# *In-vitro* Antioxidant Activity of Endophytic Fungi isolated from medicinal plants

**A**
**Dissertation**

Submitted in partial fulfillment of the requirement
for the degree of

**Masters of Science**
In
**Biochemistry**

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# CERTIFICATE

This is to certify that the thesis entitled **"*In-vitro* Antioxidant Activity of Endophytic Fungi isolated from medicinal plants"** submitted by Palak Middha is an authentic record of work carried out as requirement for the award of the degree of **Masters of Science** in **Biochemistry** at **Thapar University, Patiala** under the supervision of **Dr. M. Vasundhara**, Assistant Professor, Department of Biotechnology, Thapar University, Patiala during the period of six months from January 2017 to July 2017. No part of the matter embodied in this report has been submitted to any other university or institute for the award of any degree.

It is certified that the above statement made by the student is correct to the best of my knowledge and belief.

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# CANDIDATE'S DECLARATION

I hereby declare that the work being presented in the dissertation entitled "***In-vitro* Antioxidant Activity of Endophytic Fungi isolated from medicinal plants**" in the partial fulfillment of the requirements for the award of the degree of Masters in Biochemistry and being submitted to School of Chemistry and Biochemistry, Thapar University, Patiala, is my own work during the period of six months from January 2017 to July 2017, under the supervision of **Dr. M. Vasundhara,** Assistant professor, Department of Biotechnology, Thapar University, Patiala.

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**Date:** 08-09-2017
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Palak Middha

**Palak Middha**

***Dedicated to my Parents.....***

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# LIST OF ABBREVIATIONS

| Abbreviation | Name |
|---|---|
| BHA | Butylated hydroxyanisole |
| BHT | Butylated hydroxytoluene |
| DCM | Dichloromethane |
| DNA | Deoxyribonucleic acid |
| DNase | Deoxyribonuclease |
| DPPH | 2,2-diphenyl-1-picrylhydrazyl |
| $e^-$ | Electron |
| EtOAc | Ethyl acetate |
| ELISA | Enzyme-linked immunosorbent assay |
| FDA | Food and Drug Administration |
| $FeCl_3$ | Iron(III) chloride |
| $H^*$ | Proton |
| KOH | Potassium hydroxide |
| MeOH | Methanol |
| NaOCl | Sodium hypochlorite |
| Nm | Nanometer |
| O.D. | Optical density |
| PDA | Potato dextrose agar |
| PDB | Potato dextrose broth |
| ROS | Reactive oxygen species |
| RNA | Ribonucleic acid |
| RNase | Ribonuclease |
| SOD | Super Oxide Dismutase |
| Sp. | Species |
| TLC | Thin-layer chromatography |
| UV | Ultraviolet |
| WHO | World Health Organization |

# ABSTRACT

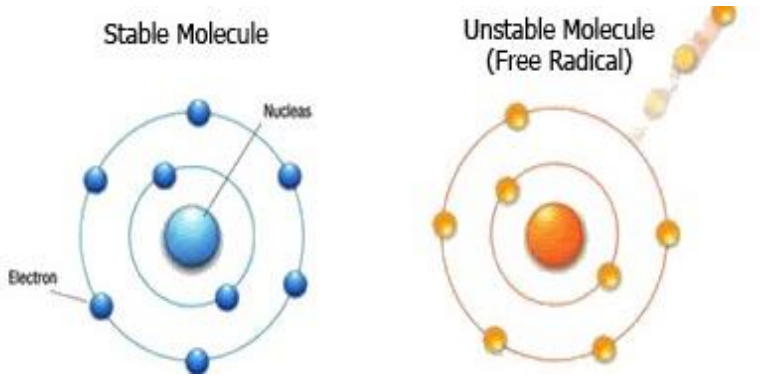
Endophytic fungi are microorganisms living inside plants are rich source of novel metabolic compounds. The aim of this project was to isolate the endophytic fungi from the stem of *Tinospora cordifolia* and the bark of *Terminalia arjuna,* screening for antioxidant activity of the crude fungal extracts obtained from the endophytic fungi and fractionation of these fungal extracts along with antioxidant assay of eluted fractions. Antioxidant activity of methanolic fungal extracts was evaluated by DPPH free radical scavenging assay. A total of 11 endophytic fungi were isolated from *Tinospora cordifolia* and 5 from *Terminalia arjuna*. All the obtained fungal extracts were investigated for antioxidant activity from which 5 isolated fungal extracts from *Tinospora cordifolia*, 1 from *Terminalia arjuna* and 2 from *Xylaria* species showed positive results for antioxidant activity. G1 and G3 fungal extracts obtained from *Tinospora cordifolia* showed significant scavenging activity with the value of 40% and 45% at a concentration of 1000µg/ml. The fungal extract ARJ1 from *Terminalia arjuna* showed 55% scavenging activity at the concentration of 1000µg/ml. XF-2 and XF-4 from *Xylaria* sp. showed maximum antioxidant activity. XF-2 was observed to have 71% antioxidant activity even at lower concentration of fungal extracts. As the concentration of fungal extract increased the scavenging activity also increased significantly for all the fungal extracts except XF-2 as it did not show much significant increase in the scavenging activity with the varying concentration. The preparative TLC of 2 fungal extracts from *Tinospora cordifolia* and *Terminalia arjuna* was done to separate the fractions. All Fractions were screened for antioxidant activity, only two fractions D and F from *Tinospora cordifolia* and b and c from *Terminalia arjuna* showed positive results for antioxidant activity. Phytochemical analysis was also done. Endophytic fungi were isolated from the medicinal plants *Tinospora cordifolia* and *Terminalia arjuna*. The phenolics and flavonoids were present in the fungal extract could be the reason for antioxidant activity. Hence the above results indicate that endophytic fungus obtained from above mentioned source can be exploited as a potential source of antioxidants.

# 1 Introduction

## 1.1 Free radicals

The area of free radical chemistry is hot news now a day. These free radicals are reactive oxygen and nitrogen species which are produced inside our body by so many physiological processes. Free radicals are produced during normal cell metabolism and result from the metabolism of certain drugs or xenobiotics. Exposure to UV light, cigarette and other environmental pollutants also increases the body's free radical burden (Halliwell 1994).

Atoms contain nucleus and electrons that move around the nucleus, usually in pairs. Free radical is any atom or molecule that contains one or more unpaired electrons. This unpaired electron can affect the chemical nature or reactivity of the molecule, by making the molecule more active than the corresponding non-radical (figure 1).




a b

**Figure 1: (a) Stable molecule and (b) Unstable molecule or free radical**

Uncontrolled free radical production in body system can lead to attack on membranes, lipids, proteins, enzymes and DNA causing oxidative stress and ultimate cell death. Free radicals are the main reason for many human diseases like diabetes mellitus, cancer, neurodegenerative disorders, ageing and inflammatory diseases. Free radicals and oxidants generate a phenomenon called oxidative stress that is when cells cannot destroy the excess of free radicals formed in body. In other words, oxidative stress is the result of imbalance between formation and neutralization of reactive oxygen species or reactive nitrogen species (ROS/RNS) (Cui et al., 2004).

## 1.2 Antioxidants

To counter the effects of free radicals the body system is endowed with another category of compounds called antioxidants (MEIER et al., 1998). An anti-oxidant is a stable molecule which donates an electron to a rampaging free radical and terminates the chain reaction before vital molecules are damaged. The ability of antioxidants to destroy free radicals or free radical scavenging property protects the structural integrity of cells and tissues (Frank et al., 2000). These antioxidant substances inhibit or delay oxidative processes, while often being oxidized themselves (Singh et al., 2002). Antioxidant constituents of plant material act as radical scavengers and help in converting the radicals to less reactive species.

In human body, there are various enzyme systems for free radical scavenging but micronutrients present in diet work as naturally occurring anti-oxidant compounds which can stabilize the highly reactive, harmful free radicals. A variety of free radical scavenging antioxidants are found in dietary sources like fruits, vegetables and tea (Mandal et al., 2009). Vitamin E, β-carotene and vitamin C are the major antioxidants, but these must be provided in diet as body cannot produce these nutrients. Exogenous antioxidants taken in sufficient quantity can enhance the protection against free radicals.

### 1.2.1 Classification of antioxidants

There are two types of antioxidants present in nature

a. Natural antioxidants
b. Synthetic antioxidants

a. Natural antioxidants: Naturally occurring antioxidants can differ in their composition, physical and chemical properties and also in their mechanisms. They can be enzymes such as super-oxides (SOD), catalase and glutathione peroxidase which can terminate the chain reactions by transferring ROS/RNS into stable compounds (Ursini et al. 1997)**.** Other naturally occurring anti-oxidants are of low molecular weight and can be sub-divided in lipid soluble anti-oxidants such as tocopherols, carotenoids, quinones, bilirubin and water soluble anti-oxidants such as polyphenols and carbic acid.
b. Synthetic anti-oxidants: These are more efficient and chemically synthesized anti-oxidants. They can be toxic, so there is proper mechanism of approval of these synthetic anti-oxidants by food and diet administration for addition to food example BHA (butylated hydroxyl anisole), BHT (butylated hydroxyl toluene) etc.

Free radicals produced in our body system may contribute to a variety of pathological effects and also cause many diseases (Addis et al., 2012). To reduce the ROS mediated

damage, both natural and synthetic anti-oxidants are used. However synthetic anti-oxidants are considered to be toxic. Therefore, it is necessary to develop natural non-toxic antioxidants. The natural antioxidants, more recently have attracted considerable attention (Rao et al., 2007). In this content, many medicinal plants which are easily approachable are the potent source of antioxidants as they contain a mixture of different chemical compounds that may act individually or in synergy to cure diseases and improve health. A single medicinal plant may have a variety of phenolic compounds for antioxidant property and many other pharmacological properties such as anti-bacterial, antifungal, anti-cancer etc. (Miguel 2010). A wide range of diversity of naturally occurring antioxidants is found in medicinal plants which are different in their composition, physical and chemical properties. For the past two years, there has been an increasing interest in the investigation of different novel natural products from plants. Medicinal plants and their endophytes are important resources for discovery of natural products. Several previous studies have found a positive correlation between total antioxidant capacity and total phenolic content (TPC) of many medicinal plant extract.

As slightly opened reservoir of bio-resources, endophytes have been shown to be excellent producers of bioactive and novel metabolites (Strobel et al., 2003).

## 1.3 Endophytes

All higher plants are host to one or more endophytic microbes. Endophytes that live in living plant tissues without causing any harm to the host, are highly diverse micro-organisms which are known as dependable sources of novel natural products (Gond et al., 2007). These novel bioactive compounds from endophytes have huge potential for exploitation in a wide variety of medical, agricultural and industrial areas (Tan and Zou 2001). A lot of bioactive products such as anti-biotic, anti-viral, anti-cancer and anti-diabetic agents have been isolated from endophytes (Guo et al., 2008). It has been reported that endophytes can produce useful antioxidants (Liu et al., 2009). Endophytes are the micro-organisms that include different group of organisms such as fungi, bacteria, mycoplasma etc. The production of novel bioactive metabolites by endophytic fungi by building an endo-symbiont relation with host plant depend on the independent evolution of the endophytes, which may have incorporated genetic information from higher plants thus they can prevent the host from successfully attacking fungi and pests by producing secondary metabolites and in return demanding nutrition (Strobel et al., 2003) .

There are various natural products that are derived particularly from medicinal plants and exploited by humans for their use for about more than thousands of years. The drugs used

in cancer therapy may contain 34% of natural products or their derivatives and also about 68% of anti-bacterial compounds are either natural products or their derivatives (Newman et al., 2012).

Knowing all these facts, there is a need to develop many more new bioactive compounds that are useful for humans and provide relief to them in any condition. So, there is need to discover more endophytic fungi to solve the health related problems.

The naturally occurring bioactive compounds from endophytic fungi isolated from the plants having medicinal values, have a wide spectrum of biological activities such as anti-fungal, anti-cancer, anti-oxidants and these bioactive products can be grouped into various different categories like alkaloids, steroids, flavonoids, glycosides, terpenoids, xanthones, isocoumarins, quinones, phenyl propanoids, lignans, aliphatic metabolites, lactones etc. (figure 2) (Aly et al., 2010). These endophytic fungi are poorly studied micro-organism group which are capable of producing enormous dependable bioactive and novel compounds.

