

**STUDIES ON THE BIODIVERSITY, CONSERVATION, BIO ACTIVE COMPOUNDS IDENTIFICATION FROM SELECTIVE WILD MACROFUNGI COLLECTED FROM WESTERN GHATS OF TIRUNELVELI REGION, TAMILNADU, INDIA**

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**ABSTRACT**

Western Ghats of Tamilnadu contains varieties of wild macro fungi. Three selected wild fungi were collected from Kalakad Mundanthurai Tiger Reserve & Range in Tamilnadu to document their distribution and diversity. Gilled mushroom are short lived, abundant soon after the monsoon where as the Polyporus species. This study also envisages further study on the usefulness of most predominant three fungi are *Calocybe indica*, *Laetiporus sulphureus*, *Gleophyllum sepiarium* collected. Then Cultivation parameters, Optimization, Separation, Characterization and estimation of Mycochemicals by TLC and GCMS studies were analysed for selected wild fungus.

**KEYWORDS:** Wild macro fungi, TLC, GC-MS, Antioxidant analysis, Antimicrobial activity.

**INTRODUCTION**

Fungi are present almost everywhere, in a spectacular array of shapes, sizes and colours and performing a wide variety of different activities. In 1991 David Hawksworth, a mycologist estimated the world's fungal diversity at 1.5 million species (equal to the estimated number of all known other living organisms). This was thought at the time to be a radical over estimate, but now other researchers have proposed figures in excess of 13 million. Fungi perform essential roles in every terrestrial and many aquatic, ecosystems, eg. decomposing

dead organic matter to release nutrients, supporting plant life on poor soils by improving the absorption of nutrients when they form mycorrhizal associations with roots, living inside plants as endophytes.

*Calocybe indica* is a tropical domesticated edible mushroom; native to India was first described by Purkayastha and Chandra. It is also known as *dudh chatta* / milky mushroom because of its attractive milky whitish appearance with excellent shelf life and large sized basidiocarp with fleshy stipe and broadly adnate to decurrently gills. The dried sporophores of this mushroom contain 17.69% protein, 4.1% fat, 3.4% crude fiber and 64.26% carbohydrate. Mature sporocarp contains 4% soluble sugar, 2.95% starch and 7.43 % ash. In addition to this, it has most of the mineral salts such as potassium, sodium, phosphorus, iron, calcium and amino acid namely alanine, aspartic acid, glutamine, glutamic acid, glycine, hydroxyproline, histidine, lysine, threonine, tyrosine, valine, arginine and proline (Tripathi, 2005; Bhatt, 2007). Due to its alkaline ash and high fiber content it is highly suitable for the people with hyper acidity and constipation. It can be easily grown at a temperature ranged between 25-35°C and its cultivation can be best fitted in early cropping when no other mushroom can grown except *Volvariella* spp. at such a higher temperature. Keeping above importance in view different substrates as well as their combination were organically tested in present investigation to increase its biological efficiency.

In Ethiopia, wild mushrooms are used as both food and medicine by various ethnic groups in the country. The habit of eating mushrooms differs from region to region and among the different ethnic groups of the same region. However, no clinical studies have been conducted to validate the medicinal claims ascribed to this mushroom and no chemical compound (biomarker) unique to the mushroom has been identified as a possible agent responsible for the mushroom's metabolic effects. The field of metabolomics, aiming at global analysis of numerous targeted or non-targeted low molecular compounds (metabolites) in a biological sample, has recently found its application in diverse research areas, biomarker discovery, functional genomics (Saito & Matsuda 2010).

*Gloeophyllum sepiarium* (Rusty gilled polypore) is a wood decay fungus that causes a brown rot. *Gloeophyllum sepiarium* grow in thin, dark brown/green brackets on coniferous trees. Fruiting bodies can be seen throughout the year, but it sporulates in late summer to autumn. *Gloeophyllumsepiarium* is infrequent and inedible. Decomposing the deadwood of conifers across North America, *Gloeophyllum sepiarium* is fairly easily recognized. When fresh and

very young its cap is more or less orange, but as it matures brown colors replace the orange from the center outwards. Other distinguishing features include the brown flesh and the black reaction to KOH, as well as microscopic features.

## MATERIALS AND METHODS

**Study area:** Tamilnadu encompasses diverse hills and plains in Western Ghats. These areas are rather treasure of diverse flora and fauna. wild mushrooms were collected from different areas of Western Ghats areas like Mundanthurai and Karayar regions in Tirunelveli district. Latitude  $8^{\circ} 25'$  and  $8^{\circ} 53'$  N and longitude  $77^{\circ} 10'$  and  $77^{\circ} 35'$  E, about 45 km west of Tirunelveli. Equator with an annual rainfall of 3,097.8 mm, annual relative humidity of 78% and annual mean temperature of 30.6 degree C ). The preserved specimen and photographs of the mushrooms were taken for identification the mushrooms were identified morphologically Mushrooms have been collected from a few locations from various places of Western Ghats(KMTR) of Tirunelveli District.



**Fig. 1: Western Ghats (KMTR).**

**Collection and identification:** Regular survey and collection of macrofungi were carried out in Western Ghats of KMTR especially Karaiyar, Kannikatti, Kattalaimalai, Pandiyankottai, Karuthalipudavu, Palodai, Poongulam, Seyaru, Ullaru, Thulukamattai, Tunnel forest in the year of 2015. While collecting information with regard to the host, their habitat, colour of caps, associated features were also noted down carefully. The specimens were collected from different location from tree branches, moisture soil, and dead trees of forest. According to the differences we learn the mushrooms morphological characters from selected area.

### Macroscopic examination

Macroscopic characters of fresh specimens were noted after the collection. Photographs of the fresh specimens were taken both in the collection place as well as in the laboratory (Atri *et al.*, 2003; Kaviyarasan *et al.*, 2009). Colour Status, Overall shape, pileus shape from side, size, total height, head height, substrate attachment, habitat.

Each specimen was carefully labelled before transporting to the laboratory. The specimens were air-dried and stored in transparent boxes that were loosely kept to allow for proper aeration of the specimens. (Largent, 1986). Those specimen were stored in the lab for further study. Identification of the specimens was carried out by standard microscopic methods and also considering various morphological and anatomical features into account.

### Sample Collection

The selected two different species *L. sulphureus* and *Gloeophyllum sepiarum* were collected from Western Ghats of Tamil Nadu. Pure cultures were isolated from internal tissues into potato dextrose agar (PDA) and incubated at 30°C for 14 days. After incubation, the agar surface was fully covered with a white mycelium and the plate choosed for the spawn preparation further the plate stored at 40C.

