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Original Research Article

Antibacterial Activity of Ethanol Extract of the Marine Sponge (*Agelas* sp) Symbiont *Bacillus cereus* MH997647 IA5 against *Klebsiella pneumoniae* ESBL

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ABSTRACT

Klebsiella pneumoniae ESBL is the major causative organism of pneumonia, a lower respiratory tract infection that could be lethal. In 2019, pneumonia was ranked the fourth-leading cause of death worldwide. This study aims to investigate the antibacterial activity of the ethanol extract of *Bacillus cereus* MH997647 IA5, a symbiont of the marine sponge *Agelas* sp. against *Klebsiella pneumoniae* ESBL as well as to identify the secondary metabolites present in the extract. The antibacterial activity of the sponge symbiont was assessed using the disc diffusion assay. The ethanol extract of the sponge symbiont was subjected to liquid chromatography-mass spectrometry (LC-MS-MS) to identify the compounds present in the extract. The results of the antibacterial activity screening showed that the sponge symbiont had growth inhibitory activity against *Klebsiella pneumoniae* ESBL with an average inhibition zone diameter of 7.72 mm. The LC-MS/MS analysis identified seven compounds in the ethanol extract of the sponge symbiont *Bacillus cereus* MH997647 IA5, namely DL-phenylalanine, L-(+)-leucine, DL-tyrosine, L-(+)-valine, choline, noradrenaline, and tributyl citrate acetate. These compounds could be responsible for the antibacterial activity of the ethanol extract of *Bacillus cereus* MH997647 IA5 against *Klebsiella pneumoniae* ESBL. Therefore, *Bacillus cereus* MH997647 IA5 could be a potential source of active compounds for the treatment of bacterial infection particularly pneumonia.

***Keywords*:** *Bacillus cereus*, *Agelas* sp., Antibacterial activity, *Klebsiella pneumonia*.

Introduction

Pneumonia is the 4th leading cause of death worldwide. About 1.9 million of the estimated 2.5 million pneumonia deaths occurred in children under the age of 5 and adults over the age of 69. The main cause of pneumonia is the pathogenic bacteria *Klebsiella pneumoniae*.[1] *Klebsiella pneumoniae* colonization causes respiratory tract epithelial cell damaged.[2,3]

Various types of synthetic antibiotics have been developed to treat infections caused by *K. pneumonia*, but the irrational use of these synthetic antibiotics has led to the problem of antibiotic resistance e.g. antibiotic resistant *K. pneumoniae*.[4,5] A 2018 study showed that *Klebsiella pneumoniae* was resistant to the antibiotics carbapenem and colistin.[6] Meanwhile, another study in 2020 regarding the resistance of *Klebsiella pneumoniae* showed an increase in the resistance of *K. pneumoniae* to the antibiotics carbapenem, ertapenem, and meropenem.[7]

Efforts made to overcome antibiotic resistance in *K. pneumoniae* have been geared towards exploring new bioactive compounds as potential antibiotics.[8] One of the sources of potential bioactive compounds include bacteria that are in symbiotic association with sponges.[9]

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The results of a 2019 study showed that bacteria in symbiotic association with sponges were proven to have antibacterial activity against *Klebsiella pneumoniae* RSDK.[10] Likewise, a 2021 study reported that isolates of sponge symbiont bacteria have the potential to be used as antibacterial agents against *Klebsiella pneumoniae* ESBL.[11,12] The potential of sponge symbiont bacteria as an antibacterial agent is due to their ability to produce active secondary metabolites.[13] There is scarcity of information regarding bacteria-sponge symbiotic association, the metabolites produced by the symbionts, and the antibacterial activity of the bacteria symbiont. There is the need therefore to investigate these symbionts for the purpose of discovery and development of drug molecules that could be potential candidates against antibiotic-resistant *Klebsiella pneumonia*. On the basis of the foregoing, the aim of the present study is to determine the antibacterial activity of the ethanol extract of the sponge symbiont *Bacillus cereus* MH997647 IA5 against *Klebsiella pneumoniae* ESBL, as well as to identify the bioactive compounds of the ethanol extract of the sponge symbiont.

Materials and Methods

Fermentation of Sponge Symbiont Bacteria

The sponge symbiont bacteria isolates were obtained and made into a starter medium of 15 mL maltose yeast broth (MYB), which was incubated at 37ºC for 24 h. The starter medium was then fermented into 300 mL of production medium using a 500 mL Erlenmeyer flask, incubated at 37ºC for 6 days, and shaken using a shaker at a speed of 150 rpm. After 6 days of fermentation, the bacterial growth medium was sonicated and then filtered to separate the biomass and fermentation liquid. The fermented liquid was extracted with ethanol (1:1) in a separatory funnel for 20 minutes. The extract obtained was stored in a desiccator for use in the next experiment.[1,12]

Column chromatography

The bacterial extract was subjected to column chromatographic seperation using silica gel: alumina (2:3) with methanol:chloroform (8:2) as the eluent.

