Chapter 3

MATERIALS AND METHODS
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3.1 Study Area

Assam is located in the North - Eastern part of India surrounded by Arunachal Pradesh in the north, Nagaland in the east, Mizoram and Tripura in the south and West Bengal in the west. The countries viz. China and Bangladesh form international borders along with Assam. Assam is surrounded by a ring of blue hills. It is an amalgamation of plains and river valleys. Its principal geographical regions are Brahmaputra valley in the north, the Barak region in the south and Mikir and Cachar hills divide the two regions. These two valleys, the Brahmaputra and Barak are the richest species zone in the map of India. The climate of Assam is tropical monsoon type and a temperate region which is uncomfortably humid especially during rainy season. Winter season starts from the end of October and lasts till late February. The minimum temperature during this period varies between 6°C to 8°C. Nights and early mornings are foggy and rain is scanty. Summer starts in mid May which is accompanied by high humidity and rainfall. The maximum temperature ranges from 35 to 38°C. The monsoon season starts from mid June and continues till August. Thunderstorms known as Bordoiicila are frequent during afternoons. Spring and autumn seasons are characterized by moderate temperatures. The state has six agro climatic zones which are divided based on soil, rain fall and crop characteristics. The climatic zones are Upper Brahmaputra Valley, Central Brahmaputra Valley, lower Brahmaputra Valley, Hill Zones, Barak
Valley and North Bank zones. Barak valley in Assam consisting of three district i.e. Cachar, Karinganj and Hailakandi with a geographical area of 6922 sq. km., (according to 2001 census).

The study area comprises of Cachar district (Fig. 3.1) is located in the Barak range, is situated in the southern part of the Assam with a geographical area of 3,77,610 ha. The district is bounded by North Cachar Hills in the north, Mizoram in the south, Karinganj and Hailakandi districts and a part of Meghalaya state in the east and Manipur state in the west. The climate of the district is warm in humid during summer where humidity ranges from 85 to 90% and 65 to 70% in winter. The District receives about 3200-3500 mm rainfall during the year. Cachar district of Assam covers 15 community Development Blocks having geographical area of 377610 Ha. The District has 163 numbers of G.P., 15 numbers of A.P. and 19 numbers of A.D.O. Circles with a population 15.62 lakhs (according to 2001 census). The district is situated at 36.5 (MSL) and altitude (92°24'E and 93°15'E) and latitude (24°22N and 25°08'N). The district is having two civil sub divisions viz. Silchar and Lakhipur and three Agricultural sub divisions viz. Silchar, Sonai and Lakhipur. The district headquarter is located at Silchar. The district is at present comprise only one sub-division i.e. Silchar and five circles viz. Katigora, Silchar, Udarbond, Lakhipur and Sonai. It has seven Community Development Blocks. The district has 1050 villages, of which 1024 are inhabited villages and 26 are uninhabited villages.

Silchar is the gateway to Mizoram, Manipur and Tripura and therefore it plays a vital role so far supplies of essential commodities to those states are concerned. Silchar is connected by air from Calcutta and Guwahati, by rail from Guwahati via Lumding and by road to Guwahati via Shillong. Total road length in the district is 945 kms. The Cachar district is a heterogeneous land composed of high hills, low lands and level plains. The general appearance of this district is extremely picturesque. On the north, east and south it is shut by ranges of purple hills whose forest clad sides are seamed with beautiful landscapes. The Silchar sub-division includes the southern section of the Assam range and a belt of hilly country with an average width of six to seven miles, containing peaks between three to four thousand feet in height. The Bhutan range on the eastern frontier covers a considerable area, and rises in places to over 3000 feet above the level of the sea. Almost the whole of the country north and south of the river Barak is dotted with low-ranged isolated hills called tilahs rising from the level of alluvial soil. Most parts of the Barak banks are lined with villages concealed in groves of slender areca palms, broad-leaved
plantains and feathery bamboos, and in all seasons of the year, the country looks fresh and green. The hills on the southern part of this district are covered with dense ever-green forest and bamboo jungle. Barak is the main river, which passes through the district origins from Angami Naga hills in Manipur and travels in curved route cutting through the heart of Cachar district. Jiri, Chiri, Madhura, Jatinga, Dhalesweri, Ghagra, Longai, Shingla, Sonai are the major rivers in Barak Valley. Barail, Bhuban, Panchgram, Chatacherra, Mohonpur, Saraspur are the major hills with numerous hillocks in their vicinities. This plain track of Barak valley is a geographical extension of Gangetic Bengal. The valley is predominantly inhabited by the Indo- Aryan population and the demography is formed in early times by integrating the Indo-Mongoloid, Austric and other non- Aryan ethnic groups in a long historical process. With the formation of two more districts truncating Cachar, the area in the south of Assam is collectively termed as Barak valley.

