3. MATERIALS AND METHODS

The present study centered on evaluating the antioxidant and anticancer properties of the compounds isolated from methanolic extract of Pleurotus florida mushroom in vitro and also the adsorption of ferrous iron (Fe²⁺) using spent mushroom substrate of P. florida. The overall designs of this study are represented schematically in the Fig. 3.1.

This study was performed in five different aspects in order to complete the objectives. In the first aspect, the P. florida mushroom was cultivated using groundnut husk, maize powder, horse gram powder and coconut oilcake powder as nutrient supplements. In that horse gram supplemented batch showed the highest yield of mushroom. Hence, the P. florida mushroom was cultivated using horse gram as nutrient supplement in huge amount for further study. The phytochemical and biochemical properties of the cultivated mushroom were characterized.

In the second aspect, the aqueous, methanolic, ethanolic, ethyl acetate and hexane extracts of P. florida were probed for their antibacterial and antioxidant activity. The methanolic extract of P. florida showed significant antibacterial activity against the bacterial pathogens, hence further pharmacological studies and phytochemical analysis was carried out by using methanolic extract of this mushroom.

The third aspect deals with the identification of the major active components present in the methanolic extract responsible for antioxidant and anticancer effect. In the fourth aspect, antioxidant and anticancer response evoked by the isolated compounds from P. florida methanolic extract were analyzed against a battery of in vitro experimental systems.

The final aspect comprised of the novel approach in the adsorption of Fe²⁺ from ground water using spent mushroom substrate of P. florida. The detailed study plan was categorized and they are discussed as following:

3.1. Cultivation and characterization of P. florida mushroom

The P. florida mushroom was cultivated by using different nutrient supplements and the aqueous, methanolic, ethanolic, ethyl acetate and hexane extracts of this mushroom were subjected into biochemical analysis and further the phytochemical constituents of this extract were analyzed by qualitative and quantitatively.
3.1.1. Cultivation of P. florida mushroom using different nutrient supplements

3.1.1.1. Preparation of substrate

Paddy straw (Oryza sativa) was collected from in and around of Panamarathupatti, Salem, Tamil Nadu, India which was bounded by the longitude of 78.14°E and the latitude of 11.66°N. The collected straw was chopped into desirable size of 3 cm with a hand cutter for oyster mushroom cultivation. The chopped straw was weighed and soaked in water for overnight. The soaked straw was removed from water and sterilized by autoclaving at 121 °C, for 15 min. Nutrient supplements viz., groundnut husk (Arachis hypogea), maize powder (Zea mays), horse gram powder (Macrotyloma uniflorum) and coconut oilcake powder (Cocos nucifera) were used at the concentration of 5% on wet weight basis of the substrate. Finely the raw materials were powdered and sterilized in an autoclave at 121°C for 15 min and they were thoroughly mixed well with already sterilized paddy straw and finally sorghum grain (Sorghum bicolor) spawn of P. florida was used to inoculation in order to cultivate the oyster mushroom (Marimuthu et al., 1993). The authenticated spawn packets of P. florida were obtained from Tamil Nadu Agriculture University (TNAU) Coimbatore, Tamil Nadu, India. The horse gram supplemented batch showed the highest yield of mushroom. Hence, the P. florida mushroom was cultivated using horse gram as nutrient supplement in huge amount for further study.

3.1.1.2. Preparation of cultivation bags

The straw with 80% moisture content was weighed and filled in plastic bags (60 x 30 size and 80 x 100 gauge thickness) with the addition of freshly prepared nutrient supplements and mushroom spawn. The sterilized substrates were cooled (24°C) under clean and sterile conditions inside the cultivation room and mixed with supplements. Layering method was adopted for cultivation process. Separate plastic bags were taken and filled by ½ kg of nutrient supplemented substrate and 20 g of spawn was uniformly spreaded over the layered substrate and again further 3 layers were made in the same manner and tied tightly. For the experimental conditions four bags were used with nutrient supplements and a control bag
kept without any nutrient supplements. The packed bags were pin holed for aeration and weighed before keeping in cultivation room.

3.1.1.3. Cultivation conditions and harvesting

The inoculated bags were incubated in a cultivation room and maintained at 25-30°C with relative humidity of 85 ± 5%. The temperature and relative humidity was maintained with wet jute bags in the walls of the cropping room and by sprinkling water in the floor of the cropping room covered with sand at the period of September to November 2010 for ramification of the mushroom mycelium. Growth of the mushroom was recorded daily for all the five treatments. While the substrate was covered with ramified mycelium and appearance of pin-heads, the mouth of the bag was mouth opened to facilitate the development of fruiting bodies. After development of fruiting bodies they were harvested with sterile sharp knife or blade. The harvesting was done in 4 flushes at a regular interval of seven days. After the 2nd flush, the substrate was turned upside down and regularly watered to harvest the 3rd and 4th flushes. The yield of mushrooms was recorded (Plate 3.1).

3.1.2. Proximate analysis

3.1.2.1. Determination of moisture content

About 5 g of P. florida mushroom (W₁) was dried using a silica crucible in an oven temperature of 105°C for 3 h and weighed. It was allowed to cool in an airtight desiccator and reweighed. Again it was heated in the oven for 30 min, cooled and weighed. The process was repeated until constant weight was obtained (W₂) (AOAC, 1990).

