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Evaluation of antibacterial activity and identification of bioactive metabolites by GCMS technique from Rhizospheric Actinomycetes

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Since thousands of years, plants are used as a source for medicine as they are established to possess a reservoir of bioactive compounds. Plants have been considered a great natural source of medicinal properties and can be used as either in the form of a pure bioactive compound or as traditional preparations. Plants and soil micro-organisms both have been considered as a precursor of therapeutically helpful metabolites because of their natural production of various bioactive secondary metabolites. Evolving drug resistance these days increase the need to isolate new drugs for the treatment of many diseases caused by multidrug-resistant organisms. To combat these multidrug-resistant pathogens, studies were carried out to isolate antibiotic compounds from actinomycetes. In the present study, a total of 65 isolates were isolated from rhizospheric actinomycetes. Isolation, characterization and biochemical tests of actinomycetes were carried out and subjected to fermentation and solvent extraction by four solvents as- benzene, pet ether, ethyl acetate, and chloroform of AIA26 isolate. After extraction crude obtained was checked for their antimicrobial activity on Mueller Hinton agar (MHA) media against five indicator organisms but, activity was recorded against *S. aureus* and *P. aeruginosa*, and no activity was obtained against *P. vulgaris*, *K. pneumonia*, and *B. subtilis*. By the help of GCMS technique, major compounds present have been identified in AIA26 isolate.

Keywords: Actinomycetes, Antimicrobial activity, Bioactive metabolites, GCMS.

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Introduction

It has been recognized for long that various plants and microorganisms have provided noteworthy contribution to the development and discovery of newer antibacterial drugs. Therefore, plant and bacterial crude extracts have been studied *in vivo* and *in vitro* for past many years against several multi drug-resistant (MDR) bacteria like *P. aeruginosa*[1,2]. Various secondary metabolites have been discovered from soil organisms which have contributed to the Medical, Agriculture and Pharmaceutical industries[2]. The resistance of pathogens against current antibiotics and the increasing burden of infectious diseases is a matter of concern. There is a vast potential for natural product-based discovery and development of drug candidates with good therapeutic efficacy and low toxicity[3]. In the region of the rhizosphere, plants and microorganisms live together to make an extraordinary biological community; which involves activity like carbon and water cycling, supplement and mineral trapping etc. Taking into consideration, the plant-microbes association, their cooperation produces a broad scope of bioactive metabolites, which are amazingly significant and are known to encourage different purposes. The actinomycetes, primarily those belonging to Streptomyces sp., make up a large significant group of soil microorganisms and are excellent in the degradation of complex molecules to basic or simple substances for plant development and promotion and control plant pathogens[4]. There are plenty of compounds which have been isolated and characterized, many of which have been developed into drugs for various treatment of broad range of diseases in human, veterinary, and agriculture sectors[5]. For this reason, the actinomycetes have been considered to be the most powerful source for the production of secondary metabolites, antibiotic drugs and other bioactive compounds. There is substantial evidence confirming that actinomycetes are remarkable as an antibiotic and other active metabolites producers, making 75% of all well-known products, and the Streptomyces has an exceptional role in antibiotic production. Streptomycetes and related actinomycetes

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persist to be valuable sources of novel bioactive secondary metabolites with a broad range of biological performance which may ultimately find applications as anti-infectious, anticancer agents and other pharmaceutically helpful compounds[6]. There are yet many unexplored or uninvestigated regions, which require to be investigating for novel active compounds. Since emergence and re-emergence of (MDR) multi drug resistant pathogenic organisms, there is a necessity to expand additional powerful antimicrobial metabolites with wide-spectrum antimicrobial activity. Hence, the actinomycetes were grown for the production of antimicrobial metabolites using Actinomycetes Isolation Agar (AIA) media.

Materials and Methods

Rhizospheric sample collection

For the collection of samples, four rhizospheric regions of Rajasthan were selected like Kota, Jaipur, Alwar, and Udaipur. Samples were collected with dried, clean hand gloves and debris was removed from samples and transferred it to sterile covers. Around 12-15 cm, deep rhizospheric samples were taken and brought to Research laboratory of JECRC University, Jaipur, Rajasthan. Presence of moisture was removed from samples by keeping them at room temperature for 24-48 hours. Each sample was stored at 4 ^{o}C till further processing[7]. Samples were processed by drying, crushing and sieving for starting the isolation process. After air-drying at room temperature, dried soil was crushed into motor and pestle. Thereafter, the soil was sieved through a plastic sieve (2-3 times) and passed samples were collected for further process[8].

