JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

ANTIMICROBIAL, ANTIOXIDANT, GC-MS ANALYSIS AND MOLECULAR DOCKING ANALYSIS OF BIOACTIVE COMPOUNDS OF ENDOPHYTE *ASPERGILLUS FLAVUS* FROM *ARGEMONE MEXICANA*

Pooja Singh[1], *Angkita Sharma*[1], *Manobjyoti Bordoloi*[2], *Shoma Paul Nandi*[1*]

Address(es):
[1] Amity Institute of Biotechnology, Amity University, Noida- 201313, Uttar Pradesh, India.
[2] Department of Chemistry, Cotton University, Panbazar, Guwahati- 781001, Assam, India.

*Corresponding author: spaul@amity.edu

https://doi.org/10.55251/jmbfs.9970

ARTICLE INFO

Received 12. 3. 2023
Revised 17. 5. 2023
Accepted 17. 5. 2023
Published 1. 8. 2023

Regular article

OPEN ACCESS

ABSTRACT

Studies of potent antibiotics from plant endophytes have become a new interest among researchers. Plant derived antibiotics are costly, laborious, time-consuming and require extensive area of land. Endophytes can be used as an alternative source of antibiotics. Endophytes are microbes (bacteria and fungi) that live inside healthy plant tissues and do not show any antagonistic symptoms. They produce a variety of secondary metabolites having various commercial properties, medicinal, environmental, and agronomic purposes. In present work, endophytic fungi *Aspergillus flavus* associated with *Argemone mexicana*, was tested for its antimicrobial activity. The secondary metabolites of *A. flavus* were extracted using ethyl acetate solvent and tested for their antibacterial potential against certain Gram-negative bacteria, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Vibrio cholerae*, *Escherichia coli*, and Gram-positive bacteria *Staphylococcus aureus* using Agar well diffusion assay. Thin layer chromatographic bands were also checked further to know the bioactive spot. Gas chromatography- mass spectroscopy (GC-MS) was used to identify the bioactive compounds in TLC fraction and eighteen bioactive compounds were identified. Molecular docking and MM-GBSA were performed for the bioactive compounds with the respective proteins of the *S. aureus* (5JG0, 5JQ6, 1T2W, 3WYI, 3TFP, 3G7B, and 3WQU) and *E. coli* (1AJ0, 1GG4, 1 AJ6, 5ZHE and 7D6G) by using Schrodinger Maestro. *In silico* analysis suggested that Methyl (3-oxo-2-pentylcyclopentyl) acetate (MOPA) and 13-Hexyloxacyclotridec-10-en-2-one (HCT) exhibited the best binding affinity with most of the receptors. Pharmacokinetics and physicochemical analysis also showed that HCT and MOPA are the best drug candidate for the *S. aureus* and *E. coli*.

Keywords: *Argemone mexicana*, antimicrobial activity, bioactive secondary metabolites, *Aspergillus flavus*, Molecular docking, ADMET property, GC-MS

INTRODUCTION

Antimicrobial resistance is a very serious public health concern in the world. The overuse of antibiotics leads to resistance in the bacteria. Antimicrobial resistance may result due to mutation in the genome of microbes therefore antibiotics cannot fight them. Antibiotic resistance in pathogenic strains of microbes (fungi, bacteria, and viruses) is reaching dangerously high levels around the world. New resistance mechanisms are emerging and spreading globally, threatening our ability to treat common infectious diseases. Growing AMR infections are increasing difficulty to treat the patients therefore discovery of new drugs is a global need. Medicinal plants are known for being the source of several lifesaving drugs. The large number of world's population depends on the medicinal plants for curing many diseases. Several medicinal plants belong to *Papaveraceae* family which has 775 species in 42 genera. *Argemone mexicana* also belongs to *Papaveraceae* family known as Mexican prickly poppy. It is indigenous to South America but is found in many tropical and subtropical countries including India. *A. mexicana*'s compounds have ability to cure gastrointestinal disorders as well as possess wound healing activity, cytotoxic activities, anti-inflammatory, anti-stress, anti-allergic activity, fungi toxic, anticancer, antidiabetic activity and many more are to mention a few (**Iqbal *et al*., 2021; Brader *et al*., 2014; More *et al*., 2017**).

In addition to plant's metabolites, plant associated microorganisms contribute significantly to secondary metabolite production and medicinal properties of the plants (**Aly *et al*., 2013; Brahmachari *et al*., 2013; Kusari *et al*., 2013; Gandhi *et al*., 2015**). Endophytes are the bacterial or fungal organisms which live inside a plant without causing any harm or damage to the plant itself (**Hardoim *et al*., 2008, 2015; Bhattacharjee *et al*., 2010; Ludwig-Muller *et al*., 2014; Chowdhary and Kaushik, 2015**). Symbiotic relationship between plant and endophytes varies from mandatory to obligatory/ facultative (**Lorena *et al*., 2021**). Mandatory endophytes are those who spend their whole life inside the plant and obligatory/ facultative endophytes can survive outside the plant. Some bacterial strains like *Bacillus* sp. and *Pseudomonas* sp. promote induced systemic resistance in plants against pathogens whereas the fungal endophytes inhibit pathogenic growth by production of several metabolic compounds like alkaloids, flavonoids, phenols *etc*. (**Nie *et al*., 2017**). In our previous study, consortium of endophytes (bacteria and fungi) triggered Induced Systemic Resistance in the *A. mexicana* as well as model plant *Arabidopsis thaliana* (**Singh *et al*., 2021**). Endophyte provides help for cellular defense responses, oxidative brust, defense-related enzymes formation, and required secondary metabolites production in host plant (**Rania *et al*., 2016; Ludwig-Muller, 2015; Rahman *et al*., 2014; Ahn *et al*., 2007; Wojtaszek, 1997**). Studying novel metabolites and novel strains may help in various fields of agriculture and pharmacology.

Typhoid is one of the major health problems in the developing world. It spreads rapidly within the population, where there is lack of sanitation and pure water supply (**Wain *et al*., 2015**). It spreads from the intestine through blood to the intestinal lymph nodes, liver, and spleen where the number of bacterial colonies increases. **Majowicz *et al*., (2010**) reported that the disease's morbidness and mortality possibly affect over 90 million people worldwide each year. Typhoid fever is caused by *Salmonella typhimurium* in humans (**Mweu and English, 2008**). The *S. typhimurium* is non-capsulated, Gram-negative, anaerobic bacilli and facultative intracellular human pathogen. The only effective treatment for typhoid is antibiotics. However, *S. typhimurium* bacteria have become resistance to various antibiotics by altering its characteristics and are becoming hard to analyze and treat Typhoid fever (**Dahiya *et al*., 2014; Kariuki *et al*., 2015**).

Klebsiella pneumoniae is an encapsulated, Gram-negative, rod- shaped, aerobic, and non-motile bacterium. It causes pneumonia in humans. Lack of treatment can make the disease dangerous and chronic. *K. pneumoniae* infects respiratory system of human. It colonizes on mucosal surfaces of gastrointestinal tract and oropharynx. It can spread from person to person through breathing and blood contact. After entering the human body, it shows highly virulent because of its AMR property (**Jiang *et al*., 2020**). *K. pneumoniae* can acquire antibiotic resistance elements that are responsible for making various β-lactamases and efflux pumps (**Jiang *et al*., 2020**). Multidrug resistant *K. pneumoniae* shows resistance to colistin antibiotic by mutational inactivation of *mgrB* regulatory gene (**Kidd *et al*., 2017**).

Vibrio cholerae are Gram-negative, comma-shaped, motile, and aerobic bacterium. *V. cholerae* cause cholera disease, leading to mild fever, vomiting, watery stool, and serious diarrhea. *V. cholerae* generally grows in the small intestine. It is a waterborne bacterium that can spread by poor sanitization. Oral rehydration therapy, intravenous rehydration, antibiotics, and good hygiene are used for the treatment of pneumonia disease. Almost 200 years ago, pneumonia became

pandemic due to poor hygiene (**Sharma *et al*., 2021**). *V. cholerae* also shows antimicrobial resistance to the existing antibiotics by facilitating one of the following three mechanisms, reducing the permeability or active efflux of antibiotics, changing the antibiotic targets by introducing post-transcriptional or post-translational modifications, or hydrolyzing or chemically altering antibiotics (**Das *et al*., 2020**).

Escherichia coli are Gram-negative, rod- shaped and facultative anaerobic bacterium. Most of the *E. coli* strains are harmless but some strains are very dangerous for humans and warm-blooded animals. *E. coli* can be found in food, water and intestine of people and animals. It causes dehydration, vomiting, severe diarrhea, and abdominal cramps. Due to lack of treatment, it may cause complications such as, anemia, severe dehydration, kidney failure and death. Its treatment is like that of *V. cholerae*, which includes oral and intravenous rehydration, antibiotics and consumption of clean food and water.

Staphylococcus aureus is Gram-positive, round- shaped, beta-lactamase positive and facultative anaerobic bacterium. It can cause a wide variety of disease related to blood (sepsis), skin (staphylococcal scalded skin syndrome, cellulitis, impetigo, furuncles, and abscesses), respiratory system (pneumonia), heart (endocarditis), stomach (food poisoning) and bones (osteomyelitis) (**Ghalehnoo *et al*., 2018**). The emergence of MRSA (Methicillin-resistant *Staphylococcus aureus*) takes place when the native *S. aureus* strain gain MecA gene and becomes resistant to beta-lactam antibiotics. In the past years, there has been a significant use of drugs. That has led to significant evolution in bacteria. That evolution has caused the resistance of *S. aureus* to increase drastically making its curability and deciding the line of treatment more difficult.

