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Screening of Actinomycetes from Turmeric (*Curcuma longa* L.) and Ginger (*Zingiber officinale*) Rhizosphere for Antifungal Activity

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Authors' contributions

This work was carried out in collaboration among all authors. Author RCOM designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors FSI and NFP managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The present study was carried out to isolate actinomycetes from rhizospheric soil of *Curcuma longa* and *Zingiber officinale* and evaluate their antifungal potential.
Methods: Actinomycetes were isolated from the rhizosphere soil of two Zingiberaceae plants (*Curcuma longa* and *Zingiber officinale*), using four different culture media. Isolates were screened for antifungal activity using dual culture technique against two reference strains *Colletotrichum coccodes* (DSM 2492) and *Alternaria pimpriana* (DSM 62023). The most potent strain was identified based on 16S rRNA gene sequence and the bioactive components of the strain were identified using GC-MS chromatography.
Results: Fifteen strains of actinomycetes were isolated, SCA (starch casein agar) was found best for cultivation of actinomycetes. The 15 strains were grouped into three genera *Norcadia* 8(54%), *Streptomyces* 5(33%) and *Janibacter* 2(13%) based on morphological, biochemical and physiological identification. Among the 15 isolates 6(40%) strains showed activity against either of the test organisms, while 1(6.7%) was active against the both test organisms. Comparative

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analysis of the 16S rRNA gene sequences identified the potent isolates as *Janibacter* sp. strain RC18. GC-MS analysis revealed the presence of 20 compounds with 10 identified as potent antimicrobial metabolites.
Conclusion: This study has revealed that rhizosphere of ginger and turmeric harbours rare actinomycetes that are valuable tool for sustainable agriculture.

Keywords: Actinomycetes; antifungal; GC-MS analysis; rhizosphere; 16S rRNA gene sequencing.

1. INTRODUCTION

Rhizosphere is a term designated for the description of contact region between root and soil [1]. The root affects the soil in the rhizopshere, enhances the biological activity taking place in this region by providing nutrients for the microorganisms [2]. In return the rhizospheric microorganisms play a vital role by protecting the plant (biocontrol) and promoting the plant growth [3,4].

Zingiberaceae plants especially turmeric and ginger (*Curcuma longa* L. and *Zingiber officinale)* are commonly used rhizomes in Nigeria as spices and also for medicinal purpose [5]. As a result of the antimicrobial properties of these plants, the plant exudates will be more favourable to beneficial microorganisms thereby increasing the chances of isolating novel and rare actinomycetes [6].

Fungal phytopathogens are problematic worldwide, are casual agent of rots, rusts, smuts, blight and wilt which destroys the crops [7]. In order to enhance food security plant diseases need to be controlled and managed effectively [8]. Anthracnose and *Alternaria* blight are prime fugal rots reported worldwide and have been implicated annually as a major factor contributing to losses in agriculture [9]. *Alternaria pimpriana* causative agent of *Alternaria* blight characterized by leaf spots, sterm lesion and fruit rot was first implicated by Rao [10]. *Colletotrichum coccodes* cause anthracnose in fruits and vegetables [11].

Actinomycetes are Gram positive bacteria with high G+C content, they are group of microorganisms on a borderline between fungi and bacteria that are mostly free living saprophytes [6,12]. Actinomycetes are major producers of bioactive compounds for industrial and pharmaceutical purposes, they are widely distributed and are also known to represent the larger part of rhizosphere microbiota [13].

In view of the fact that there is an urgent need to replace chemical pesticides that has long term effect on the environment with environmentally friendly pesticides (biopesticides) [14]. Actinomycetes became the best bet for this function, hence the need to screen the environment for novel actinomycetes [15].

2. MATERIALS AND METHODS

2.1 Test Phytopathogens

Alternaria pimpriana DSM 62023 and *Colletotrichum coccodes* DSM 2492 were obtained from DSMZ (German collection of microorganism and cell cultures).

