CHAPTER III

MATERIALS AND METHODS

3.1. Study area:

Kokrajhar is among the four districts of BTAD, Assam. Surrounded by Bhutan in the north, Bongaigaon district in the east, Dhubri district in the south and West Bengal on the west. It is situated in the western bank of Brahmaputra and western part of Assam. The area is mainly inhabited by the ethnic tribal groups (Bodos, Rabhas, Garos) besides, Assamese, Adivasis, Bengalis, Nepalese and Santhalis etc.

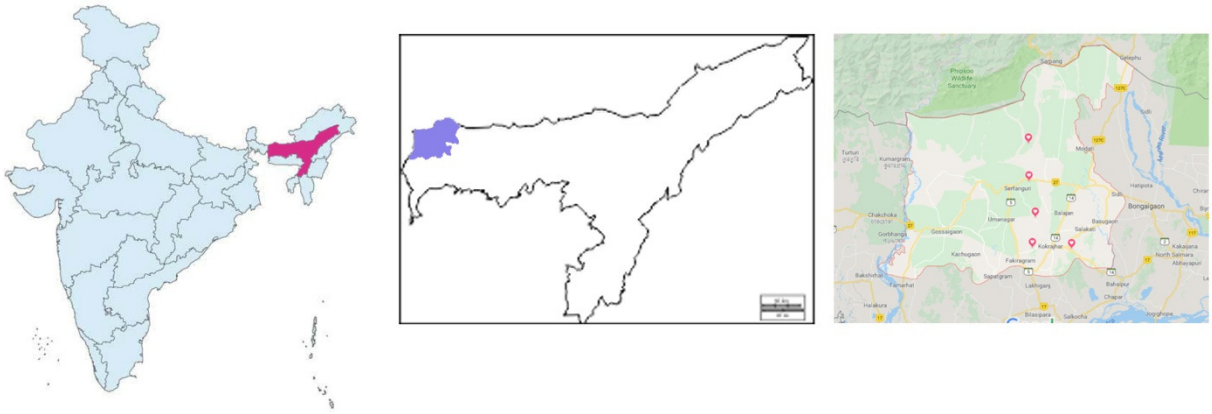

Figure 3.1: Study Area.

3.2. Climatic conditions:

Kokajhar witnesses a subtropical climate having four seasons in a year, average rainfall ranges from 2300-3000 mm. The rainy seasons starts from April and lasts till September with temperature ranging from 28-35 ºC during summer and from 9- 20 ºC during winter. Some parts have dense forest with mixed vegetation, dominated by trees of different species. Community managed forests dominated by *Shorea robusta* can be witnessed in many areas of Kokrajhar.

3.3. Sample collection:

The samples were collected from different parts of Kokrajhar during summer from April to September of 2013-2017. The forests were surveyed on regular basis during this period following rainfall. The collected specimens were photographed and most of the morphological characters and details like place of collections, date, temperature, solitary or in groups, collection number, forest type, shape, substrate, and colour were recorded on the

field. The specimens were then dug out or cut with a sharp knife and kept in a paper bag and taken to laboratory for detail study. In laboratory the samples were wiped (cleaned) for any external dust and other debris with paint brush. Precise photographs were taken and recorded the macroscopic characters and kept for spore print in white and black art papers. It was also cultured after surface sterilization with 70% alcohol or sodium hypo-chloride in PDA and MEA media. Then the samples were dried in oven for further studies.

Collection sites

1. *Volvariella volvacea*	26˚46'74.7" N, 90˚22'41.1" E
2. *Termitomyces heimii*	26˚39'16.1" N, 90˚21'60.1" E
3. *Lentinus sajor-caju*	26˚65'30.9" N, 90˚20'45.1" E
4. *Chlorophyllum hortense*	26˚55'83.7" N, 90˚20'60.5" E
5. *Cantharellus subamethysteus*	26˚38'86.4" N, 90˚32'50.3" E

3.4. Taxonomic study:

3.4.1. Macroscopic characters:

Basidiome: the shape, colour, height, substrate, temperature, latex if any, volva if present, remains of volva, annulus ring or coverings of gills were recorded.

