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# Unlocking the bioactive prowess and *in-silico* prediction of pharmacokinetics and toxicity profiling of endophyte *A. fumigatus*

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***Abstract:*** **The endophytic fungus, rich in potent bioactive compounds, exhibited notable antibacterial efficacy against pathogenic microorganisms. Our study delved into the efficacy of *A. fumigatus*, using *in-vitro* techniques to assess the bioactive potential (antibacterial and anticancer activity) of ethyl acetate extract from fermented broth, containing secondary metabolites. The crude extract demonstrated significant antibacterial activity against Multi-Drug Resistant (MDR) strains *S. aureus* and *K. pneumoniae*, and promising anti-cancer activity against HeLa cells. Chemical analysis of fermented extract via GC-MS unveiled a diverse array of constituents. *In-silico* analysis highlighted two compounds *i.e.* 1,3,6,9 b-tetraazaphenalene-4-carbonitrile and 4-isothiazolecarboxylic acid, 3,5-bis(methylthio)-, methyl ester, that are actively participating in biological activities, corroborated by *in-vitro* experiments. These findings underscore *A. fumigatus*' potential as a natural source of antibacterial and anticancer agents, with favorable pharmacokinetics and docking profiles, promising as drug candidates with minimal toxicity. Further research should explore their potential therapeutic applications through pre-clinical *in-vivo* studies.**

***Index Terms:*** **ADMET, *Aspergillus fumigatus,* Cancer, Endophyte, Molecular docking, Multi-drug resistance, Natural compounds**

## I. Introduction

The emergence of drug-resistant human pathogens poses a formidable challenge in the fields of biomedicine and pharmaceuticals. Among these pathogens, Methicillin-resistant *Staphylococcus aureus* (MRSA) stands out due to its high prevalence and well-documented capability for drug resistance (Kobayashi, 2015). *Staphylococcus aureus* infects 50-60% of individuals, leading to a relatively high risk of infections (Kobayashi, 2015). The widespread prevalence and notorious drug resistance capability of *S. aureus* have triggered epidemic waves of antibiotic resistance (Chambers, 2009). Initial reports of MRSA from the early 1960s were followed by decades of global dissemination, highlighting the pathogen's capacity for rapid spread and persistence. Beyond healthcare-associated MRSA infections, this pathogen affects individuals with predisposed risk factors such as antibiotic use, open wounds, hemodialysis, and HIV infection (Yasmin, 2016). It can also cause illness in apparently healthy people. Resistance to β-lactam antibiotics is a major issue in treating *S. aureus* infections, and the bacteria can develop resistance to other antibiotics, including vancomycin, a crucial therapeutic option for severe MRSA infections (DeLeo, 2010). In addition to MRSA, the opportunistic pathogen *Klebsiella pneumoniae* poses significant challenges, primarily affecting individuals with compromised immune systems and often resulting in nosocomial infections. Before causing these infections, opportunistic *K. pneumoniae* typically colonizes the gastrointestinal tract, but colonization can also occur in the blood, urinary tract, and respiratory system (Li, 2014). Infections originating from *K. pneumoniae* tend to be chronic, as this pathogen frequently exhibits multidrug-resistant phenotypes. These resistant phenotypes are often due to the presence of extended-spectrum β-lactamases or carbapenemases, which complicate the selection of appropriate antibiotics for therapy (Paterson, 2004; Munoz-Price, 2013).

Cancer represents another global health issue, causing significant mortality worldwide, often in conjunction with deadly bacterial infections (Ferlay, 2015). Despite advancements in cancer therapies, treatments may not completely eliminate cancer but instead aim to reduce its impact and extend patients' overall lifespan (Banu, 2009). The current treatment regimens,

although improved over time, are still considered less favorable. This underscores the critical need for effective medications to treat cancer, which remains a vital aspect of global health research (Anderson, 2019). Alongside chemotherapeutic drugs, natural compounds have shown promise in cancer treatment. Some natural or modified natural compounds, such as taxol, are preferred over traditional chemotherapeutics for cancer treatment due to their unique properties and effectiveness (Rai, 2022). However, the development of resistance in cancer cells has necessitated a continuous search for new anti-cancer natural compounds (Longley, 2005). Moreover, not all natural compounds exhibit selectivity towards cancer cells alone, which is a significant consideration when screening natural compounds for anticancer drug development (Dallavalle, 2020).

To address these challenges, natural bio-active compounds with therapeutic potential are being explored as a promising alternative to traditional medicine. The field of natural bio-active compounds has gained considerable traction in recent years, primarily due to the increasing understanding of their potential benefits over traditional synthetic drugs (Mesa, 2020). Natural compounds often exhibit a broad spectrum of bioactivities, targeting various pathways and mechanisms simultaneously, which can be particularly advantageous in the treatment of complex diseases such as cancer and multi-drug-resistant infections (Ferreira, 2023). Moreover, the relatively lower incidence of adverse effects associated with natural compounds makes them a preferable choice in many therapeutic contexts. These compounds offer unique properties and diverse modes of action, making them highly effective candidates in the field of therapeutics (Fadiji, 2020). Some natural bio-active components have the ability to target multiple molecular sites simultaneously (Feng, 2021), which increases the likelihood of achieving therapeutic efficacy while reducing the risk of resistance development. This polypharmacology approach can play a crucial role in combating drug resistance and other diseases. Furthermore, natural bioactive substances often boast a superior safety profile compared to synthetic compounds, making them highly appealing for pharmaceutical applications. These bioactive compounds can be derived from a variety of sources, including fruits, vegetables, seeds, herbs, spices, and different microbial species (Samtiya, 2021). Of particular interest are bio-active compounds from microbial sources, especially endophytes. Endophytes are microorganisms that reside internally within plant tissue and serve as a source of renewable and pharmacologically active compounds (Shukla, 2014). Many plant species can host endophytes, providing a sustainable and eco-friendly supply of bioactive chemicals. Utilizing endophytic biodiversity as an alternative medication can reduce reliance on harmful natural resources and minimize adverse environmental effects associated with conventional drug development methods (Pandey, 2018). Endophytic fungi, in particular, have emerged as a focal point in the search for bioactive compounds relevant to antimicrobial therapy. Screening antimicrobial agents from endophytes holds significant promise in addressing the growing threat of disease-causing organisms, including drug-resistant strains (Tan, 2001).

