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GC-MS ANALYSIS AND PASS-ASSISTED PREDICTION OF BIOLOGICAL ACTIVITY SPECTRA OF EXTRACT OF *PHOMOPSIS* SP. ISOLATED FROM *ANDROGRAPHIS PANICULATA*

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ABSTRACT

Endophytic fungi isolated from medicinal plants have been recognized to be a valuable source of metabolites and novel bioactive compounds. The present investigation was carried out to determine the possible bioactive components produced by an endophytic fungus *Phomopsis* sp. isolated from *Andrographis paniculata* (Burm. f.) Wall. ex Nees (Acanthaceae) using GC-MS. Eleven compounds have been identified from ethyl acetate extract of *Phomopsis* sp., of which tetradecane (23.43%), hexadecane (20.21%), and Dodecane (19.41%) were found to be predominant compounds. PASS (Prediction of Activity Spectra for Substances) has been employed as a strong potential tool to predict the biological activity spectrum of synthetic substances for the discovery of new drugs. But the potential of PASS to predict the biological activity spectra of natural products is still underestimated. Therefore, the present study was undertaken to investigate the biological activity spectrum of the ethyl acetate extract of the fungus, *Phomopsis* sp. isolated from *Andrographis paniculata*.

KEYWORDS: Endophytic fungus, *Phomopsis* sp., *Andrographis paniculata*, GC-MS, PASS, Ethyl acetate extract.

INTRODUCTION

Endophytic fungi are a group of fungi that reside inside the living tissues of the plant without showing any distinctive symptoms and they are the chemical synthesizers residing inside plants.[1, 2] It was reported that endophytic fungi has enormous potential to produce

biologically active metabolites.[3, 4] Hence, in recent years there is a growing interest in studying compounds synthesized by endophytic fungi. Fungal metabolites are known to have diverse structural groups possessing antibacterial, antiviral, anticancer, antioxidant, insecticidal, antidiabetic and immunosuppressive properties.[5, 6, 7, 8, 9, 10, 11, 12, 13, 14] The genus *Phomopsis* belongs to the family Diaporthaceae and consists of approximately 900 species from a wide range of hosts.[15, 16] Several species of *Phomopsis* have been reported as plant pathogens,[17, 18, 19] endophytes,[20, 21, 22, 23, 24, 25, 26] saprobes,[27, 28, 29, 30, 31, 32, 33] and even causing health problems in humans and other mammals.[34, 15, 35, 36, 37, 38] The compound Dicerandrols A, B and C isolated from *Phomopsis longicolla* have antibacterial property.[39] Phomoxanthones isolated from *Phomopsis sp* exhibited antitumor activity.[40] Phomopsidone and isobenzofuranones isolated from *Phomopsis* sp possess antioxidant, antifungal and cytotoxic activity.[41] Considering the medicinal importance of the fungi, the ethyl acetate extract of the *Phomopsis* sp was analyzed in GC-MS. This study will help to identify the bioactive compounds present in the extract that possess abundant therapeutic potential.

A. paniculata has been reported as having antibacterial, antifungal, antiviral, choleretic, hypoglycemic, hypocholesterolemic and adaptogenic effects.[42, 43] The bioactive nature of *A.paniculata* needs to be reexamined in the light of chemicals produced by endophytic fungus *Phomopsis* sp. The ethyl acetate extract obtained from *Phomopsis* sp. living in *Andrographis paniculata* leaves was studied for detailed chemical investigation, using Gas Chromatography/Mass Spectroscopy (GC-MS).

MATERIALS AND METHODS

Collection of plant samples: The leaves of *Andrographis paniculata* were collected from State Horticultural Department, Chennai, Tamilnadu, India. Plants free from insect and disease infestation were selected and marked. Healthy leaves from these healthy plants were collected and processed separately within 48 h of collection.

Isolation of endophytic fungi: The mature and healthy leaves collected from *Andrographis paniculata* (Burm.f.) Wall. ex Nees, were surface sterilized as described by Dobranic *et al*.[44] Surface sterilized plant materials were placed in Potato dextrose agar (PDA) medium containing Chloramphenicol (150mg). Petri dishes were incubated at 30°C for a week under regular monitoring. The fungal colonies that grow out from the leaf segments were sub cultured repeatedly on PDA medium until a pure culture is obtained. The endophytic fungal cultures were identified based on their morphology and spore character. Pure fungal cultures

of the endophytic isolates were obtained by the hyphal tip method in test tube slants. The isolated fungus was numbered and identified based on the morphological characters and the same sent to Dr. S. Muthumary, the Centre for Advance Studies in Botany, University of Madras, Chennai (India) for the confirmation. The individual cultures were then transferred to PDA slants and stored at 4^0C until further use.

