Journal Homepage: -www.journalijar.com
INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)
Article DOI:10.21474/IJAR01/8302
DOI URL: http://dx.doi.org/10.21474/IJAR01/8302

RESEARCH ARTICLE

ANTIMICROBIAL AND GCMS ANALYSIS OF CHLOROFORM EXTRACT OF PSEUDARTHRIA VISCIDA (L.) WIGHT AND ARN. AND ASSOCIATED MAJOR FUNGAL ENDOPHYTE.

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Manuscript Info

Manuscript History
Received: 01 November 2018
Final Accepted: 03 December 2018
Published: January 2019

Key words:-
Pseudarthria viscida, Endophyte, Chloroform extract, Antimicrobial, GC-MS.

Abstract

Pseudarthria viscida a member of Fabaceae family possess great medicinal attributes.The present study dealt with antimicrobial ability of chloroform extract P. viscida and its associated major fungal endophyte and their phytochemical analysis via GCMS. Pseudarthria viscida yielded two endophytes Colletotrichum gloeosporioides (Pv-1) and C. lindemuthianum (Pv-2) and isolation frequency resulted C. gloeosporioides (Pv-1) to be major one. Chloroform extracts at different concentrations (25, 50, 75 and 100µl) of P. viscida leaves, mycelial mat (µg/ml)and culture filtrate (v/v) of major endophyte C. gloeosporioides(Pv-1) were tested for their antimicrobial activity against two bacterial species, Escherichia coli and Staphylococcus aureus and two fungal species, Fusarium oxysporum and Cladosporium cladosporioides respectively. The extracts were found to show inhibitory activity against all the studied pathogens. GC-MS analysis of both plant and fungal endophyte resulted in the presence of 16 compounds from P. viscida plant extracts, 16 from 16 from Pv-1 C.F. and 20 from Pv-1 M.E. In case of P. viscida and its endophyte Pv-1, 2, 4-Ditert-butylphenol, E-14-Hexadecenal, E-15-Heptadecenal, 1-Hexadecene, Cyclotetracosane, Octadecyl trifluoroacetate were similarbut their area percentage varied. Majority of the compounds from extracts were identified to possess therapeutic properties and further chemical evaluation is needed for its successful application.

Introduction:-

Medicinal plants and products derived from themhave been in use for the treatment of various ailments. Plant synthesis hundreds of chemical compounds and have been tested for biological, antimicrobial, hypoglycemic, antiulcerogenic, antihelminthic, hepatoprotective, analgesic, antipyretic, antileishmania and insecticidal activities (Slutsker et al., 1998). Plants possessing these traits have been attributed to secondary metabolites synthesized by plants (Selvamohan et al., 2012).

Pseudarthria viscida (L.) Wight & Arn. is a semi-erect sub-shrub, with slender, reddish brown branch lets, leaves are alternate, tri-foliate leaflets, distributed in the Indo-Malesian region and SriLanka. In India it is recorded in the states of Gujarat, Orissa, Karnataka, Tamil Nadu and Kerala. It is used in treatment of rheumatic arthritis, intestinal worm,

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asthma, fever, dysentery, cardiac problems, tuberculosis, diabetes, piles, insect bites and to curebone fracture (Shanthakumar et al., 2009).

A number of endophytes have been found associated with various plant species which comprise bacterial and fungal colonies (Petrini and Fisher, 1990 and Arnold, 2000). Fungal endophytes form symbiotic associations and thrive in millions of unique ecological niches in many unusual environments almost ubiquitously throughout the plant kingdom and serves as an indirect defense against herbivores (Baccon and white 2000, Schulz et al., 2002; Strobel and Daisy 2003; Strobel et al., 2004; Bandara et al., 2006).

They have proven to be a promising source of new and biologically active natural products which are of interest for specific medicinal or agrochemical applications (Strobel, 2002). Several studies have demonstrated the ability of endophytic fungi to produce various compounds such as enzymes (Teske and Trentini, 1995; Bezerra et al., 2012b), antitumor substances (Chandra, 2012), antimicrobial substances (Souza et al., 2004; Siqueira et al., 2011) and plant growth hormones (Hwang et al., 2011). Industrial application resulted endophytes as an alternative tool for the mass production of the biologically active compounds (Zhao et al., 2010).