Numerous reports have highlighted the remarkable antimicrobial and anticancer properties of fungal endophytes in tropical regions (Carvalho, 2012; Rajagopal, 2012; Banerjee, 2011). The rich and diverse ecosystem of tropical regions provides a unique habitat for various microorganisms, including fungi, and especially endophytes. Endophytic fungi, such as *Aspergillus fumigatus* (*A. fumigatus*), represent a largely untapped reservoir of bioactive compounds with significant pharmaceutical potential (Manganyi, 2020). These microorganisms have co-evolved with their plant hosts over millions of years, leading to the production of a diverse array of secondary metabolites with potent bioactivities. The symbiotic relationship between endophytes and their host plants often results in the production of unique compounds that are not found in other microorganisms (Wani, 2015). This unique chemical diversity makes endophytic fungi a promising source of new drugs. However, searching for new drugs and pinpointing the mechanism of action is a crucial part that requires much attention. The integration of *in-silico* methods with traditional bioactivity assays represents a powerful approach to the discovery of new drugs. *In-silico* methods, such as molecular docking and virtual screening, allow for the prediction of the interactions between bioactive compounds and their target proteins. These methods can also be used to identify potential off-target effects and optimize the pharmacokinetic properties of the compounds (Olğaç, 2017). By combining *in-silico* methods with experimental validation, researchers can significantly accelerate the drug discovery process and increase the likelihood of identifying effective therapeutic agents (Shaker, 2021).

This study aims to assess the bioactivities of *A. fumigatus*, specifically its potential as an antibacterial and anticarcinogenic agent against cancer cell lines, building upon existing knowledge. Through meticulous analysis using gas chromatography-mass spectrometry (GC-MS), the crude culture extract of *A. fumigatus* was scrutinized to identify the bioactive components responsible for its efficacy. Isolated bioactive components were then tested for their antibacterial and anticancer activities. The antibacterial activity was evaluated using *in-vitro* assays against various drug-resistant bacterial strains, while the anticancer activity was assessed using cell viability assays on HeLa cells. Furthermore, *in-silico* approaches were employed to study the specific antibacterial efficacy of the endophytic product following *in-vitro* validation. The experimental findings were robustly supported by the *in-silico* study, emphasizing the promising therapeutic applications of *A.*

*fumigatus* in combating drug-resistant bacteria and cervical cancer cells.

## II. Results and Discussion

### A. Isolation and identification of endophytic fungi

Multiple fungal endophytes were isolated from *Capsicum sp.*. Depending upon the preliminary experimental results against test organisms, we selected LS4 for further identification and investigation (Fig. 1a). The endophyte underwent identification based on ITS region sequencing. BLASTN nucleotide database facilitated matching LS4's ITS region to its genomic DNA, PCR amplified and sequenced. Prior to the search, adjustments were made to conform to database annotation conventions, including trimming nucleotide sequences, correcting feature spans, and adjusting GT and AG sequences. The data demonstrated a 100% match with existing records in the database (accession number MT267796), detailed in Table S1. Molecular identification confirmed the genus *Aspergillus* and species *fumigatus* due to 100% similarity with the closest match. Following confirmation, the gene sequence was submitted to GenBank (accession number OQ410309).

### B. Screening of biological activity

#### 1) Anti-bacterial activity

MDR strains of *S. aureus* (gram-positive bacteria) and *K. pneumoniae* (gram-negative bacteria) were used to assess the ability of *A. fumigatus* extract for antibacterial activity using the radial inhibitory zone formation assay onto Muller Hinton (MH) agar plates. Post-incubation, the inhibition zone was measured. The result revealed that the inhibition zone for *S. aureus* was 16 mm, which is a bit smaller than the inhibition zone of 18 mm for *K. pneumoniae* (Fig. S1). Observation confirms the significant efficacy of the extract against both the test organisms as compared to the untreated control.

#### 2) Minimum inhibitory concentration (MIC) and growth curve study:

To determine the potency as a naturally occurring bio-active compound, the fungal extract was chosen and examined for its MIC feature along with gentamicin as positive control by broth dilution susceptibility test against *S. aureus* and *K. pneumoniae* as model organisms. The resistant profile of the model organisms is described in Table S2. The growth inhibition curve from the unveiled data of MIC indicated dose-dependent antibacterial activity against *S. aureus* and *K. pneumoniae* (Fig. 1b) and comparative data with positive control are presented in Table I. Further, the growth curve analysis revealed remarkable retardation of the growth pattern for both organisms when compared to the untreated control. The sigmoidal curve was observed in untreated control of both bacterial strains (*K. pneumoniae* and *S. aureus*) whereas the treated group at the desired concentrations exhibited a non-monotonic retardation in the curve pattern (Fig 1c).

Comparison between the control and treated indicated that crude extract arrests both the organisms at the lag phase (Chatterjee, 2015). Importantly, the lag phase generally allows bacterial cells to be accustomed to new environments. Consequently, the elongated lag phase can alternatively be defined as the time requisite for the bacteria to enter the reproductive stage. Data exhibits (Fig. 1c) an astonishing rise in delay time with MIC concentration of crude extract for both bacteria. However, the rise in the delay time of *K. pneumoniae* is more as compared to that of *S. aureus*.

Table I. Anti-bacterial activity of the *A. fumigatus* crude extract

| Bacterial strain | *A.fumigatus* crude extract (µg/mL) (MIC) | Gentamicin (µg/mL) (MIC) | Negative control (10% DMSO) |
|---|---|---|---|
| *S.aureus* | 1200 | 230 | - |
| *K.pneumoniae* | 800 | 120 | - |

#### 3) Scanning electron microscopy (SEM):

Scanning electron microscopy was performed to monitor the morphological changes that occur after the treatment of bacterial strains with isolated crude extract (LS4). The SEM photomicrographs demonstrated the loss of membrane integrity in both organisms after treatment with the MIC concentration. An inconsistent distortion in the membrane structure was observed in treated *K. pneumoniae* (Fig. 1d) whereas pores were observed in the membrane of *S. aureus* (Fig. 1d). Therefore, the SEM study revealed that crude extract was found significant effect on both the *Klebsiella* and *Staphylococcus* cells.

#### 4) Cell viability assay:

Different compounds for instance plant extracts, endophytic extracts, or any biologically active compounds are been studied a lot to monitor their toxicity or interaction with biological entities. The cellular toxicity of crude extract was evaluated on HeLa cells at different concentrations by MTT assay. The result revealed that there were significant decreases in the percentage of proliferative HeLa cells in a dose-dependent manner when treated with the extract (Fig. 1e). The lower concentration (0.84 mg/mL) showed 53% viability in the population of cancer cells when compared to untreated control cells. Thereby, the cell viability results demonstrated the crude ethyl acetate extract of *A. fumigatus* has good cytotoxic activity against HeLa cells. Moreover, the isolated crude extract LS4 showed anticancer activity even at the lowest concentration (0.42 mg/mL) hence LS4 proved to be a potent anticancer drug candidate and could be further explored for its scope in the area of biomedical therapeutics and drug discovery.

