3. Methodology

3.1. Materials

3.1.1. Glassware
All the glassware used, were of Borosil make and were sterilized prior to use.

3.1.2. Chemicals and culture media
Agar-agar, β-carotene, 6-Benzylaminopurine (BAP), Chloramphenicol, Czapek-Dox broth (CDB) medium, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4-dichlorophenoxyacetic acid (2,4-D), Folin-Ciocalteu (FC) reagent, Gallic acid (GA), Linoleic acid, Malt extract agar (MEA) Medium, Murashige and Skoog (MS) media, Potato dextrose agar (PDA) media, Sodium hypochlorite, Sucrose, and Tween 80 of HiMedia make were used. Methanol (analytical grade), Butylated hydroxytoluene (BHT), Chloroform, Ferric chloride (FeCl₃), Phosphate buffer, Potassium ferricyanide, [K₃Fe(CN)₆], Sodium carbonate (Na₂CO₃) and Trichloroacetic acid of Merck were used. Bavistin® of BASF India Ltd. was used. Double distilled water was used for preparation of solutions and other purpose.

Corn sand meal medium was prepared by mixing soil, sand and corn granules in proportion of 66:28:6 (Appendix-1) and sterilized in autoclave at 121°C for 6 h per day in three successive days.

3.1.3. Experimental Soil
A mixture of garden soil and sand in ratio of 7:3 with N, P, K and OC of 0.021, 0.0076, 0.017 and 2.03% was used for glass/net house experiments.

3.1.4. Instruments
All the media were used for isolation, cultivation, and storage of fungi were sterilized in an Autoclave (Caltor, India) at 121°C for 15 min and all glassware were sterilized in a hot air oven (Wieber, India) at 160°C for 2 hrs. Before autoclaving the pH of media were tested using pH meter (Eutech, Singapore) and were adjusted by buffers whenever
required. Experimental soil were sterilized by heavy duty Autoclave (NAT, India) at 121°C for 6 hrs per day in three successive days. The extract solutions were filtered through bacterial filter (0.45 μm) (Himedia) before use. The isolation and cultivation of fungi and experiments of tissue culture were carried out under aseptic condition in a laminar air flow (Saveer Biotech., India). The fungal cultures were incubated for requisite days in BOD incubator (Caltor, India) and seeds of test plants were germinated in a seed germinator (Seedburo Equipment Company, Chicago). The PDA slants of fungal cultures were maintained at 4 to 8°C in a refrigerator (Zenith, India). For extracts preparation mycelia were cultivated in Czapek-Dox liquid medium in an orbital shaker incubator (Metex®, India). During the extract preparation methanol was evaporated in vacuum using Evaporator (Equitron Roteva, India). While testing Total Phenolic Contents and antioxigenic capacity of extracts absorbance was measured by UV-visible spectrophotometer (ELICO SL-159 UV-Visible Spectrophotometer, India). The extract treatments were sprayed over the test plants using TLC glass sprayer (Merck India). Binocular microscope (Leica, Germany) was used for the micro-morphological studies of fungi and the images were clicked by Motic Digital Microscope (China). Electronic weighing balance (Anamed, India) was used for the weighing of chemicals, extracts and harvested plant materials. Fluorescent tubes (Philips, India) at an intensity of 2500 lux were used as light source in tissue culture room.

