Discussion

DISCUSSION

Lichen

Most of the lichen have huge number of secondary metabolites due to biotic and abiotic interactions of lichens with their environment and they may help to protect thallus of the lichens against some external factors, herbivores and pathogens (Masthan, *et al*., 2014). Lichen substances contain variety of compounds such as depsides, depsidones, depsones, terpenoids, steroids, carotenoids and diphenyl ethers, amino acid derivatives, sugar alcohols, aliphatic acids, macrocyclic lactones, mono-cyclic aromatic compounds, quinines and xanthones which possess many biological applications (Clix *et al*., 1984).

The present study revealed that concentration and percentage yield of water extraction of *Hypotrachyna infirma* was from 1.07mg/ml and 2.14% to methanol extraction of *H.infirma* 1.48 mg/ml and 2.96%. Methanol had higher extraction yield followed by ethyl acetate, petroleum ether, ethanol, chloroform and water.

Similar results were found in concentration and percentage yield of ethanol extraction of *Heterodermia leucomelos* (26mg/ml and 2.1%) and methanol extraction of *Parmotrema tinctorum* (250mg/ml and 20.8%). Murugan and Parimelazhagan (2014) studied different extraction method and established out that in successive soxhlet extraction, methanol had higher extraction yield followed by ethanol, ethyl acetate and n-hexane.

The extraction yield of *Limnophila aromatica* increased with increasing water content in acetone, ethanol and methanol system. Here, methanol with 38% of extraction yield was the applicable solvent to extract more yields and also had higher phytochemical content. These were found by Diem Do *et al*. (2014).

In another study, the extraction yield of *Herpothallon* sp., *Parmotrema reticulatum, Parmotrema tinctorum, Parmotrema crinitum, Leptogium sp., Ramalina celastri, Cladonia subradiata* and *Heterodermia leucomella* was found to be high in methanol than the other solvents such as hexane, methanol, ethyl acetate and ethanol. Among these, methanol extract found to produce higher extractive value whereas hexane was found to be with less extractive value (Hengameh Parizadeh and Rajkumar, 2017).

Phytochemical study

Qualitative analysis

Parmeliaceae represents the huge and widespread family of lichens and include species that attract great interest regarding pharmacological activities, due to the production of unique secondary metabolites.

For the preliminary qualitative phytochemical analysis of the lichen *H.infirma*, 6 different solvents such as ethyl acetate, chloroform, methanol, water, ethanol and petroleum ether were used. Qualitative phytochemical analysis of *H.infirma* showed the presence of alkaloids in all tested extracts. The ethyl acetate and chloroform extracts showed the presence of tannins, flavonoids, steroids, glycosides and phenols. Proteins, carbohydrates, terpenoids and flavonoids were present in the water and petroleum ether extracts. Methanol and ethanol extract showed the presence of Tannins, flavonoids, terpenoids, steroids, carbohydrates, glycosides and phenols. These results revealed that maximum number of bioactive compounds were present in the methanolic extract of *H.infirma*.

Similarly, Rashmi *et al*. (2014) reported qualitative phytochemical analysis of 45 different solvent extracts of nine lichen species. From these, methanolic extract showed the presence of all tested phytochemicals such as tannins, alkaloids, saponins, glycosides, flavonoids, proteins, triterpenes, carbohydrates and steroids in all the lichen samples.

In the present study also, the above qualitative analysis revealed that the maximum number of phytochemicals were present in the methanolic extract of *H.infirma.* Minimum number of phytochemicals compounds were shown in the water and petroleum ether extracts. Methanol extract was more active than the other solvents because of the active compounds in the lichens.

Quantitative analysis

The major active compounds of *H.infirma* have been identified as tannins, flavonoids, alkaloids, saponins and phenols. All of these constituents have been reported to be intimately associated with the health improving effects (Sheng *et al*., 2007). In quantitative analysis, six different extracts of *H.infirma* were tested to quantify tannins, flavonoids, alkaloids, saponins and phenols.

Alkaloids inhibit pathogenic bacteria (Ebana *et al*., 1991). As medicinal importance of the secondary metabolites, alkaloids possess various phytochemical activities such as anti-HIV, antiparasitic, anticancer and antimalarial activity (Leitao Da-cunha *et al*., 2005; Bouayad *et al*., 2011). Alkaloids content of methanol extract of *H.infirma* is 2.66±0.23mg was higher than ethyl acetate (2.01±0.39), water (1.98±0.56), ethanol (1.73±0.16), petroleum ether (1.62±0.12) and chloroform (1.41±0.25).

Tannins are crucial in herbal medicine in treating wounds which contain severe burns and to arrests bleeding (Nguyi, 1988). Tannins are medicinally used as diuretics, astringents, antitumours, antiseptic, antioxidant, haemostatic pharmaceuticals and anti-inflammatory (De Bruyne *et al*., 1999; Dolara *et al*., 2005). Tannin content of methanol extract of *H.infirma* is 3.59±0.02 mg that was higher than ethyl acetate (2.26±0.12), ethanol (1.78±0.11), petroleum ether (1.75±0.07), chloroform (1.73±0.13) and water (1.35±0.13) extracts.

Saponins are medically used for hyperglycaemia, hypercholesterolemia, anticancer, antioxidant, anti-inflammatory and weight loss (De-lucca *et al*., 2005). Saponin content of methanol extract of *H.infirma* is 1.40±0.11 mg was higher than ethanol (1.35±0.13), water (1.32±0.10), ethyl acetate (1.13±0.08), petroleum ether (0.84±0.12) and chloroform (0.82±0.11) extracts.