Fermentation and solvent extraction: The peripheral hyphae of the pure isolate was inoculated into Czapek – Dox Broth (CDB) medium containing glucose and then incubated for 24 days at room temperature. After incubation the culture was filtered through four-layered cheese cloth and the filtrate was added with equal volume of ethyl acetate and kept in rotary shaker (180rpm) at 4^0C for 24 hours and allowed to settle in a separating funnel. The ethyl acetate fraction was collected and evaporated at 50^0C using rotary evaporator. The dried sample was collected weighed and stored at 4^0C for further use.

GC-MS analysis: Ethyl acetate extract of the fungus was analyzed by GC-MS. Gas chromatograph linked to mass spectrometer system equipped with a capillary column DB-5ms (30.0m x 0.25mm, 0.25µm film thickness) was used. The GC column oven temperature was programmed from 70^0 C to 300^0 C. The initial temperature was 70^0 C (hold time 2min) and it rose to 300^0 C (hold time 7min) at the rate of 10^0 C min^{-1}. The total run time was 32.0 min. Helium with 99.9995% purity was used as carrier gas with a constant flow of 1.51ml/min. The GC-MS interface temperature was at 280^0 C. Injector and detector temperatures were set at 200^0 C. 1µl of sample was injected in split ratio of 1:10. The MS scan range was set from 40-1000 Da. Identification of compounds was obtained by comparing the retention times with those of authentic compounds and with the spectral data obtained from data library of the corresponding compounds. Quantities of the compounds are represented as relative area percentage in derived from the integrator.

Biological Activity: The biological activity spectra of these phytoconstituents were obtained by PASS version (version 9.1, http://195.178.207.233/PASS). This software estimates the predicted activity spectrum of a compound as probable activity (Pa) and probable inactivity (Pi). Prediction of this spectrum by PASS is based on qualitative structure-activity relationships (SAR) analysis of the training set containing more than 205,000 compounds exhibiting more than 3750 kinds of biological activities. Being probabilities, the Pa and Pi values vary from 0.000 to 1.000 and, in general, $Pa+Pi \neq 1$, since these probabilities are calculated independently. The PASS prediction results were interpreted and used in a flexible

manner: (i) only activities with Pa>Pi are considered as possible for a particular compound; (ii) if Pa>0.7, the chance to find the activity experimentally is high; (iii) if 0.5<Pa<0.7, the chance to find the activity experimentally is less, but the compound is probably not so similar to known pharmaceutical agents; (iv) if Pa<0.5, the chance to find the activity experimentally is less, but the chance to find a structurally new compound, that is, NCEs is more.[45, 46]

RESULTS AND DISCUSSION

Gas chromatography and mass spectrometry (GC-MS) analyses of the *Phomopsis* sp. extract lead to the identification of 11 components. The prevalent compounds were Tetradecane (23.43%), Hexadecane (20.21%) and Dodecane (19.41). The ethyl acetate extract of *Phomopsis* sp. exhibited 11 major peaks in GC analysis (Fig. 1). 11 peaks accounting for 100% of the extract, were identified and listed along with respective retention time, percentage of compound in the extract, molecular formula, molecular weight and nature of compound are given in Table 1.

Table 1: Chemical constituents of the ethyl acetate extract of *Phomopsis* sp. (GC-MS analysis)

Constituent	RT	%	MF	MW	Nature of Compound
Decane	4.685	1.72	$C_{10}H_{22}$	142	Alkane hydrocarbon
2 Ethyl hexanol	5.145	1.93	$C_8H_{18}O$	130	Fatty alcohol
Dodecane	7.759	19.41	$C_{12}H_{26}$	170	Alkane hydrocarbon
Tetradecane	10.637	23.43	$C_{14}H_{30}$	198	Alkane hydrocarbon
Hexadecane	13.122	20.21	$C_{16}H_{34}$	226	Alkane hydrocarbon
3-Methylheptadecane	15.041	1.76	$C_{18}H_{38}$	254	Hydrocarbon
Octadecane	15.348	9.67	$C_{18}H_{38}$	254	Hydrocarbon
Dibutyl phthalate	17.070	9.99	$C_{16}H_{22}O_4$	278	Ester
Eicosane	17.365	5.31	$C_{20}H_{42}$	282	Alkane
Heneicosane	19.209	3.70	$C_{21}H_{44}$	296	Aliphatic hydrocarbon
Dioctyl hexanedioate	20.914	2.89	$C_{22}H_{42}O_4$	370	Diester of adipic acid

