Anti-microbial Activities of *Pseudomonas* Metabolites Methanolic Extracts against Different MDR Bacterial Pathogens

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ABSTRACT---

Background: ***A Multiple drug resistance (MDR) in pathogenic bacteria has become a significant public health issue for treatment. Among bacteria, Pseudomonas is another important genus except Streptomyces and Bacillus in production of antimicrobial compounds. The current study aim to to determine the antibacterial activity and preliminary characterization of antibacterial compounds produced by Pseudomonas species such as BB1D11, BN2D41, TG1D11, TR1D41, LH1D11 and TN1D41.***

Method: ***The antibacterial activity was checked by using bio autography method as well as agar well diffusion method, against four multiple drug resistant bacteria including three Gram negative bacteria (E.coli, Acinetobacter and Pseudomonas) and one Gram positive bacterium (Methicillin Resistant Staphylococcus aureus). Isolation test showed good activity against all the four MDR bacteria, by producing clear zone of diameter from 2mm up to 20mm. Optimum temperature for growth and antibiotic production of Pseudomonas BB1D11, BN2D41, TG1D11, TR1D41, LH1D11 and TN1D41 was 37C^0. It produces more metabolites when subjected to shaking incubation. In thin layer chromatography, the extracts were repeatedly inserted on a silica gel coated plate, which was run in mobile phase. Normal HPLC was perform to reveal the presence of antibacterial compounds.***

Results: ***By well diffusion assay a zone of inhibition range from 2-18 mm of diameter against different test bacteria. The components were separated, resulting in the formation of bands with different colors, each showing a different compounds. Biological screening was performed by bio autography, metabolites showed a significant activity at retention factor of 0.89. While HPLC at retention time 2.50-2.90 showed presence of significant antibacterial compounds.***

Conclusion: Pseudomonas BB1D11, BN2D41, TG1D11, TR1D41, LH1D11 and TN1D41 showed promising anti-microbial activity against different MDR bacteria. It is concluded that HPLC revealed the presence of DAPG at retention time 2.90 which inhibit the growth of MDR bacterial strains.

Keywords: MDR, *Pseudomonas, Streptomyces, Bacillus* and TLC

1. INTRODUCTION

Multidrug resistant bacteria is one of the most important public health problems currently. In American society such as infectious disease society of America (IDSA) identifies an antimicrobial resistance to treats the human health worldwide (1). Several problems trigger the severe vulnerability which is stood due to high level of MDR bacteria (2). The most important results in patient infected by MDR bacteria tend to be badly as compared to patient infected with susceptible organism. In this way, the high level of antibiotics resistant's effect of the new medicine and danger to reduce the activities of cancer disease, kidney transplantation and many other surgeries as well as their price are increased day by day(3,4).
However, MDR are related with nosocomial infection which leads to a community-acquired infection. The increasing level of MDR bacteria cause risk at population and mostly increase the quantity of infection in community population(5).
Furthermore, MDR bacteria that are effective in the community have to be capable to continue lacking of biofilm on inorganic compound. The biofilm production is important pathophysiologic constituent of periodontal infection, stomach infection with *H.pylori* middle ear infection, UTI, throat infection and other infection. MDR bacteria have the capability to develop the biofilm without of foreign particles in a community (6).

When microorganisms start growing in the presence of antibiotics, it is known as microbial resistance to antibiotic. The potential for antibiotic resistance was recognized in the early 1940s, almost immediately after the first large-scale clinical applications of penicillin, the first antibiotic [7]. Antibiotic resistance is gained by two types of genetic mechanisms: mutation and acquisition of new genetic material [8,9]. Examples of the former type are chloramphenicol-acetylating *staphylococci* and penicillin-inactivating *staphylococci* [10]. In the last three decades, as the number of antibiotics increased, bacterial resistance against antibiotics also reached to its peak, which leads to serious problems [11].
Antibacterial agents showed good activity, initially, but its effect reduced slowly with time, as many bacteria became resistant to these antibiotics. There are also bacteria which are resistant to multiple antibiotics. These bacteria are called super bugs [12]. The most well-known example of super bug is Methicillin resistant *Staphylococcus aureus* (*MRSA*). 53 million people are carriers of *MRSA* globally according to survey conducted in 2006 and this number is increasing day by day [13]. Vancomycin has been the therapeutic option to treat *MRSA*, but vancomycin resistant *S. aureus* (VRSA) have emerged clinically very rapidly because *MRSA* also develops resistant to vancomycin [14]. The development of "pan-resistant" of bacterial strains, specially strains of *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, occurred more recently, after most major pharmaceutical companies stopped the development of new antibacterial agents [15]. It was proposed in 1973 that the microorganisms which produce antimicrobial components have a main role in producing antibiotic-inactivating enzymes for their own protection [16]. Some environmental bacteria contain natural antibiotic resistance genes, like the soil *Pseudomonas aeruginosa* [17]. The main reason of multiple drug resistance is the misuse (overuse) of antibiotics [18].
Pseudomonas spp. also produces bioactive metabolites. Mupirocin free acid (produced by *Pseudomonas*), and its salts and esters are agents currently used in creams, ointments, and sprays as a treatment of Methicillin-resistant *Staphylococcus aureus* infection [19].
Antibiotic producing bacteria are gaining importance by many investigators as bio control agents against pathogenic bacteria [20,21]. *Pseudomonas* sp. isolated from soil showed antimicrobial activity against some pathogenic bacteria *S. aureus* [22]. Similarly, other MDR strains are also found to be susceptible to metabolites, produced by *Pseudomonas putida* [23].
The objective of this study is to the protective effect of anti-microbial activities of *Pseudomonas* methanolic extracts against different MDR bacterial pathogens.

