In Vitro process optimisation and isolation of antibacterial agents from marine *Aspergillus flavus* against *Klebsiella pneumoniae*

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Abstract

The present study is focused on *Aspergillus flavus*-originated compounds that can be used to treat future infections. *Aspergillus flavus* CBS 593.65 was isolated from a seawater sample. Agar well diffusion method and bioautography were used to assess the antibacterial potential of broth-fermented chloroform extract of *Aspergillus flavus* CBS 593.65 against bacterial culture *Klebsiella pneumoniae* MTCC 432. The compound from *A. flavus* that had been purified was identified by its chemical name as 2(5H)-Furanone with formula $C_4H_4O_2$ by using GC-MS and NMR spectroscopy showed an inhibitory activity against *Klebsiella pneumoniae* MTCC 432. The acquired results lead to the conclusion that this molecule is a strong candidate for antibacterial use, but more research is required before it can be employed as an antibiotic.

Key Words: *Aspergillus flavus*, Bioautography, Secondary metabolites, Metabolomics, Furanone

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1. Introduction

Many naturally occurring secondary metabolites have been discovered to date, showing a wide range of scaffolds, making natural sources an exceptional store of bioactive compounds. The most ubiquitous producers of bioactive molecules have been discovered to be fungi. According to certain research, fungi are crucial for every terrestrial ecosystem's sustainability and biodiversity as well as its health and prosperity (Pang et al., 2016). Unlike terrestrial microorganisms, which can create a variety of secondary metabolites (SMs) with significant structural diversity and biological activity, marine microorganisms have unique physicochemical features because of the unique characteristics of the marine environment. Recently, a substantial number of structurally unique secondary metabolites have been produced by the marine-derived fungus. Some of these metabolites have demonstrated anticancer, antibacterial, anti-inflammatory, antioxidant, and enzymatic activity and could represent attractive sources for brand-new therapeutic medications. In addition, over the past 20 years, there has been a dramatic increase in the rate of identification of new fungal metabolites. Fungi have the benefit of producing bioactive substances during growth and metabolism, which makes it possible to track their mycelia and by-products in their various substrates and habitats (Debbab et al., 2010). There is an almost infinite potential for metabolic variation, as evidenced by the richness of filamentous fungi, particularly in the Ascomycetes and Basidiomycetes, and the corresponding evolution of metabolic genes and gene clusters. One might make the case that the ability of the filamentous fungus to use a variety of secondary metabolites in conjunction with their absorptive life forms is largely responsible for their ecological success in colonising almost all ecosystems on the globe. On the other hand, research into the chemistry of fungi has led to the discovery of numerous significant medications (Arumugam et al., 2015).

Natural products have played a crucial role in the development of successful treatments for cancer, malaria, bacterial and fungal infections, neurological and cardiovascular diseases, and autoimmune disorders. They continue to be among the most important therapeutic agents and lead compounds in medicine (Tanwar et al., 2014).

Thus it is essential and useful to identify the individual therapeutic compounds in a mixture of metabolites using bioassay-guided compound isolation and identification. Therefore, screening bioactive substances like antibacterial from fungi is a successful method to find new and strong medications (Pinu et al., 2019). One method for directly tracing out bioactive chemicals from preparations on thin layer chromatograms is bioautography. Bioautography is the word used to describe planar chromatography analysis that has a biological detection method hyphenated (Valle et al., 2016). It is a useful and affordable method for identifying bioactive lead/scaffolds in natural extracts. Thus, it can be carried out both in advanced laboratories and in smaller research labs with limited access to expensive equipment (Balouiri et al., 2016). Current study was focused on extraction of marine fungal metabolites using different fermentation methods. Further Bioautography technique was use for analysis of bioactive compounds.

2. Materials and Methods

2.1. Study site and sample collection:

The location of the water sample collection site was the Gorai Sea of Mumbai coastal region from Maharashtra, India. From the sea region, 50ml of water sample was collected in a sterile stoppered bottle around 200 meters away from the beach side and of 3 feet depth. Samples were properly labelled and bought to a lab and further stored at room temperature for isolation (Athira et al., 2016).

2.2. Isolation and identification of fungi:

Fungal isolation was done using the serial dilution method on a potato dextrose agar plate. Further identification was carried out using the slide culture method, lactophenol cotton blue staining and 16S rRNA sequencing (Shamly et al., 2014).

2.3. Fermentation and Extraction of Metabolites:

2.3.1. Broth fermentation method and extraction:

The fungal strain was cultivated on Potato dextrose agar. A little block of fungal culture from agar was cut and inoculated in 50ml of Potato dextrose broth in a 250ml conical flask. Flask was incubated at room temperature for 14 days on a rotary shaker set at 150rpm. After the incubation period, 50ml of each Ethyl acetate, Chloroform and Ethanol were added to a separate flask of broth. This was further incubated on a rotary shaker set at 150 rpm for 24 hours at room temperature. Further whole content was filtered

using the Whatman filter paper. The solvent layer was separated in a beaker and allowed to evaporate. After evaporation leftover sticky substance was collected in vials, weighed and refrigerated at 4ºC for further studies (Arumugam et al., 2015).

