# Chapter-3

# Materials and Methods

## 3.1 Fungal material

Fungal collections were done from various places during the rainy season as fungi are available more at that time. The materials were collected in sterile bags and stored at 4° C for the study. *S. stipitatum* is a rare fungus and are seen only in termites' nest under the soil (Figure 1).

The cross-section of this species shows a dark black outer covering. It is amorphous in nature. Inner to the black surface, there is a layer of elongated rectangular cells representing the endodermis. Internal to the endodermis, is closely packed club-shaped cells showing bright white color and representing the pith region. In the early stages of this fungus, the pith region is vacuolated, but it is filled with white tissue at its maturity.

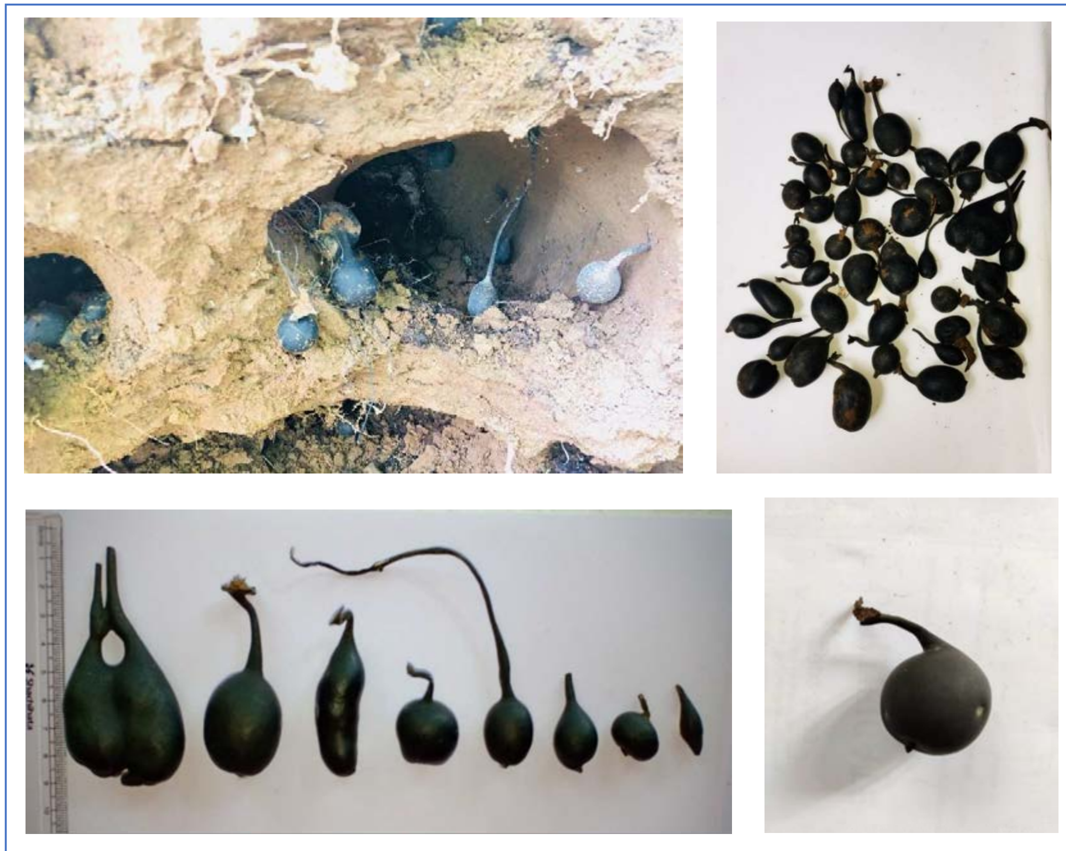
Their posterior end becomes notched and flattened at their maturity. At their early young stage, the posterior end becomes pointed and produces a beak-like structure. In its early stage, it stands erect singly in an underground termite hole. The inner white part of the fungus is edible and taste like a mushroom. It is also a healthy, nutrient-dense meal. Many chronic illnesses and nutritional deficits are cured by it. It remains fresh at the time of collection, and gradually freshness diminishes in contact with an open environment and becomes dry and dead within few days.

## 3.2 Molecular characterization

*DNA extraction*

DNA was extracted from fungal specimens using the modified CTAB method (Lee *et al*., 1988; Wu *et al*., 2001). The DNA extracted was quantified with a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA) at 260 nm.

