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Extraction and identification of bioactive compounds (eicosane and dibutyl phthalate) produced by *Streptomyces* strain KX852460 for the biological control of *Rhizoctonia solani* AG-3 strain KX852461 to control target spot disease in tobacco leaf

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Abstract

Streptomyces strain KX852460 having antifungal activity against *Rhizoctonia solani* AG-3 KX852461 that is the causal agent of target spot disease in tobacco leaf. The aim of the study was to determine the antifungal activity of *Streptomyces* strain KX852460 extract against *R. solani* AG-3 and to identify bioactive antifungal compounds produced by strain KX852460. Crude substance was produced by submerged fermentation process from *Streptomyces* strain KX852460. Various solvent was used to extract the culture filtrate. Among all, ethyl acetate extracted supernatant showed great potency against *R. solani* AG-3 KX852461. The active fractions were purified by silica gel column chromatography having 52 mm zone of inhibition against *R. solani* AG-3 KX852461. The purified fractions were identified by gas chromatography–mass spectrometry technique. Twenty-seven compounds were identified and most of the compounds were the derivatives of aromatic compounds. Eicosane (C₂₀H₄₂) and dibutyl phthalate (C₁₆H₂₂O₄) were found antifungal compounds in this study. While morphinan, 7,8-didehydro-4,5-epoxy-17-methyl-3,6-bis[(trimethylsilyl)oxy]-, (5. Alpha. 6. Alpha)—(C₂₃H₃₅NO₃Si₂), cyclononasiloxane, octadecamethyl—(C₁₈H₅₄O₉Si₉) and benzoic acid, 2,5-bis(trimethylsiloxy) (C₁₆H₃₀O₄Si₃) were the major compounds with highest peak number. These results suggested that *Streptomyces* strain KX852460 had good general antifungal activity and might have potential biocontrol antagonist against *R. solani* AG-3 KX852461 to cure the target spot in tobacco leaf.

Keywords: *Streptomyces* strain KX852460, *Rhizoctonia solani* AG-3, Gas chromatography–mass spectrometry, Aromatic compounds, Target spot

Introduction

In the Liaoning province of China in 2006 target spot disease of tobacco was investigated, *Rhizoctonia solani* was the causal agent and caused heavy economic loss regarding to the production and quality of the tobacco (Wu et al. 2012). Anastomosis AG-3 of the *R. solani* is the

causal agent of target spot in tobacco (Johnk et al. 1993). To conflict with phytopathogens biocontrol is most potent and environment friendly practice (Castano et al. 2013). Against the *R. solani* biocontrol is better strategy even though environmental conditions affected its efficacy (dos Reis Almeida et al. 2007). *Actinomycetes* are extensively present microorganisms in the environment that have potential to produce bioactive compounds against the phytopathogens (Xue et al. 2013; Zeng et al. 2013). Among the *Actinomycetes*, *Streptomyces* is the

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largest genus and belongs to the family *Streptomycetaceae* (Kämpfer 2006).

Streptomyces have potential to produce curial compounds for antibiotics and agro-antibiotics (Demain 2009). *Streptomyces* species are the source of 75% of antibiotics (Bhavana et al. 2014). Different solvents and elucidation utilized to extract the secondary metabolites and their structure can be found by different techniques for example gas chromatography–mass spectrometry (GC–MS), liquid chromatography–mass spectrometry (LC–MS), and nuclear magnetic resonance (NMR) (Tiwari et al. 2015). Volatile and semi-volatile compounds with lower molecular mass can be separated by using GC–MS (Snyder et al. 2012). The GC–MS is novel technology for isolation of the compounds that present in the secondary metabolites. Recently antibacterial (Khatab et al. 2016), antifungal compound against *Lycopersici* and *Fusarium oxysporum* (Jalaluldeen et al. 2015), antifungal compounds against *Pyricularia oryzae* (Awla et al. 2016), and broad spectrum pharmaceutical compounds (Narsaiah et al. 2014) were extracted by GC–MS. The main objective of this study was extraction, purification and identification of bioactive antifungal compounds produced from *Streptomyces* strain KX852460 grown under submerged fermentation.

