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Identification of phytochemical constituents from biologically active pet ether and chloroform extracts of the flowers of *Allamanda violacea* A.DC (Apocynaceae)

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ABSTRACT

Allamanda violacea is an ornamental plant of the genus Allamanda belongs to family Apocynaceae. Different flower extracts of the plant have been reported to possess anti-hyperlipidemic, anti-hyperglycemic and anti-oxidant activities. Present study deals with the identification of phytochemical constituents of the petroleum ether (pet ether) and chloroform extract by GC-MS analysis, and the constituents responsible for difference in the biological activity of these extract. Number of underivatized constituents identified in the pet ether and chloroform extracts, by comparing with the reference spectra's of NIST library, were found to be 11 and 10 respectively. The respective extracts on derivation with N, O- bis (trimethylsilyl) trifluoroacetamide (BSTFA) + trimethylchlorosilane (TMCS) [99:1 v/v] yielded their trimethyl silane derivatives. Sixteen constituents from the pet ether fraction and fourteen from chloroform fractions were identified after derivation.

Keywords: *Allamanda violacea*, pet ether, chloroform extract, phytochemical constituents, GC-MS analysis.

INTRODUCTION

Allamanda violacea (Purple Allamanda, violet Allamanda, Syn *Allamanda blanchetii*) is an ornamental plant of the genus Allamanda belongs to the Apocynaceae family. Flowers of *Allamanda violacea* are reddish purple in color and blooms during summer. Previous phytochemical examination of the roots of this plant indicated the presence of plumericin, Isoplumericin and 5, 6-dimethoxycoumarin [1]. Ethanolic extract of the root, leaves and stems of plant have been reported to possess cytostatic activities [2]. Previously we have synthesized novel pregnane derivatives as anti- oxidant and anti-dyslipidemic agent[3,4] and also isolated new drugs having potential anti-oxidant and hypolipidemic properties [5,6], in continuation of our research for the isolation of new drugs having potential anti-oxidant and hypolipidemic properties, and as no work was reported on the flowers of *Allamanda violacea* , hence different extracts of the flowers of this plant were first evaluated for their hypolipidemic, hypoglycemic and anti-oxidant activities [7]. Chloroform extract was reported to possess significant hypolipidemic, anti-oxidant and hypoglycemic activities [7]. In order to identify the constituents responsible for the significant hypolipidemic, anti-oxidant and hypoglycemic activities of chloroform extract in comparison to pet ether extract, in the present study GC-MS analysis of both the extracts was carried out.

Less polar constituents were analyzed by GC-MS method without derivation, however polar constituents present in the extract were analyzed by silylation, which is one of the best and most common methods used for converting polar constituents into less polar, more volatile and thermally more stable, thereby making them suitable for GC-MS analysis. Some of the common silylating reagents are, hexamethyldisilazane (HMDS), trimethylchlorosilane

(TMCS), trimethyl silylimidazole (TMSI), bis(trimethylsilyl)acetamide (BSA), N-methylsilyltrifluoroacetamide (MSTFA), trimethylsilyldiethylamine (TMS-DEA), N-methyl,N-t-butyldimethyl-silyltrifluoroacetamide (MTBSTFA) and N,O- bis(trimethylsilyl)trifluoroacetamide (BSTFA). In the present study BSTFA along with TMCS have been used.

MATERIALS AND METHODS

Collection of plant

The whole plant of the *Allamanda violacea* was collected in the month of October 2010 from Lucknow, India. The identity of the plant was confirmed by Dr. Tariq Hussain, Head of the Department of Taxonomy and Herbarium, National Botanical Research Institute, Lucknow, India where voucher specimen, no-97108 was deposited.

Preparation of extracts

Extracts were prepared as reported earlier by Sethi et al [7]

Thin Layer Chromatography of pet ether extract and chloroform extract

Both the extracts were analyzed with the help of thin layer chromatography. TLC was performed on silica gel G (purchased from Merck) using 2% chloroform/methanol as mobile phase. Corresponding spots were developed using 10% H_2SO_4 as a spraying reagent.

Only 13 and 11 spots were observed in pet ether and chloroform extract respectively **[Fig.1.]**

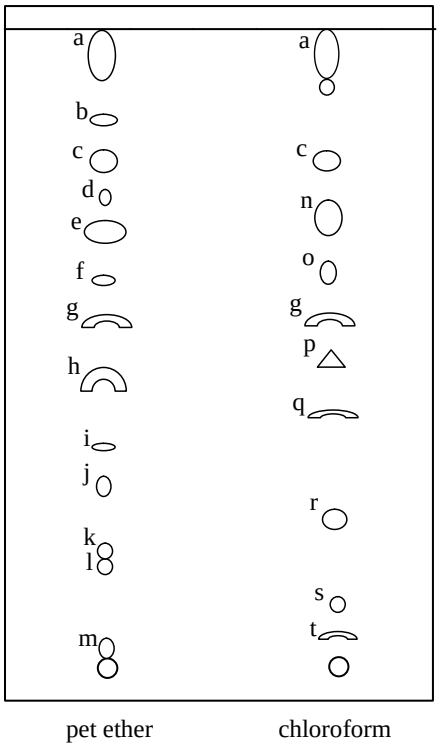

Fig.1.

Both the extracts showed some common spots denoted as ‘a’, ‘c’ and ‘g’, which is probably in good correlation with the similar mass spectrum of three constituents as obtained in GC-MS analysis.

Preliminary Phytochemical screening of extracts

Both the extracts were subjected to phytochemical tests for the detection of carbohydrates and/ or glycosides, tannins, flavonoids, sterols and/or terpenes. pet ether, and chloroform extracts gave positive Liebermann Burchardt test (for sterol and/or terpenes) [8], Liquid ammonia, NaOH and Shinoda test (for flavones) [9, 10] Feigl test (for

normal sugars) [11] Vanillin perchloric acid test (for the presence of 2-deoxy and 6-deoxy sugars) [12] indicating the presence of flavones/sterols/ terpenes in their free state or in form of their glycosides.