Flavonoids possess various pharmacological effects including anti-inflammation, antiplatelet, anticancer, antiallergic, heart disease and antioxidant (Asif and Khodadadi, 2013). Flavonoid content of methanol extract of *H.infirma* is 2.59±0.14 mg higher than ethyl acetate (1.78±0.11), chloroform (1.76±0.11), water (1.57±0.15), petroleum ether (1.46±0.13) and ethanol (1.33±0.11) extracts.

Phenol content of methanol extract of *H.infirma* is 3.6±0.21 mg was higher than ethyl acetate (2.85±0.12), ethanol (2.03±0.14),water (1.84±0.10), petroleum ether (1.84±0.10) and chloroform (1.44±0.10) extracts. Comparison of phenols and flavonoids enumerated that there is a perfect remarkable correlation between these two groups. Both phenol and flavonoid compounds revealed strong correlation with antioxidant activity, that means amount of these phytochemicals are linearly correlated. So, it was showed that higher number of phenols and flavonoids (which both were directly correlated), caused higher antioxidant range in studied natural extracts. All the phenolic classes have approved reasonable attention because of their physiological

functions, including free radical scavenging, antioxidants (Bandoniene and Murkovic, 2002) and antimicrobial activity (Nychas *et al*., 2003). The naturally occurring phenolic compounds may play an important role in the maintenance of human health and prevention of several diseases (Rose and Kasum, 2002).

Antimicrobial activity

Biological activities of lichen metabolites have described broad range and most of the common metabolites were powerful agent against bacterial and fungal human pathogenic microorganisms (Molnar and Farkas, 2010). Since ancient times lichens are known to have potential antimicrobial factors and many research studies all over the world showed the efficiency of lichen extracts and purified compounds to inhibit broad range of microorganisms including bacteria and fungi (Prabhu and Sudha, 2015; Yilmaz *et al*., 2004; Vinayaka *et al*., 2009; Kekuda *et al*., 2011).

The difference in the sensitivity among the extracts against the selected bacteria may due to the differences in the morphology of the organisms (i.e.,) differences in the cell wall composition of the organisms (Rankovic *et al*., 2012). Gram positive bacteria have a thick peptidoglycan layer and no outer lipid membrane whilst Gram negative bacteria have a thin peptidoglycan layer and have an outer lipid membrane.

The variance in antimicrobial activity between the extracts is dependent on the type of extracting solvent used which confirms the previous reports (Turk *et al*., 2003; Yilmaz *et al*., 2004; Oloke *et al*., 1998). *Parmotrema tinctorum* and *Parmotrema grayanum* revealed notable antibacterial activity against *Staphylococcus aureus* (Ramya and Thirunalasundari, 2011). *Parmotrema tinctorum* was detected to be active against *Escherichia coli* (Ayyappadasan *et al*., 2015).

In the present study, antimicrobial activity of *H. infirma* has been analysed against gram positive bacteria, gram negative bacteria and two fungal strains such as *Escherichia coli, Streptococcus* sp., *Staphylococcus aureus, Candida albicans* and *Aspergillus niger*.

In antibacterial activity, methanol extract of *H.infirma* showed the maximum inhibition against *Streptococcus* sp., (17.03±0.24 mm), minimum inhibition against *Staphylococcus aureus* (9.57±0.20 mm) and moderate inhibition against *Escherichia coli* (12.83±0.23mm).

In antifungal activity, methanol extract of *H.infirma* showed the maximum inhibition against *Candida albicans* (7.87±0.15 mm) and moderate inhibition against *Aspergillus niger* (6.63±0.22 mm)

Similarly, the methanolic extract of *Usnea ghattensis* have been analysed to be active against *Bacillus subtilis, Bacillus licheniformis* and *Staphylococcus aureus* (Behera *et al*., 2005). Methanolic extracts of lichen showed stronger antibacterial activity against Gram positive bacteria than gram negative bacteria (Huneck, 1999; Halama P.and C.Haluwin *et al*., 2004 and Gulluce *et al*., 2006).

Methanol extract of *Herpothallon* sp., *Parmotrema reticulatum, Parmotrema tinctorum, Parmotrema crinitum, Leptogium sp., Ramalina celastri, Cladonia subradiata (full form)* and *Heterodermia leucomella* showed high range of inhibition against tested bacteria compared to other solvents such as ethyl acetate, hexane and ethanol (Hengameh parizadeh and Rajkumar, 2017a).

Antibacterial activity of methanolic extract of *Parmotrema tinctorum* was effective against *Staphylococcus aureus* and their zone of inhibition was 13-19 mm reported by Quinto and Santos (2005).

Antimicrobial activity of lichen extracts of *Parmotrema tinctorum, Parmotrema grayanum and Parmotrema praesorediosum* showed good growth inhibition against *Aspergillus flavus, Helminthosporium sp., Sclerotium rolfsii* and *Alternaria sp*., (Vivek *et al*., 2014).

