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CHARACTERISATION OF BIO-ACTIVE COMPOUNDS OF *Penicillium chrysogenum* AAJ2 , a MARINE FUNGI ISOLATED FROM THE MANGROVE SOIL OF NIZAMPATNAM

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Abstract:
The fungal strain AAJ2 isolated from Sediment samples of the mangrove ecosystem of Nizampatnam, Andhra Pradesh was explored for bioactive metabolites with anti-microbial, anti-inflammatory and anti cancer potential. The fungal strain AAJ2 has shown potential biological activities in the preliminary screening of secondary metabolites, and identified as *Penicillium chrysogenum* AAJ2 (OQ569546) by ITS region analysis **.** Methanolic crude extract of AAJ2 reported anti-oxidative activity with DPPH with an IC_{50} value of 28.67 µg/ml and superoxide free radical scavenging activity with an IC_{50} value 12.41 µg/ml , 5LOX enzyme inhibition by 19.62% at 5mg/ml, 78.20 % of HRBC membrane stability and 41.10% of inhibition of MCF-7 cell lines at 120 µl. GC-MS and HPLC metabolite fingerprinting of methanolic extract of AAJ2 followed by *insilico* molecular docking studies of the identified bioactive compounds were checked for affinity with human proteome using Swiss target prediction and also predicted potential anti-cancer and anti-inflammatory properties. Our investigation revealed that 7-hydroxyjanthinone, Shearinines B, Citrinin, Janthinone and Tetradecane are significant compounds of *P. chrysogenum* AAJ2 strain, a novel marine fungus and are responsible for various pharmacological activities.

Keywords: Marine fungus, *Penicillium chrysogenum*, Secondary metabolites, Anti microbial activity, Anti oxidation, Protein docking.

1. Introduction

Mangroves are distinct ecosystems with various species in tropical and subtropical latitudes along the intertidal zone (Barbier *et al*., 2008). They offer a diverse range of supporting, supplying, and regulating ecosystem functions and are extensively dispersed throughout 137,769 km^2 of coastlines worldwide (Getzner & Islam, 2020; Giri *et al*., 2011). Because of their ability to absorb and store carbon, mangrove ecosystems are renowned for reducing the consequences of climate change and defending coastlines from erosion and sea level rise (Howard *et al*., 2017). Mangroves are incredibly productive ecosystems that include intricate microbial communities that play vital role in the cycling of nutrients and the breakdown of organic matter into sources of nitrogen and phosphorus that plants may utilise (Guo *et al*., 2013; Mendes & Tsai, 2018). Throughout the biogeochemical cycle, soil microorganisms produce nitrous oxide, carbon dioxide, and other greenhouse gases into the atmosphere (Gomes *et al*., 2010). The ecosystem's biogeographic, ecological, and anthropogenic characteristics impact the microbial communities in mangrove soils (Gomes *et al*., 2010). This environment is extraordinarily diversified in microbial life forms because of the particular physicochemical properties of mangrove soil, such as levels of oxygen, salinity, and pH changes, and as a result, is a promising reservoir of fungi of biotechnological interest (B C. Behera, Thatoi *et al*., 2012).

Despite the high diversity of microbes, only 5% of the microbe species found in mangrove environments have been characterised. Less than 1% of the world's microbes have domesticated, and only 5% of those, including bacteria and fungi, have undergone chemical analysis (Xu *et al*., 2015). To manufacture antibacterial, anti-cancer, anti-oxidant, anti-diabetic, and other therapeutic agents have been produced by mangrove endophytic fungus,used in the pharmaceutical and nutraceuticals industries (Keller & Zengler, 2004). In addition, some mangrove fungi produce bio-pesticides that aid in the control of plant diseases and also as a feedstock for biodiesel production. As mangrove soil possess plethora of fungi with potential secondary metabolites in the current investigation, Marine fungal strain that produces secondary metabolites was isolated and its biological activities such as anti-bacterial , anti-oxidant, anti-inflammatory, and anti-cancerous action was explored along with metabolite fingerprinting using GCMS and HPLC and *insilico* molecular docking.

2. Material and Methods:

2.1. Isolation of Fungi :

Soil samples collected from five different sites of Nizampatnam mangrove forest, Andhra Pradesh. 2 gm of each soil sample was diluted by serial dilution method. 100µg/ml antibiotic streptomycin was used to prevent bacterial growth (J H War cup, 1950). 20 µl soil sample of 10^{-4} dilution spread on a Czapekdox yeast extract agar (CYA) medium. The plates were grown at 30ºC for 72-96 hrs. The plates that showed good fungal growth were selected and further purified for pure cultures.

2.2. Identification of fungal strains:

2.2.1. Microscopic observation by slide culture technique:

Sterilized Czapekdox yeast extract agar medium (CYA) medium poured into the petriplates and allowed to solidify. After solidification, 1cm agar blocks of solidified medium transferred the glass slide and inoculated with fungal isolate on the corners of the block, and covered with a cover slip. The inoculated agar block was placed in the wet chamber and incubated at 37ºC for 24 to 48 hrs. After incubation, the cover slip was taken from the wet chamber and placed on the sterilised glass slide containing a lacto phenol cotton blue drop (Riddell, 1950). The mycelium, presence of conidia and conidiophores were observed using an Olympus binocular digital microscope (model Motic-BA410).

2.3. Molecular identification by ITS region analysis

DNA extracted from fully grown fungal culture was centrifuged with alkaline solution as described (Wright et al., 2001) and DNA pellet was washed with 70% ice-cold ethanol and dried and resuspended in TE Buffer. Isolated DNA was confirmed on 0.8% agarose gel. Further partial gene analysis was carried out using ITS1 primer with the help of Macrogen inc, Seoul, South Korea. The ITS region of gene sequence was identified using BLAST (N) of NCBI and pair-wise evolutionary distances were computed by MEGA-11 software. The phylogenetic analysis was conducted using the maximum parsimony method. The ITS region of the gene sequence of the isolate was registered in the GenBank database.

2.4. Screening of bioactive metabolites

Crude fungal broth (CFB) of 14 days old fungus was grown on Czapekdox yeast extract broth (CYB) broth at 35°C was used to screen bioactive metabolites using standard protocol. For qualitative confirmation CFE was mixed with a few drops of diluted HCl and filtered and the

filtrate was tested with Mayer's, Dragendorff's, Hager's, and Wagner's reagents to confirm alkaloids (Farnsworth et al., 1966). CFE was subjected to Molisch's test to detect the presence of Glycosides. Tannins were confirmed as yellow-brown precipitate with 10% aqueous potassium dichromate solution (Schandrei, 1970). Ellagic acid test for *phenol, Reducing sugar* by Dinitro salicyclic acid method (Miller, 1972) and *Protein* by Lowry method (Lowry *et al.,* 1951).

2.4.1. Anti-microbial Activity:

Primary Screening:

The crude fungal extract (CFE) was tested against the clinical pathogens (*Xanthomonas campestris* (MTCC 2286), *Bacillus megaterium* (NCIM 2187), *Escherichia coli*(ATCC 9027), *Pseudomonas aeruginosa* (ATCC 9027), and the fungus *Candida albicans* (MTCC 183)) separately using Agar well diffusion method, after 48hr, inhibition zones were measured. 20 µl of CFE was introduced into the prepared wells and incubated at 35°C (Silva et.al, 2011).

Secondary screening:

CFE separated from the mycelium by filtration through Whatman no1 filter paper, and the filtrate was mixed with methanol (1:1, v/v). The methanol extract (ME) was dissolved in 500 µL of dimethylsulfoxide (M R O Silva, A C Almeida, 2011). 20 µL of extract was placed in Muller-Hinton agar medium inoculated with test bacterial suspensions adjusted according to 0.5 McFarland standard solutions 0.5. Plates were incubated at 35°C, and inhibition zone diameter was measured after 24/48 hr.

2.5. Optimization of Physico chemical factors of bioactive metabolites:

Efficacy of secondary metabolite production was measured in terms of zone of inhibition against tested pathogens using agar well diffusion method (Taritla S 2021). Putative fungal isolate grown in CY broth was subjected to optimization studies at different incubation periods (2,4,6 and 8 days) , pH (ranging from 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0), temperature (26ºC, 28ºC,30ºC,35ºC, and 40ºC), carbon sources (Galactose, Maltose, Fructose, Starch, Sucrose, and Cellulose at a rate of 1% (w/v)), nitrogen sources (Yeast extract, Tryptone, Ammonium sulphate, Peptone and Malt extract at a rate of 1% (w/v)),metal ions ($MgSO_4$, $MnSO_4$, $FeSO_4$, $ZnSO_4$ and $CuSO_4$ @ 0.5 %) and different rpm (0, 100, 120, 150). Experiments were done in triplicates and values were statistically analysed. Potential chemical factors efficacy was also analysed at different concentrations (Carbon sources -

1,2,3,4,5), nitrogen sources –0.1,0.2,0.3,0.4,0.5)). By optimization of physical factors on one parameter one time (OPOT) method. Optimized chemical factors (carbon and nitrogen) were further analysed at different concentrations.

2.6. Extraction of bioactive secondary metabolites :

Solvent specificity of secondary metabolites was analysed using different solvents such as ethyl acetate, Methanol, Chloroform, and Acetone (1:1, v/v). The crude extract of different solvents ethyl acetate (EAE), Methanol (ME), Chloroform (CE) were dissolved separately with 500 µL of dimethyl sulfoxide (DMSO). 20 µL of each extract was placed on Mueller-Hinton agar medium plates inoculated with test bacterial suspensions adjusted according to McFarland standard solution 0.5 (Thanh Van Ngo et al., 2017) and plates were incubated at 35°C, and the diameter of the inhibition zones was measured after 24/48 hours. Organic solvent extraction which showed maximum inhibition was used for further investigation.

