CHAPTER-3

MATERIALS AND METHODS

The present study entitled "Studies on genetic variability and bioactive molecules production by *Ganoderma* species" was carried out at Shoolini University of Biotechnology and Management Sciences, Solan, Himachal Pradesh, during the year 2009-2014. The plan of work and the methodology adopted are presented in this chapter and materials and methods are described under following heads:

3.1 Culture source/collection of *Ganoderma* isolates
3.2 Identification
3.3 Maintenance of pure cultures
3.4 Cultural characteristics
3.5 Analysis of genetic variability
3.6 Effect of various nutritional conditions on mycelial growth
3.7 Effect of various environmental parameters on mycelial growth
3.8 Assessment of antimicrobial activities of *Ganoderma* isolates
3.9 Estimation of polysaccharide content
3.10 Molecular approach to confirm the identity of selected isolate
3.11 Basidiocarp cultivation studies
3.12 Extraction and analysis of bioactive compounds
3.13 Evaluation of bioactivities
3.14 Statistical analysis

3.1 CULTURE SOURCE/COLLECTION OF *GANODERMA* ISOLATES

A total of twenty three *Ganoderma* isolates designated as GL1, GL2, GL3, GL4, GL5, GL6, GL7, GL8, GL9, GL10, GL11, GL12, GL13, GL14, GL15, GL16, GL17, GL18, GL19, GL20, GL21, GL22 and GL23 were collected during the period from March 2009 to March 2010 from different regions of Himachal Pradesh and Punjab (Figure 3.1). Six mycelial cultures of *Ganoderma* were procured from Directorate of Mushroom Research (DMR), Solan (Himachal Pradesh) and two cultures were procured from University of Horticulture and Forestry (UHF),

Nauni, Solan (Himachal Pradesh). Details of all the thirty one collected/procured isolates are depicted in Table 4.1.

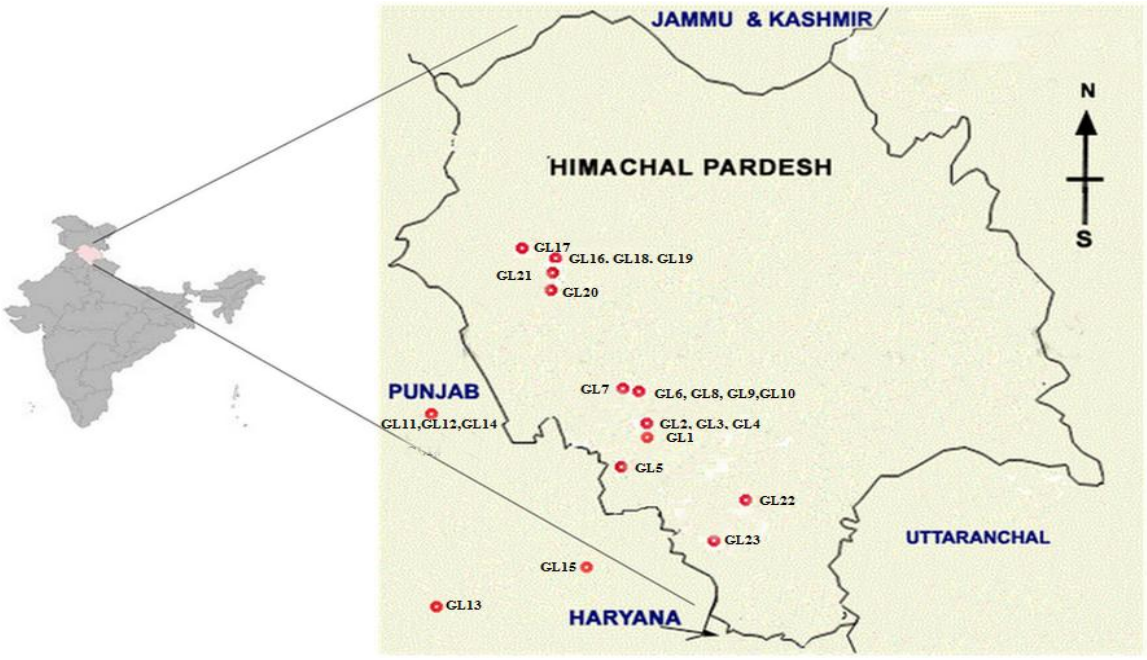


Figure 3.1: Map of Himachal Pradesh and Punjab showing different sampling sites

3.2 IDENTIFICATION

Collected samples were identified on the basis of macroscopic and microscopic traits of basidiocarp and cultural characteristics.

3.2.1 Macroscopic Traits

Various macroscopic traits (morphological features and dimensions) such as shape, size and colour of pileus, presence or absence of stipe, context colour, length and thickness of stipe of fresh fruit bodies were recorded for accurate identification of isolates.

3.2.2 Microscopic traits

Basidiospores size, colour and shape were also recorded with the help of projection microscope (Radical RXLr-4) (Van der Westhuizen and Eicker, 1994).

3.2.2.1 Spore collection and slide preparation

The spores were collected on clean filter paper by keeping the fruit bodies directly on it. After a thick deposition of spores, these were folded and then pinned into polythene bags labelled by different isolate identification codes and their microscopic study was done. Spore colour, length, width and spore index (length/width ratio) were determined for twenty spores of each specimen (Steyaert, 1980).

A clean and dry microscope slide was placed on a flat surface. One drop of spore sample (in 3% potassium hydroxide) was dispensed gently onto the slide. Grasped the cover slip gently by the edges with forefinger and thumb and placed one edge of the cover slip near the edge of sample on the slide. The cover slip over the sample with the edges in line with the edges of the slide was lowered gently. Air bubbles were removed by gently pressing on the cover slip. Blotting paper was used to absorb excess potassium hydroxide and flattened the wet mount slide by placing it at the edge of the cover slip. Spore slides were observed by bright field microscope (Radical RXLr-4) at 40X and 100X magnifications.

3.3 MAINTENANCE OF PURE CULTURES

3.3.1 Preparation of Culture Medium

Potato dextrose agar (PDA) was used to raise the pure cultures of isolates. Preparation of medium is given below:

About 250 g of good quality potatoes were washed with tap water, peeled off, cut into small pieces and boiled in 500-600 ml of water for about 15 minutes or till they were tender. The extract was obtained after filtration through a muslin cloth. Dextrose (20 g) was dissolved in the extract and the volume was made to 1000 ml. The final pH was adjusted to 5.0. Solidifying agent agar-agar (15 g) was then dissolved in the filtrate by heating. The hot medium was dispensed into clean test tubes to $1/4^{th}$ of their capacity, plugged with non-absorbent cotton and wrapped tightly with paper. These medium containing tubes were then sterilized by autoclaving at 15 psi pressure (121°C) for 20 minutes and were thereafter cooled and tilted on a support so as to get slants. The slants were kept at room temperature for 2 days and only contaminant free slants were used for culture maintenance.