Endophytic fungus *Phomopsis* has gained attention in the discovery of novel biochemically and physiologically active compounds and their direct use in agricultural biotechnology and medicine.[47, 48] The ubiquity, diversity and biology of the species of *Phomopsis* encourage the need for evaluation of potential applications of this fungus.[16]

The first 15 biological activity spectrum of the 11 ethyl acetate extract compounds identified using GC-MS predicted by PASS was represented in the tables 2-12. Mass spectrum of

identified compounds is given in the figures 2-12. Tetradecane (23.43%) was the major compound, which is the active principle compound in *Phomopsis* sp. (Fig. 2). Tetradecane has nematicidal activity against *Bursaphelenchus xylophilus* at 0.5 mg/ml when measured after 48 hr under microscope.[49]

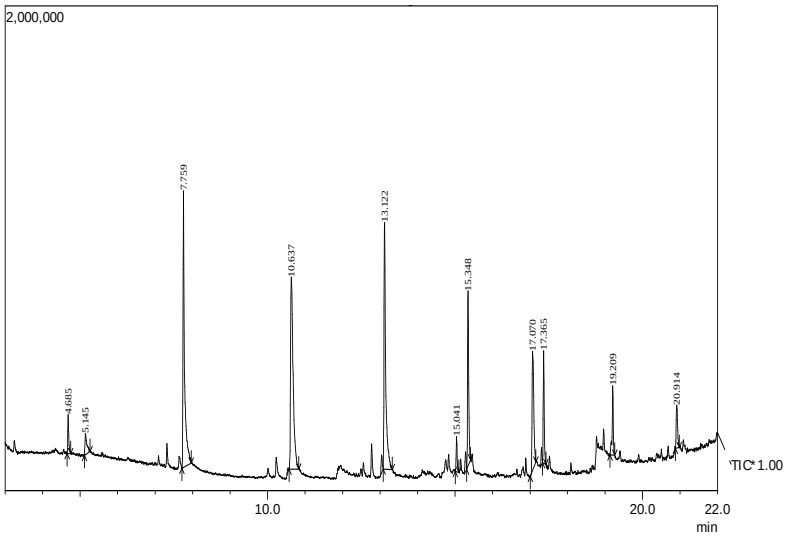

Fig. 1: GC-MS spectrum of ethyl acetate extract of endophytic fungus *Phomopsis* sp.

Hexadecane is another important component of ethyl acetate extract of *Phomopsis* sp. (Table 1; Fig 3). It is reported that hexadecane present in crude extracts of *Spirulina platensis* showed antibacterial activity.[50] Hexadecane is an organic oxygen vector that enhances oxygen transfer and carotenoid production in liquid fermentation of *Phaffia rhodozyma*.[51] Whereas Dodecane identified by GC-MS may possess diverse biological activity including neurotropic action. However, no *in vivo* studies could confirm the predicted biological activity. Derivatives of dodecane confirmed some activity upon central nervous system.[52]

Dibutyl phthalate was another important constituent of *Phomopsis* sp. shows antimicrobial property.[53, 54] Cytotoxic activity of dibutyl phthalate against tumor cell lines has been worked out by Mabrouk *et al*.[55] This compound is also an antimetabolite of proline.[56] Octadecane is another constituent of extract of *Phomopsis* sp. The inhibition by octadecane boronic acid was competitive when measured against the hydrolysis of dissolved tripropionin in the presence of siliconized glass beads.[57] S-1-*O*-phosphocholine-2-*N*-acetyl-octadecane induces apoptosis in T cells.[58] Whereas Eicosane identified in the present analysis has inhibitory effect on food borne pathogens isolated from essential oil and organic extracts of *Cestrum nocturnum*.[59] Antimicrobial activity of flower of *Allium atroviolaceum* attributed to

the presence of Eicosane.[60] In the *Aloe vera*, eicosane and phytol are reported to be the main constituents that are responsible for elevated antimicrobial activity against clinical pathogens.[61]

