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Qualitative assessment of bioactive compounds of actinomycetes AIA25a isolate using HPTLC and GCMS technique

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In the present study AIA25a strain has been isolated from the rhizospheric soil actinomycetes from selected locations of Rajasthan. Antimicrobial activity test of AIA25a against bacterial strains *S. aureus* (MTCC 3160), *P. aeruginosa* (MTCC 1688), *P. mirabilis* (ITCC 425) has been carried out. A good activity is shown by chloroform crude extract against *P. aeruginosa* (IZ=13.4±0.3mm), moderate activity (IZ=11.1±0.1) is shown against *S. aureus* and no activity is observed against *P. mirabilis* strain. Different compounds isolation is executed with the help of chloroform solvent. Using GC-MS identification has been carried out for compounds present in AIA25a strain followed by partial purification of compounds. With the help of HPTLC method partial purification of compounds has been achieved and followed by GC-MS analysis for identification of separated compounds. The Bioautography technique has been applied for the detection of antimicrobial spot of partial purified compounds of crude extract of AIA25a isolate. Results of this study show the presence of various compounds in the rhizospheric soil of Rajasthan.

Keywords: Actinomycetes, AIA25a, Bioautography, Florescent light, HPTLC, Partial purification

A critical public health challenge has been the continuous rise in global resistance to existing antibiotics, the prevalence of multidrug-resistant pathogens, and the rapid growth of cross-resistance with new antibiotics[1,2]. It is possible to acquire bacterial antibiotic resistance by mutation or horizontal transfer of a resistant gene, with environmental and genetic factors affecting phenotypic expression[3]. There has been an emergence of resistance in all groups of antibiotics that compromises their use[4]. Research efforts are therefore geared to the discovery and development of novel and successful therapeutic agents[5]. Despite attempts to create new synthetic antibiotics[6], natural environment bioprospecting has been responsible for discovering, developing and selling the majority of antibiotics[7] and continues to provide key drug production scaffolds[8]. Consequently, the key sources for the discovery of novel antibiotics are still natural products, primarily bacteria and fungi[7]. The chances of new discoveries of bioactive molecules from different well-known bacteria have dropped significantly recently. In the discovery of new antibiotic molecules, there are some explanations for this decrease, including the large number of secondary metabolites that are cryptic or silent under popular culture conditions, thus inhibiting the recognition of the total collection of isolated molecules[9] and regular genetic transfers between species that share similar chemical and physical selection factors in environment[10,11], making the probability of obtaining new molecules from the same environment more difficult. Actinomycetes are gram positive species that are saprophytic bacteria and are widely distributed in soil and other terrestrial environments. They are important contributors to complex organic matter biopolymers, such as chitin and lignocellulose, for example. Many well-known secondary metabolites, with many essential uses in the medical, agricultural and pharmaceutical sectors, such as antibiotics, antitumor agents, anti-infection agents and other novel natural products, serve as manufacturing facilities. There are many researches on the biological regulation of plant pathogenic bacteria and fungi using actinomycetes[12,13]. So the present study is focussed on the bioactive compounds isolated from the actinomycetes strain from rhizospheric soil of Rajasthan.

Experimental Section

Sample collection site

The rhizospheric soil samples were collected from four different locations (Kota, Jaipur, Alwar, and Udaipur) of Rajasthan, India. The debris present in soil samples were removed before collection. The collection site was dug into 12–15 cm in and around, and approximately 15-20 gm of the rhizospheric soil was collected in a sterile bag and brought to Research laboratory of JECRC University, Jaipur, Rajasthan. Rhizospheric soil was oven dried for 24-48h due to presence of moisture. Samples were processed and then stored at 4°C for further testing[14].

Isolation and maintenance

Isolation process was carried out using Actinomycetes isolation agar (AIA) medium with composition; Sodium caseinate -2.0 g, L-Asparagine 0.1 g, Sodium propionate 4.0 g, Dipotassium phosphate 0.5 g, Magnesium sulphate 0.1 g, Ferrous sulphate 0.001 g, Agar 15.0 g, and final *p*H 8.1±0.2. Suspended 21.70 g of media into 1000 mL distilled water containing 5 mL of glycerol. On AIA medium, a total of 65 strains were isolated and the strain AIA25a was isolated from the soil of Alwar, Rajasthan. Saline solution (100 mL) was prepared in a 250 mL flask. Sodium chloride (0.8 g) in 100 mL of distilled water was mixed and autoclaved. 1g of each dry soil sample was suspended in 9 mL of sterile distilled water and shaken well. Diluted aliquots (0.2 mL) of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were spread on the plates. The autoclaved AIA medium containing petri-plates were prepared aseptically so that it minimizes the fungal and bacterial contaminations. Dilutions were spread with a 'L' shaped glass spreader containing actinomycetes isolation agar (AIA) and Plates were incubated inverted at 37°C for 7–14 days. Immediate after the incubation period, morphologically dissimilar colonies were picked up from the Petri plates and re-streaked every colony in suitable media and pure cultures were obtained and stored for further testing[15].