Statistical analysis

Data are expressed as the mean ± standard deviation. Statistical significance was defined as a two-tailed $p < 0.05$.

2.7. Quantitative analysis of Biological activities

2.7.1. DPPH radical scavenging assay

The antioxidant activity of crude extract was evaluated by 2, 2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) free radical. The solvent extract of fungi (5 – 50 µg/ml) was prepared in methanol. 0.002% of DPPH in ethanol was used as free radical. The 3ml of DPPH solution was mixed with different concentrations (5 – 50 µg/ml) of methanolic culture crude extract. The flasks were incubated in dark conditions at room temperature for 30 minutes. The optical density was measured at 520nm using UV-Vis spectrophotometer. Ascorbic acid was used as standard (Yamasaki et al., 1994 and Blois, 1958). The DPPH scavenging activity was expressed in percentage by using the following formula

$$\textbf{DPPH scavenging activity (\%)} = [\,(A_o - A_e) / A_o] \times 100$$

Where Ao is absorbance of the control; Ae is absorbance of the sample / standard. The half-inhibitory concentration (IC_{50}) values were calculated.

2.7.2. Superoxide radical scavenging assay

The reaction mixture contained 0.2 ml of Nitro blue tetrazolium (NBT) (1 mg/ml of solution in DMSO), different concentrations of test solution (5-50 µg/ml), 2 ml of alkaline DMSO (1 ml DMSO containing 5 mM NaOH in 0.1 ml H2O) in a final volume of 2.8 ml. The absorbance was recorded at 520 nm using a UV–VIS spectrophotometer. The blank consisted of pure DMSO instead of alkaline DMSO. The results have been expressed as ascorbic acid equivalent, which was used as a standard (Hyland et al., 1983; Zhou J et al., 2020).

$$\textbf{\% Inhibition} = (A0\text{-}A1) / A0 \times 100$$

Where A0 is absorbance of the control; A1 is absorbance of the culture extract

2.7.3.. In vitro 5-Lipoxygenase inhibition:

5-LOX enzyme inhibitory activity of MEAAJ2 was measured according to Reddanna *et al* 1999 and Ulusu *et. al*., 2002.The assay mixture contains 80 mM linoleic acid and 10 µl potato 5-LOX in 50 mM phosphate buffer (pH 6.3). The reaction mixture consists of enzyme buffer mix to linoleic acid and absorbance was measured at 234 nm. The inhibitory potential of crude compounds was measured at various concentrations (5-50 µg/ml) for 2min before addition of linoleic acid. Triplicates were maintained. Reaction was calculated by using the following formula.

$$\textbf{\% inhibition} = 100 \times (A0 - A1)/A0$$

Where A0 is absorbance of the control; A1 is absorbance of the culture extract

2.7.4.Human red blood cell (HRBC) membrane stabilisation:

In-vitro anti-inflammatory activity was tested through Membrane stabilisation method of human red blood cells (HRBC). A healthy human volunteer's blood was drawn and combined with an equal volume of Alsever solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid, and 0.42% NaCl) before being tested. Prior to usage, all blood samples were kept at 4°C for 24 hours. The supernatant was collected after 5 minutes of centrifugation at 2500 rpm. The cell suspension was centrifuged at 2500 rpm for 5 minutes after being cleaned with 0.9% w/v NaCl. Until the supernatant was clear and colourless, this process was repeated three times.

Crude extracts (at concentrations of 100 and 2000 µg/ml, respectively) in hypotonic phosphate buffer saline were added with 0.5 ml of HRBC solution and centrifuged for 20 min at 3000 rpm after 30min @ 37°C. At 560 nm, the supernatant solution's haemoglobin content was calculated spectrophotometrically (Sathuluri Vineela et al., 2021). Aspirin (100 g/ml) used as a standard and methanol as a control. The modified approach was used to calculate the percentage inhibition of hemolysis or membrane stabilisation.

$$\textbf{\% Inhibition of hemolysis} = 100 \times \{OD1 - OD2/OD1\}$$

where: OD1 = Optical density of hypotonic-buffered saline solution. OD2 = Optical density of test sample in hypotonic solution.

2.7.5. Anti-cancer activity:

Cytotoxic potential was evaluated by the MTT Assay, cells were trypsinised, and the trypan blue assay was performed to know viable cells in cell suspension. Cells were counted by haemocytometer and seeded at a density of 5.0 X $^{10-3}$ cells / well in 100 µl media in 96 well plate culture medium and incubated overnight at 37°C. After incubation, discarded the old media and add fresh media 100µl with various concentrations of crude compounds in represented wells in 96 plates. After 48 hrs, Discard the drug solution and add the fresh media with MTT solution (0.5 mg / mL-1) to each well and plates were incubated at 37°C/3 hr. After incubation, precipitates are formed as a result of the reduction of the MTT salt to chromophore formazan crystals by the cells (K. Daniel and M. Guravaiah, 2018). The optical density of solubilised crystals in DMSO was measured at 570 nm. The % growth inhibition was calculated using the following formula.

$$\% \text{ inhibition} = 100 \times (A0 - A1)/A0$$

Where A0 is absorbance of the control; A1 is absorbance of the culture extract

2.8.Spectral analysis of secondary metabolites

2.8.1.GCMS analysis

Gas chromatography-mass spectroscopy (GC-MS) is one of the so-called hyphenated analytical techniques. GC separates the components of a mixture, and mass spectroscopy characterises each of the components individually. By combining the two techniques, an analytical chemist can both qualitatively and quantitatively evaluate a solution containing many chemicals**.**

The secondary metabolites present in the methanol crude extract were profiled using GCMS analysis. The methanol was run through the GC column and it connected with a mass spectroscope. 2µl of crude extract was injected into the GC sample inlet. The sample was allowed to mix with carrier gas and travel through the column. Run multiple samples of supernatant from the fully grown isolated fungal species. The sample was run via column and detected by a detector (Wang et al., 2014). The MS remained connected with GC, so output was recorded via mass spectroscope. The data was collected and analysed based on output files. JASCO offer raw data that can be analysed using multiple software.

2.8.2. HPLC analysis

Shimadzu Prominence SPD-20A UV/Vis detector, Shimadzu CTO-10AS column oven, and Shimadzu LC-20D dual binary pumps were used in the high-performance liquid chromatography (HPLC) analysis. A C-18 reversed-phase column (Phenomenex, Gemini 5, 150 mm length, 4.6 mm internal diameter) was used for the analysis. With minor adjustments, the gradient elution profile and solvent composition used in this research were those previously described (Chew et al., 2009; Kaisoon et al., 2011). With a flow rate of 0.8 mL/min, acetonitrile served as solvent B, and acetic acid-acidified deionised water (pH 2.8) served as solvent A in the mobile phase. Here is how gradient elution was carried out: 5-15 min (9-11% solvent B); 22-38 min (11-18% solvent B); 38-43 min (18-23% solvent B); 43-44 min (23-90% solvent B); 45-55 min (80% solvent B); 55-60 min (80-5% solvent B). 0-5 min, 5-9% solvent B; 5-15 min, 9% solvent B; 15-22 min, 9-11% solvent B; 38-43 min, 18-23% solvent B; 43-44 min, 23-90% solvent B After each injection of samples, the column was equilibrated with 5% solvent B/20 min. The injection volume was 20 L, and the column temperature was adjusted to 38°C. In order to detect flavonoids, alkaloids and polyphenolic compounds, the wavelengths were chosen at 280 nm, 370 nm and 420 nm, respectively. By comparing individual retention times and peak areas with pure compounds, phenolic compounds were carried out. The range of standard quantities employed for the calibration curve was 0.01 mM to 3 mM.

2.9. *In silico* Molecular Docking Study:

The compounds isolated and characterised from the current study were subjected to the affinity check with human proteome. All major five molecules were checked for affinity with human proteome using Swiss target prediction—the following strategy was used for the affinity check between characterised molecules and human proteome.

SMILES for the drug (https://dev.drugbank.com/guides/terms/smiles); InChIKey (http://inchi.info/inchikey_overview_en.html) and PDB for proteins m (https://www.rcsb.org/) are the data sources used for the data collection. Computational tools such as MODELLER https://salilab.org/modeller/, AUTODOCK 4.2, and Ligand Scout (https://pubs.acs.org/doi/10.1021/ci049885e) were used for Molecular modelling molecular docking (https://vina.scripps.edu/) and simulations (https://www.gromacs.org/).

The binding site region was created using a range of distance 5 Å, and the grid was focused on the centre of the binding site region. As a result, a map of 50 x 50 x 50 points and a grid

spacing of 0.375 Å around the active site. The docking parameters used were based on the default software values, and only the number of GA runs set at 100 (https://vina.scripps.edu/) for each drug prediction of drug targets to the human proteome were done using servers (Swiss docking server http://www.swissdock.ch/). Docked clusters were selected based on docking score and binding energy. Additionally, higher probable docked clusters (drugs and affinity with human proteome) were tested individually using Auto Dock Vina (https://vina.scripps.edu/) and server-based docking (https://mcule.com/apps/1-click-docking/).

3. Results and Discussion:

3.1.Identification of marine fungus:

Out of nine morphologically distinct fungal strains isolated from mangrove soil sediment, one of the strains named AAJ2 was the subject of the present investigation. Based on morphological and microscopic characteristics (Figure 1), AAJ2 tentatively identified as *Penicillium* sp.

