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Vol. 14(43), pp. 2972-2994, 28 October, 2015
DOI: 10.5897/AJB2015.14906
Article Number: EDE5FF355935
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Antibacterial activity of secondary metabolites isolated from *Alternaria alternata*

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Received 9 August, 2015; Accepted 13 October, 2015

The aims of this study were the analysis of the secondary metabolites and evaluation of the antibacterial and antifungal activity of *Alternaria alternata*. Twenty six bioactive compounds were identified in methanolic extract of *Alternaria alternata*. The identification of bioactive chemical compounds is based on the peak area, retention time molecular weight and molecular formula. GC-MS analysis of *A. alternata* revealed the existence of the α-acetyl-L-serine, 2(5H)-furanone, 6-oxa-bicyclo[3.1.0]hexan-3-one, D-glucose,6-O-α-D-GALACTOPYRANOSYL, DL-arabinose, ε-N-fommyl-L-lysine, 2-[4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES), thrietol, 2-O-heptyl, 2-deoxy-2-fluoro-1,6-anhydro-ß-d-glucopyranose, d-ribo-hexos-3-ulose, A-D-glucopyranoside, O-α-D-glucopyranosyl-(1.fwdarw.3)-ß-D-fru, maltose, 4H-pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl, desulphosinigrin, uric acid, midazole-4-carboxylic acid, 2-fluoro-1-methoxymethyl-ethyl ester, geranyl isovalerate, 1-nitro-ß-d-arabinofuranose, tetraacetate, glycyl-D-asparagine, α-D-xylofuranose, cyclic 1,2:3,5-bis(butylboronate), estra -1,3,5(10)-trien-17ß-ol, glucobrassicin, N-2,4-Dnp-L-arginine, dasycarpidan-1-methanol, acetate(ester) and 5alpha-androstane-3,17-monooxime. The fourier transform infrared (FTIR) analysis of *A. alternata* proved the presence of aromatic rings, aliphatic fluoro compounds, tetiary amine, C-N stretch, ammonium ions, organic nitrate, methylene-CH. asym, and normal polymeric O-H stretch which shows major peaks at 711.73, 846.57, 873.75, 1026.13, 1149.57, 1205.51, 1238.30, 1409.96, 1631.78, 2517.10, 2854.65, 2924.09, 3059.75 and 3271.27. *A. alternate* had maximum zone formation (5.04 ± 0.29) mm against *Klebsiella pneumonia.*

Key words: *Alternaria alternata*, bioactive compounds, gas chromatography mass spectrometry (GCMS), fourier transform infrared (FTIR).

INTRODUCTION

Alternaria spp. are cosmopolitan mould fungi and can be found in soils, plants, food, feed and indoor air (Thomma, 2003). *Alternaria* species are frequently found on small grains, causing yield losses in production and processing

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Abbreviations: AOH, Alternariol; **AME,** alternariol monomethyl ether; **ALT,** altenuen; **TEA,** tenuazoic acid; **ATX,** altertoxins; **PDB,** potato dextrose broth.

(Ostry, 2008). Many *Alternaria* species are mycotoxin producers with different toxicological properties. The most important Alternaria toxins are alternariol (AOH), alternariol monomethyl ether (AME), altenuen (ALT), tenuazoic acid (TEA) and altertoxins (ATX-I, II, III) (Logrieco et al., 2009; Hameed et al, 2015a). *Alternari*a spores are considered to be one of the most prolific fungal allergens, which have been associated with respiratory allergies and skin infections (Corden et al., 2003; Kilic et al., 2010; Pavon et al., 2010).

A. alternata is considered as the most important toxin producing species. *A. alternata* is a widespread saprophytic species which produces a wide variety of different secondary metabolites, among which are the mutagenic mycotoxins alternariol (AOH) and altertoxin (ATX) (Pfeiffer et al., 2007). The altertoxins ATX-I, -II, and -III are mutagenic in the Ames test and are more potent and acutely toxic to mice than AOH and AME. *A. alternata* is known as producer of a large spectrum of secondary metabolites. The effect of light on the amount of secondary metabolites by GC-MS and FT-IR was analyzed (Altameme et al., 2015; Hameed et al., 2015b).

The main purpose of this research was the screening of the secondary metabolites products from *A. alternata* and evaluation of the antibacterial activity.

MATERIALS AND METHODS

Collection and growth condition

A. alternata species were isolated from dried fruit and the pure colonies were selected, isolated and maintained on potato dextrose agar slants (Usha and Masilamani, 2013). 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.

Production, extraction and determination of metabolites

The metabolites were determined and extracted for GC analysis using the method of Hussein et al. (2015) with some modifications. 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 were 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 (Hameed et al., 2015c; Jasim et al., 2015). The identification of the components was based on comparison of their mass spectra with those of NIST mass spectral library as well as on comparison of their retention indices either with those of authentic compounds or with literature values.

Gas chromatography-mass spectrometry (GC-MS) analysis

Bioactive compound were examined for the chemical composition using GC-MS (Agilent 7890 A) equipped with a DB-5MS column (30 m × 0.25 mm i.d., 0.25 um film thickness, J&W Scientific, Folsom, CA). Helium was used as the carrier gas at the rate of 1.0 mL/min (Imad et al., 2014a; Kareem et al., 2015). Effluent of the GC column was introduced directly into the source of the MS via a transfer line (250°C) (Tabaraie et al., 2012). Ionization voltage was 70 eV and ion source temperature was 230°C. Scan range was 41 to 450 amu. The constituents were identified after comparison with available data in the GC-MS library in the literatures (Mohammed and Imad, 2013).

Fourier transform infrared spectrophotometer (FTIR)

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

Determination of antibacterial activity of crude fraction of *A. alternata* compounds

The test pathogens (*Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Staphylococcus aureus)* were swabbed in Muller Hinton agar plates. 90 µl of fungal extracts was loaded on the bored wells. The wells were bored in 0.5 cm in diameter (Suja et al., 2013; Huda et al., 2015b; Imad et al., 2015). The plates were incubated at 37°C for 24 h and examined. After the incubation the diameter of inhibition zones around the discs were 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.

RESULTS AND DISCUSSION

Isolation of fungi from dried fruit

The fungi were isolated from dried fruit by serial dilution method (Perfect et al., 2001; Mogensen et al., 2003). Based on morphological characteristics, fungi was isolated in selective media of potato dextrose agar media. Morphological and microscopical characteristics of fungal strains were determined using specific media light and compound microscope (Figure 1).

Production of secondary metabolites

The 400 ml of fermentation broth (PDA broth) which contained 200 µl of the standardized fugal suspensions were used to inoculate the flasks and incubated at 37°C on a shaker at 90 rpm for 7 days. After fermentation, the secondary metabolites were produced by isolated microorganisms.

Identification of secondary metabolites from the methanolic crude extract of *A. alternata* by gas chromatography and mass spectrometry

Gas chromatography and mass spectroscopy analysis of

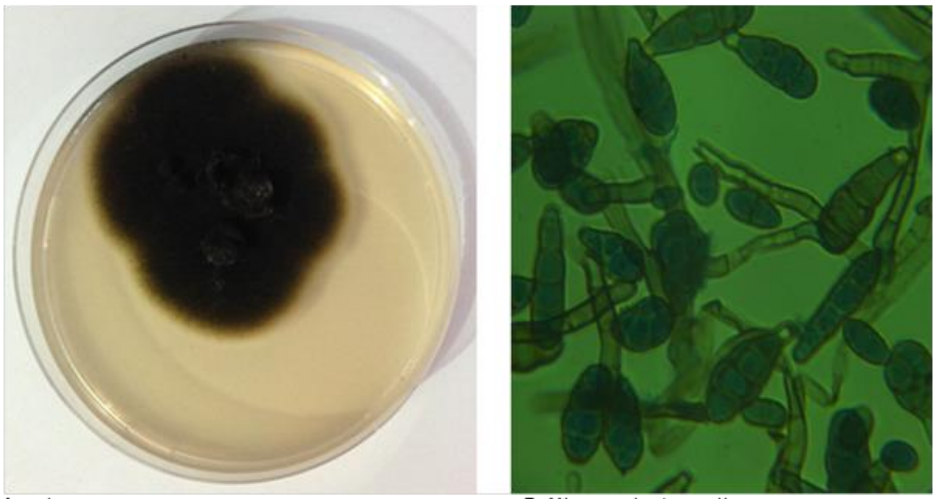
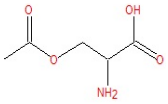
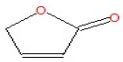
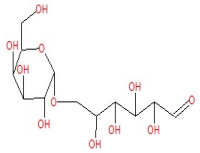
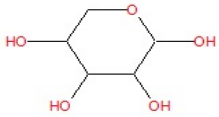


Figure 1. Morphological characterization of *Alternaria alternata*.

Table 1. Major bioactive chemical compounds identified in methanolic extract of *Alternaria alternata*.

Serial No.	Phytochemical compound	RT (min)	Formula	Molecular weight	Exact mass	Chemical structure	MS Fragment- ions
1	α-Acetyl-L-serine	3.23	$C_5H_9NO_4$	147	147.0532		60,74,87,102,129
2	2(5H)-Furanone	3.419	C4H4O2	84	84.02113		55,84
3	6-Oxa-bicyclo[3.1.0]hexan-3-one	3.505	C5H6O2	98	98.03678		50,55,69,81,98
4	D-Glucose,6-O-α-D-galactopyranosyl	3.533	C12H22O11	342	342.1162		60,73,85,110,126,182,212,261
5	DL-Arabinose	3.945	C5H10O5	150	150.0528		60,85,133

compounds was carried out in methanolic extract of *A. alternate* as shown in Table 1. The GC-MS chromatogram of the 26 peaks of the compounds detected are shown in Figure 2. The first set up peak was determined to be α-acetyl-L-serine (Figure 3). The second peak indicated to be 2(5H)-furanone (Figure 4). The next peaks were considered to be, 6-oxa-bicyclo[3.1.0]hexan-3-one, D-glucose,6-O-α-D-galactopy-ranosyl, DL-arabinose, ε-N-fommyl-L-lysine, HEPES, thrietol, 2-O-heptyl, 2-deoxy-2-fluoro-1,6-anhydro-ß-d-glucopyranose, d-ribo-hexos-3-ulose, A-D-glucopyranoside, O-α-D-gluco-pyranosyl-(1.fwdarw.3)-ß-D-fru, maltose, 4H-pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl, desulphosinigrin, uric acid, midazole-4-carboxylic acid, 2fluoro-1-methoxymethyl-,ethyl ester, geranyl isovalerate, 1-nitro-ß-d-arabinofuranose, tetraacetate, glycyl-D-asparagine, α-D-xylofuranose, cyclic 1,2:3,5-bis(butylboronate), estra -1,3,5(10)-trien-17ß-ol, glucobrassicin, N-2,4-Dnp-L-arginine, dasycarpidan-1-methanol , acetate(ester) and 5α- androstane-3,17-monooxime (Figures 5 to 28).