3.3.2 Propagation of Cultures

All the isolates of *Ganoderma* were maintained on PDA slants. To raise a pure culture, a piece of fresh mushroom was taken from stipopileal region and dipped into mercuric chloride solution (0.1%) for 20-30 seconds for proper disinfection in order to avoid contamination and dried on sterilized filter paper. The dried pieces were placed on potato dextrose agar slants using flame sterilized, cooled inoculating needle in laminar air flow. These inoculated slants were incubated at 28±2ºC until substantial mycelial growth was seen. These mycelial cultures were used as inoculum for maintaining subcultures of isolates and for spawn production.

3.4 CULTURAL CHARACTERISTICS

Mycelial morphology (colour, density and type of growth), microscopic features (clamp connections, mycelia type) and chlamydospore shape and size were also examined after purifying the cultures on potato dextrose agar. Chlamydospore shape and size were examined for twenty spores of each isolate using lactophenol cotton blue as a stain.

3.5 ANALYSIS OF GENETIC VARIABILITY

Genetic variability among 31 isolates was characterized using RAPD primers based on polymerase chain reaction (PCR) technique and consists of the following steps:

3.5.1 Raising Mycelial Cultures

Pure cultures of all isolates of *Ganoderma* were raised in potato dextrose broth (PDB) for 6 days at 28±2ºC. These actively growing mycelial mats were then filtered through Whatman No. 1 filter paper and used for DNA extraction following methodology described by Vankan *et al.* (1991), with certain modifications in the protocol.

3.5.2 Isolation of Genomic DNA

Approximately 100 mg of mycelium was taken and ground in pestle and mortar using liquid nitrogen. A micro-centrifuge tube (1.5 ml) was filled 1/3rd with freeze dried mycelial powder. To the powdered mycelium in tube, 0.5 ml of freshly prepared prewarmed (65°C) extraction buffer (Appendix III) was added. Samples were mixed well and incubated at 65°C in

water bath for 30 minutes. Afterwards, samples were cooled on ice for 3-5 minutes. Then, an equal volume (0.5 ml) of phenol was added and mixed well and kept for 15 minutes at room temperature. To this mixture, 0.5 ml of chloroform: isoamyl alcohol (24:1) was added and mixed by inversion. Then the tubes were incubated at room temperature for 15 minutes and centrifuged for 20 minutes at 12,000 rpm. The upper aqueous layer was transferred to new eppendorf tubes. To the aqueous layer, 400 µl of chloroform: isoamyl alcohol (24:1) was added and mixed by inverting gently and centrifuged for 10 minutes at 12,000 rpm and transferred the supernatant to a new centrifuge tube. Then 0.54 volumes of isopropanol was added to precipitate DNA and incubated for 15 minutes and centrifuged for 10 minutes at 12,000 rpm. The pellet was washed with 100 µl of 70% (v/v) ethanol. DNA pellet was resuspended in 300 µl of ammonium acetate and incubated overnight at 4ºC. Next day, DNA was precipitated by adding 600 µl of ethanol and centrifuged at 10,000 rpm for 15 minutes at 4ºC, and supernatant was discarded. The pellet was washed with 300 µl of 70% ethanol. The pellet was dissolved in 50 µl of TE buffer (pH 8.0) (Appendix III). DNA samples were stored at -20°C.

3.5.3 Analysis of DNA

3.5.3.1 Assessment of quality and quantity of extracted DNA

The extracted DNA was assessed in terms of both quality and quantity. The quality of extracted DNA was checked by agarose gel electrophoresis. For this, 0.8 g of agarose was dissolved in 100 ml of 0.5X TBE buffer (45 mM Tris base, 45 mM boric acid, 1 mM EDTA) and the mixture was heated till agarose was completely dissolved, i.e., when solution becomes clear and transparent. It was cooled to 60°C with constant stirring and ethidium bromide solution (10 mg/ml) was added to a final concentration of 0.5 µg/ ml of buffer. The warm solution was poured into an already prepared gel mould, the comb was immediately clamped and the gel was allowed to solidify at room temperature for 35-40 minutes. DNA samples were prepared by mixing 2 µl DNA, 1 µl of 1X loading dye and 7 µl of distilled water. After the gel was completely set, the comb was removed carefully and the gel was mounted in the electrophoresis tank (Cleaver Sci. Ltd.). The DNA samples were then loaded into the wells with the help of micropipette and the DNA fragments were separated on gel by applying 50 volts for 45-60 minutes. The electrophoresis was stopped after the samples had run the required distance and the

gel was visualized under UV transilluminator. Using gel documentation system (AlphaImager® EP), the DNA bands were photographed under UV. The intensity of fluorescence of each sample was compared and the DNA concentration of each sample was ascertained. The quality of DNA samples was judged on the basis of whether the DNA forms a single high molecular weight band (good quality) or a smear (poor quality). Composition of the stock solutions is given in Appendix III.

3.5.3.2 Spectrophotometric quantification of DNA

The concentration and purity of the extracted DNA samples was determined by spectrophotometric method (Leninger, 1975). The instrument was first calibrated by placing 1 µl of sterile distilled water on the sample reading port with the help of micropipette. The values for optical density 260/280 nm and the concentration of DNA in samples were recorded. The purity of DNA samples was judged on the basis of value of optical density 260/280 nm. The DNA samples having ratio of 1.6-1.8 were regarded as pure while those having significantly less values were regarded as being contaminated with proteins or phenol (Hoisington *et al.*,1994).

3.5.3.3 Selection of primers

A set of ten decamer primers (Operon Technologies Inc. (Alameda, CA, USA) were first tested on one of the 31 samples. Out of these ten primers screened for amplification of DNA of *Ganoderma* species, four resulted in either sub-optimal or non distinct amplification products. Therefore, these were discarded and the remaining six primers (OPP 07, OPP 08, OPP 14, OPP 18, OPP 19 and OPP 20) which gave clear and reproducible products were used to amplify the template DNA of all the isolates. The list of six primers along with their sequences is given below (Table 3.1).

