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# Spectroscopic and chromatographic identification of bioprospecting bioactive compounds from cow feces: Antimicrobial and antioxidant activities evaluation of gut bacterium *Pseudomonas aeruginosa* KD155

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ABSTRACT

The prime objective of our study was to evaluate antimicrobial and antioxidant activities of *Pseudomonas aeruginosa* KD155 isolated from cow dung. For identification of the isolate KD155, molecular techniques were employed and obtained 16S rRNA gene sequence was deposited in the NCBI GenBank under the accession number MK801234. Extracellular crude extract of *P. aeruginosa* KD155 displayed significant antimicrobial activity against *Bacillus subtilis* (MTCC 441) and *Staphylococcus aureus* (MTCC 7443) in comparison to tetracycline and ketoconazole. The resistance of extracellular crude chloroform extract to DPPH scavenging activity was also observed with 77.49% inhibition rate reflecting strong antioxidant activity. In addition, HP-TLC, FT-IR and GC-MS analysis of extracellular chloroform crude extract was done which revealed phenolic compound (quercetin) as major bioactive metabolite being produced by our isolate KD155. Further, the stability of 16S rRNA sequence of the strain was studied using bioinformatics tools viz. mfold and NEB cutter indicating the thermodynamic stability of its gene sequence.


## 1. Introduction

Disease causing pathogens are constantly become resistant towards commercially available antimicrobial agents [1,2]. This problem is of great concern for clinicians, public health officials and researchers due to substantial morbidity, mortality and increasing cost of treatment [3–6]. World health organisation (WHO) has already warned that the world is heading back to the pre-antibiotic era regarding therapy for these multiple antibiotic-resistant pathogens [7]. There is a gradual increase in infectious diseases caused by multidrug-resistant bacteria [8–10]. Because of the production of extended-spectrum β-lactamases (ESBLs) by bacteria, even fourth generation of cephalosporin and other antibiotics are now being threatened [6,11,12]. Due to the development of pathogen resistance against existing medicines and appearance of new diseases leads towards an alarming scarcity of new antibiotics [13,14]. Bacteria living in stressful ecological habitats may give rise to novel and potent metabolites which may serve as broad-spectrum antibiotics, targeting the medically important pathogens. Researchers around the world are continuing their effort to isolate bacteria capable of producing bioactive compounds from a range of different habitat such as Camel milk [15], soil [16], marine habitats [17], fermented foods [18], sponges [19] and endophytes [20–22]. Thus, continuous search of bacteria capable of producing useful bioactive compounds from untapped sources especially natural ones like wild plants, endophytic microorganisms, marine environment and even from the gut of animals and insects is a significant aspect of ongoing research.

Gut of ruminant animals such as cows are rich in microbial diversity and have stable microbial ecological balance system [23]. Cow dung is a biological waste of bovine herbivorous animals excreted after digestion of consumed food material, mainly consisting of lignin, cellulose, hemicelluloses and 24 different minerals. Cow dung can be considered as gold mine of micro-organisms as it posses wide variety of microorganisms comprising over 60 different bacterial species and 100 species of protozoa and yeast [19,24–27]. Culture-dependent techniques have revealed the presence of many different genera such as *Citrobacter koseri, E. aerogenes, E. coli, K. oxytoca, K. pneumoniae, Kluyvera spp., Morganella morganii, Pasteurella spp., Providencia alcaligenes, P. stuartii* and *Pseudomonas* spp. [28].

In view of this, the present work describes isolation, characterization and identification of bioactive strain *P. aeruginosa* KD155 from an easily available biological resource i.e., cow dung. The study was also extended to evaluate the antimicrobial and antioxidant potential of strain KD155. Furthermore, spectroscopic and chromatographic techniques such as HP-TLC, FT-IR and GC-MS

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were used for identification and characterization of different bioactive metabolites produced by the isolated strain KD155.

## 2. Materials and methods

### 2.1. Isolation of bacterial strain KD155 from cow dung sample

Strain KD155 was isolated from cow dung sample collected aseptically from cow shed located in Kazipura, Saharanpur (Latitude, 29.9671 °N; Longitude, 77.5494 °E; Altitude 278 m), Uttar Pradesh, India. Standard serial dilution technique was employed for commencement of isolation using nutrient agar (NA) and luria bertani (LB) agar supplemented with Cyclohexamide (25 μg/ ml). Aliquots (0.1 ml) of each dilution were spread on agar plates in 3 replicates. The plates were incubated at 35 ± 1 °C for 24–48 hrs. Bacterial isolate was propagated twice on NA plate and was stored at 4 °C for further investigation [29].

### 2.2. Determination of antagonistic potential of KD155

#### 2.2.1. Bacterial and fungal test strains

The following bacterial and fungal test strains were used for antimicrobial activity in present work. *Vibriocholera* (MTCC 3904), *Salmonella typhi* (MTCC) 3216, *Staphylococcus aureus* (MTCC 7443), *Bacillus subtilis* (MTCC 441), *Bacillus cereus* (MTCC 6728), *Proteus vulgaris* (MTCC 426), *Enterococcus faecalis* (MTCC 439), *Pseudomonas aeruginosa* (MTCC 424), *Escherichia coli* (MTCC 118), *Shigella flexneri* (MTCC 1457), *Salmonella typhimurium* (MTCC 3231), *Streptococcus pyogenes* (MTCC 442), *Staphylococcus aureus* (MTCC 3160), *Listeria monocytogenes* (MTCC 657) and *Candida albicans* (MTCC 227). All the test strains were procured from Microbial Type Culture Collection (MTCC), IMTECH Chandigarh, India. All the test strains were cultured and maintained on nutrient agar at 35 ± 1 °C and 4 ± 1 °C respectively.

#### 2.2.2. Cross-streak method

The antagonistic activity of isolated strain was evaluated using cross-streak method. KD155 was streaked in the centre of plate and incubated at 35 ± 1 °C. After 48 h, test strains were streaked perpendicularly to the edge of initial streak and further incubated at 35 ± 1 °C for 24–48 h. The antagonistic activity was measured by distance of inhibition between the test isolate KD155 and test strains [30].

### 2.3. Characterization of isolate KD155

Cultural and morphological characteristics of strain KD155 were analysed by growing on nutrient agar and broth using traditional methods reported in the Bergey's manual of determinative bacteriology [31]. The bioactive strain was subsequently analysed for macroscopic (colony form, margin, colour, elevation and opacity) and microscopic analyses (motility, spore formation, cell shape and gram staining). For assessment of carbohydrate utilization by strain KD155, different carbon sources were supplemented in basal medium at a concentration of 1% and incubated at 35 ± 1 °C for 24–48 h. Physiological characters for growth were determined on NA medium by growing at different temperatures (15 ± 1 °C, 35 ± 1 °C and 45 ± 1 °C), pH (5–9) and NaCl (1 %–9 %) ranges. Hydrolysis of starch, liquefaction of gelatine and other biochemical tests were also evaluated as criteria given in Uzair et al. [32]. All the results were recorded after 24–48 h of incubation.

