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Research Article

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Bioactive Compounds from Marine Fungus *Penicillium citrinum* Strain ND7c by Gas Chromatography-Mass Spectrometry

Nguyen Tan Binh, Phan Van Ha Lam, Cao Ngoc Diep*

Biotechnology R&D Institute, Can Tho University, Viet nam

Abstract The objectives of this study were analysis of the secondary metabolite products from extract of marine *Penicillium citrinum* strain ND7a which isolated from sponges at Ha Tien Sea, Kien Giang province, Vietnam. Sixteen bioactive compounds were identified in the organic solvent hexan-aceton and aceton-methanol. The identification of bioactive chemical compounds is based on the peak area, retention time, molecular weight and molecular formular. GC-MS analysis of *Penicillium citrinum* strain ND7a revealed the existence of the 9-Hexadecenoic acid, Hexadecane, n-Hexadecanoic acid, Heptacosane, Octadecane, 3-ethyl-5-(2-ethylbutyl), Tributyl acetylcitrate, 17-Pentatriacontene, Hexanedioic acid, bis(2-ethylhexyl) ester, Bis(2-ethylhexyl) phthalate (in organic solvent hexan-aceton) and Oxime-, methoxy-phenyl, 2-Hydroxy-gamma-butyrolactone, 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione, Pentadecanoic acid, 14-methyl-, methyl ester, Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester, Stigmasterol, γ-Sitosterol (in organic solvent aceton-methanol).

Keywords bioactive compounds, GC-MS, Ha Tien Sea, Marine sponge, *Penicillium citrinum*

1. Introduction

In the last decade, marine microorganisms, such as bacteria, microalgae and fungi, have become increasingly important as sources for new bioactive natural products [1-5]. 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. Fungi have proven to be particularly prolific sources of new compounds when compared to other microbial sources isolated from the sea. The first report of a bioactive natural product from a marine-derived fungus dates back to the 1940s when the fungus *Acremonium chrysogenum* Gams 1971 was isolated from a sewage outlet in the Mediterranean Sea close to the island of Sardinia. The fungus was the source of cephalosporin C, the parent compound of modern cephalosporin antibiotics that are indispensible for the treatment of numerous bacterial infections [6]. Initially, progress with rigorous evaluation of marine fungal metabolites was slow. This situation changed dramatically in the 1990s when there was a sharp rise in interest in marine microbial metabolites that continues until today. Up to the year 2002, 272 new natural products had been isolated from marine-derived fungi [7]. A dramatic increase in the number of elucidated marine fungal structures began afterwards, illustrated by the fact that between 2002 and 2004, 240 additional compounds were described [8]. Even though the value of marine-derived fungi as a source of new bioactive metabolites is now commonly accepted, there is still much debate on the nature of fungi that are isolated from various marine substrates, such as drifting wood, algae or invertebrates. Due to the fact that numerous, if not most, of these fungi belong to genera already well known from the terrestrial environment, such as *Aspergillus, Penicillium, Cladosporium, Phoma, and Fusarium*, a true marine origin of these fungal strains is frequently doubted [9-10]. It is possible that several, if not many, marine-derived fungi thus far investigated originated from terrestrial habitats (e.g., soil) from which they were

washed to the sea and survived (as spores) until they were recovered by a marine chemist looking for new compounds from the sea. On the other hand, in the last few years more and more evidence has accumulated indicating an adaptation of these ''ubiquitous'' fungi to the marine environment [11-15]. Nevertheless, the fact remains that regardless of their true origin, marine-derived fungi have developed into an important source of new and structurally unprecedented metabolites [8]. Interestingly, sponges continue to be one of the most important sources for the isolation of metabolite-producing marine-derived fungi [7-9, 16], even though the presence of fungal mycelia growing in sponges has not yet been proven. Meanwhile, the search of bioactive secondary metabolites from marine microorganisms is not widely explored in Vietnam [17-18].

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 [19]. Taking into consideration the medicinal importance of this plant, the ethyl acetate root extract of medical plant [20] and/or leaves as Neem (*Azadirachta Indica* A. Zuss) [21], flowers *Holarrhena antidysentrica* Wall [22] 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. [20].

In the course of our screening program, the EtOAc extract of a *Penicillium citrinum* strain ND7a 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 *Penicillium citrinum* strain ND7a of in two kinds of organic solvent. The present study was aimed to identify the chemical constituents in ethyl acetate extract of marine fungus was analyzed by the GC-MS technique.

2. Materials and methods

2.1. Fungus 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 Glucose Peptone Yeast Extract Agar (GPY) media. The media containing 50% of sterile sea water were supplemented with ampiciline and streptomycine (100 µg/mL) (Himedia Mumbai) to inhibit bacterial contamination, respectively. The petriplates were incubated up to 3 weeks at 28°C. The isolated discrete colonies were observed and used for identification.

The obtained fungus strain was identified by using 18S rRNA gene sequencing method. The universal primers including forward primer, ITS1 and reverse primer, ITS4 were used for amplifying nearly full length of 18S rRNA gene sequence from 550 to 800 bp [23]. The obtained sequence was analyzed by comparing with bacterial 18S rRNA sequences in GenBank by BlastN, which showed 99% similarity with *Penicillium citrinum* strain TDD (GenBank Accession No. JX192960).

2.2. Fermentation, extraction and isolation

Penicillium citrinum strain ND7a 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 PDA medium and 10% fungal inoculum at 30^{o}C for 52 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 8.1 g of crude extract which was chromatographed on a silica gel column using a gradient of 1 - 100% acetone in hexane to afford three fractions F1-9, after that it was continuously chromatographed on a silica gel column using a gradient of 1 - 100% acetone in methanol to afford three fractions F10-16. Therefore, sixteen fractions were received from 2 kinds of organic solvent (hexane – acetone and acetone – methanol).

