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Full Length Research Paper

Determination of metabolites products by *Penicillium expansum* and evaluating antimicobial activity

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The objectives of this study were to analyze the secondary metabolites of *Penicillium expansum* and evaluate antibacterial activity. Twenty eight bioactive compounds were identified in the methanolic extract of *P. expansum*. The identification of bioactive chemical compounds is based on the peak area, retention time molecular weight and molecular formula. Gas chromatography mass spectrometry (GC/MS) analysis of *P. expansum* revealed the existence of the Levoglucosenone, Edulan II, 4-[Dichloromethyl]-2-[[2-[1-methyl-2-pyrrolidinyl]ethyl]amino-6-trichloro, 1,2-Cyclopentanedione, Ethanethiol, 2-(5-(4-methyl-2-pyridyloxy)pentyl)amino-hydrogen su, Imidazole,2-amino-5-[(2-carboxy)vinyl], D-Glucose,6-O-α-D-galactopyranosyl, Eicosanoic acid , phenylmethyl ester, Dodecanoic acid, 3- hydroxyl, DL-Leucine,N-glycyl, Cyclohexene,1,5,5-trimethyl-6-acetylmethyl, 1,2-Nonadecanediol, Bicyclo[2.2.1]heptane-2-carboxylic acid isobutyl-amide, 6-Acetyl-ß-d-mannose, α-D-Glucopyranoside,O-α-D-glucopyranosyl-(1.fwdarw.3)-ß-D-fruc, propanedioic acid, amino-diethyl ester, 4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl, valeric acid, dodecyl ester, deoxyspergualin, l-Gala-l-ido-octonic lactone, 5-Hydroxymethylfurfural, paromomycin, 16-Nitrobicyclo[10.4.0]hexadecane-1-ol-13-one, cis-Vaccenic acid, 2-Bromotetradecanoic acid, phthalic acid, butyl undecyl ester, picrotoxin, D-Fructose and diethyl mercaptal. The FTIR analysis of *P. expansum* proved the presence of aliphatic fluoro compounds, tetiary amine, C-N stretch and methylene-CH. asym which shows major peaks at 1028.06, 1151.50 and 2852.72, respectively. Methanolic extract of bioactive compounds of *P. expansum* were assayed for *in vitro* antibacterial activity against *Proteus mirabilis, Pseudomonas aerogenosa, Escherichia coli*, *Staphylococcus aureus* and *Klebsiella pneumonia* by using the diffusion method in agar. The zones of inhibition were compared with different standard antibiotics. *S. aureus* has maximum zone formation (7.01±0.141) mm.

Key words: Antimicrobial activity, metabolites, bioactive compounds, GC-MS, FT-IR, *P. expansum*.

INTRODUCTION

The genus *Penicillium* is known worldwide for the production of secondary metabolites (Pitt and Hocking, 1997). *Penicillium expansum* has been demonstrated to produce extracellular enzymes of commercial value, including the pectinases, utilized in fruit juice industry during the stage of pulp maceration, juice liquefaction or depectinization (Rosenberger et al., 1991; Baracat-Pereira et al., 1989), Patulin, a mutagenic, immunoitoxic

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and neurotoxic mycotoxin, particularly unacceptable to apple juice industry (Bracket and Marth, 1979). Most *Penicillium* species are considered ubiquitous, opportunistic saprophytes. Blue mold decay caused by *P. expansum* link is the most important postharvest disease of apple worldwide (Pierson et al., 1971). The genus *Penicillium* is subdivided into four subgenera (*Aspergilloides, Penicillium, Biverticillium* and *Furcatum*), determined by the number of branch points between phialide and stipe, down the main axis of the penicillus and others characters, like ratio of metula length to phialide, length and colony diameter on G25N and when the number of branch points is the same (Grassin and Fauquembergue, 1996).

Nutritionally, they are supremely undemanding being able to grow in almost any environment with a sprinkling of mineral salts, any but with the most complex forms of organic carbon, and a wide range of physical-chemical environments, temperature, pH and redox potential. The taxonomy of this genus is hard as its classification is based mainly on conidiophore and conidia structure, although the colony diameter after incubation under standardized conditions has greater importance for classification (Amiri and Bompeix, 2005; Morales et al., 2008; Hameed et al., 2015a). *P. expansum* is one of most important fungal pathogen of stored pome fruits and responsible for 50% of losses in all pome fruit and pear (Mattheis and Roberts, 1992; Andersen et al., 2004; Murphy et al., 2006; Hameed et al., 2015b).

It is reported to produce different secondary metabolites such as expansolides A and B (Massias et al., 1990), citrinin, ochratoxin A, chaetoglobosins A and C (Frisvad, 1992), rubratoxin B (Paterson et al., 1987; Hameed et al., 2015), roquefortine C, penitrem A (Bridge et al., 1989), patulin (Andersen et al., 2004; Altameme et al., 2015) and others like cyclopiazonic acid, brevianamide A, gentisyl alcohol, griseofulvin, mycophenolic acid and raistrick phenols (Frisvad and Filtenborg., 1989). The aims of this study were to analyze the secondary metabolites and evaluation of antibacterial activity.

MATERIALS AND METHODS

Collection, extraction and determination of metabolites

P. expansum was isolated from dried fruit. After the species were identified by the identification key, spores were grown in a liquid culture of potato dextrose broth (PDB) and incubated at 25°C in a shaker for 16 days at 130 rpm (Usha and Masilamani, 2013). The metabolites were determined and extracted for GC analysis using the method of Jasim et al. (2015). The extraction was performed by adding 25 ml methanol to 100 ml liquid culture in an Erlenmeyer flask after the infiltration of the culture. The mixture was incubated at 4°C for 10 min and then shook for 10 min at 130 rpm. Metabolites was separated from the liquid culture and evaporated to dryness with a rotary evaporator at 45°C. The residue was dissolved in 1 ml methanol, filtered through a 0.2 µm syringe filter, and stored at 4°C for 24 h before being used for GC-MS.

Gas chromatography-mass spectrometry (GC-MS)

Bioactive compound were examined for the chemical composition using GC-MS (Agilent 789N) equipped with a DB-5MS column (30 m×0.25 mm i.d., 0.25 um film thickness, J&W Scientific, Folsom, CA). The oven temperature was programmed (Imad et al., 2014a; Hussein et al., 2015). Helium was used as the carrier gas at the rate of 1.0 ml/min. Effluent of the GC column was introduced directly into the source of the MS via a transfer line (250°C). Ionization voltage was 70 eV and ion source temperature was 230°C. Scan range was 41 to 450 amu. The constituents were identified after being compared with available data in the GC-MS library in the literatures of Kareem et al. (2015) and Imad et al. (2014b).

Fourier transform infrared spectrophotometer (FTIR)

The sample was run at infrared region between 400 and 4000 nm. The powdered sample of the *P. expansum* specimen was treated for fourier transform infrared spectroscopy (Shimadzu, IR Affinity 1, Japan) (Imad et al., 2014c).

Determination of antibacterial activity of crude fraction of *P. expansum* compounds.

The test pathogens (*E. coli, Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Staphylococcus aureus)* were swabbed in Muller Hinton agar plates. 50µl of fungal extracts was loaded on the bored wells. The wells were bored in 0.5 cm in diameter. The plates were incubated at 37°C for 24 h and examined. After the incubation the diameter of inhibition zones around the discs was measured.

Statistical analysis

Data were analyzed using analysis of variance (ANOVA), and differences among the means were determined for significance at $P < 0.05$ using Duncan's multiple range test (by SPSS software)Version 9.1 (Mohammed and Imad, 2013)

RESULTS AND DISCUSSION

Fungus identification and secondary metabolites production

The fungi were isolated by serial dilution method. Morphological, microscopical and microscopical characteristics of fungal strains were determined using specific media light and compound microscope (Figures 1 and 2). After fermentation, the secondary metabolites were produced by isolated microorganisms.

Identify the secondary metabolites from the methanolic crude extract of *P. expansum* by (GC-MS)

Gas chromatography and mass spectroscopy analysis of compounds was carried out in methanolic extract of *P. expansum*, shown in Table 1. The GC-MS chromatogram of the twenty eight peaks of the compounds detected was shown in Figure 2. Chromatogram GC-MS analysis of the

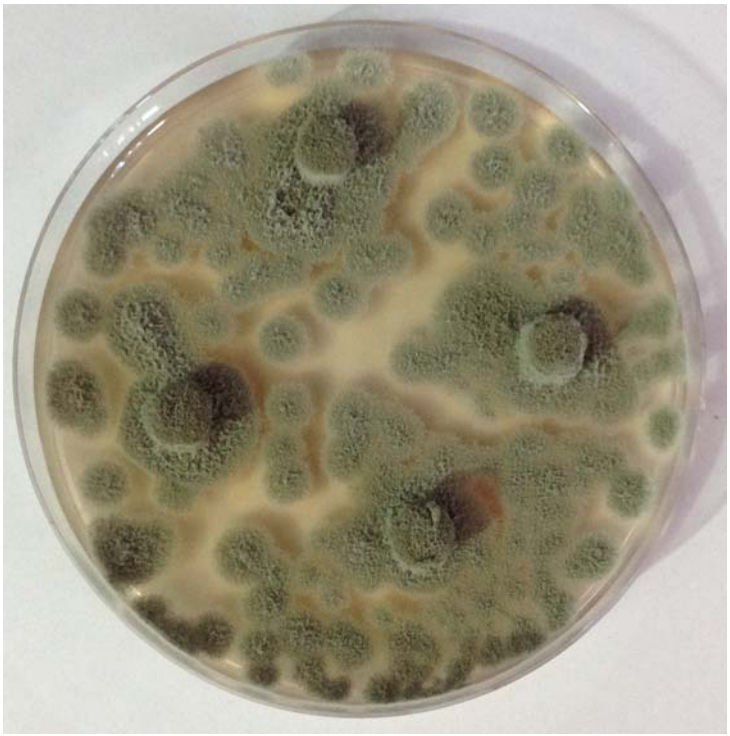


Figure 1. Morphological characterization of *P. expansum*.