Table 1. Contd.

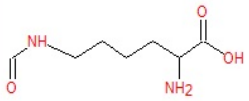
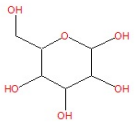
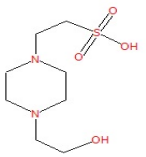
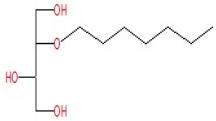
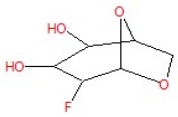
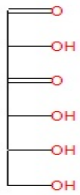
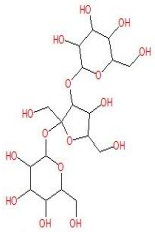
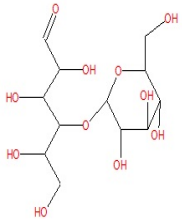
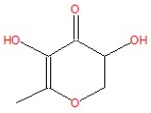
6	ε-N-Fommyl-L-lysine	4.105	$C_7H_{14}N_2O_3$	174	174.1004		56,84,100,112,128,138,156,173
7	ß-D-Glucopyranose	4.329	C6H12O6	180	180.0634		60,73,85,103,131,149
8	HEPES	4.437	$C_8H_{18}N_2O_4S$	238	238.0987		55,84,112,143,157,207,237
9	Thrietol, 2-O-heptyl	4.546	C11H24O4	220	220.1675		57,70,91,159,189,221
10	2-Deoxy-2-fluoro-1,6-anhydro-ß-d-glucopyranose	4.655	C6H11FO4	164	164.0485		56,74,102,118,147
11	d-Ribo-hexos-3-ulose	4.821	C6H10O6	178	178.0477		60,73,89,101,118,130,160
12	A-D-Glucopyranoside, O-α-D-glucopyranosyl-(1.fwdarw.3)-ß-D-fru	5.313	C18H32O16	504	504.169		60,73,85,97,113,126,145,163,179,199
13	Maltose	5.559	C12H22O11	342	342.1162		60,73,85,97,126,163,191,215
14	4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl-	6.028	C6H8O4	144	144.0423		55,72,85,101,115,144

Table 1. Contd.

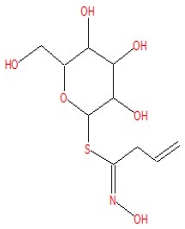
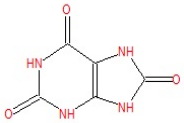
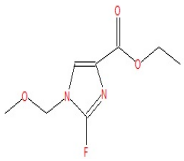
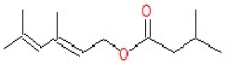
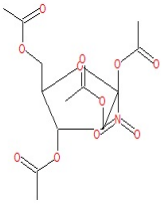
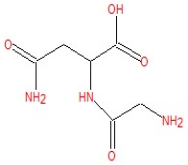
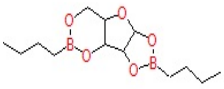
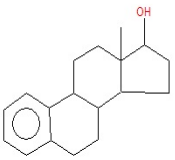
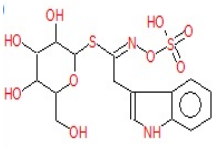
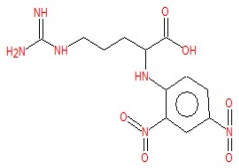
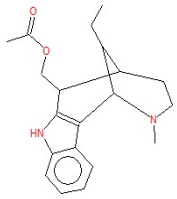
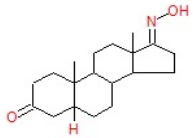
15	Desulphosinigrin	6.549	$C_{10}H_{17}NO_6S$	279	279.0777		60,73,85,103,127,145,163,213,262
16	Uric acid	9.701	$C_5H_4N_4O_3$	168	168.0283		54,69,82,97,125,140,168
17	Imidazole-4-carboxylic acid , 2fluoro-1-methoxymethyl- ,ethyl ester	10.085	$C_8H_{11}FN_2O_3$	202	202.0754		56,72,100,114,127,157,182
18	Geranyl isovalerate	10.194	C15H26O2	238	238.1933		57,69,85,93,121,136,168,198,238
19	1-Nitro-ß-d-arabinofuranose , tetraacetate	12.168	C13H17NO11	363	363.0802		60,85,103,115,145,170,217,234, 264,289,320
20	Glycyl-D-asparagine	14.937	$C_6H_{11}N_3O_4$	189	189.075		##55,113,154
21	α-D-Xylofuranose , cyclic 1,2:3,5-bis(butylboronate)	14.8	$C_{13}H_{24}B_2O_5$	282	282.181		55,83,97,111,127,139,152,167,182,197,225,253,282
22	Estra -1,3,5(10)-trien-17ß-ol	17.02	C18H24O	256	256.1827		57,73,85,97,107,129,157,185,213,241,256
23	Glucobrassicin	17.186	$C_{16}H_{20}N_2O_9S_2$	448	448.061		58,102,117,130,142,155 ,175,256,281,308

Table 1. Contd.

24	N-2,4-Dnp-L-arginine	17.872	$C_{12}H_{16}N_6O_6$	340	340.1131		69,80,107,131,153,177,226,256, 269,296340
25	Dasycarpidan-1-methanol , acetate(ester)	18.777	$C_{20}H_{26}N_2O_2$	326	326.1994		58,102,117,130,142,155,175,256 ,281,308
26	5Alpha-androstane-3,17-monooxime	19.354	C19H29NO2	303	303.2198		55,96,119,161,231,286

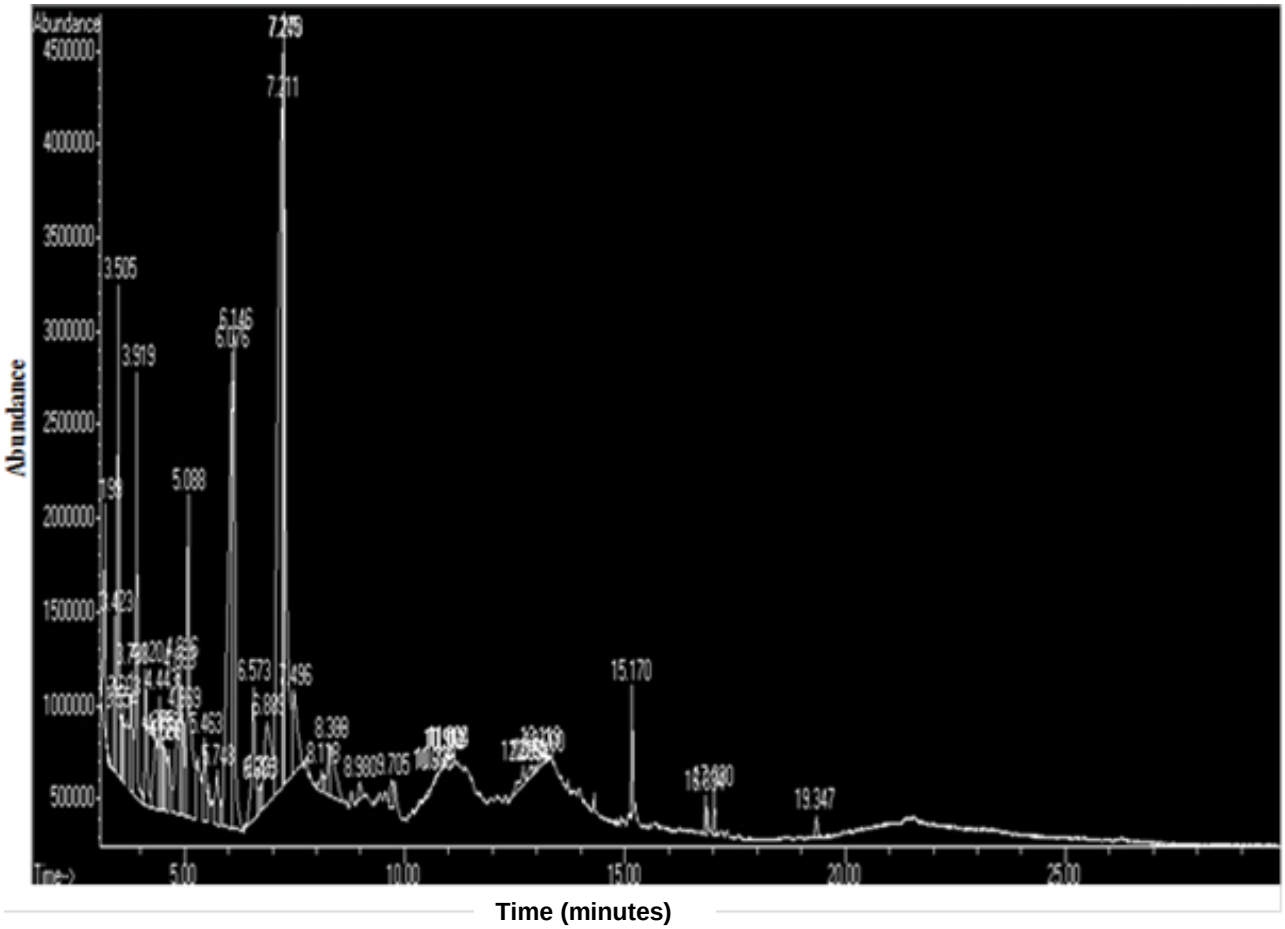


Figure 2. GC-MS chromatogram of methanolic extract of *Alternaria alternata*.

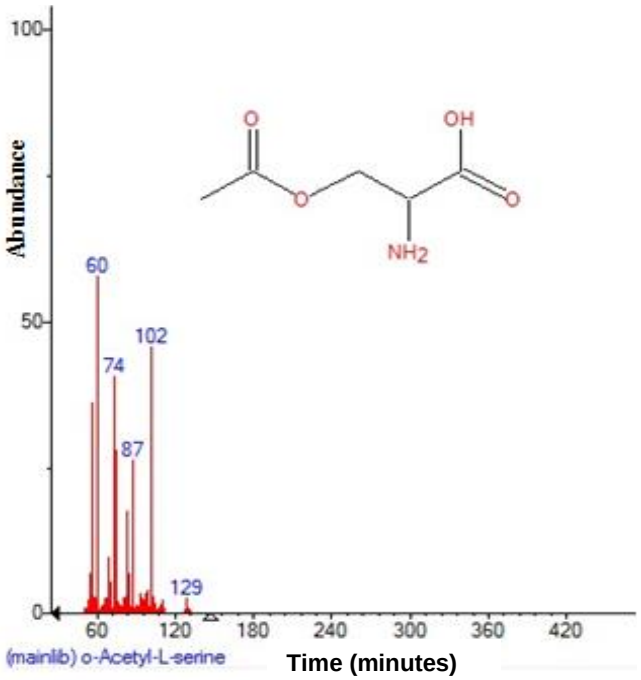


Figure 3. Mass spectrum of α-Acetyl-L-serine with retention time (RT) = 3.230.