3.5.3.4 DNA amplification by polymerase chain reaction and fractionation of RAPD amplified products

Genomic DNA was amplified through polymerase chain reaction (PCR) using six RAPD primers. The PCR amplification was carried out in a total volume of 25 µl reaction mixture which contained 2.5 µl of 10X buffer (20 mM Tris-HCl, pH 8.0, 50 mM KCl) and 1.5 µl of 1.5mM $MgCl_2$, 2 µl of dNTPs (0.2

Table 3.1: Primers with their sequences used for RAPD

Sr. No.	Primer	Sequence
1.	OPP-07	GTCCATGCCA
2.	OPP-08	ACATCGCCCA
3.	OPP-14	CCAGCCGAAC
4.	OPP-18	GGCTTGGCCT
5.	OPP-19	GGGAAGGACA
6.	OPP-20	GACCCTAGTC
7.	OPP-09	GTGGTCCGCA
8.	OPP-10	TCCCGCCTAC
9.	OPP-16	CCAAGCTGCC
10.	OPP-17	TGACCCGCCT

mM each), 0.2 µl of Taq polymerase (5 U/µl), 2 µl of DNA template (20 ng), 1.0 µl primer (10 µM) and 15.8 µl of nuclease free Milli-Q water. While setting a PCR reaction DNA of each sample (20 ng/ µl) was added separately while all other components were mixed in a cocktail and then dispensed. All the components of PCR reaction were stored at -20°C. These components were taken out from -20 °C when used and were kept at 4°C in a mini cooler so as to thaw them. After thawing properly, a short spin was given to all the components. Then cocktail was prepared by adding calculated amount of water, buffer, dNTPs, and $MgCl_2$, primers and Taq polymerase in an eppendorf tube. 23 µl of the cocktail was added to each PCR tube. 2 µl of template DNA from each isolate was put separately in PCR tubes. The PCR tubes were then placed in a thermal cycler, appropriate PCR amplification was performed in thermal cycler (Labnet International) using temperature profile (Williams *et al.,* 1990) given in the Table 3.2.

Table 3.2: Temperature profile for PCR (RAPD)

Sr. No.	Steps	Temperature (°C)	Time (min.)	No. of cycles
1.	Initial denaturation	98	4	1
2.	Denaturation	95	1	45
3.	Annealing	35/37 *	1	
4.	Elongation	72	2	
5.	Final Extension	72	10	

* Varies with the primer used

3.5.3.5 Visualization of the PCR products

After amplification 5 µl aliquot of each resultant PCR product was mixed with 5 µl of bromophenol blue dye and electrophoresed on 1.5% agarose gel. PCR products were resolved by running at 50 volts. The gels were visualized under UV light and photographed using gel documentation system (AlphaImager® EP) with AlphaImager View software programme.

3.5.3.6 Scoring of RAPD bands

Band sizes were analysed by the position of bands relative to the ladder. Binary matrix was constructed based upon the presence or absence of RAPD fragments.

3.5.3.7 Estimation of genetic similarity and cluster analysis

In order to represent graphically the genetic divergence patters, the similarity matrix was submitted to a group analysis i.e., UPGMA (Unweighted Pair-Group Method Analysis), using the software programme MVSP version 3.22 and the similarity matrix value based on Jaccard's coefficient of similarity was used to generate dendrogram.

3.6 EFFECT OF VARIOUS NUTRITIONAL CONDITIONS ON MYCELIAL GROWTH

The aim of this study was to determine the best suitable medium for mycelial growth of *Ganoderma* isolates. Most vigorously growing isolates were taken for further study.

3.6.1 Preparation of Inoculum on Agar Plates

About 20 ml sterilized PDA was poured in each sterilized petri plate and was allowed to solidify. A mycelial bit was taken from the pure culture slants with the help of inoculating needle and was placed on the agar surface, in the centre of plate such that the mycelial end of the bit touches the surface of the medium. The entire process of pouring and inoculation was carried out under aseptic conditions. After inoculation, the sides of plates were sealed with parafilm and were incubated at 28±2°C in inverted position till the agar surface was covered with mycelia.

3.6.2 Effect of Different Liquid Culture Media on Mycelial Growth

Different liquid media, which are being widely used for studying growth pattern in different fungi, were used. All media were prepared as per the instructions given by manufacturers and their composition has been detailed out in Appendix I.

3.6.2.1 Media used

Liquid media used were Potato Dextrose Broth (PD), Richards' solution (RS), Sabourd's Broth (SB), Malt Extract Broth (ME), Czapek Dox Broth (CZ), Mineral Salt Broth (MS), Modified Melin Norkrans Broth (MN), Yeast Maltose Glucose Broth (YM), and Minimal Mineral Broth (MM). Prepared media were autoclaved at 121ºC and 15 psi pressure for 20 minutes. The pH of each medium was set to 5.0 with 1N HCl or 1N NaOH (as required).

3.6.2.2 Biomass growth

Fifty ml of each medium was dispensed into 250 ml Erlenmeyer flasks and autoclaved. Flasks were inoculated with equal number of mycelial discs of 8 mm diameter cut from actively growing culture plates of different isolates of *Ganoderma* and incubated at 28±2°C for 15 days in shaker incubator at 100 rpm.

3.6.2.3 Harvesting, drying and weighing of biomass

Mycelial biomasses were recovered by filtration through pre-weighed Whatman filter papers (No. 1) and the filter paper along with recovered biomass was dried in an oven at 60±1ºC until a constant weight was obtained. The mycelial dry weight was obtained by following formula:

Biomass = Weight of (filter paper + biomass) -Weight of filter paper (Karthikeyan *et al.,* 2007)

The dried biomass was stored in pre-sterilized screw-tight vials for future use.

3.7 EFFECT OF VARIOUS ENVIRONMENTAL PARAMETERS ON MYCELIAL GROWTH

The effect of various growth parameters such as temperature, pH and light on mycelial growth of *Ganoderma* isolates were investigated on best suited medium in above experiment, i.e., Malt extract broth. All following experiments were set in triplicates.

3.7.1 Effect of Incubation Temperatures

Fifty ml of Malt extract broth was added to 250 ml Erlenmeyer flasks. The pH of the medium was adjusted to 5.0 and autoclaved, after cooling the medium all flasks were inoculated with discs of mycelia (8 mm) cut from four days old culture plate of *Ganoderma* isolates. These flasks were incubated in temperature stress at different temperatures ranging from 20-40ºC at interval of 5ºC (i.e., 20, 25, 30, 35, 40°C) for 15 days in shaker incubator at 100 rpm. Then, the mycelial biomass yield was calculated as previously described under section 3.6.2.3.

3.7.2 Effect of Different pH Regimes

Fifty ml of Malt extract broth was added to 250 ml Erlenmeyer flasks. Medium was adjusted to different pH values (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0) by using 1N HCl and 1N NaOH (as required) and sterilized by autoclaving, after cooling the medium all flasks were inoculated with discs of mycelia (8 mm) cut from culture plates of *Ganoderma* isolates. All flasks were incubated at 30°C for 15 days. At the end of 15^{th} day mycelial biomass yield was calculated as previously described under section 3.6.2.3.

3.7.3 Effect of Light and Dark Conditions

Fifty ml of Malt extract broth was added to 250 ml Erlenmeyer flasks. The pH of the medium was adjusted to 5.5 and sterilized by autoclaving, after complete sterilization and cooling the medium, all flasks were inoculated with discs of mycelia (8 mm) cut from culture plates of *Ganoderma* isolates. One set of flasks was incubated in complete light condition and one set was incubated in complete darkness at 30ºC for 15 days. Then, the yield of biomass was calculated as previously described under section 3.6.2.3.

