*Chapter 6*

# DISCUSSION

# 6. DISCUSSION

## 6.1 FREE RADICAL SCAVENGING ACTIVITY

Free radicals are constantly generated in the body as a result of aerobic metabolism in cells. $O_2^{\bullet}$ are generated slowly by the leakage of electrons from electron carriers of the electron transport chain of mitochondria to $O_2$. Beside this other endogenous and exogenous factors such as infection, exercise, aging, radiations, pollutants, etc. contribute to the generation of free radicals which ultimately leads to the development of oxidative stress (Zima *et al*., 2001). Different types of ROS includes singlet oxygen, nitric oxide ($NO^{\bullet}$), superoxide anion ($O_2^{\bullet}$), hypochlorite radical and hydrogen peroxide ($H_2O_2$). For protection against free radicals, human system has developed a complex and sophisticated antioxidant protection system. Antioxidants are endogenous and exogenous in origin. Endogenous antioxidants are different antioxidant enzymes like glutathione peroxidase, superoxide dismutase (SOD), and catalase. Apart from this, non enzymatic molecules including thiols, thioredoxin and disulfide bonding also constitute antioxidant defense system. Exogenous antioxidants are obtained from food in the form of vitamin C, vitamin E and beta carotene. In addition, a number of plant derived substances were also proved to contain antioxidant activity. Phytochemicals such as flavonoids, tannins and phenolic compounds are considered as the main antioxidant contributing compounds of plants (Wong *et al*., 2006). Bryophytes, a second largest group of plant kingdom have demonstrated potent antifungal, antimicrobial, cytotoxic and other biological activities however documentation of information regarding their biological activities is rather less as compared to higher groups of plants. So the main aim of this study was to explore the pharmacological properties and phytochemical constituents of liverworts.

Eastern Himalaya is a home to large number of bryophyte species (Liverwort, hornwort and moss). Eleven liverwort species were selected from this area based on their uses in traditional medicine and abundance; they are *Pellia epiphylla* (L.) Corda, *Conocephalum japonicum* (Thunb.) Grolle, *Lunularia cruciata* (L.) Dumort. Ex Lindb, *Dumortiera hirsuta* (Sw.) Nees, *Marchantia emarginata* Reinw., Blume & Nees subsp. *emarginata*, *M subintegra* Mitt., *M polymorpha* L. subsp. *ruderalis* Bischl. & Boisselier-Dubayle, *Plagiochasma cordatum* Lehm. & Lindenb, *Asterella wallichiana* (Lehm.) Grolle, and *Plagiochila nepalensis* Lindenb. and *Marchantia paleacea* Bertol.

Extraction is an important step for pulling out desired phyto-compounds from plants for further analysis. Depending on the nature of bioactive compounds, solvent system for their extraction was selected. For extraction of hydrophilic compounds, polar solvents like methanol, ethanol or ethyl acetate is used (Cosa *et al.*, 2006). Akinmoladun *et al.* (2007), working on *Ocimum gratissimum* found the presence of anthraquinones only in aqueous extract while alkaloids were detected in methanolic extract only. Bryophytes are composed of different types of polysaccharides, lipids, polyphenols and other secondary metabolites (Klavina, 2014). In the present work both non polar and polar substances were extracted almost in equal amount indicating the presence of lower and higher polarity phytocomponds in equal amount. Klavina *et al*. (2015) reported the presence of polar substances in lower amount in case of mosses than the non polar compounds. While in case of higher group of plant the presence of polar substances was found in higher amount than non polar substances (Saeed *et al.*, 2012). Thus, the result obtained in the present work is in contradiction with those found in higher group of plants. This highlights the uniqueness of phytochemicals in this group of plants; so, further chemical analysis on the secondary metabolites of liverworts can identify a number of different new compounds which could be useful in pharmaceutical, cosmetic or agricultural fields**.**

In recent years wide ranges of spectrophotometric assays were used to measure the antioxidant properties of plants (Thaipong *et al*., 2006). These assays are based on the principle of generation of coloured synthetic radicals, their chelation by antioxidants present in the studied plant and monitoring the free radical scavenging activity spectrophotometrically. These assays are categorised into two groups. One approach involves transfer of electron and reduction of coloured free radicals, like the case of $DPPH^{\bullet}$ and $ABTS^{+}$. In another approach antioxidants and substrate compete for thermally generated peroxyl radicals such as the case of oxygen radical absorbance ability and ferric reducing ability (Rodriguez- Amaya, 2010). $DPPH^{\bullet}$ and $ABTS^{+}$ are stable free radical method which is a rapid, easy and sensitive way to study the antioxidant activity of plant extracts (Koleva *et al*., 2002). $DPPH^{\bullet}$ is a well known stable organic nitrogen free lipophilic radical which has strong absorption band at about 520 nm. $DPPH^{\bullet}$ radical in solution has a deep violet colour and becomes pale yellow when it is neutralized. Antioxidants neutralize $DPPH^{\bullet}$ free radical either by transferring an electron or hydrogen atom. Studied liverworts demonstrated $DPPH^{\bullet}$ radical scavenging activity in a concentration dependent manner. Liverworts like *Marchantia paleacea*, *Plagiochasma cordatum*, *Plagiochila nepalensis, Dumortiera hirusta* ($IC_{50}$ value 60 µg/ml -510 µg/ml) showed significant potential to scavenge free radicals which are as good as DPPH radical scavenging potential of Algerian medicinal plants (4.30 µg/ml - 486.6 µg/ml) (Soumia *et al*., 2014). $DPPH^{\bullet}$ scavenging activity of studied liverworts was also found to be more or less similar to wetland medicinal plants (Ho *et al*., 2012). Significant $DPPH^{\bullet}$ radical scavenging activity can be attributed to hydrogen or electron donating ability of the phytochemicals present in these liverworts.

$ABTS^+$ is another commonly used assay to assess the antioxidant property of the plant. $ABTS^+$ scavenging assay is based on the generation of green $ABTS^+$ by reacting $ABTS^+$ and potassium persulfate. Addition of samples under study to $ABTS^+$ solution reduces it depending on the antioxidative potential of the samples. In the present work diethyl ether extract of *Plagiochasma cordatum* showed highest $ABTS^+$ scavenging activity. *Marchantia polymorpha*, *M. paleacea*, *M. subintegra*, *Pellia epiphylla*, *Plagiochila nepalensis* and *Asterella wallichiana* also showed strong $ABTS^+$ scavenging activity. Diethyl ether and ethyl acetate extract of all the liverworts have shown the highest activity than other solvent extracts. Methanolic fraction after diethyl ether and ethyl acetate extract showed good $ABTS^+$ activity. This result showed that for liverworts, diethyl ether and ethyl acetate are best solvents for extracting phytochemicals with significant $ABTS^+$ scavenging activity.

Superoxide anion is one among the most powerful reactive species generated by the auto-oxidation reactions, enzymatic processes and nonenzymatic electron transfer reactions (Michelson *et al*., 1977). They are mostly produced within the mitochondria and are usually produced by enzymes xanthine oxidase (Kuppusamy *et al*., 1989), cyclooxygenase (McIntyre *et al*., 1999), lipooxygenase and NADPH dependent oxidase. Superoxide is considered more harmful because it leads to the production of other harmful radicals such as hydroxyl ion. Superoxide ion exists in two forms, $O_2^-$ or hydroperoxyl radical ($HO_2$) at low pH (Bielski and Cabelli, 1996). Under physiological pH superoxide exists in $O_2^-$ form and reduces iron complexes such as cytochrome c and ferric-thylene diaminetetra acetic acid reducing $Fe^{+3}$ to $Fe^{+2}$. Hydroperoxyl radical formed can easily enter the phospholipid bilayer causing its oxidation. Studied liverworts have shown significant potential in scavenging SO radicals. Liverworts like *Marchantia subintegra* and *M. paleacea* showed SO scavenging activity ($IC_{50}$ value of 109 µg/ml and 160 µg/ml respectively) that was similar to the SO scavenging activity of medicinal plants like *Prunella vulgaris*, *Saurauia oldhamii*, *Rubus parvifolius*, *Jassiaea repens* showing ($IC_{50}$ value 113-159 µg/ml) (Shyur *et al*., 2005). SO scavenging activity of *Marchantia subintegra* ($IC_{50}$ value 109 µg/ml) was comparable to that of the aqueous extract of medicinal plant *Acorus calamus* ($IC_{50}$ value 101 µg/ml) which is used as a central nervous system relaxant, an appetite stimulant, an anthelmintic, a sedative and an antibacterial agent (Barua *et al.*, 2014). In the present case, moderate polarity solvent extracts scavenged SO more efficiently than extreme polar and non polar solvent extracts.

Free radicals are grouped into two group namely reactive oxygen species and reactive nitrogen species. Most important reactive nitrogen species is nitric oxide. However nitric oxide is an important signalling molecule in living organisms. Enzyme nitric oxide synthase that converts L-arginine to L-citrulline generates nitric oxide (Andrew and Mayer, 1999). It is soluble in both lipid and water thus can diffuse easily through cytoplasm and plasma membrane and act as an important intracellular second messenger (Chiuch, 1999). It helps in muscle relaxation in blood vessel by stimulating guanylate cyclase and protein kinase. It is also an important redox regulator of the cells (Wink and Mitchell, 1998). Other biological activities like blood pressure regulation, neurotransmission and smooth muscle relaxation also requires $NO^{\bullet}$, however its sustained level in the biological system is harmful. Result of this work showed the presence of NO scavenging property in the studied liverworts. Among studied liverworts *Marchantia paleacea*, *Dumortiera hirsuta* and *Lunularia cruciata* ($IC_{50}$ 20-82 µg/ml), scavenged the *in vitro* originated $NO^{\bullet}$ more efficiently than medicinal plants *Phyllunthus fraternus*, *Triumfetta rhomboidea*, *Casuarina littorea* ($IC_{50}$ 48.27-196.89 µg/ml) (Parul *et al*. (2013). Similarly, *Marchantia paleacea*, *Dumortiera hirsuta* and *Lunularia cruciata* displayed higher $NO^{\bullet}$ scavenging ability than medicinal plant *Guettarda speciosa* having $IC_{50}$ value 77.22 µg/ml in its aqueous extract (Revathi *et al* 2015). Study of the effect of solvent on extraction of $NO^{\bullet}$ scavenging phytochemicals from liverworts revealed that non polar solvents to moderately polar solvents are best for the extraction of phytochemicals with potential NO scavenging activity. This result is in disparity with that of higher group of plants where better NO has been shown by the extracts of polar solvent.

The ferric reducing ability of plasma (FRAP) assay is different from all other antioxidant assays as no free radicals is generated here. Instead in this assay reduction of ferric ion ($Fe^{+3}$) to ferrous ion ($Fe^{+2}$) is monitored. Reducing potential is the measure of the ability of antioxidants to transfer electron (Meir *et al.*, 1995) and thus, are considered as the direct measure of antioxidant activity. In the present study, *Plagiochasma cordatum* showed the highest ferrous ion reducing potential.