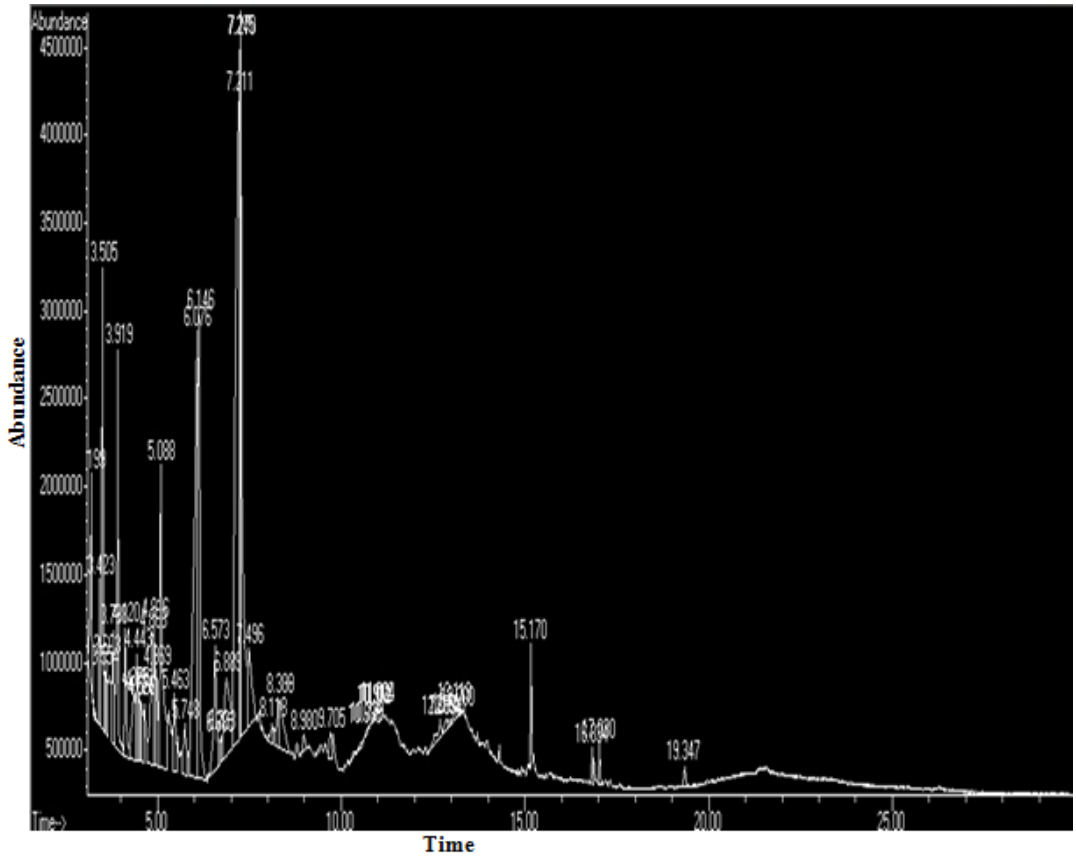


Figure 2. GC-MS chromatogram of methanolic extract of *P. expansum.*

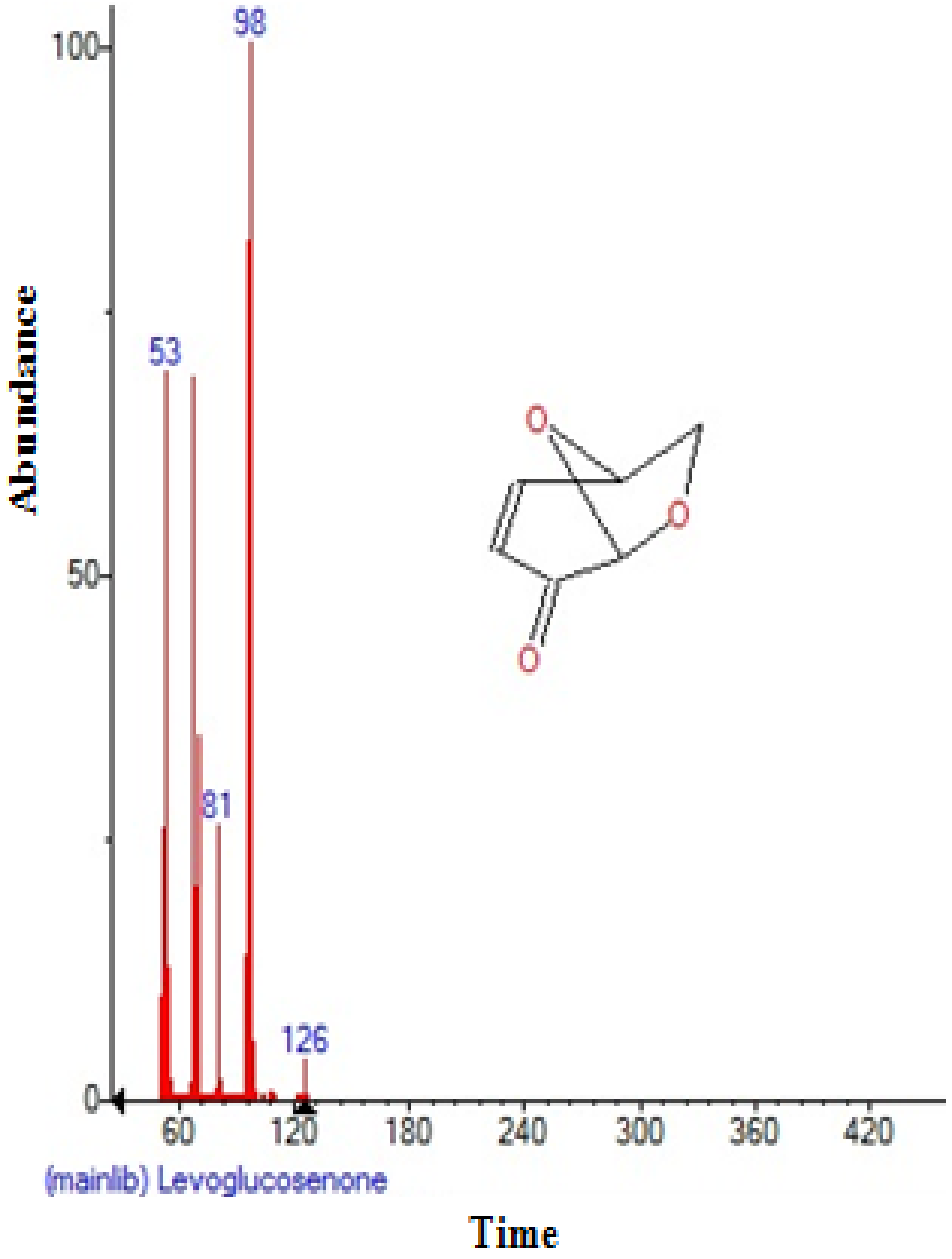


Figure 3. Mass spectrum of Levoglucosenone with Retention Time (RT)= 3.230.

methanol extract of *P. expansum* showed the presence of twenty eight major peaks, and the components corresponding to the peaks were determined as follows. The first set up peak was determined to be levoglucosenone, Edulan II, (Figure 3). The second peak indicated to be 4-[Dichloromethyl]-2-[[2-[1-methyl-2-pyrrolidinyl]ethyl]amino-6-trichloro (Figure 4). The next peaks were considered to be 1,2-Cyclopentanedione, ethanethiol, 2-(5-(4-methyl-2-pyridyloxy)pentyl) amino,hydrogen su, imidazole, 2-amino-5-[(2-carboxy)vinyl], D-Glucose,6-O-α-D-galactopyranosyl, eicosanoic acid, phenylmethyl ester, dodecanoic acid, 3-hydroxyl, DL-Leucine, N-glycyl, Cyclohexene,1,5,5-trimethyl-6-acetylmethyl, 1,2-Nonadecanediol, Bicyclo[2.2.1]heptane-2-carboxylic acid isobutyl-amide, 6-Acetyl-ß-d-mannose, α-D-Glucopyranoside,O-α-D-glucopyranosyl-(1.fwdarw.3)-ß-D-fruc, Propanedioic acid, amino-, diethyl ester, 4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl, valeric acid, dodecyl ester, deoxyspergualin, I-Gala-I-ido-octonic lactone, 5-Hydroxymethylfurfural, Paromomycin, 16-Nitrobicyclo [10.4.0]hexadecane-1-ol-13-one, cis-Vaccenic acid, 2-

