**CHAPTER 3**

# MATERIALS AND METHODS

## 3.1 Study area

The area selected for the present work is Ultapani Forest Range, one of the oldest reserve forests and biodiversity rich area, that falls under Manas Biosphere Reserve, Assam. Manas Biosphere Reserve is famous for its rich flora and fauna in North-east India. It spans across two districts, Bongaigaon and Barpeta. It lies in between E89°51.45′ to E92°70.00′ and N26°30.00′ to N26°56.43′ with a total area of 2837 sq. kms. (Paul *et al.*, 2011). It is located in foothills of Eastern Himalayas and shares its international border with Bhutan. In 1985, it was recognized as a UNESCO World Heritage site and named "Manas Wildlife sanctuary". It serves as a vital conservation area for various endangered species, both flora and fauna. Within few years in 1989, it was expanded and designated as Manas Biosphere Reserve by the Government of India.

Ultapani Forest Range is located in Kokrajhar district, Bodoland Territorial Region, Assam. The forest range is a part of Chirang Reserve Forest under Haltugaon Forest Division that covers an area of 462.87 sq. kms. In compliance with Act VII of 1865, the CRF was established on 20$^{th}$ of November, 1875 by the government of Assam. The UFR is geographically positioned between E90°14.551′ to E90°21.801′, and N26°40.377′ to N26°52.753′, covering an area of 225 sq. kms (Fig. 3.1). It is situated 56 kms away from Kokrajhar town and covers three forest villages, viz. Labanyapur, Saralpara, and Ultapani.

The forest range is bordered by foothills of Bhutan, an international border on north side, on south side by Jharbari range (a reserve forest) which looks like a transition zone between Jharbari and Ultapani Forest Range, on east side by Laopani river and Saralbhanga river on west.

The name "Ultapani Forest Range", is derived from its river, "Ultapani", that flows in reverse direction from west to east (Plate 1A). The most notable and peculiar features of