Although higher concentration of secondary metabolites might result in a more resistant plant, the production of secondary metabolites thought to be costly and reduces plant growth and reproduction. Hence plant have evolved induced defense, where concentration generally increase only in stress conditions (Simms et al., 1992). Hence the present study has been carried out to study antimicrobial ability of chloroform extract P. viscida and its associated major fungal endophyte and their phytochemical analysis using GCMS.

Materials and Methods:-

Sample collection:-

Healthy leaf sample of Pseudarthria viscida were randomly collected from in and around Kerala Forest Research Institute (KFRI) campus.

Isolation of Endophytic Fungi:-

The healthy leaf sample of P. viscida were washed 2-3 times under running tap water. Surface sterilization was performed by sequentially rinsing leaves with 70% ethanol for 2 minutes, then with 4% sodium hypochlorite for 60 seconds followed by ethanol for 30 seconds and finally rinse with sterile distilled water for 2-3 times.

The surface sterilized leaves were blotted and cut into small pieces (2mm) and about 10 leaf segments were placed at equal distance on 5 plates of potato dextrose agar (PDA) and oat meal agar (OMA) supplemented with antibioticand incubated at 25±2° C for 5-7 days (extended to two weeks) under 12hrs alternating light and dark conditions to promote the growth of endophytes. Fungal colonies were isolated and identified on the basis of colony morphology, mycelium, fruiting-body, spore shape and size by referring standard manuals (Arx, 1981; Ellis and Ellis, 2001; Gilman, 1994; Ramarao and Manoharachary, 1990; Subramanian, 1983) and isolation frequency of various isolates were calculated using the formula:

$$\text{Isolation frequency (IF)} = \frac{\text{Number of single endophytic fungus isolated in each bits}}{\text{Total number of bits observed}} \times 100$$

Preparation of Chloroform Extracts:-

Plant Extract Preparation:-

The selected plant (P. viscida) leaves were washed in running tap water, followed bysterile distilled water. They were shade dried at room temperature and ground into coarse powder. Powdered leaf samples were thoroughly mixed with chloroform and kept for three days provided with rotatory shaker and were filtered through Whatman No.1filter paper. The chloroform extracts of leaf samples were stored in darkness.

Preparation of Fungal Extract:-

The major endophytic fungus from the plant species was selected for extract preparation. Endophytic fungus was grown on PDA at 25±2° C for 7 days and ten mycelial discs (7mm) were inoculated into 500 ml Erlenmeyer flasks

containing 200ml of antibiotic amended potato dextrose broth (PDB) and incubated at 25±2° C for 15 days. The broth cultures were filtered through Whatman No.1filter paper and centrifuged to separate mycelial fragmentsand were denoted as mycelial mat extract (M.E.) and broth was denoted as culture filtrate (C.F.). Both the mycelial mat and culture filtrates were extracted with chloroform.

Antimicrobial Assay:-

Chloroform extracts of plant (P. viscida), mycelial mat and culture filtrate were tested for their antimicrobial activity against two bacteria (Escherichia coli and Staphylococcus aureus) and two fungi (Fusarium oxysporum and Cladosporium cladosporioides) at different concentrations (25, 50, 75 and 100µl) and incubated on Nutrient Agar (NA) medium at 37° C for bacteria and 25±2° C on PDA medium for fungi. The plates were observed after 24 hours of incubation for bacteria and after 7 days for fungi to determine minimum inhibitory concentration.

GC – MS Analysis:-

GC – MS analysis was carried out using QP2010S Shimadzu GC-MS instruments (30m x 0.25mm x 0.25µm, Rxi-5Sil MS). 1µl of chloroform extract was injected into the GC-MS instrument. Initially the column temperature was maintained at 80°C for 2 minutes, followed by a temperature gradient from 80°C to 200°C and held constant for 2 minutes and finally raised temperature to 260°C and held constant for 2 minute. The instruments operated in a split mode and Libraries used for analysis were NIST 11& WILEY 8.