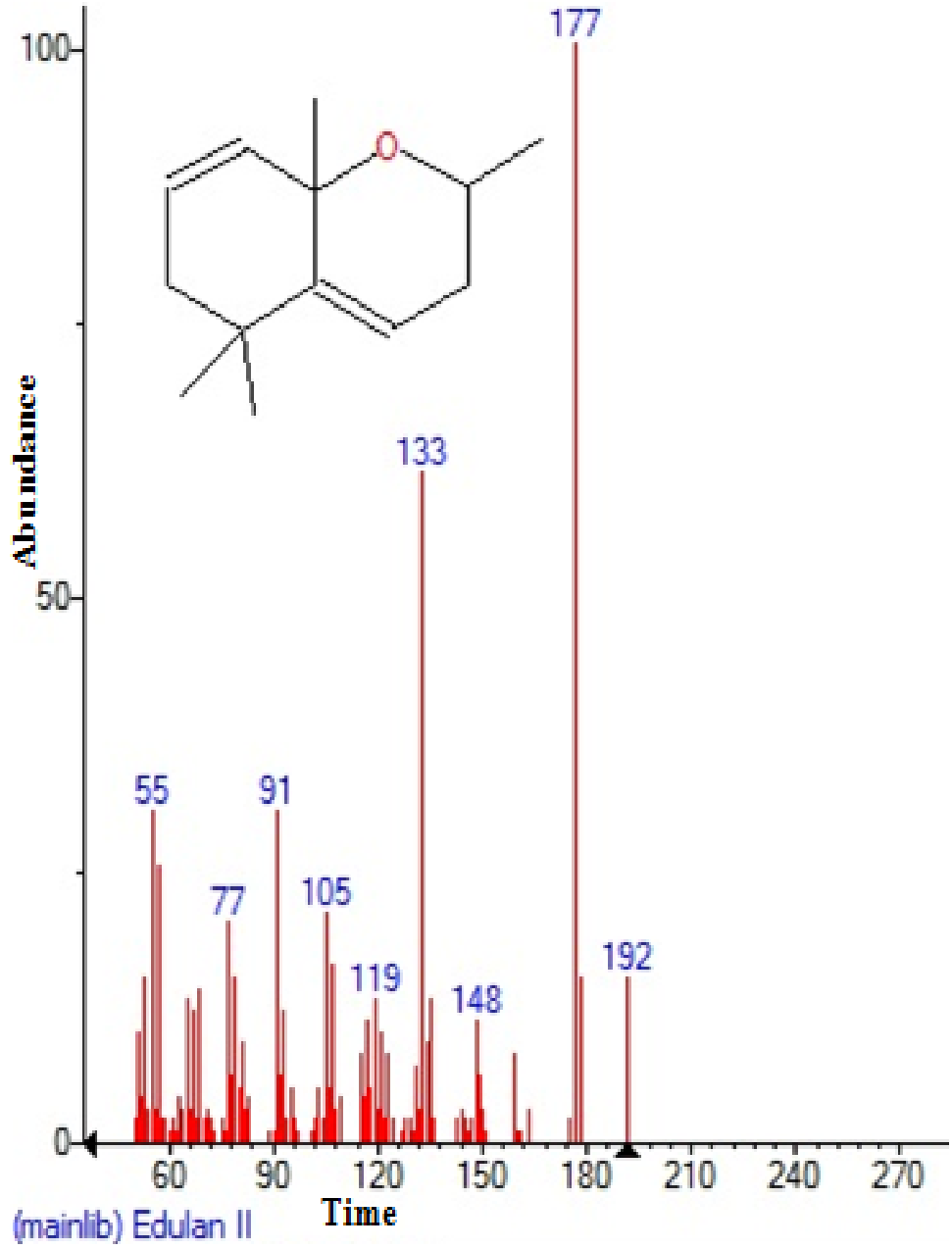


Figure 4. Mass spectrum of Edulan II with retention time (RT)= 3.613.

Bromotetradecanoic acid, Phthalic acid, butyl undecyl ester, Picrotoxin, D-Fructose and diethyl mercaptal (Figure 5 to 30).

Identify the secondary metabolites from the methanolic crude extract of *P. expansum* by (FTIR)

Fourier-transform infrared analysis of dry methanolic extract of *P. expansum* proved the presence of aliphatic fluoro compounds, tetiary amine, C-N stretch and methylene-CH. asym which shows major peaks at 1028.06, 1151.50 and 2852.72, respectively (Table 2 and Figure 31).

Antibacterial activity

K. pneumoniae, Pseudomonas aeroginosa, E. coli and *S.aeureus* are four clinical pathogens selected forantibacterial activity, and maximum zone formation against *S. aureus* (Table 3 and Figure 32).

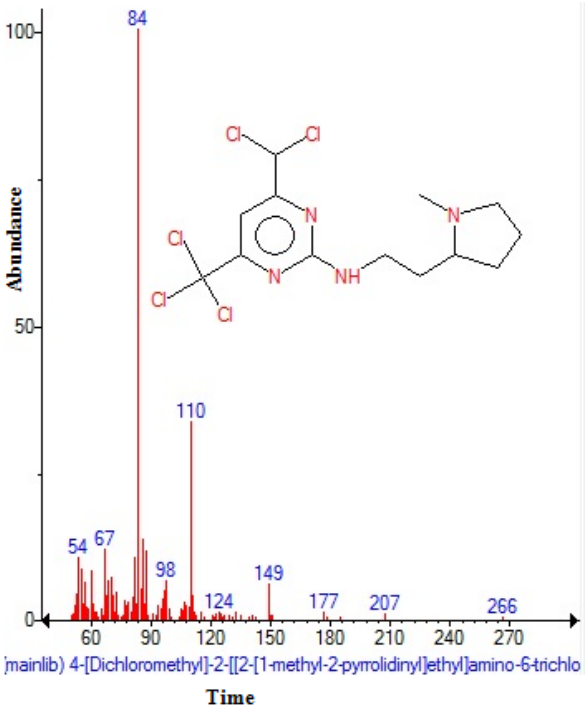


Figure 5. Mass spectrum of 4-[Dichloromethyl]-2-[[2-[1-methyl-2-pyrrolidinyl]ethyl]amino-6-trichloro with retention time (RT)= 3.693.

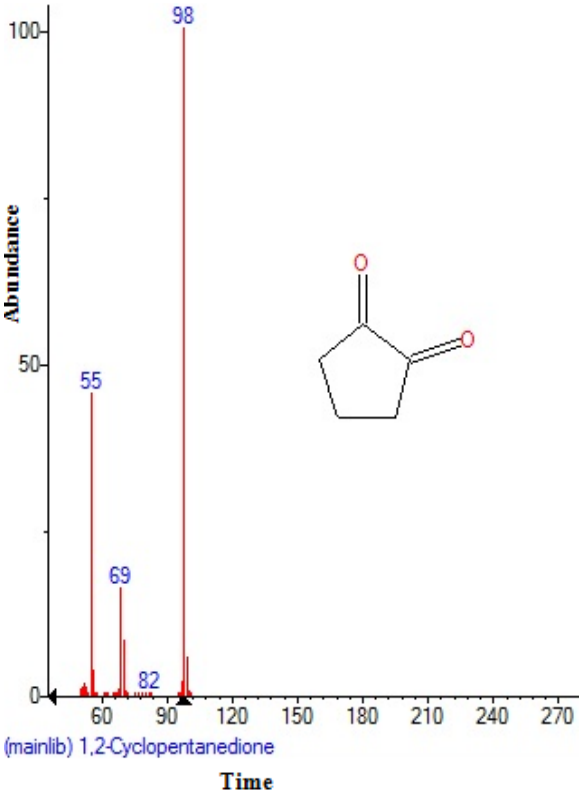


Figure 6. Mass spectrum of 1,2-Cyclopentanedione with retention time (RT)= 3.750.

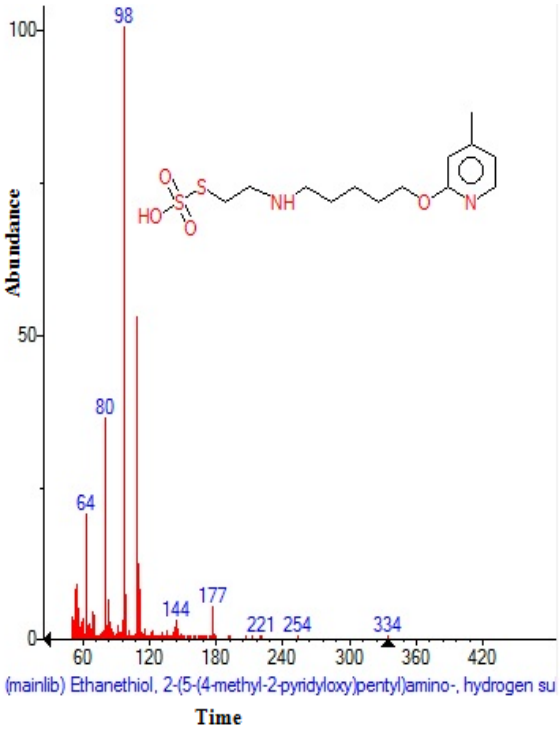


Figure 7. Mass spectrum of Ethanethiol , 2-(5-(4-methyl-2-pyridyloxy)pentyl)amino-,hydrogen su with retention time (RT)= 3.779.

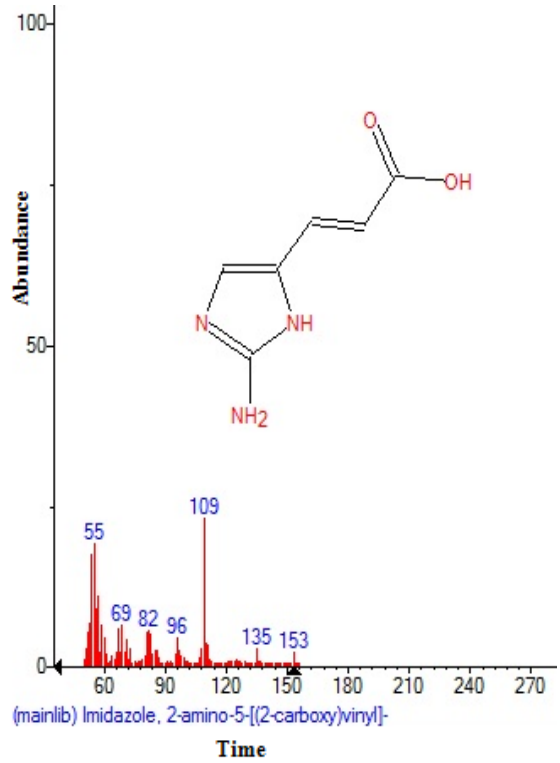


Figure 8. Mass spectrum of Imidazole,2-amino-5-[(2-carboxy)vinyl] with retention time (RT)= 3.859.

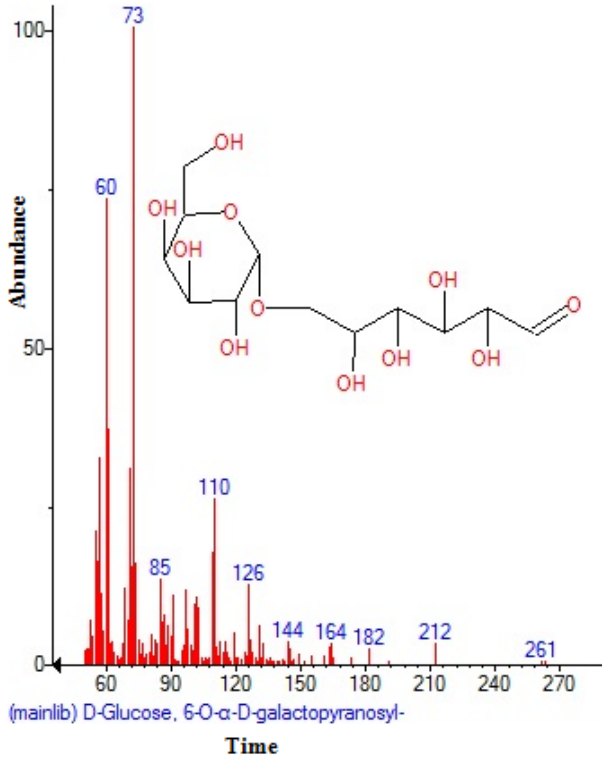


Figure 9. Mass spectrum of D-Glucose,6-O-α-D-galactopyranosyl with retention time (RT)= 3.997.