Antifungal activity of *Parmotrema grayanum, Parmotrema praesorediosum* and *Parmotrema tinctorum* revealed the growth inhibition against *Colletotrichum capsici* (Kekuda *et al*., 2014)

Antioxidant activity

Free radicals are oxygen containing hugely reactive superoxide anion radical, perox nitric radical, hydroxyl radical, singlet oxygen, hypochloride, nitric oxide and hydrogen peroxide etc., produced during numerous metabolic responses in the human body. Free radicals contribute to more than one hundred disorders in humans including atherosclerosis, arthritis, ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDS (Kumpulainen and Salonen, 1999; Cook and Samman, 1996).

The synthetic antioxidants like BHA, BHT, gallic acid esters etc., have been suspected to cause or prompt negative health effects. Strong restrictions have been placed on their applications (Barlow, 1990; Branen, 1975). In recent years much attention has been devoted to natural antioxidants and their association with health benefits (Ali *et al*., 2008).

In the present study, the free radical scavenging activity of all the lichen extracts was remarkably diverse and was dose dependent as well as concentration dependent (Solvent concentration). Antioxidant activity may be due to the presence of tannins, terpenes and flavonoids (EI-Massry *et al*., 2009; Maestri *et al*., 2006). The strong antioxidant activity of analysed extracts is correlated with a high-level content of total phenols. We can note a number of reports for the antioxidant properties of sample extracts with high content of phenolic compounds (Behera *et al*., 2009). Phenols are important antioxidants in most lichens because of their capacity to scavenge free radicals such as super oxide, hydroxyl radicals and singlet oxygen (Kosanic *et al*., 2012).

Maestri *et al*. (2006) observed that the antioxidant activity may be due to the presence of terpenes, tannins and flavonoids. Similarly, Sharma and Kalikotay (2012) studied radical scavenging activity in ethanol ad methanol extract of *Parmotrema reticulatum*. It was observable that the lichen extracts revealed hydrogen donating ability and therefore the lichen extracts may serve as free scavengers and act as better primary antioxidant against oxidative stress (Vivek *et al*., 2014).

Triggiani *et al*. (2009) revealed strong anticancer activity of *Xanthoria parietina* and also Monojlovic *et al*. (2012) reported the antioxidant effects of *Umbilicaria cylindria*. Antioxidant activity of lichens was exhibited by the presence of phenols (Behera e*t al*., 2005; and Jayaprakasha and Rao, 2000). The lichens *Parmotrema austrosinense* and *Parmotrema tinctorum* had highest amount of phenolic substances that possess the highest capacity of antioxidant activity (Ayyappadasan *et al*., 2015).

Vivek *et al*. (2014) reported strong scavenging activity using the lichen extracts *Parmotrema grayanum, Parmotrema tinctorum* and *Parmotrema praesorodiosum*. DPPH activity of the lichen extracts revealed that absorbance decreased with increasing concentration of extract. The discovery of free radical scavenging activity by the DPPH method is also one of the main and broadly used for lichen extracts (Kekuda *et al*.,

2011and Kekuda *et al*., 2013) that provided an easy and fast way to estimate the antioxidants potential of the extracts (Bondet *et al*.,1997).

In this study, DPPH was effective in methanol extract of *H.infirma.* Percentage of inhibition increased from 51.47±1.83% at 20µg/ml to 85.02±0.45 at 100µg/ml. The IC_{50} value of *H.infirma* was 19.67±1.76 µg/ml. The IC_{50} value of standard (ascorbic acid) was 18.67±1.20µg/ml.

In the present study, hydrogen peroxide activity of methanol extract of *H.infirma* was high. Percentage of inhibition increased from 43.25± 0.12 at 20µg/ml to 86.36±1.67 at 100µg/ml. The IC_{50} value of *H.infirma* was 26.33±1.20 µg/ml. The IC_{50} value of standard (ascorbic acid) was 17.33±0.88µg/ml.

The chloroform extract of *Parmotrema austrosinense* and ethyl acetate extract of *Parmotrema tinctorum* showed strong free radical scavenging activity with lower IC_{50} value 67.79 µg/ml and 49.60 µg/ml respectively.

The radical scavenging and ferric reducing power of solvent extracts may be attributed to the presence of phenolic compounds (Pavithra *et al*., 2013) and Sharma *et al*. (2013) reported antioxidant activity of *Parmotrema reticulatum* with remarkable radical scavenging activity.

Total antioxidant assays reflect the capacity of a nonenzymatic antioxidant protection system. In the phosphomolybdenum method, molybdenum VI (Mo^{6+}) decrease to form a green phosphate/ Mo^{5+} complex at acidic pHs. High absorbance values show that the sample possesses significant antioxidant activity. The method is used for the spectrophotometric quantification of total antioxidant capacity and employs cost-effective reagents (Prieto et al.,1999).

In the present study, total antioxidant activity of methanol extract of *H.infirma.* percentage of inhibition increased from 66.05±3.6 at 20µg/ml to91.38 at 100 µg/ml. The IC_{50} value of *H.infirma* was 24.33±1.20 µg/ml. The IC_{50} value of standard (ascorbic acid) was 18.67±1.20µg/ml.

Superoxide anions are the most common free radicals *in vivo* and are produced in different biological systems and the concentration of superoxide anions increases under conditions of oxidative stress (Lee *et al.*,2002). It was hence proposed to measure the comparative interposing ability of the methanolic extract to scavenge the superoxide radical.