GC-MS analysis and conditions

GC-MS analysis was performed on a Trace GC ultra gas chromatography coupled to TSQ quantum XLS mass spectrometer (Thermo Scientific, USA). constituents were separated on a Column TG-5MS (5% phenyl:95% dimethyl polysiloxane) having dimensions of 0.25 µm film thickness x 0.25 i.d x 30 m length using Helium as a carrier gas with the flow rate of 1 mL/min.

1µmL of each samples were injected in splitless mode where injector temperature and ion source temperature were 280^{o}C and 220^{o}C respectively.

Oven temperature was initially maintained at 70^{o}C for 2 minutes and then increased to 150^{o}C, the rate of an increase being 10^{o}C/min and this temperature was maintained for 3 minutes and finally the temperature was increased upto 280^{o}C for 25 minutes. Mass spectrum was recorded at 70ev with a scan interval of 0.5 seconds and mass range from 50-650 amu.

The non-derivatized samples of both pet ether and chloroform extract were dissolved in chloroform and run as mentioned above.

Trimethylsilyl derivatives of extracts were prepared by dissolving in chloroform and then added 60µl pyridine + 40µl BSTFA [BSTFA + TMCS; 99:1 v/v] diluted with ethyl acetate followed by heating at 70^{o}C for 30 minutes. Derivatized extracts were then subjected to GC-MS analysis following the same procedure as discussed above.

Identification of constituents

Mass spectra of each constituent were compared with the database of National Institute of Standard and Technology (NIST). Molecular mass, name and structure of the constituents of both the extracts were ascertained [13, 14]

RESULTS AND DISCUSSION

Phytochemical constituents present in the pet ether and chloroform extracts of the flowers of *Allamanda violacea* were identified by GC-MS analysis and mass spectra of constituents were compared with the NIST library. In pet ether extract 11 peaks were identified without silylation indicating the presence of 11 phytochemical constituents. Active constituents with their retention time (RT), name, molecular formula, molecular weight and % peak area are listed in (**Table.1.)**. Two constituents namely hexadecanoic acid ethyl ester and ethyl 9-cis 11- trans-octadecadienolate were found to be major with 24.55% and 36.39% peak area respectively.

Sixteen constituents were identified in their silyl form, except for hexadecanoic acid ethyl ester, which was also identified in its underivatized form; all other 15 constituents were only identified after silylation. Amongst the sixteen constituents, seven steroids namely, androsta-5, 7-diene 4, 4 dimethyl, hydrocortisone acetate, ergosta-5, 7, 22-trien-3ol (3β, 22E), 11-methylspirostan-3, 11-diol, campesterol, stigmasterol, β sitosterol were identified (**Table.2.**). Three constituents of the pet ether extract after silylation namely hexadecanoic acid ethyl ester, 9-octadecenoic acid ethyl ester and 9. 12- octadecadienoic acid were identified as major, with % peak areas being 15.63, 47.26 and 15.21 respectively. Biological activities of compounds identified in pet ether extract are listed in **(Table.3)**.

In the chloroform extract, eleven constituents were detected without silylation (**Table.4**). Naphthalene was the major with 54.09% peak area. After silylation fourteen other compounds were identified including 6 steroids, out of which 3 steroids namely, β sitosterol, campesterol, stigmasterol, were also present in pet ether extract while other 3 steroids namely, cholesterol, corticosterone and cholest-2-eno [2, 3-b] naphthalene, 7'-methoxy identified were new **(Table.5).** Biological activities of constituents identified in chloroform extract are listed in **(Table.6)**.

Except β sitosterol, campesterol and stigmasterol all the other constituents, identified in pet ether and chloroform extracts were different.

Thus in pet ether extract, eleven constituents were identified in underivatized form while sixteen constituents were identified after derivation (with one common constituent) hence total 26 constituents were identified in pet ether extract by GC-MS analysis.

Similarly in chloroform extract, ten constituents were found to be present in underivatized form while fourteen constituents were identified after derivation. Hence a total of 24 constituents were identified in chloroform extract by GC-MS analysis.

Four constituents (hexadecanoic acid ethyl ester, campesterol, stigmasterol and β-sitosterol) in pet ether extract were found to possess anti-oxidant activity while in chloroform extract seven constituents (1-hexadecanol, hexadecanoic acid, 1, 2-benzenedicarboxylic Acid, mono (2-ethylhexyl) ester, β-sitosterol, cholesterol, campesterol and stigmasterol) were found to possess anti-oxidant activity. These finding correlates significant anti-oxidant activity of chloroform extract in comparison to pet ether extract.

In pet ether extract one constituent namely, tetradecanoic acid ethyl ester was found to be hypercholesterolemic while six constituents, namely, hexadecanoic acid methyl ester, hexadecanoic acid ethyl ester, 9, 12 octadecadienoic acid (Z,Z), campesterol, stigmasterol and β sitosterol were found to possess hypocholesterolemia, out of these campesterol, stigmasterol and β sitosterol were present in very negligible proportion, while in chloroform extract four constituents namely, hexadecanoic acid, campesterol, stigmasterol and β sitosterol were found to possess hypocholesterolemia. High hypocholesterolemic activity of chloroform extract can be attributed to the presence of these constituents in higher proportion and also due to some constituents present in the extract which could not be identified by GC-MS analysis.

High hypoglycemic activity of chloroform extract is attributed to the presence of corticosterone that has been previously reported to possess intractable hypoglycemic activity [15]

Constituents that could not be identified by GC-MS analysis of the respective extracts are being isolated by column chromatography.

Table.1. Phytochemical constituents present in pet ether extract of the flowers of *Allamanda violacea* detected by GC-MS without silylation.

