BIOCHEMICAL AND MOLECULAR CHARECTERISATION OF A MANGROVE -*Excoecaria agallocha.*

PROJECT REPORT

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FINAL SEMESTER PROJECT IN

CELL & MOLECULAR BIOLOGY

FOR THE AWARD OF THE DEGREE OF

MASTERS OF SCIENCE IN BOTANY

SUBMITTED BY

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CELL BIOLOGY & BIOTECHNOLOGY

POST GRADUATE DEPARTMENT OF BOTANY

LADY BRABOURNE COLLEGE

AFFILIATED BY UNIVERSITY OF CALCUTTA

KOLKATA, WEST BENGAL

2019

CERTIFICATE

This is certifying that ***Sukanya Saha*** *has carried out this project (A Comprehensive Review and Research Work) for the fulfilment of the* ***Master's Degree in Botany****, with special paper* ***Cell Biology & Biotechnology*** *2019, under my supervision.*

The little of the project on which she has worked on is, ***BIOCHEMICAL AND MOLECULAR CHARECTERISATION OF A MANGROVE FERN-Excoecaria agallocha.***

The student has completed her work sincerely and to my satisfaction.

_____

Dr. Debleena Roy

ASSISTANT PROFFESSOR

POST GRADUATE DEPRTMENT OF BOTANY

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KOLKATA; WEST BENGAL

DATE: _____

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DATE: _____

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SUKANYA SAHA

CHAPTER: 1

INTRODUCTION

1. INTRODUCTION:

Mangroves are diverse and highly productive ecological communities which provide important ecosystem functions. Located at the land-sea interface, they protect coastal areas against natural hazards such as cyclones and tsunamis they retain terrestrial sediment and recycle nutrients, thus supporting clear offshore waters, which in turn favors the photosynthetic activity of phytoplankton as well as growth and robustness of coral reefs, seagrass beds and reef fish communities they serve as an important habitat, nursery and refuge, providing food for countless organisms including humans . These ecosystems are also vital carbon sinks, either storing carbon temporarily within organic peat soils, or as dissolved organic carbon in ocean sediments at greater depths, offsetting climatic-active greenhouse gasses for longer periods. Because of these collective ecosystem services, mangroves are also having great economic value. However, owing to both anthropogenic and climatic factors, mangroves the world over are severely threatened, and, with current global annual loss rates of 1%–2%, no such forests may be left by the end of the 21st century, if the current trend continues.

1.1 DISTRIBUTION:

The Sundarbans stretches along the coast of Bangladesh (6,017 km2) and India (4,000 km2) and forms the largest single block of halophytic mangrove forest in the world. Covering approximately 10,000 km the Sundarbans in the Northern Bay of Bengal is the largest contiguous mangrove forest on earth. Mangroves forests are highly productive and diverse ecosystems, providing a wide range of direct ecosystem services for resident populations. In addition, mangroves function as a buffer against frequently occurring cyclones; helping to protect local settlements including the two most populous cities of the world, Kolkata and Dhaka, against their worst effects. Mangroves constitute among the most dominant intertidal ecosystem along the tropical and subtropical coastlines. The distribution of mangrove populations is considered to be largely shaped by their responses to colder climate and arid conditions at the limit of their ranges, and have been expanding their ranges along changing coastal zones since the last glacial period (Duke et al., 1998; Saenger, 1998; Dodd et al., 2002; Saintilan et al., 2014). Most mangrove species have viviparous propagules that are buoyant and can be dispersed by ocean currents. These make mangrove species good candidates for the study of population genetic structures influenced by founder effects resulting from frequent local extinction, recolonization, and

long-distance dispersal (LDD) of propagules by water currents along coastlines of different forms.

It was designated a Ramsar site under the Ramsar Convention in 1927. UNESCO declared the Sundarbans a World Heritage Site in 1997, because of its 'Outstanding Universal Value', biological diversity and the ecosystem services the area provides.

- **1.2 HABIT:**

The plant ***Excoecaria agallocha*** from the family **Euphorbiaceae** is a dioecious tree to 15 m high with abundant white latex; It is an evergreen shrub commonly along with higher estuarine banks, cannels, tidal forest and mangrove swamps; bark grayish, lenticulate. Lateral roots spreading and intermingled with each other, supraterranean bands produce elbow-shaped pegs instead of pneumatophores; leaves alternate, ovate-elliptic or orbicular, apex shortly acuminate, base narrowed, margin entire or sinuate crenate, 3-8 × 1.5-3 cm, glabrous, petiolate; Flowers are Unisexual, Male flowers in catkin spikes, fragrant, yellow, 2-3 mm across; stamens 3, filaments free. Female flowers in axillary raceme, pale green, 2.5-3.5 mm across, pedicellate; calyx 3-lobed; ovary 3-celled, trifid style; Fruits Capsule, globose 3-lobed, seeds sub-globose; Flowers are pollinated by insects; germination is epigeal or modified epigeal type.

- This evergreen mangrove species has traditionally been used to treat sores and stings from marine creatures, and ulcers, as a purgative and an emetic, and the smoke from the bark to treat leprosy They are well-known as extreme skin irritants and tumor promoter
- Recent ethnobotanical survey on Kodiyampalayam coastal village, Nagapattinam district, Tamilnadu, India depicted the presence and traditional usage of *E. agallocha* to blood glucose level reduction and fish poison.

- **FIGURE 1: INFLORESCENCE;**
- **FIGURE 2: FRUITS;**
- **FIGURE 3: ELBOW-SHAPED ROOTS;**
- **FIGURE 4: LEAVES**

OF *Excoecaria agallocha*

FIGURE - 1

FIGURE - 2

FIGURE - 3

FIGURE - 4

Excoecaria agallocha

SYSTEMATIC POSITION

KINGDOM: Plantae

ORDER: Malpighiales

FAMILY: Euphorbiaceae

TRIBE: Hippomaneae

GENUS: ***Excoecaria***

SPECIES: ***Excoecaria agallocha***

CHAPTER: 2

RATIONALE OF WORK

➢ 2. RATIONALE OF WORK:

We choose to working on the Restriction Digestion of Chloroplast DNA molecules and protein profiling by using SDS-PAGE Gel electrophoresis of total protein and chloroplast protein of *Excoecaria agallocha.*

RESTRICTION DIGESTION can be used for easy visualization of sequence dissimilarity of multiple species of the same genus. just by amplifying the isolated **cp DNA** with its conserve sequence dependent primers and double digestion with easily available **RE**s **HIND III** and **ECoR**1 and by simple gel electrophoresis differencing the banding pattern could be generated.

A **restriction enzyme** or **restriction endonuclease** is an enzyme that cleaves DNA into fragments at or near specific recognition sites within molecules known as restriction sites. Restriction enzymes are one class of the broader endonuclease group of enzymes. To cut DNA, all restriction enzymes make incisions, through each sugar-phosphate backbone (i.e. each strand) of the DNA double helix. Over 3,000 restriction enzymes have been studied in detail, and more than 600 of these are available commercially. These enzymes are routinely used for DNA modification in laboratories, and they are a vital tool in molecular cloning. Here we are using EcoR1 & Hind III to cleave the chloroplast DNA of *Excoecaria agallocha.*

SDS-PAGE (**sodium dodecyl sulfate–polyacrylamide gel electrophoresis**) is a variant of polyacrylamide gel electrophoresis, an analytical method in biochemistry for the separation of charged molecules in mixtures by their molecular masses in an electric field. It uses sodium dodecyl sulfate (SDS) molecules to help identify and isolate protein molecules. SDS-PAGE is an electrophoresis method that allows protein separation by mass. The medium (also referred to as ′matrix′) is a polyacrylamide-based discontinuous gel. In addition, SDS (sodium dodecyl sulfate) is used. About 1.4 grams of SDS bind to a gram of protein, corresponding to one SDS molecule per two amino acids. SDS acts as a surfactant, covering the proteins intrinsic charge and conferring them very similar charge-to-mass ratios. The intrinsic charges of the proteins are negligible in comparison to the SDS loading, and the positive charges are also greatly reduced in the basic pH range of a separating gel. Upon application of a constant electric field, the protein

migrates towards the anode. each with a different speed, depending on its mass. This simple procedure allows precise protein separation by mass.

As we know mangroves are world over severely threatened .so we are trying to know the DNA sequence, proteomics and molecular details of the mangrove fern *Excoecaria agallocha* for further in-situ conservation in future.

❖ 2.1 OBJECTIVES:

- **Quantify the total protein content and chloroplast specific protein content by using Different Biochemical Assays.**
- **Extraction of total proteins and chloroplast proteins from leaf tissue.**
- **By Using SDS-PAGE profiling to generate the proper banding pattern of the proteins of the plant under study.**
- **Restriction Digestion of Chloroplast DNA by using REs - EcoR1 and HindIII.**

CHAPTER: 3

REVIEW OF LITERATURE

➢ REVIEW OF LITERATURE:

A mangrove is a tree, shrub, palm or ground fern, generally exceeding one half meter in height, that normally grows above mean sea level in the intertidal zone of marine coastal environments and estuarine margins. The term "Mangroves", plants which exist in muddy, wet soil in tropical or subtropical tidal waters. (Kaliamurthi and Selvaraj, Nat Prod Chem Res 2016,*) Excoecaria agallocha* is one of the predominant mangrove species in the Indo-West Pacific (IWP) region with an extensive range of distribution.

Molecular studies on plants like these could be helpful for their conservation. Knowing the molecular and biochemical characters can be useful tool for better utilization of these plants. In depth studies can uncover various crucial important facts about their salinity tolerance and their native mode of livelihoods. That knowledge can further be exploited for creating new transgenic crop lines.

Some studies already suggest that the antioxidant potential of the leaves of the mangrove plant *Excoecaria agallocha* Linn. using in vitro assay, the methanol extract of *E. agallocha* Linn has exhibited potent free-radical scavenging activity particularly against DPPH and water fraction observed to be effective in protecting oxidative DNA damage due to presence of flavonoids. The water and methanol fraction of the extract has revealed a significant protective effect against Fenton's reaction on supercoiled pCAMBIA assayed by agarose gel electrophoresis. The extract also exhibited strong lipid peroxidation inhibition nitric oxide radical inhibition and a metal chelating effect in a concentration dependent manner. (C. Asha et al.2012). Some researchers also found that *Excoecaria lucida* leaves (Euphorbiaceae) are used by the Cuban population due to their antimicrobial activity. (A.Ochoa-Pacheco et al.2017). Some studies also suggest that *Excoecaria agallocha* has anti-cancer activity. The methanol extract and chloroform extract of leaves were treated with under various concentrations extract. (B. Ahmed et al.2013). Some researches also suggest that *Excoecaria agallocha* also has anti-tumor-promoting activities. Eight new diterpenoids have been isolated from the wood of *Excoecaria agallocha* (Euphorbiaceae) and their inhibitory effects on the induction of Epstein–Barr virus early antigen (EBV-EA) in Raji cells were examined to search for potent anti-tumor-promoters from natural resources.) (K.Takao et al.2001).An article also suggests that *Excoecaria agallocha* have some radical scavenging effects. The alkaloids were identified

from crude alkaloids fraction of *E.agallocha* leaves using GC-MS analysis. GC-MS results identify 21 Phyto-constituents, include 12 alkaloid derivatives are 1-[a-(1-Adamantyl) benzyl idene] thio, Propenamide, Benzene methanol, 2-(2-aminopropoxy, 2-5-Dimethoxy-4-(methyl thionyl), 1-Amino-2-(hydroxymethyl) anthraquinone, DL-Cystine, 2-Propen-1-one, 3-(4-nitrophenyl)-, Phenylephrine and 5 higher alkanes viz., Hexadecane, Tetradecane, Pentadecane and Heptafluorobutyric acid acids/alcohols are present in significant concentration.(K Satyavani et al.2013) .It was also found from an article that Three new halimane-type diterpenoids and three clerodane-type diterpenoids were isolated from the twigs of *Excoecaria formosana*. (Lin.B et al. 2016) The neuropharmacological, microbiological and toxicological studies on the ethanol extract of the bark of *Excoecaria agallocha* are also reported. The extract was found to produce a profound decrease in exploratory activity in a dose dependent manner. It also showed a marked sedative effect as evidenced by a significant reduction in gross behavior and potentiation of sodium thiopental-induced sleeping time. The totality of these effects showed that the extract possesses depressant action on the central nervous system (CNS). The extract of *E. agallocha* exhibited significant in vitro antibacterial activity against Staphylococcus aureus, Shigella dysenteries, Shigella sonnies and Enterococci with the zones of inhibition it displayed only low level of toxicity in mice.(N.Subhan et al. 2008).It is also used traditionally in the treatment of various diseases such as epilepsy, ulcers, leprosy, rheumatism, and paralysis. The latex obtained from the bark is poisonous in nature and may cause temporary blindness, thus it is also known as the blind-your-eye mangrove plant. Many phytoconstituents were isolated from the plant, which were mainly diterpenoids, triterpenoids, flavonoids, sterols, and few other compounds. The plant also showed many pharmacological activities such as antioxidant, antimicrobial, anti-inflammatory, analgesic, antiulcer, anticancer, antireverse transcriptase, antihistamine-release, antifilarial, DNA damage protective, antidiabetic, and antitumor protecting activities. (Mondal S, Ghosh D, et al. 2016).A novel biological protocol for the synthesis of silver nanoparticles was also found using aqueous extract of *Excoecaria agallocha* . The newly synthesized silver nanoparticles were characterized using physico-chemical techniques UV, XRD and TEM analysis. The transmission electron microscopy result indicates that the most of the formed silver nanoparticles were spherical in nature. (Arun.S et al. 2014). *Excoecaria agallocha* (L.). Leaves extract were also evaluated for its effect on blood sugar level in normal and alloxan induced wistar albino mice at various time

points comparing it with standard drug metformin. The studies indicated that the crude ethanolic extract exhibited significant hypoglycemic and anti- hyperglycemic activities in normal and alloxan ñ induced diabetic albino mice respectively. The study reports for the first time the hypoglycemic activity of *Excoecaria agallocha* (L.) in mice. (Thirumurugan.G et al. 2010). Another paper suggests that Three tigliane-type diterpenoids named excoecafolins and two daphnane-type diterpenoids named excoecafolins and together with 13 known compounds, were isolated from the EtOAc extract of *Excoecaria acerifolia Didr*. Their structures were elucidated through the analysis of the spectroscopic data. And found The anti-HIV-1 activity. (Huang S.Z et al. 2014). From some another work we came to know that four new polyphenols containing both phenolic bisabolane and diphenyl ether units, expansols and one new diphenyl ether derivative, 3-*O*-methyldiorcinol as well as twelve known compounds were isolated from *Penicillium expansum* endogenous with the mangrove plant

Excoecaria agallocha (Euphorbiaceae).(Wang.J et al.2012) .Some researchers suggest that excoecarianin isolated from the whole plant of Phyllanthus urinaria Linnea (Euphorbiaceae) through acetone extraction, and investigated its anti-HSV-1 and HSV-2 activities. (Cheng HY et al. 2018).

Some results also show that *Excoecaria agallocha* of Indian Sundarbans region can tolerate and adapt to high saline condition. The effect of salinity on chlorophyll a, chlorophyll b and total chlorophyll levels of hydroponically grown seedlings of *Excoecaria agallocha* was studied. The selected seedlings were exposed to five different salinity levels (2, 5, 10, 15 and 20 psu) for a period of 30 days and observations were done at a regular interval of 7, 14, 21 and 30 days respectively. The concentrations of chlorophyll exhibited significant positive correlations with salinity ($p < 0.01$). The chlorophyll a:b ratio in the seedlings varied between 2.65 to 4.92 throughout the period of investigation. (Pal.N et al. 2014)

E. agallocha was also found across its distribution range and investigated the phytogeography of this species using four chloroplast DNA (cpDNA) fragments. The results showed that *E. agallocha* possessed a high degree of species-level genetic diversity, while the average genetic diversity within populations was much lower. The presence of population genetic structure was supported by the estimates of genetic differentiation and the analysis of molecular variance (AMOVA). Of the ten haplotypes identified, no

haplotypes were shared between the East Indian Ocean (EIO), the West Pacific Ocean (WPO), and the North Australian (NA) regions. Genealogy analysis, haplotype distribution patterns, and the principal coordinate analysis (PCoA) consistently suggested the existence of three haplotype groups distributed in distinct geographical locations. (Guo.W et al.2017). The responses of anti-oxidative enzymes and stress related hormones was also found in *E. agallocha* to different levels of Pb stresses at different exposure time. Pb stress posed higher toxic effects on root than leaf. (Yan.Z & Tam.N 2012). Another article suggests that the growth of *Excoecaria agallocha* seedling decreases as the NaCl salinity increases in the soil. It was observed that the germination percentage were decreased with increasing NaCl salinity. The maximum percentage (100%) of seed germination was recorded in fresh water and the minimum percentage (36%) of germination was observed in 200 mM NaCl concentration. (S. Sivasankaramoorthy et.al 2010).The total blue carbon stock of the Bangladesh Sundarbans mangroves was evaluated and the probable future status after a century was predicted based on the recent trend of changes in the last 30 years and implementing a hybrid model of Markov Chain and Cellular automata. The prediction shows that after a hundred years the blue carbon would be lost from the low saline regions accompanied by increase in the high saline regions dominated mainly by *Excoecaria sp.* and *Avicennia spp*. The net carbon loss would be due to both mangrove area loss and change in species composition of CO2 emission within the year 2115. (Chanda. A et al. 2016).

CHAPTER 4

BIOCHEMICAL ANALYSIS

4.1 INTRODUCTION:

Molecular and Biochemical studies on plants like these could be helpful for their conservation. Knowing the molecular and biochemical characters can be useful tool for better utilization of these plants. In depth studies can uncover various crucial important facts about their salinity tolerance and their native mode of livelihoods. That knowledge can further be exploited for creating new transgenic crop lines.