Fermentation, Centrifugation and Solvent extraction for bioactive metabolites

Production of antimicrobial compounds was carried out by 500 mL Luria broth. Broth was prepared and distributed into two 250 mL clean flasks and autoclaved the broth. After sterilization process upon cooling each broth flask was inoculated with the strain aseptically in laminar air flow hood and kept on shaker incubator at 150 Rpm at 30°C for around 21 days. After incubation period was over, the cell suspension was kept for centrifugation at 5000 Rpm for 15-20 min for separation of the supernatant and the biomass. Biomass was discarded and supernatant was collected separately in a beaker for further tests. After fermentation, clear filtrate was used for the detection of antimicrobial measures and solvent extraction method was used for the isolation of antimicrobial compounds from chloroform and Ethyl acetate solvent. Solvent extraction was followed by mixing of solvent and broth in 1:1 (v/v) ratio and shaken well for some time for complete extraction of metabolites[16]. This mixture was kept undisturbed till 2 dissimilar layers get separated and clearly visible. Two layers when separated, were collected in different beakers and solvent was evaporated by keeping beakers on water bath at 50-60°C and crude remaining in beakers were measured and used for further antimicrobial testing[17].

Antimicrobial screening against standard pathogens

Primary and secondary screening of strain was performed against bacterial and fungal indicator pathogens. Disc diffusion method was applied for primary and secondary screening of isolate on Mueller Hinton Agar (MHA) petri-plates[17]. Plates were kept at 37°C for 24–48 h and results were observed and complete inhibition zone (IZ) was measured in mm. Isolate was screened for their antimicrobial activity against selected indicator bacterial and fungal cultures. Studies were conducted on antimicrobial activity of isolate against 3 bacterial strains such as *S. aureus* (MTCC 3160), *P. aeruginosa* (MTCC 1688), and *P. mirabilis* (ITCC 425) (Fig. 1).

Identification of bioactive compounds with Gas Chromatography–Mass Spectrometry (GC–MS)

The analysis of the active chloroform extract of

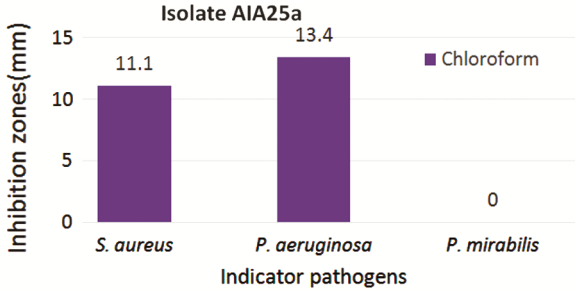

Fig. 1 — Antimicrobial activity of cholroform extract of AIA25a strain against indicator pathogens *S. aureus, P. aeruginosa* and *P. mirabilis*

isolate was executed by Gas Chromatography–Mass Spectrometry (GC–MS). Shimadzu QP2010 ultra was used for identification of compounds and gas chromatography interfaced to a mass spectrometer. The appliance was built with Elite-1 fused silica capillary. Helium gas (99.99%) was the carrier gas with a constant flow rate of 1.21 mL/min and with split ratio: 10. Temperature of Injector was set on 260°C; Ion-source temperature was 200°C. The oven temperature was intended from 60°C (constant for 3 min.) with an increment of 280°C for around 22 min. Mass spectra were taken at 70eV; a scan interval of 0.5 sec. The chemical composition of the extract was determined by measuring the peak area and the retention time by comparing the NIST 14 library[17,18].

Partial purification of compounds by HPTLC method

Instrument HPTLC-CAMAG Linomat5 ("Linomat5_192428" S/N 192428 (1.00.13)) was used for this process. Before starting of procedure and application of sample spot, aluminium sheet was kept in between two glass plates and placed in the oven at 110- 120°C for 15 min. Plate size was - 20.0 × 10.0 cm, syringe size- 100µL, total number of tracks were 12 (3 for each sample), application position Y-8mm, application volume-5µL, band length-8mm. Automatic applicator- nitrogen gas sprays sample and standard from a syringe on TLC plates as bands. Band wise application led to better separation.