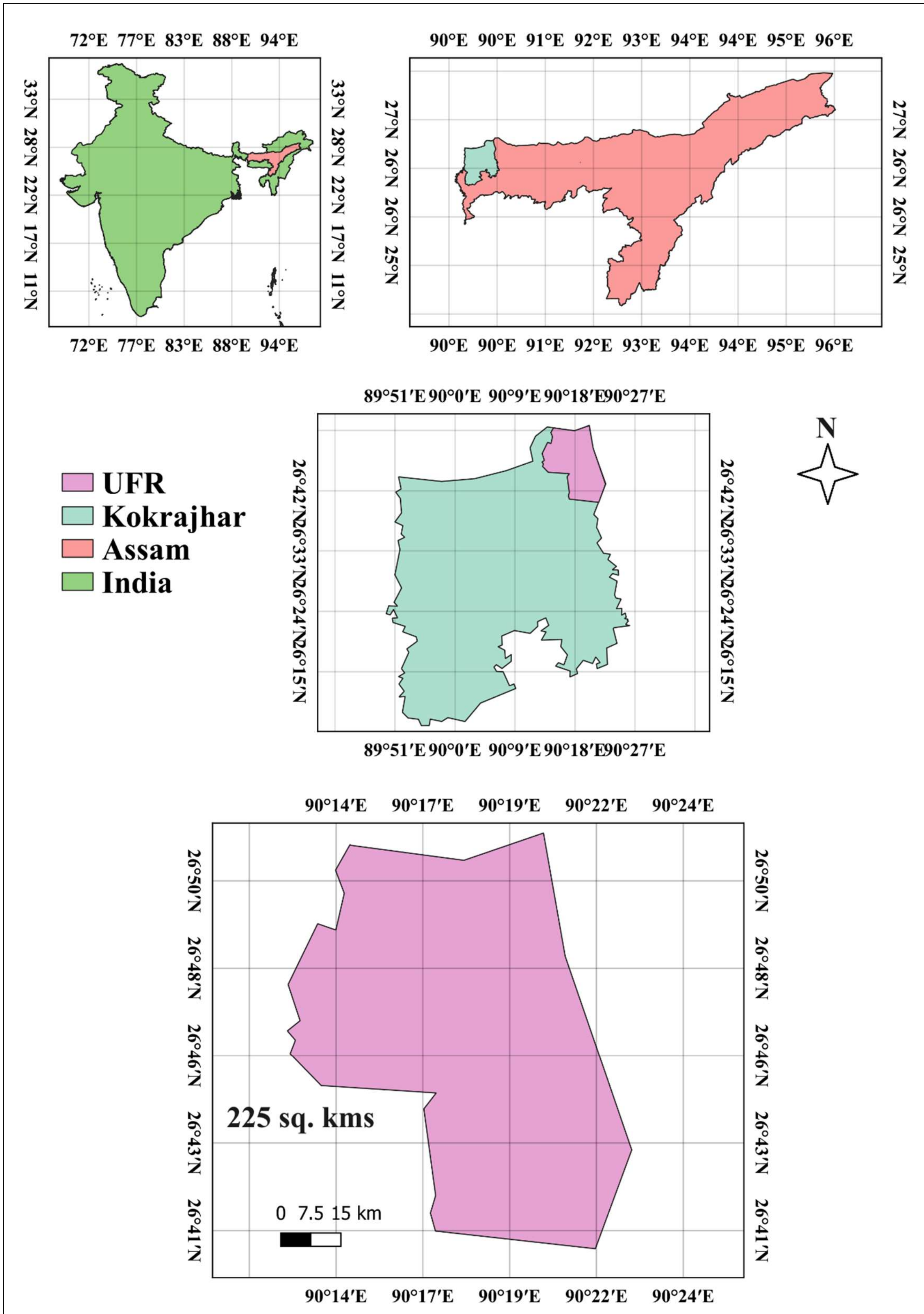


**Fig. 3.1.** Map showing the location of Ultapani Forest Range (UFR)

**Plate 1**

A - Ultapani river (flows in reverse direction), B - Photograph inside Ultapani forest

**Plate 2**

A - Naa Bhandhar (Mach Bhandhar), B - Saralbhanga river

**Plate 3**

A - Laopani river, B - Semi-evergreen forest

**Plate 4**

A - Photograph from outside forest, B - Golden langur on tree

the forest is swampy and mangrove-like vegetation (Plate 1B) that can be seen in stream bank of “Naa Bhandhar” (Mach Bhandhar) within Ultapani (Plate 2A). The area is well recognized as a hub of orchid diversity as it cradles good number of many threatened orchid species. Saralbhanga is a narrow river which originates from Bhutan hills and flows through Saralpara and is one of the most visited tourist spots of the region (Plate 2B). The river Laopani, flows through forest of Labanyapur separating forest area of Chirang and Kokrajhar district (Plate 3A).

The forest type of UFR is distinctive and comprises semi-evergreen (Plate 3B) and moist deciduous forest (Plate 4A), riparian forest and degraded and scrub forests. Most of the plants are semi-evergreen, with medical value; some are edible, oil and timber yielding, and have significant economic advantage. The people of the forest village basically depend on the forest resources for their day-to-day life. The region is renowned for being the “heaven of butterflies” and the home of Golden Langurs (Plate 4B).

The forest area is inhabited mainly by the Bodo, Nepali and Santhal community. Among them, Bodos are the oldest and dominant inhabitants. As per the census of 2011, Ultapani Forest Range has a total population of 3,900 individuals living in 446 households (Baruah *et al*., 2016). The natural beauty, uniqueness and wildlife of UFR attract nature lovers. But now it is under threat due to anthropogenic activities.

## 3.2 Collection of the lichen specimens

Lichen specimens were collected during the period 2019–2023 from three areas, Ultapani, Saralpara and Labanyapur of Ultapani Forest Range. For the collection of lichen specimens, a boundary map of UFR were digitised using online google earth. The locations of the collection sites were plotted with GPS points on google earth map and the final map was generated using the QGIS 3.26.1 software. The entire area was explored for the collection of lichen specimens. The specimens were collected at an elevation of 100–300 m from the bark of trees using hammer and chisel. Collected specimens were wrapped with blotting papers and air dried to remove moisture content and prevent it from fungal infection and made it suitable for herbarium preparation. The herbarium specimens contain all the detailed informations like name of the species, family, location, date, collector and other notes.

