Introduction

INTRODUCTION

The word lichen is of Greek origin, which is pronounced as “Lie kens” and was first used by Theophrastus (370-285) who is authorized as “Father of Botany” to indicate the superficial growth on the bark of olive trees (Pulak das, 2008). Erik Acharius (1803, 1810, 1814) a Swedish botanist designed the first National System for lichens and referred to as “Father of Lichenology”. He coined several terms for the structures distinct to lichens and described many new genera and numerous new species on the basis of external morphology in his monumental works Methodica Lichenum, Lichenographa Universalis and Synopsis Methodica Lichenum (Pooja gupta, 2004).

Lichens are special in having at least two microorganisms in a single plant, a photobiont (alga) and a mycobiont (fungus), forming a thallus (Boonpragob, 2013). In addition, definite groups of bacteria have recently been found to be present in lichen thalli. Sometimes lichens grow on several insects and animals (Upreti, 1998). Lichens are the most exceptional composite symbiotic organisms that are built up from members of as many as three kingdoms (Pulak das, 2008).

Lichens are one of the important constituents of the Indian flora. In India, early lichen research started just with collection and description of lichen taxa (Awasthi, 1988; Singh, 1997; Hariharan, 1991). In general, in India the lichen studies have always been at a slow step in comparisonto higher plants. So far about 20,000 species of lichens have been recorded; in it, India shares 10% with only about 2% of global land surface (Awasthi, 1988; Awasthi, 1991; Groom bridge, 1992; Upreti, 1998). Out of these evaluated 20,000 lichen species in the world, 95% belong to the Ascomycetes group of fungi while basidiomycetes and deuteromyetes groups are represented by only 3% and 2% of species respectively (Pooja gupta, 2004).

In lichen thallus (body), the mycobiont (fungus) predominates with 90% of the thallus volume and provide water, mineral substances, vitamins, structure, colour and physical protection for the phytobiont (Pulak das, 2008). In the same way, phytobiont supplies photosynthetically produced carbohydrates to the mycobiont. In turn the phytobiont gets protection from desiccation by sheltering its delicate cells within the thallus body of the latter partner. Lichens lack cuticle and epidermis; therefore, it employs biochemical defense mechanism to protect delicate thalloid structures against high solar fluxes (Bachereau and Asha, 1997).

Based on the substratum, lichens can be classified as:

Terricolous	-	Soil
Humicolous	-	Humus
Saxicolous	-	Stones, rocks, brick
Calcicolous	-	Lime plaster
Folicolous	-	Leaves
Corticolous	-	Tree trunk
Lignicolous	-	Decaying wood and Man-made substratum

Based on their morphology and size, lichens are categorized into three major types (Pulak das, 2008)

i. Crustose (crust like)
ii. Foliose (Leaf like)
iii. Fruticose (shrubby)

Crustose lichens:

Crustose lichen thallus is closely attached to the substratum without leaving any free edge. Usually, cructose lichen thalli lacks lower cortex and rhizines (root like structure).

Foliose lichens:

The lichen thallus appears similar to a leaf. It is loosely attached to the substratum at least at the edge.

Fruticose lichens:

Fruticose lichen thallus is attached to the substratum at a single part and the remaining major portion is either growing erect or hanging and usually appear as small shrub on bark (Pooja gupta, 2004).

There are about 2,040 species of lichens available in India (Awasthi, 1991). Indian lichen biota is dominated by crustose lichens with 1518 taxa, followed by foliose (705 taxa) and fruticose (205 taxa) lichens. In India, Tamil Nadu records the maximum numbers of lichens with 785 taxa followed by Uttarakhand (581 sp.), West Bengal (531 sp.), Sikkim (503 sp.), Jharkhand by (10 sp.), Lakshadweep (9 sp.), Bihar and Chattisgarh (6 sp. each), Punjab (3 sp.) and Gujarat, Mizoram and Puducherry (2 sp. each) (Nayaka and Asthana, 2014).

Generally, lichens are considered as a symbiotic association between algae and fungi, also houses fungi as endophytes and these are generally termed as endolichenic fungi. The term “Endophyte” (from Greek “endo” – within and “phytos” – plant) was named by de Bary (1866) including each and every individual from virulent foliar pathogens to mycorrhizal root symbionts.

The fungal kingdom is one of the most diverse groups of eukaryotes on earth (Blackwell, 2011; Hibbett and Taylor, 2013). The whole fungal wealth is projected around million (Blackwell, 2011). In this, only about 1,00,000 species have been documented (Blackwell, 2011). Those missing fungi were found from following three places which were recognized by Hawksworth and Rossman (1997).