4.2 BACKGROUND:

Some studies already suggest that the antioxidant potential of the leaves of the mangrove plant *Excoecaria agallocha* Linn. using in vitro assay, the methanol extract of *E. agallocha* Linn has exhibited potent free-radical scavenging activity particularly against DPPH and water fraction observed to be effective in protecting oxidative DNA damage due to presence of flavonoids. The water and methanol fraction of the extract has revealed a significant protective effect against Fenton's reaction on supercoiled pCAMBIA assayed by agarose gel electrophoresis. The extract also exhibited strong lipid peroxidation inhibition nitric oxide radical inhibition and a metal chelating effect in a concentration dependent manner. (C. Asha et al.2012). Some researchers also found that *Excoecaria lucida* leaves (Euphorbiaceae) are used by the Cuban population due to their antimicrobial activity. (O.Ania, et al.2017). Some studies also suggest that *Excoecaria agallocha* has anti-cancer activity. The methanol extract and chloroform extract of leaves were treated with under various concentrations extract. (B. Ahmed et al.2013). Some researches also suggest that *Excoecaria agallocha* also has anti-tumor-promoting activities. Eight new diterpenoids have been isolated from the wood of *Excoecaria agallocha* (Euphorbiaceae) and their inhibitory effects on the induction of Epstein–Barr virus early antigen (EBV-EA) in Raji cells were examined to search for potent anti-tumor-promoters from natural resources.) (K.Takao et al.2001).An article also suggests that *Excoecaria agallocha* have some radical scavenging effects. The alkaloids were identified from crude alkaloids fraction of *E.agallocha* leaves using GC-MS analysis. GC-MS results identify 21 Phyto-constituents, include 12 alkaloid derivatives are 1-[a-(1-Adamantyl) benzyl idene] thio, Propenamide, Benzene methanol, 2-(2-aminopropoxy, 2-5-Dimethoxy-4-(methyl thionyl), 1-Amino-2-(hydroxymethyl) anthraquinone, DL-Cystine, 2-Propen-1-one, 3-(4-nitrophenyl)-, Phenylephrine and 5 higher alkanes viz., Hexadecane, Tetradecane, Pentadecane and Heptafluorobutyric acid acids/alcohols are present in significant

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4.3 MATERIALS & METHODS:

4.3.1 PLACE OF SAMPLE COLLECTION:

The collection spot Kumirmari was selected based on the abundance of the *Excoecaria agallocha* specimens. From the identified cluster a healthy young plant was selected and juvenile leaves of the sample were served at the base. They were then washed with distilled water and segregated into airtight polypropylene bags. Once in the lab they were stored for further use in -20°C freezer. From this collected set 5 gm of sample was weighed and crushed using a mechanical homogenizer. The homogenized sample was then separated into five sets 1 gm each and further analysis was performed using the individual sets.

FIG 4.3.1: PLACE OF SAMPLE COLLECTION

4.3.2 BIOCHEMICAL ASSAYS:

TABLE: 4.3.2.1: TOTAL FLAVONOIDS ASSAY

REAGENTS		STRENGTH	QUANTITY
i.	**CRUSHED LEAF**	-	1gm
ii.	**METHANOL**	100%	10ml
iii.	**QUERCETIN (Standard curve)**	(0.1, 0.2,0.3,0.4,0.5)	5ml
iv.	**METHANOL STOCK**	-	0.1ml
v.	**DISTILLED WATER**	-	1.2ml+1.6ml
vi.	**Na-NITRITE**	5%	0.12ml
vii.	**ALLUMINIUM CHLORIDE**	10%	0.12ml
viii.	**NaOH**	1mM	0.8ml

- **PREPARATION OF STOCK:**1gm of crushed plant sample was taken and then 10ml of methanol was added to it. After that, the solution was filtered and it was kept.
- **PROTOCOL:**

0.1ml of methanol stock solution of plant extract was taken (0.1 ml methanol for blank preparation was used). Then 1.2 ml of D.H_2O was added. Further 0.12 ml 5% Na-nitrite was added to it. Then wait for 5minutes and 0.12ml of $Alcl_3$ was added to the solution. After that, kept for 5minutes.Finally,0.8ml of 1mM NaOH and 1.6ml D.H_2O was added. At last, absorbance was recorded at 510 nm.

- **CALCULATION**

Plot against Quercetin standard curve to get the gm equivalent (Quercetin) value.

TABLE: 4.3.2.2: ABTS ASSAY

REAGENTS	STRENGTH	QUANTITY
1. **METHANOL STOCK**	-	0.1ml
2. **DISTILLED WATER**	-	50ml
3. **AQ. ABTS SOLUTION**	7Mm	5ml
4. **POTASSIUM PERSULFATE**	140mm	88ml
5. **ALCOHOLIC BHT SOLUTION**	0.1g/ml	10ml

- **PREPARATION OF RADICAL CATION SOLUTION:** 5ml of aq. ABTS solution was added to 88µl of 2.45 mM Potassium-persulfate solution. Then, it was kept for 16 hours for incubation.
- **PREPARATION OA DILUTE RADICAL CATION SOLUTION:** 1000µl of radical cation solution was added to 10ml of alcohol (methanol) for preparing stock solution.
- **PROTOCOL:** 2ml of dilute radical cation solution was taken and 20µl of methanol stock solution of plant extract was added to it. (20µl Methanol for blank preparation was used /20µl of alcoholic BHT solution was used for positive control). The mixture was stirred for 30minutes.Absorbance was recorded at 744nm at an interval of 30minutes for 120 minutes stared from 0 minute. ABTS (% inhibition) was calculated and average was taken.
- **CALCULATION**
  - ABTS (%inhibition) = [(AS/AB) *100]
  - AS = Absorbance of sample
  - AB = Absorbance of Blank

TABLE: 4.3.2.3: TOTAL PHENOL ASSAY

REAGENTS	STRENGTH	QUANTITY
1. **GALLIC ACID MONOHYDRATE (**STANDARD CURVE)	1 M	10ml
2. **FOLIN CIOCALTEAU**	-	10ml
3. **Na_2CO_3**	7.5%	1.6ml
4. **DISTILLED WATER**	-	100ml
5. **FC REAGENT**	-	50ml

- **PREPERATION OF CIOCALTEU REAGENT: -**

10ml of commercial Folin-Ciocalteu reagent was taken. 1.6ml of 7.5% Na_2CO_3 was added. The volume was made up to 50ml by adding distilled water.

- **PROTOCOL: -**

0.1ml of Methanol Stock Solution of plant extracts were taken. [0.1ml Methanol was used for Blank preparation]. To it 2ml of prepared Folin Ciocalteau reagent solution was added. The absorbance was recorded at 765nm.

- **CALCULATION: -**

Total phenol content from the Gallic Acid Standard curve was plotted in gm equivalent (Gallic Acid equiv.).

TABLE: 4.3.2.4: CHLOROPHYLL ESTIMATION

REAGENTS	STRENGTH	QUANTITY
1. **CRUSHED LEAF**	-	1gm
2. **ACETONE STOCK**	-	1ml
3. **ACETONE**	80%	10ml
4. **DISTILLED WATER**	-	2ml

- **PREPERATION OF STOCK: -**

ACETONE STOCK OF PLANT EXTRACT: 1g of crushed plant sample was taken. 10ml of 80% acetone was added to it. The solution was filtered and kept.

- **PROTOCOL: -**

1ml of Acetone Stock Solution plant extract was taken. It was then centrifuged for 5000 rpm for 1min. The supernatant was collected and the pellet was discarded. 2ml of distilled water was added to the supernatant. Absorbance was taken at **645nm, 652nm** and **653nm**. 80% acetone was used as blank.

- **CALCULATION: -**

Chlorophyll a = 12.7 **(A663) -** 2.69 **(A645)**
Chlorophyll b = 22.9 **(A663) -** 4.68 **(A645)**

Total Chlorophyll = Chlorophyll a + Chlorophyll b

- **TOTAL CHLOROPHYLL IN ORIGINAL TISSUE SAMPLE-**

Chl a × final volume (here 3ml)

Chl b × final volume (here 3ml)

Total Chl × final volume (here 3

TABLE: 4.3.2.5: DPPH ASSAY

REAGENTS	STRENGTH	QUANTITY
1. DPPH SOLUTION (light sensitive)	0.075mg/30ml	30ml
2. METHANOL STOCK	-	0.1ml
3. METHANOL	100%	20ml
4. BHT (positive control)	200 mg in 2ml Methanol	2ml

- **PREPERATION OF STOCK: -**

- **METHANOLIC STOCK OF PLANT EXTRACT-** 1g of crushed plant sample was taken. 10ml of methanol solution was added to it. The solution was filtered and kept.

- **PROTOCOL: -**

0.1 ml of Methanol Stock Solution of plant extract was taken. [0.1ml Methanol for Blank preparation /0.1 ml BHT solution for positive control was used].3.9 ml 5% DPPH Solution was added to it. The solution was allowed to stand for 30 mins in dark at room temperature. The absorbance was recorded at 517 nm.

- **CALCULATION: -**

DPPH [% of scavenging radical (% inhibition)] = [(AB-AA)/AB] ×100

AB = Absorbance of Blank

AA = Absorbance of Sample.

TABLE: 4.3.2.6: CARBOHYDRATE TEST

REAGENTS	STRENGTH	QUANTITY
1. ANTHRONE	-	100mg
2. H_2SO_4	95%	50ml
3. GLUCOSE	-	100mg/100ml

- **PROTOCOL:**

0.5ml plant extract of sample was taken and then o.5ml of distilled water was added to it. Further 4ml of anthrone reagent was added. At last, solution was kept stand for 8minutes for boiling water bath Finally, absorbance was recorded at 630nm.

- **CALCULATION**

Calibrate present in 100mg of the sample = [mg of glucose volume of the test sample) x100

TABLE: 4.3.2.7: IRON CHELATION TEST

REAGENTS	STRENGTH	QUANTITY
HEPES BUFFER SOLUTION	**20mM**	**-**
$Feso_4$	**-**	**12.5µM**
Ferozin	**-**	**75 µM**
EDTA	**1M**	**1ml**

- **PROTOCOL:**

1gm plant extract of sample was taken and then 1ml of EDTA was added to it. And then, 5ml of working solution was added and solution was kept stand for 20 minutes in dark at room temperature. At last, the absorbance was recorded at 562 nm.

- **CALCULATION**

A_U = Unknown sample

A_B = Blank

A_S = Standard

$= [A_U - A_B / A_S - A_B] x\ 100$

TABLE: 4.3.2.8: TOTAL PROTEIN ASSAY

REAGENTS	STRENGTH	QUANTITY
1. **PROTEIN**	-	**1ml**
2. **Na2CO3 (A)**	**2%**	**48ml**
3. **Na-K (B)**	**1%**	**1ml**
4. **CuSO4 (C)**	**0.5%**	**1ml**
5. **FOLIN CIO-CALTEAU REAGENT**	-	**4ml**
6. **DISTILLED WATER**	-	**30ml**
7. **BSA STOCK** (STANDARD CURVE)	**1mg/ml**	**20ml**

- Reagent (I) = [48 ml (A) + 1 ml (B) + 1 ml (C)]
- Reagent (II) = [Folin-Ciocalteau Reagent(commercial): d.H2O] =1:1

➢ **PROTOCOL: -**

0.5ml of protein sample was taken. 0.5ml of distilled water was added to it. 4.5ml of Reagent (I) was added to it. Then we waited for 10minutes. To it 0.5ml of Reagent (II) was added. The mixture was incubated in dark for 30minutes. Absorbance was recorded at 660nm. Distilled water was used as blank.

➢ **CALCULATION: -**

It was plotted against BSA standard curve to get the mg/ml (BSA standard) value.

4.4 RESULT:

The profiles of the antioxidant assays from the standardized extract indicate that the free radical scavenging activity is present ubiquitously and in considerable amount. In our results we have been able to understand the biochemical functioning of the plant *Excoecaria agallocha* using the standard content analysis of total phenol content and total flavonoids both important by-product of metabolism. Total carbohydrate concentration is important regarding studying photosynthetic efficiency. Further, the antioxidant potentials of the leaf extracts were also tested using standard protocols of DPPH assay, ABTS assay, and Fe chelation activity. The results show that the leaf extraction of the plant *Excoecaria agallocha* contains total phenol 6.237 gm and flavonoids 0.0829 gm as shown in the (TABLE:4.4.2) (FIG: 4.4.2.2B) total chlorophyll content 8.49 (TABLE:4.4.1) (FIG:4.4.1), total carbohydrate concentration 20.089 gm eqv.(TABLE: 4.4.2) (FIG:4.4.2C), DPPH assay 24.34% inhibition, ABTS assay 72.43 % inhibition and iron chelation activity 63.023 mg/ml as shown in the (TABLE: 4.4.2) (FIG: 4.4.2A) The high content of chlorophyll and carbohydrates are important indicators of higher photosynthetic efficiency which may be attributable to the total incident light that the plants receive through the twelve hours photoperiod in low canopy areas. ABTS cation radical concentrations were found to be significantly reduced during the assay where butylhydroxytoluene served as positive control.

In our results we have been able to understand the biochemical functioning of the plant using the standard content analysis of total phenol contents, DPPH test, total protein content, total flavonoid contents, ABTS assay, carbohydrate contents, Fe chelation test etc. And the results are showing that the leaf extractions of the *Excoecaria agallocha* contain total phenol 6.237 gm and flavonoids 0.0829 gm (TABLE:4.4.1.2) (FIG:.4.4.2B) total chlorophyll content 8.49 (TABLE:4.4.1.1) (FIG:4.4.1) total carbohydrate concentration 20.089 mg/ml ,Total protein content 6.237 gm eqiv. (TABLE: 4.4.2) (FIG:4.4.2C), DPPH assay 24.34% inhibition, ABTS assay 72.43% inhibition and Iron chelation activity 63.023 mg/ml. (TABLE:4.4.1.2) (FIG: 4.4.2A).

TABLE 4.4.1.:

Sl. No	TEST	Chl A /gm	Chl B/gm	Total Chl (A+B)
1.	**Chlorophyll Estimation**	4.95	3.54	8.49

TABLE: 4.4.2

Sl. No	TEST	RESULT	UNIT
1	**DPPH Assay**	24.34	% of inhibition
2	**Total Phenol**	6.237	gm equivalent (Gallic acid equiv)
3	**Total Protein**	0.492	mg /ml (BSA Standard)
4	**Total Flavonoid**	0.0829	gm equivalent. (Quercetin equiv)
5	**ABTS Assay**	72.43	% of inhibition
6	**Carbohydrate**	20.089	mg /ml
7	**Fe-Chelation**	63.023	mg /ml

