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Original Research Article

Identification of Antibiotic-Producing Bacteria against Selected MDR pathogens from Pharmaceutical Waste Soil

Malik Abdul Wahab[1], Syeda Asma Bano[*1], Abdul Tawab[2], Akrama[3], Sadia Alam[1], Sana Munir[1]

[1]*Department of Microbiology, the University of Haripur, Khyber Pakhtunkhwa, Pakistan*
[2]*Department of Agronomy, the University of Haripur, Khyber Pakhtunkhwa, Pakistan*
[3]*Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan*
**Corresponding author`s email address:* asma_baano@yahoo.com

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Abstract: *Antibiotics or antimicrobial agents are the most significant utilized secondary metabolites, which are commonly synthesized by soil bacteria and fungi and found to be effective. Most of the antibiotics used are derived from the soil bacteria and Actinomycetes. Antibiotic resistance poses a serious global health threat, making infections harder to treat, potentially leading to longer illnesses, more complications, increased healthcare costs, and even death. The present research project has been designed to identify and characterize the antibiotic-producing bacteria from the waste soil samples collected from ten different Pharmaceutical Industrial waste soils of Hattar, Haripur, using standard microbiological techniques.* ***Objective:*** *To isolate, identify, and characterize antibiotic-producing bacterial strains from pharmaceutical industrial waste soils, and to evaluate their antibacterial activity against selected MDR pathogens.* ***Methods:*** *Overall, 10 bacterial strains were isolated from waste soil of Pharmaceutical industries to examine their antibacterial activity against four tested pathogens, such as Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, and Staphylococcus aureus, by using the well diffusion method. Only four bacterial strains out of ten showed high antibacterial activity against these selected MDR pathogens. The isolated bacterial strains were initially identified through Gram staining and biochemical tests. For molecular identification, the MALDI TOF technique was used.* ***Results:*** *The isolated strains S1 and S6 were Enterobacter cloacae, S2 was Enterobacter asburiae, and S8 was identified as Pseudomonas aeruginosa. Then the crude extracts of S1, S2, S6, and S8 were further analyzed by GC-MS to identify the compounds present, in which almost 300 compounds were identified, and out of 300 compounds, some of them (D-Glycero-D-Gulo-Heptonic Acid, D-(+)-Ribonic Acid.Gamma.-Lac, Oxalic Acid, Ethyl Neopentyl E, D-Glycero-D-Gulo-Heptonic Acid, Hentriacontane, Neopentyl Glycol, Strychane, 1-Acetyl-20.Alpha, 3,4-Altrosan, Carbamic Acid, Hydroxy-, Ethyl, etc) were found in previous literature showing anti-microbial, anti-bacterial, anti-fungal, anti-inflammatory, anti-tumor, and anti-cancerous activities. Some new compounds were also detected. Further research of the identified compounds may detect some more effective antibacterial agents against selected MDR pathogens (Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, and Staphylococcus aureus).* ***Conclusion:*** *Bacteria present in waste soil samples of Pharmaceutical industries have the potential to show antibacterial activity against selected MDR pathogens (Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, and Staphylococcus aureus), and biologically active compounds having antibacterial properties may be extracted from these bacterial isolates.*

Keywords: Antibiotic Producing Bacteria, Antibacterial Activities, GC-MS Analysis, Submerged Fermentation, Well Diffusion Method, Biochemical Tests

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Introduction

Antibiotics are widely used for the treatment of bacterial diseases in humans, as well as in non-medical applications. Antibiotics have been highly beneficial for treating some bacterial, fungal, helminthic, inflammatory, and immunosuppressant diseases in humans (1). The total production of antibiotics found worldwide is more than one million tons per annum. Due to continuing research in this field, over 5,500 antibiotics have been discovered (1). Among the most significant commercially available and commonly utilized secondary metabolites are antibiotics, which are widely manufactured by microorganisms (bacteria and fungi) in the soil and have been demonstrated to be potent and broad-spectrum. Microorganisms produce metabolic products that act as antimicrobial agents (2). A large proportion of antibiotic classes in use are derived from microbes. More research is needed to develop new antimicrobial treatments with distinctive compositions that are reliable, toxicity-free, and cheaper against microbial illnesses (2).

Antibiotics are synthesized internationally between 100 and 200 thousand tons annually, with over a billion tons produced since 1940. Despite a persistent search for new effective antibacterial medications, nearly 100 years before Fleming discovered penicillin as a life-saving treatment in 1928, humanity is once again plagued with a powerful tool for combating infections (3). Natural soil provides the best environment for these microorganisms to produce bioactive compounds, which are used in defense and survival mechanisms against infectious diseases caused by bacteria. Most antibiotics administered today are identified by screening soil-isolated bacteria for antibacterial properties (4). Researchers previously isolated the antibiotic-producing bacteria from municipal solid waste dumpsite soils (5). Antibiotic resistance and hospital-acquired diseases can result from chronic antibiotic exposure. Soil is found to be an important source of antimicrobial-producing bacteria. Soil is a very heterogeneous habitat and is rich in diverse microorganisms. There is also a high variation in biotic and abiotic conditions in soils that challenge the microorganisms.

The present research project aimed to detect antibiotic-producing bacteria from waste soil samples from ten different pharmaceutical industries in Hattar. The antibacterial activities of isolated bacterial strains were tested against different multidrug-resistant pathogenic bacteria such as Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, and Bacillus subtilis through the Agar Well Plate Method. After that, bioactive compounds produced by the isolated bacterial strains were identified through Gas Chromatography-Mass Spectrometry (GC-MS).

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) mass spectrometry has emerged as a pivotal tool in the molecular

characterization and identification of bacterial strains. This sophisticated technique offers rapid, accurate, cost-effective identification of isolated bacterial strains. Its application in identifying antibiotic-producing bacteria holds a significant role for advancing understanding of microbial diversity and potential antimicrobial sources (7).

Multi-drug-resistant pathogens have resulted in an increased number of diseases, so there is a need to isolate and identify bacteria producing antibacterial compounds from the soil environment of Pharmaceutical waste soil areas and screen them against MDR strains of selected pathogens. The GC-MS analysis of crude extracts obtained from those isolates could identify the promising compounds responsible for antibacterial activities against the selected MDR pathogens (*Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, and Staphylococcus aureus*).