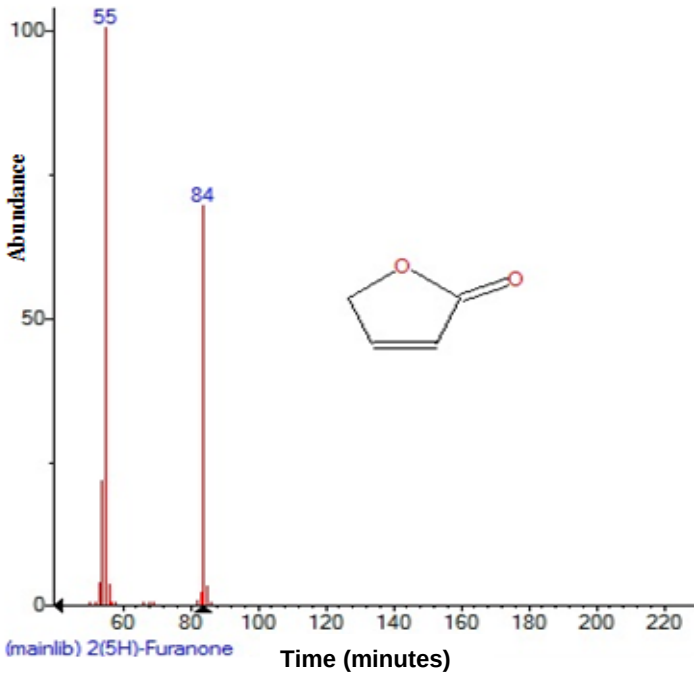


Figure 4. Mass spectrum of 2(5H)-Furanone with Retention Time (RT) = 3.419.

Some of them (thrietol, 2-O-heptyl, desulphosinigrin, Imidazole-4-carboxylic acid, 2fluoro-1-methoxymethyl-ethyl ester and geranyl isovalerate) are biological compounds with antimicrobial activities (Anupama et al.,

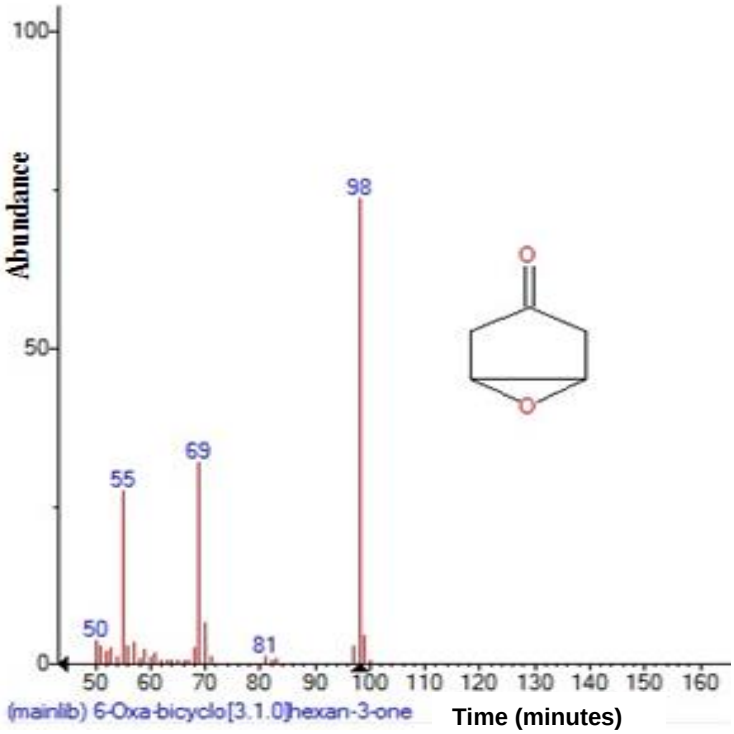


Figure 5. Mass spectrum of 6-Oxa-bicyclo[3.1.0]hexan-3-one with Retention Time (RT) = 3.505.

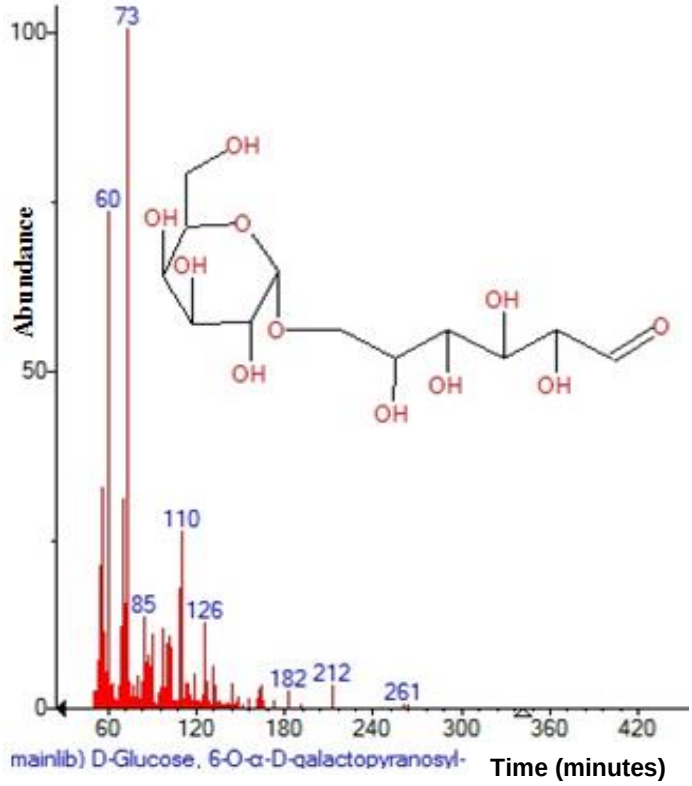


Figure 6. Mass spectrum of D-Glucose,6-O-α-D-galactopyranosyl with Retention Time (RT) = 3.533.

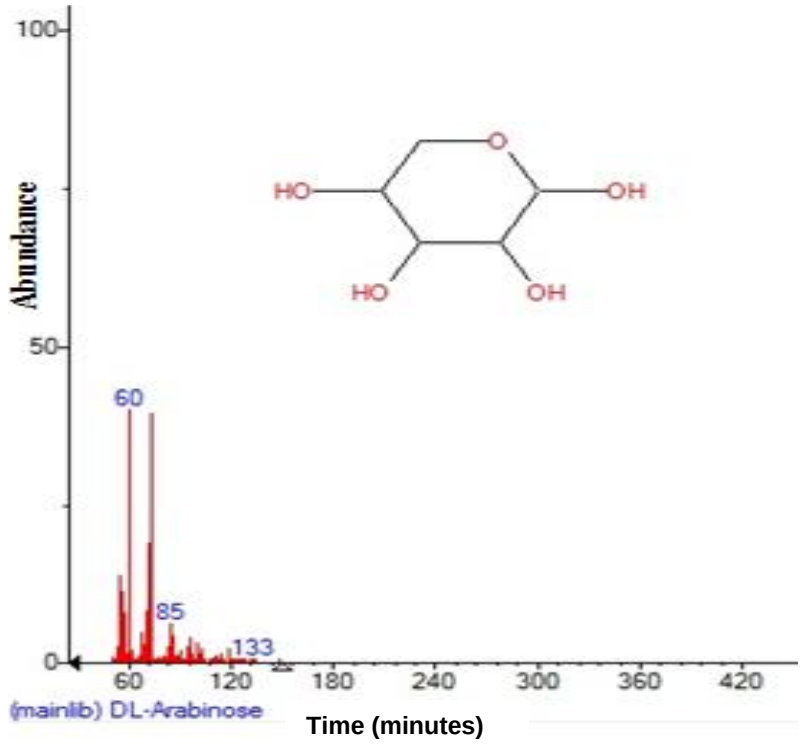


Figure 7. Mass spectrum of DL-arabinose with Retention Time (RT) = 3.945.

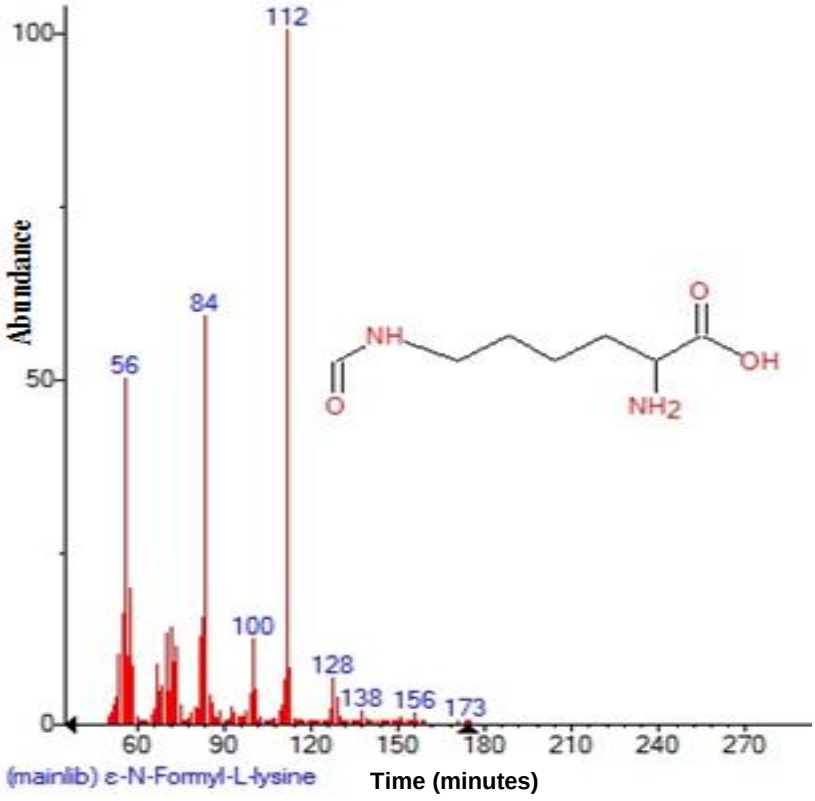


Figure 8. Mass spectrum of ε-N-fommyl-L-lysine with retention time (RT) = 4.105.