- **FIG: 4.4.1: GRAPHICAL REPRESENTATION OF CHLOROPHYLL ESTIMATION**

- **FIG 4.4.2A: GRAPHICAL REPRESENTATION OF BIOCHEMICAL ASSAY**

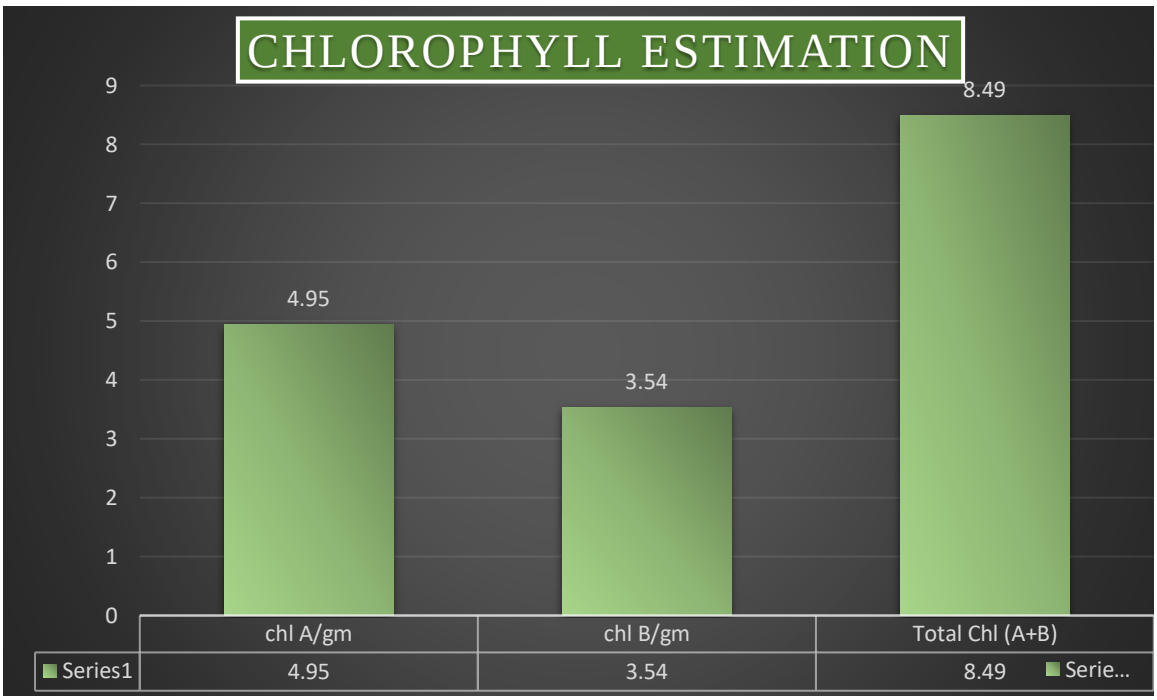


FIG: 4.4.1

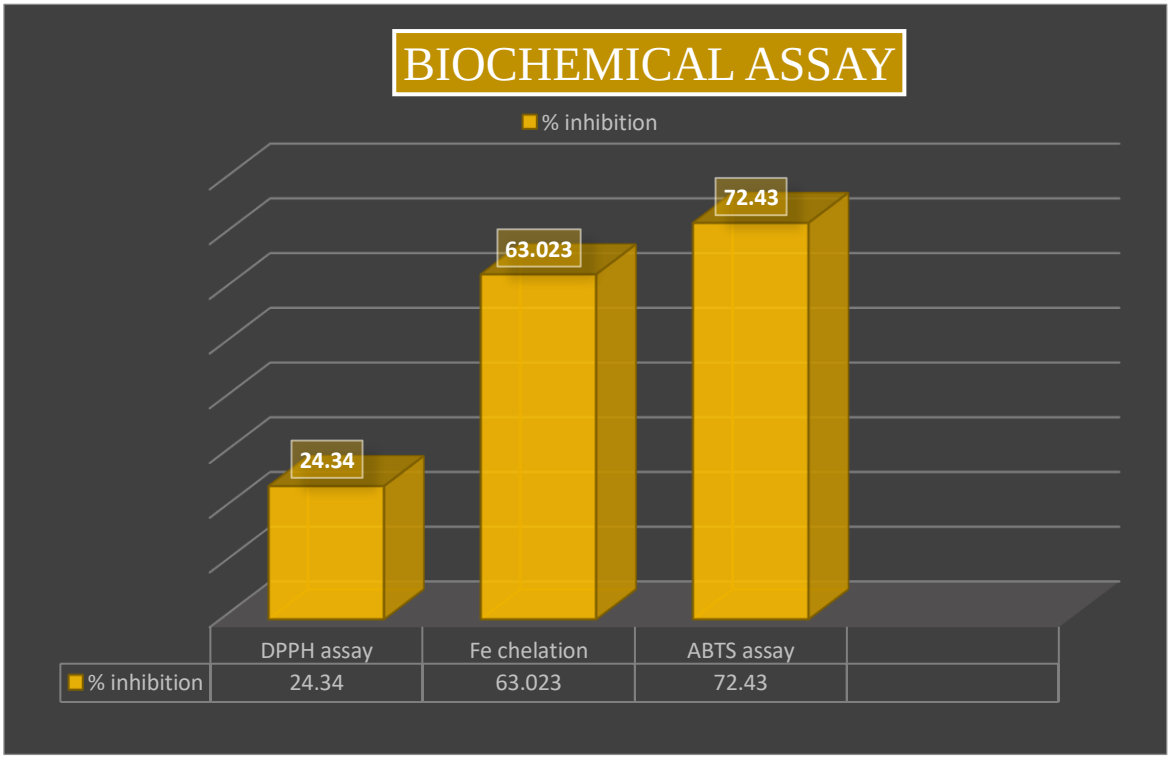


FIG: 4.4.2.A

- **FIG 4.4.2B: GRAPHICAL REPRESENTATION OF BIOCHEMICAL ASSAY**

- **FIG 4.4.2C: GRAPHICAL REPRESENTATION OF BIOCHEMICAL ASSAY**

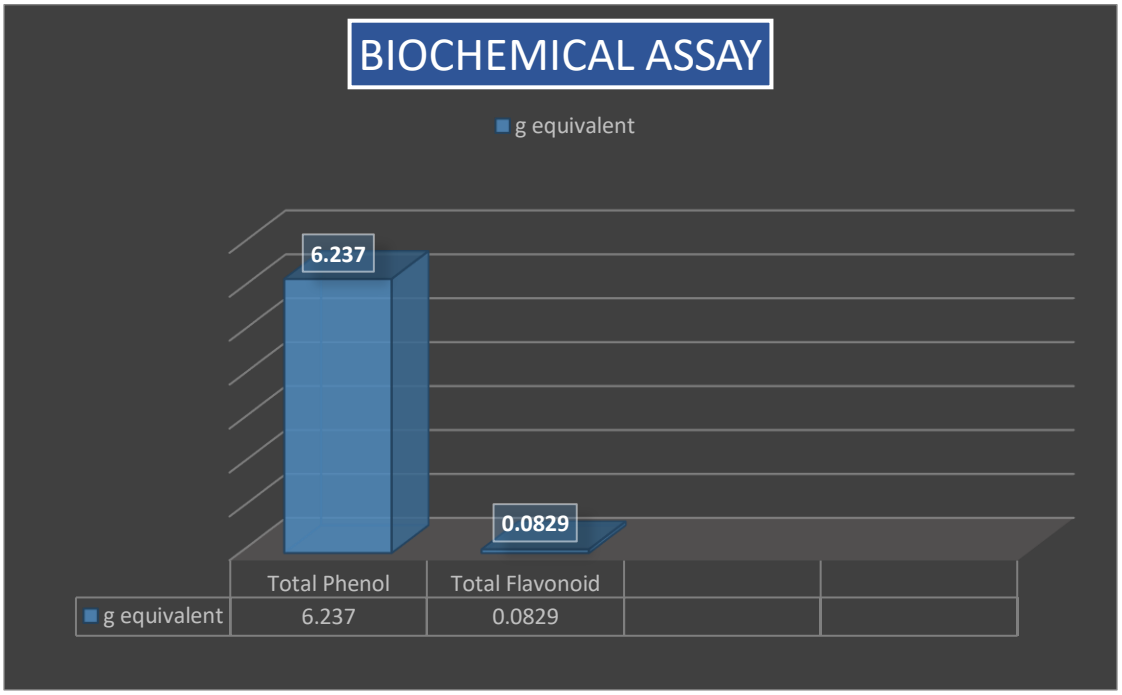


FIG: 4.4.2B

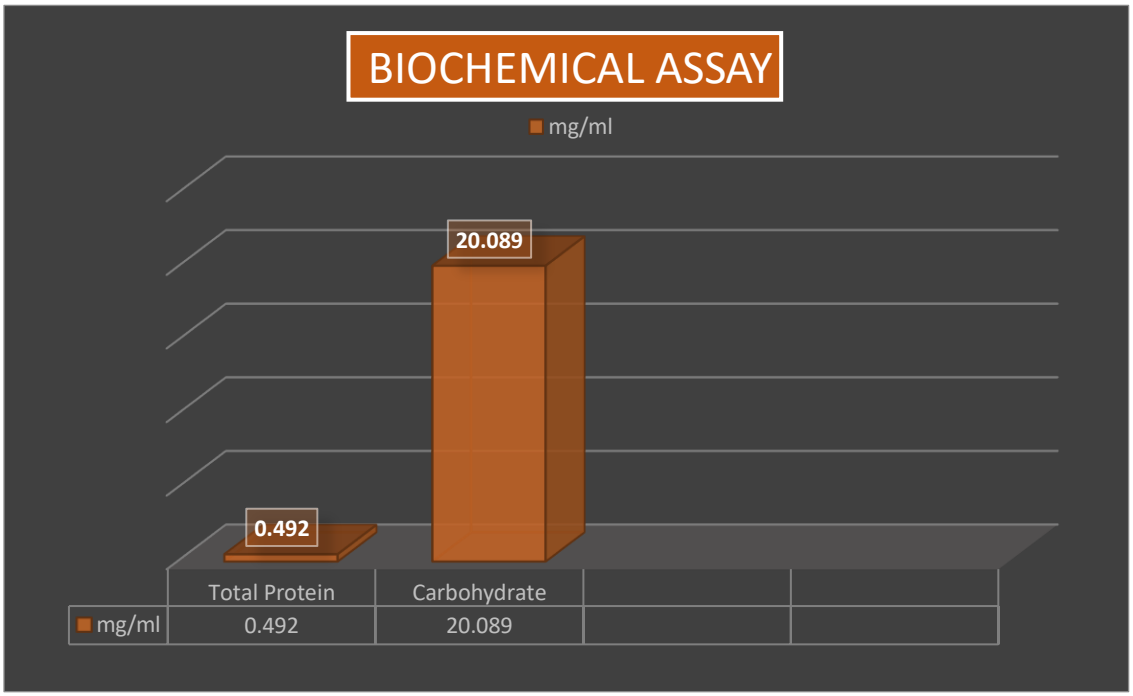


FIG: 4.4.2C

4.5 DISCUSSION:

Over the years there has been several reports on the soil characters of the sundarban area both from the chemical as well as from the microbial content. This is as a result of the fact that the sundarban delta presents a unique transitional zone which also harbors anthropogenic influences. Due to the gradual erosion of the banks of the islands and landmasses of the Indian Sundarbans, more and more deforestation has taken place as the settlers have gradually moved inwards and have cut down large areas of the forest cover. This represents two significant ecological pressures, - first since these mangrove forests offers the first barrier for tsunamis and other cyclones – their erosion has resulted in the loss of more and more property. Secondly the habitat of important fauna is also lost as a result of the illegitimate felling of trees. Following the declaration of the Indian Sundarbans as the world heritage site there has been concerted efforts from both the government and local dwellers towards afforestation by planting of true mangroves. Unfortunately, the expected success rate has not been achieved as a majority of the saplings have died at the initial stages. Numerous workers (Nandy et.al.) have attributed this observation to the lack of acclimatization of these newly planted saplings to the environment. The biochemical analysis results present important insights into the potential role of the microbial members abundant in the rhizosphere. The profiles of the antioxidant assays from the standardized extract indicate that free radical scavenging activity is present ubiquitously and in considerable amount. However, current ideas regarding the plant rhizosphere associations indicate that each plant possesses a core microbiome which is constant for a particular plant. Thus, it is important that these core microbiomes be identified and used as possible standardized supplements wherever that plant is being replanted (Mendes et. al 2013, Toju et. al 2018). In our results we have been able to understand the biochemical functioning of the plant using standard content analysis of phenol (6.237 gm) and flavonoids (0.0829 gm) both important by products of metabolism. In studies regarding photosynthetic efficiency, total chlorophyll content was measured and found to be significantly higher (8.49) along with total carbohydrate concentration (20.089mg/ml). The high content of both chlorophyll and carbohydrates are important indicators of higher photosynthetic efficiency which may be attributable to the total incident light in the range of 45000 to 55000 lux

that the plants receive through the 12-hour photoperiod in low canopy areas. Further, the antioxidant potentials of the leaf extracts were also tested using standard protocols of DPPH assay (24.34%), ABTS assay (72.43%) and Iron chelation activity (63.023 mg/ml). ABTS cation radical concentrations were found to be significantly reduced during the assay where butylhydroxytoluene served as positive control. This leads us to conclude, that the plant under study harbors reservoirs of biologically active compounds which can be explored further.

CHAPTER 5

PROTEIN PROFILING OF TOTAL & CHLOROPLAST PROTEIN CONTENT

5.1 INTRODUCTION:

SDS-PAGE (**sodium dodecyl sulfate–polyacrylamide gel electrophoresis**) is a variant of polyacrylamide gel electrophoresis, an analytical method in biochemistry for the separation of charged molecules in mixtures by their molecular masses in an electric field. It uses sodium dodecyl sulfate (SDS) molecules to help identify and isolate protein molecules. SDS-PAGE is an electrophoresis method that allows protein separation by mass. The medium (also referred to as ′matrix′) is a polyacrylamide-based discontinuous gel. In addition, SDS (sodium dodecyl sulfate) is used. About 1.4 grams of SDS bind to a gram of protein, corresponding to one SDS molecule per two amino acids. SDS acts as a surfactant, covering the proteins intrinsic charge and conferring them very similar charge-to-mass ratios. The intrinsic charges of the proteins are negligible in comparison to the SDS loading, and the positive charges are also greatly reduced in the basic pH range of a separating gel. Upon application of a constant electric field, the protein migrates towards the anode. each with a different speed, depending on its mass. This simple procedure allows precise protein separation by mass.

5.2 BACKGROUND:

Some results also show that *Excoecaria agallocha* of Indian Sundarbans region can tolerate and adapt to high saline condition. The effect of salinity on chlorophyll a, chlorophyll b and total chlorophyll levels of hydroponically grown seedlings of *Excoecaria agallocha* was studied. The selected seedlings were exposed to five different salinity levels (2, 5, 10, 15 and 20 psu) for a period of 30 days and observations were done at a regular interval of 7, 14, 21 and 30 days respectively. The concentrations of chlorophyll exhibited significant positive correlations with salinity ($p < 0.01$). The chlorophyll a:b ratio in the seedlings varied between 2.65 to 4.92 throughout the period of investigation. (Pal.N et al. 2014)

Any other works on this plant related to protein profiling is hardly found.

5.3 MATERIALS & METHODS:

5.3.1 PLACE OF SAMPLE COLLECTION:

The collection spot Kumirmari was selected based on the abundance of the *Excoecaria agallocha* specimens. From the identified cluster a healthy young plant was selected and juvenile leaves of the sample were served at the base. They were then washed with distilled water and segregated into airtight polypropylene bags. Once in the lab they were stored for further use in -20°C freezer. From this collected set 5 gm of sample was weighed and crushed using a mechanical homogenizer. The homogenized sample was then separated into five sets 1 gm each and further analysis was performed using the individual sets.

FIG 5.3.1: PLACE OF SAMPLE COLLECTION

5.3.2 ANALYSIS OF TOTAL PROTEIN AND CHLOROPLAST PROTEIN CONTENT BY USING SDS-PAGE:

5.3.2.1 ISOLATION OF TOTAL PROTEIN:

- **PREPERATION OF EXTRACTION BUFFER (PEB):** 23.96g sucrose, 6.05g TRIS-base, 10ml 0.5M EDTA (pH=8.0), 0.75g KCl, 250 µl HCl, 2ml 2-mercaptoethanol was added and the final volume was made up to 100ml by adding distilled water (dH_2O).

  [NOTE: This buffer should be prepared freshly and pre-cooled at 4°C.]

- **TOTAL PROTEIN EXTRACTION:**

  Ice was obtained, and used to chill the extraction buffer and the mortar and pestle. Two centrifuge tubes were labelled for the plant sample from which we wanted to obtain protein.1g of fresh plant tissue was taken and cut it as fine as we can with a razor blade. It was placed into a mortar with approximately 4ml of the cold extraction buffer (PEB). Grinded until smooth with a pestle. It is very important to grind the tissue well. We may need to add more buffer but the final product should be about the consistency of slightly watery texture. The mortar and pestle and plant tissue were kept as cold as possible during this process. Plant leaf extraction buffer was transferred into a centrifuge tube using clean spatula. The tube was placed on ice. Then the sample was centrifuged at 15000rpm for 15 minutes at 4°C.All the centrifuge tubes were balanced before spinning. The liquid supernatant was transferred into a second (new) centrifuge tube. Then the supernatant was collected and stored at -20°C.The samples were stored in ice only until the Total protein Estimation Assay, was done. Then the samples were stored in -20°C refrigerator.

5.3.2.2 ISOLATION OF CHLOROPLAST PROTEIN:

- **REAGENTS:**

MEDIUM A (grinding buffer): 50mM HEPES-KOH (pH-8.0), 330 mM sorbitol, 2mM EDTA-Na_2 (pH-8.0), 5mM ascorbic acid, 5mM cysteine, 0.05% bovine serum albumin (BSA)

MEDIUM B (wash medium): 50mM HEPES-KOH (pH-8.0), 330 mM sorbitol, 2mM EDTA-Na2 (pH-8.0)

MEDIUM C (lysis medium)-10mM HEPES-KOH (pH- 8.0), 5mM $MgCl_2$, Protease inhibitor stock-Leupeptin- 1mg in 1ml ddH_2O and Chymostatin- 1mg in 50µl ddH_2O.

- **PROCEDURE:**

- **PREPERATION OF MEDIUM A-** 6.01g sorbitol (330mM), 1.19g HEPES (50mM), 0.0584g EDTA (2mM), 0.088g Ascorbic acid (5mM), 0.825g cysteine (5mM), 50µl BSA (0.05%) were mixed in 60ml ddH_2O. then the pH was adjusted to 8.0 with KOH (1M) and the final volume was made up to 100ml.
- **PREPERATION OF MEDIUM B-** 1.19g HEPES (50mM), 6.01g sorbitol (330mM), 0.0584g EDTA (2mM) were dissolved in 60ml ddH_2O and pH was pH was adjusted to 8.0 and the final volume was made up to 100ml.
- **PREPERATION OF MEDIUM C-** 0.02383g HEPES (10mM), 0.1g $MgCl_2$ (5mM) were added to 40ml of ddH_2O. In Medium C 10µl of Leupeptin and Chymostatin stocks were added and pH was adjusted to 8.0 and the final volume was made up to 100ml.

- **CHLOROPLAST PROTEIN EXTRACTION:**

Ice was obtained, and used to chill the extraction buffer and the mortar and pestle. Two centrifuge tubes were labelled for the plant sample from which we wanted to obtain protein.0.5g of fresh plant tissue was taken and It was placed into a mortar with approximately 1ml of the Medium A. Grinded until smooth with a pestle. It is very important to grind the tissue well. The mortar and pestle and plant tissue were kept as cold as possible during this process. Plant leaf extract in extraction medium was transferred into a centrifuge tube using a clean spatula. Then the sample was centrifuged at 15000rpm for 30 minutes at 4°C. It should be remembered that all the centrifuge tubes containing the samples are of equal weight. The liquid supernatant

was transferred into a second (new) centrifuge tube.1ml of Medium B was added to each of the centrifuge tubes and again centrifuged at 15000rpm at 4°C for 20minutes.Then again, the supernatants were collected into a third centrifuge tube and 1ml of Medium C was added to each of them and further centrifuged at 15000rpm for 20mins at 4°C. then the supernatant was collected. Then the supernatant was stored at -20°C and used as protein soup until the SDS-PAGE Protein Estimation Assay was done.

5.3.2.3 SDS-PAGE PROTOCOL FOR TOTAL AND CHLOROPLAST PROTEIN PROFILING:

- **MATERIALS: -**
  - **STOCK SOLUTION**

1) **Acryl-bisacryl (30%):**

   29 mg of acrylamide and 1 g of bisacrylamide were dissolved in 100ml of warm distilled water and the pH was kept at 7.00. The solution was kept in dark.

2) **Sodium dodecyl sulphate (SDS 10%):**

   10 g of sodium dodecyl sulphate powder was dissolved in warm distilled water and kept at room temperature.

3) **Tris buffer for preparing of resolving and stacking gels:**

   Tris buffers were prepared with Tris base. After dissolving Tris base in water, the pH of the solution was adjusted by HCl.

- **1M Tris buffer with pH 6.8 –**

12.11g of Tris base was dissolved in 80 ml of water and the pH was adjusted to 6.8. The final volume of the solution was adjusted to 100ml by H_2O.

- **1.5 M Tris buffer with pH 8.8 –**

18.16 g of Tris base was dissolved in 80 ml of water and the pH was adjusted to 8.8. The final volume of the solution was adjusted to 100ml by H_2O.

4) **Ammonium per sulphate solution (APS 10%):**

   1g of ammonium per sulfate (APS) powder was dissolved in 10 ml of H_2O. The solution was freshly prepared.

5) Tris –glycerin electrophoresis buffer (5x):

15.1 g of Tris base and 94g of glycine were dissolved in 900 ml of H_2O. After that 50 ml of 105 (W/V) stock solution of SDS was added to it and final volume was adjusted to 1000ml by H_2O.