The percentage moisture was calculated from the following equation,

\[ \text{Moisture (\%)} = \frac{W₁-W₂}{W₁} \times 100 \]

3.1.2.2. Processing of sample

An electronic blender was used for milling the P. florida mushroom and sieved with 0.2 to 0.6 mm pore sizes to get fine powder. This powder sample was used for further proximate analysis.
3.1.2.3. Crude fat (AOAC, 1990)

The soxhlet apparatus was used for the analysis of crude fat in the P. florida mushroom. The round bottomed flask was weighed and 2 g of the accurately weighed sample was taken in the open end of the thimble which was then plugged with absorbent cotton. The open end of the thimble was fixed up into the extractor of the apparatus. The cold water circulation was provided for condensing the solvents. The petroleum ether (60 - 80°C boiling range) was poured from the top of the condenser so as to have sufficient excess in the round bottom flask after filling the extraction chamber.

The flask was heated over a heating mantle and the extraction was allowed to continue for 4 hrs at a condensation rate of 5-6 drops/sec. The assembly was dismantled after recovery of the solvent and the round bottom flask was weighed. The final weight of the round bottom flask infers the percentage of crude fat content in the sample.

\[
\text{Grain in weight of the flask} \\
\text{Percentage} \ (%) \ of \ crude \ fat \ = \ \frac{\text{-----------------------------}}{\text{Weight of sample}} \times 100
\]

3.1.2.4. Total ash (AOAC, 1990)

Total ash of the P. florida mushroom was carried out in muffle furnace. About 2 g of sample was weighed in a silica crucible and the sample was ignited in a burner till smoke ceases. The crucible was placed in a muffle furnace and heated to 600°C for 2h. At this temperature all organic matter will be burnt leaving behind minerals. The crucible was removed from the furnace carefully and cooled in a desiccator and weighed again.

\[
\text{Weight of the ash} \\
\text{Percentage} \ (%) \ of \ total \ ash \ = \ \frac{\text{-----------------------------}}{\text{Weight of the sample}} \times 100
\]

3.1.2.5. Estimation of carbohydrate and protein

The total carbohydrate and protein content from the P. florida mushroom was estimated with various concentrations (0.2, 0.4, 0.6, 0.8 and 1 ml) at 490 and 660 nm using
UV-VIS-spectrophotometer (Cyberlab UV100, USA) by Phenol- sulphuric acid method (Sadasivam and Manickam, 1992) and the method of Lowry et al. (1951) respectively.

3.1.3. Preparation of the mushroom extract

Freshly-harvested P. florida mushroom was shade dried and finely powdered. About 25 g of the powder was extracted with 250 ml of 95% solvents like aqueous, methanol, ethanol, ethyl acetate and hexane using soxhlet apparatus (Plate 3.2). The residue was filtered and concentrated to a dry mass by vacuum distillation; the filtrate thus obtained was used as mushroom extract for pharmacological and phytochemical studies (Jayakumar et al., 2009).

3.1.4. Preliminary phytochemical characteristics

Preliminary biochemical tests such as carbohydrates, tannins, saponins, proteins, phenols, flavonoids, steroids, terpenoids, alkaloids, glycosides, resins and fixed oil were carried out in the crude aqueous, methanolic, ethanolic, ethyl acetate and hexane extract using standard procedures described by Trease and Evans, (1994) and Harborne, (1973). Based on the intensity of colour variation in all the phytochemical analysis experiments they were denoted into high (+++), moderate (++), low (+) and no reaction (-).

3.1.5. Quantitative phytochemical analysis

3.1.5.1. Determination of total phenolic contents

The total phenolic content of the P. florida mushroom extract was determined according to the method described by Siddhuraju and Becker (2003). Aliquots of 0.1 ml each extracts were taken in test tubes and made up to the volume of 1 ml with distilled water. Then 0.5 ml of folin-ciocalteu phenol reagent (1:1 with water) and 2.5 ml of sodium carbonate solution (20%) were added sequentially in each tube. Soon after vortexing the reaction mixture, the test tubes were placed in dark for 40 min and the absorbance was recorded at 725 nm comparing with the reagent blank. The analysis was performed in triplicate and the results were expressed as catechol equivalents.

3.1.5.2. Estimation of total flavonoid content

The total flavonoid content of the mushroom extract was determined by the use of a slightly modified method described by Zhishen et al. (1999). About 0.5 ml mushroom extract
was mixed with 2 ml of distilled water and subsequently with 0.15 ml of 5% sodium nitrate solution. After 6 min, 0.15 ml of 10% aluminium chloride solution was added and allowed to stand for 6 min, then 2 ml of 4% sodium hydroxide solution was added to the mixture. Distilled water was added to make up the final volume of 5 ml and then the mixture was thoroughly mixed and allowed to stand for another 15 min. Absorbance of the mixture was determined at 510 nm. Catechol was used as a standard compound for the quantification of total flavonoid content.