2.3. 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 *Penicillium citrinum* strain ND7a with organic solvent Hexan-Aceton as shown in Figure 1.

Chromatogram GC-MS analysis of hexan-aceton extract of *Penicillium citrinum* strain ND7a showed the presence of nine major peaks (Table 1) and the components corresponding to the peaks were determined as follows.

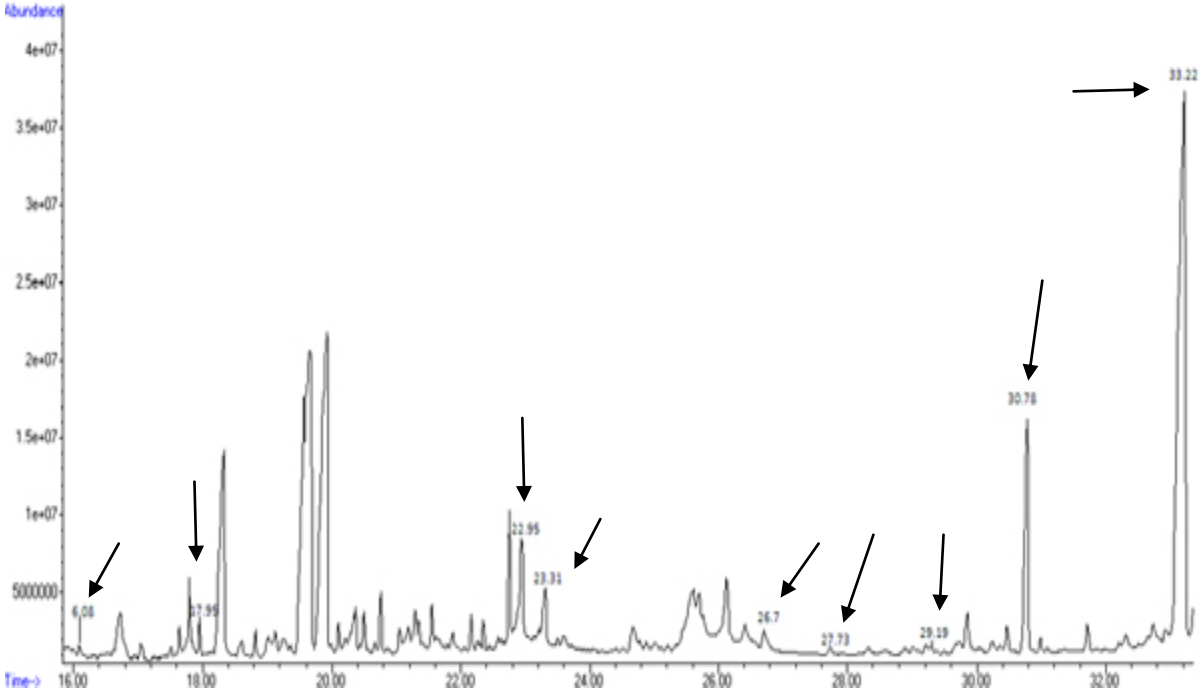
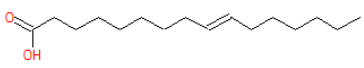
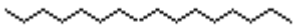
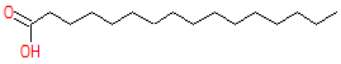
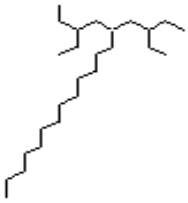
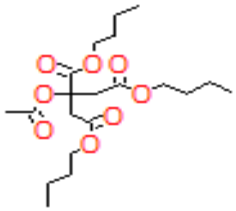
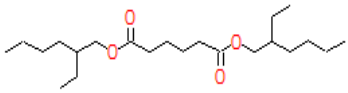
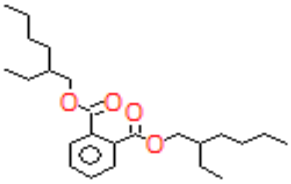


Figure 1: GC-MS chromatogram of extract of Penicillium citrinum strain ND7a in organic solvent Hexan-Aceton

Table 1: Major compounds identified in extract of *Penicillium citrinum*.strain ND7a (hexan-aceton)

Retention time	Name of the compound	Molecular weight (g/mol)	Molecular formula	Peak %	Bioactivity
16.08	9-Hexadecenoic acid 	254	$C_{16}H_{30}O_2$	6.74	Pesticide and antibiotic

17.95	Hexadecane 	226	$C_{16}H_{34}$	18.6	Antimicrobial, antioxidant, antidiabetic
22.95	n-Hexadecanoic acid 	256	$C_{16}H_{32}O_2$	44.6	Antibacterial
23.31	Heptacosane	380	$C_{27}H_{56}$	7.58	Antimicrobial
26.70	Octadecane, 3-ethyl-5-(2-ethylbutyl) 	366	$C_{26}H_{54}$	14.6	Antimicrobial antifungal
27.73	Tributyl acetylcitrate 	402	$C_{20}H_{34}O_8$	43.2	Anticancer activity Antimicrobial activity
29.19	17-Pentatriacontene	490	$C_{35}H_{70}$	8.72	Antibacterial, antiviral
30.78	Hexanedioic acid, bis(2-ethylhexyl) ester 	370	$C_{22}H_{42}O_4$	56.1	Antioxidant antimicrobial, antiproliferative
33.22	Bis(2-ethylhexyl) phthalate 	390	$C_{24}H_{38}O_4$	38.5	Antibacterial and antifungal agent

The first set up peak was determined to be 9-Hexadecenoic acid (Figure 2).

