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In vitro and *in vivo* efficacy of natural plant extracts against biofilm-forming clinical isolates of *Pseudomonas aeruginosa*

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ABSTRACT

Introduction: *Pseudomonas aeruginosa*, a gram-negative pathogen, is among the most persistent nosocomial pathogen. *Pseudomonas aeruginosa* over the years has become antibiotic resistant and difficult to treat. There is an imminent need to discover new and alternate agents that can effectively inhibit *P. aeruginosa* growth under physiological conditions. In this regard, natural sources offer a wide variety of antimicrobial agents that can be exploited for this purpose. In this study, we evaluated the anti-microbial and anti-biofilm activity of three natural compounds (*Camellia sinensis*, *Hippophae rhamnoides*, and *Juglans regia*) under *in vitro* and *in vivo* (burn mouse model) conditions.

Materials and methods: We screened *Pseudomonas aeruginosa* 60 clinical isolates for susceptibility/resistance to eight commonly used antibiotics (Cephalexin, Chloramphenicol, Gentamicin, Vancomycin, Erythromycin, Tetracycline, Ampicillin, and Ofloxacin). Subsequently, the biofilm formation ability of *Pseudomonas aeruginosa* clinical isolates was tested using microtiter plate assay. The *in vitro* antimicrobial activity of *Camellia sinensis*, *Hippophae rhamnoides*, and *Juglans regia* was tested using the agar well plate method, while *in vivo* activity was determined using the burn mouse model.

Results: The *Pseudomonas aeruginosa* clinical isolates (n=60) were found to be dominant biofilm formers and were resistant particularly against Ampicillin and Erythromycin, while susceptible to Gentamicin and Ofloxacin. The minimum-inhibitory concentration (MIC) of three natural compounds ranged from 0.5-1 mg/ml. Under *in vivo* conditions, *Juglans regia*, and *Hippophae rhamnoides* extracts were found effective in controlling the infection, with 0-4 CFU/ml from the organ homogenate obtained from the infected mice. These compounds also showed appreciable properties of healing burn wounds in mice.

Conclusions: The natural plant extracts used in the study revealed anti-microbial and anti-biofilm activity against *Pseudomonas aeruginosa* under *in vitro* and *in vivo* conditions, suggesting plants to be an excellent source for providing new and effective antimicrobial agents.

Keywords: Burn mouse model, *Pseudomonas aeruginosa*, biofilm, plant extracts

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INTRODUCTION

Biofilms are microbial networks comprising of at least one type of microorganisms in exceptionally organized surface-adherent structures. Studies published in the last three decades suggest that the bacteria in most settings reside in the biofilm mode of growth, whereas the planktonic form is regarded as a transition phase (1). The National Institutes of Health

(NIH) suggests that bacterial biofilms are responsible for over 80% of chronic diseases and about 65% of microbial infections (2). Among these microbes, *Pseudomonas aeruginosa* is viewed as the most threatening microorganism, capable of biofilm capable of forming biofilms on a wide variety of surfaces (3).

The biofilm matrix of *Pseudomonas aeruginosa* comprises principally of polysaccharides, extracellular DNA, lipids and proteins (4). The critical arsenal of *Pseudomonas aeruginosa* is the resilient biofilm, which can survive, and compete particularly in the polymicrobial environment in cystic lung fibrosis (5). *Pseudomonas aeruginosa* can colonize on various surfaces such as indwelling medical devices (urinary catheter, central venous catheters, intrauterine devices, etc.), and equipment in the food industry (tubing, vats, and mixing tanks). The quorum-sensing system of *Pseudomonas aeruginosa las* and *rhl*, regulates various virulence factors production (6). Biofilms act as significant barriers to antibiotic penetration, thus finding new drugs that can prevent biofilm formation or adhesion would be of considerable attention (7).