## 3.3 Identification of the specimens

The specimens were initially segregated based on their growth forms and grouped based on presence, absence and types of fruiting bodies. The specimens were identified by studying their morphology, anatomy, and chemistry (Orange *et al*., 2010; Nayaka, 2014).

### 3.3.1 Morphological observation

Under morphological observations, characters like thallus shape, size, colour, texture, lobes pattern, isidia, soredia, pruina, pycnidia, cyphellae, pseudocyphellae, cilia, rhizines were observed. In case of apothecia, shape, size, attachment, colour and texture of the disc and margin, disc shape and pruina were noted. Whereas in perithecia, colour, shape, size, single or grouped, position of the ostiole were studied under stereo-zoom microscope (Leica EZ4W).

The fluorescence of lichen thallus was examined under UV light by keeping the specimens in a closed UV chamber. The thallus flourishes yellowish, bluish, greenish and whitish colour on exposure to UV light at the wavelength of 254 nm.

### 3.3.2 Anatomical observation

The various layers of thallus, upper and lower cortex, medulla, algae type and their distribution (heteromerous or homeomerous) were examined. Thin sections of ascomata (fruiting bodies) mounted with water and lactophenol cotton blue on glass slide were studied. Different spore types (simple, septate, submuriform to muriform), colour (hyaline or brown), its shapes, size, number of ascospores in ascus, colour and type of excipulum, colour and height of different layers (hymenium, epihymenium and hypothecium), branching pattern, arrangement and thickness of paraphyses, presence or absence of crystals and agal cells within have been considered under trinocular microscope (Leica DM750).

### 3.3.3 Spot test

The spot tests were usually done on lichen thallus, cortex and medulla to identify the presence or absence of lichen substances. The chemical reagents used for the spot tests

include 10% potassium hydroxide solution (K), aqueous solution of calcium hypochlorite or bleaching powder (C), aqueous solution of para-phenylenediamine, sodium sulphite and liquid detergent (P), iodine solution (I) and KC test (application of K reagent immediately followed by C reagent over it) were performed.

### 3.3.4 Microcrystallography

In microcrystallography, small fragments of lichen thallus were placed on a glass slide and few drops of acetone was put over it for extraction of lichen substances. To the concentric rings, few drops of crystallizing reagents (GE and GAW) was added. A cover slip was placed over it and warmed gently over the spirit lamp, cooled and observed under trinocular microscope (DM750) for studying the crystals.

### 3.3.5 Thin Layer Chromatography

TLC was performed to identify the lichen substances. The acetone extracts of the specimens were loaded on thin aluminium plate pre-coated with silica gel (Merck 60F) with the help of capillary tubes. Then the spotted plate was run with solvent system A (Toluene-dioxane-acetic acid, 180:45:5). After 40–45 min, plate was taken out of the jar and allowed to dry at room temperature. Sulphuric acid (10 %) was sprayed over TLC plate, dried and put in preheated hot air oven at 110°C for 3–5 min. Thereafter, the spots on the plate were identified based on the colour and Rf value, calculated as followed:

$$\text{Rf value} = \frac{\text{distance travelled by substances}}{\text{distance travelled by solvent}} \times 100$$

*Buellia morehensis* were used as control, it gives atranorin, norstictic and salazinic acid at Rf 7, 4 and 2 class.

### 3.3.6 Identification of lichen taxa and endemic species

Relevant literatures, Awasthi, 1991; Upreti, 1994, 1998; Thor, 2002; Kalb *et al*., 2004; Nayaka 2004; Rivas Plata *et al*., 2006; Awasthi, 2007; Makhija and Adawadkar, 2007; Lücking *et al*., 2009; Mishra *et al*., 2011; Upreti *et al*., 2011; Aptroot, 2012; Sharma *et al*., 2012; Sipman *et al*., 2012; Rivas Plata *et al*., 2012; Frisch *et al*., 2014; Breuss and Lücking, 2015; Sobreira *et al*., 2015; Aptroot and Lücking, 2016; Jagadeesh Ram and Sinha, 2016; Joshi *et al*., 2017, 2018; Bajpai *et al*., 2018; Breuss, 2020; Joseph *et al*.,

2021; Joseph and Nayaka, 2021, were consulted for identifying the specimens upto species level. For the nomenclature of the lichen species and family, indexfungorum.org and Wijayawardene *et al*. (2020) were followed.