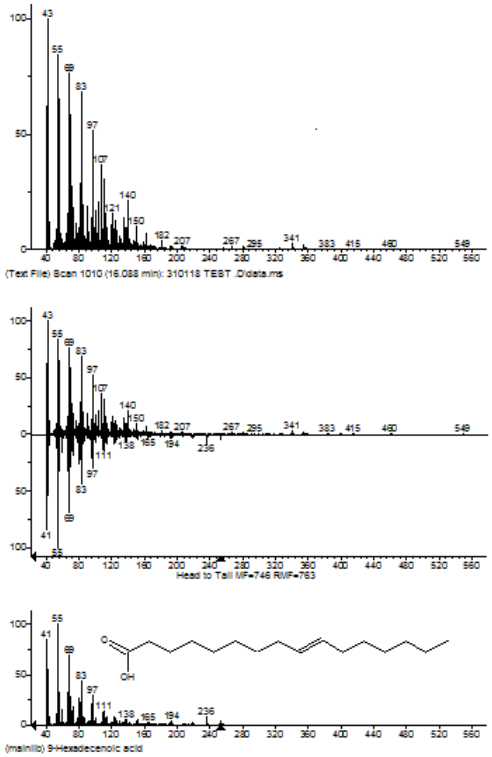


Figure 2: Mass spectrum of 9-Hexadecenoic acid with retention time (RT) = 16.08

The second peak indicated to be Hexadecane (Figure 3) and the third peaks considered to be n-Hexadecanoic acid (Figure 4).

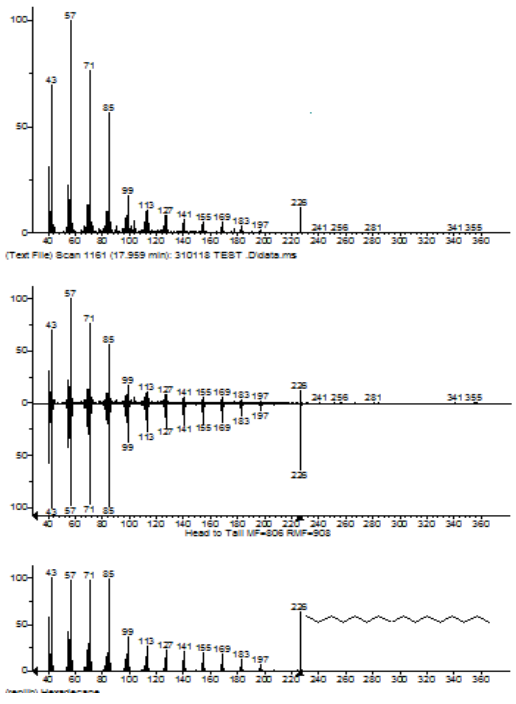


Figure 3: Mass spectrum of Hexadecane with RT = 17.95

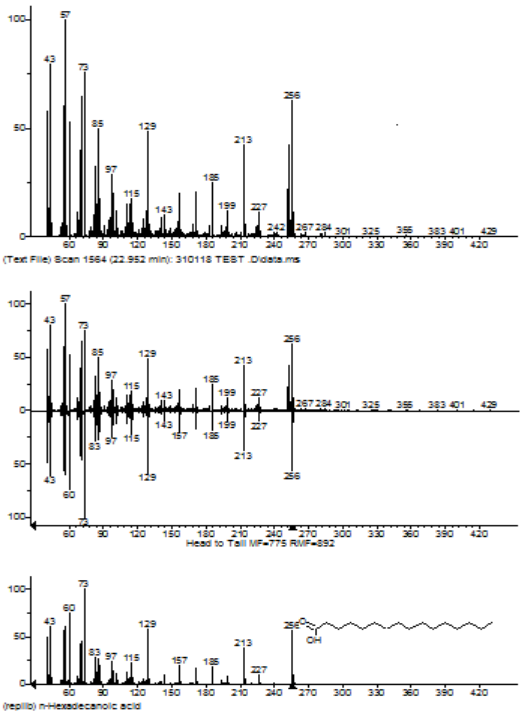


Figure 4: Mass of spectrum of n-Hexadecanoic acid with RT = 22.95

The fourth peak indicated to be Heptacosane (Figure 5) and the fifth peaks considered to be Octadecane, 3-ethyl-5-(2-ethylbutyl) (Figure 6).