The places are:

(1) Fungi in tropical forests

(2) Fungi in unexplored habitats and

(3) Lost or hidden.

Colonization of fungi as endophytes has a long evolutionary history. From a 400-million-year-old land plant, plant fossil remains containing fungal endophytes have been reported (Krings *et al*., 2007). These fungi need not to be confused with mycorrizal fungi. Because they differ from the mycorrhizal fungi in both ecology and infection allocation. While mycorrhizas are restricted to the rhizosphere with particular importance for plant growth in nutrient-stressed situations, endophytes can be found in both above and below- ground plant-tissues and seem to be present even when nutrients are abundant (Smith and Read, 1997). The high proportion of endophytes belong to Dothideomycetes, Leotiomycetes, Pezizomycetes, and Sordariomycetes, which was highlighted by most studies with the help of culture-based methods (Petrini, 1985; Petrini *et al*., 1990; Stone *et al*., 2000; Stone *et al*., 2004; Arnold and Lutzoni, 2007; Shipunov *et al*., 2008; Arnold *et al*., 2009).

The term ‘endolichenic fungi’ was named by Miadlikowska *et al*. (2004) for the fungi that live inside lichen thalli without producing any apparent disease symptoms. They represent lineages of Ascomycota which are distinct from lichen mycobionts (the primary fungal component of the lichen thallus), lichenicolous fungi/secondary fungi/accessory fungi and incidental fungi on thallus surfaces (Lutzoni *et al*., 2001; Lawrey and Diederich, 2003; Arnold *et al*., 2009).

Hawsksworth (1982) divided these secondary fungi into three categories:

1. Parasites
2. Saprophytes
3. Parasymbionts

However, he didn't mention anything about the placement of endolichenic fungi. The endophytes fall in the second category i.e., fungi in unexplored habitats. They are thought to be one of the most essential evolutionary steps from an aquatic to a terrestrial lifestyle (Selosse and Le Tacon, 1998; Heckman *et al*., 2001). In the history of endophytic research, the year 1977 is important landmark year, because of the discovery of endophytic fungus *Epichloe coenophiala* (=*Neotyphodium coenophialum*) from *Festuca arundinacea* which is the cause of "fescue toxicosis" (Bacon *et al*., 1977).

The fungal endophytes are gaining more attention rather than bacterial or other microbial endophytes because they are more applicable in various fields. Endophytes have been isolated from plants found in alpine, boreal, temperate and tropical forests, including extreme arctic (Petrini, 1987; Fisher *et al*., 1995) and xeric environments (Muhsin and Booth, 1987) and from mesic temperate and tropical forests.

Endophytic fungi have been obtained from several plant lineages e.g. algae (Cubit, 1975; Hawksworth, 1988; Krohn *et al*., 2005; Wang *et al*., 2006; Yang *et al*., 2006; Thirunavukkarasu *et al*., 2011), bryophytes (Dobbeler, 1979; Ligrone, 1988; Ligrone *et al.*, 1993; Chambers *et al*., 1999; Davis *et al.*, 2003; Kauserud *et al.*, 2008; U'Ren *et al*., 2010; Zhang *et al*., 2013), pteridophytes (Fisher *et al.*, 1992; Schmid and Oberwinkler, 1993; Swatzell *et al*., 1996; Sati and Belwal 2005; Sati *et al*., 2009; Kumaresan *et al*., 2013), gymnosperms (Carroll and Carroll, 1978; Carroll and Petrini,1983; Sahashi *et al.*, 1999; Tan and Zou, 2001; Strobel and Daisy, 2003; Sieber, 2007; Rodriguez *et al.*, 2009; Thongsandee *et al.*, 2012), angiosperms (Clay, 1991; Elmi and West, 1995; Saikkonen *et al*., 1998; Rajagopal and Suryanarayanan, 2000; Clay and Schardl, 2002; Nalini *et al.,* 2005; Raviraja, 2005; Tejesvi *et al.,* 2005; Lin *et al*., 2007; Verma *et al.,* 2007; Kharwar *et al.,* 2008, 2010, 2011; Mishra *et al*., 2014; Verma *et al.,* 2014) but their existence within lichens was reported recently (Petrini *et al*., 1990; Girlanda *et al*., 1997; Suryanarayanan *et al*., 2005, 2017; Li *et al.,* 2007; Tripathi *et al*., 2014, 2014a; Tripathi and Joshi, 2015; Wang *et al*., 2016; Maduranga *et al*., 2018).