In this study, Superoxide radical scavenging activity of methanol extract of *H.infirma* was found effective. Percentage of inhibition increased from 39.16±1.22 at 20µg/ml to 93.67±0.28 at 100 µg/ml. The IC_{50} value of *H.infirma* was 28±1.53 µg/ml. The IC_{50} value of standard (ascorbic acid) was 17±1.73 µg/ml.

Nitric oxide is an abundant reactive radical that act as an important oxidative biological signalling molecule in diverse physiological processes, including neurotransmission, blood pressure regulation, defence mechanisms, smooth muscle relaxation and immune regulation (Bergendi *et al*., 1999).

In the present study, methanol extract of *H.infirma* was effective in nitric oxide scavenging activity and showed the percentage of inhibition increased from 63.78±0.80 at 20µg/ml to 89.45±1.45 at 100µg/ml. The IC_{50} value of *H.infirma* was 23±2.08 µg/ml. The IC_{50} value of standard (ascorbic acid) was19±1.53 µg/ml.

GC-MS analysis

To date, a large number of different compouds have been isolated in Parmeliaceae family, mostly by high pressure liquid chromatography (HPLC) methods. These methods however require standards for compounds' identification or isolation of extract compounds and their structure explanation.

The economic aspect of very expensive HPLC grade solvents and long analysis time should not be abandoned. Moreover, the volatile part of the extracts might represent a slight proportion of the compounds and consequently identification by HPLC could be difficult or even impossible.

On the other hand, good software for searching different MS libraries qualify compounds identification by GC-MS for many known compounds without isolation and standards. GC-MS gave best results in analysis of volatile constituents of selected Parmeliaceae family (Stojanovic *et al*., 2011).

In the present study, GC-MS analysis of methanol extract of *H.infirma* displayed 40 phyto-constituents. The identified compounds have various biological activities. The major compounds present in the *H.infirma* were Benzoic acid, 2,4-dihydroxy-3, 6-dimethyl-,methyl ester, Dodecanoic acid, methyl ester and Hexadecanoic acid, methyl ester which were found at retention time at 15.619, 13.430 and 17.86 and peak area 27.34%, 22.12% and 10.9%.

GC-MS analysis of *Parmelia perlata* was carried out in acetone, chloroform and Petroleum ether extracts. They showed the presence of 21, 16 and15 major compounds respectively. From these, petroleum ether showed few phyto compounds compared to other solvents (Pratibha and Sharma Mahesh, 2016). GC-MS analysis of *Parmotrema austrosinense* revealed the presence of Hexadecanoic acid and Heptane, 3, 3, 5-trimethyl- with retention time of 13.98 min and 11.67 min respectively (Rajendran *et al*., 2020). Major compounds of *Parmelia reticulata* and *Usnea subflorida* were detected by GC-MS (Adesalu and Agadagba, 2016).

The identified major compounds such as cyclopentanecarboxylic acid, 2-oxo-, methyl ester, Dodecanoic acid, methyl ester, Bis(2-ethylhexyl) phthalate, Hexadecanoic acid, methyl ester, Octadecanoic acid, methyl ester have several biological activities such as antibacterial, antifungal, antiallergic and antiviral.

Isolation

Fungi are hugely diverse and established colonized in varied environments. Lichen thalli produce an unknown ecological niche for a broad variety of microorganisms including fungi. Fungal symbionts favour endophytes also inhabit inner healthy lichen thalli forming symptomless infections and are termed as endolichenic fungi (Arnold *et al*., 2009).

Since not much research work have been carried out to isolate the endolichenic fungi, the present study aimed to isolate and characterize the endolichenic fungi from the selected lichen *Hypotrachyna infirma*. Endolichenic fungi isolating methods are very similar to those of isolating endophytic fungi. Earlier Ahmadjian (1993) and Yoshimura *et al*. (2002) reported that among the three methods tried, lichen mycobiont (endolichenic fungi) was isolated from *Parmotrema austrosinense* by thallus fragmentation method that was found to be the best. The lichens are mostly being aseptically cultured from two main parts i.e., thallus and apothecium. Spore discharge method was ineffective for the isolation, since the apothecia is found less clear and immatured in the lichen (Jayalal *et al*., 2013).

The most distinct culture method is to fragmentize parts of a lichen thallus appropriately which then would be inoculated into a particular mineral medium (Yamamoto *et al*., 2002).

Manisha *et al*. (2010) isolated endolichenic fungi from *Parmelinella simplicior* using MGYP medium and Behera *et al* (2012) also isolated endolichenic fungi from lichen *Usnea complanata*.

Earlier, Suryanarayanan *et al*. (2005) discovered that there is little overlap between the fungal endophytic accumulation of the leaves of trees and the endolichenic fungal assemblages found in the lichens which grew on these trees, vouching the unlikeness of endolichenic fungi.

In the present study, 28 endolichenic fungi were isolated from the lichen *H. infirma*. For the isolation different media (PDA, SDA, MYEA), pH (6, 7, 8) and temperature (25ºC, 35ºC, 45ºC) were maintained. Similarly, Tripathi (2016) isolated 43 endolichenic fungi from different lichens collected from Kumaun Himalaya.

PHYTOCHEMICAL ANALYSIS

The phytochemical analysis was carried out for the isolated 29 endolichenic fungi. Secondary metabolites of lichen, mainly synthesised from fungal metabolism. They are crystal accumulation on the surface of lichens which poorly dilute in water and can usually be isolated from lichen by organic diluents. (Otzurk *et al*., 1999). The studies propose that like fungal endophytes of plants, endolichenic fungi are present in virtually all lichen species that have been examined to date and represent a vital yet poorly studied branch in lichenology.