Methodology

Sample collection and preparation of bacteria

Waste soil samples were collected from 10 different Pharmaceutical industries of Hattar to isolate bacteria that produce antibiotics. With the help of a sterile spatula, samples were taken from the waste soil at a 5–10 cm depth in sterile polythene zip-lock bags, then kept in the refrigerator at 4°C.

Isolation and Identification of Antibiotic-Producing Bacteria

The serial dilution process was performed in a laminar flow hood. Five sterile test tubes were collected, marked, and labeled for each sample. 1g of dried soil sample is then dissolved in 10 mL of distilled water to create a suspension, which is then vortexed in the first test tube. Then, this stock solution was serially diluted up to 10^{-5}. 0.1 mL of the 10- 4 dilution sample was incubated on the sterile nutrient agar at 37 °C for 24 hours. Sub-culturing was done by selecting bacterial colonies with clear boundaries using a sterile loop to isolate pure cultures of bacterial strains. These colonies were then streaked on freshly prepared nutrient agar plates using the streak plate method and cultured at 37°C for 24 hours. Standard preservation techniques were used to preserve these pure colonies.

Microscopic and Bacterial Characterization of Selected Bacteria

After bacterial strains were isolated on Nutrient agar plates, they were identified and characterized using Bergey's manual as a reference and morphological, cultural, and biochemical assays (8). The isolated strains were examined on a microscopic and biochemical level. The Gram staining technique was used for microscopic identification. According to Bergey's manual, biochemical assays, such as catalase, citrate, oxidase, urease, indole, etc., have been utilized to identify the bacterial isolates.

Primary Screening

Muller-Hinton agar (MHA) was used to perform the well diffusion method to primarily screen antibiotic-producing bacterial isolates. The test pathogens were swabbed over the MHA surface, and bored wells of 6 mm diameter were made using sterile well borers. Then 100µlof 24hrs incubated isolated bacterial strains in Nutrient broth were poured in each well. These plates were left to settle in an incubator at 37°C without inverting for 24 hours (9). Then, after 24 hours of incubation of the isolated bacterial strains and test pathogens, the antibacterial activity was checked.

Extraction of metabolites

Submerged Fermentation: Based on the results from primary screening, isolates that exhibited inhibitory activity towards test pathogens were assigned for submerged fermentation. The shake flask method was employed for submerged fermentation (10). Isolated bacterial strains were inoculated in Nutrient broth and kept in a shaker incubator for 7 days at 28°C at 150 rpm. *Separation of crude extract*: After fermentation, the broth was centrifuged at 4000 rpm for 30 minutes, and the pellet was discarded. The supernatant was filtered. The pH of the supernatant was adjusted to neutral and mixed with double the amount of Ethyl Acetate. Next, the solvent portion harboring secondary metabolites was separated using a separating funnel and reduced to 10 mL using a rotary evaporator (11). The concentrated solvent was then used to perform the assay.

Secondary Screening

As part of a secondary screening procedure, the obtained bacterial strains were tested for their antibacterial properties against four selected MDR pathogens, including Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, and Staphylococcus aureus. As test pathogens, they were cultured in the nutrient broth to test the antibacterial activity. The test pathogens and isolated bacterial strains were added to liquid broth and then incubated for 24 hours at 37°C. Turbidity was adjusted to 0.5 McFarland. After that, these test pathogens were swabbed over the MHA surface, and sterile well borers were used for drilling 6 mm diameter bore holes. After that, 100 µL of a concentrated solvent was added to several wells on each plate. Ethyl acetate and antibiotic (Gentamicin) were employed as controls and kept at 37°C without inversion for 24 hours. After 24 hours of incubation, the zone of inhibition was examined. The zones were subsequently analyzed using the scale (9).

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis:

Samples S-1, S-2, S-6, and S-8 were selected for GC-MS analysis of bioactive compounds in their crude extracts of isolated antibiotic-producing bacteria. The crude extract was evaporated at room temperature until the volume was reduced to 2 mL. Walls of falcon tubes were washed with Ethyl acetate and again reduced to 2 mL. Samples were concentrated to a 2 mL volume; the selected samples were subjected to Gas Chromatography-Mass Spectrometry. The crude extracts were analyzed using the Perkin-Elmer GC Clarus 500 system.

Samples were loaded in GC columns, and an ionizing energy of 70 eV was utilized in the ionization system. Helium gas was utilized as the carrier. The flow rate of helium was set to 1ml/min. Raw scan files were processed using Turbo mass version 5.4.0 and a Perkin-Elmer GC Clarus 500 system. Mass spectrum interpretation of GC-MS was compared with the NIST14 library. Compared with previous literature, compounds detected in a crude extract of Samples S-1, S-2, S-3, and S-4 possessed broad-spectrum antimicrobial activity (6, 12).

Identification of Bacterial Samples using the MALDI-TOF Technique:

MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight) is an efficient method used for molecular identification of bacteria. The sample is mixed with a matrix solution, which absorbs laser energy and converts the proteins into ions. The ionized proteins are accelerated by an electric field and travel through a vacuum chamber to a detector. The detector generates a mass spectrum, a "fingerprint" of the bacterial protein profile. The mass spectrum is compared to a reference database containing spectra of known bacterial species. The system uses algorithms to identify the bacterial species or strains. Bacterial samples S1, S2, S6, and S8 were examined to identify their bacterial species or strains at the molecular level (7).

Results

Isolation and identification of bacterial isolates having antibacterial activity against selected MDR pathogens (*Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, and Staphylococcus aureus*)

The soil samples of 10 Pharmaceutical industries in Hattar, Haripur, showed a pH range of 5.5-7.4. The soil characteristics also varied due to different pH values, including color and consistency. The collected soil sample descriptions are presented in Table 1.