### Spawn preparation

Sorghum is found to be the best substrates for spawnproduction (Purkayastha, *et.al.*, 1976; Krishnamoorthy, *et.al.*, 1997). Spawn was prepaired according to Pandey, *et.al.*, 2000.

### Substrate optimization

Most popular species under cultivation are naturally wood inhabiting fungi. So, growingon sawdust is a logical choice. Sawdust is mixed with wheat bran (or another nitrogen source) at 5% and Calcium Carbonate (CaCO<sub>3</sub>) (a buffering agent) at 2-3% and filled into autoclavable bags. The bags are sterilized for a minimum of 1 hour at 121°C (15 psi when at sea level). Note sterilization exposure times vary depending on pressure and elevation. Finally, the substrate is cooled to at least 25°C (approx. 80°F) then, inoculated with grain or liquid spawn and incubated at appropriate temp for the species until colonization is complete.

### Supplemented substrate used for cultivation

Four different types of substrates and agricultural wastes, namely sorghum, corn cobs, wheat grain and sawdust substrate, were used to determine which substrate is the best for cultivation

of *Calocybe indica*, *Laetiporus sulphureus* and *Gloeophyllum sepiarium*. The spawn media were prepared as described by Nwanze *et.al.*, (2005). The ingredients included 1 kg of cereal grain/sawdust, 12 g of  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ , 3 g of  $\text{CaCO}_3$ . The supplemented cereal grain/sawdust was divided into two types (Type 1: all ingredients were mixed with gypsum. Each cereal grain/sawdust type was washed and soaked overnight, water was drained off and boiled for 15 minutes, and left to cool down for 20 minutes. Fifty grams of each cereal grain/sawdust were filled into 4-ounce media bottles, autoclaved at  $121^\circ\text{C}$  for 15 minutes and left to cool. Before mycelia of *Calocybe indica*, *L. sulphureus* and *Gloeophyllum sepiarium* were inoculated, the cereal grain/sawdust bottles were shaken to prevent clump formation and to mix the mycelial discs with the substrate."Spawn bottles were incubated in dark condition at  $30^\circ\text{C}$  for 10 days. The linear mycelium length was measured every 2 days for 10 days to calculate the growth rate.

### **Cultivation conditions and harvesting**

The inoculated bags were incubated in a cultivation room and maintained at  $25\text{-}30^\circ\text{C}$  with relative humidity of  $85 \pm 5\%$ , for ramification of the mushroom mycelia. Growth of mushroom mycelium was recorded daily. When the spawn run was complete and pinheads started appearing, the bags were mouth opened to facilitate the development of fruiting bodies. As soon as the fruiting bodies developed and attained their full size, they were cut just above surface of the substrate with sterile sharp knife or blade. The harvesting was done in 4 flushes of 1 week intervals. After the 2nd flush, the substrate was turned upside down and regularly watered to harvest the 3rd and 4th flushes. The yields of mushroom were recorded.

### **Mycochemical analysis**

The selected mushroom extracts were subjected to qualitative chemical investigation to test for the presence of various phytochemicals in extracts.

### **Preparation of the mushroom extract**

Freshly-harvested whole mushrooms were shade dried and finely powdered. Twenty five grams of the powder were extracted with 250 ml of 95% solvents like methanol, ethanol, ethyl acetate, hexane and aqueous using Soxhlet apparatus. The residue was filtered and concentrated to a dry mass by vacuum distillation; the filtrate thus obtained was used as mushroom extract.

**Test for carbohydrates**

- **Molisch's Test:** To 2ml of extract 2-3 drops of alpha naphthalene solution in alcohol was added, shaken for 2 min and 1 ml of concentrated sulphuric acid was added slowly from the sides of the test tube. A deep violet colour at the junction of two layers indicates the presence of carbohydrates.

**Test for reducing sugars**

- **Fehling's Test:** Fehling's A and Fehling's B solutions, each 1ml were mixed and boiled, for 1ml and 2 ml of extracts were added heated in boiling water bath for 10 min, appearance of yellow and then brick red precipitate indicates the presence of reducing sugars.

- **Benedict's Test:** Equal volume (2ml each) of Benedict's solution and extracts were mixed in a test tube and heated in boiling water bath for 10min the changes in colour to yellow, green and red indicates the presence of reducing sugars.

**Test for monosaccharide**

- **Barfoed's Test:** Equal volume (2ml each) of Barfoed's reagent and extract were mixed in a test tube heated in boiling water bath for 2 min and cooled. The appearance of red precipitate shows the presence of monosaccharides.

**Test for pentose sugar**

- **Bial's Test:** To 1 ml of boiling bial's reagent, 1ml of solution extracts were added. The appearance of green or purple colour shows the presence of pentose sugar.

**Test for Hexose Sugars**

- **Seliwanoff's Test:** To 3 ml of seliwanoff reagent 1ml of fungal extract was added and heated on water bath for 2 min. The change in colour to red indicates the presence of hexose sugar.

- **Cobalt chloride Test:** To 3 ml of extract 2% cobalt chloride solution was added, boil or 2 min and cooled. To this 2-3 drops of sodium hydroxide solution was added and observed for the change in colour. The appearance of greenish blue it indicates presence of the glucose and purplish colour indicates the fructose are upper layer greenish blue and lower layer purplish indicates the mixture glucose and fructose respectively.

**Test for proteins**

- **Biuret's Test:** To 3 ml of extract 1 ml of 4% w/v sodium hydroxide and 1ml of 1% w/v copper sulphate were added. The change in colour of the solution to violet or pink indicates the presence of proteins.
- **Millon's Test:** To 3 ml extract 5 ml of millon's reagent was added and heated the appearance of white precipitate which changed to brick red on heating indicates the presence of proteins.
- **Xanthoprotein Test:** To 3 ml of extract 1ml of concentrated sulphuric acid was added. The appearance of white precipitate which turn to yellow on boiling and orange on addition of ammonium hydroxide (1ml) indicates the presence of proteins containing tyrosine tryptophan.
- **Ninhydrin Test:** To 3 ml extract 3 drops of 5% v/w lead acetate solution was added and boiled on water bath for 10 min. The change in colour of solution to purple or blue indicates the presence of amino acids.

**Test for Tannins and Phenols**

- **Ferric chloride Test:** To 3 ml of extract, 3 ml of 5% w/v ferric chloride solution was added. The blue – black colour indicates the presence of tannins and phenols.
- **Lead acetate Test:** To 3ml of extract, 3 ml of lead acetate solution was added. The occurrence of white precipitates indicates the presence of tannins and phenols.

