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Research Article

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GC-MS Profiling of Secondary Metabolites from Endophytic Fungi Aspergillus terreus of Eichhornia crassipes (Mart.) Solms: A Way to Novel Therapeutic Compound Discovery by In-Vitro and In-Silico Bio-activity

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ABSTRACT

Endophytes fungi have made a potential contribution in producing of various volatile secondary metabolites to combat unexplored wide area for novel therapeutic and industrial biotechnology applications. Methods: In this work, we reported in-vitro and in-silico analysis of secondary metabolites crude extract from an endophytic fungi Aspergillus terreus for novel therapeutic compound discovery from the fresh water aquatic weed Eichhornia crassipes. Results: From in-vitro analysis, the results revealed a high potent anti-bacterial activity (± 39 mm) against Escherichia coli, Klebsiella pneumoniae and Pseudomonas aeruginosa only. The fungal extract also showed anti-inflammatory activity (50 mg / ml) by protein denaturation and also exhibited lipase-like activity in Castor Oil - Phenol Red Agar media. The sequenced ITS 1 and 4 regions were deposited in GenBank (accession number: MT158738). In-silico molecular docking analysis showed the compounds o-Tolylamino-acetic acid [1-(2,5-dihydroxy-phenyl)-ethylidene]-hydrazide, diisobutyl phthalate, physcion showed minimum binding energy when compared with Vemurfenib and determined as potent anti-cancer activity against BRAF V600E Kinase. FT-IR spectrum of secondary metabolites depicts that presence of Halo compounds, Alkene, Phenol, Sulfone functional groups. Conclusion: The GC-MS spectrum resulted and identified 92 secondary metabolites volatile compounds with their own characteristics applications. As far as, this is the first study on Eichhornia crassipes endophytic fungi Aspergillus terreus and hence we suggested the identified secondary metabolites showed high bio-activities.

Keywords: *Eichhornia crassipes*; volatile secondary metabolites; In-vitro analysis

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INTRODUCTION

Eichhornia crassipes (Mart.) Solms

Eichhornia crassipes (commonly called water hyacinth) belongs to Pontederiaceae family and world worst weeds. An erect herb which reproducing by seed and origin of Amazonia. Moreover, this plant is having habit of aquatic source and excessive growth of this invasive weed blocking the water supplying lines, canal, pipes. Dense mats formataion prevent light penetration into the water bodies and there by developing biological oxygen demand to other species in the same habitat. Now, Eichhornia crassipes is found in all over the world and considered as one of top most fast growing plant in the world.

Endophytic Fungi Secondary Metabolites Uses

Endophytic fungi are excellent natural resources, living in many plants and they can be obligate or facultative without causes no damage or infection to the host plant organism. Their interactions with their plant physiological system develop mutualism and antagonism [1]. Endophytes are reported to produce wide range of secondary metabolites in a plant that have habitat and reservoir of therapeutic compounds for many diseases. These secondary metabolites were classified into many phytocompounds includes alkaloids, pyranose, flavonoids, phenol compounds, Phyto steroids, terpenoids and so on. The production of secondary metabolites from endophytes, has been associated with host microorganism's evolution by incorporating their nucleic acids and making adapt to the host plant system and performed various antagonistic activities such as insect, plant pathogen inhibition and grazing of animals. Hence, the endophytic fungi can also act as latent pathogens in host plant to protect it against harmful pathogens. The major focus of this work to isolate, production, extraction of secondary metabolites using endophytic fungi of Eichhornia crassipes to explore novel therapeutic volatile compound identification [2].

Protein Kinase as Targeted Protein

There are 538 protein kinases are encoded in human genome and many of are associated with human cancer. Kinase proteins plays an essential function in cellular growth and metabolic pathway activation. In most type of cancers, the kinases are the proteins which are commonly dysregulated in abnormal cellular functions. Dysregulated, overexpressed, or mutated protein kinases are identified in many cancers, and they are considered as drug targets for the development of new antineoplastic drugs. Kinases are belonging to a large group of enzymes that involved in catalysing of phosphate group from Adenosine Triphosphate (ATP) to substrates molecules including protein-tyrosine kinases, the serine-threonine specific kinases), lipids such as (phosphatidylinositol kinases, sphingosine kinases), carbohydrates, DNA and RNA. Phosphorylation modulates its substrate activity and interaction with other molecules resulted in different physiological and biological responses. It is demonstrated that 50% of all proteins are constantly involved in reversible phosphorylation and dephosphorylation to either cellular metabolic pathway activation or deactivation including proliferation, cellular growth, apoptotic pathway, and signal transduction pathways [3].

Braf Inhibitor- Vemurfenib

Vemurfenib is selective inhibitor of mutated BRAF V600E codon crucial for RAS/RAF/MEK/ERK mitogen-activated protein kinase (MAPK) signalling pathway activation in metastatic melanoma. In clinical trial, vemurfenib overcome dacarbazine inhibition effect (first line treatment in melanoma). Current clinical trials in melanoma treatment involve combinatorial drug therapy method of using vemurfenib with immunomodulatory candidates (ipilimumab) and MAP Kinase inhibitors (GDC-0973) to combat the disease aggressive state.