Due to increase antibiotic resistance and failure to treat biofilm-related infections, many researchers have focused on alternate agents, such as natural plant extracts and several plant-based biologically active pharmaceutical substances, for the inhibition of biofilm development and detachment of existing biofilms. Medicinal herbs have been utilized as traditional remedies for human ailments (including infections) for thousands of years. Dandasa (*Juglans regia*) is commonly used as a lip beautifying and teeth cleaning agent by women of Khyber Pakhtunkhwa province of Pakistan. Similarly, among many drinks used in Asian and Middle Eastern countries green tea (*Camellia sinensis)* is one of the most popular. Likewise, Sea buckthorn (*Hippophae rhamnoides*) is commonly used in northern areas of Pakistan as a food item. The non-toxicity and efficacy of these extracts against biofilm-forming pathogens make them a good candidate for controlling biofilm-related infections.

Burn mouse is an ideal model to study systemic and localized infections (8). In this study, we used in vitro and in vivo (burn mouse model) assays to determine the inhibitory activity of *Juglans regia, Camellia sinensis and Hippophae rhamnoides* against *Pseudomonas aeruginosa*.

METHODS

Sample collection

In this study, 60 previously collected *Pseudomonas aeruginosa* isolates, parts of Immunology and Infectious Diseases Research Laboratory (IIDRL), Department of Microbiology, University of Karachi, isolate bank was used.

Quantitative biofilm analysis

The microtiter-plate method was utilized to analyze the biofilm-forming potential of the *Pseudomonas aeruginosa* isolates (O'Toole, 1999). In brief, cultures of *Pseudomonas aeruginosa* and *Pseudomonas aeruginosa* ATCC 27857 (used as control) were inoculated in 5-ml of Tryptic soy broth (TSB) and cultures were allowed to attain a stationary phase. Cultures were diluted (1:100) in TSB. Diluted culture (100ul) was pipetted into each of two wells in a non-tissue culture treated fresh microtiter plate with the lid closed and incubated for 48 hours at 37 ºC. After incubation, the planktonic bacteria were removed by washing the wells with water. Subsequently, the wells were stained with 0.1% Crystal Violet (CV; 125ul) solution. Plates were incubated at room temperature for 10 minutes. The plates were washed with water, and 95% ethanol (200ul) was added to each stained well. Solubilization of dye was performed by covering plates and incubating for 10 min at room temperature. Subsequently, the content of each well was mixed by pipetting, and 125ul of the crystal violet/ethanol solution was shifted to a new flat-bottom 96-well plate (optically clear). The optical densities (OD) of each well were estimated at a wavelength 630 nm.

Antibiotic resistance/susceptibility profile

Pseudomonas aeruginosa isolates were tested for antibiotic resistance/sensitivity against eight commonly used antibiotics (Cephalexin, Chloramphenicol, Gentamicin, Vancomycin, Erythromycin, Tetracycline, Ampicillin, and Ofloxacin) using the Kirby-Bauer disk diffusion method. Isolates were grown in the Mueller Hilton Broth (MHB) at 37 ºC for 2 hours in a shaking water bath. Following incubation, cultures were diluted to match the McFarland index 0.5. The diluted cultures were plated on the Mueller Hilton Agar plates, and the bacterial lawn was prepared with the help of sterile cotton swabs. The antibiotic disks (commercially available) were carefully placed on the lawn and incubated at 37 ºC for 24 hours. The next day, resistance or sensitivity was assessed by measuring the zones of inhibition around each disk. Antibiotics giving zone of inhibition of 12 mm or more were considered susceptible.

Antimicrobial activity of the plant extracts

For the antimicrobial activity, 1 mg/ml aqueous stock solution of natural plant extracts (*Camellia sinensis, Juglans regia,* and *Hippophae rhamnoides*) were prepared. The antimicrobial activity of the extracts was measured using the agar well diffusion method. Briefly, the 0.5 McFarland index-matched bacterial cultures were plated on the Mueller Hilton Agar plates, and the bacterial lawn was prepared with the help of sterile cotton swabs. Three,