Materials and methods

Microorganism

Streptomyces strain was isolated from soil and identified by 16S rRNA gene sequence technology and sequence was submitted to Gene bank under accession number of KX852460 and also submitted to Chinese general microbial collection center (CGMCC4.7384). The strain was used for the production of antifungal compounds in submerged fermentation. *Rhizoctonia solani* AG-3 was obtained from naturally infected tobacco leaves in Dandong of China which was also identified by 16S rRNA gene sequence technique and sequence obtained was submitted to the Gene bank and under Accession Number of KX852461 and also submitted to Chinese general microbial collection center (CGMCC3.18223). Other test pathogens obtained from the plant pathology lab of college of plant protection, Shenyang agricultural University China. Fungus pathogens were stored on potato dextrose agar (PDA) at 4 °C.

Preparation of inoculum

Fermentation was performed in two stages, seed growth and production of active antifungal substance. *Streptomyces* Strain KX852460 was grown on plates of Gause's synthetic agar medium at 28 °C for 5 days after spore production used in liquid fermentation medium. Two

spore cakes (5 mm) were used to inoculate a 250 ml flask having medium volume of 40 ml and then incubated at 28 °C with agitation speed of 160 rpm for 48 h.

Fermentation technique

For the production of antifungal compounds, 40 ml of fermentation medium [47 g soluble starch, 3 g yeast extract, 22 g peanut meal, 2.7 g (NH₄)₂ SO₄, 2.7 g NaCl, 2.7 g CaCO₃ dissolved in 1 L distilled water and pH was adjusted to 6.8–7.2] was taken in 250 ml flask and sterilized. After sterilization, the medium was inoculated with 5% (v/v) seed culture and incubated at 28 °C in rotatory shaker with agitation speed of 160 rpm for 96 h. After the termination of fermentation process, the culture was centrifuged and the supernatant was stored at –4 °C for further work (Gao et al. 2015).

Antifungal activity

Antifungal activities were determined by oxford cup method (Wang et al. 2010a, b) and measured the inhibition zone.

Stability test of the cultural filtrate of *Streptomyces* KX852460

Thermal stability, pH stability, illuminated light stability, and UV light stability were performed according to Zhao and Wu (2006). All the experiments were performed in triplicates and antifungal activity determined by oxford cup method mentioned above.

Extraction of the culture filtrate

The culture filtrate (500 ml) was extracted two times with ethyl acetate as solvent. The solvent was added to the filtrate in the ratio of 1:1 (v/v) and shaken vigorously for 20 min. The ethyl acetate phase that contains antibiotic was separated from the aqueous phase using separating funnel. Ethyl acetate layer was concentrated by evaporating to dryness at 50 °C and residue obtained was purified using methanol to (1.8 g) brown crude extract (Ahmed 2007).

Purification and identification of the compound

The purification of the antimicrobial compound was carried out using silica gel column chromatography as described by Atta et al. (2009). Ethyl acetate was used as eluting solvent. The column was packed with silica gel (60–120 mesh). The sample to be separated was loaded on the packed column and eluted with the solvent at the flow rate of one drop per minute. A conical flask was placed at the bottom of the column to collect the eluted fractions. Antifungal activity was checked and most active fractions were used for further analysis. The antifungal compounds

were identified by using gas chromatography–mass spectrometer technique (GC–MS). Agilent technologies 6890–5973 N with capillary column TG-5 ms Phenyl Methyl Siloxane (30 m × 250 μm × 0.25 μm) system were used. Mass detector used in split mode, and helium gas with flow rate of 1.0 ml/min was used as a carrier. Injector was operated at 230 °C and oven temperature for initial setup was 60 °C for 2 min, ramp 10/min to 280 °C for 8 min.

Results

Streptomyces strain KX852460 was isolated from the soil and screened against the *R. solani* AG-3 that is the causal agent of target spot in tobacco leaf. This strain had great potency against the pathogen. Twenty liter fermentation broth was produced by the *Streptomyces* strain KX852460 and broth was active against different plant pathogens including *R. solani* AG-3 having inhibition zone diameter of 45.78 mm. The strain also showed strong activity against *Sclerotinia sclerotiorum* with inhibition zone diameter of 50.4 mm (Table 1). Antifungal activity of the fermentation broth was found stable at various temperature levels from 60 to 90 °C, while at 100 °C activity was decreased (Fig. 1a). At different pH values antifungal activity of fermentation broth was observed, having peak activity at pH 6, while extreme pH conditions (pH 2, 14) resulted decreased antifungal activity (Fig. 1b). Fermentation broth treated with illuminated light showed stability in the activity against the pathogen (Fig. 1c). Under UV light treated broth was affected within duration of treatment and activity decreased abruptly (Fig. 1d). Solvent extraction method used to extract the bioactive compounds with several organic polar and non-polar solvents. All the extracts showed some inhibition effect against the pathogen ranging from 1.43 to 44.36 mm inhibition zone. But extract with ethyl acetate showed strong antifungal activity against the *R. solani* AG-3 KX852461 (Fig. 2). For further work based on this result ethyl acetate was selected and resulted antifungal