Metal ions accelerate lipid peroxidation by decomposing hydrogen peroxide to form alkoxyl and peroxyl radicals. Transition metal also reacts with hydrogen peroxide to form hydroxyl ion. Metal chelating activity has high perceptibility in living system as it lowers the concentration of transition metals in the process like lipid peroxidation. Result showed that among studied liverworts *Marchantia paleacea*, *Conocephalum japonicum*, *Plagiochasma cordatum* and *Plagiochila nepalensis* have high metal chelating potential with $IC_{50}$ value ranging between 90 µg/ml to 380 µg/ml. The metal chelating activity of medicinal plants ($IC_{50}$ 80- 250 µg/ml) found in Jordan (Bilto, 2015) were somewhat similar to the result obtained in the present study.

Present work showed that different solvent extracts of a single plant have wide variation in the radical scavenging activity. Extraction of phytochemicals having different polarity might have resulted in the variation of the activity. It was noticed that in all studied liverworts diethyl ether and ethyl acetate extracts have showed best antioxidat activity. Butanol and acetone fractions have shown an average activity while least activity was recorded in heptane and methanol extracts. However, transition metal chelating activity was better in case of heptane extract. It has been reported that terpenoids and aromatic compounds present are responsible for different biological activities by the liverwort (Asakawa, 2007). Terpenoids are naturally occurring non polar compounds derived from isoprene unit that can be extracted by using non polar solvents (Harman-Ware *et al*., 2016). In the present study, better antioxidant activity of diethyl ether and ethyl acetate extracts can be assumed due to the presence of non-polar compounds like terpenoids in the extract which is similar to the findings of other workers (Asakawa, 2007).

## 6.2 ANTI-DIABETIC ACTIVITY

Diabetes mellitus (DM) is one amongst the major health problems worldwide. Reduction in insulin sensitivity and postprandial hyperglycemia are the characteristics of type 2 diabetes (Mousinho *et al.*, 2013). Lowering postprandial hyperglycemia can be an important measure to control diabetes. Postprandial hyperglycemia can be controlled by inhibiting the activity of enzymes α-amylase and α-glucosidase (carbohydrate hydrolyzing enzymes) (Ali *et al.*, 2006). Inhibition of these enzymes will delay carbohydrate digestion which will result in the reduction of rate of glucose absorption and consequently blunts the postprandial hyperglycemia. Liverworts like *Conocephalum japonicum*, *Pellia epiphylla*, *Marchantia emarginata*, *M. subintergra* and *Plagichasma cordatum* showed high α-amylase and α-glucosidase enzyme inhibitory activity. Diethyl ether extract of *Marchantia subintegra* inhibited the α-glucosidase activity with greater efficiency than that of standard metformin. α-amylase and α-glucosidase inhibitory activity of medicinal plant *Solanum surratense* L (Manoj, 2012) was somewhat similar to the result obtained in the present study. Traditionally used plant *Calamus erectus* for treatment of diabetes have α -amylase and α-glucosidase inhibitory activity ($IC_{50}$- 1.69 and 2.00 mg/ml) similar to the result obtained in the present work. Hence, this result supports the potential of liverworts as an active source of phytochemicals having anti-diabetic activity. Bis(benzyls) namely marchantin C found in liverworts are reported to be responsible for α-glucosidase inhibitory activity (Harinantenaina and Asakawa, 2007).

In *Asterella wallichiana*, *Pellia epiphylla*, *Marchantia emarginata*, *M. polymorpha* and *Plagiochasma cordatum* ethyl acetate extract showed the highest α-glucosidase inhibitory activity. In *Dumortiera hirsuta* and *Marchantia subintegra* diethyl ether, in *Conocephalum japonicum* and *Lunularia cruciata* acetone and in *Plagiochila nepalensis* butanol extract showed the highest α-glucosidase inhibitory activity. It has been noticed that solvent having moderate polarity i.e. solvents having polarity between two extreme polarity values are more efficient in extraction of phytochemicals with significant α-glucosidase reducing potential in liverworts. While, many studies (Zarena and Sankar, 2009; Mohsen and Ammar, 2009) reports the greater efficiency of polar solvents in extraction of phytochemicals with better pharmacological activities. Extraction of phenolic group in polar solvents is responsible for the said biological activities. The result of present study is different from these findings suggesting that in liverworts non polar to moderately polar phytochemicals are pharmacologically more active. Many new compounds uncommon in higher plant groups are found to be present in bryophytes (Sabovljevic and Sabovljevic, 2008). Result of the present study is in conformity with the earlier findings suggesting the presence of unique phytochemicals in liverworts than those found in higher group of plants. Less polar solvents like acetone was also found to be more suitable for extraction of phenolics compounds from flowers (Liu *et al*., 2009). Phenolics varying from simple (anthocyanins, phenolic acids) to highly polymerized form (tannins) are present in plants. Higher α-glucosidase inhibitory activity of moderate polarity extracts of studied liverworts might be due to the presence of more active moderate polarity phytochemicals than highly polar one in liverworts.

In *Asterella wallichiana*, *Pellia epiphylla* , *Marchantia emarginata* and *M. polymorpha* ethyl acetate fraction showed the highest α-amylase inhibitory activity. In *Dumortiera hirsuta* and *Marchantia subintegra* diethyl ether extract, in *Conocephalum japonicum* and *Lunularia cruciata* acetone extract and in *Plagiochila nepalensis* butanol extract displayed the highest α-amylase inhibitory activity. Diethyl ether, ethyl acetate, acetone and butanol extracts also showed good α–amylase inhibitory activity suggesting moderate polarity phytochemicals to be more active against α–amylase. It has been reported that terpenoids and aromatic compounds present in the liverwort are responsible for different biological activities (Asakawa, 2007). Better anti-diabetic activity of diethyl ether and ethyl acetate extracts shown in the present study might be due to the presence of non-polar compounds like terpenoids in the extract which is similar with the findings of other workers like Asakawa (2007).

Oxidative stress increases largely under sustained hyperglycaemia. During hyperglycaemia, free radical generates from self-oxidation of glucose, increased glycation and alteration of polyol pathway. These free radicals cause damage to β-cells and leads to the development and progression of complications associated with diabetes such as memory impairment, neuropathy, nephropathy and retinopathy (Maritim *et al*., 2003). Therefore, antioxidant therapies targeting diabetes induced oxidative stress can be considered to prevent downstream diabetic complications (Araki and Nishikawa, 2010). Thus, considering the potential free radical scavenging activity, studied liverworts can be used for reducing downstream diabetic complications.

## 6.3 CYTOTOXIC ACTIVITY

Renal cell carcinoma (RCC) is the third most frequent malignancy of genitourinary organs (Cheng *et al*., 2017). In most of the cases, RCC is diagnosed at the later stages (Jemal *et al*., 2009). It is found to be usually resistant to chemotherapy and radiotherapy. Recently certain drugs have been used in targeted therapeutic approach (Escudier *et al*., 2012) but this approach also faced some limitations of development of drug resistance (Cheng *et al*., 2017). So, there is a need to find novel source of chemotherapeutic agents that can target renal cell carcinoma without acquiring drug resistance. Bryophytes have been investigated for active biomolecules having potential cytotoxicity against cancer cells. Both isolated compounds and crude extracts from bryophytes were found to have cytotoxic properties. Active biomolecules like terpenoids (Perry *et al*., 1996), bi- bisbenzyl (Asakawa *et al*., 1982), marchantin (Asakawa *et al*., 1982), plagiochn (Shi *et al*., 2008), perrottetin (Asakawa *et al*., 1982), lunularin (Lu *et al.,* 2006) found in liverworts are reported to have potential cytotoxic activities. In our study, liverworts *P. cordatum, A. wallichiana, L. cruciata, M. paleacea* and *M. nepalensis* were studied for their anti-proliferative activity against human kidney cancer cell line (ACHN). Studied liverworts showed potent cytotoxic effects in ACHN human renal cancer cells. *P. cordatum* showed highest cytotoxic effect ($IC_{50}$ 69.15 µg/ml) against ACHN among studied liverworts. The cytotoxic activity of *P. cordatum, P. nepalensis* and *M. paleacea* against ACHN was found to be better than that of Semen Euphorbiae (dried ripe seed of *Euphorbia lathyris*) extract ($IC_{50}$ 185.2 µg/ml) studied by Cheng *et al*. (2017). Extracts having cytotoxicity value ranging between ≤ 20 and1000 µg/ml ($IC_{50}$) are considered active and extracts having cytotoxicity value more than 1000 µg/ml are considered inactive according to the reports of Atjanasuppat *et al*. (2009). Studied liverworts have anti-proliferative activity value ranging between 69.15 µg/ml and 308.98 µg/ml ($IC_{50}$). Extracts of studied liverworts exhibited promising cytotoxic activity. The result of this study suggested that liverworts also have the anti-proliferative potential and thus can act as the source of novel bioactive compounds with therapeutic and anti-cancer properties. Till date, liverworts are less explored for their bio-prospective values and were just ignored, but the result of this study provides the basis for further investigation on this group of plants as a source of potent bioactive compounds having anti-cancer property.

## 6.4 PHYTOCHEMICAL ESTIMATION

The phytochemicals present in plant are responsible for the biological activities displayed by plant. An insight into chemical nature can present rich data in understanding correlation between phytochemicals and their biological activities. Plant phenols are an important group of natural antioxidants having tremendous therapeutic potential. Variations in the structure of phenolic compounds lead to their solubility in solvents of different polarity. Thus, nature of extraction solvent has a significant impact on the extraction of polyphenols from plants. In this work, six solvents namely heptane, diethyl ether, ethyl acetate, acetone, butanol and methanol were used. Optimization of extraction conditions for polyphenolic compounds indicated that the most suitable solvent is usually different for different plant samples. Ngo *et al.* (2017) and Yang *et al*. (2017) reported acetone as the optimal extraction solvent for extraction of phenolic compounds from their studied plants. Addai *et al*. (2013) and Ferhat *et al*. (2017) found methanol to be optimal extraction solvent for phenols. Singh *et al*. (2014) found mixture of ethanol, diethyl ether, water (8:1:1) to be optimal solvent mixture for phenolic compound extraction. In this study, variation was noticed in the phenol content among extracts obtained from liverworts using solvents of different polarity. In *Marchantia polymorpha*, *M. paleacea* and *Pellia epiphylla*, diethyl ether, ethyl acetate and methanol showed high phenol content. In *Marchantia subintegra, M. emarginata, Plagiochila nepalensis, Dumortiera hirsuta, Lunularia cruciata* and *Conocephalum japonicum* ethyl acetate and diethyl ether showed optimal extraction of phenolic compounds. Phenol content of studied liverworts varied from 4.8 mg Gallic acid equivalent / g extractive weight to 377.9 mg GAE/g EW. The phenol content of bryophytes such as *Pellia endiviifolia* (80.3 µg/mg) [Dey *et al*., 2013] was found to be lower than that of studied liverworts except *Conocephalum japonicum* (66.4 µg/mg) and *Pellia epiphylla* (49.5 µg/mg). Phenol content of *Barbula javanica* (30 µg/ ml) [Vats and Alam, 201] was lower than that of liverworts studied in this work. The phenol content of moss *Bryum moravicum* (356.4 µg/mg) was slightly lesser than the phenol content of *Marchantia emarginata* (377.9 µg/mg) obtained in present study. Similarly screening of phenol content of higher group of plants with medicinal property showed phenol content (69.89-382.57 mg GAE/g) somewhat similar to result obtained in present work (4.8 to 377.9 mg GAE/g).