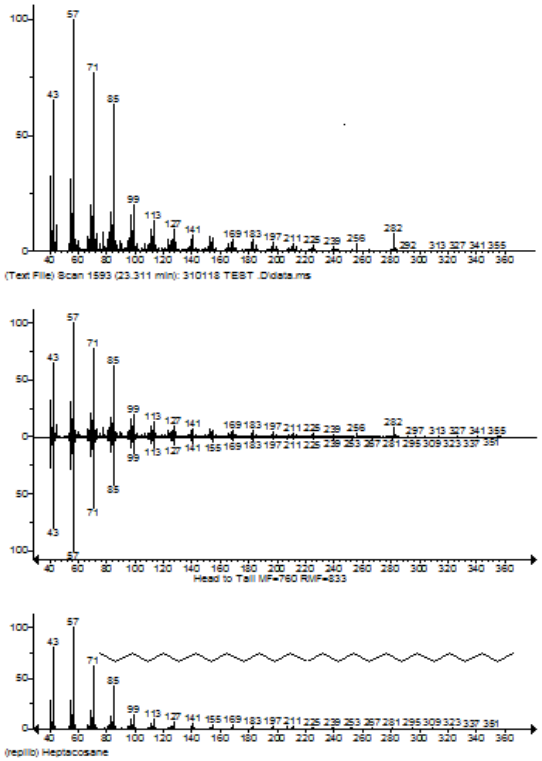


Figure 5: Mass spectrum of Heptacosane with RT = 23.31

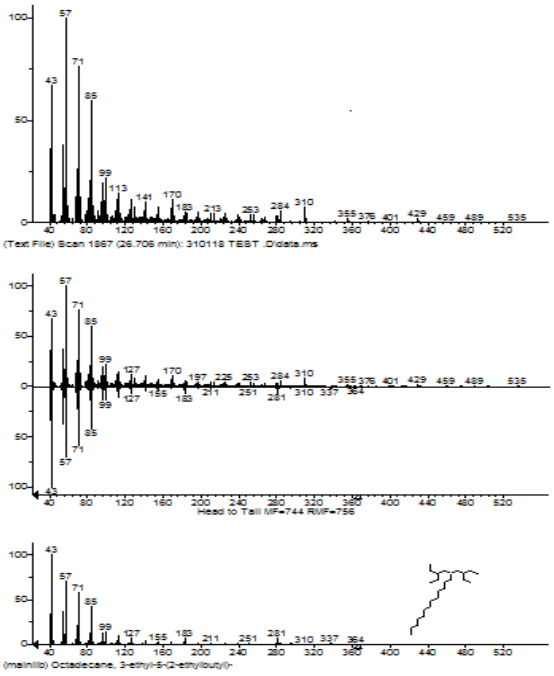


Figure 6: Mass spectrum of Octadecane, 3-ethyl-5-(2-ethylbutyl) with RT = 26.70

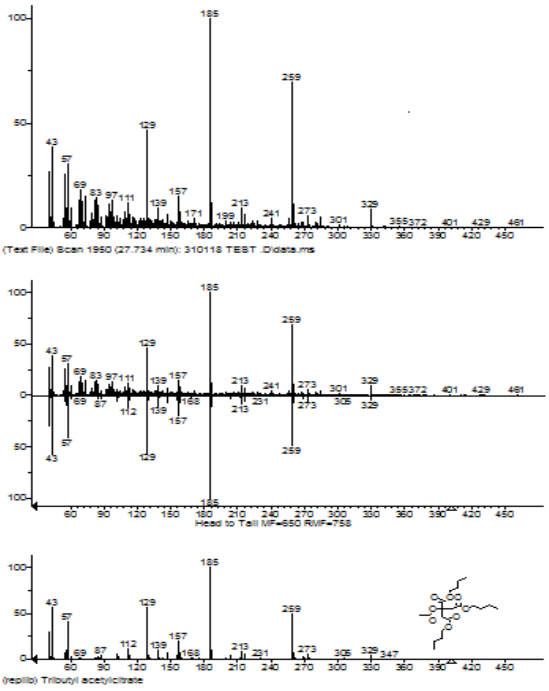


Figure 7: Mass spectrum of Tributyl acetylcitrate with RT = 27.73

The sixth peak determined to be Tributyl acetylcitrate (Figure 7) and seventh peakconsidered to be 17-Pentatriacontene (Figure 8)

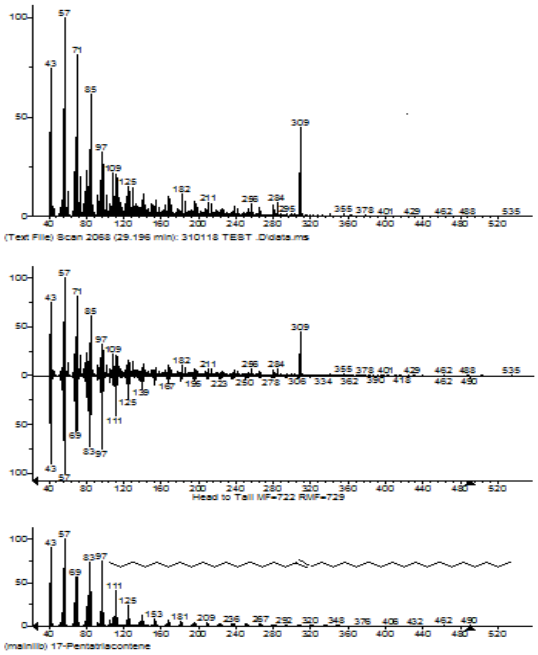


Figure 8: Mass spectrum of 17-Pentatriacontene with RT = 29.19

The eighth peak indicated to be Hexanedioic acid bis(2-ethylhexyl) ester (Figure 9), and the ninth peak determined to be Bis(2-ethylhexyl) phthalate (Figure 10).