The extracellular crude extract was dissolved in chloroform and spotted by means of a syringe on TLC plates (TLC 20×10 cm). By using diverse mobile phase solvents for instance in order to establish the best solvent system for separation of the bioactive compounds, in addition to determining the R_f values of the bioactive compounds with the suitable mobile phase. The mobile phase used was Toluene: Ethyl acetate: Formic acid in 5:5:1 ratio was used for running the spots. After this, the plate was dried and observed under UV light, to detect the spots position[18,19] (Fig. 2). R_f values were recorded for each spot. R_f value for different spots were recorded as; spot a= 0.08, spot b= 0.47, spot c= 0.51, spot d= 0.54 and spot e=0.72 (Table 1) obtained on the TLC plate under UV light at 254 nm and also observed under fluorescent light at 366nm.

The Bioautography

The Bioautography technique includes the TLC to determine the active antimicrobial metabolites position[20]. In this method, antimicrobial compounds presence was tested by placing them (TLC plate) on Petri-plate containing media and test pathogens. By "establishing direct contact of the TLC plate on the surface of an MHA plate occupied with the microbial pathogen, then, incubated for 24 hours at 30°C. After incubation, the inhibition zones (IZ) around the active spots present on the plate was examined by our naked eyes. The inhibition zones on the media plate point towards the presence of active antimicrobial compounds that causes inhibition of the tested microbial organism.

Result and Discussion

The focus of present study was investigation of antimicrobial compounds from actinomycetes strain from Rajasthan soil. Antimicrobial activity test of AIA25a against bacterial strains *S. aureus* (MTCC 3160), *P. aeruginosa* (MTCC 1688), *P. mirabilis* (ITCC 425). A good activity was shown by chloroform crude extract against *P. aeruginosa* (IZ=13.4±0.3mm), moderate activity (IZ=11.1±0.1) was shown against *S. aureus* and no activity was observed against *P. Mirabilis* strain (Fig. 1).

In HPTLC method the mobile phase used was Toluene: Ethyl acetate: Formic acid in 5:5:1 ratio was used for running the spots. R_f value for different

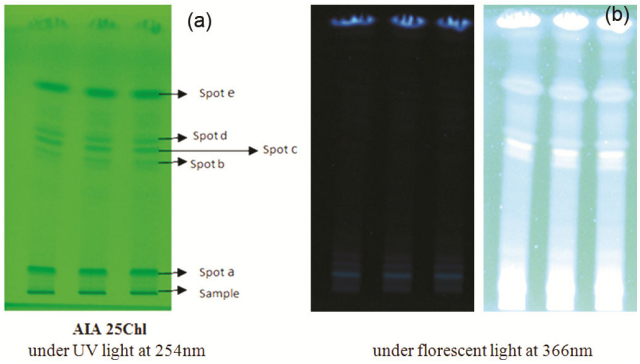

Fig. 2 — Partial purification of bioactive compounds by HPTLC of chloroform extract of AIA25a isolate under different wavelength

Table 1 — Mobile phase, solvent ratio, R_f values, spraying reagents and UV visibility for bioactive compounds of AIA25a isolate

Sample	Extract	Mobile phase	Sol-vent ratio	R_f values	Spraying reagent	UV visibility
AIA25a	Chloroform	Toluene: Ethyl acetate: Formic acid	5:5:1	spot a=0.08 spot b=0.47 spot c=0.51 spot d=0.54 spot e=0.72	-	UV visible

spots were recorded as; Spot a=0.325, spot b=0.546, spot c=0.662, and spot d=0.686 under UV light at 254 nm (Fig. 2).

AIA25a isolate was subjected to separation and identification of the compounds by HPTLC GCMS method. Three spots of chloroform crude extract were spotted on a pre-coated silica gel sheet and run to obtain more amounts of compounds. Different mobile phases were checked to attain the good separation and R_f values of the separated spots. GCMS analysis of chloroform crude of AIA25a were shown in the chromatogram (Fig. 3) with some major compounds (Table 2) having antimicrobial activity such as-pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-(61.62%) as anti-bacterial and anti-fungal, Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3 (phenylmethyl)-(8.43%) as antifungal, Phenol, 2,4-bis(1,1-dimethylethyl)-, phosphite (3:1)(6.06%) as antioxidant, Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, octadecyl ester(5.04%) as