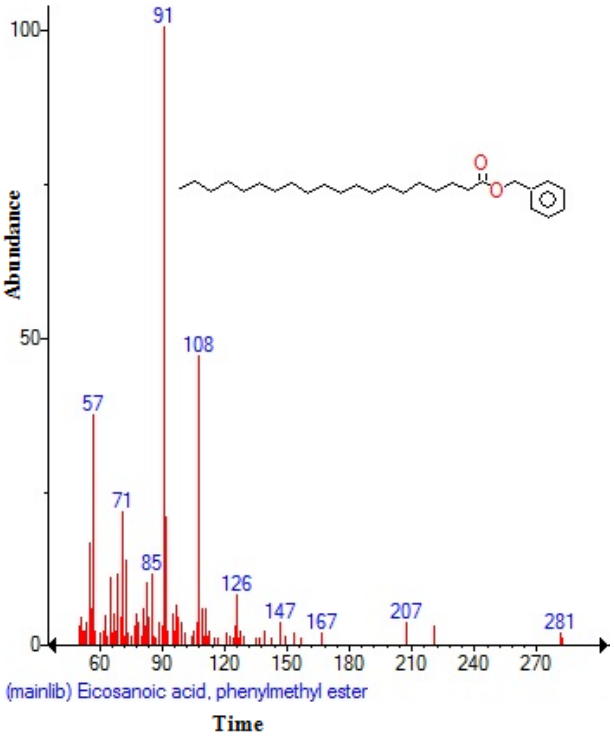


Figure 10. Mass spectrum of eicosanoic acid, phenylmethyl ester with retention time (RT)= 4.546.

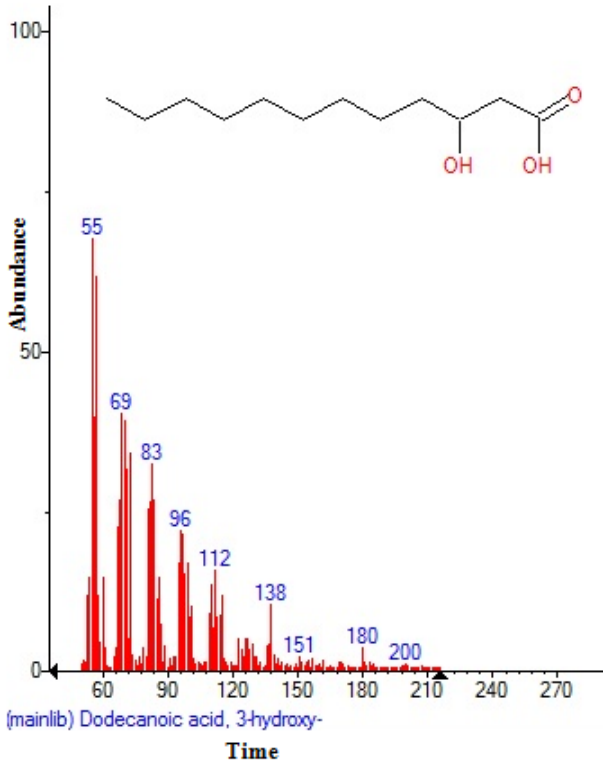


Figure 11. Mass spectrum of dodecanoic acid , 3-hydroxyl with retention time (RT)= 4.626.

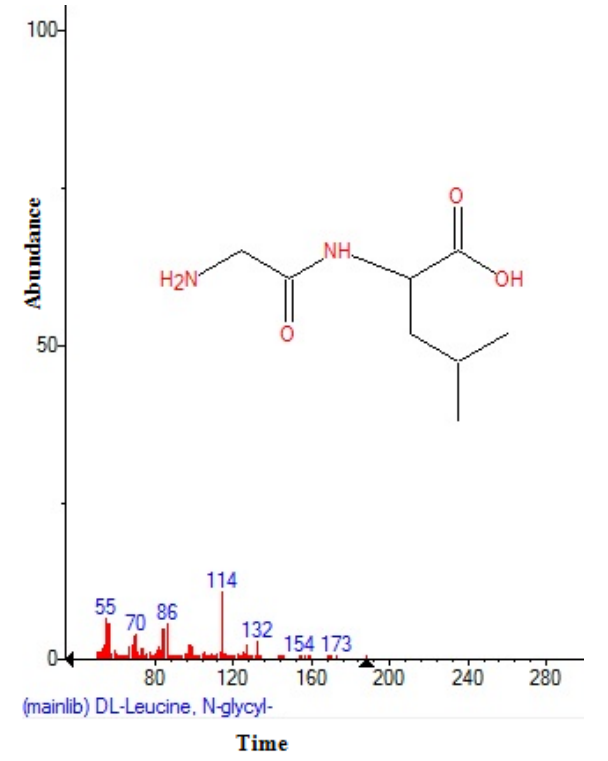


Figure 12. Mass spectrum of DL-Leucine,N-glycyl with retention time (RT)= 4.763.

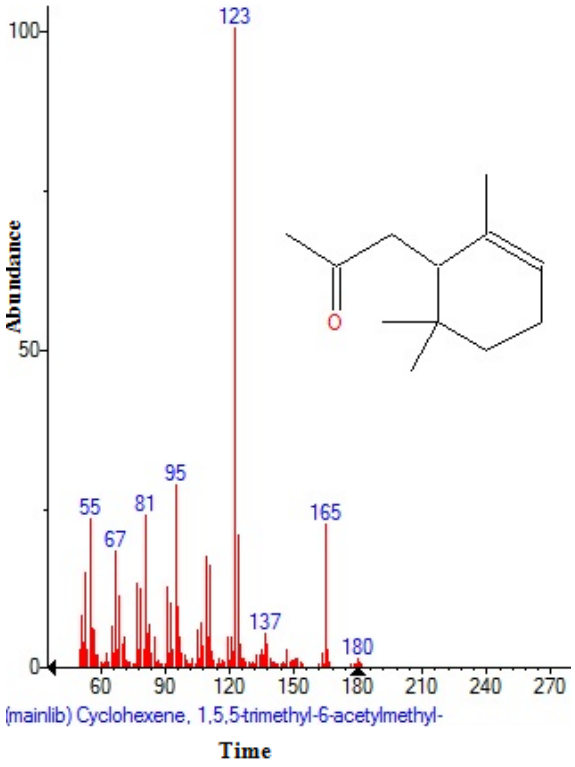


Figure 13. Mass spectrum of cyclohexene,1,5,5-trimethyl-6-acetylmethyl with retention time (RT)= 5.124.

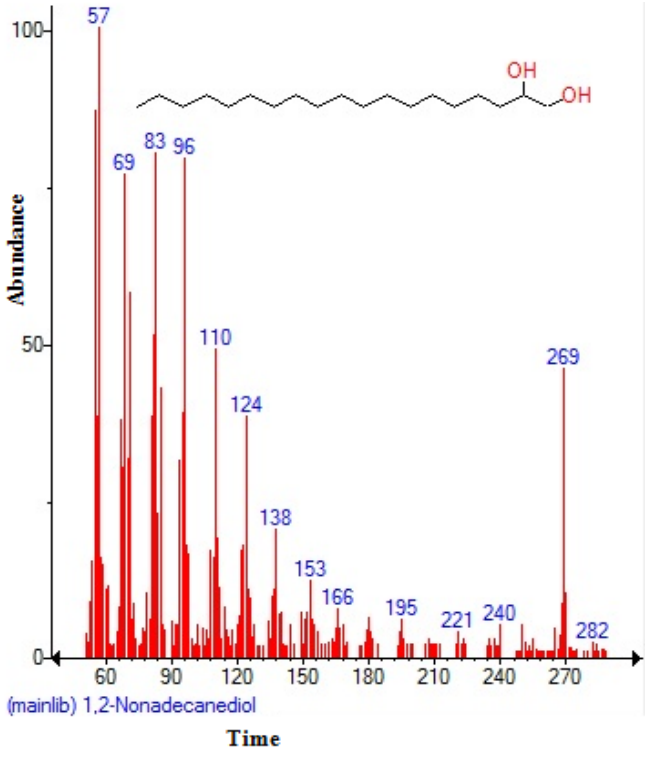


Figure 14. Mass spectrum of 1,2-Nonadecanediol with retention time (RT)= 5.095.

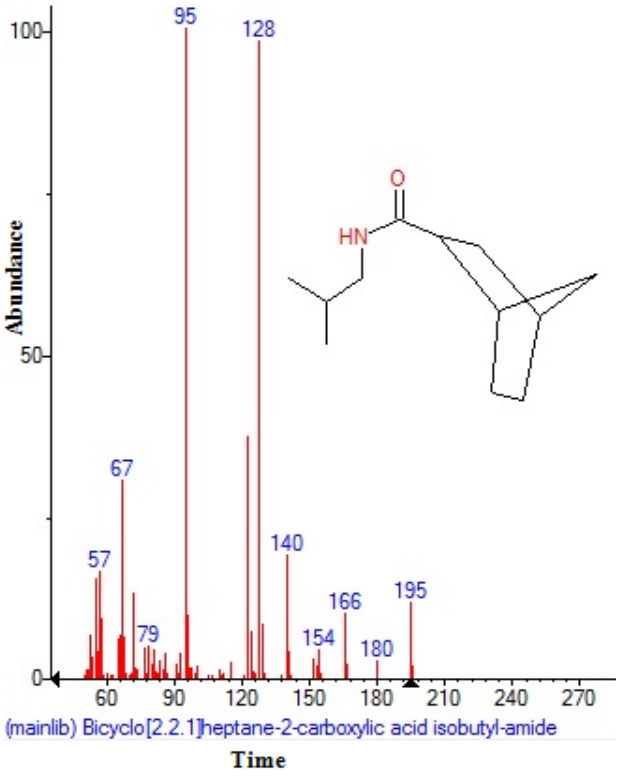


Figure 15. Mass spectrum of bicyclo[2.2.1]heptane-2-carboxylic acid isobutyl-amide with retention time (RT)= 5.232.