one cm holes were punched in the plates and each of the plant extracts was added to each hole. The plates were incubated at 37 ºC for 24 hours. The next day, the antimicrobial activity of the plant extract was assessed by measuring the zones of inhibition around each well. The minimum inhibitory concentration (MIC) of three plant aqueous extracts was determined using the micro broth dilution method in the 96-well microtiter plate (Aboaba, 2006). Briefly, 1 mg/ml of plant extracts (stock solution) were serially diluted (two-fold) in TSB broth (100ul) and the 0.5 McFarland index-matched culture (10ul) was added to each well. One well served as blank, whereas others served as control of the culture. Plates were incubated for twenty-four hours at 37ºC. The MIC was noted when the well display no noticeable growth.

***In vivo* activity of the plant extracts using the burn mouse model**

The virulence of various *Pseudomonas aeruginosa* mutants has been examined in the modified Burned-Mouse Model as described by Stieritz and Holder (Stieritz and Holder 1975). For our study, male, BALB/c mice weighing 25 g each were used. Mice were divided into two groups, with 3 mice in each group. Anesthetic ether was used to anesthetize the mice, and their abdominal cavity was shaved. The shaved skin was exposed to hot water (90 °C) for 10 seconds to induce thermal injury. This type of injury is non-fatal, however, causes a 3rd-degree burn of full-thickness. The first group of mice was subcutaneously injected with 100 ul of *Pseudomonas aeruginosa* culture directly below the burn, followed by injection of 500 ul (5%) of plant extracts of *Camellia sinensis, Hippophae rhamnoides*, and *Juglans regia*, while the second group of mice was subcutaneously injected with 100 ul of *Pseudomonas aeruginosa* culture only (and no extract) directly below the burn. Mice were comfortably placed for observation for 48 hours, after which the mortality rate of infected mice was recorded. After 48-hour, living mice were killed by cervical dislocation, and heart, liver, and spleen from all three groups were homogenized in sterile saline. Subsequently, 1 ml of suspension was cultured onto the blood agar. Wound swabs were also cultured on the blood agar. The plates were incubated at 37 °C for 24 hours and the next day plates were examined for growth and the colony forming units (CFU)/ml count was calculated.

RESULTS

Quantitative Biofilm Analysis

In the present work, the biofilm-forming potential was examined for all *Pseudomonas aeruginosa* isolates as well as for the *Pseudomonas aeruginosa* ATCC 27857 (used as control). The clinical *Pseudomonas aeruginosa* isolates were found to be dominant biofilm formers (OD_{630} >0.5-1) as compared with *Pseudomonas aeruginosa* ATCC 27857 isolate which exhibited an OD of 0.2 (Figure 1).

Antibiotic resistance/susceptibility profile of the *Pseudomonas aeruginosa* isolates

We tested the antibiotic resistance/susceptibility profile for all the clinical isolates of *Pseudomonas aeruginosa* against eight antibiotics (Ofloxacin OFX, Chloramphenicol C, Erythromycin E, Tetracycline TE, Gentamicin CN Chloramphenicol C, Ampicillin AMP, Cephalexin CL and Vancomycin V). Most of the isolates were resistant to at least one or more antibiotics. Most isolates were susceptible to Gentamicin and Ofloxacin, while resistant to Ampicillin and Erythromycin (Table 1).

Antimicrobial activity of the plant extracts against *Pseudomonas aeruginosa* isolates

The antimicrobial activity of *Camellia sinensis, Hippophae rhamnoides, and Juglans regia* aqueous extracts was evaluated using the agar well diffusion method. All three extracts were found to be quite effective against all the isolate of *Pseudomonas aeruginosa* (Table 2). MIC values of the plant extracts were tested and found to be in the range of 250-1000 ug/ml.

***In vivo* activity of the plant extracts using the burn mouse model**

The antimicrobial activity of aqueous extracts of *Juglans regia, Camellia sinensis, and Hippophae rhamnoides* was determined using the burn mouse model. In this analysis, *Juglans regia*, and *Hippophae rhamnoides* extracts were found to be most effective in controlling the *Pseudomonas aeruginosa* infection with 0-4 CFU/ml in organ homogenate obtained from the treated mice (Figure 2A). Interestingly, the treated mice also showed appreciable healing of the burn wounds (Figure 2B). Mice given no treatment died of infection within 24 hours, and CFU was too numerous to count.