6) SDS-gel loading buffer (4x) – The components are

- 2 ml of 1m Tris-HCl (pH 6.8)
- 8 ml of 10% SDS
- 4 ml of 10% glycerol
- 1.2% β mercaptoethanol
- 8 mg of bromophenol blue

7) Amount of solution for preparing Resolving gels:

TABLE:5.3.2.3.1

Gel Concentration	Components	Volume (ml) of components required to cast gels of indicated volumes	
		10ml	**15ml**
	D.D H2O	3.3	5.9
	30% Acrylamide mix	4.0	5
12% Gel	1.5 M Tris (pH 8.8)	2.5	3.8
	10% SDS	0.0	0.15
	10% APS	0.1	0.15
	TEMED*	0.004	0.006

8) Amount of solution for preparing stacking gels:

TABLE: 5.3.2.3.2

Gel concentration	Components	Volume (ml) of components required to cast gels of indicated volumes	
		4ml	**5ml**
	H2O	2.7	3.4
	30% Acrylamide mix	0.67	0.83
5% Gel	1.5 M Tris (pH 8.8)	0.5	0.63
	10% SDS	0.04	0.05
	10% APS	0.04	0.05
	TEMED*	3.4	0.005

*Tetra methyl ethylene di amine.

9) Staining and distaining solution:

- **Staining solution: (0.25%)**

  0.25 g of Coomassie dye was dissolved in a 100 ml of 5:4:1 alcohol, water and glacial acetic acid.

- Distaining solution:

  A 500 ml mixture of 5:4:1 alcohol, water and glacial acetic acid.

10) Gel fixing solution:

20% glycerol solution.

- **PROCEDURE:**

The gels generally consisted of acrylamide, bis-acrylamide, sodium dodecyl sulphate (SDS) and a Tris-HCl buffer with adjusted pH. Ammonium persulphate (APS) and TEMED were added when the gel was ready to be polymerized. The separating or resolving gel (TABLE: 5.3.2.3.1) was usually more basic and had higher polyacrylamide content than the loading gel. Gels were polymerized in a gel caster. First the separating gel (12%) was poured and allowed to polymerize. Next a thin layer of isopropanol was added. The loading gel (TABLE: 5.3.2.3.2) was then poured after decanting the isopropanol and a comb was placed to create the wells. After the loading the gel was ready for electrophoresis. Samples (minimum 50 μg of protein) were mixed with equal amount of 1X SDS-gel loading buffer (SLB). The mixture was boiled for 3 minutes in a water bath and vortexed well before loading. Next, the denatured sample proteins were added to the wells at one end of the gel with a pipette. The loaded gel was set up with Tris-Glycine electrophoresis buffer (1X).

Finally, the apparatus was hooked up to a power source under the appropriate running conditions (at constant voltage of 60 V) to separate the protein bands. An electric field was applied across the gel, causing the negatively charged proteins to migrate across the gel towards the anode. Depending on their size, each polypeptide would move differently through the gel matrix. After a set time period (usually 3-4 hours) the polypeptide differentially migrated based on their size. Smaller ones travelled further down the gel, while larger ones remained closer to the point of origin and were separated roughly according to size. After electrophoresis, gel was immersed in staining solution for overnight and distained with the solution of 5:4:1 mixture of methanol, water and acetic acid. The image was taken under illuminator and analyzed in a computer with software Gel Analyzer 2.0.

The electropherograms were evaluated on the basis of band mobility and relative intensity. Protein profile of each sample showed its own electrophoresis pattern with subunits of varied molecular weight. Differences were observed in both presence and absence of a particular band.

➢ 5.4 RESULT:

▪ QUALITATIVE ANALYSIS OF TOTAL PROTEIN:

Qualitative analysis of total protein was done through SDS –PAGE gel electrophoresis. Here the protein molecules were segregated according molecular mass and charge. In case of total protein 3 bands (LANE:5) were observed as shown in the FIG: 5.4.1A). After that relative mobility of each band was calculated. The R_m value of Band-1, Band-2, Band -3, were 0.36, 0.92, and 0.97 respectively (FIG: 5.4.1B)

▪ QUALITATIVE ANALYSIS OF CHLOROPLAST PROTEIN:

Qualitative analysis of chloroplast protein was also done through SDS –PAGE gel electrophoresis. Here the protein molecules were also segregated according molecular mass and charge. In case of total protein one band was observed. After that relative mobility of band was calculated. The R_m value of Band was 0.65.

After the SDS-PAGE gel electrophoresis Lane 5 (FIG 5.4.1A & FIG:5.4.1B) of the SDS-PAGE gel showing 3 bands of total protein contents and the Lane 3 (FIG 5.4.3A & FIG:5.4.3B) of the SDS-PAGE gel showing the only band of the chloroplast protein content of *Excoecaria agallocha.*

FIG 5.4.1: LANE 5 SHOWING TOTAL PROTEIN PROFILING OF *Excoecaria agallocha*

- **FIG 5.4.1A: TOTAL PROTEIN PROFILE AFTER ELECTROPHORESIS.**
- **FIG 5.4.1B: ANALYZED GEL PROFILE USING GEL ANALYZER SOFTWARE.**

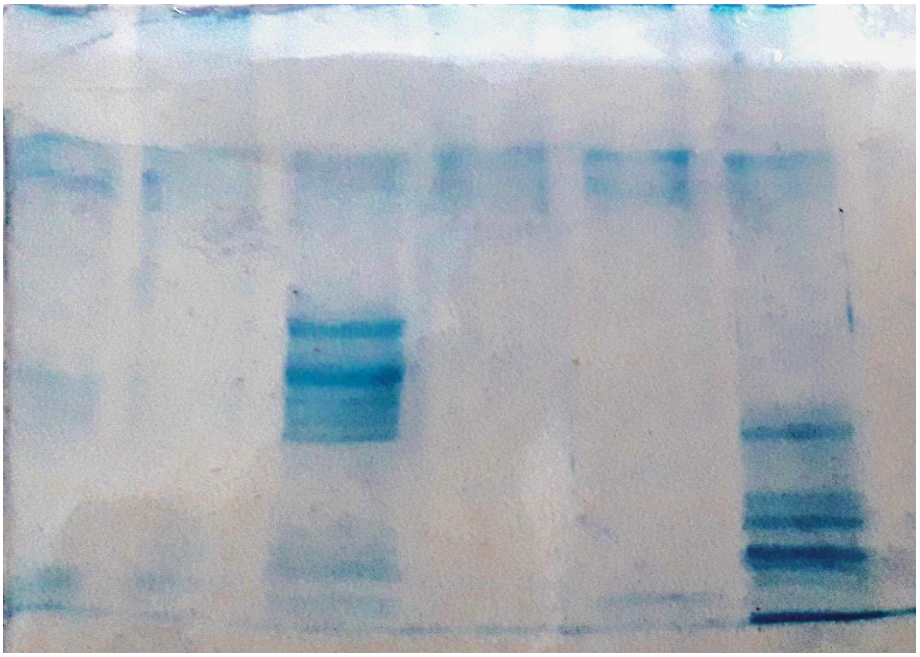
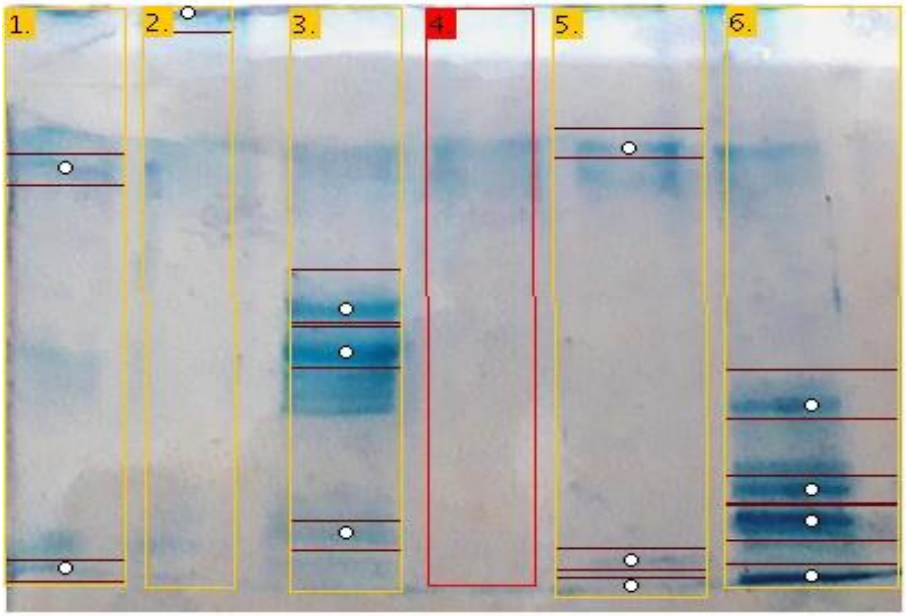
Lane number 1 2 3 4 5 6 7	Position of electrode
 **FIG:5.4.1A** **E**  **FIG:5.4.1B** **E**	**-** **+**

FIG:5.4.1

FIG 5.4.2: TOTAL PROTEIN PROFILING OF *Excoecaria agallocha*

- **FIG 5.4.2A: ANALYSIS OF PROTEIN LADDERS BAND INTENSITIES.**
- **FIG 5.4.2B: ANALYSIS OF PROTEIN PROFILE OF *Excoecaria agallocha***

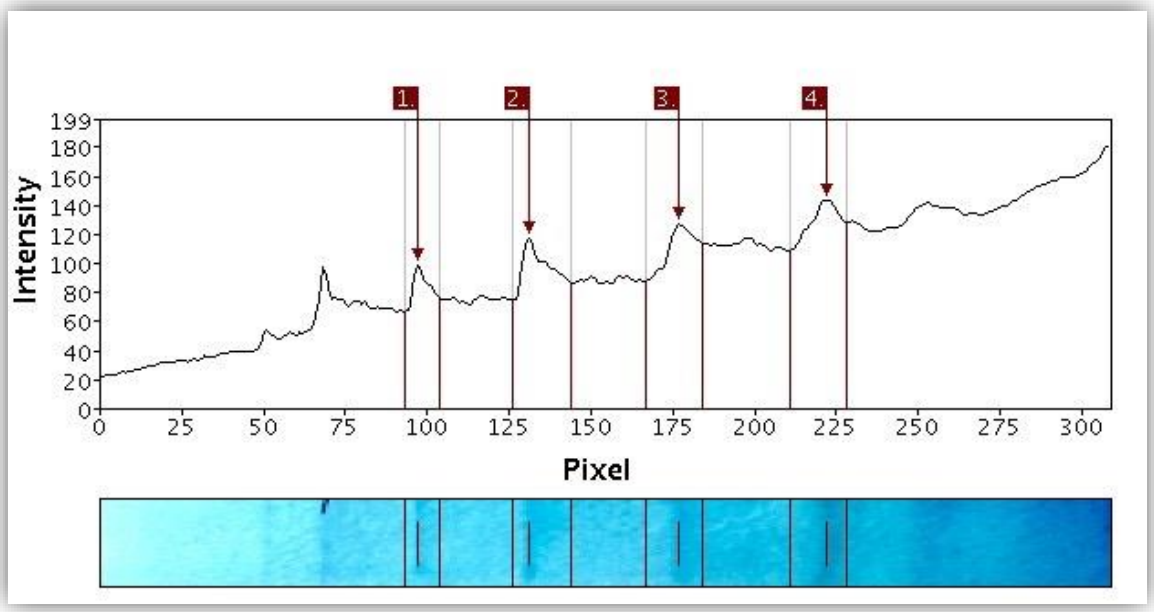


FIG: 5.4.2A

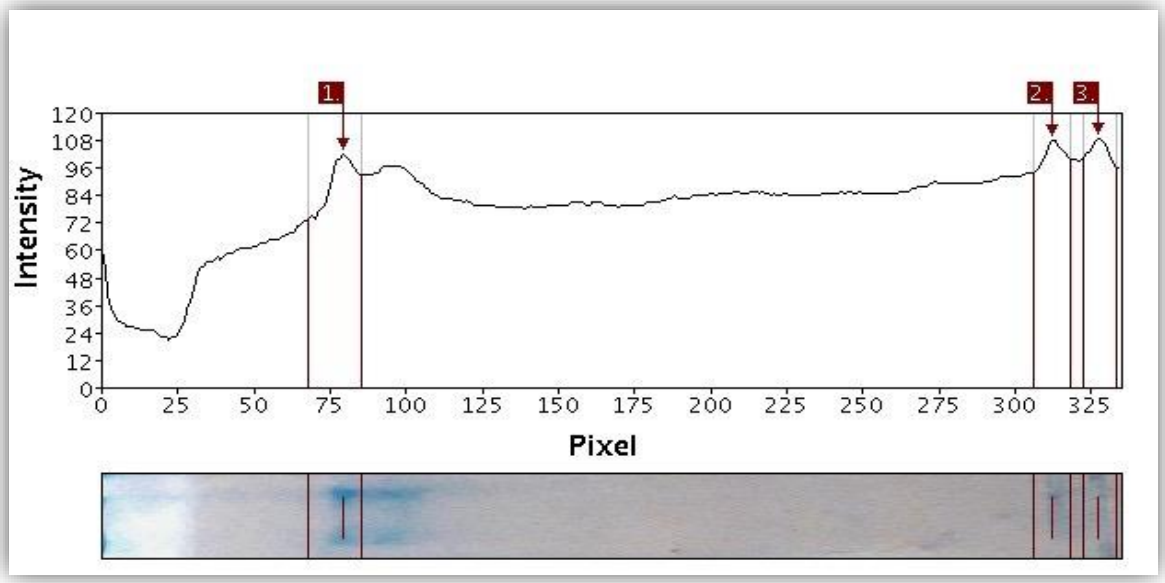


FIG:5.4.2B

FIG 5.4.3: LANE 3 SHOWING CHLOROPLAST PROTEIN PROFILING OF *Excoecaria agallocha*

- **FIG 5.4.3A: CHLOROPLAST PROTEIN PROFILE AFTER ELECTROPHORESIS.**
- **FIG 5.4.3B: ANALYZED GEL PROFILE USING GEL ANALYZER SOFTWARE**

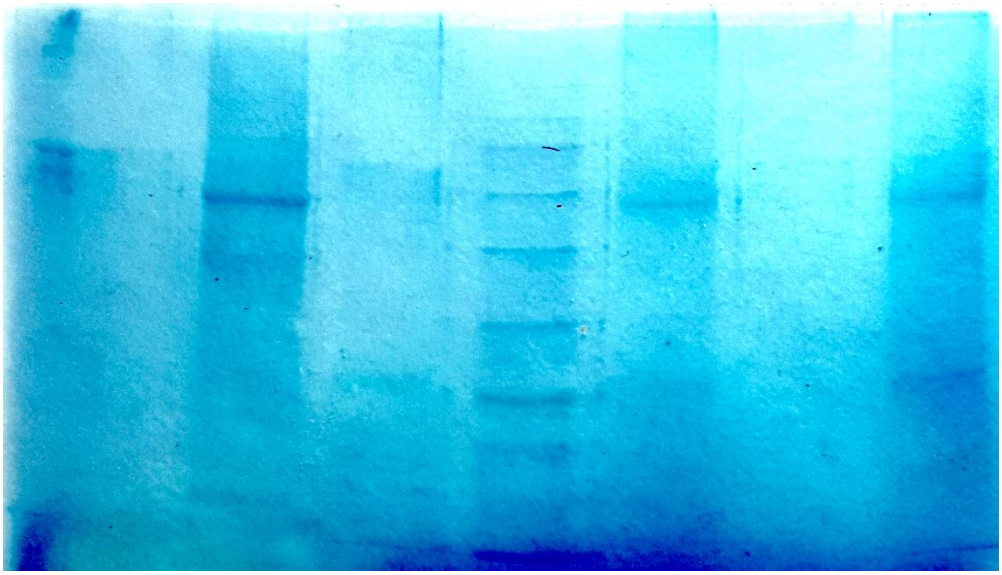
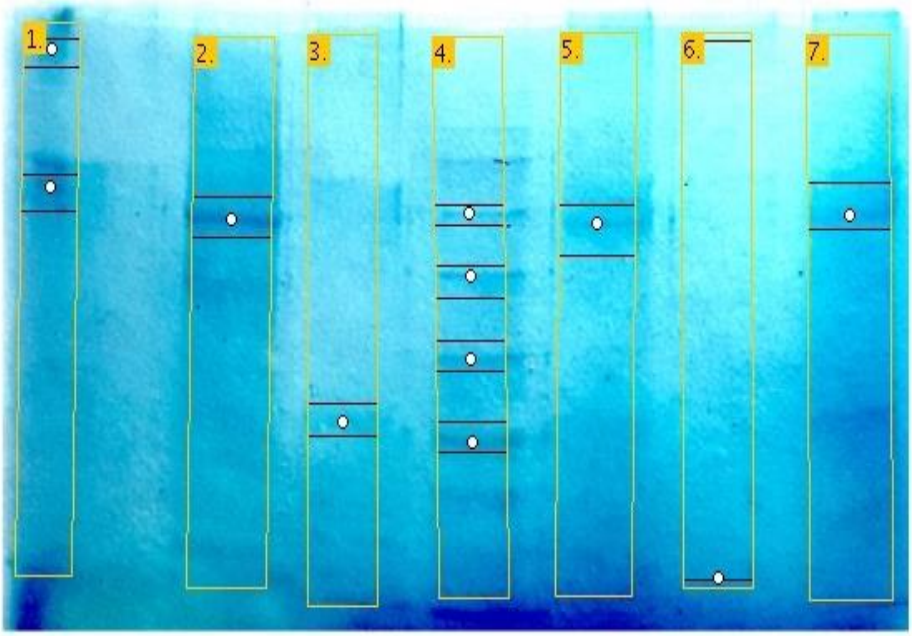
Lane number 1 2 3 4 5 6 7	Position of electrode
 E FIG:5.4.3A  E FIG:5.4.3B	- +