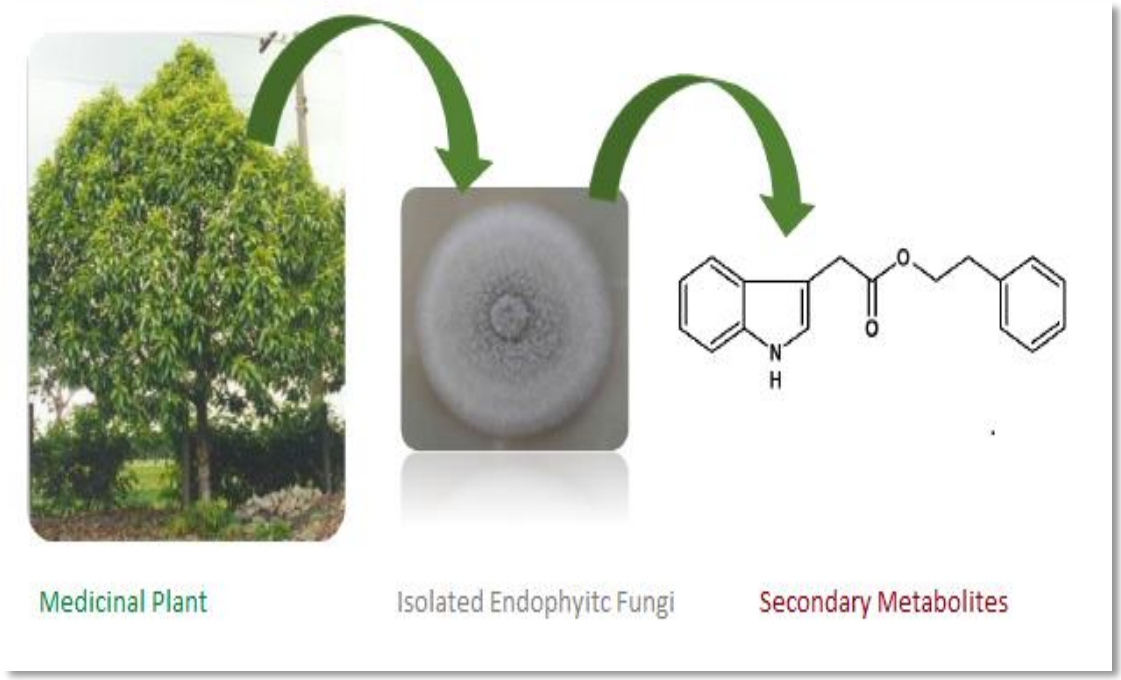



**Figure 2: Secondary metabolites from medicinal plants by isolating endophytic fungi**

The main objectives of this project were isolation of endophytic fungi from the medicinal plants, *Tinospora cordifolia* and *Terminalia arjuna*, antioxidant screening of crude fungal extracts obtained from the isolated endophytic fungi and fractionation of crude fungal extracts along with the antioxidant assay of the eluted fractions.

# 2 Review of Literature

## 2.1 What is a free radical?

Nucleus is present in atoms having electrons revolving around it in pairs. If any electron is unpaired in the atom then it becomes free radical. The chemical reactivity of atoms altered in case of free radicals, as atoms start showing more reactivity than non- radical (Bagchi et al., 1998). The simplest free radical is hydrogen atom, having one electron and one proton. Initiation of chain reactions of free radical takes place through removal of H* present in another molecule. Among highly reactive intermediates free radicals are the one having a surplus of one or more unpaired electrons rather than having matched pairs. Due to the presence of these free floating electrons, free radicals are highly unstable and reactive molecules.

### 2.1.1 Sources of free radicals

Normal metabolic processes and external sources like x-rays, ozone, smoke from cigarette, pollutants present in the air, chemicals in the industry etc form reactive species and free radicals.

Radiations from environment, received by both natural and manmade resources can cause generation of free radicals. Hydroxyls radical $OH^-$ is generated by low wavelength radiations like gamma rays, splits the water and also generates superoxide. By adding an extra electron to the oxygen super oxides are generated and are poorly reactive in nature (Lobo et al., 2010). These molecules generate accidently in body due to reaction of oxygen with different molecules like tetrahydrofolates, catecholamine's and other mitochondrial constituents and other of electron transport chains. Phagocytes like monocytes, neutrophils, macrophages and eosinophils etc generate superoxide in large amount in order to kill foreign organisms. In case of chronic inflammation this mechanism becomes cell damaging. Super-oxides are mainly formed by the 1-3% oxygen inhaled during breathing.

The internal sources of the generation of free radicals are enzymatic reactions that occur in human body. Free radical sources include respiratory reactions, synthesis of prostaglandin, phagocytosis, P450 cytochrome system, mitochondria, phagocytes, xanthenes oxidase, arachidonate pathways, peroxisomes, ischaemia and inflammation.

Free radical formation takes place due to emotion, disease conditions and stress etc. Oxygen which is an indispensable element for life, have severe deleterious effects under certain situations on the human body. Potentially harmful effects are mostly due to the formation of highly active chemical compounds, known as ROS, which have oxygen donation

tendency to the other substances. Collective term, ROS is used to include radicals of oxygen and other non radical oxidizing agents such as HOCI or Hypochlorous acid, $H_2O_2$, ozone, etc. In human body due to wide range of food system full of oxygen free radicals, reactive species are formed (Halliwell et al., 1995). Damage by free radical accelerates the transition of metal ions. As a result of this oxidative damage occur and this is responsible for carcinogenesis, aging and atherosclerosis.

Exogenous and endogenous originated free radicals generate ROS. When antioxidants are present in low concentration there is a delay in oxidative processes significantly. Antioxidants are better understood by their reactivity.

### 2.1.2 Damages caused by free radicals

Oxidative damage occur due to activation of free radicals, all cellular macromolecules like carbohydrates, lipids, proteins and nucleic acids are damaged by their chemical reactivity and are also responsible for different kinds of diseases like muscle contraction, cancer, LDL and heart diseases. Ageing is also caused by free radicals (Bagchi et al.,1998).

**Oxidative damages to proteins**

Oxidative attack on protein is due to site specific modifications in amino acid, peptide chain fragmentation, cross linked reaction products aggregation, electrical charges alterations and more susceptible to proteolysis (Farr et al.,1991).

**Oxidative damage to DNA**

Deletion, mutations and other lethal genetic effects induce numerous lesions in DNA. Characterization of DNA damage has indicated that both base moieties and sugar are susceptible to oxidation, causing degradation of bases and cross linking to proteins (Imlay et al., 1986).

Adverse changes of free radical reactions are expected to produce aging effect throughout the body progressively. In sub sarcoma region of muscles, pigments of aging like lipofusin granules starts accumulate and increase with increase in age.

Free radicals results in number of degenerative diseases and also affect a number of physiological functions like diabetes, atherosclerosis, ischemic injury, inflammatory diseases like arthritis, bowel diseases and pancreatitis, different types of cancers, neural diseases, hypertension etc. (Cui et al., 2004). Free radicals are not harmful always but also have useful purposes in human body. In living system, oxygen radicals are necessary compounds in the cellular structure maturation process. Free radicals also released by white blood in order to destroy microbes or invading pathogenic as a part of defence system of body in order to treat

disease. Hence, radical's complete elimination is harmful to living system (Bagchi et al., 1998).

## 2.2 Antioxidants

Antioxidants act as an inhibitor of oxidation process, even at relatively low concentration. Plant materials extract having antioxidant constituents behave like radical scavengers and can convert the radicals to less reactive species than free radicals. In dietary sources such as fruits, vegetables and tea etc. a variety of antioxidants are present (Mandal et al., 2009).

Literature survey shows that substance if present in less concentration compared to oxidizable substrates (proteins, lipids and nucleic acids) inhibits or delays the oxidation of substrates (Singh et al., 2002).

Antioxidants are defined as compounds which have capability to inhibit oxygen mediated oxidation of various substances from simple molecule to complex bio-systems and to polymers (Chen et al.,1995).

According to FDA (Food and Drug Administration), antioxidants are explained as substances present in food for their preservation from oxidation by deterioration, retardation, discoloration or rancidity (Gutteridge et al., 1990).

The food industry has interest in antioxidants because antioxidants can prevent food rancidity (Loliger 1991). Clinicians and biologists are also interested in antioxidants because they can protect human body against damage by ROS (reactive oxygen species).

Antioxidants defence in enzymatic and non enzymatic reactions, protect the body against damage due to oxidation. Non enzymatic antioxidants are added to food frequently to prevent oxidation of lipids.

Cell damage is also prevented by the antioxidants. For health improvement, naturally derived antioxidant and diet containing antioxidants are recommended which can delay the damage or provide protection to human cells from the damage. ROS like $O_2^-$ and $OH^-$are by products of the metabolism and are found in different types of organisms from microbes to higher plants and animals. (Halliwell 2012). It has been found that ROS reactions which are free radical mediated can cause oxidative damage of bio molecules and Lead to aging, atherosclerosis, cancer, diabetes, coronary heart ailment, Alzheimer's disease, and other disorders of nerves system etc. ROS-mediated tissue impartment is effectively managed by antioxidants (Finkel et al., 2000).

## 2.3 Medicinal Plants: A source for bioactive metabolites

Since times immemorial therapeutic plants have been utilized as a source of pharmaceutical. In ancient literature, for example in Vedas and Bible, the widespread utilization of herbal medicines and healthcare preparations has been portrayed. Natural products have been exploited for human use for many years and plants have been a good source of compounds used for medicine.

In industrialized nations, more than 7,000 compounds are contributed by the plants to the pharmaceutical industry counting those used in heart drugs, laxatives, anti-cancers, contraceptives, hormones, analgesics, antibiotics and diuretics etc. WHO defines a medicinal plant as any plant organ contains substances that can be used for therapeutic purposes or which are precursors for the pharmaceutical chemo semi synthesis (Zheng et al., 2001). This definition distinguishes those plants that are already experimentally tested from those which do not have any scientific study but used traditionally in medicine system. The beneficial effects of the medicinal plants in health care can be well judged from the WHO estimate that around 80 % of the world population uses them in some form or the other (Balick et al., 1996).

The people using medicinal plants are mostly those living in the remote areas and rural and indigenous people who depend heavily on the natural resources. An ethno biological survey revealed that about 8,000 species of medicinal plants are used as food supplements, medicines, biocides and other phyto-chemicals (Cai et al., 2004).

Traditional health remedies usage from medicinal plant reported to have minimum side-effects (Salama et al., 2010).

Radical scavenging molecules like phenolic compounds i.e. quinines, lignin, flavonoids, coumarins, lignans, stilbenes, phenolic acids, tannins, nitrogen compounds like alkaloids and amines, vitamins, terpenoids, and other endogenous metabolites are present in medicinal plants (Kahkonen et al., 1999). Microorganisms along with plants also have been regarded as an important source of metabolites with promising anti-mitotic, anti-bacterial and anti-viral activity (Berdy 2005). Endophytes are found as rich sources of various bioactive metabolites having importance in agriculture, medicine, and industries. Many endophytic fungi from nature have been reported to produce novel antifungal, antiviral, anti-inflammatory, antitumor, antibacterial and other compounds belonging to the alkaloids, flavonoids, steroid, and terpenoids derivatives etc (Tan et al., 2001). Endophytes of medicinal plants are important resources for the natural products discovery.

## 1.3 Endophytic Fungi

De Bary, introduce the term endophyte and defined as any organism which is occurring within tissues of plants (Stepniewska et al., 2013).

Fungi are present mainly on the organic compounds. The majority of fungi are saprobes and decompose organic matter which is dead. Many are specialized to attack and infect the living organisms. Some of these fungi may be pathogenic; symptoms of disease may become prominent after incubation for the short period of time. Other ones can infect the living organisms but do not develop the symptoms, because once fungi present inside tissue, it forms a latent state either for the whole lifetime of the infected plant tissue or for extended time i.e. until conditions of environment are favourable for growth of the fungus or for the disposition phase of the host changes in order to advantage the fungus, these fungi are referred as the entophytes (Yu et al., 2010). If symbiosis takes place in the leaves and endophytic fungi, the fungus entity is sometimes called mycophylla along with the term mycorrhiza which is for the symbiosis of roots and fungi.

All the plant species (300,000) developing in unexplored range on the earth are host to at least one endophytic living beings (Strobel et al., 2003). To date, just a couple of plants are explored for their endophytic biodiversity and their capability to deliver bioactive auxiliary metabolites. Studies have been led at various parts of the world about the endophytic biodiversity, scientific categorization, proliferation (Bandra et al., 2001).