2. MATERIAL AND METHODS

Pseudomonas spp. (*BB_1D_11, BN_2D_41, TG_1D_11, TR_1D_41, LH_1D_11 and TN_1D_41*) were checked. The samples were identified by Gram staining and biochemical tests.

***Extraction of crude secondary metabolites with organic solvent**:* Crude metabolites were extracted from culture of *six different strain of pseudomonas* grow in 200 ml of nutrient broth in a shaking incubator for 48 hours at 37 ^oC then mixed with ethyl acetate in the ratio of 1:1 sonicated for 15 minutes in a sonicating bath (UltrasonsMedi-II) to break all the cells. The dead cells layer were separated by separating funnel Cell free layer having ethyle acetate and metabolites were collected in a flask and subjected to the rotary evaporator (Heidolph Laborata 4000). The crude extracts of active compounds remained stuck to the bottom of rotatory evaporator, which were mixed in methanol and were collected in clean glass tubes.

Antibacterial activity (well diffusion method): *Antibacterial activity of crude extracted metabolites:* Test bacteria *MRSA*, *Escherichia coli*, *pseudomonas aeruginosa* and *acintobacter baumannii* were obtained from department microbiology KUST and maintained by sub-culturing on Nutrient Agar. Methanolic extract extracted from *six different strain of pseudomonas* were tested by Agar well diffusion method for their activity against MDR bacterial pathogens (Valgas *et al.* 2007). The prepared culture plates were inoculated with different test bacteria using plate method. Wells were made on the agar surface with 6mm cork borer. Bacterial lawn of test organisms were formed by sterile swab on muller hinton agar, each. Six wells of diameter 6mm were made in each plate by cork borer. The 50 µl of methanol extracts was poured in each well by micropipettes. The plates were left for 5 minutes and then incubated at 37^oC for 24 hours. The zone were observed around the wells.

Detection of metabolites by thin layer chromatography: 20 µl of sample were spotted on thin layer chromatographic column coated with silica gel. The spotted plate were transfer to gel tank having organic solvent chloroform: acetic acid (95:5) after 30 minutes of capillary movement of organic solvent on silica plate the spotted plate were observed under UV of 260nm and 280nm as well sprayed by Spraying reagent Anisaldehyde/H_2SO_4 and Spraying Ehrlich reagent

High performance liquid chromatography (HPLC): HPLC were performed to detect the retention time of secondary metabolites of various strains of pseudomonas bacterial. Before the investigation methanolic extract were dissolved in a DMSO. 20 µl methanolic crude extract were injected into the HPLC equipped with silica column (5 µm; 4.6×150 mm) and

eluted at 1 ml/min. initially, 80% acetonitrile/ water (v/v, both acidified with 0.1% acetic acid), was run for 2 min then 80% acetonitrile (acidified with 0.1% acetic acid) run for 32 minute period. The HPLC mobile-phase consisted of Acetonitrile: Water (80:20) at a flow rate of 0.8 ml/min and detected at a wavelength of 254 nm. Resolutions results may vary from column to column.

3. RESULTS AND DISCUSSION

Extraction of secondary metabolites: protocols for most of the secondary metabolites extraction are well known and they are produce during growth and stationary phases at higher rate [24]. Reported literature presented that *Pseudomonas* strains are at third number producers of secondary metabolites with highest antimicrobial activities [25, 26]. In current study, different *pseudomonas* strains were found that produces Secondary Metabolites (SM).

Already describing protocols for the extraction of secondary metabolites making it more easy with choice of extraction procedure such as the process of liquid-liquid extraction are vastly applicable for compounds isolation by the use of different solvents such as ethyl acetate and chloroform [27]. In current the methanol was found best solvent to dissolve metabolites of pseudomonas strains which show protection against bacterial MDR.

