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Microbial Status and Resistance Profile of Bacterial Species Isolated from Recreational and Agricultural Sites in Multan

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Abstract

Background: Humans are in continuous contact with recreational areas and agricultural fields. However, recent discovery of resistant bacteria in these sites have posed serious threats to the community.

Methods: This study is designed to investigate prevalence of resistant bacteria in agricultural and recreational areas i.e., parks and rivers, to investigate their resistant profiling against antibiotics and metals by culture dependent agar techniques and to investigate effect of local medicinal plants on isolated resistant bacteria.

Results: A total of 106 isolates from 35 water and sediments samples were isolated. A resistant rate of 46.05% was reported against antibiotics; the highest against ampicillin (75%) and the lowest against streptomycin (8.9%). Identified resistant strains were *Bacillus megaterium*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *E. coli*, *Micrococcus luteus*, *Staphylococcus* spp., *Pseudomonas* spp., *Streptococcus* spp. and *Staphylococcus aureus*. Co-occurrence of metal resistance with antimicrobial resistance was also reported. Ethanolic leaves extracts showed promising results against isolated resistant bacteria. The highest and lowest zone of inhibition was reported in *Syzygium cumini* (37mm) and *Ziziphus mauritiana* (10mm).

Conclusion: The finding of this study has demonstrated presence of resistant bacteria in publicly accessed areas that support various needs. The potential of *Syzygium cumini, Ficus benghalensis* and *Psidium guajava* against resistant bacteria in combination can be explored as an alternative green method against resistant bacteria.

Introduction

Resistance to antibiotics is a leading dilemma. With every passing day the number of resistant microorganisms is increasing, thus original medication is becoming ineffective in an affected host body [1]. These microorganisms may shortly become untreatable and hazardous due to development of antibiotic resistance and/or and multidrug resistance. Till 2050, it is expected that deaths due to resistant microorganisms will be higher than others [2].

Seventy-one nations observed a 36% expansion in antibiotic utilization between 2000 to 2010. Similarly, a rise of 67% in the worldwide utilization of antibiotics from the year 2010 to 2030 is expected [2]. Uncontrolled utilization of antimicrobial agents is the burning cause of these resistant strains of bacteria [3]. Cross-resistance is the term used to describe the co-occurrence of metal resistance as well as antibiotic resistance in strains of bacteria. Microorganisms exposed to areas rich in metals typically exhibit resistance to such metals [4].

In underdeveloped nations, most people are unable to buy medicine. Tropical medicinal plants are an important source of bioactive agents. The presence of a variety of bioactive compounds makes them a suitable candidate as a natural remedy. Antioxidants found in plants have extraordinary applications in the prevention and treatment of wellbeing-related problems brought by these free radicals [5]. The developing pathogenic antibiotic-resistant microorganisms inspire the use of therapeutic plants for the discovery of new bioactive substances [6]. Therefore, this is the need of the hour that higher plants be investigated and screened for bioactive phytocompound as an effective and affordable natural cure [7].

Recreational areas and irrigational fields are public sites closer to human population, people can severely be affected by Multi Drug Resistant (MDR) bacteria present in these sites. The prevalence of these isolates established in the city waterways indicate to their potential spread to the environment and the aquatic ecosystem through waste disposal. The present study assessed the antibiotic residues in the sediment and rivers and discovered a widespread dispersion of antibiotics. Significant bacterial biodiversity was present across all sediment locations, and the community composition of multi resistant bacterial varied by site, particularly high diversity being identified in high-pollution areas [8]. Therefore, this study's goal was to look at the possibility of multidrug-resistant (MDR) bacteria occurrence in irrigational and public places and investigation of the effect of different medicinal plants on isolated MDR bacteria. The goals of the current study were: (1) to ascertain the presence as well as the level of resistant bacteria in recreational and agricultural sites in Multan; (2) to determine the incidence of metal resistance along multi-drug resistance in bacteria; and (3) to explore the impact of medicinal plants on resistant bacteria.