2.2 Collection of Soil Sample

Rhizospheric soil of two Zingiberaceae plants: Turmeric (*Curcuma longa* L.) and ginger (*Zingiber officinale*), were obtained from NRCRI (National Root Crop Research Institute) Umudike farm. After removing about 3 cm soil from the surface, samples were taken from actively growing roots of the plants up to 5 cm depth.

2.3 Isolation of Actinomycetes

Pretreatment of samples were carried out for 1 hour at 100°C, serial dilution 10^{-5} were plated onto SCA (Starch casein agar) Hi media Mumbia india), TSCA (trypton soy casein Agar) (Hi media Mumbia india), AIA (actinomycetes isolation agar) (Hi media Mumbia india), and ASA (acidify starch agar) (Starch 15 g/l, $(NH_4)_2SO_4$ 3 g/l, NaCl 1 g/l, $CaCO_3$ 2 g/l Agar 15 g/l, pH 5.5) supplemented with 25 µg/ml nalidixic acid, 50 µg/ml nystatin and 2 g/l $CaCO_3$ by spread plate method. Incubation was carried out at 28°C ±2 for 1 to 10 weeks, isolated strains were preserved in 20% glycerol for further studies [15].

2.4 Phenotypic Characterization

Morphological characteristics of the isolates were studied with respect to their colony colour, shape and texture. Various biochemical and physiological tests (Gram staining, catalase, oxidase, casein hydrolysis, gelatine hydrolysis,

starch hydrolysis, hydrogen sulphide reduction, indole, methyl red, carbohydrate fermentation test) were carried out as illustrated by Shirling and Gottlieb [16] and Witman, et al. [17].

2.5 Molecular Characterization

Deoxy ribonucleic acid (DNA) extraction was done using bacterial genomic DNA extraction kit (Zymo Quick). The 16s rRNA region of the rRNA genes of the isolate was amplified using 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' universal primers. The PCR (polymerase chain reaction) product was resolved on a 1% agarose gel at 120V for 20 minutes and visualized on a blue light transilluminator. Sequencing was done using the BigDye Terminator kit on on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The resulting 16S rRNA gene sequences were compared with those available in the GenBank using the BLAST. Using the MEGA version 5.0 [18] package phylogenetic and molecular evolutionary analyses were carried out. Alignment of the 16S rRNA gene sequences of the actinomycetes strain against neighbouring nucleotide sequences was done with CLUSTAL W program [19]. Neighbour-joining method was used to construct the phylogenetic tree [20] with Jukes and Cantor [21] model. To evaluate the reliability of the tree topology Bootstrap analysis [22] was performed. The 16S rRNA gene sequences have been deposited in the genbank database under accession numbers MK473882.

2.6 Extraction of Bioactive Compounds

The most potent isolates was cultivated in starch casein broth as a fermentation medium and incubated 7 days in shaker incubator at 28°C. Then it was centrifuged for 20 mins at 10,000 rpm and the supernatant was collected, mixed with an equal volume of chloroform in the ratio of 1:1(v/v) and was incubated in shaker (180 rpm) for 1 hr for complete extraction. The chloroform phase contains bioactive compounds was separated from the aqueous phase. The extracted crude compounds were dried to get dry powder by using heating mantle at 40°C [23].

2.7 GC-MS (Gas Chromatography Mass Spectrophotometric) Analysis

Chloroform extract of *Janibacter* sp. RC18 were analyzed by GC-MS technique performed by using GC Shimadzu QP2010 system and gas chromatograph interfaced to a Mass Spectrometer. (GC-MS) equipped with Elite-1 fused silica capillary column. Helium gas (99.99%) was used as the carrier gas at a constant flow rate of 1.51 ml/min and an injection volume of 2 µl was employed (split ratio: 20). Injector temperature was 200°C; Ion-source temperature 200°C. The oven temperature was programmed from 70°C (Isothermal for 2 min.) with an increase of 300°C for 10 min. Mass spectra were taken at 70eV; a scan interval of 0.5 seconds with scan range of 40 -1000 m/z. Total GC running time was 35 min.