Heneicosane is another compound identified from the extract of *Phomopsis* sp. shows oviposition-attractant pheromone of larval origin in *Aedes aegypti* mosquito.[62] Application of n-heneicosane in breeding habitats will be a useful method to attract the gravid mosquitoes using ovitraps for surveillance and monitoring.[63] Dioctyl hexanedioate isolated from *Conyza dioscoridis* is also active against pathogenic pests.[64, 65] Whereas Alcohol 2-ethyl 1-hexanol identified from this study is an antifungal agent and prevents infections.[66, 67]

2-methyl heptadecane is another component of extract of *Phomopsis* sp. and it is an attractant. It attracts male moths and also used as a pest repellant. It is also a major component of the sex pheromones of several sibling species in the *Holomelina aurantiaca* complex.[68] Derivatives of decane are show potential anti-inflammatory activity.[69] The other derivatives of decane proved to inhibit the apomorhine-induced locomotor hyperactivity and stereotypy, hypoxic damage of the memory (nootropic effect), hypobaric hypoxia and cerebral (brain) edema.[70]

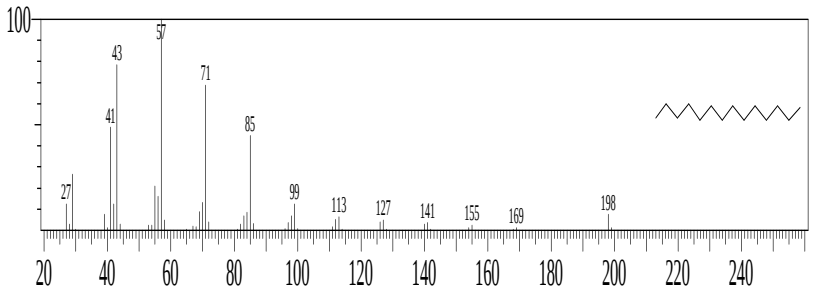

Fig. 2: Mass spectrum of Tetradecane

Table 2: Tetradecane

Pa	Pi	Activity
0.918	0.005	Antineoplastic
0.871	0.019	Membrane integrity agonist
0.850	0.017	Ubiquinol-cytochrome-c reductase inhibitor
0.821	0.028	Aspulvinone dimethylallyltransferase inhibitor
0.811	0.018	CYP2J substrate
0.814	0.022	Testosterone 17beta-dehydrogenase (NADP+) inhibitor
0.779	0.009	Complement factor D inhibitor
0.767	0.004	CYP2A4 substrate

0.799	0.037	CYP2C12 substrate
0.763	0.004	Antineoplastic (ovarian cancer)
0.757	0.005	Cardiovascular analeptic
0.754	0.006	CYP2B5 substrate
0.757	0.020	Nicotinic alpha6beta3beta4alpha5 receptor antagonist
0.746	0.014	Glucan endo-1,6-beta-glucosidase inhibitor
0.744	0.023	CYP2J2 substrate

44 possible activities at Pa>70%

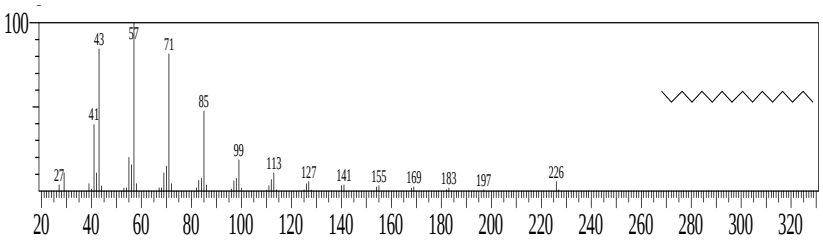

Fig. 3: Mass spectrum of Hexadecane

Table 2: Hexadecane

Pa	Pi	Activity
0.902	0.003	Cognition disorders treatment
0.899	0.003	Antianginal
0.761	0.019	Nicotinic alpha6beta3beta4alpha5 receptor antagonist
0.697	0.004	Albendazole monooxygenase inhibitor
0.706	0.025	Nicotinic alpha2beta2 receptor antagonist
0.647	0.005	Leukopoiesis inhibitor
0.638	0.013	CYP2A8 substrate
0.626	0.020	Chloride peroxidase inhibitor
0.589	0.005	Taurine-2-oxoglutarate transaminase inhibitor
0.572	0.006	PfA-M1 aminopeptidase inhibitor
0.570	0.026	Ovulation inhibitor
0.540	0.018	5 Hydroxytryptamine uptake stimulant
0.544	0.047	Platelet aggregation stimulant
0.505	0.032	(S)-6-hydroxynicotine oxidase inhibitor
0.537	0.074	Kidney function stimulant