Flavonoids are important group of phenolic compounds that are reported to have strong antioxidant activity (Pietta *et al*., 2000). In the present work presence of significant amount of flavonoids was recorded. Flavonoid content ranged between 0.3 to 94.2 mg quercetin equivalent/ g extractive weight. Flavonoid content in some liverworts like *Plagichasma cordatum*, *Dumortiera hirsuta*, *Lunularia cruciata*, *Marchantia emarginata* and *M. subintegra* was higher than that of Macedonian medicinal plants *Digitalis ferruginea* and *Digitalis lanata* having flavonoid content 2.86 and 11.19 mg CE/g DW, respectively (Tusevski *et al*., 2014). However, variation in flavonoid content was noticed with the polarity of solvent. Presence of high amount of flavonoids was recorded in non polar, moderately polar and polar solvent. This result differs from the findings of Yumrutas and Saygideger, (2012) and Ghasemzadeh *et al*. (2011) who reported higher flavonoid content in polar solvents only. Therefore, it can be inferred that in liverworts less polar flavonoid aglycones are present in high amount which results in its extraction in higher amount in non polar solvents. Polar flavonoids like flavonoid glycosides might have extracted in polar solvents. Ortho-dihydric phenol was also found to be present in studied liverworts. Presence of ortho-hydric phenol was recorded in almost equal amount in all the six extracts.

Steroids are group of secondary metabolites having many medicinal applications (Sultan and Raza, 2015). It has cardiotonic; anticancer, anti-viral, insecticidal and antimicrobial activities (Kokpol *et al*., 1984). Steroid content in studied liverworts ranged between 0.002 to 1.64 mg solasodine equivalent/ gram extractive weight. Madhu *et al*. (2016) studied the content of steroid in ten medicinal plants which ranged between 30.45 to 69.20 µg/ml. Steroid content of these medicinal plant was found to be lesser than that of liverworts studied in the present work. Steroid compounds are non polar in nature and are expected to be extracted in non polar solvents (Nuryanti and Puspitasari, 2017). Estimation of steroids using Libermann-Burchad reagent showed that steroids was extracted in higher amount in non polar solvents. Least amount of steroids has been detected in polar solvents.

Tannins are polyphenols having high molecular weight. It has many therapeutic potential like cardio-protective, anti-diabetic, anti-carcinogenic potential (Kumari and Jain, 2015). The presence of tannins in *Litsea cubeba* and *Zanthoxylum acanthopodium* supports the traditional medicinal use of these plants in the treatment of different diseases. Trease and Evans (1989) stated that tannins have potent antimicrobial, anticancer as well as antioxidants activities. Studied liverworts showed the presence tannins ranging between 0.02 to 2.56 mg Tannic acid equivalent/g extractive weights. Tannin content of *Halimium halimifolium* (Rebaya *et al*., 2015) was lesser (0.05-2.20 g/100 g) than that of studied liverworts. While fifty three trees which are traditionally used in the treatment of diarrhoea contained tannin in higher amount (6-10 mg/ml) than studied liverwort (Wurger *et al*., 2013). In *Marchantia subintegra*, *M. emarginata*, *Conocephalum japonicum*, *Pellia epiphylla* , *Plagiochasma cordatum*, *Plagiochila nepalensis* and *Lunularia cruciata* dietyl ether extract contained highest tannin content than other solvent. While in case of *Marchantia polymorpha*, *M. paleacea*, *Asterella wallichiana* and *Dumortiera hirsuta* moderately non polar solvents like methanol, butanol and acetone has extracted tannin in greater amount than the other solvents. Pansera *et al*., 2004 and Yuliana *et al*., 2014 have also reported polar solvents to be more suitable for extraction of tannins.

## 6.5 CORRELATION BETWEEN PHYTOCHEMICAL CONTENT AND PHARMACOLOGICAL ACTIVITIES

### 6.5.1 Pearson correlation test

Plants were used as medicines since prehistoric times until synthetic drugs were developed in 19$^{th}$ century. The health beneficial and health promoting effects of plants are due to the active principles they possess. Plant secondary metabolites are mainly responsible for displayed pharmacological properties. Phenolics are ubiquitous bioactive compounds present in plants (Robards *et al*.1999). Phenolic acids like chlorogenic, coumaric, caffeic and ferulic acids are strong antioxidants (Larson, 1988). Phenolic compound β-carotene acts as antioxidants by quenching singlet oxygen. *p*- coumaric acids act by inhibiting the generation free radicals (Laranjinha *et al*., 1995). Flavonoids are other group of phenolic compounds that donate electrons and quench free radicals. However, data in the literature on the correlation between the phenolic compounds and their antioxidant activities are sometimes contradictory. Some researchers have noticed good correlation while some others have reported very weak or no correlation between antioxidant activity and phenolic compounds. Qusti *et al.* (2010) found good correlation between the phenolics and antioxidant activity of fruits. High correlation between the antioxidant activity and the phenolic compound was also observed in case of flax seeds and cereals (Velioglu *et al*., 1998). Similarly, phenolic compounds contributing to the antioxidant activity was also reported by Wong *et al.* (2005) in case of 30 Chinese medicinal plants.

Like higher group of plants, high correlation between antioxidant activity and total phenolic content was also noticed in liverworts studied in present work. Manoj *et al.* (2012) observed linear correlation between antioxidant activity and phenol content of bryophytes like *Plagiochila beddomei*, *Leucobryum bowringii* and *Octoblepharum albidum*. In this work, correlation between the antioxidant activities, anti-diabetic activity and phenolic content was studied using Pearson Correlation coefficient and Principle Component Analysis. Data showed correlation between studied pharmacological activity (antioxidant and anti-diabetic activity) and phenolic compounds present in plants (Fig 5.5.1). Correlation was analyzed by comparing $IC_{50}$ value of the assays and phenolic content of the plants. Lower the $IC_{50}$ value, higher is the antioxidant activity. Negative value of Pearson correlation test in our study indicates positive correlation between phenol content and $DPPH^{\cdot}$ scavenging activity. Value is negative because higher phenolic content results in lowering of $IC_{50}$ value (i.e. $DPPH^{\cdot}$ scavenging activity increases) showing negative correlation between the two parameters. Phenols present in the studied liverworts were also found to have a strong correlation with the superoxide scavenging activity. Ferric reducing activity is considered equivalent to free radical scavenging potential of plant. The result of the present work also showed positive correlation between ferric reducing ability and phenolic content. It was suggested that phenolic compounds are main free radical scavengers that are produced by studied liverworts as secondary metabolites. Suganya Devi *et al.* (2012) also found strong positive correlation between total phenol and FRAP assay. Flavonoids having many hydroxyl group are very good scavengers of pyroxyl radicals (Robards *et al.*, 1999). Flavonoid content of the liverworts was positively correlated with the reducing power suggesting flavonoids group of phenolic compounds to be another active free radical scavengers.

Furthermore orthodihydic phenol in the present study showed positive correlation with metal iron chelating activity and α-amylase inhibitory activity (anti-diabetic activity). The inhibition of the activity of carbohydrate hydrolyzing enzymes, α-amylase and α-glucosidase is an important strategy in treating diabetes. Result of the study showed that orthodihdric phenols were involved in inhibition of enzyme α- amylase thus contributing to the anti-diabetic activity displayed by the studied liverworts. Steroids a group of secondary metabolites are low molecular weight compounds having many medicinal applications. In our case, steroid present in the liverworts was positively correlated with the metal chelating activity.

**6.5.2 Principal Component Analysis**

Principal component analysis (PCA) was done to further confirm relationships between the studied variables from the eleven liverworts species. The loading of the first principal component (PC1) accounted for 19.84% of the variance and second principal components (PC2) accounted for 13.59% of the variance. In PC1, phytochemicals content and reducing power of the plants were separated from the antioxidant and anti-diabetic activities of plants. Loadings of PC1 were phenol, flavonoids, cardiac glycoside, alkaloid content, reducing power, metal chelating, nitric oxide scavenging, superoxide scavenging, α-amylase inhibitory and α-glucosidase inhibitory activities. Phytochemicals like phenol, flavonoids, alkaloid and cardiac glycoside showed negative correlation with the $IC_{50}$ value of different antioxidant and anti-diabetic assays. This indicated that phytochemicals like phenol, flavonoids, alkaloid and cardiac glycoside present in the studied plants were responsible for free radical scavenging and α-amylase, α-glucosidase enzyme inhibiting activities. Ribeiro *et al.* (2013) also found a positive correlation between total phenolic content and antioxidant activity. Result of the PCA analysis showed positive correlation of phytochemicals with reducing power (FRAP assay). Kang *et al.* (2014) also found positive correlation between the flavonoids content and FRAP assay. Reducing power (FRAP) was negatively correlated with studied pharmacological activities suggesting that at higher metal ion concentration, reducing potential of plant results in better antioxidant and anti-diabetic activities. Loadings of PC2 were triterpene, resine, amino acid content and $ABTS^+$ scavenging activity as revealed from their squared cosine values. By PC2 triterpene, resine and amino acid were separated from

$ABTS^+$ scavenging activity suggesting the contribution of these phytochemicals on $ABTS^+$ scavenging property of liverworts.

## 6.6 SEASONAL CHANGES IN ANTIOXIDANT ACTIVITY AND PHENOLIC CONTENT IN LIVERWORTS

Life strategies of bryophytes are the system of co-evolved adaptive qualities. They can survive extreme environmental conditions and are considered as successful colonizers. Plants die when the relative water level becomes lesser than 20-50%. Selective plants, including bryophytes, dries up to 4-13% and can still survive and resurrects when favourable condition prevails. Hence they are referred as the desiccation tolerant plants. At the time of excessive dryness, there prevails a condition when there is little intracellular water content and reduction in metabolic activity resulting in irrevocable damage to lipids, protein and nucleic acids by the production of RS. Different physiological and chemical adaptations made by bryophytes help them to recover from water loss and resurrect under favourable environmental conditions. One of the important strategies for limiting damage in cellular membrane and organelles to a repairable level is the production of strong antioxidant defence system composed of enzymatic and non enzymatic mechanisms (Van Breusegem *et al*., 2001). Plant secondary metabolites are strong non enzymatic antioxidative machinery which helps them cope with different biotic and abiotic stresses (Dey and De, 2012).