3.2. Determination of the antibacterial and antioxidant property of P. florida mushroom extracts

The efficacy of the different extracts of P. florida mushroom was analyzed for antibacterial activity against Gram-positive and Gram-negative bacteria by well diffusion method and antioxidant activity. The results of the phytochemical analysis, antibacterial activity and antioxidant activity showed that the methanolic extract of P. florida mushroom was effective in evoking an antioxidant response. Hence, the free radical scavenging activity was performed in detail only with methanolic extract of P. florida against DPPH (2,2-diphenyl-1- picrylhydrazyl), superoxide scavenging activity, nitric oxide radical scavenging activity, reducing power, hydroxyl radical scavenging activity and chelating ability.

3.2.1. Antimicrobial activity of P. florida mushroom extracts

Test microorganisms

A total of 13 bacterial cultures namely Bacillus sp. (MTCC1133), Pseudomonas sp. (MTCC2295), Escherichia coli (MTCC1302), Klebsiella pneumoniae (MTCC109), Shigella sp. (MTCC1457), Proteus sp. (MTCC1771), Salmonella typhi (MTCC1254), Staphylococcus aureus (MTCC737), Campylobacter sp. (ATCC33560), Streptococcus sp. (MTCC1924), Vibrio sp. (MTCC3906), Shigella sonnei (MTCC2957) and Cornyebacterium sp. (ATCC31090) were used in this study.

Antimicrobial activity of the different extracts of P. florida mushroom was evaluated using well diffusion technique using nutrient agar (Aida et al., 2001). The test bacterial strains were inoculated in nutrient broth and incubated at 37°C for 3 to 6 h. A sterile cotton
swab was immersed in the bacterial suspension and swabbed aseptically on the surface of sterile Mueller-Hinton agar plates. Wells of 5 mm diameter were cut by sterile cork borer in the agar medium and filled with mushroom extracts (10 mg/ml) prepared from respective solvents. The plates were incubated at 37°C for 24 h, after incubation, a clear zone around the well was noted. A zone of inhibition around the well was measured in millimetres. Each test was performed in triplicates and each solvent was used as a negative control.

3.2.2. Antioxidant activity of P. florida mushroom extracts

Antioxidant activity of the different extracts of P. florida mushroom was investigated by using 1,1-Diphenyl-2-picrylhydrazyl (DPPH) scavenging assay and reducing power assay (Refer Section 3.2.3).

3.2.3. Antioxidant activity of the methanolic extract of P. florida mushroom

3.2.3.1. DPPH radical scavenging assay

The antioxidant activity of the methanolic extract of P. florida mushroom was determined in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH carried out by using the method of Molyneux, (2004). About 1 ml of 100 μM DPPH solution in methanol and equal volume of different concentrations (20 - 100 μg/ml) of the extract in methanol was added and incubated in dark for 30 min and 1 ml of methanol served as control. The change in colour was observed in terms of absorbance using a spectrophotometer at 517 nm. The different concentrations of ascorbic acid were used as reference compound. Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the formula:

\[
\text{Percentage of DPPH assay} = \left(\frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}}\right) \times 100.
\]

3.2.3.2. Superoxide radical scavenging assay

The superoxide radical scavenging activity of the methanol extract was studied by using the method of Liu et al. (1997). Superoxide radicals are generated in phenazine methosulphate (PMS) - Nicotinamide adenine dinucleotide (NADH) systems by oxidation of NADH and assayed by the reduction of Nitro Blue Tetrazolium (NBT). About 200 μl of methanol extract of different concentrations were taken in a series of test tubes. Superoxide
radicals were generated by adding 1 ml of Tris-HCl buffer (16.0 mM, pH - 8.0), 1 ml of NBT (50.0 µM), 1 ml of NADH (78.0 µM) solution and 1 ml of PMS (10 µM). The reaction mixture was incubated at 25°C for 5 min and the absorbance was measured at 560 nm. A control tube containing Tris-HCl buffer was also processed in the same way without methanolic extract of P. florida mushroom. Different concentration of ascorbic acid was used as reference compound. The percentage inhibition of superoxide anion generation was calculated as follows:

\[
\text{Percentage Inhibition} = \left( \frac{A_0 - A_1}{A_0} \right) \times 100
\]

where \( A_0 \) is the absorbance of the control; \( A_1 \) is the absorbance of the sample extract/standard.

3.2.3.3. Nitric oxide radical scavenging assay

Nitric oxide radical scavenging activity was measured spectrophotometrically according to the method described by Govindharajan et al. (2003). When sodium nitroprusside was mixed with aqueous solution at physiological pH, suddenly it generates nitric oxide, which reacts with oxygen to produce nitrite ions that can be estimated using Greiss reagent. Nitric oxide scavengers compete with oxygen leading to reduced production of nitrite ions. About 1 ml of sodium nitroprusside (5 mM) in phosphate buffer (pH 7.4, 0.1 M) was mixed with different concentrations of the P. florida methanolic extract (200 - 1000 µg/ml) in phosphate buffer (pH 7.4, 0.1 M). The tubes were then incubated at 25°C for 2 h. After incubation, 1.5 ml of reaction mixture was removed and diluted with 1.5 ml of Greiss reagent [1% sulphanilamide, 2% O-phosphoric acid and 0.1% of N-(1- naphthyl) ethylenediamine dihydrochloride]. The absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with N-(1- naphthyl) ethylenediamine dihydrochloride) was measured at 546 nm. Control tube was maintained with all chemicals excluding P. florida extract.