**Test for Glycosides**

- **Legal test:** To 2ml of the extract, 1ml of pyridine and 1 ml of sodium nitro prusside were added. The change in colour pink or red indicates the presence of cardiac glycosides.
- **Keller-killiani test:** To the test tubes containing 2 ml of extract 1 ml of glacial acetic acid, 3 drops 5% W/V ferric chloride and concentrated sulphuric acid were added and observed, disappearance of reddish brown colour at the junction of two layers and bluish green in upper layer indicates the presence of cardiac glycosides.
- **Borntrager's Test:** To the test tubes containing 2 ml of extract 2 ml of dilute sulphuric acid was added, boil for 5 min and filtered. To the filtrates, equal volumes of chloroform was

added and mixed well. organic layers were separated and ammonia was added to this. Pinkish red colour of the ammonia layer indicated the presence of anthraquinone glycosides.

### Test for Saponins

- **Foam Test:** The extract (2g) was shaken vigorously with 20 ml of water and observed for persistent foam, which indicates the presence of saponins.
- **Haemolysis Test:** Sodium chloride (18% v/w, 2 ml) was shaken in six test tubes. The three test tubes 2 ml of chloroform, methanol and ethanol- water (8:2) were added, to the other three test tubes 2 ml of extract were added, few drops of blood was added to all the test tubes, mixed and observed for the haemolysis under microscope.

### Test for flavonoids

- **Shinoda Test:** To the dry extract (2g), 5 ml of ethanol (95% v/v), 5 drops of hydrochloric acid and 0.5g of magnesium were added. Appearance of pink colour indicates the presence of flavonoids.

### Test for Triterpenoids

- **Salkowaski Test:** To 2 ml of extract 5 drops of concentrated sulphuric acid was added, shaken and allowed to stand. Appearance of greenish blue colour indicates the presence of triterpenoids.
- **Libermann burchard Test:** To 2 ml of the test solution, 10 drops of acetic anhydride was added and mixed well. To this 5 ml of concentrated sulphuric acid was added from the sides of the test tube, appearance of greenish blue colour indicates the presence of triterpenoids.

### Test for Alkaloids

To the 10 g of dry extracts, 20 ml of dilute hydrochloric acid was added, shaken well and filtered. The following tests were performed using the filtrate.

- **Mayer's Test:** To 3 ml of the filtrates, 1ml of Mayer's reagent (potassium mercuric iodide) was added. The appearance of white precipitate indicates the presence of alkaloids.
- **Wagner's Test:** To 3 ml of filtrate, 1ml of Wagner's reagent (iodine in potassium iodide) was added. The appearance of reddish brown precipitate indicates the presence of alkaloids.
- **Hager's Test:** To 3 ml of filtrate, 1ml of Hager's reagent (saturated picric acid solution) was added. The appearance of yellow precipitate indicates the presence of alkaloids.



• **Dragendroff's Test:** To 3ml of the filtrate, 1ml of Dragendroff's reagent (potassium bismuth iodide) was added. The appearance of brick red precipitate indicates the presence of alkaloids.

### **Test for lipids**

To 10 ml of extract 0.5N alcoholic potassium hydroxide was added along with a drop of phenolphthalein. This mixture was heated on water bath for 1 h and observed for the formation of soap or partial neutralisation of alkali.

### **Identification of phenol by using TLC**

#### **Thin layer chromatography (TLC)**

##### *Preparation of TLC plates*

The glass plate, which measured 20 × 20 cm, was prepared for TLC by coating it to a thickness of 0.25 mm with silica gel HF254 (Article no. 7739, Merk) using a TLC spreader.

**Spotting the plates:** The samples were spotted on the TLC plates with microcapillary tubes and the prepared (spotted) TLC plates were eluted using a solvent system comprising Chloroform: Methanol (27: 0.3) (Ulubelen, 2000).

##### **Visualization of the TLC plates**

The plates were examined under UV at 254 and 365 nm. The spots were marked with a pencil. The value for each compound as evident from the blue and green fluorescent spots under UV was calculated as the R<sub>f</sub> value (retention factor) for that compound

**R<sub>f</sub> value = Distance traveled by the compound / Distance traveled by the solvent front**

### **Identification of flavonoid by using TLC**

##### *Preparation of TLC plates*

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**Spotting the plates:** The samples were spotted on the TLC plates with microcapillary tubes and the prepared (spotted) TLC plates were eluted using a solvent system comprising chloroform and methanol (19:1).

### Visualization of the TLC plates

The plates were examined under UV at 254 and 365 nm. The spots were marked with a pencil. The value for each compound as evident from the blue and green florescent spots under UV was Calculated as the Rf value (retention factor) for that compound

**Rf value = Distance traveled by the compound / Distance traveled by the solvent front**

The TLC Analysis result of three mushroom extract had the phenol and flavonoid.

### Moisture analysis

20 g of fresh mushroom was weighed into a weighed moisture box (A&D company ltd. N 92; P1011656; Japan) and dried in an oven at 100~105°C and cooled in a dessicator. The process of heating and cooling was repeated till a constant weight was achieved.

The moisture content was calculated as following equation

**Moisture (%) = (initial weight – final weight) × 100/weight of sample** (Raghuramulu *et.al.*, 2003).

### Determination of total protein

Five gram of grinded mushroom was taken with 50 ml of 0.1 N NaOH and boiled for 30 min. The solution was cooled in room temperature and centrifuged at 1000 × g by a DSC-200T tabletop centrifuge (Digisystem Laboratory Instruments, Taipei, Taiwan). The supernatant was collected and total protein content was measured according to the method of Lowry *et.al.*, (1951). For the determination of protein content from fresh mushroom, 5 g was taken with 50 ml phosphate buffer and homogenized with a tissue homogenizer (Polytron, Lucerne, Switzerland). Five milliliter of homogenized was taken with 50 ml of 0.1 N NaOH and protein content was determined as mentioned above.