Study of changes in antioxidant activity and phytochemical content of liverwort *M. paleacea* during rainy season (May to October) and dry season (October to March) showed that antioxidant activities increased significantly during dry season when the environmental conditions are not suitable for the bryophytic growth. This may be due to increase in enzymatic and non enzymatic defence mechanism against reactive oxygen species (ROS) produced under stressful conditions. The generation of reactive oxygen species during desiccation was also reported in vascular plant *Atrichum androgynum* by Mayaba *et al.*, 2002 and in algae *Scytosiphon arbuscula* by Burritt *et al*., 2002. In drought tolerant moss *Otoblepharum albidum* too desiccation stress resulted in sharp increase of $H_2O_2$ as result of enhanced production of ROS (Lubiana *et al.*, 2013). These drought induced ROS acts as a signal to induce antioxidant mediated defence system (Weisany *et al*., 2012).

Result of the present work showed that the $DPPH^{\cdot}$ scavenging activity of *M. paleacea* increased significantly during dry season in all extracts (heptane, diethyl ether, ethyl acetate, butanol, acetone) except methanolic extract. $ABTS^+$ scavenging potential was also found to increase in heptane, diethyl ether, ethyl acetate and acetone extracts during dry season; while methanol and butanol extracts of *M. paleacea* collected during rainy season showed better $ABTS^+$ scavenging activity. $ABTS^+$ scavenging potential of diethyl extract was similar during rainy and dry season.

Reductions of Free ferrous ions are important for protection against oxidative damage and lipid peroxidation through Fenton reaction. Result of present study showed that metal chelating potential of *M. paleacea* was increased significantly during dry season in diethyl ether, ethyl acetate and acetone extract. While heptane, methanol and butanol extracts showed better ferrous iron chelating activity during rainy season. Superoxide is a strong radical which leads to the generation of other RS like $H_2O_2$, $O_2^{\cdot-}$ and $OH^{\cdot}$ that are extremely reactive and capable of damaging bio-molecules of living system. Superoxide scavenging activity was also found to increase in heptane, diethyl ether, ethyl acetate and acetone during dry season. However all other studied free radicals scavenging activity increased during drought condition, the result of nitric oxide scavenging assay was rather contradictory. *M. paleacea* showed increased potential to scavenge nitric oxide during rainy season than dry season. Nitric oxide ($NO^{\bullet}$) is an important bio-molecule, but its sustained level is toxic to tissue and thus is required to scavenge from the body.

From the results of different antioxidant assays it was observed that among six solvents used for extraction of bioactive phytochemicals from *M. paleacea* during rainy and dry season, radical scavenging activities increased in all the extracts during dry season except in methanol and butanol. This result suggested that during drought stress there is an increase in the synthesis of phenolic compounds that provide crucial protection against oxidative damage caused by almost no metabolic activity due to water loss. This was evident from the result of phenolic compound screening where the phenolic content in all the extracts except methanolic extract increased during dry season. During rainy season higher phenol content in methanolic extract indicated the synthesis of polar phenolic compounds in higher concentration during rainy season imparting protection to the plants against reactive species. Flavonoid content was also found to increase during dry season. Flavonoid content increased significantly in heptane extract. This result indicated that flavonoids were synthesized in high amount by *M. paleacea* as a result of chemical adaptations during the unfavorable condition. Whereas, ortho-dihydric phenol content of plant was found to be present in higher concentration in *M. paleacea* during rainy season. This result suggests the less involvement of orthodihydric phenol compounds in imparting protection against reactive species to plants under stress.

## 6.7 BIOASSAY GUIDED PURIFICATION

Assay guided purification are usually implemented for the extraction and identification of principal active components from the crude extract. A large number of bioactive substances have been isolated from crude extract following assay guided purification. Panichayupakarananta *et al*. (2010) by antioxidant assay guided purification have isolated ellagic acid that is responsible for antioxidant activity displayed by the pomegranate peel. Similarly, through bioassay guided purification method Ejele *et al*. (2012) isolated three phyto-compunds from *Garcinia kola* having significant antimicrobial activity. Phytochemicals having anti-ulcer activity was isolated from *Cassia singueana* by Ode *et al.* (2011). Similarly, four novel cyclic bisbenzyl dimmers were isolated from liverwort *Blasia pusilla* by Yoshida *et al,* (1996). In this work also, an attempt was made to perform an *in vitro* antioxidant and anti-diabetic assay guided purification of bioactive substances from liverwort *Marchantia paleacea*. Plant contains a wide array of phytochemicals varying from polar to non-polar compounds. For isolating major groups of phytochemicals a sequential elution method can be used for separating lipids initially and more polar substances afterwards. In the present work, solvent from lower to higher polarity were sequentially used in the elution process. Lower to higher polarity solvents was also used by Jassbi *et al*. (2016) for isolation of bioactive phytochemicals. Two bioactive fractions showing high pharmacological activity and phytochemical content were finally selected and the phytochemical present in the active fraction was detected by using Gas Chromatography Mass Spectrometry.

### 6.7.1 GC-MS analysis and identification of phytochemicals

GCMS result of bioactive fraction obtained from *M. paleacea* in this study showed the presence of phytochemicals like phenols, flavonoids, terpenoids and alkanes. Thus the promising antioxidant and anti-diabetic activity of this plant can be attributed to these phytochemicals. Through spectrum analysis 2,6-bis (1,1-dimethylethyl)-4-ethyl phenol was found to be present in the bioactive fraction. The identified compound 2,6-bis(1,1-dimethylethyl)-4-ethyl phenol is a strong antioxidant generally used for food, cosmetics and pharmaceuticals. Antioxidant and anti-diabetic activities of compound 2,6-bis(1,1-dimethylethyl)-4 -ethyl phenol was earlier reported by Unnikrishnan *et al*. (2015). Plant polyphenols are important group of metabolites that have a number of beneficial therapeutic effects including their role as free radical scavengers. Researchers have found a positive correlation between the antioxidant activity and polyphenolic content of plants (Oki *et al*., 2002). Thus promising antioxidant activity shown by the purified fraction can be due the presence of compound 2,6-bis (1,1-dimethylethyl)-4-ethyl phenol in *M. paleacea*. Krishnan and Murugan (2014b) also reported the presence of 2,6-bis (1,1-dimethylethyl)-4-ethyl phenol in liverwort *Marchantia linearis*. This compound was also reported to be present in *Mesembryanthemum crystallinum* (Bouftira *et al*., 2007). Among identified phytochemicals, 7-hydroxy-2-phenyl 4H-1-benzopyran-4-one; 5-hydroxy-7-methoxy-2-phenyl 4H-1-benzopyran-4-one; 4',5,7 trihydroxy isoflavone and 5-hydroxy-2-(-4 methoxypheny) 4H-1-Benzopyran-4-one belonged to flavonoid group of compound which possess various activities such as anti-mitotic (Hadjeri *et al*., 2004), estrogenic (Vitale, 2013), antioxidant activities (Dowling *et al*., 2010) etc. Suba *et al*., 2004 from the result of their work suggested that flavonoids present in plants mainly contribute to its anti-diabetic activities. GC-MS analysis of *M. paleacea* confirms the presence of large amount of flavonoids which suggests that *M. paleacea* can be a promising source of pharmacologically active bio-molecules.

Terpenoids are most abundant and structurally diverse products of plants. This class of compounds have displayed a wide range of important pharmacological properties. Mass spectrometry of the most active purified fraction from *M. paleacea* had shown the presence of different kind of terpenoid compounds like Caryophyllene, Myrcene, Pimaric acid, Phytol and Palustric acid. Caryophyllene, also known as beta-caryophyllene is a natural bicyclic sesquiterpene which is a constituent of many essential oils of some aromatic plants. When assessed for its pharmacological properties caryophyllene was found to have an anticancer, analgesic properties (Fidyt *et al*., 2016), etc. Caryophyllene was also reported from liverwort *Marchantia convulata* (Xiao *et* al., 2006) which showed cytotoxicity against human liver and lung cancer cells. Presence of caryophyllene in bryophytes was also confirmed by Sorbo *et al*. (2004). Caryophyllene was also found to present in higher group of plants like *Polygonum minus* (Baharum *et al*, 2010).

Another constituent of purified fraction was myrcene which belongs to the class of organic compounds known as acyclic monoterpenoids and are reported to have anti-inflammatory properties (Jakovlev *et al*., 1979). Sonwa and Konig, (2002) also found the presence of myrcene in the essential oil of hornwort *Anthoceros caucasicus.* 4-methyl-1 -(1-methylethyl), 3-cyclohexen-1-ol, is a menthane monoterpenoids having a strong antioxidant activity (Chung, 2011). Phytol belongs to the class of organic compounds known as acyclic diterpene alcohol used as a precursor in the manufacturing of synthetic forms of vitamin K1 and vitamin E[1]. Phytol is a biologically active compound which were reported to have many pharmacological activities such as anti-nociceptive; antioxidant (Santos *et* al., 2013) etc. Presence of phytol was also reported from other mosses such as *Rhytidiadelphus triquetrus* and *Polytrichum commune* (Klavina *et al*., 2015). Other compounds present belonging to terpene family were pimaric acid and palustric acid. Pimaric acid is a diterpene which was recorded to have an antimicrobial (Ali *et al*., 2012), anti-atherosclerotic activity (Suh *et al*., 2012). Pimaric acid among the bryophyte family has been found to be present in high concentration in *Ptilium crista-castrensis* (Klavina *et al*., 2015). Pimaric acid was also isolated from *Aralia cordata* which showed strong anti-atherosclerotic activity. Palustric acid belonging to the organic compounds class diterpenoids are generally reported of having an anti-microbial activity (Savluchinske-Feio *et al*., 2006).

Another important bioactive compound found was 3-Eicosene, an aliphatic hydrocarbon. 3-eicosene was reported to have a strong anti-antioxidant activity (Adeosum *et al.,* 2013). Other compounds like 2-tetradecanoyl 2-Cyclohexen-3,6-diol-1-one; Methyl 2-(4-hydroxy-3-isopropylbenzoyl) benzoate; 7 (diethylamino) 2H-1-benzopyran-2-one; (17α)-estra-1,3,5(10),6-tetraene-3,17-diol, diacetate and 9-thiocyanato androstan-4-en,3,17-diol-11-one were also present in the purified fractions. These compounds are not reported to have biological activities till date. Thus from the GC-MS analysis of purified fractions FF-1 and FF-3 it can be interpreted that promising antioxidant and anti-diabetic activities displayed by the fraction were mostly due to the presence of bioactive phytochemicals belonging to class phenols, flavonoids, terpenoids and alkanes. The result of the bioassay guided purification of *M. paleacea* confirms that this liverwort can serve as a potent source of phytochemicals having many pharmacological properties.