11 possible activities at Pa>70%

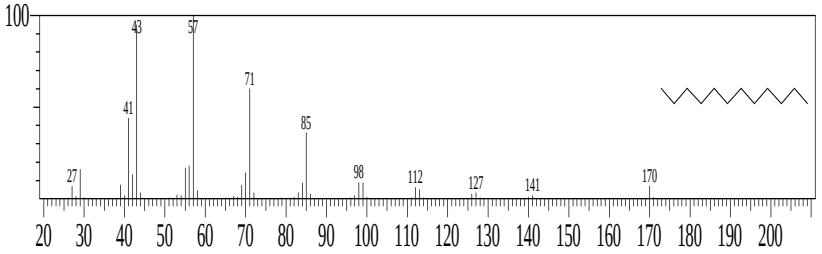

Fig. 4: Mass spectrum of Dodecane

Table 4: Dodecane

Pa	Pi	Activity
0.949	0.004	Antineoplastic
0.892	0.006	Ubiquinol-cytochrome-c reductase inhibitor
0.876	0.013	Aspulvinone dimethylallyltransferase inhibitor
0.858	0.003	Antineoplastic (ovarian cancer)
0.852	0.004	Glucan endo-1,6-beta-glucosidase inhibitor
0.854	0.023	Membrane integrity agonist
0.827	0.002	Phosphatase inhibitor
0.821	0.005	Complement factor D inhibitor
0.815	0.003	General pump inhibitor
0.828	0.019	Testosterone 17beta-dehydrogenase (NADP+) inhibitor
0.819	0.010	NADPH peroxidase inhibitor
0.810	0.006	5-O-(4-coumaroyl)-D-quinate 3'-monooxygenase inhibitor
0.825	0.025	Phobic disorders treatment
0.813	0.014	Sugar-phosphatase inhibitor

132 possible activities at Pa>70%

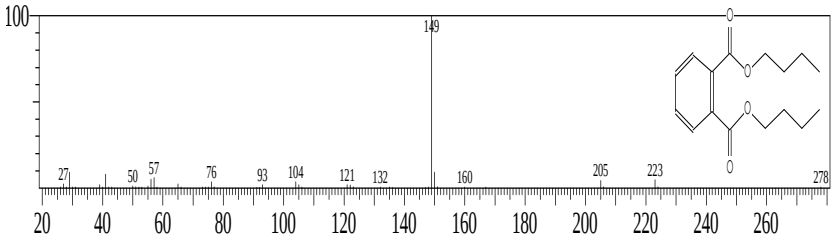

Fig. 5: Mass spectrum of Dibutyl phthalate

Table 5: Dibutyl phthalate

Pa	Pi	Activity
0.939	0.003	Eye irritation, inactive
0.931	0.003	Sugar-phosphatase inhibitor
0.919	0.004	Alkenylglycerophosphocholine hydrolase inhibitor
0.899	0.003	Pullulanase inhibitor
0.891	0.003	Phosphatidylcholine-retinol O-acyltransferase inhibitor
0.894	0.006	Phobic disorders treatment
0.890	0.002	Gluconate 5-dehydrogenase inhibitor
0.883	0.004	Skin irritation, inactive
0.884	0.005	Antiseborrheic
0.865	0.003	All-trans-retinyl-palmitate hydrolase inhibitor
0.873	0.014	Aspulvinone dimethylallyltransferase inhibitor
0.863	0.004	IgA-specific serine endopeptidase inhibitor
0.861	0.004	Carboxypeptidase Taq inhibitor
0.855	0.003	Phenol O-methyltransferase inhibitor
0.867	0.02	Membrane integrity agonist