3.1.5. Test plants species
The seeds of Dalbergia sissoo and Withania somnifera were obtained from Rang Office, FRI, Dehradun, Uttarakhand, India. The seeds of Valeriana wallichii were collected from single plant in natural forest in Mussoorie Forest Division, Uttarakhand, India and identified by monographs, keys and available expertise. The seedlings of all test plants were prepared in Net-house. The brief description of these plants is given below:
i. *Dalbergia sissoo*: *Dalbergia sissoo* Roxb. (Family Fabaceae - Papilionoideae) commonly known as Shisham is an important multipurpose tree species of Northern India. It is native to the plains, low hills and mountain valleys of the sub-Himalayan region, however, it is cultivated and grown in plantations, in avenues, along canals, roadside, and field bunds in private lands, thus playing an important role in supplying the socio-economic needs of the growers (Harsh *et al.*, 2006). Shisham mortality is spread throughout its distribution zone, but more severe in eastern Bihar, Uttar Pradesh, Haryana, Punjab and West Bengal in India and the responsible pathogens are *Ganoderma lucidum* causing root rot and *Fusarium solani* f.sp. *dalbergiae* causing vascular wilt disease (Bakshi and Singh, 1959; Rajput *et al.*, 2008; Shukla *et al.*, 2008; Shukla and Harsh, 2010).

ii. *Valeriana wallichii* (Indian valerian): *Valeriana wallichii* DC (Family: Valerianaceae) is one of the most widely used herbal medicines. It is used as treatment of epilepsy and various other nervous disorders. It is native found in the Himalaya (India) although also distributed in damp woods, ditches, and along streams in Europe, and is cultivated as a medicinal plant, especially in Belgium, England, Eastern Europe, France, Germany, India, the Netherlands, the Russian Federation, and the United States of America.

iii. *Withania somnifera*: *Withania somnifera* (L). Dunal (Family: Solanaceae) commonly known as *Ashwagandha*, Indian ginseng, or winter cherry is a herb used in Ayurvedic medicine for number of diseases. Many people use this herb for general vitality, although the effects are not similar to *Panax ginseng* rather it causes relaxation. It is also used to treat a number of disorders that affect human health including central nervous system (CNS) disorders, particularly in epilepsy, prevent disease in athletes, stress and neurodegenerative diseases such as Parkinson’s and Alzheimer's disorders, tardive dyskinesia, cerebral ischemia, and
even in the management of drug addiction (Mishra and Singh, 2000). The most useful usage is to reduce stress and perhaps aid in sleep. However, high dosage or a concentrated extract may not effect as much. It was reported that *Fusarium solani* caused vascular wilt disease in this herb which results in water blockage and death (Bhandari, 2008; Sharma and Trivedi, 2010).

### 3.1.6. Fungal pathogen
The virulent strains of *Fusarium solani* 1149 was obtained from National Type Culture Collection, Forest Pathology Division, FRI, Dehradun, India.

### 3.2. Sampling
In the present study the key samples for antioxidant studies were endophytic fungi and mushrooms.

#### 3.2.1. Collection of mushrooms
Mushrooms were randomly collected from forest nearby Dehardun, Uttarakhand, India in paper bags and brought to laboratory. These were identified on the basis of monographs, keys (Christensen, 1966; Bakshi, 1971; Miller, 1979; Pegler, 1983) and available expertise and were isolated on PDA or MEA plates. Their pure cultures were maintained in slants of PDA at 4-8°C. Poisonous mushrooms were not considered for the study.

#### 3.2.2. Isolation of endophytic fungi
The leaves, stem, flowers and roots of healthy plants were brought in new paper bags and processed immediately in laboratory.

i. The plant samples were washed thoroughly under running tap water to remove adhering soil particles and the majority of microbial surface epiphytes. The surface of samples was decontaminated by the method described by Sita and Kevin (2004). For this plant materials were immersed consecutively in 96% ethanol (v/v) for one minute and then in 4% aqueous sodium hypochlorite for 10 min and finally in 96% ethanol for 30
sec and then rinsed thoroughly thrice with sterilized distilled water.

ii. The samples were dried under laminar airflow and with a sterile blade the outer tissues were removed and cut into small pieces \( \sim 1.0 \text{ cm}^2 \). Ten to fifteen pieces were placed on the surface of Malt extract agar (MEA) medium (appendix-I) in 90 mm bottom sized Petri plates supplemented with chloramphenicol (100 mg l\(^{-1}\)). For each sample 10 replicates were used. All plates were incubated at 25±1°C at 12h light/12h dark cycles for 6 days and were observed periodically for immersion of fungal mycelia.