### 6.7.2 Network pharmacology

*Marchantia paleacea,* a liverwort belonging to family Marchantiaceae has been used in many traditional medicines. It is used in treatment of hepatitis, in skin tumefacation, antipyretic etc. liverworts has been used as ethnomedicine worldwide. Recent advancement in scientific research have proved that plant belonging to this group have many important pharmacological properties. Present work aims to study the role of *M. paleacea* as anti-diabetic agent. In Liverwort only Marchantin C has so far has shown anti-diabetic activity (Harinantenaina and Asakawa, 2007). In the present work, *in-vitro* anti-diabetic activity guided column chromatography has lead to the isolation of 19 phytochemicals from *M.*

*paleacea* having potential anti-diabetic activity. After identifying of phytochemicals, the underlying mechanism of action at cellular level of these compounds was studied by network pharmacology. Network pharmacology is an important approach for drug discovery and development process. This approach involves data base mining, phytochemical-protein target – target disease relationship study and drug likeliness study. The therapeutic effect of phytochemical is correlated with the binding of these compounds to particular protein or nucleic acid target. Therapeutic target database (TTD) allows the user to find protein and nucleic acid targets of concerned protein sequence or drug structure. In total 81 human proteins was targeted by the 15 out of 19 phyto-compounds. No human protein target was found for the other five phyto-compounds. Further the diseases associated with the target proteins have been identified and classified into 22 broad categories according to ICD 10 codes. The highest number of diseases found to be associated with target proteins was those belonging to class malignant neoplasm. While second highest number of disease found to be associated with target proteins was endocrine, nutritional and metabolic diseases i.e. disease related with diabetes. To explore the involvement of isolated phytochemicals from *M. paleacea* in diabetes disease a tripartite network was constructed with phytochemicals, their protein targets and target disease. In total 20 protein targets: PPARG, VEGFA, AKR1B1, PTPN1, HMGCR, EGFR, HSD11B1, MIF, BCHE, GSK3B, ESR1, CYP19A1, AR, ALOX15, BACE1, DRD2, NTRK2, ESR2, ADORA2A and ALOX5 were found to be involved in endocrine, nutritional and metabolic diseases. CooLGen data mining of all diabetes targets ultimately identified 8 targets having high percentage of Heat and Centrality Betweenness value. Protein targets PTPN1, AKR1B1, PPARG, ADORA1, MIF, GSK3B, HMGCR and VEGFA were identified as the main targets of phytochemicals to regulate the diabetes. Among them, PTPN1, PPARG and GSK3B directly affect the insulin signalling pathway (KEGG ID: has04910, hsa04931).

In adipose and muscle cells, PPAR nuclear receptors and winged-helix-forkhead box class O (FOXO) are main transcription factors that regulate GLUT4 gene expression (Fig 6.1). Impaired regulation of GLUT4 gene is directly associated with type 2 diabetes mellitus. PPAR is also associated with the regulation of pro-inflammatory factors and increased level of free fatty acid. Effect of PPARG depends on the presence of ligands, kinases, cofactors and growth factors. In unliganded state, PPARG forms a heterodimer with retinoid X receptor (RXR) and binds to PPAR response elements (PPREs) of target gene (GLUT4). The PPARG-RXR heterodimer then binds with co-repressor and represses the expression of GLUT4 gene.

However, when ligand binds with PPARG, the corepressor will be replaced by a coactivator complex and leads to transcriptional activation of GLUT4. Thus, PPARG ligands have strong role in insulin responsiveness. PPARG is also associated with the regulation of pro-inflammatory factors. In the anti-inflammatory action, PPAR interferes with nuclear factor (NF) κB pathway and causes trans-repression of the concerned gene.

FOXO1 is a negative regulator of insulin sensitivity (Nakae *et al*., 2002). FOXO1 represses the transcription of PPAR-G1 or PPAR-G2 by repressing their promoter. Insulin completely or partially reverses the effect of FOXO1 by causing PKB (Protein kinase B) or AKT activation which leads to hierarchical phosphorylation of FOXO1 at three sites. FOXO1 cycles between the cytoplasm and the nucleus but mostly are localized in the nucleus. Phosphorylation by PKB causes its exclusion from the nucleus and de-repression of PPARG1 or PPARG2 promoter. PPARG are considered as an important target for anti-diabetic drug development. Therefore, PPARG agonists are clinically used as insulin sensitizers (Kletzien *et al*., 1992). Synthetic drugs like thiazolidinediones (rosiglitazone and pioglitazone) or glitazones are used to activate PPARG and decrease serum glucose level by decreasing insulin resistance in patients suffering from type-2 diabetes mellitus. In the present study, Peroxisome proliferators activated receptor gamma (PPARG) is targeted by phytochemicals, 5-hydroxy-2-(4-methoxyphenyl), 4-H-1 Benzopyran-4-one and 5 hydroxy-7-methoxy-2-phenyl, 4H-1-Benzoyran-4-one present in the studied liverwort. BindingDB (Binding batabase) has shown that these compounds have structural similarity with Bavachinin obtained from *Psoralea corylifolia*. Bavachinin has been reported to act as agonist to PPARG and improve glucose uptake by sensitizing insulin signalling pathway and AMPK activation (Lee *et al*., 2016). Thus, it can be interpreted that, 5-hydroxy-2-(4-methoxyphenyl), 4-H-1 Benzopyran-4-one and 5 hydroxy-7-methoxy-2-phenyl, 4H-1-Benzoyran-4-one present in *M. paleacea* might have therapeutic potential to activate insulin signalling pathways and cure type 2 diabetes. Thus, further detailed work can be focused to understand the series of events leading to interaction of these phytochemicals and PPARG receptor.

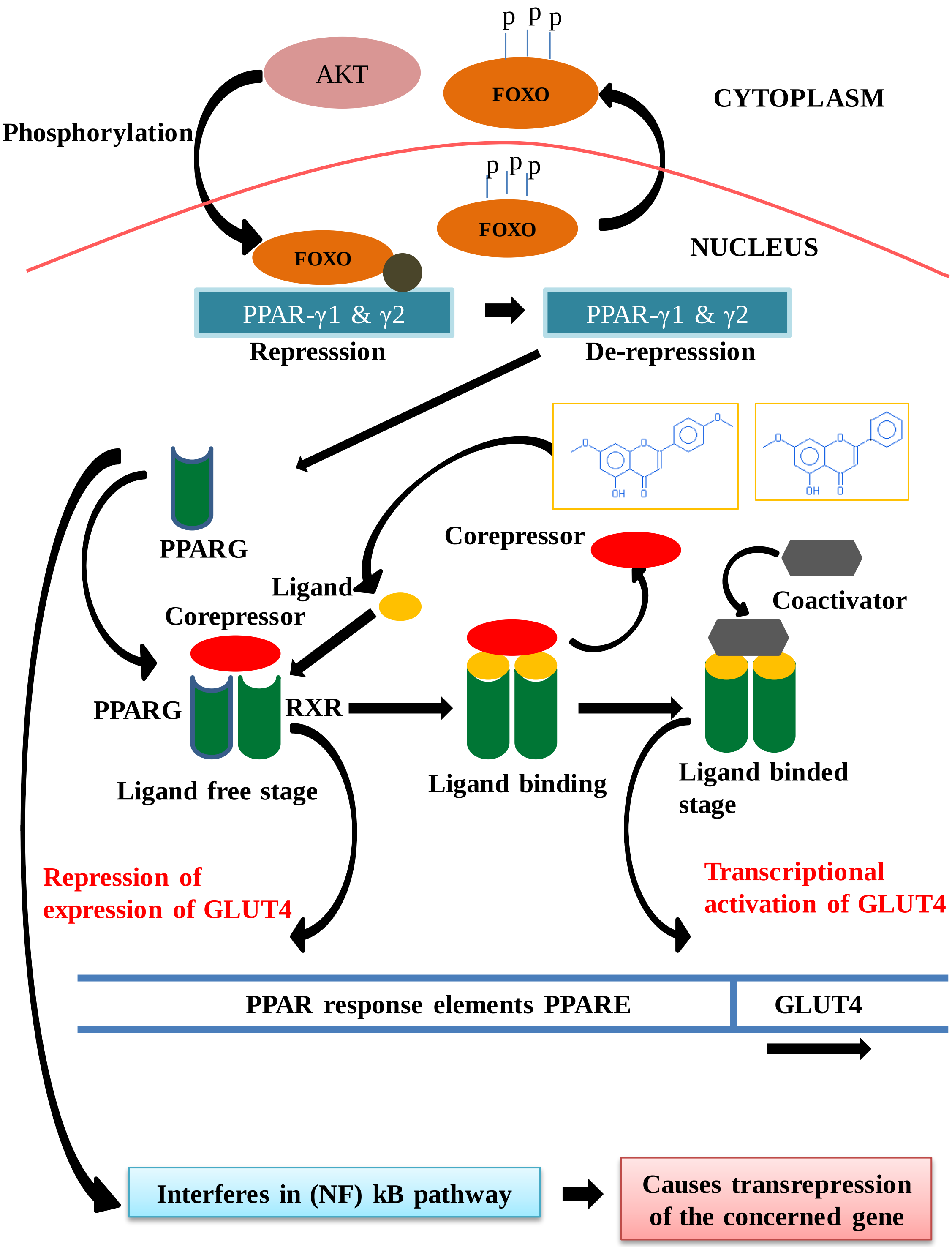


Fig 6.1: Assumed cellular effects of PPARG activation by 5-hydroxy-2-(4-methoxyphenyl), 4-H-1 Benzopyran-4-one; 5 hydroxy-7-methoxy-2-phenyl, 4H-1-Benzoyran-4-one

Glycogen synthase kinase (GSK3) is regulated by 4′,5,7-Trihydroxy isoflavone and 5 hydroxy-7-methoxy-2-phenyl, 4H-1-Benzoyran-4-one. GSK plays a key role in insulin resistance by regulating glycogen synthesis. GSK3 was identified as the key regulator in glycogen synthesis by Woodgett and Cohen (1984). GSK3 is a serine/threonine protein kinase that acts as suppressor of many signalling pathway when present in the active form (Fig 6.2). Key target of insulin signalling, glycogen synthase and insulin receptor substrate 1 are inactivated by GSK. Thus, GSK3 becomes an important target for therapeutic interference for insulin resistance. Kaidanovich and Finkelman (2002) reported GSK3B as the main drug target for diabetes type 2. Lappas (2014) reported the increase of GSK3β in adipose tissue and skeletal muscle in gestational diabetes. Result of network analysis showed two phytochemicals extracted from *M. paleacea*, 4',5,7-Trihydroxy isoflavone and 5 hydroxy-7-methoxy-2-phenyl, 4H-1-Benzoyran-4-one targets GSK3B. Natural drug targeting GSK3B are of high significance thus, further study on the interaction of phytochemicals and GSK3B can be useful to explore role of these phytochemicals in insulin resistance.

