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Bioactive Compounds from Marine Bacterium *Bacillus subtilis* Strain HD16b by Gas Chromatography-Mass Spectrometry

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Abstract The objectives of this study were analysis of the secondary metabolite products from extract of marine *Bacillus subtilis* strain HD16b which isolated from sponges at Ha Tien Sea, Kien Giang province, Vietnam. Eight bioactive compounds were identified in the organic solvent ethyl-acetate. The identification of bioactive chemical compounds is based on the peak area, retention time, molecular weight and molecular formular. GC-MS analysis of *Bacillus subtilis* strain HD16b revealed the existence of the phenylacetic acid, pentadecanoic acid, pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl), n-Hexadecanoic acid, cis-10-Heptadecenoic acid, octadecanoic acid, didemnin B and 1,2-Benzenedicarboxylic acid, diisooctyl ester.

Keywords *Bacillus subtilis*, bioactive compounds, GC-MS, Ha Tien Sea, marine sponge

1. Introduction

Marine sponges (phylum Porifera) are among the most ancient multicellular animals (metazoans). These sessile, filter feeding animals are a rich source of novel biologically active metabolites and offer great potential for drug discovery and, in the long term, for treatment of cancer and infectious diseases [1]. Sponges are also known to have intimate contact with various types of microorganisms such as viruses, bacteria, archaea, fungi, protozoa and single-celled algae, and the nature of the sponge-microbe interaction is manifold [2,3]. In the last decade, marine microorganisms, such as bacteria, microalgae and fungi, have become increasingly important as sources for new bioactive natural products [4-8]. Marine microorganisms have been the important study in recent years because of production of novel metabolites which represent various biological properties such as antiviral, antitumor or antimicrobial activities. These secondary metabolites serve as model systems in discovery of new drugs [4, 5]. Metabolites from microorganisms is a rapidly growing field, due, at least in part, to the suspicion that a number of metabolites obtained from algae and invertebrates may be produced by associated microorganisms [8]. Marine bacteria are considered to play a central role as symbionts of most marine invertebrates and also represent one of the most novel biomedicial resources remaining to be explored [1]. Among these bacteria, the groups *Pseudoalteromonas, Bacillus,* Proteobacteria, and Actinobacteria are common producers of bioactive metabolites [9,10,11]. These bacteria produce diverse compounds, such as polyketides, alkaloids, fatty acids, terpenes, terpenoids, and peptides, which exhibit antiviral, antitumor, antimicrobial, and antiinfective activities [10]. However, the isolation of bioactive compounds from some microbial symbionts could be difficult because of their dependency on the host [12].

In the past few years, Gas chromatography Mass spectrometry (GC-MS) is used as one of the technological platform for finger print analysis of secondary metabolites in both plant and non-plant species [13]. Taking into consideration the medicinal importance of this plant, the ethyl acetate root extract of medical plant [14] and/or leaves as Neem (*Azadirachta Indica* A. Zuss) [15] , flowers *Holarrhena antidysentrica* Wall [16] were analyzed using GC-MS. This

work will help to identify the bioactive components. GC-MS is the best technique to identify bioactive constituents of long chain hydrocarbons, alcohols, acids, ester, alkaloids, steroids, amino and nitro compound etc. [17].
In the course of our screening program, the EtOAc extract of a *Bacillus subtilis* strain HD16b from marine sponge of Ha Tien Sea, Kien Giang province, Vietnam exhibited an inhibition activity against *Salmonella typhymurium, Echerichia coli, Bacillus cereus* and *Candida albicans*. In this paper, we reported the isolation and structural elucidation of secondary metabolites from the cultures broth of *Bacillus subtilis* strain HB16b, is the best strain with against 4 pathogenic microbes, in ethyl-acetate. The present study was aimed to identify the chemical constituents in ethyl acetate extract of marine bacterium was analyzed by the GC-MS technique.