FIG:5.4.3

FIG 5.4.4: CHLOROPLAST PROTEIN PROFILING OF *Excoecaria agallocha*

- **FIG 5.4.4A: ANALYSIS OF PROTEIN LADDERS BAND INTENSITIES.**

- **FIG 5.4.4B: ANALYSIS OF PROTEIN PROFILE OF *Excoecaria agallocha***

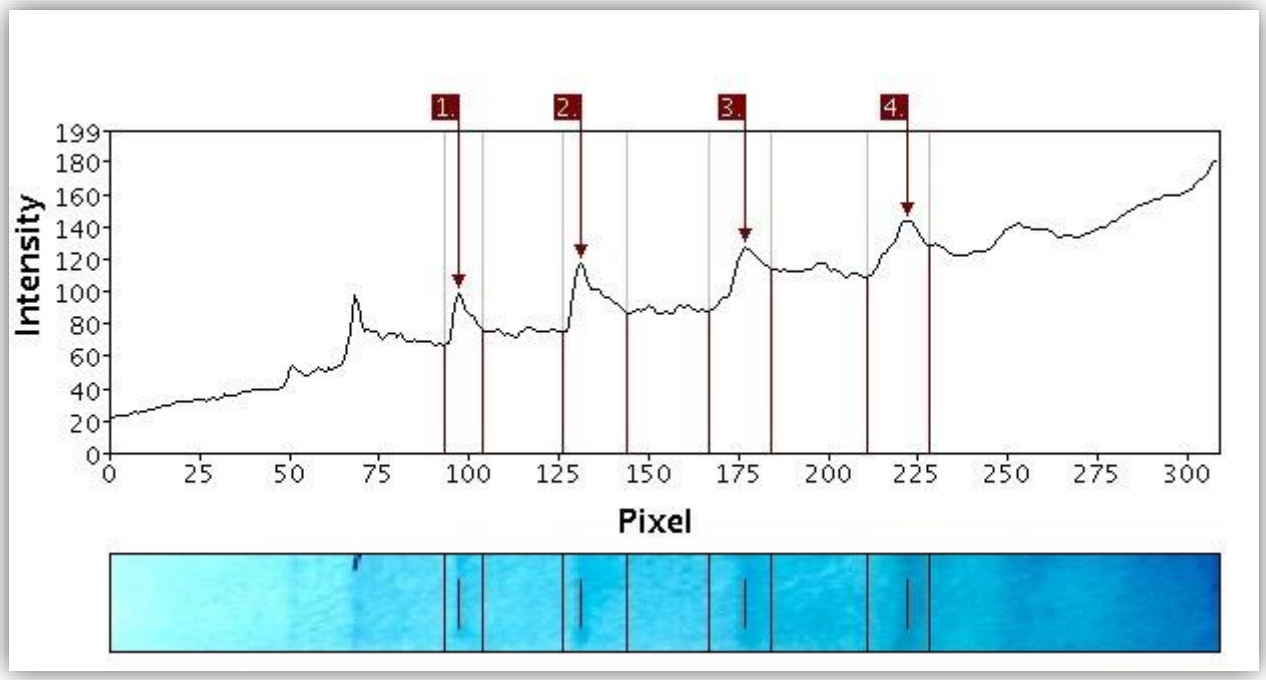


FIG:5.4.4A

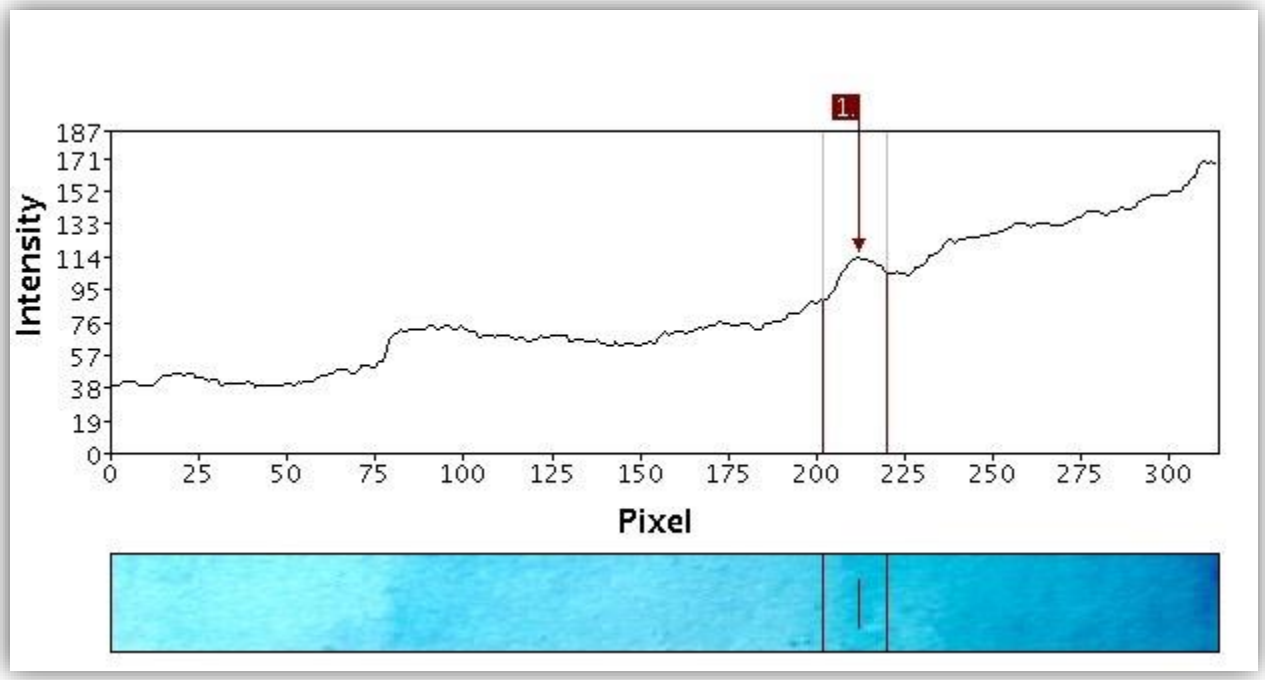


FIG:5.4.4B

5.5 DISCUSSION:

Over the years there has been several reports on the soil characters of the Sundarban area both from the chemical as well as from the microbial content. This is as a result of the fact that the Sundarban delta presents a unique transitional zone which also harbors anthropogenic influences. Due to the gradual erosion of the banks of the islands and landmasses of the Indian Sundarbans, more and more deforestation has taken place as the settlers have gradually moved inwards and have cut down large areas of the forest cover. This represents two significant ecological pressures, - first since these mangrove forests offers the first barrier for tsunamis and other cyclones – their erosion has resulted in the loss of more and more property. Secondly the habitat of important fauna is also lost as a result of the illegitimate felling of trees. Following the declaration of the Indian Sundarbans as the world heritage site there has been concerted efforts from both the government and local dwellers towards afforestation by planting of true mangroves. Unfortunately, the expected success rate has not been achieved as a majority of the saplings have died at the initial stages. Numerous workers (Nandy et.al.) have attributed this observation to the lack of acclimatization of these newly planted saplings to the environment. Characterization of the plant at the molecular level yielded mixed results. Protein isolation was found to be affected by the presence of higher polyphenols which when treated with PVP yielded better results. Chloroplast protein complexes were isolated and analyzed in SDS - PAGE and distinct banding patterns were observed. Further plant specific standardization is required to produce better results.

CHAPTER 6

RESTRICTION PROFILE OF CHLOROPLAST DNA

6.1INTRODUCTION:

Chloroplasts have their own DNA, often abbreviated as **cp DNA** It is also known as the **plastome** when referring to genomes of other plastids. the organellar DNA is easy to be handle because of its smaller size than the genomic DNA and could be useful for inter specific identification. cp DNA is smaller, easy to isolate and is conserved in multiple regions throughout the phylogenetic evolution. Over time, many parts of the chloroplast genome were transferred to the nuclear genome of the host, a process called ***endosymbiotic gene transfer.*** As a result, the chloroplast genome is heavily reduced compared to that of free-living cyanobacteria. Chloroplasts may contain 60–100 genes whereas cyanobacteria often have more than 1500 genes in their genome. Endosymbiotic gene transfer is how we know about the lost chloroplasts in many chromalveolate lineages. Even if a chloroplast is eventually lost, the genes it donated to the former host's nucleus persist, providing evidence for the lost chloroplast's existence. The cp DNA possess some intergenic regions that varies in their sequences. And could be utilize as a tool for systemic identification.

RESTRICTION DIGESTION can be used for easy visualization of sequence dissimilarity of multiple species of the same genus. Double digestion with easily available RE s viz HIND III and ECoR1 and by simple gel electrophoresis thus differentiating the banding pattern.

There are various molecular analytic tools and techniques that can be utilized directly or with some or little modifications for uncovering important information about mangrove plants like these.

Some such procedures include **Restriction digestion,** is the type of enzyme that cleaves DNA into fragments at or near specific recognition sites within molecules known as restriction sites. Restriction enzymes are one class of the broader endonuclease group of enzymes. To cut DNA, all restriction enzymes make incisions, through each sugar-phosphate backbone (i.e. each strand) of the DNA double helix. Over 3,000 restriction enzymes have been studied in detail, and more than 600 of these are available commercially. These enzymes are routinely used for DNA modification in laboratories, and they are a vital tool in molecular cloning. We choose to use the EcoR1 & Hind III to cleave the chloroplast DNA of *Excoecaria agallocha.*

6.2 BACKGROUND:

E. agallocha was also found across its distribution range and investigated the phytogeography of this species using four chloroplast DNA (cpDNA) fragments. The results showed that *E. agallocha* possessed a high degree of species-level genetic diversity, while the average genetic diversity within populations was much lower. The presence of population genetic structure was supported by the estimates of genetic differentiation and the analysis of molecular variance (AMOVA). Of the ten haplotypes identified, no haplotypes were shared between the East Indian Ocean (EIO), the West Pacific Ocean (WPO), and the North Australian (NA) regions. Genealogy analysis, haplotype distribution patterns, and the principal coordinate analysis (PCoA) consistently suggested the existence of three haplotype groups distributed in distinct geographical locations. (Guo.W et al.2017).The responses of anti-oxidative enzymes and stress related hormones was also found in *E. agallocha* to different levels of Pb stresses at different exposure time. Pb stress posed higher toxic effects on root than leaf. (Yan.Z & Tam.N 2012). Another article suggests that the growth of *Excoecaria agallocha* seedling decreases as the NaCl salinity increases in the soil. It was observed that the germination percentage were decreased with increasing NaCl salinity. The maximum percentage (100%) of seed germination was recorded in fresh water and the minimum percentage (36%) of germination was observed in 200 mM NaCl concentration. (S. Sivasankaramoorthy et.al 2010).The total blue carbon stock of the Bangladesh Sundarbans mangroves was evaluated and the probable future status after a century was predicted based on the recent trend of changes in the last 30 years and implementing a hybrid model of Markov Chain and Cellular automata. The prediction shows that after a hundred years the blue carbon would be lost from the low saline regions accompanied by increase in the high saline regions dominated mainly by *Excoecaria sp.* and *Avicennia spp*. The net carbon loss would be due to both mangrove area loss and change in species composition of CO2 emission within the year 2115. (Chanda. A et al. 2016).

6.3 MATERIALS & METHODS:

6.3.1 PLACE OF SAMPLE COLLECTION:

The collection spot Kumirmari was selected based on the abundance of the *Excoecaria agallocha* specimens. From the identified cluster a healthy young plant was selected and juvenile leaves of the sample were served at the base. They were then washed with distilled water and segregated into airtight polypropylene bags. Once in the lab they were stored for further use in -20°C freezer. From this collected set 5 gm of sample was weighed and crushed using a mechanical homogenizer. The homogenized sample was then separated into five sets 1 gm each and further analysis was performed using the individual sets.

FIG 6.3.1: PLACE OF SAMPLE COLLECTION

6.3.2 PROCEDURE OF RESTRICTION DIGESTION OF CHLOROPLAST DNA USING EcoRI AND HindIII ENZYMES:

- **Requirements:**

  - **Reagents**-

    Chloroplast DNA**,** Agarose**,** Restriction Enzyme: EcoRI**,** Restriction Enzyme: HindIII**,** 10X Assay Buffer for EcoRI**,** 10X Assay Buffer for HindIII**,** Molecular Biology Grade Water**,**50X TAE**,** 6X Gel Loading Buffer**,** Ethidium Bromide (10mg/ml).

  - **Glass Goods-** Beakers, Measuring Cylinders, Reagent Bottles.
  - **Instruments-** Electrophoresis Apparatus, UV Trans-illuminator, Digital balance, Heater, Vortex Mixture, Micropipettes and Tips, Adhesive tape.

- **PROCEDURE:**

Before starting the experiment, the ice was being crushed and placed in the vials containing Chloroplast DNA, Restriction Enzymes and Assay buffers onto it. In this experiment Chloroplast DNA was digested with two restriction enzymes: EcoRI and Hind III.

- **The reaction mixture is set up as followed:**

REACTION 1: (ECoR1)

TABLE: 6.3.2.1

i.	**Chloroplast DNA**	**5.0 µl**
ii.	**10X Assay Buffer of ECoR1**	**2.5 µl**
iii.	**Milli Q Water**	**16.5 µl**
iv.	**ECoR1**	**1.0 µl**
	Total	**25 µl**

REACTION 2: (Hind III)

TABLE:6.3.2.2

i.	**Chloroplast DNA**	**5.0 µl**
ii.	**10X Assay Buffer of Hind III**	**2.5 µl**
iii.	**Milli Q Water**	**16.5 µl**
iv.	**Hind III**	**1.0 µl**
	Total	**25 µl**

After preparing the two reaction tubes, the components were mixed by gentle pipetting and tapping. The tubes were then incubated at 37°C for 1 hour. After 1-hour incubation, immediately the vials were placed at room temperature (15°C – 25°C) for 10 minutes.

6.3.3 AGAROSE GEL ELECTROPHORESIS:

Preparation of 1X TAE: To prepare 500 ml of 1XTAE buffer, 10 ml of 50 X TAE Buffer was added to 490 ml of sterile distilled water and was mixed well before use.

Preparation of Agarose Gel: To prepare 50 ml of 1% agarose gel, 0.5 g Agarose was measured in a glass beaker or flask and 50 ml 1XTAE buffer was added to it. The mixture was heated on a microwave of hot plate or burner, swirling the glass beaker/ flask occasionally until the agarose was dissolved completely (It was to be ensured that the lid of the flask was loose to avoid buildup of pressure). The solution was allowed to cool to about 55-60°C. Next, 0.5 µl Ethidium bromide was added, mixed well and poured into the gel solution into the gel tray. The gel was allowed to cool to solidify for about 30 minutes at room temperature.

Loading of DNA sample: 3µl of ready to use DNA marker was loaded into the well 1. To prepare sample for electrophoresis, 2µl of 6X loading buffer to 10 l of DNA(TABLE: 6.3.2.1 & TABLE:6.3.2.2) samples. The sample was mixed well by pipetting and loaded into the well.

Electrophoresis: The power cord was connected to the electrophoretic power supply according to the conventions. (RED-anode, BLACK- cathode) and electrophoresis was continued at 100V-120V and 90 mA until dye markers had migrated to an appropriate distance depending on the size of DNA to be visualized.

- **Precautions:** Ethidium bromide is a powerful mutagen and is very toxic. Appropriate safety precautions should be taken by wearing latex gloves. However, use of nitrite gloves is recommended.

6.4 RESULT:

Restriction digestion and Electrophoresis was successful as exemplified by the distinct DNA bands observed using UV transilluminator on digestion with EcoRI and Hind III. After 35 mins of the completion of the electrophoresis, the band intensity analyses were performed using the GEL Analyzer Software. Restriction patterns obtained on digestion with EcoRI and Hind III are markedly different, demonstrating the fact that each restriction enzyme recognizes and cuts only a particular base sequence unique to it.

An intercalating dye like Ethidium bromide added to agarose gel examining under UV light, results to detect location of bands, wherein DNA fluoresces. Thereafter, the gel is observed against a light background wherein DNA appears as dark colored bands.

After the Gel electrophoresis Lane 7 & 8 (FIG 6.4.1) of the agarose gel showing restriction digestion of chloroplast DNA of *Excoecaria agalocha*. LANE 7 is showing restriction digestion of the restriction enzyme EcoR1 which contains 2 bands. And the LANE 8 is showing restriction digestion of restriction enzyme HIND III which contains 4 bands. (FIG: 6.4.1).