MATERIALS AND METHODS

Samples Collection

The healthy whole plants of Eichhornia crassipes (common water hyacinth) was collected at Pozhichaloor location in Chennai, Tamil Nadu, India in the month of August, September 2019. The plant samples were taken in a clean plastic container. The collected samples are transferred to the laboratory within 2 hours.

Endophytic Fungi Isolation

The collected plant sample was washed thoroughly with tap water to detach the attached epiphytes from the plant parts Initially the sample was cut into 1 - 2 cm pieces and washed with distilled water for 2 minutes to remove the unwanted contaminants. The distilled washed plant samples are washed with 70 % ethanol for about a minute [4]. Followed by ethanol treatment, the plant samples are washed with 3% Sodium hypochlorite for about 4 minutes. After the treatments, the plant samples were subjected to a wash with 70% ethanol for 30 - 60 seconds. Then the sample was washed with autoclaved demineralised water for about 1 minute to remove the surface sterilized solvents trace and dried under a laminar air flow chamber. The surface sterilized samples are aseptically transferred to freshly prepared PDA culture media. Addition of Chloramphenicol (50 mg / mL) in PDA plate to suppress the

bacterial growth. The last washed water 80 micro litre (µl) was spread on PDA plates separately by spread plate technique. Then incubate the plates at room temperature for 4 to 5 days for endophytic fungi to validate growth and it is considered as the control sample. The isolated strain was sub-cultured in PDA slants and were preserved with glycerol and stored in - 20℃ for future usage.

Colony Morphology and Microscopic Identification

LPCB stained prepared sample slides were subjected to microscopic examination in 40X magnification to detailed view of fungal cells structure.

Phyto-Compounds Extraction for Marker Compound Prediction by TLC

By following the maceration extraction method, 5 grams of grounded plant parts leaves, stem and root were firstly immersed in 50 mL of ethyl acetate in room condition. The crude extracts were obtained by filtration and the remaining dried residual parts were again immersed in 50 mL. The ethyl acetate and carbinol filtrates were air dried under room temperature. The recovered solid crude extracts were properly labeled and stored at 4℃ until further analytical use.

Small Scale Production of Secondary Metabolites

The isolated endophyte EFSP909 from Eichhornia crassipes were inoculated in 400 mL of PDA culture broth. The inoculated culture broth was incubated at room temperature for an interval periods of about 25 days under a static conditions and TLC were checked at intermittent day of incubation period. After the 25 days of liquid fermentation process, the broth culture was subjected to filtration process using an autoclaved four layered cheese cloth to separate the secondary metabolites from broth filtrate. To the broth filtrate, an equal volume of ethyl acetate was used.

Extraction of Secondary Metabolites

The added ethyl acetate broth filtrate was allowed to stand for few for extraction of secondary metabolites. Then upper organic phase containing secondary metabolites were separated by using separating funnel with vigorous shaking. Finally, the separated organic phase was evaporated under room temperature to yield secondary metabolites crude extract. The dried crude extract was weighed, labeled and stored at -20 ℃ until further use. For further investigation studies the dried crude extracts were dissolved in DMSO solvent to reach 50 mg/mL concentration.

TLC Fractionation Profiling of of Plant Extracts and Broth Secondary Metabolites Crude Extracts

The DMSO dissolved EFSP909 crude extract and plant crude extracts were separated by thin layer chromatography to identify the number of compounds presence. 5 - 10 µL of crude extracts was spotted in TLC plate (Merck TLC plate, Silica gel 60 F254). The crude extract loaded plates were analyzed by running using two different solvent system of Chloroform: Carbinol (4:1) and Dicloromethane: Carbinol (9:1), Hexane: Ethyl Acetate (8.5 : 1.5). After compounds were separated, the TLC plates were visualized under UV Illuminator (Deep Vision - UV inspection cabinet) at 365 nm. The solvent system Chloroform: Carbinol was chosen as an ideal mobile phase for isolate (EFSP909) crude extract and Hexane: Ethyl Acetate for plant crude extracts based on compound separation efficiency.

Anti-Bacterial Activity

The DMSO dissolved crude extract (EFSP909) was tested in clinical isolates of Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus collected from "The King Institute of Preventive Medicine and Research", Guindy, Chennai, Tamil Nadu 600032. To study anti-bacterial activity of crude extract agar well diffusion method was followed. For the lowering high cell density, 24 hours cultures was inoculated in a freshly prepared broth to prepare 3 hours inoculum cultures. The prepared bacterial cultures were spread on Muller Hinton Agar (MHA) using sterile wooden swabs. After that, different volumes (50 µL and 70 µL) of crude extract (50 mg/ ml) were loaded into a impregnated 6 mm sized wells on the plate with a positive control of

Chloramphenicol and Ampicillin (50mg/mL in DMSO). After incubation, the plates were observed for Zone of Inhibition (ZOI) in mm.