### 2.4. Amplification of 16S rRNA sequence and construction of phylogenetic tree

The isolate was recognized by 16S rRNA gene sequencing method. The genomic DNA of KD155 was isolated as described by Ganesan et al. [33]. (50AGAGTTTGATCMTGGCTCAG30) and 1492R (50TACGGYTACCTTGTTACGACTT 30) were used to amplify 16S ribosomal sequence from genomic DNA in thermal cycler. The amplified product (~1400 bp) was sequenced using ABI PRISM [34]. The similarity of obtained sequences was compared using the BLAST tool. CLUSTAL W program was used for multiple alignment of DNA sequence and phylogenetic tree was constructed using neighbour-joining method from MEGAX software. The 16S rRNA sequences were submitted to the GenBank, NCBI, USA.40.

### 2.5. RNA secondary structure prediction and determination of restriction sites

The secondary structure of the 16S rDNA was predicted by Mfold software (http://unafold.rna.albany.edu/) to analyze the structural stability in terms of Gibb's free energy [35]. The sequences of *Pseudomonas aeruginosa* KD155 were submitted to the Mfold web server. Different parameters such as exterior loop type, bulge loop size, base numbering frequency, structure draw mode, structure annotation and structure rotation angle were fixed. The folding process was predicted at 37 °C and under ionic conditions of 1 M NaCl deprived of divalent ions and restriction site was identified using NEB cutter online tool version 2.0 (nc2.neb.com/nebcutter2/) [33].

### 2.6. Bioactive profiling of P. aeruginosa *KD155*

#### 2.6.1. Cultivation and recovery of bioactive compound

For bioactive metabolite production, *P. aeruginosa* KD155 culture was transferred into the flask containing 500 ml of beef peptone broth and incubated in shaker incubator at 150 rpm for 2 days at 35 ± 1 °C. Following incubation, the liquid culture and biomass was separated through centrifugation at 5000 rpm for 20 min, and cell-free supernatant was collected separately. Metabolites from supernatant were extracted by manual shaking thrice with equal volume of ethyl acetate and chloroform (1:1) in a separating funnel. The organic layer was concentrated using the vacuum rota evaporator. The resulting crude extract was recuperated in DMSO and bioassayed against test strains [36].

#### 2.6.2. Antimicrobial activity

Antimicrobial activity of crude extracts was carried out against test strains using agar well diffusion method [37]. Petri plates were prepared and test strains were swabbed on solidified media. Agar wells (4 mm diameter) were prepared and 100 μl of both crude extracts and positive controls were carefully dispensed into each well, allowed to diffuse for 2 h, and incubated at 35 ± 1 °C for 24–48 hrs. After incubation, zone of inhibition around each well was recorded. Solely DMSO was included as negative control while tetracycline (30 mcg) and ketoconazole (10 mcg) were incorporated as positive control. Each experiment was performed in duplicates.

#### 2.6.3. Determination of minimum inhibitory concentrations (MIC)

MIC of crude extracts was determined by two fold dilution methods with minor modifications as described by Wu et al. [38]. The sterile 96 well micro-titre (12 × 8) plate was labelled accordingly and crude extract (200 μl) was pipetted into the first well acting as stock solution. Further, 100 μl of Mueller Hinton broth fortified with 0.01 % of 2, 3, 5 triphenyltetrazolium chloride salt (TTC) as growth indicator was added in remaining wells. 100 μl of crude extract were transferred from stock to the next well subsequently so that each well had test material in decreasing concentrations. The test strains were added at a concentration of 10 μl to each well [39]. The MIC was determined as the lowest dilution that inhibited the growth of test organism after 24–48 h post incubation [40]. Antimicrobial agents such as tetracycline (30 mcg) and ketoconazole (10 mcg) were incorporated as positive control.

**Table 1**
Cultural, morphological, physiological and biochemical characterization of *Pseudomonas aeruginosa* KD155.

| Properties | Result |
|---|---|
| Cultural characteristics | |
| Colony form | Irregular |
| Colony margin | Entire |
| Colony colour | Blue green |
| Colony elevation | Convex |
| Optical density | Opaque |
| Morphological characteristics | |
| Gram-reaction | – |
| Shape | Rod |
| Endospore | – |
| Motility | Motile |
| Physiological characterisation | |
| Temperature range for growth | 15OC-35OC |
| pH range for growth | 5–8 |
| NaCl tolerance | 3% |
| Biochemical characterisation | |
| Catalase | – |
| Oxidase | + |
| Citrate | + |
| Nitrate | + |
| Indole production | – |
| MR test | – |
| VP test | – |
| Casein hydrolysis | + |
| Gelatine hydrolysis | + |
| Starch hydrolysis | + |
| Urea | – |
| Carbohydrate utilization | |
| Glucose | + |
| Sucrose | – |
| Lactose | – |
| Mannitol | – |

Abbreviations Used: (+)= Positive, (-)= Negative.

#### 2.6.4. Evaluation of minimum bactericidal and fungicidal concentrations (MBC and MFC)

From the above wells, where no visible turbidity was observed, 0.1 ml aliquot was spreaded over the surface of Mueller Hinton agar plates. After incubation at 35 ± 1 °C for 24–48 hrs, the colonies were observed and MBC and MFC were determined [37].

### 2.7. Antioxidant assay

Free radical scavenging activity of crude extract was measured using the procedure as described by Zhu et al. [41]. 500 μl of the crude extract was added with 3.0 ml of freshly prepared solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH). 500 μl of methanol added to 3 ml DPPH solution mixed in dark and incubated for 30 min, served as control. Absorbance was recorded at 517 nm after 30 min of incubation under dark condition. All the readings were recorded in triplicates and the average absorbance value was calculated. The percentage DPPH scavenging activity was calculated according the equation given below.

$$\text{Free radical scavanging activity } \% = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Where $A_{control}$ is the absorbance of control reaction (without test sample) and $A_{sample}$ is the absorbance in the presence of crude extract.

### 2.8. Hemolytic assay

Hemolytic activity was detected using blood agar with 5 % (v/v) human blood, prepared by adding 7.4 g of blood agar base powder in 95 ml of distilled water. This mixture was sterilized and left until it reached the temperature around 45 ± 1 °C. After that, 5 ml of the defibrinated blood was added aseptically in the blood agar base with constant shaking in such a way that froth formation prevented. The blood agar was poured in sterile petri-plates and was solidified. Wells were cut with sterile cork borer, loaded with 100 μl of crude extract and incubated at 35 ± 1 °C for 18–24 hrs. After incubation, the plates were inspected for hemolysis pattern around the wells [42].

### 2.9. Partial characterization of bioactive compound

#### 2.9.1. High performance thin layer chromatography (HP-TLC)

HPTLC analysis was performed with CAMAG-Linomat and TLC Scanner in pre-coated silica gel 60 F254 of uniform thickness 0.2 mm and a size of 5 × 10 cm. The method was standardized by running different solvents at 254 and 366 nm. Approximately 10 μl of samples and the standard solution was used for the spotting. After saturation time, the spots were analyzed in UV spectra for the similarity of peaks and retardation factor (Rf) value [43].

#### 2.9.2. Fourier transform infrared spectroscopy (FT-IR)

The IR spectra of chloroform extract was determined on Perklin Elmer spectrophotometer in the mid IR region from 4000–400 $cm^{-1}$ at a resolution of 4 $cm^{-1}$ using FTIR spectrophotometer coupled with TGS (Triglycine Sulfate) detector. For FTIR analysis, 1 mg of dried sample was mixed with 100 mg of pure Potassium bromide (KBr) of AR grade, dried under vacuum at 100 °C and compressed to prepare salt disc (3 mm). Intensity was plotted against wave number to get the desired spectrum [44].