2.8 Identification of Compounds

The database of National Institute of Standards and Technology (NIST) was used for the identification of components Interpretation of mass spectra of GC-MS. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST.

3. RESULTS AND DISCUSSION

3.1 Isolation and Preliminary Identification of Actinomycetes

Actinomycetes being saprophytic in nature are abundant in the rhizosphere soil of plants which are enriched environment because of root exudates and plant litters that serve as source of nutrient for activity of microorganisms [24]. Total numbers of 15 isolates were recovered from rhizosphere soil of two Zingiberaceea (Turmeric and Ginger) *Curcuma longa* L. (turmeric) gave the highest number of rhizospheric actinomycetes isolate [8]. From the result of the morphological, physiological and biochemical identification [16,17], as shown in Table 1, all the 15 isolates have genetic affiliation to three genera (*Norcadia*, *Streptomyces* and *Janibacter*) Previous studies on screening of soil for actinomycetes recorded these genera in their results [25,6,26]. *Norcardia* was the highest occurring genus 8 (54%) and *Janibacter* the least occurring 2 (13%) as shown in Fig. 1. Contrary to most studies [27-29] where *Streptomyces* were mostly reported as the dominant actinomycetes isolated, in this study the most dominant strains were non- *Streptomyces* (66.67%). The abundance of rare actinomycetes in this study might be attributed to the creativity of supplementing the isolation media with $CaCO_3$.

The findings is in accordance with the result reported by other investigators, Ashokvardhan, et al. [6] recorded the presence of actinomycetes from rhizospheric soil of *Capsicum annum* L., Duangmal, et al. [30] reported an active rare actinomycete *Allokutzneria oryzae* sp.nov from rhizospheric soil of *Oryza sativa* L. Also Adegboye and Babalola [31] reported 88 rare actinomycetes from 17 rhizospheric soil.

On trying to recover diverse and as much culturable actinomycetes as possible, four differ culture media were adopted and SCA gave the highest number and variety of actinomycetes strain [7] and all the three genera were represented, this is followed by TSCA 4 strains and the least was ASA where only 1 isolate was obtained, this result is presented in Fig. 2. The highest number and diversity of actinomycetes was obtained with SCA, this might be as result of its high nitrogen to carbon content. The least number of actinomycetes was obtained with ASA might be as a result of the acidic nature of the acidify starch agar (pH 5.5) owing to the fact that most actinomycetes thrive well in environment near neutrality. This result is in agreement with result obtained by Rhada, et al. [32] and Paul, et al. [32], different medium have different ability of recovering actinomycetes from the soil. Also backing the result that SCA was the best in enumeration and diversity are findings of Malek, et al. [33] and also that of Naikpatil and Rathod [34].

3.2 Screening for Antifungal Activity

Fungal phytopathogens *Alternaria pimpriana* DSM 62023 and *Colletotrichum coccodes* DSM 2492 were used for antagonistic assay. Of all the 15 isolates screened using dual culture technique only 7 (46.7%) exhibited antifungal activity against either of the test organisms, while 1(6.6%) isolate $R_1$03 displayed antifungal activity against the both phytopathogens. The result also revealed that amongst the positive isolates the antifungal potential varied with 5 (71.4%) isolates showing activity against *C. coccodes* and 4 (57.1%) against *A. pimpriana*. Similar studies conducted by Mariastuti, et al. [35] and Ahsan, et al. [36], found that actinomycetes isolated from soil displayed antifungal activities against fungal phytopathogens. However, it was only 1 (6.7%) strain ($R_1$03) a *Janibacter* from *Curcuma longa* L. that displayed antifungal activity against the both test fungi, this could be as a result of *Janibacter* as a rare actinomycetes having less encounter with pathogens thereby reducing resistance. The studies of Goudjal, et al. [37], Deshmukh and Vidhale [38] recorded 16.7%, 3.65% respectively of isolated actinomycetes that exhibited strong antifungal activity against test organisms, therefore supports the results of this study .