Isolation and maintenance of actinomycetes

Isolation of actinomycetes AIA26 isolate was carried out with 1 g of dry soil sample which was suspended in 9 mL of sterile distilled water and mixed well. Dilutions were prepared up to 10^{-5}. Prepared dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} were spread on the Petri plates containing autoclaved AIA medium. 'L' shaped glass spreader was used for spreading of dilutions on plates filled with AIA media. Media plates were incubated inverted at 37 ^{o}C for 7-14 days. Immediately after the incubation period, colonies were selected from the Petri plates and re-streaked every colony on AIA media. Pure colonies were obtained and stored for further testing[9].

Morphological characteristics of the isolate

Morphological characterizations of the isolated colony were carried out using gram's staining method. Twenty four hours old Luria broth culture of isolate AIA26 was used for gram's staining followed by biochemical characterization[10].

Primary screening

Indicator pathogens

Isolate AIA26 was screened for their antimicrobial activity against selected indicator bacterial cultures. Indicator bacterial cultures were brought from IMTECH Chandigarh. Studies were conducted on antimicrobial activity of isolate against *Staphylococcus aureus* (MTCC 3160), *Pseudomonas aeruginosa* (MTCC 1688), *Klebsiella pneumonia* (MTCC 432), *Proteus vulgaris* (MTCC 7306), *Bacillus subtilis* (MTCC 441). The method used for the activity tests were both disc diffusion and agar well method carried out in the Research laboratory of JECRC University Jaipur, Rajasthan. These pathogenic bacterial strains were regularly maintained in the nutrient agar (NA) media slants for routine laboratory use and for long time uses, were stored at 4 ^{o}C[11].

Fermentation and extraction of antibiotics

For the production of antibiotics or antimicrobial compounds, 500 mL of Luria broth was prepared and dispersed into 2 flasks of 250 mL and each flask was autoclaved for sterilization. Flasks were sterilized and inoculated with isolate and kept in the shaker incubator at 30 ^{o}C at 150 rpm for 2-3 weeks. After appropriate incubation, the broth culture was centrifuged for 20 minutes at 5,000 rpm for the separation of supernatant and the biomass. For the separation of metabolites, four solvents were used like Pet ether, Benzene. Ethyl acetate and chloroform were used for bioactive components extraction from AIA26 culture broth. Each solvent and isolate culture was mixed to 1:1 (v/v) amount and shaken well for proper mixing. Flasks were kept for 30 minutes without interruption till two diverse layers (organic and inorganic layer) seen were completely separated from each other. Beakers with solvent (containing bioactive metabolites) were kept on the water bath for absolute disappearance of solvents at 60 ^{o}C and the beaker was then left with secondary metabolites[2]. A mixture of bioactive compounds was transferred from beaker to small Eppendorf tube for further identification of compounds by Gas chromatography Mass spectroscopy (GCMS) technique[12,13].

Antibacterial activity against indicator pathogens

The antibacterial test of AIA26 isolate was carried out against *S. aureus*, *P. aeruginosa*, *K. pneumonia*, *P. vulgaris*, and *B. subtilis* indicator pathogens. Inhibition zone (IZ) of antibacterial activity of the crude extracts of benzene, pet ether, ethyl acetate, chloroform was measured. Petri-plates were prepared with 20 mL of Mueller Hinton Agar (MHA) media and indicator strains were spread on the solidified MHA media and covered plates after holding for 10 minutes for drying of plates. For the activity test, standard Kirby-Bauer disc diffusion and agar well diffusion method was used. This activity test was conducted with each crude extracts. The sterilized discs with crude extract were positioned on the MHA media plates and kept for 30 minutes at room temperature so that complete diffusion of compounds can take place. The plates were incubated overnight at 30 ^{o}C and IZ was measured (mm) against each pathogen[14,15].

Identification of main bioactive components using GC–MS

The composition of the crude extracts of AIA26 isolate was identified by GCMS analysis by Shimadzu QP 2010 ultra and gas chromatograph interfaced to a mass spectrometer GCMS. The instrument was built with Elite-1 fused silica capillary. Helium gas (99.99%) was used as carrier gas. Flow rate was set as 1.21 mL/min and with a split ratio: 10. Injector temperature was 260 °C; ion-source temperature 200 °C. The oven temperature was intended from 60 °C (constant for 3 minutes) with an increment as of 280 °C for 22 minutes. Mass spectra were taken at 70 eV; scan time 0.5 seconds. The chemical composition of the crude extract was analyzed by measurement of the peak area and the retention time by NIST 14 library[16].