Singh and Sinha (2010) and Sinha *et al*. (2018) was referred for the confirmation of the endemic species collected from Ultapani Forest Range.

## 3.4 Lichen herbarium

A voucher specimen of the identified species was deposited at CSIR-National Botanical Research Institute, Lucknow, and Department of Botany, Bodoland University Botanical Herbarium (abbreviated here as BUBH), Bodoland University, Kokrajhar, Assam, India.

## 3.5 Preparation of Keys

Artificial dichotomous bracketed keys were prepared for the identified lichen taxa. Key to the families, genus and species are provided, when it had more than one genus in a family and more than one species in a genus. The taxonomic descriptions of all the species are enumerated.

## 3.6 Ecological parameters

The following significant quantitative parameters were utilised to describe community structure:

### 3.6.1 Frequency

It is represented in terms of percentage occurrence and indicates the distribution and degree of dispersion of individual species within a region. The following formula can be used to determine the frequency:

$$\text{Frequency} = \frac{\text{No.of quadrats in which the species occur}}{\text{Total no.of quadrats studied}} \times 100$$

### 3.6.2 Relative abundance

It determines the distribution pattern of species within an area. Additionally, it reveals information about a species rarity or prevalence. It is calculated as follows:

$$\text{Abundance} = \frac{\text{No.of individuals of that particular species}}{\text{Total individuals of all species}}$$

## 3.7 Phytochemical analysis of *Anzia ornatoides*

### 3.7.1 Preparation of lichen extract

The lichen specimen, *A. ornatoides*, was allowed to air dry at room temperature after washing and kept in paper bags. Then the material was pulverized in a mixer grinder. Using a Soxhlet apparatus, finely ground 50 g of dried ground thalli were extracted for 8–16 h with 1000 ml of hexane, diethyl ether, ethyl acetate, methanol, and water in increasing polarity. Thereafter, the crude extracts were concentrated in a rotary evaporator with reduced pressure (IKON, IK-154) and preserved at 4°C for further experiments.

### 3.7.2 Preliminary phytochemical screening

*Anzia ornatoides* extracts were subjected to a preliminary phytochemical study, which involved standard laboratory procedures to identify the presence of various secondary metabolites. The different tests (Mayer's and Wagner's test for alkaloid, Benedict's and Fehling's test for carbohydrate, NaOH and lead acetate test for flavonoid, Keller-kilani and Salkowski's test for glycoside, Ferric chloride and Lead acetate test for Phenol, foam and honey comb test for saponin) were performed (Singh and Bag, 2013; Rashmi and Rajkumar 2014; Devi *et al.*, 2017; Shaikh and Patil, 2020).

## 3.8 Antioxidant activity of *Anzia ornatoides*

### 3.8.1 Total phenolic contents

The total soluble phenolic compounds in extracts were determined by Folin-Ciocalteu reagent (Slinkard and Singleton, 1977), using gallic acid as a standard. Briefly, 50 µl of the extracts (5 mg/ml in 5% DMSO) were diluted with 1.8 ml of water, then 1 ml of 10% $Na_2CO_3$ was added and carefully mixed. After 2–3 min, 150 µl of Folin-Ciocalteu reagent was added, and the solutions were left at room temperature for 10–20 minutes. The absorbance was measured at 765 nm in UV-VIS spectrophotometer (UV-1900i, Shimadzu) against blank (all the reactions without extract). The equation derived from the gallic acid standard graph was used to determine the concentration of phenolic content in extracts as milligrams of gallic acid equivalent per gram of dry weight (mg GAE $g^{-1}$).

