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Original article

Antimicrobial and antinematicidal metabolites from *Streptomyces cuspidosporus* strain SA4 against selected pathogenic bacteria, fungi and nematode

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ABSTRACT

The exploration of novel therapeutic agents and other bioactive secondary metabolite from *Streptomyces* species, for possible agricultural farming, pharmaceutical and industrialized applications, has been, and still is, essential. The existing studies were aimed with biologically potential *Streptomyces* species and its antagonistic activity against dreadful microorganisms. Totally, morphological three different actinomycetes were selected from the fertile agricultural lands. Among the three, the isolate SA4 exhibited significant antimicrobial and anti-nematicidal activity towards selected microbial pathogens such as E *Escherichia coli, Klebsiella pneumonia, Staphylococcus aureus, Salmonella typhi, Bacillus subtilis, Proteus vulgaris, Shigella flexineri, Candida albicans,* and *Fusarium* sp. The prospective strain SA4 was identified as *Streptomyces cuspidosporus.* The isolate SA4 optimized for secondary metabolites production with International *Streptomyces* project 4 (ISP 4) medium, pH 7.0 at 37 °C for 14 days. Gas Chromatography-Mass spectrometry (GCMS) analysis of strain SA4 bioactive extract publicized the existence of 1,2-Benzenedicarboxylic acid, bis(2-Methylpropyl) ester compound and occupied by high peak area and its possessed significant biological properties.

1. Introduction

Antimicrobial resistance (AMR) is the emergent universal threat which accounts for nearly 700,000 annual deaths in the world (CDC, 2019). In addition, also occurs wide spreading of infectious diseases from various sources which increases the clinical burden. The multi-drug resistance demands the world to explore novel antimicrobial drugs which are effective against drug resistant pathogenic microorganisms. Accordingly, searching and generating unique antimicrobial drugs, as well as combined antibiotic treatment were shown to delay the emergency of microbial resistance and can also produce desirable synergistic effects in the treatment of diseases caused by microorganisms.

In developing countries discovery and production of new and effective antimicrobials will make a voluminous impact on the clinical insight and can save millions of lives across the world. It is better known that soil microorganisms serve as a better source for the separation and identification of therapeutically active products. Among those the predominant is actinomycetes, a group of saprophytic bacteria known to generate a broad range of secondary metabolites, bioactive compounds and antibiotics that control microbial growth. It is estimated that 70–80% of secondary metabolites and one-third of antibiotics available commercially till date have been obtained from actinomycetes (Ding et al., 2019).

Mostly, *Streptomyces* genus was reports for approximately 80% of the natural products documented to date (Subramani and Sipkema, 2019). But the risk factor in discovering and producing new bioactive compounds is that it might be a commercially existing compound from known species. Thus, a group of actinobacteria

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from unexamined habitats could be sources of novel biologically active compounds (Periyasamy et al., 2019).

The contemporary study address with the collection and examination of prospective actinomycetes from soil sample and evaluate the antagonistic activity of the bioactive metabolites against various bacterial, fungal and nematode pathogens. Finally, the bio-prospective compound was identified and elucidate the structure by using Fourier Transform Infra-Red spectroscopy (FTIR) and Gas Chromatography and Mass Spectrometry (GCMS) analysis.

2. Materials and methods

2.1. Soil samples

Soil samples were aseptically collected from agricultural lands. The soil samples were attained from 5 cm below the soil surface with a clean, dry bag by using sterile spatula and transferred to lab. The air dried soil samples were pulverised using a sterilized mortar and pestle. Then, the soil samples sieved to take off the unwanted debris and finely sieved soil samples were kept in a dry sterile container for actinomycetes isolation.

2.2. Actinomycetes isolation

Enumeration and isolation of actinomycetes from soil samples were performed with serial dilution methods (Njenga et al., 2017). Concisely, 1 g of soil sample was serially diluted with 9 ml of sterile saline water up-to 10^{-5} dilutions. Then, 0.1 ml aliquot of diluted samples was used for plating with Actinomycetes isolation agar (AIA) (Sodium caseinate – 2.00, L-Asparagine – 0.100, Sodium propionate – 4.000, Dipotassium phosphate – 0.500, Magnesium sulphate – 0.100, Ferrous sulphate – 0.001, Agar – 15.000 and final pH at 8.1 ± 0.2) and the Petri plates were incubated at 30° C for 5 days. Lastly, purified isolates were sub- cultured with AIA and Starch casein nitrate (SCN) agar. All the isolates and stored in SCN agar slants at 4 °C.

2.3. Microbial pathogens

The tested bacterial pathogens were *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella typhi* and *Shigella flexneri*. The pure and identified fungal pathogens used for this study were *Aspergillus niger, Candida albicans,* and *Fusarium* sp. Antinematicidal activity was tested on plant-parasitic roundworm named as *Meloidogyne incognita* nematode.

2.4. In vitro screening of actinomycete strains for antimicrobial activity

2.4.1. Preliminary screening

The isolated actinomycetes were screened for their antimicrobial activity by Cross streak method against bacterial and fungal pathogens (Velho-Pereira and Nandkumar, 2012). The actinomycetes were streaked on centre of the SCN agar and the bacterial pathogens were streaked perpendicular near to it and incubated at 37 °C for 24 h. For the antifungal screening, the actinomycetes cultures were streaked on the sides of the Potato Dextrose Agar (PDA) and the mycelia agar block of the fungal pathogens were placed in centre of the Petri plates and incubated at 30 °C for 3 days.

2.5. Identification and characterisation of perspective isolate

2.5.1. Colony and spore morphology

The colony morphology, colour and pigment production of the actinomycetes isolates were considered by growing them on AIA, SCN agar and International *Streptomyces* Project (ISP4) medium. The plates were inoculated with isolates and incubated at 30 °C for 3 days and the growth of isolates was observed at regular intervals. The nature of the specific colony, colour and pigmentation were recorded.

Spore morphology was observed by cover slip method described by Williams and Davies, (1967). Briefly, sterilized coverslip was placed at an angle of 45° in a Petri plates containing SCN agar medium. A loop of inoculum was streaked along the coverslip line and then the plates were incubated at 30 °C for 3 days. Later, the coverslip was carefully withdrawn from the medium and examined their morphological features of aerial mycelium, substrate mycelium, spores and sporangia were examined with light microscopy (x1500).

2.5.2. Biochemical characteristics

Biochemical characteristics of the selected strain were performed to identify and confirm the genus and species level of the organism according to the procedure of Cappuccino and Sherman, (2013). The different biochemical tests *viz*., Indole test, MR-VP test, Citrate test, Triple Sugar iron agar test, Starch hydrolysis test, Gelatin liquefaction test, Urease test, Nitrate reduction test, Oxidase test and Catalase test were performed (Shirling and Gottlieb, 1966). The sugar utilization characteristics of actinomycetes were determined carbohydrate fermentation test with various sugars like glucose, sucrose, fructose, lactose, maltose and mannitol as a carbon source (Mayfield et al., 1972).