#### 2.9.3. Gas chromatography–mass spectroscopy (GC-MS)

GC-MS analysis of the crude extracellular extract of cow dung bacterial isolate KD155 was performed using the Thermo Scientific TSQ 8000 Gas Chromatograph - Mass Spectrometer, Thermo MS DSQ II. The equipment has a DB 35-MS Capillary Standard non-polar column with dimensions of 30 mm × 0.25 mm ID × 0.25 μm film. The carrier gas used is Helium with at low flow of 1.0 ml/min. The injector was operated at 350 °C and the oven was operated at an range of 60 °C–280 °C for 15 min-30 min. The identification of components was based on NIST libraries by comparing their retention indices. The constituents were identified with those available in the computer library (NIST and Willey) attached to the GC-MS instrument [45].

## 3. Results

### 3.1. Characterization and taxonomy of KD155

The bacterial strain KD155 was found to be Gram-negative rod shaped bacterium. The colony showed blue green colouration having entire margins. The cell was found to be motile and non-spore former. The strain KD155 could grow up to 35 °C and pH 8 with 3 % NaCl tolerance. KD155 demonstrated positive result towards oxidase production, gelatine hydrolysis, citrate utilisation, nitrate reduction and could only utilized glucose as the carbon source out of four tested carbohydrate sources. The isolate showed clear zone in casein and starch hydrolysis indicating the production of extracellular enzymes i.e., gelatinase and amylase respectively. The cultural, morphological, physiological and biochemical characteristics of the strain KD155 are shown in Table 1. The 16S rRNA gene sequence of the strain KD155 was analysed using NCBI GenBank database. The phylogenetic tree of the strain KD155 was constructed from the sequences of closely related strains using neighbor-joining method is presented in Fig. 1. The strain has got maximum 16S rRNA gene sequence homology (100 %) with *Pseudomonas aeruginosa* strain BUFF12 16S ribosomal RNA gene, partial sequence (MH375412). Therefore, on the basis of cultural, morphological, physiological, biochemical and analysis of the 16S rRNA gene sequence, the isolate KD155 was designated as

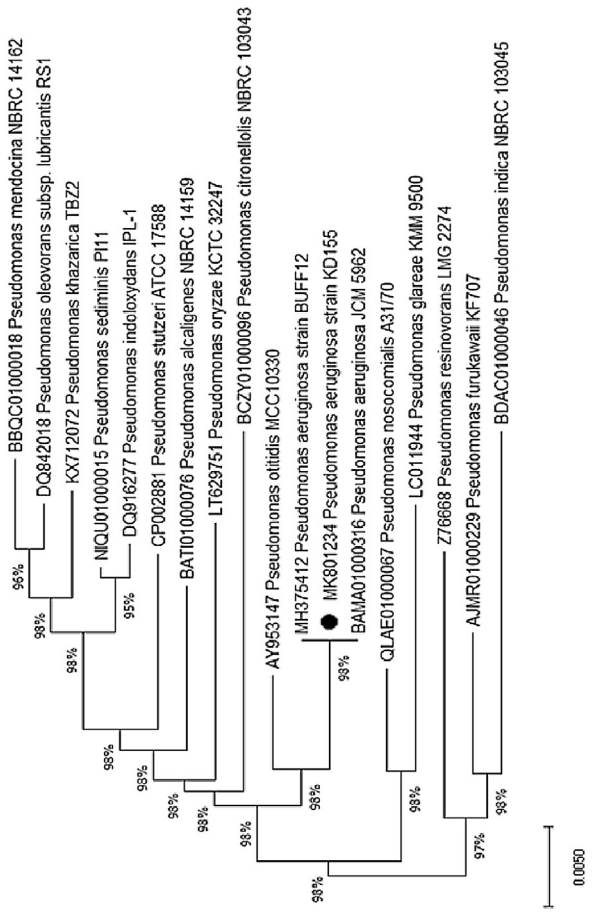


**Fig. 1.** The evolutionary history was inferred using the Neighbor-Joining method. There were a total of 1493 positions in the final dataset.

*Pseudomonas aeruginosa* strain KD155. The 16S rRNA gene partial sequence of the isolate KD155 has been deposited in the GenBank database under the accession number MK801234.

### *3.2. 16S rRNA secondary structure prediction and restriction sites analysis*

The 16S rRNA folding was predicted to comprehend the thermodynamic stability of the gene sequence (Fig. 2a). The free Gibb's energy of 16S rRNA in its folded form for KD155 was observed to be −480.10 kcal/mol. This study suggested the minimal energy level of 16S rRNA sequences, specifying high folding stability of nucleotides in the organisms. The restriction analysis of 16S rRNA sequence of KD155 indicated the presence of GC-AT content to 54 % and 46 % respectively. However, a total of 54 restriction sites were observed in the obtained gene sequence of the strain KD155. (Fig. 2b)

### *3.3. Determination of antagonistic potential of* P. aeruginosa *KD155*

In the present study, *P. aeruginosa* KD155 was found active against 74 % of test organisms. The strain KD155 showed a broad spectrum activity since it not only inhibited the growth of 5 Gram-positive and 5 Gram-negative bacteria but also restricted the growth of fungal test strain (Table 2).

### *3.4. Quantitative bioassay of crude extracts*

The bioactive strain was cultivated in BPB medium for the production of bioactive metabolite. BPB medium was extracted using two organic solvents i.e., ethyl acetate (EA) and chloroform (CHL). Ethyl acetate and chloroform are widely used as suitable solvent for extraction of bioactive compounds. *P. aeruginosa* KD 155 showed yield of 1.315 g and 0.954 g of crude metabolite from chloroform and ethyl acetate respectively. The antimicrobial activity of both the extracts was assessed by agar well diffusion method against 7 Gram-positive, 7 Gram-negative bacteria including one fungal pathogen, compared with standard drugs. Here we found that both the obtained crude extracts have shown broad spectrum bactericidal and fungicidal action. However, chloroform extract obtained from KD155 was found more potent than ethyl acetate extract as it inhibited 12 test pathogens out of 15 whereas, ethyl acetate extract could only able to inhibit only 11 test organisms. Chloroform extract depicted improved activity by 7 % as compared to ethyl acetate extract. Chloroform extract showed better activity against *B. cereus* (25 ± 1 mm) in comparison with tetracycline (16 ± 1 mm). Isolate KD155 maximally inhibited the growth of *S.aureus* (27 ± 1 mm) as compared to tetracycline (24 ± 1 mm). Crude extract of KD155 (24 ± 1 mm), showed improved activity in comparison with ethyl acetate extracts against *C. albicans* which is quite comparable with ketoconazole (20 ± 1 mm). Both the extracts were found inactive against *E. faecalis* (MTCC 439) and *S. pyogenes* (MTCC 442). Although, it was also noted that apart from improved bioactivity of chloroform extract, one test organisms i.e., *P. vulgaris* (MTCC 426) was inhibited by ethyl acetate extract only (Table 3) (Fig. 3a, b and c).