2. Materials and methods

2.1. Bacterial material

The marine sponge was collected in Ha Tien Bay – Kien Giang province in April 2016. The sponge sample (1 g) was added to the 10 mL of sterile sea water in a conical flask. The flask was agitated for about one hour. The marine sponge was filtered and the filtrate was serially diluted to obtain 10^{-1} to 10^{-7} dilutions using the sterilized sea water. An aliquot of 100 µL of each dilution was spread on the media. Starch-yeast extract-peptone-sea water (SYP-SW) and Marine Agar (WA) [18] were used for isolation of bacteria. The SYP-SW and MA media containing 90% of sterile sea water were supplemented with nystatin (25 µg/mL) (Himedia Mumbai) to inhibit fungal contamination. The petriplates were incubated up to 24 – 48 hours at 28°C. The isolated discrete colonies were observed and used for identification.

2.2. Screening assays for antibacterial activity

The search for bioactivity was performed with isolated bacterial isolates from sponges followed by the method of Manikandan *et al.,* [19] and Gopi *et al.,* [20] with pathogenic bacteria namely *Edwardsiella ictaluri, Bacillus cereus, Escherichia coli, Salmonella enterica* and *Candida albicans* which were obtained from College of Aquaculture, Biotechnology R&D Institute (Can Tho University) and Can Tho Center for Technology, Standard, Quality (Department of Science and Technology, Can Tho city).

2.3. 16S rDNA Gene Amplification and Sequencing

Bacterial DNA was isolated following published protocols [21]; Amplification of 16S rDNA by PCR was carried out using the universal primers 8F and 1492R [22]. The 50 µL reaction mixture consisted of 2.5 U Taq Polymerase (Fermentas), 50 µM of each deooxynucleotide triphosphate, 500 nM of each primer (Fermentas) and 20 ng DNA. The thermocycling profide was carried out with an initial denaturation at 95^{o}C (5 min) followed by 30 cycles of denaturation at 95^{o}C (30 s), annealing at 55^{o}C (30 s), extension at 72^{o}C (90 s) and a final extension at 72^{o}C (10 min) in C1000 Thermal Cycler (Bio-Rad). Aliquots (10 µl) of PCR products were electrophoresed and visualized in 1% agarose gels using standard electrophoresis procedures. Partial 16S rRNA gene of selective isolates in each group were sequenced by PHUSA Company, Vietnam. Finally, 16S rRNA sequence of the isolate was compared with that of other microorganisms by way of BLAST (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi). The obtained sequence was analyzed by comparing with bacterial 16S rDNA sequences in GenBank by BLAST N, which showed 99% similarity with *Bacillus subtilis* strain CR26 (GenBank Accession No. KR780430).

2.4. Fermentation, extraction and isolation

Bacillus subtilis strain HD16b was cultured in 250 ml flasks at 30^{o}C for 24 hours with shaking at 150 rpm. Fermentation was carried out in 100 L fermenter with 50 L medium SYP-SW and 10% bacterial inoculum at 30^{o}C for 48 hours. Neutral pH was maintained automatically by NaOH or HCl 1N. The obtained culture broth (50 L) was extracted with ethyl acetate (25 L × 3 times). The combined organic solutions were then decanted, filtered and concentrated under reduced pressure to yield 3.2 g of crude extract ethyl-acetate organic solvent to afford one fraction with eight bioactive compounds.

2.5. GC/MS analysis

The samples were analysed at GC/MS of Chemistry Laboratoty, Department of Chemistry, College of Natural Science, Can Tho University. GC-MS analysis of the sample was carried out using Shimadzu Thermo with column TG-SQC; 15m x 0.25mm x 0.25µm. Helium was used as the carrier gas and the temperature programming was set as follows:

	Speed (^oC/min)	Temperature (^oC)	Keep (min)
Initial		50	1.00
Ramp 1	2.00	70	2.00
Ramp 2	10.00	150	2.00
Ramp 3	10.00	250	10.00
Total time		43 minutes	

10 µl sample was injected with split less mode. Mass spectra was recorded over 35-400 amu range with electron impact ionization energy 70 eV, total running time for a sample was 43 min. Quantitative determination were made by relating respective peak areas to TIC areas from GC-MS.

3. Results and Discussion

GC-MS analysis of compounds from extract of *Bacillus subtilis* strain HD16b with organic solvent ethyl acetate as shown in Figure 1.