132 possible activities at Pa>70%

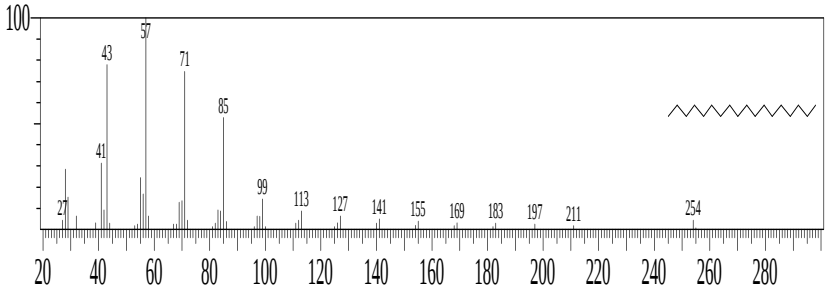

Fig. 6: Mass spectrum of Octadecane

Table 6: Octadecane

Pa	Pi	Activity
0.949	0.002	Sugar-phosphatase inhibitor
0.933	0.002	Fucosterol-epoxide lyase inhibitor
0.93	0.004	Phobic disorders treatment
0.925	0.001	Glucan 1,4-alpha-maltotriohydrolase inhibitor
0.926	0.004	Alkenylglycerophosphocholine hydrolase inhibitor
0.924	0.002	IgA-specific serine endopeptidase inhibitor
0.923	0.002	Carboxypeptidase Taq inhibitor
0.925	0.004	Polyporopepsin inhibitor
0.922	0.003	Alkylacetylglycerophosphatase inhibitor
0.916	0.004	Acrocylindropepsin inhibitor
0.916	0.004	Chymosin inhibitor
0.916	0.004	Saccharopepsin inhibitor
0.912	0.003	Pullulanase inhibitor
0.908	0.002	Gluconate 5-dehydrogenase inhibitor
0.901	0.003	Cutinase inhibitor

175 possible activities at Pa>70%

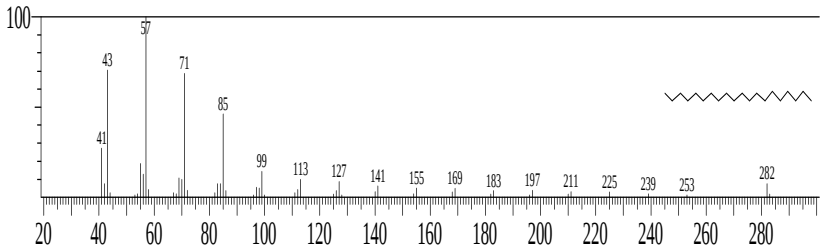

Fig. 7: Mass spectrum of Eicosane

Table 7: Eicosane

Pa	Pi	Activity
0.947	0.003	Acrocylindropepsin inhibitor
0.947	0.003	Chymosin inhibitor
0.947	0.003	Saccharopepsin inhibitor
0.941	0.003	Phobic disorders treatment

0.931	0.004	Polyporopepsin inhibitor
0.916	0.005	Testosterone 17beta-dehydrogenase (NADP+) inhibitor
0.913	0.004	Pro-opiomelanocortin converting enzyme inhibitor
0.909	0.002	Cutinase inhibitor
0.907	0.005	Ubiquinol-cytochrome-c reductase inhibitor
0.898	0.003	Acetylesterase inhibitor
0.897	0.005	5 Hydroxytryptamine release stimulant
0.893	0.005	Sugar-phosphatase inhibitor
0.882	0.005	Acylcarnitine hydrolase inhibitor
0.875	0.004	Alkylacetylglycerophosphatase inhibitor
0.858	0.004	Carboxypeptidase Taq inhibitor

151 possible activities at Pa>70%

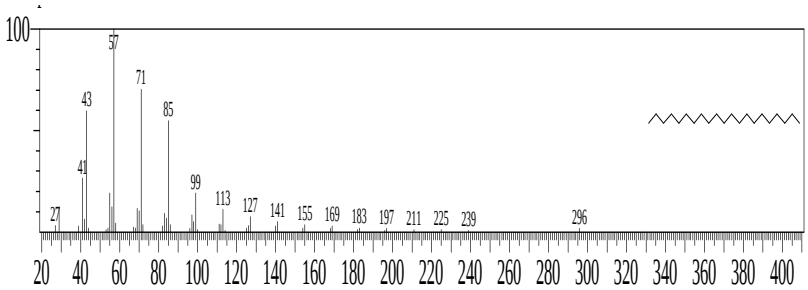

Fig. 8: Mass spectrum of Heneicosane

Table 8: Heneicosane

Pa	Pi	Activity
0.909	0.001	Complement C5a chemotactic receptor antagonist
0.874	0.002	Antieczematic atopic
0.865	0.004	Glyceryl-ether monooxygenase inhibitor
0.846	0.002	Phosphatase inhibitor
0.836	0.002	Myosin-light-chain-phosphatase inhibitor
0.841	0.019	Phobic disorders treatment
0.83	0.01	Beta-adrenergic receptor kinase inhibitor
0.83	0.01	G-protein-coupled receptor kinase inhibitor
0.82	0.009	Antineoplastic
0.817	0.009	Nicotinic alpha6beta3beta4alpha5 receptor antagonist
0.827	0.024	Ubiquinol-cytochrome-c reductase inhibitor
0.819	0.016	Acrocylindropepsin inhibitor
0.819	0.016	Chymosin inhibitor
0.819	0.016	Saccharopepsin inhibitor
0.827	0.026	Aspulvinone dimethylallyltransferase inhibitor