***Table* 1: Soil samples with the number of isolates**

Area of the Soil sample		Physical characteristics of soil		
Soil sample	**Name of Pharmaceutical Industry**	**Color**	**Consistency**	**P^H**
S-1	Weather Folds Pharmaceutical Industry	Brown	Moist	6.6
S-2	Genomics Pharmaceutical Industry	Dark Yellow	Dry	5.9
S-3	Welborne Pharmaceutical Industry	Dark Brown	Dry	7.3
S-4	Shahzeb Pharmaceutical Industry	Black	Dry	5.6
S-5	Qarshi Industries (Pvt.) Ltd.	Grey	Moist	6.9
S-6	Shazal's Pharmaceutical, IE, Hattar	Brown	Dry	5.7
S-7	World Pharmaceutical Industry	Black	Dry	7.1
S-8	Sayyed Pharmaceutical (Pvt) Ltd	Yellow	Moist	7.0
S-9	Genome Pharmaceutical Industry Hattar	Whitish	Moist	6.7
S-10	Cherwel Pharmaceutical (Pvt) Ltd	Black	Dry	6.8

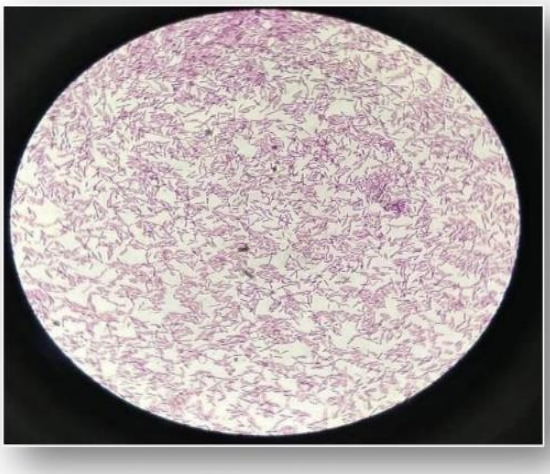


Figure 1. Colony morphology of isolated bacterial strains.

Figure 1: Microscopic observation of isolated bacteria under a 100X objective after Gram staining.

10 Bacterial strains were isolated and were further distinguished into subclasses based on their microscopic, macroscopic appearance, and biochemical observations (Table 2). Out of ten bacterial isolates, S1 & S6 were classified as *Enterobacter cloacae*, S2 as *Enterobacter asburiae*, and S8 as *Pseudomonas aeruginosa*.

Table 2: All the isolated strains were catalase and citrate positive, while they were negative for the methyl red test. Only two strains, S4 and S7, were positive for oxidase, and all others were negative.

Table 2: Biochemical characteristics of isolates

S. No	Catalase	Methyl Red	Citrate	Oxidase
S-1	+	-	+	-
S-2	+	-	+	-
S-3	+	-	+	-
S-4	+	-	+	+
S-5	+	-	+	-
S-6	+	-	+	-
S-7	+	-	+	+
S-8	+	-	+	-
S-9	+	-	+	-
S-10	+	-	+	-

Identified isolates were primarily screened for their antibiotic-producing ability using the well diffusion method (Figure 2). Isolates exhibited antagonistic activity when screened against the selected MDR pathogens (*Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, and Staphylococcus aureus*) depicted in Table 3.

Table 3. Antibacterial activity of isolated strains as depicted by clear zones of bacterial isolates. S1, S2, S4, S6, and S8: Isolated bacterial strains from industrial waste samples for testing antibacterial activities against *P. aeruginosa, E. coli, K. pneumoniae*, and *S. aureus*.

Table 3: Primary Screening of Isolates

Zone of inhibition (mm)				
S. No	***Staphylococcus aureus***	***Klebsiella pneumoniae***	***Escherichia coli***	***Pseudomonas aeruginosa***

S-1	**19**	**16**	**22**	**21**
S-2	**21**	**20**	**19**	**20**
S-3	9	15	13	10
S-4	10	14	12	13
S-5	15	10	14	13
S-6	**21**	**25**	**24**	**26**
S-7	11	10	9	12
S-8	**22**	**23**	**26**	**23**
S-9	11	16	18	9
S-10	13	16	14	13

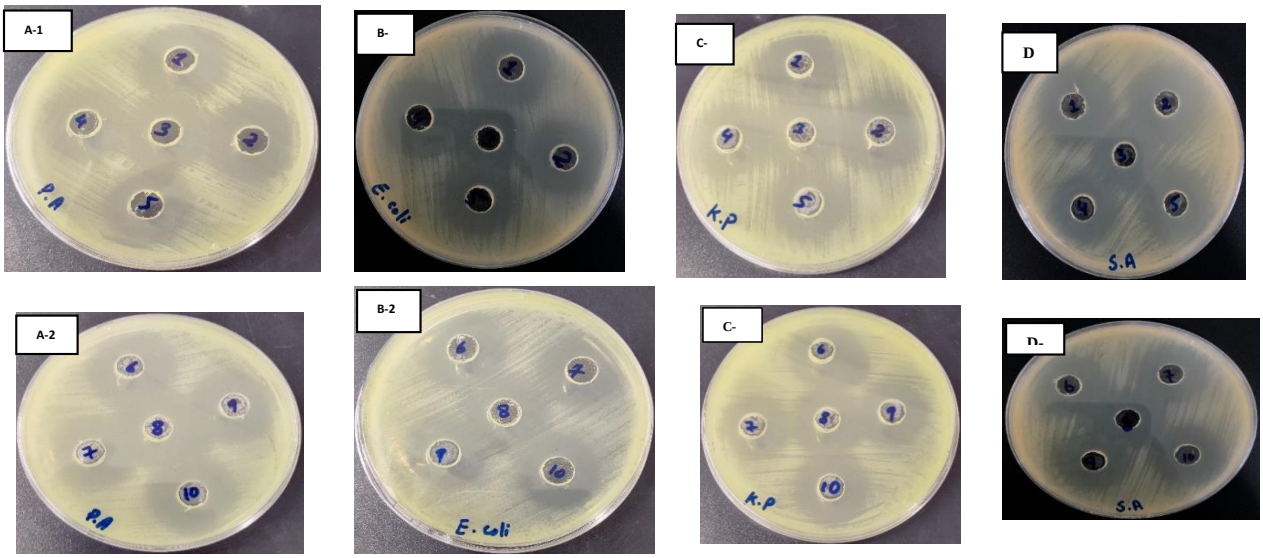


Figure 2: Primary Zones of Inhibition by:

A-1 & A-2: S1 – S10 against *P.aeruginosa*

B-1 & B-2: S1 - S10 against *E.coli*

C-1 & C-2: S1 – S10 against *K.pneumonae*

D-1 & D-2 S1 – S10 against *S. aureus*

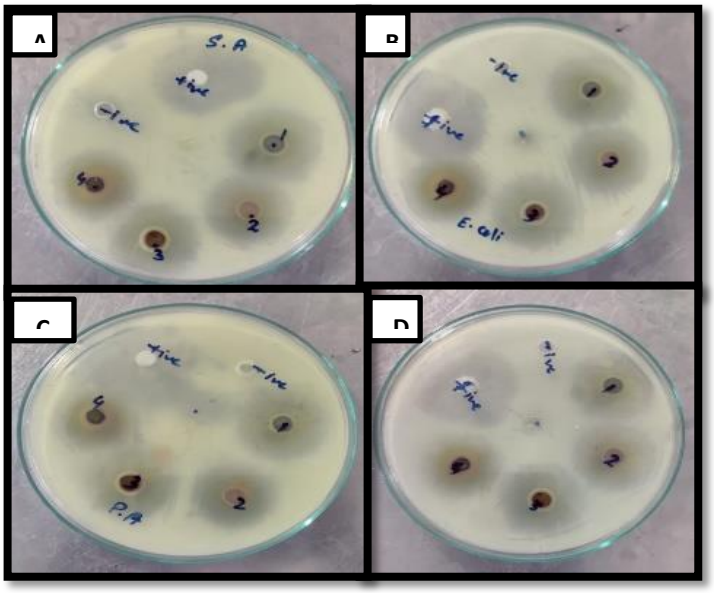


Figure 3: Secondary Zones of Inhibition by:

A:S1 – S4 against *S.aureus*

B:S1 – S4 against *E.coli*

C: S1 – S4 against *P.aeruginosa*

D:S1 – S4 against *K.pneumonae*

Molecular identification

For molecular identification, the MALDI TOF technique was used. The isolated strains S1 and S6 were Enterobacter cloacae, S2 was Enterobacter asburiae, and S8 was identified as *Pseudomonas aeruginosa*.

Table 4. Zones of inhibition formed by the Crude extracts of isolated bacterial strains with antibacterial activities against *P. aeruginosa*, *E. coli*, *K. pneumoniae*, and *S. aureus*. The names of bacterial samples for secondary screening were changed from S1 to S1, S2 to S2, S6 to S3, and S8 to S4.

Table 4 Secondary Screening Results of Isolates

Zone of inhibition (mm)				
S. No	***Staphylococcus aureus***	***Klebsiella pneumoniae***	***Escherichia coli***	***Pseudomonas aeruginosa***
S-1	22	24	21	23
S-2	23	20	22	21
S-3	24	22	23	23
S-4	21	20	24	22

Table 5: Maldi TOF analysis

Bacterial Sample	**Identified Bacteria**	**Percentage**
S-1 & S-6	*Enterobacter cloacae*	50
S-2	*Enterobacter asburiae*	25
S-8	*Pseudomonas aeruginosa*	25

MS analysis

GC-MS analysis of ethyl alcohol extracts of Bacterial samples S1, S2, S3 and S4 exhibited the presence of bioactive compounds as confirmed by previous literature. The bacterial extracts that were tested showed the presence of many compounds with confirmed biological activities like antibacterial, antifungal, anticancer, anti-inflammatory and antioxidants etc. Tables 6, 7, 8, and 9 contain references to previous research where these compounds were identified.

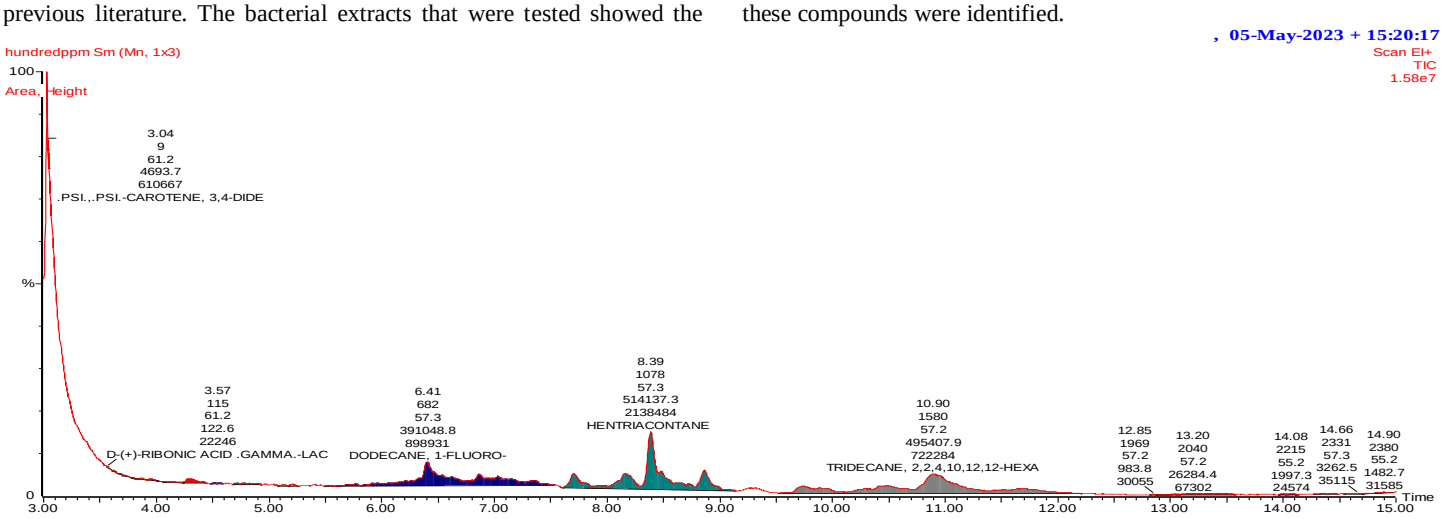


Figure 4: GC-MS analysis of S-1 for the detection of compounds produced by the isolated strains from waste soil of Pharmaceutical industry