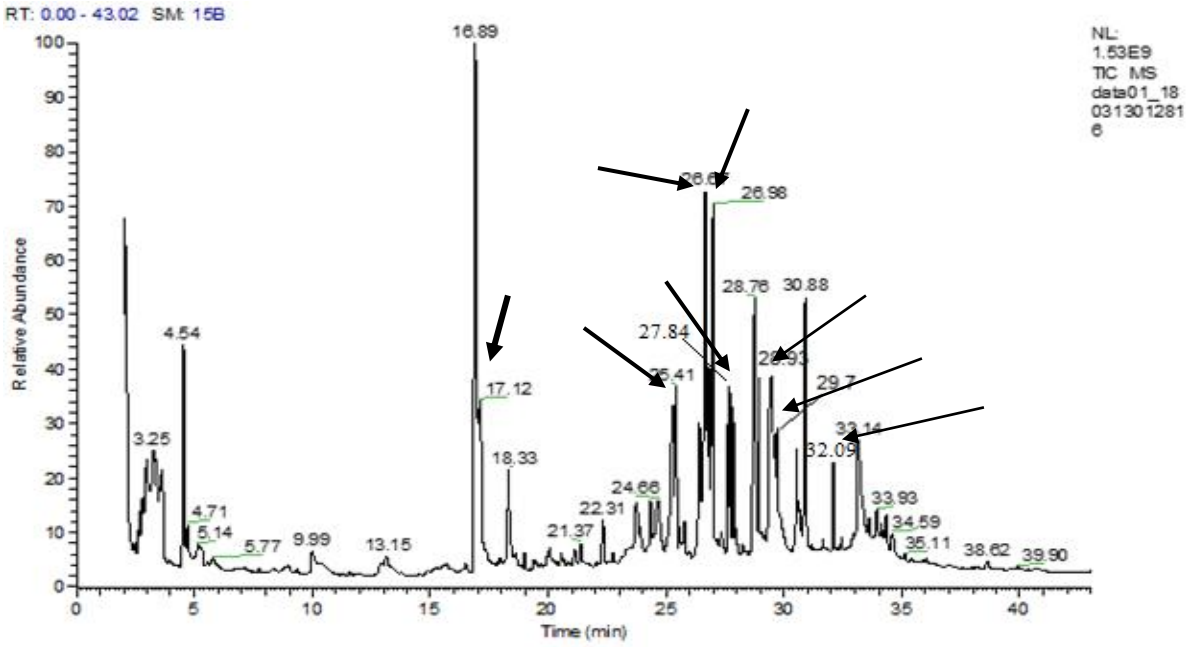

Figure 1: GC-MS chromatogram of extract of Bacillus subtilis strain HD16b in Ethyl-Acetate

Chromatogram GC-MS analysis of Ethyl Acetate extract of *Bacillus subtilis* strain HD16b showed the presence of eight major peaks (Table 1) and the components corresponding to the peaks were determined as follows:

Table 1: Major compounds identified in extract of *Bacillus subtilis* strain HD16b in Ethyl Acetate

S/N	R.T (min)	Name of the compound	Peak (%)	Molecular weight (g/mol)	Molecular formula	Bioactivity
1	17.12	Phenylacetic acid	88.8	136	$C_8H_8O_2$	Antifungal

2	25.41	Pentadecanoic acid	55.2	242	$C_{15}H_{30}O_2$	Antimicrobial
3	26.67	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)	77.3	210	$C_{11}H_{18}N_2O_2$	Antibacterial activity
4	26.98	n-Hexadecanoic acid	86.2	256	$C_{16}H_{32}O_2$	Antimicrobial, antioxidant, antiinflammatory and cancer preventive
5	27.84	cis-10-Heptadecenoic acid	11.6	268	$C_{17}H_{32}O_2$	Anticancer
6	28.93	Octadecanoic acid	22.9	284	$C_{18}H_{36}O_2$	Antibacterial Antifungal
7	29.70	Didemnin B	50.7	1111	$C_{57}H_{89}N_7O_{15}$	Antineoplastic, Antiviral and Subsequently immunosuppresive activities
8	32.09	1,2-Benzenedicarboxylic acid, diisooctyl ester	26.8	390	$C_{24}H_{38}O_4$	Antimicrobial

The first set up peak was determined to be 2- Phenylacetic acid (Figure 2).