About 180 filamentous fungal species make up the diverse genus *Aspergillus*, which is well known for producing a variety of secondary metabolites with therapeutic uses, such as anticancer and antimicrobial properties (**Youssef and Singab, 2021**). *Aspergillus flavus*, a saprophytic fungus, may produce metabolites with a wide range of biological activities, including antimicrobial activity against *Streptococcus gordonii, Bacillus subtilis, S. aureus, E. coli, Salmonella enteric, Pseudomonas aeruginosa, P. florescens and Candida albicans* (**Dudeja *et al*., 2021**). Many antimicrobial compounds have already been reported from fungi belonging to *Aspergillus* species (**Youssef and Singab, 2021**). *A. flavus* fungal endophyte was isolated from the *A. mexicana* plant in our previous study (**Singh *et al*., 2020**). In a current study, we checked antibacterial activity of ethyl acetate (EA) extract of it. We also examined antibacterial properties of the bioactive compounds.

In view of such a problem of emerging antimicrobial resistance, curing of disease becomes a priority for public health. In present study, we aim to focus on *A. flavus* fungal endophyte associated with wild *A. mexicana* plants occurring in natural habitat of northeast regions of India (**Singh *et al*., 2020**) and their antibacterial potential against Gram-positive and Gram-negative bacteria. In addition, bioactive compounds were purified using thin layer chromatography (TLC) and identified by gas chromatography – mass spectroscopy (GC-MS). Obtained bioactive compounds were further tested for Molecular docking and MM-GBSA analysis by using Schrodinger Maestro. Physicochemical and Pharmacokinetics study of these bioactive compounds was performed at pkCSM web server.

MATERIALS AND METHODS

Plant sample collection

Endophyte *A. flavus* associated with *A. mexicana* plants were collected from Assam, India, were selected for the antimicrobial study. Isolation and identification of endophytes were published in our previous study (**Singh *et al*., 2020**).

Secondary metabolites extraction

The endophytic fungi were grown in Potato dextrose broth (PDB) at 28 ± 2°C and 120 rpm for 2 weeks. The fungus was filtered through Whatman filter paper and filtrate was used for extraction of secondary metabolites. An equal volume of EA was added to filtrate and left on shaker for 1 day. EA extracts were separated using separatory funnel and dried using Rota evaporator (Buchi, Rotavapor R210, Switzerland). Different dilutions were prepared by using dried bacterial extracts in EA solvent. The fungal extract was further tested for their antibacterial activity using agar well diffusion method.

Antibacterial potential

The antibacterial activities of *A. flavus* were performed against human pathogenic bacteria; Gram negative bacteria, *Salmonella typhimurium* (ATCC14028), *Klebsiella pneumoniae* (AIIMS-5), *Vibrio cholerae* (P5), *Escherichia coli* (MTCC729), and Gram positive bacteria *Staphylococcus aureus* (MTCC96). The actively growing pathogenic bacteria culture was spread evenly on the nutrient media and cups were made by using sterilized cork borer (diameter 8 mm). The 100 µL of EA extract were filled in agar cup. Similarly, fungal endophytes were also tested, and zone of inhibition (ZOI) was measured.

Thin layer chromatographic separation of bioactive compounds

The EA fractions of the fungal isolates were loaded onto pre-coated thin layer chromatography (TLC) plate (MERCK TLC Silica gel 60 F_{254}) for separation of secondary metabolites. The 25µl of EA extracts (20mg/ml) of fungus was loaded on pre-coated TLC plates. Toluene: chloroform: acetone (45:25:35) was used as running solvent. For the visualization of bioactive spots, TLC strip was seen under Visible and UV light as well as iodine fumigation was performed, and retention factor (Rf) were calculated. Rf values were calculated by the formula (Rf = Distance of TLC band from extract loading point (cm)/Distance travelled by the solvent front from extract loading point (cm). Each bioactive fraction was scrapped off from TLC plate and used in bioassay to check the antimicrobial activity. Preparatory TLC slides were used for purification of bioactive compounds required for GCMS analysis.

Minimum inhibitory concentration

Minimum inhibitory concentration (MIC) was tested by standard protocol (**Gamal *et al*., 2021**). Four concentrations (10mg/ml, 5mg/ml, 1mg/ml and 0.5mg/ml) of TLC fraction B were prepared for MIC test. 100µl of EA extract was filled in the wells and incubated at 37°C for 24 hours. ZOI of extract was recorded after incubation.

Antioxidant activity

The antioxidant activity of the TLC fraction B of EA extract was determined by using 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay (Brand-Williams et al. 1995). The 0.5ml of TLC fraction B (10mg/ml) was added with 0.5ml of 0.3mM DPPH reagent and left for 30 min at room temperature. The absorbance of the reaction mixture was taken at 517 nm. The Ascorbic acid was taken as positive control. The results are concluded as free radical scavenging activity in percentage (**Yadav *et al*., 2014**). Formula is used:

$$\text{DPPH radical scavenging (\%)} = \left(\frac{\text{Abs (control)} - \text{Abs (test)}}{\text{Abs (control)}}\right) \times 100$$

Statistical analysis

The experiments were done in three replicates and statistically analyzed for standard error.

GC-MS analysis

TLC fraction- B was chosen for GC-MS analysis on the basis of its antimicrobial activity. It gave the maximum ZOI against the pathogenic bacteria. GC-MS analysis was performed on GCMS-QP2010 Ultra (SHIMADZU Serial no. 0205251) at Advanced Instrumentation Research Facility (AIRF), Jawaharlal Nehru University (JNU), New Delhi. GC-MS system contained auto sampler (AOC-20_{i+S}) with gas chromatography and mass spectrophotometer. The size of the column Rtx-5Sil-MS of the system was (length 30.0 m × diameter 0.25 mm, thickness 0.25 µm). Helium gas (99.99%) was employed as carrier gas in GC-MS system. Two µl of the sample was used to analyze the compounds in the system. The temperature of the injector was programmed at 80°C and temperature of ion-source was maintained at 220°C. The column oven temperature was maintained initially from 80 °C to final 280°C. The total running time for the sample was 40 min. Pressure of the column was programmed at 81.9kPa. The GC-MS analysis provides chemical name, molecular weight, structures, and retention time of the compounds. Evaluation of the mass spectra of the compounds was conducted by using the database of WILLEY8 and National Institute Standard and Technology (NIST14s).

Molecular Docking study and MM-GBSA

The *in- silico* study of obtained chemical compounds was carried out by using Schrodinger Maestro, including protein preparation (protein preparation wizard), ligand preparation (LigPrep), site mapping (sitemap), grid generation, and receptor-ligand docking (GlideXP docking). The Centos Linux operating system was used for computational study.

The bioactive compounds (ligands) were obtained from GC-MS analysis of the bioactive TLC fraction B and downloaded from the NCBI (http://www.ncbi.nlm.nih.gov/pccompound). All ligands were prepared using LigPrep, which can produce low energy isomer of the ligand in optimization by using the OPLS_2005 force field. The ligand was prepared by adding hydrogen atoms, removing unwanted molecules, generating ionization states at pH7, tautomers, geometric characteristics, and low-energy ring conformations. The X-ray crystal structures of bacterial proteins (listed in table 5) were retrieved from the Protein Data Bank (PDB). Protein preparation wizard has three steps, preprocess, optimization, and minimization. During the preprocessing step, water molecules were deleted, and hydrogens were added from the protein. Active sites of the protein were analyzed by using Site Mapping**.** The OPLS_2005 force field was

used for generating Grid on protein receptors. During grid generation, Van der Waals radii of protein (1.00 Å) were adjusted with a 0.25 atomic charge. The grid generation process was provided square block at the active site of the protein for the accurate binding score with thermodynamic optimal energy. Protein- grid out file was used for the molecular docking study. Schrodinger 2019-2 version was used to predict the binding affinity, ligand competence, and inhibitory candidate to the protein by performing molecular docking. Molecular docking was conducted by using the Glide XP (extra precision) module. The optimal ligand selection for the receptor was done based on the docking score. Binding energy of the docked protein- ligand interaction was predicted by using MM-GBSA tool.

ADMET properties prediction

ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) properties of the bioactive compounds with highly negative docking scored against various *S. aureus* and *E. coli* proteins were analyzed by using pkCSM web server. Physicochemical (Lipinski's rules) and Pharmacokinetics (ADMET) properties of most active compounds were performed to know their drug like properties (table 9).

RESULTS

Endophytic fungus *A. flavus* (accession no MT322245) was used for the antimicrobial study. The EA extracts of endophytic fungus *A. flavus* isolated from *A. mexicana* have showed the antimicrobial activity against water and foodborne human pathogens. Agar cup diffusion assay of fungal EA extracts were performed and showed ZOI against all the pathogens (Table 1, Fig S1).

Table 1 Antimicrobial activity of ethyl acetate extract of *A. flavus* isolated from *A. mexicana*

EA extract	*E. coli*	*S. typhimurium*	*V. cholerae*	*K. pneumoniae*	*S. aureus*
10mg/ml	17±1	16±2	18±2	15±3	17±2
5mg/ml	15±2	16±2	16±1	14±2	15±2
1mg/ml	14±2	13±2	15±1	12±1	0
0.5mg/ml	12±2	13±2	14±2	12±3	0
Ampicillin (10mg/ml)	22±2	20±1	20±2	21±3	21±2

TLC Analysis

The TLC study of the EA extract of fungal endophyte revealed various bands of bioactive compounds after iodine fumigation of the TLC plate (Fig 1). The appearance of a huge range of bands is an indication that there are several bioactive metabolites produced by the fungal strain *A. flavus*. All the spots had substantial potential to inhibit the growth of the pathogenic bacteria. TLC fraction A (Rf value 0.16) showed maximum ZOI against the pathogenic bacteria *S. aureus* (Fig S2, table 2). TLC fraction C (Rf value 0.45) did not show antimicrobial activity against *E. coli* and *K. pneumonia*. TLC fraction D and E (Rf values 0.625 and 0.88, respectively) did not show any ZOI against *K. pneumonia* and showed minimum ZOI on *S. aureus* agar plate. The TLC fraction B (Rf values 0.29) was selected for further studies because it showed inhibition activity against all the bacteria except *E. coli*.