activity against *R. solani* AG-3 KX852461 (Fig. 3). Crude extract dried at 50 °C and brown color powdery substance obtained that further purified by silica gel column chromatography by using ethyl acetate as eluent. Several fractions were obtained and most active fractions against the *R. solani*. In (Fig. 4) fraction number 8 had strongly inhibited the *R. solani* AG-3 with diameter of inhibition zone 52 mm. Active fraction was further analyzed by gas chromatography–mass spectrometer (GC–MS). GC–MS analysis detected 27 bioactive compounds (Table 2). By comparison of mass spectra of the constituent with NIST library twenty-seven peaks obtained (Fig. 5a). Among 27 different compounds, 16 compounds were the constituent of aromatic compounds while others were derivatives of different hydrocarbons. Eicosane (C₂₀H₄₂) and dibutyl phthalate (C₁₆H₂₂O₄), having retention time of 13.641 (Fig. 5b) 18.852 (Fig. 5c) respectively were two antifungal compounds identified.

Discussion

In this study *Streptomyces* strain KX852460 was screened against the *R. solani* AG-3 that is the causal agent of target spot disease in tobacco. Strain KX852460 belongs to *Streptomyces* which strongly inhibits the pathogen and could be curing the target spot in tobacco. The effects of the *R. solani* diseases are very severe throughout the world and affected the quality and yield of the several crops. For the control of *R. solani*, bacterial antagonist could be an environment friendly substituent. Various bacterial antagonists against the *R. solani*, including *Bacillus subtilis* CA32 in eggplant (Abeyasinghe 2009), *Pseudomonas fluorescens* In5 (Michelsen and Stougaard 2011), *Burkholderia cepacia* T1A-2B and *Pseudomonas* sp. T4B-2A in tomato (De Curtis et al. 2010), inoculum of GB7 and 3Re4-18 in lettuce (Grosch et al. 2012), endophytic *Streptomyces* damping off growth promotion in tomato (Goudjal et al. 2014) have been reported as effective biological control agents. Antagonist activity of *Streptomyces* sp. CACIS-1.16CA against different phytopathogens including *R. solni* was also investigated by Evangelista-Martínez (2014).

In this study stability of the active cultural filtrate was determined at various temperatures, pH values, and treated with illuminated light and UV light. At 60–90 °C the antifungal activity of cultural filtrate of the *Streptomyces* strain KX852460 remained same, however above these conditions, the activity become decreased. pH values remained stable between 5.0 and 8.0 pH, while pH values above and lower beyond this were not stable. Uddin et al. (2013) reported stability of antimicrobial filtrate at different temperature and pH values. Treated with illumination light cultural filtrate was stable and showed good activity. Treated with UV light for long

Table 1 Antimicrobial effects of the cultural filtrate

Test pathogens	Inhibition spectrum (mm) of cultural filtrate
<i>Alternaria alternata</i>	33.96
<i>Botrytis cinerea</i>	40.16
<i>Alternaria solani</i>	38.62
<i>Rhizoctonia solani</i> AG-3	45.78
<i>Fusarium oxysporum</i>	24.78
<i>Sclerotinia sclerotiorum</i>	50.4
<i>Bipolaris maydis</i>	33.72
<i>Colletotrichum capsici</i>	25.48