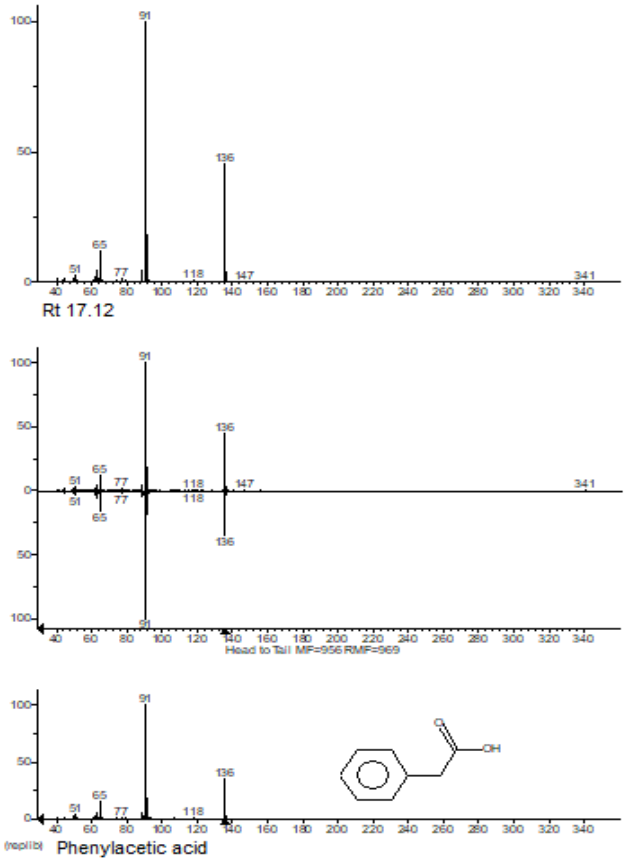

Figure 2: Mass spectrum of phenylacetic acid with retention time (RT) = 17.12

The second peak indicated to be pentadecanoic acid (Figure 3) and the next peaks considered to be pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) (Figure 4).

The fourth set up peak was determined to be n-Hexadecanoic acid (Figure 5). The fifth peak indicated to be cis-10-Heptadecenoic acid (Figure 6), the sixth peak considered to be octadecanoic acid (Figure 7). The seventh peak indicated to be Didemnin B (Figure 8) and final peak considered to be 1,2-Benzenedicarboxylic acid, diisooctyl ester (Figure 9).

