BIODIVERSITY PROSPECTING OF ENDOPHYTIC FUNGI FROM *SOLANUM VIOLACEUM* ORTEGA. FRUITS AND ANTAGONISTIC ACTIVITY OF THE FILTRATES AGAINST PATHOGENS

Thesis submitted for the award of the degree of

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In

Cell Biology and Molecular Genetics

Under the Faculty of Allied Health and Basic Sciences

by

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TABLE OF CONTENTS

ABBREVIATIONS

CFS	Cell Free Supernatant
CF	Colony Frequency
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribo Nucleic Acid
dNTPs	Deoxyribonucleotide triphosphate
DPPH	1, 1 Diphenyl Picryl Hydrazyl
EDTA	Ethylenediaminetetraacetic acid
GC-MS	Gas Chromatography Mass Spectrophotometer
IC	Inhibitory Concentration
ITS	Internal Transcribed Spacer
LC-MS	Liquid chromatography Mass Spectrophotometer
MIC	Minimum Inhibitory Concentration
MHA	Muller Hinter Agar
NB	Nutrient Broth
NaOCl	Sodium hypochlorite
OD	Optimum density
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PBS	Phosphate buffered saline
SDA	Sabouraud Dextrose Agar
SDS	Sodium Dodecyl Sulphate
TE	Tris EDTA buffer
UV-Vis	Ultra- Violet Visible Spectroscopy

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Background

Antimicrobial resistance is the second most serious health complication affecting millions of people every year globally. Pathogenic organisms like bacteria, fungi, and viruses cause serious infections. Antibiotic-resistant microbes are difficult to treat. In most cases, last resort is using toxic chemicals and this burdens the patient. Their effect is huge in developing countries due to the relative unavailability of drugs and the development of extensive antibiotic resistance (Prestinaci *et al.,* 2015). Combinational antibiotic treatments as well as higher doses are required to treat the resistant microbes. Due to this, normal physiological processes natural gut microflora and immune systems get disturbed.

1.1 Nosocomial infections

Nosocomial infections are also called healthcare-related infections. The infection is absent in the patient at the time of admission, but appears during hospital stay and continues after the discharge from the hospital (AK *et al.,* 2011). Prolonged stay in the hospital leads to increased vate of infections, subsequently increasing mortality and antibiotic resistance. Intensive Care Units (ICU) in hospitals are the breeding places for the growth and spread of antibiotic-resistant pathogens (Struelens 1998; Peters *et al.,* 2019).

The Centers for Disease Control and Prevention (CDC) state that, *S. aureus*, *P. aeruginosa,* and *E. coli* are the communal nosocomial bacterial pathogens. In many cases, along with bacteria, fungi, to name a few, *Candida albicans*, *Candida tropicalis* and *Aspergillus,* also take part in causing infections. (Magill *et al.,* 2018).

1.2 Biofilm

Biofilm is a sticky, surface-attached, complex structured microbial communities formed due to communal interaction of pathogen attached to substratum surface and submerged into extracellular slimy accumulations. Biofilm has significantly diverse properties and also involves in a wide diversity of microbial infections in the body.

Established biofilm by microorganisms has significant role in the bionetwork. Pathogenic microorganism of the biofilm, cause infection even in the presence of a high concentration of antibiotics. The level of antibiotic resistance is greater than that of non-biofilm pathogens. This leads to the spread of disease and antibiotic resistance. Hence, exploration of new effective molecules to tackle this problem is a main concern (Cepas *et al.*, 2019).

1.3 Antioxidants

Plants are the main source of antioxidants like flavonoids, phenolic acids and polyphenols. Synthetic antioxidants (Butylated hydroxytoluene (BHT) and propyl gallate) have remarkable side effects. The natural antioxidants derived from plants source are safe for use in food and drug industries (Lourenci *et al.*, 2019). Lately, microbes are documented as rich sources of biologically active metabolites that find wide-ranging exploitation in medicine.

Oxidative stress is another risk factor that causes many diseases including aging in humans. In many human diseases, oxidative stress acts as a primary or secondary causative agent by damaging cellular and tissue components. (Pizzino *et al.*, 2017).

1.4 Historic review and recent scenario on medicinal plants

Since ancient times, human beings are dependent on natural products essentially from plants for their primary health requirements. India is the land of Ayurvedic medicine which has a long history of controlling/curing diseases. In Ayurveda, plant-based preparations perform a key role (Yuan *et al.,* 2016). Many medicinal plants have been utilized worldwide and are an economical source as well as having a strong therapeutic action with minimal side effects. Nearly 90% of ayurvedic preparations are plant based. Ayurvedic plants have a strong positive action on the body.

Bioactive compounds also defined as secondary metabolites present in all plants and have the impact on modulating metabolic processes and resulting in the promotion of good health, may be positive or negative depending on the nature of the substance, its dose, and its bioavailability (Batista *et al.,* 2021). Natural bioactive compounds are getting additional attention because of numerous therapeutic properties and huge exploitation in numerousprofitable sectors such as food, chemical, pharmaceutical industries (Gonzalez 2020) and soon.

These bioactive products not only obtained from plants but also as by-products from other living organisms. These natural products exhibit an extensive variety of pharmaceutical activities and perform a significant role in drug discovery and development. Plants are considered as one of the foremost sources of biologically active compounds in natural products research (Thomford *et al.,* 2018; Anand *et al.,* 2019).

Natural products differ from synthetic compounds having the following properties (Atanasov *et al.*, 2021):

- Unique structural complexity
- Diverse in chemical nature
- Suitable for combination in drug delivery
- Ability to optimize and alter to synthesize
- Unique medicinal properties

Since the discovery of penicillin in 1929, "Golden Age of Antibiotics" (1940- 1970) era has started.Metabolites from microbial source are well recognized as valuable supply of novel compounds and lead structures in the quest for drug applicants against infectious diseases, cancer and many other illnesses (Nicolaou *et al.*, 2018).Recognizing the ability of microbes to synthesize wide range of bioactive compounds and the presence of unexplored microbial diversity, research is underway to isolate and screen microbes of diverse habitat and unique environment for discovery of novel metabolites (Figure 1).

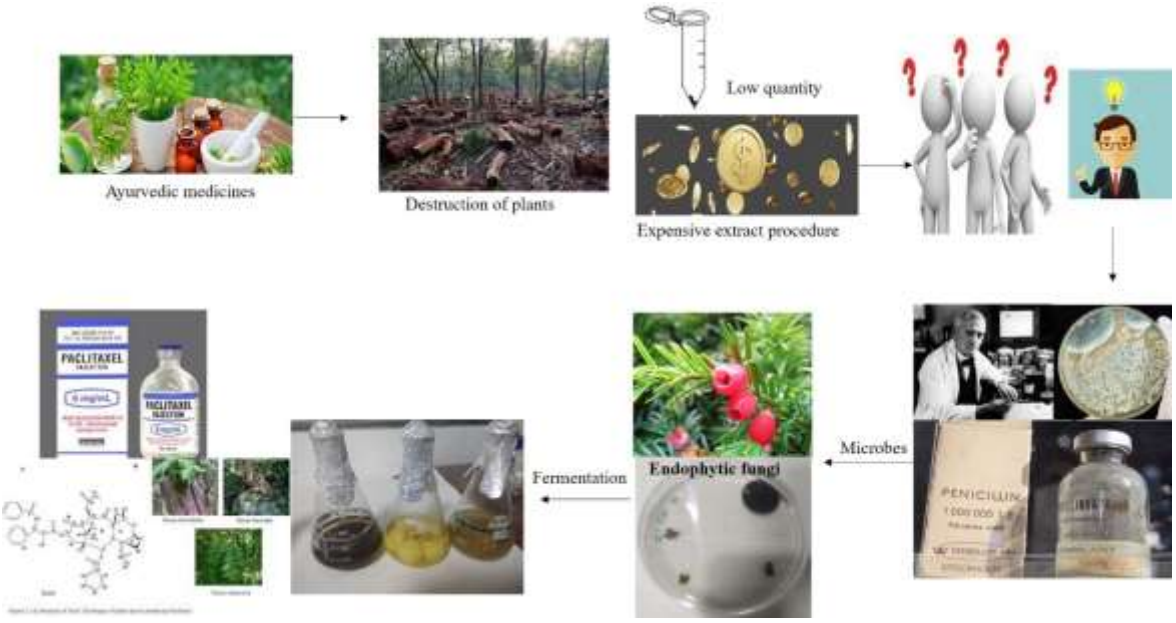


Figure 1: Diagrammatic representation of endophytic fungi

The extraction of bioactive compounds from plants leads to the species extinction, ecosystems destruction, longer maturation, and lesser production. Since the synthesis is based on the plant's need, recovering the available compound is always at its lower side (David *et al.,* 2015). A good example is taxol from *Taxus brevifolia*, a drug used for cancer treatment. The taxol concentration in each matured bark is about 0.001-0.05%. Based on the statistical analysis, the recovery from 15kg of bark is only 1g, which is insufficient to treat a cancer patient as each patient on an average requires 2.5g of body weight (Cragg *et al.,* 1993; Malik *et al.,* 2011).

To overcome the above issues, new bioactive compounds from different natural sources are screened by researchers to make them unique drugs (Paterson *et al.,* 2016). One such area is screening and looking for plant associated counterparts. Host and its counterparts co-ordinately or individually synthesize compounds or small molecules efficiently against pathogens with multiple modes of action (Ludwig-Muller 2015). Recently, phytochemicals, for example, alkaloids, flavonoids, phenolic acid, saponins, and glycosides etc., have been extensively investigated to treat many microbial infections (Khare *et al.,* 2021). This is a better option as they also produce similar molecules for pharmaceutical use.

These counterparts are endophytes/ microorganisms; those can be isolated, identified, and grown easily on an artificial medium under laboratory conditions. Inspite of these advantages, only a few plants have been studied for their endophytes.

1.5 Endophytes

It was the German scientist Heinrich Anton De Bary (1866) who coined the term endophyte. Bacteria, fungi and actinomycetes are the important endophytes that can colonize throughout the plant without showing any negative symptoms.

Endophytes are unique in nature. These become part of any plant either by choice or chance (Strobel *et al.,* 2018). The selection of the host plant plays a vital role in isolating endophytic microorganisms. So, understanding and knowing the ethnopharmacology of a host plant is important as hosts and endophytes are always mutually associated.

Microorganisms present inside the plant body are closely associated with each other. Microbes help in maintaining the plant's fitness, and thus, they are ecologically significant traits of their hosts (Hardoim *et al.,* 2015). Microbes are an important source for isolating bioactive natural products and have vast possibilities for finding novel compounds beneficial for medicine, industrial use, agricultural domains (Newman and Cragg 2016) and soon.

1.6 Biological inference of endophytic fungi

An endophytic fungal domain is one of the potent domains. These are extremely common and highly diverse microbes. These group of microbes signify an abundant and dependable high value of bioactive metabolites. The metabolites are chemically novel with the potential exploitation in the field agriculture, livestock, pharmaceutical and cosmeceutical industries, but are poorly investigated and are also of the largest untapped resources (Fouda *et al.,* 2015). Fungal endophytes are phylogenetically varied category of microbes. They can flourish symptomless inside the plant parts including stems, leaves, fruits, and roots. Various bioactive metabolites are produced by many endophytes either independent or dependent on the host or when plant is at stress and these can be used as therapeutic agents to treat or control many diseases (Fadiji and Babalola 2020). Secondary metabolites are active, novel

phytochemicals that possess antibacterial, anticancer, antifungal, antiviral, and anti-inflammatory activity belonging to the phytochemical category. This activity has been reported from many endophytic fungi (Anyasi and Atagana 2019) and the list includes Paclitaxel (synonym: Taxol) (Stierle *et al.,* 1993), Campothesin and its structural analogs (Puri *et al.,* 2006; Kusari *et al.,* 2009, 2011; Shweth *et al.,* 2008, 2013), Podophyllotoxin (Eyberger *et al.,* 2006; Puri *et al.,* 2006), Deoxypodophyllotoxin (Kusari *et al.,* 2009), Hypericin, Emodin and Azadirachtin (Kusari *et al.,* 2008, 2009b, 2012). Some biologically active compound listed below (Table 1).

Table 1: Biologically active compounds isolated from endophytic fungi

Host plant	Fungal endophytes	Compound isolated	Study status	Reference
Taxus brevifolia Nutt.	*Taxomyces andreanae*	Taxol	Cancer treatment	Stierle *et al.,* 1993
Catharanthus roseus L.	*Alternaria* sp.	Vinblastine	Lymphoid cancer drug	Guo *et al.,* 1998
Catharanthus roseus L.	*Fusarium* sp.	Vincristine	Children's leukaemia	Zhang *et al.,* 2000
Melia azedarach L.	*Penicillium janthinellum*	Citrinin	Cancer cell lines	Marinho *et al.,* 2005
Salvia officinalis L.	*Chaetomium* sp.	Cochliodinol and Isocochliodinol	*In vitro* studies	Debbab *et al.,* 2009
Camptotheca acuminate Decne.	*Fusarium* sp.	Campothecin	Phase 1 clinical trial	Kusari *et al.,* 2009
Pongamia pinnata L.	*Phomopsis glabrae*	Depsipeptide	Cancer cell lines	Shilpa *et al.,* 2014

1.7 Plant selection criteria

Though many criteria are involved in plant selection for fungal endophytes' isolation (Gakuubi *et al.,* 2021) the following are a few:

1. Plants growing in an adverse ecological environment (temperature, drought, salinity, and light)

2. Plants surrounded by pathogen-infected plants with no symptoms

3. Plants with a background of having medicinal properties used in traditional medicine

4. Plants that are known to have restricted growth in certain landmass (Example- *Maytenus hookeri* grown only in China)

1.8 Selected host plant

Solanum violaceum Ortega has an ethnobotanical history, belongs to Solanaceae family. Since ancient times, people used this plant for various medicinal applications which are currently in the practice. Plant parts like fruits, leaves, and roots are used to treat loss of appetite, anorexia, asthma, colic, digestive disorders, heart diseases, nasal ulcers, body pains, toothache, vomiting, and worm infestation caused by roundworms, whipworms, hookworms, tapeworms, and flukes (Jain and Borthakur, 1986 and Raju *et al.*, 2013).

S. violaceum is commonly called as poison berry, wide spread in humid and sub-tropical areas of India and Nepal up to an elevation of 1500 m. Plants are usually found on wastelands, roadsides, and in open scrublands (Baharul and Muhammad 2018). The plant is a spiny, highly branched shrub with raft branches, and grows up to 5 m. The younger branches are heavily covered with minute star-shaped hair, the pale purple flowers are arranged in racemose and extra-axillary cymes. Unripe (green) and ripe (dark orange-yellow) berries are about 8 mm in diameter and starts begins to appear from October. Matured fruits can be collected from February.

Taxonomical Classification and selected plant material

Kingdom	Plantae
Order	Solanales
Family	Solanaceae
Genus	*Solanum*
Species	*Violaceum*
Botanical name	*Solanum violaceum* Ortega

1.9 Pharmaceutical use

Traditional practitioners used different plant parts like fruits, leaves, and roots to treat many diseases (Karim *et al.,* 2017; Raju *et al.,* 2013). The table 2 shown below describes the medicinal properties of the plant.

Table 2: Medical properties of in *S. violaceum*

Sl.no	Plant part	Phytochemicals	Aliments	References
1.	Whole plant (Juice)	Alkaloids, carbohydrates, proteins, glycosides, saponins, gums, and di-terpenes	Gastrointestinal disorders and respiratory infections	(Karim *et al.,* 2017 and Meyer *et al.,* 2014, Raju *et al.,* 2013)
2.	Fruits (Juice)	Steroidal alkaloids, glycosides	Asthma, indigestion, leukoderma	Ghorbani *et al.,* 2011
3.	Roots and leaves (Dry Powder)	Solanine, solanidine and solasodine	Vomiting, colic, and toothache	Motaleb *et al.,* 2015, Ghani, 2003, Thongchai *et al.,* 2010 and Karim *et al.,* 2017
4.	Seeds (Dry powder)	Steroidal glycosides	Intestinal worms and hypertension	Raghavendra *et al.,* 2015 and Ehilen *et al.,* 2017

Globally, the traditional system of medicinal plants and their therapeutic abilities are helpful to cure many different ailments. Plants are the best natural sources for isolating endophytic fungi those have therapeutic properties. *S. violaceum* fruits are edible, and seeds are the seat of diverse endophytes and will be transmitted from one generation to another.

REVIEW OF LITERATURE

4.1 Natural products

“Human happiness and development are always depending on good health”. Many factors such as lifestyle, hygiene, long exposure to toxins will cause certain diseases include but not limited to asthma, cancer, chronic diarrhea and obesity (Egger & Dixon 2014; Dovjak & Kukec 2019). Since ancient times, plants and their products have been used as food supplements, nutraceuticals, modern medicines, folk medicines, pharmaceutical intermediates, chemical entities for synthetic drugs and so considered them as the richest bioresources. Further, plant-based compounds are the lead molecules to produce synthetic analogs (Yuan *et al.,* 2016). Different countries conducted many studies to explore their efficacy, which are claimed to have medicinal properties (Salmeron-Manzano *et al.,* 2020; Oyebode *et al.,* 2016; Fitzgerald *et al.,* 2020). On earth more-then 500,000 plant species are estimated, but till date, only 10% have been exploredfor human need.

Natural products play a specific role to control and prevent diseases, help in drug discovery and development, due to whichnatural products gained increased attention in the last few years (Atanasov 2021). Traditionally many plants are used in treating microbial infections. Many research works have proved that plants could be a good source of new, safe, biodegradable and less side-effective in discovering antimicrobial drugs (Anand *et al.,* 2019). However, in Indian herbal medicine, only a few plants are used scientifically and are evaluated systematically for their clinical application to manage human pathogenic microbes (Rupani and Chavez 2018). The extraction of bioactive components from plants results in the extinction of species and the destruction of ecosystems, requires prolonged maturation and a very low yield of quantity compound. The limiting factor in the commercial success of some natural products from plants is, the essential compound synthesis is based on the plant’s need (David *et al.,* 2015). The option other than using plants is to look for a superior microbe that is associatedwith the plant. During co-evolution, these creatures acquire the ability to

synthesise plant-based chemicals or comparable molecules through genetic recombination with the host plant.

4.2 Endophytes

Endophytes are abundant in every plant. Bacteria, fungi and actinomycetes are different group of microbes present inside as endophytes. Because of their interactions with their host plants, they are able to establish a microbial community in the plant's endosphere.From the available literature, more than 100 ancient (Ayurveda) medicinal plants have been studied concerning endophytes. Endophyte colonisation provides a novel biological niche that promotes their growth and grants them a distinct ability to synthesize new/ unknown bioactive metabolites. As a results, extensive research is being conducted to discover their diversity and utility in different sectors (Thomas *et al.,* 2017; Jia *et al.,* 2016). Plants' age, genotype, specific plant tissues, topographical site, and ecological circumstances influence the distribution pattern of endophytic microbiota (Gouda *et al.,* 2016).

4.3 Definitions given for endophytes

Petrini (1991) defined endophytes as "All organisms inhabiting plant organs that at some time in their life, can colonize internal part tissues without causing apparent harm to the host".

Sturz and Nowak (2000) have reported that micro-organisms are in a communalistic and symbiotic relationship with their host plants. Bacon and White (2000); Strobel and Daisy (2003) explained that "microorganisms like bacteria and fungi colonize inside the healthy plant tissues and documented as the potential source for discovering unique bioactive metabolites". This definition was further expanded by Fesel and Zuccaro (2016) "endophytes present as mutualistic symbionts in their hosts, but their relationships are not very specific". They may exist as saprophytes or pathogens depending on the conditions.

4.4 Endophytic fungi

Fungi are a heterotrophic group of microbes with various life cycles that includes symbiotic association with a wide variety of autotrophic organisms. Endophytic fungi live in mutualistic nature and maintain harmony with the plant tissues. Endophytes produce a unique pattern of secondary metabolites as a result of their mutualistic interaction with the plant along with the metabolites produced by plants (Fan *et al.,* 2020). An endophytic fungal domain is one of the potent domains, extremely common, highly diverse, largely untapped resource and poorly investigated microbes. And capable of synthesizing, high value bioactive and chemically novel compounds with the potential for exploitation in the field agriculture, livestock, pharmaceutical, and cosmeceutical industries (Fouda *et al.,* 2015).

4.5 Types of endophytic fungi

Based on the history and phylogenesis, fungal endophytes have been classified into two major groups (Rodriguez *et al.,* 2009; Bamisile *et al.,* 2018).

a) Clavicipitaceous – also called as Balansiaceous group under Class 1 endophytes, which represents a small number of phylogenetically associated Clavicipitaceous species. Restricted to cool and warm season grasses.
b) Non- Clavicipitaceous –polyphyletic groups, majorly present in the vascular and non-vascular plants.This groups are belonged to Ascomycota fungi. Species belonging to this group are biotic and abiotic stress tolerant.

4.6 Ecology of fungal endophytes

According to the literature, every plant species has been stated for the existence of endophytes until now. They are typically found in the intercellular (apoplast) and intracellular (symplast) spaces of different plant species. These endophytes protect plants from biotic, abiotic stresses and also helps in adapting to new environment (Murphy *et al.,* 2019).

Endophytic fungi may initiate the degradation of dead host plants then further leads to nutrient recycling (Ben *et al.,* 2016).

4.7 Host plant-endophyte interaction

The plant and endophyte relationships were evolved 60 million years ago. Their interactions are well established and both are beneficial from each other. Extended association between the hosts and microbes helps microbes adapt to the microenvironments of the host plant (Jia *et al.,* 2016). During this process, the host plant protects and provides essential nutrients for endophyte survival and in turn, endophytes produce metabolites for plant's growth, absorption of nutrients and fight against both biotic and abiotic stresses (Khare *et al.,* 2018). Endophytes also protect their hosts by either killing the pathogen directly (predation) or penetrating and parasitizing it by twisting and secreting lyase todecompose pathogen's cell wall (Gao et al., 2010). This property of endophyte is used to control pathogens.

The question now arises is how endophytes make their way into the host plant? The two possible ways of transmission of endophytes are vertical or horizontal. Horizontal transmission takes place by sexual or *via* spores, which can be spread by vector and agents, for example, insects, air, water and pollen. In vertical transmission, endophytes transmit through asexual and *via* vegetative organ penetration such as roots, leaves and flowers (Tiwari & Bae 2020; Wiewiora et al., 2015).

Plants and their counterparts possess both primary and secondary metabolic pathways. Based on this, metabolites are divided into the following categories a) primary metabolites those help in growth, development, and reproduction b) secondary metabolites comprise of both high and low molecular weight polymeric compounds such as lignin, proteins, and cellulose those help in defence and activities other than growth and development. The most stable

secondary metabolites are species-specific and derived from the intermediates of primary metabolites (Singh *et al.,* 2017; Collemare *et al.,* 2020).

Endophytes synthesize certain biologically active compounds either together or independently; hence endophyte-specific compounds could be generated in artificial fungal culture media in absence of host plants. These compounds have a lot of potential in terms of disease prevention and protection (Ravnikar *et al.,* 2015). Independence is a resourceful means of bioactive compounds, frequent studies have reported of high antimicrobial active metabolites from cultured endophytic fungi to produce variety of drugs which cure numerous diseases either in a raw or derived form (Ncube and van Staden 2015; Edreva *et al.,* 2008).

4.8 Biodiversity of fungal endophytes

Biodiversity can be defined as variability of life at the genetic, species and ecosystem levels of organization.Microbial diversity (bacteria, fungi and viruses) is a large part of the diversity and these are essential for maintaining a healthy ecosystem (Thatoi *et al.,* 2013).

As living being is also part of this ecosystem, damage to the diversity leads to ill health (Panizzon *et al.,* 2015). Scientists use a formula called the biodiversity index to describe the amount of species diversity in a given area. Because uniform population of a single species of plants adapted to a particular environment is more at risk if environmental changes occur (Supriatna 2018).

Fungal endophytes are a diverse taxonomic and ecological cluster. The efficacy of each endophytic species is related to the distribution and diversity. High diversity is also related with the ability of fungal endophytes to deal with environmental conditions and the exchange of information between the plant (Yadav *et al.,* 2016; Sahay *et al.,* 2017) and the endophyte.

Fungal endophytes are phylogenetically varied category of microbes. They can flourish symptomless inside the plant parts including stems, leaves, fruits.

Shubha and Srinivas, (2017) isolated a total of 165 fungal endophytes from 240 segments of leaf, root, and flowers of *Cymbidium aloifolium* and studied their diversity and bioactivity. The isolated fungi belonged to 22 different species, where in roots consisted of 67 strains, while leaf and flower samples contained 53 and 45 strains respectively. Colonization (40.6%) and isolation rate (0.83%) were higher in roots compared to leaf and flower. The diversity was calculated by different indices, resulted that the roots had highest Shannon-Wiener index (H' = 2.64) and Simpson diversity index (D' = 0.93) whereas leaf and flower had H' = 2.12, D'= 0.88; H' = 1.5, D' = 0.78, respectively. Shannon-evenness index (J') was highest in leaf (J' = 0.96) when compared to root (J'= 0.95) and flower (J' = 0.93).The results demonstrated that the colonization and isolation rate greatly vary from part to part in same plant.

Fan M. *et al.,* (2020) investigated the presence of fungal endophytes in the *Vaccinium dunalianum* leaves. A total of 239 fungal endophytes were isolated and identified based on morphological and molecular characteristics. By comparing the relative abundance (RA) values, the most frequent species belonged to *Phyllosticta* and *Guignardia* with RA of 26·78 and 14·22% respectively. Of which, the strains *P. capitalensis* and *G. mangiferae* with potential antimicrobial activity were the dominant endophytes to the sampling of leaves. A high diversity of endophytic fungi from *V. dunalianum* leaves was observed with high species richness S (62), Margalef index D' (11·1386), Shannon-Wiener index H' (3·2588), Simpson's diversity index Ds (0·9179), probability of interspecific encounter index (0·9218), and evenness Pielou index J (0·7896). Based on the above facts, it can be concluded that the *V. dunalianum* leaves possess high diversity of endophytic fungi. This two taxa *P. capitalensis* and *G. mangiferae* showed potent antimicrobial activity. This is a promising source of natural bioactive compounds for future agro-industry applications.

Dhayanithy *et al.,* (2019) conducted the study hypothesizing that the microbial communities in the coastal regions would tolerate a range of abiotic stress and may produce new

metabolites. Twenty fungal endophytes were isolated from different parts of *Catharanthus roseus* and identified using molecular technique and calculated the species diversity. *Colletotrichum, Alternaria,* and *Chaetomium* were more dominant species with colonization frequencies of 8.66%, 7.00%, and 6.33%, respectively. The overall species richness and diversity were highest in the bark (1.89) when compared to the leaf (1.5) and stem (1).

4.9 Growth media and fungal endophyte isolation

Media used for endophytic fungi isolation are Potato dextrose agar (PDA), malt extract agar (MEA), Sabouraud dextrose agar (SDA), Synthetic nutrient-poor agar (SNA), Oatmeal agar (OA) and Water agar (WA). With varying culture conditions such as pH, temperature, light, incubation time and aeration (VanderMolen *et al.,* 2013; Murphy *et al.,* 2015).

The most important step in studying fungal endophytes is effective surface sterilization. The proper surface sterilization of the plant tissues is required to eliminate epiphytes and other microorganisms (Greenfield *et al.,* 2015). Sterilizing agent concentration and time of the exposure play an important role, because it penetrates into plant tissue and kill endophytes. The effectiveness of the surface-sterilization must be confirmed (Yang *et al.,* 2018).

4.10 Identification of fungal endophytes

Morphological and molecular techniques are the two important methods for identifying fungi. Routinely and traditionally mycologists use phenotypic characters for identification, such as colony, colour, shape, elevation of colony and spore characteristics. Due to limited characters, species level identification may mislead by taking morphological characters into consideration. Molecular techniques are much more reliable compared to morphological identification (Raja *et al.,* 2017).

In Amsterdam (2011), multinational syndicate of mycologists assessed six DNA regions, *SSU, LSU, ITS, RPB1, RPB2, MCM7* for identification of fungi. Among these, ITS region

was officially nominated as fungal barcode. ITS region is about 600 bp long and contains ITS-1 and ITS-2, two variable spacers. The ITS region is flanked by the 18S r DNA gene at the 5'-end of the ITS-1 spacer and by the 28S r DNA gene at the 3' of the ITS-2 spacer. The highly conserved 18S, 5.8S, and 28S r DNA genes allow to design universal primers for ITS-1 and ITS-2 amplification (Xu 2016). In this study we used nuclear ribosomal ITS region (rDNA) for species-level identification, ITS is the most useful, the fastest evolving portion of the rDNA cistron and exhibits the highest variation (difference between interspecific and infraspecific variation) (Schoch *et al.*, 2012). The ITS region is too variable to address higher rank phylogeny, that is, at the level of families and orders.The intervening 5.8S rDNA and the adjacent ITS1 andITS2 regions were amplified using the ITS1 and ITS4 primer pair (Figure 2).

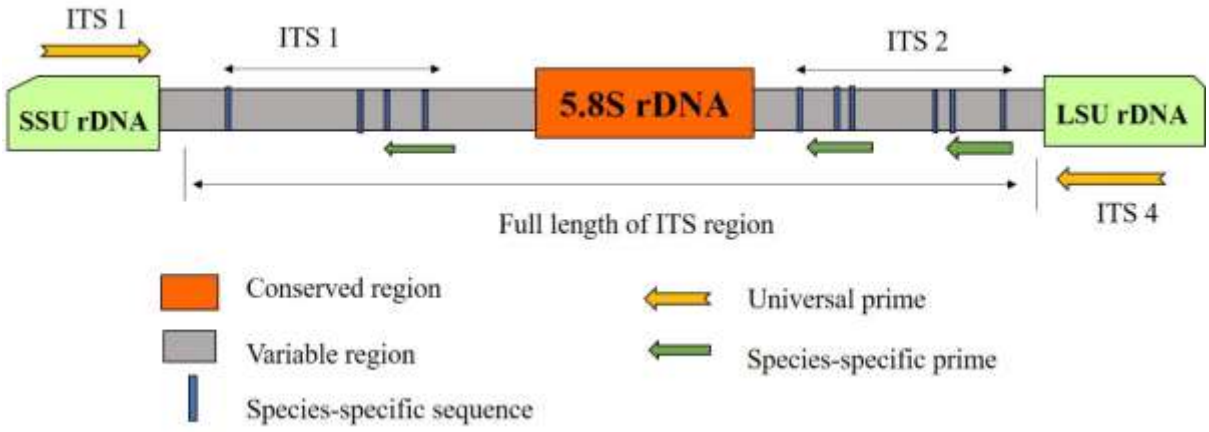


Figure 2: Schematic representation of ITS region between small subunit rDNA (SSU) and large subunit rDNA (LSU)

4.11 Fungal Metabolites

Endophytes are potential to synthesize metabolites with beneficial values and they can be successfully cultivated in the laboratory. To increase the synthesis of secondary metabolites, many studies have considered optimizing the culture conditions, such as solid-state fermentation and submerged fermentation (Son *et al.*, 2018). According to Subramaniyam &

Vimala (2012), secondary metabolites have been predominantly produced using submerged fermentation.

4.12 Characterization of metabolites from fungal isolates

VanderMolen *et al.,* (2013), Gao *et al.,* (2017) extracted fungal secondary metabolites using different solvents such as ethyl acetate, methanol, *n*-butanol. In their experiment, they sorted the mat and the fermented products, i.e., cell free supernatants (CFS) by using filter paper and performed solvent extraction of both CFS and mats and the resulted crude solvent extract were used for analyses (Verma *et al.,* 2014). Treating both CFS and dried mycelium individually results in to get intracellular and extracellular bioactive compounds (Zhang *et al.,* 2012; Bhardwaj *et al.,* 2015). Based on their solubility, the dried extracts be dissolved in DMSO, MeOH and PBS; those can also be used as vehicle controls in analyses experiments (Sharma *et al.,* 2016).

Further, bioactive compounds are subjected to chromatographic techniques like TLC, HPTLC and column chromatography. Silica gel or Sephadex LH 20 was used as stationary phase and different solvents in different gradient as mobile phase. Eluent was collected from different solvents. (Lu *et al.,* 2000; Verma *et al.,* 2014; Bogner *et al.,* 2017).

Purity and purification of compounds were determined using reverse phase High Performance Liquid Chromatography (RP-HPLC) and Ultra-Performance Liquid Chromatography (UP-HPLC) (Gao *et al.,* 2017).

Molecular mass and structural elucidation of unknown compounds can be achieved by LC-MS, GC-MS, Nuclear Magnetic Resonance (NMR), X- ray crystallography and High-resolution Electrospray Ionisation Mass Spectrometry (ESI-MS) (Ju *et al.,* 2016; Gao *et al.,* 2017; Wang *et al.,* 2017; Zhu *et al.,* 2017).