S.NO	RT	Name of compound	Mol. Formula	Mol. Wt	peak area %
1	14.56	fumaric acid ethyl Undec-2en-1yl-ester	$C_{17}H_{28}O_4$	296	0.43
2	16.00	tetradecanoic acid ethyl ester	$C_{16}H_{32}O_2$	256	2.75
3	16.65	hexadecanoic acid methyl ester	$C_{17}H_{34}O_2$	270	2.86
4	16.99	hexadecanoic acid ethyl ester	$C_{18}H_{36}O_2$	284	24.55
5	17.66	ethyl 9-cis 11-trans octadecadienoate	$C_{20}H_{36}O_2$	308	36.39
6	17.73	octadecanoic acid ethyl ester	$C_{20}H_{42}O_2$	312	5.36
7	18.11	tricosane	$C_{23}H_{48}$	324	4.99
8	19.01	tricontane	$C_{30}H_{62}$	422	7.42
9	19.55	nonadecanoic acid ethyl ester	$C_{21}H_{42}O_2$	326	5.70
10	20.26	tetratriacontane	$C_{34}H_{70}$	478	6.98
11	21.07	ethyl tetracosanoate	$C_{26}H_{52}O_2$	396	2.56

Chromatogram of pet ether fraction without silylation is shown in **Fig2**. Mass spectrum of fumaric acid ethyl undec-2en-1yl-ester and tetradecanoic acid ethyl ester are shown in **Fig3.** and **Fig4.** respectively.