Endophytes are classified into two broad groups based on phylogeny and life history Traits.

1. Clavicipitaceous

2. Non-clavicipitaceous

The first group includes endophytes infecting some grasses confined to cool regions, and the second group non-clavicipitaceous includes endophytes from asymptomatic tissues of non-vascular plants, ferns and allies, conifers and angiosperms and are limited to the Ascomycota or Basidiomycota group (Jalgaonwala *et al*., 2011; Bhardwaj and Agrawal, 2014).

Endolichenic fungi vary from the lichenicolous fungi in both symptoms and phylogeny. Outward symptoms of infection are showed by Lichenicolous fungi and not by Endolichenic fungi. When phylogeny is considered, endolichenic fungi were found to be most common among primarily non-lichenized lineages of euascomycetes (Sordariomycetes, Dothideomycetes, Leotiomycetes and Pezizomycetes) but absent among the lichen-dominated clades (Lecanoromycetes, Arthoniomycetes, Lichinomycetes), while lichenicolous fungi were thought to be closely related to lichens and found mainly in lichen-forming clades (Arnold *et al*., 2009; Diederich *et al*., 2018). Other than this, the commensalistic lichenicolous fungi would be nutritionally similar to primary mycobionts (Hawksworth, 1988), while endolichenic fungi are associated mainly with the photobiont (Arnold *et al*., 2009).

Lichen has economic benefits to human being in various applications such as biomedical, environmental monitoring and natural dye extraction purposes. Search for drugs derived from different natural resources is gaining importance. Lichen can produce a broad array of both intracellular and extracellular compounds. These lichen compounds are called secondary metabolites which are extracellular in nature often called lichen acids (Ponmurugan *et al.,* 2016). Lichens can be used for monitoring the pollution level in the atmosphere and lichen samples can be used to estimate the extent and pollutant emissions around an industry or particular locality (Negi, 2003). It has folkloric repute of cosmetics for skin bleach and has been prescribed for the management of diarrhoea, dyspepsia, spermatorrhoea, amenorrhoea, dysentery and wound healing (Lindley, 1981; Kirtikar and Basu, 1996).

A number of lichen species are reported to be used in traditional or folk medicines (Llano, 1956; Chandra and Singh, 1971; Saklani and Upreti, 1992;

Gonzalez-Tejero *et al*., 1995; Upreti and Negi, 1996), sold as condiments in the Indian markets, cooked as vegetable curry by the tribal people of Sikkim Himalayas particularly during the scarcity of food and are even utilized as common livestock fodder in some places of South India (Saklani and Upreti, 1992).

The use of lichens in medicine is due to their unique, relatively low molecular weight and varied secondary metabolites that are unique compared to those of higher plants (Turk, *et al.*, 2003; Upadhyay *et al.*, 2017). These different metabolites along with other chemical compounds can be utilized for curing aches and diseases. While numerous activities of lichen metabolites are now recognized, their therapeutic potential has not been fully explored and remains pharmaceutically unexploited (Pant and Rao, 2018). Lichen metabolites have wide variety of biological actions including antibiotic, antimycotic, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative, and cytotoxic effects (Grube *et al*., 2009).

An important elevated proportion of fungal endophytes (80%) manufacture secondary metabolites possessing biologically active compounds (Schulz *et al*., 2002) synthesized via various metabolic pathways (Tan and Zou, 2001). These secondary metabolites be owned to different structural groups i.e., aliphatics, alkaloids, cytochalasines, depsipeptides, furandiones, isocoumarines, phenols, quinines, steroids, terpenoids, sulfur-containing chromenones and xanthones that have been commercially utilized for pharmaceutical, medical and agricultural purposes (Tan and Zou, 2001; Castillo *et al*., 2002, 2003; Strobel and Daisy, 2003; Ezra *et al*., 2004; Li *et al.*, 2005; Park *et al.*, 2005; Gunatilaka, 2006; Wang *et al.*, 2007; You *et al.*, 2009). These metabolites also possess various biological activities like anticancer, antiviral, antibacterial, antifungal and anti-Alzheimer's activities (Kaul *et al*., 2012; Biosca *et al*., 2016; Muggia *et al.,* 2016; Suryanarayanan and Thirunavukkarasu, 2017).