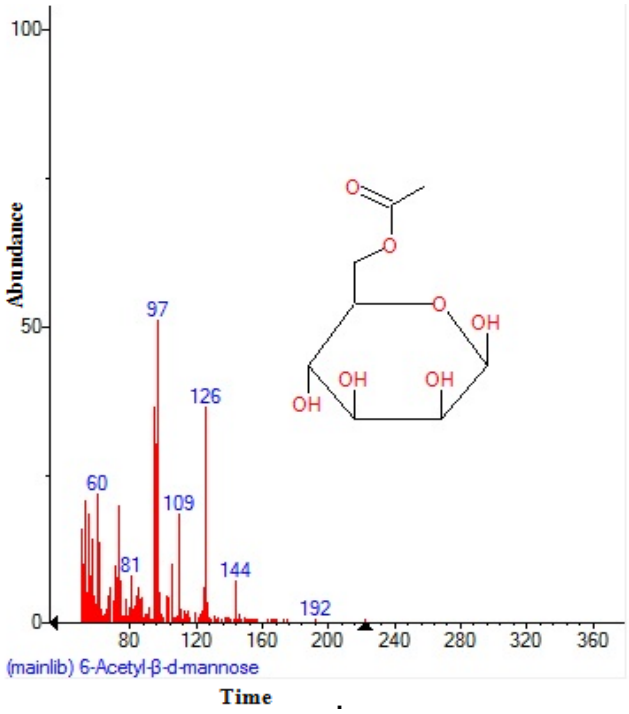


Figure 16. Mass spectrum of 6-Acetyl-ß-d-mannose with retention time (RT)= 5.536.

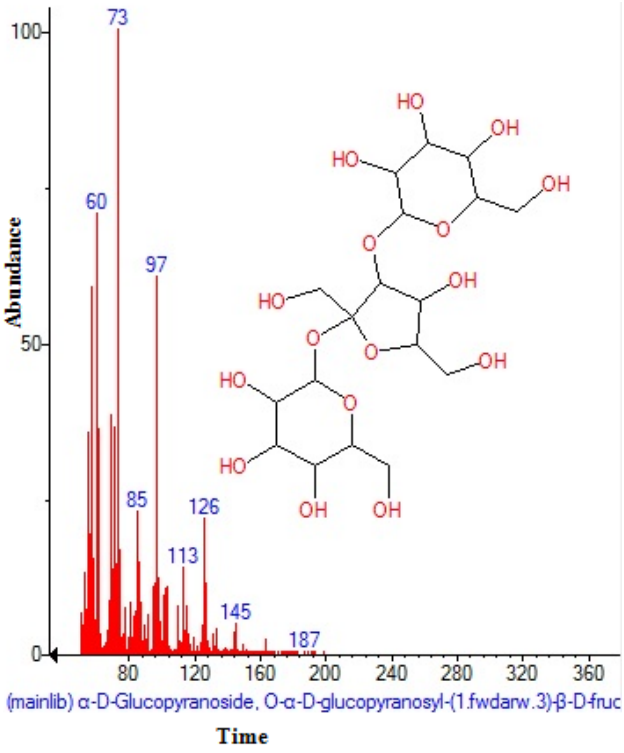


Figure 17. Mass spectrum of α-D-Glucopyranoside,O-α-D-glucopyranosyl-(1.fwdarw.3)-ß-D-fruc with retention time (RT)= 5.805.

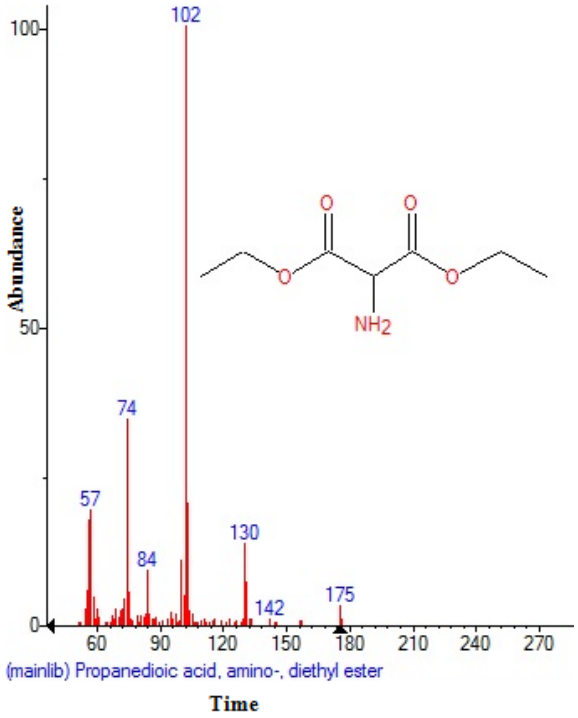


Figure 18. Mass spectrum of Propanedioic acid , amino -, diethyl ester with retention time (RT)= 5.862.

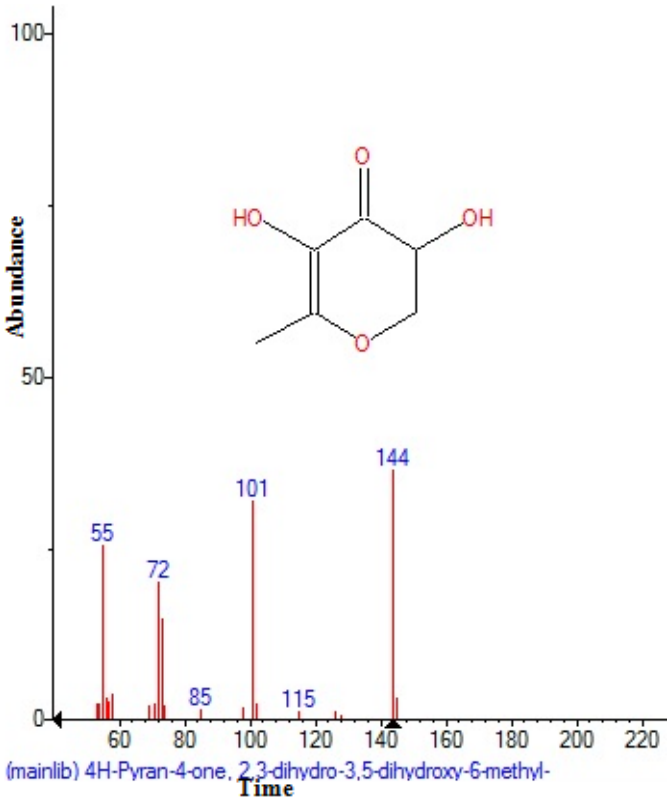


Figure 19. Mass spectrum of 4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl with retention time (RT)= 5.942.

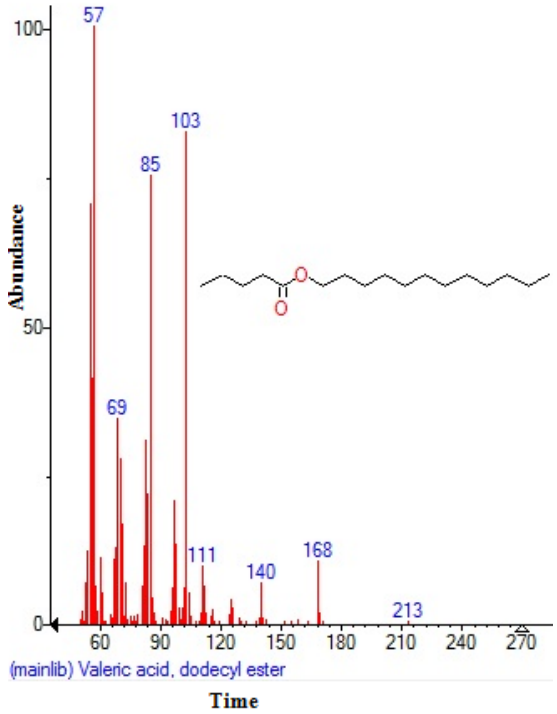


Figure 20. Mass spectrum of valeric acid, dodecyl ester with retention time (RT)= 6.600.

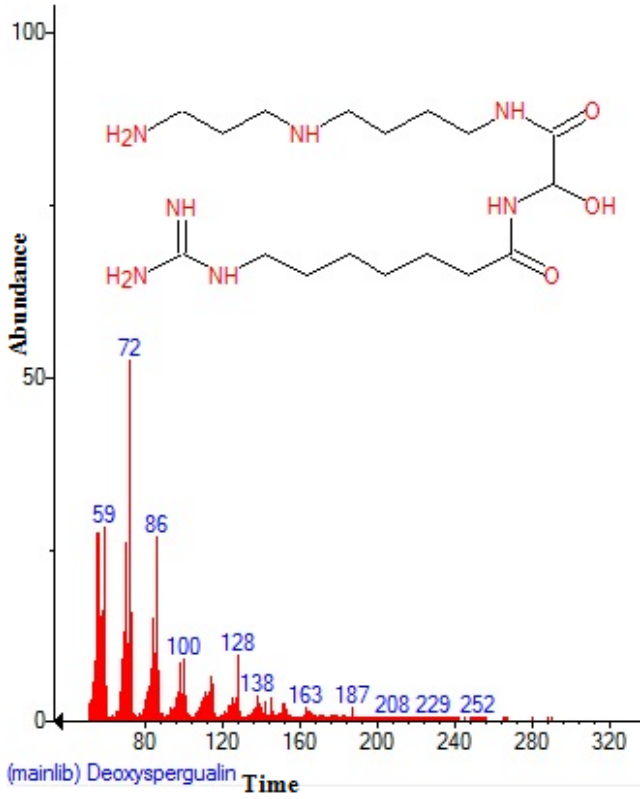


Figure 21. Mass spectrum of Deoxyspergualin with retention time (RT)= 6.852.