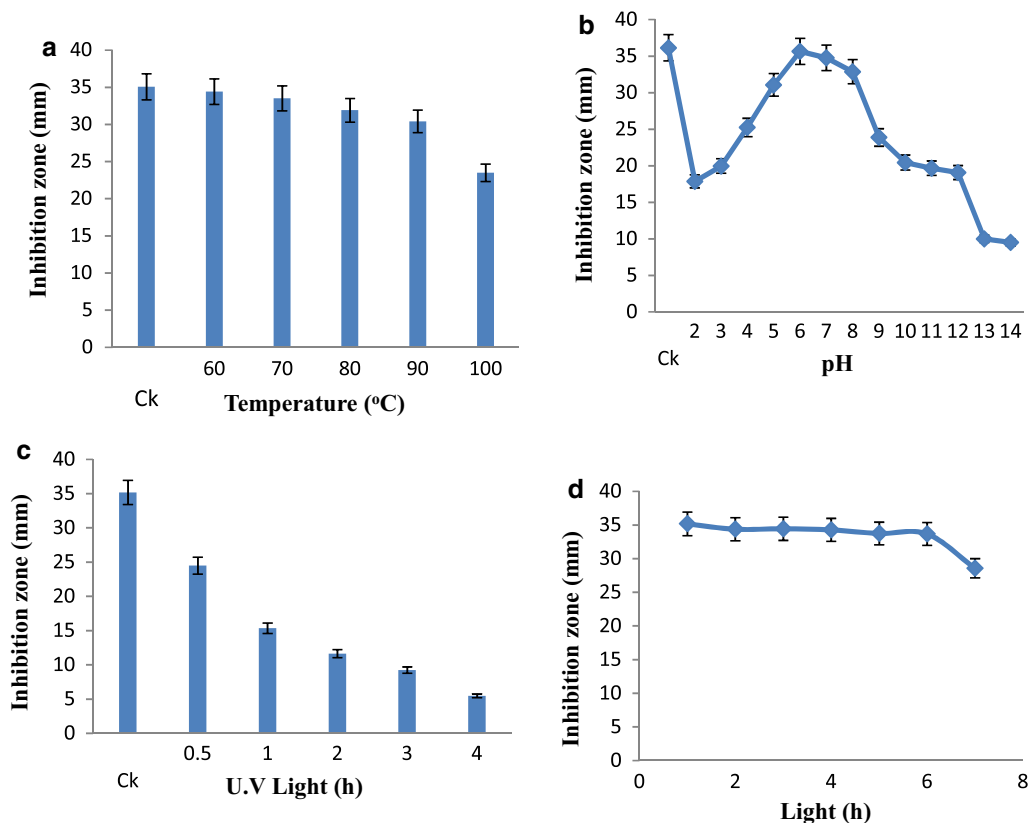


Fig. 1 Effect of temperature (a), pH (b), illuminated light (c) and ultra violet light (d) on the stability of fermentation broth. Ck represents control of each treatment

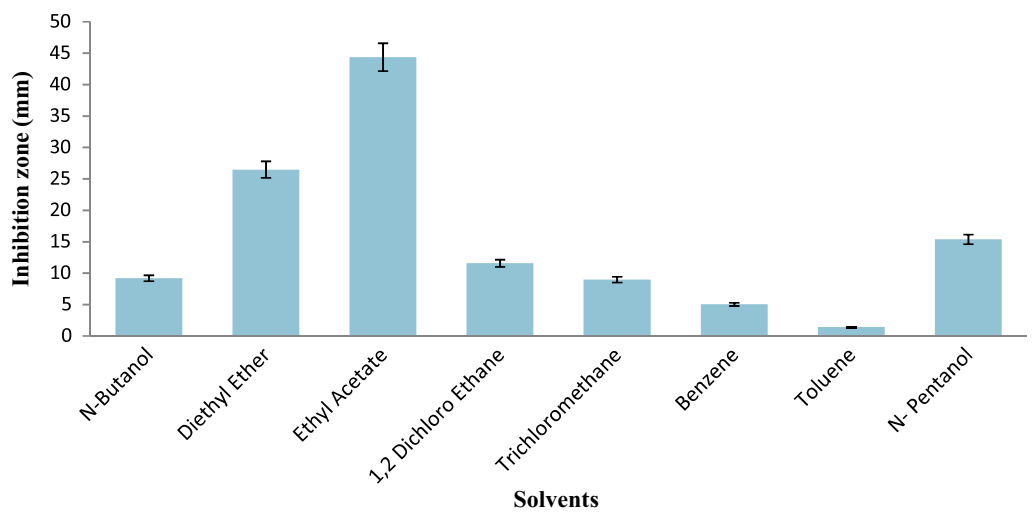


Fig. 2 Effect of different solvents on activity of bioactive compound produced from *Streptomyces* strain KX852460



Fig. 3 Activity of solvent extracted supernatant with ethyl acetate against *R. solani* AG-3

time was not stable, while treated for 30 min and for 1 h was stable. Stability of antimicrobial cultural filtrate from *Streptomyces* was also reported by Zhao and Wu (2006).