Molecular Identification of Symbiont Bacteria

Molecular identification of the symbiont bacteria was carried out by identifying the 16S rRNA gene marker. The 16S rRNA gene was amplified using a PCR machine from the Gradian Lab cycler brand (SENSOQUEST). The primers used for the PCR were universal primer pairs; 9F (forward) and 1492R (reverse). The primer pairs are 16S rRNA primers with a 9F primer base sequence; 5'-GAGTTTTGATCCTGGTCCAG and a 1492R primer base sequence; 5'-GGCTACTTGTTACG ACTT.[14] The amplicons were then analysed using electrophoresis with a concentration of 1% agarose gel. Agarose gel (0.4 g) was put into a 40 mL TBE (Thris-Borate EDTA). The agarose gel was completely immersed in a running buffer, namely TBE 5 l. PCR results of DNA, molecular ballast loading dye, and marker were inserted into the gel wells. The voltage used for the electrophoresis process was 75 volts with a current strength of 100 mA for 60 minutes. Visualization was done with UV light on a UV trailluminator. Isolates that encode the 16S rRNA gene form a 1500-bp band.[15] Sequencing was performed to determine the nucleotide sequence of the detected DNA fragment from the visualization of amplified DNA in the PCR process using an automatic DNA sequencing machine. The results of DNA sequencing were analysed using the BLAST method via the NCBI online medium to look for similarities in the nucleotide sequence of the 16S rRNA gene in the molecular identification of the sponge symbiont bacteria using 16S rRNA markers. The results of 16S rRNA sequences and several reference 16S rRNA sequences were analysed using the MEGA X application. The phylogenetic tree was constructed using maximum parsimonial analysis with a 1000x bootstrap.[16]

Antibacterial activity test

The antibacterial activity test of the crude extract of *Bacillus cereus* MH997647 IA5 as well as the column fractions was carried out using the disc diffusion method. The extract/fraction was dissolved in methanol. Oxoid paper discs were immersed in the extract/fraction solution for 10 - 15 min. A suspension of *Klebsiella pneumoniae* ESBL isolates was inoculated in Muller Hinton Agar (MHA) using the swab method with a sterile cotton bud. Oxoid discs that had been impregnated with the extract/fraction solution were placed on the surface of the media which had been inoculated with *Klebsiella pneumoniae* ESBL isolate, and then incubated at 35 - 37°C for 24 h. Afterwards, the inhibition zone formed was measured.[17,18]

LC-MS/MS analysis

Preparation of Extracts for LC-MS Analysis

Liquid media were inoculated with bacteria and incubated for 5 - 7 days with occasional shaking. Then, 5 mL of the liquid cultures were extracted twice with equal volume of ethyl acetate. The organic layers were pooled, and the organic solvent was removed in vacuo. The extract was then resuspended in 1 mL methanol with sonication and clarified by centrifugation. The clarified extracts were then used for LC/MS analysis. Extracts of the culture media were prepared in a similar fashion.[19]

Sponge tissues were frozen and lyophilized to dryness. The dried sponge tissues were crushed and extracted in 1:1 dichloromethane/methanol (1 mL solvent/5 mg biomass) for 48 h with agitation. The solvent was filtered and dried in vacuo. The extracts were resuspended in methanol with sonication and clarified by centrifugation. The clarified extracts were used for LC/MS analysis.[20]

LC-MS Data Collection and Processing

All samples were analyzed with an Agilent 1290 Infinity II UHPLC system (Agilent Technologies, Santa Clara, CA, USA) using a Kinetex 1.7 µm C18 reverse-phase UHPLC column (50 × 2.1 mm, Phenomenex, Torrance, CA, USA) coupled to an ImpactII ultrahigh resolution Qq-ToF mass spectrometer (Bruker Daltonics, Billerica, MA, USA) equipped with an electrospray ionization (ESI) source for a mass spectrometry (MS/MS) analysis. MS spectra were acquired in the positive mode with an m/z range of 50 - 2000 Da. The eight most intense ions per MS1 spectra were selected for further acquisition of MS2 data. An active exclusion of two spectra was used; implying that an MS1 ion would not be selected for fragmentation after two consecutive MS2 spectra had been recorded for it in a 0.5 min time window. The exclusion was reconsidered, and an additional MS2 spectrum was acquired if a fivefold enhancement in intensity was observed. The chromatography grades olvents A: H_2O (Fisher Chemical, LC/MS grade) + 0.1% v/v formic acid (Fisher Scientific, Waltham, MA, USA LC/MS grade) and B: MeCN (Fisher Chemical, LC/MS grade) + 0.1% v/v formic acid were used for the separation. The flow rate was held constant at 0.5 mL/min. The gradient applied for chromatographic separation was 5% solvent B and 95% solvent A for 3 min, a linear gradient of 5% B - 95% B over 17 min, held at 95% B for 3 min, 95% B - 5% B in 1 min, and held at 5% B for 1 min, 5% B - 95% B in 1 min, held at 95% B for 2 min, 95% B -5% B in 1 min, then held at 5% B for 2.5 min. Following the acquisition of data on 12 samples, data on a mixture of six compounds (amitryptiline, sulfamethazine, sulfamethizole, sulfachloropyridazine, sulfadimethoxine, coumarin-314) were acquired as a quality control step to ensure instrument consistency and column performance.[21]