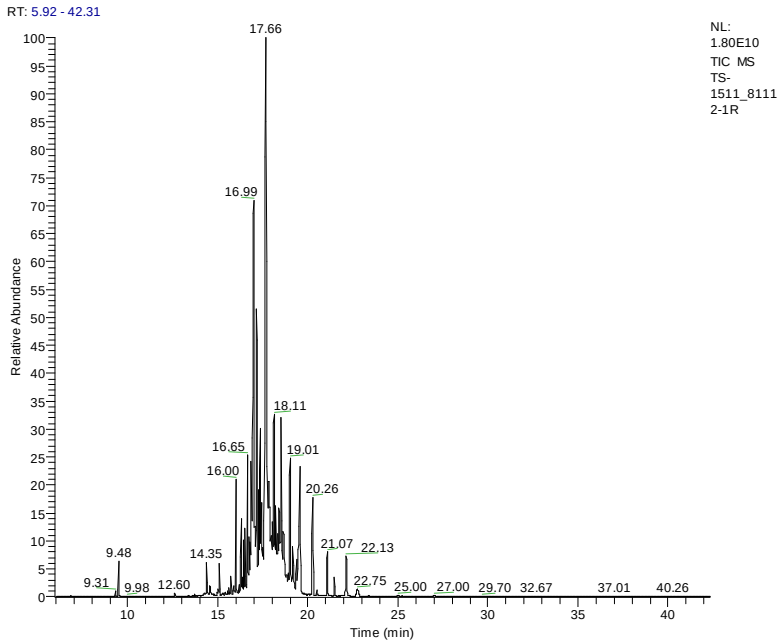

Fig.2.total ion chromatogram of pet ether fraction without silylation

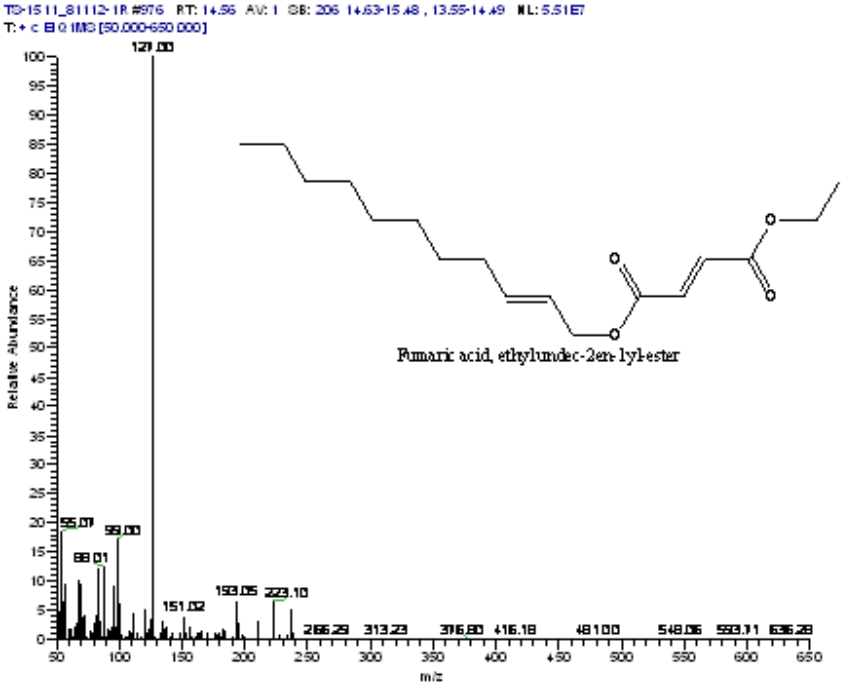

Fig.3. mass spectrum of fumaric acid ethylundec-2en-1yl-ester

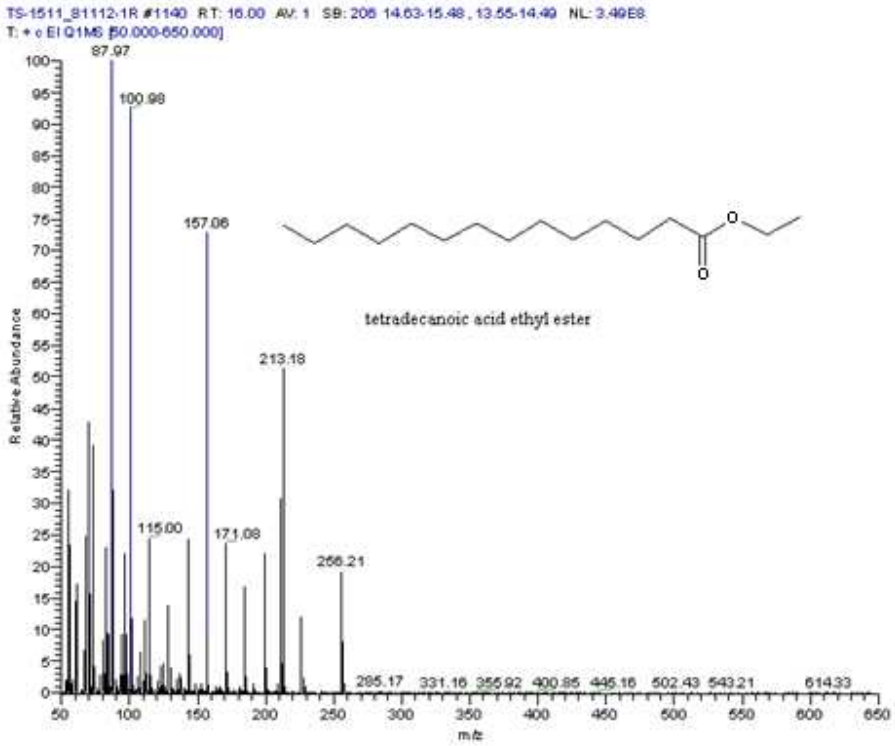

Fig.4. mass spectrum of tetradecanoic acid ethyl ester

Table.2. Phytochemical constituents present in pet ether extract of the flowers of *Allamanda violacea* detected by GC-MS after silylation,

S.No	RT	Name of compound	mol. formula	mol. wt	peak area %
1	16.94	hexadecanoic acid ethyl ester	$C_{18}H_{36}O_2$	284	15.63
2	17.60	9-octadecenoic acid (Z) ethyl ester	$C_{20}H_{38}O_2$	310	47.26
3	17.76	9,12-octadecadienoic acid(Z,Z)	$C_{18}H_{32}O_2$	280	15.21
4	18.19	androsta-5,7-diene,4,4-dimethyl	$C_{21}H_{31}$	284	2.64
5	18.29	hydrocortisone acetate	$C_{23}H_{32}O_6$	404	2.17
6	18.38	ergosta-5,7,22-trien-3ol(3β,22E)	$C_{28}H_{44}O$	396	1.40
7	18.47	methyl 19-methyl-eicosanoate	$C_{22}H_{42}O_2$	340	3.34
8	18.67	eicosanoic acid	$C_{20}H_{42}O_2$	312	3.04
9	18.96	11-methyl spirostan-3,11-diol	$C_{28}H_{46}O_4$	446	1.63
10	19.12	hexadec-2-enylsuccinic Anhydride	$C_{20}H_{34}O_3$	322	1.23
11	19.34	1,2-benzenedicarboxylic, bis(2-ethylhexyl)ester	$C_{24}H_{38}O_4$	390	2.07
12	19.50	hexadecanoic acid, 2-(hydroxy)-1,3- propanediol ester	$C_{35}H_{68}O_5$	568	3.83
13	19.78	docosanoic acid	$C_{22}H_{42}O_2$	340	0.40
14	28.81	campesterol	$C_{28}H_{48}O$	400	0.02
15	29.59	stigmasterol	$C_{29}H_{48}O$	412	0.07
16	31.15	β-sitosterol	$C_{29}H_{50}O$	414	0.05

Chromatogram of pet ether fraction after silylation is shown in **Fig.5.** Mass spectrum of 9, 12-octadienoic acid (Z,Z)- trimethylsilyl ester and stigmasterol trimethylsilyl ester are shown in **Fig.6.** and **Fig.7.** respectively. Biological activities of the constituents present in pet ether extract are shown in table.3.