Minimum Inhibitory Concentration Determination

The minimum and maximum inhibitory concentration of crude extracts were determined by broth dilution method. Different concentration of crude extracts (50, 25, 12.5 and 6.25 mg/mL) respectively were added into nutrient broth of freshly inoculated overnight bacterial cultures prepared in a test tube. Whereas the nutrient broth without crude extracts were considered as negative control and with Chloramphenicol as reference control. All the test tubes were incubated for 24 hours in incubator. After 24 hours of incubation, the nutrient broths were examined for growth inhibition percentage by analyzed using UV spectrophotometer (Hitachi UH5300) at 600 nm. The bacterial growth inhibition percentage was identified by the below equation:

Lipase-Like Activity

The lipase-like activity of crude extracts was studied by using phenol red indicator. Initially Castor Oil - Phenol Red Agar plate composition of 0.01 % phenol red, 0.1 % of castor oil, 0.1 % of Calcium Chloride, 2 % of agar agar powder was prepared with pH of 7.3 to 7.4 and sterilized. After then 6mm sized well was cut into a prepared plate and 100 µL of crude extract was loaded into the well and incubated for 24 hours in incubator. Positive results observed by a yellow colour zone formation around the well confirmed the presence of lipase-like activity in crude extract.

Anti-inflammatory activity

The anti-inflammatory activity was determined by Bovine Serum Albumin (BSA) protein anti-denaturation activity by followed [31]. The reaction mixture of 5mL contains 0.2 mL of 1 % of BSA, 4.78 mL of Phosphate Buffer Saline (PBS) at pH 6.4 and 0.02 mL of crude extracts. The reaction mixture was then mixed gently and incubate it for 37 ℃ for 15 minutes. After incubation, reaction mixture was heated at 90 ℃ for 5 minutes. Finally reaction mixture analyzed by UV spectrophotometer (Hitachi UH5300) at 600 nm after cooling. PBS (pH 6.4) was considered as control and distilled water as blank. For positive control or standard, Diclofenac sodium drug (5mg/mL) was used. The percentage of inhibition of protein denaturation is calculated by the below formula [5]

Fourier Transform Infrared (FTIR) Analysis

The general working condition of FT-IR spectroscopy is relies on the absorption of vibrating chemical bonds in sample at certain specific frequencies indicated by infrared ray spectrum. The amount of radiation observed by the sample was converted into vibrational energy by molecules presence in a sample. After then, the FTIR detector detect the resulted spectrum of 400 cm-1 to 4000 cm-1 regions. In this study, we used FTIR analysis for the identification of chemical compounds presence in crude extract (EFSP909) of secondary metabolites. A common alkali halide potassium bromide (KBr) is used pellet method. Approximately 100 µL of crude extract were completely mixed with 150 to 250 mg of KBr powder and pulverize into 200 mesh maximum. Then the sample-KBr mixture was placed in pellet forming die for few minutes. 8 tons force is exerted on the sample-KBr mixture under a vacuum of several mm (Hg) to form transparent pellet formation for few minutes. Before measurement, KBr pellet with no sample were measured to avoid infrared light scattering loss. The prepared sample-KBr mixture pellet of endophytic fungal crude extract EFSP909 was placed in sample chamber of FTIR spectroscope (Shimadzu - QP2010 PLUS, Japan) for 50 scans.

Gas Chromatography - Mass Spectrometer (GC - MS) Analysis

Gas Chromatography Program set up: Column Oven temperature (50.0 ℃), Injection Temperature (280.0 ℃), Injection Mode (split), Flow Control Mode (Pressure), Split ratio (5.0). (2) Mass Spectrophotometer Program Set up: Start time (3 min), end time (39 min), ACQ mode (Scan), Event time (30 sec), Scan speed (3333), Start m/z (50.00), End m/z (1000.00).

Genetic Identification

Genomic DNA isolation from isolate following the EXpure Microbial DNA isolation kit developed by Bogar Bio Bee stores Pvt Ltd. Isolated DNA concentrations was measured by Qubit 3.0 and analyzed in 1 % agarose gel electrophoresis for band identification. The Internal Transcribed Spacer (ITS) 1 and 4 region (ITS 1 - 5' TCCGTAGGTGAACCTGCGG 3' 5' and ITS 4 - TCCTCCGCTTATTGATATGC 3') was amplified using Polymerase Chain Reaction (PCR) was carried out in 5 µL of isolated DNA using 25 µL of PCR reaction mixture.

PCR Product purification

Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore). The PCR product was sequenced using the primers. Sequencing reactions were performed using a ABI PRISM® Big DyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).

Single-Pass Sequencing

Each template using ITS 1 and 4 rRNA universal primers. The fluorescent-tagged fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Molecular phylogeny

Molecular phylogeny analysis was done after acquired amplified ITS 1 and 4 region from Sanger Sequencing method. The blast was run using NCBI blast web tool. The phylogeny analysis of blast results was performed followed by multiple sequence alignment. The program MUSCLE 3.7 was used for multiple alignments of sequences. The framed aligned sequences were cured using the program Gblocks 0.91b to eliminate removes alignment noise. Finally, the program PhyML 3.0 aLRT curated for phylogeny analysis and HKY85 as Substitution model. PhyML was shown to other existing phylogeny programs using simulated data. The Tree Dyn 198.3 for tree rendering. The sequenced ITS 1 and 4 regions deposited to GenBank and acquired accession number (MT158738).