3.8 ASSESSMENT OF ANTIMICROBIAL ACTIVITIES OF *GANODERMA* ISOLATES

In-vitro antimicrobial susceptibility studies of alcoholic (methanol) extracts of the fruit bodies of *Ganoderma* isolates (collected from different regions of Himachal and Punjab) were performed against known pathogens.

3.8.1 Preparation of Methanol Extracts

The fruit bodies were cut into bits and dried at 40ºC in oven. These dried fruit bodies were pulverized in a blender. Ten gram of the pulverized fruit bodies was soaked in 100 ml of absolute methanol in conical flasks. This was covered with aluminium foil and allowed to stand for 7 days for extraction. The mixture was filtered using Whatman filter paper No. 1 and the filtrate was concentrated under a reduced pressure in a rotator evaporator until a semi solid substance was obtained. It was dried inside a crucible under controlled temperature (45ºC) to obtain solid extract. The left residues were kept in refrigerator until used (Jonathan and Fasidi, 2003).

3.8.2 Revival/ Maintenance of Lyophilized Culture of Test Organisms

Antimicrobial effect of the prepared extracts was studied against four human pathogenic bacteria procured from Institute of Microbial Technology Chandigarh; these include *Escherichia coli* (MTCC 739), *Staphylococcus aureus* (MTCC 737), *Klebsiella pneumoniae* (MTCC 109), *and Salmonella typhimurium* (MTCC 98). Environmental isolate of *Cryptococcus* species procured from Microbiology laboratory of Shoolini University was used as test organism among fungi. Lyophilized bacterial cultures were emulsified in normal saline and then spread on nutrient agar slants. These slants were incubated at 37±1°C for 18-24 hrs.

Bacterial and fungal cultures were sub-cultured and maintained on nutrient agar and sabouraud's dextrose agar slants, respectively. Media were prepared as per the manufacturer's instructions and was dispensed into sterilized test tubes to $1/4^{th}$ of their capacity, plugged with non-absorbent cotton and wrapped tightly with paper. These medium containing tubes were then sterilized by autoclaving at 15 psi pressure (121°C) for 20 minutes and were thereafter tilted on a support so as to get slants. Loopful of bacterial and fungal cultures were streaked on their

respective medium and incubated (bacteria at 37±1°C and fungus at 30±1°C). These cultures were stored under refrigeration.

3.8.3 Preparation of Inoculum

The bacterial inoculum used for testing the activity was prepared in nutrient broth. Five ml of broth was suspended in test tubes and autoclaved. The medium was cooled and inoculated with a loopful of bacterial cultures from the NA slants under aseptic conditions and then incubated at 37±1°C for 24 hrs. In order to standardize the inoculums density for test, a $BaSO_4$ turbidity standard equivalent to 0.5 McFarland (turbidity equals to 0.5= 1 to 2×10^{8} cfu/ ml) was used. Same procedure was followed for preparation of *Cryptococcus* species inoculum.

3.8.4 Agar Well Diffusion Assay

Muller hinton agar (for bacteria) and Sabouraud's dextrose agar (for yeast) plates were used for susceptibility tests. Cultures were swabbed on agar surfaces and wells were cut using sterile cork borer. Extract residues were reconstituted at 100 mg/ ml concentration using sterile distilled water. Sixty microliters of this concentration from each sample was dispensed in wells. Gentamycin (for bacteria) and amphotericin B (for fungi) were used as positive control and methanol was used as negative control. The plates were incubated at 37±1ºC (bacteria) and 30±1°C (*Cryptococcus* species); activity was measured in terms of zone of inhibition size (mm) obtained after 24 hrs (Jonathan and Fasidi, 2003).

3.9 ESTIMATION OF POLYSACCHARIDE CONTENT

3.9.1 Extraction of Polysaccharides

Polysaccharides were isolated from fruit bodies of all isolates by the method proposed by Pillai *et al.* (2008) with some modifications. Fruit bodies were dried and powdered using blender. 15 g of this fungal material was transferred to a 500 ml round bottom flask and 200 ml of distilled water was added to it. The round bottom flasks were fitted with condensers and samples were refluxed on a heating mantle at 100°C for 10 hrs. The refluxed samples were cooled and then filtered through Whatman filter paper No. 1. The filtrates obtained were used for

the extraction of polysaccharides while the residues left were discarded. The filtrates were concentrated to 100 ml by evaporation.

3.9.2 Precipitation of Polysaccharides

300 ml of chilled ethanol (4°C) was added to filtrate and left in refrigerator (4°C) for 24 hrs so as to allow them to precipitate. After precipitation, the samples were centrifuged in a refrigerated centrifuge (Eppendorf AG) at 4°C for 20 minutes at 6000 rpm. The residue so obtained was the first fraction of polysaccharides which was collected in a pre-weighed glass vial and stored at room temperature. The supernatant left after centrifugation was used for the extraction of another fraction of polysaccharides. Again equal volume of ethanol was added to the supernatant, kept in refrigerator for 24 hrs for precipitation and was then centrifuged at 4°C in a refrigerated centrifuge at 6000 rpm for 20 minutes. The residue so obtained was collected as the second fraction of polysaccharides in glass vials and stored at room temperature while the supernatant left after centrifugation was discarded.