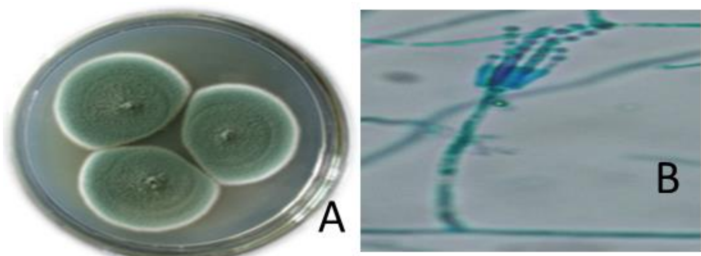


Figure 1. A).Colony morphology of AAJ2 in blue-green colour with yellow pigmentation in CYA medium and B) typical filamentous hyphae with conidia with conidiophores originated as long thick tubes with swollen vesicles under light microscope**.**

18s rRNA partial gene sequencing with amplification of ITS1 region followed by phylogenetic analysis revealed that AAJ2 was *Pencillium chrysogenum* (Plate 2 and Figure 1) and deposited in Gen bank, NCBI *as Pencillium chrysogenum* AAJ2 with Accession no OQ569546.

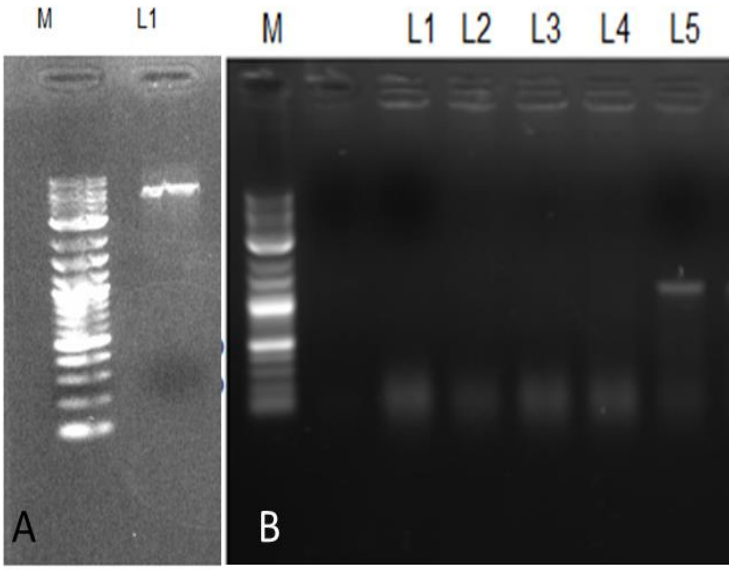


Figure 2: A. Total Genomic DNA of AAJ2 on 0.8% Agarose gel(Lane M represent marker lane while lane1 represents genomic DNA of AAJ2) B. Visualization of ITS1 region for 600bp for fungal isolate. (Lane M represent the marker lane, Lane 5 represents the AAJ2) .

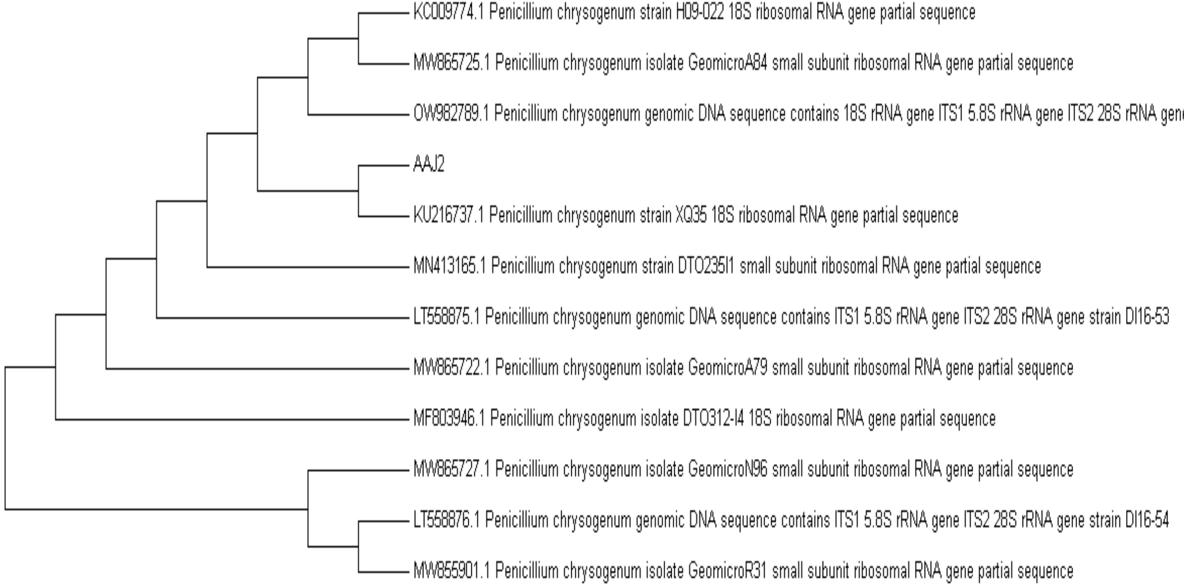


Figure 3. Phylogenetic analysis of ITS region sequence of AAJ2 showing sequence similarity by neighbor-joining (NJ) method

3.2.Bioactive Secondary metabolite analysis

Preliminary screening of methanolic crude extract(MCE) of AAJ2 showed positive confirmation of flavonoids, saponins, tannins, and phenolic compounds(Table 1).

Table 1. Analysis of bioactive secondary metabolites

Secondary Metabolites	Alkaloids	Flavanoids	Saponins	Quinones	Tannins	Steroids	Phenolis	Glycosides
AAJ2	-	+++	++	-	+	-	+++	-

3.3.Antimicrobial activity

Crude fungal broth of 14 days old AAJ2 grown in CYA medium was showed positive anti microbial activity against five clinical pathogens (*Xanthomonas campestris* (MTCC 2286), *Bacillus megaterium* (NCIM 2187), *Escherichia coli* (ATCC 9027), *Pseudomonas aeroginosa* (ATCC 9027), and the fungus *Candida albicans* (MTCC 183) in primary screening and showed a significant zone of inhibition against the growth of bacteria such as *Xanthomonas campestris* (MTCC 2286), *Bacillus megaterium*(NCIM 2187), *Escherichia coli* (ATCC 9027), *Pseudomonas aeruginosa*(ATCC 9027), and the fungus *Candida albicans* (MTCC 183) in secondary screening as shown in Figure 4, Table 2.

Table 2. Antimicrobial activity of crude extract of *P. chrysogenum* AAJ2

S.No	Fungal isolate	Zone of inhibition (mm)				
		Xanthomonas campestris	*Escherichia Coli*	*Bacillus megaterium*	*Pseudomonas aeroginosa*	*Candida Albicans*
1.	AAJ2	24±0.57	14.5±1.45	17.3±0.88	19±1.52	11±1.15

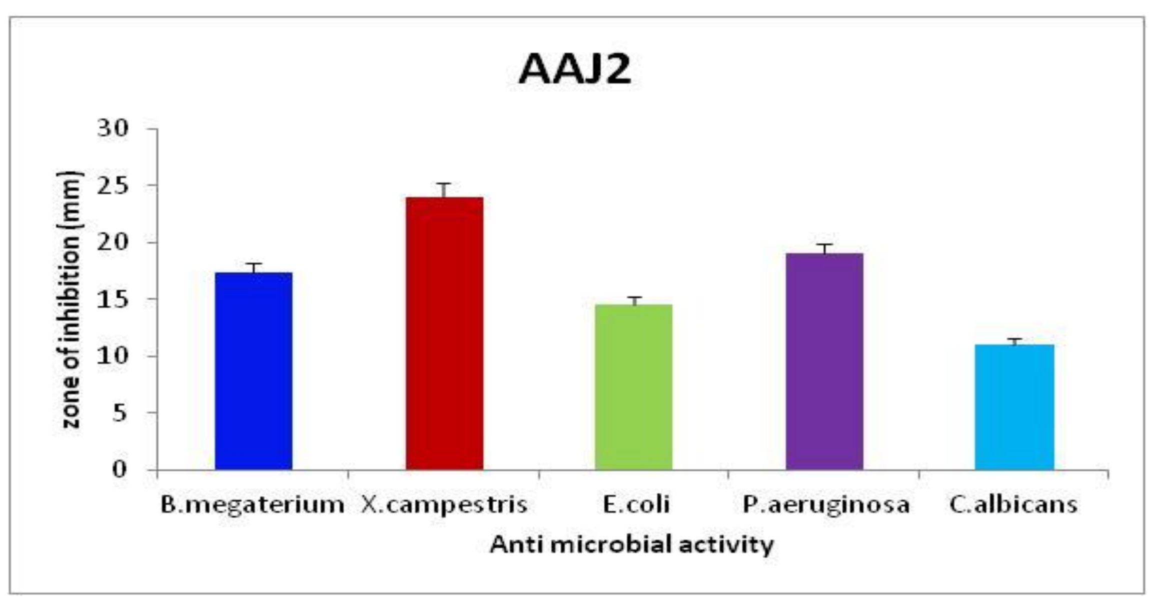


Figure 4. Anti microbial activity of AAJ2 against clinical test pathogens

3.4.Optimization of physico chemical factors of bioactive metabolites

Physico chemical factors of culture conditions were optimized to understand the efficacy of bioactive secondary metabolite production of AAJ2 in terms of anti microbial activity against five specified test pathogens (*Xanthomonas campestris*(MTCC 2286), *Bacillus megaterium* (NCIM 2187)*, Escherichia coli*(ATCC 9027)*, Pseudomonas aeroginosa*(ATCC 9027)*,* and the fungus *Candida albicans* (MTCC 183). AAJ2 showed a maximum zone of inhibition after 8 days of incubation at 35° C and pH 7 against five test pathogens . Chemical factors such as carbon and nitrogen sources and metal ions have also showed significant variation on anti microbial activity of AAJ2. Out of six carbon sources (Galactose, Maltose, fructose, starch, sucrose and cellulose) and five nitrogen sources (Yeast extract, Trptone, ammonium sulphate, Peptone and malt extract), Sucrose and Yeast extract showed greater influence on anti microbial activity against five test pathogens with higher values of zone of inhibition. Further reported that 3% of sucrose and 0.3% yeast extract showed signifgicant inhibition compared to other concentrations (Figure 5 and 6).