Protein Preparation

Deregulated oncogenic BRAF (V600E) kinase has been reported major cause in almost 50% of patients with malignant melanomas and 5 to 15 % of colorectal cancers with involved in increase and spreading of caner cells. There are various BRAF (V600E) inhibitors are currently using in clinical activities to treat metastatic melanoma but their treating efficacy is not up to much impressive. In this study, we docked secondary metabolites of endophytic fungi Aspergillus terreus from Eichhornia crassipes against BRAF (V600E) kinase target (PDB ID : 4R5Y). These findings support future discovery of anti-cancer drug candidate to inhibit BRAF(V600E) kinase.

Ligands Library Preparation

Totally 20 candidates were obtained from our previous work and selected for molecular docking. The chosen ligands were proceeded to virtual screening of potential drug candidate against BRAF (V600E) kinase target by molecular docking.

Energy Minimization of Protein and Ligands

Energy minimization is important to remove local constraints for a residue of proteins. Swiss-Pdb viewer is a version of GROMOS 43B1 force field which allows to evaluate the energy of a protein and ligand structures to repair distorded geometries by energy minimization step. The energy minimization step was done in vacuo without including reaction field. The distorded geometries are repaired by releasing of internal constraints in order by moving the atoms in protein and ligand structures. The moving direction of atoms to reach with the colour indication of lower energy state displaced (dotted lines), minimal deplacement (dark, blue) and a large displacement (blue-green-red gradient). After energy minimization, instead of MD simulation of about 200 cycles of Steepest Descent

program repaired the geometries, configuration, orientation in protein and ligand structures . The final refined protein model was created by removed spdbv coordinates for further studies.

Molecular Docking by Auto Dock 4.0 Version

For comparison the positive reference drug vemurfenib (BRAF Kinase targeted Drug) was included in our study for better understand in identification of leads compounds. Ligands library was prepared in MDL mol files and Open Babel free software was used for converting the .mol file to pdb format for molecular docking step. Targeted protein was converted to PDBQT format using installed MGL Tools (version - 1.5.6rc3). The polar hydrogen atoms, kollman charge (24.688) and atoms as assigned radii was added. A ligand binding poses generated by AutoDock shows that the highest accuracy is achieved when the search space dimensions are 2.9 times larger than the radius of gyration. The x,y and z coordinates (21.536, -0.035 and -11.56), current total grid points per map (531441) and spacing angstrom (1.000) of grid box parameters were automatically generated with the help of installed MGL tools to increase the accuracy of docking studies. Calculating 5 grids over 597051 elements, around 4912 receptor atoms. Every pairwise-atomic interaction was clamped at 100000.00. Docking study employed Lamarckian genetic algorithm to generate a significant conformation of a ligands and protein docking. Totally Ten docking runs were performed and the scoring of the generated docking poses was based on the RMSD (Root Mean Square Deviation) empirical scoring function approximating the binding affinity in kcal/mol and inhibition constant in µM and nM. The docked ligands were observed in an order of minimum binding energy and the created .pdb files of Ligand - amino acid residues interaction profiles were visualized by using UCSF Chimera (3D visualizing software).

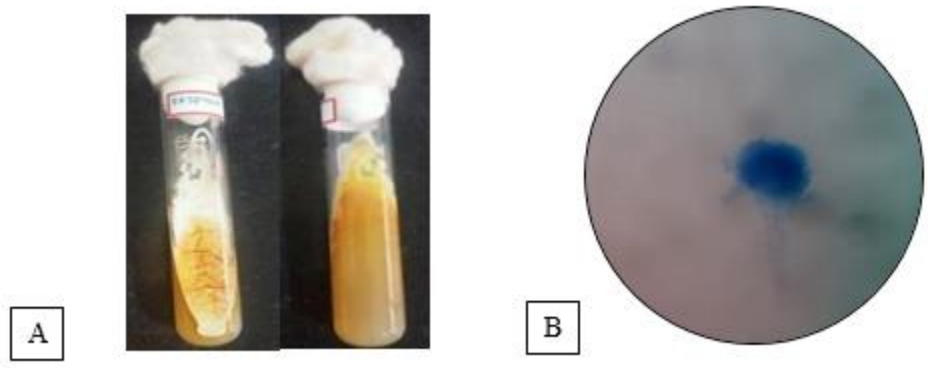

Figure 1: A. *Aspergillus terreus* - Colonies on Potato Dextrose Agar (PDA) are white in young and beige to cinnamon colour colony at mature. Reverse yellow pigment production and powdery mycelium mat was observed. B. The fungal isolate (EFSP909) was examined under 40X magnification followed by LPCB staining. The observation conformed the isolate was belongs to *Aspergillus* species.

Hydrogen Bond Analysis

The hydrogen bond formation contributed as the strong interacting stability of protein-ligand complex in a drug development and it is an important in enzymatic catalysis reaction to stabilize a ligand in a binding pocket of targeted protein. The hydrogen bond analysis determined hydrogen bond donor - acceptor pair in protein-ligand complex. More the amino acids residues in a protein, the greater number of hydrogen bond formation. The hydrogen bond analysis was done by using Protein-Ligand Interaction Profiler (PLIP) web tool.