3.1.1. Geology and rocks

Cachar district of Assam is a huge storehouse of limestone. Limestone which is basically calcium carbonate is primarily a sedimentary rock which is used in a plethora of purposes namely construction, interior decoration, etc. Karbi Anglong district and North Cachar hills have substantial reserves of coal. Of the four types of coal namely peat, lignite, bituminous and anthracite, the third one is readily available out here. The most important characteristic of this area in relationship to Brahmaputra valley is its geological newness and water stagnation.

3.1.2. Land use and forestry

The topography of the district varies from small hillocks to plain areas and low lying areas locally known as haors, beels, etc. About 33% of total geographical area is used for cultivation of various agricultural purposes. More than 20% of the geographical areas of the district cannot be grown crop during April to September due to water stagnation. On the other hand lack of rain from November to April most of the cultivable lands remain fallow during the period. There are 2037 ha cultivable wasteland in the district. Hence, tanks and ponds, check dams and storage reservoirs can be thought of for the purpose of water management and water conservation. Although Govt. attaches considerable importance to improving the quality and productivity of
our land and soil resources by reclamation of degraded and fallow lands as well as problem soils under its National Watershed Development Project for Rain fed Areas, the progress/benefits derived from the project is yet to be noticeable in the district.

Farm mechanism is one of the tools to accelerate the growth of agriculture productivity. Farm mechanism not only help in increasing the productivity of agriculture sector by best use of the lands but also saves valuable time of labour force for other productive purposes. The National Policy on Agriculture seeks to actualize the vast untapped growth potential for agriculture, strengthen rural infrastructure to support faster agricultural development, promote value addition, accelerate growth of agro business, create employment in rural areas, secure a fair standard of living for the farmers and agricultural workers and their families, discourage migration to urban areas and face the challenges arising out the economic liberalization and globalization and it aims to attain a growth rate in excess of 4% per annum in the agricultural sector over the next two decades. Hence, mechanism of farms viz. uses of tractors, power tillers, etc. is necessary to increase production and productivity of agriculture. There are large utilized tilah lands in the district where bamboo, jatropha, etc. can be grown profitably. Further existence of Cachar paper mill in the adjacent district of Hailakandi is added advantage for bamboo growing. The mil requires about 5 lakh MTG of bamboo for its full capacity utilization. To produce 5 lakh MTG bamboos it is estimated that there is a requirement of 10000 ha of land. At present, the mill fed by bamboo grown in the forest of Barak Valley, Mizoram and N.C Hills. The mill has taken initiatives for large scale bamboo plantation in the valley. According to NABARD, in consultation with mill and bankers prepared a banking plan for cultivation of bamboo in Barak Valley. Credit requirement for the year 2007-08 towards this sector has been estimated at Rs. 50.90 lakh. The landscape of Cachar tea areas is composed of small hillocks, plains and low lands. The stagnation of water in low lying areas led to the formation of bheel i.e., bog or true peat. The tilah of Cachar shows considerable variation in nature of the underlying rocks. The approximate percentages of tea under different topographies are tilahs 35%, flat and plateau 60% and bheels 5%. The soils in most of the tilahs are coarse, loam in texture and most plateau and flat areas are silty clay loam. The peat soil (bheel’s soil) is classified as mineral soil with high proportion of organic matter. The mineral fraction mainly constitutes fine particles of silt and clay. The soils are acidic with a large percentage showing low organic matter content and potash.
Wide variability in yield is observed while comparing hot (south facing) and cold (north facing) slopes on teelas. Yield of cold slopes is generally higher however under good condition of shade with proper drainage crop in the hot slope can also be improved.