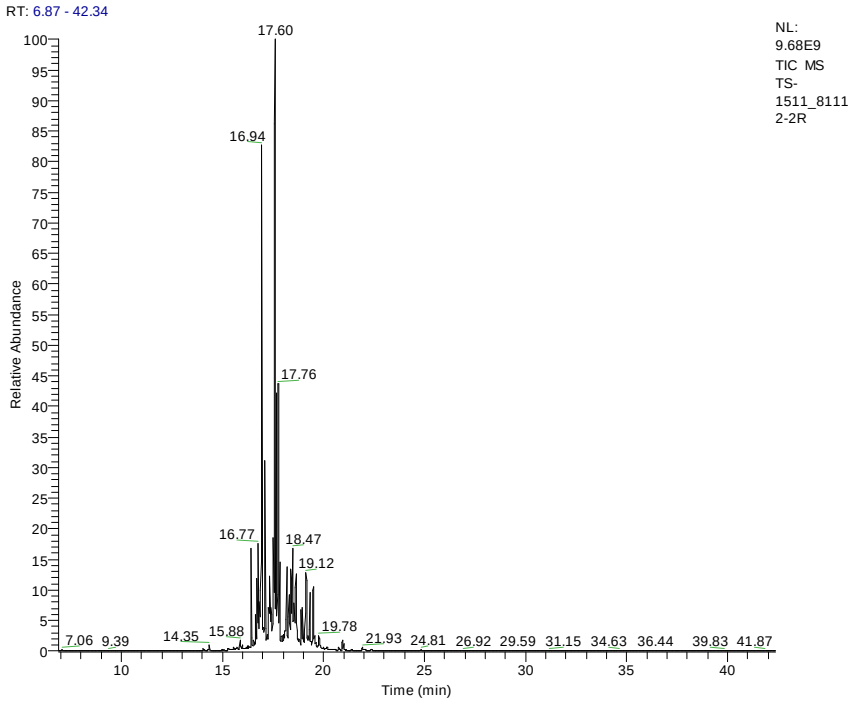

Fig.5.total ion chromatogram of pet ether fraction after silylation

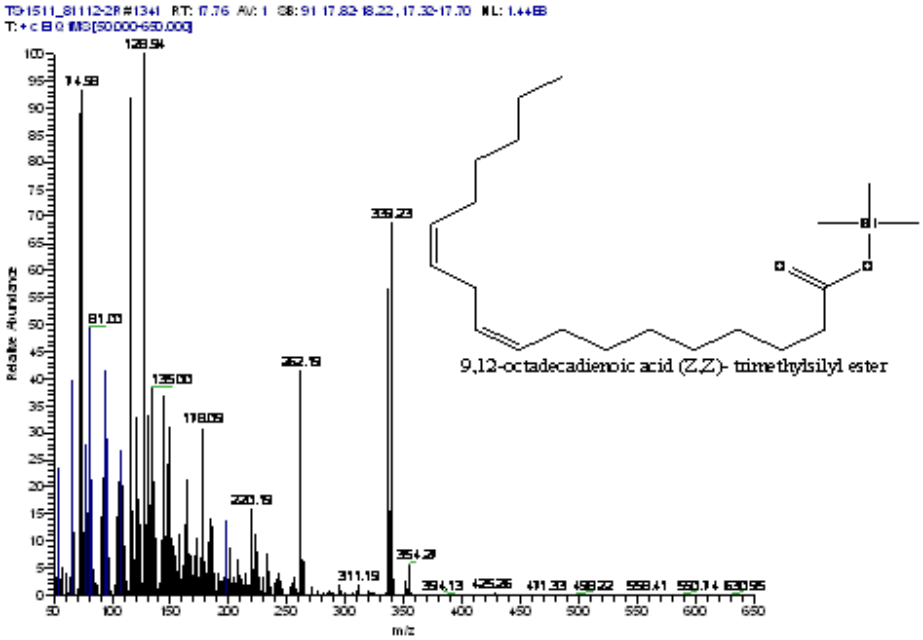

Fig.6. mass spectrum of 9, 12-octadienoic acid (Z,Z)- trimethylsilyl ester

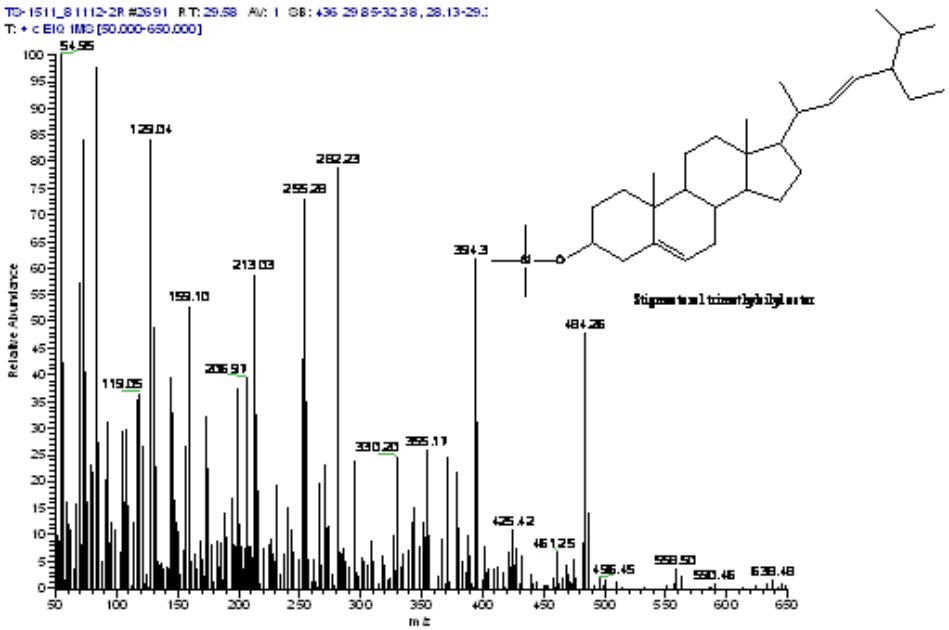

Fig.7. mass spectrum of stigmasterol trimethylsilyl ester

Table.3. Biological activity of constituents identified in pet ether extract of the flowers of *Allamanda violacea* by GC-MS.

Compound	Biological activity
Fumaric acid ethyl Undec-2en-1yl-ester	no activity reported
Tetradecanoic acid ethyl ester	Hypercholesterolemic [16]
Hexadecanoic acid methyl ester	expression of cyclooxygenase-2 inhibition of NF- κB[11]anti-oxidant, hypocholesterolemic [17]
Hexadecanoic acid ethyl ester	anti-oxidant nematicide and hypocholesterolemic*
Ethyl 9-cis 11-trans octadecadienoate	no activity reported
Octadecanoic acid,ethyl ester	perturbs the cell cycle and induces apoptosis in Hep-G_2 cell [16]
Tricosane	no activity reported
Tricontane	no activity reported
Nonadecanoic acid ethyl ester	no activity reported
Tetratriacontane	no activity reported
Ethyl tetracosanoate	no activity reported
9-octadecenoic acid (Z) ethyl ester	steroids and primer pheromone [16]
9,12-octadecadienoic acid (Z,Z)	anti-inflammatory, nematicide, hypocholesterolemic, antiarthritic, antieczemic*
Androsta-5,7-diene,4,4-dimethyl	no activity reported
Hydrocortisone acetate	anti-inflammatory [17]
Ergosta-5,7,22-trien-3ol(3β,22E)	antiangiogenic, antiflu, anti tumor, and antiviral*
Methyl 19-methyl-eicosanoate	no activity reported
11-methyl spirostan-3,11-diol	no activity reported
hexadec-2-enylsuccinic Anhydride	no activity reported
1,2-benzenedicarboxylic, bis(2-ethylhexyl)ester	oral toxicity during pregnancy and suckling in the long-evans rat [18]
Hexadecanoic acid, 2-(hydroxy)-1,3- propanediol ester	no activity reported
Docosanoic acid	no activity reported
Campesterol	anti-oxidant and hypocholesterolemic*
Stigmasterol	antihepatotoxic, anti-oxidant hypocholesterolemic, anti-inflammatory [19] estrogenic, antiviral*
β-sitosterol	anti-oxidant [20], analgesic anti-inflammatory [21], hypocholesterolemic [22]