Methods

Sample Collection

Sites of sampling were chosen based on accessibility and types of land use (agricultural and recreational). These sites included parks, lakes, and Chenab River in Multan, Pakistan by dip sampling technique (US EPA 1994). These sites were popular destinations that remain open around the year for the general public and no endangered or protected species were involved. No specific permission for sampling was required at any site. Briefly for water sampling, a clean sterilized labelled bottle with a rope tied around its neck was dipped into the surface water. For sediment sampling, with the help of a sterilized spatula, samples were collected in a plastic zip bag aseptically. Samples were transported to the lab aseptically within 12hours of collection under cold conditions. Physiological parameters of sampling sites i.e., temperature, pH, and texture were noted. Additionally, sampling dates coincide with times of high recreational use. A minimum quantity of 25ml / 10g sample was collected.

Bacterial Isolation

Samples were serially diluted and 100µl from 10^{-0}, 10^{-3}, $10^{-5,}$ and 10^{-7} was spread on the nutrient agar and let to grow for 24 hours at 37 degrees. Following incubation, bacterial colonies were selected based on their morphological characters i.e., margins, color, pigments, elevation, consistency, shape, and size of colonies. By the means of streak plate approach, single well-isolated colonies were sub-cultured 3 times under aseptic conditions on the nutrient agar and then incubated at 37 °C for 24 hours. Preliminary Gram staining was performed to categorize strains as Gram-negative and Gram-positive bacteria as well as to observe their shape, size, and arrangements. They were further identified biochemically and confirmed by API strips.

Antibiotic Resistance Profiling

Preparation of Inoculums

Muller Hinton broth was inoculated with isolated bacterial strains and under moderate shaking (150 rpm) conditions were incubated for 24 hours at 37°C. The bacterial culture was adjusted to 0.5 McFarland's standard [9].

Determination of Antibacterial Activity

The disc diffusion technique was used to assess the antibacterial susceptibility using Muller Hinton (MH) agar against a panel of 9 antibiotics namely Ampicillin

(10µg), Streptomycin (10µg), Cefuroxime (30µg), Ceftazidime (30µg), Amoxicillin (15µg), Erythromycin (15µg), Vancomycin (10µg), Gentamicin (10µg) and Tetracycline (30µg) (Bauer et al. 1966). The bacterial lawn was prepared using 0.5 McFarland's standard adjusted broth. Commercial antibiotic discs were placed on agar plates in an aseptic manner and incubated for 24 hours at 37°C. The inhibition zone (mm) was calculated following 24 hours. According to the Clinical and Laboratory Standards Institute's criteria, bacterial isolates were categorized as susceptible, intermediate, or resistant. (CLSI 2021). Only resistant strains were biochemically characterized following Bergey's testing scheme and further confirmed by API-20 strips [9-10].

Metal Resistant Profiling

The heavy metals employed to test the isolates' various resistivities to those other heavy metals were namely mercury chloride, chromium chloride, arsenic chloride, and molybdenum oxide of range 250µg/100ml to 1500µg/100ml. 10 percent of stock solutions were made of distilled autoclaved water and all these metal stock solutions were kept at 4°C. At various concentrations, the resistance of MDR bacteria to the tested metals was examined. The desired quantity of stock solution was added to the L-agar media under sterile circumstances. Using heavy metal treated plates, MDR bacteria were inoculated and then incubated at 37 degrees for a period of 24 to 48 hours. Negative or positive results were documented for each test. The presence of growth indicates positive results.

Activity of Plant Extracts against Resistant isolates

Plant Collection, Extract Preparation and Phytochemical Screening

Locally available medicinal plant leaves of *Ficus carica*, *Ziziphus mauritiana*, *Ficus benghalensis*, *Psidium guajava*, *Morus alba*, *Moringa oleifera* and *Syzygium cumini* were collected from different areas of Multan (Figure 1). The leaves were appropriately washed with sterilized water to eliminate dirt and then air-dried for approximately seven days under ambient conditions. The plant material (10g) was extracted in 100ml of ethanol (50%) at room temperature for 72 hours at 120 rpm. Extracts were filtered and concentrated to one-fourth volume from the original. Extracts were stored under cold conditions for future use [11-12]. Preliminary phytochemical screening was analyzed in plant extracts using already established protocols i.e., carbohydrates, alkaloids, phenolic compounds, saponins, reducing sugars, phytosterols, tannins, cardiac glycosides, terpenoids, proteins, and amino acids were analyzed in plant extracts using already established protocols [13].