iii. The Percent colonization frequency (CF) and Relative Dominance of fungal isolates were calculated by the formula given by Fisher and Petri (1987) mentioned below:

\[
\text{Colonization frequency (CF)} = \frac{N_{\text{col}}}{N_t} \times 100 \hspace{1cm} \text{.................. (23)}
\]

Where, \( N_{\text{col}} \) is Number of pieces colonized by specific fungus within a replicate, and \( N_t \) is total number of segments per replicate examined

\[
\text{Relative Dominance (RD)} = \frac{\text{No. of isolates of specific fungi}}{\text{Total no. of isolates}} \times 100 \hspace{1cm} \text{.... (24)}
\]

iv. Additionally, the host plants were also picked and after washing thoroughly under running tap water transferred in sterilized soil in Glass house. The fungal isolates were re-inoculated into their host plants and observed for any disease symptoms in subsequent days.

v. The fungal isolates causing disease in host plants were rejected and that which were re-isolated from healthy pants was considered as true endophyte.

vi. All the endophytic fungi were identified on the basis of monographs, keys (Gilman, 1957; Booth, 1971; Ellis, 1971; Barnett and Hunter, 1972) and available expertise and maintained on PDA slants at 4-8°C in a refrigerator.
3.3. Sample preparation from mycelia

Pure cultures of endophytic fungi and mushrooms were cultivated in CDB medium in conical flasks kept in rotatory shaker maintained at 25±1°C for 15 days. Mycelia of separate fungi were filtered through sieve and washed in sterilized distilled water thrice. These were later freeze-dried and crushed to prepare fine powder. Similarly, fruiting bodies of mushrooms were also freeze-dried and crushed to prepare fine powder.

3.4. Extracts preparation

It was suggested that polar constituents were rich in antioxidants (Vaskovsky et al., 1998) and hence, aqueous methanol was used for extract preparation from powder of mycelia and fruiting bodies.

i. Ten gram of pulverized fruiting bodies was mixed with 100 ml of aqueous methanol (80%) and stirred at 25±1°C and 130 rpm for 48 hrs. Similarly, 2 g of pulverized mycelia was extracted in 20 ml aqueous methanol. After 48 hrs both samples were filtered separately through Whatman No. 1 filter paper.

ii. The residue of each sample was then extracted with two additional portions of methanol and combined before evaporation.

iii. The individual extracts of fungi were evaporated separately using vacuum evaporator at 40°C to dryness.

iv. The yields were calculated after evaporation. The remaining residue from each extract was redissolved in methanol at a concentration of 100 mg ml⁻¹, and stored at 4°C for further use.

3.5. Determination of Total Phenolic Contents (TPC)

Total phenolic contents of all extracts were determined using Folin-Ciocalteu (FC) reagent as described by Singleton and Rossi (1965).

i. Different dilutions (0.001, 0.01, 0.1 and 1.0 mg ml⁻¹) of fungal extract were prepared in aqueous methanol.

ii. One ml extract of each dilution was transferred into a test tube and mixed thoroughly with 5.0 ml of previously diluted (10 times with distilled water) FC reagent.
iii. After 5 min incubation at 25±1°C, 4.0 ml of 7.5% Na₂CO₃ was added and allowed to react for 2 h at room temperature (25±1°C).

iv. The absorbance was measured at 740 nm using UV-vis spectrophotometer. Samples were taken in three replicates.

v. Standard curve of gallic acid solutions (25, 50, 75, 100, 125 and 150 ppm) was prepared using the similar procedure. Content of total phenolic compounds has been expressed as mg of gallic acid equivalent (GAE) per g of mushroom/mycelial extract (mg GAE g⁻¹).