**Figure 1.** Photographs of *S. stipitatum*



*PCR amplification and sequencing of ITS barcode region*

Working concentration of genomic DNA from fungal specimens was prepared by diluting the stock solution at a concentration of 25 ng/µL. PCR amplification of the fungal DNA was performed using the Internal Transcribed Sequence (ITS) nuclear barcode region of ribosomal DNA (rDNA). 25 µL of PCR reaction mixture comprised of 2.5 µL PCR buffer at 1X (supplied with10X concentration), 1 µL each of forward (5'-TCCGTAGGTGAACCTGCGG-3') and reverse primers (5'-TCCTCCGCTTATTGATATGC-3') (5 pmol), 2.5 µL of dNTPs from 10 mM stock, 2 U/25 µL of Taq-polymerase, 1 µL template DNA with the concentration of 25 ng/µL and the final volume of the PCR reaction mixture was made up to 25 µL with sterile distilled water. PCR reaction was performed with the following conditions, initial denaturation of 5 mins at 94° C, cycle denaturation of 1

min at 94° C, cycle annealing of 1 min at 60° C and cycle extension of 1 min at 72° C for 35 cycles and a final extension at 72° C for 10 mins. PCR products were resolved by 2% agarose. Electrophoresis was performed on agarose gel by applying a constant voltage to resolve the products and documented with Alpha Imager (Alpha Innotech, USA). PCR reaction was scaled up to 50 µL volume for the purpose of elution. Elution of the PCR product was done by Nucleospin gel and PCR clean-up kit as per the manufacturer's protocol (Machery-Nagel, U.S.A.).

DNA sequencing was performed using Sanger's dideoxy method for the eluted PCR products in both forward and reverse directions. Sequences were edited using Bioedit software (Biological sequence alignment editor) (Hall, 1999). They were aligned using Clustal X (version 2.0.11) with default parameters for species-specific nucleotide changes, including SNPs (single nucleotide polymorphism) in each of the analysed sequences (Larkin *et al*. 2007). The consensus maximum likelihood (ML) tree was constructed using the aligned ITS nucleotide sequences from 2 specimens each of *Sclerotium stipitatum* and *Xylaria hypoxylon* (both collected in this study) (*Xylaria hypoxylon* is used, as *Xylaria acuminatilongissima* is not available in India) with default parameters in Molecular Evolutionary Genetics Analysis (MEGA) software package version 4.0 (https://www.megasoftware.net/). Bootstrap percentages from 1000 replications are indicated.

**3.3 Preparation of fungal extracts**

The collected fungi were cut into small pieces and dried using a hot air oven for 24 hrs at 75°C. Then it was grinded and powdered using sterile mortar and pestle and a mixer. The powder was then used for the extraction using a soxhlet apparatus in petroleum ether, chloroform, acetone, and ethanol. Also, aqueous extract was prepared. These extracts were stored in refrigerator at 4°C.

### 3.3.1 Chemical analysis

Qualitative estimation

The powdered drugs were subjected to different qualitative tests to identify phytoconstituents (Kokate, 1990). Extraction is done by the soxhlet apparatus. The solvents were removed by distillation over a water bath. The extracts got were subjected to chemical screening. They are based on the chemical reaction between drug (in powder form or extracted) and selected chemical reagents resulting in a clearly distinguishable color or a precipitate or fluorescence (Kokate, 1993). The different extracts were subjected to preliminary chemical screening for the determination of major chemical groups by standard procedures.

**Test for Alkaloids**

*Mayer's test*: Alkaloids produce a cream-colored precipitate when mixed with Mayer's reagent (potassium mercuric iodide solution).

*Wagner’s test*: Wagner’s reagent (Iodine potassium iodide solution) gives reddish-brown precipitate when mixed with alkaloids.

**Test for Tannins**

*Braemer’s test:* 2 mg of the extract and 3 mL of 1% ferric chloride solution when mixed, develops blue color if hydrolyzable tannin is present, produces green color if condensed tannins are present.

*Lead acetate test:* 5 mL of the extract and 1 mL of lead acetate solution is mixed. Tannins give a flocculent white precipitate.

*Test for catechin:* A matchstick is dipped in the test solution, dried, and moisten with Conc. HCl. Then stick is warmed near the flame. Phloroglucinol changes the wood color to pink.

**Test for Flavonoids**

*Alkaline reagent test:* Few drops of sodium hydroxide solution are added to the test solution, an intense yellow color is produced. When HCl has been added, it turns colorless. It indicates the presence of flavonoids.

*Lead acetate test:* Few drops of 10% solution of lead acetate are added to the alcoholic solution of the extract. Flavonoids give a yellow precipitate.