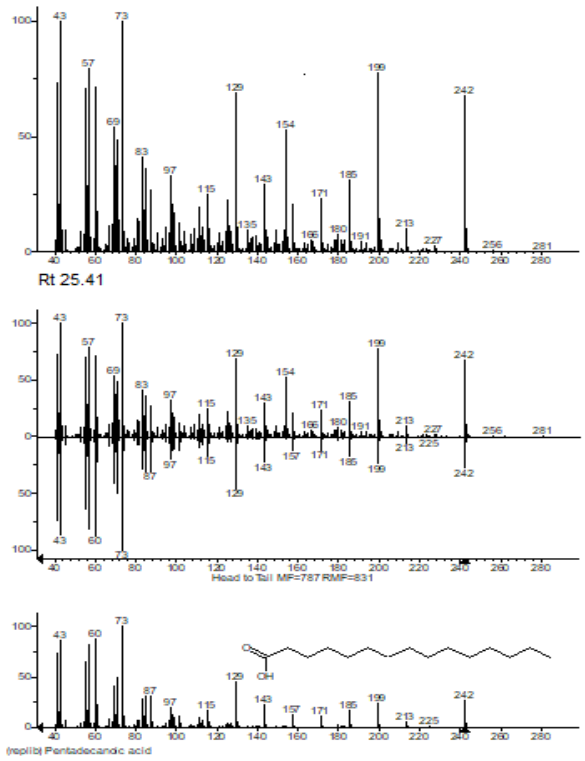

Figure 3: Mass spectrum of pentadecanoic acid with retention time (RT) = 25.41

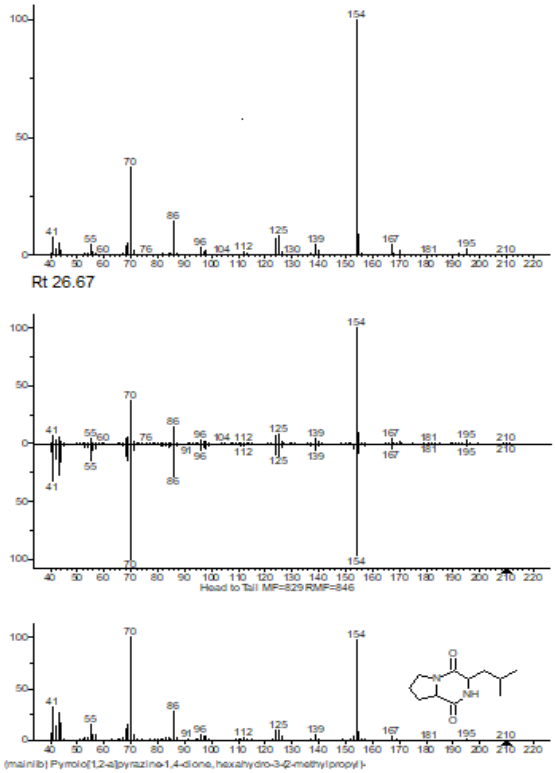

Figure 4: Mass spectrum of pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) with RT = 26.67