3.6. Antioxidant capacity of extracts
The comprehensive methods were normally used for food grade materials (Prior et al., 2005; Apak et al., 2007). In the present study, DPPH scavenging assay was used because many authors (Alam et al., 2013) had agreed that it is the most easy, simple and reasonable cost effective method which can be used to make an outline of antioxigenic capacity of any kind of sample satisfactorily. The other methods i.e., reducing power and inhibition of β-carotene bleaching assay were also used. These methods were extensively used by various researchers (Table 2.4.).

3.6.1. DPPH radical-scavenging assay
DPPH Radical-scavenging method: This method was evidently introduced by Blois in 1958 (Molyneux, 2004) is a faster, sensitive and requires small sample amount and can be helpful in the investigation of novel antioxidants (Kulisica et al., 2004). The assay involves preparation of test sample in polar organic solvents e.g., methanol and allow to react with DPPH radicals at room temperature. The UV-spectrophotometer is used to following the decreasing in the absorbance of DPPH free radicals due to reduction by antioxidant (AH) or reaction with a radical species (R•) of at 517 nm (Brand-Williams et al., 1995).

\[
\begin{align*}
\text{DPPH}^+ + \text{AH} & \rightarrow \text{DPPH}^- + \text{A}^+ \quad \text{.......................... (25)} \\
\text{DPPH}^+ + R^• & \rightarrow \text{DPPH}^- + \text{R}^\cdot \quad \text{.......................... (26)}
\end{align*}
\]
The DPPH radical (Fig 3.1) react with antioxigenic phenol (equation, 25) but slow down the reaction with R* (equation, 26) which cause a progressive decrease in absorbance, so that the steady state may not be reached for several hours. However, standard time duration of 30 min is considered enough for testing a potential compound by most of the researchers (Table 2.4) for scavenging reaction. The data is commonly reported as EC$_{50}$, which is the concentration of antioxidant required for 50 % scavenging of DPPH radical in the specified time period. This parameter was apparently introduced by Brand-Williams and his colleagues (Brand-Williams et al., 1995). This is a most common method used for detection of antioxigenic capacity of a compound.

In the present study the hydrogen atom or electron donation abilities of the extracts were measured from bleaching of the purple-coloured methanol solution of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) by standard method of Mau et al. (2002) with few modifications.

i. In separate test tubes, 4.0 ml of various concentrations of extracts (0.2 to 2.0 mg ml$^{-1}$ of fruiting body extracts and 1.0 to 10.0 mg ml$^{-1}$ of mycelial extracts) were mixed with 1.0 ml of methanolic solution of DPPH radicals, resulting in a final concentration of 0.2 mM DPPH.

ii. The mixture was shaken vigorously and left to stand for 30 min at room temperature in the dark.

iii. The reduction of the DPPH radicals was determined by measuring the absorbance at 517 nm using UV-vis spectrophotometer.
iv. The DPPH radical scavenging capacity (DSC) was calculated as a percentage of DPPH discoloration using the equation:

\[
\text{% DSC} = \left( \frac{A_{\text{DPPH}} - A_S}{A_{\text{DPPH}}} \right) \times 100
\]

(27)

Where, \( A_S \) was the absorbance of the mixture solution when the sample extract has been added at a particular concentration and \( A_{\text{DPPH}} \) was the absorbance of the DPPH solution.

v. The Butylated hydroxytoluene (BHT) was used as standard antioxidant and was similarly tested. The assays were carried out in triplicate. For each extract the EC\(_{50}\) value (milligrams of extract per milliliter) i.e., effective concentration at which DPPH radicals were scavenged by 50% was calculated by probit analysis using software SPSS 16.0 (SPSS Inc.).

3.6.2. Reducing power assay

The reducing power (REP) of a compound is related to its electron transfer ability and may therefore serve as a significant indicator of its antioxidant activity (Oyaizu, 1986; Prior et al., 2005).