### *3.5. Determination of minimum inhibitory concentration (MICs), minimum bactericidal (MBCs) and fungicidal concentration (MFCs)*

On the basis of the results of antimicrobial assays performed on both the extracellular crude extracts, chloroform extract was selected to carry out further studies. The results presented in Table 4 showed that the MIC values varied from 41–164 μg/ ml against the entire test strains. The inhibitory effect of chloroform extract obtained from strain KD155 started at 41 μg/ ml against *S. aureus* (MTCC 7443), *B. cereus* (MTCC 6728) and *P. aeruginosa* (MTCC 424) whereas higher MIC value of 328 μg/ ml was found against *B. subtilis* (MTCC 441). However, most of the test bacterial organisms were inhibited at concentration of 164 μg/ ml. Apart from this, MIC range of chloroform extract against *C. albicans* (MTCC 227) was found to be 164 μg/ ml. Chloroform extract of *P. aeruginosa* KD155 showed potentially bactericidal activity against *S. aureus* (MTCC 7443), *B. cereus* (MTCC 6728) and *P. aeruginosa* (MTCC 424) with MBC of 86 μg/ ml. The fungal pathogen i.e., *C. albicans* (MTCC 227) was observed sensitive towards higher values of MFCs i.e., 410 μg/ ml (Table 4) (Fig. 4).

### *3.6. DPPH radical scavenging and hemolytic assay*

DPPH is a stable free radical having absorption maxima at 517 nm. A free radical scavenging property of strain KD155 was measured in terms of hydrogen donating or radical scavenging ability using the stable radical determination of 1, 1-diphenyl-2-picryl hydrazyl (DPPH). The isolate KD155 inhibited 76.597 % DPPH. *P. aeruginosa* KD155 was also cultured on 5 % blood agar and showed no zone of clearance around the bacterial colonies depicting their non-hemolytic nature. Similarly, zone of clearance was also not observed in extracellular crude extract obtained from this isolate.

### *3.7. HP-TLC*

The chloroform extract of *P. aeruginosa* KD155 was subjected to HPTLC analysis by specific solvent system ethyl acetate: acetone: AcOH: Water = 60:20:10:10. The bioactive compounds were clearly

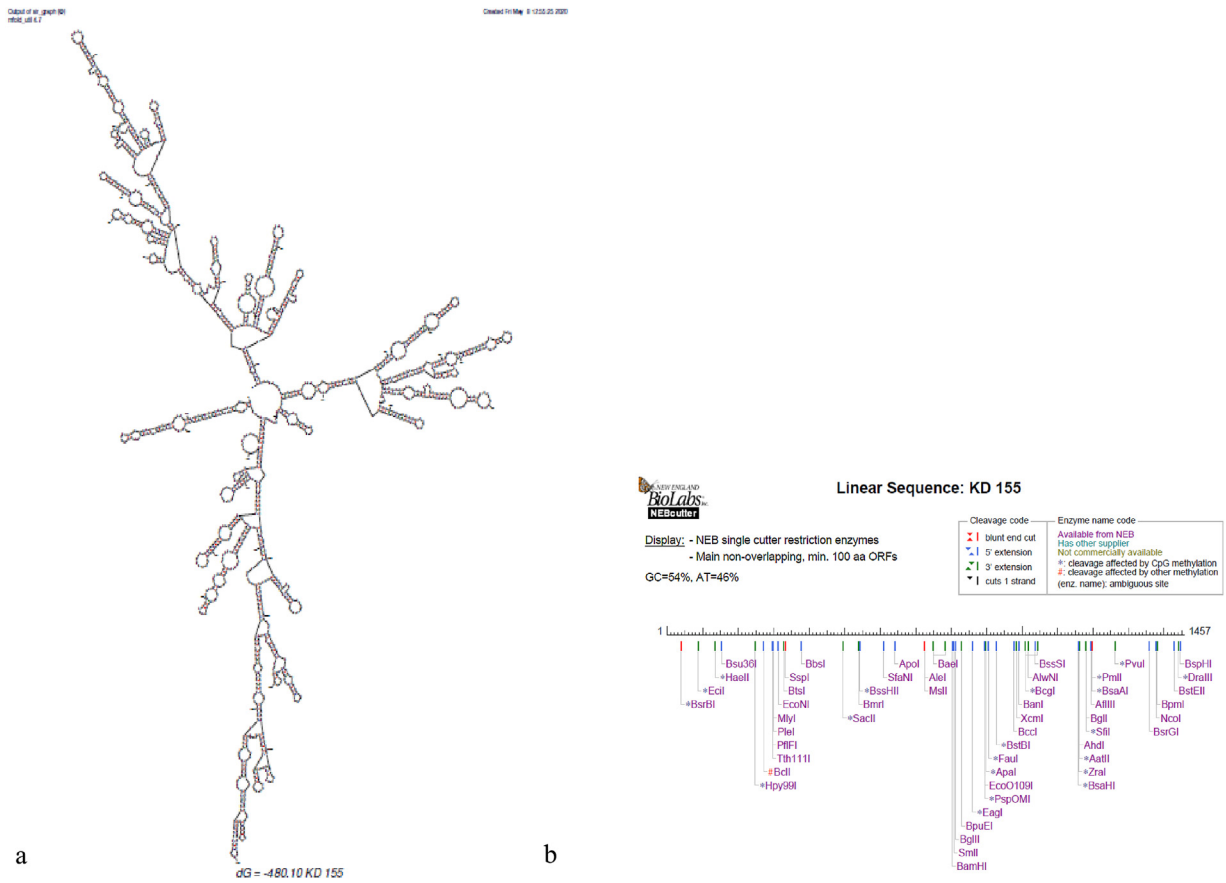


**Fig. 2.** (a) Predicted secondary structure of 16S rRNA isolated from *P. aeruginosa* strain KD155 (Gibb's free energy −480.10 kcal/mol). (b) Restriction sites on the 16S rRNA sequence of *P. aeruginosa* strain KD155.

**Table 2**
Antagonistic activity of *Pseudomonas aeruginosa* KD155.

| Target Organisms | Cross-streak method |
|---|---|
| *V. cholera* (MTCC 3904) | + |
| *S. typhi* (MTCC 3216) | + |
| *S. aureus* (MTCC 7443) | + |
| *B. subtilis* (MTCC 441) | – |
| *B. cereus* (MTCC 6728) | + |
| *P. vulgaris* (MTCC 426) | – |
| *E. faecalis* (MTCC 439) | + |
| *P. aeruginosa* (MTCC 424) | + |
| *E. coli* (MTCC 118) | + |
| *S. flexneri* (MTCC 1457) | – |
| *S. typhimurium* (MTCC 3231) | + |
| *S. pyogenes* (MTCC 442) | – |
| *S. aureus* (MTCC 3160) | + |
| *L. monocytogenes* (MTCC 657) | + |
| *C. albicans* (MTCC 227) | + |

Abbreviations Used: (+) = denotes the presence of antagonistic potential; (-) = denotes absence of antagonism.

separated without any tailing and diffuseness. The analysis showed total four peaks with respective Rf values 0.01, 0.65, 0.87 and 0.98. The concentration of Quercetin (flavnoid) was found to be the highest through the densitometric analysis of the different peaks as shown in Fig. 5a and b. TLC plates at 254 nm are shown in Fig. 6 while plates at 366 nm are shown in Fig. 7.