3.9.3 Calculation of Percentage Yield

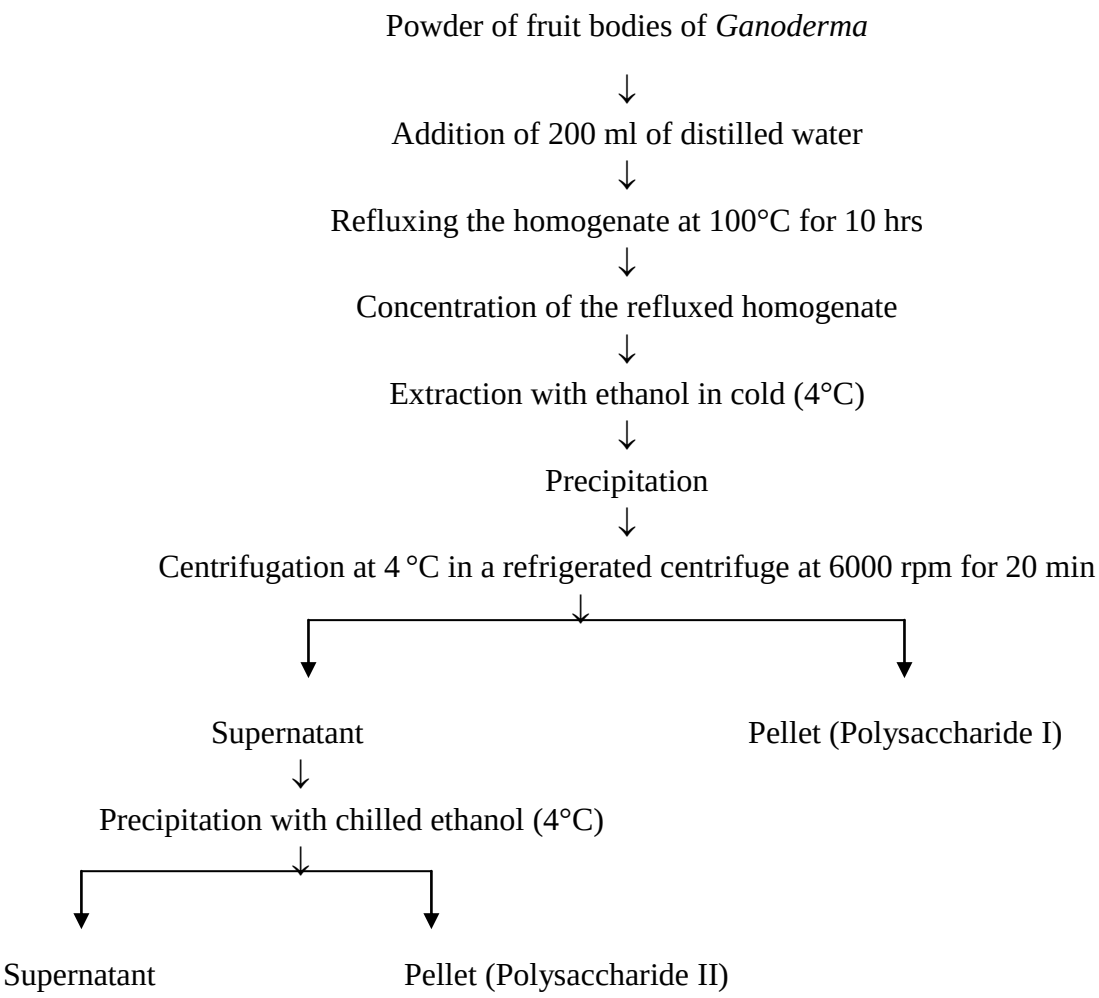
The percentage yield obtained in all the samples was then calculated by the following formula:

$$\%\ \text{yield} = \frac{\text{Amount of polysaccharide obtained after extraction (mg)}}{\text{Amount of sample used for extraction (mg)}} \times 100$$

3.10 MOLECULAR APPROACH TO CONFIRM THE IDENTITY OF SELECTED ISOLATE

It is evident from literature that traditional classification systems of *Ganoderma* based on morphological characteristics should be reviewed in light of molecular data. Therefore, the identity of isolate (GL4) with good antimicrobial activities and highest polysaccharide content was confirmed by amplifying and sequencing the 5.8S rRNA gene or Internal Transcribed Spacer (ITS) DNA (Park *et al.*, 2012), and chosen for further study. Composition of the solutions and buffers used for the extraction and purification is given in Appendix III.

Extraction process for polysaccharides of *Ganoderma* species:



3.10.1 Raising Mycelial Cultures

Pure culture of *Ganoderma* isolate (GL4) showing good antimicrobial activities and polysaccharide content was sub-cultured in potato dextrose broth for 6 days at 30±1°C. This actively growing mycelial mat was then filtered through Whatman filter paper No. 1 and used for DNA extraction.

3.10.2 Isolation of Genomic DNA

Total genomic DNA was extracted by following the methodology described by Vankan *et al.* (1991) with certain modifications, as described under section 3.5.2.

3.10.3 Analysis of DNA

The extracted DNA was assessed in terms of both quality and quantity, as described under section 3.5.3.

3.10.4 Gene Annotation

5.8S rRNA gene was amplified through polymerase chain reaction (PCR) using ITS 1 and ITS 4 primers (Park *et al.,* 2012). PCR reactions were performed in a 50 µl reaction mixture containing 1 µl of DNA (ca. 10 ng), 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 2.5 units of Taq DNA polymerase, 0.5 µM of each primer i.e., ITS1 (5'-TCCGTAGGTGAACCTGCGG- 3') and ITS4 (5'-TCCTCCGCTTATTGATATGC- 3'). DNA was amplified according to the following protocol depicted in Table 3.3.

Table 3.3: Temperature profile for PCR of ITS region

Sr. No.	Steps	Temperature (°C)	Time	No. of cycles
1.	Initial denaturation	94	5 min.	1
2.	Denaturation	94	30 Sec.	35
3.	Annealing	62	30 Sec.	
4.	Elongation	72	1 min.	
5.	Final Extension	72	5 min.	

3.10.5 Visualization of the PCR Products

After amplification resultant PCR product was visualized by method as described under section 3.5.3.5.

3.10.6 5.8S r RNA Gene Sequencing

PCR product was sent to Xcelris Genomics, Ahmadabad for sequencing.

3.10.7 Gene Annotation (Sequence analysis)

ITS sequences obtained were compared with other reported *Ganoderma* ITS nucleotide sequences in GenBank database using nucleotide BLAST (Basic Local Alignment Search Tool) search (accessible through NCBI (National Centre for Biotechnology Information).

3.11 BASIDIOCARP CULTIVATION STUDIES

Isolate which was identified and confirmed as *Ganoderma lucidum* (GL4) was taken for cultivation studies. Objectives of this section of study were to check feasibility of different cost effective alternative substrates, to find optimum spawn level and to evaluate the effect of different supplements at different rates. All the cultivation studies were conducted at the Department of plant pathology, UHF Nauni, Solan, Himachal Pradesh and at a private mushroom farm. It was a five step process which includes:

1. Spawn preparation
2. Substrate preparation
3. Spawning (inoculation)
4. Opening of bags
5. Harvesting

3.11.1 Spawn Preparation

Master spawn was prepared on wheat grains in glass bottles (500 ml capacity). Good quality wheat grains free from pests and moulds were selected for spawn production using standard technique.