2.5.3. 16S rRNA gene sequencing

Taxonomic relationships between isolates were determined by comparative 16S rRNA gene sequences of existing databases. The 16srRNA was amplified using with primer (27F 5′-AGAGTTT GATCCTGGCTCAG-3′ and 1492R 5′-TACGGYTACCTTGTTACGACTT-3′) in BioRad T100 thermal cycler. The PCR conditions performed 35 cycles of 95 °C for 2 min, 42 °C for 30 s and 72 °C for 4 min, and one additional cycle with 20 min for chain elongation. The amplified products were purified and sequenced at Macrogen, South Korea. The resulting 16 s rRNA region sequences were evaluated by comparing with those sequences with submitted sequence in NCBI databases using, Basic Local Alignment Search Tool (BLAST) and Ezbiocloud (Park et al., 2012).

2.6. Optimization of parameters for bioactive compound production

The optimization of production media, carbon sources, nitrogen sources, salt concentrations, temperature, pH and agitation were conceded for the mass cultivation and secondary metabolites production of the actinomycete strain SA4 according to methods documented by Aliero et al. (2018).

2.6.1. Effect of media, carbon, nitrogen sources and salt concentrations

In order to select a basal medium for bioactive compound production, the strain SA4 was inoculated in four different media *viz*., ISP 4 broth (Inorganic salt starch), Modified nutrient glucose (MNG) broth, Yeast malt extract (YME) broth and SCN broth. The inoculated media were kept for fermentation on rotary shaker incubator at 30 °C and 200 rpm for 7 days. After 7 days, the biomass concentration was measured using UV/Vis spectrophotometer and the fermentation broth was centrifuged at 10,000 rpm, at 4 °C and culture filtrate was analysed for antimicrobial activity

(Al-Ghazali and Omran, 2017). Based on the results, ISP 4 broth medium was selected for further mass production studies.

The SA4 strain was inoculated in the ISP 4 medium with five different carbon sources. The different sugars in 1% (w/v) used along with medium were glucose, fructose, glycerol, starch and lactose. The inoculated flasks were incubated at 200 rpm for 7 days. After 7 days, the fermentation medium optical density (OD) were measured and assayed for its antimicrobial activity.

The different organic nitrogen sources at 1% (w/v) were used *viz*., yeast extract, beef extract and peptone with ISP 4 production medium. The inorganic nitrogen sources used were ammonium sulphate and potassium nitrate. The SA4 strain was inoculated with various nitrogen sources and incubated at 200 rpm for 7 days. OD value was measured after incubation and the fermentation medium was centrifuged and examined for antimicrobial activity.

To determine the impact of salinity on growth and secondary metabolites production by the SA4 strain, ISP 4 production medium was amended with different NaCl concentration ranging from 0.5 to 5%. The medium was incubated in the rotary shaker for 7 days at 200 rpm. The fermentation medium was processed and assayed for antimicrobial activity after incubation periods.

2.6.2. Effect of pH, temperature and agitation

Optimum pH required for maximum production of strain SA4 was defined by adjusting the pH of the ISP 4 broth from 5, 7, 9 and 11, then it was incubated at 30 °C for 7 days in rotary incubator shaker (200 rpm). Later the incubation period, culture growth was observed at 600 nm using UV/Vis spectrophotometer and the fermentation medium was centrifuged and assayed for antimicrobial activity.

Optimal temperature for mass cultivation and production the strain SA4 was determined by incubating ISP 4 broth at different temperatures of 4 °C, 15 °C, 30 °C and 45 °C for 7 days in rotary incubator shaker (200 rpm). After 7 days, growth was determined by OD at 600 nm using UV/Vis spectrophotometer and the culture filtrate was evaluated for antimicrobial activity.

To study the effect of agitation on ISP 4 medium (150 ml) was prepared and inoculated with SA4 strain. The production flasks were incubated on rotary incubator shaker at different rpm such as 50, 100, 150, 200 and 250 for 7 days at 30 °C. After 7 days, the biomass concentration of the fermented broth was measured and broth was centrifuged and assayed for antimicrobial activity.

2.7. Optimized production of bioactive compounds and extractions

2.7.1. Fermentation process

Shake flask fermentation process was used for the production of bioactive compounds from selected potential strain SA4. A loopful of strain SA4 spore was inoculated into a four 500 ml flask each containing 250 ml of ISP 4 broth medium and finally production medium volume was 1200 ml. All the flasks were incubated for 14 days at 30 °C in a rotary shaker with 200 rpm (Yun et al., 2018).

2.8. Extraction and purification of bioactive compounds

The spent media were spinning at 5000 rpm for 20 min to confiscate biomass and other cell debris. The solvent extraction method was employed to separate bioactive composites from the culture filtrates (Berdy, 2005). Concisely, culture filtrate was blended with ethyl acetate was in the ratio of 1:1 (v/v) and dynamically shakes up for an hour. The aqueous and solvent phase was separated with separating funnel and solvent phase (ethyl acetate phase) comprises bioactive compounds was separated out from the aqueous phase. Then, the collected and concentrated with hot air oven at 40 °C (Raghava Rao et al., 2017). The concentrated, dried compounds were scrapped and dissolved in dimethyl sulfoxide (DMSO).

Thin layer chromatography (TLC) was accomplished to separate the active compound. Accordingly, the extract was loaded on 1 mm thickness TLC plates and chromatogram was developed using solvent chloroform: methanol (4:1). Then the plate was exposed to iodine vapour for 30 min. After 30 min brown colour spot was appeared on TLC plate and the Rf value was calculated (Omran and Kadhem, 2017). The spots were scraped from TLC plates, dissolved in DMSO and centrifuged at 10,000 rpm for 5 min in order to remove silica. The supernatant was collected and evaluated for its antimicrobial and nematicidal activities.

2.9. Antimicrobial activity of partially purified compound

The partially purified compound was examined for its antimicrobial activity by agar well diffusion method (Saadoun and Muhana, 2008) with Muller Hinton agar (MHA) plates. The bacterial and fungal test pathogens were swabbed onto the agar surface and the compound was dissolved in DMSO were added into the wells at various concentrations (25, 50 and 75 µg). The bacterial plates were incubated at 37 °C for 24 h and fungal plates at 30 °C for 3 days. Later, the period of incubation, inhibition zones were observed and documented.

2.10. Anti-nematicidal activity

To study the *M. incognita* nematode mortality, 50 µl larval suspension containing 100 freshly hatched root knot nematodes were taken in Petri plates. Three dilutions viz., 1:05, 1:10 and 1:20 were prepared from stock solutions of partially purified compound and from each dilution 5 ml was poured into the larval suspension. Distilled water was taken as control. The mortality of the nematode was observed after 24, 48 and 72 h by counting the live and dead larvae under the stereoscopic microscope, and mortality % was calculated and recorded (Sun et al., 2006).

2.11. Identification of bioactive compound

2.11.1. Fourier Transform infrared (FTIR) spectroscopy

The partially purified yellow coloured compound was subjected to FTIR spectroscopy analysis. The infrared spectra were scanned in the range of 4000 cm^{-1} to 400 cm^{-1} (Shimadzu IR-470) and result was documented (Singh et al., 2018).