2.3.2. Mycelial Biomass Extraction:

Broth with mycelial growth was poured onto a muslin cloth and boiling water was added until the whole broth disappears to get clear mycelia. Repeat it twice. Keep the cloth with mycelia in a hot air oven at 50 ºC. Further dried mycelia were collected, crushed, and weighed and powdered mycelia were stored in the refrigerator at 4 ºC for further studies. For the extraction process, 10g of mycelial powder was added in each 50ml of solvent (Ethyl acetate, Chloroform and Ethanol) and incubated for 24 hours at room temperature. The next day it was filtered using Whatman's filter paper. The solvent was allowed to evaporate. After evaporation leftover sticky substance was collected in vials, weighed and refrigerated at 4 ºC for further studies (Luo et al., 2020).

2.3.3. Solid state fermentation (ssf) method and extraction:

10g of each soya meal, rice bran and corn meal were taken in 100ml flask. 50 ml of sterile saline was added to it. Each fungal isolate was inoculated in the flask and incubated for 5-7 days at room temperature. After incubation 50ml of each solvent (Ethyl acetate, Chloroform and Ethanol) was added and kept at room temperature for 24 hours. Further extraction was done using Whatman filter paper, and the solvent was allowed to evaporate. The sticky substance was collected in vials and stored in the refrigerator at 4 ºC for further studies (Nayak & Mishra, 2016).

2.3.4. Submerged fermentation (SmF) and Extraction:

50ml of soya meal, rice bran and corn meal (1:10 ratio) were taken to a 250 ml flask and inoculated with fungal culture and incubated at room temperature for 14 days. After incubation sample was centrifuged at 5000 rpm for 10 min. 50ml of each solvent (Ethyl acetate, Chloroform and Ethanol) was added to a flask and kept at room temperature for 24 hours. Further extraction was done using Whatman filter paper and the solvent was allowed to evaporate. The left sticky substance was collected, weighed and stored in a refrigerator at 4 ºC for further studies (Arumugam et al., 2015).

2.4. Antibacterial Activity of crude extract by Well diffusion method:

Bacterial culture *Escherichia coli* MTCC 64, *Enterococcus faecalis* MTCC 439, *Enterococcus faecium* MTCC 9728, *Klebsiella pneumoniae* MTCC 432, *Bacillus subtilis* MTCC 441, and *Staphylococcus aureus* MTCC 96 was adjusted to 0.5 McFarland standard for the assay. Further, spread onto Mueller Hinton agar plates using a sterile swab moistened with bacterial culture. Wells of 6mm diameter were punched onto an agar plate using a cork borer. This well was filled with the crude extract obtained through various fermentation processes in a range of 2-10µg/ml and 10-150 µg/ml with diluent DMSO. Then it was allowed to diffuse for 2h at room temperature. Plates were incubated in an upright position at 37 ºC for 24 hours. Wells of positive control (antibiotic) and negative control (DMSO) were also set. After incubation zone of inhibition was measured in mm and noted (Perez, 2020)

2.5. Minimum Inhibitory Concentration (MIC) using Tube method:

2 fold dilutions of effective crude extract in the range of 2-10µg/ml were prepared along with positive (antibiotic) and negative control (DMSO). Bacterial inoculum of *Escherichia coli* and *Klebsiella pneumoniae* was prepared at 0.5 McFarland standard and inoculated in Mueller Hinton broth along with dilution of crude extract. Tube was mixed properly and incubated at 37 ºC for 24 hours. After incubation results was analysed by looking for turbidity in the tube (Garo et al., 2003).

2.6. TLC-Agar-Overlay Bioautography Technique:

Aluminium TLC silica plates were used in this technique. The effective crude extract was spotted onto the plate and placed in the solvent for development. After the run, TLC plates were placed in a petri dish and molten Luria Bertani agar seeded with bacterial culture was overlaid. This TLC plate was then placed in an incubator at 37 ºC for 24 hours. After incubation TLC plates were sprayed with (2.5 mg/mL) of methyl thiazole tetrazolium and again incubated for 3-4 hours at 37 ºC. Result noted on basis of clear zone against the red background (Nielsen & Larsen, 2015).

2.7. Column Chromatography:

The crude extract was mixed with silica (60:120 mesh sizes) powder. The admixture (5g) prepared was charged in the glass column (25x2cm) packed

with a slurry of silica gel (60-120 mesh) pre-activated at 120ºC for 4 hrs. After the column was successfully eluted with chloroform and ethanol. The fractions collected were used for further activity and analytical studies (Erecta et al., 2015).