3.2.3.4. Hydroxyl radical scavenging assay

Hydroxyl radicals (OH\(^*\)) are generated from Fe\(^{2+}\) - ascorbate - EDTA - H\(_2\)O\(_2\) system (Fenton’s reaction) which attack the deoxyribose and set off a series of reactions that
eventually result in the formation of malondialdehyde (MDA), measured as a pink MDA-TBA chromogen at 535nm. The hydroxyl radical scavenging activity of P. florida methanolic extract was measured according to a modification of the method of Halliwell et al. (1992). Stock solutions of EDTA (1 mM) were prepared in Dimethyl sulfoxide (DMSO) and FeCl$_3$ (10 mM), ascorbic acid (1 mM), H$_2$O$_2$ (10 mM) and deoxyribose (10 mM) were prepared in distilled deionized water. The method was carried out by adding 100 µl of EDTA, 10 µl of FeCl$_3$, 100 µl of H$_2$O$_2$, 360 µl of deoxyribose, 1000 µl of the extract (100 to 500 µg/ml) dissolved in distilled water, 330 µl of phosphate buffer (50 mM, pH 7.4) and 100 µl of ascorbic acid. This mixture was then incubated at 37°C for 1 h. About 1 ml of incubated mixture was mixed with 1 ml of 10% TCA and 1 ml of 0.5% TBA (in 0.025 M NaOH containing 0.025% butylated hydroxyl anisole (BHA)) and the development of pink chromogen was measured spectrophotometrically at 532 nm. The hydroxyl radical scavenging activity of the mushroom extract was reported as percentage inhibition of deoxyribose degradation and was calculated by using the formula

\[
\text{OH}^* \text{ scavenged (\%)} = \frac{(A_0 - A_1)}{A_0} \times 100
\]

where $A_0$ was the control absorbance and $A_1$ was the mixture containing the extract absorbance or the standard absorbance.

3.2.3.5. Reducing power assay

The reducing power was determined according to the method of Berker et al. (2010). About 2.5 ml P. florida methanolic extract (100-500 µg/ml) was mixed with 2.5 ml of 200 mM sodium phosphate buffer and 2.5 ml of 1% potassium ferricyanide, and the mixture was incubated at 50°C for 20 min. After the addition of 2.5 ml of 10% trichloroacetic acid the reaction mixture was centrifuged at 3000 rpm for 10 min. About 5 ml of the upper layer was mixed with 5 ml of deionised water and 1 ml of 0.1% ferric chloride and the absorbance was measured at 700 nm against a blank. A higher absorbance indicated a higher reducing power.
IC$_{50}$ value (µg extract/ml) is the effective concentration at which the 0.5 absorbance for reducing power and ascorbic acid was used as a standard.

3.2.3.6. Metal chelating ability of ferrous ions

The chelating of ferrous ions by methanol extract of P. florida was estimated by the method of Hinneburg et al. (2006). The different concentration of extract was added to a solution of 0.05 ml FeCl$_2$ (2 mM/L). The reaction was initiated by the addition of 0.2 ml ferrozine (5 mM/L) and the mixture was shaken vigorously and left at 37°C for 10 min and the absorbance was measured UV-Vis spectrophotometrically at 562 nm. The chelating activity of the extracts was evaluated using EDTA as standard and the results were expressed as µg EDTA equivalent/g extract.

3.3. Isolation, purification and characterization of the active compounds from methanolic extract of P. florida

In phase III, the active components of methanolic extract of P. florida was separated and purified by subjecting the extract to chromatographic techniques (column and thin layer chromatography) and spectral studies including $^1$H - NMR and GC/MS.

3.3.1. Column Chromatography (CC)

Column chromatography was performed by using silica gel 60 - 120, 230 - 400 mesh (Merck) to isolate the active compound from the crude extracts. A column was prepared by adding a mixture of 30 g of silica gel and 40 ml petroleum ether to a column (3 x 18 cm). The silica gel was allowed to settle while petroleum ether was slowly passed through the column. The dried crude extracts of 1 g were dissolved in a minimal volume of petroleum ether and mixed with 5 g of pure silica and added in the column. Later, it was eluted with the solvent systems starting with petroleum ether followed by increasing polarity up to methanol, until all the fractions was eluted (Table 3.1). For each eluent mixture about 20 ml was used and each fraction was collected in a beaker.

3.3.2. Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) analyses were performed with silica gel precoated on aluminium plates (60, F254 Merck) with the dimension of 20 x 20 cm. Crude extract of P.
Florida in methanol was spotted 2 cm away from the bottom of a 20 x 20 cm pre-coated aluminium sheet of silica gel. Glass jars were saturated overnight by the different solvent systems, such as n-hexane, dichloromethane, diethyl ether, ethyl acetate and methanol with following proportions: n-hexane: ethyl acetate: methanol (7:2:1 and 7.5:2:0.5), n-hexane: diethyl ether (8:2) and n-hexane: dichloromethane: methanol (6:3:8:0.2). The spotted aluminium sheets were placed in the glass jar containing solvents and it was run completely. After few min TLC spots were visualized under UV light with short (254 nm) and long (360 nm) wave and in addition, adequate TLC reagents were used to detect the phenolic compounds. Individual Retention factor (Rf) values for each spot were determined (Stahl, 1969).

\[
\text{Retention factor (Rf)} = \frac{\text{Distance travelled by substance}}{\text{Distance travelled by solvent front}}
\]

3.3.2.1. Visualizing agents/ locating agents

TLC plates were visualized by using various reagents like Iodine, Vanillin sulphuric acid reagent and Folin-Ciocalteu reagent and illumination on UV- 254 and 360nm.