In the present study, above mentioned isolated 28 endolichenic fungi were screened for the presence of preliminary phytochemicals namely tannins, flavonoids, terpenoids, saponins, steroids, carbohydrates, glycosides, alkaloids, proteins and phenols were tested in two different solvents ethyl acetate and chloroform. Totally 56 extracts were prepared and tested.

From these endolichenic fungi, 13 showed good results for the presence of phyto constitutents. Based on these results, 13 endolichenic fungi were morphologically identified such as *Trichoderma piluliferum, Trichoderma harzianum, Scytalidium lignicola, Geotrichum candidum, Aspergillus niger, Aspergillus stellatus, Aspergillus oryzae, Aspergillus flavus, Nigrospora oryzae, Nodulisporium gregarium* and *Microascus cirrosus.*

Similarly, Preliminary phytochemical analysis of ethyl acetate extracts of endophytic fungi associated with *E.jambolana* establish the presence of alkaloids, phenols, flavonoids, saponins and terpenes. Phenols are the major chemical compounds

responsible for reducing lipid peroxidation and hence function as primary and secondary antioxidants (Gulcin, 2006; Hajdu *et al*., 2007).

Quantitative analysis

The main phyto components of endolichenic fungi have been identified as tannins, phenols, flavonoids, alkaloids and saponins. All of these compounds have been revealed to be closely associated with the health enhancing effects (Sheng *et al*., 2007).

In this study, based on the qualitative analysis 13 endolichenic fungi were selected for the quantitative analysis. From these 13 endolichenic fungi 26 extracts were prepared using two ethyl acetate and chloroform.

From these two solvents, ethyl acetate extracts have good results compared to chloroform extracts. The ethyl acetate extracts of endolichenic fungi *Nigrospora oryzae, Geotrichum candidum, Aspergillus oryzae, Aspergillus niger, Scytalidium lignicola* have good results compared to other endolichenic fungi.

Antimicrobial activity

Endolichenic fungi are hugely beneficial source of novel natural drugs with a broad range of biological activities. The eluted fraction of endolichenic fungi showed considering antimicrobial activity against human pathogens comprising gram-positive and gram-negative bacteria and pathogenic fungi. It is normally trusted that secondary metabolites, also known as lichens substances, are produced mainly by the fungus and produced onto the surface of the lichen's hyphae either in unstructured forms or as crystals (Molnar and Farkas, 2010).

Manisha *et al*. (2010) studied the antimicrobial activity of isolated endolichenic fungi from the lichen *Parmelinella simplicior,* wherein the growth of endolichenic fungi was observed to be rapid than the natural lichen thallus

In the present study, antimicrobial activity of ethyl acetate extract of endolichenic fungi such as *Nigrospora oryzae, Geotrichum candidum, Aspergillus oryzae, Aspergillus niger, Scytalidium lignicola* was studied against two gram positive, two gram negative and two pathogenic fungi were used for this study.

Ethyl acetate extract of *Nigrospora oryzae* showed the maximum inhibition against *Escherichia coli* (23±0.88mm), *Streptococcus sp.,* (20.67±1.86mm),

Staphyococcus aureus (16.33±0.88mm), *Aspergillus niger* (20.67±0.88mm) *and Candida albicans* (7.47±0.20mm) than the other tested endolichenic fungi.

Similarly, Logesh *et al*. (2012) described antimicrobial activity of endolichenic fungi *Aspergillus niger*, *Penicillium citrinum*, *Rhizopus oryzae and Fusarium oxysporium.* This is the first report described in India about antimicrobial activity of endolichenic fungi against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhi* and *Vibrio cholera.*

Recently, Padhi, S., & Tayung, K. (2015) revealed the *in vitro* evaluation of antimicrobial activity against clinically significant pathogens of bacteria and fungi (*Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Shigella flexneri* and *Klebsiella pneumonia*, and three pathogenic fungi (*Candida albicans*, *Candida krusei* and *Trichophyton mentagrophytes*).

Antioxidant activity

DPPH free radical scavenging activity

DPPH free radical scavenging assay is a basic and most popularly used assay. This method is observed as most accurate screening method to estimate the antioxidant activity of samples (Brand-Williams.,1995). In the present study, the endolichenic fungal extracts having high phenolic content also revealed good antioxidant activity. Earlier studies also revealed that there is a linear correlation between total phenolic content and antioxidant potential of any sample (Sultana *et al*., 2007).

Ethyl acetate extract of *Nigrospora oryzae* exhibited increase in percentage inhibition from 60.98±0.69 at 20µg/ml to 86.95±0.28 at 100µg/ml. *Nigrospora oryzae* extract have highest antioxidant activity and IC_{50} value of 26±1.15 µg/ml higher than that of the positive control ascorbic acid (19.67±1.76 µg/ml). The DPPH scavenging assay revealed effective activity present in ethyl acetate extract of *Nigrospora oryzae* compared to other endolichenic fungi.

Hydrogen peroxide scavenging activity

Hydrogen peroxide itself is not very active, but it can sometimes be toxic to cell because it may give rise to hydroxyl radical in the cells (Halliwell, 1991). Thus, the removing of H_2O_2 is very crucial for antioxidant protection in cell or food systems.