2.8. Gas chromatography-mass spectrometry (GC/MS) for analysis of bioactive compounds

GC-MS (Agilent 5977B) with a DB-5MS column was used to analyze the chemical makeup of bioactive substances. The carrier gas, helium, was employed at a rate of 1.0 ml/min. Ion source temperature was 230°C, and the ionization voltage was 70 eV. 41 to 400 amu was the scan range. The constituents were determined after being compared to data from the literature and the GC-MS library (Sholkamy et al., 2020).

2.9. Nuclear Magnetic Resonance (NMR) Spectroscopy:

1H NMR spectra of pure compounds present in the active fractions of chloroform extract of the isolate were recorded using SA-AGILENT operating at 400 MHz with solvent DMSO (Dona et al., 2016).

3. Results and Discussion

3.1. Study site:

The sea water sample was collected from Gorai beach located in the city of Malad, Mumbai, Maharashtra (Lat. Long. - 19.197634, 72.795712). The sample location has been shown in **Fig 1.**

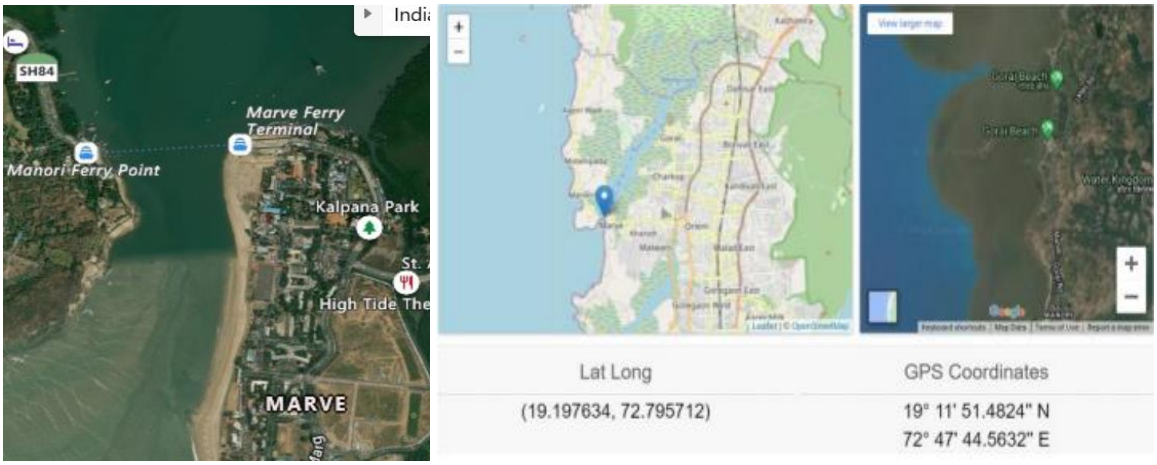
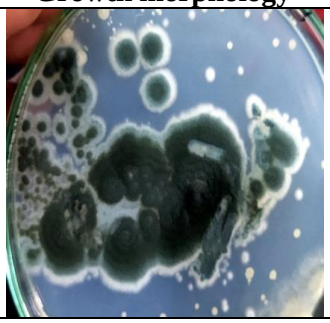
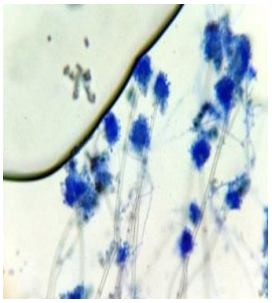


Fig 1. Map of sample collected site

3.1. Isolation and identification of fungi:

Fungal identification was done using the slide culture method and lactophenol cotton blue staining. For genus identification, 16S rRNA Sequencing was carried out. Fungus *Aspergillus flavus* CBS 593.65 were isolated from the sample. Fungal microscopy and sequence are shown in **Table 1** and **Fig 3**.

Table 1 Isolated fungi and Microscopy

Isolate	Growth morphology	Microscopic examination	
Aspergillus flavus CBS 593.65			

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TGCCGCCGCTGCCTTTCGGGCCCGTCCCCCGGGGGGGGACGACGACCCAACACACAAGCCGGGCTTGATGGGCAGC
AATGACGCTCGGACAGGCATGCCCCCCGGAATGCCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACTG
AATTCTGCAATTCACATTACTTATCGCAGTTCGCTGCGTTCTTCATCGATGCCGGAACCAAGAGATCCATTGTTG
AAAGTTTTGACTGATTTTATATTCAGACTCAGACTGCATCACTCTCAGGCATGAAGTTCAGTAGTCCCCGGCGGC
TCGCCCCCGAGGGGGTTCCCCGCCGAAGCAACAGTGTTAGGTATTCACGGGTGGGAGGTTGGGCGCCCGGAGGCA
GCCCGCACTCAGTAATGATCCTTCCGCAGGTTCACCCTACGGAA
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Fig 3. Sequence text of *Aspergillus flavus* CBS 593.65 in FASTA format font: courier new 10