The crude extract obtained by solvent extraction with ethyl acetate showed strong activity against the *R. solani*. Isolation of crude extract by solvent extraction is very

important phenomenon, to find a good solvent that have the potential to extract high yield and most potent bio-active compounds. Studies demonstrated that the extract of ethyl acetate have wide antimicrobial spectrum against the bacterial and fungus pathogens (Khamna et al. 2009; Kobayashi et al. 1994). Extract from *Streptomyces* EF37141 contains 27 different organic compounds. GC-MS analysis showed that the majority of the compounds were derivatives of the aromatic compounds. These compounds were antimicrobial and antifungal. Volatile organic compounds and polycyclic aromatic derivatives have the antifungal potential (Müller et al. 2009; Memic et al. 2011).

GC-MS is a novel technique to identify the secondary metabolites from the *Streptomyces* fermentation broth and analysis of GC-MS is very reliable to identify the compound in complex biochemical product. From current study some compounds were reported antifungal. Eicosane reported as antifungal compound (Karanja et al. 2012; Nandhini 2015) and dibutyl phthalate also reported antifungal compound (Nandhini 2015; Roy et al. 2006). Morphinan, 7,8-didehydro-4,5-epoxy-17-methyl-3,6-bis[(trimethylsilyl)oxy]-, (5.alpha, 6.alpha)-, cyclononasiloxane, octadecamethyl- and 1,2,4-benzenetricarboxylic acid, 4-butyl 1,2-dimethyl ester showed highest peaks and the area percent of these compounds also more than other compounds. On the base of GC-MS analysis highest peak number and area percent indicated that these three compounds