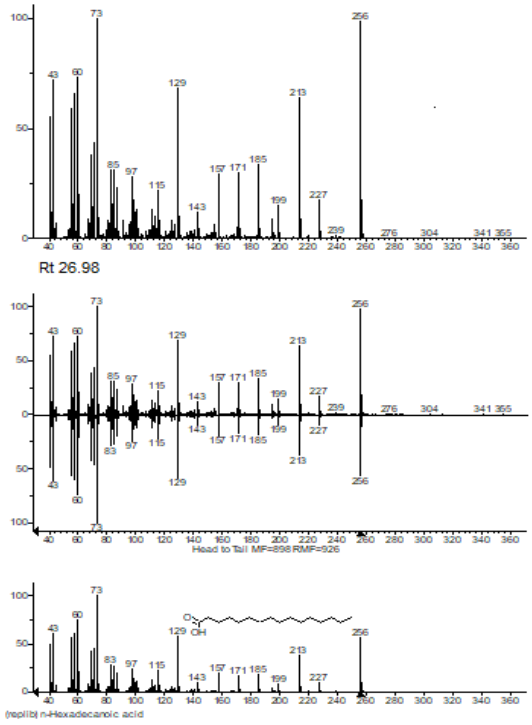

Figure 5: Mass spectrum of n-Hexadecanoic acid with RT = 26.98

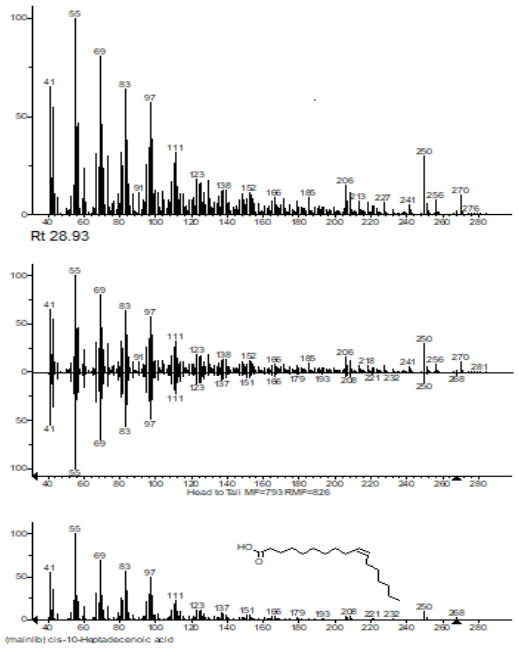

Figure 6: Mass spectrum of cis-10-Heptadecanoic acid with RT = 27.84

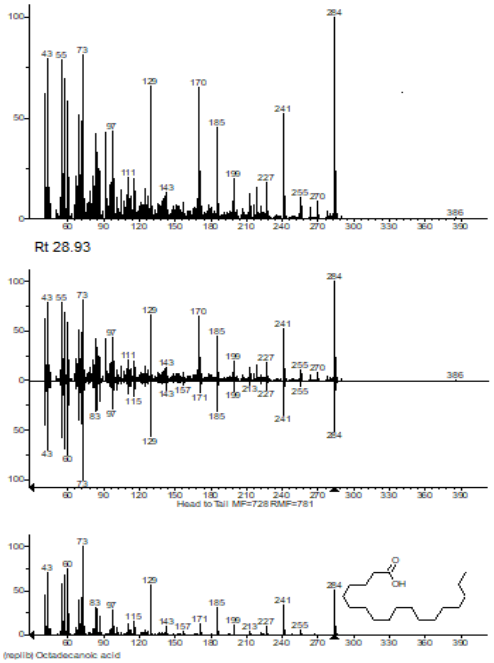

Figure 7: Mass spectrum of Octadecanoic acid with RT = 28.93

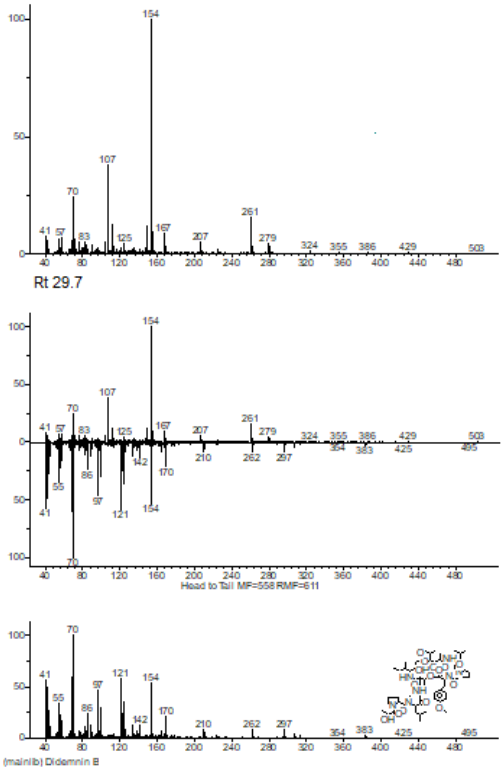

Figure 8: Mass spectrum of Didemine with RT = 29.7

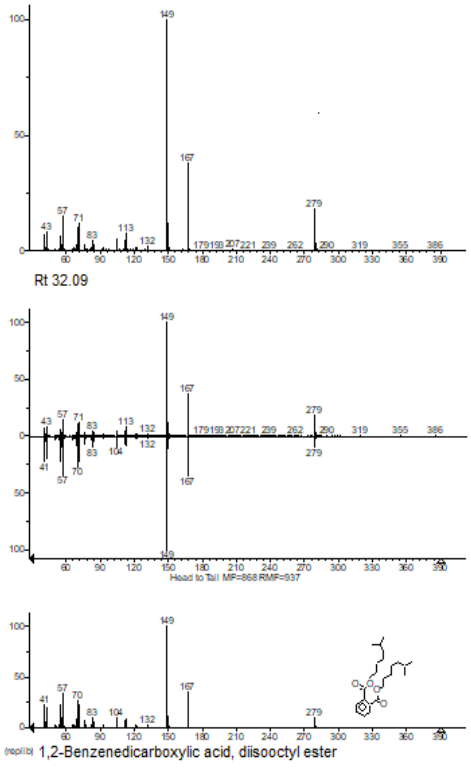

Figure 9: Mass spectrum of 1,2-Benzenedicarboxylic acid, diisooctyl ester with RT = 32.09