Statistical Analysis

The raw data were converted to the mzXML format using vendor software. Metabolite features were extracted using MZmine to perform steps for mass detection, chromatogram building, chromatogram deconvolution, isotopic grouping, retention time alignment, duplicate removal, and missing peak filling.[20] Prior to statistical analysis, blank subtraction was performed as previously described on the quantification tables using a Lenovo notebook.[21]

Results and Discussion

The isolation of bacteria from marine invertebrates, especially sponges, can be potential source of active secondary metabolites that will be of clinical benefit.[22] The study of sponge-microbe relationship have shown a high diversity of bacteria associated with sponges. Marine sponges are potential sources of antibacterial compounds.[10] Sponge bioactive compounds are potential sources of antibacterial, antitumor, anticancer, antifungal, anti-inflammatory, antimicrobial, antiviral, antimalarial, antifouling, and immunosuppressive drugs.[8] The existence of sponge associations with bacteria occurs to prevent pathogenic bacteria.[17]

The results of the antibacterial activity test of the ethanol extract of *Bacillus cereus*, a symbiont of the marine sponge *Agelas* sp against *Klebsiella pneumoniae* ESBL showed that the ethanol extract of *Bacillus cereus* has growth inhibitory activity against the pathogenic bacteria *K. pneumoniae* with an average inhibition zone diameter of 7.72 mm compared to the positive control with inhibition zone diameter of 20.45 mm (Figure 1). This value indicates that the symbiont bacterial extract has antibacterial activity, although this activity is significantly lower compared to that of the positive control. The antibacterial activity of the symbiont bacterial extract is thought to be due to the alkaloidal compounds present in the extract. Alkaloids have been shown to exhibit antibacterial action by destroying the peptidoglycan component in pathogenic bacterial cells so that the cell wall layer of the bacteria is not completely formed, resulting in the death of the bacteria.[18] Antibacterial action of alkaloids has also been thought to be by DNA intercalation and inhibition of topoisomerase enzymes in pathogenic bacterial cells. Alkaloids isolated from marine bacteria generally have potential as antibiotics.[23] Several studies reported that the alkaloids contained in *Bacillus cereus*, including quinazolinones and their derivatives, are known to have antifungal ability.[24]

Bacillus is an important genus that produces a number of ribosomal and non-ribosomal bioactive peptides.[25] A small spectrum of antibacterial metabolites have been purified from free supernatants of the genus *Bacillus*. These metabolites have been shown to have strong antimicrobial activity against several drug-resistant pathogens, including vancomycin-resistant *Staphylococcus aureus* (MVRSA), methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-resistant *Staphylococcus epidermidis* (MRSE), and vancomycin-resistant *Enterococcus faecalis* (VRE).[26] Other observations showed

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that the antimicrobial compounds produced by *Bacillus* tend to be stable, such as the antibiotic bacilysocin.[27]

Chromatographic seperation of *Bacillus cereus* extract yielded six fractions (1 – 6) with fraction 5 having the highest yield of 1617 mg (Figure 2). Most of the fractions had a liquid consistency except for fractions 5 and 6. The colour of the fractions also varied greatly suggesting variations in the bacterial metabolites present in the different fractions.[19] A study has indicated the presence of flavonoids in a chromatographic column fraction of *Bacillus cereus* extract.[28]

The results of the antibacterial activity test of the column chromatographic fractions are presented in Figure 3. Fractions 2, 4, and 5 did not show inhibitory activity against *K. pneumoniae.* Whereas fractions 1, 3, and 6 exhibited antibacterial activity against *K. pneumonia* with fraction 6 showing the highest activity with inhibition zone diameter of 11.25 mm. This indicated that the extract of sponge symbiont bacteria is a potential source of antibacterial compounds.

The LC-MS/MS analysis of the ethanol extract of *Bacillus cereus* MH997647 IA5 identified seven compounds namely; DL-Phenylalanine, L-(+)-Leucine, DL-tyrosine, L-(+)-Valine, Choline, noradrenaline, and Tributyl citrate acetate (Figure 4). Phenylalanine and valine have been shown to have potential as antibacterials by virtue of their ability to disrupt cell membranes of pathogenic bacteria.[5] The mechanism of action involves electrostatic interaction of the cationic head groups with the negatively charged bacterial lipid membrane, followed by incorporation into the bacterial membrane which lead to the disruption of cell membrane structure and irreversible cell damage.[29,30]

Conclusion

The results of the study have shown that the marine sponge (*Agelas* sp.) symbiont *Bacillus cereus* MH997647 IA5 have antibacterial activity against *Klebsiella pneumoniae* ESBL with an average inhibition zone diameter of 7.72 mm. LC-MS/MS analysis of the ethanol extract of *Bacillus cereus* MH997647 IA5 identified seven compounds, namely; DL-phenylalanine, L-(+)-leucine, DL-tyrosine, L-(+)-valine, choline, noradrenaline, and tributyl citrate acetate. These compounds could be responsible for the antibacterial activity of the bacterial extract against *Klebsiella pneumoniae* ESBL.