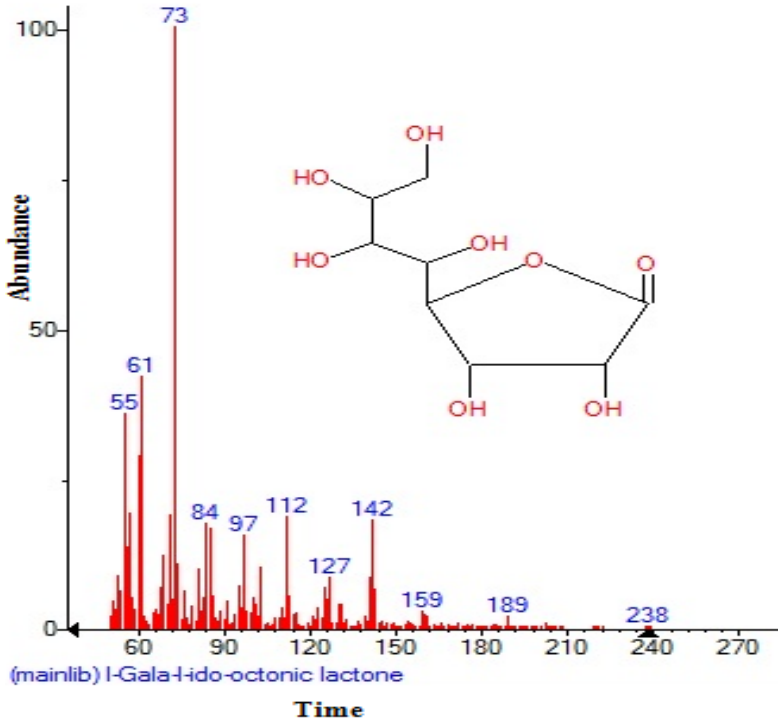


Figure 22. Mass spectrum of l-Gala-l-ido-octonic lactone with retention time (RT)= 6.766.

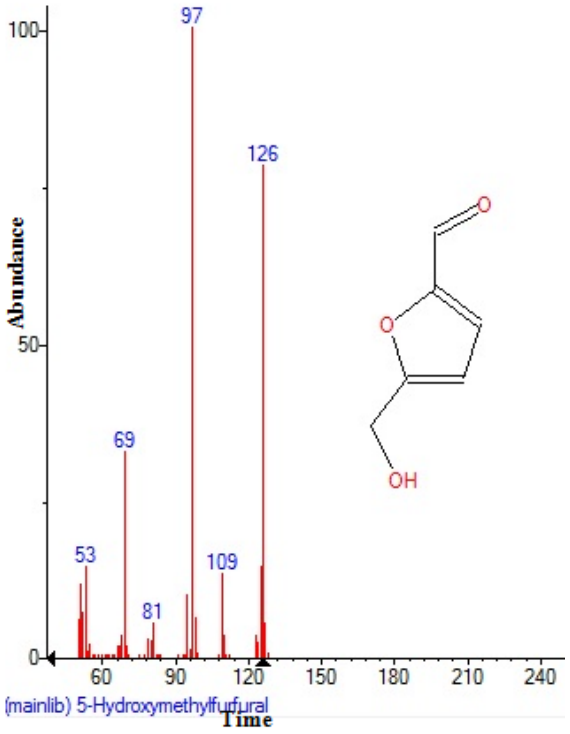


Figure 23. Mass spectrum of 5-Hydroxymethylfurfural with retention time (RT)= 6.983.

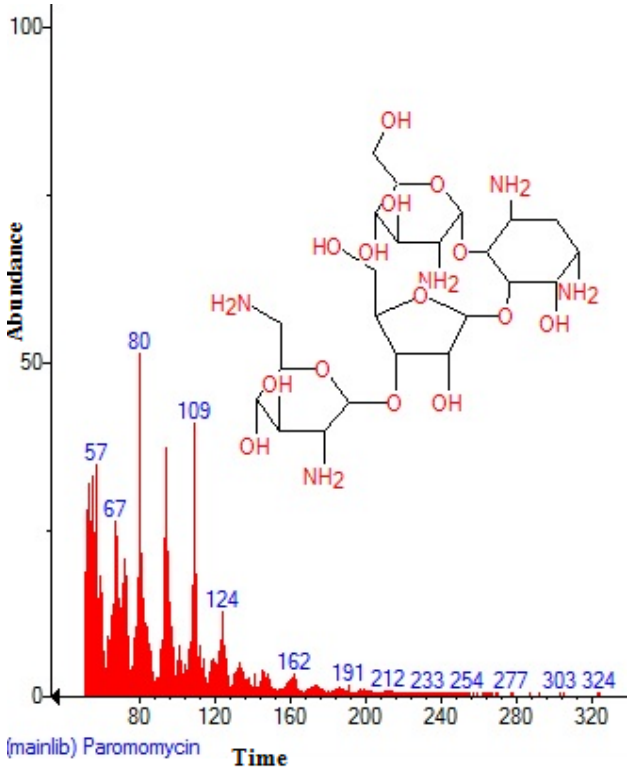


Figure 24. Mass spectrum of paromomycin with retention time (RT)= 7.807.

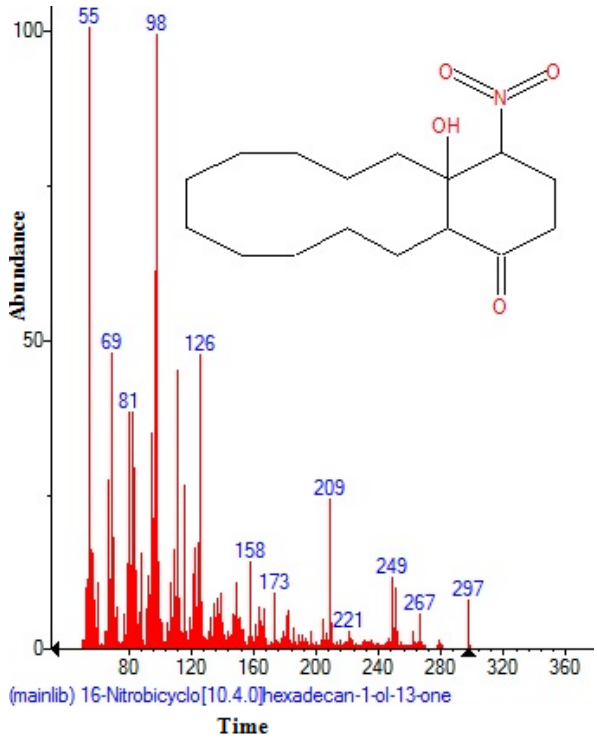


Figure 25. Mass spectrum of 16-Nitrobicyclo[10.4.0]hexadecane-1-ol-13-one with retention time (RT)= 8.797.

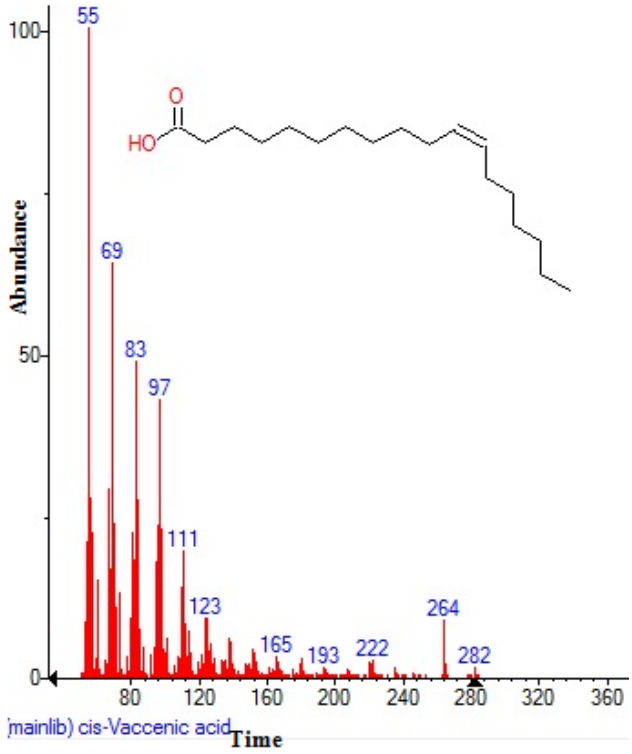


Figure 26. Mass spectrum of Cis-Vaccenic acid with retention time (RT)= 10.085.

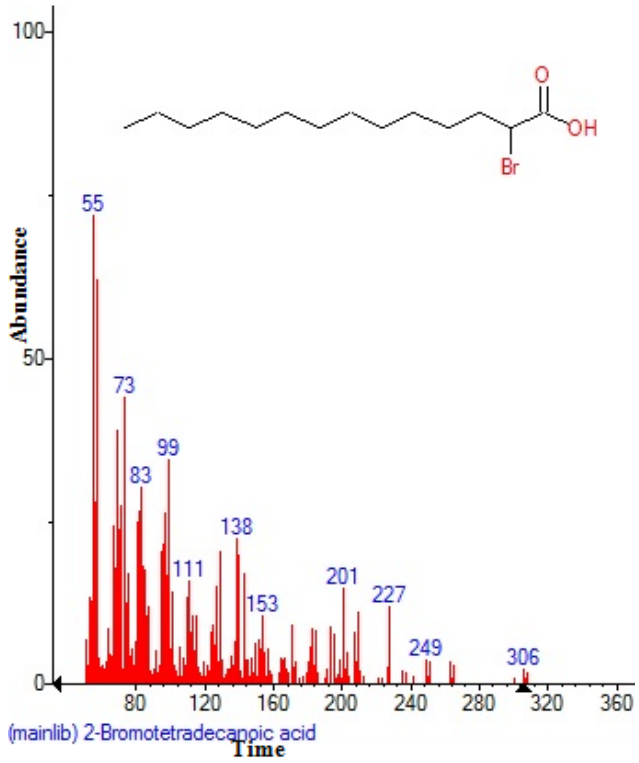


Figure 27. Mass spectrum of 2-Bromotetradecanoic acid with retention time (RT)= 11.567.