Results and Discussions:-

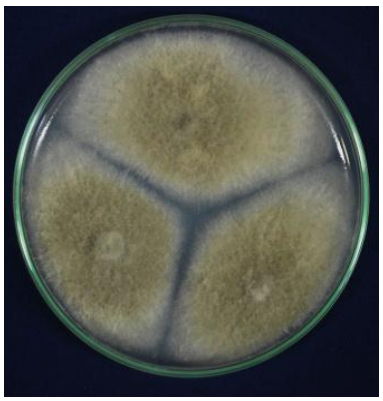
Isolation and Identification of Endophytic fungi:-

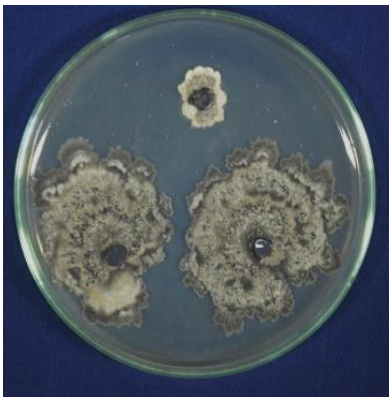
Pseudarthria viscida leaves on PDA and OMA medium, resulted in the isolation of two endophytes namely Colletotrichum gloeosporioides (Pv-1) and C. lindimuthianum (Pv-2) (Table 1, Fig 1). Colletotrichum gloeosporioides was used to analyze its antimicrobial ability and for secondary metabolites. Various species of Colletotrichum have been isolated as endophytic fungi from various medicinal plants (Rakotonirianaet al., 2007, Gangadevi et al. 2008, Bhagyaet al., 2011).

Table 1:-Isolation frequency of Endophytic fungi from selected medicinal plants

Sl.no.	Endophytic fungi	Morphological characteristics	P .viscida Isolation frequency (IF) %	
			PDA	OMA
1	C. gloeosporioides	Upper surface of colony white colony with orange oozes at center, cottony in nature with striated margin, hyaline. Reverse grayish black colony, center hyaline and undulate margin, white or hyaline.	32	46
2	C. lindemuthianum	Upper surface of colony greyish green colony velvety in nature with undulate margin. Reverse greyish black colony center hyaline with alternate circles and undulate margin, white or hyaline.	24	34

Fig 1:-Endophytes isolated form Pseudarthria viscida.


Pv-1- *C.* gloeosporioides


Pv-2- C. lindimuthianum

Antimicrobial assay of chloroform extracts:-

Chloroform extracts of the plant P. viscida, mycelial mat (µg/ml) and culture filtrate (v/v) of major endophyte Pv-1 (Fig 2, 3 & 4) were tested for their antimicrobial activity against two pathogenic bacterial species, Escherichia coli and Staphylococcus aureus and two fungal species, Fusarium oxysporum and Cladosporium cladosporioides. Different concentrations of extracts (25, 50, 75 and 100µl) were studied. All the chloroform extracts were found to show good inhibitory activity against all the studied pathogens. Antibacterial activity of P. viscida showed an inhibition of 8-9 mm at all concentrations of extracts. On the other hand endophytic fungi Pv-1, both C.F. and M.E. showed inhibitory activity against E. coli at all concentrations and 25µl found to be minimum inhibitory concentration but against S. aureus, 75µl of C.F. and 50 µl of M. E. showed minimum inhibitory activity (Table 2).

Antifungal activity resulted in zone of inhibition against F. oxysporum by both, plant and fungal chloroform extracts. The Pseudarthria viscida plant extract showed an inhibition of 9-10mm at all concentrations. In case of Pv-1, both C.F. and M.E. all concentrations showed inhibitory activity against F. oxysporum. An inhibition of 8mm in C.F. and 9 mm in M.E. at 25µl were found to be minimum inhibitory concentration. For C. cladosporioides, none of the concentrations of plantextractwere effective in the management of fungus. For endophyte Pv-1, C.F. and M. E. at concentration 100 µl showed inhibition activity whereas rests of the concentrations were ineffective (Table 3). P. viscida methanol extract has been characterized by various workers for the identification of compounds and its antimicrobial activity (Baskar et al., 2012; Hemlal and Subban, 2012). Studies have shown potentiality of plant extracts against various microbial agents and standardization of solvent system for efficient activity.