3.2. Fermentation and Extraction of Metabolites:

Broth fermentation has previously been used for the extraction of secondary metabolites from many fungi like *Penicillium roqueforti* (Mioso et al., 2014). Several comparison studies between different fermentation processes have been done. Every research has its own conclusion as some find submerged fermentation to be showing good activity (Suparmin et al., 2019). As per our study, the yield of metabolite was high in solid and submerged fermentation but it was unable to show any activity because of delay in the fermentation process and continuous passaging of culture. Some studies have proven that continuous passaging of culture tends to lose the production of metabolite (Keller, 2019). Extraction of metabolites from fungus *Aspergillus flavus* CBS 593.65 was done using broth fermentation, mycelial extraction, solid state and submerged fermentation. For extraction three solvents with increasing polarity were used ethyl acetate, chloroform, and ethanol. For broth fermentation single substrate was used while for solid state and submerged fermentation, three different substrates like soya meal, rice bran and corn meal were used. The major crude extract was obtained by ethanol solvent. Submerged fermentation has shown greater yield as compared to other fermentation processes. Yield of metabolite by each fermentation has been shown in **Table 2**.

Table 2 Yeild of crude extract by fermentation process

Sr. No	Type of fermentation	Substrate	Solvent		
			Chloroform	Ethyl acetate	Ethanol
			Yeild (mg/50ml)		
1	Broth fermentation	Potato dextrose broth	32.9	0	57.2
2	Mycelial biomass extraction	-	107.3	0	128.3
3	Solid state fermentation	Soya meal	56.65	344.0	57.87
		Rice bran	0	0	88.7
		Corn meal	44.0	107.3	142.3
4	Submerged fermentation	Soya meal	90.5	90.0	1248
		Rice bran	0	0	0
		Corn meal	410	0	2608

3.3. Antibacterial activity of crude extracts using well diffusion method:

Antibacterial activity of all crude extracts was tested against bacteria *Escherichia coli, Klebsiella pneumoniae, Enterococcus feacalis, Enterococcus faecium, Bacillus subtilis* and *Staphylococcus aureus* at range 2-10 µg/ml using well diffusion method. Major activity was shown against *Staphylococcus aureus* and *Klebsiella pneumoniae*. The highest activity among all extracts was shown by chloroform extract of *Aspergillus flavus* CBS 593.65 obtained using broth fermentation process against *Klebsiella pneumoniae a*s shown in **Fig.2**. No activity was seen by crude extracts obtained using solid-state and submerged fermentation

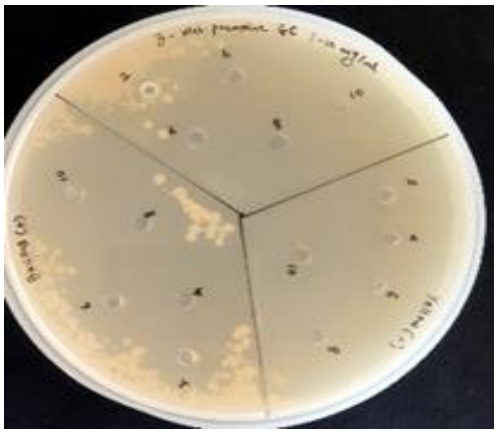


Fig 2. Antibacterial activity of chloroform extract of fungi *Aspergillus flavus* CBS 593.65 obtained by broth fermentation process

3.4.Minimum Inhibitory Concentration (MIC):
MIC was tested using the tube method against *Klebsiella pneumoniae* in the range of 2-4 µg/ml because too big inhibitory zones were observed at all 2-10 µg/ml concentrations. MIC of the extract against *Klebsiella pneumoniae* was seen at 3µg/ml as shown in **Fig 3**.

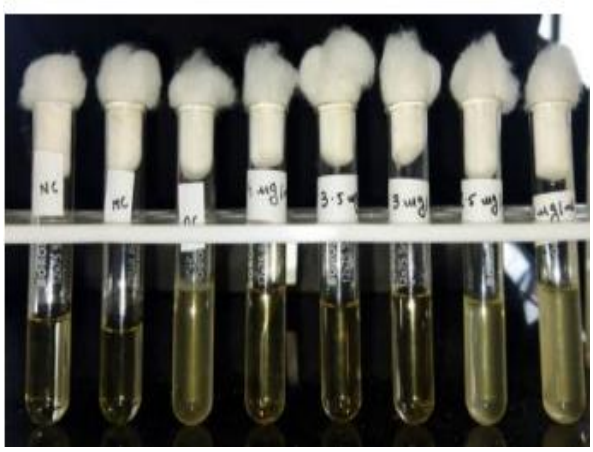


Fig 3. MIC of *Aspergillus flavus* CBS 593.65

3.5.TLC-agar-overlay Bioautography Technique:
TLC-agar-overlay Bioautography method is said to be a bioassay-guided separation technique as it helps in identification as well as separating the compounds even at a minute level (Rakshith et al., 2016). Several fungi have been studied using bioautography method endophytic fungi have shown inhibition and extract were separated using the TLC overlay bioautography method (Fathoni et al., 2021). Two similar TLC plate of crude extract was run using solvent chloroform: ethyl acetate : butanol: glacial acetic acid (25:15:2:10). On one plate Luria Bertani molten agar along with *Klebsiella pneumoniae* culture was overlaid and incubated. After incubation zone of inhibition was observed after spraying methyl thiazole tetrazolium as bacteria turns colourless methyl thiazole tetrazolium into red colour formazone compound. The formation of a colourless zone indicates the inhibition by the crude extract. With another TLC plate solvent was allowed to evaporate and it was exposed to UV light for band identification as shown in **Fig. 4**.