3.1.3. Topography and agro-climatic characteristics

Cachar district comes under Barak valley zone this zone comprising 8.9% of the state area and 11.7% of the state population. Apart from the tea gardens located at the hill slopes, the entire zone is growing rice as a major crop occupying about 93% of the net crop area. The climate is warm and humid during the summer and lowest temperature is recorded during December and January. The humidity ranges from 65-70% during winter and 85-90% in the rainy season.

3.1.4. Ground water and irrigation

The district experience sufficient rainfall as a result of which most of the crops are grown in rain fed condition where irrigation is done manually with low lift pump. As a result assured irrigation was available to 2% of cultivable land in the district. There is plenty of scope to boast up the production and crop area if irrigation facilities are provided effectively. Assured irrigation can raise the regular Ahu cultivation under controlled flood situation.

3.1.5. Study sites

A total of 16 study sites were selected in different zones (north, south, east, and west) of Cachar district depending on the abundant growth of Euglena blooms(Plate 3.1) as a red (Plate 3.1, a, c-f) or green scum (b). The bloom is abundant to very abundant throughout the year except during periods of heavy rainfall. These ponds are located far away from the city in the rural areas where people are mainly engaged with fishery and other farming practices (e.g. poultry, piggery, homegarding etc.) and sale in rural market. Most of the studied ponds (Arkatipur, Baskandi, Karikandi, Kashipur, Madhuramukh, Madhuraghat, Sonai, Silcoorie, Irangmara, Barjalenga) are surrounded by tea plantations and paddy fields and are very much prone to allochthonous input. Other ponds (Machpara, Bagpur, Dudhpatil, Durgabari, Udharband, Dargakona) are mainly fishery ponds where fish foods are applied to increase the nutrient level. Among the sixteen
ponds Arkatipur, Baskandi, Karakandi, Kashipur, Madhuramukh, Madhuraghat, Sonai are located in flood prone areas.

**Table 3.1:** Geographical location different study sites

<table>
<thead>
<tr>
<th>SL. NO.</th>
<th>SITES</th>
<th>LATITUDE</th>
<th>LONGITUDE</th>
<th>ELEVATION(m)</th>
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<tr>
<td>1</td>
<td>ARKATIPUR</td>
<td>24°12'N</td>
<td>92°17'E</td>
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</tr>
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<td>2</td>
<td>BASKANDI</td>
<td>24°10'N</td>
<td>92°15'E</td>
<td>34</td>
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<tr>
<td>3</td>
<td>KARIKANDI</td>
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<td>92°28'E</td>
<td>23.6</td>
</tr>
<tr>
<td>4</td>
<td>MACHPARA</td>
<td>24°11'N</td>
<td>92°23'E</td>
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</tr>
<tr>
<td>5</td>
<td>KASHIPUR</td>
<td>24°24'N</td>
<td>92°11'E</td>
<td>22.2</td>
</tr>
<tr>
<td>6</td>
<td>BAGPUR</td>
<td>24°32'N</td>
<td>92°13'E</td>
<td>32.6</td>
</tr>
<tr>
<td>7</td>
<td>MADHURAMUKH</td>
<td>24°23'N</td>
<td>92°31'E</td>
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</tr>
<tr>
<td>8</td>
<td>MADHURAGHAT</td>
<td>24°29'N</td>
<td>92°29'E</td>
<td>21</td>
</tr>
<tr>
<td>9</td>
<td>DUDHPATIL</td>
<td>24°37'N</td>
<td>92°32'E</td>
<td>27</td>
</tr>
<tr>
<td>10</td>
<td>DURGABARI</td>
<td>24°39'N</td>
<td>92°37'E</td>
<td>34</td>
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<tr>
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<td>UDHARBAND</td>
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<td>SONAI</td>
<td>24°27'N</td>
<td>92°55'E</td>
<td>24.7</td>
</tr>
</tbody>
</table>
3.1.6. Meteorological data of Cachar district

The meteorological data were obtained from Cachar College (Meteorological office, Cachar College, Silchar-Assam) and described in Fig. 3.2.