Table 2:-Antibacterial activity of chloroform extracts of selected medicinal plants and fungal endophyte.

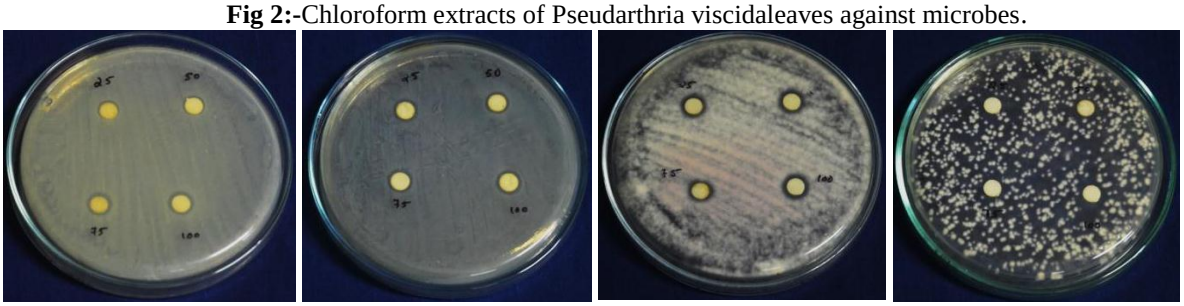
Chloroform extract	Antibacterial activity							
	S. aureus				E. coli			
	Inhibition zone diameter(IZD) (mm)				Inhibition zone diameter(IZD) (mm)			
	25µl	50µl	75µl	100µl	25µl	50µl	75µl	100µl
Plant extracts								
P. viscida	8	9	9	9	9	9	9	9
Fungal extracts								
Pv-1 C.F.	_	_	8	8	7	8	8	9
Pv-1 M.E.	_	8	8	9	9	9	9	10

Chloroform	Antifungal activity

extract	Fusarium oxysporum				Cladosporium cladosporioides			
	Inhibition zone diameter (IZD) (mm)				Inhibition zone diameter (IZD) (mm)			
	25µl	50µl	75µl	100µl	25µl	50µl	75µl	100µl
Plant extracts								
P. viscida	9	9	9	10	_	_	_	_
Fungal extracts								
Pv-1 C.F.	8	9	10	11	_		_	_	10
Pv-1 M.E.	9	10	10	11	_		_	_	10

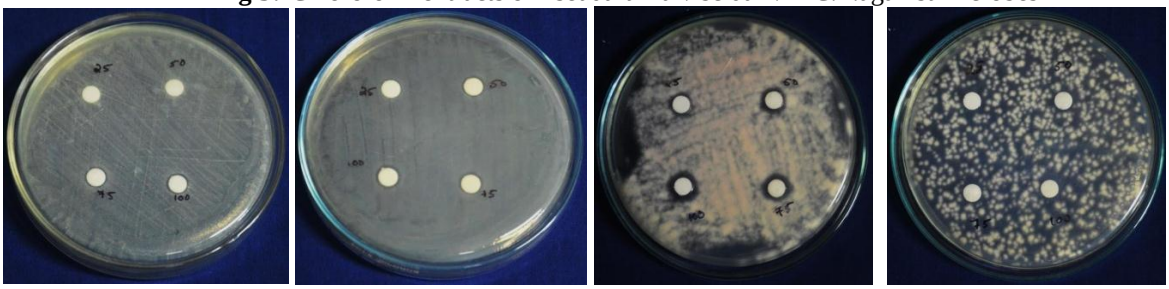
Table 3:-Antifungal activity of chloroform extracts of selected medicinal plants and fungal endophyte.

Fig 2:-Chloroform extracts of Pseudarthria viscidaleaves against microbes.