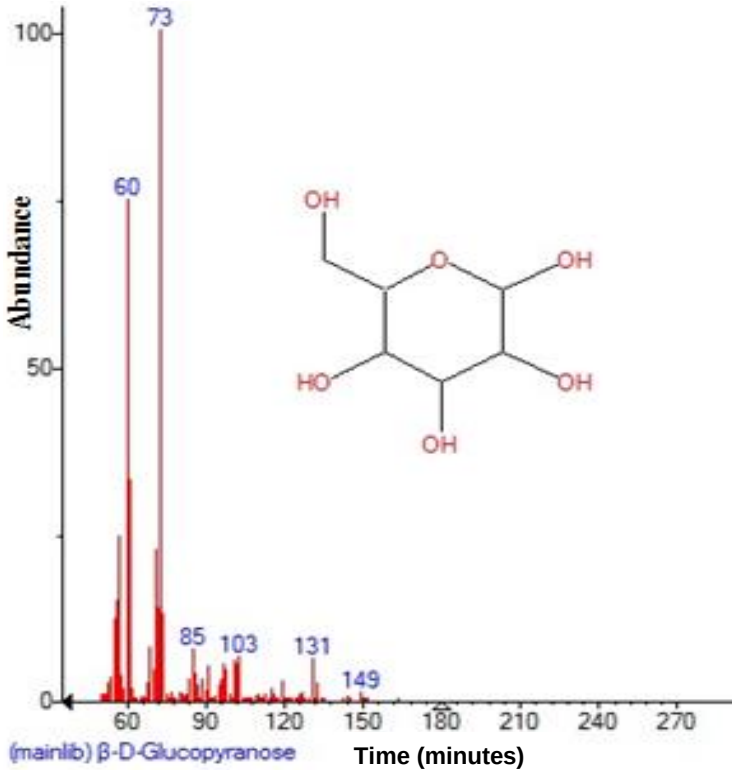


Figure 9. Mass spectrum of ß-D-glucopyranose with retention time (RT) = 4.329.

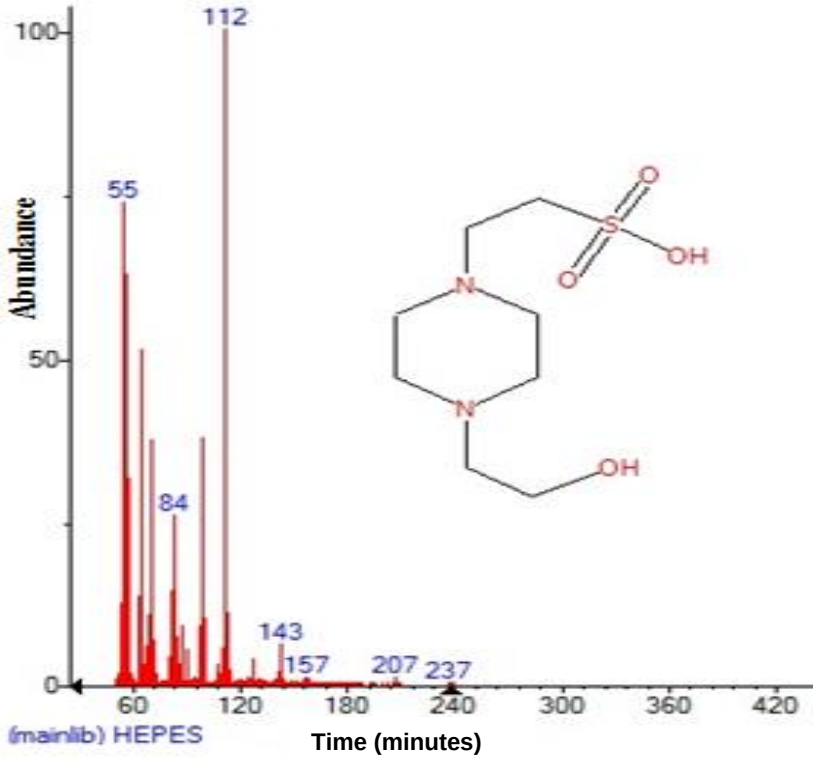


Figure 10. Mass spectrum of HEPES with retention time (RT) = 4.437.

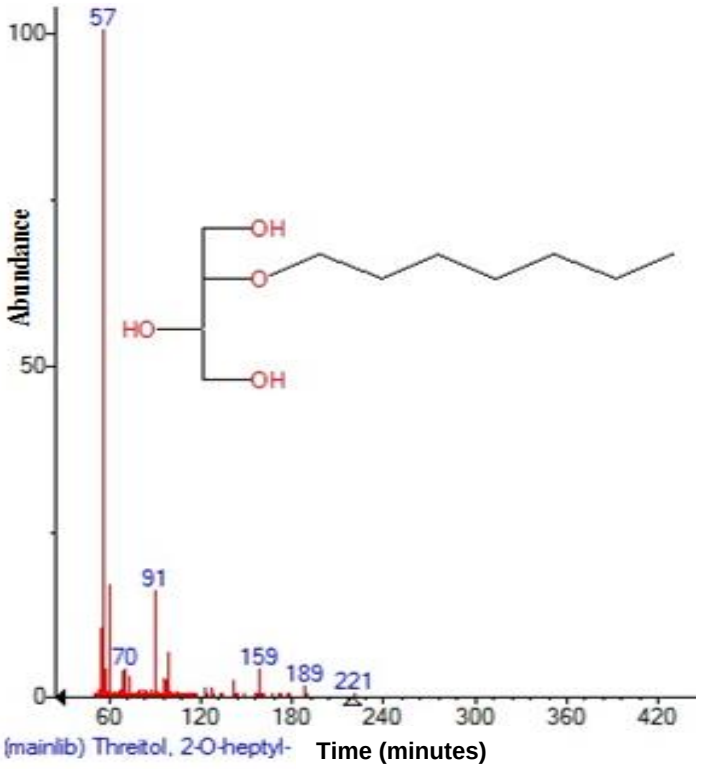


Figure 11. Mass spectrum of thrietol, 2-O-heptyl with retention time (RT) = 4.546.

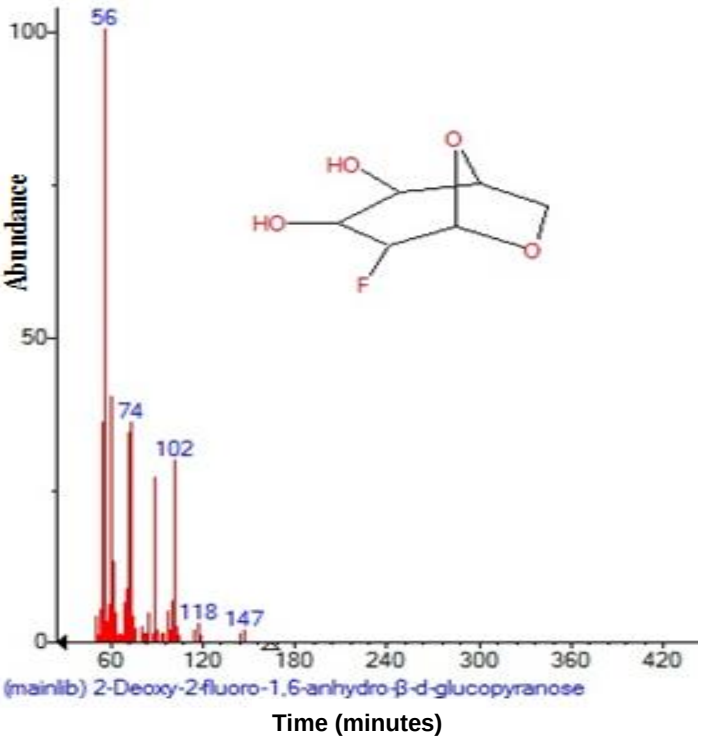


Figure 12. Mass spectrum of 2-deoxy-2-fluoro-1,6-anhydro-ß-d-glucopyranose with retention time (RT) = 4.655.

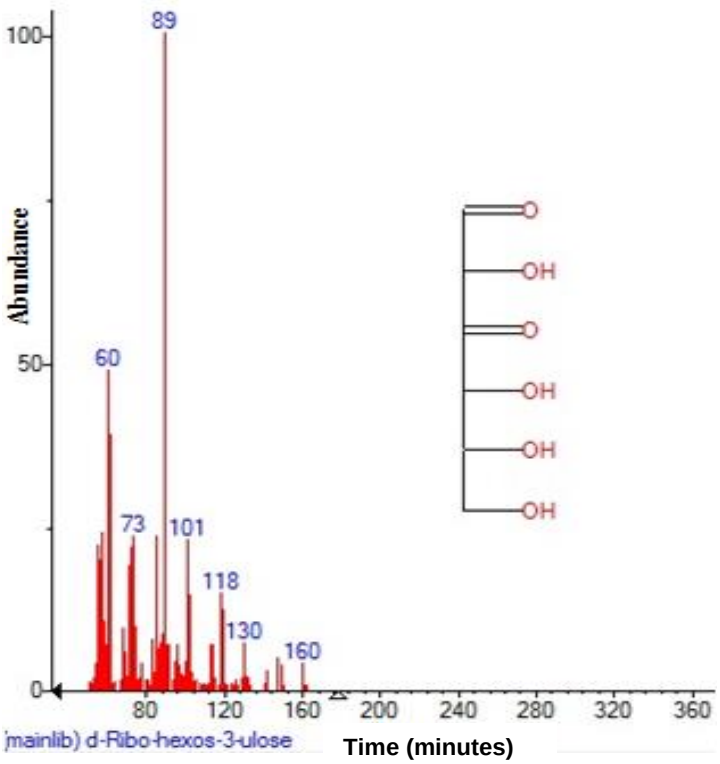


Figure 13. Mass spectrum of d-ribo-hexos-3-ulose with retention time (RT) = 4.821.