Further each band from TLC was scraped separately and tested for inhibition using well diffusion as well as bioautography. Both crude extract and eluted compound showing antibacterial activity was given for GC-MS and NMR analysis.

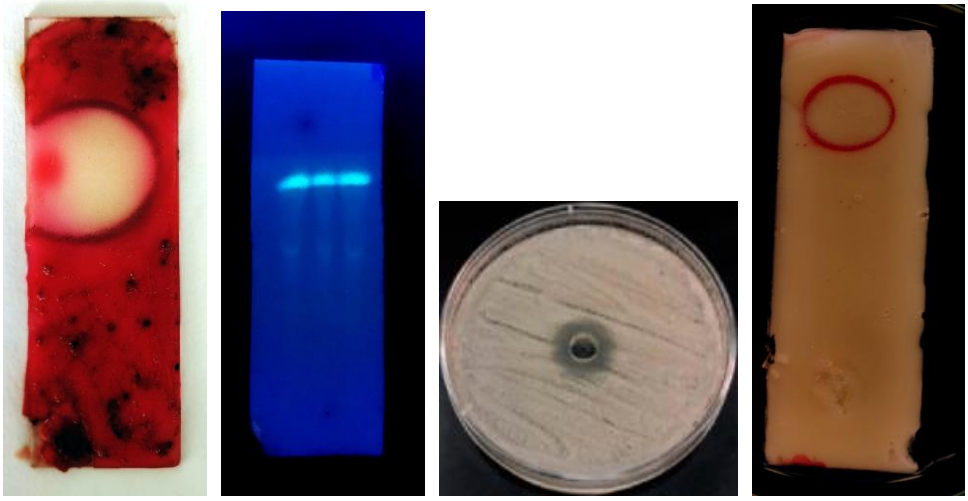


Fig 4. Bioautography of crude extract and eluted compound along with antibacterial activity by well diffusion method

3.6.Gas chromatography-mass spectrometry (GC/MS) for analysis:
GC-MS of crude extract and eluted compound obtained from chloroform extract of *Aspergillus flavus* CBS 593.65 was done. GC-MS chromato gram of compounds detected is shown in **Fig 5**. Six compounds were obtained from crude extract as 2(5H)-Furanone, D-Glucose,6-O-α-D-galacto pyranosyl, 1,2-Cyclopentanedione,3- methyl, 6-Acetyl-ß-d-mannose, Octadecanal,2 –bromo, 2 Furancarboxaldehyde,5-Methyl as shown in **Fig 6** and **Table 3**. Also, the GC-MS of the eluted compound was done. Derivative of Furanone was identified as shown in **Fig 8**.

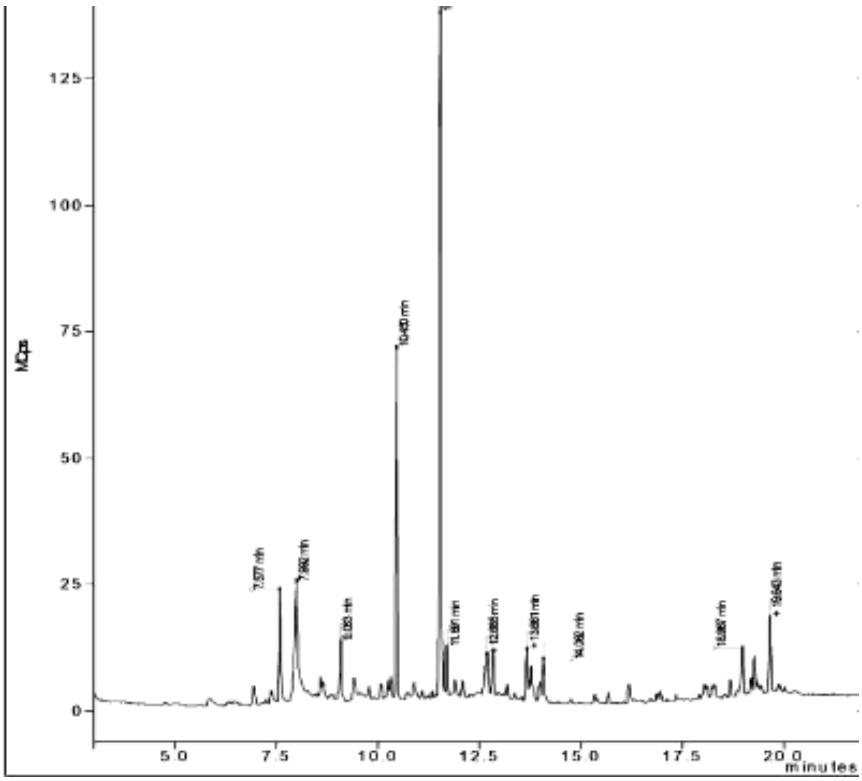


Fig 5. GC-MS Chromatogram of chloroform extract of *Aspergillus flavus* CBS 593.65 (Crude extract)