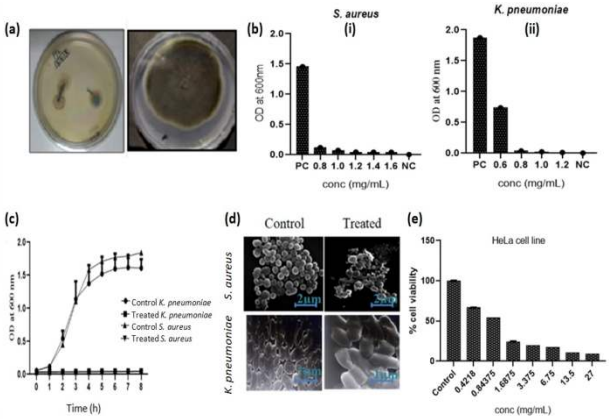


Fig. 1.Isolation and bio-activity of *A.fumigatus:* (a) Isolation plate showing mixed growth of fungal endophytes from the leaf of *Capsicum sp.*(left) and the pure culture plate of LS4 exhibits its morphological feature (right) (b) Growth inhibition graph to determine MIC against i) *S. aureus* and ii) *K. pneumoniae*; (c) Growth curve analysis of the treated organisms alone and with untreated control; (d) SEM photomicrographs of bacterial cells demonstrating the morphological changes in the treated cells; (e) Graph showing the cytotoxicity pattern of HeLa cells treated with the fungal extract

*C. Identification of compounds of crude extracts from A. fumigatus using GCMS*

The GCMS describes that 17 chemical compounds with distinct classes are present in the crude extract of *A. fumigatus* which are associated with diverse biological activities (Fig. 2).

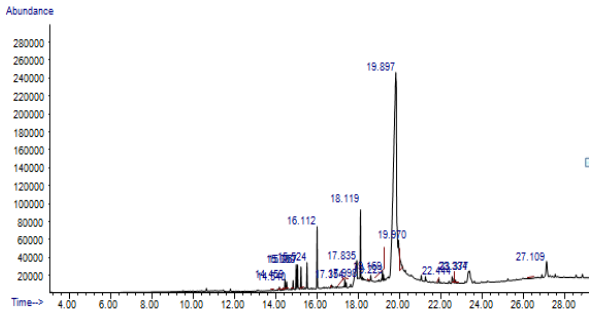


Fig. 2.GCMS chromatogram of ethyl acetate extract of *A. fumigatus*

Discrete classes of chemical compounds namely amine, pyrimidine, hydrocarbon, phenol, indole, quinoline, morpholine, etc were found in the crude extract (Fig. 3). Natural quinoline, amine, and phenol are one of the most occurring groups of fungal secondary metabolites with pharmacological potential. From the GCMS results, a phenol (9(10H)-Acridinonedihydroxy-10methyl), quinoline (Isoxazolo[4,3-a]phenazin-1(3H)-one and [1]Benzothieno[2,3-b]quinoline) and amine (Benzenamine, 2-chloro-5-(trifluoromethyl) were detected from the crude extract. These classes of molecules are known to possess therapeutic potential including antibacterial, antimalarial, anti-cancerous, and anti-inflammatory activities. These compounds may prevent bacterial growth by inhibiting microbial enzymes and/or DNA replication (Hussein, 2020) while arresting cancer through different molecular levels such as gene regulation, inhibition of cellular differentiation, and proliferation thereby inducing apoptosis in cells (Adnan, 2021).

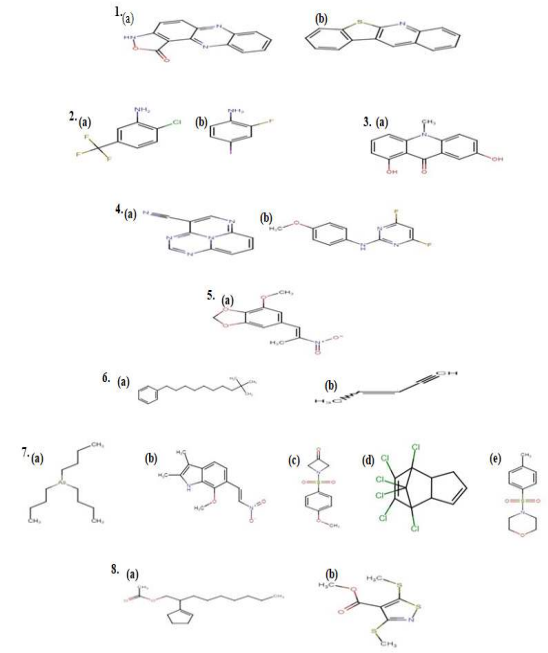


Fig. 3.Structure of chemical components obtained from GCMS analysis of ethyl acetate extract of *A. fumigatus*: 1. quinoline compounds: a. Isoxazolo[4,3-a]phenazin-1(3H)-one, b. [1]Benzothieno[2,3-b]quinoline ; 2. Amines: a. Benzenamine, 2-chloro-5-(trifluoromethyl), b. 2-Fluro-4-iodoaniline; 3. phenolic compound: a. 9(10H)-Acridinone, 1,7-dihydroxy-10methyl; 4. Pyrimidines: a. 1,3,6,9b-Tetraazaphenalene-4-carbo nitrile, b. 4,6-Difluoro-2-(4-methoxyphenylamino)pyrimidine; 5. Nitrostyrene: a.trans3-Methoxy-4,5-methylenedioxy-.beta.-methyl-.beta.-nitrostyrene; 6. Hydrocarbons: a. Benzene, (9,9dimethyldecyl), b. 3-Penten-1-yne; 7. Arsine, indole, azetidine, indene, and morpholine: a. Arsine, tributyl, b. 7-Methoxy-6-(E-2-nitroethenyl)-2,3 dimethylindole, c. N-(4-Methoxybenzenesulfonyl)azetidin-3-one, d. Chlordene, e. Morpholine, 4-[(4-methylphenyl)sulfonyl]; 8. esters a. 2-Cyclopentene-1-nonanol, acetate, b. 4-Isothiazolecarboxylic acid, 3,5- bis(methylthio)-, methyl ester

Detailed information along with the code name of the component present in the crude extract has been given in Table II.

Table II. Chemical compositions present in the crude ethyl acetate extract of *Aspergillus fumigatus*