Endophytic microbes are of biotechnological interest because of their potential as a source of auxiliary metabolites that has been demonstrated valuable for novel medication revelation (Tu 2011). Antifungal and antibacterial activities of plant endophytic fungi have been accounted for some parasitic and bacterial strains (Sharma et al., 2016). Endophytic fungi has been appeared to produce some pharmacologically essential compounds, for example, antimycotics steroid 22-triene-3β-ol (Helaly et al., 2014), anticancer cajanol (Biava et al. 2014), podophyllotoxin and kaempferol (Huang et al., 2014), calming ergoflavin (Deshmukh et al., 2009), cancer prevention agent lectin (Sadananda et al., 2014), insecticidal heptelidic corrosive (Keswani et al. 2014), immunosuppressive sydoxanthone A, B (Song et al. 2013) and cytotoxic radicicol (Wang et al., 2008).

Highly diverse polyphyletic group of endophytic fungi mainly of ascomycetous are functionally defined by their presence in plant tissues without causing any significant effects (Naik et al., 2008). Ferns, equisetopsids, horn and liver words, lycophytes and plants having seeds in from tropics to the tundra arctics (Aly et al., 2010). Inside plant the endophytic fungus forms latent state either for lifetime in tissues of infected plant or for long time period

until the conditions of environment becomes suitable for the growth of the fungus. The host ontogenetic state changes according to the advantages of the fungi which turn into pathogen (Aly et al., 2010). Higher plants nearly 300,000 species exists on the earth, which are host to one or more endophytes (Strobel et al.,2003). Within the plant the fungal diversity analysis is fast germinating and growing but the species which are rare are less strengthen and have specific requirements during cultivation and analysis practices (Aly et al., 2010).

Secondary sources like alternative metabolites are found from the plants, having a lot of endophytes which are active biologically and (Suryanarayanan et al., 2009) important for the discovery of the new drugs and for the agriculture (Mitchell et al., 2010). It is found that the endophytic fungi having different chemical structures are modified by evolution and involved in protection and communication of the host plant (Han et al., 2012).

### 1.3.1 Endophytic Fungi: Secondary metabolite sources of plants

Secondary metabolites produced by endophytic fungi associated with plants, have pharmaceutical values. By Alexandra Fleming, discovery of penicillin from the *Penicillium notatum* is responsible for the realisation of the significance of the fungi as a source of bioactive compound. In search for the discovery of the drug sources which is novel, endophytic fungi shows promising results, with wide distribution and diversity. Secondary metabolites discoveries form endophytic fungi raise the use of these as a alter source of these metabolites (Kusari et al., 2012). From endophytic fungus, named *Taxomyces andreanae* which is used to get taxcol or paclitaxel from the plant *Taxus brevifolia* (Strobel et al., 1996). Paclitaxel (taxol) is a gold bioactive compound, has been proved with an efficient action against prostate, ovarian, breast and lung cancers. Naphthodianthrones, like hypericin are the secondary metabolites also obtained from endophytic fungi of *Hypericum* species, and useful to treat depression and anxiety (Kusari et al., 2008). Many kind of natural products are synthesised by the endophytes which occur in plants, Attempts have been made to isolate and identify these bioactive or natural compounds from endophytic fungus.

### 1.3.2 Antioxidants associated with endophytic fungi

The significance of bioactive compounds bearing antioxidant activity lays in the fact that they are exceptionally effective against damage caused by reactive oxygen species (ROSs) and oxygen-determined free radicals for example, DNA damages, carcinogenesis, and cell degeneration (Huang et al., 2007). Antioxidants have been viewed as promising treatment for prevention and treatment of ROS-linked infections as tumor, cardiovascular diseases, atherosclerosis, hypertension, ischemia/reperfusion injury, diabetes mellitus, neurodegenerative disorders (Alzheimer and Parkinson sicknesses) and ageing (Huang et al.,

2007). Numerous antioxidant compounds have antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial, or antiviral activities in higher or lower level (Sala et al., 2002).

Natural antioxidants are regularly found in restorative plants, vegetables, and natural products. Nonetheless, it has been reported that metabolites from endophytes can be a potential source of novel natural antioxidants. Liu and colleagues assessed the antioxidant activity of an endophytic Xylaria sp. isolated from the medicinal plant Ginkgo biloba (Liu et al., 2007). The outcomes collected showed that the methanol extract displayed strong antioxidant capacity because of the presene of "phenolics" and "flavonoids" compounds. Huang and collaborators explored the antioxidant capacity of endophytic fungal cultures of medicinal plants and its connection to their total phenolic substance (Kaul et al., 2012). They recommended that the phenolic content were the real antioxidant constituents of the endophytes (Huang et al., 2007).

“Pestacin" (C15H14O4) and "isopestacin", 1,3-dihydro isobenzofurans, were acquired from the endophytic fungus Pestalotiopsis microspora isolated from a plant developing in the Papua New Guinea, *Terminalia morobensis* (Harper et al., 2003). Other than antioxidant activity, pestacin and isopestacin likewise showed antimycotic and antifungal activities, respectively. Pestacin is accepted to have antioxidant activity 11 times more prominent than Trolox, a vitamin E subsidiary, essentially via cleavage of an abnormally receptive C-H bond and to a lesser degree, O-H deliberation (Harper et al., 2003). Isopestacin have antioxidant activity by scavenging both superoxide and hydroxyl free radicals in solution, added to the way that isopestacin is structurally same to the flavonoids.

### 1.3.3 Importance and need to explore new sources of endophytic fungi

Natural products are naturally derived by-products or metabolites from plants, animals or microorganisms (Strobel et al., 2003) . These natural products are the source of novel metabolites which are bioactive and have significant impact on the different modern medicines. Natural products or their derivatives contribute around 68% of antibacterial compounds and 34% of products used in cancer therapy (Newman et al., 2007).

Natural products have been exploited for human use, derived particularly from medicinal plants for thousands of years to make the life easy. Because of these facts, there is a need to explore useful and new bioactive compounds to provide relief and assistance in all aspects of human conditions.

Endophytic fungi are the one which can help to eliminate the problems faced by the living organisms. The term endophytic indicates the fungi within tissues of the plants for at

least a part of their life cycle and causes no side effects, it does not involve the mycorrhizal fungi (Bacon et al., 2000). By definition, an endophytic fungus grows in the mycelial form in the association with plant at least for some times. Therefore, the minimal requirement to termed a fungus, an endophyte should be the demonstration of its hyphae in the living tissue.

Endophytes show synergistic effects to their host. They also prevent the host from successfully attacking fungi and pests by secreting special substances such as secondary metabolites and in return demanding the nutrients (Strobel et al., 2003). The endophytes from medicinal plants synthesize a number of chemicals, metabolites with more resistance to livestock, nematodes and insects. Plants having specific endophytic fungi often grow fast because of the production of phyto hormones and show domination in specific environment.

The endophytes have benefit of their symbiotic relationship with the host plant, due to the ability of host plant to supply the necessary compounds and nutrients essential for the growth of endophyte in order to complete its life cycle. Under quite extreme and inhospitable conditions many endophytes are able to survive unlike to the host plant (Stone et al., 2000).

Fungal endophytes and microbes produce a lot of secondary metabolites (Redon et al., 2006). Secondary metabolites which are novel are concentrated on organisms, inhibiting exceptional and unique biotopes. The interaction between plants and fungus is mainly responsible for the generation of the secondary metabolites with their novel properties (Bednarek et al., 2009). This may be possible due to the consequence of high frequency isolation of endophytic fungi from the plants. Fungal endophytes produce natural products which have a wide spectrum of biological activity and can be grouped into various categories like alkaloids, steroids, xanthons, lactones, glycoside etc. These microorganisms from medicinal plants are poorly investigated although these endophytic fungi show an abundant and dependable source of bioactive and chemical compounds.

# 3 Methodology

## 3.1 Materials, chemicals and instruments

In this project the materials and instruments used were wash-bottles, petri-plates, flasks, separation funnel, test tubes, test tube stands, TLC plates, Chromatographic chamber, micro- titre plates (tarson), spray bottle, auto-pipettes (1ml, 20-200 µl, 0.5-10µl), Calton B.O.D. Incubator at 27°C, Laminar air hood, Spectrophotometer, TLC applicator, Autocalve, IKA RV 10 Rotary evaporator, Rota flask, Centrifuge, ELISA micro-plate reader, Vortex.

Different chemicals used in this project were distilled water, Milli-Q water, Tween -20 detergent, Sodium hypochlorite (NaOCl), Acetone, Ethyl acetate, Anti-oxidant as control (ascorbic acid), absolute methanol, DPPH (2,2 –diphenyl-1-picrylhydrazyl), DCM (di-choromethane), chloroform, ninhydrin, conc. $H_2SO_4$, Molisch reagent, 5% $FeCl_3$, Wagner reagent.

## 3.2 Source of endophytic fungi

Two different medicinal plants were selected for the isolation of endophytic fungi and two different fungal endophytes which were isolated from the different host plants of Western Ghats of India were selected for the screening of antioxidant activity in this project.

These two plants were as follows- *Tinospora cordifolia* common name Giloy or Guduchi and *Terminalia arjuna* common name Arjuna tree.

The fungal isolates from plants of Western Ghats used in this project were XF-2 and XF-4 (table 1).

**Table 1: Host and family name of fungal isolates of *Xylaria* sp.**

| CODE NO. | HOST | FAMILY |
|---|---|---|
| XF-2 | *Memcylon* | *Melastomaceae* |
| XF-4 | *Lasianthus venulosus* | *Rubiaceae* |

These XF-2, XF-4 fungal cultures used in this project for screening of anti-oxidant activity were provided by TIFAC- CORE, Thapar University, Patiala.

## 3.3 Plant sample collection

The stem of ***Tinospora cordifolia*** and the bark of ***Terminalia arjuna*** were collected from the plant collection of Thapar University Campus, Patiala, Punjab (figure 3, 4). Samples of the selected plants were collected by cutting the desired part with a sterile blade and putting it in sterile bags. As mentioned above, two plants were selected from which endophytic fungi

had to be isolated and two fungal isolates XF-2 and XF-4 had to be sub-cultured from slants to the solid media.

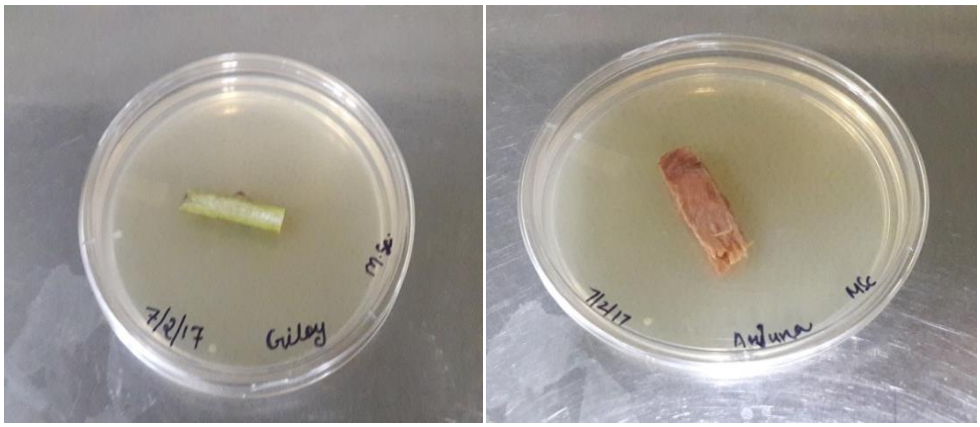
**Figure 3: *Tinospora cordifolia* (Giloy) from Thapar University campus, Patiala.**

**Figure 4: *Terminalia arjuna* (Arjuna) from Thapar University campus, Patiala.**

## 3.4 Isolation and secondary metabolite extraction from endophytic fungi

### 3.4.1 Isolation of endophytic Fungi

Isolation of endophytic fungi was carried out using a modified method (Schulz et al., 1993). Endophytic fungi were isolated from the selected samples of *Tinospora cordifolia* and *Terminalia arjuna* by washing the sample collected in sterile bag with tap water to remove the dust and debris, followed by washing with Tween-20 detergent and distilled water. Then surface sterilization was done by soaking the sample in 70% ethanol for 1 minute. After that the samples were immersed in 1% sodium hypochlorite for 30 sec. Then the sample was rinsed with sterile distilled water for 3-4 times and allowed to surface dry on filter paper. After proper drying the stem parts were cut and inoculated on a petri-plate containing PDA medium (figure 5 a, b)




(a) (b)

**Figure 5: Inoculation of surface sterilized (a) *Tinospora cordifolia* stem and (b) *Terminalia arjuna* bark on PDA.**

After inoculation, plates were incubated at 27°C for 7- 14 days. Growth of fungi was seen on 7th day. After the endophytic fungi had grown on the plate, pure fungal cultures of endophytic isolates were acquired by hyphal tip method.