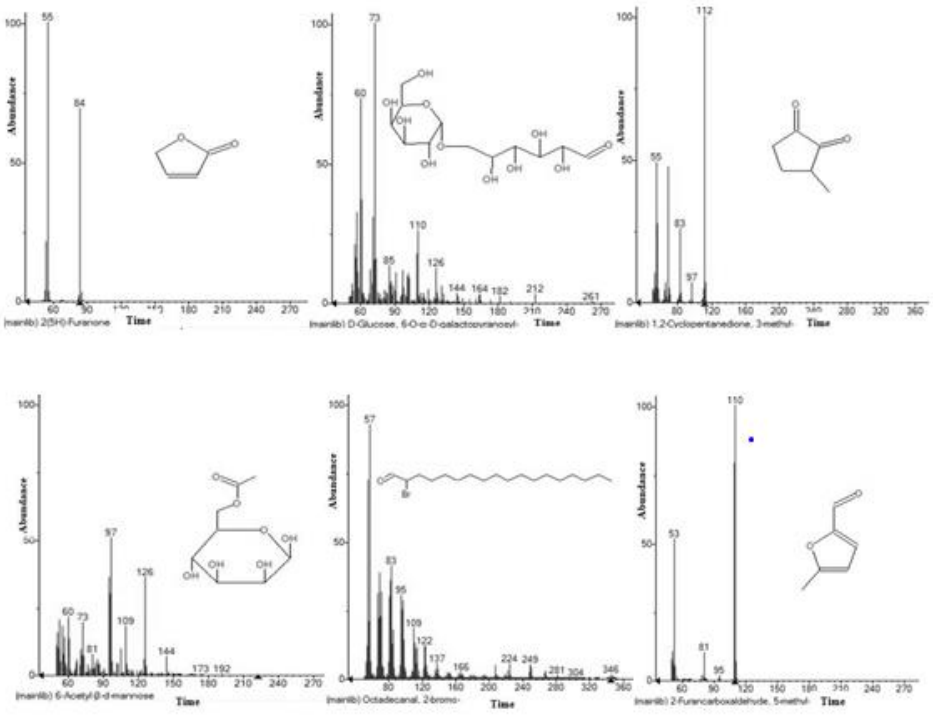
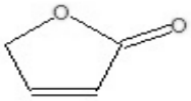
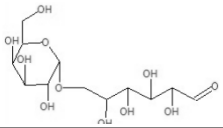
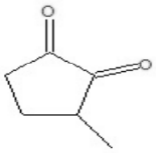
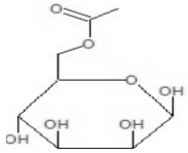
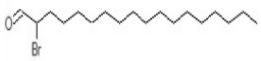
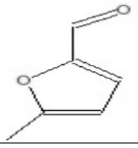


Fig 6. GC-MS Chromatogram of compounds characterize from chloroform extract of *Aspergillus flavus* CBS 593.65. 2(5H)-Furanone, D-Glucose, 6-O-α-D-galactopyranosyl, 1,2-Cyclopentanedione,3- methyl, 6-Acetyl-ß-d-mannose, Octadecanal ,2 –bromo, 2 Furancarboxaldehyde,5-Methyl (crude extract)

Table 3 Compounds with structure characterize from chloroform extract of *Aspergillus flavus* CBS 593.65 (crude extract)

Sr. No.	Compound name	Molecular formula	Compound structure	Retention time (min)
1	2(5H)-Furanone	$C_4H_4O_2$		3.83
2	D-Glucose,6-O-α-D-galactopyranosyl	$C_{12}H_{22}O_{11}$		3.99
3	1,2-Cyclopentanedione,3-methyl	$C_6H_8O_2$		4.70
4	6-Acetyl-ß-d-mannose	$C_8H_{14}O_7$		6.28
5	Octadecanal ,2 –bromo	$C_{18}H_{35}BrO$		17.02
6	2-Furancarboxaldehyde,5-Methyl	$C_6H_6O_2$		3.7

1 H NMR studies confirmed the purified compound's identity as 2(5H)-Furanone, D-Glucose,6-O-α-D-galactopyranosyl, 1,2-Cyclopen tanedione,3- methyl, 6-Acetyl-ß-d-mannose, Octadecanal,2 –bromo, 2 Furancarboxaldehyde,5-Methyl at 400 MHz, DMSO-d6 δ (ppm), shows carboxylic acid, aromatic compounds, aldehydes, unsaturated groups etc. as shown in **Fig 7**.