### Determination of crude fiber

Ten grams of moisture and fat-free sample was taken in a beaker and 200 ml of boiling 0.255 N H<sub>2</sub>SO<sub>4</sub> was added. The mixture was boiled for 30 minutes keeping the volume constant by the addition of water at frequent intervals. The mixture was then filtered through a muslin cloth and the residue washed with hot water till free from acid. The material was then transferred to the same beaker and 200 ml of boiling 0.313 N NaOH added. After boiling for 30 minutes (keeping the volume constant as before) the mixture was filtered through a muslin cloth and the residue washed with hot water till free from alkali, followed by washing with some alcohol and ether. It was then transferred to a crucible, dried overnight at 80~100°C and weighed (We) in an electric balance (Keyi: JY-2003; China). The crucible was heated in

a muffle furnace (Nabertherm: Mod-L9/ 11/c6; Germany) at 600°C for 5~6 hours, cooled and weighed again ( $W_a$ ). The difference in the weights ( $W_e - W_a$ ) represents the weight of crude fiber. Crude fiber (g/100 g sample) =  $(100 - (\text{moisture} + \text{fat}) \times (W_e - W_a) / W_t$  of sample (Raghuramulu *et al.*, 2003).

#### Determination of total ash

One gram of the sample was weighed accurately into a crucible. The crucible was placed on a clay pipe triangle and heated first over a low flame till all the material was completely charred, followed by heating in a muffle furnace for about 5~6 hours at 600°C. It was then cooled in a desiccator and weighed. To ensure completion of ashing, the crucible was then heated in the muffle furnace for 1 h, cooled and weighed. This was repeated till two consecutive weights were the same and the ash was almost white or grayish white in color. Then total ash was calculated as: Ash content (g/100 g sample) = weight of ash  $\times$  100/weight of sample taken (Raghuramulu *et al.*, 2003).

#### Mineral analysis

Total ash was taken for the analysis of mineral contents. Two ml of conc.  $\text{HNO}_3$  was added to the ash and heated for 2 minutes. One drop of hydrogen peroxide was added into the solution. The solution was then transferred into a volumetric flask and total volume was made 50 ml by adding deionized distilled water. This was then used to analyze the contents of calcium (Ca), iron (Fe), manganese (Mn), magnesium (mg), zinc (Zn), Selenium (Se) and arsenic (As) by flame and graphite method with atomic absorption spectrophotometer (Perkin Elmer: AS 80).

#### Estimation of phenol

The amount of total phenolics (TP) in the extracts was determined by the modified Folin-Ciocalteu method (Minnusi *et al.*, 2003). Mushroom extract of 100  $\mu\text{l}$  was added to 4ml of 20% sodium carbonate mixed thoroughly and allowed to stand for 2 minutes; to this mixture 100  $\mu\text{l}$  of Folin-Ciocalteu and 500  $\mu\text{l}$  of distilled water were added. The mixture was vortexed for 15 sec and allowed to stand for 30 min at 40°C for color development. Absorbance was then measured spectrophotometrically at 670nm. Gallic acid was used as the standard in order to create a calibration curve by plotting absorbance versus concentration. TP content was standardized against gallic acid and the data was expressed as Gallic Acid Equivalent (GAE) per dry weight of the mushroom.

**Estimation of flavonoid**

The concentrations of total flavonoid in the extracts were determined as described previously (Chai, *et.al.*, 2012). Mushroom extract (0.2 ml) was added to 0.15 ml of NaNO<sub>2</sub> and the mixture was incubated at room temperature for 6 min. Next, 0.15 ml of AlCl<sub>3</sub>.6H<sub>2</sub>O (10% w/v) was added to the mixture, which was then left at room temperature for 6 min. Next, 0.8 ml of NaOH (10% w/v) was added and the absorbance of the mixture was read at 510 nm after standing at room temperature for 15 min. For the blank, the extracts were replaced with water. To correct for background absorbance, a blank was prepared for each sample in which the AlCl<sub>3</sub>.6H<sub>2</sub>O was replaced with water. A standard curve was prepared from 0 - 500mg/ml quercetin dissolved in 80% ethanol. Total flavonoid content was expressed in mg quercetin equivalents/g dry matter.

**Estimation of protein**

- The Lowry solution should be prepared fresh, at the day of measurement. Though the individual solutions for the Lowry solution can be prepared in advance and then mixed at the day of measurement.
- Solution A is a dilute alkali solution. 2N Folin and Ciocalteu's Phenol Reagent contain HCl and H<sub>2</sub>PO<sub>4</sub>.

**Solution A:** (alkaline Solution) (for 100 ml) 0.572gm NaOH 2.862gm Na<sub>2</sub>CO<sub>3</sub>

**Solution B:** (for 20 ml) 0.285gm CuSO<sub>4</sub>. 5(H<sub>2</sub>O)

**Solution C:** (for 20 ml) 0.571gm Na<sub>2</sub> Tartrant .2(H<sub>2</sub>O)

**Lowry Solution:** (freshly prepared, 0.7/ml sample) Sol. A + Sol. B +Sol. C with a ratio (vol : vol) of 100:1:1.

**Folin Reagent** (instant fresh, 0.1 ml/sample): 5ml of 2N Folin and Ciocalteu's Phenol Reagent + 6ml odouble distilled water - this solution is light sensitive. So it should be prepared at least 5min of the first sample incubation and kept in an amber container.

**BSA Standard Protein Solution (fresh)**

Although BSA is a water soluble protein, it takes time to dissolve it completely. So, prepare this stock solution and keep it mixed i.e., for 1 hour before starting the experiment.

**Stock Standard solution**

100mg of BSA is dissolved in 100ml of distilled water.

**Working Standard solution**

In the ratio of 1 ml of stock solution was made upto 2ml with distilled water.

**Protein estimation**

- In a series of clean test tube pipetted out 0.2, 0.4, 0.6, 0.8 and 1.0ml of BSA stock standard solution.
- 1.0ml of unknown solution was taken in another test tube. All the test tubes were made upto 1ml with distilled water. To all the test tubes are added with 5ml of Lowry's reagent followed by addition of 0.5ml of Folin's reagent and vortex the tubes immediately and let it for 20 minutes at room temperature.
- The blue colour developed was read at 650nm against the blank.
- The standard graph was drawn by taking concentration of BSA on x-axis and their corresponding optical density on y-axis from the graph the amount of protein was calculated and measured the optical density.

**Estimation of carbohydrate**

The series of standard solution of 0.2, 0.4, 0.6, 0.8 and 1.0ml were taken in a series test tubes. Then the test tubes were made upto 1ml with distilled water. 1ml of distilled water on another test tube served as blank. 4ml of anthrone reagent was added to all test tubes and incubated for 5 to 10 minutes in a boiling water bath. The colour was burned from green to dark green. The intensity of colour formed was read by using calorimeter at 625nm. A Standard graph was drawn by taking the concentration of glucose on x-axis and absorbance on y-axis. A straight line was obtained from this standard graph. The amount of glucose in the unknown solution was calculated.