| Sl No. | Compound | Code | RT | Area % | Formula | MW (g/mol) | Class |
|---|---|---|---|---|---|---|---|
| 1. | Benzenamine, 2-chloro-5-(trifluoromethyl) | C1 | 14.541 | 0.53 | $C_7H_5ClF_3N$ | 195.57 | Amine |
| 2. | 1,3,6,9b-Tetraazaphenalene- | C2 | 14.5 | 0.59 | $C_{10}H_5N_5$ | 195.1 | Pyrimidine |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | 4-carbo nitrile | | 47 | | | 8 | (Fused) |
| 3. | 7-Methoxy-6-(E-2-nitroethenyl)-2,3 dimethylindole | C3 | 15.007 | 1.47 | $C_{13}H_{14}N_2O_3$ | 246.266 | Indole |
| 4. | Benzene, (9,9dimethyldecyl) | C4 | 15.231 | 1.36 | $C_{18}H_{30}$ | 246.438 | Hydrocarbon |
| 5. | Arsine, tributyl | C5 | 15.527 | 1.64 | $C_{12}H_{27}As$ | 246.26 | Arsine |
| 6. | 9(10H)-Acridinone, 1,7-dihydroxy-10methyl | C6 | 16.013 | 3.37 | $C_{14}H_{11}NO_3$ | 241.246 | Phenol |
| 7. | Isoxazolo[4,3-a]phenazin-1(3H)-one | C7 | 17.934 | 4.28 | $C_{13}H_7N_3O_2$ | 237.21 | Quinoxaline |
| 8. | 3-Penten-1-yne | C8 | 17.995 | 0.19 | $C_5H_6$ | 66.1 | Enyne |
| 9. | N-(4-Methoxybenzenesulfonyl)azetidin-3-one | C9 | 18.113 | 3.51 | $C_{10}H_{11}NO_4S$ | 241.27 | Azetidin |
| 10. | Chlordene | C10 | 19.167 | 0.53 | $C_{10}H_6Cl_6$ | 338.9 | Indene |
| 11. | 2-Cyclopentene-1-nonanol, acetate | C11 | 19.221 | 0.29 | $C_{16}H_{28}O_2$ | 252.398 | Ester |
| 12. | 4-Isothiazolecarboxylic acid, 3,5-bis(methylthio)-, methyl ester | C12 | 19.893 | 61.98 | $C_7H_9NO_2S_3$ | 235.4 | Isothiazole (Ester) |
| 13. | [1]Benzothieno[2,3-b]quinoline | C13 | 19.972 | 3.80 | $C_{15}H_9NS$ | 235.31 | Quinoline |
| 14. | Morpholine, 4-[(4-methylphenyl)sulfonyl] | C14 | 22.540 | 0.13 | $C_{11}H_{15}NO_3S$ | 241.31 | Morpholine |
| 15. | trans-3-Methoxy-4,5-methylenedioxy-.beta.-methyl-.beta.-nitrostyrene | C15 | 23.335 | 1.59 | $C_{11}H_{11}NO_5$ | 237.21 | Nitrostyrene |
| 16. | 4,6-Difluoro-2-(4-methoxyphenylamino)pyrimidine | C16 | 23.374 | 2.11 | $C_{11}H_9F_2N_3O$ | 237.21 | Pyrimidine |
| 17. | 2-Fluoro-4-iodoaniline | C17 | 27.105 | 1.88 | $C_6H_5FIN$ | 237.01 | Amine |

*D. Pharmacokinetics and ADMET profiles analysis of identified bio-active components from ethyl acetate extract of A. fumigatus*

ADMET (absorption, distribution, metabolism, excretion, and toxicity) is a crucial tool in the field of drug discovery as the poor qualities of ADMET can impair pharmacological activity (Lin, 2003). The misleading of drug discovery in the clinical phase is exceedingly expensive and also frequently causes undesirable pharmacokinetics and toxicity. Thus, ADMET parameters have been evaluated with the help of *in-silico* studies to determine whether or not *A. fumigatus* ethyl acetate crude extract will make a good choice for a viable medication (Table III). It's fascinating to note that all of the detected bioactive constituents except two (C5, C10) followed Lipinski's rule of five, and some of them, including C6, C7, C9, C12, C13, C15, and C16 also follow Ghose, Veber, and Egan filters with a good bioavailability score (55-85%). Thus, these compounds were selected further for an *in-silico* toxicity study.

Based on AMES toxicity, hERG potassium channel blockage, skin sensitization, and hepatotoxicity criteria, the toxicity profile of the previously chosen constituents has been anticipated. Results showed that just four of the previously selected compounds namely C2, C8, C12 and C15 have not a deviating mutagenic and hepatic toxicity potential.

Further, these four constituents (C2, C8, C12, and C15) were adopted to carry out *in-silico* ADME profile analysis. Initially, human cytochrome P450 (CYP) isoforms were implicated in the liver's drug metabolism. Among these, CYP3A4 is the prime and most clinically significant enzyme responsible for drug metabolism in the human body. Drug toxicity, drug-drug interactions, and other negative outcomes could result from its inhibition. The analysis confirmed that C2, C8, and C12 were neither inhibitors nor substrates of any isoenzymes.

Additionally, solubility is a crucial component for a molecule's absorption and distribution in the body. These three substances (C2, C8, and C12) are highly soluble in water. To increase the efficacy of a transdermal drug administration, the skin permeability should be examined. All three chosen compounds appear to have good to moderate skin penetrability. The human epithelial colorectal adenocarcinoma cells that make up the Caco-2 cell line. The permeability of Caco-2 can be used to foresee oral medication ingestion. All substances had Caco-2 permeability values that ranged from moderate to powerful (log Papp values >0.90 cm/s). The strongest permeability was anticipated for all C2, C8, and C12.

Another parameter P-gp is known as the most significant member of the ABC-transporters, or ATP-binding cassette transporters, employed to shield the central nervous system (CNS) from xenobiotics and is crucial to estimating active efflux through biological membranes. None of the chosen compounds but C8 were P-gp inhibitors or substrates. So, the rest of them, *i.e.* C2 and C12 were tested and promoted for their ability to pass the brain-blood barrier (BBB).

Additionally, the total clearance (CLTOT) for both the hepatic and renal organic cation transporter 2 (OCT2) substrates, was predicted in order to figure out the excretion routes. The findings showed that around 98% of the detected bioactive

components had positive overall clearance values and were easily eliminated. Thus, C2 and C12 were selected finally for further *in-silico* studies.

Table III. Absorption, distribution, metabolism, excretion, and toxicity properties of selected best profile bio-active components

| Entry | C2 | C12 |
|---|---|---|
| **Drug likeness** | | |
| Lipinski | Yes | Yes |
| Ghose | Yes | Yes |
| Veber | Yes | Yes |
| Egan | Yes | Yes |
| Muegge | No | Yes |
| Bioavailability score | 0.55 | 0.55 |
| **Toxicity** | | |
| AMES | No | No |
| Hepatotoxicity | Yes | No |
| HERG I inhibitor | No | No |
| Skin sensitization | No | No |
| **Absorption** | | |
| Water solubility | -2.326 | -2.869 |
| Caco2 permeability | 0.624 | 1.487 |
| Intestinal absorption | 100 | 94.449 |
| Skin permeability | -3.345 | -2.662 |
| p-glycoprotein substrate | No | No |
| p-glycoprotein I inhibitor | No | No |
| p-glycoprotein II inhibitor | No | No |
| **Distribution** | | |
| VDss | 0.055 | -0.384 |
| BBB permeability | 0.63 | 0.352 |
| CNS permeability | -0.317 | -2.837 |
| **Metabolism** | | |
| CYP2D6 substrate | No | No |
| CYP3A4 substrate | No | No |
| CYP1A2 inhibitor | No | No |
| CYP2C19 inhibitor | No | No |
| CYP2C9 inhibitor | No | No |
| CYP2D6 inhibitor | No | No |
| CYP3A4 inhibitor | No | No |
| **Excretion** | | |
| Total clearance | 0.734 | 0.431 |
| Renal OCT2 substrate | No | No |

Through ADMET analysis selection for bio-active components that can act as a potent alternative for drug development, the top two eligible components (C2 and C12) were evaluated further for better drug-likeness and bioavailability (Fig. 4a & b). The lipophilicity has shown as the bioavailability radar results that are (XLOGP3 between 0.7 and +5.0; size was MW between 150 and 250 g/mol; the polarity of TPSA between 60 and 120; solubility was found to be log S not higher than 5, and flexibility was no more than 5 rotatable bonds with the colored zone) indicating desired bio-active component hold good drug-like properties and physicochemical space for good oral bioavailability.