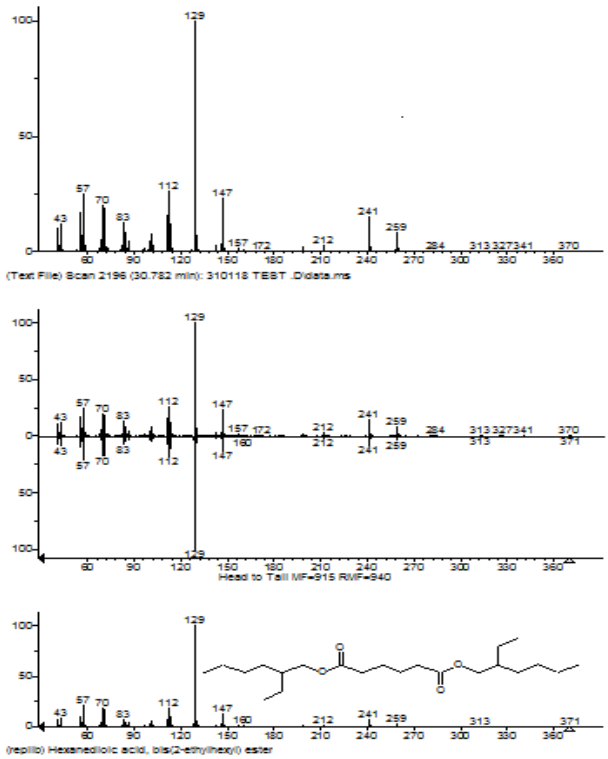


Figure 9: Mass spectrum of Hexanedioic acid bis(2-ethylhexyl) ester with RT = 30.78

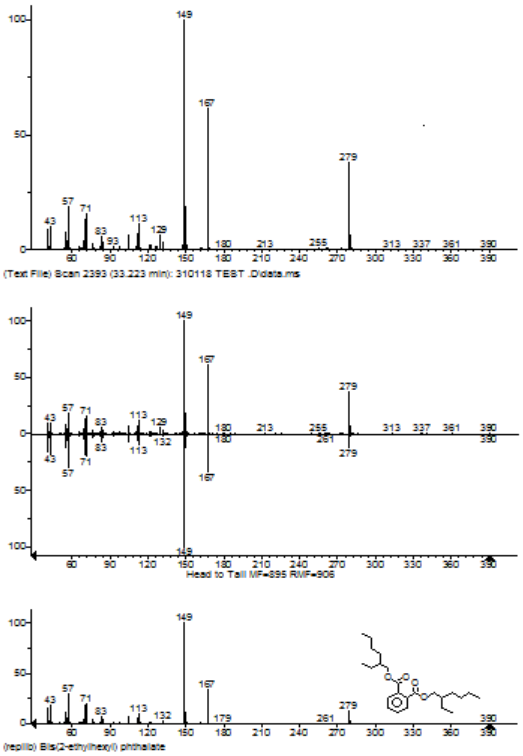


Figure 10: Mass spectrum of Bis(2-ethylhexyl) phthalate with RT = 33.22

Shettima *et al.* [24] discovered nine compounds from the ethyl acetate root extract of *Guiera senegalensis* were identified by Gas-chromatography–Mass spectrometry (GC-MS) analysis. The biological activities of each of the identified phytocomponents range from antimicrobial, antioxidant and antitumoral activities. The nature of the identified compounds are mostly organic acids and they also found 9-Hexadecenoic acid, is one in nine compounds from the root of *Guiera senegalensis*. Paramanantham and Murugesan [22] analysed the flowers extract show the presence of 30 phyto compounds and they found Hexadecane and Hexanedioic acid, bis(2-ethylhexyl) ester as various bioactive compounds justifies the use of the plant flower for various ailments by traditional practitioners. When using GC-MS analysis of one Ayurvedic medicine Talisapatradi Churnam, this medicine is used to treat respiratory and digestive disorders. The medicine was subjected to DPPH, FRAP and Hydrogen Peroxide scavenging assays and it was found that it has good antioxidant potential. The GC MS analysis results indicated the presence of twenty six bio molecules and n-Hexadecanoic acid and Octadecane, 3-ethyl-5-(2-ethylbutyl) in this medicine [25]. When study on the phytochemical composition of *Adiantum capillus-veneris* and to evaluate the isolates for possible in vitro antifungal and antibacterial activities. The compound obtained were screened by GC-MS method. While agar-well diffusion method was employed to measure antimicrobial activity against five bacteria and fourteen fungi and yeast. Thirty-one bioactive phytochemical compounds were identified in the methanolic extract of *Adiantum capillus-veneris.* Tributyl acetylcitrate found was one in 31 bioactive phytochemical compounds [26]. The presence of five secondary bioactive components with 17-pentatriacontene has been identified in *Turbinaria ornata*, brown alga, of which four bioactive compounds [27]. Al-Bari *et al.* [28] isolated *Streptomyces bangladeshensis* sp. nov., from soil, which produces bis-(2-ethylhexyl)phthalate, as antimicrobial agent.

GC-MS analysis of compounds from extract of *Streptomyces* sp. strain ND7a with organic solvent Aceton-Methanol as shown in Figure 11.