### 3.8.2 Total flavonoid contents

The total flavonoid content was determined according to the method of Dowd (Meda *et al.*, 2005). In brief, 1 ml of aluminium trichloride (2% $AlCl_3$ in methanol) was added to 50 µl of the extract solution (5 mg/ml in 5% DMSO) that had been diluted in 1.95 ml of distilled water. The mixtures were left for 10–12 min at room temperature. Afterwards, the absorbance was measured in UV-VIS spectrophotometer (UV-1900i, Shimadzu) at 415 nm. As a blank, all of the reactions without extract were utilised as the negative control. Equation derived from quercetin standard graph was used to quantify the concentration of flavonoid content as milligrams of quercetin equivalent (QE) per gram of dry weight (mgQE$g^{-1}$ DW).

### 3.8.3 Phosphomolybdenum assay

The phosphomolybdenum method was used to evaluate total antioxidant compounds (Prieto *et al.*, 1999). The experiment is used to ascertain how Mo(VI)−Mo(V) is reduced by an antioxidant molecule, resulting in the formation of a green phosphate Mo(V) complex at low pH. Briefly, 1 ml of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) was mixed with 0.1 ml of the aliquot solution (5 mg/ml in 5% DMSO) in a 3 ml vial, and the mixture was diluted up to 3 ml with distilled water. The mixed solution was incubated for 90 min at 95°C in a water bath. The absorbance was measured at 765 nm in UV-VIS spectrophotometer (UV-1900i, Shimadzu) against a blank (0.1 ml of methanol instead of extract) once the sample had cooled to room temperature. Using the standard ascorbic acid graph, the data was computed as milligrams of ascorbic acid equivalent (AAE) per gram of dry weight (mg AAE$g^{-1}$DW).

### 3.8.4 Ferric-reducing antioxidant power assay

The reducing power of the extracts was determined by the method of Oyaizu (Oyaizu, 1986), using ascorbic acid as a standard compound. 10–50 µl of extracts (5 mg/ml in 5% DMSO) were combined with 1 ml of potassium ferricyanide [K3Fe(CN)6] at 1% and 1 ml of phosphate buffer (0.2 M, pH 6.6); and incubated at 50°C for 30 min in water bath. Then, 1 ml of 10% trichloroacetic acid was added to the mixture to rest the reaction, and the mixture was centrifuged at 3000 g for 10 min. Thereafter, 0.5 ml of $FeCl_3$ (0.1%) and 1.5 ml of distilled water were mixed with 1 ml of supernatant. Using a blank (all reaction

agents without extract), the absorbance was measured at 700 nm in UV-VIS spectrophotometer (UV-1900i, Shimadzu). When the reaction mixture exhibits higher absorbance, the reducing power is increased.

### 3.8.5 DPPH radical scavenging

The 1,1-diphenyl-2,2-picrylhydrazil test was used to measure the capacity of extracts to scavenge free radicals (Gadow *et al*., 1997; Dorman *et al*., 2004). Different concentrations of extracts (5 mg/ml in 5% DMSO) and standards, namely 10–50 µl were prepared in test tubes, and 2 ml of methanolic solution of DPPH radicle (0.05 mg/ml) was added to it and diluted up to 3 ml with distilled water. After that, the mixture was allowed to stand at room temperature for approximately 30 min. Using UV-VIS spectrophotometer (UV-1900i, Shimadzu), the absorbance was measured at 517 nm in comparison to methanol as a blank. The following equation was used to calculate it:

$$\text{DPPH scavenging effect (\%)} = (A_0–A_1/A_0) \times 100$$

Where, $A_0$ is the absorbance of the control (2 ml of methanolic solution of DPPH radicle and 1 ml of 5% DMSO) and $A_1$ is the absorbance of reaction mixture or standard (ascorbic acid). The results of inhibitory activity towards DPPH are presented as IC50 values.