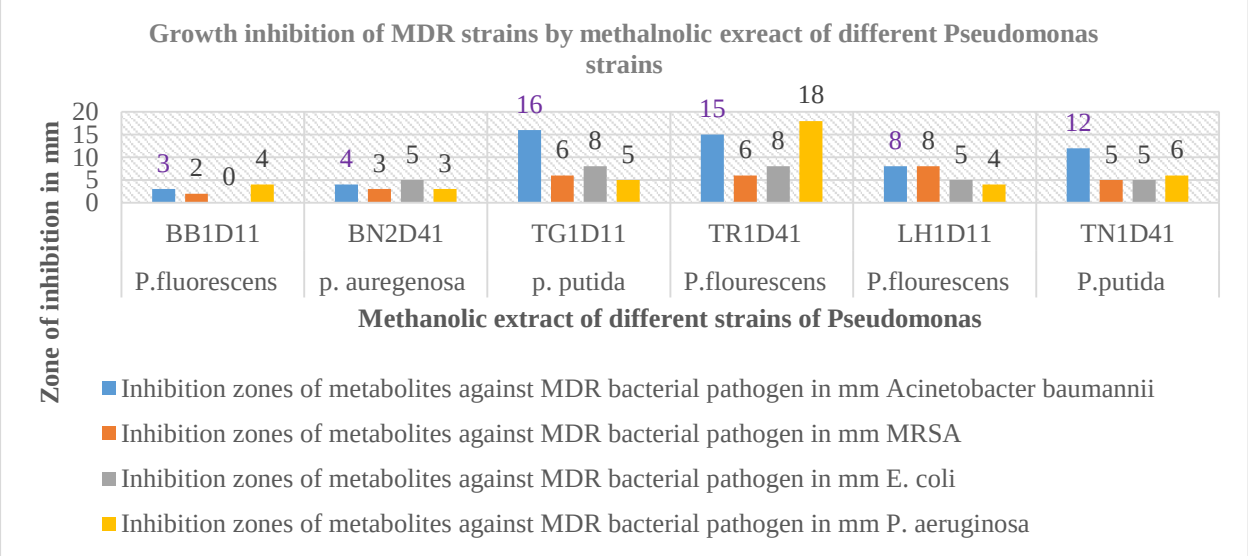
Antibacterial activity of secondary metabolites (SM) The crude methanolic extract of SM isolated from *P.fluorescens* (*BB1D1*) showed a zone of inhibition, i.e 03 mm against *A. baumannii*, 02 mm against MRSA, 04 mm against *P. aeruginosa* and having no activity against *E. coli* while the methanolic extract of *p. auregenosa* (BN2D41) present zone of inhibition i.e. 04 mm against *A. baumannii*, 03mm against MRSA, 05 mm against E. coli and 03mm again *p. aeruginosa* whereas *p. putida* (*TG_1D_11*) produced zone of inhibition i.e 16mm against *A. baumannii*, 06 against MRSA, 08 against E. coli and 05 against *p. aeruginosa*. As shown in figure 1.

The crude methanolic extract obtained from *P.flourescens* (TR1D41) showed a zone of inhibitions i.e 15mm against *A. baumannii*, 06mm against MRSA, 08mm against *E. coli* and 18mm *against P. aeruginosa*. While another *P.flourescens* (LH1D11) strain metabolites presented zone of inhibition i.e. 08 mm against *A. baumannii*, 08mm against MRSA, 05mm against E. coli and 04mm against *p. auregenosa* as shown in figure 1.

Another *P. putide* (TN1D41) strain extracted SM showed inhibition zones i.e. 12mm against *A. baumannii*, 05 mm against MRSA, 05mm against *E. coli* and 06mm against *p. auregenosa* as shown in figure 1.