FIG 6.4.1: LANE 7 & 8 SHOWING RESTRICTION DIGESTION OF CHLOROPLAST DNA

- **FIG 6.4.1A: GEL IMAGE OF RESTRICTION DIGESTION PROFILE OF CHLOROPLAST DNA ISOLATED FROM *Excoecaria agallocha***

- **FIG 6.4.1B: ANALYZED GEL IMAGE USING GEL ANALYZER SOFTWARE SHOWING SPECIFIC BANDS AND THEIR POSITION.**

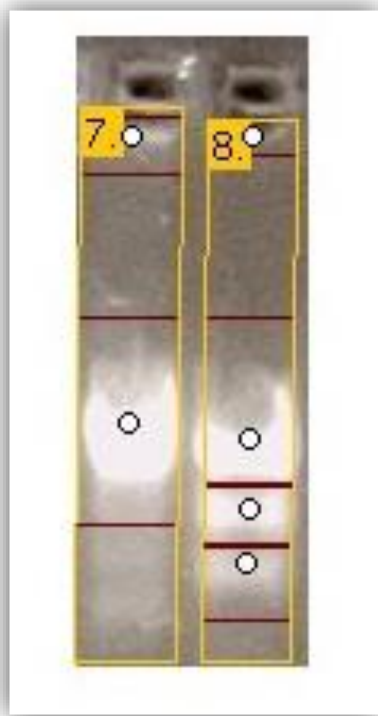
POSITION OF ELECTRODE	LANE NUMBER
– +	 EcoR1 HIND III A  EcoR1 HIND III B

FIG:6.4.1

FIG 6.4.2: RESTRICTION DIGESTION OF CHLOROPLAST DNA OF *Excoecaria agallocha*

- **FIG 6.4.2A: RESTRICTION DIGESTION PROFILE OF ECoR1**

(TOTAL BANDS-2)

- **FIG 6.4.2B: RESTRICTION DIGESTION PROFILE OF HindIII**

(TOTAL BANDS- 4)

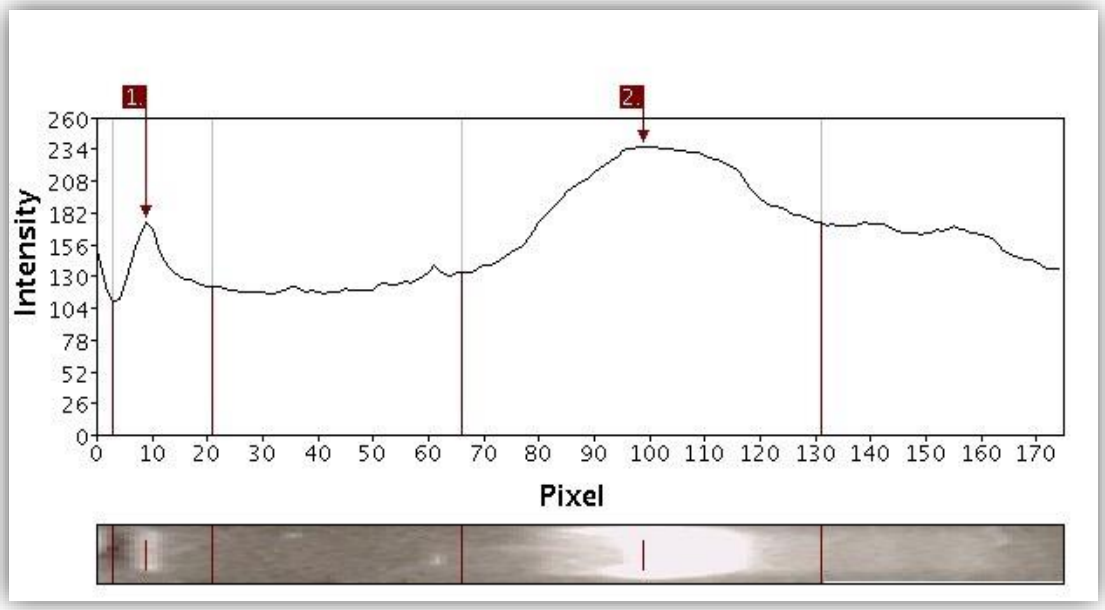


FIG:6.4.2A

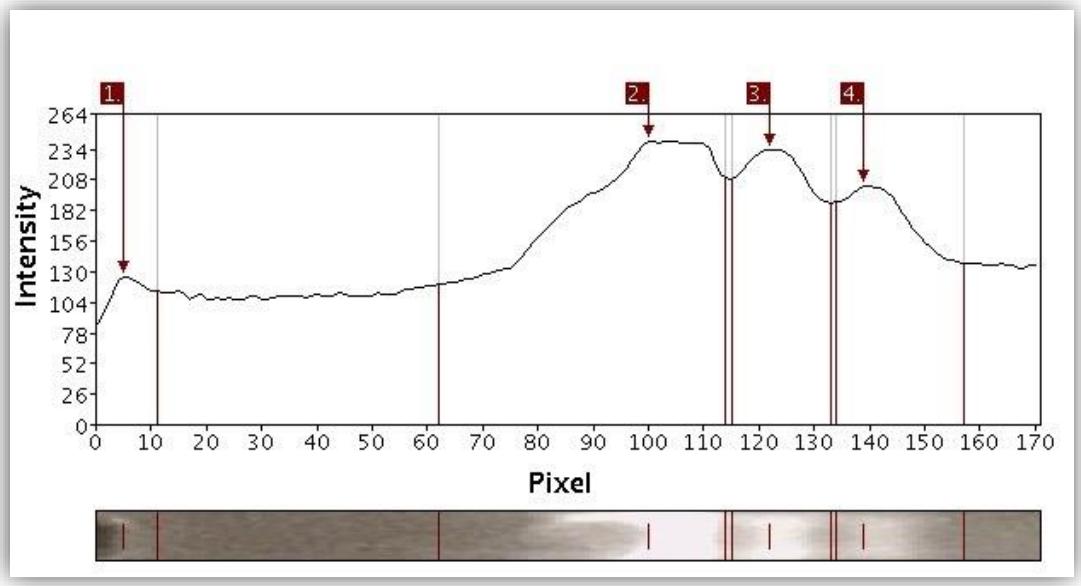


FIG: 6.4.2B

6.5: DISCUSSION:

Over the years there has been several reports on the soil characters of the Sundarban area both from the chemical as well as from the microbial content. This is as a result of the fact that the Sundarban delta presents a unique transitional zone which also harbors anthropogenic influences. Due to the gradual erosion of the banks of the islands and landmasses of the Indian Sundarbans, more and more deforestation has taken place as the settlers have gradually moved inwards and have cut down large areas of the forest cover. This represents two significant ecological pressures, - first since these mangrove forests offers the first barrier for tsunamis and other cyclones – their erosion has resulted in the loss of more and more property. Secondly the habitat of important fauna is also lost as a result of the illegitimate felling of trees. Following the declaration of the Indian Sundarbans as the world heritage site there has been concerted efforts from both the government and local dwellers towards afforestation by planting of true mangroves. Unfortunately, the expected success rate has not been achieved as a majority of the saplings have died at the initial stages. Numerous workers (Nandy et.al.) have attributed this observation to the lack of acclimatization of these newly planted saplings to the environment. It is thus important to note that this work presents some important insights into the actual biochemistry and molecular biology of (*Excoecaria agallocha*) - Biochemical profiling to analyses contents of metabolism as well as potential antioxidant properties were noted which should pave way for ethnomedicine based bioactive compound research; the identification of chloroplast DNA banding patterns should provide important landmarks into DNA barcoding studies and profiling of the chloroplast protein complexes which may pave the way for standardization of molecular protocols for in depth studies using halophytic vegetation.

CHAPTER:7

CONCLUSION

- **Conclusion:** All these works were performed and the Biochemical & Molecular Study of this plant can help us in further in future by in depth analysis of its DNA sequence and banding pattern of proteins. More in depth studies on the plants whole metabolome and other aspects are needed for proper utilization and conservation of the plant. Molecular and Biochemical as well as other aspects of the plants are required to be studied in a holistic manner. RESTRICTION DIGESTION can be used for easy visualization of sequence dissimilarity of multiple species of the same genus. Double digestion with easily available RE s viz HIND III and ECoR1 and by simple gel electrophoresis thus differentiating the banding pattern. Proper identification is the key to the conservation. The mangroves like *Excoecaria* is one of the most important species of the region so proper identification as well as proper understanding of its proteome and other important phytochemical properties are necessary to understand its position in the ecosystem as well as for proper exploitation of its adaptive features in molecular level. Molecular Study of this plant can help us in further future by in depth analysis of its DNA sequence and banding pattern of proteins. It can be concluded that this information will help us conserve the plant species in in-vitro & in-vivo condition.

CHAPTER: 8

FUTURE PROSPECT

- **Future Prospects:** Future prospects includes acclimatization of this plant in no saline environment to exploit its Phyto biomedical- properties, no nanobiotechnological studies so far had not been reported on this plant. That could open new window of opportunities. Novel proteins if identified from the plant that is responsible for the plant's salinity tolerance, proper identification and cloning of the protein could be helpful for raising salinity tolerance in new transgenic crop plant lines. Proteins and other bioactive compounds could be screened for their anti-viral properties. Cell culture and/or in vitro studies on anti-viral property of this plant is yet not reported. The salt tolerant plant houses special swarm of endophytes inside them. Proper studies and development of proper media for in vitro culture of the endophytes from the plants is incomplete. Medicinal properties of the plant if can be linked with its endophytes then the production, purification and isolation of those components could be performed in a commercial scale in fermenter system after proper bio-optimization processes. And moreover, proper understanding of the proteome and metabolome is helpful for proper conservation of this particular plant and other mangrove plants which have major operating function in their biome.

CHAPTER: 9

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Evaluation of Genotoxic Effects of Sodium Benzoate on The Root Meristem Cells of *Allium cepa* (L.)

PROJECT REPORT

2019

FINAL SEMESTER PROJECT IN

CELL BIOLOGY & BIOTECHNOLOGY

FOR THE AWARD OF THE DEGREE OF

MASTERS OF SCIENCE IN BOTANY

SUBMITTED BY

SUKANYA SAHA

ROLL NO: LBC-PG-BOTANY-17411

CELL BIOLOGY & BIOTECHNOLOGY

POST GRADUATE DEPARTMENT OF BOTANY

LADY BRABOURNE COLLEGE

AFFILIATED BY UNIVERSITY OF CALCUTTA

KOLKATA, WEST BENGAL

2019

CERTIFICATE

This is certifying that ***Sukanya Saha*** *has carried out this project (A Comprehensive Review and Research Work) for the fulfilment of the* ***Master's Degree in Botany****, with special paper* ***Cell Biology & Biotechnology*** *2019, under my supervision.*

The little of the project on which she has worked on is, ***Evaluation of Genotoxic Effects of Sodium Benzoate On The Root Meristem Cells of Allium cepa (L.)***

The student has completed her work sincerely and to my satisfaction.

_____

Dr. SUDIPTA DAS

ASSOCIATE PROFFESSOR

POST GRADUATE DEPRTMENT OF BOTANY

LADY BRABORNE COLLEGE

KOLKATA; WEST BENGAL

DATE: _____

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DATE: _____

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SUKANYA SAHA

CONTENTS	PAGE

➢ INTRODUCTION:

Safe storage of food and beverages for a prolonged time is one of the oldest human needs. It is achieved by different conservation methods which improve over time. In general, for preservation purposes, different chemical agents are used. Sodium benzoate, also known as benzoate of soda, is a food additive used as a preservative for its ability to effectively kill most yeasts, bacteria and fungi. SB is a bacteriostatic and fungistatic agent in acidic environments which, after being absorbed into cells, interacts with the anaerobic energy production pathways and suppresses the development of food-spoilage microorganisms. Owing to this feature, SB is broadly used in other fields and is one of the indispensable components of pharmaceutical and cosmetic products in order to preserve a product, assuming it has been correctly prepared and adjusted to a pH of 4.5 or less, an approximate SB concentration of 0.1% is usually enough. Today, it is difficult to imagine a food product without any food additives. Even in small concentrations, preservatives affect the health of long-term consumers, which may present as a migraine, nausea, vomiting, diarrhoea, rhinitis, bronchospasm, anaphylaxis and hyperactivity in children. What is more, there may also be induced damage at the molecular level, including chromosome damage. Because of reports of such side effects of these chemicals, especially in the gastrointestinal system, the allowable concentration is placed under the regulation of the Food and Drug Administration (FDA).

The *Allium* assay was introduced by Levan in 1938; it is a short-term biological assay and has been proposed as a standard method for toxicity testing. Some advantages of *Allium* assay include the fact that *A. cepa* is readily available all year round, it is relatively easy to handle, it provides good mitotic spreads for analysis, it is economical and shows a good correlation with a number of other test systems (Shahaby et al., 2003; Fiskesjo, 1985). *Allium cepa* roots contain oxidase enzyme which activates the conversion of promutagens into mutagens.

FIG: 1.1 ROOTS OF *Allium cepa* (L.)

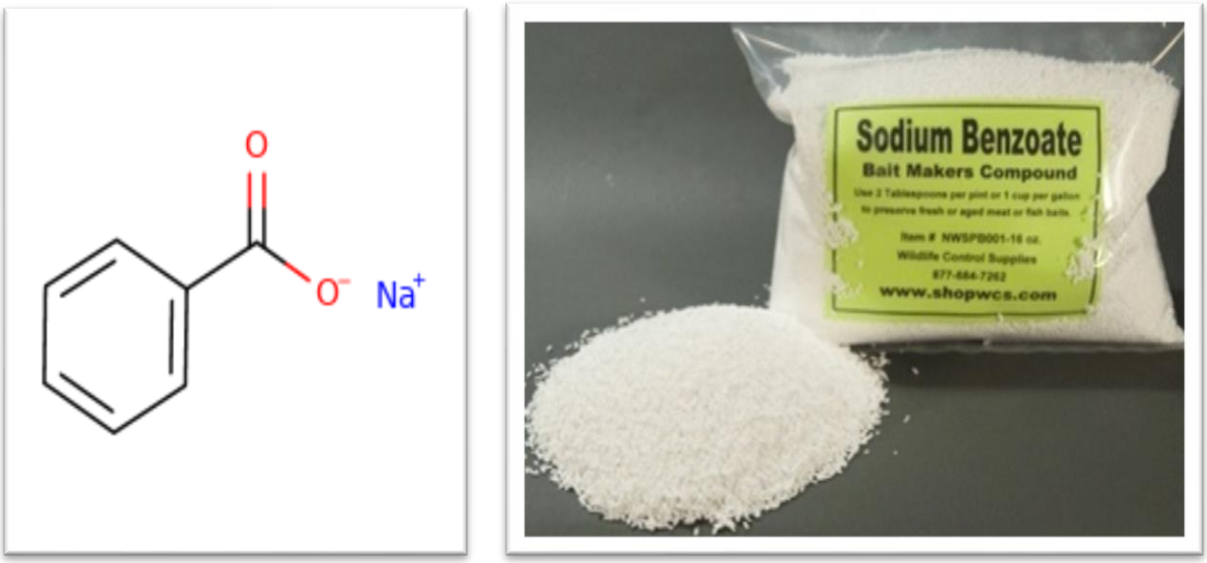

FIG 2: FOOD PRESERVATIVE SODIUM BENZOATE.

➢ OBJECTIVES OF WORK:

- To observe the genotoxic effects of Sodium Benzoate in the mitotic cells of *Allium cepa.*
- To identify the abnormalities in the mitotic cells of *Allium cepa* during mitotic cell division which are treated with different percentages of Sodium Benzoate.

➢ REVIEW OF LITERATURE:

Researchers found that Sodium benzoate and sodium sulphite are two of the most commonly used food preservatives in Kenya. Their effects on mitosis in onion root meristems, growth and development in Drosophila and sex recessive lethal induction in Drosophila are being studied. Both sodium sulphite and sodium benzoate do not cause any gross chromosome aberrations. Sodium sulphite (65 mM)" treatment for 27 hours results in a 95% inhibition of mitotic figures. 30 h treatment results in total inhibition. The effects are both time- and concentration dependent. Sodium benzoate treatment (0.065 mM; 9--18 h) of roots results in premature chromosome condensation and near complete mitotic inhibition. Preliminary data indicate that sodium sulphite-treated cells accumulate in late interphase or early prophase. Both chemicals, in feeding experiments with Drosophila, cause delay in development and altered sex ratios. Mutagenicity screening using Muller-5 chromosomes are in progress. (Gopalan and Njagi.,1987)

Some researchers also found the effects of different treatments with food preservatives, sodium propionate (SP), calcium propionate (CP) and potassium propionate (PP), on the cytology and DNA content of Allium cepa were investigated. Five concentrations of these additives – 1000, 1500, 2000, 2500, and 3000 ppm – were applied for 24, 48, and 72 h. All concentrations of these chemicals showed an inhibitory effect on cell division in root-tips of A. cepa and caused a decrease in mitotic index values. Additionally, all treatments changed the frequency of mitotic phases when compared with the control groups. These compounds increased chromosome abnormalities in test material. Among these abnormalities were C-mitosis, anaphase bridges, micronuclei, binucleated cells, stickiness, laggards, and chromosome breaks. The nuclear DNA contents decreased when compared with control groups. (Tu¨rkog˘lu,2008)

The effects of the food preservatives sodium benzoate (SB), boric acid (BA), citric acid (CA), potassium citrate (PC) and sodium citrate (SC) have been also studied on root tips of *Allium*

cepa L. Roots of *A. cepa* were treated with a series of concentrations, ranging from 20 to 100 ppm for 5, 10 and 20 h. The results indicate that these food preservatives reduced mitotic division in *A. cepa* compared with the respective control. Mitotic index values were generally decreased with increasing concentrations and longer treatment times. Additionally, variations in the percentage of mitotic stages were observed. The total percentage of aberrations generally increased. (Tu¨rkog˘lu,2006) with increasing concentrations of these chemicals and the longer period of treatment. Different abnormal mitotic figures were observed in all mitotic phases. Among these abnormalities were anaphase bridges, C-mitosis, micronuclei, lagging, stickiness, breaks and unequal distribution. (Tu¨rkog˘lu,2006)

International Programme on Chemical Safety found no adverse effects of SB on humans at doses of 647–825 mg/kg of body weight per day and the acceptable dose of SB declared in 2003 by the Turkish Ministry of Health is 500–1000 mg/kg. Although SB is widely used as a preservative in several products like pickles, sauces, milk and meat products and fruit juices, there is also a high demand in the soft drink industry due to the usage of high-fructose corn syrup in carbonated beverages. Benzoic acid, which is a precursor of SB, is found naturally in cranberries, prunes, greengage plums, cinnamon, ripe cloves and apples. Benzoates have been detected in groundwater, but not in drinking water. In the literature, there is only a limited number of studies that have been conducted on SB. According to Nair, although previous works of other researchers reported some toxic effects of benzoates, no genotoxic effects of benzyl alcohol, benzoic acid and sodium benzoate have been found in studies on rats and mice. The results from carcinogenicity studies have also been found negative. However, Turko € glu et al have also reported some genotoxic effects of antimicrobial additives on root tips of Allium cepa. Since there is scarce information about the genotoxic effects of preservatives, and an insufficient number of in vivo studies in mammals, testing the potential of SB to induce genomic alterations in mitotic cells of pregnant rats can help to increase our knowledge of the biological effect of SB from a different point of view. The detection of chromosome fragments or whole chromosomes without migration potential during cell division (micronuclei) using the micronucleus test is one of the methods for determination of the possible genotoxic effect of SB. Thus, the aim of this study was to evaluate the effects of three different concentrations of SB on the weight gain, food and water intake of pregnant rats; on the micronuclei (MN) formation and mitotic index (MI) in Tlymphocytes of pregnant rats and their foetuses on the fatal body weight, perinatal mortality and malformations; and (4) the potential of SB to induce DNA breaks in the liver cells of pregnant rats and their new-borns.(Saatci, 2016)

A study was conducted to investigate the effects that plant extracts from 5 medicinal plants may have on mitosis in *Allium cepa*. Root of *A. cepa* were immersed in alcoholic extracts at the concentrations of 0, 25, 50, 75 and 100 mg/mL, respectively for each of the following plants: *Gnetum africanum* Welw., *Lasianther aafricana* P. Beauv, *Ocimum gratissimum* Linn., *Telfairia occidentalis* Hook F. and *Vernonia amygdalina* Del. Leafy vegetable which are commonly used in herbal medicine. Results obtained show that the various concentrations of the extracts from test plants had toxic effects on the cells, which caused significant reduction ($p<0.05$) in the mitotic index when compared with the control. Other effects were prophase inhibition, the delay of mitosis and nuclear lesion. The cytotoxic effect makes a case for a precaution in the use of the leafy extracts in herbal medicine practice. (Udo,2013)