ADMET Analysis

To compute ADMET analysis, SwissADME web tool was used . From this computation, physiochemical properties, lipophilicity, water solubility, pharmacokinetics, druglikeness and medicinal chemistry were predicted. The toxicity models were predicted by using Prediction of Toxicity of Chemicals (ProTox-II), a virtual web tool lab for predicting toxicity category of ligands. The ligands toxicity prediction enabled researchers to reduce and replace an experiment on animals and also reduce animal testing with suggesting animal Ethics 3R.

RESULTS AND DISCUSSIONS

In-vitro activity of endophyte EFSP909 crude extracts include potential anti-bacterial, anti-inflammatory, lipase-like activity. Moreover, insilico drug design involved targeted protein preparation, ligands preparation, virtual screening and molecular docking studies plays an efficient role in better understand of interaction between targeted protein and drug (ligands) candidates. The GC-MS resulted volatile secondary metabolites were proceeded molecular docking using AutoDock 4.0 version. Totally 20 compounds were selected as drug candidates and checked for ADMET properties (Figure 1,2,3,4,5,6,7,8,9,10,11 and 12) (Table 1,2,3,4,5,6,7,8,9,10 and 11).

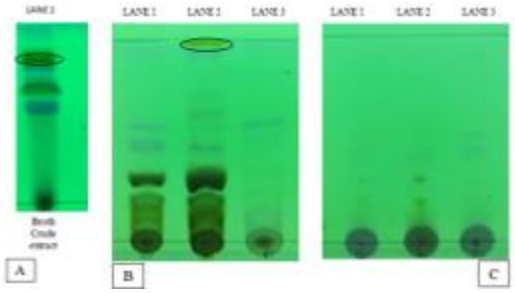

Figure 2: (A) Lane 1 - Broth crude extract was separated by Chloroform: Methanol in the ratio of 4 :1. (B,C) The lane 1 - stem crude extract, lane 2 - leaf crude extract and lane 3 - root crude extract represents the labeled plant parts. Ethyl acetate (B) and Carbinol (C) extracted plant parts crude extract were analyzed by TLC plates for separation of compounds

Table 1: Zone of Inhibition formed by the extracted secondary metabolites crude extract

Endophytic fungi with Isolate Code	Tested Clinical Pathogens	Zone of Inhibition (ZOI) in mm			
		50 µl	70 µl	Negative control (50 µl)	Positive control (50 µl)
EFSP909	Escherichia coli	39 mm	39 mm	-	39 mm
(*Aspergillus terreus*)	Staphylococcus aureus	Merge zone	Merge zone	-	Merge Zone
	Pseudomonas aeruginosa	24 mm	34 mm	-	29 mm
	Klebsiella pneumoniae	31 mm	34 mm		39 mm

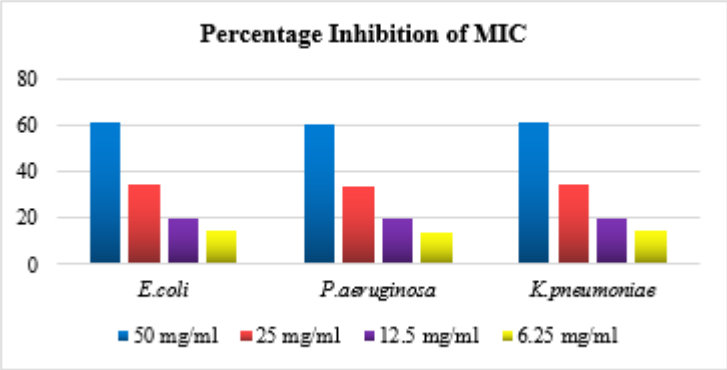

Figure 3: From the MIC data, the concentration 25 mg/mL was selected as the ideal minimum concentration to inhibit the bacterial growth and 50 mg/mL concentration was selected as the maximum concentration to inhibit the bacterial growth

Figure 4: The lipase-like activity was studied using Castor Oil - Phenol Red Agar medium. From the observed yellow colour zone formation was confirmed that crude extract has potent lipase-like activity

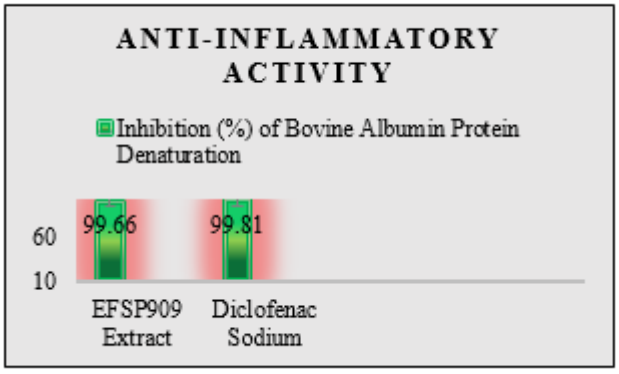

Figure 5: Secondary metabolites crude extract (conc. in mg / ml) - Total Volume refers to the final solution volume (i.e., Combines volume of solute and solvent). Anti-inflammatory analysis results revealed that crude extract (50 mg/mL) exhibited high activity by compared it with standard drug Diclofenac Sodium (5 mg/mL)