Iodine vapour was used for the detection of compounds with conjugated double bonds. A TLC plate was placed into a TLC tank, which had been previously saturated with iodine vapour by the addition of iodine crystals. The TLC plate was removed and evaluated in visible light.

Vanillin sulphuric acid reagent was used for the unspecific detection of flavonoids and saponin like compounds. This reagent consisted of two parts: a) 5% (w/v) ethanolic H₂SO₄ solution and b) a 1% (w/v) ethanolic vanillin solution. The TLC plate was sprayed with 10 ml of solution (a) followed by 5 to 10 ml of solution (b), warmed at 100°C for 5 to 10 min and evaluated under visible light.

The detection of phenolic compounds was attained by using Folin-Ciocalteu reagent and was evaluated in visible light for the presence of blue zones.
3.3.3. Preparative Thin Layer Chromatography

For preparative thin layer chromatography (PTLC) separations, glass plates coated with silica gel with 1mm thickness (Merck) were employed.

For the preparation of PTLC, about 25 g of silica gel was suspended in 50 ml of deionised water and shaken vigorously for 45 sec in rubber-stopper Erlenmeyer flask. The thickened slurry was poured into the glass and pulled with a ruler in two sides at 1mm trailing edge to prepare similar plates. Then, the plates were allowed to air dry for 30 min in the oven at 50°C until they turn white. After preparation of the plates, a few drops of the P. florida methanol extract was applied using a capillary tube to the bottom of activated glass plates. After 5 min of drying, each plate was placed in the separate glass chamber with solvent system of n-hexane: dichloromethane: methanol (6:3.8:0.2) as the mobile phase. After the development, the plates were removed from the glass chamber and air-dried separately. After air-drying, the spots were located by their fluorescence under long and short wave UV light (254 and 360 nm respectively). The fluorescent spots visualized upon UV exposure were scraped off and eluded with methanol, filtered and allowed to dry. Finally, each fraction was tested for antioxidant activity as before to know the active fraction.

3.3.3.1. Isolation of active compound

Preparative Thin layer chromatography (PTLC) was applied for the isolation of the extracted metabolites using silica gel of 10 x 10 cm and Rf values were measured. A further purification of fraction compounds was made by a repeated column chromatography technique. The identification of the purified compounds was made by using GC/MS technique.

3.3.4. GC/MS analysis

Gas chromatography - Mass Spectrum (GC/MS) was adopted for the identification and determination of the molecular weights and structure of the purified bioactive compounds. The methanolic fractions of P. florida were subjected to GC/MS analysis. It was performed on GC Clarus 500 Perkin Elmer systems under the following conditions: Injector temperature 250°C, using a capillary column Elite 5 (100% dimethyl poly siloxane), mass
range 40-500 m/z, oven temperature from 110-280°C at 5°C/min, with initial hold of 2 min and it was detected by using turbo mass detector.

3.3.4.1. Identification of active compounds

Interpretation on mass-spectrum GC/MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The unknown components spectrum was compared with known components spectrum stored in the NIST library. The name, molecular formula, molecular weight, retention time and structure of the components of the test materials were ascertained.

3.3.5. Nuclear Magnetic Resonance (NMR)

The powdered isolated compounds of methanolic extract of P. florida were also subjected to ¹H-NMR. The NMR experiments were partly performed with a Bruker Avance 200 in chloroform (CHCl₃), internal standard TMS or alternatively on a JEOL Eclipse 400 NMR spectrometer. ¹H chemical shifts (δ, ppm) are relative to residual solvent signals. The abbreviation s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet and br = broad are used throughout; coupling constants (J) are reported in Hz.

3.4. Analysis of antioxidant and anticancer activity for the purified active fractions of P. florida mushroom

The purified compounds were subjected to free radical scavenging activity and anticancer activity. MTT (3-(4, 5 dimethyl thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide), SRB (sulforhodamine B) and trypan blue dye assays were used to test the cytotoxicity of the purified compound of P. florida mushroom against human lung cancer (A549) cell line.

3.4.1. Determination of antioxidant activity for the active fractions

Antioxidant activity of the P. florida mushroom fractions was investigated by using DPPH scavenging assay and reducing power of the extract as mentioned in Section 3.2.3.

3.4.2. Determination of anticancer activity of 3-hydroxy-4-methoxy cinnamic acid on A549 cell line

A549 cell line was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in Dulbecco's Modified Eagle Medium (DMEM)
supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml),
streptomycin (100 μg/ml) and amphotericin B (5 μg/ml) in an humidified atmosphere of 5% CO₂ at 37°C until confluent. The cells were dissociated with Trypsin Phosphate Versene Glucose (TPVG) solution (0.2% trypsin, 0.02% EDTA and 0.05% glucose in PBS). The stock cultures were grown in 25 cm² culture flasks (Tarsons India Pvt. Ltd., Kolkata, India).