Antibacterial Activity of Plant Extracts

The antibacterial activity of plants against isolated resistant bacteria was tested by agar well diffusion assay [14]. Bacterial lawn was prepared by 0.5 McFarland's standard adjusted broth. Briefly, wells (6-8mm diameter) were made by using a sterilized cork borer and 75µl plant extract was introduced into wells and incubated for 24 hours at 37°C. Presence of zone around well showed that plant extract is effective against tested resistant isolates. Maximum the inhibition zone, maximum will be the activity of plants. For control, extraction solvents i.e., ethanol was used. All experiments were run in triplicates.

Results

Bacterial Isolation and Characterization

Total 35 samples (20 water and 15 wet soil samples) were taken from river Chenab, recreational parks and agricultural fields in Multan, Pakistan. The average temperature and pH of water samples were30.4 ^{0}C, 8.5, and of wet soil was 32.4 ^{0}C, 8.0, respectively (Table 1). The pH of both types of samples lied in alkaline range. A total of 106 bacterial isolates of distinct morphological characteristics were isolated. These isolates were *Bacillus megaterium, Bacillus subtilis*, *Micrococcus luteus*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Isolates were identified by analytical profile index (API). Out of 106 strains, 35 strains (46%) were resistant bacteria of which 17(48.6%) were gram-positive and 18(51.4%) were gram-negative.

Antimicrobial Susceptibility of Bacterial Isolates

Analysis of the antimicrobial susceptibility of the 106 isolates revealed that resistance to ampicillin (75%), amoxicillin (68%), ceftazidime (67%), vancomycin (35%) and cefuroxime (33%) was most frequent. Furthermore, resistance to erythromycin (14%) and streptomycin (8.5%) was also observed, although at slightly lower levels (Table 2) resistance to multiple antibiotics was more commonly present in samples. It has been observed that microbial load in water bodies is significantly linked with seasonal variations. In heavy water flow, the bacterial load was comparatively low as compared to static low water flow. Moreover, it has been observed that MDR bacteria were observed more commonly in agricultural land sample than in river or recreational samples.

Metal Resistant Assay

The linear trend of metal resistance was observed; with increasing metal concentration low resistance was present. Bacterial isolates were able to resist metals at lower concentrations in vitro. However, with increasing

Type of Sample (n)	Average Temperature (range)	Average pH (range)	CFU log10 (range)
Water (20)	30.4ºC (24ºC-36ºC)	8.8 (7.9 to 10.7)	3.59 (3.42-3.80)
Wet Soil (15)	32.4ºC (25ºC-37ºC)	9.0 (7.6 to 9.9)	3.55 (3.19-3.84)

Table 1: Sample Characteristics including Average Temperature and pH of Samples and CFU obtained from these samples.

Species	Total Strains	Percentage inhibition (%)								
		S	E	VA	AMP	CN	CAZ	CXM	AML	TE
Streptococcus spp.	05	100	100	100	100	100	100	100	100	100
Staphylococcus spp.	10	0	40	0	80	0	80	40	0	20
Bacillus megaterium	09	22	0	54	78	0	88	78	54	0
Micrococcus luteus	05	0	0	0	100	0	0	100	0	100
Escherichia coli	08	0	0	0	0	100	100	0	0	0
Bacillus subtilis	10	0	0	100	0	0	100	0	100	0
Staphylococcus aureus	08	0	50	0	100	0	100	100	100	100
Pseudomonas spp.	13	0	0	54	100	0	100	0	100	0
Gram negative cocci	27	15	37	54	75	07	22	31	67	45
Klebsiella pneumoniae	11	0	0	0	100	55	100	46	100	0
Total	106	8.5	14	35	75	20	67	33	68	30

[1] S: Streptomycin, E: Erythromycin, VA: Vancomycin, AMP: Ampicillin, CN: Gentamicin, CAZ: Ceftazidime, CXM: Cefuroxime, AML: Amoxicillin, TE: Tetracycline.