Some researchers also found that Sodium benzoate is food preservative that inhibits microbial growth. The effects of sodium benzoate preservative on micronucleus induction, chromosome break, and Ala40Thr superoxide dismutase gene mutation in lymphocytes were studied. Sodium benzoate concentrations of 0.5, 1.0, 1.5, and 2.0mg/mL were treated in lymphocyte cell line for 24 and 48 hrs, respectively. Micronucleus test, standard chromosome culture technique, PCR, and automated sequencing technique were done to detect micronucleus, chromosome break, and gene mutation. The results showed that, at 24- and 48-hour. incubation time, sodium benzoate concentrations of 1.0, 1.5, and 2.0mg/mL increased micronucleus formation when comparing with the control group ($P < 0.05$). At 24- and 48-hour. incubation time, sodium benzoate concentrations of 2.0mg/mL increased chromosome break when comparing with the control group ($P < 0.05$). Sodium benzoate did not cause Ala40Thr (GCG→ACG) in superoxide dismutase gene. Sodium benzoate had the mutagenic and cytotoxic toxicity in lymphocytes caused by micronucleus formation and chromosome break. (Pongsavee,2015)

Allium cepa assay has been used extensively to determine the cytotoxicity and genotoxicity of compounds on plants and animals. The cytogenetic effects of two commonly used food preservatives, sodium benzoate and sodium metabisulphite were evaluated using the *A. cepa* assay. The parameters scored for the different concentrations of the compounds tested are: root length, chromosomal aberrations and Mitotic Index. The Mitotic Index (MI) decreased with increasing concentration of both sodium benzoate and sodium metabisulphite. Cytological

aberrations observed were clumping, fragmentation, pulverization, lagging, binucleate cells and reduction in chromatin materials. Clumping and fragmentation were the most frequent aberrations. The percentage of chromosomal aberrations at mitosis increased with increase in concentration of the food preservatives. The effects of sodium metabisulphite at the different concentrations in this study were very detrimental as more aberrations were recorded even after the recovery experiment. The results of this experiment show that these additives had irreversible cytotoxic effects at some levels of dosage. It supports the call for the banning of these substances as food preservatives. (Onyemaobi,2012)

The effects of sodium metabisulfite (SMB) on mitosis were investigated in Allium cepa L also have been studied.The roots of A. cepa were treated with SMB concentrations of 7.5 mg/lt, 15 mg/lt and 30 mg/lt for 10- and 20-hour treatment periods. SMB significantly decreased the mitotic index (MI) at all concentrations and treatment periods. While the decreasing of the MI was dose-dependent at 10 hours treatment time, SMB increased the mitotic abnormalities dose dependently. (ZOÚULLARI,2000)

➢ MATERIALS AND METHODS:

❖ MATERIALS REQUIRED:

- **Plant material:**
  - Fresh onion bulb (without shoot root)- *Allium cepa*
- **Chemicals-**
  - Sodium Benzoate
  - Acetic acid (45%)
  - Orcein
  - Distilled water
- **Others:**
  - Sand

❖ METHODS:

- **PREPARATION OF CHEMICAL SOLUTION:**
- **Preparation of Sodium Benzoate (SB) Solution:**

  For preparing 0.2%,0.4%,0.6%,0.8% and 1.0% SB solution 0.2gm ,0.4gm,0.6gm,0.8gm,1.0gm MSG was dissolved in 100ml distilled water in case of preparing each concentration.

- **Preparation of 45% Acetic-acid:**

  To prepare 500ml of 45% (V/V) acetic acid, 225 ml of glacial acetic acid was measured using a glass measuring cylinder and the final volume was made upto 500 ml using 275 ml distilled water.

- **Preparation of 2% Aceto-orcein:** To prepare 2% aceto-orcein 4gm of orcein powder was weighed with a digital balance.200ml of 45% acetic acid was brought to boilin conical flask using a ekectric heater .Orcein powder was gradually added to the heated acetic acid solution which was kept simmering and was regularly stirred with a glass rod for one and half hour .Solution was allowed to cool down to room temperature and then filtered .The filtrate obtained was mixed with 1(N) HCl in proportion of 9:1 and stored in glass bottle to be used in staining of root tips.

- **Procedure**: All bulbs were carefully collected and unscaled properly and the old roots were excised. Then a sand bed was made using sand. After that these bulbs were put there for the growth of root. When the roots were about 2-2.5cm then these bulbs were kept on the small beakers containing different concentration of SB solution (0.2%, 0.4%, 0.6%, 0.8% and 1.0 %water solution) room temperature (25±2° C).The root the onion bulbs were treated with the series of SB concentrations on the small beakers for different time periods 5hr, 7hr and 22 hr. Control group were also maintained parallel for the same period of time. After the treatment with the respective concentrations at particular time period the healthy grown root tips were excised from each bulb, washed in water and fixed in the glacial acetic acid (45%). Then the root tips were washed in distilled water and stained in 2% aceto-orcein. Five slides were made for each treatment and mean of each concentration was tabulated. Picture of the most frequent abnormalities in different concentrations were taken.

- **Statistical Analysis:** The data was collected and constructed graphs for the comparison of mitotic index in different time periods and abnormality percentage (Number of abnormal cells in microscopic field/ Total number of cells in microscopic field).

➢ RESULT:

In this investigation the effect of Sodium Benzoate on the root growth of meristematic cells of *Allium cepa* was analysed by treating the root cells at different concentrations of food additive SB for different time periods. There was significant difference observed between the control and other concentrations of SB in which time of exposure also made key role in causing the abnormalities of the chromosomes. The maximum effect to the mitotic index was observed at highest concentration 1.0% SB for 7 hours' exposure recorded the most significant chromosomal aberrations. In case of exposure of 22hrs time period it was found that no division was observed in case of exposure to each concentration.

CONCENTRATION OF SB (% SOLUTION)	DURATION OF EXPOSURE (hrs.)	MITOTIC INDEX	PERCENTAGE OF ABNORMALITY (%)
0.2	5	33.41	10.02
0.4	5	32.17	11.89
0.6	5	27.06	12.34
0.8	5	25.82	17.05
1.0	5	21.96	19.53

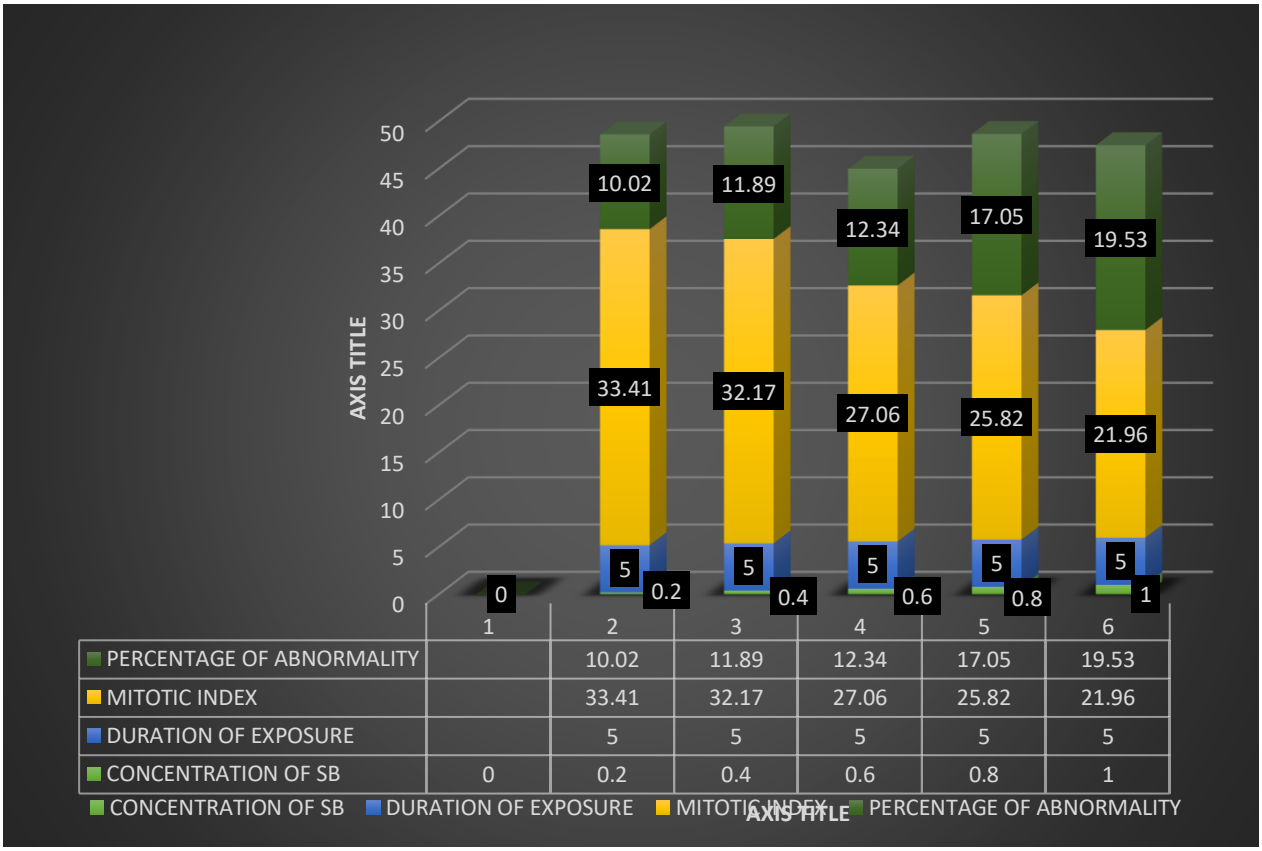

FIG 3: PERCENTAGE OF ABNORMALITY IN DIFFERENT CONC. OF SB

CONCENTRATION OF SB (% SOLUTION)	DURATION OF EXPOSURE (hrs.)	MITOTIC INDEX	PERCENTAGE OF ABNORMALITY(%)
0.2	7	32.07	11.73
0.4	7	27.64	15.42
0.6	7	23.18	18.09
0.8	7	21.46	23.25
1.0	7	19.13	25.64

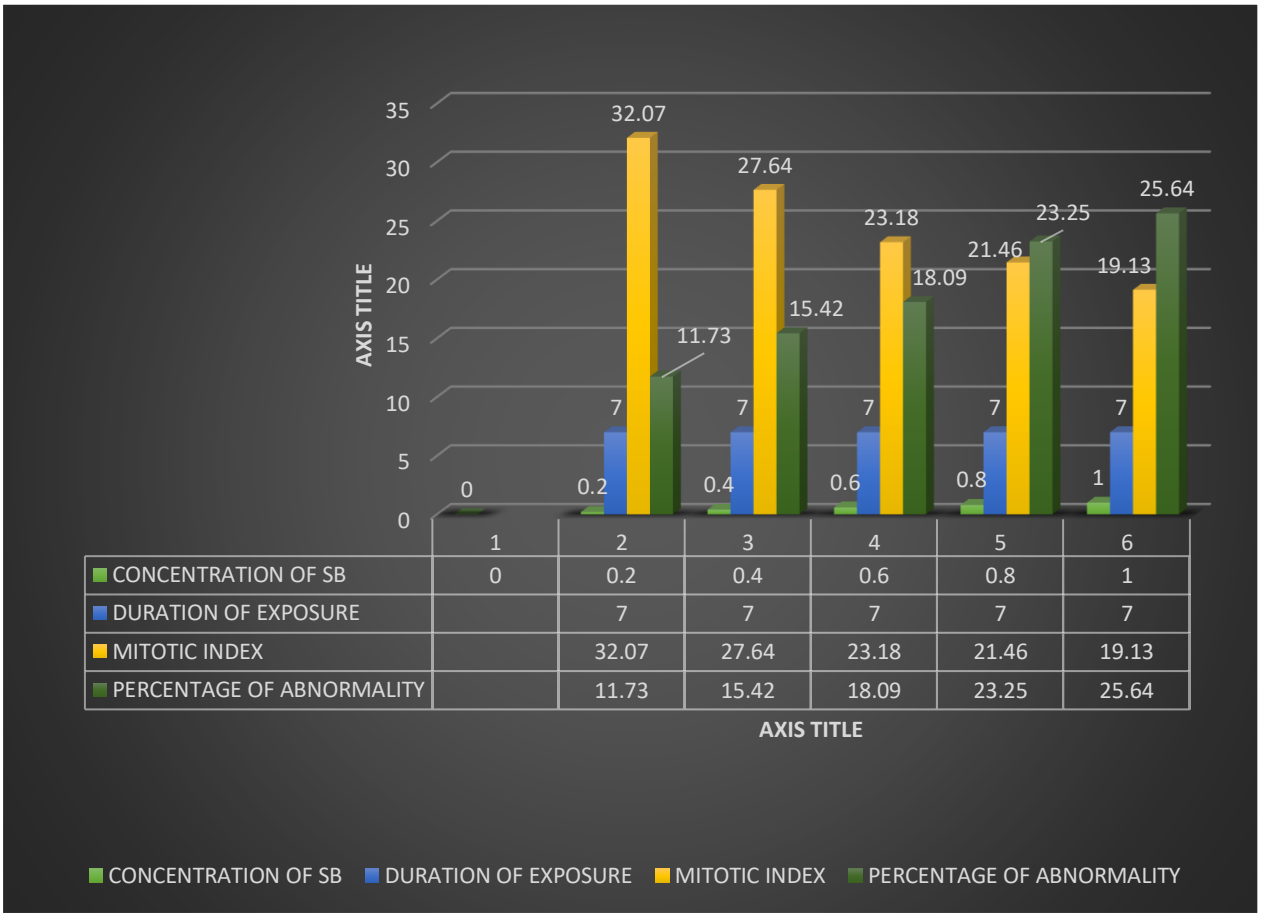

FIG 4: PERCENTAGE OF ABNORMALITY IN DIFFERENT CONC. OF SB

CONCENTRATION OF SB (% SOLUTION)	DURATION OF EXPOSURE (hrs.)	MITOTIC INDEX	PERCENTAGE OF ABNORMALITY (%)
)0.2	22	17.26	27.45
0.4	22	15.19	29.62
0.6	22	12.46	34.31
0.8	22	9.20	37.05