Ethyl acetate extract of *Nigrospora oryzae* exhibited increase in percentage inhibition increased from 65.51±0.89 at 20µg/ml to 86.45±1.63 at 100µg/ml. *Nigrospora oryzae* extract showed highest antioxidant activity and IC_{50} value of 27.67±1.76 µg/ml higher than that of the positive control ascorbic acid (26.67±1.20 µg/ml). Hydrogen peroxide scavenging activity revealed effective activity present in ethyl acetate extract of *Nigrospora oryzae* compared to other endolichenic fungi.

The total antioxidant activity

Total antioxidant activity can be reported as the ability of a compound to suppress the oxidative degradation of lipid. Lipid peroxidation action demand oxidative deterioration of lipid with unsaturation. This peroxidation known the initiative mechanism starts with the production of conjugated trienes and dienes, called as primary oxidation results due to the abstraction of a hydrogen molecule. This leads to the evolution of new radical which triggers a number of different diseases in the mankind (Etim *et al*., 2015).

Ethyl acetate extract of *Nigrospora oryzae* exhibited increase in percentage inhibition from 62.30±0.21 at 20µg/ml to 88.62±0.17 at 100µg/ml. *Nigrospora oryzae* extract showed highest antioxidant activity and IC_{50} value of 27.67±1.20 µg/ml higher than that of the positive control ascorbic acid (24.33±1.20 µg/ml). The total antioxidant activity revealed effective activity present in ethyl acetate extract of *Nigrospora oryzae* compared to other endolichenic fungi.

The superoxide anion radical scavenging activity

Superoxide anions play important part in the formation of ROS such as hydroxyl radical, singlet oxygen and hydrogen peroxide, which get oxidative damage in lipids, proteins and DNA (Pietta, 2000). Over production of superoxide anion radical, donate to redox imbalance and associated with harmful physiological reaction (Pervaiz and Clement, 2007).

Ethyl acetate extract of *Nigrospora oryzae* exhibited increase in percentage inhibition increased from 67.76±0.46 at 20µg/ml to 89.26±0.24 at 100µg/ml. *Nigrospora oryzae* extract possessed highest antioxidant activity and IC_{50} value of 26.33±1.20 µg/ml higher than that of the positive control ascorbic acid (28±1.53 µg/ml). The superoxide anion radical scavenging activity revealed effective activity present in ethyl acetate extract of *Nigrospora oryzae* compared to other endolichenic fungi.

The nitric oxide scavenging activity

Nitric oxide is a chemical moderator of physiological functions such as neuronal signalling, smooth muscle relaxant, regulation of cell mediated toxicity and inhibition of platelet accretion. It is encompassing free radical that regulates many roles as an effectors molecule in different biological system as well as antimicrobial, neuronal messenger vasodilation and antitumour function (Miller *et al*., 1993).

Ethyl acetate extract of *Nigrospora oryzae* exhibited increase in percentage inhibition from 60.88±0.25 at 20µg/ml to 84.50±0.67 at 100µg/ml. *Nigrospora oryzae* extract showed highest antioxidant activity and IC_{50} value of 30.33±0.88 µg/ml higher than that of the positive control ascorbic acid (23.67±1.08 µg/ml). The nitric oxide scavenging activity revealed effective activity present in ethyl acetate extract of *Nigrospora oryzae* compared to other endolichenic fungi.

From *Ocimum sanctum,* endophytic fungi *Aspergillus terreus* was isolated and ethyl acetate extract of this endophyte exhibited 34.83% antioxidant activity with 14.96±0.07 mg/g GAE phenolic content (Sharma, 2013).

GC-MS ANALYSIS

GC-MS analysis is a common verification test used to make virtual chemical analysis. GC-MS is the best technique to recognize the component of volatile matter, branched chain hydrocarbons, alcohols, acids, long chain esters, etc. Application of GC-MS involve environmental analysis, drug detection, explosives investigation and identification of unknown samples.

Tripathi (2016) revealed the GC-MS analysis of *Mucor* sp., *Trichoderma viride, Penicillium citrinum* and *Aspergillus niger*. *Mucor* sp. showed the predominance of Hexadecane; Octadecanoic acid; 8(E)- Octadecenoic acid, methyl ester; 6-Hydroxy-7-isopropyl-1,4a-dimethyl 1, 2, 3, 4, 4a, 9, 10, 10a. *Trichoderma viride* showed the

predominance of 9-Octadecanoic acid, methyl ester, (E)-; Simvastatin, trimethylsilyl ether; Cylobutanecarboxylic acid, 2,6- dimethylnon-1-en-3-yn-5-; Docosa-8, 14-diyn-cis-1, 22-diol, bis (trimethylsilyl) ether.

Penicillium citrinum contained Spiro[benzofuran-2(3H),1ˈ- [2] cyclohexene]-3,4ˈ-dione, 2, 4, 6; Spiro[benzofuran-2(3H),1ˈ- [2] cyclohexene]-3,4ˈ; 9- Octadecenoic acid (Z)-, methyl ester; Anthralin. *Aspergillus niger* exhibited the key existence of Octadecane; n-Hexadecanoic acid; 9-Octadecenoic acid, methyl ester, (E)-; Phenol, 3,5-dimethoxy-, acetate as chief chemical constituent.

