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GC-MS Based Metabolomic Profiling of *Streptomyces clavuligerus* Isolated from *Ocimum gratissimum* Rhizosphere

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Authors' contributions

This work was carried out in collaboration among all authors. Author RGI carried out this laboratory study as part of her PhD dissertation. Author COA was the main supervisor for the research work. Author MOI co-supervised the work and carried out the ideation and manuscript preparation. Author CUE carried out the data curation and proof-reading. All authors read and approved the final manuscript.

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ABSTRACT

Streptomyces clavuligerus is a member of the Actinobacteria family primarily known for its production of clavulanic acid antibiotic. The need for identification of new antimicrobials led to the identification of volatile components of *S. clavuligerus* metabolites using GC-MS. The isolate was obtained from *Ocimum gratissimum* rhizosphere using starch casein agar, and identified using molecular typing. The preliminary antibacterial screening of the isolate was carried out using some indicator bacteria from wound sites and urinary tract infection. Its bioactive metabolites were obtained using sub-merged fermentation over a four-day period, and the volatile compounds

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identified using GC-MS. The organism showed significant ($p<0.05$) inhibition on *Pseudomonas aeruginosa* and *Escherichia coli*. Metabolomics study revealed the presence of compounds of alkanone and alkene functional groups. Eicosene was the major antimicrobial compound identified. Likewise, a non-antimicrobial, steroidal metabolite – pregnelonone, was also dominant in the metabolite mixture produced by the organism. Identifying volatile constituents of microbial metabolites may be a route for obtaining new antimicrobials and the GC-MS is a useful tool for achieving such aim.

Keywords: Streptomyces; rhizosphere; antimicrobial activity; GC-MS.

1. INTRODUCTION

Antimicrobial resistance cases across the world have been a course for health concern due to its negative implications. Measures adopted for its control span from controlled use, monitoring patient antibiotic exposure profile, monitoring of antibiogram of pathogens, maintaining good antimicrobial stewardship and also sourcing for alternative/natural ways for infection control by pathogens. The quest for novel antimicrobial substances from natural sources has seen the increase in the studies of probiotics, prebiotics, and metabolomic studies of beneficial microorganisms with antimicrobial susbstance production capacities.

The Genus *Streptomyces* has been known to contain some antimicrobial producing species and are ubiquitous in terrestrial and aquatic habitats [1]. They are known to be Gram positive, spore forming, filamentous bacteria. They are known to possess a complex secondary metabolite production capacity and thus have been extensively used for the production of novel antimicrobial substances. They produce about 70-80% of novel and natural bioactive compounds that serve agrochemical and pharmacological purposes [2],3]. These metabolites produced by species of *Streptomyces* could be volatile or non-volatile in nature. These metabolites can be antimicrobial, antitumor, antiviral, antihypertensive and also cytotoxic in nature [4]. *Streptomyces clavuligerus* is known to produce clavulanic acid which is a potent broad spectrum antibiotic, commonly used in combination with amoxicillin to give the well known amoxiclav antibiotic [5]. Clavulanic acid along with some other antimicrobial compounds produced by this organism is usually non-volatile in nature. This present study sought to employ Gas Chromatography-Mass spectrometry (GC-MS) technique to identify the volatile antimicrobial metabolites produced by *S. clavuligerus*.

The rhizosphere is a zone of soil found surrounding the roots of plants. The rhizosphere is known to be a favourable habitat for diverse microbes with antimicrobial activities including the *Streptomyces* species [1]. *Ocimum gratissimum* is a herbaceous plant known as scent leaf which in its own possess diverse antimicrobial activities alongside other pharmacological advantages. Its rhizosphere has been reported to harbor diverse microorganisms with antimicrobial capabilities as well, and thus this present study sought to isolate *Streptomyces* species from this rhizosphere and evaluate the volatile bioactive compounds present in its secondary metabolites.

2. METHODS

2.1 Isolation of Microorganism from Rhizosphere

This was done using serial dilution and plating technique on starch casein agar. A 1 ml aliquot of the rhizosphere sample was suspended in 9 ml sterile water in a test tube. Ten fold serial dilution was done up to fifth dilution, and then one ml was collected from each 10^{-2} dilution and plated on the different agar medium. The nutrient agar plates were incubated for 24 h at 30^{o}C aerobically and anaerobically for 7 days at room temperature [6].