Molecular characterization of isolate $R_1$03: Further 16S rRNA of isolate $R_1$03 which was previously identified by morphological and biochemical characterization to belong to genus *Janibacter*, confirmed the genetic affiliation of the isolate to *Janibacter* strain RC18 with accession number MK473882 as shown in Fig. 3. The result is in agreement with that obtained from biochemical and physiological identification. The 16S rRNA sequence of strain RC18 although shared 99% similarity with the closest related specie *Janibacter* Actino-24 did not cluster with it, this is an indication of a novel strain. According to Dyson and Schrempf [39] employing phylogenetic analysis in identification of microorganisms gives better ruling.

3.3 GC-MS Analysis

The GC-MS analysis result revealed the presence of 20 compounds (Fig. 3), the list of the identified compounds with their associated biological activity is presented in Table 3. GC-MS analysis result confirmed strain RC18 as a potent antifungal strain, among the NIST identified compounds 10 were previously reported as antimicrobial agents: Cyclohepta-siloxane, teradecamethyl (12.20%) has been reported with antimicrobial, immuno-modulatory and antitumour properties [40,41, 42]. Dodecanal (5.22%), Donega, et al. [43] reported antileishmania activity of this compound also studies by Chanprapa, [44] reported the antifungal activity of dodecanal against *Rhizoctonia solani* and *Xanthomonas oryzae*. Oxalic acid, allyl nonyl ester (4.81%) has antimicrobial properties [45], it was also identified in GC MS analysis of *Streptomyces albus* culture filtrate [46]. 2-Decen-1-ol (3.80%) is a volatile compound that is vital in cosmetic industry as a fragrance agent. Most volatile compounds often posses valuable biological activity, they serve as allelochemicals [47]. 9-Dodecanoic acid methyl ester is a fatty acid methyl ester (FAME). Presence of FAME compound have been reported in actinomycetes, El-Naggar, et al. [48] reported it in *Streptomyces anulatus* NEAE94, Narendharan, et al. [49] found it in *Streptomyces cavouresis* KUV39 and also Dickschat, et al. [50] recorded it in *Micromonospra aurantiaca*.

Dodecanoic acid methyl ester possesses antimicrobial and anticancer property [51].

Naphthalene,1,2-dihydro-1-phenyl (4.34%) as a naphthalene derivative is a compound of potential medical interest [52]. So many naphthalene containing compound have been studied for their antimicrobial activity, there are also many existing naphthalene containing drugs such as nafacillin, tolnaftate and terbinafine etc. Therefore, the presence of naphthalene derivative in chloroform extract of *Janibacter* sp RC18 makes it a candidate that occupy a central point among actinomycetes due to diverse and interesting antibiotic properties of naphthalene derivatives [53]. Thiocarbamic acid, N, N – dimethyl,S-1,3-diphenyl-2-butenyl ester (7.01%) was the fourth most abundant identified compound, although the biological activity is yet unknown [54], but the presence of this compound has been recorded in the GC-MS analysis of rhizosphere associated microorganisms [55]. N-(2-Phenylethyl)undeca-(2Z,4E)diene-8,10-diynamide (2.54%) an N-alkylamide, N-alkylamide has enormously attracted biomedical interest in the last two decades [54]. From the GC-MS analysis result 6 compounds, peak 6, 9, 10, 11, 12, 15 with retention time 24.85 min, 25.79 min, 26.09 min, 26.81 min, 28.11 min, and 34.00 min respectively were not identified from the NIST library, this might be that these compounds are novel compounds.