The BOILED-Egg model (Brain or Intestinal Estimated permeation technique) has been used as an example to predict the gastrointestinal (GI) absorption and BBB permeability of the same selected substances. The data signifies that the components with a red point in the yellow ellipse (Fig. 4c) have a high likelihood of penetrating the brain and are not P-gp (PGP) substrate.

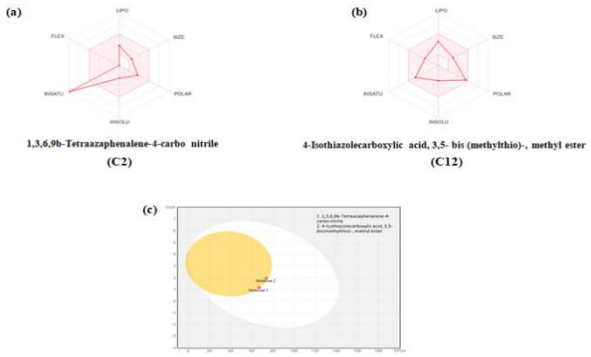


Fig. 4.The bioavailability radar of bio-active components is primarily based on physicochemical indicators that are optimal for oral bioavailability (a & b) and boiled egg model for selected components using the Swiss ADME web tool (c).

### *E. In-silico molecular docking studies*

To understand the promising efficacy of the selected components against *S. aureus* and *K. pneumoniae* as well as to investigate the potential binding and interactions of ligands within the active sites of the enzymes, a docking study of the two most active metabolites was performed. It was evident from a thorough review of the docking data that compound C2 has a higher binding affinity at the active site of the target enzymes than compound C12 in the case of both bacterial strains. The list of the selected enzymes with their docking score is described in Table S3.

Compound C2, the most active substance in the series, had the highest binding affinities of -7.2 and -7.0 to the target enzyme DNA Gyrase and penicillin-binding protein (PBP2A) respectively in cases of MRSA. The docked complex C2 and DNA Gyrase (Fig. 5b) revealed three hydrogen bonds whereas the docked complex of C2 and penicillin-binding protein (PBP2A) (Fig. 5a) showed one H-bond, one π-σ bond, and one π-alkyl bond. Again, the *in-silico* molecular docking data revealed that *K. pneumonia* with the C2 molecule showed the highest binding affinities of -7.5 and -6.6 to the target KPC-3 and OmpA. The docked complex between enzyme and bioactive compounds and their bonding interaction with the active site was demonstrated in Fig. 5c & d. Table IV demonstrates the actual bonding positions of amino acids at the active site of these selected enzymes with bioactive compounds and the rest of the

enzyme binding position and docked complex details are given in Table S4 and Fig. S2, and S3 respectively.

Table IV. Table demonstrating the bond interaction details between the ligand and targets (enzymes)

| Enzyme | Docking score (Kcal/mol) | H-bond | Alkyl | Vander Waals | C-H bond |
|---|---|---|---|---|---|
| MRSA | | | | | |
| Penicillin-binding protein (PBP2A) | -7.0 | Lys,B:215 | Val,B:217 Pro,B370 | Tyr,B369 Lys,B218 Lys,b219 Thr,B216 Asp,B211 Leu,B224 | - |
| DNA Gyrase | -7.2 | Arg, C:92 Gln, C:267 Val,c:268 | - | Gln, C:95 Ser, C:98 Ile, C:264 Pro, C:265 Thr, C:220 Gly, C:115 Met, C:113 Ser, C:112 Asn, C:269 | - |
| ***Klebsiella pneumonia*** | | | | | |
| KPC-3 | -7.5 | Thr, A:237 | - | Glu, A:166 Lys, A:73 Ser, A:70 Thr, A:235 Gly, A:236 Ser, A:130 Trp, A:105 Pro, A:104 Leu, A:167 | Asn, A:170 Asn, A:132 |
| OmpA | -6.6 | Ser, A:518 Ser, B:508 Asp, B:507 | Lys, A:482 | Glu, A:517 Glu, A:517 Tyr, A:515 Pro, B:506 Gln, A:485 | |

Finally, in an initial effort to pinpoint the mechanism of the promising inhibitory activity against *S. aureus*, C2 was shown the highest inhibition as opposed to PBP2A and DNA gyrase. The PBP2A is a critical factor in the broad-spectrum β-lactam resistance MRSA strains. Transpeptidase activity is offered by PBP2A to enable cell wall synthesis at β-lactam concentrations that inhibit the β-lactam-sensitive PBPs typically generated by *S. aureus* due to its low affinity for β-lactams (Lim, 2002). DNA gyrase is responsible for the negative super-coiling of double-stranded closed-circular DNA. The inhibitor against (PBP2A) and DNA gyrase can block the normal transpeptidation reaction and interfere with the DNA cleavage/resealing function of the enzyme respectively, resulting in bacterial death. So, the data suggested that the tested compound may function in a manner consistent with an inhibitor of reference two.

According to *in-silico* docking analysis, KPC-3 and OmpA are the two target molecules in *K. pneumoniae* with which the binding affinity of selected bioactive components is high. Among these two target molecules, KPC-3 is the most prevalent variant with varying geographic distribution (Rodrigues, 2016). KPC-type carbapenemases in *K. pneumoniae* and other gram-negative bacteria are emerging resistance factors, which represent a significant process of obtaining resistance to carbapenems and other β-lactams (Giani, 2009). Again, OmpA plays crucial pathogenic roles in the adhesion, invasion, or intracellular survival of bacteria as well as in the evasion of host defense. Inhibition of the particular enzyme and/or protein can make the bacteria more susceptible to a selected active component. Theoretically, molecular docking supported the activity of the C2 compound on the outer membrane of *K. pneumoniae* which can be further confirmed by the *in-vitro* experiment as well (Fig. 1d).