Pileus: shape, size, colour, hairs if present, context, gills edge, gills colour, flesh colour were noted.

Stipe: colour, shape, diameter, length, veil, volva, striated or not, colour change with touch or cut, latex if present, hollow or fleshy, point of attachment with the pileus, flesh colour and any change of diameter along the length of stipe were recorded.

Lamellae: characters like size, shape, thickness, interwoven or not, consistency, distinctness, arrangement, attachment, abundance, colour, edge, brittleness were noted.

Spore: the spore print was taken in paper (black and white) to note the colour of spores shedding and then the papers were folded and kept in envelope for further studies.

Smell: the smell was recorded in the field.

Taste: a small piece of fruit was analyzed by touching it with the tip of tongue, following which mouth was rinsed three to four times.

3.4.2. Microscopical characters:

For microscopic studies the dried sample were cut into thin sections with free hand using thermocol pith and sharp razor blade. The sections were then treated with 5 % KOH,

Phloxin and Congo red for five to ten minutes and visualized under biological microscope Olympus CX 43 at magnification up to 1000X. The structures and cells that are visualized were then photographed with the camera attached to microscope and drawn into papers with the help of drawing attachment. All the captured images were then visualized and measured with the software package Olympus viewer and LC micro.

Basidia: they are the fertile sporangium cells that lines throughout the hymenophore, they are specialized cells and important element of mushrooms, with small projections called sterigma which bears basidiospores.

Basidiospores: the reproductive spores that are produced after meiosis and are dispersal units in fungi, differ in shape and size across the species. They may be oval, ellipsoid, cylindrical, elongated, thin or thick walled.

Cystidia: there are different types of cystidia based on their shape, function, content, size and location of their presence. They are usually taller than basidia providing mechanical strength and help in air trap. They vary in their abundance across the species and in some species they are even absent.

Hyphal system: The different hyphal systems are present and it is important to note the types of hyphae viz. skeletal, generative and vegetative.

Stipitipellis: the cells on the surface of stipe were studied.

Pileipellis: the cuticular layer with cell lining and its arrangement was recorded.

Hymenophoral trama: The arrangements of cell are also studied.

Clamp connections: the presence or absence can differentiate between species and have an important part in taxonomy.

Cells from annulus ring and volva were also considered.

3.5. Molecular studies:

3.5.1. DNA isolation:

The DNA isolation was done with CTAB/ chloroform-isoamyl alcohol DNA extraction protocol (Doyle and Doyle, 1987) with minor modifications.

a. 50 mg of fruit body was taken in mortar and kept at -20 ºC for 20 minutes.

b. 500 µL of CTAB buffer was added and ground with pestle.

c. The paste was then transferred to 1.5 mL eppendroff tube.

d. 4 µL of RNAse was added to the tube and mixed by inverting.

e. The tubes were then incubated for 1 hour at 55ºC.

f. After incubation 500 µL of Chloroform-Isoamyl alcohol at the ratio of 24:1 was added and mixed.

g. Then the tubes were centrifuged at 15000 rpm for 10 minutes.

h. The aqueous layer at the top was then placed into a new tube and 0.08 volume of cold ammonium acetate (7.5 M) was added and 0.54 volume of total combined volume of ice cold isopropanol was added to the tube and mixed well by inverting several times.

i. The tubes were then left at -4ºC, after 1 hour it was centrifuged at 15000 rpm for 3 minutes, the top aqueous phase was discarded and the pallets were washed with 70% ethanol, mixed and centrifuged at maximum rpm for one minute, discarded the upper layer and washed with 95% ethanol.

j. The top layer was discarded after centrifugation at maximum rpm for 1 minute and the pallet was allowed to dry, after drying it was resuspended in 100 µL of Milli Q water.

3.5.2. Electrophoresis:

The extracted DNA was then run in 0.8% agarose gel prepared in 1X TAE buffer with 6X loading dye and 1 µg/mL Ethidium bromide. The gel was subjected to electrophoresis for 1 hour at volt 80 and current 80. After 1 hour the gel was visualized in gel documentation (E gel imager, Life technologies).