**Test for Phytosterols**

*Salkowski Test:* In a test tube 1mL of conc. sulphuric acid is added to 0.5 mL chloroform extract through the sides of the test tube. Phytosterols gives reddish-brown color in the chloroform layer.

**Test for Phenols**

To 1 mL of water, about 5 mg of the extract was dissolved and 5 drops of 10% ferric chloride solution was added to it. Phenol indicates the development of dark brown color.

**Test for Glycosides**

*Keller- Killiani Test:* Chloroform extract is brought to dryness and added about 0.4 mL of glacial acetic acid having a trace amount of ferric chloride. It is transferred to a small test tube. Then about 0.5 mL of conc. sulphuric acid ($H_2SO_4$) is added carefully through the sides of the test tube. Glycosides indicate blue color on the acetic acid layer.

**Test for Saponins**

*Foam test:* About 0.5 g of the extract and 5 mL of distilled water are mixed and shaken well and observed for stable, persistent froth. Saponins indicate the presence of emulsion.

**Test for Coumarins**

2 mL of extract taken in a test tube was treated with alcoholic NaOH or KOH. Coumarins give dark yellow color.

**Test for Anthraquinones**

*Borntrager's test:* Extract is boiled with 1mL of sulphuric acid for 5 mins in a test tube. Filter it while hot. Cool it and shake well with an equal volume of chloroform. Separate the lower layer of chloroform and shake it with half of its volume of dil. ammonia. A rose-pink to red color is produced in the ammoniacal layer indicate the presence of Anthraquinones.

**Test for Resins**

The extract is treated with conc. sulphuric acid gives red or reddish-brown color, when mixed with 50% $HNO_3$, forms green color.

**Test for Triterpenoids**

*Libermann- Burchard test:* Treat the extract with few drops of acetic anhydride, boil and cool. Then add conc. sulphuric acid ($H_2SO_4$) through the side of the test tube, the brown ring is formed at the junction between two layers and upper layer turns green which shows the presence of steroids and formation of brown color indicates presence of triterpenoids.

**Test for Lactones**

*Legal's Test:* To the extract mixtures, add sodium nitroprusside & pyridine. Then the mixture is treated with NaOH. The appearance of blood-red color indicates the presence of lactones.

**Test for Volatile oil**

To a thin section of drug, add Sudan lll solution, red color obtained by globules indicates volatile oil.

**Test for Naphthoquinones**

*Juglone test:* Take 2 mL of chloroform extract and 2 mL of ethyl ether with dil. ammonia solution. The pink color indicates naphthoquinones.

**Test for Aleurone grains**

Add few drops of alcoholic iodine solution to the aqueous test solution. Yellowish-brown to brown color indicates the presence of aleurone grains.

**Test for Sterol**

*Libermann- Burchard test:* A few quantities of powder are dissolved in chloroform & a few mL of acetic anhydride. After that one or two drops of conc. $H_2SO_4$ is added. Reddish-brown color indicates the presence of sterol.

**Test for Carbohydrates**

*Molisch's test:* Add a few drops of alcoholic α-naphthol to the test solution, then a few drops of conc. sulphuric acid through the walls of the test tube. A purple to violet hue ring emerges at the junction of two layers, indicating the presence of carbohydrates.

*Benedict's test:* Take 5 mL of Benedict's reagent & add few drops of 1% glucose solution. Boil & note the color change. The color of the mixture turns red-orange after heating. The depth of the color of the mixture may vary depending on the concentration.

*Barfoed's Test:* 1 mL of test solution is heated with 1 mL of Barfoed's reagent on water bath, if brick red cupric oxide is formed, monosaccharide is formed. Disaccharides on prolong heating may also cause reduction, owing to partial hydrolysis to monosaccharides.

*Seliwanoff's test:* To an equal volume of test solution added 5 mL of Seliwanoff's reagent & boiled. A cherry red color was obtained.

**Test for Polysaccharides**

*Iodine test:* To a small quantity of test solution, is added few drops of dilute iodine solution. As a result, a blue-colored solution was formed. Starch gives blue color with iodine due to the formation of starch- iodine complex.

*Molisch's test:* To a 5 mL of hydrolyzed starch solution, added two drops of Molisch's reagent & 2 mL of conc. $H_2SO_4$, without shaking, sulphuric acid along the sides of the test tube forms a layer of acid beneath the solution. A purple ring is formed in the junction of two layers.