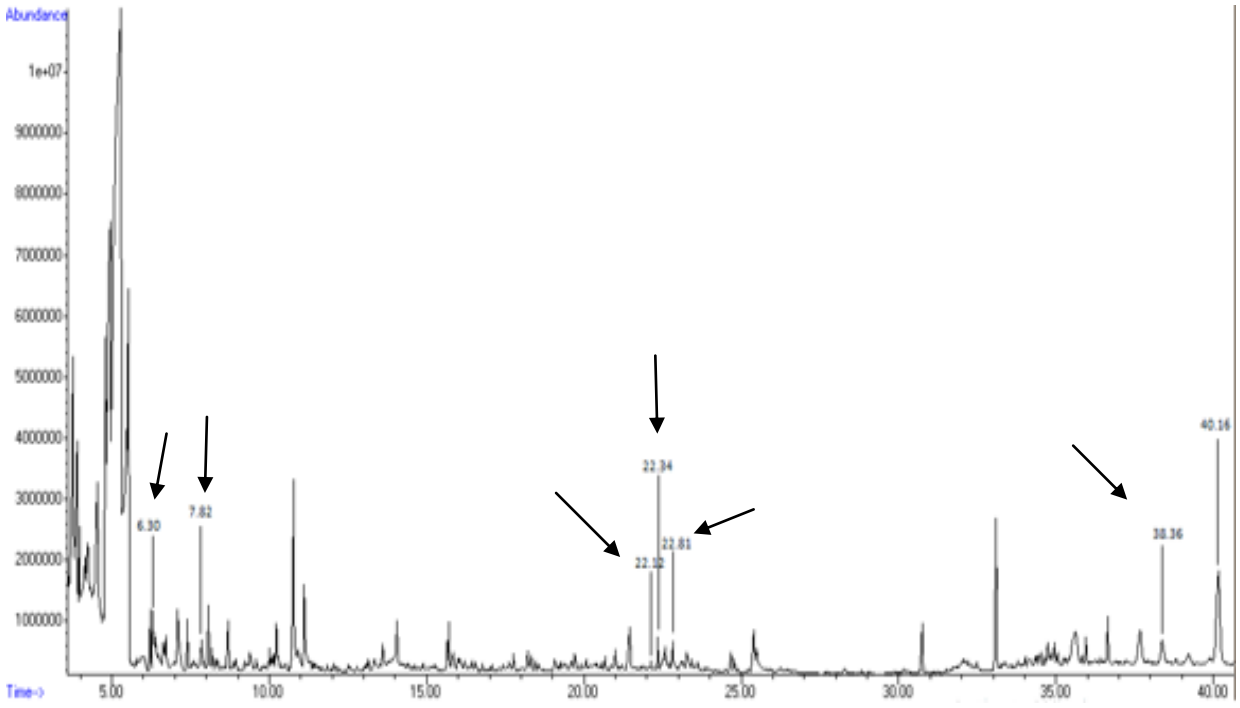
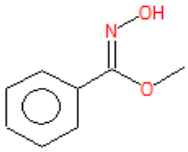
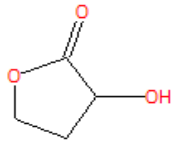
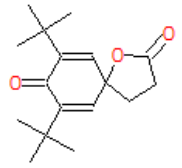
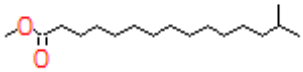
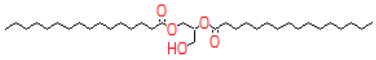
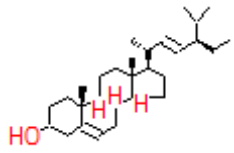
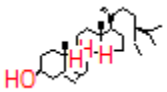


Figure 11: GC-MS chromatogram of extract of Penicillium citrinum strain ND7a in organic solvent Aceton-Methanol

Chromatogram GC-MS analysis of hexan-aceton extract of *Penicillium citrinum* strain ND7a showed the presense of seven major peaks (Table 2) and the components corresponding to the peaks were determined as follows.

Table 2: Major compounds identified in extract of *Penicillium citrinum* strain ND7a (aceton-methanol)

Retention time	Name of the compound	Molecular weight (g/mol)	Molecular formula	Peak %	Bioactivity
6.30	Oxime-, methoxy-phenyl 	151	$C_8H_9NO_2$	81.5	Antifungal, Antibacterial, Anticancer and Antitumor
7.82	2-Hydroxy-gamma-butyrolactone 	102	$C_4H_6O_3$	47.4	Antioxidant, analgesic, anti-diabetic, antibacterial, and antifungal activity
22.13	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione 	276	$C_{17}H_{24}O_3$	68.7	Antimicrobial
22.34	Pentadecanoic acid, 14-methyl-, methyl ester 	270	$C_{17}H_{34}O_2$	26.6	Antifungal, Antimicrobial
22.81	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester 	568	$C_{35}H_{68}O_5$	34.1	Antimicrobial activity
38.36	Stigmasterol 	412	$C_{29}H_{48}O$	50.4	Antimicrobial
40.16	γ-Sitosterol 	414	$C_{29}H_{50}O$	85.3	Antioxidant, antibacterial and prophylactic activities

The first set up peak was determined to be Oxime-,methoxy-phenyl (Figure 12) and the second peak considered to be 2-Hydroxy-gamma-butyrolactone (Figure 13).

