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

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 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.

Key words: *Streptomyces*, Rhizosphere, Antimicrobial activity, GC-MS.

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 (Donald *et al*., 2022). 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 (Alam *et al*., 2022; Komaki *et al*., 2023). 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 (Liras and Martin, 2021). *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 (Alvarez-Alvarez *et*

al., 2017). 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 (Donald *et al*., 2022). *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.

Methods

Isolation of Microorganism from Rhizosphere

This was done using serial dilution and plating technique on starch casein agar. A 1 ml aliquot of the sewage 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 (Atsede and Fassil, 2018).

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

Bacterial isolates were isolated from urine samples according to the method described by Tanzina *et al*. (2016). 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 MacFadden (2000). 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.

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 (2006); Umeh and Odibo (2014).

Molecular Identification

Isolates were characterized using 16srDNA molecular typing

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).

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

This was analyzed according to AOAC 1990

Preparation of standard

A 10 ul aliquot of accu standard was injected in the chromatography and the retention time compared with retention time of standard.

Extraction of bioactive compounds from samples

1ml of filtered residue was dissolved in 50ml of chloroform, transferred to a 100ml volumetric flask and diluted to the mark. Most of the chloroform was evaporated at room temperature, 1 ml of the reagent {20 vol% benzene and 55 vol% methanol} was added, Sealed and heated at 40^0c water bath for 10 minutes. After heating, the organic sample was extracted with hexane and water, so that the final mixture of the reagent, hexane and water, is in proportion of 1:1:1 (i.e., 1ml each of hexane and water was added to the reaction mixture). The mixture was shaked vigorously by hand for 2min. A stable emulsion that was formed was broken by centrifugation. Then about half of the top hexane phase was transferred to a small test tube for injection. Proper care was taken in ensuring that only the organic layer was removed. And injection was not done directly from the reaction vial because of the risk of injecting water. Water can ruin the GC column.

Gas Chromatographic conditions for bioactive compound determination

The final extracts were analyzed by **Gas Chromatograph-Buck M910 scientific gas chromatography equipped with Electron capture detector** that allowed the detection of contaminants even at trace level concentrations (in the lower µg/g and µg/kg range) from the matrix to which other detectors do not respond. The GC conditions used for the analysis were capillary column HP 88 capillary column (100m x 0.25µm film thickness,) CA, USA

The injector and detector temperature were set at 250 ºC and 290ºC respectively. The oven temperature was programmed as follows: 110 ºC held for 10 min, ramp at 10 ºC/ min to 200 ºC, held for 5min, and finally ramp at 10 ºC/ min to 320 ºC. Helium was used as carrier gas at a flow rate of 1.0 mL/ min and detector make-up gas of 29 Ml min-1. The injection volume of the GC was 8.0 µL. The total run time for a sample was 48 min.

Quantification of bioactive compound residues.

The residue levels of the bioactive compounds were quantitatively determined by the external standard method using peak area. Measurement was carried out within the linear range of the detector. The peak areas whose retention times coincided with the standards were extrapolated on their corresponding calibration curves to obtain the concentration.

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.

Results

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 Figure 1. It significantly ($p<0.05$) inhibited the growth of *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Escherichia coli* when compared to the ciprofloxacin standard (Figure 1).

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 Figure 2 and Table 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	+

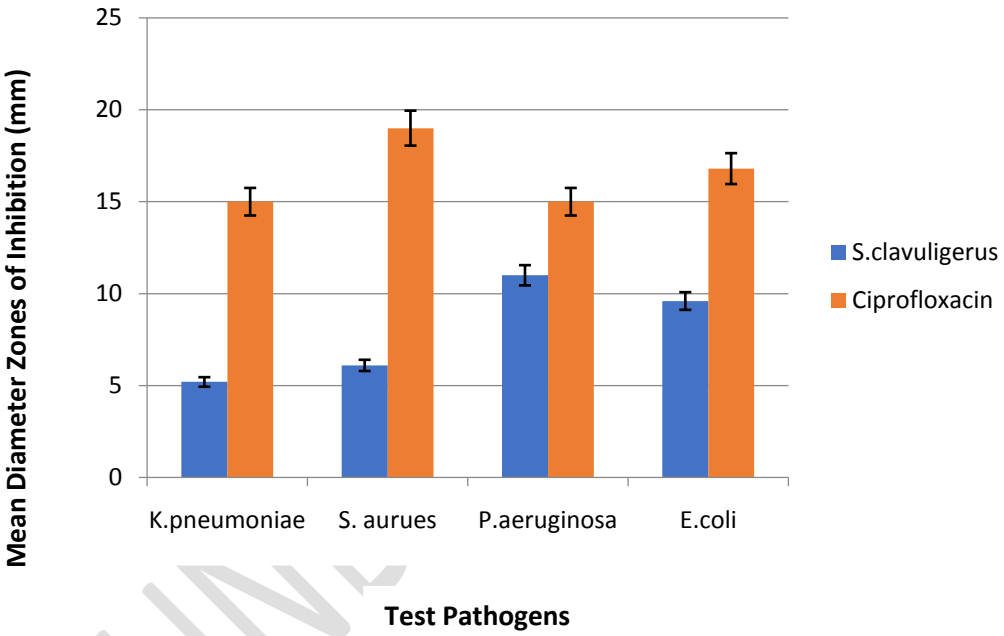

Figure 1: Antimicrobial Screening of *S. clavuligerus* against Test Pathogens.