### 3.4.2 Sub-culturing

The above obtained fungal cultures were sub-cultured on petri- dish containing PDA medium and incubated at 25° ± 2° C. This sub-culturing from one plate to another was repeated until the pure cultures were successfully obtained.

Master cultures were preserved on PDA slants at 4°C. PDA slants were made by heating agar to the boiling point and pouring it into test tubes. Prior to the agar cools and solidifies, the test tube is set to its side. Once the agar was cooled, the test tubes were inoculated with fungal isolates and stored.

The reference master cultures XF-2 and XF-4 were kept at TIFAC- CORE, Thapar University, Patiala, Punjab. These cultures were maintained on PDA slants and sub-cultured regularly. For sub-culturing, mycelial disk of XF-2 and XF-4 were inoculated on the Petri-plate containing PDA medium and incubated at 27°C ± 2°C.

### 3.4.3 Fermentation of endophytic isolates

After getting the purified cultures through sub-culturing and cultivation of given fungal isolates fermentation was done for the extraction of secondary metabolites. This process was done by inoculation of four discs of ($0.5\times0.5cm^2$) mycelial agar into 500 ml of Erlenmeyer flasks containing 250 ml of potato dextrose broth (PDB).

Composition of media used for culturing of endophytic fungi:-

PDB (Potato Dextrose Broth) [Himedia Pvt Ltd.]

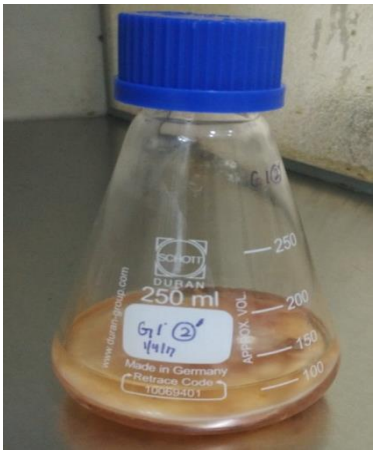
Suspend 24g of PDB in 1000ml of distilled water. Boiled till PDB dissolve completely. Autoclaved the media for 15 minutes at 15 lbs pressure at 121°C.

The culture flask was incubated for 21 days at 27° C in a static condition.

### 3.4.4 Filtration and Extraction

After 21 days of incubation, the fermented broths were filtered to separate the mycelia by filtration through sterile muslin cloth in laminar air flow bench. The culture filtrate was extracted with ethyl acetate in the separating funnel by shaking with equal volume for 10 mins and then allowed to settle for phase separation (figure 6). Ethyl acetate phase consisting of the constituents from the extracted broth was collected in a bottle. The process was repeated twice. Then the combined ethyl acetate extracts were concentrated using rotary evaporator to obtain dry residue. Weight of the residue was determined.

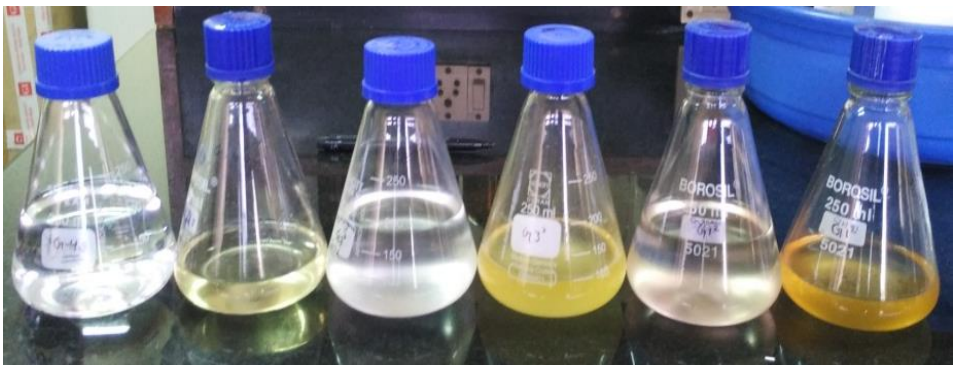
The dried residue was mixed with methanol and stored at 4°C. Stocks of crude endophytic extracts were made in methanol at a concentration of 5mg/ml. These extracts were further investigated for their anti-oxidant activities.




Fermented Broth

Filtration

Extraction




Organic Fungal Extract

**Figure 6: Extraction of crude compounds from endophytic fungal cultures.**

## 3.5 Anti-Oxidant Screening: DPPH Radical Scavenging Activity Assay

The DPPH radical scavenging method was conducted based on previous developed method with some minor modifications (Duan et al. 2006). This assay was done in 96 well microplate. DPPH is a stable, nitrogen centered free radical which produces violet colour in methanol solution and during reaction with antioxidant, the substrate can donate electrons and can reduce the DPPH from violet to yellow colour by producing the reduced product diphenylpicrylhydrazine.

Fungal extracts ranging from 100-1000 µg/ ml concentration in methanol were mixed with methanolic solution of DPPH (0.1 mM/L). Ascorbic acid was taken as standard antioxidant as control. Methanol was taken as blank and DPPH solution without the extracts was taken as control. The 96 well micro-plate was left to stand in the dark for 45 minutes. After incubation the reduction in the DPPH radicals was determined by taking absorbance in microplate reader at 517 nm (figure 7). Free radical scavenging activity was expressed in percentage by using the equation:

$$\text{Percent inhibition of DPPH radicals} = \frac{\text{Control absorbance} - \text{Sample absorbance}}{\text{Control absorbance}} \times 100$$

A standard template was made for performing the assay in 96 well microplate for different concentrations of fungal extracts. The experiment was performed in triplicates (table 2).

**Table 2: Template for DPPH assay in 96 well micro-plate with fungal extracts.**

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| M | MD | AAS | AAC | G3` | $G2^2$ | G2” | $G4^1$ | $G4^3$ | $G1^2$ | $G9^3$ | $G7^3$ |
| M | MD | AAS | AAC | G3` | $G2^2$ | G2” | $G4^1$ | $G4^3$ | $G1^2$ | $G9^3$ | $G7^3$ |
| M | MD | AAS | AAC | G3` | $G2^2$ | G2” | $G4^1$ | $G4^3$ | $G1^2$ | $G9^3$ | $G7^3$ |
| | | | | | | | | | | | |
| | | | | | | | | | | | |
| $G9^2$ | $G8^2$ | $G8^4$ | XF2 | XF4 | ARJ | | | | | | |
| $G9^2$ | $G8^2$ | $G8^4$ | XF2 | XF4 | ARJ | | | | | | |
| $G9^2$ | $G8^2$ | $G8^4$ | XF2 | XF4 | ARJ | | | | | | |

Total well capacity: 200µl

M : methanol 200µl

MD : methanol 50µl + DPPH 150µl

AAS : methanol 20µl + DPPH 150µl + ascorbic acid 30µl

AAC : methanol 20µl + DPPH 150µl + distilled water 30µl

Fungal extracts : methanol + DPPH 150µl + fungal extract

Volumes added were according to the concentration of fungal extracts (table 3).

**Table 3: Volume of each component according to different fungal extract concentration.**

| Sample concentration(µg/ml) | Total volume(µl) | Fungal extract(µl) | Methanol (µl) | DPPH (µl) |
|---|---|---|---|---|
| 100 | 200 | 4 | 46 | 150 |
| 250 | 200 | 10 | 40 | 150 |
| 500 | 200 | 20 | 30 | 150 |
| 1000 | 200 | 40 | 10 | 150 |

All assays were carried out in triplicates and the inhibition of DPPH free radicals were expressed in percentage.

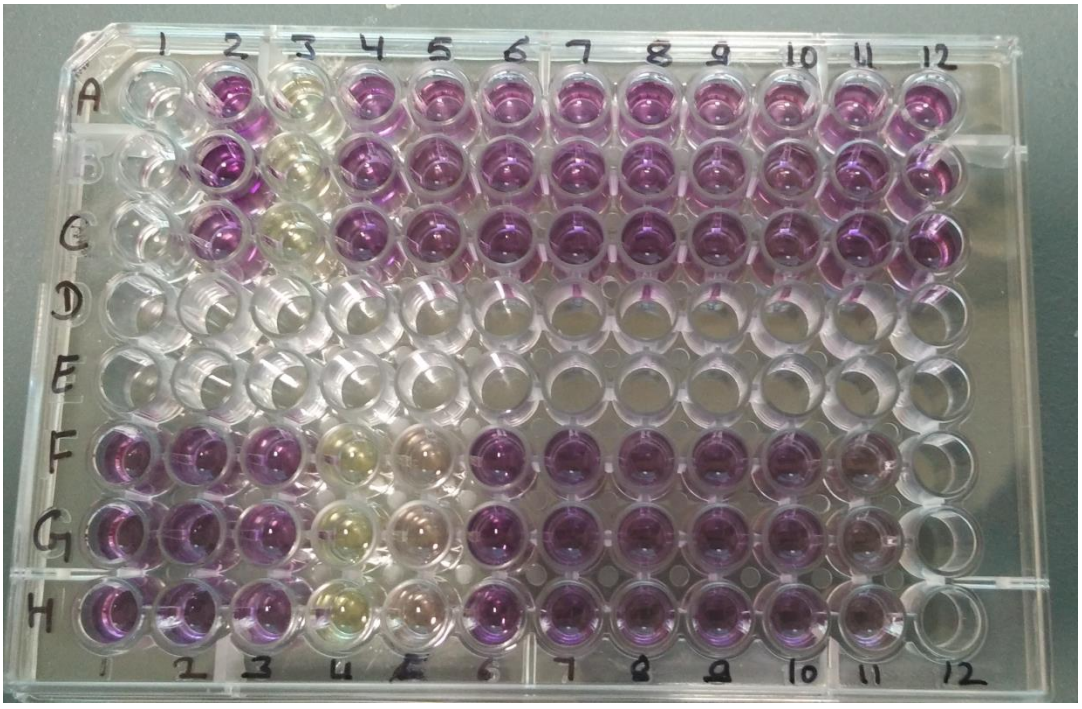



**Figure 7: DPPH assay in 96 well micro-plate.**

## 3.6 Phytochemical analysis

Preliminary phytochemical screening of the crude fungal extracts was carried out for the presence of the following metabolites: alkaloids, flavonoids, tannins, phenols, saponins, terpenoids and carbohydrates using standard methods with modification (Devi *et al*., 2012; Bhardwaj *et al*., 2015).

**Test for amino acids**

1ml of water dissolved extract was added in 1ml of 5% ninhydrin solution and then heated on boiling water bath for 10 minutes. Appearance of violet colour indicated the presence of amino acids in fungal extracts.

**Test for alkaloids (Wagner`s reagent test)**

Wagner`s reagent was prepared by mixing 1.25g iodine and 2g of potassium iodide (KI) in 5ml of water and made up the volume up to 100ml. Few drops of the Wagner`s reagent was added in 1ml of extract. Reddish precipitate in test tube indicated the presence of alkaloids.

**Test for carbohydrates (Molisch`s test)**

Molisch's reagent is 1g of α-naphthol dissolved in 60ml 95% alcohol. Few drops of Molisch's reagent was added in 1 ml of extract after that few drops of concentrated hydrochloric acid (HCl) was added down the sides of the test tube. Appearance of a purple coloured ring at the interface of HCl and test layer indicated the presence of carbohydrates in the fungal extract.

**Test for flavonoids**

1ml of the fungal extract was dissolved in diluted sodium hydroxide (NaOH) and then 1ml Hydrocloric acid was added. Yellow colour disappears and solution turns into colourless which indicates the presence of flavonoids.

**Test for fats and fixed oils (Saponification test):**

To 1ml of the extract, few drops of 0.5N alcoholic potassium hydroxide (KOH) were added along with a drop of phenolphthalein. Heated on boiling water bath for 1 hour. The formation of soap indicated the presence of oils and fats.

**Test for glycosides**

Glacial acetic acid was added in 2ml of extract along with the one drop of 5% ferric chloride ($FeCl_3$) and 0.5ml of concentrated sulphuric acid. A brown ring at the interface indicated the presence of cardiac glycosides.