71 possible activities at Pa>70%

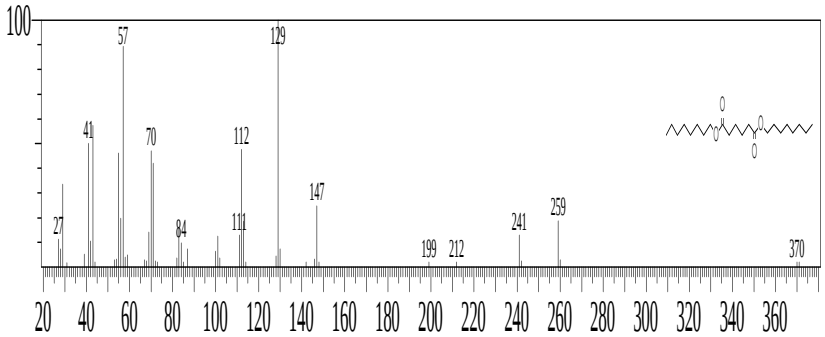

Fig. 9: Mass spectrum of Dioctyl hexanedioate

Table 9: Dioctyl hexanedioate

Pa	Pi	Activity
0.953	0.001	All-trans-retinyl-palmitate hydrolase inhibitor
0.94	0.003	Sugar-phosphatase inhibitor
0.939	0.003	Alkenylglycerophosphocholine hydrolase inhibitor
0.934	0.002	Alkylacetylglycerophosphatase inhibitor
0.934	0.003	Acylcarnitine hydrolase inhibitor
0.933	0.003	Eye irritation, inactive
0.931	0.002	Cutinase inhibitor
0.928	0.002	Carboxypeptidase Taq inhibitor
0.929	0.004	Phobic disorders treatment
0.924	0.002	Dextranase inhibitor
0.924	0.004	Polyporopepsin inhibitor
0.919	0.004	Acrocylindropepsin inhibitor
0.919	0.004	Chymosin inhibitor
0.919	0.004	Saccharopepsin inhibitor
0.915	0.003	Pullulanase inhibitor

144 possible activities at Pa>70%

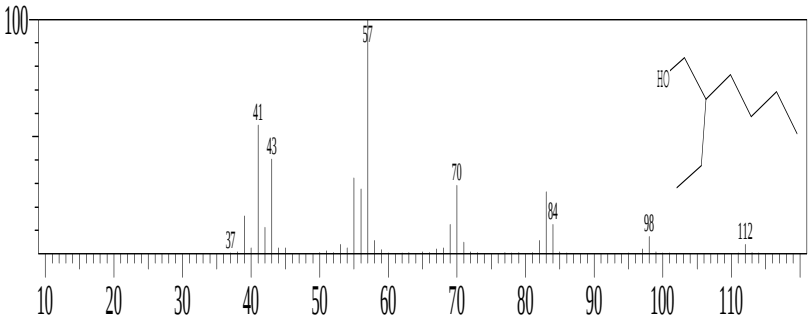

Fig. 10: Mass spectrum of 2 Ethyl hexanol

Table 10: 2 Ethyl hexanol

Pa	Pi	Activity
0.941	0.002	Cutinase inhibitor
0.927	0.003	Sugar-phosphatase inhibitor
0.926	0.004	Ubiquinol-cytochrome-c reductase inhibitor
0.921	0.004	Alkenylglycerophosphocholine hydrolase inhibitor
0.914	0.004	Acrocylindropepsin inhibitor
0.914	0.004	Chymosin inhibitor
0.914	0.004	Saccharopepsin inhibitor
0.909	0.003	Alkylacetylglycerophosphatase inhibitor
0.904	0.005	Polyporopepsin inhibitor
0.899	0.003	Carboxypeptidase Taq inhibitor
0.901	0.005	5 Hydroxytryptamine release stimulant
0.897	0.001	Sclerosant
0.896	0.006	Phobic disorders treatment
0.891	0.003	Acetylesterase inhibitor
0.888	0.006	Sphinganine kinase inhibitor