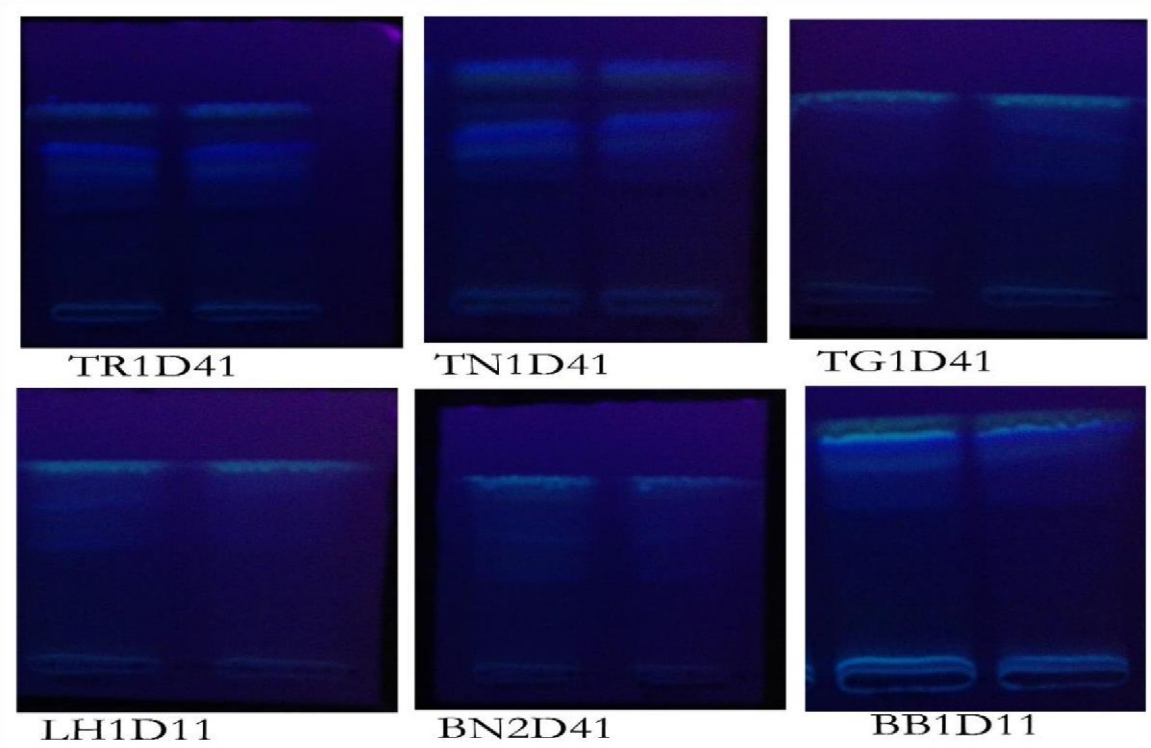
Recent finding presented that *pseudomonas* metabolites have efficient activity against *bacillus subtiles, Paracoccus paratrophus, Pseudomonas diminutus* and *Micrococcus luteus* with zones of inhibition of 15 mm, 25 mm, 8 mm and 3 mm respectively [28]. According to Sunker and Nachiyar (2013) [29] showed that *Pseudomonas aeruginosa have* promising bacterial inhibition activity against *Staphylococcus aureus* , *Escherichia coli* and *Salmonella typhi*. The antibacterial activity of 2,4-diacetylphloroglucinol (2,4-DAPG) extracted from *Pseudomonas fluorescens* was interpreted against 23 vancomycin resistant *Staphylococcus aureus* (VRSA) and *Enterococcus* spp. [30].

Figure 1. Antibacterial inhibition of pseudomonas metabolites against MDR.