The endolichenic fungal species investigated till date for the isolation of bioactive secondary metabolites belonged to several geographical locations. The estimated global lichen diversity is about 20,000 species (Feuerer and Hawksworth 2007). From this, only a small number of lichen species have been screened for harvesting the endolichenic fungi with the potential to offer bioactive metabolites. Therefore, one can assume the magnitude of prospective lichen diversity which is waiting to be unveiled.

Lichen forming fungi produce antibiotic secondary metabolites that protect many animals from pathogenic microorganisms like *Staphylococcus aureus, Bacillus subtilis, Mycobacterium tuberculosis, Mycobacterium smeymatis, Streptococcus pyogenes, Pneumococcus* sp.*, Salmonella typhimurium,* etc. (Lawrey, 1989).

Endophytes have been implicated in decreased herbivory, increased tolerance against drought, salinity, heat, metals etc., and improvement of plant growth (Frohlich, and Petrini, 2000; Schardl *et al.*, 2004; Sieber, 2007; Redman *et al*., 2011). Therefore, plants that have been removed from their natural environment and cultivated are thought to become depleted in their specific or coevolved endophytes (Taylor *et al.,* 1999).

In the field of endophytic research, discovery of anticancer drug is an important mile stone. The drug "taxol" was isolated, from *Taxomyces andreanae*, an endophytic fungus of *Taxus brevifolia* (Stierle *et al*., 1993). This discovery led to the search for endophytes worldwide from each and every group of organisms for a better understanding of their ecologicalfunction and capability to create prospective bioactive natural products for the betterment of the human.

Lichens have been also found to contain a variety of secondary substances with strong antioxidant activity. These lichen substances which have high ability to scavenge toxic free radicals due their phenolic groups. Antioxidants must be non-toxic, inexpensive, effective at low concentrations, capable of surviving processing, solid in the finished products, and devoid of unpleasant flavour, colour and odour effects (Shahidi *et al.,* 1992). Antioxidants are molecules that can delay or avert an oxidative reaction (Velioglu *et al*., 1998) catalysed by free radicals. This antioxidant effect is mainly due to the presence of phenolic components which are flavonoids (Pietta *et al*., 1998), phenolic acids and phenolic diterpenes (Shahidi *et al.,* 1992).

Natural and synthetic antioxidants are extensively used in the food and pharmaceutical industry. In addition, antioxidants neutralize free radicals ensure normal biological activities of living organisms (Oliveira *et al.,* 2008). The role of free radicals in many disease conditions has been well confirmed. Based on the bioactive potential of endophytes, lot of work have been done such as anticancer, antiviral, antimicrobial and antidiabetic effects but very little is known about their antioxidant capacity (Strobel *et al*., 2002).

Antioxidant substances can block the harmful action of the free radicals by scavenging the free radicals. Natural antioxidants are broadly distributed in plants, animal tissues and microorganisms. These compounds save the organisms from damage by oxidative stress caused by reactive oxygen species (ROS) and reactive nitrogen species (RNS), that damage proteins, lipids, and DNA inside the cells (Perron and Brumaghim, 2009).

From filamentous fungi, antioxidant compounds were isolated which are carotenoids, flavonoids, phenolic acids, and their derivatives (Akilandeswari and Pradeep, 2016; Wang *et al.*, 2007). The endolichenic fungi *Parmotrema hababianum*, *Parmotrema* austrosinense and *Parmotrema reticulatum* possess potential antioxidant and antibacterial activities that makes them useful in various disease treatments in human beings (Poornima *et al.*, 2016).

In European folk medicine, it is used primarily in cancer treatment (Muller, 2001). For cancer research and drug discovery, cancer cell lines are broadly used which are valuable *in vitro* model systems (Masters, 2000). The importance of human continuous cell lines in the enlargement of new drugs has been observed by the studies of the United States (US) National Cancer Institute (NCI) (Takimoto, 2003; Shoemaker, 2006). In 19 86, the NCI established the human tumor cell line anticancer screening (NCI60) project to propose a novel research approach for supplanting the use of transplantable animal tumors in anticancer drug screening (Shoemaker, 2006).

Human cancer cell lines continue to play an important role in modern cancer research. Actually, they are widely used as preclinical model systems for gaining mechanistic and therapeutic insight. Particularly, with the advent of -omics technologies (Hasin *et al*., 2017), recent studies have provided extensive databases dedicated to the characterization of most existing cell lines (Dan *et al.*, 2002). Lichens are promising sources for anticancer and antibiotic drugs (Kulshrestha *et al*., 2009). It was also showed that a few Umbilicaria lichen species have an inhibitory effect against several 3 bacteria as well as human melanoma FemX and human colon carcinoma LS174 cell lines (Kosanic *et al*., 2013a).