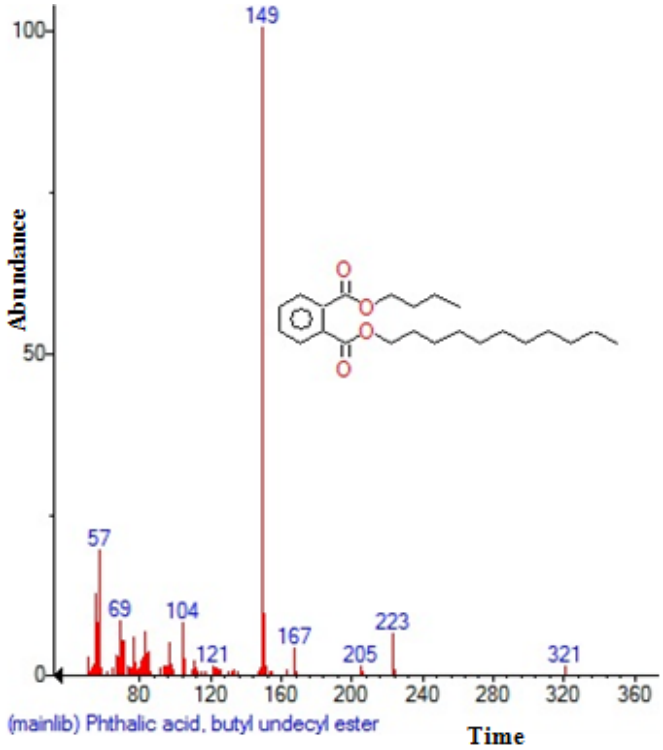


Figure 28. Mass spectrum of Phthalic acid , butyl undecyl ester with retention time (RT)= 14.279.

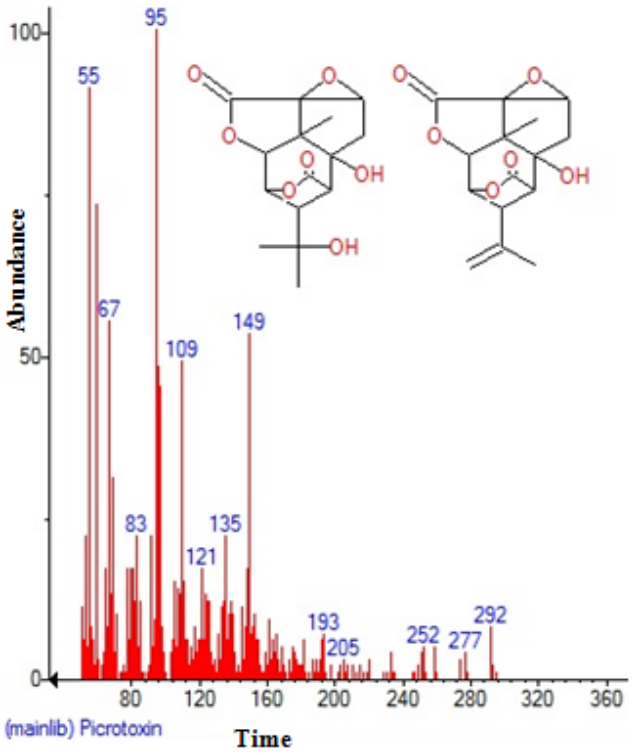


Figure 29. Mass spectrum of picrotoxin with retention time (RT)= 14.582.

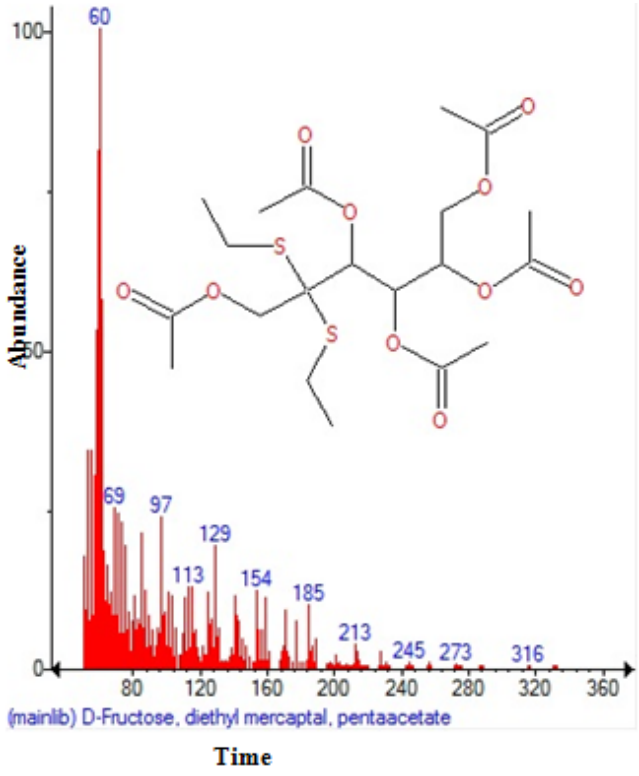


Figure 30. Mass spectrum of D-Fructose, diethyl mercaptal and pentaacetate with retention time (RT)= 15.046.

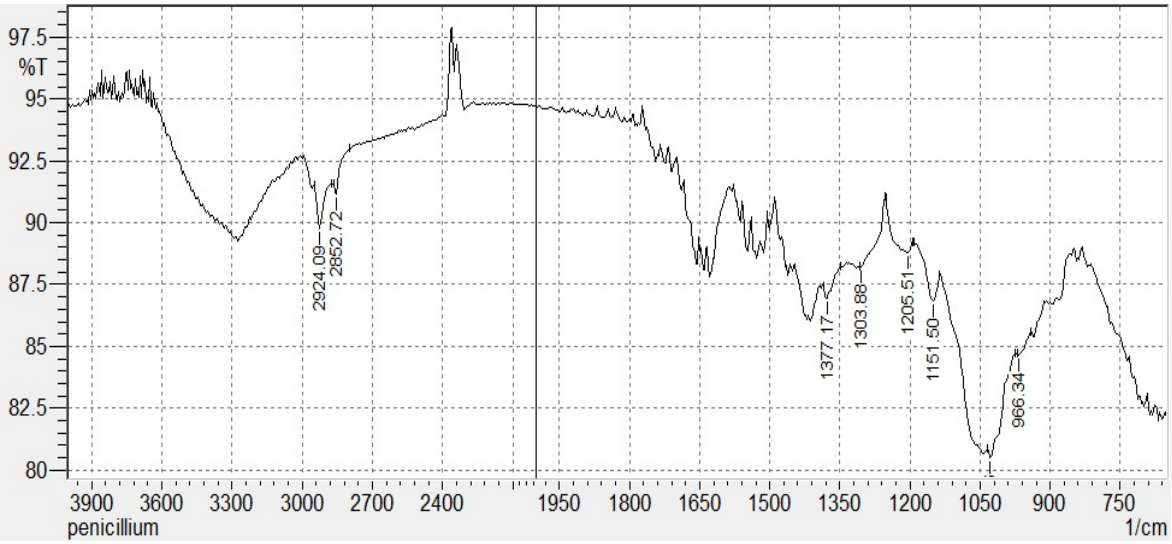


Figure 31. Fourier-transform infrared spectroscopy peak values of *P. expansum.*

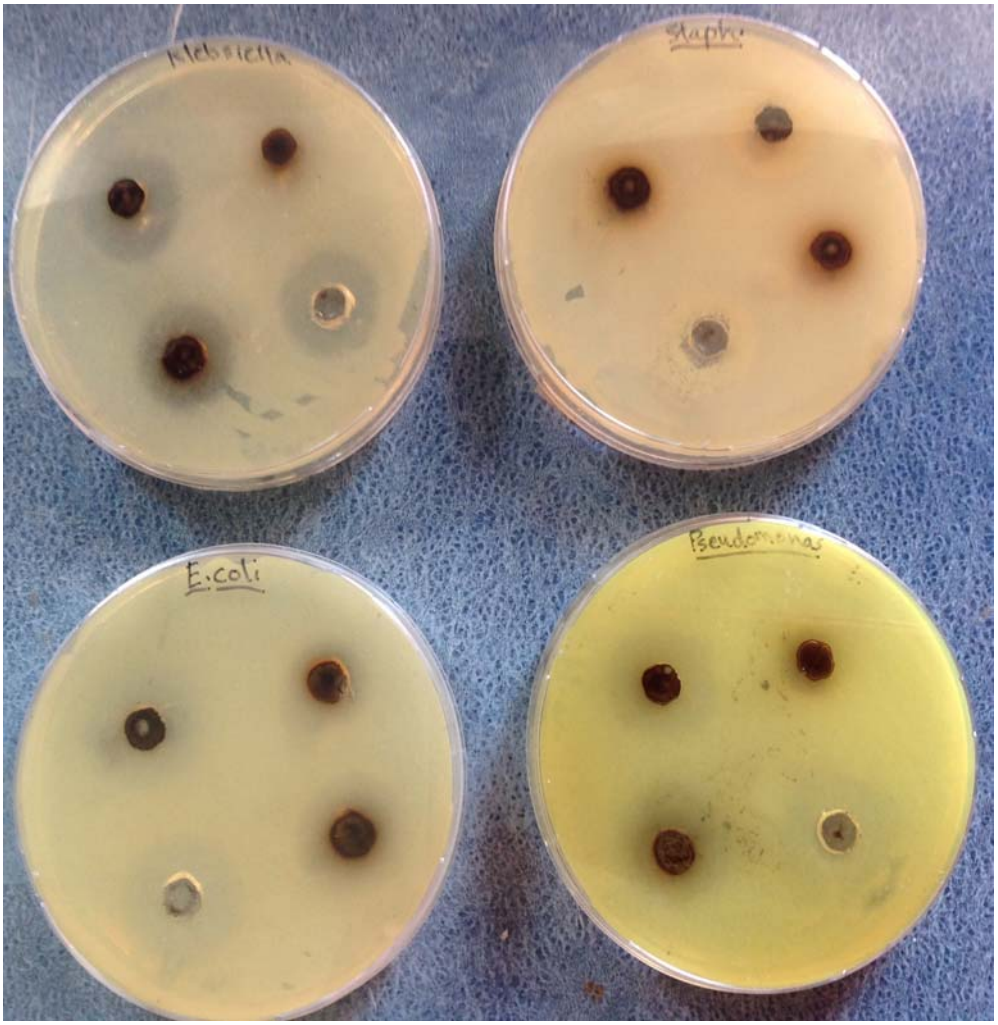


Figure 32. Antimicrobial activity of *P. expansum.*

Table 1. Bioactive chemical compounds identified in methanolic extract of *P. expansum*.