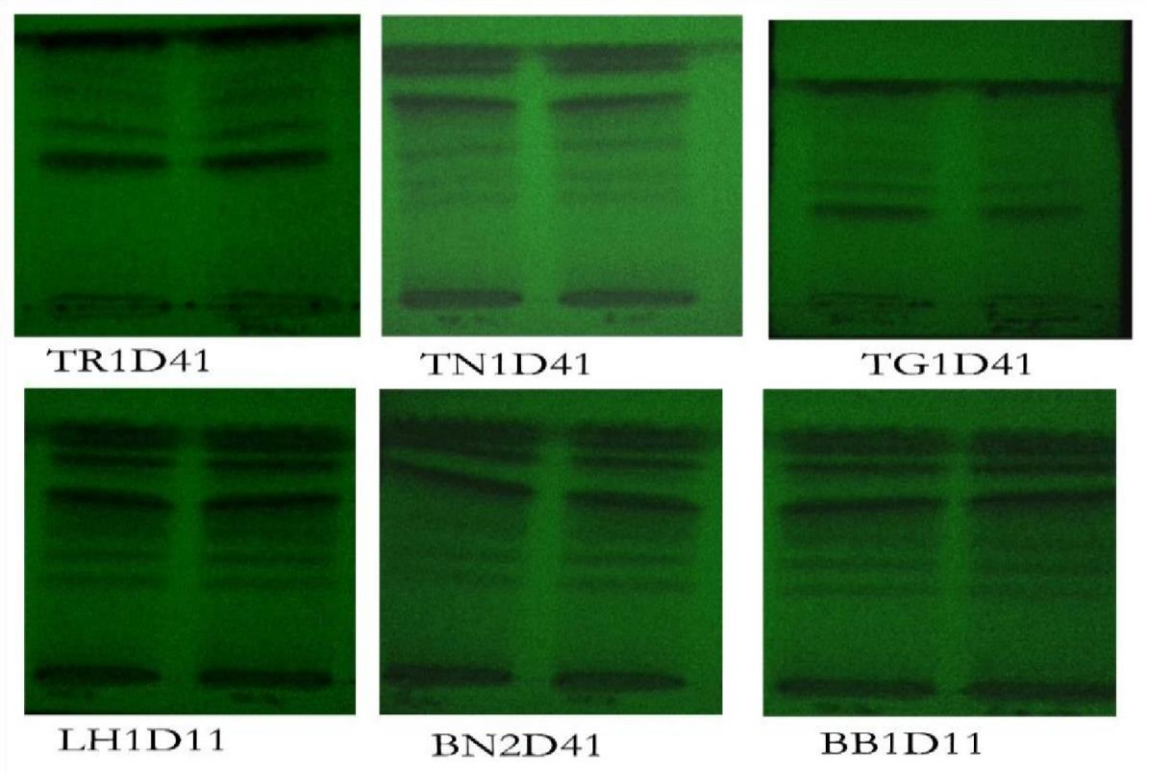


Identification of metabolites by TLC

TLC bands visualized under 280nm light



TLC bands visualized under 260nm light



Bio-autography

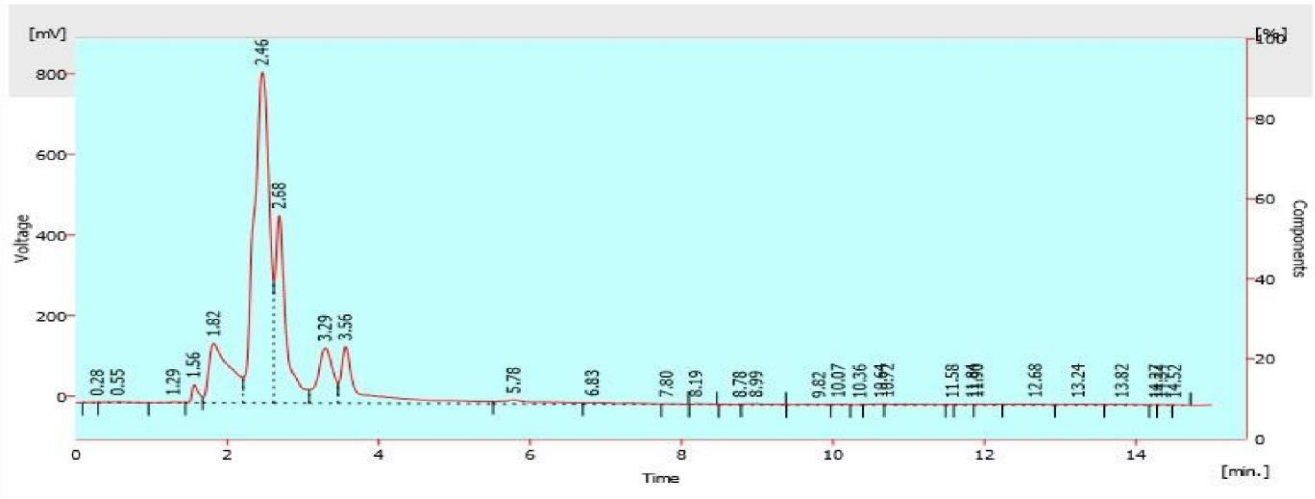
The methanolic extract of various strains of pseudomonas species were screened by thin layer of silica gel the crude secondary metabolites of different strains showed different spot at retention factors (Rf) i.e TR_1D_41 Rf range from 0.89 to 0.97, TN_1D_41 Rf 0.89 and BB_1D_11 Rf 0.98 showed significant results while rest of the compounds have non-significant values. The antibacterial compounds were confirm i.e . 2, 4-DAPG, Pyrrolnitrin and Phenazine at the Rf range of 0.89 to 0.98 produces by TR_1D_41, TN_1D_41 and BB_1D_11 respectively. They present closed relevance to the R*f* value of synthetic 2,4-diacetyl phloroglucinol (0.81-0.89) and spots R*f* value vary from 0.5 to 0.80 have closed R*f* value of synthetic phenazine (0.10 and 0.89) Earlier, three strains (PF1, FP7 and PB2) were stated to yield 2,4-DAPG which had the similar R_f value as synthetic phloroglucinol (0.76) [31]. Distinct spots of 2,4-DAPG were reported to have R*f* of 0.88 that visualized on the TLC plate after spraying by diazotized sulphanilic acid [32]). Secondary metabolite secreted by *Pseudomonas brassicacearum* J12 with *Rf* value of 0.10 was characterized as 2,4-DAPG which inhibited the growth of phytopathogenic bacteria *Ralstonia solanacearum* [33]. The zone of inhibition shown by TLC column of TR_1D_41 on agar plate i.e 12mm against *Acinetobacter baumannii, 8mm against E. coli, 4mm against MRSA, 2mm against P. aeruginosa while* TN_1D_41 and BB_1D_11 shown zone of inhibition against *Acinetobacter baumannii 2mm and 4mm respectively. The significant results shown by* TR_1D_41 TLC column on agar plates as shown table 1. The bio autographic analysis of pseudomonas metabolites against MDR strains.