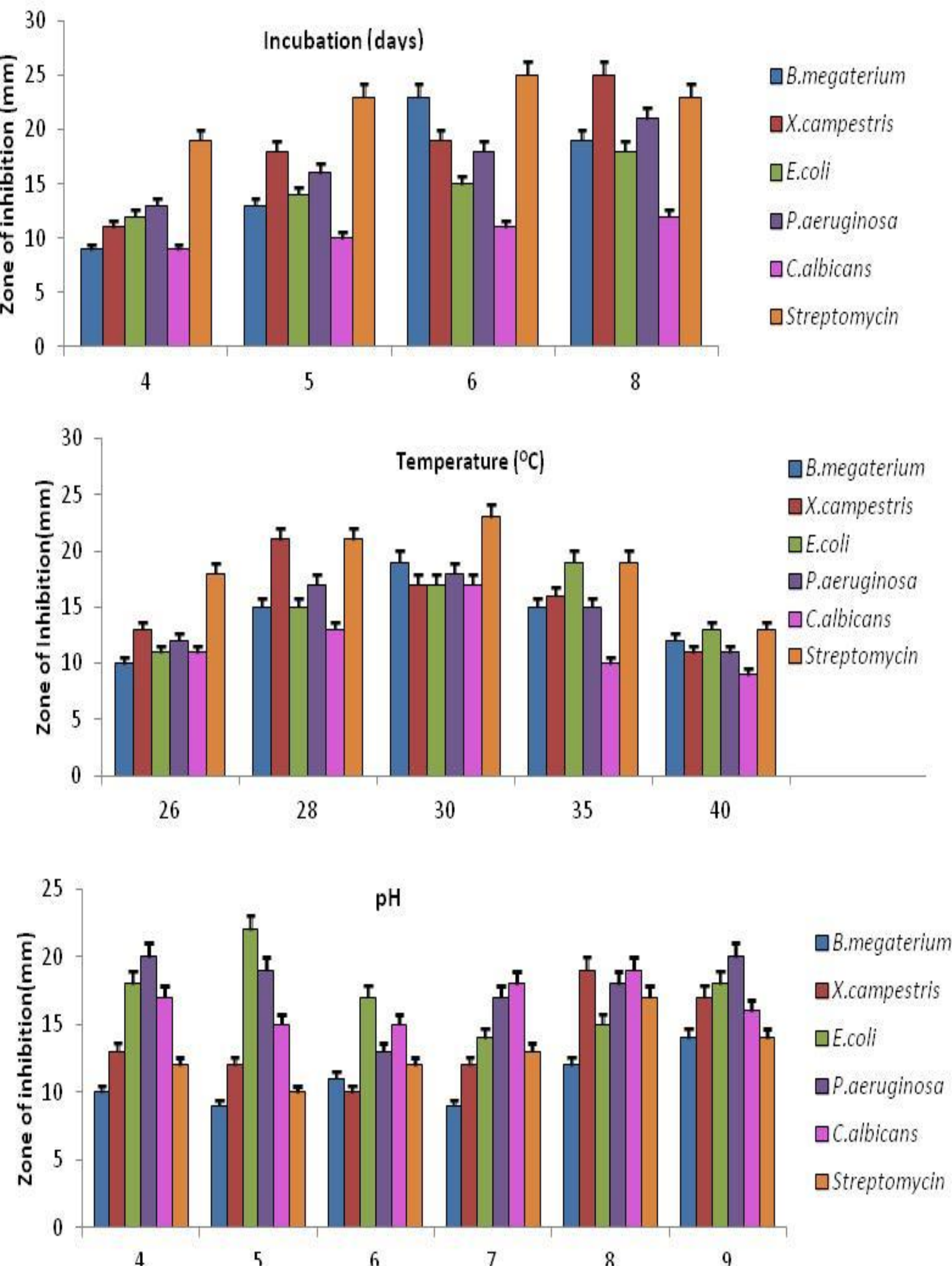


Figure 5. Effect of incubation, Temperature (^{O}C) and pH on anti fungal activity of AAJ2

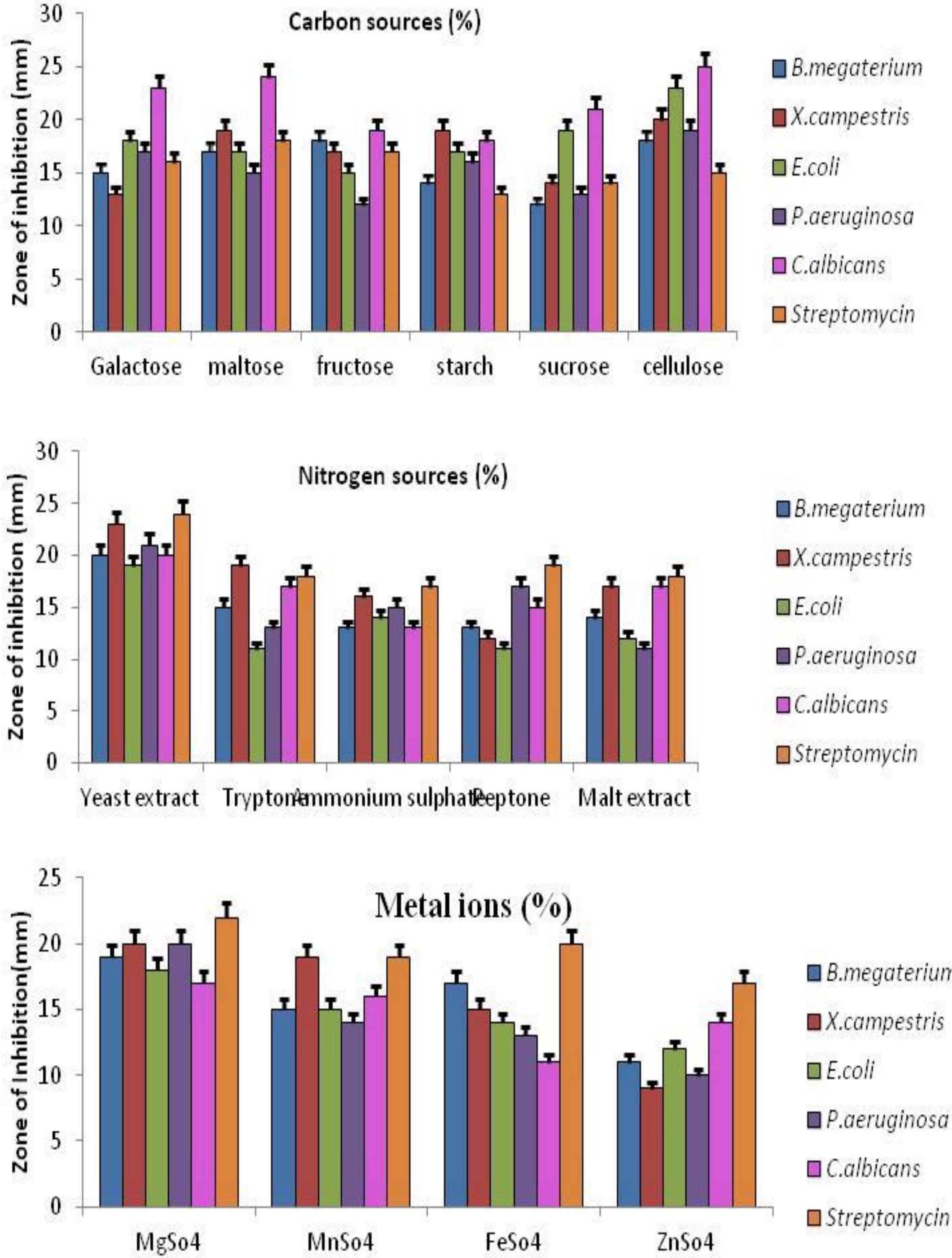


Figure 6. Effect of carbon sources, nitrogen sources and metal ions on anti fungal activity of AAJ2

3.5. Solvent specific anti microbial activity:

Out of four different solvents acetone, ethyl acetate, methanol and chloroform , Methanol showed greater influence on extraction of bioactive metabolites against five test pathogens (*Xanthomonas campestris*(MTCC 2286) (Figure 7), *Bacillus megaterium* (NCIM 2187), *Escherichia coli*(ATCC 9027), *Pseudomonas aeruginosa*(ATCC 9027), and the fungus *Candida albicans* (MTCC 183) with higher values of zone of inhibition