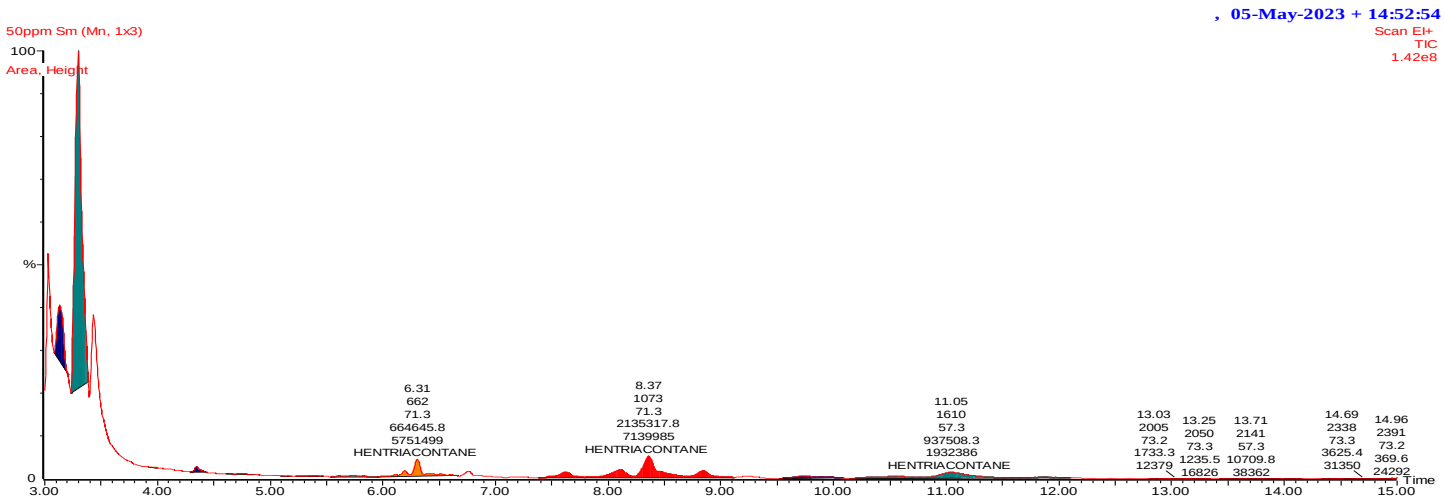


Figure 5: GC-MS analysis of S-2 for the detection of compounds produced by the isolated strains from waste soil of Pharmaceutical industry

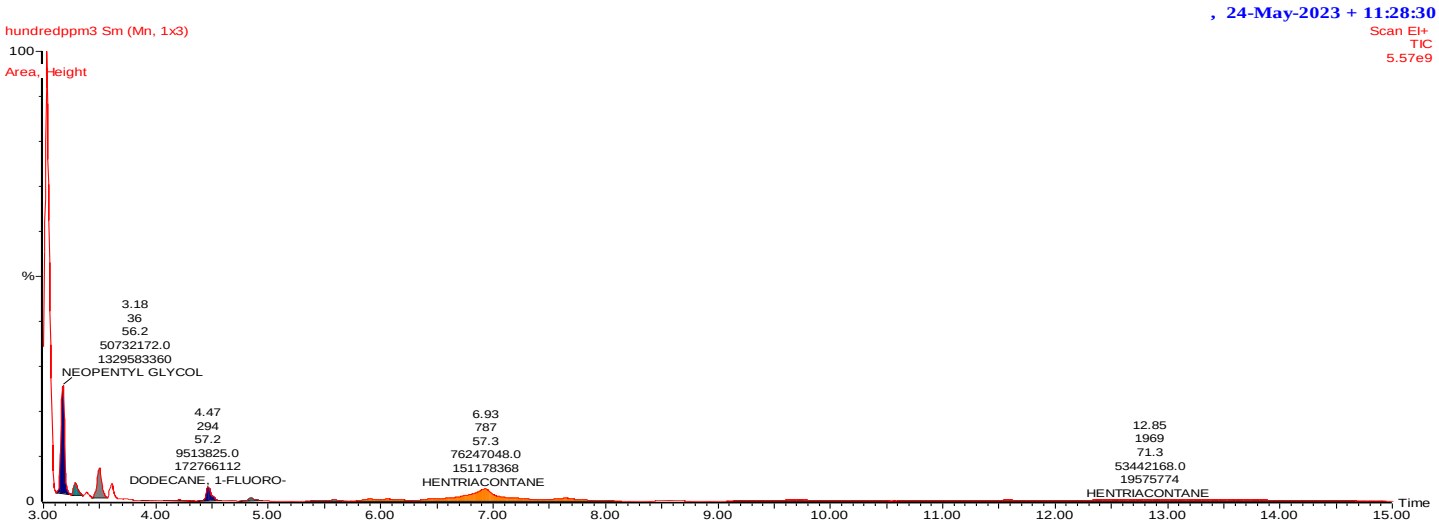


Figure 6: GC-MS analysis of S-6 for the detection of compounds produced by the isolated strains from waste soil of Pharmaceutical industry