2.11.2. Gas Chromatography mass spectrometry (GC–MS)

Partially purified active compound was analysed through the GCMS system equipped with Thermo GC-Trace Ultra Version: 5.0. Column condition was programmed with initial oven temperature at 70 °C raised to 260 °C at 6 °C/min. Helium gas was used as a carrier gas at the flow rate of 1.0 ml/min. The obtained peaks were analysed with mass spectral analysis. The spectrum was analysed from the available database in National Institute and Standard Technology (NIST) and the results were interpreted (El-Naggar et al., 2017).

3. Results and discussion

3.1. Isolation of actinomycete isolates

Isolation of actinomycetes was carried out by serial diluting the soil sample and inoculating on SCN agar by spread plate technique. Currently, three actinomycete isolates were selected based on distinct characters and they were designated as SA3, SA4 and SA5 (Fig. 1). These three strains were further processed to identify their

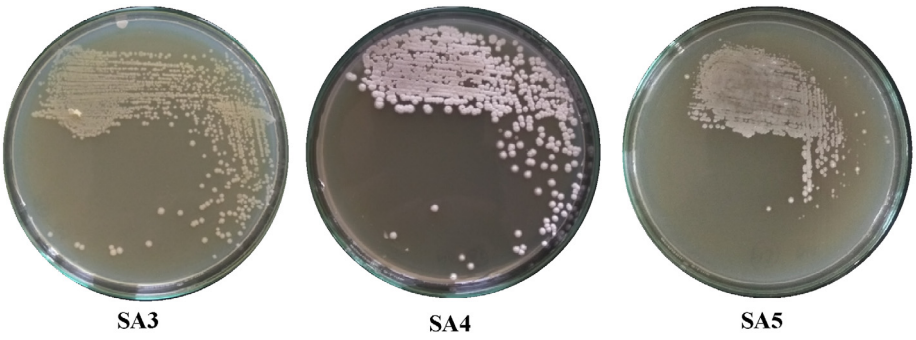

Fig. 1. Actinomycete strains isolated from soil samples on starch casein nitrate agar medium.

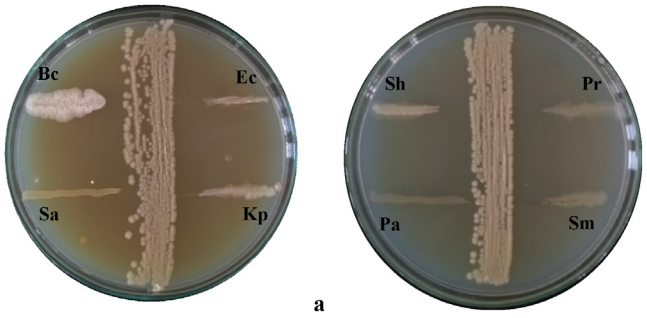

Fig. 2a. Antibacterial activity of isolate SA4; a) against bacterial pathogens. Bc- *Bacillus* sp.; Sa- *Staphylococcus* sp.; Ec- *E. coli*; Kp- *Klebsiella* sp. Sh- *Shigella* sp.; Pa- *Pseudomonas* sp.; Pr- Proteus sp.; Sm- *Salmonella* sp.

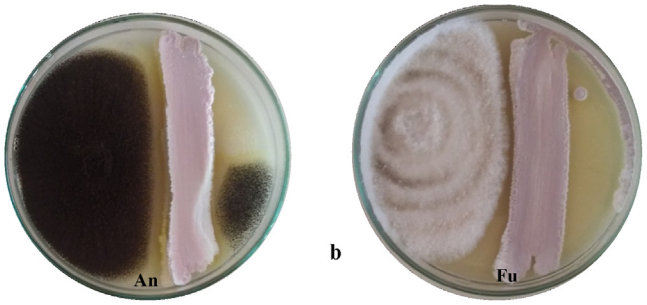

Fig. 2b. Antifungal activity of isolate 04; a) against fungal pathogens. An- *A. niger*; Fu- *Fusarium* sp.

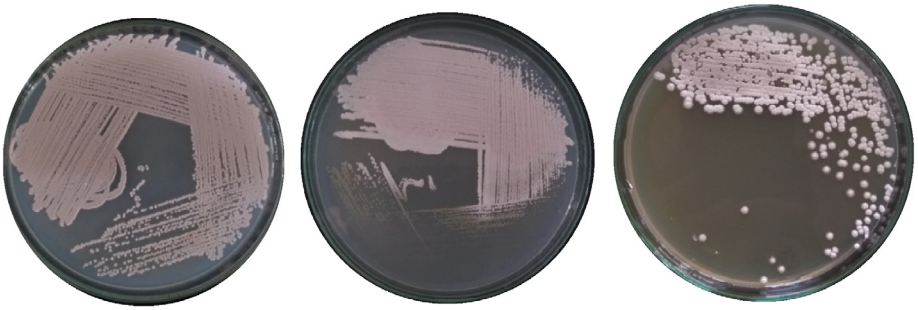

Fig. 3. Colony morphology of actinomycete isolate SA4; a) on SCN agar, b) ISP 4, c) AIA.

Table 1
Colony morphology of isolated actinomycete strains on Different media.

S. No.	Strains	Colony morphological features		
		SCN Agar	**AIA**	**NA**
1	SA3	White colony, flat margin	White, elevated colony	Creamy White colony
2	SA4	White to pink, elevated colony, with earthy odour	White to Lavender, with flat margin	White elevated colony
3	SA5	White to grey colony	White to light grey	White Colony

morphological, cultural, biochemical and antimicrobial activities. A characteristic rough colony with branched or aerial mycelia and arrangement of spores on aerial mycelia were recognized as actinomycete species.

3.2. In vitro preliminary screening of actinomycete strains

The three actinomycete strains SA3, SA4 and SA5 were screened for their antimicrobial activity against various bacterial and fungal pathogens by cross streak method. Among the three strains, the strain SA4 was found to be potential isolate and it exhibited a widest range of antimicrobial activity against the bacterial pathogens such as *E. coli*, *K. pneumonia*, *S. typhi*, *P. vulgaris*, *S. flexineri*, *S. aureus*, *B. subtilis* (Fig. 2a) and fungal pathogens such as *C. albicans, A. niger* and *Fusarium* sp., (Fig. 2b), Whereas the other two strains SA3 and SA5 were not exhibited significant antimicrobial activity against the tested microbial pathogens. Henceforth, the SA4 strain was picked as the potential candidature among the three strains and SA4 strain was considered for further exploration of bioactive metabolites production. Comparable results were described by Thakur et al. (2007) and Vijayakumar et al. (2012).

3.3. Identification and characterizations of prospective strain SA4

3.3.1. Cultural and colony morphology

The isolates SA3, SA4 and SA5 were cultured on three different media such as AIA, SCN agar and ISP 4 as shown in Table 1 where the three isoates showed different morphological charaterizations on three media. These isolates were developed as round, powdery, convex colonies with spreading edges. The morphological characteristics exhibited by the isolates were presented in Fig. 3. The results were corresponding with earlier studies of Baskaran et al. (2011) and Naine et al. (2012). The SCN agar was selected, because the medium suppressed the growth of bacterial and fungal colony and allowing the growth of actinomycetes (Ramesh and Mathivanan, 2009). Amongst three isolates, the isolate SA4 extensively studied and it was gram positive with long filamentous mycelia and spores arranged as long chains (Fig. 4).