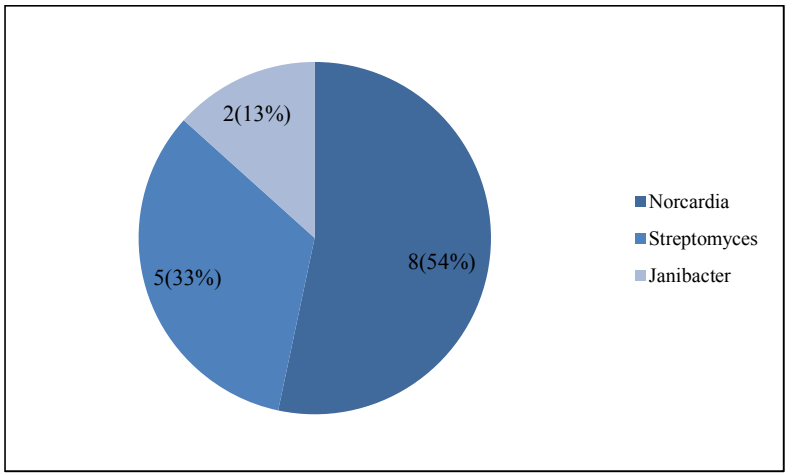

Fig. 1. Percentage distribution of actinomycetes genera within the total number of isolates