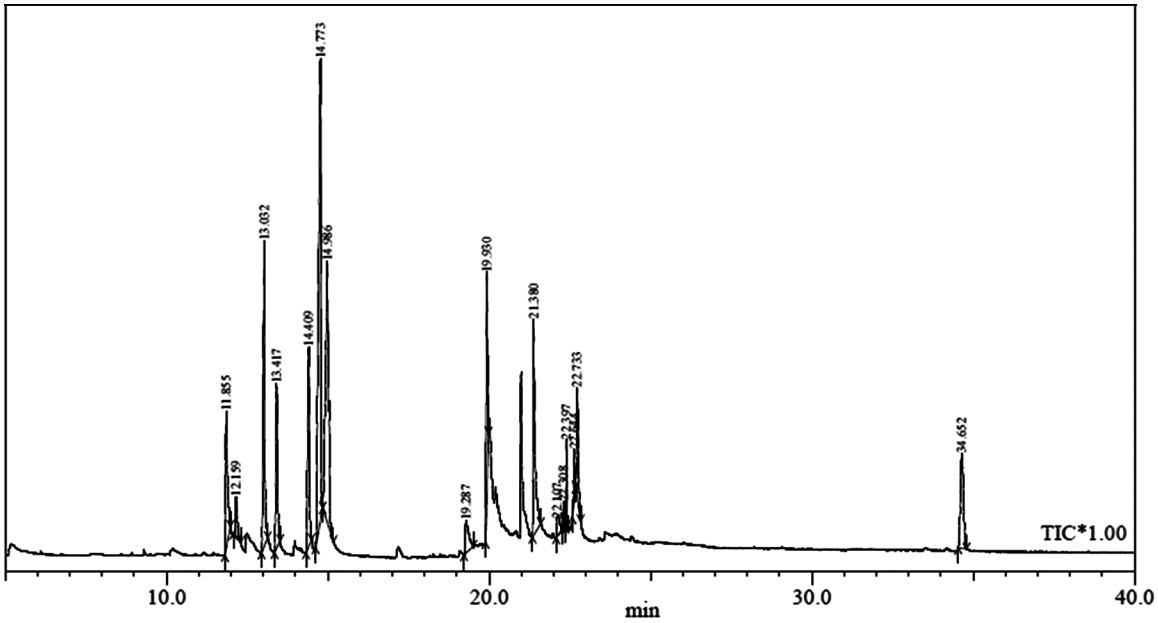

Fig. 3 — Chromatogram of chloroform crude extract of AIA25a isolate

Table 2 — Compounds, Retention time, % area, molecular formula and molecular weight present in chloroform crude extract of AIA25a isolate detected from GCMS technique

S. No	Compounds	Chloroform extract		Antimicrobial activity	Mol. formula	Mol. weight	References
		R. Time	Sum of Area%				
1	Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	13.032	61.62	Anti-bacterial Anti-fungal	$C_{11}H_{18}N_2O_2$	210	Awla *et al*., 2016
2	Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	21.38	8.43	Anti-fungal	$C_{14}H_{16}N_2O_2$	244	Awla *et al*., 2016
3	3-Methyl-1,4-diazabicyclo[4.3.0] nonan-2,5-dione, N-acetyl-	11.855	7.1	Not reported	$C_{10}H_{14}N_2O_3$	210	-
4	N-Formyltryptamine	19.93	6.49	Not reported	$C_{11}H_{12}N_2O$	188	-
5	Phenol, 2,4-bis(1,1-dimethylethyl)-, phosphite (3:1)	34.652	6.06	Antioxidant	$C_{42}H_{63}O_3P$	646	Wassenaar *et al*., 2017
6	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, octadecyl ester	22.644	5.04	Antifungal & Antioxidant	$C_{35}H_{62}O_3$	530	Elnawawy *et al*., 2017
7	2,5-Piperazinedione, 3,6-bis (2-methylpropyl)-	19.287	2.33	Antifungal	$C_{12}H_{22}N_2O_2$	226	Raut and Hamde, 2018
8	1,2-benzenedicarboxylic acid	22.397	1.30	Antimicrobial	$C_{24}H_{38}O_4$	390	Gunalan *et al*., 2014
9	1H-indene, 1-hexadecyl-2,3-dihydro-	22.107	1.10	Anticancer	$C_{25}H_{42}$	342	Arora & Kumar, 2018
10	Benzyldiethyl-(2,6-xylylcarbamoylmethyl)-ammonium benzoate	22.308	0.53	Not reported	$C_{28}H_{34}N_2O_3$	446	-

antifungal and antioxidant, 2,5-Piperazinedione, 3,6-bis(2-methylpropyl)- (2.33%) as antifungal, 1,2-benzenedicarboxylic acid (1.30%) as antimicrobial, 1H-indene, 1-hexadecyl-2,3-dihydro-(1.10%) as anticancer. The activity of compound, 3-Methyl-1,4 diazabicyclo [4.3.0] nonan-2,5-dione, N-acetyl-(7.1%), N-Formyltryptamine (6.49%), and Benzyldiethyl-(2,6-xylylcarbamoylmethyl)-ammonium benzoate is still not reported. The presence of compounds in sample extract on the basis of area % have been shown in Fig. 4.