Therefore, *Bacillus cereus* MH997647 IA5 Symbiont of the Sponge *Agelas* sp. can be explored as a potential source of antibacterial compounds against *Klebsiella pneumoniae* ESBL

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

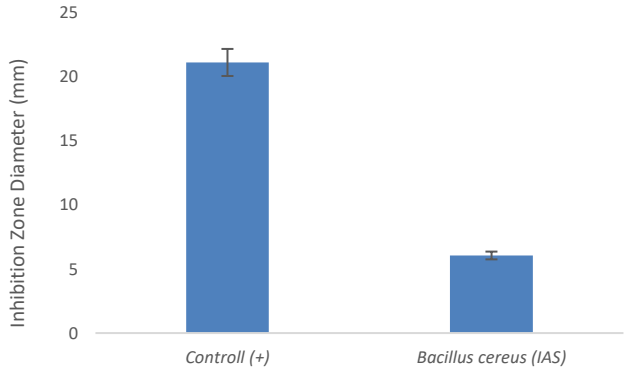

Figure 1: Antibacterial activity of the ethanol extract of *Bacillus cereus* MH997647 IA5 against *K. pneumoniae*

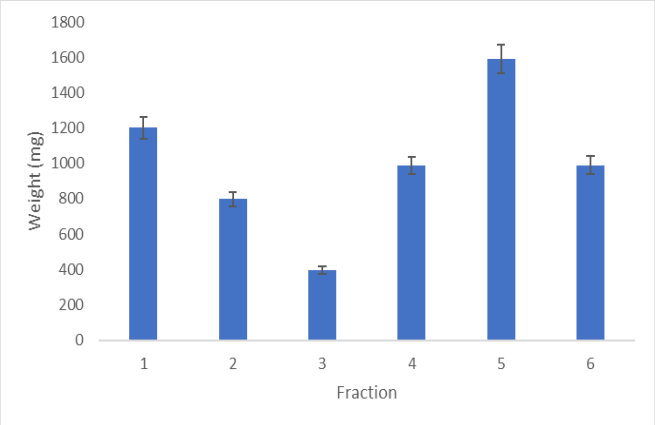

Figure 2: Weight of fractions of the ethanol extract of *Bacillus cereus* MH997647 IA5 obtained from column chromatography

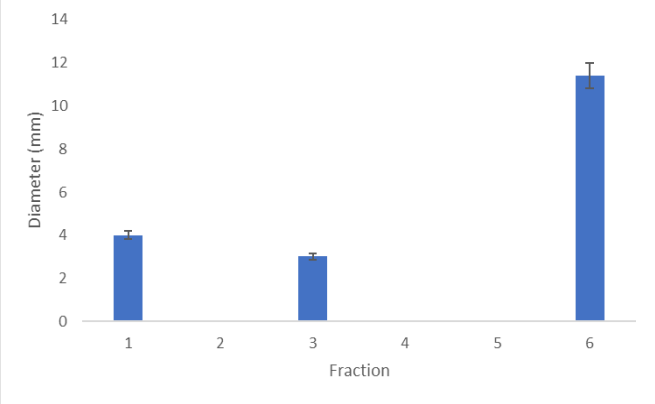

Figure 3: Antibacterial activity fractions of the ethanol extract of *Bacillus cereus* MH997647 IA5 against *K. Pneumoniae*