**Test for terpenoids**

2 ml of chloroform was added in 2ml of fungal extract. Concentrated HCl was added carefully to form a layer. Appearance of reddish brown colour at the interface indicated the

presence of terpenoids.

**Test for tannins and phenolic compounds**

1ml of the fungal extract was treated with 5% ferric chloride ($FeCl_3$) and while observation if blue colour appeared then hydrozylated tannins were present and if dark green colour appeared then condensed tannins were present.

**Test for steroids**

2ml of acetic anhydride was added in 2ml of fungal extract and heated on water bath for a minute. After that few drops of conc. sulphuric acid was added. Violet colour solution turned into blue or green colour indicates the positive results for steroids.

## 3.7 Chromatographic analysis of fungal extracts

Qualitative determination of fungal extracts from *Terminalia arjuna* and *Tinospora cordifolia* was done by thin layer chromatography A straightforward, exact and quick elite thin layer chromatographic strategy has been created for the synchronous qualititative assurance of secondary metabolites present in fungal extracts. The separation of these compounds was done on TLC plate with mobile phase of chloroform and ethyl acetate for fungal extracts from *Tinospora cordifolia*, and mobile phase of chloroform and ethyl acetate for fungal extract from *Terminalia arjuna* and the results of analysis were observed through TLC viewing cabinet.

The composition of the mobile phase for TLC was optimised by testing different solvent mixtures of varying polarity and then best results were obtained for both fungal extracts (table 4 and 5).

**Table 4: Optimization of mobile phase of varying polarity for *Tinospora cordifolia***

| Mobile phase for *Tinospora cordifolia* | Percentage | Total volume |
|---|---|---|
| Pure chloroform | 100% | 4ml |
| Chloroform + Ethyl acetate | 90% | 3.6ml+0.4ml |
| Chloroform + Ethyl acetate | 70% | 2.8ml+1.2ml |
| Chloroform + Ethyl acetate | 50% | 2ml+2ml |
| Chloroform + Ethyl acetate | 30% | 1.2ml+2.8ml |
| Pure Ethyl acetate | 100% | 4ml |

**Table 5: Optimization of mobile phase of varying polarity for *Terminalia arjuna***

| Mobile phase for *Terminalia arjuna* | percentage | Total volume |
|---|---|---|
| Pure chloroform | 100% | 4ml |
| Chloroform + Methanol | 90% | 3.6ml+0.4ml |
| Chloroform + Methanol | 70% | 2.8ml+1.2ml |
| Chloroform + Methanol | 50% | 2ml+2ml |
| Chloroform + Methanol | 30% | 1.2ml+2.8ml |
| Pure Methanol | 100% | 4ml |

Upon the development of a TLC plate and visualization under UV light, the starting point and solvent front were marked and all spots observed on the plate were circled with lead pencil. The location of each spot on the plate is then represented numerically by calculating a Retention Factor (Rf).

$$\text{Rf Value} = \frac{\text{Distance from baseline travelled by solute}}{\text{Distance from baseline travelled by solvent}}$$

## 3.8 Preparative thin layer chromatography

Preparative thin layer chromatography is a useful technique for the purification of the small quantities of samples. This technique allows the rapid separation of a number of compounds in a reaction mixture. This method is used for obtaining a profile of the compounds present in the natural extract. By doing the preparative TLC of the fungal extracts, the compound will be separated. This separation is determined by analyzing the bands that occur on TLC plate, which are visible under UV visible light. By scraping the bands, fractions can be separate out from the fungal extract and then the confirmation screening of antioxidant activity can be done with different fractions.

**Analytical procedure**

- A full size plate with a thick layer of silica gel was used for preparative separation by thin layer chromatography.
- Gently marked a pencil line roughly 1-1.5 inches from one side of the plate but carefully, not to scrape the silica. This was taken as line of origin.
- Prepared a relatively concentrated solution of crude fungal extract in ethyl acetate. Volume of the sample applied on the plate was less so that clear separation of band occurs.

- By using a capillary, sample was applied across pencil line on TLC plate and an inch away from the edges. This was done slowly and uniformly, without contacting the capillary too much to silica so that silica does not scrape out. The entire remaining sample was deposited on the original line and then plate was allowed to air dry.
- A preparative TLC chamber was used after filling with the mobile phase. A total of 150ml of this mobile phase was filled in TLC chamber and a filter paper was kept in it to saturate the chamber.
- Plate was placed in the chamber and chamber was sealed with lid. Plate was allowed to run for 40 minutes to 1 hour.
- Plate was taken out from the TLC chamber after the run and allowed to air dry. The plate was visualized under UV light to check for the separation of various fractions present in the extract.
- Marked the bands and scraped off each band with the help of spatula.
- To retrieve the compound from the scraped off silica, the silica powder was poured into a syringe sealed with glass cotton plug. Washings with 10% methanol in dichloromethane were given to the silica so that the fractions were eluted and collected in a glass vial.
- After that each fraction was dissolved in methanol and antioxidant screening was done by DPPH radical scavenging assay.

## 3.9 Identification

### 3.9.1 Morphological identification

The fungal isolates from giloy and arjuna were observed for colony characterization and minute examination under microscope. For colonial characterization, parameters like shade of the colony, filamentous and mat sort development was considered. The slides of both old and new fungal cultures were readied by using lactophenol cotton blue stain and seen under microscope (Olympus CH20i) at ×40 and ×100 amplification.

### 3.9.2 Molecular identification

Fungal cultures were sub cultured again and growth was developed on PDA plates overlaid with cellophane sheets for 5 days. After incubation of 5 days the mycelium was scratched from the cellophane sheets and pounded or crushed with liquid nytrogen. Crushed tests were kept at - 80°C for continuing further steps of the isolation of genomic DNA.

**Genomic DNA isolation**

100 mg of liquid nitrogen crushed mycelium of giloy and arjuna were taken in 1.5 ml centrifuge tube and 600µl of extraction buffer was included in both the tubes and vortex the tube well for 1 minute. These tubes were then kept at 65°C for 30 minutes to 1 hour. After that tubes were centrifuged at 12000g for 10 minutes and supernatant were collected in new tubes. 5µl RNase was added in both the tubes and then incubated at 37°C for 30 minutes. Equal volume of isoamyl alcohol (24:1) was included and mixed well by inversion. Then tubes were kept at room temperature for 20 minutes. After that tubes were centrifuged at 14000g for 20 minutes and the upper fluid layer was collected into another tubes. Equal volume of isopropanol was added in both tubes and kept at -20°C for 30 minutes to 1 hour. The samples were then centrifuged at 14000g for 10 min. Supernatant was discarded from both the tubes and the pellet was washed with 500µl of 70% chilled ethanol. The tubes were centrifuged again at 7500g for 10 minutes and pellet was allowed to air dry. Pellet was re-suspended in 30µl to 50µl of Milli-Q water and stored at 4°C.

**Agarose gel electrophoresis for DNA**

Gel electrophoresis is the standard lab technique for differentiating DNA by its size (e.g., length in base sets) for representation and purification. Electrophoresis utilizes an electrical field to move the adversely or negatively charged DNA through an agarose gel network toward a cathode. Shorter DNA fragments relocate through the gel more rapidly than the longer ones. In this manner, length of a DNA section can be measured by running it on an agarose gel close by a DNA laddder (an accumulation of DNA parts of known lengths).

**Procedure of agarose gel electrophoresis**

For 1% agarose gel 0.4 gm agarose was mixed in 40 ml of 0.5x TBE buffer. Agarose is insoluble at room temperature so the agarose solution was warmed in microwave oven until the point that it broke down totally and progress toward becoming clear. The solution was chilled off and 2µl of ethidium bromide was added and mixed appropriately. The comb was set over the casting plate in a gel caster and agarose gel was poured. The gel was permitted to solidify for around 30 to 40 minutes at room temperature. The comb was deliberately removed so that the wells are not damaged, and the intact wells are formed for the counteractive action of a spillage. The gel was then put deliberately on to the electrophorosis chamber. The electrophorosis buffer was added to cover the gel. Ensured that each well was loaded with electrophorosis buffer.

# 4 Results and Discussion

The aim of this present study is to isolate endophytic fungi from *Tinospora cordifolia* and *Terminalia arjuna* for screening of antioxidant activity. Antioxidant screening was done by DPPH free radical scavenging assay. Separation of crude fungal extracts was done by chromatographic technique and the separated bands were further screened for antioxidant activity.

## 4.1 Isolation of endophytic fungi from *Tinospora cordifolia*

A total number of 11 fungal isolates were obtained from the stem of *Tinospora cordifolia*, collected from campus of Thapar University, Patiala, Punjab (figure 8). Isolation was done by the inoculation of surface sterilized plant stem on PDA followed by incubation at 27°C ± 2°C for 7-14 days. The isolated endophytic fungi were coded as G1 to G11.

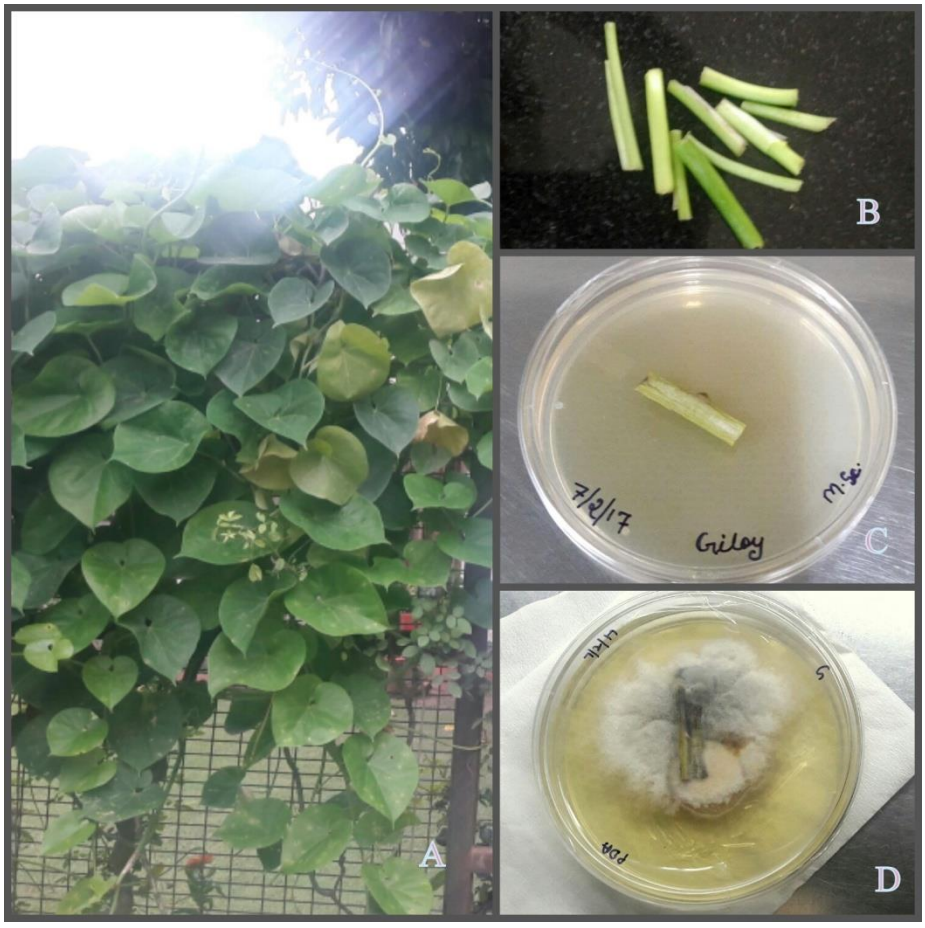



**Figure 8: A: sample source *Tinospora cordifolia* from Thapar university campus, Patiala, B: stem of *Tinospora cordifolia*, C: inoculation of surface sterilized stem on PDA medium, D: isolated endophytic fungi**

## 4.2 Isolation of endophytic fungi from *Terminalia arjuna*

A total number of 5 endophytic fungi were isolated from the bark of *Terminalia arjuna* after surface sterilization and incubation for 7-14 days at 27±2°C (figure 9). The plant sample was collected from campus of Thapar University, Patiala, Punjab. The isolated endophytic fungi were coded as A1 to A5.