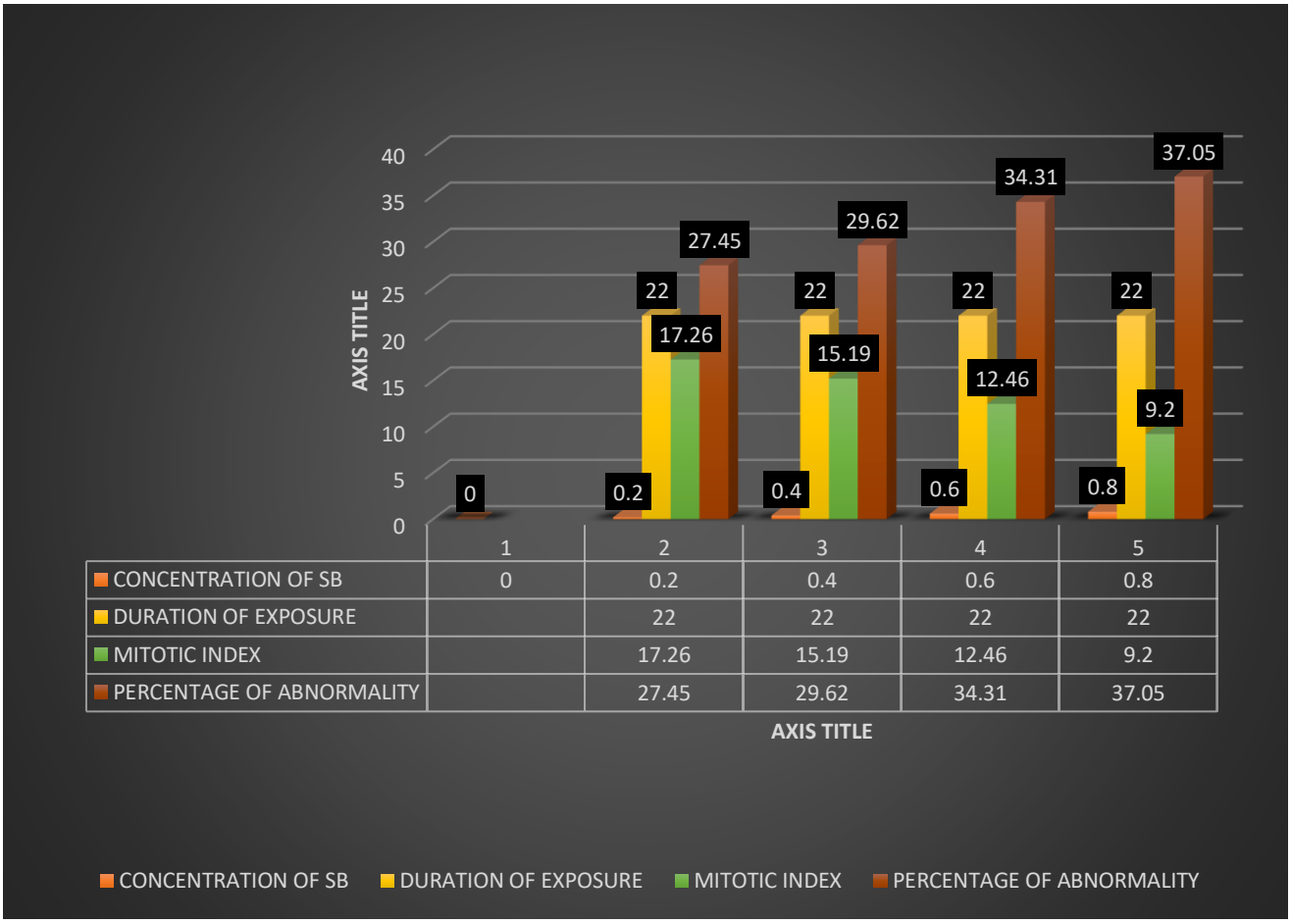

FIG 5: PERCENTAGE OF ABNORMALITY IN DIFFERENT CONC. OF SB

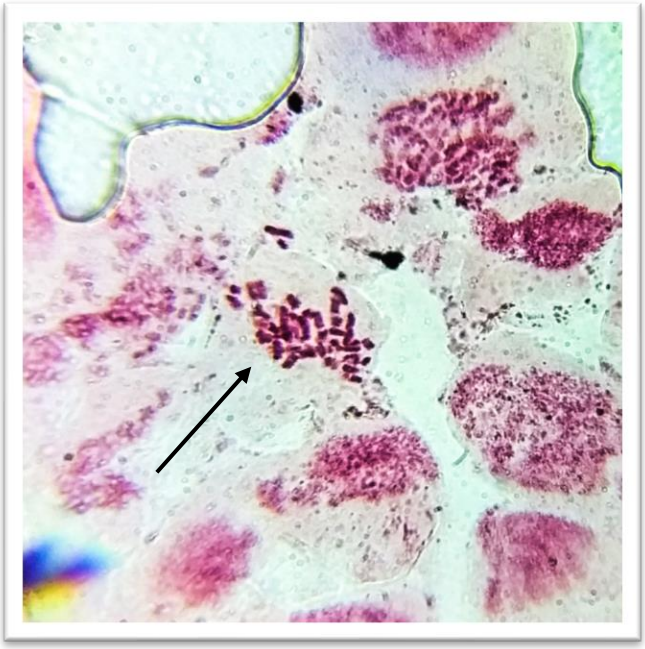

FIGURE 6

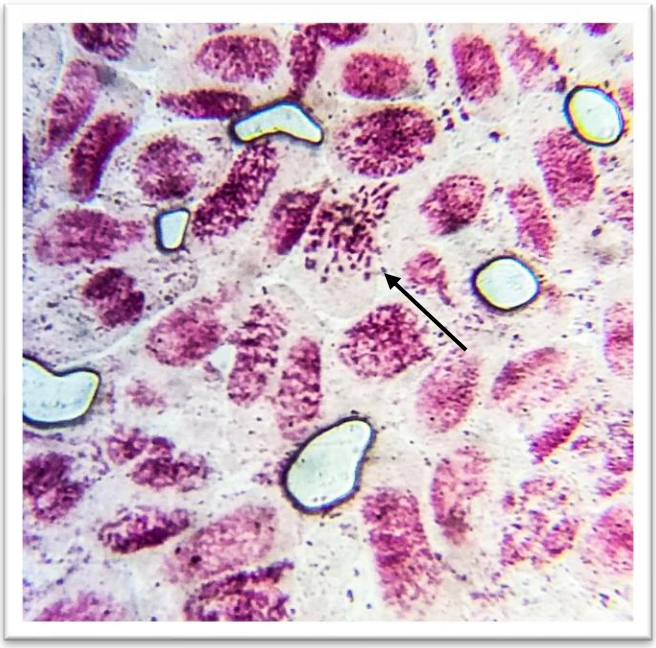

FIGURE 7

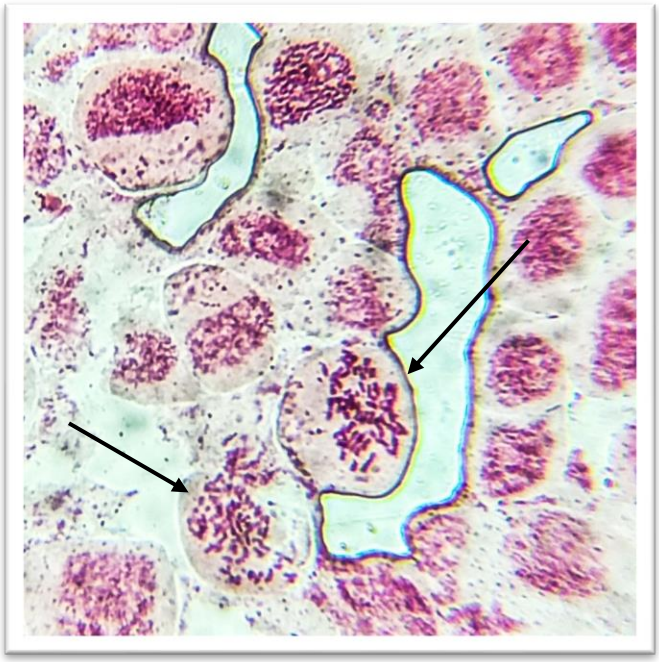

FIGURE 8

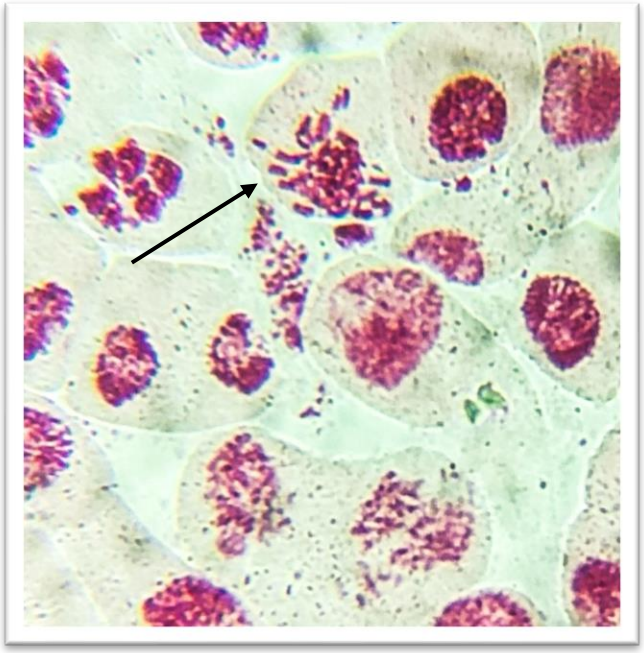

FIGURE 9

FIG 6,7,8 & 9: CHROMOSOME BREAKAGES CAUSED BY SODIUM BENZOATE

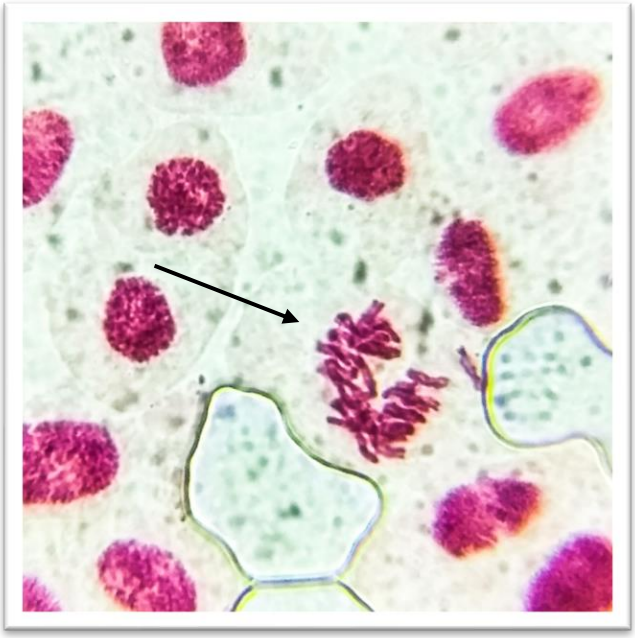

FIGURE 10

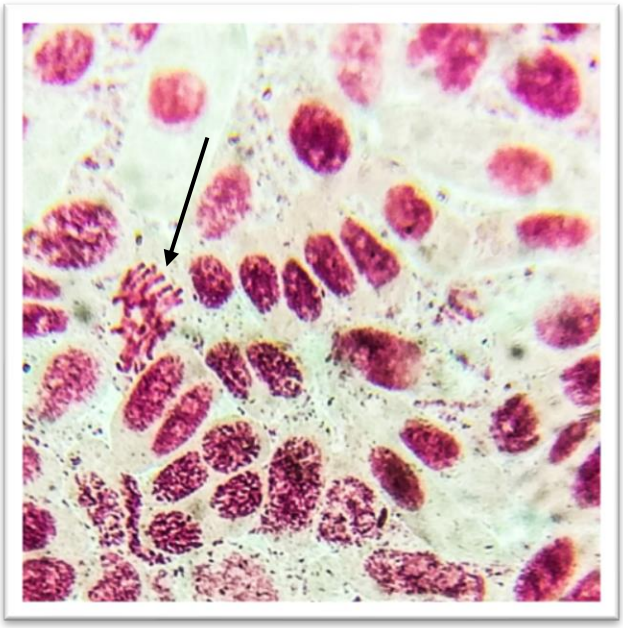

FIGURE 11

FIG 10 & 11: ABNORMAL METAPHASE CELL DIVISION CAUSED BY SODIUM BENZOATE

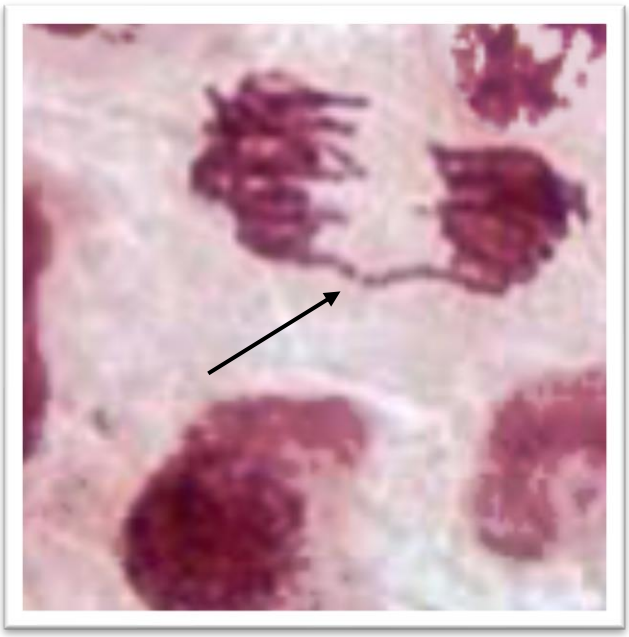

FIGURE 12

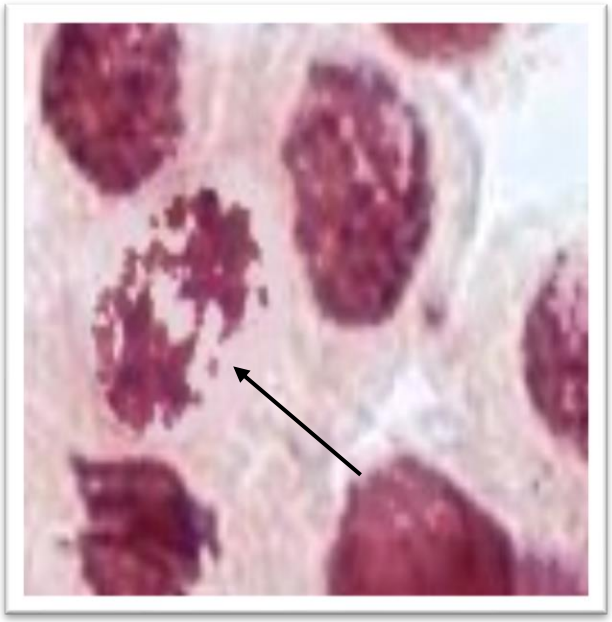

FIGURE 13

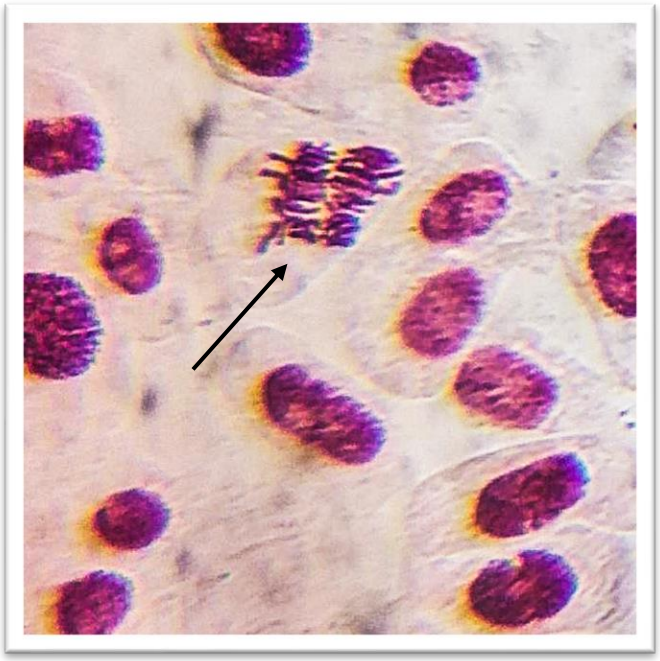

FIGURE 14

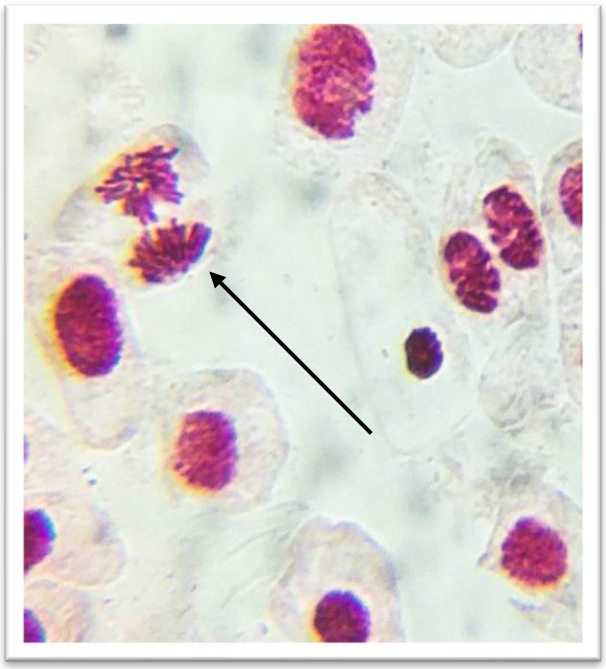

FIGURE 15

FIG 12,13,14 & 15: ABNORMAL ANAPHASE CELL DIVISION CAUSED BY SODIUM BENZOATE

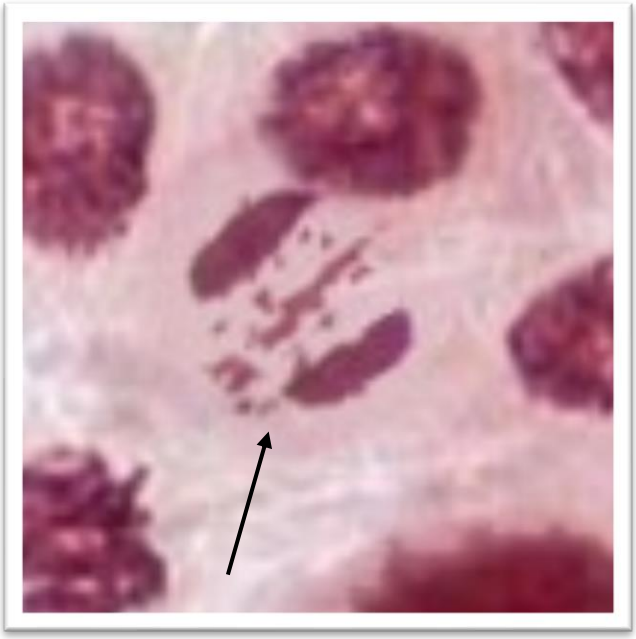

FIG 16: ABNORMAL TELOPHASE CELL DIVISION CAUSED BY SODIUM BENZOATE

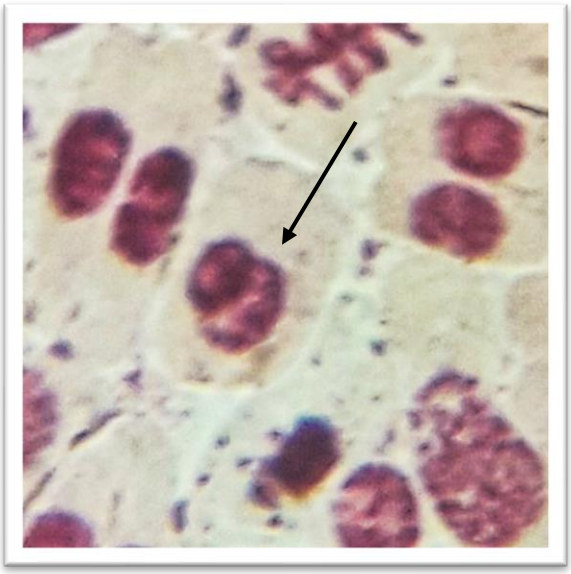

FIGURE 17

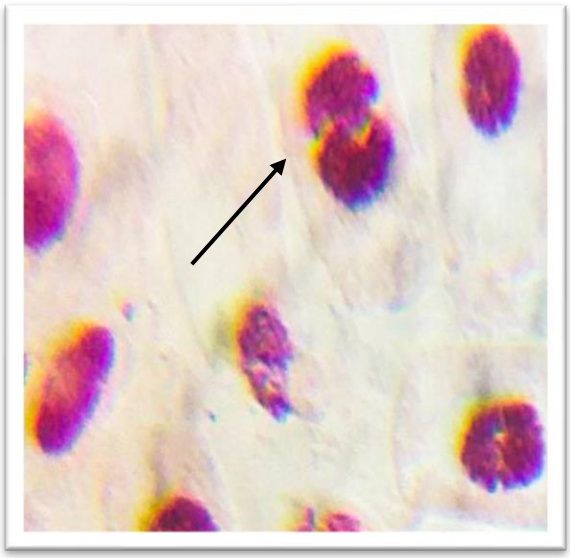

FIGURE 18

FIG 17 & 18: ABNORMAL BINUCLEATE STAGES CAUSED BY SODIUM BENZOATE

➢ CONCLUSION:

The effects of the different concentration's treatments of SB, on the amounts of DNA are studied in this project. Several amounts of DNA were reduced in *A. cepa* under investigation as a result of root treatments with SB when compared with control groups. The degree of the reduction was greater when the concentration was increased and the period of treatment was prolonged. The reduction in mitotic activity was accompanied with a depressive action on the amounts of DNA. This inhibition could be due either to blocking of G1 suppressing DNA synthesis or a blocking in G2 preventing the cell from entering mitosis Most studies on food additives toxicity in several test materials have focused on cytotoxic effects but there are not many reports on the effects of food additives on the activity of nuclear DNA. Sodium benzoate inhibited DNA synthesis in *Allium cepa* root meristems. The investigations on the DNA contents were reported after treatment with other chemicals the effect of these food additives on DNA content, on the other hand, needs further investigations which may involve a study on the DNA polymers. In conclusion, from the present study, it appears that SB is used commonly in the food industry have clear chromotoxic effects. For this reason, it is necessary to be careful when using these chemicals as food additives. In addition, further cytogenetic studies dealing with clastogenicity and genotoxicity of these food additives may reveal further interesting results.

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