3.5.3. Quantification:

The concentration of the genomic DNA was recorded using Qubit 4.0 using high sensitivity standards. Two tubes for standard and one tube for each sample was taken, working solution was prepared by diluting Qubit reagent with buffer at the ratio of 1:200. For standard 10 µL of high sensitivity standards and was added to 190 µL of buffer to make the final volume of 200 µL and for samples 20 µL of each sample was added to 180 µL of working solution. The tubes were then vortexed for 2-3 seconds and were incubated for 2 minutes. Then it was quantified (Qubit 4.0 Flurometer invitrogen).

3.5.4. Purity check:

The isolated DNA was then checked for purity using UV-Visible spectrophotometer at 260/280 nm. The absorbance of the sample solution gives a precise idea about protein and RNA contamination, a double stranded DNA has an OD of 1 for 50 µg/mL with cuvette of 1

cm at 260 nm, the standard value of good purity must be between 1.8-2 OD. Greater than or less than this value indicates contamination.

3.5.5. Amplification:

Hot start polymerase chain reaction (PCR) was employed to exponentially amplify the target region of ITS (primer ITS1 5'- TCCGTAGGTGAACCTGCGG-3', ITS 4 5'-TCCTCCGCTTATTGATATGC-3') and LSU (LR05 5'-ATCCTGAGGGAAACTTC-3', ITS4R 5'-GCATATCAATAAGCGGAGGA-3'). PCR reaction mixture was of 25 µL containing 15 µM $MgCl_2$, 2 unit Taq polymerase, PCR buffer 10X, dNTPs 2.5 mM each, BSA 5%, primers 10 picomole, DNA template 120-160 ng. The parameters was set as initial denaturation at 94ºC for 7 minutes, denaturation at 94ºC for 30 seconds, annealing at 55ºC for 2 minutes, Extension at 72ºC for 2 minutes, final extension at 72ºC for 10 minutes and hold at 4ºC. Here, the Taq DNA polymerase is added to the reaction mixture after completion of initial denaturation of the PCR programme. This is done to add substantial life to Taq DNA Polymerase.

3.5.6. Sequencing:

The PCR amplified products were subjected to Sanger sequencing with DNA Sequencer AB13730XL-15104-028 make Applied Biosystem with Sequence scanner 2 V2.0. The obtained sequence was then refined using Bioedit and BLAST was done in NCBI using nucleotide BLAST. The sequences were then deposited to GenBank using Bankit.

3.6. Nutritional properties:

3.6.1. Moisture content:

Moisture content was done according to (AOAC, 1995). 5 grams of samples were taken, initial weight was recorded and then kept in hot air oven at 105ºC till the weight was constant. The moisture content was calculated with the formula:

$$Moisture\ \% = \frac{fresh\ weight\ (g) - dry\ weight\ (g)}{fresh\ weight\ (g)} \times 100$$

3.6.2. Ash content:

Ash content was estimated by taking 1 gram of sample in silica crucible and then the crucibles were kept in muffle furnace at 500ºC for 2 hours. The initial weight and final weight of the crucibles were recorded. Ash content was calculated as:

$$Ash\ content\ \% = \frac{initial\ weight\ of\ crucible\ (g) - final\ weight\ of\ crucible\ (g)}{Amount\ of\ sample\ taken} \times 100$$

3.6.3. Protein content:

Protein content of the samples was studied using Lowry's method.

0.5 gram of dried sample was ground in mortar and pestle with 10 mL of distilled water. The solution was filtered and used for protein estimation (Lowry *et al.,* 1951).

Reagents Used:

a. Solution A- 2 gram of sodium carbonate was dissolved in 100 mL of 0.1N NaOH.

b. Solution B- 0.5% copper sulphate ($CuSO_4.5H_2O$) was dissolved in 1% sodium potassium tartrate.

c. Solution C was prepared by 50 mL of solution A mixed with 1 mL of solution B.

d. Folin- Ciocalteau reagent was mixed with equivolume of distilled water before use.

e. Standard curve was prepared using BSA at the concentration of 200 µg/mL.

5 mL of solution C was then mixed with 0.5 mL of Folin-Ciocalteau reagent and 0.1 mL of standard solution or sample. The absorbance was taken using Double beam UV Visible spectrophotometer (Systronics 2202).