Similar to the above study, in the present work also, GC-MS analysis of ethyl acetate extracts of *Scytalidium lignicola, Nigrospora oryzae, Geotrichum candidum, Aspergillus niger* and *Aspergillus oryzae* confirmed the presence of 30 different compounds in each endolichenic fungal extracts. The identified compounds possess many biological properties.

The major compounds present in ethyl acetate extract of *Scytalidium lignicola* was Bis(2-ethylhexyl) phthalate (74.38%), 2- Chloropropionic acid, octadec. (4.61), 1-Tricosene (2.97), Pentafluoropropionic acid, penta... (2.79), Cyclotrisiloxane, hexamethyl-(1.63).

The major compound present in ethyl acetate extract of *Nigrospora oryzae* was Bis(2-ethylhexyl) phthalate (52.67%), Methyltris (trimethylsiloxy) silane (16.34%), 1 1-Octadecene (4.54%), Pentafluoropropionic acid, tetra (2.36%), 1-Docosene (2.67%).

The major compound present in ethyl acetate extract of *Geotrichum candidum* was Bis(2-ethylhexyl) phthalate (29.24%), Squalene (25.11%), 3-Buten-2-one, 4-(2,2,3-trimethy (19.78%),1-Docosene (4.33%), Dichloroacetic acid, heptadecyl ... (3.49%).

The major compound present in ethyl acetate extract of *Aspergillus oryzae* was 4H-Pyran-4-one, 5-hydroxy-2- (hyd... (58.88%), Bis(2-ethylhexyl) phthalate (14.71%), 4H-Pyran-4-one, 5-(acetyloxy)-2-... (6.42%), 1, 1'-Biphenyl, 3-methyl-(4.77%), 1, 2 Cyclohexane dicarboxylic acid... (3.27%).

The major compound present in ethyl acetate extract of *Aspergillus niger* was Naphthalene (16.42%), Bis(2-ethylhexyl) phthalate (11.42%), Piperidine, 1-acetyl-(3.58%),7,9-Di-tert-butyl-1-oxaspiro (4, 5... (1.75%), Octadecane, 1-iodo-(1.35%).

Anti-inflammatory activity

Since 15th century the usage of some lichen species such as *Usnea, Cladonia, Xanthoria, Evernia, Physica, Parmelia, Lobaria, Pertusaria, Peltigera* and *Rocella* was reported in Ayurveda, Western medical Herbalism, Traditional Chinese Medicine, Unani, Doctrine of Signature and Homeopathy. The denaturation of biological proteins due to any one of the following denaturation pathways such as acidic (or) alkaline reactions, heat treatment, radiation reactions, etc cause the proteins lose their complex tertiary structure. Because of the externally induced stress under the above-mentioned conditions, the proteins are denaturated.

Inflammation is indicated by the stimulation of the hosts non-immune and immune cells in response to infection and /or toxins in order to destroy pathogens and stimulate tissue healing and recovery (Furman *et al*., 2019).

Evernia furfuracea belongs to the Parmeliaceae family was marketed as the first drug for curing microbial infections in 18th century. During middle age, medicinal supporters mainly used lichens as herbs for inflammation, skin problems, rabies and coughs (Pereira, 2014).

Vinay and Girija (2018) studied the acetone extract and isolates from *Dirinaria consimilis, Ramalina leiodea, Roccella montagnei* and benzohydrazides derivatives for inhibition of protein denaturation induced by heat.

In this present study, maximum absorbance of 75.14±0.81 in *H.infirma* and 68.28±0.05 in *Nigrospora oryzae a*t 500 (µg/ml) concentration were noted. IC_{50} of *H.infirma* and *Nigrospora oryzae* was 298.27 and 371.44. Aspirin used as standard. The extract of *H.infirma* showed absorbance values similar to the standard value when compared to *Nigrospora oryzae* extract.

Molecular docking

To identify novel compounds, rational drug design uses a variety of computational methods. One of these methods is molecular docking. Here interactions between protein receptors and ligands are predicted and analysed (Akhila and Aleykutty,2012).

Molecular docking studies of five lichen metabolites namely usnic acid, atranorin, diffractic acid, lecanoric acid and salazinic acid were carried out with COX-

2 enzyme using Autodock vina to identify the binding mode of ligands and the intermolecular hydrogen bond interaction between ligands and the target protein.

In the present study, lichen and endolichenic fungal compounds namely Dodecanoic acid, Methyl ester and Bis(2-ethylhexyl) phthalate, was carried out with active protease (FXa)-Breast Cancer Drug Target, Vascular Endothelial Growth Factor Receptor 1(VEGFR)-Liver Cancer drug target using Autodock. The *in-silico* studies revealed the potency of Bis(2-ethylhexyl) phthalate as an alternative to treat liver cancer rather than breast cancer.

Cytotoxic activity

Until now, only a few researchers evinced that the lichens have anticancer activity. (Kosanic *et al*., 2013) reported a significant anticancer effect of *Evernia prunastri* and *Pseudoevernia furfuraceae* against FemX (human melanoma) and LS174 (human colon carcinoma) cell lines using MTT assay.

Antiproliferative activity of lichen extracts such as *Parmelia sulcata, Cladonia foliacea, Evernia prunastri, Flavoparmelia caperata* and *Hypogymnia physodes* against colon cancer adenocarcinoma cell line HCT-116 by MTT assay and acridine orange/ethidium bromide staining. From these, methanolic extracts of *C. foliacea* (IC_{50} 265 µg/ml) and *H. physodes* (IC_{50} 253 µg/ml) revealed better cytotoxic activity than other extracts (Mitrovic *et al*., 2011).