For the compounds separation mobile phase used was Toluene: Ethyl acetate: Formic acid (5:5:1) and R_f values recorded for five spots; spot a=0.08, spot b=0.47, spot c=0.51, spot d=0.54 and spot e=0.72 observed at 254nm and 366nm. Further analysis of four out of five spots of AIA25a isolate were carried out by GCMS method and spots were analysed for presence of compounds at different retention time.

Chromatogram shows [Fig. 5(a)] four compounds like; Docosyl behenate (78.19%), Eicosane (3.19%), 1,2-Benzenedicarboxylic acid, ditridecyl ester (2.09%), and Hexacosyl heptafluorobutyrate (16.53%) were found on spot a (Table 3A). Spot c has four compounds [Fig. 5 (b)] (Table 3B) as; Phenol, 2,4-bis (1,1-dimethylethyl)-, phosphite (3:1) (89.35%), Dodecane, 4,6-dimethyl-(2.64%), Heneicosane (2.76%), and Eicosane (1.76%).Spot d of isolate was identified with three compounds [Fig. 5 (c)] (Table 3C) viz;1,2-benzenedicarboxylic acid (43.24%), Alloaromadendrene (16.98%), and Dodecane, 4,6-dimethyl- (25.84%) and four compounds [Fig. 5 (d)]

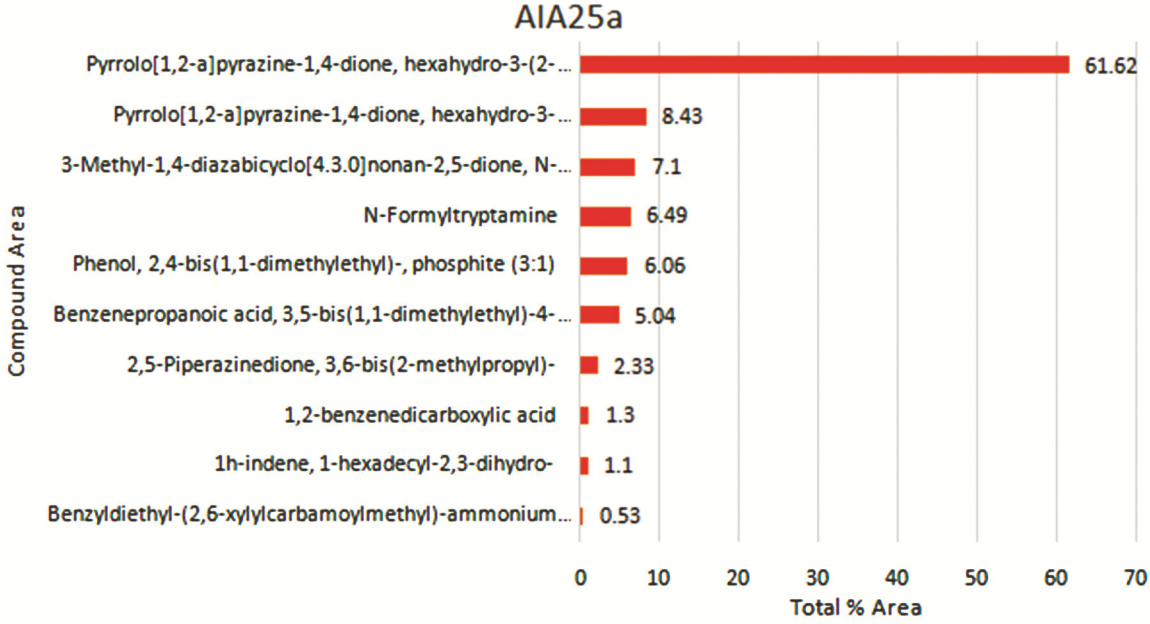

Fig. 4 — Bioactive compounds present in highest to the lowest % amount in AIA25a isolate

Table 3A — Partially purified compounds of chloroform extract of spot a, of isolate AIA25a, % area, molecular formula, and molecular weight present in sample at different retention time detected from GCMS technique