Results and Discussion

Antimicrobials have been considered crucial for medical as well as agricultural purposes. Many secondary metabolites have been produced and applied as a source of therapeutic drugs for the past many years. On the other hand, drug resistance has increased and as a result, diverse human diseases have risen. With this changing atmosphere and necessity of antimicrobials, screening with validation of antibiotics in the form of precious drugs needs to be updated. Isolation of actinomycetes was carried out on AIA media followed by Gram staining and some biochemical test like Gelatin liquefaction, Starch hydrolysis, Triple sugar iron (TSI) agar, Casein hydrolysis, Citrate Utilization, Methyl red, Voges-Proskauer, Nitrate reduction, Indole production, Catalase test, Urease production, Hydrogen sulphide production (H_2S), Motility test, Mannitol, Lactose, Dextrose, Sucrose and Mannose (Table 1) were executed with isolate AIA26.

After biochemical characterization of the pure colony, culture broth fermentation process was executed. After the incubation period, extraction of bioactive components from four selected solvents such as pet ether, benzene, ethyl acetate and chloroform was completed. The isolate was examined for antimicrobial activity against some of the selected indicator pathogens. Extraction of components was preceded by layer separation method followed by sterilization of discs. Discs with compounds were applied on prepared plates of MHA media already occupied with indicator organisms growth. Antimicrobial susceptibility assessment was performed against indicator strains *S. aureus*, *P. aeruginosa*, *K. pneumonia*, *P. vulgaris*, and *B. subtilis* procured from IMTECH Chandigarh. IZs of antibacterial activity of crude extracts were recorded against *S. aureus* (IZ=Ben-24mm)

Table 1 — Characterization of AIA26 isolate

Characteristics	Result
Gram staining	+ve
Aerobic growth	+ve
Anaerobic growth	-ve
Sugar fermentation test Gelatin liquefaction	+ve
Starch hydrolysis	-ve
Triple sugar iron (TSI) agar	+ve
Casein hydrolysis	+ve
Citrate Utilization	-ve
Methyl red	-ve
Voges-Proskauer	-ve
Nitrate reduction	-ve
Indole production	-ve
Catalase test	+ve
Other tests	
Urease production	-ve
Hydrogen sulphide production (H_2S)	+ve
Motility test	+ve
Carbon utilization	
Mannitol	-ve
Lactose	-ve
Dextrose	-ve
Sucrose	-ve
Mannose	+ve

P. aeruginosa (IZ=Ben-42 mm), and *P. aeruginosa* (IZ=Chl-18mm) and no activity was recorded against *P. vulgaris, K. pneumonia* and *B. subtilis* (Fig. 1).

From analysis obtained from GCMS, Structure, Molecular weight, Molecular formula, Retention time (RT) and Area percentage of components were identified and matched from NIST14 library. Fatty acids, phenolic compounds, alkaloids, alcoholic compounds and amide compounds etc. were present in good to a moderate amount (Table 2). Presence of compounds in the crude extract of AIA26 isolated colony was characterized by GCMS technique. Major compounds obtained in Chloroform extract of AIA26 isolate, Phenol, 2,4-bis(1,1-dimethylethyl)- at RT- 14.54, 3-methyl-1,4-diazabicyclo[4.3.0]nonan-2,5-dione, n-acetyl at RT- 19.539, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) at RT-21.939, 5h,10h-dipyrrolo[1,2-a:1',2'-d]pyrazine-5,10-dione, octahydro-, (5as-cis)- at RT- 24.121, Acetamide, n-[2-(1h-indol-3-yl)ethyl]- at RT- 29.21, O o'-biphenol, 4,4',6,6'-tetra-t-butyl- at RT- 31.541, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl) at RT- 31.755, 1-heptacosanol at RT-31.872, N-tetracosanol-1 at RT-28.463, Docosyl heptafluorobutyrate- at RT-39.83. A relative concentration of compounds was shown by chromatogram of AIA26 isolate (Fig. 2). In chromatogram height of every peak showed a proportional concentration of compounds present in the sample.