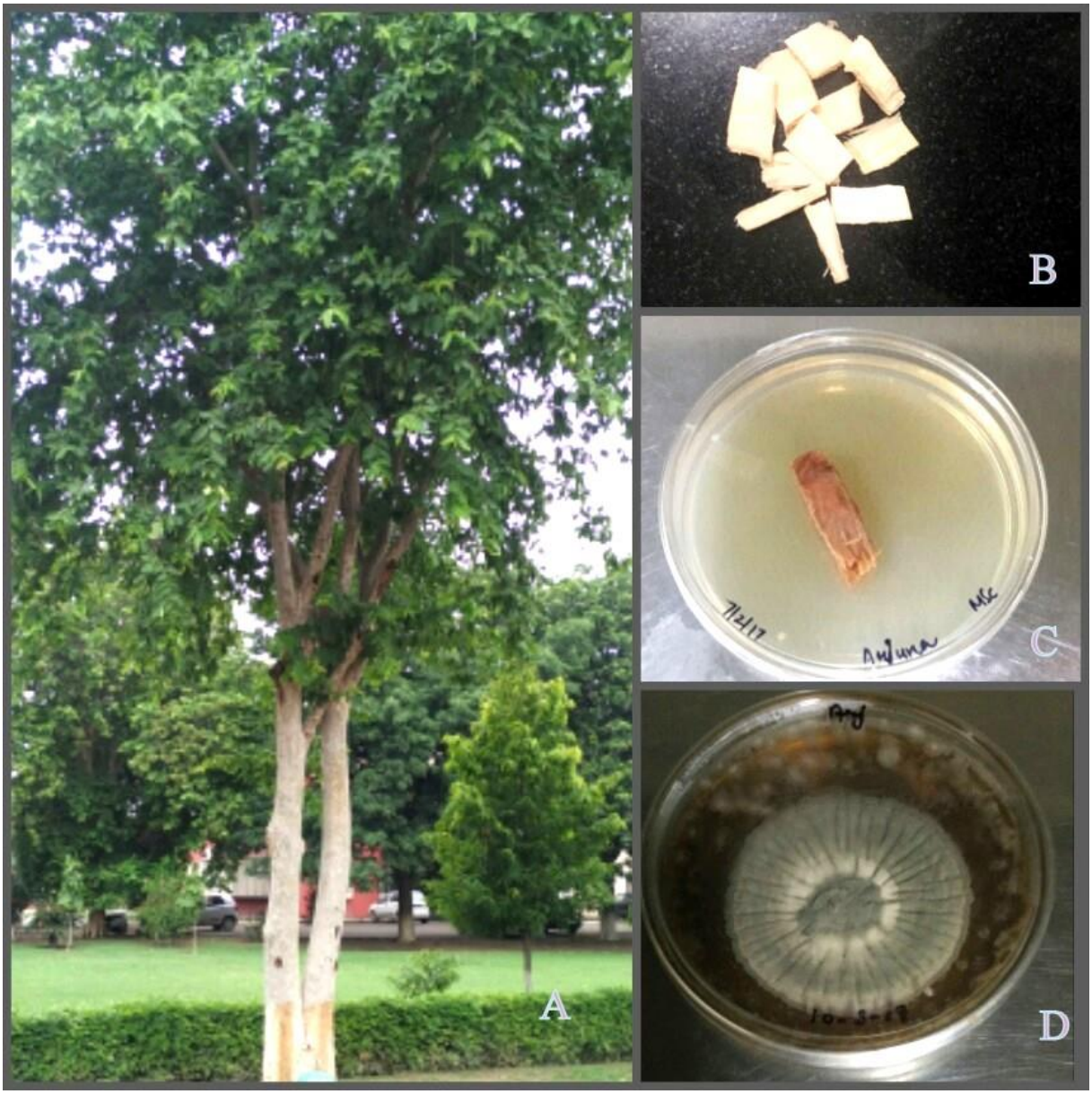
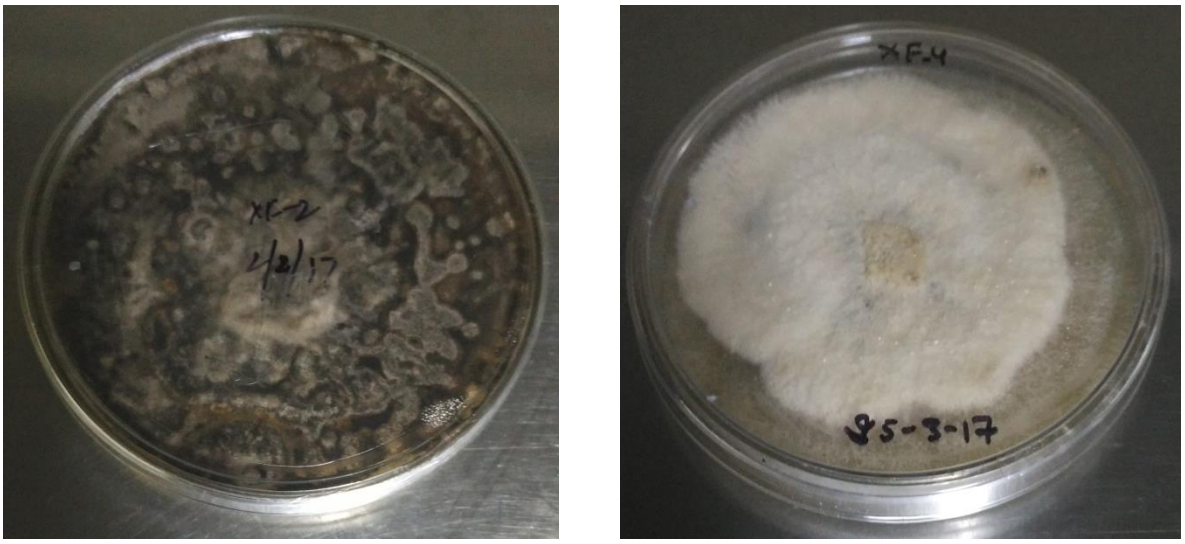



**Figure 9: A: sample source *Terminalia arjuna* from Thapar University campus, Patiala, B: bark of *Terminalia arjuna*, C: inoculation of surface sterilized bark on PDA medium, D: isolated endophytic fungi**

## 4.3 Sub-culturing of XF-2 and XF-4

Two different fungal isolates of endophytes from *Xylaria* sp. XF-2 and XF-4 were sub-cultured from master cultures provided by TIFAC Core, Thapar University, Patiala, Punjab.




**(a)** **(b)**

**Figure 10: (a) XF-2 fungal isolate (b) XF-4 fungal isolate from *Xylaria sp*.**

A total number of 17 fungal isolates were differentiated by giving the numbering to the respective plant source from which fungal isolates were obtained.

## 4.4 Fermentation and extraction

The cultures of *Tinospora cordifolia, Terminalia arjuna, Xylaria* sp., were sub-cultured on PDA plates before fermentation. Mycelial discs of the isolated fungal cultures grown on PDA plates were cut and transferred into 500 ml PDB and kept at 27±2°C at static conditions.

After 21 days fermented broth of all the fungal isolates were filtered using sterile muslin cloth and extracted with organic solvent. After ethyl acetate extraction, the organic solvent was removed by evaporation under reduced pressure at 35°C using Rotary Vacuum Evaporator.

The dry residues were obtained and weighed to constitute a fungal crude extract. Samples were re-dissolved in methanol for the screening of antioxidant activity and for subsequent chromatographic separation.

## 4.5 Preliminary screening of antioxidant activity (DPPH Assay)

DPPH scavenging activity was tested with different concentration of crude extract of fungal isolates. DPPH strategy depends on the reduction of DPPH in the presence of a

hydrogen-donating antioxidant due to the development of the non-radical form DPPH-H. Eleven methanolic fungal extracts were screened for preliminary antioxidant activity of fungal isolates from *Tinospora cordifolia*, from all tested methanolic fungal extracts five showed antioxidant activity and were selected for further screening.

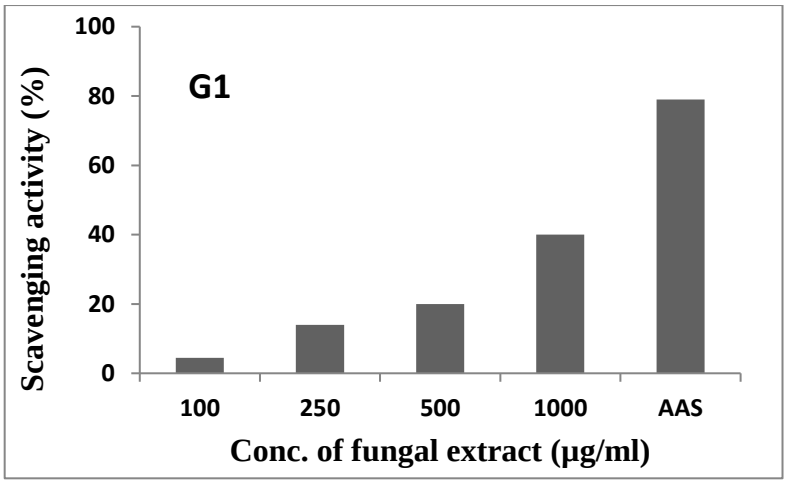
Five methanolic fungal extracts obtained from fungal isolates of *Terminalia arjuna* were screened and 2 from *Xylaria* sp .were tested for preliminary antioxidant activity.

### 4.5.1 Antioxidant screening of fungal extract from *Tinospora cordifolia*

Preliminary antioxidant screening was done by DPPH assay for eleven methanolic stocks of 5mg/ml fungal extracts obtained from the master culture of *Tinospora cordifolia* (G1-G4 and G8). Out of these fungal extracts 5 showed antioxidant activity (table 6).

**Table 6: Scavenging activity of fungal extracts from *Tinospora cordifolia***

| | Scavenging activity (%) | | | | |
|---|---|---|---|---|---|
| Fungal extract | 100µg/ml | 250 µg/ml | 500 µg/ml | 1000 µg/ml | AAS |
| G1 | 4.5 | 14 | 20 | 40 | 79 |
| G2 | 2.2 | 7 | 15 | 20 | 79 |
| G3 | 4.5 | 14 | 20 | 45 | 79 |
| G4 | 2.2 | 7 | 15 | 20 | 79 |
| G8 | 4.5 | 7 | 20 | 30 | 79 |

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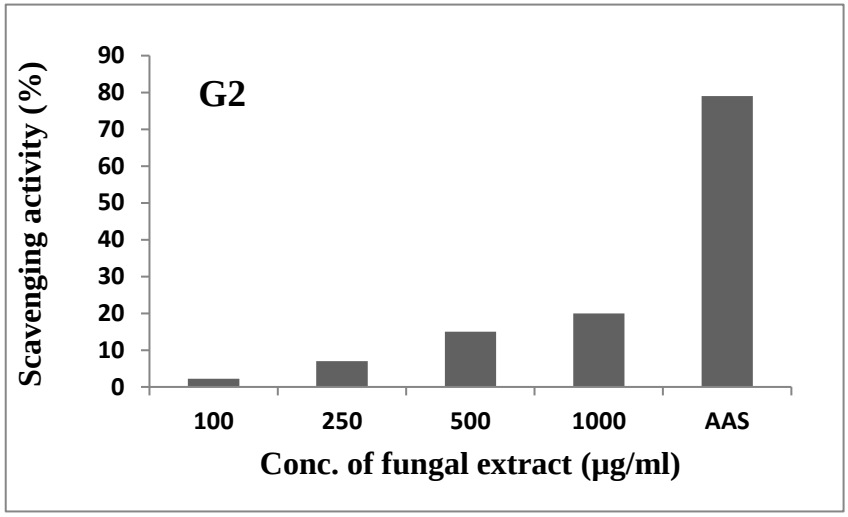
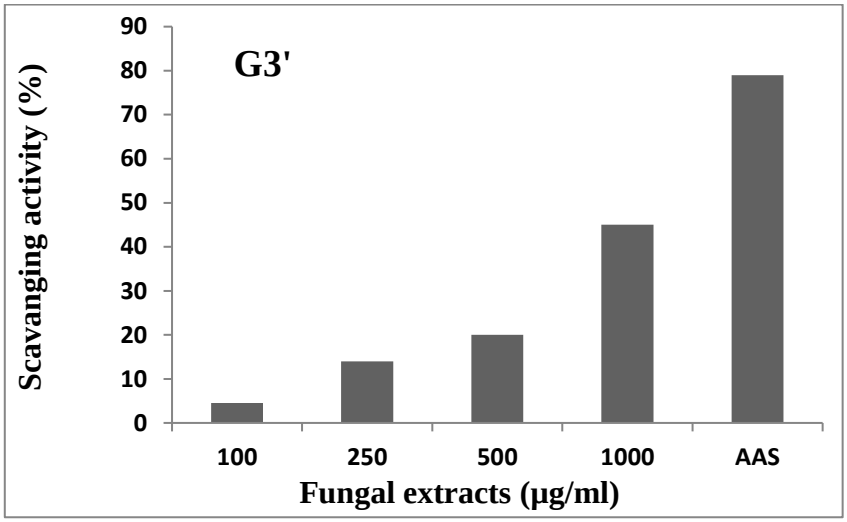
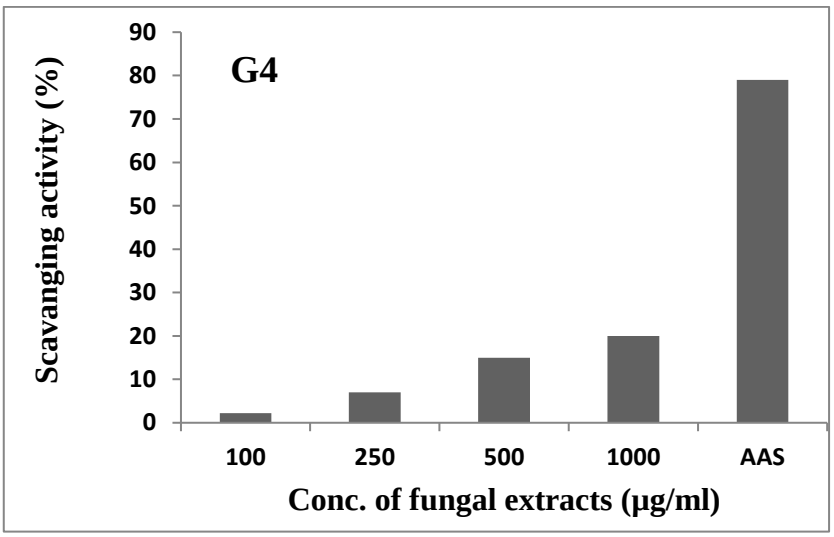