DISCUSSION

In this study, we evaluated the biofilm forming potential as well antimicrobial susceptibility/resistance profile of *Pseudomonas aeruginosa* isolated from clinical samples. We subsequently evaluated the anti-microbial and anti-biofilm activity of three natural compounds (*Camellia sinensis, Hippophae rhamnoides,* and *Juglans regia*) under *in vitro* and *in vivo* (burn mouse model) conditions. Biofilm formation has been reported in many pathogenic and multi-drug resistant organisms such as *Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus,* and *Staphylococcus epidermidis.*

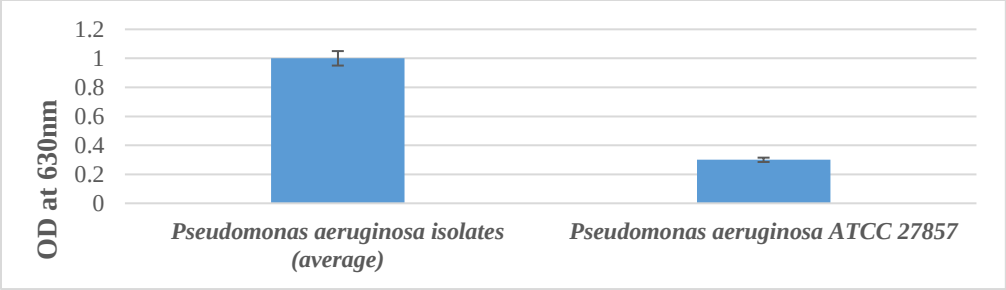

Figure 1: Quantitative biofilm analysis of *Pseudomonas aeruginosa* clinical isolates (n=60) as well as *Pseudomonas aeruginosa* ATCC 27857 strain. The biofilm-forming potential was determined using OD_{630} nm.

Table 1: Antibiotic susceptibility/resistance profile for *Pseudomonas aeruginosa* isolates against eight antibiotics. Key: V = Vancomycin, E = Erythromycin, TE = Tetracycline, C= Chloramphenicol, AMP = Ampicillin, OFX = Ofloxacin, CL = Cephalexin and CN = Gentamicin.

Percentage of isolates resistant or susceptible to antibiotics								
Percent resistant isolates								
	CL	CN	OFX	E	VA	TE	C	AMP
Pseudomonas aeruginosa isolates (n=60)	53	38	20	73	67	58	57	83
Pseudomonas aeruginosa - ATCC 27857	0	0	0	0	0	0	0	0
Percent susceptible isolates								
	CL	CN	OFX	E	VA	TE	C	AMP
Pseudomonas aeruginosa isolates (n=60)	47	62	80	27	33	42	43	17
Pseudomonas aeruginosa - ATCC 27857	100	100	100	100	100	100	100	100

Table 2: Zones of inhibition and minimum inhibitory concentration aqueous extracts of *Juglans regia*, *Camellia sinensis*, *and Hippophae rhamnoides.*

Culture	**An aqueous plant extract used**					
	Juglans regia		***Camellia sinensis***		***Hippophae rhamnoides***	
	Average zone of inhibition (mm)	**Average MIC (ug/ml)**	**Average zone of inhibition (mm)**	**Average MIC (ug/ml)**	**Average zone of inhibition (mm)**	**Average MIC (ug/ml)**
Pseudomonas aeruginosa (n=60)	17-20	500	18-20	1000	15-19	500