### 3.8. FT-IR spectroscopy

The FTIR spectra, peak values and probable functional group of chloroform crude extract obtained from *P. aeruginosa* KD155 are presented in Fig. 8 and Table 5. The identification of functional group present in the active components is identified based on the region of IR radiation displayed as peak values in FT-IR spectrum. When the extract was passed into the FT-IR, the functional groups of the components were separated based on its peaks ratio. The absorbance bands analysis in bioreduction process are observed in the region between 400–4000 $cm^{-1}$. The FTIR analysis of the crude extract revealed peak of different functional groups at 766 $cm^{-1}$ showing bending in C—H bond, 930 $cm^{-1}$ (C=C Bending), peak at 1043 $cm^{-1}$ shows stretching in S=O bond, 1236 $cm^{-1}$ (C-O Stretching), 1516 $cm^{-1}$ (C-N Stretching), 1883 $cm^{-1}$ (C-H Bending), 1992 $cm^{-1}$ (C=N Stretching), 2140 $cm^{-1}$ (C=O Stretching), intramolecular forces between O-H group was evident from the peak 2424–2623 $cm^{-1}$ (O-H Intramolecular forces), 2623 $cm^{-1}$ (S-H Stretching), 2856–3280 $cm^{-1}$ (C-H Stretching), 3677 $cm^{-1}$ (O-H Stretching) (Table 5, Fig. 8).

### 3.9. GC-MS analysis

The gas chromatogram shows that the relative concentration of various compounds getting fractionated at their specific retention time. A total set of 40 peaks were obtained from the chloroform crude extract of KD155 run for 26.59 min (Fig. 9). All the compounds and their molecular weights present at respective peaks were analysed using NIST database to locate the probable compound as well as their molecular weight (Table 6). The heights of the percentage of peak area indicate the relative concentrations of the components present in the chloroform crude extract of *P. aeruginosa* KD155. 4 peaks at RT 15.24, 16.21, 18.49 and 20.55 showed more % area coverage i.e., 23.27, 17.69, 12.95 and 6.82

**Table 3**
Antimicrobial activities (in mm) of chloroform and ethyl acetate crude extracts obtained from *P. aeruginosa* KD155.

| Test organisms | CHL (mm) | EA (mm) | KET (mm) | TET (mm) |
|---|---|---|---|---|
| *V. cholera* (MTCC 3904) | 20 ± 1 | 15 ± 1 | NA | 25 ± 1 |
| *S. typhi* (MTCC 3216) | 16 ± 1 | 13 ± 1 | NA | 22 ± 1 |
| *S. aureus* (MTCC 7443) | 27 ± 1 | 10 ± 1 | NA | 24 ± 1 |
| *B. subtilis* (MTCC 441) | 14 ± 1 | 11 ± 1 | NA | 15 ± 1 |
| *B. cereus* (MTCC 6728) | 25 ± 1 | 22 ± 1 | NA | 16 ± 1 |
| *P. vulgaris* (MTCC 426) | 0 | 15 ± 1 | NA | 35 ± 1 |
| *E. faecalis* (MTCC 439) | 0 | 0 | NA | 20 ± 1 |
| *P. aeruginosa* (MTCC 424) | 25 ± 1 | 15 ± 1 | NA | 18 ± 1 |
| *E. coli* (MTCC 118) | 13 ± 1 | 13 ± 1 | NA | 18 ± 1 |
| *S. flexneri* (MTCC 1457) | 15 ± 1 | 15 ± 1 | NA | 16 ± 1 |
| *S. typhimurium* (MTCC 3231) | 11 ± 1 | 11 ± 1 | NA | 20 ± 1 |
| *S. pyogenes* (MTCC 442) | 0 | 0 | NA | 23 ± 1 |
| *S. aureus* (MTCC 3160) | 22 ± 1 | 0 | NA | 24 ± 1 |
| *L. monocytogenes* (MTCC 657) | 17 ± 1 | 0 | NA | 18 ± 1 |
| *C. albicans* (MTCC 227) | 24 ± 1 | 11 ± 1 | 20 ± 1 | NA |

Abbreviations Used: CHL- Chloroform; EA- Ethyl acetate; KET- Ketoconazole; TET- Tetracycline; mm- millimetre; Values are means ±SD; NA- Not applicable.

respectively. Phenol, 3,5 bis (1,1dimethylethyl), Phenol, 2,4 bis (1,1dimethylethyl) and Phenol, 2,5 bis (1,1dimethylethyl) were identified as major contributor at retention time of 15.24 min.

## 4. Discussion

For the development of novel or more potent bioactive metabolites, screening of microbial strains belonging to underexplored or extreme environments is among one of the most efficient approach [46,47]. The ability of these strains for producing antimicrobials may be associated with defensive or aggressive roles in order to maintain their ecological niche in such environments, which on the other hand is known to be strongly influenced by natural selection [48]. With this perspective, an antimicrobial agent producing bacterial strain KD155 was isolated from the cow dung of desi breed from Uttarpradesh (India). Previously, this bacterium was reportedly isolated from cow dung slurry [49]. The concept of studying free energy associated with the folding of 16S rDNA gene sequence might provide preliminary information to make a concurrent prediction on stabilities of the genes. In our study, *P. aeruginosa* KD155 showed −480.10 kcal/mol free energy of secondary structure which is more or less in accordance with the previous studies [50,51].

The preliminary screening results clearly showed that KD155 possessed very high antimicrobial activity against all the tested human pathogens. In a previous study, *P. aeruginosa* was found antagonistic against *S. aureus, E. coli, P. vulgaris, Bacillus sp.* in cross streak method [52,53].

Among both of the tested extracellular extracts, chloroform extract showed better inhibition against *S. aureus* in comparison to