Peak	RT	Area%	Compounds	Mol. formula	Mol. weight
1	11.477	3.19	Eicosane	$C_{20}H_{42}$	282
2	22.438	2.09	1,2-Benzenedicarboxylic acid, ditridecyl ester	$C_{34}H_{58}O_4$	530
3	23.698	16.53	Hexacosyl Heptafluorobutyrate	$C_{30}H_{53}F_7O_2$	578
4	26.798	78.19	Docosyl behenate	$C_{44}H_{88}O_2$	649.2

Table 3B — Partially purified compounds of chloroform extract of spot c of isolate AIA25a, % area, Molecular formula, and Molecular weight present in sample at different retention time detected from GCMS technique

Peak	RT	Area%	Compounds	Mol. formula	Mol.weight
1	6.137	2.64	Dodecane, 4,6-dimethyl-	$C_{14}H_{30}$	198
2	8.952	2.76	Heneicosane	$C_{21}H_{44}$	296
3	11.479	1.76	Eicosane	$C_{20}H_{42}$	282
4	34.834	89.35	Phenol, 2,4-bis(1,1-dimethylethyl)-,phosphite (3:1)	$C_{42}H_{63}O_3P$	646

(*Contd.*)

Table 3C─ Partially purified compounds of chloroform extract of spot d of isolate AIA25a, % area, Molecular formula, and Molecular weight present in sample at different retention time detected from GCMS technique (*Contd*.)

Peak	RT	Area%	Compounds	Mol. formula	Mol.weight
1	6.138	25.84	Dodecane, 4,6-dimethyl-	$C_{14}H_{30}$	198
2	8.412	16.98	Alloaromadendrene	$C_{15}H_{24}$	204
3	22.45	43.24	1,2-benzenedicarboxylic acid	$C_{24}H_{38}O_4$	390

Table 3D─Partially purified compounds of chloroform extract of spot e of isolate AIA25a, % area, molecular formula, and molecular weight present in sample at different retention time detected from GCMS technique

Peak	RT	Area%	Compounds	Mol.formula	Mol. weight
1	6.136	21.5	Dodecane, 4,6-dimethyl-	$C_{14}H_{30}$	198
2	8.954	17.18	Octadecane, 1-iodo-	$C_{18}H_{37}I$	380
3	11.491	4.81	Octadecane	$C_{18}H_{38}$	254
4	22.445	27.96	1,2-Benzenedicarboxylic acid, ditridecyl ester	$C_{34}H_{58}O_4$	530

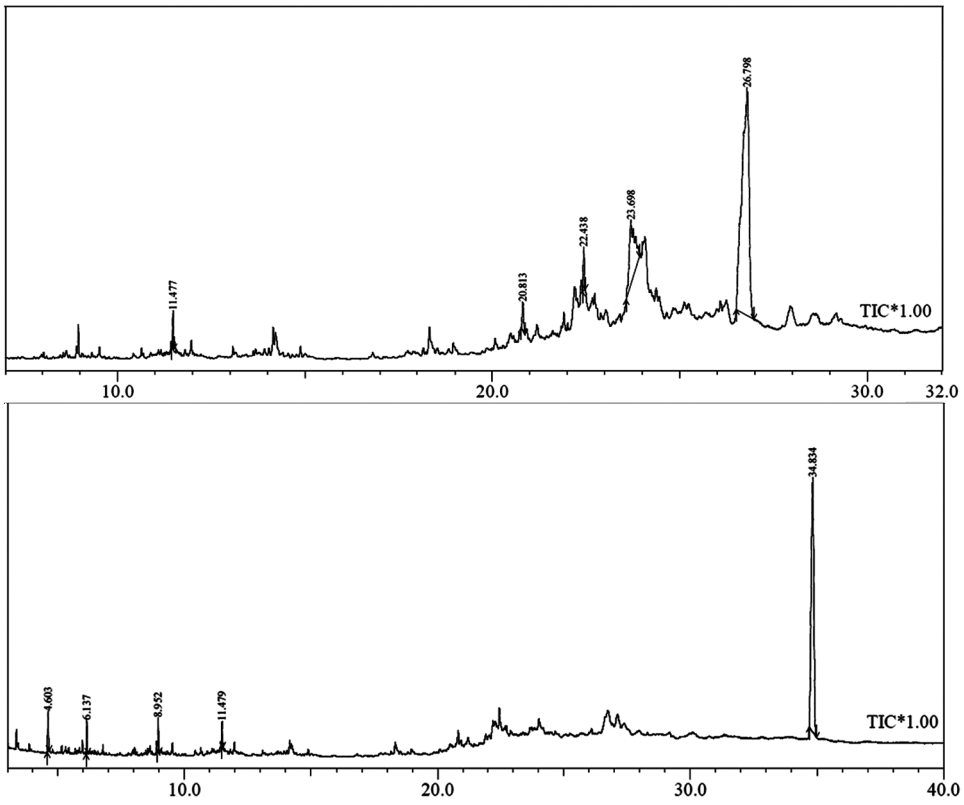

Fig. 5 — (a) Chromatogram of spot a of AIA25a and (b) Chromatogram of spot c of AIA25a (*Contd*.)