The most versatile tool for detecting and identifying active secondary metabolites/ natural products is LC-MS and GC-MS. Their high sensitivity and specificity allow them to distinguish between isobaric compounds with different elemental compositionat microgram concentrations (Altemimi *et al.,* 2017). The search for bioactive secondary metabolites is still going on, and it has resulted in the discovery of tens of thousands of substances, expanding the list of fungal secondary metabolites. GC-MSis applied for the separation of complex mixtures with low polarity or volatile substances on capillary columns. Because of the increased fragmentation provided by GC/MS, unknowns can be identified by searching a mass spectral database (Reber 2014). To get better chromatographic separation, samples can be derivatized which further enhances the sensitivity of the system (Sellers *et al.,* 2007).

Techaoei *et al.,* (2020) isolated endophytic fungi from *Nelumbo nucifera*, an aquatic plant. From different plant segments, six endophytes were isolated. An ethyl acetate extract of*Aspergillus cejpii* sample showed potent antibacterial activity against MRSA. Bacteriostatic mode of action was observed from a time-kill assay with a 7.5 mg/ml concentration. Samples were subjected to GC-MS for compound identification and major component identified was 5-(1H-Indol-3-yl)-4,5-dihydro- [1,2,4] triazin-3-ylamine ($C_{11}H_{11}N_5$).

Meenambiga and Rajagopal (2018) isolated *Aspergillus nidulans*, a fungal endophyte from *Acacia nilotica*, and tested its activity against the oral pathogen *Candida albicans*. Chloroform extract displayed anticandidal activity and 74.86% of *C. albicans* biofilm was inhibited by the extract. Flavonoids like saltillin, taxifolin, and 6-methoxyflavone are rich in the extract and it is revealed by GC-MS analysis. The study concluded that search of the new active compounds of the endophyte to be used as a lead drug against *C. albicans*.

Da Silva *et al.,* (2020) isolated 315 fungal endophytes from *Passiflora incarnata*.Endophytic fungi *Chaetomium globosum* (LAM 1793) and *Aspergillus nidulans* var. *dentatus* (LMA

1705) showed higher antioxidant. GC-MS has been done to identify active compounds present in crude solvent samples. Sorbicillin, Ergosterol, and Oreinol were the major compounds. the study concludes that the microbial extract possesses rich phenolic and flavonoids, the source for antioxidant compounds.

Tazik *et al.,* (2020) studied endophytic fungi from *Ferula ovina,* and screened for bioactive metabolites. A total of 40 fungi were isolated. Among all, *Ochrocinis ferulica* and *Pithoascus persicus* were able to produce tschimgine and stylosin, revealed by LC-MS analysis. These two compounds are majorly found in the host plant. The study concludes that plant-associated microbes can produce similar compounds that are only synthesized in the host plant.

Manganyi *et al.,* (2019) fungal endophyte a *Fusarium oxysporum* isolated from *Sceletium tortuosum* L. and its CFS subjected for GC-MS analysis. Ethanol, saturated hydrocarbons, acetamide and 5-hydroxymethylfurfural was the most abundant secondary metabolite produced by *F. oxysporum*.

From the leaves of *Cupressus torulosa* D. Don. Sharma *et al.,* (2016) isolated *Pestalotiopsis neglecta* (BAB-5510) fungal endophyte. They found that 5-hydroxymethylfurfural was one of the major bioactive compounds analysed by GC-MS.

Manganyi *et al.,* (2019) studied endophytic fungifrom *Pelargonium sidoides*. among all, *Alternaria* sp. exhibited antibacterial activity against test pathogen. Linoleic acid (9,12-octadecadienoic acid (Z,Z)) and cyclodecasiloxane might be the reason for its activity.

4.13 Phytochemicals

Bioactive metabolites from fungal endophytes (phytochemicals)such as alkaloids, glycosides, flavonoids, tannins, saponins and so on have been used in a wide range of commercial and industrial applications. The research and use of phytochemicals are increasing simultaneously

because of the harmful side effects of synthetic compounds (Ramesha & Chowdappa 2014). The following are a few studies on phytochemical identification by researchers (Table 3).

Table 3: List of the phytochemical from endophytic fungi

Host plant	Fungal endophytes	Extracts	Phytochemicals	Reference
*Aegle marmelos*L.	*Curvularia australiensis Alternaria citrimacularis*	Cell free supernatant	Flavonoids, phenols, cardiac glycosides, alkaloids, saponins	Mani *et al.*, 2018
Cupressus torulosa D.Don	*Pestalotiopsis neglecta* BAB-5510	Ethyl acetate	Alkaloids, flavonoids, tannins, phenols, saponins, terpenoids and carbohydrates	Sharma *et al.*, 2016
Pinus rouxburghii Sarg.	*Penicillium frequentans*	Ethyl acetate	Alkaloids, flavonoids, tannins, phenols, saponins, terpenoids	Bhardwaj *et al.*, 2015
*Centella asiatica*L.	*Penicillium* sp.	Ethyl acetate and methanol extract	Alkaloids, phenols, flavonoids, tannin and glycosides	Devi *et al.*, 2012
*Warburgia ugandensis*Sprague	*Phomopsis mali, Fusarium oxysporum* and *Alternaria alternata*	Cell free supernatant	Saponins, tannins, alkaloids, flavonoids, sterols and glycosides	Mbilu *et al.*, 2018
Ceriops decandra (Griff.) W. Theob	*Fusarium oxysporum, Chlonostachys* sp. and *Fusarium solani*	Crude extract	Flavonoids, terpenoids, carotenoids, and anthocyanins	Munshi *et al.*, 2021

4. 14 Extracellular enzymes

Valuable enzymes are produced from the endophytic fungi. Enzymes originated from endophytes are more stable than the other sources (Rajput *et al.,* 2016). Microbial enzymes gained more importance because of their consistency, ease of process modification, economic production and purification. In addition to this they are eco-friendly (Singh *et al.,* 2016). Beverage, textile, food and leather industries require enzymes to simplify the raw materials (Prasad and Roy 2018). Microbial enzymes are gradually substitute the conventional chemical catalysts in industrial processes. Enzymes have numerous applications in human health. Extracellular enzymes produced by fungal endophytes could help to start the host symbiosis process. Fungal endophytes isolated from numerous plant sources have been reported for the production of different types of extracellular enzymes since last few years (Khan *et al.,* 2016; Esteves *et al.,* 2014).Some of the industrially important extracellular enzymes are produced from endophytic fungi. Amylase, asparaginase, cellulase, protease and lipase are some of the key enzymes (Monteiro *et al.,* 2020). The below table 4 explains the production of extracellular enzymes by endophytes of various host plants.

Table 4: List of the extracellular enzymes from endophytic fungi

Host plant	Fungal endophytes	Extracellular enzymes Present	Reference
Aegle marmelos L.	*Curvularia australiensis Alternaria citrimacularis*	Amylase, protease, cellulase, lipase	Mani *et al.,* 2018
*Cymbidium aloifolium*L.	Total isolates:165	Amylase, protease, cellulase, lipase and laccase	Shubha and Srinivas 2017
Boswellia sacra Flueck	Total isolates: 77 *Thielavia microspore,Preussia* sp., *Phoma medicaginis* and *Pencillium citrinum*	Cellulase, phosphatase, and glucosidases	Khan *et al.,* 2016
Dillenia indica L.	Total isolates: 798 *Fomitopsis meliae, Schizophyllum commune* and *Lasiodiplodia*	Amylase, lipase, protease, laccase, and asparaginase	Vijay kumar *et al.,* 2021

	theobromae		
Sapindus saponaria L.	*Diaporthe* sp. *Diaporthe phaseolorum* *Phomopsis* sp.	Amylase, phosphatase, and pectinase	Santos *et al.*, 2019

4.14 Biological activities of endophytic fungi

4. 14a Antimicrobial agent production

The increased use of pesticides and antibiotics (drugs) makes the pathogens stronger against the available antibiotics, which leads to antibiotic resistance in pathogens. Pharmaceutical industries have developed several new antibiotics over the last three decades but fails to achieve control on it. These situations encouraged scientists to explore diverse natural sources. Research on natural sources is gaining importance for controlling the disease or infection and is much needed in the present situation (Taware *et al.,* 2015). However, there is a demand for new active compounds from a natural source to treat drug resistance pathogens, uncurable diseases and new diseases.

Biofilm are complex surface attached microbial communities, held together by self-produced polymer extracellular matrix. Biofilms are responsible for chronic illness, nosocomial infections and life-threatening infections (Muhammad *et al.,* 2020). Biofilm show an increased survival and resistance to environmental and chemical stressors. They are more susceptible to specific antimicrobial agents (Balcazar *et al.,* 2015). Finding new source for controlling and prevention of biofilm is essential.

Numerous current discoveries have been focused on extraction of bioactive compounds from a distinctive group of less studied and unexplored endophytic fungi (Newman and Cragg 2020). They are an inspiring basis for scientists due to their enormous structural diversity and complexity in the production of bioactive metabolites, antimicrobial, anticancer compounds, bio-control agents and pigments (Rai *et al.*, 2021; Aslam *et al.*, 2018).

Antimicrobial compounds like Piperine, Javanicin, Hydericin, Fusapyridon A, Isopestacin, Clavatol, Phomopsin A, Pestacin, Penijanthine A, Pestalone and Altersolanol A are also isolated from different fungal endophytes, those having anti-bacterial, antifungal, anti-viral and anti-protozoan activities (Gupta *et al.*, 2020). In an axenic culture condition, many fungal endophytes are capable of producing bioactive compounds having broad-spectrum activity and lower toxicity as compared to synthetic products (Gakuubi *et al.*, 2021). Antimicrobial compounds synthesized from some fungal endophytes are listed below in Table 5.

Table 5: Antimicrobial activity of fungal endophytes

Host plant	Endophytic fungi	Extracts	Test pathogens	Test method	Mode of action	Reference
Cupressaceae	*Trichoderma koningii* CSE_{32} *Trichoderma atroviride* JCE_{33}	CFSs	*A. niger* *A. fumigatus*	Dual plate assay Fumigation assay Agar -well diffusion assay	Microbicidal	Erfandoust R. *et al.*,(2020)
Ocimum tenuiflorum L.	*Alternaria tenuissima*	Crude ethyl acetate extract	*C. albicans*	Agar -well diffusion assay	Fungicidal	Chatterjee *et al.,* (2021)
Taxus wallichiana Zucc.	*Annulohypoxylon* sp.	Crude ethyl acetate extract	*P. aeruginosa* *C. albicans*	Agar -well diffusion assay	Bactericidal	Gauchan *et al.,* (2021)
Sceletium tortuosum L.	*Fusarium oxysporum*	CFSs	*E. faecalis, E. gallinarum, Bacillus cereus*	Disk diffusion assay	Bactericidal	Manganyi *et al.,* 2019
Azadirachta indica A. juss	*Alternaria alternata*	Crude ethyl acetate extract	*B. subtilis* *L. monocytogenes* *S. aureus* *E. coli* *S. typhimurium*	Agar well diffusion assay	Bactericidal	Chatterjee *et al.,* (2019)

4. 14b Antioxidant capacity

Antioxidants are also known as oxidation inhibitors and are extremely active in combating against reactive oxygen species (ROS) likehydrogen peroxide, nitric oxide, superoxide and organic hydroperoxides, this is due to an imbalance in pro-oxidant/antioxidant homeostasis (Ivanov *et al.,* 2017; Pizzino *et al.,* 2017). In modern medicine, antioxidants are becoming a promising and alternative natural biological therapy to treat diseases. The antioxidant compounds from endophytic fungi are of great significance which can reduce the risk of diseases caused by oxidative damage.The natural antioxidants derived from plants source is safe for use in food processing and /or drugs industries (Lourenci *et al.,* 2019). Asymptomatic fungi as mediators can produce antioxidants that can interrupt the chain reaction of reactive oxygen species to help host plants respond to numerous biotic and abiotic stresses. As a result, some endophytes with scavenging ROS activity *in vitro* are isolated from plants.The antioxidant compounds produced by fungal endophytes likely help the host plant to neutralise ROS. Existing research showed that fungal endophytes can confer effective tolerance to ROS under abiotic stress conditions, and also promote growth *via* biosynthesis of plant hormones and nutrient acquisition. Pestcin, Isopestacin, Rutin, Salidroside and *p*-Tyrosol are some of the natural known antioxidants (Panossiana *et al.,* 2014). The antioxidants isolated from endophytic fungi were given in the below table 6.

Table 6: Antioxidant activity of fungal endophytes

Host plant	Endophytic fungi	IC_{50} Value	Reference
Ceriops decandra (Griff.) W. Theob	*Fusarium oxysporum*	31.07 µg/mL	Munshi *et al.*, 2021
*Justicia gendarussa*Burm.f.	*Seudopestalotiopsis camelliae*	37.14 µg/mL	Mahmud *et al.*, 2020
Eugenia jambolana Lam	Chaetomium sp. and *Aspergillus* sp.	41.2 µg/mL	Yadav *et al.*, 2014
Fritillaria unibracteata var. wabuensis	*Fusarium sp.* *Fusarium redolens*	68.45 µg/mL 834.30 µg/mL	Pan *et al.*, 2017
Asiatic pennyworth L.	*A. oryzae* *Aspergillus austroafricanus*	10.29 ppm 12.08 ppm	Susilowati *et al.*, 2021
Ceriops decandra W.	*Fusarium oxysporum*	31.07 µg/mL	Munshi *et al.*, 2021
*Catharanthus roseus*L.	*Chaetomium nigricolor*	22 µg/ml	Dhayanithy *et al.*, 2019

4.15 The family Solanaceae

Solanaceae is also recognized as the nightshade family or potato family. Members of Solanaceae are flowering plants including weeds, herbs, shrubs, trees, ornamental plants, and farming crops comprising above 98 genera and approximately 3,000 species which are spread globally (Bohs and Olmstead *et al.,* 1999). Plants belonging to this family are distributed in tropical and temperate weather. It has a great diversity of locality (variety), morphology (long and short), and ecosystem. The plants of this family are well known for their alkaloids (Eich 2008).

4.16 Fungal endophytes from Solanaceae family

Solanaceae, a well-known nightshade/potato family. Plants belong to this family are known for their medicinal properties and contain toxic alkaloids. Studies shown that plants and endophytes belong to this family possess therapeutic properties (Samuels 2015). A study conducted by Pelo *et al.,* (2020) isolated nine endophytic fungi from *Solanum mauritianum* and all the fungal crudeextracts exhibited broad spectrum antimicrobial activity against tested pathogens. Among all fungal endophytes, *Paracamarosporium leucadendri* exhibited potent inhibitory activity at 6 µg/mL against *Mycobacterium bovis*, *K. pneumoniae* and *P. aeruginosa*. El-Hawary *et al.,* (2016) isolated Solamargine, a cytotoxic steroidal alkaloid from *Solanum nigrum* fungal endophytes, characterized by NMR and high-resolution mass spectrometry. This study demonstrated that plant derived compounds can also be derived from fungal endophytes. Another study isolated and identified nine known metabolites belong to sterols, γ- lactone, auxin and diketopiperazine from *Solanum nigrum* endophytic fungi, suggested that these might be reason for survival of host plant in desert conditions (El-Hawary *et al.,*2017). Vieira *et al.,* (2016) isolated 246 fungal isolates in winter (122) and summer (124) season from *Solanum cernuum* Vell. a Brazilian medicinal plant, among all, 64 extracts displayed antimicrobial activity, 18 isolates exhibited anti-fungal activity, and only 42 isolates displayed antibacterial activity. Study concludes that endophytes from host plant have potential antimicrobial compounds.

As plants and endophytes of this family are well known for their medicinal properties, it was decided to select *Solanum violaceum* Ortega for isolation and identification fungal endophytes with potential bio-efficacy.

Solanaceae are flowering plants including weeds, herbs, shrubs, trees, ornamental plants, and farming crops comprising above 98 genera and approximately 3,000 species which are spread

globally (Bohs and Olmstead *et al.,* 1999). Plants belonging to this family are distributed in tropical and temperate weather. It has a great diversity of locality (variety), morphology (long and short), and ecosystem. The plants of this family are well known for their alkaloids (Eich 2008).

4. 17 Plant material selection

Solanum violaceum Ortega, used as a vegetable, is widely distributed and belongs to Solanaceae family. For many years, entire plant or plant parts have been used to treat many diseases, like loss of appetite, anorexia, asthma, colic, digestive disorders, heart diseases, nasal ulcers, body pains, toothache, vomiting, and worm infestation caused by roundworms, whipworms, hookworms, tapeworms, and flukes (Jain and Borthakur, 1986 and Raju *et al.*, 2013).

Studies shown that phytochemicals present in this plant have biologically active compounds, and reviewed literature has revealed that there has been no report from endophytes isolation from the plant. This current scenario focuses on the isolation and identification of endophyticfungi as well as to find their pharmacological activities.

4. 18 Chemical constituents of *S. violaceum*

The whole plant contains carbohydrates, alkaloids, di-terpenes, glycosides, proteins, saponins, tannins, and flavonoids. Fruits, leaves, and roots are rich in steroidal alkaloids along with solanine, solasodine, and solanidine (Thongchai *et al.,* 2010 and Raju *et al.*, 2013). Some of the isolated steroidal sapogenins are indioside L, indioside M, indioside N and indioside O. Other steroids isolated from the plant are: 7-oxostigmasterol, 7-oxositosterol, diosgenin, yamogenin, diosgenone and (25S)-neospirost-4-en-3-one. Steroidal glycosides, indioside G to K, and some other molecules such as borassoside D, 7-hydroxysitosterol-3-O-β-D glucopyranoside, N-p- coumaroyl tyramine, trans-feruloyl

octopamine, and tricalysioside U were also isolated from the plant (Yen *et al.*, 2012). In addition, coumarin (scopoletin) and lignin (syringaresinol) were also isolated (Chang *et al.*, 2013).

4. 19 Research hypothesis

The hypothesis is "a friendly relation exists between endophytic fungi and plant". Ideally, a system mimicking the mutualistic or antagonistic symbiosis conditions have the capability of producing bioactive compounds.

4. 20 Lacunae in knowledge

From the review of literature, it was found that there is a need to explore the biological role of endophytic fungus isolated from this particular plant, as there are no studies available. Hence, the present study is aimed to isolate fungal endophytes from the fruit of *Solanum violaceum* and to explore their bioactive potential like antioxidant and antimicrobial activities towards biomedical applications.

4. 21 Research question

- Are fungal endophytes present in *Solanum violaceum*Ortega?
- Do these fungal endophytes contain remedial bioactive compounds?

SCOPE, RATIONALE AND RECOMMENDATIONS

2.1 Scope

New diseases, increased drug resistance in pathogens, the appearance of life-threatening viruses, and complications in patients undergoing organ transplantation are just a few of the challenges that researchers, physicians and the patients face.

This situation has compelled researchers to look for safe and effective agents in natural sources in order to meet the demands of the developing world.For thousands of years, natural products, particularly those derived from medicinal plants, have been exploited for safety use to living beings.

Endophytic microorganisms live in plant internal tissues and are a rich and symbiotic source of bioactive and chemically unique compounds with the potential for desecration in a variety of agricultural, medical, and industrial fields. Currently, there are several metabolites obtained from fungal endophytes that have antimicrobial properties.

Endophytes make up a significant portion of the fungal diversity that has yet to be discovered. In 1990s and 2000s, secondary metabolites from endophytic fungi gained a lot of attention, over taxol (anticancer drug) production from *Taxus* sp. As a results endophytes emerged as a keen interest for researches, since then paper have been published focusing on isolation and characterization of putative secondary metabolites from fungal endophytes.

These issues have been thoroughly discussed, as evidenced by current scientific knowledge and future perspectives.

2.2 Rationale

Endophytic fungi isolated from plants possess medicinal values. As whole plant, *S. violaceum* used in traditional practice to cure many diseases. It was found that there is a need to explore the biological role of endophytic fungus isolated from *S. violaceum* as there are no studies available.*S. violaceum* fruits are edible, and seeds are the seat of diverse endophytes and will be transmitted from one generation to another.

Hence, the present study is aimed at to isolate fungal endophytes from the fruit of *S. violaceum* and to explore their bioactive potential like antioxidant and antimicrobial activities towards biomedical applications.

2.3 Recommendations

- As fruits endophytic fungi possess potential bioactive compounds, remaining plant parts can be subjected for the isolation of endophytes
- Based on the results, fruits can be included in daily diet helps in reducing the risk of diseases
- Different solvents can be used for extraction of crude metabolites
- Crude solvent extracts contain active metabolite, individual compounds can be purified for further analysis for novel drugs development

AIM AND OBJECTIVES

3.1 Aim of the study

The present investigation was undertaken to isolate and identify culturable endophytic fungi from fruits of *S. violaceum* Ortega and screen them for their bio-active potential against pathogenic microorganisms. The current investigation was performed with the following objectives in order to achieve the aim:

3.2 Objectives of the study

1. Isolate, identify endophytic fungi from fruits of *S. violaceum* Ortega by morphological and molecular techniques
2. Determine fungal diversity within fruit tissues
3. Screened for phytochemicals and extracellular enzyme activity of the fungal isolates
4. Evaluate antioxidant activity and antimicrobial activity of crude solvent extracts and their mode of action (a mechanism) on human pathogens
5. Identify active compounds by Gas Chromatography and Liquid Chromatography-Mass-Spectrometry

MATERIALS AND METHODS

Materials and methods

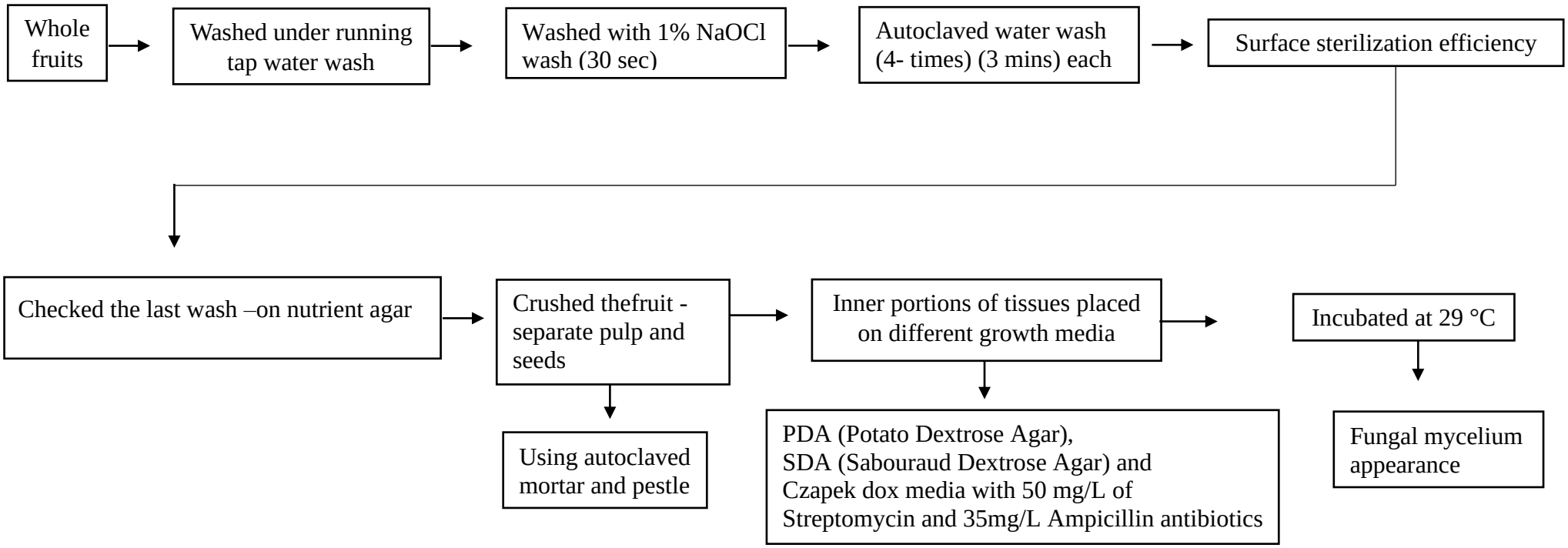
All chemicals and reagents are used in this research were of analytical grade.

5.1 Sample collection

Healthy (showed of no visible disease symptoms), matured and uniformly ripened (orange-yellow) and unripened (green) fruits of *S. violaceum* Ortega were collected from the Dhanvantri herbal garden of Sri Devaraj Urs Academy of Higher Education and Research (SDUAHER). The sample was identified and authenticated by the Department of Forestry and Environmental Science, University of Agriculture Science, Gandhi Krishi Vignana Kendra (GKVK), Bangalore with voucher specimen number (UASB-4589).

5.2 Sample preparation

Surface sterilization was carried out for collected samples as described by protocol Jinu *et al.*, (2015). Isolation from surface-sterilized host tissues is the most common method for detecting and quantifying fungal endophytes. Surface sterilization of plant material frequently involved a brief action with a powerful oxidant or general disinfectant, followed by a sterile washing to remove any leftover sterilant.



The tissue segments were observed periodically and the fungi growing out of them were scored, isolated and cultured on their corresponding medium. NaOCl- Sodium hypochlorite

5. 3 Stock culture storage

The following methods were used to store the isolates (Paul *et al.,* 2015).

A. Punched the actively growing endophytic fungal hyphae and kept at 4°C.
B. Four-day old slants at -20°C.
C. Four-day old slants with 50% glycerol at 4°C.

5. 4 Identification of fungal endophytes

The fungal endophytes isolated from *S. violaceum* were identified based on microscopic and molecular methods.

5.4a Microscopic identification

Fungal mycelium and spores were stained with lactophenol cotton blue, viewed and captured the images under the light microscope (40x) attached with a digital camera (Zeiss- Primo Star) (Petrini *et al.,* 1992).

5. 4b Molecular identification

Fungal genomic DNA was isolated by CTAB (Cetyl Trimethyl Ammonium Bromide) method (Camacho *et al.,* 1997 and Chiang *et al.,*2001). It is rapid, simple and in-expensive method for DNA extraction and DNA can be used directly for PCR amplification without further processing (Figure 3).

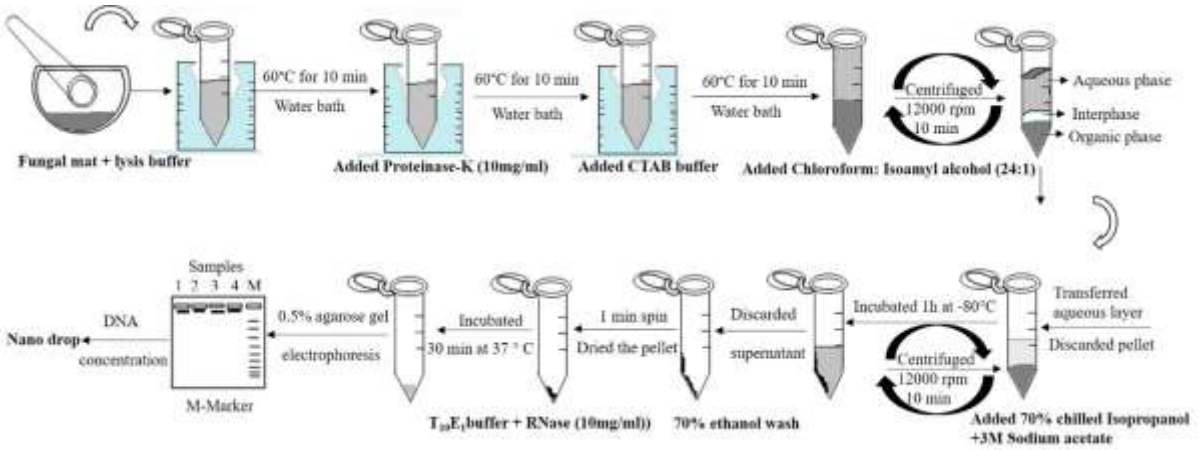


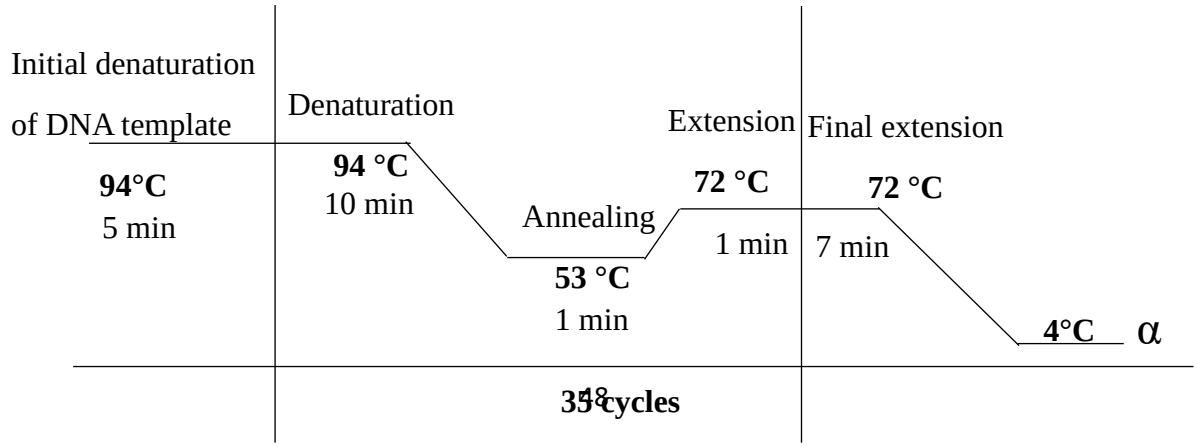
Figure 3: Pictorial presentation of fungal DNA isolation by CTAB method

5.5 PCR amplification conditions

Due to the limitations of traditional isolation techniques and to avoid technical bias, molecular techniques have been employed in the detection of fungal endophytes. In this study we used nuclear ribosomal ITS region (rDNA) because the ITS evolves the fastest and exhibited the highest variation.

Components	Concentration	Volume
PCR master mix (dNTPs, Taq buffer, $MgCl_2$ and Taq DNA polymerase)	1X	15 µl
ITS 1 - Forward primer (5'-TCC GTA GGT GAA CCT GCG G-3')	0.50 µM	1.5 µl
ITS 4 – Reverse primer (5'-TCC TCC GCT TAT TGA TAT GC-3')	0.50 µM	1.5 µl
Templet DNA	10 ng	3 µl
Molecular grade water		9 µl
Total volume		30 µl

ITS- Internal Transcribed Spacer



A small volume of the amplified DNA was loaded in 1% agarose gel along with DNA ladder (1kb) (Kuriakisa *et al.,* 2014), followed by one-directional sequencing of DNA using ITS1 primer at Biokart sequencing service (Bangalore). These sequences were used as the queries for mega-BLAST algorithm similarity searches in GenBank database. The isolates were discovered based on the best hit in the similarity search by BLAST. Sequences were dropped in NCBI-GenBank (http://www.ncbi.nlm.nih.gov) database and accession numbers obtained.

5.6 Phylogenetic analysis

By using the obtained sequences and from the sequences available in GenBank, a dendrogram was made for the phylogenetic analysis. Clustal X alignment was used to make a dendrogram in MEGA5 (Tamura *et al.,* 2013) by neighbour joining.

5.7 Data analysis of the fungal endophytes

Colonization frequency/rate [1] of fungal endophytes were calculated using the following formula and divergence was evaluated using indices such a, Simpson's diversity index (D) [2], Sorensen's index of similarity (QS) [3] Margalef (D_{mg}) [4] and Menhinick's index (D_{mn}) [5] (Dhayanithy *et al.,* 2019, Katoch *et al.,* 2017, Uzma *et al.,* 2016).

$$\textbf{Colonization frequency}\ (\%) = \frac{\text{No. of segments with fungal growth}}{\text{Total number of segments incubated}} \times 100 \longrightarrow [1]$$

$$\textbf{Simpson's dominance (D)} = \frac{\Sigma\, n\,(n-1)}{N\,(N-1)} \longrightarrow [2]$$

Wheren- number of individuals of each species, N- Total number of individuals of all species

$$\textbf{Sorensen's index of similarity (QS)} = \frac{2(a)}{2(a) + b + c} \longrightarrow [3]$$

Where ‘a’ is the number of common species in both ripe and unripe.

‘b’ and ‘c’ are the number of species specified too ripe and unripe.

Margalef’s diversity (D_{mg}) = $\dfrac{S-1}{\ln N}$ ⟶ [4]

Menhinick’s diversity (D_{mn}) = $\dfrac{S}{\sqrt{N}}$ ⟶ [5]

where N = the total number of individuals in the sampleand S = the number of species recorded.

5.8 Extracellular enzyme screening from endophytic fungi

Fungal enzymes are gaining importance in various sectors, as they are often more stable at extreme circumstances than the enzymes obtained from plants and animals. As part of their mode of action to overcome the defence of the host during invasion and to obtain nutrients for their development fungal endophytes produce enzymes such as amylases, lipases and proteases etc... Qualitative techniques (Sunitha *et al.,* 2013, Mani *et al.,* 2018 and Moharram *e t al.,* 2016) were used for the screening of extracellular enzymes such as amylase, lipases, protease, laccase, and asparaginase from the isolated fungi (Table 7).