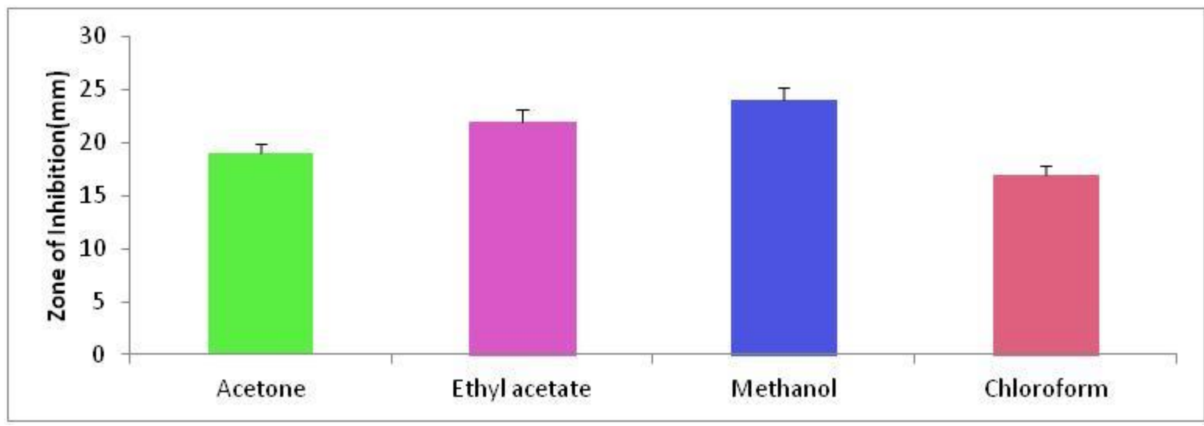


Figure 7. Anti microbial activity of AAJ2 on Xanthomonas campestris with different solvents

3.6.Quantitative analysis of biological activities:

Fungi are the critical source of diverse and bioactive phytochemicals for different applications in nutraceuticals and pharmaceutical industries. These molecules exert several therapeutic and protective properties on fungi, including anti-microbial, anti-oxidant, anti-inflammatory and anti-cancer properties.

3.6.1.Anti-oxidant activity:

The anti-oxidant activity of MEAAJ2 by DPPH and Superoxide free radical scavenging activities (Figure 8 and 9). showed 51.32 % DPPH inhibition with an IC_{50} value of 28.67µg/ml compared to ascorbic acid (IC_{50}-32.73µg/ml) and 76.32% of superoxide free radical scavenging activity with IC_{50} value of 12.41µg/ml compared to Gallic acid standard (IC_{50} value 6.16 µg/ml.)

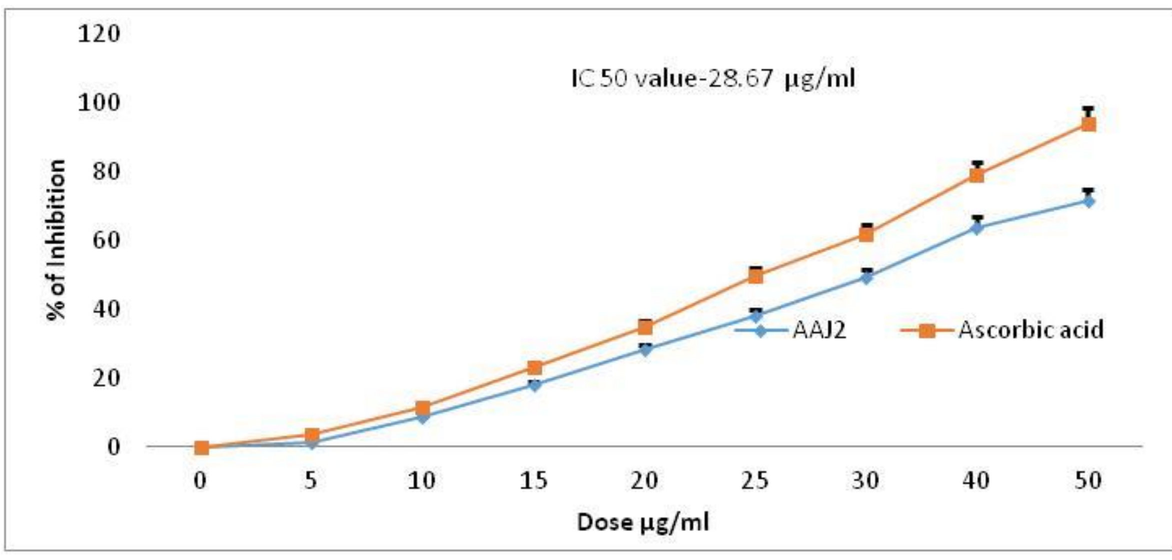


Figure 8. Anti-oxidant activities of Methanol extracts of AAJ2
DPPH radical scavenging activity

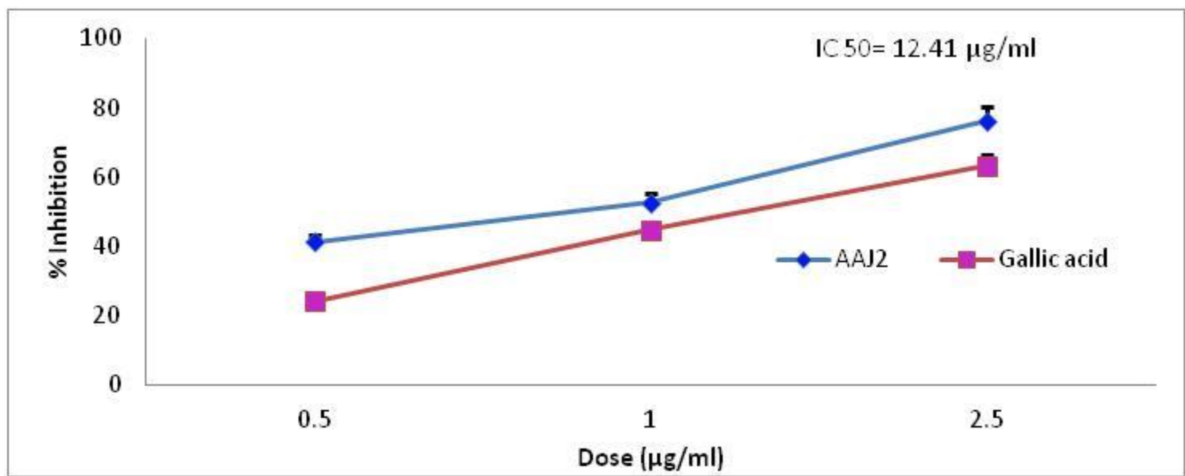


Figure 9 Anti-oxidant activity of Methanol extracts of AAJ2 by
Superoxide free radical scavenging activity

3.6.2.Anti-inflammatory activity:

The anti-inflammatory activity of MEAAJ2 was analysed by HRBC Membrane Stabilization and 5-Lipoxygenase inhibition(Figure.10 and 11) using gallic acid as standard and as per the data shown in the figure standard drug demonstrated maximum % HRBC membrane reported 81.44 at 50 µg/ml while MEAAJ2 reports 78.20 respectively. These results demonstrate that MEAAJ2 possess bioactive secondary metabolites/bio-molecules. In the table shown below,

MEAAJ2 demonstrates significantly higher activity. The IC_{50} value of the standard (Gallic acid) was 6.16 mg/ml. The percentage inhibition was 58.69 at 5mg/ml concentration. MEAAJ2 extracts showed dose-dependent 5-LOX inhibiting activity with a percentage inhibition 19.62 at 5mg/ml. The IC_{50} value of MEAAJ2 with the standard was 30.70 and 39.65µg/ml(HRBC and 5-LOX) compared to the standard(IC_{50} value- 5.12 mg/ml). Gallic acid is a naturally occurring phenolic chemical with a variety of industrial uses as well as biological properties that include anti-oxidant, anti-inflammatory, anti-microbial, and anti-cancer properties

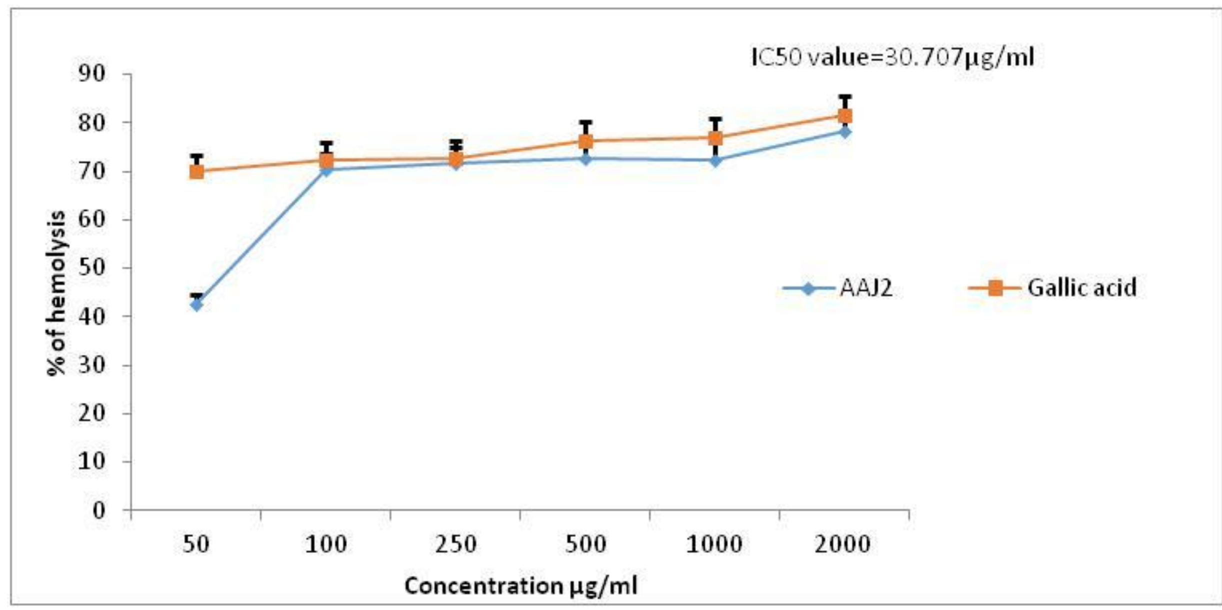


Figure 10. Anti-inflammatory activity of Methanol extracts of AAJ2 by HRBC membrane stabilization activity