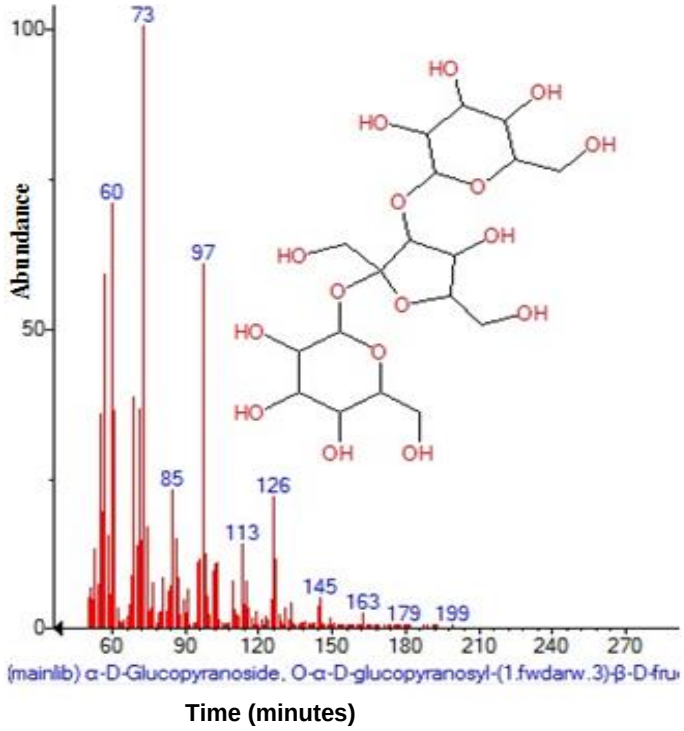


Figure 14. Mass spectrum of A-D-Glucopyranoside, O-α-D-glucopyranosyl-(1.fwdarw.3)-ß-D-fru with Retention Time (RT)= 5.313.

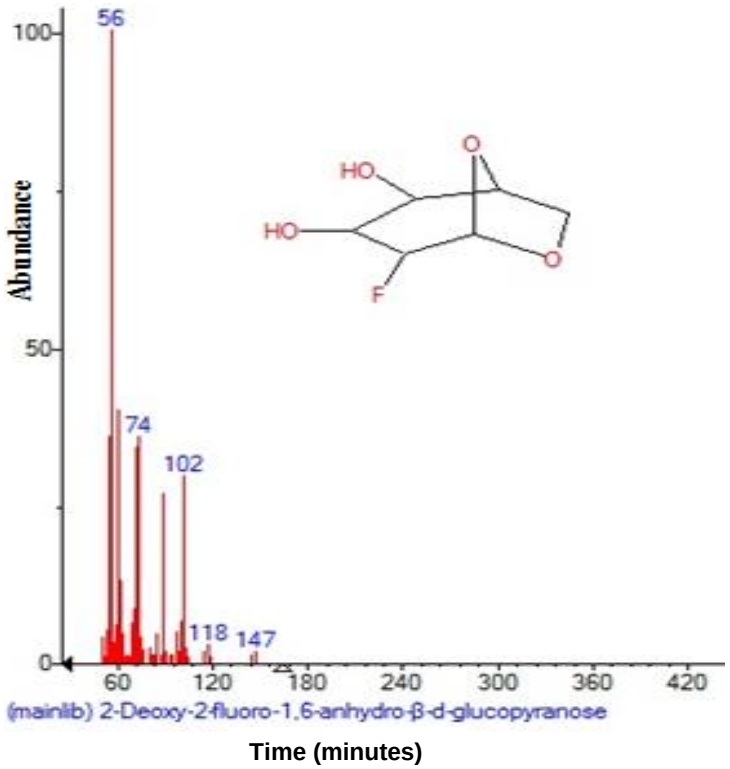


Figure 12. Mass spectrum of 2-Deoxy-2-fluoro-1,6-anhydro-ß-d-glucopyranose with retention time (RT) = 4.655.

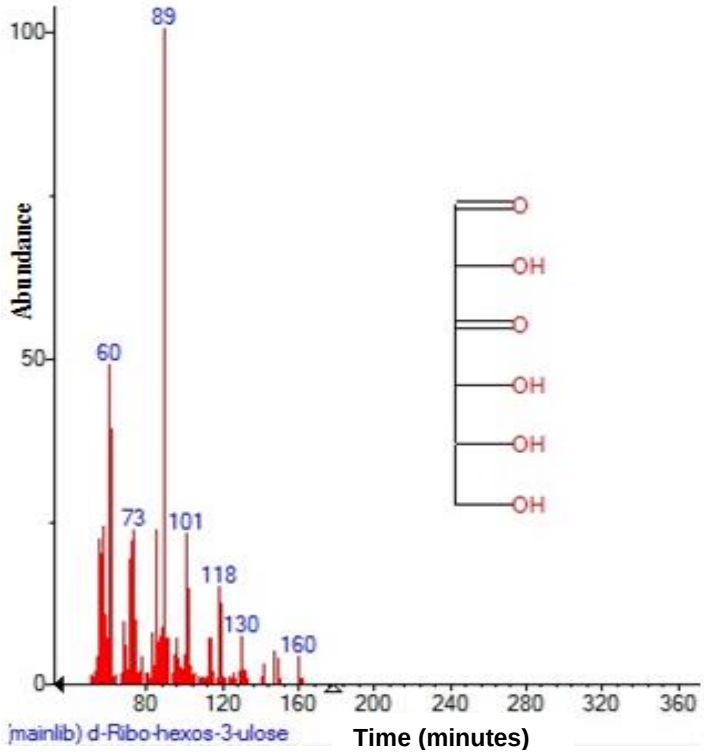


Figure 13. Mass spectrum of d-ribo-hexos-3-ulose with retention time (RT) = 4.821.

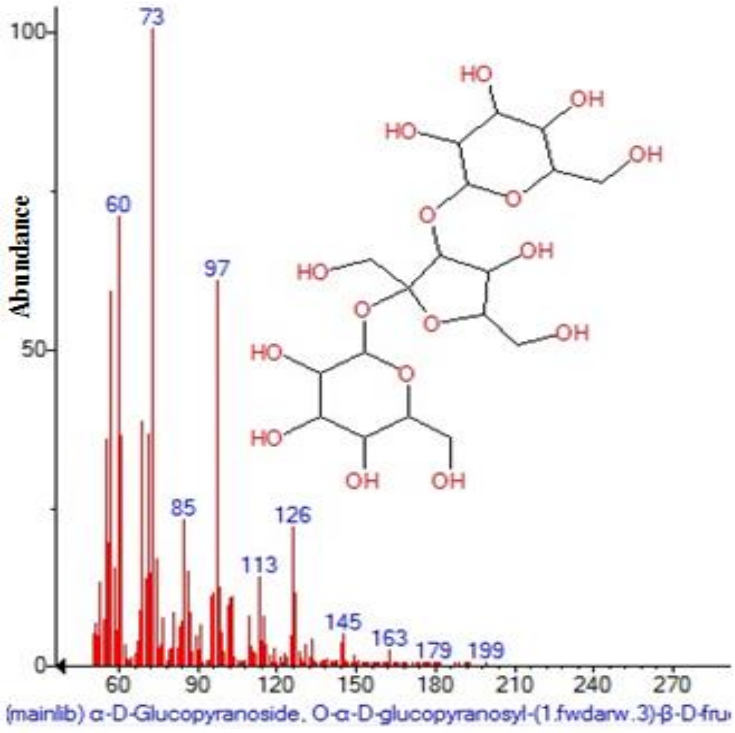


Figure 14. Mass spectrum of A-D-Glucopyranoside, O-α-D-glucopyranosyl-(1.fwdarw.3)-ß-D-fru with retention time (RT) = 5.313.

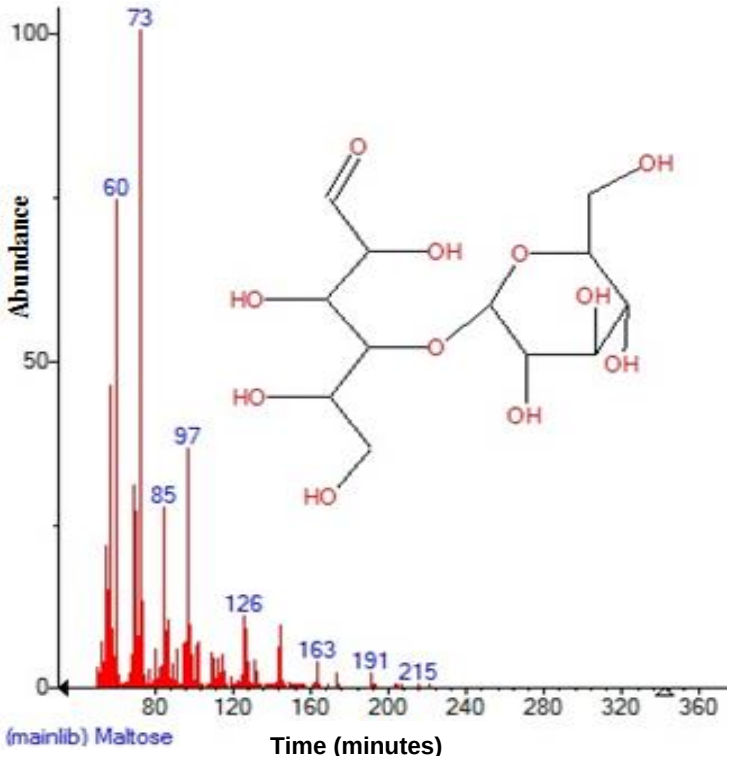


Figure 15. Mass spectrum of maltose with retention time (RT) = 5.559.

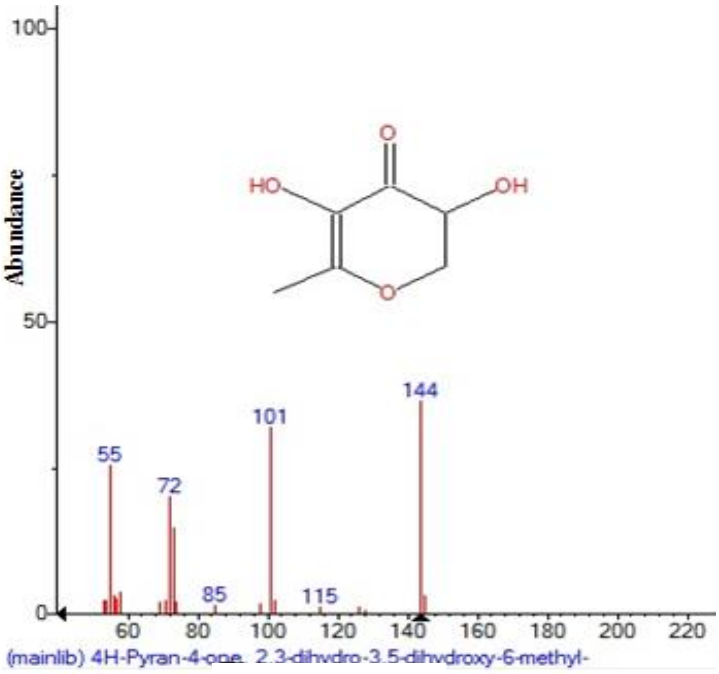


Figure 16. Mass spectrum of 4H-pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl with retention time (RT) = 6.028.