*Benedict's test:* To 5 mL of benedict's reagent added 8 drops of the hydrolyzed starch solution add heated in a water bath for 5 mins. A brick-red precipitate was obtained.

*Seliwanoff's test:* To an equal volume of hydrolyzed starch solution added 5 mL of Seliwanoff's reagent & boiled. No cherry-colored solution was obtained.

**3.3.2 GC-MS analysis**

GC-MS analysis was performed on a Thermo GC-Trace Ultra Version: 5.O, Thermo MS DSQ II and gas chromatograph linked to a mass spectrometer (GC-MS) instrument under the following conditions: column DB 5- MS Capillary standard non-polar column (Sample ID: EM - 473), helium (HE) was used as carrier gas at a static flow of 1.0 mL/min, dimension was used as 30 MTS, ID: 0.25 mm Film: 0.25 μM. The oven temperature was set from 70° C raised to 260° C at 6° C/min. Injection volume taken was 1 μL.

**3.3.3 LC-MS analysis**

The components in the extract were identified using the LC-MS method. HR-LCMS analysis was performed at IIT Bombay's Sophisticated Analytical Instrument Facility. Agilent Technologies 1290 Infinity UHPLC system and liquid

chromatograph interfaced to a Mass Spectrometer (LC-MS) equipped with electro-spray ionization source and zorbax eclipse column (C18, 2.1 150 mm, 5- micron) were used for the analysis. The injection volume was 5 µL. The sample was introduced through direct infusion at a flow rate of 0.200 mL/min, and it was analyzed in both positive and negative ionization modes from 50 to 1000 m/z. A TOF/Q-TOF mass spectrometer with a dual AJS ESI ion source was utilized for LC-MS detection. The following tuning parameters were used in the analysis: gas temperature (250° C), gas flow (13 L/min), and nozzle voltage (1000 V). The total time spent running the LC was 30 mins. The compounds' structure, molecular weights, and retention times were calculated and compared to the METLIN database for identification.

### 3.4 In vitro cytotoxicity study

In vitro cytotoxic assays were done in prior to in vivo antitumor studies. The in vitro cytotoxicity studies were conducted in Amala Cancer Research Centre, Thrissur. First, the ethanol fungal extract was studied for short term in vitro cytotoxicity using Dalton’s Lymphoma Ascites cells (DLA), Ehrlich Ascites Carcinoma (EAC) cells. Then about 5 mg of extracts were dissolved in 1 mL of distilled water. This was used as a stock solution.

The tumor cells (DLA and EAC) were aspirated from tumor-bearing mice's peritoneal cavity. They were washed thrice with PBS or normal saline. Cell viability was studied by trypan blue dye exclusion method (Babu et al. 1995). From the stock solution, different dilutions were made in different test tubes: 10, 20, 30, 50, 100 and 200 µL, respectively. In those tubes, phosphate-buffered saline (PBS) was used to make up to 1 mL of viable cell suspension ($1x10^6$ cells in 0.1 mL). Control tube contained only cell suspension. These assay mixtures were incubated for 3 hrs at 37º

C. Further, 0.1 mL of 1% trypan blue was added to the cell suspension and allowed to sit for 2-3 mins before being put into a hemocytometer. The dead cells take up the blue color of trypan blue, but live cells do not take up the dye. The number of unstained and stained cells were counted separately.

Percentage of cytotoxicity = Number of dead cells / (Number of live cells + Number of dead cells) × 100.

**3.5 Animal Experiments**

All the animal experiments were conducted at Amala Cancer Research Centre, Thrissur. They were bought from Small Animal Breeding Station, Kerala Veterinary and Animal Sciences University, Mannuthy, Kerala, India. Swiss albino mice (20–25 g b.wt.) of both sexes were used for the experiments. They were brought and housed at Amala Cancer Research Centre with standard experimental conditions (Temperature: 22-30º C, relative humidity: 60 -70% and 12 hrs of dark/light cycle). They were maintained in polypropylene cages with stainless steel top for providing food and water and paddy husk for bedding. They were fed with normal standard rat feed and water ad libitum.

*Preparation of drug*

The ethanol extract was concentrated until they were completely dry, and the residue was dissolved in water and it was used as the drug.

**3.5.1 Toxicity Study**

Before moving on to the in vivo animal experiments toxicity study was done to understand the toxicity of the drug. For that 6 swiss-albino mice were used. 3 males and 3 females. Acute toxicity study was conducted. ie, a higher concentration of the drug was given as a single dose. The concentration used was 2 gm/kg

b.wt. *ie,* 50 mg/animal as per OECD guidelines. After administering the drug orally, the mortality rate of the mice was noted.