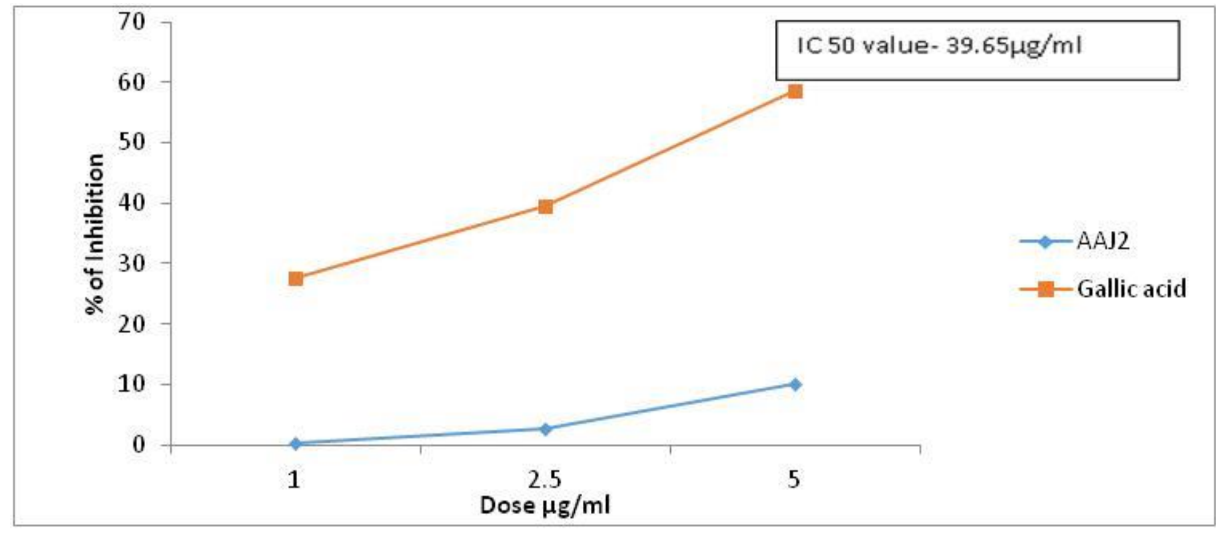


Figure 11. Anti-inflammatory activity of Methanol extracts of AAJ2 by 5-LOX inhibiting activity

3.6.3.Anti-cancer activity:

The antitumor/anti-cancerous activity of MEAAJ2 (Figure 12) showed significant inhibition of the growth of MCF7 cancer cells examined via MTT assay. In this study, MCF-7 cells were exposed to different volumes of MEAAJ2 and reported 41.10 % inhibition at 120 µl.

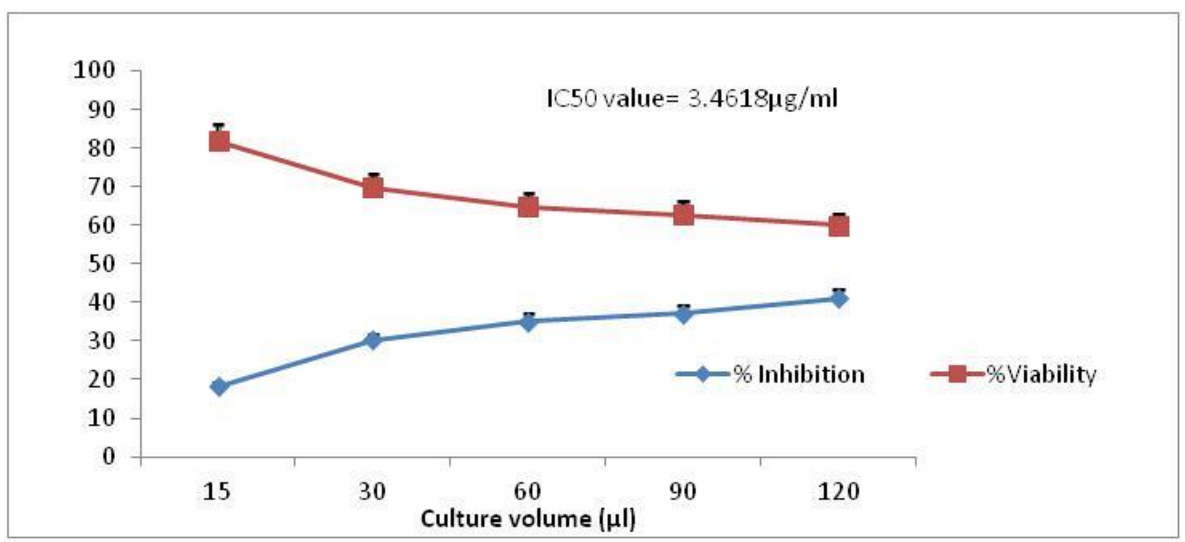


Figure 12. Anti-cancer activity of Methanol extracts of AAJ2 by MCF7cell line cytotoxicity

3.7. Spectral analysis of secondary metabolites :

3.7.1. GC-MS analysis :

GC-MS study of MEAAJ2 (Table 3) revealed that most of the compounds present in MEAAJ2 belong to polyphenols and flavonoids such as Benzoic acid, 2-methoxy-methyl ester, oxime- methyl-phenyl,7-hydroxyjanthinone, Shearinines B, Citrinin, Janthinone and Tetradecane. Methyl ester, Oxime-, methoxy-phenyl, Cyclotrisiloxane, hexamethyl, Benzoic acid, methyl ester, 1, 2-Benzenedicarboxylic acid, butyl octyl ester, 8-Octadecenoic acid, methyl ester (E), Heptadecanoic acid, 16-methyl-, methyl ester (Figure 13 A Table 3)

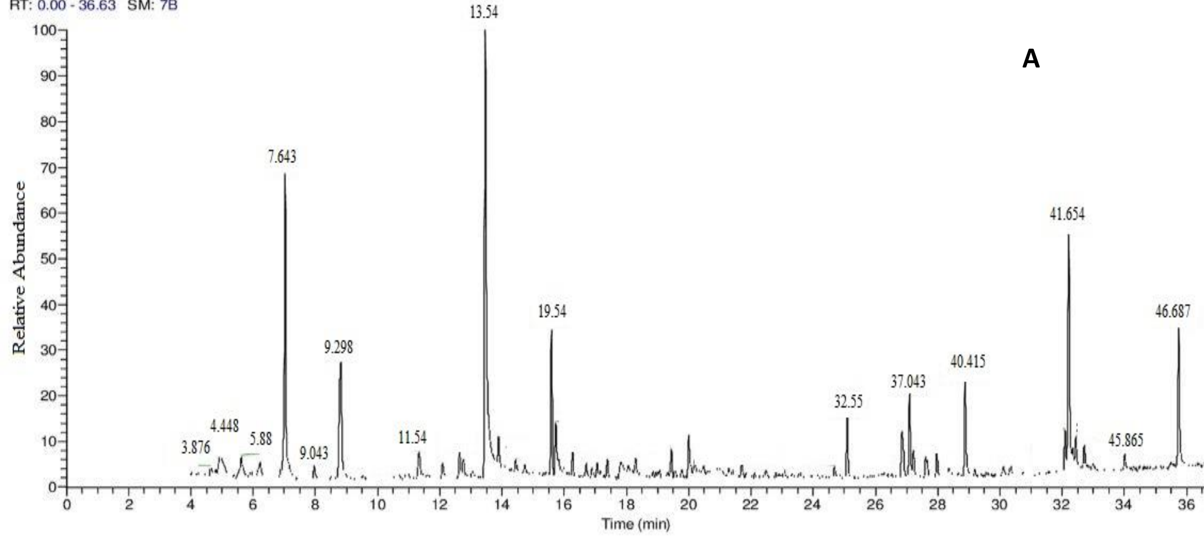
Table 3. GC –MS Analysis of derived compounds of MEAAJ2

Peak No.	Retention time	Name of the compound	Molecular formula	Molecular weight (gmol−1)
1	3.876	Benzoic acid, 2-methoxy-, methyl ester	$C_6H_{10}O_3$	166.17
2	4.448	Oxime-, methoxy-phenyl	$C_8H_9NO_2$	151.16
3	5.88	Cyclotrisiloxane, hexamethyl	$C_6H_{18}O_3Si_3$	242.33
4	7.643	7-hydroxyjanthinone*	$C_{14}H_{10}O_4$	222.46
5	9.043	Undecane	$C_{11}H_{24}$	156.31
6	9.298	Shearinines B*	$C_{37}H_{45}NO_6$	136.15
7	11.54	Benzoic acid, methyl ester	$C_6H_5COOCH_3$	583.08
8	13.54	Citrinin*	$C_{13}H_{14}O_5$	250.25
9	19.54	Janthinone*	$C_{16}H_{12}O_5$	284.06
10	32.55	1, 2-Benzenedicarboxylic acid, butyl octyl ester	$C_{20}H_{30}O_4$	198.38
11	37.043	Isopropyl myristate	$C_{17}H_{34}O_2$	270.5
12	40.415	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.45
13	41.654	Tetradecane*	$C_{14}H_{30}$	333.45
14	45.865	8-Octadecenoic acid, methyl ester (E)	$C_{19}H_{36}O_2$	296.5
15	46.687	Heptadecanoic acid, 16-methyl-, methyl ester	$C_{19}H_{38}O_2$	298.50

Molecules marked with * showed prominent peaks

3.7.2. HPLC analysis:

The HPLC analysis of MEAAJ2 confirmed five significant molecules in the chromatogram(Figure 13B, Table 4), and their structures were validated with the literature of PubChem and reported as 7-hydroxyjanthinone, Shearinines B, Citrinin, Janthinone and Tetradecane.