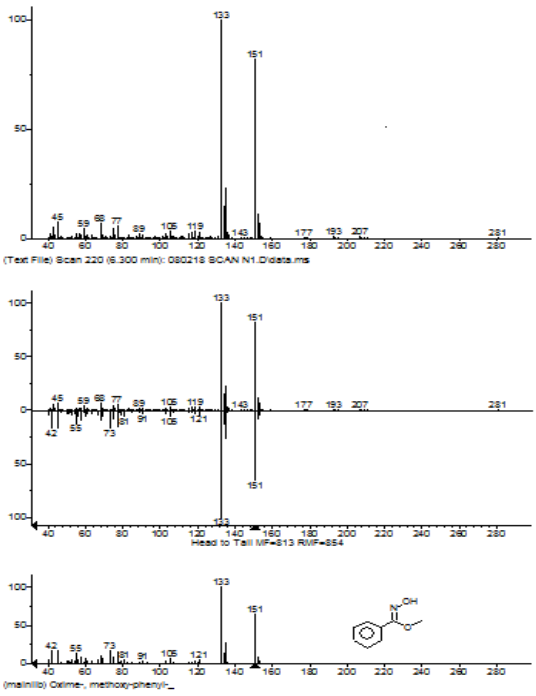


Figure 12: Mass spectrum of Oxime-,methoxy-phenyl with RT = 6.30

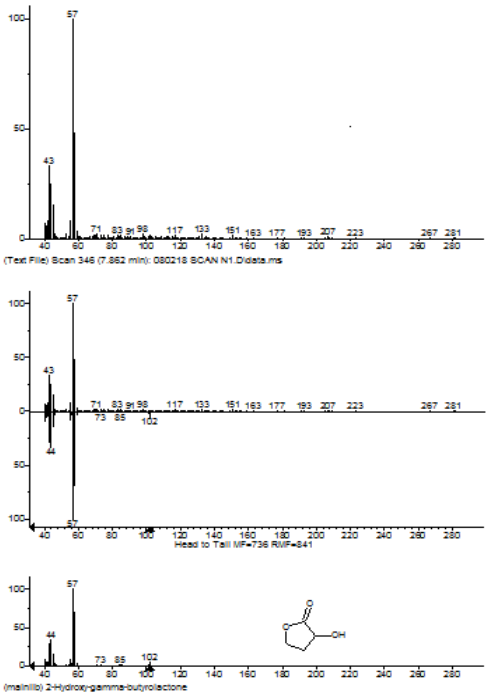


Figure 13: Mass spectrum of 2-Hydroxy-gamma-butyrolactone with RT = 7.82

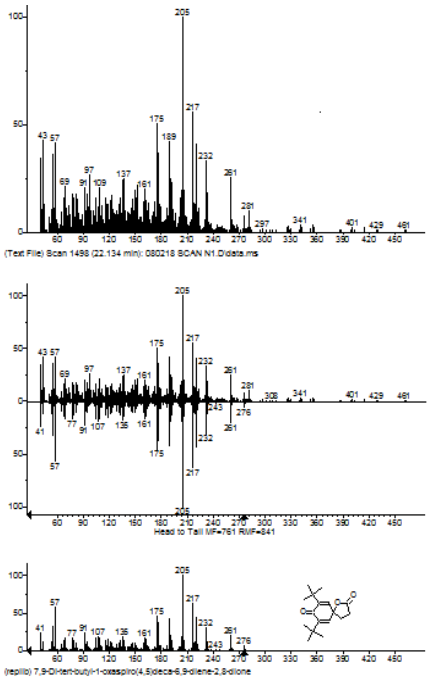


Figure 14: Mass spectrum of 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione with RT = 22.13

The third peak determined to be 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione (Figure 14), the fourth peak indicated to be Pentadecanoic acid, 14-methyl-, methyl ester (Figure 15), the fifth peak considered to be Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester (Figure 16).

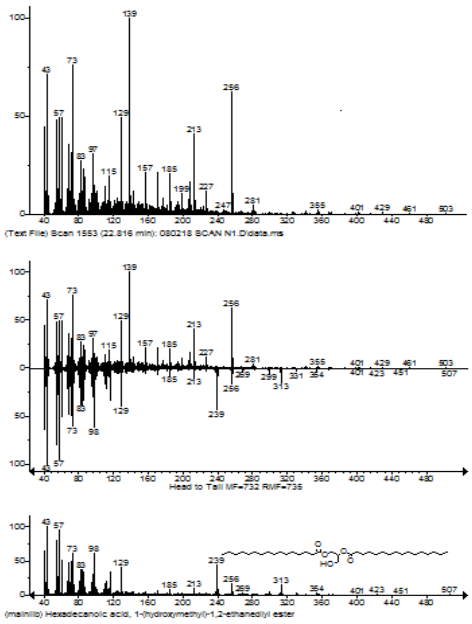


Figure 15: Mass spectrum of Pentadecanoic acid, 14-methyl-, methyl ester with RT = 22.34

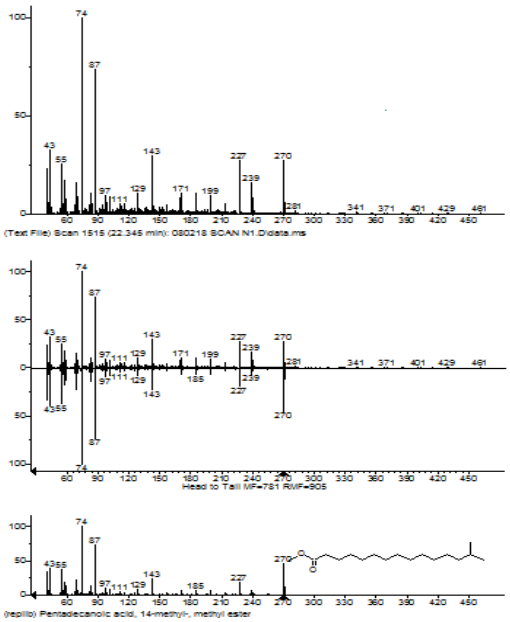


Figure 16: Mass spectrum of Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester with RT = 22.81

The sixth peak indicated to be Stigmasterol (Figure 17) and the seventh peak determined to be γ-Sitosterol (Figure 18).