Growing interest has been shown in endophytic organisms that have been isolated from plants and are known to produce a variety of secondary metabolites with various biological functions. According to reports, the genus *Capsicum* is a great source of endophytic fungi *A. fumigatus* with beneficial bioactivities like cytotoxic against human leukemia cells (Ge, 2009), and antibacterial (Bala, 2013) properties. Since in West Bengal, the isolation of endophytic *A. fumigatus* from *Capsicum sp.* has not previously been documented, we conducted a bio-prospecting study to find this plant's endophytic fungus with promising antibacterial properties. The broad-spectrum antibacterial efficacy of *A. fumigatus* extract against *S. aureus* and *K. pneumoniae* was the most encouraging among all isolated endophytes. Also, we identified 17metabolites from the

crude extract using mass spectrometry (Table 3). The anti-bacterial effect of isolated metabolite was noted against *S. aureus* and *K. pneumoniae* as these both are the major human pathogens. This inspired us to investigate the working strategies of the fungus extract against test organisms. According to the outcome of pharmacokinetics and toxicity profiling analysis, C2 and C12 are the two most promising compounds among all identified components. These two compounds are also reported for the first time from the ethyl acetate extract of *A. fumigatus.*

Those proteins (PBP2a, OmpA) and enzymes (DNA gyrase, KPC-3) play an important role in showing pathogenicity and regulating bacterial cell cycle progression are attractive targets for exploring more potent antibiotics. The compound 1,3,6,9b-tetraazaphenalene-4-carbonitrile was effectively incorporated into the PBP2a, DNA gyrase, KPC-3, and OmpA, as shown by docking studies. This study was considered to have new findings since no other study has relies on a molecular docking approaches to demonstrate how these bioactive compounds interact mechanistically with the selected proteins and enzymes. The outcomes enable a logical explanation of how these compounds carry out their antibacterial activity.

The *in-silico* findings shed light on *A. fumigatus'* isolation as an endophyte from that particular region. By using molecular docking experiments, we also looked more closely at the potential mechanisms by which the secondary metabolite affects PBP2A, DNA gyrase, KPC-3, and OmpA. The bioactive component of *A.fumigatus* can potentially be employed as a source of alternative medications. *A. fumigatus* ethyl acetate extract is a viable therapeutic candidate for the treatment of various disorders since *in-silico* analyses showed that the bioactive ingredients had an excellent pharmacokinetic and safety profile.

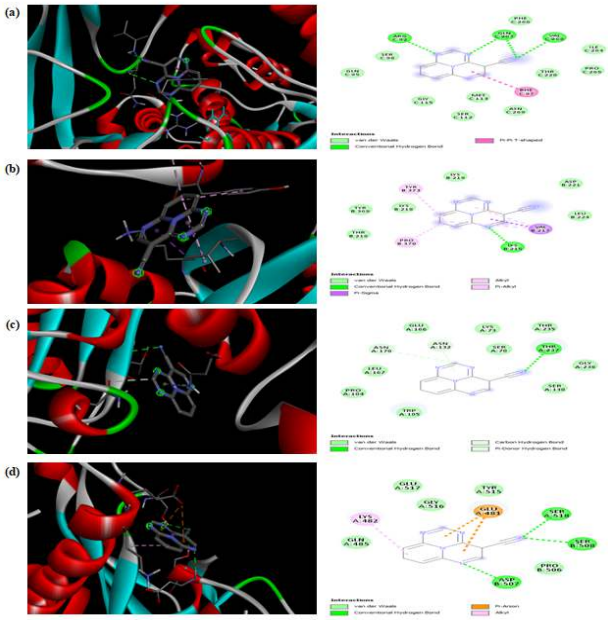


Fig. 5.(a-b) diagram illustrating a 3D and 2D binding pattern of compound C2 with PBP2A and DNA gyrase against *S. aureus.* (c-d) diagram illustrating 3D and 2D binding pattern of compound C2 with KPC-3 and OmpA against *K. pneumonia*.

## III. Conclusion

The exploration of endophytic fungi, like *A. fumigatus*, as sources of bioactive compounds represents a promising avenue for drug discovery. Endophytes, which reside symbiotically within plant tissues, produce a wide array of secondary metabolites with diverse bioactivities. These organisms are particularly promising due to their ability to synthesize unique compounds that are often not found in other microorganisms or plants. The findings of this study unequivocally demonstrated the significant potential of the endophytic fungus *A. fumigatus* as a source of both antibacterial and anticancer agents. A detailed GC-MS analysis of the ethyl acetate extract of *A. fumigatus* culture led to the isolation of 17 distinct metabolites. These metabolites were scrutinized for their bioactive properties against MDR bacterial strains and cancer cells, and the results were promising. The fungal extract exhibited a significant reduction in the growth of *S. aureus* and *K. pneumoniae*, highlighting their potential for development into therapeutic agents. The pharmacokinetic characteristics of the isolated metabolites were assessed against *S. aureus* and *K. pneumoniae*, leading to the identification of two metabolites, designated as C2 and C12, which demonstrated significant pharmacological activity. These compounds also demonstrated favorable safety profiles, which is a crucial consideration in drug development and underscores the potential of C2 and C12 as novel antibacterial agents. While the primary focus of this study was on the antibacterial potential and mechanisms of action of C2 and C12, the anticancer potential of *A. fumigatus* also merits further investigation. Preliminary data suggest that these compounds might have significant anticancer properties. However, comprehensive *in-vivo* pharmacological and toxicological evaluations are necessary to fully elucidate their therapeutic potential and safety profile. Such investigations will be crucial for translating these findings into viable clinical applications, potentially leading to the development of new anticancer therapies.

In conclusion, the exploration and utilization of natural bioactive compounds from endophytic fungi such as *A. fumigatus* present a compelling avenue for the development of new therapeutic agents. These compounds not only offer potential solutions to the challenges posed by drug-resistant pathogens and cancer but also align with the growing emphasis on sustainable and eco-friendly pharmaceutical practices. As research in this field continues to advance, new, effective, and safer treatments for various diseases will likely emerge, ultimately improving global health outcomes. The integration of traditional bioactivity assays with advanced *in-silico* methods

holds great promise for accelerating the drug discovery process and increasing the likelihood of success. By harnessing the potential of natural bioactive compounds, we can address some of the most pressing health challenges of our time and pave the way for a healthier future.

## IV. EXPERIMENTAL SECTION

### A. Collection of plant materials

Fresh and healthy samples from different parts of *Capsicum sp.* (Chili pepper) such as leaves, stems, and roots were collected from the garden of the Department of Microbiology, University of Kalyani, and other nearby residential areas of Kalyani, West Bengal ( 22° 58' 30.3024" N and 88° 26' 4.2324" E). After collection, each sample was kept separately in sealed plastic bags and brought to the laboratory on the same day, processed for fungal endophyte isolation.