3.4.2.2. Preparation of test solutions

For cytotoxicity studies, weighed 3-hydroxy-4-methoxy cinnamic acid was separately dissolved in distilled DMSO and the volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this to carry out cytotoxic studies.

3.4.2.3. Determination of cell viability by trypan blue dye exclusion technique

The monolayer cell culture was trypsinized and the cell count was adjusted to 1 x 10⁵ cells/ml using DMEM medium containing 10% FBS. To each of 40 mm petridish, 1 ml of the diluted cell suspension approximately 1, 00,000 cells was added. After 24 hrs, when a partial monolayer was formed, the supernatant was removed, washed the monolayer once with medium and 1 ml of different concentrations of 3-hydroxy-4-methoxy cinnamic acid was added on to the partial monolayer in culture dishes. Dishes were incubated at 37°C for 3 days in 5% CO₂ atmosphere and microscopic examination was carried out and observations were noted every 24 hrs time interval. After 72 hrs, the drug solutions in the wells were removed and cells were trypsinized. The cells were suspended in PBS and centrifuged to separate cell pellet, resuspended in 1 ml of fresh medium and performed dye exclusion test i.e. equal quantity of the drug treated cells and trypan blue (0.4%) were mixed and left for 1 min. It was then loaded in a haemocytometer and viable and non-viable cell count was recorded within 2 min. The percentage growth inhibition was calculated and CTC₅₀ value was generated from the dose-response curves for each cell line (Unnikrishnan and Ramadasan, 1988).

\[
\text{Percentage growth inhibition} = 100 - \left( \frac{\text{Total Cells} - \text{Dead Cells}}{\text{Total Cells}} \right) \times 100
\]
3.4.2.4. Determination of cell viability by MTT and SRB Assays

The monolayer cell culture was trypsinized and the cell count was adjusted to $1 \times 10^5$ cells/ml using medium containing 10% FBS and were used for the determination of cell viability by MTT and SRB assays as described by Francis and Rita (1986) and Philip et al. (1990). The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC$_{50}$) value was generated from the dose-response curves for each cell line.

$$\text{Percentage growth inhibition} = 100 - \left( \frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \right) \times 100$$

Statistical analysis

The biochemical parameters studied were subjected to statistical analysis using SPSS (Version 16). One way ANOVA followed by post–hoc analysis using Duncan’s LSD was adapted to all the other parameters under study to test the level of statistical significance. The results obtained for the various parameters analyzed in the four major aspects and the salient findings made during the study are presented in the next chapter.

3.5. Environmental applications of P. florida mushroom

3.5.1. Removal of Fe$^{2+}$ from ground water using P. florida spent mushroom substrate

Mushroom industries generate a virtually inexhaustible supply of a co-product called spent mushroom substrate (SMS). SMS was used as novel biosorbent for the adsorption of Fe$^{2+}$ in aqueous solution. Spent mushroom substrate (SMS) of P. florida on the removal of environmental contaminant Fe$^{2+}$ was carried out using live, dead and chemically (sodium hydroxide, formaldehyde and ortho-phosphoric acid) modified SMS. These adsorbents were used in different dosage levels such as 0.25, 0.50, 1.0 and 1.50 g/50 ml at different time intervals (10, 20, 30, 40, 50, 60, 70, 80 and 90 min).

3.5.2. Preparation of adsorbents

The spent mushroom substrate (SMS) left after the harvest of P. florida mushroom was collected from Panamarathupatti, Salem, Tamil Nadu, India and it was air-dried and
manually chopped into small pieces. This Pleurotus SMS was stored at room temperature in a sealed plastic bag which was then placed in a dry and dark container (Plate 3.3).

3.5.2.1 Collection of metal contaminated water

The present study area covers the ground water around Salem, Namakkal and Dharmapuri districts. The site is located at the longitude of 78.14°E and the latitude of 11.66°N (Salem), the longitude of 78.17°E and the latitude of 11.23°N (Namakkal) and the longitude of 78.17°E and the latitude of 12.13°N (Dharmapuri) in Tamil Nadu, South India. Nearly 14 water samples were collected and analyzed for significant physico chemical characteristics and Fe$^{2+}$ content using standard method (APHA, 2005). Table 3.2 illustrate the sampling area of Fe$^{2+}$ contaminated water.

Table 3.2. Sampling area and number of Fe$^{2+}$ contaminated water

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Name of the districts</th>
<th>Sampling area</th>
<th>Sample No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Salem</td>
<td>Chalk hills</td>
<td>SW1</td>
</tr>
<tr>
<td>2</td>
<td>Salem</td>
<td>Kanjamalai</td>
<td>SW2</td>
</tr>
<tr>
<td>3</td>
<td>Salem</td>
<td>Thalamalai</td>
<td>SW3</td>
</tr>
<tr>
<td>4</td>
<td>Salem</td>
<td>Steel plant</td>
<td>SW4</td>
</tr>
<tr>
<td>5</td>
<td>Salem</td>
<td>Sithar kovil</td>
<td>SW5</td>
</tr>
<tr>
<td>6</td>
<td>Salem</td>
<td>Dalmia board</td>
<td>SW6</td>
</tr>
<tr>
<td>7</td>
<td>Salem</td>
<td>Panamarathupatti</td>
<td>SW7</td>
</tr>
<tr>
<td>8</td>
<td>Salem</td>
<td>Bothamalai</td>
<td>SW8</td>
</tr>
<tr>
<td>9</td>
<td>Salem</td>
<td>Poolampatti</td>
<td>SW9</td>
</tr>
<tr>
<td>10</td>
<td>Salem</td>
<td>Mecheri</td>
<td>SW10</td>
</tr>
<tr>
<td>11</td>
<td>Namakkal</td>
<td>Kolli hills</td>
<td>NW1</td>
</tr>
<tr>
<td>12</td>
<td>Namakkal</td>
<td>Nainormalai</td>
<td>NW2</td>
</tr>
<tr>
<td>13</td>
<td>Namakkal</td>
<td>Rasipuram</td>
<td>NW3</td>
</tr>
<tr>
<td>14</td>
<td>Dharmapuri</td>
<td>Theerhamalai</td>
<td>DW1</td>
</tr>
</tbody>
</table>