(Table 3D) like, Dodecane, 4,6-dimethyl-(21.5%), Octadecane, 1-iodo-(17.18%), Octadecane(4.81%), 1,2-Benzenedicarboxylic acid, and ditridecyl ester (27.96%)were found on spot e. Overall the results showed that total 15 compounds were separated from partial purification from AIA25a strain isolated from rhizospheric sample of Rajasthan.

The Bioautography technique was applied for partial purified compounds of chloroform crude extract of AIA25a isolate. Out of which only spot a was having active spot and having antimicrobial activity tested by bioautography method (Fig. 6). Spot a of chloroform extract of the isolate was found to have four compounds as Docosyl behenate, Eicosane, 1,2-Benzenedicarboxylic acid, ditridecyl ester, and Hexacosyl heptafluorobutyrate. The compound Hexacosyl heptafluorobutyrate present only in the spot a of chloroform extract of AIA25a. Hexacosyl heptafluorobutyrate has been isolated from AIA25a isolate from soil of Rajasthan. In the previous research Hexacosyl heptafluorobutyrate has not been demonstrated antimicrobial activity against strain

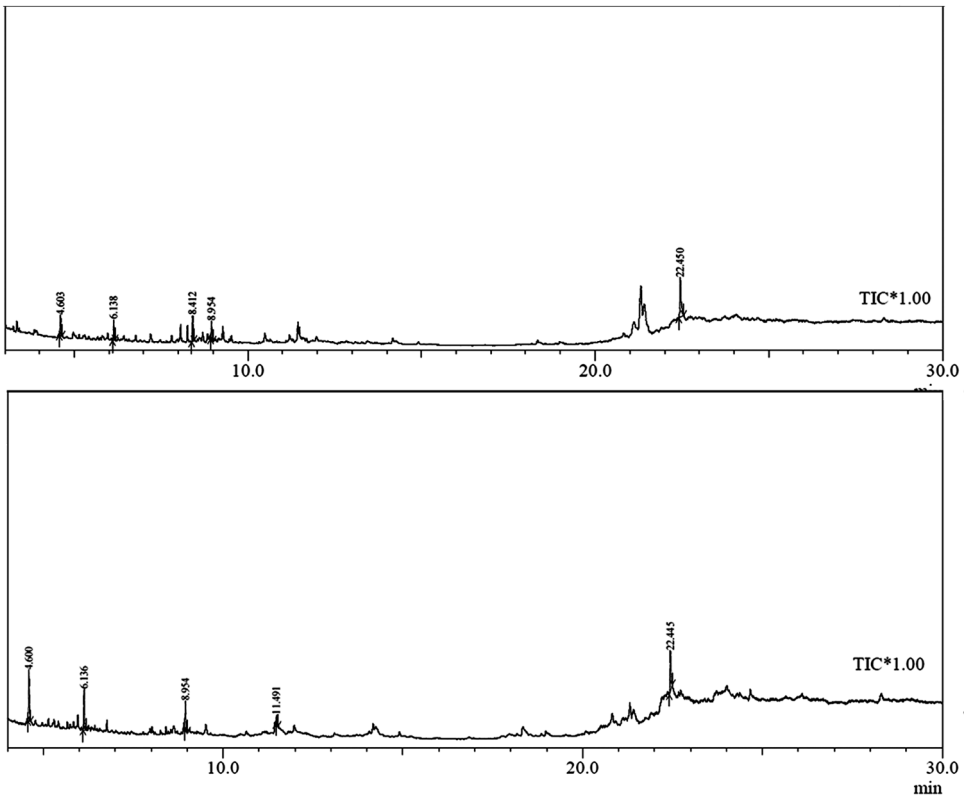

Fig. 5 — 5 (c) — Chromatogram of spot d of AIA25a and (d) Chromatogram of spot e of AIA25a

p.aeruginosa.