### B. Isolation of endophytic fungi

To isolate fungal endophytes, different parts including leaves, stems, and roots of the collected plant were washed thoroughly under running tap water followed by sterile Milli-Q water. The washed plant parts were transferred into an aseptic environment and surface sterilized by using a hypochlorite solution (Pelo, 2020). Briefly, the surface was sterilized by immersing the pieces into 70% ethanol for 1 min followed by 2% sodium hypochlorite solution for 5 min, and finally rinsed twice in sterile Milli-Q water for 5 min. Then plant parts were sectioned into small pieces (approx 2 mm × 2 mm) with a sterilized blade. The samples were air-dried and then transferred onto potato-dextrose agar (PDA) plates. The PDA plates were sealed and incubated at 28 °C for 4-7 days and monitored regularly for the visual growth of fungi. Following that, every fungal isolate was moved from mixed isolation plates to pure culture plates. Transferring hyphae to an agar slant and incubating for 6-12 days at 28 °C allowed for the preparation of stock from the pure culture. Bioactive secondary metabolites were isolated by fermentation using active stock culture as the start culture. Isolates were growing in duplicate every 2 weeks to maintain stock culture and stored at 4 °C until further use.

### C. Fermentation and extraction of secondary metabolites from endophytic fungi

To obtain secondary metabolites from the fungal isolates, fermentation was carried out by a single-step process with slight modifications (Kjer, 2010). Briefly, mycelial agar pieces of fungus from the stock culture were transferred aseptically in a 250 mL conical flask containing 100 mL of sterile potato dextrose broth (PDB) incubated at 28 °C for 14-20 days at 120 rpm. After the incubation period, the fungal broth was filtered using Whatman® filter papers, and the filtrate was collected in another conical flask. The secondary metabolite was extracted from this filtrate using the solvent extraction method. The filtrate and the solvent *i.e.* ethyl acetate (EtOAc) were taken in a separating funnel at a ratio of 1:1 and mixed well by shaking the funnel vigorously several times. The funnel was kept in resting condition for around 30 min to allow the solvent and aqueous phase to be separated and the aqueous phase was extracted twice with the same volume of EtOAc. Finally, the solvent phase was collected and evaporated under vacuum using a vacuum drier at 40 °C to get a semi-solid residue of fungal extract. The dried extract was stored in a deep freezer at -20 °C until further use.

### D. Screening of biological activity

Test organisms: Two multi-drug-resistant (MDR) bacterial strains were used as test organisms namely gram-positive bacteria Methicillin-resistant *Staphylococcus aureus* (ATCC 700699) and gram-negative bacteria *Klebsiella pneumoniae* (ATCC10031).

#### 1) Antibacterial assay

The antibacterial assay was performed to monitor the potency of isolated fungal endophytic extracts against bacterial strains. Briefly, dried extracts were dissolved in DMSO and tested against MDR organisms using the agar well diffusion method. Suspension of bacteria was grown overnight at 37 °C in Muller Hinton (MH) broth. The turbidity of the suspension was adjusted to ~$10^7$ CFU/mL using an O.D. value of 0.5. The MH agar plates were prepared and wells (6 mm diameter) were cut aseptically using a sterile cork borer on the agar medium seeded with bacterial culture. In each well 60 µL of test compound was added and DMSO (60 µL) served as a negative control. To allow the diffusion of the substances into the agar, plates were in pre-incubation for 1 h at 4 °C and then incubated at 37 °C for 24 h. Post incubation, the diameter of the inhibition zone around the wells was measured to record the antibacterial activity of fungal endophytic isolates. The isolate showing the highest activity namely LS4 was selected for further study.

#### 2) Minimum inhibitory concentration (MIC) and growth curve analysis

The minimum inhibitory concentration (MIC) of the crude extract against *S. aureus* and *K. pneumoniae* was determined following the protocol of broth dilution method with slight variation (Christofilogiannis, 2001). Briefly, sterile media was inoculated with diluted 20 µL ($5\times10^5$ CFU/mL) overnight grown bacterial suspension in the test tube containing crude extract solution ranging from 3000-5000 µg/mL. In this experiment, gentamicin was used as positive control and DMSO (10%) was used as negative control. The test tubes were then incubated at 37 °C for 24 h, and the optical density (O.D.) was measured at 600 nm using a spectrophotometer (UV2300 II Spectrophotometer, Techcom). MIC of the crude extract was considered the maximum concentration that inhibits the growth of the bacterial population ($OD_{600}$ nm < 0.05).

The bacterial growth curve of each tested organism was estimated in the presence of crude extract. A set of conical flasks, containing 20 mL of nutrient broth and crude extract, was

inoculated with 0.2 mL overnight cultured of each bacterial strain of interest. A control was carried out for each test organism under the same conditions but without any crude extract. Conical flasks were then incubated at 37 °C in stirring of 120 rpm on a shaker. Cell turbidity was recorded by measuring the optical density ($OD_{600}$ nm) with a spectrophotometer (UV2300 II Spectrophotometer, Techcom ).

*3) Scanning Electron Microscopy (SEM)*

Scanning electron microscopy (SEM) was performed to observe the morphological changes in the bacterial cells after the treatment with the test compound. The sample was prepared according to the procedure with some alterations (Hartmann, 2010). *S. aureus* and *K. pneumoniae* bacterial strains were treated with the test compound at MIC concentration. To prepare the sample, bacterial cells were centrifuged at 10,000 rpm for 10 min and the collected pellet was washed twice with 1× PBS followed by the addition of 0.1% glutaraldehyde to the cell pellet and incubated for 3 h in the dark. Further, glutaraldehyde was discarded following centrifugation and the pellet was again washed with 1× PBS. Thereafter cells were dehydrated with graded ethanol series. Then the sample was drop-casted on coverslips (12 mm, Blue Star®, Mumbai, India), air-dried, and photomicrographs were captured in a scanning electron microscope (Zeiss EVO LS10).

*4) Cell culture*

The cervical cancer cell line (HeLa) was purchased from the National Centre for Cell Science (NCCS), Pune, India. HeLa cell cultures were maintained in DMEM media supplemented with 10% fetal bovine serum and antibiotic solution (100 U/mL penicillin G and 100 µg/mL streptomycin sulfate) at 37 °C with a constant supply of 5% $CO_2$ in an incubator.

*a) Cell viability assay*

Using the MTT assay, the antiproliferative activity of endophytic extract on HeLa cells was examined (Mosmann, 1983). A 96-well plate was seeded with $1\times10^5$ cells/mL, and the cells were treated with a 2-fold dilution of LS4 extract (0-27 mg/mL) for 24 h. Post-24 h incubation, the media was then discarded and wells were rinsed with 1×PBS. Following treatment, 80 µL of new DMEM media was added to each well along with 20 µL of dissolved MTT (5 mg/mL) in media and placed in the $CO_2$ incubator and left for 4 h. Using a microplate reader (Thermo Scientific, Multiskan ELISA, USA), the absorbance of the solution was determined at 570 nm after the intracellular formazan crystals were dissolved in DMSO. Samples were plated in triplicate for each set of experiments. The cell viability percentage was calculated by the following formula:

$$\text{Cell viability (\%)} = \frac{\text{Mean absorbance of LS4 extract}}{\text{Mean absorbance of untreated control cells}} \times 100$$

