotic effects were observed in vitro.

The purpose of this experimental study was to evaluate the synergistic antibacterial effect of Tabebuia impetiginosa against Staphylococcus aureus. The research was performed by taking, collecting and identification of samples of P. aeruginosa. Minimum inhibitory concentration (MIC) of ethanolic extract of Tabebuia impetiginosa was determined; against *P. aeruginosa*; in the microbiology laboratory.

S.no.	Antibiotic/plant	Antibacterial class	Targets	Structure
1	Ciprofloxacin	A broad-spectrum antimicrobial carboxyfluoroquinoline	Inhibition of the enzymes topoisomerase II (DNA gyrase) and topoisomerase	
2	Tabebuia impetiginosa	Flavonoids/Phenolic acid	Generalized Mechanism of action include enzyme inhibition	



MATERIAL

Following materials were used in the research:

Sterilized loop, Mueller Hinton agar, CLED agar with indicator, Eppendorf tubes, pure glycerol, blood culture broth,dimethyl sulfoxide (DMSO), 0.2% triphenyltetrazolium chloride (TTC) dye,petroleum ether, nutrient broth, ethanol, deionized water, 0.18 mol/L H_2SO_4, $BaCl_2$ standard solution, hydrogen peroxide. distilled water and Vancomycin 500mg inj.

METHODOLOGY

Test Drug

In order to access the anti-bacterial activity against P. aeruginosa, ethanolic extract of Tabebuia impetiginosa was used.

Standard Drugs
In order to compare the results of test drug and also as a positive control, antibiotic discs of Methicillin, cefoxitin and vancomycin were used in standard concentrations of 10μg, 30μg and 30μg respectively.

Test Organism
P. aeruginosa was obtained from clinical specimens of pus at Microbiology Laboratory of Shaikh Zayed Hospital, Lahore and was used as the test animal.

Preparation of ethanolic extract of Tabebuia impetiginosa
Tabebuia impetiginosa were obtained, washed (with distilled water), dried (in hot air oven) powdered, dried again and then stored in an air tight container. The extract was prepared in Pakistan council of Scientific and Industrial Research (PCSIR) Lab Lahore. The powder was then defatted and extracted with petroleum ether and 95% ethanol respectively in soxhlet apparatus.

500ml of petroleum ether was taken in a conical flask and about 100gm of dried powder was added in it and then it was placed in rotary shaker for 24 hours at 120 rpm. Extract was then filtered (using 0.22mμ filter membrane) and centrifuged for 10 minutes at 5000rpm.

Preparation of Mueller-Hinton & CLED Media
Instructions leaflet of oxoid was used to prepare the Mueller Hinton and CLED media. Both petri dishes and media were autoclaved before pouring. Petri plates containing media were stored in refrigerator and can be used within 7 days of preparation. The risk of contamination was reduced by drying the petri dishes in oven before use.

Determination of anti-bacterial sensitivity
Disk diffusion method was used to access the synergism between the extract and drugs.

Inoculation of plates for sensitivity and synergism
Fresh inoculum was prepared and a sterile cotton swab was immersed into it. Excess inoculum was then removed from the swab by pressing it with the margins of test tube. Before inoculated, the moisture in agar plates was removed by drying them in oven. Cotton swab was the streaked over the entire surface of dried agar plates. Plates were then rotated $60^0$for two times and each time the agar plates were streaked with the swab. In this way plates were streaked three times with the swab in three direction. Likewise all the plates were inoculated and labelled with the respective inoculum and isolate No.

The prepared discs of extract and standard drug were then placed on the agar plates after drying them near the flame for 3-4 minutes. Discs must be in close contact with the agar surface otherwise press the discs with sterilized needle or syringe. 2.4cm distance must be there in between two discs while 1.5 cm distance must be there in disc and margins of plate. While <20 mm distance must be there between the drugs and extract. The discs plates were covered with the lid and were kept in the incubator in inverted position at 35^0C for 18-24 hours. Each isolate was tested thrice times.

Methodology for reading results
Each plate was examined after 24 hours of incubation. Diameter of disc and diameter of the zone of inhibition was measured with a ruler. If there is any visible colony within zone of inhibition, it was considered as resistance or regrowth. Synergism plates were observed for any increase in zone diameter or the enlarged combine zone between drug and extract disc.

Interpretation of zone sizes
CLSI Standard recommendations were used to interpret the diameters of zone of inhibition as shown in following Table-I.

RESULTS
Results of Tabebuia impetiginosa extract
All the isolates were susceptible to extract with average zone diameter of 19 mm. Mean inhibition zone was 18.75±0.96 mm, with minimum of 14.70 mm and maximum of 21 mm zone diameter was observed (Table-II). Results of individual isolates

are also given in details (Table-II). One way ANOVA test was used to compare mean zones of inhibition.