**Fig. 3.2:** Variation of temperature, humidity and rainfall in the study area from 2008-2010
3.2 Analysis of Physico-Chemical properties of water sample

Both air and water temperatures of the ponds’ water were measured using a thermometer in unit °C. Water analysis was performed in triplicate following standard procedures (APHA, 2005; Wetzel and Likens, 1979; Suess, 1982). Field determination included pH (Electrometric method), conductivity (Electrometric method), dissolved oxygen (Winkler’s method), free CO₂ (Titrimetric method), Alkalinity (Titration with colour indicator solution), silica (Molybdate blue method), Soluble Reactive Phosphorus (SRP) (Ascorbic acid method) and nitrate (Brucine method).

3.3 Analysis of soil properties

Soil samples were collected randomly to a depth of 0-10cm using soil corer. A composite sample was obtained by pooling all soil samples site wise to account spatial variation of the ponds. All soil samples were passed through 2mm sieve and made air dry except bulk density for which soil samples were dried in 105°C in an oven. Soil pH and conductivity were measured in1:2.5 soil-water (w/v) suspensions by “Electrometric method” (Anderson and Ingram, 1993). Soil moisture was estimated by the standard procedure mentioned by Allen (1989). Bulk density was determined by “Soil core method” (Anderson and Ingram, 1993). Water holding capacity was measured by “Keen’s Box method” (Piper, 1942). Soil organic carbon was determined by Walkley & Black’s rapid titration method (Jackson, 1973).

Total nitrogen of the sediment soil was estimated following Micro-Kjeldahl method by acid-digestion, distillation and titration (Jackson 1958). Total phosphorus (P) was determined by Vanadomolybdate method (Jackson 1958). Micronutrients Cu, Mn, Zn, Fe were determined by AAS (Atomic Absorption Spectrophotometer).

3.4 Algal bloom analysis

The algal samples were collected from different study sites by using plankton net made of No. 25 bolting silk. The strained sample was concentrated to a constant volume. Several drops from
each sample were examined from 50 random fields of the mounds. The organisms were counted under the microscope and expressed accordingly. Microscopic observations of the cell morphology such as size of the organism, presence or absence of chloroplast, eyespot, flagella, paramylon etc. were taken into account and identified following standard keys (Prescott, 1951; Leedale, 1967; Phillipose, 1982).

3.4.1 Algal fresh weight and dry weight estimation

1 litre of algal samples collected by the plankton net was filtered and weighed for its fresh weight. The samples were air dried and reweighed for its dry weight.

3.4.2.1 Algal enumeration

1 litre of algal sample was filtered and preserved in 4.5% formalin solution and cell counting was done by drop count method (Lackey, 1938).

3.4.2.2 Diurnal behavior of Euglena tuba

For observing diurnal periodicity of Euglena blooms bright sunny days were selected. Experimental observation was started at 7 a.m. and concluded at 5 p.m. A total of 11 observations were made to estimate the diurnal variation. Samples were collected in centrifuge tubes and mixed with 90% acetone at every 1 h interval.

3.4.2.3 Behavioural study of Euglena tuba

Observation of fresh Euglena tuba was done under the microscope and the changes in structure were recorded. The Euglena tuba was placed in an environment where dim light is coming from one direction for 30 minutes and changes were recorded. For shock reaction treatment, Euglena sample was kept in a bottle with water and was shaken. Changes were noted down after observing it under the microscope.

3.4.2.4. Estimation of pigment profile

Pigment profile of the algal samples was estimated by analyzing chlorophyll (a and b) and carotenoid content by extracting the samples with 90% acetone following cold extraction method (Strickland and Parsons, 1968).
3.4.2.4 Measurement of trophic state of pond ecosystem

Uttormark and Wall (1975) scheme was followed to develop a lake condition index LCI by using penalty points for characteristics like DO, transparency, fish kills and recreational use impairment. The total of all the ponds was used to categorize the water bodies.

3.4.2.5 Estimation of biochemical properties (Carbohydrate and Protein)

Total carbohydrate was estimated by Anthrone method (Spiro, 1966). Protein was measured by Lowry’s method (Lowry, 1951).