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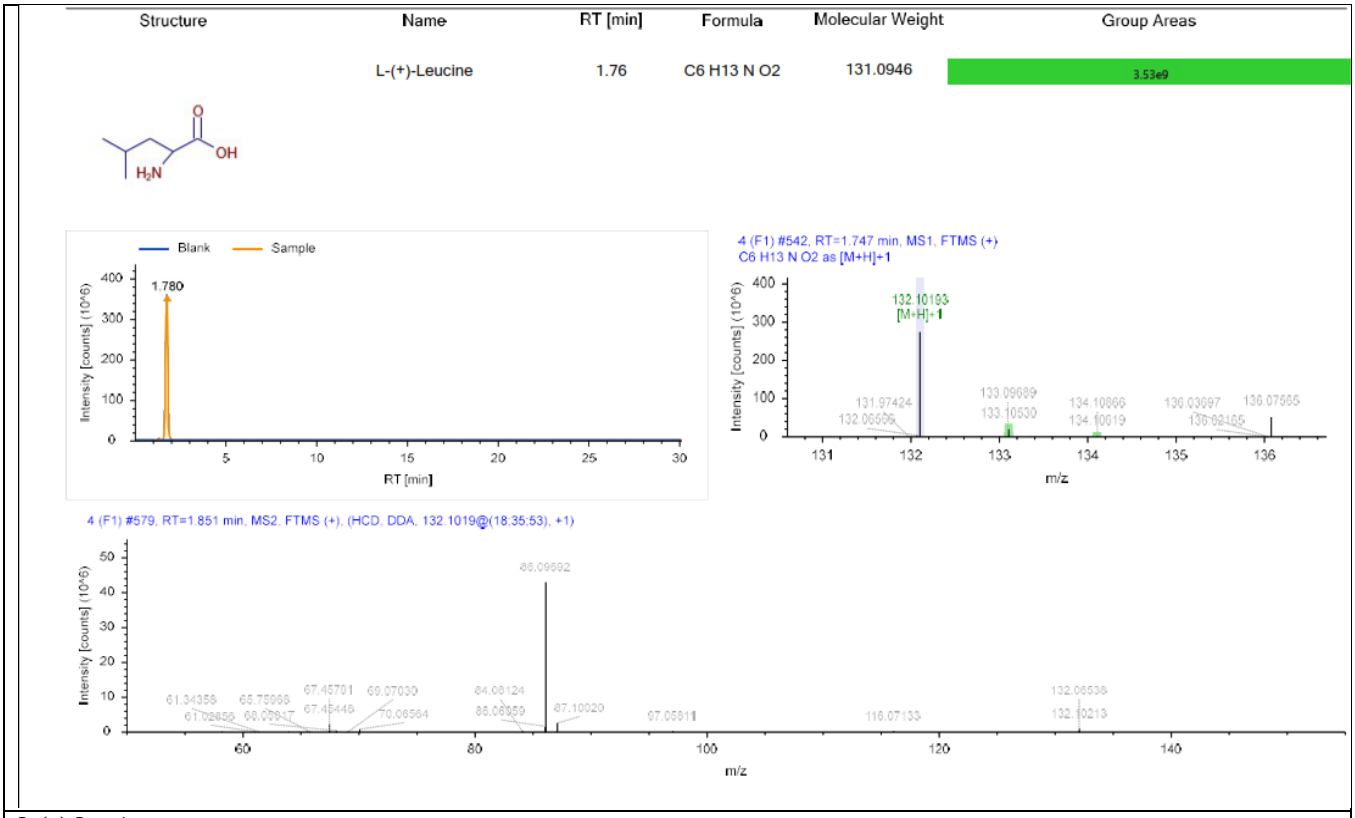
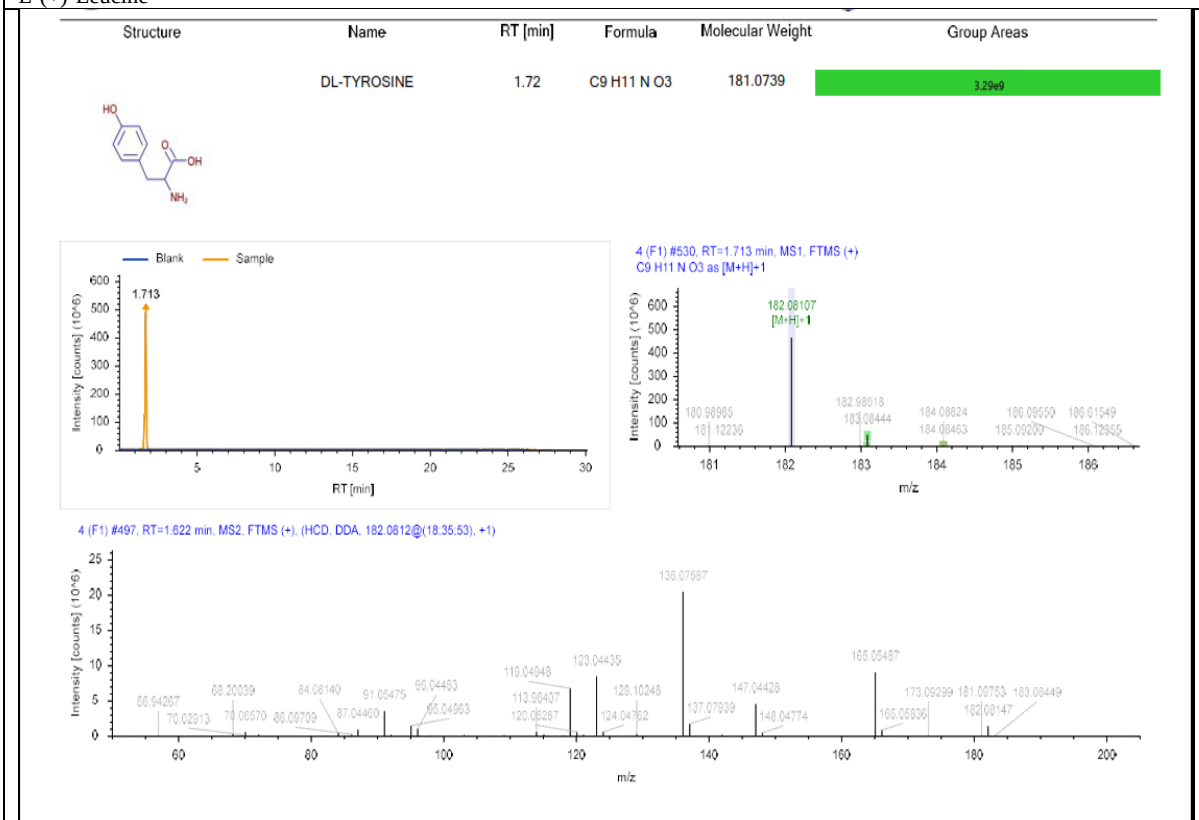