G2
Scavenging activity (%)
90
80
70
60
50
40
30
20
10
0
100
250
500
1000
AAS
Conc. of fungal extract (µg/ml)

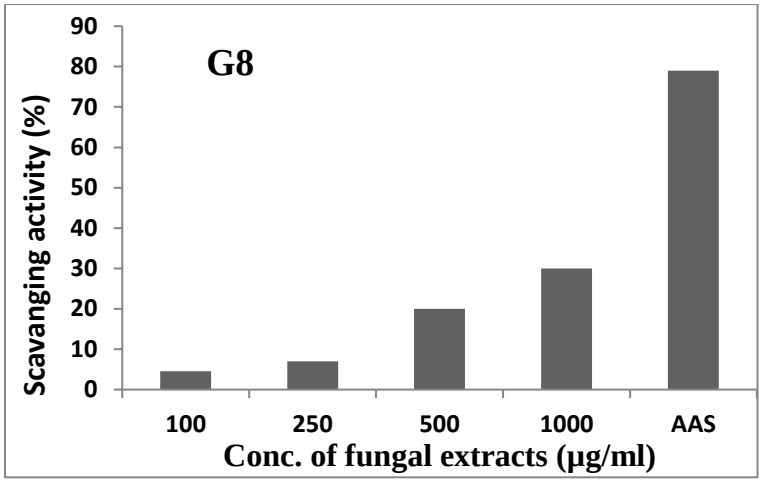
b


G3'
Scavanging activity (%)
90
80
70
60
50
40
30
20
10
0
100
250
500
1000
AAS
Fungal extracts (µg/ml)

c


G4
Scavanging activity (%)
90
80
70
60
50
40
30
20
10
0
100
250
500
1000
AAS
Conc. of fungal extracts (µg/ml)

d




e

**Figure 11: (a-e) Antioxidant activity of of fungal extracts and ascorbic acid standard at various concentration. a: G1, b: G2, c: G3', d:G4 and e: G8**

The reaction was visible as colour of DPPH changes from violet to yellow. The radical scavenging activity in percentage of crude fungal extracts at various concentration of 100, 250, 500, 1000µg/ml are shown in figures 11 (a- e).

From the figure 11, observation indicated that free radical scavenging activity in percentage increases with increasing extract concentration. Scavenging percentage in concentration of 100µg/ml was equal to or less than 5% but as the concentration of fungal extracts increases, the antioxidant activity also increases. The significantly remarkable scavenging percentage was showed by G1 and G3, with highest antioxidant activity value of 40% and 45% in 1000 µg/ml cocentration where as showed the least antioxidant activity value of 4.5% in the concentration of 100 µg/ml. The other tested extracts did not show significant antioxidant activity.

Ascorbic acid was taken as standard antioxidant, showing 79% antioxidant activity. In comparison to standard the potential of crude extract of G1 and G3 for quenching the free radical (DPPH) could be considered as a potential source of antioxidants.

### 4.5.2 Antioxidant screening of fungal extracts from *Terminalia arjuna*

A total of five methanolic fungal extract from *Terminalia arjuna* with ascorbic acid standard was screened for antioxidant activity and remarkable antioxidant activity is shown by one fungal extract represented in (table 1).

**Table 7: Scavenging activity of crude fungal extract from *Terminalia arjuna***

| | Scavenging activity (%) | | | | |
|---|---|---|---|---|---|
| Fungal extract | 100µg/ml | 250 µg/ml | 500 µg/ml | 1000 µg/ml | AAS |
| ARJ1 | 4.5 | 14 | 45 | 55 | 79 |

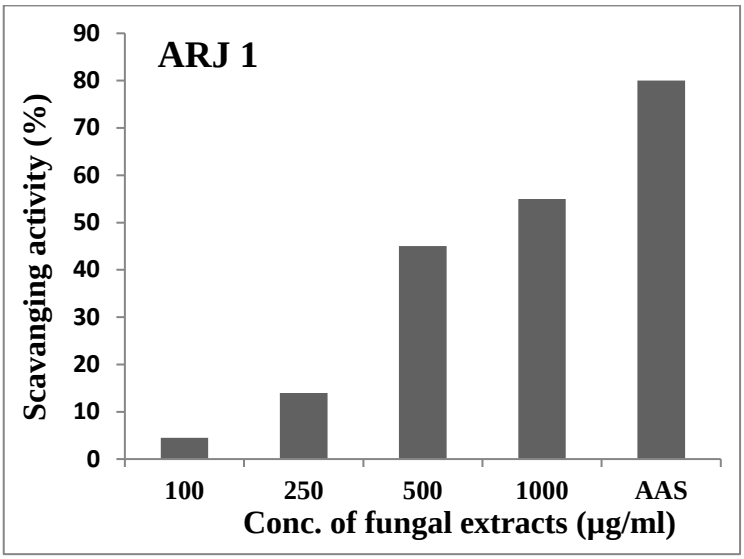



**Figure 12: Scavenging activity of fungal extracts from Terminalia arjuna and asorbic acid at various concentrations**

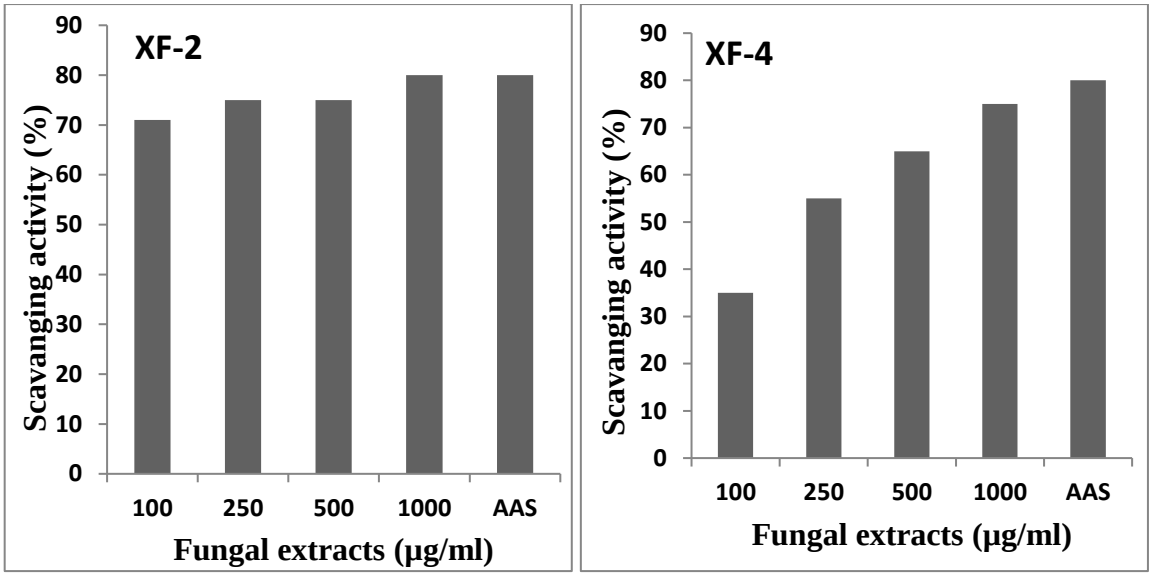
It was observed that the methanolic extract from *Terminalia arjuna* notably reduce the DPPH free radicals. It results showed that scavenging activity in percentage increases with the increase in fungal extract concentration. The highest scavenging activity with the value of 55% at 1000µg/ml concentration and lowest with the value of 4.5% at 100µg/ml was observed. As this fungal extract showed significant antioxidant activity of 55%, can be considered as an important and novel source of natural antioxidants (figure 12).

### 4.5.3 Antioxidant screening of fungal extracts from *Xylaria sp.*

XF-2 and XF-4 methanolic fungal extract from *Xylaria sp.* with ascorbic acid standard was screened for antioxidant activity and presented in figure 13.

**Table 8: Scavenging activity of XF-2 and XF-4**

| | Scavenging activity (%) | | | | |
|---|---|---|---|---|---|
| Fungal extract | 100µg/ml | 250 µg/ml | 500 µg/ml | 1000 µg/ml | AAS |
| XF-2 | 71 | 75 | 75 | 80 | 80 |
| XF-4 | 35 | 55 | 65 | 75 | 80 |




**a** **b**

**Figure 13: Scavenging activity of fungal extracts from *Xylaria sp.* and asorbic acid at various concentrations. a: XF-2, b: XF-4**

XF-2 and XF-4 methanolic fungal extract from *Xylaria sp.* with ascorbic acid standard was screened for antioxidant activity and presented in figure 13. It was observed that the methanolic extract from *Xylaria sp.* notably reduced DPPH free radicals even at a low cocentration of fungal extrats. XF-2 and XF-4 show 70% and 35% scavenging activity respectively at the concentration of 100µg/ml . It was observed that there is not much significant increase in scavenging percentage of XF-2 with the increase in fungal extract concentration as it is ranging from 71-80% in 100-1000µg concentration. The highest scavenging activity was obtained with the value of 80% and 75% in 1000µg/ml concentration of XF-2 and XF-4 respectively. From the results obtained for XF-4 it was observed that the scavenging activiy increases as the fungal extracts concentration increase as it showed scavenging activity in percentage of 35% at 100µg/ml, 55% at 250µg/ml, 65% at 500µg/ml and 75% at the highest concentration of 1000µg/ml. The scavenging performance of methanolic extracts from Xylaria sp. XF-2 was almost comparable as ascorbic acid standard as it shows 70% antioxidant activity at 100µg/ml concentration Ascorbic acid was observed to have the highest activity in DPPH assay, which is in agreement with previous study (Duan et al., 2006).

## 4.6 Phytochemical analysis

Phytochemical analysis was carrid out of fungal crude extrats to determine the presence of chemical components as a prospective source for medicinal and industrial use.

**Table 9: Phytochemical screening of fungal extracts.**

| Biochemical compounds present in fungal extract | G3 | G1 | ARJ | XF-2 | XF-4 |
|---|---|---|---|---|---|
| Tannins and phenolics | - | + | + | - | - |
| Amino acids | + | + | + | ++ | + |
| Carbohydrates | ++ | ++ | + | +++ | +++ |
| Alkaloids | + | + | ++ | ++ | ++ |
| Fats and fixed oils | - | - | - | - | - |
| Steroids and terpwnoids | + | - | + | + | + |
| Flavonoids | + | ++ | +++ | +++ | +++ |
| Glycosides | - | - | - | - | - |

(- ): Negative test, (+): Weak positive test, (++): Positive test, (+++): Test firmly positive.