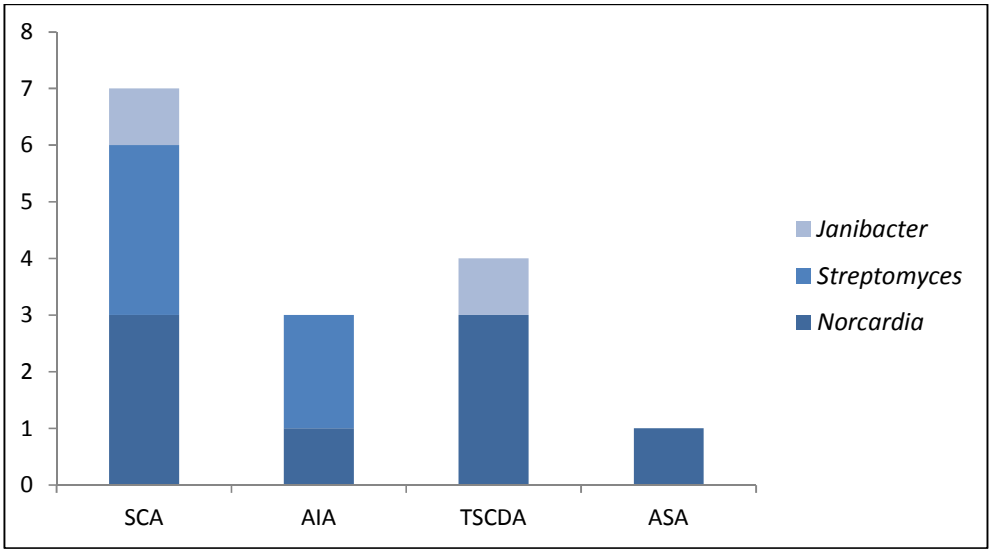

Fig. 2. Number of actinomycetes isolates according to the culture media used

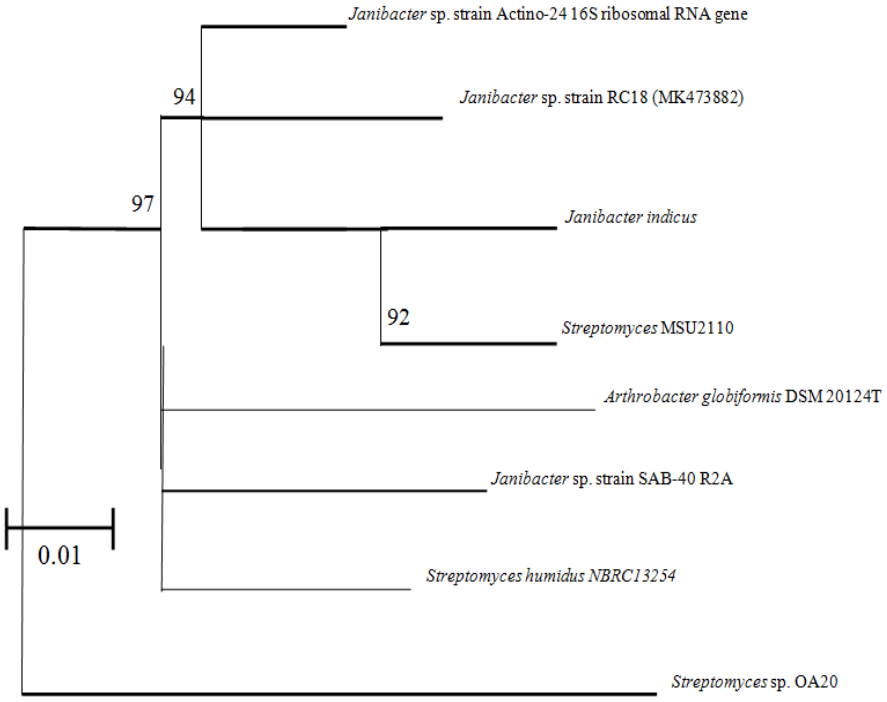

Fig. 3. Neighbor-joining tree based 16S RNA gene sequence showing relationship between *Janibacter* sp. strain RC18 and related members of actinomycetes group

Numbers at nodes indicates the level of bootstrap support (%), only values above 50% are shown. Score bar represents 0.01 substitution per site