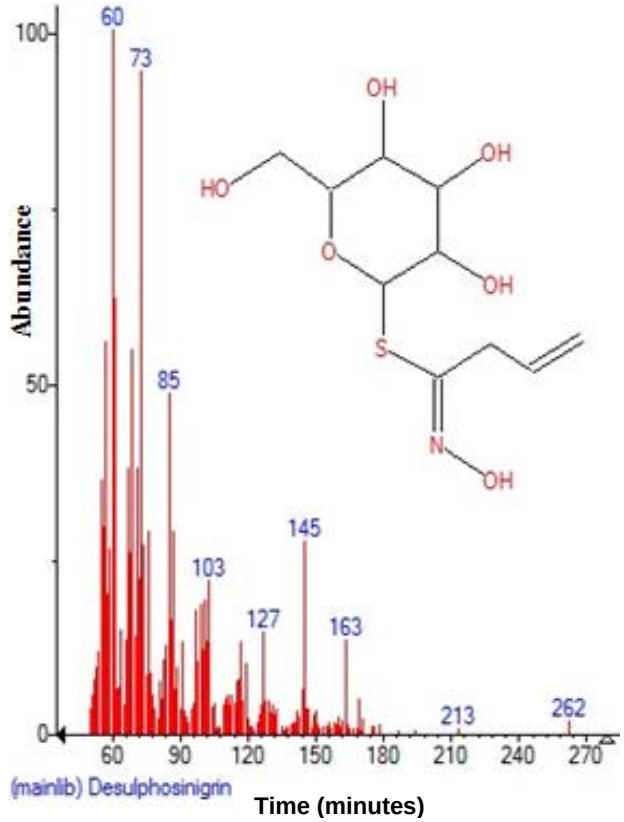


Figure 17. Mass spectrum of desulphosinigrin with retention time (RT) = 6.549

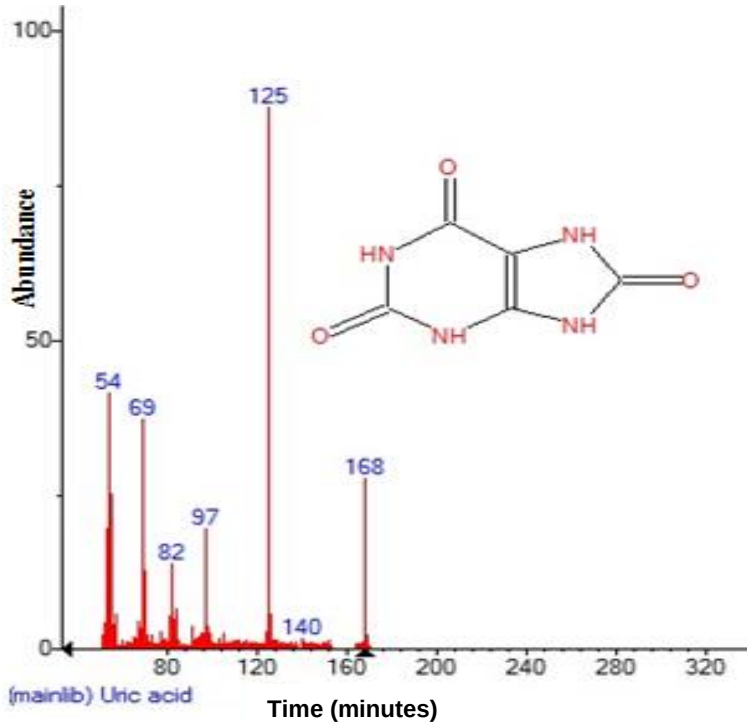


Figure 18. Mass spectrum of uric acid with retention time (RT) = 9.701.

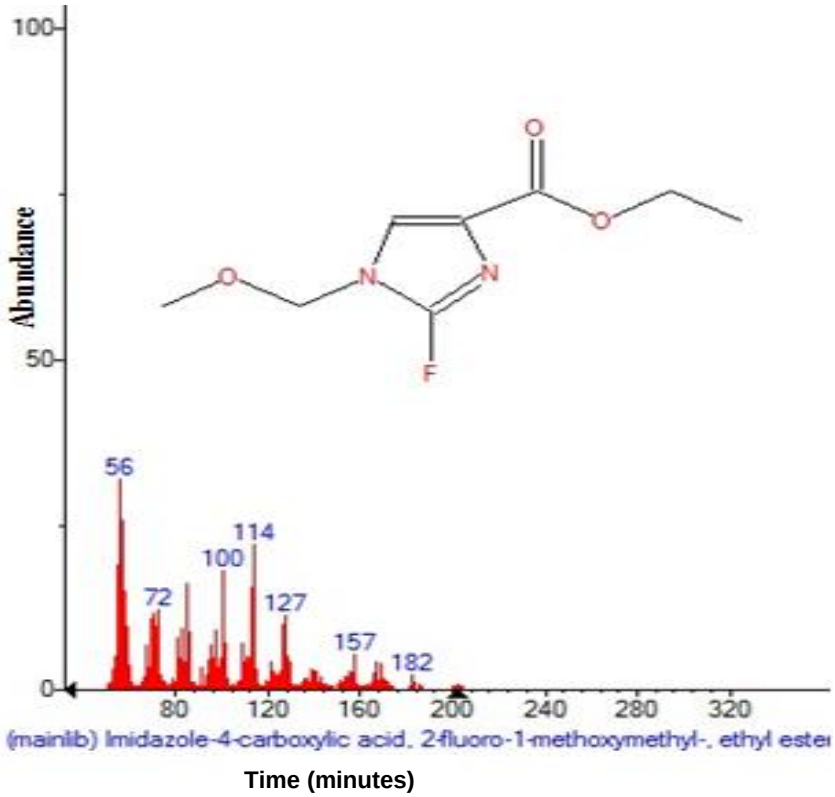


Figure 19. Mass spectrum of midazole-4-carboxylic acid, 2fluoro-1-methoxymethyl-,ethyl ester with retention time (RT) = 10.085.

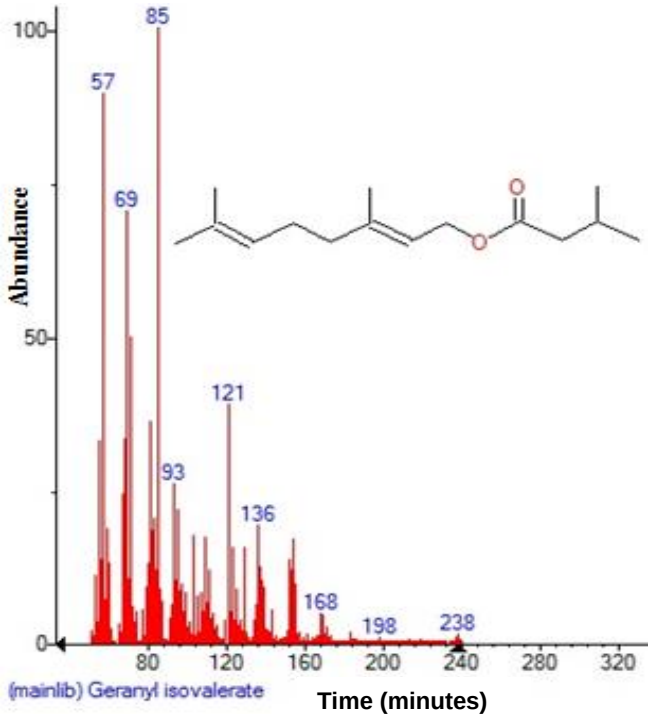


Figure 20. Mass spectrum of geranyl isovalerate with retention time (RT) = 10.194.

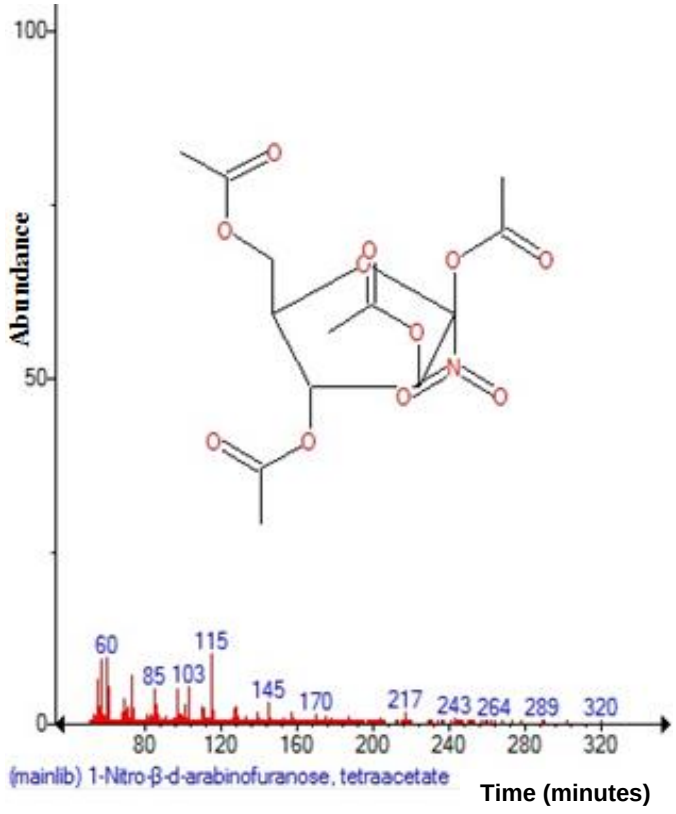


Figure 21. Mass spectrum of 1-Nitro-ß-d-arabinofuranose, tetraacetate with retention time (RT) = 12.168.