**GC/MS ANALYSIS****Gas chromatography/Mass spectrometry analysis**

Qualitative and quantitative GC/MS analyses were carried out on a Hewlett-Packard 5890 Series II Plus gas chromatograph interfaced to an HP 5989B mass spectrometer. Separation was done on a 25 m x 0.25 mm HP5-MS capillary column coated with 0.50  $\mu\text{m}$  5% phenyl 95% methylpolysiloxane. Temperature programming was set at 70-250°C, at a rate of 3°C/min. The carrier gas used was helium at a constant flow rate of 1.9 ml/min. Injector and interface temperature were adjusted to 250°C and 280°C, respectively. EI mass spectra were recorded at 70 eV ionization voltage (source temperature 250°C). Compounds were identified

by mass spectral comparison with a commercial database (Wiley8 and NISTO5 mass spectral library) and the laboratory's own database. Spectral data were compared with linear retention indices published in the literature.

## BIOACTIVITY OF SELECTED MUSHROOMS

### Antioxidant Activity

The Phenol and Flavonoid extracts were prepared by the following methods. The 0.5g of sample was immersed in 50ml of methanol. Then the sample was filtered by using whatman filter paper. The filtered extract was used for antioxidant activity. The 0.1g of sample was immersed in 10ml of methanol. The methanol extract for waterbath for 5min. The water and ethylacetate ratio (10:1) was added to the sample. Then the sample was filtered by using whatman filter paper. The filtered extract was used for antioxidant activity. 1ml of phenol extract and 1ml of flavonoid extract was mixed and it was used for antioxidant activity.

### Determination of DPPH radical scavenging activity

The 1, 1- diphenyl-2- picryl- hydrazyl (DPPH) assay was performed by using the method of (Braca, De Tommasi Nunziatina, Di Bari Lorenzo, Pizza Cosimo, Politi Mateo & Morelli Ivano, 2001). Various concentrations of mushrooms extract (100-600µg/ml) were added to 1ml of the 0.004% methanol solution of DPPH, and the mixture was vortexed vigorously. The tubes were then incubated at room temperature for 30 minutes in dark and the absorbance was taken at 517nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. Catechin was taken as known free radical scavenger. Percentage inhibition activity was calculated by using the formula

$$\% \text{ inhibition} = \frac{A_0 - A_1}{A_0} \times 100$$

Where A0 was the absorbance of the control and A1 was absorbance in the presence of mushroomextract/ known antioxidant.

### Methodology for Antimicrobial Activity

#### Micro-organisms

Escherichia coli MTCC 1687, Bacillus subtilis MTCC 441, Salmonella MTCC 3858, Shigella MTCC 11947, Pseudomonas aeruginosa MTCC 1688, Streptococcus pyogenes MTCC 2608, MRSA MTCC 2940, Proteus mirabilis MTCC 2813, Klebsiella MTCC 5328. The bacteria were subcultured in nutrient agar medium and the culture of each bacterium was

preserved on the same medium at 40C. The cultures were sub cultured periodically on the same medium at 370C ± 20C.

### Anti-microbial screening

*In vitro* antibacterial activity of the different compounds was studied against seven bacterial strains by the agar well diffusion method as described by Perez *et.al.*, 1990 with certain modifications. Nutrient agar (Hi Media, India) was used as the bacteriological medium. The antibacterial activity of different compounds was taken at different concentrations (500 and 250 µg/well). The nutrient agar was melted and cooled to 48-500C and a standardized inoculum of  $1 \times 10^6$  CFU/ml was then added aseptically to the molten agar and poured into sterile Petri dishes to give a solid plate. Wells were prepared in the seeded agar plates. The test compound was introduced in the well (5 mm). The plates were incubated overnight at 37°C. The antimicrobial spectrum of the compounds was determined for the bacterial species in terms of zone sizes around each well.

### Antibacterial Activity

In the present study five different extract of (Water, Hcl, Chloroform, Phenol and Flavonoid) three mushroom extract showed activity against the organisms of *E.coli*, *Salmonellatyphi*, *Staphylococcus aureus*, *Bacillus sp*, *Pseudomonas sp*, *Klebsiella sp*, *Salmonella*, *Proteusand Streptococcus* were documented in below. These results confirm that bioactive components of mushroom may differ in their solubility depending on the extractive solvents. Antimicrobial activity in natural source extracts depends not only on the presence of phenolic compounds but also on the presence of various secondary metabolites (Gordana SC, *et.al.*, 2007).

### RESULTS

Tthe most common 3 species was collected, it was introduced in to cultivation, substrate optimisation and cultivation parameters analysis tests. then the Mycochemical test was taken, according to this test Results GC-MS and Mass spec test was taken, antimicrobial tests were taken.

**Table 2: Effect of media on mycelial growth by using three selected mushrooms.**

Culture media	Colony diameter			Growth rate			Mycelial density		
	<i>C.indica</i>	<i>L.sulpureus</i>	<i>G.sepiarum</i>	<i>C.indica</i>	<i>L.sulpureus</i>	<i>G.sepiarum</i>	<i>C.indica</i>	<i>L.sulpureus</i>	<i>G.sepiarum</i>
PDA	1.06	0.06	0.04	1.08	0.03	0.06	4+	-	-
MEA	1.02	0.04	0.02	1.10	0.01	0.04	4+	-	-

**Effect of pH for mycelial growth**

All pH from 5–8 were suitable for growth of mycelium of *C.indica*. The optimal pH on mycelia growth of *L. sulphureus* was in the range of pH 7, however. The optimal pH for *Gloeophyllum sepiarum* was in the range of pH 7 (Lai *et.al.*, 2011; Kumla *et.al.*, 2013).