L-(+)-Leucine



DL-TYROSINE

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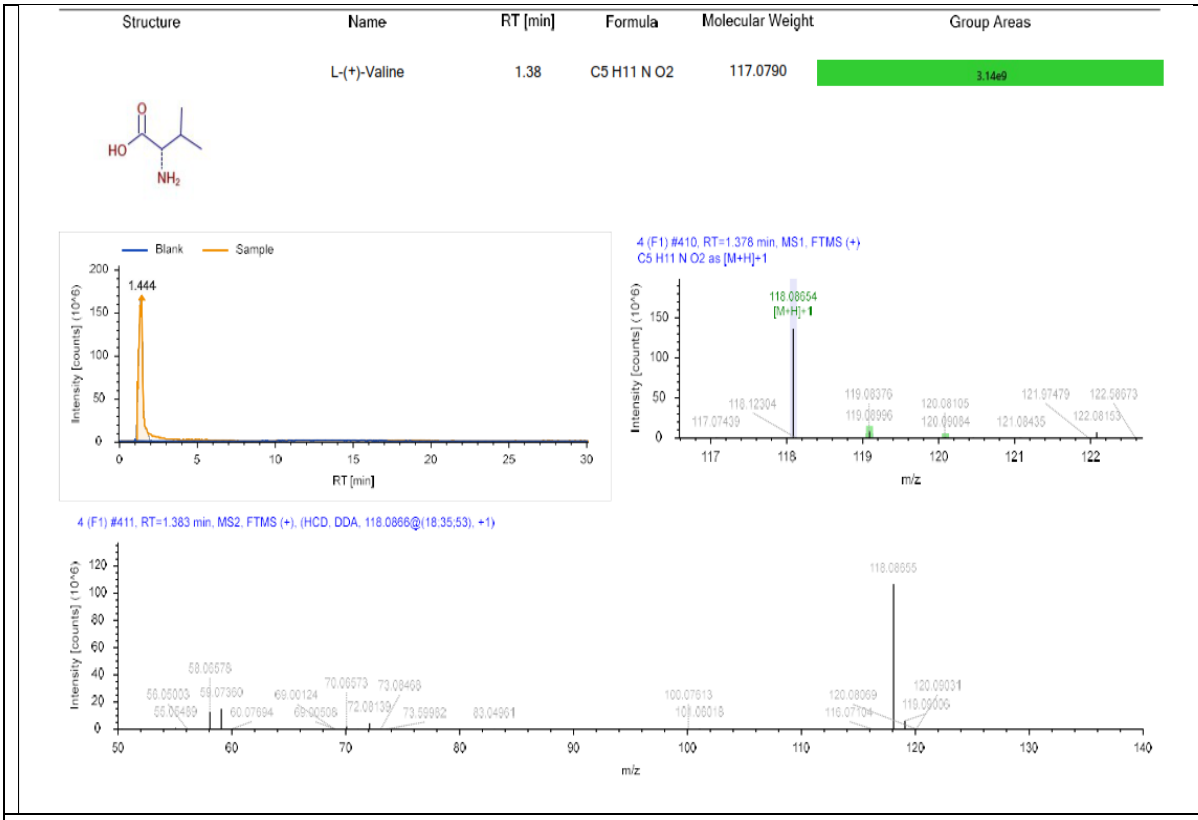
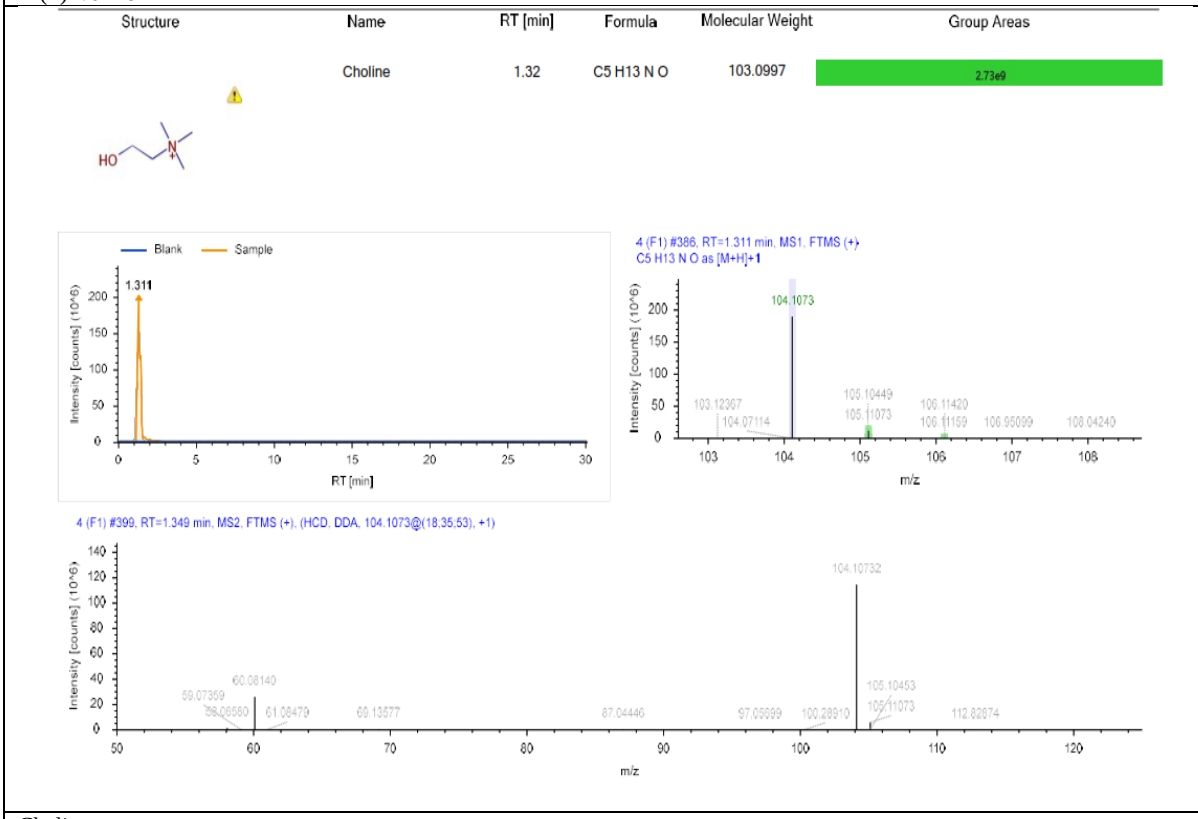