**Dr Dukes phytochemical and ethnobotanical database*

Table.4. Phytochemical constituents present in chloroform extract of the flowers of *Allamanda violacea* detected by GC-MS without silylation

S.NO	RT	Name of compound	mol. formula	mol. wt	peak area %
1	8.31	naphthalene	$C_{10}H_8$	128	54.09
2	11.17	cyclotetradecane	$C_{14}H_{28}$	196	3.41
3	13.58	2-tert-butyl-4isopropyl 5- methyl phenol	$C_{14}H_{22}O$	206	4.94
4	14.52	1-hexadecanol	$C_{16}H_{34}O$	242	8.06
5	15.50	4[2,4,4 trimethyl pentan-2-yl] Phenol	$C_{14}H_{22}O$	206	3.79
6	15.69	4-[4-ethyl-5-(4-hydroxyphenyl) hexan-3yl]phenol	$C_{20}H_{26}O_2$	298	0.94
7	15.97	nonadecene	$C_{19}H_{38}$	266	8.07
8	16.89	1-docosene	$C_{22}H_{44}$	308	7.83
9	17.63	9-hexacosene	$C_{26}H_{52}$	364	5.73
10	18.40	17- pentatriacontene	$C_{35}H_{70}$	491	3.13

Chromatogram of chloroform fraction without silylation is shown in **Fig.8**. Mass spectrum of Naphthalene and cyclotetradecane are shown in **Fig.9**. and **Fig.10.** respectively.