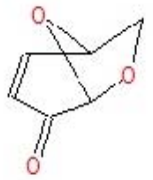
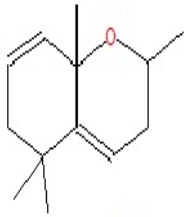
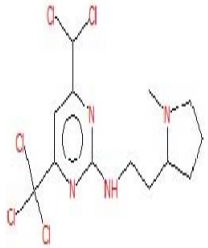
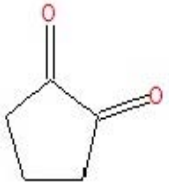
S/N	Phytochemical compound	RT (min)	Formula	Molecular weight	Exact mass	Chemical structure	MS Fragment- ions
1.	Levoglucosenone	3.230	$C_6H_6O_3$	126	126.031694		53,81,98,126
2.	Edulan II	3.613	$C_{13}H_{20}O$	192	192.151415		55,77,91,105,119,133,148,177,192
3.	4-[Dichloromethyl]-2-[[2-[1-methyl-2-pyrrolidinyl]ethyl]amino-6-trichloro	3.693	$C_{13}H_{17}Cl_{15}N_4$	403	403.989586		54,67,84,98,110,124,149,177,207,266
4.	1,2-Cyclopentanedione	3.750	$C_5H_6O_2$	98	98.0367794		55,69,82,98

Table 1. Cont'd.

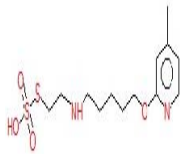
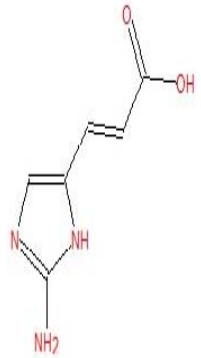
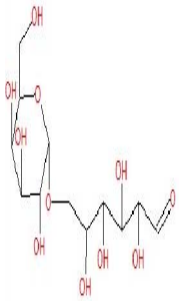
5.	Ethanethiol , 2-(5-(4-methyl-2-pyridyloxy)pentyl)amino-,hydrogen su	3.779	$C_{13}H_{22}N_2O_4S_2$	334	334.102098		64,80,98,144,177,221,254,334
6.	Imidazole,2-amino-5-[(2-carboxy)vinyl]	3.859	$C_6H_7N_3O_2$	153	153.053826		55,69,82,96,109,135,153
7.	D-Glucose,6-O-α-D-galactopyranosyl	3.997	$C_{12}H_{22}O_{11}$	342	342.11621		60,73,85,110,126,144,164,182,212,261
8.	Eicosanoic acid , phenylmethyl ester	4.546	$C_{27}H_{26}O_2$	402	402.349781		57,71,85,91,108,126,147,167,207,281

Table 1. Cont'd.

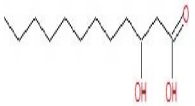
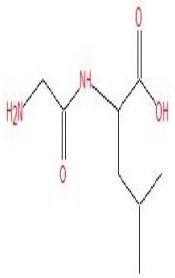
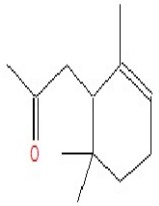
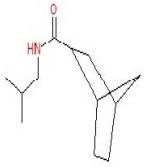
9.	Dodecanoic acid , 3- hydroxyl	4.626	$C_{12}H_{24}O_3$	216	216.1725445		55,69,83,96,112,138,151,180,200
10.	DL-Leucine,N-glycyl	4.763	$C_8H_{16}N_2O_3$	188	188.116093		55,70,86,114,132,154,173
11.	Cyclohexene,1,5,5-trimethyl-6-acetylmethyl	5.124	$C_{12}H_{20}O$	180	180.151415		55,67,81,95,123,137,165,180
12.	1,2-Nonadecanediol	5.095	$C_{19}H_{20}O_2$	300	300.30283		57,69,83,96,110,124,138,153,166,195,221,240,269,282
13.	Bicyclo[2.2.1]heptane-2-carboxylic acid isobutyl-amide	5.232	$C_{12}H_{21}NO$	195	195.162314		57,67,79,95,128,140,154,166,180,195

Table 1. Cont'd.

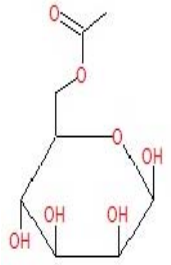
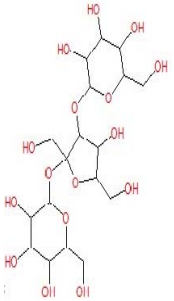
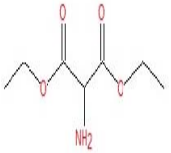
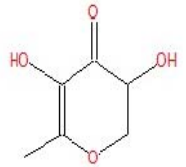
14.	6-Acetyl-ß-d-mannose	5.536	$C_8H_{14}O_7$	222	222.073953		60,81,97,109,126,144,192
15.	α-D-Glucopyranoside,O-α-D-glucopyranosyl-(1.fwdarw.3)-ß-D-fruc	5.805	$C_{18}H_{23}O_{16}$	504	504.169035		60,73,85,97,113,126,145,187
16.	Propanedioic acid , amino -, diethyl ester	5.862	$C_7H_{13}NO_4$	175	175.084458		57,74,84,102,130,142,175
17.	4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl	5.942	$C_6H_8O_4$	144	144.042258		55,72,85,101,115,144
18.	Valeric acid , dodecyl ester	6.600	$C_{17}H_{34}O_2$	270	270.25588		,1

Table 1. Cont'd.

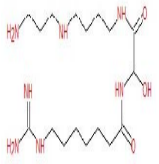
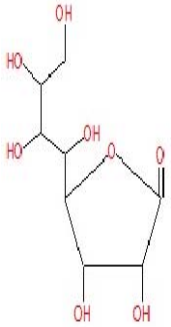
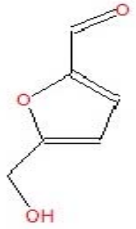
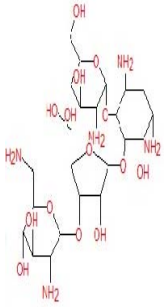
19.	Deoxyspergualin	6.852	$C_{17}H_{37}N_7O_3$	387	387.295788		59,72,86,100,128,138,163,187,208,229,252
20.	l-Gala-l-ido-octonic lactone	6.766	$C_8H_{14}O_8$	238	238.068868		55,61,73,84,97,112,127,142,159,189,238
21.	5-Hydroxymethylfurfural	6.983	$C_6H_6O_3$	126	126.031694		53,69,81,97,109,126
22.	Paromomycin	7.807	$C_{23}H_{45}N_5O_{14}$	615	615.296303		57,67,80,109,124,162,191,212,233,254,277,303,324

Table 1. Cont'd.

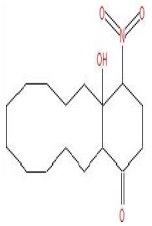
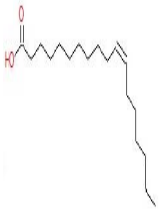
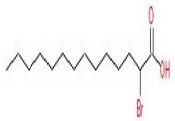
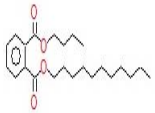
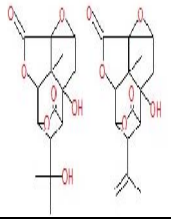
23.	16-Nitrobicyclo[10.4.0]hexadecane-1-ol-13-one	8.797	$C_{16}H_{27}NO_4$	297	297.194008		55,69,81,98,126,158,173,209,221,249,267,297
24.	cis-Vaccenic acid	10.085	$C_{18}H_{34}O_2$	282	282.25588		55,69,83,97,111,123,165,193,222,264,282
25.	2-Bromotetradecanoic acid	11.567	$C_{14}H_{27}Bro_2$	306	306.119442		55,73,83,99,111,138,153,201,227,249,306
26.	Phthalic acid , butyl undecyl ester	14.279	$C_{23}H_{36}O_4$	376	376.26136		57,69,104,121,149,167,205,223,321
27.	Picrotoxin	14.582	$C_{30}H_{34}O_{13}$	602	602.19994		55,67,83,95,109,121,135,149,193,205,252,277,292

Table 1. Cont'd.

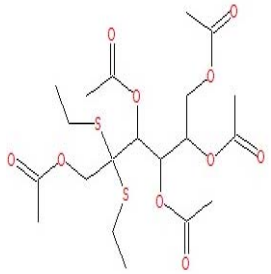
28.	D-Fructose , diethyl mercaptal , pentaacetate	15.046	$C_{20}H_{32}O_{10}S_2$	496	496.14369		9,97,113,129,185,213,245,273

Table 2. FT-IR peak values of *P. expansum*.

No.	Peak (Wave number cm-1)	Intensity	Bond	Functional group assignment	Group frequency
1.	966.34	84.642	-	Unknown	-
2.	1028.06	80.509	C-F stretch	Aliphatic fluoro compounds	1000-10150
3.	1151.50	86.828	C-H	Tetiary amine, C-N stretch	1150-1207
4.	1205.51	88.792	C-H	Tetiary amine, C-N stretch	1150-1207
5.	1303.88	88.221	-	Unknown	-
6.	1377.17	86.934	-	Unknown	-
7.	2852.72	91.145	-	Methylene-CH. asym	2840-2860
8.	2924.09	89.745	-	Methylene-CH. asym	2915-2935

Table 3. Antibacterial activity of bioactive compounds of *Penicillium expansum* against bacterial strains.

Fungal products antibiotics	Zone of inhibition (mm)				
	Bacteria				
	K. pneumonia	*P. eurogenosa*	*S. aureus*	*P. mirabilis*	*E. coli*
Fungal products	5.94±0.491	3.88±0.913	7.01±0.141	5.64±0.203	5.97±0.192
Streptomycin	0.91±0.711	1.41±0.282	1.50±0.407	1.29±0.32	1.00±0.46
Kanamycin	0.93±0.162	0.49±0.500	0.70±0.195	0.50±0.097	0.90±0.204
Rifambin	1.10±0.303	1.09±0.201	0.99±0.496	0.78±0.41	0.76±0.300
Cefotoxime	1.29±0.502	0.91±0.310	1.40±0.203	0.98±0.841	1.47±0.180

Conclusion

This study showed that *P. expansum* produce many important secondary metabolites with high biological activities. Based on the significance of employing bioactive compounds in pharmacy to produce drugs for the treatment of many diseases, the purification of compounds produced by *P. expansum* can be useful.

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Conflict of interest

Authors have none to declare.

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