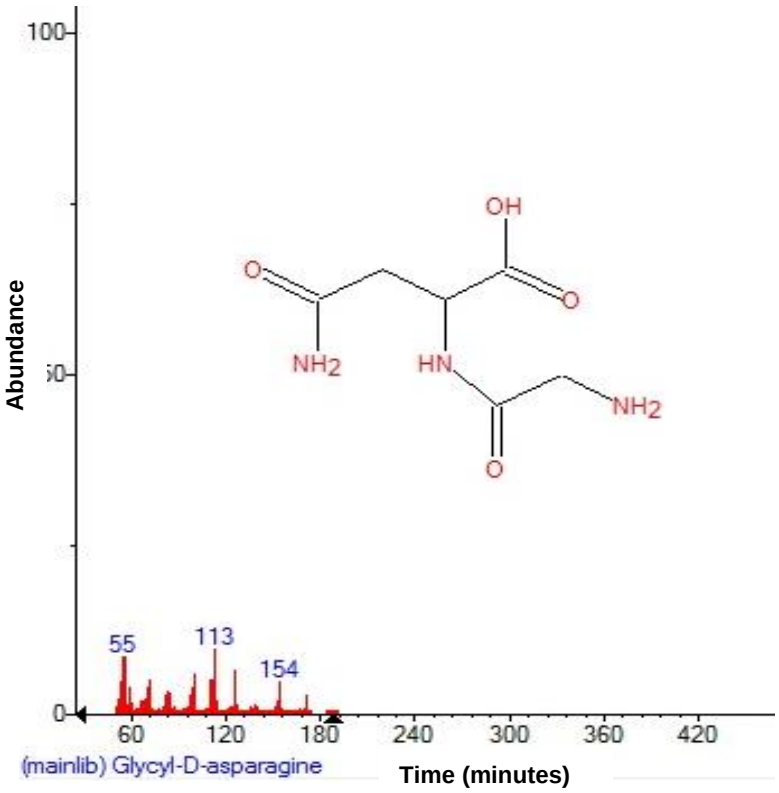


Figure 22. Mass spectrum of glycyl-D-asparagine with retention time (RT) = 14.937.

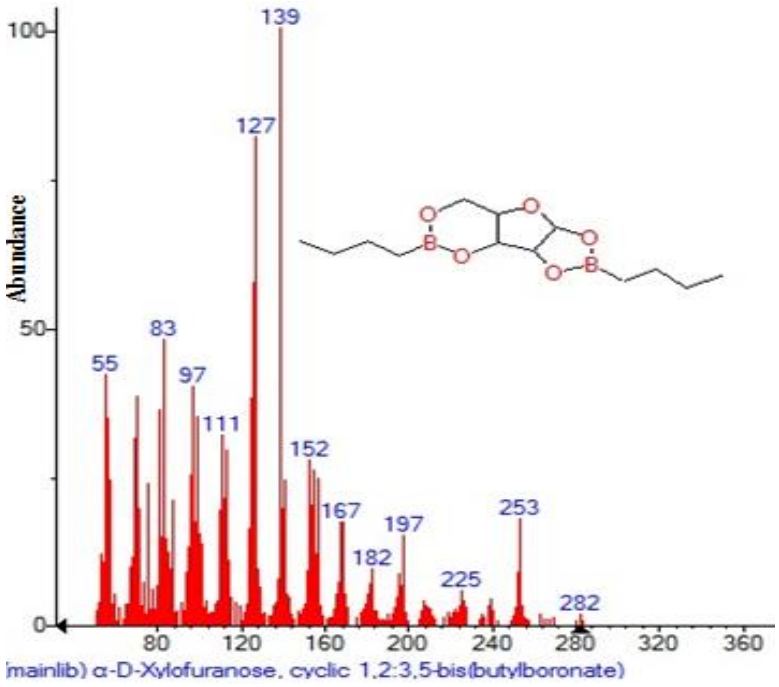


Figure 23. Mass spectrum of α-D-xylofuranose, cyclic 1,2:3,5-bis(butylboronate) with retention time (RT) = 14.800

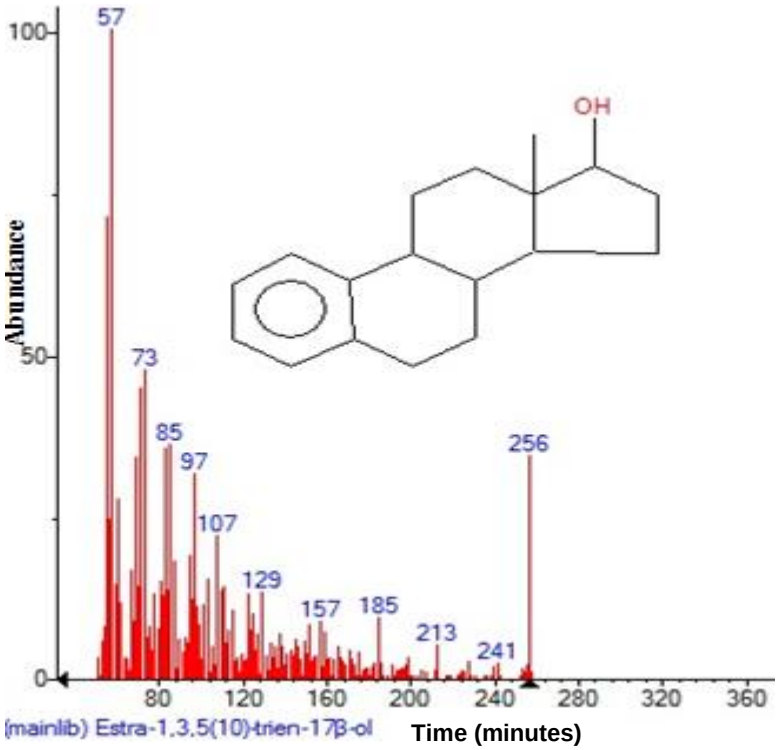


Figure 24. Mass spectrum of Estra -1,3,5(10)-trien-17ß-ol with Retention Time (RT)= 17.020.

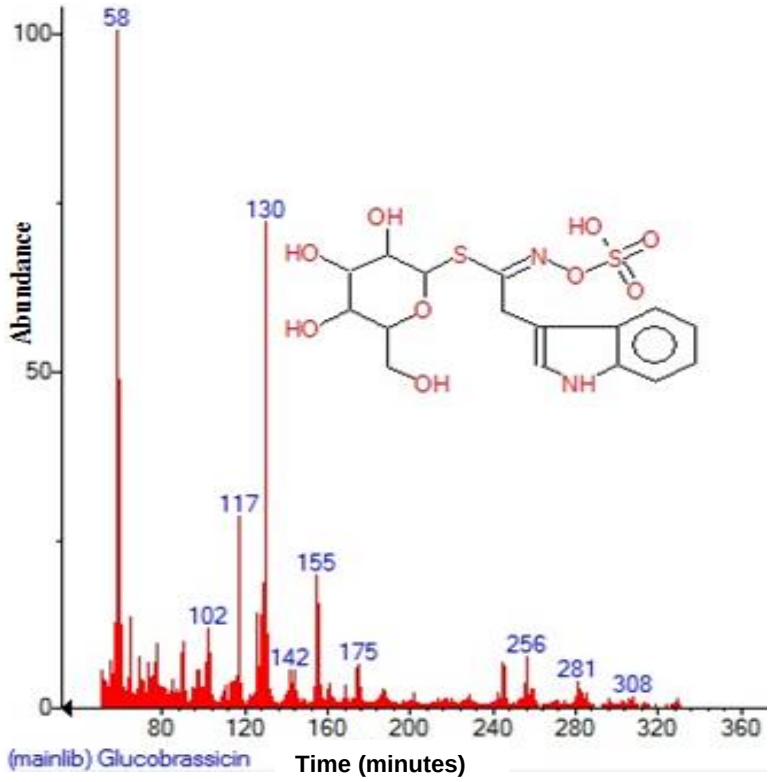


Figure 25. Mass spectrum of glucobrassicin with retention time (RT) = 17.186.

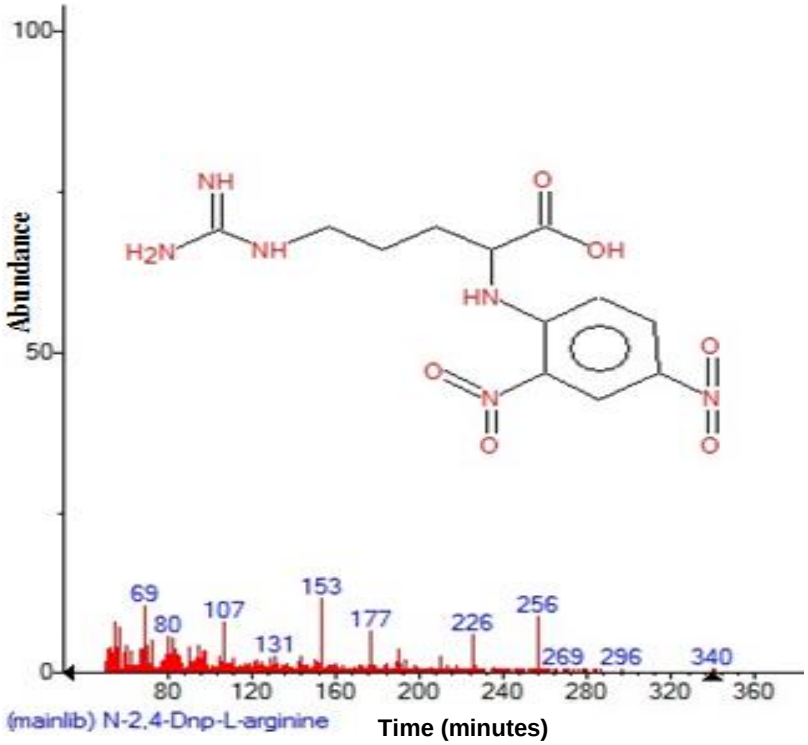


Figure 26. Mass spectrum of N-2,4-Dnp-L-arginine with retention time (RT) = 17.872.

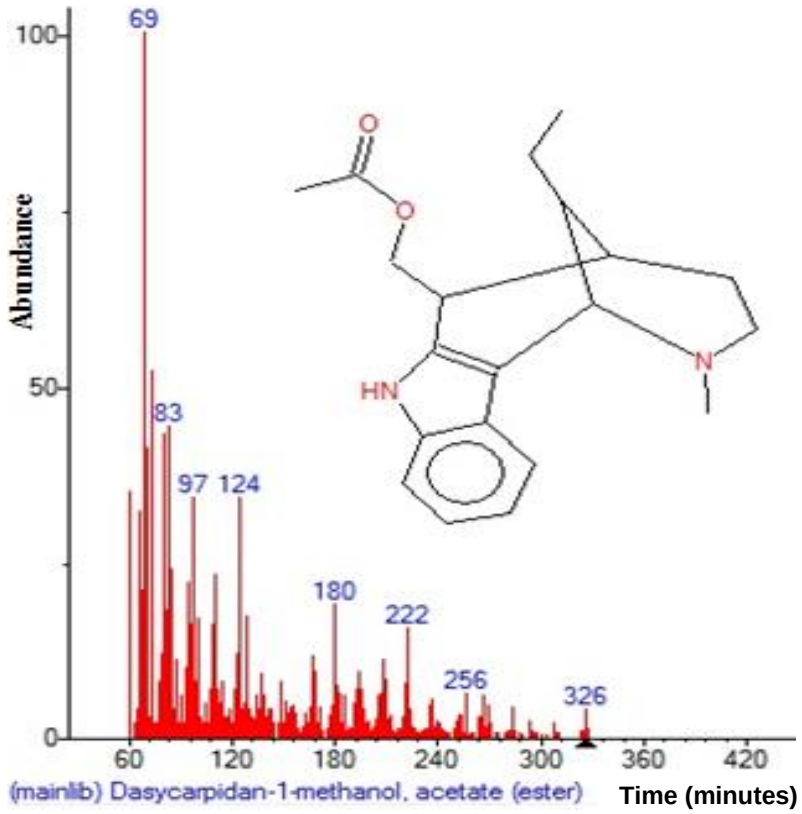


Figure 27. Mass spectrum of Dasycarpidan-1-methanol, acetate(ester) with retention time (RT)= 18.777.