In this assay antioxygenic substances, which have reduction potential, react with potassium ferricyanide (Fe\(^{3+}\)) to form potassium ferro cyanide (Fe\(^{2+}\)), which then reacts with ferric chloride to form ferric ferrous complex (equation, 28) that has an absorption maximum at 700 nm. Depending on reducing power of an extract, the yellow color of test solutions become change into various shades of green and blue.

\[
\begin{align*}
K_3[\text{Fe(CN)}_6] &+ \text{FeCl}_3 &\overset{\text{Antioxidant}}{\rightarrow} K_4[\text{Fe(CN)}_6]\cdot3\text{H}_2\text{O} + \text{FeCl}_2 \\
(\text{Potassium ferricyanide}) & (\text{Ferric chloride}) & (\text{Potassium ferrocyanide}) & (\text{Ferrous chloride})
\end{align*}
\]

(28)

In the present study the ability of extracts to reduce FeCl\(_3\) was determined by the standard method of Pal et al. (2010) with some modifications.

i. In separate test tubes 2.5 ml of different dilutions (0.5 to 10.0 mg ml\(^{-1}\)) of extract was mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of potassium ferricyanide \([K_3\text{Fe(CN)}_6](1\%)\).
ii. The mixtures were incubated at 50°C for 20 min, after which 2.5 ml of trichloroacetic acid (10%) was added.

iii. The mixture was then centrifuged at 3000 rpm for 10 min. Two and half milliliters of supernatant of the mixture was taken and mixed with 2.5 ml water and 0.5 ml 0.1% FeCl₃.

iv. After allowing the solution to stand for 30 min the absorbance at 700 nm was measured to determine the amount of ferric ferrocyanide (Prussian blue) formed.

v. A higher absorbance indicates a higher reductive capability.

vi. A graph of absorbance v/s extract concentration was plotted and the extract concentration showing 0.5 value of absorbance (EC₅₀) was calculated. The BHT was used as a standard antioxidant and tested similarly.

3.6.3. **β-carotene bleaching inhibition assay**

Test for β-carotene bleaching inhibition: This assay method investigates the hydrogen donation ability of an antioxidant. In the absence of a suitable antioxidant the β-Carotene rapidly decolorized by linoleic acid free radicals (conjugated diene hydroperoxides) that are generated by abstraction of a hydrogen atom from one of its diallylic methylene groups. These radicals oxidize the highly unsaturated β-carotene molecules and broken down in parts and subsequently lose its chromophore and the characteristic orange colour. The presence of different antioxidants can hinder the extent of β-carotene bleaching by neutralizing the linoleate free radical and other free radicals formed in the system.

In the present study the inhibition of β-carotene bleaching was determined by the method of Lillian *et al.* (2007) with some modifications.

i. A fresh solution of β-carotene was prepared by dissolving 2 mg of β-carotene in 10 ml of chloroform.

ii. Two millilitres of this solution were pipetted into a round-bottom flask and mixed with 40 mg of linoleic acid and 400 mg of Tween 80 as emulsifier and then chloroform was removed at 40°C under
vacuum. Followed by addition of 100 ml of oxygenated distilled water the flask was vigorously shaken for a minute.

iii. Aliquots of 4.8 ml of this emulsion were immediately transferred into separate test tubes and mixed with 0.2 ml of various concentrations of extract fruiting body/mycelia. The tubes were shaken vigorously and incubated for 2 h at 50±1°C in a water bath. In control tube methanol was used in place of sample whereas only methanol was used as blank.

iv. At the addition of the sample/control to the tube, the zero-time and after 2 h incubation absorbance was measured at 470 nm using a blank. The assays were carried out in triplicate. The bleaching rate (R) of β-carotene was calculated according to equation 29.

\[
R = \frac{\ln(a/b)}{t}
\]

Where, \( \ln \) is natural log; ‘a’ is absorbance at time \( t=0 \); ‘b’ is absorbance at \( t=120 \) min; and ‘t’ is time in min (120min)

v. The percent β-Carotene bleaching inhibition (INBCB) was calculated using the following equation:

\[
% \text{INBCB} = \frac{(R_{\text{control}} - R_{\text{sample}})}{R_{\text{control}}} \times 100
\]

Where, \( R_{\text{control}} \) and \( R_{\text{sample}} \) are the bleaching rate of β-carotene with control and sample, respectively

vi. The extract concentration providing 50% antioxidant activity (EC_{50}) was calculated by probit analysis using software SPSS 16.0 (SPSS Inc.). BHT was used as reference compound in similar way in place of samples.