Table 7: Methods used for screening extracellular enzymes

Enzymes screened	Media with supplements	After 7 days of incubation	Reaction in medium	Confirmation
Amylase	GYP – Starch		Flooded with 1% iodine in 2% potassium iodide – to digest starch	Clear halos around the colony
Laccase	GYP – α-naphthol		Oxidation of 1-naphthol	Colourless to blue colour around the colony
Lipase	PA – 1% Tween 20		Formation of calcium salts of the lauric acid liberated by enzymes	Visible precipitation around the colony
Protease	GYP agar media - 0.4% gelatin		Flooded with saturated aqueous ammonium sulphate-gelatin degradation	The clear zone around the colony
Asparaginase	Modified Czapex Dox's agar – L-Asparagine		Formation of ammonia increases pH (acid to alkaline)	Change of media colour yellow to pink

(GYP- Glucose Yeast Extract Peptone; PA- Peptone Agar)

Appendices 1- Media composition.

5.8 Phytochemical screening

The CFSs and dry mat power were screened for the qualitative presence of phytochemicals *viz.* alkaloids, flavonoids, phenols, tannins, saponins, steroids, reducing sugars, cardiac glycosides, gums and mucilage, fats, and oils by standard protocols Table 8 (Devi *et al.*, 2012 and Harborne *et al.*,1998)**.**

Table 8: Tests used to detect the phytochemicals in CFSs and dry mat powder

Sl. No	Phytochemicals	The test used to detect	Protocol	Confirmation
1.	Alkaloids	Wagner's test	Test samples + Wagner's reagent (Iodine-potassium iodide)	Brownish-yellow precipitation
2.	Steroids	Lieberman-Burchad method	Test samples + 2 mL of chloroform solution + acetic anhydride + a few	A blue-green ring in the solution

			drops of concentrated H_2SO_4	
3.	Phenols	Ferric chloride test	Test samples + a few drops of 10% ferric chloride	Presence of blue-green colour
4.	Reducing sugars	Benedict's reagent	Test samples + a few drops of Benedict's reagent	Change in colour in 4-10 min. Absence - blue colour. Presence - green, yellow, orange, dark red, and brown
5.	Saponins	Foam test	Test samples + water was shaken vigorously	Stable foam for 10 min
6.	Glycoside	Killer-kallani test	Test samples + 2ml of glacial acetic acid + 1-2 drops of 2% $FeCl_3$+ 2ml of conc. H_2SO_4	A brown ring at the interphase
7.	Fat and fatty acid	Spot test	Test samples pressed strongly between two filter papers	Oil strain on paper
8.	Flavonoids	Alkaline reagent test	Test samples + a few drops of NaOH + a few drops of dilute acid	Yellow colour to colourless after adding dil. Hcl
9.	Tannins	Ferric chloride test	Test samples + equal amount of alcoholic $FeCl_3$ reagent + two drops of dilute H_2SO_4	Yellowish-brown precipitate.

5.9 Test pathogens, standard antibiotics and media used in this study

Test organisms	**Pathogenic bacteria**	**Pathogenic fungi**
	Staphylococcus aureus (ATCC 25923)	*Candida albicans* (ATCC 10231)
	Pseudomonas aeruginosa (ATCC 27853)	*Candida tropicalis* (ATCC 201380)
	Escherichia coli (ATCC 25922)	
	Klebsiella pneumoniae (ATCC 35657)	
Media used	Mueller Hinton Agar medium (MHA)	Potato Dextrose Agar (PDA)
Standard antibiotics used	chloramphenicol	Fluconazole
Negative control	DMSO	

The overnight pathogen suspensions were adjusted to 0.5 McFarland standard for the test analysis. This is to produce a standardized microbial inoculum of approximately 1x 10^8 CFU/ ml.

5.10 Bioactive metabolites from fermentation broth

To maintain uniformity, and for fermentation experiment all the fungi were cultured on PDA. Actively growing hyphae tips were inoculated in 250ml flasks contained PDB (Potato Dextrose Broth) and cultivated for three weeks and incubated on an orbital shaking incubator (120 rpm) at 27 ± 2°C. After incubation, broth was filtered through Whatman filter paper 1 to separate the mycelial mat from fungal filtrate. The filtrates were further clarified by spinning at 4°C at 12,000 rpm for 10 min.Cell-free supernatants (CFSs) were stored at 4°C till further use. The collected mycelium was kept at 37°C till dry, made to a fine powder, and stored for further use at 4°C in a tightly sealed tubes (Sharma *et al.,* 2016).

5.11 Preliminary antimicrobial activity by different diffusion methods

Due to the inability of currently available antimicrobials to treat infectious diseases, many researchers have turned to natural products as a novel source for bioactive compounds. A variety of methods are found for this purpose and since not all of them are based on same principles and the test systems should ideally be simple, rapid, reproducible, and inexpensive. In the present study for primary screening of bioactivity of isolates was performed using five different culture assays. 1. Agar well diffusion assay, 2. Agar plug diffusion assay, 3. Cross streak plate assay, 4. Dual plate assay and 5. Fumigation assay.

5.11a Agar well-diffusion method for CFSs

All the filtrates were filter sterilized before performing the experiments. Petri dishes were prepared for both bacteria and fungi using MHA and PDA respectively. After solidification, wells were made with sterilized cork borer (5mm). lawn cultures were prepared using sterile cotton swabs and poison food technique were followed for fungi. 30 µl of crude CFSs were added to each well. To facilitate proper diffusion, plates were refrigerated for half an hour,

later incubated at 37°C for 24 h (bacteria) and 28°C for 48-72 h (fungi) and measured the inhibition zone. The experiment was carried out for three times (Pai *et al.*, 2018, Yasser *et al.*, 2018).

5. 11b Agar plug diffusion assay

All isolated fungi were grown on PDA for three weeks at 27°C. Fungal agar plugs (8 mm) were cut from them, pressed against the test bacteria/ fungi which were already spread on the respective plates, and the plates were sealed. The plates were refrigerated for 12 h to facilitate metabolite diffusion and left undisturbed at 37°C to enable bacterial and fungal growth, and measured zone of inhibition (Marcellano *et al.,* 2017).

5. 11c Cross-streak plate method

Method employed was based on the protocol of Aljuraifani *et al.,* (2019). Blood agar medium was used in this experiment because, this medium is suitable for both bacteria and fungi. Briefly, the actively growing endophytic fungal strains were seeded in centre of the blood agar plate as a single streak. After an incubation period for 2-3 days, depending upon growth of the fungal strain, test pathogens were streaked on either side of endophytic fungus. After incubation for 48 h at 37°C, the antimicrobial interactions were analysed by observing the growth.

5. 11d Dual plate assay

PDA-contained plates were co-cultured with pathogenic fungi and fungal endophytes at a distance of 3 cm from the plate end. Plates were sealed using parafilm and incubated at 25 ± 2°C. Only pathogens in the plate were served as control. After a week-day incubation, pathogen growth in the presence/absence of the fungal endophyte was noted. The percentage antagonism was calculated as mentioned below (Katoch *et al.*, 2017).

Where, $$A\,(\%) = \frac{(DCC\text{-}DCT)}{DCC} \times 100$$

A (%) – Per cent antagonism

DCC- Diameter of the colony in control (without of endophytic fungi).

DCT- Diameter of the colony in test (with endophytic fungi).

5. 11e Fumigation assay

In separate PDA plates, allowed endophytic and pathogenic fungi to grow at 28°C for five days. In sterile conditions, both the plates (without lids) with respective fungi were kept in such a way that both the fungi were facing each other and tightened with layers of parafilm (Erfandoust *et al.* 2020). Organisms were allowed to grow further for seven days at 28°C. Individual petri plates with only endophyte and only pathogen served as controls. The pathogenic fungal growth in each plate was recorded.

5. 12 Effect of CFSs on pathogenic fungal colony frequency and morphology

The extracts that showed activity against pathogenic fungi (*C. albicans* and *C. tropicalis*) were selected for this assay. Method employed is based on the protocol of Chatterjee *et al.,* (2019). The pathogenic fungi, PDB medium (supplemented with 50 mg/L of Streptomycin to avoid bacteria contamination) and CFSs were mixed in 1:1:1 proportion. Pathogenic fungi without CFSs were considered as controls. Treated and untreated cultures were kept undisturbed for 24 h, 48 h, 72 h and 96 h at 37°C. Colony forming units were counted by spreading on PDA. Microscopic observations were recorded for morphological changes under 40X.

5. 13 Stability test for cell free supernatants

In order to establish the thermal stability, based on the antimicrobial potential, the selected CFSs were kept in a boiling water bath (98-100°C) for 10 min. In order to check its protease stability, the CFSs were treated with Proteinase-K (1 mg/mL) for 2 h at 37°C. These treated

CFSs and positive control (antibiotic discs) were tested for antimicrobial activity (Chatterjee *et al.,* 2019).

5. 14 Solvent extraction

The CFS (25ml) and dry powdered mycelium (5gms) were separately mixed with three different solvents, Solvents used for solvent extraction were hexane (non-polar), ethyl acetate (medium polar), and *n*-butanol (polar).In a separating flask, equal proportions of solvent and CFS were shaken vigorously for 10 min and kept undisturbed until the two clear immiscible layers were found. The CFS and solvent holding crude metabolites were parted and the solvent holding fractions were kept for drying at room temperature. The dry mycelium was grinded with solvents, until it completely mixed. transferred to separating flask kept undisturbed until the two clear immiscible layers were found. solvent holding crude metabolites were parted. The dried extract from CFS and dry powdered mycelium was collected and kept at 4°C for further use (Taufiq and Darah 2020).

5. 15 Antimicrobial activity of solvent extracts

Antimicrobial activities of different solvent fractions of CFS and powdered mat of isolateswere checked against the nosocomial pathogenic fungi and bacteria using agar well diffusion assay (Nakamura *et al.,* 2015). Different concentrations (1mg/mL, 2, 3, and 4mg/mL) of solvent fractions were prepared by dissolving in DMSO (dimethyl sulphoxide), dispensed 20µL to each well (5mm), incubated at 37^{o}C, and measured the diameters after 24h.

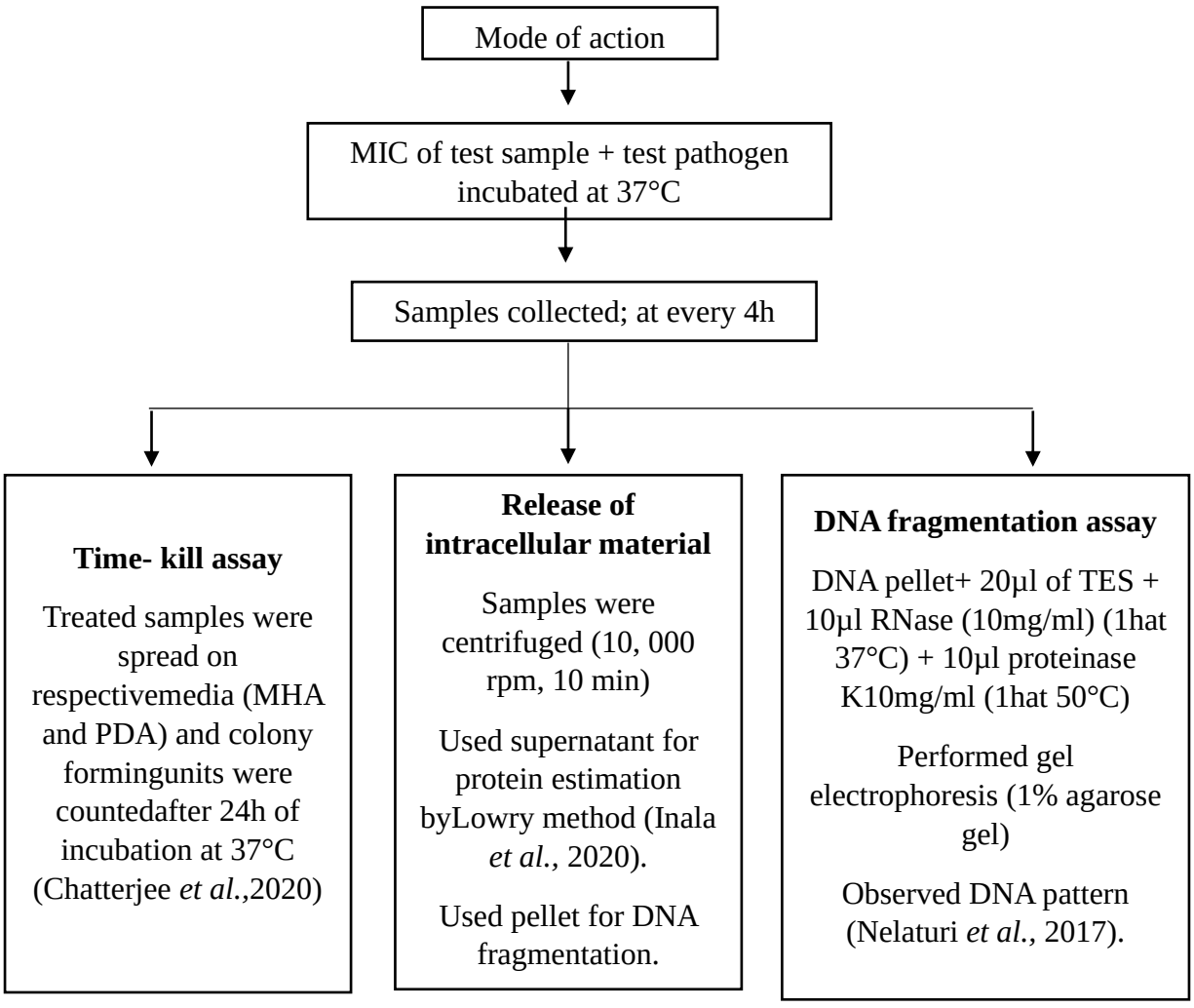
5. 16 Serial dilution assay to determine the minimum inhibitory concentration (**MIC)**

Using the serial dilution method, MIC was determined by counting the colony-forming units (CFUs) of treated pathogens present on plates and compared with untreated controls

(Bhardwaj *et al.*, 2015). The fungal crude extracts (4 mg/ml) were dissolved in DMSO, and two-fold serial dilutions were made. The plates were incubated at 37°C for 24h, the dilution that shows complete inhibition of tested strain was considered as the MIC for the respective organisms.

5. 17 Mechanism of action

Based on the evidenced article, in *in vivo*, the microorganism required a minimum of 4h to start acting as pathogens. Hence, the experiment was so planned so that all the parameters of this study would start at 4h or after 4h, 8h, 12h, 16h, 20h and 24h (Gary Kaiser, 2021).



Appendices 2- TES composition

5. 18 Antibiofilm screening

Prevention of initial and matured (pre-formed) cell attachment

To check the crude solvent extract potential to prevent primary (4h) and matured (48h) biofilm formation, biofilm inhibition assay was performed in a 96-well plate. PDB and fresh culture of pathogens were added to each well and incubated for 4 h and 48 h at 37 ^{o} C. Crude solvent extract dissolved in DMSO with different concentrations (200-1000 µg/ml with a difference of 200 µg/ml) was added to wells. Without disturbing the developed biofilm, the broth was decanted and washed with sterile distilled water. After air drying, 1% crystal violet was used for staining and kept for 10mins, followed by washing with sterile distilled water and again air dried. Added to each well the 70% ethanol with 30 min incubation with low agitation. Optical density (OD) of ethanol was measured at 595 nm using UV-Vis spectrophotometer and the inhibition percentage was determined using the equation (Theodora *et al.,* 2019; Famuyide *et al.,* 2019).

$$\text{Per cent inhibition} = \frac{\text{OD negative control} - \text{OD experimental}}{\text{OD negative control}} \times 100$$

5. 19 Antioxidant activity

In order to know the antioxidant potential of fungal extracts, DPPH (2, 2-Diphenyl-1-picrylhydrazyl) method was performed (Chatterjee *et al.,* 2019; Dhayanithy *et al.,* 2019.). The dried crude solvent extracts and gallic acid standard were dissolved at a 2mg/ml concentration in methanol. Aliquots of different concentrations of extracts (50 µg/ml to 250 µg/ml with a difference of 50 µg/ml) were mixed with 3 ml of methanolic DPPH solution (0.1mM). The samples were mixed thoroughly, followed by 30 min incubation in the dark. The absorption was read at 517 nm against DPPH as blank. Using the given formula, the

percentage of inhibition (%) was calculated, a graph was plotted and IC50 values were calculated.

Blank: Absorbance of DPPH without test sample, Sample: Absorbance of the test sample with DPPH.

$$\text{Per cent inhibition} = \frac{\text{Blank} - \text{Sample}}{\text{Blank}} \times 100$$

5. 20 Chemical profiling by GC-MS and LC-MS analysis

Gas Chromatography-Mass Spectrometry (GC-MS) and Liquid Chromatography-Mass Spectrometry (LC-MS) have been described as valuable tools for metabolite identification of endophytic fungi including the expression of metabolites produced in major quantities. The solvent extract was subjected to LC-MS and GC-MS (Song *et al.,* 2019), to identify the active compounds.

5. 20a GC-MS analysis

To characterize the antimicrobial compounds of solvent extract, the same was subjected to GC-MS analysis using DB 5 MS (instrument) following ion trap technology. Helium, as a carrier gas, at a flow rate of 1.5 mL/min and an injection volume of 1 µL was used (split ratio 1:10). The injection port base temperature was maintained at 250°C. Oven initial temperature was kept at 40°C for 2 min and elevated up to 280°C and maintained for 7 min. The identification of bioactive compounds was carried out by comparing the mass spectra with data from the NIST 2017 (National Institute of Standard and Technology, US) library.

5. 20b LC-MS analysis

The dried extract was resolved in methanol for LC-MS analysis. This was performed in Bruker's impact HD Q-Tof. The micro-LC is equipped with a Thermo Syncronis C18 column (150 mm×4.6 mm×5 µm particles). Column temperature maintained at 40°C. The analysis was done at a flow rate ranging from 1 µL/ min to 2.5 mL/min with acetonitrile and water

(90:10) mixer and the MS spectra were acquired in positive ion mode. In this study, mass feature extraction of the acquired LC-MS data and maximum detection of peaks were done using the Bruker analysis software.

5. 21 Statistical analysis

In the present study, all the experiments were performed in triplicates, and mean values are calculated by using Microsoft Excel-2019. Using species as the statistical unit, the number of isolates (N) and isolation frequency for fungal species in different tissues were calculated. Species richness index (S), Margalef index (D_{mg}), Simpson's diversity index (Ds), and Menhenik (D_{mn}) were used to calculate diversity. Results were expressed as mean ± standard deviation (SD) of triplicate of measurements for the antimicrobial activity, DPPH and biofilm assay.Data were analysed using one-way analysis of variance (ANOVA) and A *P*-value of <0.05 was considered.

RESULTS

Results

6.1 Isolated fungi from the source plant

After several rounds of the surface sterilization procedure, the isolation on different media resulted in the purification of twelve endophytic fungi from 255 segments of *S. violaceum* healthy unripe and ripe fruits (fruit pulp, whole fruit, and seed) (Table 9 and Figure 5).

Table 9: Number of endophytic fungi isolated from different regions of fruits on different media

Sl. No.	Source	Media used		
		PDA	SDA	Czapek dox
1.	Crushed whole fruit	-	7	-
2.	Seed	1	-	-
3.	Fruit pulp	1	-	3

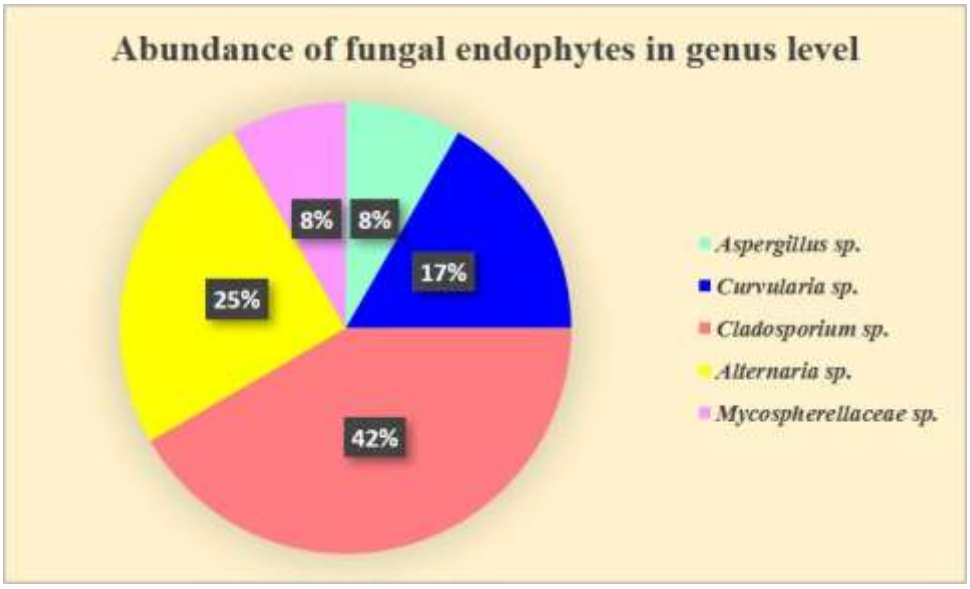


Figure 4: Relative abundance of endophytic fungi isolated from ripe and unripe fruits of *S. violaceum*

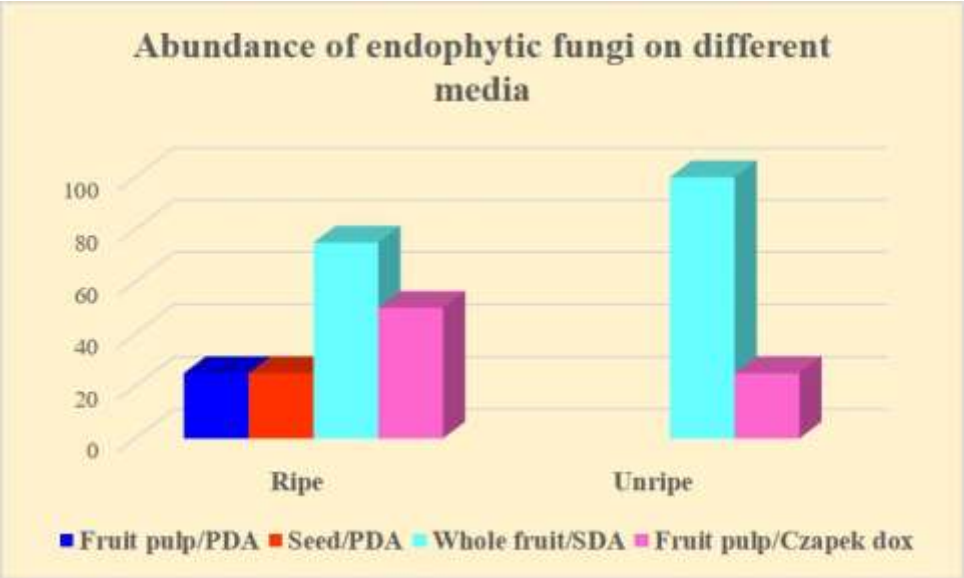


Figure 5: Per cent abundance of endophytic fungi on different media from ripe and unripe fruits of *S. violaceum*

6. 2 Identification of isolated endophytic fungi

The extracted quality DNA from twelve endophytic fungi were used for gene amplification by using ITS 1 (forward prime) and ITS 4 (reverse primer). The amplified PCR product was loaded in 1.5% agarose gel and the approximate size noted to be between 500 and 600 bp (Figure 6).

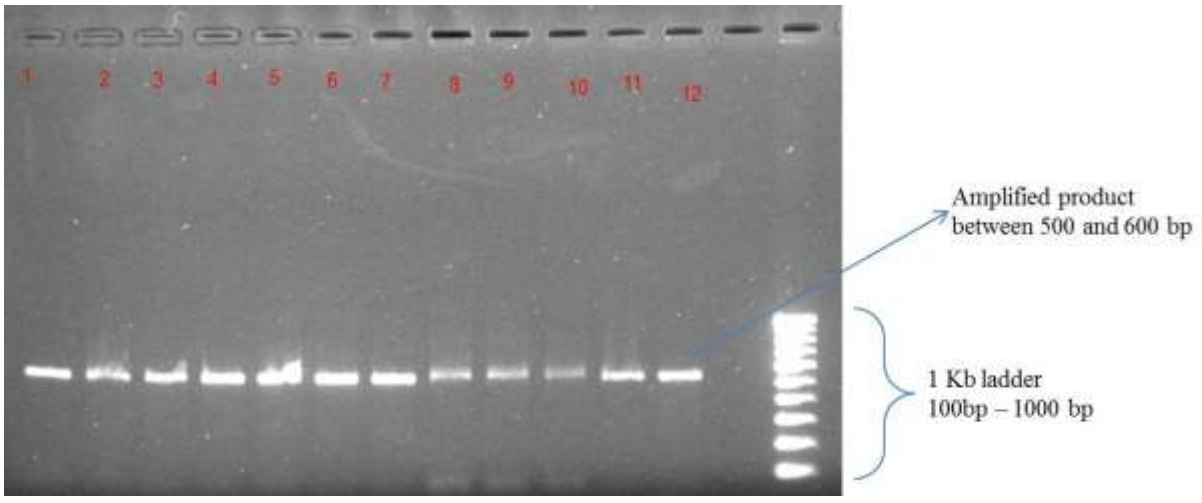
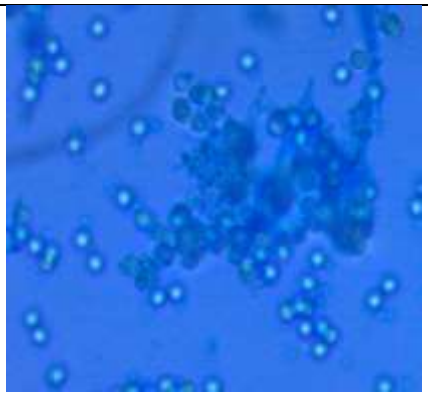
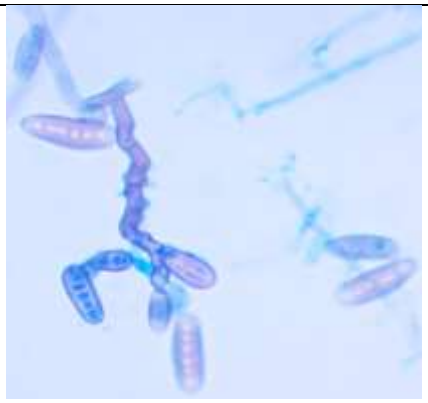


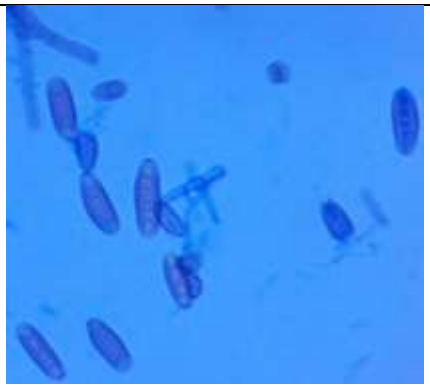
Figure 6: ITS 1 (F) and ITS 4 (R) PCR amplified product from 12 endophytic fungi isolated from *S. violaceum* in 1.5% agarose gel

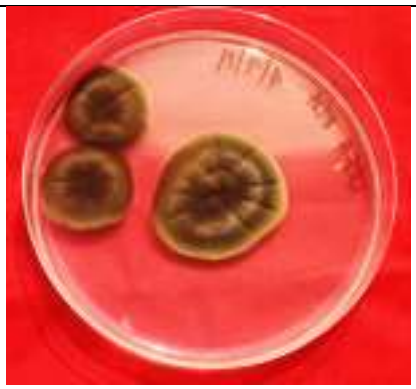
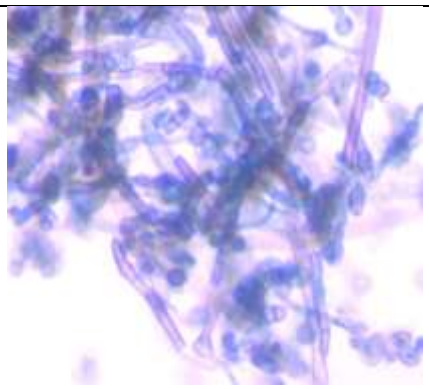
DNA sequencing of the ITS regions of rRNA, followed by comparative sequence analysis, has been the ‘gold standard’ for molecular identification of culturable fungal endophytes. In the present study molecular identification was carried out from the obtained sequences. The obtained ITS sequence of each endophyte was BLASTed against the nucleotide database of NCBI for the >95% homologous sequence. Taking into consideration the BLAST results, the species to which each endophyte belonged were determined accordingly. The identified fungal endophytes’ data is as shown in table 2. The neighbor-joining phylogenetic tree also showed its close relationship with the strains (Figure 7 and Table 10).