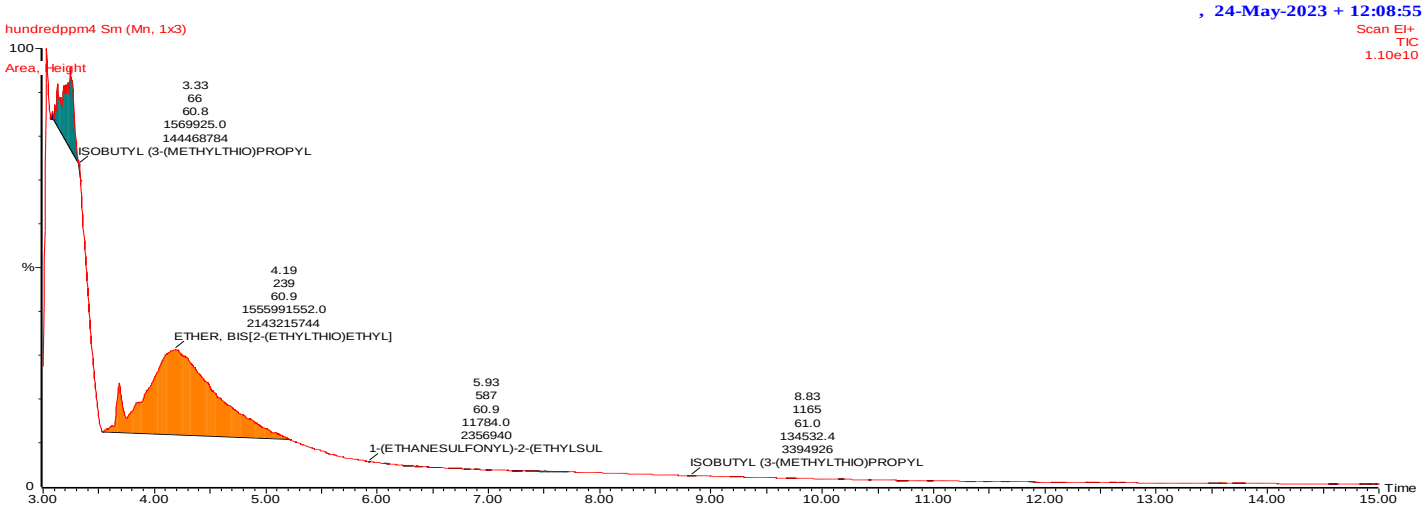


Figure 7 : GC-MS analysis of S-8 for the detection of compounds produced by the isolated strains from waste soil of Pharmaceutical industry.

Table 6: Major compounds detected by GS-MS analysis in sample S 1

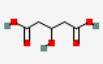
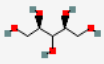
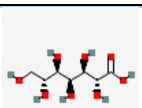
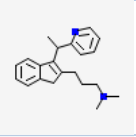
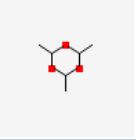
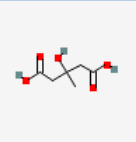
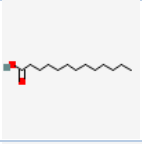
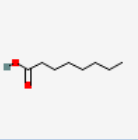
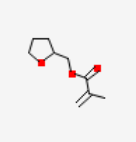
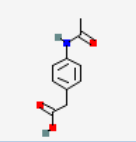
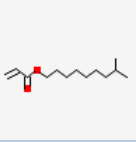
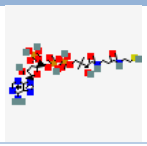
Name	Molecular Formula	Molecular Weight	Structural Formula	RT	Area	%comp	Activity
. PSI.,. PSI. - CAROTENE, 3,4-DIDE	$C_{20}H_{24}O_4$	328.4g/mol		3.043	4693.688	0.315229	Anticancer, antioxidant (23).
D- (+)-RIBONIC ACID. GAMMA. -LAC	$C_5H_8O_5$	148.11g/mol		3.574	122.642	0.008237	Important building block for chiral acyclic, cyclopentenones, and oxabicyclic system (24).
XYLITOL	$C_5H_{12}O_5$	152.15g/mol		3.619	69.558	0.004672	Xylitol is used for its antibacterial activity and to increase palatability (25)

Table 7: Major compounds detected by GS-MS analysis in sample S 2

D-GLYCERO-D-GULO-HEPTONIC ACID	$C_7H_{14}O_8$	226.18g/mol		3.689	305.655	0.020528	Used as a multisite ligand in tungstate complexes (26).
STRYCHANE, 1-ACETYL-20. ALPHA. -	$C_{21}H_{26}N_2O_2$	338.4g/mol		3.834	185.069	0.012429	Potent anti-microbial compound with the lowest binding energy (27)
BUTOXYACETIC ACID	$C_6H_{12}O_3$	132.16g/mol		3.954	2719.711	0.182656	Known as a urinary metabolite of the corresponding alkoxy ethanol solvents (28)
3,4-ALTROSAN	$C_6H_{10}O_5$	162.14g/mol		4.074	146.359	0.009829	Bacteriostatic, fungicide (29)
3-METHYLBUTYL 2-ETHYLHEXANOATE	$C_{13}H_{26}O_2$	214.34g/mol		4.314	16326	1.096456	Flavor compounds (30)
2-T-BUTYL-5-METHYL (1,3) DIOXOLA	$C_8H_{16}O_2$	144.21g/mol		4.714	6869.991	0.461389	Used in the preparation of an anti-malarial and anti-trypanosome agent (31).
OXALIC ACID, ETHYL NEOPENTYL E	$C_9H_{14}O_3$	170.21g/mol		5.144	2928.391	0.196671	Antimicrobial, Antioxidant, Anti-inflammatory functions (32).
PROPIONIC ACID, THIO-, S-ISOPE	$C_{10}H_{11}NO_3$	193.2g/mol		5.554	2531.276	0.170001	Anti-bacterial and anti-fungal properties (33), (34).
DODECANE, 1-FLUORO-	$C_{12}H_{25}F$	188.32g/mol		6.41	391048.8	26.26288	used as a cross-linking agent in the production of biodegradable polymers and can be used in the production of probiotic bacteria (35, 36, 37)
HENTRIACONTANE	$C_{31}H_{64}$	436.8g/mol		8.391	514137.3	34.52952	Possess different effects such as antitumor, antibacterial and anti-inflammatory effects (21).
TRIDECANE, 2,2,4,10,12,12-HEXA	$C_{13}H_{24}O_2$	212.33g/mol		10.90	495407.9	33.27165	Antimicrobial (29).

Name	Molecular Formula	Molecular Weight	Structural Formula	RT	Area	%com	Activity
PROPANOIC ACID, 3-HYDROXY-2,2-	$C_{21}H_{36}N_7O_{16}P_3S$	767.5g/mol		3.138	1162278	8.908396	Antimicrobial potential (15).