The phytochemical analysis of the crude fungal extracts have shown the presence of phytochemical profile in the table 9 . The presence of phenols in G1 and ARJ1 and presence of flavanoids in high intensity in all tested fungal extracts could be the reason for the significant antioxidant activity.

## 4.7 Chromatographic analysis

Thin layer chromatography (TLC) was performed on a silica gel plate (Silica Gel GF254, Merck), two microliters (2µl) of each crude extract were spotted on TLC plate. In this manner mixture of chloroform : ethyl acetate for fungal extract from *Tinospora cordifolia* and chloroform : methanol for fungal extract from *Terminalia arjuna* as mobile phase for separation of extracts, were employed. After development of the chromatogram, the plates were dried at room temperature, and the detection was basically done utilizing UV radiation at 365 nm. The Rf values were measured.

The variation in Rf values of crude extracts provided an important clue in understanding their polarity and helps in selection of appropriate solvent system for the separation of compounds.

The best results for *Tinospora cordifolia* derived crude fungal extracts were obtained at 7:3 of chloroform:ethyl acetate with the Rf of 0.76 and 0.73 and for *Terminalia arjuna* derived crude fungal extracts showed maximum separation at 6:4 of chloroform:methanol with the Rf value of 0.79 (table 10).

**Table 10: Rf values of crude fungal extract.**

| Crude fungal extract | Mobile phase | Rf value |
|---|---|---|
| *Tinospora cordiflia* (G1,G3) | Chloroform:Ethyl acetate 7:3 | G1 - 0.76<br>G3- 0.73 |
| *Terminalia arjuna* (ARJ) | Chloroform:Methanol 6:4 | ARJ - 0.7 |

## 4.8 Preparative thin layer chromatography:

Preparative TLC was done for 2 fungal extracts, one from *Tinospora cordifolia* (G1) and one from *Terminalia arjuna* (ARJ). Preparative TLC was also performd on silica plates and by depositing the crude extract and developing the plate in TLC chamber with respective solvent system.

Six fractions were obsereved for G1 fungal extract in TLC viewing cabinet under UV light and five were observed for ARJ1. Obtained fractions were marked by lead pencil and separated. Elution of various separation from silica was done for obtaining the separated fractions with 10% methanol in DCM.

**Table 11: Fractions eluted from preparative TLC of crude fungal extract of G1**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **G1** | Eluted fractions | A | B | C | D | E | F |
| | Rf values | 0.13 | 0.17 | 0.33 | 0.45 | 0.55 | 0.73 |

fractions were analyzed for the antioxidant screening by DPPH method.

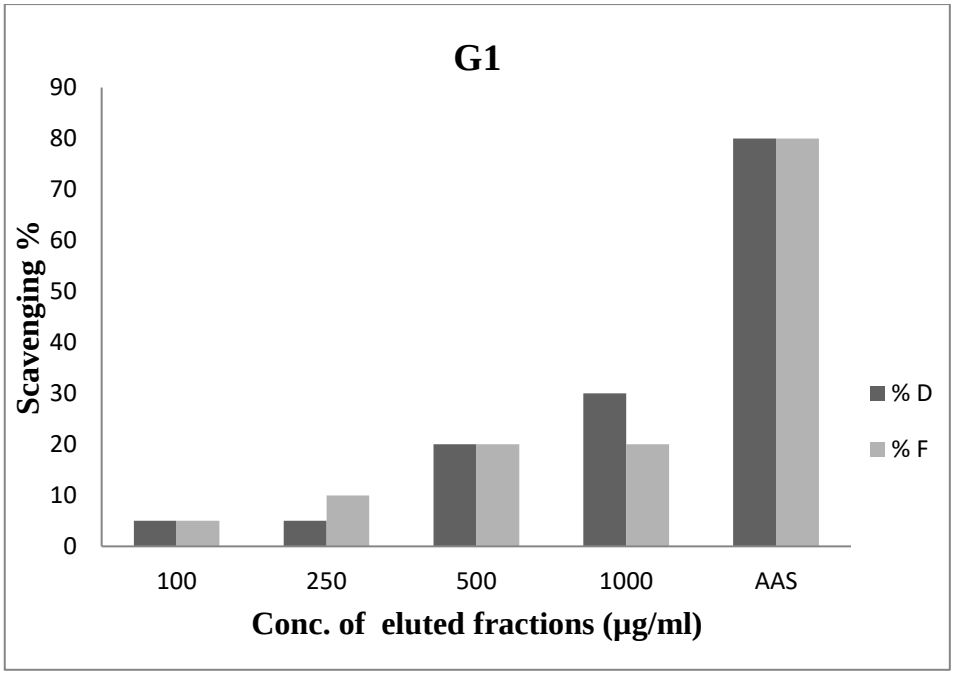



**Figure 14: Scavenging activity of eluted fractions from G1 by preparative TLC**

A total of 6 fractions were observed when developed in chloroform : ethyl acetate (7:3) on silica gel TLC plate. These 6 fractions were designated as A,B,C,D,E and F. Rf values were determined for each fraction and shown in table 11. These fractions of G1 crude fungal extract from *Tinospora cordifolia* were eluted out by running in a column with 10% methanol in DCM. All fractions with the different were screened for antioxidant activity. Scavenging activity was shown by two fractions D and F having the Rf values 0.45 and 0.73 respectively (figure 14). Significant antioxidant activity was exhibited by the D fraction with 30% scavenging activity.

**Table 12: fractions eluted from preparative TLC of crude fungal extracts of ARJ1**

| | | | | | | |
|---|---|---|---|---|---|---|
| **ARJ1** | Eluted fractions | a | b | c | d | e |
| | Rf values | 0.08 | 0.2 | 0.30 | 0.48 | 0.71 |

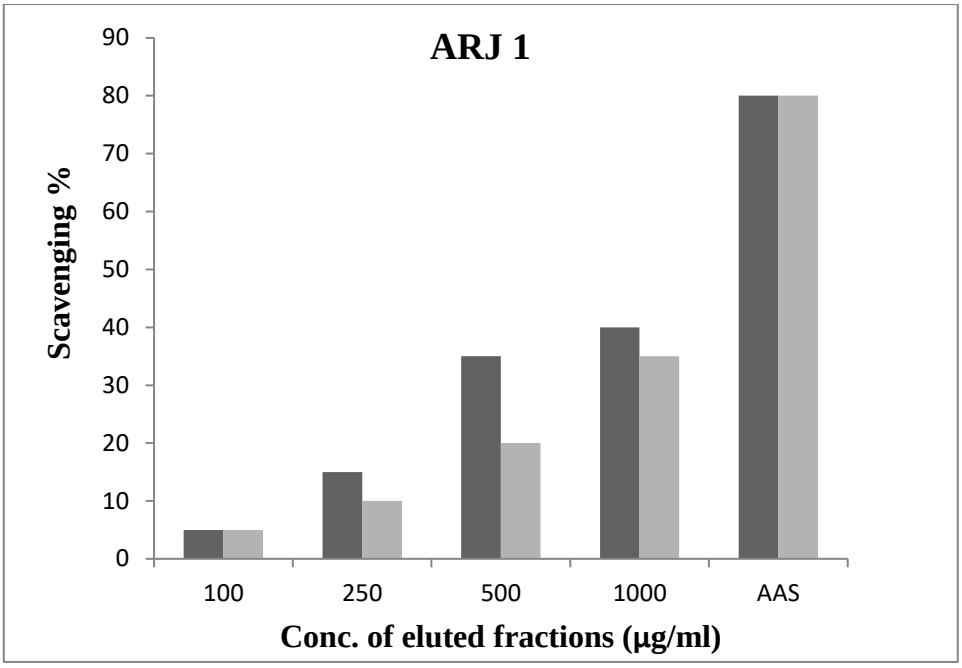



**Figure 15: Scavenging activity of eluted fractions from ARJ1 by preparativ TLC**

As mentioned above preparative tlc was carried out to obtain different fractions from crude fungal extract for further screening of antioxidant activity. Five fractions coded as a, b, c, d, and e were observed when developed in appropriate solvent system in appropriate ratio. The Rf values were measured and eluted out and checked for antioxidant activity (table 12). Only the 2 fractions b and c, having Rf values of 0.2 and 0.3 exhibited the antioxidant activity (figure 15).

## 4.9 Identification

### 4.9.1 Molecular identification

Endophytic fungi were isolated from *Tinospora cordifolia* and *Terminalia arjuna*. The isolates were identified on the basis of their colony morphology. Two to three distinct colonies were observed. The colony morphology of fungal isolates from *Terminalia arjuna* on PDA showed green and white surface initially but turn into dark green later on. Colonies formed were powdery with rapid growth.

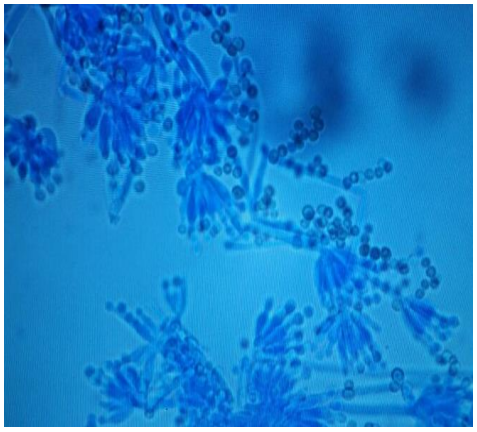


**Figure 16: Microsopic view of endophytic fungi isolated from *Terminalia arjuna***

Based on macro and microscopic characteristics the endophytic fungal isolates from *Terminalia arjuna* was identified as "Penicillium species" by showing the presence of conidia and hyphae as shown in the figure 16.

### 4.9.2 Molecular identification

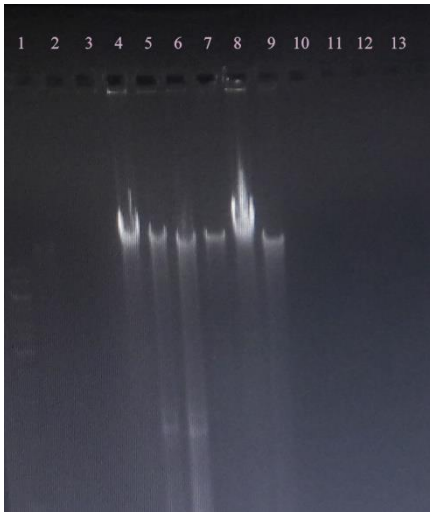



**Figure 17: Electrophoresis of the isolated DNA from endophytic fungi of *Terminalia arjuna***

After the microscopic analysis the DNA was isolated from the endophytic fungi of *Terminalia arjuna* and its agarose gel electrophoresis was done to know that the DNA has been isolated or not. After electrophoresis bands of DNA was visible in the UV transilluminator. Lane 4 has the DNA the endophytic fungi isolated from *Terminalia arjuna*.

# 5 Conclusions

Plant endophytic fungi are considered as a novel resource for having the ability to produce similar compounds originated from their host plants. This feature of endophytic fungi have increased interest in both basic research and applied fields. Till now hundreds of endophytic fungi from medicinal plants have been investigated and many of them have proved to be rich with bioactive or secondary metabolites. Discovery of the new compounds from natural resources like endophytic fungi will play an important role in the development of pharmaceuticals.

The data presented in this study have demonstrated that the extracts from different endophytic fungi isolated from the stem of *Tinospora cordifolia* and the bark of *Terminalia arjuna* have significant antioxidant activity. The methanolic fungal extract showed excellent antioxidant activity by DPPH assay. The antioxidant activity of methanolic fungal extracts increased with the increasing concentration of the fungal extracts. G1 and G3 showed good antioxidant activity of 40 and 45 percent at the concentration of 1000 µg/ml. ARJ1 showed 55 percent antioxidant activity at the highest concentration of 1000µg/ml. XF-2 showed significant antioxidant activity even at the lower concentration of fungal extract and did not show any significant change with the increased concentration. The fractionation was done for G1 and ARJ1 fungal extract with preparative TLC along with screening of fractions for antioxidant activity. Two fractions D and F from *Tinospora cordifolia* and two fractions b and c from *Terminalia arjuna* showed positive results for antioxidant activity.

Endophytes have proven to be a rich source of novel natural compounds with a wide range of bioactivities and usefulness in safety and human health concerns. It can be concluded that endophytic fungi could be a reliable source for antioxidants for pharmaceuticals.

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