Actinomycetes have potential that produces diverse compounds showing a good range activity against the selected variety of indicator organisms. 1,2 – Benzene dicarboxylic acid, bis (2-methyl propyl) ester compound was isolated from chloroform extract of *Portieria hornemannii* (red algae) and leaves of the plant *Begonia malabarica* Lam. *B. malabarica* Lam. is medicinally significant herbs belonging to the family of Begoniaceae. Its leaves have been reported to be effective in the treatment of respiratory infections, diarrhoea, blood cancer, and skin diseases. The entire plant has a diverse variety of secondary metabolites. 1,2 – Benzene dicarboxylic acid, bis (2-methyl propyl) ester has antibacterial and anticancer activity[17,18]. Phenol, 2, 4-bis (1,1-dimethylethyl) – compound has antibacterial and anti-inflammatory activity reported in leaves of *Nimbapatradi Choornam* plant. *Nimbapatradi Choornam* is a well known Ayurvedic medicine for various diseases like; Leprosy, eczema, gout, leukoderma, skin eruptions and psoriasis. The different ingredients of *Nimbapatradi choornam* are neem leaves, sulphur, and turmeric, which are used for their antiseptic properties[19]. N-tetracosanol-1 compound was isolated from Chloroform extract of the *Croton bonplandianum* leaves. N-tetracosanol-1 has anti-bacterial activity, nematicidal, anticancer, antioxidant, and antimicrobial activity. *Croton bonplandianum* belongs to Euphorbiaceae family and has been used to

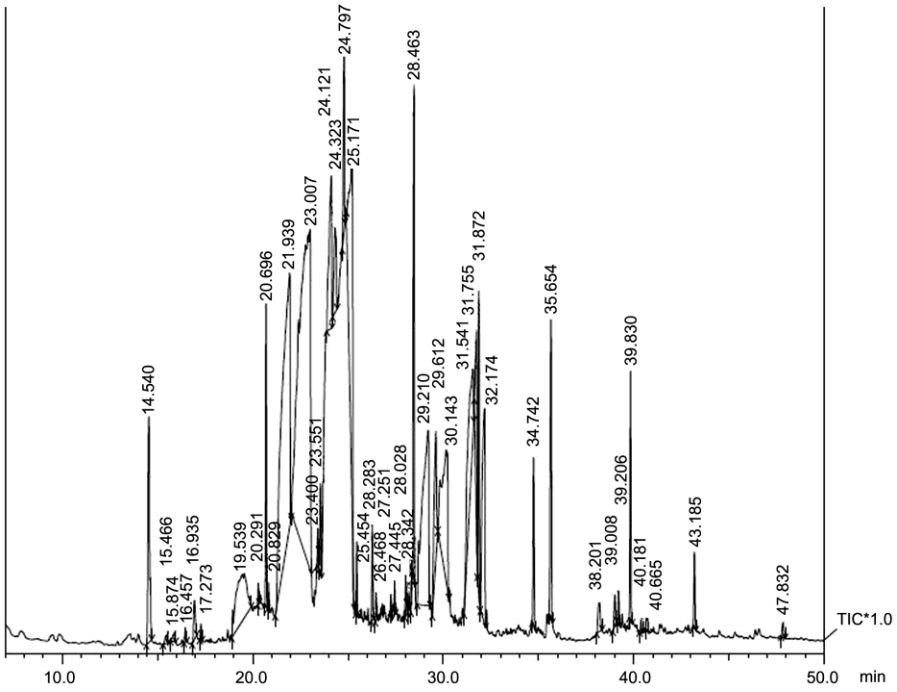

Fig. 1 — Antibacterial activity of chloroform crude extract of AIA26 isolate

Table 2 — Composition of chloroform extract, RT, molecular weight and molecular formula of compounds of rhizospheric AIA26 isolate by GCMS analysis