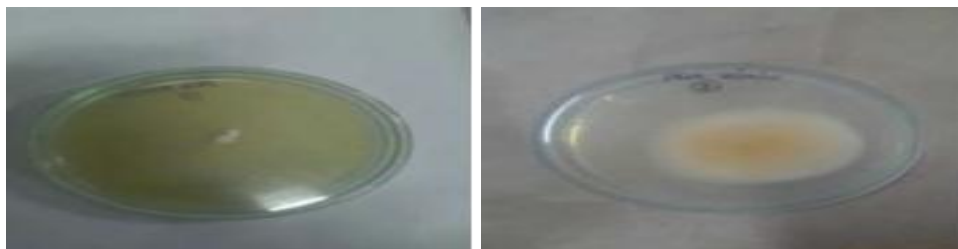
**Table 3. Effect of temperature on mycelial growth by using three selected mushrooms.**

Temperature	Mycelial Growth RATE		
	<i>C.indica</i>	<i>L.sulpureus</i>	<i>G.sepiarum</i>
20	0.98	0.12	0.18
25	1.65	0.46	0.36
30	0.84	0.32	0.28
35	0.62	0.21	0.24

**Supplemented substrates used for cultivation**

Our study examined the use of different cereal grain/sawdust media for promoting mycelia growth and spawn production. After 10 days of incubation, the mycelium was able to colonize all the cereal grains and agricultural waste medium (sawdust). The data for mycelium growth on different cereal grain media/sawdust was investigated. Therefore, the results showed that the various cereal grain media can be used in order to promote mycelia growth of *Calocybeindica*, *L. sulphureus* and *Gloeophyllum sepiarum*.





A tissue of milky mushroom on PDA plate



Cultivation Chamber



Different Substrates



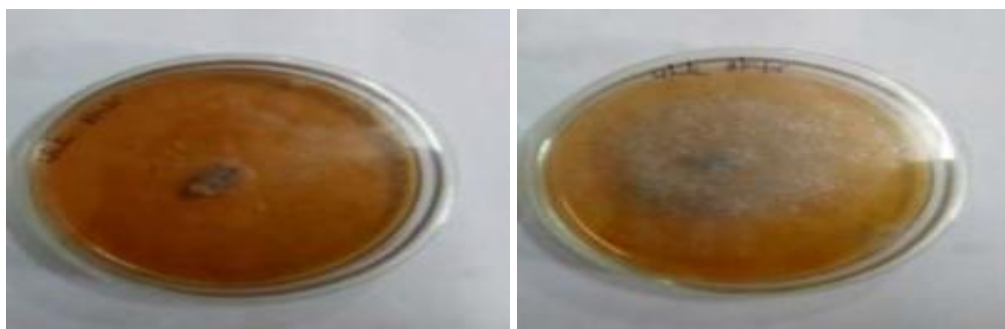
Casing soil applied on bed

Pin head formation



Production of Fruiting Body

Fig. 3: Cultivation of *Calocybe Indica*.



The growth of *Laetiporous sulphureus* on PDA plate (5 days).

The growth of *Laetiporous sulphureus* in PDA agar (14 days).

**Fig. 4: Cultivation of *Laetiporous Sulpureus*.**



The growth of *Gloeophyllum sepiarum* on PDA plate (5 days)

The growth of *Gloeophyllum sepiarum* on PDA plate (14 day)

**Fig. 5: Cultivation of *Gloeophyllum Sepiarum*.**

**Nutritional studies:** The results of the studies carried out on the three species of *Calocybe indica*, *Laetiporous sulphureus* and *Gloeopyllum sepiarum* on dry weight basis is depicted. In these mushrooms Crude fibres were found to be maximum in *C. indica* (14.7) while lowest in *L.sulpureus* (13.4) and *G. sepiarum* (10.9) ash content in *C. indica* (9.3), *L.sulpureus* (8.7) and *G. sepiarum* (6.9). The average moisture content in dried samples was highest in *C. indica* (88.6) and minimum in *L.sulpureus* (84.3) and *G. sepiarum* (76.1).

#### Evaluation for mineral elements

During the present investigation mineral elements evaluation was estimated on dry weight basis and results obtained are documented in respectively. Out of 3 wild samples of *C.indica*, *L. sulphureus* and *G.sepiarum* examined for mineral estimation, Fe content of mushrooms had 258mg/100 g in *C. indica*, 242mg/100g in *L. sulphureus* and *G. sepiarum*. Cu content of mushrooms had 8mg/100g in *C. indica*, 6mg/100g in *L. sulphureus* and 3mg/100g in *G. sepiarum*. Mn content in *C. indica*, *L.sulpureus* and *G. sepiarum* were 3, 2 and 1 mg/ 100 g,

respectively. Mg content in *C. indica*, *L.sulpureus* and *G. sepiarum* were 247, 204 and 132 mg/ 100 g, respectively. Ca content in *C. indica*, *L.sulpureus* and *G. sepiarum* were 13, 21 and 3 mg/ 100 g, respectively. The results of mineral values of the three edible species of mushrooms clearly indicate the potential for their use as sources of good quality food.

**Table. 4. Qualitative Mycochemical Screening of Selected Three Mushrooms.**

Test	<i>Calocybe indica</i>	<i>Leotiphorous sulphureus</i>	<i>G. sepiarum</i>
Alkaloids	-	-	-
Glycosides	-	-	-
Saponins	+	-	-
Steroids and Sterols	+	-	-
Phenols	+	+	+
Tannins	+	-	-
Proteins and aminoacids	+	+	+
Fixed oils and fats	-	-	-
Carbohydrates	+	+	+
Gums and Mucilage	-	-	-
Flavonoids	+	+	+

**Table. 5: Name of species and Mineral elements (mg/100gm) of dry samples.**

S.NO	Fe	Mg	Cu	Mn	Ca
<i>C. indica</i>	258±0.76	247±1.93	8±0.28	3±0.26	13±0.53
<i>L. sulphureus</i>	242±1.59	204±0.39	6±0.64	2±0.48	21±0.33
<i>G. sepiarum</i>	225±1.41	132±0.87	3±0.71	1±0.16	3±0.72

(mean ± SD; n = 3)

#### Evaluation for nutraceutical components

The present study indicates the presence of phenolics, flavonoids, Proteins and Carbohydrates in the extracts of selected mushrooms. The selected mushroom extract of *Calocybe indica* has the presence of saponins, tannins, Phenols, Flavonoids, Proteins, Carbohydrate, steroids and sterols. *Laetiporous sulphureus* has the presence of phenols, proteins, flavonoids and carbohydrate. *Gloeophyllum sepiarium* has the presence of phenols, proteins, carbohydrate and flavonoids.

**Identification of phenol by using TLC:** In TLC analysis the Rf value of *C. indica*, *L.sulpureus* and *G. sepiarum* were 0.955, 0.822 and 0.866 respectively.

**Identification of flavonoid by using TLC:** In TLC analysis the Rf value of *C. indica*, *L.sulpureus* and *G. sepiarum* were 0.927, 0.583 and 0.527 respectively.