3.4.2.6 Analysis of macro and micronutrients of *Euglena* sp.

Total nitrogen of the *Euglena* sample was estimated following Micro-Kjeldahl method by acid-digestion, distillation and titration (Jackson, 1958). Total phosphorus (P) was determined by Vanadomolybdate method (Jackson 1958). Micronutrients Cu, Mn, Zn, Fe were determined by AAS (Atomic Absorption Spectophotometer).

3.4.2.7 *Euglena* intake by indigenous fish varieties

Ponds were selected from different study sites and were based on the presence of thick growth of *Euglena*. A questionnaire was prepared and answers were listed down (Saxena, 1995; Rastogi et al., 1998; Mendez et al., 2001; Vogl et al., 2004). Gut contents of the indigenous fishes were dissected out, weighed and preserved in 5% formalin (Sivan and Radhakrishnan, 2011).

3.4.2.8 Antioxidant activity of *Euglena tuba*

**Chemicals**

2,2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was obtained from Roche diagnostics, Mannheim, Germany. 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was obtained from Fluka, Buchs, Switzerland. Potassium persulfate (K₂S₂O₈), 2-deoxy-2-ribose, mannitol, trichloroacetic acid (TCA), nitro blue tetrazolium (NBT), reduced nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), lipoic acid, quercetin, sodium nitroprusside, sulfanilamide, naphthyl ethylenediamine dihydrochloride (NED), L-histidine, sodium pyruvate, 1,10-phenanthroline and ferrozine were obtained from
Sisco Research Laboratories Pvt. Ltd, Mumbai, India. Hydrogen peroxide, potassium hexacyanoferrate, Folin-Ciocalteu reagent, sodium carbonate, aluminium chloride, ammonium iron (II) sulfate hexahydrate, potassium nitrite, xylene orange, sodium hypochlorite, mercuric chloride, potassium iodide, anthrone, vanillin, 2,4-dinitrophenyldrazine, Thiourea and N,N-dimethyl-4-nitrosoaniline were obtained from Merck, Mumbai, India. DPPH, gallic acid, (+) catechin and curcumin were obtained from MP Biomedicals, France. Catalase, reserpine, Ferrous sulfate and Sodium bi carbonate were obtained from HiMedia Laboratories Pvt. Ltd, Mumbai, India. Evans blue, Lead acetate, were purchased from BDH, England. Manganese dioxide was obtained from SD Fine Chemicals, Mumbai, India. D-glucose, sodium nitrite was procured from Qualigens Fine Chemicals, Mumbai. Diethylene-triamine-pentaacetic acid (DTPA) was obtained from Spectrochem Pvt. Ltd, Mumbai, India. Thiobarbituric acid (TBA) was obtained from Loba Chemie, Mumbai, India.

**Plant material and extraction**

The *Euglena* algal sample was collected from the Cachar district of the state of Assam, India and authenticated as described in Chapter 3. The fresh algal sample was dried at room temperature for 7 days, finely powdered and used for extraction. The powder (100 g) was mixed with 1000 ml methanol:water (7:3) at 37°C for overnight using shaker incubator, then the mixture was centrifuged at 2850 × g and the supernatant was decanted. The pellet was mixed again with 1000 ml methanol-water and the entire extraction process was repeated. The supernatants collected from the two phases were mixed in a round bottom flask and concentrated under reduced pressure in a rotary evaporator. The concentrated extract was then lyophilized. The residue was kept at -20°C for future use. MilliQ grade water was used to dissolve the extract and for further dilution during the experiments.

**Animals**

The Animal Ethical Clearance Committee of the Institute (Registration number: 95/1999/ CPCSEA) approved the use of adult male Swiss Albino mice (*Mus musculus*), weighing 20-25 g for experimentation. Each polypropylene cage contained 4 mice at a time; supplied with *ad libitum* laboratory diet and water. The mice were kept at 25 ± 2°C and 60 ± 5% humidity and normal photo cycle (12 h dark/12 h light).
Phytochemical Analysis

Qualitative tests
Phytochemical analysis of 70% methanol extract of *Euglena tuba* (ESME) was carried out using standard qualitative methods as described previously by Harborne and Baxter, Kokate *et al.*, (1995). The components analysed for phytochemicals were alkaloids, carbohydrates, flavonoids, glycosides, phenols, saponins, tannins, terpenoids, anthraquinones, and triterpenoids.