R.Time	Compound name	Molecular weight	Molecular formula
14.54	Phenol, 2,4-bis(1,1-dimethylethyl)-	206	$C_{14}H_{22}O$
15.466	P-tert-butyl catechol	166	$C_{10}H_{14}O_2$
15.874	Bis(2,4-dimethylamino)pyrimidine	166	$C_8H_{14}N_4$
16.457	1,2-benzenedicarboxylic acid, didecyl est	446	$C_{28}H_{46}O_4$
16.935	2-d,2-pentadecyl-1,3-dioxepane	212	$C_{20}H_{39}DO_2$
17.273	Sedoheptulose, 2,3:4,5-dimethylene-	234	$C_9H_{14}O_7$
19.539	3-methyl-1,4-diazabicyclo[4.3.0]nonan-2,5-dione, n-acetyl	210	$C_{10}H_{14}N_2O_3$
20.291	3,5-di-tert-butyl-4-hydroxybenzaldehyde	234	$C_{15}H_{22}O_2$
20.696	1-octadecanol	270	$C_{18}H_{38}O$
20.829	Eicosane	282	$C_{20}H_{42}$
21.939	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	210	$C_{11}H_{18}N_2O_2$
23.551	7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	276	$C_{17}H_{24}O_3$
24.121	5h,10h-dipyrrolo[1,2-a:1',2'-d]pyrazine-5,10-dione, octahydro-, (5as-cis)-	194	$C_{10}H_{14}N_2O_2$
24.323	1,2-benzenedicarboxylic acid, dibutyl ester	278	$C_{16}H_{22}O_4$
24.797	Octacosanol	410	$C_{28}H_{58}O$
25.454	2,4,6-tri-t-butylbenzenethiol	278	$C_{18}H_{30}S$
26.283	Cyclopenta[c]pyran-7-carboxaldehyde, 4-[(acetyloxy)methyl]-	218	$C_{12}H_{10}O_4$
26.468	Benzenebutanoic acid, .alpha.,2-diamino-.gamma.-oxo-, (s)-	208	$C_{10}H_{12}N_2O_3$
27.251	Triacontanoic acid, methyl ester	466	$C_{31}H_{62}O_2$
28.028	Octadecanoic acid	284	$C_{18}H_{36}O_2$
28.184	Tetratetracontane	618	$C_{44}H_{90}$
28.342	Dodecanoic acid, 3-methylbutyl ester	270	$C_{17}H_{34}O_2$
28.463	N-tetracosanol-1	354	$C_{24}H_{50}O$
29.21	Acetamide, n-[2-(1h-indol-3-yl)ethyl]-	202	$C_{12}H_{14}N_2O$
31.541	O o'-biphenol, 4,4',6,6'-tetra-t-butyl-	410	$C_{28}H_{42}O_2$
31.755	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylme	244	$C_{14}H_{16}N_2O_2$
31.872	1-heptacosanol	396	$C_{27}H_{56}O$
34.742	1,2-benzenedicarboxylic acid	390	$C_{24}H_{38}O_4$
38.201	2,4,6-trihydroxybenzaldehyde	154	$C_7H_6O_4$
39.206	1,2-bis(3,5-di-tert-butyl-4-hydroxyphenyl)-ethylene	436	$C_{30}H_{44}O_2$
39.83	Docosyl heptafluorobutyrate	522	$C_{26}H_{45}F_7O_2$
40.665	Cycloprop[e]indene-1a,2(1h)-dicarboxaldehyde, 3a,4,5,6,	232	$C_{15}H_{20}O_2$
47.832	Nonadecyl pentafluoropropionate	430	$C_{22}H_{39}F_5O_2$

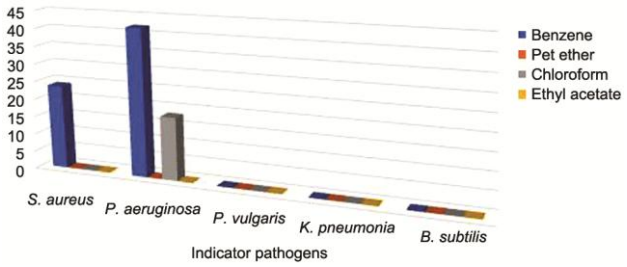

Fig. 2 — Chromatogram Chloroform of AIA26 isolate showing peaks of compounds in GCMS analysis

cure liver diseases, swelling of the body, ringworms, and skin diseases[20]. Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) compound has antibacterial and antifungal activity in ethyl acetate extract isolated from *Streptomyces strain.* Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) and Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl) have been isolated from *Epichloe* spp. and *Neotyphodium* spp. Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl) also has antifungal property in ethyl acetate extract isolated from *Streptomyces* strain[21,22]. Tetratetracontane is an antioxidant compound and has cytoprotective activities. Tetratetracontane was isolated from the plant *Quisqualis indica*, commonly known as Rangoon creeper belonging to family *Combretaceae*. Some medicinal properties of *Q. indica* L. have been known in *Ayurveda*. Nearly all of the plant parts are used independently or mixed with other ingredients as a remedy to different ailments like anti flatulence, coughs, diarrhoea, body pains, and toothache. Herbs are very rich in flavonoids and vitamin C that may enhance immune function. The plant *Q. indica* L.has immunomodulatory, antibacterial, antioxidant, antipyretic, anthelmintic, antirheumatic, antiviral, antifungal, anti- inflammatory,

Table 3 — Components with their biological activity present in chloroform extract of isolate AIA26