Another important protein target of phytochemical isolated from *M. paleacea*, involved in insulin signalling pathway was PTPN1. In the present study, tyrosine protein phosphatase non-receptor type 1 (PTPN1) was targeted by palustric acid and pimaric acid. PTPN1 is an enzyme that acts as negative regulator of insulin signalling pathway (Fig 6.2). PTP1B (protein tyrosine phosphatase-1B) encoded by PTPN1 inactivates insulin receptor (INSR) and insulin receptor substrate 1 (IRS1) by dephosphorylation and leads to insulin resistance (type 2 diabetes mellitus). Mackawy *et al.* (2015) found PTPN1 promoter variant 1023> A to be associated with type 2 diabetes patient. PTPN1 is considered another very important therapeutic target for drug discovery. Further work to explore the role of palustric acid and pimaric acid on PTPN1 gene expression can be of great significance.

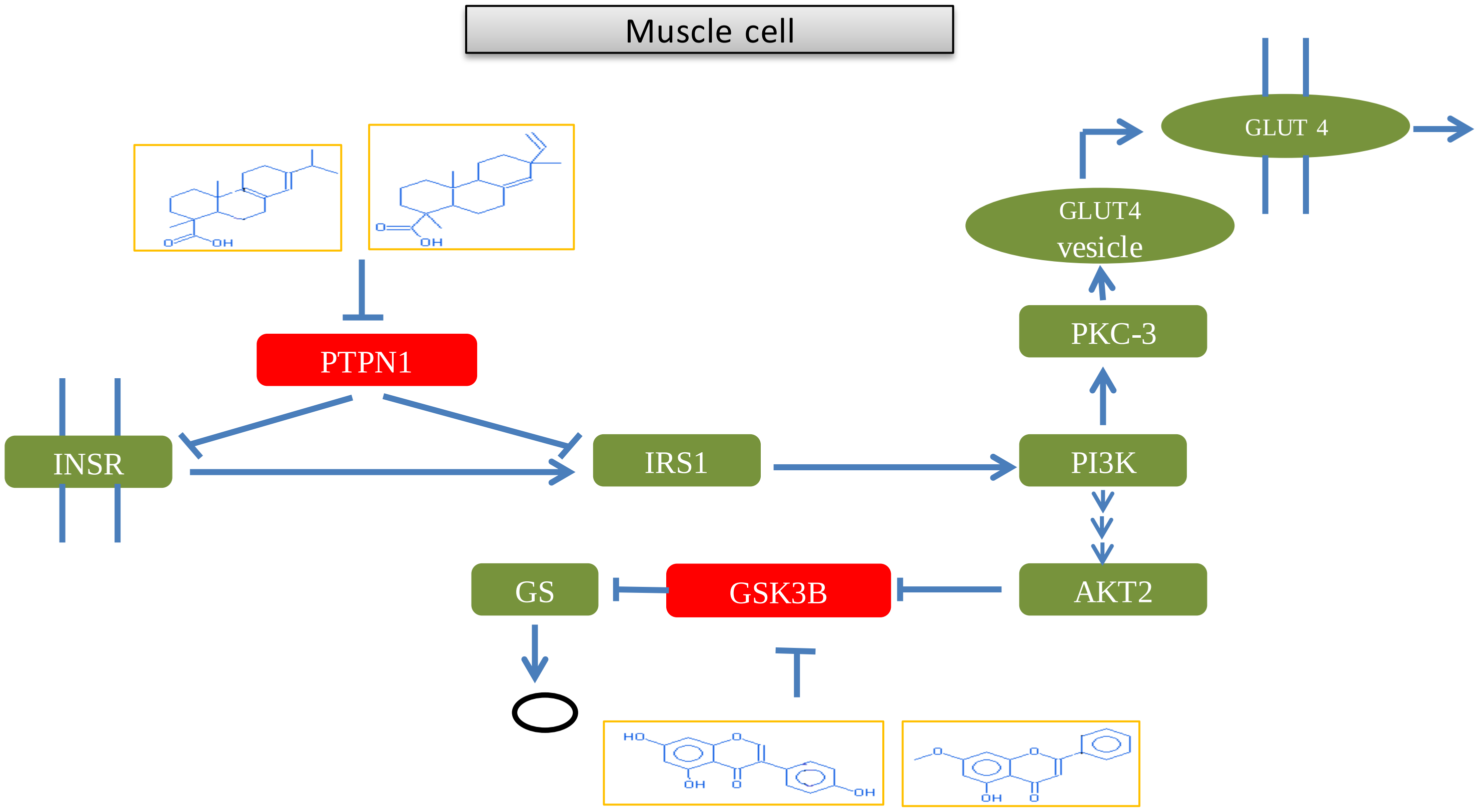


Fig 6.2: Assumed cellular effects of PTPN1inactivation by Palustric and Pimaric acid; GSK3 by 4’,5,7-Trihydroxy isoflavone, 5 hydroxy-7-methoxy-2-phenyl, 4H-1-Benzoyran-4-one

Adenosine receptor A1 (ADORA) causes diabetes retinopathy by blocking the activity of adenyl cyclase through regulatory Gi protein. Inactivated adenyl cyclase fails to activate protein kinase A (PKA) which has an important role in fatty acid degradation (Fig 6.3). Abrogation of ADORA1 might improve the metabolic disorder causing diabetes retinopathy. Attenuation of metabolic dysfunction by abrogating the adenosine mediated activation of ADORA1 was earlier reported by Yang *et al.* (2015). In the present study ADORA1 was found to be targeted by phytochemicals, 5-hydroxy-2-(4-methoxyphenyl), 4-H-1 Benzopyran-4-one and 5 hydroxy-7-methoxy-2-phenyl, 4H-1-Benzoyran-4-one.

Insulin resistance pathway has lead to the identification of five active phytochemicals namely, 5-hydroxy-2-(4-methoxyphenyl), 4-H-1 Benzopyran-4-one; 4',5,7-Trihydroxy isoflavone and 5 hydroxy-7-methoxy-2-phenyl, 4H-1-Benzoyran-4-one, palustric acid and pimaric acid present in *M. paleacea* that are important for curing insulin resistance in human. Druaggability analysis is important for drug discovery process. Estimation of ADMET properties is highly important for considering a drug candidate for further clinical trials (Waterbeemd and Gifford, 2003). Studied five phytochemical in *in silico* test showed high intestinal absorption percentage and Caco2 (human epithelial colorectal adenocarcinoma) cell permeability. Blood brain barrier permeability and Central Nervous System penetrating potential was also shown by the studied phytochemicals. *In silico* test for excretion found two phytochemical, 5-hydroxy-2-(4-methoxyphenyl), 4-H-1 Benzopyran-4-one and Palustric acid are found to be capable of acting as a substrate of Organic Cation Transporter 2 (OCT2) that helps in renal clearance of drugs. Toxic potency of a drug is an important parameter to consider for a drug. Toxicity was measured by Ames test, maximum recommended tolerated dose (MRTD), hERG I and II inhibitory activity and skin sensitization. Phytochemicals studied cleared the entire toxicity test. 5-hydroxy-2-(4-methoxyphenyl), 5 hydroxy-7-methoxy-2-phenyl, 4-H-1 Benzopyran-4-one, 5-hydroxy-2-(4-methoxyphenyl), 4-H-1 Benzopyran-4-one, and 4',5,7-Trihydroxy isoflavone showed no hepatotoxicity in *in silico* test while hepatoxicity was shown by pimaric acid and palustric acid. Druaggability of five phytochemicals was also analyzed by Lipinski's "rule of five" criterion. Studied drugs clearly passed Lipinski's "rule of five" criterion.

Information of drugs regarding diabetes was collected from DrugBank database and target of these drugs are collected from Therapeutic Database (TTD). On matching the synthetic drug targets from Drugbank and centralized protein targets, 1 taget i.e. PPARG was matched. So it can be concluded that PPARG is one of the main therapeutic target in diabetes.

Thus, as mentioned earlier agonist of PPARG can act as good candidate for increasing insulin sensitivity and curing type 2 diabetes. In this study PPARG was targeted by 5 hydroxy-7-methoxy-2-phenyl, 4-H-1 Benzopyran-4-one and 5-hydroxy-2-(4-methoxyphenyl), 4-H-1 Benzopyran-4-one. Thus, these phytochemicals can be explored further to find the entire mechanism by which these drug like molecules can activate the PPARG.

Therefore, this network assisted work has paved a new way to find the potential of liverworts in curing diabetes. Thus, this study will prove important for understanding phytochemical-protein level interaction that will eventually help in the identification of novel natural drug compound from liverworts against diabetes mellitus.

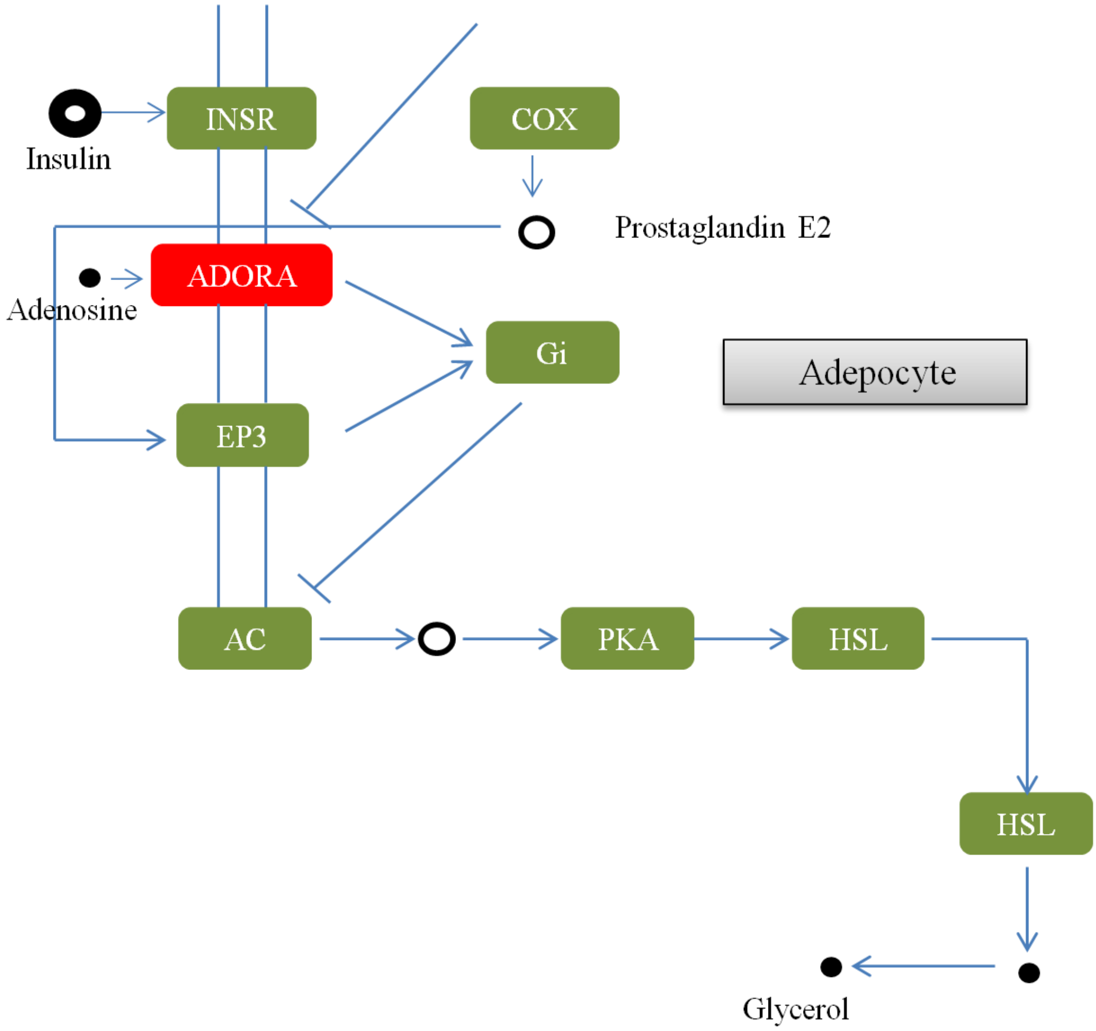


Fig 6.3: Assumed cellular effects of ADORA1 inactivation by 5-hydroxy-2-(4-methoxyphenyl), 4-H-1 Benzopyran-4-one; 5 hydroxy-7-methoxy-2-phenyl, 4H-1-Benzoyran-4-one