Quantification of total phenolic content
The total phenolic content present in the 70% methanol extract of *Euglena tuba* was determined using Folin-Ciocalteu (FC) reagent by a formerly reported method (Hazra *et al.*, 2008). All tests were performed six times. The phenolic content was evaluated from a gallic acid standard curve.

Quantification of total flavonoid content
The amount of total flavonoid content was determined with aluminium chloride (AlCl₃) according to a modified method (Hazra *et al.*, 2008). All tests were performed six times. The flavonoid content was calculated from a quercetin standard curve.

Quantification of carbohydrate content
Quantification of carbohydrate content was carried out using previously described method by Sadashivam *et al.*, (1997) with slide modification. 100 mg of the extract was weighed into a test tube, hydrolysed by keeping it in a boiling water bath for three hours with 5 ml of 2.5 N HCl and cooled to room temperature. The volume was made to 100 ml and centrifuged. 0.25 ml sample extract made up to 0.5ml distilled water and mixed with Anthrone reagent (4 ml) and was incubated at 95°C for 8 min. After incubation, cooled rapidly and absorbance was measured of green to dark green color at 630 nm. All tests were performed six times. The carbohydrate content was evaluated from a glucose standard curve.

Quantification of ascorbic acid content
The amount of total ascorbic acid content was determined with 2,4-dinitro-phenylhydrazine reagent according to the method of Joseph(1961) with slide modification. 1 ml aliquots of extract (1 mg/ ml) in water were mixed with 1 ml of ‘2,4-dinitro-phenylhydrazine reagent’(2% 2,4-dinitrophenylhydrazine and 4% thiourea in 9(N) H₂SO₄) and was incubated at 95°C water bath for 15 minutes. After incubation 5ml of 85% H₂SO₄ was added drop wise in an ice bath. Then the mixture was stood for 30 minutes, the absorbance was measured at 520 nm. All tests were
performed six times. The ascorbic acid content was evaluated from a L-ascorbic acid standard curve.

**Quantification of alkaloid content**

Quantification of alkaloid content was carried out using previously reported by Singh *et al.* (2004) with slide modification. To the 1 ml of extract (1 mg/ml) in water 0.1 ml of FeCl₃ (2.5 mM FeCl₃ in 0.5 M HCl) was added followed by addition of 0.1 ml 1,10 phenanthroline. After incubation for 30 min at 70°C the absorbance was taken at 500 nm. All tests were performed six times. The alkaloids content was evaluated from the reserpine standard graph.

**Quantification of tannin content**

This was assayed as described by Robert (1971) with a slight modification. 0.1 ml aliquots of extract (1 mg/ml) in water were mixed with the 0.5 ml vanillin hydrochloride reagent (Mix equal volumes of 8% hydrochloric acid in methanol and 4% vanillin in methanol. The solutions were mixed just before use, and avoid using even if it is slightly colored). After incubation for 20 min at room temperature the absorbance was measured of magenta-pink color at 500 nm. All tests were performed six times. The tannin content was evaluated from the catechin standard graph.

**DPPH radical scavenging assay**

The complementary study for the antioxidant capacity of the fruit extract was confirmed by the DPPH scavenging assay according to Mahakunakorn *et al.*, (2004) with slight modification. Different concentrations (0-100 μg/ml) of the extracts and the standard trolox were mixed with equal volume of ethanol. Then 50 μl of DPPH solution (1mM) was pipetted into the previous mixture and stirred thoroughly. The resulting solution was kept standing for 2 minutes before the optical density (OD) was measured at λ = 517 nm. The measurement was repeated with six sets. The percentage radical scavenging activity was calculated from the following formula:

\[
\%
\text{scavenging} \ [\text{DPPH}] = \left[\frac{(A_0 - A_t)}{A_0}\right] \times 100.
\]

where \(A_0\) was the absorbance of the control and \(A_t\) was the absorbance in the presence of the samples and standard.