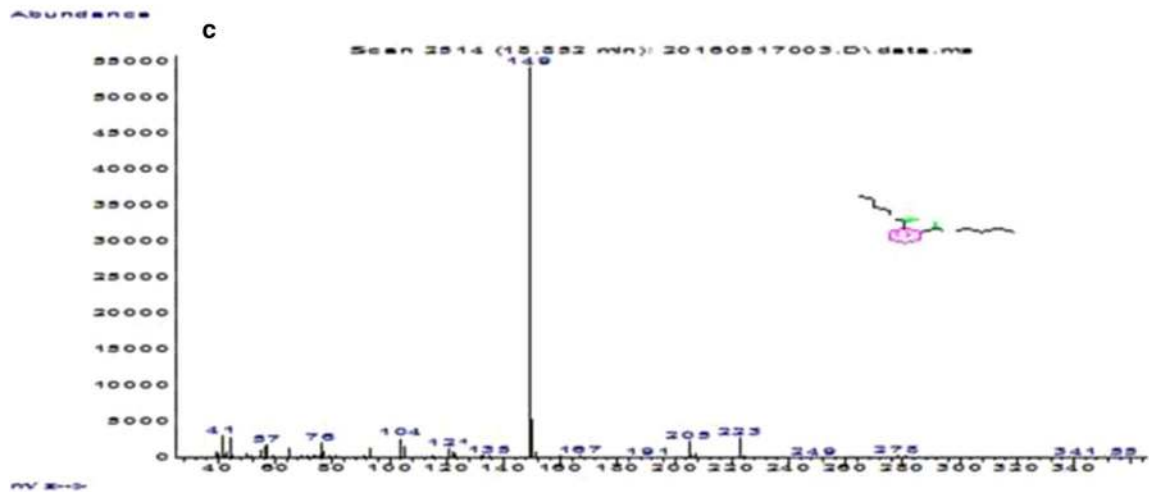
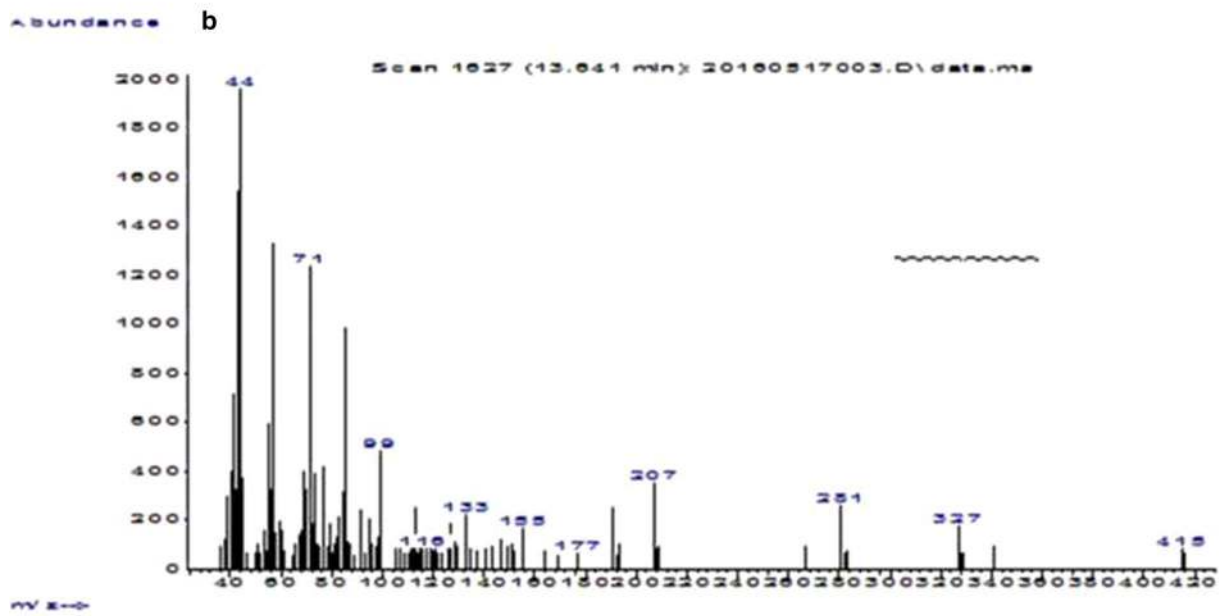
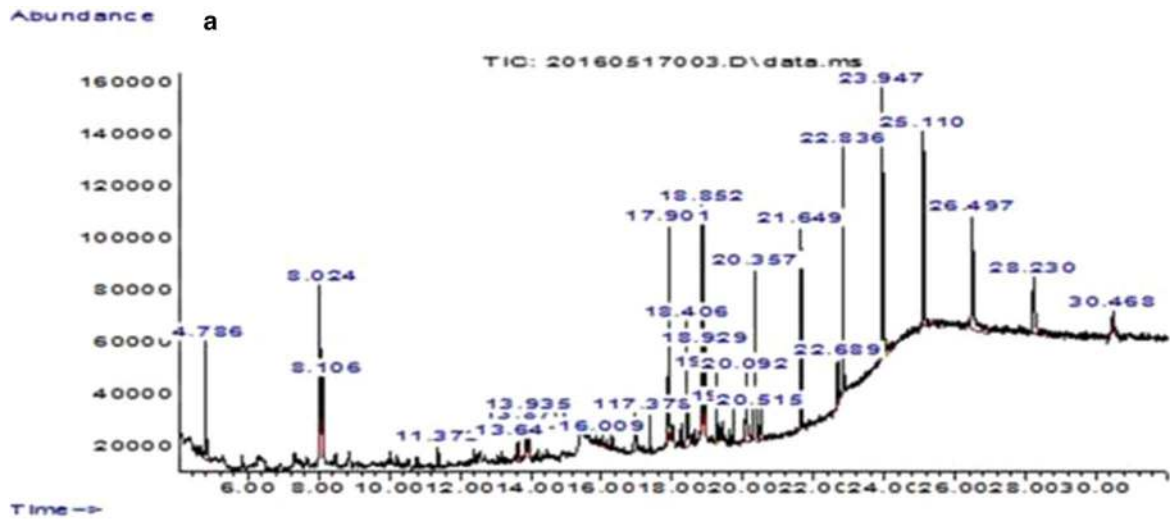
Fig. 4 Antifungal activity of purified fractions by silica gel column chromatography and 1, 2, 3, 4, 5, 6, 7, 8, and 9 represented the numbers of purified fractions, obtained by silica gel column chromatography

Table 2 Compounds identified in ethyl acetate extract of *Streptomyces* KX852460 by GC–MS