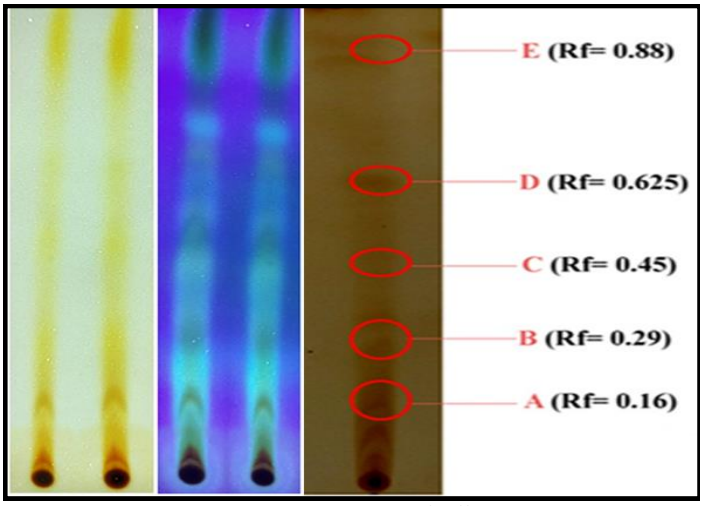

Figure 1 TLC analysis showed the occurrence of different bioactive metabolites of the EA extract of fungus run in toulene: chloroform: acetone (45:25:35) running solvent system. TLC plates were visualized under Visible light (white), Ultraviolet light (blue) and iodine fumigation (brown).

Table 2 Antimicrobial activity of the TLC fractions of *A. flavus's* ethyl acetate extract

Fractions / Pathogens	Fraction A	Fraction B	Fraction C	Fraction D	Fraction E
S. typhimurium	10±1	13±2	14±2	12±1	12±2
K. pneumonia	14±2	11±3	-	-	-
E. coli	-	-	-	09±1	09±2
S. aureus	15±3	14±2	17±2	20±2	20±2
V. cholerae	-	10±2	12±2	14±2	-

Minimum inhibition concentration (MIC)

The MIC was calculated as the lowest concentration of antimicrobial compounds that inhibit the growth of pathogenic microbes. The MIC of the TLC fraction B was tested by using agar well diffusion assay and ZOI were taken for the record. The MIC values of EA extracts were against *S. typhimurium* (5mg/ml), *K. pneumoniae* (1mg/ml), *V. cholerae* (0.05mg/ml), *E. coli* (1mg/ml) and *S. aureus* (0.01mg/ml).

Antioxidant (DPPH) assay

The free radical scavenging capacities of the TLC fraction B of the endophytic fungus *A. flavus* was estimated by using DPPH method. DPPH assay is the simple method to estimate antioxidant activity of the compounds. Antioxidant compounds reduce the absorbance of DPPH radical at 517nm. TLC fraction B changed the purple color of DPPH radical to the yellow colored diphenylpicrylhydrazicine. The IC_{50} values of the TLC fraction B was 22.9 ± 0.2 µg/ml, which is significantly different from ascorbic acid (13.7 ± 0.2 µg/ml) at $P < 0.05$.

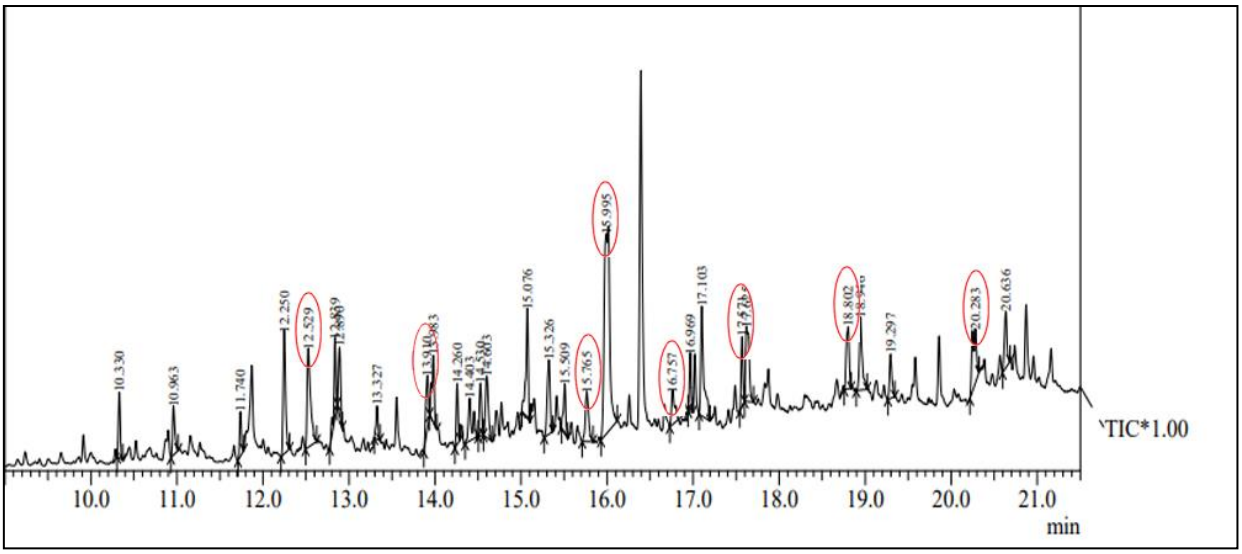

Figure 2 GC-MS analysis of the TLC fraction B of ethyl acetate extract of *A. flavus.* Red circles show the bioactive compounds having good docking score with the various proteins of *S. aureus* and *E. coli*

Table 3 GC-MS analysis of bioactive compounds in the TLC fraction B of ethyl acetate extract of *A. flavus* endophyte

PubChem CID	Name of the compounds	Retention Time	Molecular Formula	Molecular weight	Area %
11006	Hexadecane	10.330	$C_{16}H_{34}$	226.44	3.22
102861	Methyl (3-oxo-2-pentylcyclopentyl) acetate	12.529	$C_{13}H_{22}O_3$	226.31	2.77
12523	Hexadecane, 2,6,10,14-tetramethyl-	12.839	$C_{20}H_{42}$	282.5	2.77
527459	2-Methyltetracosane	12.890	$C_{25}H_{52}$	352.7	2.47
107166	Naphtho[2,1-b] furan, dodecahydro-3a,6,6,9a-tetramethyl-	13.910	$C_{16}H_{28}O$	236.39	2.47
12401	Nonadecane	13.983	$C_{19}H_{40}$	268.5	2.90
8042	Isopropyl myristate	14.260	$C_{17}H_{34}O$	270.5	1.85
5365759	E-11(12-Cyclopropyl) dodecen-1-ol	14.403	$C_{15}H_{28}O$	224.38	2.64
8222	Eicosane	15.076	$C_{20}H_{42}$	282.5	14.40
8181	Hexadecanoic acid, methyl ester	15.326	$C_{17}H_{34}O_2$	270.5	3.70
3026	1,2-benzenedicarboxylic acid, dibutyl ester	15.765	$C_{16}H_{22}O_4$	278.34	2.81
13849	Pentadecanoic acid	15.995	$C_{15}H_{30}O_2$	242	19.21
6536948	13-Hexyloxacyclotridec-10-en-2-one	16.757	$C_{18}H_{32}O_2$	280.4	1.27
5284421	9,12-Octadecadienoic acid (Z, Z)-, methyl ester	16.969	$C_{19}H_{34}O_2$	294.5	1.73
18432395	trans, trans-9,12-Octadecadienoic acid, propyl ester	17.571	$C_{21}H_{38}O_2$	322.5	2.14
537300	Erythro-9,10-dibromopentacosane	18.802	$C_{25}H_{50}Br_2$	510.5	3.12
23494	Tetratetracontane	18.946	$C_{44}H_{90}$	619.2	3.62
9817754	9,12-Octadecadienoyl chloride, (Z, Z)-	20.283	$C_{18}H_{31}ClO$	298.9	4.17

Table 4 Pharmacological activities of identified bioactive compounds of TLC fraction B of ethyl acetate extract of *Aspergillus flavus*

Name of compounds	Pharmalogical activity
Dibutyl phthalate	Antimicrobial, antifungal
Tetratetracontane	Antibacterial, Antifungal
Isopropyl myristate	Pediculicide for lice
9,12-octadecadienoic acid (z,z)-, methyl ester	Analgesic, anti-inflammatory, ulcerogenic, anti-melanogenic effect (skin whitening effect)
Hexadecanoic acid, methyl ester	Antimicrobial, Antioxidant, anti-inflammatory, decrease blood cholesterol
Pentadecanoic acid	Antioxidant activity
Naphtho[2,1-b] furan, dodecahydro-3a,6,6,9a-tetramethyl-	Antimicrobial activity
Eicosane	Antifungal
13-Hexyloxacyclotridec-10-en-2-one	Antimicrobial activity
9,12-Octadecadienoyl chloride, (Z, Z)-	Antibacterial, anti-tuberculosis, Anti-dengue-2 virus, anticancerous, antioxidant and thyroid inhibitor, anti-diabetic
Hexadecane	Cytotoxicity, Antimicrobial, Antioxidant, Antipyretic, Anthelmentic, Tumour,Bronchitis, Asthma, Tuberculosis, Dyspepsia, Constipation, Anemia, Throat diseases,Elephantiasis, Antidiabetic, Anti-inflammatory, Antidiarrhoeal
Nonadecane	Anti-HIV, Antioxidant, Antibacterial, Antimicrobial,Cytotoxic effect, Antimicrobial Antimalarial, Unini uses likeweakness of the principal organs like heart, Brain, liver, General weakness, Haemoptysis, Palpitation, Conjunctivitis, Earache, Stomatitis