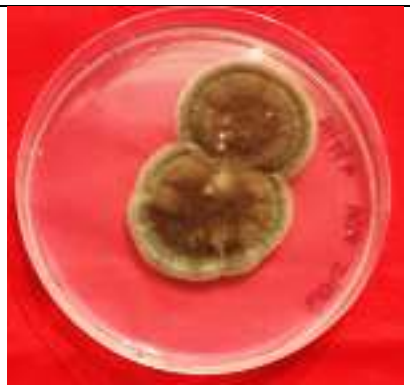
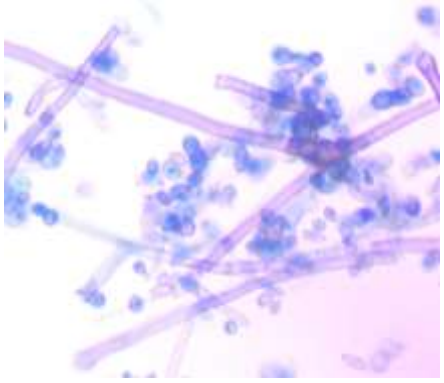
Table 10: Micro, macroscopic and molecular observation of *S. violaceum*endophytic fungal isolates with GenBank accession numbers

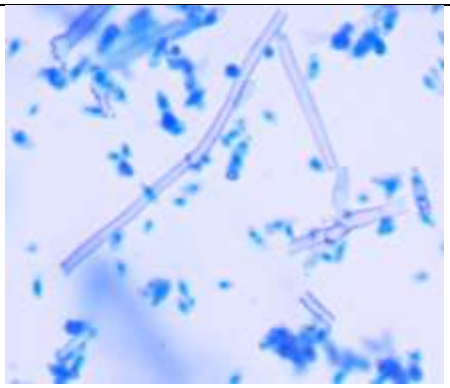
1.	GenBank accession number and name of the isolate	Homologous organism and accession number from NCBI	E- value	Per cent similarity	Division/Class
	MN784457 *Aspergillus hiratsukae*	*Aspergillus hiratsukae* strain MK336575.1	0.0	100%	Ascomycota/ Eurotiomycetes
	Fungi on plate		Stained spore under microscope		
					

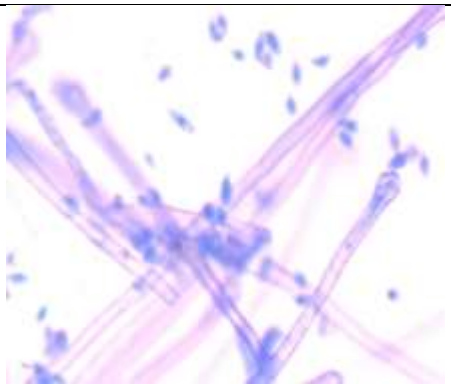
2.	GenBank accession number and name of the isolates	Homologous organisms and accession number from NCBI	E- value	Per cent similarity	Division/Class
	MN784459 *Curvularia beasleyi*	*Curvularia beasleyi* NR158442.1	0.0	100%	Ascomycota/ Dothideomycetes
	Fungi on plate		Stained spore under microscope		
					

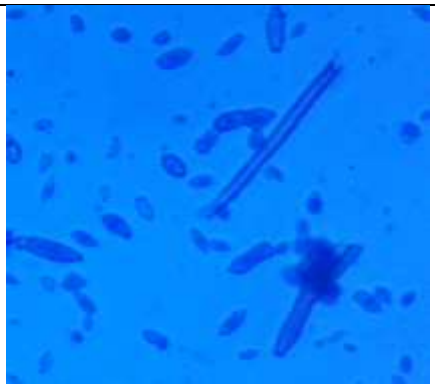
3.	GenBank accession number and name of the isolates	Homologous organisms and accession number from NCBI	E- value	Per cent similarity	Division/Class
	MN784458 *Curvularia hawaiiensis*	*Curvularia hawaiiensis* MG571422.1	0.0	100%	Ascomycota/ Dothideomycetes
	Fungi on plate		Stained spore under microscope		
					

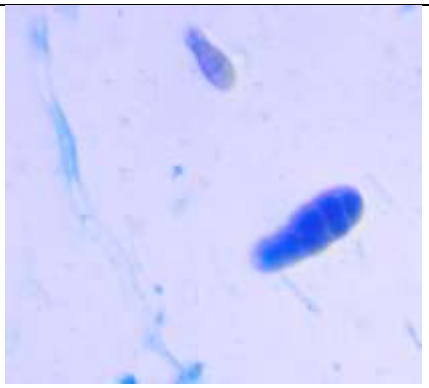
4.	GenBank accession number and name of the isolates	Homologous organisms and accession number from NCBI	E- value	Per cent similarity	Division/Class
	MN784448 *Cladosporium sphaerospermum*	*Cladosporium sphaerospermum* KP794157.1	0.0	99.00%	Ascomycota/ Dothideomycetes
	Fungi on plate		Stained spore under microscope		
					

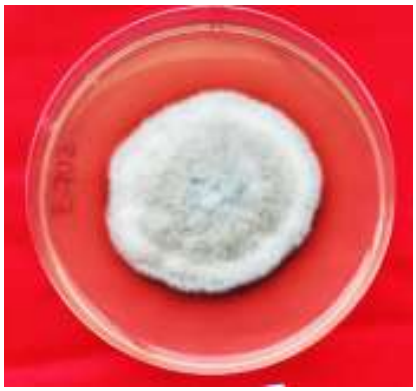
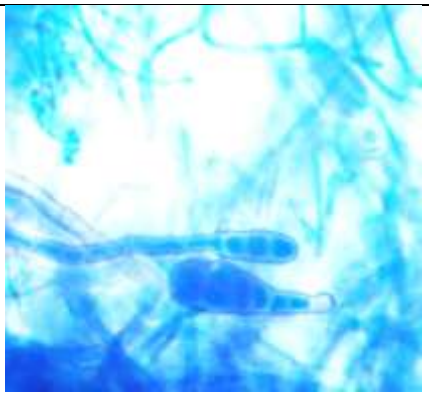
5.	GenBank accession number and name of the isolate	Homologous organism and accession number from NCBI	E- value	Per cent similarity	Division/Class
	MN784453 *Cladosporium tenuissimum*	*Cladosporium tenuissimum*strain MK957179.1	0.0	99.26%	Ascomycota/ Dothideomycetes
	Fungi on plate		Stained spore under microscope		
					

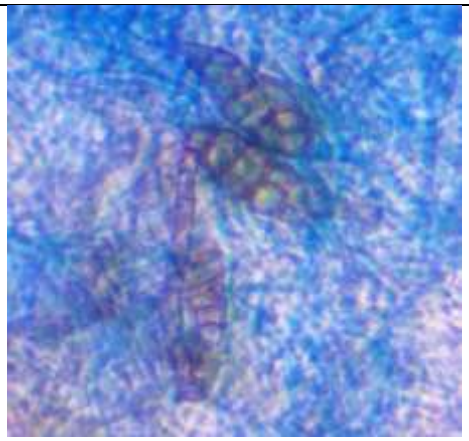
6.	GenBank accession number and name of the isolates	Homologous organisms and accession number from NCBI	E- value	Per cent similarity	Division/Class
	MN784454 *Cladosporium tenuissimum*	*Cladosporium tenuissimum* isolateMN700643.1	0.0	100%	Ascomycota/ Dothideomycetes
	Fungi on plate		Stained spore under microscope		
					

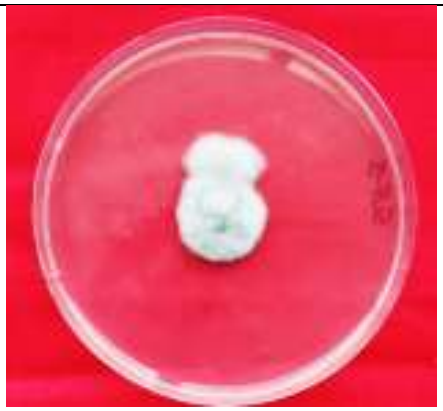
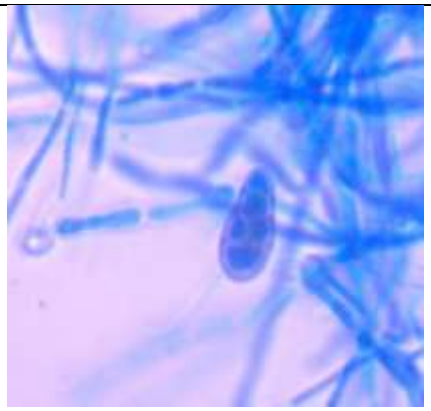
7.	GenBank accession number and name of the isolates	Homologous organisms and accession number from NCBI	E-value	Per cent similarity	Division/Class
	MN784455 *Cladosporium* sp.	*Cladosporium* sp.isolate MH325926.1	0.0	100%	Ascomycota/ Dothideomycetes
	Fungi on plate		Stained spore under microscope		
					

8.	GenBank accession number and name of the isolates	Homologous organisms and accession number from NCBI	E- value	Per cent similarity	Division/Class
	MN784456 *Cladosporium Cladosporiodes*	*Cladosporiumcladosporiodes* MG228421.1	0.0	100%	Ascomycota/ Dothideomycetes
	Fungi on plate		Stained spore under microscope		
					

9.	GenBank accession number and name of the isolates	Homologous organisms and accession number from NCBI	E- value	Per cent similarity	Division/Class
	MN784449 *Alternaria* sp	*Alternaria* sp. KC707558.1	0.0	99.02%	Ascomycota/ Dothideomycetes
	Fungi on plate		Stained spore under microscope		
					

10.	GenBank accession number and name of the isolates	Homologous organisms and accession number from NCBI	E- value	Per cent similarity	Division/Class
	MN784450 *Mycosphaerellaceae* sp.	*Mycosphaerellaceae* sp. EF060417.1	3e- 161	92.06%	Ascomycota/ Dothideomycetes
	Fungi on plate		Stained spore under microscope		
					

11.	GenBank accession number and name of the isolates	Homologous organisms and accession number from NCBI	E- value	Per cent similarity	Division/Class
	MN784451 *Alternaria alternata*	*Alternaria longipes* strain MN853398.1	0.0	100%	Ascomycota/ Dothideomycetes
	Fungi on plate		Stained spore under microscope		
					

12.	GenBank accession number and name of the isolates	Homologous organisms and accession number from NCBI	E- value	Per cent similarity	Division/Class
	MN784452 *Alternaria gaisen*	*Alternaria alternata* MT077170.1	0.0	100%	Ascomycota/ Dothideomycetes
	Fungi on plate		Stained spore under microscope		
					

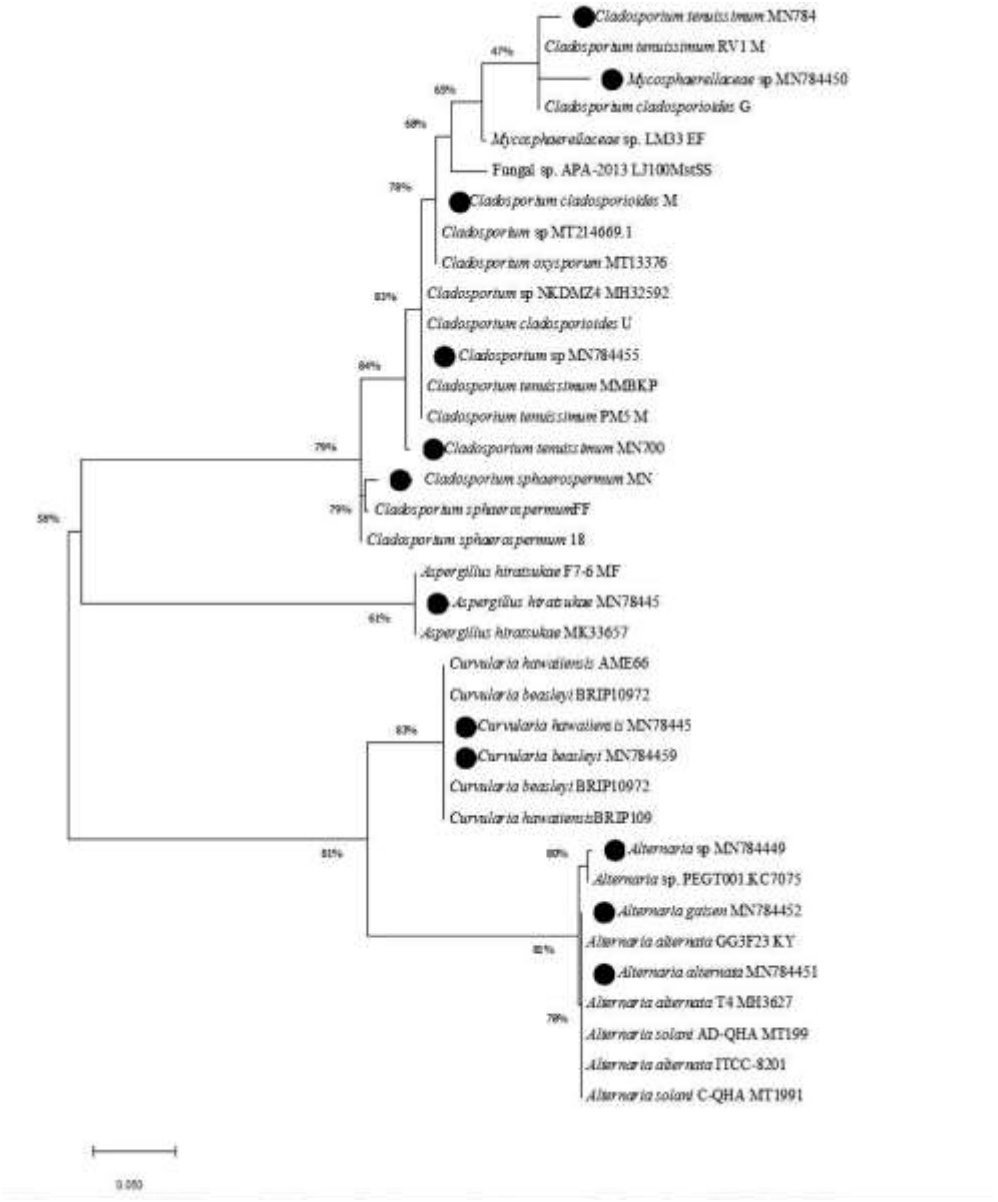


Figure 7: Neighbour-joining phylogenetic tree based on ITS rDNA sequence

6. 3 Fungal diversity analysis

All the identified organisms belonged to Ascomycota phylum. Fungi were inoculated on PDA to maintain uniformity. The rate of fungal growth was higher in ripe fruits (7) than the unripe fruits (5). *Cladosporium* sp., was having the highest colonization frequency in ripe

fruit, whereas *Alternaria* sp., was with the highest colonization frequency in unripe fruit (Figure 4).

6. 4 Diversity analysis among the fruits

Table 11 summarizes the indices related to the diversity of fungal endophytes in *S. violaceum.* In this experiment, species diversity was evaluated based on Simpson's diversity index (1-D) which is the strength of the individuals in different taxa. Along, Margalef and Menhinick indices, would reflect the richness of endophytic fungal species. The similarity between the species was calculated based on Sorensen's index of similarity.

Table 11: Common ways to measure the diversity of isolated fungi from *S. violaceum*

Indices	**Ripe**			**Unripe**		
	FP	S	WF	FP	S	WF
Species richness	3	1	2	1	0	2
Simpson's diversity	0	0	0.834	0	0	0.667
Menhenik (R1 for WF)	1.229			1		
Margalef (R2 for WF)	2.567			1.661		
Sorensen's index	0.538			0.666		

(FP-Fruit pulp, S- Seed and WF- Whole fruit)

6. 5 Qualitative analysis of phytochemicals

Dry mat powder and CFSs of the isolated endophytes were subjected to qualitative phytochemical analysis to determine the presence and absence of chemical constituents,

Dry mat powders of isolates, did not show the presence of phytochemicals except alkaloids (*A. hiratsukae, C. beasleyi, C. tenuissimum, Cladosporium* sp., *C. cladosporiodes, Alternaria* sp.,) and carbohydrates (*C. tenuissimum, C. cladosporiodes* and *A. gaisen*).

Alkaloids, carbohydrates, glycosides and saponins were noted as the major phytochemical constituents of the isolated fungal cell-free supernatants.

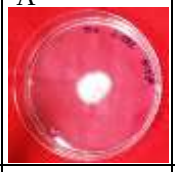
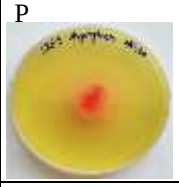
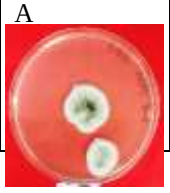
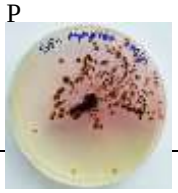
6. 6 Extracellular enzyme productions by the isolates

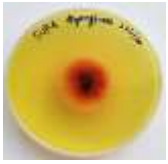
In order to establish the functional role of endophytes, it would be beneficial to establish their patterns of substrate utilization and the enzymes they produce. In the study, results revealed that each strain was able to produce one or the other enzymes. None was capable to synthesize all five (amylase, lipase, laccase, protease and asparaginase) enzymes. Out of 12 isolates, only four isolates produced a clear zone around the colony by digesting the starch, a positive result for amylase. Among these, *Cladosporium* sp. was the major amylase producer with a maximum halo of 13mm. White precipitate around the colony was indicative of lipase production in seven isolated endophytes. Among these seven isolates, *A. gaisen* was found to be a major lipase producer based on the clear zone around the colony. Asparaginase enzyme was produced by 80% of the isolates, majorly the isolate, *C. sphaerospermum*. The relative enzyme activity index is listed in Table 12. None of the isolates produced laccase and protease (Table 13).

Table 12: List of extracellular enzymes and enzyme index from isolated endophytic fungi

Sl. No	Endophytic fungi	Amylase	Lipase	Laccase	Protease	Asparaginase
		Enzyme activity (mm)				
1.	*A. hiratsukae*	0	0	0	0	20
2.	*C. beasleyi*	0	0	0	0	09
3.	*C. hawaiiensis*	0	0	0	0	10
4.	*C. sphaerospermum*	0	12	0	0	11
5.	*C. tenuissimum*	11	0	0	0	21
6.	*C. tenuissimum*	07	15	0	0	12
7.	*Cladosporium* sp.	13	16	0	0	20
8.	*C. cladosporiodes*	10	20	0	0	23
9.	*Alternaria* sp.	0	13	0	0	0
10.	*Mycosphaerellaceae* sp.	0	0	0	0	11
11.	*A. alternata*	0	20	0	0	0
12.	*A. gaisen*	0	15	0	0	05

Table 13: Screening of endophytic fungi for extracellular enzymes on solid medium

Sl. No	Isolated name	Amylase	Lipase	Laccase	Protease	Asparaginase
1.	*A. hiratsukae*	A 	A 	A 	A 	P 
2.	*C. beasleyi*	A 	A 	A 	A 	P 
3.	*C. hawaiiensis*	A 	A 	A 	A 	P 
4.	*C. sphaerospermum*	A 	P 	A 	A 	P 
5.	*C. tenuissimum*	P 	A 	A 	A 	P 
6.	*C. tenuissimum*	P 	P 	A 	A 	P 
7.	*Cladosporium* sp.	P 	P 	A 	A 	P 
8.	*C. Cladosporiodes*	P 	P 	A 	A 	P 

9.	*Alternaria* sp.	A 	P 	A 	A 	A 
10.	*Mycosphaerellaceae* sp.	A 	A 	A 	A 	P 
11.	*A. alternata*	A 	P 	A 	A 	A 
12.	*A. gaisen*	A 	P 	A 	A 	P 

A-Absent; P-Present

6. 7 Antimicrobial activity of isolates

All the obtained CFSs and agar plugs were assessed for the initial antimicrobial activity on human pathogenic nosocomial organisms. *Alternaria* sp, *C. tenuissimum*, *Mycosphaerellaceae* sp., and *A. alternata* CFSs acted against *S. aureus*, *E. coli*, and *K. pneumoniae*. Only CFS of *C. beasleyi* displayed both antibacterial and antifungal activity and the range of 10 to 22 mm in diameter inhibition was noted by the agar well diffusion method. (Figure 8).

In the agar plug diffusion method, the fungal agar plugs showed activity against only with *E. coli* and *S. aureus* (Figure 9).*A. hiratsukae against E. coli* showed 16 mm zone of inhibition. *C. beasleyi, A. hiratsukae, Mycosphaerellaceae* sp., and *Cladosporium* sp., showed a range of inhibition between 16 and 19 mm. No activity was observed against *K. pneumoniae, P. aeruginosa, C. albicans and C. tropicalis.*

In the cross-streak method, only *A. gaisen* and *Alternaria* sp.showed potent activity against the test bacteria (*E. coli* and *S. aureus*), results are similar to the agar plug diffusion assay (Figure 10) none of the endophytic fungi showed activity on pathogenic fungi in agar plug and cross-streak methods.Fungal positive control, Fluconazole, showed 22mm diameter, whereas bacterial positive control Chloramphenicol showed 20 mm diameter as inhibition zone.

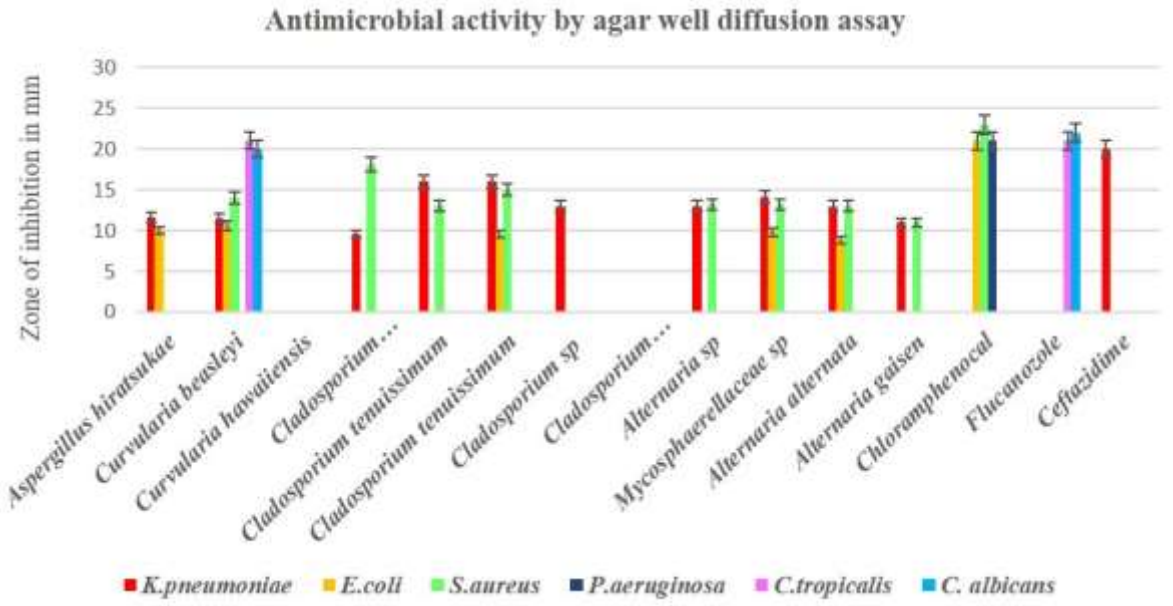


Figure 8: Antimicrobial activity of cell free supernatants of endophytic fungi isolated from *S. violaceum* on pathogenic bacteria and fungi

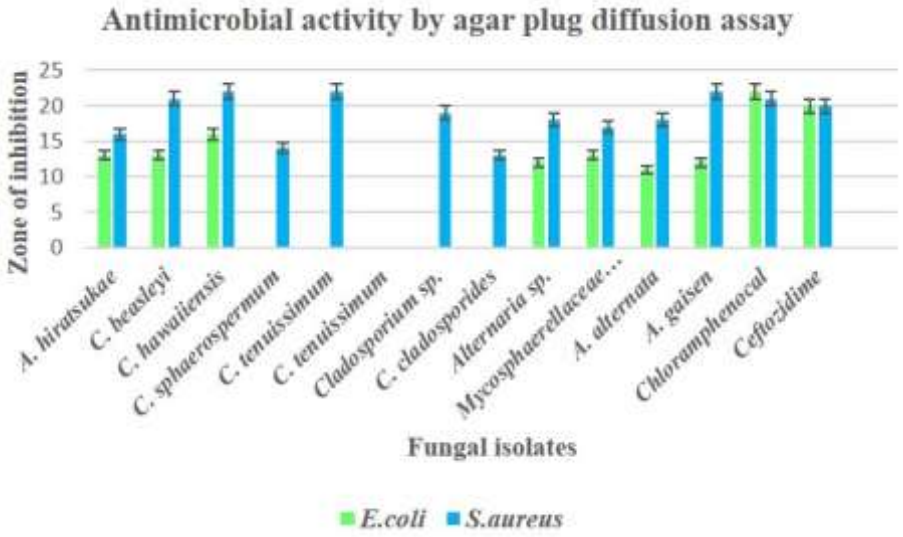
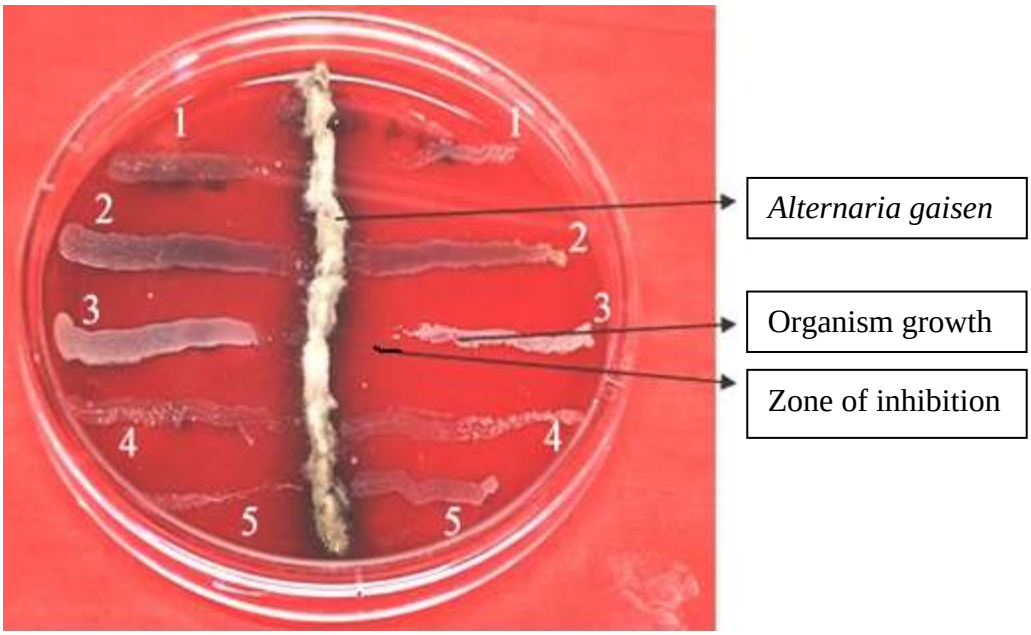


Figure 9: Antimicrobial activity of agar plug of endophytic fungi isolated from *S. violaceum* on pathogenic bacteria and fungi



1) *E. coli*, 2) *K. pneumonia*, 3) *S. aureus*, 4) *C. albicans*, 5) *C. tropicalis*

Figure 10: Cross streak method of testing antimicrobial activity of pathogenic organisms on blood agar medium

6.8 Outcome of *C. beasleyi* CFS on fungal colony forming units and morphology

Of the identified organisms, CFS of *C. beasleyi* demonstrated antifungal activity. When treated with CFS of *C. beasleyi*, the number of colonies of *C. albicans* and *C. tropicalis* was reduced in a time dependent manner. Incubation for 96 h with CFS showed no growing cell of *Candida* species. Growth was noted in 24 h and 48 h, then further declined in growth was observed in terms of number of colonies and the morphological changes of *Candida* species after 96 h treatment. No such changes were noted in controls. The lactophenol cotton blue stained mycelia of *Candida* species were observed under 40X magnification (scale bar 100µm) after 96 h to find out the changes in mycelial and spore morphology. Controls

(without any antibiotic, only pathogen in medium) of both the species were not affected, whereas both the treated mycelia became fragmented and shrunken (Figure 11a, 11b).

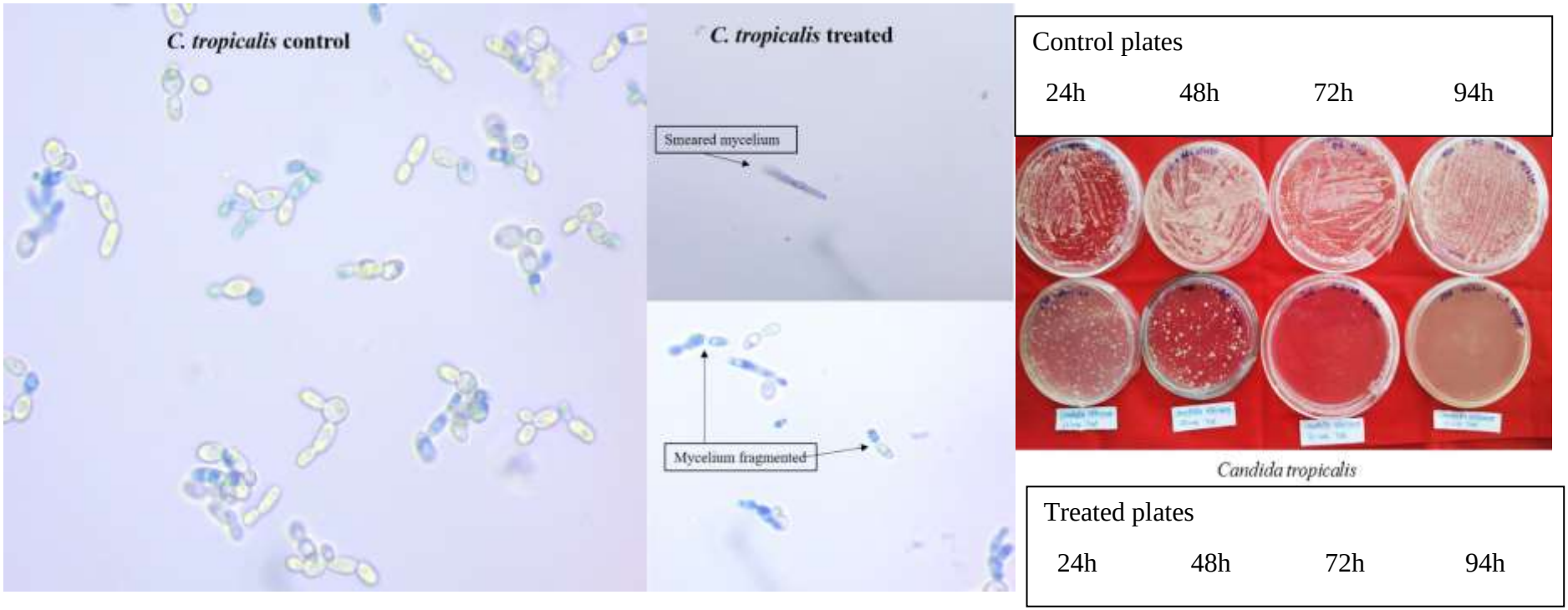


Figure 11a: Images of *C. tropicalis* fungal mycelium at 40 X after 96 hours of incubation with *C. beasleyi* extract

Arrows indicate the mycelia nature

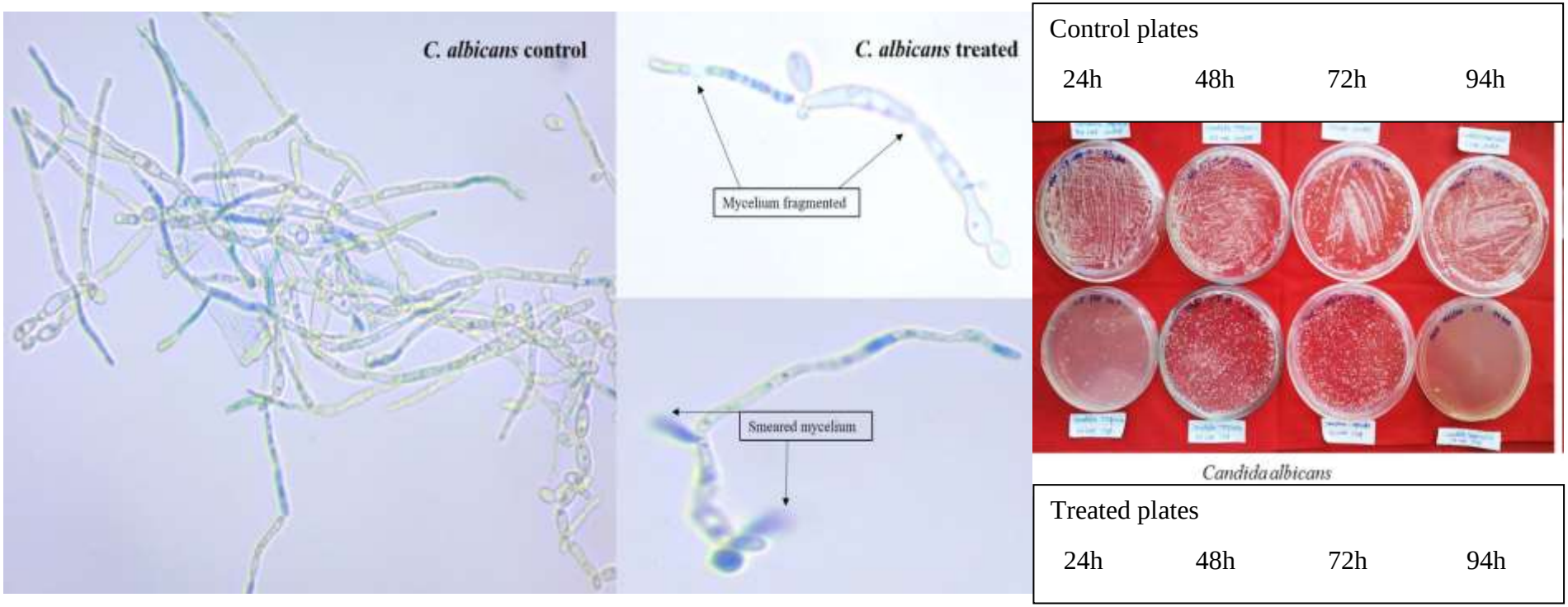


Figure 11b: Images of *C. albicans* fungal mycelium at 40 X after 96 hours of incubation with *C. beasleyi* extract

Arrows indicate the mycelia nature

6. 9 Nature of antimicrobial principle of stable cell-free supernatants

In the untreated CFS (without heat inactivation or Proteinase-K treatment), clean zones of inhibitions were noted for all the pathogens tested. No halos were observed in the control (uninoculated broth and 1 mg/ml of Proteinase-K). Halos produced by both heat-inactivated and Proteinase-K treated CFS indicated the thermo and enzyme stability nature of the antimicrobial principle(s). Based on these results CFSs were subjected to solvent extraction for further use.

6. 10 Dual plate assay

Of the 12 isolates, only *C. beasleyi* and *Cladosporium* sp. showed significant growth inhibition against the *C. albicans* Between *Cladosporium* sp. and *C. albicans*, < 1 mm clear zone, slower growth of test pathogen (*C. albicans*) and unaffected growth of endophyte (*Cladosporium* sp.) were noted.

C. beasleyi inhibited the growth of *C. albicans* significantly in dual plate assay after seven days of incubation. In a control petri dish (without endophytes) the pathogen, *C. albicans*, grew at a faster rate (2.8cm), while *C. albicans* displayed comparatively slower growth (1.5cm), when both have grown together and a clear zone was formed between the two organisms (Figure 12).