3.7. Relationship between TPC and antioxidant activity
The statistical method i.e., correlation study was used to determine the relationship between TPC and antioxidant activity of the extracts.

3.8. Screening of fungal extracts
Among the extracts of mushrooms and endophytic fungi, the most potent extract(s) was/were screened on the basis of above tests.
3.9. Test for growth enhancing properties of extracts
Screened extracts were tested on *Dalbergia sissoo, Valeriana wallichii* and *Withania somnifera* plants for their growth enhancer properties i.e., effect on seed germination, seedling growth and callus induction and regeneration, respectively.

Five treatments were prepared from one extract in sterilized distilled water and applied on test plants in Completely Randomized Design (CRD) in three replicates. The treatments were:

- \( T_1 = \text{sterilized distilled water} \) (control)
- \( T_2 = 0.05\% \text{ aqueous solution of fungal extract} \)
- \( T_3 = 0.1\% \text{ aqueous solution of fungal extract} \)
- \( T_4 = 0.2\% \text{ aqueous solution of fungal extract} \)
- \( T_5 = 0.4\% \text{ aqueous solution of fungal extract} \)

3.9.1. Effect on seed germination
Effect of fungal extracts on the seed germination test was performed by the standard method of Kumar and Singhal (2009) with few modifications.

i. Five hundred healthy seeds of roughly equal size and shape of one test plant were selected and were surface sterilized with 1% sodium hypochloride solution for 5 min and washed thoroughly with sterilized distilled water.

ii. Five sets were prepared from each 100 seeds selected randomly, soaked in different treatment solution for 30 min and transferred aseptically on filter paper (Whatman No. 1) in 10 Petri dishes (each 7 cm) in diameter so that each dish contained 10 seeds. The paper was also soaked with 5 ml of solution of same treatment.

iii. All Petri dishes were incubated in a seed germinator (25±1°C), 70% humidity and continuous darkness.

iv. Seeds were considered germinated upon radicle emergence (at least 2 mm). Germination was determined by counting the number of germinated seeds at 24 h intervals over the 3 weeks period. The numbers of germinated seeds were recorded after
subsequent days and percent germination and germination index (GI) was calculated. The test was performed in triplicates.

### 3.9.2. Effect on wilt disease control

Effect of fungal extracts on wilt disease caused by *Fusarium solani* was tested by the standard method of Sakr and Arafa (2009) with few modifications.

i. The pathogenic ability to induce root rot and wilt in the selected plant species by virulent strains of *Fusarium solani* on test plants was confirmed according to Koch postulates.

ii. Roughly equal size and age (two months old) 75 healthy seedlings of one plant species were selected and their roots were washed thoroughly with tap water.

iii. In each treatment solution 15 seedlings were soaked for 30 min and after that immediately transferred in sick soil containing virulent *F. solani* (10⁴ to 10⁵ spores g⁻¹ of soil) in root trainers (150 ml capacity). The spores of *F. solani* were previously prepared in corn sand meal medium and mixed in soil.

iv. The soil of seedlings was treated with 50 ml of respective treatments immediately after transferring and after every 7th day. On same day leaves of seedlings were also applied with the foliar spray of treatments. All seedlings were observed for their survivals.

v. The experiment was performed in 3 replicates.

vi. TPC was also determined from the crude extracts of leaves collected randomly from seedlings on day first and after 7 weeks (from survived seedling(s)) similarly as mentioned in section 3.5. and compared with other untreated seedlings grown in sterilized soil.