Healthy undamaged, unshrivelled, unbroken and clean wheat grains were selected, washed with fresh water, put in open pan and boiled in water for 30-40 minutes (wheat grains: water, 2:1 w/v) so as to cook them soft enough to be pressed within fingers. Excess water was removed from the boiled grains through sieving and the grains were allowed to cool. Grains were then well mixed with calcium carbonate and calcium sulphate @ 4 and 2% (w/w) respectively, for adjusting pH (5.5) and to keep the grains loose. Empty glass bottles (500 ml) were filled with about 250 g of these grains, plugged with non-absorbent cotton, wrapped with paper strips and then sterilized by autoclaving at 22 psi for 90 minutes. These autoclaved bottles were allowed to cool overnight to room temperature and were thereafter shaken to restore transparent visibility of the glass. The bottles were then inoculated aseptically with mycelial bits from the slant cultures of *Ganoderma lucidum.* The bits were placed near the wall of the glass bottle in such a way that their mycelium end touched the grains and it is easy to check for the mycelium run. Thereafter,

these bottles were incubated at 30±1°C till the mycelia covered the grains. In between, after 5 days of inoculation period these bottles were once shaken so as to enhance uniform mycelial growth and as a check for contamination. The culture bottles thus produced were the "master cultures". After complete mycelial run 'master cultures' were shaken so that grains impregnated with mycelium were homogenously mixed and then grains were transferred aseptically to fresh grain bottles. These bottles were incubated and on complete mycelial run were used for spawning the substrate.

3.11.2 Substrate Preparation

Ten different substrates i.e., sawdust of sheesham (*Dalbergia sissoo*), mango (*Mangifera indica*), poplar (*Populus* species), pear (*Pyrus* species), oak (*Quercus*) and kikar (*Acacia nilotica*) and wheat straw, paddy straw, soybean waste and Lantana weed were used for the cultivation of *G. lucidum* because of their availability, suitability and economics. These agro-forestry wastes were procured from farmers of Himachal Pradesh and Punjab.

Substrate preparation includes setting pH, moisture content and sterilization. Substrate materials were stacked on a cemented floor and wetted thoroughly for 24 hrs to raise the moisture content to approximately 65%. Moisture was set with the help of palm test method (Kwon and Kim, 2004). A fistful of sawdust mixture was taken and squeezed tightly. Just a few drops of water were released with pressure which indicated that the substrate mixture had proper water content. Calcium sulphate (gypsum, approx. 2%) and calcium carbonate (chalk powder, approx. 4%) were added to keep the grains loose and get a pH of 5.5. Test substrate mixtures with a final moisture content of about 65% were filled in test tubes up to two-third capacities, and plugged with cotton to prevent possible contamination by airborne organisms while allowing air exchange. Tubes were autoclaved at 22 psi for 2 hrs and allowed to stand for cooling so that spawn can be mixed. In order to avoid use of large amount of substrate in plastic bag tests, preliminary tests were performed in test tubes to investigate the feasibility of these agro-forestry wastes as a substrate.

3.11.3 Spawning (inoculation)

Three-four grains of spawn were added to each test tube and incubated at 30±1°C under darkness. All the tubes were observed visually for checking mycelial run rate on different substrates.

3.11.4 Screening of the Substrates for Fruiting Behaviour

To avoid environment conditional defects, cultivation was carried out in a room where temperature, relative humidity and light could be precisely controlled.

3.11.5 Analysis of Best Cultivation Substrate

Best six substrates with maximum mycelial run rates were evaluated for most suitable substrate. Each treatment had 3 replicates resulting in 18 experimental units.

3.11.5.1 Substrate preparation and bag filling

Substrate was prepared following same methodology as described under section 3.11.2. After substrate preparation, substrates with a final moisture content of about 65% were filled into the heat resistant polypropylene bags (up to two-third capacities) each weighing 1 kg (350 g dry weight basis). Further, the bags were labelled and closed with PVC (polyvinyl chloride) rings and plugged with non-absorbent cotton. These were autoclaved at 22 psi for 2 hrs.

3.11.5.2 Spawning (inoculation)

The autoclaved bags were left at room temperature for one day for cooling. After cooling, the bags were inoculated @ 3% spawn level in laminar air flow, to avoid contamination. The work surface, inoculation room and gloves were disinfected with 70% ethanol. Spawn was mixed throughout the sterile substrate by shaking the bags properly (“thorough spawning”) as quickly as possible for secure sterile operation. The bags were then incubated at 30±1°C under darkness. Top of all inoculated bags were kept closed until the substrate in the bags was fully colonized. Days taken for complete spawn run were recorded. After complete colonization (thick mycelial sheet was developed), bags were shifted to cropping room.

Cultivation technique of *Ganoderma lucidum*:

Substrate materials
↓
Spreading of substrate materials on clean cemented floor
↓
Wetting overnight
↓
Mixing with supplements (4% $CaCO_3$ and 2% $CaSO_4$)
↓
Filling of substrate in test tubes/ polypropylene bags
↓
Autoclaving at 22 psi for 2hrs
↓
Cooling of substrate tubes/ bags
↓
Inoculation of substrates under aseptic conditions
↓
Incubation at 30±1°C in dark and approx. 65% humidity
↓
Completion of spawn run
↓
Opening of polypropylene bags
↓
Incubation at 28±1°C in light and approx. 90% relative humidity
↓
Pinning
↓
Cap formation with light exposure
(28±1°C, RH 80%)
↓
Cap development with light exposure
(25±1°C, RH 90%)
↓
Maturation
(28±1°C, RH 60%)
↓
Harvesting
↓
Drying (<45°C)

3.11.5.3 Opening of bags

Completely colonized bag tops were cut at the level of the substrate to allow the development of fruit bodies. Days taken for primordium initiation were recorded.

3.11.5.4 Cropping conditions

Inoculated bags were arranged on pest resistant shelves in a cropping room to attain the five stages of mushroom growth, i.e., spawn run, primordial initiation, stalk formation, pileus differentiation and cap maturation. The temperature and relative humidity were controlled at 30±1ºC and 65%, respectively, during the first stage (spawn run period was completed without artificial lighting); at 28±1ºC and 90-95% during the second stage with light exposure (10 hrs, provided by white fluorescent bulbs); 28±1ºC and 70-80% during the third stage with light exposure; 25±1ºC and 85-90% during the fourth stage with light exposure and 28±1°C and 60% during the last stage. Relative humidity was maintained by spraying clean water on the substrate bags and walls of the room 2 to 3 times daily (Fasidi, 2006) to minimize drying of the substrate surfaces.

3.11.5.5 Harvesting and calculation of biological efficiency

After complete maturation of fruit bodies, these were harvested by cutting at the base of stipe. The weight of fruit bodies from each bag was recorded. The biological efficiency (BE) percentage was calculated using formula:

$$\text{BE } \% = \frac{\text{Weight of fresh harvested mushrooms}}{\text{Dry weight of the substrate}} \times 100$$

Samples of fruit bodies were collected in polyethylene bags for further analysis.

3.11.6 Effect of Different Spawn Levels

Top three substrates with high fruit body yield (g/kg of wet substrate) and biological efficiency were taken for optimization of spawn level. Each treatment had 3 replicates resulting in 36 experimental units.

3.11.6.1 Substrate preparation

Substrate was prepared by same methodology described under section 3.11.2 and bags were filled as described under section 3.11.5.1.