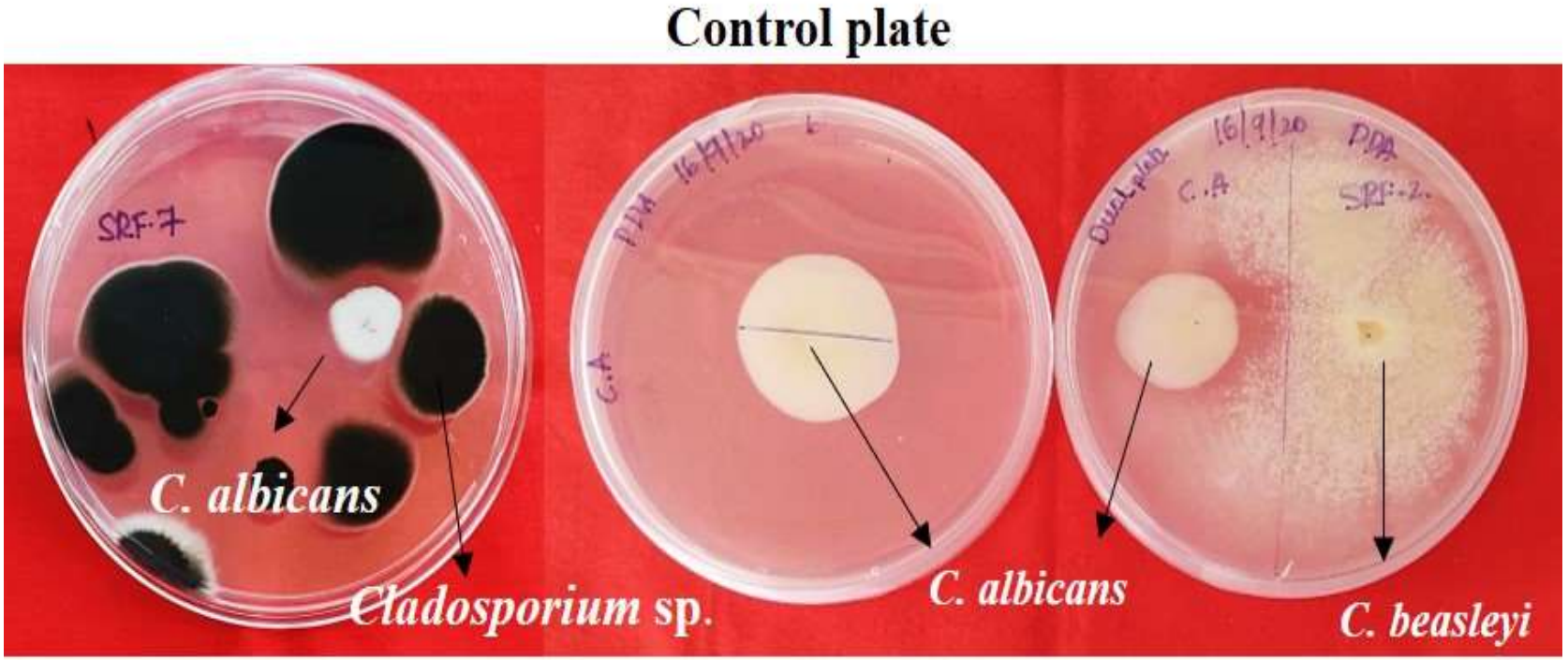


Figure 12: *Cladosporium* sp. and *C. beasleyi* inhibited the growth of fungal pathogens in dual plate assay

6. 11 Effect of volatile compounds

None of the isolated endophytic fungi were able to show inhibition against the test pathogenic fungi *C. albicans* and *C. tropicalis*. (Data not shown).

6. 12 Antimicrobial activity of solvent extracts against test pathogens

Based on the stability nature of the CFSs, the same were further subjected to solvent extraction. The outcome of antimicrobial activity of crude solvent extracts revealed that *Cladosporium* sp., *C. beasleyi*, *C. tenuissimum*, *Mycosphaerellaceae* sp., *Alternaria* sp., *A. alternata* and *A. gaisen*, showed potential antimicrobial activity (Figure 13a, 13b, 13c). The *n*-butanol extracts of *Cladosporium* sp. showed a broad spectrum and effective antibacterial and antifungal activity. The *n*-butanol extract of *C. tenuissimum* and *Mycosphaerellaceae* sp., *Alternaria* sp., *A. alternata,* and *A. gaisen*, showed activity only against *S. aureus* (11.05± 12.01) and ethyl acetate extract of *C. beasleyi* exhibited antimicrobial activity against *S. aureus* (12±0.5), *C. albicans* (13±0.2) and *C. tropicalis* (13±0.8). The *n*-butanol and ethyl acetate extracts showed a distinctive zone of inhibition than hexane extracts. None of the hexane crude extracts showed inhibitory activity against test pathogens. Chloramphenical (19±0.5), Fluconazole (22±0.8) was observed, intestingly *Cladosporium* sp. extract displayed similar zone of inhibition which is equal to standard antibiotic. The outcome gave a clear conclusion that isolates possess potent antimicrobial activity and further research was carried out to know the exact mode of action against pathogens.

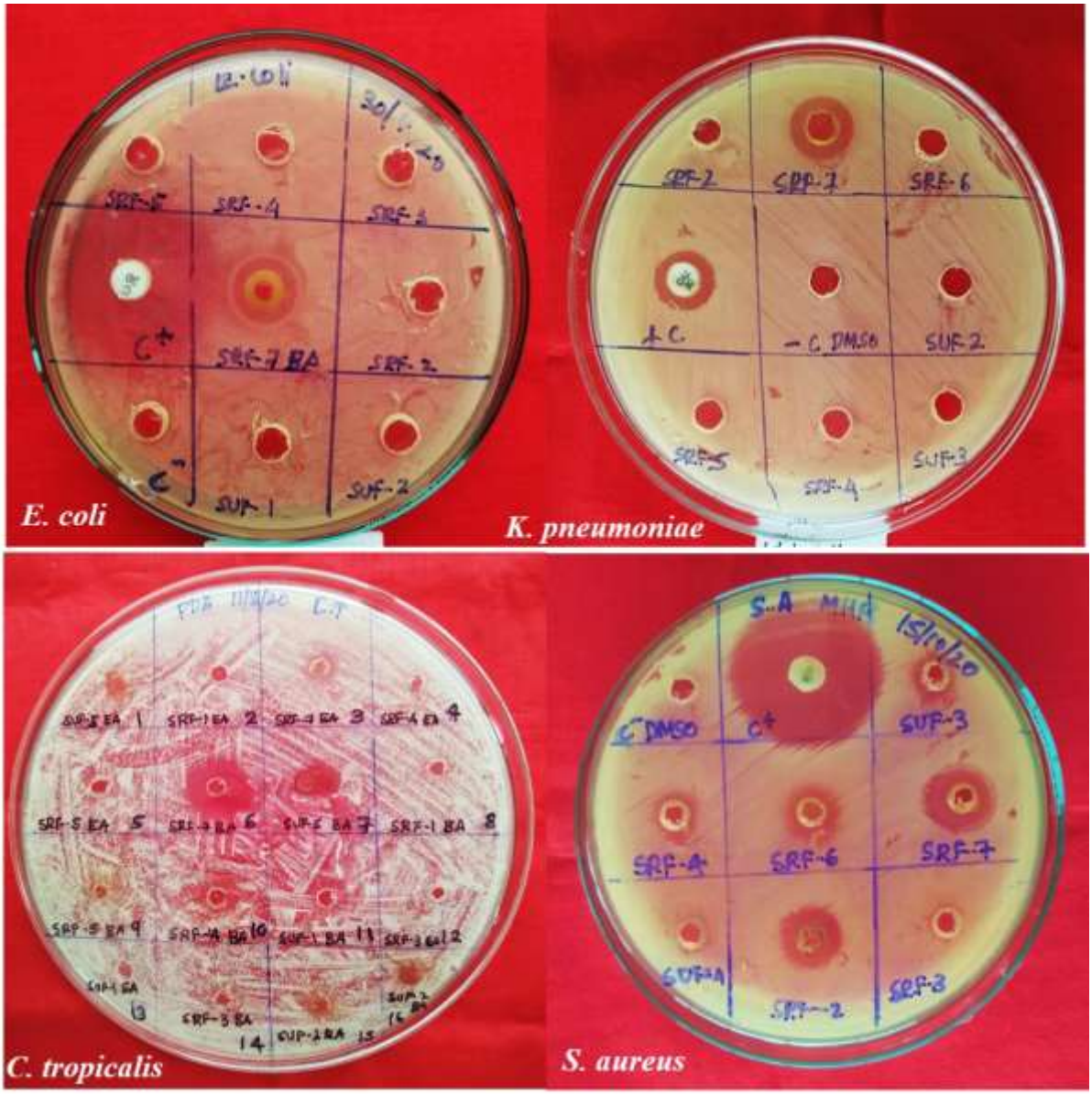


Figure 13a: Antimicrobial activity of crude solvent extracts of CFSs of *Cladosporium* sp. against test pathogens

BA- *n*-butanol, EA- Ethyl acetate, H- Hexane. SRF-1 (*A. hiratsukae*); SRF-2 (*C. beasleyi*); SRF-3 (*C. hawaiiensis*); SRF-4 (*C. sphaerospermum*); SRF-5 (*C. tenuissimum*); SRF-6 (*C. tenuissimum*); SRF-7 (*Cladosporium* sp.); SUF-1 (*C. cladosporiodes*); SUF-2 (*Alternaria* sp.); SUF-3 (*Mycosphaerellaceae* sp.); SUF-4 (*A. alternata*); SUF-5 (*A. gaisen*). Positive control (C+) Chloramphenicol and Fluconazole. Negative control (C-) DMSO

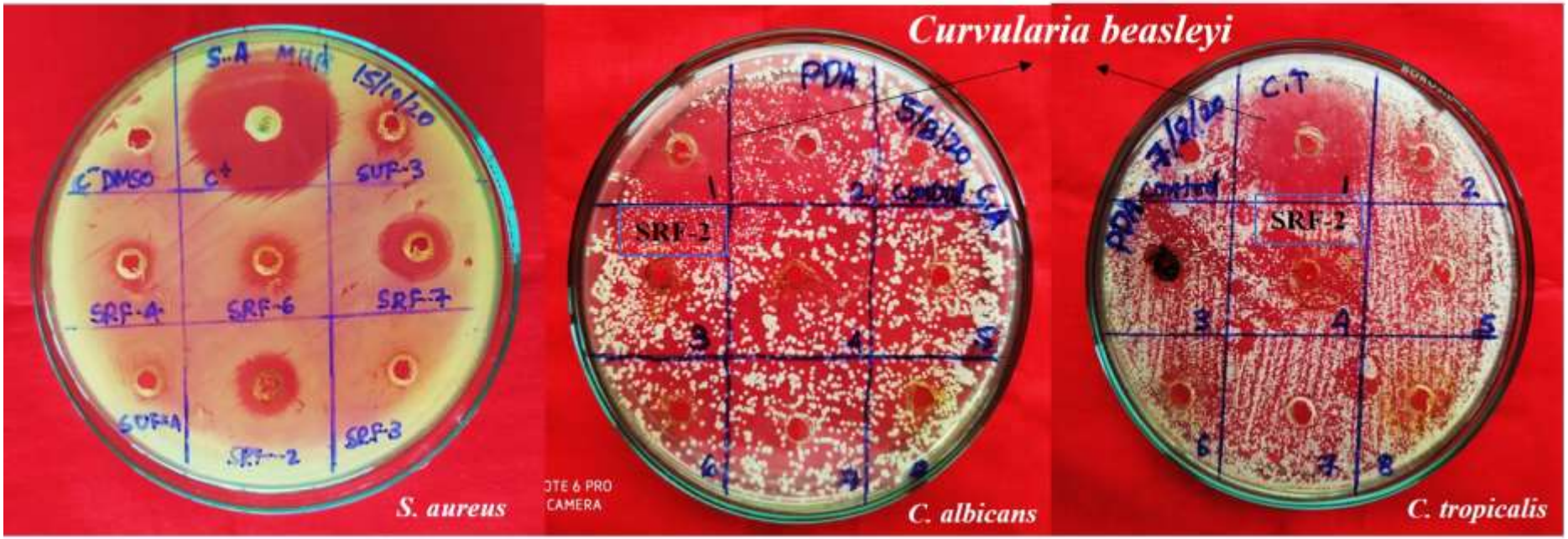


Figure 13b: Antimicrobial activity of crude solvent extracts of CFSs of *Curvularia beasleyi* against test pathogens

SRF-2 (*C. beasleyi*); SRF-3 (*C. hawaiiensis*); SRF-4 (*C. sphaerospermum*); SRF-5 (*C. tenuissimum*); SRF-6 (*C. tenuissimum*); SRF-7 (*Cladosporium* sp.); SUF-3 (*Mycosphaerellaceae* sp.); SUF-4 (*A. alternata*), Positive control (C+) Chloramphenicol, Fluconazole, Negative control (C-) DMSO

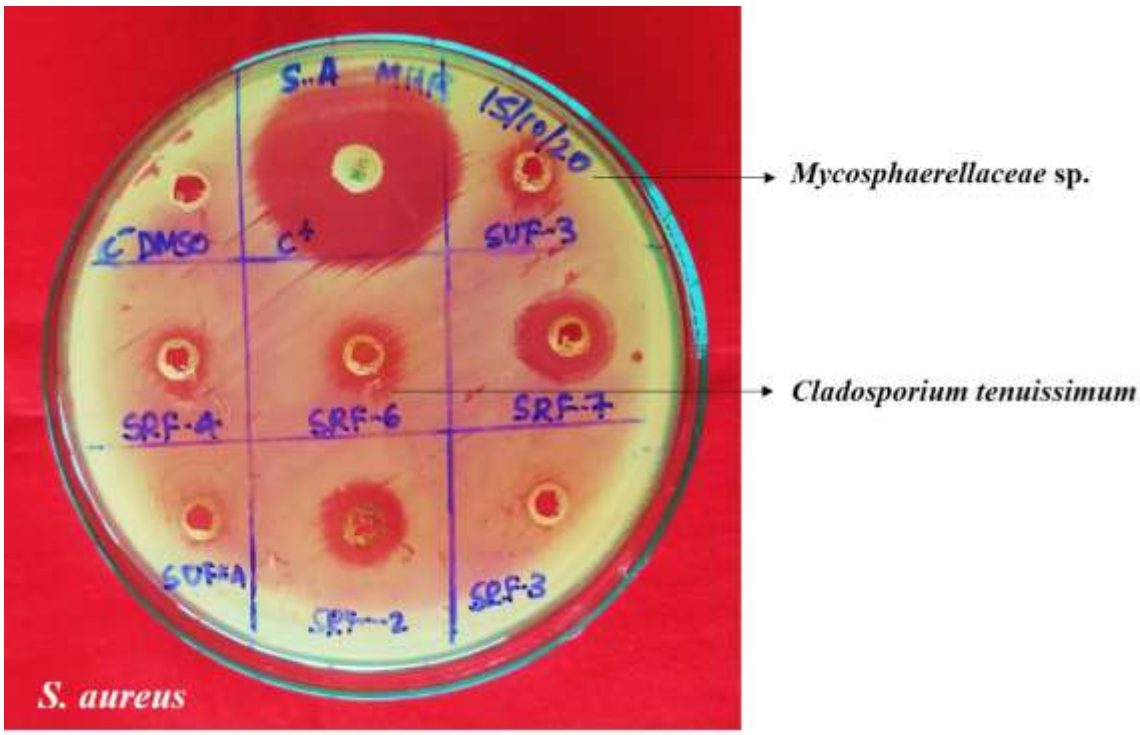


Figure 13c: Antimicrobial activity of crude solvent extracts of CFSs of *Mycosphaerellaceae* sp. and *C. tenuissimum* against test pathogens

SRF-2 (*C. beasleyi*); SRF-3 (*C. hawaiiensis*); SRF-4 (*C. sphaerospermum*); SRF-6 (*C. tenuissimum*); SRF-7 (*Cladosporium* sp.); SUF-3 (*Mycosphaerellaceae* sp.); SUF-4 (*A. alternata*); Positive control (C+) Chloramphenicol. Negative control (C-) DMSO

6. 13 Antimicrobial activity of dry mycelium solvent extracts

However, the intracellular compounds extracted by all the three solvents from dried mycelium did not inhibit the growth of tested pathogens (Figure 14a, 14b, 14c). There was no antimicrobial activity displayed by different solvent extracts which were pulled out from the fungal dry mycelium powder.

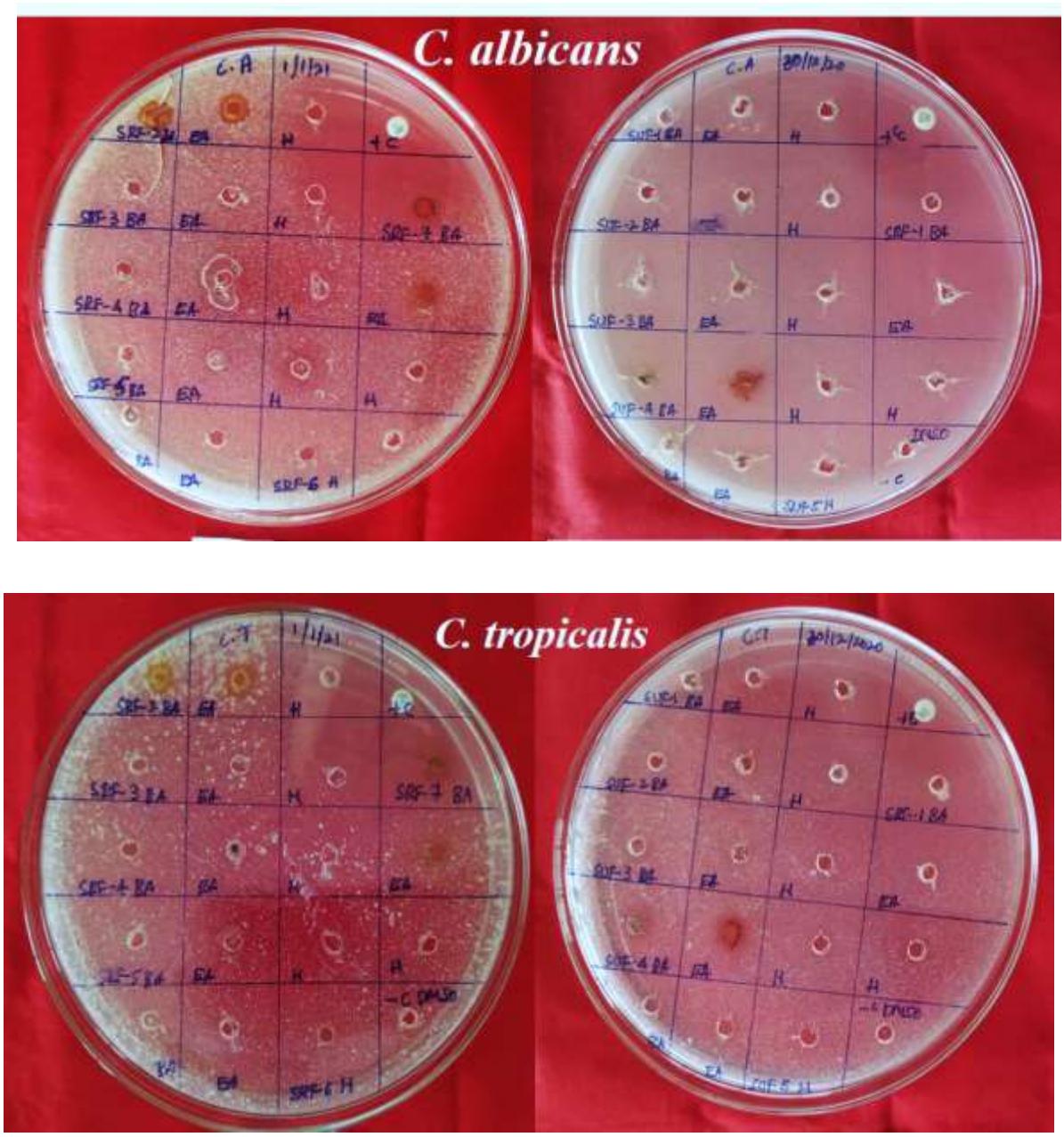


Figure 14a: Antimicrobial activity of crude solvent extracts of dry mat against *C. albicans* and *C. tropicalis*

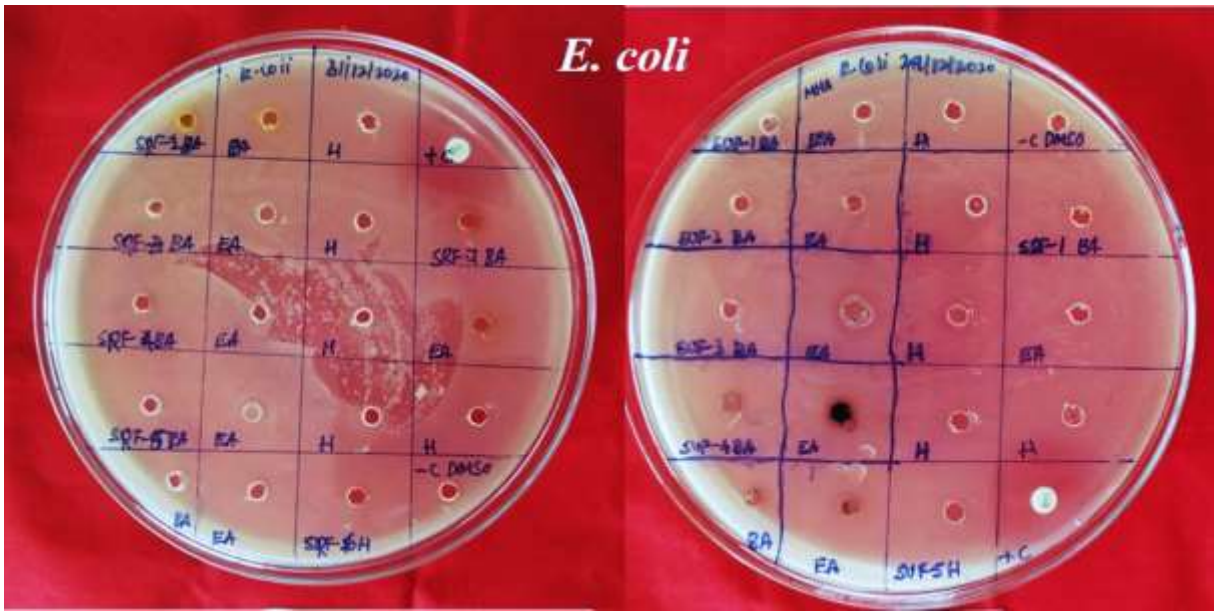


Figure 14b: Antimicrobial activity of crude solvent extracts of dry mat against *E. coli*

BA- *n*-butanol, EA- Ethyl acetate, H- Hexane. SRF-1 (*A. hiratsukae*); SRF-2 (*C. beasleyi*); SRF-3 (*C. hawaiiensis*); SRF-4 (*C. sphaerospermum*); SRF-5 (*C. tenuissimum*); SRF-6 (*C. tenuissimum*); SRF-7 (*Cladosporium* sp.); SUF-1 (*C. cladosporiodes*); SUF-2 (*Alternaria* sp.); SUF-3 (*Mycosphaerellaceae* sp.); SUF-4 (*A. alternata*); SUF-5 (*A. gaisen*). Positive control (+) Chloramphenicol. Negative control (-) DMSO

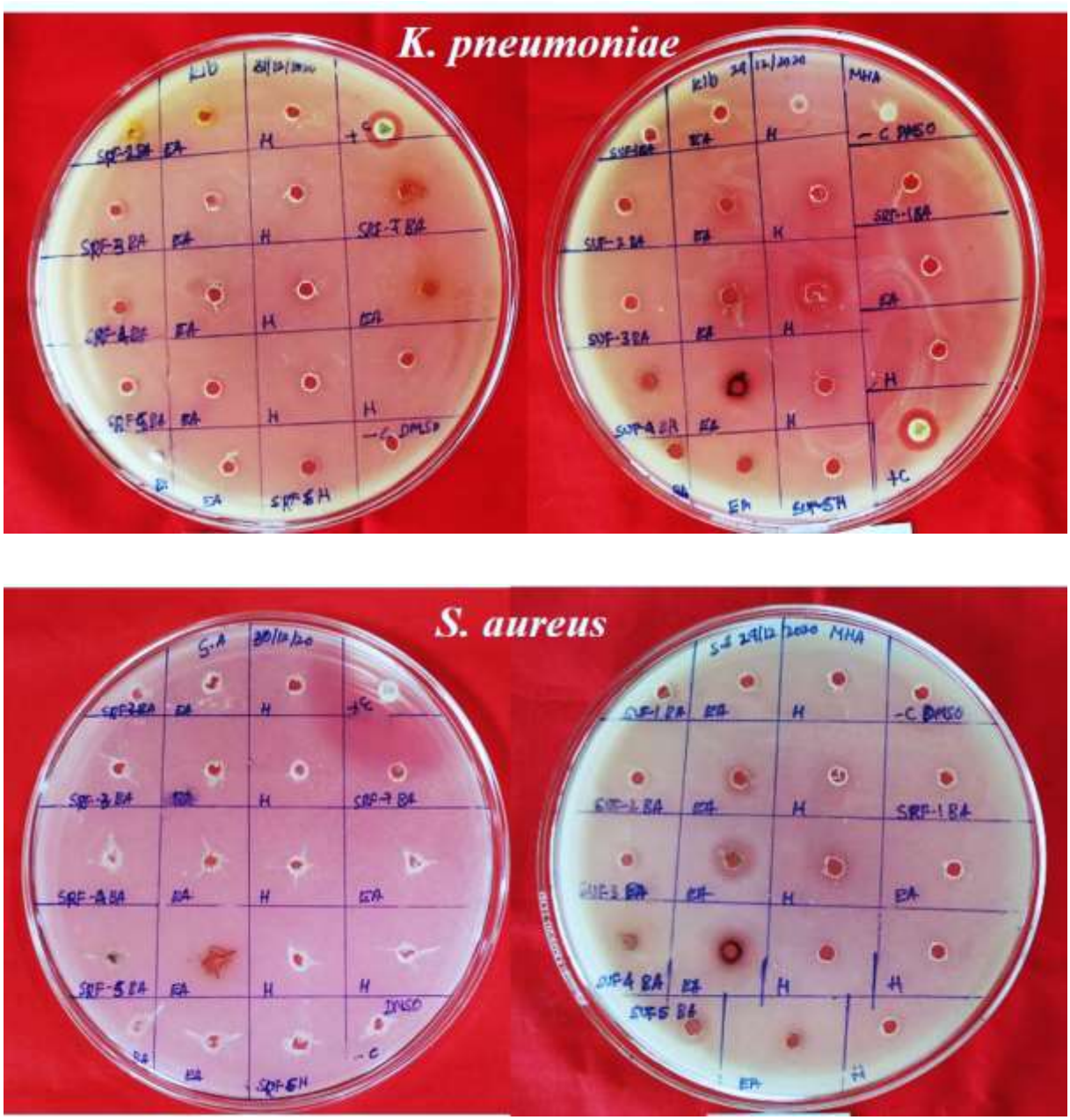


Figure 14c: Antimicrobial activity of crude solvent extracts of Dry mat against *S. aureus*and *K. pneumoniae*

BA- *n*-butanol, EA- Ethyl acetate, H- Hexane. SRF-1 (*A. hiratsukae*); SRF-2 (*C. beasleyi*); SRF-3 (*C. hawaiiensis*); SRF-4 (*C. sphaerospermum*); SRF-5 (*C. tenuissimum*); SRF-6 (*C. tenuissimum*); SRF-7 (*Cladosporium* sp.); SUF-1 (*C. cladosporiodes*); SUF-2 (*Alternaria* sp.); SUF-3 (*Mycosphaerellaceae* sp.); SUF-4 (*A. alternata*); SUF-5 (*A. gaisen*). Positive control (+) Chloramphenicol. Negative control (-) DMSO

6. 14 Selected fungal endophytes for further experiments

Based on the results of antimicrobial activity of fungal endophytes, *Cladosporium* sp., *C. beasleyi, C. tenuissimum,* and *Mycosphaerellaceae* sp., was selected for further studies. These organisms exhibited a strong broad-spectrum antimicrobial activity against a panel of nosocomial pathogens. This feature makes these species an ideal candidate to explore desirable antimicrobial compounds.

6. 15 Minimal inhibitory concentration

In experimental practice, knowing about MIC is very important for the complete bio-activity calculation of antimicrobial drugs to treat a particular pathogenic infection and to avoid drug resistance. In initial screening on the pathogens, serially diluted solvent extracts of the fermentation broth showed inhibitory activity when assayed for their MIC. The increased concentration significantly reduced the number of CFUs. The lowest concentration of solventextracts where no growth of microorganisms was observed upon visual observation after incubating at 37°C for 18h is considered as MIC value for that particular organism. The MIC of the respective organisms was given in Table 14.

Table 14: The MIC (mg/ml) values of endophytic fungi solvent extracts against pathogens

Sl.no	Fungal isolates	Test organisms	*n*-butanol crude extract	Ethyl-acetate crude extract
1.	*Cladosporium* sp.	*S. aureus*	1mg/ml	-
		E. coli	2mg/ml	-
		K. pneumoniae	4mg/ml	-
		C. tropicalis	2mg/ml	-

2.	*C. beasleyi*	*S. aureus*	-	4mg/ml
		C. albicans	-	1mg/ml
		C. tropicalis	-	4mg/ml
3.	*C. tenuissimum*	*S. aureus*	2mg/ml	-
4.	*Mycosphaerellaceae* sp.	*S. aureus*	4mg/ml	-

6. 15 Time-kill kinetics against pathogens

The respective MIC concentration was added to cultures to establish the microbicidal or microbistatic nature of the extract. Aliquots were collected at every 4h interval (4h, 8h, 12h, 16h, 20h and 24h), plated on MHA medium and CFUs were counted. The results revealed a reduced number of CFUs after adding the test compound. After 20 and 24 h treatment, the number of CFUs became zero in all the test pathogens and displayed microbicidal action. These results revealed that the number of CFUs were significantly ($P<0.05$) reduced compared to the control (Figure 15).

6. 16 Leakage of intracellular materials after treatment

For observing the effect of the solventextract on pathogen cellular integrity, protein concentration in the extracellular medium was measured. Initially, the protein concentration was almost the same in both test and control group. An increase in extracellular protein concentration was noticed in the all-treated pathogens within 8 - 12 h of incubation. However, *C. tropicalis* showed no release of proteins within 4h of incubation when treated with *Cladosporium* sp. crude solvent extract. Significant increase ($P<0.0001$) of proteins' concentration was observed upon treatment compared to 4 h to 24 h. In extracellular medium three-time more protein concentrations were observed after incubating for 24h in a treated set when compared with the control set (Figure 16). The outcome of this study displays that

crude solvent extracts could damage the structure of cell membrane, increase permeability and affect membrane transport.