*E. Molecular identification of endophytic fungi*

To identify the selected endophytic strain ITS (Internal Transcribed Spacer) sequencing was performed. At first, the fungal DNA was isolated according to the procedure described by Cenis (Fillat, 2016) with few required alterations. Briefly, fungal cells were grown in a 250 mL conical flask containing 100 mL Potato dextrose broth (PDB) at 120 rpm, 28 °C for 5 days. The cell mass was taken in an eppendorf tube and washed with TE buffer (1×, pH 8.0) for the isolation of fungal DNA. Then the cells were centrifuged at 13,000 rpm for 5 min. DNA extraction buffer (200 mM Tris-HCl pH 8.5, 250 mM NaCl, 25 mM EDTA, and 0.5% SDS) was added to the pellet and the cell suspension was sonicated (2 times at 80% amplitude for 30 s) on ice. After completion of mechanical rupturing via sonication, 3 µL proteinase K (20 mg/mL) was added and the tube was incubated at 65 °C for 10 min followed by the addition of sodium acetate (pH 5.2) and further incubation at -20 °C for 10 min. After incubation, tubes were centrifuged at 15,000 rpm and the supernatant was taken to precipitate DNA by adding an equal volume of isopropanol and centrifuged at 15,000 rpm for 10 min. Finally, the DNA pellet was washed with 70% ethanol, air-dried, and dissolved in 50 µL nuclease-free water. Isolated DNA was run in 0.8% agarose gel. The purity and quantification were measured by NanoDrop spectrophotometer (NanoDrop 2000, Thermo Scientific) and stored at 4 °C in nuclease-free water.

After the extraction of fungal DNA, the entire ITS region was PCR amplified by using primer pairs *i.e.* ITS-1 forward primer (5′ TCCGTAGGTGAACCTGCGG 3′) and ITS-4 reverse primer (5′ TCCTCCGCTTATTGATATGC 3′) (Singh, 2020). The amplification cycle was run under the thermocycler (Biorad cycler) with initial denaturation for 1 min at 95 °C, annealing for 45 s at 55.5 °C, and extension for 1 min at 72 °C. The final extension was performed for 1 min at 72 °C. The amplified product was visualized using 1.5% agarose gel stained with ethidium bromide. The PCR amplified product was consigned to SciGenom, Kochi, Kerala for sequencing. The retrieved sequences were compared with other relevant sequences using BLAST search (Henry, 2000) in GenBank (NCBI). Matched sequences were further aligned with ClustalW (Maharana, 2021) of MegaX software (Kumar, 2018) based on the nucleotide homology and analyzed by the Bootstrap model method and the obtained sequence was submitted to GenBank.

*F. Detection of the bioactive compound by GC-MS analysis*

The extract which has shown its efficacy against both the gram-negative and gram-positive pathogenic bacteria as well as in cancer cells was further analyzed using a gas chromatogram coupled with mass spectrophotometry (GC-MS) for identification of its components. The study was conducted in the National Test House, Guwahati, Assam by using Trace Ultra DSQ II gas chromatography-mass spectroscopy (Thermo Fisher

Scientific) fitted with TG 5 MS capillary column (30 mm × 0.25 mm × 0.25 µm). In order to separate the existing component in crude extract 1 µL of the sample (1 mg/mL stock) was injected in the column of gas chromatography and at 280 °C injector temperature was maintained. An electron ionization system was used in the evaluation process. The ion source temperature was sustained at 280 °C. Helium gas was used as carrier gas at a constant flow rate of 1 mL/min. The oven temperature of gas chromatography was maintained initially at 50 °C for 1 min then the temperature was increased by 6 °C/min to 280 °C which was terminated with an isothermal of 5 min at 280 °C. Reading of mass spectroscopy or mass spectra was taken at ionization voltage 70 eV with a scanning interval of 0.2 s and fragment ranges between 50-350 Da. The total running time of GCMS was 28 min. The percentage of different compounds that were present in the sample was detected by calculating the peak size and/or peak area. The Xcalibur software was taken on to figure out mass spectra. The name, molecular weight, and structure of bioactive compounds were ascertained by comparing the mass spectra with data from NIST (National Institute of Standard and Technology, US) libraries.

### G. *ADMET analysis*

Chemical absorption, distribution, metabolism, excretion, and toxicity (ADMET) are crucial factors in the platform of new drug development. Thus, pharmacokinetics and toxicity of the bio-active component from ethyl acetate extract of *A. fumigatus* were predicted by using SwissADME (Daina, 2017) and pkCSM (Lohohola, 2021) online predictor. Briefly, ChemDraw Professional 16.0 was used to creat the corresponding structure of the chemical compound present in crude extract. The compounds were then transformed into the standard SMILES (simple molecular-input line- entry) format. Further, using the SwissADME and pkCSM software for ADMET, Lipinski's infamous Rule of Five drew a connection between physiochemical and pharmacokinetics proterties.

### H. *In-silico molecular docking studies*

The principal mechanism of computer-assisted drug design is the interaction between the receptor and ligand. The list of selected virulent components as the target for both the MRSA and *K. pneumoniae* infections were described with their respective PDB ID in Table 1. The crystal structure of all the targets was downloaded from the Protein Data Bank. The downloaded protein structures act as receptor molecules and bioactive compounds of crude extract *i.e*. C2 and C12 act as ligand molecules for *in-silico* docking studies. The receptor parameterization and grid box specifications were performed using AutoDock Tools (V 1.5.6) (Mothay, 2020). The binding relationship between the active components and the 3D structure of targets was visualized using PyMol and BIOVIA Discovery Studio Visualizer (Magaji, 2022).

Table I. List of the selected enzymes for docking analysis with their PDB ID and references

| Enzyme | PDB ID | Reference |
|---|---|---|
| **Methicillin-resistant *Staphylococcus aureus*** | | |
| Penicillin-binding protein (PBP2A) | 1VQQ | (Skariyachan, 2011) |
| Panton-valentine leukocidin (PVL) | 1T5R | (Skariyachan, 2011) |
| DNA Gyrase | 5CDP | (Collin, 2011) |
| Topoisomerase IV | 2INR | (Collin, 2011) |
| Penicillin-binding protein (PBP2A) | 4CJN | (Santhaseelan, 2022) |
| Penicillin-binding protein (PBP4) | 3HUM | (Santhaseelan, 2022) |
| ***Klebsiella pneumonia*** | | |
| OmpA | 7RJJ | (Abdulrazik, 2022) |
| Carbapenemase KPC-2 | 2OV5 | (Malathi, 2019) |
| KPC-3 | 6QWA | (Malathi, 2019) |
| TEM 72 | 3P98 | (Dhara, 2013) |
| SHV-2 | 1N9B | (Dhara, 2013) |
| CTXM-9 | 1YLJ | (Dhara, 2013) |

### I. *Statistical analysis*

Each experiment was run in triplicate. We used GraphPad Prism 8 for Windows (GraphPad Software, La Jolla, CA, USA) to conduct descriptive statistical analyses. Two-way analysis of variance (ANOVA) was used to examine the statistical significance between the control sets and treatment groups. P values of 0.05 were regarded as significant.

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