**3.5.2 In vivo antitumor Study**

The antitumor activity of the extract was studied by DLA induced solid tumor model and EAC induced ascites tumor model.

**3.5.2.1 DLA-induced solid tumor model**

The experimental Swiss albino mice were categorized into four groups, each with six animals.. On the first day, DLA cells were aspirated from the peritoneal cavity of the tumor-bearing mice, and the solid tumor was induced by injecting 0.1 mL of the cell lines ($1\times10^6$ cells/animal) intramuscularly to the right hind limb of the animals. The control group received DLA cells only, the standard with DLA cells and cyclophosphamide drug while the treated groups were administered with DLA cells and low and high doses of fungal extract. Animals were grouped as follows. (6 nos in each group)

Group I - Control: DLA cells alone

Group II - Standard: Cyclophosphamide (10 mg/kg b.wt)

Group III - Low dose of *S. stipitatum* (50 mg/kg b.wt)

Group IV - High dose of *S. stipitatum* (200 mg/kg b wt)

The drugs were orally administered for 10 consecutive days. Using Vernier calipers the diameter of the developing tumor was measured at fixed interval of 3 days for 30 days and the tumor volume was calculated using the formula:

$$\text{Tumor Volume} = 4/3\ \pi\ r_1^2 \times r_2$$

Where $r_1$ represents the minor radius and $r_2$ represents the major radius of the tumor in two different planes (Rajesh Kumar *et al*. 2002). Then the percentage inhibition of tumor volume was calculated using the formula:

Percentage of inhibition =

[(Tumor volume of the control on 30th day – Tumor volume of the treated on 30th day) / Tumor volume of the control on 30th day] x 100

**3.5.2.2 EAC-induced ascites tumor model**

The experimental Swiss albino mice were divided into four groups, each with six animals. On the first day, EAC cells were aspirated from the peritoneal cavity of the tumor-bearing mice, and the ascites tumor was induced by injecting 0.1 mL of the cell lines ($1\times10^6$ cells/animal) intraperitoneally to the animals. The control group received EAC cells only, the standard with EAC cells and cyclophosphamide drug while the treated groups were administered with EAC cells and low and high doses of fungal extract. Drug was administered for 10 consecutive days. Then the life span of mice was monitored.

Animals will be grouped as follows. (6 nos in each group)

Group I - Control: EAC cells alone

Group II - Standard: Cyclophosphamide (10 mg/kg b.wt)

Group III - Low dose of *S. stipitatum* (50 mg/kg b.wt)

Group IV - High dose of *S. stipitatum* (200 mg/kg b.wt)

Drug was administered for 10 consecutive days after the implantation of the tumor. Then the life span of mice was monitored. The percentage of increase in the life span was calculated with the formula:

Percentage increase in life span = [(T- C) / C] x 100

Where T and C are mean survival of treated and control mice respectively (Joy *et al*., 2000)

### 3.5.3 In vivo antioxidant Study

It is done by evaluating the protective effect of the drug against sodium fluoride (NaF) induced oxidative stress. Male Swiss albino mice of weight 20-25 g were chosen for the study. Animals were divided into 5 groups. Each contain 6 animals.

Group 1: Untreated/ Normal

Group 2: Control/ NaF alone

Group 3: Standard/ Vitamin C (15 mg/kg b.wt) + NaF

Group 4: Drug high dose + NaF

Group 5: Drug low dose + NaF

Standard drug and fungal extract drug were given orally for 7 days. From 8th day onwards along with the drug, NaF (600 ppm, 60 mg/100 mL) was administered in the drinking water and continued for 7 consecutive days. Then the final weight for animals were taken. Then on $15^{th}$ day animals were sacrificed after overnight fasting and blood and liver of animals were collected by cardiac puncture. Then the antioxidant status of enzymes like catalase, superoxide dismutase, glutathione in the liver and blood were analysed.

Determination of haemoglobin (Hb) content – Cyanmethaemoglobin method

Haemoglobin when reacts with Drabkin’s fluid containing ferricyanide forms methaemoglobin, which is converted to cyanmethaemoglobin by cyanide. The haemoglobin count was determined by mixing 10 μL of the blood sample with 2.5 mL of Drabkin’s fluid which was allowed to react for 15 mins. The optical density was determined at 546 nm and compared with the standard. The standard solution depicts optical density corresponding to 60 mg/dL haemoglobin while the optical density of the sample is directly proportional to the amount of haemoglobin present in blood.