3.3.2. Spore morphology

The spore chain morphology of strain SA4 grown in coverslip was observed under high power and oil immersion objective. The spore morphology of the SA4 strain was observed as rectiflexibles (Straight to flexuous) with simple branching and straight spore bearing hyphae. The results coordinated with earlier results of Sathiyaseelan and Stella (2011), who used coverslip technique to find out the spore chain morphology of different actinomycete isolates.

3.3.3. Biochemical test

Biochemical tests were performed to observe the biochemical and physiological activities of the SA4 strain. The strain SA4 showed diversified results and displayed in Table 2. The SA4 strain showed positive for methyl red test, citrate utilisation while indole test vogus proskauer and melanin production were negative; and enzymatic activity of SA4 strain for starch hydrolysis, urease production, catalase and oxidase test was positive while nitrate reduction was negative. In sugar fermentation, SA4 strain utilised glucose, sucrose, mannitol and exhibited positive colour change, whereas the fructose sugar was not fermented and the TSIA test also confirmed ability of SA4 starin on fermentaion of glucose, lactose and sucrose (Table 2).

3.3.4. 16S rRNA gene sequencing

The 16S rRNA gene sequence analysis was performed to elucidate the taxonomic position of isolated prospective strain SA4. The SA4 strain showed up closely related to *Streptomyces cuspidosporus* because it sharing 99.2% 16S rRNA gene similarity (Fig. 5). Clarridge (2004), reported that the genotypic methods were accurate then the phenotypic based identification. Hence, 16S rRNA gene sequence considers are choice of experiment for

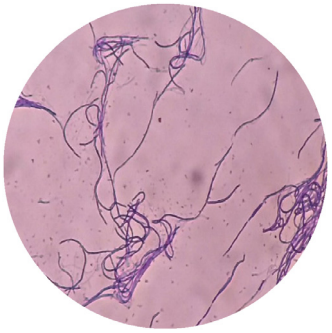

Fig. 4. Microscopic observation of isolate SA4 (1500X).

Table 2
Biochemical and physiological characteristics of strain SA4.

Characteristics test	SA4 Strain
Biochemical	
Indole	Negative
Methyl red	Positive
Vogus Proskauer	Negative
Citrate	Positive
Melanin production	Negative
Triple sugar iron agar	A/A
H_2S production	Negative
Urease	Positive
Catalase	Positive
Oxidase	Positive
Nitrate reduction	Negative
Starch hydrolysis	Positive
Carbohydrate utilization	
Glucose	Positive
Sucrose	Positive
Fructose	Negative
Mannitol	Positive

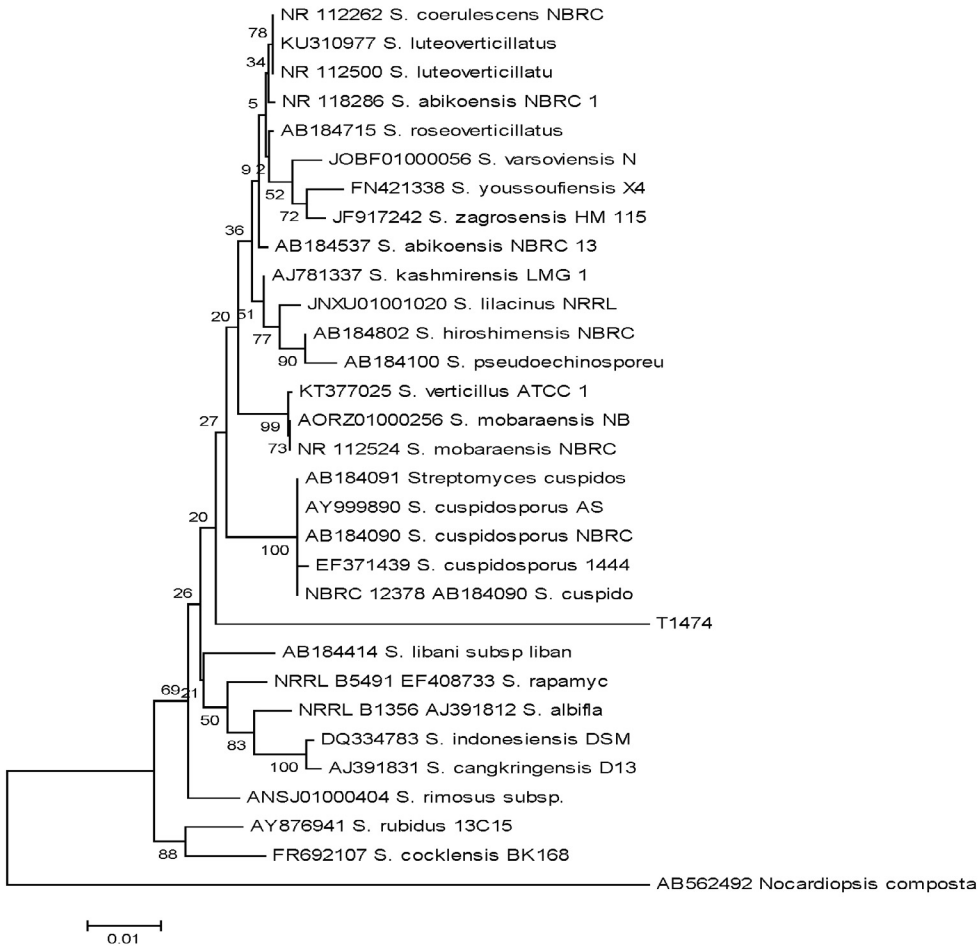

Fig. 5. Phylogenetic tree and relationships of closely associated actinomycetes species. Isolate SA4 code: T1474, and it was identified as *Streptomyces cuspidosporus* stain SA4.