L-(+)-Valine



Choline

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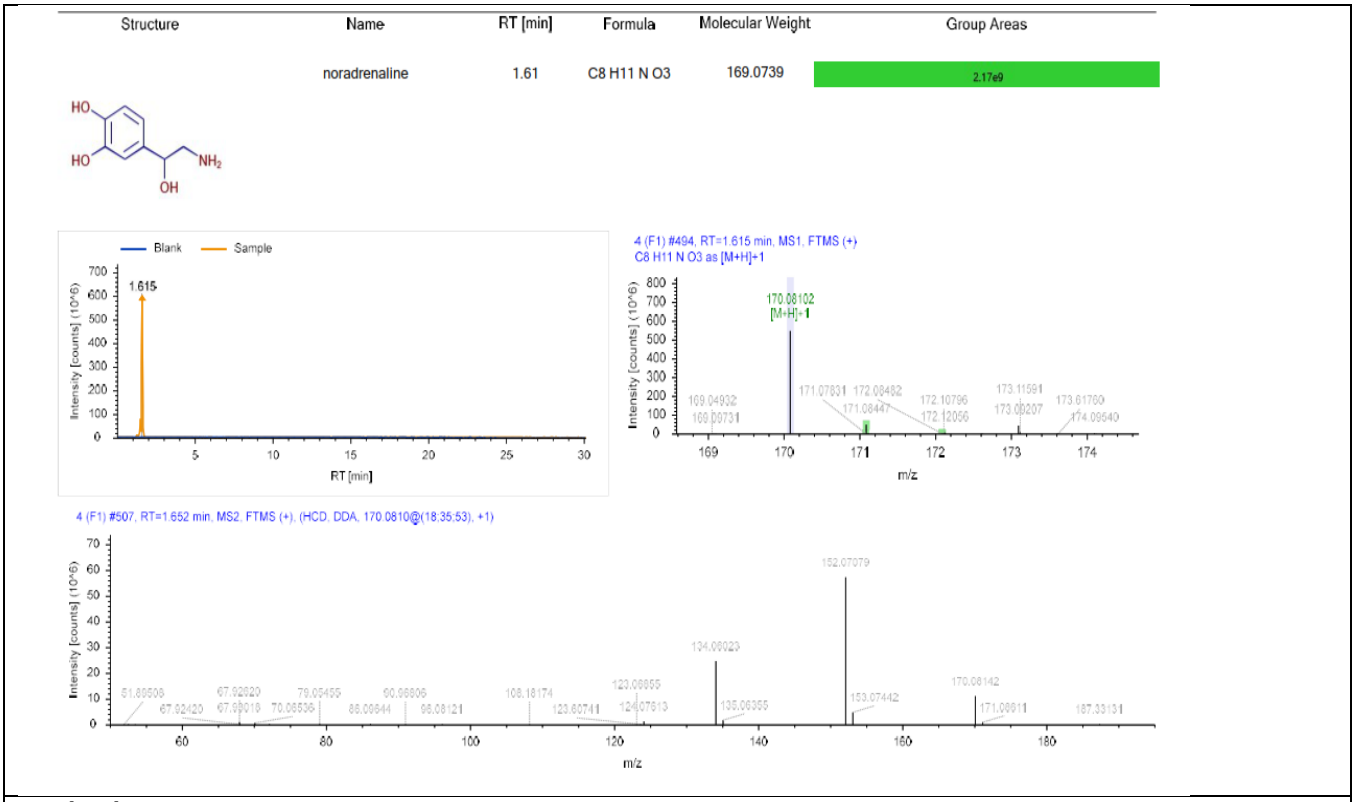
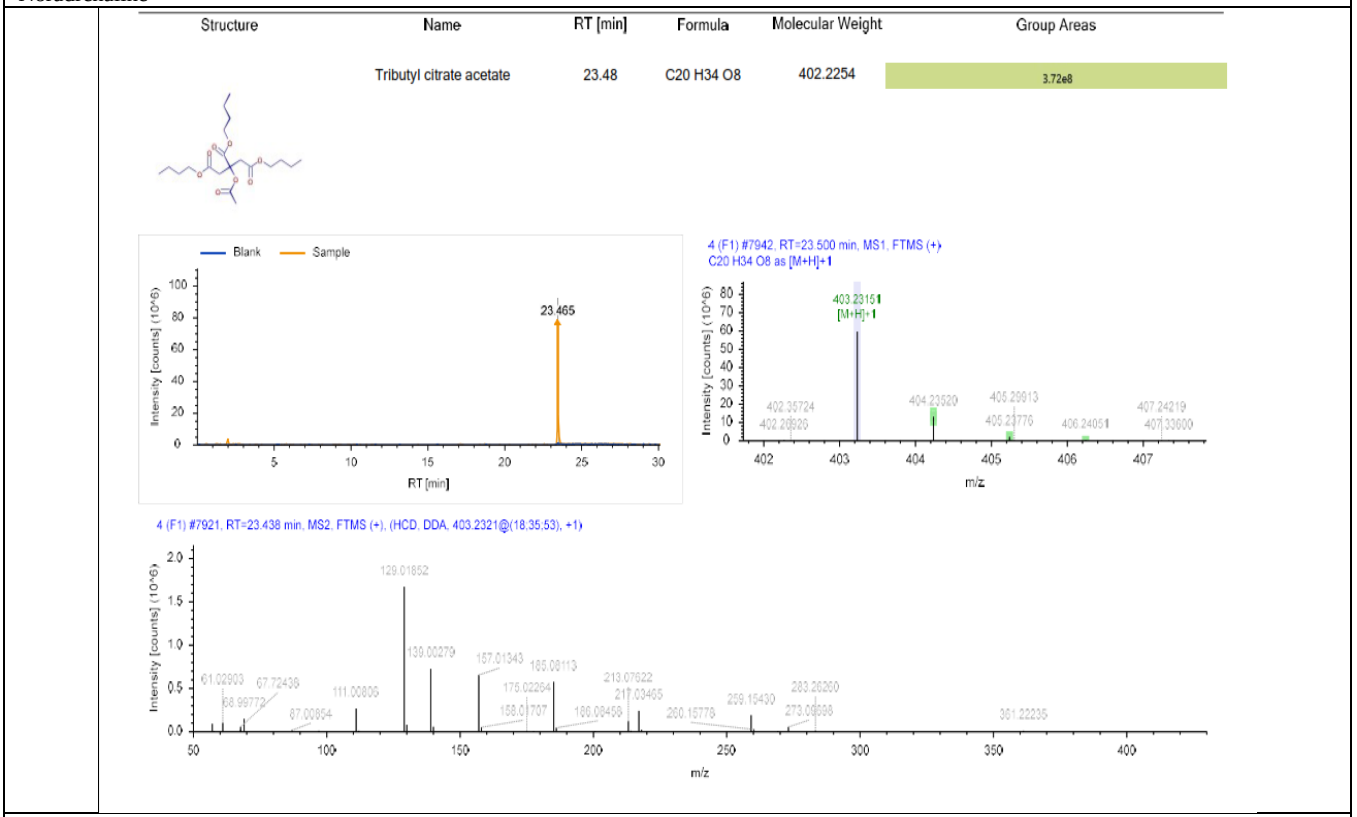


Noradrenaline



tributyl citrate acetate

Figure 4: Compounds identified in the ethanol extract of the *Bacillus cereus* MH997647 IA5 symbiont of the sponge *Agelas* sp.

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