2.2 Isolation and Identification of the Test Bacterial Isolates from Urine and Wound Samples

Bacterial isolates were isolated from urine samples according to the modified method of Alshomrani et al. [7]. Clean-catch midstream morning urine specimen was collected using sterile wide mouth glass containers. Until laboratory analysis, the samples were kept cooled in a refrigerator. The time between sample collection and the sample analysis did

not exceed one hour. Using sterile wire loops, 0.01 ml urine sample was then plated onto blood agar and MacConkey agar plates, incubated aerobically at 37℃ for 24 h. This was used for the isolation of *E. coli*, *Klebsiella* and *Staphylococcus aureus* from urine samples.

Pseudomonas aeruginosa was isolated from wound sites using method described by Al-Mathkhury and Al-Aubeidi [8]. With sterile swab sticks, wound swabs were taken carefully from the site of infection and placed in tubes containing normal saline to maintain the swab wet during transferring to laboratory. Each specimen was inoculated on cetrimide agar plates supplemented with 1% glycerol and allowed to incubate for 24 h at 28°C.

2.3 Biochemical Characterization for *Streptomyces* Species

Isolates were characterized using Gram Staining, catalase, citrate, gelatin hydrolysis, nitrate reduction test, urease test and starch hydrolysis characteristics as described by Cheesbrough [9], Umeh and Odibo [10].

2.4 Molecular Identification

Isolates were characterized using 16srDNA molecular typing.

2.5 Production of Antibacterial Metabolites from the *Streptomyces* Isolate

The *Streptomyces* isolate was grown in Yeast Malt extract broth (YMEB) with continuous shaking at 120 rpm for 4 days at 28℃. Broth cultures were filtered firstly with whatman No.1 filter paper and secondly with nitrocelloluse membrane (0.45 μm pore diameter), after incubation. The filtrate was chilled and kept for gas chromatography and mass spectrometry analyses (Gebreyohannes et al., 2013).

2.6 Extraction and Identification of Bioactive Compounds Present in the Produced Antibacterial Metabolites Using Gas Chromatography and Mass Spectrometry

This was carried out according to the method described by Buss and Butler [11]. A 1ml aliquot of the sample was extracted with 5ml of acetonitrile, the mixture was centrifuge for 1hr and the acetonitrile layer (upper layer) was collected and evaporated to dryness. 1ml of pyridine was added prior to GC analysis.

The GC–MS analysis of bioactive compounds from the different extracts was done using Agilent Technologies GC systems with GC-7890A/MS-5975C model (Agilent Technologies, Santa Clara, CA, USA) equipped with HP-5MS column (30 m in length × 250 µm in diameter × 0.25 µm in thickness of film). Spectroscopic detection by GC–MS involved an electron ionization system which utilized high energy electrons (70 eV). Pure helium gas (99.995%) was used as the carrier gas with flow rate of 1 mL/min. The initial temperature was set at 50 –150 °C with increasing rate of 3 °C/min and holding time of about 10 min. Finally, the temperature was increased to 300 °C at 10 °C/min. One microliter of the prepared 1% of the extracts diluted with respective solvents was injected in ansplitless mode. Relative quantity of the chemical compounds present in each of the extracts of was expressed as percentage based on peak area produced in the chromatogram.

2.7 Identification of chemical Constituents

Bioactive compounds extracted from different extracts were identified based on GC retention time on HP-5MS column and matching of the spectra with NIST library (Replib and Mainlab data of GC–MS systems).

2.8 Statistical Analyses

Statistical Analyses was done using GraphPad Prism version 8. Mean values were compared using one way Analyses of Variance (ANOVA), at 95% confidence interval.

3. RESULTS

3.1 Isolation, Characterization and Antibacterial Screening of *Streptomyces clavuligerus* from Rhizosphere Sample

Streptomyces sp. was characterized as shown in Table 1 and was confirmed using molecular typing as *Streptomyces clavuigerus*. The isolate was screened for antibacterial activity using the organisms isolated from urine and wound sites as shown in Fig. 1. It significantly ($p<0.05$) inhibited the growth of *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Escherichia coli* when compared to the ciprofloxacin standard (Fig. 1). The colony morphology of the isolate is shown in Fig. 2.