3.6.4. Total soluble sugars:

Total soluble sugar was estimated using Anthrone method (Bisen, P. S. 2014).

For estimation of total sugars 100 mg of samples were digested with 5 mL of 2.5 N HCl. The volume was made up to 10 mL and then 0.1 mL of the digested solution (after filtration) was used for quantification. Anthrone reagent was prepared by dissolving 200 mg of Anthrone in 100 mL of concentrated H_2SO_4. The absorbance was read at 630 nm using spectrophotometer.

3.6.5. Total Fat content:

For total fat content 10 gram of the dried powdered samples were extracted in chloroform methanol and water. The non fatty components are removed in methanol and water and the chloroform layer was collected in a dry container and its weight was recorded. The solvent was allowed to evaporate and the final weight of the container was recorded. The fat content was quantified as:

$$Fat\ content\ \% = \frac{Final\ weight\ of\ container - initial\ weight}{Weight\ of\ sample} \times 100$$

3.6.6. Amino acid content:

Samples were prepared by digesting with 6N HCl. The tubes were sealed and placed in dry bath at 60ºC under N_2 gas for 15 minutes to maintain inertness. Then the temperature was increased to 110ºC and incubated for 24 hours to evaporate all the solvents. The reagents were added and incubated for 10 minutes at 55ºC. After incubation 1µL was loaded to the instrument (WATERS Acquity UPLC), which was quantified using 42 standard amino acids with flow rate of 0.5 mL per minute. The column temperature was maintained at 55ºC and PDA detector was maintained at 260 nm.

3.6.7. Fatty acid content:

The analysis of fatty acids was carried out with standard protocol (Bligh and Dyer, 1959). For the analysis fatty acid were extracted from the samples using solvents chloroform: methanol: water at the ratio of 1:2:0.8 with thorough mixing in magnetic stirrer and then further solvents were added to make the ratio to 2:2:1.8. Two layers were formed when allowed to stand for some time, the upper aqueous layer was discarded and chloroform layer was taken which contains purified lipids. The fatty acid were subjected to analysis using GC-MS- Schimadzu GCMS-QP2010 plus, column DB5 MS (0.25×30×0.25). The injection temperature was set at 280ºC and oven temperature was maintained at 100ºC (hold for 4 min) raised to 280ºC (4ºC per minute) hold for 12.95 min. Mass spectra was recorded at 2 scans per second with a mass to charge ratio of 50-600 scanning range.

3.6.8. Mineral estimation:

Mineral estimation was carried out according to (AOAC, 1990) for elemental estimation. Oven dried fruit body of the collected mushrooms were powdered and 1 gram was taken into glazed porcelain crucible which was digested at 500ºC for two hours in muffle furnace. The digested sample was allowed to cool and then 10 drops of deionised Milli Q water was added followed by 3-4 drops of HNO_3. The excess HNO_3 was allowed to evaporate at 100-120ºC. After evaporation it was further digested for 1 hour at 500ºC, allowed to cool and then dissolved the ash in 10 mL of HCl. The solution was then analysed by AAS (Thermo scientific ICE-3500, Software- solar) and the results are expressed as mg/ g dry weight.

3.7. Antioxidant Assay:

3.7.1. Total phenolic content:

The samples were extracted in three different solvents by cold extraction method, 10 grams of dry powdered samples of respective mushrooms were extracted in ethanol,

methanol and water as a solvent. The three different extracts were then analysed for total phenolic content by Folin-Ciocalteu method as described by (Singleton and Rossi, 1965 & Olajire and Azeaz, 2011) with modifications. 1 mL of each extracts of the samples was mixed with 1 mL of Folin and Ciocalteu's phenol reagent which was prepared by mixing at the ratio of 1:9 with distilled water. The solution mixture was allowed to stand for 5 minutes then further mixed with 1 mL of 13% sodium carbonate. The final volume was made up to 10 mL with distilled water. The mixture was then allowed to stand for 90 minutes in dark before recording the absorbance at 725 nm using spectrophotometer. For the standard curve Gallic acid was prepared at the concentration of mg/mL and results were expressed as gallic acid equivalent (GAE).