Ground water samples were collected in sterilized plastic container from the iron contaminated areas of Salem, Namakkal and Dharmapuri Districts of Tamil Nadu viz., Kolli hills, Chalk hills, Kanjamalai, Thalamalai, Steel plant, Sithar kovil, Dalmia board, Mecheri,
Panamarathupatti, Bothamalai, Nainarmalai, Theerthamalai, Poolampatti and Rasipuram. The samples were carefully transported to the laboratory and stored at 4°C in refrigerator until for the further analysis.

3.5.3. Removal of Fe$^{2+}$ by adsorption method using P. florida SMS

3.5.3.1. Adsorption of Fe$^{2+}$ by SMS of P. florida

Prior to treat the ground water, a batch mode study was carried out to optimize the efficiency of adsorbent and different environmental factors on Fe$^{2+}$ removal from aqueous solution. The P. florida SMS was used as a potential adsorbent to remove Fe$^{2+}$ from aqueous solution.

3.5.3.2. Preparation of Fe$^{2+}$ aqueous solution

The stock solution of Fe$^{2+}$ was prepared by dissolving 3.04 g of Ferrous Ammonium Sulphate to 100 ml of double distilled water. From this stock solution, 1000 and 100 ppm of ferrous stock was prepared by dissolving with required volume of deionized water. From 100 ppm stock solution, various concentration of Fe$^{2+}$ solution was prepared.

3.5.4. Effect of adsorbent dosage on Fe$^{2+}$ removal

3.5.4.1. Use of live P. florida SMS

For determining the optimal adsorbent dosage for Fe$^{2+}$ removal, 50 ml of aqueous solution containing Fe$^{2+}$ at various concentrations of 4, 6 and 8 ppm were prepared in 250 ml conical flasks and inoculated with various concentration (0.25, 0.5, 1.0 and 1.5 g/50 ml) of adsorbent dosages. Then the flasks were kept in a rotary shaker with 120 rpm at room temperature for up to 90 min. About 1 ml of Fe$^{2+}$ solution was taken at a regular interval of 10 min and analysed Fe$^{2+}$ by standard method using UV-Vis Spectrophotometer (Cyber lab UV100, USA) at 400 nm (APHA, 2005).

3.5.4.2. Use of dead P. florida SMS

To enhance the adsorption rate of Fe$^{2+}$, the SMS of P. florida was autoclaved at 15 lbs for 20 min. Then the SMS was washed with generous amounts of deionized water, filtered using muslin cloth and used as adsorbent for the biosorption process. The study was carried out as the protocol shown in the section 3.5.4.1.
3.5.5. Pretreatment of SMS adsorbent for Fe$^{2+}$ removal studies (Sathishkumar et al., 2008)

3.5.5.1. Preparation of alkali treated P. florida SMS

The 15 g of SMS was transferred into 100 ml of 0.5 N NaOH solution for 30 min and washed with sterile distilled water and used as adsorbent. Various Fe$^{2+}$ concentrations (4, 6 and 8 ppm) were prepared from 100 ppm of Fe$^{2+}$ stock solution and inoculated with various NaOH treated adsorbent dosages (0.25, 0.50, 1.0 and 1.5 g/50 ml) in 250 ml conical flasks. Then the flasks were kept in rotary shaker with 120 rpm at 30°C for up to 90 min. At a regular interval of 10 min, 1 ml of treated solution was taken and used to estimate the Fe$^{2+}$ level.

3.5.5.2. Preparation of acid treated P. florida SMS

Pipette out the 10 ml of orthophosphoric acid mixed with 90 ml of double distilled water. The 15 g of P. florida SMS was transferred into 100 ml of orthophosphoric acid (10%) solution for 30 min. After proper washing with strile distilled water the substrate was used for adsorption study. Various concentrations of 4, 6 and 8 ppm of Fe$^{2+}$ were prepared from 100 ppm of ferrous stock solution and inoculated with various (acid treated SMS) adsorbent dosages (0.25, 0.5, 1.0 and 1.5 g/50 ml) in conical flasks. Then the flasks were kept under agitation with 120 rpm at 30°C for 90 min. At a regular interval of 10 min, 1 ml of Fe$^{2+}$ solution was taken and estimated for the level of Fe$^{2+}$.