Table 1. Biochemical Characteristics of *Streptomyces clavuligerus*

Cultural/Biochemical Characteristics	Results
Colony morphology on starch casein agar	Red coloured punctiform colonies that grow into a mycelia mat, and produce red dye in the medium after 14 days.
Catalase test	+
Oxidase test	-
Citrate test	-
Gelatin hydrolysis test	+
Nitrate reduction test	+
Urease test	+
Starch hydrolysis	+

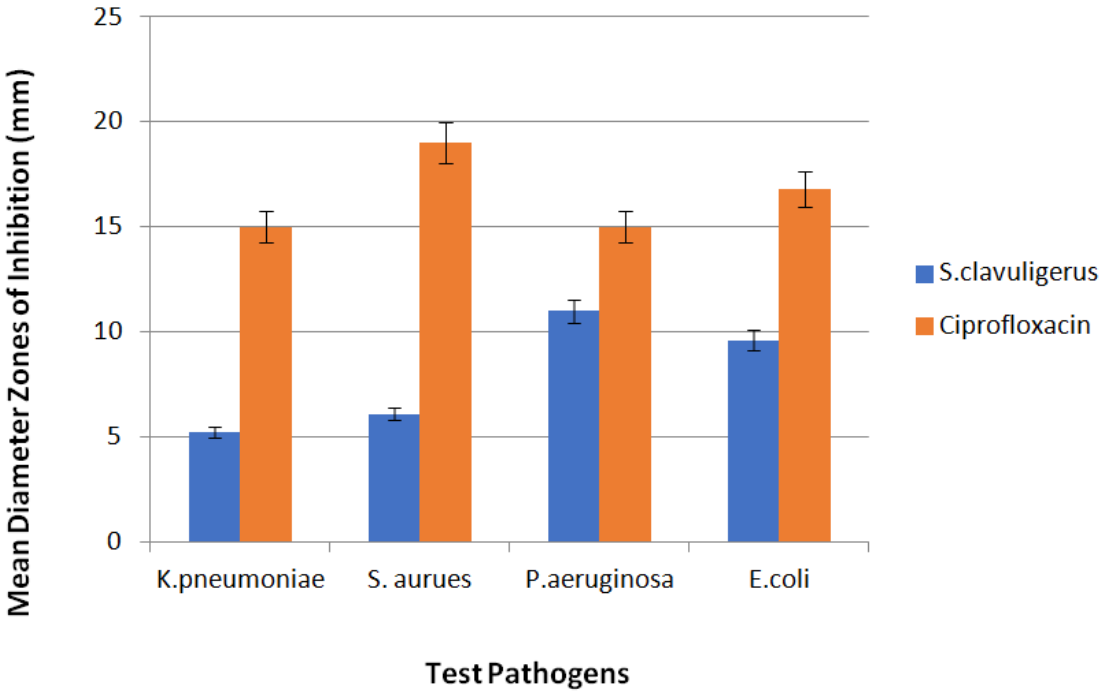

Fig. 1. Antimicrobial screening of *S. clavuligerus* against test pathogens

Fig. 2. Colony morphology of *S. clavuligerus* showing red pigmented rhizoid margine colonies

The isolate was identified with molecular typing as *Streptomyces clavuligerus* strain F1D10.

3.2 Production and GC-MS Evaluation of Antibacterial Substances from *S. clavuligerus*

Gas chromatography and mass spectrometry analyses showed twenty-three volatile bioactive compounds present in the metabolites. The top five bioactive compounds that possibly contributed predominantly in the antibacterial activities of *S. clavuligerus* are shown in Fig. 3 and Table 2.