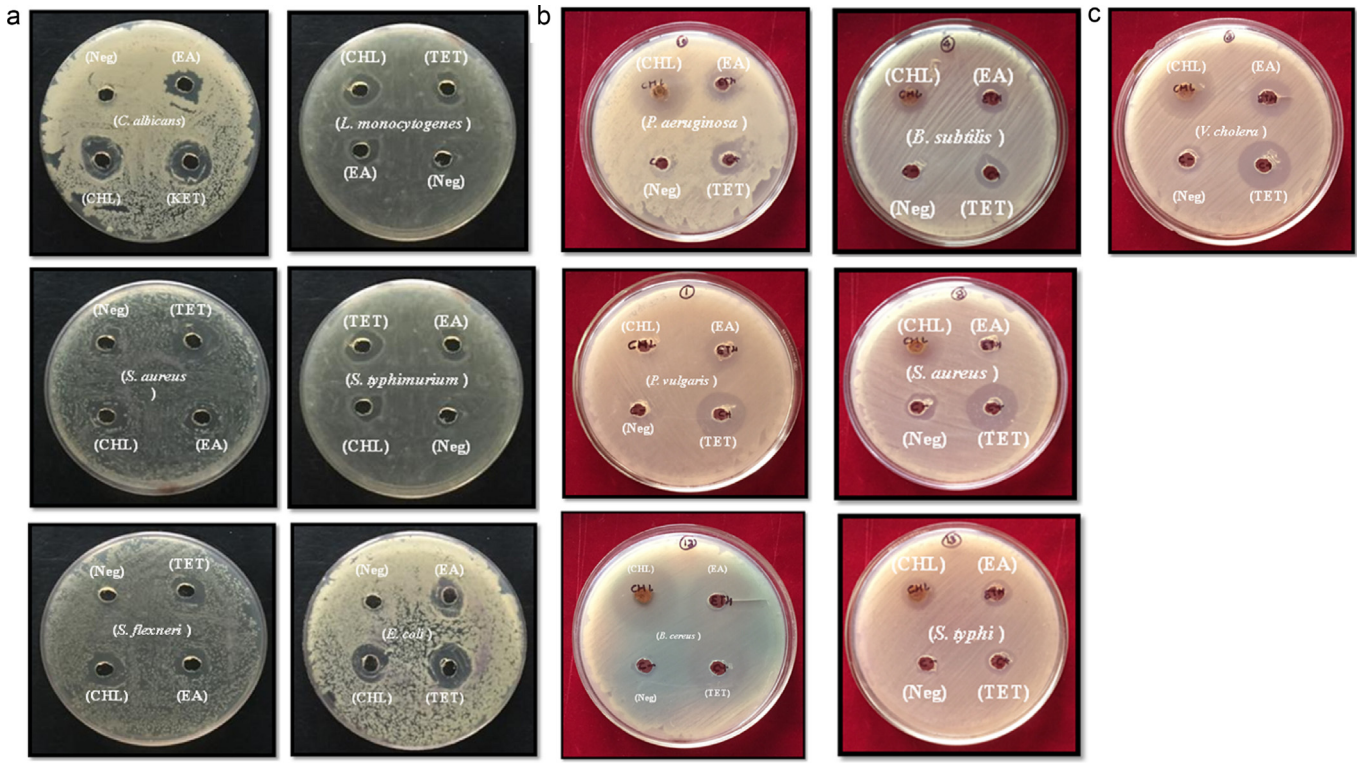


**Fig. 3.** (a), (b) and (c) Antimicrobial activity of chloroform (CHL) and ethyl acetate extract (EA) obtained from *P. aeruginosa* KD155.

**Table 4**
MICs, MBC, and MFCs of chloroform extract obtained from *P. aeruginosa* KD155 (μg/ ml).

| Test organisms | MIC | MBC/MFC | TET (MIC/MBC) | KET (MIC/MBC) |
|---|---|---|---|---|
| *V. cholera* (MTCC 3904) | 164 | 410 | 15/30 | NA |
| *S. typhi* (MTCC 3216) | 164 | 410 | 1.87/3.75 | NA |
| *S. aureus* (MTCC 7443) | 41 | 86 | 0.93/3.75 | NA |
| *B. subtilis* (MTCC 441) | 328 | 656 | 15/30 | NA |
| *B. cereus* (MTCC 6728) | 41 | 86 | 1.87/3.75 | NA |
| *P. vulgaris* (MTCC 426) | – | – | – | NA |
| *E. faecalis* (MTCC 439) | – | – | – | NA |
| *P. aeruginosa* (MTCC 424) | 41 | 86 | 7.5/30 | NA |
| *E. coli* (MTCC 118) | 164 | 410 | 7.5/30 | NA |
| *S. flexneri* (MTCC 1457) | 164 | 410 | 30 | NA |
| *S. typhimurium* (MTCC 3231) | 164 | 410 | 30 | NA |
| *S. pyogenes* (MTCC 442) | – | – | – | NA |
| *S. aureus* (MTCC 3160) | 82 | 180 | 0.93/3.75 | NA |
| *L. monocytogenes* (MTCC 657) | 164 | 410 | 15/30 | NA |
| *C. albicans* (MTCC 227) | 164 | 410 | NA | 2.5/5 |

Abbrevations Used:- (-)= Not determined; Not applicable.

ethyl acetate extract. Metabolites obtained from *P. aeruginosa* showed good antimicrobial property against *S. aureus, E. coli, Klebsiella sp, S. typhi, Shigella sp.* and *C. albicans* [52]. Apart from this, some pathogens were not inhibited by the crude extracts which may be due to the disintegration of some metabolites during extraction process [37,52]. The results clearly demonstrate difference between the activity of crude extracts and pure antibacterial and antifungal drug (tetracycline and ketoconazole) showing higher, equivalent or lesser zone of inhibition. Rex et al. [54] observed a marked difference in the crude (unpurified) extracts in comparison to pure drug that was already in clinical use. Kennedy et al. [55] isolated *P. putida* from rhizospheric soil, which produces 5-methyl phenazine-1-carboxylic acid betaine (MPCAB) showing considerable amount of antimicrobial potential against human pathogen. *Pseudomonas* sp. isolated from wheat rizosphere soil was known for its antifungal potential which is due to the production of phenazine-1-carboxylic acid [56,57].

The MIC (41 μg/ ml) of bioactive compounds of our strain KD155 against *S. aureus, B. cereus* and *P. aeruginosa* was found more or less similar to those reported in case of other species of genus *Pseudomonas* for example, Ahmed [58] reported MIC of *P. stutzeri* CMG1030 in the range of 50–125 μg/ mL against *B. cereus, S. aureus* (MSSA and MRSA), S. *epidermidis, E. faecalis and E. faecium* etc. Comparable findings were also demonstrated by Wratten et al. [59] who isolated *Pseudomonas* sp. 102−3 displaying inhibitory activity against *S. aureus* at a concentration range of 50 μg/disk-5 mg/disk. In another report by Levenfors et al. [60] who isolated *P. protegens* UP46 from soil samples reported the MIC value of 1−2 μg/ ml for *S. aureus* while in same study *P. aeruginosa* showed inhibition at a concentration of 32 μg/ ml. Therefore, it can be concluded that MIC values of present study showed variation than those of earlier reports which could be due to the differences between the tested bacterial strains or may be due to the different bioactive compounds.

The DPPH inhibition (75.17 %) by the bioactive metabolites of *P. aeruginosa* KD155 was quite comparable with previous study in which, *P. aeruginosa* HSS-6 showed 74.9 % free radical scavenging activity [61], indicating an efficient antioxidant potential.

The presence of non-hemolytic activities among bacterial strains as well as in their active compounds is considered as a safety prerequisite prior to consumption or industrial and pharmacological applications [62,63]. It is noteworthy that any

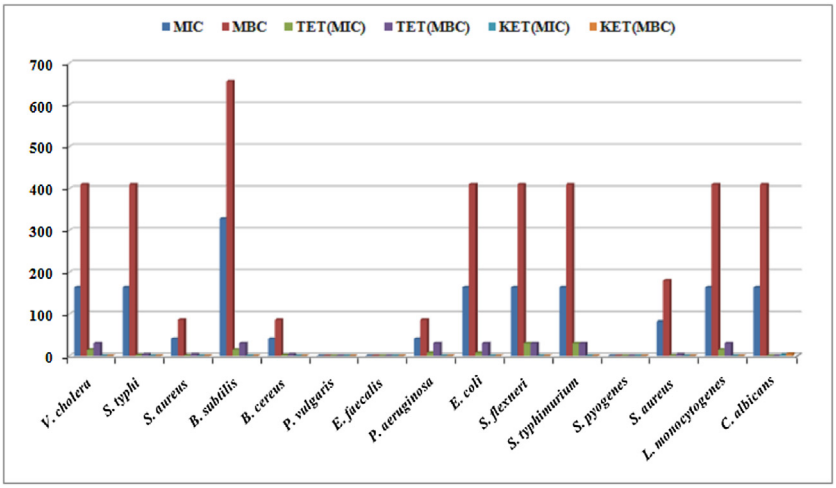


**Fig. 4.** MIC of extracellular crude extract obtained from *P. aeruginosa* KD155. Tetracycline (TET) and Ketoconazole (KET).