Table 2: Percentage Antimicrobial Susceptibility of Bacterial Isolates against Different Antibiotics

Plant	Phytochemicals									
	AL	CAR	RS	S	PHY	P	CG	F	TER	PA
ZM	-	-	-	+	-	+	+	+	+	-
FC	-	-	-	-	-	+	-	+	+	-
FB	+	-	+	+	-	+	-	-	+	-
PG	+	-	+	+	+	+	+	-	+	+
SC	+	+	+	+	+	+	-	-	+	+
MA	-	-	+	-	-	+	-	+	+	-
MO	-	+	+	+	+	+	-	-	+	-

[1]**Plants** ZM: *Ziziphus mauritiana*, FC: *Ficus carica*, FB: *Ficus benghalensis*, PG: *Psidium guajava*, SC: *Syzygium cumini*, MA: *Morus alba*, MO: *Moringa oleifera;* **Phytochemicals** AL: Alkaloids, CAR: Carbohydrate, RS: Reducing Sugar, S: Saponins, PHY: Phytosterols, P: Phenol, CG: Cardiac Glycosides, F: Flavonoids, TER: Terpenoids, PA: Proteins and amino acids.

Table 3: Qualitative Phytochemical Screening of *Ziziphus mauritiana*, *Ficus carica*, *Ficus benghalensis*, *Psidium guajava*, *Syzygium cumini*, *Morus alba*, and *Moringa oleifera* Plant Extracts

concentrations, all bacterial isolates displayed resistance against mercuric chloride and zinc sulfate whereas, all strains were found sensitive to increasing concentrations of ferric chloride. Only four isolates were resistant to chromium chloride. *Staphylococcus aureus* exhibited growth on all concentrations of tested metal salts except Ferric chloride (3000µg/ml). The trend of metal resistance among the isolated bacterial strains was Ferric Chloride> Chromium Trichloride>Cobalt Chloride>Mercuric Chloride and Zinc Sulphate from least to most resistant.

Qualitative Phytochemical Screening

Preliminary screening had shown the presence of a variety of phytochemical substances, including phytosterols, alkaloids, proteins, amino acids, carbohydrates, terpenoids, tannins, saponins, phenols, flavonoids, cardiac glycosides, and reducing sugars while phenol and terpenoids compounds were present in all plant extracts (Table 3).

Antibacterial Activity of Plant Extracts

Antibacterial activity of the plant extracts was performed and it has shown that highest antibacterial activity was observed with the extracts of *Syzygium cumini* (37mm) whereas, *Ziziphus mauritiana* (10mm) showed the least activity (Figure 1). On average, *Psidium guajava* and *Syzygium cumini* were highly effective against most of the MDR strains and *Morus alba* and *Moringa oleifera* were least effective in exhibiting antibacterial activity. Against *Streptococcus* spp. all the plant extracts displayed antibacterial activity except *Ziziphus mauritiana*. The trend of plant extract antibacterial activity was *Morus alba* and *Moringa oleifera*>*Ficus carica* and *Ziziphus mauritiana*>*Ficus benghalensis*>*Psidium guajava*>*Syzygium cumini* from least to most effective antibiotic.

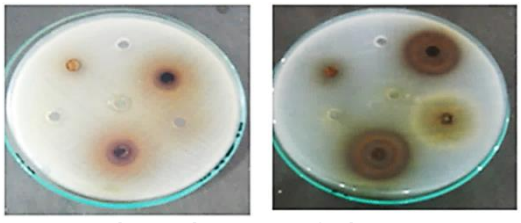

Figure 1: Antibacterial Activity of Plant Extracts against Multidrug Resistant Strains of Bacteria by Well Diffusion Method.

Discussion

Rapid industrialization and urbanization with ever-increasing antibiotic resistance have posed significant

harm to the world and its inhabitants in the twenty-first century. It is generally considered that MDR isonly present in hospital areas however this research had shown that MDRs are even present in public places including parks, rivers, and irrigational areas [14, 15]. In the present study multidrug and metal-resistant bacteria were isolated from the irrigational and recreational lands which pose threat to humans as well as to other living that live in these habitats. The alkaline nature of soil samples decreases metal mobility and increases their retention in soil [12, 16]. As these sites are closer to the human population and people can severely be affected bythese resistant bacteria like *Staphylococcus* species, *Bacillus megaterium*, *Pseudomonas* species, *Staphylococcus aureus*, *Klebsiella pneumoniae, Micrococcus luteus*, *E. coli, Bacillus subtilis,*and *Streptococcus* species. One study revealed presence of *Aeromonas*, *Stenotrophomonas*, *Comamonas*, *Enterobacter*, *Staphylococcus* and *Bordetella* species in irrigational fields of rice [14]. Moreover, inthe present study resistance to ampicillin (45.8%), streptomycin (33.9%), chloramphenicol (31.6%), and nalidixic acid (23.2%) were also reported in bacterial isolates from recreational sites [17].