3.7.2. Total flavonoid content:

Total flavonoid content was performed by following Aluminium Chloride colorimetric method according to (Barros *et al.,* 2008).

1 mL of different extracts of the samples was added with 500 µL of distilled water and 30 µL of 5% sodium nitrate. Mixture was then left for 5 minutes followed by addition of 60 µL of 10% aluminium chloride solution. After 6 minutes again 200 µL of 1 M NaOH solution was added to the mixture and the final volume was made up to 3 mL followed by measurement of the solution spectrophotometrically at 510 nm. Standard solution of quercetin was prepared to serve as standard and the results were presented as mg of quercetin equivalent per gram of extract (QE).

3.7.3. ABTS radical scavenging activity:

ABTS radical scavenging activity was carried out according to the method described by (Re *et al.,* 1999). Firstly, ABTS of 7 mM concentration was dissolved in deionised water then 2.45 mM potassium persulphate was added. The mixture was then left overnight in dark. The colour developed by $ABTS^{*+}$ was diluted to get the absorbance of 0.75 OD set at 734 nm for which it was diluted with ethanol and PBS. Then different concentration of extracts were mixed with 1 mL of the diluted solution with absorbance 0.75 OD and the absorbance was recorded at 734 nm using spectrophotometer. The percentage inhibition was calculated according to the equation below then the graph was plotted for % inhibition and from the regression equation the IC_{50} value was calculated.

$$\text{ABTS} * + \text{radical scavenging } \% = \frac{Abs.of\ control - Abs.of\ extract}{Abs.of\ control} \times 100$$

3.7.4. DPPH radical scavenging activity:

The potential of mushroom extracts to scavenge DPPH radicals were estimated by the process as described by (Shimada *et al.,* 1992). Stock solution of DPPH was prepared to the concentration of 100 µM and from the stock solution 2.9 mL was mixed with different concentration of extracts. The mixture was allowed to stand for 30 minutes and then the absorbance was recorded at 515 nm in spectrophotometer. Control was prepared without extract and the extraction solvent served as blank. The scavenging potential was calculated based on the equation then the graph was plotted for % inhibition and from the regression equation the IC_{50} value was calculated.

$$\text{DPPH radical scavenging } \% = \frac{Abs.of\ control - Abs.of\ extract}{Abs.of\ control} \times 100$$

3.7.5. FRAP assay:

Ferric Reducing Antioxidant Power assay was carried out with the process described by (Benzie and Strain, 1996 and Huang *et al.,* 2005).

Reagents:

FRAP reagent: 2.5 mL of TPTZ 10 mmol in 40 mmol HCl.

25 mL of Acetate buffer 300 mmol.

2.5 mL of $FeCl_3.H_2O$ 20 mmol.

Freshly prepared FRAP reagent was taken in a test tube and incubated for 10 minutes in water bath set at 30 ºC. The absorbance was recorded. Then to the reagent 1 mL of extract was added and again incubated for 30 minutes. The absorbance was recorded after 30 minutes at 593 nm using spectrophotometer. Ferrous sulphate was taken to serve as standard and the results were expressed as µmol Fe^{2+} equivalent. FRAP value was calculated with the equation:

FRAP value= *Abs FRAP+ extract – Abs FRAP reagent*.

3.7.6. Nitric Oxide Scavenging Activity:

Nitric oxide scavenging potential of mushroom extracts was evaluated by the process of (Rice- Evans *et al.,* 1996). Different concentrations of extracts were mixed with 5 mM sodium nitroprusside prepared in phosphate buffer saline. The mixture was incubated for 30 minutes at 25ºC. It resulted in the production of nitric oxide. Then 1.5 mL of Griess reagent was added to the mixture. The formation of azo dye due to diazotization of sulphanilamide and nitrite which further coupled with N-1-naphthylenediamine dihydrochloride. The absorbance of the chromophore was recorded at 546 nm. Control was prepared without

extract and ascorbic acid served as standard. Then the graph was plotted for % inhibition and from the regression equation, the IC_{50} value was calculated.

$$\text{Nitric oxide scavenging } \% = \frac{Abs.of\ control - Abs.of\ extract}{Abs.of\ control} \times 100$$