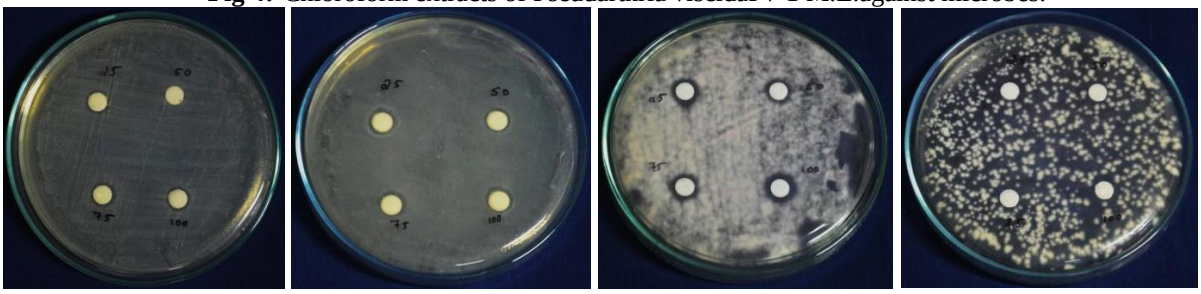
Staphylococcus aureus Escherichia coli Fusariumoxysporum Cladosporium cladosporioides

Fig 3:-Chloroform extracts of Pseudarthria viscidaPv-1 C.F.against microbes



Staphylococcus aureus Escherichia coli Fusariumoxysporum Cladosporium cladosporioides

Fig 4:-Chloroform extracts of Pseudarthria viscidaPv-1 M.E.against microbes.



Staphylococcus aureus Escherichia coli Fusarium oxysporum Cladosporium cladosporioides

Qualitative analysis of metabolites of plant and fungal extracts by GC-MS:-

Phytochemical analysis of chloroform extracts of plant P. viscida (Table 4, Fig 5), culture filtrates and mycelial mat of endophyte Pv-1 (Table 5 & 6, Fig 6 & 7) resulted in the identification of different bioactive compounds. In the case of P. viscidaand its endophyte Pv-1, 2,4-Ditert-butylphenol, E-14-Hexadecenal, E-15-Heptadecenal, 1-Hexadecene, Cyclotetracosane, Octadecyl trifluoroacetate were found to be same. But the amounts of bioactive compounds (Area %) present in the extracts varied. Methanol extracts of P. viscida plant have been studied by various authors and a number of different chemical compounds have been isolated (Baskar et al., 2012; Hemlal and Subban, 2012). The results suggested that most of the compounds present in the plant and associated endophyte possess potential therapeutic activities, the amount and purity of the compounds present need to be analysed further chemically for its effective isolation and application in various medicinal fields.

Table 4:-List of metabolites of P. viscida chloroform extract by GC-MS

Peak#	R. Time	Area	Area%	Height	Height%	Name	Base m/z
1	14.392	2844495	7.12	587929	5.31	1-TETRADECENE	55.10
2	16.760	479237	1.20	151406	1.37	NONADECANE	57.10
3	17.352	7219352	18.06	1767712	15.97	2,4-DITERT-BUTYLPHENOL	191.20
4	19.296	4832781	12.09	1226694	11.08	1-HEXADECANE	55.10
5	19.465	450692	1.13	129797	1.17	UNDECANE,4,7-DIMETHYL	57.05
6	23.758	5509259	13.78	1676797	15.15	E-15-Heptadecenal	55.10
7	23.897	388754	0.97	122824	1.11	PENTADECANE	57.10
8	24.644	2355748	5.89	848945	7.67	Phytol, acetate	68.10
9	25.152	369695	0.92	136476	1.23	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	57.10
10	26.051	459876	1.15	159475	1.44	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	57.10
11	26.120	499102	1.25	181798	1.64	EICOSANE	77.10
12	27.047	620886	1.55	192499	1.74	Tridecanol, 2-ethyl-2-methyl-	57.10
13	28.085	5281916	13.21	1537257	13.89	E-14-Hexadecenal	55.10
14	34.463	4150939	10.38	975023	8.81	Cyclotetracosane	57.10
15	39.526	2715658	6.79	821108	7.42	1-TRICOSENE	57.10
16	43.402	1794828	4.49	553605	5.00	Octadecyl trifluoroacetae	57.10