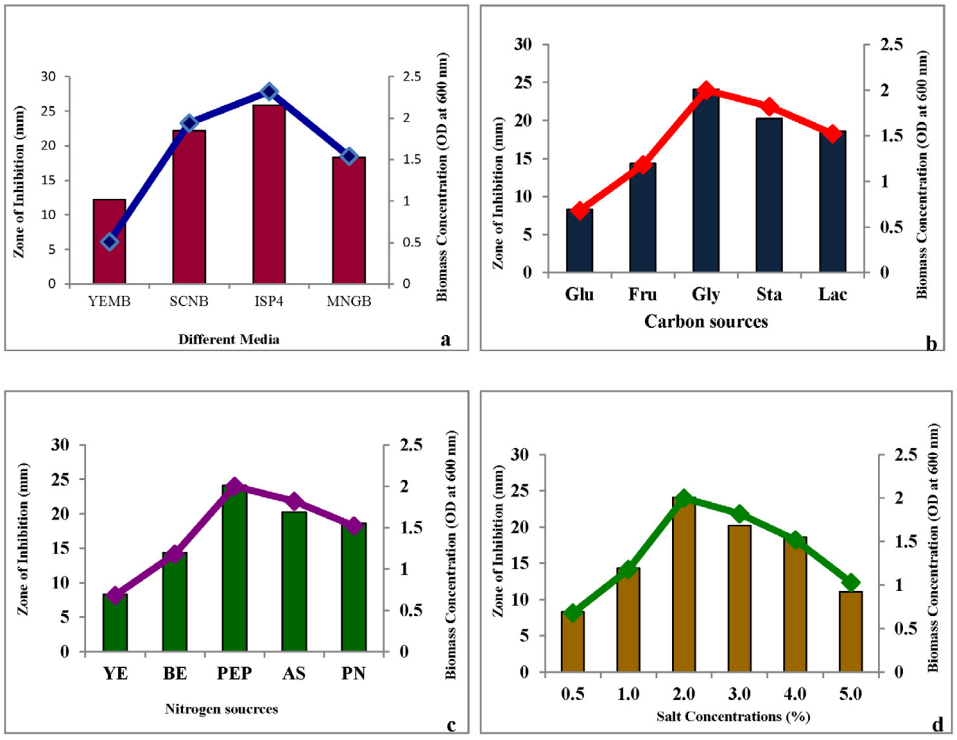

Fig. 6. Effect of bioactive metabolites production by *S. cuspidosporus* SA4; a) different media, b) carbon sources, c) nitrogen sources, d) salt concentrations.

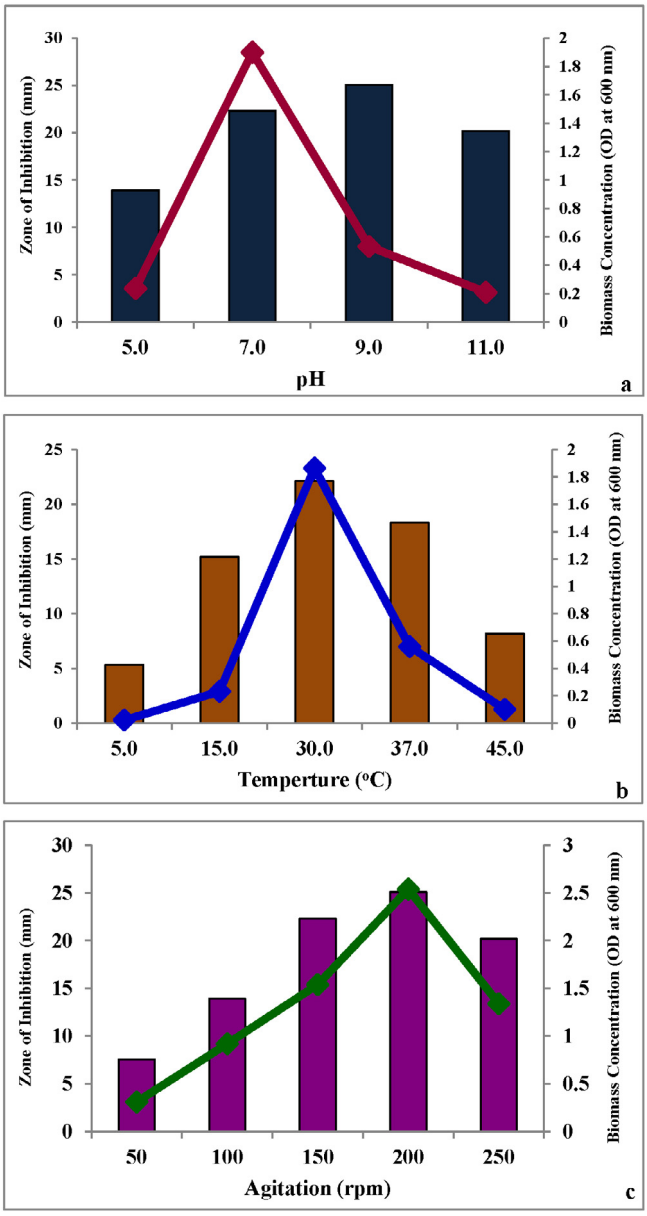

Fig. 7. Effect of bioactive metabolites production by *S. cuspidosporus* SA4; a) pH, b) temperature, c) agitation.

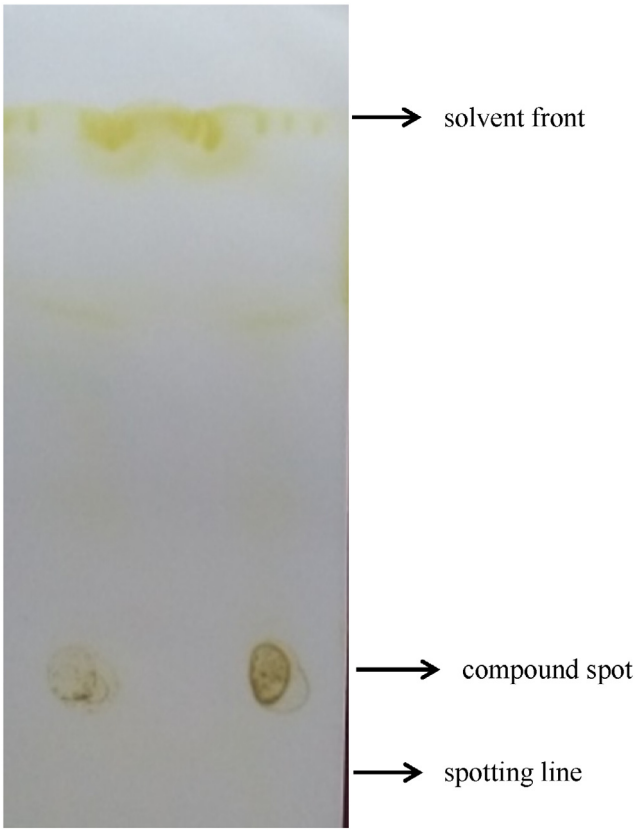

Fig. 8. Spot of bioactive compound from *S. cuspidosporus* SA4 on TLC plate.

Table 3
Antimicrobial activity of *S. cuspidosporus* SA4 crude extract.

Microbial pathogens	Zone of inhibition (mm)/Concentration (µg)		
Bacterial	**25**	**50**	**75**
E. coli	11.00	14.00	19.00
K. pneumoniae	9.00	13.00	17.00
P. vulgaris	8.00	12.00	15.00
P. aeurigenosa	0.00	0.00	0.00
S. flexineri	9.00	11.00	16.00
S. typhi	10.00	15.00	19.00
S. aureus	8.00	11.00	14.00
B. subtilis	10.00	13.00	17.00
Fungal			
A. niger	–	8.00	15.00
A. flavus	–	–	8.00
C. albicans	–	10.00	18.00
Fusarium sp.	–	9.00	14.00
Unknown Fungi	–	11.00	19.00

rarely isolated, phenotypically aberrant isolates for identification of microorganisms (Souagui et al., 2019).

3.4. Optimisation and production of bioactive compound with *S. cuspidosporus SA4*

The production medium constituents and concentrations are nearly associated with the metabolic capabilities of the bioactive metabolites producing strain and also prominently influence the biogenesis of metabolic compounds (Elleuch et al., 2010). In general, the *Streptomyces* species are owned its antimicrobial activities, in addition in this studies were provided appropriate cultural parameters for mass production.