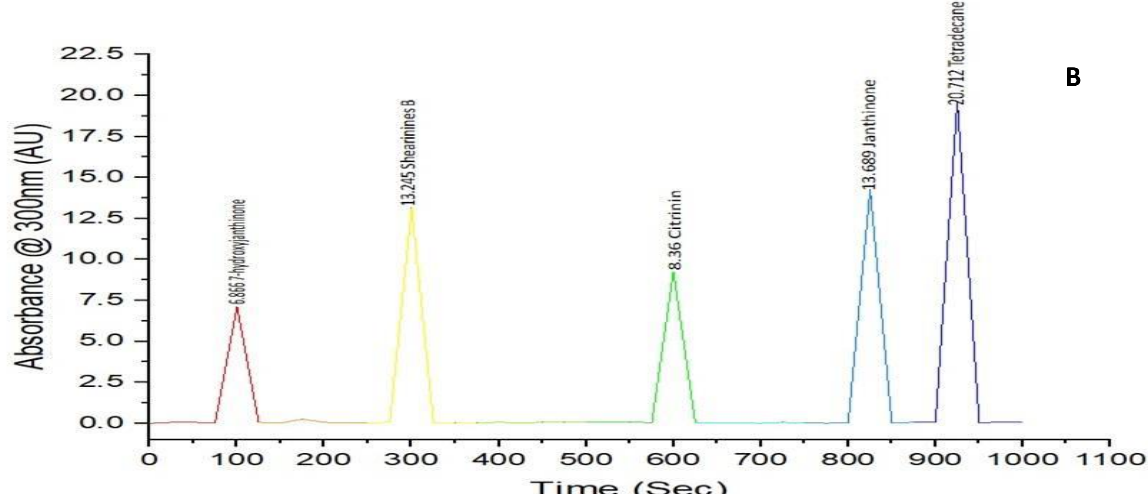


Figure 13 A)GC-MS chromatogram of AAJ2 ; B) HPLC Chromatogram of 7-hydroxyjanthinone, Shearinines B, Citrinin, Janthinone and Tetradecane of ME AAJ2

Table.4 HPLC analysis of five secondary metabolites of MEAAJ2

Peak No.	Retention time	Compound name	Structure of the compound
1.	6.856	7-hydroxyjanthinone	
2.	13.245	Shearinines B	
3.	8.36	Citrinin	
4.	13.689	Janthinone	
5.	20.712	Tetradecane	

3.8. Insilico molecular docking :

1. **Docking studies using Seam dock** (https://bioserv.rpbs.univ-paris-diderot.fr/services/SeamDock/) for the five listed compounds, 7-hydroxyjanthinone,

Shearinines B, Citrinin, Janthinone and Tetradecane. Proteins such as Monoamine Oxidase A, Cyclooxygenase 2, Gamma Secretase, 5 Lipoxygenase, Aldose Reductase, Fructose 1,6, Biophosphase, VEGDR-2, *Thymidylate* synthetase (TS), Testis specific binding protein and CXC chemokines receptor type 3 as receptors. The given setup showed Docking analysis(Figure 14 ,Table 5 and 6)

1. 7-hydroxyjanthinone with Monoamine Oxidase A (PDB id; 2Z5X) and Cyclooxygenase 2 (PDB id; 1CX2)
2. Shearinines B with Gamma Secretase (PDB id; 5A63) and 5 Lipoxygenase (PDB id; 3O8Y)
3. Citrinin with Aldose Reductase (PDB id; 2R24) and Fructose 1,6, Biophosphase (PDB id; 1FPK)
4. Janthinone with VEGDR-2 (PDB id; 2XIR) and *Thymidylate* synthetase (TS) (PDB id; 1HZW)
5. Tetradecane with Testis-specific binding protein (PDB id 4A60) and CXC chemokines receptor type 3 (PDB id; 3ODU)

Figure .14. Enzyme docking of Five active metabolites of *Penicillium chrysogenum* AAJ2

7-hydroxyjanthinone with Monoamine Oxidase A (pdb id; 2Z5X)	7-hydroxyjanthinone with and Cyclooxygenase 2 (pdb id; 1CX2)
Shearinines B with Gamma Secretase (pdb id; 5A63)	Shearinines B with 5 Lipoxygenase (pdb id; 3O8Y)
Citrinin with Aldose Reductase (pdb id; 2R24)	Citrinin with Fructose 1,6, Biophosphase (pdb id; 1FPK
Janthinone with VEGDR-2 (pdb id; 2XIR)	Janthinone with *Thymidylate* synthetase (TS) (pdb id; 1HZW)
Tetradecane with TSBP (pdb id 4A60)	Tetradecane with CXC chemokines receptor type 3 (pdb id; 3ODU)

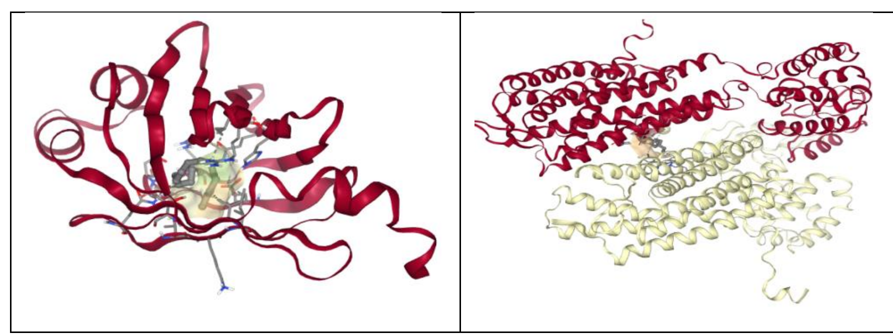


Table 5. Docking analysis of five active secondary metabolites of *Penicillium chrysogenum* AAJ2

Docking	Property	Ligand	Receptor
7-hydroxyjanthinone with Monoamine Oxidase A (pdb id; 2Z5X)	Hydrophobic contact	C11	I180(A) CG2
		C9	Q215(A) CG
		C9	F352(A) CE1
		C3	Y444(A) CE2
	Pi pi stacking	C5	Y407(A) CE2
	Hydrogen bonding	O3	Y444(A) OH
	Weak Hydrogen bonding	C12	Q215(A) OE1
		C13	Q215(A) OE1
7-hydroxyjanthinone with and Cyclooxygenase 2 (pdb id; 1CX2)	Hydrophobic contact	C3	F556(A) CE2
	Pipi stacking	O4	E346(A) OE2
		O3	F361(A) O
		O3	N560(A) OD1
		O1	W545(A)NE1
		O4	K342(A) NZ
	Hydrogen bonding	C2	D362(A) OD1
		C1	E364(A) OE2
	Weakhydrogen bonding	O4	D362(A) CA
Shearinines B with 5 Lipoxygenase (pdb id; 3O8Y	Hydrophobic contact	C8	P43(A) CB
		C6	P43(A) CG
		C28	W289(A)CE3
		C28	I656(A) CG1
		C30	I656(A) CD
	Hydrogen bonding	O4	I656(A) O
		O1	W653(A)N

			E1
Shearinines B with 5 Lipoxygenase (pdb id; 3O8Y)	Hydrophobic contact	C27	L244(A) CD2
		C28	L288(A) CB
		C32	D442(A) CB
		C36	D442(B) CB
		C36	T444(B) CG2
	Hydrogen bonding	O4	D442(A) OD2
Citrinin with Aldose Reductase (pdb id; 2R24)	Ionic interaction	O3	K21(A) NZ
	Hydrophobic contact	C5	W20(A)CE3
		C13	Y48(A) CE1
		C2	Y209(A) CB
		C12	I260(A) CD
	Hydrogen bonding	O2	S210(A) OG
		O5	I260(A) O
		O1	C298(A) SG
		O2	S210(A) OG
		O2	S214(A) OG

		O4	K262(A) N
	WeakHydrogenbonding	C4	S210(A) OG
		O5	G18(A) CA
Docking	Property	Ligand	Receptor
Citrinin with Fructose 1,6, Biophosphase (pdb id; 1FPK)	Ionic interaction	O3	R243(A) NE
	Hydrophobic contact	C12	I208(A) CD
		C9	R254(A) CG
		C2	Y258(A) CE1
		C12	Y258(A) CE2
	Hydrogen bonding	O2	N125(B) O
		O4	V245(B) O
	WeakHydrogenbonding	C5	N125(B) OD1
		C10	N125(B) OD1
		C4	C128(B) SG
		O2	N125(B) CA
		O3	R254(A) CD
		O5	R254(A)