Hwang *et al*. [23] identified the antifungal substances SH-1 and SH-2 which were isolated from *Streptomyces humidus* strain S5-55 cultures by various purification procedures and they were identified as phenylacetic acid and sodium phenylacetate, respectively, based on the nuclear magnetic resonance, electron ionization mass spectral, and inductively coupled plasma mass spectral data. SH-1 and SH-2 completely inhibited the growth of *Pythium ultimum, Phytophthora capsici, Rhizoctonia solani, Saccharomyces cerevisiae,* and *Pseudomonas syringae* pv. *syringae* at concentrations from 10 to 50 mg/ml. Ibrahim *et al.* [24] used GC-MS to analyse seed shell pericarp of *Chrysophyllum albidum* fruit and they discoved pentadecanoic acid (Antimicrobial activity), n-Hexadecanoic acid (Antioxidant Hypocholesterolemic activity) and octadecanoic acid (Antifungal, antitumor activity). Fifty one bioactive compounds were identified in the methanolic extract of *Acinetobacter baumannii by* GC-MS and Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropy is one among 51 bioactive compound with antibacterial activity [25]. Twenty one bioactive phytochemical compounds were identified in the methanolic extract of *Mentha viridis* using GC-MS method and n-Hexadecanoic acid was determined as herbal formulation exhibiting cardiac tonic, analgesic, antiasthamatic, anti-inflammatory and antipyretic properties [26]. Besides,. Paramanantham and Murugesan [15] and Kadhim *et al.* [27] identified hexadecanoic acid, is bioactive compound, antimicrobial activity, it was extracted from flower of *Holarrhena antidysentrica* Wall and *Vitis vinifera* tree.

Srivastava, *et al*. [28] used GC-MS to analyse *Wrightia tinctoria* R.Br. (Family: Apocynaceae) seed, and 22 components were identified from seed alcoholic extract, pet ether fraction, cis-10-Heptadecenoic acid is one 22 bioactive compounds, it was identified as anticancer agent. Eight bioactive compounds from the methanolic extract of *Annona muricata* leaves by Gas chromatography and Mass spectroscopy (GC-MS) were identified and n-hexadecanoic acid, (Antioxidant, hypocholesterolemic nematicide, pesticide, anti-androgenic flavor, hemolytic, 5-Alpha reductase inhibitor) and Octadecanoic acid (Antibacterial Antifungal activity) are two in 8 compounds in the methanolic extract of *Annona muricata* [29]. Didemnin B is one of a number of related depsipeptides isolated from the Caribbean tunicate *Tridemnum solidum* (Didemnidae). It was later found to display antineoplastic, antiviral and subsequently immunosuppresive activities [30]. Mechanistically, didemnin B acts at the GTP-binding protein elongation factor [31]. This compound, though, is too toxic to be useful as antiviral or immunosuppressive agent, it has been in Phase I clinical trials as an anticancer agent. Phase II clinical trials are underway. A close relative of didemnin B - dehydrodidemnin B, isolated from a Mediterranean tunicate. *Aplidium albicans*, is currently in Phase II studies in the United States and Europe, to determine its anticancer properties. PharmaMar SA of Spain owns the rights to the compound, which has been shown to be six times more effective than didemnin B in animal tests [32].

Shettima *et al.* [16] used ethyl acetate root extracts of *G. senegalensis* have been shown to be effective against diarrhoea and also have antibacterial activity. The plant was therefore investigated for its bioactive components. The ethyl acetate root extract was investigated using Gas Chromatography-Mass Spectrometry (GC-MS) analysis. Nine components were identified, n-Hexadecanoic acid (46.6%) as the major component followed by 9-Hexadecenoic acid (20.93%), methyl ester (7.75%), 7-Octadecenoic acid- methyl ester, 1, 2-benzene dicarboxylic acid – diisoctyl ester having (6.97%) respectively;

4. Conclusion

In the present study eight compounds from the ethyl acetate extract of *Bacillus subtilis* strain HD16b extract were identified by Gas-chromatography–Mass spectrometry (GC-MS) analysis. The biological activities of identified components range from antimicrobial, antioxidant and antitumoral activities. The research findings have shown that the is extensively rich in secondary metabolites and they have been reported as bioactive compounds and they have been used in the world.

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