**Hydroxyl radical scavenging assay**

The scavenging assay for hydroxyl radical was performed by a standard method (Hazra, 2008). Hydroxyl radical was generated by the Fenton reaction using a Fe³⁺-ascorbate-EDTA-H₂O₂ system. The assay quantifies the 2-deoxyribose degradation product, by its condensation with
TBA. All tests were carried out six times. Mannitol, a classical OH scavenger, was used as a standard compound. Percent inhibition was evaluated by the following equation:

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

where \( A_0 \) was the absorbance of the control and \( A_1 \) was the absorbance in the presence of the samples and standard.

**Superoxide radical scavenging assay**

Measurements of superoxide anion scavenging activities of the samples and standard quercetin were done based on the reduction of NBT according to a previously described method (Hazra et al., 2008). Superoxide radical is generated by a non-enzymatic system of phenazine methosulfatenicotinamide adenine dinucleotide (PMS/NADH). These radicals reduce nitro blue tetrazolium (NBT) into a purple colored formazan which was measured spectrophotometrically at \( \lambda = 562 \) nm. All tests were performed six times. The percentage inhibition of superoxide anion generation was calculated using the following formula:

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

where \( A_0 \) was the absorbance of the control and \( A_1 \) was the absorbance in the presence of the samples and standard.

**Nitric oxide radical scavenging assay**

Sodium nitroprusside (SNP) gives rise to nitric oxide that under interaction with oxygen produce nitrite ions measured by Griess Illosvoy reaction (Hazra et al., 2008). The chromophore generated was spectrophotometrically measured at \( \lambda = 540 \) nm against blank sample. All tests were performed six times. Curcumin was used as a standard. The percentage inhibition of nitric oxide radical generation was calculated using the following formula:

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

where \( A_0 \) was the absorbance of the control and \( A_1 \) was the absorbance in the presence of the samples and standard.

**Hydrogen peroxide scavenging assay**

FOX-reagent method was used to determine this activity of the sample and the reference compound sodium pyruvate, as previously described (Hazra et al., 2008). The absorbance of the ferric-xylitol orange complex was measured at \( \lambda = 560 \) nm. All tests were carried out six times. The percentage of scavenging of hydrogen peroxide of fruit extracts and standard compound:
% scavenged [H₂O₂] = [(A₀ - A₁) / A₀] × 100

where A₀ was the absorbance of the control and A₁ was the absorbance in the presence of the samples and standard.

**Peroxynitrite scavenging assay**

Peroxynitrite (ONOO-) synthesis was done 12 h before the assay, according to Beckman *et al.*, (1994) Acidic solution (0.6 M HCl) of 5 ml H₂O₂ (0.7 M) was mixed with 5 ml of 0.6 M KNO₂ on an ice bath for 1 s and 5 ml of ice cold 1.2 M NaOH was added to the reaction mixture. Excess H₂O₂ was adsorbed by granular MnO₂ and the reaction mixture was left at -20°C. The concentration of the peroxynitrite solution was measured spectrophotometrically at λ = 302 nm (ε = 1670 M⁻¹ cm⁻¹). Evans blue bleaching assay was used to measure the peroxynitrite scavenging activity (Hazra *et al.*, 2008). The percentage of scavenging of ONOO⁻ was calculated by comparing the results of the test and blank samples. All tests were performed six times. Gallic acid was used as reference compound. The percentage of scavenging of peroxynitrite anion was calculated using the following equation:

% scavenged [ONOO⁻] = [(A₀ - A₁) / A₀] × 100

where A₀ was the absorbance of the control and A₁ was the absorbance in the presence of the samples and standard.

**Singlet oxygen scavenging assay**

Singlet oxygen (¹O₂) production, and at the same time, its scavenging by the samples and the reference compound lipoic acid can be monitored by N, N-dimethyl-4- nitrosoaniline (RNO) bleaching, using an earlier reported method (Hazra *et al.*, 2008). Singlet oxygen was generated by a reaction between NaOCl and H₂O₂ and the bleaching of RNO was read at λ = 440 nm. All tests were performed six times. Singlet oxygen scavenging was calculated by the following formula:

% scavenging = [(A₀ - A₁) / A₀] × 100

where A₀ was the absorbance of the control and A₁ was the absorbance in the presence of the samples and standard.