3.4.1. Effect of media, carbon, nitrogen source and salt concentrations

Among the three different broth media examined for biomass cultivation and bioactive compound production in shake flask conditions, ISP 4 medium was found to be the most applicable medium for the mass cultivation and also production active antimicrobial compounds by *S. cuspidosporus* SA4, while lowest production was seen with MNGB and YEM (Fig. 6a). Thus, ISP 4 broth served as the choice medium for mass production bioactive metabolites.

Highest biomass production of strain SA4 was obtained in the medium consists of glucose and starch as carbon source (Fig. 6b). This outcome was quite comparable with the study stated by Singh et al. (2014). Since, the carbon source with simple sugars like glucose, fructose, and sucrose as sole carbon source improved growth and also active metabolite production rather than complex carbon sources (Oskay, 2011).

Table 4
Antimicrobial activity of partially purified extract from *S. cuspidosporus* SA4.

Microbial pathogens	Zone of inhibition (mm)/Concentration (μg)			Streptomycin (10 μg)
Bacterial	**25**	**50**	**75**	
E. coli	12.00	15.00	22.00	25.00
K. pneumoniae	10.00	14.00	19.00	19.00
P. vulgaris	10.00	13.00	17.00	17.00
P. aeurigenosa	0.00	0.00	7.00	15.00
S. flexineri	10.00	13.00	18.00	19.00
S. typhi	12.00	17.00	20.00	21.00
S. aureus	9.00	12.00	16.00	18.00
B. subtilis	11.00	15.00	20.00	19.00
Fungal				**Nystatin (100 units)**
A. niger	–	9.00	17.00	18.00
A. flavus	–	10.00	13.00	15.00
C. albicans	–	11.00	22.00	20.00
Fusarium sp.	–	10.00	16.00	18.00

Fig. 9. Antimicrobial activity of partially purified bioactive compounds of *S. cuspidosporus* SA4; a) *E. coli,* b) *K. pneumoniae*, c) *P. vulgaris*, d) *S. aureus*, e) *S. typhi*, f) *B. subtilis*, g) *Fusarium* sp., h) *A. niger*.

The outcomes of the nitrogen sources indicated that strain SA4 was utilized various forms both organic and inorganic nitrogen sources. Biomass concentration was higher in the medium containing ammonium sulphate and peptone as nitrogen source (Fig. 6c). Similar reports were obtained by Lee et al. (1997) and Farid et al. (2000). In general glucose, potassium dihydrogen orthophosphate, ammonium sulphate, sodium nitrate and beef extract were favourable ingredients for the production of bioactive compounds from *Streptomyces* species.

It was experimental that the production of antimicrobial metabolites was shown maximal existence of 2% NaCl. The growth of the strain SA4 gradually decreased with the increase of NaCl

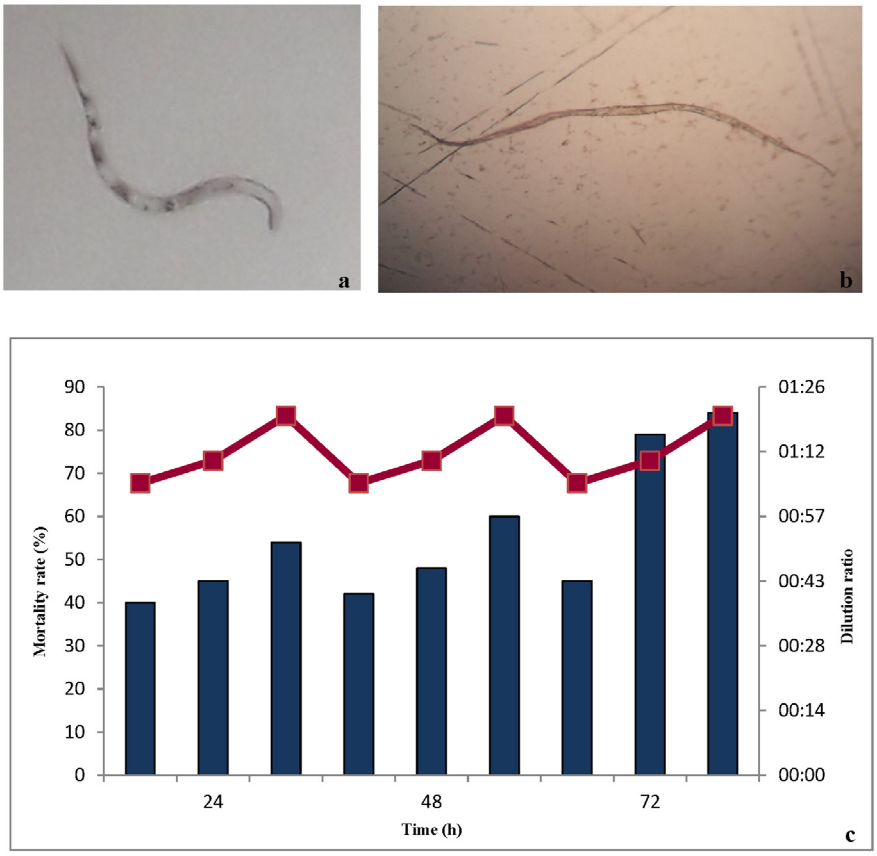

Fig.10. Nematicidal activity of partially purified bioactive extract of *S. cuspidosporus* SA4 against *M. incognita*; a) Live nematode, b) dead nematode, c) nematicidal activity.

Table 5
FTIR Absorption frequencies of functional groups in partially purified extract of *S. cuspidosporus* strain SA4.

S. No.	Characteristic Absorption (cm^{-1})	Functional group	Name of Functional group
1	3618	O—H	Alcohol
2	3444	N—H	Amine
3	2970	O—H	Carboxylic acid
4	2866	C—H	Alkane
5	2522	O—H	Carboxylic acid
6	1635	C=C	Alkene
7	1157	C—F	Alkyl halide
8	1087	C—O	Ester
9	1018	C—O	Ester

concentration (Fig. 6d). The NaCl is also essential elements for the growth of microorganisms for its water activity and osmotic pressure of the surrounding environments (Singh et al., 2009).

3.4.2. Effect of pH, temperature and agitation

The optimum pH of strain SA4 for biomass and bioactive compound production was pH 9.0. Poor growth was observed below and above 7.0 pH (Fig. 7a). Similarly, the maximum production of antimicrobial compound from *S. albidoflavus* was found at pH 9.0 Narayana and Vijayalakshmi, 2008; Saurav and Kannabiran, 2010). The results proposed that the broth pH is playing a critical role in the secondary metabolites production.

The strain showed highest level of biomass and bioactive compound production was noticed at 30 °C incubation. The strain was found to be strictly mesophilic, extreme temperature was unfavourable for bioactive production (Fig. 7b). Jacob et al. (2017), reported that 30 °C and 7.0 was the optimum temperature and pH for growth and metabolites assembly by *S. nogalater* NIIST A30. Naragani et al. (2014), recorded high yields of antimicrobial metabolites by *Rhodococcus erythropolis* VLK-12 when cultured in ISP 2 broth supplemented with lactose and asparagine incubated with initial pH 7.0 at 30 °C.