3.11.6.2 Spawning (inoculation)

Spawning was done following same methodology described under section 3.11.5.2. In order to determine optimum spawn level, different doses of spawn were inoculated at 1, 2, 3 and 4% dry weight basis of mango, sheesham and poplar substrates under aseptic conditions.

3.11.6.3 Opening of bags

Bags were opened following same methodology described under section 3.11.5.3.

3.11.6.4 Cropping conditions

Cropping conditions were same as described under section 3.11.5.4.

3.11.6.5 Harvesting and calculation of biological efficiency

Crop was harvested and biological efficiency was calculated as described under section 3.11.5.5.

3.11.7 Effect of Different Supplements

Best three substrates with high fruit body yield (g/kg of wet substrate) and biological efficiency were taken for evaluation of effect of different supplements at different concentrations. Each treatment had 3 replicates resulting in 81 experimental units. The substrates without supplements were used as control in these experiments.

3.11.7.1 Substrate preparation and supplementation

Substrates preparation was same as described under section 3.11.5.2. To find a proper combination of medium and to assess effect of supplementation, sawdusts and supplements were mixed in different ratios (sawdust: supplement ratios, 90: 10, 80: 20 and 70: 30).

3.11.7.2 Spawning (inoculation)

Substrates were inoculated at optimum spawn level which has been obtained from the previous experiment i.e., 3 % level.

3.11.7.3 Opening of bags

Bags were opened following same methodology described under section 3.11.5.3.

3.11.7.4 Cropping conditions

Cropping conditions were same as described under section 3.11.5.4.

3.11.7.5 Harvesting and calculation of biological efficiency

Crop was harvested and biological efficiency was calculated as described under section 3.11.5.5.

3.12 EXTRACTION AND ANALYSIS OF BIOACTIVE COMPOUNDS

Polysaccharides are one of the major bioactive compounds of *G. lucidum*. The extraction of these biomolecules i.e., polysaccharides, was done from harvested fruit bodies of *G. lucidum.*

3.12.1 Extraction of Polysaccharides

Fruit bodies were powdered using liquid nitrogen in pestle and mortar. Polysaccharides were extracted by the method proposed by Pillai *et al.* (2008), described under section 3.9.

3.12.2 Analysis of Polysaccharides

3.12.2.1 Determination of total sugars in crude polysaccharides

Total sugars were estimated by phenol-sulphuric acid method of Sawetsuwannakun (2011) using glucose as a standard. Preparation of used reagents is given in Appendix IV. 3 mg of crude polysaccharide was dissolved in 1 ml of water. 0.5 ml of this was taken to glass tube. 0.5 ml of 5% phenol solution was added to sample and mixed by vortex mixer. 2.5 ml of concentrated sulphuric acid was added to it and incubated at room temperature for 10 minutes.

The sample was mixed by mixer and incubated for 20 minutes. The absorbance of mixture was measured at 490 nm using spectrophotometer. D-Glucose was used as a standard.

3.12.2.2 Determination of reducing sugar in crude polysaccharides

Reducing sugars were estimated by the DNS assay as described by Sawetsuwannakun (2011). 3 mg of crude polysaccharide was dissolved in 1 ml of water. 1.0 ml of this was taken to glass tube. 1 ml of DNS solution was added in the tube and mixed by mixer. The sample was boiled in hot water for 10 minutes. Then the sample was cooled down in cold water for 3 minutes. 10 ml of distilled water was added into the tube and mixed. The absorbance of mixture was measured at 540 nm in spectrophotometer. D-Glucose was used as a standard.

3.12.2.3 Determination of protein content in crude polysaccharides

Protein content was measured by the Bradford method as described by Sawetsuwannakun (2011). Three mg of crude polysaccharide was reconstituted in 1 ml distilled water in 1.5 ml eppendorf tube. 40 µl of this suspension was transferred to a test tube and 2 ml of Bradford reagent was added and mixed in a mixer. The absorbance of mixture was measured at 595 nm in spectrophotometer. BSA (bovine serum albumin) was used as a standard.

3.12.2.4 Determination of total phenol in crude polysaccharides

Total phenol content was tested by the Folin-Ciocalteu colorimetric method as described by Sawetsuwannakun (2011). Three mg of crude polysaccharide was reconstituted in 1 ml distilled water in 1.5 ml eppendorf tube. 0.5 ml of this suspension was transferred to a test tube and 0.5 ml of Folin-Ciocalteu reagent was added to it and incubated for 3 minutes. 0.5 ml of 7% Na_2CO_3 was added to the sample and mixed by vortex mixer. The distilled water was added to the tube to adjust the total volume to 5 ml. The tube was kept in dark for 90 minutes. The absorbance of mixture was measured at 725 nm. Gallic acid was used as a standard.

3.12.2.5 Fourier transform infrared spectroscopy (FTIR) spectroscopy

FTIR spectroscopy of *G. lucidum* polysaccharides was tested using FTIR Spectrometer (Agilent Cary-630) (Grosev *et al.*, 2001). 10 mg of polysaccharide sample was put on mirror

stage of spectrometer and spectrum was obtained by tightening the lens over sample. After analysis, the lens was cleaned to remove the sample completely.

3.12.2.6 Monosaccharide/oligosaccharide composition of *Ganoderma lucidum*

3.12.2.6.1 Acid hydrolysis of sample

Ten mg of the crude polysaccharide was put in a screw capped tube and 20 ml of 2M hydrochloric acid was added to it. It was kept at 105ºC for 8 hrs to hydrolyse the polysaccharides (Zhang *et al.,* 2002).

3.12.2.6.2 Paper chromatography of hydrolysate

The hydrolysate was analysed for presence of various sugars by paper chromatography and compared with standard sugars. A mixture of n-butanol, acetic acid and water in ratio 4:1:5 (n-butanol: acetic acid: water) was used as mobile phase and Whatman filter paper No. 1 sheet was used as a stationary phase.

3.12.2.6.3 Saturation of chromatography chamber

A mixture of n-butanol, acetic acid and water in ratio 4:1:5 (n-butanol: acetic acid: water) was put in chromatography chamber to a height of around 1-2 cm and chamber was closed tight so as to saturate it with vapours of mobile phase.

3.12.2.6.4 Loading samples on chromatogram

Whatman filter paper No. 1 sheet of about 18×27 cm was taken and folded 3 cm distant from lower edge and marked the equally spaced sample spots using pencil avoiding excessive touching of sheet and contact with dirty surfaces. The test sample in middle and standard monosaccharides at its sides was loaded using a fine capillary. Spots were dried immediately with the help of a hair dryer, so that spot diameter should not exceed 6-10 mm. Then put the sheet in saturated chromatography chamber, dipping the lower edge of sheet in solvent to about 0.5 cm. Solvent was allowed to move for around 4 hrs. Marked the solvent front immediately after run and dried chromatogram immediately at 105°C for 5 minutes. Sheet was dipped in dye aniline hydrogen phthalate to locate the spots/ develop the chromatogram. Dried the chromatogram for 5-10 minutes at 110°C and marked the spot positions immediately.