Hb Concentration (gm/dL) = (Abs. of sample/Abs. of standard) x 15

*Preparation of tissue homogenate*

The collected liver was thoroughly rinsed with normal saline and kept on a filter paper and blotted gently and the weight was noted. Then prepared 10% tissue homogenate by homogenizing 1g tissue and 10 mL 0.1 M tris- HCl buffer using a polytron homogenizer. A portion of the homogenate was collected and preserved and used directly for the estimation of lipid peroxidation assay. The other portion of the homogenate was centrifuged at 8000 rpm for 30 mins in a cooling centrifuge for eliminating the cell debris, unbroken cells, nuclei and mitochondria. Then the supernatant was collected and preserved for the estimation of CAT, SOD, GSH, GPx, GR etc.

*Estimation of superoxide dismutase (SOD) activity in blood and Tissue*

SOD was determined by the (McCord and Fridovich, 1969) method with slight modifications.

Principle:

During the reaction of photo reduced riboflavin with oxygen, superoxide radical is generated which can inhibit the reduction of nitro blue tetrazolium (NBT). The assay relies on the capacity of SOD for this inhibition.

Procedure

A volume of 100 µL of tissue supernatant or blood was mixed with 200 µL of 0.0015% KCN in .1 M EDTA, 100µL of 1.5 mM NBT and 2550 µL of 67 mM K-Na phosphate buffer (pH-7.8). Then 50 µL of 0.12 mM riboflavin was added in dark. And the absorbance was read immediately at 560 nm. It is considered as the initial reading. A tube devoid of test sample with extra volume of phosphate buffer was kept as the control. The tubes were then kept under an incandescent lamp for 15 mins

and uniformly illuminated. Then the absorbance was read and recorded as the final reading. The percentage inhibition was then estimated by comparing the percentage change in absorbance between the test and control groups. For blood and tissue samples, the concentration of the sample necessary to scavenge 50% of the produced superoxide anion was considered as 1 unit of enzyme activity and was represented as U/g Hb and U/mg protein, respectively. The percentage difference between AT (absorbance of the test) and control can be used to calculate percentage inhibition (AC). The activity can be obtained by the equation,

SOD activity in blood (U/g Hb) = % inhibition x 1750/ 100 x 50 x Hb

SOD activity in tissue (U/mg protein) = % inhibition / 50 x mg protein (in 100 μL)

*Determination of catalase (CAT) activity in the blood and tissue*

With minor modifications, the activity of catalase in the blood and tissue was assessed using the protocol of (Beers and Sizer, 1952).

Principle

The decomposition or breakdown of hydrogen peroxide by catalase can be observed as the absorbance decreases in the ultraviolet range, 240 nm spectroscopically.

Procedure

The estimation of tissue catalase: Dilute hydrogen peroxide solution (13μL, 30 mM peroxide/ 10 mL phosphate buffer) was used as control. And its absorbance was read against buffer blank. It is considered as the initial OD. After adding 15μL of cleared tissue sample, the reduction in light absorption of peroxide solutions was evaluated at 25° C in 30 sec intervals for 3 mins at 25° C. The activity of catalase in tissue can be calculated as:

Specific activity of Catalase (U/mg protein) = ΔA/min x 3000 / 43.6 x mg protein

Where, ΔA/min= Decrease in absorbance per minute

3000 = dilution factor

43.6 = molar extinction coefficient of $H_2O_2$

Catalase in hemolysate: About 10 µL of hemolysate was diluted to 2.5 mL of phosphate buffer, pH 7. In a quartz cuvette, add 2 mL of this and 1 mL $H_2O_2$ (30 mM). The decrease in light absorption of peroxide solutions was measured in the 30 sec interval for 3 mins at 25 ° C. The difference in absorbance is termed as ΔA. The activity of catalase in hemolysate was calculated as:

Catalase activity (U/g Hb) = A/min x 1000 x 3/ 43.6 x g Hb

*Estimation of lipid peroxidation in tissue*

The degree of lipid peroxidation in tissue was assessed using the Ohkawa *et al*., (1979) protocol, which assessed the formation of thiobarbituric acid reactive substances.

Principle

During the peroxidation of polyunsaturated fatty acids, lipid carbonyls are formed, which can react with the thiobarbituric acid (TBA) reagent in an acidic reaction mixture to generate a pink-colored product with a maximum absorbance at 532 nm.