Fig 5:-Chromatogram of chloroform extract of P. viscida

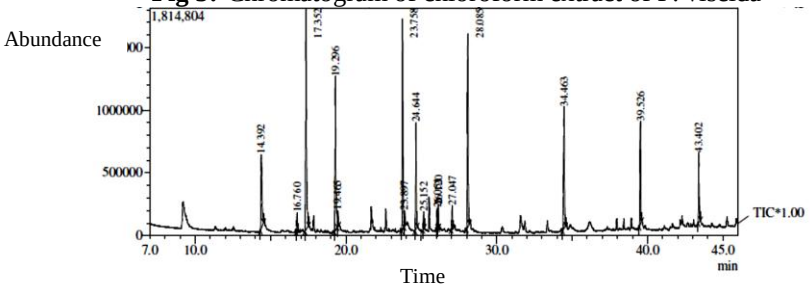


Table 5:-List of metabolites of chloroform extract of P. viscida C.F.of C. gloeosporioides Pv-1 by GC-MS.

Peak#	R. Time	Area	Area%	Height	Height%	Name	Base m/z
1	14.394	2128890	4.83	460741	4.50	1-PENTADECENE	55.10
2	17.358	9994117	22.65	2556925	24.98	2,4-DITERT-BUTYLPHENOL	191.15
3	19.294	3610069	8.18	1010046	9.87	1-HEXADECANE	55.05
4	19.468	528072	1.20	171602	1.68	HEXADECANE	57.10
5	23.758	4091234	9.27	1305079	12.75	E-14-Hexadecenal	55.05
6	23.901	545476	1.24	180860	1.77	PENTADECENE	57.10

7	26.061	752121	1.70	239018	2.33	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	57.10
8	27.051	408314	0.93	138469	1.35	Tridecanol, 2-ethyl-2-methhyl-	57.10
9	28.089	4175241	9.46	1080099	10.55	E-15-Heptadecenal	55.05
10	28.251	383331	0.87	102195	1.00	HEPTADECANE	57.10
11	34.463	3086662	7.00	742498	7.25	Cyclotetracosane	57.10
12	36.285	6966190	15.79	437456	4.27	Squalene	69.10
13	39.530	2144687	4.86	655848	6.41	Heptadecyl trifluoroacetate	57.10
14	42.178	828912	1.88	234567	2.29	1,2-BENZENEDICARBOXYLIC ACID	149.05
15	43.039	3343026	7.58	489615	4.78	9(11)-Dehydroergosteryl benzoate	251.20
16	43.409	1130770	2.56	432335	4.22	Octadecyl trifluoroacetae	57.10

Fig 6:-Chromatogram of chloroform extract of P. viscida C.F.of C. gloeosporioides Pv-1

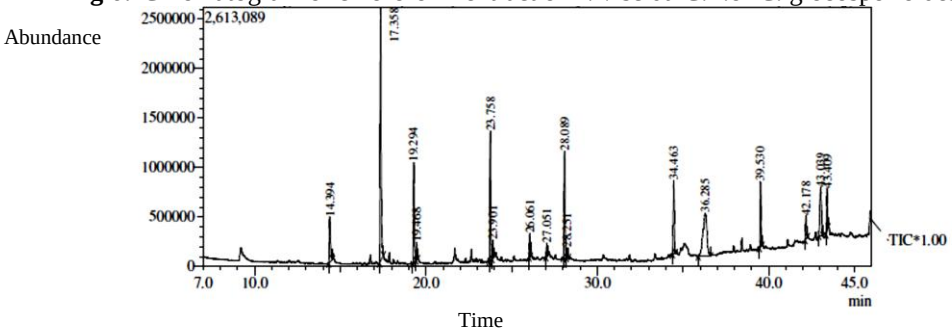
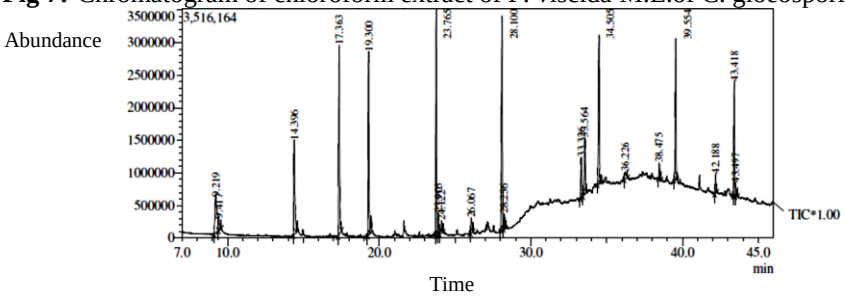