3.12.2.6.5 Identification of sample spots

Distance of solvent front and different spots was measured in 'cm' from start line and retardation factor was calculated for all spots using following formula:

$$\text{Retardation factor ('Rf')} = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

'Rf' values of sample spots were matched with standard spots. Two spots with similar 'Rf' values were similar. This way by matching the 'Rf' values of test sample spots with 'Rf' values of standards, various sugars were identified in sample.

3.13 EVALUATION OF BIOACTIVITIES

3.13.1 Measurement of Antioxidant Activity

Antioxidant activity of fruiting body extract, mycelial extract and polysaccharide sample of harvested *G. lucidum* (GL4) was evaluated using antioxidant activity (AOA %) by conjugate diene method, scavenging activity on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals and reducing power assay.

3.13.1.1 Antioxidant activity (AOA %) by conjugate diene method

The antioxidant activity was determined by the methodology of Lingnert *et al.* (1979). Various concentrations of samples were made ranging from 1 to 20 mg/ml in water. 100 µl of each extract was mixed with 2 ml of 10 mM linoleic acid emulsions in 0.2 mol/l sodium phosphate buffer (pH 6.5) in test tubes. All the tubes were properly marked with type and concentration of sample. All the tubes were placed in darkness at 37°C for 15 hrs to accelerate oxidation. After incubation for 15 hrs, 6 ml of 60% (600 ml/l) methanol in deionized water was added and the absorbance of the mixture was measured at 234 nm against a blank in a spectrophotometer. The antioxidant activity (AOA) was calculated as follows:

$$\text{AOA (\%)} = [(\Delta A_{234} \text{ of control} - \Delta A_{234} \text{ of sample})/\Delta A_{234} \text{ of control}] \times 100$$

An AOA value of 100% indicates the strongest antioxidant activity. EC_{50} value (mg extract/ml) is the effective concentration at which the antioxidant activity was 50% and was

obtained by interpolation from linear regression analysis. Butylated hydroxyanisole (BHA, Hi Media) was used as control.

3.13.1.2 Scavenging activity on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals

The potential to scavenge the DPPH free radical was determined following the method of Shimada *et al.* (1992). First made the dilutions of extract ranging from 1-20 mg/ ml. Took 4 ml of each dilution in test tubes and marked appropriately. To these tubes 1 ml of methanolic solution containing DPPH (Hi Media) radicals was added (final concentration of 0.2 m mol/ l $DPPH^{\bullet}$). The mixture was shaken vigorously and left to stand for 30 minutes in dark to obtain stable absorption values. Reduction of the $DPPH^{\bullet}$ radicals was determined by recording the absorption at 517 nm. The scavenging ability was calculated as follows:

$$\text{Scavenging ability (\%)} = [(\Delta A_{517} \text{ of control} - \Delta A_{517} \text{ of sample})/\Delta A_{517} \text{ of control}] \times 100$$

EC_{50} value (mg extract/ml) is the effective concentration at which $DPPH^{\bullet}$ radicals were scavenged by 50% and were obtained by interpolation from linear regression analysis. BHA was used as control.

3.13.1.3 Reducing power assay

The reducing power was determined according to the method of Oyaizu (1986). Various concentrations of samples were made ranging from 1 to 20 mg/ml in deionized water. 2.5 ml of each extract was mixed with 2.5 ml of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml of 10% (10 mg/ml) potassium ferricyanide. The mixture was incubated at 50 °C for 20 minutes. Then, 2.5 ml of 10% (100 mg/ml) trichloroacetic acid (w/v) were added, the mixture was centrifuged at 200 g for 10 minutes. Took 5 ml of the upper layer and it was mixed with 5 ml of deionized water and 1ml of 0.1% (1 mg/ml) ferric chloride. The absorbance of this mixture was measured at 700 nm against a blank in a spectrophotometer. A higher absorbance indicates a higher reducing power. EC_{50} value (mg extract/ml) is the effective concentration at which the absorbance was 0.5 for reducing power and was obtained by interpolation from linear regression analysis. BHA was used as control.

3.13.2 Comparative Evaluation of Antimicrobial Activities

The antimicrobial activity of fruit bodies and mycelial extracts and polysaccharide of harvested *G. lucidum* (GL4) was evaluated. Fruit bodies and mycelia were powdered using liquid nitrogen in pestle and mortar.

3.13.2.1 Extract preparation

Fruiting body and mycelial extracts were prepared using methanol as a solvent following same methodology explained under section 3.8.1 and polysaccharides were extracted following methodology explained under section 3.9. Extracts were reconstituted at 100 mg/ ml in dimethyl sulphoxide (DMSO).

3.13.2.2 Preparation of microbial inoculums

Test organisms were same as used previously (Section 3.8.2) and microbial inoculum was prepared following same methodology explained under section 3.8.3.

3.13.2.3 Agar well diffusion assay

Activity was determined by agar well diffusion assay (explained under section 3.8.4). 60 µl of reconstituted extracts (100 mg/ml) were dispensed in each well to determine the inhibitory activities of extracts.

3.13.2.4 Determination of minimum inhibitory concentration and minimum bactericidal concentration of polysaccharide extract

Minimal inhibitory concentration (MIC) of the polysaccharide fractions obtained from harvested *G. lucidum* fruit bodies for four reference strains, including *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae* and *Salmonella typhimurium* was performed by the micro-dilution broth method according to Skalicka-Wozniak *et al.* (2010). The stock solutions of polysaccharides fractions were prepared in dimethyl sulphoxide (DMSO).

The series of two-fold dilution of these stock solutions, ranging from 0.075 to 5 mg/ml, were prepared in Mueller-Hinton broth in 96-well micro-titre plates. Bacterial inoculum was added to each well to obtain final optical density corresponding to 5×10^5 CFU/ml. After

incubation at 37±1°C for 24 hrs, the MIC was assessed visually as the lowest concentration of polysaccharides showing total inhibition of bacterial growth. Appropriate DMSO, growth and sterile controls were carried out. Minimal bactericidal concentration (MBC) of polysaccharide fractions towards all bacterial strains tested was defined by sub-culturing 0.1 ml from each well that showed bacterial growth inhibition onto Mueller-Hinton agar plates. The plates were incubated at 35±1°C for 24 hrs and the MBC was defined as the lowest concentration of polysaccharides at which there was no bacterial growth. Each treatment was repeated in triplicate.

3.14 STATISTICAL ANALYSIS

All experimental treatments were performed in triplicates and analysis of variance (ANOVA) was performed using software programme COSTAT, to determine the significance of differences between different study groups.