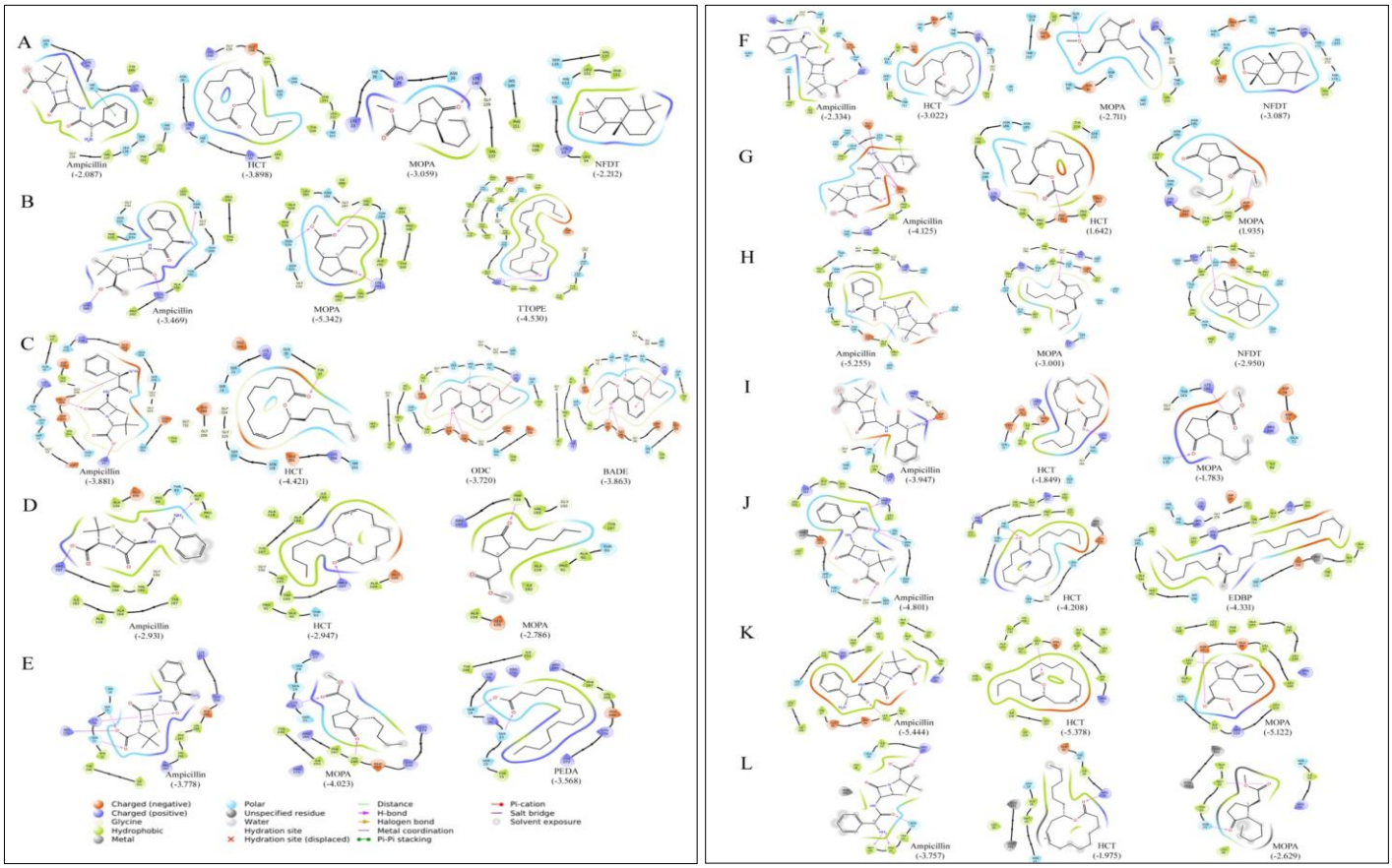

Figure 3 2D diagrams of protein and ligands interaction with docking score. A. 5JGO, B. 5JQ6, C. 3WQU, D. 1T2W, E. 3TFP, F. 3G7B, G. 3WYI, H. 1AJ0, I. 1AJ6, J. 1GG4, K. 5ZHE, 7D6G

Bioactive compound profiling by GC-MS

GC–MS chromatogram of TLC fraction B showed various peaks indicating the presence of bioactive compounds (Fig 2). Bioactive compounds were identified and characterized by comparison of mass spectra of fractions with Wiley and NIST library. Some bioactive compounds are reported for their biological activities (Table 4). Total 18 compounds were detected in fraction B of *A. flavus* endophyte and 13 compounds were found with medicinal properties, such as Naphtho[2,1-b]furan, dodecahydro-3a,6,6,9a-tetramethyl- (2.47%), 9,12-Octadecadienoyl chloride, (Z,Z)- (4.17%), Eicosane (14.40%), Pentadecanoic acid (19.21%), Hexadecanoic acid, methyl ester (3.70%), Nonadecane (2.90%), Phenol 2,4-bis(1,1-dimethylethyl)- (1.93%), 9,12-octadecadienoic acid (z,z)-, methyl ester (1.73%), Tetratetracontane (3.62%), Isopropyl myristate (1.85%) and 1,2-benzenedicarboxylic acid, dibutyl ester (2.81%) were known for various biological properties (Table 3-4).

Molecular docking and MM-GBSA analysis of bioactive compounds

Molecular docking studies give important information regarding the orientation of the ligands or inhibitors in the active site of the target proteins. Molecular docking is performed to predict the potential inhibitors of the target proteins. Docking studies provide important information about the orientation of the inhibitor in the target protein binding pocket. Eighteen bioactive compounds were identified by the GC-MS analysis. These compounds were used for the molecular docking and MMGBSA analysis with *S. aureus* (5JG0, 5JQ6, 3G7B, 3WYI, 1T2W, 3TFP and 3WQU) and *E. coli* (7D6G, 1AJ6, 5ZHE, 1AJ0 and IGG4) proteins (table 6, S1). Ampicillin antibiotic was used as positive control (standard ligand) for the protein- ligand docking study. Eleven secondary metabolites out of eighteen are reported for their biological activity. Methyl (3-oxo-2-pentylcyclopentyl) acetate (MOPA) and 13-Hexyloxacyclotridec-10-en-2-one (HCT) showed a highly negative glide score against *S. aureus* and *E. coli* proteins. HCT gave better glide score against the *S. aureus* proteins (5JG0, 1T2W, 3G7B, 3WQU) and *E. coli* proteins (1GG4, 5ZHE, 1AJ6, 7D6G) (table 8). HCT compound showed a better glide score than ampicillin against 5jg0 (-3.898), 1t2w (-2.947), 3g7b (-3.022), 3wqu (-4.421) with binding energy -63.43, -52.98, -55.11, and -46.9 kcal/mol, respectively. HCT gave good glide score -5.378 and -4.208 against *E. coli* proteins 5ZHE and 1GG4, respectively but less than ampicillin antibiotic. HCT gave a good glide score. Similar results were achieved by the MOPA. MOPA and HCT showed good binding score against 5ZHE protein with -5.122 and, respectively. MOPA showed good binding activity against *S. aureus* proteins 5jg0 (-3.059), 5jq6 (-5.342), 3tfp (-4.023), 3g7b (-2.711) with binding energy -37.03, -53.81, -21.31 and -41.81 kcal/mol. Other than MOPA and HCT, Naphtho[2,1-b]furan, dodecahydro-3a,6,6,9a-tetramethyl (NFDT), trans,trans-9,12-Octadecadienoic acid, propyl ester (TTOPE), Pentadecanoic acid (PEDA), 9,12-Octadecadienoyl chloride, (Z,Z)- (ODC), 1,2-benzenedicarboxylic acid, dibutyl ester (BADE) were showed excellent binding score against *S. aureus* and *E. coli* proteins (table 6, Fig 3).