Procedure

The tubes with reaction mixture contain 200 µL tissue homogenate, 100 µL of 8% SDS, 750 µL of 20% acetic acid (pH-3.4) and 750 µL of TBA. This is incubated at 95ºC for 1hr. Then the tubes are cooled underwater and 300 µL of distilled water is added to the mixture. Mixed well and centrifuged at 2000 rpm for 5 mins. Then the absorbance of the supernatant is read at 532 nm against the blank. The concentration

of MDA was measured from the standard graph plotted at concentrations ranging from 1-10 nmol and expressed as nmol of MDA equivalent/mg protein.

*Estimation of glutathione peroxidase (GPx) activity in tissue*

Glutathione peroxidase was estimated by the method of Hafeman *et al*., (1974).

Principle:

In the presence of glutathione (GSH), glutathione peroxidase depletes H2O2. The GSH remaining is measured using 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), which gives a colored complex.

Procedure:

A volume of 50 μL tissue homogenate, 50 μL of 5mM GSH, 50 μL of 25 mM NaN3 and 50 μL of 2.5 mM $H_2O_2$ and 800 μL of phosphate buffer (pH-7) was mixed well and then incubated for 15 mins at 37º C. Then the reaction was stopped by adding 1 mL of 1.67% of $H_2PO_3$. Then the above reaction mixture is centrifuged for 10 mins at 8000 rpm. Then to 1 mL of supernatant 1 mL of $Na_2HPO_4$ and 500 μL of DTNB is added. It is incubated for 10 mins. The intensity of yellow color formed was read at 412 nm. The enzyme activity is expressed as units/mg protein.

*Estimation of glutathione (GSH) content in blood and tissue*

Followed the methodology of Moron *et al.* (1979) was with slight changes.

Principle:

GSH is calculated by its reaction with DTNB, which produces a yellow colored complex with maximum absorption at 412 nm wavelength.

Procedure:

500 μLof the test sample (hemolysate or tissue sample) was combined with 125 μL of 25% TCA and cooled for 5 mins on ice. 600 μL of 5% TCA was added to the mixture and centrifuged for 10 mins at 2000 rpm. The supernatant was combined

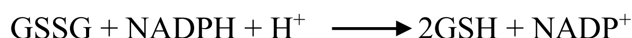
with 700 µL of 0.2 M sodium phosphate buffer (pH 8.0) and 2.0 mL of newly produced 0.6 mM DTNB in a total volume of 300 µL. At 412 nm, the absorbance was measured. The GSH content of the sample was determined using a standard graph drawn for different concentrations of GSH values ranging from 10 to 50 nmoles. The results are given in nmol/mL of blood or nmol/mg of tissue protein.

*Estimation of glutathione reductase (GR) activity in tissue*

Glutathione reductase was estimated by the method of Racker (1955).

Principle:

GR activity was estimated by the amount of NADPH consumed in the conversion of oxidised glutathione (GSSG) to reduced glutathione (GSH). The reaction catalyzed by GR.



$$GSSG + NADPH + H^+ \longrightarrow 2GSH + NADP^+$$

Procedure:

The reaction mixture contains 100 µL EDTA (10 mM), 100 µL GSSG (10 mM), 50 µL of serum and sodium phosphate buffer (1 M, pH-7.0) in a total volume of 900 µL. It was then incubated for 5mins at 37˚C, followed by the addition of 100 µL NADPH (2 mM) and decrease in optical density was measured at 340 nm for 5 mins with an interval of one minute. The activity of GR was calculated using the molar extinction coefficient of 6.22 mm-1cm-1 and expressed as nanomoles of NADPH consumed/min/mg protein.

**3.5.4 In vivo anti-inflammatory study**

For understanding the anti-inflammatory activity of the drug 2 models were studied. Acute carrageenan-induced model and chronic formalin-induced model.

### 3.5.4.1 Acute carrageenan-induced model

Male Swiss albino mice were divided into four groups. First group was kept as vehicle control. The second group was treated with standard drug (diclofenac). Third and fourth group was treated with higher and lower dose of fungal extract respectively.