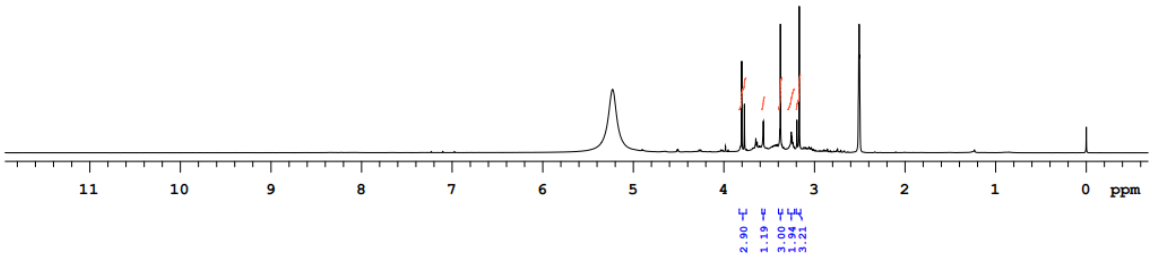


Fig 7. NMR spectra of chloroform extract of *Aspergillus flavus* CBS 593.65 (crude extract)

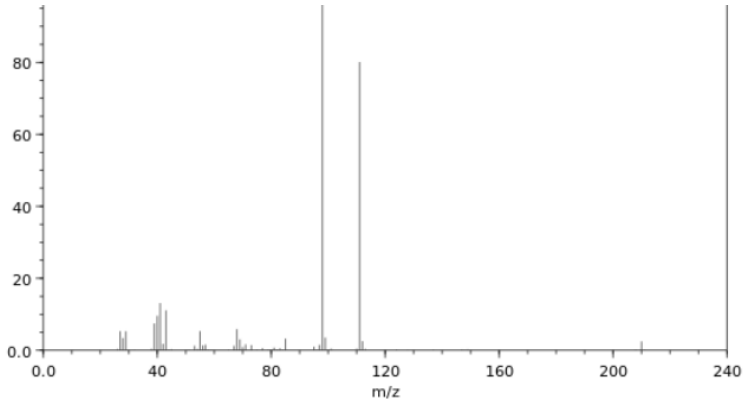


Fig 8. GC-MS analysis of TLC eluted - 2(5H)-Furanone

4. Conclusion

The *chloroform extract of Aspergillus flavus* CBS 593.65 showed greater antibacterial potential against *Klebsiella pneumoniae* MTCC 432 compared to other extracts. This extract was obtained from the broth fermentation process while solid-state and submerged fermentation did not show any inhibitory activity due to the continuous passaging of isolates. GC-MS chromatogram analysis of the chloroform crude fungal extract showed many peaks which indicates the presence of many bioactive compounds. 2(5H)-Furanone was found to be the bioactive compound showing major inhibitory activity.