Peak #	Retention time	Area %	Name of the compound	Chemical formula	Molecular weight
1	4.786	3.92	Benzoic acid, 2-methoxy-, methyl ester	C ₉ H ₁₀ O ₃	166
2	8.024	5.36	Undecane	C ₁₁ H ₂₄	156
3	8.106	1.8	Undecane	C ₁₁ H ₂₄	268
4	11.373	0.98	Cyclohexasiloxane, dodecamethyl-	C ₁₂ H ₃₆ O ₆ Si ₆	444
5	13.641	0.98	Eicosane	C ₂₀ H ₄₂	282
6	13.87	0.72	Phenol, 2,4-bis(1,1-dimethylethyl)	C ₁₄ H ₂₂ O	206
7	13.935	1.02	Butylated hydroxytoluene	C ₁₅ H ₂₂ O	220
8	16.009	0.74	<i>N</i> -(Glycyl)alanine	C ₅ H ₁₀ N ₂ O ₃	146
9	16.955	1.98	1,3-Diphenyl-4H-1,2,4-triazoline-5-thione	C ₁₄ H ₁₁ N ₃ S	253
10	17.378	1.09	Cyclononasiloxane, octadecamethyl-	C ₁₈ H ₅₄ O ₉ Si ₉	666
11	17.901	5.9	1,2-Benzenedicarboxylic acid, bis (2-methylpropyl) ester	C ₁₆ H ₂₂ O ₄	278
12	18.406	4.67	Hexadecanoic acid, methyl ester	C ₁₇ H ₃₄ O ₂	270
13	18.852	6.44	Dibutyl phthalate	C ₁₆ H ₂₂ O ₄	278
14	18.929	1.63	Cyclodecasiloxane, eicosamethyl-	C ₂₀ H ₆₆ O ₁₀ Si ₁₀	740
15	19.264	2.04	<i>p</i> -Dicyclohexylbenzene	C ₁₈ H ₂₆	242
16	19.74	1.1	<i>p</i> -Dicyclohexylbenzene	C ₁₈ H ₂₆	242
17	20.092	3.44	9,10-Anthracenedione, 2-ethyl-	C ₁₆ H ₂₀ O ₂	236
18	20.357	5.95	Cyclodecasiloxane, eicosamethyl-	C ₂₀ H ₆₀ O ₁₀ Si ₁₀	740
19	20.515	1.4	Octahydrotripheylene	C ₁₈ H ₂₀	236
20	21.649	5.35	7-Chloro-10-ethyl-1-[[[2-[[[2-hydroxyethyl] amino] ethyl] amino]-3-[4-	C ₂₆ H ₂₅ ClF ₃ N ₃ O ₂	503
21	22.689	1.48	Hexanedioic acid, bis(2-ethylhexyl) ester	C ₂₂ H ₄₂ O ₄	370
22	22.836	6.56	Morphinan, 7,8-didehydro-4,5-epoxy-17-methyl-3, 6-bis [(trimethylsilyl)oxy]-, (5.alpha. 6. Alpha.)-	C ₂₃ H ₃₅ NO ₃ Si ₂	429
23	23.947	14.46	Cyclononasiloxane, octadecamethyl-	C ₁₈ H ₅₄ O ₉ Si ₉	666
24	25.11	7.55	Benzoic acid, 2,5-bis(trimethylsiloxy)-, trimethylsilyl ester	C ₁₆ H ₃₀ O ₄ Si ₃	370
25	26.497	8.32	1,2,4-Benzenetricarboxylic acid, 4-butyl 1,2-dimethyl ester	C ₁₅ H ₁₈ O ₆	294
26	28.23	4.15	Cyclotrisiloxane, hexamethyl-	C ₆ H ₁₈ O ₃ Si ₃	222
27	30.468	0.97	Cyclotrisiloxane, hexamethyl-	C ₆ H ₁₈ O ₃ Si ₃	222

(See figure on next page.)

Fig. 5 Gas chromatography-mass spectrometer (GC–MS) analysis of the purified active fraction **(a)**, detection of eicosane **(b)** and dibutyl phthalate **(c)** from purified active fraction



were major in the extract of *Streptomyces* EF37141. These compounds consider active substances against the *R. solani*. Extract from the *Streptomyces* shows the same effectiveness as the oxine benzoate and fungicide (Sabaratnam and Traquair 2002).

Abbreviations

PDA: potato dextrose agar; GC–MS: gas chromatography–mass spectrophotometry; NMR: nuclear magnetic resonance.

Authors' contributions

Planning and designing of study: YW; Experimentation: TA; Result Analysis: JC, XZ; Manuscript Drafting: MI. All authors contributed in the final approval of manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Ethical approval

No data was used in this article which needs approval.

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