Table .1. Bio autographic analysis of metabolites of pseudomonas against MDR

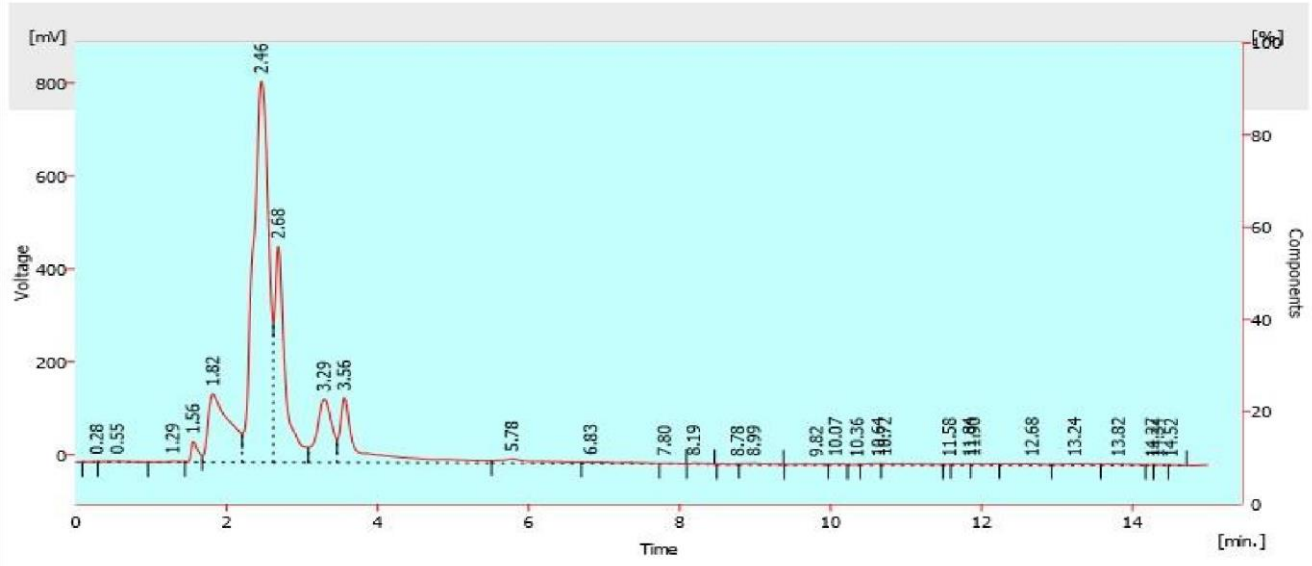
Isolate code	Rf value	Zone of inhibition	Susceptible MDR
TR_1D_41	0.91	12 mm	Acinetobacter *baumannii*
TR_1D_41	0.89	8 mm	Escherichia *coli*
TR_1D_41	0.97	4 mm	MRSA
TR_1D_41	0.10	2 mm	*Pseudomonas aeruginosa*
TN_1D_41	0.89	2 mm	Acinetobacter *baumannii*
BB_1D_11	0.98	4 mm	Acinetobacter *baumannii*

High performance liquid chromatography

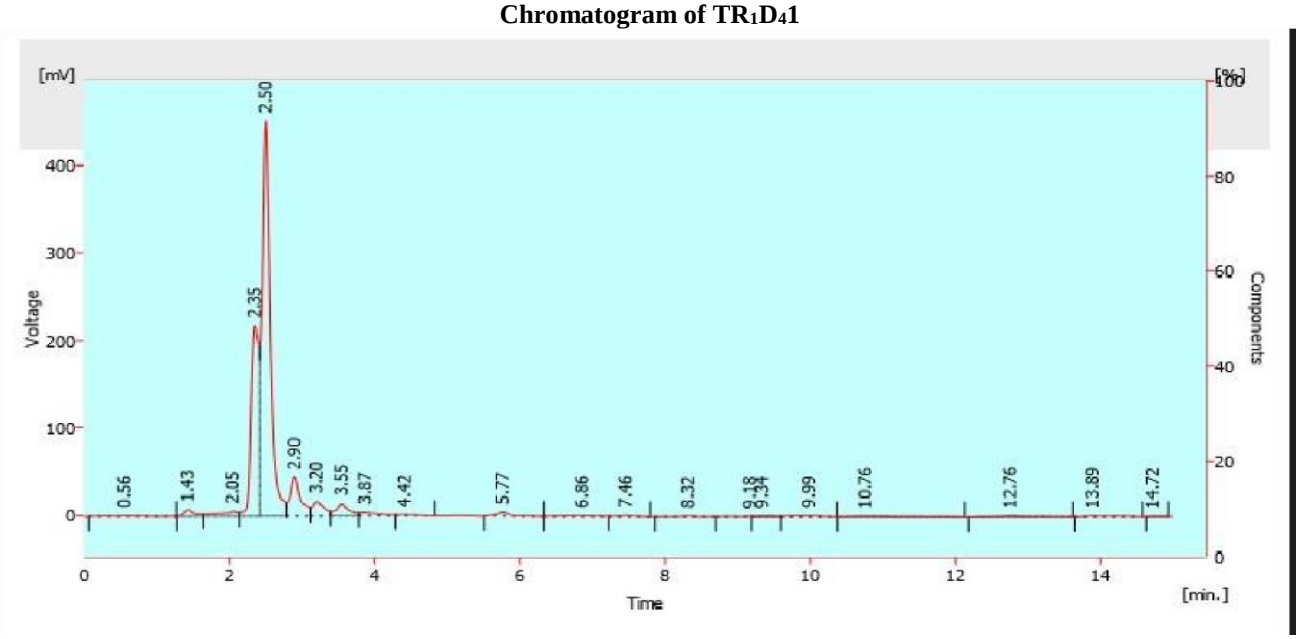
The crude methanolic extract shown promising activity against different test MDR organism. The secondary metabolites were subjected to HPLC for further identification and characterization. The key compound revealed at the retention time at HPLC 2.50, 2.6 and 2.90 and Rf factor of metabolites range from 0.89 to o.98 revealed the presence of DAPG as shown in below figures. *P. fluorescens* and challenge metabolites eluted at retention time ranging from 20.00 min to 21.30 min in HPLC and *Rf* value 0.88 in TLC developed by acetonitrile : methanol : water (1:1:1) solvent system [34].