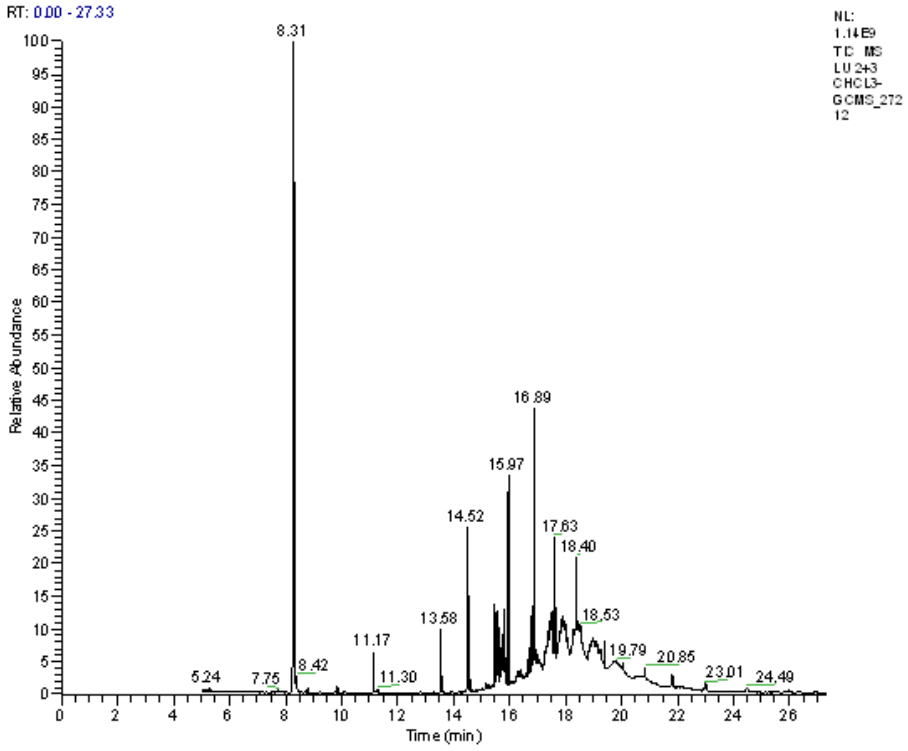

Fig8.total ion chromatogram of chloroform fraction without silylation

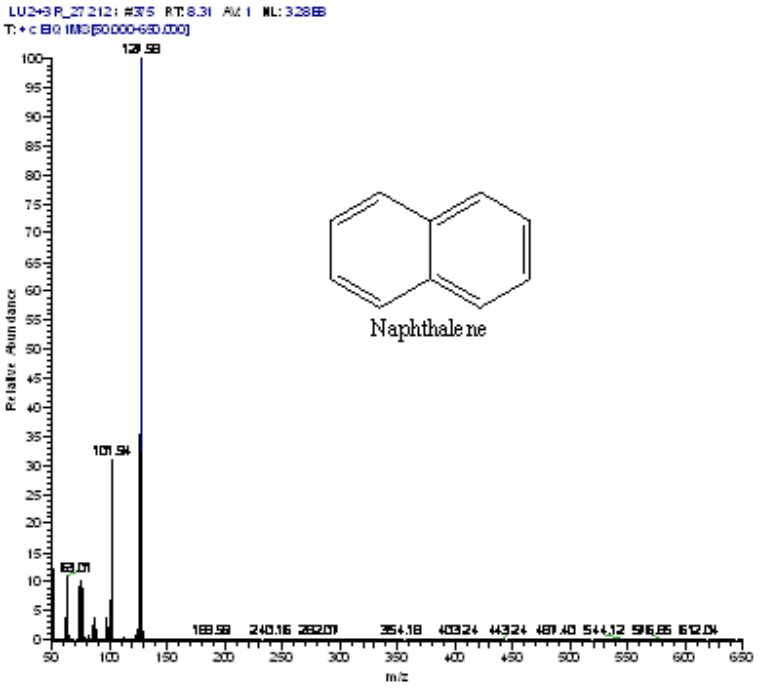

Fig9. mass spectrum of naphthalene

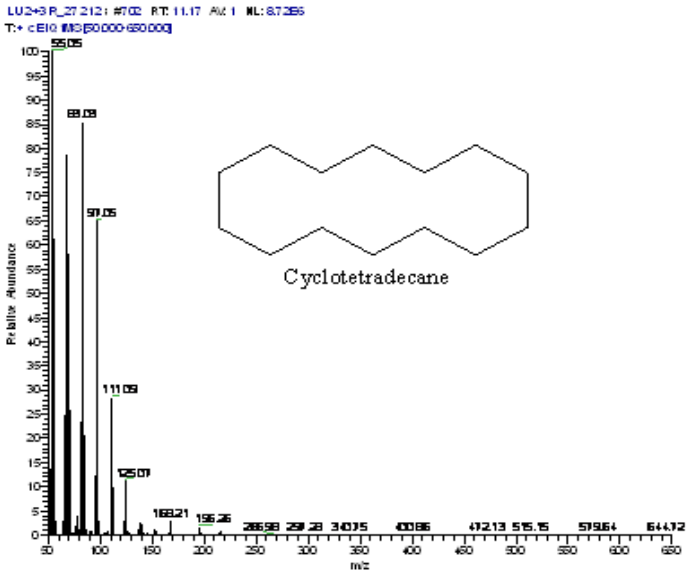

Fig10. mass spectrum of cyclotetradecane

Table.5. Phytochemical constituents present in chloroform extract of the flowers of *Allamanda violacea* detected by GC-MS after silylation

S.NO	RT	Name of compound	mol. formula	mol. wt	peak area %
1	7.98	3- hydroxybutanoic acid	$C_4H_8O_3$	104	1.28
2	8.45	Tricyclo[4,3,2,0(1,4)undeca-2,4(5),8,10 tetraen-7-one	$C_{11}H_8O$	156	0.58
3	9.51	propane 1,2,3 triol	$C_3H_8O_3$	92	1.56
4	17.10	hexadecanoic acid	$C_{16}H_{32}O_2$	256	12.29
5	17.81	3'-(3''-nitrophenyl)-spiro(indoline (3,2')-thiazolidine)- 2,3'(1H)-dione	$C_{16}H_{11}N_3O_4S$	341	54.80
6	19.28	1,2-Benzenedicarboxylic Acid, mono(2-ethylhexyl) ester	$C_{16}H_{24}O_3$	264	9.38
7	19.70	β-sitosterol	$C_{29}H_{50}O$	414	5.89
8	26.07	cholesterol	$C_{27}H_{46}O$	386	0.42
9	28.37	campesterol	$C_{28}H_{48}O$	400	1.12
10	29.23	stigmasterol	$C_{29}H_{48}O$	412	4.82
11	29.61	N,N'-Dicyclohexyl-1-cyano7-pyrrolidinylperylene-3,4,9,10 tetracarboxylic acid bismide	$C_{41}H_{36}N_4O_4$	648	0.66
12	38.85	corticosterone	$C_{21}H_{30}O_4$	346	0.15
13	40.61	2,11,13,22,23,25-Hexaoxa-1,12(1,3,2)dibeza-24(2,9)-1,10-phenanthrolinabicyclo[10,10,3] pentacosaphane	$C_{40}H_{44}N_2O_6$	648	1.19
14	41.54	cholest-2-enol[2,3]b naphthalene, 7' methoxy	$C_{35}H_{52}O$	501	0.25

Chromatogram of chloroform fraction after silylation is shown in **Fig11**. Mass spectrum of butanoic acid, 3-[(trimethylsilyl) oxy]-,trimethylsilyl ester and β sitosterol trimethylsilyl ester are shown in **Fig12.** and **Fig.13**. respectively. Biological activities of the constituent present in chloroform extract are shown in **Table.6**.