Table 6:-List of metabolites of chloroform extract of P. viscida M.E.of C. gloeosporioides Pv-1 by GC-MS

Peak#	R. Time	Area	Area%	Height	Height%	Name	Base m/z
1	9.219	5613391	5.83	549016	2.27	Cyclohexane,2-propyl-1,1,3-trimethyl-	128.10
2	9.417	961531	1.00	188381	0.78	Octane, 2,6-dimethyl-	57.05
3	14.396	6927889	7.20	1454947	6.01	1-TETRADECENE	55.05
4	17.363	11289778	11.73	2903970	12.00	2,4-DITERT-BUTYLPHENOL	191.15
5	19.300	9644866	10.02	2824034	11.67	1-HEXADECANE	55.10
6	23.765	10420674	10.82	3461329	14.30	E-14-Hexadecenal	55.05
7	23.905	1016460	1.06	321629	1.33	PENTADECENE	57.10
8	24.122	1149117	1.19	189265	0.78	1S,3R,4S,5R,6S-1-Hydroxy-2,2,3,4,5,6-hexamethyl-8-oxo-7,9-dioxatricyclo[4.2.1.0(3,5)]nonane	153.15
9	26.067	1185726	1.23	232381	0.96	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	149.10
10	28.100	13579814	14.11	3278862	13.55	E-15-Heptadecenal	55.05
11	22.256	836380	0.87	229479	0.95	EICOSANE	57.10
12	33.326	2983122	3.10	619781	2.56	ETHYL(9Z,12Z)-9,12-OCTADECADIENOATE #	67.10
13	33.564	4149536	4.31	869044	3.59	(E)-9-Octadecenoic acid ethyl ester	55.05
14	34.505	10262087	10.66	2290738	9.47	Cyclotetracosane	57.10
15	36.226	977802	1.02	101174	0.42	2,6-FARNESOL (CIS,TRANS)	69.10
16	38.475	943339	0.98	243435	1.01	O O'-BIPHENOL,4,4',6,6'-	57.10

						TETRA-T-BUTYL-	
17	39.554	7291618	7.57	2174668	8.99	1-TRICOSENE	57.10
18	42.188	1018261	1.06	297827	1.23	1,2-BENZENEDICARBOXYLIC ACID	149.05
19	43.418	5098354	5.30	1765604	7.30	Octadecyl trifluoroacetae	57.10
20	43.497	918262	0.95	205728	0.85	OCTADECANE	57.10

Fig 7:-Chromatogram of chloroform extract of P. viscida M.E.of C. gloeosporioides Pv-1.



Conclusions:-

Medicinal plants and associated endophytes possess numerous bioactive compounds which forms an important source of new therapeutics. In the present study P. viscida were inoculated for the isolation of endophytes. Two endophytic fungi, Colletotrichum gloeosporioides and C.lindemuthianum were isolated and identified from P. viscida. Among these C. gloeosporioides from plant species was selected for further study. The chloroform extracts of plants and fungus, mycelia mat and culture filtrate were studied for antimicrobial activity and were found to be effective against selected microbes, E. coli, S. aureus, F. oxysporum and C. cladosporioides. Furthermore, active crude extract were subjected for the isolation and identification of biologically active secondary metabolite via GC-MS which may provide better source for developing new therapeutic agents. The obtained metabolites need evaluation and further attempts may be required in order to isolate and identify the secondary metabolites responsible for antimicrobial activity reported here.

Acknowledgements:-

The authors are grateful to the Director, Kerala Forest Research Institute for encouragement and also all the Forest pathology department lab members, KFRI.

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