4. DISCUSSION

The present study assessed novel compounds produced by *Streptomyces clavuligerus*. This actinobacteria member has been known to produce a beta-lactam antimicrobial compound known as clavulanic acid. This antibiotic alongside other similar bioactive compounds reported from this microorganism after sub-merged fermentation has been from HPLC-MS, LC-MS, UV-VIS and Infra red [12]. These methods are known to identify the non-volatile compounds that exhibit the bioactivity against pathogens. However, the present study made use of GC-MS to identify the volatile compounds from sub-merged fermentation broth of *S. clavuligerus* which also possess antibiotic activity; with the aim of identifying new antimicrobials.

Streptomyces clavuligerus produced the following antibacterial compounds: Methyl dihydroisosteviol, Nonadecane, octadecene and eicosene (Fig. 2, Table 2). This finding partly corresponds with that of Naragani et al. [13]. who reported eicosene, nondecane and octadecene as one of the antibacterial GC-MS components of metabolites produced by *Streptomyces cheonanensis* isolated from mangrove soil samples. Although, their finding was from a different *Streptomyces* species, it still suggests that *Streptomyces* genus possibly produce these lists of volatile antimicrobials. The microorganism's metabolites also showed resistance to *E. coli*, *S. aureus* and *P. aeruginosa* which corresponds with the findings of this study. Hsouna et al. [14] described the antibacterial roles of eicosene and nondecane contained in *Ceratonia siliqua* essential oil, against *L. monocytogenes.* Adeyemo et al. [12] also characterized the GC-MS spectra of three different *Streptomyces* species and also reported they all produced eicosene which partly corresponds with the findings of the present study, and also produced other metabolites which differed from that of *S. clavuligerus*. Kawuri and Darmayasa [15] also identified eicosene as a major metabolite from another species of *Streptomyces – S. capoamus*, using GC-MS. Kumar et al. [16] opined that substrates used for the sub-merged fermentation of these *Streptomyces* species also contribute to the nature of biosynthesis they undergo, as well as the nature of the final metabolites they produce. Volatile metabolites produced by *S. clavuligerus* had antibacterial activities against *Klebsiella pneumonia*e, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*; while the study of Adeyemo et al. [12] showed

Table 2. Predominant metabolites produced by *Streptomyces clavuligerus*

Peak numbers	Retention time	Area%	Metabolite names
22	31.331	15.39	a) Methyl dihydroisosteviol
			b) 16-Pregnenolone
			c) Estra-5(10)-en-3-one-17-ol, acetate
23	31.355	11.19	a) Methyl dihydroisosteviol
			b) 16-Pregnenolone
			c) Pregn-16-en-20-one, 3-hydroxy-, (3.beta.,5.beta.)-
8	13.165	9.87	a) 10-Methylnonadecane
			b) Octadecane, 1-chloro-
			c) Nonadecane
7	13.016	9.76	a) 1-Octadecene
			b) 1-Octadecene
			c) 3-Eicosene, (E)-
15	15.763	8.01	a) 1-Octadecene
			b) 1-Docosene
			c) 5-Eicosene, (E)-