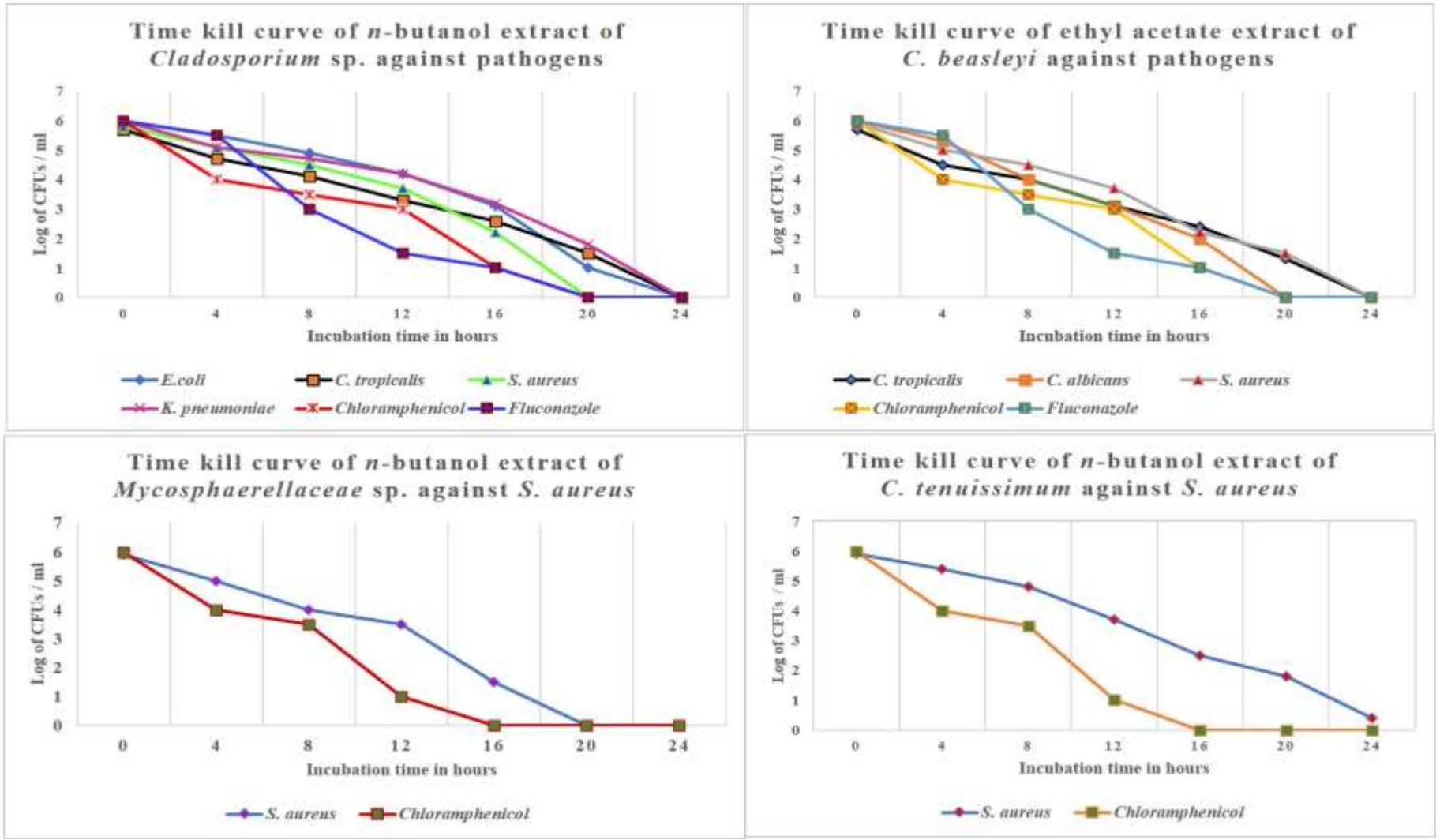


Figure 15: Time kill curve of fungal crude solvent extracts against test pathogens

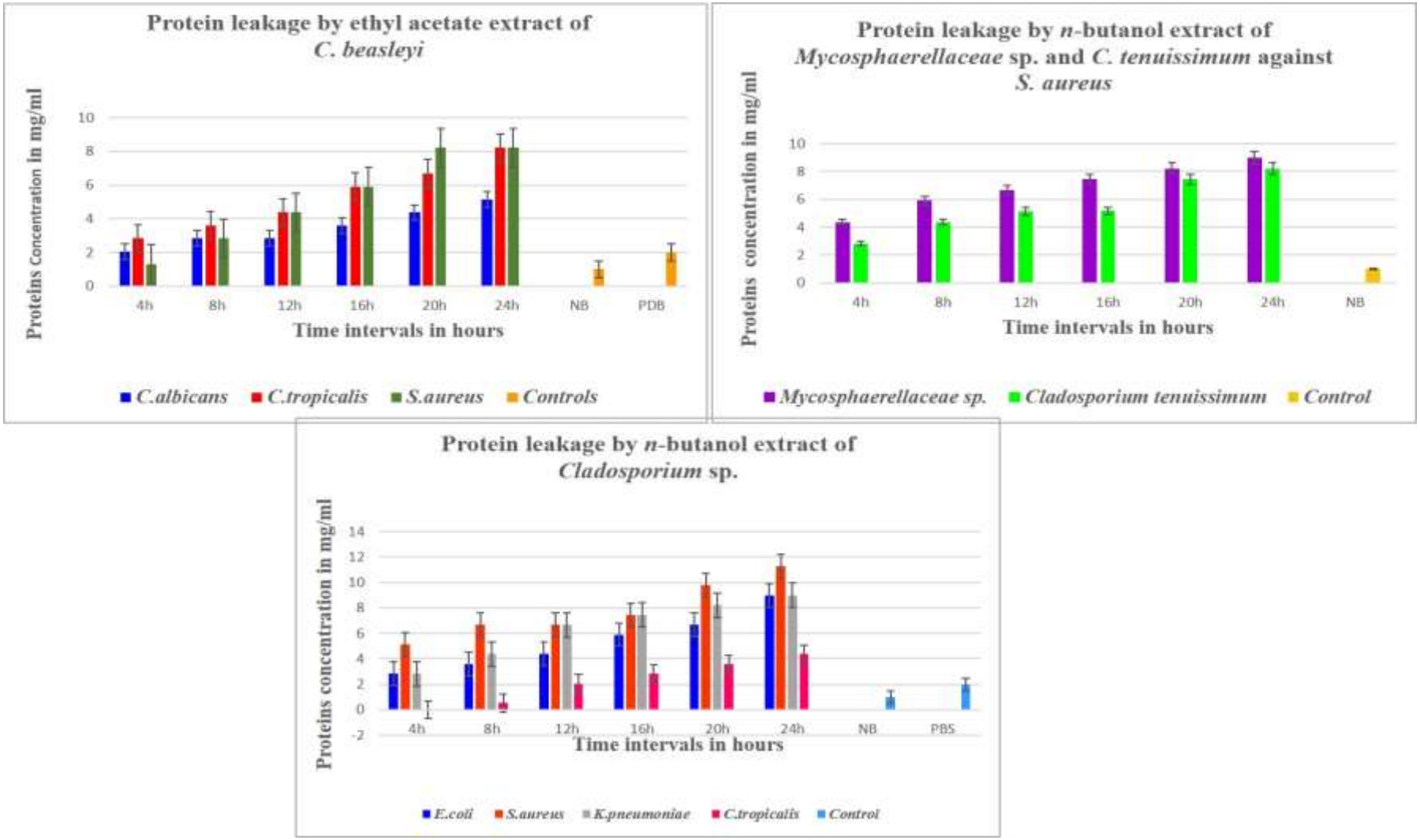


Figure 16: Release of proteins into extracellular media

6. 17 DNA Fragmentation assay

Upon treatment with solvent extracts of isolates at respective MICs, the shredded genomic DNA was observed from *E. coli*, *S. aureus,* and *K. pneumoniae*, when treated with *Cladosporium* sp., extract and compared with 24h control sets. *Curvularia beasleyi* extract showed fragmented DNA after 16h of treatment in *Candida* sp., and the extract is not effective against *S. aureus* DNA. *Mycosphaerellaceae* sp. and *C. tenuissimum*displayed smeared *S. aureus* DNA patterns at 20 and 24h respectively. This confirms the extract has effectively crossed the boundaries of the cell and fragmented the DNA (Figure 17a,17b, 17c).

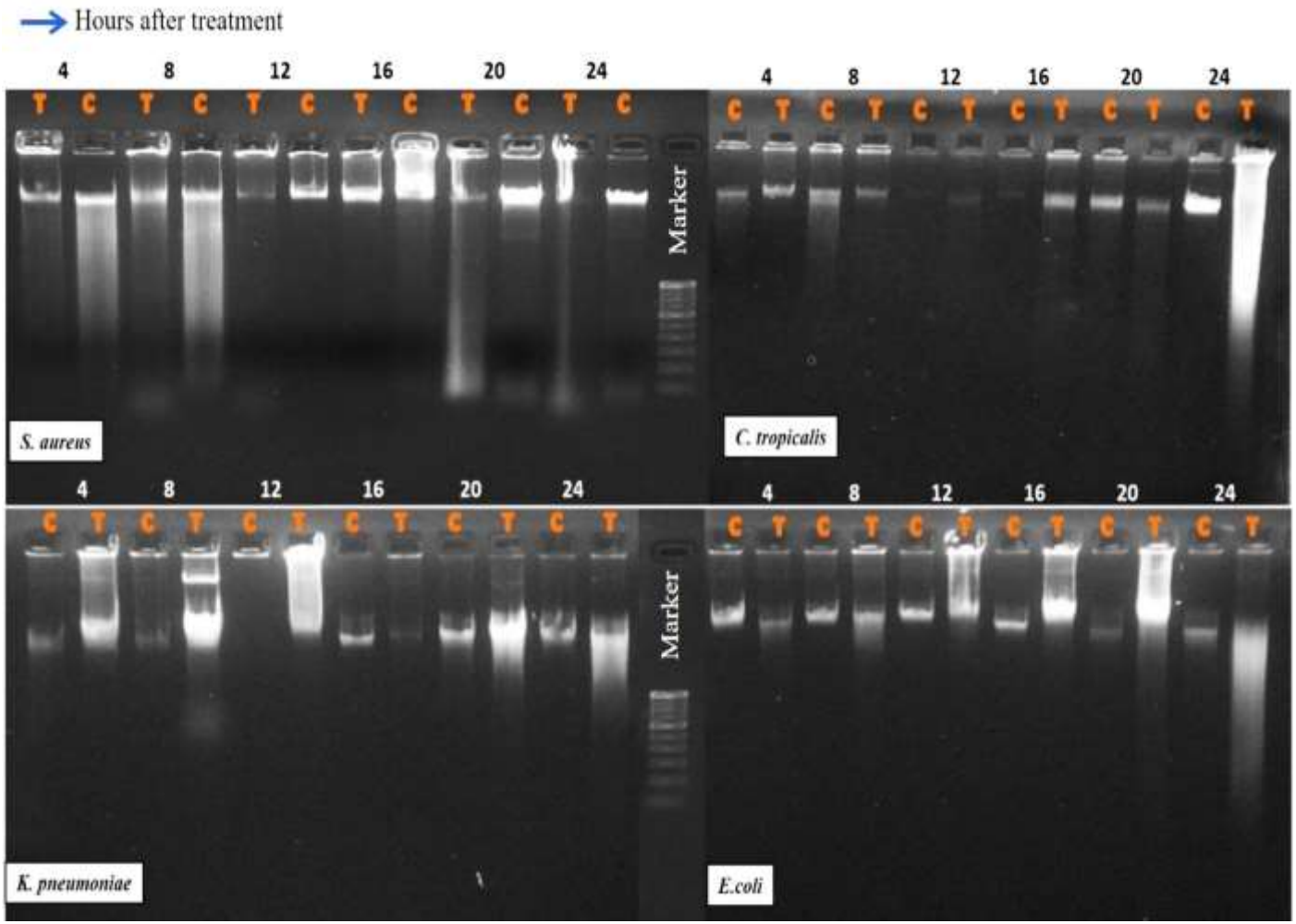
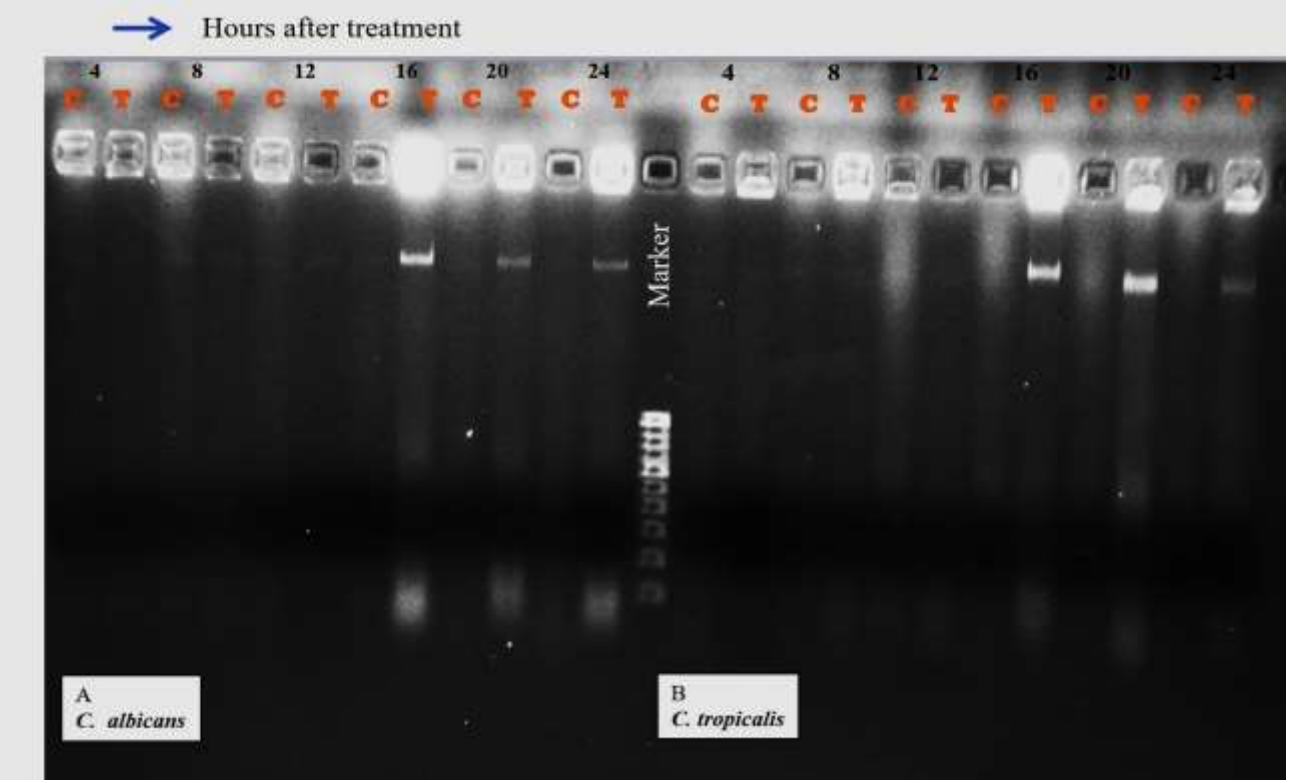


Figure 17a: Effect of crude *n*-butanol extract of *Cladosporium* sp. against a panel of pathogens DNA


Hours after treatment
4
8
12
16
20
24
Marker
A
C. albicans
B
C. tropicalis

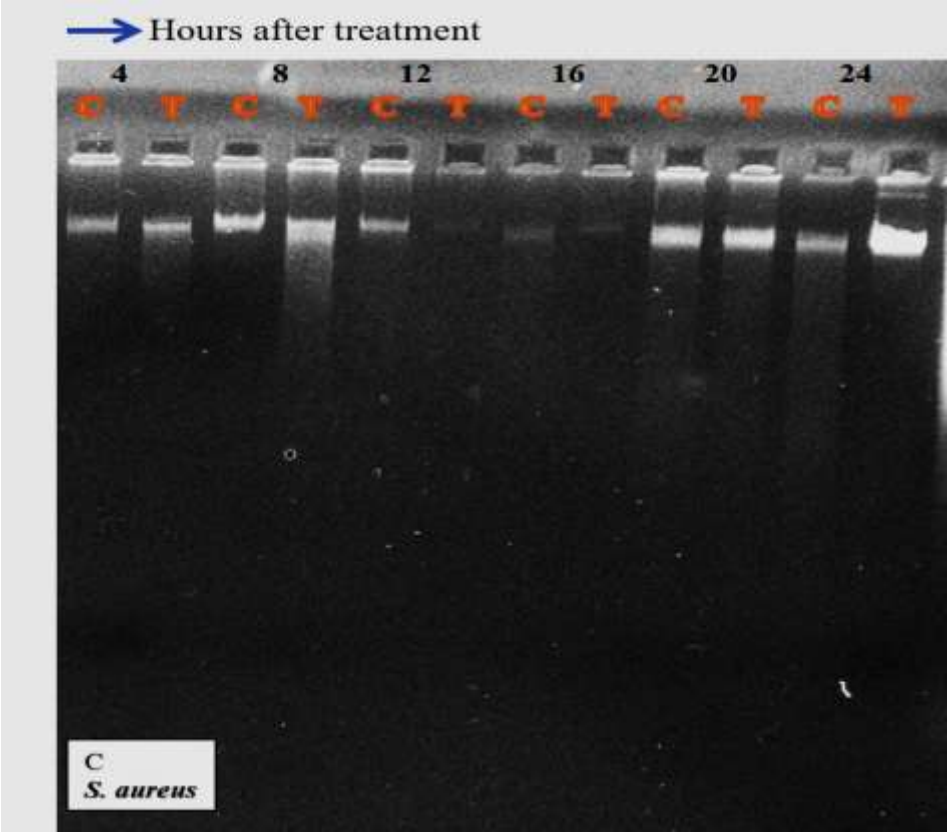


Figure 17b: Effect of crude ethyl acetate extract of *Curvularia beasleyi* against a panel of pathogens DNA

A- *C. albicans*, B- *C. tropicalis* and C- *S. aureus*

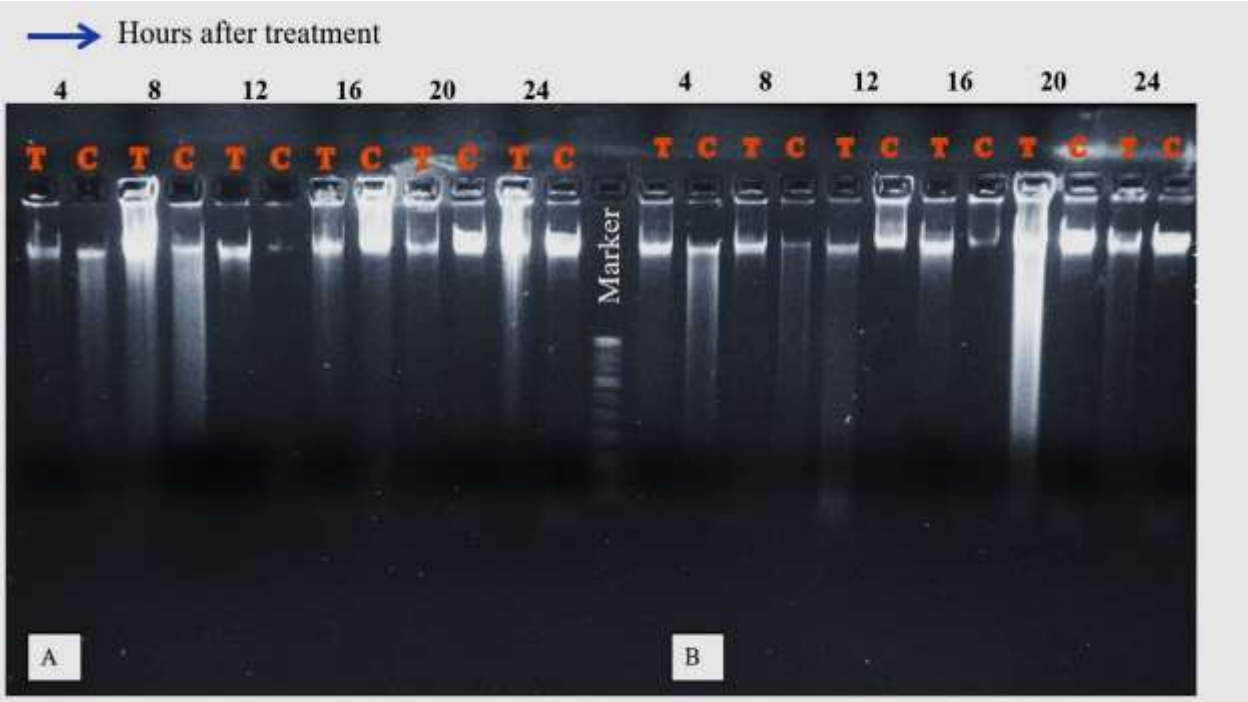


Figure 17c: Effect of crude *n*-butanol extracts of *Mycosphaerellaceae* sp. and *C. tenuissimum*against *S. aureus* DNA

6. 18 Prevention of initial and mature biofilm formation

Biofilm formation by microorganisms has an important role in the bionetwork. If the pathogenic microorganisms are present in the bio-film they can cause infection even in the presence of high a concentration of antibiotic resistance can lead to one of the main sources of disease. Controlling biofilm is an important approach.

A decrease of biofilm development after being treated with solvent extracts was observed by using crystal violet stain and the intensity was measured by UV-VIS spectrophotometer (595 nm). The crystal violet staining suggested significant ($P<0.003$) decrease of initial biofilm and no patchy colonies were observed in the treated set when compared to the control (figure 18). The extracts were effective against the initial biofilm than the matured biofilm. It proves that the developed biofilms are more resistant to antimicrobial agents than initial biofilm.

The lowest biofilm inhibition was observed in *C. tropicalis* biofilm when treated with *Curvularia beasleyi* and *Cladosporium* sp., extracts in both 4h and 48h. The highest biofilm inhibition was observed from *Cladosporium* sp. extract against *S. aureus, K. pneumoniae,* and *E. coli* with 90- 97% initial and 82- 95% of matured biofilm in 1mg/ml concentration. Extract of *C. beasleyi* effectively inhibited the initial biofilm (97%) of *C. albicans*. *Curvularia beasleyi*, *Mycosphaerellaceae* sp., and *Cladosporium tenuissimum* inhibited 82% of mature and 93% of initial *S. aureus* biofilm. The overall study concludes that extracts of isolates could effectively inhibit initial biofilm rather than matured biofilm.

(4h-Initial biofilm and 48h- Matured)

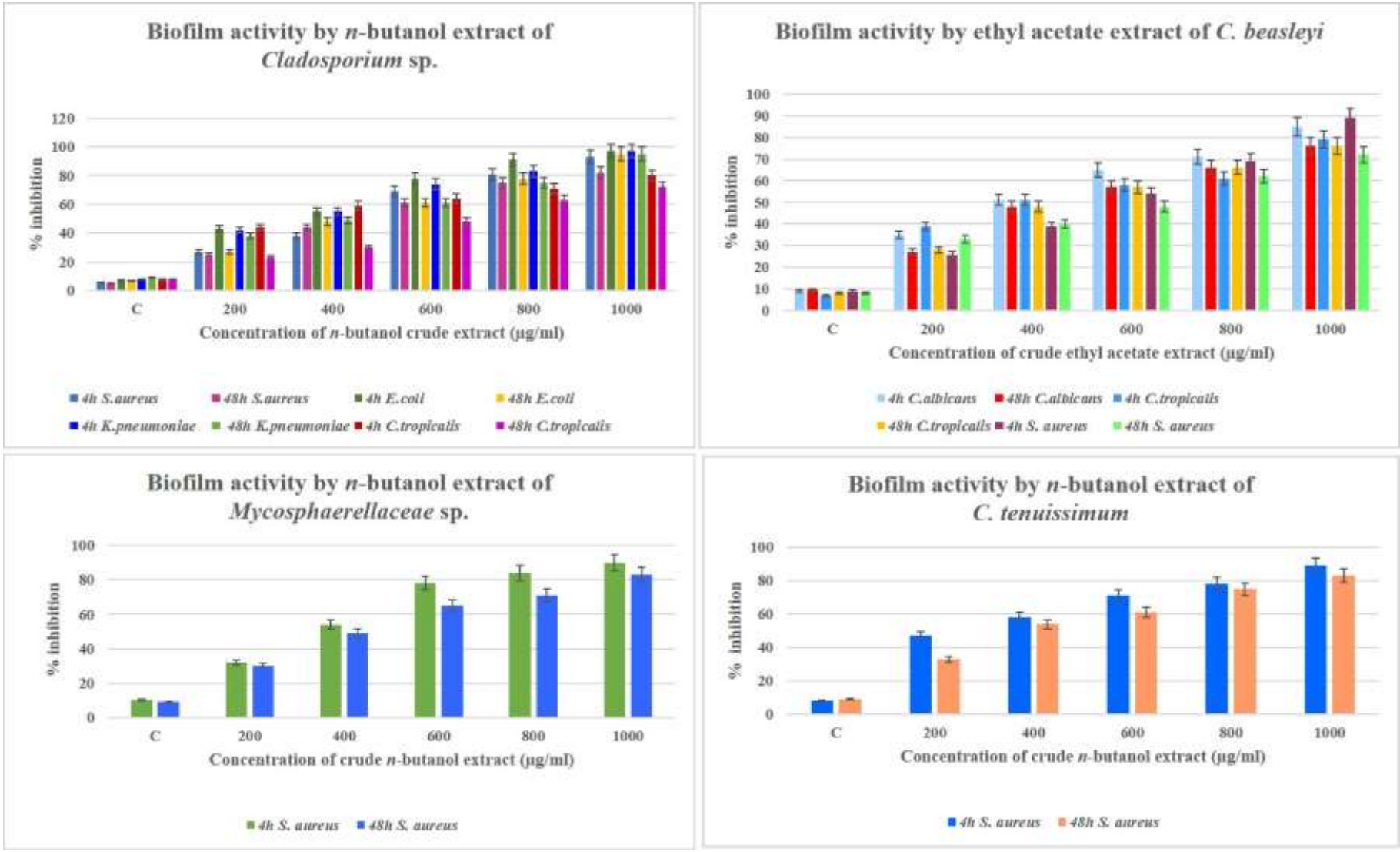


Figure 18: Anti-biofilm activity of solvent extracts against pathogens

6. 19 Free radical scavenging activity of solvent extracts

Oxidative stress is another risk factor that causes many diseases and aging in humans. The natural antioxidants derived from plants source is safe for use in food processing and /or drugs industries. To explore *in vitro* antioxidant capacity of solvent extracts of isolates DPPH scavenging percentage was calculated. The scavenging activity increased significantly with the increased concentration of fungal extract. Gallic acid was used as positive control, and it demonstrated 305.05 % antioxidant activity. Among the isolates, ethyl acetate extract of *C. beasleyi* demonstrated higher antioxidant activity than the others (Figure 19). The extracts IC_{50} (concentration of sample required to scavenge 50% of free radicals).

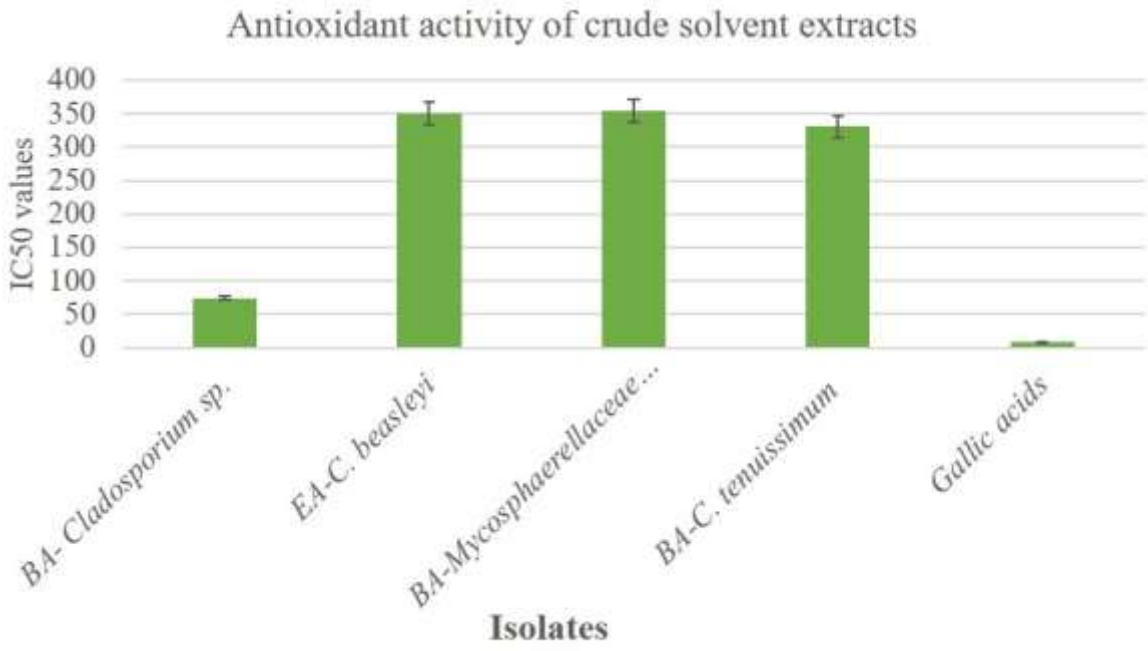


Figure 19: DPPH scavenging activity of solvent extracts (IC_{50} Value)

BA- n-butanol, EA- ethyl acetate

Identification of compounds by GC-MS and LC-MS analysis

LC-MS and GC-MS have been described as valuable tools for metabolite identification of any organism including the expression of metabolites produced in major quantities. GC-MS and LC-MS analysis was carried out to identify known and unknown metabolites of extract based on the available literature from internet. These analyses specified the occurrence of severalactive compounds. An appearance of peak and met-frag web search (LC-MS) and NIST (GC-MS) indicated the occurrence of different compounds. Retention time (RT), chemical formula, molecular weight (MW), biological activity, and chemical structure of each compound are reported.

Chemical profiling by LC-MS analysis

The majority of the metabolites were tentatively predicted using the spectral information of ions; were compared with range compounds and their *in-silico* fragments in online databases. The identified compounds are listed in table 15a, 15b, 15c,15d, and figures 12, which provide detailed information on the said compounds.

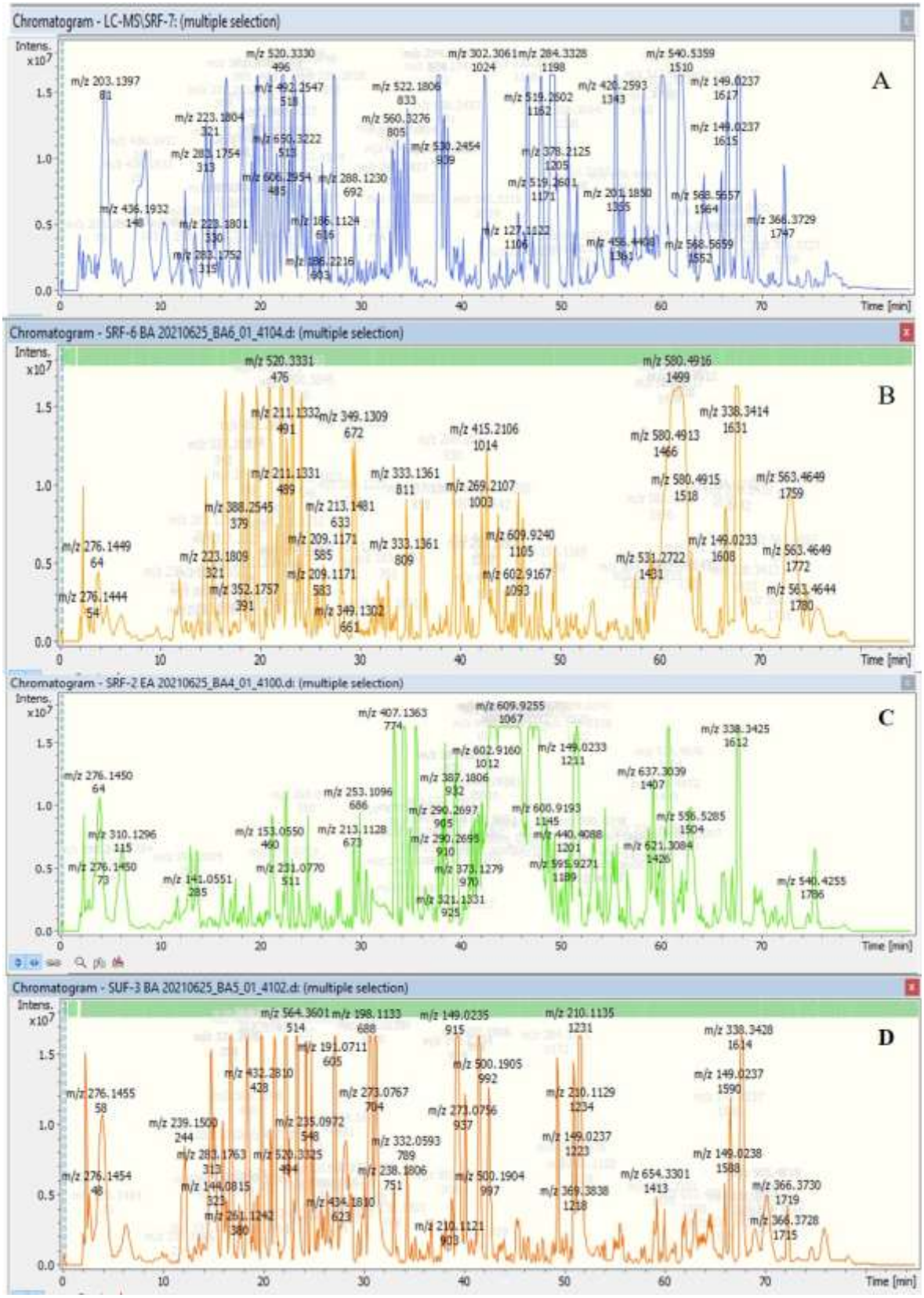


Figure 20: The total ion chromatogram of solvent extracts of isolates obtained by positive mode LC-MS analysis (A- *Cladosporium* sp., B- *Cladosporium tenuissimum*, C- *Curvularia beasleyi* D- *Mycosphaerellaceae* sp.)

Chemical profile by GC-MS analysis

The bioactive components present in the extract have been analysed using GC-MS. The chromatogram discloses the presence of various biologically active compounds (figure 21 and table 16a,16b,16c,16d).