Drug	Disc Content	Zone diameter (mm)	
		S	R
Methicillin (ME)	10μg	>10mm	<10mm
Cefoxitin (FOX)	30μg	>22mm	<22mm
Vancomycin (VA)	30μg	>7mm	<7mm
extract	----μg(MIC)		-

Table-I. Interpretation of zones of inhibition according to CLSI standard.

Drug	N	Mean ± SD	Minimum	Maximum	95% Confidence Interval for Mean	
					Lower Bound	Upper Bound
Methicillin 10μg	70	0.00 ± 0.00	0.00	0.00	0.00	0.00
Cefoxitin 30μg	70	15.57 ± 8.08	0.00	25.00	13.65	17.50
Vancomycin 30μg	70	15.04 ± 1.33	9.30	17.00	14.73	15.36
Tabebuia impetiginosa extract 340μg	70	18.75 ± 0.96	14.70	21.00	18.52	18.98
Total	280	12.34 ± 8.35	0.00	25.00	11.36	13.32

Table-II. Effect of Methicillin 10μg, Cefoxitin 30μg, Vancomycin 30μg and Tabebuia impetiginosa extract 340μg on mean inhibition zone

S/N	Isolate no.	Plate no.	Methicillin (ME-10μg)	Cefoxitin (FOX-30μg)	Vancomycin (VA-30μg)	Tabebuia impetiginosa extract (340μg)
1	449-P	SA-1	0	12	16	18
		SA-2	0	12.5	16.5	18.5
		SA-3	0	12	16	18
		Mean±SD	0	12.2±0.3	16.2±0.3	18.2±0.3
2	739-P	SA-1	0	10	16	19
		SA-2	0	10.5	16	19
		SA-3	0	10	16	19.5
		Mean±SD	0	10.2±0.3	16.0±0.0	19.2±0.3
3	558-P	SA-1	0	22	16	20
		SA-2	0	22	16	20
		SA-3	0	22	16	20
		Mean±SD	0	22.0±0.0	16.0±0.0	20.0±0.0
4	551-P	SA-1	0	10	16	19
		SA-2	0	10.5	16.5	19.5
		SA-3	0	10	16	19
		Mean±SD	0	10.2±0.3	16.2±0.3	19.2±0.3

Table-III. Comparative study of Tabebuia impetiginosa extract with antibiotics against Pseudomonas aeruginosa. Zones of Inhibition (mm)

Legend: P stands for pus, 449,739, 558, 551 are patient result report numbers.

Frequency of Resistance and Sensitivity of Drugs against Pseudomonas aeruginosa					
Drug	**Resistance n (%)**	**Sensitive n (%)**	**No Response n (%)**	**Re-growth n (%)**	**Total**
Methicillin 10μg	70 (100%)	0 (0%)	0 (0%)	0 (0%)	70 (100%)
Cefoxitin 30μg	40 (57%)	30 (43%)	0 (0%)	0 (0%)	70 (100%)
Vancomycin 30μg	0 (0%)	70 (100%)	0 (0%)	0 (0%)	70 (100%)
Tabebuia impetiginosa Extract 340μg	0 (0%)	70 (100%)	0 (0%)	0 (0%)	70 (100%)
p-value	< 0.001				

Table-IV. Frequency of resistance and sensitivity of drugs against pseudomonas aeruginosa. Graphical comparison of mean inhibition zone of drugs Methicillin 10μg, Cefoxitin 30μg, Vancomycin 30μg and Tabebuia impetiginosa extract 340μg: Chi-Square test value= 208.13

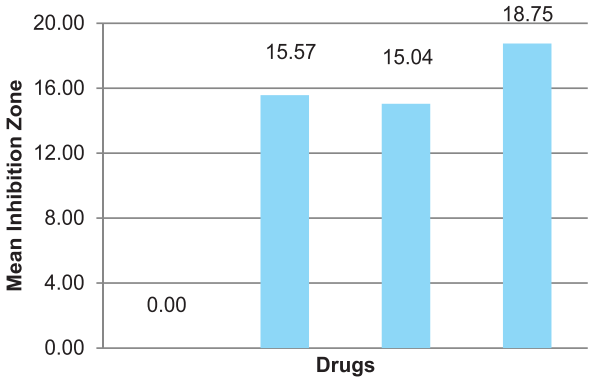


Figure-1. Mean inhibition zone of drugs Methicillin 10μg 0.00, Cefoxitin 30μg 15.57, Vancomycin 30μg 15.04 and Tabebuia impetiginosa shows 18.75