			CD
Janthinone with VEGDR-2 (pdb id; 2XIR)	Hydrophobic contact	C7	E885(A) CG
		C2	I888(A) CG2
		C15	L889(A) CD2
		C13	L1019(A) CD2
	Hydrogen bonding	O3	H1026(A) O
		O5	H1026(A) O
		O3	H1026(A) NE2
		O2	D1046(A) O
		O3	D1046(A) OD1
		O3	H1026(A) O
Tetradecane with TSBP (pdb id 4A60)	Hydrogen Bond	C12	I52(A) CG2
		C5	T54(A) CG2
		C13	I63(A) CG1
		C11	I63(A) CD
		C1	T75(A) CG2
		C3	A76(A) CB
		C9	I105(A) CD
Tetradecane with CXC chemokines receptor type 3 (pdb id; 3ODU)	Hydrophobic contact	C1	W125(A) CD2
		C3	W125(A) CZ3
		C10	W125(A) CH2
		C9	L210(A) CD1
		C12	L210(A) CD2
		C10	V214(A) CG2
		C14	W125(B) CH2
		C14	L210(B) CB
		C9	L210(B) CD2
		C14	V214(B) CG2

Table 6. Docking parameters of Five compounds with various proteins

Compound	Protein	Pose	Docking Score	Binding Energy kCal/Mol
7-hydroxyjanthinone	Monoamine Oxidase A	1	-8.8	-9.4
		2	-8.7	-9.2
	Cyclooxygenase 2	1	-8.8	-5.7
		2	-8.6	-5.6
Shearinines B	Gamma Secretase	1	-7.6	-9.2
		2	-7.4	-8.9
	5 Lipoxygenase	1	-6.2	-7.6
		2	-5.8	-7.0
Citrinin	Aldose reductase	1	-8.9	-6.5
		2	-8.6	-6.5
	Fructose 1,6, Biophosphase	1	-8.6	-4.2
		2	-6.1	-3.9
Janthinone	VEGDR-2	1	-6.2	-8.4
		2	-5.8	-7.7
	Thymidylate synthetase (TS)	1	-6.5	-51.4
		2	-6.1	-49.45
Tetradecane	Testis specific binding protein	1	-9.5	-4.2
		2	-9.1	-4.0
	CXC chemokines receptor type 3	1	-7.4	-4.2
		2	-7.0	-4.2

Five key molecules examined via HPLC analysis demonstrated 7-hydroxyjanthinone, Shearinines B, Citrinin, Janthinone and Tetradecane. These molecules are responsible for the anti-oxidant, antibacterial and anti-cancer activity. As the data showed above, 7-hydroxyjanthinone inhibits Monoamine Oxidase A and cyclooxygenase 2 and demonstrates anti-inflammatory activity. Shearinine B inhibits gamma-secretase, and 5 lipoxygenase enzymes also offer anti-inflammatory activity. Similarly, Citrinin targets Aldose Reductase and Fructose 1,6, and Biophosphase offer antibacterial activity. Other two compounds reported that Janthinone selectively inhibits vascular endothelial growth factors

and *Thymidylate* synthetase (TS). In contrast, Citrinin inhibits Testis-specific binding protein, and CXC chemokines receptor type 3 offers intense anti-inflammatory activity In the molecular docking studies, we have selected the most probable targets for finding affinity with human proteome.

Discussion:

Microbes remain a critical natural source of bioactive compounds as secondary metabolites where the habitat of growing microbes plays a pivotal role in the biosynthesis of bioactive compounds. Fungi are the most crucial microbes that set an ecosystem potentially rich in bioactive compounds.

Marine fungus *Penicillium chrysogenum* AAJ2 isolated from mangrove forest soil showed prominent growth in CYA media(plate1). Morphological screening by Lactophenol blue staining followed by biochemical and genomic characterisation confirmed AAJ2 fungal isolate as *Penicillium chrysogenum* AAJ2 with accession number OQ569546 (Figure1, 2, and 3) .

Penicillium-related fungi are saprophytic filamentous fungi, although they are prevelant, contribute significantly to bioremediation processes by breaking down various xenobiotics of which, *Penicillium chrysogenum* is widespread and found in practically all habitats and produce a variety of primary and secondary metabolites. Due to the presence of bioactive substances such as flavonoids, alkaloids, minerals, proteins, phenols, tannins, vitamins, *P.chrysogenum* shows potential anti-oxidant properties. In more recent years, research has focused heavily on this species' physiologically active secondary metabolites. *P. chrysogenum* has yielded a variety of bioactive compounds, including tannins, alkaloids, terpenoids, and tannins. *Penicillium* species also produced DPPH radical scavengers. As a possible anti-oxidant, penicillenols, secalonic acid D, and atrovenetin were identified from *Penicillium* sp. But the presence of biologically active chemicals and myco-chemical analyses have yet to understand .Earlier studies reported the presence of physiologically active substances in fungal strains to control the anti-oxidant functions (Thomas et al., 2014; Priyanka et al., 2015).

In our research, *Penicillium chrysogenum* AAJ2 has reported to possess alkaloids, flavanoids, saponins and phenolic compounds as primary secondary metabolites (Table1). After optimization of physico chemical characters *P.chrysogenum* AAJ2 and bioactive secondary metabolites extracted from AAJ2 with methanol showed potential anti-microbial activity against *Bacillus megaterium*, *Xanthomonas campestris*, Escherichia coli, *Pseudomonas*

aeruginosa, and *Candida albicans* (Table 2). The antibacterial activity of MEAAJ2 using the zone of inhibition assay showed significant zone of inhibition with all clinical test pathogens, including *Bacillus megaterium, Pseudomonas aeruginosa, Escherichia coli, Xanthomonas campestris* and fungi *Candida albicans* as a marker of the presence of active secondary metabolites and also reported that significant bioactive secondary metabolites in methanol extract (MEAAJ2)compared to other solvents (Figure 4). Biological activities such as antioxidant, anti-inflammatory, and anti-cancer studies revealed that *Penicillium chrysogenum* AAJ2 possess significant bioactive secondary metabolites (Figure 8-12).

Previous studies stated that non-steroidal anti-inflammatory medicines can be made from benzoic acid, 2-methoxy-, methyl ester, or ortho-anisic acid. Hexadecanoic, oxime, and methoxy-phenyl acids all exhibit anti-oxidant and antibacterial properties. The anti-oxidant potential is present in cyclotrisiloxane, hexamethyl, and isopropyl myristate and wide range of antibacterial actions exhibited by undecane (Mostafiz et al., 2018; Chen et al., 2018). Insects like *Bemisia tabaci* were susceptible to the repellent properties of benzoic acid methyl ester and Methyl benzoate as insecticidal against mites and pest insects by Chen et al.2018. Butyl octyl ester of 1, 2-benzene dicarboxylic acid can be employed as an anti-oxidant and antibacterial agent in addition to being used in the development of drugs for cancer, microbial allergies, and arthritis. The methyl ester of 8-octadecenoic acid (E) demonstrated anti-oxidant and antibacterial properties and effects on serum lipids in lactating women. Heptadecanoic acid, 16-methyl methyl ester, has anti-inflammatory, antibacterial, and anti-oxidant effects.

GCMS, followed by HPLC analysis also confirmed he presence of phenolics and flavonoids as distinct bioactive metabolites, further reported that 7-hydroxyjanthinone, Shearinines B, Citrinin, Janthinone and Tetradecane are prominent metabolites which show significant biological activities, anti-oxidant, anti-inflammatory and anti-cancer. (Figure 13 and 14 Table 3 and 4).

The anti-oxidant, anti-microbial, and anti-inflammatory activity of MEAAJ2 was primarily due to these compounds. Additionally, the anti-cancer activity of these isolates is due to the wide range of compounds and precise esters present in the fungal isolates. Undecane is one compound reported in the present study already been established for its anti-inflammatory activity. The HPLC study of four essential compounds revealed 7-hydroxyjanthinone, Shearinines B, Citrinin, Janthinone, and Tetradecane. These compounds charge the antibacterial, anti-cancer, and anti-oxidant action. The reported compounds are responsible for the anti-microbial and anti-cancerous activity. Earlier, Suryanarayanan and Kumaresan (2020) profiled a diverse list of compounds from fungi isolated from soil;

however, the reported fungi were *Penicillium* sps. *and Aspergillus* sps. posses anti-oxidant compounds from the fungi isolated from mangrove soil and several polyphenolic and flavanoid compounds. HPLC analysis demonstrated Shearinine B as a critical molecule for anti-oxidant activity. Eight novel indole triterpenes with the names shearinines D–K, shearinine A, paspalitpem A, and paspaline were discovered in a mangrove endophytic fungus called *Penicillium* sp. significant invitro blocking activity was shown by shearinines D, E, and G for large conductance calcium-activated potassium channels. The purified secondary metabolites (7-hydroxyjanthinone, Shearinines B, Citrinin, Janthinone and Tetradecane) present in AAJ2 validated by *insilico* docking studies to confirm their potential bioactivities. Our docking results also confirmed that (7-hydroxyjanthinone, Shearinines B, Citrinin, Janthinone and Tetradecane) are highly significant bioactive metabolites of *Penicillium chrysogenum* AAJ2(Figure 14;Table 5 and 6)

4. Conclusion

Penicillium chrysogenum AAJ2 (OQ569546) isolated from mangrove soils exhibited anti-microbial activity with a significant zone of inhibition against clinical pathogens and the secondary metabolite profile molecular docking revealed that 7-hydroxyjanthinone, Shearinines B, Citrinin, Janthinone and Tetradecane are five essential compounds responsible for the anti-oxidant, antibacterial and anti-cancer activity.

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6. Conflict of Interest:

The authors declare that there is no conflict of interest

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