In current study, highest antibacterial activity was observed with the extracts of *Syzygiumcumini*. On average, *Psidium guajava* and *Syzygiumcumini* were highly effective against most of the MDR strains and *Morus alba* and *Moringa oleifera* were least effective in exhibiting antibacterial activity.Guillén-Román et al. [17] study discovered that the extract of leaves of *Moringa oleifera* exhibited antibacterial action against all bacteria displaying the most noteworthy inhibition zone against *S. epidermidis* and *S. aureus* and least diameter of zone against *K. pneumonia.* While in the present study,*Moringa oleifera* extract was only effective in inhibiting *Staphylococcus* spp[6]. The results of Al Ghasham et al. [18] antimicrobial activities of the methanolic extract of *Ziziphus mauritiana* leaves have revealed that the plant extract has a significant level of antimicrobial activities against bacteria *Bacillus cereus, Streptococcus pneumonia,*and *Staphylococcus aureus.* The results of the present study showed *Ziziphus mauritiana* extract effective against Gram-negative bacteria, *Bacillus megaterium*, *Pseudomonas sp.* and *Klebsiella pneumoniae* [19]. Asghar et al. [19] revealed that leaf extracts of the medicinal plant *Syzygiumcumini*displayed antibacterial activity against the vancomycin-resistance and methicillin-resistance *Staphylococcus aureus* while the present study also showed inhibition against *Streptococcus* sp., gram-negative *cocci, Staphylococcus* sp., *Bacillus megaterium, Micrococcus luteus, Staphylococcus aureus, Pseudomonas* sp.,and *Klebsiella pneumonia.* Because of the rise in resistance to antibiotics, there is an urgent need to create new and creative antimicrobial agents.

A study conducted by Bagade et al., [20] revealed that *Bacillus firmus* showed growth in presence of metal arseniteAs(III) fixation and this bacteria did 100% oxidation process of arsenite As(III) having a concentration of 5mM in a brief time of 9 hours while in 24 hours, *Bacillus* oxidized total concentration of 20mM of this metal [21]. The phenomena of co-selection mechanism (co-resistance and cross-resistance) is a progressing threat to human well-being owing to the increase in metal-compelled several antibiotic-resistant pathogens [16, 22]. According to Zhang et al., [22] (2020), bacterial resistance to antibiotic vancomycin (Van), cephalosporin (Cep), rifampin (Rif), chloramphenicol (Chl), erythromycin (Ery) and tetracycline (Tet) increased up to approximately 1.5 times after exposure to metal salts [21].

Nowadays, the dire problem of antibiotic usage frequently and for the wrong reasons is causing multi-drug resistant bacteria phenomena globally. Alternatively, using natural products such as plant extractscan help reduce this problem. Not only will these plant extracts diminish these infections inflicted by MDR bacteria but also will lessen the rate of intake of antibiotics [23].

Our work emphasizes the necessity to find out the occurrence of the co-resistance of MDR resistance as well as metal resistant bacteria in agricultural and recreational areas i.e., parks and rivers as well as to characterize them and shows the immense potential of the polluted urban waterways as sites of emergence and methods of transmission of these resistant isolates. Plant extracts under study, *Ficus benghalensis* and especially *Syzygiumcumini* and *Psidium guajava* exhibited antibacterial activities against many multi-drug and metal-resistant strains and these natural products should be explored to control the spread of resistant bacteria. Further research should be done on naturally occurring plant extracts rather than relying on synthetically made antibiotic drugs that result in detrimental side effects.

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Competing Interest

The authors declare that there is no conflict of interest.

Author Contributions

HY designed the study, supervised the experiments and finalized the draft, RS carried out all experimental work and wrote first draft of paper, IM assisted in manuscript write up.

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