**Table. 6: TLC Analysis of phenol in three selected mushrooms.**

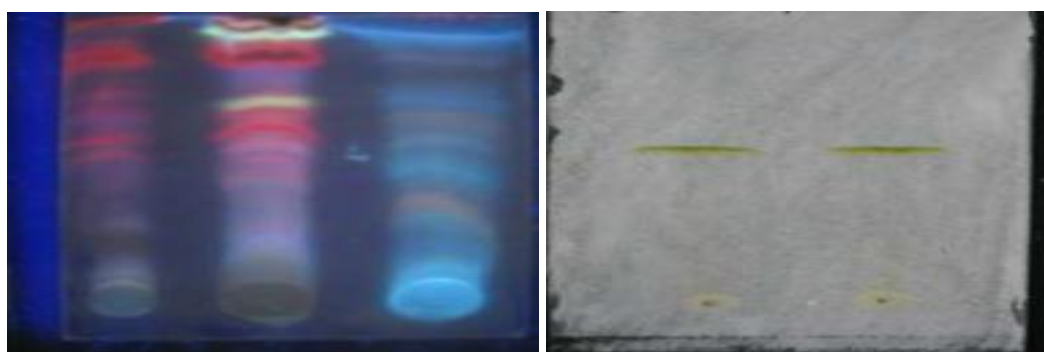
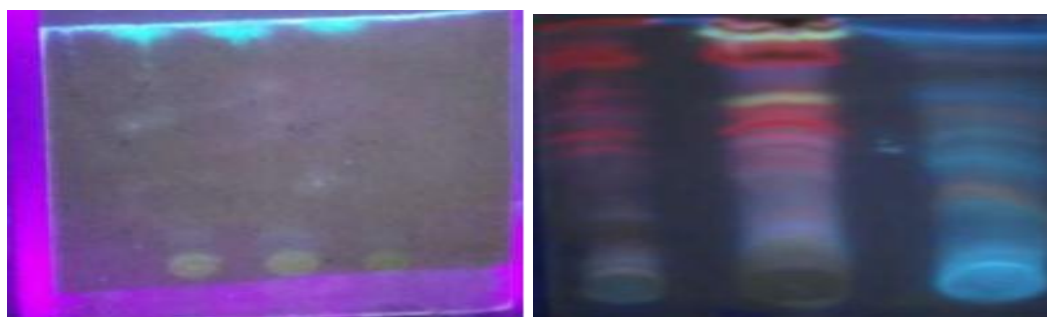
Samples	Distance traveled by the compound (cm)	Distance traveled by the solvent front (cm) Rf value	Rf Value
<i>C.indica</i>	17.2	18	0.955
<i>L.sulpheurus</i>	14.8	18	0.822
<i>G.sephiarium</i>	15.6	18	0.866

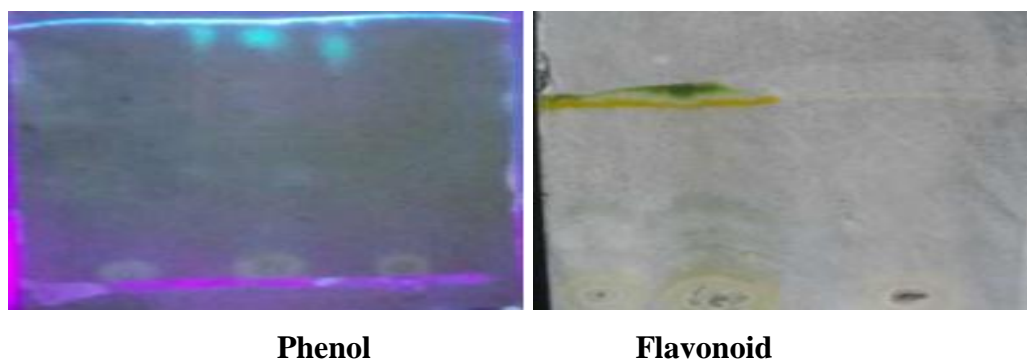
**Estimation of phenol:** The selected mushrooms had phenol content in *C. indica*, *L.sulpureus* and *G. Sepiarum* were 0.580, 0.450 and 0.095 respectively.

**Estimation of flavonoids:** The selected mushrooms had flavonoid content in *C. indica*, *L.sulpureus* and *G. Sepiarum* were 0.290, 0.180 and 0.120 respectively.

**Estimation of carbohydrate:** The selected mushrooms had flavonoid content in *C. indica*, *L.sulpureus* and *G. Sepiarum* were 90, 10 and 20 respectively.

**GCMS Analysis:** Qualitative and quantitative GC/MS analyses were done in *Laetiporous sulphureus* and *Gloeophyllum sepiarum*.

**Phenol****Flavonoid****Fig. 6: Tlc Analysis In *Calocybe Indica*.****Phenol****Flavonoid****Fig. 6: Tlc Analysis In *Laetiporous Sulpureus*.**



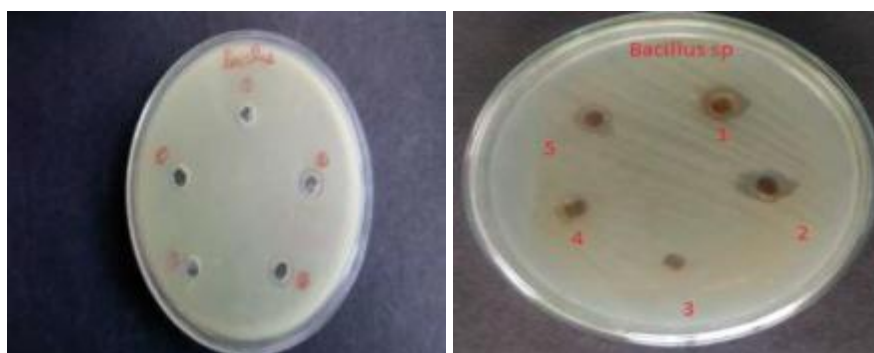
**Fig. 7: Tlc Analysis In *Gloeophyllum Sepiarum*.**