Agitation helps in facilitating greater aeration to the cells, thus provides favourable conditions for the greater availability of the nutrients to the culture. The maximum growth and production of bioactive compound by *S. cuspidosporus* SA4 was observed at 200 rpm (Fig. 7c). Beyond 200 rpm, the growth and antimicrobial compound production was decreased gradually. Abdel-Aal et al. (2011), reported that the cell growth and anisomycin production of *S. griseolus* was exhibited maximum at 200 rpm. Geetha Ramani and Vinoth Kumar (2012), reported that the 200 rpm of agitation was optimum for active metabolites biogenesis by *Streptomyces* sp. SH7. Ivana et al. (2017), was observed the production of antimicrobial compound from *S. hygroscopicus* was found maximum at 100 and 200 rpm in a lab scale bioreactor which showed that agitation rates in shake flask method results in maximum production of secondary metabolites from *Streptomyces* sp.

3.5. Antimicrobial activity of crude extract

The crude extract containing the secondary metabolite was examined for its antimicrobial activity alongside the bacterial and fungal tested pathogens by agar well diffusion method. The inhibition zone was measured and the results were tabulated in Table 3. Bioactivity of the crude extract at concentration 75 µg was the highest against bacterial and fungal tested pathogens compared to the crude extract bioactivity at a concentration 25 and 50 µg, and also low bioactivity of the crude extract at a concentration

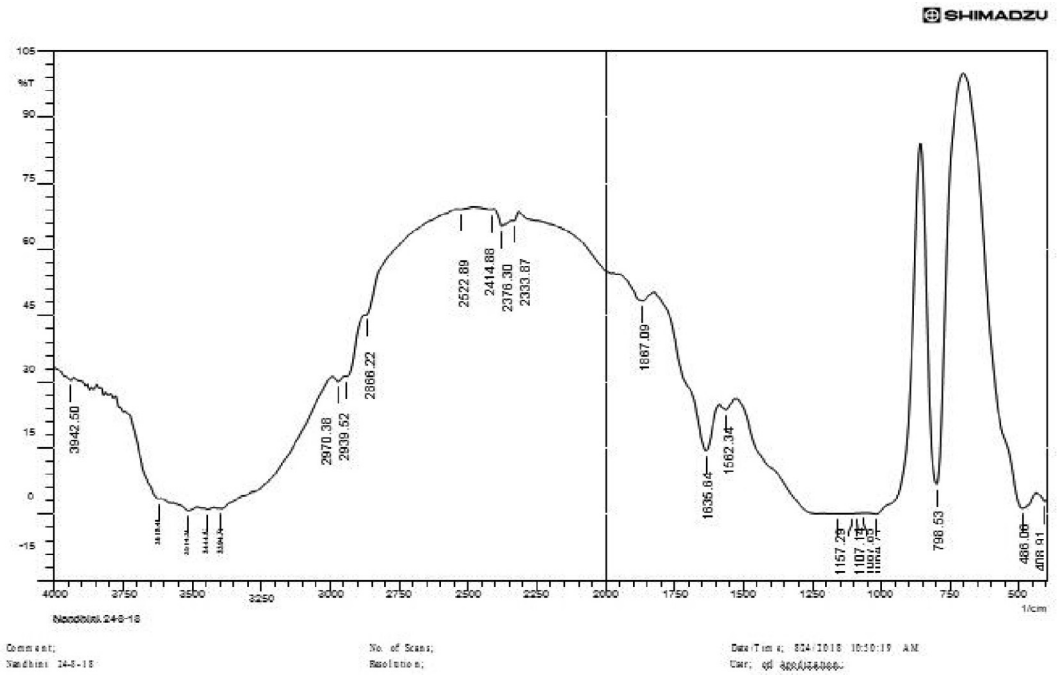

Fig. 11. FTIR absorption spectrum of partially purified extract of *S. cuspidosporus* SA4 and functional groups.

Table 6
GC–MS peaks of partially purified compounds from *S. cuspidosporus* SA4.

S. No.	Retention time	Area %	Compound Name	Mol. Formula	Mol. weight
1	18.762	5.96	1-Heptadecene	C17H34	238
2	18.866	5.74	Phthalic acid, bis (7-methyloctyl) ester	C26H42O4	418
3	20.308	2.32	Heptadecane	C17H36	240
4	20.464	3.04	Icosane	C20H42	282
5	21.576	7.52	Hexadecane, 2,6,10,14_Tetramethyl	C20H42	282
6	21.662	2.42	1-Octadecene	C18H36	252
7	23.210	2.77	1,2-Benzenedicarboxylic acid, dibutyl ester	C16H22O4	278
8	23.275	2.32	Dotriaconatane	C32H66	450
9	23.808	8.20	1,2-Benzenedicarboxylic acid,Bis(2-Methylpropyl) ester	C16H22O4	278
10	24.055	4.27	Phthalic acid, butyl 2-pentyl ester	C17H24O4	292
11	24.114	6.82	Dibutyl phthalate	C16H22O4	278
12	24.947	3.50	1,2-Benzenedicarboxylic acid, butyl 2-ethylhexyl ester	C20H30O4	334
13	25.809	3.25	1-Butyl2-(8-Methylnonyl) Phthalate	C20H30O4	362
14	26.427	5.47	n-Nonadecanol-1	C19H40O	284
15	28.547	3.75	Eicosyl trifluroacetate	C22H41F3O2	394
16	30.120	4.15	1,2-Benzenedicarboxylic acid, diisooctyl ester	C24H38O4	390
17	30.513	4.01	Docosyl Heptaflurobutyrate	C26H45F7O2	522

25 μg against bacterial tested pathogens while no bioactivity against fungal tested pathogens.The crude extract of *S. cuspidosporus* SA4 was shown antibacterial activity against *E. coil, K. pneumoniae, P. vulgaris S. typhi, S. flexneri, S. aureus* and *B. subtilis* and no activity against *P. aeruginosa,* range of antifungal activity was observed for *A. niger, A. flavus, C. albicans, Fusarium sp.* and Unknown Fungi at concentration 75 μg of crude extarct as shown in Table 3. Similar kind of outcomes was described by Selvameenal et al. (2009) and Thakur et al. (2007).

3.6. Extraction and purification of bioactive compound

The purification and separation of bioactive compound was performed by TLC. The retention factors (Rf) of moved spot was 0.83 (Fig. 8). The spot was scrapped and composed in a vial, then evaluated for its antimicrobial activity. The results were coordinated with the earlier studies of Augustine et al. (2005), who purified bioactive substance from *S. albidoflavus* PU23 by using TLC.