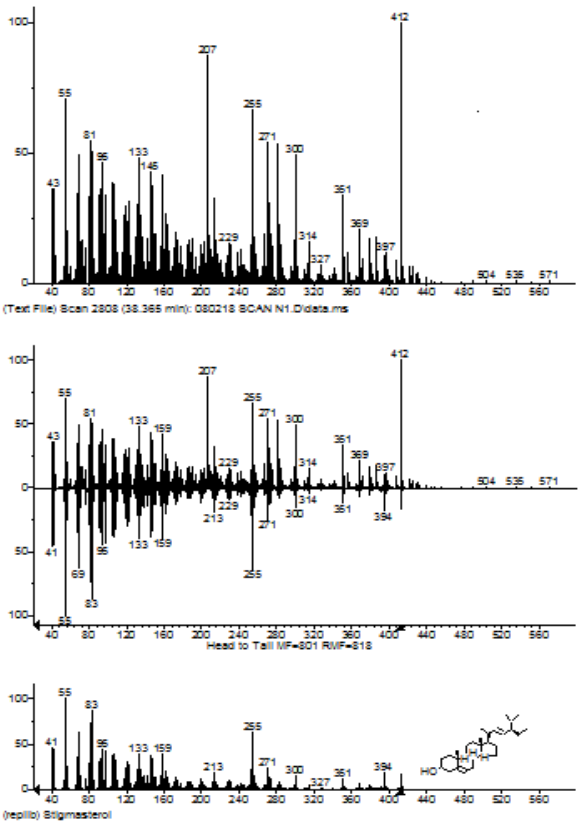


Figure 17: Mass spectrum of Stigmasterol with RT = 38.36

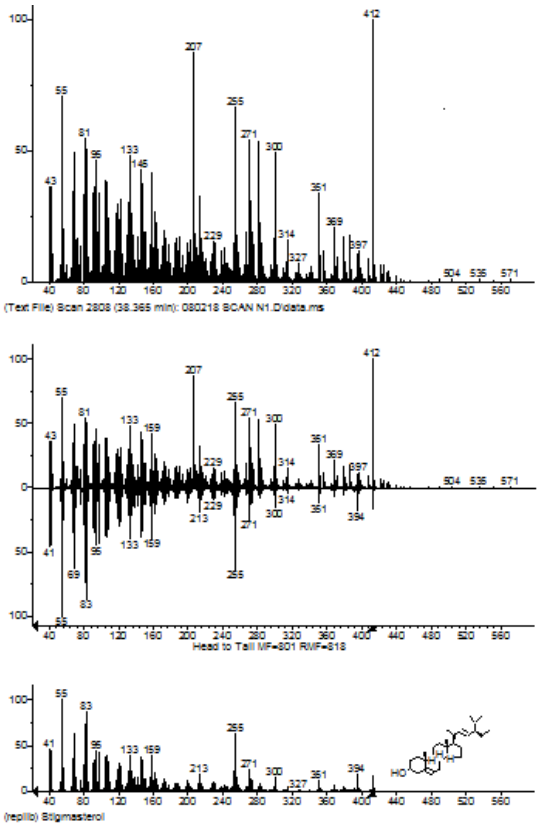


Figure 18: Mass spectrum of γ-Sitosterol with RT = 40.16

Based on the data analysis of GC/MS identified 45 bioactive compounds in the n-Hexane extract of *Azadirachta indica* leaves out of which 33 have antimicrobial and antifungal activity; Akpuaka *et al.* [21] found Oxime-, methoxy-phenyl-, Pentadecanoic acid, 14-methyl-, methyl ester and γ-Sitosterol in *Azadirachta indica* leaves. Furthermore, Al-Tamene *et al.* [29] and Altaee *et al.* [30] also found Oxime-, methoxy-phenyl-3, Oxime-, methoxy-phenyl-, Oxime-, methoxy-phenyl-2, in *Urtica dioica* leaves and Volatile Compounds produced by *Pseudomonas aeruginosa* Isolated from UTI Patients, respectively. Altaee *et al.* [30] showed that *Pseudomonas aeruginosa* produce many important secondary metabolites with high biological activities. Park *et al.* [31] used GC/MS analysis confirmed the predominant components of garlic extract to be 2-Hydroxy-gamma-butyrolactone in 75% ethanol extract was the most efficient in terms of the recovery rate and antimicrobial and antioxidant activities. Arora and Kumar [32] used GC–MS analyses root and stem of *Cenchrus setigerus* Vahl (Poaceae) showed that majority of these identified compounds in various crude extracts contain the high percentage of compounds that were identified in the crude extracts shows chemical and biological importance are bioactive compounds as 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione in methanolic and ethyl acetate extractant which had high antimicrobial. Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester was in thirty three bioactive compounds were identified in the methanolic extract of *Vitis vinifera* and it was evaluated high antimicrobal [33]. Al-Rubaye [34] used GC-MS analysis of *Malva sylvestris* revealed the existence of the bioactive compounds and Stigmasterol was found in these bioactive compounds. Seven bioactive compounds presented above, we discovered in the organic solvent aceton-methanol from *Penicillium citrinum* strain ND7a extract (ethyl acetate). Subramani *et al.* [35] reported a bioactive compound identified from the *Penicillium* sp. FF001 and this fungus was isolated from marine sponge at Fiji island; Citrinin isolated from sponge associated *Penicillium* sp. from this study and described antibiotic especially against Gram positive pathogens. Recently reported that citrinin was also active against Gram positive pathogens Gram negative pathogens and its antifungal properties [36]. However, we isolated and identified *Penicillium citrinum* strain ND7a from marine sponge at Ha Tien Sea, Vietnam but we did not find citrinin as bioactive compound from *Penicillium citrinum* strain ND7a.

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4. Conclusion

In the present study sixteen compounds from the ethyl acetate extract of *Penicillium citrinum* strain ND7a extract were identified by Gas-chromatography–Mass spectrometry (GC-MS) analysis in two kinds of organic solvent (hexan-aceton and aceton-methanol). The biological activities of each of the identified components range from antimicrobial, antioxidant and antitumoral activities. The nature of the identified compounds are mostly organic acids. 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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