The extract of *Parmotrema tinctorum* showed cytotoxic activity against Hep-2 larynx carcinoma, MCF7,786-0 and B16-F10 cell lines (Bogo *et al*., 2010) and Moriano *et al*. (2016) also reported the remarkable cytotoxic activity in methanolic extracts of *Flavoparmelia euplecta, Bulbothrix setschwanensis and Lethariella canariensis* against MCF-7 cell line.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide) colorimetric assay is broadly used in determination of cytotoxicity, proliferation studies in cell biology and cell viability (Ferrari *et al*., 1990; Van de Loosdrecht *et al*., 1991). In the assay, the mitochondrial enzyme dehydrogenase of the living viable cells cleaves the MTT and produce a purple product called formazan that can be measured employing computer-based evaluation system. Here, the number of viable cells or cleaved cells is measured using spectrophotometer in terms of concentration of the formazan

(Rubinstein *et al*., 1990; Takimoto, 2003; Covell *et al*., 2007; Madhuri and Pandey, 2009).

In the present study, the methanol extract of *H.infirma* and ethyl acetate extract of *Nigrospora oryzae* were investigated for cytotoxic activity using MTT assay. The methanol extract of *H.infirma* and ethyl acetate extract of *Nigrospora oryzae* showed significant inhibitory activity in 5µg/ml, that exhibited 75.33±0.65% and 78.33±1.86% of inhibition. The concentraction required for 50% inhibition (IC_{50}) was found to be 19±1.2 µg/ml and 24± 0.5 µg/ml.

Synthesis of bionanoparticles using silver nitrate

The production of AgNPs using *Aspergillus ochraceus* cell filtrate at 40ºC temperature pH 6 and 0.75 mM concentration of AgNO3 was reported by Magdi *et al*. (2014). Hafez *et al*. (2016) and Chandrappa *et al*. (2016) have revealed that 5mM concentration of $AgNO_3$ solution was optimum for the synthesis of AgNPs through *Cladosporium sphaerospermum* and *Penicillium* sp. respectively. These results are in consonance with the present findings.

The decrease in production of silver nanoparticles, as the concentration of AgNO3 solution increase in case of AgNPs synthesis by the fungal extract of *Penicillium atramentosum* was reported (Sarsar *et al*., 2015). Li *et al*. (2012) also described synthesis of AgNPs at room temperature by extracellular enzymes of *Aspergillus terreus*.

UV Visible Spectroscopy

While elevated by light of specific wavelength, the metal nanoparticles (MNPs) would give a characteristic surface phenomenon called surface plasmon resonance (SPR), which may produce a specific peak for kind of MNPs by UV-Vis spectroscopy.

The fungal cell filtrate of *Aspergillus niger*, *Penicillium ochrochloron* and *Aspergillus tamarii* served with silver nitrate (1.0mM) solution reported a characteristic broad band at 419nm and 430nm respectively (Devi and Joshi, 2015).

Maliszewska *et al*. (2009) reported that broad peaks were obtained on 420nm-450nm for *Penicillium* sp. In case of *Aspergillus flavus* characteristic peak was obtained at 410nm (Ninganagouda *et al*., 2013).

During the present investigation, colour change in extracts of lichen (*H.infirma*) and their endolichenic fungi (*Nigrospora oryzae*) indicates the presence of silver nanoparticles and primary confirmation was done by UV-Vis spectrophotometer. From these broad peaks obtained on 420nm-450nm.

Additionally, FT-IR spectroscopy has established that amino acid residues and peptides have well-built ability to affix with metal to avoid agglomeration of the particles and stabilized the medium (Sandhu *et al*., 2017).

In AgNPs solution, *H.infirma* showed stretches at 3332.99, 2036.83, 1319.31, 601.79 referred to Phenolic groups (O-H), Carboxylic acids (O-H), Alcohols, Esters, Carboxylic acids, Ethers (C-O), Alkyl halides (C-Br). Similarly, *Nigrospora oryzae* showed stretches at 3379.29, 1296.16, 810.10, 555.50 referred to a Phenolic group (O-H), Alcohols, Esters, Carboxylic acids (C-O), Primary and secondary amines (N-H), Alkyl halides (C-Br) respectively.

SEM ANALYSIS

Both Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) can be used for the morphological analysis of the nanospheres.

The comparative investigation of the SEM analysis of the lchen and endolichenic fungus showed spherical shape and nano sized particles. Analysis of the size of silver nanoparticles showed homogeneously distributed AgNps and size in the range of 65.92 and 64.64 nm for *Penicillium* sp. (Govindappa *et al*., 2016).

Similarly, Devi and Joshi (2015) reported the arrangement of spherical nano sized Ag particles with dissimilar sizes.

The AgNPs were characterized by SEM and it was reported that shape of NPs was spherical shape with size ranging from 20-55nm. During the characterization studies, *Aspergillus terreus* revealed size in the range of 35-59 nm and *Myceliophthora thermophila* revealed 18-30nm. During the comparative exploration of the SEM characterization, all mentioned fungi reported spherical shape and nano sized particles. During the characterization studies of *H.infirma* and *Nigrospora oryzae* also revealed the shape of NPs was spherical and cylindrical shape with size range from 3-20µm.