3.5.5.3. Preparation of formaldehyde treated P. florida SMS

About 15 g of filtered P. florida SMS was transferred into 100 ml of 15% formaldehyde solution for 30 min. For adsorption study, various Fe$^{2+}$ concentrations (4, 6 and 8 ppm) were prepared from 100 ppm of Fe$^{2+}$ stock solution and inoculated with various (formaldehyde treated SMS) adsorbent dosages (0.25, 0.5, 1.0 and 1.5 g/50 ml) in conical flasks. Then the flasks were kept under agitation with 120 rpm at 30°C for up to 90 min. At a regular interval of 10 min, 1 ml of Fe$^{2+}$ solution was estimated for the level of Fe$^{2+}$.

3.5.6. Effect of various pH on biosorption of Fe$^{2+}$

Adsorption experiments were carried out at a range of pH of the solutions at 3, 5, 7, 9 and 11. The acidic and alkaline pH of the medium has been maintained by adding the necessary amounts of hydrochloric acid and sodium hydroxide solutions. The
parameters like particle size of the adsorbents and temperature were kept constant while carrying out the experiments.

3.5.7. Effect of various temperature on biosorption of Fe$^{2+}$

The adsorption experiments were carried out at four different temperatures viz., 30, 35, 40 and 45°C in a thermo stated shaker machine (Neolab, India). The constancy of the temperature was maintained with an accuracy of ± 0.5°C. The parameters like particle size of the adsorbents and temperature were kept constant while carrying out the experiments.

3.5.8. Determination of Fe$^{2+}$ and significant physicochemical parameters in ground water

The environmental Fe$^{2+}$ contaminated water samples were collected from Salem, Namakkal and Dharmapuri Districts of Tamil Nadu viz., Kolli hills, Chalk hills, Kanjamalai, Thalamalai, Steel plant, Sithar kovil, Dalmia board, Mecheri, Panamarathupatti, Bothamalai, Nainarmalai, Poolampatti and Rasipuram. The significant physicochemical parameters of the Fe$^{2+}$ contaminated water such as colour, odour, pH, total solids (TS), total suspended solid (TSS), total dissolved solid (TDS) and Fe$^{2+}$ were analyzed by using standard prescribed methods (APHA, 2005).

3.5.9. Fe$^{2+}$ removal from ground water SW10 sample using different concentration of live SMS of P. florida mushroom

Among the different samples collected from various places, sample site SW10 showed high amount of Fe$^{2+}$. Further, SW10 sample was processed for adsorption in batch mode and column studies. For determining the optimal adsorbent dosage for Fe$^{2+}$ removal, 50 ml of Fe$^{2+}$ contaminated water SW10 was taken in 250 ml conical flasks and inoculated with various adsorbent dosages (0.25, 0.5, 1.0 and 1.5 g/50 ml). Then the flasks were kept under agitation with 120 rpm at 30°C for up to 90 min. Every 10 min time interval 1 ml of solution was pipette out from all conical flasks and Fe$^{2+}$ was estimated.

3.5.10. Adsorption of Fe$^{2+}$ by P. florida SMS packed in column studies

3.5.10.1. Column experiments

Column studies were carried out at constant bed heights and constant flow rates with the collected Fe$^{2+}$ contaminated water sample (SW10) on the column of 4 cm internal
diameters and 35 cm height of column. In column, the SMS bed height was adjusted in to 10 cm and flow rate was fixed at 2.5 ml/min. The experiment was carried out with Fe$^{2+}$ contaminated sample. The samples were collected from the outlet of column at a time interval of 10 min up to 3 h and analyzed for Fe$^{2+}$ concentration by standard method using UV-Vis Spectrophotometer (Cyber lab UV100, USA) at 400 nm.

3.5.11. Adsorption kinetics study

Kinetic experiments using untreated (live) P. florida SMS was conducted under similar conditions already mentioned and the samples were withdrawn at regular intervals for analysis. The pH of the solution was monitored continuously with a pH electrode and adjusted with 0.1N HCl or 0.1N NaOH solution, if deviations were observed.

In order to investigate the sorption kinetics of Fe using live SMS of P. florida, the most commonly used pseudo first order and pseudo second order rate equations (Xu et al., 2012) were employed to fit the experimental data obtained from batch metal iron removal experiments.

A simple pseudo first order rate equation is expressed as

$$\log (q_e - q_t) = \log q_e - \frac{k_1 t}{2.303}$$

------------ (1)

Where $q_e$ and $q_t$ are the amount of Fe$^{2+}$ (mg/g) adsorbed at equilibrium and at time $t$ at time $t$ respectively, and $k_1$ is the first-order rate constant (/min). The value of $k_1$ was calculated from the slope of the plot of log ($q_e - q_t$) versus $t$.

In addition, the pseudo second order model is also widely used. There are four types of linear pseudo second order kinetic models the most popular linear form is

$$\frac{t}{q_t} = \frac{1}{h b} + \frac{t}{q_e}$$

------------ (2)

Where $k_2$ is the second order adsorption rate constant (g/mg/min), and $q_e$ is the adsorption capacity calculated by the pseudo second order kinetic model (mg/g). The constant $k_2$ is used to calculate the initial sorption rate $h$= $k_2 q_e^2$ (mg/g/min). The application of the pseudo second order kinetics by plotting $t/q_t$ versus $t$ yielded the second order rate constant $k_2$. 

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