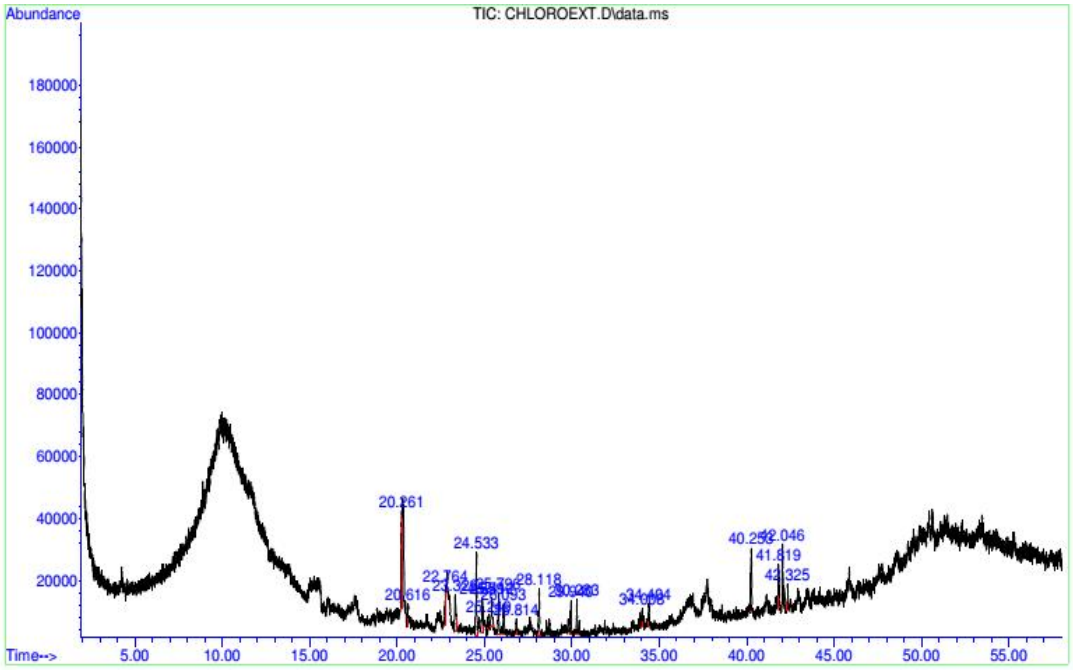

Fig. 4. GC-MS chromatogram of *Janibacter* sp. Strain RC18

Table 1. Morphological and biochemical characteristics of isolates

Isolates	Colour	Morphology	Indole	Methyl red	Catalase	Gram reaction	Oxidase	Hydrogen sulphide	Starch hydrolysis	Casein hydrolysis	Gelatine hydrolysis	Glucose	Fructose	Sucrose	Mannitol	Lactose	Actinobacteria
$R_1$03, $R_4$01	White, Cream	Rod, coccoid	_	_	+	+	_	+	+	+	_	_	D	_	_	_	*Janibacter*
$R_1$04,$R_2$02, $R_2$03, $R_3$03 and $R_4$02	White, grey	Filamentous	_	_	+	+	+	+	D	+	+	+	+	D	D	_	*Streptomyces*
$R_1$01, $R_1$02, $R_1$05, $R_2$01, $R_3$01, $R_3$02, $R_3$04 and $R_4$03	White, pale orange	Rods	_	_	+	+	_	+	D	_	_	D	_	D	_	D	*Norcardia*

D= Different isolates gave different reaction; _= Negative; +=Positive

Table 2. Antagonistic potential of the Isolates

S/no	Samples	Isolates codes	*C. cocoides*	*A. pimpriana*
1	Tumeric	$R_1$01	_	+
2		$R_1$02	_	_
3		$R_1$03	+	+
4		R_l04	_	_
5		$R_1$05	_	_
6		$R_2$01	+	+
7		$R_2$02	_	_
8		$R_2$03	_	_
9	Ginger	$R_3$01	_	+
10		$R_3$02	+	_
11		$R_3$03	+	_
12		$R_3$04	_	_
13		$R_4$01	+	_
14		$R_4$02	_	_
15		$R_4$03	_	_
Total no (%)		**15(100)**	**5(71.4)**	**4(57.1)**

+ Inhibition, _ no inhibition

Table 3. Bioactive compounds identified in the chloroform extract of *Janibacter* sp. RC18 by GC-MS

S/n	Retention time	Compound name	Molecular formula	Area %	Molecular weight (g/mol)	Activity
1	20.26	Cycloheptasiloxane, tetradecamethyl	$C_{14}H_{42}O_7Si_7$	12.20	519	Antimicrobial, anticancer, fragrance and skin condition
2	22.61	3-Nonyn-2-ol	$C_9H_{16}O$	2.94	140	Unknown
3	22.76	Cyclopentaundecanoic acid	$C_{16}H_{30}O_2$	4.68	254	Unknown
4	23.32	Dodecanal	$C_{12}H_{24}O$	5.22	184	Cosmetics, antimicrobial, antileishmanial
5	24.53	Cyclooctasiloxane, hexadecamethyl-	$C_{16}H_{48}O_8Si_8$	10.63	593	Antimicrobial
6	25.24	3-Decyn-2-ol	$C_{10}H_{18}O$	1.83	154	Unknown
7	25.41	Oxalic acid, allyl nonyl ester	$C_{14}H_{24}O_4$	4.81	256	Antimicrobial
8	29.94	8-Hydroxy-2-octanone	$C_8H_{16}O_2$	3.73	144	Unknown
9	30.28	2-Decen-1-ol	$C_{10}H_{20}O$	3.80	156	Fragrance
10	34.40	9-Dodecenoic acid, methyl ester, (E)-	$C_{13}H_{24}O_2$	2.24	212	Antimicrobial, anticancer
11	40.25	Benzene, 1,1-[3-(2-phenylethylidene)-1,5-pentanedily]bis-	$C_{25}H_{26}$	7.96	326	Fungicide.
12	41.81	Naphthalene,1,2-dihydro-1-phenyl	$C_{16}H_{14}$	4.34	206	Antitumor, anti-inflammatory, antiviral, antimicrobial.
13	42.04	Thiocarbamic acid, N,N –dimethyl,S-1,3-diphenyl-2-butenyl ester	$C_{19}H_{21}NOS$	7.01	311	Unknown
14	42.32	N-(2-Phenylethyl)undeca-(2Z,4E)diene-8,10-diynamide	$C_{19}H_{19}NO$	2.54	277	Fungicide, insectide and antibacterial.

4. CONCLUSION

The study revealed that rhizosphere harbours rare actinomycetes such as *Norcardia* and *Janibacter*. *Janibacter* sp. RC18 reported in this study is a potential biocontrol agent and should be exploited.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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