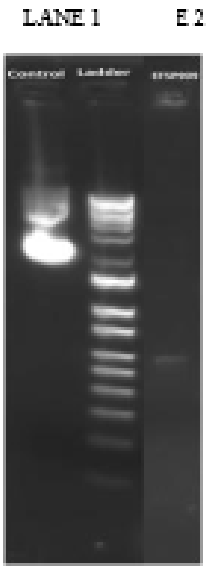

Figure 6: After amplification of PCR products ITS 1 and ITS 4 were run on 1 % of Agarose Gel electrophoresis to conform the amplified PCR products. Lane 1 is Molecular ladder of 500 bp. Lane 2 is ITS 1 and ITS 4 PCR products of 481 bp

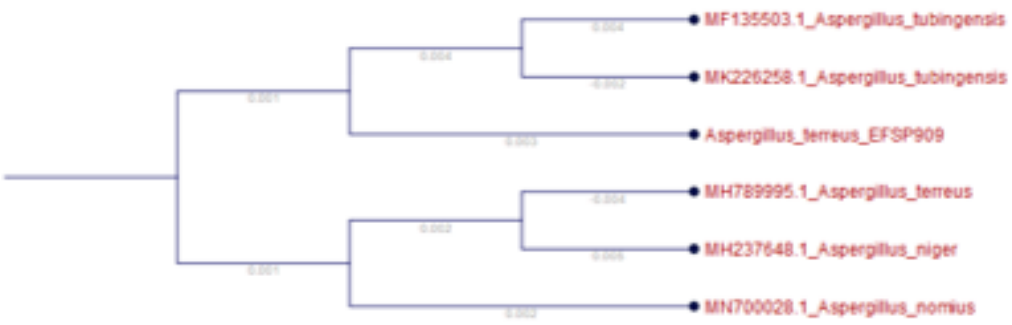

Figure 7: The above figure represents the isolate EFSP909 amplified and sequenced ITS 1 and 4 variable region matched with *Aspergillus terreus* of 98.15 % sequence similarity

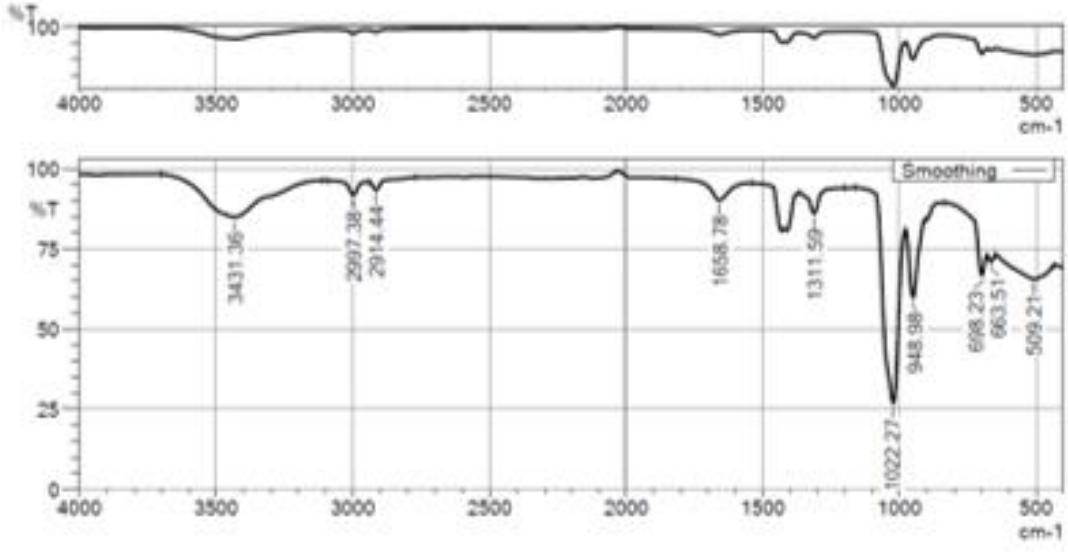

Figure 8: FT - IR result depicts that secondary metabolites crude extracts have Halo compounds, Alkene, Phenol, Sulfone functional groups by followed KBr pellet method

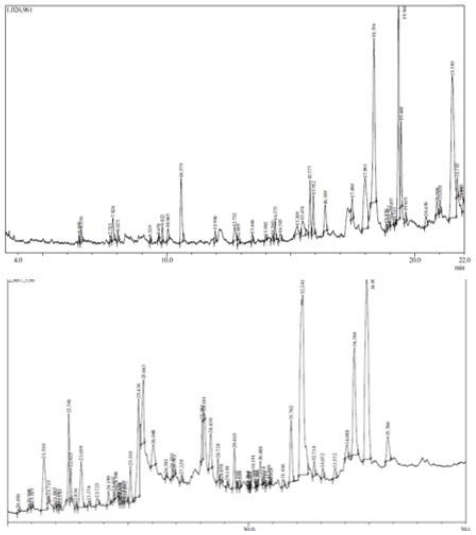

Figure 9: The analysis run time was 40 minutes to explore most of the separation. The molecular 2D structure of ligands were determined and used to understanding the chemical skeleton. Selectively 20 secondary metabolites were chosen for in-silico anti-cancer activity