**Hypochlorous acid scavenging assay**

According to a previously described method (Hazra *et al.*, 2008), hypochlorous acid (HOCl) was prepared just before the experiment by adjusting the pH of a 10% (v/v) solution of NaOCl to
pH 6.2 with 0.6 M H$_2$SO$_4$ and the concentration of HOCl was determined by taking the absorbance at $\lambda = 235$ nm using the molar extinction coefficient of 100 M$^{-1}$ cm$^{-1}$. The scavenging activities of the extract and the standard, ascorbic acid, a potent HOCl scavenger was evaluated by measuring the decrease in the absorbance of catalase at $\lambda = 404$ nm. All tests were performed six times. The percentage of scavenging of HOCl was calculated using the following equation:

$$\% \text{ scavenged [HOCl]} = \left( \frac{A_0 - A_1}{A_0} \right) \times 100$$

where $A_0$ was the absorbance of the control and $A_1$ was the absorbance in the presence of the samples and standard.

Reducing power assay

The Fe$^{3+}$-reducing power of the extract was determined by a standard method (Hazra et al., 2008). In a phosphate buffer solution (0.2 M, pH 6.6), different concentrations (0.0-0.4 mg/ml) of the extract were mixed with potassium hexacyanoferrate (0.1%), followed by incubation. After incubation, the upper portion of the solution was diluted, and FeCl$_3$ solution (0.01%) was added. The reaction mixture was left for 10 min at room temperature for colour development and the absorbance was measured at $\lambda = 700$ nm. All tests were performed six times. A higher absorbance of the reaction mixture indicated greater reducing power ascorbic acid was used as a positive control.

Lipid peroxidation inhibition

The inhibition of lipid peroxidation of mice brain homogenate was assayed by measuring malondialdehyde (MDA), according to the method of Kızıl et al., (2008) with slight modification. The homogenate was prepared by centrifuging the brain (20 ± 2 gm) with 50 mM phosphate buffer (pH 7.4) and 120 mM KCl, at 3000 rpm for 10 min. A 100 µl aliquot of the supernatant homogenate was mixed with plant extract of various concentrations (2.5-25 µg/ml), followed by addition of 0.1 mM FeSO$_4$ and 0.1 mM ascorbic acid, each of 100 µl and incubated for 1 hr at 37 °C. 500 µl 28% TCA was used to stop the reaction and then 380 µl 2% TBA was added with heating at 95 °C for 30 min, to generate the colour. The samples were cooled on ice, centrifuged at 8000 rpm for 2 min and the absorbance of the supernatant was taken at 532 nm. All tests were repeated 6 times. Trolox was used as the standard.

3.4.2.9 Synthesis of Carbon Nanomaterials from Euglena tuba
A quartz boat loaded with 10 g of biomass known as CVD precursor was kept inside the horizontal quartz tube of CVD furnace. Argon gas was allowed to flow with a flow rate of 6ml/min. Furnace was then switched on to attain the set temperature of 900°C. When the temperature reached, furnace was left on for a set time of 2 hours and then allowed to cool. After cooling the furnace, carbon materials were taken out and purified by heating at 400°C for half an hour to remove the amorphous carbon from the synthesized material.

A detail methodology on carbon nanoparticles from *Euglena* are described in Chapter 8.

### 3.5. Statistical Analysis

All statistical analyses (one-way ANOVA; correlation; Principal Component Analyses (PCA); Hierarchical cluster analysis) were done by SPSS 15.0 software package. The statistical analysis for antioxidant property of *Euglena* sp. was performed by KyPlot version 2.0 beta 15 (32 bit). The IC50 values were calculated by the formula, \( Y = 100 \times A_1/(X + A_1) \) where \( A_1 = IC_{50} \), \( Y \) = response (\( Y = 100\% \) when \( X = 0 \)), \( X \) = inhibitory concentration. The IC50 values were compared by paired t test. \( p < 0.05 \) was considered significant.
Fig. 3.1: Map showing the locations of collection sites in Cachar District
Plate 3.1: Bloom of *Euglena* sp. observed in the study sites during 2008-2010