### 3.8.6 ABTS assay

The antioxidant activity of the extracts was measured by the 2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) $ABTS^+$ radical cation decolorization assay (Re *et al*., 1999). To make an ABTS, an aqueous solution containing 7 mM $ABTS^+$ and 2.4 mM potassium persulfate was left in the dark for 12–16 h at room temperature. When kept at room temperature in the dark for longer than two days, this radicle remained constant. After diluting the ABTS solution with ethanol (1:84 v/v) at 30°C, the absorbance was adjusted to 0.70000.02 at 734 nm. Subsequently, 2 ml of diluted $ABTS^+$ solution was added to standard trolox (10–50 µl) or different sample concentrations (5 mg/ml in 5% DMSO) and diluted up to 3 ml with distilled water. With a blank (ethanol) in place, the absorbance was measured at 734 nm using UV-VIS spectrophotometer (UV-1900i, Shimadzu) following 30 min incubation period at room temperature. The following formula was used to determine the percentage inhibition of ABTS:

$$\text{ABTS scavenging effect (\%)} = (A_0–A_1/A_0) \times 100$$

Where, $A_0$ is the absorbance of control and $A_1$ is the absorbance of standard or extracts. The results of the inhibitory activity of ABTS are presented as IC50 values.

### 3.8.7 Determination of inhibitory activity toward lipid peroxidation

The antioxidant activity of the extracts was determined by the thiocyanate method (Hsu *et al*., 2008). The linoleic acid emulsion (2.5 ml, 40 mM, pH 7.0) was mixed with 0.5 ml of the stock solution (5 mg/ml of extracts in 5% DMSO). An emulsifier of 0.2804 g Tween 20 was combined with 50 ml of 40 mM phosphate buffer, pH 7.0, to create an emulsion of linoleic acid. This mixture was left in the dark for approximately 72 h at 37 °C. A series of 10–50 µl aliquots was then mixed with 2.3 ml of ethanol, 50 µl of $FeCl_2$ (20 mM), and 50 µl of 30% ammonium thiocyanate. After 3 min of stirring, the mixture was measured at 500 nm using UV-VIS spectrophotometer (UV-1900i, Shimadzu). As a reference substance, ascorbic acid was employed. The inhibition percent of linoleic acid peroxidation was calculated with the given formula:

$$\% \text{ inhibition} = (A_0 – A_1/A_0) \times 100$$

Where, $A_0$ is the absorbance of control and $A_1$ is the absorbance of standard or extracts. The results of inhibitory activity towards lipid peroxidation are presented as IC50 values.

## 3.9 Cytotoxic activity of *Anzia ornatoides*

### 3.9.1 Cell culture and reagents

The cancer cell lines were acquired from the National Cell Repository, NCCS, Pune, India and included prostate cancer (PC-3), ovarian cancer (OVCAR-3), breast cancer (MCF-7), cervical cancer (HeLa), hepatic cancer (Hep-G2), and lungs cancer (h-1299). The cell lines were maintained at 37°C in 5% $CO_2$ culture conditions with RPMI-1640, 10% foetal bovine serum, and antibiotics (streptomycin and penicillin). Complete RPMI-1640 media was used to cultivate the cells in T75 culture flasks.

### 3.9.2 Determination of cytotoxicity by MTT assay

The cancer cell lines PC-3, OVCAR-3, MCF-7, HeLa, Hep-G2, and h-1299 were seeded in 96-well plate ($3 \times 10^4$ cells/well) and incubated for 24 h. Then, the cells were treated with a 5 µg/ml to 160 µg/ml concentration of the extracts (dissolved in 1% DMSO) and again incubated for 72 h. At the end of experiment MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide), 0.5 mg/ml (dissolved in 1% DMSO) was added to

each well and incubated for 3 h followed by discard of content and addition of 100 µl DMSO and absorbance was measured at 570 nm using ELISA plate reader (Saluja *et al*., 2020; Meher *et al*., 2021a). Using the AAT bio quest calculator, the IC50 value of extracts against the cell lines was ascertained.