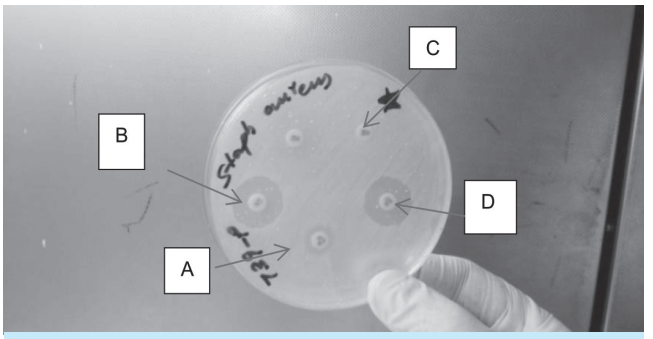


Fig 2: Antibiotic disc of cefoxitin 30μg(A) showing resistant zone of inhibition, Pseudomonas aeruginosa disc(B) showing susceptible zones of inhibition, methicillin 10μg disc (C) showing no inhibitory zone, vancomycin30μg disc (D) showing susceptible zone of inhibition.

S/N	Isolate no.	Plate no.	Methicillin (ME-10μg)	Cefoxitin (FOX-30μg)	Vancomycin (VA-30μg)	Tabebuia impetiginosa -340μg)
1	449-P	SA-1	0	12	16	18
		SA-2	0	12.5	16.5	18.5
		SA-3	0	12	16	18
		Mean±SD	0	12.2±0.3	16.2±0.3	18.2±0.3
2	739-P	SA-1	0	10	16	19
		SA-2	0	10.5	16	19
		SA-3	0	10	16	19.5
		Mean±SD	0	10.2±0.3	16.0±0.0	19.2±0.3
3	558-P	SA-1	0	22	16	20
		SA-2	0	22	16	20
		SA-3	0	22	16	20
		Mean±SD	0	22.0±0.0	16.0±0.0	20.0±0.0
4	551-P	SA-1	0	10	16	19
		SA-2	0	10.5	16.5	19.5
		SA-3	0	10	16	19
		Mean±SD	0	10.2±0.3	16.2±0.3	19.2±0.3

Table-V. Comparative study of Tabebuia impetiginosa extract with antibiotics against Pseudomonas aeruginosa. Zones of Inhibition (mm)

Legend: P stands for pus, 449,739, 558, 551 are patient result report numbers.

DISCUSSION

In the present study, 70 Pseudomonas aeruginosa isolates were obtained from Microbiology Laboratoryof Shaikh Zayed Hospital, Lahore. The laboratory obtained these organisms from wound specimens. This study was compared to the study that was conducted in Peshawar, Pakistan. 723 samples of Pseudomonas aeruginosa were collected by the researches in twelve months. 699 samples were obtained from pus while 16 samples were obtained from blood and urine.[98] High prevalence of Pseudomonas aeruginosa was found in wound infections, because it readily causes wound infection by penetrating through damaged skin.

Samples were collected from both males and females and all age group patients were included in the study. 41 samples from males and 29 samples from females (58.6% and 41.1% respectively) were collected. Qarshi Research International Labs and Kohat University of Science and Technology also performed this research in 2013 and similar results were obtained. In their results 57.79% prevalence of Pseudomonas aeruginosa were found in males and 48.21% was found in females i.e. more in males as compared to females. Similar results were found in the study conducted by University of Lahore i.e., more prevalence of Pseudomonas aeruginosa in males (58%) as compared to females (42%). It may because of the reason that males have more hairs on their body, more sweat and sebum secretions by the testosterone. The calculated mean age was 38.3 ± 18.5 years. 65.7% data was collected from patients between 40-77 years and 34% was collected from patients less than 40 years. It was evident from the study that elderly patients were more infected with Pseudomonas aeruginosa.

CONCLUSION

It was concluded that Tabebuia impetiginosa seed extract possessed activity against Pseudomonas aeruginosa with 340μg of minimum inhibitory concentration. It was found that 340μg concentration of Tabebuia impetiginosa seed extract shows greater therapeutic response against bacteria as compared to Vancomycin. Therefore Tabebuia impetiginosa extract may be used as an alternate of expensive antibacterial drugs as remedy for wound infections.

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Do what is right, not what is easy.

– Unknown –

AUTHORSHIP AND CONTRIBUTION DECLARATION

Sr. #	Author-s Full Name	Contribution to the paper	Author=s Signature
1	Yasir Mehmood	Theme, Methodology & Discussion.	
2	Muhammad Imran Ashraf	Methodology & Statistical analysis.	
3	Shazana Rana	Statistics analysis & Discussion.	
4	Humayun Riaz	Literature Review & Supervision.	
5	Syed Atif Raza	Literature Review & Supervision.	
6	Zia mohy-ud-din Khan	Literature Review & Supervision.	