Table 5 Target proteins of *S. aureus* and *E. coli* were used *in silico* study and their roles in the pathogenicity and bacterial proliferation

PDB ID	Resolution of Proteins	Organism's Name	Name of Protein	Functions
5JG0	1.88 Å	*Staphylococcus aureus*	Dihydrofolate Reductase (DHFR)	- Synthesis of purines, thymidylate, methionine etc - Synthesis of RNA, DNA and proteins - Cell proliferation
5JQ6	2.40 Å	*S. aureus*	Surface Protein Clumping Factor A (ClfA)	- Virulence factor - Binds with blood plasma glycoprotein fibrinogen - Facilitate the infection
3WYI	2.00 Å	*S. aureus*	Undecaprenyl diphosphate synthase (UPPS)	- Cell wall synthesis - Formation of peptidoglycans - Not present in human
1T2W	1.80 Å	*S. aureus*	Sortase A (SrtA)	- Extracellular transpeptidase enzymes - Found in Gram positive bacteria - Catalyses cell wall sorting reaction for surface proteins
3TFP	2.00 Å	*S. aureus*	Dehydrosqualene Synthase	- Carotenoid pigment staphyloxanthin synthesis - Virulence factor
3WQU	2.80 Å	*S. aureus*	Filamentous temperature sensitive A (FtsA)	- Cell division protein
3G7B	2.30 Å	*S. aureus*	Gyrase B	- Unwinding double stranded DNA - Belongs to type II topoisomerase
1AJ0	2.00 Å	*Escherichia coli*	Dihydropteroate synthase (DHPS)	- Synthesis dihydropteroate - Biosynthesis of Pyrimidines, purines and amino acids, - Cell proliferation - Virulence factor
1AJ6	2.30 Å	*E. coli*	Gyrase B	- Unwinding double stranded DNA - Belongs to type II topoisomerase
7D6G	1.65 Å	*E. coli*	DHFR	- Synthesis of purines, thymidylate, methionine etc - Synthesis of RNA, DNA and proteins - Cell proliferation
5ZHE	2.18 Å	*E. coli*	UPPS	- Cell wall synthesis - Formation of peptidoglycans - Not present in human
1GG4	2.30 Å	*E. coli*	MurF	- Cell wall synthesis

Table 6 Molecular docking and MM-GBSA results of *S. aureus* and *E. coli* proteins with bioactive compounds of *A. flavus* endophyte

Protein-Ligand Complex	**Docking score (kcal/mol)**	**Binding Energy (kcal/mol)**	**Amino acids associated in hydrogen bonds**	**Amino acids associated with hydrophobic interactions**
Staphylococcus aureus				
5JG0_AMP	-2.087	-43.08	-	ASN26, LYS29, HIE30, TYR109, LYS33, LEU34, HIE153, LEU152, PHE151, SER135, SER136, VAL137, GLY139
5JG0_HCT	-3.898	-63.43	-	ASN26, LYS29, HIE30, LYS33, LEU34, TYR109, SER135, VAL137, GLU138, GLY139, LYS140, HIS149, PHE151, LEU152, HIE153
5JG0_MOPA	-3.059	-37.03	LYS140	ASN26, LYS29, HIE30, LYS33, VAL137, GLY139, LYS140, HIS149, PHE151,
5JG0_NFDT	-2.122	-38.29	-	HIE30, LYS33, LEU34, TYR109, SER135, VAL137, PHE151, LEU152, HIE153
5JQ6_AMP	-3.469	-15.04	ASN286, LYS293, LYS389	LEU285, GLY287, THR289, THR291, ALA292, PRO295, PRO336, TYR338, LYS389, PHE529, ASN530, ASN531, GLY532
5JQ6_MOPA	-5.342	-53.81	VAL288, LYS293, ASN530	LEU285, ASN286, GLY287, THR289, ALA292, VAL294, PRO295, ILE309, MET335, PRO336, TYR338, ALA528, PHE529, ASN531, GLY532
5JQ6_TTOPE	-4.53	-76.48	LYS293	PRO251, HIE252, ALA254, GLY255, TYR256, ASN284, LEU285, ASN286, GLY287, VAL288, THR289, ALA292, VAL294, PRO295, MET335,

				PRO336, TYR338, ILE339, ASP340, PRO341, TYR369, ASP385, ALA528, PHE529, ASN530, ASN531, GLY532
1T2W_AMP	-2.931	-36.74	ALA92, ARG197	PRO91, THR93, PRO94, ALA104, GLU105, ALA118, ILE182, ALA184, TYR187, GLY192, VAL193, TRP194
1T2W_HCT	-2.947	-52.98	ARG197	PRO91, ALA92, THR93, ALA104, GLU105, ALA118, ILE182, ALA184, TYR187, GLY192, VAL193, TRP194
1T2W_MOPA	-2.786	-49.48	TRP194	PRO91, ALA92, THR93, ALA104, GLU105, ALA118, ILE182, TYR187, GLY192, VAL193, ARG197
3WYI_AMP	-4.125	-14.21	GLU181, ASN185, ASP195(4), TYR218	ASN186, THR190, LYS191, TYR193, PRO194, TRP214, GLN215, SER217
3WYI_HCT	-1.642	-29.85	ASP195	ASN185, ASN186, LEU188, THR190, LYS191, TYR193, PRO194, PRO196, GLU197, TYR218, SER219
3WYI_MOPA	-1.935	-16.09	ASP195	ASN185, ASN186, LEU188, THR190, LYS191, ASP192, TYR193, PRO194
3TFP_AMP	-3.778	-19.12	SER21, LYS20, SER19, ARG171	LYS17, PHE22, TYR248, ILE251, ARG265, VAL266, PHE267, VAL268, GLU269, LYS270, LYS273
3TFP_MOPA	-4.023	-21.13	LYS20, VAL268	LYS17, HIS18, SER19, SER21, TYR248, ILE251, ARG171, ARG265, PHE267, GLU269, LYS270, LYS273
3TFP_PEDA	-3.568	-20.31	SER19, SER21	LYS16, LYS20, SER23, TYR24, TYR248, ILE251, ARG171, ARG265, PHE267, VAL268, GLU269, LYS270, LYS273
3G7B_AMP	-2.334	-14.29	Lys78	GLN66, THR80, ASH81, ASN82, TYR141, HIS143, ILE148, LYS170, THR171, GLY172, THR173, VAL174
3G7B_HCT	-3.022	-55.11	-	GLN66, ILE67, GLU68, THR80, ASH81, ASN82, HIS143, THR168, LYS170, THR171, GLY172, THR173, VAL174, GLN210, ILE211, THR212
3G7B_MOPA	-2.711	-41.81	GLN66	ILE67, GLU68, THR80, ASH81, ASN82, HIS143, LYS170, THR171, GLY172, THR173, VAL174, GLN210, THR212
3G7B_NFDT	-3.087	-34.76	-	GLN66, ILE67, GLU68, THR80, ASH81, ASN82, HIS143, THR168, LYS170, THR171, GLY172, THR173, VAL174
3WQU_AMP	-3.881	-3.45	LYS77, GLU209(2)	ASN10, GLY12, SER13, SER14, SER15, LYS17, GLN35, TYR37, TYR189, ASP206, ILE207, GLY208, GLU209, ASP210, VAL211, GLN213, GLU251, LYS254, HIE255, GLY324, GLY325, SER326, ASN328, SER361, GLU358
3WQU_HCT	-4.421	-46.9	TYR37	SER14, SER15, LYS17, GLN35, GLY208, GLU209, GLY232, GLU251, LYS254, HIE255, GLY324, GLY325, SER326, ASN328, GLU358
3WQU_ODC	-3.720	-72.91	SER14	GLY12, SER13, SER15, LYS17, GLN35, TYR37, GLY208, GLU209, ASP210, VAL211, LYS254, HIE255, GLY258, GLY325, SER326, ASN328, LEU329, LYS356, GLU358
3WQU_BADE	-3.863	-66.07	SER14, LYS17, GLU209, ASP210	ILE11, GLY12, SER13, SER15, GLN35, TYR37, ILE41, GLY44, LYS77, PRO79, ILE81, MET168, TYR189, ASP206, GLY208, VAL211, GLN213, GLY324, GLY325, SER326, GLU358, SER361
Escherichia coli				
1AJ0_AMP	-5.255	-19.11	GLN149, ALA151, GLN226	ASN193, LYS192, GLY191, PHE190, GLY189, THR62, PRO64, MET141, ASN144, PRO145, THR147, MET148, GLU150, PRO152, SER222
1AJ0_MOPA	-3.001	-23.85	GLY191	PRO64, MET141, GLN142, GLY143, ASN144, PRO145, THR147, GLN149, GLU150, ALA151, GLY189, PHE190, LYS192, ASN193, ASN197, SER222, LYS221, GLN226
1AJ0_NFDT	-2.95	-21.65	GLY191	PRO64, GLN142, GLY143, ASN144, PRO145, THR147, GLN149, GLU150, ALA151, PRO152, GLY189, PHE190, LYS192, ASN193, LEU194, SER222, GLN226
7D6G_AMP	-3.757	-26.83	MET20, PRO21, ASN23, ARG52	TRP22, LEU28, SER49, ILE50
7D6G_HCT	-1.975	-34.9	ARG52	ASN18, ALA19, MET20, ASN23, LEU28, GLU48, SER49, ILE50
7D6G_MOPA	-2.629	-39.67	MET20, ASN23	ALA19, TRP22, LEU28, SER49, ILE50, ARG52
1GG4_AMP	-4.801	-47.07	GLY110, HIE281, ASN282, ARG316	SER109, THR112, SER113, GLU116, MSE117, LEU277, ASN285, LEU310, LYS311, ALA312, VAL313, LEU317
1GG4_HCT	-4.208	-60.44	HIE281, ASN282	SER109, GLY110, SER113, GLU116, MSE117, LEU277, PRO278, ASN285, LEU310, LYS311, ALA312, VAL313, ARG316, LEU317
1GG4_EDBP	-4.331	-95.33	-	SER113, GLU116, MSE117, ALA119, ALA120, TYR130, LEU277, GLY279, ARG280, HIE281, ASN282, LEU310, LYS311, ALA312, VAL313, ARG316, LEU317, ASP331, SER339, ALA342, ALA343, GLN345, VAL346
5ZHE_AMP	-5.444	-60.21	LEU93	MET25, ALA47, VAL50, ARG51, LEU67, ALA69, ALA92, ASH94, GLU96, VAL97, LEU107, ILE109, LEU120, ARG123, ILE124, SER127
5ZHE_HCT	-5.378	-67.21	LEU93	MET25, ALA47, VAL50, ARG51, LEU67, ALA69, ALA92, ASH94, LEU107, ILE109, PHE116, LEU120, ILE124, ILE141, ALA142, ALA143
5ZHE_MOPA	-5.122	-66.36	LEU93, ASH94	ARG51, ALA92, GLU96, VAL97, LEU100, LEU107, ILE109, PHE116, LEU120, ARG123, ILE124, SER127, LEU139, ILE141, ALA143
1AJ6_AMP	-3.947	-34.51	HIE55, ASP74	GLY54, CYC56, LYS57, GLY75, ARG76, THR163
1AJ6_HCT	-1.849	-33.29	LYS162	LYS57, GLU58, ILE59, ILE60, GLN72, ASP73, ASP74, GLN135, THR163, GLY164
1AJ6_MOPA	-1.783	-33.89	GLN135, LYS162	ILE60, GLN72, ASP73, ASP74, THR163, GLY164, ARG206

This table shows only those protein-ligand interactions, which have the highest docking score for each protein. Whole results of molecular docking and MM-GBSA are given in supplementary file.