3.7.7. Superoxide scavenging activity: (Elmastas *et al.,* 2007, Nishikimi *et al.,* 1972)

Superoxide scavenging potential of extracts from mushrooms were analysed according to Elmastas *et al.,* 2007. The oxidation of NADH in PMS generated superoxide anions. $O_2{}^{*-}$ was generated with the addition of 0.05 mL of 60 µM of PMS to a tube containing 0.5 mL of 156 µM NBT and 0.5 mL of 468 µM NADH. All the reagent mixtures were prepared in phosphate buffer. After adding different concentration of extracts, it was left for 5 minutes at 25 ºC. The blue formazan formed due to reduction of NBT was measured for absorbance at 560 nm. Control was prepared without extract and the scavenging activity was calculated. Then the graph was plotted for % inhibition and from the regression equation the IC_{50} vale was calculated.

$$\text{Superoxide scavenging } \% = \frac{Abs.of\ control - Abs.of\ extract}{Abs.of\ control} \times 100$$

3.8. Antimicrobial assay:

The anti microbial assay was carried out with 4 different extracts (aqueous, ethanolic, methanolic and petroleum ether). The dried samples were crushed to powder and extracted in respective solvents at the ratio of 1:9. The samples were extracted for 48 hours. The extracts were filtered through Whattman filter 41, the residue was re-extracted 3 times and the extracts were then dissolved in DMSO to a concentration of 1 mg/mL for analysis. The antimicrobial properties of different mushrooms were determined against five bacterial species following disc diffusion method (Balouri *et al.,* 2016).

The bacterial species were procured from IMTECH, Chandigarh and were cultured in nutrient broth accordingly for 24 hours and used for the study. The colony forming unit and minimum inhibitory concentration (MIC) was also determined during the study. Different antibiotics were taken as positive control.

3.9. GC-MS analysis of Bioactive compounds:

The dried sample was extracted with methanol for 48 hours at the ratio of 1:9. The extraction process was repeated 3 times. The solvent was then left to dry. The residue was then analysed for the presence of bioactive compounds by GC-MS, Perkin Elmer clarus 680, MS-clarus 600(EI), software-TurboMass ver 5.4.2, Library-NIST-2008.

3.10. Molecular Docking studies:

The mushroom samples were oven dried, analysed for the presence of bioactive compounds using GC-MS. The detected compounds were then browsed in PubChem (https://pubchem.ncbi.nlm.nih.gov/) and their structures were downloaded.

3.10.1. Ligand Preparation:

Ligand preparation was carried out using LigPrep from Schrödinger. Hydrogens were added and unwanted molecules were removed. The charged groups were desalted. Ionization states, tautomers, alternative chiralities and stereoisomers at 16 per ligand and low energy conformations were generated at pH 7 ± 2 using Epik and OPLS3e force field. The geometries of the structures are optimized and relaxed.

3.10.2. Protein preparation:

Antimicrobial proteins were downloaded from RCSB (https://www.rcsb.org/). PDB files are not suitable for molecular docking (as it may include only heavy atoms, co-crystallized ligands, other molecules, metal ions, co factors which, multimeric and may need reduction). The protein were subjected to fixation for any missing atoms and side chains, missing hydrogen atoms, charge states and orientation of various groups. This is done along with assignment of bond orders and formal charges. Refinement is done at neutral pH with RMSD of 0.30 Å using OPLS3e force field after reviewing overlapping and alternate position of residues to its minimal energy state.

3.10.3. Sitemap and Grid generation:

The receptor grid was generated from the co-crystallized ligand in proteins using Glide from Schrödinger, parameters like VdW radii with the scaling factor of 1.0 and partial charge cut-off at 0.25 which is default in the Glide grid generation.

3.10.4. Ligand docking:

Ligand docking was carried out using Glide with extra precision and Epik state penalties were added to the docking score. Parameters like VdW radii with scaling factor of 0.8 and cut-off charge at 0.15 and other settings were kept as default in the software, ΔG of binding was analysed using Macromodel from Schrödinger. ADME was calculated using QikProp from Schrödinger.