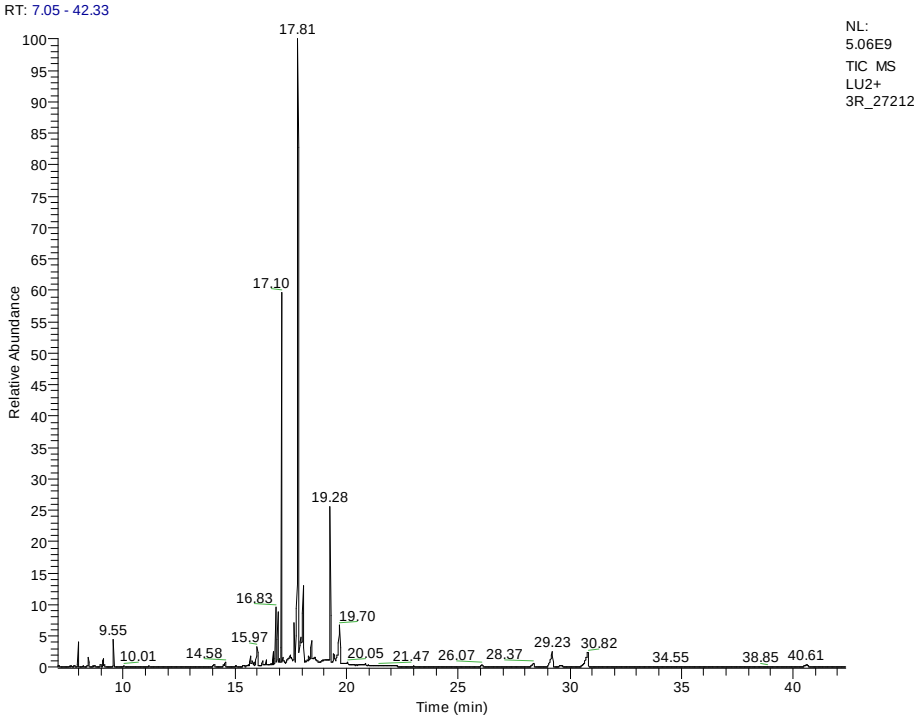

Fig11.total ion chromatogram of chloroform fraction after silylation

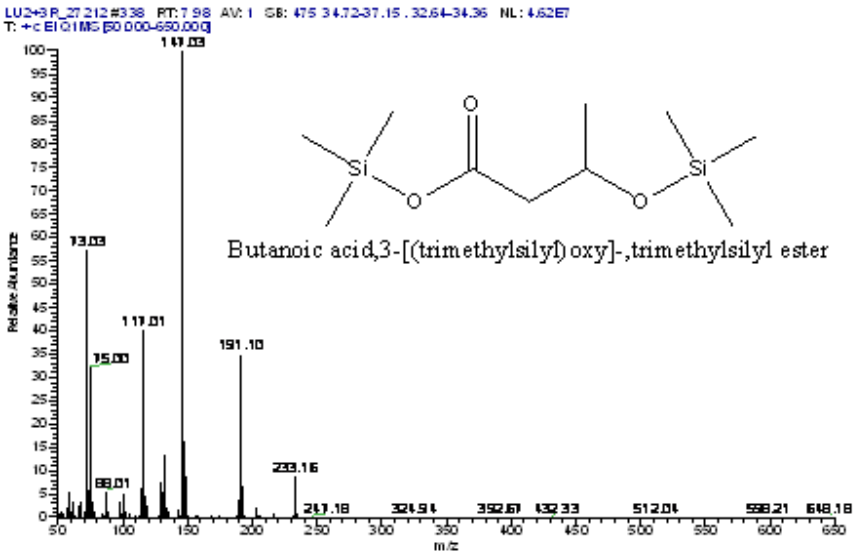

Fig12. mass spectrum of butanoic acid, 3-[(trimethylsilyl) oxy]-,trimethylsilyl ester

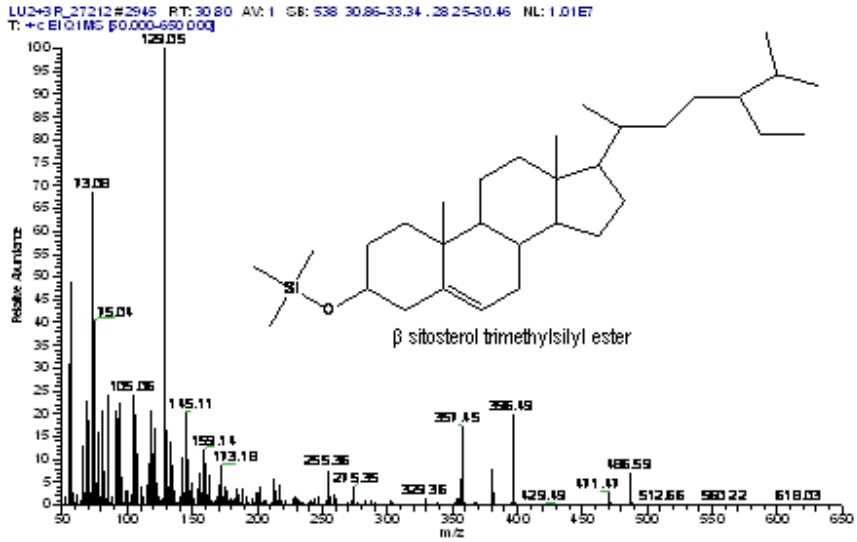

Fig13. mass spectrum of β sitosterol trimethylsilyl ester

Table.6. Biological activity of constituents identified in chloroform extract of the flowers of *Allamanda violacea* detected by GC-MS.

Compound	Biological activity
Naphthalene	cataractagenic*, anti- insect, anti- microbial [23, 24]
Cyclotetradecane	no activity reported
2-tert-butyl-4-isopropyl 5- methyl phenol	no activity reported
1-hexadecanol	anti-oxidant [25]
4[2,4,4 trimethyl pentan-2-yl] Phenol	no activity reported
4-[4-ethyl-5-(4-hydroxyphenyl) hexan-3yl]phenol	no activity reported
Nonadecene	no activity reported
1-docosene	no activity reported
9-hexacosene	analgesic and anti-inflammatory [26]
17- pentatriacontene	no activity reported
3- hydroxybutanoic acid	selective GABAB receptor agonist [27], treatment of alcoholism [28]
Tricyclo[4,3,2,0(1,4)undeca-2,4(5),8,10 tetraen-7-one	no activity reported
Propane 1,2,3 triol	anticataract, antinueralgic, antirythmigenic, hyperglycemic*
Hexadecanoic acid	5α reductase inhibitor, hemolytic, hypocholesterolemic, anti-oxidant* nematicide, pesticide [29]
3'-(3''-nitrophenyl)-spiro(indoline (3,2')-thiazolidine)- 2,3'(1H)-dione	no activity reported
1,2-Benzenedicarboxylic Acid, mono(2-ethylhexyl) ester	anti-oxidant and anti-inflammatory [30]
β-Sitosterol	anti-oxidant [20], analgesic and anti-inflammatory[21], hypocholesterolemic [22]
Cholesterol	anti-oxidant*
Campesterol	anti-oxidant and hypocholesterolemic*
Stigmasterol	antihepatotoxic, anti-oxidant hypocholesterolemic, anti-inflammatory [19] estrogenic, antiviral*
N,N'-Dicyclohexyl-1-cyano7-pyrrolidinylperylene-3,4,9,10 Tetracarboxylic acid bismide	no activity reported
Corticosterone	Hypoglycemic [15]
2,11,13,22,23,25-Hexaoxa-1,12(1,3,2)dibeza-24(2,9)-1,10 Phenanthrolinabicyclo[10,10,3]-Pentacosaphane	no activity reported
Cholest-2-eno[2,3-b] naphthalene, 7' methoxy	no activity reported

**Dr Dukes phytochemical and ethnobotanical database*

CONCLUSION

GC-MS analysis has helped in exploring phytochemical constituents present in pet ether and chloroform extracts of the flowers of *Allamanda violacea* and showed the presence of 26 known components in pet ether extract and 24 known components in the chloroform extract. In pet ether extract one component, hexadecanoic acid ethyl ester was detected in both underivatized and derivatized samples while in chloroform extract no component was found to be common in derivatized and underivatized samples. Activities possessed by different flower extracts as reported earlier by Sethi et al [7] are in good agreement with GC-MS analysis.

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