Drug- likeness properties study

Eight bioactive compounds were selected to analyze drug- likeness analysis based on their molecular docking score. According to Lipinski's rule, molecules have passed the drug- likeness analysis when they have values of MW ≤ 500, HBD ≤ 5, HBA ≤ 10, log P ≤5, and PSA (<140). The molecular weight is an important parameter of drug molecules because membrane transportation of drug depends on the size. The physicochemical parameters (HBD, HBA, PSA and logP) affect the absorption, bioavailability, metabolism, receptor-drug interactions, and toxicity of drug molecules. The physicochemical property of bioactive compounds analysis is a preliminary screening to determine an ideal drug. However, a drug does not have to follow all the Lipinski's rules to be a potential drug candidate. According to **Bickerton *et al.* (2012)**, the oral bioavailability of molecules does not affect biological or pharmacological activity. In our study,

EFDT was observed to follow almost all Lipinski's rules, while other than this, HCT and MOPA follow the ≥4 drug similarity rule (table 7). Thus, among the chosen bioactive compounds, EFDT, HCT and MOPA were ideal drugs because they showed excellent structural properties.

Table 7 Physicochemical properties of potential bioactive compounds of *A. flavus* from *A. mexicana* medicinal plant

Descriptor	Amp	HCT	MOPA	NFDT	TTOPE	BADE	PEDA	EDBP	ODC
Molecular Weight (<500 Da)	349.412	280.452	226.316	236.399	322.533	278.348	242.403	510.483	298.898
Partition coefficient (LogP) (<5)	0.3181	5.5592	2.7251	4.408	6.7531	3.6004	5.1622	10.7453	6.5653
Number Rotatable Bonds (<3)	4	5	6	0	16	8	13	22	14
HBA (<10)	5	2	3	1	2	4	1	0	1
HBD (<5)	3	0	0	0	0	0	1	0	0
PSA (<140 Å^2)	143.121	124.523	97.549	106.309	143.934	119.631	106.804	189.233	130.029

ADMET analysis

The ADMET property analysis of compounds is important in the early stage of drug innovation. Based on the best docking score, eight compounds were selected for the ADMET analysis. Water solubility, Caco2 and skin permeability, intestinal absorption (> 30%) and P- glycoprotein substrate and inhibitor are significant absorption properties in the drug innovation (table 8) (**Dahlgren and Lennernas, 2009**). Intestinal absorption means good absorption of the drug. MOPA showed the highest percentage (96.3%) after NFDT (95.2%), PEDA (95.0%), BADE (95.0%) and HCT (92.5%), TTOPE (92.1%), ODC (91.2%) and EDBP (86.2%) (Table-7). An ideal drug shows the skin permeability (< −2.5 cm/h) and almost all compounds showed suitable skin permeability. All bioactive compounds showed Caco2 permeability greater than 1.0cm/s. All the compounds showed non-substrate to the P-glycoproteins (table 7). The VDss, BBB and CNS permeability are important parameters in Drug distribution. Greater than 0.45 log VDss value is considered relatively high, HCT and NFDT showed 0.403 and 0.623 respectively. BBB permeability value between > -0.3 to < -1 is considered that the drug molecule can cross the blood brain barrier permeability. The Cytochrome 450 enzyme have an important role in metabolism of drug in liver system. The metabolism scores demonstrated that MOPA did not inhibit CYP2A4, CYP2C9, CYP2C19, CYP2D6, CYP2D6, and CYP3A4 enzymes. The total drug excretion rate is measured by the hepatic and renal clearance. In drug development, toxicity of the drug is a most important factor and takes part in the screening of most appropriate drug compound. The hERG I, II, AMES test, LD50, lowest-observed-adverse-effect level (LOAEL), hepatotoxicity, skin sensitization, and *Tetrahymena pyriformis* toxicity were performed on pkCSM web server (table 7). Consequently, according to ADMET results, HCT, MOPA, and NFDT can be used as potential antimicrobial candidates against *S. aureus* and *E. coli*.

Table 8 Absorption, Distribution, Metabolism, Excretion and Toxicity of potential bioactive compounds of *A. flavus* using pkCSM web server.

Property	Descriptor	Unit	Amp	HCT	MOPA	NFDT	TTOPE	BADE	PEDA	EDBP	ODC
Absorption	Water solubility	Numeric (log mol/L)	-2.396	-5.458	-2.938	-5.152	-7.555	-4.169	-4.169	-7.875	-7.835
	Caco2 permeability (<0.9cm/s)	Numeric (log Papp in 10-6 cm/s)	0.395	1.619	1.331	1.524	1.419	1.622	1.622	1.121	1.5
	Intestinal absorption (human) (<30%)	Numeric (% Absorbed)	43.034	92.537	96.339	95.287	92.135	95.044	95.044	86.273	91.195
	Skin Permeability (<-2.5cm/h)	Numeric (log Kp)	-2.735	-2.015	-2.328	-1.986	-2.796	-2.655	-2.655	-2.745	-2.691
	P-glycoprotein substrate	Categorical (Yes/No)	No	No	No	No	No	No	No	No	No
	P-glycoprotein I inhibitor	Categorical (Yes/No)	No	No	No	No	No	No	No	No	No
	P-glycoprotein II inhibitor	Categorical (Yes/No)	No	No	No	No	Yes	No	No	Yes	No
Distribution	VDss (human) (< 0.45)	Numeric (log L/kg)	-1.23	0.403	0.056	0.623	0.253	-0.007	-0.007	0.305	0.399
	Fraction unbound (human)	Numeric (Fu)	0.752	0.232	0.363	0.223	0	0.148	0.148	0	0.01
	BBB permeability (> -0.3 to < -1)	Numeric (log BB)	-0.767	0.518	-0.088	0.689	0.794	-0.054	-0.054	1.084	0.81
	CNS permeability (> -2 to < -3)	Numeric (log PS)	-3.166	-2.751	-2.347	-2.235	-1.515	-2.408	-2.408	-1.017	-1.394
Metabolism	CYP2D6 substrate	Categorical (Yes/No)	No	No	No	No	No	No	No	No	No
	CYP3A4 substrate	Categorical (Yes/No)	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes
	CYP1A2 inhibitior	Categorical (Yes/No)	No	No	No	No	Yes	Yes	Yes	No	Yes
	CYP2C19 inhibitior	Categorical (Yes/No)	No	Yes	No	Yes	No	Yes	Yes	No	No
	CYP2C9 inhibitior	Categorical (Yes/No)	No	No	No	Yes	No	No	No	No	No
	CYP2D6 inhibitior	Categorical (Yes/No)	No	No	No	No	No	No	No	No	No
	CYP3A4 inhibitior	Categorical (Yes/No)	No	No	No	No	No	No	No	No	No
Excretion	Total Clearance (− 0.6 to 1.1 mL/min/kg)	Numeric (log ml/min/kg)	0.337	1.599	1.477	0.822	2.092	0.93	0.93	0.968	0.237
	Renal OCT2 substrate	Categorical (Yes/No)	No	No	No	No	No	No	No	No	No
Toxicity	AMES toxicity	Categorical (Yes/No)	No	No	No	No	No	No	No	No	No
	Max. tolerated dose (human)	Numeric (log mg/kg/day)	0.952	0.071	0.336	-0.08	0.102	1.536	1.536	-0.262	-0.172
	hERG I inhibitor	Categorical (Yes/No)	No	No	No	No	No	No	No	No	No
	hERG II inhibitor	Categorical (Yes/No)	No	No	No	No	No	No	No	Yes	Yes
	Oral Rat Acute Toxicity (LD50)	Numeric (mol/kg)	1.637	1.967	1.808	1.583	1.588	1.806	1.806	2.708	1.769
	Oral Rat Chronic Toxicity (LOAEL)	Numeric (log mg/kg_bw/day)	2.398	2.195	2.111	1.236	3.107	2.326	2.326	0.699	1.048
	Hepatotoxicity	Categorical (Yes/No)	Yes	No	No	No	No	No	No	No	No
	Skin Sensitisation	Categorical (Yes/No)	No	Yes	Yes	Yes	Yes	No	No	Yes	Yes
	T.Pyriformis Toxicity	Numeric (log ug/L)	0.285	1.814	0.998	1.402	1.362	1.1	1.1	0.321	0.637
	Minnow toxicity	Numeric (log mM)	4.232	0.038	0.791	0.518	-1.978	0.09	0.09	-4.302	-1.717

Cytochrome P450 enzymes (CYP2A4, C YP2C9, CYP2C19, CYP2D6 and CYP3A4)