Inflammation is a complex biological reaction of the immune system that can be induced in the tissue by various factors in response to harmful stimulants such as pathogens, damaged cells or irritation and is characterized by swelling, pain, redness, fever and impaired function the tissue level (Ferrero miliani *et al.,* 2007; Takeuchi and Akira, 2010).

Molecular docking is a method applied to study molecular behaviour on target proteins binding. It is a tool which is used highly in drug discovery. The topmost software used for best scores in docking are AutoDock, Vina, MOE-Dock, FlexX and GOLD respectively. GOLD and LeDock are used for predicting the exact binding poses (Singh *et al.*, 2017).

By the needs of structural molecular biology and structure-based drug discovery, the field of molecular docking has emerged during the last three decades. It has been greatly helped by the dramatic growth in availability and power of computers, and the growing ease of access to small molecule and protein databases (Hendlich, 1998; Berman *et al.*, 2000; Hu, *et al.*, 2005; Irwin and Shoichet, 2005). To understand and predict molecular recognition, both structurally, finding likely binding modes, and energetically, predicting binding affinity, these are the main goal of automated molecular docking software.

Molecular docking has a wide variety of uses and applications. The applications are assisting x-ray crystallography in the fitting of substrates and inhibitors to electron density, chemical mechanism studies, combinatorial library design, drug discovery, finding potential leads by virtual screening, including structure–activity studies, lead optimization, providing binding hypotheses to facilitate predictions for mutagenesis studies (Pozzan, 2006).

Nanotechnology is an emerging field of science that involves synthesis and development of different nanomaterials. At present, different types of metal nanomaterials are being produced using zinc, gold, copper, titanium, magnesium, silver and alginate. Thus, nanomaterials are used in different fields such as bactericidal, electronic, optical devices, sensor technology, biological labelling, catalytic and treatment of some cancers. The nanotechnology is an immense field which deals with the structure, dimension and the size of the particles ranging from 1-100 nm.

One of the main dominant criteria of nanotechnology is that of the development of nontoxic, clean and eco-friendly green chemistry procedures (Sharma *et al.*, 2009). Green nanotechnology is the approach for silver nanoparticles (AgNP’s) synthesis from microorganisms and plant extract although there are various methods including chemical, physical, physicochemical reduction, electrochemical techniques used for synthesizing AgNP’s (Verma and Maheswari, 2019). Numerous micro-organisms such as fungus, bacteria, yeasts and plants either intra or extracellular that are of higher production yield and with low cost have been found to be capable of synthesizing nanoparticles.

Many researches were undergone based on endophytic fungi but less investigation was made on endolichenic fungi and their bioactive compounds. Therefore, the present study was carried out with the following objectives:

- To analyze the secondary metabolites of the Ethyl acetate, Chloroform, Methanol, Water, Petroleum ether and Ethanol extracts of the identified lichen *Hypotrachyna infirma* (Kurok.) Hale using qualitative and quantitative phytochemical screening.
- To identify the unknown compounds in the methanolic extracts of *Hypotrachyna infirma* using GC-MS analysis.
- To evaluate the antimicrobial, antioxidant, anti-inflammatory and *in-vitro* cytotoxic activities of the methanolic extract of the lichen.
- To isolate the endolichenic fungi from the lichen *Hypotrachyna infirma* (Kurok.) Hale.
- To perform the qualitative and quantitative phytochemical studies in the ethyl acetate and chloroform extracts of the isolated endolichenic fungi.
- To identify the unknown compounds in the ethyl acetate extracts of selected five endolichenic fungi using GC-MS analysis.
- To evaluate the antimicrobial and antioxidant activities of ethyl acetate extracts of five selected endolichenic fungi.
- To evaluate the anti-inflammatory and *in-vitro* cytotoxic activities of ethyl acetate extracts of selected endolichenic fungus *Nigrospora oryzae*.
- To retrieve the molecular docking of lichen and selected endolichenic fungal structure to the target.
- To determine the bio nanoparticles in the methanol extract of lichen and ethyl acetate extract of selected one endolichenic fungus using UV-VIS spectroscopy, FTIR analysis and SEM analysis.