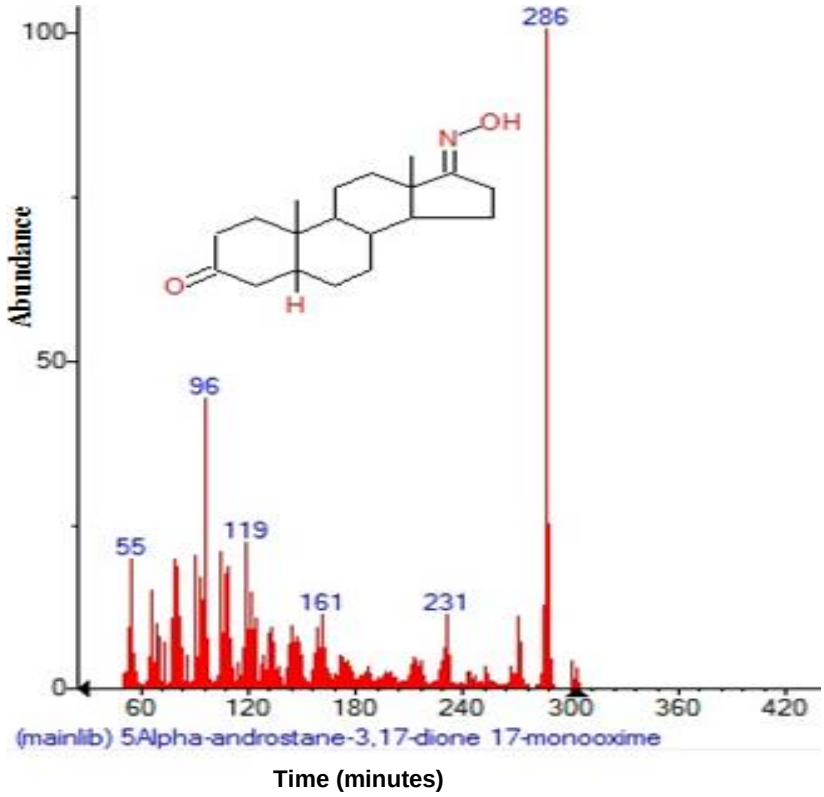


Figure 28. Mass spectrum of 5α-androstane-3,17-monooxime with retention time (RT) = 19.354.

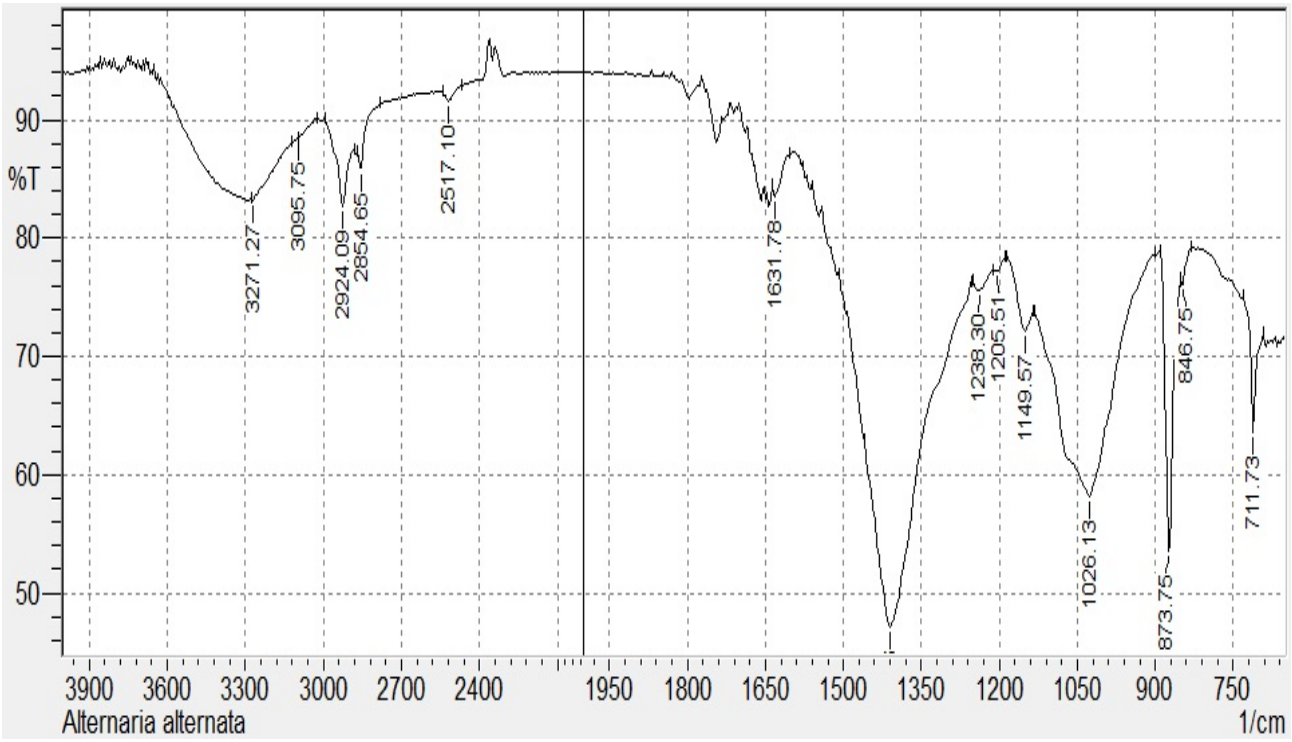


Figure 29. Fourier-transform infrared spectroscopy peak values of *Alternaria alternata.*

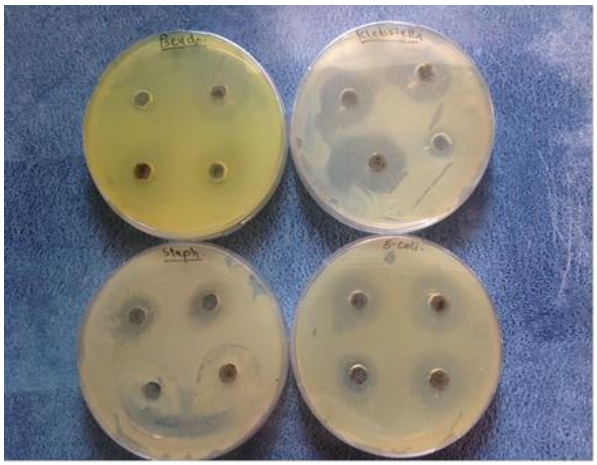


Figure 30. Antimicrobial activity of *Alternaria alternata.*

Table 2. Fourier-transform infrared spectroscopy peak values of *Alternaria alternata*.

No.	Peak (Wave number cm-^{l})	Intensity	Bond	Functional group assignment	Group frequency
1	711.73	63.635	C-H	Aromatic rings	690-900
2	846.57	76.018	C-H	Aromatic rings	690-900
3	873.75	35.592	C-H	Aromatic rings	690-900
4	1026.13	58.22	C-F stretch	Aliphatic fluoro compounds	1000-10150
5	1149.57	72.214	C-F stretch	Aliphatic fluoro compounds	1000-10150
6	1205.51	77.167	C-H	Tetiary amine, C-N stretch	1150-1207
7	1238.3	57.611	-	Unknown	-
8	1409.96	47.136	-	Ammonium ions	1390-1430
9	1631.78	83.433	-	Organic nitrate	1620-1640
10	2517.1	91.623	-	Unknown	-
11	2854.65	85.872	-	Methylene-CH. asym	2840-2860
12	2924.09	82.676	-	Methylene-CH. asym	2915-2935
13	3059.75	88.43	-	Unknown	-
14	3271.27	83.174	O-H	Normal polymeric O-H stretch	3200-3400

2007; Sharma et al., 2011; Chacko et al., 2012).

Identification of secondary metabolites from the methanolic crude extract of *A. alternata* by fourier-transform infrared analysis

Fourier-transform infrared analysis of dry methanolic extract of *A. alternata* proved the presence of aromatic rings, aliphatic fluoro compounds, tetiary amine, C-N stretch, ammonium ions, organic nitrate, methylene-CH. Asym, and normal polymeric O-H stretch showed major peaks at 711.73, 846.57, 873.75, 1026.13, 1149.57, 1205.51, 1238.30, 1409.96, 1631.78, 2517.10, 2854.65, 2924.09, 3059.75 and 3271.27 (Table 2 and Figure 29).

Antibacterial activity

Four clinical pathogens were selected for antibacterial activity namely, *K. pneumoniae, P. aeroginosa, E. coli* and *S. aeureus.* Maximum zone formation against *K. pneumonia* was found (5.04 ± 0.29) as shown in Table 3 and Figure 30.

Conclusion

The results of this study showed that *A. alternata* have high biological activities and produce many important secondary metabolites.

Conflict of interests

The author(s) did not declare any conflict of interest.

Table 3. Zone of inhibition (mm) of test bacterial strains to fungal products and standard antibiotics.

Fungal products antibiotics	Bacteria			
	Klebsiella pneumonia	***Pseudomonas eurogenosa***	***Staphylococcus aureus***	***Escherichia coli***
Kanamycin	1.98±0.73	0.79±0.26	0.74±0.28	1.04±0.22
Rifambin	1.01±0.50	1.081±0.37	1.59±0.36	0.90±0.54
Cefotoxime	0.95±0.84	1.06±0.55	1.19±0.40	1.19±0.62
Streptomycin	1.09±0.61	1.09±0.59	0.91±0.72	1.40±0.27
Alternaria alternata bioactive compounds	5.04±0.29	3.98±0.41	4.99±0.68	5.00±0.71

ACKNOWLEDGEMENT

The authors wish to thank Dr. Hussein for providing us the opportunity to work on this project. Also, they would like to thank O. Ali for his guidance and help in the laboratory work.

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