DISCUSSION

Endophyte *A. flavus* was identified in the root and shoot of the *A. mexicana* plant. *A. mexicana* is a medicinal plant, belonging to *Papaveraceae* family of plant kingdom. Some fungal endophytes produce antibacterial compounds. Penicillin antibiotic was identified by Alexander flaming for the first time in *Penicillium* fungus in 1928. Later, much research has been done on antimicrobial properties of the various fungi. Some literatures support the concept that fungal endophytes can produce the plant-based drugs. Therefore, it can be said that fungal endophytes of medicinal plants can produce good quality of drugs. It has been reported that secondary metabolites produced by endophytes are sometimes present in the same medicinal plants. Microorganisms are the rich source of as many as 20,000 metabolite production which directly influences the plants survival and its characteristics (**Brader et al., 2014**). Endophytes also produce other compounds like steroids, terpenoids, xanthones, xantocouramins (**Ludwig-Muller et al., 2014**). *A. mexicana* is a medicinal plant showing antimicrobial property against various pathogens. Therefore, we performed antimicrobial experiments of ethyl acetate extracts of *A. flavus* against various bacterial pathogens. *A. flavus* were selected for the antimicrobial study on the basis of agar well diffusion assay. It gave good inhibition result against five pathogens *S. typhimurium*, *K. pneumoniae*, *V. cholerae*, *E. coli* and *S. aureus*. A low IC50 value of the drug indicates a great radical scavenging activity. Ascorbic acid (13.7 ± 0.2 µg/ml) was used as a positive control because of its antioxidant activity. Ethyl acetate extract of *A. flavus* showed antioxidant activity at 22.9 ± 0.2 µg/ml). In another study, Botryosphaeria fabicerciana showed antioxidant activity (**Silva et al., 2022**). **Silva et al**. (**2022**) reported that Botryosphaeria fabicerciana (associated with leaves of Morus nigra) extract gave best MIC of 15.6 µg/mL (B. cereus), 62.5 µg/mL (*S. aureus* and *B. subtilis*). Ethyl acetate extract of fungal endophytes from Garcinia plant showed antimicrobial activity against methicillin- resistant *S. aureus*, *Cryptococcus neoformans*, *Microsporum gypseum*, and *Candida albicans* with MIC 32-512µg/ml, 64-200µg/ml, 2-64µg/ml, and 64-200µg/ml, respectfully (**Souwalak et al., 2006**).

Antimicrobial resistance in human pathogens is a major problem to combat the antimicrobial infection. Therefore, new drugs innovation is become important. HCT is a fatty acid, isolated previously from *Andrographis paniculata* (**Aysha *et al*., 2014**) and leaves of *Phyllanthus debilis Klein* (**Malayaman *et al*., 2019**). It has been reported antimicrobial agent against *E. coli* and *K. pneumoniae*. MOPA is isolated from the leaves of *Glycyrrhiza glabra* (**Vijayalakshmi, 2019**). It has not been reported its biological activity. MOPA showed very good inhibition activity against the *S. aureus* and *E. coli* proteins in *In-silico* analysis.

Bioactive compounds were purified through the TLC and its fractions were tested against bacterial pathogens. TLC fraction B gave the maximum ZOI against all four pathogens except *S. typhimurium*. **Sharma *et al*.** (**2021**) reported antibacterial activity of ethyl acetate extract of *A. versicolor* against *V. cholerae* strains. GC-MS analysis was performed to identify the potent antimicrobial compound in TLC fraction B. A total of eighteen compounds were identified by GC-MS study however eleven compounds have previously been reported for their biological activity. There is no report found on the biological properties of seven compounds. Even though, they gave good binding scores in computational analysis against the proteins of *S. aureus* and *E. coli*. Molecular docking results revealed that HCT and MOPA have the potential to inhibit the growth of the *S. aureus* and *E. coli* by blocking the active site of proteins related to various pathways (table 5).

CONCLUSION

In the present study, endophytic fungus *A. flavus* checked for antimicrobial activity against human pathogens, *S. typhimurium* (ATCC14028), *V. cholerae* (P5), *K. pneumoniae* (AIIMS-5), *E. coli* (MTCC729) and *S. aureus* (MTCC96). EA extract of endophytic fungus *A. flavus* showed antibacterial activity. In our study, it was observed that crude extract of fungal endophytes was given small ZOI then the TLC purified bioactive spots. The chemical name of bioactive compounds of the TLC purified fraction-B were determined by GC-MS analysis. Molecular docking and MM-GBSA results concluded that MOPA and HCT could be used as a potential antimicrobial candidate to treat different diseases related to *S. aureus* and *E. coli*. Physicochemical and pharmacokinetics analysis also support this statement. Other than these two compounds, NFDT, TTOPE, PEDA, ODC, BADE showed good binding affinity with the proteins of pathogen. Based on the results, we concluded that MOPA and HCT are better inhibitor then the Ampicillin antibiotic for combating of *S. aureus* and *E. coli* bacterial infection. In the era of AMR, our compounds can be used as effective antibiotics for the *S. aureus, E. coli, K. pneumoniae, V. cholerae and S. typhimurium bacteria*. The novelty of the study is mentioned above as biologically important compounds which were first time identified in *A. flavus*. Maybe the reason behind it is that plant-microbe interaction between *A. mexicana* and *A. flavus* helped with the production of these important compounds.

Conflict of interest: The authors declare that there are no conflicts of interest.

Acknowledgement: All authors want to acknowledge Retd. Scientist from ICAR Prof. Amithabh Bandopadhyay for his guidance. Thanks to all collectors for plant sample collection from the Assam, India.

Funding information: The work was conducted with the support of NE-DBT (Application No: AGRI/2015/48; year 2017). Pooja Singh is grateful to CSIR-UGC NET fellowship.

REFERENCES

Ahn, I. P., Lee, S. W., & Suh, S. C. (2007). Rhizobacteria-induced priming in *Arabidopsis* is dependent on ethylene, jasmonic acid, and NPR1. *Molecular Plant Microbe Interaction, 20*, 759–768. https://doi.org/10.1094/MPMI-20-7-0759

Aly, A. H., Debbab, A., and Proksch, P. (2013). Fungal endophytes— secret producers of bioactive plant metabolites. *Pharmazie, 68,* 499–505. https://doi.org/10.1691/ph.2013.6517

Aysha, N. M., Mumtaj, P. M., & Dhamotharan, R. (2014). Insilico Identification of Potent Inhibitor from *Andrographis Paniculata* for ESBLs. *International journal of Advances in Applied Sciences, 3*(3), 632-639. http://doi.org/10.11591/ijaas.v3.i3.pp151-157

Bhattacharjee, I., Chatterjee, S. K., & Chandra, G. (2010). Isolation and identification of antibacterial components in seed extracts of *Argemone mexicana* L. (*Papaveraceae*). *Asian Pacific Journal of Tropical Medicine, 3*(7), 547-551. https://doi.org/10.1016/S1995-7645(10)60132-0

Bickerton, G. R., Paolini, G. V., Besnard, J., Muresan, S., Hopkins, A. L. (2012). Quantifying the chemical beauty of drugs. *Nature Chem*istry, 90–98. https://doi.org/10.1038/nchem.1243

Brader, G., Compant, S., Mitter, B., Trognitz, F., & Sessitsch, A. (2014). Metabolic potential of endophytic bacteria. *Current Opinion Biotechnology*, *27*, 30-37. https://doi.org/10.1016/j.copbio.2013.09.012

Brahmachari, G., Gorai, D., & Roy, R. (2013). *Argemone mexicana*: chemical and pharmacological aspects. *Revista Brasileira de Farmacognosia*, *23*(3)**,** 559-575. https://doi.org/10.1590/S0102-695X2013005000021

Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *Food Science and Technology, 28*(1), 25-30. https://doi.org/10.1016/S0023-6438(95)80008-5

Chowdhary, K., & Kaushik, N. (2015). Fungal Endophyte Diversity and Bioactivity in the Indian Medicinal Plant *Ocimum sanctum* Linn. *PLoS ONE, 10*(11), e0141444. https://doi.org/10.1371/journal.pone.0141444

Dahiya, S., Kapil, A., Lodha, R., Kumar, R., Das, B. K., Sood, S., & Kabra, S. K. (2014). Induction of resistant mutants of *Salmonella enteric* serotype *typhi* under ciprofloxacin selective pressure. *Indian Journal of Medical Research, 139*, 746–753. https://pubmed.ncbi.nlm.nih.gov/25027085/

Dahlgren, D., & Lennernas, H. (2019). Intestinal permeability and drug absorption: predictive experimental, computational and in vivo approaches. *Pharmaceutics, 11*(8), 411. https://doi.org/10.3390/pharmaceutics11080411

Das, B., Verma, J., Kumar, P., Ghosh, A., & Ramamurthy, T. (2020). Antibiotic resistance in Vibrio cholerae: Understanding the ecology of resistance genes and mechanisms. *Vaccine, 38(1)*, A83-A92. https://doi.org/10.1016/j.vaccine.2019.06.031

Dudeja, S., Chhokar, V., Beniwal, V., Badgujjar, H., Chauhan, R., Soni, S., & Kumar, A. (2021). Optimization and production of antimicrobial compounds by *Aspergillus flavus* MTCC 13062 and its synergistic studies. *Biocatalysis and Agricultural Biotechnology, 35*, 102065, https://doi.org/10.1016/j.bcab.2021.102065

Gamal, A. I., Osama, M. S., Mamdouh, S. A., Ahmed, M.Y., Nadia, M. D., & Mohamed, F. E. (2021). Extraction, Evaluation and Structure Elucidation of Bioactive Metabolites of *Lactobacillus helveticus* CNRZ 32. *Biointerface Research in Applied Chemistry, 11*(1), 7677-7688. https://doi.org/10.33263/BRIAC111.76777688

Gandhi, S. G., Mahajan, V., & Bedi, Y. S. (2015). Changing trends in biotechnology of secondary metabolism in medicinal and aromatic plants. *Planta, 241*, 303–317. https://doi.org/10.1007/s00425-014-2232-x.