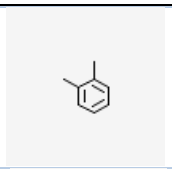
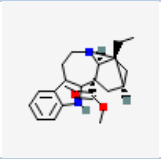
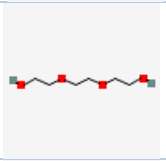
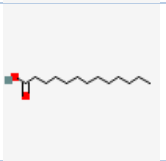
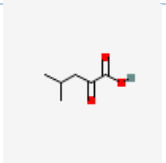
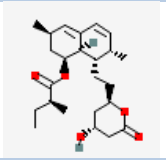
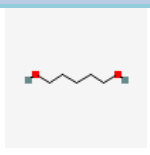
O-XYLENE	C_8H_{10}	106.16g/mol		3.304	7739345	59.31901	Antiparasitic (41), (42).
STRYCHANE, 1-ACETYL-20. ALPHA. -	$C_{21}H_{26}N_2O_2$	338.4g/mol		3.934	228.637	0.001752	Most potent anti-microbial compound with the lowest binding energy (27).
1,6-DIDEOXYDULCITOL	$C_6H_{14}O_4$	150.17g/mol		3.964	1396.84	0.010706	Anticancer agent (40).
3-METHYLBUTYL 2-ETHYLHEXANOATE	$C_{13}H_{26}O_2$	214.34g/mol		4.734	25577.84	0.196044	Antibacterial and antifungal agent (45), Flavor compounds (46).
PENTANOIC ACID, 3-METHYL-4-OXO	$C_6H_{10}O_3$	130.139g/mol		5.464	204.83	0.00157	Antiproliferative, anti-aging (47), and antimicrobial therapeutic properties (48).
ISOBUTYL 3-METHYLBUTAN-2-YL CA	$C_6H_{14}O$	102.17g/mol		5.745	91068.06	0.698001	Antibacterial Activity (49).
HENTRIACONTANE	$C_{31}H_{64}$	436.8g/mol		6.31	664645.8	5.094246	Possess different effects such as antitumor, antibacterial and anti-inflammatory effects (21).
BUTANOIC ACID, 2-METHYL-, 1,2-	$C_{24}H_{36}O_5$	404.5g/mol		9.766	191786.4	1.469967	Antimicrobial (38).

Table 8: Major compounds detected by GS-MS analysis in sample S

Name	Molecular Formula	Molecular Weight	Structural Formula	RT	Area	%comp	Activity
NEOPENTYL GLYCOL	$C_5H_{12}O_2$	104.15g/mol		3.178	50732172	23.10974	Used in the production of polyester resins, coatings, lubricants, plasticizers, and other industrial products [39].
1,6-DIDEOXYDULCITOL	$C_6H_{14}O_4$	150.17g/mol		3.289	6797815	3.09657	Anticancer agent [40]

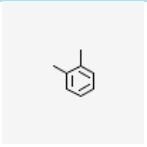
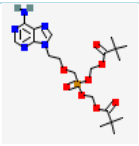
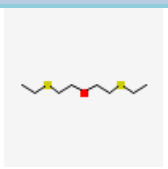
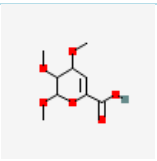
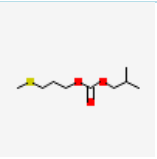
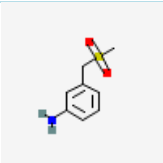
P-XYLENE	C_8H_{10}	106.16g/mol		3.504	17613620	8.023433	Antimicrobial active compound [41], Antiparasitic [42]
PROPANOIC ACID, 2,2-DIMETHYL-,	$C_{20}H_{32}N_5O_8P$	501.5g/mol		3.904	196936.484	0.089709	Antimicrobial [21].
OCTANE, 3,3-DIMETHYL-	$C_{10}H_{22}$	142.28g/mol		4.214	844777.438	0.384817	Antimicrobial Agent [43], Anti-inflammatory and antioxidant [44].
DODECANE, 1-FLUORO-	$C_{12}H_{25}F$	188.32g/mol		4.469	9513825	4.333779	used as a cross-linking agent in the production of biodegradable polymers and can be used in the production of probiotic bacteria [37]
HENTRIACONTANE	$C_{31}H_{64}$	436.8g/mol		12.847	53442168	24.34421	Possess different effects such as antitumor, antibacterial and anti-inflammatory effects [21].

Table 9: Major compounds detected by GS-MS analysis in sample S 4

Name	Molecular Formula	Molecular Weight	Structural Formula	RT	Area	%comp	Activity
ETHER, BIS[2-(ETHYLTHIO)ETHYL]	$C_8H_{18}OS_2$	194.4g/mol		3.083	2683976	0.150216	Antibacterial agent [50].
METHYL-4-DEOXY-2,3-DI-O-METHYL	$C_9H_{14}O_6$	218.2g/mol		3.249	222499088	12.45277	Antibiotic [51].
ISOBUTYL (3-(METHYLTHIO)PROPYL	$C_9H_{18}O_3S$	206.3g/mol		3.329	1569925	0.087865	Antibacterial Activity [49].
1-(ETHANESULFONYL)-2- (ETHYLSUL	$C_8H_{11}NO_2S$	185.25g/mol		5.935	11784.024	0.00066	Used in mitsunobu reactions excellent [52], antibacterial activity against pathogens [53].

Discussion

Ten isolated strains were used for the screening purpose against *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, and Staphylococcus aureus*. These samples produced different zones of inhibition against the selected MDR bacteria. Finally, the four isolated strains S1, S2, S6 and S8 showed biggest zones of inhibition against the selected pathogenic bacteria were selected. These four strains were further processed for their identification by using PCR and MALDI-TOF technique. The isolated strains S1 and S6 were *Enterobacter cloacae* and

S2 was *Enterobacter asburiae* while S8 was identified as *Pseudomonas aeruginosa.* Also, those showing an antibiotic property were applied for the antibiotic production process by extracting crude extract through the submerged fermentation process, and finally, its activity was checked by the well diffusion method.