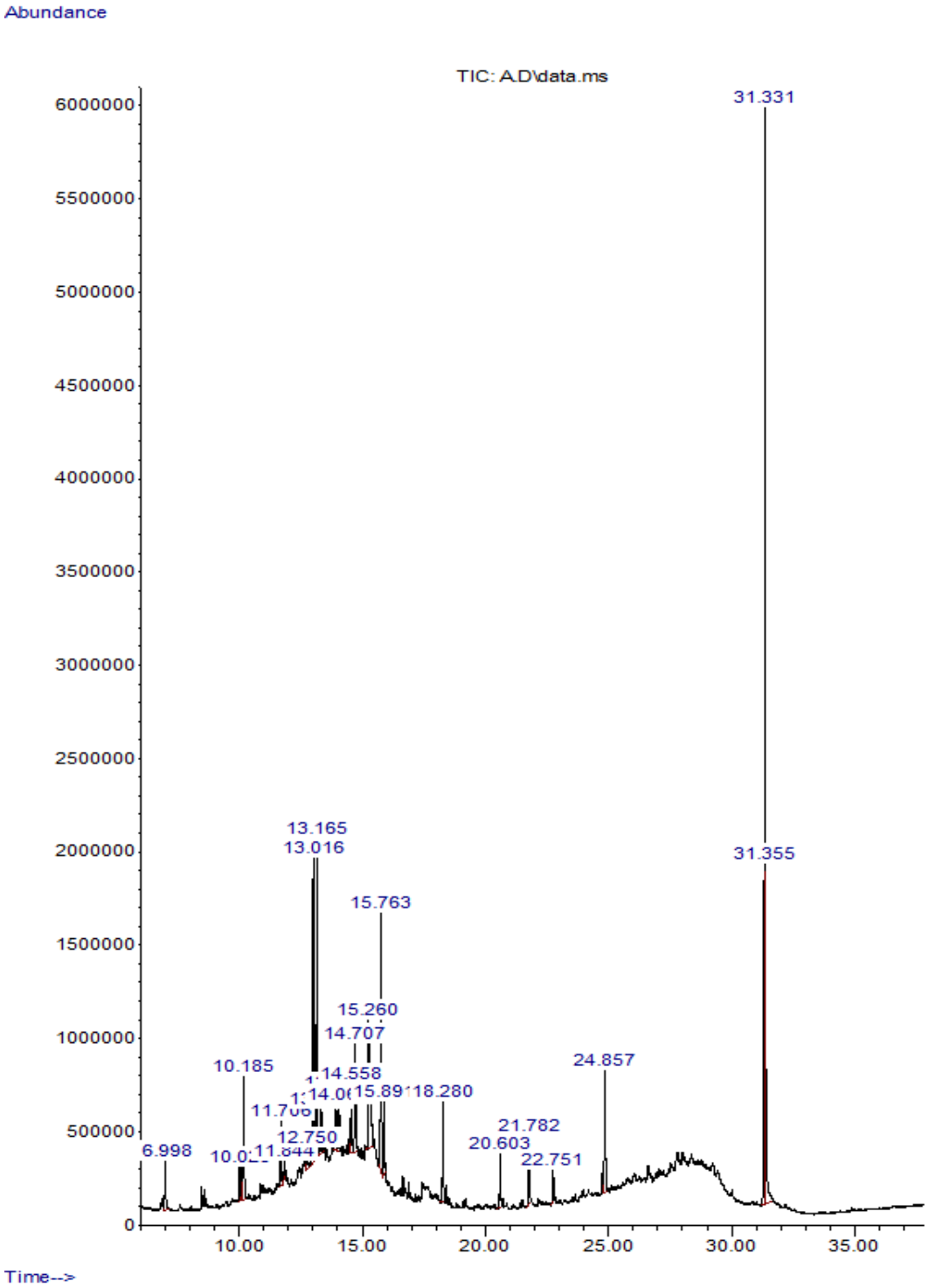

Fig. 3. Gas chromatogram showing elution peaks of identified bioactive compounds produced by *S. clavuligerus*

their *Streptomyces* isolates had antibacterial activities against *S. aureus* and *P. aeruginosa.* Abusara et al. [17] identified non-volatile bioactive compounds of *S. clavuligerus* using Liquid chromatography-Mass spectrometry (LC-MS) and reported the presence of dithiolopyrrolone, tunicamycin and naringenin, all known to possess antibacterial properties. This goes to show that employing different metabolite evaluation mechanism shows different

metabolites that all contribute to antibacterial capacities of microorganisms. This present study has shown that GC-MS identifies potent volatile bioactive compounds different from what LC-MS or HPLC-MC may identify. This suggests that for comprehensive information on new antimicrobials produced by microorganisms to be known, methods that identify both the volatile and non-volatile components should be adopted. The present study however, adopted GC-MS for volatile compound identification from *S. clavuligerus*.

5. CONCLUSION

Streptomyces species are known antimicrobial substance producing genus. Although different identification methods reveals different antimicrobial components of *Streptomyces* species, it has been seen from this finding and that of other compared in this study, that GC-MS mostly identifies eicosene as mostly occurring volatile bioactive compound from *Streptomyces* species, including *S. clavuligerus*. This possibly means that for future bio-prospecting of antibacterial compounds from *Streptomyces* species, eicosene is most likely a volatile compound not to be ignored. Since most identified antibiotics from *Streptomyces* species are non-volatile, identifying new bioactive substances may require looking into the volatile components as well using GC-MS.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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