Table 2: Identified functional groups in the secondary metabolites crude extract

Functional Group Region	**Peak Intensity Interpretation**	**Fingerprinting Region**	**Peak Interpretation**
1658.78 cm-1	Medium, C = C stretching, Alkene, dissubstituted (cis) bond type	509.21 cm-1	Strong, C-I stretching, Halo compound
2914.44 cm-1	Weak, Broad, O - H stretching, Alcohol, Intramolecular bonded	663.51 cm-1	Strong, C-Br stretcing, Halo compound
2997.38 cm-1	Weak, Broad, O - H stretching, Alcohol, Intramolecular bonded	698.23 cm-1	Strong, C=C bending, Alkene, Disubstituted (cis)
3431.36 cm-1	Strong, Broad, O - H stretching, Intermolecular bonded	948.98 cm-1	Vibration of C - O stretching
____		1022.27 cm-1	Phenol
		1311.59 cm-1	Strong, S=O stretching, Sulfone

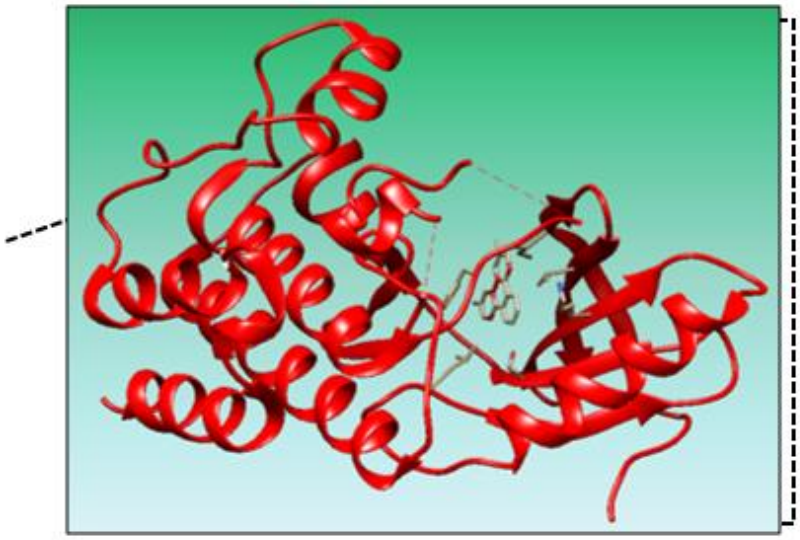

Figure 10: Diisobutyl phthalate bound with chain B of protein molecule

Table 4: The docked ligands and their minimum binding energy

S. No.	Ligands	Minimum binding energy
1	1-Octanesulfonyl chloride	-4.25
2	3,5-Dimethylbenzaldehyde	-5.18
3	Trans-Hexahydro-2(3H)-benzofuranone	-4.96
4	4-Nitrobenzaldehyde	-5.6
5	2,4-Ditert-Butylphenol	-6.65
6	(3RS,6R)-3-METHYL-6-(1'-methylethenyl)dec-9-en-1-yl ACETATE	-5.51
7	o-Tolylamino-acetic acid [1-(2,5-dihydroxy-phenyl)-ethylidene]-hydrazide	-9.37
8	Diisobutyl phthalate	-7.86
9	1H-2-Benzopyran-1-one	-5.44
10	5-(Morpholino)Pent-2-en-4-ynal	-4.68
11	Methyl 2-O-benzyl-d-arabinofuranoside	-5.08
12	(3-methoxymethoxy-1,4-dimethyl-pent-1-enyl)-benzene	-4.83
13	Heptadecyl heptafluorobutyrate	-4.8
14	Tetracosyl heptafluorobutyrate	-5.5
15	Physcion	-7.59
16	2,2,7,7-Tetramethyl-5-octyn-4-one	-5.15
17	2-Chloro-5-methyl-1,3-benzenediol	-5.94
18	1H-Pyrazole	-3.9
19	Tert-Butylpropanedial	-4.57
20	4,6,8-Trimethyl-1-nonene	-3.93
21	Vemurfenib	-7.12