### 3.9.3 Fluorescence imaging for the detection of apoptosis

Acridine orange and ethidium bromide stains were employed in fluorescence microscopy studies of cells. OVCAR-3 were grown on 6-well plates with coverslips, and were treated with methanolic extract at an IC50 concentration for 72 h. Following the incubation period, the plates were fixed in 3% formaldehyde, rinsed with phosphate buffered saline, stained with AO and EtBr at a concentration of 10 µg/ml, and then rinsed again with PBS after 10 minutes to eliminate any remaining stain. The coverslip was put on a slide and images were taken with a confocal microscope (Carl Zeiss). Apoptotic cells were identified based on their shape in comparison to untreated cells (Meher *et al*., 2021b; Mir *et al*., 2023).

### 3.9.4 Flow cytometry for the detection of apoptosis

OVCAR-3 was treated with methanolic extract at IC50 concentration and berberine individually in order to induce apoptosis, and they were then incubated for 48 h. After inducing apoptosis, the cells were labelled with Hoechst 33342 (15 µg/ml) for 45 min. Ho33342 was stimulated by UV or 405 nm lasers, and 30,000 events were recorded. Emission was captured at 440/40 nm.

### 3.9.5 In-vitro toxicity analysis

The cytotoxicity evaluation was performed using the normal cell lines human embryonic kidney cell line (HEK-293), murine fibroblast (L-929), human mesenchymal cells (hMSCs), and human breast epithelial cell line (MCF-10A). The cells were permitted to grow in culture medium (MEM, DMEM) that was kept in 5% $CO_2$ at 37°C and supplemented with 10% FBS, 1% penicillin/streptomycin. In a 96-well plate, the cells were seeded at a density of $5 \times 10^3$ cells/well and were treated with methanolic extract for 72 h at progressively higher doses (10 µg/ml to 400 µg/ml). Sulphorodamine B (0.56% in 1% acetic acid) was used to stain the cells. To wipe away the unbound stain by washing, 1% acetic acid was employed. A solution of 10 mM Tris base at pH 10.5 was added to the 96-well plate that contained fixed cells with protein-bound stain. Then the absorbance

was determined using a Bio Red 96-well plate reader at a wavelength of 495 nm. The IC50 values of the extract were determined from the plate reader data using an online IC50 value calculator.

## 3.10 Antimicrobial activity of *Anzia ornatoides*

The antimicrobial potential of *A. ornatoides* was assessed using disc-diffusion, agar-well diffusion assay, minimum inhibitory concentration, minimum bactericidal concentration, minimum fungicidal concentration. One strain from each bacterial and yeasts species having resistance to maximum numbers of antibiotics was used for monitoring antimicrobial activities of lichen extracts.

### 3.10.1 Test microorganisms

Human pathogenic Multi-drug resistance (MDR) bacterial and yeast strains were obtained from department of Medical Research Laboratory, Sum hospital, Bhubaneswar. The pathogens included two gram-positive bacteria, *Enterococcus faecalis* (3885), *Staphylococcus aureus* (9797), two gram-negative bacteria, *Escherichia coli* (8585), *Klebsiella pneumoniae* (8108) and four yeasts namely *Candida albicans* (56), *C. glabrata* (12), *C. krusei* (53), and *C. tropicalis* (54).

### 3.10.2 Disc-diffusion assay

Sterile petriplates were filled with Muller-Hinton agar for bacteria and Sabourard dextrose agar for yeast, and the media was allowed to solidify. Microbial cultures were seeded in NB (for bacteria) and YPD broth (for yeasts) for 24 hours at 37°C. Suspension of microorganisms were inoculated on the entire surface of MHA and SDA media using sterile cotton swabs. Sterile discs (6 mm diam.) were laid on the inoculated substrate after being soaked with 20 µl of the lichen extracts (15 mg/ml, 20 mg/ml, 25 mg/ml prepared in 10% DMSO) and incubated at 37°C for 18–24 h. Standard antibiotics gentamicin (30%) for bacteria and fresocan for yeast were used as control. Every test was carried out three times. The sensitivity of the microorganisms to the studied lichen was revealed by measuring the inhibition zone around the discs (Bauer *et al*., 1966; NCCLS, 1993).