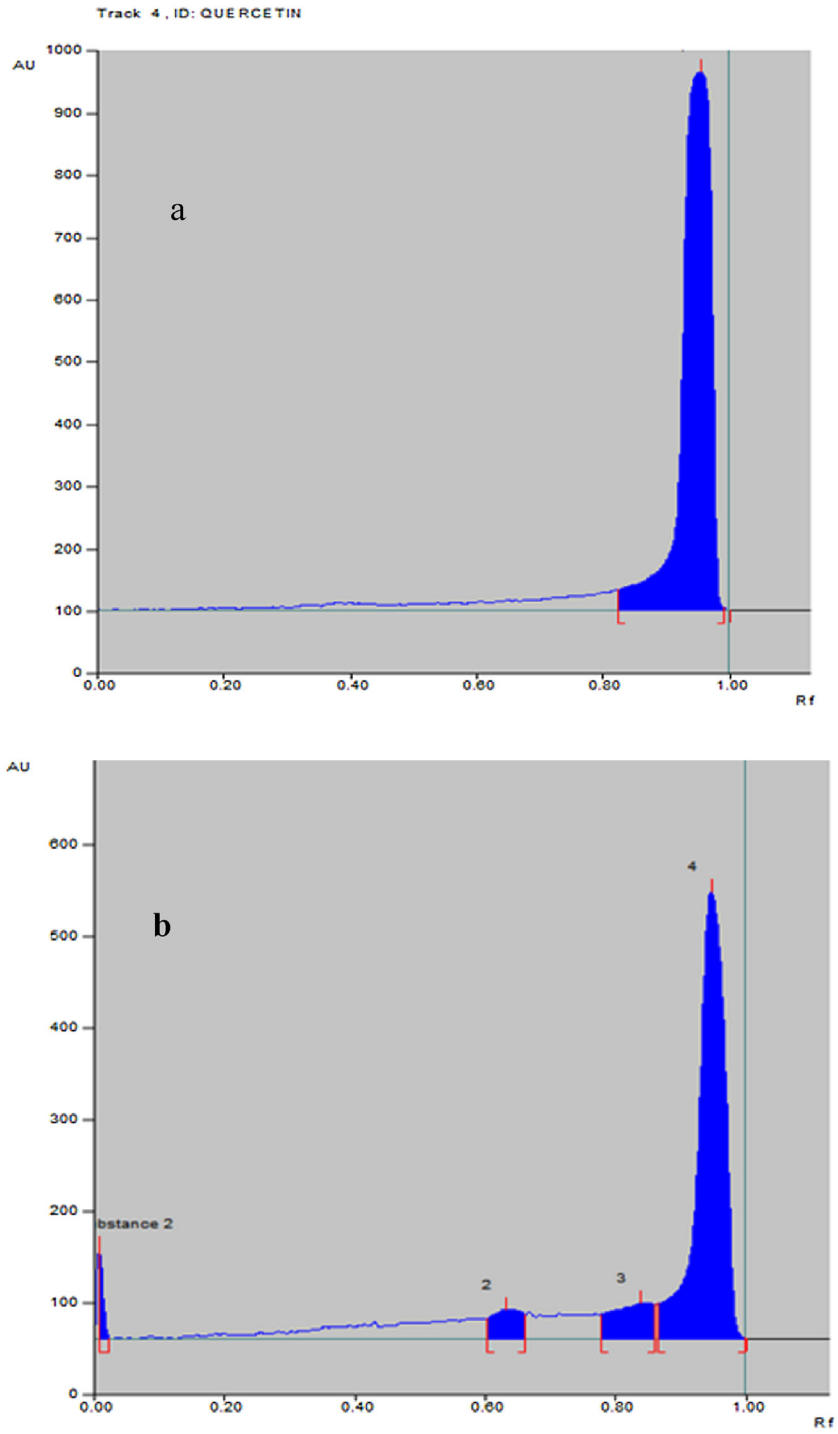


**Fig. 5.** (a)- HPTLC chromatogram of standard Quercetin, (b)- HPTLC chromatogram of CHL extract of KD155.

antimicrobial compound needs to be devoid of hemolytic/cytotoxic activity and present results are in accordance with this.

HP-TLC investigations of chloroform extract showed high levels of flavonoids (quercetin) as a major constituent in the extract. Quercetin, a common flavonol found in plants [64] comprising diverse pharmacological activities, including antioxidant [65,66], antihyperglycemic [67], antiinflammatory [68] and hepatoprotective activities [69]. However, as far as literature is concerned, flavonoid content in the crude extract of *P. aeruginosa* is very less explored and only a single report was available in which *P. aeruginosa* BS25 was detected with quercetin as an active compound [70]. However, this is the first report on quercetin producing gut bacterium *P. aeruginosa* isolated from cow dung.

The FT-IR analysis of chloroform extract of KD155 proved the presence of aliphatic compounds, alcohols, nitro compounds,

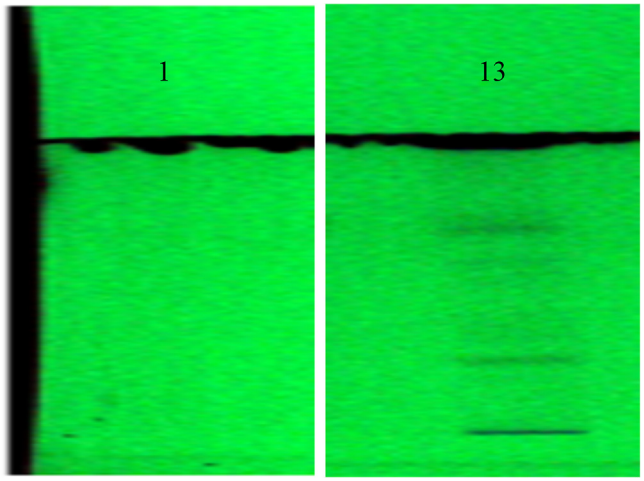


**Fig. 6.** TLC plates at 254 nm (1)- Quercetin, (13)- CHL extract of KD155.

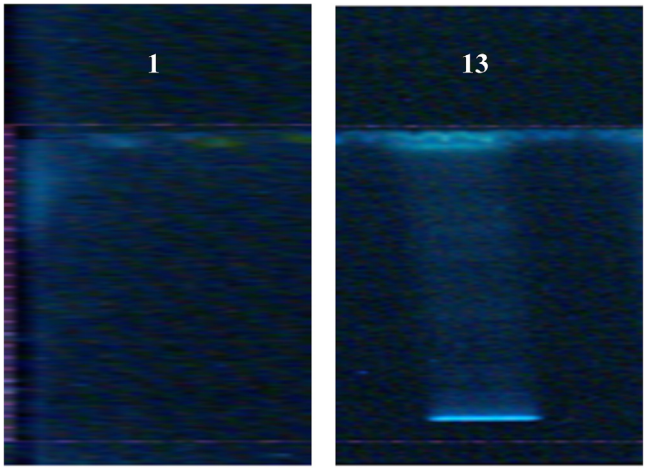


**Fig. 7.** TLC plates at 366 nm (1)- Quercetin, (13)- CHL extract of KD155.

**Table 5**
Functional groups and peak characters of chloroform crude extract of *P. aeruginosa* KD155.

| Functional group | FTIR peaks ($cm^{-1}$) | Peak characters |
|---|---|---|
| C-H | 766 | Bending |
| C=C | 930 | Bending |
| S=O | 1043 | Stretching |
| C-O | 1236 | Stretching |
| C-N | 1516 | Stretching |
| C-H | 1883 | Bending |
| C=N | 1992 | Stretching |
| C=O | 2140 | Stretching |
| O-H | 2424–2623 | Intramolecular Force |
| S-H | 2623 | Stretching |
| C-H | 2856–3280 | Stretching |
| O-H | 3677 | Stretching |