### 6.8 *IN VITRO* PROPAGATION AND COMPARISON OF BIOACTIVITIES OF AXENICALLY AND NATURALLY GROWN LIVERWORT *LUNULARIA CRUCIATA*

Research on the study of phytochemicals and pharmacological activities of bryophytes has been neglected for long in spite of being treasure house for large number of photochemical. Restriction in their study is mainly due to i) habitat preference allowing them to grow only within a selected geographic area ii) seasonal dependence for sample collection and iii) less availability in natural habitat. So, the aim of this work is *in vitro* propagation and evaluation of phytochemical and pharmacological activity of artificially grown and naturally grown *Lunularia cruciata*.

*In vitro* propagation of *L. cruciata* was initiated from gemmae which started germinating after 8-12 days of inoculation. Spores of moss *Erythrodontium julaceum* and liverwort *Marchantia linearis* also found to require the similar time period for germination (Awasthi *et al*., 2012; Krishnan and Murugan, 2014). Awasthi *et al*. (2013) found diluted nutrient medium to be most suitable for germination of the spores of endangered liverwort *Cryptomitrium himalayensis*. In present work different culture media such as Gamborg B-5, Knop, MS and diluted MS/2 were used for optimizing micro propagation of *L. cruciata*. Among the media used, germination of gemmae was successfully obtained in half strength Murashige and Skoog medium. Profound effect of growth regulators BAP and NAA was noticed on the growth and multiplication of thalli. Krishnan and Murugan (2014a) and Senarath *et al*. (2017) used 2 mg/L Benzylaminopurine (BAP) and 0.5 mg/L Naphthaleneacetic acid (NAA) for thallus and rhizoids differentiation from the spore of *Marchantia linearis*. In the present work, thallus and rhizoids differentiation from callus was initiated by supplementing basal salt media with 2 mg/L BAP and 0.5 mg/L NAA. Alteration of hormone concentration at later stage of growth and development was found to be helpful. Increased BAP (4 mg/L) and decreased NAA concentration (0.2 mg/L) restricted the excessive growth of rhizoids and restored normal growth and multiplication of the thallus in the present work. Alternation of concentration of growth regulators was also found to be helpful by other researchers in achieving proper growth and proliferation of bryophytes in *in vitro* condition. Krishnan and Murugan (2014a) achieved root induction by transferring the differentiating leafy thalli of *Marchatia linearis* in a rooting medium containing 2 mg/L Indolebutaric acid. This result highlighted the importance of plant hormones in controlling the growth and proliferation of plants in *in-vitro*. Young thalli then grew continuously to develop dichotomously branched fully grown thalli after 12 weeks.

*In-vitro* propagation provides the means for rapid multiplication of plants which helps to overcome various exertions related to isolation of phytochemicals from wild varieties due to factors like seasonal dependence, lower availability in nature, low rates of growth and over exploitation of wild varieties. However, there is a possibility that there occurs alteration in the phytochemical composition of naturally grown plants due to change in biochemical pathways resulting from artificial growth system (Senarath *et al*., 2017). Thus, comparison of phytochemicals in *in-vitro* and naturally grown plants are essential if the axenically grown plants are to be used as alternative to naturally grown plants (Manivannan *et al*., 2015). GC-MS analysis of methanolic extract showed the presence of ten compounds in naturally grown plants and nine compounds in *in-vitro* grown plants. Presence of similar kind of phytochemicals was found in naturally and artificially grown plants. Synthesis of similar kind of phytochemicals in naturally and naturally grown plants was also reported by Nikolova *et al*. (2013); Seranath *et al*. (2017). Alkaloids, flavonoids, terpenes, fatty acid, aliphatic hydrocarbon and acyclic alkanes were the phyto-compounds that had been detected in the studied plant extracts. Compounds like flavone, phytol, piperidine were common in both the plant extracts. While many other compounds were detected that differed slightly in structure, but belonged to same metabolite class and had similar retention time.

Ethyl tetradecanoate and cyclopentaneudecanoic acid present in *in-vitro* and naturally grown plants respectively were fatty acids detected in similar retention time (18.03 and 18.08 respectively). Alkaloid group was detected in both the extracts, in *in-vitro* grown plant alkaloid was present in the form of 1-1-butyl piperidine, while slightly modified ester form, 4-piperdineacetic acid, 1 acetyl 5 ethyl-2[3-(2 hydroxy ethyl)-1H-indol-2-yl]-a methyl; methyl ester was detected in naturally grown plants. Studies suggest that alkaloids present in plants have many medicinal properties like anti-microbial (Kumar *et al*., 2009), cytotoxic (Rinaldi *et al*., 2017), antimalarial activities (Wirasathien *et al*., 2006), etc. Apart from alkaloids, fatty acid esters was also found to be present, tricosane 2,4-dione was detected in *in-vitro* grown plants and 13,16-octadecanoic acid, methyl ester; cyclopentaneudecanoic acid, methyl ester; 13-decasenoic acid, methyl ester were obtained in naturally grown plants. Another class of compounds detected were terpenes; phytol was present in both extracts while thujopsene –[12] was found to be present only in naturally grown plants. Researchers while exploring plants for their medicinal properties found terpenes to be responsible for many important health beneficial activities like antimicrobial (Cardoso *et al*., 2012), anti-inflammatory (Khiev *et al*., 2011), anti cancer (Islam, 2017), cardiovascular and diuretic activity (Somova *et al*., 2001).

Flavonoid group was also found to be present in *L. cruciata* extract. Flavonoids like flavones were present in both extracts while 4H-1-Benzopyran-4-one, 3-hydroxy-2-phenyl was detected in naturally grown plant extract only. Flavonoids are another important class of phytochemicals in plants having many important activities like antioxidant (Pietta, 2000), hepatoprotective (Akachi *et al*., 2010), antibacterial (Cushnie and Lamb, 2005), anti-inflammatory (Serafini *et al*., 2010), anticancer (Zhang *et al*., 2008) and antiviral activity (Lani *et al*., 2016). GC-MS analysis has showed the presence of alkane only in *in-vitro* grown plants. Result of this study showed that the remarkable substances like phenols, terpenes and alkaloids that are present in naturally grown plants were also been synthesized in plants grown under artificial condition. *In vitro* grown *Fossombronia pusilla* has produced the same terpenoid group as is produced by its natural counterparts (Sauerwein and Becker, 1990). Different fatty acid, fatty acid ester, fatty acid alcohol and alkane have also found to be synthesized in *in-vitro* grown plants like their natural counterparts. This suggests possible application of *in-vitro* cultured plants for clinical validation, bioprospection studies and commercial exploitation of novel compounds through bio farming without over harvesting plants from their natural habitats. The presence of similar phytochemicals and biological activities in *in-vitro* and naturally grown plants were also reported by Kumari (2013); Vujcic *et al*. (2017); Senarath *et al*. (2017).

Liverworts are moisture loving plants with a restricted pattern of distribution growing mostly during rainy season under natural condition (Singh, 1999; Bhatacharyya, 2011); these drawbacks are responsible for their least use in research purposes. Also their low availability in nature is the biggest challenge for identification and isolation of biologically active phytochemicals in sufficient amount from these plants (Klavina *et al*., 2015). Our finding supports the potential of *in-vitro* grown plants to overcome the challenges of seasonal dependence and low availability of liverworts for structural elucidation of compounds and biological assay. Potential of *in-vitro* grown plants to be used in place of naturally grown plants has also been reported by other researchers, like two times increase in sesquiterpene was recorded by Otha *et al*. (1990) in cell culture of liverwort *Calypogeia granulata* and *Marchantia polymorpha*. Better production of effective phytochemical in artificial condition rather than natural habitat was also noticed by Sabovljevic *et al.* (2011).

Different health beneficiary properties shown by herbal medicines are due to bioactive phytochemicals present in the plant (Sahoo *et al*., 2013; Saravanam and Parimelazhagan, 2014). Presence of many phytochemicals in naturally and *in-vitro* grown plants were recorded through GC-MS analysis. In this work, antioxidative and anti-diabetic assays were performed to check if there are any biological activities of these phytochemicals, and if so, study was done to find out whether any change takes place in the activity of phytochemicals if grown in artificial habitat. DPPH is the stable organic nitrogen free lipophilic radical commonly used to investigate the free radical scavenging properties. Both naturally and *in-vitro* grown plant extracts has shown DPPH radical chelating property which might be due to the donation of electron or hydrogen atom to DPPH radical by phytochemicals (Shirwaikar, 2006) present in the studied plants. Naturally grown plants were recorded to have slightly better ($IC_{50}$= 0.45 mg/ml) DPPH radical scavenging activity than *in-vitro* grown plants ($IC_{50}$= 0.62 mg/ml). Slightly better DPPH activity of *in-vitro* grown plants was also reported by Mohan *et al*. (2011) in his work with *Bacopa monnieri*. In ABTS radical scavenging assay it was found that artificial condition of growth does not hinder the ABTS radical scavenging property, rather little improvement in the activity was noticed. Little improvement in free radical scavenging activity by *in-vitro* shoot extract was also reported by Manivannan *et al.* (2015). In living system, free ferrous ions are the powerful pro-oxidants which cause oxidative damage and lipid peroxidation by Fenton reaction (Saravanam and Parimelazhagen, 2014). It is important to scavenge such radicals from the body of a living organism. On testing the *in-vitro* and naturally grown *L. cruciata* extracts for metal chelating property we have found plant grown on both habitat have developed ability to scavenge ferrous ion. In this assay $Fe^{2+}$ and ferrozine forms complexes to produce hydroxyl radical, chelating effect observed might be due to interference of plant phytochemicals in the complex formation. Free radical scavenging property might be attributed to the presence of phenolic compounds like flavone, 4H-1-Benzopyran-4-one, 3-hydroxy-2-phenyl present in the extracts. Phenolic compound acting as radical scavengers has also been reported by workers like Wong *et al*. (2006); Tusevski *et al*. (2014). Apart from phenolic group, other bioactive metabolites like alkaloids, terpenoids identified might have contributed to biological activities.