3.7. Antimicrobial activity of bioactive compound from S. cuspidosporus SA4

The zone of inhibition was measured at different concentrations of purified compound (25, 50 and 75 μg) against tested microbial pathogens and the results presented in Table 4 showed that bioactivity of purifed compound at 75 μg was more effective than its activity at other concentrations used. The antibacterial activity of compound extracted from *S. cuspidosporus* SA4 reported effective against *S. aureus* (16.00 mm), *B. subtilis* (20.00 mm), *E. coli* (22.00 mm), *K. pneumoniae* (19.00 mm), *P. vulgaris* (17.00 mm), *S.*

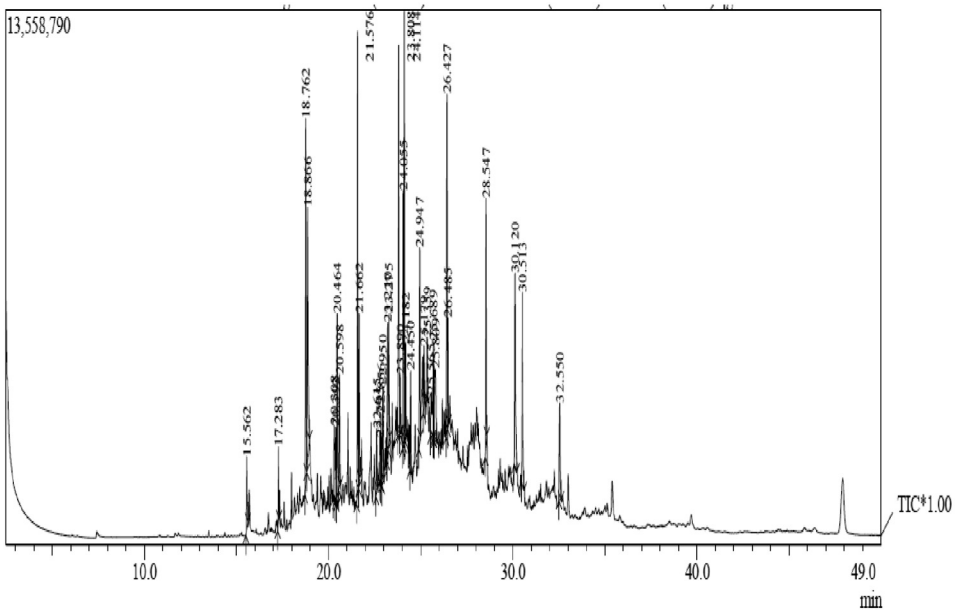

Fig. 12. GC–MS chromatogram of partially purified bioactive extract from *S. cuspidosporus* strain SA4.

flexineri (18.00 mm) and *S. typhi* (20.00 mm) compared to activity of stretomycin, while *P. aeurigenosa* (7.00 mm) showed very less zone of inhibition (Table 4 and Fig. 9). The antifungal activity of compound of strain SA4 was as follow as; *A. niger* (17.00 mm), *A. flavus* (13.00 mm), *C. albicans* (22.00 mm) and *Fusarium* sp. (18.00 mm) compared to activity of nystatin as showwn in Table 4 and Fig. 9. In accordance with previous studies, several actinomycetes isolated and characterized from soil were proven to have antimicrobial activities (Saleh et al., 2014; Al-Ansari et al., 2019; Ouchari et al., 2019; Malisorn et al., 2020).

3.8. Nematicidal activity of S. cuspidosporus SA4 against M. incognita

Nematicidal activity of partially purified bioactive extract of *S. cuspidosporus* SA4 was tested against the second stage juveniles of the root-knot nematode. The result showed that, with an increase in the concentration of partially purified bioactive extract concentrations from 1:20 to 1:5, there was corresponding significant increase in the mean larval mortality. As the exposure time increase from 24 to 72 h, there was significant corresponding increase in larval mortality (Fig. 10). The higher concentration of active compound (1:5) recorded 84% mortality, whereas lower concentration (1:20) of culture filtrate recorded only 40% mortality.

There are numerous species of *Streptomyces* that can be selected for nematode control. The investigations of the current study were in line with Sharma et al. (Park et al., 2020), who tested nematicidal activity of culture filtrates of six different strains of *S. antibioticus* strain M7 on *M. incognita*. Park et al. (2020), reported nematicidal activity of *Streptomyces yatensis* KRA-28 showed mortality against *M. incognita* ranging from 75 to 85% in *in vitro* assay.

3.9. Identification of bioactive compounds

The FTIR results revealed the presence of alcohol (3618 cm^{-1}), amine (3444 cm^{-1}), carboxylic acid (2970 or 2522 cm^{-1}), alkane (2866 cm^{-1}), alkene (1635 cm^{-1}), alkyl halide (1157 cm^{-1}) and ester (1087 or 1018 cm^{-1}) functional groups acccording to the absorption present in the partially purified bioactive extract of *S. cuspidosporus* SA4 as shown in Table 5 and Fig. 11, which were renowned to have antimicrobial properties. Alike results were witnessed by Mathur et al. (2015), they confirmed the FTIR spectrum of ethyl acetate extracts of *Streptomyces* sp. In general, the functional groups such as "alcohol, phenol, alkanes, aldehyde, aromatic compound, secondary alcohol, aromatic amines and halogen compounds" are detected from FTIR analysis (Sahu and Saxena, 2013).

Conferring, the GCMS chromatogram peak area, molecular weight and molecular formula, the derived compound was identified and presented in Table 6 and Fig. 12. The peak area is directly proportional to magnitude of the bioactive compounds existing in the partially purified composites. GCMS analysis showed the presence of 17 compounds, most of which were earlier documented for its antibacterial, antifungal, antitumor, anticancer and antioxidant activities (Al-Dhabi et al., 2014; Tan et al., 2015; Law et al., 2019; Priyanka and Debajit, 2020; Sholkamy et al., 2020).

The GCMS analysis of *S. cuspidosporus* SA4 indicated the presence highest peak area percentage of Phthalate derivatives. It includes 8.20% of 1,2-Benzenedicarboxylic acid, Bis (2-Methyl propyl) ester, 6.82% of Dibutyl phthalate, 5.74% of Phthalic acid, bis (7-methyloctyl) ester, 5.74 of Phthalic acid, bis (7-methyloctyl) ester, 4.27 % of Phthalic acid, butyl 2-pentyl ester, 4.15 % of 1,2-Benzenedicarboxylic acid, diisooctyl ester, 3.5 % of 1,2-Benzenedicarboxylic acid, butyl 2-ethylhexyl ester and 2.77 % of 1,2-Benzenedicarboxylic acid, dibutyl ester. These derivatives and components are biologically active compounds and are effective against various microbial pathogens.

4. Conclusion

The present study was concluded that, actinomycetes exclusively *Streptomyces* sp. are recognized to generate an immense range of composites with varied biological attributes. The present investigation publicized the presence of bioactive compounds such as Phthalic acid, bis (7-methyloctyl) ester, Hexadecane,2,6,10,14-Tetramethyl, 1,2-Benzenedicarboxylic acid, Bis (2-methylpropyl) ester, Dibutyl phthalate, n-Nonadecanol-1 were demonstrated a broad range of microbiocidal activity against the bacterial and fungal pathogens. Hence, this outcome indicates that the metabolite

produced by *S. cuspidosporus* strain SA4 has a potential controlling efficiency and it can be used as a promising candidate for antimicrobial drug in near future.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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