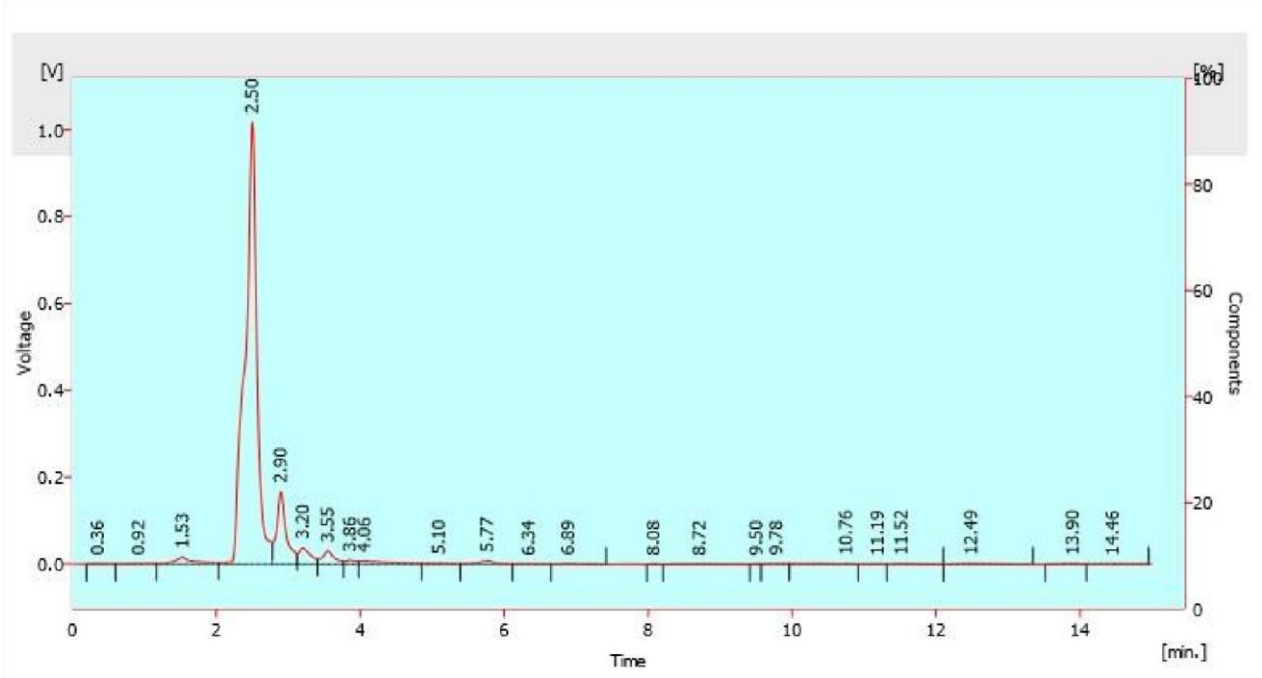
Chromatogram of BB_1D_11



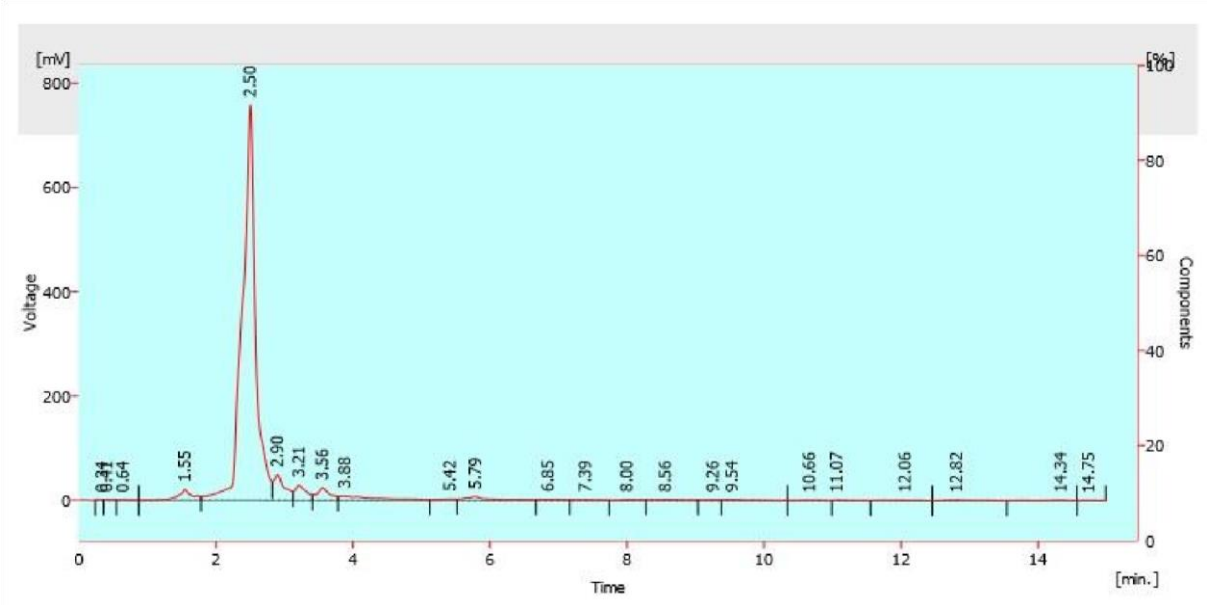
Chromatogram of TR_1D_41



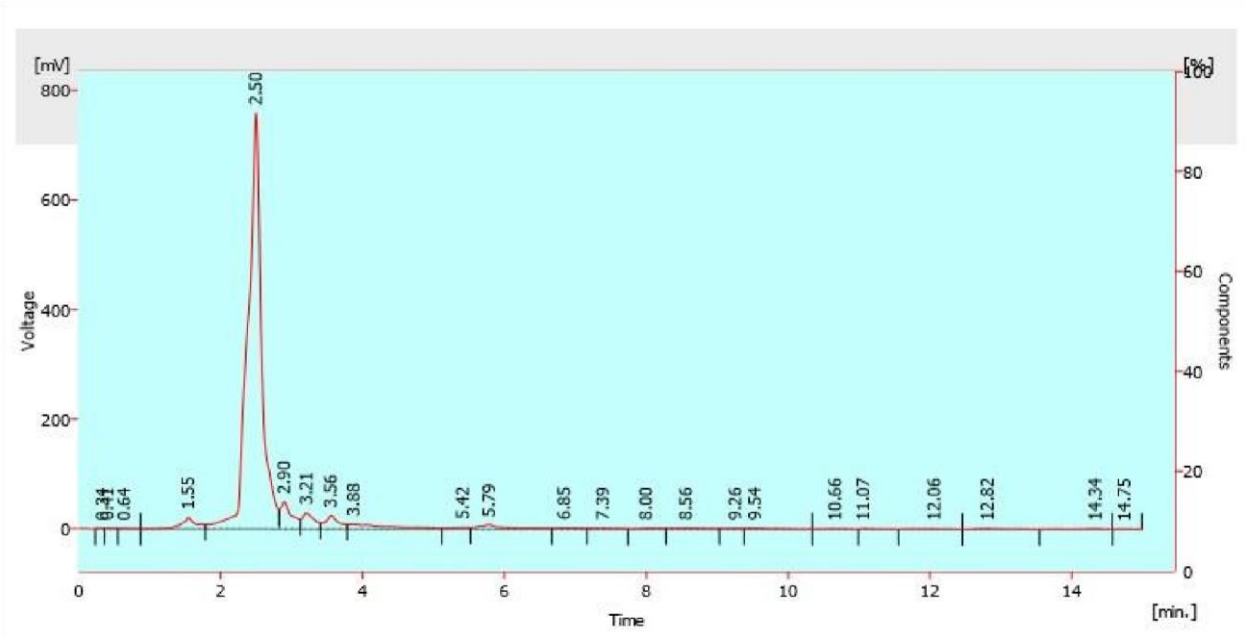
Chromatogram of BN_2D_41



Chromatogram of LH_1D_11



Chromatogram of TG_1D_41



Chromatogram of TN_1D_41

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