### 3.10.3 Agar-well diffusion assay

For agar-well diffusion assay, bacterial and yeasts lawns were prepared with agar of 6 mm thick that was fully punched and four wells per plate were prepared when lawn was 30 min old. Further, wells were filled with 50 µl aliquots of solvent extracts for bacteria and 30 µl aliquots of solvent extracts for yeast (15 mg/ml, 20 mg/ml, 25 mg/ml prepared in 10% DMSO). The plates were incubated for 18–24 h at 37°C. The diameter of zone of inhibition was measured to assess the antimicrobial activity. All the tests were performed in triplicate. An aliquot of 50 µl of gentamicin (30 %) for bacteria and fresocan for yeasts were used as reference control (Perez *et al*., 1990).

### 3.10.4 Determinations of MIC, MBC, and MFC values

The original stock solution of each extract (25 mg/ml prepared in 10% DMSO) was diluted to an appropriate dilution to determine the minimum inhibitory, minimum bactericidal, and minimum fungicidal concentrations of all five extracts. An aliquot of 80 µl NB and YPD broth (HiMedia) was added to a well on a 96-well (12 × 8) micro-titer plate. This was followed by an aliquot of 100 µl of each dilution of a solvent extract (25 mg/ml in 10% DMSO) into the third well and two-fold serial dilutions were made till the $12^{th}$ well and discarded 100 µl to get the dilutions from 0, 1.562, 3.125, 6.25, 12.5, 25, 50 and 100 mg/ml in aliquots of solution. The well was supplemented with 20 µl of bacterial and yeast inoculum ($10^9$ CFU/ml) and a 5 µl aliquot of 0.5% 2,3,5-triphenyl tetrazolium chloride. The micro-titre plate was incubated for 18–24 h at 37°C. The formation of a pink coloration as a result of TTC in a well indicated bacterial and yeast growth was interpreted, and the lack of colouration was taken as growth inhibition. The first well of the micro-titre plate was without any extract, second well was with gentamicin in place of extracts (Eloff *et al*., 1998). The MIC value of the well was recorded, where pink colour was not manifested. Furthermore, MHA and SDA were used to subculture the bacteria and yeast from each well of the micro-titre plate; the dilution level at which no bacterial or yeast growth was detected on the MHA and SDA, as shown by the MBC and MFC values.

## 3.11 GC-MS analysis

The methanolic extract of the sample, *A. ornatoides* was processed for the GC-MS analysis, with a Perkin Elmer (USA) GCMS instrument, Model Clarus 680 d amp,

CalruMS,600C MS comprising a liquid auto-sampler. TurboMass Ver 6.1.2. software was used and the peaks were analysed by data analysis software NIST-2014. The capillary column used was ‘‘Elite-5MS’ having dimensions-length-60 m, ID-0.25 mm and film thickness-0.25 µm. The 5% diphenyl 95% dimethyl polysiloxane is used for the stationary phase. In the GC-Protocol, helium gas (99.99%) was used as a carrier gas (i.e., mobile phase) at a flow rate of 1 ml/min. An injection volume of 2 µl was employed in splitless mode. Injector temperature is 280°C, and ion-source temperature is 180°C. The oven temperature was programmed at 60°C (for 1 min), with an increase at the rate of 7°C/min to 200°C (hold for 3 min), then again increased at a rate of 10°C/min to 300°C (hold for 5 min). The total run time is ~39 min. Solvent delay was kept for 8 min. A MS protocol mass spectral measurement was taken in electron impact positive (EI+) mode at 70 eV. A solvent delay of 8 min was there for MS scan. Mass range i.e., *m/z* range is 50-600 amu. Interpretation of the peaks appeared in the GC chromatogram were done by library search of the mass spectrum of corresponding peaks using the database software NIST-2014. The mass spectra of unknown components were compared with the known components of the NIST library, and compounds were identified by name, molecular weight, and empirical formula.

## 3.12 Photo plates

All the photo plates were arranged in photoshop 7.0

## 3.13 Statistical analyses

Statistical analyses were performed in excel by doing Kruskal-Wallis test in OriginPro 9.0, because the data was not normally distributed. All values were represented as mean ± SD of three parallel measurements. The IC50 value (µg/ml) was calculated using the AAT Bioquest IC50 value calculator (AAT Bioquest, Inc., Sunnyvale, CA, USA).