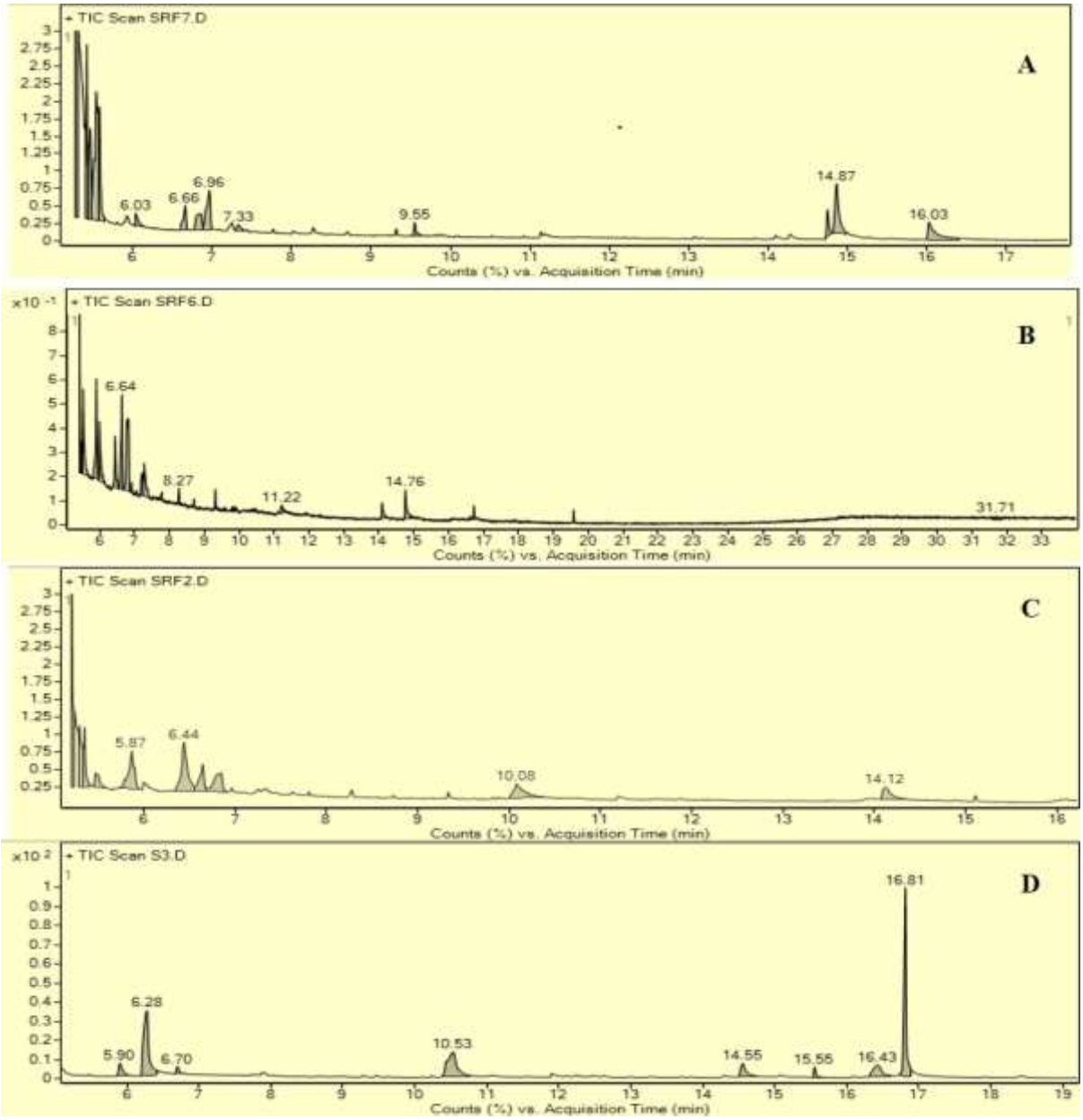
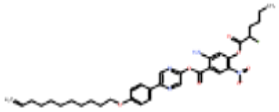
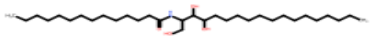
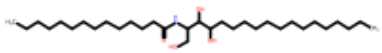
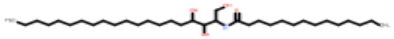
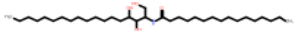
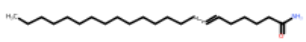
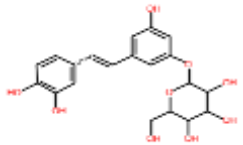
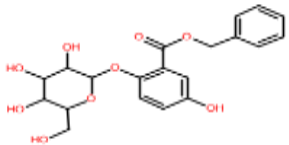
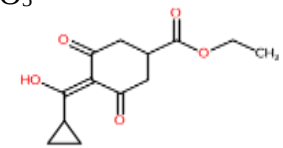


Figure 21: The total ion chromatogram of solvent extracts of the isolates from GC-MS

Table 15a: Chemical structure, molecular formula and their applications of the compounds found in ethyl acetateextract of *Curvularia beasleyi* from LC-MS analysis

Sl. No	Compound Name	Structure, Molecular mass m/z and Molecular formula	Properties
1.	[5-(4-undec-10-enoxyphenyl) pyrazin-2-yl] 2-amino-4-[(2R)-2-fluorohexanoyl] oxy-5-nitro-benzoate	$C_{34}H_{41}FN_4O_7$ 636.29 	• No activity reported
2.	N-tetradecanoyl-4-hydroxysphinganine	$C_{32}H_{65}NO_4$ 527.49  $C_{32}H_{65}NO_4$ 527.49	• Used to produce ceramides
3.	N-(2-hydroxytetradecanoyl) sphinganine		• Used to produce ceramides

4.	N-tetradecanoylicosaphytosphingosine	$C_{34}H_{69}N\ O_4$ 555.52 	• Antibacterial activity
5.	N-hexadecanoyl phytosphingosine	$C_{34}H_{69}N\ O_4$ 555.52 	• Anti-inflammatory activity. Antimicrobial and anti-biofilm activity

6.	6-cis-Docosenamide	$C_{22}H_{43}N\ O$ 337.33 	Cytotoxic activity • Isolated from *Asimina parviflora* fruit, • Cytotoxic activity against - cell line A-549
7.	Astringin	$C_{20}H_{22}\ O_9$ 406.12 	• Antioxidant activity and cancer-chemo-preventive activity.
8.	Trichocarpin	$C_{20}H_{22}\ O_9$ 406.12 	Antibacterial and antioxidant activity • Isolated from *Salix babylonica*L. Leaves
9.	Trinexapac-ethyl	$C_{13}H_{16}\ O_5$ 252.09 	Plant growth regulator. • Gibberellin biosynthesis inhibitor that decreases leaf elongation rates

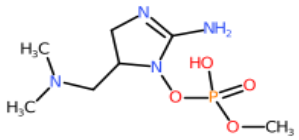
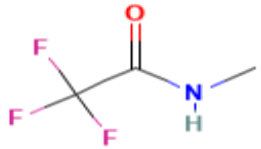
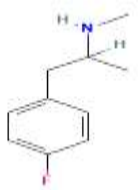
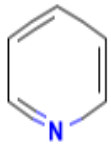
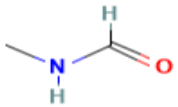
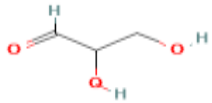
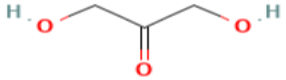
10.	Anatoxin a(s)	$C_7H_{17}N_4O_4P$ 252.09 	Neurotoxic alkaloid • **Anatoxin**-A(**s**) is the only naturally occurring organophosphorous cholinesterase inhibitor

Table 16a: **Compounds identified fromethyl acetateextract of *Curvularia beasleyi* by GC-MS analysis**

Sl. No	Compound Name	Structure, Molecular mass m/z and Molecular formula	Properties
1.	Acetamide, 2,2,2-trifluoro-N-methyl-	$C_3H_4F_3N$ 127.07 	No activity reported
2.	4-fluoromethamphetamine	$C_{10}H_{14}F$ 167.22 	No activity reported

3.	Pyridine	C_5H_5N 79.10 	Antibacterial and Antifungal activity • Targets- Lipopolysaccharide in bacteria and DNA (Koszelewski*et al.*, 2021) • Cdr1 p and Cdr2 p efflux pumps was sensitive and reduce ergosterol synthesis in *C. albicans* (Buurman*et al.*, 2004)
4.	Formamide, N-methyl-	C_2H_5NO 59.07 	• Isolated from red algae*Portieriahornemanii.* • Active against two plant bacteria (Shivakumar*et al.*, 2017)
5.	Glyceraldehyde	$C_3H_6O_3$ 90.08 	• Natural metabolite
6.	Dihydroxyacetone	$C_3H_6O_3$ 90.08 	• Used as self-tanning ingredient • Produced from *Gluconobacteroxydans* (Vu *et al.*, 2016)

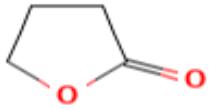
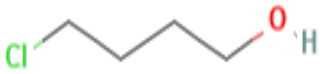
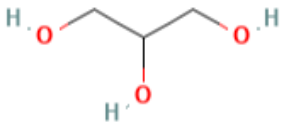
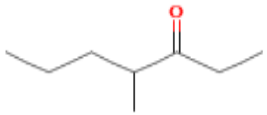
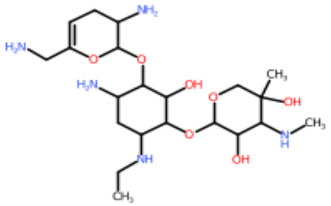
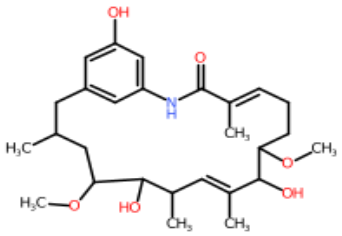
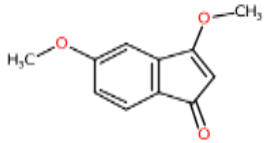
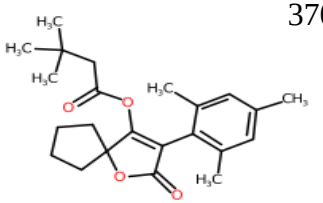
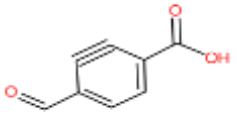
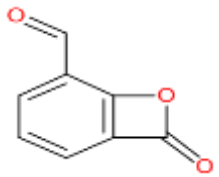
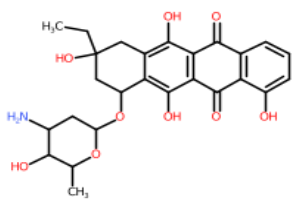
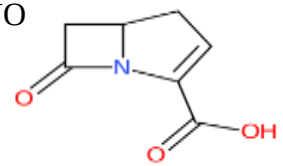
7.	gamma-Butyrolactone	$C_4H_6O_2$ 86.09 	• Helps in regulating antibiotic production and differentiation (Eriko Takano) • Bind to acyl-homoserine lactone receptor controls quorum sensing (Liu *et al.*, 2021)
8.	4-Chloro-1-butanol	C_4H_9ClO 108.57 	Antimicrobial activity
9.	Glycerin	$C_3H_8O_3$ 92.09 	Antimicrobial activity • It is generally known that glycerin can benefit the fixation of bacteria on nitrocellulose (NC) membranes. • Addition of glycerin in the spotting buffer can maintain the complete structure of the protein, retaining the activity of bacterial surface proteins by inhibiting cell dehydration.
10.	3-Heptanone, 4-methyl	$C_8H_{16}O$ 128.21 	No activity reported

Table 15b: Chemical structure, molecular formula and their application of the coumponds found in *n*-butanol extract of *Mycosphaerellaceae* sp. from LC-MS analysis

Sl. No	Compound Name	Structure, Molecular mass m/z and Molecular formula	Properties
1.	Netilmicin	$C_{21}H_{41}N_5O_7$ 475.3 	Aminoglycoside antibiotic **Target**- outer cell membrane **Results**- leakage and increased antibiotic uptake
2.	Progeldanamycin	$C_{27}H_{41}NO_6$ 475.2 	Precursor for antitumor drug – Geldanamycin **Target**- N-terminal ATP-binding domain of heat shock protein90 **Results**- inhibits its ATP-dependent chaperone activity (Martin *et al.*, 2019)

3.	Coixinden A	$C_{11}H_{10}O_3$ 190.06 	Antimicrobial activity
4.	Spiromesifen	$C_{23}H_{30}O$ 370.2 	Synthetic insecticide Targets lipid biosynthesis in insects (Bretschneider*et al.*, 2003)
5.	4-formylcyclohexa-1,3-dien-5-yne-1-carboxylic acid	$C_8H_4O_3$ 148.11 	No activity detected
6.	8-oxo-7-oxabicyclo [4.2.0] octa-1,3,5-triene-5-carbaldehyde	C_8H_4O 148.11 	No activity detected

7.	13-Deoxycarminomycin	$C_{26}H_{29}N$  499.5	Cytotoxic anthracycline antibiotic Synthesized- *Streptomyces peucetius* var. *caesius*
8.	N-methyl-N-octyl-decan-1-amine	$C_{19}H_{41}N$  299.5	No activity reported
9.	N-methyloctadecan-1-amine	$C_{19}H_{41}N$  324.5	No activity reported
10.	1-carbapenem-3-carboxylic acid	C_7H_7NO  153.14	β- lactam antibiotic **Target**- Penicillin binding proteins **Results**- continues autolysis, cell burst due to osmotic pressure

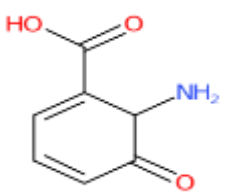
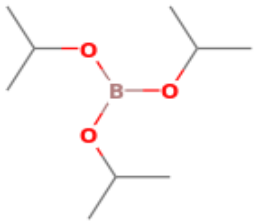
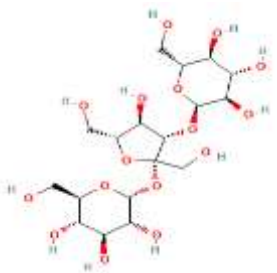
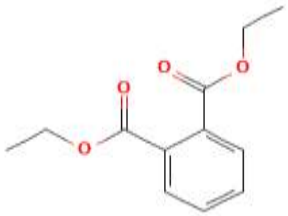
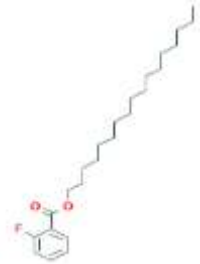
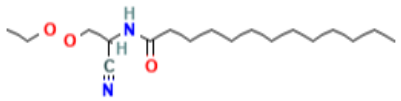
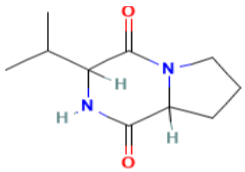
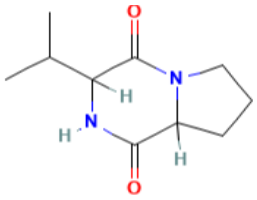
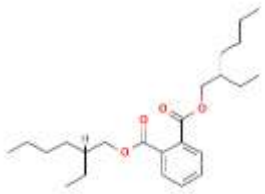
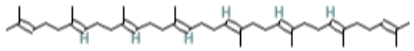
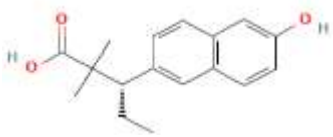
11.	2,3-dihydro-3-oxoanthranilic acid	$C_7H_7NO_3$  153.14	Precursor for antifungal agent PCN (Phenazine-1-carboxamide)

Table 16b: Compounds identified from *n*-butanol fractions of *Mycosphaerellaceae* sp. by GC-MS analysis

Sl. No	Compound Name	Structure, Molecular mass m/z and Molecular formula	Properties
1.	Boric acid (H3BO3), tris (1-methylethyl) ester	$C_9H_{21}BO_3$  188.07	No activity reported

2.	Melezitose	$C_{18}H_{32}O_{16}$  504.4	Carbohydrate
3.	Diethyl Phthalate	$C_{12}H_{14}O_4$  222.24	• Widely used as a plasticizer and softener, pharmaceutical coatings, cosmetic additives and also as an insecticide
4.	2-Fluorobenzoic acid. heptadecyl ester	$C_{24}H_{39}FO_2$  378.6	• Cause growth inhibition and apoptosis induction in human gastric cancer cells (Yu *et al.*, 2005).

5.	1-Pyridineacetic acid, 4-(aminocarbonyl)hexahydro-, decyl ester	$C_{18}H_{34}N_2O_3$ 326.5 	No activity reported
6.	Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro	$C_7H_{10}N_2O_2$ 196.2 	Antioxidant
7.	2,4(1H,3H)-Pyrimidinedione, dihydro-1-(2-(4-ethoxy-3-methylphenyl) ethyl)-	$C_{15}H_{20}N_2O_3$ 276.33 	Herbicide
8.	4,4'-Thiobis(2-methylphenol)	$C_{14}H_{14}O_2S$ 358.5 	No activity reported

9.	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$ 390.6 	No activity reported
10.	.psi.,.psi.-Carotene, 7,7',8,8',11,11',12,12',15,15'-decahydro	$C_{40}H_{66}$ 547.0 	Antimutagen, Antioxidant activity
11.	3,4,4a,5,6,7-Hexahydro-9,10-dimethoxy-2-oxo-2H-dibenzo(a,c)cycloheptene	$C_{17}H_{20}O_3$ 272.34 	No activity found
12.	Alternariol	$C_{14}H_{10}O_5$ 258.23 	Anticancer and antibacterial activity phototoxic **Target**- DNA **Results**- DNA cross-linking mycotoxin in UV light, DNA-intercalating agent (DiCosmo& Straus (1985)

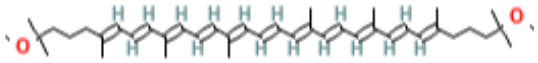
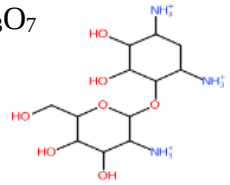
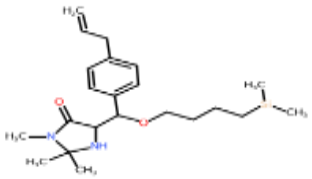
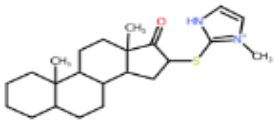
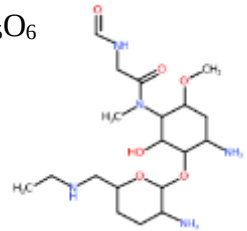
13.	.psi.,.psi.-Carotene, 1,1',2,2'-tetrahydro-1,1'-dimethoxy-	$C_{42}H_{64}O_2$ 601.0 	No activity found

Table 15c: Chemical structure, molecular formula and their application of the coumponds found in *n*-butanol extract of *Cladusporium tenuissimum* from LC-MS analysis

Sl. No	Compound Name	Structure, Molecular mass m/z and Molecular formula	Properties
1.	Heptaethylene glycol	$C_{14}H_{30}O_8$ 327.20	No activity reported
2.	paromamine(3+)	$C_{12}H_{28}N_3O_7$ 327.20 	Antibacterial activity

3.	4-[(4-allylphenyl)-[(4S)-1,2,2-trimethyl-5-oxo-imidazolidin-4-yl] methoxy] butyl-dimethyl-silicon	$C_{22}H_{35}N_2O_2Si$ 388.25 	No activity reported
4.	(10R,13R,16S)-10,13-dimethyl-16-[(3-methyl-1H-imidazol-3-ium-2-yl) sulfanyl]-1,2,3,4,5,6,7,8,9,11,12,14,15,16-tetradecahydrocyclopenta[a]phenanthren-17-one	$C_{23}H_{35}N_2OS$ 387.2 	No activity reported
5.	Istamycin C1	$C_{19}H_{37}N_5O_6$ 432.28 	Aminoglycoside analog, Antibacterial activity • **istamycinC1** ligand, which resulted in the binding energy of -8.23kcal/mol, • determined as the molecules with the best anti-cancer effect.

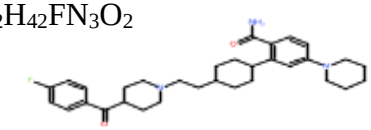
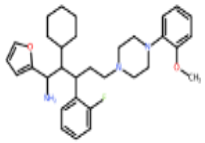
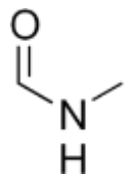
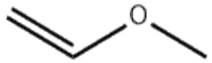
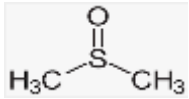
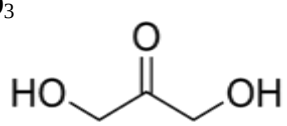
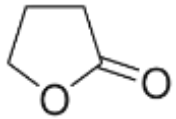
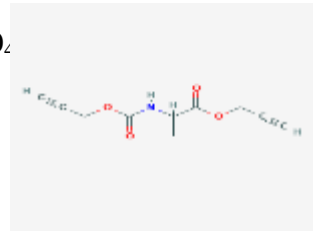
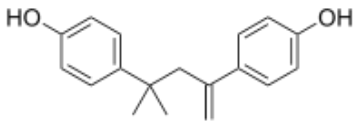
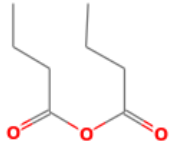
6.	2-[4-[2-[4-(4-fluorobenzoyl)-1-piperidyl] ethyl] cyclohexyl]-4-(1-piperidyl) benzamide	$C_{32}H_{42}FN_3O_2$ 520.33 	No activity reported
7.	2-cyclohexyl-3-(2-fluorophenyl)-1-(2-furyl)-5-[4-(2-methoxyphenyl) piperazin-1-yl] pentan-1-amine	$C_{32}H_{42}FN_3O_2$ 519.32 	No activity reported

Table 16c: **Compound identification from BA fraction of *Cladosporium tenuissimum* by GC-MS analysis**

Sl. No	Compound Name	Structure, Molecular mass m/z and Molecular formula	Properties

1.	Formamide, N-methyl-	C_2H_5NO  59.07	Antineoplastic agent, antioxidant
2.	Ethene, methoxy	C_3H_6O  58.07	Antimicrobial activity
3.	Dimethyl Sulfoxide	C_2H_6OS  78.14	Antioxidant activity
4.	Dihydroxyacetone	$C_3H_6O_3$  90.07	Anti fungal and Antibacterial activity
5.	Butyrolactone	C_4H_6O2  86.09	Neurotoxin ● derivatives showed the most potent antiproliferative activity against MH60 cells ● the activity was suggested

			to be due to apoptosis
6.	4-Chloro-1-butanol	C_4H_9ClO 108.57 	Antimicrobial activity
7.	Alanine, N-methyl-n-propargyl oxycarbonyl-, heptyl ester	$C_{15}H_{25}NO_4$  209.20	No activity reported
8.	-Methyl-2,4-bis(p-hydroxyphenyl) pent-1-ene, 2TMS derivative	$C_{24}H_{36}O_2Si_2$ 268.35 	No activity reported
9.	Butanoic acid, anhydride	$C_8H_{14}O_3$  158.19	No activity reported

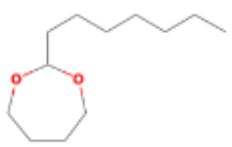
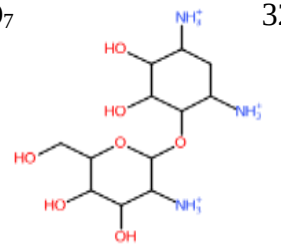
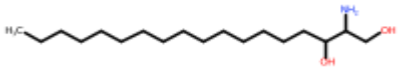
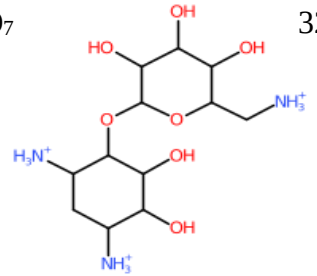
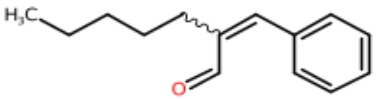
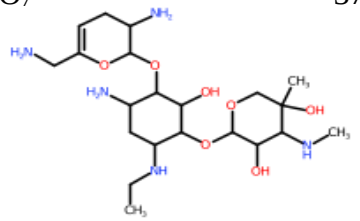
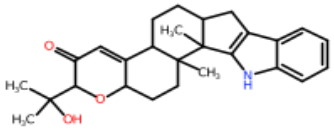
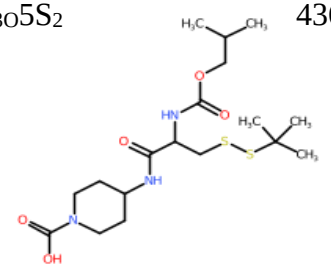
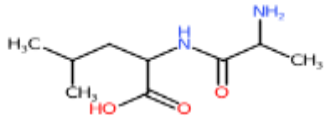
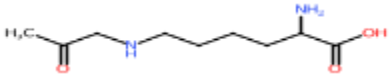
10.	1,3-Dioxepane, 2-heptyl-	$C_{12}H_{24}O_2$  200.32	Antibacterial activity

Table 15d: Chemical structure, molecular formula, and their applications of the compounds found in *n*- butanol extract of *Cladosporium* sp. from LC-MS analysis

Sl. No	Compound Name	Structure, Molecular mass m/z and Molecular formula	Properties
1.	Paromamine (3+)	$C_{12}H_{28}N_3O_7$  327.2013	Antibacterial activity

2.	Sphinganine	$C_{18}H_{39}NO_2$ 302.3063 	Antimicrobial, anti-biofilm agents and antiproliferative activity
3.	2"-deamino-2"-hydroxyneamine (3+)	$C_{12}H_{25}N_3O_7$ 327.2013 	Antimicrobial agent
4.	Alpha-amyl cinnamaldehyde	$C_{14}H_{18}O$ 203.1397 	Antibacterial, Antibiofilm, Anti-inflammatory and antitumor activity **Alpha-amylcinnamicaldehyde** caused UVA- and UVB-induced photohaemolysis.

5.	Netilmicin	$C_{21}H_{41}N_5O_7$ 376.3062 	Antimicrobial activity
6.	13-Desoxypaxilline	$C_{27}H_{33}NO_3$ 420.2593 	Antimicrobial activity
7.	4-[[3-(tert-butyldisulfanyl)-2-(isobutoxycarbonylamino) propanoyl] amino] piperidine-1-carboxylic acid	$C_{18}H_{33}N_3O5S_2$ 436.1933 	No activity reported

8.	Octaethylene glycol	$C_{16}H_{34}O_9$ 371.2270 	No activity reported
9.	Ala-Leu	$C_9H_{18}N_2O_3$ 203.13 	No activity reported
10.	N (6)-acetonyllysine	$C_9H_{18}N_2O_3$ 203.13 	No activity reported

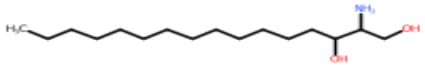
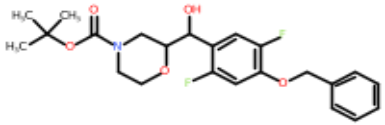
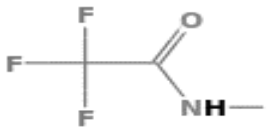
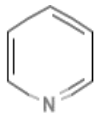
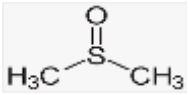
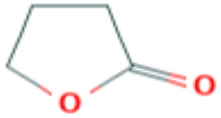
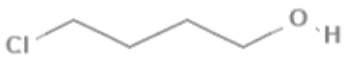
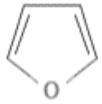
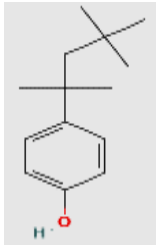
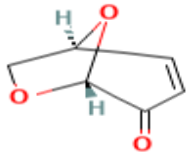
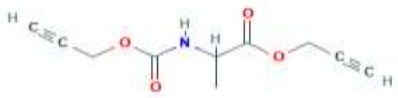
11.	Hexadecasphinganine (3+)	$C_{16}H_{35}NO_2$ 274.27 	No activity reported
12.	tert-butyl 2-[(4-benzyloxy-2,5-difluoro-phenyl)-hydroxy-methyl] morpholine-4-carboxylate	$C_{23}H_{27}F_2NO$ 436.1933 	No activity reported

Table 16d: Compounds identified from *n*-butanol fractions of *Cladosporium* sp by GC-MS analysis

Sl. No	Compound Name	Structure, Molecular mass m/z and Molecular formula	Properties

1.	Acetamide,2,2,2-trifluoro-N-methyl	$C_3H_4F_3NO$ 127.06 	No activity reported
2.	Pyridine	C_5H_5N 79.10 	Antimicrobial, Antioxidant, Antidiabetic and Antitumor activity
3.	Dimethyl Sulfoxide	C_2H_6OS 78.14 	Antioxidant activity
4.	gamma-Butyrolactone	$C_4H_6O_2$ 86.09 	Antimicrobial, Antioxidant and Antitumor activity
5.	4-Chloro-1-butanol	C_4H_9ClO 108.57 	Antimicrobial activity

6.	Furan	C_4H_4O 68.07 	Insecticidal, phytocidal, Antimicrobial, Antiproliferative and antioxidant activity • isolated from*Aspergillus flavus*, endophytic fungi from*Cephalotaxusfortunei*.
7.	4-tert-Octylphenol	$C_{17}H_{30}OSi$  206.32	No activity reported
8.	Levoglucosenone	C_6H_6O 126.11 	Antibacterial activity, Antiproliferative and antioxidant activity
9.	D-Alanine, N-propargyloxycarbonyl-, propargyl ester	$C_{10}H_{11}NO_4$ 209.20 	No activity reported

10.	Beta-D-Glucopyranose, 1,6-anhydro	$C_6H_{10}O_5$  162.14	No activity reported
11.	1,3-Dioxolane	$C_3H_6O_2$  74.078	Antibacterial, antifungal and anticancer activity • Dimeric bithiophenes containing 1,3-dioxolane or 1,4-dioxane rings could be used as novel antifungal and nematicidal agents

DISCUSSION

Discussion

7.1 Importance of fungal endophytes

Endophytic fungal secondary metabolites have been getting additional attention nowadays. The isolated compounds from these endophytes have a variety of unique structures and biological functions, indicative of having the potential to treat some diseases. Among the common endophytic microbes, the endophytic fungiare most often isolated. This solved the resource shortage problem and ecological destruction caused by the slow growers (plants) and a large amount of artificial exploitation. The most beneficial thing, they are eco-friendly.

7. 2 Biological activities of isolates

As endophytes of reproductive organs have been rarely addressed, we analysed fungal endophytes in fruits and seeds of *S. violaceum*, due to the fact that only a sub-population of endophytes colonizing the below-ground plant tissues, i.e., the root interior, can translocate to above- ground plant parts, to reproductive organs and proliferate in these tissues as discussed in Compant *et al.*, (2011).

One of the major criteria for isolating fungal endophytes was to obtain plant parts in fresh condition. In this study, isolation of endophytes was made on the day of sample collection. The standard surface sterilization method was used (Waheeda & Shyam 2017).

A total of 12 cultivable fungal colonies were isolated using three different media (SDA, PDA and Czapek Dox media). Different media support the growth of many different organisms and also the growth of individual isolates varied significantly when grown on different media. According to Murphy *et al.*, (2015) selection of suitable culture media for initial isolation and the growth of endophytes is very important. This was performed in order to assess the growth

of endophytes on different media. In a study conducted by Deka and Jha (2018) used two different media (PDA and Czapek Dox Agar) for isolation of endophytes from *Litsea cubeba* Pers. Among the two media used, PDA was found to be more suitable for endophyte isolation and growth. Since, the primary focus of our study was to find novel fungal endophytes, using three distinct fungal media, but only normal endophytes were observed. These isolates thrive in PDA and so it was selected for this study because it is largely used in mycological laboratories.

Colonies forming rate of endophytic fungi was higher in ripe fruit (seven) followed by unripe (five) in the study and all isolates belonged to the Ascomycota phylum. Porter (1991) in his findings demonstrated that the average size of the ITS region in the fungal kingdom belongs to Ascomycota, which is in agreement with our results. This result supports the finding of Rakotoniriana *et al.*, (2008) wherein fungal endophytes mainly belong to the Ascomycetes. It is the most prevalent phylum with diverse morphology. *Cladosporium* sp., had the highest colonization frequency in ripe fruit, whereas *Alternaria* sp., had the highest colonization frequency in unripe fruit. *Cladosporium*, *Alternaria* sp., were dominant in our work and it might be due to their cosmopolitan nature, which increases their chance to get accepted as endophytes (Uzma *et al.,* 2016).

The ways to measure fungal diversity are richness (number of species), relative abundance (proportion of a species in a community), and evenness (well representation of a species). Diversity at different levels were analysed by Simpson index which indicated differences in endophytic fungal isolates and species richness. These results are in line with the work done on endophytic fungi isolated from *Piper nigrum* by Uzma *et al.*, (2016), wherein high Simpson's diversity index was noted in ripe fruit pulp. In the present study, phytochemical

analysis of CFSsand dry mycelium from all fungal isolates was carried out to know the existence of chemical components as a potential source for medicinal and industrial use. CFSs revealed the presence of alkaloids, carbohydrates, saponins, and glucosides and the absence of flavonoids, tannins, and phenols. On contrary, dry mat powder showed only presence of alkaloids and carbohydrates. According to Gasecka *et al.,* (2020) drying temperature may affects the phytochemicals in the mycelium. In another study, total phenolic contents are compared in vacuum dried, oven dried (70 °C, 24h) and fresh mycelium. Observation suggested that slight lost in phenolic content compared to other, indicates that drying approach play a vital role in harvesting bioactive compounds (Sim *et al.,* 2017).