alkanes and hydrogen bond alcohols at their respective peaks. The OH group detected is able to form hydrogen bonding that probably related with the higher inhibitory activities of alcohols and phenolics against microorganisms [71–73]. Devnath et al. [52] also indicated the presence of –OH group and the appearance of bands between 3000–2900 $cm^{-1}$ is an indication of C aromatic compound [74]. Due to the presence of aromatic compound, vibrations of carbon stretching in the aromatic ring shows absorptions in the regions 1618–1660 $cm^{-1}$. It is suggested that, less hydroxyl groups in the molecular structure of compound may reduce the antimicrobial activity [75]. On the other hand, phenolic compounds containing hydroxyl groups increase the affinity of compounds towards microbial lipid membranes resulting in higher antibacterial activity [76].

The GC-MS chromatogram of chloroform extract obtained from *P. aeruginosa* KD155 showed presence of bioactive compounds such as Phenol, 2,4-bis(1,1dimethylethyl) [77,78]. As a compound of natural origin, phenol, 2, 4-bis (1, 1-dimethylethyl) and its derivatives displayed wide range of activity in the field of medicine, food and agriculture [79]. Several workers have reported a wide range of bioactivities i.e., antibacterial [80], antifungal [81], anticancer [82] and antioxidant activity [83,84] associated with phenol, 2, 4-bis (1, 1-dimethylethyl). The bioactive compound Phenol, 2, 4-bis (1, 1-dimethylethyl) reported in this study is also known to be originated from different microorganisms such as *P.*

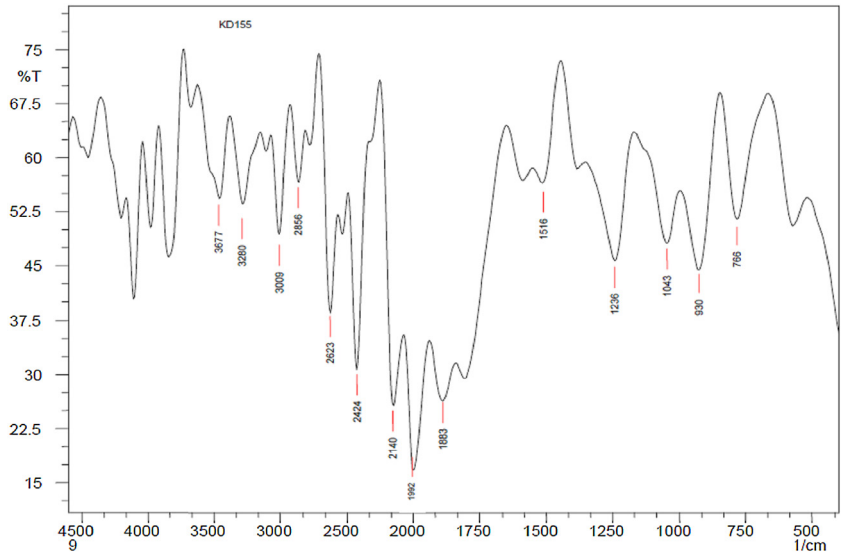


**Fig. 8.** FTIR spectra.

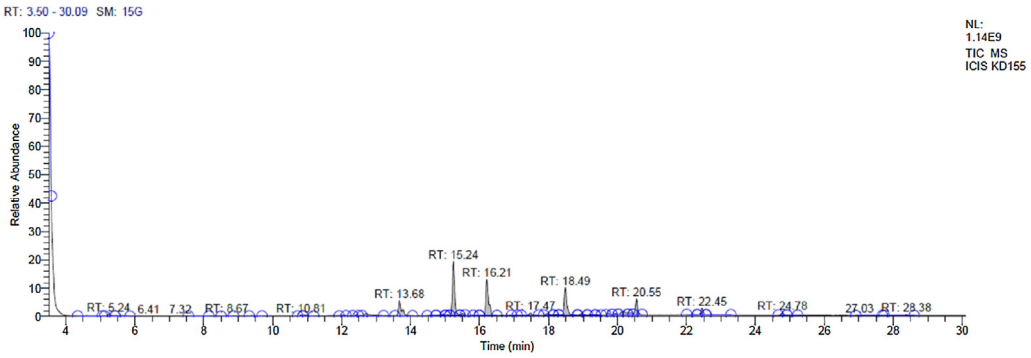


**Fig. 9.** GC-MS chromatogram of chloroform extract of KD155.

**Table 6**
Major identified compound from chloroform extract of *P. aeruginosa* KD155.

| Compound Name | RT | Molecular Formula |
|---|---|---|
| Pentanoic acid, 5hydroxy, 2,4ditbutylphenyl esters | 15.24 | $C_{19}H_{30}O_3$ |
| Pentanedioic acid, (2,4ditbutylphenyl) monoester | 15.24 | $C_{19}H_{28}O_4$ |
| Phenol, 3,5 bis(1,1dimethylethyl) | 15.24 | $C_{14}H_{22}O$ |
| Phenol, 2,4 bis(1,1dimethylethyl) | 15.24 | $C_{14}H_{22}O$ |
| Phenol, 2,5 bis(1,1dimethylethyl) | 15.24 | $C_{14}H_{22}O$ |

*fluorescens* (79) and *Streptomycetes albidoflavus* [85] displaying antifungal and antidermatophytic activities.

Cow dung, an easily available bioresource harbours a wide variety of microorganisms which are still underexplored for their antimicrobial potential. The present investigation elucidated strong antagonistic potential in KD155 which was taxonomically identified as *P. aeruginosa*. The extra cellular chloroform crude extract of *P. aeruginosa* KD155 showed broad spectrum antimicrobial activity and found highly effective against *S. aureus*, *B. cereus* and *V. cholera*. The produced metabolite demonstrated strong antioxidant potential and was found to be non-toxic that may be considered as safe for human consumption. An attempt was made to recognize the chemical nature of the produced bioactive compounds revealing the presence of phenolic compounds (quercetin). The GC-MS confirmed the extracellular secretion of quercetin compounds by *P. aeruginosa* KD155. As far as the literature is concerned, this is the first report on isolation of quercetin producing *P. aeruginosa* from cow dung. The findings established the fact that bacterial strains from underexplored sources such as cow dung in this study have an enormous potential for the biosynthesis of bioactive compounds that may lead to the development of antimicrobial agents against microorganisms of medical importance.

## CRediT authorship contribution statement

**Kartikey Kumar Gupta:** Conceptualization, Supervision, Methodology, Writing - original draft, Writing - review & editing, Validation. **Deepanshu Rana:** Methodology, Data curation, Visualization, Investigation, Software, Writing - review & editing.

## Declaration of Competing Interest

The authors declared no conflict of interest.

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