### 3.9.3. Effect on seedling growth

Effect of fungal extracts on the growth of seedlings was tested by the standard method of Bhatia and Ashwath (2008) with few modifications.
i. Roughly equal size and age (two months old) 75 healthy seedlings of one plant species were selected and their roots were washed thoroughly with tap water.

ii. In each treatment solution 15 seedlings were soaked for 30 min and after that immediately transferred in sterilized soil in root trainers (150 ml capacity).

iii. Solutions of 5 treatments were sprayed over the respective sets of seedlings after transfer and at 7 days intervals. The soil of each seedling was also treated with 50 ml of respective treatment.

iv. All seedlings were observed for their growth.

v. The seedlings were harvested after 10 months and their height, collar diameter, No. of leaves, fresh and dry weight were recorded.

vi. In case of *Valeriana wallichii* 12 seedlings were used for one treatment and thus total 60 seedlings were used for the experiment. These seedlings were tested in root trainers of 240 ml capacity.

3.9.4. Effect on callus induction

Effect of fungal extracts on callus induction was performed by the standard method of Jain *et al.* (2008) with few modifications.

i. The healthy foliar explants were obtained randomly from a test plant species and sterilized initially immersing in aqueous solution of fungicide Bavistin® (Carbendazim WP; 1 % w/v) for 10 min and after washing with sterilized distilled water again immersed in aqueous solution of HgCl$_2$ (0.05%) for 10 min and then washed 4-5 times with sterilized distilled water in a laminar air flow.

ii. Using sterilized blade small segments (~ 1 to 1.5 cm$^2$ sized) of explants were made. Randomly 5 segments were immersed separately in each treatment solution for 30 min. In this experiment, treatment T$_1$ i.e., control was replaced with sterilized aqueous solution of plant hormone 2,4-D (0.2%).

iii. All the segments were transferred on the surface of MS medium containing sucrose (30 g l$^{-1}$) and agar (5.8 g l$^{-1}$) in separate test
tubes and were incubated in a culture room at temperature range of 26 to 30°C with 16 h light and 8 h dark periods in 24 h cycle. Cultures were maintained under fluorescent light having 2000 to 2500 lux intensity.

iv. Observations were made till the callus induction. Experiment was performed with 3 replicates for each treatment.

v. In case of *Valeriana wallichii* T1 contained additional plant hormone BAP (0.5%) with 2,4-D (0.2%).

### 3.9.5. Effect on regeneration of callus

Effect of fungal extracts on callus regeneration was performed by the standard method of Jain *et al.* (2008) with few modifications.

i. Previously cultivated active callus (one week old) from test plant species was cut into small pieces. Forty pieces ~ 1.0 g were selected and randomly grouped into 5 sets containing 8 pieces each.

ii. Each set of callus segment was immersed in respective treatment solution for 30 min at 25±1°C.

iii. All segments were then transferred on the surface of MS medium supplemented with agar (5.8 g l⁻¹), sucrose (30 g l⁻¹) and plant hormone 2,4-D (0.2%) in separate test tubes.

iv. The test tubes were incubated in a culture room at temperature range of 26°C to 30°C with 16 h light and 8 h dark periods in every 24 h cycle. Cultures were maintained under fluorescent light having 2000 to 2500 lux intensity.

v. Five tubes in each set of treatment were randomly selected and observed till the callus proliferation or up to 3 weeks. Weight ratio was calculated from the final fresh weight of proliferated callus to its initial weight. Remaining 3 tubes in each treatment set were observed till the browning and necrosis of callus appeared. Experiment was performed with 3 replicates for each treatment.

vi. In case of *Valeriana wallichii* MS medium contained additional plant hormone BAP (0.5%) with 2,4-D (0.2%).
3.10. Statistical analysis
The data were expressed as mean±SD values. Statistical analyses were performed using Statistica 7 (Stat Soft. Inc., Tulsa, USA). Pearson’s correlation analysis was used to determine the correlation between TPC and antioxidant capacity. The level of statistical significance was taken at P<0.05.