104 possible activities at Pa>70%

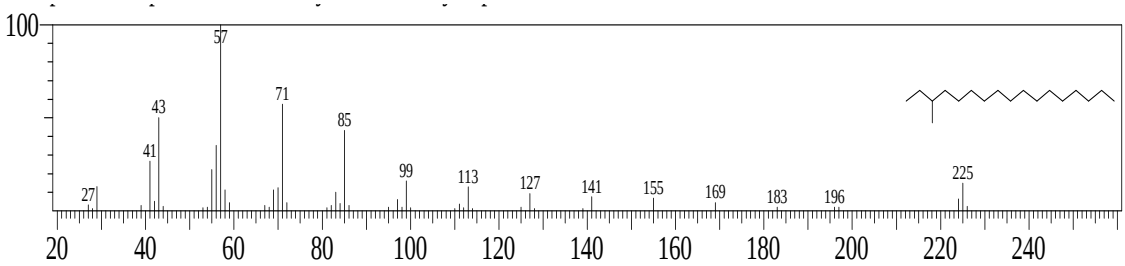

Fig. 11: Mass spectrum of 3- Methyl heptadecane

Table 11: 3- Methyl heptadecane

Pa	Pi	Activity
0.941	0.003	Acrocylindropepsin inhibitor
0.941	0.003	Chymosin inhibitor
0.941	0.003	Saccharopepsin inhibitor
0.938	0.002	Cutinase inhibitor
0.938	0.004	5 Hydroxytryptamine release stimulant
0.922	0.004	Polyporopepsin inhibitor
0.918	0.004	Ubiquinol-cytochrome-c reductase inhibitor
0.915	0.004	Phobic disorders treatment
0.911	0.002	Acetylesterase inhibitor
0.905	0.004	Sugar-phosphatase inhibitor
0.906	0.005	Testosterone 17beta-dehydrogenase (NADP+) inhibitor
0.903	0.004	Pro-opiomelanocortin converting enzyme inhibitor
0.896	0.005	Acylcarnitine hydrolase inhibitor

0.889	0.004	Alkylacetylglycerophosphatase inhibitor
0.875	0.004	Carboxypeptidase Taq inhibitor

165 possible activities at Pa>70%

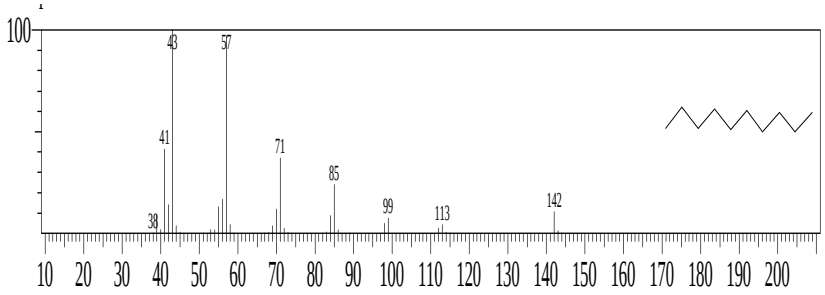

Fig. 12: Mass spectrum of Decane

Table 11: Decane

Pa	Pi	Activity
0.8	0.002	Polarisation stimulant
0.587	0.006	Ophthalmic drug
0.587	0.054	Phosphatase inhibitor
0.601	0.087	Membrane permeability inhibitor
0.484	0.006	Antineoplastic (pancreatic cancer)
0.433	0.013	Antihelmintic
0.406	0.019	Platelet aggregation inhibitor
0.408	0.029	Antiparasitic
0.41	0.039	Antihelmintic (Nematodes)
0.336	0.005	Platelet activating factor beta antagonist
0.293	0.016	Orexin receptor 1 antagonist
0.294	0.019	Dual specificity phosphatase inhibitor
0.32	0.06	Neuropeptide Y2 antagonist
0.316	0.059	Transcription factor STAT3 inhibitor
0.228	0.004	Antiacromegalic

9 possible activities at Pa>70%

CONCLUSION

Our systematic investigation reveals the potential of *Phomopsis* sp. isolated from *Andrographis paniculata* as a good source of bioactive compounds such as alkanes, alkenes and hydrocarbons. The PASS study reveals that compounds identified from the present study have various potential biological activities. Further studies are needed to validate these predictions. However, our study also gives a better understanding for identification and comparison of volatile and non-volatile compounds in fungal extracts by GC-MS and further interest to researchers for the study and isolation of bioactive compounds.

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