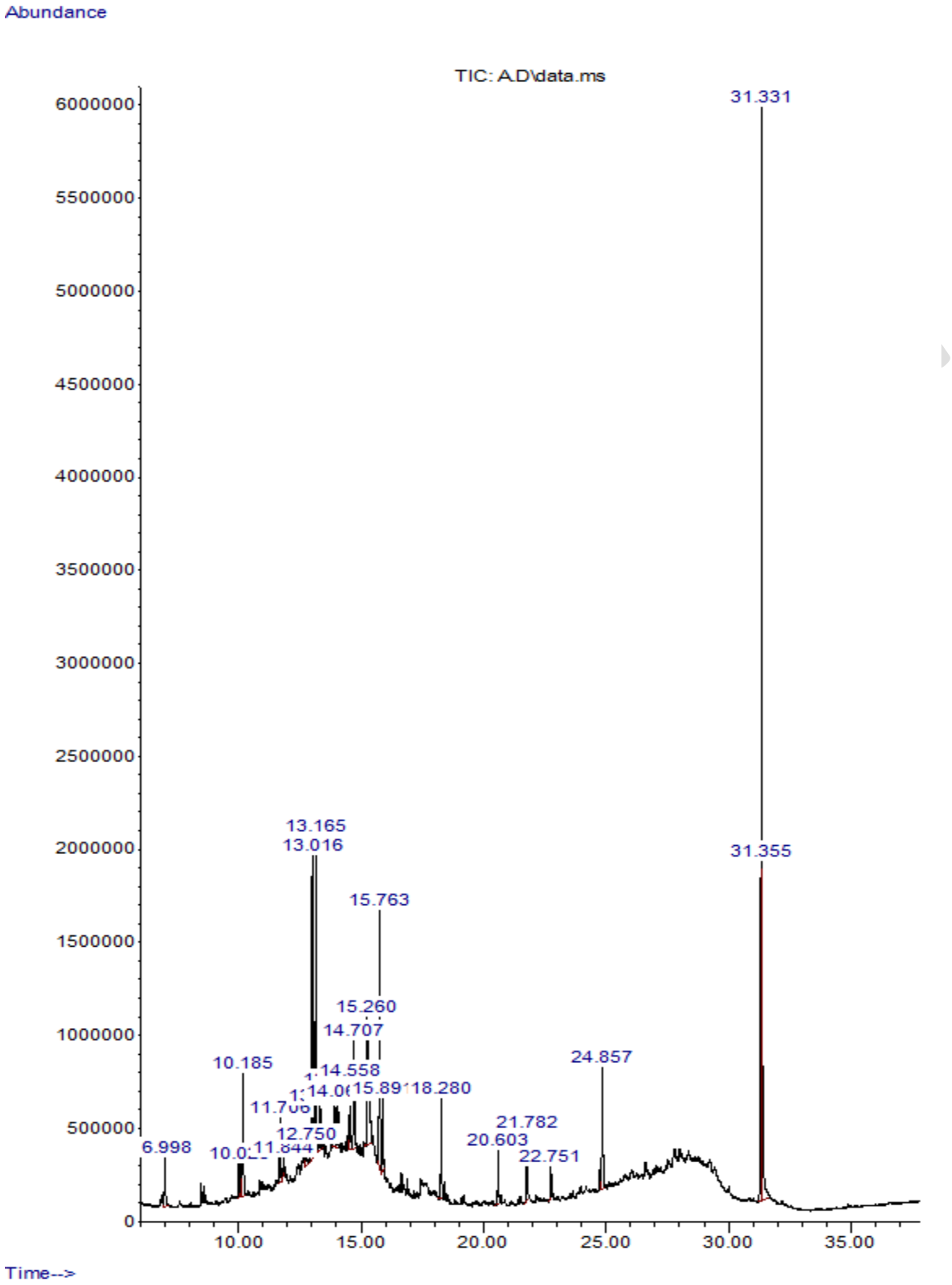

Figure 2: Gas Chromatogram showing Elution Peaks of Identified Bioactive Compounds Produced by *S. clavuligerus*.

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)-

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 (Adeyemo *et al*., 2021). 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*. (2016) 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*. (2011) described the antibacterial roles of eicosene and nondecane contained in *Ceratonia siliqua* essential oil, against *L. monocytogenes.* Adeyemo *et al*.

(2021) 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 (2019) also identified eicosene as a major metabolite from another species of *Streptomyces* – *S. capoamus*, using GC-MS. Kumar *et al*. (2023) 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*. (2021) showed their *Streptomyces* isolates had antibacterial activities against *S. aureus* and *P. aeruginosa*. Abusara *et al*. (2019) 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*.

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.

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