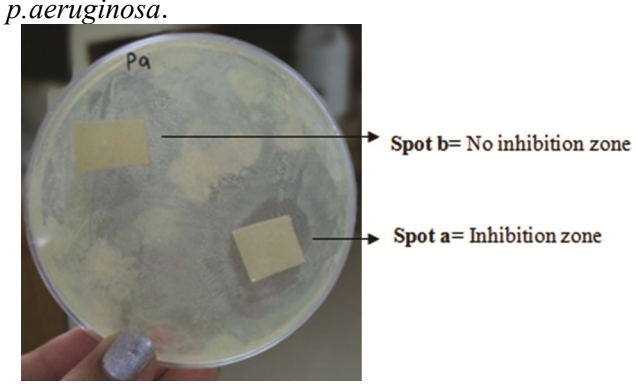

Fig. 6 — The Bioautography technique for partially purified compounds of chloroform extract of AIA25a isolate and inhibition zone against *P. aeruginosa*

Conclusion

As research has been made to isolate antibiotic compounds that contribute to prevent threats of global health, food security, and high-cost antibiotic drugs. Present study indicates the presence of good antimicrobial compounds which shows antimicrobial activity. AIA25a strain was recovered from rhizospheric soil of Rajasthan and has abundance of antimicrobial compounds like pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-is present in highest amount (61.62%) and has anti-bacterial and anti-fungal activity. Hexacosyl heptafluorobuty rate has been isolated from AIA25a isolate from soil of Rajasthan and has antimicrobial activity against the strain *P. aeruginosa*. The research has not been published previously for this compound having activity against *P. aeruginosa* strain. Overall study was successful in finding antimicrobial components from the rhizospheric soil and to continue the search for more antibiotics or antimicrobial components from these unexplored locations of Rajasthan.

Conflict of interest

No conflict of interest has been declared.

References

1 Livermore D M, *J Antimicrob Chemother*, 64 (2009) 64.
2 Roca I, Akova M, Baquero F, Carlet J, Cavaleri M, Coenen S, Cohen J, Findlay D, Gyssens I & Heure O E, *New Microbes New Infect*, 6 (2015) 22.
3 Hughes D & Andersson D I, *FEMS Microbiol Rev*, 41 (2017) 374.
4 Genilloud O, *Antonie van Leeuwenhoek,* 106 (2014) 173.
5 Bax R, Mullan N & Verhoef J, *Int J Antimicrob Agents*, 16 (2000) 51.

6 Hamedi J, Kafshnouchi M & Ranjbaran M, *Saudi J Biol Sci*, 26 (2019) 1587.
7 Newman D J, Cragg G M & Snader K M, *J Nat Prod*, 66 (2003) 1022.
8 Katz L & Baltz R H, *J Ind Microbiol Biotechnol*, 43 (2016) 155.
9 Onaka H, *J Antibiot*, 70 (2017) 865.
10 Bredholt H, Fjærvik E, Johnsen G & Zotchev S B, *Mar Drugs*, 6 (2008) 12.
11 Lee L H, Cheah Y K, Nurul Syakima A M, Shiran M S, Tang Y L, Lin H P & Hong K, *Genet Mol Res*, 11 (2012) 1627.
12 Kekuda P T R, Shobha K S & Onkarappa R, *J Pharm Res*, 3 (2010) 250.
13 Donadio S, Maffioli S, Monciardini P, Sosio M & Jabes D, *J Antibiot*, 63 (2010) 423.
14 Kumari N, Menghani E & Mithal R, *Indian J Tradit Knowl*, 19 (2020) 111.
15 Kumari N, Menghani E & Mithal R, *Indian J Chem Technol*, 26 (2019) 362.
16 Kumari N, Menghani E & Mithal R, *J Sci Indust Res*, 78 (2019) 793.
17 Kumari N, Menghani E & Mithal R, *J Sci Indust Res*, 79 (2020) 712.
18 Kumari N, Menghani E & Pandey S, *Indian J Geo Mar Sci*, 50 (2021) 29.
19 Kumari N & Menghani E, *Indian J Nat Prod Resour*, 11 (2020) 287.
20 Rios J L Recio M C & Villar A, *J Ethnopharmacol*, 23 (1988) 127.
21 Awla K H, Kadir J, Othman R, Rashid T S & Wong M Y, *Am J Plant Sci*, 7 (2016) 1077.
22 Wassenaar P N H Janssenz N De, Poorter L R M & Bodar C W M, *National Inst Public Health Environ RIVM Lett Report*, (2017) 0071.
23 Elnawawy A S, Khalil M S & Mohareb R M, *J Sci Eng Res*, 4 (2017) 421.
24 Raut L S & Hamde V S, *Int J Pharm Biol Sci*, 8 (2018) 611.
25 Gunalan G, Krishnamurthy V & Saraswathy A, *World J Pharm Res*, 3 (2014) 1313.
26 Arora S & Kumar G, *J Pharmacogn Phytochem*, 7 (2018) 1445.