Reduction in insulin sensitivity and postprandial hyperglycemia are the characteristics of type 2 diabetes (Mousinho *et al*., 2013). Lowering postprandial hyperglycemia can be an important measure to control diabetes. Postprandial hyperglycemia can be controlled by inhibiting the activity of enzymes α-amylase and α-glucosidase (carbohydrate hydrolyzing enzymes) (Ali *et al.,* 2006). *In-vitro* and naturally grown plants inhibited the activity of α-amylase and α-glucosidase enzymes to an impressive level. Betterment of α-glucosidase inhibitory activity was recorded *in-vitro* grown ($IC_{50}$ 0.24 mg/ml) plants in comparison to in-vivo grown ($IC_{50}$ 0.51 mg/ml) while, α-amylase activity was better in case of naturally grown plants (naturally grown: $IC_{50}$ 0.49 mg/ml; *in-vitro* grown: $IC_{50}$ 0.59 mg/ml)

Here we report more or less similar biological activity and presence of similar phytochemicals in *in-vitro* and naturally grown plants. All these results showing similar biological activity of in-vitro grown plants supported the objective of our work to use tissue cultured plants as a substitute for naturally grown plants to overcome the shortcomings restricting the use of liverworts for analytical purposes, despite of being the storehouse of many unique phytochemicals.

### 6.9.1 VARIATION IN THE ABUNDANCE OF EPIPHYTIC LIVERWORTS IN RELATION PHYSICO-CHEMICAL ATTRIBUTES

Bryophytes are integral part of forest ecosystem having strong functional relationship with many ecosystem processes. Darjeeling is home to large variety of epiphytic bryophytes. Nowadays increasing anthropogenic activities are posing serious threats to natural habitats of liverworts. They are generally considered as weed and are being ignored. Lack of proper knowledge and appreciation by the masses, including general botanists are serious threats leading to extinction of these plants. However, through facilities like ‘Bryophyte Gardens’, ‘Moss Houses and ‘Species Specific Sites’, liverworts can be familiarized to students, researchers and the common people, which may inculcate interest among them and help to reduce threats to this unique group of plants. Therefore study of habitat requirements of liverworts are important to consider for their conservation.

Study of growth and proliferation of epiphytes on trees occurring in more or less similar environment is a feasible method for analyzing the effect of physical and biochemical variables on proliferation of epiphytes. Several factors may account for epiphytic liverwort density on trees. The diameter at breast height (dbh) exerts a profound influence on distribution of epiphytic liverworts. Density of epiphytic liverwort increased with the increase in the diameter of the tree indicating positive correlation between two variables. Dbh is the indicator of habitat quality of epiphytes. Growth anomaly becomes more frequent and the bark texture becomes more fissured with the increase in dbh. Similar to the findings of present work, McGee and Kimmere (2002), Friedel *et al*. (2006) and Orjan *et al.* (2009) also observed an increase in epiphytic species with the increase in tree diameter. Positive correlation was found between dbh and the age of the tree by Lukaszkiewicz and Kosmala (2008). So it can be stated that old trees are most favoured site by epiphytic liverworts. Suitable substrate formation with the increase in tree age might have favoured the increase in density of liverworts. Exceptional occurrence of epiphytic liverwort on tree with much small bark area (40 – 48.3 inch) suggested that abundance might not be solely dependent on tree girth. This highlights complex interplay of role of different factors on distribution pattern.

Moisture is an important physical factor affecting species composition and diversity (Hettenbergerova *et al.*, 2013). Most epiphytic species of bryophytes are stenoecious demanding shady and highly humid condition (Friedel *et al*., 2006). Result of the present study also highlighted the importance of moisture in sustaining the diversity and density of epiphytic liverworts. Positive correlation between two variables shows significant impact of increasing moisture content on increasing density of epiphytes.

Most epiphytic liverworts suffer from abrupt exposure to radiations. Light condition exerted profound influence on the species density. Statistical analysis of data revealed an interesting fact about the correlation between light intensity and epiphytic liverwort cover. Liverwort abundance reduced dramatically above and below particular threshold limits of light. Growth of epiphytic liverworts was recorded in light intensity range 1900–4600 lux, above this range density of epiphytic liverworts decreased dramatically. It was also noticed that light of very low intensity also reduced liverwort abundance. Thus, it was inferred that shade to half shade condition was favoured by the studied epiphytic liverworts. Connection between richness of epiphytes and the pH of tree bark has been recorded by various authors (Mezaka and Znotina, 2006; Lobel *et al*., 2006). But in our study variation in epiphytic cover was not significantly characterized by the pH of the bark.

Apart from physical attributes, biochemical characteristics of tree bark might also affect the epiphytic richness and distribution. Probable role of plant secondary metabolites on the density of epiphytic liverwort cover was studied in present work. *C. japonica* exudes resin, the major components of which are terpenes. Resin protects plants from invading pathogens. So we studied possible effect of terpenes on epiphytic abundance. We have noticed an increase in the liverwort abundance with the decrease in terpenoid content of the trees, suggesting that the two attributes are correlated in a negative manner. Similarly, tree age and terpenoid content were found to be negatively correlated. Kim (2001) also noticed negative correlation between terpenes and age of the tree. Unlike pathogen, bryophytes do not invade the sieve or blast tissues of the tree; they remain attached to cork layers through rhizoids. Nevertheless they are subjected to bark allelochemicals that are protective against plant pathogens. This study therefore confirmed our hypothesis that bark phytochemical might have some allelopathic effect on epiphytic liverworts and the resistance action of *Cryptomeria japonica* against these liverworts might be due to higher accumulation of terpenoid compounds particularly at young age.

Phenolic group of phytochemicals are mostly involved in plant defense. However, we found that phenolic group didn't exert allelopathic influence on liverwort cover. Epiphytic abundance varied irrespective of the total phenolics content of the bark. Other researchers have noticed decrease in phenol content with the increase in tree age. In our study, no correlation was observed between the tree age and phenolic compounds. Various biotic and abiotic factors affect the composition and concentration of secondary metabolites of a plant species. Sites selected for analysis being natural, trees growing within this area may experience wide variation in biotic and abiotic stresses. Different environmental stresses experienced by the trees might have contributed for variation in the secondary metabolite content irrespective of age of the plant.

For better understanding of complex interplay of the roles of different physical and biochemical factors, Principal Component Analysis (PCA) and Heatmap analysis was performed. In Principal component analysis, variables were clustered in four groups, *viz.* Cluster A, B, C and D. It was noticed that the variables of cluster A were negatively correlated with the variables of cluster C. Variables like moisture, abundance and girth of the tree were closely related. With increase in diameter of the trees, the physical and chemical characteristics of bark changes continuously (Barkman, 1958). With the increase in tree size and age, the bark of the tree becomes more porous, absorbent and resinous facilitating the settlement of epiphytic species (Schumacher, 2000) and thus abundance increases with the increase in the phorophyte diameter. With the increase in tree age, also the bark composition changes and becomes more absorbent favouring luxuriant growth of epiphytes. Furthermore, cover of the tree layer and tree density are also important parameters affecting the moisture and thus the abundance of the bryophytes.

Heatmap test result interprets that high moisture content in air as well as substrate are required to ensure the occurrence and luxuriance of epiphytes. PCA and heatmap focuses on the significant impact of light condition on density of epiphytic cover. Abrupt exposure to light affects the species requiring consistent humidity and shady condition (Friedel *et al*., 2006). Moreover, tree density as a substitute for light condition, also affects the density of liverworts, as thinning of the forest after logging may lead to the exposure of species, demanding shady and humid condition, to radiation. Old growth forest stands containing large old trees, dead logs and canopy cover preventing exposure to sunlight are important for epiphytic growth and conservation. Light of higher intensity are found to be detrimental for moisture loving epiphytic species. Heatmap and PCA suggested that among plant secondary metabolites terpenes content of the plant has a negative impact on epiphytic growth. It can be interpreted that not only the favourable substrate developed with the increase in tree age favours epiphyte abundance but also the reduced terpene content with the increasing plant age is highly important for maintenance the epiphytic cover.

Senchal Wildlife Sanctuary, is one among the important tourist places of Darjeeling. Nowadays increasing tourist activity is imposing serious threat to the biodiversity of this area and chances are there that many plant species might disappear from this area unnoticed due to increasing anthropogenic activity. Bryophytes have always been neglected form the ecological as well as biochemical point of view as compared to other plant group. So, the result of the present study reports about the habitat preference of epiphytic liverworts in Senchal Wildlife Sanctuary. Dbh, moisture of tree bark were the important factors that influenced the abundance of epiphytic liverworts. In addition light condition was of great importance for the proliferation of epiphytic liverworts. Terpenoid content of the tree on which the liverwort was found to grow influenced the epiphytic liverwort abundance immensely. The terpenoid content was found to be inversely related to the age of trees suggesting old trees to be the preferred habitat of epiphytic liverworts. Thus, forest management or the responsible authority therefore should ensure the presence of old, large and rough barked trees to sustain the epiphytic liverwort cover in this area. Liverworts are moisture loving plants so care should also to be taken to maintain the tree density and canopy cover as abrupt exposure to light may prove lethal for epiphytic liverwort diversity and density.

### 6.9.2 VARIATION IN THE ABUNDANCE OF LIVERWORTS GROWING ON SOIL IN RELATION TO PHYSICAL FACTOR AND SOIL PROPERTIES

Determination of factors that controls the relative abundance of liverworts is of great significance for understanding their habitat preference. Studies have showed that nutrient supply rates can influence the abundance of species in communities. Much less is known about the role of physical factors and biochemical attributes of soil on liverwort species abundance. Thus in this study an effort was made to study role different environmental factors and biochemical attributes of soil on liverwort abundance. It was found that moisture content of the soil is very important for the sustenance and growth of liverworts. High moisture content is favourable for growth and proliferation of liverworts. Another important factor that was found to have significant influence on liverwort abundance was light intensity. It was observed that low light intensity was mostly preferred by the liverworts. Liverwort abundance decreased with the increase in the intensity of light. Among different biochemical properties of soil that has been analyzed, carbon to nitrogen ratio found to be positively correlated with the abundance of liverworts while organic carbon, organic nitrogen, available phosphorous, potassium and sulphur content of the soil didn't influence the abundance of liverworts in the present study. However, Virtanen *et al*. (2000) found positive correlation between the soil pH and the presence of liverworts but in the present study pH of the soil didn't influence the liverwort abundance. Thus, proper understanding of this plant group is necessary for its proper conservation.