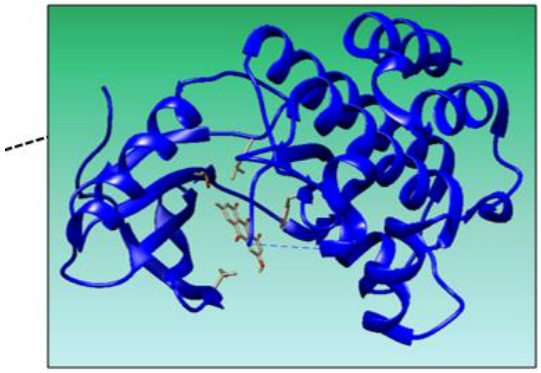

Figure 11: Physcion bound with chain A of protein molecule

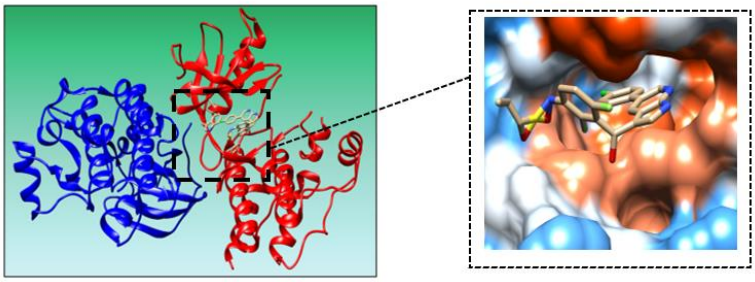

Figure 12 : Vemurafenib bound with chain B of protein molecule

Table 5: Amino acids of targeted protein interacting with bounded ligands

S.No.	Ligands	Interacting chains	Interacting Amino acids	No. of Hydrogen Bond formed
1	Diisobutyl phthalate	B	Phe 583, Cys 532, Trp 531, Gln 530, Leu 514, Thr 529, Asp 594, Ala 481	3
2	Physcion	A	Gly 534, Phe 583, Leu 514, Phe 595, Cys 532, Glu 533, Gln, Trp 531, Thr 529, Ala 481, Ile 463, Val 471	11
3	o-Tolylamino-acetic acid [1-(2,5-dihydroxy-phenyl)-ethylidene]-hydrazide	A & B	Asn 512, Lys 591, Ile 513, Val 511, His 510, Val 511, Leu 515, Leu 514.A, Thr 508.A, Arg 509, Phe 516.A, Arg 509.A, Gln 530.A, Leu 515.A	12
4	Vemurfenib	B	Val 471, Ala 481, Gly 464, Leu 597, Gln 530, Ile 463, Phe 595, Trp 531, Cys 532, Ser 535, Ser 536	6

Protein-ligand binding affinity was evaluated by hydrophobic interaction and hydrogen bond pairing. Hydrogen bond pairing in protein-ligand complex support the discovery and optimization of lead compounds targeting drug designing methods.

Table 6: Hydrophobic interaction profile of Diisobutyl phthalate

S.NO.	Residue	Amino Acids	Distance	Ligand atom	Protein atom
1	463B	ILE	3.53	4005	2112
2	471B	VAL	3.59	3991	2156
3	483B	LYS	3.59	3998	2252
4	514B	LEU	3.31	3996	2493
5	531B	TRP	3.77	4003	2627
6	595B	PHE	3.96	4001	3140

Table 7: Hydrophobic interaction profile of Physcion

S.NO.	Residue	Amino Acids	Distance	Ligand atom	Protein atom
1	463A	ILE	3.7	3999	120
2	471A	VAL	3.8	3990	163
3	481A	ALA	3.73	3994	246
4	514A	LEU	3.46	3988	504
5	529A	THR	3.97	3988	616
6	595A	PHE	3.63	3991	1144
7	595A	PHE	3.7	3992	1146

Table 8: Hydrophobic interaction profile of o-Tolylamino-acetic acid [1-(2,5-dihydroxy-phenyl)-ethylidene]-hydrazide

S.NO.	Residue	Amino Acids	Distance	Ligand atom	Protein atom
1	509A	ARG	3.58	4008	458
2	515A	LEU	3.34	3999	512
3	515A	LEU	3.88	4001	512
4	515A	LEU	2.82	4008	511
5	515B	LEU	3.66	3994	2501

Table 9 : Hydrogen Bond interaction between targeted protein and ligand complex in diisobutyl phthalate

S.NO.	Residue	Amino Acids	Distance H-A	Distance D-A	Donor atom	Acceptor atom
1	529B	THR	2.78	3.54	2605	3987

Table 10: Hydrogen Bond interaction between targeted protein and ligand complex in Physcion

S.NO.	Residue	Amino Acids	Distance H-A	Distance D-A	Donor atom	Acceptor atom
1	510A	HIS	2.32	2.94	464	4004
2	510A	HIS	2.52	2.68	4004	467
3	530A	GLN	3.13	3.61	625	4002
4	591A	LYS	3.09	4.09	1117	3989
5	591A	LYS	2.89	3.73	3992	1117
6	591B	LYS	2.7	3.33	3111	4002

Table 11: Hydrogen Bond interaction between targeted protein and ligand complex in o-Tolylamino-acetic acid [1-(2,5-dihydroxy-phenyl)-ethylidene]-hydrazide

S.NO.	Residue	Amino Acids	Distance H-A	Distance D-A	Donor atom	Acceptor atom
1	530A	GLN	2.26	153.64	3995	620
2	532A	CYS	3.18	129.26	4003	643
3	532A	CYS	3.19	174.22	640	3995

CONCLUSION

The absorption, distribution, metabolism, excretion and toxicity properties were evaluated to the potent ligands which showed most minimum binding energy with docked protein in order to understand the ADMET properties. The parameters include in this study are oral toxicity, organ toxicity, carcinogenicity, immunotoxicity, mutagenicity, cytotoxicity, Tox21 - nuclear receptor signalling pathways and stress response pathways. All predictions showed inactive state of candidates in ADMET properties. Hence, we reported to use this compounds as a BRAF V600E inhibitor to combact melanoma in patients.

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