RT	Name of Compound	Nature of compound	Other Sources of compounds	Parts/extract	Activity
34.742	1,2 – Benzene dicarboxylic acid, bis (2-methyl propyl) ester	Pale yellow oily substance	Red algae *Portieria hornemannii*	Chloroform extract	Antibacterial activity[17]
			Begonia malabarica Lam.	Leaves	Antibacterial and anticancer activity[18]
14.54	Phenol, 2,4-bis (1,1-dimethylethyl)-	Alkylbenzene	*Nimbapatradi Choornam*	leaves extract	Antibacterial and anti-inflammatory activities[19]
28.463	N-tetracosanol-1	Alcoholic compound	*Croton bonplandianum*	Chloroform leaf extract	Anti-bacterial activity, nematicidal, anticancer, antioxidant and antimicrobial[20]
21.939	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)	Aromatic compounds	*Streptomyces* strain *Epichloe* spp. and *Neotyphodium* spp.(fungus)	Ethyl acetate extract	Antibacterial and antifungal[21]
31.755	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)	Aromatic compounds	*Streptomyces* strain *Epichloe* spp. and *Neotyphodium* spp.(fungus)	Ethyl acetate extract	Antifungal[21,22]
28.184	Tetratetracontane	Hydrocarbons	*Quisqualis indica*	Ethyl acetate extract	Antioxidant and cytoprotective activities[23]
20.829	Eicosane	Alkane	*Streptomyces* strain	Ethyl acetate extract	Antifungal[24]
20.696	1-octadecanol	Long chain alcohol	*Caralluma indica*	Stem	Antibacterial, antifungal & anti-larva[25]
27.251	Triacontanoic acid, methyl ester	Fatty acid	*Gracilaria corticata* (Red Seaweed)	Methanolic exract	Antibacterial[26]
24.797	Octacosanol	Long chain alcohol	*Sabicea grisea* var. *grisea*	Leaves	Antinociceptive and anti-Inflammatory[27]
28.028	Octadecanoic acid	Fatty acid	*Biophytum sensitivum*	Leaf and callus extracts	Antibacterial[28]
31.872	1-Heptacosanol	Alcoholic compound	*Eupatorium odoratum*	Methanol extracts	Nematicidal, anticancer, antioxidant and antimicrobial[29]

anti-staphylococcal, and antiseptic properties[23]. The plant has also been reported to helpful in relief in stomach pain, cold, skin parasites, and rickettsia[23]. Eicosane compound has antifungal activity against *Rhizoctonia solani*, which is a causal agent of target spot in tobacco and caused serious economic loss regarding the production and quality of the tobacco[24]. 1-Octadecanol was isolated from the stem of *Caralluma indica* having antibacterial, antifungal, and anti-larva properties[25]. Triacontanoic acid, methyl ester compound has antibacterial activity and isolated from the plant *Gracilaria corticata* having medicinal properties[26]. Octacosanol compound isolated from *Sabicea grisea* has antinociceptive and anti-Inflammatory activity. *Sabicea* species have been used in the treatment of fever and malaria, its chemical components help relieve pain and inflammation[27]. Octadecanoic acid has antibacterial activity and is reported from the plant *Biophytum sensitivum.* Its leaf and callus extracts have potent antibacterial actions. *B. sensitivum* known as life plant has many medicinal properties such as it helps in reducing diabetes, is effective in the treatment of inflammatory diseases, and has antiseptic properties[28]. 1-Heptacosanol has nematicidal, anticancer, antioxidant, and antimicrobial activity and was isolated from *Eupatorium odoratum* (*Asteraceae*). The plant has been used for the treatment of various microbial diseases[29] (Table 3).

Conclusion

The present study showed that rhizospheric soil actinomycetes have the capability of producing antimicrobial compounds exhibiting antibacterial activity against selected pathogens. A good antibacterial activity (24 and 42 mm) was found against pathogen *S. aureus* and *P. aeruginosa* from benzene crude extract. GCMS analysis showed the

presence of a diverse group of bioactive compounds. Chloroform extract showed various compounds important as antimicrobials. These compounds have importance in agricultural and medical sector and are significant in biotechnological purposes. Inhibition zones of antibacterial activity of the chloroform extract of actinomycetes isolate against the multi drug resistant pathogens were realized. The unexplored rhizospheric regions of Rajasthan actinomycetes is the source for the revival of bioactive secondary metabolites with an extensive amount of activities that may lead to the development of newer antibiotics or antimicrobial substances.

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Conflict of interest

No conflict of interest has been declared.

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