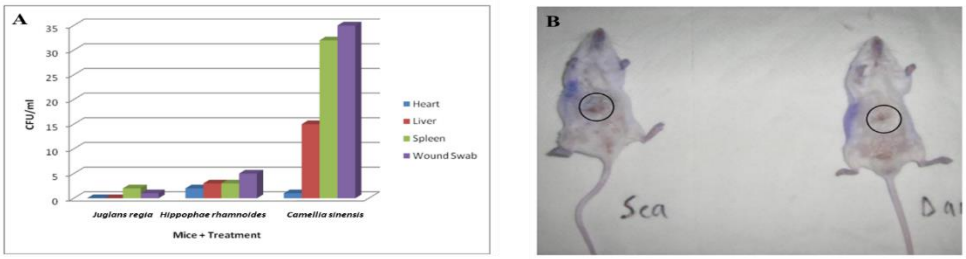

Figure 2: *In vivo* activity of aqueous extracts of *Juglans regia*, *Camellia sinensis*, and *Hippophae rhamnoides* against *Pseudomonas aeruginosa* in the burn mouse model. A)

Furthermore, emerging antimicrobial resistance in clinically significant pathogens, such as *Helicobacter pylori* (causative agent of gastroduodenal pathologies) and *Mycobacterium* species involved in serious medical conditions may also be attributed to their biofilm-forming potential and thus increasing the severity of the infection. In this study, we found out that almost all the clinical isolates of *Pseudomonas aeruginosa* were potent biofilm former which is in accordance with studies conducted by Hussain and Raad (9). We also found that most of the isolates were resistant against the generally used antibiotics particularly, Erythromycin and Ampicillin. This observation is also supported by other studies conducted in Pakistan, such as by Lubna et al, (2019) and Waheed et al (2016), who demonstrated resistance against multiple antibiotics in clinical isolates of *Pseudomonas aeruginosa* (10, 11). Infections instigated by multi-drug resistant *Pseudomonas aeruginosa* isolates are increasing frequently, primarily due to the injudicious use of antibiotics.

Increasing antibiotic resistance also warrants the need to search for antimicrobial agents from alternate sources, such as plants. In our study, we screened three aqueous plants extracts for their antimicrobial and antibiofilm potential. Our results revealed that extracts of *Juglans regia* (Dandasa), *Camellia sinensis* (Green Tea), and *Hippophae rhamnoides* (Sea Buckthorn) were quite effective in inhibiting the growth of biofilm-forming isolates of Pseudomonas aeruginosa, with MIC in the range of 500-1000 ug/ml. Our results are in agreement with previous studies that have also shown the efficacy of *Juglans regia* (12, 13), *Camellia sinensis* (14, 15), and *Hippophae rhamnoides* (16) against *Pseudomonas aeruginosa*.

In vivo drug interaction and its efficacy are important parameters, which should be evaluated prior to the use of a drug to treat infections in humans or animals. Animal models are effective and important tools to study *in vivo* as well as pre-clinical efficacy of antimicrobial agents. In this study, we developed a burn mouse model, which is an ideal model to study systemic and localized infections, as well as to test the efficacy of different antimicrobial agents (17). We found aqueous extracts of *Juglans regia* and *Hippophae rhamnoides* to be effective in not only inhibiting/controlling *Pseudomonas aeruginosa* infection but also showed healing of the burn wound. These results were in line with previous studies showing *in vivo* efficacy of plant extracts against clinically significant pathogens (18, 19). In summary, our findings support the role of plant-derived antimicrobial agents as effective alternatives to curb the emerging antibiotic resistance.

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References:

1. Tolker-Nielsen T. Biofilm development. Microbiology spectrum. 2015;3(2):3.2. 21.
2. Muhsin J, Wisal A, Saadia A, Fazal J, Muhammad I, Muhammad A, et al. Bacterial biofilm and associated infections. Journal of the Chinese Medical Association. 2018;81(1):7-11.
3. Das MC, Sandhu P, Gupta P, Rudrapaul P, De UC, Tribedi P, et al. Attenuation of Pseudomonas aeruginosa biofilm formation by Vitexin: A combinatorial study with azithromycin and gentamicin. Scientific reports. 2016;6(1):1-13.
4. Strempel N, Neidig A, Nusser M, Geffers R, Vieillard J, Lesouhaitier O, et al. Human host defense peptide LL-37 stimulates virulence factor production and adaptive resistance in Pseudomonas aeruginosa. PloS one. 2013;8(12):e82240.
5. Oluyombo O, Penfold CN, Diggle SP. Competition in biofilms between cystic fibrosis isolates of Pseudomonas aeruginosa is shaped by R-pyocins. MBio. 2019;10(1):e01828-18.
6. Swetha TK, Pandian SK. Role of Bacteria in Dermatological Infections. Pocket Guide to Bacterial Infections. 2019:279.
7. Murugan K, Selvanayaki K, Al-Sohaibani S. Antibiofilm activity of Andrographis paniculata against cystic fibrosis clinical isolate Pseudomonas aeruginosa. World Journal of Microbiology and Biotechnology. 2011;27(7):1661-8.
8. Brandenburg KS, Weaver AJ, Karna SR, You T, Chen P, Van Stryk S, et al. Formation of Pseudomonas aeruginosa biofilms in full-thickness scald burn wounds in rats. Scientific reports. 2019;9(1):1-12.
9. Al-Obaidi RD, Al-Dahmoshi H. Biofilm and antibiotic resistance profile among Pseudomonas aeruginosa isolated from clinical samples. Eurasia J Biosci. 2020;14(1):1135-9.
10. Ullah W, Qasim M, Rahman H, Bari F, Khan S, Rehman ZU, et al. Multi drug resistant Pseudomonas aeruginosa: Pathogen burden and associated antibiogram in a tertiary care hospital of Pakistan. Microbial pathogenesis. 2016;97:209-12.
11. Farooq L, Memon Z, Ismail MO, Sadiq S. Frequency and antibiogram of multi-drug resistant pseudomonas aeruginosa in a Tertiary Care Hospital of Pakistan. Pakistan journal of medical sciences. 2019;35(6):1622.
12. Khan I, Khan U, Khan K, Nawaz M, Khan N, Ali F. In vitro anti-pseudomonal potential of Juglans regia and Otostegia limbata leaves extract against

planktonic and biofilm form of Pseudomonas aeruginosa. Pak J Bot. 2018;50(2):827-33.
13. Dolatabadi S, Moghadam HN, Mahdavi-Ourtakand M. Evaluating the anti-biofilm and antibacterial effects of Juglans regia L. extracts against clinical isolates of Pseudomonas aeruginosa. Microbial pathogenesis. 2018;118:285-9.
14. Flayyih MT, Yousif HS, Subhi IM. Antimicrobial effects of black tea (Camellia sinensis) on Pseudomonas aeruginosa isolated from eye infection. Iraqi Journal of Science. 2013;54(2.2013):255-65.
15. Kumar A, Kumar A, Thakur P, Patil S, Payal C, Kumar A, et al. Antibacterial activity of green tea (Camellia sinensis) extracts against various bacteria isolated from environmental sources. Recent Research in Science and Technology. 2012;4(1):19-23.
16. Jamehdor S, Zarabi M, Mehrnejad F. In vitro Evaluation of antibacterial efficacy of aqueous extracts of Iranian Native Plants on the Standard Strains of Pseudomonas aeruginosa. Iranian Journal of Medical Microbiology. 2014;8(2):51-4.
17. Calum H, Høiby N, Moser C. Burn mouse models. Pseudomonas methods and protocols: Springer; 2014. p. 793-802.
18. DeLeon K, Balldin F, Watters C, Hamood A, Griswold J, Sreedharan S, et al. Gallium maltolate treatment eradicates Pseudomonas aeruginosa infection in thermally injured mice. Antimicrobial agents and chemotherapy. 2009;53(4):1331-7.
19. Montie T, Doyle-Huntzinger D, Craven R, Holder I. Loss of virulence associated with absence of flagellum in an isogenic mutant of Pseudomonas aeruginosa in the burned-mouse model. Infection and immunity. 1982;38(3):1296-8.