Animals were pretreated orally with respective drug for 5 days. On the $6^{th}$ day, animals were administered with drugs and to the standard group, diclofenac was induced intraperitoneally (10 mg/kg b.wt.). Diclofenac was administered only on the $6^{th}$ day. Carrageenan was induced in the right hind paw of each mouse after 1 hour, by a subplantar injection of 0.02 mL of 1 % w/v carrageenan in 0.1% carboxymethylcellulose (CMC) (Winter *et al*., 1962). The paw thickness was measured with a vernier caliper, 1 hr before and up to the $6^{th}$ consecutive hour followed by $24^{th}$ and $48^{th}$ hours respectively after carrageenan administration. The percentage inhibition of paw thickness was calculated by the formula:

Percentage inhibition of thickness = [(tCn-tC0) – (tTn-tT0) / tCn – tC0] x 100

Where, tCn- paw thickness at particular time period of control animal

tC0- paw thickness before induction of control animal

tTn- paw thickness at particular time period of treated animal

tT0- paw thickness before induction of treated animal

### 3.5.4.2 Chronic formalin-induced model

As in the case of carrageenan-induced model, here also the animals were grouped into 4. Six animals in each group. And they are pretreated with drugs for 5 consecutive days. And on the 6th day after administering the drug, freshly prepared 2% formalin was injected in the right hind paw. The paw thickness was measured with a vernier caliper before and after injecting the formalin. The edema was

measured everyday up to 7 days. Then the percentage of inhibition was calculated using the below formula.

Percentage inhibition of thickness = [(tCn-tC0) – (tTn-tT0) / tCn – tC0] x 100

Where, tCn- paw thickness at particular time period of control animal

tC0- paw thickness before induction of control animal

tTn- paw thickness at particular time period of treated animal

tT0- paw thickness before induction of treated animal

### 3.5.5 Statistical analysis

All the in vitro and in vivo experiments were repeated at least thrice, and the values were expressed in Mean ± SE. The data obtained were statistically analyzed with significance difference of values ($P<0.0001$, $P<0.001$, $P<0.01$and $P<0.05$) using one-way ANOVA followed by an appropriate post hoc test (Dunnett's multiple comparison test) using Prism 9.0 GraphPad software Inc. USA.

## 3.6 Mycosynthesis of silver nanoparticles

30 g of dried powder of fungi were weighed and transferred into a 500 mL beaker containing 300 mL distilled water and boiled for about 20 mins. The extracts were then filtered thrice through muslin cloth to remove particulate matter and get clear solutions that were then refrigerated in 250 mL Erlenmeyer flasks for further experiments. In every step of the experiment, sterility conditions were maintained for the effectiveness and accuracy in results without contamination.

*Procedure*

For the preparation of silver nanoparticles (AgNPs), a 1 mM aqueous solution of silver nitrate was prepared. The reaction medium contained 30mL of aqueous extract of *S. stipitatum* and 270 mL of 1 mM silver nitrate aqueous solution, The reaction was then allowed to continue for 24 hrs at room temperature.

Characterization of silver nanoparticles are essential in order to determine the functional aspects of produced particles. UV- Visible spectroscopy, Scanning Electron Microscopic (SEM) analysis, Transmission electron microscope (TEM) analysis and X-ray diffraction technique are among the analytical techniques used for characterization (Zhang *et al.,* 2016).

**3.6.1 UV-Visible Spectra analysis of silver nanoparticles**

UV- Visible spectroscopy is commonly used to examine the intensity of nanoparticles in aqueous suspensions (Wiley *et al*., 2006). The reduction of pure $Ag^+$ ions was monitored by measuring the UV-Vis spectrum of the reaction medium after 24 hrs, after diluting a small aliquot of sample into distilled water. UV-Vis spectral analysis was done using UV-Vis Spectrophotometer (ELICO-SL-191).

**3.6.2 SEM analysis of silver nanoparticles**

Scanning Electron Microscopic SEM can be used to determine the size and shape of nanoparticles (Fissan *et al*., 2014). SEM analysis was done using the PHILIPS-XL-30SEM machine. The films of sample were prepared on a carbon-coated copper grid by just dropping a minute quantity of the sample on the grid, the extra solution was removed using a blotting paper, and then the films on the SEM grid were allowed to dry by putting under a mercury lamp for 5 mins.

**3.6.3 TEM analysis of silver nanoparticles**

Transmission electron microscope (TEM) technique was used to visualize the morphology of the AgNps (Lin *et al.,* 2014). This method allows to observe the particle size of material in nano-dimensions and study the crystal structure meticulously. In a high-resolution transmission electron microscope, a thin sample or specimen is irradiated with a sharp, high electron beam (usually in the range of 100-200 KV).

### 3.6.4 XRD Analysis of silver nanoparticles

X-ray diffraction is an analytical technique that provide detailed information about the chemical composition, crystallographic structure and physical properties of particles. XRD was used to examine the silver nanoparticles' crystalline structure (Lin *et al.,* 2014). XRD patterns were recorded on Phillips PW- 1710 automated diffractometer using a Cu tube operated at 40 kV and 35 mA.