Ghalehnoo, Z. (2018). Diseases caused by *Staphylococcus aureus*. *Albanian Journal of Medical and Health Sciences, 11*, 65-67.

Hardoim, P. R., van Overbeek, L. S., & van Elsas, J.D. (2008). Properties of bacterial endophytes and their proposed role in plant growth. *Trends in Microbiology, 16*(10), 463-471. https://doi.org/10.1016/j.tim.2008.07.008.

Hardoim, P. R., Van Overbeek, L. S., Berg, G., Pirttila, A. M., Compant, S., Campisano, A., Doring, M., & Sessitsch, A. (2015). The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. *Microbiology and Molecular Biology Reviews, 79*(3), 293-320. https://doi.org/10.1128/MMBR.00050-14.

Iqbal, R., Hamid, I., Janbaz, K. H., Akhtar, M. F., Saleem, A., Sharif, A., Peerzada, S., Akhtar, B., Sohail, K., & Ali, S. (2021). *Argemone mexicana* extract alleviates gastrointestinal disorders by stimulating muscarinic receptors and blocking voltage-gated L-type calcium channels. *Asian Pacific Journal of Tropical Biomedicine*, *11*, 214-21. https://doi.org/10.4103/2221-1691.311769

Jiang, W., Yang, W., Zhao, X., Wang, N., & Ren, H. (2020). *Klebsiella pneumoniae* presents antimicrobial drug resistance for β-lactam through the

ESBL/PBP signaling pathway. *Experimental and Therapeutic Medicine*, *19*(4), 2449-2456. https://doi.org/10.3892/etm.2020.8498

Kariuki, S., Gordon, M. A., Feasey, N., & Parry, C. M. (2015). Antimicrobial resistance and management of invasive Salmonella disease. *Vaccine*, *33*(03), C21-9. https://doi.org/10.1016/j.vaccine.2015.03.102

Kidd, T. J., Mills, G., Sá-Pessoa, J., Dumigan, A., Frank, C. G., Insua, J. L., Ingram, R., Hobley, L., & Bengoechea, J.A. (2017). A *Klebsiella pneumoniae* antibiotic resistance mechanism that subdues host defences and promotes virulence. *EMBO Molecular Medicine*, *9(4)*, 430-447. https://doi.org/10.15252/emmm.201607336.

Kusari, S., Pandey, S. P., & Spiteller, M. (2013). Untapped mutualistic paradigms linking host plant and endophytic fungal production of similar bioactive secondary metabolites. *Phytochemistry*, *91*, 81–87. https://doi.org/10.1016/j.phytochem.2012.07.021.

Lorena, B. B., Javiera, O., & Franco, C. J. (2021). Chapter 1 - Facultative fungal endophytes and their potential for the development of sustainable agriculture. Editor(s): Ajay Kumar, Samir Droby, *Microbial Management of Plant Stresses*, Woodhead Publishing, 1-12. https://doi.org/10.1016/B978-0-323-85193-0.00014-0

Ludwig-Muller, J. (2015). Plants and endophytes: equal partners in secondary metabolite production? *Biotechnology letters*, *37*(7), 1325-1334. https://doi.org/10.1007/s10529-015-1814-4.

Ludwig-Muller, J., Jahn, L., Lippert, A., Puschel, J., & Walter, A. (2014). Improvement of hairy root cultures and plants by changing biosynthetic pathways leading to pharmaceutical metabolites: strategies and applications. *Biotechnology Advances*, *32*, 1168–1179. https://doi.org/10.1016/j.biotechadv.2014.03.007

Majowicz, S. E., Musto, J., Scallan, E., Angulo, F. J., Kirk, M., O'Brien, S. J., Jones, T. F., Fazil, A., & Hoekstra, R. M. (2010). The Global Burden of Nontyphoidal *Salmonella Gastroenteritis*. *Clinical Infectious Diseases*, 50(6), 882-889. https://doi.org/10.1086/650733

Malayaman, V., Mohamed, S. S., Senthilkumar, R. P., Basha, M. G. (2019). Analysis of phytochemical constituents in leaves of Bhumyamalaki (*Phyllanthus debilis* Klein ex Wild.) from Servaroy hills, Tamil Nadu, India. *Journal of Pharmacognosy and Phytochemistry*, *8*(1), 2678-2683.

More, N. V., Kharat, K.R., & Kharat, A. S. (2017). Berberine from *Argemone mexicana* L. exhibits a broadspectrum antibacterial activity. *Acta Biochimica Polonica*, *64*(4), 653-660. https://doi.org/10.18388/abp.2017-1621

Mweu, E., & English, M. (2008). Typhoid Fever in Children in Africa. *Tropical Medicine and International Health*, *13*(4), 532-540. https://doi.org/10.1111/j.1365-3156.2008.02031.x

Nie, P., Li, X., Wang, S., Guo, J., Zhao, H., & Niu, D. (2017). Induced Systemic Resistance against *Botrytis cinerea* by *Bacillus cereus* AR156 through a JA/ET- and NPR1-Dependent Signaling Pathway and Activates PAMP-Triggered Immunity in *Arabidopsis*. *Frontiers in Plant Science*, *8*, 238. https://doi.org/10.3389/fpls.2017.00238

Rahman, A., Uddin, W., & Wenner, N.G. (2014). Induced systemic resistance responses in perennial ryegrass against *Magnaporthe oryzae* elicited by semi purified surfact in lipopeptides and live cells of *Bacillus amyloliquefaciens*. *Molecular Plant Pathology*, *16*, 546–558. https://doi.org/10.1111/mpp.12209

Rania, A. B. A., Jabnoun-Khiareddine, H., Nefzi, A., Mokni-Tlili, S., & Daami-Remadi, M. (2016). Endophytic bacteria from *Daturametel* for plant growth promotion and bioprotection against *Fusarium* wilt in tomato. *Biocontrol Science and Technology*, *26*(8), 1139-1165. https://doi.org/10.1080/09583157.2016.1188264

Sharma, A., Singh, P., Rajkhowa, S., Nath, T., Sarmah, B. K., Ghosh, S., Swarnalakshmi, K., & Nandi, S. P. (2021). Anti-vibrio potential of bacterial and fungal endophytes isolated from *Datura metel*. *Indian Journal of Biotechnology*, *20*, 43-53.

Silva, A. A. D., Polonio, J. C., Bulla, A. M., Polli, A. D., Castro, J. C., Soares, L. C., Oliveira-Junior, V. A., Vicentini, V. E. P., Oliveira, A. J. B., Gonçalves, J. E., Gonçalves, R. A. C., Azevedo, J. L., Abreu-Filho, B. A., & Pamphile, J. A. (2022). Antimicrobial and antioxidant activities of secondary metabolites from endophytic fungus *Botryosphaeria fabicerciana* (MGN23-3) associated to *Morus nigra* L. *Natural Product Research*, *36(12)*, 3158-3162. https://doi.org/10.1080/14786419.2021.1947272.

Singh, P., Sharma, A., Bordoloi, M., & Nandi, S. P. (2020). Molecular identification of endophytic fungi isolated from Medicinal plant. *Biointerface Research in Applied Chemistry*, *2020*, 10(5), 6436 – 6443. https://doi.org/10.33263/BRIAC105.64366443

Singh, P., Sharma, A., Nandi, A. K., & Nandi, S. P. (2021). Endophytes from *Argemone mexicana* and *Datura metel* activate induced-systemic resistance in multiple hosts and show host- and pathogen-specific protection. *Journal of Plant Biochemistry and Biotechnology*, *30*, 1016–1019. https://doi.org/10.1007/s13562-021-00734-5

Souwalak, P., Nattawut, R., Vatcharin, R., Jariya, S. (2006). Antimicrobial activity in cultures of endophytic fungi isolated from *Garcinia* species, *FEMS Immunology & Medical Microbiology*, *48(3)*, 367–372. https://doi.org/10.1111/j.1574-695X.2006.00155.x

Vijayalakshmi, U., & Shourie, A. (2019). Comparative GC–MS analysis of secondary metabolites from leaf, stem and callus of *Glycyrrhiza glabra*. *World Journal of Pharmaceutical Research*, *8*(7), 1915-1923. https://doi.org/10.20959/wjpr20197-15125

Wain, J., Hendriksen, R. S., Mikoleit, M. L., Keddy, K. H., & Ochiai, R. L. (2015). Typhoid fever. Lancet, 385(9973), 1136–1145. https://doi.org/10.1016/s0140-6736(13)62708-7

Wojtaszek, P. (1997). Oxidative burst: an early plant response to pathogen infection. *Biochemistry journal*, 322(3), 681–692. https://doi.org/10.1042/bj3220681

Yadav, M., Yadav, A., & Yadav, J. P. (2014). In vitro antioxidant activity and total phenolic content of endophytic fungi isolated from *Eugenia jambolana* Lam. *Asian Pacific Journal of Tropical Medicine*, *7S1*, S256-61. https://doi.org/10.1016/S1995-7645(14)60242-X

Youssef, F. S. & Singab, A. N. B. (2021). An Updated Review on the Secondary Metabolites and Biological Activities of *Aspergillus ruber* and *Aspergillus flavus* and Exploring the Cytotoxic Potential of Their Isolated Compounds Using Virtual Screening. *Evidence-Based Complementary and Alternative Medicine*, 8860784. https://doi.org/10.1155/2021/8860784