Enzymes are potential biocatalysts for a wide range of reaction. Microbes are a viable substitute source of enzymes, they can be cultured in large quantities in a short period of time via fermentation, are biochemically diverse, and can be genetically manipulated. Further, fungal enzymes play an important role in biodegradation and hydrolysis, both of which are important in protecting against invading pathogens and in obtaining nutrition from the host plant (Sunitha *et al.,* 2013). Indeed, their primary functions include hydrolysis of food substances and pathogen defence (Desire *et al.,* 2014). Fungal enzymes are used to simplify raw material processing in the beverage, food, confectionary, textiles and leather industries. Furthermore, such enzymes are frequently more stable than those derived from other sources. Alberto *et al.,* in 2016 and Choi *et al.*,in 2005 described the endophytes as potential enzyme synthesizers. The extracellular enzymes vary from fungi to fungi; also influenced by the hosts and their growing conditions. Out of 12 isolated endophytic fungi, four of them were able to degrade starch by producing amylase, an enzyme that has huge industrial applications. Our results specify that isolates might produce large amounts (high activity) of amylase enzyme upon large-scale fermentation is performed. Sunitha *et al.,* (2013) stated thatfungal

endophytes of *Bixa orellana* and *Catharanthus roseus* were not active producers of the amylase enzyme. In our study, out of seven isolates showed lipase activity, out of them, *A. gaisen* was able to produce visibly more lipase enzyme when compared to others. Studies by Chow & Ting (2015) revealed that endophytes are good alternative sources for L-asparaginase production. Out of 12, ten isolates were able to produce asparaginase. Isolates of *C. cladosporiodes* and *A. hiratsukae* exhibited maximum asparaginase enzyme activity in the present study. *Aspergillus* sp. showed maximum enzyme activity as that of our study. An interesting finding was that none were able to produce laccase and protease enzymes. These results are in line with Uzma *et al.,* (2016) also stated the absence of laccase enzyme producers from the endophytic isolates from all six wild medicinal plants. So, it can be concluded that enzyme production by endophytic microbes depends on the type of host and its habitats.

Antibiotic resistance is significantly increased and extended beyond the clinical application of drugs. There is now a ray of hope that new drugs obtained can be used to from microbes that fight against the other microbes (Malik & Bhattacharyya 2019). The hypothesis says that microbes associated with medicinal plants could be a potent source of bioactive secondary metabolites for medicinal applications. Secondary metabolites from endophytic fungi of plants have been receiving the utmost attention. A few secondary metabolites exhibit significant biological activities; hence, they have potential and could be considered as lead compounds in drug discovery (Zheng 2021).

The behaviour of endophytic fungi and pathogens was studied using various assay techniques like dual plate assay, fumigation assay, agar well diffusion (CFSs and solvent extracts), agar plug diffusion and cross-streak plate assay; the relation between two strains could be

antagonistic or competitive.In this study, four different culture conditions were employed to know the behaviour of endophytic fungi against test pathogens. Results revealed that isolates displayed diverse inhibitory effects against test pathogens in all the tested methods. This could be attributed to the chemo-diversity of fungal endophytes under different culture conditions. These results support the OSMAC (One Strain Many Compounds) concept. The outcome is in line with Kotach *et al.,* who observed varying degrees of antagonistic behaviour in three different assay conditions. The findings suggested that changes in culture conditions with test pathogens could have resulted in producing the different 'cryptic' metabolites from endophytes.

A good number of methods are in practice to estimate or monitor the antimicrobial activity of extracts. The most common ones are well and disc diffusion methods for bacteria and poisoned food methods for fungi. Every method has its own merits and demerits. In widely used well method, the chosen concentration of test sample is introduced into a known size hole and as the organisms are thoroughly mixed with the agar (pour plate), so that the organisms are in close proximity to the test sample. The suitable method to know the antagonistic activity is the agar plug diffusion method, though it is similar to the disk diffusion method. For the cross-streak method, in the present study, commercially available blood agar has been used and is considered as a standard media for the analysis of food samples (Coman *et al.,* 1997).

In the present study, CFSs of *Alternaria* sp., *C. tenuissimum*, *Mycosphaerellaceae* sp., and *A. alternata* endophytic fungal isolates demonstrated pathogenic bacterial controlling capacity (*S. aureus*, *E. coli,* and *K. pneumoniae*). *P. aeruginosa* known for its antibiotic drug-resistant potential and we also noted that no CFS of endophytic fungi-controlled *P. aeruginosa*. *P.*

aeruginosa (ATCC 27853) contains virulence genes, *lecA*, *lecB*, and quorum sensing regulators, *LasI/R* (Cao *et al.*, 2017). And Garey *et al.*, (2008) stated that *P. aeruginosa* strains possessing exoenzyme U gene were significantly more resistant to several antibiotics. Also, transcriptional regulator *Psr A* plays significant role in establishment of biofilm, intrinsic and adaptive antibiotic resistance. *E. coli* and *K. pneumoniae* lack the exoenzyme U gene and transcriptional regulator *Psr A* (Shen *et al.*, 2005; Barbosa and Lery 2019). This might be the possible reason for antimicrobial activity of CFSs against *E. coli* and *K. pneumoniae*.

According to Pai & Chandra (2018), *C. cladosporiodes* was the most effective endophytic fungus against *P. aeruginosa*. On the contrary, in our study, *C. cladosporiodes* did not show any activity on test pathogens. Rani *et al.*, (2017) studied the endophytic fungal extracts of *Aspergillus nidulans, Curvularia hawaiiensis, Chaetomium arcuatum,* and *Chaetomium atrobrunneum* and demonstrated significant microbial controlling capacity. The broad-spectrum antimicrobial property of *C. beasley*iis significantly inhibited the tested microorganisms and it is nearly equivalent to the standard antibiotics tested. These results are on par with the results of Pavithra *et al.*, (2012).

As we noted, in agar plug diffusion assay, all the isolates inhibited *S. aureus*(19mm) and a few isolates inhibited *E. coli* (13mm). The outcomes corroborate with that of Marcellano *et al.*, (2017) studied agar plug assay, *S. aureus* was the most inhibited by the isolates and *E. coli* was the least inhibited organism. Other study reported by Mahadevamurthy *et al.*, (2016) displayed endophytes *A. fumigatus* and *Fusarium* sp. showed highest zone of inhibition against the *S. aureus* (25mm and 16mm respectively).

Discussion

The cross-streak method is simple and quick way to screen for novel antibiotics. Balouiri *et al.,* (2016) reported the common and different methods for evaluating antimicrobial activity, one of which was cross streak. The main disadvantage of the 'cross streak method' was difficulty in obtaining quantitative data, because the zone of inhibition in margins was frequently uncertain and unclear (Velho-Pereira *et al.,* 2011). Aljuraifani *et al.,* (2019) discovered antimicrobial activity of endophytic bacteria, *Bacillus licheniformis* and *Proteus mirabilis* using cross streak method. This is the first attempt that endophytic fungal antimicrobial activity has been reported using blood agar as a medium to grow both bacteria and fungi.

In dual culture assay, results revealed that isolate displayed diverse inhibitory effects against test pathogens. There are several types of antagonism that fungal antagonists could exhibit. In this study aggressive inhibition against *C. albicans* by *Curvularia beasleyi* and *C. tropicalis* by *Cladosporium* sp. was observed.

The role of volatile compounds in fumigation assay was almost negligible as none of the *fungal*isolates exhibited growth inhibition of the testpathogens. Radha *et al.,* (2014) observed no inhibitory activity of volatile compounds of *C. gloeosporioides* strains, a fungal endophyte against the phytopathogenic fungi *P. theae* and *C. camelliae*. In another study, within 3 days, *Trichoderma koningii* and *Trichoderma atroviride*endophytic fungi exhibitediof nhibition complete growth on of the human pathogenic fungi*A. niger*and *A. fumigatus*within 5 days.The findings suggest that the effects of endophyte's volatile compounds became more effectiveas time passed (Erfandoust et al., 2020).

Antimicrobial activity by the CFSs suggested secretion of active metabolites to the extracellular media by the isolates. Holding of antimicrobial activity after heat and proteinase

K- treated helps to know the thermostable, non-proteinaceous nature of the antimicrobial principle. Due to non-proteinaceous nature, the CFSs was subjected to solvent extraction for purification of the antimicrobial metabolites. If the proteinaceous part of CFSs having antimicrobial activity, when they bond to solvent active compounds will get degraded and release the high energy.

Taufiq &Darah (2020) stated that organic solvents with different polarities were frequently used to gain diverse bioactive substances from the crude extract. According to Synytsya*et al.*, (2017), the successful isolation of fungal metabolites demonstrating antibacterial activity depends on solvents, strains, and test microorganisms.Polar (*n*-butanol), medium polar (ethyl acetate) and non-polar (hexane) solvents are used to attain the active metabolites from CFSs. In the present, the obtained crude solvent extracts were dissolved in DMSO. DMSO is an organosulfur compound, and polar aprotic solvent. DMSO used as a vehicle control in *in-vitro* and *in-vivo* studies of test compounds because that dissolves both polar and non-polar compounds. In addition, it has the ability to cross cell membranes and bypass hydrophobic barriers in both eukaryotes and prokaryotes (Brito *et al*. 2017).

Only ethyl acetate and *n*- butanol solvent extracts exhibited potent antimicrobial activity when compared to hexane solvent used. Metabolite's analysis is a very important aspect of all life sciences and particularly where cells are used as living factories to produce a wide range of chemicals.Due to over flow of the intracellular metabolites to the extracellular medium, secretion of certain metabolic intermediates is detected with specific metabolic pathways.Thus, based on the metabolic overflow concept, a given metabolite is secreted to the extracellular medium when its intracellular levels increase, reflecting an increase in the metabolic flux of the pathway(s) associated to the production of that metabolite. But this

phenomenon can truly be observed under time series experiments (Kell*et al.,* 2005). Carneiro *et al.,* (2011) published time-series metabolomics experiments of *E. coli* during the production of recombinant proteins and while assessing their data, they observed that the concentrations of some extracellular metabolites increased, despite their intracellular levels being decreased.

Our results are in line with this metabolic overflow, the intracellular compound extracted from dry mycelium of the isolated organisms was less effective against the tested pathogens, whereas extracellular metabolites from CFS is more effective against tested pathogens. It shows that isolates released metabolites into extracellular broth.

The antimicrobial activity of the crude solvent extract showed that hydrophilic and lipophilic compounds present in the crude extract are attracted mainly by the type of solvent used (Minarni *et al.,* 2017). The above findings uncover the culture filtrates of isolatesare having antimicrobial activity. The present results correlate with the finding of an earlier report by Chatterjee *et al.*,(2019, 2020) have initially used different solvents, but ethyl acetate was chosen as it has maximum efficiency to extract the bioactive compounds from endophytes.

Several antimicrobial compounds have been explored, but are failed to achieve the control of many diseases. In the current situation, such discovery is relatively significant because pathogen infections have become a serious problem in patients with impaired defence mechanisms (Muazzam and Darah 2021).

Among all the isolates only *Cladosporium* sp., *Curvulariabeasleyi*possess antibacterial and antifungal activity, *Mycosphaerellaceae* sp., and *Cladosporiumtenuissimum* are the organisms that demonstrated antibacterial activity and were chosen for further research based on their activity.

Discussion

The present results were in agreement with the study of Wang *et al.,* (2007) who had isolated 67 fungal endophytes from *Quercus variabilis*. Among all the isolates, *Cladosporium* sp. produced Brefeldin A, a cytotoxic compound, and demonstrated effective inhibition against the panel of pathogens tested in this study particularly, on *C. albicans* (20±04mm). And in another study conducted by Khan *et al.,* (2016) isolated *Cladosporium* sp., from *Rauwolfia serpentina* L. ethyl acetate extract showed presence of two naphthoquinones, and exhibited antibacterial activity by disc diffusion assay with prominent zone of inhibition observed against *S. aureus* with 27mm.

Kaaniche *et al.,* (2019) identified three active compounds from *Curvularia* sp. from *Rauwolfia macrophylla*using 1 and 2D NMR spectroscopy and mass spectrometry, which demonstrated antibacterial activity, and also exhibited antioxidant activity (EC_{50} ranges from 0.56- 1.09). Data from the literature revealed that isolated compounds have biological activity.

The ethyl acetate extract of *Curvularia lunata* isolated from *Cymbopogon caesius*showed antimicrobial activity against *S, aureus* (20 ±1.5) and *C. albicans* (18 ± 1.2) when compared to other solvents such as hexane and methanol. Similarly, in our study also only ethyl acetate extract showed activity against the *S, aureus* and *C. tropicalis* and *C. albicans* (Avinash *et al.,* 2015),which was in agreement with the information reported in an earlier study that confirmed methanolic and ethyl acetate extract of *Cladosporium tenuissimum* isolated from*Sonneratia apetala* displayed potent antimicrobial activity against all test pathogens (Nurunnabi *et al.,* 2020). A few studies also reported *Mycosphaerellaceae* sp., possess potent antimicrobial activity (Vaz *et al.,* 2012 and Wong Chin *et al.,* 2021).

Varying growth-inhibitory percentages were observed suggesting the chemo-diversity of endophytic fungi under three different assay conditions. These results obliged the concept of OSMAC, wherein, production of different microbial molecules depends on the culture conditions (Bode *et al.,* 2002). Observations showed that isolates are less powerful on Gram-negative bacteria when compared to Gram-positive bacteria. This may be due to the cell wall components of bacteria (Scherrer & Gerhardt 1971). Because Gram-positive bacteria have thick layer of peptidoglycan. Whereas thin layer of peptidoglycan observed in Gram-negative bacteria with additional outer membrane (Epand *et al.,* 2016).

In experimental practice, knowing about MICs are very important for the complete bio-activity calculation of antimicrobial drugs to treat a particular pathogenic infection and to avoid drug resistance (Johan *et al.,* 2018). *In vitro* determination of the rate, concentration, and actions of active compounds will be achieved by time kill assay beyond that of MIC. This method was commonly used as the basis in the examination of antimicrobial drugs in pharmacodynamics (Appiah *et al.,* 2017). The antimicrobial activity of the crude solvent extract was also confirmed by a significant reduction in the number of CFUs in the time-kill assay at its MIC. A rapid decline in the CFU count of test pathogens in the treated set indicated the strong microbicidal nature of the endophytic compounds.

For any antimicrobial compound, the main target of action is the cell membrane, its permeability, and cell wall biosynthesis (Muazzam and Darah 2021). In the present study, we accessed the action of the extract on the cell by measuring the release of protein and DNA damage. Losses of cellular contents are also evidence of cell death upon treatment with isolated solvent extracts. The release of protein and DNA into extracellular media from pathogenic fungi was also stated previously by Chatterjee *et al.,* (2020). *Aspergillus*

welwitschiae isolated from *Aloe ferox* Mill crude solvent extract exhibited significantly (P< 0.05) leakage of absorbing materials from *S. aureus* and *E. coli* when treated with MIC of the extract (Maliehe *et al.*, 2022). Antimicrobial treatment indicates cell wall breakage and membrane leakage of pathogens, which leads to high levels of protein concentration in extracellular medium. The solvent extract metabolite actively inhibiting the pathogens is the suggested application to control clinical pathogens. Cell death is indicated by the breakdown of double and single standard DNA. There have been a few studies on the detection of DNA fragmentation by secondary metabolites from endophytic fungi. In this study the isolated bioactive compound caused DNA fragmentation of pathogen cells.When pathogens were treated with the crude solvent extracts, it showed smeared DNA at constant concentration and it is time dependent. It was evident that the crude solvent extracts interfere with the nucleic acid system at several different levels, thereby preventing the DNA from functioning as proper template.

The free radicals produced due to oxidative stress and this could be a key feature for DNA damage. And most importantly, crude solvent extract of *C. beasleyi* displayed DNase inhibiting kind activity, where treated samples DNA band was brighter compared to control.

Zhao *et al.*, (2017) isolated 10 natural compounds from *Cyclocarya paliurus* leaves and screened for DNase 1 inhibitors, among all five compounds are DNase activators and few are inhibitors. And concludes DNase inhibitors will be highly useful tools as chemical probes.

Biofilm remains a global health threat because of the higher degree of management required for a cure and the capacity to aggravate nosocomial infections. Hence, the search for unique useful molecules to block this problem is a priority. Treatment of biofilm-based infection is difficult due to unavailability of biofilm specific drugs. The capability of antimicrobial agents

to prevent the development or destruction of biofilms holds promise for reducing colonization on surfaces by microorganisms. The increased concentration of the crude extract displayed the reduction of biofilm thickness and disappearance of patchy microcolonies. These results are in line with Kaur (2020) who had isolated *Chaetomium globosum* from *Moringa oleifera* having potent antibiofilm activity against the pathogenic fungi. The results revealed that the anti-biofilm activity was dose-dependent and a higher dose could eliminate more pathogen growth for both initial and pre-formed biofilm (Awasthi 2020). In this study, the pre-formed biofilm (48h old culture) was less susceptible to the extract than the initial biofilm (4h culture).During initial biofilm formation, weak association of microorganisms to surface will be observed, when it reaches to matures, biofilm eventually adheres to surface and starts clustering to form microcolonies that mature over time this might be the reason, extracts are effective against initial than mature biofilm. Some studies also noted that it is less difficult to inhibit cell attachment than to get rid of matured biofilm (Famuyide *et al.,* 2019).

Plants used in diet are one of the most promising sources of antioxidants and can be used to treat a variety oxidative stress- related diseases (Rajput *et al.,* 2017). Oxidative damage is induced by free radicals and causes numerous damages to the body like aging, tumour formation, and neurological problems, which can be prevented by antioxidant compounds. Radical scavenging activity assay (DPPH) is a fast and sensitive method for testing antioxidant activity. The donated electrons from natural products react with DPPH, thus converting into purple colour. The degree of de-coloration relies on the concentration of antioxidants in the test material. Under test, more of the absorbance values signify the scavenging activity (Saeed 2012). The percentage inhibition was observed at the lowest concentration (16.66 to 35.58%), which considerably elevated with increased concentration. This suggested the extracts contained electron-donating phytochemicals for scavenging

activity. This agrees with the previous observations (Khiralla 2015) where in endophytic fungus *Aspergillus* sp. ethyl acetate extract of *Trigonella foenumgraecum* demonstrated strong antioxidant activity.

LC-MS and GC-MS have been described as valuable tools for metabolite identification of endophytic fungi including the expression of metabolites produced in major quantities (Chithra *et al.,* 2014). As far we are not observedany plant specific metabolites from the analysis, CFSs subjected for the solvent extraction, this might be the possible reason for missing some plant synthesizing compounds.

The bioactive components present in the crude solvent extract have been analysed using GC-MS and LC-MS which revealed the presence of various compounds that might be accountable for its bioactivities. Some of the important antimicrobial agents which are commercially available are identified from the isolates by GC-MS and LC-MS (Table 17).

Table 17: Some active compounds identified from the Crude solvent extracts by GC-MS and LC-MS

Compound name	Identified solvent extract	Nature of the compound	Isolated	Target and action	Reference
Netilmicin	*Cladosporium* sp., and *Mycosphaerellaceae* sp**.**	semisynthetic aminoglycoside antibiotic	an actinomycete, *Streptomyces fradiae* (1943)	• Outer bacterial membrane is the first site of action. The cationic antibiotic molecules cause cracks in the outer cell membrane, resulting in leakage and increased antibiotic uptake. This rapid action at the outer membrane probably accounts for most of the bactericidal activity • Netilmicin is less effective against *P. aeruginosa*. In the present study, CFSs did not inhibit *P. aeruginosa*.	Montie & Patsmasucon (1995). Briedis *et al.,* (1976)
Paromamine (3+) (Synonym-Neomycin)	*Cladosporium* sp. and *Cladosporiumtenuissimum*	Intermediate compound		• If converted to either 4,6-disubstituted 2DOS aminoglycosides, or 4,5-disubstituted 2DOS aminoglycosides, Paromamine acts like kanamycin and gentamicin; neomycin and butirosin respectively	(Kudo *et al.,* 2009).
Progeldanamycin	*Mycosphaerellaceae* sp.	Precursor for	*Streptomyces*	• Binds to the N-terminal ATP-	Hermane *et al.,*

		antitumor drug – Geldanamycin	*hygroscopicus*	binding domain of heat shock protein 90 (Hsp90) and inhibits its ATP-dependent chaperone activity • Reported as weak antibacterial and antifungal agent	2019 Martin *et al.*, 2019
Sphinganine	*Cladosporium* sp.			• specific diagnostic biomarker for *T. marneffei* infection •	Li *et al.*,(2021)
Anatoxin a(s)	*Curvularia beasleyi*		Freshwater cyanobacteria	• Bacterial genus *Pseudomonas* was capable of degrading anatoxin-a	Kiviranta and colleagues (1991)
1-carbapenem-3-carboxylic acid	*Mycosphaerellaceae* sp.	unique β-lactam antibiotics	*Pectobacterium carotoyorum* and *Streptomyces*	• capability of inhibiting β-lactamases • Bind to multiple different PBPs (penicillin binding proteins • Peptidoglycan weakens, due to osmotic pressure cell burst will take place	Papp-Wallace *et al.*, 2011 Mettetis *et al.*,2012 Meletis 2016
1,3-Dioxolane	*Cladosporium* sp.	Antiseptics	Bark extract of *Juglans regia*	• Antimicrobial activity of 1.3-Dioxolane is associated with its antiradical activity, depending on their hydrophilic-hydrophobic balance allows targeted variation in structural parameters	

Levoglucosenone	*Cladosporium* sp.	Chiral building block, templet for synthesizing biologically active intermediates	*Kappaphycus alvarezii*, red seaweed	• Antibacterial and antioxidant activity	Comba *et al.*, 2018). Bhuyar et al., (2020
Alternariol	*Mycosphaerellaceae* sp.	Toxic	*Alternaria* sp.	• DNA-intercalating agent and is a DNA cross-linking mycotoxin in UV light	

SUMMARY AND CONCLUSION

Summary and conclusion

- *Solanum violaceum* unripe and ripe fruits were selected for isolation and identification of fungal endophytes
- A total twelve endophytic fungi were isolated and identified by microscopic and molecular techniques (ITS) and obtained sequences were deposited to GenBank
- This is the first study to discover fungal endophytes from this plant
- Isolates screened for extracellular enzymes, showed amylase, lipase and asparaginase positive. None of the isolated produced laccase and protease
- All the cell free supernatants were effectively inhibited test pathogens except, *P. aeruginosa*
- All the cell free supernatants and dry mycelium were subjected for solvent extraction, to obtain crude metabolites
- Crude solvent extracts obtained from dry mycelium did not show any activity, well potential antimicrobial activity was observed in *n*-butanol and ethyl acetate extracts of CFSs
- Results displayed those isolates released several antimicrobial compounds to the extracellular environment
- Crude solvent extract of*Cladosporium* sp., *Curvularia beasleyi*, *Mycosphaerellaceae* sp., and *C. tenuissimum*dissolved in DMSO (vehicle control) showed potent antibacterial and antifungal activity
- Among all, *Cladosporium* sp., solvent extract displayed time kill assay and protein leakage were observed at 4 h after treatment with MIC, fragmentation of *K. pneumoniae*, *E. coli* and *S. aureus* DNA after 20 h, while fragmentation of *C. tropicalis* DNA observed at 24h treatment with MIC, when compared with controls

- *Cladosporium* sp., extract exhibited microbicidal nature against the pathogens
- All the extracts displayed 100% initial biofilm inhibition than the pre-formed biofilm on dose dependent manner.
- *Curvularia beasleyi*, *Mycosphaerellaceae* sp., and *Cladosporium tenuissimum* inhibited 82% of mature and 93% of initial *S. aureus* biofilm.
- Among all solvent extracts, *Cladosporium* sp., displayed potential antioxidant activity with IC_{50} 72 µg/mL.
- LC-MS and GC-MS results revealed that extracts possess biologically active compounds with antimicrobial activity.
- Some are Netilmicin, Paramamine, Sphinganine,13-Desoxypoxilline, Astringin, Anatoxin, Levoglycosenome and so on.

LIMITATIONS OF THE STUDY

Limitations

- Growth conditions optimization
- Generation test for consistency to get the compounds
- Active compound purification
- *in vitro* and *in vivo*elucidation

NEW KNOWLEDGE GENERATED

New knowledge generated

- *S. violaceum* fruits contain endophytic fungi which are known for their bioactivity
- Isolated endophytes are capable of producing industrially important extracellular enzymes like, Amylase, Lipase and Asparaginase
- Alkaloids, glucosides, saponins and carbohydrates are present in both cell free supernatant and dry mycelium mat
- Isolates *Cladosporium* sp., *Curvularia beasleyi*., *Mycosphaerellaceae* sp., *Alternaria alternata*and *Cladosporium tenuissimum*showed antibacterial activity
- *Cladosporium* sp. and*Curvularia beasleyi*displayed antifungal activity against *C. albicans* and *C. tropicalis*
- Crude solvent extracts (ethyl acetate and *n*-butanol) have microbicidal activity against test pathogens
- *Cladosporium* sp.*n*-butanol possess potent antioxidants
- Biologically active compounds are present in the isolates extract confirmed from biophysical characterization

RECOMMENDATIONS

Recommendations

- As fruits endophytic fungi possess potential bioactive compounds, remaining plant parts can be subjected for the isolation of endophytes
- Based on the results, fruits can be included in daily diet helps in reducing the risk of diseases
- Different solvents can be used for extraction of crude metabolites
- Crude solvent extracts contain active metabolite, individual compounds can be purified for further analysis for novel drugs development

Appendices

SRI DEVARAJ URS ACADEMY OF HIGHER EDUCATION & RESEARCH

SRI DEVARAJ URS MEDICAL COLLEGE

Tamaka, Kolar

INSTITUTIONAL ETHICS COMMITTEE

Members

1. **Dr. D.E.Gangadhar Rao, (Chairman)** Prof. & HOD of Zoology, Govt. Women's College, Kolar,
2. **Dr. Sujatha.M.P, (Member Secretary),** Assoc. Prof. of Anesthesia, SDUMC,
3. Mr. Gopinath Paper Reporter, Samyukth Karnataka
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13. Dr. Munilakshmi U Asst. Prof. of Biochemistry, SDUMC

No. SDUMC/KLR/IEC/40/2019-20 **Date: 06-06-2019**

PRIOR PERMISSION TO START OF STUDY

The Institutional Ethics Committee of Sri Devaraj Urs Medical College, Tamaka, Kolar has examined and unanimously approved the study entitled **"Biodiversity prospecting of endophytic fungi from *Solanum violaceum* Ortega fruits and the antagonistic activity of the filtrate against pathogens "** being investigated by **Ms. Manasa A P** Dr.Kiranmayee P, Dr.Deena C Mendez[1] in the Departments of Cell Biology & Molecular Genetics & Biochemistry[1] at Sri Devaraj Urs Medical College, Tamaka, Kolar. **Permission is granted by the Ethics Committee to start the study.**

Sujatha.M.P
Member Secretary
Institutional Ethics Committee
Sri Devaraj Urs Medical College
Tamaka, Kolar.

Chairman
CHAIRMAN
Institutional Ethics Committee
Sri Devaraj Urs Medical College
Tamaka, Kolar

131.

Presentation and Publication

NAAC Accredited with 'A' Grade

National Conference on Phytochemicals and Microbial Bioactive Compounds – Role in Agriculture and Human Welfare (PMBC-2019)

ज्ञान-विज्ञान विमुक्तये
UGC
University Grants Commission

Certificate

This is to certify that Prof./Dr./Mr./Miss....Manasa A P.....has presented (Invited talk/Oral/Poster) a paper entitled..Antimicrobial & extracellular enzyme activities of endophytic fungi.....::...Solanum violaceum L.....in a National Conference on Phytochemicals and Microbial Bioactive Compounds – Role in Agriculture and Human Welfare (PMBC-2019) held on 3rd and 4th October, 2019 organized by Department of Microbiology and Biotechnology, Bangalore University, Bangalore.

Prof. C. Srinivas
Organizing Secretary

Prof. D. Manjulakumari
Organizing Chairman

International Virtual Conference on

Plant Specialized Metabolism & Metabolic Engineering (PSMME-2020)

14th-16th October, 2020

PSMME-2020

CSIR-CIMAP

CSIR-Central Institute of Medicinal and Aromatic Plants, India

CERTIFICATE

This is to certify that

Ms ... Manasa AP ... has participated in PSMME-2020 from 14th-16th October, 2020, and her Abstract was selected for Oral Talk

Dr. Prabodh Kumar Trivedi
Conference Chairman &
Director, CSIR-CIMAP

Dr. Dinesh A. Nagegowda
Conference Convenor &
Organizing Secretary

SRI PADMAVATI MAHILA VISVAVIDYALAYAM

(WOMEN'S UNIVERSITY)

TWO DAY VIRTUAL INTERNATIONAL CONFERENCE

ON

"CURRENT TRENDS ON TRANSDISCIPLINARY RESEARCH"

(5th AND 6th JANUARY 2022)

TRANSDISCIPLINARY RESEARCH INITIATIVE CENTRE IN ADOLESCENT HEALTH (TRICA) UNDER UGC-STRIDE

CERTIFICATE OF PARTICIPATION

This certificate is awarded to Ms./Prof./Dr. MANASA A.P for his/her participation during the conduct of Two Day International Conference on "Current Trends on Transdisciplinary Research" held on 5th and 6th January 2022 and organized by Transdisciplinary Research Initiative Centre in Adolescent Health (TRICA) under UGC-STRIDE,Tirupati, Andhra Pradesh, India.

PROF. R. USHA
Co PI-TRICA

PROF. P. UMA MAHESWARI DEVI
Co PI-TRICA

PROF. P. VIJAYA LAKSHMI
PI-TRICA

Presentation and Publicationss

Presentation and Publicationss

Publications

1. Manasa AP, Moutusi S, Mendez DC, Kiranmayee P, Prasannakumar MK. (2020). Phytochemical distribution, antimicrobial activity, enzyme production of phylogenetically differentiated endophytes from *Solanum violaceum* Ortega fruits. Current Research in Environmental and Applied Mycology (Journal of Fungal Biology) 2020; 11(1): 210–229.

2. Reservoir of extrolites with cidal and antioxidant properties from *Cladosporium* sp. of *Solanum violaceum* Ortega fruits (Manuscript submitted)

3. Chemical profile, antifungal and antioxidant activity of *Solanum violaceum* Ortega fruit endophyte: *Curvularia beasleyi* (Manuscript submitted)

1. Different Media used for fungal endophytes isolation and its composition

PDA	SDA	Czapek Dox Medium
Potato Extract - 4g Dextrose - 20g Agar - 15g Distilled water - 1000ml	Dextrose - 40g Peptone - 10g Agar - 15g Distilled water - 1000ml	Sucrose - 30g Sodium nitrate - 2g Dipotassium phosphate - 1g Magnesium sulphate - 0.500g Potassium chloride - 0.500g Ferrous sulphate - 0.010g Agar - 15g Distilled water - 1000ml

2. Media used for screening extracellular enzymes

Amylase activity GYP agar	Laccase Activity GYP agar	Lipase activity PA media
Glucose - 1g Yeast extract - 0.1g Peptone - 0.5g Agar - 16g Distil. H_2O - 1000ml Soluble starch -2% pH - 6	Glucose - 1g/L Yeast extract - 0.1g/L Peptone - 0.5g/L Agar - 16g/L 1-naphthol - 0.005% pH - 6	Peptone - 10g/L Nacl -5g/L $CaCl_2.7H_2O$ - 0.1g/L Agar - 16g/L Tween 20 - 1%

Protease activity GYP agar	Asparaginase activity
Glucose - 1g Yeast extract -0.1g Peptone - 0.5g Agar - 16g Gelatin - 0.4% pH - 6	Glucose - 2.0g L-asparagine - 10g KH_2PO_4 - 1.52g KCl - 0.52g $MgSO_4.7H_2O$ - 0.52g $CuNO_3.3H_2O$ - 0.001g $ZnSO_4.7H_2O$ - 0.001g $FeSO_4.7H_2O$ - 0. 001g Agar - 16g Phenol red - 0.009%

3. Media used for bio-activities

Muller Hinton Agar (MHA)	Nutrient Agar (NA)	Nutrient Broth (NB)	PDB (Potato dextrose broth)
Beef infusion solids-300 g Casein acid hydrolysate -17.5 g Starch - 1.5 g Agar - 20g pH- 7.4 ± 0.2	Peptone - 10g/L Nacl -5g/L Yeast extract - 1.5 g Beef extract - 1.5 g Agar - 20g Distilled water- 1L pH - pH- 7.4 ± 0.2	Peptone - 10g/L Nacl -5g/L Yeast extract - 1.5 g Beef extract - 1.5 gS Distilled water- 1L pH - pH- 7.4 ± 0.2	Potato infusions - 4g Dextrose - 20g Distilled water -1L pH- 5.1± 0.2

4. Lysis buffer

1M Tris-Hcl	2.5 ml
0.5 M EDTA	1 ml
5M Nacl	3.5 ml
1% SDS	1g
PVP	Pinch

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RECOMMENDATIONS

Recommendations

- As fruits endophytic fungi possess potential bioactive compounds, remaining plant parts can be subjected for the isolation of endophytes
- Based on the results, fruits can be included in daily diet helps in reducing the risk of diseases
- Different solvents can be used for extraction of crude metabolites
- Crude solvent extracts contain active metabolite, individual compounds can be purified for further analysis for novel drugs development