Sever public health problems have been increasing due to acquired multi-drug resistant pathogens. Many antimicrobial agents have been discovered, which are effective against a variety of contagious diseases (15). Industrial waste (effluent) contains a large number of pathogenic micro-organisms along with their other microbes which are resistant to many antibiotics. The biomass of the microorganisms also acts as activated sludge by breaking down the sewage content, which is complex in nature. The saprophytic bacteria, some metazoans and along with some protozoa, together made a biological floc (16). Present research project showed the identification of bacteria from waste soil samples of Pharmaceutical industries. Some of these bacteria showed antibacterial activities against *P. aeruginosa, E. coli, K. pneumoniae,* and *S. aureus*. Similar research was performed who reported that the antibacterial activities of 115 isolated bacterial strains against *Escherichia coli, Enterococcus mutans, Pseudomonas aeruginosa, Streptococcus aureus* and *salmonella typhi* (17).

Many microorganisms, now a days, show resistance to several antibiotics, and its ratio is increasing day by day to form multidrug resistance which might be due to the modification of antibiotics during the manufacture process in the industry. The use of naturally isolated antibiotics would significantly reduce the MDR property of the microorganism, thereby providing safe antibiotic therapy against infection. The isolated antibacterial compounds from natural sources could be used for the treatment of different type of infections since it does not use most of the costly materials for its preparations. Research performed in Bangalore also demonstrated the identification of antibiotic producing microorganisms from soil against several pathogenic bacteria. They identified *Bordetella, Achromobacter and Streptomyces*, which is in accordance with the present research project (7, 18).

Bacteria having antimicrobial properties against MDR pathogens could also be isolated from contaminated water environment. Extended-spectrum beta-lactamase (ESBL) producing isolates were detected from water sources (7). The Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) and Polymerase Chain Reaction (PCR) was proved to be an effective technique for the identification of bacteria (7). *Acinetobacter calcoaceticus, Enterobacter bugandensis, Acinetobacter pittii, Pseudomonas aeruginosa, Acinetobacter junii, Pseudomonas oleovorans,* and *Enterobacter ludwigigii* were identified by using these techniques (19, 20).

Variety of compounds were identified through GC-MS analysis of crude extract of S1, S2, S3 and S4. Literature search of these compounds confirmed anti-bacterial, anti-fungal, anti-inflammatory, anti-tumor and anti-cancerous properties which were confirmed by different researches conducted previously. D-(+)-Ribonic Acid. Gamma. Lac, Oxalic Acid, Ethyl Neopentyl E, D-Glycero-D-Gulo-Heptonic Acid, Hentriacontane, Neopentyl Glycol, Strychane, 1-Acetyl-20. Alpha, 3,4-Altrosan, Carbamic Acid, Hydroxy-, Ethyl etc were the most important antimicrobial compounds. Hentriacontane were detected in S1, S2 and S3 which possess different effects such as antitumor, antibacterial and anti-inflammatory effects stated by (21). Octane, 3,3-Dimethyl were detected in sample S3 which was also an antimicrobial agent against several bacterial pathogens (22).

Present study may help to increase the benefits of antibiotics and improve the economy of nation which could be utilized by the different governmental health sectors for maintaining the quality of life of people. This research project was confined to few samples, it does not reveal the picture of whole identification process with confident finding. Therefore, covering a wide geographical area for the identification of antibacterial compounds producing microorganisms along with an advance identification process could be an effective strategy to identify useful bacteria which may produce antimicrobial compounds against wide range of pathogenic bacteria.

Conclusion

Waste soil samples of pharmaceutical industries of Hattar, Haripur, Pakistan contains antibiotic producing bacteria. Ten bacterial strains were isolated on nutrient agar plates, out of which only four strains showed clear zones and high antibacterial activity against both gram positive and gram negative selected MDR pathogens e.g. *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae,* and *Staphylococcus aureus*. The results of gram staining and their biochemical tests identified all the strains as gram negative rod like bacteria. The MALDI TOF analysis confirmed the results of isolated bacterial strains. The isolated strains S1 and S6 were *Enterobacter cloacae* and S2 was *Enterobacter asburiae* while S8 was identified as *Pseudomonas aeruginosa*

The compounds *D-Glycero-D-Gulo-Heptonic Acid, D- (+)-Ribonic Acid. Gamma. -Lac, Oxalic Acid, Ethyl Neopentyl E, D-Glycero-D-Gulo-Heptonic Acid, Hentriacontane, Neopentyl Glycol, Strychane, 1-Acetyl-20. Alpha, 3,4-Altrosan, Carbamic Acid, Hydroxy-, Ethyl etc*having anti-microbial, anti-bacterial, anti-fungal, anti-inflammatory, anti-tumor and anti-cancerous functions were identified among the extracts of S1, S2, S3 and S4 isolates. Along with those, many new compounds were also detected which were not biologically tested before, which may have antibacterial activities against several other selected MDR pathogens (*Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, and Staphylococcus aureus*). These finding could be beneficial, if utilized at large scale of for manufacturing of antibiotics and for other research in the development of antibiotics against selected MDR pathogens (*Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, and Staphylococcus aureus*).

Declarations

Data Availability statement

All data generated or analysed during the study are included in the manuscript.

Ethics approval and consent to participate

Approved by the department concerned

Consent for publication

Approved

Funding

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Conflict of interest

The authors declared the absence of a conflict of interest.

Author Contribution

MAW, SAB (Associate Professor)
Coordination of collaborative efforts.
Study Design, Review of Literature.
Conception of Study, Development of Research
Methodology Design, Study Design, Review of manuscript,
Conception of Study
AT, A
Sampling, Study Design, Manuscript revisions, critical input.
Data acquisition and Data analysis,
Coordination of collaborative efforts.
Statistical Analysis.
Data acquisition, analysis.
Manuscript drafting.
SA, SM (Associate Professor)
Study Design, Review of Literature.
Data entry and Data analysis, drafting article.

Data acquisition, analysis.
Coordination of collaborative efforts.

All authors reviewed the results and approved the final version of the manuscript. They are also accountable for the integrity of the study.

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