5. Acknowledgment

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6. References

1. Arumugam, G. K., Srinivasan, S. K., Joshi, G., Gopal, D., & Ramalingam, K. (2015). Production and characterization of bioactive metabolites from piezotolerant deep sea fungus Nigrospora sp. in submerged fermentation. Journal of Applied Microbiology, **118**(1), 99–111. https://doi.org/10.1111/jam.12693
2. Athira, M, S. A. L., & Jayadev, and A. (2016). Isolation and partial characterization of fungal strains from marine samples. Internation Journal of Applied and Pure Science and Agriculture, **2**(8), 195–199.
3. Balouiri, M., Sadiki, M., & Ibnsouda, S. K. (2016). Methods for in vitro evaluating antimicrobial activity: A review. Journal of Pharmaceutical Analysis, **6**(2), 71–79. https://doi.org/10.1016/j.jpha.2015.11.005
4. Debbab, A., Aly, A. H., Lin, W. H., & Proksch, P. (2010). Bioactive compounds from marine bacteria and fungi: Minireview. Microbial Biotechnology, **3**(5), 544–563. https://doi.org/10.1111/j.1751-7915.2010.00179.x
5. Dona, A. C., Kyriakides, M., Scott, F., Shephard, E. A., Varshavi, D., Veselkov, K., & Everett, J. R. (2016). A guide to the identification of metabolites in NMR-based metabonomics/metabolomics experiments. Computational and Structural Biotechnology Journal, **14**, 135–153. https://doi.org/10.1016/j.csbj.2016.02.005
6. Erecta, T., Devika, L. R., & Koilpillai, J. (2015). Column Chromatographic Separation of Bioactive Compounds From. International Journal of Pharmaceutical Sciences and Research, **6**(2), 762–766. https://doi.org/10.13040/IJPSR.0975-8232.6(2).7 62-66
7. Fathoni, A., Hudiyono, S., Budianto, E., & Herry, C. (2021). Metabolite Detection and Antibacterial Activity of Fungal Endophytic Extracts Isolated from Brotowali (Tinospora crispa) Plants using TLC-Bioautography Assay Metabolite Detection and Antibacterial Activity of Fungal Endophytic Extracts Isolated from Bro. January. https://doi.org/10.1088/1757-899X/1011/1/012041
8. Garo, E., Starks, C. M., Jensen, P. R., Fenical, W., Lobkovsky, E., & Clardy, J. (2003). Trichodermamides A and B, cytotoxic modified dipeptides from the marine-derived fungus Trichoderma virens. Journal of Natural Products, **66**(3), 423–426. https://doi.org/10.1021/np0204390
9. Keller, N. P. (2019). discovery. **17(**3), 167–180. https://doi.org/10.1038/s41579-018-0121-1.Fungal
10. Luo, Y., Wei, X., Yang, S., Gao, Y. H., & Luo, Z. H. (2020). Fungal diversity in deep-sea sediments from the Magellan seamounts as revealed by a metabarcoding approach targeting the ITS2 regions. Mycology, **11**(3), 214–229. https://doi.org/10.1080/21501203.2020.1799878
11. Mioso, R., Javier, F., Marante, T., Herrera, I., & Laguna, B. De. (2014). Chemical constituents of the fermentation broth of the marine-derived fungus Penicillium roqueforti. Revista Iberoamericana de Micología, 1–6. https://doi.org/10.1016/j.riam.2014.01.004
12. Nayak, S. K., & Mishra, P. C. (2016). Application of Nagchampa biodiesel and rice husk gas as fuel. Energy Sources, Part A: Recovery, Utilization and Environmental Effects, **38**(14), 2024–2030. https://doi.org/10.1080/15567036.2015.1017672
13. Nielsen, K. F., & Larsen, T. O. (2015). The importance of mass spectrometric dereplication in fungal secondary metabolite analysis. Frontiers in Microbiology, **6**(FEB), 1–15. https://doi.org/10.3389/fmicb.2015.00071
14. ang, K. L., Overy, D. P., Jones, E. B. G., Calado, M. da L., Burgaud, G., Walker, A. K., Johnson, J. A., Kerr, R. G., Cha, H. J., & Bills, G. F. (2016). 'Marine fungi' and

'marine-derived fungi' in natural product chemistry research: Toward a new consensual definition. Fungal Biology Reviews, **30**(4), 163–175. https://doi.org/10.1016/j.fbr.2016.08.001

15. Perez, C. (2020). An antibiotic assay by the agar well diffusion method. September.
16. Pinu, F. R., Goldansaz, S. A., & Jaine, J. (2019). Translational metabolomics: Current challenges and future opportunities. Metabolites, **9**(6). https://doi.org/10.3390/metabo9060108
17. Rakshith, D., Santosh, P., Pradeep, T. P., Gurudatt, D. M., Baker, S., Rao, H. C. Y., Pasha, A., & Satish, S. (2016). Application of Bioassay-Guided Fractionation Coupled with a Molecular Approach for the Dereplication of Antimicrobial Metabolites Application of Bioassay - Guided Fractionation Coupled with a Molecular Approach for the Dereplication of Antimicrobial Metab. Chromatographia, May 2018. https://doi.org/10.1007/s10337-016-3188-8
18. Shamly, V., Kali, A., Srirangaraj, S., & Umadevi, S. (2014). Comparison of microscopic morphology of fungi using lactophenol cotton blue, iodine glycerol and Congo red formaldehyde staining. Journal of Clinical and Diagnostic Research, **8**(7), 7–8. https://doi.org/10.7860/JCDR/2014/8521.4535
19. Sholkamy, E. N., Muthukrishnan, P., Abdel-Raouf, N., Nandhini, X., Ibraheem, I. B. M., & Mostafa, A. A. (2020). Antimicrobial and antinematicidal metabolites from Streptomyces cuspidosporus strain SA4 against selected pathogenic bacteria, fungi and nematode. Saudi Journal of Biological Sciences, **27**(12), 3208–3220. https://doi.org/10.1016/j.sjbs.2020.08.043
20. Suparmin, A., Kato, T., Takemoto, H., & Park, E. Y. (2019). Metabolic comparison of aerial and submerged mycelia formed in the liquid surface culture of Cordyceps militaris. February, 1–21. https://doi.org/10.1002/mbo3.836
21. Tanwar, J., Das, S., Fatima, Z., & Hameed, S. (2014). Multidrug resistance: An emerging crisis. Interdisciplinary Perspectives on Infectious Diseases, 2014. https://doi.org/10.1155/2014/541340
22. Valle, D. L., Puzon, J. J. M., Cabrera, E. C., & Rivera, W. L. (2016). Thin Layer Chromato graphy-Bioautography and Gas Chromato graphy-Mass Spectrometry of Antimicrobial Leaf Extracts from Philippine Piper betle L. against Multidrug-Resistant Bacteria. Evidence -Based Complementary and Alternative Medicine, 2016. https://doi.org/10.1155/2016/4976791