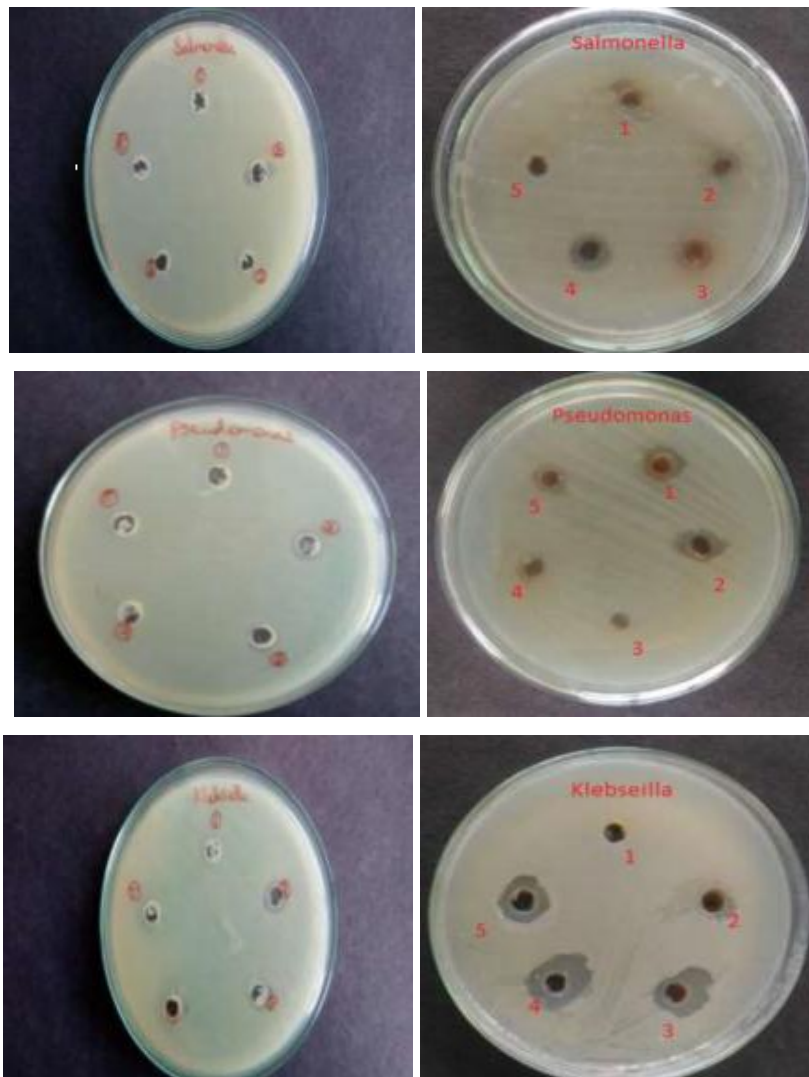
### DPPH radical scavenging activity

During the present investigation Antioxidant Activity was estimated on dry weight basis and results obtained are documented in respectively. Out of 3 wild samples of *C. indica*, *L.sulpureus* and *G.sepiarum* examined for Antioxidant Activity of phenol extract had 71.42% in *C. indica*, 49.66% in *L. sulpureus* and 30.61% in *G. sepiarum*. Flavonoid extract of mushrooms had 70.06% in *C. indica*, 63.26% in *L. sulpureus* and 36.05% in *G. sepiarum*. From above activity the more activity was present in sample B. The sample A has the reference purpose. So the sample of B was used with mixed extracts had the percentage of 82.31% was present.

### Antibacterial Activity

In the present study five different extract of (Water, Hcl, Chloroform, Phenol and Flavonoid) three mushroom extract showed activity against the organisms of *E.coli*, *Salmonellatyphi*, *Staphylococcus aureus*, *Bacillus sp*, *Pseudomonas sp*, *Klebsiella sp*, *Salmonella*, *Proteus* and *Streptococcus* were documented in below. These results confirm that bioactive component of mushroom may differ in their solubility depending on the extractive solvents. Antimicrobial activity in natural source extracts depends not only on the presence of phenolic compounds but also on the presence of various secondary metabolites (Gordana SC, *et.al.*, 2007).





Control Plates

Inhibitory Activity

Fig. 8: Antimicrobial Activity of *Calocybe Indica*.

## DISCUSSION

### Mushroom morphology and isolation

Basidiocarps of *Calocybe indica* vary considerably in size (6–12 cm wide and 10–24 cm across). The distinctive characteristics of the fruiting bodies are the milky white colour, large sized basidiocarps with fleshy stipe and broadly adnate to decurrently gills. The pores of the basidiocarps are milky white in colour. The internal tissues of *Calocybe indica* fruiting bodies were cut and placed on PDA plates and incubated at 30°C. After 2 months, the mycelia were fully covered the plates and produced young primordial fruiting bodies, observed after incubating the fully mycelia covered PDA plates for 10 days. These stock pure cultures are maintained in PDA slant tubes at 4°C while with 15% glycerol on the top of the PDA slants at –20°C for long term preservation.

### Effect of media on mycelial growth

*Calocybe indica* had the largest colony diameter on PDA medium (1.06 cm/days), density (4+); and MEA medium (1.02 cm/day), density (4+). *Laetiporus sulphureus* had the mycelial colony diameter on PDA medium (0.06 cm/days), density was not well and MEA medium (0.04 cm/day), density was not well and *Gloeophyllum sepiarium* had the mycelial colony diameter on PDA medium (0.04 cm/days), density was not well and MEA medium (0.02 cm/day), density was not well. Our results are similar to those of Siwulski *et.al.*, (2009). *C. indica* grew well on PDA medium.

### Effect of temperature on mycelial growth

The *Calocybe indica*, *L. sulphureus* and *Gloeophyllum sepiarium* strains were tested for the suitable temperature for promoting mycelial growth on PDA medium. Temperatures of 20°C, 25°C, 30°C and 35°C were used and the mycelial grew well between 25°C and 30°C, while the most unfavourable was 35°C. Even though the mycelial growth of *L. sulphureus* occurred at 30°C (Shim *et.al.*, 2005; Lai *et.al.*, 2011), growth of *Gloeophyllum sepiarium* was observed at 25–30°C (Jayasinghe *et.al.*, 2008). The optimal temperature for wood-decay fungi from temperate regions is between 25°C and 30°C; thus, we consider the suitable temperature for *L. sulphureus* between 25°C and 30°C.

### CONCLUSION

Mushroom can serve as a dietary supplement for proteins, vitamins, minerals as well as cheap and easily accessible source for natural antioxidants for both man and/or livestock. The various antioxidant mechanisms of the mushroom species extract may be attributed to strong hydrogen-donating ability, a metal-chelating ability and their effectiveness as good scavengers of superoxide and free radicals. This indicates the potential of mushrooms as panacea for many disease and also reveals a novel potential to fight against tumors in man. However, more intensive and extensive investigations are needed to exploit their valuable therapeutic potential. The present investigation can conclude that the extract of 3 edible mushrooms showed biopharmaceutical potentiality. However whether such extracts will act as effective therapeutic agents remain to be investigated, the identification of the bioactive compounds and study of mechanisms of actions are necessary prior to application.

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